IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Jeffrey B. Etter	Confirmation No.:	5370
Serial No.:	12/466,213	Art Unit:	1623
Filed:	May 14, 2009	Examiner:	Lawrence E. Crane
For:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF	Attorney Docket (CAM No.:	9516-847-999 501872-999847)

AMENDMENT AND RESPONSE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the non-final office action mailed August 1, 2011, Applicants respectfully submit the following amendments and remarks for the Examiner's consideration and entry into the record.

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Abstract begin on page 4 of this paper.

Amendments to the Claims are reflected in the listing of the claims that begins on page 5 of this paper.

Remarks begin on page 12 of this paper.

Applicants hereby authorize any required fees, including a one-month time extension fee of $150 and a fee of $180 for filing a Supplemental Information Disclosure Statement, to be charged to Jones Day Deposit Account No. 50-3013.

AMENDMENTS TO THE SPECIFICATION

Please make the following amendments to the as-filed specification:

Please replace the first paragraph on page 1 with the following amended paragraph:

[0001] This application claims priority to U.S. Provisional Patent Application Nos. 61/053,609, filed May 15, 2008, which is now expired; 61/201,145, filed December 5, 2008, which is now expired; and 61/157,875, filed March 5, 2009, which is now expired, the contents of each of which are incorporated by reference herein in their entireties.

Please replace paragraphs 35–37 on page 11 with the following amended paragraphs:

[0035] Figure 4A represents PD data from an individual patient (Subject 02008, 80 year old male, RAEB-1) collected during a multiple dose escalation study. The patient was dosed with azacitidine Formulation #3, 240 mg. Platelets (K/μL), and Hgb (g/dL), ~~ANC (K/μL), and Relative BM Blast (%)~~ are plotted versus sampling dates over the course of the study in Figure 4A. Figure 4B represents PD data from an individual patient (Subject 02008, 80 year old male, RAEB-1) collected during a multiple dose escalation study. The patient was dosed with azacitidine Formulation #3, 240 mg. ANC (K/μL) and Relative BM Blast (%) are plotted versus sampling dates over the course of the study in Figure 4B.

[0036] Figure 5A represents PD data from an individual patient (Subject 02007, 76 year old male, CMML) collected during a multiple dose escalation study. The patient was dosed with azacitidine Formulation #3, 240 mg. Platelets (K/μL), and Hgb (g/dL), ~~ANC (K/μL), and Relative BM Blast (%)~~ are plotted versus sampling dates over the course of the study in Figure 5A. Figure 5B represents PD data from an individual patient (Subject 02007, 76 year old male, CMML) collected during a multiple dose escalation study. The patient was dosed with azacitidine Formulation #3, 240 mg. ANC (K/μL) and Relative BM Blast (%) are plotted versus sampling dates over the course of the study in Figure 5B.

[0037] Figure 6A represents PD data from an individual patient (Subject 02004, 61 year old male, MDS, MDACC) collected during a multiple dose escalation study. The patient was dosed with azacitidine Formulation 1, 120 mg. Platelets (K/μL)~~,~~ and Hgb (g/dL)~~, ANC (K/μL), and Relative BM Blast (%)~~ are plotted versus sampling dates over the course of the study in Figure 6A. Figure 6B represents PD data from an individual patient (Subject 02004, 61 year old male, MDS, MDACC) collected during a multiple dose escalation study. The patient was dosed with azacitidine Formulation 1, 120 mg. ANC (K/μL) and Relative BM Blast (%) are plotted versus sampling dates over the course of the study in Figure 6B.

AMENDMENTS TO THE ABSTRACT

Please make the following amendments to the as-filed abstract:

ABSTRACT

The present disclosure provides pharmaceutical compositions comprising cytidine analogs, for example, 5-azacytidine or decitabine, for oral administration, wherein the compositions release the cytidine analog, for example, 5-azacytidine or decitabine, substantially in the stomach. Also provided are methods of treating diseases and disorders using the oral formulations provided herein.

AMENDMENTS TO THE CLAIMS

The following listing of claims will replace all prior versions, and listings, of claims in the application.

Listing of the Claims

1. (Currently amended) A pharmaceutical composition for oral administration comprising a therapeutically effective amount of a cytidine analog ~~5-azacytidine~~ and at least one pharmaceutically acceptable excipient, wherein the composition is an immediate release tablet or an immediate release capsule, and the cytidine analog is 5-azacytidine or decitabine ~~releases the 5-azacytidine substantially in the stomach following oral administration to a subject~~.

2. (Canceled).

3. (Currently amended) The composition of claim 1, ~~which is non-enteric-coated~~ wherein the composition does not comprise an enteric coating.

4. (Currently amended) The composition of claim 1, ~~which~~ wherein the composition is a tablet.

5. (Currently amended) The composition of claim 1, ~~which~~ wherein the composition is a capsule.

6. (Currently amended) The composition of claim 1, ~~which further comprises an~~ wherein the pharmaceutically acceptable excipient is selected from mannitol, microcrystalline cellulose, crospovidone, and magnesium stearate.

7. (Original) The composition of claim 1, which further comprises a permeation enhancer.

8. (Currently amended) The composition of claim 7, wherein the permeation enhancer is ~~d~~ D-alpha-tocopheryl polyethylene glycol 1000 succinate.

9. (Currently amended) The composition of claim 8, wherein the ~~d~~ D-alpha-tocopheryl polyethylene glycol 1000 succinate is present in the formulation at about 2% by weight relative to the total weight of the formulation.

10. (Original) The composition of claim 1, which is essentially free of a cytidine deaminase inhibitor.

11. (Original) The composition of claim 1, which is essentially free of tetrahydrouridine.

12. (Currently amended) The composition of claim 1, which further comprises ~~an additional therapeutic~~ a therapeutically effective amount of a second active agent.

13. (Currently amended) The composition of claim 1, wherein the amount of 5-azacytidine or decitabine is at least about 40 mg.

14. (Currently amended) The composition of claim 1, wherein the amount of 5-azacytidine or decitabine is at least about 400 mg.

15. (Currently amended) The composition of claim 1, wherein the amount of 5-azacytidine or decitabine is at least about 1000 mg.

16. (Currently amended) The composition of claim 1, which has been shown to achieves an area-under-the-curve value of at least about 200 ng-hr/mL following oral administration to a test subject.

17. (Currently amended) The composition of claim 1, which has been shown to achieves an area-under-the-curve value of at least about 400 ng-hr/mL following oral administration to a test subject.

18. (Currently amended) The composition of claim 1, which has been shown to achieves a maximum plasma concentration of at least about 100 ng/mL following oral administration to a test subject.

19. (Currently amended) The composition of claim 1, which has been shown to achieves a maximum plasma concentration of at least about 200 ng/mL following oral administration to a test subject.

20. (Currently amended) The composition of claim 1, which has been shown to achieves a time to maximum plasma concentration of less than about 180 minutes following oral administration to a test subject.

21. (Currently amended) The composition of claim 1, which has been shown to achieves a time to maximum plasma concentration of less than about 90 minutes following oral administration to a test subject.

22. (Currently amended) The composition of claim 1, which has been shown to achieves a time to maximum plasma concentration of less than about 60 minutes following oral administration to a test subject.

23. (Currently amended) A method for treating ~~a subject having~~ one or more symptoms of a disease associated with abnormal cell proliferation, comprising orally administering to ~~the~~ a subject in need thereof a pharmaceutical composition comprising a therapeutically effective amount of a cytidine analog ~~5-azacytidine~~ and at least one pharmaceutically acceptable excipient, wherein the composition releases the cytidine analog ~~5-azacytidine~~ substantially in the stomach following oral administration to the subject, the disease associated with abnormal cell proliferation is a cancer or a hematologic disorder, and the cytidine analog is 5-azacytidine or decitabine.

24. (Original) The method of claim 23, wherein the disease is myelodysplastic syndrome.

25. (Original) The method of claim 23, wherein the disease is acute myelogenous leukemia.

26. (Original) The method of claim 23, wherein the disease is non-small-cell lung cancer.

27. (Original) The method of claim 23, wherein the disease is ovarian cancer.

28. (Original) The method of claim 23, wherein the disease is pancreatic cancer.

29. (Original) The method of claim 23, wherein the disease is colorectal cancer.

30. (Original) The method of claim 23, which results in improved overall survival.

31. (Currently amended) The method of claim 23, wherein the method further comprises co-administering to the subject in need thereof ~~an additional therapeutic~~ a therapeutically effective amount of a second active agent.

32. (Original) The method of claim 23, wherein the composition is an immediate release composition.

33. (Original) The method of claim 23, wherein the composition further comprises a permeation enhancer.

34. (Currently amended) The method of claim 33, wherein the permeation enhancer is ~~d~~ D-alpha-tocopheryl polyethylene glycol 1000 succinate.

35. (Currently amended) The method of claim 34, wherein the ~~d~~ D-alpha-tocopheryl polyethylene glycol 1000 succinate is present in the formulation at about 2% by weight relative to the total weight of the formulation.

36. (Original) The method of claim 23, wherein the method further comprises not co-administering a cytidine deaminase inhibitor with the cytidine analog.

37. (Original) The method of claim 23, wherein the composition is a single unit dosage form.

38. (Currently amended) The method of claim 23, wherein the composition does not comprise an enteric coating ~~is non-enteric-coated~~.

39. (Original) The method of claim 23, wherein the composition is a tablet.

40. (Original) The method of claim 23, wherein the composition is a capsule.

41. (Currently amended) The method of claim 23, wherein the pharmaceutically acceptable ~~composition further comprises an~~ excipient is selected from mannitol, microcrystalline cellulose, crospovidone, and magnesium stearate.

42. (Currently amended) The method of claim 23, wherein the amount of 5-azacytidine or decitabine is at least about 40 mg.

43. (Currently amended) The method of claim 23, wherein the amount of 5-azacytidine or decitabine is at least about 400 mg.

44. (Currently amended) The method of claim 23, wherein the amount of 5-azacytidine or decitabine is at least about 1000 mg.

45. (Currently amended) The method of claim 23, which has been shown to achieves an area-under-the-curve value of at least about 200 ng-hr/mL following oral administration to ~~the~~ a test subject.

46. (Currently amended) The method of claim 23, which has been shown to achieves an area-under-the-curve value of at least about 400 ng-hr/mL following oral administration to ~~the~~ a test subject.

47. (Currently amended) The method of claim 23, which has been shown to achieves a maximum plasma concentration of at least about 100 ng/mL following oral administration to ~~the~~ a test subject.

48. (Currently amended) The method of claim 23, which has been shown to achieves a maximum plasma concentration of at least about 200 ng/mL following oral administration to ~~the~~ a test subject.

49. (Currently amended) The method of claim 23, which has been shown to achieves a time to maximum plasma concentration of less than about 180 minutes following oral administration to ~~the~~ a test subject.

50. (Currently amended) The method of claim 23, which has been shown to achieves a time to maximum plasma concentration of less than about 90 minutes following oral administration to ~~the~~ a test subject.

51. (Currently amended) A pharmaceutical composition comprising a therapeutically effective amount of a cytidine analog ~~5-azacytidine~~ and at least one pharmaceutically acceptable excipient, wherein the composition is for treating a disease or disorder associated with abnormal cell proliferation, wherein the composition is an immediate release tablet or an immediate release capsule prepared for oral administration, wherein the disease or disorder associated with abnormal cell proliferation is a cancer or a hematologic disorder, and wherein the cytidine analog is 5-azacytidine or decitabine ~~and wherein the composition is prepared for release of the 5-azacytidine substantially in the stomach~~.

52. (Currently amended) The pharmaceutical composition of claim 51, wherein the amount of 5-azacytidine or decitabine is about 40 mg, about 400 mg, or about 1000 mg.

53. (Currently amended) The pharmaceutical composition of claim 51, wherein the composition ~~is prepared~~ has been shown to achieve an area-under-the-curve value of at least about 200 ng-hr/mL or 400 ng-hr/mL following oral administration to a test subject.

54. (Currently amended) The pharmaceutical composition of claim 51, wherein the composition ~~is prepared~~ has been shown to achieve a maximum plasma concentration of at least about 100 ng/mL or 200 ng/mL following oral administration to a test subject.

55. (Currently amended) The pharmaceutical composition of claim 51, wherein the composition ~~is prepared~~ has been shown to achieve a time to maximum plasma concentration of less than about 60 minutes or 90 minutes after being administered to a test subject.

56. (Canceled).

57. (Currently amended) The pharmaceutical composition of any one of claims 51 to 55, wherein the composition is prepared for oral administration in combination with ~~an additional therapeutic~~ a therapeutically effective amount of a second active agent.

58. (Original) The pharmaceutical composition of any one of claims 51 to 55, wherein the disease or disorder is myelodysplastic syndrome or acute myelogenous leukemia.

59. (Currently amended) The pharmaceutical composition of any one of claims 51 to 55, wherein the composition is a tablet ~~single unit dosage form~~.

60. (Currently amended) The pharmaceutical composition of any one of claims 51 to 55, wherein the composition is ~~a tablet or~~ a capsule.

61. (Currently amended) The pharmaceutical composition of any one of claims 51 to 55, wherein the ~~composition further comprises an~~ pharmaceutically acceptable excipient is selected from mannitol, microcrystalline cellulose, crospovidone, and magnesium stearate.

62–65. (Canceled).

66. (New) The composition of claim 1, wherein the cytidine analog is 5-azacytidine.

67. (New) The composition of claim 1, wherein the cytidine analog is decitabine.

68. (New) The method of claim 23, wherein the cytidine analog is 5-azacytidine.

69. (New) The method of claim 23, wherein the cytidine analog is decitabine.

70. (New) The pharmaceutical composition of claim 51, wherein the cytidine analog is 5-azacytidine.

71. (New) The pharmaceutical composition of claim 51, wherein the cytidine analog is decitabine.

REMARKS

Amendments to the Specification

The specification is amended at page 1, first paragraph, to recite the current status of provisional applications. No new matter is added.

The specification is amended at page 11, paragraphs 35–37, to provide separate descriptions for formal Figures 4A, 4B, 5A, 5B, 6A, and 6B. Support is found, for example, in the as-filed specification at page 11, paragraphs 35–37, and the as-filed Figures 4–6. No new matter is added.

Entry of the amendments to the specification is respectfully requested.

Amendments to the Abstract

The abstract is amended to recite "5-azacytidine or decitabine" as exemplary embodiments of a cytidine analog. Support is found throughout the as-filed specification, including, for example, at page 17, paragraph 75; and page 19, paragraph 79. No new matter is added.

Entry of the amendments to the abstract is respectfully requested.

Claim Amendments

Claims 1, 3–55, 57–61, and 66–71 are pending in this application. Claims 2, 56, and 62–65 are canceled without prejudice to Applicants' right to pursue the subject matter recited therein in one or more divisional, continuation, and/or continuation-in-part applications.

Claims 1, 3–6, 8–9, 12–23, 31, 34–35, 38, 41–55, 57, and 59–61 are amended without any intention of disclaiming any equivalents thereof.

Claim 1 is amended to recite, *inter alia*, "a cytidine analog," "at least one pharmaceutically acceptable excipient," "an immediate release tablet or an immediate release capsule," and "the cytidine analog is 5-azacytidine or decitabine." Support is found, for example, at page 6, paragraph 17; pages 14–15, paragraphs 63–64; page 17, paragraph 75;

page 19, paragraph 79; and pages 36–37, paragraph 121 of the as-filed specification.

Claims 8–9 and 34–35 are amended to recite, *inter alia*, "D-alpha." Support is found, for example, at the original claims 8–9 and 34–35.

Claims 12, 31, and 57 are amended to recite, *inter alia*, "a therapeutically effective amount of a second active agent." Support is found, for example, at page 52, paragraph 155, lines 1–5 of the as-filed specification.

Claims 13–15, 42–44, and 52 are amended to recite, *inter alia*, "decitabine." Support is found, for example, at page 17, paragraph 75; and page 19, paragraph 79 of the as-filed specification.

Claims 16–22, 45–50, and 53–55 are amended to recite, *inter alia*, "has been shown to achieve" and "test subject" as recommended by the Examiner.

Claim 23 is amended. Support is found, for example, at page 6, paragraph 17; page 12, paragraph 55; page 17, paragraph 75; page 19, paragraph 79; pages 36–37, paragraph 121; pages 60–61, paragraphs 171 and 173; and pages 64–65, paragraph 182 of the as-filed specification.

Claim 51 is amended. Support is found, for example, at page 6, paragraph 17; pages 14–15, paragraphs 63–64; page 17, paragraph 75; page 19, paragraph 79; pages 36–37, paragraph 121; pages 60–61, paragraphs 171 and 173; and pages 64–65, paragraph 182 of the as-filed specification.

Claims 59 and 60 are amended. Support is found, for example, at the original claim 60.

Claims 3–6, 38, 41, and 61 are amended to more clearly recite the claimed subject matter.

Claims 66–71 are added. Support is found, for example, at page 17, paragraph 75; and page 19, paragraph 79 of the as-filed specification.

No new matter is introduced. Applicants respectfully request consideration and entry of the claim amendments.

Applicants respectfully submit that the pending claims are allowable for at least the following reasons.

A. Objections to the As-Filed Abstract and Specification

On pages 2–3 of the Office Action, the as-filed abstract and specification are objected to. Without acquiescing to the propriety of the objections, and solely to expedite prosecution, the abstract and paragraphs 1 and 35–37 of the specification have been amended. Applicants respectfully request the objections be withdrawn.

B. Claim Rejections Under 35 U.S.C. § 101

Claims 62–65 are rejected under 35 U.S.C. § 101 allegedly "because the claimed recitation of a use, without setting forth any steps involved in the process." Office Action, at page 3. Without acquiescing to the propriety of the rejection, and solely to expedite prosecution, claims 62–65 have been canceled without prejudice. Applicants respectfully request the rejection to claims 62–65 be withdrawn.

Claims 16–22, 45–50, and 53–55 are rejected under 35 U.S.C. § 101. The Office Action alleges that "the claim of an *in vivo* result in the present tense implies ownership of the host treated." Office Action, at page 3. Applicants respectfully disagree because claims 16–22, 45–50, and 53–55 are directed to pharmaceutical compositions and methods of treatment, which are clearly patentable subject matter under 35 U.S.C. § 101 as mandated by the statute and controlling case law. Contrary to the allegations of the Office Action, the claims do not imply any ownership of a treated subject. However, solely to expedite prosecution of the present application and without prejudice, claims 16–22, 45–50, and 53–55 have been amended according to the Examiner's suggestion. *See* Office Action, at page 4. Applicants respectfully request the rejection to claims 16–22, 45–50, and 53–55 be withdrawn.

C. Claim Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 23–50 and 62–65 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly not enabled. Specifically, it is alleged that "the specification, while being enabled for the treatment of a limited number of neoplastic disease conditions (see the non-prospective disclosures of Examples 1–7), does not reasonably provide enablement for the effective treatment of all other 'diseases associated with abnormal cell proliferation,' including at least cancers of pancreas (instant Example 11 is entirely prospective), liver and

brain." Office Action, at page 4. It is further alleged that the analysis of *Wands* factors as set forth in the Office Action shows that undue experimentation would be required for practicing the claimed methods. Office Action, at pages 4–5. Applicants respectfully disagree with each of these allegations and respectfully request reconsideration and withdrawal of the rejections on the ground of enablement.

The test for enablement is whether one skilled in the art could make or use the invention based on the disclosures of the specification coupled with information known in the art at the time of filing without undue experimentation. *See, e.g., U.S. v. Telectronics Inc.*, 857 F.2d 778, 785 (Fed. Cir. 1988). The test is not whether any experimentation is necessary, but whether it is undue. *See, e.g., In re Angstadt*, 190 U.S.P.Q. 214, 219 (C.C.P.A. 1976). Factors to determine whether undue experimentation is required are set forth in *In re Wands*. *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988). As the Federal Circuit explained in *Wands*, routine screening does not amount to undue experimentation. *Id.* at 736. "A considerable amount of experimentation is permissible, if it is merely routine." *Id.* at 737.

In addition, "a specification disclosure which contains a teaching of the manner and process of making and using an invention . . . must be taken as being in compliance with the enablement requirement . . . unless there is a reason to doubt the objective truth of the statements," and thus, the Office has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. M.P.E.P. § 2164.04, citing *In re Wright*, 999 F.2d 1557, 1562 (Fed. Cir. 1993).

In view of these well-settled legal principles, Applicants respectfully submit that the analysis of *Wands* factors provided in the Office Action falls far short of meeting the initial burden of establishing a legally sufficient basis to question the enablement of the instant application. Thus, the rejections on the ground of enablement are legally improper and should be withdrawn.

Applicants respectfully point out that claims 23–50[1] are enabled because the specification "contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented." M.P.E.P. § 2164.04. For example, the specification teaches that 5-azacytidine or decitabine can modulate the activity of DNA methyltransferases,

[1] Claims 62–65 have been canceled without prejudice.

thereby restoring normal functions of morphologically dysplastic, immature hematopoietic cells and cancer cells. (*e.g.*, Specification, at paragraph 11.) Indeed, 5-azacytidine and decitabine have been approved for intravenous (IV) or subcutaneous (SC) administration to treat myelodysplastic syndromes (MDS). (*e.g.*, Specification, at paragraphs 9 and 11–13.) However, at the time of the application, oral delivery of 5-azacytidine or decitabine has proven difficult. (*e.g.*, Specification, at paragraph 13.) In this regard, the specification describes in detail various embodiments of oral formulations of cytidine analogs, such as 5-azacytidine or decitabine, that release the cytidine analog substantially in the stomach following oral administration, and provides specific working examples of formulations that were shown to achieve significant drug exposure in the plasma of a subject after oral administration. (*e.g.*, Specification, at paragraphs 108–154 and Examples 1–6.) Furthermore, the specification teaches, *inter alia*, that one or more symptoms of cancers and hematological disorders can be treated by modulators of DNA methyltransferase, such as 5-azacytidine or decitabine. (*e.g.*, Specification, at paragraphs 11–12 and 196–205.) The specification further describes detailed methods of using the oral formulations provided therein to treat one or more symptoms of a disease associated with abnormal cell proliferation, including cancers and hematologic disorders. (*e.g.*, Specification, at paragraphs 170–206 and Examples 4–12.) In view of the disclosure of the instant specification, those skilled in the art can make an oral formulation of 5-azacytidine or decitabine, fully guided by the detailed description and specific examples provided, and orally administer the formulation to a subject in need thereof to treat one or more symptoms of a cancer or a hematological disorder, for example, by reversing abnormal DNA methylation in the subject. Therefore, it is clear that the application provides sufficient guidance to allow those of ordinary skill in the art to make and use the claimed invention without undue experimentation.

However, the Office Action contends that the analysis of *Wands* factors as set forth therein shows that undue experimentation would be required for practicing the claimed methods. Applicants respectfully disagree. The instant claims relate to treating one or more symptoms of particular diseases, such as cancers and hematologic diseases, using an oral formulation of 5-azacytidine or decitabine which releases the 5-azacytidine or decitabine substantially in the stomach following oral administration. As explained above, the specification provides ample guidance to a person of ordinary skill in the art to practice the claimed invention without undue experimentation. Indeed, when analyzing the *Wands*

factors, the Office Action contends that the "[t]he treatment of neoplastic disease by the administration of an effective amount of 5-azacytidine to a host in need thereof is very well known in the art . . ." and "[i]n view of the substantial and relevant teachings of . . . cited documents presently of record disclosing that 5-azacytidine has neoplastic activity supports the view that this art area is at least somewhat predictable." Office Action, at pages 4–5. Thus, it appears that the Office Action concedes that 5-azacytidine possesses anti-neoplastic activity and may be used to treat neoplastic diseases. Yet, the Office Action goes on to allege that "non-prospective exemplifications appear to end at Example 7," and "[t]he small number of instant exemplifications suggests that the instant disclosure has enabled only a very limited number of disease treatments wherein 5-azacytidine is administered as the active ingredient." Office Action, at page 5. In this regard, Applicants respectfully point out that compliance with the enablement requirement does not turn on whether an example is disclosed. M.P.E.P. § 2164.02. It is respectfully reminded that the claims are presumed to be enabled where the specification "contains a teaching of the manner and process of making and using [the claimed] invention." *In re Wright*, 999 F.2d at 1562. Such a teaching is clearly provided by the current specification, and no evidence to the contrary is provided by the Office. Furthermore, as explained above, the instant application provides detailed descriptions or specific examples on how to use oral formulations of 5-azacytidine or decitabine to treat cancers or hematologic disorders. In view of the guidance provided in the instant application, the state of the art and the level of one of ordinary skill at the time of the application, the required experimentation, if any, to practice the subject matter of claims 23–50 would be merely routine. In other word, ***no*** undue experimentation by one skilled in the art is needed to practice the claimed methods.

Therefore, Applicants respectfully submit that the rejections under 35 U.S.C. § 112, first paragraph, on the ground of enablement should be withdrawn.

D. Claim Rejections Under 35 U.S.C. § 112, Second Paragraph

Claims 1–65 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. Office Action, at pages 5–7. Without acquiescing to the propriety of the rejections, and solely to expedite prosecution, claims 1, 3–6, 8–9, 12, 23, 31, 34–35, 38, 41, 51, 57, and 61 have been amended without prejudice. Applicants respectfully request the rejections for alleged indefiniteness be withdrawn.

E. Claim Rejections Under 35 U.S.C. § 102

Claims 1–65 are rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by U.S. Patent No. 7,189,740 to Zeldis (hereinafter "the '740 Patent"). Office Action, at pages 7–8. In particular, the Office Action refers to the '740 Patent at claims 1 and 7–11; column 25, lines 8–48; and column 7, lines 16–21; and alleges that the '740 Patent teaches "pharmaceutical compositions of 5-azacytidine" and "second active ingredient" including "conventional therapy for MDS." Office Action, at pages 7–8. The Office Action also refers to Beers *et al.* (The Merck Manual of Diagnosis and Therapy, 2006, pages 1114–1116; hereinafter "Beers") and O'Neil *et al.* (The Merck Index, 2006, page 150, Entry 890; hereinafter "O'Neil"), "to provide examples of MDS and neoplastic therapy in order to provide specific definitions of the generic terms in the '740 reference." Office Action, at page 8. Applicants respectfully traverse the rejections.

A claim is anticipated "only if each and every element as set forth in the claim is found in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987) (emphasis added). Thus, Applicants respectfully submit that it is legally improper for the Office Action to rely on more than one reference to reject the instant claims for alleged anticipation. In addition, the Office Action does not provide any analysis whatsoever as to how each of the rejected claims (*i.e.*, each of claims 1–65) is anticipated by the cited references. In this regard, the Office Action falls far short of satisfying its burden of establishing that the pending claims are anticipated.

Applicants respectfully point out that none of three cited references anticipates the instant claims. For instance, none of the references teaches or suggests an immediate release tablet or an immediate release capsule comprising 5-azacytidine or decitabine prepared for oral administration.[2] Moreover, none of the references teaches or suggests a method involving orally administering 5-azacytidine or decitabine to a subject in need thereof, wherein the 5-azacytidine or decitabine is released substantially in the stomach following oral administration. For example, Applicants respectfully point out that claims 1 and 7–11 of the '740 Patent do not recite 5-azacytidine or decitabine, let alone an oral formulation of 5-azacytidine or decitabine or the methods of the instant claims. Indeed, the

[2] The term "immediate release" as used in the present application is defined in the specification at paragraph 64.

'740 Patent, including the passages at column 25, lines 8–48 and column 7, lines 16–21, does not disclose any immediate release oral formulation of a cytidine analog of the instant claims or the methods of the instant claims (*e.g.*, orally administering a cytidine analog to a subject in need thereof, wherein the cytidine analog is released substantially in the stomach following oral administration). Similarly, none of Beers or O'Neil teaches or suggests an immediate release tablet or an immediate release capsule comprising a cytidine analog of the instant claims or the methods of the instant claims. For at least these reasons, none of the cited references discloses "each and every element set forth in" the pending claims. Therefore, it is respectfully requested that the rejections be withdrawn.

F. Claim Rejections Under 35 U.S.C. § 103

Claims 1–65 are rejected under 35 U.S.C. § 103 as allegedly being unpatentable over Redkar *et al.* (U.S. Patent Publication No. 2006/0074046; hereinafter "Redkar"), in view of Dintaman *et al.* (Pharmaceutical Research, 1999, 16(10), 1550–1556; hereinafter "Dintaman") and Sands *et al.* (U.S. Patent Publication No. 2004/0162263; hereinafter "Sands"). Office Action, at pages 8–9. In particular, it is alleged that Redkar teaches that "5-azacytidine is an active ingredient and that the pharmaceutical compositions thereof are known . . . and are effective in the treatment of neoplastic diseases." *Id.* at page 8. It is also alleged that Redkar teaches "the co-administration of 5-azacytidine with a second active ingredient." *Id.* The Office Action acknowledges that Redkar "does not expressly disclose pharmaceutical compositions comprising a 'permeation enhancer' including the vitamin E derivative TPGS." *Id.* However, the Office Action contends that Dintaman "discloses the vitamin E derivative TPGS and further discloses its mechanism of action as an inhibitor of P-glycoproteins known to be part of chemotherapy failures due to multidrug resistance." *Id.* at page 9. The Office Action also alleges that Sands discloses "pharmaceutical compositions comprising 5-azacytidine . . . and carriers useful therein . . . including the carriers specified in instant claim 6," and that "the pharmaceutical compositions include the capability to rapidly dissolve in mildly acidic solutions." *Id.* The Office Action contends that Sands also teaches that "the pharmaceutical compositions may also include substances which can 'enhance the therapeutic efficacy' of the composition." *Id.* Based on these, the Office Action goes on to allege that "[i]t would have been obvious to a person of ordinary skill in the art at the time of the invention was made to add the instant claims' excipients or carriers to the pharmaceutical compositions of [Redkar] because [Redkar]

provides teachings which permits or suggests that such excipients are part of the disclosure therein," and that "the disclosures of [Sands] provide broad coverage for alternative excipients including the specific excipients claimed herein, including compounds like the compound TPGS disclosed in [Dintaman]." *Id.* The Office Action contends that "[o]ne having ordinary skill in the art would have been motivated to combine these references because all three references are directed to pharmaceutical compositions and the administration thereof to treat neoplastic diseases, including the treatment of MDS by the administration of 5-azacytidine as disclosed in the two cited U.S. patent publications." *Id.* Applicants respectfully disagree with these allegations and respectfully request reconsideration and withdrawal of the rejections.

Applicants respectfully submit that no *prima facie* case of obviousness is established by the Office Action because: (1) the Office Action fails to establish that at the time of the invention, one of ordinary skill in the art would have had a reason to combine the alleged teachings of the cited references to pursue the subject matter of the instant claims; and (2) one of ordinary skill in the art would not have had a reasonable expectation of success. *See, e.g., In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991); *accord PharmaStem Therapeutics, Inc. v. ViaCell, Inc.*, 491 F.3d 1342, 1364 (Fed. Cir. 2007) ("The burden falls on the patent challenger to show by clear and convincing evidence that a person of ordinary skill in the art would have had reason to attempt to make the composition or device, or carry out the claimed process, and would have had a reasonable expectation of success in doing so.") (internal quotation marks omitted). The unpredictable nature of the chemical arts must be considered in determining obviousness. *See, e.g., Abbott Laboratories v. Sandoz*, 544 F.3d 1341, 1352 (Fed. Cir. 2008).

"A patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art." *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 401 (2007). It is important to identify "a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does." *Id.* (emphasis added). When determining obviousness, a fact finder must "weigh the specific differences between the claimed invention—with all its limitations—and the prior art references, the so-called second Graham factor." *In re Ochiai*, 71 F.3d 1565, 1569 (Fed. Cir. 1995) (internal quotation marks omitted). The claimed "subject matter as a whole" is compared with the prior art. *Id.* (quoting 35 U.S.C. § 103).

Applicants respectfully submit that the rejection should be withdrawn because at the time of the invention, one skilled in the art would not have had any reason to combine the alleged teachings of the cited references to pursue the claimed subject matter. The present claims relate to, *inter alia*, immediate release oral formulations comprising a cytidine analog designed to effect substantial release of the compound in the stomach and methods of orally administering a cytidine analog to a subject in need thereof, wherein the cytidine analog is released substantially in the stomach following oral administration. At the time of the invention, oral delivery of 5-azacytidine or decitabine has proven difficult due to chemical instability, enzymatic instability, and/or poor permeability of the compound. *See, e.g.*, Specification, at paragraph 13; *see also*, Redkar, at paragraphs 9 and 155; Sands, at paragraphs 10–12. For example, 5-azacytidine and decitabine have been considered acid labile and unstable in the acidic gastric environment. *See, e.g.*, Sands, at paragraph 29. Certain attempts to develop oral dosage forms of 5-azacytidine or decitabine have involved enteric coating of the drug core to protect the active pharmaceutical ingredient (API) from what was understood and accepted to be therapeutically unacceptable hydrolysis in the stomach, such that the drug is targeted to be absorbed in specific regions of the lower gastrointestinal tract, such as the jejunum in the small intestine. *See, e.g.*, Sands, at paragraph 29. Thus, in view of the state of the art, a person of ordinary skill in the art would not have had a reason to pursue an oral formulation comprising 5-azacytidine or decitabine targeting substantial release of the drug in the stomach, which is a highly acidic environment, following oral administration. Indeed, none of the cited references, either alone or in combination, provides any reason to pursue the instantly claimed subject matter. For example, Redkar discloses salts of certain cytidine analogs and pharmaceutical compositions comprising the salts. However, Redkar does not teach or suggest an immediate release tablet or immediate release capsule comprising a cytidine analog which effects substantial release of the API in the stomach following oral administration or the methods of the instant claims. Indeed, paragraphs 175–182 of Redkar, cited in the Office Action, teach formulations in a liquid form that can be administered intravenously. *See, e.g.*, Redkar, at paragraph 176–177. Paragraphs 178–182 of Redkar describe conditions for intravenous infusion of formulations described herein. Moreover, Redkar discusses that 5-azacytidine and decitabine are known to be pH-sensitive. *See, e.g.*, Redkar, at paragraph 155. Thus, Redkar would not have provided a person of ordinary skill in the art any reason to pursue immediate release oral formulations of 5-azacytidine or decitabine or methods involving the release of 5-azacytidine or decitabine substantially in the stomach following oral administration. Furthermore, in view of the state

of the art at the time of the invention, those of skill in the art would not have combined Redkar with any of the cited references, to arrive at the instantly claimed subject matter.

Sands teaches oral formulations that release the drug in the upper regions of the small intestine, such as the jejunum. *See, e.g.*, Sands at page 3, paragraph 29; page 25, paragraph 305. The oral formulations disclosed in Sands are enteric-coated in order to bypass the acidic gastric environment and are designed to release the API in the small intestine. *See, e.g.*, Sands at page 3, paragraphs 30 and 34–38. In particular, Sands teaches that the enteric-coating material should selectively dissolve at a pH above about 5.2. *See, e.g.*, Sands at page 3, paragraph 34. Sands prefers that the pharmaceutical compositions do not substantially disintegrate in an acidic, aqueous medium at pH 1–3. *See, e.g.*, Sands at page 3, paragraph 37. It is well-known that the gastric environment is highly acidic (*e.g.*, pH of about 1 to 2). Thus, Sands in fact teaches away from the instant claims, because Sands emphasizes that the oral formulations should not disintegrate in the stomach.

For example, the specific formulations taught in the Example in Sands contain an enteric coating designed to release the drug in the small intestine. *See* Sands, at page 27, paragraph 324 (describing a decitabine formulation with an enteric coating that is "specific to jejunum," a region of the small intestine). Sands purportedly teaches formulations including an excipient that serves to maximize retention of the drug in the upper small intestine, thereby avoiding drug absorption anywhere other than the upper small intestine. *See* Sands at page 6, paragraph 89, lines 19-23 ("The inventive formulation may further include an excipient that serves to increase the retention time of the drug in the upper small intestine, thereby maximizing the absorption of the drug into this particular region of the GI tract.") (emphasis added). Accordingly, Sands would have directed one of ordinary skill to prepare oral formulations of 5-azacytidine or decitabine specifically targeting the small intestine, not the stomach.

Therefore, if anything, Sands teaches away from the claimed invention. Prior art that teaches away from a claimed invention is strong evidence of the nonobviousness of that invention. *See In re Geisler*, 116 F.3d 1465, 1469 (Fed. Cir. 1997) (noting that a showing "that the art in any material respect taught away from the claimed invention" can rebut an obviousness allegation) (internal quotation marks omitted); *see also* M.P.E.P. § 2141.02(VI) ("A prior art reference must be considered in its entirety, *i.e.*, as a whole, including portions that would lead away from the claimed invention."). Viewed properly as a whole, Sands destroys any reasonable expectation of success that a person of ordinary skill might otherwise have had in attempting to prepare the claimed invention. Considering the

explicit teaching in Sands to deliver cytidine analogs to the upper small intestine, one of ordinary skill in the art would have been directed away from pursuing any stomach-targeting oral formulations of 5-azacytidine or decitabine.

Moreover, Dintaman does not cure the defects of Redkar and Sands, because Dintaman would not have provided a person of ordinary skill with any reason to develop stomach-targeted formulations of cytidine analogs or the methods of the instant claims. Dintaman teaches that TPGS is an inhibitor of P-glycoprotein (PGP) and inhibition of PGP by TPGS in the intestine may enhance oral bioavailability of co-administered drugs. For example, in the abstract on page 1550, Dintaman states that "[t]hese results suggest that enhanced oral bioavailability of drugs co-administered with TPGS may, in part, be due to inhibition of P-glycoprotein in the intestine" (emphasis added). On page 1550 at the bottom of the right column, Dintaman teaches that "perhaps its effect on drug absorption is, in part, mediated by inhibition of active drug efflux in the intestine" (emphasis added). In the last paragraph on page 1555, Dintaman describes that "TPGS functioned as a bile substitute and solubilized the CsA through micelle formation, thus facilitating the absorption of the drug through the intestinal lumen," and "amphipathic TPGS may also be acting as an inhibitor of P-glycoprotein to enhance absorption and decrease transport back into the intestinal lumen" (emphasis added). Therefore, Dintaman does not teach or suggest any drug formulation targeting substantial release of the API in the stomach. Accordingly, Applicants respectfully submit that one skilled in the art would not have had a reason to pursue the invention of the instant claims in view of the cited references.

Further, one skilled in the art would not have had a reasonable expectation of successfully practicing the claimed invention. As understood by those skilled in the art at the time of the invention, oral delivery of cytidine analogs, such as 5-azacytidine and decitabine, has proven difficult due to chemical instability, enzymatic instability, and/or poor permeability of the compound. In view of the instability of 5-azacytidine and decitabine under acidic conditions, and considering the numerous possible formulations that can be explored, a skilled person would not have expected that an immediate release formulation which releases the API substantially in the stomach would provide significant systemic exposure after oral administration. Indeed, the present application discloses unexpected results, including the beneficial pharmacokinetic profiles achieved by immediate release formulations of 5-azacytidine of the instant application. (*See, e.g.*, Specification, at paragraphs 228–230, 233–237, and 242.) Such results would ***not*** have been reasonably expected by a person of ordinary skill in the art. Thus, at the time of the invention, there

would have been no reasonable expectation that the claimed subject matter would be practicable.

For at least the foregoing reasons, Applicants respectfully submit that the pending claims are not obvious over Redkar in view of Dintaman and Sands. Applicants respectfully submit that the Office has not established a *prima facie* case of obviousness, because (1) one of ordinary skill in the art would not have found a reason to pursue the subject matter as claimed in the present application; and (2) one skilled in the art would not have had any reasonable expectation of success. Thus, Applicants respectfully submit that the rejection under 35 U.S.C. §103 should be withdrawn.

CONCLUSION

For at least the foregoing reasons, Applicants respectfully submit that all of the pending claims are in allowable form, and thus, respectfully request that the rejections be withdrawn and the application proceed to allowance.

A one-month time extension fee of $150 and a fee of $180 for filing a Supplemental Information Disclosure Statement are believed due for this submission. The Director is hereby authorized to charge these fees and any other required fees to Jones Day Deposit Account No. 50-3013 (referencing CAM: 501872-999847).

Respectfully submitted,

Date December 1, 2011

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Electronic Filing

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Etter *et al.*	Confirmation No.:	5370
Serial No.:	12/466,213	Art Unit:	1623
Filed:	May 14, 2009	Examiner:	Lawrence E. Crane
For:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF	Attorney Docket No: (CAM:	9516-847-999 501872-999847)

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Commissioner for Patents
P.O. Box 1450
Washington, D.C. 22313-1450

Sir:

Pursuant to Applicants' duty of disclosure under 37 C.F.R. §§ 1.56 and 1.97, enclosed is a list of seventy-six (76) references for the Examiner's review and consideration. These references (A18–A57, B03–B09, and C42–C70) are listed on the enclosed form entitled "List of References Cited by Applicant." Copies of B03–B09 and C42–C70 are enclosed herewith.

Identification of the foregoing references is not to be construed as an admission of Applicants or Attorneys/Agents for Applicants that such references are available as "prior art" against the subject application. Applicants respectfully request that the Examiner review the references and make them of record by completing and returning the enclosed List of References.

A fee in the amount of $180.00 is believed due for the submission of this statement as it is submitted after the mailing of a first Office Action on the merits and before the mailing of any of a final action under 37 C.F.R. §1.113, a notice of allowance under 37 C.F.R. §1.311 or an action that otherwise closes prosecution in the application. The Director is authorized to charge this fee and any other required fees to Jones Day Deposit Account No. 50-3013 (referencing CAM: 501872-999847).

Respectfully submitted,

Date December 1, 2011

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LIST OF REFERENCES CITED BY APPLICANT

(Use several sheets if necessary)

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Filing Date	May 14, 2009
First Named Inventor	Jeffrey B. Etter
Art Unit	1623
Examiner Name	Lawrence E. Crane
Attorney Docket No.	9516-847-999

U.S. PATENT DOCUMENTS

*Examiner Initials	Cite No.	Document Number – Kind Code	Publication Date mm/dd/yyyy	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	A18	US 3,350,388	10/31/1967	Sorm *et al.*	
	A19	US 3,817,980	06/18/1974	Vorbruggen *et al.*	
	A20	US 3,891,623	06/24/1975	Vorbruggen *et al.*	
	A21	US 4,082,911	04/04/1978	Vorbruggen	
	A22	US 4,209,613	06/24/1980	Vorbruggen	
	A23	US 5,700,640	12/23/1997	Voss *et al.*	
	A24	US 6,642,206	11/04/2003	Ramasamy *et al.*	
	A25	US 6,887,855	05/03/2005	Ionescu *et al.*	
	A26	US 6,943,249	09/13/2005	Ionescu *et al.*	
	A27	US 7,038,038	05/02/2006	Ionescu *et al.*	
	A28	US 7,078,518	07/18/2006	Ionescu *et al.*	
	A29	US 7,189,740	03/13/2007	Zeldis	
	A30	US 7,192,781	03/20/2007	Luna *et al.*	
	A31	US 7,759,481	07/20/2010	Gevenda *et al.*	
	A32	US 7,642,247	01/05/2010	Daifuku *et al.*	
	A33	US 7,700,770	04/20/2010	Ionescu *et al.*	
	A34	US 7,772,199	08/20/2010	Ionescu *et al.*	
	A35	US 7,858,774	12/28/2010	Ionescu *et al.*	
	A36	US 8,058,424	11/15/2011	Ionescu *et al.*	
	A37	US 2004/0162263	08/19/2004	Sands *et al.*	
	A38	US 2006/0063735	03/23/2006	Redkar *et al.*	
	A39	US 2006/0069060	03/30/2006	Redkar *et al.*	
	A40	US 2006/0074046	04/06/2006	Redkar *et al.*	

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	A41	US 2006/0128654	06/15/2006	Tang *et al.*	
	A42	US 2007/0190022	08/16/2007	Bacopoulos *et al.*	
	A43	US 2008/0057086	03/06/2008	Etter *et al.*	
	A44	US 2008/0182806	07/31/2008	Pizzorno	
	A45	US 2009/0286752	11/19/2009	Etter *et al.*	
	A46	US 2010/0035354	02/11/2010	Bigatti *et al.*	
	A47	US 2010/0036112	02/11/2010	Henschke *et al.*	
	A48	US 2010/0062992	03/11/2010	Redkar *et al.*	
	A49	US 2010/0210833	08/19/2010	Jungmann *et al.*	
	A50	US 2010/0292180	11/18/2010	Ionescu *et al.*	
	A51	US 2010/0298253	11/25/2010	Ionescu *et al.*	
	A52	US 2010/0311683	12/09/2010	Beach *et al.*	
	A53	US 2011/0042247	02/24/2011	Kocherlakota *et al.*	
	A54	US 2011/0092694	04/21/2011	Ionescu *et al.*	
	A55	US 2011/0201800	08/18/2011	Cherukupally *et al.*	
	A56	US 2011/0245485	10/06/2011	De Ferra *et al.*	
	A57	13/273,127	10/13/11*	Ionescu *et al.*	

* Application filing date.

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	B03	CZ 114716	11/15/1964	Sorm *et al.*		T
	B04	CZ 116297	04/15/1965	Sorm *et al.*		T

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	B05	FR 2123632	09/15/1972	Ceskoslovenska Akademie Ved		T*
	B06	UK 1,227,691	04/07/1971	Ceskoslovenska Akademie Ved		
	B07	UK 1,227,692	04/07/1971	Ceskoslovenska Akademie Ved		
	B08	WO 2009/016617	02/05/2009	Chemagis Ltd.		
	B09	WO 2011/014541	02/03/2011	Eagle Pharmaceuticals, Inc.		

*Abstract only.

NON PATENT LITERATURE DOCUMENTS

*Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T
	C42	Beers *et al.* (eds.), Chapter 142, Section 11, *in* THE MERCK MANUAL OF DIAGNOSIS AND THERAPY, 18th Edition, pages 1114–16 (2006).	
	C43	Beisler *et al.*, "Chemistry of Antitumor Triazine Nucleosides. An Improved Synthesis of Dihydro-5-Azacytidine," *J. Carbohydrates Nucleosides Nucleotides*, 4(5): 281–99 (1977).	
	C44	Beisler, "Isolation, Characterization, and Properties of a Labile Hydrolysis Product of the Antitumor Nucleoside, 5-Azacytidine," *J. Med. Chem.*, 21(2): 204–08 (1978).	
	C45	Bergy *et al.*, "Microbiological Production of 5-Azacytidine II. Isolation and Chemical Structure," *Antimicrobial Agents and Chemotherapy*, 625–30 (1966).	
	C46	Chen *et al.*, "Highly Efficient Regioselective Synthesis of 5'-O-lauroyl-5-azacytidine Catalyzed by Candida Antarctica Lipase B," *Appl. Biochem. Biotechnol.*, 151: 21–28 (2008).	
	C47	Dintaman *et al.*, "Inhibition of P-Glycoprotein by D-α-Tocopheryl Polyethylene Glycol 1000 Succinate (TPGS)," *Pharmaceutical Research*, 16(10): 1550–1556 (1999).	
	C48	Garcia-Manero *et al.*, "A Pilot Pharmacokinetic Study of Oral Azacitidine," *Leukemia*, 22: 1680–84 (2008).	
	C49	Garcia-Manero *et al.*, "Phase 1 Study of Oral Azacitidine in Myelodysplastic Syndromes, Chronic Myelomonocytic Leukemia, and Acute Myeloid Leukemia," *J. Clin. Oncol.*, 29(18): 2521–27 (2011).	
	C50	Gaubert *et al.*, "Unnatural Enantiomers of 5-Azacytidine Analogues: Synthesis and Enzymatic Properties," *Nucleosides, Nucleotides & Nucleic Acids*, 20(4–7): 837–40 (2001).	

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	C51	Gut *et al.*, "Aza Analogs of Pyrimidine and Purine Bases of Nucleic Acids," *in* ADVANCES IN HETEROCYCLIC CHEMISTRY, Vol. 1, Katritzky ed., pages 189–251 (1963).	
	C52	Hanka *et al.*, "Microbiological Production of 5-Azacytidine I. Production and Biological Activity," *Antimicrobial Agents and Chemotherapy*, 619–24 (1966).	
	C53	Kritz *et al.*, "Pilot Study of 5-Azacytidine (5-AZA) and Carboplatin (CBDCA) in Patients with Relapsed/Refractory Leukemia," *American Journal of Hematology*, 51(2): 117–21 (1996).	
	C54	Niedballa *et al.*, "A General Synthesis of N-Glycosides. V. Synthesis of 5-Azacytidines," *J. Org. Chem.*, 39(25): 3672–74 (1974).	
	C55	Notice of Allowance dated September 20, 2011 in U.S. Patent Application No. 12/729,116.	
	C56	O'Neil *et al.* (eds.), THE MERCK INDEX, 13th Edition, page 154–55 (2001).	
	C57	O'Neil *et al.* (eds.), THE MERCK INDEX, 14th Edition, page 150 (2006).	
	C58	Office Action dated September 23, 2011 in U.S. Patent Application No. 12/787,214.	
	C59	Office Action dated November 28, 2011 in U.S. Patent Application No. 12/729,116.	
	C60	Piskala *et al.*, "Nucleic Acids Components and Their Analogues. LI. Synthesis of 1-Glycosyl Derivatives of 5-Azauracil and 5-Azacytosine," *Collect. Czech. Chem. Commun.*, 29: 2060–76 (1964).	
	C61	Piskala *et al.*, "Direct Synthesis of 5-Azapyrimidine Ribonucleosides," *Nucleic Acids Research*, Special Pub. No. 1: s17–20 (1975).	
	C62	Piskala *et al.*, "Direct Synthesis of a 5-Azapyrimidine Ribonucleoside by the Tri-methylsilyl Procedure," *Nucleic Acid Chem.*, 1: 435–41 (1978).	
	C63	Vogler *et al.*, "5-Azacytidine (NSC 102816): A New Drug for the Treatment of Myeloblastic Leukemia," *Blood*, 48(3): 331–37 (1976).	
	C64	Vorbruggen *et al.*, "Nucleoside Synthesis with Trimethylsilyl Triflate and Perchlorate as Catalysts," *Chem. Ber.*, 114: 1234–55 (1981).	
	C65	Vorbruggen *et al.*, "A New Simplified Nucleoside Synthesis," *Chem. Ber.*, 114: 1279–86 (1981).	
	C66	Vorbruggen *et al.*, *in* ORGANIC REACTIONS, Vol. 55, 100 (L.A. Paquette ed., John Wiley & Sons, New York, 2000).	
	C67	Winkley *et al.*, "Direct Glycosylation of 1,3,5-Triazinones. A New Approach to the Synthesis of the Nucleoside Antibiotic 5-Azacytidine (4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2-one) and Related Derivatives," *J. Org. Chem.*, 35(2): 491–95 (1970).	

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*Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T
	C68	Wittenburg *et al.*, "A New Synthesis of Nucleosides," *Zeitschrift fur Chemie*, 4: 303–04 (1964) (with English translation).	
	C69	Zaitseva *et al.*, "Convergent Syntheses and Cytostatic Properties of 2-Chloro-2'-Deoxy-2'-Fluoroadenosine and its N^7-Isomer," *Bioorg. & Med. Chem. Lett.*, 5(24): 2999–3002 (1995).	
	C70	Ziemba *et al.*, "Development of Oral Demethylating Agents for the Treatment of Myelodysplastic Syndromes," *American Association of Cancer Research*, 100th Annual Meeting, April 18–22, Abstract #3369 (2009).	

EXAMINER SIGNATURE	DATE CONSIDERED

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Electronic Patent Application Fee Transmittal

Application Number:	12466213
Filing Date:	14-May-2009
Title of Invention:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF
First Named Inventor/Applicant Name:	Jeffrey B. Etter
Filer:	Jihong Lou
Attorney Docket Number:	9516-847-999

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 1 month with $0 paid	1251	1	150	150

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**330**

Electronic Acknowledgement Receipt

EFS ID:	11525904
Application Number:	12466213
International Application Number:	
Confirmation Number:	5370
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First Named Inventor/Applicant Name:	Jeffrey B. Etter
Customer Number:	84802
Filer:	Jihong Lou/Eric Baclig
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Deposit Account	503013
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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

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Multipart Description/PDF files in .zip description

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Specification	2	3
Abstract	4	4
Claims	5	11
Applicant Arguments/Remarks Made in an Amendment	12	24
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Office of Patents and Inventions

PATENT SPECIFICATION 114716 a

The right to exploit the invention belongs to the state according to § 3 par. 6 of Statute No. 34/1957

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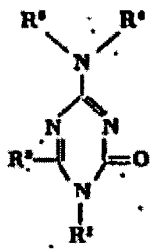
Published 15 May 1965

Patent Classification: 12 p, 10/05
IPC: C 07 d
Decimal Classification: 547.854.6/8:66

Academician FRANTIŠEK ŠORM and ALOIS PISKALA, ind. chem., cand. of sciences, both of PRAGUE

Method of preparation of 1-glycosyl-5-azacytosines

The object of the invention is a method of preparation of 1-glycosyl-5-azacytosines of the general formula:



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in which R^1 signifies glycosyl or peracylglycosyl, R^2 is hydrogen or alkyl with 1-4 carbon atoms, R^3 and R^4, the same or different, signify atoms of hydrogen or alkyls with 1-4 carbon atoms or aralkyls, such as benzyl, possibly substituted in the benzene ring.

It is known that substances belonging to the group of so-called antimetabolites are effective chemotherapy agents, suppressing the growth of malignant cancer tissue or preventing the development of viral infections, and important among these are, e.g., 6-mercaptopurine, 5-fluorouracil, 6-azauridine, 5-iododesoxyuridine, and hydroxybenzylbenzimidazole.

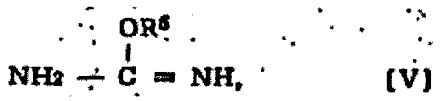
Our research has found that especially penetrating cancerostatic and virological action is possessed by 1-glycosyl-5-azacytosines, which have thus far not been described in the literature. At the same time, a method of rational preparation of these substances

has been worked out. In particular, 5-azacytidine (1-β-D-ribofuranosyl-5-azacytosine) is a substance which intervenes very effectively in the metabolism of nucleic acids of fast growing biological systems.

The present invention of a method of preparation of 1-glycosyl-5-azacytosines is characterized in that one acts on esters of glycosylisocyanates of the general formula:

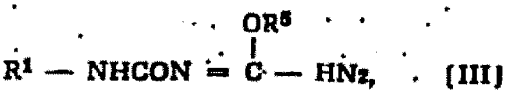
$$R^1 - N = C = O, \qquad (II)$$

where R^1 signifies peracylglycosyl, with O-alkylisoureas of the general formula:



(V)

where R^5 signifies alkyl with 1-4 carbon atoms. This produces esters of 1-glycosyl-4-alkylisobiurets of the general formula:

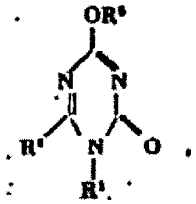


(III)

where R^1 signifies the same as in formula II and R^5 the same as in formula V. Said esters are then condensed with orthoesters of aliphatic acids of the general formula:

$$R^2 - C(OR^6)_3, \qquad (VI)$$

where R^2 signifies the same as in formula I and R^6 is methyl or ethyl, into esters of 1-glycosyl-4-alkoxy-2-oxo-1,2-dihydro-1,3,5-triazines of the general formula:



(IV)

where R^1 signifies the same as in formula II, R^2 the same as in formula I, and R^5 the same as in formula V.

The products so obtained are caused to react with a compound of the general formula:

$$R^3 - NH - R^4, \qquad (VII)$$

where R^3 and R^4 have the same meaning as in formula I, and the resulting esters of the 1-glycosyl derivates of 5-azacytosines are subjected to alcoholysis, preferably by the action of methanol in the presence of sodium methylate.

Moreover, according to the invention, it is also possible to convert the esters of the 1-glycosyl derivates of 5-azacytosine into corresponding 1-glycosyl-5-azacytosines by ammonolysis, preferably by the action of ammonia in ethanol.

Another characteristic of the invention is that the reaction of the esters of glycosylisocyanate of the general formula II with the O-alkylisoureas of the general formula V is conducted in equimolar proportions while cold in an inert solvent, preferably in chloroform.

According to the invention, the condensation of the isobiurets of the general formula III with the orthoesters of aliphatic acids of the general formula VI is done at elevated temperature, but lower than the boiling point of the orthoester used, in a stream of anhydrous inert gas.

Finally, the invention is characterized in that the reaction of the alkoxytriazines of the general formula IV with the compound of the general formula VII is carried out at room temperature in the presence of an alkanol with 1-6 carbon atoms.

The configuration of the glycoside center does not change during the synthesis and it is the same in the end product as in the starting compound of the general formula II.

Depending on the nature of the groups protecting the hydroxyls, replacing the residue $-OR^5$ (IV) with the group $-N(R^3)(R^4)$ (I) results in their simultaneous cleavage. Of course, these groups can also be removed in the end product by any of the methods known and used.

The examples given will serve as illustration, but in no way limit the procedure used.

Example 1

To a solution of 3.01 g of crude 2,3,5-tri-O-acetyl-β-D-ribofuranosylisocyanate in 50 ml of anhydrous chloroform, under cooling and mixing one adds, while excluding external moisture, a solution of 0.65 g of 2-methylisourea in 10 ml of anhydrous chloroform. The solution is left for 1 hour at laboratory temperature, extracted twice with 20 ml [sic] and after desiccation with anhydrous sodium sulfate evaporated in vacuum to a thick syrup, which is dissolved in 10 ml of benzene and precipitated with 50 ml of petroleum ether. One obtains 3.0 g, i.e., 80%, of vitreous 1-[2',3',5'-tri-O-acetyl-β-D-ribofuranosyl]-4-methylisobiuret,

$\lambda_{max}^{C_2H_5OH} = 221\ m\mu$ (log ε 4.25).

3.75 g of 1-[2',3',5'-tri-O-acetyl-β-D-ribofuranosyl]-4-methylisobiuret are dissolved while hot in 30 ml of ethyl orthoformate and the solution is heated for 10 h to 135-140° C in a slow current of anhydrous nitrogen. After cooling, the solution is precipitated with 75 ml of petroleum ether and the separated syrup is dissolved in 10 ml of anhydrous benzene and again precipitated with 50 ml of petroleum ether. After decanting and drying in a vacuum, one obtains vitreous 1-[2',3',5'-tri-O-acetyl-β-D-ribofuranosyl]-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine in a yield of 2.0 g, i.e., 52%

$\lambda_{max}^{CH_3CN} = 253\ m\mu$ (log ε 3.36).

3.85 g of the crude 1-[2',3',5'-tri-O-acetyl-β-D-ribofuranosyl]-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine is dissolved in 30 ml of a methanol solution of ammonia saturated at 0° C and left at room temperature in a sealed tube for a period of 12 h. The separated crystalline 1-β-D-ribofuranosyl-5-azacytosine (5-azacytosine) melts at 227-230° C (decomposition), yield 1.7 g, i.e., 70%.

Example 2

To a solution of 3.01 g of crude 2,3,4-tri-O-acetyl-β-D-ribopyranosylisocyanate in 50 ml of anhydrous chloroform, under cooling and mixing one adds, while excluding external moisture, a solution of 0.70 g of 2-methylisourea in 10 ml of anhydrous chloroform. The solution is then left for 1 hour at room temperature, extracted twice with 20 ml of water and after desiccation with anhydrous sodium sulfate, it is evaporated in vacuum to a thick syrup, which is dissolved in 10 ml of benzene and precipitated with 50 ml of petroleum ether and the mixture is left overnight in a refrigerator at -15° C. The crystalline 1-[2',3',4'-tri-O-acetyl-β-D-ribopyranosyl]-4-methylisobiuret is then aspirated and washed with ether. One obtains 3.2 g, i.e., 85%, of product with melting point of 189-192° C,

$\lambda_{max}^{C_2H_5OH} = 221\ m\mu$ (log ε 4.28).

3.75 g of 1-[2',3',4'-tri-O-acetyl-β-D-ribopyranosyl]-4-methylisobiuret are dissolved while hot in 50 ml of ethyl orthoformate and the solution is heated for 10 h to 135-140° C in a slow current of anhydrous nitrogen. After cooling, the solution is precipitated with 200 ml of petroleum ether. The mixture is left overnight in a refrigerator and after decanting the substance is mixed with 30 ml of hot benzene and the mixture is left overnight at room temperature. The separated crystals are aspirated and washed with benzene. One obtains 1.6 g, i.e., 40%, of 1-[2',3',4'-tri-O-acetyl-β-D-ribopyranosyl]-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine with melting point of 196-198° C (from ethanol),

$\lambda_{max}^{CH_3CN} = 253\ m\mu$ (log ε 3.39).

3.85 g of 1-[2',3',4'-tri-O-acetyl-β-D-ribopyranosyl]-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine is dissolved in 60 ml of an ethanol solution of ammonia saturated at 0° C and the solution is left for 2½ h at room temperature in a sealed tube. After evaporation in a vacuum, the residue is triturated with 100 ml of absolute ether and after this is poured off the product is mixed with 200 ml of absolute ethanol and evaporated in a vacuum. This procedure is repeated again and the residue is dissolved in 50 ml of absolute methanol. By letting the solution stand until the next day, crystalline 1-β-D-ribopyranosyl-5-azacytosine separates with melting point of 250-252° C (decomposition), in a yield of 1.48 g, i.e., 61%.

Example 3

To a solution of 3.75 g of 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylisocyanate in 50 ml of anhydrous chloroform, under cooling and mixing one adds, while excluding external moisture, a solution of 0.70 g of 2-methylisourea in 10 ml of anhydrous chloroform. The solution is then left for 1 hour at room temperature, extracted twice with 20 ml of water and after desiccation with anhydrous sodium sulfate it is evaporated in vacuum to a thick syrup, which is dissolved in 20 ml of anhydrous benzene and precipitated with 60 ml of petroleum ether. After decanting and desiccation in a vacuum, one obtains vitreous 1-[2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl]-4-methylisobiuret in a yield of 3.8 g, i.e., 85%,

$\lambda_{max}^{C_2H_5OH} = 221\ m\mu$ (log ε 4.26).

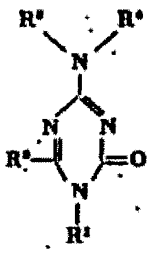
4.47 g of 1-[2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl]-4-methylisobiuret are dissolved while hot in 50 ml of ethyl orthoformate and the solution is heated for 10 h to 135-140° C in a slow current of anhydrous nitrogen. After cooling, crystalline 1-[2',3',4',6'-tetra-O-acetyl-ß-D-glucopyranosyl]-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine is separated from the solution. The mixture is left overnight in a refrigerator and the substance is aspirated and washed with ether. One obtains 1.97 g, i.e., 43% of product with melting point of 232-235° C (from ethanol),

$\lambda_{max}^{CH_3CN} = 254\ m\mu$ (log ε 3.40).

4.57 g of 1-[2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl]-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine is dissolved in 80 ml of a methanol solution of ammonia saturated at 0° C and the solution is left at room temperature in a sealed tube for 1½ h. After evaporation in a vacuum, the residue is triturated with 100 ml of absolute ether and after this is poured off, the product is mixed with 80 ml of absolute methanol and left to crystallize freely until the next day. One obtains 1.65 g, i.e., 60%, of 1-ß-D-glucopyranosyl-5-azacytosine with melting point of 259-260° C (decomposition).

CLAIMS

1. Method of preparation of 1-glycosyl-5-azacytosines of the general formula:



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in which R^1 signifies glycosyl or peracylglycosyl, R^2 is hydrogen or alkyl with 1-4 carbon atoms, R^3 and R^4, the same or different, signify atoms of hydrogen or alkyls with 1-4 carbon atoms or aralkyls, such as benzyl, possibly substituted in the benzene ring, characterized in that one acts on esters of glycosylisocyanates of the general formula:

$$R^1 - N = C = O, \qquad II$$

where R^1 signifies peracylglycosyl, with O-alkylisoureas of the general formula:

$$NH_2 - C(OR^5) = NH \qquad V$$

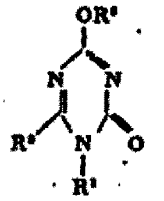
where R^5 signifies alkyl with 1-4 carbon atoms, and the resulting esters of 1-glycosyl-4-alkylisobiurets of the general formula:

$$R^1 - NHCON = C(OR^5) - NH_2 \qquad III$$

where R^1 signifies peracylglycosyl and R^5 the same as in formula V, are condensed with orthoesters of aliphatic acids of the general formula:

$$R^2 - C(OR^6)_3 \qquad VI$$

where R^2 signifies the same as in formula I and R^6 is methyl or ethyl, into esters of 1-glycosyl-4-alkoxy-2-oxo-1,2-dihydro-1,3,5-triazines of the general formula:



IV

where R^1 and R^5 signify the same as in formula III, R^2 the same as in formula I, which are caused to react with a compound of the general formula:

$$R^3 - NH - R^4 \qquad VII$$

Where R^3 and R^4 have the same meaning as in formula I, and the resulting esters of the 1-glycosyl derivates of 5-azacytosines are subjected to alcoholysis, preferably by the action of methanol in the presence of sodium methylate.

2. Method of preparation according to point 1, characterized in that the resulting esters of the 1-glycosyl derivates of 5-azacytosine are subjected to ammonolysis, preferably by ammonia in ethanol.

3. Method of preparation according to claim 1, characterized in that the reaction of the esters of glycosylisocyanate of the general formula II with the O-alkylisoureas of the general formula V is conducted in equimolar proportions while cold in an inert solvent, preferably in chloroform.

4. Method of preparation according to claim 1, characterized in that the condensation of the isobiurets of the general formula III with the orthoesters of aliphatic acids of the general formula VI is done at elevated temperature, but lower than the boiling point of the orthoester used, in a stream of anhydrous inert gas.

5. Method of preparation according to claim 1, characterized in that the reaction of the alkoxytriazines of the general formula IV with the compound of the general formula VII is carried out at room temperature in the presence of an alkanol with 1-6 carbon atoms.

ČESKOSLOVENSKÁ SOCIALISTICKÁ REPUBLIKA

ÚŘAD PRO PATENTY A VYNÁLEZY

PATENTOVÝ SPIS 114716 a

Právo k využití vynálezu přísluší státu podle § 3 odst. 6 zák. č. 34/1957 Sb.

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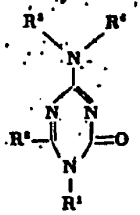
Způsob přípravy 1-glykosyl-5-azacytosinů

1

Předmětem vynálezu je způsob přípravy 1-glykosyl-5-azacytosinů obecného vzorce:



I

ve kterém značí R^1 glykosyl nebo peracylglykosyl, R^2 vodík nebo alkyl s 1—4 atomy uhlíku, R^3 a R^4, stejné nebo různé, značí atomy vodíku nebo alkyly s 1—4 atomy uhlíku nebo aralkyly, např. benzyl, popř. substituovaný v benzenovém jádře.

Je známo, že jako účinná chemoteraupeutika, potlačující růst zhoubné nakovinné tkáně nebo zabraňující rozvoji virové infekce, se jeví látky patřící do skupiny tzv. antimetabolitů, z nichž významné jsou např. 6-merkaptopurin, 5-fluorouracil, 6-azauridin, 5-jododesoxyuridin a hydroxybenzylbenzimidazol.

Naším výzkumem bylo zjištěno, že zvláště pronikavé kancerostatické a virologické účinky mají 1-glykosyl-5-azacytosiny, které dosud nebyly v literatuře popsány. Současně

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byl vypracován způsob racionální přípravy těchto látek. Látkou, která velmi účinně zasahuje do metabolismu nukleových kyselin rychle rostoucích biologických systémů, zejména se jeví 5-azacytidin (1-β-D-ribofuranosyl-5-azacytosin).

Předmětný vynález přípravy 1-glykosyl-5-azacytosinů je vyznačen tím, že na estery glykosylisokyanatů obecného vzorce:

$$R^1 - N = C = O, \qquad (II)$$

ve kterém R^1 značí peracylglykosyl, se působí O-alkylisomočovinami obecného vzorce:

$$NH_2 - \overset{\displaystyle OR^5}{\overset{|}{C}} = NH, \qquad (V)$$

v němž R^5 značí alkyl s 1—4 atomy uhlíku. Tím vznikají estery 1-glykosyl-4-alkylisobluretů obecného vzorce:

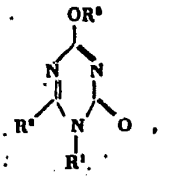
$$R^1 - NHCON = \overset{\displaystyle OR^5}{\overset{|}{C}} - HN_2, \qquad (III)$$

v němž R^1 značí totéž co ve vzorci II a R^5 totéž co ve vzorci V. Zmíněné estery se poté kondensují s ortoestery alifatických kyselin obecného vzorce:

$$R^2 - C(OR^6)_3, \qquad (VI)$$

v němž R^2 značí totéž co ve vzorci I a R^6

methyl neb ethyl, na estery 1-glykosyl-4-alkoxy-2-oxo-1,2-dihydro-1,3,5-triazinů obecného vzorce:



(IV)

v němž R^1 značí totéž co ve vzorci II, R^2 totéž co ve vzorci I a R^5 totéž co ve vzorci V.

Takto získané produkty se uvádějí do reakce se sloučeninou obecného vzorce:

$$R^3 — NH — R^4, \qquad (VII)$$

v němž R^3 a R^4 značí totéž co ve vzorci I a vzniklé estery 1-glykosyl-derivátů 5-azacytosinů se podrobí alkoholyse, s výhodou působením methanolu v přítomnosti methylátu sodného.

Podle vynálezu je dále možno převést estery 1-glykosylderivátů-5-azacytosinu na příslušné 1-glykosyl-5-azacytosiny i ammonolysou, s výhodou působením amoniaku v ethanolu.

Dalším význakem vynálezu je, že reakce esterů glykosylisokyanátu obecného vzorce II s O-alkylisomočovinami obecného vzorce V se provádí v ekvimolárních poměrech za chladu v inertním rozpouštědle, s výhodou ve chloroformu.

Podle vynálezu se kondensace isobiuretů obecného vzorce III s ortoestery alifatických kyselin obecného vzorce VI provádí za zvýšené teploty, avšak nižší, než je bod varu použitého ortoesteru, v proudu suchého inertního plynu.

Konečně význakem vynálezu je, že reakce alkoxytriazinů obecného vzorce IV se sloučeninou obecného vzorce VII se provádí při teplotě místnosti v prostředí alkanolu s 1—6 atomy uhlíku.

Konfigurace glykosydického centra se během sythesy nemění a je v konečném produktu stejná jako ve výchozí sloučenině obecného vzorce II.

Podle povahy skupin chránících hydroxyly dochází při záměně zbytku —OR^5 (IV) za skupinu —$N(R^3)(R^4)$ (I) k jejich současnému odštěpení. Samozřejmě lze tyto skupiny v konečném produktu odstranit též některou ze známých a používaných metod.

Uvedené příklady slouží k ilustraci, nikoliv však k vymezení použitého postupu.

Příklad 1

K roztoku 3,01 g surového 2,3,5-tri-O-acetyl-β-D-ribofuranosylisokyanátu v 50 ml suchého chloroformu se za chlazení a míchání přidá, za vyloučení vnější vlhkosti, roztok 0,65 g 2-methylisomočoviny v 10 ml suchého chloroformu. Roztok se ponechá 1 hodinu za laboratorní teploty, vytřepe dvakrát 20 ml a po vysušení bezvodým síranem sodným odpaří ve vakuu na hustý sirup, který se rozpustí v 10 ml benzenu a vysráží 50 ml petroletheru. Získá se 3,0 g, tj. 80 %, sklovitého 1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-methylisobiuretu,

$\lambda_{max}^{C_2H_5OH}$ = 221 mμ (log ε 4,25).

3,75 g 1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-methylisobiuretu se rozpustí za horka ve 30 ml ortomravenčanu ethylnatého a roztok se zahřívá 10 hodin na 135 až 140° C v pomalém proudu suchého dusíku. Po ochlazení se roztok vysráží 75 ml petroletheru a vyloučený sirup se rozpustí v 10 ml suchého benzenu a znovu vysráží 50 ml petroletheru. Po dekantaci a vysušení ve vakuu se získá sklovitý 1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazin ve výtěžku 2,0 g, tj. 52 % $\lambda_{max}^{CH_3CN}$ = 253 mμ (log ε 3,36).

3,85 g surového 1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazinu se rozpustí ve 30 ml methanolického roztoku amoniaku nasyceného při 0° C a ponechá při teplotě místnosti v zatavené trubici po dobu 12 hodin. Vyloučený krystalický 1-β-D-ribofuranosyl-5-azacytosin (5-azacytidin) taje při 227—230 °C (rozklad), výtěžek 1,7 g, tj. 70 %.

Příklad 2

K roztoku 3,01 g surového 2,3,4-tri-O-acetyl-β-D-ribopyranosylisokyanátu v 50 ml suchého chloroformu se za chlazení a míchání přidá za vyloučení vnější vlhkosti roztok 0,70 g, 2-methylisomočoviny v 10 ml suchého chloroformu. Roztok se pak ponechá 1 hodinu při teplotě místnosti, vytřepe dvakrát 20 ml vody a po vysušení bezvodým síranem sodným odpaří ve vakuu na hustý sirup, který se rozpustí v 10 ml suchého benzenu a vysráží 50 ml petroletheru a směs se ponechá přes noc v chladicím pultu při —15° C. Pak se krystalický 1-(2',3',4'-tri-O-acetyl-β-D-ribopyranosyl)-4-methylisobiuret odsaje a promyje etherem. Získá se 3,2 g, tj. 85 % produktu o bodu tání 189 až 192° C, $\lambda_{max}^{C_2H_5OH}$ = 221 mμ (log ε 4,28).

3,75 g 1-(2',3',4'-tri-O-acetyl-β-D-ribopyranosyl)-4-methylisobiuretu se rozpustí za horka v 50 ml ortomravenčanu ethylnatého a roztok se zahřívá 10 hodin na 135—140° C v pomalém proudu suchého dusíku. Po ochlazení se roztok vysráží 200 ml petroletheru. Směs se ponechá přes noc v chladicím pultu a po dekantaci se látka rozmíchá se 30 ml horkého benzenu a směs se ponechá přes noc při teplotě místnosti. Vyloučené krystaly se odsají a promyjí benzenem. Získá se 1,6 g, tj. 40 % 1-(2',3',4'-tri-O-acetyl-β-D-ribopyranosyl)-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazinu o bodu tání 198

až 198° C (z ethanolu), $\lambda_{max}^{CH_3CN}$ = 253 mμ (log ε 3,39).

3,85 g 1-(2',3',4'-tri-O-acetyl-β-D-ribopyranosyl)-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazinu se rozpustí v 60 ml ethanolického roztoku amoniaku nasyceného při 0° C a roztok se ponechá 2½ hodiny při teplotě místnosti v zatavené trubici. Po odpaření ve vakuu se zbytek rozetře se 100 ml absolutního etheru a po jeho odlití se produkt rozmíchá s 200 ml absolutního ethanolu a odpaří se ve vakuu. Tento postup se znovu opakuje a zbytek se rozpustí v 50 ml absolutního methanolu. Stáním roztoku do příštího dne se vyloučí krystalický 1-β-D-ribopyranosyl-5-azacytosin o bodu tání 250 až 252° C (rozklad) ve výtěžku 1,48 g, tj. 61 %.

Příklad 3

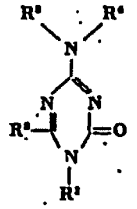
K roztoku 3,75 g 2,3,4,6-tetra-O-acetyl-β-D-glukopyranosylisokyanátu v 50 ml suchého chloroformu se za chlazení a míchání přidá za vyloučení vlhkosti roztok 0,70 g 2-methylisomočoviny v 10 ml suchého chloroformu. Roztok se pak ponechá 1 hodinu při teplotě místnosti, vytřepe dvakrát 20 ml vody a po vysušení bezvodým síranem sodným odpaří ve vakuu na hustý sirup, který se rozpustí ve 20 ml suchého benzenu a vysráží 60 ml petroletheru. Po dekantaci a vysušení ve vakuu se získá sklovitý 1-(2',3',4',6'-tetra-O-acetyl-β-D-glukopyranosyl)-4-methylisobiuret ve výtěžku 3,8 g, tj. 85 %, $\lambda_{max}^{C_2H_5OH}$ = 221 mμ (log ε 4,26).

4,47 g 1-(2',3',4',6'-tetra-O-acetyl-β-D-glukopyranosyl)-4-methylisobiuretu se rozpustí za horka v 50 ml ortomravenčanu ethylnatého a roztok se zahřívá 10 hodin na 135 až 140° C v pomalém proudu suchého dusíku. Po ochlazení se z roztoku vyločuje krystalický 1-(2',3',4',6'-tetra-O-acetyl-β-D-glukopyranosyl)-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazin. Směs se ponechá přes noc v chladicím pultu a látka se odsaje a promyje etherem. Získá se 1,97 g, tj. 43 % produktu o bodu tání 232—235° C (z ethanolu), $\lambda_{max}^{CH_3CN}$ = 254 mμ (log ε 3,40).

4,57 g 1-(2',3',4',6'-tetra-O-acetyl-β-D-glukopyranosyl)-4-metoxy-2-oxo-1,2-dihydro-1,3,5-triazinu se rozpustí v 60 ml metanolického roztoku amoniaku nasyceného při 0° C a roztok se ponechá 1½ hodiny při teplotě místnosti v zatavené trubici. Po odpaření ve vakuu se zbytek rozetře se 100 ml absolutního eteru a po jeho odlití se produkt rozmíchá s 80 ml absolutního metanolu a ponechá volné krystalisaci do příštího dne. Získá se 1,65 g, tj. 60 % 1-β-D-glukopyranosyl-5-azacytosinu o bodu tání 259—260° C (rozklad).

PŘEDMĚT PATENTU

1. Způsob přípravy 1-glykosyl-5-azacytosinů obecného vzorce



I

v němž R^1 značí glykosyl nebo peracylglykosyl, R^2 atom vodíku nebo alkyl s 1—4 atomy uhlíku, R^3 a R^4 stejné nebo různé atomy vodíku nebo alkyly s 1—4 atomy uhlíku nebo aralkyly například benzyl popř. substituovaný v benzenovém jádře, vyznačený tím, že se na estery glykosylisokyanátů obecného vzorce

$$R^1 - N = C = O, \qquad II$$

ve kterém R^1 značí peracylglykosyl, se působí O-alkylisomočovinami obecného vzorce

$$NH_2 - \overset{OR^5}{\overset{|}{C}} = NH \qquad V$$

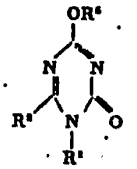
v němž R^5 značí alkyl s 1—4 atomy uhlíku, a vzniklé estery 1-glykosyl-4-alkylisobiuretů obecného vzorce

$$R^1 - NHCON = \overset{OR^5}{\overset{|}{C}} - NH_2 \qquad III$$

v němž R^1 značí peracylglykosyl a R^5 totéž co ve vzorci V, se kondensují s orthoestery alifatických kyselin obecného vzorce

$$R^2 - C(OR^6)_3 \qquad VI$$

v němž R^2 značí totéž co ve vzorci I a R^6 methyl nebo ethyl, na estery 1-glykosyl-4-alkoxy-2-oxo-1,2-dihydro-1,3,5-triazinů obecného vzorce



IV

v němž R^1 a R^5 značí totéž co ve vzorci III, R^2 totéž co ve vzorci I, které se uvedou do reakce se sloučeninou obecného vzorce

$$R^3 - NH - R^4 \qquad VII$$

v němž R^3 a R^4 značí totéž co ve vzorci I a vzniklé estery 1-glykosyl-derivátů 5-azacytosinů se podrobí alkoholyse, s výhodou působením methanolu v přítomnosti methylátu sodného.

2. Způsob přípravy podle bodu 1 vyznačený tím, že vzniklé estery 1-glykosyl-derivátů 5-azacytosinů se podrobí amonolyse s výhodou amoniakem v ethanolu.

3. Způsob přípravy podle bodu 1, vyznačený tím, že reakce esterů glykosylisokyanátu obecného vzorce II s O-alkylisomočovinami obecného vzorce V se provádí v ekvimolárních poměrech za chladu v inertním rozpouštědle, s výhodou ve chloroformu.

4. Způsob přípravy podle bodu 1 vyznačený tím, že kondensace isobiuretů obecného vzorce III, s orthoestery alifatických kyselin obecného vzorce VI se provádí za zvýšené teploty, avšak nižší, než je bod varu použitého orthoesteru, v proudu suchého inertního plynu.

5. Způsob přípravy podle bodu 1 vyznačený tím, že reakce alkoxytriazinů obecného vzorce IV se sloučeninou obecného vzorce VII se provádí při teplotě místnosti v prostředí alkanolu s 1—6 atomy uhlíku.

severografia, n. p., provozovna 32, Most

Czechoslovakian Socialist Republic
Office of Patents and Inventions

PATENT SPECIFICATION 116297 a

The right to exploit the invention belongs to the state according to § 3 par. 6 of Statute No. 34/1957

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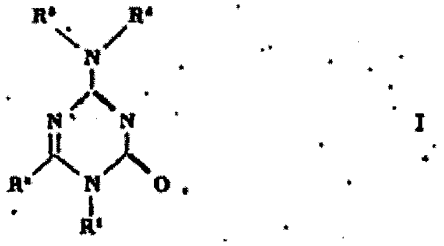
12 p, 10
IPC: C 07 d
Decimal Classification: 547.85:66

Academician FRANTIŠEK ŠORM and ALOIS PISKALA, ind. chem., both of PRAGUE

Method of preparation of 1-glycosyl-5-azacytosines

The object of the invention is a method of preparation of 1-glycosyl-5-azacytosines of the general formula:

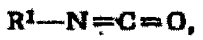


where R^1 represents glycosyl or peracylglycosyl, R^2 signifies hydrogen or alkyl with 1-4 carbon atoms, R^3 and R^4 (which can be the same or different) signify atoms of hydrogen or alkyls with 1-4 carbon atoms, possibly aralkyls, such as benzyl, which can be substituted in the benzene ring.

The method thus far known and used for preparation of 1-glycosyl-5-azacytosines starts with peracylglycosylisocyanates, which after addition of 2-methylisourea and after condensation of the resulting isobiurets with acid orthoesters are converted into the corresponding methoxytriazines, which then provide the corresponding glycosyl derivates with ammonia, or with primary or secondary amines. In the course of further research it has been found that in certain instances it is advantageous to increase the yield of the end product, i.e., 1-glycosyl-5-azacytosines, in that the 2-methylisourea is replaced during the production by suitable substituted derivates of carbonic acid with

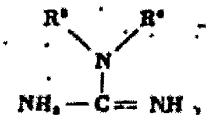
combinations of a sulfhydryl group and an amino group, or by substituted derivates of carbonic acid with amino groups.

The object of the proposed invention is a method of preparation of 1-glycosyl-5-azacytosines, wherein one acts on acyl glycosylisocyanates of the general formula:



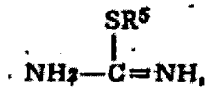
II

where R^1 signifies peracylglycosyl, with guanidine, possibly substituted guanidine of the general formula:



III

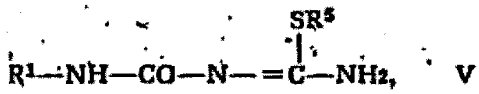
where R^3 and R^4 have the same meaning as in formula 1, or one acts on it with S-alkylisothioureas of the general formula:



IV

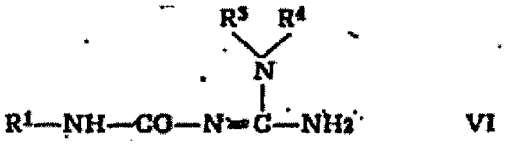
where R^5 signifies alkyl with 1-4 carbon atoms.

This produces 1-acylglycosyl-4-alkyl-4-isothiobiurets of the general formula:



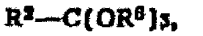
V

where R^1 signifies the same as in formula II and R^5 the same as in formula II, or acylglycosylcarbamylguanidines of the general formula:



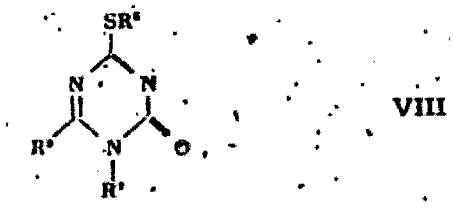
VI

where R^1 signifies the same as in formula II and R^3 and R^4 the same as in formula I. Said substances of formula V and VI are then condensed with orthoesters of aliphatic acids of the general formula:



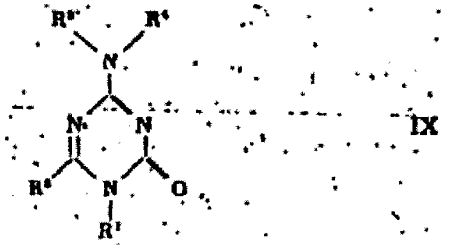
VII

where R^2 signifies the same as in formula I and R^6 is methyl or ethyl, into 1-acylglycosyl-4-alkylmercapto-2-oxo-1,2-dihydro-1,3,5-triazines of the general formula:



VIII

where R^1 signifies the same as in formula II, R^2 the same as in formula I, and R^5 the same as in formula III, or into 1-acylglycosyl-4-amino-2-oxo-1,2-dihydro-1,3,5-triazines, possibly 4-alkylamino or 4-dialkylamino derivates of the general formula:



IX

where R^1 signifies the same as in formula II, R^2, R^3 and R^4 the same as in formula I.

The substances of the general formula VIII are caused to react with a compound of the general formula:

$$R^3—NH—R^4 \qquad X$$

where R^3 and R^4 have the same meaning as in formula I, and the resulting 1-acylglycosyl derivates are subjected to alcoholysis, preferably by the action of methanol in presence of sodium methylate.

Moreover, according to the invention, it is also possible to convert the 1-acylglycosyl-5-azacytosines of the general formula IX into corresponding 1-glycosyl-5-azacytosines by ammonolysis, preferably by the action of ammonia in methanol.

Another characteristic of the invention is that the reaction of the acylglycosylisocyanates of the general formula II with the S-alkylisoureas of the general formula III or with guanidines of the general formula IV is conducted in equimolar proportions while cold in an inert solvent, preferably in chloroform.

According to the invention, the condensation of the isobiurets of the general formula V or the carbamylguanidines of the general formula VI with the orthoesters of aliphatic acids of the general formula VII is done at elevated temperature, but lower than the boiling point of the orthoester used, in a stream of anhydrous inert gas, if need by brief simmering in the presence of acetanhydride. Finally, the invention is characterized in that the reaction of the alkylmercaptotriazines of the general formula VIII with the compound of the general formula X is carried out at room temperature in the presence of an alkanol with 1-6 carbon atoms.

The configuration of the glycoside center does not change during the synthesis and it is the same in the end product as in the starting compound of the general formula II.

Depending on the nature of the groups protecting the hydroxyls, replacing the residue $-SR^5$ (VIII) with the group $-N(R^3)(R^4)$ results in their simultaneous cleavage. Of course, these groups can also be removed in the end product by any of the methods known and used.

The examples given will serve as illustration, but in no way limit the procedure used.

Examples

Example 1

To a solution of 3.01 g of crude 2,3,5-tri-O-acetyl-β-D-ribofuranosylisocyanate in 50 ml of anhydrous chloroform, under cooling and mixing one adds, while excluding external moisture, a solution of 0.81 g of S-methylisothiourea in 10 ml of anhydrous chloroform. The solution is then left for 1 hour at laboratory temperature, evaporated in vacuum to a thick syrup, dissolved in 10 ml of anhydrous benzene and precipitated with 50 ml of petroleum ether. The precipitation is repeated again. One obtains 3.2 g, i.e., 82%, of amorphous 1-[2,3,5-tri-O-acetyl-β-D-ribofuranosyl]-4-methyl-4-isothiobiuret.

3.91 g of the preceding product are dissolved while hot in 30 ml of ethyl orthoformate and the solution is heated for 10 h to 135° C in a slow current of anhydrous nitrogen. After cooling, the solution is precipitated with 75 ml of petroleum ether and the separated syrup is dissolved in 10 ml of anhydrous benzene. The solution is again precipitated with 50 ml of petroleum ether. One obtains vitreous 1-[2,3,5-tri-O-acetyl-β-D-ribofuranosyl]-4-methylmercapto-2-oxo-1,2-dihydro-1,3,5-triazine in a yield of 2.0 g, i.e., 50%.

4.01 g of the preceding product are dissolved in 30 ml of a methanol solution of ammonia saturated at 0° C and left at room temperature in a sealed tube for a period of 2 h and then overnight in a refrigerator at -15° C. The separated 5-azacytidine is aspirated, washed with methanol and dried in a vacuum. One obtains 1.6 g, i.e., 59% of product with melting point of 227-230° C (decomposition).

Example 2

To a solution of 3.01 g of crude 2,3,5-tri-O-acetyl-β-D-ribofuranosylisocyanate in 50 ml of anhydrous chloroform, under cooling and mixing one adds, while excluding external moisture, a solution of 0.54 g of guanidine in 10 ml of anhydrous chloroform. The solution is then left for 1 hour at room temperature and concentrated in vacuum to a thick syrup, which is dissolved in 20 ml of anhydrous benzene and precipitated with 80 ml of petroleum ether. After another precipitation, one obtains amorphous 2,3,5-tri-O-acetyl-β-D-ribofuranosylcarbamylguanidine in a yield of 3.1 g, i.e., 86%.

3.6 g of the preceding product are dissolved while hot in 60 ml of ethyl orthoformate and the solution is heated for 10 h to 135° C in a current of anhydrous nitrogen. After cooling, the solution is precipitated with 200 ml of petroleum ether, dissolved in 20 ml of ethyl acetate, and again precipitated with 100 ml of petroleum ether. One obtains amorphous 1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-5-azacytosine in a yield of 2.2 g, i.e., 81%.

3.7 g of the preceding product are dissolved in 30 ml of a methanol solution of ammonia saturated at 0° C and left for 1.5 h at room temperature in a sealed tube and then overnight in a refrigerator. The separated crystalline 1-β-D-ribofuranosyl-5-azacytosine (5-azacytidine) was obtained in a yield of 1.7 g, i.e., 70%, and had melting point of 227-230° C (decomposition).

Example 3

To a solution of 3.72 g of 3,5-di-p-toluyl-2-desoxy-β-D-ribofuranosylisocyanate in 50 ml of anhydrous chloroform, under cooling and mixing one adds, while excluding external moisture, a solution of 0.54 g of guanidine in 10 ml of anhydrous chloroform. The solution is then left for 1 hour at room temperature and concentrated in vacuum to a thick syrup, which is dissolved in 30 ml of benzene and precipitated with 100 ml of petroleum ether. After another precipitation, one obtains amorphous 2,3-di-p-toluyl-2-desoxy-β-D-ribofuranosylcarbamylguanidine in a yield of 4.0 g, i.e., 93%.

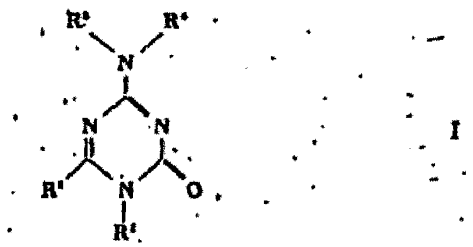
4.31 g of the preceding product are dissolved while hot in 60 ml of ethyl orthoformate and the solution is heated for 10 h to 135° C in a slow current of anhydrous nitrogen. After cooling, the solution is precipitated with 200 ml of petroleum ether, dissolved in 20 ml of ethyl acetate, and again precipitated with 100 ml of petroleum ether. One obtains amorphous 1-(2,3-di-p-toluyl-2-desoxy-β-D-ribofuranosyl)-5-azacytosine in a yield of 2.8 g, i.e., 65%.

4.41 g of the preceding product are dissolved in 200 ml of absolute methanol and after adding 10 ml of 1N sodium methylate the solution is left at laboratory temperature in a sealed tube for 8 h. After neutralizing the solution with carbon dioxide, the solution is evaporated and the residue is purified by chromatography on a column of cellulose in suitable composition. One obtained 1.42 g, i.e., 55%, of amorphous 2'-desoxy-5-azacytidine.

λ_{max}^{1N-HCl} = 257 mμ (log ε 3.6).

CLAIMS

1. Method of preparation of 1-glycosyl-5-azacytosines of the general formula:



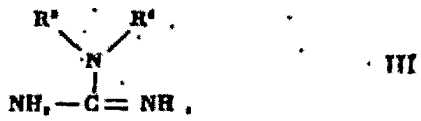
I

where R^1 represents glycosyl or peracylglycosyl, R^2 signifies hydrogen or alkyl with 1-4 carbon atoms, R^3 and R^4, the same or different, signify atoms of hydrogen or alkyls with

1-4 carbon atoms, or aralkyls, such as benzyl, which can be substituted in the benzene ring, characterized in that one acts on acylglycosylisocyanates of the general formula:

II

where R^1 signifies peracylglycosyl, with guanidines of the general formula:

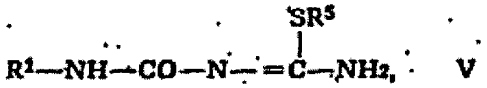


III

where R^3 and R^4 have the same meaning as in formula 1, or one acts on it with S-alkylisothioureas of the general formula:

IV

where R^5 signifies alkyl with 1-4 carbon atoms, and the resulting acylglycosylisothiobiurets of the general formula:



V

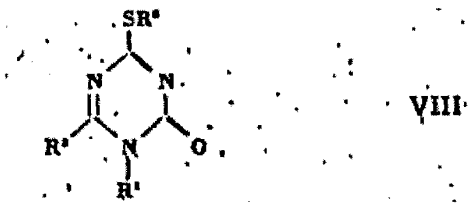
where R^1 signifies peracylglycosyl and R^5 the same as in formula III, or acylglycosylcarbamylguanidines of the general formula:

VI

where R^1 signifies the same as in formula V and R^3 and R^4 the same as in formula IV, are condensed with orthoesters of aliphatic acids of the general formula:

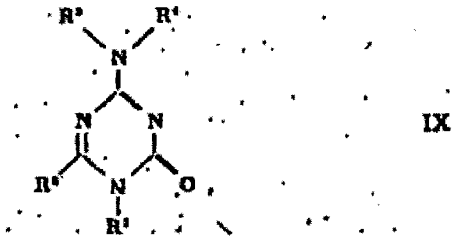
VII

where R^2 signifies the same as in formula I and R^6 is methyl or ethyl, into acylglycosyl-4-alkylmercapto-2-oxo-1,2-dihydro-1,3,5-triazines of the general formula:



VIII

where R^1 signifies the same as in formula V, R^2 the same as in formula I, and R^5 the same as in formula III, or into acylglycosyl-5-azacytosines of the general formula:



IX

where R^1 signifies the same as in formula V, R^2, R^3 and R^4 the same as in formula I, and the substances of the general formula VIII after being made to react with a compound of the general formula:

$$R^3—NH—R^4 \qquad X$$

where R^3 and R^4 have the same meaning as in formula I, and having formed substances of the general formula IX, are then subjected to alcoholysis, preferably by the action of methanol in the presence of sodium methylate, or ammonolysis, preferably by ammonia in methanol.

2. Method of preparation according to claim 1, characterized in that the reaction of the substances of the general formula II with the compounds of the general formulas III and IV is conducted in equimolar proportions while cold in an inert solvent, preferably in chloroform.

3. Method of preparation according to claim 1, characterized in that the condensation of the substances of the general formulas V and VI with the orthoesters of aliphatic acids of the general formula VII is done at elevated temperature, but lower than the boiling point of the orthoester used, in a stream of anhydrous inert gas.

4. Method of preparation according to claim 1, characterized in that the condensation of the substances of the general formulas V and VI with the orthoesters of aliphatic acids of the general formula VII is done at elevated temperature, in the presence of acetanhydride.

5. Method of preparation according to claim 1, characterized in that the reaction of the substances of the general formula VIII with the compound of the general formula X is carried out at laboratory temperature in the presence of an alkanol with 1-6 carbon atoms.

ČESKOSLOVENSKÁ SOCIALISTICKÁ REPUBLIKA

ÚŘAD PRO PATENTY A VYNÁLEZY

PATENTOVÝ SPIS 116297 a

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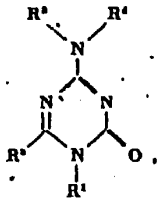
a ALOJS PÍSKALA, prom. chemik,
oba PRAHA

Způsob přípravy 1-glykosyl-5-azacytosinů

1

Předmětem vynálezu je způsob přípravy 1-glykosyl-5-azacytosinů obecného vzorce:



I

kde R^1 představuje glykosyl nebo peracylglykosyl, R^2 značí vodík nebo alkyl s 1 — 4 atomy uhlíku, R^3 a R^4 (které mohou být stejné nebo odlišné) značí atomy vodíku nebo alkyly s 1 — 4 atomy uhlíku, popřípadě aralkyly, jako benzyl, jenž může být substituován v benzenovém jádře.

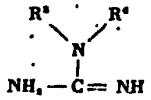
Doposud známý a používaný způsob přípravy 1-glykosyl-5-azacytosinů vychází z percylglykolsylisokyanátů, které po adici 2-methylisomočoviny a po kondensaci vzniklých isobiuretů s ortoestery kyseliny jsou převáděny na příslušné metoxytriaziny, které pak s amoniakem, popřípadě s primárními nebo sekundárními aminy poskytují příslušné glykosiliderivváty. V průběhu dalšího výzkumu se ukázalo, že v některých případech je výhodné zvýšit výtěžek konečného výrobku, to jest 1-glykosyl-5-azacytosinů tím, že při výrobě 2-methylisomočovina je nahrazena vhodnými substituovanými deriváty kyseliny uhličité s kombinacemi skupiny sulfhydrylové a aminoskupiny nebo substituovanými deriváty kyseliny uhličité s aminoskupinami.

2

Předmětem předloženého vynálezu je způsob přípravy 1-glykosyl-5-azacytosinů, při kterém na acylglykosylisokyanáty obecného vzorce:

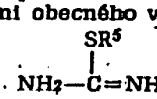
$$R^1—N=C=O, \qquad II$$

ve kterém R^1 značí peracylglykosyl, působí se guanidinem, popřípadě substituovaným guanidinem obecného vzorce:



III

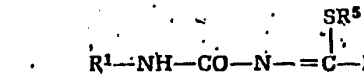
v němž R^3 a R^4 mají stejný význam jako ve vzorci I, nebo působí se na něj S-alkylisothiomočovinami obecného vzorce:



IV

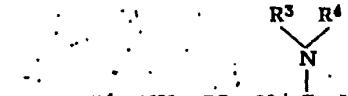
v němž R^5 značí alkyl s 1 — 4 atomy uhlíku.

Tím vznikají 1-acylglykosyl-4-alkyl-4-isothiobiurety obecného vzorce:



V

v němž R^1 značí totéž co ve vzorci II a R^5 totéž co ve vzorvi II, nebo acylglykosylkarbamylguanidiny obecného vzorce:

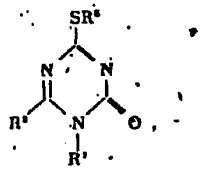


VI

v němž R^1 značí totéž co ve vzorci II a R^3 a R^4 totéž co ve vzorci I. Zmíněné látky obecného vzorce V a VI se poté kondensují s ortoestery alifatických kyselin obecného vzorce:

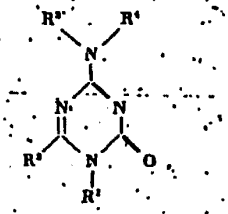
$$R^2—C(OR^6)_3, \qquad VII$$

v němž R^2 značí totéž co ve vzorci I a R^6 metyl nebo etyl, na 1-acylglykosyl-4-alkylmerkapto-2-oxo-1,2-dihydro-1,3,5-triaziny obecného vzorce:



VIII

v němž R^1 značí totéž co ve vzorci II, R^2 totéž co ve vzorci I a R^5 totéž co ve vzorci III, nebo na 1-acylglykosyl-4-amino-2-oxo-1,2-dihydro-1,3,5-triaziny, popřípadě 4-alkylamino nebo 4-dialkylaminoderiváty obecného vzorce:



IX

v němž R^1 značí totéž co ve vzorci II, R^2, R^3 a R^4 co ve vzorci I.

Látky obecného vzorce VIII se uvádějí do reakce se sloučeninou obecného vzorce:

$$R^3—NH—R^4, \qquad X$$

v němž R^3 a R^4 značí totéž co ve vzorci I a vzniklé 1-acylglykosylderiváty se podrobí alkoholyse, s výhodou působením metanolu v přítomnosti metylátu sodného.

Podle vynálezu je dále možno převést 1-acylglykosyl-5-azasytosiny obecného vzorce IX na příslušné 1-glykosyl-5-azacytosiny i amonolysou, s výhodou působením amoniaku v metanolu.

Dalším významem vynálezu je, že reakce acylglykosylisokyanátů obecného vzorce II s S-alkylisomočovinami obecného vzorce III nebo s guanidiny obecného vzorce IV se provádí v ekvimolárních poměrech za chladu v inertním rozpouštědle, s výhodou v chloroformu.

Podle vynálezu se kondensace isobiuretů obecného vzorce V nebo karbamylguanidinů obecného vzorce VI s ortoestery alifatických kyselin obecného vzorce VII provádí za zvýšené teploty, avšak nižší, než je bod varu použitého ortoesteru v proudu suchého inertního plynu, popř. krátkodobým povařením v přítomnosti acetanhydridu. Konečně významem je, že reakce alkylmerkaptotriazinů obecného vzorce VIII se sloučeninou obecného vzorce X se provádí při teplotě místnosti v prostředí alkamolu s 1 až 6 atomy uhlíku.

Konfigurace glykosidického centra se během syntesy nemění a je v konečném produktu stejná jako ve výchozí sloučenině obecného vzorce II.

Podle povahy skupin chránících hydroxyly dochází při záměně zbytku —SR^5 (VIII) za skupinu —$N(R^3)$ (R^4) k jejich současnému odstranění. Samozřejmě lze tyto skupiny v konečném produktu odstranit též některou ze známých a používaných metod.

Uvedené příklady slouží k ilustraci, nikoliv však k vymezení použitého postupu.

Příklady provedení

Příklad 1

K roztoku 3,01 g surového 2,3,5-tri-O-acetyl-β-D-ribofuranosylisokyanátu v 50 ml suchého chloroformu se za chlazení a míchání přidá za vyloučení vnější vlhkosti roztok 0,81 g S-metylisothiomočoviny v 10 ml suchého chloroformu. Roztok se pak ponechá 1 hodinu za laboratorní teploty, odpaří na hustý sirup ve vakuu, rozpustí v 10 ml suchého benzenu a vysráží 50 ml petroleteru. Srážení se ještě jednou opakuje. Získá se 3,2 g, tj. 82 % amorfního 1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-4-metyl-4-isothiobiuretu.

3,91 g předchozího produktu se rozpustí za horka ve 30 ml ortomravenčanu etylnatého a roztok se zahřívá 10 hodin na 135° C v pomalém proudu suchého dusíku. Po ochlazení se roztok vysráží 75 ml petroleteru a vyloučený sirup rozpustí v 10 ml suchého benzenu. Roztok se znovu vysráží 50 ml petroleteru. Získá se sklovitý 1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-4-metylmerkapto-2-oxo-1,2-dihydro-1,3,5-triazin ve výtěžku 2,0 g; tj. 50 %.

4,01 g předchozího produktu se rozpustí ve 30 ml metanolického roztoku amoniaku, nasyceného při 0° C, a ponechá při teplotě místnosti v zatavené trubici po dobu 2 hodin a dále přes noc v chladicím pultu při —15° C. Vyloučený 5-azacytidin se odsaje, promyje metanolem a vysuší ve vakuu. Získá se 1,6 g, tj. 59 % produktu o b. t. 227 až 230° (rozklad).

Příklad 2

K roztoku 3,01 g surového 2,3,5-tri-O-ace-

tyl-β-D-ribofuranosylisokyanátu v 50 ml suchého chloroformu se za chlazení a míchání přidá za vyloučení vnější vlhkosti roztok 0,54 g guanidinu v 10 ml suchého chloroformu. Roztok se pak ponechá 1 hodinu při teplotě místnosti a zahustí ve vakuu na hustý sirup, který se rozpustí ve 20 ml suchého benzenu a vysráží 80 ml petroleteru. Po opakovaném přesrážení se získá amorfní 2,3,5-tri-O-acetyl-β-D-ribofuranosylkarbamylguanidin ve výtěžku 3,1 g, tj. 86 %.

3,6 g předchozího produktu se rozpustí za horka v 60 ml ortomravenčanu etylnatého a roztok se zahřívá 10 hodin na 135° v proudu suchého dusíku. Po ochlazení se roztok vysráží 200 ml petroleteru, rozpustí se v 20 ml etylacetátu a znovu vysráží 100 ml petroleteru. Získá se amorfní 1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-5-azacytosin ve výtěžku 2,2 g, tj. 61 %.

3,7 g předchozího produktu se rozpustí ve 30 ml metanolického roztoku amoniaku, nasyceného při 0° C, a ponechá při teplotě místnosti v zatavené trubici po dobu 1,5 hodin a pak v chladicím pultu přes noc. Vyloučený krystalický 1-β-D-ribofuranosyl-5-azacytosin (5-azacytidin) byl získán ve výtěžku 1,7 g, tj. 70 %, a měl b. t. 227 až 230° C (rozklad).

Příklad 3

K roztoku 3,72 g 3,5-di-p-toluyl-2-desoxy-β-D-ribofuranosylisokyanátu v 50 ml suchého chloroformu se za chlazení a míchání přidá za vyloučení vnější vlhkosti roztok 0,54 g guanidinu v 10 ml chloroformu. Roztok se pak ponechá 1 hodinu při teplotě místnosti a zahustí ve vakuu na hustý sirup, který se rozpustí ve 30 ml benzenu a vysráží 100 ml petroleteru. Po opakovaném přesrážení se získá amorfní 2,3-di-p-toluyl-2-desoxy-β-D-ribofuranosylkarbamylguanidin ve výtěžku 4,0 g, tj. 93 %.

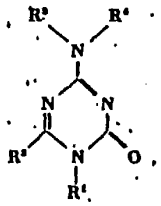
4,31 g předchozího produktu se rozpustí za horka v 60 ml ortomravenčanu etylnatého a roztok se zahřívá 10 hodin na 135° C v pomalém proudu suchého dusíku. Po ochlazení se roztok vysráží 200 ml petroleteru, rozpustí ve 20 ml etylacetátu a znovu vysráží 100 ml petroleteru. Získá se amorfní 1-(2,3-di-p-toluyl-2-desoxy-β-D-ribofuranosyl)-5-azacytosin ve výtěžku 2,8 g, tj. 65 %.

4,41 g předchozího produktu se rozpustí ve 200 ml absolutního metanolu a po přidání 10 ml 1N-metylátu sodného se roztok ponechá za laboratorní teploty 8 hodin v uzavřené baňce. Po zneutralisování roztoku kysličníkem uhličitým se roztok odpaří a zbytek přečistí chromatografií na sloupci celulosy ve vhodné soustavě. Získáno bylo 1,42 g, tj. 55 % amorfního 2'-desoxy-5-azacytidinu.

λ_{max}^{1N-HCl} = 257 mμ(log ε 3,6).

PŘEDMĚT PATENTU

1. Způsob přípravy 1-glykosyl-5-azacytosinů obecného vzorce:

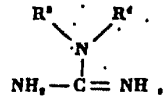


I

v němž R^1 značí glykosyl nemo peracylglykosyl, R^2 atom vodíku nebo alkyl s 1 — 4 atomy uhlíku, R^3 a R^4 stejné nebo různé atomy vodíku nebo alkyly s 1 — 4 atomy uhlíku nebo aralkyly, například benzyl, popřípadě substituovaný v benzenovém jádře, vyznačený tím, že na acylglykosylisokyanáty obecného vzorce:

$$R^1—N=C=O, \qquad II$$

v němž R^1 značí peracylglykosyl, se působí guanidiny obecného vzorce:



III

v němž R^3 a R^4 značí totéž co ve vzorci I, nebo se působí S-alkylisothiomočovinami obecného vzorce:

IV

v němž R^5 značí alkyl s 1 — 4 atomy uhlíku a vzniklé acylglykosylisothiobiurety obecného vzorce:

V

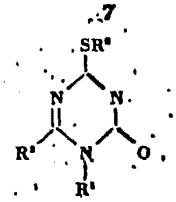
v němž R^1 značí peracylglykosyl a R^5 totéž co ve vzorci III, nebo acylglykosylkarbamylguanidiny obecného vzorce:

VI

v němž R^1 značí totéž co ve vzorci V a R^3 a R^4 totéž co ve vzorci IV, kondenzují s ortoestery alifatických kyselin obecného vzorce:

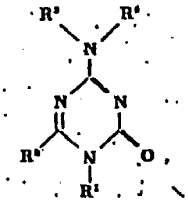
$$R^2—C(OR^6)_3, \qquad VII$$

v němž R^2 značí totéž co ve vzorci I a R^6 metyl nebo etyl, na acylglykosyl-4-alkylmerkapto-2-oxo-1,2-dihydro-1,3,5-triazíny obecného vzorce:



VIII

v němž R^1 značí totéž co ve vzorci V, R^2 totéž co ve vzorci I a R^5 totéž co ve vzorci III, nebo na acylglykosyl-5-azacytosiny obecného vzorce:



IX

v němž R^1 značí totéž co ve vzorci V, R^2, R^3 a R^4 totéž co ve vzorci I, a látky obecného vzorce VIII se po uvedení do reakce se sloučeninou obecného vzorce:

$$R^3—NH—R^4 \qquad X$$

v němž R^3 a R^4 značí totéž co ve vzorci I,

a vzniku látek obecného vzorce IX se pak podrobí alkoholyse s výhodou působením metanolu v přítomnosti metylátu sodného nebo amonolyse, s výhodou amoniakem v metanolu.

2. Způsob přípravy podle bodu 1, vyznačující se tím, že se látky obecného vzorce II uvádějí do reakce se sloučeninami obecných vzorců III a IV, za chladu, v ekvimolárních poměrech v inertním rozpouštědle, s výhodou v chloroformu.

3. Způsob přípravy podle bodu 1, vyznačující se tím, že se kondenzace látek obecných vzorců V a VI s ortoestery alifatických kyselin obecného vzorce VII provádí za zvýšené teploty, avšak nižší, než je bod varu užitého ortoesteru v proudu suchého inertního plynu.

4. Způsob podle bodu 1 vyznačující se tím, že se kondenzace látek obecných vzorců V a VI s ortoestery alifatických kyselin obecného vzorce VII provádí za zvýšené teploty v přítomnosti acetanhydridu.

5. Způsob podle bodu 1 vyznačující se tím, že se reakce látek obecného vzorce VIII se sloučeninou obecného vzorce X provádí za laboratorní teploty v prostředí alkanolu s 1 — 6 atomy uhlíku.

Severografia, n.p., provozovna 32, Most

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[File 351] **Derwent WPI** 1963-2009/UD=200979


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?  s pn=(fr 2123632 or cz 114716 or cz 116297)
             1    PN=FR 2123632
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Fulltext available through: Order File History
Derwent WPI

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WPI Acc no: 1972-81719T/ 197252
Prepn of 5-azacytosines and 5-azacytidines - from bis trimethylsilyl-5-azacytosine
Patent Assignee: CESKOSLOVENSKA AKADEMIE V (CESK)

Patent Family (1 patents, 1 countries)

Patent Number	Kind	Date	Application Number	Kind	Date	Update	Type
FR 2123632	A	00000000	FR 19712441	A	19710126	197252	B

Priority Applications (no., kind, date): FR 19712441 A 19710126
Alerting Abstract FR A
The prepns. of the following are described: 2',3',5'-tri-O-benzoyl-5-azacytidine (I), 5-azacytidine (II), 1,(3,5-di-O-tolyl-2-desoxy-alpha-D-ribofuranosyl)-5-azacytosine (III) and 1-(2-desoxy-alpha-D-ribofuranosyl)-5-azacytosine (IV). (I) and (III) are prepd. by condensation of bis(trimethyl silyl)-5-azacytosine (V) with dry acetonitrile and the appropriate acid chloride. (II) and (IV) are prepd. from (I) and (III) by treatment with sodium methoxide.

Class Codes

International Patent Classification

IPC	Class Level	Scope	Position	Status	Version Date
C07D-055/00			Secondary		"Version 7"

DWPI Class: B03; E13

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(15) CERTIFICAT D'ADDITION A UN BREVET D'INVENTION

PREMIÈRE ET UNIQUE PUBLICATION

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(51) Classification internationale (Int. Cl.) C 07 d 55/00.

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(73) Titulaire : *Idem* (71)

(74) Mandataire : Cabinet J. Bonnet Thirion, L. Robida & G. Foldès.

(54) Procédé de préparation de 1-glycosyl 6-azacytosines.

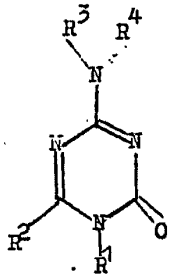
(72) Invention de :

(33) (32) (31) Priorité conventionnelle :

(61) Références du brevet principal : Brevet d'invention n. 69.22727 du 4 juillet 1969.

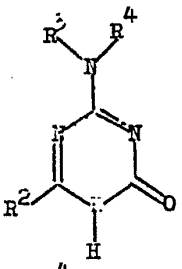
Certificat(s) d'addition antérieur(s) :

Le brevet principal décrit un procédé de préparation de 1-glycosyl 5-azacytosines répondant à la formule générale I :



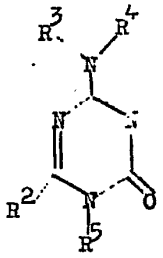
I.

dans laquelle R^1 est un radical glycosyle, R^2 l'hydrogène ou un radical alkyle ayant dans sa molécule de un à quatre atomes de carbone, R^3 et R^4, identiques ou différents pouvant être l'hydrogène, un alkyle de un à quatre atomes de carbone, ou un radical aralkyle, ce procédé étant caractérisé en ce que l'on soumet à une réaction de triméthylsilysation une 5-azacytosine répondant à la formule générale II :



II.

dans laquelle R^2, R^3 et R^4 ont les mêmes significations que dans la formule I, le produit ainsi obtenu étant ensuite mis en réaction dans un solvant inerte avec un halogénure de peracyl glycosyle avec formation de 1-peracylglycosyl 5- azacytosines correspondant à la formule III :



III.

dans laquelle R^2, R^3 et R^4 ont les mêmes significations que dans

la formule I, R^5 étant un radical peracylglycosyle, et en ce que le produit obtenu est soumis à une alcolyse ou à une ammonolyse.

La présente invention vise divers modes opératoires, qui trouvent leur place dans le cadre de ce procédé, ainsi que certaines de ses applications.

Ces modes opératoires et applications ressortiront de la description qui va suivre.

Préparation de 2', 3', 5' - tri-O-benzoyl 5-azacytidine :

La bis (triméthyl-silyl) 5-azocytosine brute, préparée à partir de 1,13 g de 5-azacytosine comme décrit au brevet principal est agitée avec 20 ml d'acétonitrile sec. On ajoute ensuite une solution 0,5 M du chlorure de 2, 3, 5-tri-O-benzoyl-D-ribofuranosyle dans l'acétonitrile. Le mélange est agité pendant trois jours par un système magnétique à la température ambiante à l'abri de l'humidité de l'air, puis étendu avec 150 ml de chloroforme. On filtre, on agite avec 50 ml d'une solution à 5 % de bicarbonate de potassium, on sèche sur sulfate de sodium et on évapore sous vide. Le résidu sirupeux est redissous dans 20 ml de benzène et laissé une nuit à la température ambiante. On obtient 2,20 g, soit un rendement de 40 % en 2', 3', 5'-tri-O-benzoyl 5'- azacytidine, qui subit une transformation à 135-140° et fond à 179-182°. Après recristallisation dans l'alcool éthylique, le produit fond à 185-187° sans changement préalable.

Préparation de 5-Azacytidine :

On agite à l'abri de l'air humide un mélange formé par 0,556 g de 2', 3', 5' -tri-O-benzoyl 5-azacytidine, 4 ml d'alcool méthylique absolu de 1 ml d'une solution N de méthylate de sodium dans l'alcool méthylique, jusqu'à passage en solution. Le mélange est laissé 20 minutes à la température ambiante, puis 3 heures à 0°C. Le produit est essoré par aspiration, lavé à l'alcool éthylique et séché à 100° sous vide, jusqu'à poids constant.

On obtient 0,230 g, soit un rendement de 94 % en 5-Azacytidine, fondant avec décomposition à 231-233°.

En variante, on agite à l'abri de l'air humide, au moyen d'un système magnétique, pendant deux heures à la température ambiante, un mélange formé par 0,556 g de 2', 3', 5'-tri-O-benzoyl 5-azacytidine et 4 ml d'une solution à 10 % d'ammoniaque anhydre dans l'alcool méthylique absolu.

On laisse reposer une nuit à 0°C et on essore le produit précité ; on le lave à l'alcool méthylique et on le sèche dans le vide

à 100° jusqu'à poids constant.

On recueille 0,225 g, soit un rendement de 92 %, de 5-azacytidine , fondant avec décomposition à 230-232°.

Préparation de 1-(3,5-di-O-p.toluyl-2-désoxy-α-D-ribofuranosyl)-5-azacytosine-

On agite à l'abri de l'air humide, au moyen d'un système magnétique, un mélange formé par 0,385 g de bis-(triméthyl-silyl)-5-azacytosine, 10 ml d'acétonitrile sec et 0,584 g de chlorure de 3,5-di-O-p.toluyl-D-ribofuranosyle. On étend avec 50 ml de chloroforme, on filtre et on agite avec 20 ml d'une solution à 5 % de bicarbonate de sodium. On sèche sur sulfate de sodium et on évapore sous vide. On fait bouillir le résidu avec 5 ml de benzène, on sépare rapidement par essorage la partie insoluble de la solution encore chaude et on recristallise dans 25 ml d'alcool éthylique. On obtient 0,305 g, soit un rendement de 44 %, de 1-(3,5-diO-p.toluyl-2-désoxy-α-D-ribofuranosyl)-5-azacytosine, fondant à 220-221° C.

Préparation de 1-(2-désoxy-α-D-ribofuranosyl)-5-azacytosine.

On agite en ballon fermé à la température ambiante un mélange formé par 0,232 g de 1-(3,5-di-O-p.toluyl-2-désoxy-α-D-ribofuranosyl)-5-azacytosine, 2 ml d'alcool méthylique absolu et 0,5 ml d'une solution N de méthylate de sodium dans l'alcool méthylique, jusqu'à dissolution des produits de départ. Au bout de deux heures, on met le mélange au réfrigérateur jusqu'au lendemain.

Le produit précité est essoré, lavé à l'alcool méthylique et séché sous vide. On recueille 0,085 g, soit un rendement de 74 %, de 1-(2-désoxy-α-D-ribofuranosyl)-5-azacytosine, fondant à 176-178°C.

REVENDICATIONS

1. On agite un mélange de bis (triméthyl-silyl) 5-azacytosine, et d'acétonitrile sec, on ajoute du chlorure de 2,3,5-tri-O-benzoyl-D-ribofuranosyle, on agite à l'abri de l'humidité de l'air, on étend de chloroforme, on filtre, on agite dans du bicarbonate de potassium en solution, on sèche sur sulfate de sodium, on évapore, on dissout le résidu dans du benzène pour obtenir de la 2',3',5'-tri-O-benzoyl 5'-azacytidine.

2. On mélange de la 2',3',5'-tri-O-benzoyl 5-azacytidine, avec de l'alcool absolu et du méthylate de sodium en solution N, on refroidit à 0°C, on essore, on lave à l'alcool éthylique et on sèche pour recueillir de la 5-Azacytidine.

3. On procède selon 2, mais en remplaçant, d'une part, l'alcool absolu et le méthylate de sodium par de l'ammoniaque anhydre en solution dans l'alcool méthylique absolu, et d'autre part, l'alcool éthylique de lavage par de l'alcool méthylique.

4. On agite un mélange de bis-(triméthyl-silyl)-5- azacytosine, d'acétonitrile et de chlorure de 3,5-di-O-p-toluyl-D-ribofuranosyle, on étend de chloroforme, on filtre, on agite avec une solution de bicarbonate de sodium, on sèche sur sulfate de sodium, on évapore, on fait bouillir le résidu avec du benzène, on sépare l'insoluble par essorage et on recristallise dans de l'alcool éthylique pour recueillir de la 1-(3,5-di-O-p.toluyl-2-désoxy-α-D-ribofuranosyl)-5-azacytosine.

5. On agite un mélange de 1-(3,5-di-O-p.toluyl-2-désoxy-α-D-ribofuranosyl)-5-azacytosine, d'alcool méthylique absolu et d'une solution N de méthylate de sodium dans l'alcool méthylique, on conserve au réfrigérateur, on essore le précipité, on lave à l'alcool méthylique et on sèche pour recueillir de la 1-(2-désoxy-α-D-ribofuranosyl)-5-azacytosine.

PATENT SPECIFICATION (11) 1227691

1227691

NO DRAWINGS

(21) Application No. 21132/69 (22) Filed 24 April 1969

(31) Convention Application No. 3399 (32) Filed 8 May 1968 in

(33) Czechoslovakia (CS)

(45) Complete Specification published 7 April 1971

(51) International Classification C 07 d 99/04

(52) Index at acceptance

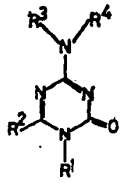
C2C 173—199—272 177—271—279 215 247 250 252 253 25Y 28X 30Y 321 322 323 32Y 351 352 360 361 362 36Y 3A12A4A 3A12A4B 3A12B2 3A12C4 652 670 672 761 766 790 LK

(54) A PROCESS FOR PREPARING 1-GLYCOSYL-5-AZACYTOSINES

(71) We, ČESKOSLOVENSKÁ AKADEMIE VĚD, a Corporation organised and existing under the laws of Czechoslovakia of No. 3 Narodni, Prague 1, Czechoslovakia, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a process for preparing 1 - glycosyl - 5 - azacytosines.

More particularly this invention relates to a process for preparing 1 - glycosyl - 5 - azacytosines of the general formula I:



(1)

wherein R^1 designates a glycosyl residue, R^2 designates a hydrogen atom or an alkyl group having from 1 to 4 carbon atoms and R^3 and R^4, which are identical or different, designate hydrocarbon atoms, alkyl groups having from 1 to 4 carbon atoms or aralkyl groups having from 7 to 10 carbon atoms.

Two compounds of the above type show significant biological effects, namely, 1 - β - D - ribofuranosyl - 4 - amino - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one (or 5 - azacytidine) and 1 - (2 - deoxy - β - D - ribofuranosyl) - 4 - amino - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - one (or 5 - aza - 2' - deoxycytidine). 5 - Azacytidine, a pyrimidine antimetabolite, in low concentrations inhibits bacterial growth and exhibits a high antileukemic effect with mice. In the case of *V. faba meristen*, 5-azacytidine causes a mitosal inhibition and chromosomal aberrations.

[*Price 25p*]

A mutagenic effect of 5 - azacytidine has been reported. Furthermore, 5 - azacytidine suppresses the formation of inductive enzymes in mammalian cells and regeneration of rat liver after heptatectomy and, on the other hand, protects mice against the effects of X-rays.

5 - Aza - 2' - deoxycytidine, similarly to 5 - azacytidine, suppresses considerably the formation of experimental leukemia and shows, even at low concentrations, significant bacteriostatic properties.

A similar biological activity can be expected also with some further 1 - glycosyl - 5 - azacytosines.

The preparation of 1 - glycosyl - 5 - azacytosines was previously reported in British patent specifications Nos. 1,046,181 and 1,050,899. Per-acylglycosyl isocyanates are added to O - alkylisoureas or S - alkyl - isothioureas to produce the corresponding per-acylglycosylisobiurets or peracylglycosyliso - thiobiurets. Condensation of the latter compounds with ortho-esters of aliphatic acids affords 1 - per - acylglycosyl - 4 - alkoxy - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - ones or 1 - per - acylglycosyl - 4 - alkylthio - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - ones, the treatment of which with ammonia or amines in alcohols produces the 1 - glycosyl - 5 - azacytosines. One disadvantage of this procedure is that the reaction of ammonia or amines with 1 - per - acylglycosyl - 4 - alkoxy (or alkylthio) - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - ones usually produces the required 1 - glycosyl - 5 - azacytosines in very low yields because of the instability of the aforementioned intermediates under amination conditions. Furthermore, the 4 - alkylthio derivatives are less stable than the corresponding 4-alkoxy derivatives and their reactivity towards ammonia or amines is very low.

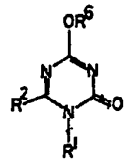
An object of the present invention is to

obviate or mitigate the aforesaid disadvantages.

We have discovered that the free 1 - glycosyl - 4 - alkoxy - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - ones are much more stable than their per-acyl derivatives. The amination of 1 - glycosyl - 4 - alkoxy - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - ones is very rapid and affords high yields of the required 1 - glycosyl - 5 - azacytosines.

The free 1 - glycosyl - 4 - alkoxy - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - ones are readily accessible by alcoholysis of the corresponding per-acyl derivatives as well as by alcoholysis of 1 - per - acylglycosyl - 4 - alkylthio - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - ones which, in this special case, is accompanied by a conversion of the 4 - alkylthio group to the 4 - alkoxy group.

According to the present invention there is provided a process for preparing a 1 - glycosyl - 5 - azacytosine of the general formula I defined above comprising effecting reaction of a 1 - glycosyl - 4 - alkoxy - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one of the general formula III



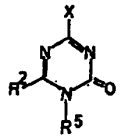
(3)

wherein R^1 and R^2 are as defined for general formula I and R^6 is an alkyl group having from 1 to 6 carbon atoms, with ammonia or an amine of the general formula IV

$$R^3—NH—R^4 \qquad IV$$

wherein R^3 and R^4 are as defined for general formula I.

The alkoxytriazinone of the general formula III may be prepared by effecting reaction of an alkali metal alkoxide having from 1 to 6 carbon atoms with a compound of the general formula II

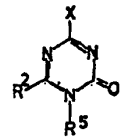


(2)

wherein R^2 is as defined for general formula I, R^5 is a peracylglycosyl group wherein the acyl group has from 2 to 10 carbon atoms and X is an alkoxy or alkylthio group having from 1 to 4 carbon atoms.

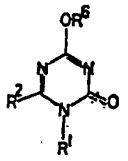
Conversion of the alkoxytriazinone or the alkylthiotriazinone of the general formula II to the 1 - glycosyl - 5 - azacytosine of the general formula I preferably is performed without isolation of the alkoxytriazinone of the general formula III.

Hence further according to the present invention there is provided a process for the preparation of a 1 - glycosyl - 5 - aza - cytosine of the general formula I, comprising effecting reaction of a compound of the general formula II



(2)

wherein R^2 is as defined for general formula I, R^5 designates a per - acylglycosyl residue wherein the acyl group has from 2 to 10 carbon atoms and X designates an alkoxy or alkylthio group having from 1 to 4 carbon atoms, with an alkali metal alkoxide having from 1 to 6 carbon atoms, preferably in methanol, thus effecting the formation of a 1 - glycosyl - 4 - alkoxy - 1,2 - dihydro - 1,3,5 - triazin - 2 - one of the general formula III



(3)

wherein R^1 and R^2 are as defined for general formula I and R^5 designates an alkyl group having from 1 to 6 carbon atoms, and effecting reaction of the alkoxytriazinone of the general formula III with ammonia or an amine of the general formula IV

$$R^3—NH—R^4 \qquad IV$$

wherein R^3 and R^4 are as defined above for general formula I.

The reaction of compounds of the general formula II with the alkali metal alkoxide preferably is performed at room temperature, preferably in an alkanol containing from 1 to 6 carbon atoms, and more preferably in methanol. This reaction preferably is carried out in the absence of atmospheric moisture. In the case of alkylthio derivatives, optimum yields are obtained with the use of 1.2 moles of the alkoxide per 1 mole of the starting compound.

The reaction of the alkoxytriazinone of the general formula III with ammonia or an amine of the general formula IV preferably is performed at room temperature, preferably in the medium of an alkanol containing

from 1 to 6 carbon atoms, and more preferably in methanol.

The conversion of the compound of the general formula II to the 1 - glycosyl - 5 - azacytosine of the general formula I may be performed in one step, namely, by the simultaneous action of the alkali metal alkoxide and of an alkanol solution of ammonia or an amine of the general formula IV.

The invention will be illustrated further by the following examples, although it is not limited thereto.

5 - Azacytidine
(1 - β - D - Ribofuranosyl - 4 - amino - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one)

EXAMPLE 1

A mixture of 1 - (2, 3, 5 - tri - O - benzoyl - β - D - ribofuranosyl) - 4 - methylthio - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one 0.5875 g), absolute methanol (5 ml) and a normal methanolic sodium methoxide solution (1.2 ml) is stirred at room temperature with the exclusion of atmospheric moisture (a guard tube filled with potassium hydroxide pellets is fitted to the reaction vessel). The starting compound passes into solution in the course of 5 minutes. The resulting solution is allowed to stand at room temperature for 45 minutes and then the cations are removed by passage of the solution through a column packed with 10 ml of a weakly acidic cation exchange resin in the H^+ form prewashed with water and methanol. The methanolic effluent (60 ml) is evaporated under reduced pressure at 30° C, the residue is dissolved in methanol (20 ml) and the solution once again is evaporated. The residual crude crystalline 1 - β - D - ribofuranosyl - 4 - methoxy - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one is dissolved (without any additional purification) in a 10% solution of dry ammonia in absolute methanol (4 ml) and the whole reaction mixture is allowed to stand in a stoppered flask for 30 minutes at room temperature (the product begins to deposit in the course of 5 minutes) and for 12 hours in a refrigerator at −10° C. The resulting 5 - azacytidine is collected with suction, washed with methanol and dried under reduced pressure. A yield of 0.216 g (88.6%) of 5 - azacytidine, m.p. 232—234° C (decomposition), is obtained.

EXAMPLE 2

A mixture of 1 - (2, 3, 5 - tri - O - benzoyl - β - D - ribofuranosyl) - 4 - methylthio - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one (0.5875 g), a 10% solution of dry ammonia in methanol 4 ml) and a normal methanolic sodium methoxide solution (1.2 ml) is stirred at room temperature with the exclusion of atmospheric moisture (a guard tube filled with potassium hydroxide pellets is fitted to the reaction vessel) until the starting compound dissolves (3 minutes). The resulting solution is allowed to stand for 45 minutes at room temperature after 30 minutes, the product begins to deposit) and for 12 hours in a refrigerator at −10° C. The crystals are collected with suction, washed with methanol and dried under reduced pressure. A yield of 0.162 g (66.4%) of 5 - aza - cytidine, m.p. 232—234° C (decomposition), is obtained.

EXAMPLE 3

A mixture of 1 - (2, 3, 5 - tri - O - benzoyl - β - D - ribofuranosyl) - 4 - methoxy - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one (0.5715 g), absolute methanol (5 ml) and a normal methanolic sodium methoxide solution (1 ml) is stirred at room temperature with the exclusion of atmospheric moisture (a guard tube filled with potassium hydroxide pellets is fitted to the reaction vessel) until the starting compound dissolves (5 minutes), and the resulting solution is allowed to stand for one hour at room temperature and then is processed as in Example 1. A yield of 0.219 g (89.7%) of 5 - azacytidine, m.p. 232—234° C (decomposition), is obtained.

EXAMPLE 4

A mixture of 1 - (2, 3, 5 - tri - O - benzoyl - β - D - ribofuranosyl) - 4 - methoxy - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one (0.5715 g), at 10% solution of dry ammonia in methanol (4 ml) and a normal methanolic sodium methoxide solution (1 ml) is stirred at room temperature with the exclusion of atmospheric moisture (a guard tube filled with potassium hydroxide pellets is fitted to the reaction vessel) until the starting compound dissolves (5 minutes). Work-up of the resulting solution is performed as in Example 2. A yield of 0.174 g (71.3%) of 5 - azacytidine, m.p. 232—234° C (decomposition), is obtained.

5 - Aza - 2' - deoxycytidine
1 - (2 - Deoxy - β - D - ribofuranosyl) - 4 - amino - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - one

EXAMPLE 5

A mixture of 1 - (3, 5 - di - O - p - toluyl - 2 - deoxy - β - D - ribofuranosyl) - 4 - methylthio - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one (0.4956 g), absolute methanol (10 ml) and a normal methanolic sodium methoxide solution (1.2 ml) is stirred magnetically at room temperature with the exclusion of atmospheric moisture (a guard tube filled with potassium hydroxide pellets is fitted to the reaction vessel) for 2 hours and 45 minutes (after 2 hours, the starting compound dissolves). The cations are then removed by passing the resulting solution

through a column of a weakly acidic cation exchange resin (10 ml) in the H^+ form (prewashed with water and methanol). The methanolic effluent is evaporated under reduced pressure at 30° C, the residue is dissolved in methanol (10 ml) and the solution once more is evaporated. The resulting crude viscous 1 - (2 - deoxy - β - D - ribofuranosyl) - 4 - methoxy - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - one is dissolved in a 10% solution of dry ammonia in methanol (2 ml) and the resulting reaction mixture is allowed to stand in a stoppered flask for 30 minutes at room temperature (after 15 minutes, the crystalline product begins to separate) and for 12 hours in a regrigerator at −10° C. The crystals are collected, washed with methanol and dried under reduced pressure. A yield of 0.180 g (79%) of 5 - aza - 2′ - deoxycytidine, m.p. 196—198° C (resolidification), is obtained.

EXAMPLE 6

A mixture of 1 - (3, 5 - di - O - p - toluyl - 2 - deoxy - β - D - ribofuranosyl) - 4 - methylthio - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - one (0.4956 g), a 10% solution of dry ammonia in methanol (4 ml) and a normal methanolic sodium methoxide solution (1.2 ml) is stirred magnetically at room temperature with the exclusion of atmospheric moisture (a guard tube filled with potassium hydroxide pellets is fitted to the reaction vessel) until the starting compound dissolves (45 minutes). The resulting solution is allowed to stand at room temperature for 1 hour (during this period of time, the crystalline product begins to separate) and then in a refrigerator at −10° for an additional 12 hours. The crystals are collected with suction, washed with methanol and dried under reduced pressure. A yield of 0.132 g (58%) of 5 - aza - 2′ - deoxycytidine, m.p. 198—199° C (resolidification), is obtained.

EXAMPLE 7

A mixture of 1 - (3, 5 - di - O - p - toluyl - 2 - deoxy - β - D - ribofuranosyl) - 4 - methoxy - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - one (0.4796 g), absolute methanol (10 ml) and a normal methanolic sodium methoxide solution (1 ml) is stirred magnetically at room temperautre with the exclusion of atmospheric moisture (a guard tube filled with potassium hydroxide pellets is fitted to the reaction vessel) until the starting compound dissolves (45 minutes). The reaction mixture then is processed as described in Example 6. A yield of 0.120 g (52.7%) of 5 - azo - 2′ - deoxycytidine, m.p. 198—199° C (resolidification), is obtained.

EXAMPLE 8

A mixture of 1 - (3, 5 - di - O - p - toluyl - 2 - deoxy - β - D - ribofuranosyl - 4 - methoxy - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - one (0.4796 g) a 10% solution of dry ammonia in methanol (4 ml) and a normal methanolic sodium methoxide solution (1.0 ml) is stirred magnetically at room temperature with the exclusion of atmospheric moisture (a guard tube filled with potassium hydroxide pellets is fitted to the reaction vessel) until the starting compound dissolves (45 minutes). The reaction mixture then is processed as described in Example 6. A yield of 0.120 g (52.7%) of 5 - aza - 2′ - deoxy - cytidine, m.p. 198—199° C (resolidification), is obtained.

1 - β - D - Ribofuranosyl - 4 - methoxy - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - one

EXAMPLE 9

A mixture of 1 - (2, 3, 5 - tri - O - benzoyl - β - D - ribofuranosyl) - 4 - methylthio - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - one (0.5875 g), absolute methanol (5 ml) and a normal methanolic sodium methoxide solution (1.2 ml) is stirred at room temperature with the exclusion of atmospheric moisture (a guard tube filled with potassium hydroxide pellets is fitted to the reaction vessel) until the starting compound dissolves (5 minutes). The resulting solution is allowed to stand at room temperature for an additional 40 minutes and is processed as described in Example 1. The crude crystalline product is recrystallised from absolute methanol. A yield of 0.210 g (81%) of 1 - β - D - ribofuranosyl - 4 - methoxy - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one, m.p. 177—179° C, is obtained.

1 - β - D - Ribofuranosyl - 4 - methylamino - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one

EXAMPLE 10

A mixture of 1 - β - D - ribofuranosyl - 4 - methoxy - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one (0.259 g) and a 7% solution of dry methylamine in absolute methanol (2 ml) is stirred at room temperature with the exclusion of atmospheric moisture (a guard tube filled with potassium hydroxide pellets is fitted to the reaction vessel) for 5 minutes, kept at room temperature for an additional 10 minutes and finally in a refrigerator at −10° C for 15 minutes. The crystals are collected with suction, washed with ice-cool methanol and dried under reduced pressure. A yield of 0.210 g (81.4%) of 1 - β - D - ribofuranosyl - 4 - methylamino - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - one, m.p. 148—150° C., is obtained.

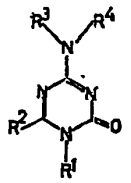
1 - β - D - Ribofuranosyl - 4 - dimethyl - amino - 1, 2 - dihydro - 1, 3, 5 - triazine - 2 - one

EXAMPLE 11

A mixture of 1 - β - D - ribofuranosyl - 4 - methoxy - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one (0.259 g) and a 7% solution of dry dimethylamine in absolute methanol (2 ml) is stirred at room temperature with the exclusion of atmospheric moisture (potassium hydroxide guard tube) for 5 minutes, kept at the same temperature for additional 15 minutes and evaporated under reduced pressure at 30° C. The residue is coevaporated with absolute methanol (5 ml) and finally dissolved in absolute ethanol (1 ml). The solution is cooled and stirred with a sharp edged rod to deposit crystals which are kept in a refrigerator at —10° C overnight, collected with suction, washed with ethanol and dried under reduced pressure. A yield of 0.216 g (79.4%) of 1 - β - D - ribofuranosyl - 4 - dimethyl - amino - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one, m.p. 128—130° C, is obtained.

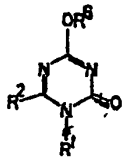
WHAT WE CLAIM IS:—

1. A process for preparing 1 - glycosyl - 5 - azacytosine of the general formula I



(1)

wherein R^1 designates a glycosyl residue, R^2 designates a hydrogen atom or an alkyl group having from 1 to 4 carbon atoms and R^3 and R^4, which are identical or different, designate hydrogen atoms, alkyl groups having from 1 to 4 carbon atoms or aralkyl groups having from 7 to 10 carbon atoms, comprising effecting reaction of a 1 - glycosyl - 4 - alkoxy - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one of the general formula III



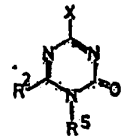
(3)

wherein R^1 and R^2 are as defined for general formula I and R^6 is an alkyl group having from 1 to 6 carbon atoms, with ammonia or an amine of the general formula IV

$$R^3—NH—R^4 \qquad IV$$

wherein R^3 and R^4 are as defined for general formula I.

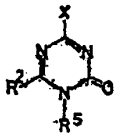
2. A process as claimed in claim 1, wherein the alkoxytriazinone of the general formula III is prepared by effecting reaction of an alkali metal alkoxide having from 1 to 6 carbon atoms with a compound of the general formula II



(2)

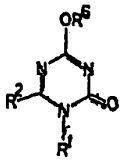
wherein R^2 is as defined for general formula I, R^5 is a per-acylglycosyl group wherein the acyl group has from 2 to 10 carbon atoms and X is an alkoxy or alkylthio group having from 1 to 4 carbon atoms.

3. A process for the preparation of a 1 - glycosyl - 5 - azacytosine of the general formula I defined in claim 1, comprising effecting reaction of a compound of the general formula II



(2)

wherein R^2 is as defined in claim 1 for general formula I, R^5 designates a per-acylglycosyl residue wherein the acyl group has from 2 to 10 carbon atoms and X designates an alkoxy or alkylthio group having from 1 to 4 carbon atoms, with an alkali metal alkoxide having from 1 to 6 carbon atoms, thus effecting the formation of a 1 - glycosyl - 4 - alkoxy - 1, 2 - dihydro - 1, 3, 5 - triazin - 2 - one of the general formula III



(3)

wherein R^1 and R^2 are as defined in claim 1 for general formula I and R^6 designates an alkyl group having from 1 to 6 carbon atoms, and effecting reaction of the alkoxytriazinone of the general formula III with ammonia or an amine of the general formula formula IV

$$R^3—NH—R^4 \qquad IV$$

wherein R^3 and R^4 are as defined in claim 1 for general formula I.

4. A process as claimed in Claim 2 or

claim 3 wherein the reaction between the compound of general formula II and the alkali metal alkoxide is carried out in a medium of an alkanol having from 1 to 6 carbon atoms.

5. A process as claimed in any one of claims 1 to 4 wherein the reaction of the alkoxytriazinone of general formula III with the ammonia or amine of general formula IV is carried out in a medium of an alkanol having from 1 to 6 carbon atoms.

6. A process as claimed in claim 4 or claim 5, wherein the alkanol is methanol.

7. A process as claimed in any one of claims 2, 3 and 4 or in claim 5 or claim 6 as appendant to any one of claims 2, 3 and 4, wherein in the compound of general formula II X is an alkylthio group and the molar ratio of the compound of the general formula II to the alkali metal alkoxide is 1:1.2.

8. A process as claimed in any one of the preceding claims, wherein the reaction of the alkoxytriazinone of general formula III with the ammonia or amine of general formula IV is performed at room temperature.

9. A process as claimed in any one of claims 2, 3 and 4, or in any one of claims 5 to 8 as appendant to any one of claims 2, 3 and 4, wherein the reaction of the compound of the general formula II with the alkali metal alkoxide is carried out at room temperature.

10. A process as claimed in claim 9, wherein the reaction of the compound of the general formula II with the alkali metal alkoxide is carried out in the absence of atmospheric moisture.

11. A process as claimed in any one of claims 2, 3 and 4, or in any one of the claims 5 to 10 as as appendant to any one of the claims 2, 3 and 4, wherein conversion of the compound of general formula II to the 1 - glycosyl - 5 - azacytosine of general formula I is carried out in one step by the simultaneous action of the alkali metal alkoxide and an alkanol solution of the ammonia or amine of general formula IV.

12. A process for preparing a 1 - glycosyl - 5 - azacytosine according to claim 1, substantially as hereinbefore described.

13. A process for preparing a 1 - glycosyl - 5 - azacytosine according to claim 3, substantially as hereinbefore described.

14. A process for the preparation of a 1 - glycosyl - 5 - azacytosine as described in any one of the Examples 1 to 9.

15. A process for the preparation of a 1 - glycosyl - 5 - azacytosine according to Example 10 or Example 11.

16. A 1 - glycosyl - 5 - azacytosine whenever prepared by the process claimed in any one of Claims 1 to 15.

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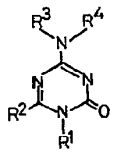
(54) A PROCESS FOR PREPARING A 1-GLYCOSYL-5-AZACYTOSINE

(71) We, CESKOSLOVENSKA AKADEMIE VED, a Corporation organised and existing under the laws of Czechoslovakia, of No. 3 Narodni, Prague 1, Czechoslovakia, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a process for preparing a 1 - glycosyl - 5 - azacytosine of the general formula I:



I

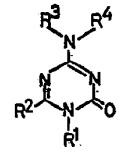
wherein R^1 designates a glycosyl residue, R^2 designates a hydrogen atom or an alkyl group and R^3 and R^4, which are identical or different, designate hydrogen atoms, alkyl groups or aralkyl groups.

British Patent Application No. 21,132/69 reports two compounds of the above mentioned general formula I, namely, 1 - β - D - ribofuranosyl - 4 - amino - 1,2 - dihydro - 1,3,5 - triazin - 2 - one (5 - azacytidine) and 1 - (2 - deoxy - β - D - ribofuranosyl) - 4 - amino - 1,2 - dihydro -1,3,5 - triazin - 2 - one (5 - aza - 2' - deoxycytidine). Both these compounds show a considerable biological activity, especially a high antileukemic effect. A similar biological activity can be expected also with some further 1 - glycosyl - 5 - azacytosines.

The preparation of 1 - glycosyl - 5 - azacytosines is reported in British Patent Specification Nos. 1,050,899 and 1,046,181 and in British Patent Application No. 21,132/69 (Serial No. 1227691). The per-acylglycosyl isocyanates are used as the starting materials. Addition of the latter compounds to *O* - alkylisoureas, S - alkylisothioureas or guanidines produces the corresponding derivatives of isobiuret, isothiobiuret or guanylurea, respectively. Condensation of the latter intermediates with orthoesters of aliphatic acids produces the corresponding alkoxy-, alkythio- and amino - triazinones. Transesterification of the latter compounds (or amination in the case of alkoxytriazinones and alkylthiotriazinones) produces the required 1 - glycosyl - 5 - azacytosines. The alkylthiotriazinones can be preferably converted to aminotriazinones via alkoxytriazinones. All these procedures are somewhat laborious and time consuming.

In connection with a detailed study of 1 - glycosyl - 5 - azacytosines we have discovered that the per - acylglycosyl isocyanates can be converted to 1 - (per - acylglycosyl) - 5 - azacytosines more advantageously by reaction with acylguanidines with the intermediary formation of N - (per - acylglycosyl) - N' - acylguanidines which are then (without isolation) subjected to cyclisation. The protecting acyl groups are removed in the usual manner, i.e. by ammonolysis or alcoholysis.

According to the present invention there is provided a process for preparing a 1 - glycosyl - 5 - azacytosine of the general formula I:



I

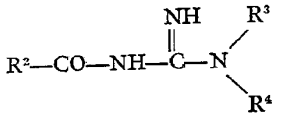
wherein R^1 designates a glycosyl residue, R^2 designates a hydrogen atom or an alkyl group,

[*Price 25p*]

and R^3 and R^4, which are identical or different, each designate a hydrogen atom, an alkyl group or an aralkyl group, which process comprises reacting a per - acylglycosyl isocyanate of the general formula II:

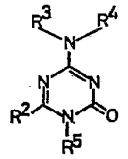
$$R^5—N=C=O \qquad II$$

wherein R^5 designates a per - acylglycosyl residue, with an acylguanidine of the general formula III:



III

wherein the symbols R^2, R^3 and R^4 designate the same as in the general formula I, to produce a 1 - (per - acylglycosyl) - 5 - azacytosine of the general formula IV:



IV

wherein the symbols R^2, R^3 and R^4 designate the same as in the formula I and R^5 designates the same as in formula II, and subjecting the latter compound to an alcoholysis or an ammonolysis.

Preferably the alkyl groups represented by R^2, R^3 and R^4 contain from 1 to 4 carbon atoms.

Preferably also the reaction between the compound of general formula II and the compound of general formula III is carried out in an inert solvent selected from acetonitrile, dimethylformamide and dimethyl sulfoxide, in the presence of a drying agent, preferably a molecular sieve.

Preferably also the alcoholysis is performed by means of an alkali metal alkoxide in an alkanol, containing preferably 1 to 6 carbon atoms, as solvent at room temperature, preferably sodium methoxide in methanol; and preferably the ammonolysis is performed by means of ammonia in an alkanol, preferably containing 1 to 6 carbon atoms, and more preferably in methanol.

The present invention will now be described by way of illustration, by the following Examples:—

EXAMPLE I

2',3',5' - Tri - O - benzoyl - 5 - azacytidine

A suspension containing formylguanidine (0.87 g), dry acetonitrile (40 ml) and molecular sieve Potassit 3 (5 g) was treated under magnetic stirring with a solution of crude 2,3,5 - tri - O - benzoyl - β - D - ribofuranosyl isocyanate (5.5 g) in dry acetonitrile (20 ml). The whole mixture was allowed to stand at room temperature overnight, refluxed for 30 minutes, and filtered. The filtrate was evaporated to dryness under reduced pressure, the residue was dissolved in chloroform, and the chloroform solution passed through a column packed with silica gel (200 g). To remove the contaminants, the column was washed with a solvent mixture (800 ml) of benzene and ethyl acetate (1:1). The product was eluted with ethyl acetate (1000 ml). The eluate was evaporated to dryness under reduced pressure and the residue crystallised from benzene (10 ml) to produce 2.22 g (40%) of 2',3',5' - tri - O - benzoyl - 5 - azacytidine. On heating, reports two compounds of the above mentioned at 135—140°C and melted at 179—182°C. After recrystallisation from ethanol, the product melted at 185—187°C without any previous change.

EXAMPLE II

5 Azacytidine

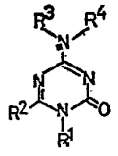
A suspension of 2',3',5' - tri - O - benzoyl - 5 - azacytidine (0.556 g) in absolute methanol (4 ml) was treated with 1N methanolic sodium methoxide (1 ml) and the mixture stirred at room temperature with exclusion of atmospheric moisture (potassium hydroxide guard tube) until the starting compound dissolved. The resulting solution was allowed to stand at room temperature for 20 minutes (after 5 minutes, the solution began to deposit the product) and then at −5°C for 3 hours. The product was collected by suction filtration, washed with methanol, and dried under reduced pressure. A yield of 0.235 g (96.3%) of 5 - azacytidine, m.p. 231—232°C (decomposition), was obtained.

EXAMPLE III

A suspension of 2',3',5' - tri - O - benzoyl - 5 - azacytidine (0.556 g) in a 10% solution (4 ml) of dry ammonia in absolute methanol was allowed to stand at −5°C in a stoppered flask with occasional stirring for 12 hours to deposit the product which was collected, washed with methanol, and dried under reduced pressure. A yield of 0.228 g (93.4%) of 5 - azacytidine, m.p. 229—230°C (decomposition), was obtained.

WHAT WE CLAIM IS:—

1. A process for preparing a 1 - glycosyl - 5 - azacytosine of the general formula I:

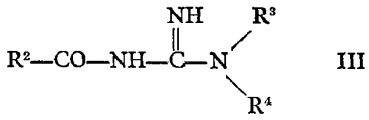


I

wherein R^1 designates a glycosyl residue, R^2 designates a hydrogen atom or an alkyl group, nd R^3 and R^4, which are identical or different, each designate a hydrogen atom, an alkyl group or an aralkyl group, which process comprises reacting a per - acylglycosyl isocyanate of the general formula II:

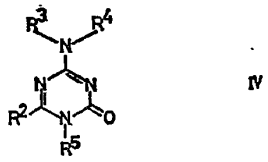
$$R^5—N=C=O \qquad II$$

wherein R^5 designates a per - acylglycosyl residue, with an acylguanidine of the general formula III:



III

wherein the symbols R^2, R^3 and R^4 designate the same as in the general formula I, to produce a 1 - (per - acylglycosyl) - 5 - azacytosine of the general formula IV:



IV

wherein the symbols R^2, R^3 and R^4 designate the same as in the formula I and R^5 designates the same as in the formula II; and subjectin the latter compound to an alcoholysis or an ammonolysis.

2. A process according to Claim 1, wherein the reaction between the compound of general formula II and the compound of general formula III is carried out in an inert solvent selected from acetonitrile, dimethylformamide and dimethyl sulfoxide.

3. A process according to Claim 2, wherein the inert solvent is used in the presence of a drying agent.

4. A process according to Claim 3, wherein the drying agent is a molecular sieve.

5. A process according to Claims 1 to 4, wherein the alkyl groups represented by R^2, R^3 and R^4 contain from 1 to 4 carbon atoms.

6. A process in accordance with any preceding claim, wherein the alcoholysis is performed at room temperature by the action of an alkali metal alkoxide in an alkanol.

7. A process according to Claim 6, wherein the alkanol contains from 1 to 6 carbon atoms.

8. A process according to any one of Claims 1 to 7, in which the alcoholysis is performed by the action of methanolic sodium methoxide.

9. A process in accordance with Claim 1, wherein the ammonolysis is performed by the action of ammonia in an alkanol.

10. A process according to Claim 9, in which the alkanol contains from 1 to 6 carbon atoms.

11. A process according to Claim 10, wherein the alkanol is methanol.

12. A process according to Claim 1, for the preparation of a 1 - glycosyl - 5 - azacytosine, substantially as hereinbefore described.

13. A process for the preparation of a 1 - glycosyl - 5 - azacytosine of the general formula I as defined in claim 1, as described in any one of the Examples.

14. A 1 - glycosyl - 5 - azacytosine whenever prepared by the process claimed in any one of the preceding claims.

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(54) Title: STABLE HIGHLY PURE AZACITIDINE AND PREPARATION METHODS THEREFOR

(57) Abstract: Disclosed herein are methods of obtaining highly pure 5-azacytidine, which contains minimal amounts of degradation impurities and methods of assessing the impurity profile of the degradation of cytidine analogues, such as 5-azacytidine

STABLE HIGHLY PURE AZACITIDINE AND PREPARATION METHODS THEREFOR

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This patent application claims the benefit of U.S. Provisional Patent Application No. 60/963,113, filed August 2, 2007, which is incorporated herein by reference.

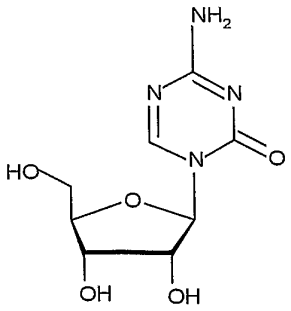
TECHNICAL FIELD

[0002] The present invention relates to methods of obtaining highly pure azacitidine containing minimal quantities of degradation impurities.

BACKGROUND OF THE INVENTION

[0003] Azacitidine, (5-azacytidine, Compound I), marketed by Pharmion under the trademark VIDAZA™, is the first drug approved by the United States Food and Drug Administration (FDA) for treating myelodysplastic syndromes (MDS), a diverse collection of hematological conditions united by ineffective production of blood cells and varying risks of transforming into acute myelogenous leukemia. Azacitidine is an anticancer medicine that exerts its antineoplastic effect by causing hypomethylation of DNA and direct cytotoxicity on abnormal hematopoietic cells in the bone marrow and thus is used for treating certain types of bone marrow cancers and blood cell disorders.

[0004] Azacitidine is an azacytosine nucleoside, having the chemical name 4-amino-1-β-D-ribofuranosyl-1,3,5-s-triazine-2(1H)-one, and the chemical structure:



5-azacytidine (I)

[0005] Azacitidine is a white to off-white solid, which is insoluble in acetone, ethanol, and methyl ethyl ketone; slightly soluble in ethanol/water (50/50), propylene glycol, and polyethylene glycol; sparingly soluble in water, water saturated octanol, 5% dextrose in water, N-methyl-2-pyrrolidone, normal saline, and 5% Tween 80 in water; and soluble in dimethylsulfoxide (DMSO).

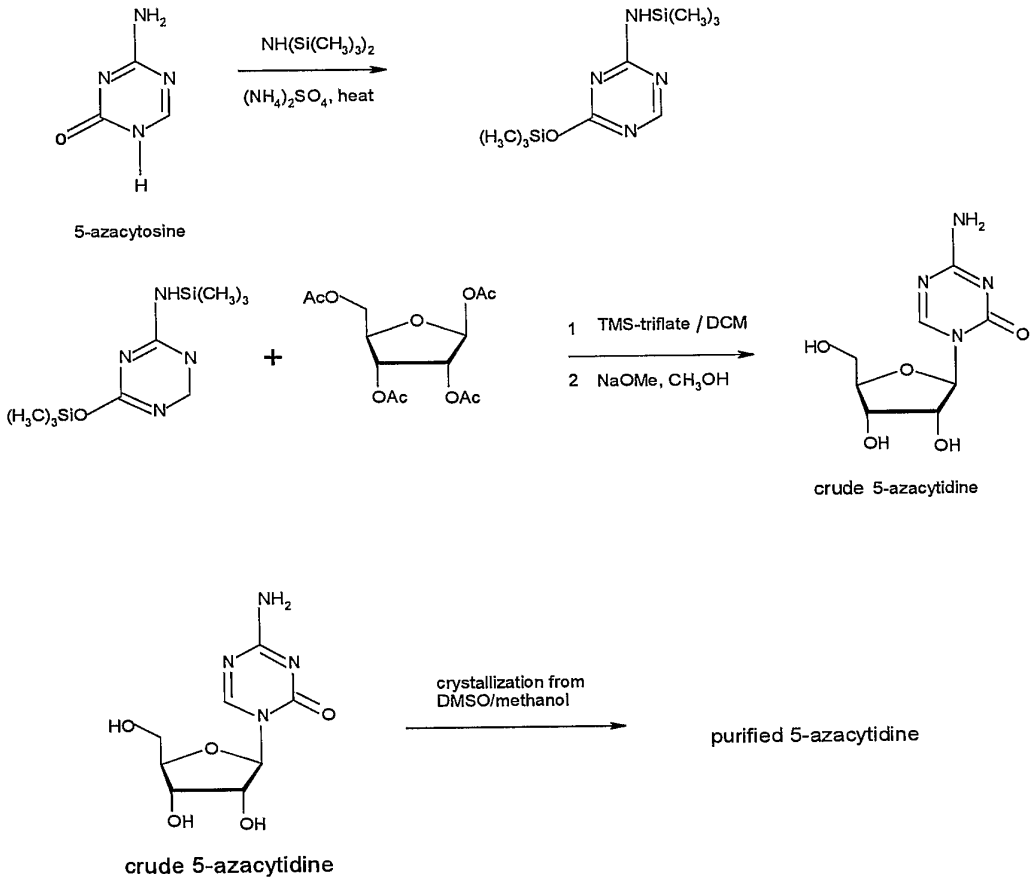
[0006] VIDAZA™ may be administered subcutaneously, wherein the drug is supplied in the form of a sterile powder for reconstitution and subcutaneous injection in vials containing 100 mg of azacitidine and 100 mg of mannitol as a lyophilized powder. Another route of administration is through a slow intravenous infusion over a period of 10-40 minutes.

[0007] 5-Azacytidine first was prepared via a multi-step synthesis starting from peracetylated 1-glycosyl isocyanate by Piskala and Sorm (*Collect. Czech. Chem. Commun.*, 29, 2060, 1964). Subsequently, 5-azacytidine was isolated as a new antibiotic by Hanka, et al. (*Antimicrob. Ag. Chemother.*, 619, 1966) from *Streptoverticillium ladakanus*.

[0008] U.S. Patent No. 7,038,038 (hereinafter the '038 patent) describes a process for preparing 5-azacytidine, which comprises the steps of: (a) reacting 5-azacytosine with a silylating reagent, e.g., 1,1,1,3,3,3-hexamethyldisilazane (HMDS), in the presence of ammonium sulfate at elevated temperature to yield a silylated 5-azacytosine, (b) coupling the reaction mixture of step (a) with 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose in dichloromethane in the presence of TMS-triflate followed by treatment with a mixture of sodium carbonate and sodium bicarbonate, (c) deprotecting the silylated azacitidine product of step (b) by adding sodium methoxide in methanol, and (d) purifying crude 5-azacytidine by crystallization from mixture of DMSO and methanol. The '038 patent does not disclose the purity of the obtained 5-azaytidine.

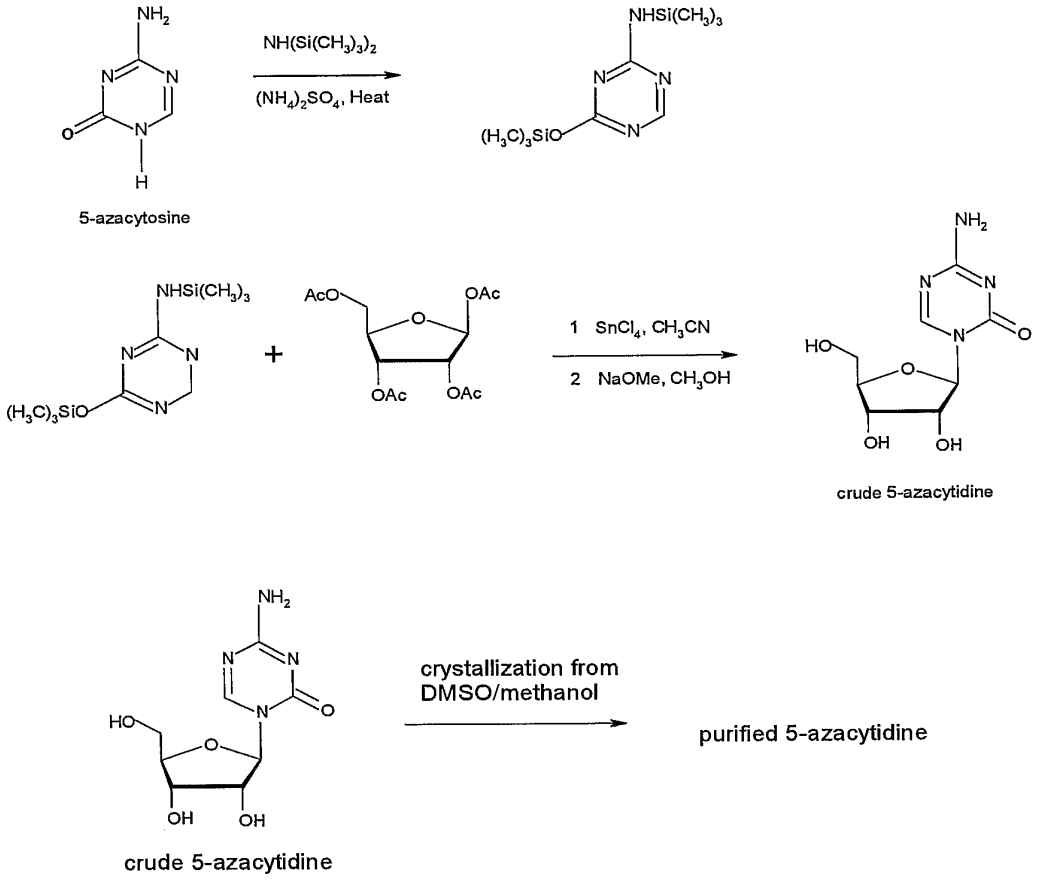
[0009] The following Scheme 1 illustrates the process of the '038 patent:

Scheme 1



[0010] A different procedure for preparing 5-azacytidine, which is based on the procedure of Vorbrueggen et.al., *J.Org.Chem.* Vol. 39, No.25, 1974, is described in Scheme 2 below. The process comprises the steps of: (a) reacting 5-azacytosine with a silylating reagent, e.g., 1,1,1,3,3,3-hexamethyldisilazane (HMDS), in the presence of ammonium sulfate at elevated temperature to yield a silylated 5-azacytosine, (b) coupling the reaction mixture of step (a) with 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose in acetonitrile in the presence of stannic chloride ($SnCl_4$), and (c) deprotecting the silylated azacitidine product of step (b) by adding sodium methoxide in methanol.

Scheme 2

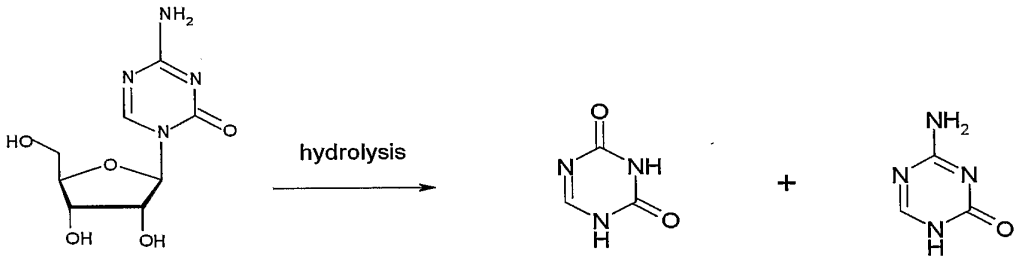


[0011] U.S. Patent No. 6,887,855, U.S. Patent No. 6,943,249 (hereinafter the '249 patent), and U.S. Patent No. 7,078,518 (hereinafter the '518 patent) describe eight crystalline forms of 5-azacytidine designated as forms I-VIII, along with an amorphous form. According to the examples of the '249 patent, Form I of 5-azacytidine is obtained by crystallization from solvent mixtures comprising a primary solvent (DMSO) and a co-solvent (e.g., ethanol, isopropanol, acetonitrile, etc.), but the '249 patent is silent with regard to the purity of the obtained product. It is mentioned in Example 1 of the '518 patent that the crude azacitidine was dissolved in DMSO preheated to about 90°C, then methanol was added to the DMSO solution. The co-solvent mixture was cooled to allow crystallization of 5-azacytidine crystals and the product was collected by filtration and dried. According to Examples 2, 3, and 4 of the '518 patent, 5-azacytidine was re-

crystallized from solvent mixtures of DMSO/toluene, DMSO/methanol, and DMSO/chloroform, and from N-methyl-2-pyrrolidone as a single solvent, but in no example was the purity or yield of the obtained product reported.

[0012] R.E. Notari and J.L. DeYoung in *Pharmaceutical Science*, Vol. 64, No. 7, July 1975, p 1148-1157, investigated the stability of 5-azacytidine in aqueous solution, concluding that it was relatively instable in comparison to cytidine. The hydrolytic degradation of 5-azacytidine was studied as a function of pH, temperature, and buffer concentration. For example, at pH 1, the main degradation products were 5-azacytosine and 5-azauracil, while at higher pH values, the degradation products were different. However, these degradation products were not detectable while being examined in acidic solutions as they were non-chromophoric. The following Scheme 3 describes the degradation products:

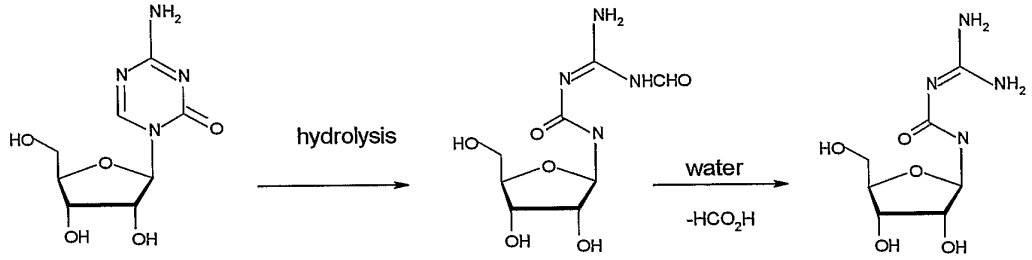
Scheme 3



5-azacytidine 5-azauracil 5-azacytosine

[0013] In another study, conducted by J.A. Beisler, *Journal of Medicinal Chemistry*, Vol. 21, No. 27, 1978, p 204-208, it is mentioned that during the prolonged intravenous infusion time of 5-azacytidine, facile drug decomposition occurs in aqueous formulations giving rise to products of unknown toxicity. Thus, HPLC analysis of 24 hours old aqueous solutions of 5-azacitidine revealed that the main degradation products are N-(formylamidino)-N'-β-D-ribofuranosylurea (Compound IV, RGU-CHO) and 1-β-D-ribofuranosyl-3-guanylurea (Compound V, RGU). The following Scheme 4 depicts the degradation products:

Scheme 4



5-azacytidine

N-(formylamidino)-N'-beta-D-ribofuranosylurea (Compound IV, RGU-CHO)

1-beta-D-ribofuranosyl-3-guanylurea (Compound V, RGU)

[0014] Thus, it is evident that 5-azacytidine is not stable and is prone to degradation in aqueous formulations. Furthermore, it is likely that purification of 5-azacytidine from a solvent that contains water will be not effective, due to a high level of instability in the presence of water. Hence, it is likely to find relatively high levels of degradation products in the commercial product. Therefore, there is a need for improved methods of preparing highly pure 5-azacytidine, which contains minimal amounts of degradation products, such as N-(formylamidino)-N'-β-D-ribofuranosylurea, particularly on a commercial scale. The present invention provides such methods, as will be apparent from the description of the invention provided herein.

SUMMARY OF THE INVENTION

[0015] It has been found by the inventors of the present invention, that while analyzing a sample of the drug VIDAZA™, which was purchased as a ready-to-use dosage form for pharmaceutical use, the purity of the compound 5-azacytidine was only 98.45%. Furthermore, the sample analysis showed that significant quantities of impurities were contained in the sample, which were identified as degradation products of 5-azacytidine.

[0016] Thus, the present invention provides methods of preparing highly pure 5-azacytidine, i.e., containing minimal amounts of degradation products, which is suitable for prolonged intravenous infusions, comprising:

(a) heating a solution of crude 5-azacytidine to at least about 45°C;

(b) allowing the solution of step (a) to cool to precipitate crystals of purified 5-azacytidine from the solution;

(c) optionally isolating, washing, and drying the crystals of step (b); and

(d) optionally slurrying the crystals of step (c) in a solvent, and filtering and drying the filtered crystals. In some embodiments, the isolating of step (c) comprises filtering.

[0017] In some cases, 5-azacytidine obtained by the methods provided herein, has a purity of at least 99% by weight, or at least 99.6% by weight.

[0018] In various cases, 5-azacytidine obtained by the methods provided herein contains less than about 0.2% by weight of at least one degradation product. In specific cases, the 5-azacytidine contains less than about 0.2% by weight N-(formylamidino)-N'-β-D-ribofuranosylurea (Compound IV, RGU-CHO) and/or less than about 0.1% of 1-β-D-ribofuranosyl-3-guanylurea (Compound V, RGU).

[0019] The present invention also provides a method of analyzing the impurity profile of 5-azacytidine, typically using chromatography, such as liquid or gas chromatography. Methods of liquid chromatography include, for example, Thin Layer Chromatography (TLC), High Pressure Liquid Chromatography (HPLC), and/or Liquid Chromatography/Mass spectrometry (LC-MS).

[0020] The method of analyzing the impurity profile of azacitidine typically comprises:

separating a sample comprising 5-azacytidine in an eluent using a liquid chromatography system (LC), wherein the LC system is equipped with a suitable stationary phase and is capable of separating the 5-azacytidine and any degradation products present in the sample; and

identifying, detecting, or both the presence of any degradation products in the sample using mass spectrometry (MS).

[0021] A sample of 5-azacytidine, which was withdrawn from the VIDAZA™ packaging for injectable suspension, was analyzed using the HPLC method detailed in Example 8, below. Three impurities were identified: RGU (Compound V), RGU-CHO (Compound IV) and Compound VI.

[0022] The present invention further provides a method of analyzing the degradation products of cytidine analogues, such as 5-azacytidine, 5-aza-2'-deoxycytidine, and zebularine (which is reported as stable in aqueous solution), that can be useful to establish a

degradation pathway of the cytidine analogue, 5-azacytidine, when exposed to degradation-inducing conditions.

[0023] According to one embodiment of the present invention, an induced degradation study on 5-azacytidine can be carried out in solid state conditions, as well as in liquid state conditions. Solid state conditions that can be used include, but are not limited to, storage conditions, ambient conditions, elevated temperature conditions, UV light conditions, and accelerated conditions. The liquid state conditions that can be used include, but are not limited to, photolysis conditions, acidic conditions, basic conditions, and oxidative conditions.

BRIEF DESCRIPTION OF THE FIGURES

[0024] Figure 1 depicts the thermogravimetric analysis (TGA) curve of the 5-azacytidine obtained according to Reference Example 1A

[0025] Figure 2 depicts the thermogravimetric analysis (TGA) curve of the 5-azacytidine obtained according to Reference Example 1B, entry 1.

[0026] Figure 3 depicts the thermogravimetric analysis (TGA) curve of the 5-azacytidine obtained according to Reference Example 1B, entry 2.

[0027] Figure 4 depicts the thermogravimetric analysis (TGA) curve of the 5-azacytidine obtained according to Reference Example 1B, entry 3.

DETAILED DESCRIPTION OF THE INVENTION

[0028] In one embodiment, the present invention provides methods of preparing pure 5-azacytidine, containing less than 0.2% by weight of at least one degradation product, which can be used for prolonged intravenous infusions, comprising:

(a) heating a solution of crude 5-azacytidine to at least about 45°C;

(b) allowing the solution of step (a) to cool to precipitate crystals of purified 5-azacytidine from the solution;

(c) optionally isolating, washing, and drying the crystals of step (b); and

(d) optionally slurrying the crystals of step (c) in a solvent, and filtering and drying the filtered crystals.

[0029] As used herein, the term "crude 5-azacytidine" refers to a 5-azacytidine sample having a purity up to 98.9% by weight, preferably up to about 98.5% by weight of 5-azacytidine. As used herein, the term "pure 5-azacytidine" or "purified 5-azacytidine" refers to a 5-azacytidine having a purity of at least 99.0% by weight, preferably at least 99.5% or at least 99.6% by weight of 5-azacytidine.

[0030] The solutions of crude 5-azacytidine can be heated to a temperature of at least about 45°C. The temperature can be at least about 50°C, at least about 55°C, at least about 60°C, at least about 65°C, at least about 70°C, at least about 75°C, at least about 80°C, at least about 85°C, at least about 90°C, at least about 95°C, or at least about 100°C. The temperature to which the solution is heated depends upon the solvent used to prepare the solution and the solvent's physical properties (e.g., boiling point), a determination of which is within the skill of a person of the relevant art.

[0031] Preferably, the solution of the crude 5-azacytidine is prepared using an organic solvent, non-limiting examples of which are N,N- dimethylformamide (DMF), N,N-dimethylacetamide (DMA), ethylene glycol, N-methyl-2-pyrrolidone, dimethylsulfoxide (DMSO), and mixtures thereof. In more preferred embodiments, the solvent is N,N-dimethylformamide (DMF), N,N-dimethylacetamide (DMA), or mixtures thereof.

[0032] Preferably, the solvents used for slurrying the crystals of 5-azacytidine include, but are not limited to, acetone, methyl ethyl ketone, methyl isobutyl ketone, ethyl acetate, n-propyl acetate, isoproyl acetate, n-butyl acetate, isobutyl acetate, ethanol, and mixtures thereof.

[0033] Preferably, the ratio of the crude 5-azacytidine to the solvent used in step (a), i.e., 5-azacytidine : solvent ratio, is about 1 gram (g) 5-azacytidine per at least 2 milliliter (ml) of solvent, preferably the ratio is about 1 g 5-azacytidine per about 10 to about 20 ml of solvent.

[0034] Preferably, 5-azacyitidine obtained by methods provided herein has a purity of at least 99% by weight, or at least 99.6% by weight. Preferably, 5-azacytidine obtained by methods provided herein contain less than about 0.2% by weight of N-(formylamidino)-N'-β-D-ribofuranosylurea (Compound IV, RGU-CHO) and/or less than about 0.1% by weight of 1-β-D-ribofuranosyl-3-guanylurea (Compound V, RGU).

[0035] According to the guidance "Q3C: Residual Solvents" published by the "International Conference on Harmonization of Technical Requirements of Registration of Pharmaceuticals for Human Use (ICH)" [A copy of this guidance can be found in the US Federal Register Volume 62, No. 247 (December 24, 1974) Docket 97D-0148, Appendixes 5-7: toxicological data for class 1-3 solvents respectively], the use of industrial solvents in active pharmaceutical ingredients is restricted according to their toxicity and safety features. The industrial solvents are divided into three main classes:

Class 1: Solvents to be avoided. These are solvents that should not be employed in the manufacture of drug substances or drug products because of their unacceptable toxicity or their deleterious environmental effect. Solvents that belong to this class are: benzene, carbon tetrachloride, 1,2-dichloroethane and others.

Class 2: Solvents to be monitored. These are solvents that should be limited in pharmaceutical products because of their inherent toxicity. Important industrial solvents that belong to this class are chlorinated solvents such as chloroform, dichloromethane, hydrocarbons such as hexane and aromatic solvents such as toluene.

Class 3: Solvents that are regarded as less toxic and of lower risk to human health. Important industrial solvents that belong to this class are certain ketones, esters, alcohols and others.

For example, according to the above mentioned Q3C guidance, the maximal concentration limit of some relevant solvents is summarized in Table 1.

Table 1

Solvent	Class	Maximal permitted concentration, ppm
Chloroform	2	60
Methanol	2	3000
Toluene	2	890
DMSO	3	5000 *
DMF	2	880
Acetone	3	5000 *

* The permitted level of a class 3 solvent is 5000 ppm (0.5%).

[0036] It has been found by the inventors of the present invention that the purification of 5-azacytidine by crystallization according to Example 2 or 3 of Patent US 7,078,518 yielded high levels of residual solvents (see Reference Examples 1A and 1B). On the other hand the

5-azacytidine of the present invention contains low levels of residual solvents. The inventors of the present invention also have found that when purification of 5-azacytidine was carried out overnight by crystallization from DMF at ambient temperature, the final product contained (after slurrying in acetone) 1780 ppm of DMF (Example 2). However, when purification of 5-azacytidine was carried out overnight by crystallization from DMF at a temperature of -20°C, the final product contained (after slurrying in acetone) only 165 ppm of DMF (Example 2A).

[0037] The 5-azacytidine obtained by the methods provided herein is stable under typical storage conditions for a solid, such as ambient temperatures (e.g., about 20°C to about 30°C) and relative humidities of up to about 60%. The term "stable" is used to refer to 5-azacytidine that retains at least about 85% of its initial amount under various storage conditions. In certain cases, the 5-azacytidine is stable after 1 month storage, after 2 months storage, after 3 months storage, after 4 months storage, after 5 months storage, or after 6 months storage. In some cases, the 5-azacytidine retains at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% of its initial amount.

[0038] 5-Azacytidine obtained by the methods provided herein can be used in pharmaceutical compositions for intravenous infusion or injection together with other acceptable additives and excipients, one non-limiting example of which is mannitol.

[0039] It has been found by the inventors of the present invention that a ready-to-use dosage of VIDAZA™ has a purity of the active pharmaceutical ingredient (API) (5-azacytidine) of only 98.7%. Furthermore, the sample analysis showed that significant quantities of 5-azacytidine degradation impurities were contained in the sample.

[0040] Thus, the present invention provides a method of analyzing a sample of 5-azacytidine to determine its purity and to identify and/or measure the impurities present in the sample. These analytical methods comprise the use of chromatography. The analyses of the samples are typically carried out using gas chromatography or liquid chromatography. Methods of liquid chromatography are, for example, Thin Layer Chromatography (TLC), High Pressure Liquid Chromatography (HPLC), and/or Liquid Chromatography/Mass spectrometry (LC-MS).

[0041] The method of analyzing a sample containing 5-azacytidine comprises:

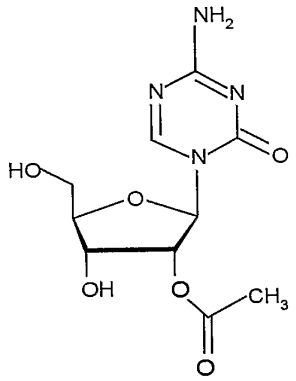
separating 5-azacytidine and 5-azacytidine degradation products in the sample using a liquid chromatography system (LC), wherein the LC system is equipped with a suitable stationary phase and is capable of separating the 5-azacytidine and 5-azacytidine degradation products; and

identifying and/or detecting the presence and/or amount of the 5-azacytidine degradation products in the sample using mass spectrometry (MS).

[0042] The suitable stationary phase of the LC system, which facilitates separation of the constituents of the 5-azacytidine sample, typically is a Reverse Phase (RP) stationary phase column, which can be a C4, C8, C14, C18, phenyl, or polymeric packing, e.g., polyamide, polymethacrylate, polystyrene, and the like. In some specific embodiments, the LC is equipped with a C18 stationary phase.

[0043] The sample of 5-azacytidine can be any sample, including, for example, those used for injectable suspensions and commercially synthesized 5-azacytidine.

[0044] Thus, a sample of 5-azacytidine, which was withdrawn from the VIDAZA™ packaging for injectable suspension, was analyzed by using the method disclosed herein (see Example 7, below). Three impurities were identified, that is RGU, RGU-CHO and Compound VI



Compound VI,

the results of which are summarized in Table 2.

Table 2

RRT*	Area %	m/z, M^{+1}	MS major fragments	Identified compound	Molecular weight
0.35	0.12	235.1	150.1, 132.6, 86.1, 72.2	RGU	234.2
0.64	1.31	263.1	150.1, 132.6, 114.1, 87.9, 72.2	RGU-CHO	262.2
2.06	0.13	286.9	174.7,113.1	Compound VI	286.2
1.00	98.45	244.9	133.0,113.0, 85.9	5-azacytidine	244.2

RRT=Relative Retention Time, where 1.00 is the retention time of 5-azacytidine

[0045] The results provided herein clearly demonstrate that the commercial 5-azacytidine sample, which was withdrawn from the VIDAZA™ packaging, has a purity of only 98.45%.

[0046] The present invention further provides a method of analyzing the structure of degradation products of a cytidine analogue, such as 5-azacytidine, to establish a degradation pathway of the cytidine analogue when exposed to degradation-inducing conditions.

[0047] The analysis of the impurity profiles of cytidine analogues, such as 5-azacytidine, formed under conditions of induced degradation can be performed using the methods disclosed herein, and, more specifically, using High Pressure Liquid Chromatography (HPLC), and/or Liquid Chromatography/Mass spectrometry (LC-MS), Fourier Transform Infra Red (FT-IR) spectroscopy, and a combination of methods thereof.

[0048] An induced degradation study on 5-azacytidine can be performed in solid state conditions, as well as in liquid state conditions. Solid state conditions include, but are not limited to, storage conditions, ambient conditions, elevated temperature conditions, UV light conditions, and accelerated conditions (e.g., high humidity and/or temperature). The liquid state conditions include but are not limited to, photolysis conditions, acidic conditions, basic conditions, and oxidative conditions.

[0049] Table 3 summarizes the various experimental conditions of induced degradation of 5-azacytidine. The diluent comprises a mixture of 30% 10 mM ammonium acetate and 70% THF.

Table 3

Entry	Degradation condition	State	Experimental conditions	Sample preparation
1	Storage	Solid	none	The sample was withdrawn directly from the package
2	Ambient	Solid	Exposure to visible light for 48 hours at 25°C	The sample of 5-azacytidine was used as is
3	Elevated temperature	Solid	Exposure to a temperature of 105°C for 48 hours	The sample of 5-azacytidine was used as is
4	UV light	Solid	Exposure to UV light for 48 hours at 25°C	The sample of 5-azacytidine was used as is
5	Accelerated conditions	Solid	Exposure to a temperature of 40°C and 75 relative humidity for 48 hours	The sample of 5-azacytidine was used as is
6	Photolysis	Liquid	Exposing a sample to UV light for 48 hours at 25°C	50 mg of 5-azacytidine was dissolved in 50 ml of the diluent
7	Acid hydrolysis	Liquid	Exposing a sample at 25°C for one hour	50 mg of 5-azacytidine was dissolved in 50 ml of 0.01M HCl
8	Basic hydrolysis	Liquid	Exposing a sample at 25°C for one hour	50 mg of 5-azacytidine was dissolved in 50 ml of 0.01M NaOH
9	Oxidation	Liquid	Exposing a sample at 25°C for one hour	50 mg of 5-azacytidine was dissolved in 25 ml of 10% hydrogen peroxide solution

[0050] Example 9 tests the induced degradation analysis of 5-azacytidine in solid state, wherein a slight change in color of the sample was observed when exposed to an elevated temperature. The FT-IR spectra did not show any significant changes. Furthermore, the HPLC analysis shows that the material is stable to heat and UV light as long as it is in solid state, as detailed in Tables 7 and 8 respectively.

[0051] Example 10 tests the induced degradation analysis of 5-azacytidine in liquid state, wherein the HPLC analysis shows significant degradation, as detailed in Table 9.

[0052] Example 11 tests the solution stability of the 5-azacytidine in the experimental conditions of the HPLC method, as disclosed herein. The results, which are summarized in Table 10 below, indicate that 5-azacytidine is stable within the average time period needed to complete the HPLC method, while being dissolved in the HPLC diluent.

[0053] Example 12 tests the solution stability of the 5-azacytidine in water. The results, which are summarized in Table 11 below, indicate that 5-azacytidine is unstable in water over prolonged time periods.

[0054] The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention and, in the following claims, are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0055] Preferred embodiments of this invention are described herein. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto.

EXAMPLES

Reference Example 1 (Prior Art Preparation)

[0056] This example demonstrates the preparation of 5-azacytidine according to prior art examples, e.g., Vorbrueggen et.al., *J.Org.Chem.* Vol. 39, No.25, 1974 and US Patent No. 7,038,038.

[0057] 5-Azacytosine (200 g, 1.8 mol) was mixed with 1,1,1,3,3,3-hexamethyldisilazane (HMDS) (800 ml, 619.36 g, 3.837 mol) and ammonium sulfate $(NH_4)_2SO_4$ (5 g, 37.8 mmol). The resulting mixture was heated to reflux for a period of 5 hours. Then, the

mixture was cooled to 60°C, and the excess HMDS was distilled off under reduced pressure. The residue was heated to 135°C for 30 minutes, and the product was cooled to ambient temperature to afford bis(trimethylsilyl)-5-azacytosine (404 g, 1.58 mol). The 5-azacytosine was dissolved in dry 1,2-dichloroethane (125 ml), and 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose (47 g, 0.1476 mol) was added. The reaction mixture was cooled to 5-10°C and a solution of $SnCl_4$ (42.18 g, 0.162 mol) in 1,2-dichloroethane (25 ml) was added dropwise over 15 minutes. The resulting mixture was stirred for 2 hours, during which time the temperature was allowed to reach ambient temperature. Sodium bicarbonate ($NaHCO_3$) (70 g) was added under constant mixing and the reaction mixture was cooled to 15°C. Purified water (140 ml) was added drop wise and mixing was maintained for additional 20 minutes, then 1,2-dichloroethane was added and mixing was maintained for 10 additional minutes. The organic and aqueous phases were separated, and the organic phase was filtered through a layer of Celite, washed with 1,2-dichloroethane, and dried over sodium sulfate (Na_2SO_4).

[0058] The organic solvent was evaporated, and the residue was dissolved in methanol (120 ml), then heated to 60°C to afford a clear solution. Charcoal (1.6 g) was added and the resulting mixture was stirred for 2 hours at ambient temperature. The charcoal was filtered off, and methanol/ammonia solution (200 ml of a 16% solution) was added to the filtrate and stirring was maintained for 20 hours at ambient temperature, during which time the reaction mixture solution gradually became viscous. Vacuum was applied to remove the excess ammonia, and the reaction mixture was cooled to 5°C. The resulting solid was filtered off, washed with methanol (3 X 30 ml) and dried to obtain crude 5-azacytidine (8 g, 21% yield) having purity of 98.7% (according to HPLC).

Reference Example 1A (Prior Art Preparation)

[0059] This example demonstrates the purification of 5-azacytidine by crystallization according to Example 2 of Patent US 7,078,518.

[0060] 5-azacytidine (5 g), having a purity of 98.7% and containing, inter alia, 0.14% by weight RGU-CHO and 0.09% by weight RGU, was dissolved in DMSO preheated to 90°C (100 ml), and toluene preheated to 50°C was added (900 ml) to the solution and mixed. The solution was cooled to ambient temperature overnight to form crystals. The resulting crystals were collected by filtration and air-dried to yield 5-azacytidine having a purity of

98.9% by weight, containing 0.33% by weight RGU-CHO. The sample contained 23.13% residual solvents, according to the TGA curve.

Reference Example 1B (Prior Art Preparation)

[0061] This example demonstrates the purification of 5-azacytidine by crystallization according to Example 3 of Patent US 7,078,518.

[0062] 5-azacytidine (5 g), having a purity of 98.7% and containing, inter alia, 0.14% by weight RGU-CHO and 0.09% by weight RGU, was dissolved in DMSO preheated to 90°C (100 ml), and a co-solvent (methanol, toluene, or chloroform) preheated to 50°C was added (900 ml) to the solution and mixed. The solution was cooled to -20°C overnight to form crystals. The resulting crystals were collected by filtration and air-dried to yield 5-azacytidine having purity and residual solvents content as detailed in Table 4.

Table 4

Entry	Solvent combination	Purity *	RGU-CHO content *	Residual solvents content **
1	DMSO/methanol	99.4%	0.06%	13.77%
2	DMSO/toluene	97.8%	0.36%	20.64%
3	DMSO/chloroform	97.6%	0.17%	31.65%

*According to HPLC. **According to TGA curve

Example 2

[0063] This example demonstrates the purification of 5-azacytidine by crystallization from N,N-dimethylformamide (DMF) at ambient temperature and slurrying in acetone.

[0064] In a 100 ml round flask, crude 5-azacytidine (0.5 g), having a purity of 98.7% and containing, inter alia, 0.14% by weight RGU-CHO and 0.09% by weight RGU, was mixed with DMF (10 ml), and the mixture was heated to 65°C to afford complete dissolution. The solution was cooled to ambient temperature overnight to form crystals. The resulting crystals were collected by filtration, washed twice with DMF, and filtered to obtain a wet solid. The solid was slurried for four hours in dry acetone (20 ml), filtered, washed with acetone and dried under reduced pressure to yield 5-azacytidine having a purity of 99.6% by weight, containing 0.1% by weight RGU-CHO and 0.3% by weight of other impurities (as

measured by HPLC). No traces of RGU were found in this sample. The sample contained 1780 ppm of DMF and 1340 ppm of acetone.

Example 2A

[0065] This example demonstrates the purification of 5-azacytidine by crystallization from N,N-dimethylformamide (DMF) at a temperature of -20°C and slurrying in acetone.

[0066] Crude 5-azacytidine (115 g), having a purity of 98.7% and containing, inter alia, 0.14% by weight RGU-CHO and 0.09% by weight RGU, was mixed with DMF (1725 ml), and the mixture was heated to 100°C to afford complete dissolution. The solution was cooled under mixing to a temperature of -20°C over a period of two hours and left at that temperature overnight to form crystals. The resulting crystals were collected by filtration, washed twice with acetone (2X50 ml) and filtered to obtain a wet solid. The solid was slurried at ambient temperature for 4 hours in acetone (3000 ml), filtered, washed twice with acetone (2X100 ml) and dried at a temperature of 80°C under reduced pressure to yield 5-azacytidine having a purity of 99.95% by weight, containing 0.01% by weight RGU-CHO and 0.02% of RGU. The sample contained 165 ppm of DMF and 781 ppm of acetone.

Example 3

[0067] This example demonstrates the purification of 5-azacytidine by crystallization from N,N-dimethylformamide (DMF).

[0068] In a 250 ml round flask, crude 5-azacytidine (5 g), having a purity of 98.7% by weight and containing, inter alia, 0.14% by weight RGU-CHO and 0.09% by weight RGU, was mixed with dry DMF (100 ml), and the mixture was heated to 100°C to afford complete dissolution. The solution was cooled to ambient temperature, then to 5°C overnight to form crystals. The resulting crystals were collected by filtration, washed twice with DMF, and dried at 80°C under reduced pressure to yield 1.5 g of 5-azacytidine having a purity of 99.7% by weight and containing 0.27% by weight RGU-CHO and 0.03% by weight of other impurities (as measured by HPLC). No traces of RGU were found in this sample.

Example 4

[0069] This example demonstrates the purification of 5-azacytidine by crystallization from N,N-dimethylacetamide (DMA).

[0070] In a 250 ml round flask crude 5-azacytidine (5 g), having a purity of 98.7% by weight and containing, inter alia, 0.14% by weight RGU-CHO and 0.09% by weight RGU, was mixed with dry DMF (50 ml), and the mixture was heated to 100°C to afford complete dissolution. The solution was cooled to ambient temperature, then to 5°C overnight to form crystals. The resulting crystals were collected by filtration, washed twice with DMF, and dried at 80°C under reduced pressure to yield 5-azacytidine having a purity of 99.7% by weight and containing 0.22% by weight RGU-CHO and 0.08% by weight of other impurities (as measured by HPLC). No traces of RGU were found in this sample. The sample contained 2000 ppm of DMA

Example 5

[0071] This example demonstrates the purification of 5-azacytidine by first crystallization from N,N-dimethylacetamide (DMA) and second crystallization from N,N-dimethylformamide (DMF).

[0072] In a 250 ml round flask crude 5-azacytidine (5 g), having a purity of 98.7% by weight and containing, inter alia, 0.14% by weight RGU-CHO and 0.09% by weight RGU, was mixed with dry DMA (50 ml), and the mixture was heated to 100°C to afford complete dissolution. The solution was cooled to ambient temperature overnight to form crystals. The resulting crystals were collected by filtration and triturated twice with dry acetone. The wet material was mixed with dry DMF (50 ml), and the mixture was heated to 100°C to afford complete dissolution. The solution was cooled to ambient temperature overnight to form crystals. The resulting crystals were collected by filtration, washed twice with DMF and dried at 80°C under reduced pressure to yield 5-azacytidine having a purity of 99.7% by weight and containing 0.02% by weight RGU-CHO, 0.04% RGU by weight and 0.24% by weight of other impurities (as measured by HPLC).

Example 6

[0073] This example demonstrates the purification of 5-azacytidine by crystallization from dimethylsufoxide (DMSO) and slurrying in acetone.

[0074] In a 100 ml round flask crude 5-azacytidine (1 g), having a purity of 98.7% by weight and containing, inter alia, 0.14% by weight RGU-CHO and 0.09% by weight RGU, was mixed with DMSO (2 ml), and the mixture was heated to 100°C to afford complete

dissolution. The solution was cooled to ambient temperature overnight to form crystals. The resulting crystals were collected by filtration, washed twice with DMSO, and filtered to obtain a wet solid. The solid was slurried for an hour with dry acetone (20 ml), filtered, and dried under reduced pressure to yield 5-azacytidine having a purity of 99.1% by weight and containing 0.26% by weight RGU-CHO and 0.64% by weight of other impurities (as measured by HPLC). No traces of RGU were found in this sample.

Example 7

[0075] This example demonstrates the purification of 5-azacytidine by slurrying in acetone.

[0076] In a 100 round flask, crude 5-azacytidine (2g), having a purity of 98.7% by weight and containing, inter alia, 0.14% by weight RGU-CHO and 0.09% by weight RGU, was mixed with dry acetone (10 ml) at ambient temperature and left overnight to form a solid. The solid was collected by filtration, washed twice with acetone, and dried to yield 5-azacytidine having a purity of 99.5% by weight and containing 0.11% by weight RGU-CHO and 0.39% by weight of other impurities (as measured by HPLC), as depicted in Entry 5 of Table 3. No traces of RGU were found in this sample. The impurities profile which was obtained in several experiments which were carried out for purification of 5-azacytidine by slurrying in acetone, are further detailed in Table 5 marked as entries 1-4.

Table 5

Entry	Relative Retention Time (RRT)										Total other impurities by % area (excluding RGU-CHO)
	0.44	0.49	0.80	1.23	2.32	2.50	2.57	2.65	2.84	2.96	
	Area (%)										
1	0.01	0.04		0.01	0.22		0.03				0.3
2								0.02	0.01		0.03
3	0.03	0.04	0.01								0.08
4	0.04	0.10	0.08	0.02	0.06		0.11	0.19	0.04		0.64
5		0.03	0.02		0.07	0.08				0.04	0.39

Example 8

[0077] This example details HPLC method parameters for analyzing 5-azacytidine samples.

[0078] The HPLC measurements were performed using a system equipped with an Inertsil C18 column (ODS-2, 5 microns, 250X4.6 mm (ODS-167)). Other parameters of the system were as follows:

Detection:	UV detector operated on 242 nm
Column temperature:	20°C
Run time:	45 minutes
Injection volume:	10µl
Flow rate:	1.0 ml/minute
Sample set temperature:	5°C
Sample concentration:	about 1.65 mg/ml
Diluent:	Mixture of 30% 10 mM ammonium acetate and 70% THF

[0079] Analyses were performed using the following mobile phase

Mobile Phase (Eluent) A:	10 mM ammonium acetate
Mobile Phase (Eluent) B:	60% 10 mM ammonium acetate, 40% MeOH

[0080] The HPLC gradient is detailed in Table 6.

Table 6

Time (minutes)	Eluent A %	Eluent B %
0	95	5
10	95	5
20	30	70
45	30	70
45.1	95	5
52	95	5

Example 9

[0081] This example details the preparation of samples for the induced degradation analysis in solid state.

[0082] **Ambient conditions** A 5-azacytidine sample (about 0.2 g) was spread uniformly in a Petri dish and exposed to visible light in the laboratory for 48 hours.
Elevated temperature A 5-azacytidine sample (about 0.2 g) was spread uniformly in a Petri dish and exposed to 105°C for 48 hours.
UV light (Photolysis) A 5-azacytidine sample (about 0.2 g) was spread uniformly in a Petri dish as a thin layer and was covered with a transparent glass Petri dish lid. The sample was placed in a UV chamber and exposed to UV light for 48 hours.
Accelerated conditions [40±2°C/75±5% Relative Humidity (RH)]. A 5-azacytidine sample (about 0.2 g) was spread uniformly in a Petri dish and exposed to 40±2°C/75±5% relative humidity for 48 hours.
At the end of the stipulated time period, the physical descriptions of each sample were noted down. Identification tests were performed by FT-IR, and purity checks were performed by HPLC analysis. The protected sample, as defined herein, is the reference storage material used for carrying out the experiments detailed in Tables 7 and 8.

[0083] The results of induced degradation study of 5-azacytidine in solid state by observation as well as FT-IR tests is summarized in Table 7.

Table 7

Degradation conditions	Period of exposure (hours)	Observation	
		Description	IR spectrum
Protected sample	---	White to off white powder	-----
Ambient conditions	48	White to off white powder	Comparable with protected sample IR spectrum
Elevated temperature	48	Off white to cream color powder	Comparable with protected sample IR spectrum
UV light	48	White to off white powder	Comparable with protected sample IR spectrum
Accelerated conditions	48	White to off white powder	Comparable with protected sample IR spectrum

[0084] Table 8 below details the results obtained by HPLC measurements for solid state degradation.

Table 8

Degradation conditions	Test results (by HPLC)				
	Relative Retention Time (RRT) *				Total impurities (%)
	0.63	1.00	2.01	2.05	
	Area (%)				
Protected sample (storage)	1.55	96.72	1.37	0.24	3.28
Exposure to ambient conditions	1.52	96.75	1.36	0.27	3.25
Exposure to elevated temperature (105°C)	2.00	96.61	1.01	0.17	3.39
Exposure to UV light	1.66	96.64	1.35	0.24	3.36
Accelerated conditions (40±2°C, 75±5% RH)	1.90	97.04	0.80	0.14	2.96

* RRT of 5-azacytidine (set at 1.00). RH= Relative Humidity. The differences in the results are within the experimental error.

Example 10

[0085] This example details the preparation of samples for the induced degradation analysis of liquid conditions.

[0086] **Acidic hydrolysis - blank preparation**: Hydrochloric acid (5ml, 0.01M HCl) was diluted to 10 ml with the diluent. **Acidic hydrolysis - Preparation of sample solution**: A 5-azacytidine sample (50 mg) was dissolved in 0.01M HCl (25 ml) and mixed at room temperature for about 1 hour. An aliquot (5 ml) was diluted to 10 ml with the diluent. The blank preparation and sample preparation were injected to the HPLC system by using the chromatographic conditions as mentioned in example 8.

Basic hydrolysis - blank preparation: Sodium hydroxide (5ml, 0.01M NaOH) was diluted to 10 ml with the diluent. **Basic hydrolysis - preparation of sample solution**: A 5-azacytidine sample (50 mg) was dissolved in 0.01M NaOH (25 ml) and mixed at room temperature for about 1 hour. An aliquot (5 ml) was diluted to 10 ml with diluent. The blank preparation and sample preparation were injected to the HPLC system using the chromatographic conditions as detailed in example 8.

Oxidation - blank preparation: Hydrogen peroxide (5 ml, 10% solution) was poured into a clean and dry 10 ml volumetric flask and filled up to the mark with the diluent.

Oxidation -preparation of sample solution: A 5-azacytidine sample (50 mg) was dissolved in 10% hydrogen peroxide solution (25 ml) and mixed at room temperature for

about 1 hour. An aliquot (5 ml) was diluted to 10 ml with the diluent. The blank and sample preparations were injected to the HPLC system using the chromatographic conditions as detailed in example 8.

Photolysis - blank preparation: The diluent (50 ml) was mixed under UV light for 48 hours. **Photolysis - preparation of sample solution**: A 5-azacytidine sample (50 mg) was dissolved in the diluent (50 ml) and the solution was exposed to UV light under mixing for 48 hours. The blank preparation and sample preparation were injected to the HPLC system using the chromatographic conditions as mentioned in example 8.

[0087] Table 9 below details the results obtained for liquid state degradation

Table 9

Degradation conditions	Test results (by HPLC)									
	Relative Retention Time (RRT) *									Total impurities (%)
	0.33	0.39	0.42	0.63	1.00	1.18	1.65	2.01	2.05	
	Area (%)									
Storage	-	-	-	1.55	96.72	-	-	1.37	0.24	3.28
Acidic	0.17	-	-	21.1	71.96	5.35	0.14	1.10	-	28.04
Basic	89.92	-	3.97	-	3.38	-	-	-	-	96.62
Oxidation	0.84	-	0.06	0.23	97.18	-	-	1.62	-	2.82
Photolysis	0.85	0.11	0.20	35.24	61.64	0.46	0.39	0.86	0.17	38.36

Example 11

[0088] This example details the solution stability of the 5-azacytidine in the experimental conditions of the HPLC method.

[0089] A sample of 5-azacytidine in the diluent (about 1.65 mg/ml) was withdrawn from the flask (which was kept at the HPLC conditions as detailed in example 7) on every consecutive hour and injected to the HPLC system. The results, which are summarized in Table 10, demonstrate the stability of 5-azacytidine in prolonged dilution in the HPLC diluent.

Table 10

Time (Hours)	Relative Retention Time (RRT) *				Total impurities by % area
	0.63	1.00	2.01	2.05	
	Area (%)				
0	1.55	96.72	1.37	0.24	3.28
4	2.04	96.21	1.41	0.24	3.79
5	2.16	96.11	1.37	0.24	3.89
6	2.30	96.00	1.36	0.24	4.00
7	2.43	95.85	1.37	0.23	4.15
8	2.55	95.74	1.37	0.24	4.26
9	2.66	95.63	1.37	0.24	4.37
10	2.77	95.50	1.37	0.24	4.50
11	2.87	95.40	1.37	0.24	4.60

* RRT of 5-azacytidine

Example 12

[0090] This example details the solution stability of the 5-azacytidine in water.

[0091] A sample of 5-azacytidine was dissolved in water in a flask to form a solution having concentration of about 1.65 mg/ml. Samples were withdrawn from the flask every consecutive hour and injected to the HPLC system. The results, which are summarized in Table 11, demonstrate the instability of 5-azacytidine in prolonged dilution in water.

Table 11

Time (Hours)	Relative Retention Time (RRT) *				Total impurities by % area
	0.63	1.00	1.70	2.01	
	Area (%)				
0	0.64	97.47	-	1.59	2.53
1	2.16	95.97	-	1.57	4.03
4	6.86	91.38	-	1.46	8.62
8	14.68	83.61	0.23	1.36	16.39
12	21.28	77.01	0.35	1.25	22.99

* RRT of 5-Azacytidine

What is Claimed:

1. A method of purifying 5-azacytidine comprising:

(a) heating a solution of crude 5-azacytidine to at least 45°C;

(b) allowing the solution of step (a) to cool to precipitate crystals of purified 5-azacytidine from the solution;

(c) optionally isolating, washing, and drying the crystals of step (b); and

(d) optionally slurrying the crystals of step (c) in a solvent, and filtering and drying the filtered crystals,

wherein the crystals of 5-azacytidine of step (b), (c), or (d) have a purity of at least 99.0% by weight of 5-azacytidine and contain up to 0.2% by weight of any individual degradation product of 5-azacytidine.

2. The method of claim 1, wherein the crystals of 5-azacytidine of step (b), (c), or (d) contain less than 0.1% by weight of any individual degradation product of 5-azacytidine.

3. The method of claim 1, wherein the solution of crude 5-azacytidine comprises a solvent selected from the group consisting of N,N- dimethylformamide, N,N-dimethylacetamide, ethylene glycol, N-methyl-2-pyrrolidone, dimethylsulfoxide, and mixtures thereof.

4. The method of claim 3, wherein the solution of crude 5-azacytidine comprises N,N-dimethylformamide, N,N-dimethylacetamide, or a mixture thereof.

5. The method of claim 1, wherein the solvent of step (d) comprises acetone, methyl ethyl ketone, methyl isobutyl ketone, ethyl acetate, n-propyl acetate, isoproyl acetate, n-butyl acetate, isobutyl acetate, ethanol, or a mixture thereof.

6. The method of claim 1, wherein the ratio 5-azacytidine : solvent of the crude 5-azacytidine to the solvent of step (a) is about 1 g 5-azacytidine per at least 2 ml solvent.

7. The method of claim 6, wherein the 5-azacytidine : solvent ratio is 1 g 5-azacytidine per 10 to 20 ml solvent.

8. The method of claim 1, wherein the 5-azacytidine has a purity of at least 99.0% by weight.

9. The method of claim 8, wherein the 5-azacytidine has a purity at least 99.6% by weight.

10. 5-azacytidine having less than 0.2% by weight of N-(formylamidino)-N'-β-D-ribofuranosylurea.

11. The 5-azacytidine of claim 10 having less than 0.1% by weight of N-(formylamidino)-N'-β-D-ribofuranosylurea.

12. 5-azacytidine having less than 0.1% by weight of 1-β-D-ribofuranosyl-3-guanylurea.

13. 5-azacytidine containing less than 200 ppm DMF and/or less than 1000 ppm acetone as residual solvents.

14. A pharmaceutical composition comprising the 5-azacytidine of claim 8 and a pharmaceutically acceptable excipient.

15. The pharmaceutical composition of claim 14, further comprising mannitol.

16. The method of claim 1, wherein the crystals of step (b), (c), or (d) are stable under storage conditions for at least 3 months.

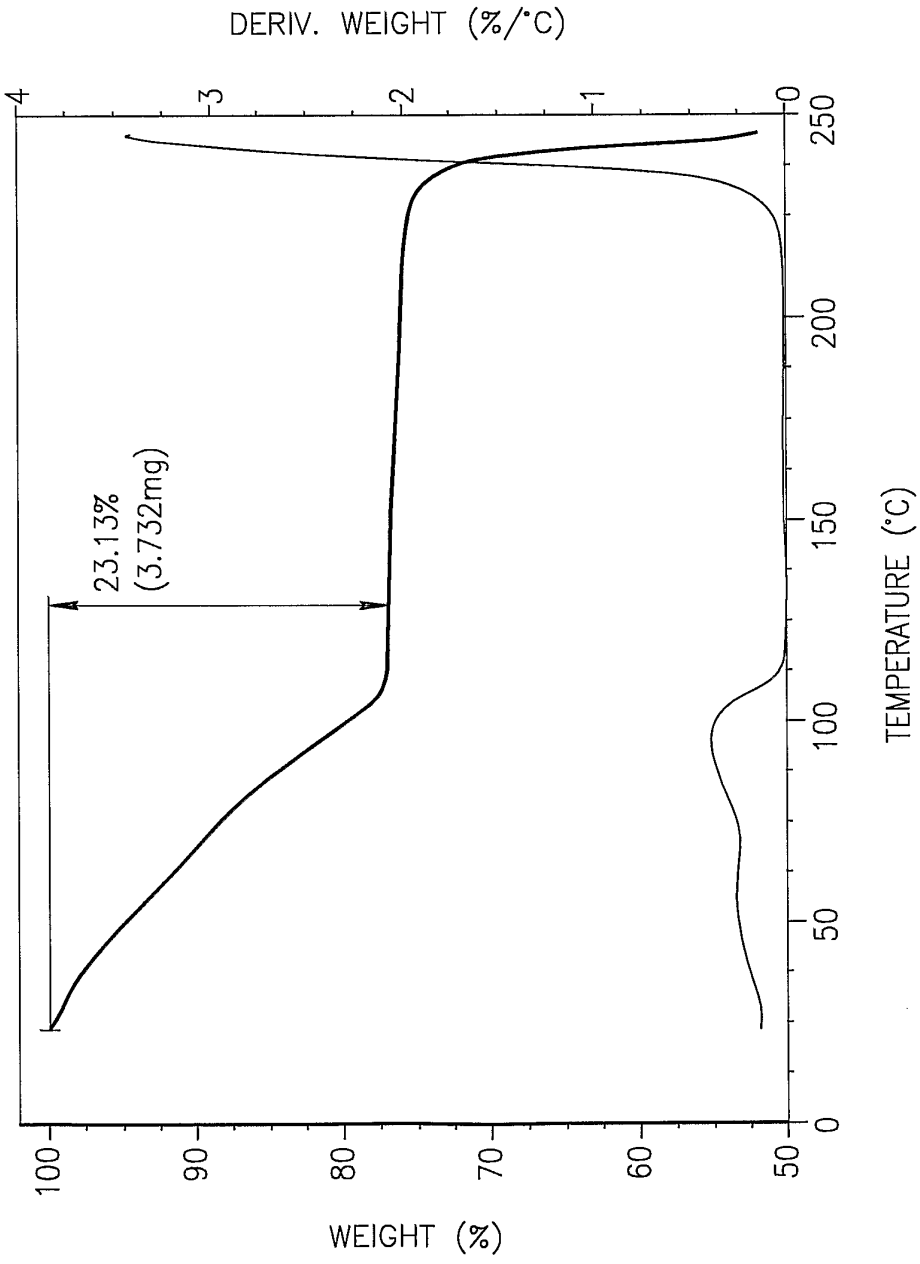


FIG.1

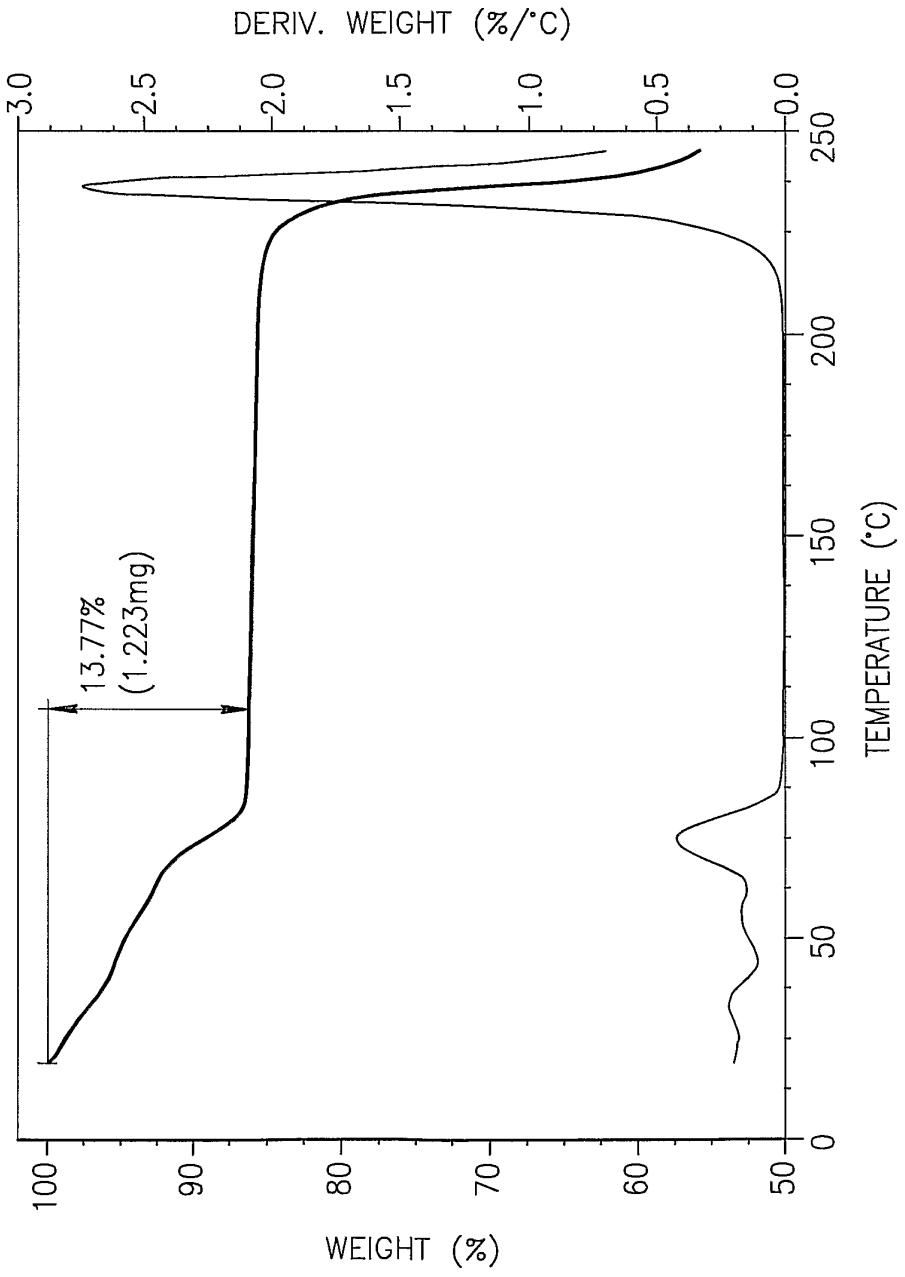


FIG.2

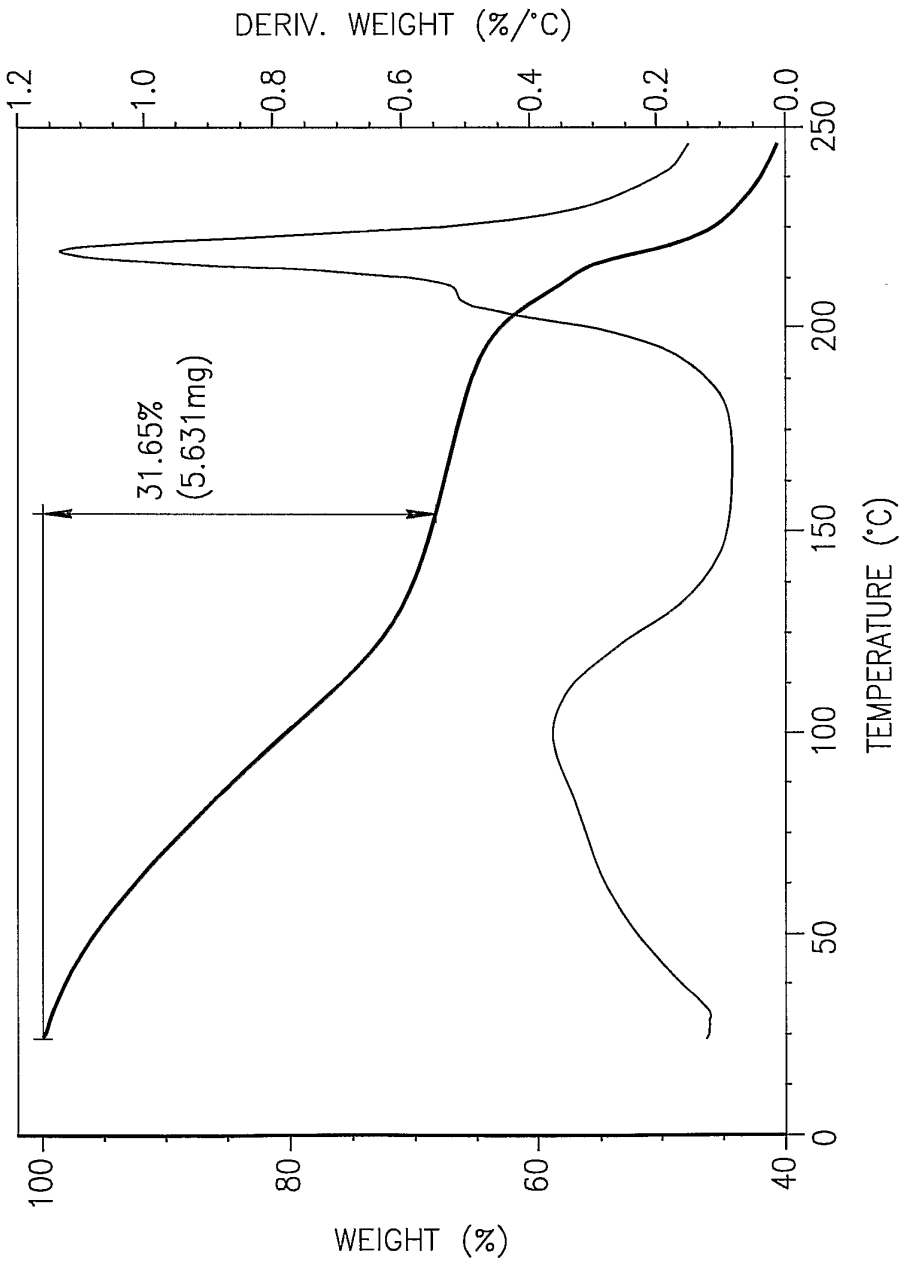


FIG.3

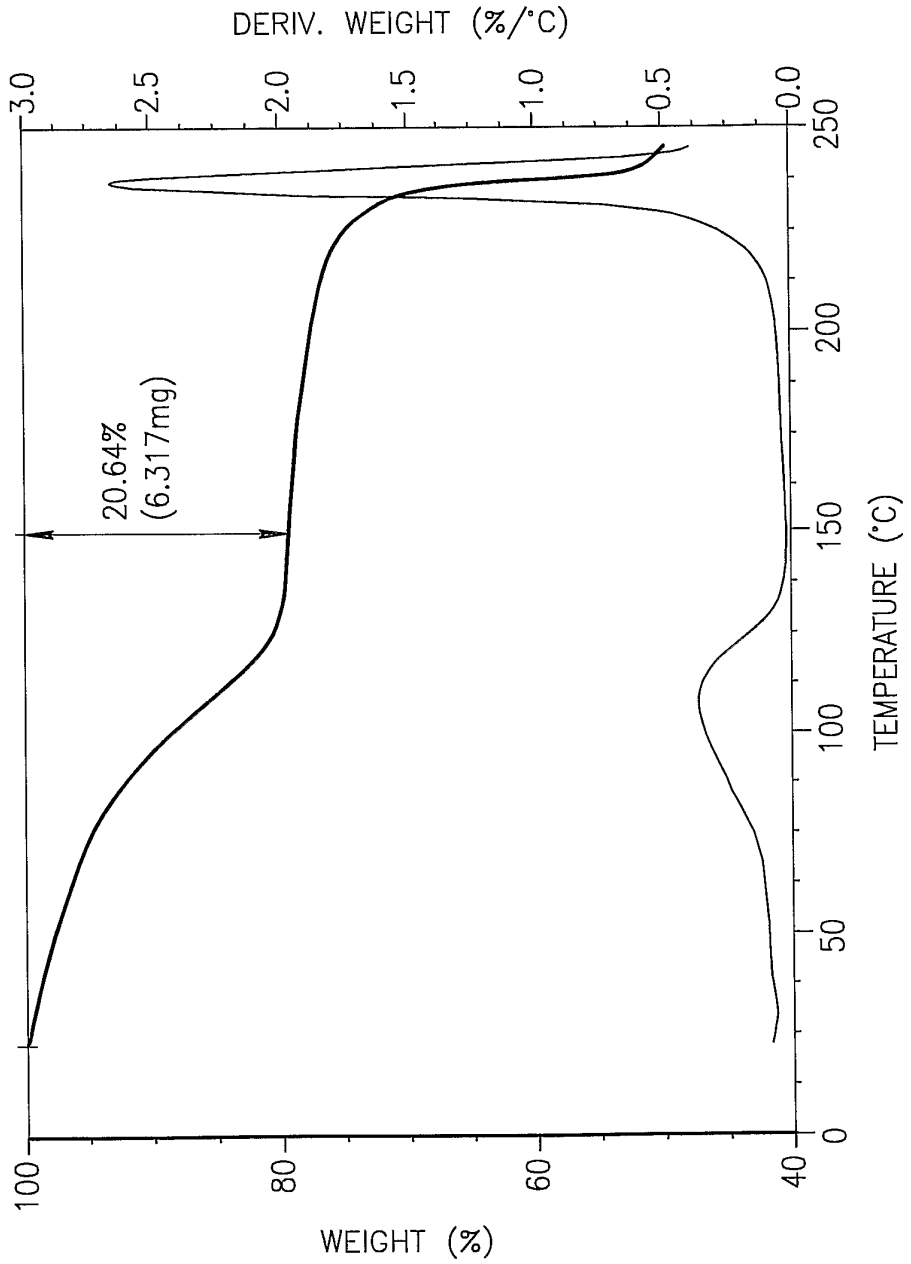


FIG.4

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(54) **Title**: STABLE FORMULATIONS OF AZACITIDINE

(57) **Abstract**: Compositions including azacitidine and a pharmacologically suitable fluid are disclosed. The pharmacologically suitable fluid is preferably non-aqueous and includes propylene glycol, polyethylene glycol, or mixtures thereof; and a surfactant.

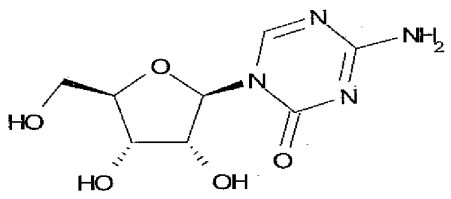
STABLE FORMULATIONS OF AZACITIDINE

Cross-reference to Related Applications

This application claims the benefit of priority from U.S. Provisional Patent Application No. 61/229,832, filed July 30, 2009, entitled "STABLE FORMULATIONS OF AZACITIDINE," the contents of which are incorporated herein by reference.

Background of the Invention

Azacitidine is a pyrimidine nucleoside analog. The structure of azacitidine is



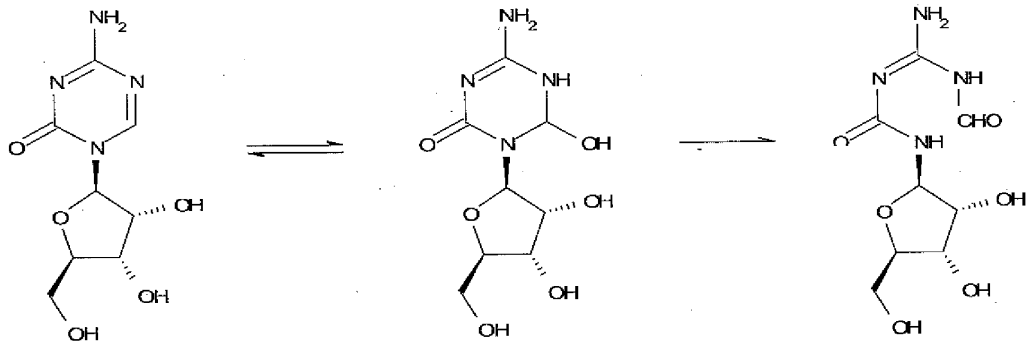
Azacitidine

Azacitidine is used in the treatment of myelodysplastic syndrome subtypes: refractory anemia or refractory anemia with ringed sideroblasts (if accompanied by neutropenia or thrombocytopenia or requiring transfusions), refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, and chronic myelomonocytic leukemia. It is believed that azacitidine causes hypomethylation of DNA and direct cytotoxicity on abnormal hematopoietic cells in bone marrow.

Azacitidine (ACD) use is not widespread due to limitations of the formulation, specifically the chemical stability of the reconstituted lyophile is very short. A simple solution in water loses around 30% of its drug content over a period of 16 hours and at least 9% over the first 2 hours.

Azacitidine is commercially available as Vidaza™ for intravenous or subcutaneous treatment of acute forms of leukemia. Vidaza™ which is formulated at a pH of about 6.5, contains a small quantity of sodium hydroxide and delivers 100mg of azacitidine in 10ml of water when the lyophile is reconstituted. This concentrate is further diluted with normal saline or Ringer's Lactate to yield a finally administered concentration of 1 mg/ml. The presence of sodium hydroxide in Vidaza™ also results in limited stability.

Degradation Mechanism of Azacitidine



I II III

Azacitidine, in common with other nitrogen-containing heterocycles, is prone to hydration across the 5,6 carbon-nitrogen bond yielding the transient intermediate (II) which immediately collapses by ring opening to the formamide derivative (III) which appears as the main hydrolysis product at Relative Retention Time (RRT) 0.66. If the reaction is forced further by extended reaction times or by the use of

more extreme conditions, III hydrolyses to give guanidino or urea based derivatives. Prevention or retardation of this process is necessary to provide a product with a viable shelf life and sufficient in-use stability to allow administration to take place.

The reconstituted azacitidine product is stable only for about 1 hour under ambient conditions and about 8 hours at 5 °C. A hospital environment requires a minimum of about 8 hours of reconstitution stability under ambient conditions. Thus, there is a need for azacitidine formulations with increased stability after reconstitution, especially at ambient conditions.

Summary of the Invention

The invention is generally directed to azacitidine-containing compositions that are stable for at least about 24 hours at room temperature, i.e. temperatures of less than or equal to about 25 °C. In several aspects of the invention, the inventive compositions contain azacitidine or a pharmaceutically acceptable salt thereof and a pharmacologically suitable fluid which includes (i) propylene glycol (PG), polyethylene glycol (PEG), or mixtures thereof and (ii) an effective amount of a surfactant which, in many preferred embodiments, is at least /w). In some aspects of the invention, the fluid can be about 99.5% propylene glycol or PEG and about 0.5% of a surfactant such as polysorbate 80. Still further aspects include fluids with about equal portions of PG and PEG, with about 0.5% surfactant. In most embodiments, the amount of surfactant included is at least about 0.5% and less than about 10% with amounts of less than or equal to 2.5% being preferred.

In an alternative embodiment, there are provided kits containing an amount of azacitidine or a pharmaceutically acceptable salt thereof, preferably in lyophilized form, in a first vial or container and a pharmacologically suitable fluid which

contains at least one of PG or PEG, or mixtures thereof and an effective amount of a surfactant, i.e. at least about 0.5%, in a second vial or container.

The inventive compositions can also include dispersions of a lyophilized azacitidine in the pharmacologically suitable fluids, forming homogenous suspensions. The inventive compositions are substantially free of hydrolysis products, including formamide, guanidine and urea based derivatives for periods of at least about 24 hours or more.

In accordance with some particularly preferred embodiments, stable compositions according to the invention include those in which the formulation is substantially free of, i.e. less than about 2% but preferably less than about 0.2-0.5%, hydrolysis products, including formamide, guanidine and urea based derivatives for a period of at least about 24 hours at temperatures of less than or equal to about 25°C, hereinafter "room temperature".

Thus, most formulations in accordance with the present invention will have less than about 0.5% total hydrolysis products after about 24 hours when stored at temperatures of less than or equal to 25°C. Surprisingly, it has now been discovered that the stability of azacitidine as measured at lea t by the absence of substantially all hydrolysis products, is much improved by dispersing azacitidine (preferably lyophilized) in the pharmacologically suitable fluids described herein. Moreover, it has also been surprisingly found that the dispersibility of azacitidine is markedly increased in the aforementioned pharmacologically suitable fluids. As a result, when appropriate storage conditions are employed, i.e. room temperature or under refrigerated conditions, a shelf-life of at least 24 hours for reconstituted azacitidine is attained.

Still further aspects of the invention include methods for making azacitidine formulations suitable for parenteral administration. The methods include dispersing a lyophilized azacitidine such as that described herein into a

pharmacologically suitable, non-aqueous fluid as defined herein and forming a dispersion that is suitable for intramuscular, subcutaneous or subdermal injection and an extended shelf life.

Detailed Description of the Invention

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. In the event that there are a plurality of definitions for a term herein, those in this section prevail unless stated otherwise.

As used herein, stable refers to azacitidine-containing compositions in which there is not more than about 2% hydrolysis products after a period of at least about 24 hours at a temperature of less than or equal 25°C.

As used herein, pharmaceutically suitable fluid is a solvent suitable for pharmaceutical use and, if desired, as part of a parenteral formulation.

As used herein, azacitidine also includes pharmaceutically acceptable salts thereof or derivatives of the compound, including esters, enol ethers :ids, bases, solvates, hydrates or prodrugs thereof. Such salts and derivatives may be readily prepared by those of skill in the art using known methods. The salts and derivatives produced may be administered to animals or humans without substantial toxic effects and are either pharmaceutically active or are prodrugs.

In some aspects of the invention, the azacitidine can be prepared and lyophilized in conventional methods known in the art, such as that described in the package insert for Vidaza™, the contents of which are incorporated herein by reference. In another aspect of the invention, the azacitidine is obtained from commercial sources. In either case, the azacitidine is understood by those of ordinary skill to be suitable for inclusion in the formulations described herein.

In accordance with one aspect of the invention, there are provided azacitidine-containing compositions which have improved stability and dispersibility. In one embodiment, the invention includes azacitidine-containing compositions, including:

a) azacitidine or a pharmaceutically acceptable salt thereof; and

b) a pharmacologically suitable fluid which contains

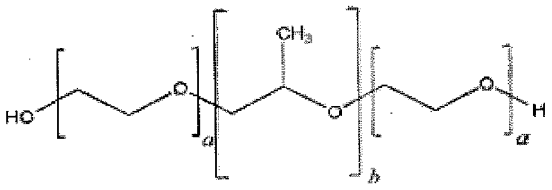
i) one of propylene glycol or polyethylene glycol, or a mixture thereof; and

ii) an effective amount, i.e. preferably at least about 0.5%, of a surfactant (w/w).

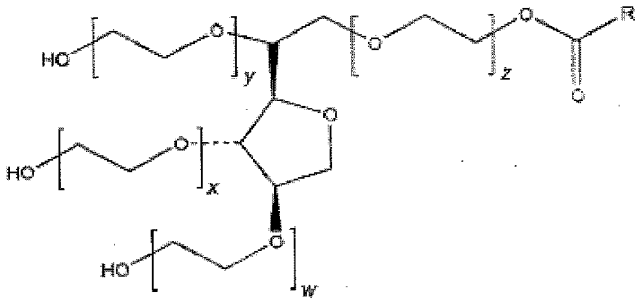
For purposes of the present invention, "effective amount" shall be understood to be an amount which is sufficient, when combined with the propylene glycol or polyethylene glycol to confer not only suitable dispersability upon the azacitidine included in the inventive formulations, but will also sufficient to impart the unexpected increase in stability thereon. While in many preferred aspects, the amount of surfactant is at least about 0.5%, it will be appreciated that some surfaciants, when included in amounts below 0.5% will nonetheless be sufficient to impart the desired qualities upon the formulation.

Preferably, the surfactant is a poloxamer of the general formula (I), polyoxyethylene sorbitan fatty acid ester surfactants of general formula (II), polyoxyethylene sorbitan fatty acid ester surfactants of general formula (III), or mixtures thereof. See below.

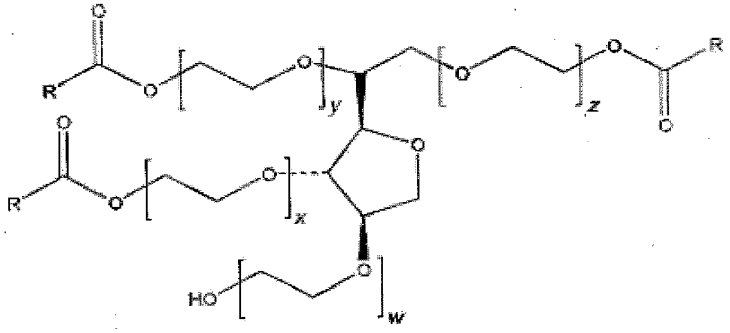
Formula (I)



Formula (II)



Formula (III)



wherein:

R = fatty acid;

$w + x + y + z = 20$; 5; or 4.

Preferably, the surfactant is selected from the non-limiting list of materials which includes polysorbate 80, poloxamer 188, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 65, polysorbate 85, polysorbate 81, polysorbate 21, polysorbate 61 and Cremophor EL™. In some other preferred aspects of the invention, the pharmacologically suitable fluid includes polysorbate 80 as the

surfactant. In other preferred aspects of the invention, the pharmacologically suitable fluid includes poloxamer 188 as the surfactant.

The pharmaceutically suitable fluids used herein are preferably non-aqueous or substantially non-aqueous. The fact that the surfactant or other ingredients included therein may include a small amount of water, the non-aqueous nature of the fluids herein is not altered.

The PG and PEG used in the fluids will be pharmaceutically acceptable (as such term is understood by those of ordinary skill in the art) and meet all necessary pharmacopeial (USP, JP, PhEur, etc.) standards.

Preferably, the molecular weight of polyethylene glycol is from about 190 to about 9,000 or higher, as known to be used by those of ordinary skill in the art. More preferably, the polyethylene glycol is PEG 400. This invention is not limited to the aforementioned fluids and can optionally also include all pharmacologically acceptable organic solvents that are miscible in water such as ethanol, benzyl alcohol, etc. The surfactants described herein allow the azacitidine to be more readily dispersed in the preferably non-aqueous fluids.

In some preferred aspects of the invention, the pharmacologically suitable fluid includes a combination of propylene glycol and a surfactant. The ratio of propylene glycol to surfactant can be from about 99.6/0.4 to about 90/10 (w/w). Preferably, the ratio of propylene glycol to surfactant is from about 99.5/0.5 to about 97.5/2.5 (w/w) or, more preferably, from about 99.5/0.5 to about 98.5/1.5 (w/w). One particularly preferred fluid useful herein includes 99.5% PG and 0.5% surfactant.

In one embodiment of the invention, the azacitidine-containing composition includes azacitidine or a pharmaceutically acceptable salt thereof; a pharmacologically suitable fluid including propylene glycol, and polysorbate 80;

wherein the ratio of propylene glycol to polysorbate 80 is about 99.5/0.5 (w/w). One particularly preferred fluid useful herein includes 99.5% PG and 0.5% surfactant.

Other preferred aspects of the invention are those in which the pharmacologically suitable fluid includes a combination of polyethylene glycol and surfactant. The ratio of polyethylene glycol to surfactant can be from about 99.6/0.4 to about 90/10 (w/w). Preferably, the ratio of polyethylene glycol to surfactant is from about 99.5/0.5 to about 97.5/2.5 (w/w) or from about 99.5/0.5 to about 98.5/1.5 (w/w). One particularly preferred fluid useful herein includes 99.5% PEG and 0.5% surfactant.

In one embodiment of the invention, the azacitidine-containing composition includes azacitidine or a pharmaceutically acceptable salt thereof; a pharmacologically suitable fluid including polyethylene glycol, and polysorbate 80; wherein the ratio of polyethylene glycol to polysorbate 80 is about 99.5/0.5 (w/w).

In another alternative embodiment, the pharmacologically suitable fluid includes a mixture of propylene glycol and polyethylene glycol. The ratio of propylene glycol to polyethylene glycol is from about 1/99 to about 99/1 (w/w). Preferably, the ratio of propylene glycol to polyethylene glycol is about 50/50 (w/w). After inclusion of the surfactant, the fluid will include PG:PEG:surfactant in a ratio of about 49.25:49.25:0.5.

In one embodiment of the invention, the azacitidine concentration is from about 0.5 mg/ml to about 120 mg/mL. In another embodiment of the invention, the azacitidine concentration is from about 1 mg/ml to about 34 mg/ml. Preferably, the azacitidine concentration is from about 20 mg/ml to about 31 mg/ml. More preferably, the azacitidine concentration is from about 25 to about 30 mg/ml. In

alternative embodiments, there are provided formulations in accordance with the invention where the azacitidine concentration is about 100 mg/ml.

Still further aspects of the invention include methods for making azacitidine formulations suitable for parenteral administration. The methods include dispersing azacitidine as described herein, i.e. lyophilized or not, into a pharmacologically suitable fluid as defined herein and forming a dispersion that is suitable for intramuscular, subcutaneous or subdermal injection and an extended shelf life. The steps are carried out under pharmaceutically acceptable conditions for sterility and manufacturing. In another aspect of the invention, the dispersion can be transferred into an IV container or bag containing infusion fluid such as normal saline, dissolved and administered as IV infusion. The dosage form of azacitidine can be packaged with the Add-vantage™ system for dispersion and administration by IV. The principle indications and uses and amounts administered will be apparent to those of ordinary skill in the art without undue experimentation. A further aspect of the invention includes a reconstituted dispersion that is ready to administer.

Another embodiment of the invention includes methods of treating an azacitidine sensitive disease in mammals, including administering an effective amount of an azacitidine-containing composition as described herein, i.e. including azacitidine or a pharmaceutically acceptable salt thereof; and a pharmacologically suitable fluid including propylene glycol, polyethylene glycol, or mixtures thereof; and an effective amount of a surfactant, to a mammal in need thereof. In some embodiments, the azacitidine sensitive disease is, but not limited to, myelodysplastic syndrome, refractory anemia, refractory anemia with ringed sideroblasts, refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, and chronic myelomonocytic leukemia.

Another embodiment of the invention provides a kit including:

(a) a first container including an amount of lyophilized azacitidine or a pharmaceutically acceptable salt thereof; and

b) a second container which includes a pharmacologically suitable fluid including

i) propylene glycol, polyethylene glycol, or mixtures thereof; and

ii) an effective amount of a surfactant, which, in most cases, is at least about 0.5%.

The kit may also include instructions for use in/administration of the drug and/or ancillary materials useful in the administration of the drug to a patient in need thereof. The amount of azacitidine included in the container will vary, depending upon need but generally is an amount suffient for one or more therapeutic doses to a patient.

Examples

The following examples serve to provide further appreciation of the invention but are not meant in any way to restrict the effective scope of the invention.

Comparative Example 1

100% water was added to vials containing lyophilized azacitidine (ACD) obtained from commercial sources. The final ACD concentration was 0.5mg/ml. The vials were sealed and stored at room temperature (25 °C) for 16 hours. The samples were tested via HPLC, and the relative retention time (RRT) is reported in Table 1 below. RRT is calculated by dividing the retention time of the peak of interest by the retention time of the main peak, 10 minutes for azacitidine. Any peak with an RRT <1 elutes before the main peak, and any peak with an RRT >1 elutes after the main peak. Table 1 shows that azacitidine in water loses about 30% of its drug content over a period of 16 hours, and at least 9% over the first two hours. Thus, azacitidine exhibits poor stability in water.

Table 1: Stability of ACD (0.5mg/ml) in Water (Ambient Laboratory Temperature, about 25 °C)

Time - hrs	% Initial	%RRT 0.42	%RRT 0.66	%RRT 1.14	Total Imp
0	100	ND	3.88	ND	3.88
1	94	ND	18.7	ND	18.7
2	91	ND	25.4	ND	25.4
4	83	ND	39.5	ND	39.5
7	78	0.07	44.7	0.24	45.0
10	76	0.12	47.7	0.44	48.3
13	73	0.16	49.4	0.52	50.1
15	72	0.25	50.0	0.58	51.0
16	71	0.28	50.3	0.54	51.1

Analysis by validated HPLC method, ND- Not Detected

Comparative Example 2

Normal saline and lactated Ringers Solution were added to vials containing Vidaza™, which contains lyophilized azacitidine and sodium hydroxide. The final concentration of azacitidine was 1mg/ml and the pH was 6.5 re sealed and stored at room temperature (25 °C) for 2 hours. T... ere tested via HPLC, and the relative retention time (RRT) is reported in Table 2 below. Table 2 shows that azacitidine plus sodium hydroxide is stable only for about 1 hour at room temperature in saline and in lactated Ringers Solution, and thus, exhibits poor stability.

Table 2: Stability of Vidaza™ (1 mg/ml) in Normal Saline and Lactated Ringers Solution

(25 °C and pH 6.5)

Time	Normal Saline			Ringers Lactate		
(min)	% Initial	% HP	% Imp	% Initial	%HP	% Imp
0	100	4.05	0.28	100	3.68	0.48
20	98	10.2	0.28	98	9.13	0.46
40	96	13.8	0.28	96	13.4	0.48
60	95	16.8	0.28	94	17.1	0.49
80	94	19.5	0.28	93	20.3	0.48
100	93	21.7	0.28	92	23.3	0.50
120	92	24.0	0.28	90	25.8	0.5

Note: % Imp is the sum total of all other recorded impurities

% HP is the area normalized percentage of the hydrolysis product at RRT 0.67

Comparative Example 3

Normal saline and lactated Ringers Solution were added to [illegible] Vidaza™, which contains lyophilized azacitidine and sodium [illegible]. The final concentration of azacitidine was 1mg/ml and the pH was 6.5. The vials were sealed and stored at 5 °C for over 13 hours. The samples were tested via HPLC, and the relative retention time (RRT) is reported in Table 3 below. Table 3 shows that azacitidine plus sodium hydroxide is stable only for about 8 hours at 5 °C in saline and in lactated Ringers Solution, and thus, exhibits poor stability.

Table 3: Stability of Vidaza™ (1mg/ml) in Normal Saline and Lactated Ringers Solutions

(5 °C and pH 6.5)

Time	Normal Saline			Ringers Lactate		
(mins)	% Initial	% HP	% Imp	% Initial	%HP	% Imp
0	100	4.05	0.26	100	3.68	0.48
200	94	19.7	0.26	93	20.6	0.50
240	93	21.7	0.26	92	22.4	0.52
280	92	23.7	0.26	91	24.0	0.5
360	90	27.1	0.26	90	26.6	0.51
440	89	29.6	0.26	90	28.0	0.51
540	88	31.5	0.26	89	29.6	0.49
800	86	36.5	0.26	87	33.6	0.51

Note: Times recorded for Ringer's lactate after initial were 10 minutes longer than indicated

% Imp is the sum total of all other recorded impurities

% HP is the area normalized percentage of the hydrolysis product at RRT 0.66

Example 4

100% propylene glycol was added to vials containing lyophilized ACD. The resulting ACD concentration was 21.6 mg/mL. The vials were sealed and stored at room temperature for 24 hours. The samples were tested via HPLC, and the relative retention time (RRT) is reported in Table 4 below. Although the samples do not show substantial degradation, the reconstitution time to disperse the azacitidine in the samples was long and required vigorous mixing.

Table 4: 24 hour stability of ACD lyophile reconstituted with 100% Propylene glycol

Time point	Content or Assay of ACD (mg/ mL)	Impurities RRT & Degradant Area %			
		RRT 0.46	RRT 0.57	RRT 0.69	RRT 1.14
Initial	21.6	ND	ND	0.65	ND
24 hours	21.6	ND	ND	0.69	ND

ND: None detected

Example 5

A mixture of 90% propylene glycol and 10% water (w/w), instead of 100% propylene glycol of Example 4, was added to vials containing lyophilized ACD to obtain an ACD concentration of 31.5 mg/mL. The amount of degradation over a 24 hour period at room temperature is reported in Table 5 below. The addition of 10% water provided excellent reconstitution and stability.

Table 5: 24 hour stability of ACD lyophile reconstituted with 90% Propylene Glycol/ 10% Water (w/w)

Time point	%Assay of label claim	Impurities RRT & De Area %			
		RRT 0.46	RRT 0.57	RRT 0.69	RRT 1.14
Initial	31.5	ND	ND	0.13	ND
24 hours	30.6	ND	ND	0.62	ND

Example 6

The ACD-containing preparation of Example 4 was made by adding a mixture of 80% propylene glycol and 20% water (w/w) instead of 100% propylene glycol and the lyophilized ACD was reconstituted at a concentration of 27.3 mg/mL. The amount of degradation over a 24 hour period at room temperature is reported in Table 6 below. The addition of 20% water provided excellent reconstitution and stability.

Table 6: 24 hour stability of ACD lyophile reconstituted with 80% Propylene Glycol/ 20% Water (w/w)

Time point	%Assay of label claim	Impurities RRT & Degradant Area %			
		RRT 0.46	RRT 0.57	RRT 0.69	RRT 1.14
Initial	27.3	ND	ND	0.22	ND
24 hours	26.1	ND	ND	2.58	

Example 7

The ACD-containing preparation of Example 4 was made by adding a mixture of 70% propylene glycol and 30% water (w/w) instead of 100% propylene glycol and the lyophilized ACD was reconstituted at a concentration of 29.1 mg/mL. The amount of degradation over a 24 hour period at room temperature is reported in Table 7 below. The addition of 30% water provided excellent reconstitution and stability.

Table 7: 24 hour stability of ACD lyophile reconstituted with 70% Propylene Glycol/ 30% Water (w/w)

Time point	Content or Assay of ACD (mg/ mL)	Impurities RRT & Degradant Area %			
		RRT 0.46	RRT 0.57	RRT 0.69	RRT 1.14
Initial	29.1	ND	ND	0.53	ND
24 hours	23.4	ND	ND	2.88	ND

Examples 5-7 demonstrate that alternative embodiments in which some water is included can provide useful formulations.

Example 8

100% polyethylene glycol 400 was added to vials containing lyophilized ACD. The lyophilized ACD was reconstituted at a concentration of 30.9 mg/mL. The vials were sealed and stored at room temperature for 24 hours. The samples were tested via HPLC, and the relative retention time (RRT) is reported in Table 8 below. Although the samples do not show substantial degradation, the reconstitution time to disperse the azacitidine in the samples required vigorous mixing.

Table 8: 24 hour stability of ACD lyophile reconstituted with 100% PEG 400

Time point	Content or Assay of ACD (mg/ mL)	Impurities RRT & Degradant Area %			
		RRT 0.46	RRT 0.57	RRT 0.69	RRT 0.79
Initial	30.9	ND	ND	0.12	0.32
24 hours	30.4	ND	ND	0.12	0.34

Example 9

A mixture containing 50% propylene glycol and 50% PEG 400 was added to vials containing lyophilized ACD. The lyophilized ACD was reconstituted at a concentration of 29.4 mg/mL. The vials were sealed and stored at room temperature for 24 hours. The samples were tested via HPLC, and the relative retention time (RRT) is reported in Table 9 below. Although the samples do not show substantial degradation, the reconstitution time to disperse the azacitidine in the samples was long and required vigorous mixing.

Table 9: 24 hour stability of ACD lyophile reconstituted with PEG/ Propylene Glycol 50/50 (w/w)

Time point	Content or Assay of ACD (mg/ mL)	Impurities RRT & Degradant Area %			
		RRT 0.46	RRT 0.57	RRT 0.69	RRT 1.14
Initial	29.4	ND	ND	0.12	ND
24 hrs	29.4	ND	ND	0.12	ND

Example 10

99.5% propylene glycol and 0.5% polysorbate 80 (w/v) were added to vials containing lyophilized ACD. The lyophilized ACD was reconstituted at a concentration of 31.2 mg/mL. The vials were sealed and stored at room temperature for one week. The samples were tested via HPLC, and the relative retention time (RRT) is reported in Table 10 below. The data shows that the

presence of small amount of polysorbate 80 improved the reconstitution time as well as dispersibility of the lyophile.

Table 10: One week stability of ACD lyophile reconstituted with 99.5% Propylene Glycol containing 0.5% polysorbate 80 (w/v)

Time point	Content of ACD (mg/ mL)	Impurities RRT & Degradant Area %			
		RRT 0.46	RRT 0.57	RRT 0.69	RRT 1.14
Initial	31.2	ND	ND	ND	ND
7 days	30	ND	ND	ND	ND

Example 11

The ACD-containing preparation of Example 10 was made with 98.5% propylene glycol and 1.5% polysorbate 80 (w/v) instead of 99.5% prop[illegible] id 0.5% polysorbate 80 (w/v), and the lyophilized ACD was reconsti[illegible] concentration of 31.8 mg/mL. The amount of degradation over a one week period at room temperature is reported in Table 11 below. The data shows that the presence of small amount of polysorbate 80 improved the reconstitution time as well as dispersibility of the lyophile without negatively affecting long-term stability.

Table 11: One week stability of ACD lyophile reconstituted with 98.5% Propylene Glycol containing 1.5% polysorbate 80 (w/v)

Time point	Content or Assay of ACD (mg/ mL)	Impurities RRT & Degradant Area %			
		RRT 0.46	RRT 0.57	RRT 0.69	RRT 1.14
Initial	31.8	ND	ND	ND	ND
7 days	30.6	ND	ND	ND	ND

Example 12

The ACD-containing preparation of Example 10 was made with a mixture of 97.5% propylene glycol and 2.5% polysorbate 80 (w/v) instead of 99.5% propylene glycol and 0.5% polysorbate 80 (w/v), and the lyophilized ACD was reconstituted at a concentration of 31.2 mg/mL. The amoun n over a one week period at room temperature is reported in Table 12 below. The data shows that the presence of small amount of polysorbate 80 improved the reconstitution time as well as dispersibility of the lyophile without negatively affecting long-term stability.

Table 12: One week stability of ACD lyophile reconstituted with 97.5% Propylene Glycol containing 2.5% polysorbate 80 (w/v)

Time point	Content or Assay of ACD (mg/ mL)	Impurities RRT & Degradant Area %			
		RRT 0.46	RRT 0.57	RRT 0.69	RRT 1.14
Initial	31.2	ND	ND	ND	ND
7 days	30.3	ND	ND	ND	ND

When water is not present in the formulation, the reconstituted dispersion may be stable for 18 months either under ambient storage conditions or under refrigerated conditions.

Examples 13-17

The procedures above were repeated using additional inventive formulations in accordance with the invention. In each case, it can be seen that the inventive formulations have sufficient long term stability so that the amount of degradation products after 24 hours at a temperature of = 25°C is less than about 2.0%.

Table 13

Ex. No.	Formulation	Temp.	Time period	Content (mg/mL)	% of Initial	RRT of degradants			% of total degradation
						0.48	0.67 (hydrolyte)	0.81	
13	ACD - 30mg/mL Tween-80 (0.5%) in PEG - 400	Initial		30.1	100	BDL	BDL	BDL	0.00
		40°C	1 week	28.9	96.0	BDL	0.06	BDL	0.06
			15 days	32.3	107.3	BDL	0.10	BDL	0.15
			1 month	29.0	96.3	BDL	0.07	BDL	0.26
14	ACD - 30mg/mL Tween-80 (0.5%) in PEG-400:PG(1:1)	Initial		28.9	100	BDL	BDL	0.16	0.16
		40°C	1 week	29.6	102.4	BDL	0.05	BDL	0.05
			15 days	31.7	109.7	BDL	0.08	BDL	0.08
			1 month	29.5	102.1	BDL	0.11	BDL	0.11
		25°C	24 hrs	30.0	103.8	BDL	0.15	0.45	0.60
			48 hrs	31.2	108.0	BDL	0.14	BDL	0.14
			1 month	29.7	102.8	BDL	0.08	BDL	0.08
			2 months	28.3	97.9	BDL	BDL	BDL	0.00

BDL: Below Detectable Levels

Table 14

Ex. No.	Formulation	Temp.	Time period (months)	Content (mg/mL)	% of Initial	RRT of degradants		% of total degrad-ation
						0.55	0.67 (HP)	
15	ACD - 30mg/mL Tween 80(0.5%) in PEG 400:PG (1:1) qs to 1mL Vial volume - 3.3mL	Initial		29.6	100	BDL	BDL	0.00
		40°C	1	25.6	86.5	BDL	0.07	0.07
			2	24.7	83.4	BDL	0.05	0.05
			3	23.5	79.4	0.26	0.09	0.35
		25°C	1	27.7	93.6	BDL	0.06	0.06
			3	27.5	92.9	BDL	0.07	0.07
			6	24.2	81.8	BDL	BDL	0
			9		0.0			0
			12		0.0			0
			24		0.0			0
16	ACD - 50mg/mL Tween 80(0.5%) in PEG 400:PG (1:1) qs to 1mL Vial volume - 2mL	Initial		45.9	100	BDL	BDL	0.00
		40°C	1	41.2	89.8	BDL	BDL	0.00
			2	40.6	88.5	BDL	0.05	0.05
			3	38.6	84.1	0.16	0.08	0.24
		25°C	1	45.8	99.8	BDL	0.05	0.05
			3	44.6	97.2	BDL	0.05	0.05
			6	39.2	85.4		)L	0
			9		0.0			0
			12		0.0			0
			24		0.0			0
17	ACD - 100mg/mL Tween 80(0.5%) in PEG 400:PG (1:1) qs to 1mL Vial volume - 2mL	Initial		81.3	100	BDL	BDL	0.00
		40°C	1	82.4	101.4	BDL	BDL	0.00
			2	80.6	99.1	BDL	BDL	0.00
			3	73.5	90.4	0.07	0.05	0.12
		25°C	1	81.4	100.1	BDL	BDL	0.00
			3	85.2	104.8	BDL	BDL	0.00
			6	76.5	94.1	BDL	BDL	0.00
			9		0.0			0.00
			12		0.0			0.00
			24		0.0			0.00

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We claim:

1. An azacitidine-containing composition, comprising:
a) azacitidine or a pharmaceutically acceptable salt thereof; and
b) a pharmacologically suitable fluid comprising
i) propylene glycol, polyethylene glycol or mixtures thereof; and
ii) an effective amount of a surfactant .

2. The azacitidine-containing composition of claim 1, wherein the pharmacologically suitable fluid comprises propylene glycol and the surfactant is polysorbate 80.

3. The azacitidine-containing composition of claim 1, wherein the pharmacologically suitable fluid comprises polyethylene glycol and the surfactant is polysorbate 80.

4. The azacitidine-containing composition of claim 1, wherein the molecular weight of the polyethylene glycol is 400.

5. The azacitidine-containing composition of claim 1, w actant is selected from the group consisting of polysorbate 80, poloxamer 188, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 65, polysorbate 85, polysorbate 81, polysorbate 21, polysorbate 61 and Cremophor EL™ and mixtures thereof.

6. The azacitidine-containing composition of claim 1, wherein said fluid includes propylene glycol and a surfactant in a ratio of about 99.5 to about 0.5 (w/w).

7. The azacitidine-containing composition of claim 1, wherein said fluid includes propylene glycol and a surfactant in a ratio of from about 99.5/0.5 to about 97.5/2.5 (w/w).

8. The azacitidine-containing composition of claim 7, wherein said fluid includes propylene with one of when one of what one of one of one withglycol and a surfactant in a ratio of from about 99.5/0.5 to about 98.5/1.5 (w/w).

9. The azacitidine-containing composition of claim 1, wherein said fluid includes propylene glycol and polyethylene glycol.

10. The azacitidine-containing composition of claim 9, wherein said fluid further comprises up to about 2.5% (w/w) of a surfactant.

11. The azacitidine-containing composition of claim 10, wherein the fluid comprises about 0.5% (w/w) of a surfactant.

12. The azacitidine-containing composition of claim 1, wherein the azacitidine concentration is from about 0.5 mg/ml to about 120 mg/ml.

13. The azacitidine-containing composition of claim 12, wherein the azacitidine concentration is from about 20 mg/ml to about 31 mg/ml.

14. The azacitidine-containing composition of claim 13, wherein the azacitidine concentration is from about 25 to about 30 mg/ml.

15. An azacitidine-containing composition according to claim 1, comprising:
a) azacitidine or a pharmaceutically acceptable salt thereof;
b) a pharmacologically suitable fluid consisting essentially of
i) propylene glycol, and

ii) a surfactant; wherein

the ratio of propylene glycol to surfactant is about 99.5 to about 0.5 (w/w).

16. An azacitidine-containing composition according to claim 1, comprising:

a) azacitidine or a pharmaceutically acceptable salt thereof;

b) a pharmacologically suitable fluid consisting essentially of

i) polyethylene glycol, and

ii) a surfactant; wherein

the ratio of polyethylene glycol to surfactant is about 99.5 to about 0.5 (w/w).

17. An azacitidine-containing composition according to claim 1, comprising:

a) azacitidine or a pharmaceutically acceptable salt thereof;

b) a pharmacologically suitable fluid consisting essentially of

i) propylene glycol,

ii) polyethylene glycol, and

ii) a surfactant; wherein

the ratio of PG:PEG:surfactant is 49.25:49.25:0.5.

18. A method of treating of an azacitidine sensitive disea s, comprising administering an effective amount of an azacitidine-containing composition of claim 1 to a mammal in need thereof.

19. A kit comprising:

(a) a first container including lyophilized azacitidine or a pharmaceutically acceptable salt thereof; and

b) a second container including a pharmacologically suitable fluid comprising

i) propylene glycol, polyethylene glycol, or mixtures thereof; and

ii) at least about 0.5% surfactant (w/w).

20. An azacitidine-containing composition according to claim 1 wherein the amount of hydrolysis products after 24 hours at a temperature of = 25°C is less than about 2.0%.

EIGHTEENTH EDITION

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Diagnosis is confirmed by presence of the Ph chromosome on cytogenic or molecular studies, although it is absent in 5% of patients.

During the accelerated phase of disease, anemia and thrombocytopenia usually develop. Basophils may increase, and granulocyte maturation may be defective. The proportion of immature cells and the leukocyte alkaline phosphatase score may increase. In the bone marrow, myelofibrosis may develop and sideroblasts may be seen on microscopy. Evolution of the neoplastic clone may be associated with development of new abnormal karyotypes, often an extra chromosome 8 or isochromosome 17.

Further evolution may lead to a blast crisis with myeloblasts (60% of patients), lymphoblasts (30%), and megakaryocytoblasts (10%). In 80% of these patients, additional chromosomal abnormalities occur frequently.

Prognosis

Before imatinib was used, with treatment 5 to 10% of patients died within 2 yr of diagnosis; 10 to 15% died each year thereafter. Median survival was 4 to 7 yr. Most (90%) deaths follow a blast crisis or an accelerated phase of the disease. Median survival after blast crisis is about 3 to 6 mo but can be up to 12 mo with remission.

Ph chromosome–negative CML and chronic myelomonocytic leukemia have a worse prognosis than Ph chromosome–positive CML. Their clinical behaviors resemble a myelodysplastic syndrome (see below).

Treatment

Except for some cases in which stem cell transplantation can be used successfully, treatment is not curative; however, survival can be prolonged by treatment with imatinib.

Imatinib inhibits the specific tyrosine kinase that results from the *ABL-BCR* gene product. It is dramatically effective in achieving complete clinical and cytogenetic remissions of Ph chromosome–positive CML and is clearly superior to other regimens (eg, interferon ± cytosine arabinoside). Imatinib also is superior to other treatments in the accelerated and blastic phases. Combinations of chemotherapy with imatinib in blast crisis have a higher response rate than does therapy with either approach alone. Treatment tolerance is excellent. The high level of durable complete remissions associated with imatinib therapy has led to the prospect of the cure of the disease.

Older chemotherapy regimens are reserved for *ABL-BCR*–negative patients, those who relapse after receiving imatinib, and those in blast crisis. The main agents are busulfan, hydroxyurea, and interferon. Hydroxyurea is easiest to manage and has the fewest adverse effects. The starting dosage is generally 500 to 1000 mg po bid. Blood counts should be followed q 1 to 2 wk and the dosage adjusted accordingly. Busulfan often causes unexpected general myelosuppression, and interferon causes a flu-like syndrome that often is unacceptable to patients. The main benefit of these therapies is reduction in distressing splenomegaly and adenopathy and control of the tumor burden to reduce the incidence of tumor lysis and gout. None of these therapies prolongs median survival > 1 yr compared with untreated patients; thus, reduction in symptoms is the major goal, and therapy is not continued in the face of significant toxic symptoms.

Although splenic radiation is rarely used, it may be helpful in refractory cases of CML or in terminal patients with marked splenomegaly. Total dosage usually ranges from 6 to 10 Gy delivered in fractions of 0.25 to 2 Gy/day. Treatment should begin with very low doses and careful evaluation of the WBC count. Response is usually disappointing.

Splenectomy may alleviate abdominal discomfort, lessen thrombocytopenia, and relieve transfusion requirements when splenomegaly cannot be controlled with chemotherapy or irradiation. Splenectomy has not proved to play a significant role during the chronic phase of CML.

MYELODYSPLASTIC SYNDROME

Myelodysplastic syndrome involves a group of disorders typified by peripheral cytopenia, dysplastic hematopoietic progenitors, a hypercellular bone marrow, and a high risk of conversion to AML. Symptoms are referable to the specific cell line most affected and may include fatigue, weakness, pallor (secondary to anemia), increased infections and fever (secondary to neutropenia), and increased bleeding and bruising (secondary to thrombocytopenia). Diagnosis is by blood count, peripheral blood smear, and bone marrow aspiration. Treatment with 5-azacytidine may help; if AML supervenes, it is treated per the usual protocols.

Etiology and Pathophysiology

Myelodysplastic syndrome (MDS) is a group of disorders, often termed preleukemia, refractory anemias, Philadelphia chromosome–negative chronic myelocytic leukemia, chronic myelomonocytic leukemia, or agnogenic myeloid metaplasia, resulting from a somatic mutation of hematopoietic precursors. Etiology is often unknown, but risk is increased with exposure to benzene, radiation, and chemotherapeutic agents (particularly long or intense regimens and those involving alkylating agents and epipodophyllotoxins).

MDS is characterized by clonal proliferation of hematopoietic cells, including erythroid, myeloid, and megakaryocytic forms. The bone marrow is normal or hypercellular, and ineffective hematopoiesis can cause anemia (most common), neutropenia, and/or thrombocytopenia. The disordered cell production is also associated with morphologic cellular abnormalities in bone marrow and blood. Extramedullary hematopoiesis may occur, leading to hepatomegaly and splenomegaly. Myelofibrosis is occasionally present at diagnosis or may develop during the course of MDS. Classification is by blood and bone marrow findings (see TABLE 142–4). The MDS clone is unstable and tends to progress to AML.

Symptoms and Signs

Symptoms tend to reflect the most affected cell line and may include pallor, weakness, and fatigue (anemia); fever and infections (neutropenia); and increased bruising, petechiae, epistaxis, and mucosal bleeding (thrombocytopenia). Splenomegaly and hepatomegaly are common. Symptoms may also be referable to other underlying diseases; eg, in an elderly patient with preexisting cardiovascular disease, anemia from MDS may exacerbate anginal pain.

Diagnosis

MDS is suspected in patients (especially the elderly) with refractory anemia, leukopenia, or thrombocytopenia. Cytopenias secondary to congenital disorders, vitamin deficiencies, or drug adverse effects must be ruled out. Diagnosis is by examining peripheral blood and bone marrow and identifying morphologic abnormalities in 10 to 20% of cells of a particular lineage.

Anemia is the most common feature, associated usually with macrocytosis and

TABLE 142–4. MYELODYSPLASTIC SYNDROME BONE MARROW FINDINGS AND SURVIVAL

CLASSIFICATION	CRITERIA	MEDIAN SURVIVAL (YR)
Refractory anemia	Anemia with reticulocytopenia, normal or hypercellular marrow with erythroid hyperplasia and dyserythropoiesis; blasts ≤ 5%	≥ 5
Refractory anemia with sideroblasts	Same as refractory anemia with ringed sideroblasts > 15% of NMC	≥ 5
Refractory anemia with excess blasts	Some cytopenia of ≥ 2 cell lines with morphologic abnormalities of blood cells; hypercellular marrow with dyserythropoiesis and dysgranulopoiesis; blasts = 5–20% of NMC	1.5
Chronic myelomonocytic leukemia	Same as refractory anemia with excess blasts with absolute monocytosis in blood; significant increase in marrow monocyte precursors	1.5
Refractory anemia with excess blasts in transformation	Refractory anemia with excess blasts and one or more of the following: ≥ 5% blasts in blood, 20–30% blasts in marrow, Auer rods in granulocyte precursors	0.5

NMC = nucleated marrow cells.

anisocytosis. With automatic cell counters, these changes are indicated by an increased MCV and RBC distribution width. Some degree of thrombocytopenia is usual; on blood smear, the platelets vary in size, and some appear hypogranular. The WBC count may be normal, increased, or decreased. Neutrophil cytoplasmic granularity is abnormal, with anisocytosis and variable numbers of granules. Eosinophils also may have abnormal granularity. Pseudo Pelger-Huët cells (hyposegmented neutrophils) may be seen. Monocytosis is characteristic of the chronic myelomonocytic leukemia subgroup, and immature myeloid cells may occur in the less well differentiated subgroups. The cytogenetic pattern is usually abnormal, with one or more clonal cytogenetic abnormalities often involving chromosomes 5 or 7.

Prognosis and Treatment

Prognosis depends greatly on classification and on any associated disease. Patients with refractory anemia or refractory anemia with sideroblasts are less likely to progress to the more aggressive forms and may die of unrelated causes.

Azacitidine improves symptoms, decreases the rate of transformation to leukemia and the need for transfusions, and probably improves survival. Other therapy is supportive, including RBC transfusions as indicated, platelet transfusions for bleeding, and antibiotic therapy for infection. In some patients, erythropoietin to support RBC needs, granulocyte colony-stimulating factor to manage severe symptomatic granulocytopenia, and, when available, thrombopoietin for severe thrombocytopenia can serve as important hematopoietic support but have not proved to increase survival. Allogeneic stem cell transplantation is useful, and nonablative allogeneic bone marrow transplantations are now being studied for patients > 50 yr. Response of MDS to AML chemotherapy is similar to that of AML, after age and karyotype are considered.

143 LYMPHOMAS

Lymphomas are a heterogeneous group of neoplasms arising in the reticuloendothelial and lymphatic systems. The major types are Hodgkin lymphoma and non-Hodgkin lymphoma (NHL)—see TABLE 143–1.

Lymphomas were once thought to be absolutely distinct from leukemias. However, better understanding of cell markers and keen tools with which to evaluate those markers now show that the differentiation between these 2 cancers is often vague. The notion that lymphoma is relatively restricted to the lymphatic system and leukemias to the bone marrow, at least in early stages, is also not always true.

HODGKIN LYMPHOMA

(Hodgkin's Disease)

Hodgkin lymphoma is a localized or disseminated malignant proliferation of cells of the lymphoreticular system, primarily involving lymph node tissue, spleen, liver, and bone marrow. Symptoms include painless lymphadenopathy, sometimes with fever, night sweats, unintentional weight loss, pruritus, splenomegaly, and hepatomegaly. Diagnosis is based on lymph node biopsy. Treatment is curative in about 75% of cases and consists of chemotherapy and/or radiation therapy.

In the US, about 7500 new cases of Hodgkin lymphoma are diagnosed annually. The male:female ratio is 1.4:1. Hodgkin lymphoma is rare before age 10 and is most common between ages 15 and 40.

Etiology and Pathophysiology

Hodgkin lymphoma results from the clonal transformation of cells of B-cell origin, giving rise to pathognomic binucleated Reed-Sternberg cells. The cause is unknown, but genetic susceptibility and environmental associations (eg, occupation, such as woodworking; history of treatment with phenytoin, radiation therapy, or chemotherapy; infection with Epstein-Barr virus, *Mycobacterium tuberculosis*, herpesvirus type 6, HIV) play a role. Risk is slightly increased in people with certain

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CHEMISTRY OF ANTITUMOR TRIAZINE NUCLEOSIDES. AN IMPROVED SYNTHESIS OF DIHYDRO-5-AZACYTIDINE.

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5-Azacytidine[3] (7, aza-C) is a nitrogen bioisostere of cytidine which has been used effectively for the clinical treatment of leukemia[4]. The severe gastrointestinal toxicity of 7 is best minimized by prolonged continuous infusion,[5,6] but working in opposition to this mode of administration is the instability of aza-C in aqueous formulations[4] which results in a continuously decreasing aza-C concentration and the production of increasing concentrations of hydrolysis products of unknown biological effects. A reduced analog, 5,6-dihydro-5-azacytidine hydrochloride (6, DHaza-C), was

recently synthesized by reduction of aza-C with sodium borohydride with the goal of eliminating the solution instability while retaining the antitumor activity of the parent drug[7]. Relative to the parent drug, DHaza-C proved to have a greatly enhanced aqueous stability over a broad pH range. DHaza-C also possessed substantial antitumor activity in mouse leukemia test systems.[7,8,9] In order to ensure sufficient quantities of DHaza-C to initiate preclinical pharmacology studies, it was necessary to develop a more direct, economical synthesis. Accordingly, an improved synthesis of DHaza-C is described in this report, and in addition, the results of chemical investigations directed toward reactions at the critical 6-position of the aza-C triazine ring, the vulnerable locus for hydrolytic attack, are presented.

RESULTS

Synthesis of Dihydro-2-azacytidine Hydrochloride (6).

The earlier[9] synthesis of DHaza-C (6) via a borohydride reduction required the use of aza-C (7) as a starting material. However, the synthetic sequence leading to 6 can be compressed and the overall yield increased by reducing the appropriate blocked nucleosides (4 or 5) directly with sodium borohydride. The nucleosides (4 and 5) were prepared essentially according to literature[10] procedures utilizing the Friedel-Crafts catalyzed N-ribosylation of trimethylsilylated 5-azacytosine (1) with per-O-acylribofuranose intermediates (2 or 3). Treatment

of the tri-O-benzoyl nucle
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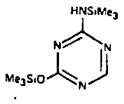

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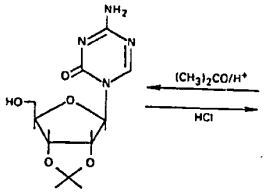

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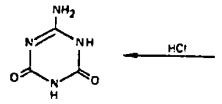

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aza-C with sodium boro-
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proved to have a greatly
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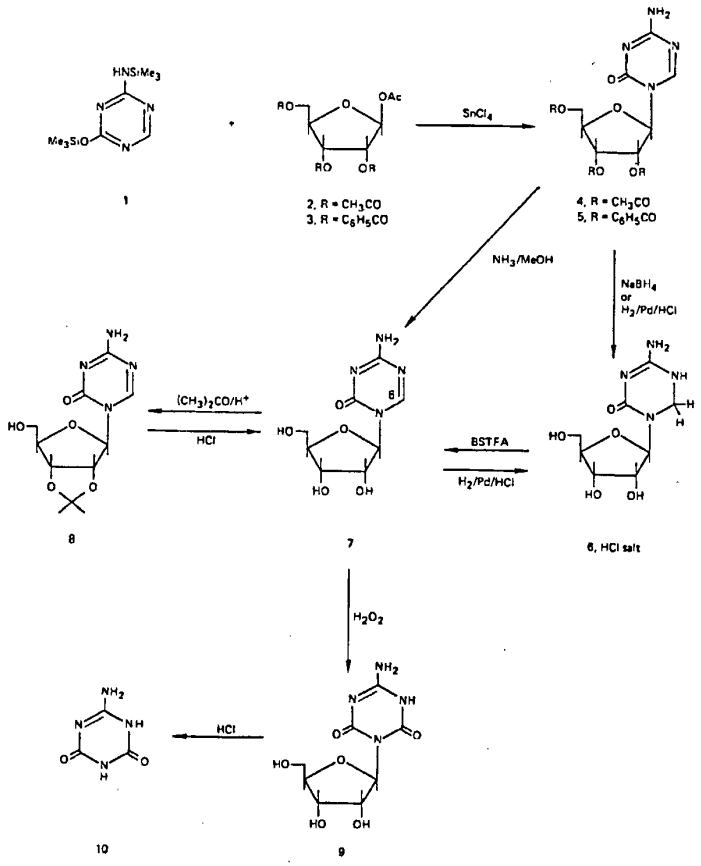
of the tri-O-benzoyl nucleoside (5) with sodium borohydride in hexamethylphosphoramide (HMPA) solution led to the reduction of the imine linkage accompanied by the removal of the benzoyl



protecting groups to provide the reduced and deblocked nucleoside in the form of a boron complex. Without further purification the boron-containing product was hydrolyzed with aqueous acid[9] to give 6 in 72% yield (58% overall yield based on 3). Similarly, the tri-O-acetyl nucleoside (4) was reduced with borohydride to afford an almost identical (73%) yield of 6 (50% overall yield based on 2). Therefore, of the borohydride reductions the former sequence is the method of choice.

It is also possible to reduce and deblock the tri-O-acetyl derivative (4) by catalytic hydrogenation in ethanol under acidic conditions to give 6 in 92% yield (63% overall yield based on 2). However, similar hydrogenation of the tri-O-benzoyl derivative (5) resulted in reduction of the imino group, but the reduction unfortunately was not also accompanied by ethanolysis of the blocking groups.

We have found that the hydrolysis of 5 with methanolic ammonia[11] to give 7 proceeds in 62% yield. Since we demonstrated earlier[9] that 7 can be converted to 6 in 72% yield using a borohydride procedure, a 36% overall yield of 6 based on 3 can be realized with our original synthesis requiring three synthetic steps (3→5→7→6). Alternatively, the reduction of 7 by hydrogenation in ethanol-hydrochloric acid gave 6 in a similar (39%) overall yield based on 3. The present synthesis of DHaza-C requires two steps using the borohydride method (3→5→6) or the hydrogenation method (2→4→6) with similar overall yields of 58% and 63%, respectively.

The Oxidation of 5-Azacyti

As part of our effort analogs of 7, we also inve derivative having a higher than aza-C. It was found oxidation when treated wit acid solution to give the The NMR spectrum of 9 lack proton characteristic of 7 siderably from that of 7. silylated (TMS) and triflu were consistent with the a triazine ring of 7. Acid of 9 gave ammelide (10) wh analysis and the mass spec 9, which has the novelty o triazine moiety than 7, is with our expectations, it L1210 leukemia assay[12] whe

Since it has been post form[14] of aza-C, it was of conversion of DHaza-C (6) t likelihood of this conversi metabolism. An acetic acid hydrogen peroxide at room t days as shown by TLC. At t

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The Oxidation of 5-Azacytidine (7) to the Oxo-analog (9).

As part of our effort to synthesize hydrolytically stable analogs of 7, we also investigated the conversion of 7 to a derivative having a higher oxidation state at the 6-position than aza-C. It was found that 7 was susceptible to selective oxidation when treated with 30% hydrogen peroxide in acetic acid solution to give the high-melting oxo-5-azacytidine (9). The NMR spectrum of 9 lacked the singlet due to the C-6 aromatic proton characteristic of 7 and the uv spectrum differed considerably from that of 7. The mass spectra of both the trimethylsilylated (TMS) and trifluoroacetylated (TFA) derivatives of 9 were consistent with the addition of one oxygen atom to the triazine ring of 7. Acid hydrolysis of the glycosidic bond of 9 gave ammelide (10) which was identified by elemental analysis and the mass spectrum of its TMS derivative. Although 9, which has the novelty of a higher degree of symmetry in the triazine moiety than 7, is stable in aqueous solution in accord with our expectations, it is devoid of antitumor activity in the L1210 leukemia assay[12] wherein aza-C exhibits high activity.

Since it has been postulated[8,9] that DHaza-C is a pro-drug form[14] of aza-C, it was of interest to investigate the chemical conversion of DHaza-C (6) to aza-C (7) in order to probe the likelihood of this conversion occurring as a result of in vivo metabolism. An acetic acid solution of 6 when treated with hydrogen peroxide at room temperature contained 7 after three days as shown by TLC. At the end of twelve days, the oxidized

nucleoside (9) was also detectable in the reaction solution suggesting a sequential oxidation process: 6 → 7 → 9. After fifteen days, the reaction was worked-up and the products were derivatized with trifluoroacetic anhydride for MS analysis. Probe introduction of the TFA mixture into the spectrometer gave a composite spectrum of 6 · TFA_4, 7 · TFA_4, 9 · TFA_4 and 10 · TFA. When a few crystals of ferrous sulphate were added as a catalyst to the oxidation system, the reaction rate increased considerably but only 9 and 10 were found (TLC, MS) as products, with 10 as the major product. As well as lending support to the pro-drug hypothesis of 6, these experiments suggest the possibility that the oxidation of 7 to 9 (or 6 to 9) could serve as a detoxification mechanism in vivo although 9 has not been identified and reported as a metabolite of 7.

The Dehydrogenation of 6 to Give 5-Azacytidine (7).

Treatment of 6 with bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) in acetonitrile solution at room temperature led smoothly to the introduction of five TMS groups into the nucleoside. The TMS_5 derivative gave a single peak in the GC, which produced a mass spectrum consistent with the proposed structure upon subsequent GC-MS analysis[9]. However, if the silylation solution was heated, a second peak began to appear in the chromatogram due to tetrakis - (trimethylsilyl)-5-azacytidine (7 · TMS_4). This material was identified by MS and was identical to the product of 7 with this silylation reagent. Continued heating ultimately resulted in a complete conversion of 6 · TMS_5 into

7 · TMS_4 with the forma
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silyl function in synt
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7 · TMS_4 with the formal loss of the elements of $(CH_3)_3SiH$. This unexpected event seemed sufficiently unusual to warrant further investigation to determine its synthetic potential for the preparation of triazine nucleosides which might otherwise be inaccessible and thereby further extend the use of the trialkylsilyl function in synthetic organic chemistry. Therefore, the reaction of 6 with the silylation mixture was repeated on a preparative scale and its progress monitored with GC. When the reaction was complete, the product, 7 · TMS_4, was de-trimethylsilylated by methanolysis to give a 76% yield of 7. The aza-C synthesized in this way was identical to an authentic sample[15]. Applying the silylation reaction to 6 containing a deuterium atom at the 6-position[9] of the triazine resulted in a 2.9:1 mixture (GC-MS) of 6-deutero-5-azacytidine-TMS_4 and 7 · TMS_4 apparently due to a primary deuterium isotope effect. Our earlier[9] positional assignment for the deuterium atom based on NMR spectral characteristics is, therefore, confirmed. Additional synthetic work making use of the silylation-dehydrogenation reaction to prepare nucleosides of biological interest will be reported at a later date.

Preparation of the 2',3'-O-isopropylidene derivative of 7 obtained from the silylation-dehydrogenation procedure was accomplished in acetone solution containing 2,2-dimethyoxypropane and catalyzed by perchloric acid[16]. A good yield of 8 was obtained which was identical in melting point and spectral properties with the isopropylidene derivative prepared in the

same way from authentic[15] 7. The difference in chemical shift ($\Delta\delta$) of the pair of singlets due to the isopropylidene methyl groups of 8 was observed to be 20 Hz. Since it has been demonstrated that a $\Delta\delta$ > 15 Hz is proof of the β-configuration of the aglycone at C_1, carbon of ribofuranosyl nucleosides[17], spectral confirmation is now at hand for the β-configuration originally assigned[18] to aza-C as well as all of the nucleoside derivatives synthesized during the course of the present investigation.

DISCUSSION

With the synthesis of 9 we have added a third member in a series of triazine nucleosides differing only in the oxidation state of a carbon atom in the heterocyclic moiety. Thus, DHaza-C (6) represents the lowest oxidation state, aza-C (7) is intermediate, and 9 is at the highest oxidation state in the series. In a structure-activity relationship context it is interesting to note that the intermediate oxidation state, 7, has the greatest antitumor potency against murine L1210 leukemia, the lowest oxidation state, 6, has good activity but at a lesser potency, and 9, the highest oxidation state, is completely inactive in the L1210 leukemia test system.

EXPERIMENTAL

Electron impact mass spectra were obtained on a GC-MS system consisting of a Varian Aerograph 2740 gas chromatograph coupled to a DuPont 21-492 mass spectrometer by a glass transfer line and single-stage jet separator; operating conditions were as

previously described[9].
mixtures and determinati
(IRI)[19] were accomplishe
packed with 3% SE-30 on
at 220°. Nucleosides ar
GC or MS analysis by eit
(direct probe MS) or by
using previously reporte
Proton NMR spectra were
HA-100D spectrometer usi
standard. A Cary Model
UV spectra and a Perkin-
infrared spectra. Optic
cell with a Perkin-Elmer
analyses were performed
Services and Instrumenta
Laboratories, Inc., Knox
routinely checked by TLC
Baker 1B2-F silica gel.
butanol-ethanol-water (40
(5:2:3), isopropanol-ammo
ammonia-water (66:33:1.5)
a Thomas-Hoover capillary
5-Azacytosine, 2 and 3 we
Co., Milwaukee, Wisc. and

ference in chemical shift
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Since it has been demon-
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e obtained on a GC-MS system
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by a glass transfer line
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previously described[9]. Gas chromatographic separation of mixtures and determination of isothermal retention indices (IRI)[19] were accomplished on a 1.83m x 2mm i.d. glass column packed with 3% SE-30 on 100/120 mesh Gas-Chrom Q and operated at 220°. Nucleosides and their aglycones were derivatized for GC or MS analysis by either per-trifluoroacetylation[9,20] (direct probe MS) or by per-trimethylsilylation[9] (GC-MS) by using previously reported procedures unless otherwise indicated. Proton NMR spectra were recorded with a Varian T-60 or a Varian HA-100D spectrometer using tetramethylsilane as an internal standard. A Cary Model 15 spectrophotometer was used to obtain UV spectra and a Perkin-Elmer Model 621 was used to record infrared spectra. Optical rotations were measured in a 1-dm cell with a Perkin-Elmer Model 141 polarimeter. Elemental analyses were performed by the Section on Microanalytical Services and Instrumentation, NIAMDD, NIH and by Galbraith Laboratories, Inc., Knoxville, Tenn. Compound purity was routinely checked by TLC using 5 x 20 cm plates coated with Baker 1B2-F silica gel. Four solvent systems were employed: butanol-ethanol-water (40:11:19), butanol-acetic acid-water (5:2:3), isopropanol-ammonia-water (7:1:2), isobutyric acid-ammonia-water (66:33:1.5). Melting points were determined with a Thomas-Hoover capillary apparatus and are uncorrected. 5-Azacytosine, 2 and 3 were purchased from the Aldrich Chemical Co., Milwaukee, Wisc. and were used without further purification.

4-Amino-1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)1,3,5-triazin-2(1H)-one (4). With some modifications, which led to an improved yield, the procedure followed was that described by Niedballa and Vorbruggen[10]. To a solution of 1[21] (21.4 mmol) and 2 (5.0 g, 15.7 mmol) in dry acetonitrile (150 ml), cooled to 0°, was added a solution of stannic chloride (3.2 ml, 27.8 mmol) in acetonitrile (80 ml) under anhydrous conditions. The solution temperature was slowly allowed to increase from 0° to 22° over 2 h, then maintained at 22° for 30 min. After work-up[10], concluded by treatment with charcoal and crystallization from ethyl acetate (75 ml), 3.92 g (68%) of 4 was obtained mp 160-161° (lit.[10] mp 160-161°). Using 1,2-dichloroethane as the reaction solvent gave identical results.

4-Amino-1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-1,3,5-triazin-2(1H)-one (5). The condensation of 1 and 3 according to the procedure of Niedballa and Vorbruggen[10] gave the O-benzoylated 5-azacytidine (5) in 81% yield, mp 184-186° (lit.[10] mp 186-187°).

4-Amino-5,6-dihydro-1-β-D-ribofuranosyl-1,3,5-triazin-2(1H)-one Hydrochloride (6). Method A. Borohydride Reduction of Compound 4. A solution of 4 (1.11 g, 3.0 mmol) in 7 ml HMPA was stirred while 300 mg (7.8 mmol) of sodium borohydride was added. The reaction mixture was heated at 50° for 1 h then left at room temperature for 3 h. Excess borohydride was decomposed with water (10 ml) and methanol (10 ml) and the solvent was removed under reduced pressure (bath 30°) after standing

for 1 h at room tempera
successively with ethe
white solid which was
After stirring the soli
6N hydrochloric acid, t
to one third its initia
ethanol. The precipita
filtration and the filt
0.48 g of 6 was obtaine
The mother liquor, when
from methanol-ethanol
(total yield, 73%), mp
lit.[9] mp 180-181° dec,

Method B. Borohyd
of 5 (557 mg, 1.0 mmol)
with sodium borohydride
was heated at 50° for 6
combined with water (10
was worked-up as descri
6, mp 180-181° dec; [α]
was identical to that o
undepressed.

Method C. Hydroge
(1.0 g, 2.7 mmol) in 16
of ethanolic hydrogen c
with 1.0 g of 10% palla

-ribofuranosyl)1,3,5-
fications, which led to
wed was that described
olution of 1[21] (21.4 mmol)
itrile (150 ml), cooled
: chloride (3.2 ml, 27.8
hydrous conditions. The
d to increase from 0° to
or 30 min. After work-up[10],
nd crystallization from
4 was obtained mp 160-
-dichloroethane as the
ts.

-D-ribofuranosyl)-1,3,5-
ion of 1 and 3 according
bruggen[10] gave the
% yield, mp 184-186°

ranosyl-1,3,5-triazin-2(1H)-
rohydride Reduction of
3.0 mmol) in 7 ml HMPA
f sodium borohydride was
ed at 50° for 1 h then
cess borohydride was de-
nol (10 ml) and the solvent
ath 30°) after standing

for 1 h at room temperature. The resulting syrup was triturated successively with ether (50 ml) and acetone (50 ml) to give a white solid which was washed thoroughly with ether and dried. After stirring the solid for 4 h at room temperature with 10 ml 6N hydrochloric acid, the solution was concentrated under vacuum to one third its initial volume and diluted with 50 ml of absolute ethanol. The precipitated inorganic materials were removed by filtration and the filtrate stored overnight at 0° from which 0.48 g of 6 was obtained as white crystals, mp 180-181° dec. The mother liquor, when evaporated and the residue crystallized from methanol-ethanol (1:1), provided an additional 0.14 g of 6 (total yield, 73%), mp 180-181° dec; $[\alpha]^{26}_{D}$ -29° (c 1.0, H_2O); lit.[9] mp 180-181° dec, $[\alpha]^{29}_{D}$ -29° (c 1.0, H_2O).

Method B. Borohydride Reduction of Compound 5. A solution of 5 (557 mg, 1.0 mmol) in 3 ml HMPA was stirred and treated with sodium borohydride (120 mg, 3.1 mmol). The reaction mixture was heated at 50° for 6 h then cooled to room temperature and combined with water (10 ml) and methanol (10 ml). The reaction was worked-up as described in Method A to give 202 mg (72%) of 6, mp 180-181° dec; $[\alpha]^{26}_{D}$ -29.0° (c 1.0, H_2O). The NMR spectrum was identical to that of an authentic[9] sample and the mmp was undepressed.

Method C. Hydrogenation of Compound 4. A solution of 4 (1.0 g, 2.7 mmol) in 160 ml absolute ethanol containing 10 ml of ethanolic hydrogen chloride (saturated at 0°) was slurried with 1.0 g of 10% palladium on charcoal catalyst and hydrogenated

at 50 psi for 16 h. After removing the catalyst the reaction solution was evaporated under vacuum. The resulting syrup, when triturated with ether, gave a solid which was recrystallized from ethanol to give 0.7 g (92%) of 6, mp 180-181° dec; $[\alpha]^{26}_{D}$ -29° (c 1.0, H_2O). The product was identical with an authentic sample[9] in mp, mmp, NMR and UV.

Method D. Hydrogenation of Compound 7 (aza-C). A solution of 7 (5.0 g, 20.4 mmol) in a mixture of 6N hydrochloric acid (50 ml) and ethanol (50 ml) was combined with 5.0 g of 10% palladium on charcoal and hydrogenated at an initial pressure of 50 psi for 8 h. The catalyst was removed by filtration through a Celite pad which was subsequently washed with ethanol. The filtrate and washings were evaporated under vacuum (30°) and the residue was crystallized from methanol-ethanol (1:1) to give 4.43 g (77%) of 6, mp and mmp 180-181° dec; $[\alpha]^{26}_{D}$ -28° (c 1.0, H_2O). NMR and UV spectra were identical to an authentic sample[9] of 6.

Hydrolysis of 5. 4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1H)-one (7). At room temperature 3 ml of methanolic ammonia (saturated at 0°) was added to 557 mg (1.0 mmol) of 5 giving a complete solution from which crystals began to deposit after a few minutes. After maintaining the temperature at 21° for 90 min in a stoppered flask the solution was stored at -16° overnight. The crystals of 7 (132 mg, mp 230-232° dec) were collected by filtration and washed with methanol. Concentration

of the mother liquor g
yield to 62%. The mix
sample of 5-azacytidin
were superimposable.

6-Amino-3-β-D-rib
dione (9). A mixture
glacial acetic acid (8
for 8 days at 25°. Th
about one third under
(50 ml), and the result
The precipitate was wa
give 2.60 g (50%) of 9
mp > 360°; UV λ_{max} (H_2O
Me_2SO-d_6) δ 5.94 (d, J
ribosyl protons); MS (te
(rel intensity) 625 (M-
2.7), 461 (M-TFAOH-CF_3,
69 (100); MS (pentakis
intensity) 605 (M-CH_3,
344 (4.4), 273 (5.4), 2

Anal. Calcd for C_8H
N, 21.53. Found: C, 36

6-Amino-1,3,5-triaz
of 9 (600 mg, 2.3 mmol)
heated on a steam bath f
removed by filtration an

ıe catalyst the reaction

The resulting syrup,

lid which was recrystallized

, mp 180-181° dec; $[\alpha]^{26}_{D}$

entical with an authentic

ound 7 (aza-C). A solution

of 6N hydrochloric acid

ned with 5.0 g of 10%

d at an initial pressure

removed by filtration

uently washed with ethanol.

ated under vacuum (30°) and

hanol-ethanol (1:1) to

0-181° dec; $[\alpha]^{26}_{D}$ -28°

e identical to an authentic

ribofuranosyl-1,3,5-

rature 3 ml of methanolic

o 557 mg (1.0 mmol) of 5

ı crystals began to deposit

ng the temperature at 21°

olution was stored at -16°

, mp 230-232° dec) were

ith methanol. Concentration

of the mother liquor gave a further 18 mg bringing the total yield to 62%. The mixture melting point of 7 with an authentic[15] sample of 5-azacytidine showed no depression and the NMR spectra were superimposable.

6-Amino-3-β-D-ribofuranosyl-1,3,5-triazine-2,4(1H,3H)-dione (9). A mixture of 30% hydrogen peroxide (80 ml) and glacial acetic acid (80 ml) was stirred with 7 (4.88 g, 20 mmol) for 8 days at 25°. The reaction mixture was concentrated to about one third under vacuum (bath 30-35°), diluted with water (50 ml), and the resulting precipitate removed by filtration. The precipitate was washed with water and methanol and dried to give 2.60 g (50%) of 9 which was recrystallized from water, mp > 360°; UV λ_{max} (H_2O) 221 nm (ε 16300); NMR (D_2O exchanged, Me_2SO-d_6) δ 5.94 (d, J = 4Hz, 1, C_1,H), 4.57-3.30 (m, 5, ribosyl protons); MS (tetrakis - trifluoroacetyl derivative) m/e (rel intensity) 625 (M-F, 0.3), 575 (M-CF_3, 1.1), 530 (M-CF_3CO_2H, 2.7), 461 (M-TFACH-CF_3,43), 208 (8.9), 155 (8.9), 112 (8.7), 69 (100); MS (pentakis - trimethylsilyl derivative) m/e (rel intensity) 605 (M-CH_3, 1.2), 403 (4.1), 348 (10), 345 (4.5), 344 (4.4), 273 (5.4), 272 (3.1), 245 (29), 217 (21), 73 (100).

Anal. Calcd for $C_8H_{12}N_4O_6$ (260.2): C, 36.92; H, 4.64; N, 21.53. Found: C, 36.66; H, 4.38; N, 21.73.

6-Amino-1,3,5-triazine-2,4(1H,3H)-dione (10). A solution of 9 (600 mg, 2.3 mmol) in 6N hydrochloric acid (15 ml) was heated on a steam bath for 3 h, cooled, and the dark precipitate removed by filtration and washed thoroughly with hot water. The

filtrate and washings were combined, decolorized with charcoal, and concentrated under vacuum (bath 35-40°) to yield 60 mg (20%) of ammelide (10) as a white powder. The product in 1N ammonium hydroxide (10 ml) solution was treated with 1N hydrochloric acid to pH 7 which gave analytically pure 10, mp > 360°; GC-MS (tris - trimethylsilyl derivative, IRI 1730) m/e (rel intensity) 344 (M^+, 53), 329 (M-CH_3, 23), 171 (38), 100 (17), 73 (100).

Anal. Calcd for $C_3H_4N_4O_2$ (128.1): C, 28.13; H, 3.15; N, 43.72. Found: C, 28.44; H, 3.18; N, 43.63.

The Peroxide Oxidation of 6. Method A. Without Catalyst. A solution of 283 mg (1.0 mmol) of 6 in a mixture of 30% hydrogen peroxide (4 ml) and glacial acetic acid (4 ml) was stirred at room temperature. At daily intervals samples were removed, spotted without work-up on TLC plates, and analyzed using four different solvent systems. After 3 days the reaction solution contained 7 as well as starting material, and after 12 days the 2-oxo nucleoside (9) was evident as a third spot. Spot identifications were made by comparison with authentic samples run on the same plate. After a total of 15 days the white precipitate (10 mg) which had accumulated was removed by filtration, washed with water, and dried, mp > 300°. Mass spectroscopic analysis (direct probe) of the product following trifluoroacetyl derivatization showed the product to be a mixture of 6 • TFA_4 (M^+, m/e 630), 7 • TFA_4 (trace, M-19, m/e 609), 9 • TFA_4 (M-19, m/e 625) and 10 • TFA (M^+, m/e 224).

Method B. Ferro
(1.4 mmol) of 6 in 2
sulphate was added 2
After the addition th
for 10 min then allow
The white precipitate
removed by filtration
probe) of the precipi
acetic anhydride indi
TLC analysis of the f
the absence of 6 and

4-Amino-1-[2,3-O
1,3,5-triazin-2(1H)-o
was used to convert 2
dehydrogenation react
isopropylidene deriva
from dimethylformamid
3285, 3090, 1688, 160
(s, 1, C_6H), 7.56 (br
1.37 (d, J=20Hz, 6, M
IRI 2580) m/e (rel in
370 (M-Me_2CO), 1.5),
73 (100).

Anal. Calcd for
N, 19.71. Found: C,

decolorized with charcoal,
35-40°) to yield 60 mg
der. The product in 1N
as treated with 1N hydro-
tically pure 10, mp > 360°;
ive, IRI 1730) m/e (rel
23), 171 (38), 100 (17),

): C, 28.13; H, 3.15;
; N, 43.63.
ethod A. Without Catalyst.
in a mixture of 30% hydrogen
cid (4 ml) was stirred at
s samples were removed,
s, and analyzed using four
days the reaction solution
rial, and after 12 days the
a third spot. Spot identi-
th authentic samples run on
days the white precipitate
moved by filtration, washed
ass spectroscopic analysis
ing trifluoroacetyl deriva-
mixture of 6 • TFA_4 (M^+,
609), 9 • TFA_4 (M-19, m/e

Method B. Ferrous Catalyst. To a stirred solution of 400 mg (1.4 mmol) of 6 in 2 ml water containing a few crystals of ferrous sulphate was added 2 ml of 30% hydrogen peroxide dropwise at 10°. After the addition the reaction solution was maintained at 10° for 10 min then allowed to come to room temperature for 2 h. The white precipitate (81 mg, mp > 300°) which had separated was removed by filtration and washed with water. MS analysis (direct probe) of the precipitate following treatment with trifluoroacetic anhydride indicated 10 • TFA contaminated with some 9 • TFA_4. TLC analysis of the filtrate from the reaction solution showed the absence of 6 and 7.

4-Amino-1-[2,3-O-(1-methylethylidine)-β-D-ribofuranosyl]-1,3,5-triazin-2(1H)-one (8). The procedure of Zderic et al.[16] was used to convert 244 mg (1.0 mmol) of 7 (obtained via the dehydrogenation reaction described below) to 240 mg (84%) of the isopropylidene derivative (8), mp 279-280°. Recrystallization from dimethylformamide gave 180 mg, mp 279-280°; IR (Nujol) 3410, 3285, 3090, 1688, 1609, 1110, 854, 804 cm^{-1}; NMR (Me_2SO-d_6) δ 8.39 (s, 1, C_6H), 7.56 (broad s, 2, NH_2), 5.72 (d, J=2Hz, 1, C_1,H), 1.37 (d, J=20Hz, 6, Me_2C); GC-MS (bis-trimethylsilyl derivative, IRI 2580) m/e (rel intensity) 428 (M^+, 1.4), 413 (M-CH_3, 6.5), 370 (M-Me_2CO), 1.5), 256 (3.6), 185 (22), 103 (24), 100 (91), 73 (100).

Anal. Calcd for $C_{11}H_{16}N_4O_5$ (284.3): C, 46.47; H, 5.67; N, 19.71. Found: C, 46.66; H, 5.68; N, 19.76.

An authentic sample[15] of 7 treated similarly gave an isopropylidene derivative with identical spectral properties, mp and mmp as the compound described above.

5-Azacytidine hydrochloride (7 · HCl) was obtained from 8 (100 mg) on treatment with 6N hydrochloric acid (2 ml) at 25° for 4 h. Addition of absolute ethanol (5 ml) to the reaction solution caused the crystallization of 7 · HCl (95 mg, mp 179-181° dec) which was recrystallized from ethanol-benzene, mp 180-181°dec.

Anal. Calcd for $C_8H_{12}N_4O_5 \cdot HCl$ (280.7): C, 34.23; H, 4.67; N, 19.96; Cl, 12.63. Found: C, 33.88; H, 4.89; N, 19.58; Cl, 12.39.

A sample of 7 · HCl prepared from authentic 7 gave the same UV, NMR, mp and mmp as the material described above.

The Dehydrogenation of 6: 4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1H)-one (7). A mixture of BSTFA (17 ml) and dry acetonitrile (30 ml) was refluxed gently with 566 mg (2 mmol) of 6 under anhydrous conditions. As the reaction proceeded the single peak in the gas chromatogram due to $6 \cdot TMS_5$ (IRI 2465) was accompanied by increasing concentrations of a second compound with a longer retention time. By GC-MS analysis it was shown to be $7 \cdot TMS_4$ (IRI 2620). After refluxing 18 h, the latter compound was the only peak in the chromatogram indicating a complete conversion had occurred. The solvent was removed under vacuum and the syrupy residue was taken up in 100 ml of absolute

methanol. Removal of
product was effected
containing materials
renewed twice over the
which time the boiling
crystals began to sepa
zation was allowed to
give 370 mg (76%) of 7
$[\alpha]^{26}D$ + 24.4° (c 1.0,
Mixture melting point
depression and the NMR

The free base of
using the identical re
salt.

SUMMARY

Dihydro-5-azacyti
biologically-active an
5-azacytidine (7). In
sufficient quantities
a shorter synthesis of
improvement in overall
gation of the reactive
bearing an oxygen atom
peroxide oxidation of 6
possible metabolic tran

ed similarly gave an iso-
spectral properties, mp
re.
HCl) was obtained from 8
loric acid (2 ml) at 25°
l (5 ml) to the reaction
7 · HCl (95 mg, mp 179-
m ethanol-benzene, mp 180-

30.7): C, 34.23; H, 4.67;
); H, 4.89; N, 19.58;

authentic 7 gave the same
escribed above.

no-1-β-D-ribofuranosyl-
re of BSTFA (17 ml) and dry
ly with 566 mg (2 mmol) of
reaction proceeded the
ue to 6 · TMS_5 (IRI 2465)
rations of a second compound
MS analysis it was shown
xing 18 h, the latter
matogram indicating a
solvent was removed under
n up in 100 ml of absolute

methanol. Removal of the trimethylsilyl groups from the desired product was effected by slow co-distillation of volatile silicon-containing materials with methanol. The methanol volume was renewed twice over the course of the distillation (~3 h) during which time the boiling point increased from 51° to 65° and crystals began to separate from the boiling solution. Crystallization was allowed to continue at room temperature overnight to give 370 mg (76%) of 7, mp 232-234° dec (lit.[18] mp 228-230° dec); $[\alpha]^{26}D$ + 24.4° (c 1.0, H_2O) (lit.[21] $[\alpha]^{26}D$ + 26.6° (c 1.0, H_2O). Mixture melting point with an authentic[15] sample of 7 showed no depression and the NMR and UV spectra were superimposable.

The free base of 6 could also be dehydrogenated to give 7 using the identical reaction conditions described above for the salt.

SUMMARY

Dihydro-5-azacytidine (6) is a hydrolysis-resistant, biologically-active analog of the clinical antitumor agent, 5-azacytidine (7). In order to facilitate the acquisition of sufficient quantities of 6 for preclinical pharmacology studies, a shorter synthesis of 6 was devised giving a substantial improvement in overall yield. As part of a chemical investigation of the reactive 6-position of 7 (*i*) an analog of 7 bearing an oxygen atom at C-6 (9) was synthesized; (*ii*) the peroxide oxidation of 6 was studied and the results related to possible metabolic transformations of 6 and 7; (*iii*) via a per-

trimethylsilylated derivative, 6 was converted to 7 utilizing a novel thermal elimination reaction. From an NMR analysis of an isopropylidene derivative (8) the β-configuration for 7, and all the nucleoside derivatives described in this report, was confirmed.

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12. Protocols[13] established by the Division of Cancer Treatment, National Cancer Institute were followed. The test compound (9) was administered by intraperitoneal (i.p.) injection to mice on days 1, 5 and 9 following i.p. implantation of 10^5 L1210 leukemia cells. At dose levels of 200, 100, 50, 25, 12.5, 6.25 and 3.12 mg/kg, no significant increase in survival time over untreated control animals was observed.

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Antimicrobial Agents and Chemotherapy—1966

Microbiological Production of 5-Azacytidine
II. Isolation and Chemical Structure

M. E. BERGY and R. R. HERR

The Research Laboratories, The Upjohn Company, Kalamazoo, Michigan

A new antitumor antibiotic produced by a new species of *Streptoverticillium* was shown to be 5-azacytidine by physical and chemical characteristics, degradation studies, and comparison with a synthetic sample. The antibiotic was isolated from a culture liquid by adsorption on carbon. It was purified by partition chromatography on diatomaceous earth and silica gel.

This paper describes the isolation, characterization, and elucidation of the structure of an antitumor antibiotic produced by a new *Streptoverticillium* species, *S. ladakanus* var. *ladakanus*. This antibiotic, initially designated as U-18,496, was subsequently found to be identical with 5-azacytidine.

Isolation

Crystalline antibiotic was obtained by the following procedure. Whole broth was filtered at *p*H 7.8, and the active material was adsorbed from the clear beer onto 6% Darco G-60. The carbon was eluted with 50% acetone, and eluates were concentrated to an aqueous solution. Inactive materials were precipitated from this solution by the addition of 5 volumes of acetone, and they were removed by filtration. The filtrate was concentrated to an aqueous solution and freeze-dried.

Crude antibiotic was purified by partition chromatography on a support of diatomaceous earth with the solvent system 1-butanol-ethyl acetate-buffer (*p*H 6.0), 75:25:35. Fractions which contained active material were combined and mixed with 2 volumes of petroleum hexane. Solvent phases were separated, and the aqueous phase was freeze-dried.

This dry material was purified further by chromatography on silica gel buffered with Na_2HPO_4 and KH_2PO_4. The solvent system for development consisted of chloroform-methanol (7:3). The product crystallized from a concentrate of active fractions.

Characterization

The antibiotic was obtained as white crystals which are soluble to the extent of approximately 1 mg/ml in methanol, acetone, chloroform, hexane, and dimethylsulfoxide, and to approximately 40 mg/ml in water.

Normal potentiometric titration data could not be obtained because of rapid degradation in both acid and base.

The antibiotic shows an ultraviolet-absorption maximum in water at 241 mμ, a = 35.9 (ϵ = 8,767); in 0.01 N HCl at 249 mμ, a = 12.6 (ϵ = 3,077); in 0.01 N KOH at 223 mμ, a = 99.1 (ϵ = 24,200), shoulder at 253 mμ, a = 6.3 (ϵ = 1,538), as shown in Fig. 1. It melts sharply at 228 to 230 C and has a specific rotation of $[\alpha]_D^{25}$ + 39 (1%, water).

Structure

Although molecular or equivalent weight data could not be readily obtained for this antibiotic, measurement of the areas in the nuclear magnetic resonance spectrum (Fig. 2) indicated the presence of 12 to 13 protons. Elemental analyses

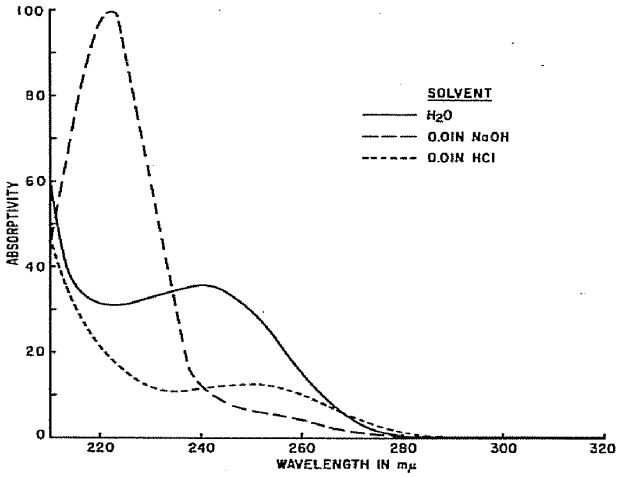


Fig. 1. *Ultraviolet absorption spectra of 5-azacytidine.*

supported the formula $C_8H_{12}N_4O_5$. Further examination of the nuclear magnetic resonance spectrum strongly suggested a nucleoside structure, with a singlet at 8.57 δ (measured from sodium 4,4-dimethyl-4-silapentanesulfonate) attributed to the proton of a -CH=N- grouping in the aglycone, a doublet at 5.80 δ to the anomeric proton, a strong singlet at 4.63 δ to exchangeable protons, and multiplets at 3.67 to 4.50 δ to the protons of the sugar moiety. The infrared spectrum (Fig. 3) supported a nucleoside structure, with strong absorptions at 3,300, 1,600 to 1,680, and 1,030 to 1,200 cm^{-1}.

The mass spectrum of the compound showed no molecular ion peak, but a major peak at 111 mass units corresponded to an aglycone fragment of formula $C_3H_3N_4O$. Acid hydrolysis gave the aglycone and a sugar identified as ribose by thin-layer chromatography. Analytical and spectral data on the aglycone suggested it to be 5-azacytosine, and this identity was confirmed by direct comparison with a synthetic sample (1).

The total structure and stereochemistry of this compound were established by comparison with a sample of synthetic 5-azacytidine (6). Melting points, optical rotations, and the ultraviolet, infrared, and nuclear magnetic resonance spectra of the two samples were identical. The structure of our compound is therefore 1-β-D-ribofuranosyl-5-azacytosine as shown in structure I.

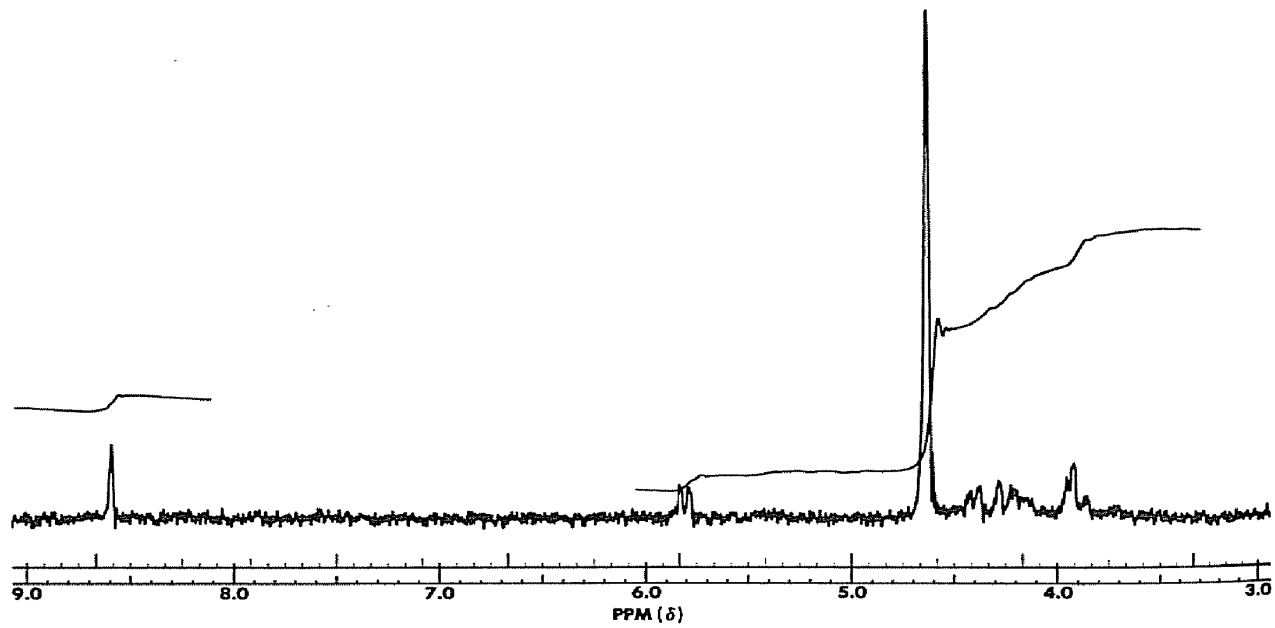


Fig. 2. *Nuclear magnetic resonance spectrum of 5-azacytidine in D_2O (Varian A60).*

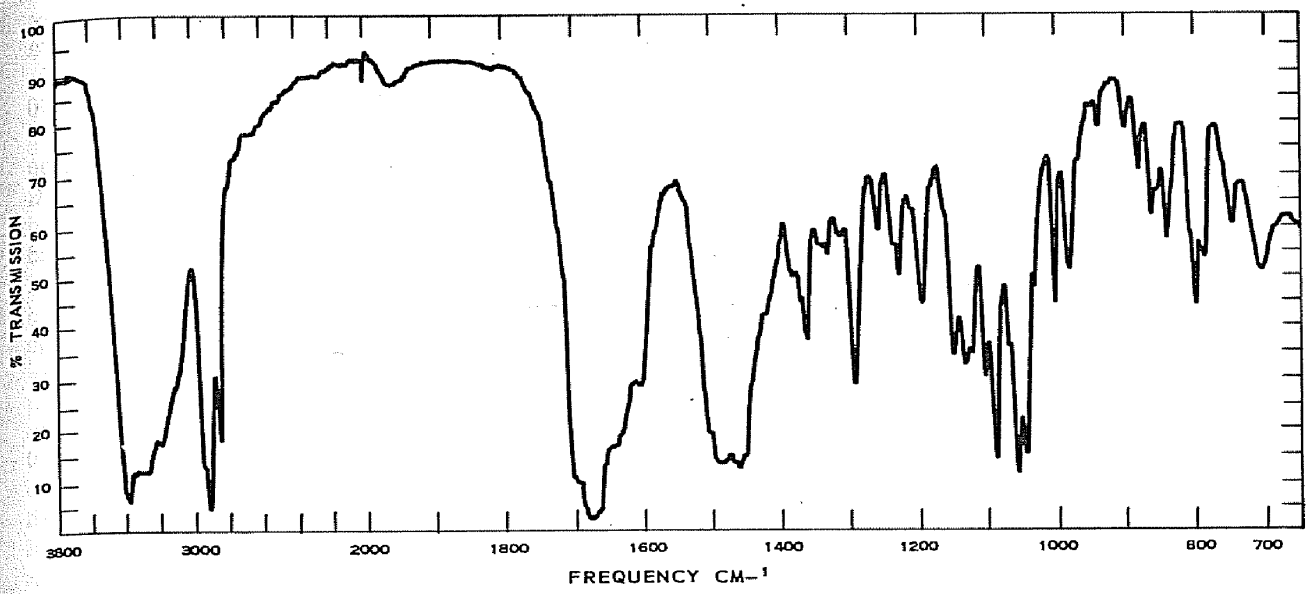
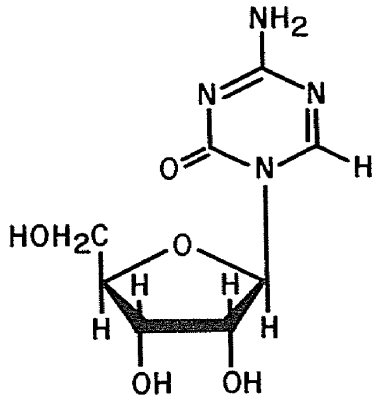


Fig. 3. *Infrared absorption spectrum of 5-azacytidine as a Nujol Mull.*



I

(5-AZACYTIDINE)

Experimental

Isolation from fermentation broth. The whole broth (265 liters) was filtered at *p*H 7.8 with 7% (w/v) diatomaceous earth filter aid. The filtered broth was mixed with 6% (w/v) Darco G-60 for 45 min. Carbon was removed by filtration, washed with 1/10 broth-volume of water, and eluted by suspending the carbon cake in 1/3 broth volume of 50% aqueous acetone. Three elutions were done. The carbon eluates were combined and concentrated in vacuo to an aqueous solution (*p*H 6.6) (15 liters) which was added to 75 liters of acetone. The precipitate which formed was removed by filtration and discarded. The clear filtrate was concentrated in vacuo to an aqueous solution (*p*H 6.6) which was freeze-dried. This dried preparation weighed 606 g and contained approximately 10 μg of 5-azacytidine per mg.

Partition chromatography. Impure 5-azacytidine, in aqueous solution, is most stable in the range of *p*H 5.0 to 7.5. It rapidly degrades in acidic or basic environments. For this reason, the following partition chromatography, as well as later silica gel chromatography and thin-layer chromatography, was conducted under buffered conditions.

A solvent system composed of 136 liters of 1-butanol, 45.2 liters of ethyl acetate, and 62.8 liters of *p*H 6.0 buffer (4) was mixed, and the two phases were separated. A 10.8-kg quantity of Dicalite 4200 was homogenized with 35 liters of the upper phase and 4.32 liters of the lower phase, and poured into a column 30.5 cm in diameter. Upper phase was passed through the column until the bed was at a constant height.

Crude 5-azacytidine (1,258 g; carbon eluate) was dissolved in 1,258 ml of lower phase. This solution was blended with 2,516 g of Dicalite 4200 and enough upper phase to impart flow, and was poured onto the top of the prepared column bed. The column was developed with upper phase at a flow rate of 500 ml/min. Fractions (4 liters) were collected from the time the feed was added, and they were spotted on an agar tray which had been seeded with *Salmonella schottmuelleri*. Fractions 24 through 34 contained 5-azacytidine. They were combined and mixed with 80 liters of petroleum hexane. The phases were separated and the aqueous phase was freeze-dried. The dried preparation (125 g) contained approximately 22 μg of 5-azacytidine per mg.

Silica gel chromatography and crystallization. The 5-azacytidine present in a dried preparation obtained by partition chromatography was purified by silica gel chromatography and crystallized. A 15-kg amount of silica gel #7734 (E. Merck AG, Darmstadt, Germany) was mixed with 12 liters of buffer solution which contained 408 g of KH_2PO_4 and 426 g of Na_2HPO_4. Unbound water was removed by evaporation at <100 C. The gel-buffer mixture was activated at 120 to 130 C for approximately 2 hr. After cooling, the buffered silica gel was slurried in chloroform and poured into a glass column (15-cm diameter) and packed to a constant height with flowing chloroform.

A 110-g preparation, obtained by partition chromatography, was dissolved in 400 ml of methanol and mixed with 900 g of buffered and activated silica gel #7734 which had been prepared as above. Methanol was removed by evaporation at room temperature. The dried mixture was slurried in approximately 3 liters of chloroform and added to the top of a prepared column bed. Chloroform-methanol (7:3) was layered onto the chloroform head, and the column was developed at a rate of 10 liters per hr.

Fractions were collected from the time the feed was added. They were assayed against *Escherichia coli* in a synthetic medium (2). The 5-azacytidine was present in fractions 106 to 130 liters. These fractions were combined and concentrated in vacuo to a volume of 100 ml which was stored at -20 C overnight. The crystals were removed by filtration, washed with methanol, and dried in vacuo to a constant weight of 975 mg (melting point, 218 to 225 C).

Recrystallization. A 250-mg portion of the crystalline 5-azacytidine was dissolved in 250 ml of boiling methanol. The solution was clarified by filtration, concentrated in vacuo to a volume of 10 ml, and held overnight at 5 C. Crystals were removed by filtration, washed with 5 ml of methanol followed by 5 ml of chloroform, and dried in vacuo to a constant weight of 191 mg. Ultraviolet spectrum: (0.01 N HCl) maximum at 249 mμ, a = 12.6. Analysis: $C_8H_{12}N_4O_5$. Calculated: C, 39.34; H, 4.95; N, 22.95; O, 32.77. Found: C, 39.25; H, 5.04; N, 22.87; O, 31.44. Melting point, 228 to 230 C.

A 600-mg portion of crystalline 5-azacytidine was dissolved in 6 ml of warm water. The solution was clarified by filtration and held overnight at 5 C. Crystals were removed by filtration and dried in vacuo to a constant weight of 404 mg. Ultraviolet spectrum: (0.01 N HCl) maximum at 250 mμ, a = 13.0.

Analyses of 5-azacytidine by thin-layer chromatography on buffered and activated silica gel HF_{254} plates (see next section for preparation) with solvent systems composed of methyl ethyl ketone-acetone-water (150:50:20)(5-azacytidine, R_F = 0.4), chloroform-methanol (1:1), (R_F = 0.2), or methanol (R_F = 0.55) revealed only 5-azacytidine. Visualization was with ultraviolet light (254 mμ), anisaldehyde spray (7), and potassium permanganate-sodium metaperiodate spray (3).

Acid hydrolysis. Crystalline 5-azacytidine (122 mg; 0.5 mmole) was hydrolyzed with 0.5 ml of 6 N HCl in a steam bath for 15 min, cooled to room temperature, and freeze-dried. Silica

gel #7734 (400 g) which had been buffered and activated as for the purification of 5-azacytidine was mixed with chloroform and packed in a 4.5-cm glass tube to a constant height of 46 cm. The entire hydrolysis mixture was dried from methanol onto 4.0 g of buffered and activated silica gel #7734, mixed with chloroform, and added to the top of the column bed. A solvent system composed of chloroform-methanol (75:25) was layered onto the chloroform, and the column was developed at a flow rate of 5 ml/min. Fractions (20 ml) were collected.

The fractions 105 to 145 were combined and concentrated in vacuo to a 3.0-ml volume and refrigerated overnight. Crystals were removed by filtration and dried to a constant weight of 6.5 mg. This crystalline preparation showed an ultraviolet absorption maximum at 250 mμ (4 N HCl), a = 62.3. The melting point was >350 C. The infrared absorption spectrum was identical to the 5-azacytosine which was produced synthetically.

The above hydrolysis proved to be too severe for the sugar moiety. Therefore, a milder hydrolysis was done for the following identification. 5-Azacytidine (12 mg) was hydrolyzed in a steam bath with 0.05 ml of 6 N HCl for 4 min, cooled to room temperature, and diluted to a volume of 6.0 ml with methanol. The presence of ribose was confirmed by thin-layer chromatography on plates (4 by 8 inches) which were prepared with silica gel HF_{254} (E. Merck AG, Darmstadt, Germany) suspended in a solution of buffer salts consisting of equal volumes of 0.2 M Na_2HPO_4 and 0.2 M KH_2PO_4 (5). The plates were activated at 120 to 130 C for 2 hr before use. Three different solvent systems were used for development: methyl ethyl ketone-acetone-water (150:50:20; ribose, R_F = .15); chloroform-methanol (1:1; ribose, R_F = .12); methanol (ribose, R_F = .23). The sugars were visualized with anisaldehyde spray (7). In each solvent system, the sugar in the hydrolysis mixture did not separate from authentic ribose but did separate easily from xylose and arabinose.

Synthesis of 5-azacytosine. The synthesis of 5-azacytosine was done according to Grundmann et al. (1). Formic acid (98%; 30 ml) was added to dicyanamide (cyanoguanidine, 21.0 g) in a round-bottom flask and heated (60 to 70 C) to start the reaction. As the reaction lessened, the mixture was heated in an oil bath at 120 to 130 C for 1 hr. After cooling, the crystalline mass was triturated with 95% ethanol, separated by filtration, and dried to a constant weight of 23.1 g. Ultraviolet spectrum (4 N HCl): maximum at 250 mμ, a = 12.2.

The 5-azacytosine contained in this product was further purified by a silica gel chromatography procedure used for purifying the hydrolysis product of 5-azacytidine. Briefly, 5.0 g of reaction product was dried onto 40 g of buffered and activated silica gel #7734 from acidic (HCl) methanol. This was placed on a 400-g column of buffered and activated silica gel and developed with chloroform-methanol (75:25). Fractions which contained solids with an ultraviolet absorption maximum at 250 mμ (4 N HCl) were combined and concentrated to a small volume which was then refrigerated. Crystals were removed by filtration and were dried in vacuo to a constant weight of 550 mg. Ultraviolet spectrum (4 N HCl): maximum at 250 mμ, a = 45.

A combination of preparations [647.6 mg, ultraviolet spectrum (4 N HCl): maximum at 250 mμ, a = 46.8] which included the above preparation was dissolved in 92.5 ml of water at 80 C. The solution was clarified by filtration and was cooled to 50 C. Methanol (185 ml) was added, and the mixture was refrigerated overnight. The white crystals which were collected by filtration and dried in vacuo (343 mg) had the following characteristics.

Analysis. Found: C, 31.91; H, 3.50; N, 49.12; O, 15.09. Calculated for $C_3H_4N_4O$: C, 32.14; H, 3.60; N, 49.98; O, 14.27. Ultraviolet absorption (4 N HCl): maximum at 249 mμ, a = 60.58. Electrometric titration (water): equivalent weight, 115 and pK_a, 8.3. Melting point, >350 C. The infrared and nuclear magnetic resonance spectra (D_2O, DCl) support the structure for 5-azacytosine.

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Highly Efficient Regioselective Synthesis of 5′-*O*-lauroyl-5-azacytidine Catalyzed by *Candida antarctica* Lipase B

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Abstract Enzymatic regioselective acylation of 5-azacytidine with vinyl laurate was successfully conducted with an immobilized lipase from *Candida antarctica* type B (i.e., Novozym 435) for the first time. The acylation of 5-azacytidine took place at its primary hydroxyl group and the desired product 5′-*O*-lauroyl-5-azacytidine could be prepared with high reaction rate, high conversion, and excellent regioselectivity. The influences of several key variables on the enzymatic acylation were also systematically examined. Pyridine was found to be the best reaction medium. The optimum initial water activity, the molar ratio of vinyl laurate to 5-azacytidine and reaction temperature were 0.07, 30:1, and 50 °C, respectively. Under the optimized conditions described above, the initial reaction rate, the substrate conversion, and the regioselectivity were as high as 0.58 mM/min, 95.5%, and >99%, respectively, after a reaction time of around 5 h.

Keywords 5-Azacytidine · Novozym 435 · Organic solvent · Regioselective acylation · Vinyl laurate

Introduction

5-Azacytidine, an analogue of the natural pyrimidine nucleoside cytidine, is employed for the treatment of myelodysplastic syndrome (MDS) [1]. However, it has some disadvantages in clinical application, such as difficulty to traverse biological membranes and skin layers due to poor lipophilicity [2], spontaneous hydrolysis in aqueous solutions and rapid deamination by cytidine deaminase, etc. [1, 3–6]. In order to overcome these problems, 5′-monoester of 5-azacytidine could be used, which is primarily based on the fact that the molecules containing hydroxyl or carboxyl groups can be converted into the corresponding esters with the desired lipophilicity by the selection of an appropriate ester side chain [7].

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5′-Monoester of 5-azacytidine could be synthesized through regioselective acylation of 5-azacytidine. On the other hand, the regioselective acylation of nucleoside is one of the important ways of introducing protecting groups as well as obtaining valuable nucleoside derivatives, and therefore will find wide applications in nucleoside chemistry.

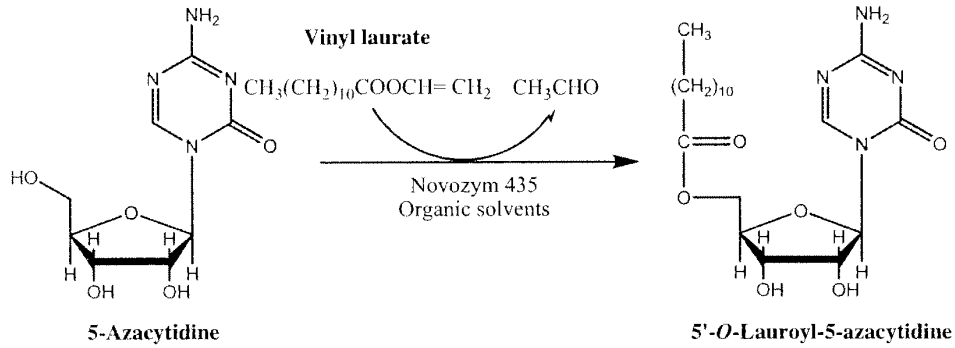
Several strategies for regioselective acylation of nucleosides have been reported using conventional chemical methods [8], but their applications are somewhat hampered due to the relatively low regioselectivity, the lack of easy access to some important intermediates, the tedious product isolation, and the environmental concerns of the process.

To date, enzymatic acylation of nucleosides in organic media has emerged as a promising procedure, due to its advantageous properties including high regioselectivity, mild reaction conditions, and environmental benign [9]. Besides, the use of organic solvents is especially advantageous when substrates or products are unstable in water. Furthermore, at a low water activity, many other water-dependent side-reactions can be prevented [10]. Ferrero and Gotor [11] have reviewed the utility of biocatalysts for the modification of nucleosides.

Various kinds of enzymes have been proven to be capable of catalyzing the acylation of nucleosides with desirable regioselectivity. Among them, Novozym 435, a commercially available lipase from *Candida antarctica* type B (CAL-B) immobilized on a macroporous resin of poly-(methyl methacrylate) (Lewatit VP OC 1600), is well recognized for its extraordinary ability to catalyze the esterification of nucleosides with substantially high regioselectivity [12, 13]. For example, Novozym 435 has been shown in our previous work to be highly active and regioselective for the enzymatic acylation of 1-β-D-arabinofuranosylcytosine [14].

Generally, fatty acid vinyl esters are preferable acyl donors in acyl transfer reactions [15]. In the course of our ongoing investigation, it was found that a great amount of undesired by-products were produced when short-chain fatty acid vinyl esters were used as acyl donors for the acylation of 5-azacytidine, while the use of long-chain fatty acid vinyl esters such as vinyl laurate yielded little by-products. Therefore, vinyl laurate, a typical long-chain fatty acid vinyl ester, is here adopted as an acyl donor for the enzymatic acylation of 5-azacytidine.

As an extension of our ongoing research program on efficient synthesis of various valuable nucleoside derivatives via enzymatic acylation, we herein for the first time report the successful regioselective acylation of 5-azacytidine with vinyl laurate catalyzed by Novozym 435 (Scheme 1) in organic solvents. The enzymatic acylation process might become a new route to the preparation of 5′-*O*-lauroyl-5-azacytidine, which is more lipophilic and might be more bio-available than 5-azacytidine. Also, the effects of several crucial factors on the enzymatic acylation are described in this paper.



Scheme 1 Novozym 435-catalyzed regioselective acylation of 5-azacytidine with vinyl laurate in organic solvents

Materials and Methods

Biological and Chemical Materials

Novozym 435 (an immobilized lipase from *Candida antarctica*, type B, 10,000 U g^{-1}) was kindly donated by Novozymes (Denmark). 5-Azacytidine and vinyl laurate were purchased from Fluka (Germany). All other chemicals were from commercial sources and were of the highest purity available.

Control of the Initial Water Activity

The reaction media, the substrate, and the enzyme were equilibrated to fixed initial water activities (α_w) over saturated salt solutions in closed containers at 25 °C separately [16–20]. The following salts were used: LiBr (α_w=0.07), LiCl (α_w=0.11), CH_3COOK (α_w=0.23), $MgCl_2$ (α_w=0.33). Molecular sieve was used to generate the nearly anhydrous reaction medium ($\alpha_w \sim 0$).

General Procedure for Enzymatic Reaction

In a typical experiment, 2 ml of pyridine containing 0.02 mmol 5-azacytidine, 0.6 mmol vinyl laurate, and 1,000 U Novozym 435 was incubated in a 10 ml Erlenmeyer shaking-flask capped with a septum at 200 rpm and 40 °C. Aliquots (20 μl) were withdrawn at specified time intervals from the reaction mixture, and then diluted by 50 times with a co-solvent mixture of water and methanol prior to HPLC analysis. To obtain larger amounts of product for its structural characterization, the synthesis was scaled up (~25 mg 5-azacytidine and 520 μl vinyl laurate). Upon the completion of the reaction, the reaction mixture was filtered to remove the immobilized enzyme and was evaporated under vacuum. The crude product was then purified by silica gel chromatography with the mixture of methanol and chloroform (25/75, *v*/*v*) as an eluant. After crystallization from ethanol, the product was obtained as a white powder (yield >90%).

HPLC Analysis

The reaction mixture was analyzed by RP-HPLC on a 4.6×250 mm (5 μm) Zorbax SB-C18 column (Agilent Technologies Industries Co., USA) using an Agilent G1311 A pump and a UV detector at 241 nm. The mobile phase was a mixture of ammonium acetate buffer (0.01 M, pH 4.27) and methanol (22/78, *v*/*v*) at a flow rate of 0.9 ml min^{-1}. The retention times for 5-azacytidine and 5′-*O*-lauroyl-5-azacytidine were 2.6 and 11.7 min, respectively. Regioselectivity was defined as the ratio of the HPLC peak area corresponding to the indicated product to that of all the products formed upon a certain reaction time according to the literature [21]. The initial rate (V_0) and the substrate conversion (c) were calculated from the HPLC date. The average error for this assay is less than 0.7%. All reported data are averages of experiments performed at least in duplicate.

Structure Determination

Mass spectrometric analysis in the negative ion mode was performed on an ion trap analyzer (Bruker HCTplus, Bruker Co., Germany). The capillary voltage was set at −113.5 V. ESI temperature and ion trap analyzer voltage were 300 °C and −40.0 V, respectively. The product

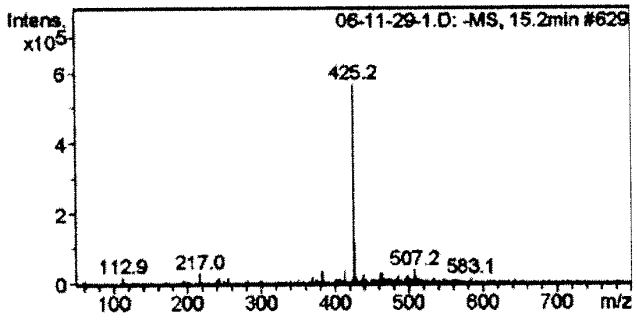


Fig. 1 Representative LC-MS/MS spectra of the main product with negative-ion mode

structure was determined by ^{13}C NMR (Bruker DRX-400 NMR Spectrometer, Bruker Co.) at 100 MHz. DMSO-d6 was used as a solvent and chemical shifts were expressed in ppm shift.

Results and Discussion

Product Characterization

As can be seen in Fig. 1, the molecular weight detected is around 425.2, which indicates that the product obtained is identical with mono lauroyl ester of 5-azacytidine (MW 426).

The ability of Novozym 435 to catalyze regioselective transformation has been exploited in the modification of polyhydroxy compounds [21, 22]. According to the published literature by Yoshimoto et al. [23], the acylation of a hydroxyl group of sugar results in a downfield shift of the peak corresponding to the *O*-acylated carbon atom and an upfield shift of the peak corresponding to the neighboring carbon atom. As evident from the data listed in Table 1, the ^{13}C NMR spectrum of the product shows a shift of 3.01 ppm on C5′ towards the lower fields as compared to the same carbon atom in the unmodified 5-azacytidine. Also, the directly neighboring carbon atom (C4′) gave a shift of about 3.27 ppm towards the higher fields due to the acylation of the hydroxyl group of C5′. In addition, 12 sharp peaks of $-CH_3$, $-CH_2$ and C=O appeared with the determinate chemical

Table 1 ^{13}C NMR spectral data for 5-azacytidine and its acylated derivative (δ, ppm)[a].

Carbon numbers	5-Azacytidine	5′-*O*-Lauroyl-5-azacytidine
Base moiety		
2	153.67	153.29
4	166.18	166.10
6	156.69	156.44
Sugar moiety		
1′	89.64	90.39
2′	74.26	73.79
3′	69.30	69.76
4′	84.65	81.38
5′	60.47	63.48
Acyl moiety		
C=O		169.54
$-CH_3$		13.97
$-CH_2$		22.54~33.91

[a] All samples were measured in DMSO-d_6.

Table 2 Novozym 435-catalyzed regioselective acylation of 5-azacytidine with vinyl laurate in different organic solvents[a].

Media	Solubility of 5-azacytidine (mM)[b]	lg*P*	V_0 (mM min^{-1})	C^c (%)	Regioselectivity (%)
DMSO	254.0	−1.35	0	0	0
DMF	112.9	−1.01	0	0	0
Pyridine	21.8	0.71	0.30	67.0	>99

[a] The reactions were carried out in 2 ml of different organic solvents (α_w=0.11) containing 0.02 mmol 5-azacytidine, 0.4 mmol vinyl laurate and 1000 U Novozym 435 at 200 rpm and 40 °C.

[b] The solubility of 5-azacytidine in each reaction medium was determined by HPLC analysis of the saturated solution at 30 °C.

[c] Maximum substrate conversion

shifts. So the product was proved to be 5′-*O*-lauroyl-5-azacytidine. And Novozym 435 was proved to display a startling regioselectivity up to 99% towards the 5′-hydroxyl group of 5-azacytidine.

It has been reported that *Candida antarctica* lipase B has a rather narrow and deep channel leading to an open active site [24]. The 5′-OH of the sugar moiety of 5-azacytidine may have an easier access to the active site of CAL-B to attack the acyl-enzyme intermediate than other −OH groups at C-3′ and C-2′ due to less steric hindrance, thus resulting in preferential acylation of the 5′-OH of 5-azacytidine.

Effect of Reaction Medium

One of the most troublesome limitations in the acylation of hydrophilic nucleosides is their poor solubility in most organic solvents. In fact, only polar organic solvents, such as pyridine and DMF, have been commonly used to solve the problem [25]. However, polar organic solvents usually strip the essential water off the enzyme molecules and then inactivate the biocatalyst, which greatly limits the application of enzymatic procedures in this area [10]. A less polar solvent does not inactivate the enzyme as much as a more polar one. As shown in Table 2, no reaction occurred in DMSO and DMF, although 5-azacytidine showed high solubility in these solvents. Only in pyridine could the lipase-catalyzed acylation of 5-azacytidine be efficiently carried out. Thus, pyridine was selected as the most suitable solvent for the reaction.

Table 3 Effect of initial water activity on Novozym 435-catalyzed regioselective acylation of 5-azacytidine in pyridine[a].

Initial water activity (α_w)	V_0 (mM min^{-1})	C^b (%)	Regioselectivity (%)
≈0	0.34	80.7	>99
0.07	0.39	84.4	>99
0.11	0.30	67.0	>99
0.23	0.14	14.2	>99
0.33	0.09	9.3	>99

[a] The reactions were performed in 2 ml of pyridine with different initial water activity containing 0.02 mmol 5-azacytidine, 0.4 mmol vinyl laurate and 1000 U Novozym 435 at 200 rpm and 40 °C.

[b] Maximum substrate conversion

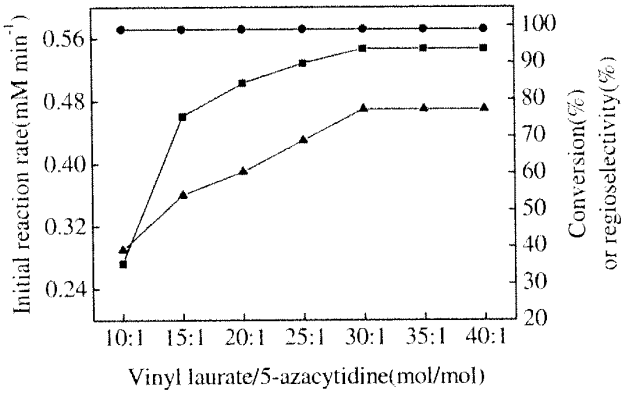


Fig. 2 Effect of the molar ratio of vinyl laurate to 5-azacytidine on the enzymatic regioselective acylation of 5-azacytidine. The reactions were performed in 2 ml of pyridine (α_w=0.07) containing 0.02 mmol 5-azacytidine, 1,000 U Novozym 435 and different amounts of vinyl laurate at 200 rpm and 40 °C. *Filled triangle*, initial reaction rate; *filled square*, conversion; *filled circle*, regioselectivity

Effect of Initial Water Activity

Generally speaking, water activity (α_w) plays a crucial role in enzymatic reactions in non-aqueous media [26–30]. In the case of the enzymatic acylation of 5-azacytidine, the presence of water may foster the competitive hydrolysis of both the desired product 5′-*O*-lauroyl-5-azacytidine and the acyl donor vinyl laurate. Therefore, it is of great importance to investigate the effect of initial water activity on the enzymatic acylation.

As shown in Table 3, Novozym 435-mediated acylation of 5-azacytidine with vinyl laurate shows a clear dependence on the α_w of the reaction system. Both the initial reaction rate and the substrate conversion increased rapidly with increasing α_w value up to 0.07, beyond which further rise in α_w value gave rise to a sharp drop in the initial reaction rate and the substrate conversion. This is because the presence of water in the reaction medium is essential for the enzyme to keep its catalytic conformation. On the other hand, water can promote the hydrolysis reactions of both the product and the acyl donor. Therefore, there exists an optimal water activity for the enzymatic acylation. The lower water activity does not provide sufficient water for the buildup of the essential water shell for the enzyme, and the higher water activity implies excessive water and thereby the lower product yield and more inactivation of the enzyme caused by the acid from the competitive hydrolysis of vinyl laurate [31, 32]. Additionally, α_w showed no significant effect on the regioselectivity, which kept above 99% within the range examined. Obviously, the optimum initial water activity for the reaction was 0.07.

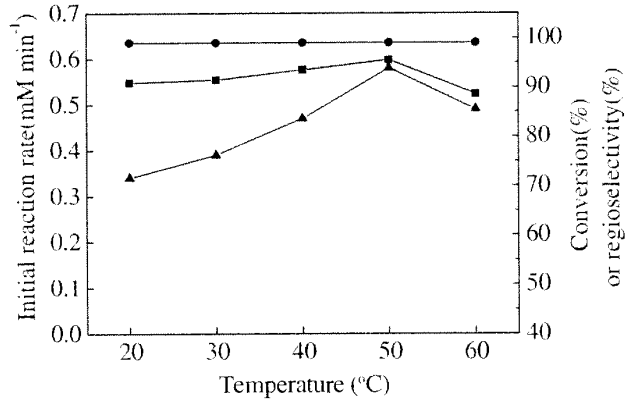


Fig. 3 Effect of reaction temperature on the enzymatic regioselective acylation of 5-azacytidine. The reactions were conducted in 2 ml of pyridine (α_w=0.07) containing 0.02 mmol 5-azacytidine, 0.6 mmol vinyl laurate and 1,000 U Novozym 435 at 200 rpm and various temperatures. *Filled triangle*, initial reaction rate; *filled square*, conversion; *filled circle*, regioselectivity

Effect of the Molar Ratio of Vinyl Laurate to 5-azacytidine

Thermodynamically, high molar ratio of vinyl laurate to 5-azacytidine may push the reaction towards the acylation of 5-azacytidine and speed up the reaction. As depicted in Fig. 2, the enzymatic acylation of 5-azacytidine was greatly affected by the molar ratio of vinyl laurate to 5-azacytidine. Remarkable enhancement in both the initial rate and the substrate conversion was observed with the increase of the ratio up to 30:1, beyond which both the initial rate and the substrate conversion showed no appreciable improvement with further increase in the molar ratio. It was also worth noting that throughout the range of molar ratio of vinyl laurate to 5-azacytidine tested, the regioselectivity manifested no variation and kept above 99%. Therefore, 30:1 was selected as the favorable molar ratio of vinyl laurate to 5-azacytidine for the enzymatic acylation. It is obvious that the excessive amount of vinyl laurate was necessary for the lipase-catalyzed acylation, which was in good accordance with our previous report [30]. Also, it has been proved experimentally that the presence of excessive amount of vinyl laurate inhibits the hydrolysis of the desired product (5′-*O*-lauroyl-5-azacytidine). In addition, the hydrolysis of vinyl laurate might consume considerable amount of vinyl laurate and lower the acylation rate and substrate conversion [32, 33].

Effect of Reaction Temperature

Temperature has great effect on the activity, selectivity and stability of a biocatalyst and the thermodynamic equilibrium of a reaction as well [34]. As show in Fig. 3, within the range from 20 to 50 °C, higher temperature resulted in both higher initial rate and higher substrate conversion. Further rise in temperature, however, led to a drastic drop in both the initial rate and substrate conversion. The regioselectivity of the reaction constantly maintained above 99% at temperatures ranging from 20 to 60 °C. The partial inactivation of the lipase in pyridine at a higher temperature (above 50 °C) may partly account for the drop in both the initial rate and the substrate conversion, which was further supported by assaying the residual activity of the enzyme after being incubated at temperatures higher than 50 °C. Thus, the optimum reaction temperature was shown to be 50 °C.

Conclusions

In summary, the regioselective acylation of 5-azacytidine with vinyl laurate could be successfully performed. Under the optimized conditions, the initial rate, the substrate conversion and the regioselectivity were as high as 0.58 mM/min, 95.5%, and >99%, respectively, after a reaction time of around 5 h. The results described here further highlights the versatility of lipases and show that the enzymatic acylation of nucleosides is a promising area.

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Research Paper

Inhibition of P-Glycoprotein by D-α-Tocopheryl Polyethylene Glycol 1000 Succinate (TPGS)

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Purpose. To investigate whether d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) functions as an inhibitor of P-glycoprotein (P-gp), the multidrug resistance transporter.

Methods. Two assays were used to measure the function of TPGS on P-gp function. First, we examined the ability of TPGS to modulate the cytotoxicity of established, cytotoxic, P-glycoprotein substrates. Parental NIH 3T3 cells and NIH 3T3 cells transfected with the human MDR1 cDNA (G185) were exposed to doxorubicin, paclitaxel, colchicine, vinblastine and 5-fluorouracil (5FU) in the presence or absence of TPGS. Cytotoxicity was assessed with the MTT assay. Second, polarized transport of the P-gp substrates rhodamine 123 (R123), paclitaxel and vinblastine was measured using the human intestinal HCT-8 and Caco-2 cell lines grown in Transwell dishes. Drug flux was measured by liquid scintillation counting or fluorescence spectroscopy of the media.

Results. G185 cells were 27–135 fold more resistant to the cytotoxic drugs doxorubicin, vinblastine, colchicine and paclitaxel than the parental NIH 3T3 cells. In contrast 5FU, which is not a P-gp substrate, is equally cytotoxic to parental and G185 cells. Co-administration of TPGS enhanced the cytotoxicity of doxorubicin, vinblastine, paclitaxel, and colchicine in the G185 cells to levels comparable to the parental cells. TPGS did not increase the cytotoxicity of 5FU in the G185 cells. Using a polarized epithelial cell transport assay, TPGS blocked P-gp mediated transport of R123 and paclitaxel in a dose responsive manner.

Conclusions. These data demonstrate that TPGS acts as a reversal agent for P-glycoprotein mediated multidrug resistance and inhibits P-gp mediated drug transport. These results suggest that enhanced oral bioavailability of drugs co-administered with TPGS may, in part, be due to inhibition of P-glycoprotein in the intestine.

KEY WORDS: P-glycoprotein; TPGS; drug transport; bioavailability.

INTRODUCTION

The multidrug transporter, P-glycoprotein (P-gp), is a 170 kDa membrane protein which functions as an ATP-dependent drug efflux pump. One activity of this protein is to lower the intracellular concentration of drugs thereby reducing the cytotoxic activity of anticancer drugs. Increased expression of this protein has been observed in human tumors and is often associated with failure of chemotherapy due to drug resistance (1–5). P-gp removes a large number of chemically unrelated drugs extending over many therapeutic indications such as anticancer drugs, steroids, antihistamines, antibiotics, calcium channel blockers and anti-HIV peptidomimetics (2,4,5).

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ABBREVIATIONS: P-gp, P-glycoprotein; R 123, Rhodamine 123; CsA, Cyclosporine A; TPGS, d-α-tocopheryl polyethylene glycol 1000 succinate.

The P-gp drug transporter is encoded by one gene, MDR1, in humans whereas in rodents two genes, *mdr1a* and *mdr1b* encode highly similar drug transporters (6,7). P-gp is primarily expressed on the luminal surface of epithelial cells from several tissues including the intestine, liver, kidney, and the endothelial cells comprising the blood-brain and blood-testes barriers (8–10). The ability of this protein to export toxic compounds combined with this localization led to the hypothesis that a physiological function of the MDR1 encoded P-gp may be as a protective barrier or export mechanism for xenobiotics. Indeed, recent investigations with knockout mice in which the *mdr1a* gene was disrupted have confirmed such a protective role for P-gp (11–14). Exposure of *mdr1a* deficient mice to vinblastine or ivermectin results in significantly higher tissue and plasma levels compared to wild-type animals. Moreover, these compounds are toxic in the knockout mice at doses which are innocuous to heterozygous and wild-type mice. These experiments further suggested a role for P-gp in the blood brain barrier since the ivermectin accumulated in the brain of the *mdr1a* deficient animals but not animals with an intact *mdr1a* gene. The knockout mice displayed ivermectin toxicity at doses 50 to 100 fold less than wild-type mice.

Additional data have supported a role of P-gp in the intestine as both a barrier to absorption as well as a mechanism of disposition of drugs such as vinblastine, etoposide, paclitaxel and digoxin. For example, Su and Huang observed that inhibition of P-gp increased bioavailability of digoxin by increasing absorption as well as reducing excretion (15). A similar phenomenon was observed with etoposide (16). P-glycoprotein has recently been suggested to be critical in oral drug absorption (17–19). In concert with the drug metabolizing enzyme CYP3A, P-gp may limit oral drug bioavailability in the gut by controlling drug transport from the intestinal lumen and by affecting access to CYP3A (19).

Vitamin E TPGS, d-α-tocopheryl polyethylene glycol 1000 succinate, is a derivative of vitamin E consisting of a hydrophilic polar head group (tocopherol succinate) and a lipophilic alkyl tail (polyethylene glycol) resulting in amphiphilic properties (Eastman Kodak, technical bulletin EFC-226). TPGS has a relatively low critical micelle concentration, 0.02 wt%, and acts to solubilize lipophilic compounds. Bordreaux et al. reported a two-fold increase in cyclosporine CsA area under the plasma concentration-time-curve (AUC) when co-administered with LiquiE, a glycerol and water solution of TPGS (20). Sokol et al. similarly observed increases up to 71% in CsA AUC in subjects who received concomitant TPGS (21). Both Sokol and Bordreaux suggested that the increased drug absorption was due to enhanced micelle formation, resulting in improved CsA solubilization. Chang *et al.* later reported a 61% increase in CsA AUC when dosed with 20–25% of the TPGS previously used in the Sokol or Bordreaux studies (22). Chang *et al.* also suggested that TPGS may interact with P-gp in the intestine to increase CsA absorption.

In the current investigation we examine the effect of TPGS on P-gp mediated drug resistance and transport of established P-gp substrates. If this agent functions as a P-gp reversal agent then perhaps its effect on drug absorption is, in part, mediated by inhibition of active drug efflux in the intestine. Our data show TPGS to be an effective inhibitor of P-gp mediated drug

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resistance and transport at concentrations well below the reported critical micelle concentration and suggests that its reversal activity is due to an effect on transport activity.

MATERIALS AND METHODS

Cell Culture

The NIH3T3 Swiss mouse embryo cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD) and was grown in Dulbecco's Modified Eagles Medium (Biowhittaker, Walkersville, MD) supplemented with 4.5 g/L glucose, 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine (Advanced Biotechnologies Incorporated (ABI), Columbia, MD), and 0.01 mg/ml gentamicin (ABI). The drug resistant, NIH-MDR-G185, cell line, derived by transfection of the human MDR1 gene into NIH3T3 cells (23), was obtained from M. M. Gottesman (NCI, NIH) and was maintained in similar medium supplemented with 60 ng/ml of colchicine (Sigma, St. Louis, MO). HCT-8 cells (ATCC), isolated from a human ileocecal adenocarcinoma cell line, were grown in RPMI-1640 medium (Biowhittaker) supplemented with 10% horse serum (Biowhittaker), 1 mM sodium pyruvate (Gibco BRL, Grand Island NY) and 0.01 mg/ml gentamicin. Caco-2 cells (ATCC), derived from a human colonic adenocarcinoma, were grown in Eagle's MEM (Biowhittaker) supplemented with 10% fetal bovine serum, and 0.01 mg/ml gentamicin. All cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Cytotoxicity Assay

Cells were plated at a density of $2.5–3.0 \times 10^3$ cells/well in 96-well microtiter plates (PGC, Gaithersburg, MD) and were exposed to 1–5000 nM of doxorubicin, vinblastine, colchicine, paclitaxel, 0.1–25 nM 5-fluorouracil (Sigma) and 0.001–.005% TPGS (Eastman, Kingsport, TN) for 72 hours. To ensure solubilization of the TPGS, a 1% solution of TPGS in ethanol was prepared fresh for each experiment and diluted further in cell culture medium to the indicated concentrations. Cell viability was determined with the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium, Sigma) assay as previously described (24,25) and the absorbance was measured with a Dynex MRX Microplate Reader (Chantilly, VA) at 570 nm. This assay is based on the reduction of MTT by mitochondria in viable cells to water insoluble formazan. The data presented are the mean ±SD of at least 3 independent experiments, each performed in quadruplicate.

Rhodamine 123 Transport

Rhodamine 123 (R123; Sigma) transport was examined as previously described (26,27) using both HCT-8 and Caco-2 cells. Briefly, cells were grown in 6 well Corning Transwell dishes (HCT-8) or collagen coated Transwell dishes (Caco-2) until a tight monolayer was formed as measured by transepithelial electrical resistance or lucifer yellow impermeability. The integrity of the monolayers following the transport experiments was similarly evaluated. Typical TEER values were > 300 Ohms/cm^2. R123 was added at a final concentration of 13 μM to the basal or apical compartments and 200 μl samples were taken at the indicated times from the opposite chamber. TPGS was added as an inhibitor to both compartments. Fluorescence of R123 in the media samples was measured using a Biotek FL500 Fluorescence Plate Reader (Winooski, VT) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All experiments were performed in triplicate; the data presented are the mean ±SD and are representative of multiple experiments.

Paclitaxel, Vinblastine and Cyclosporine Transport

Inhibition of [^{3}H] paclitaxel (Moravek Biochemical, Brea, CA), [^{3}H] vinblastine (Amersham, Arlington Heights, IL), and [^{3}H] cyclosporine (CsA; Amersham) efflux by TPGS was examined in a manner similar to R123. The transported drug, 0.1 μM (0.25 μCi/ml), was added to either the basal or apical compartment and 200 μl aliquots were taken at the indicated times from the opposite chamber. Radioactivity was measured by liquid scintillation counting.

Western Blot Analysis

Western blot analysis was performed as previously described 28. Briefly, crude cell membranes were isolated by lysing the cells in 10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 1 mM $MgCl_2$ supplemented with pepstatin (1.5 μg/ml), leupeptin (1.5 μg/ml) and 0.2 mM pefabloc. Cells were homogenized with 20 strokes of Dounce "B" (tight) pestle (Wheaton, Millville, NJ), nuclei and cell debris were removed by centrifugation for 10 minutes at 400 × g. The supernatants were then centrifuged at 100,000 × g for 30 minutes at 4°C and the pellets were resuspended in lysis buffer and stored at −80°C. 20 μg samples were fractionated in 8% polyacrylamide-SDS gel and transferred to 0.45 μm nitrocellulose membrane. The membranes were blocked in PBS-T (0.1% Tween-20 in PBS) containing 5% skim milk for 1 hour and then probed with 1 μg/ml of C219 antibody (Signet Laboratories, Dedham, MA) in PBS overnight. The membranes were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL).

RESULTS

Western Blot Analysis

We first measured the relative levels of P-gp expression in the NIH3T3 and G185 cell lines by western blot analysis using the C219 antibody, which recognizes all P-gp isoforms (29). Consistent with previous data, high P-gp expression was observed in the G185 cells relative to that in the parental NIH 3T3 cells (Fig. 1). We also examined P-gp expression in two human intestinal carcinoma cell lines, Caco-2 and HCT-8 which have been previously used for investigation of drug transport and to have polarized expression of P-gp (26). We observed that each of these intestinal cell lines have moderate P-gp expression albeit lower than the G185 cells (Fig. 1).

Cytotoxicity Experiments

The interaction of TPGS with P-gp was initially examined with cytotoxicity assays using parental NIH3T3 and MDR1 transfected G185 cells to cytotoxic anticancer drugs. Consistent with previous reports (23,30), G185 cells were more resistant

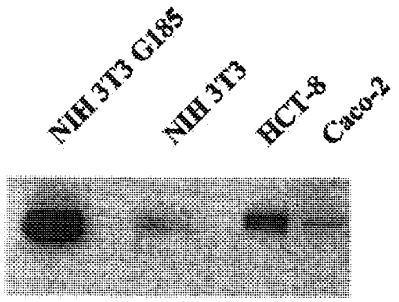

Fig. 1. Western blot analysis of P-glycoprotein expression. Twenty micrograms of total cell membrane proteins were separated by SDS-PAGE, transferred to PVDF filters which were subsequently probed with the C219 antibody and visualized using chemiluminescence as described in Materials and Methods. Lane 1, NIH-3T3 G185; lane 2, NIH-3T3; lane 3, HCT-8; lane 4, Caco-2.

Table 1. EC_{50} in NIH3T3 and NIH3T3-G185 Cells

	NIH3T3	NIH3T3-G185	NIH3T3-G185 (1 μM Verapamil)	NIH3T3-G185 (5 μM CsA)	NIH3T3-G185 (.0025% TPGS)
Doxorubicin	35	950	35	35	35
Vinblastine	2	270	20	6	40
Paclitaxel	60	>5000	100	40	1070
Colchicine	30	1000	ND	100	45

Note: NIH 3T3 and G185 cells were treated with 0–5000 nM doxorubicin, vinblastine, paclitaxel or colchicine in the absence or presence of 1 μM verapamil, 5 μM CsA or 0.0025% TPGS. The concentration of the drug that reduces cell viability by 50% (EC_{50}) was determined using the MTT cytotoxicity assay as described in Materials and Methods. Each experiment was performed in quadruplicate and repeated in at least 3 independent experiments. ND, not determined.

to doxorubicin, paclitaxel, vinblastine and colchicine compared to parental NIH3T3 cells (Fig. 2). EC_{50} values were 27 to 135 fold higher in G185 cells relative to the parental NIH3T3 cells (Table 1). Established P-gp reversal agents, such as cyclosporine A (CsA) and verapamil, reduced the resistance to doxorubicin cytotoxicity in G185 cells to levels comparable to parental NIH3T3 cells (Fig. 3). The reversal effect of CsA on doxorubicin, vinblastine, taxol and colchicine mediated toxicity in parental NIH3T3 cells was modest as previously reported. This is consistent with their low level of P-gp expression (data not shown, (27,30). Co-administration of CsA or verapamil caused a similar reversal of G185 resistance to vinblastine, paclitaxel, and colchicine (Table 1 and data not shown).

The effect of TPGS on P-gp mediated drug resistance was investigated by treating G185 cells with doxorubicin, vinblastine, paclitaxel, and colchicine concomitantly with varying doses of TPGS. The presence of TPGS increased drug sensitivity of the G185 cells to doxorubicin in a dose dependent manner

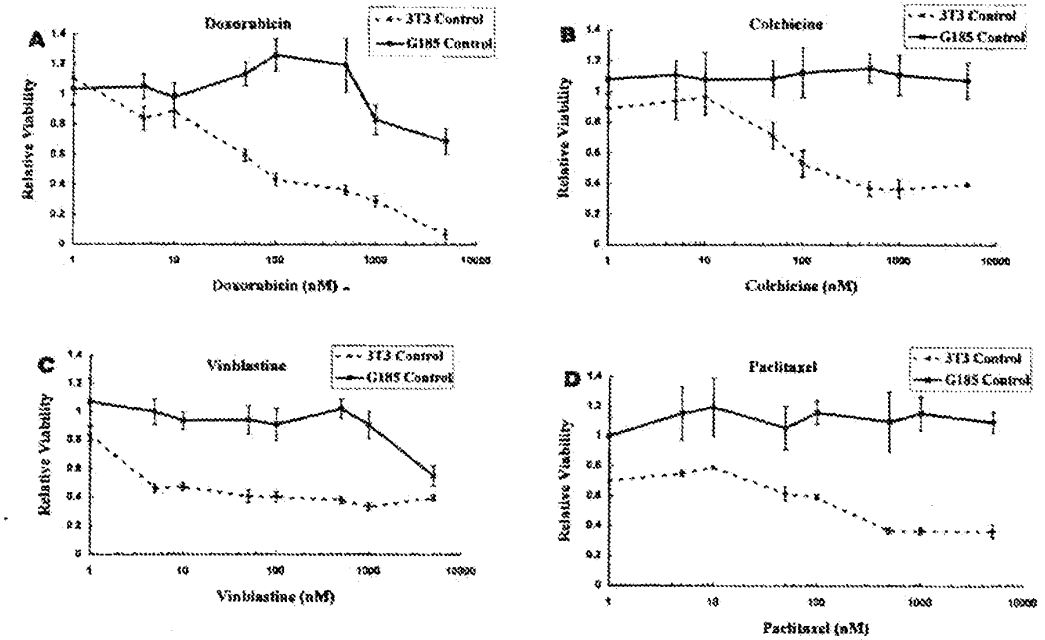

Fig. 2. Cytotoxicity of doxorubicin (A), colchicine (B), vinblastine (C), and taxol (D) in parental NIH-3T3 cells (circles), and MDR1-transfected NIH-3T3 G185 cells (squares). Cells were treated with the indicated concentrations of drugs and the viability was measured by the MTT assay as described in Materials and Methods. Data are expressed relative to untreated control cells. Each experiment was performed in quadruplicate and the data presented represent the mean ± SD of four independent experiments.

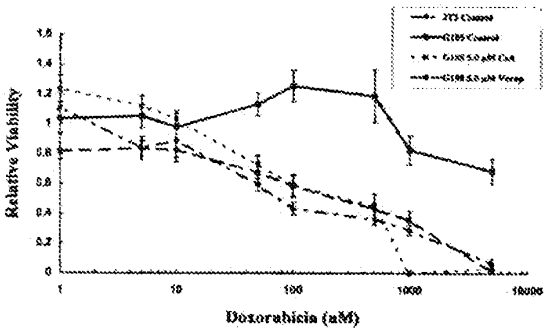

Fig. 3. Effect of CsA and Verapamil on the cytotoxicity of doxorubicin. Parental NIH-3T3 (diamonds) and G185 (squares) cells were exposed to the indicated concentrations of doxorubicin in the absence or presence of CsA, 5.0 μM, or verapamil, 5.0 μM.. Data are expressed relative to untreated control cells. Each experiment was performed in quadruplicate and the data presented represent the mean ± SD of four independent experiments.

(Fig. 4). Treatment of the drug resistant G185 cells with TPGS lowered the EC_{50} concentrations for doxorubicin, vinblastine, paclitaxel and colchicine (Table 1). TPGS, 0.0025%, sensitized the G185 cells to all four of these P-gp substrate cytotoxic drugs to levels comparable to the parental NIH 3T3 cells. The highest dose of TPGS, 0.005%, resulted in decreased viability of both NIH3T3 and G185 cells and is likely due to toxicity associated with the high concentration of TPGS. At concentrations below 0.005% TPGS itself did not affect cell viability. These data suggest that TPGS modulates drug resistance by inhibiting P-gp activity in cells which over-express the MDR1 gene.

5-Fluorouracil Cytotoxicity

Treatment of parental NIH 3T3 and G185 cells with 5-fluorouracil (5FU), a chemotherapeutic agent not transported by P-gp, results in a similar level of cytotoxicity in both cell lines (Fig. 5A) (31). Furthermore, co-incubation of 5FU with CsA had no effect on the cytotoxicity of 5FU in either G185 or NIH3T3 cells (Fig. 5B). Similarly, co-incubation of TPGS with 5FU did not increase the cytotoxicity of 5FU in either of these cell lines (Fig. 5C).

Rhodamine 123 Transport

The fluorescent dye R123, an established substrate of P-glycoprotein (32,33), was used to examine the ability of TPGS to block P-gp mediated transport. HCT-8 and Caco-2 cells have previously demonstrated directional transport of established P-gp substrates such as vinblastine, paclitaxel, CsA and R123 in the basolateral to apical direction (26,34–36). Expression of P-gp in these cells was confirmed by western blot analysis using the C219 antibody (Fig. 1). R123 was transported approximately 7 and 9 fold greater flux in the basolateral to apical direction in HCT-8 and Caco-2 cells, respectively (Fig. 6). Consistent with this transport being mediated by P-gp, R123 flux was inhibited approximately 80% by co-incubation with 5 μM CsA. Similarly, 0.001–0.0025% TPGS blocked the basolateral to apical transport of R123 in a dose responsive manner further suggesting that TPGS inhibits transport mediated by P-gp (Fig. 6).

Paclitaxel Transport

The ability of TPGS to inhibit P-gp was confirmed by measuring polarized transport of pachtaxel. [^{3}H] Paclitaxel is a good substrate for P-gp with approximately 14 and 40 fold greater transport from the basolateral to the apical compartment in HCT-8 and Caco-2 cells, respectively (Fig. 7). Addition of 5 μM CsA blocked the polarized flux of paclitaxel by 80–90%. Similarly, co-incubation with TPGS resulted in a dose dependent decrease in paclitaxel transport (Fig. 7). The IC_{50} of TPGS for inhibition of paclitaxel transport is approximately 0.001% (v/v) in HCT-8 cells and 0.005% in Caco-2 cells. Polarized

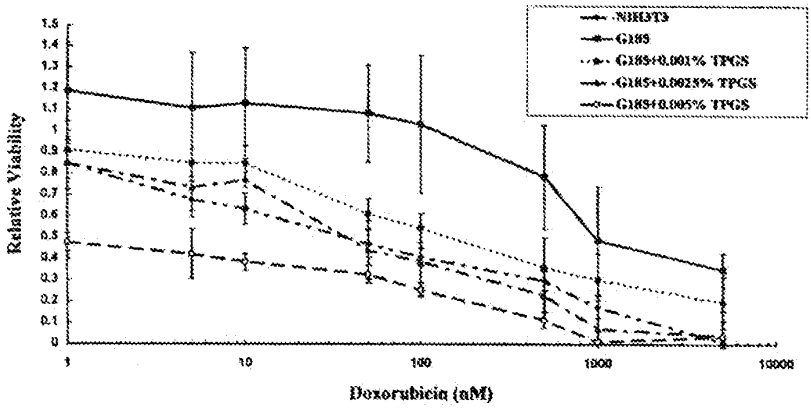

Fig. 4. TPGS reversal of P-gp mediated resistance to doxorubicin. Parental NIH-3T3 (diamonds) and G185 cells were exposed to the indicated concentrations of doxorubicin with 0% TPGS (squares), 0.001% TPGS (circles), 0.0025% TPGS (triangles) or 0.005% TPGS (open circles, ○). Data are expressed relative to untreated control cells. Each experiment was performed in quadruplicate and the data presented represent the mean ± SD of four independent experiments.

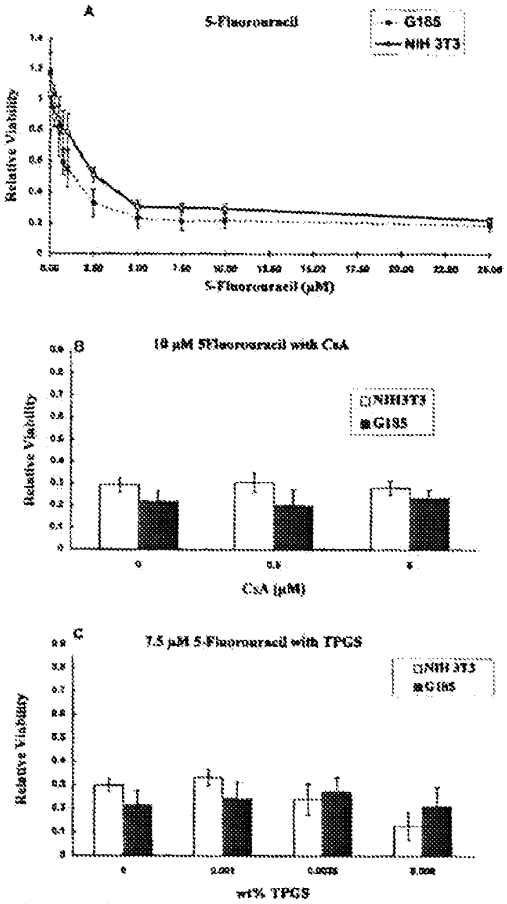

Fig. 5. Cytotoxicity of 5-fluorouracil to parental NIH-3T3, and drug resistant NIH-3T3 G185 cell lines in the presence and absence of CsA and TPGS. A. NIH-3T3 (diamonds) and NIH-3T3 G185 (squares) cells were exposed to the indicated concentrations of 5-fluorouracil. Viability was measured by the MTT assay as described in Materials and Methods. Data are expressed relative to untreated control cells. Each experiment was performed in quadruplicate and the data presented represent the mean ±SD of three independent experiments. B. Parental NIH-3T3 (open bars) and G185 (closed bars) cells were exposed to 10 μM of 5FU with the indicated concentrations of CsA. C. Parental NIH-3T3 (open bars) and G185 (closed bars) cells were exposed to 7.5 μM of 5FU and the indicated concentrations of TPGS.

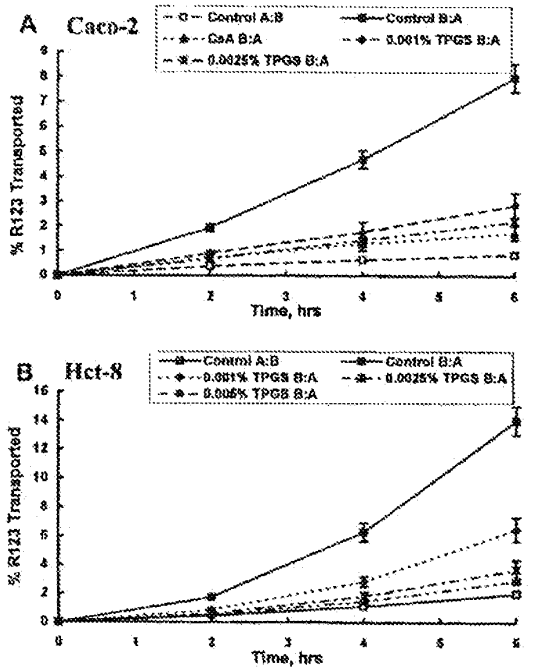

Fig. 6. Rhodamine 123 transport in Caco-2 and HCT-8 cells. Caco-2 (A) and HCT-8 (B) were grown on Transwell dishes as described in Materials and Methods. Rhodamine 123, 13 μM, was added to the apical or basolateral compartment in the absence or presence of CsA, 5 μM, or 0.0025, 0.005, 0.001% TPGS and media aliquots were taken from the opposite chamber at the indicated times. The data presented are the mean ±SD of triplicate wells and are representative of at least three independent experiments.

transport of [^{3}H] vinblastine and [^{3}H] CsA were also inhibited by addition of TPGS (data not shown). These data, combined with the cytotoxicity and R123 transport data suggest that TPGS is an effective P-gp reversal agent.

DISCUSSION

A major effort has been undertaken by many laboratories to identify inhibitors of P-glycoprotein to increase the efficacy of cancer treatment and to enhance the absorption of orally administered drugs. The data presented here support the hypothesis that TPGS functions as one such P-gp inhibitor. TPGS increased the sensitivity of P-gp expressing cells to several widely used cytotoxic drugs which are well established P-gp substrates. TPGS also effectively blocked polarized transport of R123 and paclitaxel in an epithelial cell transport assay. The reduction of directional transport provides strong evidence for TPGS functioning as an inhibitor of P-gp. Conversely, no effect was observed with 5FU, a cytotoxic drug not associated with P-gp mediated drug resistance or transport. 5FU is not transported by the P-gp pump thus, its cytotoxicity is unaffected by the addition of established P-gp inhibitors such as quinine, quinidine or verapamil (31,37). In the experiments presented here neither TPGS nor CsA impacted the cytotoxicity of 5FU in either the NIH 3T3 or G185 cells.

Previously it has been suggested that co-administration of TPGS with CsA enhanced absorption of the immunosuppressant due to micelle formation (21). Concentrations of TPGS administered in the current work are well below the critical micelle concentration, 0.02 wt% in water at 37°C, therefore it is unlikely that micelle formation is responsible for the observed effects. In fact, the IC_{50} required to inhibit R123 and paclitaxel transport across HCT-8 or Caco-2 cell monolayers is 20 fold less than the critical micelle concentration. Further, 0.001 wt% TPGS also significantly reversed the multidrug resistant phenotype of the NIH3T3-G185 cell line to doxorubicin, vinblastine, taxol

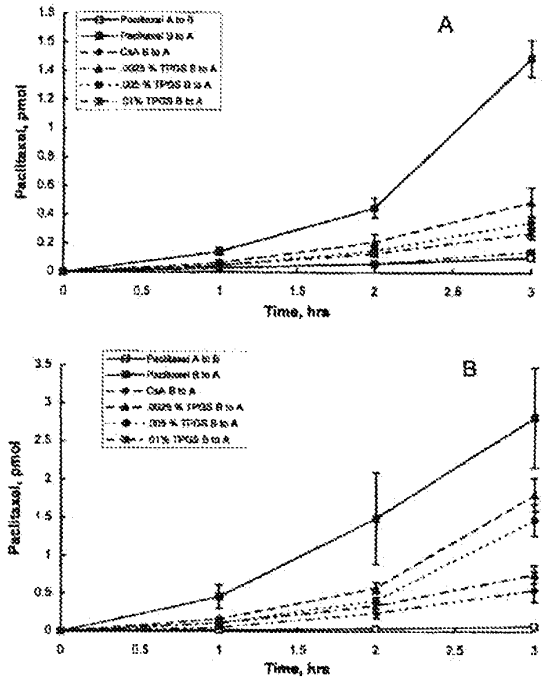

Fig. 7. Paclitaxel transport in HCT-8 and Caco-2 cells. Cells were grown on in Transwell dishes as described in Materials and Methods. [^{3}H]Paclitaxel, 0.1 μM (0.2 μCi/ml) was placed in the basolateral (B) or apical (A) chamber; media aliquots were taken at the indicated times and radioactivity was measured by liquid scintillation counting. Control Caco-2 (A) or HCT-8 (B) cells B to A (closed squares), control A to B (open squares), 5 μM CsA B to A (diamonds), 0.0025% TPGS B to A (triangles), 0.005% TPGS B to A (circles).

and colchicine, all established P-gp substrates. These data suggest that TPGS micelle formation in the intestinal lumen may not be the sole factor behind the increase in CsA absorption previously observed (20–22).

Several other surfactants, e.g. polysorbates, Cremophor EL, and Solutol 15, have been observed to be inhibitors of P-gp (38–41). These compounds are frequently added to pharmaceutical formulations to enhance solubility. These agents may also function to inhibit P-gp to add to their effect of enhancing drug absorption. Indeed the plasma concentrations of Cremophor EL in patients administered paclitaxel, which is formulated with this surfactant, reach levels sufficient to inhibit P-gp *in vitro* (42). The efficacy of this drug may, in part, be due to the activity of the Cremophor EL. Pluronic P85 has also recently been observed to block P-gp mediated rhodamine 123 efflux in Caco-2 and bovine brain microvessel endothelial cells (43). These data suggested that this agent may be useful for formulations to enhance brain and oral absorption.

TPGS has been used to enhance the bioavailability of CsA in liver transplant patients with the effects of significantly improving absorption and reducing daily drug cost. Sokol *et al.* and Boudreaux et al. reported increases in CsA absorption in pediatric transplant recipients treated with oral TPGS, 12.5 IU/kg and 10 IU/kg, respectively. The majority of patients receiving TPGS had previously experienced chronic cholestasis resulting in decreased bile flow suggesting poor solubilization of the lipophilic CsA. It was hypothesized that TPGS functioned as a bile substitute and solubilized the CsA through micelle formation, thus facilitating the absorption of the drug through the intestinal lumen. Similarly, Pan *et al.* reported a 28 and 32% decrease in CsA daily dose when co-administered with Liqui-E, a water soluble form of TPGS and a 26% decrease in daily CsA cost (44). Using normal healthy volunteers, Chang *et al.* observed a 60% rise in CsA area under the curve (AUC) in subjects receiving a TPGS-CsA cocktail. Decreased oral clearance and volume of distribution were also observed in those subjects. These authors proposed that the large, amphipathic TPGS may also be acting as an inhibitor of P-glycoprotein to enhance absorption and decrease transport back into the intestinal lumen. The current data support the hypothesis that one mechanism through which TPGS may enhance oral bioavailability is via inhibition of P-gp. Clearly further study on the effect of TPGS on oral drug delivery is required to confirm such a role.

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ORIGINAL ARTICLE

A pilot pharmacokinetic study of oral azacitidine

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Azacitidine is a pyrimidine nucleoside analog of cytidine with hypomethylating and antileukemia activity. Azacitidine has been shown to have survival benefits in patients with high-risk myelodysplastic syndrome (MDS), and has activity in the treatment of acute myelogenous leukemia (AML). It is administered by subcutaneous (s.c.) or intravenous (i.v.) injection daily at a dose of 75 mg/m^2 for 7 days every 4 weeks. An oral formulation would facilitate dosing, reduce administration side effects and potentially maximize azacitidine pharmacologic action. Previously, oral formulations of this class of agent have failed due to rapid catabolism by cytidine deaminase and hydrolysis in aqueous environments. Development of a film-coated formulation has circumvented this difficulty. In a formulation feasibility pilot study, four subjects with solid malignant tumors, AML or MDS received single oral doses of 60 or 80 mg azacitidine. Subjects demonstrated measurable plasma concentrations of azacitidine, allowing bioavailability comparisons to be made to historical pharmacokinetic data for s.c. azacitidine. Subjects safely tolerated 80 mg, a dose for which the mean bioavailability was 17.4% of historic s.c. exposure. No severe drug-related toxicities were observed. These data suggest that oral azacitidine is bioavailable in humans and should be studied in formal phase 1 trials.
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Introduction

Azacitidine is a ring analog of the pyrimidine nucleoside cytidine, with therapeutically useful effects on cell differentiation, gene expression and DNA synthesis and metabolism.[1] Azacitidine inhibits methylation of newly synthesized DNA by inhibiting DNA methyltransferase activity.[2–4] Aberrant DNA methylation (hypermethylation) results in silencing of genes responsible for cell growth control and differentiation, and has been associated with cancers, including MDS, and other hematologic malignancies.[5–9] Azacitidine is believed to exert its antineoplastic effects, in part, by inducing DNA hypomethylation.[10–14] Induction of DNA hypomethylation may restore normal function to genes that are critical for differentiation and proliferation.[5,15,16]

The goal of pharmaceutical intervention is to provide maximum therapeutic effectiveness with minimum risk to subjects. Orally administered medications allow for convenient dosing, but, because of gastrointestinal absorptive barriers, enzyme activity and interactions with other ingested substances, achieving therapeutic plasma levels of active drug by this route is sometimes challenging. This is particularly important for azacitidine, which is thought to be degraded in the intestinal tract by spontaneous hydrolysis and, enzymatically, by cytidine deaminase.[17] Until recently, attempts to develop an oral formulation of pyrimidine nucleosides have been unsuccessful because of intestinal degradation. An oral formulation of azacitidine will be more convenient for patients, facilitate long-term dosing, reduce the potential for local side effects associated with subcutaneous (s.c.) injection, and, allow for the evaluation of alternative doses and schedules. It is hypothesized that continuous (low-dose) oral azacitidine administration may provide more persistent DNA hypomethylation, translating to superior or differentiated efficacy compared to that seen with the parenteral formulation.

Recently, a new formulation of oral azacitidine has been developed that is orally bioavailable in a canine model.[18] Based on these data, a pilot phase 1 feasibility study was conducted in subjects with MDS, leukemia or solid tumors to assess the oral bioavailability and safety of this new oral formulation of azacitidine.

Methods

The objectives of this study were: (1) to obtain initial information on the oral bioavailability of azacitidine administered as a film-coated tablet; (2) to assess the safety and tolerability of escalating doses of orally administered azacitidine and (3) to gather preliminary information on the single-dose pharmacokinetics of azacitidine after oral administration.

Drug formulation

Oral azacitidine was supplied as 20 mg film-coated tablets manufactured by Azopharma in Miramar, FL, USA.

Study design

This was an open-label, single-treatment, escalating-dose, pharmacokinetic study in which single-subject cohorts were treated with escalating oral doses of azacitidine in 20 mg increments. Subjects were to receive only one dose of azacitidine. Dosing began with the first subject receiving a dose of 60 mg (three 20 mg tablets). If tolerated, the dose was to be escalated in a second subject by 20 mg (total of 80 mg) and then subsequent subjects until one of the following conditions was met: (1) drug administration was deemed intolerable; (2) the appropriate concentrations of drug were found in plasma or (3) dose escalation reached the 200 mg level, which is approximately equivalent to the maximum approved daily dose of subcutaneous azacitidine (that is, 100 mg/m^2).

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'Appropriate concentrations of drug in plasma' were defined as at least four consecutive timed samples containing quantifiable concentrations of azacitidine from which pharmacokinetic assessments could be made. After appropriate concentrations of azacitidine were found in plasma, the same dose was administered to 1–2 additional subjects to verify results.

Azacitidine treatment was deemed intolerable if a subject experienced any Grade 3 or 4 adverse event possibly related to azacitidine or a significant safety concern was identified following treatment. If a subject was found to be intolerant, a second or third subject was treated at the same dose level to confirm intolerance. If no safety concerns were identified and no Grade 3 or 4 adverse events occurred, then dose escalation could continue until appropriate concentrations of plasma azacitidine were found (or a maximum dose of 200 mg was reached).

Pharmacokinetics

Blood samples for the determination of plasma azacitidine concentrations were collected on day 1 prior to dosing and at the following time points after dosing: 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8 and 10 h, (and 12 h, if possible). Plasma was harvested and subsequently analyzed for azacitidine concentrations using a validated high-performance liquid chromatography/tandem mass spectrometric method (LC-MS/MS) (unpublished observation).

Azacitidine plasma concentration versus time data were used to calculate pharmacokinetic parameters using non-compartmental methods. Parameters calculated included C_{max}, T_{max}, $T_{1/2}$, AUC(0-t) and AUC(0-∞). Bioavailability (F%) was determined relative to historic pharmacokinetic data following s.c. treatment with azacitidine at a dose of 75 mg/m^2, approximately equal to 135 mg/day, based on a 1.8 m^2 body surface area.[18]

Subjects

Male or female subjects with MDS, AML or malignant solid tumors who were $\geqslant$18 years of age, had an Eastern Cooperative Oncology Group performance status of 0–2 and who signed a study-specific informed consent document were eligible for participation in the study. For subjects with AML or malignant solid tumors, eligibility was limited to those for whom standard curative or palliative measures did not exist or were no longer effective. Renal, hepatic and gastrointestinal parameters were required to be within normal limits. The study did not include subjects who were pregnant, had a history of severe cardiac or pulmonary disease, had advanced malignant hepatic tumors or who had received radiation therapy, chemotherapy or investigational drugs within 30 days of study onset. The study met institutional guidelines for both clinic centers.

Results

Four subjects were treated with single doses of oral azacitidine, one subject at 60 mg and three at 80 mg. Azacitidine concentrations were detected in plasma of all four subjects. Thus, the primary study endpoint was met and the study was complete (Table 1).

Pharmacokinetic results

Quantifiable plasma concentrations of azacitidine were found in the 60 mg subject, but plasma samples measured post-C_{max} were too sparse to calculate pharmacokinetic parameters. Thus, dose escalation to 80 mg followed. After determining adequate blood levels in this second subject, two additional subjects were treated at the 80 mg level. All three subjects treated at 80 mg had quantifiable azacitidine in plasma at multiple time points, allowing for valid ascertainment of pharmacokinetic parameters (Tables 2 and 3).

Maximum azacitidine plasma concentrations were reached at 3 h for the subject dosed at 60 mg and at a mean time of 1.5 h (range 1–2 h) for those dosed at 80 mg. The historical T_{max} for a s.c. dose of 135 mg is 0.5 h (the first sampling point that was employed in the study).[19] The mean maximum plasma concentration for those treated orally with 80 mg azacitidine was 64.4 ng/ml (range 26.9–91.1), which is 8.5% of the C_{max} for a s.c. dose of 135 mg (Table 2) and approximately fourfold higher than that for the 60 mg oral dose. Mean plasma half-life for the oral 80 mg dose was 0.389 h compared to 0.68 h for the s.c. 135 mg dose. Bioavailability of the oral dose was 17.4% relative to the s.c. 135 mg dose (Table 3).

Figure 1 illustrates in log scale the plasma azacitidine concentration versus time profiles of the orally treated subjects in the present study compared to the profile following s.c. 75 mg/m^2 (135 mg) dosing.

Using computer simulation of the plasma concentrations and overall drug exposure observed in this study, the azacitidine pharmacokinetic profile at doses of up to 600 mg remains below that seen with s.c. administered azacitidine at 75 mg/m^2/day.

Safety

Oral azacitidine was well tolerated by all four subjects. Serious adverse reactions were not observed in any subject during the 10 $\pm$ 3 day post-dose observation period, and most adverse events emerging during this time frame were not considered related to azacitidine. Two exceptions were headache and vomiting (possibly related) reported for subject 1 (60 mg azacitidine). Most adverse events were rated mild or moderate and resolved with no treatment. One subject treated at 80 mg experienced serious adverse events (staphylococcal bacteremia, decreased platelets and decreased hemoglobin) 24 days

Table 1 Subject characteristics

Subject number	*Age*	*Sex*	*Tumor type*	*ECOG status*	*Oral aza dose*
1	43	M	Metastatic thymic carcinoid, mets to lung and skin lesion	1 (restricted)	60 mg
2	67	M	Prostate cancer	0 (fully active)	80 mg
3	57	M	AML	2 (ambulatory, capable of self care)	80 mg
4	65	M	MDS secondary to successfully treated AML (CR, complete remission, achieved)	1 (restricted)	80 mg

Abbreviations: AML, acute myelogenous leukemia; aza, azacitidine; CR, complete remission; ECOG, Eastern Cooperative Oncology Group; F = female; M = male; MDS, myelodysplastic syndrome.

Table 2 Azacitidine plasma concentrations (ng/ml)

	Oral azacitidine concentration (ng/ml)					Subcutaneous azacitidine concentration[30] (ng/ml)	
	Subject number						
Time (h)	1 (60 mg)	2 (80 mg)	3 (80 mg)	4 (80 mg)	Mean (80 mg)	Time (h)	75 mg/m^2 s.c.
0	0	0	0	0	0	0	0
0.5	BLQ	BLQ	BLQ	4.96	1.65	0.5	750.0
1.0	3.72	8.20	31.50	91.10	43.60	1	354.2
1.5	4.56	26.90	58.50	61.70	49.03	2	124.5
2.0	3.75	9.21	75.10	26.50	36.94	4	17.9
2.5	12.40	3.35	36.40	13.90	17.88	8	BLQ
3.0	15.80	1.37	14.10	4.30	6.59		
3.5	4.27	BLQ	6.10	1.97	2.69		
4.0	1.55	BLQ	2.43	BLQ	0.81		
4.5	BLQ	BLQ	1.19	BLQ	0.40		
5.0	BLQ	BLQ	BLQ	BLQ	BLQ		

Abbreviations: BLQ, below limit of quantitation (1.0 ng/ml); h, hours; s.c., subcutaneous 75 mg/m^2 is approximately 135 mg/day based on a 1.8 m^2 body surface area.

Table 3 Summary of PK

Subject number	Dose	$AUC_{(0-\infty)}$ (ng × h/ml)	C_{max} (ng/ml)	$T_{1/2}$ (h)	T_{max} (h)	F (%)[a]
1	60 mg	22.6[b]	15.8	—	3.0	6.6
2	80 mg	24.9	26.9	0.36	1.5	5.4
3	80 mg	112.6	75.1	0.42	2.0	24.5
4	80 mg	102.8	91.1	0.39	1.0	22.3
Mean ($n = 3$)	80 mg	80.1	64.4	0.389	1.5	17.4

Abbreviations: AUC, area under the plasma concentration curve; ; C_{max}, maximum plasma concentration; F, bioavailability; h, hours; PK, pharmacokinetics; $T_{1/2}$, half life; T_{max}, time to maximum plasma concentration.
[a]Percent bio-availability compared with historical s.c. azacitidine data (dose = 135 mg; median $AUC_{(0-\infty)}$ = 777 ng × h/ml).[20]
[b]$AUC_{(0-t)}$ is presented and was used for F calculations.

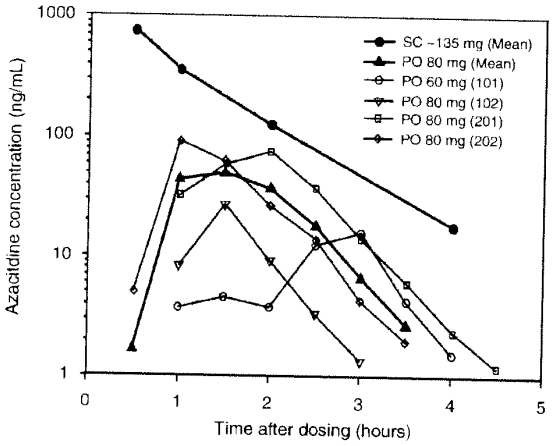

Figure 1 Concentration versus time profiles for individual subjects. Log scale comparison of plasma concentration versus time for a single dose of oral azacitidine in four subjects (mean and individual) compared to single dose subcutaneous (SC) administration (mean).

post-dosing, which were considered life threatening and required hospitalization. These were not related to azacitidine treatment. Table 4 summarizes treatment emergent adverse events observed during the study. Further investigation of increasing doses of oral azacitidine administered with sequential daily dosing is needed to determine the safety profile of oral azacitidine.

Discussion

Vidaza (azacitidine for injection) is approved in the United States for treatment of all French-American-British subtypes of MDS and has demonstrated activity in the treatment of AML.[20] Silverman *et al.*[21] demonstrated prolonged time to AML transformation in elderly patients with refractory anemia with excess blasts and refractory anemia with excess blasts in transition to AML and a trend for prolonged survival following azacitidine therapy. These results were confirmed by a recent study in 358 high-risk MDS patients, which showed statistically significant ($P = 0.0001$) greater overall survival with azacitidine treatment (24.4 months) compared to a conventional care regimen (15 months).[22] Current dosing is by s.c. injection or intravenous (i.v.) infusion with a regimen of 75 mg/m^2 for 7 days every 28 days. Several studies have shown diminished parenteral azacitidine-induced DNA demethylation by the end of a 4-week treatment cycle.[23–25]

Oral azacitidine bioavailability has been examined in dogs and rats. A comparison of oral azacitidine (6 mg/kg) to s.c. and i.v. dosing (2 mg/kg) in dogs showed rapid absorption by the oral route ($T_{max} = 15$ min), with absolute bioavailability of 67% (compared to 71% following s.c. dosing). Absolute oral bioavailability in rats was 47%.[18] Preexisting human oral bioavailability data for azacitidine is limited. Three reports of oral azacitidine administration include one patient who received 2 mg azacitidine three times daily for 5 days for the treatment of β-thalassemia,[26] and two sickle cell patients who received 0.2 mg/kg/day 3 days per week for up to 28 weeks.[27,28] In both studies, the cytidine deaminase inhibitor

Table 4 Adverse events

Subject	*Event*	*Related to study Medication*	*Grade*[a]	*Comments*
1	Headache	Possibly	2	No treatment; resolved
	Vomiting	Possibly	2	No treatment; resolved
	Chest Pain	No	1	Treated with hydromorphone HCl and morphine sulfate; not resolved
2	Hypotension	No	1	No treatment; resolved
	Nausea	No	**1**	Treated with granisetron HCl and ondansetron; not resolved
3	Constipation	No	2	No treatment; resolved
	Dizziness	No	2	No treatment; resolved
	Fatigue	No	2	No treatment; resolved
4	Hypotension	No	1	No treatment; resolved
	Indigestion	No	1	No treatment; resolved
	Decreased hemoglobin	No	3	Transfusion
	Headache	No	1	No treatment; resolved

[a]1 = mild; 2 = moderate; 3 = severe.

tetrahydrouridine was co-administered with azacitidine. In the patient with β-thalassemia, no increase in total hemoglobin levels was observed, but at the time of therapy the patient was in an advanced disease state. In the sickle cell patients, a rapid and maintained elevation of total hemoglobin, fetal hemoglobin and F cells was observed for the duration of therapy. These data suggest the drug is orally available and exerts systemic effects. At the administered dose of 0.2 mg/kg/day with co-administration of 200 mg of tetrahydrouridine, no evidence of cytotoxicity was observed.

In this pilot study, we have demonstrated that an oral formulation of azacitidine can be absorbed in humans and provide detectable levels of azacitidine in plasma. An observable delay in absorption with oral dosing was apparent and thought to be a function of the film-coated tablet formulation.

It can be concluded from this study that single oral doses of azacitidine (up to 80 mg) are bioavailable, safe and well tolerated. However, high oral doses may be required to overcome potential absorptive limitations of gastrointestinal administration and reach a clinically significant therapeutic effect. A multi-dose phase 1 study to further evaluate the safety, tolerability, pharmacokinetics and pharmacodynamics of oral azacitidine is currently ongoing. Continuous oral dosing of azacitidine may be a feasible alternative to s.c. dosing, providing greater ease of administration, allowing the possibility of a more sustained maximum therapeutic effect and eliminating local injection site reactions.

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ORIGINAL REPORT

Phase I Study of Oral Azacitidine in Myelodysplastic Syndromes, Chronic Myelomonocytic Leukemia, and Acute Myeloid Leukemia

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ABSTRACT

Purpose

To determine the maximum-tolerated dose (MTD), safety, pharmacokinetic and pharmacodynamic profiles, and clinical activity of an oral formulation of azacitidine in patients with myelodysplastic syndromes (MDSs), chronic myelomonocytic leukemia (CMML), or acute myeloid leukemia (AML).

Patients and Methods

Patients received 1 cycle of subcutaneous (SC) azacitidine (75 mg/m^2) on the first 7 days of cycle 1, followed by oral azacitidine daily (120 to 600 mg) on the first 7 days of each additional 28-day cycle. Pharmacokinetic and pharmacodynamic profiles were evaluated during cycles 1 and 2. Adverse events and hematologic responses were recorded. Cross-over to SC azacitidine was permitted for nonresponders who received ≥ 6 cycles of oral azacitidine.

Results

Overall, 41 patients received SC and oral azacitidine (MDSs, n = 29; CMML, n = 4; AML, n = 8). Dose-limiting toxicity (grade 3/4 diarrhea) occurred at the 600-mg dose and MTD was 480 mg. Most common grade 3/4 adverse events were diarrhea (12.2%), nausea (7.3%), vomiting (7.3%), febrile neutropenia (19.5%), and fatigue (9.8%). Azacitidine exposure increased with escalating oral doses. Mean relative oral bioavailability ranged from 6.3% to 20%. Oral and SC azacitidine decreased DNA methylation in blood, with maximum effect at day 15 of each cycle. Hematologic responses occurred in patients with MDSs and CMML. Overall response rate (ie, complete remission, hematologic improvement, or RBC or platelet transfusion independence) was 35% in previously treated patients and 73% in previously untreated patients.

Conclusion

Oral azacitidine was bioavailable and demonstrated biologic and clinical activity in patients with MDSs and CMML.

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Azacitidine is a cytidine nucleoside analog with a mechanism of action that involves incorporation into DNA and RNA.[1,2] Data suggest that patients must be exposed to azacitidine over several treatment cycles for optimal therapeutic effect.[4] The requirement for chronic exposure can be explained by drug pharmacokinetics, as azacitidine has a short plasma half-life, and by mechanism of action, as induction of DNA hypomethylation through incorporation into DNA is cell-cycle dependent (S-phase restricted) and DNA remethylation is observed by the end of each treatment cycle.[4]

A treatment regimen facilitating chronic administration may help achieve optimal efficacy outcomes. An oral azacitidine formulation would improve convenience of administration and expand the possibilities of exploring novel maintenance schedules, targeting different malignancies, and testing multiple combinations. A phase 0 trial demonstrated that a single oral azacitidine dose resulted in detectable levels in the blood.[5]

This phase I study sought to identify the maximum-tolerated dose (MTD), dose-limiting toxicities (DLTs), safety, pharmacokinetic and pharmacodynamic profiles, and clinical activity of oral azacitidine in patients with myelodysplastic syndromes (MDSs), chronic myelomonocytic leukemia (CMML), or acute myeloid leukemia (AML).

The trial was approved by the relevant institutional review boards and ethics committees. All patients gave written informed consent.

Patients

Eligible patients were ≥ 18 years, had an Eastern Cooperative Oncology Group performance status score of 0 to 2, and a diagnosis of MDSs, CMML, or AML according to WHO classification.[6,7] For patients with AML, eligibility was limited to those for whom standard curative measures did not exist or were no longer effective. Exclusion criteria included a diagnosis of acute promyelocytic leukemia, previous treatment with hypomethylating agents within 4 weeks before cycle 1, and anticancer therapy within 21 days before the first dose of study drug, or less than full recovery from any significant toxic effects of prior treatments.

Table 1. Baseline Patient Characteristics (N = 41)

Parameter	No. of Patients	%
Median age, years	70	
Range	31-91	
Sex		
Male	32	78
Female	9	22
MDSs (WHO classification)	29	71
RA/RARS/RCMD	11	27
RAEB-1	12	29
RAEB-2	5	12
MDSs-U	1	2
CMML	4	10
AML	8	20
De novo	4	10
Transformed from MDSs	4	10
IPSS (MDSs patients)*		
Low risk	2	7
Intermediate 1 risk	12	41
Intermediate 2 risk	13	45
High risk	1	3
Not available†	1	3
Hematology		
Median hemoglobin, g/dL	9.3	
Range	6.9-15.1	
Median white blood cell count $\times 10^9$/L	2.4	
Range	0.4-30.2	
Median absolute neutrophil count $\times 10^9$/L	0.8	
Range	0.0-21.7	
Median platelet count $\times 10^9$/L	54.0	
Range	3.0-262.0	
Cytogenetics‡		
Normal chromosomal karyotype	17	49
1 chromosomal abnormality	9	26
2 chromosomal abnormalities	3	9
≥ 3 chromosomal abnormalities	6	17
Prior treatment with hypomethylating agent	16	39
MDSs	13	32
CMML	0	0
AML	3	7

Abbreviations: AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; IPSS, International Prognostic Scoring System; MDSs, myelodysplastic syndromes; MDSs-U, MDSs unclassified; RA, refractory anemia; RAEB, RA with excess blasts; RARS, RA with ringed sideroblasts; RCMD, refractory cytopenias with multilineage dysplasia.

*IPSS score[11] was available for 28 patients with MDSs.

†Patient had a bone marrow transplantation and therefore IPSS risk was not considered applicable.

‡Cytogenetic data were available for 35 patients.

Study Design and Therapy

This open-label, phase I, dose-escalation trial was performed in four participating institutions and evaluated multiple cycles of oral azacitidine administered daily for the first 7 days of a 28-day cycle. The objectives were to determine the MTD, DLTs, and the safety profile of oral azacitidine. Pharmacokinetic and pharmacodynamic profiles of oral and subcutaneous (SC) azacitidine, administered on the same 7-day schedule, were also compared. A secondary objective was to assess the clinical activity of oral azacitidine.

During cycle 1, patients received azacitidine 75 mg/m^2 daily SC for 7 days of a 28-day cycle. During cycle 2 and beyond, patients received oral azacitidine under fasting conditions (ie, no food for 2 hours before and after dosing). The dose of oral azacitidine was escalated following a standard phase I 3 + 3 design. The starting dose was 120 mg and doses were escalated in 60 mg increments up to a dose of 360 mg, followed by 120 mg increments until the MTD was reached. Intrapatient dose escalation was permitted if the dose level to which the patient was escalated was associated with a DLT rate of ≤ 33%. Treatment continued until disease progression, lack of activity, unacceptable toxicity, or patient preference.

The MTD was defined as the highest dose at which no more than 33% of patients experienced a DLT. DLT was defined as: grade ≥ 3 nausea, diarrhea, or vomiting despite adequate/maximal medical intervention; grade ≥ 3 clinically significant nonhematologic toxicity unrelated to underlying disease or intercurrent illness; failure to recover to an absolute neutrophil count (ANC) of higher than 500/μL and/or platelet count of higher than 25,000/μL with hypocellular bone marrow (< 5%) 42 days after starting oral azacitidine (patients with a baseline ANC of ≤ 500/μL and/or platelet count of ≤ 25,000/μL were not evaluable for neutrophil or platelet toxicity); any treatment-related effect resulting in missing ≥ 3 oral azacitidine doses in the 7-day treatment period; or any treatment-related nonhematologic toxicity delaying initiation of the second oral azacitidine cycle by longer than 14 days. Only DLTs that occurred during the first oral azacitidine cycle were considered in determining the MTD. Adverse events were graded using the National Cancer Institute Common Toxicity Criteria for Adverse Events version 3.0.

Table 2. Incidence of Adverse Events According to Severity in ≥ 20% of Patients Treated With Oral Azacitidine (n = 41)

System Organ Class Preferred Term (MedDRA 10.1)	CTCAE Grade 1		2		3		4		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
Diarrhea	10	24.4	12	29.3	4	9.8	1	2.4	27	65.9
Nausea	8	19.5	10	24.4	3	7.3	0		21	51.2
Constipation	9	22.0	7	17.1	0		0		16	39.0
Vomiting	4	9.8	6	14.6	3	7.3	0		13	31.7
Abdominal pain	6	14.6	4	9.8	0		0		10	24.4
Headache	7	17.1	5	12.2	1	2.4	0		13	31.7
Fatigue	6	14.6	2	4.9	4	9.8	0		12	29.3
Peripheral edema	11	26.8	1	2.4	0		0		12	29.3
Fever	6	14.6	2	4.9	2	4.9	0		10	24.4
Cough	7	17.1	1	2.4	2	4.9	0		10	24.4
Contusion	9	22.0	0		0		0		9	22.0
Dizziness	5	12.2	3	7.3	0		0		8	19.5
Febrile neutropenia	0		0		8	19.5	0		8	19.5

NOTE. This Table includes all adverse events which started during any dosing cycle at which oral azacitidine was administered. Percentages are based on the number of patients who received at least one dose of oral azacitidine. Multiple reports of the same preferred term from a patient are counted only once, using the maximum CTCAE grade.

Abbreviations: CTCAE, National Cancer Institute Common Toxicity Criteria for Adverse Events; MedDRA, Medical Dictionary for Regulatory Activities.

Pharmacokinetic Analysis

Plasma and urine pharmacokinetic evaluation of azacitidine was performed on days 1 and 7 in cycles 1 and 2. Samples were collected up to 8 hours after administration and analyzed using a validated high-performance liquid chromatography/tandem mass spectrometric method. Parameters calculated using noncompartmental method, included maximum observed plasma concentration (C_{max}), time of maximum observed plasma concentration (T_{max}), area under the plasma concentration–time curve from zero to infinity (AUC_{inf}), apparent total clearance (CL/F), relative oral bioavailability (F), and apparent volume of distribution (Vd/F).

Pharmacodynamic Analysis

DNA methylation levels were measured to determine DNA hypomethylating activity of azacitidine when administered SC or orally. Whole blood was collected at baseline and before drug administration on days 3, 8, 15, and 22 of cycle 1, and days 1, 3, 8, 15, 22, and 28 of cycle 2. Genomic DNA was purified from each whole blood sample using the PAXgene Blood DNA System (Qiagen; Valencia, CA). DNA methylation was analyzed using the Infinium Human Methylation27 BeadArray (Illumina; San Diego, CA). In cycle 1, DNA methylation data were generated from blood samples of 15 patients. For 10 of these patients, data were also generated in cycle 2. A methylation ratio, or beta value, for each locus per sample was calculated as methylated signal/(methylated + unmethylated signal). Those with detection $P \leq .05$ were considered high-quality measures. Samples with more than 25,200 high-quality beta values and 26,304 autosomal loci with high-quality beta values in at least half of the samples were used for analyses. The low-quality beta values were reimputed using the pamr.knnimpute function from the R package pamr.[8] Wilcoxon signed-rank tests were performed to identify loci with significant methylation differences at each post-treatment time point versus baseline; $P < .01$ was considered statistically significant. All statistical analyses were carried out in R (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org).

Clinical Activity

Data for clinical activity were evaluated using International Working Group (IWG) 2006 criteria, with modifications as described below, for patients with MDSs or CMML[9] and IWG 2003 criteria for patients with AML.[10] Complete remission (CR), hematologic improvement (HI), and RBC and platelet transfusion independence (TI) were evaluated for patients with MDSs or CMML. Bone marrow CR (mCR) was also evaluated but not included in the overall response rate. RBC transfusion dependence at baseline was defined as ≥ 4 RBC units in the 56 days before cycle 1. Platelet transfusion dependence

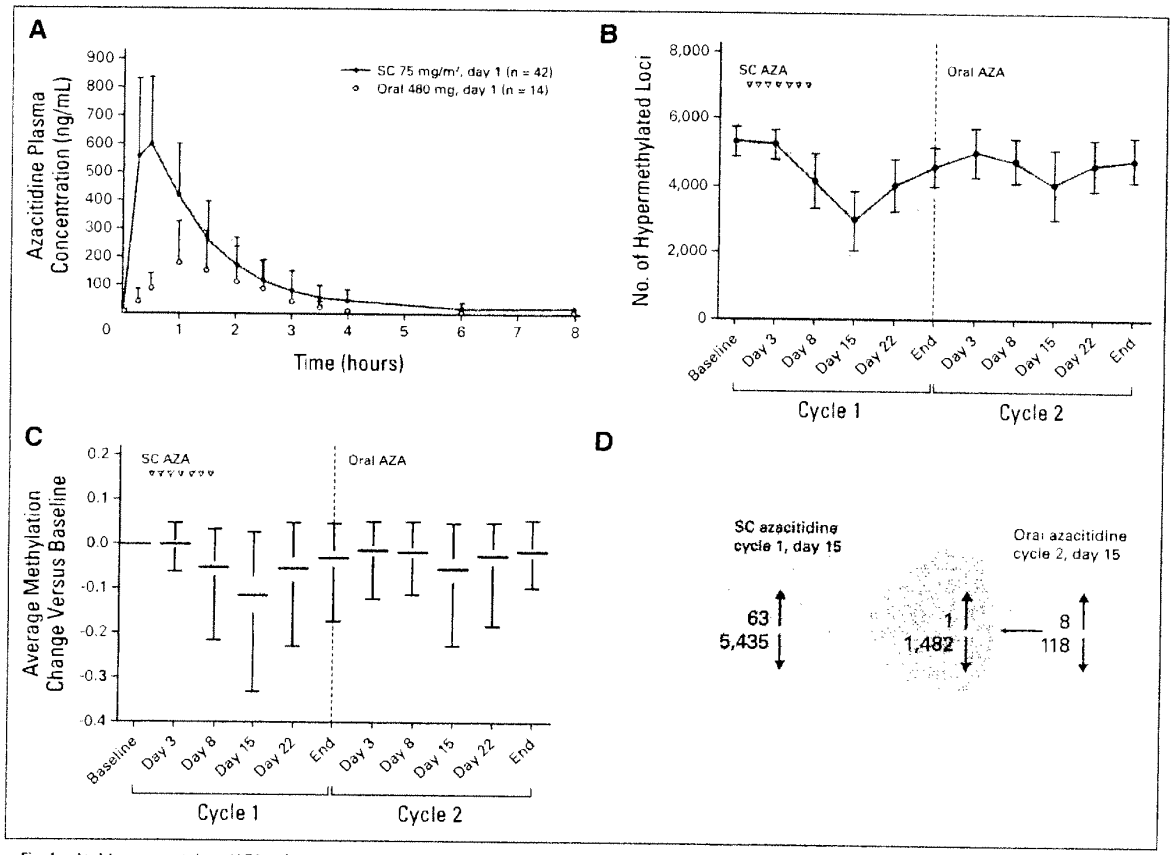

Fig 1. (A) Mean azacitidine (AZA) plasma concentration versus time profiles following single subcutaneous (SC) or oral administration (linear scale). (B) Pharmacodynamics as measured by plotting the numbers of highly methylated loci (beta ≥ 0.7; ± 95% CI) for 10 patients with DNA methylation data in cycles 1 and 2 (gold lines represent individual patients, blue line represents the average). (C) Change in methylation level during treatment with SC or oral AZA for 5,232 loci highly methylated at baseline (blue box represents the 25th to 75th percentile, horizontal band represents the median, vertical line with bars represents minimum and maximum values). (D) Number of significantly differentially methylated loci on day 15 of cycle 1 (SC azacitidine) and on day 15 of cycle 2 (oral azacitidine). Upward arrows denote hypermethylated loci and downward arrows denote hypomethylated loci

at baseline was defined as ≥ 2 platelet transfusions in the 56 days before cycle 1 (modification to IWG 2006 criteria). RBC and platelet TI were defined as no transfusions in any 56 consecutive-day period on treatment. Patients who achieved ≥ 50% reduction in platelet transfusion requirement, but not platelet TI, in any 56 consecutive-day period on treatment were counted as having achieved HI platelet (HI-P; modification to IWG 2006 criteria). Patients RBC transfusion dependent at baseline achieving a ≥ 50% reduction in RBC transfusion requirement in any 56-consecutive day period and patients not RBC transfusion dependent at baseline, but who achieved a 1.5 g/dL increase in hemoglobin in any 56-consecutive day period on treatment were considered to have achieved HI erythroid (HI-E; modification to IWG 2006 criteria). All patients who received ≥ 1 cycle of oral azacitidine were included in the response analysis. The cutoff date for data in this article was August 19, 2010.

Patient Characteristics

Forty-five patients were treated on a 7-day once-daily schedule. Four patients received the first cycle of SC azacitidine only; three discontinued due to progressive disease (including one death), and one withdrew consent. Baseline characteristics for the remaining 41 patients who received oral azacitidine are presented in Table 1.[11] Cytogenetic data were available at baseline for 35 of 41 patients treated with oral azacitidine; nearly half of the patients had normal karyotype, approximately 25% had a single abnormality, and nearly 20% had a complex karyotype (≥ 3 chromosomal abnormalities). Overall, 16 (39%) of 41 patients had received prior hypomethylating therapy.

Dose Escalation of Oral Azacitidine

No DLTs were observed at dose levels up to 480 mg. DLT was observed at the 600 mg dose, with two (66.7%) of three patients experiencing severe diarrhea, despite adequate medical intervention (grade 3 in one patient and grade 4 in the other). Per protocol, the MTD was exceeded and the previous dose level of 480 mg was determined to be the MTD.

Safety Profile

Table 2 shows the incidence of AEs (any grade) that occurred in ≥ 20% of patients treated with oral azacitidine. The most frequently observed AEs were gastrointestinal disorders, headache, fatigue, and peripheral edema. Other commonly occurring AEs included fever, cough, contusion, dizziness, and febrile neutropenia. Grade 3/4 nausea and grade 3/4 vomiting were each observed in 7% of patients. Grade 3 fatigue was observed in 10% of patients. Diarrhea occurred at grade 3 severity in 10% of patients and grade 4 severity in 2%. Grade 3 febrile neutropenia was observed in eight patients (20%), with four of those having an ANC of ≤ 500/μL at baseline.

Of the 41 patients who received oral azacitidine, 33 terminated from the study as of the date of data analysis, with 17 discontinuing before completing 6 cycles of oral therapy. Reasons for discontinuation included disease progression/treatment failure (n = 10), investigator decision primarily due to absence of observed benefit/response (n = 15), withdrawal of consent (n = 4), AEs (n = 3), and decision to pursue hematopoietic stem-cell transplantation (n = 1). There were three deaths within 28 days of last dose of study drug due to multiple organ failure (n = 1), gastrointestinal hemorrhage (n = 1), and pneumonia plus urinary tract infection (n = 1). No deaths were attributed to study drug. Eight patients remained on the study at the time of data analysis, having each received between 14 and 32 treatment cycles.

Pharmacokinetic Characteristics of Azacitidine

High interpatient variability was noted for all pharmacokinetic parameters. Azacitidine was rapidly absorbed after SC (n = 42) and oral (n = 36) administration, reaching C_{max} within 0.5 hours (range, 0.2 to 1.1 hours) and 1.0 hours (range, 0.3 to 3.6 hours) postdose, respectively. Concentration versus time profiles decreased in a pseudobiphasic manner (Fig 1A). The mean elimination half-life was 1.6 ± 0.7 hours for SC and 0.62 ± 0.25 hours for oral azacitidine. Exposure after single oral administration generally increased with dose (Table 3). For the seven oral dose levels, the mean relative azacitidine oral bioavailability (F) ranged from 6.3% to 20%. The MTD had a mean relative bioavailability of 13% ± 9%. CL/F exceeded hepatic blood flow, indicating extrahepatic metabolism, and Vd/F was greater than total body water, suggesting extensive tissue distribution. The amount of azacitidine recovered in urine relative to dose was small (< 2%) for

Table 3. Day 1 Plasma Pharmacokinetics Parameters After Single Subcutaneous or Oral Azacitidine Administration

Dose	No. of Patients	AUC_{inf} (ng × h/mL) Mean	SD	%CV	CL/F (L/h) Mean	SD	%CV	C_{max} (ng/mL) Mean	SD	%CV	T_{max} (h) Median	Range	Vd/F (L) Mean	SD	%CV	F (%) Mean	SD	Relative Oral Bioavailability
Subcutaneous, 75 mg/m²	42	1,020	440	43*	175	128	73*	650	250	39	0.50	0.2-1.1	410	410	101*	NA		
Oral, mg																		
120	4	62	43	70	4,100	4,860	118	38	24	64	1.48	1.0-2.0	2,930	3,810	130	8.1	5.6	69
180	3	112	64	58	2,330	1,890	81	72	36	50	1.50	1.0-1.5	1,700	1,580	93	6.3	2.3	37
240	3	463	221	48	598	258	43	215	102	47	1.00	1.0-1.5	814	421	52	20.0	9.6	48
300	5	282	88	31	1,180	487	41	144	13	9.2	1.48	1.0-2.0	1,090	626	57	11.5	2.6	23
360	5	311	141	45	1,360	573	42	195	79	40	1.00	0.5-3.6	947	251	27	12.8	2.4	19
480	14	362	253	70	2,140	1,620	76	211	140	66	1.00	0.3-2.5	2,010	1,910	95	12.8	9.4	74†
600	2	502	100	20	1,220	244	20	253	29	12	1.50	1.0-2.0	1,580	1,410	89	14.9	0.8	5

Abbreviations: AUC_{inf}, area under the plasma concentration–time curve from time zero to infinity; CL/F, apparent total clearance; C_{max}, maximum observed plasma concentration; F, relative oral bioavailability; NA, not applicable; T_{max}, time of maximum observed plasma concentration; Vd/F, apparent volume of distribution.
*n = 40
†n = 13

SC and oral administration, suggesting that nonrenal elimination is the predominant pathway for clearance. Results after multiple doses were similar to those obtained after a single dose for both administration routes (data not shown). There was no evidence of azacitidine accumulation.

Pharmacodynamics of Azacitidine: Effect on DNA Methylation

DNA methylation was evaluated during cycles 1 and 2 in 10 patients treated with oral azacitidine. The numbers of highly methylated loci were calculated at each time point by averaging across patients the number of loci with methylation ratios ≥ 0.7 (Fig 1B). These numbers decreased after SC and oral administration, with maximal effects at day 15 of each cycle. The reduction in levels of highly methylated loci was not maintained throughout the entire cycle and returned to near-baseline levels by the end of each cycle. SC azacitidine decreased a greater number of loci in comparison to oral azacitidine. The changes in methylation level from baseline across patients for the 5,232 highly methylated loci (average methylation ratio at baseline ≥ 0.7) are represented as box plots (Fig 1C). As with the analysis of total numbers of highly methylated loci, the median DNA methylation of these loci was reduced by 0.115 on day 15 of cycle 1 (SC azacitidine) and 0.055 on day 15 of cycle 2 (oral azacitidine), and returned to baseline levels at the end of each cycle.

Differentially methylated loci at each post-treatment time point compared with baseline were identified in cycles 1 and 2, with the maximum number observed on day 15 of each cycle; 6,981 loci were differentially methylated (6,917 hypomethylated) on day 15 of cycle 1 (SC azacitidine) and 1,609 loci were differentially methylated (1,600 hypomethylated) on day 15 of cycle 2 (oral azacitidine; $P < .01$). In total, 1,482 loci were significantly hypomethylated by both SC and oral azacitidine (Fig 1D), representing 92.6% of all loci significantly hypomethylated by oral azacitidine treatment. These data demonstrate comparable biologic activity with SC and oral azacitidine, albeit to a lesser extent with oral azacitidine.

Clinical Activity of Oral Azacitidine

The median number of oral azacitidine cycles administered to patients with MDSs, CMML, and AML was 6 (range, 1 to 32+), 12.5+ (range, 3 to 28+), and 4.5 (range, 1 to 15), respectively. Treatment duration is summarized in Figure 2. The number of patients from the MDSs, CMML, and AML groups who remained on the study at the

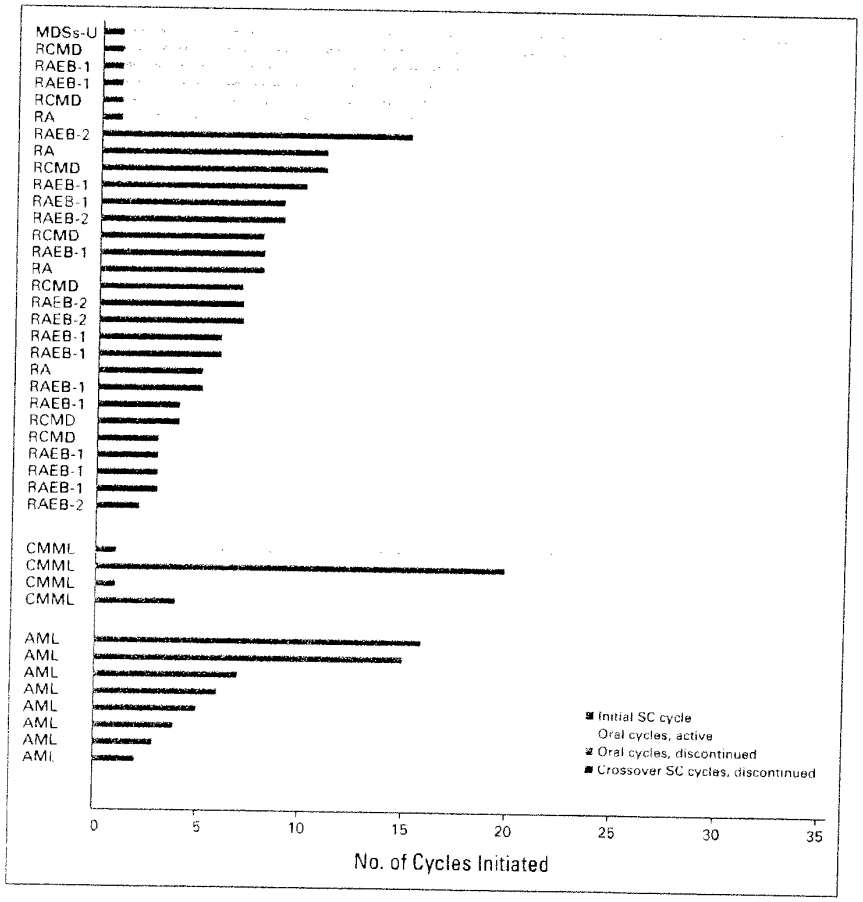

Fig 2. Treatment duration for the 41 patients treated with oral azacitidine (AZA). AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; MDSs-U, myelodysplastic syndromes-unclassified; RA, refractory anemia; RCMD, refractory cytopenias with multilineage dysplasia; RAEB, RA with excess blasts; SC, subcutaneous.

Table 4. Response in Myelodysplastic Syndromes and Chronic Myelomonocytic Leukemia Patients

Response	Previously Treated Patients[a]			First-Line Treatment			Duration of Response Range (days)
	Responders	Evaluable Patients	%	Responders	Evaluable Patients	%	
Overall response[b]	6	17	35	11	15	73	30-483[c]
CR[d]	0	17	0	6	15	40	30-152
Any HI[e]	6	16	38	5	9	56	56-483[c]
HI-E	3	10	30	2	4	50	56-483[c]
HI-N	0	10	0	2	7	29	82-321[c]
HI-P	5	14	36	2	6	33	58-351[c]
TI	0	5	0	1	3	33	76
Red blood cell	0	3	0	1	3	33	76
Platelet	0	4	0	0			NA
mCR[e,f]	6	9	67	2	6	33	63-422[g]

NOTE. At any cycle of azacitidine, International Working Group 2006 criteria were used with modifications as described in the Patients and Methods section.
Abbreviations: CR, complete remission; E, erythroid; HI, hematologic improvement; mCR, bone marrow complete remission; N, neutrophil; NA, not applicable; P, platelet; TI, transfusion independence.

[a]Includes erythropoiesis-stimulating agents, chemotherapy, hypomethylating agents, and investigational and/or other agents.

[b]Overall response rate does not include patients achieving mCR only.

[c]One or more responses, including that at upper limit of range, are ongoing. Data were censored as of last visit entered into the clinical database.

[d]Patients achieving CR were not included in any other categories.

[e]One patient with mCR in the previously treated group also achieved HI (both HI-E and HI-P). Two patients with mCR in the first-line treatment group also achieved HI (one patient with HI-P and one patient with both HI-E and HI-N). These patients have been included in both the mCR and HI categories.

[f]In the eight patients who achieved mCR, the response began in cycle 1 of subcutaneous (SC) dosing (n = 4) or very early in cycle 2 of oral dosing (n = 4). Therefore, the contribution of a single SC azacitidine cycle to the induction of these responses is likely relevant.

[g]Bone marrow aspirates were not required after 6 cycles of oral azacitidine treatment, therefore follow-up data were not available to confirm upper limit of duration. Data were censored as of last visit entered into the clinical database.

time of the analysis was 6, 2, and 0, respectively. Response and duration of response data are summarized in Table 4. In the 17 previously treated patients with MDSs and CMML, the overall response rate was 35%, without including patients who only achieved mCR; if those patients were included the response rate would be 65%. In the 15 patients with MDSs and CMML receiving first-line treatment, the overall response rate was 73% and in this group no patients achieved mCR only. Longest duration of response to date was 483 days overall. In one patient who achieved a CR, the response began before oral dosing and ended in cycle 2, thus was likely attributable to the single cycle of SC azacitidine.

No responses were observed in patients with AML. Two patients with AML (25%) had stable disease for 14 and 15 cycles, respectively, and five patients with AML (63%) received ≥ 4 oral azacitidine cycles.

An oral azacitidine formulation may bring advantages for patients (ease of administration), society (health care cost implications), and disease treatment (extended administration), provided that clinical activity and safety are similar to SC/intravenous azacitidine. This phase I trial demonstrated that oral azacitidine is associated with minimal adverse effects at doses lower than 600 mg. The MTD was 480 mg on a 7-day of 28 days treatment schedule. The 600 mg dose was associated with early onset of severe diarrhea in two of three patients. Diarrhea in patients taking oral azacitidine doses lower than 600 mg was self-limiting and manageable by treatment and/or prophylaxis with antidiarrheal agents and/or dose reduction. Azacitidine, along with one or more ingredients used in its formulation, may contribute to the diarrhea observed because it was a common adverse event at most dose levels tested. Gastrointestinal disturbances may have been exacerbated by the requirement to ingest oral azacitidine in a fasting state. Whether oral azacitidine administration with food can reduce gastrointestinal toxicity will be evaluated in ongoing studies. Grade 3 and 4 AEs consisted primarily of febrile neutropenia, gastrointestinal disturbances, and fatigue. Of the eight patients who experienced grade 3 febrile neutropenia, four entered the study with a baseline ANC of ≤ 500/μL.

After oral administration, maximum azacitidine plasma concentrations were achieved rapidly (within 1 hour), suggesting that absorption occurs from the proximal gastrointestinal tract. Azacitidine exposure increased with increasing oral doses, and the mean relative oral bioavailability ranged from 6.3% to 20%. After multiple doses, there was no evidence of azacitidine accumulation, and no apparent decline in absorption was seen between days 1 and 7. Azacitidine clearance was hepatic and extrahepatic, with little evidence of renal clearance.

Kinetics of the change in DNA methylation levels after SC and oral azacitidine were similar, with maximum hypomethylation achieved on day 15, and methylation levels returned to near-baseline values by the end of each cycle. This pattern has been observed in other azacitidine studies.[3,12] At the dosing schedule employed in this study, oral azacitidine affected fewer loci than SC azacitidine; however, 1,482 loci were identified as commonly hypomethylated by both azacitidine formulations.

Significant responses were observed in patients with MDSs and CMML, indicating that oral azacitidine has clinical activity in these settings. Although all patients received an initial cycle of SC azacitidine, which may have contributed to the clinical activity observed, it has been reported that only half of the total hematologic responses to SC azacitidine manifest within 2 cycles.[13] Continued treatment with oral azacitidine following the single cycle of SC azacitidine is therefore likely to be associated with the development and/or maintenance of clinical responses observed in this study.

Results from a study investigating alternative SC azacitidine dosing schedules in lower-risk patients with MDSs suggested that for all

dosing regimens tested, continued azacitidine treatment may be beneficial.[14] The short plasma half-life of azacitidine, S-phase restricted incorporation into DNA, and rapid remethylation of DNA, are contributing factors to the importance of chronic exposure to the drug. It is therefore likely that extended schedules of oral administration will positively affect clinical activity of azacitidine. A follow-up trial has been initiated to investigate the efficacy of such extended schedules.

In conclusion, the MTD for oral azacitidine administered daily for 7 days of a 28-day cycle was determined to be 480 mg, and oral azacitidine is bioavailable and biologically active. Clinical responses were reported in 35% of previously treated patients with MDSs and CMML, and in 73% of patients who received oral azacitidine as first-line therapy. Lower drug exposure and DNA hypomethylation seen with oral azacitidine relative to SC azacitidine provide the rationale for further study of more frequent dosing and extended schedules of oral azacitidine in MDSs, CMML, and AML. While these results show promise for an oral formulation of azacitidine, they are preliminary data and need further research so that these positive early findings can be confirmed in larger numbers of patients.

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 20(4–7), 837–840 (2001)

UNNATURAL ENANTIOMERS OF 5-AZACYTIDINE ANALOGUES: SYNTHESES AND ENZYMATIC PROPERTIES

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ABSTRACT

2′-Deoxy-β-L-5-azacytidine (L-Decitabine), β-L-5-azacytidine, and derivatives were stereospecifically prepared starting from L-ribose or L-xylose. D- and L-enantiomers of 2′-deoxy-β-5-azacytidine were weak substrates of human recombinant deoxycytidine kinase (dCK), whereas both enantiomers of β-5-azacytidine or the L-xylo-analogues were not substrates of the enzyme. None of the reported derivatives of β-L-5-azacytidine was a substrate of human recombinant cytidine deaminase (CDA).

β-D-5-Azacytidine (β-D-5-azaC), **1**, and 2′-deoxy-β-D-5-azacytidine (β-D-5-azadC, Decitabine), **2**, are important antileukemic agents used in clinical treatment [1,2]. The use of β-D-5-azaC or β-D-5-azadC suffers however from several drawbacks. The inclusion of an extra nitrogen atom into the cytosine base increases its chemical sensitivity with respect to nucleophiles and accounts for the instability of the compounds in aqueous solution [3]. Another major cause of resistance is

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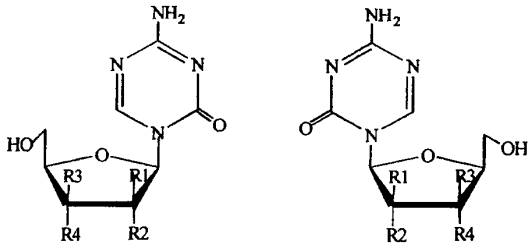

1 (R1 = R3 = H, R2 = R4 = OH)

2 (R1 = R2 = R3 = H, R4 = OH)

3 (R1 = R3 = H, R2 = R4 = OH)

4 (R1 = R2 = R3 = H, R4 = OH)

5 (R1 = R4 = H, R2 = R3 = OH)

6 (R1 = R2 = R4 = H, R3 = OH)

Figure 1. β-D- and β-L-5-azacytidine analogs studied as substrates of human dCK and human CDA.

induced by the ubiquitous cellular enzyme cytidine deaminase (CDA) since deamination of **1** or **2** results in total loss of activity [4,5]. We have previously shown that a number of cytidine analogues having the unnatural L stereochemistry are both substrates of human deoxycytidine kinase (dCK) and resistant to human cytidine deaminase [6]. We therefore stereospecifically prepared the L-enantiomers of **1**, **2** and other analogues (Fig. 1), and we studied their enzymatic properties with respect to dCK and CDA in the hope of getting phosphorylation, a lack of deamination of these compounds and possibly an efficient incorporation into DNA.

Chemistry

Most previous syntheses of 5-azacytidine analogues yield both α- and β-anomers often difficult to separate. For this reason, we used regio-and stereospecific methods to prepare the L-enantiomers **3–6** starting from L-ribose or L-xylose (Fig. 1). 1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-β-L-ribofuranose was coupled with silylated 5-azacytidine in the presence of trimethylsilyltriflate. The 2′-benzoyl group controls the stereochemistry of the substitution [7] and only the β-anomeric derivative was obtained. Deprotection catalysed by sodium methanolate in methanol afforded β-L-ribofuranosyl-5-azacytosine, **3**, in good yield. The 3′- and 5′-positions of **3** were then protected using dichlorotetraisopropyldisiloxane. A Barton-McCombie elimination of the 2′-hydroxyl group of the 3′,5′-diprotected compound followed by deprotection gave L-Decitabine, **4**, in 42% overall yield from **3**. 1,2-Di-*O*-acetyl-3,5-di-*O*-benzoyl-L-xylo-furanose was similarly condensed with silylated 5-azacytosine giving exclusively the β-anomeric derivative. Deprotection with sodium

methanolate afforded β-L-xylofuranosyl-5-azacytosine, **5**, whereas deacetylation followed by a Barton-McCombie elimination of the 2′-hydroxyl group and debenzoylation yielded 2′-deoxy-β-L-*threo*-pentofuranosyl-5-azacytosine, **6**.

Biological Results and Discussion

Several studies of the action of dCK on **1** and **2** have been previously reported, and evaluations of the substrate character of β-D-5-azaC with respect to dCK have shown that it is either low or non-existent [8]. Concerning β-D-5-azadC (Decitabine), all existing studies indicate that this compound is an average substrate of human or mammal dCK, with Km ranging from 29 to 71 μM depending on the origin of the enzyme and on the conditions [9,10]. We used HPLC to follow the kinetics of phosphorylation of **1–6** in the presence of human dCK. Under our conditions, substrate reversible decomposition was held below 2% for the duration of the kinetics. With 5 mM ATP as phosphate donor, only 2′-deoxy-β-D-5-azacytidine, **2**, and 2′-deoxy-β-L-5-azacytidine, **4**, were phosphorylated (Km: 94 and 17 μM, respectively). The efficiencies of phosphorylation were similar for the two enantiomers (Vm/Km: 0.075 and 0.05, respectively, compared to 2′-deoxy-β-D-cytidine). The value of the Km constant for 2′-deoxy-β-D-5-azacytidine is similar to the constants previously determined [9,10]. In contrast, no phosphorylation was observed in the case of both enantiomers of β-5-azacytidine, **1** and **3**. The β-L-xylo- or 2′-deoxy-xylo-derivatives **5** and **6** did not display any substrate properties.

Numerous studies have evaluated the sensitivity of β-D-5-azacytidine and 2′-deoxy-β-D-5-azacytidine to deamination catalysed by cytidine deaminase [11,12]. Using a low temperature (25°C) and short kinetic durations allowed us to limit the decomposition of the substrates as observed from HPLC analysis. Only the D-enantiomers **1** and **2** were substrates of human CDA (Km values 225 and 690 μM, respectively) whereas no deamination occurred for the L-enantiomers **3–6**, under the same conditions and in the presence of increased concentrations of enzyme.

The prepared L-nucleoside analogues **3–6** were tested as inhibitors of HIV replication in CEM-SS and MT-4 cell systems or as inhibitors of HBV replication in HepG2 cells following standard protocols. None of the compounds displayed any significant antiviral or cytotoxic effect. Our study only shows that L-Decitabine may be monophosphorylated in cells and is resistant to enzymatic deamination. The substrate properties and enantioselectivities of cellular nucleotide kinases, viral DNA polymerases or other concerned enzymes with respect to the reported compounds are not known and could be unfavourable.

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tives. Nucleophilic reagents react at the electrophilic carbinol carbon atom with the formation of cyclic derivatives, whereas the attack of electrophilic acylating and alkylating reagents necessarily occurs at the nucleophilic nitrogen atom with resulting formation of open-chain products.

Aza Analogs of Pyrimidine and Purine Bases of Nucleic Acids

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I. Introduction

Nucleic acids have recently attracted the attention of very numerous laboratories. This is because nucleic acids belong to the most important components of living matter, for genetic traits are fixed in them and transmitted through them. Nucleic acids also play the main role during biosynthesis of specific proteins.

As is well-known, nucleic acids consist of a polymeric chain of monotonously reiterating molecules of phosphoric acid and a sugar. In ribonucleic acid, the sugar component is represented by D-ribose, in deoxyribonucleic acid by D-2-deoxyribose. To this chain pyrimidine and purine derivatives are bound at the sugar moieties, these derivatives being conventionally, even if inaccurately, termed as pyrimidine and purine bases. The bases in question are uracil (in ribonucleic acids) or thymine (in deoxyribonucleic acids), cytosine, adenine, guanine, in some cases 5-methylcytosine and 5-hydroxymethylcytosine. In addition to these, a number of the so-called odd bases occurring in small amounts in some ribonucleic acid fractions have been isolated.

In view of the fact that the principal chain is formed by regularly alternating residues of phosphoric acid and sugar, it follows that the structural variety and the diversity of life functions related to it must be based on the sequence and on the kind of bases of nucleic

acids. This sequence, in which the bases are bound to the principal chain is now generally accepted as a code according to which biological information is "recorded" in a nucleic acid molecule. This fact obviously directs the attention to more detailed investigation of nucleic acid bases.

One of the lines of approach of such an investigation is the study of analogs of nucleic acid bases. The objective here is to prepare such analogs as would be incorporated into the nucleic acid molecules on the basis of their similarity to the natural species or as could interfere at some of the steps of nucleic acid biosynthesis.

If the incorporation of the analogs results in a changed property of the nucleic acid formed, it is possible to study in this way the role of nucleic acids in living systems. With the analogs of nucleic acid bases which interfere with one of the biosynthetic reactions of nucleic acids, the possibility of selective action and, thus, also of chemotherapeutical application is envisaged. Similar attention is given to other nucleic acid components, i.e., to the nucleosides (ribosyl or deoxyribosyl derivatives of the bases) and to nucleotides (nucleoside phosphates). Here there is also the possibility of altering the sugar moiety. These substances, too, can be employed in one of the ways mentioned. It should be added at this point, however, that not every analog can be considered as an antimetabolite without any further examination; the antimetabolite character of an analog must be established by studying its interactions in biological systems.

The possibility of interfering with the structure or formation of nucleic acids with the aid of such antimetabolites obviously has great practical significance. An interference with growth of neoplastic tissue and influence on the genetic properties of an organism should be mentioned in the first place.

The analogs of pyrimidine and purine bases can be derived by purely formal structural modifications or, more rationally, from the results of biochemical investigation.

An important group of antimetabolites are the aza analogs of pyrimidine and purine bases which are theoretically derived by a replacement of the methine group of a pyrimidine or purine nucleus with a nitrogen atom. This replacement represents a relatively minor alteration of the structure of these substances as it does not change the functional groups, practically preserves the molecular weight, and produces almost isosteric compounds. The replacement of the methine group with a nitrogen atom can be effected in position 5 or 6 of the pyrimidine base. This results in analogs described as "5-aza" and "6-aza." With the purine bases the replacement can take place either in position 2 or 8, this leading to 2- and 8-aza analogs, respectively.

The formal derivation of the analogs, described in the foregoing, represents, from the point of view of systematic organic chemistry, a shift to the derivatives of other heterocyclic systems. In the case of pyrimidine aza analogs we are dealing with derivatives of symmetrical or asymmetrical triazine; in the case of purine aza analogs, the derivatives produced are those of imidazo[4,5]-*v*-triazine and *v*-triazolo[4,5-*d*]pyrimidine.

For this reason dual terminology is in use for the aza analogs. The first, derived from the principal pyrimidine and purine derivatives by means of the prefix "aza-" is used almost exclusively in biochemical papers; in organic chemistry is it used (together with the systematic names) wherever it is desired to compare the properties of the natural bases and of their aza analogs. The systematic terminology is naturally used in the older literature where no biochemical aspects of the compounds were considered, and in some newer work of strictly chemical nature. Since the numbering of the substituents is in some cases different for the different systems, we shall discuss this in more detail later.[1]

The chemistry of the aza analogs of pyrimidine bases represents a part of the chemistry of the corresponding heterocyclic (triazine) group and frequently has been developed from earlier work with different objects. In keeping with its title, the present review occupies itself with those substances which were recently tested as aza analogs and further with substances which could be considered as such. Together with these, some other compounds are treated that are related to their preparation and closer study. This results in including some of the near derivatives of the aza analogs proper.

Main attention is devoted to recent papers, mostly of the last decade. In the interest of presenting a unified view of the problem,

[1] It should be stated that the nomenclature of the triazine derivatives is not uniform, e.g., in *Chemical Abstracts* the compounds are often indexed according to another system than that used in the original papers, and an additional system could have been used for this review. To avoid further complications, the author will use the commonest nomenclature in accordance with the representative monographs in this field (see references 12 and 45.)

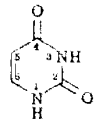
some of the older work is mentioned in passing. Since even a brief review of the studies concerning the biological activity of these substances would exceed the scope of this article, no biochemical papers are included.

II. Aza Analogs of Pyrimidine Bases

A. 5-Aza Analogs

1. *Nomenclature*

The names of these compounds as aza analogs were coined in the same way as those of the 6-aza analogs employing the frequently used numbering of uracil (1). This nomenclature is most often used for the principal aza analogs of pyrimidine bases (e.g., 5-azauracil); it is rarely used for further systematic derivatives.



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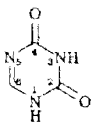

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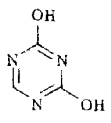


[3]

According to the triazine nomenclature, 5-azauracil is 2,4-dioxo-1,2,3,4-tetrahydro-1,3,5-triazine (2). The subject index of *Chemical Abstracts* prefers "*s*-triazine-2,4(1*H*,3*H*)-dione." Furthermore, some authors use a name derived from the lactim structure, 2,4-dihydroxy-*s*-triazine (3). The numbering of the substituents is the same for all these types of nomenclature.

2. *2,4-Dioxo-1,2,3,4-tetrahydro-1,3,5-triazine (5-Azauracil)*

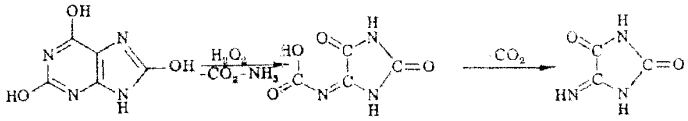
a. Methods of Preparation. The chemistry of the 5-aza analogs of the pyrimidine bases forms a relatively isolated group in the very extensive field of derivatives of *s*-triazine. It developed practically independently of the other substances of this series.

Brandenberger[1a-3] showed in 1954 that the structure of 2,4-dioxo-

[1a] H. Brandenberger, *Helv. Chim. Acta* **37**, 641 (1954).
[2] H. Brandenberger and R. Brandenberger, *Helv. Chim. Acta* **37**, 2207 (1954).
[3] H. Brandenberger, *Experientia* **12**, 208 (1956).

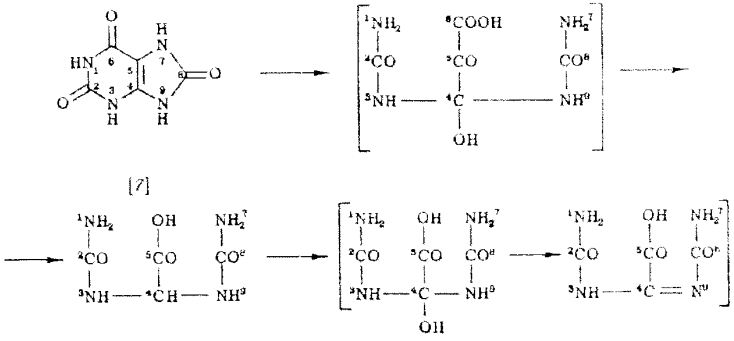
1,2,3,4-tetrahydro-*s*-triazine (2) is that of allantoxaidine which had been heretofore formulated as iminohydantoin (6) on the basis of a suggestion of Ponomarev[4] and subsequent work of Biltz and Robl.[5,6]

Allantoxaidine is formed by decarboxylation of oxonic (allantoxanic) acid (5) which is the product of alkaline oxidation of uric acid (4) with hydrogen peroxide. This reaction was studied in detail by several authors[7-9] and was expressed as follows:



[4] [5] [6]

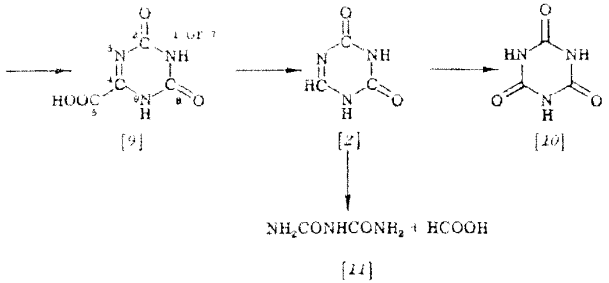
Brandenberger[1a-3] and later, independently, Canellakis and Cohen,[10] and Hartman and Fellig[11] studied the course of the oxidation with uric acid specifically labeled with C^{14}. On the basis of their work they formulated the course of the oxidation as shown in Scheme 1.



[7]

[8]

[4] J. Ponomarev, *Ber. deut. chem. Ges.* **11**, 2156 (1878).
[5] H. Biltz and R. Robl, *Ber. deut. chem. Ges.* **53**, 1967 (1920).
[6] H. Biltz and R. Robl, *Ber. deut. chem. Ges.* **54**, 2441 (1921).
[7] C. S. Venable, *J. Am. Chem. Soc.* **40**, 1099 (1918).
[8] F. J. Moore and R. M. Thomas, *J. Am. Chem. Soc.* **40**, 1120 (1918).
[9] H. Biltz and A. Schauder, *J. prakt. Chem.* **106**, 108 (1923).
[10] E. S. Canellakis and P. P. Cohen, *J. Biol. Chem.* **213**, 379 (1955).
[11] S. Hartman and J. Fellig, *J. Am. Chem. Soc.* **77**, 1051 (1955).



SCHEME 1

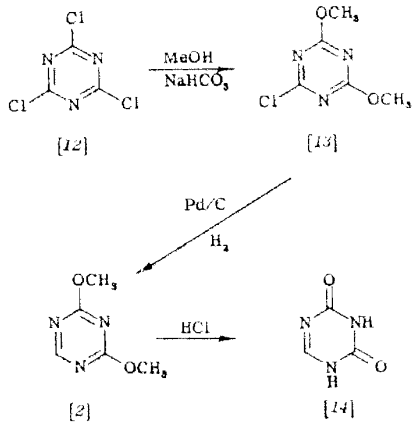
Its oxidation to cyanuric acid (**10**) and its hydrolysis to biuret (**11**) and formic acid[14] is also in agreement with the new formulation of allantoxaidine as a triazine derivative. A very strained explanation[6] would be required to make these reactions conform to the original structure.

All these findings, as well as the similarity of UV spectra[2,11] caused dioxotetrahydrotriazine to be classified as the simplest member of the formerly known 6-substituted derivatives. These derivatives are not interesting in connection with the analogs of natural pyrimidine bases and have been reviewed elsewhere.[12] The structure of allantoxaidine and its appurtenance to the triazine series have been recently demonstrated by its unequivocal synthesis.

Flament *et al.*[13] made use of the partial replacement of two chlorine atoms in cyanuric chloride (**12**) with methoxyl groups, the 2,4-dimethoxy-6-chloro-1,3,5-triazine (**13**) formed was dehalogenated to 2,4-dimethoxy-1,3,5-triazine (**14**) and this yielded the product (**2**) on gentle hydrolysis.

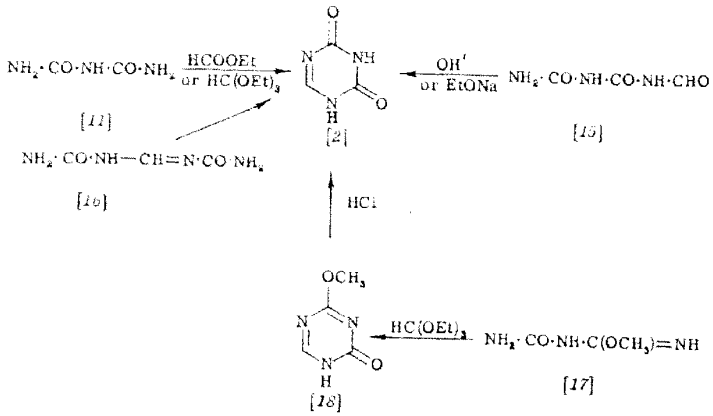
The common method of preparation of 6-alkyl-2,4-dioxotetrahydrotriazines is the cyclization of acyl-biurets by aqueous hydroxide.[12] Formyl biuret which should by analogy yield 5-azauracil had not been known until recently. Its transient formation can be expected during further synthesis of 5-azauracil. Piskala and Gut[14] achieved

[12] E. M. Smolin and L. Rapoport, "s-Triazines and Derivatives," Vol. 13 of "The Chemistry of Heterocyclic Compounds" (A. Weissberger, ed.), p. 202. Interscience, New York, 1959.
[13] J. Flament, R. Promel, and R. H. Martin, *Helv. Chim. Acta* **42**, 485 (1959).
[14] A. Piskala and J. Gut, *Collection Czechoslov. Chem. Communs.* **26**, 2519 (1961).



the cyclization of biuret (**11**) with ethyl formate in the presence of sodium ethylate. The view that formyl biuret (**15**) might be an intermediate product of these reactions was later confirmed by the cyclization[15] of the recently described formyl biuret[16] under the influence of alkalies. For further synthesis of 5-azauracil, the cyclization of *N,N'*-dicarbamylformamide (**16**) by sodium ethoxide[14] was used. The yields of both the last-named syntheses are about 60%. Runti[17] and co-workers cyclized biuret by orthoformate in the presence of sulfuric acid. The yield of the reaction was not published. It is even more convenient and productive to cyclize *O*-methylisobiuret (**17**) with ethyl orthoformate. The 4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine (**18**) formed is readily converted to 5-azauracil.[18] The possibility of hydrolysis of 5-azauracil in an alkaline medium during the syntheses described requires thoroughly anhydrous conditions.[14] This is apparently the cause of the unsuccessful attempt at cyclization of biuret with ethyl formate.[13] A recent preliminary note reports a simple but not unambiguous synthesis of 5-azauracil consisting of heating urea with trisformaminomethane or ethyl orthoformate.[18a]

[15] H. Eilingsfeld, M. Seefelder, and A. Weidinger, *Angew. Chem.* **72**, 836 (1960).
[16] Badische Anilin- & Soda-Fabrik, A. G.; German Auslegeschrift 1110625 (1961).
[17] C. Runti, L. Sindellari, and F. Ulian, *Ann. chim. (Rome)* **50**, 847 (1960).
[18] A. Piskala and J. Gut, *Collection Czechoslov. Chem. Communs.*, in press.
[18a] H. Bredereck, F. Effenberger, and A. Hofmann, *Angew. Chem.* **74**, 354 (1962).



b. Properties. According to an investigation of the IR spectra,[19] the results of which are generally identical with the spectra of uracil and 6-azauracil (e.g., Section II,B,2,b), 5-azauracil possesses the dilactam structure. The UV spectra[19] of 5-azauracil and of its *N*-alkyl and *O*-alkyl derivatives display, contrary to our expectation, only minor differences. For this reason no final conclusions can be drawn as to the tautomeric equilibrium of 5-azauracil in aqueous solutions. A similar situation was observed for the analogous compounds[20] and appears to be characteristic for these systems.

A determination of the dissociation constants[11,21,22] of the compounds reveals that 5-azauracil ($pK_a = 6.73$) is practically of the same acidity as 6-azauracil and considerably more acidic than uracil.

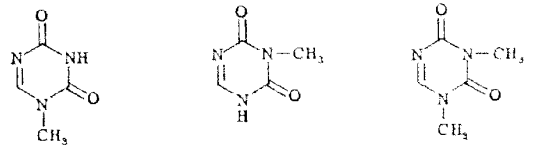
A fundamental difference between 5-azauracil, on the one hand, and 6-azauracil and uracil, on the other, lies in the low stability of 5-azauracil toward acid and especially to alkaline hydrolysis.[4] This fact appears to be in agreement with the differences in electron densities of these substances computed by the simple MO-LCAO method.[23]

[19] M. Horák, J. Jonáš, A. Píškala, and J. Gut, *Collection Czechoslov. Chem. Communs.* **27**, 2751 (1962).
[20] S. F. Mason, *in* "Ciba Foundation Symposium on the Chemistry and Biology of Purines" (G. E. W. Wolstenholme and C. M. O'Connor, eds.). J. and A. Churchill, London, 1957.
[21] J. Jonáš and J. Gut, *Collection Czechoslov. Chem. Communs.* **27**, 716 (1962).
[22] A. Albert and J. N. Phillips, *J. Chem. Soc.* p. 1294 (1956).
[23] R. Zahradník, J. Koutecký, J. Jonáš, and J. Gut, *Collection Czechoslov. Chem. Communs.*, in press.

3. *N-Alkyl Derivatives of 5-Azauracil*

The study of the *N*-alkyl derivatives of allantoxaidine was taken up by Biltz who proceeded from its formerly accepted hydantoin structure and obtained its monomethyl[24] and dimethyl[25] derivatives.

Final structure determination of the monomethyl derivatives was made possible only on performing straightforward syntheses of the compounds, analogous to syntheses of allantoxaidine. The 1-methyl (**19**) and 3-methyl (**20**) derivatives were prepared by cyclization of 1- and 3-methyl biuret,[14] respectively. The 3-methyl derivative was also obtained by cyclization of *N,N'*-bis(methylcarbamyl)formamidine.[11] The 1-methyl derivative was the same as the product obtained by Biltz[24] on methylation of silver salt of allantoxaidine with methyl iodide. The same product is formed primarily during methylation of dioxotriazine with diazomethane.[14] An excess of this agent produces the expected 1,3-dimethyl derivative[25] (**21**). These results of alkyl-



[19] [20] [21]

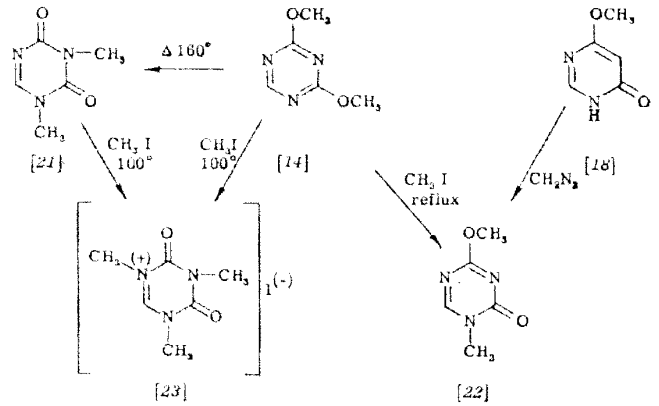
ation are in agreement with the study of the dissociation constants of 5-azauracil and of both of its *N*-methyl derivatives (1-methyl derivative $pK_a = 8.15$; 3-methyl derivative $pK_a = 6.58$). On the assumption that the effect of the methyl group is negligible, the pK_a values permit the conclusion to be drawn that the dissociation sets in first at the N-1 atom.[14,21] In this respect the behavior of 5-azauracil is identical with that of uracil but contrary to that of 6-azauracil (e.g., Section II,B,3,a).

By a more detailed study of the reaction of 5-azauracil with diazomethane[25a] it was found that this reaction is considerably accelerated by the presence of a small amount of water, methanol, or dimethylformamide. It does not proceed appreciably in absolute ether. By

[24] H. Biltz and H. Hanisch, *J. prakt. Chem.* [2] **112**, 138 (1926).
[25] H. Biltz and R. Rohl, *Ber. deut. chem. Ges.* **54**, 2448 (1921).
[25a] A. Píškala, *Collection Czechoslov. Chem. Communs.*, in press.

chromatographic separation of mixtures obtained on using excess diazomethane and the catalytic influence of the foregoing substances, it was possible to isolate small amounts of the 1-methyl-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine (**22**), in addition to the main product, the 1,3-dimethyl derivative. On adding 15% dimethylformamide, a small amount of 2,4-dimethoxy-1,3,5-triazine (**14**) was also obtained. These results demonstrate the very different behavior of 5-azauracil as compared with uracil and 6-azauracil where no formation of *O*-methyl derivatives could be observed.

The pyrimidine compounds are known to undergo a rearrangement of the *O*-alkyl derivatives to the *N*-alkyl ones.[26] The methoxy derivatives of 1,3,5-triazine display a similar behavior. On applying methyl iodide to 2,4-dimethoxy-1,3,5-triazine one of the methyl groups is shifted giving rise to 1-methyl-4-methoxy-derivative (**22**). This compound was also obtained by methylation of 4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine (**18**) with diazomethane. At higher temperature (100°C) in presence of methyl iodide a shift of both methyl groups takes place and methiodide is formed simultaneously (**23**). Similarly,



on heating 2,4-dimethoxy-1,3,5-triazine alone both methyl groups are shifted. The reactions of the methoxy derivatives of 1,3,5-triazine are

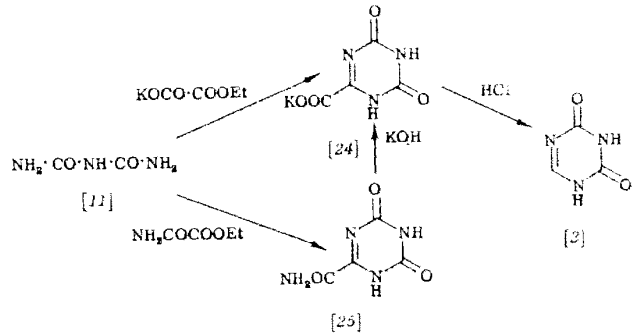
[26] G. E. Hilbert and T. B. Johnson, *J. Am. Chem. Soc.* **52**, 2001 (1930).

thus analogous to those of the analogous pyrimidine derivatives but they proceed with some difficulty and especially the thermal rearrangement gives rise to further products as yet unidentified. The formation of a methiodide is also at variance with the pyrimidine compounds, an analogous reaction being impossible with the latter.[26a]

A similar rearrangement accompanied by decarboxylation was described for 2,4-dimethoxy-1,3,5-triazine-6-carboxylic acid.[27]

4. *2,4-Dioxo-1,2,3,4-tetrahydro-1,3,5-triazine-6-carboxylic Acid (5-Azaorotic Acid)*

The name 5-azaorotic acid should be given to allantoxanic (oxonic) acid but it is not yet commonly used. The elucidation of the correct structure of this compound was closely linked to the solution of the course of oxidation of uric acid mentioned earlier.

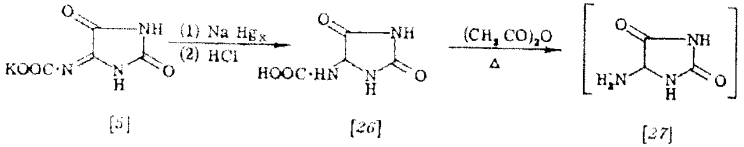


The structure (**5**) originally proposed by Ponomarev[4] appeared to be confirmed by the conversion of dihydrooxonic acid to allantoin performed by Biltz and Giesler.[28] Biltz and Robl[3] showed later that oxonic acid is identical with allantoxanic acid obtained on oxidation of allantoin.[29] Since that time both these trivial names are in usage.

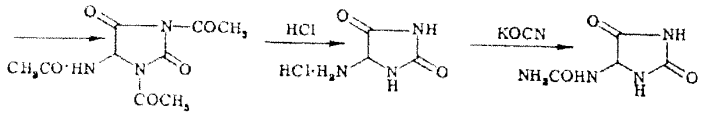
[26a] A. Piskala and J. Gut, *Collection Czechoslov. Chem. Communs.*, in press.
[27] E. Kober, *J. Org. Chem.* **26**, 5259 (1961).
[28] H. Biltz and E. Giesler, *Ber. deut. chem. Ges.* **46**, 3410 (1913).
[29] E. Mulder, *Ann. Chem. Liebigs* **159**, 365 (1871).

Works on the oxidation of uric acid has unequivocally established the triazine structure[1,10,11] (9) of oxonic acid. This is further confirmed by the straightforward synthesis described by Píškala and Gut.[30] The reaction of biuret (11) with potassium ethyloxalate yielded a potassium salt (24), that with ethyl oxamate, the amide of oxonic acid (25). Both these compounds were converted to 5-azauracil. An analogous reaction with diethyloxalate which should produce an ester of oxonic acid resulted in a mixture of urethane and parabanic acid, however.

The same authors[30] elucidated the origin of allantoin from hydroxonic acid[28] on which the original hydantoin structure (5) was based and which was formulated as shown in Scheme 2.



[5] [26] [27]

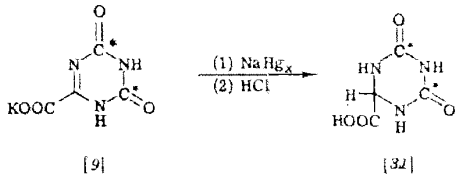


[28] [29] [30]

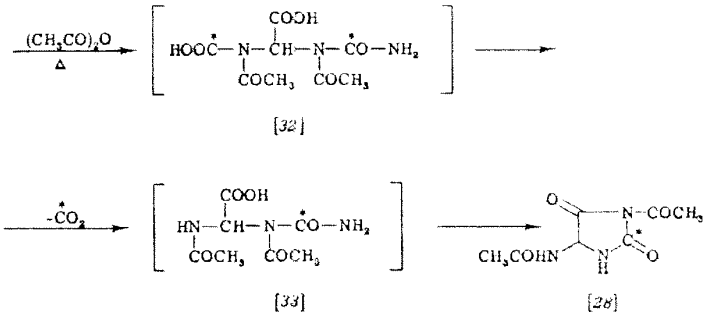
Scheme 2

For studying this reaction, oxonic acid prepared by the aforementioned synthetic procedure from biuret-(2,4-C^{14}) was used; it was converted to hydroxonic acid (31) by reduction with sodium amalgam. According to the formulation of Biltz and Giesler,[28] nonradioactive carbon dioxide would be expected from its reaction with acetanhydride. Since, however, one-half of the activity of the starting substance was present in the escaping carbon dioxide, it must be assumed that the ring is cleaved and the reaction is as shown in Scheme 3.

[30] A. Píškala and J. Gut, *Collection Czechoslov. Chem. Communs.* **27**, 1562 (1962).



[9] [32]



[33] [28]

Scheme 3

Hence, by this pathway the formation of allantoin is not at variance with the triazine structure of oxonic acid.

5. *2,4-Dioxohexahydro-1,3,5-triazine and Its Derivatives*

The 6-alkyl derivatives of 2,4-dioxohexahydro-1,3,5-triazine have been known for some time and have been reviewed earlier.[31]

The unsubstituted member of this series (34) was prepared only later, by cyclization of methylenebisurea.[32,33]

Attempts to prepare this substance by reduction of dioxotetrahydro-1,3,5-triazine with sodium amalgam,[24] hydroiodic acid, or tin in acetic acid,[28] were accompanied by hydrolytic cleavage of the ring. Only

[31] E. M. Smolin and L. Rapoport, "*s*-Triazines and Derivatives," Vol. 13 of "The Chemistry of Heterocyclic Compounds" (A. Weissberger, ed.), p. 210. Interscience, New York, 1959.

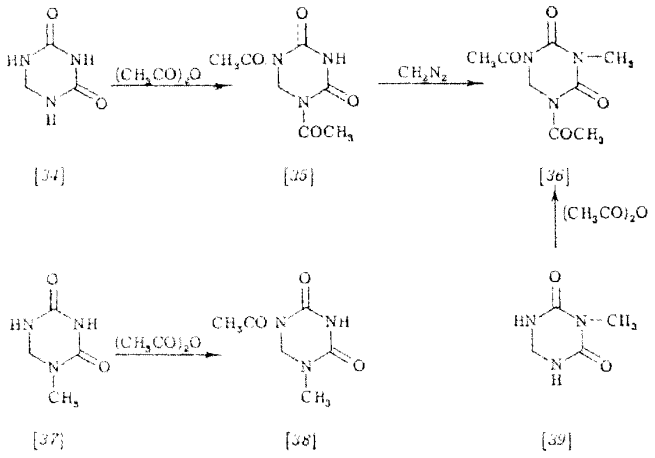
[32] O. Diels and R. Lichte, *Ber. dent. chem. Ges.* **59**, 2778 (1926).

[33] F. B. Slezak, A. Hirsch, L. J. Krimen, and H. A. McElravy, *J. Org. Chem.* **25**, 1672 (1960).

when the mixture was maintained acid during reduction with sodium amalgam[14] was it possible to prepare the desired hexahydro derivative.

Hydrogenation with Adams' catalyst took place only with the 6-alkyl derivatives.[34] Dioxohexahydrotriazine itself acted as a catalyst poison (in common with 1,3,5-triazine and cyanuric acid[35]). Dioxotetrahydrotriazine as well as its *N*-alkyl and 6-alkyl derivatives can be readily hydrogenated by using Raney nickel.[14]

The hexahydro derivatives are weakly basic substances, some of them forming hydrochlorides.[31] Dioxohexahydrotriazine yields a 1,5-diacetyl derivative (**35**), in which the positions of the acetyl groups were determined by acetylation of the *N*-alkyl derivatives and methylation with diazomethane according to Scheme 4.[30]



[34] [35] [36] [37] [38] [39]

SCHEME 4

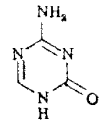
The course of acetylation here is analogous to that of dioxohexahydro-1,2,4-triazine (e.g., Section II,B,8).

6. *4-Amino-2-oxo-1,2-dihydro-1,3,5-triazine (5-Azacytosine)*

The 5-aza analog of cytosine could be taken as 4-amino-2-oxo-1,2-dihydro-1,3,5-triazine (**40**) which was prepared by the reaction of

[34] A. Ostrogovich and G. Ostrogovich, *Gazz. chim. Ital.* **26**, 18 (1936).
[35] H. Brandenberger and R. Schwyzer, *Helv. Chim. Acta* **38**, 1396 (1955).

dicyandiamide and 100% formic acid.[36] The structure of this compound was recently confirmed by an unambiguous synthesis[15] from



[40]

the methoxyderivative (**18**). The chemistry of the related 6-substituted derivatives has been studied extensively[37] but it is of no interest in the present connection.

7. *Other Related Derivatives*

Other derivatives of *s*-triazine, in particular the 2,4-disubstituted ones, are usually prepared by total synthesis and are therefore not closely linked with the chemistry of 5-azauracil unlike the analogous derivatives of 1,2,4-triazine. 2,4-Dimethoxy-1,3,5-triazine was mentioned earlier (e.g., Section II,A,2,a), the other substances are not related to the present subject.

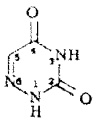
B. 6-Aza Analogs

1. *Nomenclature*

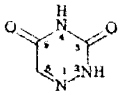
Names describing these substances as aza analogs of pyrimidine bases are generally derived from the more common type of numbering of uracil (**1**). According to this system the methine group in position 6 is replaced (**41**). The nomenclature of other derivatives is based on this principle quite systematically and is in general use. The numbering of the position of substituents by this method is the same as in the analogous pyrimidine derivatives. This is of special advantage in comparing the derivatives of 6-azauracil (especially nucleosides and nucleotides) with similar derivatives of uracil. In such cases it will also be used in this chapter.

[36] C. Grundmann, L. Schwennicke, and E. Beyer, *Chem. Ber.* **87**, 19 (1954).
[37] E. M. Smolin and L. Rapoport, "*s*-Triazines and Derivatives," Vol. 13 of "The Chemistry of Heterocyclic Compounds" (A. Weissberger, ed.), p. 189. Interscience, New York, 1959.

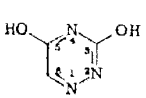
According to systematic triazine nomenclature, 6-azauracil is 3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine (**42**). The indexes of the *Chemical Abstracts* describe it as *as*-triazine-3,5(2*H*,4*H*)-dione. In addition



[41]



[42]



[43]

to this the literature contains nomenclature derived from the dilactim form (**43**), 3,5-dihydroxy-1,2,4-triazine.

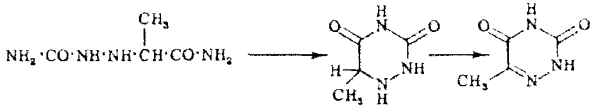
In all types of nomenclature based on triazine the numbering of the substituents is shifted by one as compared with the nomenclature of 6-aza analogs of pyrimidines.

2. *3,5-Dioxo-2,3,4,5-tetrahydro-1,2,4-triazines (6-Azauracil and Its 5-Alkyl Derivatives)*

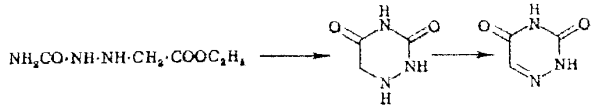
The chemistry of the 6-aza analogs of pyrimidine bases which has been developed from the biochemical aspect since about 1956 was based on work reported in relatively numerous older papers. In spite of the fact that 6-azauracil was prepared only in 1947 and suitable syntheses were described only quite recently, substances of this type and methods of their preparation had been known for a long time. The chemistry of 6-aza analogs of pyrimidine bases is therefore relatively closely linked with the chemistry of the 1,2,4-triazine derivatives.

a. Methods of Preparation. The first method of preparation of substances of this type (**45**) was described by Thiele and Bailey[38] who cyclized the amide of α-semicarbazidopropionic acid (**44**) prepared from acetaldehyde semicarbazone. Later on a similar cyclization of ethyl semicarbazidoacetate[39] (**47**) was employed to prepare dioxohexahydrotriazine (**48**). This method was later used for the preparation of the hexahydro derivatives of this type as will be seen later (e.g., Section II,B,8).

[38] J. Thiele and J. Bailey, *Ann. Chem. Liebigs* **303**, 75 (1898).
[39] J. R. Bailey and W. T. Read, *J. Am. Chem. Soc.* **36**, 1764 (1914).



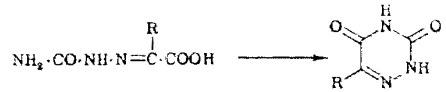
[44] [45] [46]



[47] [48] [42]

In this connection the possibility of oxidation of these substances to the tetrahydro derivatives should be mentioned. It was made use of by Thiele and Bailey[38] for the preparation of 6-methyl-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine (6-azathymine) (**46**) and only recently by Grundmann *et al.*[40] for that of 6-azauracil (**42**).

Of greater importance for the preparation of these substances is the reaction first observed by Locquin[41] in 1906 and independently treated later by Bougault.[42,43] These authors have shown that semicarbazones of α-keto acids (**49**) are cyclized under the influence of aqueous sodium hydroxide directly to the 6-substituted 2,5-dioxotetrahydro-1,2,4-triazines (**50**). The reaction was usually carried out at



[49] [50]

100°C; higher yields are obtained by working at room temperature but the reaction takes several months.[44] The cyclization proceeds best

[40] C. Grundmann, H. Schroeder, and R. Ratz, *J. Org. Chem.* **23**, 1522 (1958).
[41] R. Locquin, *Bull. soc. chim. France* [3] **35**, 964 (1906).
[42] J. Bougault, *Compt. rend. acad. sci.* **159**, 83, 631 (1914).
[43] J. Bougault, *Ann. chim. (Paris)* [9] **5**, 317 (1916).
[44] E. Cattelain, *Bull. soc. chim. France* **9**, 907 (1942).

with semicarbazones with a higher alkyl or even better an aryl or aralkyl group in the α-position. For this reason the reaction was used first of all for the preparation of numerous 6-aryl and 6-aralkyl derivatives as reviewed by Erickson *et al.*[45] Some other substances[46] of this type were prepared later by a similar method.

With semicarbazones of lower α-keto acids the reaction proceeds with some difficulty or not at all. Thus, the semicarbazones of pyruvic acid[42,43,47] cannot be cyclized and that of glyoxylic acid[48] is predominantly hydrolyzed so that the yield of the cyclization product is only 20–25%.[49] This reaction was used in work with a different object, for preparing 6-azauracil,[48] for the first time.

For unsubstituted or lower alkylated dioxotriazines, it is advantageous to cyclize semicarbazones by sodium ethylate in ethylene glycol as described by Chang and Ulbricht.[49] In this reaction 6-azauracil is obtained in 66% yield. The procedure was used for the preparation of labeled 6-azauracil[49] and later for the synthesis of a number of 6-alkyl derivatives including 6-azathymine.[50]

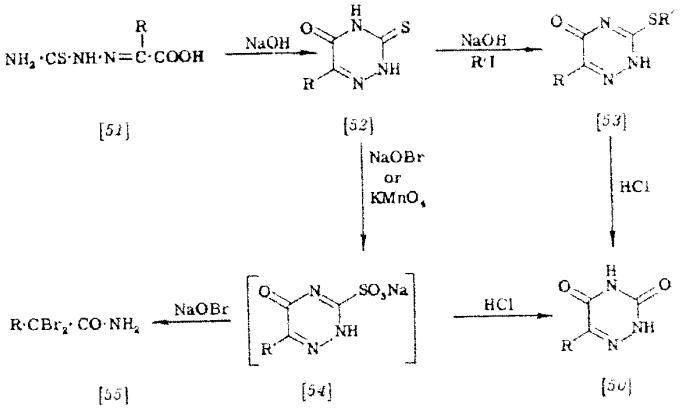
The starting semicarbazones were most often prepared directly from the α-keto acids. Godfrin[51] proceeded from α-alkyl acetoacetates, which were converted by oxidation with nitrosylsulfuric acid to α-keto-acid oximes and the latter transformed to semicarbazones or thiosemicarbazones by applying semicarbazide or thiosemicarbazide. For glyoxylic acid semicarbazone a very convenient procedure was employed, making use of the hydrolysis of nonisolated chloral semicarbazone.[49]

In continuing their previous work Bougault and Daniel[52,53] observed that thiosemicarbazones of α-keto acids (**51**) also undergo a cyclization resulting in 3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines (**52**). In contrast with the cyclization of semicarbazones this cyclization

[45] J. G. Erickson, P. F. Wiley, and V. P. Wystrach, "The 1,2,3- and 1,2,4-Triazines, Tetrazines and Pentazines," Vol. 10 of "The Chemistry of Heterocyclic Compounds" (A. Weissberger, ed.), p. 69. Interscience, New York, 1956.
[46] S. Rossi, *Gazz. chim. ital.* **83**, 133 (1953).
[47] J. R. Bailey, *Am. Chem. J.* **28**, 386 (1902).
[48] W. Seibert, *Chem. Ber.* **80**, 494 (1947).
[49] P. K. Chang and T. L. V. Ulbricht, *J. Am. Chem. Soc.* **80**, 976 (1958).
[50] P. K. Chang, *J. Org. Chem.* **23**, 1951 (1958).
[51] A. Godfrin, *J. pharm. chim.* **30**, 321 (1939); *Chem. Abstr.* **34**, 5087 (1940).
[52] J. Bougault and L. Daniel, *Compt. rend. acad. sci.* **186**, 151 (1928).
[53] J. Bougault and L. Daniel, *Compt. rend. acad. sci.* **186**, 1216 (1928).

proceeds with substantially higher yields. This reaction will be taken up in more detail later (e.g., Section II,B,4,a).

In this connection it is important to mention the finding that the thioxo derivatives (**52**) can be converted in several ways to the 3,5-dioxo derivatives. By alkylation with methyl iodide in alkaline solution, methylmercapto derivatives (**53**) are produced which are readily hydrolyzed to dioxo derivatives.[54] A similar course is followed in the reaction with ethyl chloroacetate.[55] Finally, oxidation with hypo-



bromite in an alkaline medium yields salts of the corresponding sulfonic acid (**54**) which are hydrolytically cleaved in an acid solution. However, excess hypobromite results in cleavage of the ring giving rise to α-dibromoamides[56] (**55**).

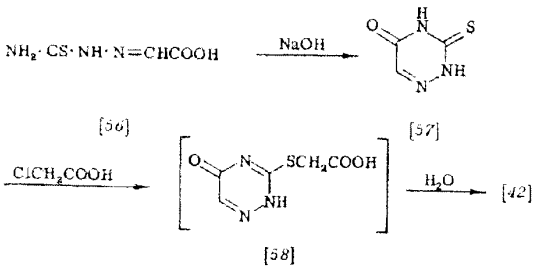
The cyclization of thiosemicarbazones has therefore recently served as the basis for further syntheses of 6-azauracil and 6-azathymine. Barlow and Welch[57] proceeded from thiosemicarbazone of mesoxalic acid (**51, 52**; R = COOH). The corresponding methylmercapto derivative (**53**; R = COOH, R′ = CH_3) was hydrolyzed and decarboxylated

[54] E. Cattelain, *Bull. soc. chim. France* **11**, 256 (1944).
[55] E. Cattelain and P. Chabrier, *Bull. soc. chim. France* p. 700 (1948).
[56] E. Cattelain, *Bull. soc. chim. France* **12**, 47 (1945).
[57] R. B. Barlow and A. D. Welch, *J. Am. Chem. Soc.* **78**, 1258 (1956).

to yield 6-azauracil. Falco *et al.*[58] used a similar procedure starting from the diester of mesoxalic acid. The replacement of sulfur with oxygen was achieved, however, by oxidation with potassium permanganate via the corresponding sulfonic acid (**54**; R = COOH) and by subsequent hydrolysis and decarboxylation.

Oxidation with potassium permanganate was also used for the preparation of derivatives with a $CH_3(CH_2)_n$ ($n = 2,4,6,8,12$) and β-substituted vinyl[58a] group in the 6-position from the corresponding thioxo derivatives.[59]

In a further synthesis, Gut[60] used the cyclization of the thiosemicarbazone of glyoxylic acid (**56**); the 2-thioxo-5-oxo-2,3,4,6-tetrahydro-1,2,4-triazine (**57**) formed was converted to 6-azauracil by applying aqueous solution of chloroacetic acid. (This reaction will be discussed later, e.g., Section II,B,4,b.) The same procedure was used

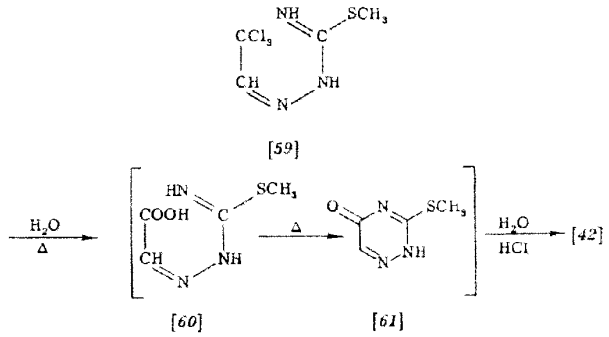


for the preparation of 6-azathymine[60] from the thiosemicarbazone of pyruvic acid. An analogous procedure was later employed for preparing 6-azauracil (4,5-C^{14})[61] and a number of other 5-substituted derivatives.[62]

Using a single-step process, 6-azauracil can be prepared from chloral 3-methylisothiosemicarbazone (**59**). The apparent intermedi-

[58] E. A. Falco, E. Pappas, and G. H. Hitchings, *J. Am. Chem. Soc.* **78**, 1938 (1956).
[58a] I. Nakata and T. Ueda, *Yakugaku Zasshi* **80**, 1068 (1960).
[59] J. Slouka *J. prakt. Chem.* **16**, 220 (1962).
[60] J. Gut, *Collection Czechoslov. Chem. Communs.* **23**, 1588 (1958).
[61] J. Moravek, *Collection Czechoslov. Chem. Communs.* **24**, 2571 (1959).
[62] J. Gut and M. Prystaš, *Collection Czechoslov. Chem. Communs.* **24**, 2986 (1959).

ate of this synthesis is the cyclic 3-methylmercapto derivative (**61**) which is immediately hydrolyzed in an acid solution (e.g., Section II,B,4,b). The yield in this synthesis is only 25%, however.[49]



b. Properties. It is well known that the 6-substituted dioxotriazines are monobasic acids titrated with phenolphthalein.[63,64] It was also known that they form salts but these have not been studied in detail.[49]

More recently the simplest member of this series, 6-azauracil, was investigated more thoroughly from this aspect.[21,65] Its dissociation constant was 7.00; it is thus considerably more acid than uracil ($pK_a = 9.43$). In the pH region above 10.0, a second hydrogen is also ionized. Uracil behaves in a similar manner.

Salts of 6-azauracil were studied in greater detail and their individual composition established.[66] 6-Azauracil, in common with uracil, is very resistant both toward acid and toward alkaline hydrolysis.

A detailed investigation of the tautomeric structure of 6-azauracil was carried out by Jonáš and Gut[66] and by Horák and Gut.[67] They measured the UV and IR spectra and compared similar systems and their derivatives in which the lactam or lactim configuration was fixed by *N*- or *O*-substitution (as will be seen later no *O*-alkyl deriva-

[63] E. Cattelain, *Bull. soc. chim. France* **12**, 59 (1945).
[64] E. Cattelain, *Ann. chim. anal.* **24**, 150 (1942); *Chem. Abstr.* **38**, 1971 (1944).
[65] J. Gut, M. Prystaš, J. Jonáš, and F. Sorm, *Collection Czechoslov. Chem. Communs.* **26**, 974 (1961).
[66] J. Jonáš and J. Gut, *Collection Czechoslov. Chem. Communs.* **26**, 2155 (1961).
[67] M. Horák and J. Gut, *Collection Czechoslov. Chem. Communs.* **26**, 1680 (1961).

tives of 1,2,4-triazine have been prepared so far). It follows from these studies that 6-azauracil possesses a dilactam structure as was demonstrated earlier for uracil. Interpretation of the pH dependence of the UV spectra elucidated the sequence of dissociations in 6-azauracil and uracil (e.g., Section II,B,3,a).[21] The IR spectra of 6-azauracil and of its alkyl derivatives display two notable maxima in the carbonyl region.[67] In similar spectra of uracil and hydantoin this phenomenon was explained by the inequality of the two carbonyl groups. According to the shifts of frequency the maxima were attributed to the individual carbonyl groups.[68] A more acceptable interpretation explains the cleavage of the carbonyl band into two by the "coupling effect" of the two carbonyl groups.[67] The molecular diagrams of 6-azauracil, 5-azauracil, and uracil were calculated by the simple MO-LCAO method.[26] On the basis of these calculations the shifts of maxima in the UV spectra and some reactions of these compounds were explained.[23]

6-Azauracil and its alkyl derivatives are readily reducible by polarography, in contrast with uracil. This makes it possible to exploit the method analytically.[69] More detailed studies of the polarographic behavior of these substances are in good agreement with the results of spectral studies about the tautomeric form and type of dissociation.[70]

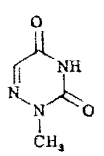
3. *N-Substituted Derivatives of 3,5-Dioxo-2,3,4,5-tetrahydro-1,2,4-triazines*

a. N-Alkyl Derivatives. The *N*-alkyl or aralkyl derivatives can be prepared by three procedures. First by direct alkylation, second by hydrolysis of *N*-alkylated 3-methylmercapto derivatives, and third, in some cases, by cyclization of substituted semicarbazones of α-keto acids.

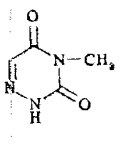
It was observed already by Bougault[43,71] that the reaction of 6-benzyl-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazines with alkyl halides in an alkaline solution yields a mixture of the 4-mono- and 2,4-dialkyl derivatives. This mixture of alkylation products can be readily separated since the monoalkyl derivative is weakly acidic and therefore soluble in alkali whereas the dialkyl derivative is alkali-insoluble.[71] It was found by hydrolysis of the monoalkyl derivatives, resulting in 4-alkylsemicarbazones, that these were the 4-alkyl derivatives.[72,73] Alkaline alkylation has been used with a number of other 6-substituted derivatives.[45]

[67] H. M. Randall, R. G. Fowler, N. Fuson, and J. R. Dangl, "Infrared Determination of Organic Structure," van Nostrand, New York, 1949.
[68] J. Krupička and J. Gut, *Collection Czechoslov. Chem. Communs.* **25**, 592 (1960).
[70] J. Krupička and J. Gut, *Collection Czechoslov. Chem. Communs.* **27**, 546 (1962).
[71] J. Bougault, *Compt. rend. acad. sci.* **159**, 83 (1914).

The alkylation of 6-azauracil will be treated later. The first, but not exactly identified dimethyl derivative was prepared by Grundmann.[40] The course of alkylation was studied in greater detail by Gut *et al.*[23] These authors found that in aqueous alkaline solution and on using alkyl halides or dialkyl sulfates, the main alkylation product is the 1,3-dialkyl derivative (**64**). Since, however, the alkylation is to some



[62]



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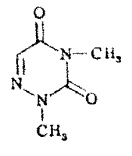


[64]

extent accompanied by hydrolysis of the alkylation products, its course is not completely clear. Depending on the reaction conditions it is possible to isolate small amounts of the 1-alkyl (**62**) or 3-alkyl (**63**) derivatives from the mixture. The structure of the monoalkyl derivatives of 6-azauracil was defined by unequivocal synthesis of 1-methyl-6-azauracil (e.g., Section II,B,4,b). During similar alkylations of uracil no monoalkyl derivatives have been isolated.

The sodium or potassium salt of 6-azauracil in aqueous ethanol, anhydrous ethanol, or ethylene glycol reacted with methyl iodide practically exclusively to give the 3-methyl derivative (**63**). In toluene the sodium, potassium, and mercuric salts produced no methylated derivatives whereas the silver salt also yielded the 3-methyl derivative.[65] Similarly, the 3-methyl derivative was prepared from the mercuric salt of 6-azathymine, and its structure was established by hydrolysis to pyruvic acid 4-methylthiosemicarbazone.[74]

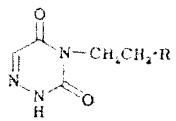
[72] E. Cattelain, *Compt. rend. acad. sci.* **208**, 1656 (1939).
[73] J. Bougault, *Compt. rend. acad. sci.* **160**, 625 (1915).
[74] R. H. Hall, *J. Am. Chem. Soc.* **80**, 1145 (1958).

During methylation of 6-azauracil with a theoretical amount of diazomethane, the 3-methyl derivative (**63**) was obtained in very good yield. Excess reagent produces the dimethyl derivative (**64**). During none of the alkylation reactions was it possible to observe the formation of *O*-alkyl derivatives of 6-azauracil.[65] This can be taken as evidence that 6-azauracil does not react in the lactim form (e.g., Section II,B,2,b).

The course of alkylations of 6-azauracil is in good agreement with the results of determination of the dissociation constants of 6-azauracil and of its two monomethyl derivatives.[70,65] On the assumption that a methyl group does not much affect the dissociation constant, and on the basis of the lactam structure, it may be concluded from the values of the dissociation constants (pK_a of 6-azauracil = 7.00, of 1-methyl-6-azauracil = 6.99, and of 3-methyl-6-azauracil = 9.52) that dissociation takes first place at the NH group in position 3. The same results are obtained independently by comparing the pH dependence of the UV spectra of these compounds.[66] These results represent an exact confirmation of the older observation by Cattelain[65] that the monoalkyl derivatives of 6-substituted dioxotriazines possess different acidity.

It should be mentioned that a similar comparison of the dissociation constant values of uracil monoalkyl derivatives does not permit the determination of the sequence of dissociation on account of the small differences between the pK_a values.[65] However, the pH dependence of the UV spectra showed that the first dissociation of uracil occurs at the NH group in position 1 and thus differently than in 6-azauracil.[66] This, together with different acidity, represents the main differences between the properties of uracil and its 6-aza analogs.

The reaction of ethylene carbonate with acid imides[75] which yields *N*-hydroxyethyl derivatives was applied to 6-azauracil. In agreement with the foregoing findings, 6-azauracil produced a 3-(2-hydroxyethyl) derivative (**65**) which was treated with thionyl chloride to convert



[65] : R = OH
[66] : R = Cl

[75] K. Yanagi and S. Akiyoshi, *J. Org. Chem.* **24**, 1122 (1959).

it to the 3-chloroethyl derivative (**66**). On applying the aforementioned reaction to uracil, a mixture of 1-(2-hydroxyethyl) and 1,3-bis-(2-hydroxyethyl) derivatives was produced.[76]

The preparation of *N*-alkyl derivatives of 6-benzyl-3,5-dioxo-1,2,4-triazine by hydrolysis of the corresponding alkylmercapto derivatives was systematically studied by Cattelain.[64,77,78] The conversion to known alkyl derivatives of dioxotriazines was used to determine the structure of alkylated methylmercapto derivatives. As will be shown later (e.g., Section II,B,4,b) this procedure has a general preparative significance for 1-alkyl derivatives of 6-azauracil.[79]

Hydrolytic cleavage of the methylmercapto group usually proceeds very readily and in practically quantitative yield even on short boiling in water acidified with a few drops of hydrochloric acid. The readiness of the hydrolysis can be affected very substantially by substitution as shown in the case of 4-methyl-3-methylmercapto-5-thioxo-4,5-dihydro-1,2,4-triazine which was hydrolyzed only with 3*N* hydrochloric acid.[80]

This method can be considered as a general one except when the preparation of methylmercapto derivatives is not possible, e.g., with 6-benzyl-2,4-dimethyl-3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazine which was converted to the corresponding dioxotriazine derivative by oxidation with bromine in an alkaline solution.[81]

The last of the procedures of preparation of *N*-alkyl derivatives of dioxotriazines is the cyclization of *N*-alkylated semicarbazones of α-keto acids. It was employed only in a few cases and it appears that its yields are very low.[72,82,83] Despite the fact that even here a fundamental effect of substitution on the yield of cyclization can be expected, as the case is with analogous thiosemicarbazones (e.g., Section II,B,4,b), the method is of no particular preparative value.

The IR and UV spectra of the *N*-alkyl derivatives of 6-azauracil[66,67]

[76] M. Prystaš and J. Gut, *Collection Czechoslov. Chem. Communs.* **27**, 1054 (1962).
[77] E. Cattelain, *Bull. soc. chim. France* **11**, 249 (1944).
[78] E. Cattelain, *Bull. soc. chim. France* **11**, 273 (1944).
[79] J. Gut, M. Prystaš, and J. Jonáš, *Collection Czechoslov. Chem. Communs.* **26**, 986 (1961).
[80] M. Prystaš and J. Gut, *Collection Czechoslov. Chem. Communs.* **27**, 1898 (1962).
[81] E. Cattelain, *Bull. soc. chim. France* **12**, 39 (1945).
[82] E. Cattelain, *Bull. soc. chim. France* **12**, 53 (1945).
[83] E. Cattelain, *Compt. rend. acad. sci.* **207**, 998 (1938).

are essentially identical with the spectra of 6-azauracil. In the IR spectrum, alkylation brings about characteristic shifts of frequency of one or the other maximum in the carbonyl region. This can be employed for defining the substitution site. The differences in the pH dependence of the UV spectra are of similar significance.

b. N-Acyl Derivatives. 6-Benzyl-[82] and 6-styryl-dioxotriazines[84] were found to form acetyl derivatives, but the position of the acetyl group has not been determined. Mono-acetyl derivatives of 6-azauracil (**67**) and of 6-azathymine were described by Prystaš *et al.*[85,86] Since methylation of these acetyl derivatives with diazomethane and subsequent hydrolysis of the methylation products (**68**) yielded the well-known 3-methyl-6-azauracil (**63**), it was concluded that the acetyl was bound in position 1. It can be assumed that the same position (2 of the triazine ring) is also occupied in the other acetyl derivatives mentioned. 1-Acetyl-6-azauracil readily loses the acetyl group on treatment with water or alcohol. This makes it possible to employ the acetylation for reversible masking of position 1.

Acetylation of 6-azauracil thus proceeds in the same way as the acetylation of uracil[87] and the properties of the acetyl derivatives are also roughly identical.

c. Nucleosides. The ribofuranosyl and 2′-deoxyribofuranosyl derivatives of 6-azauracil and 6-azathymine represent now the most important compounds of this group from the point of view of biochemistry.

Some of them were obtained for the first time by an enzymatic procedure which, of course, can result only in the aza analogs of natural nucleosides, i.e., ribofuranosyl-6-azauracil (6-azauridine) (**75**) and 2′-deoxyribofuranosyl-6-azathymine (6-azathymidine). The first of these was prepared by Škoda *et al.*[88] and a modification of their procedure was used by Handschumacher.[89] In this way it is possible to obtain the crystalline nucleoside on the large scale.

[82] S. Bodforss, *Ann. Chem. Liebigs* **639**, 125 (1961).
[84] M. Prystaš, J. Gut, and F. Sorm, *Chem. & Ind. (London)* p. 947 (1961)
[85] M. Prystaš, J. Gut, and F. Sorm, *Collection Czechoslov. Chem. Communs.* **27**, 1572 (1962).
[87] I. B. Spector and E. B. Keller, *J. Biol. Chem.* **232**, 185 (1958).
[88] Škoda, V. F. Hess, and F. Sorm, *Collection Czechoslov. Chem. Communs.* **22**, 1330 (1957).
[89] R. E. Handschumacher, *Nature* **182**, 1090 (1958).

6-Azathymidine was first prepared by Prusoff[90] and the procedure was described in more detail by Hall and Haselkorn.[91] The nucleoside was prepared here in the form of a glassy solid, but with dibenzyl-phosphochloridate it yielded a mixture of nucleotides from which crystalline 3′-phosphate, 5′-phosphate, and 3′,5′-diphosphate were prepared.

By comparing the dissociation constant of 6-azauracil and 6-azauridine with those of uracil and uridine, 6-azauridine is now considered to be 1-ribofuranosyl derivative (2-ribofuranosyl-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine).[92] The same was shown more exactly by comparing the UV and IR spectra and the dissociation constants of 6-azauridine with the two monomethyl derivatives of 6-azauracil.[79,80] Enzymatic synthesis thus, proceeds, in the same way in natural bases and in their aza analogs.

The first chemical synthesis of these substances, using a procedure which yields 1-ribofuranosyl derivatives by pyrimidine bases, was described by Hall.[93] By using the mercuric salt of 6-azathymine and tribenzoate of D-ribofuranosyl chloride, he obtained a mixture of two monoribosyl derivatives and a diribosyl derivative. He determined the structure of the 3-substituted derivative by the similarity of spectra and other properties to those of 3-methyl-6-azauracil. The structure of the 1-ribosyl derivative was then determined from the similarity of the spectra with 6-azathymine deoxyriboside obtained enzymatically.

Handschumacher[92] applied a similar procedure to 6-azauracil and obtained a mixture of two monoribosyl derivatives from the mercuric salt of 6-azauracil, with the 3-substituted derivative predominating.

Chemical and enzymatic ribosidization of the aza analogs of the pyrimidine bases thus take different routes. These results and independent earlier studies of the alkylation of 6-azauracil[65] led to the conclusion that, in order to achieve ribosidization in position 1 (i.e., position 2 of the triazine ring), the position 3 (4 of the triazine ring) must be protected.[92,83]

This procedure was verified by synthesizing 3-methyl-6-azauridine

[90] W. H. Prusoff, *J. Biol. Chem.* **215**, 809 (1955).
[91] R. H. Hall and R. Haselkorn, *J. Am. Chem. Soc.* **80**, 1138 (1958).
[92] R. E. Handschumacher, *J. Biol. Chem.* **235**, 761 (1960).
[93] R. E. Hall, *J. Am. Chem. Soc* **80**, 1145 (1958).

tribenzoate (**69**) as described by Prystaš *et al.*[89] 1-Acetyl-6-azauracil was methylated with diazomethane to obtain authentic 3-methyl-6-azauracil, and its salt was converted in the usual way to 3-methyl-6-azauridine tribenzoate. An identical product was obtained by methylation of tribenzoate of natural 6-azauridine (**70**) with diazomethane. This confirmed the assumption that 6-azauridine is actually a 1-ribofuranosyl derivative. On the assumption that the steric course of ribosidization studied in detail in the pyrimidine bases is the same as in their aza analogs, 6-azauridine is 1-β-D-ribofuranosyl-6-azauracil.

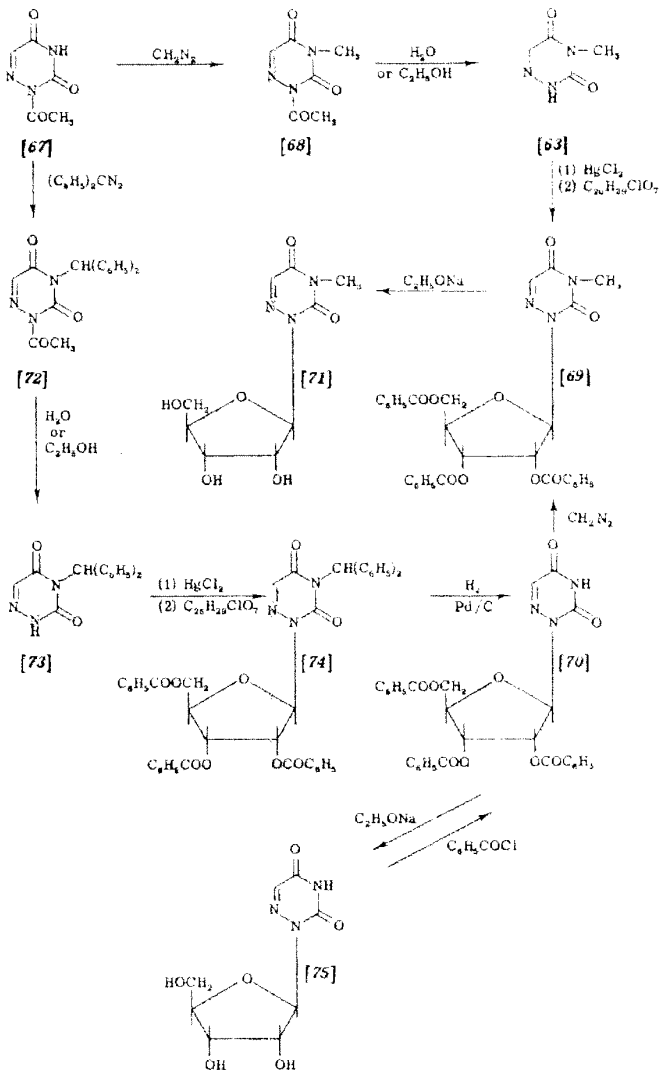
By reversibly blocking position 3 with a diphenylmethyl group (72, 73), Prystaš and Sorm[94] also prepared 6-azauridine. The diphenylmethyl group was removed after ribosidization (**74**) by selective hydrogenolysis. The crystalline 6-azauridine obtained was identical with 6-azauridine prepared enzymatically.[88]

This synthesis appears to be quite general for the preparation of 1-substituted nucleosides and was used with small modifications for the synthesis of 1-ribofuranosyl-6-azathymine[94] and 2′-deoxyribofuranosyl-6-azauracil[95] and -6-azathymine.[96] In the case of 2′-deoxyribofuranosyl a mixture of α- and β-anomers is produced, their ratio depending on the reaction conditions. In the preparation of 2′-deoxyribofuranosyl-6-azathymine only one anomer was obtained having probably the β-configuration.[96]

6-Azauridine was also synthesized using the knowledge of the course of alkylation of 6-azauracil 2-methylmercapto derivatives[79] (e.g., Section II,B,4,b). The 1-ribofuranosyl derivative obtained by reaction of the mercury salt of the 2-methylmercapto derivative with tri-*O*-benzoyl-β-D-ribofuranosyl chloride on removal of the methylmercapto and then benzoyl groups yielded crystalline 6-azauridine.[90a]

The main difference between uracil and 6-azauracil nucleosides consists in the preparation of cyclic nucleosides. It is known that uridine can be readily converted to cyclic nucleosides by the reaction of 2′(5′)-*O*-mesyl derivatives with nucleophilic agents.[97] Analogous

[89] M. Prystaš and F. Sorm, *Collection Czechoslov. Chem. Communs.* **27**, 1578 (1962).
[90] J. Plimi and F. Sorm, *Chem. & Ind. (London)*, p. 655 (1962).
[90a] J. Plimi, M. Prystaš, and F. Sorm, *Collection Czechoslov. Chem. Communs.*, in press.
[90b] A. R. Restivo and F. A. Deudzila, *J. Org. Chem.* **27**, 2281 (1962).
[97] D. M. Brown, D. B. Parihar, and A. Todd, *J. Chem. Soc.* p. 4242 (1958).

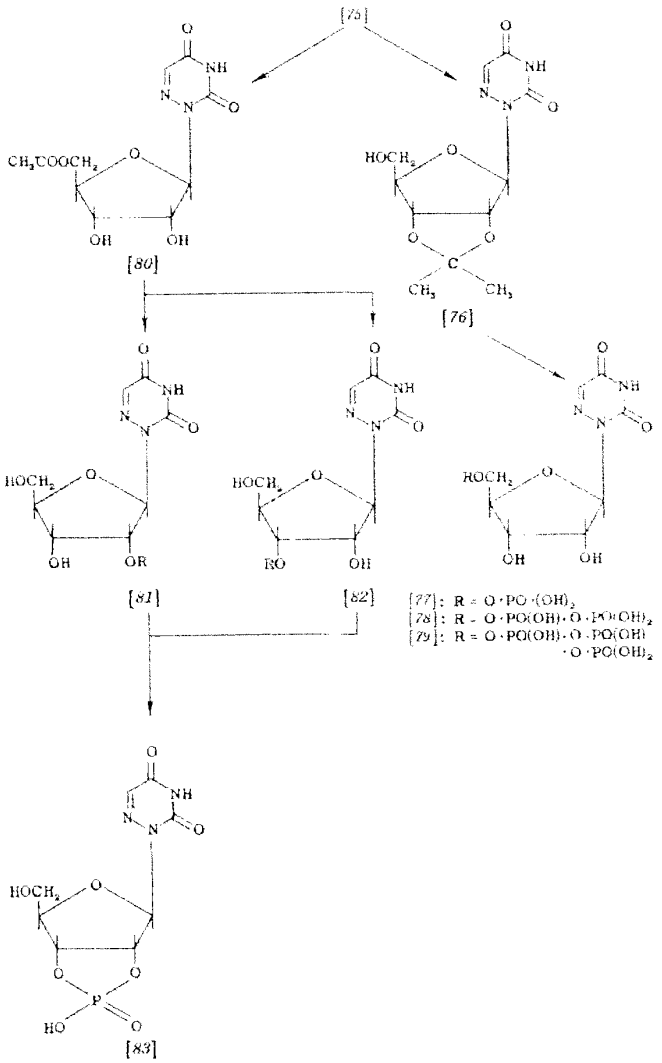


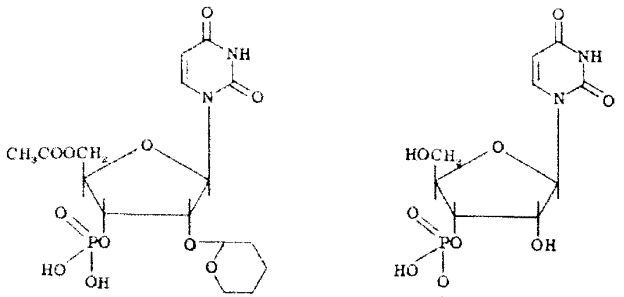
derivatives of 6-azauridine have not yet been cyclized by such agents. It was also attempted, unsuccessfully, to convert 6-azauridine[98] to an *O*^{2},2′-cyclonucleoside, which is relatively simple to achieve with uridine and cytidine by means of polyphosphoric acid.[99] These results can be taken as further evidence that 6-azauracil does not react in the lactim form.

d. Nucleotides. Beside the aforementioned preparation of 6-azathymidine nucleotides, procedures for the preparation of individual 6-azauracil nucleotides were developed by Smrt, Sorm, and coworkers. The 5′-phosphoryl derivatives were prepared from 2′,3′-*O*-isopropylidene-6-azauridine (**76**) by treatment with dibenzylphosphochloridate, with tetra-*p*-nitrophenylphosphate, or with polyphosphoric acid.[100,101] The 5′-diphosphate (**78**) was prepared from the monophosphate (**77**) by the action of dibenzylphosphochloridate,[100,101] the 5′-triphosphate (**79**) from 6-azauridine-5-phosphomorpholidate and pyrophosphoric acid.[102]

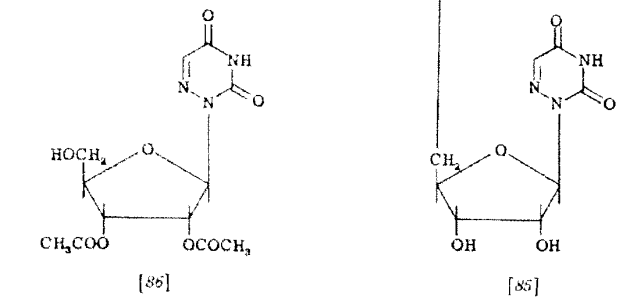
Proceeding from 5′-*O*-acetylazauridine (**80**), a mixture of 2′- and 3′-monophosphates (**81**, **82**) was prepared by phosphorylation with polyphosphoric acid, and these were converted into the 2′,3′-cyclic phosphate (**83**).[101] From the 2′,3′-*O*-isopropylidene derivative of 3-methyl-6-azauridine the 5′-phosphate was prepared by treatment with cyanoethylphosphate and the corresponding diphosphate from its morpholidate through the action of phosphoric acid.[103] Furthermore, a diribonucleoside phosphate (**85**) with a natural 3′-5′ internucleotide linkage was prepared from 6-azauridine. The starting material for the preparation of such derivatives was 5′-*O*-acetyl-2′-*O*′-tetrahydropyranyluridine-3′-phosphate (**84**) which was condensed with 2′,3′-di-*O*-acetylazauridine (**86**) or with 2′,3′-*O*-isopropylidene-6-azauridine (**76**) with the aid of dicyclohexylcarbodiimide.[104]

[98] J. Smrt, private communication (1961).
[99] E. R. Walwick, W. K. Roberts, and C. A. Dekker, *Proc. Chem. Soc.* p. 84 (1959).
[100] J. Smrt, J. Beránek, and F. Sorm, *Collection Czechoslov. Chem. Communs.* **25**, 130 (1960).
[101] J. Beránek and J. Smrt, *Collection Czechoslov. Chem. Communs.* **25**, 2029 (1960).
[102] J. Ženíšek, J. Smrt, and F. Sorm, *Collection Czechoslov. Chem. Communs.*, in press.
[103] J. Ženíšek, J. Smrt, and F. Sorm, *Collection Czechoslov. Chem. Communs.* **27**, 1462 (1962).
[104] J. Smrt and F. Sorm, *Collection Czechoslov. Chem. Communs.* **27**, 73 (1962).



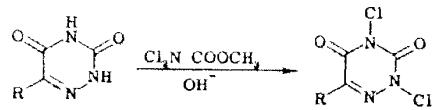


[84]



[86] [85]

e. Other N-Substituted Derivatives. The *N*-substituted derivatives also include the 6-substituted 2,4-dichloro-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazines (87) prepared by the action of esters of *N,N*-dichloro-



[87]

carbamic acid.[165,166] For 6-azauracil itself, and in more recent times for its derivatives too, no mention of such compounds can be found in the literature.

4. *Sulfur Derivatives*

The derivatives of dioxo-1,2,4-triazines in which one or both oxygen atoms are replaced with sulfur do not represent analogs of natural bases of nucleic acids. As was mentioned before, however, they occur as frequent intermediates during the preparation of dioxotriazines and are, therefore, mentioned briefly in this connection.

a. Thioxo Derivatives. Cyclization of thiosemicarbazones of α-keto acids with aqueous alkali represents the common method for the preparation of 3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines[12,93] as was mentioned earlier (e.g., Section II,B,2,a). Similarly to the semicarbazones, the cyclization is affected here by substituents in the α-position. The yields are then generally higher, so that the aryl or aralkyl derivatives are formed in a practically quantitative yield; even for the lowest homologs and glyoxylic acid thiosemicarbazone itself yields are still rather good.[60]

Formerly the required amount of hydroxide (usually in the form of 1 *N* solution) was added until alkaline to phenolphthalein plus some excess. Cyclization was achieved either at normal temperature (several days or weeks) or by boiling for several hours.

It was found recently that the cyclization requires the application of 2 moles of alkaline hydroxide; in practice, a small excess was used. It was demonstrated that the cyclization is termined within 15 min, and even sooner with α-aryl or 2-*N*-alkyl derivatives.[19]

The preparation of the 6-propyl to 6-undecyl derivatives, however, was performed by boiling for 30 min with potassium carbonate.[60] Sodium methoxide in a mixture of ethanol and benzene was used for the cyclization of the thiosemicarbazone of phenylglyoxylic acid ester.[107]

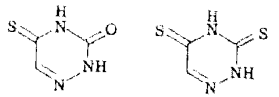
The case of cyclization of the α-monothiosemicarbazone of benzoylpyruvic acid seems to be exceptional; it was carried out either with

[165] J. Bougault and P. Chabrier, *Compt. rend. acad. sci.* **213**, 400 (1941).
[166] P. Chabrier, *Ann. chim. (Paris)* **17**, 353 (1942); *Chem. Abstr.* **38**, 3255 (1944).
[167] G. H. Hitchings, P. B. Russell, and A. D. Maggiolo, German patent 951,996 (1956); *Chem. Abstr.* **53**, 13186 (1959).

aqueous hydroxide or with dilute acetic acid or even with ethanol alone.[108]

The numerous 6-substituted 3-thioxo-5-oxo derivatives prepared by earlier authors are reviewed by Erickson *et al.*[109] Subsequently a number of alkyl,[95,61] and further 4-acetaminophenyl, 2-thienyl, 4-pyridyl,[110] 4-antipyryl,[111] and 2-aminoethyl[112] derivatives were prepared plus some others mentioned in the section on the synthesis of 6-azauracil.

5-Thioxo-3-oxo-2,3,4,5-tetrahydro-1,2,4-triazine (4-thio-6-azauracil) (**88**) and 3,5-dithioxo-2,3,4,5-tetrahydro-1,2,4-triazine (2,4-dithio-6-azauracil) (**89**) were prepared by Hitchings *et al.*[58] treating 6-azauracil with phosphorus pentasulfide. The mixture of the two substances formed simultaneously was resolved by ion-exchange chromatography. It was found later that by thiation of 6-azauracil under suitable conditions the 5-thioxo derivative can be obtained practically pure.[79] For the same reason it is advantageous to prepare the 3,5-dithioxo derivative from the 3-thioxo derivative.[79,113] The effect of the ratio of the starting compounds, the quality of phosphorus pentoxide, and the reaction time was examined by Jacquier and Lieberman.[113] The difference between the reactivity of both the carbonyl groups seems to be more pronounced in 6-azauracil than in uracil.[114]



(88)

(89)

The course of thiation (replacement of oxygen by sulfur) of dioxo-

[108] G. La Parola and C. J. Turi, *Ann. chim. (Rome)* **51**, 283 (1961).
[109] J. G. Erickson, P. F. Wiley, and V. P. Wystrach, "The 1,2,3- and 1,2,4-Triazines, Tetrazines and Pentazines," Vol. 10 of "The Chemistry of Heterocyclic Compounds" (A. Weissberger, ed.), p. 79. Interscience, New York, 1956.
[110] R. B. Hagenbach, E. Hodel, and H. Gysin, *Experientia* **10**, 62 (1954); *ibid.* **11**, 314 (1955).
[111] F. Schmidt, *Arch. Pharm.* **289**, 150 (1956).
[112] J. Hadáček and J. Slouka, *Pharmazie* **13**, 402 (1958).
[113] D. Liebermann and R. Jacquier, *Bull. soc. chim. France* p. 383 (1961).
[114] H. L. Wheeler and L. M. Liddle, *Am. Chem. J.* **40**, 547, 557 (1908).

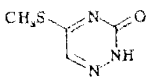
triazines is considerably affected by substitution in the ring. In the case of thiation of 6-azathymine only the dithio derivative was obtained.[58] With other 5-alkyl or aryl derivatives the reaction has not been tried. The effect of *N*-substitution is more complicated.[79] 1-Methyl-6-azauracil yielded 1-methyl-2,4-dithio-6-azauracil; 1-ribofuranosyl-6-azauracil tribenzoate[115] produced only the 4-thio derivative. Thiation of 3-methyl-6-azauracil resulted in a mixture of 4-thio and 2,4-dithio derivatives with a clear predominance of the former. 1,3-Dimethyl-6-azauracil yielded only the 4-thio derivative under the same conditions. The 2-thio and the 2-methylmercapto derivatives yield the corresponding dithio and 2-methylmercapto-4-thio derivatives irrespective of further substituents.[79]

Thiation was most frequently carried out in pyridine[58,113]; for unsubstituted compounds which are considerably polar, this is a prerequisite. The less polar *N*-alkylated derivatives can be thiated in toluene or xylene[79]; for thiation of 6-azathymine, tetralin was also used.[110]

b. S- and N-Alkyl Derivatives. The alkylation of 3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazine was studied systematically by Cattelain[54,77,78] using 6-substituted derivatives, mostly benzyl. He found that their alkylation in an alkaline medium results first in 3-alkyl derivatives and subsequently in the 2,3-dialkyl ones. He concluded that these compounds react in the thiol form and that the methylmercapto derivatives formed have structure (**96**) (with a double bond in position 3,4). In addition to the investigation of the reduction of these substances with amalgam[68] (the interpretation of which is not completely convincing), he found that 3-thioxo derivatives are oxidized to disulfides.[50,117]

Alkylation reactions were recently performed with all the thio derivatives of 6-azauracil and 6-azathymine.[79] In agreement with previous findings, the methylmercapto derivatives were obtained by alkylation of all these substances in alkaline solution. Thus, e.g., 3-methylmercapto-5-oxo-2,5-dihydro-1,2,4-triazine (**96**), 5-methylmercapto-3-oxo-2,3-dihydro-1,2,4-triazine (**90**), and 3,5-dimethylmercapto-1,2,4-triazine (**91**) were obtained. The last-named of these was

[115] V. Černěckij, S. Chládek, F. Šorm, and J. Smrt, *Collection Czechoslov. Chem. Communs.* **27**, 87 (1962).
[116] Burroughs Wellcome & Co., British patent 802,122 (1958); *Chem. Abstr.* **53**, 7216 (1959).
[117] E. Cattelain, *Compt. rend. acad. sci.* **215**, 257 (1942).



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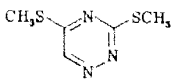
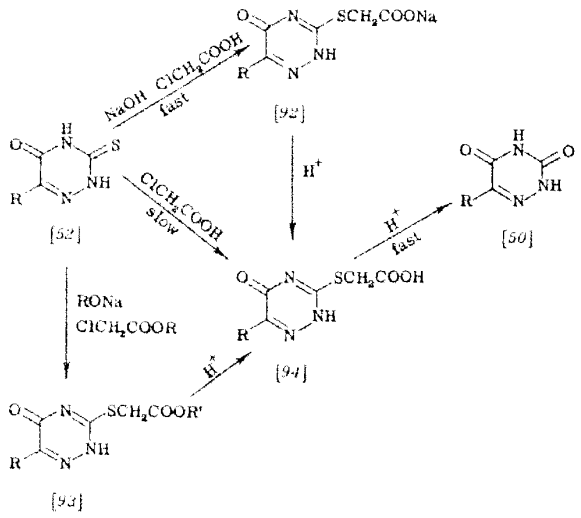


[91]

also prepared from 3,5-dichloro-1,2,4-triazine[41] (cf. Section II,B,6). All these reactions take place in aqueous hydroxide or in alcoholic sodium ethylate, at normal temperature.

In this connection the course of the reaction of 3-thioxo derivative (**52**) with chloroacetic acid was studied in detail,[92] the reaction being important for the transformation to dioxotriazine derivatives. In this reaction, the carboxymethylmercapto derivatives (**94**) must be expected as intermediates. The ethyl esters of these compounds (**93**) (R = $CH_2C_6H_5$; R′ = C_6H_5) were isolated by Cattelain after reaction with ethyl chloroacetate.[93] When the reaction is performed in the usual preparative way using 10% aqueous solution of chloroacetic acid, it requires 3–5 hr of boiling. In an alkaline solution (with a



total of 3 equivalents of NaOH), the reaction proceeds considerably more rapidly and stops at the sodium salt stage (**92**). From the substituted derivatives (R = alkyl or aryl), free carboxymethylmercapto derivatives (**94**) can be obtained, which are relatively stable in neutral solution. In an acid solution they are rapidly hydrolyzed to the dioxo derivatives (**50**). During the reaction with aqueous chloroacetic acid the slow substitution reaction is the rate-determining step. The unsubstituted carboxymethylmercapto derivative (**94**; R = H) is hydrolyzed immediately after liberation from its salt and was isolated only in the form of an ester (**93**; R = H).[92]

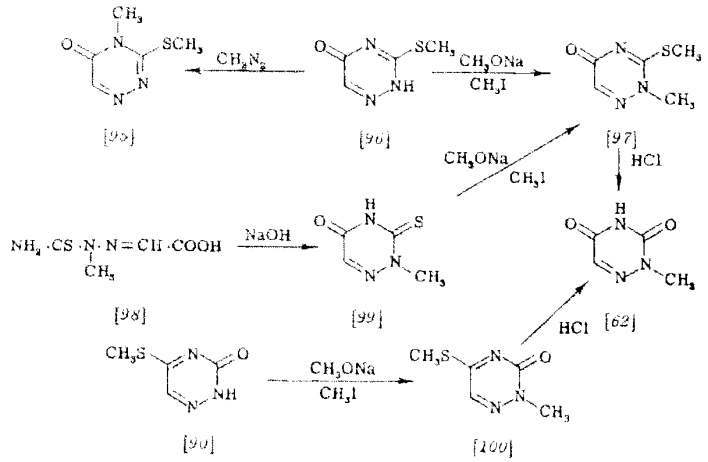
In agreement with the results of Cattelain, further methylation of the 3-methylmercapto derivative (**96**) results practically exclusively in 2-methyl-3-methylmercapto-5-oxo-2,5-dihydro-1,2,4-triazine (**97**). Further methylation of 5-methylmercapto derivative (**90**) yields 2-methyl-5-methylmercapto-3-oxo-2,3-dihydro-1,2,4-triazine (**100**). Their structure was confirmed by acid hydrolysis leading to 2-methyl-3,5-dioxo derivatives (**62**). As was already mentioned, this reaction is a suitable general procedure for preparing the 1-alkyl derivatives of 6-azauracil.[79]

The course of methylation of all the thio derivatives with diazomethane was then investigated.[79,96] These methylations generally result in mixtures of substances; it may be deduced from the products isolated, however, that this reaction proceeds first at the nitrogen atom (in contrast with alkaline methylation) and only then at the sulfur one. The methylation of the 3-methylmercapto derivative to 4-methyl-3-methylmercapto-5-oxo-4,5-dihydro-1,2,4-triazine (**95**) is of interest in this connection.

Some alkylated derivatives of 3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazines were also prepared by cyclization of the corresponding substituted thiosemicarbazones of α-keto acids. During these cyclizations a very marked effect of alkylation on the yield and course of the reaction can be observed.

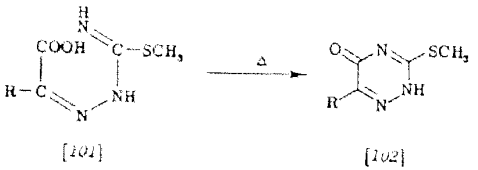
A uniformly favorable effect is displayed by an alkyl group, in position 2. Thus the 2-methylthiosemicarbazone of pyruvic acid is cyclized at normal temperature and without excess hydroxide.[54,118] The 2-methylthiosemicarbazone of glyoxylic acid (**98**) was cyclized by boiling for 5 min to 2-methyl-3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazine (**99**) (yield 93% as compared with 70–80% in the un-

[96] E. Cattelain, *Compt. rend. acad. sci.* **210**, 301 (1940).



substituted derivative[69]). This reaction was used for an unambiguous synthesis of 1-methyl-6-azauracil.[69]

Alkylation in position 3 has a still more pronounced effect. The thiosemicarbazones (**101**) obtained here were prepared either from 3-methylisothiosemicarbazide hydroiodide or by methylation of thiosemicarbazones. Their cyclization was performed either by boiling in alcohol or by heating to the melting point (**102**).[54,69a] The presence of

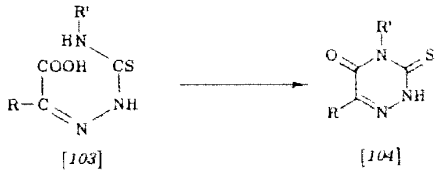


alkalies, in contrast with the preceding reactions, prevented cyclization.[54] A similar course is probably followed by the reaction of 3-methylisothiosemicarbazide with chloral[49] mentioned in the foregoing (cf. Section II,B,2,a). Hence it might be concluded that the cyclization requires the formation of the thiol form of the starting thiosemi-

carbazone which is present in an alkaline medium or which can be fixed by substitution on the sulfur atom.

Substitution in position 4 displays a more complex influence. Cyclization of the 4-methyl- and 4-ethyl-thiosemicarbazones of phenylpyruvic acid and of the 4-methylthiosemicarbazone of phenylglyoxylic acid (**103**) was readily achieved[78] (**104**), whereas it was not possible to cyclize the analogous 4-methyl derivatives of pyruvic[119] and glyoxylic acids.[63] It thus appears that cyclization is hindered by substitution in position 4 and that this unfavorable effect can be partly relieved by the known favorable effect of an aryl or aralkyl group in the α-position.

The cyclization of the 4-methylthiosemicarbazone of pyruvic acid was recently effected by refluxing in dimethylformamide.[119a]

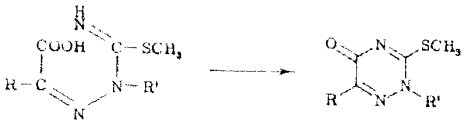


It can be concluded from further results, however, that the nature of the substituent in position 4 is even of more importance. Thus the 4-benzylthiosemicarbazone of phenylpyruvic acid is not cyclized[78] whereas 4-arylthiosemicarbazones of pyruvic acid and phenylglyoxylic acid are readily cyclized merely by boiling in ethanol, in the absence of alkalies. Attempted alkaline cyclization, on the contrary, results in the original product or (in the case of glyoxylic acid derivative) it is split to phenylthiourea.[120] Among other examples, the cyclization of the 4-methylthiosemicarbazone of cinnamoylformic acid in low yield should be mentioned. Cyclization of the 4-phenylthiosemicarbazone of the same acid proceeded readily on boiling in ethanol, and during the usual alkaline cyclization conditions the 4-phenylthiosemicarbazone of benzaldehyde was formed.[63]

As may be expected on the basis of the preceding results, the cyclization of 2,3-dialkylthiosemicarbazones (**105**) will take place

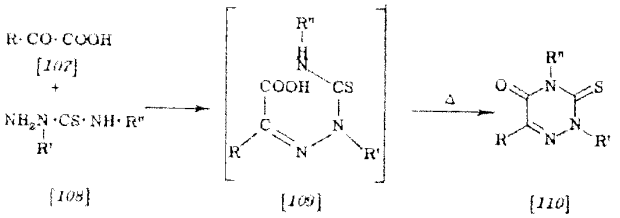
[119] R. Jacquier, private communication, (1961).
[119a] K. Y. Zee-Cheng and C. C. Cheng, *J. Org. Chem.* **27**, 976 (1962).
[120] M. Tišler and Z. Vrbaški, *J. Org. Chem.* **25**, 770 (1960).

very readily[52] (**106**). It is harder to explain why the 3,4-dialkyl derivatives are not cyclized[53] and why the 2,4-dialkyl ones, on the contrary, undergo cyclization even on mixing the substituted semi-



[*105*] [*106*]

carbazide (**108**) with phenylpyruvic acid (**107**) without isolating the thiosemicarbazone (**109**) formed.[53] In the case of 2-phenyl-4-methylthiosemicarbazide and of pyruvic acid, the cyclization set in just on boiling in ethanol or in acetic acid.[121] In the same way, the cor-



[*108*] [*109*] [*110*]

responding triazine was obtained from cinnamoylformic acid and 2,4-diphenylthiocarbazide.[54]

The cyclization of some 2- and 4-methylthiosemicarbazones and the thiation of the cyclic product was recently studied by Zee-Cheng and Cheng.[119a]

Even if no general conclusions can be drawn on the basis of existing material, it appears that further study of cyclization of these substances could elucidate the mechanism of cyclization of thiosemicarbazones of α-keto acids which is of fundamental importance in the chemistry of dioxotriazines.

c. Properties. On the basis of reactions of the 3-thioxo derivatives, especially of the ready alkylation on sulfur, a thiol structure was

[121] J. A. Elvidge and F. S. Spring, *J. Chem. Soc., Suppl.* No. 1, S 135 (1949).

formerly attributed to these substances.[15,93] Tišler and Vrbaški recently studied the UV and IR spectra of a series of 2,6-disubstituted 3-thioxo-5-oxotriazines and in neutral solution ascribed to them the thioxo form. In alkaline medium the anionic charge resides predominantly on the sulfur atom so that alkylation affords alkylmercapto derivatives.

The aforementioned detailed studies of the methylation of thio analogs of 6-azauracil[79,80] made it possible to obtain a practically complete set of all structural types, fixed in the thiolactam or thiol form. This enabled a detailed study of the structure of these substances by UV spectra.[122] It was shown that all the thio analogs of 6-azauracil possess the thioxo form in neutral solution. Similar results are indicated by the IR spectra of all the thioxo derivatives which show absorption peaks for the free NH group in the lactam region and for the thioxo grouping.[123] The UV spectra also show that the 3-methylmercapto derivative has the structure (**96**) with a double bond in position 3,4. In agreement with this finding is its further methylation in position 2 (**97**) in an alkaline medium. It is of interest that with the UV spectra, the *S*- and *N*-substituted derivatives exhibit marked differences[122]; the analogous *O*- and *N*-substituted derivatives of this type of substances display only insignificant differences (see e.g., Section II,A,2,b).

It was found already by Cattelain[63] that the 3-thioxo derivatives behave as monobasic acids that can be titrated on phenolphthalein and he considered them as more acid than the analogous 3,5-dioxotriazines. This assumption was recently confirmed by determining the dissociation constants. Just as with 6-azauracil, it was possible to demonstrate, by comparing the dissociation constants of the *N*-methyl derivatives of all the thioxo analogs, that with the 3-thioxo compounds too, dissociation proceeds first at the NH group in position 3.[122]

5. *6-Substituted Derivatives of 3,5-Dioxo-2,3,4,5-tetrahydro-1,2,4-triazine*

6-Alkyl and 6-aryl derivatives of 3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine or of its thio analogs have been mentioned before in this review (e.g., Section II,B,2,a). Some of them contained functional

[122] J. Jonáš and J. Gut *Collection Czechoslov. Chem. Communs.* 27, 1886 (1962).
[123] M. Horák and J. Gut, *Collection Czechoslov. Chem. Communs.*, in press.

groups at the more distant positions of the alkyl or aryl group. This is obviously no obstacle for their formation by cyclization of semicarbazones or thiosemicarbazones even if some exceptions are known [124]

However, for the preparation of derivatives which contain a functional group directly attached to position 6, the application of the foregoing cyclization method is considerably limited by the availability or existence of the required derivatives of α-keto acids and may also be affected by differences in their reactivity. Cyclization of thiosemicarbazones was, therefore, used for these substances only in the case of the 6-carboxylic acid[97,98] (see also Section II,B,2,a). Of the other derivatives known, the 6-acetic acid ester[92] should be mentioned. Recently some further derivatives of dioxotriazine-6-carboxylic acid were reported.[119a]

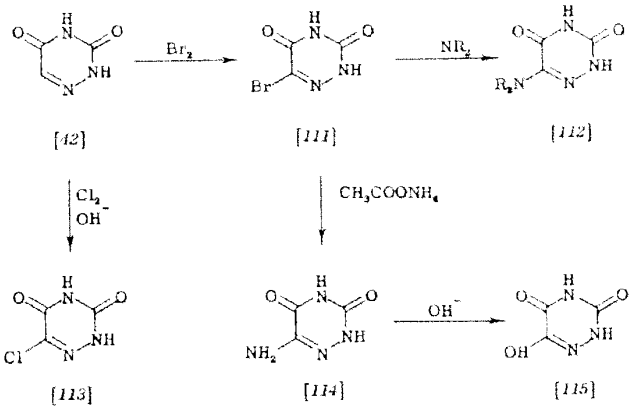
Other derivatives of this type were studied by Chang and Ulbricht[19] and were prepared by direct substitution or other secondary reactions.

Direct bromination readily yields the 6-bromo derivative (**111**), just as with uracil. Analogous chlorination and iodination requires the presence of alkalies and even then proceeds in low yield. The 6-chloro derivative (**113**) was also obtained by partial hydrolysis of the postulated 3,5,6-trichloro-1,2,4-triazine (e.g., Section II,B,6). The 6-bromo derivative (5-bromo-6-azauracil) served as the starting substance for several other derivatives.[125] It was converted to the amino derivative (**114**) by ammonium acetate which, by means of sodium nitrite in hydrochloric acid, yielded a mixture of 6-chloro and 6-hydroxy derivatives. A modified Schiemann reaction was not suitable for preparing the 6-fluoro derivative. The 6-hydroxy derivative (**115**) (an isomer of cyanuric acid and the most acidic substance of this group, $pK_a = 2.95$) was more conveniently prepared by alkaline hydrolysis of the 6-amino derivative. Further the bromo derivative was reacted with ethanolamine to prepare the 6-(2-hydroxyethyl) derivative; however, this could not be converted to the corresponding 2-chloroethyl derivative.[126] Similarly, the dimethylamino, morpholino, and hydrazino derivatives were prepared from the 6-bromo compound.[127]

In other experiments, variously substituted 6-mercapto dioxotriazines were prepared from 6-bromodioxotriazine (**111**) either by direct reaction with mercaptans or by alkylation of a mercapto derivative prepared via isothiouronium salt.[127] From these mercapto compounds alkylsulfones and sulfonamido derivatives were prepared.[127a]

It may be said in conclusion that the reactivity of position 5 (i.e., 6 of the triazine ring) is similar to that of uracil. The only difference seems to be in the failure to prepare 5-nitro-6-azauracil although this reaction proceeds readily with uracil.

[124] F. Adickes, *Ber. deut. chem. Ges.* **58**, 211 (1925).
[125] P. K. Chang, *J. Org. Chem.* **26**, 1118 (1961).
[126] P. K. Chang, private communication (1961).
[127] C. Cristescu and J. Marcus, *Pharmazie* **16**, 135 (1961).



6. *3,5-Disubstituted Derivatives of 1,2,4-Triazine*

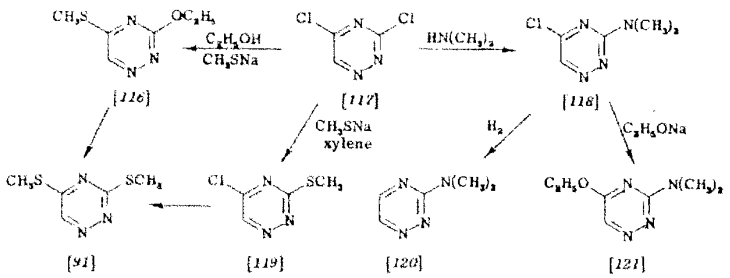
These derivatives of asymmetric triazine are rather distantly related to the group of substances reviewed here. Only those of them which are prepared from the dioxo or dithioxo derivatives will be mentioned.

On reaction with "aged" phosphoroxychloride, 6-azauracil formed 3,5-dichlorotriazine (**117**) in only a 10% yield.[19] A somewhat higher yield (30%) was obtained from the reaction of 6-bromodioxotriazine which gave 3,5,6-trichloro-1,2,4-triazine.[19,128] Similar reactions take place much more readily with uracil and in better yield.[128,129] Thus,

[127a] C. Cristescu and T. Panaitescu, *Pharmazie* **17**, 209 (1962).
[128] G. E. Hilbert and T. B. Johnson, *J. Am. Chem. Soc.* **52**, 1152 (1930).
[129] G. E. Hilbert and E. F. Jansen, *J. Am. Chem. Soc.* **56**, 134 (1934).

these reactions represent another difference in the reactivities of uracil and 6-azauracil.

Several other derivatives were prepared from dichlorotriazine according to Scheme 5, among these was also dimethylmercaptotriazine, later prepared in a different way. The position of the substituent is not defined in these cases.[10]



[116] [117] [118] [91] [119] [120] [121]

SCHEME 5

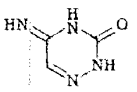
3,5,6-Trichloro-1,2,4-triazine was reacted with menthanol and subsequently crystallized from water to yield 5-chloro-6-azauracil.[49,125] A chloro-dimethoxy derivative appears to be an intermediate product, this being further cleaved by hydrogen chloride. No 2,4-dimethoxy derivatives have been prepared so far.

The thio analogs of the substances just mentioned, the alkylmercapto derivatives, are more stable and readily available (e.g., Section II,B,4,b). These derivatives were used for the preparation of the 3-hydrazino and 3,5-dihydrazino derivatives.[113]

The preparation of 3,5-diamino derivatives from 3,5-dithioxotriazines or from 6-methyl-3,5-dimethylmercapto-1,2,4-triazine was also described.[116]

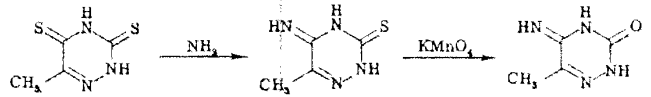
7. *3-Oxo-5-imino-2,3,4,5-tetrahydro-1,2,4-triazine (6-Azacytosine)*

Substances of this type were not studied by the earlier workers, and the first representative of this group to be investigated was 3-oxo-5-imino-2,3,4,5-tetrahydro-1,2,4-triazine (**122**) which should bear the name 6-azacytosine. It was prepared by Falco *et al.*[58] by treating 3-thioxo-5-oxo-2,3,4,5-tetrahydro-1,2,4-triazine (**88**) with alcoholic ammonia. Some *N*-substituted derivatived were prepared analogously.[119a]



[122]

The analogous 6-methyl derivative (**126**) was prepared by the same authors from 6-methyl-3,6-dithioxo derivative (**123**) as the corresponding monothioxo derivative is not easily available. Even here the substitution with ammonia took place selectively in position 4 (**124**) and the remaining sulfur atom was replaced with oxygen by oxidation with alkaline potassium permanganate (**125**).[58] A similar procedure is protected by a patent.[116]



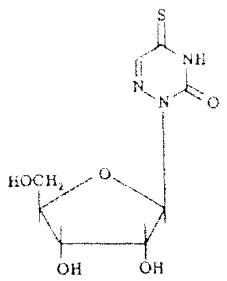
[123] [124] [125]

In contrast with cytosine, its aza analog readily undergoes hydrolysis both in acid and in alkaline solution. At 100°C hydrolysis is 50% complete within 10 min in a solution of hydrochloric acid or potassium hydroxide. The 5-methyl derivative is hydrolyzed even more readily.[58]

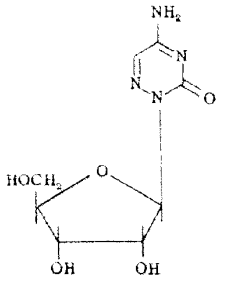
Sorm *et al.*[113] prepared azacytidine and some of its derivatives in a similar way. The 4-thio derivative was obtained from 2′,3′,5′-tri-*O*-acetyl- or 2′,3′,5′-tri-*O*-benzoyl-6-azauridine by treatment with phosphorus pentasulfide; this liberated 4-thio-6-azauridine (**126**) which was identified with 4-thio-6-azauracil on comparing the UV spectra. Treatment with ammonia produced 6-azacytidine (**127**); treatment with hydrazine, hydroxylamine, and *n*-butylamine yielded the corresponding derivatives.

The UV spectra of azacytidine are similar to those of uridine and 6-azauridine but differ from those of cytosine and cytidine. On the other hand, the spectrum of the 4-dimethylamino derivative is different from that of 6-azacytidine and similar to that of cytidine.[130]

[130] J. Zemlička, J. Beránek, and J. Smrt, *Collection Czechoslov. Chem. Communs.* **27**, 2784 (1962), and J. Zemlička, private communication (1962).



[126]



[127]

The IR spectra of chloroform solutions of 6-azacytidine and its derivatives show that these compounds, like the corresponding cytidine derivatives, exist in the amino form.[131]

Preparation of 6-azacytidine-5′-phosphate by direct phosphorylation with cyanoethylphosphate was not successful. The substance could be prepared, however, on ammonia treatment of 4-thio-6-azauridine-5′-phosphate which was obtained by phosphorylation of 2′,3′-isopropylidene-4-thio-6-azauridine with pyrophosphoryl chloride. From morpholidate of 6-azacytidine-5′-phosphate, 6-azacytidine-5′-diphosphate was prepared by the action of phosphoric acid.[132]

A new synthesis of 6-azacytidine was reported recently. Treatment of 6-azauridine tribenzoate with dimethylchloromethylammonium chloride gave the 4-chloro derivative. Reaction with ammonia and removal of the protecting groups yielded 6-azacytidine.[133]

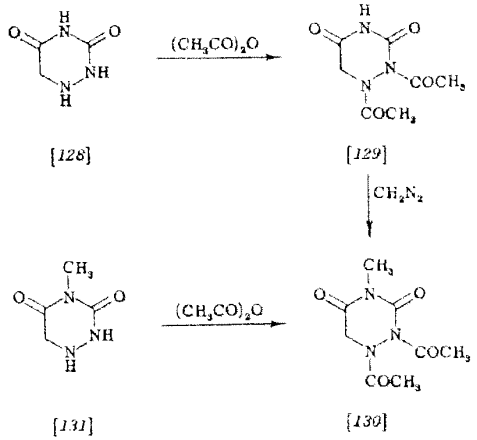
8. *Hexahydro Derivatives*

The synthesis of some 3,5-dioxohexahydro-1,2,4-triazines was described earlier (e.g., Section II,B,2,a). Other 6-substituted derivatives were prepared in the same way.[134]

[131] J. Pitha and J. Beránek, *Collection Czechoslov. Chem. Communs.*, in press.
[132] J. Beránek and F. Sorm, *Collection Czechoslov. Chem. Communs.*, in press.
[133] J. Zemlička, J. Smrt, and F. Sorm, *Tetrahedron Letters* p. 379 (1962).
[134] J. G. Erickson, P. F. Wiley, and V. P. Wystrach, "The 1,2,3- and 1,2,4-Triazines, Tetrazines and Pentazines," Vol. 10 of "The Chemistry of Heterocyclic Compounds" (A. Weissberger, ed.), p. 75. Interscience, New York, 1956.

Oxidation of the hexahydro to tetrahydro derivatives was mentioned in connection with the synthesis of 3,5-dioxo-1,2,4-triazines (e.g., Section II,B,2,a). The reverse procedure, hydrogenation of the tetrahydro derivatives, was used with 6-azauracil, 6-azathymine, and their *N*-methyl derivatives. With all these compounds hydrogenation proceeds smoothly in the presence of Adams' catalyst. Only the hydrogenation of 1-methyl-6-azathymine was not successful.[65]

3,5-Dioxohexahydro-1,2,4-triazine (dihydro-6-azauracil) (**128**) yields a diacetyl derivative (**129**) which is relatively stable toward hydrolysis. The acetylation of the *N*-methyl derivatives and the course of the reaction with diazomethane indicates that acetylation takes place here in positions 1 and 2.[123]

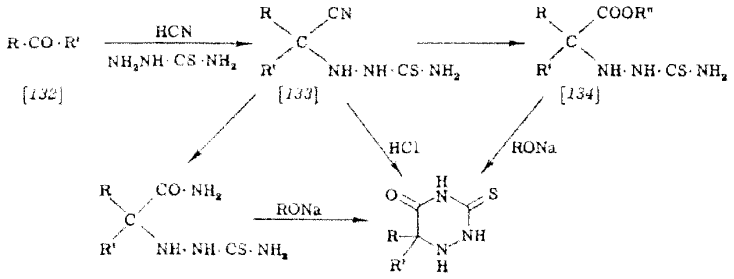


[128] [129] [131] [139]

The hexahydro derivatives are much less acid than the tetrahydro ones ($pK_a > 10$).[21] Their UV spectra naturally lack the characteristic maxima.[66] The IR spectra, however, possess similar absorption to the tetrahydro derivatives in the carbonyl region.[87] It can thus be concluded that they also possess the dilactam structure.

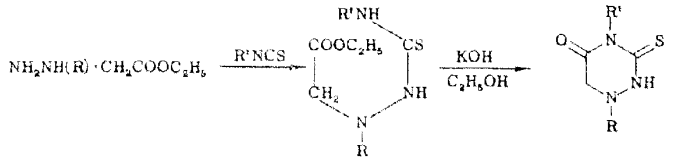
Just as for the dioxohexahydro derivatives, substituted 3-thioxo-5-oxohexahydro-1,2,4-triazines were recently prepared by cyclization

of thiosemicarbazidoacetates.[135–137] The starting thiosemicarbazidoacetic acids (**133–135, 138**) were prepared either by simultaneous addition of hydrogen cyanide and thiosemicarbazide to ketones (**132**)[135,136] or by the reaction of hydrazinoacetates (**137**) with isothiocyanates.[137,138] The esters (**134, 138**) or amides (**135**) of thiosemicarbazido-



[*132*] [*133*] [*134*] [*135*] [*136*]

acetic acids thus formed were cyclized by alkaline alcoholate or hydroxide in ethanol (**136, 139**).[135,137] The nitrile of thiosemicarbazidoacetic acid (**133**) was also cyclized by concentrated hydrochloric acid.[136] Spectra of these substances (**139**) were studied by Tišler.[137]



[*137*] [*138*] [*139*]

He found that the UV maximum exhibits a hypsochromic shift in an alkaline medium. In the IR spectra he found maxima in the carbonyl

[135] R. Fusco and S. Rossi, *Gazz. chim. ital.* **84**, 373 (1954).
[136] S. R. Safir, J. J. Hlavka, and J. H. Williams, *J. Org. Chem.* **18**, 106 (1953).
[137] M. Tišler, *Vestník Slovenu. kemi. društva* **7**, 69 (1960).
[138] M. Busch and E. Meussdörffer, *Ber. dtsl. chem. Ges.* **40**, 1021 (1907).

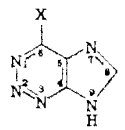
group regions but no maximum was present for a SH group. Hence he concludes that these substances possess the thiolactam form.

III. Aza Analogs of Purine Bases

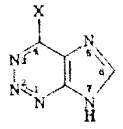
A. 2-Aza Analogs

1. *Nomenclature*

The names of 2-aza analogs are derived by formal substitution of the methine group in the 2-position of the purine skeleton by a nitrogen atom (**140**). Since this position is substituted in some purine bases, only the aza analogs of adenine or hypoxanthine are amenable to such formal derivation.



[*140*]



[*141*]

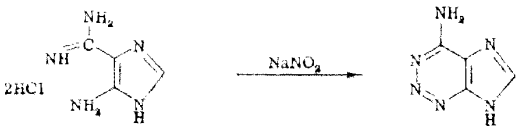
The systematic nomenclature used originally the term imidazo-1,2,3-triazine. The *Chemical Abstracts* indexes use the more accurate name imidazo[4,5-*d*]-*v*-triazine (**141**). The numbering of the substituents is different in the two systems of nomenclature as may be seen in the formulas.

2. *Preparation and Properties*

Substances of this type have hitherto received little attention. One of the reasons appears to be the limited possibilities of preparation. The only known method of preparation, described by Woolley *et al.*,[139] proceeds from the derivatives of 4-aminoimidazole-5-carboxylic acid. The amide of this acid (**142**) is treated with nitrous acid to yield 4-hydroxyimidazo[4,5-*d*]-*v*-triazine (2-azahypoxanthine) (**143**), the amidine (**144**) yielding the 4-amino derivative (2-azaadenine) (**145**) under the same conditions. 2-Azahypoxanthine was probably obtained in the same way earlier but was not identified.[139]

[139] D. W. Woolley, E. Shaw, N. Smith, and E. A. Singer, *J. Biol. Chem.* **189**, 401 (1951).

[142] [143]



[144] [145]

When it was found later that enzymatic oxidation of 2-azaadenine yields its 8-hydroxy derivative (4-amino-6-hydroxyimidazo[4,5-*d*]-*v*-triazine), its synthesis was also achieved by the procedure already described.[140]

Very few data exist on the physicochemical properties of these substances. The stability of 2-azaadenine against hydrolysis with hot hydrochloric acid and on the formation of silver salts have been mentioned; furthermore, their UV spectra have been published without detailed interpretation.[139,140]

B. 8-Aza Analogs

1. *Nomenclature*

The 8-aza analogs are formally derived by substitution of the methine group in position 8 of the purine ring. The names thus derived preserve the numbering of the purine ring (**146**), and are frequently used in papers of biochemical character, but in chemical papers only along with systematic names.

[146] [147]

[140] E. Shaw and D. W. Woolley, *J. Biol. Chem.* **194**, 641 (1952).

According to the systematic nomenclature these substances were first named 1-*v*-triazolo[*d*]pyrimidines in compliance with the general principles of the Ring Index.[141] More recent papers and *Chemical Abstracts* indexes use the term *v*-triazolo[4,5-*d*]pyrimidine (**147**) in accord with the IUPAC nomenclature. The numbering of substituents when using the last-mentioned name is different from that of the 8-aza analogs. For the formulas of oxygen and sulfur derivatives names derived from the lactim or thiolactim form are almost exclusively in use (in common with the purine derivatives). These derivatives are thus described as hydroxy and mercapto derivatives, respectively. The name 1,2,3,4,6-pentaazaindene is used only rarely for this system.

2. *Methods of Preparation*

The 8-aza analogs of purine bases were the first to be studied among all the aza analogs of nucleic acid bases (as early as 1945). Before that time the chemistry of these substances had not been treated in detail from any aspect. Thus the entire chemistry of the *v*-triazolo [4,5-*d*]pyrimidines was developed only in connection with the study of antimetabolites of nucleic acid components. Therefore all the papers involved are largely of preparative character and only rarely discuss theoretical points.

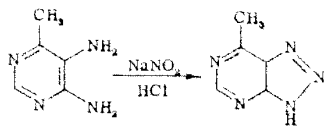
For the preparation of triazolopyrimidines three main types of synthesis are in use. The first of these proceeds from a pyrimidine derivative (especially the 4,5-diamino derivatives) and closes the triazole ring. The second method proceeds, on the contrary, from derivatives of *v*-triazole to close the pyrimidine ring. The third method finally is one which yields the derivatives through substitution or replacement of substituents in compounds prepared by one of the first-named procedures.

a. From Purine Derivatives. The only older work published on these substances is the paper by Gabriel and Colman[142] who treated 6-methyl-4,5-diaminopyrimidine (**148**) with nitrous acid and obtained a product which they designated as "4,5,6-methylazimidopyrimidine" (**149**); it appears, however, that a compound of this type was prepared even before that by Traube[143] who called it "azimid" (**150**).

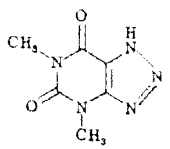
[141] R. O. Roblin, J. O. Lampen, J. P. English, Q. P. Cole, and J. R. Vaughan, Jr., *J. Am. Chem. Soc.* **67**, 290 (1945).

[142] S. Gabriel and J. Colman, *Ber. deut. chem. Ges.* **34**, 1234 (1901).

[143] W. Traube, *Ber. deut. chem. Ges.* **33**, 3035 (1900).

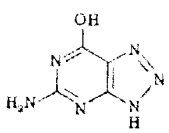


[148] [149]



[150]

This synthetic procedure was used without any significant changes for the preparation of the greatest number of derivatives of *v*-triazolo[4,5-*d*]pyrimidine. Robins *et al.*[141] prepared the aza analogs of the principal purine bases: 8-azaguanine (**151**), 8-azaadenine (**152**), 8-azaxanthine (**153**), and 8-azahypoxanthine (**154**). By similar methods,



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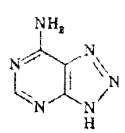

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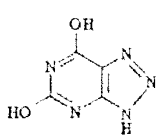

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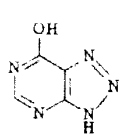
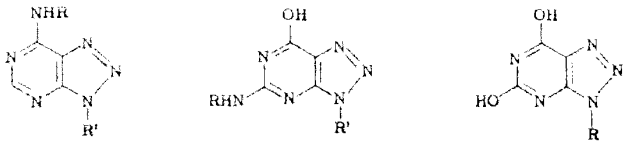


[154]

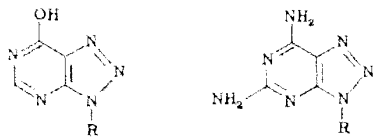
labeled 8-azaadenine[144] and 8-azaguanine were prepared.[144,145] A number of other derivatives can be grouped under the several structural types. There are 9-substituted or disubstituted derivatives of 8-azaadenine (**155**),[146-150] 9-substituted or disubstituted derivatives of 8-azaguanine (**156**)[151-155] and 9-substituted derivatives of 8-azaxanthine (**157**),[153] 8-azahypoxanthine (**158**),[149,155a] and 2,6-diamino-8-azapurine (**159**).[154,154] Further derivatives have been prepared in which one or both functional groups in positions 2 and 6 are modified[145,156-162] and a variety of mixed types.[148,151,163-167] A similar procedure was used for the preparation of the glycityl derivatives of 8-azaguanine in which the D-ribityl or D-sorbityl residue was unequivocally bound in position 3 of the triazolopyrimidine ring (i.e., analogous to position 9 of the natural purine nucleosides) (**156**; R = H, R′ = ribityl or sorbityl).[168,169]

[141] E. L. Bennett, *J. Am. Chem. Soc.* **74**, 2420 (1952).
[145] H. G. Mandel, E. L. Alpen, W. D. Winters, and P. K. Smith, *J. Biol. Chem.* **193**, 63 (1951).

[146] J. H. Lister and G. M. Timmis, *J. Chem. Soc.* p. 327 (1960).
[147] R. Hull, *J. Chem. Soc.* p. 2746 (1958).
[148] R. Weiss, R. K. Robins, and C. W. Noell, *J. Org. Chem.* **25**, 765 (1960).
[149] C. L. Leese and G. M. Timmis, *J. Chem. Soc.* p. 4107 (1958).
[150] L. Almirante, *Ann. chim. (Rome)* **49**, 333 (1959); *Chem. Abstr.* **53**, 20078 (1959).
[151] S. Yamada, I. Chibata, and D. Kiguchi, *Tanabe Seiyaku Kenkyū Nempō* **2**, 13 (1957); *Chem. Abstr.* **52**, 1177 (1958).
[152] M. J. Fahrenbach, K. H. Collins, M. E. Hultquist, and J. M. Smith, Jr., *J. Am. Chem. Soc.* **76**, 4006 (1954).
[153] H. C. Koppel, D. E. O'Brien, and R. K. Robins, *J. Am. Chem. Soc.* **81**, 3046 (1959).
[154] G. M. Timmis, D. G. I. Felton, H. O. J. Collier, and P. L. Huskinson, *J. Pharm. and Pharmacol.* **9**, 46 (1957); *Chem. Abstr.* **51**, 10531 (1957).
[155] S. Yamada and I. Chibata, Jap. patent 6338 (1958); *Chem. Abstr.* **54**, 2375 (1960).
[155a] C. Temple, R. L. McKee, and J. A. Montgomery, *J. Org. Chem.* **27**, 1671 (1962).
[156] Cilag Ltd., British patent 674,594 (1952); German patent 836,802 (1952); *Chem. Abstr.* **47**, 7553 (1953).
[157] D. S. Acker and J. E. Castle, *J. Org. Chem.* **23**, 2010 (1958).
[158] F. F. King and T. J. King, *J. Chem. Soc.* p. 943 (1947).
[159] P. Bitterli and H. Erlenmeyer, *Helv. Chim. Acta* **34**, 835 (1951).
[160] F. L. Rose, *J. Chem. Soc.* p. 3648 (1952).
[161] C. T. Bahner and D. E. Bilancio, *J. Am. Chem. Soc.* **75**, 6038 (1953).
[162] K. L. Dille and B. E. Christensen, *J. Am. Chem. Soc.* **76**, 5087 (1954).
[163] R. Hull, *J. Chem. Soc.* p. 481 (1959).
[164] K. L. Dille, M. L. Sutherland, and B. E. Christensen, *J. Org. Chem.* **20**, 171 (1955).
[165] L. F. Cavalieri, A. Bendich, J. F. Tinker, and G. B. Brown, *J. Am. Chem. Soc.* **70**, 3875 (1948).
[166] J. H. Lister, *J. Chem. Soc.* p. 3394 (1960).
[167] F. L. Rose, *J. Chem. Soc.* p. 4116 (1954).
[168] J. Davoll and D. D. Evans, *J. Chem. Soc.* p. 5041 (1960).
[169] D. L. Ross, C. G. Skinner, and W. Shive, *J. Org. Chem.* **26**, 3582 (1961).

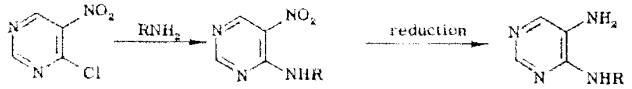


[155] [156] [157]



[158] [159]

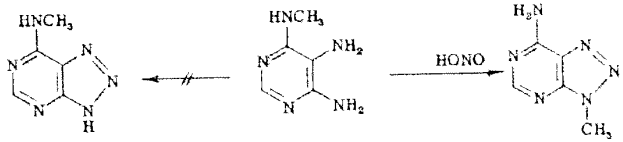
The general applicability of this synthesis follows from the great number of compounds prepared. The preparation of derivatives substituted in the pyrimidine ring and in position 3 of the triazolopyrimidine ring is principally determined by the possibility of preparing the suitably substituted derivatives of 4,5-diaminopyrimidines which represent the common starting substances. Through these starting compounds the preparation of 8-azapurines is closely related to the preparation of analogous purines which can also be prepared from 4,5-diaminopyrimidines by the action of formic acid or its derivatives. The amino group can be introduced into position 4 of the pyrimidine ring by substitution of the analogous 4-chloro derivatives[103–105] (**160**). In this manner *N*-substituted 4-amino derivatives (**162**) afford unequivocally 3-substituted triazolopyrimidines on reacting with nitrous acid. The 1-substituted derivatives would be accessible only with



[160] [161] [162]

difficulty by this method. The amino group in position 5 of the starting 4,5-diaminopyrimidines is usually prepared by the reduction of the readily available 5-nitro derivatives[156,162,164,165] (**161**). It follows

from the nature of the cyclization by nitrous acid that it is impossible to prepare 2-substituted and 1,3-disubstituted derivatives. It is known that the secondary amino group enters the cyclization more readily than does the primary one.[149] This is also borne out by the result of cyclization of 4,5-diamino-6-methylaminopyrimidine (**164**) which led to 3-methyl-7-amino-*v*-triazolo[4,5-*d*]pyrimidine (**165**) rather than to the 7-methylamino derivative[116] (**163**).



[163] [164] [165]

The mechanism of cyclization of diaminopyrimidines by nitrous acid appears not to have been studied in detail. For the preparative procedure an aqueous solution of alkaline nitrite is treated with the diaminopyrimidine either in the form of a salt or with simultaneous addition of hydrochloric or acetic acid. The first phase of the reaction is usually carried out at 0°C, in some cases the reaction being terminated by heating to 50–60°C. With diaminopyrimidines which are sparingly soluble in water, the reaction was carried out in an organic solvent using amylnitrite.[156,160] Excess nitrous acid can possibly attack the amino groups present. This was employed in some cases for the preparation of the hydroxy derivatives.[170,171]

Another synthesis of *v*-triazolo[4,5-*d*]pyrimidines described first by Benson *et al.*[172] and by Hartzel and Benson[173] also involves closing the triazole ring. It proceeds from a derivative of 4-aminopyrimidine (**166**), and making use of the aromatic character of the 5-position in the pyrimidine nucleus produces 4-amino-5-arylazo derivatives (**167**) by coupling with benzenediazonium chloride. These derivatives under-

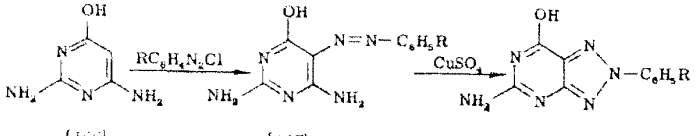
[169] F. Bergmann, G. Levin, and H. Kwietny, *Arch. Biochem. Biophys.* **80**, 318 (1959).

[170] R. P. Parker and J. S. Webb, U.S. Patent 2,543,333 (1951); *Chem. Abstr.* **45**, 7605 (1951).

[172] F. R. Benson, L. W. Hartzel, and W. L. Savell, *J. Am. Chem. Soc.* **72**, 1816 (1950).

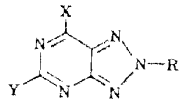
[173] L. W. Hartzel and F. R. Benson, *J. Am. Chem. Soc.* **76**, 2263 (1954)

go oxidative cyclization by cupric sulfate in pyridine to triazolopyrimidine derivatives (**168**). This synthesis is thus suitable for



[166] [167] [168]

derivatives with aromatic residues in position 2. As concerns substitution in the pyrimidine nucleus, the situation here is the same as for the preceeding synthesis. In this way it was possible to prepare a larger number of derivatives of type (**169**), where X and Y = H, alkyl, amino, hydroxy, mercapto, or alkylmercapto group and R = substituted phenyl or 3-pyridyl.[154,171-174]



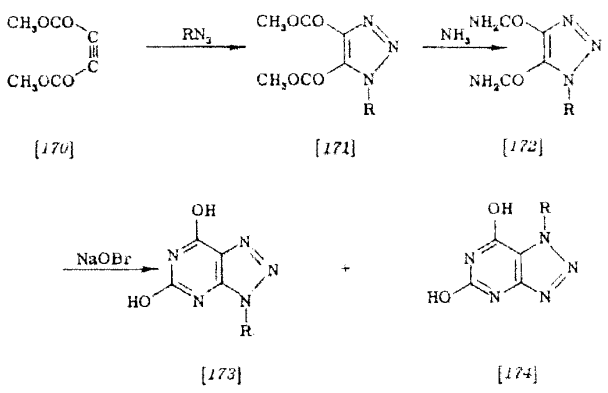
[169]

b. From Triazole Derivatives. Syntheses proceeding from triazole derivatives represent to a certain extent an analogy with syntheses of the purine derivatives. Their variability is considerably lower than for the preceding syntheses and their application has therefore been limited.

The first method was used by Baddiley *et al.*[175,176] for the synthesis of glycosyl derivatives of *v*-triazolo[4,5-*d*]-pyrimidines. Proceeding from acetylenedicarboxylic acid ester (**170**) and a glycosyl azide (R = tetra-*O*-acetyl-β-D-glucopyranosyl, 2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl, or 2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl), they prepared the diester (**171**) and further the diamide of substituted triazoledicarboxylic acid (**172**). Using a procedure developed for analogous

[154] I. Ježo and Z. Votický, *Chem. zvesti* 6, 357 (1952); *Chem. Abstr.* **48**, 7019 (1954).
[175] J. Baddiley, J. G. Buchanan, and G. O. Osborne, *J. Chem. Soc.* p. 1651 (1958).
[176] J. Baddiley, J. G. Buchanan, and G. O. Osborne, *J. Chem. Soc.* p. 3606 (1958).

purine derivatives,[177] a Hoffmann reaction (action of alkaline hypobromite) gave a mixture of the corresponding esters of 1- and 3-glycosyl-5,7-dihydro-*v*-triazolo[4,5-*d*]-pyrimidines (**173**, **174**).



[170] [171] [172]

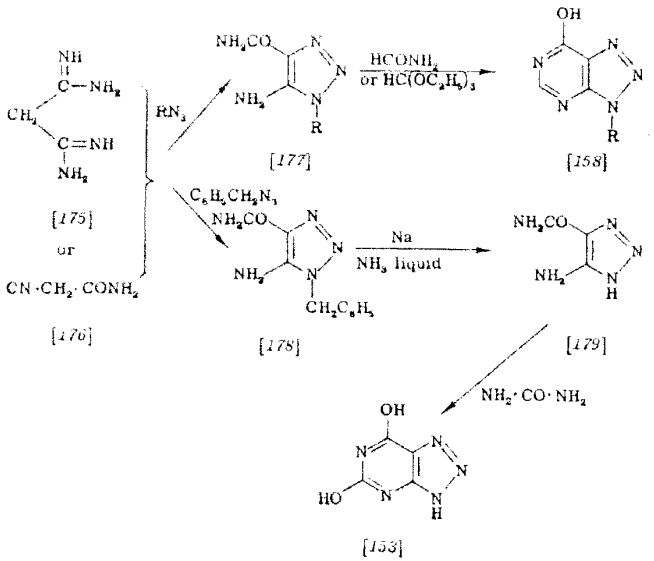
[173] [174]

This synthesis is thus suitable preparing 5,7-dihydroxy derivatives (substituted 8-azaxanthines). However, with regard to the 1- or 3-substituted derivatives its course is not unequivocal.

The second procedure of this type was first described by Yamada *et al.*[178] They used the diamidine of malonic acid (**175**) or cyanacetamide (**176**) to prepare 1-benzyl-5-amino-*v*-triazole-4-carboxamide (**178**) by treatment with benzyl azide. After removing the benzyl group with sodium in liquid ammonia they converted the product (**179**) by heating with urea to 5,7-dihydroxy-*v*-triazolo[4,5-*d*]pyrimidine (8-azaxanthine) (**153**).

A similar procedure was developed in greater detail by Dornow and Helberg.[179] They proceeded from aryl or aralkyl azid and cyanacetamide (**176**) and the 1-aryl- or 1-aralkyl-5-amino-*v*-triazole-4-carboxamide (**177**) formed was then cyclized with formamide or ethyl orthoformate. In this way they prepared 3-substituted 7-

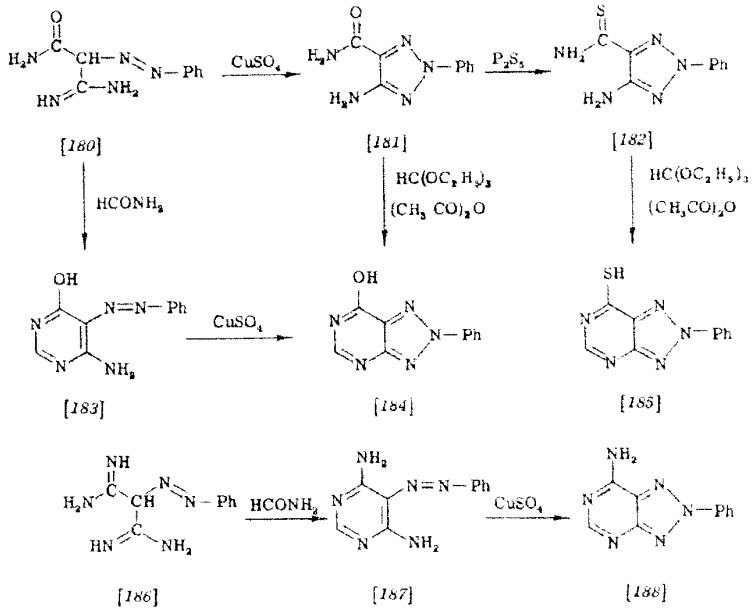
[177] R. A. Baxter and F. S. Spring, *J. Chem. Soc.* p. 378 (1947).
[178] S. Yamada, T. Mizoguchi, and A. Ayata, *Yakugaku Zasshi* **77**, 455 (1957); *Chem. Abstr.* **51**, 14698 (1957).
[179] A. Dornow and J. Helberg, *Chem. Ber.* **93**, 2001 (1960).



hydroxy-*v*-triazolo[4,5-*d*]-pyrimidines (9-substituted 8-azahypoxanthines) (**158**) in which R = phenyl, *p*-chlorophenyl, *m*-chlorophenyl, *m*-nitrophenyl, and benzyl. These examples also point to the possibility of application of this synthesis.

A combination of the preceding type of synthesis and of cyclization of 4-amino-5-arylazopyrimidine can be seen in the novel procedure of Richter and Taylor.[180] Proceeding from phenylazomalonamide-amidine hydrochloride (**180**), they actually close both rings in this synthesis. The pyrimidine ring (**183**) is closed by formamide, the triazole (**181**) one by oxidative cyclization in the presence of cupric sulfate. Both possible sequences of cyclization were used. The synthetic possibilities of this procedure follow from the combination of the two parts. The synthesis was used for 7-substituted 2-phenyl-1,2,3-triazolo[4,5-*d*]-pyrimidines (**184**, **185**). An analogous procedure was employed to prepare the 7-amino derivatives (**188**) from phenylazomalondiamidine (**186**).

[180] E. Richter and E. C. Taylor, *J. Am. Chem. Soc.* **78**, 5848 (1956).



c. By Substitution. The last method of preparation of triazolopyrimidines is the exchange of substituents or direct substitution in derivatives prepared by one of the previously described paths. During these reactions on the pyrimidine ring of the molecule, a similar course can be expected as in the corresponding pyrimidine and purine derivatives. The differences in reactivity do not appear to have been studied in detail but they can be seen from the abundant material available, i.e., derivatives prepared by substitution of the amino group of 8-azaguanine,[181-183] in some cases, ensued for preparation of the soluble derivatives.[181] Further mention should be made of the replacement

[181] K. Abe, S. Onishi, Y. Koriki, and K. Matsui, Jap. patent 9984, (1958); *Chem. Abstr.* **54**, 5713 (1960).

[182] K. Abe, S. Onishi, Y. Kunugi, and K. Matsui, *Tanabe Seiyaku Kenkyū Nempō* **2**(2), 11 (1957).

[183] Ciba Ltd., Swiss patent 279,152 (1952); *Chem. Abstr.* **47**, 8097 (1953).

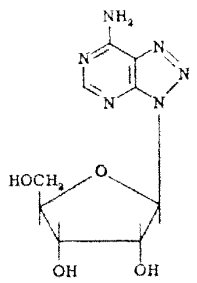
of the hydroxyl group by mercapto through the action of phosphorus pentasulfide,[184-187] conversion of the mercapto group[170] to hydroxy or amino[148] and the already mentioned replacement of amino by a hydroxy group through treatment with nitrous acid.[170,171] More attention has been devoted to the preparation of derivatives by nucleophilic substitution of the chlorine atom in position 7.[188] Preparative modification of all these reactions is analogous to the corresponding reactions in the purine and pyrimidine series.

More interesting are the substitution reactions on the triazole ring where a characteristically different course can be expected from that of analogous reactions in the purine series. These reactions were studied in more detail only in connection with the preparation of the glycosyl derivatives, and the experimental material does not permit the drawing of general conclusions.

The process of glycosidization of mercuric or chloromercuric salts used with purine bases was applied by Davoll[189] to their 8-aza analogs. He obtained two isomeric glucosyl derivatives from the 7-acetamino derivative, one of the products being identical with unequivocally prepared 3-glucosyl derivative (through cyclization with nitrous acid). An analogous reaction yielded only one ribosyl derivative to which the structure of the 3-ribofuranosyl derivative (8-azaadenosine) (**189**) was ascribed on the basis of UV spectra similarity. 5,7-Diamino-*v*-triazolo[4,5-*d*]pyrimidine yielded a ribosyl derivative which possessed a different UV spectrum from that of the 3-substituted derivatives. The 7-amino-5-mercaptomethyl derivative yielded a mixture of two ribosyl derivatives, one of which was converted in two stages to a riboside identical with 8-azaguanosine (**190**) obtained enzymatically.

Andrews and Barber[190] described the reaction of tri-*O*-benzoyl ribofuranosyl chloride with the chloromercuric salt of 7-dimethylamino-5-

[184] C. T. Bahner, D. E. Bilancio, E. B. Senter, S. Humphries, R. Nations, W. Porch, and J. Wilson, *J. Org. Chem.* **22**, 358 (1957).
[185] Wellcome Found, Ltd., British patent 765,590 (1957); *Chem. Abstr.* **51**, 12157 (1957).
[186] C. T. Bahner, D. E. Bilancio, and E. M. Brown, *J. Am. Chem. Soc.* **76**, 1370 (1954).
[187] C. T. Bahner, B. Stump, and M. E. Brown, *J. Am. Chem. Soc.* **75**, 6301 (1953).
[188] Y. F. Shealy, R. P. Struck, J. D. Clayton, and J. A. Montgomery, *J. Org. Chem.* **26**, 4433 (1961).
[189] J. Davoll, *J. Chem. Soc.* p. 1593 (1958).
[190] K. J. M. Andrews and W. E. Barber, *J. Chem. Soc.* p. 2768 (1958).



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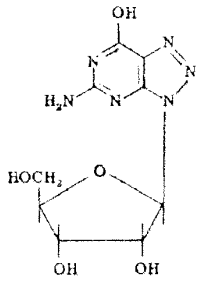

[190]

methylmercapto-*v*-triazolo[4,5,*d*]-pyrimidine. The position of the ribofuranosyl group cannot be considered as established, however.

Angier and Marsico[191] followed the course of alkylation first. The 7-dimethylamino-5-methylmercapto derivative reacted with dimethyl sulfate in an alkaline medium to yield a mixture of the 2- and 3-methyl derivatives. The reaction of the 7-dimethylamino derivative with ethyl iodide in an alkaline medium led to a mixture of all three possible monoethyl derivatives. The position of the alkyl group in all these substances was defined by comparing the UV spectra with derivatives prepared by a straightforward synthesis. After reacting the mercuric salts with tri-*O*-benzoylribofuranosyl chloride, they demonstrated the ribose residue to be bound in position 2. The same structure was shown to be valid for the derivative prepared by Andrews and Barber.[190]

It can be concluded on the basis of the present material that the course of substitution is affected both by substituents in the nucleus and by the character of the substituent to be introduced. No general rules can be formulated at present, however.

Similarly, the position of the acyl group in derivatives formed by the reaction with acetic hydride or benzoyl chloride[192] and the position of the carboxymethyl group in the derivative formed by the reaction with chloracetic acid are not established.[193]

[191] R. B. Angier and J. W. Marsico, *J. Org. Chem.* **25**, 759 (1960).
[192] F. F. Blicke and H. C. Godt, Jr., *J. Am. Chem. Soc.* **76**, 2798 (1954).
[193] N. Sugimoto and S. Imado, Jap. patent 1372 (1958); *Chem. Abstr.* **53**, 1389 (1959).

To the derivatives prepared on substitution belong also the nucleotides of 8-azaguanosine (190). From the 5′-phosphate, prepared enzymatically, the corresponding diphosphate and triphosphate were synthesized by the action of *N,N′*-dicyclohexylcarbodiimide and phosphoric acid.[194] From 2′,3′-*O*-isopropylidene-8-azaguanosine and 2-cyanoethyl dihydrogen phosphate, the 8-azaguanosine-5′-phosphate (8-azaguanylic acid)[195] resulted which was identical with the product obtained enzymatically.[190] The 2′,3′-phosphate and 2,3′-cyclic phosphate were prepared from the 5′-trityl derivative of 8-azaguanosine.[196a]

For the preparation of some of the triazolopyrimidine derivatives enzymatic reactions were also used. Thus, for example, the preparation of deoxyribofuranosyl-[197] and ribofuranosyl-8-azaguanine (8-azaguanosine)[197,198] was described. On the basis of the assumed specific course of the enzyme reactions these substances are considered to have the structure of 3-glycosyl derivatives (corresponding to position 9 of natural purine nucleosides). No chemical or physicochemical proof of the structure has been presented so far. Enzymatic deamidation of 8-azaguanine to 8-azaxanthine was also described.[199-201] 8-Azaguanosine-5′-phosphate[190] and -triphosphate[194] were also prepared enzymatically.

3. *Properties*

Triazolopyrimidines and their derivatives are relatively stable toward alkaline and acid hydrolysis. However, the action of aqueous sodium hydroxide, ammonia, or hydrazine under pressure converts them to derivatives of 1,2,3-triazole.[202,203]

[191] J. L. Way, J. L. Dahl, and R. E. Parks, Jr., *J. Biol. Chem.* **234**, 1241 (1959).
[192] J. A. Montgomery and H. J. Thomas, *J. Org. Chem.* **26**, 1926 (1961).
[193] J. L. Way and R. E. Parks, Jr., *J. Biol. Chem.* **231**, 467 (1958).
[196a] H. J. Thomas, K. Hewson, and J. A. Montgomery, *J. Org. Chem.* **27**, 192 (1962).
[197] M. Fredkin, *J. Biol. Chem.* **209**, 295 (1954).
[198] J. Kára, J. Skoda, and F. Sorm, *Collection Czechoslov. Chem. Communs.*, **26**, 1380 (1961).
[199] A. Rousch and E. R. Norris, *Arch. Biochem.* **29**, 124 (1950).
[200] E. Hirschberg, J. Kream, and A. Gellhorn, *Cancer Research* **12**, 524 (1952).
[201] J. Kream and E. Chargaff, *J. Am. Chem. Soc.* **74**, 4274 (1952).
[202] J. S. Webb and A. S. Tomcufcik, U.S. patent 2,714,110 (1955); *Chem. Abstr.* **50**, 12118 (1956).
[203] S. Yamada, T. Mizoguchi, and A. Ayata: *Yukagaku Zasshi* **77**, 441 (1957); *Chem. Abstr.* **51**, 12107 (1957).

The UV spectra were measured for practically all the numerous derivatives. Beside the analytical application of these to demonstrate the position of the substituent[191] no detailed interpretation was attempted, however. On the whole, they are similar to the spectra of analogous purine derivatives and also display a similar dependence on pH.[141,146,165] Despite the fact that the question of structure with regard to the lactim–lactam (or thiolactim–thiolactam) tautomerism has not been studied in detail, it can be assumed that oxygen and sulfur derivatives, at variance with the conventional way of writing the formulas, possess a lactam or thiolactam structure.[188] This is in agreement with the views on the analogous purine derivatives.

Among the other physicochemical studies mention should be made of the determination of the electron structure and energy of resonance[204,205] carried out for a number of the principal 8-aza analogs, and of the determination of the dissociation constants[206] and crystal structure.[207,208]

[204] A. Pullman, B. Pullman, and G. Berthier, *Compt. rend. acad. sci.* **243**, 380 (1956).
[205] B. Pullman and A. Pullman, *Bull. soc. chim. France* **7**, 973 (1958).
[206] Y. Hirata, I. Teshima, and T. Goto, *Nagoya Sangyō Kagaku Kenkyūjo Kenkyū Hōkoku No. 9*, p. 80 (1956); *Chem. Abstr.* **51**, 8516 (1957).
[207] W. Nowacki and H. Bürki, *Experientia* **7**, 454 (1951).
[208] W. Nowacki and H. Bürki, *Z. Elektrochem.* **56**, 788 (1952).

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Microbiological Production of 5-Azacytidine
I. Production and Biological Activity

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The antibiotic 5-azacytidine was produced by fermentation of *Streptoverticillium ladakanus*. It was inhibitory to some gram-negative bacteria, especially when they were grown in completely synthetic media. A microbiological assay with *Escherichia coli* was developed that can detect 5-azacytidine at a minimal level of 2 μg/ml. The inhibition by 5-azacytidine of *E. coli* ATCC 26 was reversed by several pyrimidines. The most effective reversing agent was cytidine, followed closely by uridine. 5-Azacytidine was ineffective against experimental *E. coli* infection in mice. It was inhibitory to KB cells growing in liquid medium with an ID_{50} of 0.2 μg/ml. It was active against T-4 lymphoma and L-1210 leukemia in mice.

An antibiotic (U-18,496), isolated in our laboratories, was recently identified as 5-azacytidine. The chemical synthesis of 5-azacytidine was described by Piskala and Sorm (9). Several papers dealing with its metabolism, antitumor effects, and mechanism of action have been published (3, 12, 13, 14). This paper describes the producing microorganism, fermentation conditions, microbiological assay, and biological studies. Isolation and characterization of 5-azacytidine produced by fermentation were described by Bergy and Herr (2).

Materials and Methods

Taxonomy. 5-Azacytidine is produced by a soil isolate identified as a *Streptoverticillium* species (1). Taxonomic studies were based on Ektachrome comparisons (4) as modified by Dietz and Mathews (5), sporophore type (10) electron micrographs of spores (5), reference color according to Jacobson et al. (7) and Kelly and Judd (8), and cultural characteristics.

Production. Stock cultures were maintained as spore preparations in sterile soil. The seed medium consisted of 25 g of Cerelose (Corn Product Sales Co., Detroit, Mich.) and 25 g of Pharmamedia (Traders Oil Mill Co., Fort Worth, Tex.) per liter of water. This medium was inoculated with spores from a stock culture and incubated on a rotary shaker for 48 hr at 28 C. The production medium for 5-azacytidine contained 40 g/liter of black strap molasses (Knappen Milling Co., Augusta, Mich.), 30 g of Pharmamedia, 10 g of dried whole yeast, and 20 g of dextrin. The fermentation was done in 500-ml nonstippled flasks containing 100 ml of medium on a rotary shaker at 250 rev/min (2.5-inch stroke). The optimal temperature for the fermentation was 28 C, and the peak titers were reached after 70 to 90 hr of incubation.

In vitro studies. Antibacterial studies were run at 37 C with either Nutrient Broth or a completely synthetic medium. The synthetic medium used for *Escherichia coli* consisted of $Na_2HPO_4 \cdot 4H_2O$, 2.2 g; KH_2PO_4, 1 g; $MgSO_4$, 0.1 g; $(NH_4)_2SO_4$, 1 g; glucose, 2 g; and distilled water to make 1 liter. The synthetic medium used for *Proteus vulgaris* ATCC 8427 was the medium for *E. coli* supplemented with 1% (v/v) of Eagles 100X vitamin mix (6). The synthetic medium used for *Salmonella gallinarum* USDA 8410 was that used for *Proteus* and further supplemented with 1 ml of Casamino Acids (Difco) per 100 ml of broth. The synthetic medium used for *Pseudomonas mildenbergii* ATCC 795 consisted of the base used for *E. coli* further supplemented with 50 μg of L-asparagine per ml. The activity of 5-azacytidine against growing KB cells was tested by use of the monolayer method described by Smith et al. (11).

Microbiological assay. A standard curve

assay with *E. coli* was developed for measuring 5-azacytidine. The assay medium consisted of the previously described synthetic broth supplemented with 1.5% agar. The seed consisted of 0.3 ml of culture (16 to 18 hr) per 100 ml of agar. The standard solutions were prepared by dissolving the crystalline 5-azacytidine in 0.1 M phosphate buffer (*p*H 6.0) at concentrations of 25, 12.5, 6.25, and 3.12 μg/ml. Each solution was applied to one 12.7-mm diameter paper disc (Carl Schleicher & Schuell Co., Keene, N.H.) on each of four replicate plates. The plates were incubated overnight at 30 C, and the zones of inhibition were measured. The potencies of the tested samples were estimated from a standard curve plotted as the logarithm of dose versus zone diameter.

Reversal studies. The low order of activity of 5-azacytidine when tested in nutrient broth suggested that its activity may be reversed by some of the components of this medium. Thus, some of the common pyrimidines and purines were tested for their ability to reverse the inhibition by 5-azacytidine of *E. coli* grown in a synthetic medium. All potential reversing agents were incorporated in the synthetic broth, and growth was measured turbidimetrically after 11 hr at 37 C. The stronger the reversing capacity of a compound was, the more growth was present. In a similar way the ability of several pyrimidines to reverse the inhibition by 5-azacytidine of KB cells was tested.

In vivo studies. The antibacterial properties of 5-azacytidine in vivo were studied in CFI mice against experimental infection by *E. coli* UC 311. Partially purified preparations of 5-azacytidine were administered subcutaneously for 4 days up to the maximal tolerated level equivalent to 24 mg of pure drug per kg. Animals were observed for 7 days after infection, and the number of surviving mice was recorded.

The antitumor activity of 5-azacytidine was studied in vivo with the T-4 lymphoma and two strains of leukemia L-1210. The T-4 lymphoma was implanted subcutaneously, as a tumor homogenate, into A-Heston mice received from Cumberland Farms. Groups of 10 mice were used for each treatment. The drug was administered intraperitoneally daily for 7 consecutive days, starting 5 days after implanting the tumor. The tumors were measured in two dimensions and are reported as the average of the two measurements. Leukemia L-1210 and L-1210/C95 were implanted subcutaneously in the groin, and the drug was given intraperitoneally. Treatment was started 24 hr after implanting the leukemic cells and was continued for 7 days. The efficacy of treatment was shown by a prolongation of survival time.

Results

Taxonomy. The organism that produces 5-azacytidine appears to be different from described species in the literature (15). It may be placed in the biverticillate group of Pridham (10). It is proposed that the culture be designated *Streptoverticillium ladakanus* var. *ladakanus* sp. n. Electron micrographs of spores are shown in Fig. 1. Growth characteristics and reference color characteristics are shown in Tables 1 and 2. The culture has biverticillate sporophores. It is incorporated in the Upjohn culture collection as UC 2654.

Production. The fermentation was carried out under the conditions described, and antibiotic titers were estimated by microbiological assay with *E. coli*. The results of a typical fermentation run are presented in Table 3. The isolation and characterization of 5-azacytidine was described by Bergy and Herr (2).

In vitro studies. The in vitro activity of 5-azacytidine was limited to gram-negative bacteria (Table 4). The minimal concentration which completely inhibited the growth of *E. coli* in synthetic broth for 24 hr was 0.01 μg/ml. A concentration of the drug about 5,000 times greater was needed for equivalent inhibition when *E. coli* was grown in Nutrient Broth. Inhibition of *E. coli* was bacteriostatic up to the highest concentration tested (250 μg/ml). Cells grown in the presence of the drug appeared morphologically identical with controls.

5-Azacytidine was moderately cytotoxic when tested against KB cells grown in liquid medium. The concentration required for 50% growth inhibition was 0.23 μg/ml. Inhibition of the growth of KB cells by 5-azacytidine can be reversed by either cytidine or uridine.

Microbiological assay. 5-Azacytidine can be assayed by a disc-plate assay against *E. coli* ATCC 26, and a typical dose-response line is presented in Fig. 2. The linear part of the dose-response curve was between concentrations of 3.12 and 25 μg/ml. The lowest detectable concentration was about 2 μg/ml.

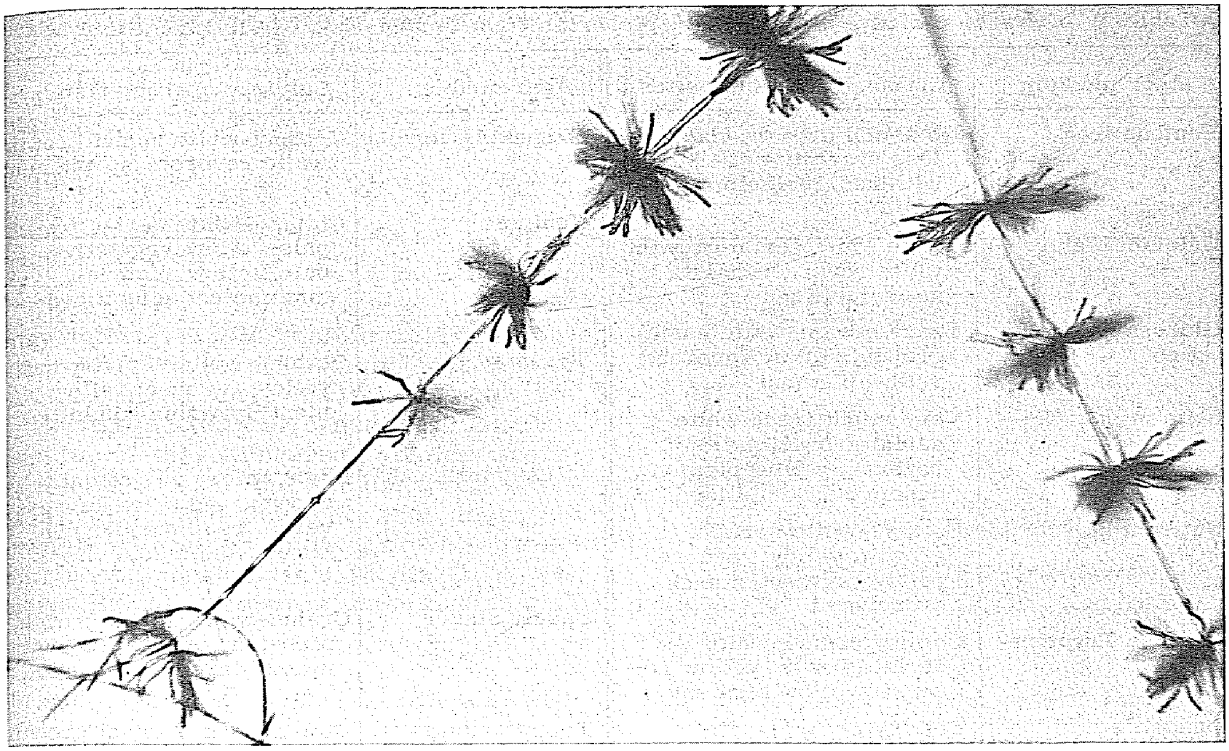

Fig. 1. *Photomicrograph of spores of Streptoverticillium ladakanus var. ladakanus.*

Reversal studies. The in vitro reversal studies showed that cytidine and uridine are the most effective compounds in this regard (Table 5).

In vivo studies. Partially purified preparations of 5-azacytidine were inactive in vivo against an experimental *E. coli* infection in mice at the maximal tolerated dose of 25 mg/kg administered subcutaneously.

The antibiotic was tested in vivo in mice by use of three tumor systems that respond to agents which affect nucleic acid metabolism. Table 6 shows the effect of a crude preparation on the T-4 lymphoma. The test is especially significant because treatment was delayed for 5 days for the tumor to become well established, and the final measurement was taken 3 days after drug treatment was discontinued. The drug was especially effective at the two highest doses. The crystalline material was tested against leukemia L-1210 and against a strain of L-1210 which was resistant to 6-mercaptopurine, Amethopterin, and partially resistant to cyclophosphamide (Table 7). The antibiotic was effective against both lines of L-1210. The most effective dose of 5-azacytidine against the leukemia is near the chronic toxic dose of the drug. The optimal dosage regimen for the drug has not been determined.

Discussion

We have shown that a new species of *Streptoverticillium* is capable of producing an antibiotic in a controlled fermentation that is identical (2) to 5-azacytidine which was synthesized by Piskala and Sorm (9). In development of the assay and the antibacterial studies, in complex nutrient media little or no antimicrobial activity could be demonstrated. Further investigation showed that cytidine and uridine would reverse the microbiological activity of 5-azacytidine. These studies suggest that 5-azacytidine

Table 1. *Cultural characteristics of Streptoverticillium ladakanus v. ladakanus UC 2654*[a]

Agar medium	Cultural characteristics	Agar medium	Cultural characteristics
Peptone Iron	No aerial growth, colorless vegetative flecked with red, melanin negative	Czapek's sucrose	Cottony-white aerial growth, white reverse, no pigment
Calcium Malate	Trace white aerial growth, white reverse, malate not solubilized	Xanthine	Cottony-white aerial growth, pale-yellow vegetative, pale-yellow pigment, xanthine not solubilized
Glucose Asparagine	Trace cream-white aerial growth, yellow reverse, yellow pigment	Tyrosine	Cottony-white aerial growth, yellow reverse, yellow pigment, tyrosine solubilized
Skim milk	Very slight trace white aerial growth, yellow reverse, yellow pigment, casein not solubilized	Litmus milk	Slight trace gray aerial growth, red lavender reverse, red lavender pigment, casein solubilized
Nutrient starch	Cottony-white aerial growth, yellow reverse, yellow pigment, starch hydrolyzed	Casein starch	Cream-white aerial growth, pink-tan reverse, no pigment, starch hydrolyzed
Maltose Tryptone	Cottony cream-white aerial growth, yellow reverse, yellow pigment		
Bennett's	Cottony cream-white aerial growth, yellow reverse, yellow pigment	Tomato paste-oatmeal	Cottony-white aerial growth yellow reverse, yellow pigment

[a]Characterized after 14 days of incubation at 28 C.

Table 2. *Reference color characteristics of Streptoverticillium ladakanus var. ladakanus UC 2654*[a]

Agar medium	*Color Harmony Manual* (7)	ISCC-NBS (8)
Bennett's		
Surface	Gray b—oyster white	263m, white; 264g, light gray
Reverse	1ba—yellow tint 2fb—bamboo, buff, straw, wheat	92m, yellowish white; 121gm, pale yellow-green 87g, moderate yellow; 89m, pale yellow
Czapek's sucrose		
Surface	Gray b—oyster white	263m, white; 264g, light gray
Reverse	Gray b—oyster white	263m, white; 264g, light gray
Maltose-Tryptone		
Surface	Gray b—oyster white	263m, white; 264g, light gray
Reverse	1ba—yellow tint 2fb—bamboo, buff, straw, wheat	92m, yellowish white; 121 gm, pale yellow-green 87g, moderate yellow; 89m, pale yellow

[a]Cultures were incubated for 14 days at 28 C.

Table 3. *Production of 5-azacytidine and pH fermentation pattern*

Time (hr)	Antibiotic titer (μg/ml)	pH
48	240	6.5
72	475	6.4
96	920	6.5
120	205	6.5

may interfere with nucleic acid metabolism prior to steps involving cytidine or uridine, since these pyrimidines can reverse the activity of 5-azacytidine. The results of these reversal studies are in agreement with the findings of Sorm et al. (12), and suggest that there is enough cytidine or uridine, or both, present in complex nutrient media (like Nutrient Broth) to reverse the antimicrobial

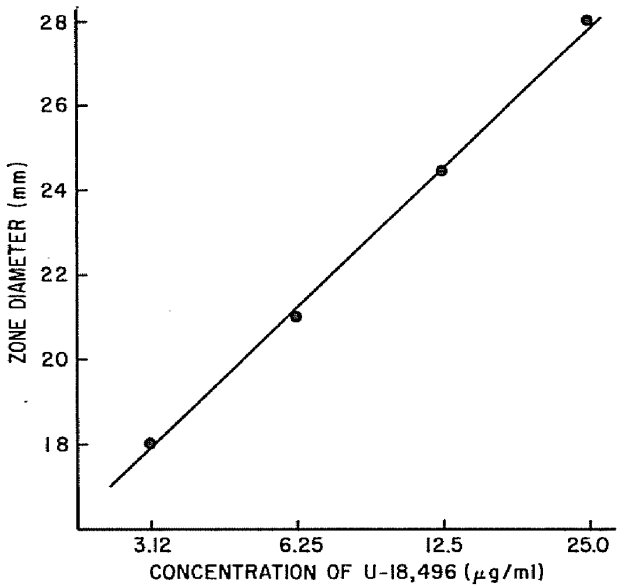

Fig. 2. *Microbiological assay for 5-azacytidine with Escherichia coli.*

Table 4. *Minimal inhibitory concentrations (MIC) of 5-azacytidine against several bacteria*

Microorganism	MIC (μg/ml) at 24 hr in	
	Synthetic Broth	Nutrient Broth
Escherichia coli ATCC 26	0.010	50
Salmonella gallinarum USDA 8410	0.08	200
S. schottmuelleri ATCC 9149	0.01	100
Pseudomonas mildenbergii ATCC 795	>200	>200
Proteus vulgaris ATCC 8427	0.005	200
Staphylococcus aureus FDA 209P		>200

Table 5. *Reversal of inhibition by 5-azacytidine (0.5 μg/ml) of Escherichia coli by several purines and purimidines*

Reversing compound[a]	Growth[b]
Orotidylic acid	0
Uridine	92
Cytidine	95
Thymidine	5
Adenosine	4
Guanosine	0
Inosine	0
Xanthosine	0
Control 1 (no reversing compound)	0
Control 2 (no 5-azacytidine)	100

[a]All compounds were at a concentration of 100 μM.
[b]Expressed in per cent of control 2.

Table 6. *Inhibition of T-4 lymphoma by crude preparation (~1% pure) of 5-azacytidine*

Daily dosage[a] (mg/kg)	Avg tumor size (mm) at day 14[b]
0	13.15
25	11.3
50	9.4
100	1.6[c]
200	None detectable

[a]A total of 10 mice were used per group. Mice were injected intraperitoneally daily for 7 days starting on the fifth day after implanting the tumor.
[b]Tumors were measured in two dimensions and averaged. Day 14 was 3 days after the last treatment.
[c]Two tumors in 10 animals.

Table 7. *Inhibition of two strains of leukemia in mice by crystalline 5-azacytidine*

Leukemia	Daily dosage (mg/kg)	Avg survival (days)	T/C ratio[a]
L-1210	0	10.1	1.00
	0.31	10.3	1.02
	0.63	12.0	1.19
	1.25	13.8	1.37
	2.50	16.3	1.61
	5.00	15.3	1.55
L-1210/C95 (triple resistant)	0	10.3	1.00
	0.31	11.2	1.09
	0.63	12.6	1.22
	1.25	14.2	1.38
	2.50	16.0	1.55
	5.00	13.9	1.35

[a]T/C ratio is the ratio of the average survival time of the treated group to that of the control group. A survival time greater than 1.25 is significant.

effect of the drug. The growth medium used for KB cells contained 10% blood serum (16), and thus it can be expected that small amounts of cytidine or uridine are present. This would explain the relatively low sensitivity of KB cells in our test system to 5-azacytidine. The efficacy of 5-azacytidine in treatment of experimental leukemia was also reported by Sorm et al. (12). It is significant that even the first, rather crude, preparations of 5-azacytidine from fermentation liquors have shown a strong activity against T-4 lymphoma in mice.

Since 5-azacytidine was effective in mice against a strain of L-1210 resistant to 6-mercaptopurine, Amethopterin, and cyclophosphamide, its mode of action is probably different from any of these three drugs.

Acknowledgments

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Pilot Study of 5-Azacytidine (5-AZA) and Carboplatin (CBDCA) in Patients With Relapsed/Refractory Leukemia

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5-azacytidine (5-AZA) and carboplatin (CBDCA) are two agents which have demonstrated antileukemic activity in a number of phase I–II trials. Their mechanisms of action and pharmacology related to cell resistance suggested suitability for combination therapy. The aim of this pilot study was to evaluate the effects of this combination in the treatment of patients with relapsed/refractory acute leukemia. A total of 21 patients was enrolled. 5-azacytidine, at doses ranging from 50–150 mg/m²/day, was administered as a 2-hr infusion for 5 consecutive days. On day 3, patients began a 5-day course of CBDCA given as a 24-hr continuous intravenous infusion of 250 mg/m²/day. There were no complete remissions with this regimen. Although there were three partial responses, these were generally of short duration. Nonhematologic toxicities were mild. No correlation was seen between response and serum platinum levels. These results demonstrate that the 5-AZA/CBDCA combination is ineffective therapy for heavily pretreated patients with acute leukemia.

Key words: 5-azacytidine, carboplatin, leukemia

INTRODUCTION

Despite initial success in achieving remission, most patients with acute leukemia relapse and eventually die from the sequelae of the disease. Furthermore, patients who are refractory to standard induction therapy rarely achieve any durable remission with additional chemotherapy alone. Therefore, efforts are continuously underway to identify new agents and combination regimens with improved activity in this disease. Along these lines, carboplatin (CBDCA) and 5-azacytidine (5-AZA) have been combined in a clinical trial for patients with relapsed/refractory acute leukemia based on the antileukemic activity and pharmacology of the individual drugs.

Platinum-containing compounds have gained wide application in the treatment of chemosensitive tumors. They have demonstrated significant therapeutic activity in a large number of metastatic solid tumors [1], and more recently have exhibited activity in hematologic malignancies such as leukemia and lymphoma. Several phase I–II trials using CBDCA as a single agent have reported response rates of 25–45% in patients with acute leukemia [2–5].

5-azacytidine, a pyrimidine analogue similar to cytosine arabinoside (Ara-C), has been used primarily for treatment of hematopoietic malignancies. As salvage therapy in acute leukemia its results have been comparable to other single agents [6–9], but it offers the advantage of a unique mechanism of action [6]. The maximal tolerated dose (MTD) defined by the phase I studies was 150 mg/m²/day for 5 days.

MATERIALS AND METHODS

Patients

Patients >15 years of age with refractory or relapsed acute leukemia and a Karnofsky performance status of >50% were eligible for this study. The study group included individuals with: 1) acute myelogenous leukemia (AML) in first or greater relapse or with primary refractory disease; 2) acute lymphocytic leukemia (ALL) in second or greater relapse or with primary refractory dis-

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ease; and 3) blast crisis of chronic myelogenous leukemia (CML) refractory to at least one course of standard induction chemotherapy. Morphologic confirmation of diagnosis at Memorial Sloan-Kettering was mandatory prior to enrolling in the study. Patients were required to have adequate hepatic (serum bilirubin <1.5 mg/dl) and renal function (creatinine clearance >60 ml/min), no serious hearing impairments, or uncontrolled infections at time of treatment. All patients gave written informed consent prior to participating in the trial, which was approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center.

Treatment Regimens

5-azacytidine was administered days 1–5 as a 2-hr intravenous (IV) infusion at doses of 50–150 mg/m²/day. Dosing was initiated at less than the MTD defined by phase I trials because of concern about possible additive gastrointestinal toxicity. At least 3 patients were treated at each dose level, and doses were escalated by 25 mg/m²/day as tolerated. CBDCA was given at a dose of 250 mg/m²/day as a continuous IV infusion, beginning on day 3 and continuing for a total of 5 days. The dose of the CBDCA infusion was based on the results and recommendations of Meyers et al. [3]. The intention of the study was to escalate the dose of 5-azacytidine to its MTD, defined as the dose level before grade 3 or greater toxicity in 2 or more patients was seen. Alternatively, we would escalate the 5-azacytidine to reach its single-agent MTD of 150 mg/m². The delayed kinetics of these agents in lowering peripheral blood counts prompted the use of hydroxyurea (HU) when necessary, to control rapidly rising peripheral blast counts or to reduce total white blood cell counts >30,000/mm³. HU was discontinued at least 24 hr prior to initiating the protocol chemotherapy.

In order to avoid renal toxicity, vigorous IV hydration was used to maintain a urine output of greater than 3 liters/day, and febrile episodes (>100.5°C) were initially treated with ticarcillin/clavulanic acid and aztreonam. Aminoglycosides and amphotericin B were added if the patient became unstable or if fever persisted longer than 48 hr. Ondanestron and decadron were used prophylactically as antiemetics. Decadron was omitted for patients with ALL. Audiograms were obtained at baseline and at completion of the study.

Bone marrow aspirations and biopsies were performed at baseline prior to therapy, 14 days after the start of the treatment, and then at 1–2-week intervals until response was documented. Responses were assessed according to the criteria established by the NCI Workshop [10]. Complete remission was defined as disappearance of all clinical evidence of leukemia for a minimum of 4 weeks with a neutrophil count >1,500 mm³, platelets >100,000 mm³, no circulating blasts and a normal marrow differential with maturation in all cell lines, no Auer rods, and <5% blasts. Partial remission was the same as complete remission, except for the presence of 5–25% bone marrow blasts. Failures included patients with resistant leukemia in their bone marrow (>25%) and circulating peripheral blood blasts, and patients who died of complications of aplasia. Clinical evaluation of patients was made daily. Serum chemistries and complete blood counts were performed at least three times a week. Specific organ toxicity was graded according to the DCT/NCI grading system. CBDCA levels were sampled on days 6–7 (96–120 hr into the CBDCA infusion) and assayed using the technique described by Menendez-Botet and Schwartz [11].

TABLE I. Patient Characteristics

Characteristic	Number
Patients enrolled	21
Evaluated for toxicity	20
Evaluated for response	20
Male/female	10/10
Age in years: median (range)	38.5 (21–69)
Karnofsky performance status: median (range)	80% (60–10%)
Type of leukemia	
De novo AML	14
ALL	2
MDS → AML	4
CML → blast crisis	1
Status of disease	
Refractory	8
First relapse	9
Second relapse	3
Prior treatment	
1 course	4
2 courses	8
3 courses	8

RESULTS

Patient Characteristics

Twenty-one patients, whose characteristics are summarized in Table I, were enrolled in the study. The first patient died from a cerebral hemorrhage on the fourth day of chemotherapy, leaving 20 patients evaluble for response. Six of the 20 study patients required treatment with HU for control of a rapidly rising peripheral blast count prior to beginning protocol therapy. All patients enrolled in this study were heavily pretreated: 76% of patients had received ≥two prior therapies and therefore had a poor prognosis for response to other standard therapies. Furthermore, several patients possessed additional unfavorable prognostic characteristics: 2 patients had relapsed after autologous bone marrow transplantation for AML, 1 patient with ALL had previously been treated with radiation and chemotherapy for lymphoma, 1 patient with AML had transformed from a secondary myelodysplastic syndrome, and 1 patient with Ph+ ALL had a number of additional cytogenetic abnormalities. Both ALL patients had relapsed while receiving chemotherapy.

TABLE II. Nonhematologic Toxicity

	Grade			
	1	2	3	4
Stomatitis	4	0	0	0
Nausea/vomiting	1	5	0	0
Diarrhea[a]	0	3	0	1
Neurootoxicity	0	3	0	0
Renal	1	0	0	0
Metabolic[b]	0	0	1	0

[a]Fatal necrotizing hemorrhagic enterocolitis.
[b]Reversible salt-losing nephropathy.

Of the patients treated in first relapse, only one remained disease free >9 months after completing consolidation.

Response

None of the patients treated in this study achieved complete remission (CR). There were 3 patients (treated with 50, 75, and 150 mg/m^2/day of 5-AZA) who obtained a partial remission (PR), making the overall response rate 15%. The median duration of PR was 42 days (range, 35–46 days). One patient who achieved PR on day 59 had normal peripheral blood counts as well as a blast count of <5% in the bone marrow aspirate, but an occasional myeloblast contained an Auer rod. A second patient had only 8% blasts in the bone marrow aspirate on day 33 but subsequently developed isolated leukemia cutis without an increase in marrow myeloblasts on day 68. The time to peripheral count recovery for the 3 patients with PR was long: 53, 28, and 37 days, respectively, for an absolute neutrophil count (ANC) >1,000/mm^3, and 57, 32, and 54 days, respectively, to achieve platelet counts >100,000/mm^3. Fifteen patients had aplastic/hypoplastic bone marrows as a result of therapy, while 5 others manifested primary drug resistance by remaining normocellular/hypercellular.

Toxicity

Therapy with the 5-AZA/CBDCA combination was generally well-tolerated (Table II). The most common extramedullary toxicities were stomatitis (4 patients, grade 1) and gastrointestinal symptoms including nausea/vomiting (1 patient, grade 1; 5 patients, grade 2) and diarrhea (3 patients, grade 2; 1 patient, grade 4). One patient who received 50 mg/m^2/day of 5-AZA, however, developed fever, abdominal pain, and grade 4 diarrhea on day 18. He subsequently expired and was found to have necrotizing and hemorrhagic colitis on autopsy, which was thought to be secondary to the chemotherapy and/or a neutropenic enteropathy.

Three patients experienced transient tinnitus of approximately 1 week duration at the completion of CBDCA. Ten patients underwent the prescribed audiograms. Two patients were symptomatic, having complained of hearing deficits. Both of these patients were found to have significant hearing loss. Two other patients were found to have insignificant high-frequency loss. The 6 remaining patients who were tested, demonstrated no change.

Renal toxicity was mild. Only 1 patient, treated with 150 mg/m^2/day of 5-AZA, developed a salt-wasting nephropathy associated with polyuria and a transient mild increase in creatinine (0.9–1.5 mg/dl). As a consequence of this, however, the patient also developed grade 3 metabolic toxicity comprised of hyponatremia, hypokalemia, hypocalcemia, and hypomagnesemia. These abnormalities resolved with fluid and electrolyte repletion within 14 days. Alopecia was difficult to assess for the entire population because of its presence at baseline in many patients.

The 5-AZA/CBDCA combination did induce a significant degree of myelosuppression. All patients were pancytopenic following therapy and required parenteral antibiotics for neutropenic fever. The time from initiation of chemotherapy until white blood count (WBC) $<1 \times 10^6$/ml was a median of 10 days (8 days after beginning carboplatin). Four patients experienced life-threatening infections (septicemia). In 2 of these patients, causative organisms were identified (*Pseudomonas auerginosa* and *Candida albicans*). Life-threatening hemorrhage occurred in 2 patients. One patient, previously mentioned, developed severe gastrointestinal bleeding in the setting of necrotizing colitis, while a second patient continued to bleed from the site of a central venous catheter which had been removed because of infection. The latter patient was refractory to platelet transfusions and subsequently developed a nonlethal CNS hemorrhage. Of the two deaths which occurred during this study, one was due to hemorrhage alone while the other was due to hemorrhage in the setting of infection.

Carboplatin Levels

Total and free platinum levels were obtained in 18 and 16 patients, respectively (Table III). The median levels of total/free platinum for the entire group were 6.1/3.3 mcg/ml (ranges, 2.0–18.0 [total]/0.3–17.0 [free] mcg/ml). For the 14 patients who achieved hypoplastic marrow, the median levels were 6.8/4.0 mcg/ml (ranges, 2.3–18.0 [total]/0.6–17.0 [free] mcg/ml). The median values for the 4 patients whose bone marrows remained cellular were 4.2/1.9 mcg/ml (ranges, 2.0–5.2 [total]/0.3–2.6 [free] mcg/ml). Platinum levels did not appear to predict for response, since there was a wide range of values in the group achieving PR (11.0/5.8 mcg/ml, 8.2/4.0 mcg/ml, 3.5/1.9 mcg/ml [total/free]).

DISCUSSION

The proposed mechanisms of action of each of these antileukemic agents differ from those of the standard drugs used in the treatment of acute leukemia. Therefore, given the resistance of relapsed/refractory disease to re-

TABLE III. Carboplatin Levels*

Patient	Diagnosis	Creative/Clearance	Total (μg/ml)	Free (μg/ml)	Cellularity (BM d21)	Response
1	AML	100			Aplastic	F
2	AML	66	8.4	3.2	Hypo	F
3	AML	121	11.0	5.8	Hypo	PR
4	AML	101	2.0	0.3	Hyper	F
5	AML	110	5.2	2.4	Hyper	F
6	AML	88	5.0	2.6	Hyper	F
7	AML	78	8.2	4.0	Hypo	PR
8	AML	70	6.8	4.1	Hypo	F
9	ALL	65	2.3	0.6	Hypo	F
10	AML	128	18.0	17.0	Hypo	F
11	MDS-T	79	6.8	4.0	Hypo	F
12	ALL	63	7.1		Hypo	F
13	AML	157	4.3		Hypo	F
14	AML	107	3.5	1.9	Hypo	F
15	AML	71	3.4	1.4	Hyper	F
16	MDS-T	69	6.3	3.3	Hypo	F
17	MDS-T	101	5.9	3.3	Hypo	F
18	MDS-T		3.5	1.9	Hypo	PR
19	CML-T	135			Hyper	F
20	AML	67	8.5	5.4	Hypo	F
Medians		6.1	3.3			

*MDS-T, myelodysplastic syndrome transformed into AML; CML-T, chronic myelogenous leukemia in blast crisis.

peated courses of conventional dose therapy, the rationale for combining 5-AZA and CBDCA is apparent. Furthermore, both drugs offer the additional advantage of having no significant cardiotoxicity. This may be important in a patient population which is heavily pretreated with anthracyclines, such as that studied here.

The study design was based on the biochemical and pharmacologic properties of the drugs. One mechanism of action of platinum-based compounds is the formation of DNA crosslinks via platinum adducts. DNA hypermethylation may protect cells from such adduct formation. In addition, certain cellular enzymes may repair these adducts. Both of these activities have been implicated as mechanisms of platinum resistance [12,13]. The active species of 5-AZA, i.e., the triphosphate, is incorporated into transfer and ribosomal RNA, resulting in direct damage to nucleic acid and indirectly inhibiting the synthesis of proteins such as cellular repair enzymes. Moreover, 5-AZA triphosphate is directly incorporated into and causes hypomethylation of cellular DNA, leading to conformational changes which may alter the DNA interaction with other agents. The sequential use of 5-AZA and CBDCA might be expected to deplete the cells of enzymes capable of repairing platinum-DNA adducts, and to prevent hypermethylation of the DNA.

The CBDCA infusion using 1,250 mg/m^2 (total dose) was based on the recommendation by Meyers et al. [3], which demonstrated a 28.5% response in previously treated patients. The dose of 5-AZA was escalated to a set maximum of 750 mg/m^2 (total) based on reports of antileukemic activity as a single agent at this dose level [9]. Dose escalation beyond this in a combination regimen was felt to be unwarranted.

Although the addition of 5-AZA to CBDCA was well-tolerated in this study, the combination was not effective in inducing remissions in this group of heavily pretreated patients. The meager 15% response rate is comparable to the results of reported studies which have combined CBDCA with other antileukemic agents such as mitoxantrone, daunorubicin, or etoposide [14–16]. The observation that CBDCA combinations produce inferior results compared to those for this drug as a single agent raises concern about the potential utility of such regimens in leukemia therapy. Such results may in part be explained by the heterogeneity of the patient populations treated with the various regimens. Seventy-six percent of patients in the present study had received at least two prior treatment regimens. In contrast, 57% of patients treated with CBDCA alone by Meyers et al., [3] were either untreated or had received minimal therapy prior to first relapse. Furthermore, the potential crossresistance between Ara-C and 5-AZA [7,17] which has been suggested, might also explain the apparent lack of benefit in adding 5-AZA to CBDCA; many patients in the present study had previously failed Ara-C-based salvage regimens.

Pharmacokinetic variability seen among patients, which resulted in differences in plasma drug levels, may also be an important factor in responsiveness to chemo-

therapy. An unexpected finding in this study was the interpatient variation in platinum levels obtained 96–120 hr following initiation of CBDCA infusion. Such levels should have represented a steady state. There appeared to be no correlation between a patient's creatinine clearance (which was required to be >60 ml/min) and platinum levels. It is notable, however, that in the 4 patients who did not achieve hypoplasia, the median total/free platinum levels (4.2/1.9 mcg/ml) were lower compared to those for the other patients (6.8/4.0 mcg/ml). Methods for defining the pathophysiology of this variability of CBDCA serum levels need to be developed in order to use this agent in a more effective manner.

CONCLUSIONS

The challenge in treating patients with refractory/relapsed leukemia lies in the development of strategies to circumvent drug resistance. New antileukemic drugs with novel mechanisms of action and combinations of older drugs may be particularly useful in this regard. CBDCA and 5-AZA have demonstrated activity as single agents, and in this study were administered in combination with an acceptable toxicity profile. The low response rate of this drug schedule, however, argues against its further investigation as a salvage therapy for heavily pretreated patients. In vitro studies may help to define mechanisms responsible for the lack of clinical efficacy of this and other CBDCA-containing regimens.

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A General Synthesis of N-Glycosides. V.[1,2] Synthesis of 5-Azacytidines

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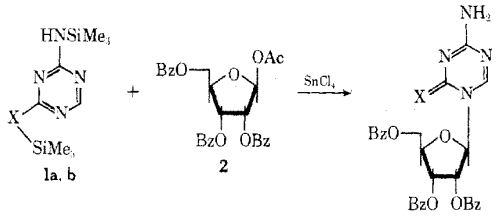
Reaction of silylated 5-azacytosines as well as their silylated 2-thio analogs with protected 1-*O*-acyl sugars in the presence of $SnCl_4$ gave the corresponding 5-azacytidines in good yields.

The cancerostatic 5-azacytidine, which is highly active against leukemia,[3] and certain analogs were first prepared *via* a multistep synthesis starting from peracetylated 1-glycosyl isocyanates by Píska la and Šorm.[4] Subsequently, 5-azacytidine was isolated as a new antibiotic by Hanka, *et al.*,[5] from *Streptoverticillium ladakanus*. More recently Winkley and Robins[6] treated silylated 5-azacytosines with acylated 1-halo sugars but obtained only fair yields of 5-azacytidine and its 2′-deoxy and other analogs.

The biological importance of 5-azacytidine[3] induced us to apply our new Friedel–Crafts catalyzed silyl Hilbert–Johnson procedure[7] to the synthesis of 5-azacytidines. Reaction of silylated 5-azacytosine (**1a**) and 2-thio-5-azacytosine (**1b**) with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**2**) in 1,2-dichloroethane or acetonitrile in the presence of $SnCl_4$ gave the corresponding O-benzoylated 5-azacytidines **3a** and **3b** in yields of up to 80%, thus making these interesting compounds readily available.[8]

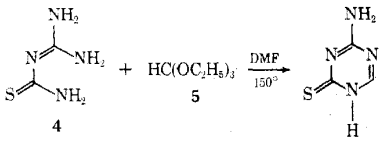


1a, b **2** **3a, b**

a, X = O
b, X = S

The preparation of the base 5-azacytosine[9] was simplified by direct synthesis from *N*-cyanoguanidine and formic acid–acetic anhydride in 35% yield.

The new 2-thio-5-azacytosine (**6**) was obtained by condensation of thiocarbamoylguanidine (**4**) with ethyl orthoformate (**5**) in dimethylformamide at 150° in analogy to Píska la[9] to give **6** in 72% yield, which could be readily silylated to the crystalline *S,N*-bissilyl compound **1b.**



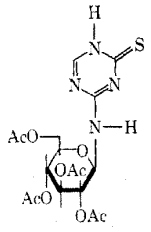
4 **5** **6**

During the reaction of **1b** with pentaacetyl-β-D-glucopyranose (**10**) the N^4-(glucosyl)-2-thio-5-azacytosine (**7**) was isolated as a side product and characterized by uv and nmr spectra. The anomeric H-1′ proton [δ 5.73 (dd, $J = J' \approx 9$ Hz)] is split by the NH group, which disappears on exchange with D_2O to give a doublet [δ 5.72 (d, $J = 9$ Hz)].

Table I
Preparation of Acylated 5-Azacytidine and Analogs

Silylated *s*-triazine	Acylated sugar	Acylated nucleoside	Yield, %
2,4-*O*,*N*-Bis(trimethylsilyl)-4-amino-1,3,5-triazine (**1a**)	1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**2**)	**3a**	81
	1,2,3,5-Tetra-*O*-acetyl-β-D-ribofuranose (**8**)	**9a**	50
	1,2,3,4,6-Penta-*O*-acetyl-β-D-glucopyranose (**10**)	**11a**	78
	1,2,3,4-Tetra-*O*-acetyl-β-D-ribopyranose (**12**)	**13a**	52
	2-Deoxy-3,5-di-*O*-*p*-toluoyl-α-D-ribofuranosyl chloride, (**14**)	**15a**	42[a]
2,4-*S*,*N*-Bis (trimethylsilyl)-4-amino-2-mercapto-1,3,5-triazine (**1b**)	1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**2**)	**3b**	82
	1,2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranose (**10**)	**11b**	59
	1,2,3,4-Tetra-*O*-acetyl-β-D-ribopyranose (**12**)	**13b**	56

[a] Yield of β anomer, total yield 77%.



7

The saponification of the O-benzoylated 5-azacytidine (**3a**) to free 5-azacytidine is difficult and best results are obtained following closely the procedure of Píska la and Šorm.[4] However, all attempts to saponify or transesterify the O-benzoylated 2-thio-5-azacytidine (**3b**) and its analogs failed. Apparently the heterocyclic ring in **3b** opens readily under basic conditions. The cleavage is accompanied by a

shift in the uv maxima from 283 to 277 nm and by disappearance of the H-6 proton at δ 8.2 in the nmr spectrum.

In Table I the preparation of acylated analogs of **3** is summarized.

Experimental Section

For instruments and the purification of solvents compare part I[7] of this series.

A. 4-Amino-1,2-dihydro-1,3,5-triazine-2-one. A mixture of 98–100% formic acid (80 ml, 2.12 mol), acetic anhydride (80 ml, 0.816 mol), and *N*-cyanoguanidine (dicyandiamide, Merck, Darmstadt) (84.08 g, 1 mol) was heated to 100°, whereupon the reaction started to boil vigorously. The solid dissolved and after a short time a colorless precipitate separated. The reaction was completed by heating to 140° for 2 hr. After cooling to 22° the solid was filtered and the crude material extracted three times with boiling ethanol to give after drying *in vacuo* a white powder (38.8 g, 34.6%) with mp 350°, which could be readily silylated in high yields according to standard procedures to give **1a**.[9]

Anal. Calcd for $C_3H_4N_4O$ (112.10): C, 32.15; H, 3.60; N, 49.99. Found: C, 31.87; H, 3.69; N, 50.13.

B. 4-Amino-1,2-dihydro-1,3,5-triazine-2-thione (6). A suspension of freshly prepared **4**[10] (118.2 g, 1 mol) and **5** (148.2 g, 1.8 mol) in dry dimethylformamide (500 ml) was refluxed (oil bath, 160°) for 2 hr with exclusion of moisture, whereupon the solid dissolved and after a few minutes a crystalline product separated. After cooling to 22° the crystalline material was filtered, washed with ethanol, and dried *in vacuo* at 50° to give 92.5 g (72%): mp >330°; nmr (NaOD) δ 8.00 (s, 1, H-6); uv (CH_3OH) λ_{max} 210 nm (ϵ 10,900), 270 (16,900).

Anal. Calcd for $C_3H_4N_4S$ (128.16): C, 28.12; H, 3.15; N, 43.72; S, 25.02. Found: C, 27.84; H, 3.31; N, 43.69; S, 24.84.

C. 2,4-*S,N*-Bis(trimethylsilyl)-4-amino-2-mercapto-1,2,5-triazine (1b). **6** (38.45 g, 300 ml) was suspended in a mixture of HMDS (400 ml), pyridine (1.2 l.), and trimethylchlorosilane (1 ml). The mixture was refluxed, whereupon the solid dissolved almost completely. The solution was filtered from the solid under nitrogen and concentrated *in vacuo* to 400 ml. **1b** crystallized from the hot solution and was filtered in an atmosphere of nitrogen and washed with a small amount of absolute benzene. The product was dried *in vacuo* at 50°: yield, 68.2 g (83.7%).

1-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)-4-amino-1,2-dihydro-1,3,5-triazin-2-one (3a). To **2** (5.0 g, 9.91 mmol) and **1a** (12.5 mmol) in 1,2-dichloroethane (150 ml) $SnCl_4$ (1.68 ml, 14.16 mmol) in 1,2-dichloroethane (20 ml) was added at 10°. After stirring at 10° for 2 hr the solution was diluted with CH_2Cl_2 and washed with ice-cold saturated $NaHCO_3$ solution. The organic phase was filtered through a layer of Celite, which was washed with a small amount of CH_2Cl_2. After drying (Na_2SO_4) and evaporation, the residue was dissolved in toluene and filtered through Celite to remove unreacted 5-azacytosine. After evaporation *in vacuo* the residue (5.2 g) was dissolved in ethanol and filtered again through Celite. **3a** crystallized from the filtrate as needles: yield, 4.45 g (80.7%); mp 186–187°; $[\alpha]^{20}D$ −33.1° (*c* 1, $CHCl_3$); nmr ($CDCl_3$) δ 8.21 (s, 1, H-6), 6.1–5.9 (m, 3, H-1′, H-2′, H-3′).

Anal. Calcd for $C_{29}H_{24}N_4O_8$ (556.54): C, 62.59; H, 4.35; N, 10.07. Found: C, 62.43; H, 4.41; N, 10.21.

3a gave on methanolysis[4] 5-azacytidine, mp 232–233° (EtOH) (lit.[4] 230–231° dec).

1-(2,3,5-Tri-*O*-acetyl-β-D-ribofuranosyl)-4-amino-1,2-dihydro-1,3,5-triazin-2-one (9a). To **8** (6.36 g, 20 mmol) and **1a** (25 mmol) in CH_3CN (200 ml) $SnCl_4$ (4 ml, 34.2 mmol) in CH_3CN (100 ml) was added at 22°. After 30 min at 22° and work-up[11] crystallization (ethyl acetate) gave 3.68 g (49.7%) of **9a**: mp 160–161°; $[\alpha]^{23}D$ 3.41° (*c* 1.04, $CHCl_3$); nmr ($CDCl_3$) δ 5.82 (d, 1, J = 3.5 Hz, H-1′).

Anal. Calcd for $C_{11}H_{18}N_4O_8$ (270.33): C, 45.41; H, 4.90; N, 15.13. Found: C, 45.55; H, 5.05; N, 15.33.

1-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-4-amino-1,2-dihydro-1,3,5-triazin-2-one (11a). To **10** (15.56 g, 40 mmol) and **1a** (50 mmol) in CH_3CN (300 ml) $SnCl_4$ (8.5 ml, 72.65 mmol) in CH_3CN (150 ml) was added. After 5.5 hr at 22° and work-up[11] recrystallization (ethyl acetate–pentane) gave 13.32 g (77.6%) of **11a**: mp 213–214°; $[\alpha]^{20}D$ 10.6° (*c* 1, $CHCl_3$); nmr ($CDCl_3$) δ 8.21 (s, 1, H-6), 5.98 (d, 1, J = 9 Hz, H-1′).

Anal. Calcd for $C_{17}H_{22}N_4O_{10}$ (442.40): C, 46.15; H, 5.01; N, 12.67. Found: C, 46.31; H, 5.19; N, 12.59.

1-(2,3,4-Tri-*O*-acetyl-β-D-ribopyranosyl)-4-amino-1,2-dihydro-1,3,5-triazin-2-one (13a). To **12** (3.18 g, 10 mmol) and **1a** (12.5 mmol) in 1,2-dichloroethane (150 ml) $SnCl_4$ (1.68 ml, 14.36 mmol) in 1,2-dichloroethane (20 ml) was added. After 2 hr at 22° and work-up[11] the residue (3.14 g) was chromatographed in ethyl acetate on silica gel (200 g). **13a** crystallized from ethanol: yield, 1.92 g (51.9%); mp 128–136° (solvated); $[\alpha]^{20}D$ 30.6° (*c* 1, $CHCl_3$); nmr ($CDCl_3$) δ 6.11 (d, 1, J = 10 Hz, H-1′).

Anal. Calcd for $C_{14}H_{18}N_4O_8$ (370.33): C, 45.41; H, 4.90; N, 15.13. Found: C, 45.26; H, 5.03; N, 15.02 [after drying for 2 hr at 50° (10^{-3} mm)].

1-(2-Deoxy-3,5-di-*O*-*p*-toluoyl-β-D-ribofuranosyl)-4-amino-1,2-dihydro-1,3,5-triazin-2-one (15a). To **14** (3.89 g, 10 mmol) and **1a** (12.5 mmol) in 1,2-dichloroethane (150 ml) $SnCl_4$ (0.84 ml, 7.18 mmol) in 1,2-dichloroethane (20 ml) was added. After 2 hr at 22° and work-up[11] crystallization (toluene) afforded a mixture of the anomeric nucleosides (3.55 g, 76.6%) from which **15a** was obtained by fractional crystallization (ethyl acetate): yield, 1.93 g (41.6%); mp 196°; $[\alpha]^{20}D$ 23.7° (*c* 1, $CHCl_3$); nmr ($CDCl_3$) δ 8.37 (s, 1, H-6), 6.27 (dd, 1, J = 8 + 6 Hz, H-1′).

Anal. Calcd for $C_{24}H_{24}N_4O_6$ (464.49): C, 62.06; H, 5.21; N, 12.06. Found: C, 62.35; H, 5.38; N, 12.08.

1-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)-4-amino-1,2-dihydro-1,3,5-triazine-2-thione (3b). To a suspension of **1b** (1.64 g, 6 mmol) in CH_3CN (50 ml) $SnCl_4$ (1.6 ml, 13.7 mmol) in CH_3CN (50 ml) was added. After addition of **2** (2.522 g, 5 mmol) and 30 min at 22°, work-up[11] gave crude **3b** (2.86 g), which crystallized as needles (ethyl acetate): yield, 2.34 g (81.7%); mp 201–203°; $[\alpha]^{20}D$ −24.2° (*c* 1, $CHCl_3$); nmr ($CDCl_3$) δ 8.52 (s, 1, H-6), 7.15 (d, 1, J = 3 Hz, H-1′).

Anal. Calcd for $C_{29}H_{24}N_4O_7S$ (572.61): C, 60.83; H, 4.23; N, 9.79; S, 5.60. Found: C, 60.75; H, 4.34; N, 9.87; S, 5.49.

1-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-4-amino-1,2-dihydro-1,3,5-triazine-2-thione (11b). To a suspension of **1b** (3.26 g, 11.9 mmol) in CH_3CN (150 ml) $SnCl_4$ (3.2 ml, 27.4 mmol) in CH_3CN (70 ml) was added (**1b** dissolved), followed by **10** (3.89 g, 10 mmol). After 30 min at 22° and work-up[11] **11b** crystallized (ethanol) as colorless needles: yield, 2.72 g (59.4%); mp 246–247°; $[\alpha]^{20}D$ 20.8° (*c* 1, $CHCl_3$); nmr ($CDCl_3$) δ 8.37 (s, 1, H-6), 7.10 (d, 1, J = 9 Hz, H-1′).

Anal. Calcd for $C_{17}H_{22}N_4O_9S$ (458.46): C, 44.54; H, 4.84; N, 12.22; S, 6.99. Found: C, 44.37; H, 4.99; N, 12.30; S, 6.88.

Reaction in 1,2-dichloroethane gave 38% **11b**. From the mother liquor the N_4-glucoside **7** was isolated by preparative tlc (silica gel, ethyl acetate): yield, 213 mg (3%); amorphous; $[\alpha]^{23}D$ 7.1° (*c* 0.48, ethyl acetate); nmr ($CDCl_3$) δ 8.45 (s, 1, H-6), 5.73 (dd, 1, $J = J'$ = 9 Hz, H-1′).

Anal. Calcd for $C_{17}H_{22}N_4O_9S$ (458.46): C, 44.54; H, 4.84; N, 12.22; S, 6.99. Found: C, 44.38; H, 4.96; N, 12.16; S, 7.11.

1-(2,3,4-Tri-*O*-acetyl-β-D-ribopyranosyl)-4-amino-1,2-dihydro-1,3,5-triazine-2-thione (13b). To **1b** (3.26 g, 11.9 mmol) in CH_3CN (100 ml) $SnCl_4$ (3.2 ml, 27.4 mmol) in CH_3CN (70 ml) was added (**1b** dissolved), followed by **12** (3.18 g, 10 mmol). After 1 hr at 22° and work-up[11] **13b** crystallized (ethanol) as needles: yield, 2.08 g (56.2%); mp 237–239°; $[\alpha]^{20}D$ 72.0° (*c* 1, $CHCl_3$); nmr ($CDCl_3$) δ 8.23 (s, 1, H-6), 7.23 (d, 1, J = 9 Hz, H-1′).

Anal. Calcd for $C_{14}H_{18}N_4O_7S$ (386.40): C, 43.52; H, 4.70; N, 14.50; S, 8.30. Found: C, 43.41; H, 4.78; N, 14.56; S, 8.26.

Acknowledgment. We are indebted to Dr. G.-A. Hoyer and Dr. D. Rosenberg for the nmr spectra and Diplom-Ing. G. Huber for the analyses.

Registry No.—**1a**, 52523-35-0; **1b**, 35782-62-8; **2**, 6974-32-9; **3a**, 28998-36-9; **3b**, 29845-69-0; **4**, 2114-02-5; **5**, 122-51-0; **6**, 36469-86-0; **7**, 52523-36-1; **8**, 13035-61-5; **9a**, 10302-78-0; **10**, 604-69-3; **11a**, 29845-67-8; **11b**, 30009-99-5; **12**, 4049-34-7; **13a**, 30370-22-0; **13b**, 30370-25-3; **14**, 4330-21-6; **15a**, 10302-79-1; 4-amino-1,2-dihydro-1,3,5-triazin-2-one, 931-86-2.

References and Notes

(1) Synthesis of Nucleosides No. 13. For a preliminary publication, compare H. Vorbrüggen and U. Niedballa, *Tetrahedron Lett.*, 3571 (1970).
(2) Part IV: *J. Org. Chem.*, **39**, 3668 (1974).
(3) (a) J. Veselý and A. Cihák, *Experientia*, **29**, 1132 (1973); (b) H. Karon, L. Sieger, S. Leimbrock, J. Z. Finklestein, M. E. Nesbit, and J. J. Swaney, *Blood*, **42**, 359 (1973).
(4) A. Piskala and F. Sorm, *Collect. Czech. Chem. Commun.*, **29**, 2060 (1964); German Patent 1922702 (1969).

(5) L. J. Hanka, J. S. Evans, D. J. Mason, and A. Dietz, *Antimicrob. Ag. Chemother.*, 619 (1966).
(6) M. W. Winkley and R. K. Robins, *J. Org. Chem.*, **35**, 491 (1970).
(7) Compare part I of this series: U. Niedballa and H. Vorbrüggen, *J. Org. Chem.*, **39**, 3654 (1974).
(8) Kilogram amounts of 5-azacytidine have been prepared using our procedures by Ash-Stevens Inc. (private communication by Professor C. S. Stevens).
(9) A. Piskala, *Collect. Czech. Chem. Commun.*, **32**, 3966 (1967).
(10) "Organic Synthesis," Collect. Vol IV, Wiley, New York, N.Y., 1968, p. 502.
(11) Compare the preparation of **3a**.

Mixed Alkylation (Methylation and Ethylation) of Adenosine by Diazoethane in Aqueous 1,2-Dimethoxyethane[1]

Lee M. Pike, M. Khurshid A. Khan, and Fritz Rottman*

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Received August 13, 1974

Synthesis of 2′-*O*-ethyladenosine by treatment of adenosine with diazoethane in aqueous 1,2-dimethoxyethane produced several unexpected alkylation products. Characterization of the products by several methods, including mass spectrometry of their trimethylsilyl ethers, indicated that methylation was occurring to approximately the same extent as ethylation. Analysis of the reaction conditions employing palmitic acid as an alkyl acceptor implicated the solvent (1,2-dimethoxyethane) as a potential source of the extraneous methyl groups since only ethylation was observed when diethyl ether was employed as an alternate solvent.

The reaction of adenosine and diazomethane in aqueous 1,2-dimethoxyethane has been used extensively to prepare 2′-Am[2] since the 2′-hydroxyl group is preferentially methylated under these conditions.[3-5] When adenosine was treated with diazoethane under similar reaction conditions, several unexpected products were observed. In this paper these products are identified and found to indicate the occurrence of mixed alkylation. Evidence is presented which is consistent with involvement of the solvent (1,2-dimethoxyethane) in the alkylation reaction under these conditions.

Adenosine and diazoethane were combined in a solvent of aqueous 1,2-dimethoxyethane, the reaction was permitted to reach completion, and the products were resolved by ion exchange chromatography.[6] Six prominent uv-absorbing fractions were observed as shown in Table I, whereas only four fractions were observed following alkylation of adenosine with diazomethane.[5]

Some preliminary conclusions concerning the nature of the six fractions may be reached from their relative yields and column retentions. By analogy to the reaction of adenosine and diazomethane, one of the major products would be 2′-Ae while lesser amounts of the 3′-ethyl ether and dialkylated products would be obtained.[3] Furthermore, the degree of retention of nucleosides on the ion-exchange column may be correlated with increasing ionization potential of available ribose hydroxyl groups.[7] Therefore, the expected order of elution is: dialkylation products, 2′-*O*-alkylation products, and finally 3′-*O*-alkylation products. Compounds with both 2′- and 3′-hydroxyl groups available are not eluted under these conditions. On the basis of yield and elution pattern, fractions 3 and 4 may contain 2′-alkyl ethers, one of which should be 2′-Ae, while fractions 5 and 6 may contain 3′-alkyl ethers.

Components of all six fractions were characterized by descending paper chromatography in the four solvent systems described in Table II. Fractions 3, 4, 5, and 6 each gave a single uv-absorbing spot in all four solvent systems and were estimated to be greater than 97% pure. Although the reaction of diazoethane with adenosine was expected to yield only ethylated products, surprisingly, the compound in fraction 4 migrated with 2′-Am in all four systems and the compound in fraction 6 migrated with 3′-Am in all four systems (Table II). The compounds in fractions 3 and 5 migrated faster than 2′-Am or 3′-Am, respectively, and were well resolved from each other by solvent D. These results were consistent with the tentative identification of fraction 3 as 2′-Ae and fraction 4 as 3′-Ae since the 3′-ethyl ether should have been retained longer than the 2′-ethyl ether on the ion exchange column as discussed above.

Both fractions 1 and 2 were resolved into several components by paper chromatography with the four solvents. Analytical studies on the first two column fractions in the comparable methylated adenosine series indicated they

Table I
Fractionation of Alkylated Nucleosides on Bio-Rad AG 1 Column[a]

Fraction no.	Identity	Registry no.	Tubes pooled in each fraction	Recovery, % of adenosine applied
1			12–24	3.8
2			38–44	1.7
3	2′-*O*-Ethyladenosine	52842-98-5	45–58	11.9
4	2′-*O*-Methyladenosine	2140-79-6	68–81	9.8
5	3′-*O*-Ethyladenosine	52928-62-8	105–120	3.8
6	3′-*O*-Methyladenosine	10300-22-8	179–210	2.8

[a] The column consisted of Bio-Rad AG 1-X2 (OH^-), 200–400 mesh, 4 × 40 cm, equilibrated with 40% ethanol prior to use. The crude reaction mixture (95,100 A_{260} units) was applied in 40% ethanol and eluted with 40% ethanol at a flow rate of 2 ml/min; the tube volume was 20 ml.

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EXAMINER
CHU, YONG LIANG

ART UNIT	PAPER NUMBER
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DATE MAILED: 09/20/2011

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/729,116	03/22/2010	Dumitru Ionescu	9516-846-999 (CAM 501872-	6614

TITLE OF INVENTION: PHARMACEUTICAL COMPOSITIONS COMPRISING CRYSTAL FORM I OF 5-AZACYTIDINE

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1510	$300	$0	$1810	12/20/2011

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

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PTOL-85 (Rev. 02/11)

Notice of Allowability	Application No.	Applicant(s)	
	12/729,116	IONESCU ET AL.	
	Examiner	Art Unit	
	YONG CHU	1626	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *08/10/2010*.

2. ☒ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are *22-61 (renumbered as 1-40)*.

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   a) ☐ All b) ☐ Some* c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.

6. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.

   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached

   1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.

   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

7. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☒ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☐ Examiner's Amendment/Comment
8. ☒ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

DETAILED ACTION

Claims 22-61 are currently pending in this Application, and are under examination on the merits.

Priority

This application is a continuation of U.S. Patent Application Serial No. 11/198,550, filed August 5, 2005, entitled Methods for Isolating Crystalline Form I of 5-Azacytidine; which is a divisional of U.S. Patent Application Serial No. 10/390,530, filed March 17, 2003, entitled Methods for Isolating Crystalline Form I of 5-Azacytidine (now U.S. Patent No. 6,943,249)

Reasons for Allowance

The present invention is directed to a pharmaceutical composition for **oral** administration comprising (crystalline) **Form I** of 5-azacytidine substantially free of other forms of 5-azacytidine. The methods of making **Form I** of 5-azacytidine have been patented in the parent patents. The XRPD characterization of (crystalline) **Form I** of 5-azacytidine is disclosed in the Example 4 of the instant specification on page 3. In addition, a pharmaceutical formulation into the form of dosage units for oral administration is also disclosed on page 9 of the specification. Since crystalline **Form I** of 5-azacytidine substantially free of other forms of 5-azacytidine is patentable, a pharmaceutical composition comprising said **Form I** of 5-azacytidine is also patentable. See *SmithKline Beecham Corp. v. Apotex Corp., 74 USPQ2d 1398,* (Fed. Appl. Cir. 2005).

The closest prior art of record is a reference by *Dover et al., Blood, (1985), Vol. 66(3), p.527-532.*

Dover et al. teaches a pharmaceutical composition comprising 5-azacytidine for oral administration. However, Dover **does not** teach a pharmaceutical composition comprising crystalline **Form I** of 5-azacytidine substantially free of other forms of 5-azacytidine. Therefore, claims 22-61 are allowed.

Conclusions

- Claims 22-61 are allowed.

Telephone Inquiry

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Yong Chu, Ph.D., whose telephone number is 571-272-5759. The examiner can normally be reached on 7:00 am - 3:30 pm EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Joseph K. McKane can be reached on 571-272-0699. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Status Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

/Yong Chu/
Primary Examiner
Art Unit 1626

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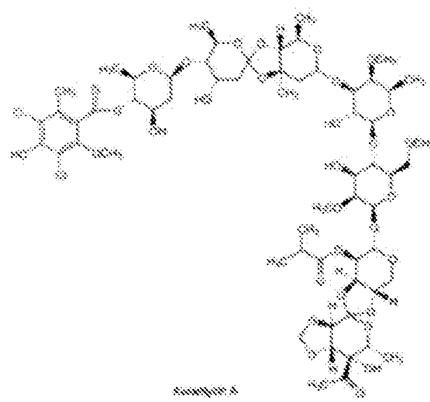


Avilamycin A

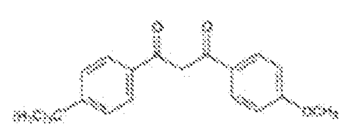
Colorless, needle-shaped crystals from acetone/ether, mp 188-189.5°. $[\alpha]_D^{20}$ +0.8° (c = 1.165 in abs ethanol) and −7.7° (c = 1.083 in chloroform).

Avilamycin A. $C_{61}H_{88}Cl_2O_{32}$; mol wt 1404.25. Colorless needles from chloroform/pet ether, mp 181-182° (1-2 H_2O). uv max (methanol): 227, 286 br nm (log ε 4.15, 3.33).

Avilamycin C. $C_{61}H_{90}Cl_2O_{32}$; mol wt 1406.27. Dihydro, colorless fine plates from acetone/ether, mp 188-189°. $[\alpha]_D^{20}$ −4.8° (c = 1.44 in chloroform). uv max (methanol): 228, 284 br nm (log ε 4.12, 3.33).

THERAP CAT (VET): Growth promotant.

891. Avobenzone. [70356-09-1] 1-[4-(1,1-Dimethylethyl)phenyl]-3-(4-methoxyphenyl)-1,3-propanedione; butyl methoxydibenzoylmethane; 4-tert-butyl-4'-methoxydibenzoylmethane; Parsol 1789. $C_{20}H_{22}O_3$; mol wt 310.39. C 77.39%, H 7.14%, O 15.46%. UV-A blocker. Prepn: K.-F. De Polo, DE 2945125; idem, US 4387089 (1980, 1983 both to Givaudan). Clinical efficacy as sunscreen: R. W. Gange et al., J. Am. Acad. Dermatol. 15, 494 (1986); K. Kaidbey, R. W. Gange, ibid. 16, 346 (1987); N. J. Lowe et al., ibid. 17, 224 (1987). Assessment of photostability: A. Deflandre, G. Lang, Int. J. Cosmet. Sci. 10, 53 (1988). Photodecomposition: N. M. Roscher et al., J. Photochem. Photobiol. A 80, 417 (1994). Photochemistry in solution: W. Schwack, T. Rudolph, ibid. B 28, 229 (1995). HPLC determn in cosmetic products: L. Gagliardi et al., J. Chromatog. 408, 409 (1987); P. Wallner, Deutsche Lebensmittel-Rund. 89, 375 (1993).



Crystals from methanol, mp 83.5°.

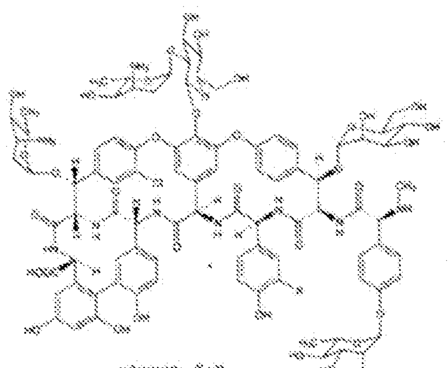
THERAP CAT: Ultraviolet screen.

892. Avoparcin. [37332-99-3] AV-290; C-254; CL-81588; LL-AV290; Avotan. Animal feed glycopeptide antibiotic complex produced by *Streptomyces candidus*. Prodn: M. P. Kunstmann, J. N. Porter, US 3338786 (1967 to Am. Cyanamid). Fermentation, isoln, characterization: M. P. Kunstmann et al., Antimicrob. Ag. Chemother. 8, 242 (1968). Isoln, purif of major components: W. J. McGahren et al., J. Antibiot. 36, 1671 (1983). HPLC separation of α-, β-avoparcin: F. Szlachetski et al., ibid. 1881. Structural studies: J. J. Hlavka et al., Tetrahedron Letters 1974, 175; W. J. McGahren et al., J. Am. Chem. Soc. 101, 2237 (1979). Structure: eidem, ibid. 102, 1671 (1980). Stereochemistry and epimerization: G. A. Ellestad et al., J. Antibiot. 36, 1683 (1983). Relationship between structure, antibacterial activity: S.W. Fesik et al., Mol. Pharmacol. 28, 375 (1984). Effect on resistance patterns in chickens: J. R. Walton, Zentralbl. Veterinaermed. (B) 25, 290 (1978); C.A. 89, 173807 (1978). Prevention of expty induced necrotic enteritis in chickens: J. F. Prescott, Avian Dis. 23, 1072 (1979). Effect on feedlot performance: R. J. Johnson et al., J. Anim. Sci. 48, 1338 (1979).

White, hygroscopic, amorphous solid; no definitive mp. uv max: 280 nm in neutral or acidic soln; 300 nm in basic soln. Sol in water, DMF, DMSO. Max stability of aq soln is at pH 4-8. Moderately sol in methanol. LD_{50} in mice, rats, and chickens: >10000 mg/kg orally (Am. Cyanamid, company literature).

α-Avoparcin. [73957-86-5] $C_{89}H_{102}ClN_9O_{36}$; $[\alpha]_D^{25}$ −96 ±2° (c = 0.62 in 0.1N HCl). uv max (0.1N HCl): 280 nm ($E_{1cm}^{1\%}$ 42.0).

β-Avoparcin. [73957-87-6] $C_{89}H_{101}Cl_2N_9O_{36}$; $[\alpha]_D^{25}$ −102 ±2° (c = 0.65 in 0.1N HCl). uv max (0.1N HCl): 280 nm ($E_{1cm}^{1\%}$ 34.0).



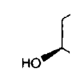
α-avoparcin R = H
β-avoparcin R = Cl

THERAP CAT (VET): Antibacterial; growth promotant.

893. Azacitidine. [320-67-2] 4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1H)-one; 5-azacytidine; 5-AzaC; ladakamycin; U-18496; NSC-102816; Mylosar. $C_8H_{12}N_4O_5$; mol wt 244.20. C 39.35%, H 4.95%, N 22.94%, O 32.76%. Pyrimidine nucleoside analog; antitumor antibiotic produced by *Streptoverticillium ladakanus*. Chemical synthesis: A. Piskala, F. Sorm, Coll. Czech. Chem. Commun. 29, 2060 (1964); M. W. Winkley, R. K. Robins, J. Org. Chem. 35, 491 (1970). Microbiological production and activity: L. J. Hanka et al., Antimicrob. Ag. Chemother. 1966, 619. Isoln and structure: M. E. Bergy, R. R. Herr, ibid. 625. HPLC determn in pharmaceutical prepns: L. D. Kissinger, N. L. Stemm, J. Chromatog. 353, 309 (1986). Clinical evaluation in β-thalassemia: C. H. Lowrey, A. W. Nienhuis, N. Engl. J. Med. 329, 845 (1993). Toxicology study: P. E. Palm, C. J. Kensler, U.S. Clearinghouse Fed. Sci. Tech. Inform., PB-194791 (1970) 191 pp., C.A. 75, 33766j (1971). Review of clinical experience in acute nonlymphocytic leukemia: A. B. Glover et al., Cancer Treat. Rep. 71, 737-746 (1987); of mechanism of action: A. B. Glover, B. Leyland-Jones, ibid. 959. Review of carcinogenic risk: IARC Monographs 50, 47-63 (1990).

Crystals fro[m]
(c = 1 in wat[er]
HCl): 249 nm
LD_{50} in mice (i
Note: This
carcinogen: *N*
2000) p III-75.
THERAP CAT:

894. Az[a]
ylamino)propy
[3-(dimethylam
25-diazacholes
77.26%, H 11.
J. Med. Pharm
3084156 (1963

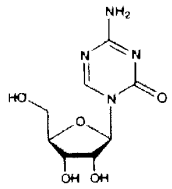


Crystals from
Dihydrochlo
$H_{44}N_2O.2HCl$;
THERAP CAT (

895. Az[a]
dinemethanol;
carbinol; gam
267.36. C 80.
hydrogenation
et al., **US 2804**

Crystals, mp
Hydrochlori
NO.HCl; mol v
Slightly bitter t
THERAP CAT:

896. Az[a]
720.71. C 58.3
isolated from tl
Juss. (*Melia az*
M. azedarach
growth regulat
feeding inhibit
Chem. Commu
M. D. Thornto

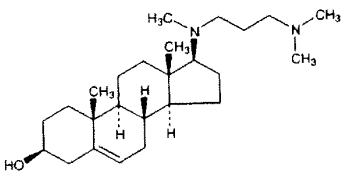


Crystals from aq ethanol, mp 235-237° (dec). $[\alpha]_D^{26}$ +22.4° (c = 1 in water). uv max (water): 241 nm (ε 8767); (0.01*N* HCl): 249 nm (ε 3077); (0.01*N* KOH): 223 nm (ε 24200). LD_{50} in mice (mg/kg): 115.9 i.p.; 572.3 orally (Palm, Kensler).

Note: This substance is reasonably anticipated to be a human carcinogen: *Ninth Report on Carcinogens* (PB2000-107509, 2000) p III-75.

THERAP CAT: Antineoplastic.

894. Azacosterol. [313-05-3] (3β,17β)-17-[[3-(Dimethylamino)propyl]methylamino]androst-5-en-3-ol; *N*-methyl-*N*-[3-(dimethylamino)propyl]-17β-aminoandrost-5-en-3β-ol; 20,25-diazacholesterol; diazasterol. $C_{25}H_{44}N_2O$; mol wt 388.63. C 77.26%, H 11.41%, N 7.21%, O 4.12%. Prepn: Counsell *et al.*, *J. Med. Pharm. Chem.* **5,** 1224 (1962); Counsell, Klimstra, **US 3084156** (1963 to Searle).

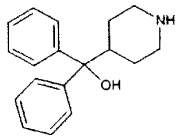


Crystals from acetone + methanol, mp 146-148°. $[\alpha]_D$ −54.5°.

Dihydrochloride. [1249-84-9] SC-12937; Ornitrol. $C_{25}H_{44}N_2O.2HCl$; mol wt 461.56. $[\alpha]_D$ −32°.

THERAP CAT (VET): Avian chemosterilant.

895. Azacyclonol. [115-46-8] α,α-Diphenyl-4-piperidinemethanol; α-(4-piperidyl)benzhydrol; diphenyl (γ-pyridyl)-carbinol; gamma-pipradrol; MER-17. $C_{18}H_{21}NO$; mol wt 267.36. C 80.86%, H 7.92%, N 5.24%, O 5.98%. Prepd by hydrogenation of α,α-diphenyl-4-pyridinemethanol: Schumann *et al.*, **US 2804422** (1957 to Merrell).

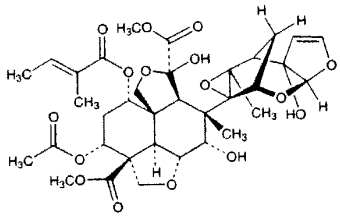


Crystals, mp 160-161°.

Hydrochloride. [1798-50-1] Frenquel; Frenoton. $C_{18}H_{21}NO.HCl$; mol wt 303.83. Crystals from butanone, mp 283-285°. Slightly bitter taste. Moderately sol in water.

THERAP CAT: Anxiolytic.

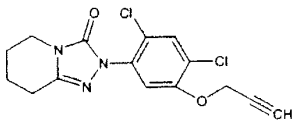
896. Azadirachtin. [11141-17-6] $C_{35}H_{44}O_{16}$; mol wt 720.71. C 58.33%, H 6.15%, O 35.52%. A tetranortriterpinoid isolated from the seeds of the neem tree, *Azadirachta indica* A. Juss. (*Melia azadirachta* L.), *Meliaceae*, and the chinaberry tree, *M. azedarach* L. Highly active insect feeding deterrent and growth regulator. Isoln from *A. indica* and identification as feeding inhibitor in locusts: J. H. Butterworth, E. D. Morgan, *Chem. Commun.* **1968,** 23; from *M. azedarach:* E. D. Morgan, M. D. Thornton, *Phytochemistry* **12,** 391 (1973). Partial synthesis: D. Pflieger *et al.*, *Tetrahedron Letters* **28,** 1519 (1987). Structural studies: J. H. Butterworth *et al.*, *J. Chem. Soc. Perkin Trans. II* **1972,** 2445. ^1H- and ^{13}C-NMR data and structure: P. R. Zanno *et al.*, *J. Am. Chem. Soc.* **97,** 1975 (1975); K. Nakanishi in *Recent Advances in Phytochemistry,* **vol. 9,** V. C. Runeckles, Ed. (Plenum Press, New York, 1975) pp 283-298. Revised structure: W. Kraus *et al.*, *Tetrahedron Letters* **26,** 6435 (1985); H. B. Broughton *et al.*, *Chem. Commun.* **1986,** 46. Isoln by HPLC: E. C. Uebel *et al.*, *J. Liq. Chromatog.* **2,** 875 (1979); J. D. Warthen, Jr. *et al.*, *ibid.* **7,** 591 (1984). Antifeedant activity in locusts: J. S. Gill, C. T. Lewis, *Nature* **232,** 402 (1971); in fall army worms, cotton bollworms: J. A. Klocke, I. Kubo, *Entomol. Exp. Appl.* **32,** 299 (1982). Insect ecdysis and growth inhibition: H. Rembold, K. P. Sieber, *Z. Naturforsch.* **36C,** 466 (1981); I. Kubo, J. A. Klocke, *Agr. Biol. Chem.* **46,** 1951 (1982); K. P. Sieber, H. Rembold, *J. Insect. Physiol.* **29,** 523 (1983). Series of articles on chemistry and activity: *Natural Pesticides from the Neem Tree,* Proc. 1st Int. Neem Conf., 1980, H. Schmutterer *et al.*, Eds. (German Agency for Technical Cooperation, Eschborn, 1981) 291 pp.



Microcrystalline powder from carbon tetrachloride, mp 154-158°. $[\alpha]_D$ −53° (c = 0.5 in $CHCl_3$). uv max (methanol): 217 nm (ε 9100).

USE: Experimentally as insect control agent.

897. Azafenidin. [68049-83-2] 2-[2,4-Dichloro-5-(2-propynyloxy)phenyl]-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]-pyridin-3(2*H*)-one; DPX-R6447; Milestone; Evolus. $C_{14}H_{13}Cl_2N_3O_2$; mol wt 338.19. C 53.27%, H 3.87%, Cl 20.97%, N 12.43%, O 9.46%. Porphyrin biosynthesis inhibitor. Prepn: A. D. Wolf, **BE 862884**; *idem,* **US 4213773** (1978, 1980 both to Du Pont). Review of physical properties, mode of action, and activity: K. Amuti *et al.*, *Brighton Crop Prot. Conf. - Weeds* **1997,** 59-66.



White powdered solid, mp 168-168.5°. Vapor pressure at 20°: 1.0×10^{-11} torr. Partition coefficient (octanol/water): 229. LD_{50} in rats, mice, bobwhite quail, mallard duck (mg/kg): >5000, >5000, >2500, >2500 orally; in rabbits (mg/kg): >2000 dermally. LC_{50} in rats, rainbow trout, blue gill sunfish (mg/l): >5.3, 33, 48 (Amuti).

USE: Herbicide.

898. Azafrin. [507-61-9] (5*R*,6*R*)-5,6-Dihydro-5,6-dihydroxy-10′-*apo*-β,ψ-carotenoic acid; escobedin. $C_{27}H_{38}O_4$; mol wt 426.59. C 76.02%, H 8.98%, O 15.00%. Carotenoid-carboxylic acid from roots of the South American plant "Azafranillo," *Escobedia scabrifolia* Ruiz & Pav., and *Escobedia laevis* Cham. & Schlecht., *Scrophulariaceae.* Isoln: R. Kuhn *et al.*, *Ber.* **64,** 333 (1931); *ibid.* **65,** 1873 (1932). Structure: R. Kuhn, A. Deutsch, *ibid.* **66,** 883 (1933); R. Kuhn, H. Brockmann, *ibid.* **67,** 885 (1934); *Ann.* **516,** 104 (1935). Absolute

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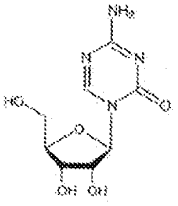
water, DMF, DMSO. Max stability of aq solns is at pH 4-8. Moderately sol in methanol. LD_{50} in mice, rats, and chickens: >10000 mg/kg orally (Am. Cyanamid, company literature).

α-Avoparcin. [73957-86-5] $C_{86}H_{102}ClN_9O_{36}$. $[\alpha]_D^{25}$ −96 ±2° (c = 0.62 in 0.1*N* HCl). uv max (0.1*N* HCl): 280 nm ($E_{1cm}^{1\%}$ 42.0).

β-Avoparcin. [73957-87-6] $C_{85}H_{100}Cl_2N_9O_{36}$. $[\alpha]_D^{25}$ −102 ±2° (c = 0.65 in 0.1*N* HCl). uv max (0.1*N* HCl): 280 nm ($E_{1cm}^{1\%}$ 44.0).

THERAPCAT (VET): Antibacterial; growth promotant.

890. Azacitidine. [320-67-2] 4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one; 5-azacytidine, 5-AzaC; ladakamycin; U-18496; NSC-102816; Mylosar (formerly); Vidaza. $C_8H_{12}N_4O_5$; mol wt 244.20. C 39.35%, H 4.95%, N 22.94%, O 32.76%. DNA methylation inhibitor; analog of the pyrimidine nucleoside, cytidine, *q.v.* Chemical synthesis: A. Piskala, F. Sorm, *Collect. Czech. Chem. Commun.* **29**, 2060 (1964); M. W. Winkley, R. K. Robins, *J. Org. Chem.* **35**, 491 (1970). Production by fermentation of *Streptoverticillium ladakanus* and activity: L. J. Hanka *et al.*, *Antimicrob. Agents Chemother.* **1966**, 619; M. E. Bergy, R. R. Herr, *ibid.* 625. HPLC determn in pharmaceutical preps: L. D. Kissinger, N. L. Stenun, *J. Chromatogr.* **353**, 309 (1986). Toxicology study: P. E. Palm, C. J. Kensler, *U.S. Clearinghouse Fed. Sci. Tech. Inform.* (PB-194791, 1970) 191 pp.; *C.A.* **75**, 33704j (1971). Review of clinical experience in acute nonlymphocytic leukemia: A. B. Glover *et al.*, *Cancer Treat. Rep.* **71**, 737-746 (1987); of mechanism of action: A. B. Glover, B. Leyland-Jones, *ibid.* 959-964. Review of carcinogenic risk: *IARC Monographs* **50**, 47-63 (1990). Clinical efficacy in β-thalassemia: C. H. Lowrey, A. W. Nienhuis, *N. Engl. J. Med.* **329**, 845 (1993); in myelodysplastic syndromes: L. R. Silverman *et al.*, *J. Clin. Oncol.* **20**, 2429 (2002); A. B. Kornblith *et al.*, *ibid.* 2441.

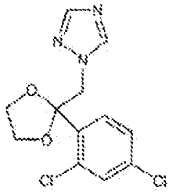


Crystals from aq ethanol, mp 235-237° (dec). $[\alpha]_D^{20}$ +22.4° (c = 1 in water). uv max (water): 241 nm (ε 8767); (0.01*N* HCl): 249 nm (ε 3077); (0.01*N* KOH): 223 nm (ε 24209). Soly (mg/mL): 46 warm water, 14 cold water, 28 0.1*N* HCl, 43 0.1*N* NaOH, 52.7 DMSO, I acetone, I chloroform, I hexane. LD_{50} in mice (mg/kg): 118.9 i.p.; 572.3 orally (Palm, Kensler).

Caution: This substance is reasonably anticipated to be a human carcinogen: *Report on Carcinogens, Eleventh Edition* (PB2005-104914, 2004) p III-24.

THERAPCAT: Antineoplastic.

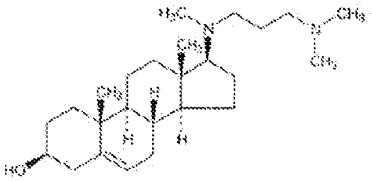
891. Azaconazole. [60207-31-0] 1-[[2-(2,4-Dichlorophenyl)-1,3-dioxolan-2-yl]methyl]-1*H*-1,2,4-triazole. R-28644; Redwood; Safetray. $C_{12}H_{11}Cl_2N_3O_2$; mol wt 300.14. C 48.02%, H 3.69%, Cl 23.62%, N 14.00%, O 10.66%. Sterol demethylation inhibitor. Prepn: G. Van Reet *et al.*, **DE 2551660**; *eidem*, **US 4079062** (1975, 1978 both to Janssen). Wood fungicide for mushroom cultivation: L. Van Leempat *et al.*, *Med. Fac. Landbouww. Rijksuniv. Gent* **52**, 703 (1987); A. Eicker, E. Strydom, *Sot. Bull. Acad. Sinica* **31**, 51 (1990). Use as a preservative for composite wood products: E. L. Schmidt, R. O. Gerjejansen, *Forest Prod. J.* **38**, 19 (1988); A. Jelihoomi *et al.*, *Wood Fiber Sci.* **26**, 178 (1994).



Crystals from diisopropylether, mp 109.9°.

USE: Fungicide for cultivation on wood; preservative for composite wood products.

892. Azacosterol. [313-05-3] (3β,17β)-17-[[3-(Dimethylamino)propyl]methylamino]androst-5-en-3-ol; *N*-methyl-*N*-[3-(dimethylamino)propyl]-17β-aminoandrost-5-en-3β-ol; 20,25-diazacholesterol; diazacterol. $C_{31}H_{58}N_2O$; mol wt 388.63. C 77.26%, H 11.41%, N 7.21%, O 4.12%. Prepn: Counsell *et al.*, *J. Med. Pharm. Chem.* **5**, 1224 (1962); Counsell, Klimstra, **US 3084158** (1963 to Searle).

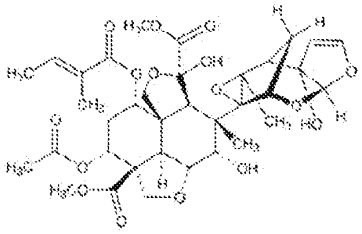


Crystals from acetone + methanol, mp 146-148°. $[\alpha]_D$ −54.5°.

Dihydrochloride. [1249-84-9] SC-12937; Ornitrol. $C_{31}H_{58}N_2O.2HCl$; mol wt 461.55. $[\alpha]_D$ −32°.

THERAPCAT (VET): Avian chemosterilant.

893. Azadirachtin. [11141-17-6] $C_{35}H_{44}O_{16}$; mol wt 720.71. C 58.33%, H 6.15%, O 35.52%. A tetranortriterpinoid isolated from the seeds of the neem tree, *Azadirachta indica* A. Juss. (*Melia azadirachta* L.), *Meliaceae*, and the chinaberry tree, *M. azedarach* L. Highly active insect feeding deterrent and growth regulator. Isoln from *A. indica* and identification as feeding inhibitor in locusts: J. H. Butterworth, E. D. Morgan, *Chem. Commun.* **1968**, 23; from *M. azedarach*: E. D. Morgan, M. D. Thornton, *Phytochemistry* **12**, 391 (1973). Partial synthesis: D. Pfleiger *et al.*, *Tetrahedron Lett.* **28**, 1519 (1987). Structural studies: J. B. Butterworth *et al.*, *J. Chem. Soc. Perkin Trans. 2* **1972**, 2445; ^1H- and ^{13}C-NMR data and structure: P. R. Zanno *et al.*, *J. Am. Chem. Soc.* **97**, 1975 (1975); K. Nakanishi, *in Recent Advances in Phytochemistry* **vol. 9**, V. C. Runeckles, Ed. (Plenum Press, New York, 1975) pp 283-298. Revised structure: W. Kraus *et al.*, *Tetrahedron Lett.* **26**, 6435 (1985); B. B. Broughton *et al.*, *Chem. Commun.* **1986**, 46. Isoln by HPLC: E. C. Uebel *et al.*, *J. Liq. Chromatogr.* **2**, 875 (1979); J. D. Warthen, Jr. *et al.*, *ibid.* **7**, 591 (1984). Antifeedant activity in locusts: J. S. Gill, C. T. Lewis, *Nature* **232**, 402 (1971); in fall army worms, cotton bollworms: J. A. Klocke, I. Kubo, *Entomol. Exp. Appl.* **32**, 299 (1982). Insect ecdysis and growth inhibition: H. Rembold, K. P. Sieber, *Z. Naturforsch.* **36C**, 460 (1981); I. Kubo, J. A. Klocke, *Agric. Biol. Chem.* **46**, 1951 (1982); K. P. Sieber, H. Rembold, *J. Insect Physiol.* **29**, 523 (1983). Series of articles on chemistry and activity: *Natural Pesticides from the Neem Tree*, Proc. 1st Int. Neem Conf., 1980, H. Schmutterer *et al.*, Eds. (German Agency for Technical Cooperation, Eschborn, 1981) 291 pp.



Microcrystalline powder from carbon tetrachloride, mp 154-158°. $[\alpha]_D$ −53° (c = 0.5 in $CHCl_3$). uv max (methanol): 217 nm (ε 9100).

USE: Experimentally as insect control agent.

894. Azafenidin. [68049-83-2] 2-[2,4-Dichloro-5-(2-propynyloxy)phenyl]-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyridin-3(2*H*)-one; DPX R6447; Milestone; Evolus. $C_{15}H_{13}Cl_2N_3O_2$; mol

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84802 7590 09/23/2011

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EXAMINER	
OLSON, ERIC	
ART UNIT	PAPER NUMBER
1623	
MAIL DATE	DELIVERY MODE
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Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	12/787,214	IONESCU ET AL.	
	Examiner	Art Unit	
	ERIC S. OLSON	1623	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *25 May 2010*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) *14-33* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *14-33* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

10)☒ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *06 August 2010* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

12)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

Detailed Action

This application is a continuation of US application 11/458365, now US patent 7772199, filed July 18, 2006, which is a continuation of US application 11/052615, now US patent 7078518, filed February 7, 2005, which is a divisional application of US application 10/390578, now US patent 6887855, filed March 17, 2003. Claims 14-33 are pending in this application and examined on the merits herein. Applicant's preliminary amendment submitted May 25, 2010 is acknowledged wherein claims 1-13 are cancelled and new claims 14-33 are introduced.

Abstract

The abstract is objected to for failing to properly represent the invention. The claimed invention is a composition of azacytidine monohydrate. However, the abstract describes the claimed material as "polymorphic and pseudopolymorphic crystalline forms of 5-azacytidine." 5-azacytidine is an anhydrous material which is a distinct chemical entity from 5-azacytidine monohydrate, And the two terms should not be used interchangeably. Furthermore, according to Seddon (Reference included with PTO-892) the term "pseudopolymorphic" is a misnomer when used to refer to solvates and hydrates of a chemical compound, which only facilitates the illusion that an anhydrate and a hydrate are somehow the same substance. The less misleading term "solvate" should be used to refer to such compounds. Appropriate correction is required.

The title of the invention is not descriptive. The current title uses the phrase, "forms of 5-azacytidine" even though the claims are drawn to a single form of 5-azacytidine monohydrate which is, as discussed above, a different compound than 5-azacytidine. A new title is required that is clearly indicative of the invention to which the claims are directed.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 14-33 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 16 is drawn to a compound (form III) characterized by a powder X-ray diffraction pattern having a limited number of identified peaks. According to Dean (Analytical Chemistry Handbook, Reference included with PTO-892) an X-ray diffraction pattern can be considered as a "fingerprint" of a particular chemical substance. (p. 10.24, last paragraph) Unlike a single crystal X-ray pattern or a NMR spectrum, a powder X-ray spectrum is not interpreted as it does not contain sufficient structural data, but is simply compared to an existing database of known powder diffraction patterns. Therefore, a diffraction pattern cannot be meaningfully interpreted by reference to an arbitrary selection of peaks judged to be sufficiently intense by a particular researcher. It is not an analytical method and cannot be used to positively identify a sample, as

opposed to merely determining that it is not the same as a different sample. Many different compounds or mixtures of compounds could reasonably produce peaks in the selected positions, especially considering that the listing of peaks is open-ended and is not an exhaustive listing of all peaks present in the pattern of the claimed crystalline form. Therefore one skilled in the art would not be able to clearly and distinctly identify a single crystalline form claimed by the instant claims, rendering said claims indefinite. It is recommended that Applicant define the diffraction pattern by reference to a diffraction pattern in the specification or drawings as originally filed, for example figure 3 in the drawings.

Regarding claims 14, 15, and 17-33, these claims refer only to “form III" of 5-azacytidine. This notation is not a standard chemical name and is not recognized in the art outside of Applicant’s own patent applications as referring to a specific crystal form of 5-azacytidine. Therefore one skilled in the art would not be able to associate Applicant's recitation of "form III" in the claims with any specific form of 5-azacytidine.

In addition, the claims use the term “5-azacytidine” (i.e. anhydrous 5-azacytidine) to describe form III which appears from the specification to be 5-azacytidine monohydrate. An anhydrous compound and its monohydrate are distinct chemical entities, and the term “5-azacytidine” does not encompass 5-azacytidine monohydrate. Thus the claims contain contradictory limitations, which would confuse one skilled in the art as to what is actually being claimed.

Therefore the claims are indefinite.

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claim 16 is rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. In particular, the claims are drawn to a compound characterized by a powder X-ray diffraction pattern having three identified peaks. According to Dean (Analytical Chemistry Handbook, Reference included with PTO-892) an X-ray diffraction pattern can be considered as a “fingerprint” of a particular chemical substance. (p. 10.24, last paragraph) Unlike a single crystal X-ray pattern or a NMR spectrum, a powder X-ray spectrum is not interpreted as it does not contain sufficient structural data, but is simply compared to an existing database of known powder diffraction patterns. It is not an analytical method and cannot be used to positively identify a sample, as opposed to merely determining that it is not the same as a different sample. Therefore, a diffraction pattern cannot be meaningfully interpreted by reference to an arbitrary selection of peaks judged to be sufficiently intense by a particular researcher. Rather, the claims as written encompass a wide variety of different compounds and mixtures that happen to produce a powder diffraction pattern having peaks in these three positions, including various mixtures of multiple crystal forms of 5-azacytidine. By contrast, Applicant’s specification discloses only eight crystal forms of

5-azacytidine, one of which has a powder X-ray diffraction pattern that includes the three recited peaks. This one example is not judged to be adequately representative of the full range of possible substances and mixtures that would possess those three peaks.

In order to meet the requirement for written description, Applicant's disclosure must meet several criteria, actual reduction to practice, disclosure of drawings or structural information, sufficient relevant identifying characteristics, methods of making the claimed invention, the level of skill and knowledge in the art, and the predictability in the art.

Actual reduction to practice: Applicant has synthesized a mixed form of 5-azacytidine crystals which produces a number of diffraction peaks including those claimed in instant claim 16.

Disclosure of drawings or structural information: No actual structural information is disclosed. The claimed crystal forms are identified by x-ray powder diffraction and thermogravimetric analysis. These two techniques do not identify the structure (e.g. unit cell configuration) of a crystal. Rather, at most they can act as a fingerprint to tell whether or not two samples of a compound have the same or different crystal structures. The claimed compositions are really only identified with reference to other crystal forms. While Applicant can say what they are not, there is no disclosure of what they actually are.

Sufficient relevant identifying characteristics: The claimed compositions are identified by the presence of a few x-ray diffraction peaks. There is no indication

whether this form is the only one that displays these peaks, or whether the peaks could be observed in other compositions containing 5-azacytidine monohydrate. Therefore the disclosed diffraction peaks are not necessarily sufficient to uniquely identify the claimed form III.

Methods of making: The specification discloses a method of making the claimed compositions.

Level of skill and knowledge and predictability of the art: The characterization of new crystal forms is difficult and unpredictable. For example Cabri et al. (Reference included with PTO-892) describes a number of different patent applications claiming various forms of Cefdinir. Upon further analysis, the authors conclude that these forms are not necessarily stable (p. 68 right column paragraphs 4-5, p. 69 left column paragraphs 1-2) Characterization solely by x-ray powder diffraction, the alleged "gold standard" for characterization, is unable to tell whether a new form of a compound is actually a salt or a solvate. (p. 71 right column) In one case a form identified by XRPD as a new crystal form was actually a pyridinium salt, and this technique is furthermore incapable of detecting the presence of solvent molecules that would render a composition unsuitable for pharmaceutical use.

In view of these factors, the undeveloped state of the art combined with the limited characterization of the claimed crystals indicates that Applicant has not uniquely described the claimed crystal form in such a way as to demonstrate possession of the claimed x-ray powder diffraction peaks. The species recited in the specification is

therefore not representative of the full scope of the claims, and therefore does not provide adequate written description for the claims.

Claims 24-33 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a compound prepared by contacting solid 5-azacytidine with water for a period of at least 19 hours, does not reasonably provide enablement for a compound prepared by contacting solid 5-azacytidine with water for shorter periods of time. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make the invention commensurate in scope with these claims.

The Applicant's attention is drawn to *In re Wands*, 8 USPQ2d 1400 (CAFC1988) at 1404 where the court set forth eight factors to consider when assessing if a disclosure would have required undue experimentation. Citing *Ex parte Forman*, 230 USPQ 546 (BdApls 1986) at 547 the court recited eight factors:
(1) The nature of the invention; (2) the state of the prior art; (3) the relative skill of those in the art; (4) the predictability or unpredictability of the art; (5) the breadth of the claims; (6) the amount of direction or guidance presented; (7) the presence or absence of working examples; and (8) the quantity of experimentation necessary.

Nature of the invention: The claimed invention is a product identified by a particular method of making the product. In order to be enabled for the full scope of the claim, one skilled in the art must be able to actually produce the claimed product using the full scope of process steps recited in the claim.

The state of the prior art: While 5-azacytidine is known in the art, the formation of hydrates of this compound has not been studied. Although Piskla et al. (Reference included with PTO-892) does mention the crystallization of a hydrate of 5-azacytidine, this product is produced by direct crystallization form aqueous acetone and not by equilibration of anhydrous 5-azacytidine in water.

The relative skill of those in the art: The relative skill of those in the art is comparatively low regarding the manufacture and characterization of specific crystal forms and solvates. Those skilled in the art are not able to predict what crystal forms or hydrates will form under what conditions.

The predictability or unpredictability of the art: According to Vippagunta et al. (Reference included with PTO-892) approximately one third of all pharmaceutically active substances are capable of forming crystalline hydrates. (p. 15 left column first paragraph) Predicting the formation of these hydrates and solvates is complex and difficult, as each compound responds uniquely to the formation of solvates and hydrates, precluding any generalizations about their structure. (p. 18 left column last paragraph) Therefore predicting which hydrates will form from a given process is difficult and unpredictable.

The Breadth of the claims: The claimed invention encompasses all crystalline hydrates (i.e. hemihydrates, dehydrates, trihydrates) of 5-azacytidine which are formed by contacting anhydrous 5-azactyidine with water.

The amount of direction or guidance presented: Applicant discloses a monohydrate of 5-azacytidine known as "form III" which is isolated from slurries of

anhydrous 5-azacytidine in water. The temperature and pressure at which the slurry was maintained is not disclosed. It is not known whether this is the only possible hydrate of 5-azacytidine or whether other forms could possibly form under different conditions.

The presence or absence of working examples: Example 8 on pp. 22-23 discloses various compositions of anhydrous 5-azacytidine equilibrating to 5-azacytidine monohydrate (form III) when slurried in aqueous conditions for 19 hours. No working examples are provided for any other reaction times less than 19 hours.

Note that lack of working examples is a critical factor to be considered, especially in a case involving an unpredictable and undeveloped art such as the formation of crystalline hydrates. See MPEP 2164.

The quantity of experimentation necessary: In order to practice the full scope of the invention, one skilled in the art must be able to make and use the full scope of products recited in the claims. In the instant case, while it is clear to one skilled in the art that Applicant's identified form III would be present after 19 hours as an aqueous slurry at some undisclosed temperature and pressure, it is entirely possible that other forms, for example hemihydrates or dihydrates, or other crystal forms of the monohydrate, are transiently formed but disappear before the full 19 hour reaction time is complete. Specifically, after 19 hours it is probable that the sample has reached equilibrium and that the product produced is thermodynamically stable. Shorter slurrying times might produce kinetic products which would disappear later in the reaction. Therefore one skilled in the art would not know what products would result

from shorter reaction times, and attempting to use shorter reaction times to produce the claimed "form III" would require an undue burden of unpredictable experimentation.

Genentech, 108 F.3d at 1366, sates that, "a patent is not a hunting license. It is not a reward for search, but compensation for its successful conclusion." And "patent protection is granted in return for an enabling disclosure of an invention, not for vague intimations of general ideas that may or may not be workable."

Therefore, in view of the Wands factors, as discussed above, particularly the nature of the invention and the unpredictability of the art, Applicants fail to provide information sufficient to practice the claimed invention for products prepared by a process involving contacting the solid 5-azacytidine with water for less than 19 hours.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 14-17 and 24-33 are rejected under 35 U.S.C. 102(b) as being anticipated by Piskala et al. (Nucleic Acid Chemistry, 1978, reference included with PTO-892)

Piskala et al. discloses the synthesis of 5-azacytidine. (p. 435 introduction) In the final step of the method, the product is recrystallized from a 1:1 water:acetone mixture

as the hydrate. (p. 440 lines 8-9) According to p. 6 of the instant specification, all forms of 5-azacytidine convert to the form known herein as form III when contacted with water. Furthermore according to p. 20 paragraphs 2-3, this form III is the monohydrate. Therefore it is concluded that the hydrate formed by Piskala et al. is inherently the same as "form III" recited in the instant claims. Furthermore, regarding claims 24-33, these claims describe a product-by-process limitation, and are therefore drawn not to the process of making the pharmaceutical composition, but rather to any composition which reasonably could have been made by the described process or which is indistinguishable from a composition made from said process. As discussed before, the specification states that any sample of 5-azacytidine contacted with water will equilibrate into form III, the hydrate. Since all of these processes have the same end point, namely a sample of form III, then form III of 5-azacytidine is seen to inherently fulfill the limitations of all of these product-by-process claims. Therefore Piskala et al. anticipates the claimed invention.

Claims 14-17 and 20-33 are rejected under 35 U.S.C. 102(a) as being anticipated by Silverman et al. (Reference included with PTO-892)

Silverman et al. discloses a clinical trial of 5-azacytidine for the treatment of leukemia. (p. 2430 left column second paragraph) The 5-azacytidine was provided as a single unit dose of 100mg of 5-azacytidine with 100mg of mannitol, which was suspended as a slurry and injected in sterile water. (p. 2430 left column last paragraph, right column first paragraph) According to the instant specification (p. 6 under the

heading “Form III” Applicant has admitted on the record that the slurry used in previous clinical trials would comprise form III as the solid. Furthermore, regarding claims 24-33, these claims describe a product-by-process limitation, and are therefore drawn not to the process of making the pharmaceutical composition, but rather to any composition which reasonably could have been made by the described process or which is indistinguishable from a composition made from said process. As discussed before, the specification states that any sample of 5-azacytidine contacted with water will equilibrate into form III, the hydrate. Since all of these processes have the same end point, namely a sample of form III, then form III of 5-azacytidine is seen to inherently fulfill the limitations of all of these product-by-process claims. Therefore Silverman et al. anticipates the claimed invention.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 18, 19, 22, and 23 are rejected under 35 U.S.C. 103(a) as being unpatentable over Piskala et al. as applied to claims 14-17 and 24-33 above and further in view of Daifuku et al. (US patent 7642247, cited on PTO-892)

The disclosure of Piskala et al. is discussed above. Piskala et al. does not specifically disclose a pharmaceutical composition in the form of a tablet or capsule, or

which further comprises a carrier, diluent, or excipient such as mannitol, microcrystalline cellulose, or magnesium stearate.

Daifuku et al. discloses the use of triazines including 5-azaacytidine as antiviral agents. (column 4 lines 45-50) For oral administration these compounds can be administered as tablets or capsules, for example, and can include excipients such as mannitol. (column 41 line 58 – column 42 line 10)

It would have been obvious to one of ordinary skill in the art at the time of the invention to formulate the 5-azacytidine hydrate described by Piskala et al. as a pharmaceutical formulation such as a tablet or capsule including an excipient such as mannitol. One of ordinary skill in the art would have been motivated to formulate them in this manner because Daifuku et al. discloses that 5-azacytidine is useful in such pharmaceutical formulations. One of ordinary skill in the art would have reasonably expected success because making specific pharmaceutical dosage forms of known therapeutic agents is well known and predictable in the art.

Therefore the invention taken as a whole is *prima facie* obvious.

Conclusion

No claims are allowed in this application.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to ERIC S. OLSON whose telephone number is (571)272-9051. The examiner can normally be reached on Monday-Friday, 8:30-5:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Shaojia Anna Jiang can be reached on (571)272-0627. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/ERIC S OLSON/
Primary Examiner, Art Unit 1623
9/21/2011

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/729,116	03/22/2010	Dumitru Ionescu	9516-846-999 (CAM 501872-	6614

84802 7590 11/28/2011

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EXAMINER	
CHU, YONG LIANG	
ART UNIT	**PAPER NUMBER**
1626	

MAIL DATE	DELIVERY MODE
11/28/2011	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	12/729,116	IONESCU ET AL.	
	Examiner	Art Unit	
	YONG CHU, Ph.D.	1626	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *18 November 2011*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) *22-61* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *22-61* is/are rejected.

8)☒ Claim(s) *22-61* is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

12)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *11/18/2011* .
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 11/18/2011 has been entered. Claims 22-61 are currently pending.

Information Disclosure Statement

Applicants' Information Disclosure Statement, filed 11/18/2011 has been considered. Please refer to Applicant's copy of the PTO-1449 submitted herewith.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to

be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 22-61 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-8 of U.S. Patent No. 7,700,770, ("the `770 patent"). The instant claims 21-61 are drawn to a pharmaceutical composition for oral administration comprising Form I of 5-azacytidine substantially free of other forms of 5-azacytidine. Claims 1-8 of the `770 patent are drawn to a method for isolating crystalline Form I of 5-azacytidine substantially free of other forms.

Although the conflicting claims are not identical, they are not patentably distinct from each other because all the claims are related to a novel crystalline Form I of 5-azacytidine. The `770 patent also teaches the crystalline Form I of 5-azacytidine is used for making a pharmaceutical composition. In view of *Sun Pharmaceutical Industries Ltd v. Eli Lilly and Co., 95 USPQ2d1797, Fed. Cir. App. (2010),* claims 22-61 would have been obviousness-type of double patenting over claims 1-8 of the `770 patent.

Claims 22-61 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-18 of U.S. Patent No. 6,943,249, ("the `249 patent"). The instant claims 21-61 are drawn to a pharmaceutical composition for oral administration comprising Form I of 5-azacytidine substantially free of other forms of 5-azacytidine. Claims 1-18 of the `249 patent are drawn to a method for isolating crystalline Form I of 5-azacytidine substantially free of other forms.

Although the conflicting claims are not identical, they are not patentably distinct from each other because all the claims are related to a novel crystalline Form I of 5-azacytidine. The `249 patent also teaches the crystalline Form I of 5-azacytidine is

used for making a pharmaceutical composition. In view of *Sun Pharmaceutical Industries Ltd v. Eli Lilly and Co., 95 USPQ2d1797, Fed. Cir. App. (2010),* claims 22-61 would have been obviousness-type of double patenting over claims 1-8 of the `249 patent.

Claim Objections

Claims 22-61 are objected to because of the following informalities: Claim 22 contains the term "Form I of 5-azacytidine", which should be replaced by "**crystalline** Form I of 5-azacytidine". Appropriate correction is required.

Conclusions

- Claims 22-61 are rejected.
- Claims 22-61 are objected to.

Telephone Inquiry

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Yong Chu, Ph.D., whose telephone number is 571-272-5759. The examiner can normally be reached between 7:00 am - 3:30 pm EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Joseph K. McKane can be reached on 571-272-0699. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

/Yong Chu/
Primary Examiner
Art Unit 1626

NUCLEIC ACIDS COMPONENTS AND THEIR ANALOGUES. LI.*
SYNTHESIS OF 1-GLYCOSYL DERIVATIVES OF 5-AZAURACIL AND 5-AZACYTOSINE

A. Píškala and F. Šorm

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague

Received January 2nd, 1964

For the preparation of 1-glycosyl derivatives of 5-azauracil and 5-azacytosine a method is described starting from peracetylglycosyl isocyanates. Addition of 2-methylisourea affords 1-peracetylglycosyl-4-methylisobiurets which are condensed with ethyl orthoformate to form 1-peracetylglycosyl-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazines. Deacetylation and demethylation of the latter compounds affords 1-glycosyl-5-azauracils whereas deacetylation followed by amination yields 1-glycosyl-5-azacytosines. By this procedure, glucopyranosyl, ribopyranosyl and ribofuranosyl derivatives of 5-azauracil as well as of 5-azacytosine have been prepared.

In the course of biochemical studies about cultivation of *Escherichia coli* in the presence of a subbacteriostatic concentration of 5-azauracil (2,4-dioxo-1,2,3,4-tetrahydro-1,3,5-triazine), ribosylbiuret[1] forming by decomposition of the unstable ribosyl-5-azauracil has been found in the medium. A detailed study of 5-azauracil anabolites has shown that cleavage of ribosyl-5-azauracil to ribosylbiuret proceeds in the culture of *E. coli via* N-formylribosylbiuret[2], *i.e.*, analogously to the cleavage of 5-azauracil to biuret[3]. Moreover, it has been demonstrated with the use of a cell-free extract of *E. coli* that 5-azauracil is a suitable substrate for microbial phosphorylases as well as pyrophosphorylases which makes possible the synthesis of ribosyl-5-azauracil and ribosyl-5-azauracil 5′-phosphate[4], resp. We have expected that ribosyl derivatives of 5-azauracil or 5-azacytosine (4-amino-2-oxo-1,2-dihydro-1,3,5-triazine) could exhibit marked biological effects similar to those of the corresponding 6-azaanalogues. In fact, preliminary experiments seem to support this assumption[5–7].

In this paper, we have studied the possibility of a chemical preparation of 1-glycosyl derivatives of 5-azauracil and 5-azacytosine which would represent the hitherto undescribed analogues of the appropriate pyrimidine nucleosides. Our attention has been focussed on the glucopyranosyl, ribopyranosyl and ribofuranosyl derivatives. The ribosyl derivatives were proposed for correlation with the above products of enzymic reactions.

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In a recent paper[8], we have described a general method for the preparation of 1-substituted 5-azauracils as well as 5-azacytosines which consists in the condensation of isobiurets with orthoesters followed by treatment with hydrogen chloride or ammonia. Since this method represents, according to our experience, the most suitable procedure for the preparation of this type of compounds, we have tried to synthesize also the required glycosyl derivatives in this manner. We have started from tetra-acetylglucopyranosyl isocyanate which was prepared by Fischer[9] in a 22% yield on treatment of 1-bromo-2,3,4,6-tetra-O-acetyl-α-D-glucopyranose (*I*) with silver cyanate. Later on, Johnson and Bergmann[10] described formation of a second, lower-melting modification. In several experiments, we isolated merely the higher-melting form. Furthermore, we were able to increase the yield (70%) by a suitable arrangement of the reaction conditions. Treatment with ammonia results in a product[9] to which the structure of 1-β-D-glucopyranosylurea was ascribed[11,12]. Consequently, the product of the treatment with silver cyanate may be considered as 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isocyanate (*II*).

Addition of 2-methylisourea afforded a high yield of a chromatographically homogeneous amorphous product analyzing in agreement with the expected 1-(2,3,4,6-

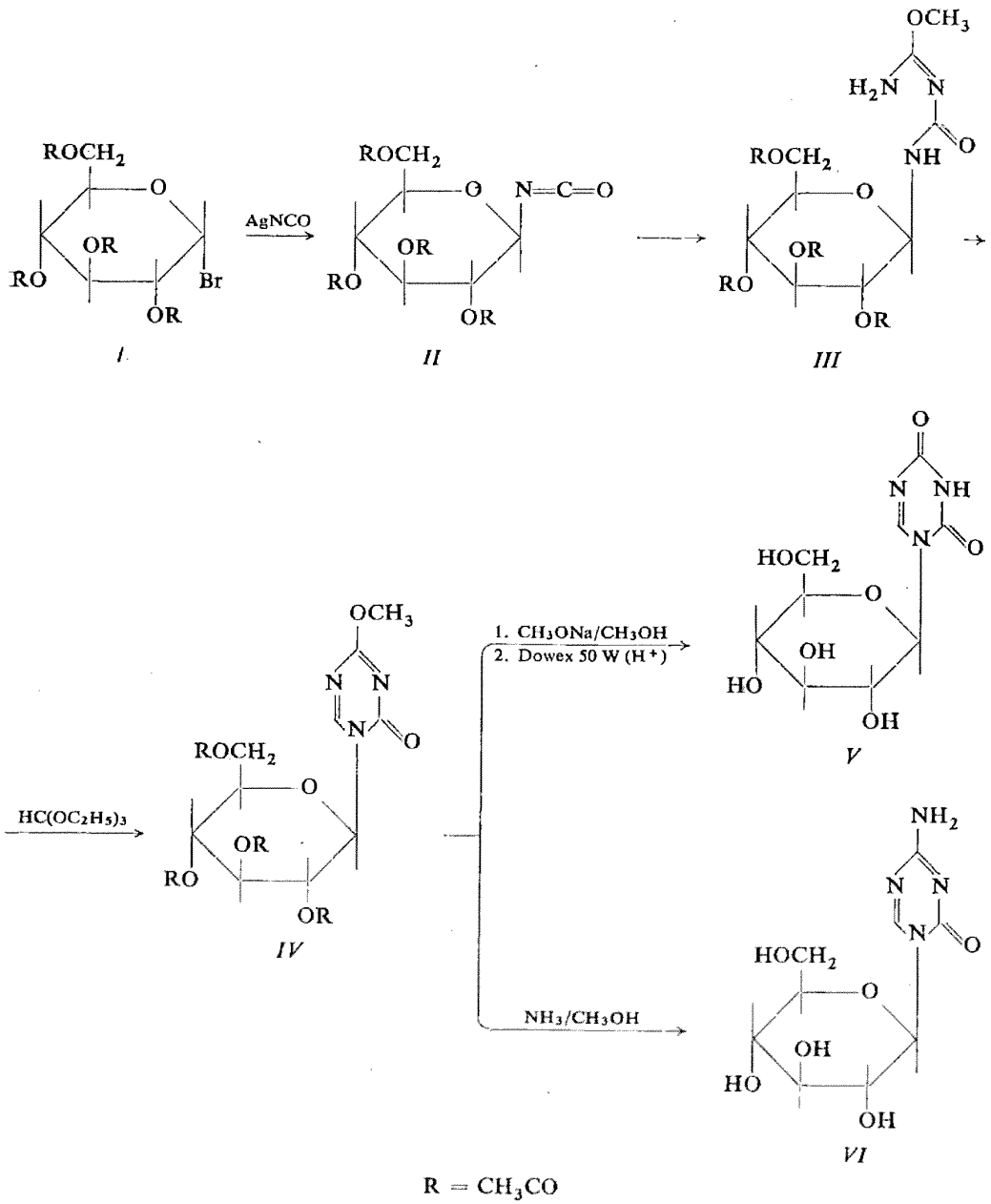
Table I

Ultraviolet Spectra

Compounds	Solvent	λ_{max} mμ	log ε
1,4-Dimethylisobiuret	96% ethanol	221	4·18
III	96% ethanol	221	4·26
X	96% ethanol	221	4·28
XVI	96% ethanol	221	4·27
1-Methyl-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine	acetonitril	254	3·34
IV	acetonitril	254	3·40
XI	acetonitril	253	3·39
XVII	acetonitril	253	3·36
1-Methyl-5-azauracil	96% ethanol	245	3·20
V[a]	96% ethanol	240	3·25
XII[a]	96% ethanol	240	3·24
1-Methyl-5-azacytosine	water, pH 5·0	247	3·74
VI[a]	water, pH 5·0	243	3·81
XIII[a]	water, pH 5·0	243	3·80
XXII[a]	water, pH 5·0	246	3·78

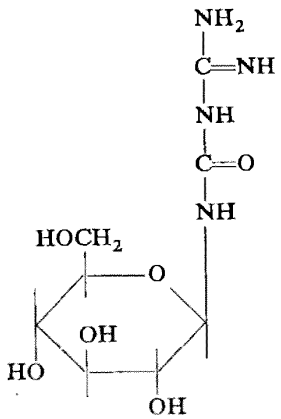
[a] Spectra of the 5-azauracil and 5-azacytosine glycosyl derivatives were measured immediately after dissolution because of the time-dependence of their extinction coefficient[13,25].

tetra-O-acetyl-β-D-glucopyranosyl)-4-methylisobiuret (*III*). Its ultraviolet spectrum is practically identical with that of the analogous 1,4-dimethylisobiuret (see Table I). The above product was condensed at 135°C with ethyl orthoformate in the stream of dry nitrogen to yield crystalline 1-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine (*IV*). Its structure was established on comparison of the ultraviolet spectrum with that of the analogous 1-methyl-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine (Table I).

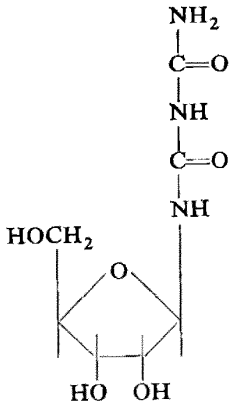


Transformation of the glucoside *IV* into the required 1-β-D-glucopyranosyl-5-azauracil (*V*) was accomplished with methanolic hydrogen chloride or Dowex 50 W(H^+) ion exchange resin in methanol or sodium methoxide followed by Dowex 50 W (H^+) resin. When crystallised from aqueous ethanol, the substances formed a solvate with ethanol and water. Water may be removed by heating *in vacuo* at 130°C for 1 hour whereas removal of ethanol requires 10 hours at the same temperature. Its structure was verified on a comparison of the ultraviolet spectrum with that of the analogous 1-methyl-5-azauracil[13], and, furthermore, by an acidic hydrolysis yielding 5-azauracil and glucose. Moreover, the formation of a stable adduct with ethanol in the case of the glucoside *V* closely resembles the behaviour of 1-methyl-5-azauracil[14]. The structure of both these adducts will be discussed in detail in a subsequent paper of this Series[15].

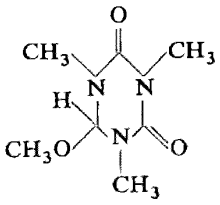
A short action of a methanolic solution of ammonia at room temperature on the glucoside *IV* afforded crystalline 1-β-D-glucopyranosyl-5-azacytosine (*VI*) in a good yield. Its structure was confirmed on comparison of the ultraviolet spectrum with that of the analogous 1-methyl-5-azacytosine (see Table I) and by acidic hydrolysis yielding 5-azacytosine and glucose. The prolonged action of the methanolic solution of ammonia led to a second product, namely, 1-β-D-glucopyranosyl-3-guanylurea (*VII*). This compound is obviously formed by cleavage of the triazine ring of the glucosyl derivative *VI*.



VII



XIX

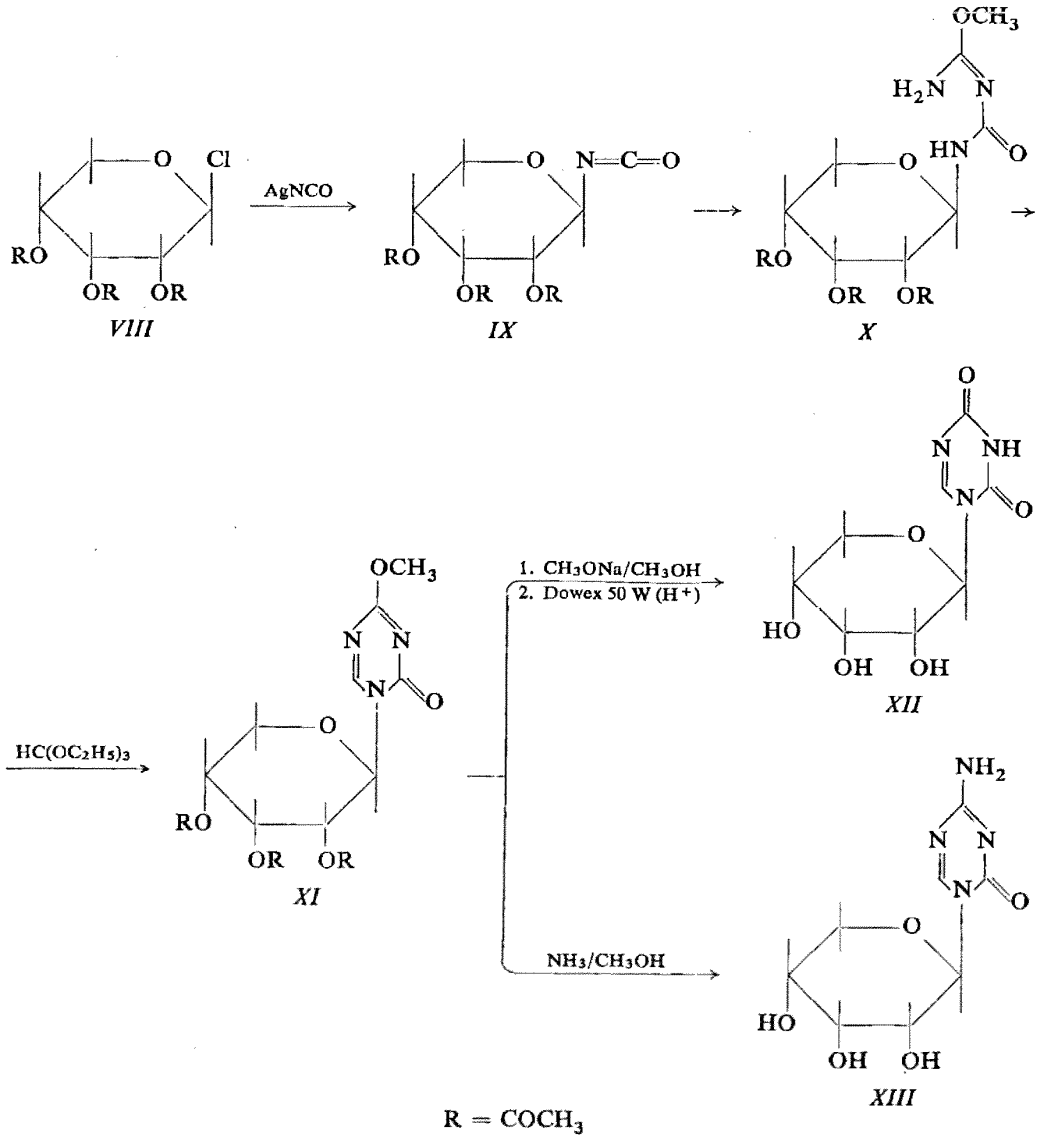


XXI

The above procedure was used also for the synthesis of 5-azauracil and 5-azacytosine ribopyranosyl derivatives. The starting 1-chloro-2,3,4-tri-O-acetyl-β-D-ribopyranose (*VIII*) was reacted with silver cyanate under similar conditions as in the preceding case. 2,3,4-Tri-O-acetyl-β-D-ribopyranosyl isocyanate (*IX*) was obtained as amorphous solid but its analysis as well as infrared spectrum, ν(N=C=O), 2252 cm^{-1}, closely resembling that of peracetylglucosyl isocyanate *II*, ν(N=C=O), 2253 cm^{-1}, both speak in favour of the supposed structure. Since in the reactions of halogenoses with silver cyanate the same steric course can be assumed as in the reactions with the

silver or mercury salts of pyrimidine and purine bases, we have used the rule of Baker[16] for allotment of the configuration at the glycosidic centre of the molecule. Treatment of the isocyanate *IX* with 2-methylisourea afforded a high yield of crystalline 1-(2,3,4-tri-O-acetyl-β-D-ribopyranosyl)-4-methylisobiuret (*X*) the structure of which was confirmed by the ultraviolet spectrum (see Table I). Condensation of this compound with ethyl orthoformate at 135°C resulted in crystalline 1-(2,3,4-tri-O-acetyl-β-D-ribopyranosyl)-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine (*XI*) the ultraviolet spectrum of which corresponded to the proposed structure (Table I).

Successive treatment of the ribosyl derivative *XI* with sodium methoxide and Dowex 50 W (H^+) ion exchange resin yielded a product to which the structure of



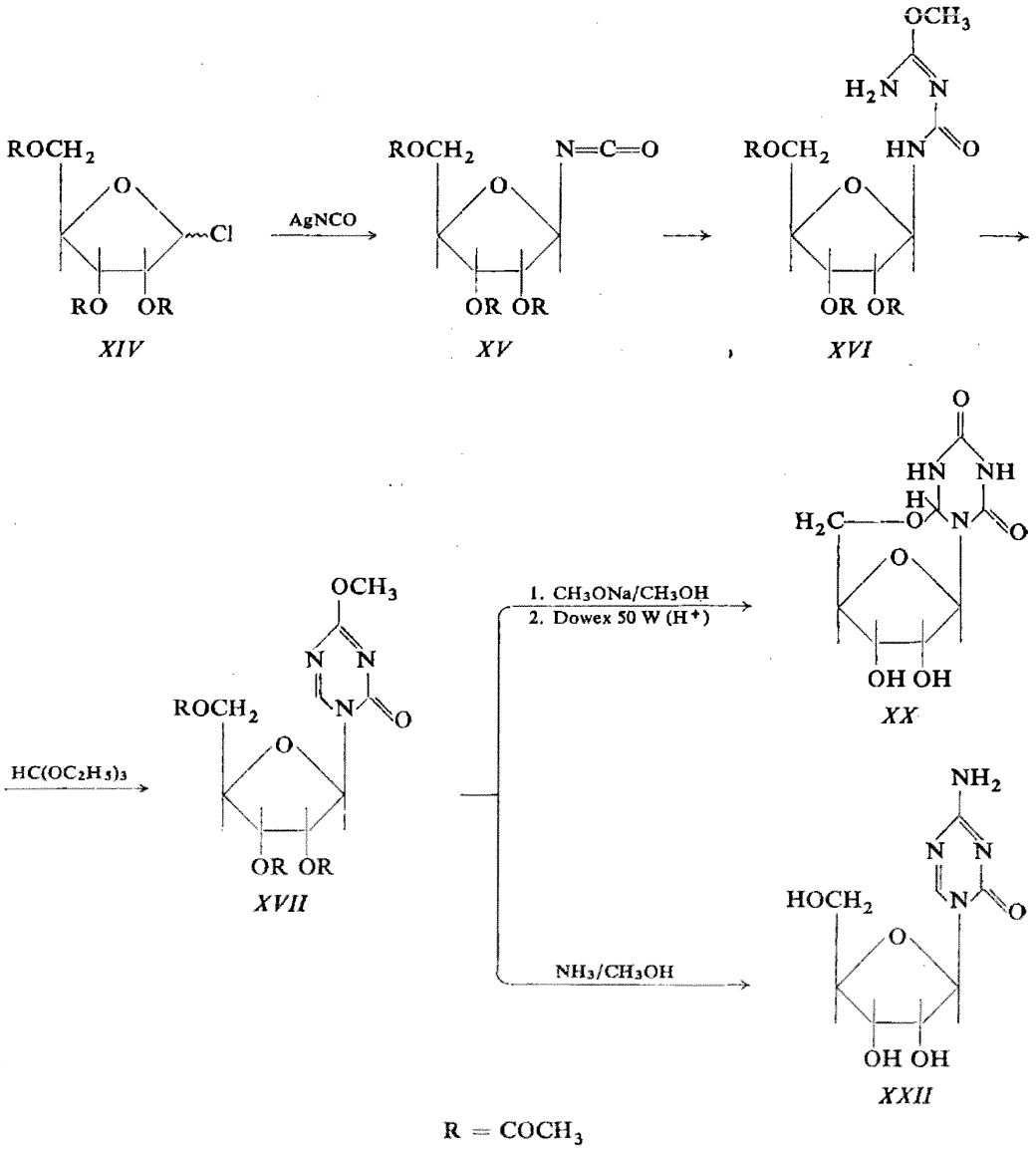
1-β-D-ribopyranosyl-5-azauracil (*XII*) was ascribed on the basis of the ultraviolet spectrum (see Table I). This compound was cleaved by the action of hydrochloric acid with the formation of ribose and 5-azauracil, and, furthermore, formed a solvate with ethanol.

Treatment of the compound *XI* with a methanolic solution of ammonia afforded the expected 1-β-D-ribopyranosyl-5-azacytosine (*XIII*) as crystals. The proposed structure was again confirmed by the ultraviolet spectrum (Table I) and acidic hydrolysis yielding ribose and 5-azacytosine.

Finally, the above method was applied also for the preparation of 5-azauracil and 5-azacytosine ribofuranosyl derivatives. The reaction of 1-chloro-2,3,5-tri-O-acetyl-D-ribofuranose (*XIV*) with silver cyanate was performed under similar conditions as in the preceding examples. 2,3,5-Tri-O-acetyl-β-D-ribofuranosyl isocyanate (*XV*) was obtained as sirup and was characterised by the infrared spectrum, ν(N=C=O), 2252 cm^{-1}. The β-configuration at the glycosidic centre of the molecule was ascribed on the basis of Baker's rule[16]. The proof of validity of this assumption follows also from the structure of ribofuranosyl-5-azauracil (*XX*) (*vide infra*). Treatment of the isocyanate *XV* with 2-methylisourea afforded crystalline 1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-4-methylisobiuret (*XVI*) the spectrum of which was similar to spectra of the preceding isobiurets (Table I). Condensation of this product with ethyl orthoformate at 135°C resulted in glassy 1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine (*XVII*) possessing the expected ultraviolet spectrum (Table I).

Deacetylation and demethylation of the ribofuranosyl derivative *XVII* was expected, in analogy to the treatment of the compounds *IV* and *XI*, to yield 1-β-D-ribofuranosyl-5-azauracil (5-azauridine; *XVIII*). The reaction was again performed by the successive treatment with sodium methoxide and Dowex 50 W (H^+) ion exchange resin. A crystalline product was obtained the elementary analysis of which corresponded to 5-azauridine. The substance, however, did not form any adduct with ethanol (in contrast to the analogous products *V* and *XII*) and, in a neutral medium, did not exhibit any maximum in the near ultraviolet region. At pH 5·0, the expected maximum (λ_{max} 241 mμ) was registered but the molar extinction coefficient was considerably lower (log ε 2·68) in comparison with other 1-substituted 5-azauracils. Moreover, the absorption disappeared rather quickly (no maximum could be registered after one hour). The infrared spectrum (in dimethyl sulfoxide) showed a broad band at approx. 1535 cm^{-1} (the triazine ring) and two carbonyl maxima at 1670 and 1715 cm^{-1}. No maximum at 1630 cm^{-1} (characteristic for the azomethine linkage[13]) could be found (also in nujol suspension). Oxidation with potassium periodate at pH 7·0 and room temperature consumed 1·5 mole of periodic acid per 1 mole of the ribosyl derivative in the course of 24 hours as determined polarographically[17] (the theory requires 1 mole of periodic acid). The higher value is obviously due to a subsequent oxidation. During the paper electrophoresis in a borate buffer (pH 7·0) the substance migrated to the anode similar to uridine. The

substance was readily cleaved with hydrochloric acid even at room temperature with the formation of ribose and 5-azauracil, and with dilute aqueous ammonia afforded 1-β-D-ribofuranosylbiuret (*XIX*). Spectral data showed that in neutral solutions and in the solid state the substance possessed no double bond in the position 5,6 of the azauracil moiety. This fact can be explained by the addition of a hydroxylic function to the azomethine linkage. Principally, any of the three hydroxylic functions in positions 2′, 3′ or 5′ can be involved intra- or intermolecularly. The possibility of a intermolecular addition can be precluded on the basis of the fact that both glucopyranosyl derivative *V* and ribopyranosyl derivative *XII* of 5-azauracil do not undergo such a reaction. Results of the oxidation with potassium periodate and the

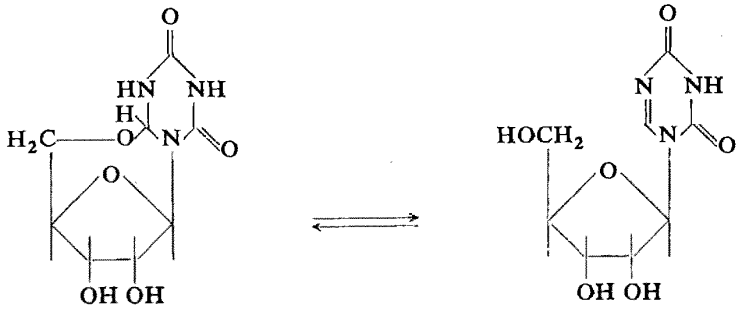


electrophoretic behaviour are in agreement with a free *cis*-diol arrangement on the carbon atoms 2′ and 3′. The intramolecular addition to the azomethine linkage is, therefore, accomplished with the primary alcoholic function and the substance may be formulated as 5′,6-anhydro-6-hydroxy-5,6-dihydro-5-azauridine (*XX*). The infrared spectrum of the substance is similar to that of the related 1,3,5-trimethyl-6-methoxy-2,4-dioxohexahydro-1,3,5-triazine[18] (*XXI*) measured in dimethyl sulfoxide (a broad band at 1520, 1680 and 1720 cm^{-1}); this fact is a further support of the proposed structure.

The spontaneous intramolecular addition of the primary hydroxylic function to the azomethine linkage represents simultaneously a proof of the β-configuration on the glycosidic centre of the molecule. Since glucosyl-5-azauracil *V* which also possesses a free primary alcoholic function does not nudergo this reaction, it may be assumed that the addition will be probably limited to furanosyl derivatives with β-configuration and a free hydroxylic function in the position 5′.

Both 5′,6-anhydro-6-hydroxy-5,6-dihydro-5-azauridine (*XX*) and ribosylbiuret *XIX* were identical with products forming in the medium in the course of cultivation of *E. coli* in the presence of 5-azauracil[1]. The identification was carried out by means of paper chromatography.

From the ultraviolet spectrum of the ribosyl derivative *XX* in a weakly acidic medium it could be assumed that the compound is partly transformed into 5-azauridine (*XVIII*) and that some equilibrium exists depending on the pH of the solution. This problem will be discussed in full detail elsewhere[15].



XX *XVIII*

Treatment of the ribofuranosyl derivative *XVII* with a methanolic solution of ammonia afforded readily crystalline 1-β-D-ribofuranosyl-5-azacytosine (5-azacytidine *XXII*). Structure of this compound was confirmed on comparison of ultraviolet (see Table I) and infrared (Fig. 1) spectra with those of the analogous 1-methyl-5-azacytosine. By means of hydrochloric acid, the product was cleaved into ribose and 5-azacytosine.

In summary it may be noted that the 5-azaanalogues of pyrimidine nucleosides differ in their properties very markedly from the pyrimidine as well as 6-azapyrimidine nucleosides, especially by the ease of cleavage of the N-glycosidic bond by the action

of mineral acids and by the instability of the triazine moiety in alkaline media. As quite exceptional may be regarded also the behaviour of the 5-azauracil ribofuranosyl derivative where an intramolecular addition of the primary alcoholic function occurs to the azomethine linkage.

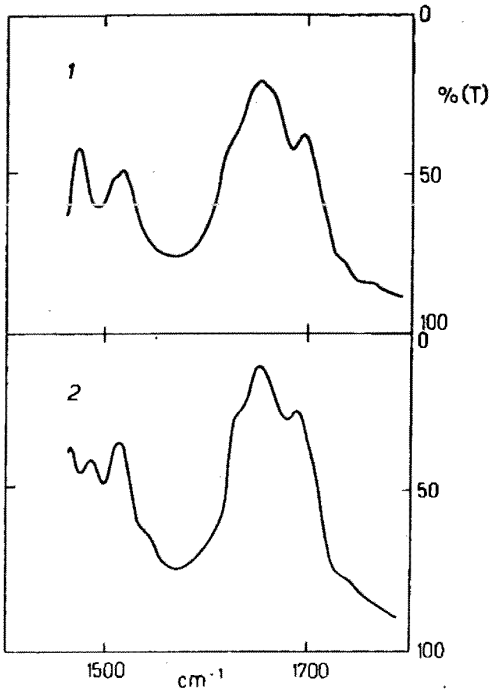


Fig. 1
Infrared Spectra of 1-Methyl-5-azacytosine (*1*) and 5-Azacytidine (*2*)
(Measured in Nujol Suspension)

Experimental

Melting points were determined on a heated microscope stage and are corrected. Unless otherwise stated, the analytical samples were dried at 0·3 mm Hg and room temperature for ten hours. Paper chromatography was performed on paper Whatman No 1 by the descending technique (without previous saturations) in solvent systems (S_1) 1-butanol–acetic acid–water (4 : 1 : 1) and (S_2) 1-butanol–ethanol–water (40: 11 : 19). Detection was carried out by viewing in ultraviolet light or according to Reindel and Hoppe[19]. The vicina *cis*-diol arrangement of the ribosyl derivatives was detected with potassium periodate and benzidine[20].

2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isocyanate (*II*)

α-Acetobromoglucose (4·11 g; 0·01 mole) was heated in dry xylene (20 ml) with thoroughly dried silver cyanate[21] (4·5 g; 0·03 mole) under efficient stirring and with exclusion of atmospheric moisture. The bath temperature was gradually increased to 100°C in the course of 30 minutes and the mixture was kept at this temperature for further 30 minutes. Additional two portions of silver cyanate (1·5 g; 0·01 mole) were then added in intervals of 30 minutes, the mixture was heated for 30 minutes, filtered off while still hot and the precipitate washed twice with 10-ml portions of hot xylene. The combined filtrates were cooled and precipitated with light petroleum (40 ml).

The supernatant was decanted (the residual precipitate was discarded), treated with additional 20 ml of light petroleum and a little active charcoal and the mixture was quickly filtered through a French filter. The clear filtrate deposited almost instantaneously the crystalline isocyanate. The next day, the product was collected, yield 2·3 g; m.p. 119 – 120°C (literature records[10] m.p. 120°C and 125°C (see[9]). Concentration of the mother liquors followed by crystallisation from the ethyl acetate–light petroleum solvent mixture afforded additional 0·37 g of the product. Total yield 2·55 g (70%). Infrared spectrum (chloroform): ν(N=C=O), 2253 cm^{-1}.

1-(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl)-4-methylisobiuret (*III*)

A stirred solution of 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isocyanate (3·73 g; 0·01 mole) in dry chloroform (50 ml) was slowly treated at −15°C under exclusion of atmospheric moisture with a solution of freshly prepared 2-methylisourea[22] (0·7 g; 9·5 millimoles) in chloroform (10 ml). The resulting solution was allowed to stand at room temperature for one hour and the chloroform was evaporated under diminished pressure (bath temperature 30°C). The residual sirup was dissolved in benzene (10 ml) and the solution treated with light petroleum (50 ml). The supernatant was decanted and discarded. The glassy residue was dissolved in hot benzene (10 ml), the solution allowed to cool and treated with light petroleum (50 ml). The resulting solid precipitate was repeatedly triturated under light petroleum and decanted. Yield 4·01 g (94%, based on 2-methylisourea), m.p. 90–100°C. Attempts to crystallise this product from various solvents failed. The R_F value 0·84 (the solvent system S_1), detected according to Reindel and Hoppe[19]. The ultraviolet spectrum is given in Table I. For $C_{17}H_{25}N_3O_{11}$ (447·3) calculated: 45·65% C, 5·64% H, 9·39% N; found: 45·48% C, 5·88% H, 9·06% N.

1-(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl)-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine (*IV*)

A solution of the isobiuret derivative *III* (4·47 g; 0·01 mole) in ethyl orthoformate (40 ml) was heated (bath temperature, 135°C) in distillation apparatus in a stream of pure dry nitrogen under exclusion of atmospheric moisture (potassium hydroxide tube) for eight hours. After five hours of heating, the crystalline condensation product began to deposit and clog the nitrogen inlet tube. For the remaining period of heating nitrogen was introduced to the surface of the mixture. After standing at −15°C overnight, the crystals were collected and washed with a small amount of ether. Yield 2·28 g (50%); m. p. 235–237°C. The melting point did not change on recrystallisation of the product from absolute ethanol. The ultraviolet spectrum is shown in Table I. For $C_{18}H_{23}N_3O_{11}$ (457·3) calculated: 47·27% C, 5·07% H, 9·19% N; found: 47·40% C, 5·09 H, 9·12% N.

1-β-D-Glucopyranosyl-5-azauracil (*V*)

A suspension of the methoxytriazine *IV* (4·57 g; 0·01 mole) in absolute methanol (300 ml) was allowed to stand at room temperature in a stoppered vessel with 50 ml of 0·5N sodium methoxide in methanol for 30 minutes. The resulting solution was treated with moist Dowex 50 W (H^+) ion exchange resin (100 g) previously washed with water and methanol. The mixture was kept at room temperature under occasional stirring for two hours, filtered off and the resin washed thoroughly with methanol. The combined filtrates were evaporated under diminished pressure (bath temperature 30°C) and the glassy residue was dissolved without any heating in 1000 ml of the ethanol. The resulting solution was concentrated under diminished pressure (bath temperature 30°C) to a small volume (30 ml). A solid was deposited in the course of the evaporation. The mixture was allowed to stand for three days at room temperature and for one day at +3°C. The crystalline product was collected and washed with a small amount of ethanol. Yield 2·41 g

(71%) of a chromatographically homogeneous product; the detection was performed by viewing in ultraviolet light and according to Reindel and Hoppe[19] (treatment of the paper with chlorine was prolonged for 30 minutes); the R_F value 0·11 (the solvent system S_1) and 0·08 (the solvent system S_2). The substance melted at 175—180°C (softening from 165°C). The analysis showed that glucosyl-5-azauracil *V* was solvated with ethanol and water. For $C_9H_{13}N_3O_7.C_2H_6O.H_2O$ (339·3) calculated: 38·94% C, 6·24% H, 12·39% N; found: 39·05% C, 6·20% H, 12·65% N. — When dried over P_2O_5 for 1 hour at 130°C/0·05 mm Hg, the substance loses the water. For $C_9H_{13}N_3O_7.C_2H_6O$ (321·3) calculated: 41·12% C, 5·96% H, 13·08% N; found: 41·30% C, 5·94% H, 13·28% N. Ethanol was removed by drying over phosphorus pentoxide at 130°C/0·05 mm Hg for ten hours. For $C_9H_{13}N_3O_7$ (275·2) calculated: 39·27% C, 4·76% H, 15·27% N; found: 38·94% C, 5·01% H, 15·03% N. The substance is sparingly soluble in ethanol and readily soluble in water. The recrystallisation was performed by dissolving the substance in a minimum amount water without any heating, addition of a tenfold volume of ethanol, inocculation and standing at +3°C overnight. The substance separated in the form of colorless fine needles solvated with ethanol and water; m.p. 183—185°C. The ultraviolet spectrum is shown in Table I.

The preparation of glucosyl-5-azauracil *V* from the methoxytriazine *IV* was accomplished also by the action of moist Dowex 50 W (H^+) ion exchange resin for 4 hours (*without* the previous removal of the protecting acetyl groups with sodium methoxide) or by treatment with an 1% solution of dry hydrogen chloride in absolute methanol for 4 hours (the hydrogen chloride was removed with silver carbonate). In both these procedures, the yield was lower than in the preparation described in the preceding paragraph. The action of methanolic hydrogen chloride was accompanied by the formation of 5-azauracil (R_F value 0·31) as shown by paper chromatography in the solvent system S_1.

Hydrolysis. Glucosyl-5-azauracil *V* (275 mg; 1 millimole) was heated in a steam bath with 1 ml of 6N-HCl for five minutes. The mixture was evaporated under diminished pressure, the residue was treated with ethanol (10 ml) and evaporated again. The residue was refluxed shortly with ethanol (2 ml) and the solution allowed to stand at +3°C overnight. The crystalline solvate of 5-azauracil with ethanol was collected and washed with ethanol. Drying over phosphorus pentoxide at 130°C/0·05 mm Hg for ten hours afforded 92 mg (81%) of 5-azauracil, m.p. 284—285°C (decomposition; sealed capillary) undepressed on admixture with an authentic specimen. The identification was further performed by comparison of the ultraviolet spectra (λ_{max} 237 mμ, log ε 3·5) and by paper chromatography. In the mother liquors, glucose was detected by means of paper chromatography. The cleavage with hydrochloric acid was found to proceed even at room temperature, though very slowly.

1-β-D-Glucopyranosyl-5-azacytosine (*VI*)

The methoxytriazine *IV* (4·57 g; 0·01 mole) was kept at room temperature for 90 minutes in a sealed tube with 50 ml of absolute methanol previously saturated at 0°C with dry ammonia gas. The mixture was shaken occasionally. In the course of 40 minutes, a clear solution was obtained. The solution was concentrated under diminished pressure (bath temperature 25°C) to a small volume (the amorphous product partly separated). The residue was triturated with absolute ether (300 ml) and the resulting powder was repeatedly decanted with additional crops of absolute ether (three 100-ml portions). The amorphous product was then collected, washed with ether and triturated with 80 ml abs. methanol. The substance gradually dissolved and separated again in the form of crystals. The mixture was allowed to stand at room temperature overnight and then filtered off. The product was washed with methanol and dried under diminished pressure. Yield 1·65 g (60%) of glucosyl-5-azacytosine *VI*, m.p. 258—260°C (decomposition). The ultraviolet spectrum is shown in Table I. The substance was chromatographically homogeneous and was detected in ultraviolet light or according to Reindel and Hoppe[19]; the R_F values 0·07 (the

solvent system S_1) and 0·05 (the solvent system S_2). The recrystallisation was performed by dissolving in a minimum amount of water (without heating), addition of a tenfold volume of methanol, inocculation and standing at +3°C overnight. The recrystallised substance melted at 260 to 262°C (decomposition). For $C_9H_{14}N_4O_6$ (274·2) calculated: 39·42% C, 5·15% H, 20·43% N; found: 39·33% C, 5·19% H, 20·15% N.

Hydrolysis. Glucosyl-5-azacytosine *VI* (274 mg; 1 millimole) was heated in a steam bath with 1 ml of 6N-HCl for five minutes. The mixture was allowed to cool, diluted with acetone (2 ml) and kept at room temperature overnight. The crystalline material was collected, washed with acetone a dried under diminished pressure to give 104 mg (70%) of 5-azacytosine hydrochloride. The identification was performed by comparison of the ultraviolet spectra (in 4N-HCl, λ_{max} 250 mμ, log ε 2·95) and by paper chromatography. The cleavage proceeds readily even at room temperature. Glucose was detected in the mother liquors.

1-β-D-Glucopyranosyl-3-guanylurea (*VII*)

The methoxytriazine *IV* (0·457 g; 1 mmole) was kept at room temperature with 5 ml of methanolic ammonia for 48 hours (*cf.* the preceding preparation). The solution was evaporated under diminished pressure (bath temperature 30°C), the residue was dissolved in methanol (50 ml) and the resulting solution evaporated again. The residue was triturated with methanol (4 ml) and allowed to stand overnight. The obtained crystals were collected, washed with methanol and dried under diminished pressure to give 0·12 g (41%) of the solvate of glucosylguanylurea *VII* with methanol as indicated by analysis; m.p. 201—203°C (decomposition). For $C_8H_{16}N_4O_6.CH_4O$ (296·2) calculated: 36·49% C, 6·81% H, 18·92% N; found: 36·33% C, 6·60% H, 18·94% N. The recrystallisation was performed by dissolving the substance in a minimum amount of water and treating the solution with a tenfold volume of hot ethanol. The resulting crystals melted at 207—209°C (decomposition) and represented the hydrate of glucosylguanylurea *VII*. For $C_8H_{16}N_4O_6.H_2O$ (282·2) calculated: 34·03% C, 6·42% H, 19·85% N; found: 34·04% C, 6·13% H, 20·00% N. The solvated water was removed on heating over phosphorus pentoxide at 100°C/0·05 mm Hg for 8 hours. For $C_8H_{16}N_4O_6$ (264·2) calculated: 36·36% C, 6·11% H, 21·20% N; found: 36·48% C, 6·35% H, 20·90% N. The substance exhibited no absorption in the near ultraviolet region. The paper chromatograms were detected according to Reindel and Hoppe[19]. The R_F values: 0·14 (the solvent system S_1) and 0·11 (the solvent system S_2). Some glucosyl-5-azacytosine *VI* was detected in the mother liquors by means of paper chromatography.

2,3,4-Tri-O-acetyl-β-D-ribopyranosyl isocyanate (*IX*)

Dry hydrogen chloride was introduced with cooling and under exclusion of atmospheric moisture into a suspension of finely ground 1,2,3,4-tetra-O-acetyl-β-D-ribopyranose (6·36 g; 0·02 mole) in absolute ether (100 ml) and acetyl chloride (1 ml) for two hours. The stoppered flask was allowed to stand at +3°C for five days. The solution was evaporated under diminished pressure (bath temperature 20°C) to the consistence of a thick sirup. The residual hydrogen chloride and acetic acid were removed by repeated codistillations (*in vacuo*) with five 40-ml portions of dry toluene (bath temperature 30°C). The colorless sirup was diluted with absolute ether (10 ml) to deposit immediately the crystalline 1-chloro-2,3,4-tri-O-acetyl-β-D-ribopyranosyl chloride (*VIII*). The mixture was allowed to stand at −15°C overnight, filtered off, the product washed with a small amount of absolute ether and dried in a desiccator over potassium hydroxide. Yield 4·9 g (83%); m.p. 93—95°C (the literature records[23] m.p. 95°C).

The above halogenose *VIII* (2·95 g; 0·01 mole) was heated with the thoroughly dried silver cyanate[21] (4·5 g; 0·03 mole) in 20 ml of dry xylene under similar conditions as in the case of glucosyl isocyanate *II*. When the reaction was finished, the silver salts were removed by filtration and washed with two 10-ml portions of hot xylene. The combined filtrates were cooled and

precipitated with dry light petroleum (80 ml). The mixture was allowed to stand in a stoppered vessel at +3°C overnight. The clear colorless supernatant was decanted (the residual glassy precipitate was discarded) and evaporated under diminished pressure (bath temperature 45°C) to the consistence of a thick colorless sirup which was dried at 0·3 mm Hg for ten hours. Yield 2·4 g (80%) of the glassy isocyanate *IX*. The analytical sample was dissolved in ether, reprecipitated with a fivefold volume of light petroleum and dried under diminished pressure. The infrared spectrum (chloroform): ν(N=C=O), 2252 cm^{-1}. For $C_{12}H_{15}NO_8$ (301·8) calculated: 47·87% C, 5·02% H, 4·65% N; found: 48·30% C, 5·10% H, 4·75% N.

1-(2,3,4-Tri-O-acetyl-β-D-ribopyranosyl)-4-methylisobiuret (*X*)

A solution of the crude ribopyranosyl isocyanate *IX* (3·01 g; 0·01 mole) in chloroform (50 ml) was gradually treated at −15°C with a solution of the freshly prepared 2-methylisourea (0·67 g; 9 mmole) in chloroform (10 ml). The solution was allowed to stand at room temperature for one hour and evaporated under diminished pressure (bath temperature 30°C). On addition of hot benzene (10 ml) to the residue the crystallisation occurred. The mixture was allowed to stand at room temperature overnight, the crystals were collected and washed with a small amount of benzene. Yield 3·1 g (92%, based on 2-methylisourea) of the isobiuret derivative *X*, m.p. 191−195°C. Recrystallisation of a concentrated solution from ethanol raised the m.p. to 194−196°C. The ultraviolet spectrum is shown in Table I. The R_F value in the solvent system S_1, 0·81 (detected according to Reindel and Hoppe[19]). For $C_{14}H_{21}N_3O_9$ (375·3) calculated: 44·80% C, 5·64% H, 11·20% N; found: 44·88% C, 5·59% H, 10·94% N.

1-(2,3,4-Tri-O-acetyl-β-D-ribopyranosyl)-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine (*XI*)

The isobiuret derivative *X* (3·75 g; 0·01 mole) was dissolved in hot ethyl orthoformate (30 ml) and the solution worked up similarly as in the case of the substance *IV*. After 8 hours, the colorless solution was cooled, precipitated with light petroleum (80 ml) and the semisolid precipitate treated with hot benzene (20 ml). The crystalline slurry was allowed to stand at room temperature overnight. The next day, the product was collected and washed with a small amount of benzene. Yield 2·14 g (55%) of the ribosyltriazine *XI*, m.p. 194−198°C. Recrystallisation from absolute ethanol raised the m.p. to 195−198°C. The ultraviolet spectrum is shown in Table I. For $C_{15}H_{19}N_3O_9$ (385·3) calculated: 46·75% C, 4·97% H, 10·91% N; found: 47·00% C, 4·86% H, 10·77% N.

1-β-D-Ribopyranosyl-5-azauracil (*XII*)

The methoxytriazine *XI* (3·85 g; 0·01 mole) in absolute methanol (300 ml) was deacetylated and demethylated similarly as in the case of glucosyl-5-azauracil *V*. The ion exchange resin was removed by filtration and the filtrate was evaporated under diminished pressure (bath temperature 30°C). The residue was dissolved (without heating) in ethanol (600 ml) and the solution concentrated under diminished pressure to a small volume (bath temperature 30°C). A small amount of an amorphous precipitate separated. The residue was triturated with ether (300 ml), the powder repeatedly decanted with three 100-ml portions of ether, collected, washed with ether and dried under diminished pressure. Yield 1·60 g (55%) of a white powder, m.p. 145−150°C, analyzing as solvate of ribopyranosyl-5-azauracil with ethanol. For $C_8H_{11}N_3O_6.C_2H_6O$ (291·3) calculated 41·23% C, 5·88% H, 14·43% N; found: 41·26% C, 6·05% H, 14·15% N. Removal of the ethanol was accomplished by heating the substance at 130°C/0·05 mm Hg for ten hours. For $C_8H_{11}N_3O_5$ (245·2) calculated: 39·19% C, 4·52% H, 17·14% N; found: 38·95% C, 4·65% H, 16·90% N. The product was chromatographically homogeneous and was detected by viewing in ultraviolet light, according to Reindel and Hoppe[19] (the treatment with chlorine was prolonged for 30 minutes),

and, finally, with potassium periodate[20]; the R_F values, 0·18 (the solvent system S_1) and 0·12 (the solvent system S_2). The ultraviolet spectrum see in Table I.

Hydrolysis. Ribopyranosyl-5-azauracil *XII* (245 mg; 1 mmole) was hydrolysed with 6N-HCl in the same manner as glucosyl-5-azauracil *V*. Yield 85 mg (75%) of 5-azauracil. By means of paper chromatography, ribose was detected in the mother liquors. The cleavage with hydrochloric acid takes place also at room temperature, but very slowly.

1-β-D-Ribopyranosyl-5-azacytosine (*XIII*)

The methoxytriazine *XI* (3·85 g; 0·01 mole) was kept at room temperature in a sealed vessel for 90 minutes with 50 ml of absolute methanol previously saturated (at 0°C) with dry ammonia. The resulting solution was evapoareted under diminished pressure (bath temperature 25°C) and the residue was triturated with absolute ether (200 ml). The amorphous powder was repeatedly decanted with absolute ether (three 100-ml portions) and dissolved in absolute methanol (50 ml). The solution which was allowed to stand at room temperature overnight, deposited crystalline ribopyranosyl-5-azacytosine *XIII*. The crystals were collected, washed with a small amount of methanol and dried under diminished pressure to give 1·44 g (59%) of the product, m.p. 248 to 250°C (decomposition). The ultraviolet spectrum is shown in Table I. The substance was chromatographically homogeneous and was detected by viewing in ultraviolet light, by the method of Reindel and Hoppe[19] and with potassium periodate[20]; the R_F values, 0·10 (the solvent system S_1) and 0·07 (the solvent system S_2). The recrystallisation was performed by dissolving the substance in a minimum amount of water (without heating), addition of a tenfold volume of methanol, inocculation and standing at +3°C overnight. The recrystallised product melted at 250—252°C (decomposition). For $C_8H_{12}N_4O_5$ (244·2) calculated: 39·34% C, 4·95% H, 22·94% N; found: 39·08% C, 5·09% H, 22·84% N.

Hydrolysis. Ribopyranosyl-5-azacytosine *XIII* (244 mg; 1 mmole) was hydrolysed with 6N-HCl in analogy with the hydrolysis of the glucosyl derivative *VI*. Yield 97 mg (65%) of 5-azacytosine hydrochloride. By paper chromatography, ribose was detected in the mother liquors. The cleavage proceeds readily even at room temperature.

2,3,5-Tri-O-acetyl-β-D-ribofuranosyl Isocyanate (*XV*)

Dry hydrogen chloride was introduced with cooling for 4 hours into a suspension of finely ground 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose[24] (31·8 g; 0·1 mole), absolute ether (500 ml) and acetic anhydride (2 ml). The mixture was allowed to stand in a stoppered vessel at +3°C for five days. The resulting solution was evaporated under diminished pressure at 20°C (bath temperature) and the residual sirup was repeatedly coevaporated (bath temperature 30°C) with five 100-ml portions of dry toluene. Finally, the yellowish sirup was dissolved in dry xylene (300 ml) and the resulting solution was treated with silver cyanate[21] (45·0 g; 0·3 mole) similarly as in the case of the glucosyl isocyanate *II*. The silver salts were removed by filtration and washed with two 50-ml portions of dry xylene. The combined filtrates were cooled and treated with dry light petroleum (1000 ml); a small amount of a glassy precipitate separated. The mixture was allowed to stand at +3°C in a stoppered flask overnight. The clear supernatant was decanted and evaporated under diminished pressure (bath temperature 45°C). The yellowish sirup was dried at room temperature and 0·3 mm Hg for ten hours to give 19·2 g of the product contaminated with a small amount of 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose (see the preparation of the isobiuret derivative *XVI*). Infrared spectrum (chloroform): ν(N=C=O), 2252 cm^{-1}.

1-(2,3,5-Tri-O-acetyl-β-D-ribofuranosyl)-4-methylisobiuret (*XVI*)

A stirred solution of the crude ribofuranosyl isocyanate *XV* (15·0 g) in chloroform (200 ml) was treated at −15°C under exclusion of atmospheric moisture with a thin stream of the freshly

prepared 2-methylisourea (2·95 g; 0·04 mole) in chloroform (50 ml). The solution was allowed to stand at room temperature for one hour and evaporated under diminished pressure (bath temperature 30°C). The residue was dissolved in dry benzene (50 ml) and reprecipitated with dry light petroleum (200 ml). After decantation, the yellowish sirup was dissolved in additional 50 ml of benzene and reprecipitated with light petroleum (200 ml). The residual solvents were removed under diminished pressure at room temperature (8 hours). The resulting faintly yellowish foam (14·3 g, *i.e.* 95% based on 2-methylisourea) was dissolved in 30 ml of hot dry benzene and the solution was allowed to stand at room temperature for six days. The crystalline product (10·2 g; 68%) was collected and washed with a small amount of benzene; m.p. 70—76°C. The recrystallisation from absolute ethanol raised the m.p. to 72—78°C. Further crystallisation did not change this m.p. The ultraviolet spectrum is shown in Table I. The R_F value in the solvent system S_1, 0·79 (detected according to Reindel and Hoppe[19]). For $C_{14}H_{21}N_3O_9$ (375·3) calculated: 44·80% C, 5·64% H, 11·20% N; found: 44·59% C, 5·67% H, 11·21% N. For the next synthetic step, the crude reprecipitated product may be used (without any crystallisation).

The supernatants after precipitation of the crude isobiuret derivative *XVI* were combined and concentrated to a volume of 10 ml. On standing at room temperatures for four days in a open vessel, the sirup deposited developed crystals of 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose (1·2 g) melting at 81—82°C without depression on admixture with an authentic specimen.

1-(2,3,5-Tri-O-acetyl-β-D-ribofuranosyl)-4-methoxy-2-oxo-1,2-dihydro-1,3,5-triazine (*XVII*)

A solution of the isobiuret derivative *XVI* (15·0 g; 0·04 mole) in ethyl orthoformate (100 ml) was heated under same conditions as in the case of the glucosyl derivative *IV*. After 8 hours, the colorless solution (when crude isobiuret was used, the solution was faintly yellow) was cooled and precipitated with 500 ml of dry light petroleum. The glassy precipitate was dissolved in hot absolute benzene (50 ml) and the solution was precipitated with light petroleum (200 ml). The precipitation was repeated once more and the product was dried *in vacuo*. Yield 9·7 g (63%) of a solid foam. The ultraviolet spectrum is shown in Table I. The analytical sample was dissolved in ethyl acetate and precipitated with light petroleum. For $C_{15}H_{19}N_3O_9$ (385·3) calculated: 46·75% C, 4·97% H, 10·91% N; found: 47·10% C, 4·99% H, 10·50% N.

5′,6-Anhydro-6-hydroxy-5,6-dihydro-5-azauridine (*XX*)

The methoxytriazine *XVII* (3·85 g; 0·01 mole) was deacetylated and demethylated in 300 ml of absolute methanol by the procedure described for the case of glucosyl-5-azauracil *V*. The treatment with the ion exchange resin was prolonged for 3 hours. The resin was removed by filtration, washed with methanol and the combined filtrates were evaporated under diminished pressure (bath temperature 25°C). The residual sirup was dissolved in acetone (50 ml). The resulting solution began to deposit the crystalline product almost immediately. After standing at +3°C overnight, the crystals were collected and washed with acetone and methanol. Yield 0·7 g (28%), m.p. 206—208°C (decomposition). The substance is insoluble in organic solvents and sparingly soluble in cold water. When heated with water, the substance is destroyed. The recrystallisation may be performed with a great loss from dilute acetic acid. The recrystallised substance melted at 210—211°C (decomposition). In the oxidation with potassium periodate[17], the substance consumed 1·5 mole of periodic acid in the course of 24 hours. The infrared spectrum (dimethyl sulfoxide): a broad band at 1535 cm^{-1} (the triazine ring); ν(CO), 1670 cm^{-1}, 1715 cm^{-1}. Paper electrophoresis (a borate buffer, pH 7·0, 30 min, 200 V/cm, Whatman paper No 1): 1·2 cm. In paper chromatography, the substance (a 5% solution of the substance in 50% acetic acid was applied) remained homogeneous; the R_F values, 0·26 (the solvent system S_1) and 0·23 (the solvent system S_2). Detections were performed with potassium periodate and benzidine[20], according to Reindel and Hoppe[19] (the action of chlorine was prolonged for 30 minutes) and, finally be viewing

in ultraviolet light (the spots absorbed only weakly). For $C_8H_{11}N_3O_6$ (245·2) calculated: 39·19% C, 4·52% H, 17·14% N; found: 39·12% C, 4·50% H, 17·08% N.

Hydrolysis. The ribosyl derivative *XX* (123 mg; 0·5 mmole) was hydrolysed with 6N-HCl by the same procedure as in the case of glucosyl-5-azauracil *V*. Yield 40 mg (71%) of 5-azauracil. By paper chromatography, ribose was detected in the mother liquors. The cleavage proceeds readily even at room temperature.

1-β-D-Ribofuranosylbiuret (*XIX*)

A solution of the ribosyl derivative *XX* (245 mg; 1 mmole) in 2% ammonia in 50% aqueous methanol (30 ml) was allowed to stand at room temperature overnight. The solvents were evaporated under diminished pressure (bath temperature 30°C) and the residual sirup was dissolved in a minimum amount of 96% ethanol. The solution was allowed to stand at −15°C for three days. The crystals were collected, washed with ethanol and dried. Yield 96 mg (41%), m.p. 176−179°C. The product was chromatographically homogeneous; detection was performed with potassium periodate and benzidine[20] and according to Reindel and Hoppe[19]; the R_F values, 0·22 (the solvent system S_1) and 0·19 (the solvent system S_2). The recrystallised sample melted at 180−181°C (water). For $C_7H_{13}N_3O_6$ (235·2) calculated: 35·74% C, 5·57% H, 17·88% N; ound: 35·58% C, 5·71% H, 17·63% N.

5-Azacytidine (*XXII*)

The methoxytriazine *XVII* (3·85 g; 0·01 mole) was kept at room temperature in a sealed vessel for 90 minutes with 30 ml of absolute methanol previously saturated (at 0°C) with dry ammonia. The substance dissolved almost immediately and, in the course of 25 minutes, the crystalline 5-azacytidine began to separate. The mixture was allowed to stand at −15°C overnight. The next day, the crystals were collected, washed with methanol, triturated with methanol, collected again, washed and dried over sulfuric acid and potassium hydroxide in a vacuum desiccator. Yield 1·4 g, m.p. 228−230°C (decomposition). A further crop (0·15 g) of the product was obtained on evaporation of the mother liquors under diminished pressure and crystallisation of the residue from 2 ml of absolute methanol. Total yield, 1·55 g (64%). The ultraviolet spectrum is shown in Table I. The infrared spectrum may be seen on Fig. 1. The substance was chromatographically homogeneous. Detection was performed by viewing in ultraviolet light or according to Reindel and Hoppe[19] or with potassium periodate-benzidine[20]. The R_F values, 0·14 (the solvent system S_1) and 0·11 (the solvent system S_2). For $C_8H_{12}N_4O_5$ (244·2) calculated: 39·34% C, 4·95% H, 22·94% N; found: 39·45% C, 5·02% H, 23·11% N. The substance was recrystallised by dissolving in water on a steam bath (5 ml of water per 1 g), addition of hot methanol (30 ml of methanol per 1 g) and standing at +3°C overnight; m.p. 230−231°C (decomposition).

Hydrolysis. 5-Azacytidine (112 mg; 0·5 mmole) was hydrolysed with 6N-HCl by the procedure as in the case of glucosyl-5-azacytosine *VI*. The time of heating was prolonged for 15 minutes (after 5 minutes, some starting compound was detected in the mixture in addition to 5-azacytosine, as shown by paper chromatography). In the mother liquors, ribose was detected by means of paper chromatography.

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Резюме

А. Пискала и В. Шорм: *Составляющие нуклеиновых кислот и их аналоги. LI. Синтез 1-гликозильных производных 5-азаурацила и 5-азацитозина*. Описан метод получения 1-гликозильных производных 5-азаурацила и 5-азацитозина, исходящий из перацетилгликозилизоцианатов. Присоединением 2-метилизомочевины полученные 1-перацетилгликозил-4-метилизобиуреты конденсируются с ортомуравьиноэтиловым эфиром с образованием 1-перацетилгликозил-4-метокси-2-оксо-1,2-дигидро-1,3,5-триазинов, которые затем дезацетилированием и деметилированием превращаются в 1-гликозил-5-азаурацилы или дезацетилированием и аминированием в 1-гликозил-5-азацитозины. Таким образом были получены глюкопиранозильное, рибопиранозильное и рибофуранозильное производные 5-азаурацила и глюкопиранозильное, рибопиранозильное и рибофуранозильное производные 5-азацитозина

Direct synthesis of 5-azapyrimidine ribonucleosides

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ABSTRACT

The ribosylation of the silver salts of 5-azapyrimidine nucleobases III and IV as well as the triazines VII, XIII and XVI has been investigated.

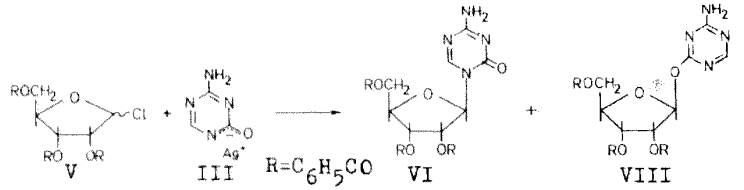
INTRODUCTION

The first 6-azaanalogues of pyrimidine nucleosides have been prepared by microbial processes[1]. An attempt at fermentative preparation[2] of 5-azauridine (I) failed, however, due to the instability of this nucleoside in aqueous medium. The first successful preparation of 5-azapyrimidine nucleosides has been carried out in 1963 by an unconventional general synthesis[3-5]. The most important nucleoside of this group is the cytostatically highly active 5-azacytidine (II). Its inhibitory effects have been described for the first time with a synthetic specimen[6]. Two years later it has been found that the antibiotic U-18,496 (ladakamycin) isolated from the medium of a culture of Streptoverticillium ladakanus was identical with 5-azacytidine[7-9]. In recent years considerable attention has been devoted to the biological effects, as well as to the mechanism of action of 5-azacytidine[1,10]. The preparation has also been used clinically against acute lymphoblastic and nyeloblastic leukemia[11-14]. Direct glycosylation of the silylated 5-azacytosine[15-17] also has been used for the preparation of 5-azacytidine and related nucleosides. In this communication we present our experiences with the preparation of the ribonucleosides of 5-azacytosine and 5-azauracil by direct glycosylation.

RESULTS

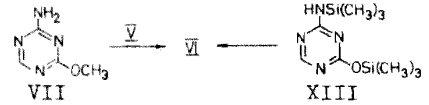
When the silver salt of 5-azacytosine (III) was boiled

with halogenose V in acetonitrile a reaction mixture was obtained from which we isolated 2',3',5'-tri-O-benzoyl-5-azacytidine (VI) in 8% yield. This was identified by comparison with

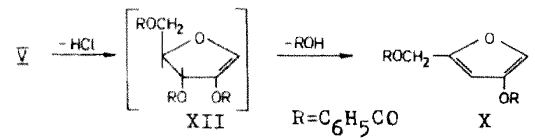


a known product[15]. In addition to this we also isolated an amorphous isomer in 15% yield, to which we assign the structure VIII on the basis of the similarity of its infrared spectrum in chloroform with the spectrum of triazine VII.

On boiling halogenose V with triazine VII in acetonitrile we obtained tribenzoate VI in 40% yield. In addition to this we also isolated an isomeric product [m.p.236-237°C (acetonitrile)] to which we assigned the structure of 1-(2,3,5-tri-O-benzoyl-α-D-ribofuranosyl)-5-azacytosine (IX) on the ba-

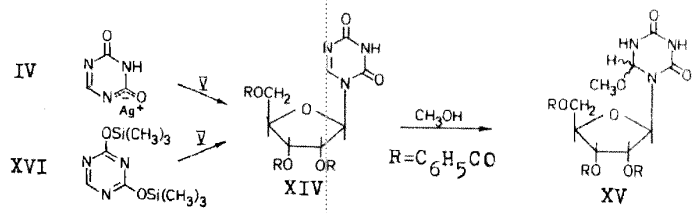


sis of spectral data. Further we isolated from this reaction furan X [m.p.75-76°C (ethanol)] in low yield (this compound has been prepared recently by an independent way[18]) and 1,2-3,5-tetra-O-benzoyl-β-D-ribofuranose (XI) which we identified by comparison with an authentic sample[19]. The formation of compounds X and XI from halogenose V may be explained by elimination of hydrogen chloride and benzoic acid and subsequent reaction of the benzoic acid set free with the still unreacted halogenose V. The instable ribofuranosene XII is formed evi-



dently as an intermediate. In connection with this we found that the side-product of the ribosylation of pyrimidines to which Prystaš[20] assigned the structure XII is in actual fact

identical with tetrabenzoylribofuranose XI which has a very close elemental composition. This conclusion followed from the agreement of the infrared spectra of authentic samples, in which the double bond absorption band is completely absent, as well as from the identity of the melting points and specific rotations measured under comparable conditions. The incorrectness of the structure XII, proposed for the side-product of ribosylation[20], was also recently indicated by Ferrier and Hurford[18] on the basis of $[\alpha]_D$ value of the enantiomeric product as well as on the basis of the well know instability of this type of compounds. In view of these facts it is very probable that the analogous derivatives of xylofuranose[21] and psicofuranose[22], will not possess the structure of furanosenes, but that also in these cases the compounds formed will be the corresponding tetrabenzoyl derivatives of xylofuranose or psicofuranose. We achieved a high yield (80%) of tribenzoate VI in the reaction of silylated 5-azacytosine XIII with halogenose V in acetonitrile in the presence of mercuric chloride as catalyst. A high yield of VI was also achieved when this reaction was carried out without the mercuric salts.



On boiling the silver salt IV with halogenose V in toluene we obtained a methanolic adduct of tribenzoyl-5-azauridine (XV) (m.p.152-158°C) in 40% yield by crystallization from methanol. We suppose a covalent bond with methanol in the crystalline adduct XV on the basis of the analogy with the behaviour of 1-methyl-5-azauracil[23]. Tribenzoate XV could also be obtained in 30% yield on reaction of halogenose V with the silylated 5-azauracil XVI in acetonitrile. Methanolysis of tribenzoate XV gave the known 5-azauridine[5] of m.p. 204-205°C (dec.) in 80% yield.

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[76] 4-AMINO-1-β-D-RIBOFURANOSYL-s-TRIAZIN-2(1H)-ONE (5-AZACYTIDINE)

Direct Synthesis of a 5-Azapyrimidine Ribonucleoside by the Trimethylsilyl Procedure

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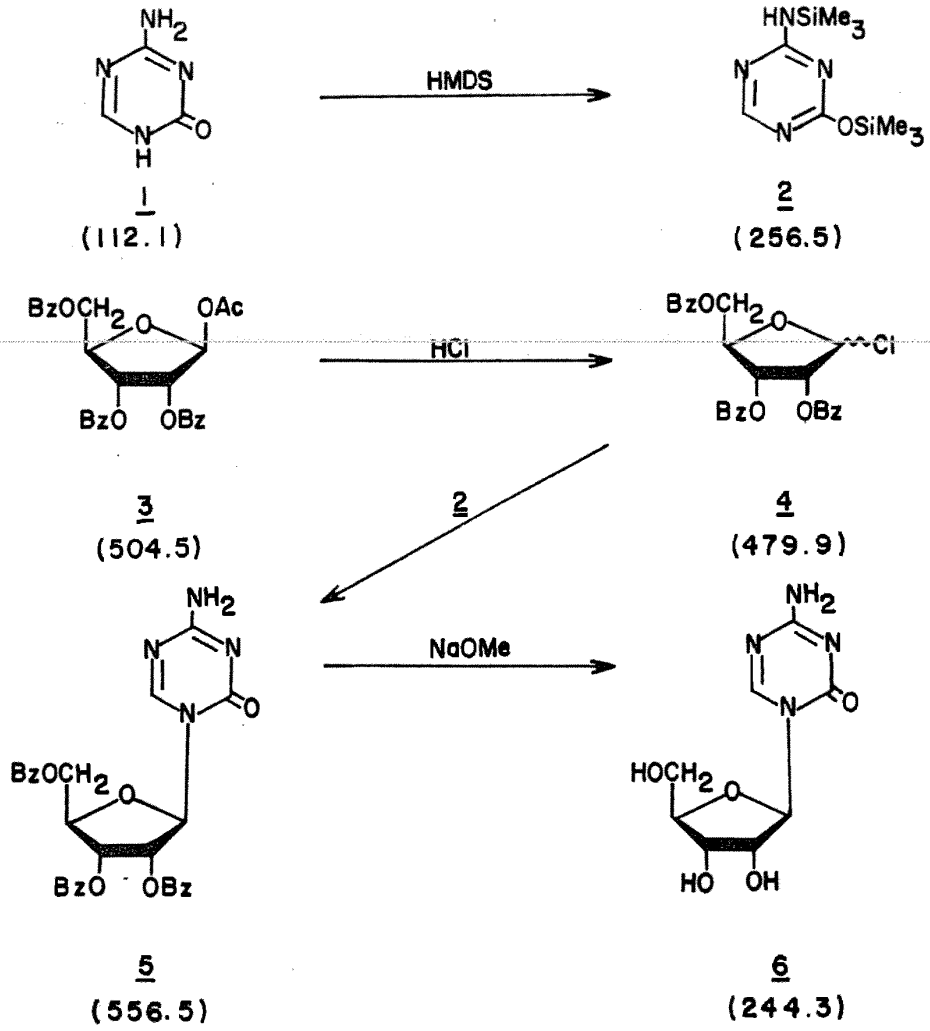
INTRODUCTION

In view of its high biological activity, 5-azacytidine (*6*) is among the most potent cytostatics. The best results have thus far been obtained in applications against the acute leukemia of children.[1] The present procedure exemplifies the use of the general trimethylsilyl method in the field of 5-azapyrimidine nucleosides,[2] and also includes an improved preparation of 2,3,5-tri-*O*-benzoyl-D-ribosyl chloride. In the synthesis of 5-azacytidine (*6*), some additional modifications of the trimethylsilyl process have recently been used.[3-5]

PROCEDURE

2-(Trimethylsilylamino)-4-(trimethylsilyloxy)-s-triazine (2)

A mixture of 5-azacytosine[6] (*1*) (11.2 g, 0.1 mol), hexamethyldisilazane (35 ml), and powdered ammonium sulfate (0.2 g) is heated



(oil bath, 160° to 170°) under a reflux condenser (potassium hydroxide tube) for 8 hr, the starting material dissolving in the course of 3 to 5 hr. The excess of hexamethyldisilazane is evaporated *in vacuo* (water pump) in a rotatory evaporator at 55° to 60° (bath temperature). The crystalline residue is thoroughly triturated with dry toluene (50 ml) and the solvent evaporated

under diminished pressure. The residue is powdered and dried *in vacuo* (water pump) in a rotatory evaporator at 55° to 60° for 1 to 2 hr; yield 25.0 to 25.4 g (97% to 99%) of compound 2, m.p. 128° to 130° (dec.; sealed capillary). An analytical sample (same m.p.) is obtained (loss, 2% to 5%) by sublimation at 100°/0.1 torr; λ_{max}^{MeCN} 224 nm, log ε (4.00), 246 (sh) nm (log ε 3.62); ν_{max}^{Nujol} 3165 (N-H), 1056 (O-Si), 912 (N-Si), and 847 cm^{-1} (C-Si).

2,3,5-Tri-O-benzoyl-D-ribosyl Chloride (4)

A solution of 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribose[a] (3) (20.16 g, 0.04 mol) in a mixture of dry 1,2-dichloroethane (80 ml) and acetyl chloride (1 ml) is saturated for 2 hr with dry gaseous hydrogen chloride under exclusion of atmospheric moisture and with external ice-cooling. The solution is kept (calcium chloride tube) overnight at room temperature, and evaporated under diminished pressure (water pump) in a rotatory evaporator at 35° to 40° (bath temperature). The almost colorless residue is coevaporated under diminished pressure at 35° to 40° with dry toluene (2 × 50 ml) to remove the remaining acetic acid. The crude chloride

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[a] For its preparation, see E. F. Recondo and H. Rinderknecht, *Helv. Chim. Acta*, 42, 1171 (1959).

4 is used directly in the next step.[b]

4-Amino-1-(2,3,5-tri-O-benzoyl-β-D-ribosyl)-s-triazin-2(1H)-one (5)

A mixture of the crude chloride 4 (prepared from 20.16 g, 0.04 mol of compound 3), dry acetonitrile (50 ml), and the trimethylsilyl derivative 2 (10.3 g, 0.04 mol) is stirred with an efficient magnetic stirrer for 30 min to 2 hr at room temperature in a tightly stoppered flask until the contents solidify. The mixture is kept overnight at room temperature, broken (in the flask) with a spatula into small pieces, and triturated with dry 1,2-dichloroethane (100 ml) under exclusion of atmospheric moisture. The strongly turbid solution resulting is run in a thin stream from a separatory funnel into 150 ml of a vigorously stirred, ice-cooled, 5% aqueous solution of sodium hydrogen carbonate, and stirring is continued for 10 min. The mixture is filtered through a layer of Celite, and the material on the filter is washed with chloroform.[c] The organic layer of the filtrate is separated, briefly (5 to

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[b] When kept in a tightly stoppered flask, the chloride 4 may be stored in a refrigerator for several weeks without any substantial decomposition. It is, however, recommended that freshly prepared material be used. The crude product 4 usually contains only 2% to 4% of the starting material. When chloride 4 is prepared by the conventional procedure in ether at low temperature, the yields in the subsequent step of the present synthesis are 15% to 20% lower.

[c] The filtration is accompanied by the formation of a sticky layer, which should be systematically disturbed by means of a spatula. To avoid hydrolysis of the product, the filtration should be as brief as possible.

10 min) dried (anhydrous sodium sulfate) and filtered, and the filtrate is evaporated under diminished pressure in a rotatory evaporator at 35° to 40° (bath temperature). The resulting thick syrup is dissolved in boiling and dry ethanol (40 ml), and the solution is nucleated and kept in a stoppered flask for 2 days at room temperature to deposit the product.[d] The crystals are collected with suction, washed successively with ethanol and ether, and dried under diminished pressure; yield,[e] 14.5 to 15.6 g (65% to 70%) of compound 5, m.p. 182° to 184° (dec.). The product is sufficiently pure for use in the next step. Recrystallization from ethanol affords an analytical sample, m.p. 186° to 187° (dec.), $[\alpha]_{\underline{\underline{D}}}^{25}$ -49.24° (c 0.526; $HCONMe_2$); ^{1}H-NMR data (Me_2SO-d_6; Me_4Si): δ 8.44 (s, 1, H-6), 6.05 (m, 3, H-1',2',3'), and 4.69 (m, 3, H-4',5',5').

4-Amino-1-β-D-ribofuranosyl-s-triazin-2(1H)-one (5-Azacytidine) (6)

A mixture of compound 5 (0.01 mol) and absolute methanol (45 ml) is heated to the boiling point, treated with M methanolic sodium methoxide (1 ml), and thoroughly swirled. The starting material rapidly dissolves, and the solution immediately begins to deposit

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[d] The turbidity, if any, may be removed by moderate heating or by the addition of a small volume of benzene.

[e] When the yield is lower than stated, some additional product (5% to 7%) may by obtained by evaporation of the mother liquor and by successive crystallization of the residue from benzene—ether and then from ethanol.

the product. The mixture is kept in a stoppered vessel for 4 hr at room temperature, and then overnight in a refrigerator. The crystals are collected with suction, washed with ice-cold methanol, and dried under diminished pressure at room temperature; yield, 2.60 to 2.64 g (94% to 96%) of the methanol solvate of compound *6*, m.p. 234° to 235° (dec.). The methanol of solvation may be removed by drying to constant weight at 110°/0.1 torr. Recrystallization[f] from 1:1 water—acetone (25 ml per g of 5-azacytidine) affords (loss, 15% to 20%) the hydrate,[g] having the same m.p. as the methanol solvate. Drying at 110°/0.1 torr affords anhydrous *6*, m.p. 232° to 233° (dec.)[h] $[\alpha]_{\underline{D}}^{25}$ +68.3 (5 min) ⟶ -16.0° (24 hr) (*c* 0.501; water); λ_{max}^{MeOH} 244 nm (log ε 3.84); CD (pH 7.1): λ 249 nm, [⊜] +11600 ⟶ λ 252 nm, [⊜] +5300 (24 hr); ^{1}H-NMR data (D_2O, DSS): δ 8.56 (s, 1, H-6), 5.76 (d, 1, H-1'), 4.37 (dd, 1, H-2'), 4.20 (m, 1, H-3'), 4.15 (m, 1, H-4'), 3.77 (dd, 1, H-5'$_B$) and 3.96 (dd, 1, H-5'$_C$), $J_{1',2'}$ 3.0 Hz; $J_{2',3'}$ 4.5 Hz; $J_{4',5'_B}$ 2.4 Hz; $J_{4',5'_C}$ 3.8 Hz; $J_{5'_B,5'_C}$ -13 Hz.

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[f]The yellow color, if any, may be removed by the addition of a small amount of active charcoal prior to the filtration. The preparation of both the solution and the filtration should be effected as quickly as possible to avoid hydrolysis of 5-azacytidine. The crystallization is completed by keeping the mixture overnight at -25°.

[g]After being dried at room temperature under diminished pressure, the product contains from 5.6% to 6.2% of water.

[h]When protected against atmospheric moisture, the product is almost completely stable.

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BLOOD

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5-Azacytidine (NSC 102816): A New Drug for the Treatment of Myeloblastic Leukemia

By William R. Vogler, Donald S. Miller, and James W. Keller

(Writing Committee for the Southeastern Cancer Study Group)

The pyrimidine analog, 5-azacytidine (NSC 102816), was administered by continuous intravenous infusion in Ringer's lactate in increasing doses to sets of patients with metastatic cancer to establish a dose sufficient to produce mild toxicity. Twenty-one patients (23 trials) were treated with doses of 50–200 mg/sq m/day for 5 days every 2–4 wk. Nausea and vomiting were moderate and easily preventable. Doses of 100–200 mg/sq m for 5 days every 14 days produced granulocytopenia, usually after two courses. Less toxicity was observed when courses were given every 21–28 days. Forty-five patients with previously treated and refractory acute myeloblastic leukemia were treated. The majority received doses of 150 mg/sq m for 5 days every 2 wk. Eleven (24%) complete remissions and four partial remissions were observed. The number of courses to achieve remission averaged three and required an average of 59 days. Nine patients with blastic crisis of chronic myeloblastic leukemia and four with refractory acute lymphoblastic leukemia failed to respond. 5-Azacytidine administered by continuous infusion is well tolerated and is an active compound in acute myeloblastic leukemia.

THE PYRIMIDINE ANALOG, 5-azacytidine (NSC 102816), has been shown to be an active compound in several animal and human neoplasms.[1-8] Its clinical usefulness has been hampered by severe nausea and vomiting and occasional diarrhea accompanying rapid intravenous injection.[9] It has been thought that the drug must be given by rapid intravenous infusion

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This paper was prepared on behalf of the Southeastern Cancer Study Group. See the Appendix for a list of participants in this study.

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because of its instability.[10] However, Israili et al.[11] have shown, using thin-layer chromatography and nuclear magnetic resonance studies, that the $T_{\frac{1}{2}}$ in buffered solutions is 60–100 hr at 25°C. Karon et al.[7] have found that gastrointestinal toxicity is less without reduction of antitumor activity if drug infusion is extended over 10–15 min or if the dose is divided. Moertel et al.[5] also have found that dividing the dose is associated with less nausea and vomiting. In an attempt to circumvent the severe gastrointestinal toxicity, the Southeastern Cancer Study Group initiated a study of continuous infusions of 5-azacytidine in patients with metastatic cancer and leukemia.

MATERIALS AND METHODS

Criteria for Patient Selection

Patients with advanced metastatic cancer who had recovered from the toxicity of any prior chemotherapy and who had a life expectancy of at least 8 wk and previously treated patients with acute leukemia or blastic transformation of chronic myelocytic leukemia giving informed consent, were eligible for study. Acute leukemia included acute lymphoblastic leukemia and acute myeloblastic leukemia (myeloblastic, myelomonocytic, monocytic). Studies were conducted at six institutions in the Southeastern Cancer Study Group (Emory University, Duke University, University of Alabama, University of Puerto Rico, Washington University, and Temple University).

Pretreatment Studies

Pretreatment studies included history, physical examination, documentation of measurable neoplastic lesions, bone marrow examination, hemogram, alkaline phosphatase, SGOT, serum proteins, BUN or creatinine clearance, plasma fibrinogen and prothrombin times.

Studies During Treatment

Blood counts were obtained at least twice weekly for the 6 wk of study and for 2 wk thereafter. Renal and liver function tests were repeated every 2 wk. Plasma fibrinogen and prothrombin times were repeated about every 4 wk. In the leukemic patients marrow examinations were done prior to subsequent courses of chemotherapy if blasts were absent from the peripheral blood.

Drug Administration

5-Azacytidine was administered by continuous intravenous infusion over a 5-day period. The drug was dissolved in 50–100 ml of Ringer's lactate and infused over a 3–12-hr period. Fresh solution was prepared every 3–12 hr.

Treatment Plan

In patients with metastatic cancers it was planned to treat three patients with 50 mg/sq m per day for 5 days followed by a 9-day rest period. If hematologic toxicity occurred, therapy was delayed. If no toxicity supervened after 6 wk of therapy (three courses), subsequent sets of three patients were to be treated with increments of 50 mg/sq m until toxicity occurred. Once toxicity occurred, six patients were to be treated at that dose before further escalation. It was planned to observe all patients for a period of 6 wk at a constant dose followed by an additional 2 wk of follow-up. Any patients showing improvement were to be continued on treatment.

In patients with acute leukemia, it was planned to start at 50 mg/sq m in the first set of three patients, but if after a 9-day rest period, there was no change or the white count had increased, the dose was to be escalated by 50 mg/sq m increments in the second and subsequent courses until a hematologic effect was noted. If no effect on the white count occurred at the 50 mg/sq m starting dose, the initial dose was to be escalated in subsequent sets of three patients by 50 mg/sq m increments.

Evaluation of Toxicity

Hematologic, hepatic, and renal toxicity were graded as previously reported.[9] Gastrointestinal toxicity was graded as follows: (1) nausea without vomiting while on therapy (mild); (2) nausea and vomiting while on therapy controlled by antiemetics (moderate); (3) nausea and vomiting not controlled with antiemetics (severe).

Criteria of Response

In metastatic cancer patients' objective responses were rated as previously reported,[9] requiring at least a 20% reduction in measurable tumors at 12 wk.

In patients with acute leukemia responses were judged according to the criteria as previously reported.[12] Those demonstrating a clearing of leukemic cells from the blood, changes in marrow status from an M-3 to 2 or less and a reduction in hepatomegaly and splenomegaly when present, but not achieving remission status, were considered to have had an antileukemic effect.

Patients with metastatic cancer were judged evaluable for toxicity if they received at least one 5-day course of treatment and had adequate follow-up information to determine the effect of 5-azacytidine on hemogram, liver, and renal function. Patients were judged evaluable for a therapeutic response if they received at least three courses of therapy.

In leukemia patients all studies were judged evaluable if they received one 5-day course of 5-azacytidine according to protocol.

RESULTS

Metastatic Cancer

Table 1 summarizes the toxicity observed in 23 trials in 21 patients entered on study with metastatic cancers.

The major hematologic toxicity was granulocytopenia. In most instances patients received two courses of therapy before toxic levels were reached. The mean nadir day was day 32 (range 22–45) with recovery (above 1500/cu mm) by day 39 (range 25–56). All six patients given infusions of 150 mg/sq m every 14 days had granulocytopenia. When the infusions were given every 21 days or later, two of four experienced granulocytopenia.

No patient had hepatic or renal toxicity which was thought to be drug related. No changes in fibrinogen or prothrombin time were noted although only a few tests were done serially.

Nausea and/or vomiting occurred in 20 patients, and was considered grade 2 in 6. Symptoms were worse the first day or two of treatment. Nausea was usually well controlled with antiemetics. Only one patient refused subsequent courses because of nausea and vomiting. An occasional patient experienced mild diarrhea.

Table 1. Metastatic Cancer Toxicity

Dose mg/sq m/day	Frequency of Course (days)	No. of Patients	Hematologic: Granulocytes < 1500	Granulocytes < 750	Platelets < 100,000	Platelets < 50,000	Hemoglobin > 3 Drop	Gastrointestinal 1	Gastrointestinal 2	Gastrointestinal > 2
50	14	4	1	0	0	0	0	2	0	0
100	14	4	0	3	0	0	2	3	1	0
100	21	1	0	0	0	0	0	1	0	0
150	14	8	1	5	0	0	0	5	3	0
150	21–28	4	1	1	1	0	1	3	1	0
175	—	1	0	0	0	0	0	0	1	0
200	—	1	0	1	1	0	0	1	0	0

Table 2. 5-Azacytidine in Leukemia (Evaluable Patients)

Leukemia*	Dose (mg/sq m/day)	No. of Patients	Dose Escalated	GI Toxicity			Response†		
				1	2	>2	CR	PR	ALE
AML	50	1	1	1	0	0	0	0	1
	100	7	5	3	2	0	3	0	3
	150	32	7	16	8	0	7	4	7
	200	5	0	3	2	0	1	0	0
	Total	45	13	23	12	0	11	4	11
Blast phase of CML	100	2	1	1	0	0	0	0	1
	150	4	0	0	0	0	0	0	3
	200	3	0	3	0	0	0	0	3
	Total	9	1	4	0	0	0	0	7
ALL	150	3	1	1	0	0	0	0	0
	200	1	0	0	0	0	0	0	0
	Total	4	1	1	0	0	0	0	0

*AML, acute myeloblastic leukemia; CML, chronic myeloblastic leukemia; ALL, acute lymphoblastic leukemia.

†CR, complete remission; PR, partial remission; ALE, antileukemic effect.

No significant therapeutic benefit was observed in this group of 21 patients, which included 6 with lung carcinoma, 5 with melanoma, 3 with breast carcinoma, 2 with hypernephroma, 2 with leiomyosarcoma, 1 with mesothelioma, 1 with colon carcinoma, and 1 with fallopian tube carcinoma. One patient with melanoma had stabilization of disease but with less than 20% regression in the size of measurable lesions.

Results in Leukemia

Acute myeloblastic leukemia. Forty-nine patients with acute myeloblastic leukemia were entered on study and 45 were evaluable. Of the four inevaluable patients, two were ineligible for the study, in one the protocol was not followed and one was lost to follow-up. A summary of these cases is given in Table 2. The initial dose of 50 mg/sq m was administered to only one patient and no hematologic effect was noted. The dose was escalated in subsequent courses. A reduction in leukocyte count was noted, but no remission occurred despite 13 courses over a year's time.

Seven patients began at 100 mg/sq m; in five the dose was escalated to 150 mg/sq m. One of these five and two others obtained a complete remission and three had an antileukemic effect. Five experienced mild nausea and vomiting.

Thirty-two patients started at 150 mg/sq m. In seven, the dose was escalated in subsequent courses and in one, it was reduced. The maximum doses given were 300 mg/sq m/day. There were seven complete remissions, four partial remissions, and seven patients who had an antileukemic effect. Mild nausea and vomiting occurred in 24 patients.

Granulocytopenia occurred in ten patients and was usually prolonged. One patient developed pleuritic chest pain with the last three of her six courses of chemotherapy, and this was thought to be drug related. Hyperglycemia occurred in two patients, and an inappropriate ADH syndrome was observed in one patient.

Five patients were started at 200 mg/sq m, and the doses were not escalated. One obtained a complete remission. Two experienced moderate nausea and vomiting.

Courses of 5-azacytidine were repeated every 2 wk except in the event of prolonged cytopenia, when treatment was delayed until evidence of recovery occurred. Bone marrows were monitored at frequent intervals and therapy was reinstituted whenever the percentage of blasts increased even though the peripheral blood count remained low. Many patients could tolerate two courses with a 9-day interval, but subsequent courses were delayed.

Thus, of 45 evaluable patients with acute myeloblastic leukemia, complete remissions occurred in 11 (24%) and partial remissions in four patients. An antileukemic effect was observed in 11 patients. Reduction of white count was achieved in almost all patients. Dose escalation in two of the patients with partial remissions failed to induce a complete remission. No gastrointestinal toxicity greater than two was observed.

Table 3 summarizes our experience in those 11 patients who achieved complete remissions. The number of courses required to achieve remission varied from one to seven with an average of three and seemed to be unrelated to dose. The beginning of repeated courses varied from 13 to 29 days with an average of 15 days. Four patients achieved remission without marrow hypoplasia, and the remainder demonstrated prolonged marrow hypoplasia after two or more courses. The time to achieve remission varied from 27 to 92 days with an average of 59 days.

Patients received varying maintenance programs, and assessment of remission duration is difficult. The duration of remission ranged from 26 to 600 days with a median of 88 days.

Blast crisis of chronic myelocytic leukemia. There were nine patients with blastic transformation treated with 5-azacytidine in doses ranging from 100 to 200 mg/sq m (Table 2). Seven patients had an antileukemic effect after one to three courses, but no remissions were obtained. Three patients had prolonged granulocytopenia.

Acute lymphoblastic leukemia. Four patients with acute lymphoblastic

Table 3. Acute Myeloblastic Leukemia—Complete Remissions

Starting Dose (mg/sq m/day)	Pt. No.	No. of Courses	Interval (days)	Aplasia (days)	Days to Remission
100	1	2	15	21	54
	2	2	20	31	59
	3	7	14	0	92
150	4	4	19,29,19	0	67
	5	2	13	0	27
	6	3	14	21	61
	7	1	—	0	38
	8	2	14	29	64
	9	3	14	22	75
	10	2	12	41	50
200	11	2	16	26	58

leukemia were treated with 150–200 mg/sq m doses, and no responses were observed (Table 2).

DISCUSSION

Administration of 5-azacytidine by continuous infusion has greatly increased patient tolerance. In a phase I study of twice weekly injections given rapidly, 100% of patients who received a dose of 100 mg/sq m or larger vomited.[9] In contrast only about 25% of patients given infusions experienced vomiting.

Clinically significant hematologic toxicity was primarily confined to granulocytes. Bone marrow samples taken during the period of neutropenia were megaloblastic. Erythropoiesis was impaired, and some patients developed anemia. Platelet toxicity was infrequent. The fact that granulocyte toxicity was common, and platelet toxicity uncommon would seem to make this drug ideal for treatment of myeloblastic leukemia.

It would appear that the frequency of administration (every 14 days) resulted in cumulative granulocyte toxicity, and less toxicity was seen when the frequency of courses was reduced to every 21 or more days. However, the every 14 day schedule appeared to be effective in treating leukemia.

From these studies the recommended starting dose for treating acute leukemia is 150 mg/sq m/day.

The full spectrum of toxicity of 5-azacytidine has not been fully delineated. Although we saw no hepatic toxicity which we could establish as due to the drug, Bellet et al.[8] described hepatic coma developing in patients given subcutaneous injections. All had hepatic metastases at the time. Patients should be watched for central nervous system toxicity. One of our patients who had been treated for central nervous system leukemia developed an inappropriate ADH syndrome.

Although no significant responses were observed in patients with metastatic cancer, the number of patients in each disease category was small, and thus the data were insufficient to draw conclusions about its efficacy.

Other studies have been reported concerning the use of 5-azacytidine in treating myeloblastic leukemia. McCredie et al.[6] administered the drug by rapid intravenous injection (15–30 min) daily in repeated 5-day courses in doses up to 400 mg/sq m and observed three complete remissions and four partial responses in 18 patients. All had been previously treated. Because of the severe toxic effects (myelosuppression, nausea, vomiting, diarrhea, fever, and occasional hypotension) they suggested that 5-azacytidine might be better tolerated if used at a smaller dose, possibly in combination with another agent. Levi and Wiernik[13] gave 5-day courses of 200 mg/sq m/day rapidly by intravenous injection and obtained five complete remissions among 18 patients. In another study Levi and Wiernik[14] gave doses of 100 mg/sq m by rapid intravenous injection in three divided doses combined with methyl-GAG[methyl glyoxal-bis (guanyl-hydrazone) (NSC-21946)] to eight patients with refractory nonlymphocytic leukemia and observed two partial remissions. They experienced similar toxicity and concluded that the dosage was too low.

These studies indicate that 5-azacytidine given by continuous infusion is an active agent in acute myeloblastic leukemia. The high degree of success in inducing remissions in patients with refractory myeloblastic leukemia requires

that further studies be done. The use of 5-azacytidine in combination with other agents seems warranted.

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APPENDIX

The following members of the Southeastern Cancer Study Group participated in this study: John R. Durant, M.D., George Omura, M.D., Richard Gams, M.D., John Carpenter, M.D., Marcel Conrad, M.D., University of Alabama in Birmingham, Birmingham, Ala.; Harold Silberman, M.D., and Donald Miller, M.D., Duke University Medical Center, Durham, N.C.; Lawrence E. Cooper, M.D., Charles C. Corley, Jr., M.D., L. Thomas Heffner, M.D., Julian Jacobs, M.D., James W. Keller, M.D., Melvin Moore, M.D., W. R. Vogler, M.D., E. F. Winton, M.D., Emory University School of Medicine, Atlanta, Ga.; Antonio Grillo, M.D., and Enrique Velez-Garcia, M.D., University of Puerto Rico School of Medicine, San Juan, Puerto Rico; Richard V. Smalley, M.D., Temple University School of Medicine, Philadelphia, Pa.; Cary Presant, M.D., and Edward H. Reinhard, M.D., Washington University School of Medicine, St. Louis, Mo.

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11) Wir danken Dr. H. Niederprüm, Anorganische Abteilung, Bayer AG, D-5090 Leverkusen, für die großzügige Überlassung des Reagenzes. Kleinere Proben von $C_4F_9SO_3K$ können kostenlos von Dr. Niederprüm erhalten werden, die Substanz ist jedoch auch in technischen Mengen verfügbar.
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Nucleoside Syntheses, XXV [1]

A New Simplified Nucleoside Synthesis [2]

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The several steps of the Friedel-Crafts catalyzed silyl-Hilbert-Johnson nucleoside synthesis – silylation of the heterocyclic base, silylation of the perfluorosulfonic acids or its salts (if $SnCl_4$ is not used as catalyst) and finally the nucleoside synthesis itself – can be combined to a simple one-step/one-pot reaction which generally affords nucleosides in high yields.

Nucleosid-Synthesen, XXV [1]

Eine neue einfache Nucleosid-Synthese

Die verschiedenen Schritte der Friedel-Crafts-katalysierten Silyl-Hilbert-Johnson-Nucleosid-Synthese – Silylierung der heterocyclischen Base, Silylierung der Perfluorsulfonsäuren oder ihrer Salze (falls nicht $SnCl_4$ als Katalysator benutzt wird) und schließlich die Nucleosid-Synthese selbst – können zu einer einfachen Einstufen-Eintopf-Reaktion vereinigt werden, die allgemein Nucleoside in hohen Ausbeuten ergibt.

1) Introduction

The reaction of persilylated heterocyclic bases with peracylated sugars in the presence of Friedel-Crafts catalysts like $SnCl_4$[3,4] or $(CH_3)_3SiSO_3CF_3$, $(CH_3)_3SiSO_3C_4F_9$ or $(CH_3)_3SiClO_4$[5] has become a standard synthetic method for the preparation of pyrimidine, purine as well as other nucleosides.

Prior to nucleoside synthesis however, the heterocyclic bases have to be silylated by heating with excess hexamethyldisilazane (HMDS) to the highly moisture sensitive persilyl derivatives which either have to be distilled or repeatedly evaporated with absol. xylene to remove the excess of HMDS.

For the synthesis of nucleosides of more basic heterocyclic systems like persilylated 5-methoxyuracil, 4-pyridone etc., the new Lewis acids or Friedel-Crafts catalysts $(CH_3)_3SiSO_3CF_3$, $(CH_3)_3SiSO_3C_4F_9$ or $(CH_3)_3SiClO_4$[5,6] must be employed as catalysts instead of $SnCl_4$ in order to obtain the natural *N*-1-nucleosides in high yields[5]. And these catalysts have also to be prepared *prior* to nucleoside synthesis by heating the free triflate or nonaflate acids with trimethylchlorosilane[7] or by treating a toluene solution of silver perchlorate with trimethylchlorosilane[8].

2) The One-Step/One-Pot Reaction

Since silylations are accelerated by Lewis acids[9] and the silylation of the heterocyclic bases would consequently be quite rapid in the presence of Friedel-Crafts catalysts, we have combined the different steps of nucleoside synthesis: a) the silylation of the heterocyclic bases, b) the silylation of the triflate or nonaflate acids or salts including the perchlorates (if $SnCl_4$ is not used as a catalyst), c) the nucleoside synthesis with acylated 1-*O*-acyl or 1-*O*-alkyl sugars in the presence of Friedel-Crafts catalysts, in a one-step/one-pot procedure employing a polar solvent like acetonitrile.

Under these conditions the amounts of trimethylchlorosilane (TCS) and hexamethyldisilazane (HMDS) have to be chosen in such a way that all reactive heterocyclic hydroxy, mercapto or amino groups as well as the free triflate or nonaflate acids $C_nF_{2n+1}SO_3H$[10], their corresponding salts[10] and perchlorate salts are silylated with formation of NH_4Cl and the corresponding alkali chlorides, NaCl or KCl. Since we are dealing with Friedel-Crafts catalyzed reactions it is crucial that only NH_4Cl is obtained, NH_3 would neutralize the Friedel-Crafts catalyst!

Because practically all of these salts are rather insoluble in acetonitrile, they precipitate when formed and therefore might shift the reactions towards the desired electrophilic trimethylsilyl esters as depicted in the following equations:

$$3\ CF_3SO_3H + HMDS + TCS \longrightarrow 3\ (CH_3)_3SiSO_3CF_3 + NH_4Cl \quad (1)$$

$$C_4H_9SO_3K^{10)} + TCS \longrightarrow (CH_3)_3SiSO_3C_4F_9 + KCl \quad (2)$$

$$NH_4ClO_4 + TCS \longrightarrow (CH_3)_3SiClO_4 + NH_4Cl \quad (3)$$

$$NaBF_4 + TCS \longrightarrow (CH_3)_3SiBF_4 + NaCl \quad (4)$$

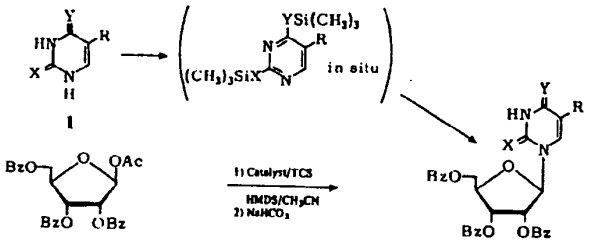
Although trimethylsilyltetrafluoroborate (equation (4)) has been described to decompose at ambient temperature to trimethylfluorosilane and BF_3[11], it might still act as a catalyst during its transient existence. It is more probable however that the BF_3 formed by decomposition of $(CH_3)_3SiBF_4$ will act as catalyst, since BF_3 has already been shown to be an effective catalyst for nucleoside formation in form of its etherate[3a].

Since potassium nonaflate ($C_4F_9SO_3K$)[10] is only partially soluble in boiling acetonitrile and potassium or ammonium perchlorate are nearly insoluble in acetonitrile, NaCl, KCl or NH_4Cl which are formed on reaction with TCS could thus occlude unreacted reagent. Therefore an excess of finely powdered $C_4F_9SO_3K$ or perchlorates was usually employed. In the case of the better soluble $NaClO_4 \cdot H_2O$ additional amounts of TCS and HMDS had to be used to eliminate the water.

As described in equation (1) for free triflic acid (CF_3SO_3H), a mixture of ca. 0.33 – 0.4 equivalents of TCS and HMDS was used whereas for potassium nonaflate ($C_4F_9SO_3K$) (equation (2)) equivalent amounts of TCS had to be employed.

For silylating uracil (1a), cytosine (1d) or a purine like N^2-acetylguanine (14b) containing two reactive oxygen, or oxygen and nitrogen functions, a mixture of a least 0.7 – 0.8 equ. each of TCS and HMDS are necessary to afford the persilylated uracil, cytosine or purine with concomitant formation of ca. 0.7 – 0.8 equ. of NH_4Cl. For a heterocyclic base like 4-pyridone (7) with only one reactive oxygen group, only half of that amount e. g. ca. 0.4 equ. each of TCS and HMDS is needed.

We studied first the formation of uridine 2',3',5'-tri-*O*-benzoate (3a) starting from uracil (1a) and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (2) in acetonitrile trying several combinations of catalysts, TCS and HMDS. In practice, this one-pot reaction is conducted by weighing the crystalline free bases, the acylated sugars and salts into a dried reaction flask connected to a reflux condenser and a drying tube, then adding the absolute solvent and HMDS and finally the liquid Lewis acids TCS, $SnCl_4$ or CF_3SO_3H with magnetic stirring.



	X	Y	R
a	O	O	H
b	S	O	H
c	O	O	OCH_3
d	O	NH	H
e	O	NAc	H

As is readily seen from Table 1, the synthesis of uridine 2',3',5'-tri-*O*-benzoate (3a), proceeded optimally in ca. 81 – 84% yield either with free triflic acid, potassium nonaflate or with $SnCl_4$ as catalysts. The stronger Friedel-Crafts catalyst $SnCl_4$[5] was effective at room temperature.

The perchlorate and tetrafluoroborate catalysts however afforded 3a only in 40 – 60% yield. But this is not of importance as the use of perchlorates should be avoided due to their explosion hazard.

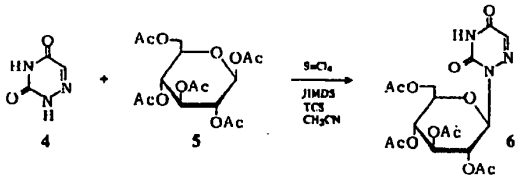
Table 1. One-step/one-pot reactions

Base/Sugar (1 equ.)	Acid or Salt	TCS/HMDS (equ.)	Reaction Time/ Temperature	Acylated Nucleoside (Yield)
1a/2	CF_3SO_3H [a]	1.2/1.1	1 h/83 °C	3a (81%)
1a/2	$C_4F_9SO_3K$ [b]	3.1/0.7	14 h/83 °C	3a (84%)
1a/2	$SnCl_4$ [a]	0.8/0.8	2 h/24 °C	3a (83%)
1a/2	NH_4ClO_4 [b]	3.1/0.7	19 h/83 °C	3a (40%)
1a/2	$NaClO_4 \cdot H_2O$ [b]	4.7/2.3	19 h/83 °C	3a (58%)
1a/2	$NaBF_4$ [b]	3.1/0.7	2 h/83 °C	3a (43%)
1b/2	$SnCl_4$ [a]	0.8/0.8	7 h/24 °C	3b (59%)
1c/2	$C_4F_9SO_3K$ [b]	3.1/0.7	20 h/83 °C	3c (71%)
1d/2	$C_4F_9SO_3K$ [b]	3.1/0.7	27 h/83 °C	3d (56%)

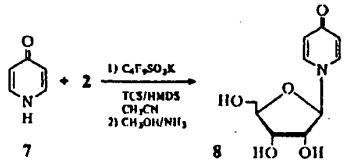
[a] 1.2 equivalents. – [b] 2.4 equivalents.

2-Thiouracil (1b) with $SnCl_4$ as catalyst gave 2-thiouridine 2',3',5'-tri-*O*-benzoate (3b) in ca. 60% yield. The more basic 5-methoxyuracil (1c)[3f,3j] as well as cytosine (1d) reacted with 2 in the presence of potassium nonaflate/TCS/HMDS to afford crystalline 2',3',5'-tri-*O*-benzoyl-5-methoxyuridine (3c) in 71% as well amorphous cytidine 2',3',5'-tri-*O*-benzoate (3d) in 56% yield. The analogous reaction of N^4-acetylcytosine (1e) followed by saponification with methanolic ammonia gave 59% of pure crystalline cytidine.

6-Azauracil (4) reacted with pentaacetyl-β-D-glucopyranose (5) in the presence of $SnCl_4$/TCS/HMDS to furnish the known[3a] crystalline nucleoside 6 in 42% yield.

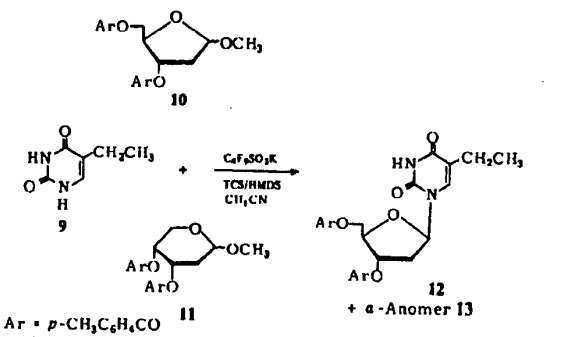


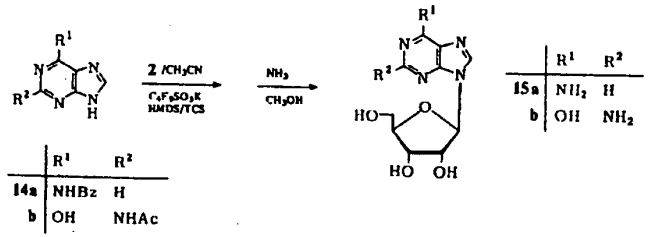
The rather basic 4-pyridone 7[5j] and 2 were converted by potassium nonaflate/TCS/HMDS and saponification in 50% yield into the known[3d] 8.



The oily anomeric mixture of 2-deoxy-1-*O*-methyl-3,5-di-*p*-toluoyl-D-ribofuranose (10) and 2-deoxy-1-*O*-methyl-3,4-di-*p*-toluoylribopyranose (11) is commonly used as starting material for the preparation of crystalline 1α-chloro-2-deoxy-3,5-di-*p*-toluoyl-β-D-ribofuranose[12]. Since the formation of the 1-cation from the furanosides 10 is kinetically favored over the 1-cation from the pyranosides 11[13], we reacted the mixture of 10 and 11 with 5-ethyluracil (9)[5j] in the presence of potassium nonaflate/TCS/HMDS and obtained after chromatography and crystallization 26% of the pure crystalline β-anomer 12 as well as 21% of the corresponding α-anomer 13. As expected[5j] no pyranoside nucleosides were formed.

The purine bases N^6-benzoyladenine (14a) and N^2-acetylguanine (14b) afforded on reaction with 2 in the presence of potassium nonaflate/TCS/HMDS and subsequent saponification with methanolic ammonia, crystalline adenosine (15a) in 63% and cristalline guanosine (15b) in 44% yield.





	R^1	R^2
14a	NHBz	H
b	OH	NHAc

	R^1	R^2
15a	NH_2	H
b	OH	NH_2

3) Solvents

Besides acetonitrile, which appears to be optimal in respect to polarity, solubility of inorganic salts and boiling point, other polar solvents like nitromethane can probably also be used.

On conducting the one-step/one-pot reaction between uracil (1a), 2 and $SnCl_4$ in the unpolar solvent 1,2-dichloroethane, ca. 30% of the *N*-3-nucleoside as well as ca. 5–10% *N*-1,*N*-3-bis-riboside were formed in addition to the desired uridine 2',3',5'-tri-*O*-benzoate (3a).

This is probably due to the increased salt concentration in the reaction mixture and enhanced σ-complex formation at N-1 of the silylated uracil[3f,6j] in 1,2-dichloroethane.

Since the polar solvent acetonitrile competes with the silylated bases for the Lewis acids, these salts usually do not interfere with nucleoside formation in acetonitrile[14].

An exception is however the one-step reaction between 6-methyluracil, 2 and potassium nonaflate/TCS/HMDS in acetonitrile which afforded only ca. 20–25% of the desired 2',3',5'-tri-*O*-benzoyl-6-methyluridine besides 20–25% of the undesired benzoylated *N*-3-riboside and 15–20% of the benzoylated *N*-1,*N*-3-bis-riboside.

In comparison, silylated 6-methyluracil reacted with 2 and trimethylsilyl triflate [$(CH_3)_3SiSO_3CF_3$] in acetonitrile to give 2',3',5'-tri-*O*-benzoyl-6-methyluridine in more than 70% yield[3j]. This striking difference in yield is due to the fact that this particular

reaction is especially sensitive towards humidity, alcohols, acetic acid or inorganic salts[3b,5] and therefore *not* suitable for this one-step/one-pot nucleoside synthesis. Apart from this exception however, the present procedure can be applied to practically any type of nitrogen heterocycle containing a reactive oxygen, sulfur or nitrogen function.

Although some of the yields obtained with this simple one-step/one-pot reaction are lower compared to the conventional two-step reaction[3-5], the new procedure is so simple and rapid that it can also be applied by biochemists, biologists and physicists with very limited chemical training. Since our preliminary publication[2] we have used this one-step nucleoside synthesis for the preparation of 3-amino-6-β-D-ribofuranosyl-6*H*-1,2,6-thiadiazine 1,1-dioxide[15] and have furthermore heard from quite a number of colleagues about successful applications of this simple nucleoside synthesis[16].

We thank Drs. *D. Rosenberg* and *A. Seeger* for the spectral data and Dr. *K. Merz* for the analyses.

Experimental Part

Materials: The solvents acetonitrile and 1,2-dichloroethane were purified as previously described[3,5]. Trimethylchlorosilane (TCS), hexamethyldisilazane (HMDS) and $SnCl_4$ were redistilled materials. The heterocyclic bases and standard sugars were commercial samples and purified by recrystallization as described[3,5]. The potassium nonaflate ($C_4F_9SO_3K$) was obtained from Dr. *Niederprüm*[10] (Bayer AG). All products were identified with authentic samples.

Thin layer systems[3,5]: A toluene/acetic acid/H_2O (5:5:1), B n-butanol/acetic acid/H_2O (5:1:4).

Workup after nucleoside synthesis: Employing ca. 75 ml absol. acetonitrile ca. 100 ml CH_2Cl_2 were added and the mixture extracted with sat. $NaHCO_3$-solution. After reextracting the aqueous phase with CH_2Cl_2, the combined organic phase was washed with sat. NaCl-solution, dried (Na_2SO_4) and evaporated.

Workup after saponification with methanolic ammonia[3]: After evaporation of the methanolic ammonia the residue was taken up in water and extracted twice with ether to remove methyl benzoate and benzamide. The aqueous phase was then evaporated and the residue recrystallized.

Uridine 2',3',5'-tri-O-benzoate (3a): a) *With CF_3SO_3H/HMDS/TCS as catalyst:* To 1.12 g (10 mmol) uracil (1a) and 5.04 g (10 mmol) 1-*O*-acetyl-2,3,5-tri-benzoyl-β-D-ribofuranose (2) in 100 ml absol. acetonitrile 2.3 ml (11 mmol) HMDS, 1.5 ml (12 mmol) TCS and 1.05 ml (12 mmol) trifluoromethane sulfonic acid were added consecutively whereupon the reaction temperature rose to ca. 35°C. After ca. 1 h the clear solution became turbid. Since acc. to t.l.c. (system A) the reaction had only partially proceeded after 4 h, the mixture was refluxed for 1½ h with exclusion of humidity. After workup the crude product (5.85 g) was crystallized partially from ethanol to afford in two crops 2.05 g pure 3a. The mother liquor was evaporated and chromatographed in toluene-ethyl acetate on a column of ca. 200 g silicagel. Elution with toluene-ethyl acetate 99:1 (1 l) and 98:2 (3 l) afforded impurities, whereas elution with 95:5 (2 l), 90:10 (2.5 l) as well as 80:20 (2 l) gave 2.55 g of crystalline 3a. Combined yield 4.50 g (80.8%) 3a.

b) *With $C_4F_9SO_3K$ as catalyst:* 0.56 g (5 mmol) uracil (1a), 2.52 g (5 mmol) 2 and 4.06 g (12 mmol) $C_4F_9SO_3K$ were refluxed in 70 ml acetonitrile for 14 h with 0.74 ml (3.5 mmol) HMDS and 1.89 ml (15 mmol) TCS. Workup, crystallization and chromatography of the mother liquor afforded 2.32 g (83.5%) 3a.

c) *With $SnCl_4$ as catalyst:* To 0.56 g (5 mmol) uracil (1a) and 2.52 g (5 mmol) 2 in 75 ml acetonitrile were added 0.84 ml (4 mmol) HMDS, 0.51 ml (4 mmol) TCS and finally 0.71 ml (6 mmol) $SnCl_4$ in 25 ml acetonitrile. After 2 h stirring at 24°C, workup gave 2.94 g crude 3a, which crystallized from ethanol to furnish in 2 crops 1.72 g 3a. Chromatography of the mother liquor as described above afforded another crop of 0.59 g 3a. Combined yield of 3a 2.31 g (83%).

2-Thiouridine 2',3',5'-tri-O-benzoate (3b): To 0.64 g (5 mmol) 2-thiouracil (1b) and 2.52 g (5 mmol) 2 in 50 ml absol. acetonitrile were added 0.84 ml (4 mmol) HMDS, 0.51 ml (4 mmol) TCS and finally 0.71 ml (6 mmol) $SnCl_4$ in 25 ml acetonitrile. After a short period of magnetic stirring everything had dissolved. The mixture was worked up after 6½ h at 24°C to give 2.75 g crude 3b which crystallized from ethanol to afford in two crops 1.68 g (58.5%) of pure crystalline 3b, mp. 105–106°C (lit. 105–106°C)[17]. Acc. to t.l.c. (system A) the mother liquor still contained some additional 3b.

5-Methoxyuridine 2',3',5'-tri-O-benzoate (3c): To 0.53 g (5 mmol) 5-methoxyuracil (1c), 2.52 g (5 mmol) 2 and 3.84 g (12 mmol) $C_4F_9SO_3K$ in 50 ml acetonitrile were added 0.74 ml (3.5 mmol) HMDS and 1.89 ml (15 mmol) TCS and the mixture refluxed for 20 h. After workup the crude product (3.72 g) crystallized in two crops from ethyl acetate-hexane to afford 2.09 g (71.3) of pure crystalline 3c, mp. 206–208°C. Acc. to t.l.c. the mother liquor still containing some 3c.

Cytidine 2',3',5'-tri-O-benzoate (3d): To 0.55 g (5 mmol) cytosine (1d), 2.52 g (5 mmol) 2 and 4.06 g (12 mmol) $C_4F_9SO_3K$ in 70 ml absol. acetonitrile were added 0.74 ml (3.5 mmol) HMDS and 1.96 ml (15.5 mmol) TCS and the mixture refluxed for 26 h. After workup, the crude product (3.26 g) gave on chromatography on 100 g silicagel and elution with toluene-ethyl acetate 4:1 → 3:2 /5 l) impurities. Elution with ethyl acetate (3.5 l) afforded 1.55 g (56%) of homogeneous amorphous 3d which was identical with an authentic sample.

Cytidine: 1.53 g (10 mmol) N^4-acetylcytosine (1e), 50.4 g (10 mmol) 2, 8.12 g (24 mmol) $C_4F_9SO_3K$ in 140 ml absol. acetonitrile were refluxed for 21 h with 1.48 ml (7 mmol) HMDS and 3.92 ml (31 mmol) TCS. After workup, the crude 3d was dissolved in 150 ml methanolic ammonia and kept for 3 days at 24°C. After workup, recrystallization from ethanol-H_2O afforded in three crops 1.68 g (59%) of pure crystalline cytidine, homogeneous in system B.

2-(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl)-1,2,4-triazine-3,5(2H,4H)-dione (6): To 1.13 g (10 mmol) 6-azauracil (4), 3.9 g (10 mmol) 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (5) and 1.68 ml (8 mmol) HMDS in 100 ml absol. acetonitrile were added 1.01 ml (8 mmol) TCS and finally 1.77 ml (15 mmol) $SnCl_4$ in 50 ml absol. acetonitrile. After 7 h stirring at 24°C and workup, the crude product (4.5 g) crystallized from ethanol to afford 0.93 g of 6, mp. 205–206°C. Chromatography of the mother liquor on 50 g silicagel gave on elution with toluene-ethyl acetate 9:1 (1.5 l) and 8:2 (1.5 l) impurities, whereas elution with 7:3 (1 l) afforded on evaporation and crystallization from ethanol in several crops 0.94 g crystalline 6. Total yield of 6 1.87 g (42.2%), mp. 205–206°C, which was identical with an authentic sample[3a].

1-(β-D-Ribofuranosyl)-4(1H)-pyridinone (8): To 0.47 g (5 mmol) 4(1*H*)-pyridinone (7), 2.52 g (5 mmol) 2 and 3.38 g (10 mmol) $C_4F_9SO_3K$ in 70 ml absol. acetonitrile were added 0.42 ml (2 mmol) HMDS and 1.52 ml (12 mmol) TCS and the mixture refluxed for 24 h, worked up and the crude product (2.7 g) saponified with 80 ml methanolic ammonia. After 2 days at 24°C and workup the residue (1.68 g) was chromatographed on 75 g silicagel with isopropyl alcohol. After a 300 ml forun the subsequent fractions (1 l) eluted slightly impure 8 (0.7 g). Crystallization from ethanol gave 0.56 g (49.6%) of pure 8, mp. 128–130°C, which was identical with an authentic sample[10].

1-(2-Deoxy-3,5-di-p-toluoyl-β-D-ribofuranosyl)-5-ethyluracil (12): To 2.60 g (6.76 mmol) anomeric sugar mixture 10/11, 0.95 g (6.76 mmol) 5-ethyluracil (9) and 5.49 g (16.22 mmol)

$C_4F_9SO_3K$ in 70 ml absol. acetonitrile were added 0.99 ml (4.73 mmol) HMDS and 2.65 ml (21 mmol) TCS and the mixture refluxed for 12 h. After workup the crude product (4.92 g) was chromatographed on 250 g silicagel. Elution with hexane-ether 8:2 (1 l), 3:2 (1 l) and 1:1 (3 l) gave only impurities, whereas elution with hexane-ether 1:1 (1 l) and 2:3 (4 l) afforded 1.48 g (47.1) of a partly crystalline mixture of 12 and the corresponding α-anomer 13 which was separated by crystallization from ethanol to give 0.81 g (25.8%) crystalline β-anomer 12, mp. 197–198 °C [3a] and about 0.67 g (21%) crystalline α-anomer 13, mp. 160–161 °C [3a,3].

Adenosine (15a): To 1.19 g (5 mmol) N^6-benzoyladenine (14a), 2.52 (5 mmol) 2, 4.06 g $C_4F_9SO_3K$ and 0.74 ml (3.5 mmol) HMDS in 70 ml acetonitrile were added 1.96 ml (15.5 mmol) TCS and the mixture refluxed for 21 h. After workup the crude product (3.9 g) was stirred with 100 ml sat. methanolic ammonia for 3 days at 24 °C. Workup and crystallization from a small volume of H_2O afforded in 4 crops 0.84 g (62.7%) pure adenosine (15a), homogeneous in system B.

Guanosine (15b): Exactly as described for the preparation of adenosine, N^2-acetylguanine (14b) was reacted with 2 and the crude acylated nucleoside saponified with methanolic ammonia to afford on crystallization from water in two crops 43.8% of crystalline guanosine (15b), homogeneous in system B.

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[257/81]

Chem. Ber. 114, 1287–1296 (1981)

Photosensibilisierte Addition von Citraconsäureanhydrid an 3,3-Dimethyl-1-butin

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Die photosensibilisierte Addition von Citraconsäureanhydrid (CA) an 3,3-Dimethyl-1-butin in Aceton führt zu den isomeren Cyclobutenen 1 und 2 sowie zum 6,6-Dimethyl-2-*cis*,4-*trans*-heptadien-2,3-dicarbonsäure-anhydrid (3). Unter den Reaktionsbedingungen photodimerisiert 3 zum thermolabilen Divinylcyclobutan-Derivat 5, dessen Konstitution aus der Röntgenstrukturanalyse folgt. Für die Bildung der Produkte 1–3 wird ein Reaktionsverlauf mit intermediären Alkyl-Vinyl-1,4-Diradikalen diskutiert. Gemeinsame Zwischenstufe für 2 und 3 ist danach das Diradikal 7. Der Mechanismus der intramolekularen Disproportionierung von 7 wird durch Isotopenmarkierung ([3-D]CA) aufgeklärt.

Photosensitized Addition of Citraconic Anhydride to 3,3-Dimethyl-1-butyne

Photosensitized addition of citraconic anhydride (CA) to 3,3-dimethyl-1-butyne in acetone solution gives rise to the isomeric cyclobutenes 1 and 2 as well as to 6,6-dimethyl-2-*cis*,4-*trans*-heptadiene-2,3-dicarboxylic anhydride (3). Under the reaction conditions 3 undergoes efficient photochemical cyclodimerization yielding the thermally labile divinylcyclobutane derivative 5. The constitution of 5 follows from *X*-ray analysis. The formation of the products 1–3 is discussed in terms of a mechanism with intermediate alkyl vinyl 1,4-diradicals. Diradical 7 is the common precursor of 2 and 3. The mechanism of the intramolecular disproportionation of 7 is elucidated by isotope labelling ([3-D]CA).

Die photosensibilisierte Addition von Maleinsäureanhydrid an terminale Alkine führt in der Regel zu Cyclobuten-Derivaten[1]. Ausnahmen sind die Reaktionen mit Acetylen bzw. Propin, bei denen neben Cyclobuten- überraschenderweise auch Bicyclopropyl-Derivate entstehen[1-3]. Für diese Photoreaktionen haben wir einen mehrstufigen Mechanismus mit intermediärer Bildung von Alkyl-Vinyl-1,4-Diradikalen formuliert[3].

Mono- wie dialkylsubstituierte Maleinsäureanhydride sind mit präparativer Zielsetzung ebenfalls photosensibilisiert an terminale Alkine addiert worden[4,5]. Bei der Addition an Acetylen entstehen außer den erwarteten Cyclobuten-Derivaten acyclische Addukte sowie in einem Fall ein Copolymeres. Die Bildungsweise der Nebenprodukte wurde nicht vollständig geklärt[5]. In zahlreichen anderen Arbeiten[4] werden Nebenprodukte, die Rückschlüsse auf den Reaktionsverlauf erlauben würden, nicht erwähnt.

Auch wir haben uns mit der photosensibilisierten Addition alkylsubstituierter Maleinsäureanhydride an terminale Alkine beschäftigt und früheren Untersuchungen folgend[1,3] uns besonders für den Mechanismus interessiert. Im folgenden berichten wir über die Ergebnisse der Reaktion von Citraconsäureanhydrid (CA) mit 3,3-Dimethyl-1-butin[6].

Chem. Ber. 114, 1234 – 1255 (1981)

Nucleoside Syntheses, XXII[1]

Nucleoside Synthesis with Trimethylsilyl Triflate and Perchlorate as Catalysts[2]

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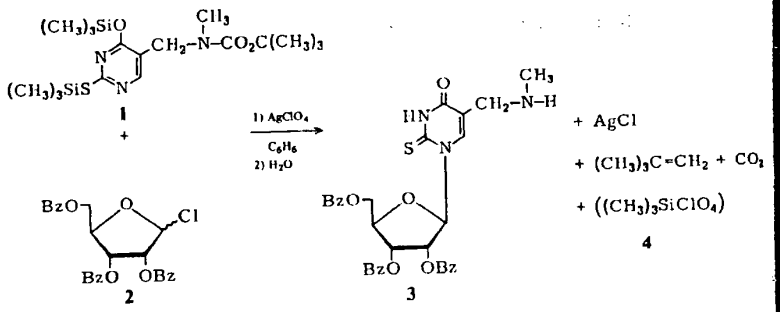
The novel Lewis acids $(CH_3)_3SiOSO_2CF_3$ (5), $(CH_3)_3SiOSO_2C_4F_9$ (6), and $(CH_3)_3SiClO_4$ (4) are highly selective and efficient Friedel-Crafts catalysts for nucleoside formation from silylated heterocycles and peracylated sugars as well as for rearrangements of persilylated protected nucleosides. With basic silylated heterocycles these new catalysts give much higher yields of the natural *N*-1-nucleosides than with $SnCl_4$.

Nucleosid-Synthesen, XXII[1]

Nucleosid-Synthese mit Trimethylsilyltriflat und Perchlorat als Katalysatoren

Die neuen Lewis-Säuren $(CH_3)_3SiOSO_2CF_3$ (5), $(CH_3)_3SiOSO_2C_4F_9$ (6) und $(CH_3)_3SiClO_4$ (4) sind sehr spezifische und effektive Friedel-Crafts-Katalysatoren für die Nucleosid-Synthese mit silylierten Heterocyclen und peracyclierten Zuckern sowie für die Umlagerung von persilylierten geschützten Nucleosiden. Insbesondere bei basischen silylierten Heterocyclen ergeben diese neuen Katalysatoren viel höhere Ausbeuten an natürlichen *N*-1-Nucleosiden als $SnCl_4$.

In the total synthesis[1] of the "rare" nucleoside 5-(methylaminomethyl)-2-thiouridine, the silylated 2-thiouracil **1** and 2,3,5-tri-*O*-benzoyl-D-ribofuranosylchloride (**2**) were reacted with $AgClO_4$[3,4,5] in absolute benzene to give unexpectedly the *O*-benzoylated nucleoside **3** in which the protecting *tert*-butoxycarbonyl (BOC) group had been lost during nucleoside formation and workup.



Investigating this reaction, the only strong Lewis acid which could have been formed as an intermediate and could have cleaved the BOC group was $(CH_3)_3SiClO_4$ (**4**). **4** had already previously been postulated as an intermediate during nucleoside synthesis by *Birkofer* et al.[4] and *Wittenburg*[5].

We could subsequently show that $(CH_3)_3SiClO_4$ (**4**) as well as $(CH_3)_3SiOSO_2CF_3$ (**5**) are very interesting new Lewis acids or Friedel-Crafts catalysts and do indeed cleave BOC groups rather selectively in protected amino acids and peptides[6]. Further interesting applications of reagents like **4** and **5** e. g. for the preparation of trimethylsilyl enol ethers and silyl ethers of *tert.* alcohols, cleavage of epoxides and cycloadditions were subsequently described[7].

A. The New Catalysts

Recently, *Marsmann* and *Horn*[8] had measured the ^{29}Si-NMR shifts of a whole series of trimethylsilyl esters of strong acids $(CH_3)_3SiX$ with X ranging from CN, Br, F, Cl to SO_4, ClO_4 and OSO_2CF_3. They estimated the p*K* values of these new Lewis acids and demonstrated that $(CH_3)_3SiClO_4$ (**4**)[9] and even more so $(CH_3)_3SiOSO_2CF_3$ (**5**)[8,10] were far stronger acids than others in these series. *Marsmann* and *Horn* however did not include higher homologues of $(CH_3)_3SiOSO_2CF_3$ (**5**) like $(CH_3)_3SiOSO_2C_4F_9$ (**6**) as well as $(CH_3)_3SiOSO_2F$[11] which probably possess about the same acidic strength than $(CH_3)_3SiClO_4$ (**4**) and $(CH_3)_3SiOSO_2CF_3$ (**5**)[12].

Encouraged by these acidity data we initially tested $(CH_3)_3SiClO_4$ (**4**) and $(CH_3)_3SiOSO_2CF_3$ (**5**) as well as other acidic silyl compounds like $(CH_3)_3SiCl$ and $[(CH_3)_3Si]_2SO_4$[8] as potential new catalysts for the synthesis of nucleosides.

$(CH_3)_3SiClO_4$ (**4**)[9], $(CH_3)_3SiOSO_2CF_3$ (**5**)[8,10], and $(CH_3)_3SiOSO_2C_4F_9$ (**6**) are readily prepared by the following reactions:

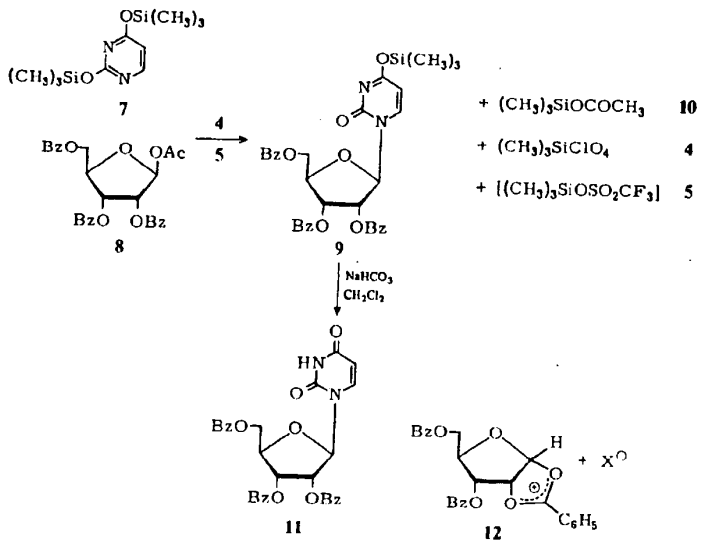
$$(CH_3)_3SiCl + AgClO_4 \xrightarrow[\text{or toluene}]{\text{benzene}} \underset{\mathbf{4}}{(CH_3)_3SiClO_4} + AgCl\downarrow$$

$$(CH_3)_3SiCl + CF_3SO_3H \xrightarrow[\Delta]{\text{neat}} \underset{\mathbf{5}}{(CH_3)_3SiOSO_2CF_3} + HCl\uparrow$$

$$(CH_3)_3SiCl + C_4F_9SO_3H \xrightarrow[\Delta]{\text{neat}} \underset{\mathbf{6}}{(CH_3)_3SiOSO_2C_4F_9} + HCl\uparrow$$

We first reacted silylated uracil **7** with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**8**) in the presence of **4** and **5** in 1,2-dichloroethane or acetonitrile and obtained the silylated intermediate **9**[13] as well as the silyl ester of acetic acid **10** and regenerated **4** or **5**. The workup with aqueous $NaHCO_3/CH_2Cl_2$ did not give rise to any emulsions (as were often obtained with $SnCl_4$) to afford pure crystalline uridine tri-*O*-benzoate (**11**) in more than 80% yield.

Due to σ-complex formation between **4** or **5** and the silylated base[14], one equivalent of **4** or **5** is inactivated during nucleoside formation. Thus application of 1.1 – 1.3 equivalents of the catalysts **4** and **5** dramatically shortens the reaction time. Such a slight excess of catalyst is therefore *preferable* to working with catalytic (0.1 equ.) amounts of **4** and **5**, because much more stringent reaction conditions must then be used (compare chapter B. 1.).



In contrast to **4** and **5**, trimethylsilyl chloride $(CH_3)_3SiCl$ as well as bis(trimethylsilyl) sulfate $[(CH_3)_3Si]_2SO_4$[8] as catalysts for the reaction of **7** with **8** *did not afford any nucleoside.* Apparently neither catalyst is a strong enough Lewis acid to convert 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**8**) into the reactive electrophilic sugar cation **12**[15].

A slight excess of trimethylsilyl fluorosulfonate[11], which we also consider to be a very strong Lewis acid[12], did not give any uridine tri-*O*-benzoate (**11**). Apparently $(CH_3)_3SiOSO_2F$ causes side reactions which prevent nucleoside formation.

Thus, we concentrated initially on $(CH_3)_3SiClO_4$ (**4**) and $(CH_3)_3SiOSO_2CF_3$ (**5**). Due to the explosive nature of pure $(CH_3)_3SiClO_4$ (**4**)[4] and the relative high cost of trifluoromethane sulfonic acid (triflic acid), we used later increasingly $(CH_3)_3SiOSO_2C_4F_9$ (**6**) which is readily prepared from the commercially available $C_4F_9SO_3K$ (potassium nonaflate)[16] either *via* the free nonaflic acid $C_4F_9SO_3H$ by heating with $(CH_3)_3SiCl$ as described above or by reaction of the stable potassium nonaflate $(KOSO_2C_4F_9)$[16] with $(CH_3)_3SiCl$ *in situ* in acetonitrile[17].

$$C_4F_9SO_3K + (CH_3)_3SiCl \xrightarrow[CH_3CN]{} \underset{\mathbf{6}}{(CH_3)_3SiOSO_2C_4F_9} + KCl\downarrow$$

$$\underset{\mathbf{13}}{HCF_2[CF_2]_3SO_3K} + (CH_3)_3SiCl \longrightarrow \underset{\mathbf{14}}{(CH_3)_3SiOSO_2C_4F_8H} + KCl$$

A further equally efficient catalyst is trimethylsilyl octaflate (**14**) which is prepared analogously from the readily available potassium octaflate **13**[16].

Persilylated polymeric perfluorinated sulfonic acids like Nafion® were not as yet tried as a catalyst. Such a catalyst would be easily recovered by filtration and reformed by heating with excess TCS.

It should be pointed out here that during workup with $NaHCO_3$, the collected mother liquors of experiments with trimethylsilyl triflate (**5**) can be evaporated and the triflate salts recrystallized from acetone[18]. In the case of trimethylsilyl nonaflate (**6**), on workup with $KHCO_3$, the slightly soluble salt $C_4F_9SO_3K$ can be easily recovered in up to 80% yield from the collected aqueous and organic phases by concentration and filtration (compare preparation of **18a**).

B. The Scope of Nucleoside Synthesis with the New Catalysts

1. Introduction

After the use of simple Friedel-Crafts catalysts like $SnCl_4$ in nucleoside synthesis had become widely accepted[19], the introduction of any new and more expensive catalysts like $(CH_3)_3SiOSO_2CF_3$ (**5**), $(CH_3)_3SiOSO_2C_4F_9$ (**6**) or $(CH_3)_3SiOSO_2C_4HF_8$ (**14**), although they can be recovered to a large extent, can only be justified if they have definite advantages over $SnCl_4$ or any of the other catalysts.

As already mentioned, in contrast to $SnCl_4$ no emulsions are formed on workup of the reaction mixture using **4**, **5** or **6** with $NaHCO_3/CH_2Cl_2$.

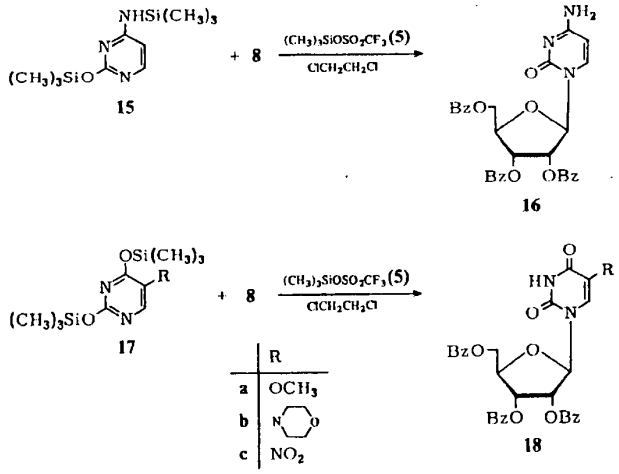
However, the major advantage which makes these new catalysts (**4**, **5**, **6**, **14**) in many cases vastly superior to $SnCl_4$ or other Friedel-Crafts catalysts[19a] is their lowered acidity as Lewis acids compared to $SnCl_4$. These new catalysts are just sufficiently acidic to form reactive sugar cations like **12** (compare also Chapter B. 6.), however they cause dramatically decreased σ-complex formation with silylated bases compared to $SnCl_4$ as discussed in the accompanying publication[14]. Consequently, much higher yields are obtained of the desired natural *N*-1-nucleosides in the case of more basic silylated heterocycles.

2. Synthesis of Pyrimidine and Pyridine Nucleosides

As we had observed and described before[19b], more basic silylated heterocycles like silylated cytosine or silylated uracils having electron donating methoxy or morpholino substituents in the 5-position lead to increasingly stable σ-complexes between the silylated bases and $SnCl_4$ and thus to longer reaction times and, most importantly, to increasing amounts of the undesired unnatural *N*-3-nucleosides.

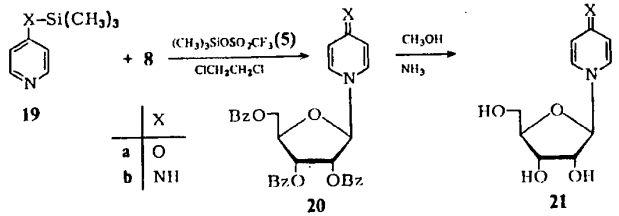
With catalytic amounts (0.1 equ.) of **5**, the basic silylated cytosine **15** reacted very slowly with **8** in boiling 1,2-dichloroethane. However, after adding a further amount of 1.1 equ. of **5** the reaction was complete after 1 h refluxing to afford a practically quantitative yield of amorphous cytidine 2',3',5'-tri-*O*-benzoate (**16**).

In the case of the rather basic silylated 5-methoxyuracil (**17a**) and 5-morpholinouracil (**17b**), using **5** instead of $SnCl_4$ dramatically improved the yield of the desired *N*-1-nucleosides **18a** and **18b** compared to $SnCl_4$. Thus, in 1,2-dichloroethane 89% **18a** were obtained compared to 53% using $SnCl_4$ and 95% **18b** compared to 39% with $SnCl_4$[19b].



As was expected, the reaction of the rather *weakly basic* silylated 5-nitrouracil (**17c**) with **8** using **5** as catalyst affords the 2',3',5'-tri-*O*-benzoyl-5-nitrouridine (**18c**) in 93% yield, thus showing no advantage over the corresponding reaction with $SnCl_4$ which gives also a nearly quantitative yield of **18c**[19a].

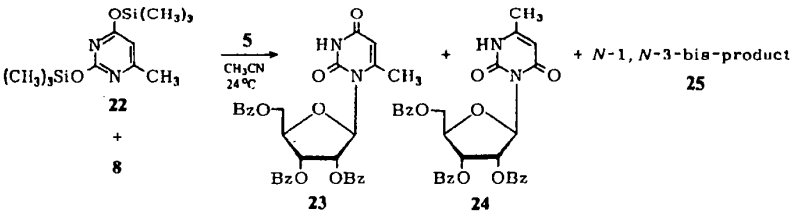
Reaction of the basic silylated 4-pyridone (**19a**) and silylated 4-aminopyridine (**19b**) with **8** and 1.2 equ. $(CH_3)_3SiOSO_2CF_3$ (**5**) afforded the nucleosides **20a** and **20b** in 83% and 80% yield. Saponification with methanolic ammonia gave the new nucleoside **21b**. The toxic 3-carboxy derivative of **21b** clitidine was recently isolated from the toadstool *Clitocybe acromelalga*[20].



It should be noted that **20a** is only formed in 63% yield under forcing conditions in the presence of $SnCl_4$[19d]. The analogous reaction of the even more basic silylated 3,4-diaminopyridine with **8** in the presence of **5** gave complicated mixtures which were not further investigated.

The less basic silylated 2-pyridone and **8** afforded with trimethylsilyl triflate (**5**) as with $SnCl_4$[19d] 85% of the desired 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-2(1*H*)-pyridinone.

A further reaction which did not proceed satisfactorily with $SnCl_4$[19b] was the synthesis of benzoylated 6-methyluridine (**23**) starting from silylated 6-methyluracil (**22**).

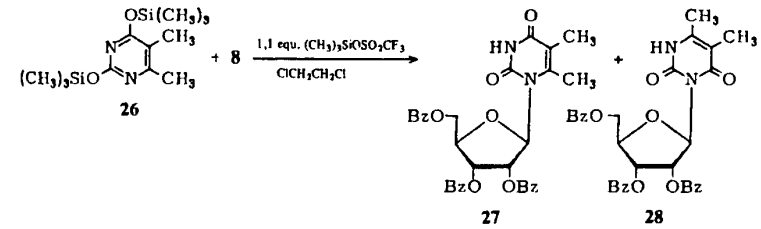


Using $(CH_3)_3SiOSO_2CF_3$ (**5**) instead of $SnCl_4$ in acetonitrile afforded 71% of **23** compared to 41% of **23** with $SnCl_4$[19b] besides varying amounts of the *N*-3 **24** as well as the *N*-1,*N*-3-bis-riboside **25**.

As described in chapter C the yield of the desired **23** can be further increased by rearrangement of silylated **24** and **25** to **23**.

In all these preparations of benzoylated 6-methyluridine (**23**)[19b], it is of paramount importance that the sugar moiety **8** is pure and absolutely free of solvent traces and the acetonitrile carefully dried by subsequent treatment with P_2O_5 and CaH_2.

The analogous reaction of silylated 5,6-dimethyluracil (**26**) with **8** and 1.1 equ. of **5** afforded in 1,2-dichloroethane 82% of the *N*-1-nucleoside **27** and only 9% of the *N*-3-nucleoside **28**.



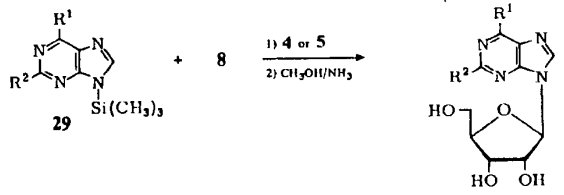
With $SnCl_4$ as catalyst[19b] in 1,2-dichloroethane *only* 10% of **27** and 60% of **28** and in acetonitrile 66% of **27** and 17% of **28** were obtained.

An additional methyl, isopropyl or nitro group[19f] in 5-position pushes the 4-*O*-trimethylsilyl group in 4-position towards the *N*-3-nitrogen which becomes therefore hindered. Thus substitution at *N*-1 is favored over substitution at *N*-3 and smaller amounts of the *N*-3-nucleoside are obtained. However, as discussed before[19f], the decreased basicity of silylated 6-methyl-5-nitrouracil favors also the formation of benzoylated 6-methyl-5-nitrouridine in the presence of **5**.

3. Synthesis of Purine and Pteridine Nucleosides

The following examples demonstrate that purine nucleosides are also readily accessable using **5** as catalyst[21]. The crude reaction mixtures obtained were saponified

directly with methanolic ammonia to the nicely crystalline free nucleosides. Adenosine (**30a**) was thus obtained in 81%, guanosine (**30b**) in 66% and xanthosine (**30c**) in 49% yield.

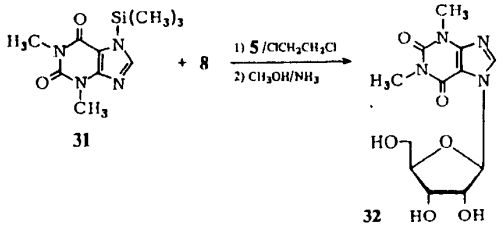


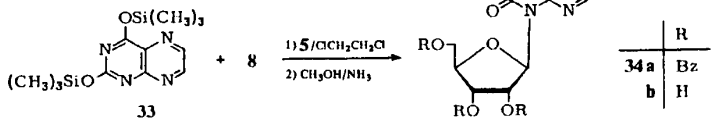
	R^1	R^2
29a	C_6H_5–CO–N–Si$(CH_3)_3$	H
b	$(CH_3)_3SiO$	$(CH_3)_3Si$–N–$COCH_3$
c	$(CH_3)_3SiO$	$(CH_3)_3SiO$

	R^1	R^2
30a	NH_2	H
b	OH	NH_2
c	OH	OH

During the synthesis of benzoylated adenosine[14], a number of intermediates are formed which are apparently gradually rearranged by **5** during the reaction to the acylated adenosine as studied in the case of the benzoylated adenine *N*-3-riboside (compare chapter C.).

Silylated theophylline (**31**) reacted analogously with **8** in the presence of **5** to give after saponification the known crystalline theophylline *N*-7-β-D-ribofuranoside[22] (**32**) in 82% yield.



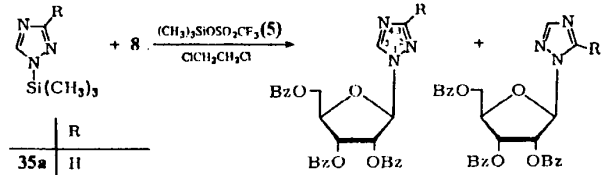


	R
34a	Bz
b	H

Silylated lumazine **33** gave after chromatography 93% of the amorphous 2',3',5'-tri-*O*-benzoylated nucleoside **34a** which had been obtained previously in 50% yield by the Wittenburg method[23]. Saponification of the tri-*O*-benzoate afforded 91% of the crystalline free nucleoside **34b**[23].

4. Synthesis of Triazole Nucleosides

Silylated 1,2,4-triazole **35a** gave on reaction with **8** in the presence of 1.2 equ. of $(CH_3)_3SiOSO_2CF_3$ (**5**) in 1,2-dichloroethane a 61% yield of the crystalline triazole riboside **36a**[24].



	R
35a	H
b	CO_2CH_3
c	CN

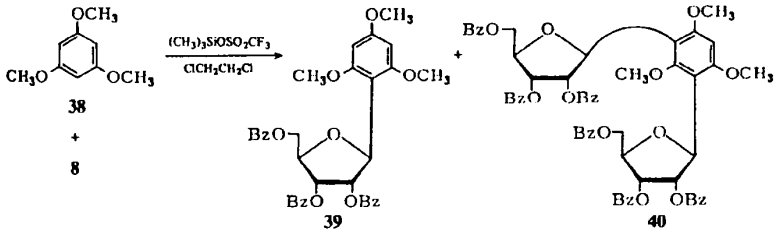
Reaction of silylated methyl 1,2,4-triazole-3-carboxylate **35b** with **8** and **5** in acetonitrile afforded 47% of the desired crystalline **36b**[25] as well as 19.6% of crystalline **37b**, 15.5% of **37c** and 2.3% of the decarboxylated product **36a**. The analogous reaction in 1,2-dichloroethane gave only 31.2% of the desired **36b** as well as 36.2% of **37b** and small amounts of **37c** and **36a**.

The mode of formation of **37c** is still unclear. It is possible that the ester moiety in methyl 1,2,4-triazolecarboxylate **35b** is partly converted during silylation, into the amide, which is subsequently dehydrated to the nitrile **37c** by HMDS or by **5** during nucleoside synthesis.

36b is readily converted by methanolic ammonia into the biologically interesting antiviral drug 1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide (ribavirin)[25].

5. Synthesis of *C*-Nucleosides

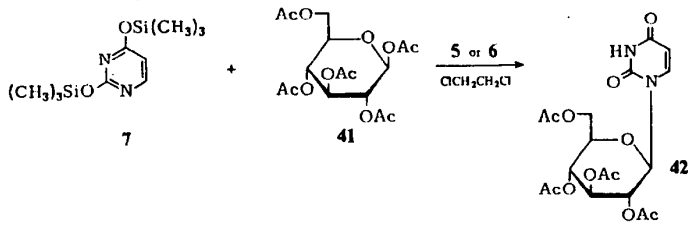
As discussed in the adjacent paper[14], the silyl-Hilbert-Johnson reaction in the presence of Friedel-Crafts catalysts is only a special version of the Friedel-Crafts reaction. Since rather stable sugar cations like **12**, which are formed during nucleoside synthesis, are only weak electrophiles, they can only react with electron-rich nucleophilic aromatic compounds. Thus while anisole did *not* react with **8**, 1,3,5-trimethoxybenzene (**38**) afforded 60% of the known crystalline β-nucleoside **39**[26] and 4.9% of the crystalline bis-product **40**. *N*,*N*-Dimethylaniline did not give any *C*-nucleoside. In the case of different di- or trimethoxybenzenes, other authors used stronger Lewis acids like $AlCl_3$ for the synthesis of such *C*-nucleosides and obtained α,β-mixtures[27].

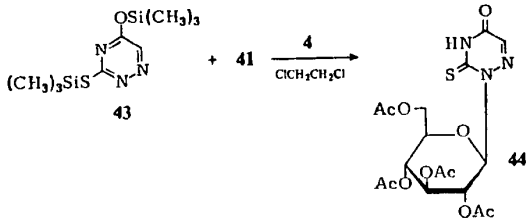


6. Variation of the Sugar Moiety

As we stated in chapter A, the new catalysts are just strong enough Friedel-Crafts catalysts to convert 1-*O*-acyl or 1-*O*-alkyl sugars into their corresponding cations e. g. 1-*O*-acetyl-2,3,5-tri-*O*-benzoylribofuranose (**8**) into the reactive intermediate **12**. Since the formation of furanosyl cations is kinetically favored over the corresponding pyranosyl cations[28], it was of interest to determine whether this difference in reactivity could be put to use.

We first reacted silylated uracil **7** with 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (**41**) and **5** and **6** as catalysts in boiling 1,2-dichloroethane and obtained the crystalline glucopyranoside **42**[29] in 89% and 92% yield respectively. This means that **5** and **6** give very similar results.





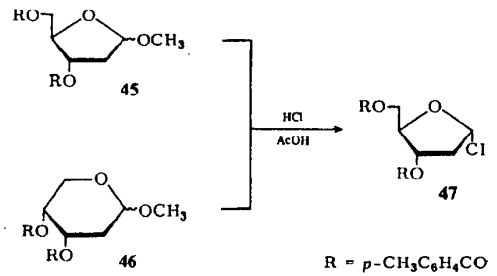
The corresponding silylated "2-thio-6-azauracil" **43** reacted analogously with $(CH_3)_3SiClO_4$ (**4**) in boiling 1,2-dichloroethane to afford a 56% yield of the nucleoside **44**[19a].

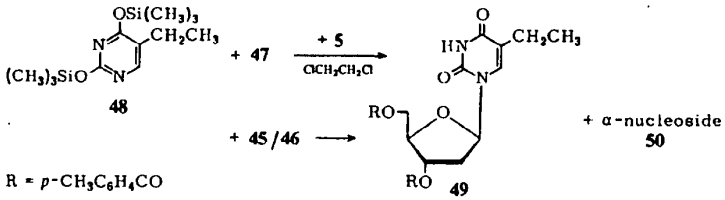
After having established that pyranosides do react with the new catalysts although under more stringent conditions than the furanosides, we turned to the synthesis of the important 2'-deoxyribosides.

The starting material for the 2'-deoxynucleosides is crude 2-deoxy-1-*O*-methyl-3,5-di-*O*-*p*-toluoylribofuranose which is actually a mixture of two furanose (**45**) and pyranose (**46**) 1-epimers. This mixture can be readily separated by chromatography and the fractions identified by NMR[30].

The mixture at hand afforded with HCl in anhydrous acetic acid 35% of the crystalline labile 1-α-chloro-2-deoxy-3,5-di-*O*-*p*-toluoylribofuranose (**47**)[31]. Silylated 5-ethyluracil (**48**) reacted readily with **47** in the presence of $(CH_3)_3SiOSO_2CF_3$ (**5**) in 1,2-dichloroethane/acetonitrile at 24°C to give a high yield of the mixture of the

desired β-nucleoside **49** and the α-nucleoside **50**. Crystallization from ethyl acetate and ethyl acetate/ether afforded 58% **49** and 31% **50**.





Since the crude mixture of the furanoside **45** and pyranoside **46** 1-epimers at hand had only furnished 35% of the crystalline 1-α-chloro sugar **47** and, as already discussed, the pyranoside 1-epimers **46** are only very slowly converted at 24°C into the corresponding pyranose cation[28], we reacted silylated 5-ethyluracil **48** directly with this mixture of **45** and **46** in the presence of **5** and obtained after simple chromatography and crystallization 27% of **49** and 15% of **50** – that means higher overall yields based on the crude 2-deoxyribose derivatives **45**/**46** than proceeding via the crystalline 1-α-chlor sugar **47**!

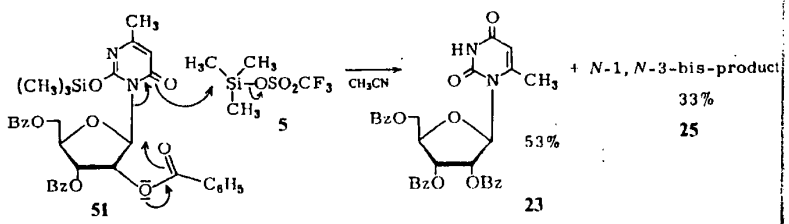
Therefore, on using the new selective catalysts like **5**, it is no longer necessary to prepare the sensitive crystalline halo sugar **47** to separate the furanose **45** from the pyranose **46** forms. $SnCl_4$[19a] as a stronger Lewis acid converts at 24°C either sugar into their corresponding cations and thus into a complex mixture of α/β-anomers of both furanosides and pyranosides[19a].

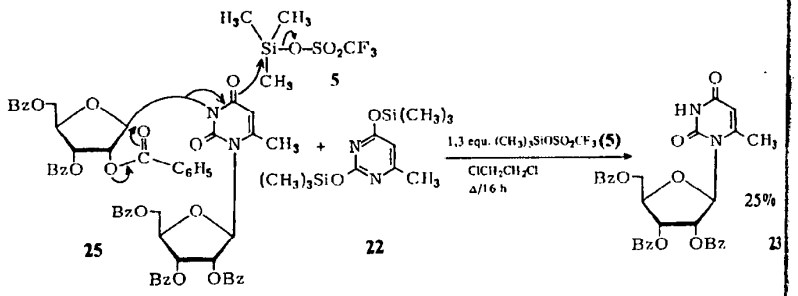
As described in the following chapter C., the yield of the desired β-anomers can be further increased by partial rearrangement of the unwanted α-anomers to the β-anomers.

C. Rearrangements of Nucleosides

During nucleoside synthesis often undesired products like the unnatural *N*-3-nucleosides are formed and isolated. If the nucleoside synthesis (cf. the accompanying paper)[14] is a reversible reaction one should be able to rearrange these undesired nucleosides in their silylated form using our new catalysts.

Heating the benzoylated 6-methyl-*N*-3-uridine (**24**) with hexamethyldisilazane (HMDS) followed by evaporative distillation with absol. xylene afforded the silylated product **51** which was treated for 2.5 h at 24 °C with 1.2 equ. of **5** to give, via *dissociation* to the silylated base **22** and reactive sugar cation **12** (as depicted by the arrows in **51**) and *resynthesis*, 53% of the desired *N*-1-product **23** as well as 33% *N*-1,*N*-3-product **25**.

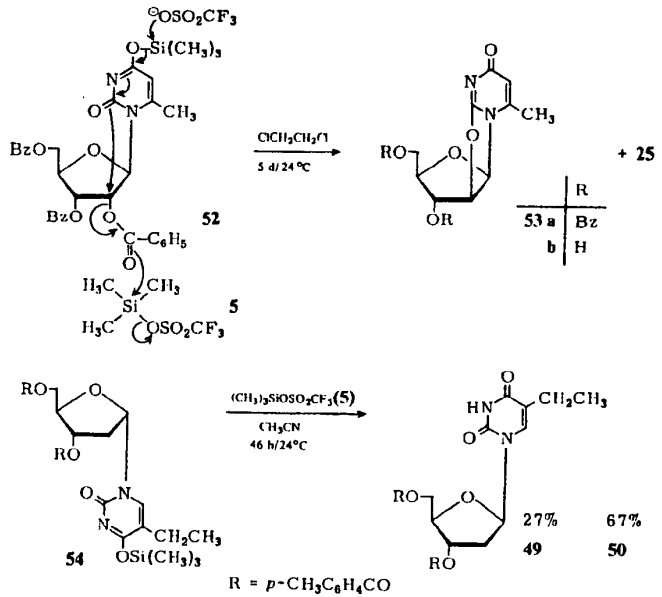




However, in the case of the undesired *N*-1,*N*-3-bis-product **25** in which the heterocyclic carbonyl groups are sterically hindered, **25** had to be heated for 16 h with silylated 6-methyluracil (**22**) and **5** in 1,2-dichloroethane to transfer a benzoylated ribose moiety from *N*-3 to **22** and thus afford 25% of the desired **23**.

To check the chemical stability of the thermodynamically most stable silylated *N*-1-nucleoside **52**, we kept **52** with 1.2 equ. **5** for 5 days in 1,2-dichloroethane at 24 °C and obtained after chromatography ca. 24% of the 3',5'-di-*O*-benzoyl-2,2'-anhydronucleoside **53a** as well as 23.5% of the *N*-1,*N*-3-bis-riboside **25**. **53a** was identified by its NMR and MS data and saponification with methanolic ammonia to the known[32] crystalline **53b**. **53a** is probably formed via electrophilic attack of **5** on the 2'-benzoate as depicted in **52**; a process probably favored by the *syn*-configuration of **52**[33].

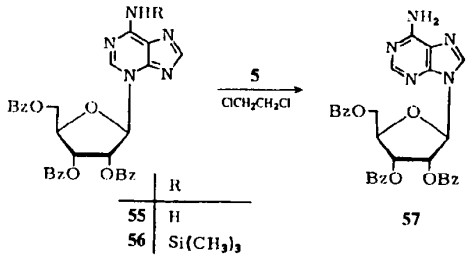
As generally observed and described in chapter B. 6., considerable amounts of the undesired α-nucleosides are always formed during the synthesis of 2'-deoxyribosides. We therefore silylated the α-nucleoside **50** by heating with HMDS and subsequent evaporative distillation with absol. xylene to **54** which was treated for 46 h at 24 °C with **5** in acetonitrile. Workup and preparative t.l.c. gave 27% of the desired β-nucleoside **49** as well as 67% recovered α-anomer **50**. In this way, the overall yield of the desired β-anomer **49** can be further increased.



Although longer reaction times might increase the yield of the β-anomer **49**, they also lead to gradual decomposition of the nucleosides e. g. with formation of furans. Thus, it is not possible to measure the equilibrium between **49** and **50** (chapter B. 6.) to determine whether the synthesis of **49** is kinetically or thermodynamically controlled.

It is interesting in this context to note that *Bardos* et al.[34] have observed that the presence of $(CH_3)_3SiCl$ during the silyl-Hilbert-Johnson reaction with 1-halo-2-deoxy sugars leads preferably to the formation of α-nucleosides.

During our studies on the synthesis of purine nucleosides we had followed the formation of N^6-benzoyladenosine 2',3',5'-tri-*O*-benzoate and isolated among other products the benzoylated *N*-3-nucleoside **55**. Consequently we silylated **55**, which is readily available by the "classical" Hilbert-Johnson reaction[35], to **56**, and rearranged **56** with **5** in boiling acetonitrile in 76% yield to the amorphous adenosine 2',3',5'-tri-*O*-benzoate (**57**)[36].



For the discussion of **56** as a potential intermediate during the synthesis of adenosine compare ref.[14,21c].

Recently, Japanese and German authors have successfully used $(CH_3)_3SiClO_4$ (**4**) and $(CH_3)_3SiOSO_2CF_3$ (**5**) for replacing the pyrimidine moiety in a nucleoside antibiotic by a purine moiety[37,38].

The authors thank Prof. Dr. *H. Schmidbaur* for a sample of $(CH_3)_3SiOSO_2F$ and Dr. *J. Farkas* for an authentic sample of **39**. We are furthermore indebted to Drs. *D. Rosenberg* and *A. Seeger* for physical measurements and Dr. *K. Merz* for microanalyses.

Experimental Part

The melting points were taken on a Kofler melting point microscope and are uncorrected. The UV spectra were recorded on a Cary Model 14 spectrometer, the NMR spectra were determined on Varian A-60 and HR-100 instruments.

The thin layer chromatography (t.l.c.) was performed on E. Merck silica plates F_{254} using systems: A toluene/acetic acid/H_2O (5:5:1)[39], B ethyl acetate/methanol (5:1), C n-BuOH/acetic acid/H_2O (5:1:4)[39].

Materials: Silicagel 60 (E. Merck) 0.063 – 02. mm (70 – 230 mesh, ASTM), as well as cellulose powder (Acivel, Merck) were used for column chromatography. 1,2-Dichloroethane as well as 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (ABR) (**8**) and 3,5-bis(*p*-toluoyl)-2-deoxyribofuranosylchloride (**47**) were purified as previously described[19a].

Acetonitrile was first refluxed several hours over P_2O_5 and destilled from P_2O_5 and finally refluxed over and distilled from CaH_2. The additional CaH_2-treatment of acetonitrile is crucial for a high yield preparation of *O*-benzoylated 6-methyluridine (**23**). Trimethylsilyl perchlorate (**4**) was prepared from silver perchlorate and trimethylchlorosilane in benzene or toluene solution[9]. Trimethylsilyl triflate (**5**), bp. 133 – 134 °C, and nonaflate (**6**), bp. 68 – 69 °C/11 torr, were obtained in 85 – 90% yield by heating of the free triflic and nonaflic acid[18a] with a slight excess of trimethylchlorosilane for ca. 7 h until the evolution of HCl ceased and subsequent distillation with careful exclusion of humidity. For the *in situ* preparation of trimethylsilyl nonaflate from potassium nonaflate ($KOSO_2C_4F_9$) and $(CH_3)_3SiCl$ in acetonitrile compare ref.[17].

Silylations: The bases were routinely silylated by heating with excess of hexamethyldisilazane (HMDS) (for 10 mmol heterocyclic base, 20 – 30 ml HMDS). In cases, where the base did not dissolve promptly after 0.5 – 2 h either 0.1 ml trimethylchlorosilane (TCS) was added (e. g. with 5-nitrouracil, lumazine) or pyridine (ca. 10 ml) (e. g. with 4-aminopyridine, N^6-benzoyladenine, N^2-acetylguanine, xanthine) to accelerate the silylation.

Although the subsequent distillation of the silylated base is crucial as in the case of the preparation of *O*-benzoylated 6-methyluridine (**23**), in most other instances the distillation step can be omitted if the excess HMDS and pyridine is removed by codistillation with 2 × 25 – 50 ml portions of absol. xylene.

The structure of the silyl compounds was only determined and confirmed in some cases and assumed to be as indicated in the formulas (compare the NMR studies in the accompanying paper on the mechanism of nucleoside synthesis[14]).

Workup

A) *After nucleoside formation:* The workup consists routinely of diluting the reaction mixture with CH_2Cl_2 and extracting the organic phase with ice-cold sat. $NaHCO_3$ or $KHCO_3$-solution. The organic phase is then dried (Na_2SO_4) and evaporated to give the crude acylated nucleoside (compare preparation of **11**).

B. *After saponification with methanolic ammonia:* The methanolic ammonia is evaporated *in vacuo*, the residue taken up in water (for 10 mmol nucleoside 25 – 100 ml) and extracted several times with ether and $CHCl_3$ to remove benzamide and methyl benzoate as well as other material. The aqueous phase is then either concentrated for crystallization from water or evaporated for crystallization from other solvents.

Recovery of $C_4F_9SO_3K$: If only a slight excess of $KHCO_3$ is used during workup, a considerable amount of $C_4F_9SO_3K$ crystallizes out (compare preparation of **18a**) which can be reused for the preparation of nonaflic acid or for the *in situ* preparation of $(CH_3)_3SiOSO_2C_4F_9$ in acetonitrile[17] (compare preparation of **18a**).

Uridine 2',3',5'-tri-O-benzoate (**11**): To a mixture of 5.15 mmol 2,4-bis(trimethylsilyloxy)-pyrimidine (**7**) (3 ml of a 1.75 N standard solution in 1,2-dichloroethane) and 2.57 g (5 mmol) ABR (**8**) in 15 ml absol. 1,2-dichloroethane, 2.5 mmol $(CH_3)_3SiClO_4$ (**4**), (16.67 ml of a 0.15 N standard solution in benzene) were added and the mixture kept for 1 week at 24 °C. The clear yellow solution was diluted with 50 ml CH_2Cl_2 and extracted with 50 ml ice-cold $NaHCO_3$-solution. After washing with 3 × 20 ml H_2O, the organic phase was dried (Na_2SO_4) and evaporated to yield 2.8 g colorless foam which crystallized from 40 ml benzene to give after 2 h at 24 °C 2.25 g (81%) crystalline **11**, mp. 138 – 140 °C. The mother liquors contained further amounts of **11** acc. to t.l.c. (system A) (**11**, R_F = 0.5).

When the reaction mixture was refluxed for 4 h instead of keeping it 1 week at 24 °C, an analogous yield of **11** was obtained.

Cytidine 2',3',5'-tri-O-benzoate (**16**): 2.56 g (10 mmol) colorless crystalline 4-(trimethylsilylamino)-2-(trimethylsilyloxy)pyrimidine (**15**) and 5.04 g (10 mmol) ABR (**8**) were dissolved in 35 ml absol. 1,2-dichloroethane and 12 mmol trimethylsilyl triflate (**5**) (24 ml of 0.5 N standard solution in benzene) added and the mixture refluxed for 1 h, cooled and diluted with 100 ml $CHCl_3$. After standard workup the brownish foam was dissolved in 150 ml hot ethanol, treated with charcoal and evaporated to give 4.5 g (98%) of colorless amorphous **16** which was homogenous on t.l.c. (system A, R_F = 0.3) and exhibited the expected NMR and UV data.

5-Methoxyuridine 2',3',5'-tri-O-benzoate (**18a**)

a) *Using triflate* **5**: To 11 mmol silylated 5-methoxyuracil (**17a**)[40] (34 ml of a 0.356 N standard solution in 1,2-dichloroethane), 5.04 g (10 mmol) ABR (**8**) in 75 ml absol. 1,2-dichloroethane, 12 mmol trimethylsilyl triflate (**5**) (22.8 ml of a 0.522 N standard solution in 1,2-dichloroethane) were added and stirred for 4 h at 24 °C. After dilution with $CHCl_3$ and standard workup the crude nucleoside afforded on recrystallization from ethyl acetate/hexane 5.24 g (89%) of pure crystalline **18a**, mp. 205 – 207 °C (lit.[41] 210 – 212 °C) which was homogenous on t.l.c. (system B).

b) *Using nonaflate* **6** (recovery of $C_4F_9SO_3K$): 33 mmol **17a**, 15.12 g (30 mmol) ABR (**8**) and 34 mmol **6** in 200 ml 1,2-dichloroethane were kept for 7 h at 24 °C, diluted with CH_2Cl_2 and worked up with an ice-cold solution of 4.95 g (49.5 mmol) $KHCO_3$ in 80 ml H_2O. On repeated extraction with CH_2Cl_2, the collected CH_2Cl_2-solution was filtered to afford a first crop of $C_4F_9SO_3K$. However the major part of $C_4F_9SO_3K$ was obtained on filtration of the aqueous phase to give a combined yield of 9 g (81%) of recovered $C_4F_9SO_3K$.

The CH_2Cl_2-phase was dried (Na_2SO_4) to give after evaporation and recrystallization of the crude product (17.1 g) from ethyl acetate-hexane in several crops 15.2 g (86%) of crystalline **18a**.

5-Morpholinouridine 2',3',5'-tri-O-benzoate (**18b**) *and 5-morpholinouridine:* To a solution of 10 mmol silylated 5-morpholinouracil[42] (**17b**) 5.04 g (10 mmol) ABR (**8**) in 70 ml absol. 1,2-dichloroethane and 11 mmol trimethylsilyl triflate (**5**) (20.9 ml of a 0.522 N standard solution in 1,2-dichloroethane) were added under argon. After 24 h stirring at 24 °C, dilution with $CHCl_3$ and standard workup the slightly impure **18b** was dissolved in 5 ml ethyl acetate and crude **18b** preci-

pitated with 500 ml hexane. The sticky amorphous **18b** was filtered to give after dissolving in ethyl acetate and evaporation 6.36 g (99%) of nearly pure amorphous **18b** which was practically homogenous on t.l.c. (system A, R_F = 0.5) and identical with an authentic sample[19f].

Saponification of 4 g crude **18b** with 125 ml methanolic ammonia for 1 week at 24°C, and workup gave practically pure nucleoside which crystallized on concentration of the aqueous phase in three crops to afford 1.69 g (82%) of 5-morpholinouridine, mp. 230–234°C (lit.[43] 229–231°C).

5-Nitrouridine 2',3',5'-tri-O-benzoate (**18c**): To a solution of 11 mmol silylated 5-nitrouracil (**17c**) (18.33 ml of a 0.6 N solution in 1,2-dichloroethane) and 5.04 g (10 mmol) ABR (**8**) in 75 ml absol. 1,2-dichloroethane, 12 mmol trimethylsilyl triflate (**5**) (23 ml of a 0.528 N standard solution in 1,2-dichloroethane) were added and the reaction mixture stirred for 2 h at 24°C. After standard workup, the crude product (6.8 g) afforded on recrystallization from ethanol in three crops 5.7 g (93%) pure crystalline **18c**, mp. 184–185°C (lit.[44] mp. 183–184°C) which was homogenous on t.l.c. (system A, R_F = 0.52).

1-(2,3,5-Tri-O-benzoyl-β-D-ribofuranosyl)-4(1H)-pyridinone (**20a**): To a solution of 11 mmol 4-(trimethylsilyloxy)pyridine (**19a**) (17.3 ml of a 0.637 N solution in 1,2-dichloroethane) and 5.04 g (10 mmol) ABR (**8**) in 100 ml absol. 1,2-dichloroethane, 12 mmol trimethylsilyl triflate (**5**) (16.4 ml of a 0.732 N standard solution in 1,2-dichloroethane) were added and the reaction mixture refluxed for 3.5 h, diluted with 100 ml CH_2Cl_2 and worked up as described above. The crude foam (5.75 g) was dissolved in ethyl acetate and chromatographed on 200 g silicagel. After elution with ethyl acetate (ca. 4 l) and ethyl acetate-methanol (97:3, 1 l) further elution with the same mixture (1.5 l) afforded 4.72 g (87%) amorphous **20a** which gave on saponification with methanolic ammonia crystalline 1-(β-D-ribofuranosyl)-4(1*H*)-pyridinone (**21a**), mp. 128–130°C, identical with a previously obtained authentic sample[19d].

1-(2,3,5-Tri-O-benzoyl-β-D-ribofuranosyl)-4(1H)-pyridinimine (**20b**): 1.89 g (11.37 mmol) redistilled, crystalline 4-(trimethylsilylamino)pyridine (**19b**) and 5.04 g (10 mmol) ABR (**8**) in 70 ml absol. 1,2-dichloroethane were treated with 16.4 ml (12 mmol) of a standard solution of trimethylsilyl triflate (**5**) in 1,2-dichloroethane under argon. After 2.5 h reflux, dilution with CH_2Cl_2 and standard workup afforded 7.15 g crude **20b**. Chromatography on 350 g SiO_2 with ethyl acetate gave after a forrun of 1 l on further elution with ethyl acetate (7.5 l) 4.34 g (80%) pure homogenous (t.l.c., system A, R_F = 0.17; system B, R_F = 0.65) amorphous **20b** which had the expected UV and NMR data.

1-(β-D-Ribofuranosyl)-4(1H)-pyridinimine (**21b**): 2 g (3.7 mmol) **20b** was stirred with 150 ml methanolic ammonia for 18 h, and worked up as usual to give the free nucleoside **21b** which has as yet refused to crystallize and was homogenous on t.l.c. (system C, R_F = 0.23). – UV (CH_3OH): λ_{max} (log ε) = 205 (3.84), 275 nm (3.97). – NMR (D_2O): δ = 5.68 (d, *J* = 5 Hz, 1'-H), 6.9 (d, *J* = 8 Hz, 3-H, 5-H), 8.18 (d, *J* = 8 Hz, 2-H, 6-H).

1-(2,3,5-Tri-O-benzoyl-β-D-ribofuranosyl)-2(1H)-pyridinone: 5 mmol 2-(trimethylsilyloxy)-pyridine (5 ml of a standard solution in 1,2-dichloroethane) and 2.52 g (5 mmol) ABR (**8**) in 25 ml absol. 1,2-dichloroethane were refluxed for 1.5 h with 5 mmol trimethylsilyl triflate (**5**) (10 ml of a 0.5 N solution in benzene). After dilution of the light brown reaction mixture with $CHCl_3$ and standard workup the crude light brown oil (2.8 g) gave on crystallization from 75 ml CCl_4 and concentration of the mother liquor to 25 ml in two crops 2.31 g (86%) of 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-2(1*H*)-pyridinone, mp. 136–138°C which was identical with an authentic sample[19d].

6-Methyl-1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-2,4(1H,3H)-pyrimidinedione (**23**) *and 6-methyl-3-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-2,4(1H,3H)-pyrimidinedione* (**24**): 11 mmol

(29.2 ml of a 0.377 N standard solution in 1,2-dichloroethane) of redistilled silylated 6-methyluracil (**22**) and 5.04 g (10 mmol) ABR (**8**) in 100 ml absol. acetonitrile (refluxed first over P_2O_5 and subsequently over CaH_2!) in a carefully dried glas apparatus were reacted at +4°C with 12 mmol **5** (25 ml of a 0.48 N standard solution in 1,2-dichloroethane). After warming up and 2 h at 24°C, no **8** could be any more detected on t.l.c. (system A). After dilution with 200 ml chloroform and standard workup the crude yellowish foam (6.38 g) was chromatographed on 350 g silicagel with chloroform (4 l). Further elution with chloroform (3 l) and chloroform-isopropyl alcohol 99:1 (1 l) and 98.5:1.5 (1 l) gave 1.1 g (10.8%) *N*-1,*N*-3-bis-riboside **25**. Elution with chloroform-isopropyl alcohol 98:2 (1.75 l) gave 4.29 g (75.3%) homogenous *N*-1-riboside **23**. Further elution with the 98:2 mixture (3 l) afforded 0.22 g (3.85% of the *N*-3-riboside **24**, mp. 165–167°C (lit.[19b] 108–109°C) from ethyl acetate-hexane which was identified with an authentic sample[45].

The *N*-1-riboside **23** was recrystallized from CH_2Cl_2-pentane to give in three crops 4.05 g (71.1%) analytically pure **23**, mp. 181–183°C (lit.[19b] 126–129°C) which was identical with an authentic sample, t.l.c. (system A, R_F = 0.55). When the authentic sample, mp. 126–129°C was recrystallized from ethyl acetate-hexane, the same higher melting crystalls, mp. 182–183°C were obtained.

5,6-Dimethyl-1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-2,4(1H,3H)-pyrimidinedione (**27**) *and 5,6-dimethyl-3-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-2,4(1H,3H)-pyrimidinedione* (**28**): 11 mmol silylated 5,6-dimethyluracil (**26**) and 5.04 g (10 mmol) ABR (**8**) in 50 ml absol. 1,2-dichloroethane were reacted with 12 mmol trimethylsilyl triflate (**5**) (25 ml standard solution in 1,2-dichloroethane) for 3 h at 24°C under argon. Dilution with chloroform and standard workup gave 6.5 g crude product which was chromatographed on 350 g neutral alumina (A III) with hexane-ethyl acetate (1:1). After discarding the first 250 ml eluate, the next 300 ml eluted 0.5 g (8.6%) *N*-3-riboside **28** (mp. 200–201°C) (lit.[19b] 200–201°C). The subsequent fractions (2 l) eluted 4.8 g (82.2%) of **27** which crystallized from hexane-CH_2Cl_2 to give analytically pure **27**, mp. 175–176°C (lit.[19b] 176–178°C).

Adenosine (**30a**): 2.393 g (10 mmol) N^6-Benzoyladenine was refluxed for 7 h with 35 ml HMDS and 0.5 ml TCS (clear solution after 2 h) and the solvents were removed at 50°C/0.1 torr. The solid yellowish silyl compound **29a** and 5.04 g (10 mmol) ABR (**8**) were dissolved in 25 ml absol. 1,2-dichloroethane and refluxed for 12 h with 1 mmol (6.7 ml standard solution in benzene) trimethylsilyl perchlorate (**4**). After dilution with CH_2Cl_2 and standard workup the crude protected adenosine (7.1 g) was dissolved in 250 ml methanolic ammonia and kept for 16 h at 24°C. After workup the residue was evaporated in vacuum to give 4.1 g crude product. Recrystallization from methanol-H_2O (2:1; 200 ml) afforded in several crops 2.16 g (80.9%) of pure crystalline adenosine (**30a**) which was homogenous on t.l.c. (system C, R_F = 0.43).

Guanosine (**30b**): 4.09 mmol (13.5 ml of a 0.303 N standard solution in absol. 1,2-dichloroethane) silylated N^2-acetylguanine (**29b**) and 1.86 g (3.7 mmol) ABR (**8**) in 35 ml absol. 1,2-dichloroethane were refluxed with 4.46 mmol (6.32 ml of a 0.705 N standard solution in 1,2-dichloroethane) trimethylsilyl triflate (**5**) for 1.5 h. After dilution with CH_2Cl_2 and the usual workup, the crude product (2.32 g) was kept for 42 h in 125 ml methanolic ammonia at 24°C. After standard workup, recrystallization from water gave two crops of pure guanosine (**30b**) (0.69 g = 66%) which was homogenous on t.l.c. (system C, R_F = 0.3) and identified with an authentic sample.

Xanthosine (**30c**): 11 mmol (22 ml of a 0.5 N standard solution in 1,2-dichloroethane) silylated xanthine **29c**, 5.04 g (10 mmol) ABR (**8**) in 80 ml absol. 1,2-dichloroethane were refluxed for 1 h with 12 mmol (17.5 ml standard solution) of trimethylsilyl triflate (**5**). After dilution with CH_2Cl_2 and the usual workup the crude product (6.18 g) showed on t.l.c. (system A) besides the main

product (R_F = 0.38) a number of minor faster moving spots. After saponification with 200 ml methanolic ammonia for 3 days/24 °C, and standard workup, concentration of the aqueous phase afforded in 6 crops 0.95 g pure xanthosine (**30c**). The mother liquor was evaporated with 2 g cellulose-powder which was packed on top of a column of cellulose-powder (40 g, Avicel, E. Merck) and chromatographed with methanol. After a forrun of 250 ml the next fractions (350 ml) eluted a further amount of 0.43 g xanthosine which was homogenous on t.l.c. (system C, R_F = 0.3) and identical with an authentic sample. Combined yield of **30c** 1.38 g (48.8%).

Theophylline 7-β-D-ribofuranoside (**32**): 2 mmol silylated theophylline **31**, 1.08 g (2 mmol) ABR (**8**) and 2.2 mmol **5** (2.2 ml of 1 M standard solution in 1,2-dichloroethane) were kept for 1 h at 24 °C. After workup, the crude product (1.35 g) was kept for 18 h at 24 °C in 50 ml methanolic ammonia to give after workup and crystallization from 5 ml H_2O 0.54 g (81.8%) pure **32**, mp. 191 – 193 °C (lit.[22] 189 °C), which had a UV spectrum quite similar to the one of coffeine. – NMR ([D_6]DMSO): δ = 3.22 (s, N – CH_3); 3.42 (s, N – CH_3); 6.05 (d, J = Hz, 1'-H); 8.4 (s, 8-H).

$C_{12}H_{16}N_4O_6 \cdot H_2O$ (333.3) Calcd. C 43.63 H 5.49 N 16.96 Found C 43.69 H 5.86 N 16.7

Lumazine riboside (**34b**): 1.64 g (10 mmol) silylated lumazine **33** and 5.04 g (10 mmol) ABR (**8**) in 30 ml absol. 1,2-dichloroethane were reacted with 12 mmol (23.7 ml of a 0.53 N standard solution in 1,2-dichloroethane) trimethylsilyl triflate (**5**) at 24 °C. After 10 min the reaction mixture turned very dark green and t.l.c. (system D) showed that only traces of starting material were left. After 1.3 h 50 ml chloroform were added and the reaction mixture worked up as usual to afford 6.34 g of foam which was practically homogenous. On chromatography on silicagel with toluene-ethyl acetate 5.68 g (93.4%) pure amorphous tribenzoate **34a** was obtained. 4.8 g (7.9 mmol) amorphous **34a** were dissolved in 350 ml methanol and sat. at + 4 °C with NH_3 and kept for 80 h at 24 °C. After standard workup and evaporation of the aqueous phase the yellowish residual foam (2.68 g) was dissolved and crystallized from ethanol-isopropyl alcohol to afford in two crops 2.13 g (91%) crystalline **34b**, mp. 152 – 155 °C. A further crystallization from ethanol-isopropyl alcohol gave the analytical sample, mp. 192 – 194 °C (lit.[23] 182 – 184 °C) which showed the same physical data (UV, NMR) as described in the literature[23].

$C_{11}H_{12}N_4O_6$ (296.4) Calcd. C 44.92 H 4.12 N 18.72 Found C 44.60 H 4.08 N 18.91

1-(2,3,5-Tri-O-benzoyl-β-D-ribofuranosyl)-1,2,4-triazole (**36a**): To a mixture of 11 mmol (24 ml of a 0.461 N standard solution in 1,2-dichloroethane) silylated triazole **35a** and 5.04 g (10 mmol) ABR (**8**) in 75 ml absol. acetonitrile 12 mmol **5** (23 ml of a 0.528 N standard solution in 1,2-dichloroethane) were added under argon. After 3 h at 24 °C and workup, the residue (6 g) was chromatographed on 300 g silicagel. Toluene (1.5 l) and toluene-ethyl acetate 19:1 (500 ml) and 4:1 (50 ml) eluted impurities. Further elution with the 4:1 mixture (750 ml) gave first 0.89 g of a product, which decomposed on saponification with methanolic ammonia. Further elution with the 4:1 mixture (5 l) afforded 3.12 g (61%) of **36a** which crystallized from ethanol to give 2.94 g pure **36a**, mp. 105 – 106 °C (lit.[24] 103 – 105 °C).

$C_{28}H_{23}N_3O_7$ (513.5) Calcd. C 65.49 H 4.51 N 8.18 Found C 65.40 H 4.57 N 8.00

Saponification of 2.02 crude **36a** with 125 ml methanolic ammonia for 24 h and usual workup gave from methanol in three crops 0.54 g (67.5%) free 1-(β-D-ribofuranosyl)-1,2,4-triazole, mp. 144 – 145 °C (lit.[24] 143 – 145 °C). – NMR (D_2O): δ = 6.0 (d, J = 5 Hz; 1'-H) 8.1 (s, 5-H) 8.63 (s, 3-H).

Methyl 1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-1,2,4-triazole-3-carboxylate (**36b**): 11 mmol (24.55 ml of a 0.488 N standard solution in 1,2-dichloroethane) silylated methyl 1,2,4-triazole-3-carboxylate **35b** and 5.04 (10 mmol) ABR (**8**) in 100 ml absol. acetonitrile were cooled to + 4 °C and 12 mmol **5** (26.76 ml of a 0.499 N standard solution in 1,2-dichloroethane) added under argon. After 4 h at + 4 °C and workup the crude product (6.1 g) was chromatographed on 300 g

silicagel. After elution with toluene (2.5 l), toluene-ethyl acetate 9.5:0.5 (2.5 l) and 9:1 (1 l) afforded only some ribose derivatives whereas the 9:1 mixture (1.5 l) eluted 0.886 g (15.5%) of the cyano derivative **37c**, mp. 158 – 160 °C (ethanol). – IR (KBr): 2250 cm^{-1} (weak, nitril). – ^{1}H-NMR ($CDCl_3$): δ = 6.2 (d, J = 1.5 Hz, 1'-H), 8.4 (s, 5-H). – MS: m/e = 538 (M^+), 445 (M – 3-cyano-1,2,4-triazole), 416 (M – $C_6H_5CO_2H$), 364 (M – $(CN)_2$), 322 (M – $C_6H_5CO_2H$ – 3-cyano-1,2,4-triazole).

$C_{29}H_{22}N_4O_7$ (538.5) Calcd. C 64.82 H 4.16 N 9.58 Found C 64.68 H 4.12 N 10.41

Further elution with the 9:1 solvent mixture (2.5 l) gave 1.12 g (19.6%) of the methyl ester **37b**, mp. 142 – 144 °C (ethanol) (lit.[25] mp. 123 – 124 °C)[45]. – NMR ($CDCl_3$): δ = 4.0 (s, CO_2CH_3).

$C_{30}H_{25}N_3O_9$ (571.5) Calcd. C 63.04 H 4.41 N 7.35 Found C 63.34 H 4.44 N 7.23

After some intermediate fractions (750 ml, 23 mg) the 9:1 mixture (1.5 l) and 4:1 mixture (1 l) gave 0.132 g (2.3%) of the benzoylated triazole riboside **36a**, mp. 144 – 145 °C. Further elution with 3 l of a 4:1 solvent mixture afforded 2.684 g (47%) of **36b**, mp. 141 – 142 °C (lit.[25] 137 – 139 °C) identical with an authentic sample. – ^{1}H-NMR ($CDCl_3$): δ = 3.98 (s, OCH_3), 6.32 (d, J = 2 Hz, 1'-H), 8.4 (s, 5-H). – MS: m/e = 571 (M^+), 540 (M – CH_3OH), 449 (M – $C_6H_5CO_2H$), 445 (M – methyl triazolecarboxylate), 390 (M – $C_6H_5CO_2H$ – CO_2CH_3), 364 (M – $C_6H_5CO_2H$ – CH_3O_2C – CN), 242 (M – 2 $C_6H_5CO_2H$ – CH_3O_2C – CN).

$C_{30}H_{25}N_3O_9$ (571.5) Calcd. C 63.04 H 4.41 N 7.35 Found C 63.40 H 4.43 N 7.50

2-(β-D-Ribofuranosyl)-1,2,4-triazole-3-carboxamide: 0.34 g (0.59 mmol) **37b** were kept in 50 ml methanolic ammonia at 24 °C and worked up as usual. The residue was homogeneous on t.l.c. (system C, R_F = 0.76) and crystallized from ethyl acetate-methanol, mp. 111 – 113 °C (lit.[25] mp. 148 – 150 °C)[45]. The ^{1}H and especially the ^{13}C-NMR data in 10% DMSO were identical with the literature data[46].

1-(β-D-Ribofuranosyl)-1,2,4-triazole-3-carboxamide: 1.0 g (1.75 mmol) **36b** was kept in 150 ml sat. methanolic ammonia over night. After the usual workup, the product was recrystallized from ethanol to give 0.39 g (91.3%) of the free amide, mp. 176 – 178 °C (lit.[25] 174 – 176 °C) which was identical with an authentic sample.

$C_8H_{12}N_4O_5$ (244.2) Calcd. C 39.34 H 4.95 N 22.94 Found C 39.66 H 5.10 N 23.25

1-(2,3,5-Tri-O-benzoyl-β-D-ribofuranosyl)-2,4,6-trimethoxybenzene (**39**) *and 1,3-bis(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-2,4,6-trimethyoxybenzene* (**40**): To 0.845 g (5 mmol) 1,3,5-trimethoxybenzene (**38**) and 2.5 g (5 mmol) ABR (**8**) in 65 ml absol. 1,2-dichloroethane 6 mmol (10.66 ml of a 0.563 N solution in 1,2-dichloroethane) trimethylsilyl triflate (**5**) were added under argon, whereupon the solution turned red. After 30 min at 24 °C and dilution with 50 ml CH_2Cl_2, standard workup gave the crude product (3.3 g) which was chromatographed on 150 g silicagel. Elution with toluene (3 l), toluene-ethyl acetate 99:1 (5 l) yielded only some impurities whereas the 98.5:1.5 mixture (2 l) gave 0.164 g of ribose derivatives. Further elution with the solvent mixtures 98.5:1.5 (1 l), 98:2 (3 l) and 97.5:2.5 (1 l) afforded 1.846 g (60.3%) of **39** which crystallized on seeding with an authentic sample[26], mp. and mixted mp. 102 – 103 °C (lit.[26] mp. 102 – 103 °C). – NMR ($CDCl_3$): δ = 3.8 (s, 4-OCH_3), 3.88 (s, 2, 6-OCH_3), 5.75 (d, J = 4 Hz, 1'-H), 6.1 (s, 3, 5-H). Elution with the 97.5:2.5 solvent mixture (4 l) and 90:10 (0.7 l) afforded 0.244 g of impurities and 0.108 g 2,3,5-tri-O-benzoyl-β-D-ribofuranose, whereas the 80:20 solvent mixture (1 l) gave 0.517 g (4.9%) of **40**, mp. 95 – 97 °C (isopropylalcohol). – NMR ($CDCl_3$): δ = 3.9 + 3.9 (s, OCH_3), 5.63 (d, J = 6 Hz, 1'-H), 6.33 (s, 3-H).

$C_{61}H_{52}O_{17}$ (1057.0) Calcd. C 69.31 H 4.96 Found C 69.29 H 5.19

1-(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl)uracil (**42**): To a solution of 11 mmol silylated uracil **7** (16.41 ml of a 0.67 N standard solution in 1,2-dichloroethane) and 3.9 g (10 mmol)

1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (**41**) in 100 ml absol. 1,2-dichloroethane 12 mmol (24.2 ml of a 0.496 N standard solution in 1,2-dichloroethane) of trimethylsilyl nonaflate (**6**) were added and the reaction mixture refluxed for 2.5 h. After dilution with CH_2Cl_2 and standard workup, the crude product (5 g) was recrystallized from ethanol to afford in two crops 4.07 g (92.1%) of pure **42**, mp. 149 – 150 °C (lit.[29b,29c] mp. 149 – 151 °C).

The analogous reaction with trimethylsilyl triflate (**5**) as catalyst gave 3.92 g (88.7%) of pure **42**.

2-(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl)-1,2,4-triazine-5(4H)-one-3(2H)-thione (**44**): To a solution of 10 mmol silylated "2-thio-6-azauracil" (**43**) (10 ml of a N standard solution in 1,2-dichloroethane) and 3.9 g (10 mmol) 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (**41**) in 25 ml absol. 1,2-dichloroethane, 1 mmol of trimethylsilyl perchlorate (**4**) (6.7 ml of a standard solution in absol. benzene) was added and the reaction mixture refluxed for 3.5 h. After dilution with $CHCl_3$ and standard workup, the crude reddish product (3.5 g) was recrystallized with charcoal from 200 ml methanol to afford in two crops 2.56 g (55.8%) of pure **44**, mp. 221 – 223 °C (lit.[19a] mp. 225 – 226 °C) which was identical with an authentic sample.

1-(2-Deoxy-3,5-di-O-p-toluoyl-β-D-ribofuranosyl)-5-ethyl-2,4(1H,3H)-pyrimidinedione (**49**): 1) To a solution of 5.5 mmol silylated 5-ethyluracil (**48**) (8.23 ml of a 0.668 N standard solution in 1,2-dichloroethane) and 1.94 g (5 mmol) crystalline 1-α-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-D-ribofuranose (**47**) in 35 ml absol. acetonitrile and 15 ml 1,2-dichloroethane, 1 mmol trimethylsilyl triflate (**5**) (1.3 ml of a 0.773 N standard solution in 1,2-dichloroethane) was added at 0 °C and the reaction mixture stirred for 3 h at 24 °C. After dilution with CH_2Cl_2 and workup the crude product (2.5 g) afforded on recrystallization from ethyl acetate in 4 crops 1.34 g (57.8%) of the pure crystalline β-anomer **49**, mp. 199 – 201 °C (lit.[19a] mp. 197 – 198 °C) which was homogenous on t.l.c. in ether (R_F = 0.83). Crystallization of the mother liquor from ethylacetate-ether and finally acetone afforded 0.073 g (31.5%) of the crystalline α-anomer **50**, mp. 157 – 159 °C (lit.[19a] mp. 160 – 161 °C) which was homogenous on t.l.c. (ether, R_F = 0.54). The total yield of both anomers was 2.07 g (89%).

2) To a solution of 3.5 mmol **48** and 1.3 g (3.38 mmol) sugar mixture **45/46** in 20 ml absol. acetonitrile was added 4.06 mmol **5** in 1,2-dichloroethane and the mixture kept at 24 °C for 3 h. After workup the crude product (1.08 g) was chromatographed on a column of 50 g silicagel with ether-hexane (1 : 1). After discarding the first 250 ml eluate, the next 500 ml afforded on recrystallization from ethanol 0.1 g of the pure β-anomer **49**. On eluting with ether-hexane (3 : 2) the first 750 ml gave a mixture of **49** and **50** from which 0.19 g of pure **49** crystallized from ethanol. The mother liquor gave a second crop of 0.14 g of **49** and 0.12 g of the α-anomer **50**[19a]. Further elution with 1.5 l of the 3 : 2 solvent mixture gave 0.1 g of the α-anomer **50**. Total yield 0.43 g (27.4%) **49** and 0.24 g (15.3%) **50**.

Rearrangement of 6-methyl-3-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-2,4(1H,3H)-pyrimidinedione (**24**): 2.85 g (5 mmol) of **24** were heated for 18 h with 15 ml HMDS and 10 ml absol. xylene at 140 °C oil bath temperature. After evaporating the solvents first at normal pressure and finally in the vac. (60 °C/12 Torr), the residue was taken up in 50 ml absol. acetonitrile and the solution stirred for 2.5 h at 24 °C after adding 6 mmol **5** (10.27 ml of a 0.584 N standard solution in 1,2-dichloroethane). After dilution with CH_2Cl_2 and the usual workup, the crude product (2.83 g) was chromatographed on silicagel as described (compare preparation of **23**) to afford 1.575 g (53.3%) of the desired *N*-1-nucleoside **23** and 0.83 g (32.8%) of the benzoylated *N*-1,*N*-3-bisriboside **25**, which were identified with authentic samples.

Cleavage of 6-methyl-1,3-bis(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-2,4(1H,3H)-pyrimidinedione (**25**): 1.0 g (0.99 mmol) **25** and 0.99 mmol **22** (2.79 ml of a 0.354 N standard solution in 1,2-dichloroethane in 40 ml absol. 1,2-dichloroethane) were refluxed for 16 h with 1.26 ml **5** (2.2 ml

of a 0.85 N standard solution in 1,2-dichloroethane). After addition of CH_2Cl_2 and workup the residue (0.74 g) was chromatographed with $CHCl_3$ on 15 g silicagel and crystallized to afford 0.287 g (25.4%) of pure crystalline **23**.

Cyclization of 6-methyl-1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-2,4(1H,3H)-pyrimidinedione (**23**) *to 2,2'-anhydro-1-(3,5-di-O-benzoyl-β-D-arabinofuranosyl)-6-methyl-2,4(1H,3H)-pyrimidinedione* (**53a**): 1.14 g (2 mmol) **23** in 10 ml HMDS and 15 ml xylene was heated until **23** had dissolved. After evaporation of the solvents *in vacuo* the silylated nucleoside was dissolved in 10 ml 1,2-dichloroethane, 2.4 mmol **5** (4.38 ml of a 0.548 N standard in 1,2-dichloroethane) added at +4 °C under argon and the reaction mixture kept for 5 days at 24 °C. After dilution with $CHCl_3$ and workup, the residue (1.5 g) was chromatographed on 150 g silicagel with $CHCl_3$ and $CHCl_3$-isopropyl alcohol. After obtaining the *N*-1,*N*-3-bis-riboside **25** (0.228 g = 23.5%) and the starting *N*-1-riboside **23** (0.215 g) the anhydronucleoside **53a** (0.225 g = 24.2%) was eluted with $CHCl_3$-isopropyl alcohol (99.5 : 0.5 → 99 : 1). – NMR ($CDCl_3$): δ = 4.35 (br. s, 6-CH_3); 5.65 (br. s, 5-H); 6.53 (*J* = 5 Hz, 1'-H). – MS: *m/e* = 448 (M^+), 343 (M – C_6H_5CO), 327 (M – C_6H_5COO), 229 (327 – $C_6H_5CO_2H$), 201, *126*.

$C_{24}H_{20}N_2O_7$ (448.4) Calcd. C 64.28 H 4.50 N 6.25 Found C 63.99 H 4.71 N 6.07

Saponification of 0.1 g (0.22 mmol) **53a** with 10 ml methanolic ammonia for 18 h at 25 °C afforded after workup crude **53b** which was recrystallized from ethanol to give 40 mg (75.5%) of **53b**, mp. 211 – 213 °C (partly 220 – 221 °C) (lit.[32a] mp. 213 – 215 °C).

UV (CH_3OH): λ_{max} (ε) = 225 (8050), 250 nm (8830). – NMR (D_2O): δ = 2.43 (s, 6-CH_3), 3.6 (d, 4 Hz, 5'-H_2), 4.38 (m, 4'-H), 5.45 (d, *J* = 6 Hz, 2'-H), 6.0 (s, 5-H), 6.52 (d, 6 Hz, 1'-H).

$C_{10}H_{12}N_2O_5$ (240.2) Calcd. C 50.00 H 5.04 N 11.66 Found C 50.04 H 4.96 N 11.43

Rearrangement of the silylated 1-(2-deoxy-3,5-di-O-p-toluoyl-α-D-ribofuranosyl)-5-ethyl-2,4(1H,3H)-pyrimidinedione (**54**): 2.3 g (5 mmol) **50** were heated in 25 ml HMDS and 25 ml xylene until **50** dissolved. The solvents were removed *in vacuo*, the residue taken up in 40 ml absol. acetonitrile and 6 mmol **5** (7.76 ml of a 0.773 N standard solution in 1,2-dichloroethane) added. After 46 h at 24 °C the dark reaction mixture was diluted with CH_2Cl_2 and worked up. 137 mg of the residue (2.26 g) were separated on two preparative (20 × 20 cm) silicagel plates with ether to afford 94.5 mg α-anomer **50** and 37.5 mg β-anomer **49** which amounts to the formation of 27% of the desired β-anomer **49** and recovery of 67% of the α-anomer **50**.

Rearrangement of 3-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)adenine (**55**) *to 2',3',5'-tri-O-benzoyladenosine* (**57**): 1.5 g (2.6 mmol) **55** was refluxed for 8 h with 75 ml HMDS and 20 ml absol. xylene. After removal of the solvents *in vacuo*, the residue was dissolved in 50 ml absol. acetonitrile and 3.12 mmol **5** (4.55 ml of a 0.685 N standard solution in 1,2-dichloroethane) added. After 15.5 h reflux with careful exclusion of humidity, the reaction mixture was diluted with CH_2Cl_2 and worked up to give 1.45 g brownish residue. Chromatography on 80 g silicagel with CH_2Cl_2-methanol 99 : 1 and 98 : 2 afforded 1.14 g (76%) of amorphous adenosine 2',3',5'-tri-*O*-benzoate (**57**), which was homogenous on t.l.c. (system A, R_f = 0.4) and gave on saponification with methanolic ammonia pure adenosine.

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[254/80]

Organic Reactions

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therefore only be used in a well ventilated hood. Acetonitrile is likewise considered a toxic hazard[523] and should therefore also be handled with care.

For a general review on the influence of solvents on reactivity, see Ref. 523a.

Workup of Friedel-Crafts-Catalyzed Silyl-Hilbert-Johnson Reactions

TMSOTf or trimethylsilyl nonaflate are converted on aqueous workup with ice-cold saturated sodium bicarbonate solution into the corresponding sodium salts, which are water soluble and do not interfere with the subsequent extraction of the aqueous phase with CH_2Cl_2. On employing potassium bicarbonate (or carbonate), crystalline potassium nonaflate can be recovered on concentration of the aqueous solution for reuse in the one step-one pot nucleoside synthesis.

In contrast to the use of TMSOTf or nonaflate, the workup of reactions employing $SnCl_4$ (or $TiCl_4$) with ice-cold aqueous sodium bicarbonate usually gives rise to emulsions, which are often difficult to extract with CH_2Cl_2. In these cases, the crude reaction mixture should be filtered through a layer of Celite (or Kieselguhr) to remove the insoluble tin salts, which should be washed thoroughly with CH_2Cl_2. The combined filtrates can then be separated and the aqueous phase extracted with additional volumes of CH_2Cl_2. In certain cases the precipitated tin salts obtained on workup with ethanol/aqueous $NaHCO_3$ solution were subsequently extracted with CH_2Cl_2 in a Sohxlet extractor to avoid any loss of precious substance.[524]

If the $SnCl_4$-catalyzed reactions are run in 1,2-dichloroethane, addition of equivalent amounts of pyridine leads to a colorless precipitate of a pyridine-$SnCl_4$ σ complex, which can be readily filtered through a layer of Celite and washed with 1,2-dichloroethane or CH_2Cl_2. The subsequent workup with ice-cold $NaHCO_3$-CH_2Cl_2 proceeds without complications.[525,526] Other authors have added an ethanolic solution of triethylamine to the reaction mixture of $SnCl_4$ in 1,2-dichloroethane and then evaporated the volatile fraction. The resulting syrup was stirred in chloroform and evaporated with silica gel, which was then placed on top of a silica gel column for subsequent chromatography.[527,528] For the synthesis of very acid-sensitive 2-oxo-6-chloropurine nucleosides employing TMSOTf in CH_2Cl_2, pyridine was added to quench the Lewis acid before chromatography on silica.[523b,c]

Removal of *O*-Acyl, *N*-Acyl, *O*-Benzyl, or *O*-Silyl Protecting Groups

After nucleoside bond formation, workup and crystallization or chromatography followed by crystallization (if they crystallize), the *O*-acyl, *N*-acyl, *O*-benzyl, or *O*-silyl groups are normally removed. *O*-Benzyl groups can be removed by BCl_3[66,344,349] or BBr_3[256] at −78°, by sodium in liquid ammonia,[343] or by hydrogenation.[68,341] *O*-Silyl groups are usually cleared by treatment with TBAF in tetrahydrofuran,[339,529] by triethylamine hydrofluoride, by pyridine hydrofluoride, or by treatment with trifluoroacetic acid.[68] For a review of the removal of silyl groups, see Ref. 529a.

The most common procedure used for the removal of *O*- and *N*-acyl groups is transesterification-saponification with saturated methanolic ammonia. On saponification of a protected nucleoside with methanolic ammonia, the progress of the saponification can be followed by TLC or HPLC, whereupon the heterocyclic chromophore can be detected by UV light and the sugar moiety by spraying with 50% H_2SO_4 in ethanol and subsequent heating to 140° to induce darkening of spots containing the carbohydrate moiety. After 72 hours at 24° normally all the *O*- and *N*-acyl groups are removed to give the free nucleoside, which can be checked for purity on SiO_2-TLC plates using the upper phase of *n*-butanol/acetic acid/H_2O (4:1:5)[81] or by RP-HPLC.

In addition to the commonly used methanolic (or ethanolic) ammonia, methylamine, diisopropylamine, triethylamine, and hydrazine have also been employed.[221,298,409,475,530–532] Saponification of 6-(2′,3′,5′-tri-*O*-acetyl-β-D-ribofuranosyl)-6-aza-5,6-dihydro-5,5-dimethyluracil with methanolic ammonia leads to rearrangement of the sugar moiety and gives 6-(β-D-ribopyranosyl)-6-aza-5,6-dihydro-5,5-dimethyluracil.[533]

However, saponification-transesterification with methanolic ammonia can sometimes be quite slow so that even after 72 hours/24° *O*-acylated nucleosides can still be detected by TLC. In the case of 2′,3′,5′-tri-*O*-benzoyl-5-fluorocytidine, the ion exchanger Amberlyst A-26 (OH^- form) in methanol was used to effect a more rapid removal of the *O*-benzoyl groups.[534] Compare also the analogous saponification-transesterification of *O*-acetyl groups with IRA-400 (OH^- form) in methanol.[535] The saponification-transesterification of *O*-acetyl or *O*-benzoyl groups in nucleosides with $NaHCO_3$, Na_2CO_3, or K_2CO_3 in absolute methanol at −50° was recently suggested.[536,536a] Alternatively, $ZnBr_2$ in chloroform-methanol removes *N*-acyl groups selectively,[536b] whereas lipases selectively saponify *O*-acyl groups.[536c]

After completion of the saponification-transesterification, the methanolic (ethanolic) ammonia (methylamine, diisopropylamine) is evaporated in vacuo. In the case of *O*-benzoyl, 4-nitro, 4-chloro, or 4-methylbenzoyl groups the rates of the saponification-transesterification differ, and the crude product contains the corresponding methyl (or ethyl) benzoates, as well as the corresponding amides, which can be readily extracted with diethyl ether or methyl *tert*-butyl ether.

On using hydrazine benzoate or hydroxylamine acetate in pyridine the more acidic 2′-*O*-acetates or benzoates can be selectively cleaved to give, with adenosine-2′,3′,5′-tri-*O*-benzoate, a 74% yield of adenosine-3′,5′-di-*O*-benzoate.[328,329,532,537] Heating with hydrazine to 100° leads to rapid removal of all *O*-acyl groups.[475]

The standard procedure with methanolic ammonia fails, however, with 5-nitrouridine-tri-*O*-benzoate,[538] which apparently decomposes via addition of NH_3 to the 6-position and subsequent ring opening. Sodium methylate in methanol, however, gives a high yield of 5-nitrouridine.[538,538a] Ammonia and primary or secondary amines in methanol (or ethanol) can aminate (or methoxylate) a 2-chloro or 2-fluoro group,[475,539] and under forcing conditions, a 6-chloro-,[298] 6-bromo-, or 6-fluoro group in purine nucleosides as well as a 6-fluoro group[540] in pyrimidine nucleosides, whereas NaOH in methanol-H_2O will introduce a methoxy group.[539] Likewise, a 5-trifluoromethyl group in pyrimidine nucleosides is transformed into

Direct Glycosylation of 1,3,5-Triazinones. A New Approach to the Synthesis of the Nucleoside Antibiotic 5-Azacytidine (4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2-one) and Related Derivatives[1]

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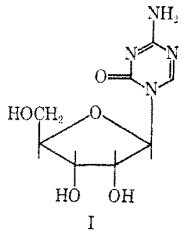
The first instance of direct glycosylation of the 1,3,5-triazine ring has been described. The synthesis of the nucleoside antibiotic 5-azacytidine (4-amino-1-β-D-ribofuranosyl-1,3,5-triazin-2-one, I) has been achieved in 34% yield by treatment of the trimethylsilyl derivative of 4-amino-1,3,5-triazin-2-one (5-azacytosine) with 2,3,5-tri-*O*-acetyl-D-ribofuranosyl bromide in acetonitrile, followed by deblocking with methanolic ammonia. Similar treatment of the trimetbylsilyl derivative of 5-azacytosine with 3,5-di-*O*-acetyl-2-deoxyribofuranosyl chloride resulted in the α and β anomers of 2′-deoxy-5-azacytidine, which were clearly distinguished by pmr. In a similar manner, 1-(β-D-ribofuranosyl)cyanuric acid (V) and 1-β-D-ribofuranosyl-3-methylcyanuric acid (VI) were prepared from cyanuric acid and 1-methylcyanuric acid, respectively. Attempts to prepare 4-amino-1-β-D-arabinofuranosyl-1,3,5-triazin-2-one (5-azaarabinofuranosylcytosine) were unsuccessful because 4-amino-1-(2,3,5-tri-*O*-benzyl-β-D-arabinofuranosyl)-1,3,5-triazin-2-one (VII) could not be deblocked without concomitant destruction of the triazine ring. The nucleoside derivatives of the 1,3,5-triazine ring present some interesting nucleosides for future biochemical and biophysical studies.

5-Azacytidine (4-amino-1-β-D-ribofuranosyl-1,3,5-triazin-2-one, I) has been isolated from *Streptoverticillium ladakanus*.[2,3] This antibiotic inhibits gram-negative bacteria and is active against T-4 lymphoma and L-1210 leukemia in mice.[2] The identity of I was



I

established[3] by comparison with authentic 5-azacytidine prepared by a lengthy procedure involving ring closure of 1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-4-methylisobiuret.[4] The remarkable biological activity of I against experimental leukemia[5–9] has resulted in the selection of 5-azacytidine for clinical trial against leukemia in human subjects.[8,9] 5-Azacytidine inhibits protein synthesis[10] and is incorporated into both RNA and DNA.[11]

Recent studies in this laboratory utilizing various trimethylsilyl pyrimidines in a direct glycosylation procedure has succeeded where other methods have failed.[12–14] The application of this study to the s-triazine ring has now resulted in the direct attachment of the D-ribofuranose moiety to the 1,3,5-triazine ring system. 5-Azacytosine[15] was treated with hexamethyldisilazane in a manner similar to that previously employed for 4-amino-6-pyrimidone.[13] The resulting trimethylsilyl derivative (II) was dissolved in acetonitrile and treated with 2,3,5-tri-*O*-acetyl-D-ribofuranosyl bromide.[16] The crude blocked nucleoside was isolated and treated with methanolic ammonia to give crude I, yield 34%. Recrystallization gave 5-azacytidine, yield 11%, mp 231–233° dec. Rigorous comparison of this product with 5-azacytidine isolated from cultures of *S. ladakanus* proved the samples to be identical.

Utilization of this procedure for the synthesis of 5-aza-2′-deoxycytidine (III) was also successful. Syrupy 1,3,5-tri-*O*-acetyl-2-deoxy-D-ribofuranose[17] was converted into 3,5-di-*O*-acetyl-2-deoxy-D-ribofuranosyl chloride and allowed to react with an excess of the trimethylsilyl derivative of 5-azacytosine in acetonitrile. After 7 days at room temperature the reaction mixture was treated as for the preparation of I and the product was purified *via* column chromatography on silica gel to give a mixture of anomers of 1-(3,5-di-*O*-acetyl-2-deoxy-D-ribofuranosyl)-5-azacytosine. This mixture was treated with ethanolic ammonia to remove the acetyl groups. The resulting α and β anomers were separated by a combination of fractional crystallization and preparative layer chromatography on silica gel to give pure 1-(2-deoxy-α-D-ribofuranosyl)-5-azacytosine (IV) and 2′-deoxy-5-azacytidine [4-amino-1-(2-deoxy-β-D-ribofuranosyl)-1,3,5-triazin-2-one, III]. Assignment of the β configuration to III was made by comparison of the pmr signals observed for the anomeric protons[17] of III and the corresponding α anomer, IV. It should be noted that a lengthy synthesis of 5-aza-2′-deoxycytidine has been reported[18] in a preliminary communication *via* 1-(3,5-di-*O*-*p*-toluyl-2-deoxy-D-ribofuranosyl)-4-methylisobiuret. However, no yield was

(1) Supported by Research Grants CA 08109-02 and CA 08109-03 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

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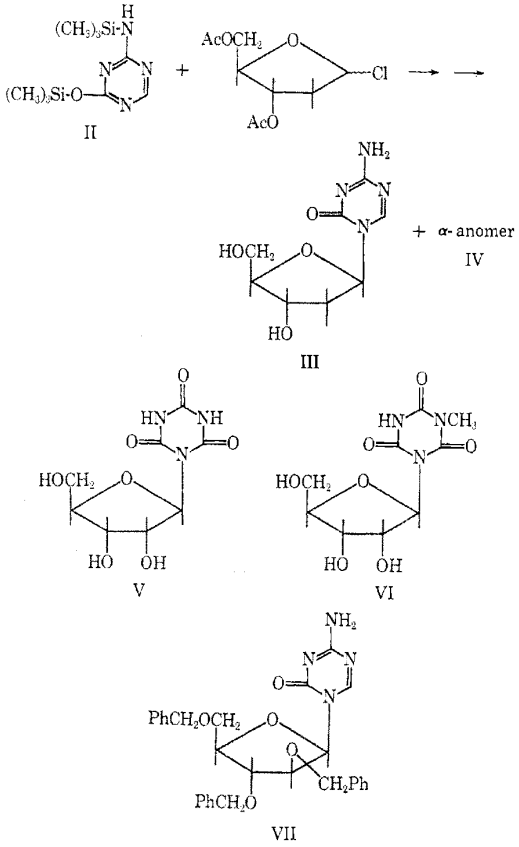
given and the authors did not distinguish between the possible two anomers, III and IV.



In an attempt to prepare 1-β-D-arabinofuranosyl-5-azacytosine, 2,3,5-tri-*O*-benzyl-D-arabinofuranosyl chloride[19] and the trimethylsilyl derivative of 5-azacytosine were dissolved in dichloromethane and kept at room temperature for 11 days to give a 47% yield of 1-(2,3,5-tri-*O*-benzyl-β-D-arabinofuranosyl)-5-azacytosine (VII). Catalytic debenzylation of VII with palladium and hydrogen resulted in destruction of the triazine ring. A fusion reaction of 2,3,5-tri-*O*-acetyl-D-arabinofuranosyl chloride with the trimethylsilyl derivative of 5-azacytosine gave only the α anomer, 1-(2,3,5-tri-*O*-acetyl-α-D-arabinofuranosyl)-5-azacytosine.

Treatment of 2,4,6-tris(trimethylsilyloxy)-1,3,5-triazine, prepared from cyanuric acid, with 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl bromide gave a 43% yield, after deblocking of 1-(β-D-ribofuranosyl)cyanuric acid (V). This molecule is of particular interest since it is symmetrical. There is essentially no *anti* form to this nucleoside. The structural resemblance to uridine is, however, indeed striking. Such a nucleoside should be of considerable theoretical interest to both biochemists and biophysical chemists, since it has been postulated that certain enzymes *prefer* either the *syn* or *anti* conformation of pyrimidine nucleosides.[20] Further studies on this nucleoside are in progress in our laboratories. For comparative purposes, the compound 1-(β-D-ribofuranosyl)-3-methylcyanuric acid (VI) was similarly prepared from 1-methylcyanuric acid[21] in 39% yield.

It would appear that the silylation procedure of nucleoside synthesis is generally applicable even to ring systems such as 1,3,5-triazine, which have not previously been alkylated by other methods of nucleoside synthesis.

Experimental Section

Melting points were determined with a Thomas–Hoover capillary melting point apparatus and are uncorrected. Specific rotations were measured in a 1-dm tube with a Perkin-Elmer Model 141 automatic digital readout polarimeter. Proton magnetic resonance (pmr) spectra were measured with appropriate internal standards of tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate with a Varian Model A-60 nmr spectrometer. Ultraviolet spectra were determined with a Beckman Model DK-2 spectrometer. Infrared spectra were determined with a Beckman Model IR-5 spectrophotometer. Detection of components on SilicAR 7 GF (Mallinckrodt) and alumina HF 254 (Brinkmann) was by ultraviolet light. Alumina used in columns was obtained from Merck & Co. (suitable for chromatographic absorption). Silica gel was purchased from J. T. Baker Chemical Co. (suitable for chromatographic use). Solvent proportions were by volume. Evaporations were performed under diminished pressure at 35° with a Buchi Rotovapor.

Trimethylsilyl derivatives of various *s*-triazines were prepared using the general procedure of Wittenburg.[17] The *s*-triazines were heated under reflux in an excess of hexamethyldisilazane with a catalytic quantity of ammonium sulfate under anhydrous conditions until complete solution was achieved. The excess hexamethyldisilazane was removed by distillation under diminished pressure and the residue (oil or crystalline solid) was used directly without further purification.

5-Azacytidine (I).—To the trimethylsilyl derivative of 5-azacytosine (prepared from 10 g of 5-azacytosine[15]) was added 2,3,5-tri-*O*-acetyl-D-ribofuranosyl bromide (prepared from 20 g of tetra-*O*-acetyl-D-ribofuranose[18]) in dry acetonitrile (180 ml). After initial stirring, the solution was left at room temperature for 3 days. The solution was evaporated to a syrup. Sodium bicarbonate, water, and ethanol were added. The mixture was evaporated to dryness. Coevaporation with absolute ethanol removed the last traces of water. The residue was extracted with chloroform (Celite used) and the extract was evaporated to dryness. The residue was extracted once more with chloroform and the solvent was removed to give 24.5 g of a foam. To this material was added methanolic ammonia solution (150 ml of methanol saturated at 0° with ammonia). The vessel was sealed and the solution was left at room temperature for 2 hr and then at 5° overnight. The mixture was evaporated to near dryness. To the residue was added methanol and the solid was collected, yield 5.2 g (34%), mp 192–209°. This material was dissolved in warm water and the solution was decolorized with charcoal. Evaporation gave crystals of 5-azacytidine, yield 1.75 g (11%), mp 231–233°. Recrystallization from aqueous ethanol (charcoal) gave pure 5-azacytidine (I), mp 235–237° dec, $[\alpha]^{26}D$ +22.4° (*c* 1.00, water).

Anal. Calcd for $C_8H_{12}N_4O_5$: C, 39.34; H, 4.95; N, 22.94. Found: C, 39.14; H, 4.99; N, 22.91.

A mixture melting point with authentic material[3] showed no depression. The $[\alpha]^{26}D$ (*c* 1, water) value recorded by us for authentic material was +26.6°.[4] The ir, uv, and pmr spectra were identical with those of authentic material.[2,3] The product was shown to be homogeneous by tlc on SilicAR 7GF with ethyl acetate–methanol (4:1) as solvent and it had the same R_f as a marker of authentic material.[3]

1-(3,5-Di-*O*-acetyl-2-deoxy-α,β-D-ribofuranosyl)-5-azacytosine.—Syrupy 2-deoxy-1,3,5-tri-*O*-acetyl-D-ribofuranose[17] (21 g) was dissolved in dry ether (400 ml) containing acetyl chloride (30 ml) and the solution was saturated with hydrogen chloride at 0° for 1 hr. The sealed solution was left at 0° for 1 day. The solution was evaporated to a syrup which was coevaporated with toluene

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to give a dark-colored, crude glycosyl halide. This product was dissolved in acetonitrile (200 ml) and transferred to the trimethylsilyl derivative of 5-azacytosine (from 15 g of 5-azacytosine). The sealed mixture was stirred at room temperature for 4 days. The mixture was evaporated to a syrup and sodium bicarbonate and ethanol were added. The mixture was evaporated to dryness and the residue was extracted with chloroform. The extract was evaporated to *ca.* 200 ml and applied to a column (40 × 5.0 cm) of silica gel prepacked in chloroform. The column was eluted with chloroform and 200-ml fractions were collected. At fraction 10 the solvent was changed to chloroform–ethyl acetate (9:1), at fraction 20 to ethyl acetate, and at fraction 31 to ethyl acetate–methanol (19:1). Fractions 33–39 were pooled and evaporated to a semicrystalline material. This mixture was extracted with chloroform and the extract was applied to a column (37 × 3.2 cm) of silica gel prepacked in chloroform. Elution was started with chloroform and 200-ml fractions were collected. The solvent was changed to chloroform–ethyl acetate (4:1) at fraction 8 and to ethyl acetate at fraction 12. Fractions 14–25 were evaporated to a small volume, whereupon crystallization occurred. Ether was added to give 2.42 g of white crystals, mp 162–165°.

Anal. Calcd for $C_{12}H_{16}N_4O_6$: C, 46.15; H, 5.16; N, 17.94. Found: C, 45.90; H, 5.07; N, 17.85.

This product, when examined by tlc on silicAR 7GF with acetone as developer, showed two very closely moving components (typical of anomers): a major, slower moving component and a minor, faster moving component. Attempts at fractional crystallization failed. The mixture exhibited the following spectral data: pmr (DMSO-d_6) δ 1.99 (s) and 2.11 (s, 6, OAc), 2.20–3.10 (m, 2, 2′ H), 4.07–4.36 ("s" at 4.15, "s" at 4.22, and "s" at 4.31, 2, 5′ CH_2OAc), 4.70–5.00 (m, 1, 4′ H), 5.08–5.42 (m, 1, 3′ H), 6.12 (rough q, 1, 1′ H), 7.55 (s) and 7.63 (s, 2, 4 NH_2), and 8.40 (s, 1, 6 H); λ_{max}^{KBr} 1740 cm^{-1} (OAc).

2′-Deoxy-5-azacytidine (III) and the α Anomer (IV).—To a solution of ammonia-saturated (at 0°) ethanol (200 ml) was added 2.76 g of the mixture of 1-(3,5-di-*O*-acetyl-2-deoxy-α,β-D-ribofuranosyl)-5-azacytosine, and the sealed mixture was stirred at 5° for 2 hr to achieve solution. The solution was maintained at −15° for 5 days and then evaporated at 25° to a syrup which was heated at 60° under oil pump vacuum to remove acetamide. A 100-mg portion of the residue (A) was applied to the short edge of a silicAR 7GF plate (2 × 200 × 400 mm) and the plate was developed several times with ethyl acetate–methanol (4:1). Two closely moving zones were observed, one major (α anomer), slower moving zone and a minor, very slightly faster moving (β anomer) zone. Extraction of the smaller zone with methanol and solvent removal gave a minute quantity of crystalline residue (crude β anomer). The remaining crude syrupy mixture of anomers (A) was dissolved in warm methanol and seeded with this material to give white needles of III, yield 0.10 g, mp 189–191°. The mother liquor and washings (ethanol and ether) deposited white prisms (1.05 g) of α anomer contaminated with a faint trace of β anomer. Recrystallization of the β anomer from methanol–2-propanol gave pure product, 2′-deoxy-5-azacytidine (III): mp 191–193°; $[\alpha]^{25}D$ +63.8° (*c* 1.00, water); λ_{max}^{KBr} 1600–1710 cm^{-1} [5-azacytosine absorptions, in the region 1200–4000 cm^{-1} the spectrum was very similar to that of 5-azacytidine (I)]; $\lambda_{max}^{pH\ 1}$ 253 mμ, $\lambda_{max}^{pH\ 4}$ 239 (ε 8200), and $\lambda_{max}^{pH\ 11}$ 253 sh (2300); pmr (D_2O) δ 2.36–2.67 (m, 2, 2′ H), 3.80–3.94 ("d" centered at 3.80 "*J*" = 2.0 cps, "s" at 3.88, 2,5′ CH_2OH), 6.29 (t, 1, *W* = 13.0 cps, $J_{1',2'}$ = 6.5 cps, 1′ H), and 8.58 (s, 1, 6 H).

Anal. Calcd for $C_8H_{12}N_4O_4$: C, 42.10; H, 5.30; N, 24.55. Found: C, 41.81; H, 5.15; N, 24.52.

Recrystallization of the crude α anomer (IV) from methanol–2-propanol gave 0.81 g of pure product: mp 177–179°; $[\alpha]^{25}D$ −40.8° (*c* 1.0, water); ν_{max}^{KBr} 1600–1660 cm^{-1} (5-azacytosine absorptions); $\lambda_{max}^{pH\ 1}$ 253 mμ, $\lambda_{max}^{pH\ 4}$ 239 (ε 8200), and $\lambda_{max}^{pH\ 11}$ 253 sh (2700); pmr (D_2O) δ 2.00–3.12 (m, 2, 2′H), 3.58–3.81 ("s" centered at 3.69 and "s" centered at 3.75, 2, 5 CH_2OH), 4.30–4.65 (m, 2, 3′, and 4′ H), 4.86 (solvent), 6.16 (q, 1, *W* = 9.0 cps, "*J*" = 2.0, 7.0 cps, 1′ H), and 8.48 (s, 1, 6 H).

Anal. Calcd for $C_8H_{12}N_4O_4$: C, 42.10; H, 5.30; N, 24.55. Found: C, 41.86; H, 5.15; N, 24.55.

The various mother liquors were evaporated and applied (*ca.* 100 mg/plate) to the short edge of silicAR 7GF plates (2 × 200 × 400 mm). The plates were developed several times with ethyl acetate–methanol (4:1) until the zones corresponding to the anomers were separated. The zones were excised and extracted with methanol. Solvent removal and crystallization from methanol–ethanol gave an additional 0.12 g of β anomer (III), mp 191–193°, and 0.40 g of α anomer (IV), mp 177–179°.

In a subsequent experiment the procedure was modified as follows. To a solution of ammonia-saturated (at 0°) ethanol (130 ml) was added 1.70 g of 1-(3,5-di-*O*-acetyl-2-deoxy-α,β-D-ribofuranosyl)-5-azacytosine and the sealed mixture was stirred at 5° for 7 hr. The solution was stored at −15° for a further 6 days. The solution was evaporated below 25° to a syrup. This material was dissolved in methanol and the solution was decolorized with charcoal. The solution was evaporated to smaller volume and then coevaporated with ethanol to give 0.77 g of white crystals, mp 175–177° (A), containing largely α anomer. The mother liquor, richer in the β anomer, was evaporated and applied (*ca.* 100 mg/plate) to the short edges of 5 silicAR 7GF plates (2 × 200 × 400 mm). The plates were developed several times with ethyl acetate–methanol (4:1) and the faster moving of the two barely separated zones was excised and extracted with methanol. Solvent removal and crystallization of the residue from methanol–ethanol gave 56.7 mg (4%) of 2′-deoxy-5-azacytidine (III), mp 193–194°. The crystalline material (A) was also subjected to a similar separation to give 33.6 mg (3%) of β anomer (III), mp 191–192°. The slower moving zone was treated similarly to give a total yield of 0.65 g (52%) of α anomer (IV), mp 181–182°.

1-(2,3,5-Tri-*O*-benzyl-β-D-arabinofuranosyl)-5-azacytosine (VII).—To the trimethylsilyl derivative of 5-azacytosine (prepared from 7.5 g of 5-azacytosine) was added 2,3,5-tri-*O*-benzyl-D-arabinofuranosyl chloride [prepared from 15.0 g of 2,3,5-tri-*O*-benzyl-1-*p*-nitrobenzoyl-D-arabinofuranose[19] in dry dichloromethane (125 ml)] and the resulting solution was protected from moisture and left at room temperature for 11 days. The solution was evaporated to dryness and the residue was treated with sodium bicarbonate, water, and ethanol. The mixture was evaporated to dryness and the residue was coevaporated with ethanol. The residue was extracted with chloroform and the chloroform extract was evaporated to dryness. The residue was extracted once more with chloroform and the extract was applied to a column (40 × 3.3 cm) of silica gel prepacked in chloroform. Fractions (200 ml each) were collected and the fractionation was monitored by tlc on SilicAR 7GF with chloroform–ethyl acetate (4:1) as developer. Elution was started with chloroform. At fraction 39 the eluting solvent was changed to chloroform–ethyl acetate (9:1). Fractions 8–43, which contained a single nucleosidic component, were evaporated to dryness. The residue was crystallized from ethanol–ether to yield 6.39 g (47%) of white crystals, mp 141–143°. Recrystallization from ethanol gave pure VII: mp 142–143°; pmr ($CDCl_3$) δ 3.60 (d, 2, "*J*" = 5.5 cps, 5′ CH_2OH), 3.93–4.32 (m, 3, 2′, 3′, and 4′ H), 4.38 (s, 2, $PhCH_2$), 4.48 (s, 2, $PhCH_2$), 4.51 (s, 2, $PhCH_2$), 6.31 (d, 1, *J* = 4.0 cps, 1′ H), 7.06–7.47 (m, 15, PhH), 7.69 (broad s, 2, 4 NH_2), and 8.26 (s, 1, 6 H).

Anal. Calcd for $C_{29}H_{30}N_4O_5$: C, 67.69; H, 5.88; N, 10.89. Found: C, 67.60; H, 5.82; N, 11.10.

1-(β-D-Ribofuranosyl)cyanuric Acid (V).—To the trimethylsilyl derivative of cyanuric acid (from 15 g of cyanuric acid) was added 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl bromide (from 40 g of 1-*O*-acetyl-2-3,5-tri-*O*-benzoyl-β-D-ribofuranose) in dry acetonitrile (250 ml). After sealing and initial stirring, the solution was left at room temperature for 8 days. The solvent was evaporated to a syrup and absolute ethanol was added to the residue. The mixture was evaporated to dryness and extracted (Celite filtration) with chloroform. The solvent was removed and the residue, redissolved in chloroform, was applied to a column (48.5 × 5.0 cm) of silica gel prepacked in chloroform. Fractions (200 ml each) were collected and the fractionation was monitored by tlc on silicAR 7 GF with ethyl acetate–chloroform (3:7) as developer. At fraction 16 the solvent was changed to chloroform–ethyl acetate (9:1) and at fraction 25 to chloroform–ethyl acetate (4:1). Fractions 14–34 were pooled and evaporated, yielding 22.60 g of a dry syrup. A portion (16.0 g) was dissolved in methanol (250 ml) saturated at (0°) with ammonia and left in a pressure vessel for 4 days. The solution was filtered and the filtrate was evaporated to smaller volume, whereupon crystallization of the product occurred. The mixture was coevaporated with absolute ethanol to yield 6.25 g (43%) of V, mp 222–223° dec. Recrystallization from water–ethanol gave pure material: mp 229–230° dec; $[\alpha]^{25}D$ 24.3° (*c* 1, water); ν_{max}^{KBr} 1680 and 1770 cm^{-1} (C=O of heterocycle); pmr (D_2O) δ 3.57–4.23 (m, 3, 4′ H and 5′ CH_2OH, 5 CH_2OH as "s" at 3.85), 4.38 (t, 1, *W*

= 12.0 cps, $J_{3',2'}$ = 6.0 cps, 3′ H), 4.55–4.85 (m, solvent and 2′ H), and 6.22 (d, 1, $J_{1',2'}$ = 3.5 cps, 1′ H).

Anal. Calcd for $C_9H_{11}N_3O_7$: C, 36.79; H, 4.25; N, 16.09. Found: C, 36.48; H, 3.92; N, 15.85.

1-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)cyanuric Acid.—The remaining portion of the crude benzoate (above, 6.60 g) was crystallized from chloroform–ethyl acetate–heptane to yield 4.44 g of white crystals, mp 211–21°. The product was dissolved in a mixture of methanol and ethyl acetate and the solution was decolorized. The solution was evaporated to small volume and heptane was added. Pure product was deposited as white needles: mp 211–213°; ν_{max}^{KBr} 1655 and 1770 cm^{-1} (C=O of heterocycle, and benzoate); pmr (DMSO-d_6) δ 4.50–5.00 [m, 3, 4′ H overlapping 5′ CH_2OH (s) centered at 4.75], 6.13–6.47 (m, 2, 2′ and 3′ H), 6.57 (s, 1, $J_{1',2'}$ < 1 cps, 1′ H), 7.22–8.26 (m, 15, benzoate), and 11.94 (s, 2, NH).

Anal. Calcd for $C_{29}H_{23}N_3O_{10}$: C, 60.73; H, 4.04; N, 7.33. Found: C, 60.91; H, 4.18; N, 7.18.

1-(β-D-Ribofuranosyl)-3-methylcyanuric Acid (VI).—To the trimethylsilyl derivative of 1-methylcyanuric acid (prepared from 5 g of 1-methylcyanuric acid[21]) was added 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl bromide (prepared from 10 g of 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribofuranose) in dry acetonitrile (150 ml). The sealed mixture was stirred initially and then left at room temperature for 2 weeks. The solution was evaporated to a syrup and the syrup was treated with absolute ethanol (50 ml). The mixture was evaporated to dryness and the residue was extracted with chloroform. Evaporation provided a syrup which was redissolved in chloroform, and the solution was applied to a column (40 × 3.3 cm) of silica gel prepacked in chloroform. The fractionation was monitored by tlc on SilicAR 7 GF with chloroform–ethyl acetate (7:3) as developer. Fractions of 200 ml each were collected up to fraction 4. Fractions 5 and 6 were of 100-ml volume. Fractions 7–12 were again of 200-ml volume. Fractions 6–12 were pooled and evaporated to give 8.37 g of a white, homogeneous foam: pmr ($CDCl_3$) δ 3.23 (s, 3, N_3CH_3), 4.50–4.88 (m, 3, 4′ H and 5′ CH_2OH), 6.05–6.30 (m, 2, 2′ and 3′ H), 6.48 (s, 1, $J_{1',2'}$ < 1 cps, 1′ H), 7.10–7.60 (m, 9, benzoate), and 7.77 (m, 6, benzoate). This material was dissolved in ammonia-saturated (at 0°) methanol (100 ml) and left at room temperature for 4 days in a sealed vessel. The solution was filtered through Celite and the filtrate was evaporated to a syrup. This syrup was dissolved in a mixture of chloroform and water. The aqueous solution was further extracted three times with chloroform and then evaporated to dryness. The residue was coevaporated with absolute ethanol and the residue was stirred in ether (200 ml) for several days. The resulting white powder (3.40 g) was collected and crystallized from methanol–2-propanol, yield 2.05 g (39%), mp 144–146°. This material was dissolved in methanol and the solution was decolorized with activated carbon. After solvent removal the syrup was crystallized from methanol–2-propanol: mp 144–146°; $[\alpha]^{31}D$ −21.5° (*c* 1, water); ν_{max}^{KBr} 1680 and 1720 cm^{-1} (C=O of heterocycle); pmr (D_2O) δ 3.26 (s, 3, N_3 CH_3), 3.80–4.15 [m, 3, 5′ CH_2OH (s) at 3.83 overlapped by 4′ H], 4.40 (t, 1, $J_{3',2'}$ = 12.3 cps, 3′ H), 4.53–5.00 (m, 2′ H and solvent), and 6.11 (d, 1, $J_{1',2'}$ = 3.5 cps, 1′ H).

Anal. Calcd for $C_9H_{13}N_3O_7$: C, 39.27; H, 4.76; N, 15.27. Found: C, 39.19; H, 4.82; N, 15.39.

1-(2,3,5-Tri-*O*-acetyl-α-D-arabinofuranosyl)-5-azacytosine.—To a solution of sodium (0.5 g) in anhydrous methanol (500 ml) was added methyl 2,3,5-tri-*O*-benzoyl-D-arabinofuranoside[22] (84 g) and the solution was heated under reflux for 45 min. To the stirred cooled solution was added portionwise Dowex 50 (H^+, X4, 200–400 mesh) until the solution was neutral. The resin was filtered off and washed with methanol. The filtrate and washings were evaporated to a syrup. The syrup was dissolved in chloroform and extracted with chloroform several times. The aqueous layer was evaporated to a syrup and the syrup was dried by coevaporation with ethanol and then with dry pyridine. The dry syrup was treated overnight with acetic anhydride (200 ml)–pyridine (200 ml). The solution was poured onto ice and the mixture was extracted with chloroform. The chloroform extract was washed consecutively with water, ice-cold 2 *N* hydrochloric acid, water-saturated sodium bicarbonate solution, and water. The dried ($MgSO_4$) solution was evaporated to give syrupy methyl 2,3,5-tri-*O*-acetyl-D-arabinofuranoside.

(22) H. G. Fletcher, Jr., in "Methods in Carbohydrate Chemistry," Vol. 2, M. L. Wolfrom and R. L. Whistler, Ed., Academic Press Inc., New York, N. Y., 1963, p 228.

This syrup was dissolved in a mixture of acetic anhydride (150 ml) and acetic acid (550 ml). Concentrated sulfuric acid (35 ml) was added dropwise to the ice-cold solution and the solution was left at room temperature overnight. The solution was poured onto ice and the mixture was extracted with chloroform. The chloroform extract was stirred with excess saturated sodium bicarbonate overnight at 5°. The extract was washed with water and dried ($MgSO_4$). Solvent removal afforded 64 g of 1,2,3,5-tetra-*O*-acetyl-D-arabinofuranose as an oil.

Dry hydrogen chloride gas was bubbled through an ice-cold solution of 53 g of the above syrup in ether (1 l.) containing acetyl chloride (100 ml) until the solution was saturated (*ca.* 1 hr). The solution was sealed and maintained at 0° for 6 days. The solution was evaporated and the residue was coevaporated with toluene.

This syrup was dissolved in toluene (150–200 ml) and transferred to the trimethylsilyl derivative of 5-azacytosine (prepared from 25 g of 5-azacytosine). An aspirator vacuum was applied to the magnetically stirred solution and the temperature was quickly raised to 195° using an oil bath. The temperature was maintained at 195° for 25 min. Ethanol and sodium bicarbonate were added to the residue. The mixture was evaporated to dryness and the residue was extracted with chloroform (Celite). The chloroform extract was evaporated to smaller volume and applied to a column (69 × 4.0 m) of silica gel prepacked in chloroform. Fractions (200 ml each) were collected and the fractionation was monitored by tlc on silicAR 7 GF with ethyl acetate–methanol (9:1) as developer. At fraction 31 the eluting solvent was changed to chloroform–ethyl acetate (9:1), at fraction 41 to chloroform–ethyl acetate (7:3), at fraction 46 to ethyl acetate, and at fraction 64 to ethyl acetate–methanol (19:1). Fractions 44–74 were collected and evaporated to a syrup which was crystallized from ethyl acetate–ether, yield 2.77 g, mp 165–168°. The mother liquor was evaporated and the residue was dissolved in chloroform. Silica gel was added and the mixture was evaporated to give a free-running powder. This material was added to a dry column of silica gel (43.5 × 4.0 cm) so that the total column size was 66.0 × 4.0 cm. Elution was started with chloroform and 200-ml fractions were collected. At fraction 5 the solvent was changed to chloroform–ethyl acetate (9:1), at fraction 9 to chloroform–ethyl acetate (7:3), at fraction 13 to ethyl acetate (1:1), at fraction 21 to ethyl acetate–chloroform (7:3), at fraction 35 to ethyl acetate, and at fraction 60 to ethyl acetate–methanol (98:2). Fractions 36–66 were evaporated to a syrup which was crystallized as above to give 1.80 g, mp 163–165°.

The mother liquor was evaporated to a syrup and dissolved in chloroform. Silica gel was added and the mixture was evaporated to give a free-running powder. This material was added to a dry column (25 × 3.3 cm) of silica gel so that the total column size was 44.0 × 3.3 cm. The elution was started with chloroform and 200-ml fractions were collected. At fraction 6 the solvent was changed to chloroform–ethyl acetate (9:1), at fraction 11 to chloroform–ethyl acetate (7:3), at fraction 15 to chloroform–ethyl acetate (3:7), and at fraction 26 to ethyl acetate. Fractions 22–30 were evaporated and crystallized as above to give 0.65 g, mp 170–171°. The various crystalline materials were combined and dissolved in chloroform. The solution was decolorized and evaporated to a syrup, which was crystallized from ethyl acetate–ether to give 4.85 g (8%), mp 166–168°. A further crystallization gave pure product: mp 167–168°; pmr ($CDCl_3$) δ 2.10 (s, 3, Ac), 2.18 (s, 6, Ac), 4.33 ("d", 2, "J" = 5.5 cps, 5′ CH_2OH), 4.61–4.91 (m, 1, 4 H), 5.20–5.38 (m, 1, 3′ H), 5.62–5.80 (m, 1, 2′ H), 5.95 (d, 1, $J_{1',2}$ = 2.5 cps, 1′ H), 6.73 (s, 2, 4 NH_2), and 8.21 (s, 1, 6 H).

Anal. Calcd for $C_{14}H_{18}N_4O_8$: C, 45.40; H, 4.90; N, 15.13. Found: C, 45.14; H, 4.74; N, 15.04.

1-(2,3-Isopropylidene-β-D-ribofuranosyl)cyanuric Acid.—1-(β-D-Ribofuranosyl)cyanuric acid (6.25 g) was dissolved in a mixture of dimethylformamide (20 ml) and dimethoxypropane (18 ml) containing 15 drops of a solution of 4 *M* hydrogen chloride in dioxane. The sealed solution was left at room temperature for 3 days. Sodium bicarbonate (5 g) was added and the mixture was stirred for 2 hours. The solution was filtered through Celite and the filter was washed with 1-butanol. The filtrate was evaporated to dryness under oil pump vacuum. The residue was dissolved in ethanol (100 ml) containing glacial acetic acid (5 ml). The solution was heated on a steam bath for 5 min and then left overnight at room temperature. The solvent was removed and the residue was coevaporated with toluene. The residue was dissolved in chloroform and silica gel (40 g) was added. The

mixture was then evaporated to give a free-running powder. This material was added to a dry column of silica gel (41 × 3.3 cm) so that the final size was 61 × 3.3 cm. The column was eluted with chloroform and 100-ml fractions were collected. At fraction 11 the solvent was changed to chloroform–methanol (9:1). Fractions 15–20, which were homogeneous as judged by tlc on SilicAR 7GF with ethyl acetate developer (detection with sulfuric acid), were evaporated to dryness. The syrupy residue was crystallized from ethanol–heptane to yield 6.36 g (88%) of white product, mp 179–181°. This material was recrystallized from ethanol–heptane to give pure product: mp 180–181°; λ_{max}^{KBr} 1680–1800 cm^{-1} (C=O of cyanuric acid); pmr (DMSO-d_6) δ 1.32 (s, 3, CCH_3), 1.52 (s, 3, CCH_3), 3.54 ("d," 2, "J" = 6.5 cps, 5′ CH_2OH), 3.80–4.18 (m, 1, 4′ H), 4.54–4.93 (m, 2, 3′ H and 5′ CH_2OH), 5.17 (d, 1, $J_{1',2'}$ = 6.0 cps, 2′ H), 6.18 (s, 1, $J_{1',2'}$ <1 cps, 1′ H), and 11.84 (broad, s, 2, NH).

Anal. Calcd for $C_{11}H_{15}N_3O_7$: C, 43.85; H, 5.02; N, 13.95. Found: C, 44.21; H, 5.45; N, 14.20.

1-(2,3-*O*-Isopropylidene-5-methylsulfonyl-β-D-ribofuranosyl)-cyanuric Acid.—To a stirred solution of 1-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)cyanuric acid (6.32 g) in dry pyridine (50 ml) at 0° was added dropwise methylsulfonyl chloride (1.80 ml) and the resulting solution was sealed and stored at 0° for 36 hr. Absolute ethanol (a few drops) was added and the solution was left overnight at 0°. The solution was evaporated to dryness and the residue was coevaporated with toluene. The dried (oil pump vacuum) residue was dissolved in methanol and silica gel was added. The mixture was evaporated to give a free-running powder which was added to a column (51 × 3.5 cm) of silica gel so that the final dimensions were 72 × 3.5 cm. Elution was started with chloroform. Fractions (200 ml each) were collected and the fractionation was monitored by tlc on SilicAR 7GF with ethyl acetate–chloroform (7:3) as developer (detection by sulfuric acid). At fraction 9 the solvent was changed to chloroform–ethyl acetate (4:1) and at fraction 14 to ethyl acetate. Fractions 16–19, which were of 100-ml volume and which contained a single component, were pooled and evaporated to a foam. Crystallization from ethyl acetate–heptane yielded 7.06 g (89%) of white crystals, mp 194–196°. These crystals were dissolved in methanol and the solution was decolorized. After solvent removal, the product was crystallized from ethanol–heptane to give pure material: mp 195–197°; ν_{max}^{KBr} 1710–1760 cm^{-1}; pmr (DMSO-d_6) 1.33 (s, 3, CCH_3), 1.52 (s, 3, CCH_3), 3.20 (s, 1, 5′ CH_3SO_2), 4.10–4.60 [m, 3, 5′ CH_2O (s) at 4.36 overlapped by 4′ H], 4.74–4.98 (m, 1, 3′ H), 5.21 (d, 1, $J_{2',3'}$ = 7.0 cps, 2′ H), 6.14 (s, 1, $J_{1',2'}$ <1 cps, 1′ H), and 11.66 (s, 2, NH).

Anal. Calcd for $C_{12}H_{17}N_3O_9S$: C, 37.98; H, 4.52; N, 11.08. Found: C, 37.88; H, 4.42; N, 11.04.

Registry No.—I, 320-67-2; III, 2353-33-5; IV, 22432-95-7; V, 22432-96-8; VI, 22432-97-9; VII, 22432-98-0; 1-(3,5-di-*O*-acetyl-2-deoxy-α,β-D-ribofuranosyl)-5-azacytosine, 22432-93-5; 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)cyanuric acid, 22432-99-1; 1-(2,3,5-tri-*O*-acetyl-α-D-arabinofuranosyl)-5-azacytosine, 22433-00-7; 1-(2,3-isopropylidene-β-D-ribofuranosyl)cyanuric acid, 22433-01-8; 1-(2,3-*O*-isopropylidene-5-methylsulfonyl-β-D-ribofuranosyl)cyanuric acid, 22433-02-9.

Synthesis of 21-Hydroxymethylprogesterone

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The synthesis of 21-hydroxymethylprogesterone was accomplished by two pathways, from progesterone and from 3β-acetoxy-5-pregnen-20-one. The preferred method involved the formylation and subsequent borohydride reduction of the 3-monoketal of progesterone. This diol was subsequently tritylated, oxidized, and hydrolyzed to yield 21-hydroxymethylprogesterone.

The C-17 side chains of the progestational and adrenocortical steroid hormones may be compared with the lowest members of the deoxy sugar and sugar series, respectively. Elongation of these side chains by addition of hydroxymethyl groups would yield homologs of the steroid-substituted carbohydrates. The higher hydroxymethyl homologs of progesterone and cortisol would have side chains which may be pictured as 1-substituted deoxy ketoses and 1-substituted ketoses, respectively. We wish to report the synthesis of 21-hydroxymethylprogesterone (**7a**), our initial objective in these studies.

A simple, direct method has been reported for the synthesis of 21-hydroxymethylcortisol by condensation of cortisol with formaldehyde.[2] When we attempted this method with pregnenolone and formaldehyde, we recovered only starting steroid. Our further studies with this method will be the subject of a separate paper. We did not obtain monohydroxymethylation in the desired position.

Very few primary aliphatic α-unsubstituted β-hydroxy ketones have been reported in the literature.[3] We presumed that 21-hydroxymethylprogesterone would be quite labile and that synthesis by indirect methods would be very sensitive to manipulations involved in protecting the other functional groups in the molecule. This did not prove to be the case.

The addition of the hydroxymethyl group on C-21 was accomplished by condensation of the 17β-acetyl group of pregnenolone acetate (**1**) with formate ester[4] followed by reduction with borohydride to the triol **2a** in the reaction medium (Scheme I). A number of routes were considered in order to utilize this condensation reaction for the synthesis of 21-hydroxymethylprogesterone. That the formate condensation occurs on C-21 has been demonstrated by Hirai, *et al.*,[5] as well as from evidence below.

One approach was to form the 20,21a-acetonide derivative[6] of the triol **2a** in order to oxidize selectively the Δ^5-3β-hydroxyl to the Δ^4-3-ketone by the Oppenauer method. Hydrolysis of the acetonide **4** yielded the diol ketone **5a**. The overall yield of this method to this point was so low that we turned to other approaches. An attempt to shortcut this pathway by tritylation of

(1) (a) Senior Postdoctoral Fellow, 1965–1967, supported by Grant 5 TU-MH6418, National Institutes of Health.

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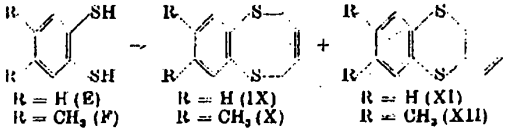
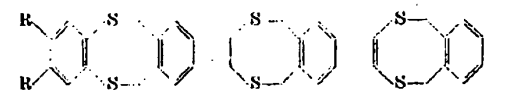
als (II) ($\lambda_{max}^{96\%iger\ Alkohol}$: 293; 251 nm; lg ε = 3,42; 3,72) und läßt somit den Schluß auf eine sterisch bedingte Mesomerieeinschränkung im R—S—CH=CH—S—R-Bereich von (III), entsprechend einer der Einebnung in diesem Bereich entgegenstrebenden spannungsfreien Wannen- oder Sesselkonformation, zu.

Ringschlußreaktionen von Dithiobrenzcatechin (E) und von 4,5-Dimercapto-o-xylol (F) [5] mit cis-1,4-Dichlorbut-2-en ergaben die Benzologen (IX) (Smp. 75 bis 76 °C) und (X) (Smp. 132 °C). In Konkurrenz wurden — über eine intramolekulare S_N'2-Reaktion [8] des nicht isolierten Monothioäthers — die jeweiligen Vinyl-1,4-dithiene (XI) ($Sdp._{0,15}$ 92 bis 93 °C) und (XII) (Smp. 42 bis 43 °C) gebildet. Lösungsmittel höherer Polarität begünstigen die Achtringbildung. Umsetzung mit trans-1,4-Dichlorbut-2-en führt ausschließlich zu (XI) bzw. (XII).



R = H (E) R = H (IX) R = H (XI)
R = CH_3 (F) R = CH_3 (X) R = CH_3 (XII)

(XIII) (Smp. 142 °C) bzw. (XIV) (Smp. 188 °C) konnte aus (E) bzw. (F) und o-Xylylenbromid, (XV) (Smp. 93 °C) aus Dithioglykol und o-Xylylenbromid einfach erhalten werden. Gemeinsam mit dem erst kürzlich erwähnten (G) [2] liegen nun auch alle Benzologen von (I) und (III) vor.



R = H (XIII) (XV) (G)
R = CH_3 (XIV)

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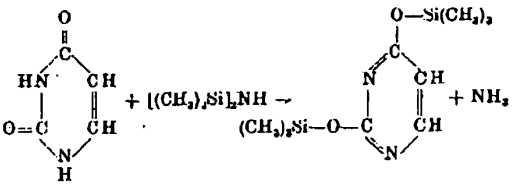
Eine neue Synthese von Nucleosiden[1])

Durch *L. Birkofer* u. Mitarb. [1] wurde gezeigt, daß N-Trialkylsilyl-Heterocyclen sehr leicht mit Acylhalogeniden zu N-Acylverbindungen reagieren. Diese große Reaktionsfähigkeit der Trialkylsilyl-Heterocyclen läßt sich auch erfolgreich zur Synthese natürlicher und in der Natur nicht vorkommender Pyrimidin-Nucleoside bzw. ihrer Derivate ausnutzen, wenn man silylierte Pyrimidine mit Acylhalogenzuckern reagieren läßt.

Die Trimethylsilyl-Pyrimidine werden dargestellt, indem man geeignete Pyrimidinderivate mit Hexamethyl-disilazan bei 145 bis 155 °C zur Umsetzung bringt. Dabei werden die reaktionsfähigen Wasserstoffatome von Gruppen wie —OH und —NHX $\left(X = H \text{ oder } -C\begin{matrix}O\\R\end{matrix}\right)$ am Heterocyclus durch den Trimethylsilylrest ersetzt, z. B.:



Die Trimethylsilyl-Pyrimidinderivate entstehen in sehr guter Ausbeute und sind destillierbar. Eine Reinigung durch Destillation ist in der Regel aber nicht erforderlich, da nach Abdestillieren des überschüssigen Hexamethyl-disilazans das zurückbleibende Trimethylsilyl-Pyrimidinderivat für weitere Umsetzungen genügend rein ist.

Die folgenden Trimethylsilylderivate wurden bisher dargestellt:

Trimethylsilyl-4-O-äthyl-uracil, Ausbeute 85%, $Sdp._{17}$ 115 bis 116 °C, UV-Absorption in absolutem Dibutyläther: λ_{max} 261 nm, λ_{min} kleiner als 233 nm[2]);

Trimethylsilyl-4-O-äthyl-thymin, Ausbeute 97%, Smp. 53 bis 54 °C, λ_{max} 265 nm, λ_{min} 236 nm;

Bis-(trimethylsilyl)-uracil, Ausbeute 94%, $Sdp._{18}$ 123 °C, λ_{max} 257 nm, λ_{min} kleiner als 233 nm[2]);

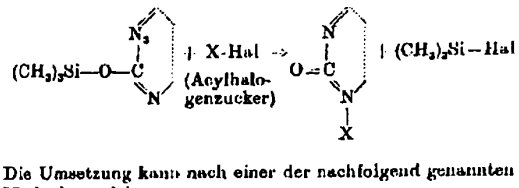
Bis-(trimethylsilyl)-thymin, Ausbeute 92%, $Sdp._{18}$ 127 °C, λ_{max} 265 nm, λ_{min} 236 nm;

Tris-(trimethylsilyl)-5-hydroxymethylcytosin, Ausbeute 95%, $Sdp._{18}$ 176 bis 180 °C, λ_{max} 274 nm, 236,5 nm, λ_{min} 251 nm;

Bis-(trimethylsilyl)-cytosin, Ausbeute 90%, λ_{max} 306 nm, 275 nm, 236,5 nm, λ_{min} 246 nm, 294 nm;

Bis-(trimethylsilyl)-N-acetylcytosin, Ausbeute 84%, $Sdp._{3}$ 155 °C.

Bei Einwirkung von Acylhalogenzuckern auf die genannten Trimethylsilyl-Pyrimidinderivate wird der Silylrest am Sauerstoff des C-Atoms 2 ersetzt durch einen acylierten Zuckerrest, der an das N-Atom in 1-Stellung gebunden wird.



Die Umsetzung kann nach einer der nachfolgend genannten Methoden erfolgen:

a) Schmolzen der Komponenten bei 90 bis 100 °C im Vakuum, wobei das entstehende Trimethylsilylhalogenid laufend abdestilliert.

b) Erhitzen der Komponenten in einem trockenen Lösungsmittel, wie Benzol, Toluol, Dimethylformamid, Nitromethan u. a.

c) Man läßt die beiden Reaktionspartner bei Zimmertemperatur in einem Lösungsmittel in Gegenwart von HgO, Hg-Acetat oder $AgClO_4$ aufeinander einwirken.

Die letztgenannte Methode liefert in der Regel die besten Ergebnisse.

In allen drei Fällen entsteht bei der Umsetzung entweder direkt das entsprechende acylierte Nucleosid [bei 4-Äthoxy-5-methyl-pyrimidinon-(2) und 4-Äthoxy-pyrimidinon-(2)] oder ein acyliertes Trimethylsilyl-Nucleosid, aus dem während der Aufarbeitung die überschüssigen Silylreste hydrolytisch abgespalten werden. Auf diese Weise wurden folgende Acyl-Nucleoside hergestellt (vgl. Tab. 1):

[1]) Vorläufige Mitteilung; ausführliche Mitteilung erfolgt an anderer Stelle.

[2]) Eine Messung bei kürzeren Wellenlängen ist auf Grund des verwendeten Lösungsmittels nicht möglich.

Tabelle 1
Acylierte Nucleoside über Silylverbindungen von Pyrimidinen

Silylester der Pyrimidinbase	Acylhalogenzucker	Ausbeute an acyl. Nucleosid in %	Smp. in °C	Lit.-Smp. in °C	Lit.
1	2	3	4	5	6
Uracil	Acetobromglucose	44	149–151	154–155	[2]
,,	Acetobromgalactose	86	amorph	—	—
,,	Acetobrom-L-arabinose	90	amorph	—	—
,,	Benzochlorribofuranose	52	144–146	142–143	[3]
,,	DOR-chlorid[a])	25	216–217	216–217	[4]
Thymin	Acetobromglucose	48	154–156[b])	156–158[b])	[5]
,,	Acetobromgalactose	50	136–138	—	—
,,	Acetobrom-L-arabinose	78	119–121[b])	137–141	[5]
,,	Benzochlorribofuranose	70	166–167	167–168	[5]
,,	DOR-chlorid[a])	31	196–197	197	[6]
4-Äthoxy-5-methyl-pyrimidinon-(2)	Acetobromgalactose	91	amorph	..	..
,,	Acetobrom-L-arabinose	53	177–179	181	[7]
N-Acetylcytosin	Acetobromglucose	38	145–146[b]) 225	225	[8]
,,	Acetobromgalactose	70	amorph	..	..
,,	Acetobrom-L-arabinose	53	amorph	..	..
5-Hydroxymethyl-cytosin	Acetobromglucose	79	amorph	..	..

Die Bindung der Glycosylreste an das N-Atom 1 des Pyrimidinringes ergibt sich durch Vergleich der physikalischen Konstanten mit den Eigenschaften von in der Literatur (vgl. Tab. 1, Spalte 6) beschriebenen Verbindungen, die auf anderen Wegen dargestellt wurden. Außerdem wurde die Struktur durch Messung der UV-Absorption wahrscheinlich gemacht.
Aus den genannten acylierten Nucleosiden lassen sich durch Abspaltung der Acylreste mit methanolischer Salzsäure oder Natrium- bzw. Barium-methylat-Lösung in bekannter Weise die entsprechenden freien Nucleoside gewinnen.
Die Untersuchungen über den Anwendungsbereich dieser Methode zur Darstellung von natürlichen und in der Natur nicht vorkommenden Nucleosiden werden fortgesetzt.

[a]) DOR-chlorid = 3,5-Di-p-toluyl-2-desoxy-D-ribofuranosylchlorid.
[b]) Smp. für Halbhydrat.

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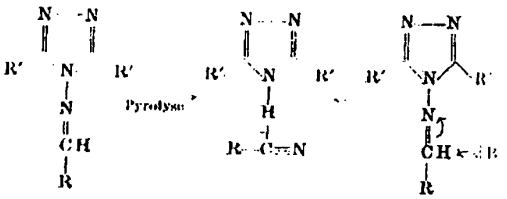
Eine neue Synthese von Nitrilen aus Aldehyden

N-Acyl-imidazole, -triazole bzw. -tetrazole sind als Analoga der entsprechenden Acylchloride anzusprechen [1]. Auf Grund der gleichen theoretischen Vorstellungen ist eine Verwandtschaft zwischen 4-Amino-1,2,4-triazol und Chloramin zu erwarten. Wir haben dies experimentell verifizieren können.
So lassen sich die leicht erhältlichen Azomethine aus aromatischen oder heterocyclischen Aldehyden und 4-Amino-1,2,4-triazol (bzw. dessen Derivaten) durch Pyrolyse oder basenkatalysierte Eliminierung glatt in die entsprechenden Nitrile überführen. Diese Reaktion entspricht durchaus der Bildung



von Nitrilen aus N-Chlor-aldiminen. Trotz zahlreicher Veröffentlichungen [2] hat dieses Verfahren keine praktische Bedeutung erlangt, da das Chloramin unbeständig und unbequem zu handhaben ist. Unser Verfahren hat den Vorteil, an Stelle des instabilen Chloramins das bequem aus Ameisensäure und Hydrazin zugängliche 4-Amino-1,2,4-triazol einsetzen zu können, so daß ein leicht anwendbares Laborverfahren resultiert.
Zur Darstellung der Azomethine vermischt man äquimolare Mengen Aldehyd und 4-Amino-1,2,4-triazol in Benzol und destilliert nach Zusatz einer katalytischen Menge p-Toluolsulfonsäure das Reaktionswasser azeotrop ab. Über die erhaltenen Azomethine unterrichtet Tab. 1. Aliphatische Aldehyde liefern Alkylidenbisamine und bleiben in dieser Publikation außer Betracht.
Die Azomethine gehen beim Erhitzen über ihren Schmelzpunkt in die entsprechenden Triazole und Nitrile über. Flüssige Nitrile können direkt aus dem Reaktionsgefäß heraus destilliert werden. Bei festen Nitrilen eignet sich besonders Glykol als Lösungsmittel, da es sich durch Auswaschen mit Wasser leicht vom Nitril abtrennen läßt.
Kinetische Untersuchungen ergaben, daß die Reaktion durch Basen katalysiert wird. Entsprechend liegt in Gegenwart einer Base die Zersetzungstemperatur viel niedriger (120 bis 130 °C gegenüber etwa 200 °C der reinen Pyrolyse).
Dadurch ist es möglich, die gesamte Umsetzung als Eintopfverfahren durchzuführen. Man stellt zunächst das Azomethin in Xylol dar, setzt dann etwa 10 Mol-% Natrium in Äthylglykol zu und kocht schließlich die Lösung noch 2 bis 3 Stunden, wobei die Eliminierung eintritt.
Die Gesamtausbeuten sind bei dieser Variante besonders gut. Neben der einfachen Durchführung und den guten Ausbeuten

TRANSLATION

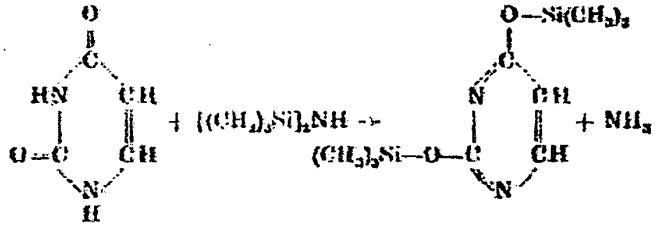
A New Synthesis of Nucleosides [1])

[1]) Preliminary communication; detailed communication follows elsewhere.

L. Birkofer and colleagues [1] showed that N-trialkylsilyl heterocycles react very easily with acyl halides to form N-acyl compounds. This high reactivity of trialkylsilyl heterocycles can also be used successfully to synthesize pyrimidine nucleosides, both natural and not found in nature, and respectively their derivatives, if silylized pyrimidines are reacted with acyl halogen sugars.

Trimethylsilyl pyrimidines are prepared by reacting suitable pyrimidine derivatives with hexamethyl disilaxane at 145 to 155°C. Thereby, the reactive hydrogen atoms of groups, such as –OH and –NHX (X = H or C=O) on the heterocycle,

are replaced by the trimethylsilyl radical, for example,:



The trimethylsilyl pyrimidine derivatives form in very good yields and are distillable. Purification by distillation is usually not required. After distilling off excess hexamethyl disilaxane, the residual dimethylsilyl pyrimidine derivate is pure enough for further reactions.

The following trimethylsilyl derivatives have been prepared so far:

Trimethylsilyl-4-O-ethyl uracil, 85% yield, B.P.$_{17}$: 115-116°C, UV absorption in absolute dibutyl ether: λ_{max} 261 nm, λ_{min} less than 233 nm *;

Trimethylsilyl-4-O-ethyl thymine, 97% yield, M.P. 53-54°C, λ_{max} 265 nm, λ_{min} 236 nm;

Bis(trimethylsilyl)uracil, 94% yield, B.P.? 123°C, λ_{max} 257 nm, λ_{min} less than 233 nm *;

Bis(trimethylsilyl)thymine, 92% yield, B.P.?127°C, λ_{max} 265 nm, λ_{min} 236 nm;

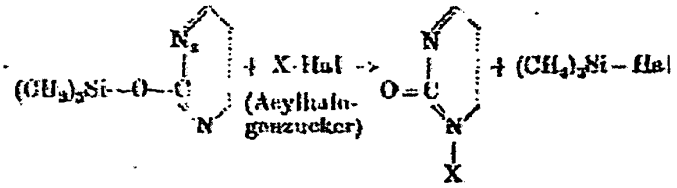
Tris-(trimethylsilyl)-5-hydroxylmethyl cytosine, 96% yield, B.P.? 176°C -180°C, λ_{max} 274 nm, 236.5 nm, λ_{min} 251 nm,

Bis-(trimethylsilyl)cytosine, yield 90%, λ_{max} 306 nm, 275 nm, 236.5 nm, λ_{min} 246 nm, 294 nm;

Bis-(trimethylsilyl)-N-acetyl cytosine, 84% yield, B.P.? 155°C.

*Measurement at short wavelengths is not possible due to the solvent used.

In the action of the acyl halogen sugars on the cited trimethylsilyl pyrimidine derivatives, the silyl radical on the oxygen of carbon atom 2 is replaced by an acylated sugar radical that is linked to the N atom in the 1 position.



Acylhalogenzucker = acyl halogen sugar

The reaction can occur according to one of the following methods:

(a) melting the components at 90 to 100°C in a vacuum, whereby the resulting trimethylsilyl halide distills off continuously,

(b) heating the components in a dry solvent, such as, benzene, toluene, dimethyl formamide, nitromethane, among others,

(c) both reactants are allowed to react with each other at room temperature in a solvent in the presence of HgO, Hg acetate, or $AgClO_4$.

The last-cited method usually yields the best results.

In all three cases, the reaction usually yields either directly the corresponding acylated nucleoside [with 4-ethoxy-5-methyl-pyrimidinone-(2) and 4-ethoxy-pyrimidinone-(2)] or an acylated trimethylsilyl nucleoside, from which the excess silyl radicals are split off hydrolytically during subsequent processing. The following acyl nucleosides were prepared in this manner (see Table 1):

Table 1
Acylated Nucleosides Via Silyl Compounds of Pyrimidines

Silyl ester of pyrimidine base	Acyl halogen sugar	Yield of acyl. nucleoside in %	M.P. in °C	Lit.- M.P. in °C	Lit.
1	2	3	4	5	6
Uracil	Acetobromoglucose	44	149-151	154-155	[2]
"	Acetobromogalactose	86	amorph.	---	---
"	Acetobromo-L-arabinose	90	amorph.	---	---
"	Benzochlororibofuranose	52	144-146	142-143	[3]
"	DOR-chloride [3])	25	216-217	216-217	[4]
Thymine	Aceetobromoglucose	48	154-156[7])	156-158[7])	[5]
"	Acetobromogalactose	50	136-138	---	---
"	Acetobromo-L-arabinose	78	119-121[7])	137-141	[5]
"	Benzochlororibofuranose	70	166-167	167-168	[5]
"	DOR chloride[3])	31	196-197	197	[6]
4-ethoxy-5-methyl-pyrimidinone-(2)	Acetobromogalactose	91	amorph.	---	---
"	Acetobromo-L-arabinose	53	177-179	181	[7]
N-acetylcytosine	Acetobromoglucose	38	145-146[7]) 225	225	[8]
---	Acetobromogalactose	70	amorph.	---	---
---	Acetobromo-L-arabinose	53	amorph.	---	---
5-hydroxymethyl-cytosine	Acetobromoglucose	79	amorph.	---	---

[7])DOR-chloride = 3,5-Di-p-toluyl-2-desoxy-D-ribofuranosyl chloride. [42]) M.P. for half hydrate

The bonding of the glycosyl radicals to the N atom 1 of the pyrimidine rings is shown by comparison of the physical constants with the properties of compounds described in the literature (see Table 1, column 6), which were prepared by other routes. In addition, the structure was probably determined by measuring UV absorption. The corresponding free nucleosides can be obtained from the cited acylated nucleosides in the known manner by splitting off the acyl radicals with methanolic hydrochloric acid or a sodium or barium respectively methylate solution.

Investigations continue in the field of applying these processes for preparing natural nucleosides and nucleosides not found in nature.

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CONVERGENT SYNTHESES AND CYTOSTATIC PROPERTIES OF 2-CHLORO-2'-DEOXY-2'-FLUOROADENOSINE AND ITS N^7-ISOMER

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Abstract. Glycosylation of trimethylsilylated 2,6-dichloropurine **2** with acetate **1** in anhydrous MeCN was investigated. In the presence of $SnCl_4$, the reaction was regio- and stereoselective affording N^7-β-glycoside **3** (86%). The use of TMS-Tfl instead of $SnCl_4$ afforded a ≈ 9:1 mixture of the N^9-β- and -α-glycosides **5** and **6** (90%, combined). The title nucleosides were tested for their cytotoxicity.

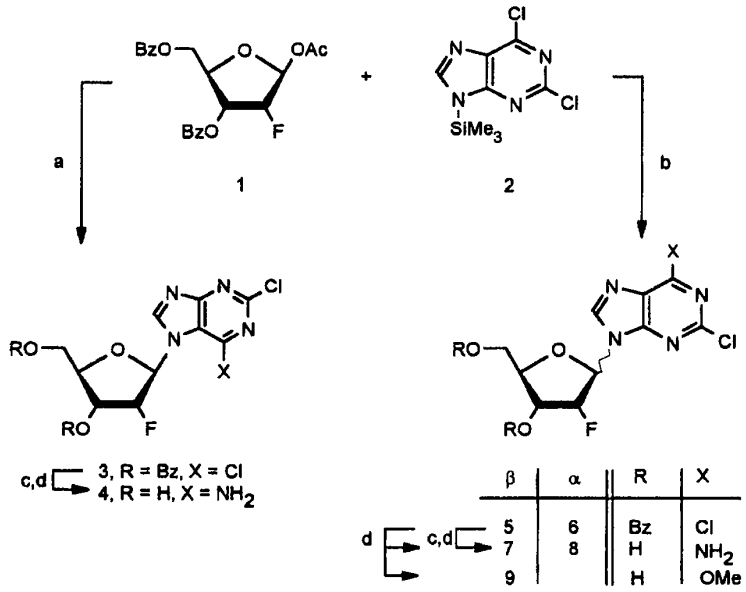
The 2-Chloro-2'-deoxyadenosine (2-CdA, 'Cladribine) is recognized as a potent anticancer and immunosuppressive drug (for reviews, see Refs 1,2) and undergoes extensive clinical trials (*e.g.*, Refs 3). Closely related analogue of 2-CdA, 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine, showed a similar *in vitro* and *in vivo* spectrum of activity (reviewed in Ref. 4). The foregoing, in conjunction with the development of practical synthesis of 1-O-acetyl-3,5-di-O-benzoyl-2-deoxy-2-fluoro-β-D-ribofuranose (**1**)[5], prompted us to synthesize 2-chloro-2'-deoxy-2'-fluoroadenosine (**7**). We describe here the synthesis and cytostatic activity of **7** and its N^7-isomer **4**[6].

Synthesis: The condensation of acetate **1** with trimethylsilylated 2,6-dichloropurine **2** in the presence of tin(IV) chloride in anhydrous acetonitrile at room temperature for 2.5 h proceeded regio- and stereospecifically and the N^7-β-nucleoside **3** was isolated in 86% yield. When tin(IV) chloride is replaced by trimethylsilyl triflate (TMS-Tfl), the reaction led to the formation of the N^9-β-glycoside **5** as principal product along with formation of the N^9-α-anomer **6** (β/α ratio ≈ 9:1 according to the ^{1}H NMR data; 90%, combined) (*cf.* the data in Ref. 7). Transformation of **3** to **4** was effected *via* successive action of saturated solution of ammonia in 1,2-dimethoxyethane at room temperature for 24 h[8] and then methanolic ammonia. After silica gel column chromatography, nucleoside **4**[9] was obtained in 56% combined yield. In a similar manner, the mixture of **5** and **6** was transformed into deblocked anomeric nucleosides and subsequent chromatographical separation gave the corresponding individual compounds **7**[10] and **8**[11] in yields of 72 and 8%, respectively. Treatment of the **5**/**6** mixture with methanolic ammonia at room temperature for 24 h followed by

chromatography afforded nucleosides of 2-chloroadenine **7** (42%) and 2-chloro-6-methoxypurine **9**[12] (39%) (Scheme).

The structures of the compounds were confirmed by ^{1}H NMR, UV, and FAB mass spectra. The ^{1}H NMR data recorded for **7** are in agreement with those reported[7]. The site of glycosylation of 2-chloroadenine was determined by comparison of the ^{1}H NMR and UV spectra of the corresponding compounds with those of the pairs of related adenine N^7- and N^9-glycosides[13]. The most informative features of the ^{1}H NMR spectrum of the α-anomer **8** are *(i)* the 0.36 ppm shift of H-4' resonance signal to a lower field when going from β- to α-anomer (see, *e.g.*, Refs 13,14), and *(ii)* the long-range coupling of H-8 to fluorine of 2.5 Hz exhibited in its ^{1}H NMR spectrum. This coupling is generally indicative of a spatial proximity of the nuclei involved[15] and is not observed in the β-anomers. The structure of the heterocyclic base of **4** was deduced from a comparison of the UV spectral data with those for the N^7-glycosides of the 6-amino-2-chloropurine and its 2-amino-6-chloro isomer[16] which display widely different UV spectra.

Scheme



a) **1**/**2**/$SnCl_4$ (1.0:2.0:3.0, mol), MeCN, 20 °C, 2.5 h (86%); b) **1**/**2**/TMS-Tfl (1.0:1.7:2.55, mol), MeCN, reflux, 20 min (**5**/**6**, ≈ 9:1; 90%, combined); c) saturated at 20 °C ammonia in 1,2-dimethoxyethane, 20 °C, 24 h; d) saturated at 0 °C methanolic ammonia, 20 °C, 24 h [**7** (42%) and **9** (39%); c + d, **4** (56%); **7** (72%) and **8** (8%)]

Cytotoxicity studies: Toxicity of nucleosides **4** and **7** was determined by their effects on protein synthesis in cells in culture. The human leukemia cell lines were obtained from the American Type Culture Collection (Rockville, MD). Test compounds were added to cultures in 96-well microplates containing 2 x 10^4 leukemia cells or 2.5 x 10^4 PHA-stimulated lymphocytes per 200 μL well. Cells were cultured in RPMI 1640 medium containing glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 μg/mL) and fetal calf serum (10%, v/v), in humidified atmosphere containing 5% CO_2 at 37 °C. [U-^{14}C]-L-leucine (specific activity 1.3 mCi/mmol and 0.5 μCi/mL) was added to the cultures for the final 24 h of the 3-day culture period of human leukemia cells and for the 4-day culture period of PHA-stimulated lymphocytes. After incubation, the proteins were precipitated with 0.2 N perchloric acid and collected on glass fiber filters with use of a multiple cell harvester (Wallac, Turku, Finland). The radioactivity incorporated into proteins was measured in a scintillation counter (1410, Wallac, Turku, Finland). The incorporation of [^{14}C]-leucine per cell remains constant during the final 24 h of culture, and a good correlation has been demonstrated between cell number and [^{14}C]-leucine incorporation[17,18].

The toxicities of nucleosides **4** and **7** were first screened at a concentration of 10 μg/mL against four human leukemia/lymphoma lines and against mitogen-stimulated human peripheral blood lymphocytes; no significant toxicity was observed with N^7-β-anomer **4**. In contrast, the N^9-β-anomer **7** was toxic against all lines. Hence, three lower concentrations were also tested. The results are presented in the Table.

Table. Toxicity of Nucleoside **7** Against Malignant and Normal Hematopoietic Cells *in vitro*.

Concentration [μg/mL]	[^{14}C]-Leucine Incorporation (% of control)[a]				
	IM-9[b]	Raji	MOLT-3	U-937	PHA-Ly
10	5.7	2.5	2.6	1.8	4.0
1.0	19.0	9.1	3.3	4.9	17.0
0.1	110	68.0	79.0	70.0	90.0
0.01	108	103	106	99.0	98.0

[a]Mean value of three determinations.
[b]Abbreviations are as follows: IM-9, myeloma cell line; Raji, Burkitt's lymphoma (B-cell); MOLT-3, acute T cell leukemia; U-937, histiocytic lymphoma; PHA-Ly, phytohemagglutinin-stimulated peripheral blood lymphocytes.

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9. Compound **4**. mp 243-244 °C (from ethanol); TLC, Kieselgel 60 F_{254} (Merck, Germany) plates, chloroform-methanol, 10:1 (triple elution), $R_f = 0.13$; 200 MHz ^{1}N NMR spectrum (DMSO-d_6), δ_{TMS} ppm, *J* Hz: 8.67 (s, 1H, H-8), 7.47 (br.s, 2H, NH_2), 6.42 (dd, 1H, $J_{1',2'} = 3.8$, $J_{1',F} = 14.0$, H-1'), 5.80 (d, 1H, $J_{3',OH\text{-}3'} = 6.0$, OH-3'), 5.39 (t, 1H, $J_{5',OH\text{-}5'} = 4.8$, OH-5'), 5.17 (dt, 1H, $J_{2',3'} = 5.0$, $J_{2',F} = 52.0$, H-2'), 4.33 (m, 1H, $J_{3',4'} = 6.0$, $J_{3',F} = 15.0$, H-3'), 4.04 (m, 1H, H-4'), 3.78 (dd, 1H, $J_{5',4'} = 2.5$, $J_{5',5''} = 12.5$, H-5'), 3.62 (dd, 1H, $J_{5'',4'} = 3.0$, H-5''). UV, (pH 7.0), λ_{max} 216.0 nm (ε 22,150), ≈ 245 sh (ε 5,300), 275.6 (ε 8,700), λ_{min} 234 nm (ε 4,600); (pH 1.0), λ_{max} 215.0 nm (ε 20,000), 274.0 (ε 9,400), λ_{min} 205 nm (ε 17,000); 237 nm (ε 4,300); FAB mass spectrum, *m/z* 304 and 306, $^{35}Cl/^{37}Cl$ ratio ≈ 3:1, $(M + H)^+$.
10. Compound **7**. mp 229-231 °C [from water; (lit.[7]: mp 220-222 °C, from methanol)]; TLC, as above, $R_f = 0.36$; ^{1}N NMR spectrum (DMSO-d_6), δ_{TMS} ppm, *J* Hz: 8.40 (s, 1H, H-8), 7.92 (br.s, 2H, NH_2), 6.19 (dd, 1H, $J_{1',2'} = 2.8$, $J_{1',F} = 16.8$, H-1'), 5.79 (d, 1H, $J_{3',OH\text{-}3'} = 6.0$, OH-3'), 5.38 (ddd, 1H, $J_{2',3'} = 4.3$, $J_{2',F} = 53.2$, H-2'), 5.25 (t, 1H, $J_{5',OH\text{-}5'} = 5.0$, OH-5'), 4.44 (ddd, 1H, $J_{3',4'} = 6.8$, $J_{3',F} = 18.5$, H-3'), 4.00 (m, 1H, H-4'), 3.78 (dd, 1H, $J_{5',4'} = 2.5$, $J_{5',5''} = 12.3$, H-5'), 3.60 (dd, 1H, $J_{5'',4'} = 4.0$, H-5''). UV, (pH 7.0), λ_{max} 264.0 nm (ε 15,500), λ_{min} 228.0 nm (ε 2,700); (pH 1.0), λ_{max} 265.6 nm (ε 14,700), λ_{min} 229.0 nm (ε 2,600); FAB mass spectrum, *m/z* 304 and 306, $^{35}Cl/^{37}Cl$ ratio ≈ 3:1, $(M + H)^+$.
11. Compound **8**. Lyophilized powder from water; TLC, as above, $R_f = 0.31$; ^{1}N NMR spectrum (CD_3OD), δ_{TMS} ppm, *J* Hz: 8.28 (d, 1H, $J_{H\text{-}8,F} = 2.5$, H-8), 6.46 (dd, 1H, $J_{1',2'} = 4.0$, $J_{1',F} = 16.5$, H-1'), 5.24 (dt, 1H, $J_{2',3'} = 4.0$, $J_{2',F} = 54.0$, H-2'), 4.51 (ddd, 1H, $J_{3',4'} = 7.0$, $J_{3',F} = 19.0$, H-3'), 4.36 (m, 1H, H-4'), 3.90 (dd, 1H, $J_{5',4'} = 2.3$, $J_{5',5''} = 12.5$, H-5'), 3.70 (dd, 1H, $J_{5'',4'} = 3.5$, H-5''). UV, (pH 7.0), λ_{max} 265.0 nm (ε 11,000), λ_{min} 229.0 nm (ε 2,500); (pH 1.0), λ_{max} 265.0 nm (ε 11,000), λ_{min} 229.0 nm (ε 2,500); FAB mass spectrum, *m/z* 304 and 306, $^{35}Cl/^{37}Cl$ ratio ≈ 3:1, $(M + H)^+$.
12. Compound **9**. mp 177-178 °C (from ethanol)]; TLC, as above, $R_f = 0.52$; ^{1}N NMR spectrum (CD_3OD), δ_{TMS} ppm, *J* Hz: 8.58 (s, 1H, H-8), 6.33 (dd, 1H, $J_{1',2'} = 2.5$, $J_{1',F} = 16.5$, H-1'), 5.39 (ddd, 1H, $J_{2',3'} = 4.0$, $J_{2',F} = 52.5$, H-2'), 4.63 (ddd, 1H, $J_{3',4'} = 7.0$, $J_{3',F} = 18.0$, H-3'), 4.18 (s, 3H, OMe), 4.14 (m, 1H, H-4'), 3.98 (dd, 1H, $J_{5',4'} = 2.5$, $J_{5',5''} = 12.0$, H-5'), 3.80 (dd, 1H, $J_{5'',4'} = 3.2$, H-5''). UV, (pH 1.0, 7.0, and 11.0), λ_{max} 258.0 nm (ε 10,000), λ_{min} 224.0 nm (ε 2,200); (pH 1.0); FAB mass spectrum, *m/z* 319 and 321, $^{35}Cl/^{37}Cl$ ratio ≈ 3:1, $(M + H)^+$.
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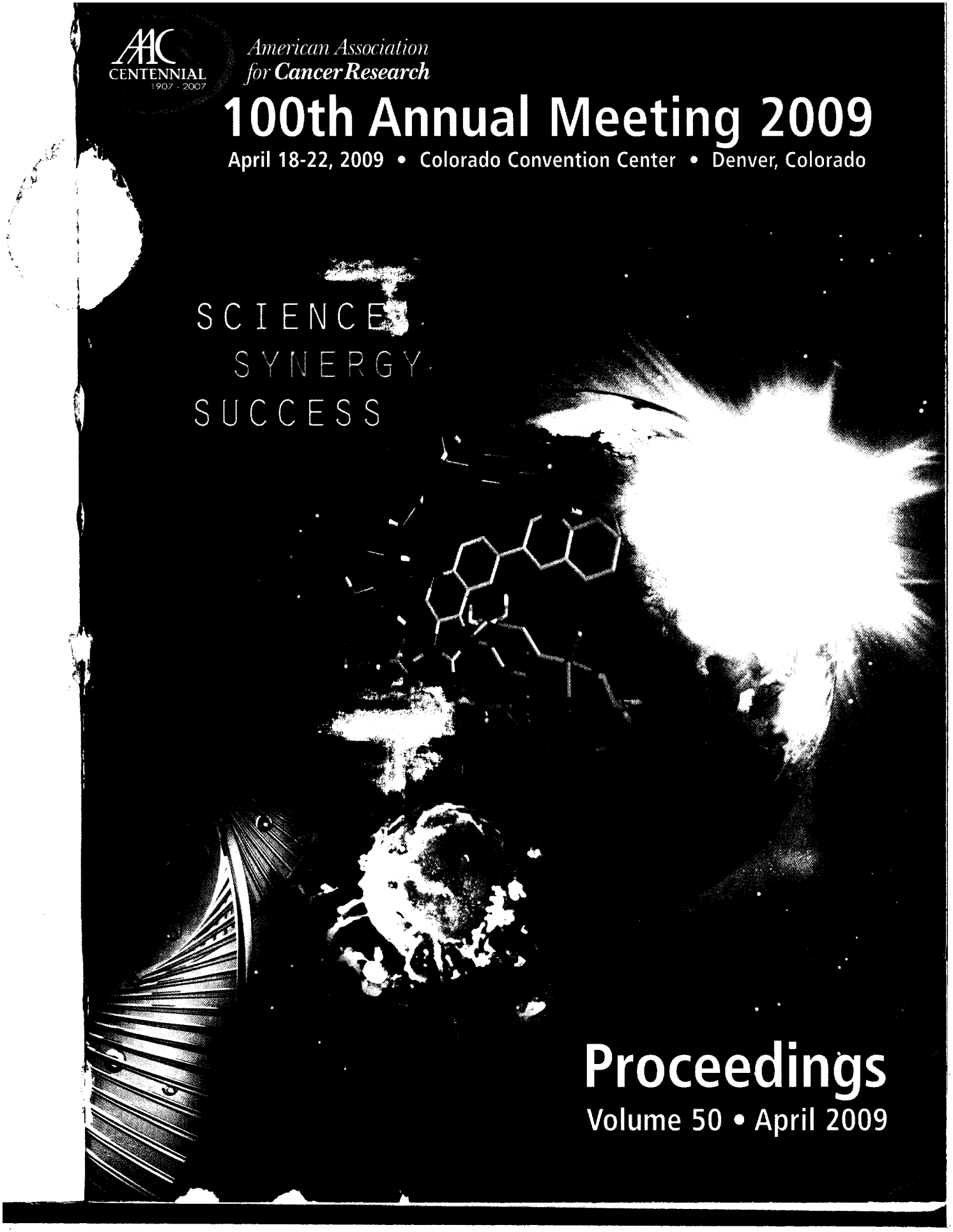

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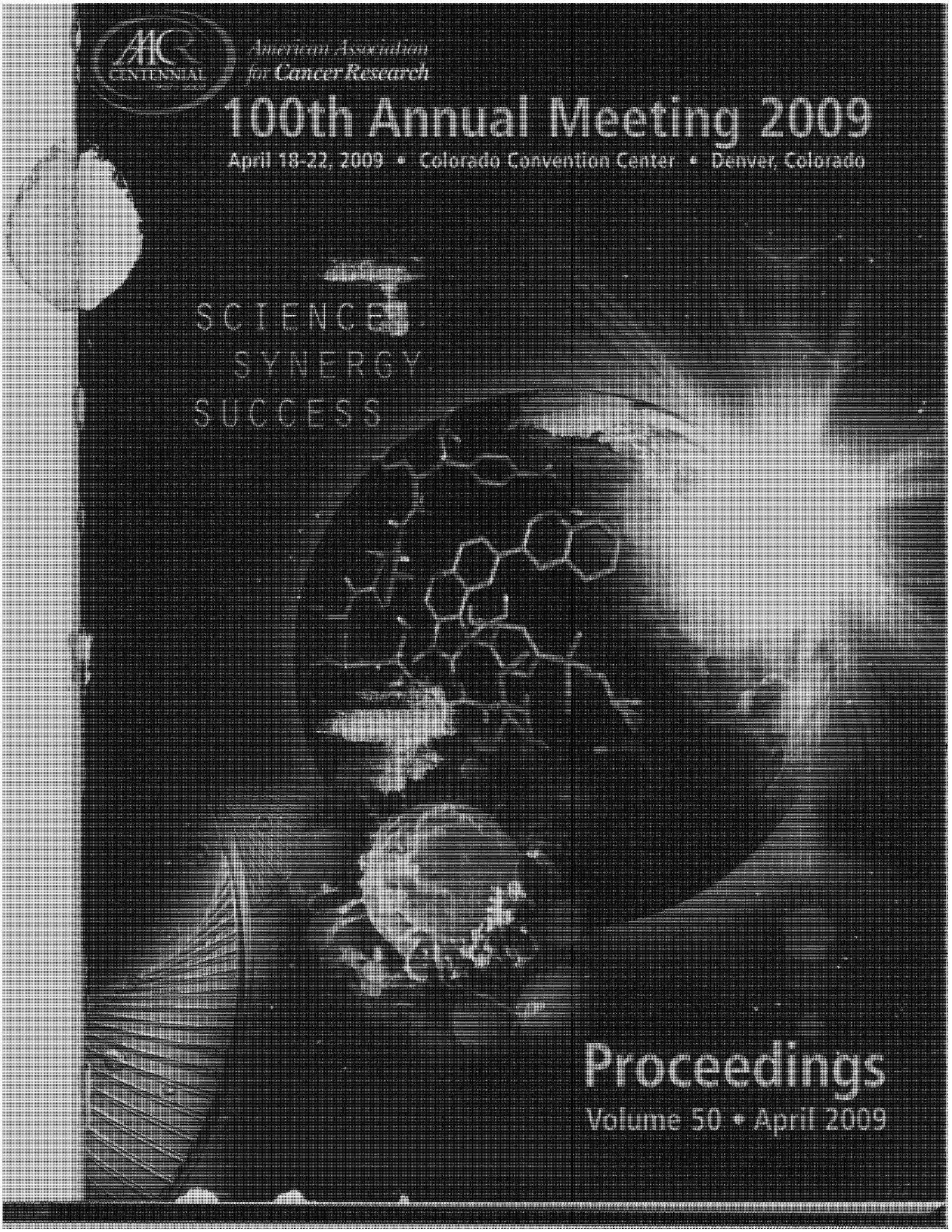

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Advances in technology, progress in prevention, and exponential developments in the field of translational cancer medicine have increased the importance of fostering dynamic collaborations among basic researchers, physicians, and clinician-scientists. Bridging the gap between what physicians understand about cancer biology and its application to clinical oncology is critical to the implementation of the most current, approved molecular-based tests to aid in the diagnosis, treatment and prevention of cancer. Moreover, facilitating the interface between physicians and scientists will increase physicians' knowledge of the epidemiological implications of cancer incidence and the contributions of laboratory research to drug development as well as patient care; transform the design and conduct of clinical research protocols; and, create a forum for the more rapid translation of laboratory research findings from "bench-to-bedside" for the benefit of improving patient outcomes.

After participating in this CME activity, participants will be able to:

1. demonstrate a clear understanding of the latest research findings in all areas of cancer research and how these findings will impact future decision making in the detection, diagnosis, prognosis, treatment, and prevention of cancer;
2. translate how the integration of information from the basic, translational, and clinical sciences applies to the development of new drugs and clinical research trials;
3. employ technological advances to accelerate the research progress, improve early detection and screening options, which will ultimately extend patients' lives and improve their quality of life;
4. integrate the use of molecular imaging and tumor profiling to more accurately predict cancer susceptibility, treatment strategies, as well as treatment outcomes;
5. evaluate the use of biomarkers and other indicators to improve patient selection (or stratification) for clinical trials in this era of personalized medicine; and,
6. formulate new strategies and forge collaborations that will further scientific and clinical research efforts towards the prevention, diagnosis, early detection, and treatment of cancer.

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Next Annual Meeting: April 17-21, 2010, Washington, DC

Table 1. Methylation and prognostic significance of SOX17, APC, KLK10, CST6 and RASSF1A in NSCLC

Methylation (%)	NSCLC tissues (n=57)	Adjacent tissues (n=57)	Cell-free circulating DNA in plasma: NSCLC (n=48)	Cell-free circulating DNA in plasma: Healthy donors (n=24)
SOX17	100.0	100.0	56.2	20.8
APC	66.6	80.7	25.0	8.3
KLK10	65.5	47.3	50.0	4.3
CST6	56.1	46.0	40.0	0.0
RASSF1A	66.0	52.0	23.0	4.3
Prognostic Significance	NSCLC Tissues (n=57)	NSCLC Tissues (n=57)	Cell-free circulating DNA (n=48)	Cell-free circulating DNA (n=48)
gene	DFI (P)	OS (P)	DFI (P)	OS (P)
SOX17	-	-	0.080	0.028
APC	0.006	0.057	0.069	0.002
KLK10	0.650	0.150	0.039	0.290
CST6	0.261	0.781	0.760	0.641
RASSF1A	0.284	0.473	0.889	0.560

Conclusions. SOX17, APC, KLK10, CST6 and RASSF1A, promoter methylation is a frequent event in NSCLC. The prognostic significance of cell-free circulating DNA in plasma of NSCLC patients should be further evaluated in a large number of clinical samples.

#3367 Synergistic effects of MBD2 knock down and 5-aza-2'-deoxycytidine (5AzadC) on inhibiting breast cancer growth and invasiveness: Blocking global hypomethylation and prometastatic effects of 5AzadC. Flora Chik, Moshe Szyf. *McGill University, Montreal, Quebec, Canada.*

The hallmarks of epigenetic changes in cancer cells are global demethylation and regional hypermethylation of tumour suppressor genes. The current focus in cancer therapy is on inhibiting DNA methylation and activating tumor suppressor genes. The DNA methylation inhibitor 5-aza-2'-deoxycytidine (5AzadC) is incorporated into DNA where it physically traps all DNMTs, leading to passive global demethylation. 5AzadC has been approved for the treatment of Myelodysplastic syndrome (MDS). Since 5AzadC is a global inhibitor of DNA methylation it is plausible that in addition to tumor suppressor genes it induces other genes that promote cancer and stimulate metastasis. We tested this hypothesis on non-invasive MCF-7 human breast cancer cells. A combination of transcriptomic and methylomic analysis shows that 5AzadC induces the promoters of several gene networks known to be involved in invasion and metastasis. In vitro invasion assays using a Boyden chamber analysis indicate that 5AzadC induces the invasibility of MCF-7 cells. These data raises the concern that 5AzadC therapy might induce metastasis in addition to its activity of inhibiting tumor growth. We therefore tested whether it is possible to block the invasion-promoting activity of 5AzadC while maintaining its growth inhibitory activity. We have previously shown that the methylated DNA binding protein MBD2 is involved in demethylation and re-expression of merastatic genes. We therefore tested whether a combination of 5AzadC and knockdown of MBD2 by siRNA would block both invasiveness and anchorage independent growth of MCF-7 cells. We show that MBD2 siRNA blocks the demethylating activity of 5AzadC as well as it reverses 5AzadC-induced invasiveness. Transcriptomic and methylomic analysis indicate that MBD2siRNA blocks the induction and demethylation of several of the genes induced by 5AzadC. Interestingly, MBD2siRNA enhances the growth inhibitory effects of 5AzadC. The results of this study demonstrate the potential of a combination of 5AzadC and siMBD2 as a therapy for cancers that could circumvent the adverse side effects of global DNA demethylation therapy.

#3368 Decitabine-induced Nox4 upregulation in leukemia cells is demethylation independent and ATM-dependent. Tamer E. Fandy, Anchalee Jiemjit, Steven D. Gore. *Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD.*

Reactive oxygen species (ROS) are known to affect cell survival, differentiation, and senescence; chemotherapeutic nucleoside analogues are known to increase ROS generation. The aim of this study is to investigate the effect of clinically relevant low doses of decitabine on ROS generation in leukemia cells and solid tumors. Decitabine (100 and 250 nM), a nucleoside analogue DNA methyltransferase (DNMT) inhibitor, increased ROS generation in different leukemia cell lines (ML1, BV173, HL60, CEM) but not in colon (HCT 116 and DLD-1) or breast (MCF-7 and MCF-10A) cancer cell lines after 48 and 72 hours. The increase in ROS was not abrogated by pan-caspase inhibitors, indicating that ROS generation is not a consequence of caspase activation and apoptosis induction. Decitabine did not increase ROS in deoxycytidine kinase (DCK)-deficient CEM leukemia cells, indicating that ROS increase is not a consequence of incorporation of decitabine in mitochondrial DNA and consequent mitochondrial toxicity. Decitabine treatment downregulated the expression of the pro-survival protein Bcl-2 and pretreatment with the ROS scavenger N-acetylcysteine (NAC) restored its expression, indicating that ROS generation modulated Bcl-2 expression. Concordantly, NAC pretreatment inhibited DAC-induced apoptosis, while the ROS inducer buthionine sulfoximine (BSO) augmented DAC-induced apoptosis. Mitochondria are considered the major source for ROS generation, however, non-mitochondrial enzymes such as NADPH oxidases (Nox) generate superoxide anion and increase ROS generation. Among the seven known isotypes of Nox, Nox4 possesses a putative CpG island and its expression can be epigenetically silenced by DNA cytosine methylation. While decitabine upregulated Nox4 protein expression in leukemia cells, Nox4 mRNA was expressed at baseline and was not induced after treatment with DAC in leukemia cells. Furthermore, methylation specific PCR analysis of Nox4 putative CpG island demonstrated absence of methylated alleles, indicating that decitabine induced Nox4 upregulation is transcription- independent and may involve Nox4 protein posttranslational modification. Concordantly, the specific DNMT1 inhibitor RG108 did not upregulate Nox4 expression or increase ROS generation in leukemia cells, further confirming the demethylation-independent Nox4 upregulation and ROS generation by decitabine. In contrast, the specific ATM inhibitor KU55933 inhibited decitabine-induced Nox4 upregulation, indicating the involvement of DNA damage signaling in Nox4 upregulation. These data highlight the importance of mechanisms other than DNA cytosine demethylation in modulating protein expression and suggest investigating their relevance to the clinical activity of decitabine.

#3369 Development of oral demethylating agents for the treatment of myelodysplastic syndrome. Amy J. Ziemba,[1] Marcela Cortes Ramirez,[2] Burgess Freeman,[1] Eugene Hayes,[1] Tao Ye,[2] Giuseppe Pizzorno[1]. *[1]Nevada Cancer Inst., Las Vegas, NV; [2]Shenzhen Graduate School of Peking University, Shenzhen, China.*

Myelodysplastic syndrome (MDS) is an incurable stem cell malignancy with about 12-20,000 new cases per year in the USA, with the highest prevalence in those over 60 years of age. The basis of therapy is supportive care, including the use of transfusions and erythropoietin or growth factors. MDS progresses to AML in approximately 30% of patients. DNA methyltransferase (DNMT) inhibitors may be beneficial therapeutics since epigenetic silencing through promoter methylation of a number of genes is present in poor-risk subtypes of MDS and often predicts transformation to AML. The two most promising DNMT inhibitors that have received FDA approval for the treatment of all MDS subtypes are azacytidine and decitabine. These agents have shown remarkable ability to inhibit DNA methylation and improved overall response with a longer time of progression to AML, although their stability in aqueous solutions and their low oral bioavailability complicate their clinical use. In an attempt to develop oral prodrugs, we have initially synthesized esterified derivatives of these pyrimidine nucleosides and other new nucleoside analogs. The lead prodrug, 2',3',5'-Triacetyl -5-azacytidine (TAC), was successfully synthesized through condensation of trimethylsilylated-5-azacytosine and 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose. TAC demonstrated favorable physical-chemical characteristics when compared to azacytidine, with increased solubility and stability in aqueous solution. To evaluate the pharmacokinetics of TAC, mice were dosed with 25 mg/kg i.v. azacytidine or an equimolar dose of 38 mg/kg oral TAC. TAC was rapidly deacetylated leading to a minimal accumulation of the prodrug that appears to be below the limit of detection (30 ng/ml) by 4 hours. TAC-derived azacytidine was present after 15 minutes reaching a peak concentration of approximately 5,000 ng/ml (20uM) at 30 minutes with a pharmacologically relevant concentration of 0.5uM after 24 hours. The half-life of TAC-derived azacytidine is 8.5 hours versus 4-5 hours for azacytidine when administered i.v. to indicate a protracted absorption at the gastro-intestinal level and conversion of the pro-drug. The in vivo toxicity of TAC was evaluated by orally administering repeated doses to CD-1 mice. TAC administered five days per week for 2 weeks resulted in no animal deaths and no weight loss, but did induce changes in hematological parameters, bone marrow, lymph nodes, and duodenal epithelium. The pharmacodynamics of TAC were also evaluated. TAC was capable of inhibiting global DNA methylation at a level comparable to AC in the spleen and gut of mice dosed daily for 5 days. TAC also exhibited antineoplastic activity in an in vivo L1210 leukemia model. We have now designed and synthesized a new series of nucleoside analogs to test for activity in MDS. The compounds currently being tested were chosen due to their encouraging theoretical solubility and bioavailability properties.

Development of oral demethylating agents for the treatment of myelodysplastic syndromes.

Amy Ziemba, Marcela Cortes, Burgess Freeman III, Eugene Hayes, Tao Ye, and Giuseppe Pizzorno.
Nevada Cancer Institute, Las Vegas, NV and Shenzhen Graduate School of Peking University, Shenzhen, China

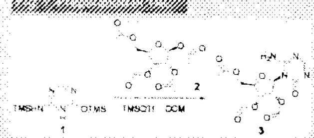
Abstract

Myelodysplastic [illegible] 20,000 new cases per year in [illegible] years of age. The basis of [illegible] transfusions and erythropoietin or [illegible] 30% of patients. [illegible] therapeutics since epigenetic [illegible] number of genes is present in [illegible] of MDS and [illegible] transformation to AML. The two [illegible] inhibitors [illegible] FDA approval for the treatment [illegible] These agents have shown remarkable [illegible] to [illegible] overall response with a longer time of [illegible] and their [illegible] oral [illegible] have [illegible]ed [illegible] of [illegible] and [illegible]
The [illegible] prodrug, 2',3',5'-[illegible] (TAC), was [illegible] through condensation [illegible] and 1,2,3[illegible] TAC [illegible] to azacitidine, [illegible] in [illegible]. To evaluate the [illegible] 25 [illegible] or an [illegible] dose of 38 [illegible] a [illegible] of [illegible] the [illegible] (30 ng/ml) by 4 hours. [illegible] present after 15 [illegible] a peak concentration of [illegible] (20uM) at 30 [illegible] after 24 hours. [illegible] half-life of TAC-derived [illegible] is 8.5 hours [illegible] 4-5 hours for [illegible] when administered [illegible] indicate [illegible] conversion of the [illegible] The [illegible] administering repeated [illegible] to CD-1 [illegible] 2 weeks resulted in no animal [illegible] did [illegible] hematological parameters, [illegible] and [illegible] The [illegible] of [illegible] was [illegible] global DNA methylation at a [illegible] to AC in [illegible] dosed daily for 5 days. [illegible] antineoplastic [illegible] leukemia model. We have [illegible] a [illegible] analogs to test for activity in [illegible] were chosen due to their [illegible]

Results

0.0	2	5-ACT	1.51
2	2	5-ADC (IS)*	1.56
5.5	90	MAC	1.65
8.0	90	DAC	5.24
8.5	2	TAC	5.35
10.0	stop		

LC/MS/MS analysis of 2',3',5'-triacetyl-azacitidine (TAC) and its metabolites ([illegible], MAC; diacetyl-5-azacitidine, DAC; and 5-azacitidine, ACT) optimized on an API 3200 instrument. *IS: internal [illegible]).



[illegible] ¹H NMR [illegible] d 8.18(s, 1H), [illegible] 5.[illegible] J = [illegible] J = 4.0Hz), 5.4[illegible] 3H), 2.11(s, 3H), [illegible] 170.3, 169.9, 169.5, 166.0, 156.0, 153.1, [illegible] 20.3 ppm.

Results

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Azacitidine (ACT)	244	-2.32	-2.17	-1.44
Decitabine	228	-1.49	ND	-1.84
Triacetyl-azacitidine (TAC)	370	-0.85	-0.606	-2.67

LogP value: [illegible] of a [illegible] partition [illegible]. The [illegible]

[illegible]	[illegible]	[illegible]
1	10 mg/ml	50 mg/ml
3	10 mg/ml	20 mg/ml
5	5 mg/ml	20 mg/ml
7.4	1 mg/ml	50 mg/ml

pH1: 2% Phosphoric acid
pH3: 0.1% Formic Acid
pH5: K_3PO_4 Buffer
pH7.4: 1x PBS

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Control	100			
pH 1- 1hr	99	1	0	0
pH 1- 3hr	74	22	4	0
pH 1- 6hr	38	41	21	0
pH 1- 29hr	1	22	31	46
pH 3- 1hr	97	3	0	0
pH 3- 3hr	96	4	0	0
pH 3- 6hr	92	6	2	0
pH 3- 29hr	93	4	2	1
pH 5- 1hr	98	2	0	0
[illegible] 3hr	94	6	0	0
[illegible] 6hr	98	2	0	0
pH 5- 29hr	94	4	1	1
pH 7.4- 1hr	96	4	0	0
pH 7.4- 3hr	91	7	2	0
pH 7.4- 6hr	85	12	3	0
pH 7.4-29hr	69	17	13	1

(TAC, 2',3',5'-[illegible], DAC, [illegible])

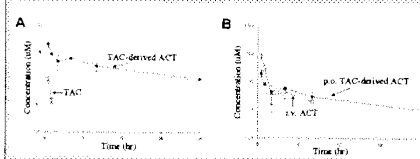
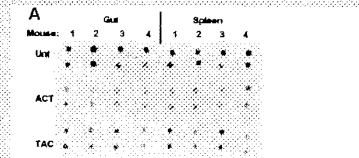


Fig. 2. A. Mean plasma [illegible] vs. time for TAC (○) and [illegible] ACT (•) in [illegible] 38 [illegible] TAC. **B.** ACT [illegible] ACT [illegible] TAC p.o. (•). [illegible]

Results



B

	Gut.1	Gut.2	Gut.3	Gut.4	Average	st dev
Unt	100	100	100	100	100	0
ACT	37	14	34	33	29	11
TAC	56	30	41	25	38	14
	Spleen.1	**Spleen.2**	**Spleen.3**	**Spleen.4**	**Average**	**st dev**
Unt	100	100	100	100	100	0
ACT	54	71	48	29	50	17
TAC	48	31	62	22	41	18

Fig. 3. Effect of [illegible] DNA methylation in tissues [illegible] ACT or TAC. **A.** [illegible] blot of [illegible] ACT or [illegible] **B.** [illegible]

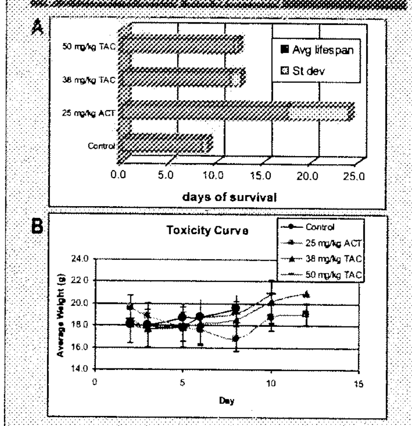


Fig. 4. [illegible] L1210 [illegible] murine [illegible] female [illegible] **A.** [illegible] **B.** [illegible]

Results

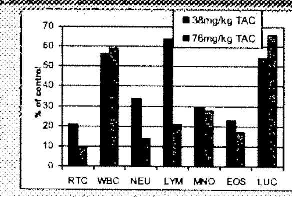
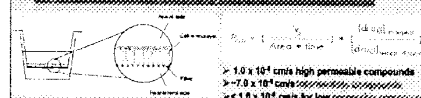


Fig. 5. TAC-associated decreases in [illegible] in CD-1 [illegible] (sterile water) or TAC via oral [illegible] on [illegible] 8–12, [illegible] for necropsy. [illegible] neutrophil [illegible] eosinophil count, [illegible]

TAC-related fi[illegible]llularity) and in the lymph [illegible] overall WBC count [illegible]. We [illegible] apoptosis [illegible] the crypt [illegible] mg/kg/day [illegible] and [illegible]partment [illegible]



> 1.0 x 10⁻⁶ cm/s high permeable compounds
> ~7.0 x 10⁻⁶ cm/s [illegible]
> < 1.0 x 10⁻⁶ cm/s for low [illegible]

	avg	st dev	% deviation
AC Papp	1.13E-07	2.16E-08	19.04
TAC Papp	1.16E-06	2.29E-07	19.80

The human colon [illegible] cell model is used to [illegible] of the [illegible]

Conclusions

- [illegible] demonstrated higher [illegible] compared to its parent compound [illegible]
- Oral TAC [illegible] to i.v. ACT [illegible] to [illegible] global [illegible]
- TAC [illegible] animal deaths and no [illegible] loss, [illegible] high-dose TAC [illegible] induce changes [illegible]
- The [illegible] predicts [illegible] in GI [illegible] TAC [illegible]

Future Directions

- [illegible]
- [illegible] methy[illegible]
- [illegible] of [illegible] oral [illegible] DNA [illegible]

Isolation, Characterization, and Properties of a Labile Hydrolysis Product of the Antitumor Nucleoside, 5-Azacytidine

John A. Beisler

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Received June 22, 1977

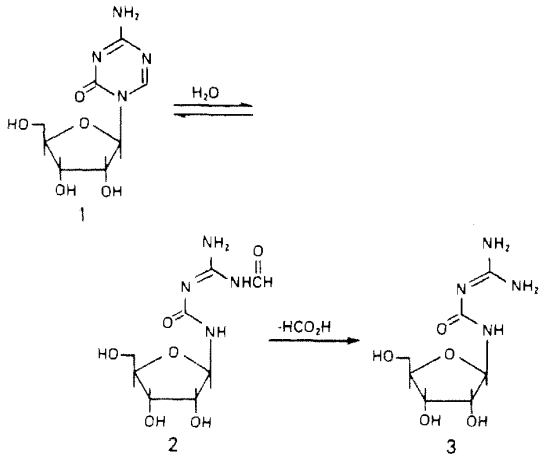
The antitumor nucleoside, 5-azacytidine (5-AC), is best administered clinically by prolonged intravenous infusion to minimize toxic effects. In opposition to this administration technique is facile drug decomposition in aqueous formulations giving products of unknown toxicity. Analysis of 24-h-old water solutions of 5-AC with high-pressure liquid chromatography (HPLC) indicated a threefold mixture of 5-AC, *N*-(formylamidino)-*N'*-β-D-ribofuranosylurea (RGU-CHO), and 1-β-D-ribofuranosyl-3-guanylurea (RGU). Preparative HPLC allowed the isolation and subsequent identification of each component in the mixture, including RGU-CHO which, until now, has not been available for chemical and biological study. It was shown that RGU-CHO in water solution readily equilibrates to 5-AC and more slowly deformylates to give RGU irreversibly. The latter hydrolysis product exhibited no pronounced toxicity when tested either in vitro or in vivo. Although RGU-CHO showed considerable antitumor activity against murine L1210 leukemia, hydrolysis studies indicated that all of the observed activity could be attributed to 5-AC formed by in vivo equilibration from RGU-CHO. Moreover, RGU-CHO seemed to impart to test animals a toxicity which was no greater than that anticipated from its ability to generate 5-AC.

5-Azacytidine (1, 5-AC) is a nucleoside antimetabolite[1] which has a clinical specificity for acute myelogenous leukemia.[2] When administered by rapid intravenous (iv) injection,[3] the drug causes severe, often dose limiting,[2] gastrointestinal toxicity which can be greatly reduced[4] or virtually eliminated[5] by slow, continuous iv infusion of the drug in lactated Ringer's solution over a 5-day period. However, the latter technique is thwarted by the facile hydrolysis of 5-AC in aqueous formulations,[6] leading not only to solutions of decreasing 5-AC potency but also to hydrolysis products having toxicological or therapeutic effects which have not been determined. Microbiological assay of 24-h-old aqueous solutions of 5-AC indicates twice the cytotoxicity as would be anticipated from the results of chemical stability data.[5,7] The discrepancy between the chemical and biological analysis of "aged" 5-AC solutions suggests that one or more hydrolysis products, in addition to 5-AC, contribute to the observed cytotoxic effect. Because of implications relevant to the clinical usage of 5-AC, it was of interest to examine partially hydrolyzed 5-AC solutions for the presence of biologically active hydrolysis products. Scheme I shows the predominant hydrolytic pathway of 5-AC (1) at room temperature and neutral pH such as would occur in a clinical formulation. The presence of the initial hydrolysis product, **2** (RGU-CHO), formed as a consequence of nucleophilic attack by water at C-6 of 5-AC followed by ring opening, was inferred by spectroscopic observations.[6] However, all attempts to isolate or synthesize RGU-CHO were unsuccessful.[6] The ribosylguanylurea (**3**, RGU), formed by an irreversible loss of the *N*-formyl group from RGU-CHO, was sufficiently stable to allow isolation but was found to be weakly cytotoxic against *Escherichia coli*[6] and inactive in vivo against murine L1210 leukemia while exhibiting no pronounced toxicity in the test animals.[5]

The present report describes the isolation [by high-pressure liquid chromatography (HPLC) in a preparative mode], characterization, and some chemical and biological properties of the labile *N*-formyl intermediate **2** from "aged" water solutions of 5-AC.

Fractionation. As shown in Figure 1, a water solution of 5-AC (**1**) exhibits a single peak in the chromatogram (trace A) when analyzed with HPLC immediately after the solution is formed. After the solution is stored at room temperature for 2 h a second peak due to RGU-CHO (**2**) can be observed (trace B). A third peak emerges from baseline noise after 6 h (trace C) which is primarily due to RGU (**3**). After 24 h (trace D), **2** is approximately at its maximum concentration in the mixture, and thereafter both **1** and **2** decrease in concentration while the ratio of their peak areas remains essentially constant. From its first appearance in the chromatogram, the concentration of RGU increases continuously until it is the only detectable material in solution after about 10 days.

Scheme I



The three peaks of the 24-h-old solution were sufficiently separated to encourage an attempt to isolate and identify the individual components of the mixture by preparative HPLC. It was found that **1** (32 mg) in water solution (2 mL), after 24-h storage at room temperature to give the threefold mixture, could be chromatographed without significant peak overlap. Multiple collection of the first eluted peak (t_R = 2.0 min), due primarily to **3**, gave a glass on lyophilization from which the picrate of **3** was isolated in 52% yield. The somewhat low yield of picrate and the presence of a small shoulder on the peak in the chromatogram suggest the presence of other materials, although triazine products can be eliminated from consideration since absorptivity in the UV was absent.

The second peak (t_R = 5.9 min) due to **2** was collected, frozen immediately, and lyophilized to give a white solid. Typically, fractions from five or six runs were combined in water solution (2 mL) and rechromatographed to provide pure samples of **2** which were used for charac-

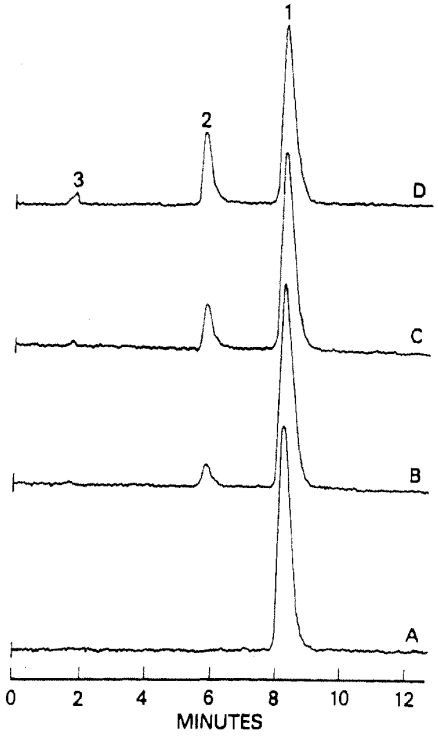


Figure 1. HPLC traces of 5-AC in aqueous solution (10 mg/mL, 25 °C) at increasing time intervals showing the response from a refractive index detector (×8) produced by 20-μL injections. The column and conditions are described in the Experimental Section. Traces A, B, C, and D were produced at time = 0, 2, 6, and 24 h, respectively. Peak 3 = RGU (3), peak 2 = RGU-CHO (2), and peak 1 = 5-AC (1).

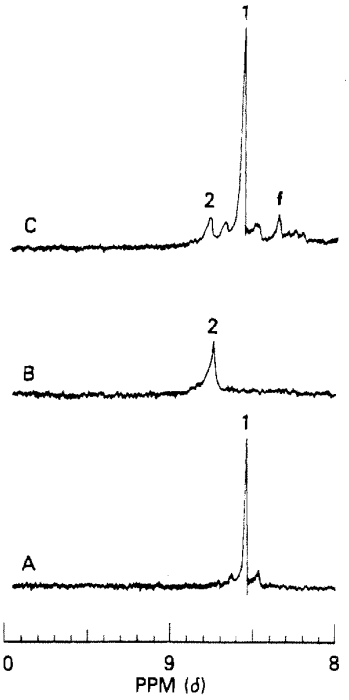


Figure 2. The low-field portions of the proton NMR spectra (Me_2SO-d_6) are shown for pure 5-AC (spectrum A), pure RGU-CHO (spectrum B), and a hydrolysis mixture obtained by lyophilization of a 5-AC solution in distilled water after storage at 25 °C for 24 h (spectrum C). Peak 1 is the C-6 aromatic proton of 5-AC (flanked by spinning side bands). Peak 2 is the formyl proton of RGU-CHO. Peak f is due to the formyl proton of formate anion.

terization and biological testing described in this report.

Examination of the δ 8–10 region of the NMR spectra of compounds 1 and 2 showed characteristic differences (Figure 2). The C-6 aromatic proton of 5-AC (1) produces a sharp singlet at δ 8.60 (Figure 2, spectrum A) which is absent from the spectrum of 2 (spectrum B). Compound 2, however, shows the formyl proton as a broadened singlet (δ 8.80). For purposes of comparison, the analogous proton of formylguanidine[9] in the same solvent (Me_2SO-d_6) was found to exhibit a singlet at δ 8.45.

NMR analysis presented a method to qualitatively corroborate our HPLC analysis of 24-h-old aqueous solutions of 5-AC. For that purpose, a water solution of 1 was stored at room temperature for 24 h, lyophilized, and dissolved in Me_2SO-d_6. The NMR spectrum of the hydrolysis mixture (Figure 2, spectrum C) showed singlets assignable to the formyl proton of 2 and the C-6 proton of 1. The formyl proton of formate anion, which indirectly indicates the presence of the ribosylguanylurea (3), appeared at δ 8.39 as a singlet. Incremental addition of formic acid caused a corresponding stepwise strengthening of the singlet with concomitant stepwise high-field shifts. The formyl proton of ammonium formate (δ 8.43, Me_2SO-d_6) exhibited the same behavior in the NMR with incrementally added formic acid. Using ^{13}C NMR, Israili et al.[10] also found evidence for formate in a similar preparation of 5-AC.

Chromatographically pure 2, run as soon as possible after making a water solution, gave a maximum in the UV at 238 nm (ε 18700). After 1 h the extinction coefficient decreased to 15200 with no change in the maximum position. It was reported[6] that 1 in aqueous solution showed an increase in UV absorbance with time from its initial value. The increase continues for several hours at room temperature before a gradual decrease is noted. With UV data for 2 in hand, these observations can now be interpreted. The early hydrolysis product, 2, because of its high extinction coefficient relative to 1 [λ_{max} 241 nm (ε 6800)] augments UV absorbance as the hydrolysis of 1 proceeds. Since the difference in the maxima of pure samples of 1 and 2 is small (3 nm), no apparent shift in maximum is observed during the hydrolysis course. A point in time is reached when 2 in turn is hydrolyzed to 3 (nonchromophoric) at a rate which exceeds the production of 2 from 1. An observed net decrease in UV absorbance then results.

It should be mentioned that as a consequence of nucleophilic attack by water at the 6 position of 1, ring opening could conceivably occur in a manner which would locate the formyl group on the nitrogen atom bearing the ribosyl moiety (N-1) and *not* as indicated in structure 2. However, it can be reasoned that 3, which has no UV absorptivity at wavelengths greater than 225 nm, if substituted at the terminal amidino residue with a formyl group, as in 2, would have the ability to extend the conjugation of the chromophore through enolization of the formyl group. Therefore, the maximum observed at 238 nm supports structure 2. On the other hand, if the formyl group is situated on N-1, enolization is not possible, and the observed bathochromic shift would not be anticipated.

The third peak eluted with water (t_R = 8.35 min) from

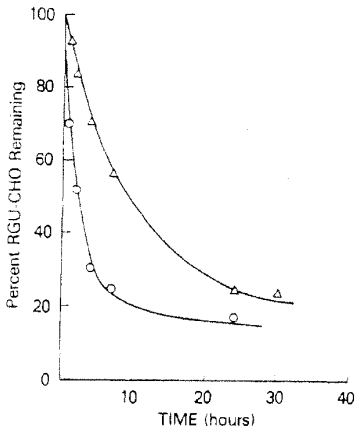


Figure 3. Time course of the disappearance of RGU-CHO in distilled water (2.6 mg/mL) at 37 °C (O-O) and 25 °C (Δ-Δ) as determined by HPLC using a UV detector (linear response in the 2.0 AUFS range used) and the conditions described in the Experimental Section.

the HPLC column was collected, lyophilized, and identified as starting material **1** by melting point, mixture melting point, UV, and NMR.

Hydrolysis Studies. The rate of disappearance of **2** at 25 and 37 °C in water solution was conveniently followed by HPLC using a UV detector having a fixed wavelength of 254 nm. Immediately after dissolving **2** in water a single peak was observed in the chromatogram. After storing the solution in a 37 °C thermostatic bath for 1 h and repeating the HPLC analysis, it was found that the peak area due to **2** had decreased to 70% of the initial value and a peak due to **1** (formed by equilibration from **2**) appeared in the chromatogram. Further aliquots were withdrawn and analyzed at intervals of 2, 4, 7, and 24 h to provide, in part, the data for Figure 3 from which it can be seen that the half-life of **2** in water at 37 °C is about 2 h. At t = 4 h, 31% of **2** remained. Almost all of the loss of **2** up to t = 4 h can be accounted for by its equilibration to **1**. In addition to loss to equilibration, **2** can also react irreversibly with water to form **3**, but very little **3** was evident in the t = 4 h chromatogram (refractive index detector, ×8). Moreover, the sum of the peak areas of **1** and **2** (UV detector), after application of a factor to account for the difference in extinction coefficients at 254 nm (ϵ_{254} RGU-CHO/ϵ_{254} 5-AC = 1.77), was nearly equal to the area of **2** at t = 0. Another interesting property of this hydrolysis system was observed at t = 7 h when 25% of the initial concentration of **2** remained. The peak area ratio of **1** and **2** became essentially constant, and, thereafter, **1** and **2** decreased in a fixed concentration ratio of 1 part of RGU-CHO to 2.43 parts of 5-AC.

In the same way, the hydrolysis of **2** was studied at 25 °C (Figure 3). The half-life occurred at t = 9 h and a constant ratio of **1** and **2** (2.42:1) was reached at t = 24 h when 24% of **2** remained.

For comparison, the hydrolysis of **1**[11] (5 mg/mL) was studied at 25 °C. Samples were withdrawn and analyzed with HPLC at 1, 3, 6, 24, 30, and 48 h. Over these intervals the percent of **1** remaining as determined by peak area comparisons was 96, 89, 82, 63, 58, and 48%, respectively. The half-life was reached after 47 h and a constant ratio of **1** and **2** (2.67:1) was established at t = 24 h when 63% of **1** remained and 24% of **2** had formed by hydrolysis.

Biological Results and Discussion. It was of interest to determine if RGU-CHO has intrinsic antitumor properties, and is the cytotoxic hydrolysis product suggested by tissue culture studies,[5,7] and if RGU-CHO imparts a host toxicity. In order to explore these two areas of enquiry, the in vivo murine L1210 leukemia model was selected to evaluate RGU-CHO biologically in a parallel assay with 5-AC. The inherent difficulty in this biological test is the interconvertibility of 5-AC and RGU-CHO by equilibration in aqueous environments, making the separation of their respective biological effects difficult since pure samples of both compounds in aqueous solution form nearly identical threefold mixtures of **1**, **2**, and **3** after the requisite period of time. Accordingly, samples of 5-AC and RGU-CHO for biological studies were purified by preparative HPLC and were administered to the test mice within 15 min after solution in physiological saline. The dose–response results of the comparative evaluation in the L1210 test system are recorded in Table I. It can be seen that both compounds have considerable antitumor activity with the optimum dose for 5-AC occurring at 20 mg/kg and that for RGU-CHO apparently is >60 mg/kg. However, the activity shown by RGU-CHO could be accounted for by its equilibration to 5-AC if the assumption is made that the findings of the above hydrolysis studies at 37 °C are approximately applicable to the behavior of RGU-CHO in a mouse. A dose of 40 mg/kg of RGU-CHO (% ILS 85) corresponds to a 10 mg/kg dose of 5-AC (% ILS 86), and 20 mg/kg of RGU-CHO (% ILS 56) corresponds to 5 mg/kg of 5-AC (% ILS 63). Therefore, RGU-CHO has one-fourth the potency of 5-AC which was confirmed by a second, identical L1210 experiment. Since it was shown that 30% of RGU-CHO is converted to 5-AC after 1 h at 37 °C, it is reasonable to conclude that RGU-CHO exhibits antitumor activity only by virtue of its ability to generate 5-AC in aqueous systems. This conclusion is strengthened if one considers that 50% of RGU-CHO equilibrates to 5-AC in 2 h at 37 °C, suggesting a potential for 5-AC to exert an even greater influence on the antitumor response following RGU-CHO administration. The observed one-fourth potency actually shown by RGU-CHO might be explained by the interplay of the following possible departures from its behavior in aqueous solution: (a) the rate of 5-AC formation is retarded in vivo, (b) RGU-CHO is more rapidly deformylated in vivo, and (c) RGU-CHO is rapidly excreted relative to 5-AC. In any event, it seems unlikely that RGU-CHO itself has antitumor properties.

Although neither drug at the dose levels tested caused acute toxicity leading to early death, the highest 5-AC dose (40 mg/kg) showed significant indications of drug-related

Table I. Comparison of 5-AC and RGU-CHO against L1210 Leukemia[a]

5-AC				RGU-CHO			
Dose, mg/kg	% ILS[b]	T − C[c] (1-5)	T − C[d] (1-9)	Dose, mg/kg	% ILS[b]	T − C[c] (1-5)	T − C[d] (1-9)
40	84	−2.3	−6.5	60	93	−1.9	−4.3
20	130	−1.6	−5.1	40	85	−0.8	−4.7
10	86	−1.8	−4.5	20	56	−1.9	−3.8
5	63	−1.0	−2.9	10	33	−1.6	−3.4
2.5	33	−1.5	−5.7	5	17	−1.2	−2.6

[a] Drug treatment was on days 1, 5, and 9 after tumor cell implantation. The untreated control group had a mean survival time of 10.1 days. See the Experimental Section for details. [b] Mean percentage increase in life span. A duplicate test gave similar results. [c] The difference of the average body weight change in grams of the test group (T) and the control group (C) as measured on days 1 and 5. [d] The same as in footnote *c* except the weight differences of T and C are those recorded on days 1 and 9.

toxicity in terms of % ILS and T – C values. On the other hand, the highest RGU-CHO dose tested (60 mg/kg) was not accompanied by the same toxic indications. Moreover, weight losses (T – C) due to RGU-CHO treatment were generally less than those recorded for the corresponding 5-AC treated group at the same dose level. Therefore, it can be concluded that RGU-CHO is apparently neither toxic nor antitumor active beyond its ability to equilibrate to 5-AC.

Although traces of additional materials were detected by scrupulous HPLC analysis, the predominant 5-AC hydrolysis products at room temperature and neutral pH are RGU and RGU-CHO. Neither of these hydrolysis products seems to possess the intrinsic cytotoxicity necessary to explain the enhanced cytotoxicity found by microbiological assay[5,7] of "aged" 5-AC solutions. Excluding the presence, in trace amounts, of a very potent cytotoxic hydrolysis product, the microbiological results could be explained by the participation of RGU-CHO as a latent form of 5-AC as the latter is removed from equilibrium by cell uptake and anabolic reaction. In a recent report Presant et al.[13] demonstrated a prolonged residual activity of 5-AC in leukemic mice lasting 1–2 days after administration. Since the drug half-life in mice is relatively short (<6 h), it was suggested that the persistent antileukemic effect was perhaps mediated by a continued availability of a 5-AC metabolite but not by the intact drug. In view of the present findings, it is interesting to speculate that RGU-CHO might reversibly react with macromolecular sulfhydryl, amino, or hydroxyl groups, resulting in a depot form of 5-AC and slowing the irreversible conversion of RGU-CHO to the inactive RGU.

The technique of administering 5-AC by continuous iv infusion offers a clinical advantage, although strict control over 5-AC dosages is operationally difficult to achieve because of hydrolysis reactions. If fresh infusion solutions are prepared every 3–12 h as has been suggested,[4] drug loss to hydrolysis of about 10–25% would occur over that period as shown by the HPLC analyses herein reported. The terminal hydrolysis product, RGU, is slowly formed and is only detectable by HPLC after 6 h. Since RGU has only a moderate toxicity, and has a concentration of <5% in a 5-AC formulation at the end of 12 h, it seems unlikely that it would contribute significantly to clinical toxicity. The initial hydrolysis product, RGU-CHO, while present in substantial concentrations in formulated 5-AC, has no salient untoward biological effects but, rather, may play an important role in the pharmacodynamics of 5-AC.

Experimental Section

Chemical. HPLC was carried out with a Waters Associates, Inc., Model ALC/GPC-244 chromatograph which included the following modular components: a U6K septumless injector, a 440 fixed-wavelength (254 nm) UV detector, a R401 differential refractometer detector, and a 6000A solvent pump. Chromatograms were recorded with an OmniScribe Model A5211-1 two-pen recorder. Both analytical and preparative work was accomplished with a 7 mm (i.d.) × 300 mm μBondapak/C_{18} column (Waters Associates, Inc.) which was eluted with distilled water at 4 mL/min. HPLC fractions were lyophilized with a Virtis Unitrap Model 10-100-C apparatus. A Cary Model 15 spectrophotometer was used to obtain UV spectra and a Perkin-Elmer Model 621 was used to record infrared spectra. Proton NMR spectra were recorded with a Varian HA-100D spectrometer. Chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane which was used as an internal standard. Melting points were determined with a Thomas-Hoover capillary apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. A generous quantity of 5-azacytidine was made available by Dr. H. B. Wood, Jr., Drug Synthesis and Chemistry Branch, NCI.

Hydrolysis Products Isolation. A solution of **1** (192 mg, 0.786 mmol) in distilled water (12 mL) was stored at 24–25 °C for approximately 24 h at which time a 2-mL sample was withdrawn and injected into the HPLC. As detected by the differential refractometer (attenuation ×64), the three eluted peaks (see Figure 1) were collected sequentially, immediately frozen, and lyophilized. The column loaded to this extent (ca. 32 mg) gave a complete separation of **3** from **1** and **2** and a near complete separation of **1** and **2** (valley = 1% of full scale) was achieved.

First Eluted Fraction: 1-β-D-Ribofuranosyl-3-guanylurea (3). The first peak eluted from the HPLC was collected, lyophilized, and pooled with the corresponding fractions from 11 earlier runs to provide a total of 32 mg (0.14 mmol) of **3** as a noncrystalline gum which showed only end absorbance in the UV. A solution of the gum in methanol (0.5 mL) was treated with picric acid (32 mg, 0.14 mmol) in absolute ethanol (0.5 mL). After allowing the solution to crystallize overnight, the picrate of **3** was removed by filtration, washed with cold absolute ethanol (three times), and dried to give 34 mg (52%) of yellow crystals, mp 172–174 °C dec (lit.[6] mp 172–174 °C dec). A mixture melting point with an authentic sample[6,8] was undepressed.

Second Eluted Fraction: *N*-(Formylamidino)-*N'*-β-D-ribofuranosylurea (2). The second peak eluted from the HPLC was collected and lyophilized. Combining the lyophilized fractions from a total of six runs in distilled water (2 mL), and rechromatographing the solution, enabled the separation and collection of pure **2** (19 mg) as a white electrostatic solid after lyophilization: mp 112–114 °C (solidifies and remelts with effervescence at ~140 °C); UV (solutions maintained at 25 °C) λ_{max} (H_2O, t = 0) 238 nm (ε 19 200); UV λ_{max} (H_2O, t = 1 h) 238 nm (ε 16 100); UV λ_{max} (pH 7 buffer, t = 0) 238 nm (ε 18 700); UV λ_{max} (pH 7 buffer, t = 1 h) 238 nm (ε 15 200); IR (Nujol) 1723, 1652, 1602 cm^{-1}; NMR δ (Me_2SO-d_6) 8.80 (s, 1, CHO), 5.14 (d, J = 5 Hz, 1, C_1'H). Anal. Calcd for $C_8H_{14}N_4O_6$ (262.2): C, 36.64; H, 5.38; N, 21.37. Found: C, 36.54; H, 5.40; N, 21.41.

Third Eluted Fraction: 4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one (1). The third peak to elute from the HPLC was collected, frozen, and lyophilized to give 15 mg of a fluffy white solid. Crystallization from ethanol–water (2:1) gave **1** as white needles: mp and mmp 231–232 °C dec; UV (solutions maintained at 25 °C) λ_{max} (H_2O, t = 0) 241 nm (ε 6800); UV λ_{max} (H_2O, t = 1 h) 241 nm (ε 7 700); UV λ_{max} (pH 7 buffer, t = 0) 241 nm (ε 6900); UV λ_{max} (pH 7 buffer, t = 1 h) 241 nm (ε 7800); NMR δ (Me_2SO-d_6) 8.60 (s, 1, C-6), 5.67 (d, J = 4 Hz, 1, C_1'H).

Biological. CDF_1 female mice, 11–12 weeks old, were each inoculated ip (day 0) with 0.1 mL of a L1210 cell suspension (Lockes balanced salt) containing 1.43 × 10^6 cells/mL (Coulter counter). Beginning 24 h after tumor implantation (day 1) drugs (purified by preparative HPLC) were administered within 15 min after dissolution in sterile 0.85% saline by ip injection in volumes of 0.2–0.3 mL. Drug treatment was continued on days 5 and 9. Ten animals were used for testing each dose level of each drug and ten untreated control animals were maintained. The mice were weighed on days 1, 5, and 9 in order to determine the correct dosage for each animal as well as to provide comparative weight gain/loss data for drug toxicity evaluations. The increase in mean life span of the test animals beyond the mean survival time of the untreated control animals expressed as a percentage increase (% ILS) was used to evaluate antitumor activity. A % ILS value of ≥25% was considered indicative of antitumor activity.

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Synthesis and Antitumor Activity of Preactivated Isophosphamide Analogues Bearing Modified Alkylating Functionalities[1]

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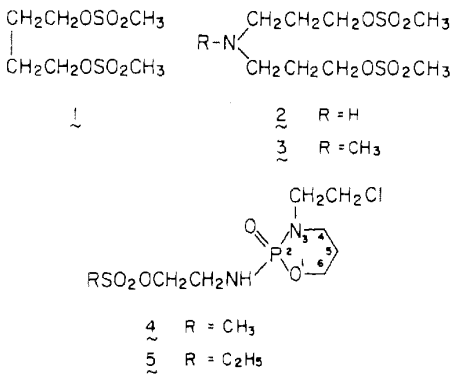
In search of cancer chemotherapeutic agents with greater efficacy than cyclophosphamide, 4-hydroperoxyisophosphamide analogues bearing modified alkylating functionalities such as 2-bromoethyl, 2-iodoethyl, 2-methylsulfonyloxyethyl, and 2-ethylsulfonyloxyethyl groups were prepared by ozonolytic cyclization reaction of N,N′-substituted 3-butenyl phosphorodiamidates. Comparative cytotoxicity against L1210 cells and antileukemic life-span activity against L1210 implanted BDF_1 mice of the newly synthesized compounds were tabulated. The 4-hydroperoxyisophosphamide analogues which have different alkylating groups in a molecule showed slightly greater cytotoxicity in vitro than those with the same alkylating groups. Most of the compounds having different alkylating groups also showed high antileukemic activity in vivo. Among them, the highest efficacy was found for 2-[*N*-methyl-*N*-(2-chloroethyl)]amino-3-(2-methylsulfonyloxyethyl)-4-hydroperoxy-1,3,2-oxazaphosphorinane 2-oxide (NSC 280122D) whose life-span activity was also greater than that of 4-hydroperoxyisophosphamide, cyclophosphamide, and isophosphamide. The superiority of this compound was especially apparent by oral administration.

Busulfan (**1**)[2] is a representative antitumor alkylsulfonate having potential alkylating activity. However, in comparison with nitrogen mustard, which is representative of antitumor alkylating agents bearing 2-chloroethylamino groups and is effective against various kinds of experimental tumors, busulfan exerts only limited activity against Walker-256 carcinosarcoma and is practically ineffective against L1210 leukemia and other animal tumors. In 1964, Sakurai and El-Merzabani[3] synthesized nitrogen-containing methanesulfonates such as **2** and **3** which are structurally related to nitrogen mustard and found that these compounds showed increased activity against some tumors which were unaffected by busulfan.[4,5] In 1974, Brock and his co-workers[6,7] reported on the antitumor activities of a number of cyclophosphamide and isophosphamide analogues bearing alkylsulfonyloxyalkyl and chloroethyl groups. Most of the compounds reported by Brock et al. showed considerable activities against Yoshida ascitic sarcoma in rats, Walker-256 carcinosarcoma in rats, and L1210 leukemia in mice, and they concluded that isophosphamide analogues bearing mixed alkylating functions such as **4** and **5** were especially effective. Recently, we[8,9] synthesized C_4-oxidized cyclophosphamide and isophosphamide derivatives and found that C_4-hydroperoxylation was as effective as C_4-hydroxylation for activating these drugs and that the C_4-hydroperoxides showed greater stability than the corresponding C_4-hydroxy derivatives. The action mechanisms of the compounds reported by Brock et al. are thought to resemble those for isophosphamide (and cyclophosphamide), i.e., the antitumor effect might be exerted after in vivo C_4-oxidation of the 1,3,2-oxazaphosphorinane ring.[10] Therefore, the C_4-hydroperoxy derivative of **4** and **5** might also exert increased activity. We now wish to report on the synthesis and antileukemic activity of C_4-hydroperoxyisophosphamide analogues bearing mixed alkylating functions related to compounds **4** and **5** (Chart I).

Chart I



The ozonolysis reaction of 3-butenyl phosphorodiamidate, which is a general synthetic method for preparing C_4-functionalized 1,3,2-oxazaphosphorinane 2-oxides and related phosphorus-containing heterocyclics,[8,9,11,12] was also used for the present syntheses. A variety of 3-butenyl phosphorodiamidates bearing different alkylating substituents at the phosphorodiamidic nitrogen atoms were prepared via three routes (see Scheme I). Route a consisted of reaction of phosphoryl halide **6** (POX_3, X = Cl or Br) with 3-buten-1-ol followed by treatment with the corresponding N-substituted 2-haloethylamine salt ($XCH_2CH_2NHR \cdot HX$, X = Cl or Br) in the presence of

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APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☐		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	12/01/2011	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 65	Minus	** 91	= 0	X $ =		OR	X $60=	0
	Independent (37 CFR 1.16(h))	* 4	Minus	***4	= 0	X $ =		OR	X $250=	0
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/MINNIE JACKSON/

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/466,213	05/14/2009	Jeffrey B. Etter	9516-847-999	5370

84802 7590 02/28/2012

JONES DAY for Celgene Corporation
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NEW YORK, NY 10017

EXAMINER	
CRANE, LAWRENCE E	
ART UNIT	PAPER NUMBER
1623	
MAIL DATE	DELIVERY MODE
02/28/2012	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)
	12/466,213	ETTER ET AL.
	Examiner	**Art Unit**
	Lawrence E. Crane	1623

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *December 1, 2011 (amendment)*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,3-55,57-61 and 66-71* is/are pending in the application.

4a) Of the above claim(s) *66,68 and 70* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1,3-55,57-61,66,68 and 70* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *14 May 2009* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)	
1) ☒ Notice of References Cited (PTO-892)	4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)	5) ☐ Notice of Informal Patent Application
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *12/01/2011*.	6) ☐ Other: _____.

Claims **2, 56 and 62-65** have been cancelled, claims **1, 3-6, 8, 9, 12-23, 31, 34, 35, 38, 41-55, 57, 69 and 61** have been amended, the Abstract has been amended as requested, the disclosure has been amended as requested, and new claims **66-71** have been added as per the amendment filed December 1, 2011. One additional or supplemental Information Disclosure Statement (1 IDS) filed December 1, 2011 has been received with all cited non-US patent references, annotated, and made of record.

Claims **1, 3-55, 57-61 and 66-71** remain in the case.

Note to applicant: when a rejection refers to a claim **X** at line y, the line number "y" is determined from the claim as previously submitted by applicant in the most recent response including ~~lines deleted by line through~~.

Newly submitted claims **67, 69 and 71** and linking claims **1, 3-55 and 57-61** in so far as they are directed to "decitabine" as an active ingredient, are directed to an invention that is independent or distinct from the invention originally claimed for the following reasons: In the first office action on the merits the claims presented were limited to pharmaceutical compositions and methods of treatment wherein 5-azacytidine was the sole active ingredient. Therefore, the prior art search conducted was limited to prior art disclosing this ingredient, with the consequence that no search was conducted directed to decitabine or the presence thereof in either a pharmaceutical composition or as the active ingredient in a medicinal method of treatment.

Since applicant has received an action on the merits for the originally presented invention, this invention has been constructively elected by original presentation for prosecution on the merits. Accordingly claims **67, 69 and 71**, and linking claims **1, 3-55 and 57-61** in so far as they are directed to "decitabine" as an active ingredient, have been withdrawn from consideration as being directed to a non-elected invention. See 37 C.F.R. §1.142(b) and MPEP §821.03.

Claims **66, 68 and 70**, and claims **1, 3-55, 57-61 and 66-71** in so far as they are directed to "5-azacytidine" as the active ingredient, remain under examination in this application.

Claim **68**, and claims **23-50** in so far as they are directed to "5-azacytidine" as the active ingredient, are rejected under 35 U.S.C. §112, first paragraph, because the specification, while being enabled for the treatment by the administration of 5-azacytidine as the sole active ingredient of a limited number of neoplastic disease conditions (see the non-prospective disclosures in Examples 1-7), does not reasonably provide enablement for the effective treatment of all other "diseases associated with abnormal cell proliferation," including at least cancers of the pancreas (instant Example 11 is entirely prospective), liver and brain wherein 5-azacytidine is administered in combination with an un-named second active ingredient (see claim **31**). The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

The fundamental issue here is whether practicing the full scope of the instant invention is possible without undue experimentation. As provided for in *In re Wands* (858 F.2d 731, 737; 8 USPQ 2d 1400, 1404 (Fed Cir. 1988) the minimum factors to be considered in determination of whether a conclusion of "undue experimentation" is appropriate are as follows:

A. The breadth of the claims: Instant claim **23** is directed to the treatment of " ... all diseases associated with abnormal cell proliferation," a scope of coverage which includes the treatment of many neoplastic and non-neoplastic disease conditions the treatment of which has not been enabled by the instant disclosed exemplifications.

B. The nature of the claimed subject matter: The instant invention is directed to the treatment of all " ... diseases associated with abnormal cell proliferation" (see claim **23**) in a host in need thereof by the administration of an effective dosage of 5-azacytidine.

C. The state of the prior art: The treatment of neoplastic diseases by the administration of an effective amount of 5-azacytidine" to a host in need thereof is very well known in the art as disclosed and taught by PTO-892 references **R**, **S**, and **T**.

D. The level of one or ordinary skill: The factor is not important in the instant analysis.

E. The level of predictability in the art: In view of the substantial and relevant teachings of both PTO-1449 and PTO-892 cited documents presently of record, teachings which disclose that 5-azacytidine has neoplastic activity. The substantial amount of highly relevant and on

point prior art already of record supports the view that this art area is at least somewhat predictable.

F. The amount of direction provided by the inventor: As noted above the instant, non-prospective exemplifications appear to end at Example 7. The small number of instant exemplifications suggests that the instant disclosure has enabled only a very limited number of disease treatments wherein 5-azacytidine is administered as the active ingredient.

G. The existence of working examples: This factor is dealt within the previous paragraph.

H. The quantity of experimentation needed to make or use the invention based on the content of the disclosure has been found to be excessive for the reasons noted above, reasons effectively summarized as an insufficient number of relevant exemplifications to adequately support the scope of subject matter presently being claimed.

Applicant's arguments filed December 1, 2011 have been fully considered but they are not persuasive.

Beginning at page 15 of the instant response applicant has argued based on citations of a number of previous judicial decisions that a reason must be provided in order to establish doubt concerning whether the instant claimed subject matter is adequately enabled. Examiner respectfully disagrees because the citations provided are not found in the relevant portion of the MPEP (MPEP §§ 2107.01-2107.03) wherein the enhanced standards for analysis of enablement in medicinal applications has been disclosed. In particular examiner respectfully requests applicant is consider one of the cited precedents therein, *Ex parte Balzarini et al.* 21, USPQ 2d 1892, 1894 (BPAI, 1991), a decision which in its first opinion stands for the proposition that claims directed to medicinal treatments of diseases in highly unpredictable art areas are properly rejected under 35 U.S.C. §112, first paragraph as lacking adequate enablement, in the absence of sufficient test data in support of the efficacy of the alleged treatment. It is examiner's understanding that the treatment of "cancers," while less unpredictable than in the past, remains unpredictable with regard to the treatment of the particular subsets of neoplastic disease noted hereinabove. In addition, the terms "abnormal cell proliferation" and "hematological disorder" are generic to both neoplastic and non-

neoplastic diseases (e.g. psoriasis, non-leukemic blood disorders, etc.), and therefore the scope of the noted claims clearly extends into areas of medicinal treatment not enabled by the non-prospective embodiments found in the instant disclosure. Additionally applicant has argued that the proposed extrapolation beyond the specific embodiments is justified by what applicant alleges, without literature support, to be a relevant prior art teaching, namely that 5-azacytidine is an inhibitor of the enzyme DNA methyltransferase. In the absence of an adequate showing of supporting prior art, and in view of the very limited number of specific embodiments, examiner does not find this argument to be any more that speculation asserted to be supportive of applicant's arguments. And lastly applicant argues that the experimentation required would only be routine, and that "***no*** undue experimentation by one of ordinary skill in the art" (emphasis in original) is needed. This argument appears to be another attempt to rely on a standard of enablement not applicable in the instant at area. Applicant is again referred to the MPEP at §§2107.01-2107.03 for a recapitulation of the enhanced standard of enablement that applies herein. For the above stated reasons the above rejection has been found to remain valid and therefore has been repeated.

Claims **66, 68 and 70**, and claims **1, 3-55, 57-61 and 66-71** in so far as they are directed to "5-azacytidine" as the active ingredient, are rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

In view of the above limitation of claimed subject matter to claims wherein "5-azacytidine" is the sole active ingredient, the term "decitabine" is respectfully requested be deleted from any of claims **1, 3-55 and 57-61** wherein it now appears. Similarly, because reference to "cytidine analog" has now become superfluous, an amendment deleting this term as well is respectfully requested.

Applicant's arguments with respect to claims **1-65** have been considered but are moot in view of the new grounds of rejection. This new ground of rejection was necessitated by applicant's amendments.

In claim **1** the term "at least one pharmaceutically acceptable excipient" appears to be inconsistent with the terms "immediate release tablet" and "immediate release capsule" because the included term "immediate release" implies the presence of "more than one

excipient." In addition the claim is incomplete because the entire contents of the "tablet" and the "capsule" have not been defined in the claim.

Applicant's arguments with respect to claims **1-65** have been considered but are moot in view of the new grounds of rejection. This new ground of rejection was necessitated by applicant's amendments.

In claim **6** the term "excipient" and the following Markush group are grammatically inconsistent. Did applicant intend the noted term to read -- the at least one excipient -- or the like? The suggested correction would also correct what appears to be improper dependence from claim **1**. See also claims **41 and 61** wherein the same issue or a very closely related issue reoccurs.

Applicant's arguments with respect to claims **1-65** have been considered but are moot in view of the new grounds of rejection. This new ground of rejection was necessitated by applicant's amendments.

In claims **16-22**, the term "achieves" is grammatically erroneous. Did applicant intend the term to read -- achieve --?

Applicant's arguments with respect to claims **1-65** have been considered but are moot in view of the new grounds of rejection. This new ground of rejection was necessitated by applicant's amendments.

Claim **30** is objected to under 37 C.F.R. §1.75(c), as being of improper dependent form for failing to further limit the subject matter of a previous claim. Applicant is required to cancel the claim, or amend the claim to place the claim in proper dependent form, or rewrite the claim in independent form.

Applicant's arguments with respect to claims **1-65** have been considered but are moot in view of the new grounds of objection.

In claim **12** the term "an additional therapeutic agent" renders the claim incomplete because the identity of the intended additional agent(s) has/have not been provided in the claim and also because the noted term lacks adequately defined metes and bounds. In addition, this

claim is superfluous in view of the term "comprising" in claim **1**, a term of art which means that any pharmaceutical composition claim invented by another which comprises 5-azacytidine and any other active ingredient would infringe the instant claim. Examiner respectfully suggests cancellation is one possible solution. See also claims **31 and 57** wherein the same issue reoccurs.

Applicant's arguments filed December 1, 2011 have been fully considered but they are not persuasive.

Examiner has noted at page 17 applicant's very brief and generic response to rejections under 35 U.S.C. § 112, second paragraph. Because said response is not specific to the instant claim, no further argument in response is possible.

In claim **23** at line 1-2, the term "a disease associated with abnormal cell proliferation" is indefinite because the disease conditions to be treated have not been specified with adequate particularity. Examiner also notes that there are non-neoplastic diseases (e.g. psoriasis) which appear to be inappropriately included within the scope of the noted term. See also claim **51** wherein the same issue reoccurs.

Applicant's arguments filed December 1, 2011 have been fully considered but they are not persuasive.

Examiner has noted at page 17 applicant's very brief and generic response to rejections under 35 U.S.C. § 112, second paragraph. Because said response is not specific to the instant claim, no further argument in response is possible.

The following is a quotation of 35 U.S.C. §103(**a**) which forms the basis for all obviousness rejections set forth in this Office action:

> "A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made."

Claims **66, 68 and 70**, and claims **1, 3-55, 57-61 and 66-71** in so far as they are directed to "5-azacytidine" as the active ingredient, are rejected under 35 U.S.C. §103(**a**) as being

unpatentable over **Redkar et al. '046** (PTO-892 ref. **B**: US **2006/0074046**) in view of **Dintaman et al.** (PTO-892 ref. **R**) and further in view of **Sands et al. '263** (PTO-1449 ref. **A09**: US **2004/01652263**).

The instant claims are directed to pharmaceutical compositions comprising 5-azacytidine, an optional penetration enhancer, and various other notoriously well known in the art excipients, and to a method of treating various neoplastic diseases as part of the indefinite genus of "diseases associated with abnormal cell proliferation" (MDS, AML non-small cell lung cancer, ovarian cancer, pancreatic cancer, psoriasis, etc.) therewith.

Redkar et al. '046 discloses at paragraphs **[0003]** and **[0011]**, that 5-azacytidine and salts thereof (paragraph **[0031]**) are active ingredients, that the pharmaceutical compositions thereof are known (paragraphs **[0175]** to **[0182]**), and that said compositions are effective following oral administration in the treatment of neoplastic diseases (Abstract, paragraphs **[0031]** to **[0034]**, and **[0106]**). At paragraph **[0009]** the **'046** reference teaches that both the aqueous solution instability plus the low water solubility of 5-azacytidine has made administration of aqueous solution thereof problematic, difficulties apparently effectively reversed by substitution of acid salts thereof. At paragraph **[0121]** the **'046** reference teaches that strong acid salts of 5-azacytidine have been found to have improved solubility and to be more stable, apparently because of ion pair formation as taught in paragraphs **[0107]** and **[0117]**. And at paragraphs **[0181]** and **[0182]** the **'046** reference teaches the co-administration of 5-azacytidine with numerous generic classes of excipients and carriers, and also with a second active ingredient.

Although **Redkar et al. '046** does disclose many varieties of carriers and excipients as part of 5-azacytidine-containing tablets, this reference does not expressly disclose pharmaceutical compositions comprising a "permeation enhancer" including the vitamin E derivative TPGS, limitations only found in instant claims **7-9 and 33-35**, or the administration thereof to a host in need thereof.

Dintaman et al. discloses the vitamin E derivative TPGS and further discloses its mechanism of action as an inhibitor of P-glycoproteins known to be part of chemotherapy failures due to multidrug resistance. Reversal of this multidrug resistance effect is illustrated

with pharmaceutical compositions comprising a known antibiotic and a known antineoplastic agent plus TPGS.

Sands et al. '263 discloses pharmaceutical compositions comprising 5-azacytidine at paragraph **[0029]**, and carriers useful therein at paragraphs **[0057]** to **[0059]** including the carriers specified in instant claim **6**. This reference also discloses at paragraph **[0038]** that the pharmaceutical compositions include the capability to rapidly dissolve in mildly acidic solutions. In addition at paragraph **[0065]** this reference also teaches that the pharmaceutical compositions may also include substances which can "enhance the therapeutic efficacy" of the composition. And finally the **'263** reference teaches at paragraphs **[0056]** -**[0061]** that multiple different excipients may be combined with 5-azacytidine in the manufacture of tablets or capsules, and wherein said tablets may be prepared without an enteric coating (see paragraph **[0062]**), an alternative that appears to be an equivalent to the instant "immediate release" type of solid dosage vehicle claimed herein.

It would have been obvious to a person of ordinary skill in the art at the time the invention was made to add the instant claims excipients or carriers to the pharmaceutical compositions of the **Redkar et al. '046** reference because the **Redkar et al. '046** reference provides teachings which permits or suggests that such excipients are part of the disclosure therein. The **'046** reference also teaches the advantages of increased solubility and stability associated with substitution of strong acid salts of 5-azacytidine for the neutral compound, a teaching which appears to render obvious the instant "immediate release" approach to the oral administration of 5-azacytidine tablets and capsules, a variation which implies the rapid conversion the the active ingredient, 5-azacytidine, into its apparently very stable and very water soluble strong acid salt in the stomach of the host being treated. In addition the disclosures of the **Sands et al. '263** reference provide broad coverage for alternative excipients including the specific excipients claimed herein, including compounds like the compound TPGS disclosed in the **Dintaman et al.** reference.

One having ordinary skill in the art would have been motivated to combine these references because all three references are directed to pharmaceutical compositions and the administration thereof to treat neoplastic diseases, including the treatment of MDS, by the administration of either 5-azacytidine, as disclosed in the two cited U. S. patent publications, or alternative medicinal agents as disclosed by **Dintaman et al.**

Therefore, the instant claimed method of treating " ... diseases associated with abnormal cell proliferation," including neoplastic diseases, by the oral administration of 5-azacytidine as the active ingredient, and optionally with TPGS as a permeation enhancer, in an "immediate release" pharmaceutical composition to a host in need thereof would have been obvious to one of ordinary skill in the art having the above cited reference before him at the time the invention was made.

Applicant's arguments filed December 1, 2011 have been fully considered but they are not persuasive.

Examiner has reviewed the previous Office action, applicant's arguments in response, and the extensive additional prior art provided. Applicant has argued as if the above rejection has alleged anticipation, when it has in fact alleged obviousness. In view of this conclusion examiner respectfully disagrees with the thrust of applicant's arguments.

Examiner has carefully reviewed the prior art of record cited above and has amended the rejection accordingly in an attempt to increase the clarity of, and therefore the strength of, the rejection. Because said amendments have cited several additional portions of the prior art documents, it is examiner's view that the above rejection now appears to have addressed all of applicant's concerns. Examiner has also concluded that the rejection remains valid and for this reason has been repeated.

Applicant's amendment necessitated the new grounds of rejection. Accordingly, **THIS ACTION IS MADE FINAL**. Applicant is reminded of the extension of time policy as set forth in 37 C.F.R. §1.136(a).

A shortened statutory period for response to this final action is set to expire THREE MONTHS from the date of this action. In the event a first response is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 C.F.R. §1.136(a) will be calculated from the mailing date of the advisory action. In no event will the statutory period for response expire later than SIX MONTHS from the date of this final action.

Papers related to this application may be submitted to Group 1600 via facsimile transmission (FAX). The transmission of such papers must conform with the notice published in the Official Gazette (1096 OG 30, November 15, 1989). The telephone number to FAX (unofficially) directly to Examiner's computer is 571-273-0651. The telephone number for sending an Official FAX to the PTO is 571-273-8300.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Examiner L. E. Crane whose telephone number is **571-272-0651**. The examiner can normally be reached between 9:30 AM and 5:00 PM, Monday through Friday.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ms. S. Anna Jiang, can be reached at **571-272-0627**.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group 1600 receptionist whose telephone number is **571-272-1600**.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status Information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see < http://pair-direct.uspto.gov >. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at **866-217-9197** (toll-free).

LECrane:lec
02/24/2012

/Lawrence E. Crane/

Primary Examiner, Art Unit 1623

L. E. Crane
Primary Patent Examiner
Technology Center 1600

Notice of References Cited	Application/Control No. 12/466,213	Applicant(s)/Patent Under Reexamination ETTER ET AL.	
	Examiner Lawrence E. Crane	Art Unit 1623	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-2007/0190022 A1	08-2007	Bacopoulos et al.	424/085.1
*	B	US-2006/0074046 A1	04-2006	Redkar et al.	514/049
*	C	US-7,189,740 B2	03-2007	Zeldis, Jerome B.	514/323
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
*	U	(R) Dintaman et al., "Inhibition of P-Glycoprotein by D-[alpha]-Tocopherol Polyethylene Glycol 1000 Succinate," Pharmaceutical Research, 16(10), 1550-1556 (1999).
*	V	(S) Beers et al. (eds.), Chapter 142, Section 11, in The Merck Manual of Diagnosis and Therapy, 18th Edition, Merck & Co., Inc., Rahway, NJ, January, 2006, only title pages and text pages 1114-1116 supplied.
*	W	(T) O'Neil et al. (eds.), "The Merck Index, 14th Edition," Merck & Co., Whitehouse Station, NJ, 2006, only title pages and p. 150 supplied (see Entry 890, "Azacitidine").
	X	(U) Silverman et al., "Randomized Controlled Trial of Azacytidine in Patients With the Myelodysplastic Syndrome: A Study of the Cancer and Leukemia Group B," Journal of Clinical Oncology, 20(10), 2429-2440 (May 15, 2002).

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Randomized Controlled Trial of Azacitidine in Patients With the Myelodysplastic Syndrome: A Study of the Cancer and Leukemia Group B

By Lewis R. Silverman, Erin P. Demakos, Bercedis L. Peterson, Alice B. Kornblith, Jimmie C. Holland, Rosalie Odchimar-Reissig, Richard M. Stone, Douglas Nelson, Bayard L. Powell, Carlos M. DeCastro, John Ellerton, Richard A. Larson, Charles A. Schiffer, and James F. Holland

Purpose: **Patients with high-risk myelodysplastic syndrome (MDS) have high mortality from bone marrow failure or transformation to acute leukemia. Supportive care is standard therapy. We previously reported that azacitidine (Aza C) was active in patients with high-risk MDS.**

Patients and Methods: **A randomized controlled trial was undertaken in 191 patients with MDS to compare Aza C (75 mg/m^2/d subcutaneously for 7 days every 28 days) with supportive care. MDS was defined by French-American-British criteria. New rigorous response criteria were applied. Both arms received transfusions and antibiotics as required. Patients in the supportive care arm whose disease worsened were permitted to cross over to Aza C.**

Results: **Responses occurred in 60% of patients on the Aza C arm (7% complete response, 16% partial response, 37% improved) compared with 5% (improved) receiving supportive care (P < .001). Median time to leukemic transformation or death was 21 months for Aza C versus 13 months for supportive care (P = .007). Transformation to acute myelogenous leukemia occurred as the first event in 15% of patients on the Aza C arm and in 38% receiving supportive care (P = .001). Eliminating the confounding effect of early cross-over to Aza C, a landmark analysis after 6 months showed median survival of an additional 18 months for Aza C and 11 months for supportive care (P = .03). Quality-of-life assessment found significant major advantages in physical function, symptoms, and psychological state for patients initially randomized to Aza C.**

Conclusion: **Aza C treatment results in significantly higher response rates, improved quality of life, reduced risk of leukemic transformation, and improved survival compared with supportive care. Aza C provides a new treatment option that is superior to supportive care for patients with the MDS subtypes and specific entry criteria treated in this study.**

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MYELODYSPLASTIC syndrome (MDS) represents a heterogeneous hematopoietic disorder in which mature blood cells are derived from an abnormal multipotent progenitor cell. The disease is characterized by morphologic features of dyspoiesis, a hyperproliferative bone marrow, and peripheral-blood cytopenias involving one or more lineages.[1] Refractory anemia (RA) with or without ringed sideroblasts can persist for years, but RA with excess blasts (RAEBs) or RAEBs in transformation to leukemia (RAEB-T) exhibit an accelerated course.[2-5] Most patients with high-risk MDS (ie, French-American-British [FAB] subtypes with excess blasts) die within 1 year from progressive bone marrow failure attributable to hemorrhage or infection. In 35% to 40% of patients, transformation to acute leukemia occurs, which is often refractory to present therapy.[1]

Therapies tried for MDS include granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin, and chemotherapy.[6-22] None has altered the natural history of the disease. Supportive care with antibiotics and transfusions is considered the standard of care. Allogeneic bone marrow transplantation, a potentially curative approach, is a realistic option for only approximately 5% of patients.[23-28]

Azacitidine (Aza C), a pyrimidine nucleoside analog, was developed as an antitumor agent.[29-31] In addition to cytotoxic effects, it induces differentiation of malignant cells in vitro.[32-35] Aza C inhibits DNA methyltransferase, the enzyme in mammalian cells responsible for methylating newly

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Table 1. Additional Eligibility Criteria for Bone Marrow Dysfunction in Patients With RA and RARS*

RBC	Symptomatic anemia requiring RBC transfusions for at least 3 months before study entry
Platelets	Thrombocytopenia with two or more platelet counts $\leq 50 \times 10^9$/L or a significant clinical hemorrhage requiring platelet transfusions
WBC	Neutropenia with an ANC $< 1 \times 10^9$/L and an infection requiring intravenous antibiotics

*Patients had to meet at least one of these criteria.

Table 2. Eligibility Criteria

- Age > 15 years
- Life expectancy ≥ 2 months
- Performance status ≤ 2 (NCI scale, 0-4)
- Total bilirubin ≤ 1.5 × ULN
- AST/ALT ≤ 2 × ULN
- Serum creatinine ≤ 1.5 × ULN
- Serum CO_2 ≥ 19 mEq/L
- No previous treatment for MDS with Aza C, G-CSF, GM-CSF, or other hematopoietic cytokines (except for erythropoietin)
- No erythropoietin, corticosteroids, interferon, or retinoids within 1 month before study
- No prior history of leukemia
- No pregnancy or uncontrolled congestive heart failure

Abbreviations: NCI, National Cancer Institute; ULN, upper limit of normal.

synthesized DNA, resulting in synthesis of hypomethylated DNA and changes in gene transcription and expression.[32-34] In patients with beta-thalassemia or sickle-cell anemia, Aza C caused hypomethylation of the gamma globin chain gene with increased production of fetal hemoglobin.[36-38]

The Cancer and Leukemia Group B (CALGB) conducted a phase II study of Aza C administered to 43 hospitalized patients as a continuous intravenous infusion for 7 days every 28 days for 4 months.[39] Responses (complete response [CR], partial response [PR], or improved) occurred in 49% of patients with high-risk MDS (RAEB and RAEB-T). A second phase II study of 67 patients with high-risk MDS showed that Aza C as a subcutaneous daily bolus injection at the same dose and schedule on an ambulatory basis produced comparable results in response rate, response duration, and survival.[40] The present phase III randomized trial compares subcutaneous Aza C treatment with supportive care.

PATIENTS AND METHODS

Patient Selection

All patients fulfilled the FAB classification criteria for MDS.[41-43] Patients with RA or RA with ringed sideroblasts (RARS) met additional criteria of significant marrow dysfunction (Table 1). Bone marrow aspiration and biopsy were required within the 2 weeks before registration. Peripheral-blood films and marrow specimens were independently evaluated through centralized pathology review (D.N.).

Patients with therapy-related MDS were eligible if they were cancer-free for at least 3 years and had not received radiation or chemotherapy for 6 months. Additional eligibility requirements are listed in Table 2. The protocol was approved by the institutional review boards of all participating institutions. Each patient provided written informed consent.

Treatment Regimen

Patients were stratified by FAB subtype and randomly assigned to supportive care or Aza C. The use of all hematopoietic growth factors was prohibited. Transfusions and antibiotics were administered as required. Marrow samples were obtained before study entry, at day 57, and at day 113.

Aza C arm. Aza C (75 mg/m^2/d) was injected subcutaneously in 7-day cycles beginning on days 1, 29, 57, and 85. Aza C, supplied by the National Cancer Institute (Bethesda, MD) in vials of 100 mg of powder plus 100 mg of mannitol, was suspended in 4 mL of sterile water and injected as a slurry with a maximum volume of 4 mL per injection site. If a beneficial effect was not demonstrated by day 57 and no significant toxicity other than nausea or vomiting had occurred, the dose of Aza C was increased by 33%. Once benefit occurred on a particular dosage, Aza C was continued unless toxicity developed. Patients were assessed after the fourth cycle. Those who achieved CR continued on Aza C for three more cycles; those with PR or improvement continued on Aza C until either CR or relapse occurred. Responses were initially evaluated by the treating physician but subsequently were scored independently by two experienced investigators (L.R.S. and R.M.S.) to validate responses. Patients who progressed (see Definitions, below) during the induction phase and those with stable disease at day 113 were classified treatment failures and removed from treatment.

Supportive care arm. After a minimum interval of 4 months of supportive care, patients whose disease was worsening (see Definitions, below) were permitted to cross over to Aza C treatment. Patients could exit supportive care before 4 months but only because of death, withdrawal of consent, transformation to acute leukemia, or a platelet count persistently less than 20×10^9/L after week 8. Patients transforming to acute myelogenous leukemia (AML) exited at any time; those with less than or equal to 40% blasts in the marrow crossed over to Aza C, whereas those with greater than 40% blasts received other treatments.

Cross-over. All data necessary to establish eligibility for cross-over from supportive care to Aza C were independently reviewed by the study chair, whose prior approval was required before cross-over (n = 46 of 49). Cross-over patients were studied and treated identically to patients initially randomized to Aza C.

Quality-of-life assessment. Quality of life (QOL), an integral component of the study, was systematically assessed during standard telephone interviews by one of two trained nurses (E.P.D. or R.O.R.) before randomization and on days 50, 106, and 182. The QOL battery included measures of four dimensions: physical symptoms and functioning, psychological state, social functioning, and sociodemographic characteristics. The questionnaire consisted of two validated scales, the European Organization for Research and Treatment of Cancer (EORTC) QOL and the Mental Health Inventory (MHI). Questionnaires were given or mailed to patients before the telephone interviews; the interview methodologies have been validated in prior CALGB studies.[44]

Table 3. Response Criteria

Trilineage response	≥ 50% restitution of the initial deficit from normal in all three peripheral-blood cell counts and elimination of all blood transfusion requirements		
Monolineage or bilineage response	≥ 50% restitution of the initial deficit from normal in one or two peripheral blood cell counts		
	CR	PR*	Improved†
Bone marrow	M_0 or M_1	≤ 50% of initial bone marrow blasts	—
Peripheral blood			
Counts	H_0	Trilineage response	Monolineage or bilineage response
Blasts	0	0	—
Transfusion	0	0	≤ 50% of baseline
Relapse	> 5% bone marrow blasts	>30% bone marrow blasts	Return to pretreatment blood values or return of RBC or platelet transfusion requirement§
	or		
	Return to pretreatment blood values or return of RBC or platelet transfusion requirement‡		

Abbreviations: M_0, normal bone marrow; M_1, < 5% blasts in the bone marrow, some dyshematopoietic features may persist; H_0, complete normalization of the peripheral-blood counts (ie, hemoglobin ≥ 133 g/L [males], hemoglobin ≥ 117 g/L [females]; WBC ≥ 4.4×10^9/L; ANC 1.8×10^9/L platelets ≥ 140×10^9/L).

*Peripheral-blood criteria alone were used for patients with RA and RARS.

†Criteria for improvement are satisfied by either monolineage or bilineage response or ≥ 50% decrease in transfusion requirement from baseline.

‡For patients with RA or RARS, relapse could be defined on peripheral-blood criteria alone.

§Changes in blood counts secondary to drug-induced myelosuppression did not constitute criteria for relapse.

Definitions

Response criteria are outlined in Table 3.

Relapse of responders. Relapse from CR was defined as greater than 5% myeloblasts in the bone marrow. Relapse from a PR was defined as ≥ 30% myeloblasts in the bone marrow (except for patients with RA and RARS, where peripheral-blood criteria alone or in conjunction with the bone marrow were used). Relapse for improved patients was defined as a decline to pretreatment levels in the blood counts, which were the criteria for response, or the recurrence of a transfusion requirement secondary to disease progression. Reversible changes in blood counts secondary to drug-induced myelosuppression did not constitute criteria for relapse.

Treatment failure in nonresponders. Treatment was considered to have failed in nonresponders receiving supportive care if they advanced to a higher FAB subtype (ie, to RAEB or RAEB-T) or to AML, remained RBC transfusion–dependent before and during study, or developed progressive bone marrow failure, defined as the following: (1) confirmed fall from baseline of greater than 25% in all three peripheral-blood cell lines or greater than 50% fall in two cell lines or a greater than 75% fall in one cell lineage or (2) development of a RBC transfusion requirement (ie, in patients not receiving RBC transfusions before study entry, if the hemoglobin fell to < 9 g/L in patients > 65 years of age or to ≤ 8 g/L if ≤ 65 years of age). Supportive care treatment was also considered to have failed if patients had a platelet count persistently lower than 20×10^9/L after week 8 (N = 9). Nonresponders taking Aza C were evaluated identically for treatment failure, and when treatment failure was present, these patients exited protocol study but were followed for survival.

Statistical Methods

Four analyses, three interim and one final, were planned using O'Brien-Fleming stopping rules. The first three analyses found a significant difference in response between the arms (undisclosed information), but the Data and Safety Monitoring Board recommended continuation of the study so that QOL, survival, and transfusion requirements could be studied in a larger sample. Twenty-six percent of the patient records were independently audited by the CALGB Data Audit Committee for protocol compliance and data quality.

Analyses were performed on an intention-to-treat basis. Patients (n = 20) determined by central pathology review to have acute leukemia at entry were noninformative for AML transformation and the time-to-treatment failure analyses. Response rates of the randomized arms were compared with the χ^2 test of proportions. Survival, time to response, and response duration were estimated with the Kaplan-Meier method and compared with the log-rank test.[45,46] In testing for differences in survival and time to transformation to AML, randomized induction treatments were compared and cross-over was ignored. The two-stage statistical methodology recommended by Gelman et al[47] was used in analyzing the time to AML to account for the competing risk of death.

Prestudy RBC transfusion requirements (present/absent) were calculated. RBC transfusion data were standardized to the number of units per month and the means across time. Differences in transfusion requirements could have been influenced by the loss of patients because of death, cross-over, and dropout (attrition bias) and by physician discretion in the administration of transfusions.

Times to initial response and to best response were measured from study entry to the date that initial and best response criteria were met, respectively. Duration of response was measured from initial response to relapse. Time to treatment failure was measured from study entry to the point of relapse (for responders) or failure (for nonresponders). The time from study entry to transformation to AML or death was chosen as the most meaningful clinical end point, because survival and QOL decline rapidly for patients with MDS after AML develops.

QOL Analysis

A pattern-mixture model was used to examine treatment differences in QOL over time.[48-51]

Table 4. Demographic and Clinical Characteristics at Study Entry

	Aza C		Supportive Care		Total	
	No. of Patients	%	No. of Patients	%	No. of Patients	%
Randomized	99	52	92	48	191	
FAB classification						
RA	17	17	20	22	37	19
RARS	5	5	3	3	8	4
RAEB	32	32	34	37	66	35
RAEB-T	27	27	18	20	45	24
CMMoL	7	7	7	8	14	7
Other*	11	11	10	11	21	11
IPSS risk group†						
Low	2	2	5	6	7	9
Intermediate-1	21	26	16	20	37	45
Intermediate-2	9	11	13	16	22	27
High	7	9	8	10	15	19
Age						
Median	69		67		68	
Range	31-92		35-88		31-92	
Sex						
Male	72	73	60	65	132	69
Female	27	27	32	35	59	31
Prior radiation therapy	8	8	3	1	11	6
Prior chemotherapy	15	15	12	13	27	14
Prior treatment for MDS	16	16	17	18	33	17
Infection requiring treatment	6	6	4	4	10	5
Active bleeding	16	16	18	20	34	18
Patients requiring platelet transfusions‡	18	18	10	11	28	15
Patients requiring RBC transfusions‡	68	69	56	61	124	65
Time from diagnosis to study entry						
Median	77 days		87 days			
Range	1 day-6.4 years		2 days-6 years			

Abbreviation: IPSS, International Prognostic Scoring System.

*Includes 19 AML, one unclassifiable acute leukemia, and one undefined MDS.

†Complete cytogenetic data to determine the IPSS score were only available for 81 patients.

‡During the 3 months preceding study entry.

RESULTS

Patient Characteristics

One hundred ninety-one patients with MDS deemed eligible by treating investigators were entered on CALGB 9221 between February 1994 and May 1996 from 26 main member institutions and their 30 affiliated hospitals. Response and toxicity were analyzed on data available through February 1999. After central pathology review, 20 patients were determined to have AML at study entry. These patients are excluded only from the AML transformation and time to progression analyses. The conclusions were unchanged if these patients were excluded from all analyses (data not shown).

The two arms were evenly balanced at study entry (Table 4). There were no differences by FAB subtype, cytogenetic analysis (n = 81), International Prognostic Scoring System score,[52] or time from diagnosis to study entry. Hematologic parameters at study registration are described in Table 5.

Analysis of Response

Among patients randomized to supportive care, 5% (n = 5) met the criteria for improvement. No patient achieved a CR or PR (Table 6). All five patients categorized as improved either had a rising WBC count or absolute neutrophil count (n = 4) or platelets (n = 1) in the process of transforming from MDS to AML. On the Aza C arm, 60% (n = 60) responded ($P < .0001$), with 7% (n = 7) achieving CR, 16% (n = 16) having PR, and 37% (n = 37) improving. In no case was improvement of cytopenia a component of transformation to AML. The trilineage response was 23% for Aza C and 0% for supportive care. Among the 37 Aza C patients categorized as improved, 35% had increases in all three cell lines (but insufficient to meet criteria for PR), 30% had increases in two cell lines, and 35% had an increase in only a single cell line (Fig 1). Response to Aza C was independent of MDS classification. Responses for patients with RA and RARS (9% CR [n = 2];

Table 5. Hematologic Parameters at Study Entry

	Aza C	Supportive Care	Total
Hemoglobin,* g/L			
Median	90	93	91
Range	53-140	57-140	53-140
WBC, × 10⁹/L			
Median	3.6	3.7	3.7
Range	0.7-124.5	0.4-41.2	0.4-124.5
ANC, × 10⁹/L			
Median	1.5	1.7	1.6
Range	0.04-90.9	0.1-27.6	0.04-90.0
Platelets, × 10⁹/L			
Median	52	72	63
Range	4-479	4-570	4-570

Abbreviation: ANC, absolute neutrophil count.
*Median hemoglobin levels at study entry may reflect transfused values.

18% PR [n = 4]; 32% improved [n = 7]) were comparable with patients with RAEB, RAEB-T, and chronic myelomonocytic leukemia (CMMoL) (8% CR [n = 5], 15% PR [n = 10], 38% improved [n = 25]) among patients classified according to central pathology review (Table 4). Median times to initial response and best response were 64 and 93 days, respectively. The median duration of response among patients who achieved CR, PR, or improvement was 15 months (95% confidence interval [CI], 11 to 20 months) (Fig 2).

Of 49 patients who crossed over from supportive care to Aza C, 47% (n = 23) then responded, with 10% (five patients) achieving CR, 4% (two patients) having PR, and 33% (16 patients) improving. The trilineage response was 14%. Neither age nor sex influenced response rates.

Time to Treatment Failure

With certain exceptions (see above), the study design intended that patients remain on the initial randomization arm for a minimum of 4 months. The median time to exit from supportive care (ie, median time to treatment failure) was 3.8 months (95% CI, 3.5 to 4.0 months; range, 0.6 to > 55 months); the median time to exit from the Aza C arm was 9.1 months (95% CI, 5.6 to 11 months; range, 0.1 to > 44 months) ($P < .0001$).

Analysis of Time-to-AML Transformation or Death

The effects of treatment on transformation to AML or death are illustrated in Fig 3. The median time to event for supportive care was 12 months (95% CI, 8 to 15 months) compared with 21 months (95% CI, 16 to 27 months) for Aza C ($P = .007$). For patients with high-risk FAB subtypes (RAEB, RAEBT, or CMMoL), the median time to AML or death for supportive care was 8 months (95% CI, 4 to 13 months) compared with 19 months (95% CI, 13 to 21 months) for Aza C ($P = .004$). There were an insufficient number of events to estimate medians in the patients with low-risk FAB. Overall, FAB subtype was a significant predictor of time to AML or death ($P = .0003$).

Transformation to AML occurred as the first event in 15% of the patients randomized to Aza C compared with 38% of patients randomized to supportive care ($P = .001$). Indeed, during the first 6 months after study entry, 3% of patients taking Aza C transformed to AML while 24% of patients on supportive care transformed ($P < .0001$). Of the patients who transformed to AML in the supportive care group, 77% were diagnosed at study entry as having RA/RARS or RAEB but not RAEB-T. Figure 4 represents the percent bone marrow myeloblasts at study entry compared with the percentage of blasts in the marrow or peripheral blood (National Cancer Institute criteria) at the time of transformation. To demonstrate the biologic impact on survival of the transformation to leukemia, we performed a landmark analysis after a 12-month date of the association of transformation to AML with survival. The two subgroups included 13 patients who had already transformed to AML by the landmark date and 93 patients who had not yet transformed, both groups independent of therapy. Patients who died before 12 months were excluded. The median additional survival (after the 12-month landmark) was 3 months (95% CI, 1 to 11 months) for patients who had already transformed and 18 months (95% CI, 14 to 26 months) for patients who had not yet transformed to AML ($P < .001$).

Table 6. Analysis of Response

	Aza C		Supportive Care		Cross-Over	
	No. of Patients	%	No. of Patients	%	No. of Patients	%
No. evaluated	99		92		49	
CR	7	7*	0	0	5	10
PR	16	16*	0	0	2	4
Improved	37	37*	5	5	16	33
Total	60	60*	5	5	23	47

*Significant differences between the arms in CR rate ($P = .01$), CR + PR rate ($P < .0001$), and CR + PR + improvement rate ($P < .0001$) were observed.

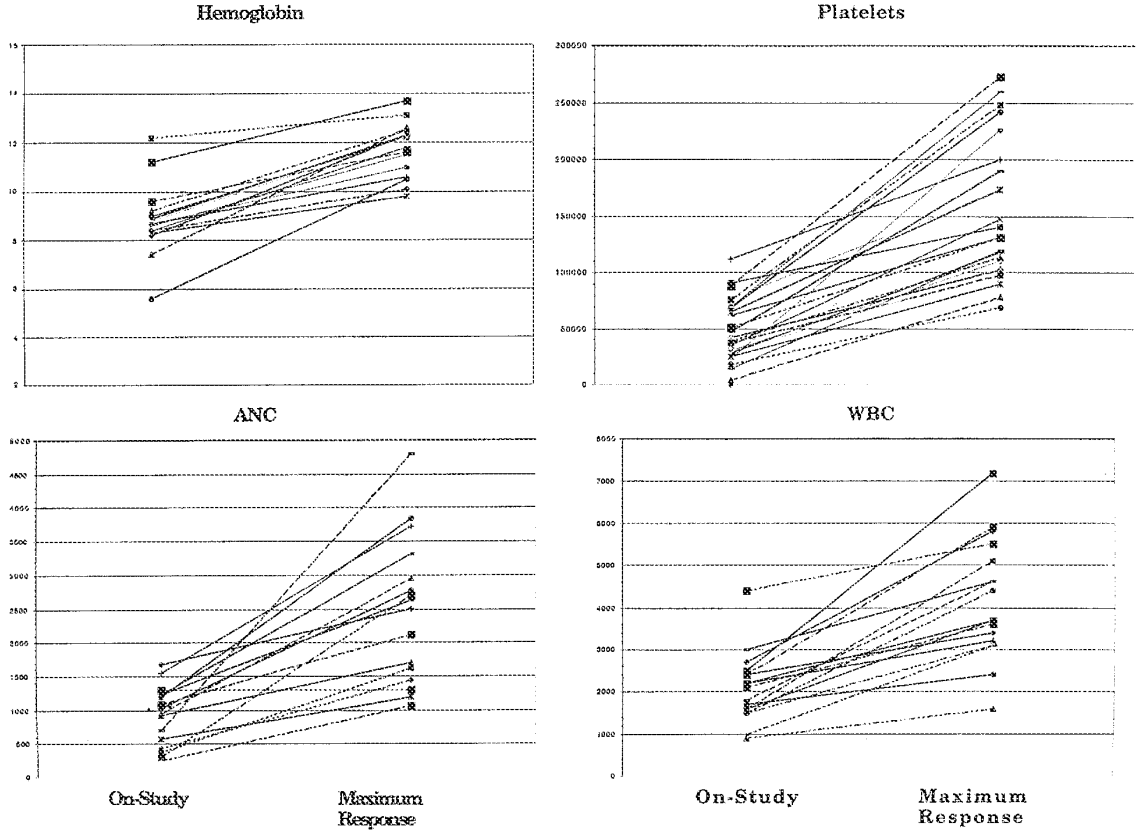

Fig 1. Changes in peripheral-blood counts at the time of response compared with study entry for 37 patients in the Aza C arm classified as improved. Patients who achieved CR or PR are not included.

Effects on RBC and Platelets

The mean number of RBC transfusions increased for the patients taking Aza C in the first month of treatment but thereafter declined, whereas the mean number of transfusions remained stable or increased for patients on supportive care. By definition (Table 3), patients achieving CR or PR had an elimination of RBC or platelet transfusion requirements. Among the 37 patients improved, 73% had an RBC response, 35% (n = 13) had a 50% or greater restitution in the RBC deficit (lineage response), 22% (n = 8) had an elimination of all RBC transfusion requirements, and 16% (n = 6) had a decrease by 50% or greater in RBC transfusions. Thus, among the 99 patients randomized to Aza C, 51% had an RBC lineage response. Among the 65 patients receiving RBC transfusions at study entry, 29 (45%) had an elimination of all transfusions and another six (9%) had a reduction in transfusions by 50%. In addition, lineage responses for platelets and WBC occurred in 47% and 40%, respectively, among those treated with Aza C.

Effects of Treatment on QOL

Patients on the Aza C arm experienced significantly greater improvement over time in fatigue (EORTC, P = .001), physical functioning (EORTC, P = .002), dyspnea (EORTC, P = .0014), psychosocial distress (MHI, P = .015), and positive affect (MHI, P = .0077) than patients in the supportive care group. Significant differences persisted after controlling for RBC transfusions. Before cross-over, the QOL of patients on supportive care was stable or worsening. After cross-over to Aza C, significant improvements occurred in fatigue (EORTC, P = .0001), physical functioning (EORTC, P = .004), dyspnea (EORTC, P = .0002), and general well-being (MHI, P = .016).[50] A complete report of QOL will be presented elsewhere.[51]

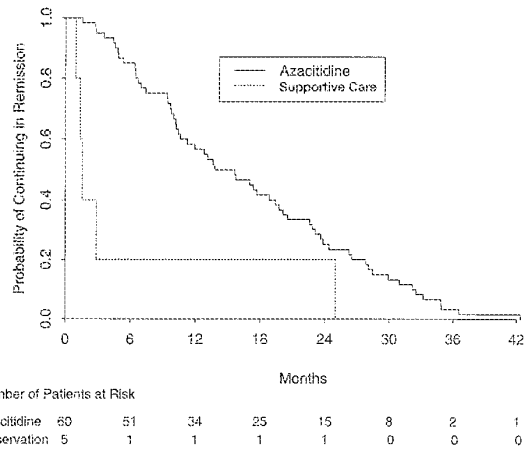

Fig 2. Duration of response. Measured from time of initial response to relapse in patients with CR, PR, or improvement and estimated according to the method of Kaplan-Meier.

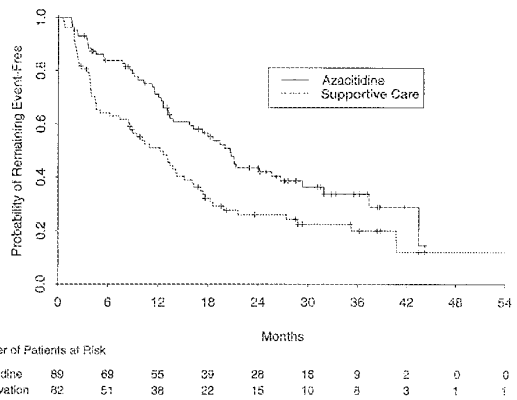

Fig 3. Time to AML transformation or death. Measured from entry on study to the time of first event, either transformation to AML or death, and estimated according to the Kaplan-Meier method.

Detailed analyses make unlikely placebo or Hawthorne[53] effects as explanations for improvements in QOL by Aza C.

Overall Survival

The median survival was 20 months (95% CI, 16 to 26 months) for patients randomized to Aza C compared with 14 months (95% CI, 12 to 14 months) for patients undergoing supportive care (53% of whom received Aza C after cross-over) ($P = .10$)(Fig 5). To eliminate the confounding effect caused by including the 49 cross-over patients in the survival analysis, a landmark analysis was done in which the survival of three subgroups of patients were compared from a 6-month landmark date. These subgroups were supportive care patients who never crossed over or who crossed over only after 6 months, supportive care patients who crossed before 6 months, and patients who were initially randomized to Aza C. The 36 patients who died before the landmark date were excluded. The median survival (after the 6-month landmark date) for these three groups was 11, 14, and 18 months, respectively (Fig 6). The Aza C group was significantly different from the supportive care subgroup who crossed over late or never ($P = 0.03$). Supportive care patients who crossed over early (subgroup 2) had a longer median survival than the patients who crossed over late or never (subgroup 1), although this did not reach statistical significance ($P = .11$). Survival by treatment arm and FAB risk group is demonstrated in Fig 7.

Treatment-Related Toxicity

The most common toxicity of Aza C was myelosuppression. In patients with severe cytopenias from their disease,toxicity was difficult to assess, rendering useless the standard criteria used for hematologic toxicity where the pretreatment marrow is normal. On the basis of standard CALGB criteria, grade 3 or 4 leukopenia occurred in 59%, granulocytopenia in 81%, and thrombocytopenia in 70% of patients receiving Aza C. When hematologic toxicity was reassessed centrally using relative changes in peripheral-blood counts compared with those at study entry, a decrease of 50% to 74% was defined as grade 3 and 75% or greater was defined as grade 4. Based on these criteria, grade 3 or 4 leukopenia occurred in 43%, granulocytopenia in 58%, and thrombocytopenia in 52% of patients receiving Aza C. Toxicity was transient, and patients usually recovered in time for the next treatment cycle. Infection was thought to have been related to treatment in 20% of patients. Nausea or vomiting occurred in 4%. There was one (≤ 1%) treatment-related death.

DISCUSSION

The present results confirm our earlier observations of the beneficial effects of Aza C on bone marrow function in high-risk MDS and extend these findings to symptomatic RA and RARS. The same stringent response criteria used in our original trials of Aza C, developed and defined in the absence of standardized criteria, were used in the present study.[39] The 5% response rate in the supportive care arm indicates that the criteria are sufficiently robust to filter out ordinary variation in blood counts. Incremental changes in peripheral-blood counts among patients improved were sizable (Fig 1). Thus, patients were not categorized as

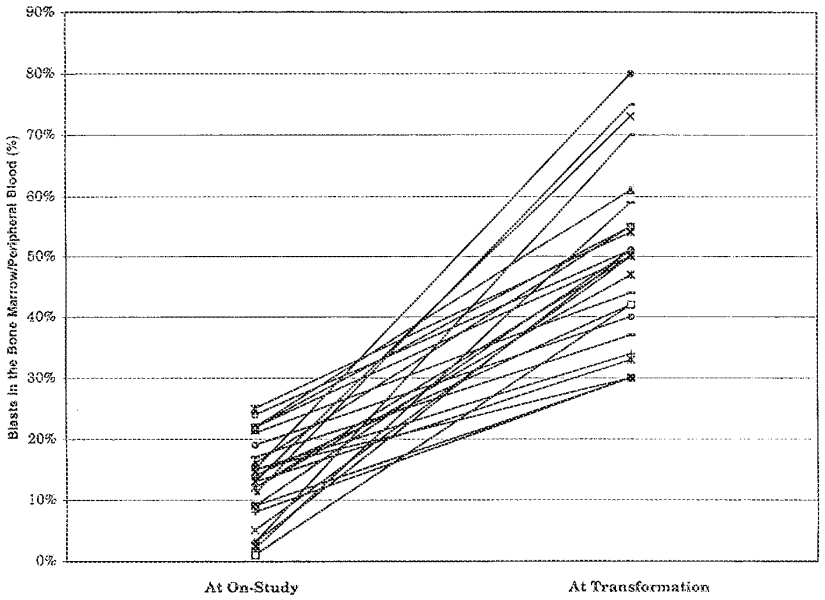

Fig 4. Comparison of myeloblasts in the bone marrow or peripheral blood at study entry and the time of leukemic transformation.

improved on the basis of only a marginal increase in counts as a potential byproduct produced by a quirk of the response criteria. The 60% response rate for Aza C shows that the criteria are sensitive and specific enough to detect biologically important changes, because they are associated with prolonged survival and improved QOL. Our patients were treated at 26 academic centers and 30 of their community affiliates. Thus, our results may predict general medical community achievement.

The number of deaths in the two arms in the first 4 months of study was comparable. The frequency of transformation to leukemia for patients on supportive care was eight-fold higher than patients treated with Aza C in the first 6 months from study entry. Over the entire course of the study, the rate was 2.5-fold higher, the lesser frequency possibly reflecting the fact that many patients were receiving Aza C after cross-over. Differences between the arms in leukemic transformation could not be explained by FAB subtype, International Prognostic Scoring System scores, or time from diagnosis to study entry. Time to leukemic transformation or death represents the purest and most objective manifestation of disease progression for MDS. The landmark analysis demonstrates that transformation to AML has a significantly adverse effect on survival. Aza C delays and decreases transformation to acute leukemia. This is the first description of a drug with this capacity.

The effect of initial treatment with Aza C on overall survival was confounded by the fact that 49 supportive care patients were crossed over to Aza C during their survival follow-up. The landmark analysis diminishes the confounding effect and demonstrates a significant survival advantage in favor of those treated with Aza C initially compared with those not treated or who received treatment only after 6 months of supportive care (Fig 6). A salvage benefit may nonetheless still be important even for patients treated late in the course of their disease.

Significant improvements in QOL, particularly for fatigue, physical functioning, dyspnea, and general well-being, were derived from Aza C treatment, even in the supportive care patients after cross-over. The data indicate that Aza C treatment is more effective in improving QOL than simply raising hemoglobin values with RBC transfusions.

Aza C appears to be superior to other drugs that have been used for MDS. Agents that can induce hematopoietic differentiation in vitro have been extensively tested, and 13-*cis*- and all-*trans*-retinoic acid, 1,25-dihydroxy vitamin D_3, butyrate, cytarabine, and hexamethylene bisacetamide have produced feeble clinical responses. Amifostine has produced responses, but its activity has yet to be fully defined.[54] None of these drugs have caused significant trilineage responses, sustained remissions, or prolonged

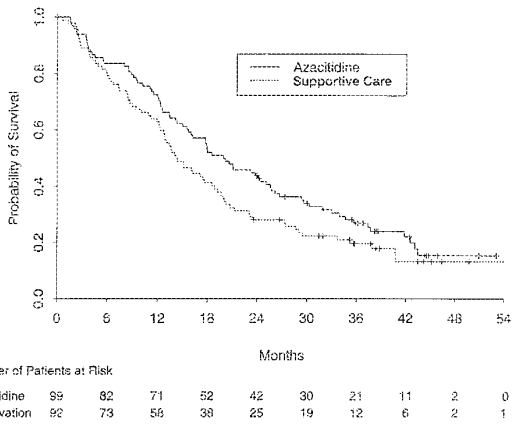

Fig 5. Overall survival by randomized arm and estimated according to the Kaplan-Meier method. Patients who were initially in the supportive care group and crossed over to treatment with azacitidine are included in the supportive care group in this plot.

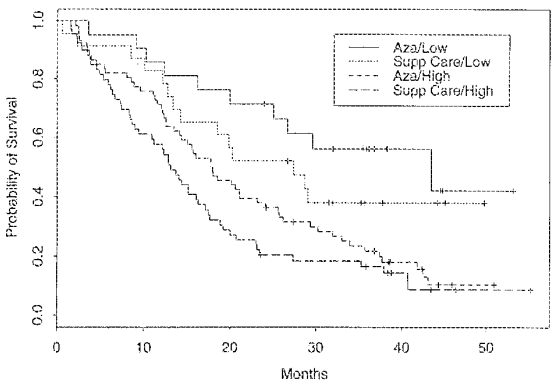

Fig 7. Survival by randomized arm and FAB subtype. FAB subgroups were divided into low-risk (RA/RARS) and high-risk (RAEB, RAEB-T, or CMMoL) groups. Median survival: Aza/Low, 44 months; supportive care (SC)/Low, 27 months; Aza/High, 18 months; SC/High, 13 months.

survival.[55-65] Aggressive antileukemic type therapy and newer agents such as topotecan alone or in combination have produced response rates up to 65% but have not been reported to alter the disease outcome.[66-71]

Four randomized controlled trials have been previously conducted in patients with MDS. *Cis*-retinoic acid demonstrated no advantage compared with placebo.[55,72] Low-dose cytarabine (10 mg/m^2 every12 hours) compared with supportive care led to a 35% hematologic response (using less stringent criteria than the present study) but no differences in time to progression, frequency of transformation to AML, or survival.[22,73] Filgrastim (G-CSF) was compared with supportive care. The death rate for patients with RAEB and RAEB-T was significantly accelerated by G-CSF, with a median survival of 10 months compared with 21 months for supportive care, leading to early termination of the study.[74] Treatment with sargramostim (GM-CSF) resulted in increases in myelomonocytic and lymphoid lineages, with a decrease in frequency of infections in those treated. There were no effects on platelets or red cells. Impact on outcome has not been reported.[11]

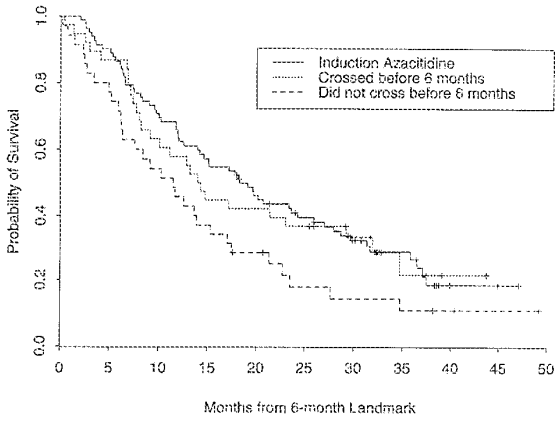

Fig 6. Survival from landmark date by cross-over status (Kaplan-Meier method). Patients were subgrouped as supportive care patients who either never crossed over or crossed over after 6 months, supportive care patients who crossed over before 6 months, and patients who were initially randomized to Aza C.

The mechanism by which Aza C produces its effects is most likely multifactorial. Aza C can produce significant myelosuppression, particularly at higher doses. The doses used in this study and the two prior phase II studies produced marrow hypoplasia in only 10% of patients. Nevertheless, myelosuppression leading to lower peripheral-blood counts and increased RBC transfusion requirements occurred during the first cycle of treatment. Continued treatment without dose reduction led to improved bone marrow function in most patients. Prolonged treatment may have inhibited the MDS clone, permitting residual normal hematopoiesis to emerge. Conversely, Aza C might have exerted a cytotoxic effect on regulatory T cells or other modulatory cells that were inhibiting hematopoiesis.

Aza C could also be acting as a biologic response modifier. The response of hematopoietic progenitors to cytokines is impaired in patients with MDS.[75] This may be attributable in part to abnormalities of the signal transduction pathway downstream from the cytokine receptors.[76-79] In vitro data suggest that Aza C can modulate the cytokine signal transduction pathway, rendering sensitive unrespon-

sive cells to the effects of cytokines, partially restoring normal hematopoietic regulation.[80-82]

As observed in our prior studies, most responding patients demonstrated response beginning in the third or fourth month. This is consistent both with a low-dose cytotoxic effect and with Aza C acting as a biologic response modifier. Incorporation of Aza C into DNA inhibits DNA methyltransferase and induces DNA hypomethylation.[32,83-86] This effect is S-phase dependent, and two or more cycles of DNA synthesis are required to alter gene transcription and expression.[32,84-86] Thus, repetitive exposure on the present low-dose intermittent schedule may have affected small numbers of cells during each treatment, requiring three to four cycles before the effects became clinically apparent. Alteration in the methylation of the *p15* gene has been implicated in transformation of MDS to AML and could be modulated by Aza C, thus reducing risk of leukemic transformation.[87,88]

Although Aza C is active in the present regimen, other doses and schedules might improve its efficacy. Additional studies of Aza C should build on these results. Besides optimization of dose and schedule, combinations with cytokines and other agents that modulate signal transduction are logical areas of exploration. The present study demonstrates that Aza C is effective therapy for patients with MDS with the subgroups and profiles treated in this study. Aza C improves their bone marrow function, decreases and delays significantly transformation to AML, and improves QOL and survival compared with supportive care. These data suggest that Aza C should be considered the treatment of choice for patients with MDS who meet the entry criteria stipulated in this study.

APPENDIX

The appendix listing participating institutions and investigators is available online at www.jco.org.

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
	Examiner	Art Unit
	LAWRENCE E CRANE	1623

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Classification for restriction purposes - 514/43; 535/28.3	7/25/2011	LEC
File CAPLUS search - see search for strategy	7/25/2011	LEC
Inventor name search - (Etter) - no basis for ODP found	7/25/2011	LEC

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

	/Lawrence E. Crane/ Primary Examiner, Art Unit 1623

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	12466213	ETTER ET AL.
	Examiner	Art Unit
	LAWRENCE E CRANE	1623

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	07/25/2011	02/24/2012							
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	36	✓	✓							

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Final	Original	07/25/2011	02/24/2012							
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(Use several sheets if necessary)

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Filing Date	May 14, 2009
First Named Inventor	Jeffrey B. Etter
Art Unit	1623
Examiner Name	Lawrence E. Crane
Attorney Docket No.	9516-847-999

U.S. PATENT DOCUMENTS

*Examiner Initials	Cite No.	Document Number – Kind Code	Publication Date mm/dd/yyyy	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	A18	US 3,350,388	10/31/1967	Sorm *et al.*	
	A19	US 3,817,980	06/18/1974	Vorbruggen *et al.*	
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	A38	US 2006/0063735	03/23/2006	Redkar *et al.*	
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d-892 - duplicate citation - see PTO-892 for original.

SDI-108146v1

EXAMINER SIGNATURE	/Lawrence Crane/	**DATE CONSIDERED**	02/23/2012

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /L.C./

LIST OF REFERENCES CITED BY APPLICANT

(Use several sheets if necessary)

Application Number	12/466,213
Filing Date	May 14, 2009
First Named Inventor	Jeffrey B. Etter
Art Unit	1623
Examiner Name	Lawrence E. Crane
Attorney Docket No.	9516-847-999

U.S. PATENT DOCUMENTS

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	A56	US 2011/0245485	10/06/2011	De Ferra *et al.*	
	A57	13/273,127	10/13/11*	Ionescu *et al.*	

d-892 - duplicate citation - see PTO-892 for original.

* Application filing date.

FOREIGN PATENT DOCUMENTS

*Examiner Initials	Cite No.	Foreign Patent Document Country Code, Number, Kind Code (if known)	Publication Date mm/dd/yyyy	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T
	B03	CZ 114716	11/15/1964	Sorm *et al.*		T
	B04	CZ 116297	04/15/1965	Sorm *et al.*		T

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SDI-108146v1

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	B09	WO 2011/014541	02/03/2011	Eagle Pharmaceuticals, Inc.		

*Abstract only.

NON PATENT LITERATURE DOCUMENTS

*Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T
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	C47	Dintaman *et al.*, "Inhibition of P-Glycoprotein by D-α-Tocopheryl Polyethylene Glycol 1000 Succinate (TPGS)," *Pharmaceutical Research*, 16(10): 1550–1556 (1999).	
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	C48	Garcia-Manero *et al.*, "A Pilot Pharmacokinetic Study of Oral Azacitidine," *Leukemia*, 22: 1680–84 (2008).	
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	C50	Gaubert *et al.*, "Unnatural Enantiomers of 5-Azacytidine Analogues: Synthesis and Enzymatic Properties," *Nucleosides, Nucleotides & Nucleic Acids*, 20(4–7): 837–40 (2001).	

d-892 - duplicate citation - see PTO-892 for original.

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SDI-108146v1

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	C52	Hanka *et al.*, "Microbiological Production of 5-Azacytidine I. Production and Biological Activity," *Antimicrobial Agents and Chemotherapy*, 619–24 (1966).	
	C53	Kritz *et al.*, "Pilot Study of 5-Azacytidine (5-AZA) and Carboplatin (CBDCA) in Patients with Relapsed/Refractory Leukemia," *American Journal of Hematology*, 51(2): 117–21 (1996).	
	C54	Niedballa *et al.*, "A General Synthesis of N-Glycosides. V. Synthesis of 5-Azacytidines," *J. Org. Chem.*, 39(25): 3672–74 (1974).	
	C55	Notice of Allowance dated September 20, 2011 in U.S. Patent Application No. 12/729,116.	
	C56	O'Neil *et al.* (eds.), THE MERCK INDEX, 13th Edition, page 154–55 (2001).	
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	Filing Date	May 14, 2009
	First Named Inventor	Jeffrey B. Etter
	Art Unit	1623
	Examiner Name	Lawrence E. Crane
	Attorney Docket No.	9516-847-999

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	C68	Wittenburg *et al.*, "A New Synthesis of Nucleosides," *Zeitschrift fur Chemie*, 4: 303–04 (1964) (with English translation).	
	C69	Zaitseva *et al.*, "Convergent Syntheses and Cytostatic Properties of 2-Chloro-2'-Deoxy-2'-Fluoroadenosine and its N^7-Isomer," *Bioorg. & Med. Chem. Lett.*, 5(24): 2999–3002 (1995).	
	C70	Ziemba *et al.*, "Development of Oral Demethylating Agents for the Treatment of Myelodysplastic Syndromes," *American Association of Cancer Research*, 100th Annual Meeting, April 18–22, Abstract #3369 (2009).	

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PTO/SB/30 (07-09)
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Request for Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	12/466,213
Filing Date	May 14, 2009
First Named Inventor	Jeffrey B. Etter
Art Unit	1623
Examiner Name	Lawrence E. Crane
Attorney Docket Number	9516-847-999

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

   a. ☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

      i. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

      ii. ☐ Other _____

   b. ☑ Enclosed

      i. ☑ Amendment/Reply

      ii. ☐ Affidavit(s)/ Declaration(s)

      iii. ☐ Information Disclosure Statement (IDS)

      iv. ☑ Other Petition for Extension of Time

2. Miscellaneous

   a. ☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

   b. ☐ Other _____

3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.

   a. ☑ The Director is hereby authorized to charge the following fees, any underpayment of fees, or credit any overpayments, to Deposit Account No. 50-3013.

      i. ☑ RCE fee required under 37 CFR 1.17(e)

      ii. ☑ Extension of time fee (37 CFR 1.136 and 1.17)

      iii. ☐ Other _____

   b. ☐ Check in the amount of $ _____ enclosed

   c. ☐ Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature		Date	August 3, 2012
Name (Print/Type)	Yeah-Sil Moon	Registration No.	52,042

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the U.S. Patent and Trademark Office on the date shown below.

Signature			
Name (Print/Type)		Date	

ELECTRONIC FILING

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Jeffrey B. Etter	Confirmation No.:	5370
Application No.:	12/466,213	Art Unit:	1623
Filed:	May 14, 2009	Examiner:	Lawrence E. Crane
For:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF	Attorney Docket (CAM No.:	9516-847-999 501872-999847)

RESPONSE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the final Office Action mailed February 28, 2012, Applicants respectfully submit the following amendments and remarks for the Examiner's consideration and entry into the record. Also submitted are (1) a Request for Continued Examination ("RCE"); and (2) a Petition for Extension of Time for three months from May 28, 2012 to and including August 28, 2012.

Amendments to the Claims are reflected in the listing of the claims that begins on page 2 of this paper.

Remarks begin on page 9 of this paper.

Applicants hereby authorize any required fees, including a three-month time extension fee of $1,270 and an RCE fee of $930, to be charged to Jones Day Deposit Account No. 50-3013.

AMENDMENTS TO THE CLAIMS

The following listing of claims will replace all prior versions, and listings, of claims in the application.

Listing of the Claims

1. (Currently amended) A pharmaceutical composition for oral administration comprising a therapeutically effective amount of 5-azacytidine ~~a cytidine analog~~ and at least one pharmaceutically acceptable excipient, wherein the composition is an immediate release tablet or an immediate release capsule~~, and the cytidine analog is 5-azacytidine or decitabine~~.

2. (Canceled).

3. (Previously presented) The composition of claim 1, wherein the composition does not comprise an enteric coating.

4. (Previously presented) The composition of claim 1, wherein the composition is a tablet.

5. (Previously presented) The composition of claim 1, wherein the composition is a capsule.

6. (Currently amended) The composition of claim 1, wherein the at least one pharmaceutically acceptable excipient is selected from mannitol, microcrystalline cellulose, crospovidone, and magnesium stearate.

7. (Original) The composition of claim 1, which further comprises a permeation enhancer.

8. (Previously presented) The composition of claim 7, wherein the permeation enhancer is D-alpha-tocopheryl polyethylene glycol 1000 succinate.

9. (Currently amended) The composition of claim 8, wherein the D-alpha-tocopheryl polyethylene glycol 1000 succinate is present in the composition ~~formulation~~ at about 2% by weight relative to the total weight of the composition ~~formulation~~.

10. (Original) The composition of claim 1, which is essentially free of a cytidine deaminase inhibitor.

11. (Original) The composition of claim 1, which is essentially free of tetrahydrouridine.

12. (Currently amended) The composition of claim 1, which further comprises a therapeutically effective amount of ~~a second active~~ an anti-cancer agent.

13. (Currently amended) The composition of claim 1, wherein the amount of 5-azacytidine ~~or decitabine~~ is at least about 40 mg.

14. (Currently amended) The composition of claim 1, wherein the amount of 5-azacytidine ~~or decitabine~~ is at least about 400 mg.

15. (Currently amended) The composition of claim 1, wherein the amount of 5-azacytidine ~~or decitabine~~ is at least about 1000 mg.

16. (Currently amended) The composition of claim 1, which has been shown to achieve[[s]] an area-under-the-curve value of at least about 200 ng-hr/mL following oral administration to a test subject.

17. (Currently amended) The composition of claim 1, which has been shown to achieve[[s]] an area-under-the-curve value of at least about 400 ng-hr/mL following oral administration to a test subject.

18. (Currently amended) The composition of claim 1, which has been shown to achieve[s] a maximum plasma concentration of at least about 100 ng/mL following oral administration to a test subject.

19. (Currently amended) The composition of claim 1, which has been shown to achieve[s] a maximum plasma concentration of at least about 200 ng/mL following oral administration to a test subject.

20. (Currently amended) The composition of claim 1, which has been shown to achieve[s] a time to maximum plasma concentration of less than about 180 minutes following oral administration to a test subject.

21. (Currently amended) The composition of claim 1, which has been shown to achieve[s] a time to maximum plasma concentration of less than about 90 minutes following oral administration to a test subject.

22. (Currently amended) The composition of claim 1, which has been shown to achieve[s] a time to maximum plasma concentration of less than about 60 minutes following oral administration to a test subject.

23. (Currently amended) A method for treating one or more symptoms of a disease associated with abnormal cell proliferation, comprising orally administering to a subject in need thereof a pharmaceutical composition comprising a therapeutically effective amount of 5-azacytidine ~~a cytidine analog~~ and at least one pharmaceutically acceptable excipient, wherein the composition releases the 5-azacytidine ~~cytidine analog~~ substantially in the stomach following oral administration to the subject, and wherein the disease associated with abnormal cell proliferation is myelodysplastic syndrome or acute myelogenous leukemia ~~a cancer or a hematologic disorder, and the cytidine analog is 5-azacytidine or decitabine~~.

24-30. (Canceled).

31. (Currently amended) The method of claim 23, wherein the method further comprises co-administering to the subject in need thereof a therapeutically effective amount of ~~a second active~~ an anti-cancer agent.

32. (Original) The method of claim 23, wherein the composition is an immediate release composition.

33. (Original) The method of claim 23, wherein the composition further comprises a permeation enhancer.

34. (Previously presented) The method of claim 33, wherein the permeation enhancer is D-alpha-tocopheryl polyethylene glycol 1000 succinate.

35. (Previously presented) The method of claim 34, wherein the D-alpha-tocopheryl polyethylene glycol 1000 succinate is present in the formulation at about 2% by weight relative to the total weight of the formulation.

36. (Currently amended) The method of claim 23, wherein the method further comprises not co-administering a cytidine deaminase inhibitor with the 5-azacytidine ~~cytidine analog~~.

37. (Original) The method of claim 23, wherein the composition is a single unit dosage form.

38. (Previously presented) The method of claim 23, wherein the composition does not comprise an enteric coating.

39. (Original) The method of claim 23, wherein the composition is a tablet.

40. (Original) The method of claim 23, wherein the composition is a capsule.

41. (Currently amended) The method of claim 23, wherein the at least one pharmaceutically acceptable excipient is selected from mannitol, microcrystalline cellulose, crospovidone, and magnesium stearate.

42. (Currently amended) The method of claim 23, wherein the amount of 5-azacytidine ~~or decitabine~~ is at least about 40 mg.

43. (Currently amended) The method of claim 23, wherein the amount of 5-azacytidine ~~or decitabine~~ is at least about 400 mg.

44. (Currently amended) The method of claim 23, wherein the amount of 5-azacytidine ~~or decitabine~~ is at least about 1000 mg.

45. (Previously presented) The method of claim 23, which has been shown to achieve an area-under-the-curve value of at least about 200 ng-hr/mL following oral administration to a test subject.

46. (Previously presented) The method of claim 23, which has been shown to achieve an area-under-the-curve value of at least about 400 ng-hr/mL following oral administration to a test subject.

47. (Previously presented) The method of claim 23, which has been shown to achieve a maximum plasma concentration of at least about 100 ng/mL following oral administration to a test subject.

48. (Previously presented) The method of claim 23, which has been shown to achieve a maximum plasma concentration of at least about 200 ng/mL following oral administration to a test subject.

49. (Previously presented) The method of claim 23, which has been shown to achieve a time to maximum plasma concentration of less than about 180 minutes following oral administration to a test subject.

50. (Previously presented) The method of claim 23, which has been shown to achieve a time to maximum plasma concentration of less than about 90 minutes following oral administration to a test subject.

51. (Currently amended) A pharmaceutical composition comprising a therapeutically effective amount of 5-azacytidine ~~a cytidine analog~~ and at least one pharmaceutically acceptable excipient, wherein the composition is for treating a disease or disorder associated with abnormal cell proliferation, wherein the composition is an immediate release tablet or an immediate release capsule prepared for oral administration, wherein the disease or disorder associated with abnormal cell proliferation is myelodysplastic syndrome or acute myelogenous leukemia ~~a cancer or a hematologic disorder, and wherein the cytidine analog is 5-azacytidine or decitabine~~.

52. (Currently amended) The pharmaceutical composition of claim 51, wherein the amount of 5-azacytidine ~~or decitabine~~ is about 40 mg, about 400 mg, or about 1000 mg.

53. (Previously presented) The pharmaceutical composition of claim 51, wherein the composition has been shown to achieve an area-under-the-curve value of at least about 200 ng-hr/mL or 400 ng-hr/mL following oral administration to a test subject.

54. (Previously presented) The pharmaceutical composition of claim 51, wherein the composition has been shown to achieve a maximum plasma concentration of at least about 100 ng/mL or 200 ng/mL following oral administration to a test subject.

55. (Previously presented) The pharmaceutical composition of claim 51, wherein the composition has been shown to achieve a time to maximum plasma concentration of less than about 60 minutes or 90 minutes after being administered to a test subject.

56. (Canceled).

57. (Currently amended) The pharmaceutical composition of any one of claims 51 to 55, wherein the composition is prepared for oral administration in combination with a therapeutically effective amount of ~~a second active~~ an anti-cancer agent.

58. (Canceled).

59. (Previously presented) The pharmaceutical composition of any one of claims 51 to 55, wherein the composition is a tablet.

60. (Previously presented) The pharmaceutical composition of any one of claims 51 to 55, wherein the composition is a capsule.

61. (Currently amended) The pharmaceutical composition of any one of claims 51 to 55, wherein the at least one pharmaceutically acceptable excipient is selected from mannitol, microcrystalline cellulose, crospovidone, and magnesium stearate.

62–71. (Canceled).

72. (New) The composition of claim 1, which is to be administered in combination with a therapeutically effective amount of an anti-cancer agent.

REMARKS

Prior to entry of this paper, claims 1, 3-55, 57-61 and 66-71 were pending in this application. Claims 24-30, 58 and 66-71 have been canceled without prejudice to Applicants' right to pursue the subject matter of the canceled claims in this application or a related application. Claims 1, 6, 9, 12-23, 31, 36, 41-44, 51, 52, 57 and 61 have been amended without any intention of disclaiming any equivalents thereof. New claim 72 has been added.

Specifically, claims 1, 23 and 51 are amended to recite a pharmaceutical composition comprising 5-azacytidine or a method of treatment comprising administering a pharmaceutical composition comprising 5-azacytidine. Claims 23 and 51 are also amended to recite that the disease or disorder is myelodysplastic syndrome ("MDS") or acute myelogenous leukemia ("AML"). Claims 13-15, 42-44 and 52, which depend on claims 1, 23 and 51 respectively, are similarly amended to recite that the pharmaceutical composition comprises 5-azacytidine. Claim 9 has been amended to substitute the term "composition" for "formulation". In addition, claims 6, 12, 16-22, 31, 57 and 61 are amended to particularly point out and distinctly claim the subject matter which Applicants regard as the invention, as discussed in details below. New claim 72 depends on claim 1 and further recites that the composition comprising 5-azacytidine is administered in combination with an anti-cancer agent. No new matter is introduced.

Applicants respectfully submit that the pending claims are allowable for at least the following reasons.

I. Status of Claims

The Examiner asserts that linking claims 1, 3-55 and 57-61 insofar as they are directed to decitabine as an active ingredient, as well as claims 67, 69 and 71, are directed to an invention that is independent or distinct from the invention originally claimed, and are therefore withdrawn from consideration. (Office Action at 2.) Without acquiescing to the Examiner's assertion and solely to expedite prosecution of the application, Applicants have deleted the recitation of decitabine in the pending claims. Claims 1, 3-23, 31-55, 57 and 59-61, which are directed to a pharmaceutical composition comprising 5-azacytidine and related method of treatment, are under consideration.

II. Claim Rejections Under 35 U.S.C. § 112, First Paragraph, Should Be Withdrawn

Claims 23-50 insofar as they are directed to 5-azacytidine as the active ingredient, and claim 68, are rejected under 35 U.S.C. § 112, first paragraph, as allegedly not being enabled. The Examiner acknowledges that the specification is enabled for the treatment by the administration of 5-azacytidine as the sole active ingredient of certain neoplastic disease conditions (*e.g.*, the conditions described in Examples 1-7), but alleges that the specification does not provide enablement for the effective treatment of all other "diseases associated with abnormal cell proliferation" such as cancers of the pancreas, live and brain. (Office Action at 3.)

For the reasons stated in the response filed on December 1, 2011, Applicants disagree with this rejection. Nevertheless, solely to expedite the prosecution of the present application, Applicants have amended independent claim 23 to recite that the disease associated with abnormal cell proliferation is MDS or AML. Administration of 5-azacytidine to treat patients with MDS or AML is described in the examples (*see*, *e.g.*, Example 4) and its enablement is acknowledged by the Examiner (Office Action at 3). The enablement rejection under 35 U.S.C. § 112, first paragraph, is therefore believed to be obviated and should be withdrawn.

III. Claim Rejections Under 35 U.S.C. § 112, Second Paragraph, Should Be Withdrawn

The following claims are rejected as allegedly being indefinite.

1. Claims 1, 3-55, 57-61 and 66-71

Claims 1, 3-55, 57-61 and 66-71 insofar as they are directed to 5-azacytidine as the active ingredient, are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. (Office Action at page 5.) The Examiner requests that the terms "decitabine" and "cytidine analog" be deleted. As discussed above, the pending claims as amended no longer recite "decitabine" or "cytidine analog". This rejection is therefore obviated.

2. Claim 1

The Examiner alleges that the phrase "at least one pharmaceutically acceptable excipient" in claim 1 appears to be inconsistent with the term "immediate release tablet" or "immediate release capsule" because the term "immediate release" implies the presence of "more than one excipients". (Office Action at 5-6.) The Examiner also alleges that this claim is incomplete

because the entire contents of the tablet or capsule have not been defined. (*Id.*)

Applicants note that the Examiner has not provided any basis for the assertion that the term "immediate release" implies the presence of "more than one excipients". Moreover, the phrase "at least one pharmaceutically acceptable excipient" is not inconsistent with and encompasses the phrase "more than one excipients" in scope. Furthermore, the transitional term "comprising" is open-ended and does not exclude additional, unrecited elements. *See* MPEP 2111.03. The Patent Office routinely grant patent claims that define a composition or a method with some, but not all, the elements of the composition or the steps of the method, using the transitional term "comprising". Therefore, the Examiner's rejection is believed to be in error.

3. Claims 6, 41 and 61

The Examiner alleges that the term "excipient" in its singular form in these claims is inconsistent with the Markush group that follows this term. (Office Action at 6.) These claims have been amended to recite "at least one pharmaceutically acceptable excipient". The rejection is believed to be obviated.

4. Claims 16-22

The Examiner states that the term "achieves" in these claims is grammatically erroneous. (Office Action at 6.) Applicants deleted the last letter "s" in this word using strike-through in the response filed on December 1, 2011. To make it easier to be perceived, this deletion is shown using double brackets in the present paper.

5. Claims 30

Claim 30 is objected to under 37 C.F.R. § 1.75(c) as being an improper dependent form for failing to further limit the subject matter of a previous claim. (Office Action at 6.) This claim has been canceled and the rejection is moot.

6. Claims 12, 31 and 57

Claim 12 is rejected as allegedly being incomplete on the ground that the term "an additional therapeutic agent" is not adequately defined and that this claim is superfluous in view of the term "comprising" in claim 1. Claims 31 and 57 are rejected on similar ground. Without

acquiescing to this rejection and solely to expedite the prosecution, Applicants have replaced this term with the term "an anti-cancer agent". As discussed above, the transitional term "comprising" is open-ended and does not exclude additional, unrecited elements. *See* MPEP 2111.03. Since these claims recite an additional element of the composition and further limit the claims they depend from, the rejection is believed to be obviated.

7. Claims 23 and 51

The Examiner alleges that the term "a disease associated with abnormal cell proliferation" is not specified with adequate particularity. (Office Action at 7.) Without acquiescing to this rejection and solely to expedite prosecution, Applicants have amended these claims to recite that the disease or disorder associated with abnormal cell proliferation is MDS or AML. The rejection is therefore believed to be obviated.

IV. Claim Rejections Under 35 U.S.C. § 103 Should Be Withdrawn

Claims 1, 3-55, 57-61 and 66-71 insofar as they are directed to 5-azacytidine as the active ingredient, are rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Redkar *et al.* (U.S. Patent Publication No. 2006/0074046; hereinafter "Redkar"), in view of Dintaman *et al.* (Pharmaceutical Research, 1999, 16(10), 1550–1556; hereinafter "Dintaman") and further in view of Sands *et al.* (U.S. Patent Publication No. 2004/0162263; hereinafter "Sands"). (Office Action, at pages 8–9.)

Specifically, the Examiner alleges that Redkar teaches the advantages of increased solubility and stability associated with substitution of strong acid salts of 5-azacytidine for the neutral compound, and that this teaching appears to render obvious the instant "immediate release" approach to the oral administration of 5-azacytidine capsules or tablets. (Office Action at 8-9.) The Examiner acknowledges that Redkar does not disclose a pharmaceutical composition comprising a permeation enhancer such as the vitamin E derivative d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS), as recited in claims 7-9 and 33-35, but asserts that Dintaman supplies Redkar with this disclosure. (*Id.*) With regard to Sands, the Examiner alleges that this reference discloses that 5-azacytidine pharmaceutical compositions may include the capability to rapidly dissolve in mildly acidic solutions. (*Id.* at 9.) The Examiner also asserts that Sands teaches that multiple different excipients may be combined with 5-azacytidine in the

manufacture of tablets or capsules, and that the tablets may be prepared without an enteric coating. (*Id.*) The Examiner alleges that this alternative (*i.e.*, tablets without enteric coating) appears to be an equivalent to the immediate release solid dosage vehicle claimed in the present application. (*Id.*)

Based on these, the Examiner asserts that one skilled in the art would have been motivated to combine these references to arrive at the presently claimed pharmaceutical compositions and methods of treatment. (Office Action at 9-10.)

Claims 24-30, 58 and 66-71 have been canceled and the rejection of these claims is therefore moot. For the following reasons, Applicants disagree with this obviousness rejection of the remaining claims.

As a preliminary matter, Applicants emphatically disagree with the Examiner's characterization of Applicants' arguments filed on December 1, 2011 as a response to an anticipation rejection but not to an obviousness rejection. (Office Action at 10.) In the December 1, 2011 response, Applicants argued that a *prima facie* case of obviousness had not been established. Specifically, Applicants discussed at length that one skilled in the art would have had neither reasons nor a reasonable expectation of success to combine the cited references to pursue the claimed subject matter, citing to *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398 (2007) and other relevant case laws. Applicants also pointed out that Sands teaches away from the presently claimed composition and method of treatment by emphasizing the drug release in the small intestine as opposed to the stomach of the subject. (*See* Response filed December 1, 2011, pages 19-24.)

Redkar is directed to salts of decitabine, a cytidine analog. (Redkar, Abstract.) Redkar notes that decitabine is most typically administered by injection; however, the length of I.V. infusion is limited by the decomposition of decitabine or azacytidine and low solubility of the drug in aqueous solutions. (*Id.* at paragraph [0009].) Redkar discloses that these shortcomings can be solved by using the salts of decitabine or azacitidine in place of the free base form. (*See, e.g. id.*; paragraph [0136].)

Contrary to the Examiner's assertion that Redkar's teaching of the advantages of increased solubility and stability associated with the salts of 5-azacytidine appears to render obvious the presently claimed composition, there is no disclosure or suggestion in Redkar of an

immediate release oral formulation comprising 5-azacytidine or an oral formulation that substantially releases 5-azacytidine in the stomach as recited in the present claims. While Redkar contains a generic disclosure that its decitabine salt formulation may be administered by various routes of administration, it is clear from its disclosure that the benefits of the greater stability and solubility in aqueous solutions provided by the salts of decitabine or azacytidine as compared to the free base form are in relation to their utility in intravenous administration. The type of acids (*e.g.*, strong or weak) being used in Redkar to prepare the salts has no bearing on the rate of drug release of an oral formulation. For instance, the 5-azacytidine free base is used to prepare the immediate release oral formulation in the present application.

Dintaman does not cure the deficiency of Redkar. Dintaman is not related to cytidine analog compositions. It studies the effect of TPGS on the inhibition of P-glycoprotein (P-gp), the multidrug resistance transporter, and concludes that co-administration of TPGS may enhance oral bioavailability of drugs due to its inhibition of P-gp in the intestine. (Dintaman, Abstract.) Thus, while the disclosure of Dintaman may be relevant to one skilled in the art who is seeking to improve the oral bioavailability of drugs in the intestine, that skilled person would not have been motivated to combine Dintaman with Redkar to attempt to arrive at an immediate release oral formulation which substantially releases the drug in the stomach, as recited in the present claims. In fact, since Dintaman focuses on the function of TPGS in the intestine as opposed to in the stomach in relation to drug bioavailability, it teaches away from the present invention.

As with Dintaman, Sands also teaches away from the presently claimed pharmaceutical composition and the related method of treatment. As Applicants pointed out in the response filed on December 1, 2011, Sands teaches oral formulations with enhanced bioavailability by targeting specific regions of the gastrointestinal tract, *i.e.*, the upper regions of the small intestine. (*See, e.g.*, Sands, Abstract.) To achieve this targeted release, Sands describes that the oral formulations comprising an acid-labile drug such as cytidine analogs are enteric-coated to bypass the highly acidic gastric environment. (*See, e.g., id.*, paragraph [0030].) The enteric coating material is pH-sensitive and preferably or selectively dissolves at a threshold pH above 5.2, which is significantly higher than the pH of gastic environment (*e.g.*, pH of about 1-2.). (*See id.*, paragraphs [0030] and [0034].)

The Examiner contends that Sands discloses that its composition is capable of rapidly dissolve in mildly acidic solutions (paragraph [0038]) and that the tablets may be prepared without an enteric coating (paragraph [0062]), an alternative embodiment that appears to be an equivalent to the "immediate release" dosage form claimed in the present application. Firstly, the environment in the stomach is highly acidic, rather than mild acidic. Secondly, paragraph [0062] of Sands does not disclose that the drug may be prepared without an enteric coating, as asserted by the Examiner. Rather, it states that the drug may be made as a drug core (or tablet blend) *with or without seal coating first and then enteric-coated with the pH-sensitive enteric coating material* to produce a tablet. As discussed above, a person skilled in the art would have understood from Sands that an enteric-coating with a pH-sensitive material that dissolves at pH above about 5.2 is essential to the targeted release of an acid-labile drug such as cytidine analogs in the upper regions of the small intestine. (*See, e.g., id.*, paragraph [0030].)

In summary, Redkar, Dintaman and Sands, either alone or in combination, do not disclose or suggest an immediate release oral formulation that substantially releases a cytidine analog such as 5-azacytidine in the stomach of a subject. Thus, a person skilled in the art would not have had any reason or a reasonable expectation of success to combine or modify these references to attempt to arrive at the presently claimed pharmaceutical compositions or methods of treatment.

Furthermore, in view of the fact that Redkar relates to the salts of decitabine or azacytidine adapted for intravenous injection and Sands and Dintaman both teach away from the present invention by emphasizing targeted drug release or the functional of a permeation enhancer in the intestine, it is respectfully submitted that the Examiner is impermissibly using the Applicants' specification and hindsight, to attempt to arrive at the claimed subject matter, where there is no reason to combine or modify the teachings of these references. *KSR Int'l Co.*, 550 U.S. at 421 (cautioning against reading the Applicant's disclosure of the claimed invention at issue into the prior art and upholding the principle of avoiding the use of hindsight reconstruction); *Panduit Corp. v. Dennison Mfg. Co.*, 810 F.2d 1561 (Fed. Cir. 1987), cert. denied, 481 U.S. 1052 (1987) (noting that using hindsight reconstruction to pick and choose among isolated disclosures in the prior art to render the claims obvious should be avoided).

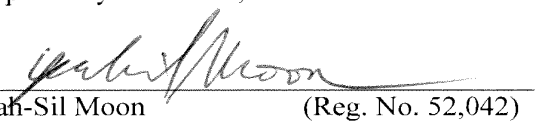
For the reasons stated above, claims 1, 23 and 51, and their respective dependent claims, are believed to be patentable over Redkar in view of Ditaman and further in view of Sands. Therefore, Applicants respectfully request that the rejection under 35 U.S.C. §103(a) be withdrawn.

CONCLUSION

For at least the foregoing reasons, Applicants respectfully submit that all of the pending claims are in allowable form, and thus, respectfully request that the rejections be withdrawn and the application proceed to allowance.

Respectfully submitted,

Date August 3, 2012



Yeah-Sil Moon (Reg. No. 52,042)

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PTO/SB/22 (09-11)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARMENT OF COMMERCE

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) 9516-847-999
Application Number 12/466,213	Filed May 14, 2009
For Oral Formulations of Cytidine Analogs and Methods of Use Thereof	
Art Unit 1623	Examiner Lawrence E. Crane

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$150	$75	$
☐	Two months (37 CFR 1.17(a)(2))	$560	$280	$
☑	Three months (37 CFR 1.17(a)(3))	$1270	$635	$ 1270
☐	Four months (37 CFR 1.17(a)(4))	$1980	$990	$
☐	Five months (37 CFR 1.17(a)(5))	$2690	$1345	$

☐ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 50-3013.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).

☑ attorney or agent of record. Registration Number 52,042

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

Signature	August 3, 2012
	Date
Yeah-Sil Moon	(212) 326-3939
Typed or printed name	Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

☑ Total of 1 forms are submitted.

Electronic Patent Application Fee Transmittal

Application Number:	12466213
Filing Date:	14-May-2009
Title of Invention:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF
First Named Inventor/Applicant Name:	Jeffrey B. Etter
Filer:	Yeahsil Moon/Rochelle Flowers
Attorney Docket Number:	9516-847-999

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with $0 paid	1253	1	1270	1270

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Request for continued examination	1801	1	930	930
		Total in USD ($)		**2200**

Electronic Acknowledgement Receipt

EFS ID:	13411880
Application Number:	12466213
International Application Number:	
Confirmation Number:	5370
Title of Invention:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF
First Named Inventor/Applicant Name:	Jeffrey B. Etter
Customer Number:	84802
Filer:	Yeahsil Moon/Rochelle Flowers
Filer Authorized By:	Yeahsil Moon
Attorney Docket Number:	9516-847-999
Receipt Date:	03-AUG-2012
Filing Date:	14-MAY-2009
Time Stamp:	12:06:41
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$2200
RAM confirmation Number	8771
Deposit Account	503013
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees) Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)	

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees) Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees) Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Continued Examination (RCE)	RCE.pdf	79367 f7d29d334532c9ce3c641d957a286bc7a7cb4675	no	1
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
2	Amendment After Final	Response.pdf	556648 64af0ddab0bf91442dad306d84def0325d7cba8f	no	16
Warnings:					
The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing					
Information:					
3	Extension of Time	EOT.pdf	58027 9dc7ca81a06a5ab914dc4733169ccf285602ea25	no	1
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	32347 bc8075680704aa781b6afa84cb4d8598c03201ea	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	726389		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
12/466,213	05/14/2009	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☐		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	08/03/2012	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 52	Minus	** 91	= 0	X $ =		OR	X $60=	0
	Independent (37 CFR 1.16(h))	* 3	Minus	***4	= 0	X $ =		OR	X $250=	0
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/JAMILAH Z. HARRIS/

LIST OF REFERENCES CITED BY APPLICANT (Use several sheets if necessary)		
	Application Number	**12/466,213**
	Filing Date	May 14, 2009
	First Named Inventor	Jeffrey B. Etter
	Art Unit	1623
	Examiner Name	Lawrence E. Crane
	Attorney Docket No.	9516-847-999

U.S. PATENT DOCUMENTS

*Examiner Initials	Cite No.	Document Number – Kind Code	Publication Date mm/dd/yyyy	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	A47	US 8,211,862	07/03/2012	Ionesco *et al.*	

FOREIGN PATENT DOCUMENTS

*Examiner Initials	Cite No.	Foreign Patent Document Country Code, Number, Kind Code (if known)	Publication Date mm/dd/yyyy	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T
	B10	WO 2004/082619	09/30/2004	Pharmion Corporation		
	B11	WO 2006/034154	03/30/2006	Supergen, Inc.		
	B12	WO 2008/088779	07/24/2008	Ivax Pharmaceuticals S.R.O. et al.		

NON PATENT LITERATURE DOCUMENTS

*Examiner Initials	Cite No.	Include name of the author (in CAPITAL LETTERS), (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T
	C71	Notice of Allowability in U.S. Patent Application No. 12/729,116, mailed May 4, 2012.	

SDI-136025v1

EXAMINER SIGNATURE	DATE CONSIDERED

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
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(22) International Filing Date: 16 March 2004 (16.03.2004)

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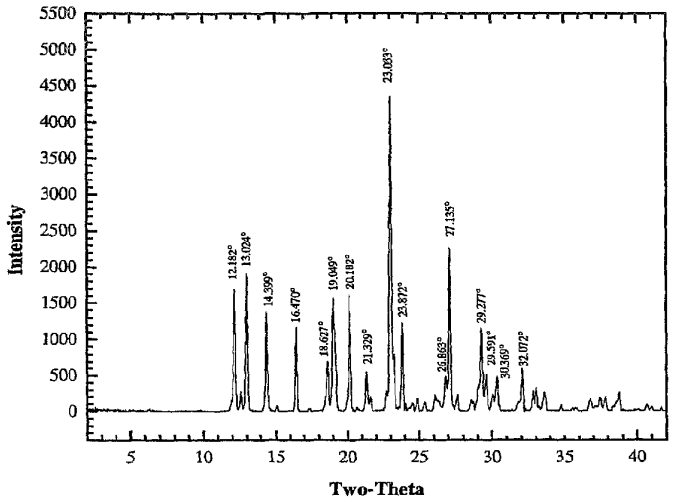
[Continued on next page]

(54) Title: FORMS OF 5-AZACYTIDINE

Figure 1. X-ray Powder Diffraction Pattern of Azacitidine, Form I, Labeled with the more Prominent 2θ Angles (Cu Kα Radiation)



(57) Abstract: The invention provides novel polymorphic and pseudopolymorphic crystalline forms of 5-azacytidine, along with methods for preparing said forms, wherein 5-azacythidine is represented by the formula (I). The invention also includes pharmaceutical compositions comprising said forms.

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FORMS OF 5-AZACYTIDINE

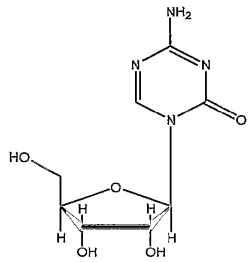
FIELD OF THE INVENTION

The invention relates to the isolation of crystalline polymorphic and pseudopolymorphic forms of 5-azacytidine (also known as azacitidine and 4-amino-1-β-D-ribofuranosyl-S-triazin-2(1*H*)-one). 5-azacytidine may be used in the treatment of disease, including the treatment of myelodysplastic syndromes (MDS).

BACKGROUND OF THE INVENTION

Polymorphs exist as two or more crystalline phases that have different arrangements and/or different conformations of the molecule in a crystal lattice. When a solvent molecule(s) is contained within the crystal lattice the resulting crystal is called a pseudopolymorph, or solvate. If the solvent molecule(s) within the crystal structure is a water molecule, then the pseudopolymorph/solvate is called a hydrate. The polymorphic and pseudopolymorphic solids display different physical properties, including those due to packing, and various thermodynamic, spectroscopic, interfacial and mechanical properties (See H. Brittain, Polymorphism in Pharmaceutical Solids, Marcel Dekker, New York, NY, 1999, pp. 1-2). Polymorphic and pseudopolymorphic forms of the drug substance (also known as the "active pharmaceutical ingredient" (API)), as administered by itself or formulated as a drug product (also known as the final or finished dosage form, or as the pharmaceutical composition) are well known and may affect, for example, the solubility, stability, flowability, fractability, and compressibility of drug substances and the safety and efficacy of drug products, (see, *e.g.*, Knapman, K Modern Drug Discoveries, March 2000: 53).

5-azacytidine (also known as azacitidine and 4-amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one; Nation Service Center designation NSC-102816; CAS Registry Number 320-67-2) has undergone NCI-sponsored clinical trials for the treatment of myelodysplastic syndromes (MDS). *See* Kornblith *et al.*, J. Clin. Oncol. 20(10): 2441-2452 (2002) and Silverman *et al.*, J. Clin. Oncol. 20(10): 2429-2440 (2002). 5-azacytidine may be defined as having a formula of $C_8H_{12}N_4O_5$, a molecular weight of 244.20 and a structure of:



The polymorphic form of 5-azacytidine drug substance and drug product has never been characterized. It is an object of the present invention to characterize the polymorphic forms of 5-azacytidine.

SUMMARY OF THE INVENTION

It has been unexpectedly found that 5-azacytidine exists in at least eight different polymorphic and pseudopolymorphic crystalline forms (Forms I-VIII), in addition to an amorphous form. Form I is a polymorph found in prior art retained samples of 5-azacytidine drug substance. Form II is a polymorph found in some prior art retained samples of the 5-azacytidine drug substance; in those samples, Form II is always found in mixed phase with Form I. Form III is a hydrate, and is formed when prior art retained and current samples of the drug product are reconstituted with water to form a "slurry" prior to administration to the patient. Form VI is found in prior art retained samples of the 5-azacytidine drug product, either substantially free of other polymorphs, or in mixed phase with Form I.

The invention provides novel crystalline forms referred to as Form IV, Form V, Form VII and Form VIII. Forms I-VIII each have characteristic X-ray power diffraction (XRPD) patterns and are easily distinguished from one another using XRPD.

Also included in the present invention are methods for robustly and reproducibly synthesizing 5-azacytidine drug substance substantially as Form IV, Form V, or Form VIII. Also provided are methods for robustly and reproducibly synthesizing a Form I/VII mixed phase. The invention also provides pharmaceutical compositions comprising the various forms of 5-azacytidine together with one or more pharmaceutically acceptable excipients, diluents, or carriers.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents the X-Ray Powder Diffraction (XRPD) pattern of 5-azacytidine, Form I, labeled with the most prominent 2θ angles (Cu Kα radiation).

Figure 2 presents the XRPD pattern of 5-azacytidine, mixed phase Form I and Form II, labeled with the most prominent 2θ angles (Cu Kα radiation).

Figure 3 presents the XRPD pattern of 5-azacytidine, Form III, labeled with the most prominent 2θ angles (Cu Kα radiation).

Figure 4 presents the XRPD pattern of 5-azacytidine, Form IV, labeled with the most prominent 2θ angles (Cu Kα radiation).

Figure 5 presents the XRPD pattern of 5-azacytidine, Form V, labeled with the most prominent 2θ angles (Cu Kα radiation).

Figure 6 presents the XRPD pattern of 5-azacytidine, Form VI, labeled with the most prominent 2θ angles (Cu Kα radiation).

Figure 7 presents the XRPD pattern of 5-azacytidine, mixed phase Form I and Form VII, labeled with the most prominent 2θ angles (Cu Kα radiation).

Figure 8 presents the XRPD pattern of 5-azacytidine, Form VIII, labeled with the most prominent 2θ angles (Cu Kα radiation).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

5-azacytidine Crystalline Forms I-VIII

It has been discovered that 5-azacytidine exists in at least eight different polymorphic and pseudopolymorphic crystalline forms, and also in an amorphous form.

Form I

A single sample of the 5-azacytidine drug substance was synthesized from 5-azacytosine and 1,2,3,5,-Tetra-O-acetyl-β-D-ribofuranose according to the prior art method provided in Example 1. The last step of this method is a recrystallization of the crude synthesis product from a DMSO/methanol co-solvent system. Specifically, the crude synthesis product is dissolved in DMSO (preheated to about 90°C), and then methanol is added to the DMSO solution. The co-solvent mixture is equilibrated at approximately -20°C to allow 5-azacytidine crystal formation. The product is collected by vacuum filtration and allowed to air dry.

The X-Ray Powder Diffraction (XRPD; see Example 5) pattern of the resulting 5-azacytidine is shown in **Figure 1** along with some of the 2θ values. Table 1 provides the most prominent 2θ angles, d-spacing and relative intensities for this material, which is designated as Form I.

2θ Angle (°)	d-spacing (Å)	Relative Intensity
12.182	7.260	39.1
13.024	6.792	44.1
14.399	6.146	31.5
16.470	5.378	27.1
18.627	4.760	16.0
19.049	4.655	35.9
20.182	4.396	37.0
21.329	4.162	12.4
23.033	3.858	100.0
23.872	3.724	28.0
26.863	3.316	10.8
27.135	3.284	51.5
29.277	3.048	25.6
29.591	3.016	11.5
30.369	2.941	10.8
32.072	2.788	13.4

Table 1: 5-azacytidine Form I - the most prominent 2θ angles, d-spacing and relative intensities (Cu Kα radiation)

Thermal analysis of Form I indicates that this form of 5-azacytidine is anhydrous. See Example 6.

Form II

Retained samples of the drug substance previously used to the formulate the drug product in the NCI-sponsored Cancer and Leukaemia Group B (CALGB) investigations (Phase 2 trial 8291 and Phase 3 trial 9221) for the treatment of MDS (Investigational New Drug (IND) 7574) were also analyzed by XRPD. The retained drug substance samples comprised either Form I, or a mixed phase of Form I and another polymorph: Form II. See Example 5.

The XRPD powder pattern of mixed phase Forms I and II is shown in **Figure 2** along with some of the 2θ values. Peaks distinctive to Form II are observed at 13.5, 17.6 and 22.3 ° 2θ. Table 2 provides the most prominent 2θ angles, d-spacing and relative intensities for this mixed phase.

2θ Angle (°)	d-spacing (Å)	Relative Intensity
12.244	7.223	34.8
13.082	6.762	37.0
13.458*	6.574	29.2
14.452	6.124	25.4
16.521	5.361	19.0
17.648*	5.022	12.1
18.677	4.747	12.7
19.093	4.645	41.3
20.231	4.386	42.1
21.353	4.158	15.5
22.309*	3.982	35.1
23.070	3.852	100.0
23.909	3.719	18.9
26.641	3.343	18.2
26.813	3.322	12.6
27.158	3.281	46.0
29.309	3.045	27.3
29.609	3.015	12.7
30.384	2.939	10.5
32.074	2.788	12.0

Table 2: 5-azacytidine, Mixed Phase Forms I and II - the most prominent 2θ angles, d-spacing and relative intensities (Cu Kα radiation)

These results indicate that the prior art 5-azacytidine synthesis procedures for the drug substance produce either Form I substantially free of other forms, or a Form I/II mixed phase *i.e.* a solid material in which 5-azacytidine is present in a mixed phase of both Form I and Form II.

Thermal analysis of mixed phase Form I/II is presented in Example 6.

Form III

An additional crystalline form of 5-azacytidine, designated Form III, is found in slurries of 5-azacytidine. See Example 8. Moreover, it has been found that all forms of 5-azacytidine (including the 5-azacytidine in the prior art drug product) convert to Form III in water. See Example 8. Thus, reconstitution of the drug product used in the aforementioned NCI trials would have led to the formation of a saturated solution (or "slurry") in which the remaining solid 5-azacytidine was Form III. The XRPD powder pattern of Form III is shown in **Figure 3** along with some of the 2θ values. Table 3 provides the most prominent 2θ angles, d-spacing and relative intensities for this crystalline material. The XRPD powder pattern for Form III is distinctly different from that of all of the other forms of 5-azacytidine.

2θ Angle (°)	d-spacing (Å)	Relative Intensity
6.566	13.450	32.9
11.983	7.380	52.5
13.089	6.758	71.0
15.138	5.848	38.9
17.446	5.079	48.2
20.762	4.275	10.8
21.049	4.147	34.8
22.776	3.901	89.5
24.363	3.651	13.7
25.743	3.458	22.8
26.305	3.385	39.9
28.741	3.104	100.0
31.393	2.847	22.5
32.806	2.728	11.8
33.043	2.709	10.1

33.536	2.670	15.1
36.371	2.468	11.0
39.157	2.299	19.3
41.643	2.167	12.1

Table 3: 5-azacytidine, Form III - the most prominent 2θ angles, d-spacing and relative intensities (Cu Kα radiation)

Thermal analysis and proton (^{1}H) NMR spectroscopy indicate that Form III is a pseudopolymorphic form of 5-azacytidine, specifically a monohydrate. See Examples 6-7.

Form IV

Form IV is a novel crystalline form of 5-azacytidine. Form IV was recovered by slow recrystallization from a DMSO/toluene co-solvent system (see Example 2) or by fast recrystallization from the DMSO/chloroform co-solvent system (see Example 3). The XRPD powder pattern of Form IV is shown in **Figure 4** along with some of the 2θ values. Table 4 provides the most prominent 2θ angles, d-spacing and relative intensities for this crystalline material. The XRPD powder pattern for Form IV is distinctly different from that of any other form.

2θ Angle (°)	**d-spacing (Å)**	**Relative Intensity**
5.704	15.408	24.9
11.571	7.642	97.8
12.563	7.040	22.2
14.070	6.289	100.0
15.943	5.555	67.4
16.993	5.213	51.0
18.066	4.906	20.1
20.377	4.355	44.7
20.729	4.281	49.0
21.484	4.132	36.30
21.803	4.073	11.2
22.452	3.957	66.7
22.709	3.913	64.0
23.646	3.760	17.3
24.068	3.695	19.4

25.346	3.526	12.0
25.346	3.511	12.5
26.900	3.312	11.0
27.991	3.185	11.4
28.527	3.126	25.7
28.723	3.106	34.1
30.124	2.964	14.7
30.673	2.912	53.6
31.059	2.877	15.7
35.059	2.557	18.1
38.195	2.354	15.0
38.403	2.342	12.6

Table 4: 5-azacytidine Form IV - the most prominent 2θ angles, d-spacing and relative intensities (Cu Kα radiation)

Thermal analysis of Form IV is presented in Example 6.

Form V

Form V is a novel crystalline form of 5-azacytidine. Form V was recovered by fast recrystallization of 5-azacytidine from a DMSO/toluene co-solvent system (see Example 3). The XRPD powder pattern of Form V is shown in **Figure 5** along with some of the 2θ values. Table 5 provides the most prominent 2θ angles, d-spacing and relative intensities for this crystalline material. The XRPD powder pattern for Form V is distinctly different from that of any other form.

2θ Angle (°)	**d-spacing (Å)**	**Relative Intensity**
11.018	8.024	40.0
12.351	7.160	29.6
13.176	6.714	28.3
13.747	6.436	42.9
14.548	6.084	18.3
15.542	5.697	14.2
16.556	5.350	47.8
17.978	4.930	18.1
18.549	4.780	83.9
19.202	4.618	25.0
19.819	4.476	12.1
20.329	4.365	28.6

21.518	4.126	100.0
21.970	4.042	65.6
22.521	3.948	11.5
23.179	3.834	66.5
24.018	3.702	13.0
24.569	3.620	40.7
27.224	3.273	50.2
28.469	3.133	24.2
29.041	3.072	24.8
29.429	3.033	15.0
30.924	2.889	15.6
31.133	2.870	22.6
37.938	2.370	10.7

Table 5: 5-azacytidine Form V - the most prominent 2θ angles, d-spacing and relative intensities (Cu Kα radiation)

Thermal analysis indicates that Form V is a solvate. See Example 6.

Form VI

The drug product used in the aforementioned NCI investigation was typically prepared by lypohilizing a solution of 5-azacytidine and mannitol (1:1 w/w). The resultant drug product comprised 100 mg of 5-azacytidine and 100 mg mannitol as a lyophilized cake in a vial and was administered by subcutaneous injection as an aqueous suspension ("slurry"). XRPD analysis of retained samples of the drug product used in the NCI investigation revealed the existence of another polymorph, Form VI. The retained drug product samples comprised either Form VI alone, or a Form I/VI mixed phase. Table 6 provides the most prominent 2θ angles, d-spacing and relative intensities for Form VI.

2θ Angle (°)	**d-spacing (Å)**	**Relative Intensity**
12.533	7.057	10.1
12.963	6.824	10.2
13.801	6.411	100.0
18.929	4.6843	10.0
20.920	4.243	34.2
21.108	4.205	49.4
21.527	4.125	47.0
22.623	3.922	10.7
22.970	3.869	13.8

24.054	3.697	77.8
26.668	3.340	23.0
27.210	3.275	33.7
28.519	3.127	12.9
29.548	3.021	27.2
30.458	2.932	50.3
33.810	2.649	11.6
35.079	2.556	12.6
37.528	2.411	24.7

Table 6: 5-azacytidine Form VI - the most prominent 2θ angles, d-spacing and relative intensities (Cu Kα radiation)

Thermal analysis and proton (^{1}H) NMR spectroscopy of Form VI is presented in Examples 6-7.

Form VII

Form VII is a novel crystalline form of 5-azacytidine. Form VII was produced by fast recrystallization from a DMSO/methanol co-solvent system (see Example 3). Form VII was always isolated by this recrystallization method as a mixed phase with Form I. The XRPD powder pattern of mixed phase Forms I and VII is shown in **Figure 7** along with some of the 2θ values and the Form VII distinctive peaks indicated with asterisks. Table 7 provides the most prominent 2θ angles, d-spacing and relative intensities for this mixed phase. Form VII exhibits distinctive peaks at 5.8, 11.5, 12.8, 22.4 and 26.6 ° 2θ in addition to peaks displayed in the Form I XRPD powder pattern. The XRPD pattern for mixed phase Forms I and VII is distinctly different from that of any other form.

2θ Angle (°)	**d-spacing (Å)**	**Relative Intensity**
5.779	15.281	14.7
11.537	7.664	8.3
12.208	7.244	28.0
12.759	6.932	21.7
13.048	6.780	34.4
14.418	6.138	22.5
16.489	5.372	21.6
18.649	4.754	13.5
19.101	4.643	34.7
20.200	4.392	34.4

20.769	4.273	10.5
21.355	4.157	11.7
22.365	3.972	29.9
23.049	3.856	100.0
23.884	3.723	23.1
26.628	3.345	13.3
27.145	3.282	52.9
29.296	3.046	26.2
29.582	3.017	11.3
32.078	2.788	12.9

Table 7: 5-azacytidine, mixed Forms I and VII - the most prominent 2θ angles, d-spacing and relative intensities (Cu Kα radiation)

Thermal analysis of Form VII is presented in Example 6.

Form VIII

Form VIII is a novel crystalline form of 5-azacytidine. Form VIII was recovered by recrystallizing 5-azacytidine Form I from a N-methyl-2-pyrrolidone (NMP) single solvent system (see Example 4). The XRPD powder pattern of Form VIII is shown in **Figure 8** along with some of the 2θ values. Table 8 provides the most prominent 2θ angles, d-spacing and relative intensities for this material. The XRPD pattern for Form VIII is distinctly different from that of any other form.

2θAngle (°)	**d-spacing (Å)**	**Relative Intensity**
6.599	13.384	2.9
10.660	8.292	2.2
12.600	7.020	23.4
13.358	6.623	2.6
15.849	5.587	2.0
17.275	5.129	4.2
20.243	4.383	5.8
20.851	4.257	7.8
21.770	4.079	74.4
22.649	3.923	32.1
25.554	3.483	100.0
25.740	3.458	7.8
29.293	3.046	3.8
32.148	2.782	8.8
35.074	2.556	7.4
38.306	2.348	2.5

Table 8: 5-azacytidine, Form VIII - the most prominent 2θ angles, d-spacing and relative intensities (Cu Kα radiation)

Amorphous 5-azacytidine

Amorphous 5-azacytidine may be recovered from equilibrium saturated solutions of 5-azacytidine in propylene glycol, polyethylene glycol and DMSO. See Example 8.

Pharmaceutical Formulations

For the most effective administration of drug substance of the present invention, it is preferred to prepare a pharmaceutical formulation (also known as the "drug product") preferably in unit dose form, comprising one or more of the 5-azacytidine forms of the present invention and one or more pharmaceutically acceptable carrier, diluent, or excipient.

Such pharmaceutical formulation may, without being limited by the teachings set forth herein, include a solid form of the present invention which is blended with at least one pharmaceutically acceptable excipient, diluted by an excipient or enclosed within such a carrier that can be in the form of a capsule, sachet, tablet, buccal, lozenge, paper, or other container. When the excipient serves as a diluent, it may be a solid, semi-solid, or liquid material which acts as a vehicle, carrier, or medium for the 5-azacytidine polymorph(s). Thus, the formulations can be in the form of tablets, pills, powders, elixirs, suspensions, emulsions, solutions, syrups, capsules (such as, for example, soft and hard gelatin capsules), suppositories, sterile injectable solutions, and sterile packaged powders.

Examples of suitable excipients include, but are not limited to, starches, gum arabic, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrup, and methyl cellulose. The formulations can additionally include lubricating agents such as, for example, talc, magnesium stearate and mineral oil; wetting agents; emulsifying and suspending agents; preserving agents such as methyl- and propyl- hydroxybenzoates; sweetening agents; or flavoring agents. Polyols, buffers, and inert fillers may also be used. Examples of polyols include, but are not limited to: mannitol, sorbitol, xylitol, sucrose, maltose, glucose, lactose, dextrose, and the like. Suitable buffers encompass, but are not limited to, phosphate, citrate, tartrate, succinate, and the like. Other inert fillers which may be

used encompass those which are known in the art and are useful in the manufacture of various dosage forms. If desired, the solid pharmaceutical compositions may include other components such as bulling agents and/or granulating agents, and the like. The compositions of the invention can be formulated so as to provide quick, sustained, controlled, or delayed release of the drug substance after administration to the patient by employing procedures well known in the art.

In certain embodiments of the invention, the 5-azacytidine forms(s) may be made into the form of dosage units for oral administration. The 5-azacytidine forms(s) may be mixed with a solid, pulverant carrier such as, for example, lactose, saccharose, sorbitol, mannitol, starch, amylopectin, cellulose derivatives or gelatin, as well as with an antifriction agent such as for example, magnesium stearate, calcium stearate, and polyethylene glycol waxes. The mixture is then pressed into tablets or filled into capsules. If coated tablets, capsules, or pulvules are desired, such tablets, capsules, or pulvules may be coated with a concentrated solution of sugar, which may contain gum arabic, gelatin, talc, titanium dioxide, or with a lacquer dissolved in the volatile organic solvent or mixture of solvents. To this coating, various dyes may be added in order to distinguish among tablets with different active compounds or with different amounts of the active compound present.

Soft gelatin capsules may be prepared in which capsules contain a mixture of the 5-azacytidine form(s) and vegetable oil or non-aqueous, water miscible materials such as, for example, polyethylene glycol and the like. Hard gelatin capsules may contain granules or powder of the 5-azacytidine polymorph in combination with a solid, pulverulent carrier, such as, for example, lactose, saccharose, sorbitol, mannitol, potato starch, corn starch, amylopectin, cellulose derivatives, or gelatin.

Tablets for oral use are typically prepared in the following manner, although other techniques may be employed. The solid substances are gently ground or sieved to a desired particle size, and a binding agent is homogenized and suspended in a suitable solvent. The 5-azacytidine form(s) and auxiliary agents are mixed with the binding agent solution. The resulting mixture is moistened to form a uniform suspension. The moistening typically causes the particles to aggregate slightly, and the resulting mass is gently pressed through a stainless steel sieve having a desired size. The layers of the mixture are then dried in controlled drying units for a pre-determined length of time to achieve a desired particle size and consistency. The granules of the dried mixture are gently sieved to remove any powder. To this mixture,

disintegrating, anti-friction, and anti-adhesive agents are added. Finally, the mixture is pressed into tablets using a machine with the appropriate punches and dies to obtain the desired tablet size.

In the event that the above formulations are to be used for parenteral administration, such a formulation typically comprises sterile, aqueous and non-aqueous injection solutions comprising one or more 5-azacytidine forms for which preparations are preferably isotonic with the blood of the intended recipient. These preparations may contain anti-oxidants, buffers, bacteriostats, and solute; which render the formulation isotonic with the blood of the intended recipient. Aqueous and non-aqueous suspensions may include suspending agents and thickening agents. The formulations may be present in unit-dose or multi-dose containers, for example, sealed ampules and vials. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets of the kind previously described.

Liquid preparations for oral administration are prepared in the form of solutions, syrups, or suspensions with the latter two forms containing, for example, 5-azacytidine polymorph(s), sugar, and a mixture of ethanol, water, glycerol, and propylene glycol. If desired, such liquid preparations contain coloring agents, flavoring agents, and saccharin. Thickening agents such as carboxymethylcellulose may also be used.

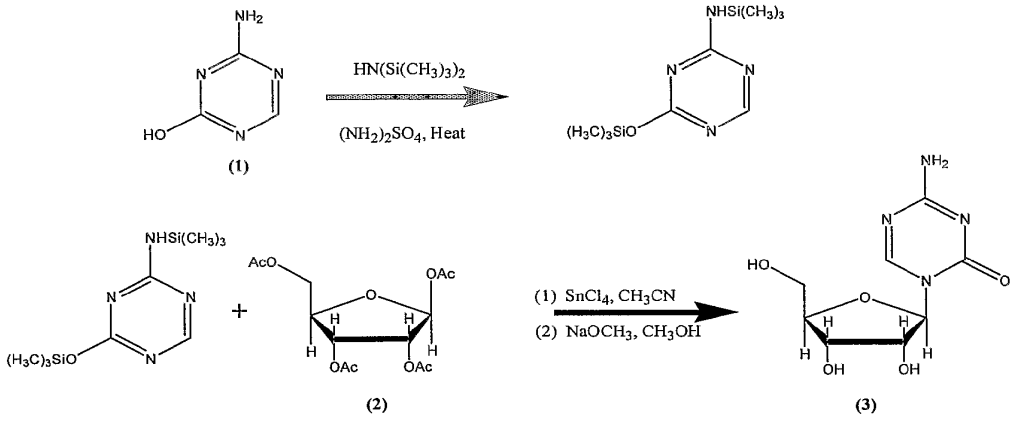
As such, the pharmaceutical formulations of the present invention are preferably prepared in a unit dosage form, each dosage unit containing from about 5 mg to about 200 mg, more usually about 100 mg of the 5-azacytidine form(s). In liquid form, dosage unit contains from about 5 to about 200 mg, more usually about 100 mg of the 5-azacytidine form(s). The term "unit dosage form" refers to physically discrete units suitable as unitary dosages for human subjects/patients or other mammals, each unit containing a predetermined quantity of the 5-azacytidine polymorph calculated to produce the desired therapeutic effect, in association with preferably, at least one pharmaceutically acceptable carrier, diluent, or excipient.

The following examples are provided for illustrative purposes only, and are not to be construed as limiting the scope of the claims in any way.

Examples

Example 1: Prior Art Procedure for Synthesis of 5-azacytidine Drug Substance

Using commercially available 5-azacytosine (1) and 1,2,3,5-Tetra-O- β-acetyl-ribofuranose (2) (RTA) , 5-azacytidine (3) may be synthesized according to the pathway



below.

The crude synthesis product is dissolved in DMSO (preheated to about 90°C), and then methanol is added to the DMSO solution. The co-solvent mixture is equilibrated at approximately -20°C to allow 5-azacytidine crystal formation. The product is collected by vacuum filtration and allowed to air dry.

Example 2: Slow Recrystallization from DMSO/toluene

Dimethyl sulfoxide (DMSO) was used as the primary solvent to solubilize Form I of 5-azacytidine and toluene was used as the co-solvent as follows. Approximately 250 mg of 5-azacytidine was dissolved with approximately 5 mL of DMSO, preheated to approximately 90 °C, in separate 100-mL beakers. The solids were allowed to dissolve to a clear solution. Approximately 45 mL of toluene, preheated to approximately 50 °C, was added to the solution and the resultant solution was mixed. The solution was covered and allowed to equilibrate at ambient conditions. The product was collected by vacuum filtration as white crystals using a Buchner funnel. The collected product was allowed to air dry.

Example 3: Fast Recrystallization from DMSO/methanol, DMSO/toluene, and DMSO/chloroform

Approximately 250 mg of 5-azacytidine was dissolved with approximately 5 mL of DMSO as the primary solvent, preheated to approximately 90 °C, in separate 100-ml beakers. The solids were allowed to dissolve to a clear solution. Approximately 45 mL of the selected co-solvent (methanol, toluene, or chloroform), preheated to approximately 50 °C, was added to the solution and the resultant solution was mixed. The solution was covered and placed in a freezer to equilibrate at approximately -20°C to allow crystal formation. Solutions were removed from the freezer after crystal formation.

The product from the methanol and toluene solutions was collected by vacuum filtration using a Buchner funnel. The resulting white crystalline product was allowed to air dry.

The chloroform product was too fine to be collected by vacuum filtration. Most of the solvent was carefully decanted from the chloroform solution and the solvent from the resultant slurry was allowed to evaporate at ambient temperature to dryness. The chloroform solution evaporated to a white product. Note that fast recrystallization using the DMSO/methanol co-solvent system has typically been used to prepare 5-azacytidine drug substance in the prior art (see the last step of the procedure provided in Example 1).

Example 4: Fast Recrystallization N-methyl-2-pyrrolidone (NMP) Single Solvent System

Approximately 500 mg of 5-azacytidine was dissolved with approximately 5 mL of NMP, preheated to approximately 90 °C, in separate 50-mL beakers. The solids were allowed to dissolve to a clear solution. The solution was covered and placed in a freezer to equilibrate at approximately -20 °C to allow crystal formation. Solutions were removed from the freezer after crystal formation, equilibrated at ambient temperature. The product was collected by vacuum filtration using a Buchner funnel. The collected product was allowed to air dry.

Example 5: X-Ray Powder Diffraction of 5-azacytidine

X-ray powder diffraction patterns for each sample were obtained on a Scintag XDS 2000 or a Scintag X_2 θ/θ diffractometer operating with copper radiation at 45 kV and 40 mA using a Kevex Psi Peltier-cooled silicon detector or a Thermo ARL Peltier-cooled solid state detector. Source slits of 2 or 4 mm and detector slits of 0.5 or 0.3 mm were used for data collection. Recrystallized material was gently milled using an agate mortar and pestle for approximately

one minute. Samples were placed in a stainless steel or silicon sample holder and leveled using a glass microscope slide. Powder diffraction patterns of the samples were obtained from 2 to 42° 2θ at 1°/minute. Calibration of the X_2 diffractometer is verified annually using a silicon powder standard. Raw data files were converted to ASCII format, transferred to an IBM compatible computer and displayed in Origin® 6.1 for Windows.

XRPD of a single sample of 5-azacytidine produced according to the method of Example 1 revealed that this sample consisted of Form I of 5-azacytidine.

NCI retained drug substance sample samples were also analyzed. These samples were all previously synthesized and recrystallized according to the method of Example 1 and were stored at 5°C since production. XRPD revealed some retained samples are comprised of Form I alone, whereas other retained samples contain a mixed phase of Form I and a different polymorph, termed Form II.

XRPD of NCI retained drug product samples revealed the existence of Form VI in some samples. In those samples, Form VI was present as a mixed phase with Form I.

XRPD of the recrystallized 5-azacytidine obtained in Example 2 revealed that slow recrystallization from a DMSO/toluene system produced Form IV. XRPD of the recrystallized 5-azacytidine obtained in Example 3 revealed that fast recrystallization from a DMSO/chloroform system produced Form IV, fast recrystallization from a DMSO/toluene system produced Form V, and fast recrystallization from a DMSO/methanol system produced mixed phased Form I/Form VII. XRPD of the recrystallized 5-azacytidine obtained in Example 4 revealed that the N-methyl-2-pyrrolidone solvent system produced Form VIII.

Example 6: Thermal Analysis of 5-azacytidine

Differential Scanning Calorimetry (DSC) measurements for each sample were collected using a Perkin Elmer Pyris 1 DSC system equipped with an Intracooler 2P refrigeration unit. The Pyris 1 DSC was purged with nitrogen. Calibration was performed prior to analysis using an Indium standard at a 10 °C minute heating rate. Each sample was gently ground in an agate mortar and pestle. Approximately 1-3 mg of the sample were individually sealed in a Perkin Elmer 30-μL universal aluminum pan with holes in the lid. Samples were heated from 25 °C to 250 °C or 350 °C at 10 °C/minute.

Thermogravimetric Analysis (TGA) measurements for each sample were collected using a Perkin Elmer TGA 7 purged with nitrogen at approximately 20 cc/minute. A 100-mg standard weight and nickel metal were used to verify balance and temperature calibrations, respectively. Samples were heated from 25 °C to 250 °C or 300 °C at 10 °C/minute.

Capillary melting point (MP) measurements were made using an Electrothermal 9300 melting point apparatus. A heating rate of 10 °C/minute was used from set point temperatures described in individual discussions. Visual melting points are reported as an average of triplicate determinations.

The results are as follows:

Form I

TGA showed a weight loss of 0.23% between ambient and 150 °C, which indicates that it is anhydrous. DSC exhibited a single event with an onset of 227.0 °C.

A capillary melting point determination was performed in triplicate on a sample of Form I of 5-azacytidine. The sample was visually observed to decompose without melting at about 215 °C using a 10 °C heating rate and a starting temperature of 200 °C. Thus, the DSC event results from decomposition of 5-azacytidine.

Form I/II Mixed Phase

The TGA for the Form I/II mixed phase showed a weight loss of 1.16% between ambient temperature and 150 °C. The DSC analysis exhibited a single event with an onset at 229.8 °C. The decomposition of the mixed phase was consistent with that observed for 5-azacytidine Form I.

Form III

The TGA showed a weight loss of between 6.56% and 8.44% when the temperature was raised from ambient and 150 °C. The loss is close to the theoretical amount of moisture,6.9 %, that 5-azacytidine monohydrate would have. The DSC analysis exhibited an endotherm, which is in the range associated with solvent loss, and a higher temperature event. The

endotherm exhibited an onset temperature in the range of 86.4-89.2 °C, peak temperatures in the range of 95.8-97.0 °C and ΔH values in the range of 73.1-100.5 J/g. The higher temperature event had onset temperatures in the range 229.1-232.1 °C and was consistent with the decomposition observed for 5-azacytidine Form I.

5-azacytidine Form III was heated at 105 °C for 4 hours in an attempt to dehydrate the material. The material did not change its physical appearance during heating. TGA was used to measure the water content of Form III before and after drying. The initial amount of moisture present in Form III was 6.31 % and was <0.1 % after drying. The XRPD powder pattern for dehydrated Form III matches that of Form I. Thus, Form III dehydrates to Form I.

Form IV

The TGA showed a weight loss of 21.80% between ambient temperature and 150 °C, which does not correspond to the solvent content for any simple solvates. It is not known whether crystalline Form IV is a polymorph or a pseudopolymorph.

The DSC analysis exhibited two endotherms and a higher temperature event. The two endotherms are in the range that is associated with solvent loss. The first endotherm exhibited an onset temperature of 87.6 °C, a peak temperature of 90.1 °C and ΔH value of 98.3 J/g. The second endotherm exhibited an onset temperature of 136.0 °C, a peak temperature of 139.0 °C and ΔH value of 81.8 J/g. The higher temperature event had an onset temperature of 230.6 °C and was consistent with the decomposition that was observed for 5-azacytidine Form I.

Form V

TGA showed a weight loss of 21.45% between ambient and 150 °C, which does not correspond to the solvent content for any simple solvate. The DSC analysis exhibited two merged endotherms, a single endotherm and a higher temperature event. The three endotherms are in the range that is associated with solvent loss. The two merged endotherms exhibit onset temperatures of 66.6 and 68.0 °C. The single endotherm exhibited an onset temperature of 88.7 °C, a peak temperature of 121.5 °C and a ΔH value of 180.3 J/g. The higher temperature event had onset temperature of 230.7 °C and was consistent with the decomposition that was observed for 5-azacytidine Form I.

Form VI

TGA showed a weight loss of 1.10% between ambient temperature and 150 °C. The DSC analysis exhibited a small endotherm, an exotherm and a higher temperature event. The small endotherm exhibited an onset temperature of 57.8 °C, a peak temperature of 77.0 °C and a ΔH value of 55.7 J/g. The exotherm exhibited an onset temperature of 149.3 °C, a peak temperature of 157.1 °C and an ΔH value of -17.9 J/g. The higher temperature event had an onset temperature of 234.7 °C and was consistent with the decomposition observed for 5-azacytidine Form I.

Form VII

TGA showed a weight loss of 2.45% between ambient temperature and 150 °C. The DSC analysis exhibited a minor endotherm and a higher temperature event. The minor endotherm had an onset temperature of 63.3 °C, a peak temperature of 68.3 °C and a ΔH value of 17.1 J/g. The higher temperature event had an onset temperature of 227.2 °C and is consistent with the decomposition observed for 5-azacytidine Form I.

Example 7: Nuclear Magnetic Resonance (NMR) Analysis of Form III and Form VI

5-azacytidine is know to be labile in water. Since Form III is found in equilibrium saturated solutions and Form VI is produced by the lyophilization of 5-azacytidine solution, it was of interest to examine the purity of these 5-azacytidine forms using NMR. The proton (^{1}H) NMR spectra of Form III and Form VI were both consistent with the structure of 5-azacytidine in all essential details.

Example 8: Polymorphic Form Conversion of 5-azacytidine

Form I of 5-azacytidine was added to various solvents in sufficient quantities to form a slurry, and the slurry allowed to equilibrate for a period of time. The solid material that was present in the slurry was recovered, dried, and analyzed using XRPD (according to the XRPD

protocol included in Example 5) with the aim of detecting new polymorphs and pseudopolymorphs during the transition to the dissolved state. Samples equilibrated for 19 hours in saline, 5% dextrose, 5% tween 80, water-saturated octanol, ethanol/water (50/50) and water alone resulted in a distinctly different form of 5-azacytidine, designated Form III (see below). Samples equilibrated for 19 hours in acetone, methyl ethyl ketone, and ethanol resulted in materials identified as Form I. Samples equilibrated for 19 hours in propylene glycol, polyethylene glycol and DMSO resulted in amorphous materials. The results are summarized in Table 9.

Solvent	XRPD Pattern Assignment
Normal Saline	Form III
5% Dextrose	Form III
Acetone	Form I
Propylene glycol	Amorphous
Polyethylene glycol	Amorphous
Methyl ethyl ketone	Form I
5 % Tween 80	Form III
DMSO	Amorphous
Water-saturated Octanol	Form III
Ethyl alcohol	Form I
50/50 EtOH/DI Water	Form III
DI Water	Form III

Table 9: X-ray Powder Diffraction Analysis Results for Solubility Samples: Form Assignment (Cu K(radiation)

The conversion of other forms of 5-azacytidine was also studied. Specifically, a Form I/II mixed phase, Form VI (the lyophilized drug product used in the prior art NCI drug trials), a Form I/VI mixed phase, and a Form I/VII mixed phase were weighed into individual small glass beakers and water was pipeted into each beaker. The sample size and water volume were scaled to maintain an approximate 25 mg/mL ratio. The resultant slurry was allowed to equilibrate for 15 minutes. After equilibration, the sample was filtered and the solid material was dried and analyzed using XRPD. In each case, Form III of 5-azacytidine was observed. The results indicate that all forms of 5-azacytidine convert to Form III during the transition to the dissolved state in water. Thus, when an 5-azacytidine suspension ("slurry") was

administered to patients in the aforementioned NCI investigation, the patients received both 5-azacytidine in solution, and Form III of 5-azacytidine.

What is claimed is:

1. Form IV of 5-azacytidine characterized by a X-Ray Powder Diffraction pattern obtained by irradiation with Cu Kα X-rays which shows main peaks with interplanar spacings at:

d(Å)
15.408
7.642
7.040
6.289
5.555
5.213
4.906
4.355
4.281
4.132
4.073
3.957
3.913
3.760
3.695
3.526
3.511
3.312
3.185
3.126
3.106
2.964
2.912
2.877
2.557
2.354
2.342.

2. A pharmaceutical composition comprising the 5-azacytidine form of claim 1 and a pharmaceutically acceptable excipient, diluent, or carrier.

3. Form V of 5-azacytidine characterized by a X-Ray Powder Diffraction pattern obtained by irradiation with Cu Kα X-rays which shows main peaks with interplanar spacings at:

d(Å)
8.024
7.160
6.714
6.436
6.084
5.697
5.350
4.930
4.780
4.618
4.476
4.365
4.126
4.042
3.948
3.834
3.702
3.620
3.273
3.133
3.072
3.033
2.889
2.870
2.370.

4. A pharmaceutical composition comprising the 5-azacytidine form of claim 3 and a pharmaceutically acceptable excipient, diluent, or carrier.

5. Form VII of 5-azacytidine characterized by a X-Ray Powder Diffraction pattern obtained by irradiation with Cu Kα X-rays which includes diffraction peaks at 5.8, 11.5, 12.8, 22.4 and 26.6 ° 2θ.

6. A pharmaceutical composition comprising the 5-azacytidine form of claim 5 and a pharmaceutically acceptable excipient, diluent, or carrier.

7. Form VIII of 5-azacytidine characterized by a X-Ray Powder Diffraction pattern obtained by irradiation with Cu Kα X-rays which shows main peaks with interplanar spacings at:

d(Å)
13.384
8.292
7.020
6.623
5.587
5.129
4.383
4.257
4.079
3.923
3.483
3.458
3.046
2.782
2.556
2.348.

8. A pharmaceutical composition comprising the 5-azacytidine form of claim 7 and a pharmaceutically acceptable excipient, diluent, or carrier.

9. A method for preparing Form IV of 5-azacytidine substantially free of other forms, the method comprising crystallizing 5-azacytidine from a solvent mixture comprising dimethylsulfoxide and chloroform by cooling said solution from a temperature selected to allow said 5-azacytidine to dissolve completely to about -20°C; and
isolating said crystals.

10. A method for preparing Form IV of 5-azacytidine substantially free of other forms, the method comprising:

crystallizing 5-azacytidine from a solvent mixture comprising dimethylsulfoxide and toluene by cooling said solution from a temperature selected to allow said 5-azacytidine to dissolve completely to about ambient temperature; and

isolating said crystals.

11. A method for preparing Form V of 5-azacytidine substantially free of other forms, the method comprising:

crystallizing 5-azacytidine from a solvent mixture comprising dimethylsulfoxide and toluene by cooling said solution from a temperature selected to allow said 5-azacytidine to dissolve completely to about -20°C; and

isolating said crystals.

12. A method for preparing a mixed phase of Form I of 5-azacytidine and Form VII of 5-azacytidine substantially free of other forms, the method comprising:

crystallizing 5-azacytidine from a solvent mixture comprising dimethylsulfoxide and methanol by cooling said solution from a temperature selected to allow said 5-azacytidine to dissolve completely to about -20°C; and

isolating said crystals.

13. A method for preparing Form VIII of 5-azacytidine substantially free of other forms, comprising crystallizing 5-azacytidine from N-methyl-2-pyrrolidone and isolating said crystals.

Figure 1. X-ray Powder Diffraction Pattern of Azacitidine, Form I, Labeled with the more Prominent 2θ Angles (Cu Kα Radiation)

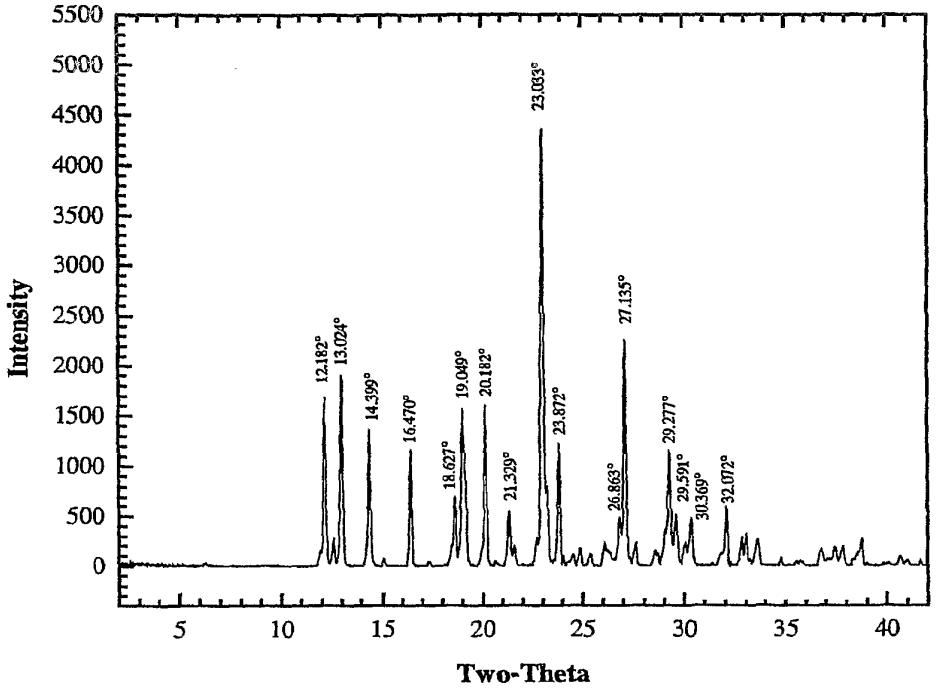


Figure 2 X-ray Powder Diffraction Pattern of Azacitidine, Mixed Phase Forms I and II, Labeled with the more Prominent 2θ Angles (Cu Kα Radiation)

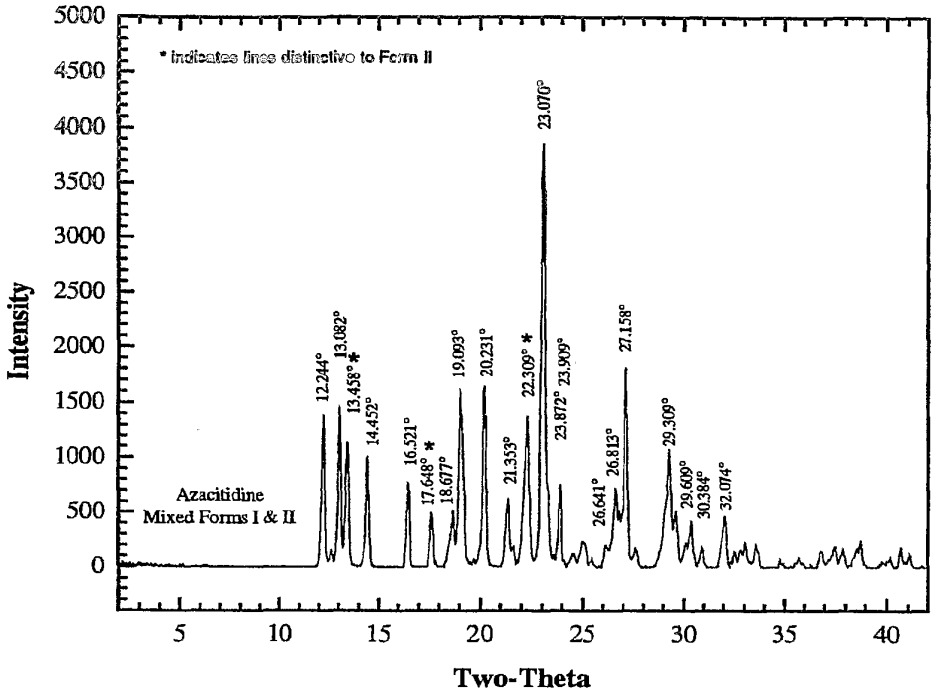


Figure 3 X-ray Powder Diffraction Pattern of Azacitidine, Form III, Labeled with the more Prominent 2θ Angles (Cu Kα Radiation)

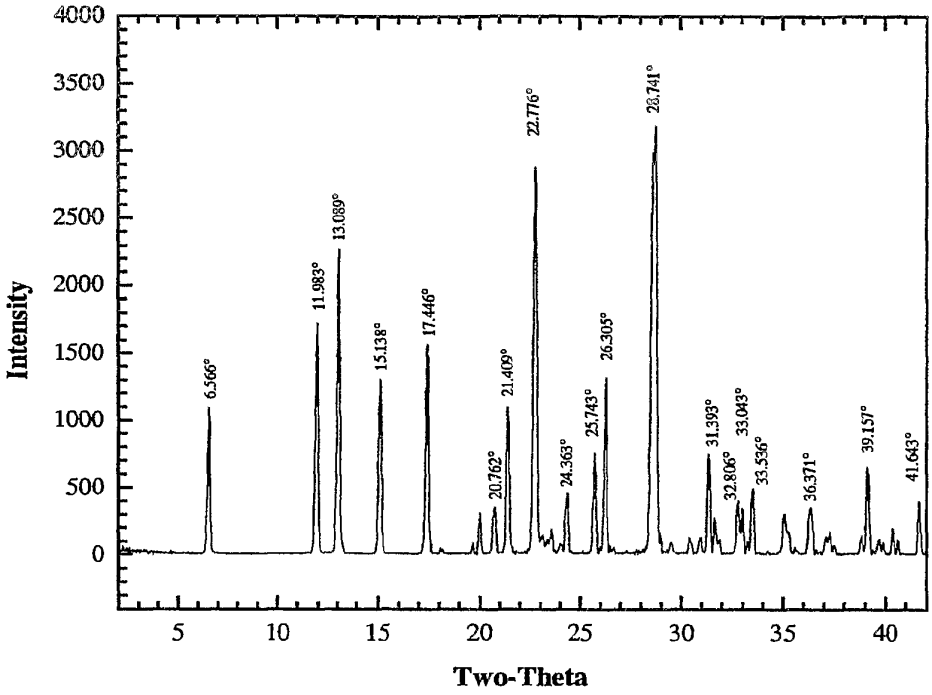


Figure 4 X-ray Powder Diffraction Pattern of Azacitidine, Form IV, Labeled with the more Prominent 2θ Angles (Cu Kα Radiation)

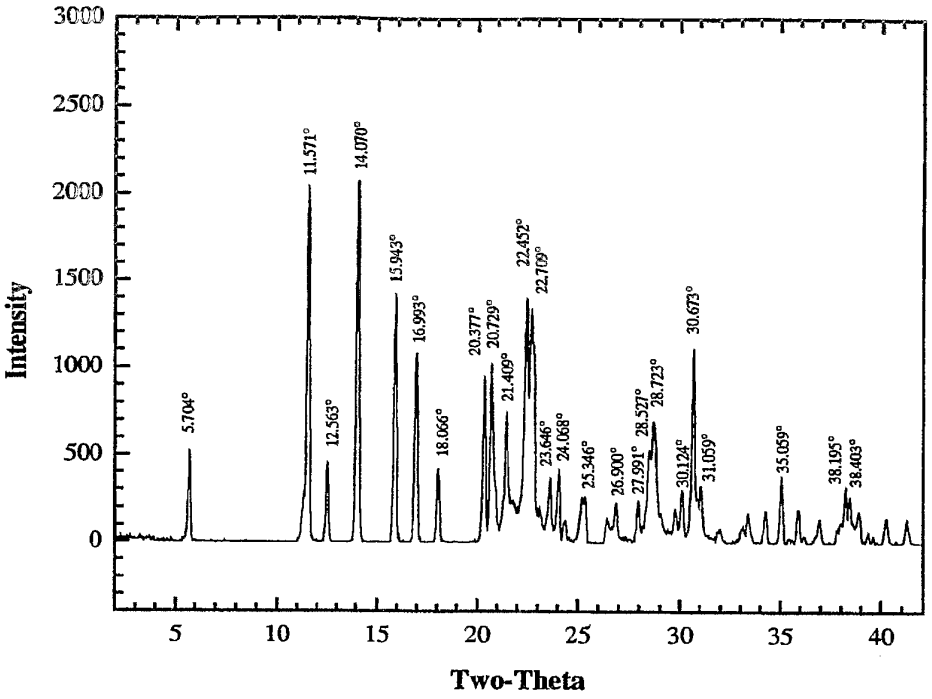


Figure 5 **X-ray Powder Diffraction Pattern of Azacitidine, Form V, Labeled with the more Prominent 2θ Angles (Cu Kα Radiation)**

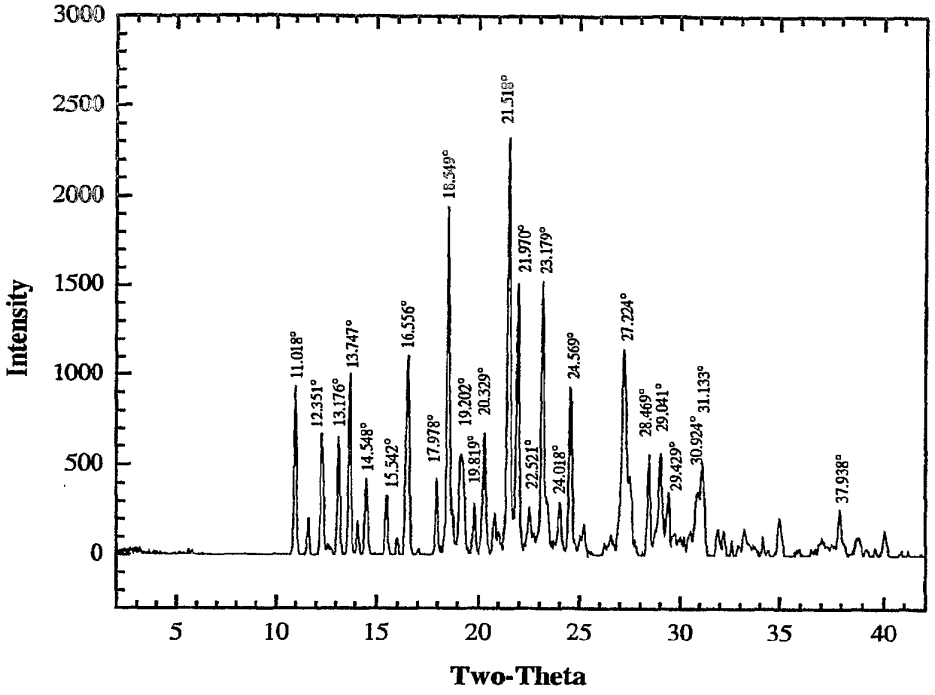


Figure 6 X-ray Powder Diffraction Pattern of Azacitidine, Form VI, Labeled with the more Prominent 2θ Angles (Cu Kα Radiation)

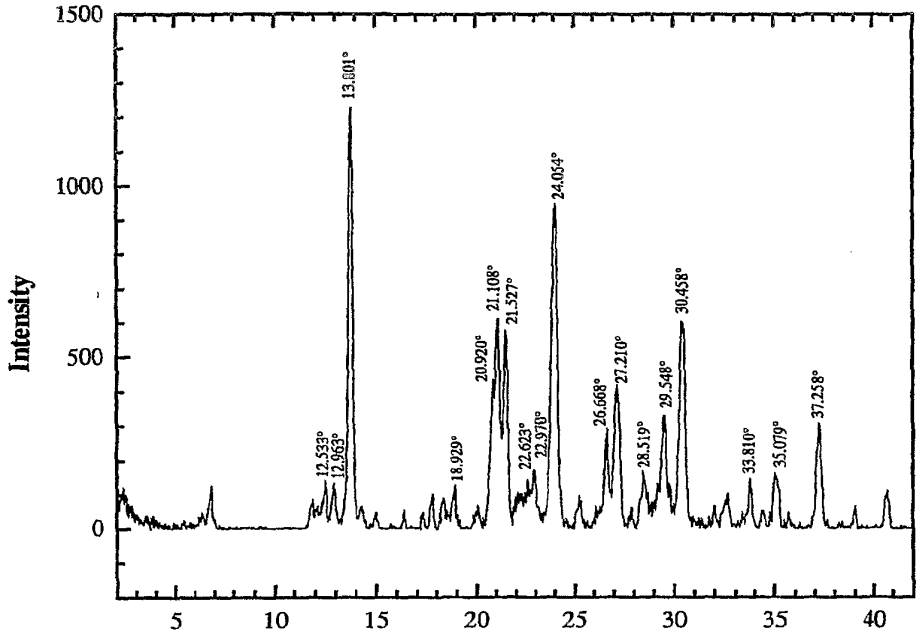


Figure 7 X-ray Powder Diffraction Pattern of Azacitidine, Mixed Phase Forms I and VII, Labeled with the more Prominent 2θ Angles (Cu Kα Radiation)

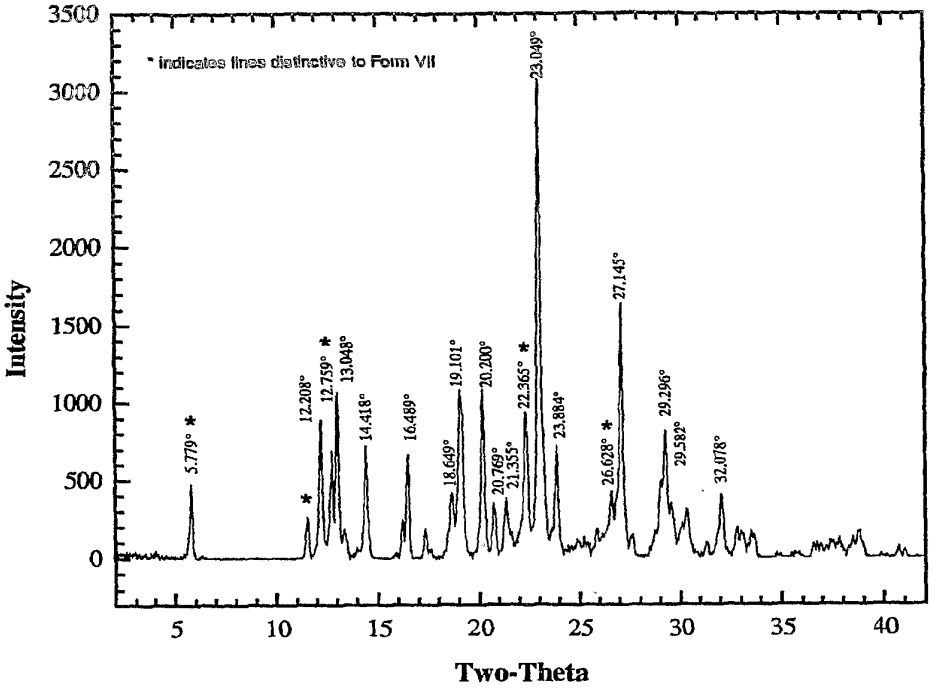
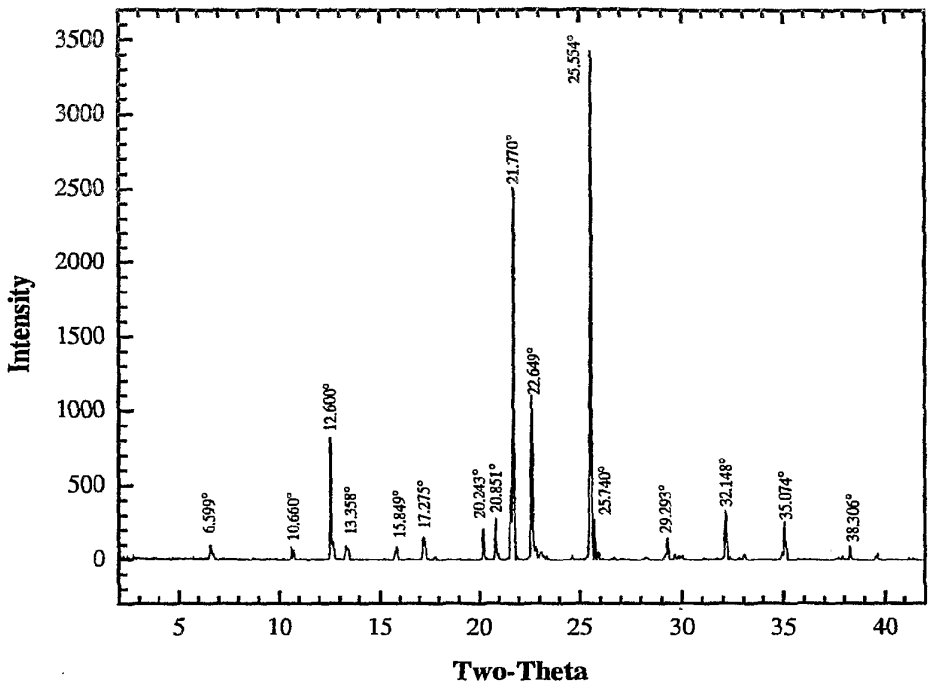


Figure 8 X-ray Powder Diffraction Pattern of Azacitidine, Form VIII, Labeled with the more Prominent 2θ Angles (Cu Kα Radiation)



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(54) Title: SALTS OF 5-AZACYTIDINE

(57) Abstract: The present invention relates to salts of 5-azacytidine as well as methods for synthesizing the salts described herein. Pharmaceutical compositions and methods of using the 5-azacytidine salts are also provided, including methods of administering the salts or pharmaceutical compositions thereof to treat conditions, such as cancer and hematological disorders.

SALTS OF 5-AZACYTIDINE

BACKGROUND OF THE INVENTION

A few azacytosine nucleosides, such as 5-aza-2'-deoxycytidine (also called decitabine) and 5-azacytidine (also called azacitidine), have been developed as antagonist of its related natural nucleoside, 2'-deoxycytidine and cytidine, respectively. The only structural difference between azacytosine and cytosine is the presence of a nitrogen at position 5 of the cytosine ring in azacytosine as compared to a carbon at this position for cytosine.

Two isomeric forms of decitabine can be distinguished. The β-anomer is the active form. The modes of decomposition of decitabine in aqueous solution are (a) conversion of the active β-anomer to the inactive α-anomer (Pompon et al. (1987) J. Chromat. 388:113-122); (b) ring cleavage of the aza-pyrimidine ring to form N-(formylamidino)-N'-β-D-2'-deoxy-(ribofuranosy)-urea (Mojaverian and Repta (1984) J. Pharm. Pharmacol. 36:728-733); and (c) subsequent formation of guanidine compounds (Kissinger and Stemm (1986) J. Chromat. 353:309-318).

Decitabine possesses multiple pharmacological characteristics. At a molecular level, it is S-phase dependent for incorporation into DNA. At a cellular level, decitabine can induce cell differentiation and exert hematological toxicity. Despite having a short half-life *in vivo*, decitabine has an excellent tissue distribution.

One of the functions of decitabine is its ability to specifically and potently inhibit DNA methylation. Methylation of cytosine to 5-methylcytosine occurs at the level of DNA. Inside the cell, decitabine is first converted into its active form, the phosphorylated 5-aza-deoxycytidine, by deoxycytidine kinase which is primarily synthesized during the S phase of the cell cycle. The affinity of decitabine for the catalytical site of deoxycytidine kinase is similar to the natural substrate, deoxycytidine. Momparler et al. (1985) 30:287-299. After conversion to its triphosphate form by deoxycytidine kinase, decitabine is incorporated into replicating DNA at a rate similar to that of the natural substrate, dCTP. Bouchard and Momparler (1983) Mol. Pharmacol. 24:109-114.

Incorporation of decitabine into the DNA strand has a hypomethylation effect. Each class of differentiated cells has its own distinct methylation pattern. After chromosomal duplication, in order to conserve this pattern of methylation, the 5-methylcytosine on the parental strand serves to direct methylation on the complementary daughter DNA strand. Substituting the carbon at the 5 position of the cytosine for a nitrogen interferes with this normal process of DNA methylation. The replacement of 5-methylcytosine with decitabine at a specific site of methylation produces an irreversible inactivation of DNA methyltransferase, presumably due to formation of a covalent bond between the enzyme and decitabine. Juttermann et al. (1994) Proc. Natl. Acad. Sci. USA 91:11797-11801. By specifically inhibiting DNA methyltransferase, the enzyme required for methylation, the aberrant methylation of the tumor suppressor genes could be prevented.

Decitabine is commonly supplied as a sterile lyophilized powder for injection, together with buffering salt, such as potassium dihydrogen phosphate, and pH modifier, such as sodium hydroxide. For example, decitabine is supplied by SuperGen, Inc., as lyophilized powder packed in 20 mL glass vials, containing 50 mg of decitabine, monobasic potassium dihydrogen phosphate, and sodium hydroxide. When reconstituted with 10 mL of sterile water for injection, each mL contain 5 mg of decitabine, 6.8 mg of KH_2PO_4, and approximately 1.1 mg NaOH. The pH of the resulting solution is 6.5 - 7.5. The reconstituted solution can be further diluted to a

concentration of 1.0 or 0.1 mg/mL in cold infusion fluids, i.e., 0.9% Sodium Chloride; or 5% Dextrose; or 5% Glucose; or Lactated Ringer's. The unopened vials are typically stored under refrigeration (2-8°C; 36-46°F), in the original package.

Decitabine is most typically administrated to patients by injection, such as by a bolus I.V. injection, continuous I.V. infusion, or I.V. infusion. Similar to decitabine, azacitidine is also formulated as aqueous solution and delivered to patients intravenously. According to clinical studies of azacitidine, longer or continuous infusions were more effective than shorter ones. Santini et al. (2001) Ann. Int. Med. 134: 573-588. However, the length of I.V. infusion is limited by the decomposition of decitabine or azacitidine and low solubility of the drugs in aqueous solutions. The present invention provides innovative solutions to such problems.

SUMMARY OF THE INVENTION

According to the present invention, a salt of a cytidine analog is provided.

In one embodiment, the cytidine analog is 5-aza-2'-deoxycytidine or 5-azacytidine.

In another embodiment, the salt of the cytidine analog is synthesized with an acid, optionally with an acid having a pKa of about 5 or less, optionally with an acid having

pKa of about 4 or less, optionally with an acid having pKa ranging from about 3 to about 0, or optionally with an acid having pKa ranging from about 3 to about -10.

Preferably, the acid is selected from the group consisting of hydrochloric, L-lactic, acetic, phosphoric, (+)-L-tartaric, citric, propionic, butyric, hexanoic, L-aspartic, L-glutamic, succinic, EDTA, maleic, methanesulfonic acid, HBr, HF, HI, nitric, nitrous, sulfuric, sulfurous, phosphorous, perchloric, chloric, chlorous acid, carboxylic acid, sulfonic acid, ascorbic, carbonic, and fumaric acid. In particular, the sulfonic acid is selected from the group consisting of ethanesulfonic, 2-hydroxyethanesulfonic, and toluenesulfonic acid.

In yet another embodiment, a salt of decitabine is provided. The salt of decitabine preferably is selected from the group consisting of hydrochloride, mesylate, EDTA, sulfite, L-Aspartate, maleate, phosphate, L-Glutamate, (+)-L-Tartrate, citrate, L-Lactate, succinate, acetate, hexanoate, butyrate, or propionate salt.

In one variation of the embodiment, the salt of decitabine is hydrochloride salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 14.79°, 23.63°, and 29.81°. The salt is further characterized by a melting endotherm of 125-155°C, optionally 130-144°C, as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In another variation of the embodiment, the salt of decitabine is a mesylate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 8.52°, 22.09°, and 25.93°. The salt is further characterized by a melting endotherm of 125-140°C, or optionally 125-134°C, as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is an EDTA salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 7.14°, 22.18°, and 24.63°. The salt is further characterized by multiple reversible melting endotherms at 50-90°C, 165-170°C, and 170-200°C, or optionally at 73°C, 169°C, and 197°C, as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is a sulfite salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 15.73°, 19.23°, and 22.67°. The

salt is further characterized by a melting endotherm at 100-140°C as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is a L-aspartate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 21.61°, 22.71°, and 23.24°. The salt is further characterized by multiple reversible melting endotherms at 30-100°C, 170-195°C, and 195-250°C, optionally at 86°C, 187°C, and 239°C, as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is a maleate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 20.81°, 27.38°, and 28.23°. The salt is further characterized by multiple reversible melting endotherms at 95-130°C and 160-180°C, or optionally at 119°C and 169°C, as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is a phosphate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 17.09°, 21.99°, and 23.21°. The salt is further characterized by a melting endotherm at 130-145°C as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is a L-glutamate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 13.33°, 21.39°, and 30.99°. The salt is further characterized by multiple reversible melting endotherms at 50-100°C, 175-195°C, and 195-220°C, or optionally at 84°C, 183°C, and 207°C as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is a (+)-L-tartarate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 7.12°, 13.30°, and 14.22°. The salt is further characterized by multiple reversible melting endotherms at 60-110°C, and 185-220°C, optionally at 91°C, and 203°C, as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is a citrate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 13.31°, 14.23°, and 23.26°. The salt is further characterized by multiple reversible melting endotherms at 30-100°C and 160-220°C, or optionally at 84°C and 201°C, as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is a L-lactate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 13.27°, 21.13°, and 23.72°. The salt is further characterized by multiple reversible melting endotherms at 30-100°C and 160-210°C, or optionally at 84°C and 198°C, as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is a succinate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 13.30°, 22.59°, and 23.28°. The salt is further characterized by multiple reversible melting endotherms at 50-100°C and 190-210°C, or

optionally at 79°C and 203°C, as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is an acetate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 7.14°, 14.26°, and 31.25°. The salt is further characterized by multiple reversible melting endotherms at 60-90°C and 185-210°C, or optionally at 93°C and 204°C, as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is a hexanoate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 13.27°, 22.54°, and 23.25°. The salt is further characterized by multiple reversible melting endotherms at 60-90°C and 190-210°C, or optionally at 93°C and 204°C, as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is a butyrate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 13.28°, 22.57°, and 23.27°. The salt is further characterized by multiple reversible melting endotherms at 40-90°C and 190-210°C, or optionally at 89°C and 203°C, as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another variation of the embodiment, the salt of decitabine is a propionate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 13.29°, 22.52°, and 23.27°. The salt is further characterized by multiple reversible melting endotherms at 50-110°C and 190-210°C, optionally at 94°C and 204°C, as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

In yet another embodiment, a salt of azacitidine is provided. The salt of azacitidine is a hydrochloride, mesylate, EDTA, sulfite, L-Aspartate, maleate, phosphate, L-Glutamate, (+)-L-Tartrate, citrate, L-Lactate, succinate, acetate, hexanoate, butyrate, or propionate salt.

According to the embodiment, the salt of azacitidine is a mesylate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 18.58°, 23.03°, and 27.97°. The salt is further characterized by multiple reversible melting endotherms at 30-80°C, 80-110°C and 110-140°C as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

Also according to the present invention, a method is provided for treating a disease associated with undesirable cell proliferation in a subject. The method comprises administering to the subject in need thereof a pharmaceutically effective amount of a salt of a cytidine analog. The disease may be benign tumors, cancer, hematological disorders, atherosclerosis, insults to body tissue due to surgery, abnormal wound healing, abnormal angiogenesis, diseases that produce fibrosis of tissue, repetitive motion disorders, disorders of tissues that are not highly vascularized, or proliferative responses associated with organ transplants. In particular, the disease is myelodysplastic syndrome, non-small cell lung cancer, or sickle-cell anemia.

The salts of present invention can be formulated in various ways and delivered to a patient suffering from a disease sensitive to the treatment with a cytidine analog via various routes of administration such as intravenous, intramuscular, subcutaneous injection, oral administration and inhalation.

The present invention also provides methods for synthesizing, formulating and manufacturing salts of a cytidine analog.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates a DSC plot of decitabine hydrochloride.

Figure 2 illustrates a DSC plot of decitabine mesylate.

Figure 3 illustrates a DSC plot of decitabine EDTA.
Figure 4 illustrates a DSC plot of decitabine l-aspartate.
Figure 5 illustrates a DSC plot of decitabine maleate.
Figure 6 illustrates a DSC plot of decitabine l-glutamate.
Figure 7 illustrates a DSC plot of decitabine sulfite.
Figure 8 illustrates a DSC plot of decitabine phosphate.
Figure 9 illustrates a DSC plot of decitabine tartrate.
Figure 10 illustrates a DSC plot of decitabine citrate.
Figure 11 illustrates a DSC plot of decitabine l-(+)-lactate.
Figure 12 illustrates a DSC plot of decitabine succinate.
Figure 13 illustrates a DSC plot of decitabine acetate.
Figure 14 illustrates a DSC plot of decitabine hexanoate.
Figure 15 illustrates a DSC plot of decitabine butyrate.
Figure 16 illustrates a DSC plot of decitabine propionate.
Figure 17 illustrates a DSC plot of azacitidine mesylate.
Figure 18 illustrates a TGA plot of decitabine hydrochloride.
Figure 19 illustrates a TGA plot of decitabine mesylate.
Figure 20 illustrates a TGA plot of decitabine EDTA.
Figure 21 illustrates a TGA plot of decitabine l-aspartate.
Figure 22 illustrates a TGA plot of decitabine maleate.
Figure 23 illustrates a TGA plot of decitabine l-glutamate.
Figure 24 illustrates a TGA plot of decitabine sulfite.
Figure 25 illustrates a TGA plot of decitabine phosphate.
Figure 26 illustrates a TGA plot of decitabine tartrate.
Figure 27 illustrates a TGA plot of decitabine citrate.
Figure 28 illustrates a TGA plot of decitabine l-(+)-lactate.
Figure 29 illustrates a TGA plot of decitabine succinate.
Figure 30 illustrates a TGA plot of decitabine acetate.
Figure 31 illustrates a TGA plot of decitabine hexanoate.
Figure 32 illustrates a TGA plot of decitabine butyrate.
Figure 33 illustrates a TGA plot of decitabine propionate.
Figure 34 illustrates a TGA plot of azacitidine mesylate.
Figure 35 illustrates an XRD pattern of decitabine hydrochloride.
Figure 36 illustrates an XRD pattern of decitabine mesylate.
Figure 37 illustrates an XRD pattern of decitabine EDTA.
Figure 38 illustrates an XRD pattern of decitabine l-aspartate.
Figure 39 illustrates an XRD pattern of decitabine maleate.
Figure 40 illustrates an XRD pattern of decitabine l-glutamate.
Figure 41 illustrates an XRD pattern of decitabine sulfite.
Figure 42 illustrates an XRD pattern of decitabine phosphate.

Figure 43 illustrates an XRD pattern of decitabine tartrate.
Figure 44 illustrates an XRD pattern of decitabine citrate.
Figure 45 illustrates an XRD pattern of decitabine l-(+)-lactate.
Figure 46 illustrates an XRD pattern of decitabine succinate.
Figure 47 illustrates an XRD pattern of decitabine acetate.
Figure 48 illustrates an XRD pattern of decitabine hexanoate.
Figure 49 illustrates an XRD pattern of decitabine butyrate.
Figure 50 illustrates an XRD pattern of decitabine propionate.
Figure 51 illustrates an XRD pattern of azacitidine mesylate.
Figure 52 illustrates an IR absorbance spectrum of decitabine hydrochloride.
Figure 53 illustrates an IR absorbance spectrum of decitabine mesylate.
Figure 54 illustrates an IR absorbance spectrum of decitabine EDTA.
Figure 55 illustrates an IR absorbance spectrum of decitabine l-aspartate.
Figure 56 illustrates an IR absorbance spectrum of decitabine maleate.
Figure 57 illustrates an IR absorbance spectrum of decitabine l-glutamate.
Figure 58 illustrates an IR absorbance spectrum of decitabine sulfite.
Figure 59 illustrates an IR absorbance spectrum of decitabine phosphate.
Figure 60 illustrates an IR absorbance spectrum of decitabine tartrate.
Figure 61 illustrates an IR absorbance spectrum of decitabine citrate.
Figure 62 illustrates an IR absorbance spectrum of decitabine l-(+)-lactate.
Figure 63 illustrates an IR absorbance spectrum of decitabine succinate.
Figure 64 illustrates an IR absorbance spectrum of decitabine acetate.
Figure 65 illustrates an IR absorbance spectrum of decitabine hexanoate.
Figure 66 illustrates an IR absorbance spectrum of decitabine butyrate.
Figure 67 illustrates an IR absorbance spectrum of decitabine propionate.
Figure 68 illustrates an IR absorbance spectrum of azacitidine mesylate.

DETAILED DESCRIPITION OF THE PRESENT INVENTION

The present invention provides salts of cytidine analogs, e.g., decitabine and azacitidine, which can be used as pharmaceuticals for the treatment of various diseases and conditions, such as myelodysplastic syndrome (MDS), non-small cell lung (NSCL) cancer, and sickle-cell anemia. This innovative approach is taken to overcome three major hurdles that have adversely impacted the commercial development of this type of drugs: hydrolytic degradation in aqueous environment; low solubility in most pharmaceutically acceptable solvents; and minimal oral bioavailability.

According to the present invention, the solid state and solution properties of a cytidine analog is modified by salt formation. The inventors believe that salt formation can lead to improved solubility and stability of this type of drugs, such as decitabine and azacitidine. Increased water-solubility can also potentially make the drug entities less toxic. Due to their easier renal clearance they are less likely to accumulate and overload the hepatic microsomes responsible for phase-one and phase-two metabolism. Further more, increased

stability can make manufacturing of the drug product more robust and facilitate development of different formulations.

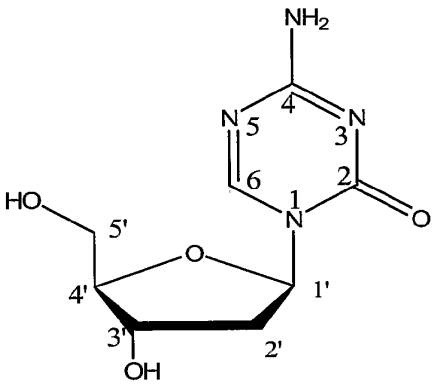
The salts of present invention can be formulated in various ways and delivered to a patient suffering from a disease sensitive to the treatment with a cytidine analog, such as hematological disorders, benign tumors, malignant tumors, restenosis, and inflammatory diseases via various routes of administration such as intravenous, intramuscular, subcutaneous injection, oral administration and inhalation.

The present invention also provides methods for synthesizing, formulating and manufacturing salts of cytidine analogs, and methods for using the salts for treating various diseases and conditions.

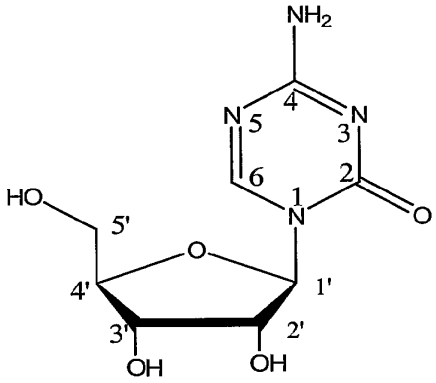
The following is a detailed description of the invention and preferred embodiments of the inventive salts, compositions, methods of use, synthesis, formulations and manufacture.

1. Salts of Cytidine Analogs and Derivatives

One aspect of the invention is the salt form of a cytidine analog or derivative, preferably a salt of 5-aza-2'-deoxycytidine (decitabine 1) or 5-azacytidine (azacitidine 2) whose chemical structures are depicted below:



Structure of decitabine (1)



Structure of azacitidine (2)

In some embodiments, to ensure sufficient proton transfer from the acid to a basic drug, the newly formed conjugate acid and conjugate base should be weaker than the original acid and basic drug, generally by at least about 2 units weaker than the pKa of the drug. Two pKa values, 7.61+0.03 and 3.58+0.06, were found for decitabine. In preferred embodiments, an acid with pKa lower than about 5, or optionally with pKa between 3 and -10, is used to synthesize a salt form of decitabine, as well as a salt form of azacitidine, and other cytidine analogs and derivatives. Examples of suitable acids are listed in Table 1a.

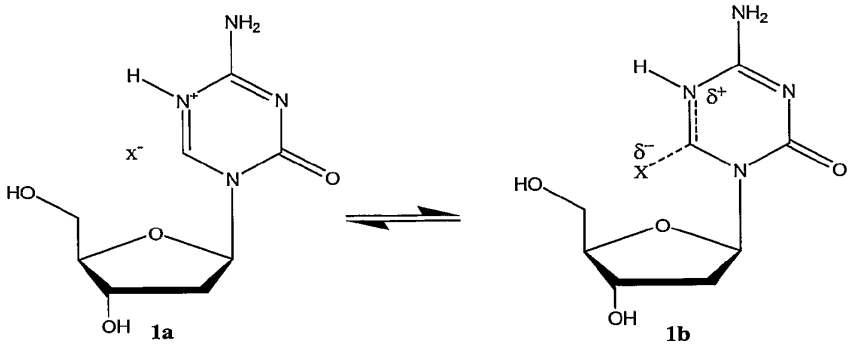
Table 1a: Examples of acids that can be used to synthesize a salt form of decitabine, azacitidine, and other cytidine analogs and derivatives.

Name	pK_{a1}	pK_{a2}	Name	pK_{a1}	pK_{a2}
Perchloric acid	-10	-	**Fumaric acid**	3.03	4.38
Hydrobromic acid	-9	-	**Galactaric acid**	3.08	3.63
Hydroiodic acid	-9	-	**Hydrofluoric acid**	3.16	-
Hydrochloric acid	-6	--	**Citric acid**	3.13	4.76
Naphthalene-1,5-disulfonic acid	-3.37	-2.64	**D-Glucuronic acid**	3.18	-
Sulfuric acid	-3	1.92	**Lactobionic acid**	3.2	-
Ethane-1,2-disulfonic acid	-2.1	-1.5	**4-Amino-salicylic acid**	3.25	10
Cyclamic acid	-2.01	-	**Glycolic acid**	3.28	-
***p*-Toluenesulfonic acid**	-1.34	-	**D-Glucoheptonic acid**	3.3	-
Thiocyanic acid	-1.33	-	**Nitrous acid**	3.3	-
Nitric acid	-1.32	-	**(-)-L-Pyroglutamic acid**	3.32	-
Methanesulfonic acid	-1.2	-	**DL-Mandelic acid**	3.37	-
Chloric acid	-1.0	-	**(-)-L-Malic acid**	3.46	5.10
Chromic acid	-0.98	6.50	**Hippuric acid**	3.55	-
Dodecylsulfuric acid	-0.09	-	**Formic acid**	3.75	-
Trichloroacetic acid	0.52	-	**D-Gluconic acid**	3.76	-
Benzenesulfonic acid	0.7	-	**DL-Lactic acid**	3.86	-
Iodic	0.80	-	**Oleic acid**	4	-
Oxalic acid	1.27	4.27	**L-Ascorbic acid**	4.17	11.57
2,2-Dichloro-acetic acid	1.35	-	**Benzoic acid**	4.19	-
Glycerophosphoric acid	1.47	-	**Succinic acid**	4.21	5.64
2-Hydroxy-ethanesulfonic acid	1.66	-	**4-Acetamido-benzoic acid**	4.3	-
EDTA	1.70	2.60	**Glutaric acid**	4.34	5.27
Phosphorous acid	1.80	6.15	**Cinnamic acid**	4.40	
Sulfurous	1.85	7.20	**Adipic acid**	4.44	5.44
L-Aspartic	1.88	3.65	**Sebacic acid**	4.59	5.59
Maleic acid	1.92	6.23	**(+)-Camphoric acid**	4.72	5.83
Phosphoric acid	1.96	7.12	**Acetic acid**	4.76	-
Chlorous acid	1.98	-	**Hexanoic acid**	4.8	-
Ethanesulfonic acid	2.05	-	**Butyric acid**	4.83	-
(+)-Camphor-10-sulfonic acid	2.17	-	**Nicotinic acid**	4.85	-
Glutamic acid	2.19	4.25	**Isobutyric acid**	4.86	-
Alginic acid	>2.4	-	**Propionic acid**	4.87	-
Pamoic acid	2.51	-	**Decanoic acid**	4.9	-
Glutaric acid	2.7	-	**Lauric acid**	4.9	-
1-Hydroxy-2-naphthoic acid	2.7	-	**Palmitic acid**	4.9	-
Malonic acid	2.83	-	**Stearic acid**	4.9	-
Gentisic acid	2.93	-	**Undecylenic acid**	4.9	-
Salicylic acid	2.97	-	**Octanoic acid**	4.91	-
(+)-L-Tartaric acid	3.02	4.36	**Malic acid**	5.05	-

In preferred embodiments, decitabine and azacitidine salts are formed with strong acids (pKa< 0). In other preferred embodiments, the decitabine salts show improved stability over decitabine free base in near neutral pH solutions. By "near neutral pH" is meant a pH at about 7+1, +2, or +3.

In preferred embodiments, salts of some cytidine analogs, e.g., decitabine salts, can show some type of protective ionic complex across the N-5 imine nitrogen and the 6-carbon in aqueous solution. Without being limited to a particular hypothesis, such an ionic complex may shield against nucleophilic attack from

surrounding water molecules. The illustration below depicts the formation of a protective ion complex (1a, 1b), hypothesized to form in some preferred embodiments of decitabine salts of the instant invention, e.g., where X is a conjugate base such as chloride, mesylate, or phosphate.

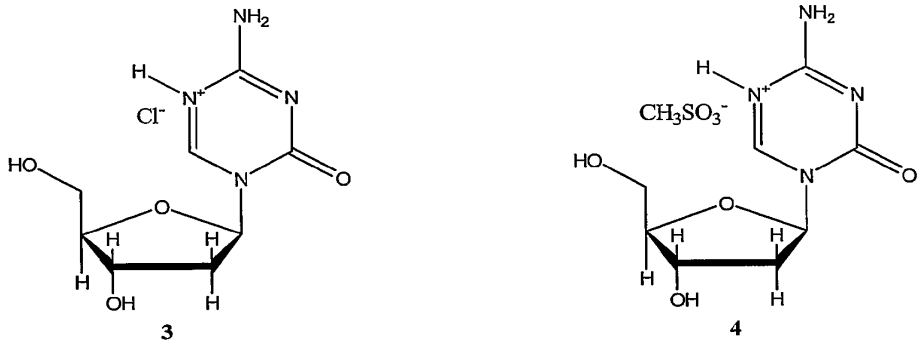


1a 1b

As illustrated, a temporary ionic adduct may form across the 5- and 6-position of decitabine, possibly helping to shield against hydrolytic cleavage in solution.

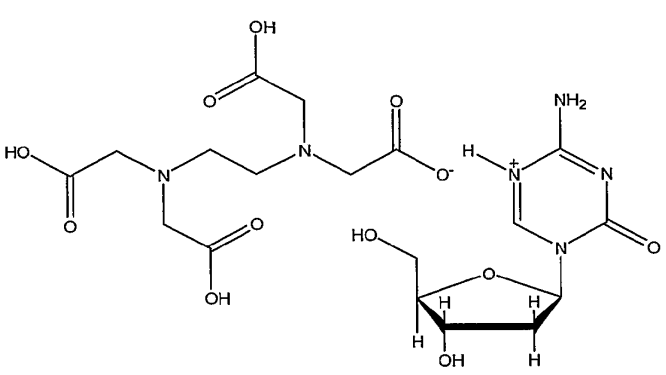
One embodiment of the invention is the salt form of decitabine synthesized with an acid. Some embodiments include salt forms synthesized with the following acids – HCl, L-lactic, acetic, phosphoric, (+)-L-tartaric, citric, propionic, butyric, hexanoic, L-aspartic, L-glutamic, succinic, EDTA, maleic, and methanesulfonic. Other embodiments include decitabine salts of other common acids. Examples of suitable inorganic acids include, but are not limited to, HBr, HF, HI, nitric, nitrous, sulfuric, sulfurous, phosphorous, perchloric, chloric, and chlorous acid. Examples of suitable carboxylic acids include, but are not limited to, ascorbic, carbonic, and fumaric acid. Examples of suitable sulfonic acids include, but are not limited to, ethanesulfonic, 2-hydroxyethanesulfonic, and toluenesulfonic acid.

Preferably, the molar ratios of acids to decitabine are about 0.01 to about 10 molar equivalents. Preferred embodiments include decitabine salts of strong acids (pKa<0). More preferred embodiments include decitabine hydrochloride (3) and decitabine mesylate (4), illustrated below, which can form in 1:1 molar equivalent (e.g., as determined from elemental analysis).



3 4

Some preferred embodiments include decitabine salts of moderate acids (0<pKa<3). Preferred salts formed with moderate acids include decitabine EDTA (5), L-aspartate (6), maleate (7) and L-glutamate (8), depicted below:



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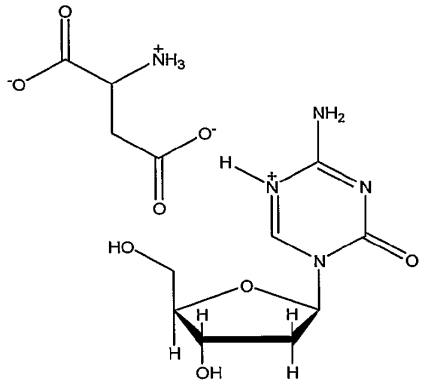

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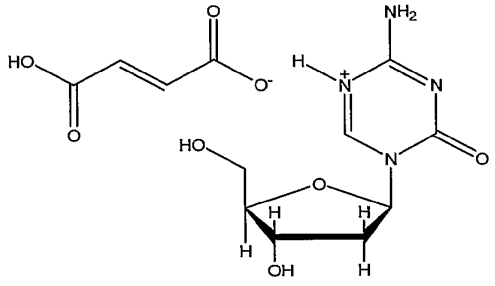

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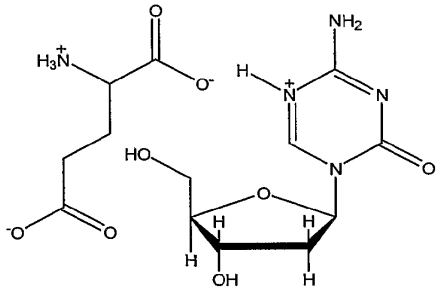
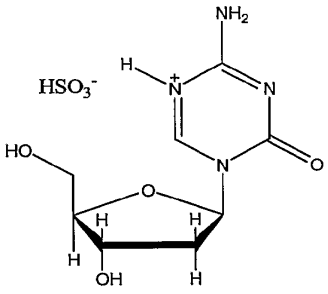


8

Still other preferred salts formed with moderate acids (0<pKa<3) include decitabine sulfite (9) or decitabine phosphate (10), depicted below:



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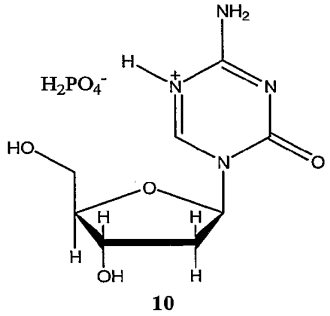
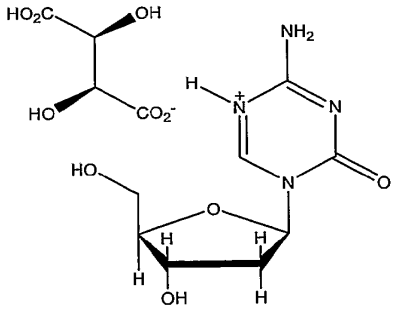


10

Some embodiments include decitabine salts of weak acids (3<pKa<5). Examples of salts formed with weak acids include decitabine (+)-l-tartrate (11); decitabine citrate (12); decitabine l-Lactate (13); decitabine succinate (14); decitabine acetate (15); decitabine hexanoate (16); decitabine butyrate (17); and decitabine propionate (18), each depicted below:



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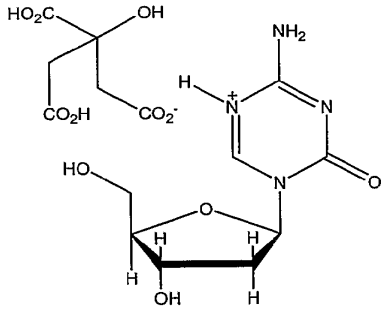

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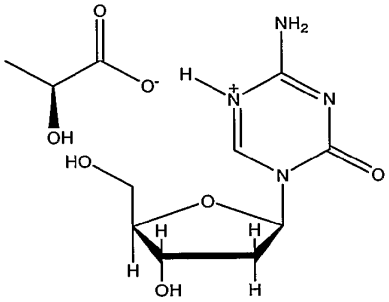

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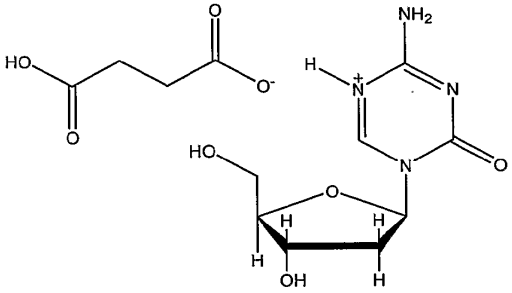

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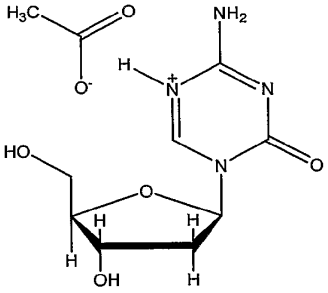

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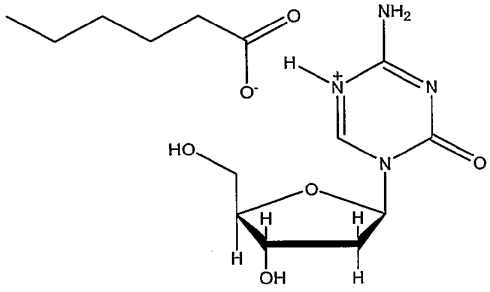

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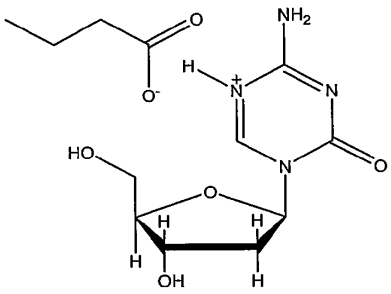

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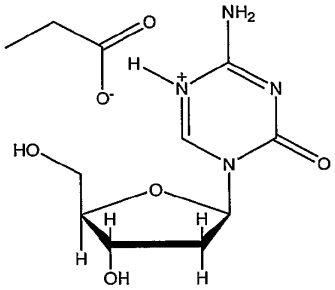
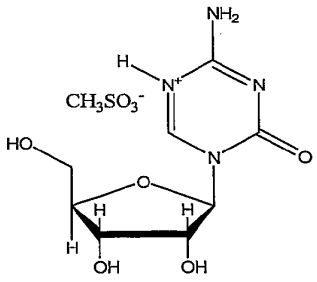


18

A second aspect of the invention is a salt form of azacitidine. One embodiment is an azacitidine salt of methanesulfonic acid, e.g., azacitidine mesylate (19), depicted below:



19

Other embodiments include azacitidine salts of inorganic or organic acids. Examples of suitable inorganic acids include, but are not limited to, HCl, HBr, HF, HI, nitric, nitrous, sulfuric, sulfurous, phosphoric, phosphorous, perchloric, chloric, and chlorous acid. Examples of suitable carboxylic acids include, but are not limited to, acetic, ascorbic, butyric, carbonic, citric, EDTA, fumaric, hexanoic, L-lactic, maleic, propionic, succinic, and (+)-L-tartaric acid. Other suitable acids for forming azacitidine salts include sulfuric and amino acids. Examples of suitable sulfonic acids include, but are not limited to, ethanesulfonic, 2-hydroxyethanesulfonic, and toluenesulfonic acid. Examples of suitable amino acids include, but are not limited to, L-aspartic and L-glutamic acid.

The present invention also embraces isolated salts of cytidine analogs. An isolated salt of a cytidine analog refers to a salt of a cytidine analog which represents at least 10%, preferably 20%, more preferably 50%, or most preferably 80% of the salt of the cytidine analog present in the mixture.

2. Pharmaceutical Formulations of the Present Invention

According to the present invention, the salts of cytosine analogs can be formulated into pharmaceutically acceptable compositions for treating various diseases and conditions.

The pharmaceutically-acceptable compositions of the present invention comprise one or more salts of the invention in association with one or more nontoxic, pharmaceutically-acceptable carriers and/or diluents and/or adjuvants and/or excipients, collectively referred to herein as "carrier" materials, and if desired other active ingredients.

The salts of the present invention are administered by any route, preferably in the form of a pharmaceutical composition adapted to such a route, as illustrated below and are dependent on the condition being treated. The compounds and compositions can be, for example, administered orally, parenterally, intraperitoneally, intravenously, intraarterially, transdermally, sublingually, intramuscularly, rectally, transbuccally, intranasally, liposomally, via inhalation, vaginally, intraoccularly, via local delivery (for example by a catheter or stent), subcutaneously, intraadiposally, intraarticularly, or intrathecally.

The pharmaceutical formulation may optionally further include an excipient added in an amount sufficient to enhance the stability of the composition, maintain the product in solution, or prevent side effects (e.g., potential ulceration, vascular irritation or extravasation) associated with the administration of the inventive formulation. Examples of excipients include, but are not limited to, mannitol, sorbitol, lactose, dextrox, cyclodextrin such as, □-, □-, and □-cyclodextrin, and modified, amorphous cyclodextrin such as hydroxypropyl-, hydroxyethyl-, glucosyl-, maltosyl-, maltotriosyl-, carboxyamidomethyl-, carboxymethyl-, sulfobutylether-, and diethylamino-substituted □-, □-, and □-cyclodextrin. Cyclodextrins such as Encapsin® from Janssen Pharmaceuticals or equivalent may be used for this purpose.

For oral administration, the pharmaceutical compositions can be in the form of, for example, a tablet, capsule, suspension or liquid. The pharmaceutical composition is preferably made in the form of a dosage unit containing a therapeutically-effective amount of the active ingredient. Examples of such dosage units are tablets and capsules. For therapeutic purposes, the tablets and capsules which can contain, in addition to the active ingredient, conventional carriers such as binding agents, for example, acacia gum, gelatin, polyvinylpyrrolidone, sorbitol, or tragacanth; fillers, for example, calcium phosphate, glycine, lactose, maize-starch, sorbitol, or sucrose; lubricants, for example, magnesium stearate, polyethylene glycol, silica, or talc; disintegrants, for example, potato starch, flavoring or coloring agents, or acceptable wetting agents. Oral liquid preparations generally are in the form of aqueous or oily solutions, suspensions, emulsions, syrups or elixirs may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous agents, preservatives, coloring agents and flavoring agents. Examples of additives for liquid preparations include acacia, almond oil, ethyl alcohol, fractionated coconut oil, gelatin, glucose syrup, glycerin, hydrogenated edible fats, lecithin, methyl cellulose, methyl or propyl para-hydroxybenzoate, propylene glycol, sorbitol, or sorbic acid.

For topical use the salts of the present invention can also be prepared in suitable forms to be applied to the skin, or mucus membranes of the nose and throat, and can take the form of creams, ointments, liquid sprays or inhalants, lozenges, or throat paints. Such topical formulations further can include chemical compounds such as dimethylsulfoxide (DMSO) to facilitate surface penetration of the active ingredient.

For application to the eyes or ears, the salts of the present invention can be presented in liquid or semi-liquid form formulated in hydrophobic or hydrophilic bases as ointments, creams, lotions, paints or powders.

For rectal administration the salts of the present invention can be administered in the form of suppositories admixed with conventional carriers such as cocoa butter, wax or other glyceride.

Alternatively, the salts of the present invention can be in powder form for reconstitution in the appropriate pharmaceutically acceptable carrier at the time of delivery.

The pharmaceutical compositions can be administered via injection. Formulations for parenteral administration can be in the form of aqueous or non-aqueous isotonic sterile injection solutions or suspensions. These solutions or suspensions can be prepared from sterile powders or granules having one or more of the carriers mentioned for use in the formulations for oral administration. The salts can be dissolved in water, polyethylene glycol, propylene glycol, ethanol, corn oil, benzyl alcohol, sodium chloride, and/or various buffers.

In an embodiment, the salt of the present invention can be formulated into a pharmaceutically acceptable composition comprising the compound solvated in non-aqueous solvent that includes glycerin, propylene glycol, polyethylene glycol, or combinations thereof. It is believed that the compound decitabine will be stable in such pharmaceutical formulations so that the pharmaceutical formulations may be stored for a prolonged period of time prior to use.

As discussed above, in current clinical treatment with decitabine, to minimize drug decomposition decitabine is supplied as lyophilized powder and reconstituted in a cold aqueous solution containing water in at least 40% vol. of the solvent, such as WFI, and diluted in cold infusion fluids prior to administration. Such a formulation and treatment regimen suffers from a few drawbacks. First, refrigeration of decitabine in cold solution becomes essential, which is burdensome in handling and economically less desirable than a formulation that can sustain storage at higher temperatures. Second, due to rapid decomposition of decitabine in aqueous solution, the reconstituted decitabine solution may only be infused to a patient for a maximum of 3 hr if the

solution has been stored in the refrigerator for less than 7 hr. In addition, infusion of cold fluid can cause great discomfort and pain to the patient, which induces the patient's resistance to such a regimen.

By modifying the solid state and solution properties of cytidine analogs, the pharmaceutical formulations comprising the inventive salts can circumvent the above-listed problems associated with the current clinical treatment with decitabine and azacitidine. The inventive salts can be formulated in aqueous solutions containing water in at least 40% vol. of the solvent, optionally at least 80%, or optionally at least 90% vol. of the solvent. These formulations of the inventive salts are believed to be more chemically stable than the free base form of decitabine or azacitidine formulated in aqueous solutions.

Alternatively, the inventive salts may be formulated in solutions containing less than 40% water in the solvent, optionally less than 20% water in the solvent, optionally less than 10% water in the solvent, or optionally less than 1% water in the solvent. In one variation, the pharmaceutical formulation is stored in a substantially anhydrous form. Optionally, a drying agent may be added to the pharmaceutical formulation to absorb water.

Owing to the enhanced stability, the inventive formulation may be stored and transported at ambient temperature, thereby significantly reducing the cost of handling the drug. Further, the inventive formulation may be conveniently stored for a long time before being administered to the patient. In addition, the inventive formulation may be diluted with regular infusion fluid (without chilling) and administered to a patient at room temperature, thereby avoiding causing patients' discomfort associated with infusion of cold fluid.

In another embodiment, the inventive salt is dissolved in a solution at different concentrations. For example, the formulation may optionally comprise between 0.1 and 200; between 1 and 100; between 1 and 50; between 2 and 50; between 2 and 100; between 5 and 100; between 10 and 100 or between 20 and 100 mg inventive salt per ml of the solution. Specific examples of the inventive salt per solution concentrations include but are not limited to 2, 5, 10, 20, 22, 25, 30, 40 and 50 mg/ml.

In yet another embodiment, the inventive salt is dissolved in a solvent combining glycerin and propylene glycol at different concentrations. The concentration of propylene glycol in the solvent is between 0.1-99.9%, optionally between 1-90%, between 10-80%, or between 50-70%.

In yet another embodiment, the inventive salt is dissolved at different concentrations in a solvent combining glycerin and polyethylene glycol (PEG) such as PEG300, PEG400 and PEG1000. The concentration of polyethylene glycol in the solvent is between 0.1-99.9%, optionally between 1-90%, between 10-80%, or between 50-70%.

In yet another embodiment, the inventive salt is dissolved at different concentrations in a solvent combining propylene glycol, polyethylene glycol and glycerin. The concentration of propylene glycol in the solvent is between 0.1-99.9%, optionally between 1-90%, between 10-60%, or between 20-40%; and the concentration of polyethylene glycol in the solvent is between 0.1-99.9%, optionally between 1-90%, between 10-80%, or between 50-70%.

It is believed that addition of propylene glycol can further improve chemical stability, reduce viscosity of the formulations and facilitate dissolution of the inventive salt in the solvent.

The pharmaceutical formulation may further comprise an acidifying agent added to the formulation in a proportion such that the formulation has a resulting pH between about 4 and 8. The acidifying agent may be an organic acid. Examples of organic acid include, but are not limited to, ascorbic acid, citric acid, tartaric acid,

lactic acid, oxalic acid, formic acid, benzene sulphonic acid, benzoic acid, maleic acid, glutamic acid, succinic acid, aspartic acid, diatrizoic acid, and acetic acid. The acidifying agent may also be an inorganic acid, such as hydrochloric acid, sulphuric acid, phosphoric acid, and nitric acid.

It is believed that adding an acidifying agent to the formulation to maintain a relatively neutral pH (e.g., within pH 4-8) facilitates ready dissolution of the inventive compound in the solvent and enhances long-term stability of the formulation. In alkaline solution, there is a rapid reversible decomposition of decitabine to N-(formylamidino)-N'-β-D-2-deoxyribofuranosylurea, which decomposes irreversibly to form 1-β-D-2'-deoxyribofuranosyl-3-guanylurea. The first stage of the hydrolytic degradation involves the formation of N-amidinium-N'-(2-deoxy-β-D-erythropentofuranosyl)urea formate (AUF). The second phase of the degradation at an elevated temperature involves formation of guanidine. In acidic solution, N-(formylamidino)-N'-β-D-2-deoxyribofuranosylurea and some unidentified compounds are formed. In strongly acidic solution (at pH <2.2) 5-azacytosine is produced. Thus, maintaining a relative neutral pH may be advantageous for the formulation comprising the inventive salt.

In a variation, the acidifying agent is ascorbic acid at a concentration of 0.01-0.2 mg/ml of the solvent, optionally 0.04-0.1 mg/ml or 0.03-0.07 mg/ml of the solvent.

The pH of the pharmaceutical formulation may be adjusted to be between pH 4 and pH 8, preferably between pH 5 and pH 7, and more preferably between pH 5.5 and pH 6.8.

The pharmaceutical formulation is preferably at least 80%, 90%, 95% or more stable upon storage at 25oC for 7, 14, 21, 28 or more days. The pharmaceutical formulation is also preferably at least 80%, 90%, 95% or more stable upon storage at 40oC for 7, 14, 21, 28 or more days.

In one embodiment, the pharmaceutical formulation of the present invention is prepared by taking glycerin and dissolving the inventive compound in the glycerin. This may be done, for example, by adding the inventive salt to the glycerin or by adding the glycerin to the inventive salt. By their admixture, the pharmaceutical formulation is formed.

Optionally, the method further comprises additional steps to increase the rate at which the inventive salt is solvated by the glycerin. Examples of additional steps that may be performed include, but are nor limited to, agitation, heating, extension of solvation period, and application of micronized inventive compound and the combinations thereof.

In one variation, agitation is applied. Examples of agitation include, but are nor limited to, mechanical agitation, sonication, conventional mixing, conventional stirring and the combinations thereof. For example, mechanical agitation of the formulations may be performed according to manufacturer's protocols by Silverson homogenizer manufactured by Silverson Machines Inc., (East Longmeadow, MA).

In another variation, heat may be applied. Optionally, the formulations may be heated in a water bath. Preferably, the temperature of the heated formulations may be less than 70°C, more preferably, between 25°C and 40°C. As an example, the formulation may be heated to 37°C.

In yet another variation, the inventive salt is solvated in glycerin over an extended period of time.

In yet another variation, a micronized form of the inventive salt may also be employed to enhance solvation kinetics. Optionally, micronization may be performed by a milling process. As an example, micronization may be performed according to manufacturer's protocols by jet milling process performed by

Malvern Mastersizer, Mastersizerusing an Air Jet Mill, manufactured by Micron Technology Inc.(Boise, ID).IncFluid Energy Aljet Inc. (Boise, IDTelford, PA).

Optionally, the method further comprises adjusting the pH of the pharmaceutical formulations by commonly used methods. In one variation, pH is adjusted by addition of acid, such as ascorbic acid, or base, such as sodium hydroxide. In another variation, pH is adjusted and stabilized by addition of buffered solutions, such as solution of (Ethylenedinitrilo) tetraacetic acid disodium salt (EDTA). As decitabine and azacitidine are known to be pH-sensitive, adjusting the pH of the pharmaceutical formulations to approximately pH 7 may increase the stability of therapeutic component.

Optionally, the method further comprises separation of non-dissolved inventive salt from the pharmaceutical formulations. Separation may be performed by any suitable technique. For example, a suitable separation method may include one or more of filtration, sedimentation, and centrifugation of the pharmaceutical formulations. Clogging that may be caused by non-dissolved particles of the inventive compound, may become an obstacle for administration of the pharmaceutical formulations and a potential hazard for the patient. The separation of non-dissolved inventive compound from the pharmaceutical formulations may facilitate administration and enhance safety of the therapeutic product.

Optionally, the method further comprises sterilization of the pharmaceutical formulations. Sterilization may be performed by any suitable technique. For example, a suitable sterilization method may include one or more of sterile filtration, chemical, irradiation, heat filtration, and addition of a chemical disinfectant to the pharmaceutical formulation.

Optionally, the method may further comprise adding one or more members of the group selected from drying agents, buffering agents, antioxidants, stabilizers, antimicrobials, and pharmaceutically inactive agents. In one variation, antioxidants such as ascorbic acid, ascorbate salts and mixtures thereof may be added. In another variation stabilizers like glycols may be added.

3. Vessels or Kits Containing Inventive Salts or Formulations Thereof

The inventive salts or their formulations described in this invention may be contained in a sterilized vessel such as syringe bottles, and glass vials or ampoules of various sizes and capacities. The sterilized vessel may optionally contain solid salt in a form of powder or crystalline, or its solution formulation with a volume of 1-50 ml, 1-25 ml, 1-20 ml or 1-10 ml. Sterilized vessels enable maintain sterility of the pharmaceutical formulations, facilitate transportation and storage, and allow administration of the pharmaceutical formulations without prior sterilization step.

The present invention also provides a kit for administering the inventive compound to a host in need thereof. In one embodiment, the kit comprises the inventive salt in a solid, preferably powder form, and a liquid diluent that comprises water, glycerin, propylene glycol, polyethylene glycol, or combinations thereof. Mixing of the solid salt and the diluent preferably results in the formation of a pharmaceutical formulation according to the present invention. For example, the kit may comprise a first vessel comprising the inventive salt in a solid form; and a vessel container comprising a diluent that comprises water; wherein adding the diluent to the solid inventive compound results in the formation of a pharmaceutical formulation for administering the inventive salt. Mixing the solid the inventive salt and diluent may optionally form a pharmaceutical formulation that comprises between 0.1 and 200 mg the inventive salt per ml of the diluent, optionally between 0.1 and 100, between 2 mg and 50 mg, 5 mg and 30 mg, between 10 mg and 25 mg per ml of the solvent.

In one embodiment, the diluent is a combination of propylene glycol and glycerin, wherein the concentration of propylene glycol in the solvent is between 0.1-99.9%, optionally between 1-90%, between 10-60%, or between 20-40%.

According to the embodiment, the diluent is a combination of polyethylene glycol and glycerin, wherein the concentration of polyethylene glycol in the solvent is between 0.1-99.9%, optionally between 1-90%, between 10-60%, or between 20-40%.

Also according to the embodiment, the diluent is a combination of propylene glycol, polyethylene glycol and glycerin, wherein the concentration of propylene glycol in the solvent is between 0.1-99.9%, optionally between 1-90%, between 10-60%, or between 20-40%; and the concentration of polyethylene glycol in the solvent is between 0.1-99.9%, optionally between 1-90%, between 10-60%, or between 20-40%.

The diluent also optionally comprises 40%, 20%, 10%, 5%, 2% or less water. In one variation, the diluent is anhydrous and may optionally further comprise a drying agent. The diluent may also optionally comprise one or more drying agents, glycols, antioxidants and/or antimicrobials.

The kit may optionally further include instructions. The instructions may describe how the solid salt and the diluent should be mixed to form a pharmaceutical formulation. The instructions may also describe how to administer the resulting pharmaceutical formulation to a patient. It is noted that the instructions may optionally describe the administration methods according to the present invention.

The diluent and the inventive salt may be contained in separate vessels. The vessels may come in different sizes. For example, the vessel may comprise between 1 and 50, 1 and 25, 1 and 20, or 1 and 10 ml of the diluent.

The pharmaceutical formulations provided in vessels or kits may be in a form that is suitable for direct administration or may be in a concentrated form that requires dilution relative to what is administered to the patient. For example, pharmaceutical formulations, described in this invention, may be in a form that is suitable for direct administration via infusion.

The methods and kits described herein provide flexibility wherein stability and therapeutic effect of the pharmaceutical formulations comprising the inventive compound may be further enhanced or complemented.

4. Methods for Administrating Inventive Salts and Formulations Thereof

The salts/formulations of the present invention can be administered by any route, preferably in the form of a pharmaceutical composition adapted to such a route, as illustrated below and are dependent on the condition being treated. The compounds or formulations can be, for example, administered orally, parenterally, topically, intraperitoneally, intravenously, intraarterially, transdermally, sublingually, intramuscularly, rectally, transbuccally, intranasally, liposomally, via inhalation, vaginally, intraoccularly, via local delivery (for example by catheter or stent), subcutaneously, intraadiposally, intraarticularly, or intrathecally. The compounds and/or compositions according to the invention may also be administered or co-administered in slow release dosage forms.

The salts/formulations of this invention may be administered or co-administered in any conventional dosage form. Co-administration in the context of this invention is defined to mean the administration of more than one therapeutic agent in the course of a coordinated treatment to achieve an improved clinical outcome. Such co-administration may also be coextensive, that is, occurring during overlapping periods of time.

The inventive salts/formulations may be administered into a host such as a patient at a dose of 0.1-1000 mg/ m2, optionally 1-200 mg/m2, optionally 1-150 mg/m2, optionally 1-100 mg/m2, optionally 1-75 mg/m2, optionally 1-50 mg/m2, optionally 1-40 mg/m2, optionally 1-30 mg/m2, optionally 1-20 mg/m2, or optionally 5-30 mg/m2.

For example, the salts of the present invention may be supplied as sterile powder for injection, optionally together with buffering salt such as potassium dihydrogen and pH modifier such as sodium hydroxide. This formulation is preferably stored at 2-8°C, which should keep the drug stable for at least 2 years. This powder formulation may be reconstituted with 10 ml of sterile water for injection. This solution may be further diluted with infusion fluid known in the art, such as 0.9% sodium chloride injection, 5% dextrose injection and lactated ringer's injection. It is preferred that the reconstituted and diluted solutions be used within 4-6 hours for delivery of maximum potency.

In a preferred embodiment, the inventive salts/formulations is administered to a patient by injection, such as subcutaneous injection, bolus i.v. injection, continuous i.v. infusion and i.v. infusion over 1 hour. Optionally the inventive compound/composition is administered to a patient via an 1-24 hour i.v. infusion per day for 3-5 days per treatment cycle at a dose of 0.1-1000 mg/m2 per day, optionally at a dose of 1-200 mg/m2 per day, optionally at a dose of 1-150 mg/m2 per day, optionally at a dose of 1-100 mg/m2 per day, optionally at a dose of 2-50 mg/m2 per day, optionally at a dose of 10-30 mg/m2 per day, or optionally at a dose of 5-20 mg/m2 per day,

For decitabine or azacitidine, the dosage below 50 mg/m2 is considered to be much lower than that used in conventional chemotherapy for cancer. By using such a low dose of the analog/derivative of decitabine or azacitidine, transcriptional activity of genes silenced in the cancer cells by aberrant methylation can be activated to trigger downstream signal transduction, leading to cell growth arrest, differentiation and apoptosis, which eventually results in death of these cancer cells. This low dosage, however, should have less systemic cytotoxic effect on normal cells, and thus have fewer side effects on the patient being treated.

The pharmaceutical formulations may be co-administered in any conventional form with one or more member selected from the group comprising infusion fluids, therapeutic compounds, nutritious fluids, anti-microbial fluids, buffering and stabilizing agents.

As described above, the inventive salts can be formulated in a liquid form by solvating the inventive compound in a non-aqueous solvent such as glycerin. The pharmaceutical liquid formulations provide the further advantage of being directly administrable, (e.g., without further dilution) and thus can be stored in a stable form until administration. Further, because glycerin can be readily mixed with water, the formulations can be easily and readily further diluted just prior to administration. For example, the pharmaceutical formulations can be diluted with water 180, 60, 40, 30, 20, 10, 5, 2, 1 minute or less before administration to a patient.

Patients may receive the pharmaceutical formulations intravenously. The preferred route of administration is by intravenous infusion. Optionally, the pharmaceutical formulations of the current invention may be infused directly, without prior reconstitution.

In one embodiment, the pharmaceutical formulation is infused through a connector, such as a Y site connector, that has three arms, each connected to a tube. As an example, Baxter® Y-connectors of various sizes can be used. A vessel containing the pharmaceutical formulation is attached to a tube further attached to one arm

of the connector. Infusion fluids, such as 0.9% sodium chloride, or 5% dextrose, or 5% glucose, or Lactated Ringer's, are infused through a tube attached to the other arm of the Y-site connector. The infusion fluids and the pharmaceutical formulations are mixed inside the Y site connector. The resulting mixture is infused into the patient through a tube connected to the third arm of the Y site connector. The advantage of this administration approach over the prior art is that the inventive compound is mixed with infusion fluids before it enters the patient's body, thus reducing the time when decomposition of the cytidine analog may occur due to contact with water. For example, the inventive compound is mixed less than 10, 5, 2 or 1 minutes before entering the patient's body.

Patients may be infused with the pharmaceutical formulations for 1, 2, 3, 4, 5 or more hours, as a result of the enhanced stability of the formulations. Prolonged periods of infusion enable flexible schedules of administration of therapeutic formulations.

Alternatively or in addition, speed and volume of the infusion can be regulated according to the patient's needs. The regulation of the infusion of the pharmaceutical formulations can be performed according to existing protocols.

The pharmaceutical formulations may be co-infused in any conventional form with one or more member selected from the group comprising infusion fluids, therapeutic compounds, nutritious fluids, anti-microbial fluids, buffering and stabilizing agents. Optionally, therapeutic components including, but are not limited to, anti-neoplastic agents, alkylating agents, agents that are members of the retinoids superfamily, antibiotic agents, hormonal agents, plant-derived agents, biologic agents, interleukins, interferons, cytokines, immuno-modulating agents, and monoclonal antibodies, may be co-infused with the inventive formulations.

Co-infusion in the context of this invention is defined to mean the infusion of more than one therapeutic agents in a course of coordinated treatment to achieve an improved clinical outcome. Such co-infusion may be simultaneous, overlapping, or sequential. In one particular example, co-infusion of the pharmaceutical formulations and infusion fluids may be performed through Y-type connector.

The pharmokinetics and metabolism of intravenously administered the pharmaceutical formulations resemble the pharmokinetics and metabolism of intravenously administered the inventive salt.

In humans, decitabine displayed a distribution phase with a half-life of 7 minutes and a terminal half-life on the order of 10-35 minutes as measured by bioassay. The volume of distribution is about 4.6 L/kg. The short plasma half-life is due to rapid inactivation of decitabine by deamination by liver cytidine deaminase. Clearance in humans is high, on the order of 126 mL/min/kg. The mean area under the plasma curve in a total of 5 patients was 408 μg/h/L with a peak plasma concentration of 2.01 μM. In patients decitabine concentrations were about 0.4 μg/ml (2 μM) when administered at 100 mg/m2 as a 3-hour infusion. During a longer infusion time (up to 40 hours) plasma concentration was about 0.1 to 0.4 μg/mL. With infusion times of 40-60 hours, at an infusion rate of 1 mg/kg/h, plasma concentrations of 0.43-0.76 μg/mL were achieved. The steady-state plasma concentration at an infusion rate of 1 mg/kg/h is estimated to be 0.2-0.5 μg/mL. The half-life after discontinuing the infusion is 12-20 min. The steady-state plasma concentration of decitabine was estimated to be 0.31-0.39 μg/mL during a 6-hour infusion of 100 mg/m2. The range of concentrations during a 600-mg/m2 infusion was 0.41-16 μg/mL. Penetration of decitabine into the cerebrospinal fluid in man reaches 14-21% of the plasma concentration at the end of a 36-hour intravenous infusion. Urinary excretion of unchanged decitabine is low, ranging from less than 0.01% to 0.9% of the total dose, and there is no relationship between

excretion and dose or plasma drug levels. High clearance values and a total urinary excretion of less than 1% of the administered dose suggest that decitabine is eliminated rapidly and largely by metabolic processes.

Owing to their enhanced stability in comparison with the free base form of decitabine or azacitidine, the inventive salts/compositions can enjoy longer shelf life when stored and circumvent problems associated with clinical use of decitabine or azacitidine. For example, the inventive salts may be supplied as lyophilized powder, optionally with an excipient (e.g., cyclodextrin), acid (e.g., ascorbic acid), alkaline (sodium hydroxide), or buffer salt (monobasic potassium dihydrogen phosphate). The lyophilized powder can be reconstituted with sterile water for injection, e.g., i.v., i.p., i.m., or subcutaneously. Optionally, the powder can be reconstituted with aqueous or non-aqueous solvent comprising a water miscible solvent such as glycerin, propylene glycol, ethanol and PEG. The resulting solution may be administered directly to the patient, or diluted further with infusion fluid, such as 0.9% Sodium Chloride; 5% Dextrose; 5% Glucose; and Lactated Ringer's infusion fluid.

The inventive salts/formulations may be stored under ambient conditions or in a controlled environment, such as under refrigeration (2-8°C; 36-46°F). Due to their superior stability in comparison with decitabine, the inventive salts/formulations can be stored at room temperature, reconstituted with injection fluid, and administered to the patient without prior cooling of the drug solution.

In addition, due to their enhanced chemical stability, the inventive compound/composition should have a longer plasma half-life compared to that of decitabine. Thus, the inventive compound/composition may be administered to the patient at a lower dose and/or less frequently than that for decitabine or azacitidine.

5. Indications for Inventive Salts or Formulations Thereof

The inventive salts/formulations described herein have many therapeutic and prophylactic uses. In a preferred embodiment, the salt forms of cytidine analogs and derivatives, including salt forms of decitabine and azacitidine, are used in the treatment of a wide variety of diseases that are sensitive to the treatment with a cytidine analog or derivative, such as the free base form of decitabine or azacitidine. Preferable indications that may be treated using the inventive salts/formulations include those involving undesirable or uncontrolled cell proliferation. Such indications include benign tumors, various types of cancers such as primary tumors and tumor metastasis, restenosis (e.g. coronary, carotid, and cerebral lesions), hematological disorders, abnormal stimulation of endothelial cells (atherosclerosis), insults to body tissue due to surgery, abnormal wound healing, abnormal angiogenesis, diseases that produce fibrosis of tissue, repetitive motion disorders, disorders of tissues that are not highly vascularized, and proliferative responses associated with organ transplants.

Generally, cells in a benign tumor retain their differentiated features and do not divide in a completely uncontrolled manner. A benign tumor is usually localized and nonmetastatic. Specific types benign tumors that can be treated using the present invention include hemangiomas, hepatocellular adenoma, cavernous haemangioma, focal nodular hyperplasia, acoustic neuromas, neurofibroma, bile duct adenoma, bile duct cystanoma, fibroma, lipomas, leiomyomas, mesotheliomas, teratomas, myxomas, nodular regenerative hyperplasia, trachomas and pyogenic granulomas.

In a malignant tumor cells become undifferentiated, do not respond to the body's growth control signals, and multiply in an uncontrolled manner. The malignant tumor is invasive and capable of spreading to distant sites (metastasizing). Malignant tumors are generally divided into two categories: primary and secondary. Primary tumors arise directly from the tissue in which they are found. A secondary tumor, or metastasis, is a tumor which is originated elsewhere in the body but has now spread to a distant organ. The

common routes for metastasis are direct growth into adjacent structures, spread through the vascular or lymphatic systems, and tracking along tissue planes and body spaces (peritoneal fluid, cerebrospinal fluid, etc.)

Specific types of cancers or malignant tumors, either primary or secondary, that can be treated using this invention include breast cancer, skin cancer, bone cancer, prostate cancer, liver cancer, lung cancer, brain cancer, cancer of the larynx, gall bladder, pancreas, rectum, parathyroid, thyroid, adrenal, neural tissue, head and neck, colon, stomach, bronchi, kidneys, basal cell carcinoma, squamous cell carcinoma of both ulcerating and papillary type, metastatic skin carcinoma, osteo sarcoma, Ewing's sarcoma, veticulum cell sarcoma, myeloma, giant cell tumor, small-cell lung tumor, gallstones, islet cell tumor, primary brain tumor, acute and chronic lymphocytic and granulocytic tumors, hairy-cell tumor, adenoma, hyperplasia, medullary carcinoma, pheochromocytoma, mucosal neuronms, intestinal ganglloneuromas, hyperplastic corneal nerve tumor, marfanoid habitus tumor, Wilm's tumor, seminoma, ovarian tumor, leiomyomater tumor, cervical dysplasia and in situ carcinoma, neuroblastoma, retinoblastoma, soft tissue sarcoma, malignant carcinoid, topical skin lesion, mycosis fungoide, rhabdomyosarcoma, Kaposi's sarcoma, osteogenic and other sarcoma, malignant hypercalcemia, renal cell tumor, polycythermia vera, adenocarcinoma, glioblastoma multiforma, leukemias, lymphomas, malignant melanomas, epidermoid carcinomas, and other carcinomas and sarcomas.

Hematologic disorders include abnormal growth of blood cells which can lead to dysplastic changes in blood cells and hematologic malignancies such as various leukemias. Examples of hematologic disorders include but are not limited to acute myeloid leukemia, acute promyelocytic leukemia, acute lymphoblastic leukemia, chronic myelogenous leukemia, the myelodysplastic syndromes, and sickle cell anemia.

In some embodiments, the salts of the instant invention are used to treat blood disorders, including inherited blood disorders and/or disorders where hemoglobin is defective, e.g., sickle cell anemia. In some embodiments, the salts of the instant invention can be used to treat cancer, including leukemia, pre-leukemia, and other bone marrow-related cancers, e.g., myelodysplatic syndrome (MDS)); as well as lung cancer, e.g., non-small cell lung cancer (NSCL). NSCL can include epidermoid or squamous carcinnoma, adenocarcinoma, and large cell carcinoma. MDS can include refractory anemia, refractory anemia with ringed sideroblasts, refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, and chronic myelomonocytic leukemia.

The present invention provides methods, pharmaceutical compositions, and kits for the treatment of animal subjects. The term "animal subject" as used herein includes humans as well as other mammals. The term "treating" as used herein includes achieving a therapeutic benefit and/or a prophylactic benefit. By therapeutic benefit is meant eradication or amelioration of the underlying disorder being treated. For example, in patient with sickle cell anemia, therapeutic benefit includes eradication or amelioration of the underlying sickle cell anemia. Also, a therapeutic benefit is achieved with the eradication or amelioration of one or more of the physiological symptoms associated with the underlying disorder such that an improvement is observed in the patient, notwithstanding the fact that the patient may still be afflicted with the underlying disorder. For example, a salt of the present invention provides therapeutic benefit not only when sickle cell anemia is eradicated, but also when an improvement is observed in the patient with respect to other disorders or discomforts that accompany sickle cell anemia, like hand-foot syndrome, fatigue, and or the severity or duration of pain experienced during a crisis (painful episode). Similarly, salts of the present invention can provide

therapeutic benefit in ameliorating symptoms associated with cancers, e.g., MDS or NSCL, including anemia, bruising, persistent infections, the size of a lung tumor, and the like.

For prophylactic benefit, a salt of the invention may be administered to a patient at risk of developing a cancer or blood disorder, or to a patient reporting one or more of the physiological symptoms of such a condition, even though a diagnosis of the condition may not have been made.

If necessary or desirable, the salt may be administered in combination with other therapeutic agents. The choice of therapeutic agents that can be co-administered with the compounds and compositions of the invention will depend, in part, on the condition being treated. Examples of other therapeutic agents include, but are not limited to, anti-neoplastic agents, alkylating agents, agents that are members of the retinoids superfamily, antibiotic agents, hormonal agents, plant-derived agents, biologic agents, interleukins, interferons, cytokines, immuno-modulating agents, and monoclonal antibodies. For example, in the case of sickle cell anemia, a salt of the instant invention may be administered with antibiotics and/or hydroxyurea; in the case of MDS or NSCL, a salt of the instant invention may be administered with a chemotherapeutic agent.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are present in an effective amount, i.e., in an amount effective to achieve therapeutic and/or prophylactic benefit in a condition being treated, including, e.g., a blood disorder, such as sickle cell anemia, MDS, and/or a cancer such as NSCL.

EXAMPLES

The following examples are intended to illustrate details of the invention, without thereby limiting it in any manner.

1. Synthesis of Salts of Cytidine Analogs

1) Decitabine Salt Formation

In some embodiments of the present invention, preparation of decitabine salts includes stirring a mixture of decitabine and acid (e.g., an acid included in Table 1a) in solvent(s) (e.g., a solvent(s) listed in Table 1b) at -70 to 100 °C for 0 to 24 hours, allowing crystallization at -70 to 25 °C, and performing filtration and purification by recrystallization from solvent(s).

Table 1b. Examples of solvent(s) that can be used for preparation of salts.

Solvent	Solubility of Decitabine free base (mg/mL)
Acetone	<1
Acetonitrile	<1
Acetonitrile:Water (1:1)	22
2-Butanone	<1
Chloroform	<1
Dichloromethane	<1
Dichloromethane: Ethanol (1:1)	<1
Dichloromethane: Methanol (1:1)	>1
Diethylamine	<1
N,N-Dimethylformamide	5
1,4-Dioxane	<2
Ethanol:Water (1:1)	3
Ethyl Acetate	<1
Ethyl Ether	<1
1,1,1,3,3,3-Hexafluoro-2-propanol	18
Hexanes	<1
Methanol	2
Methanol: 2,2,2-Trifluoroethanol (1:1)	>1
Methanol:Water (1:1)	4
Methyl Sulfide	<1
Methyl Sulfoxide	37
Nitromethane	<1
2-Propanol	<1
Tetrahydrofuran	<1
Toluene	<1
1,1,1-Trichloroethane	<1
2,2,2-Trifluoroethanol	2
2,2,2-Trifluoroethanol:Water (9:1)	5
Water	8

In some embodiments, decitabine salts were prepared from strong acids. In one embodiment, for example, decitabine hydrochloride (3), depicted above, was prepared by suspending decitabine (0.25g, 3.7 mmol) in methanol (40 mL) in a round bottom flask (100-mL). The mixture was gently stirred at 22°C. HCl gas (not less than 2-fold excess) was bubbled into the stirred methanol solution until complete dissolution was reached. The solution was concentrated to 1/3 volume, flushed with nitrogen, corked with a rubber septum and allowed to crystallize (0°C) for NLT 12 h. The first crop of crystalline product was filtered, rinsed with anhydrous ether (5 mL) and dried in vacuo for NLT 12 h. The filtrate was poured back into the 50 mL Erlenmeyer flask, and enough anhydrous ether was added to a cloudy point. The solution was flushed with nitrogen, corked with a rubber septum and allowed to crystallize (0°C) for NLT 12 h. The second crop of crystalline product was filtered, rinsed with anhydrous ether (40 mL) and dried in vacuo for NLT 12 h.

In one embodiment, for example, decitabine mesylate (4), depicted above, was prepared by suspending decitabine (1.0g, 3.7 mmol) in methanol (80 mL) in a round bottom flask (250-mL). The solution was flushed with nitrogen gas, corked with a rubber septum, and was gently stirred for 10 minutes at ambient temperature. Methanesulfonic acid (4.0 mL) was injected through the rubber septum slowly, and the mixture was gently stirred for 1 h. The suspension of decitabine immediately disappeared and the mixture became clear before

decitabine mesylate recrystallized. The crystals were allowed to completely crystallize (0°C) for NLT 4 h. The product was thoroughly washed with MeOH (50 mL) during filtration and dried in vacuo for NLT 12 h.

Decitabine salts were also prepared from moderate acids. In some embodiments, for example, decitabine EDTA (5), L-aspartate (6), maleate (7) or L-glutamate (8), depicted above, can be prepared by the following procedure. Ethylenediaminetetraacetic acid (EDTA, 1.409g, 4.8 mmol), L-Aspartic acid (641 mg), maleic acid (610 mg, 5.3 mmol) or L-glutamic acid (709 mg) was weighed in a 250 ml round bottom flask before adding methanol (100 mL) and decitabine (1.0g), and the mixture was stirred at 50 °C for 1 hr or longer until the solution was clear. The filtrate was concentrated to about 1/2 volume to allow crystallization to occur. The solution was flushed with nitrogen, corked with a rubber septum and allowed to crystallize (0 °C) for NLT 4 hrs. The first crop of crystalline product was filtered and dried in vacuo for NLT 12 hrs. In methanol, decitabine formed 1:1 molar equivalent with EDTA (5), 1:1.5 with L-aspartate (6), 0.78 molar equivalent of maleate (7), and 1:1.5 with L-glutamate (8) (see also Table 2 below).

In some further embodiments, for example, decitabine sulfite (9) or phosphate (10), depicted above, was prepared by suspending decitabine (1.0g, 3.7 mmol) in methanol (80 mL) in a round bottom flask (250 mL). The solution was flushed with nitrogen gas, corked with a rubber septum, and was gently stirred for 10 minutes at ambient temperature. Sulfurous acid (4.0 mL) or phosphoric acid (0.8 mL) was injected through the rubber septum slowly, and the mixture was gently stirred for 1 hr. The suspension of decitabine disappeared and the mixture became clear before decitabine salt recrystallized. The crystals were allowed to completely crystallize (0 °C) for NLT 4 hrs. The product was thoroughly washed with MeOH (50 mL) during filtration and dried in vacuo for NLT 12 hr. In methanol, decitabine formed 1:1 molar equivalent with sulfite (9) and phosphate (10) (see also Table 2 below).

In still some embodiments, decitabine salts were prepared from weak acids (3.0<pKa<5). For example, decitabine salts of (+)-L-tartaric, citric, L-lactic, succinic, acetic, hexanoic, butyric, or propionic acid (11-18, respectively, depicted above) were prepared by the following procedure: Decitabine (1.0 g, 4.4 mmol) was suspended in methanol (50 mL) in a round bottom flask (50 mL) and flushed and closed with nitrogen before adding acid (liquid acid: 0.4-4.4 mL; solid acid: 2-5 g) and each was heated in a sonicator at 30-55 °C until complete dissolution. If after 30 minutes complete dissolution hadn't been achieved, more methanol (5mL) was added every 10 minutes. The solution was allowed to cool to 23 °C and then stored at 0 °C for NLT 12 hrs. The first crop of crystalline product was filtered and dried in vacuo for NLT 12 hr.

Decitabine salts prepared from weak acids (3.0<pKa<5) showed less robust results. For example, in methanol, decitabine does not readily formed 1:1 molar equivalent with (+)-L-tartaric, citric, L-lactic, succinic, acetic, hexanoic, butyric, or propionic acid to form the corresponding salts (11-18, respectively, depicted above). Instead, varying ratios of acids, from 0.03 to 0.19 molar equivalents, were obtained (see also Table 2 below), which may indicate that there was partial salt formation. However, this does not necessary mean that 1:1 molar equivalent salts of these weak acids can not be prepared with other solvents.

2) Azacitidine Salt Formation

The synthesis techniques described herein for decitabine salts can also be adapted for preparation of the corresponding azacitidine salts. Analogous salts of azacitidine can also be prepared from acids used in preparation of decitabine salts. For example, in some embodiments of the present invention, preparation of azacitidine salts includes stirring a mixture of azacitidie and acid (e.g., an acid included in Table 1a).

For example, azacitidine mesylate (19, depicted above) is an azacitidine salt formed with the strong acid methanesulfonic acid. In some embodiments, azacitidine mesylate (19) was prepared by suspending azacitidine (0.5 g, 2.0 mmol) in methanol (40 mL) in a round bottom flask (100 mL). The solution was flushed with nitrogen gas, corked with a rubber septum, and was gently stirred for 10 minutes at ambient temperature. Methanesulfonic acid (2.0 mL) was injected through the rubber septum slowly, and the mixture was gently stirred for 1 h. The suspension of decitabine immediately disappeared and the mixture became clear. The volume of the mixture was reduced by half in vacuo, and azacitidine mesylate crystals were allowed to completely crystallize (0 °C) for NLT 4 h. The product was thoroughly washed with MeOH (40 mL) during filtration and dried in vacuo for NLT 12 h. Azacitidine can readily form 1:1 molar equivalent mesylate salt (19).

2. Solubility and Stability of Decitabine and Azacitidine Salts

Table 2 shows the rate of dissolution and total solubility, as well as other selected properties, for some embodiments of the instant invention compared to free decitabine and free azacitidine. Dissolution rate is based on the time it takes for 1.0 mg of sample to dissolve in water. Dissolution rates for most embodiments, e.g., most decitabine salts, are superior to that of the free base. For example, decitabine hydrochloride (3) (1 second with mixing) and decitabine mesylate (4) (3 seconds with sonication) salts are superior to decitabine free base (1) (3 minutes with sonication). Without being limited to a particular hypothesis, faster rates of dissolution may reduce hydrolytic degradation during manufacture, as well as reducing reconstitution time for powder forms. The rate of dissolution for azacitidine mesylate (19), however, was surprisingly found to be less than the free azacitidine base (2). That is, as shown in Table 2, the dissolution rate for azacitidine mesylate salt (19) (1 minute sonication) is slower than that for azacitidine free base (2) (3 second mixing).

Apparent total solubility was determined by successively adding 5 mg of a sample to a 5-mL vial containing 1.0 mL of deionized water and sonicating the mixture for 1 minute. Additional sample was added in 5-mg increments and sonication for 1 min was repeated until a suspension formed. Total solubilities of most decitabine salt forms are better than or at least as good as decitabine free base. Apparent total solubility for decitabine hydrochloride (3) (280 mg/mL) and decitabine mesylate (4) (195 mg/mL) salts, which is equivalent to 241 mg/mL and 137 mg/mL of free base, respectively, is substantially higher than decitabine free base (1) (8-10 mg/mL). Solubility for 1:1 molar ratio salts such as decitabine-HCl and decitabine-mesylate, for example, increases the solubility of decitabine by more than 10-fold. Similarly, decitabine sulfite (9) and decitabine phosphate (10) show solubilities of 80mg/mL and 50 mg/mL, respectively, or equivalent to 59 mg/mL and 35 mg/mL of free decitabine base respectively. One of skill in the art will recognize, however, that for some other decitabine salts, the total solubility measurements may not be representative of their 1:1 free base: acid molar ratio equivalents.

With respect to azacitidine mesylate (19), while its rate of dissolution was surprisingly found to be less than that of free azacitidine base (2), as noted above, the apparent total solubility is greatly enhanced, i.e., 205 mg/mL for the salt (19) (equivalent to 137 mg/mL of free azacitidine base) compared with 14 mg/mL for azacitidine free base (2).

Table 2: Summary of selected properties of decitabine and azacitidine salts

Compound #	Salt	$C_8H_{12}N_4O_4$· __ Acid Molar Ratio[#]	Appearance	Dissolution In water (1.0mg/mL)	Total Solubility (mg/mL)
1	**Decitabine free base**	--	White Powder	3 min Sonication	8-10
2	**Azacitidine free base**	--	White Powder	1 sec. Mixing	14
3	**Decitabine HCl**	1.04	White Crystalline Powder	1 sec. Mixing	280 (241)*
4	**Decitabine Mesylate**	1.00	White Crystalline Powder	3 sec Sonication	195 (137)*
5	**Decitabine EDTA**	1.10	White Powder	5 min Sonication	25-35
6	**Decitabine L-Aspartate**	1.56	White Crystalline Powder	8 sec. Sonication	25-35
7	**Decitabine Maleate**	0.078	White Crystalline Powder	5 sec. Sonication	25-35
8	**Decitabine L-Glutamate**	1.58	White Crystalline Powder	10 sec. Sonication	25-35
9	**Decitabine Sulfite**	0.99	White Crystalline Powder	1 sec. Mixing	80 (59)*
10	**Decitabine Phosphate**	1.06	White Powder	5 sec. mixing	50 (35)*
11	**Decitabine (+)-L-Tartrate**	0.091	White Powder	5 sec. Sonication	25-35
12	**Decitabine Citrate**	0.061	White Powder	5 sec. Sonication	25-35
13	**Decitabine Lactate**	0.089	Fine white Crystalline Powder	3 sec. Mixing	25-35
14	**Decitabine Succinate**	0.030	White Crystalline Powder	15 sec. Sonication	25-35
15	**Decitabine Acetate**	0.17	Fine white crystalline Powder	2 sec. Sonication	25-35
16	**Decitabine Hexanoate**	0.11	White Crystalline Powder	3 sec. Sonication	25-35
17	**Decitabine Butyrate**	0.15	White Crystalline Powder	4 sec. Sonication	25-35
18	**Decitabine Propionate**	0.19	White Crystalline Powder	2 sec. Sonication	25-35
19	**Azacitidine Mesylate**	1.02	White Crystalline Powder	1 min Sonication	205 (137)*

[#] Based on elemental analysis

* Decitabine or azacitidine free base equivalents

Table 3 shows the melting points and hydroscopicity of certain embodiments of the instant invention compared to free decitabine and free azacitidine. The observed melting (decomposition) points for decitabine hydrochloride (3) (130°C) and decitabine mesylate (4) (125°C), for example, are different from that of decitabine free base crystalline anhydrate (1) (190°C). The observed melting (decomposition) point for azacitidine mescylate (19) (138°C) was also found to be different from that of azacitidine free base (2) (230°C).

Table 3 also shows that certain salts are slightly more hydroscopic than the corresponding free base. Percent weight gained after one week in 56% relative humidity (RH) for decitabine hydrochloride (3) and decitabine mesylate (4) salts were similar to decitabine free base (1). At 98% RH, decitabine hydrochloride picked up considerably more moisture than decitabine – 65.5% compared to only 2.88% weight gain. Decitabine mesylate, however, was determined to be no more hydroscopic than decitabine at 98% RH, showing only 2.84% weight gain. Nonetheless, azacitidine mesylate (19) was shown to be more hydroscopic than free azacitidine (2).

Table 3. Stability of decitabine and azacitidine salt forms in solid state

Compound #	Sample	pK_{a1} of Acid Used	Melting Point (°C) (Decompose)	$C_8H_{12}N_4O_4 \cdot$ __ Acid Molar Ratio	Hygroscopicity-% weight gain in 1 week	
					56% RH	98% RH
1	Decitabine free base	--	190	--	0.68	2.88
2	Azacitidine free base	--	230	--	1.74	5.61
3	Decitabine HCl	-9	130	1.04	0.81	65.6
4	Decitabine Mesylate	-1.2	125	1.00	0.50	2.84
5	Decitabine EDTA	1.7	230	1.10	1.23	3.76
6	Decitabine L-Aspartate	1.9	190	1.56	3.23	4.21
7	Decitabine Maleate	1.9	210	0.078	0.76	7.2
8	Decitabine L-Glutamate	2.2	180	1.58	2.0	3.95
9	Decitabine Sulfite	1.9	220	0.99	0.29	1.46
10	Decitabine Phosphate	2.0	118	1.06	0.48	5.51
11	Decitabine (+)-L-Tartrate	3.0	202	0.091	4.12	7.71
12	Decitabine Citrate	3.1	202	0.061	5.20	7.03
13	Decitabine L-Lactate	3.9	195	0.089	0.79	11.13
14	Decitabine Succinate	4.2	210	0.030	5.56	8.25
15	Decitabine Acetate	4.8	206	0.17	0.53	4.47
16	Decitabine Hexanoate	4.8	205	0.11	0.0	2.10

17	**Decitabine Butyrate**	4.8	204	0.15	0.10	1.93
18	**Decitabine Propionate**	4.9	200	0.19	0.58	2.07
19	**Azacitidine Mesylate**	-1.2	138	1.02	6.05	38.11

Table 4 depicts the aqueous stability of certain decitabine and azacitidine salts of the present invention. Aqueous stability was determined in phosphate buffer at pH 7.0 and pH 2.5 at a drug concentration of 0.5 mg/mL. The assay conditions were: mobile phase- mixture of 40±0.5 mL of methanol and 2000 mL of 10 mM ammonium acetate; column temperature of 15+2 °C; auto sampler temperature of 5°C; flow rate of 1.7 mL/min; injection volume of 5 μL; detector wavelength of 220 nm; and analysis time of 25 minutes.

The solution stability of some of the decitabine salts in 0.05 M phosphate buffer solution at pH of 7.0 and 2.5 are at least as stable as decitabine free base. At pH of 7.0, decitabine hydrochloride (3) and decitabine free base (1) gave similar percent recoveries after approximately 30 minutes (87.59% and 87.17%) and 24 hours (81.07% and 84.07%, respectively) at ambient condition. Decitabine mesylate (4) exhibited slightly better percent recovery after 30 minutes and 24 hours (91.19% and 89.49%, respectively) at pH 7.0.

At pH of 2.5, decitabine mesylate (4) and decitabine free base (1) exhibited similar percent recovery after approximately 30 minutes (55.96% and 57.09%) and 24 hours (48.77% and 50.38%, respectively) at ambient condition. Decitabine hydrochloride (3) gave considerably better percent recovery after 30 minutes (77.89%), but eventually decreased to a value (49.90%) similar to decitabine free base (1). Decitabine L-aspartate (6) and decitabine sulfite (9) also appear to stabilize decitabine rather well. For example, the stability of decitabine sulfite (9) is improved at pH of 2.5 (95.96% after 30 minutes and 92.96% after 24 hours) compared with decitabine free base (1) (57.09% after 30 minutes and 50.8% after 24 hours).

With respect to azacitidine mesylate (19), the stability of this 1:1 salt is slightly less than the free azacitidine base (2).

Table 4: Stability of salts in 0.05 *M* phosphate buffer solution (0.5 mg/mL) at pH 7.0 and 2.5.

Compound #	**Sample**	**pK_{a1} of Acid Used**	**$C_8H_{12}N_4O_4$: Acid Molar Ratio**	**Potency Found (%) At pH 7.0**		**Potency Found (%) At pH 2.5**	
				t= 0.5 hr	**t= 24 hr**	**t= 0.5 hr**	**t= 24 hr**
1	**Decitabine free base**	--	--	87.17	84.07	57.09	50.38
2	**Azacitidine free base**	--	--	86.74	79.43	73.62	54.85
3	**Decitabine HCl**	-9	1.04	87.59	81.07	77.89	49.90
4	**Decitabine Mesylate**	-1.2	1.00	91.19	89.49	55.96	48.77
5	**Decitabine EDTA**	1.7	1.10	66.05	56.63	31.14	27.18
6	**Decitabine L-Aspartate**	1.9	1.56	97.37	87.44	71.79	63.77
7	**Decitabine Maleate**	1.9	0.078	87.56	80.54	52.14	46.54

8	Decitabine L-Glutamate	2.2	1.58	89.10	78.46	60.82	51.62
9	Decitabine Sulfite	1.9	0.99	94.90	83.78	95.96	92.96
10	Decitabine Phosphate	2.0	1.06	85.97	79.78	80.31	42.42
11	Decitabine (+)-L-Tartrate	3.0	0.091	96.31	92.53	57.10	50.96
12	Decitabine Citrate	3.1	0.061	92.01	88.35	57.50	50.64
13	Decitabine L-Lactate	3.9	0.089	88.38	88.03	62.81	55.27
14	Decitabine Succinate	4.2	0.030	87.35	80.58	62.81	54.89
15	Decitabine Acetate	4.8	0.17	89.73	84.06	56.39	50.31
16	Decitabine Hexanoate	4.8	0.11	93.77	88.24	59.40	52.84
17	Decitabine Butyrate	4.8	0.15	94.63	88.25	58.59	50.70
18	Decitabine Propionate	4.9	0.19	94.63	88.89	62.36	56.60
19	Azacitidine Mesylate	-1.2	1.02	77.47	65.79	64.56	49.94

3. Thermal Analyses of Decitabine and Azacitidine salts

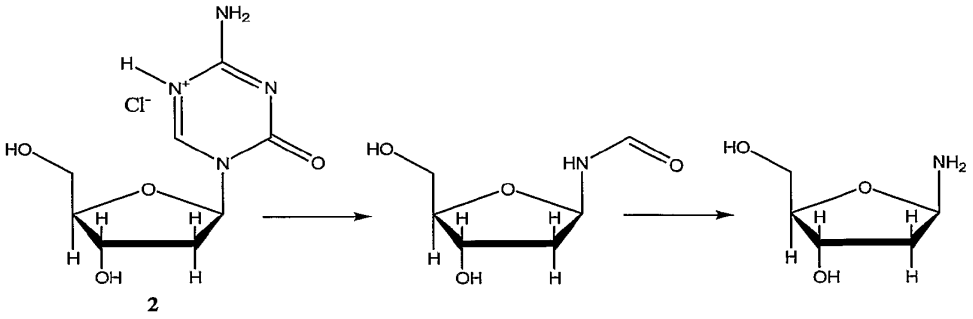
For some of the salt forms, "fingerprint" analyses that include Differential Scanning Calorimetry (DSC), Thermo Gravimetric Analysis (TGA), X-ray Diffraction (XRD) and Infrared (IR) Spectroscopic analysis are provided herein. Numerical values for DSC provided herein are intended to be each modified by "about." For example, DSC values provided herein represent the given numerical value + 1oC, + 2oC, + 3oC, + 4oC, + 5oC, + 6oC, + 7oC, + 8oC, + 9oC, + 10oC and + at least 10oC.

As mentioned above, the observed melting (decomposition) points shown in Table 3 for decitabine hydrochloride (3) (130°C) and decitabine mesylate (4) (125°C) are different from that of decitabine free base crystalline anhydrate (1) (190°C). These values were corroborated by differential scanning calorimetry (DSC) plots (at 10°C per minute, ambient to 250°C). Figures 1-17 illustrate DSC plots of decitabine hydrochloride (3), decitabine mesylate (4), decitabine EDTA (5), decitabine l-aspartate (6), decitabine maleate (7), decitabine l-glutamate (8), decitabine sulfite (9), decitabine phosphate (10), decitabine tartrate (11), decitabine citrate (12), decitabine l-(+)-lactate (13), decitabine succinate (14), decitabine acetate (15), decitabine hexanoate (16), decitabine butyrate (17), decitabine propionate (18), and azacitidine mesylate (19), respectively.

As Figure 1 illustrates, decitabine hydrochloride (3) undergoes a major thermal event starting around 130°C and culminating at 144°C. As illustrated in Figure 2, decitabine mesylate (4) has a major thermal event starting around 125°C and culminating at 134°C. These DSC endothermic events with an onset near 125-130 °C correspond to the melt, which is accompanied by an exothermic event. This behavior indicates that both decitabine hydrochloride and decitabine mesylate melt with decomposition.

Thermal analyses of these two novel salts suggest that they are anhydrate form. Figures 18 and 19 illustrate TGA plots of decitabine hydrochloride (3) and decitabine mesylate (4), respectively. TGA plot for each does not show a weight loss up to the decomposition point of the sample. As Figure 18 illustrates, the

TGA plot of decitabine hydrochloride (3) shows a steep decomposition curve appearing around 150°C and accounting for over 38% weight loss. The decomposition curve finally plateaus around 200 to 250°C. Without being limited to a particular hypothesis, it appears that loss of hydrogen chloride during decomposition is accompanied by cleavage of the triazine ring around 150°C, as depicted below.



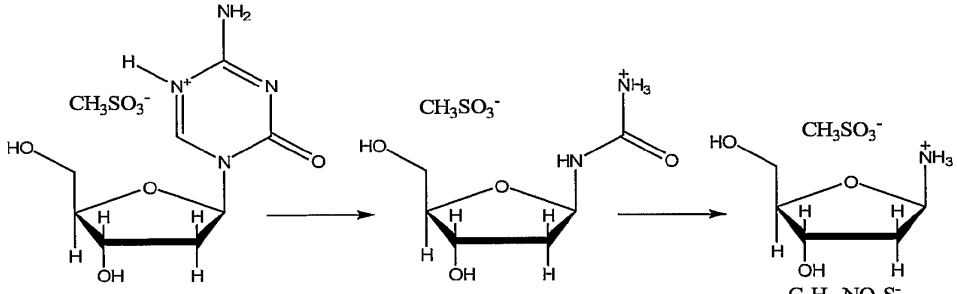
2

$C_8H_{13}ClN_4O_4$
Mol. Wt.: 264.67

$C_6H_{11}NO_4$
Mol. Wt.: 161.16

$C_5H_{11}NO_3$
Mol. Wt.: 133.15

Figure 19 illustrates the TGA plot of decitabine mesylate (4), where two major consecutive decomposition events appear around 150°C and around 200 to 250°C. The first event accounts for 15% weight lost, while the second accounts for 14%. While not being limited to a particular hypothesis, decitabine mesylate may decompose in stages similar to those of decitabine hydrochloride, as depicted below. For example, decitabine mesylate decomposition may be accompanied by cleavage of the triazine ring, as hypothesized in the case of decitabine hydrochloride. In contrast, however, cleavage of the triazine in free decitabine does not occur until around 190°C.



3

$C_9H_{16}N_4O_7S$
Exact Mass: 324.07
Mol. Wt.: 324.31

$C_7H_{16}N_2O_7S$
Mol. Wt.: 272.28

$C_6H_{14}NO_6S^-$
Mol. Wt.: 228.24

Figures 20-34 illustrate TGA plots for additional salts of the instant invention, namely decitabine EDTA (5), decitabine l-aspartate (6), decitabine maleate (7), decitabine l-glutamate (8), decitabine sulfite (9), decitabine phosphate (10), decitabine tartrate (11), decitabine citrate (12), decitabine l-(+)-lactate (13), decitabine succinate (14), decitabine acetate (15), decitabine hexanoate (16), decitabine butyrate (17), decitabine propionate (18), and azacitidine mesylate (19), respectively.

From the DSC and TGA plots for decitabine EDTA (5), decitabine l-aspartate (6), decitabine maleate (7), decitabine l-glutamate (8), decitabine sulfite (9), and decitabine phosphate (10) (Figures 3-8 and 20-25, respectively), it can be seen that these salts are not free decitabine. Accordingly, decitabine sulfite (9) and

decitabine phosphate (10) have solubility of 80 mg/mL and 50 mg/mL, respectively or equivalent to 59 mg/mL and 35 mg/mL of free base, respectively (as shown in Table 2 above). From the DSC and TGA plots for decitabine tartrate (11), decitabine citrate (12), decitabine l-(+)-lactate (13), decitabine succinate (14), decitabine acetate (15), decitabine hexanoate (16), decitabine butyrate (17), decitabine propionate (18) (Figures 9-16 and 26-33, respectivley), it can be seen that these crude salt mixtures predominantly contain decitabine. As such, solubility measurement of these crude salt mixtures shown in Table 2 may not be representative of pure 1:1 molar equivalent salts. Nonetheless, as shown in Table 4, the stabilities of these crude salt mixtures are at least as good as decitabine, if not slightly better.

As mentioned above, the observed melting (decomposition) point shown in Table 3 for azacitidine mesylate (19) (138°C) is different from that of azacitidine free base (2) (230°C). This value was corroborated by DSC plot (at 10°C per minute, ambient to 250°C), illustrated in Figure 17. As Figure 17 shows, azacitidine mesylate (19) undergoes major thermal events around 70, 95 and 118 °C. These endothermic events with an onset near 70-130 °C correspond to the melt, which is accompanied exothermic event. This behavior indicates that azacitidine mesylate can melt with decomposition.

Further, as illustrated in Figure 34, the TGA plot of azacitidine mesylate, a series of major decomposition events appear around 70°C to 250°C. The decomposition events prior to 150°C accounts for less than 10% weight lost, while consecutive decomposition up to 250°C accounts for almost 50% weight lost.

4. X-ray Diffraction and Infra-Red Spectra for Decitabine and Azacitidine Salts

Fingerprint XRD also were obtained for certain embodiments of the instant invention. Figures 35-51 illustrate XRD patterns of decitabine hydrochloride (3), decitabine mesylate (4), decitabine EDTA (5), decitabine l-aspartate (6), decitabine maleate (7), decitabine l-glutamate (8), decitabine sulfite (9), decitabine phosphate (10), decitabine tartrate (11), decitabine citrate (12), decitabine l-(+)-lactate (13), decitabine succinate (14), decitabine acetate (15), decitabine hexanoate (16), decitabine butyrate (17), decitabine propionate (18), and azacitidine mesylate (19), respectively.

IR absorbance spectra also were obtained for certain embodiments of the instant invention. Figures 52-68 illustrate IR absorbance spectra for decitabine hydrochloride (3), decitabine mesylate (4), decitabine EDTA (5), decitabine l-aspartate (6), decitabine maleate (7), decitabine l-glutamate (8), decitabine sulfite (9), decitabine phosphate (10), decitabine tartrate (11), decitabine citrate (12), decitabine l-(+)-lactate (13), decitabine succinate (14), decitabine acetate (15), decitabine hexanoate (16), decitabine butyrate (17), decitabine propionate (18), and azacitidine mesylate (19), respectively.

From the IR spectra for decitabine hydrochloride (3) (Figure 52) and decitabine mesylate (4) (Figure 53), one of skill in the art can see that all functional groups that exist in decitabine remain intact in decitabine hydrochloride and decitabine mesylate salts. A characteristically strong absorption for S=O (stretching vibration) appears at 1169 cm-1 for decitabine mesylate (4) that does not exist for decitabine free base.

5. Summary of Analytical Data

Table 5 provides a summary of analytical data for certain embodiments relating to decitabine and azacitidine salts of the instant invention, including DSC, TGA, XRD and IR spectra for decitabine hydrochloride (3), decitabine mesylate (4), decitabine EDTA (5), decitabine l-aspartate (6), decitabine maleate

(7), decitabine l-glutamate (8), decitabine sulfite (9), decitabine phosphate (10), decitabine tartrate (11), decitabine citrate (12), decitabine l-(+)-lactate (13), decitabine succinate (14), decitabine acetate (15), decitabine hexanoate (16), decitabine butyrate (17), decitabine propionate (18), and azacitidine mesylate (19), along with the corresponding Figures (discussed above). For comparison, decitabine free base (1), decitabine hydrate ('1), and azacitidine free base (2) data are also provided.

Table 5. Summary of analytical data for certain decitabine and azacitidine salts

#	Sample	Melting Point (°C) (Decompose)	DSC Endotherm[a]	TGA[b] Wt. Loss	XRD Maxima[c] (CPS @ θ-2θ°)	Distinctive Absorption (cm^{-1})
1	**Decitabine free base**	190	203 °C	0.032% @ 150 °C	--	--
1'	**Decitabine Hydrate**	--	86.0 °C, 94.9 °C, 198.4 °C	7.2% @ 150 °C	--	--
2	**Azacitidine free base**	230	--	--	--	--
3	**Decitabine HCl**	130	125 to 155 °C	38.85% @ 160 °C; 8.03% @ 200 °C; 3.95% @ 260 °C	14.79°; 23.63°; 29.81°	--
			Figure 1	Figure 18	Figure 35	Figure 52
4	**Decitabine Mesylate**	125	125 to 140 °C	15.29% @ 150 °C; 14.06% @ 260 °C	8.52°; 22.09°; 25.93°	1169 (S=O)
			Figure 2	Figure 19	Figure 36	Figure 53
5	**Decitabine EDTA**	230	50 to 90 °C; 165 to 170 °C; 170 to 200 °C	8.45% @ 200 °C; 39.14% @ 260 °C	7.14°; 22.18°; 24.63°	--
			Figure 3	Figure 20	Figure 37	Figure 54
6	**Decitabine L-Aspartate**	190	30 to 100 °C; 170 to 195 °C; 195 to 250 °C	1.86% @ 80 °C; 17.18% @ 220 °C; 18.58% @ 260 °C	21.61°; 22.71°; 23.24°	--
			Figure 4	Figure 21	Figure 38	Figure 55
7	**Decitabine Maleate**	210	95 to 130 °C; 160 to 180 °C	0.94% @ 80 °C; 1.79% @ 100 °C; 32.66% @ 185 °C; 6.97% @ 100 °C	20.81°; 27.38°; 28.23°	--
			Figure 5	Figure 22	Figure 39	Figure 56
8	**Decitabine L-Glutamate**	180	50 to 100 °C; 175 to 195 °C; 195 to 220 °C	1.92% @ 80 °C; 12.66% @ 200 °C; 24.81% @ 260 °C	13.33°; 21.39°; 30.99°	--
			Figure 6	Figure 23	Figure 40	Figure 57
9	**Decitabine Sulfite**	220	100 to 140 °C	26.31% @ 145 °C; 31.98% @ 230 °C; 2.23% @ 260 °C	15.73°; 19.23°; 22.67°	1176 (S=O)
			Figure 7	Figure 24	Figure 41	Figure 58
10	**Decitabine Phosphate**	118	130 to 145 °C	22.36% @ 150 °C; 19.18% @ 260 °C	17.09°; 21.99°; 23.21°	--
			Figure 8	Figure 25	Figure 42	Figure 59

11	**Decitabine (+)-L-Tartrate**	202	60 to 110 °C; 185 to 220 °C	2.69% @ 90 °C; 8.60% @ 200 °C; 37.31% @ 260 °C	7.12°; 13.30°; 14.22°	--
			Figure 9	Figure 26	Figure 43	Figure 60
12	**Decitabine Citrate**	202	30 to 100 °C; 160 to 220 °C	3.81% @ 80 °C; 7.55% @ 200 °C; 39.02% @ 260 °C	13.31°; 14.23°; 23.26°	--
			Figure 10	Figure 27	Figure 44	Figure 61
13	**Decitabine L-Lactate**	195	30 to 100 °C; 160 to 210 °C	3.08% @ 80 °C; 8.93% @ 200 °C; 38.64% @ 260 °C	13.27°; 21.13°; 23.72°	--
			Figure 11	Figure 28	Figure 45	Figure 62
14	**Decitabine Succinate**	210	50 to 100 °C; 190 to 210 °C	0.72% @ 185 °C; 6.89% @ 205 °C; 35.02% @ 260 °C	13.30°; 22.59°; 23.28°	--
			Figure 12	Figure 29	Figure 46	Figure 63
15	**Decitabine Acetate**	206	60 to 90 °C, 185 to 210 °C	4.70% @ 75 °C; 7.19% @ 195 °C; 39.17% @ 260 °C	7.14°; 14.26°; 31.25°	--
			Figure 13	Figure 30	Figure 47	Figure 64
16	**Decitabine Hexanoate**	205	50 to 90 °C, 190 to 210 °C	4.76% @ 75 °C; 7.01% @ 195 °C; 37.92% @ 260 °C	13.27°; 22.54°; 23.25°	--
			Figure 14	Figure 31	Figure 48	Figure 65
17	**Decitabine Butyrate**	204	40 to 90 °C, 190 to 210 °C	5.12% @ 75 °C; 6.87% @ 195 °C; 37.90% @ 260 °C	13.28°; 22.57°; 23.27°	--
			Figure 15	Figure 32	Figure 49	Figure 66
18	**Decitabine Propionate**	200	50 to 110 °C, 190 to 210 °C	4.74% @ 75 °C; 7.35% @ 200 °C; 36.07% @ 260 °C	13.29°; 22.52°; 23.27°	--
			Figure 16	Figure 33	Figure 50	Figure 67
19	**Azacitidine Mesylate**	138	30 to 80 °C; 80 to 110 °C; 110 to 140 °C	2.44% @ 70 °C; 5.56% @ 145 °C; 13.28% @ 220 °C; 13.49% @ 260 °C	18.58°; 23.03°; 27.97°	1169-1176 (S=O)
			Figure 17	Figure 34	Figure 51	Figure 68

[a] Temperature maxima of endothermic events, °C (δH, J/g)
[b] Weight changes are relative to the weight of the sample at the starting point of the specific weight change event
[c] Three integrated intensity maxima (counts) are shown

It can be appreciated to one of ordinary skill in the art that many changes and modifications can be made to the instant invention without departing from the spirit or scope of the appended claims, and such changes and modifications are contemplated within the scope of the instant invention.

All publications, patents, and patent applications, and web sites are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent, or patent application, was specifically and individually indicated to be incorporated by reference in its entirety.

CLAIMS

What is claimed is:

1. A salt of 5-azacytidine.

2. The salt of claim 1 wherein said salt is synthesized with an acid.

3. The salt of claim 2 wherein said acid has a pK_a of about 5 or less.

4. The salt of claim 2 wherein said acid has a pK_a of about 4 or less.

5. The salt of claim 2 wherein pK_a of said acid ranges from about 3 to about -10.

6. The salt of claim 2 wherein said acid is selected from the group consisting of hydrochloric, L-lactic, acetic, phosphoric, (+)-L-tartaric, citric, propionic, butyric, hexanoic, L-aspartic, L-glutamic, succinic, EDTA, maleic, and methanesulfonic acid.

7. The salt of claim 2 wherein said acid is selected from the group consisting of HBr, HF, HI, nitric, nitrous, sulfuric, sulfurous, phosphorous, perchloric, chloric, and chlorous acid.

8. The salt of claim 2 wherein said acid is a carboxylic acid or a sulfonic acid.

9. The salt of claim 8 wherein said carboxylic acid is selected from the group consisting of ascorbic, carbonic, and fumaric acid.

10. The salt of claim 8 wherein said sulfonic acid is selected from the group consisting of ethanesulfonic, 2-hydroxyethanesulfonic, and toluenesulfonic acid.

11. The salt of claim 1 wherein said salt is a hydrochloride, mesylate, EDTA, sulfite, L-Aspartate, maleate, phosphate, L-Glutamate, (+)-L-Tartrate, citrate, L-Lactate, succinate, acetate, hexanoate, butyrate, or propionate salt.

12. The salt of claim 1 wherein said salt is a mesylate salt in crystalline form characterized by an X-ray diffraction pattern having diffraction peaks (2θ) at 18.58°, 23.03°, and 27.97°.

13. The salt of claim 12 wherein said salt is further characterized by multiple reversible melting endotherms at 30-80°C, 80-110°C and 110-140°C as measured by differential scanning calorimetry at a scan rate of 10°C per minute.

14. A pharmaceutical composition comprising the salt of claim 1.

15. The pharmaceutical composition of claim 14 wherein the pharmaceutical composition is in liquid form in which the salt is dissolved.

16. The pharmaceutical composition of claim 15 wherein the salt is dissolved in a non-aqueous solvent that comprises glycerin, propylene glycol, polyethylene glycol, or a combination thereof.

17. The pharmaceutical composition of claim 14 wherein the pharmaceutical composition is an aqueous solution in which the salt is dissolved.

18. A sterilized vessel containing a pharmaceutical composition according to claim 14.

19. The vessel of claim 18, wherein the vessel is a vial, syringe or ampoule.

20. The vessel of claim 18, wherein the pharmaceutical composition is in liquid form and the vessel comprises between 1 and 50 ml of the pharmaceutical composition.

21. A kit, comprising:
a first vessel containing a salt of 5-azacytidine in solid form; and
a second vessel containing a diluent comprising water, saline, glycerin, propylene glycol, polyethylene glycol or combinations thereof.

22. The kit of claim 21, wherein salt is in a form of lyophilized powder.

23. The kit of claim 21, wherein the salt is in crytalline form.

24. The kit of claim 21, where the amount of the salt in the first vessel is between 0.1 and 200 mg.

25. The kit of claim 21, where the amount of the salt in the first vessel is between 5 and 50 mg.

26. The kit of claim 21, where the diluent is a combination of propylene glycol and glycerin, and the concentration of propylene glycol in the diluent is between 20-80%.

27. The kit of claim 21, further comprising: a written instruction describing how to mix solid salt of 5-azacytidine and the diluent to form a pharmaceutical formulation.

28. A method of treating a disease associated with undesirable cell proliferation in a subject comprising administering to the subject in need thereof a pharmaceutically effective amount of a salt of claim 1.

29. The method of claim 28 wherein the disease is selected from the group consisting of benign tumors, cancer, hematological disorders, atherosclerosis, insults to body tissue due to surgery, abnormal wound healing,

abnormal angiogenesis, diseases that produce fibrosis of tissue, repetitive motion disorders, disorders of tissues that are not highly vascularized, and proliferative responses associated with organ transplants.

30. The method of claim 28, wherein the disease is selected from the group consisting of myelodysplastic syndrome, leukemia, malignant tumors, and sickle-cell anemia.

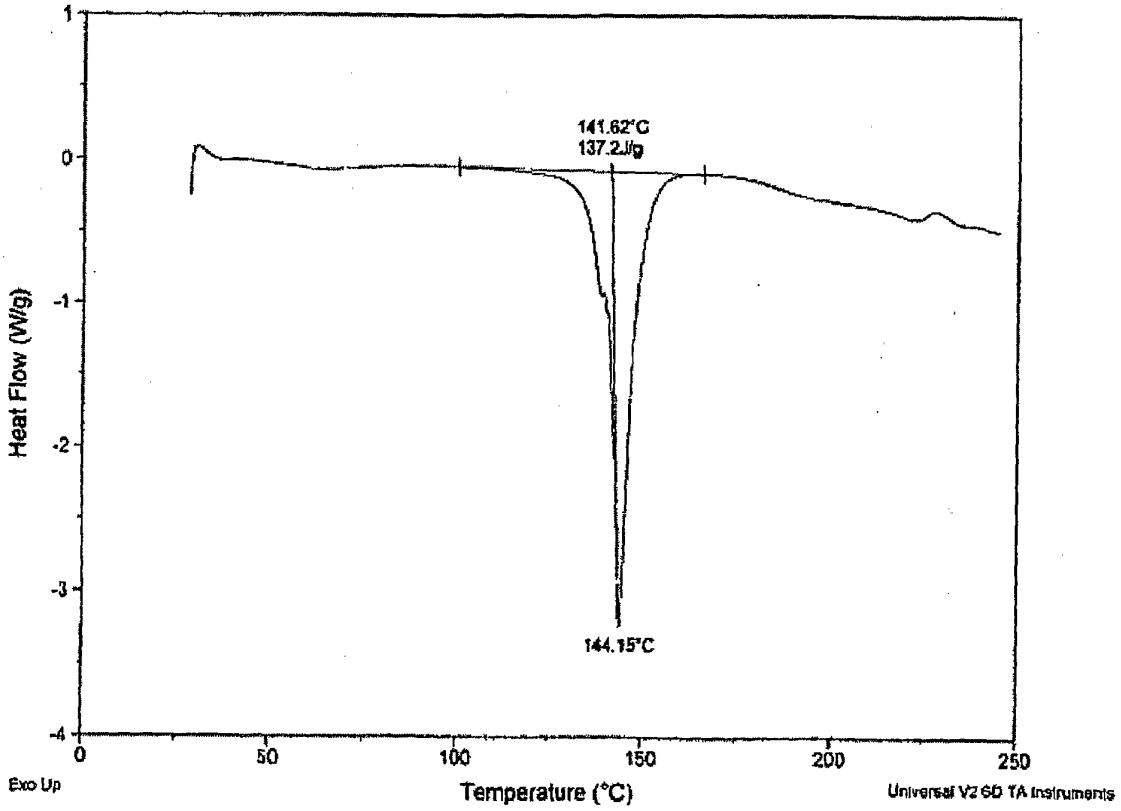


Figure 1

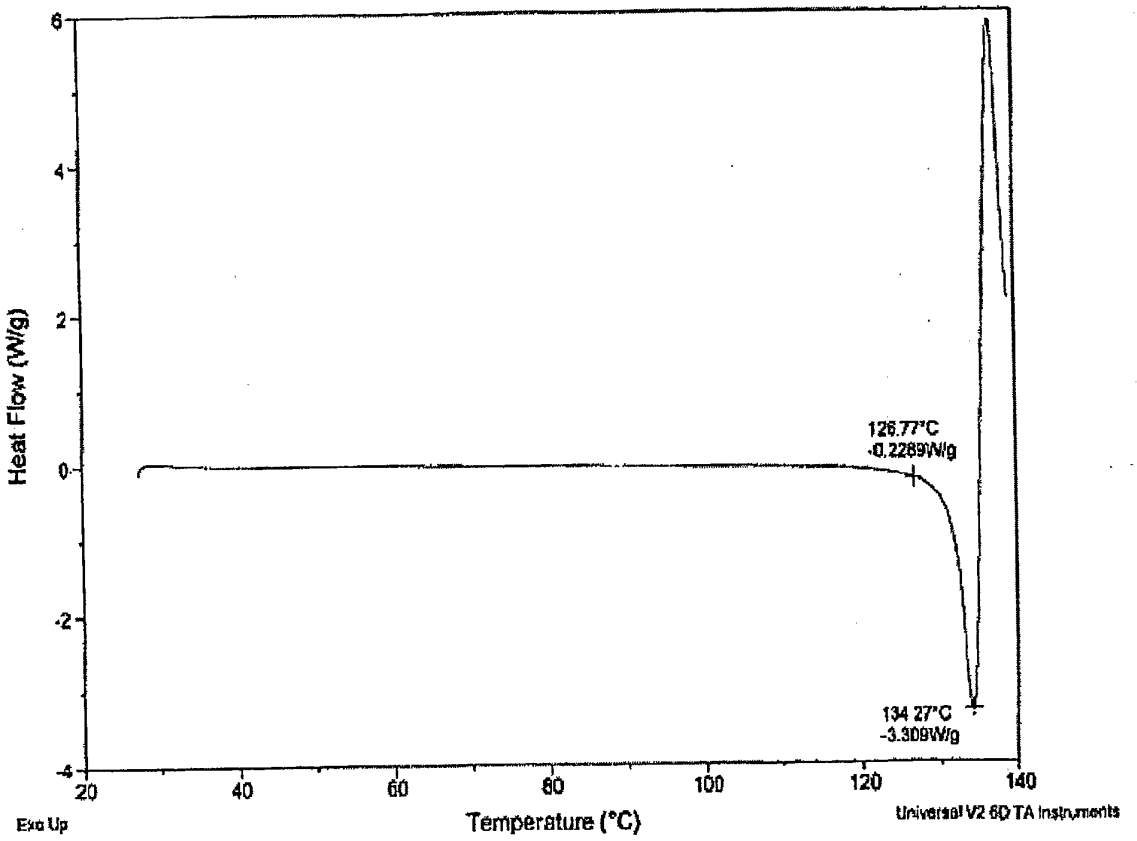


Figure 2

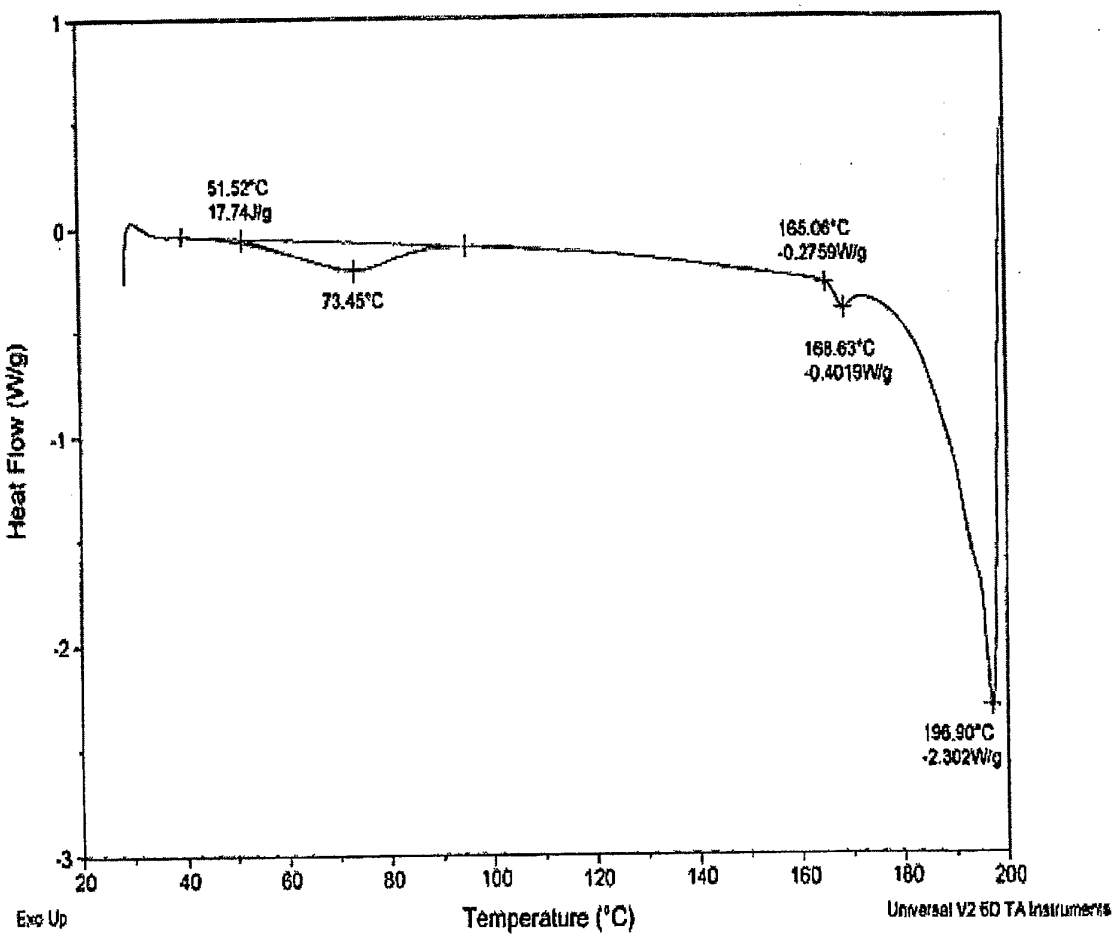


Figure 3

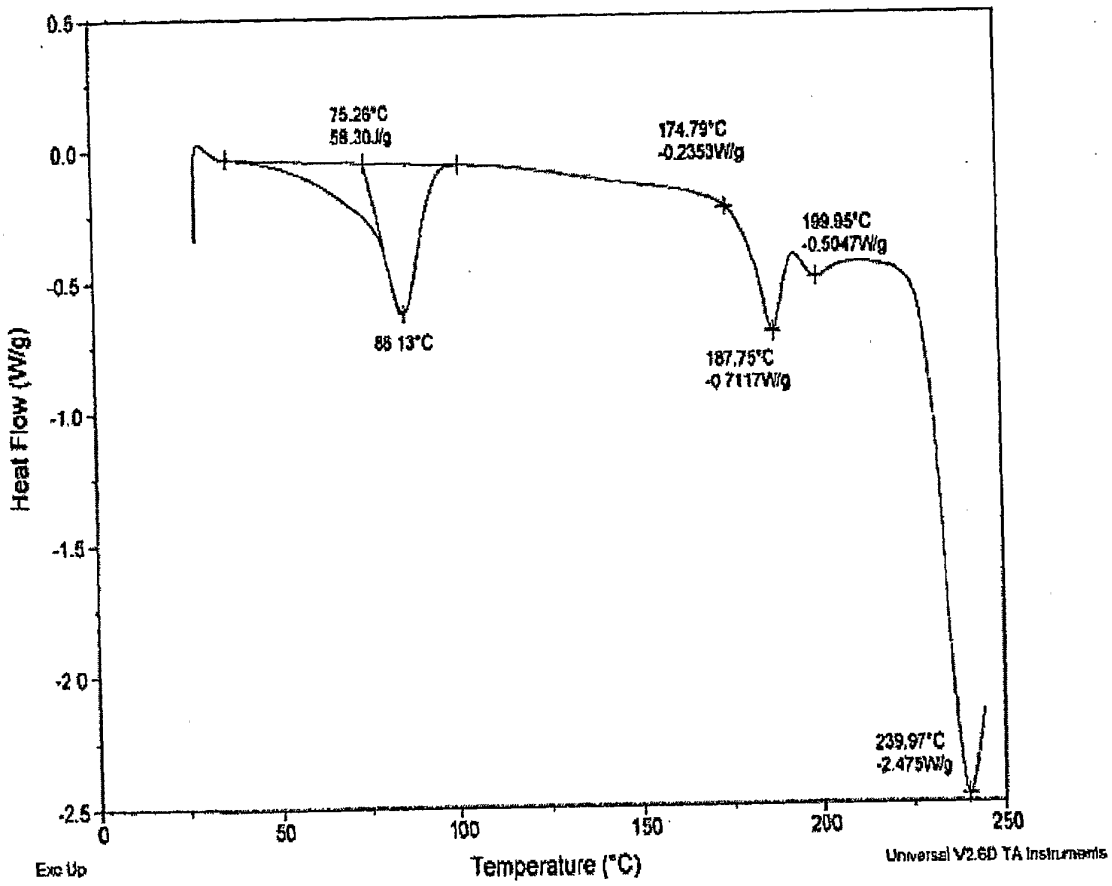


Figure 4

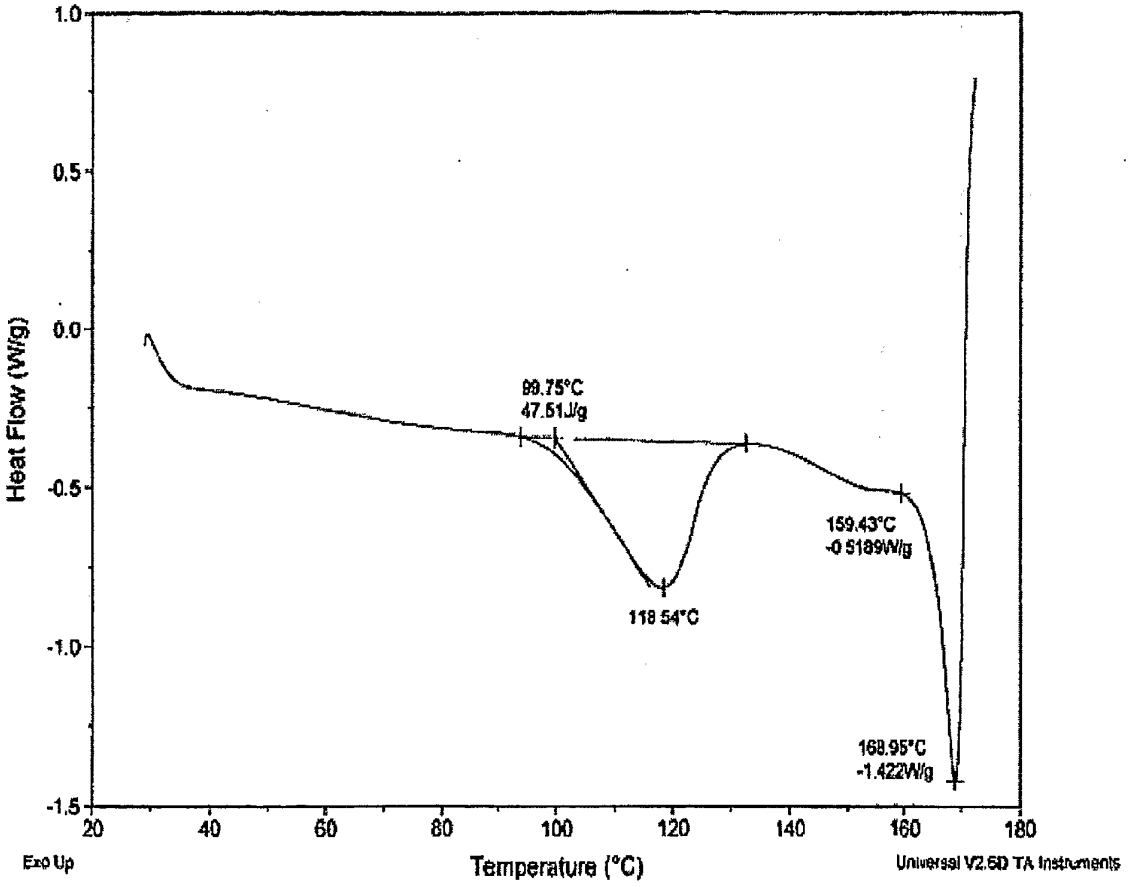


Figure 5

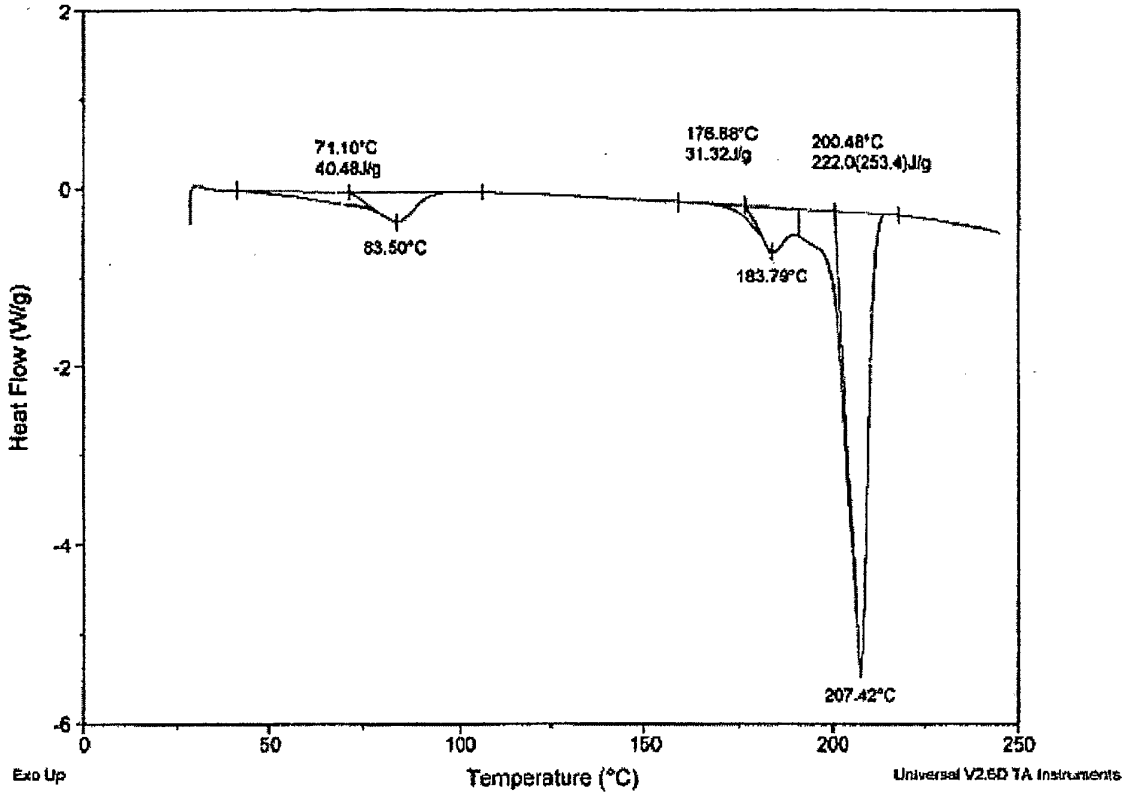


Figure 6

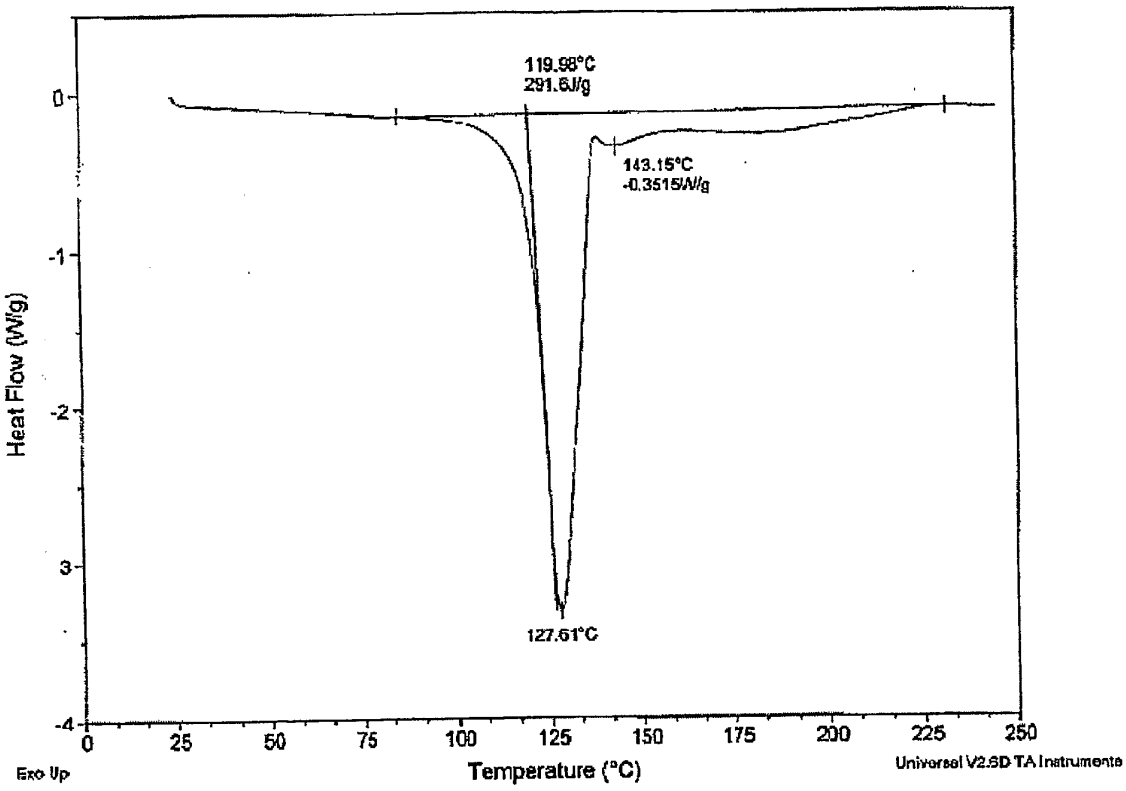


Figure 7

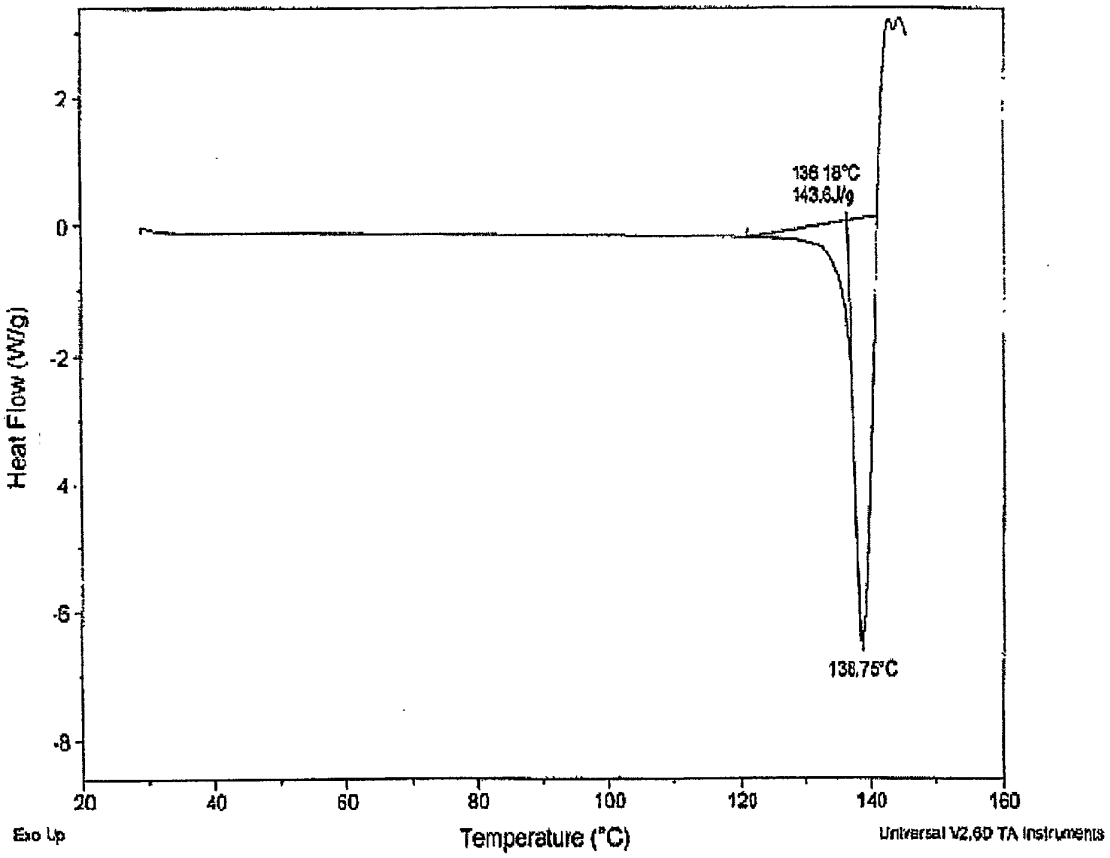


Figure 8

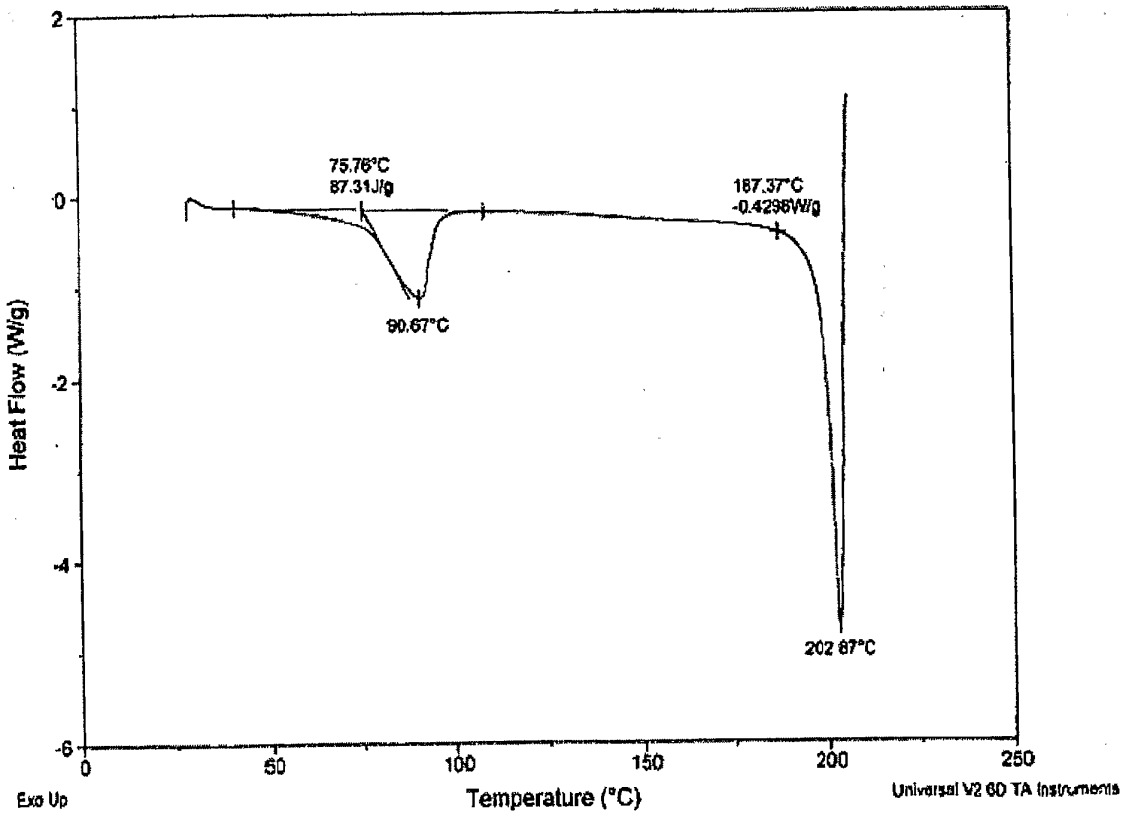


Figure 9

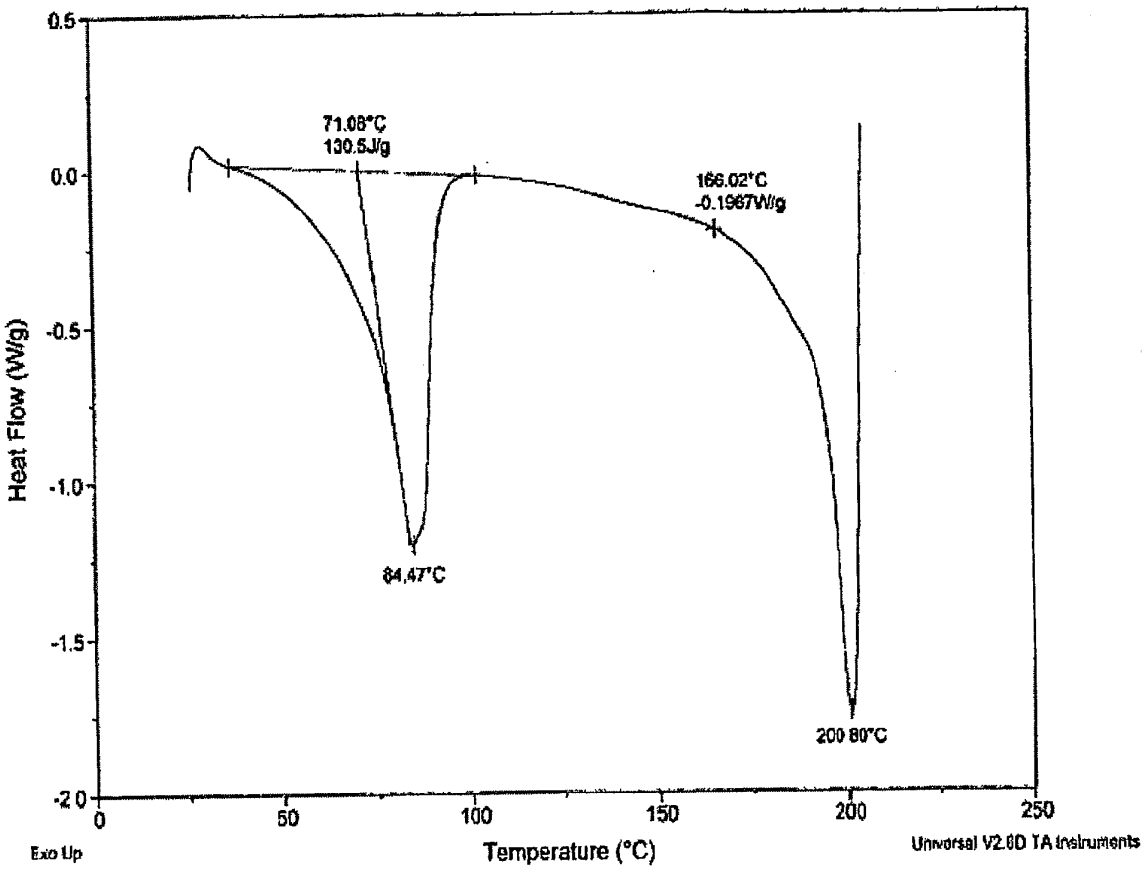


Figure 10

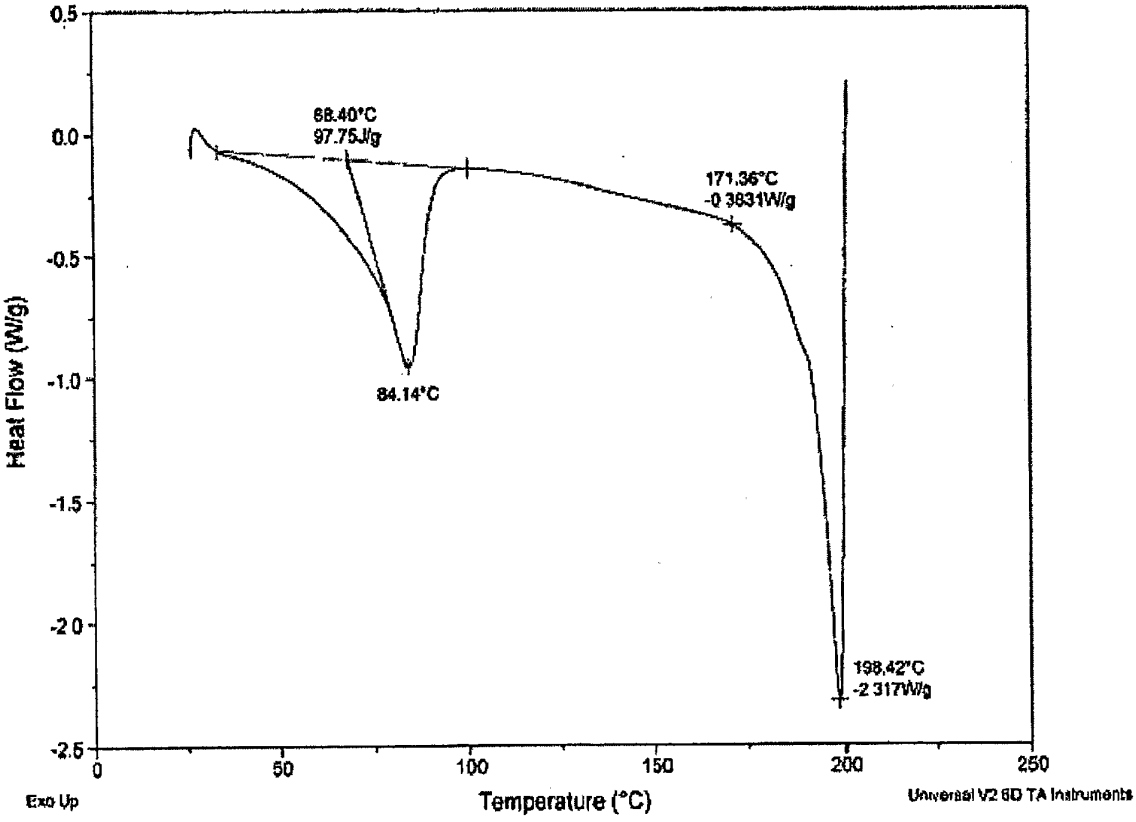


Figure 11

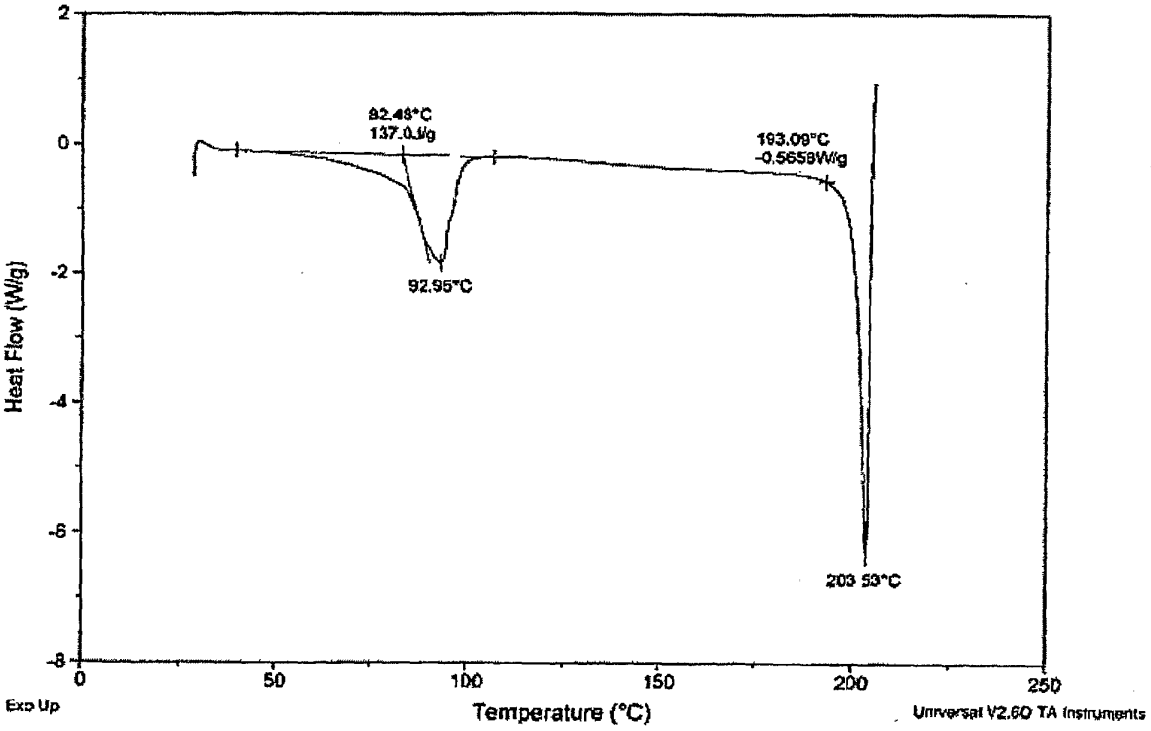


Figure 12

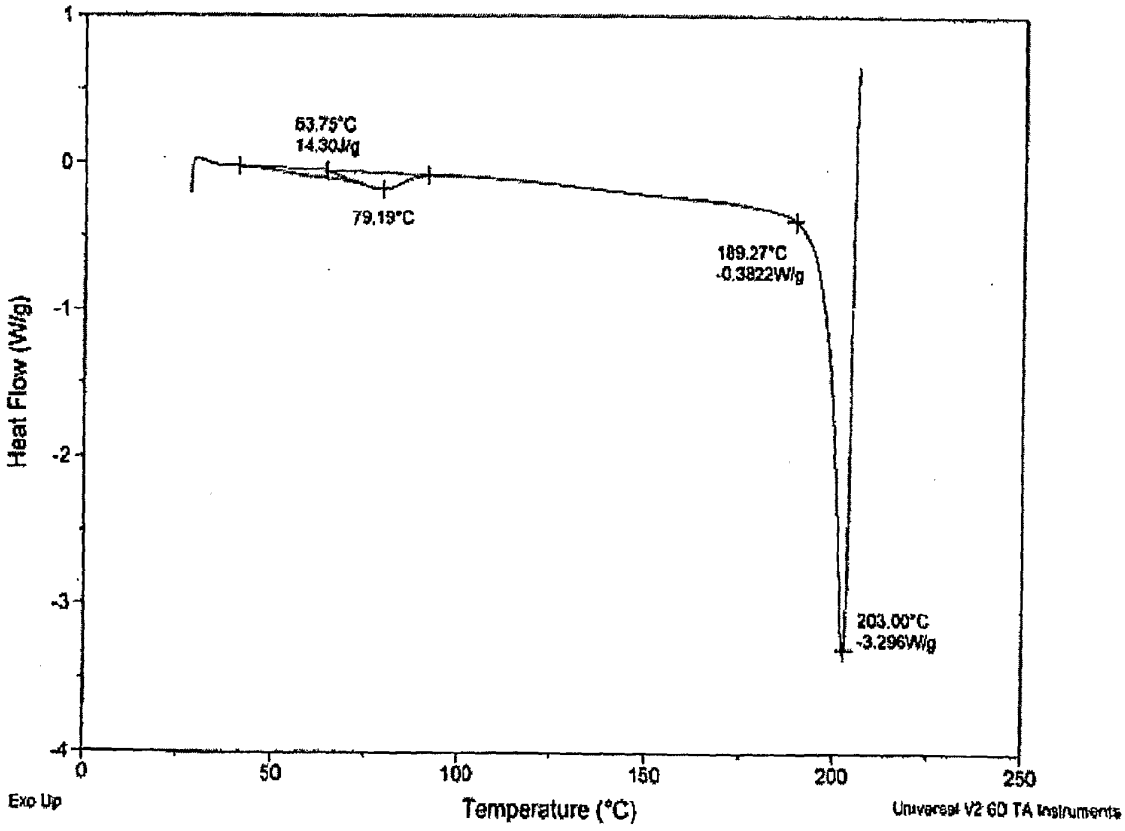
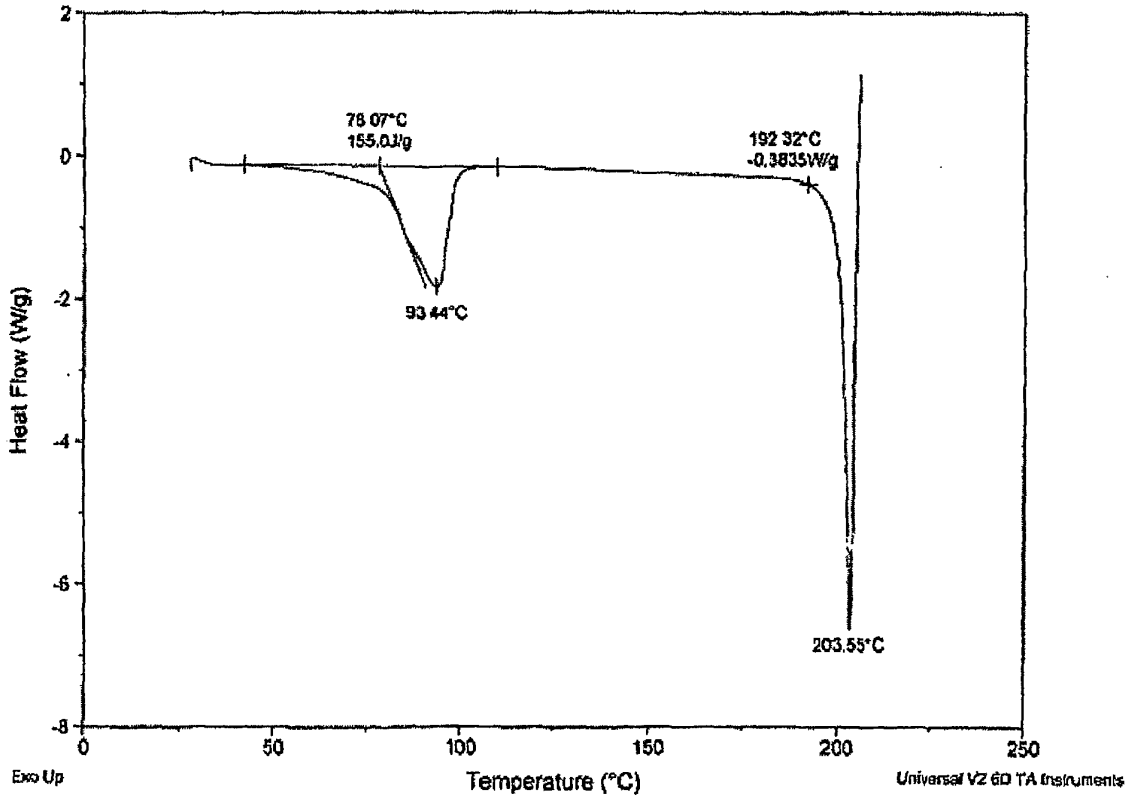


Figure 13



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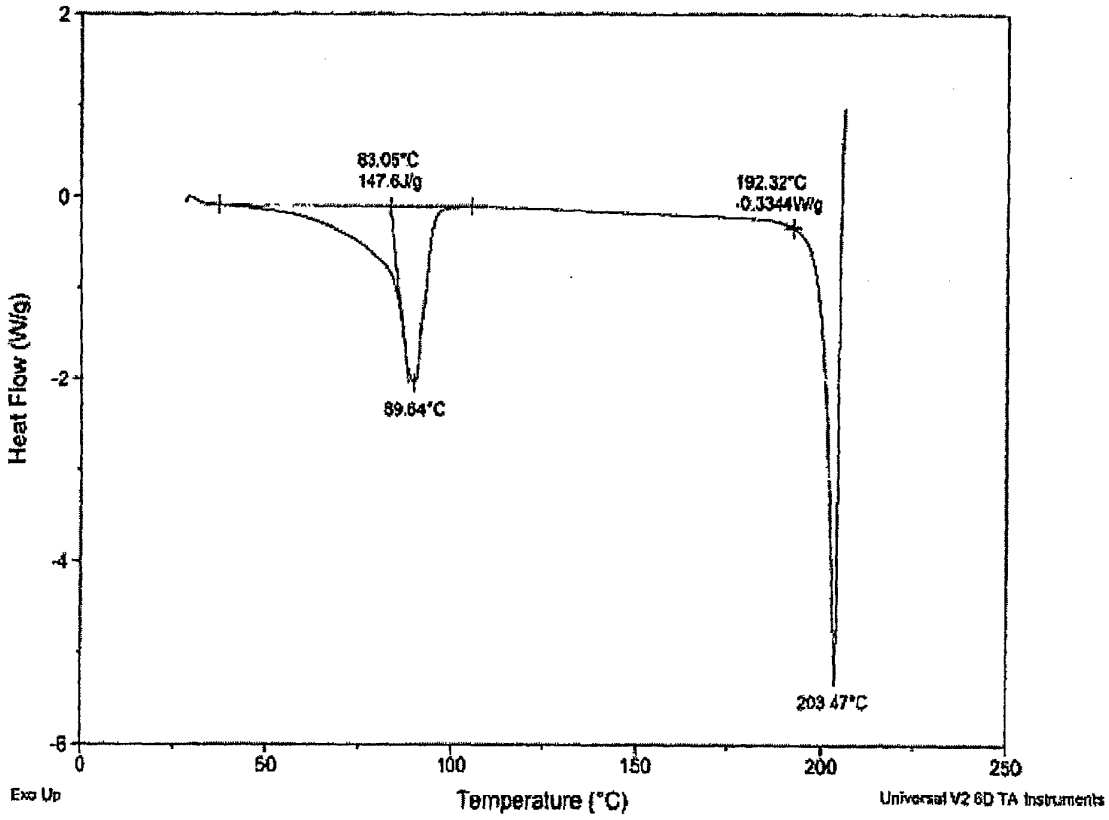

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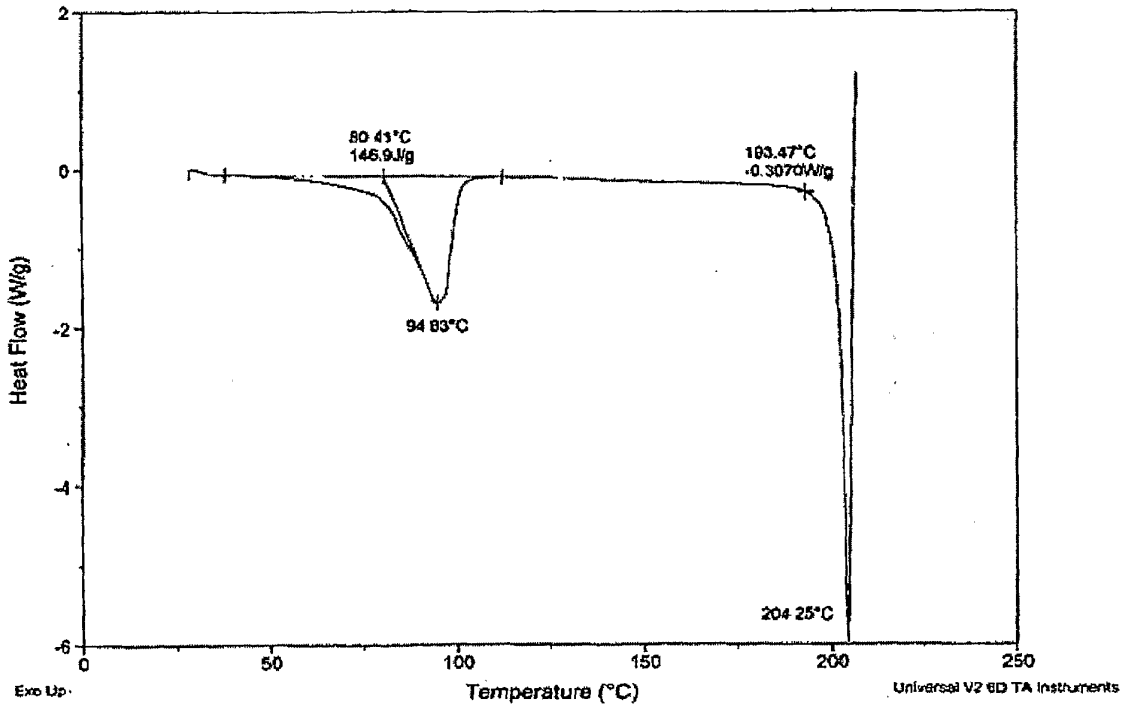

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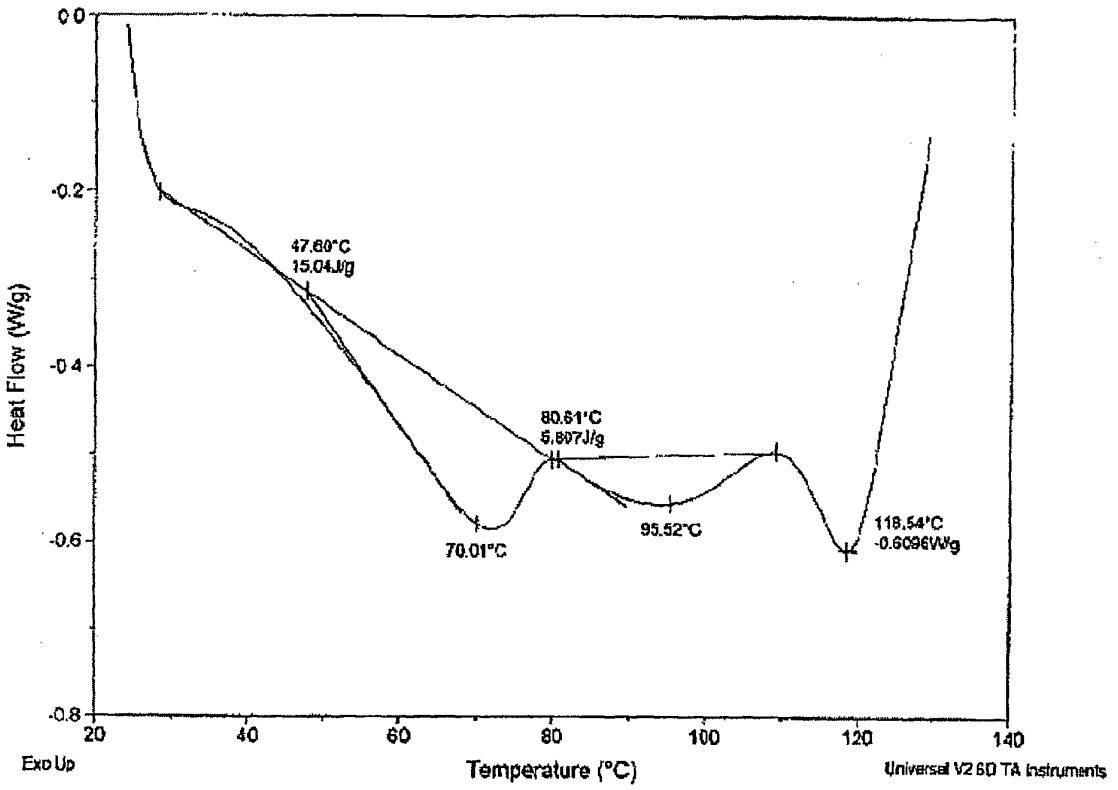

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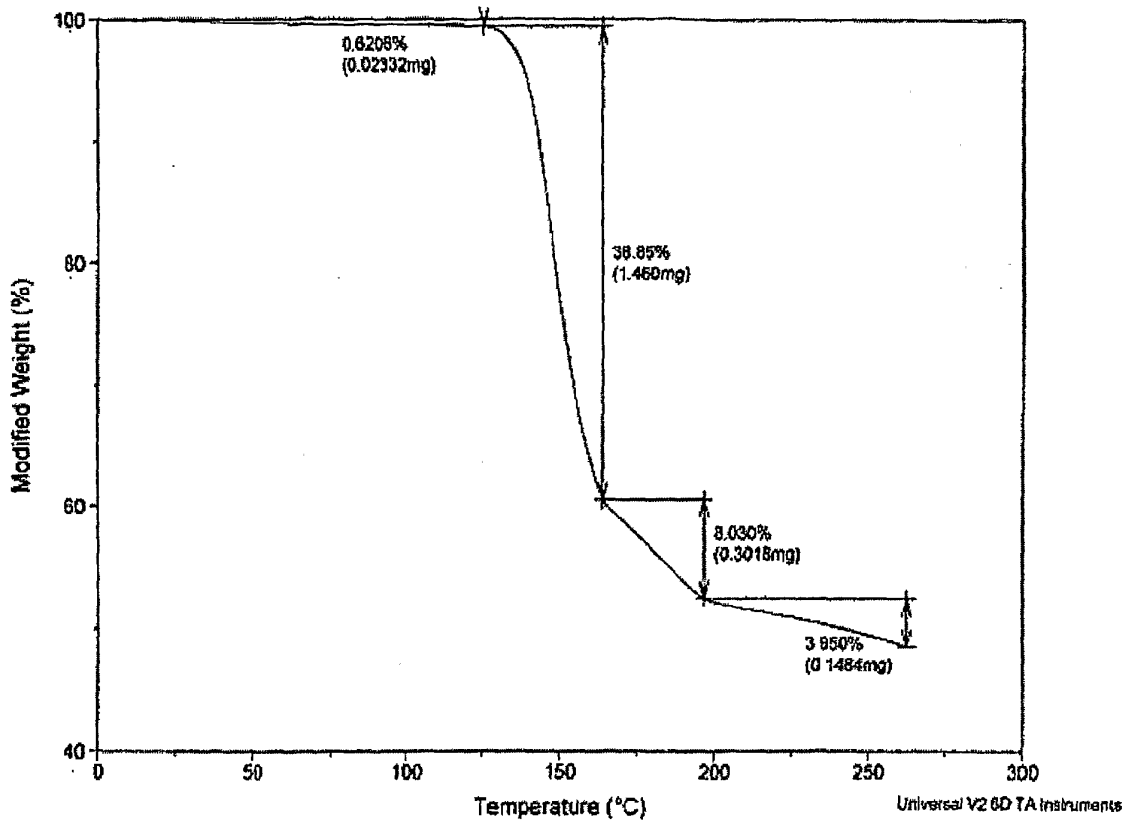

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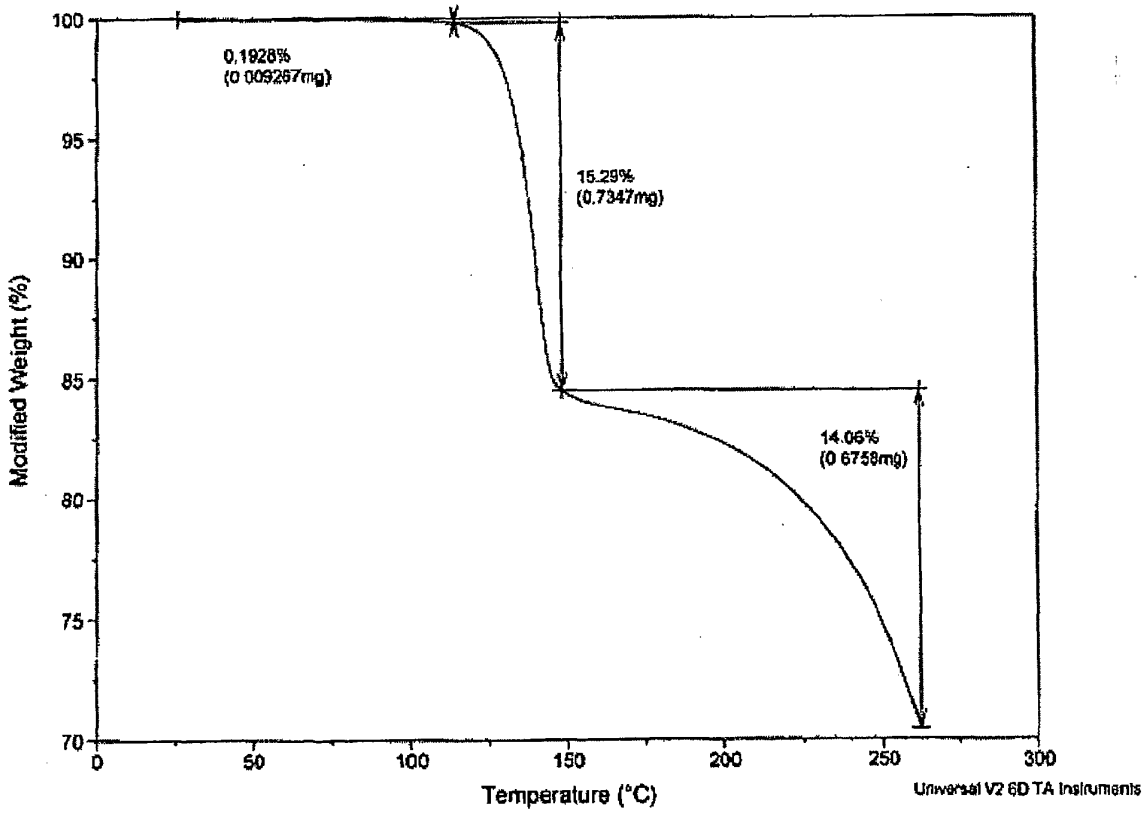

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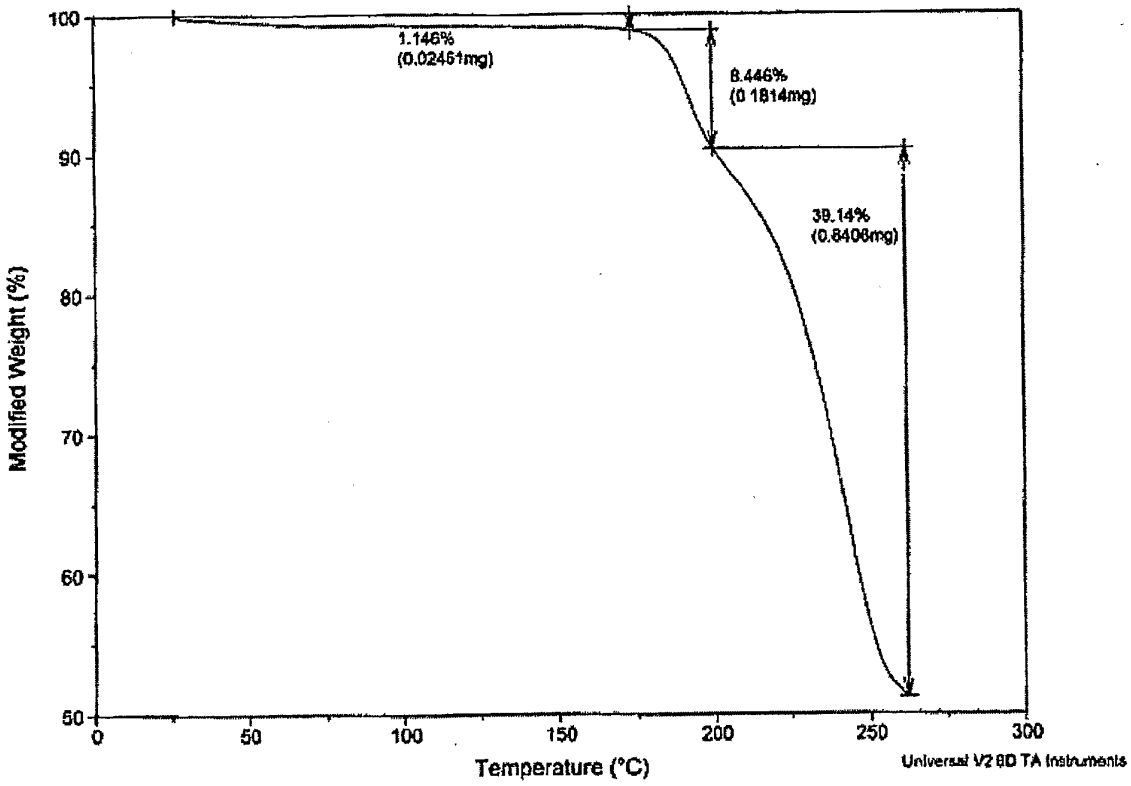

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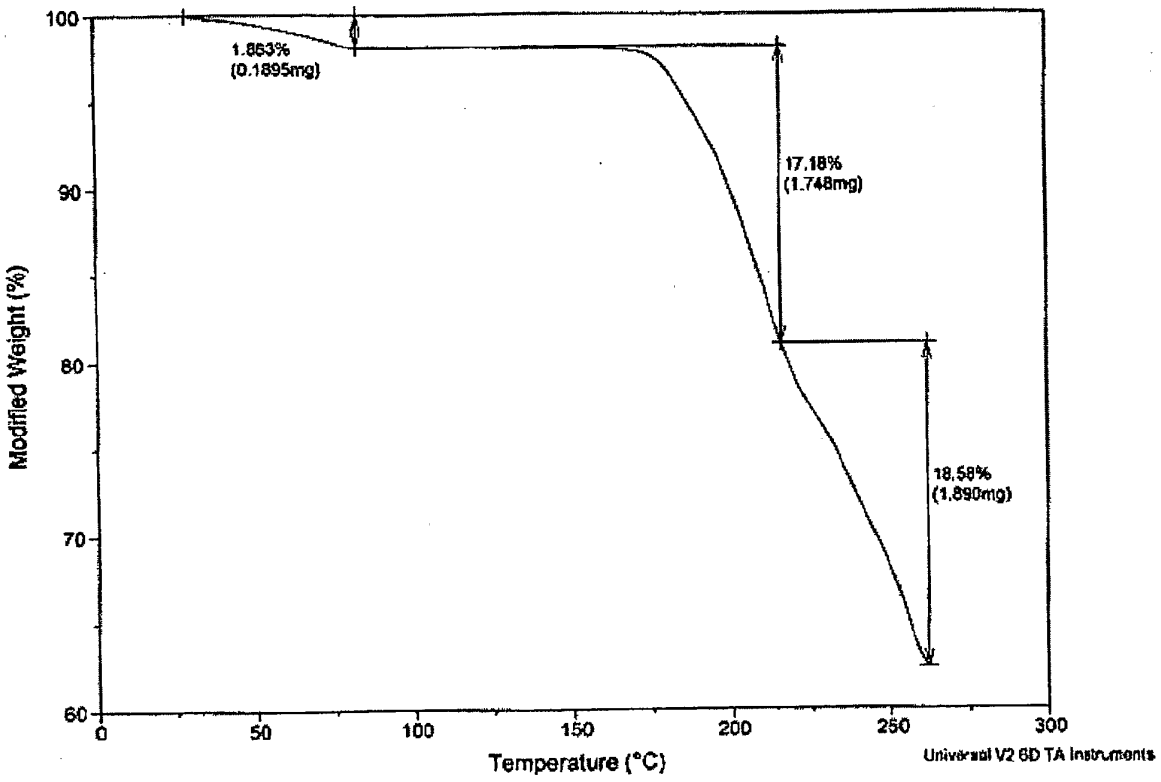

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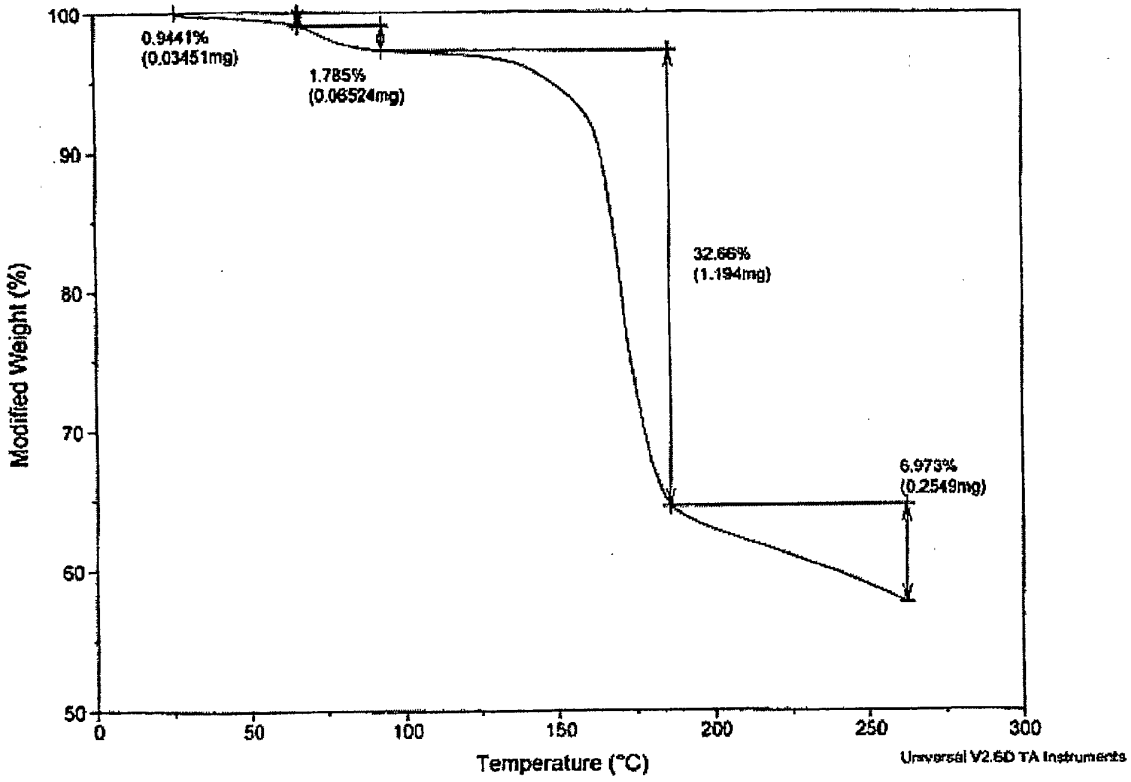

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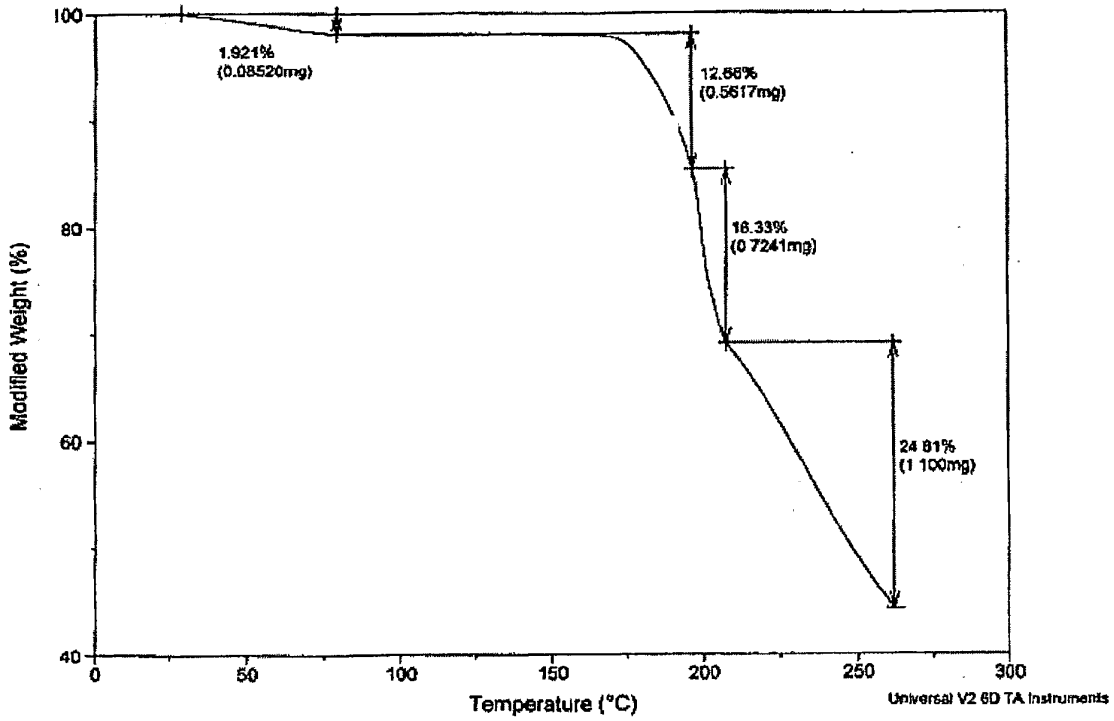

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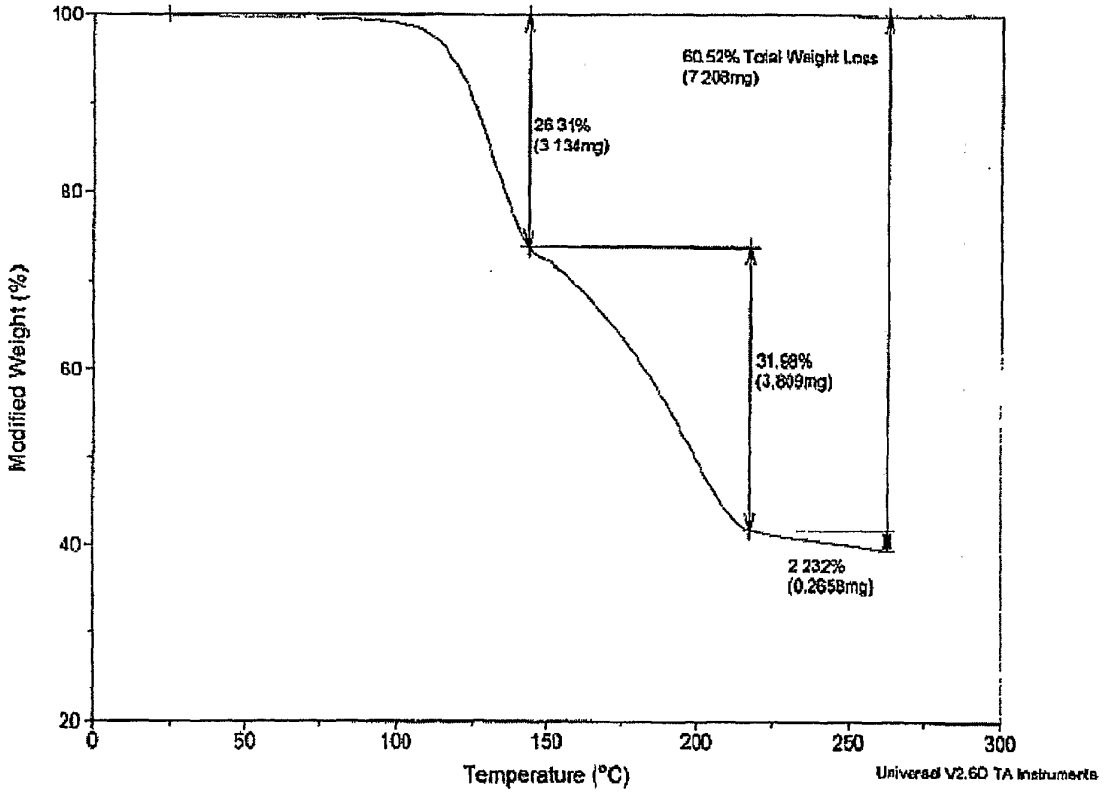

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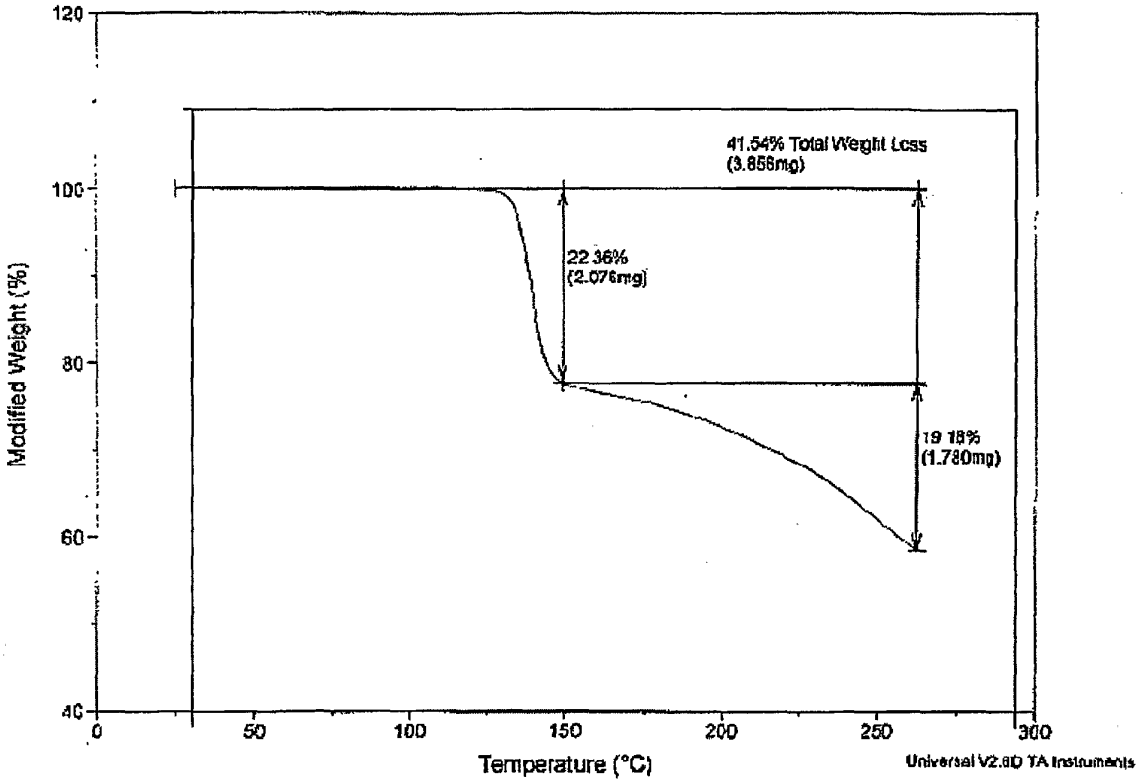

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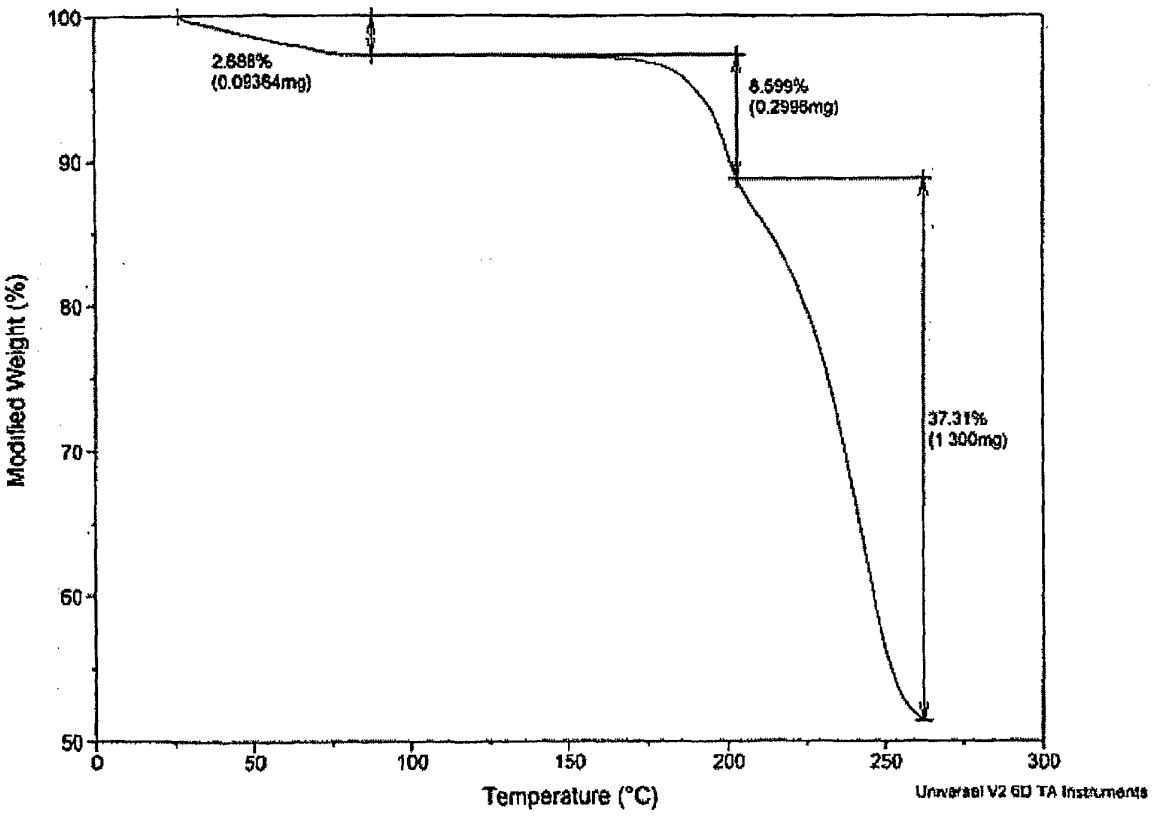

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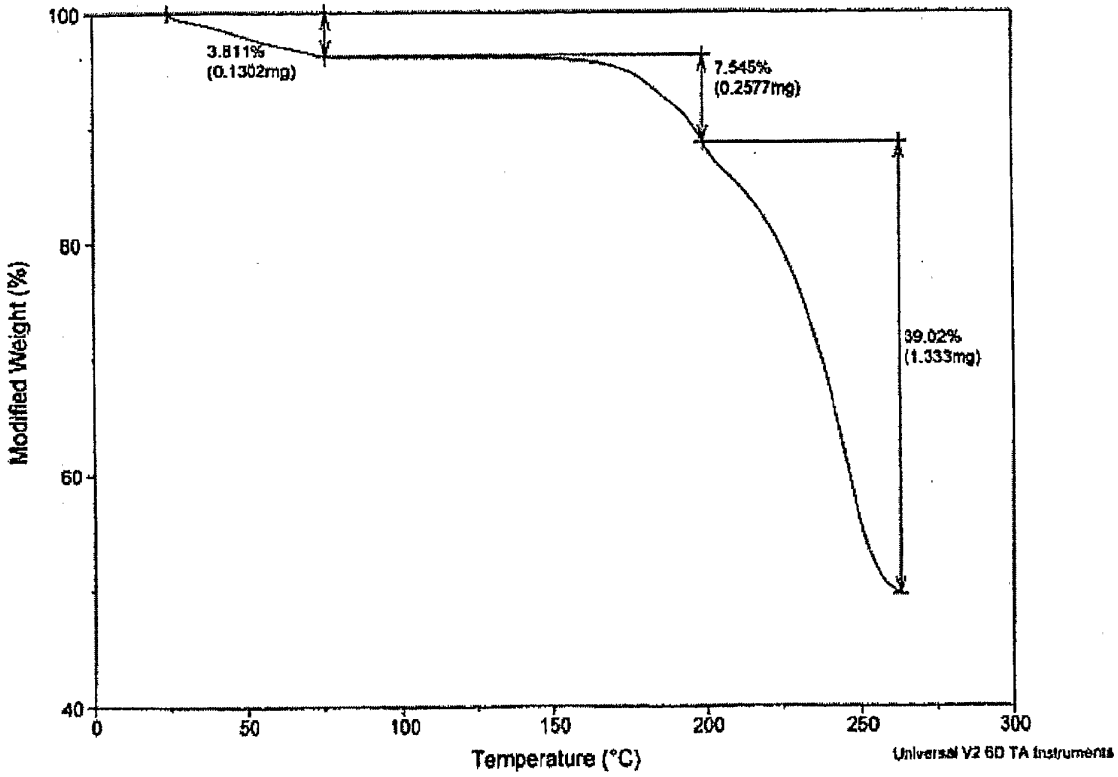

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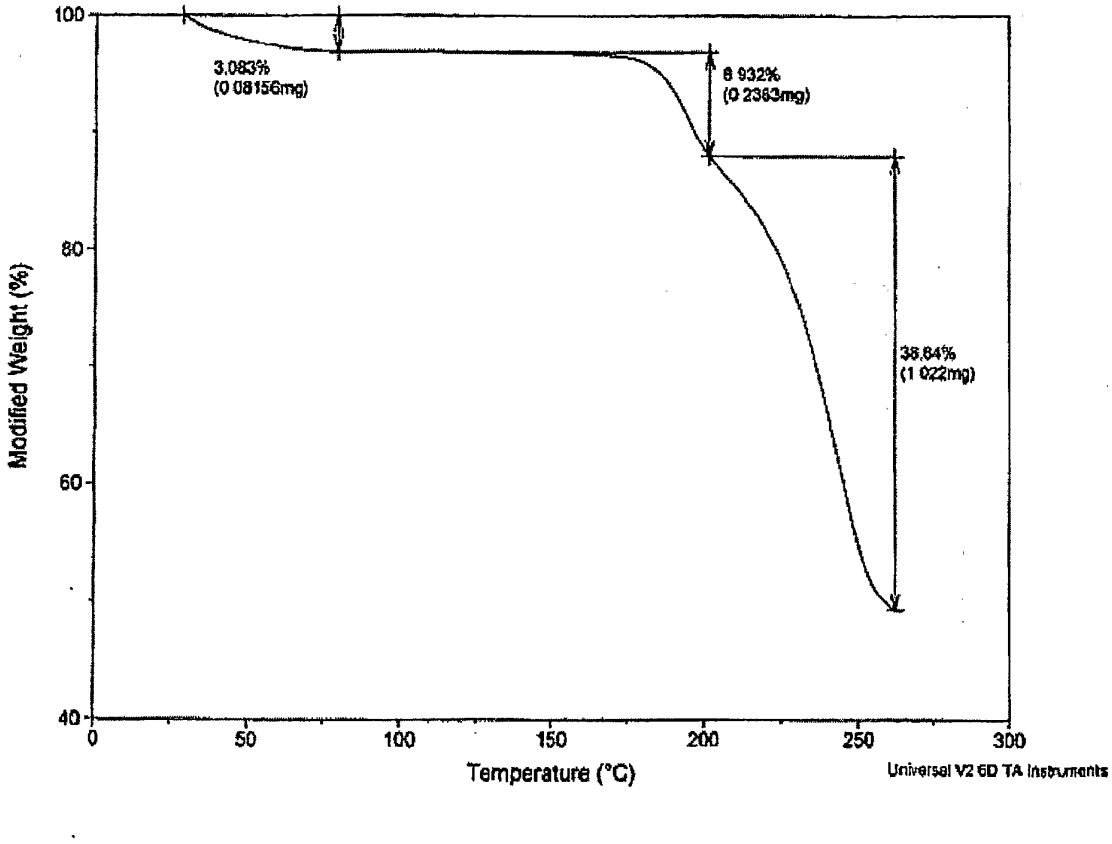

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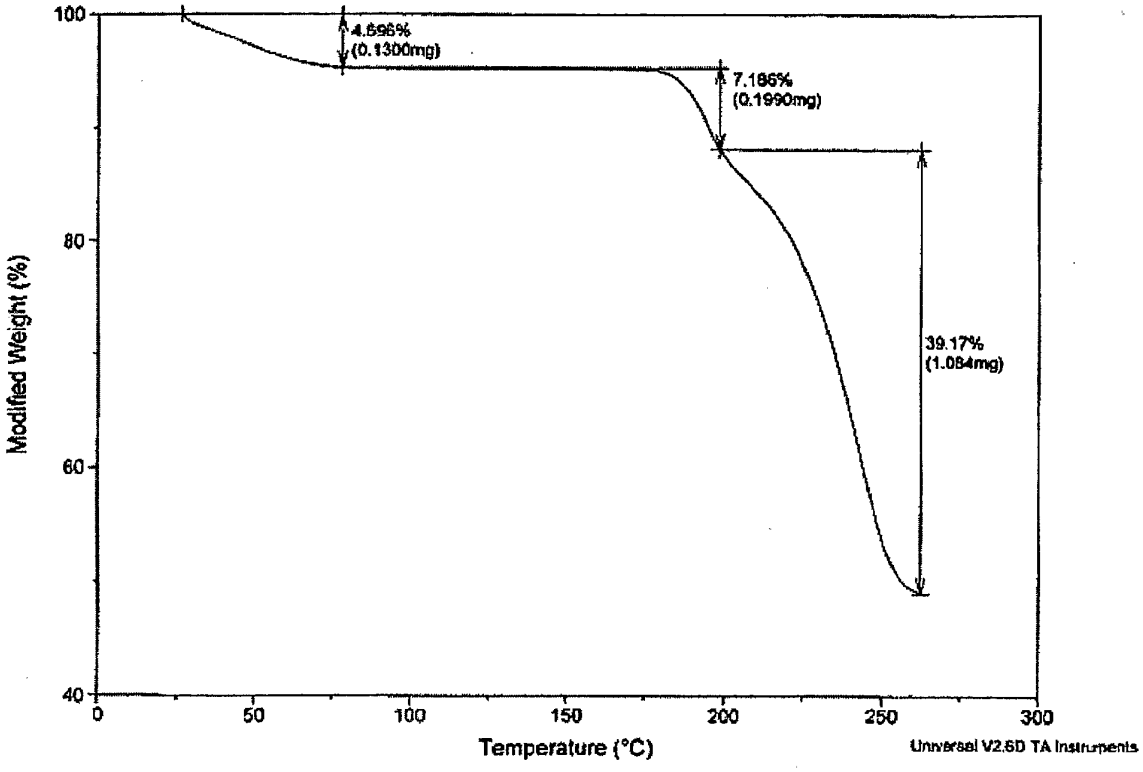

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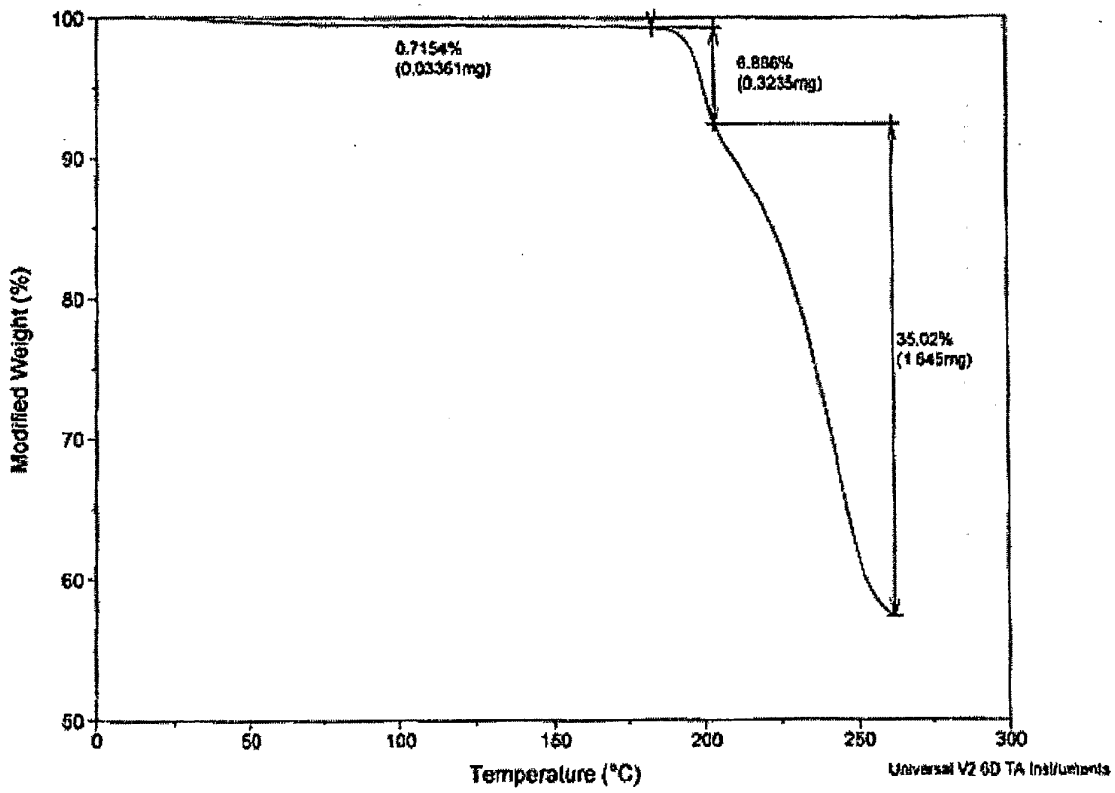

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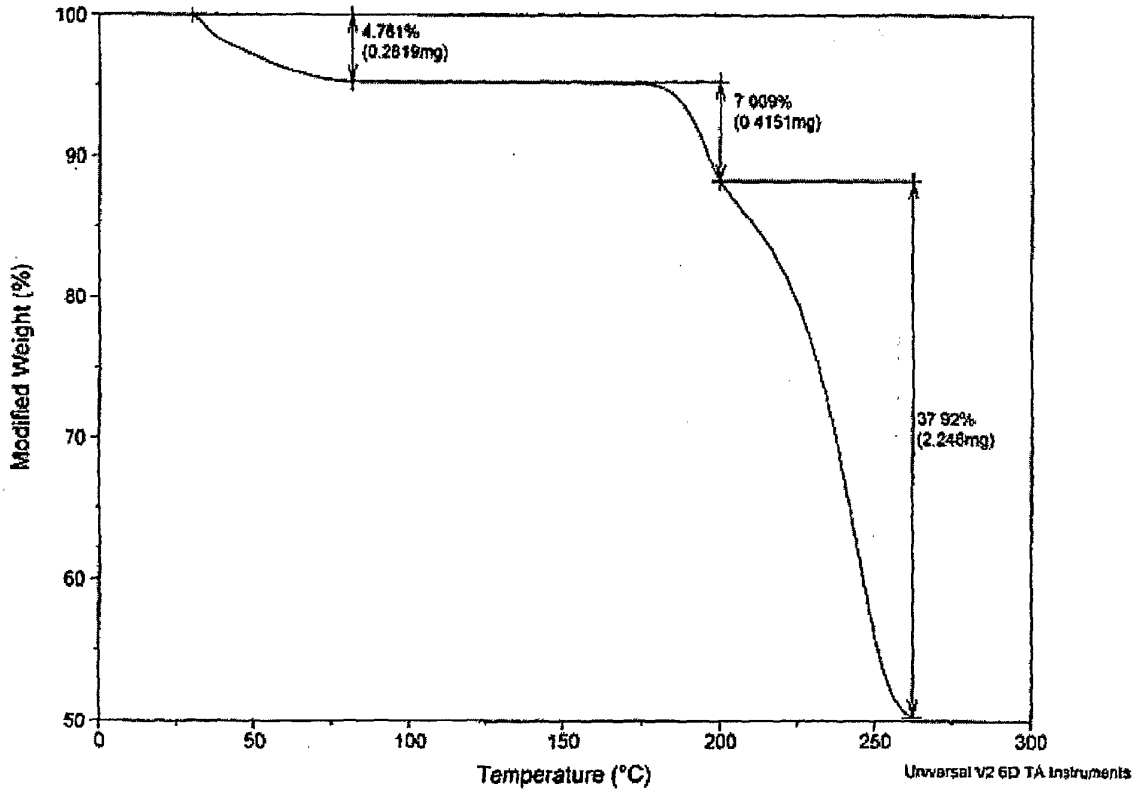

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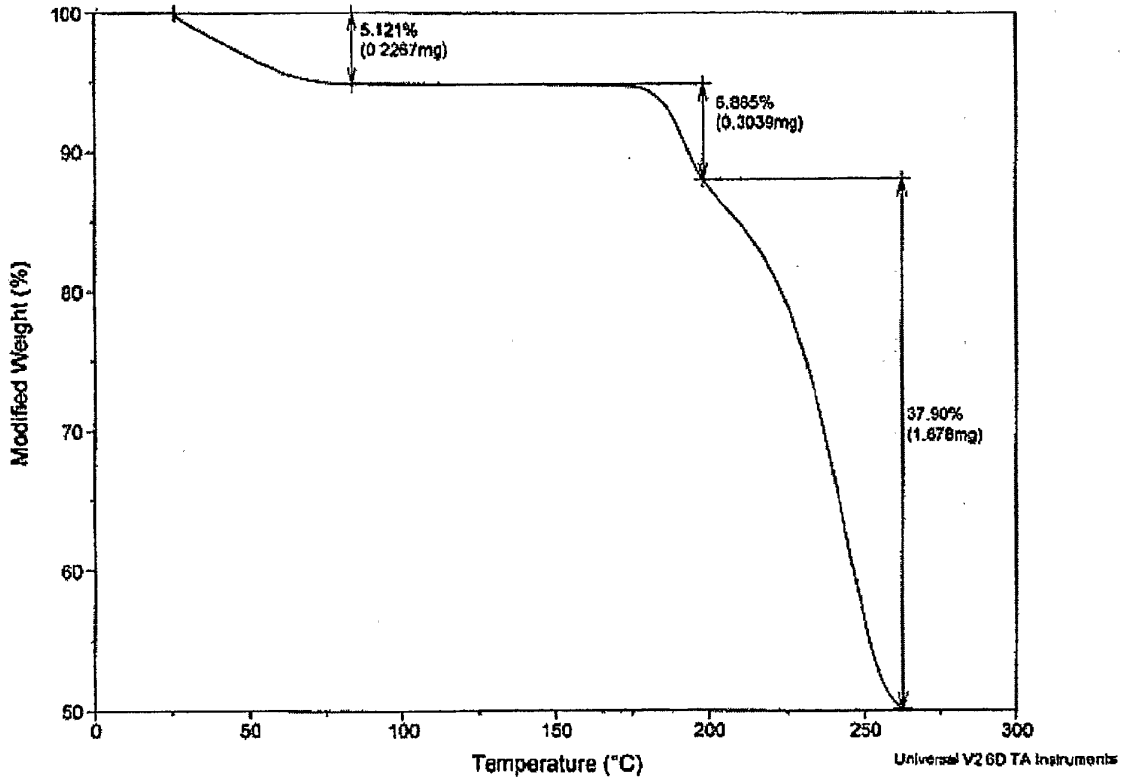

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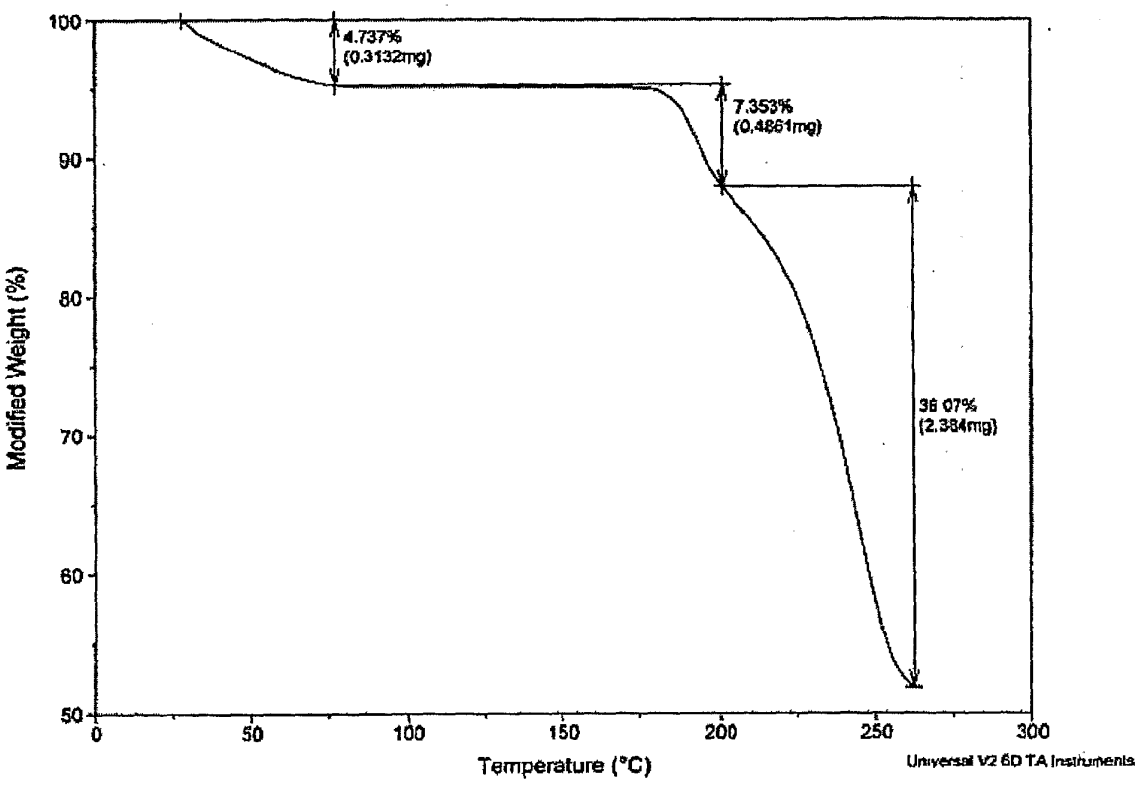

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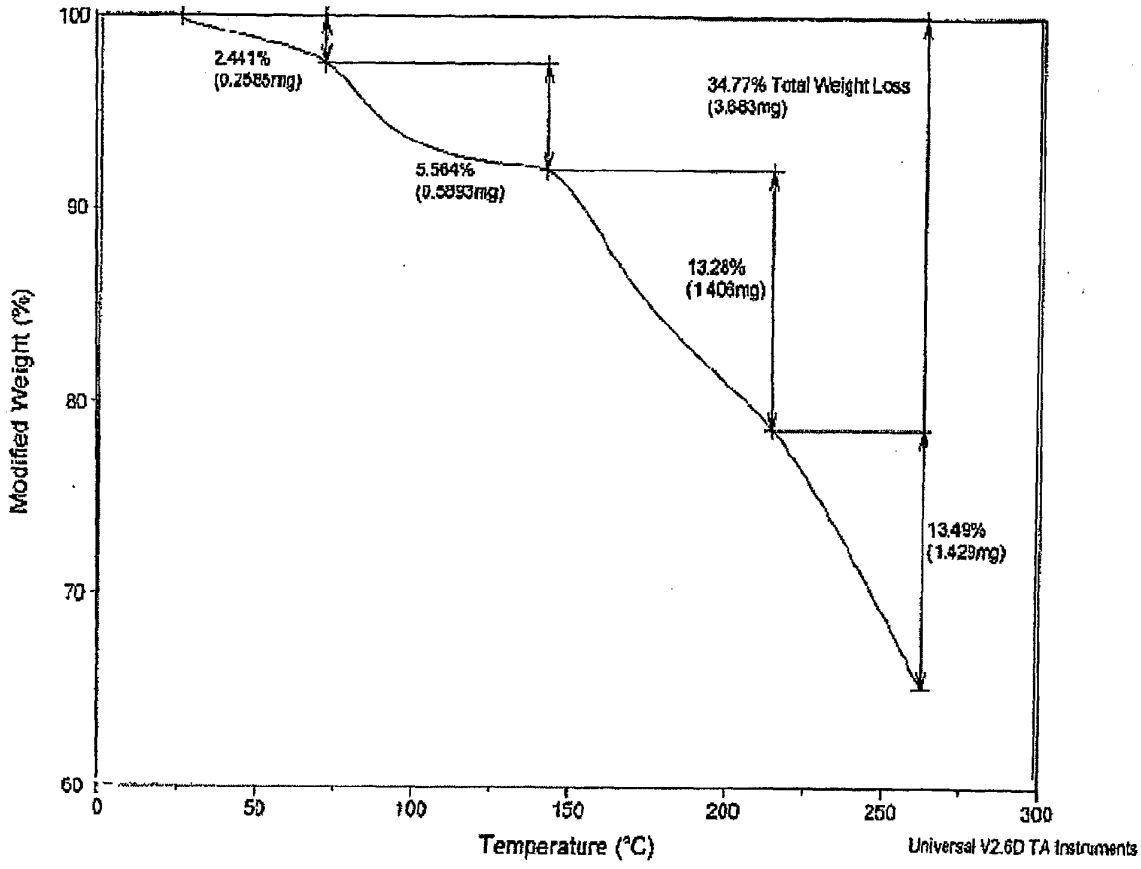

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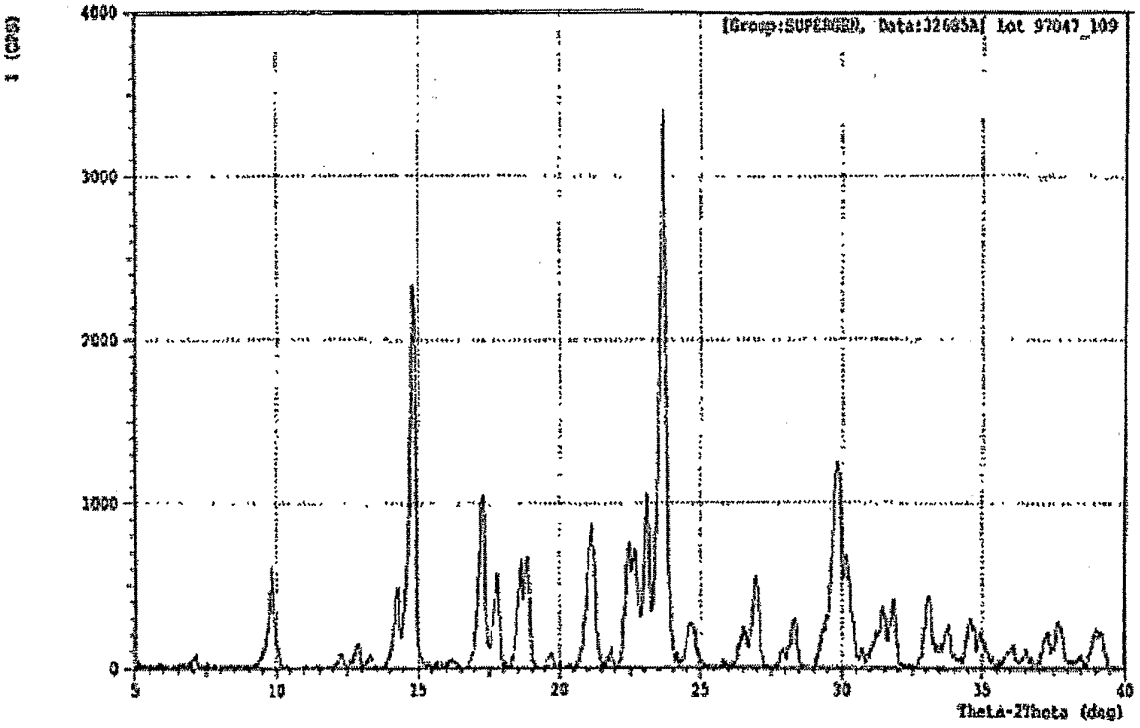

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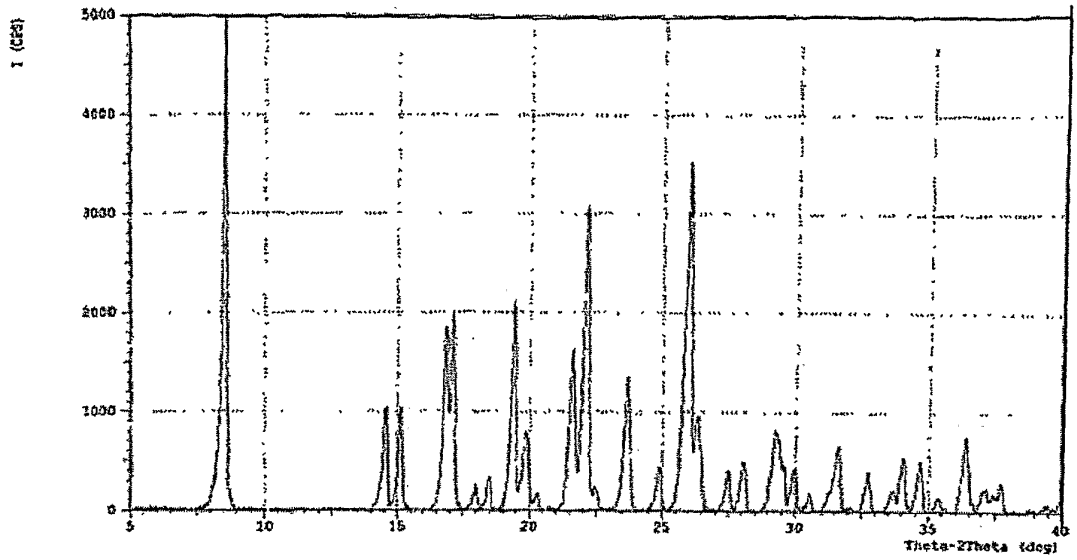

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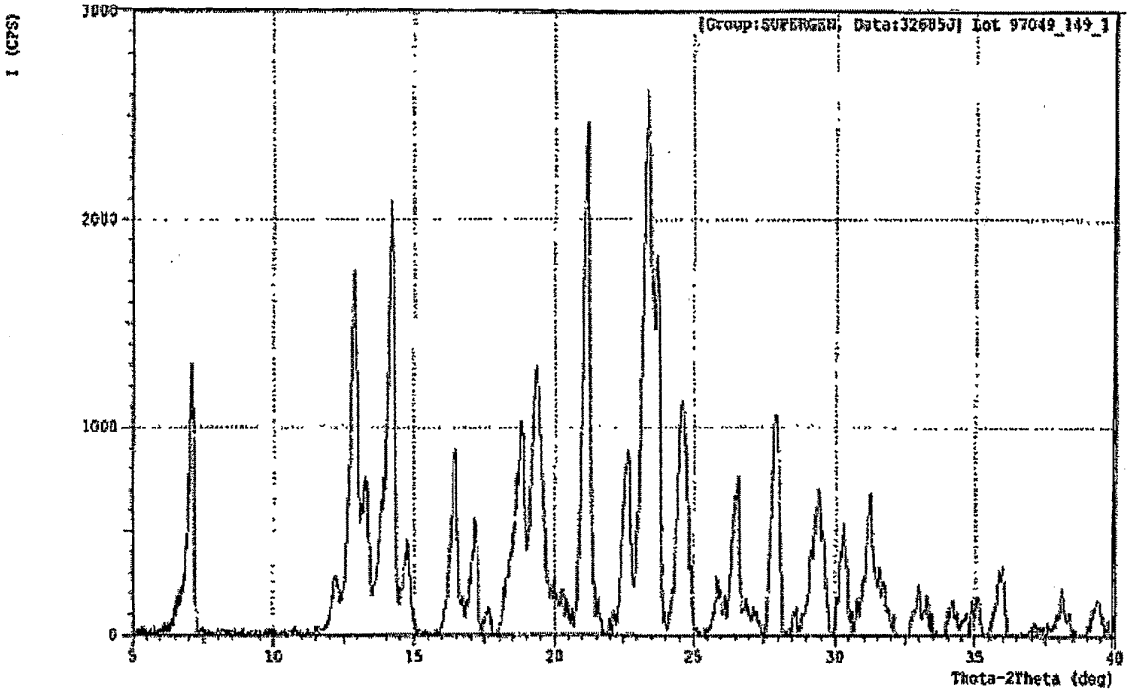

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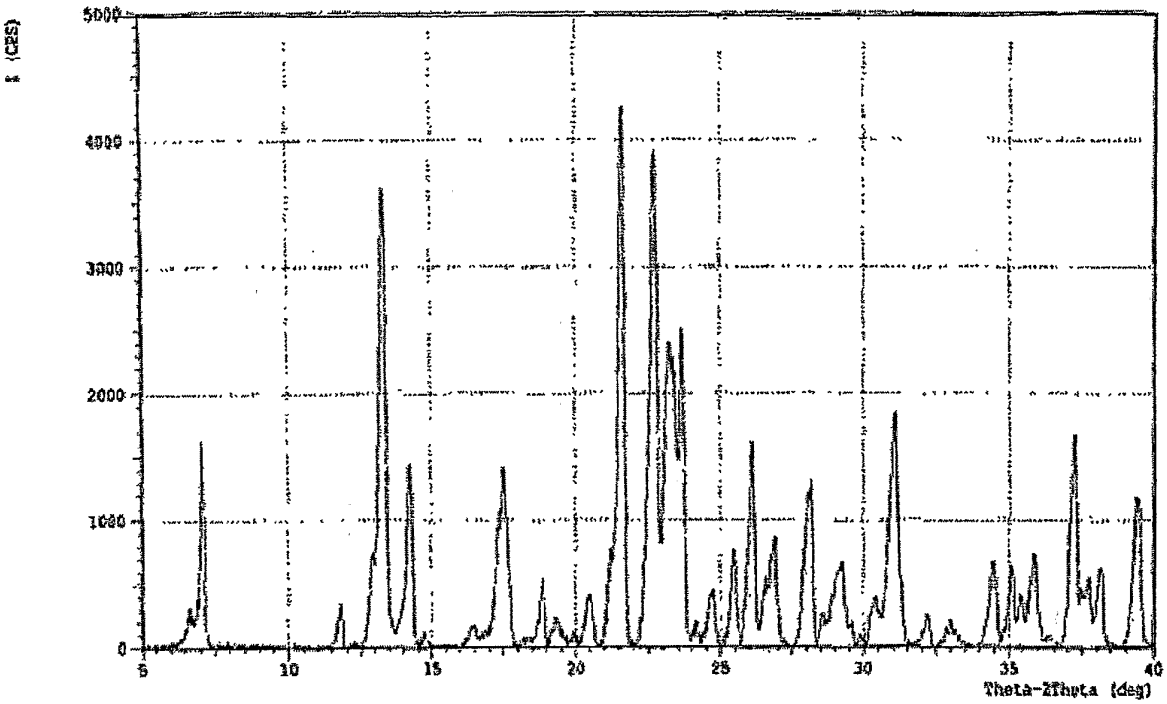

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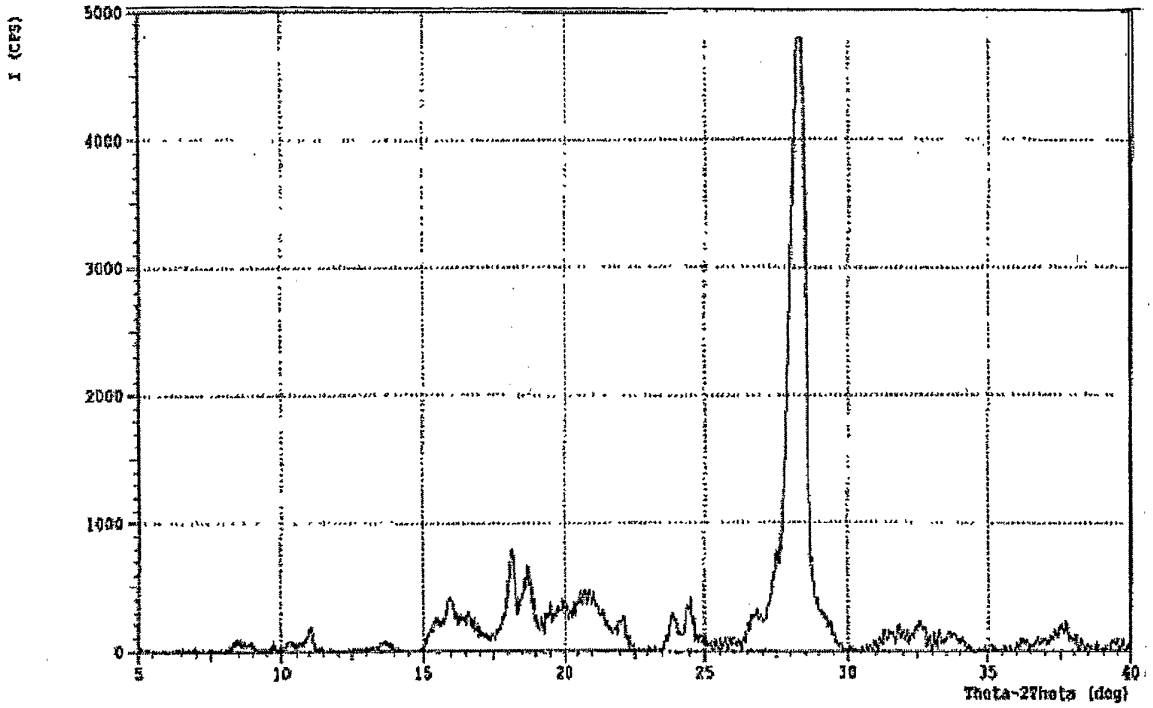

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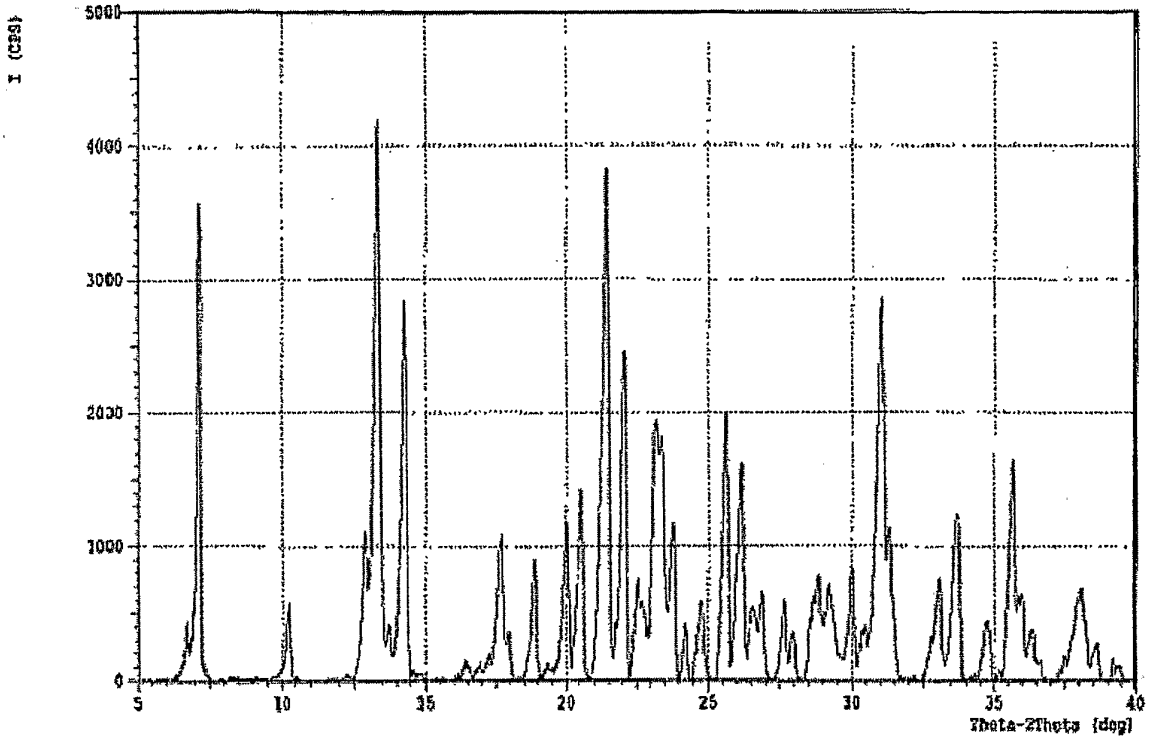

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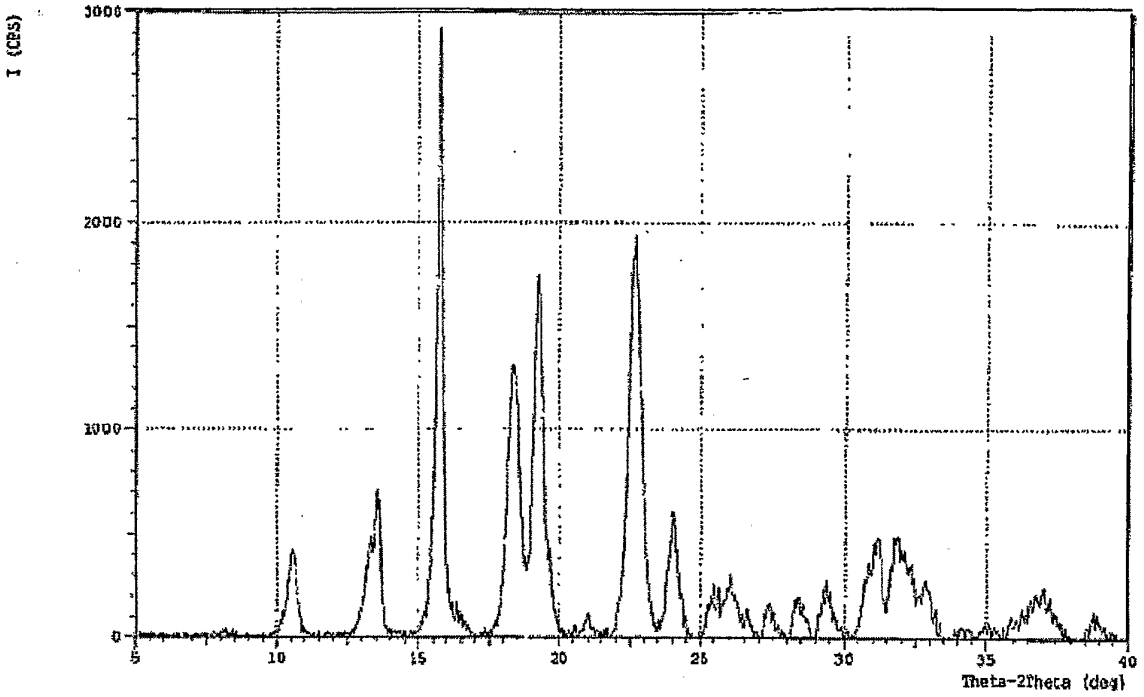

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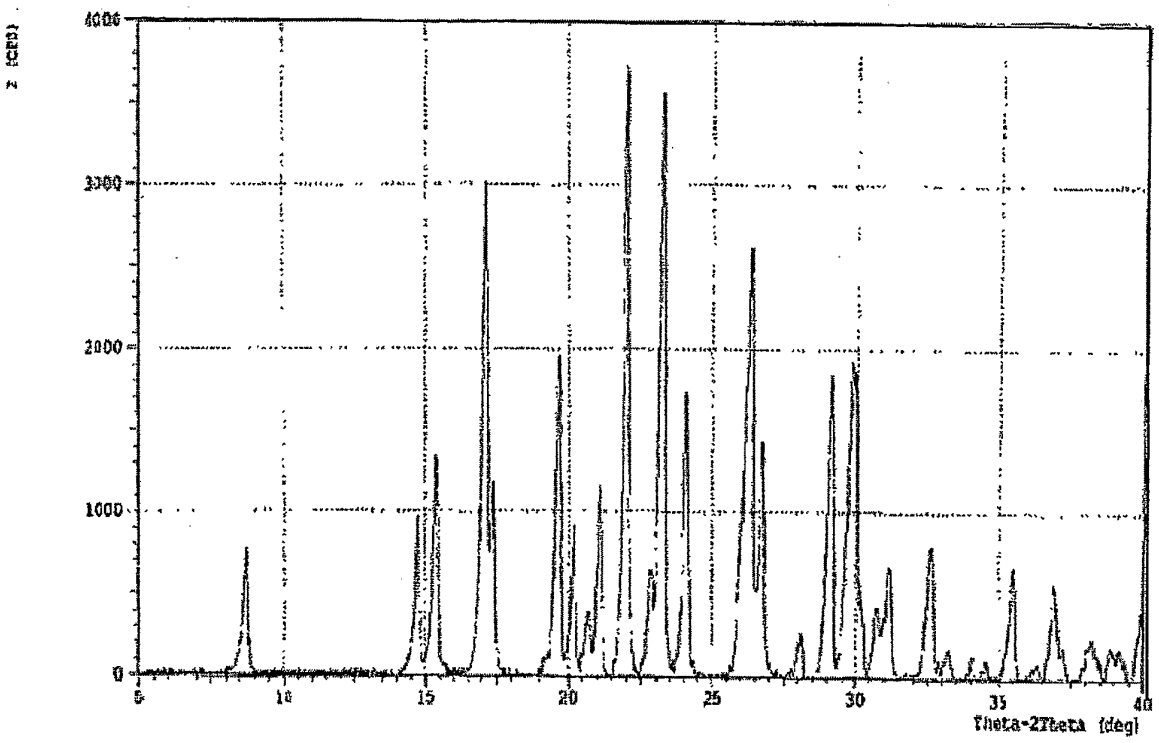

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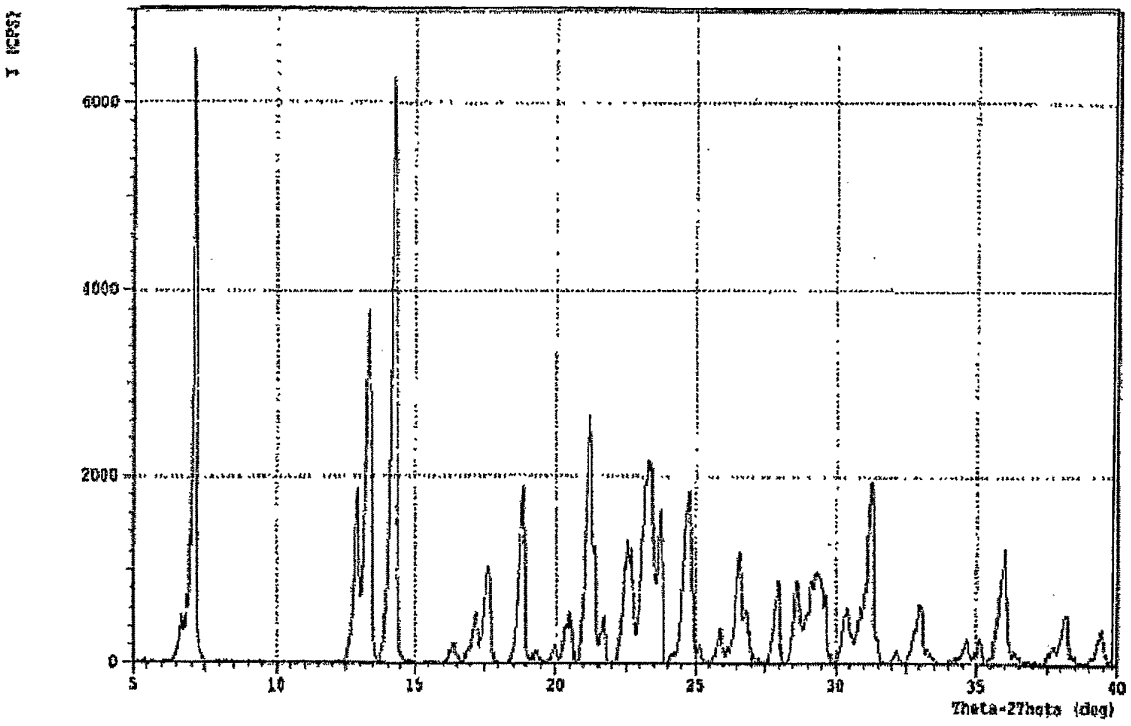

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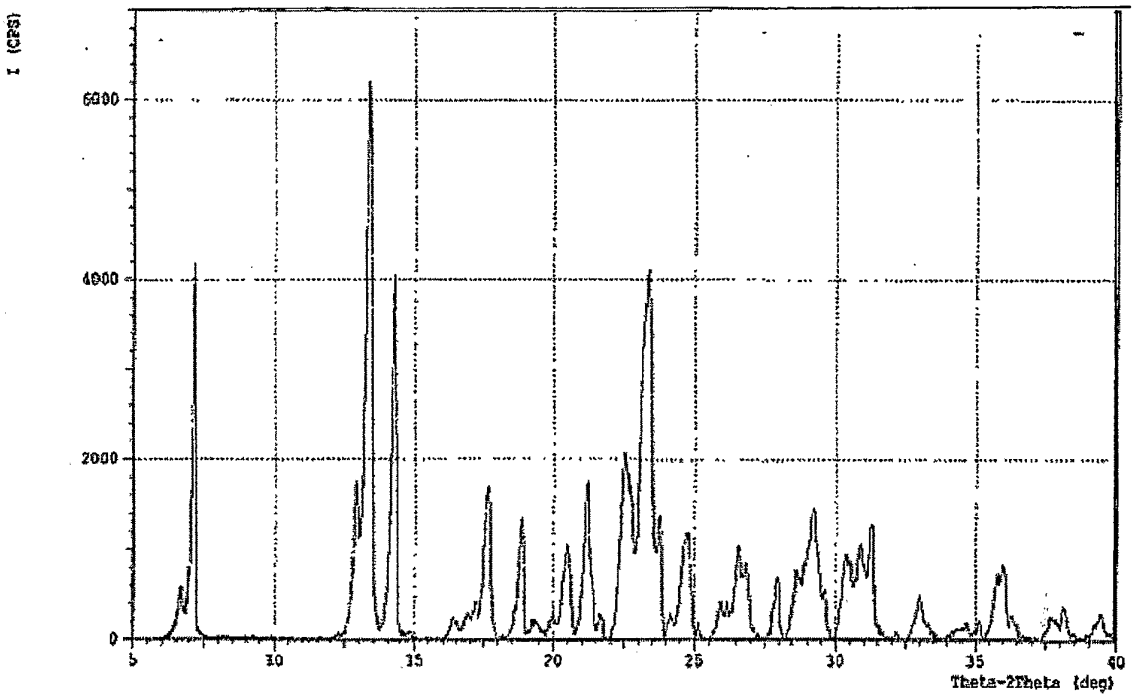

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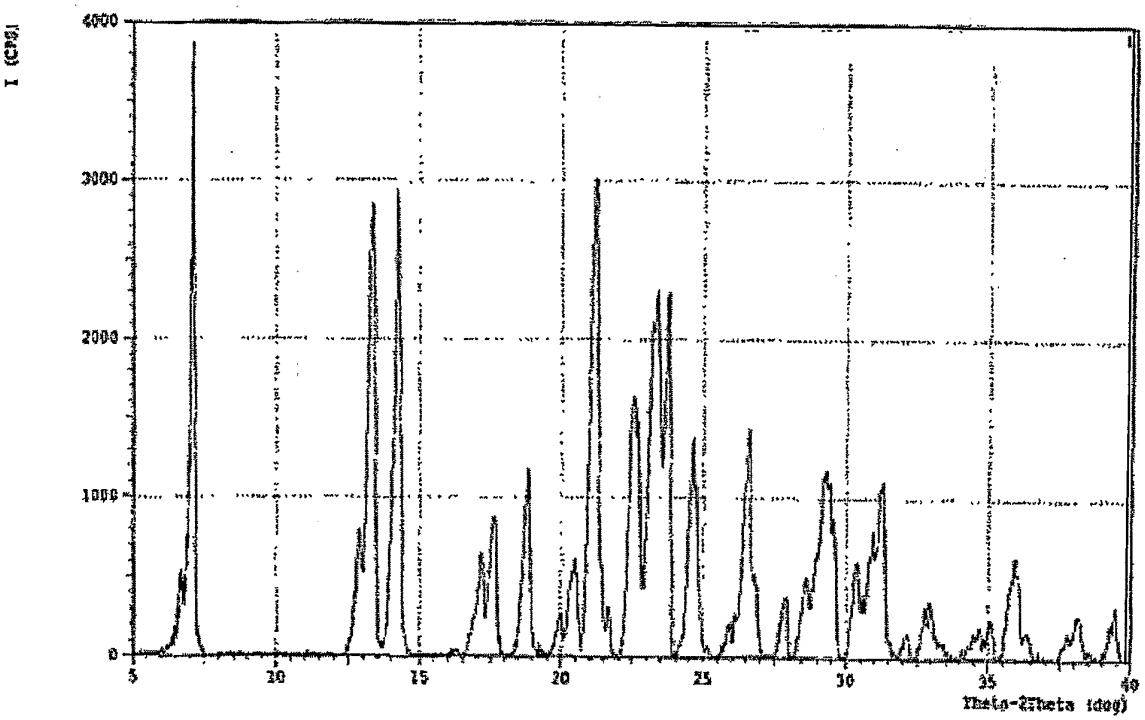

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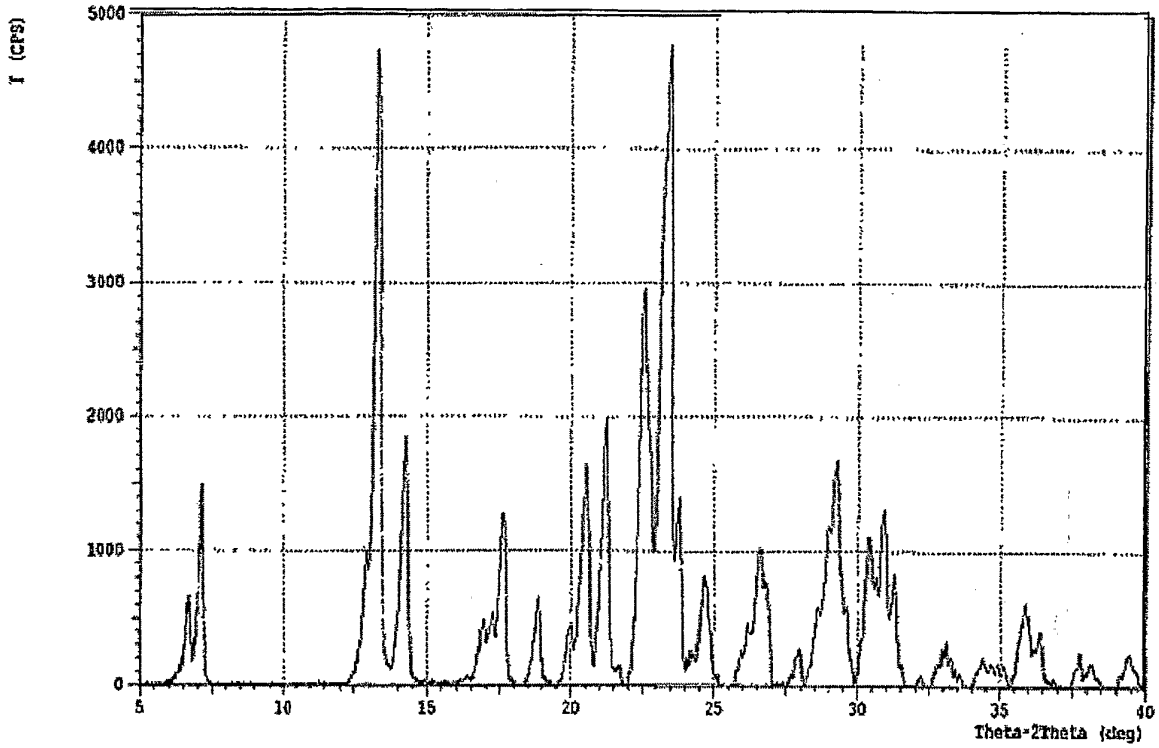

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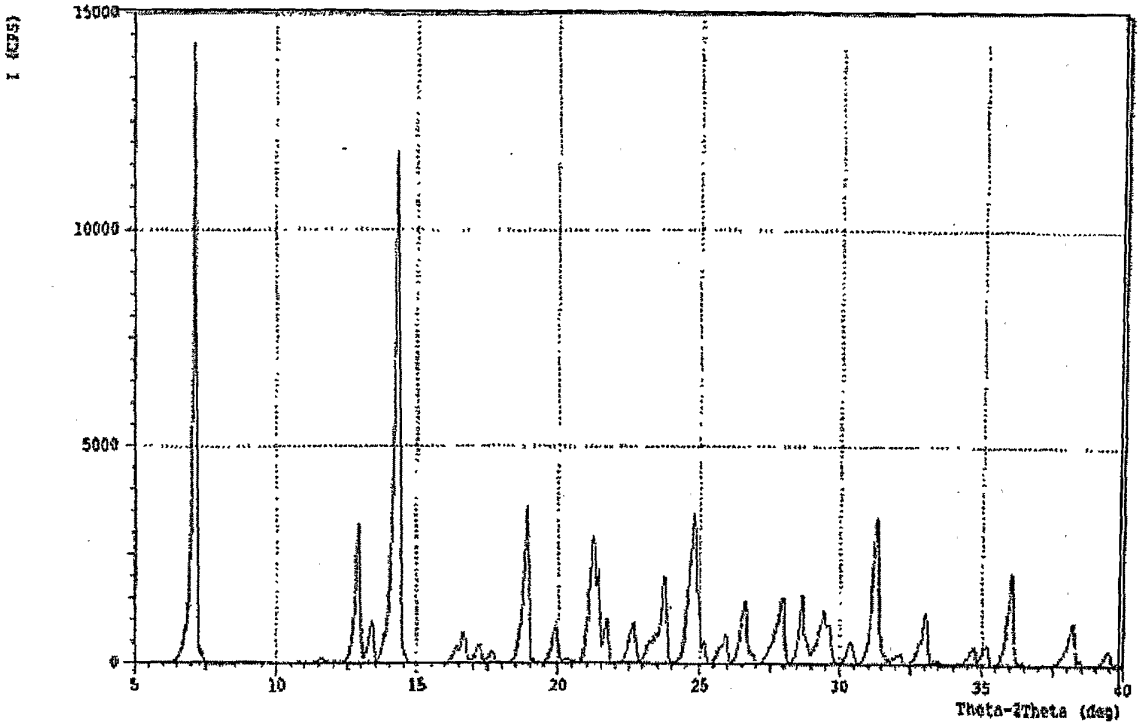

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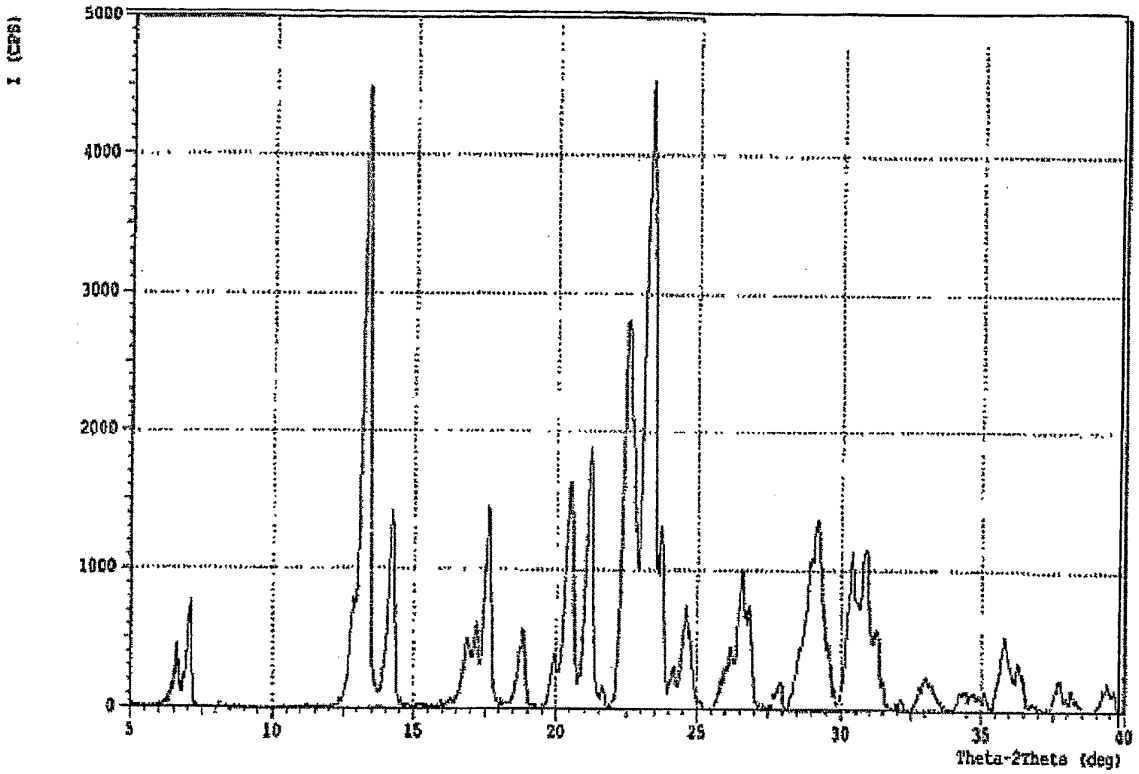

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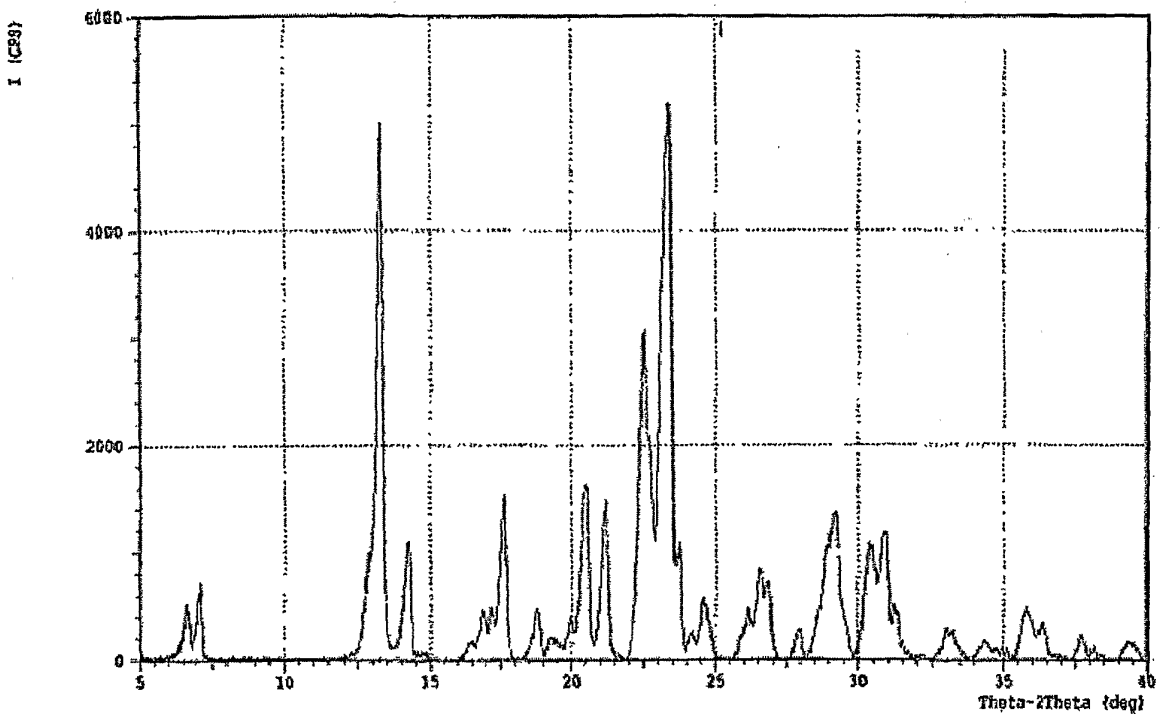

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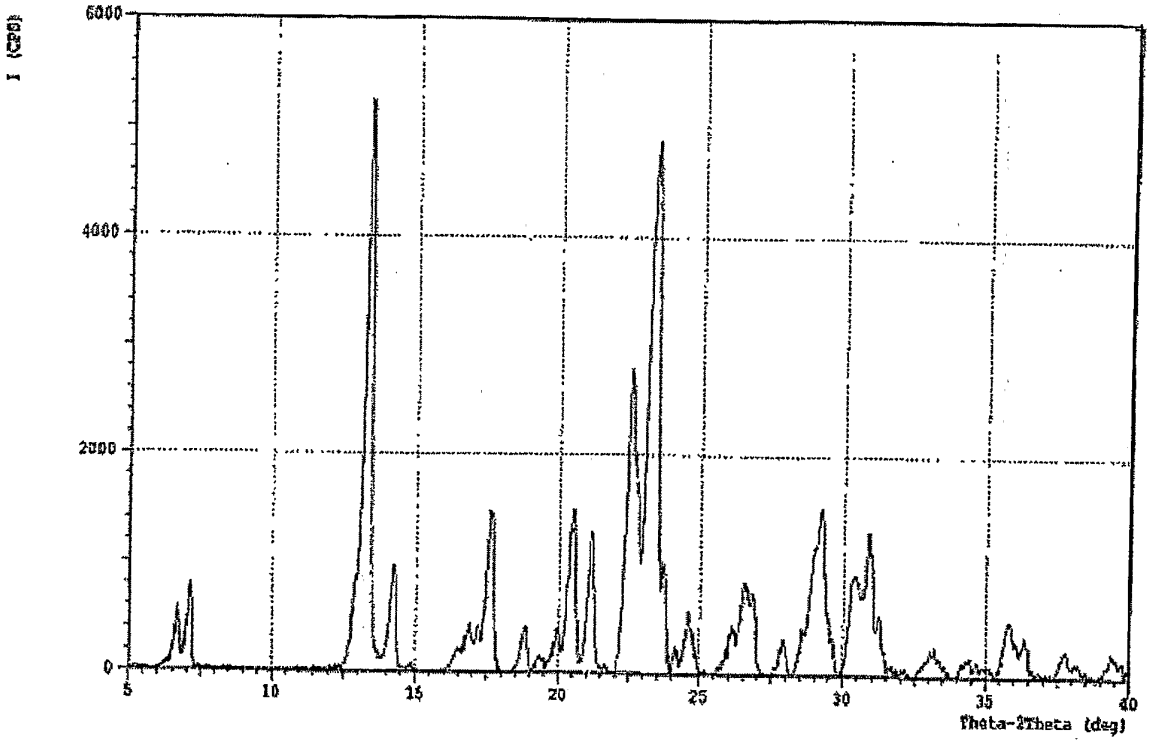

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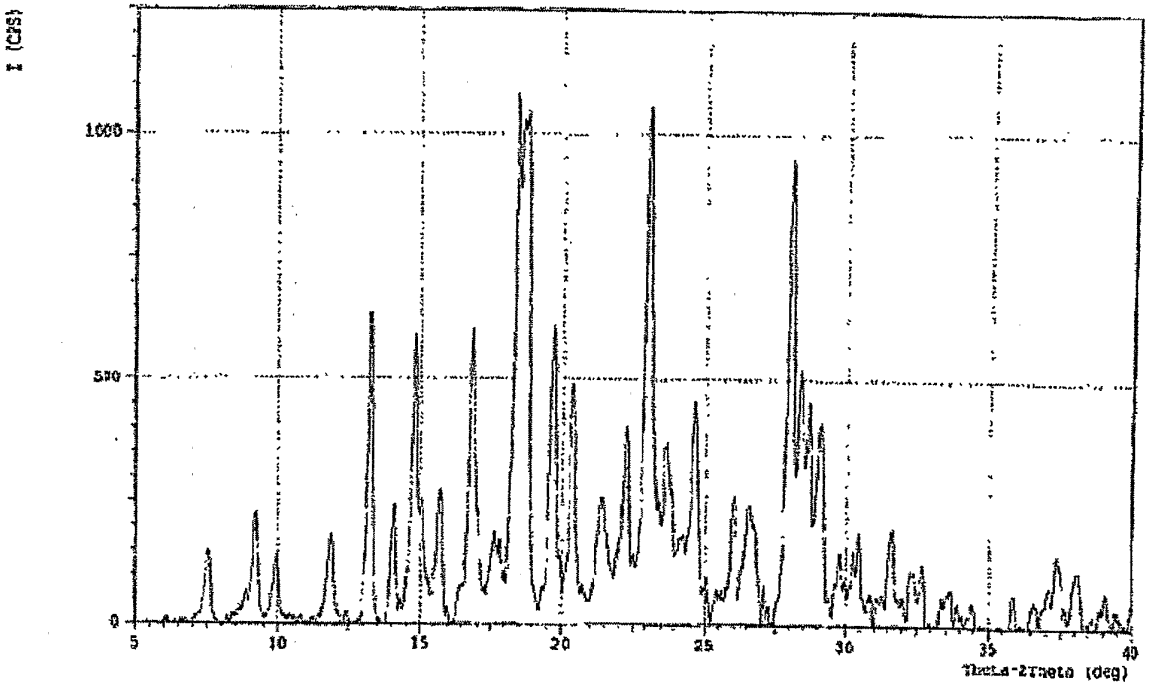

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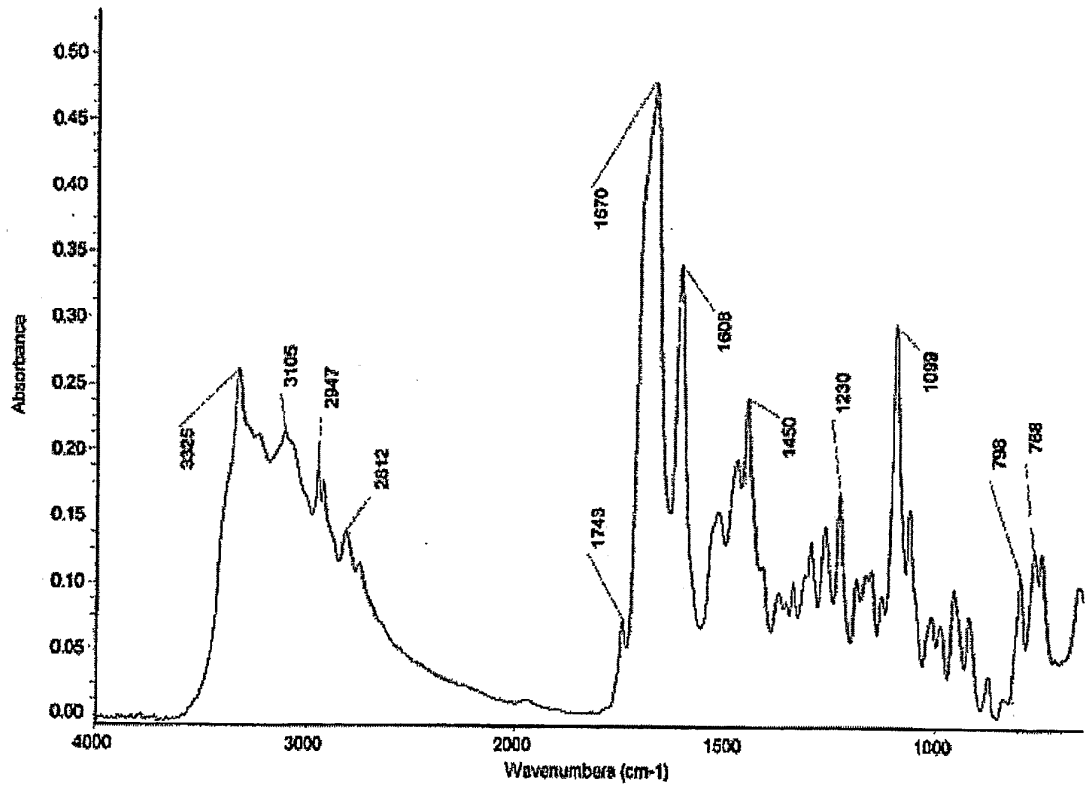

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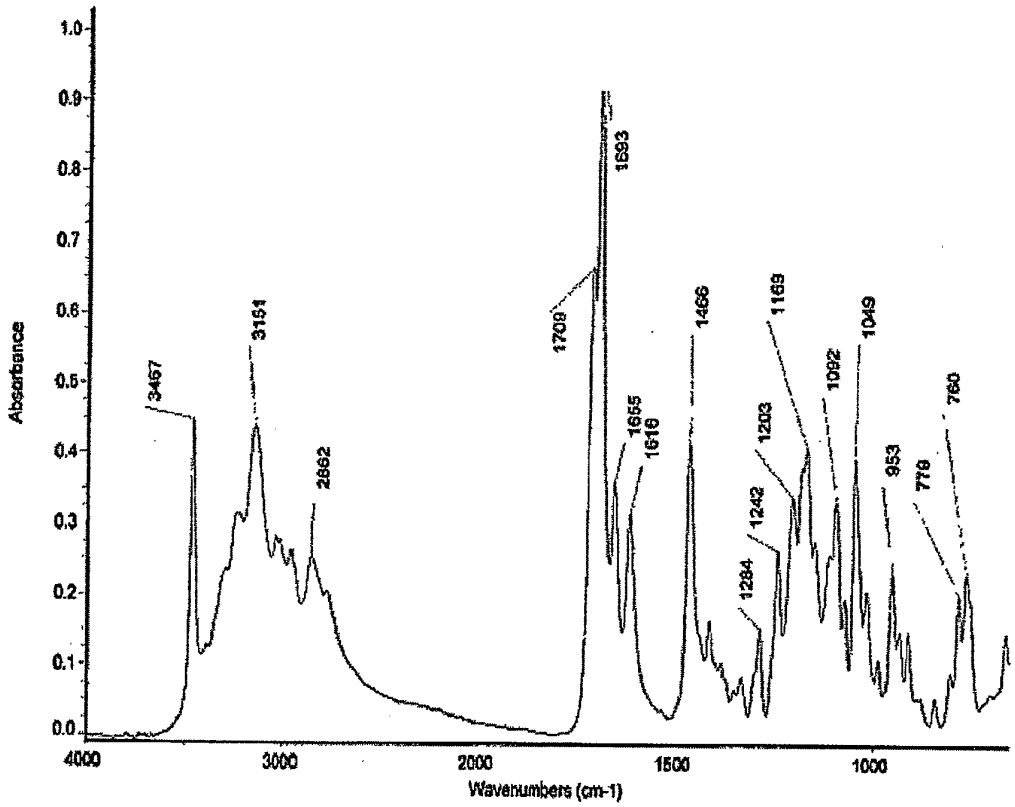

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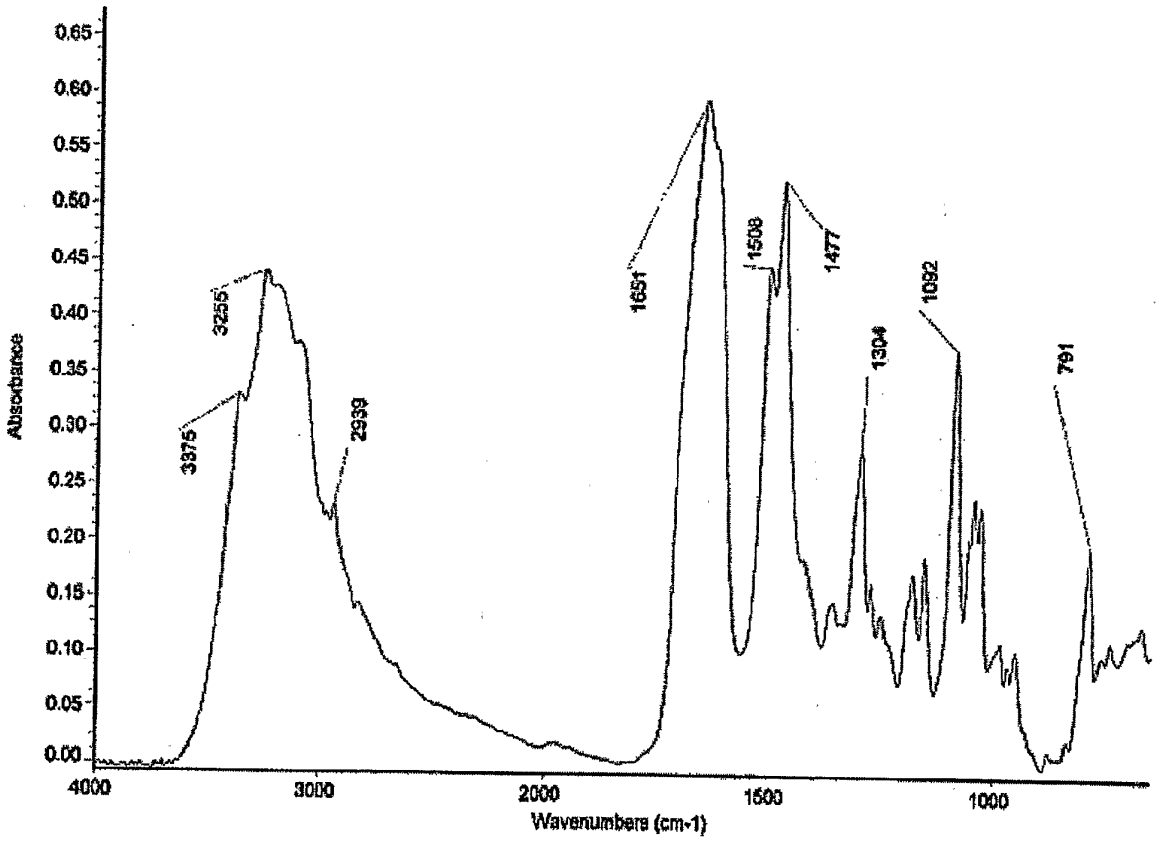

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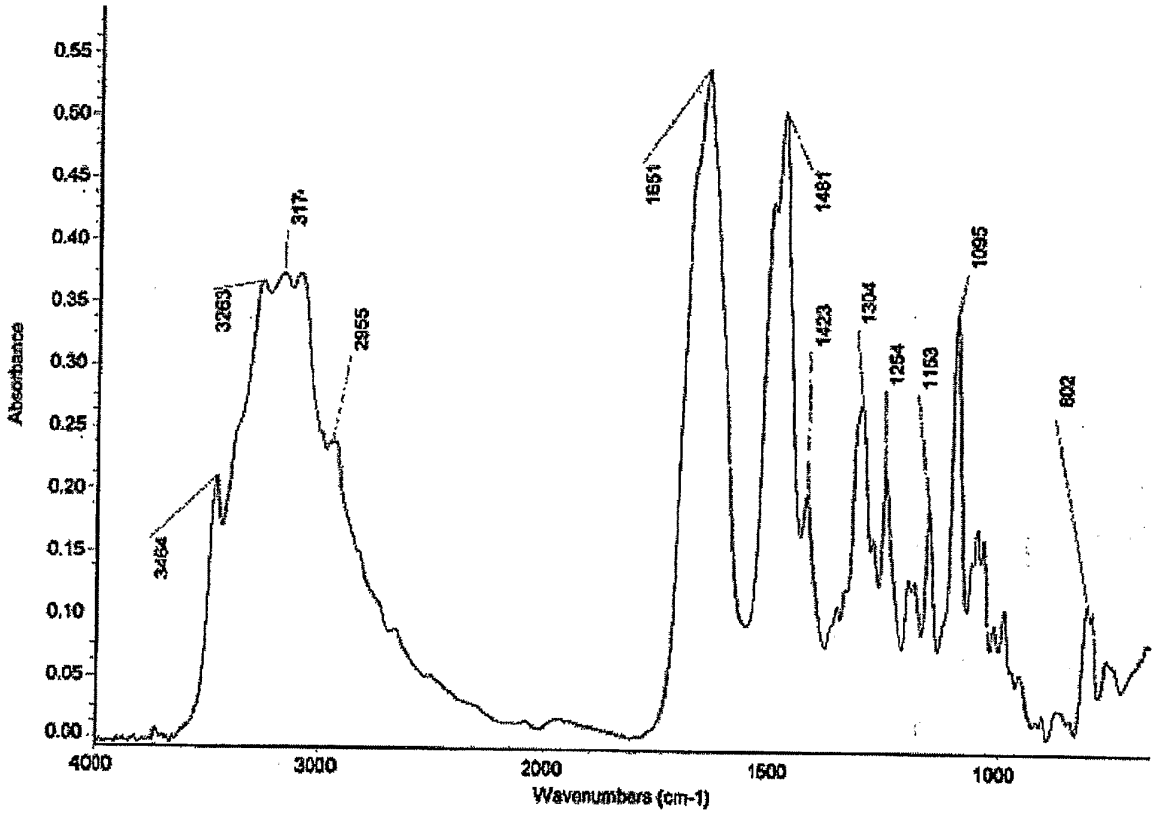

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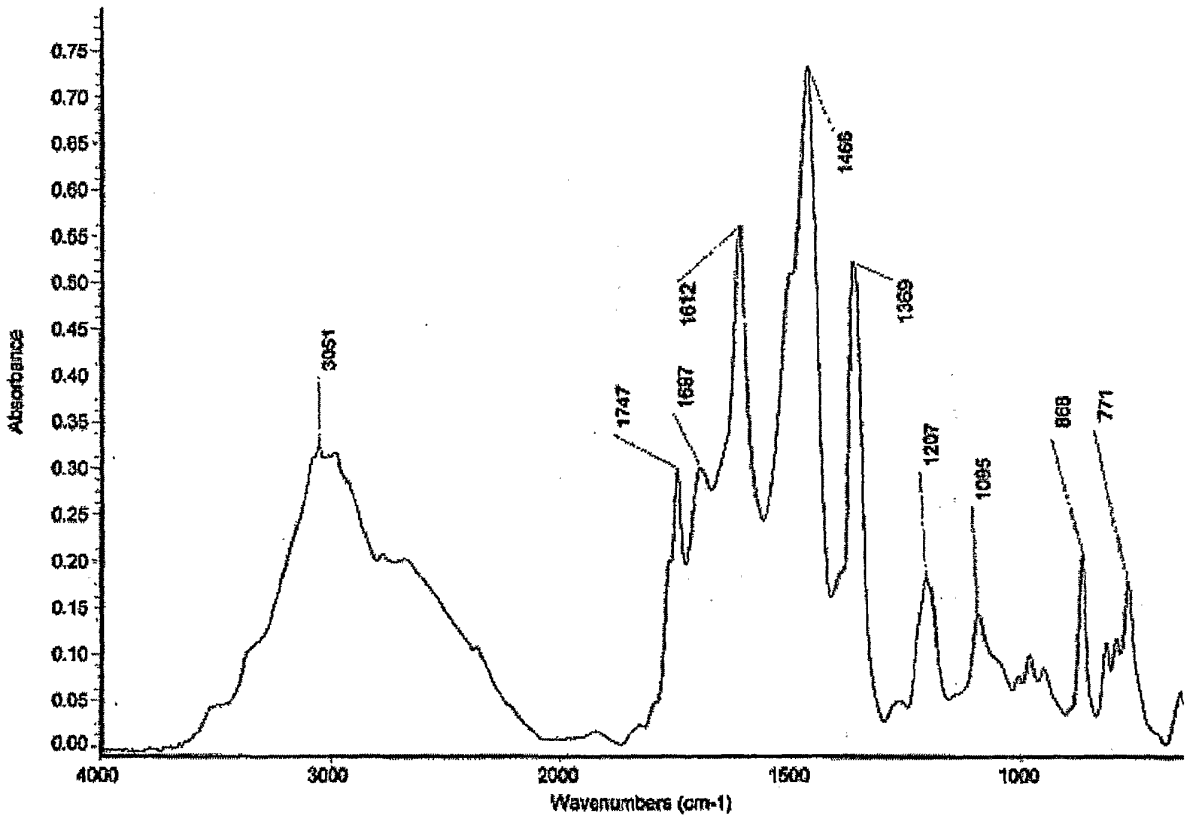

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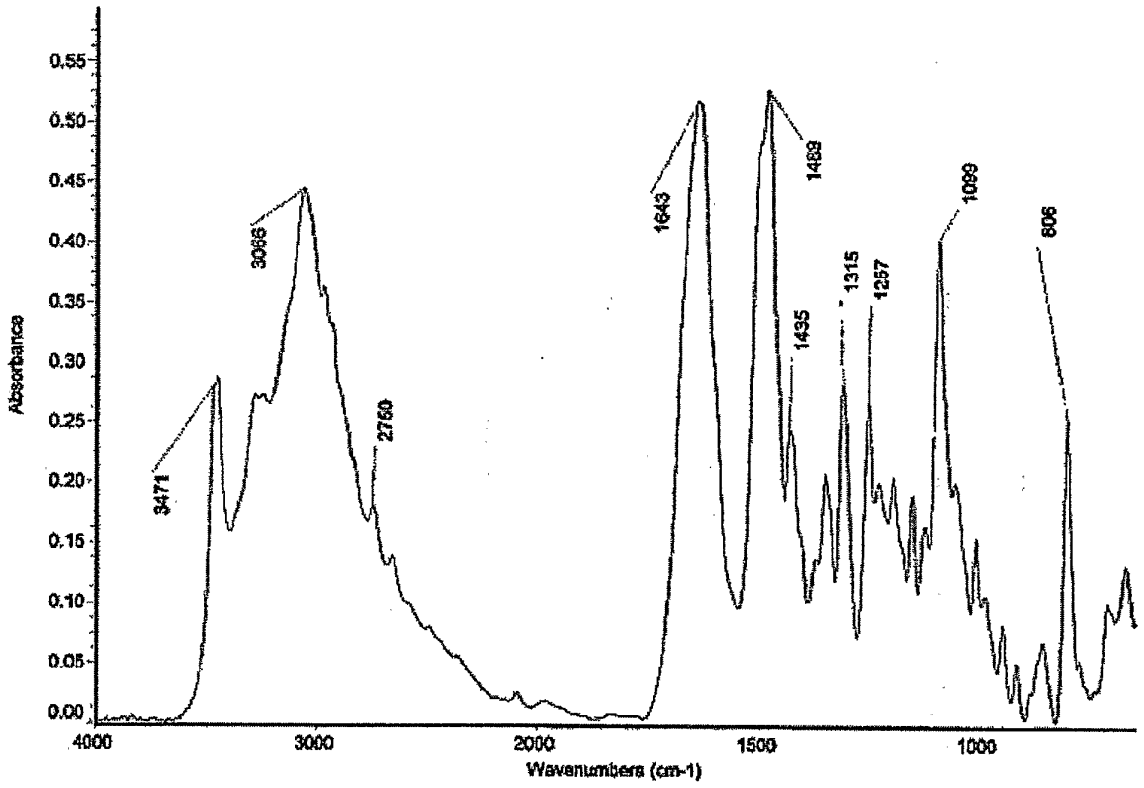

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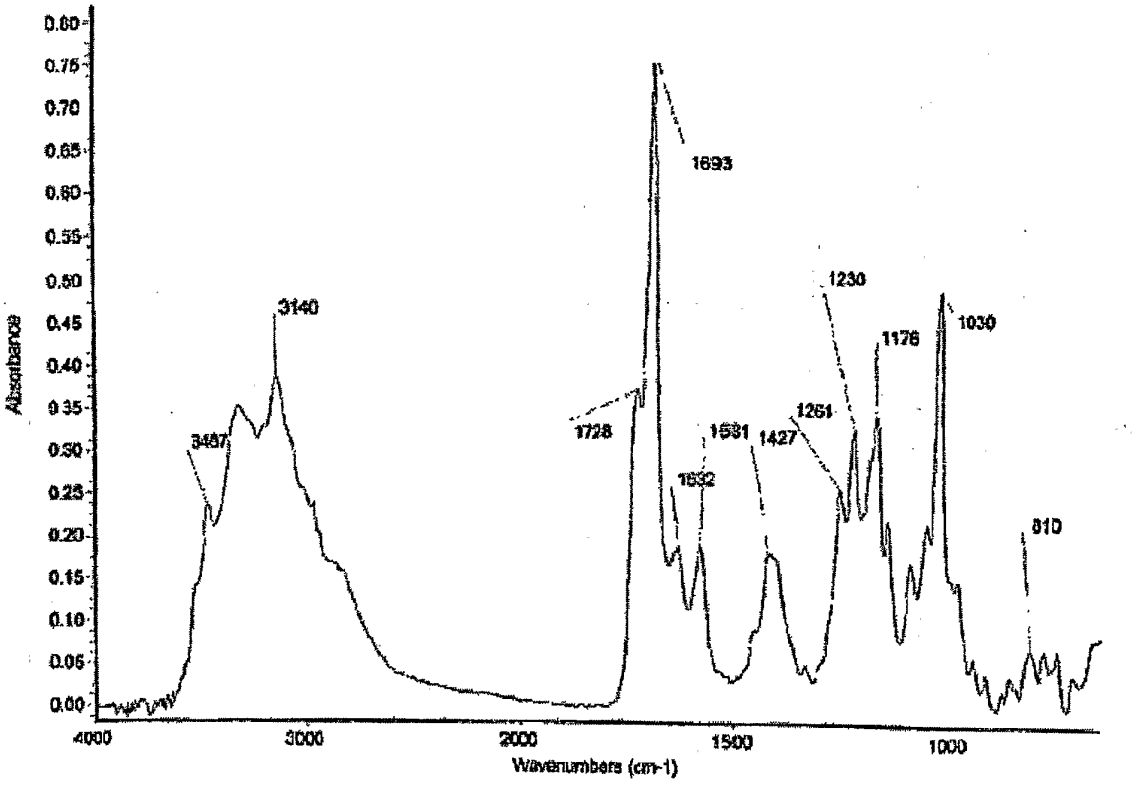

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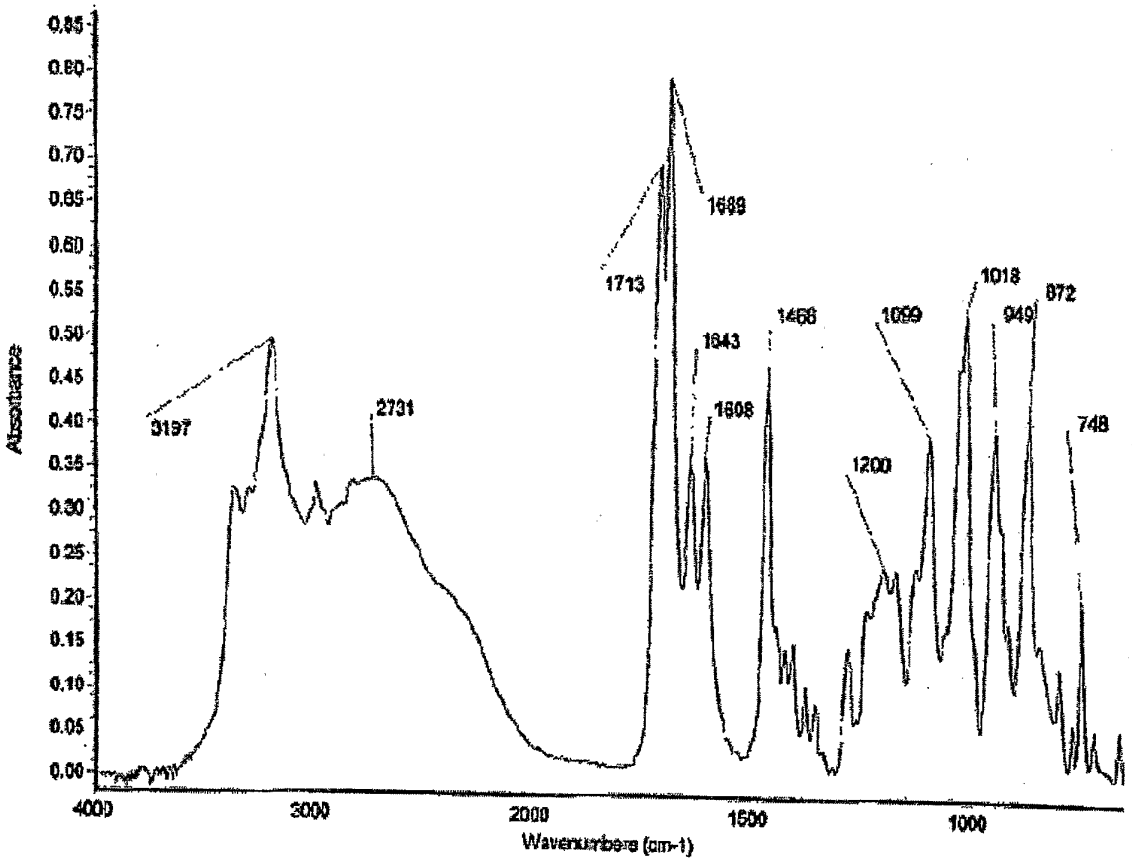

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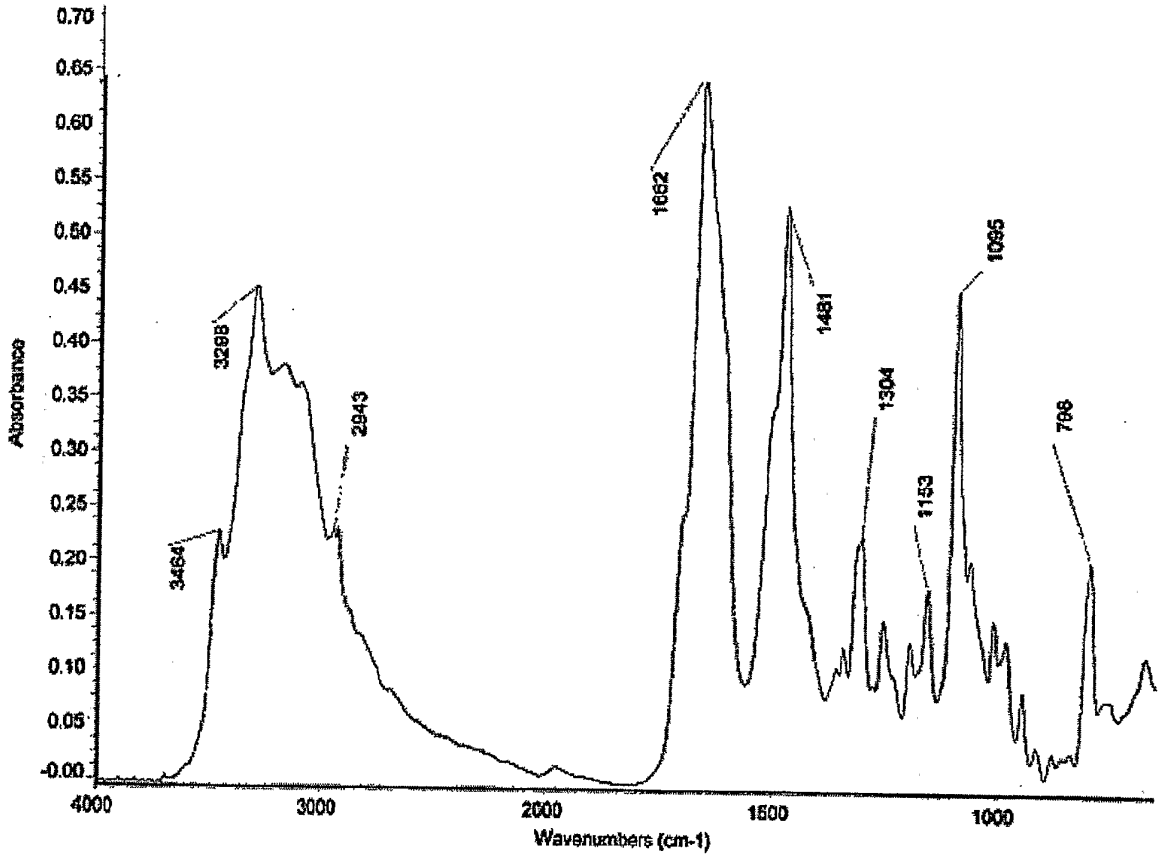

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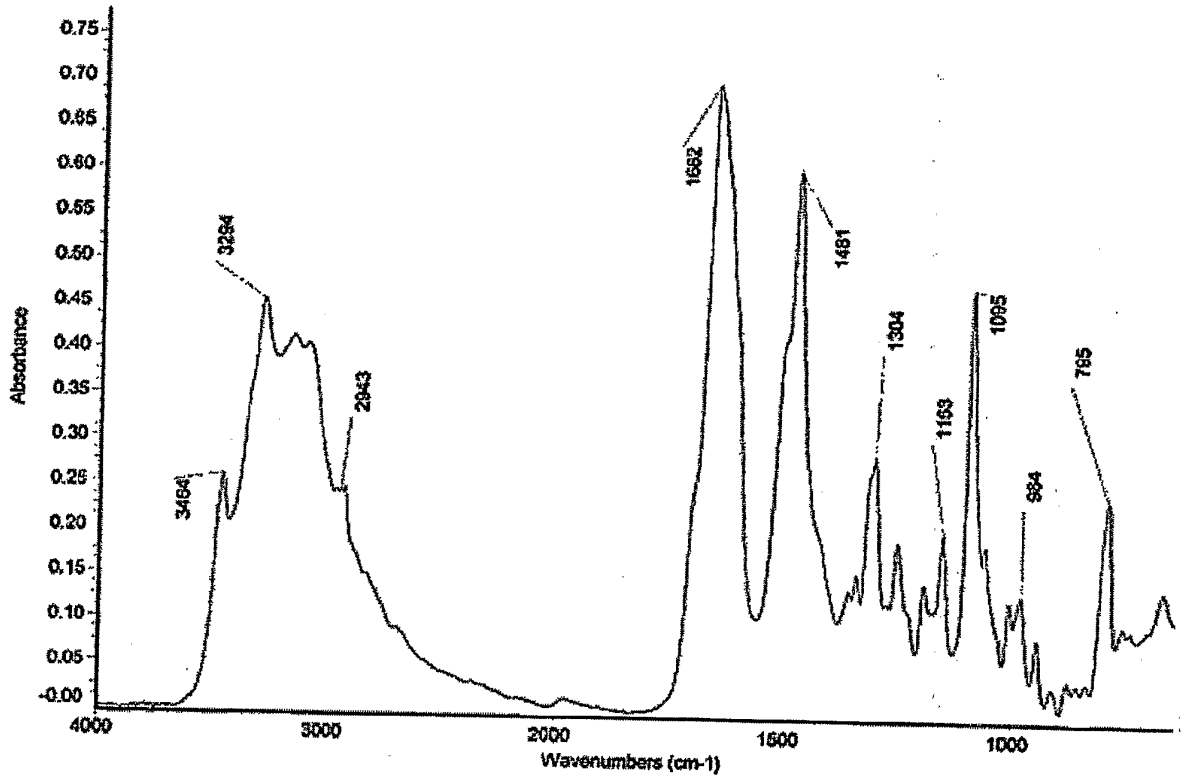

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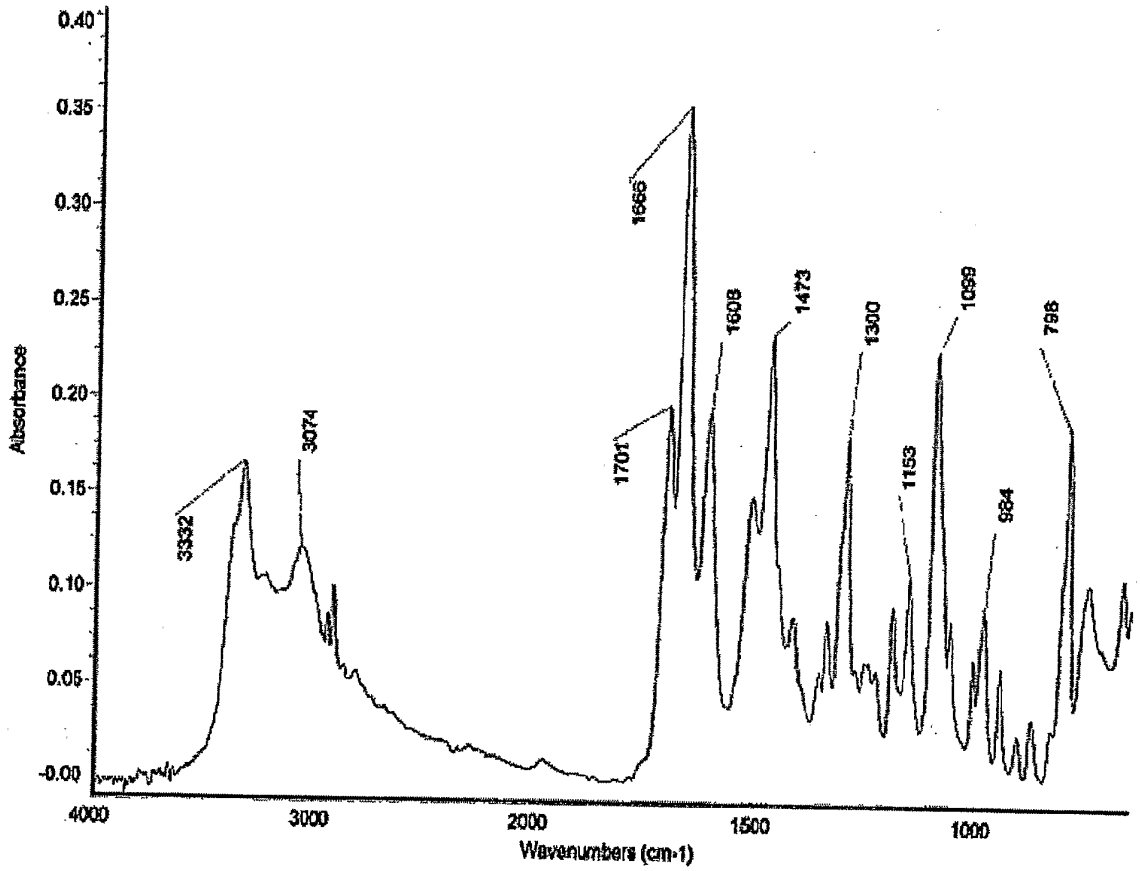

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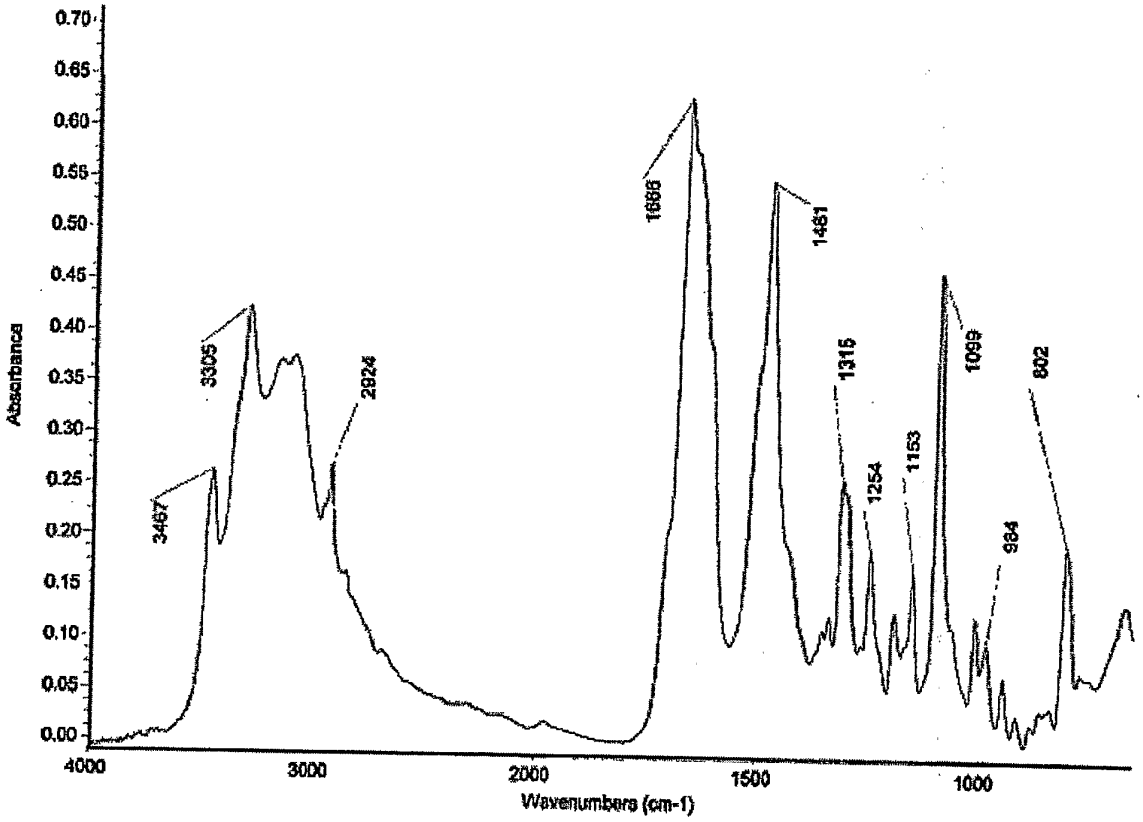

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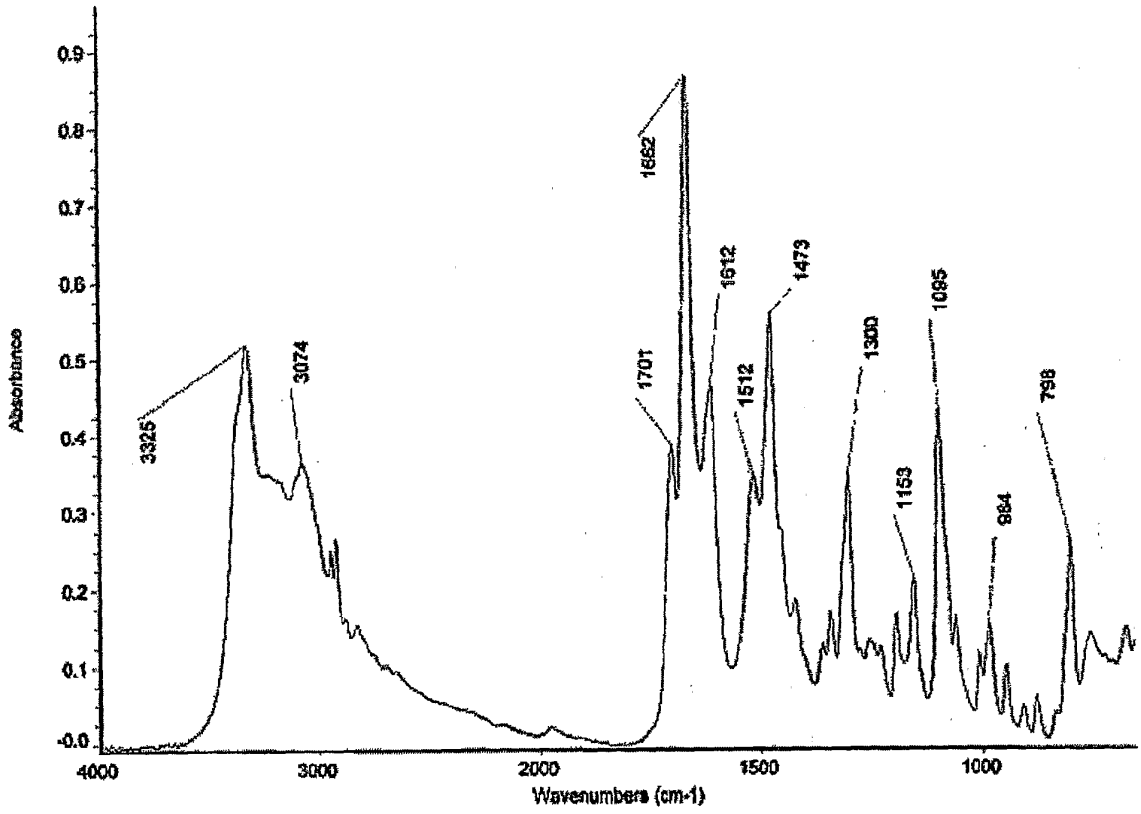

Figure 64

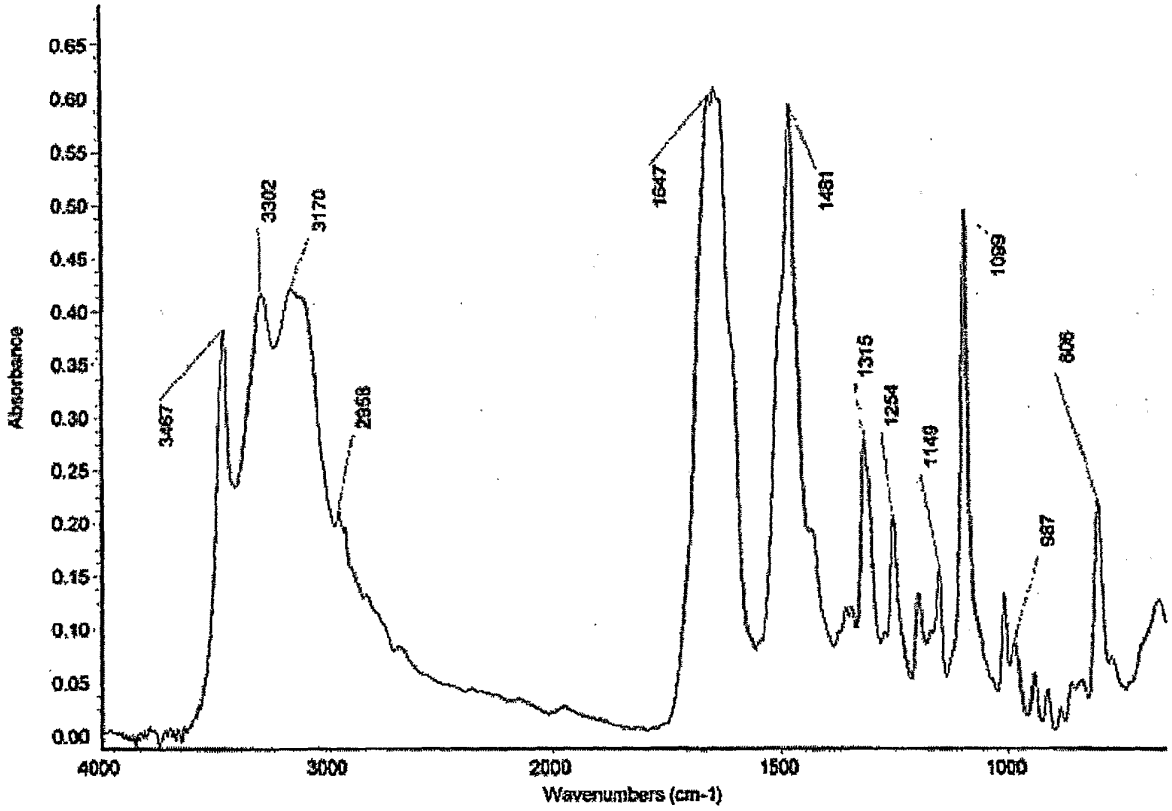

Figure 65

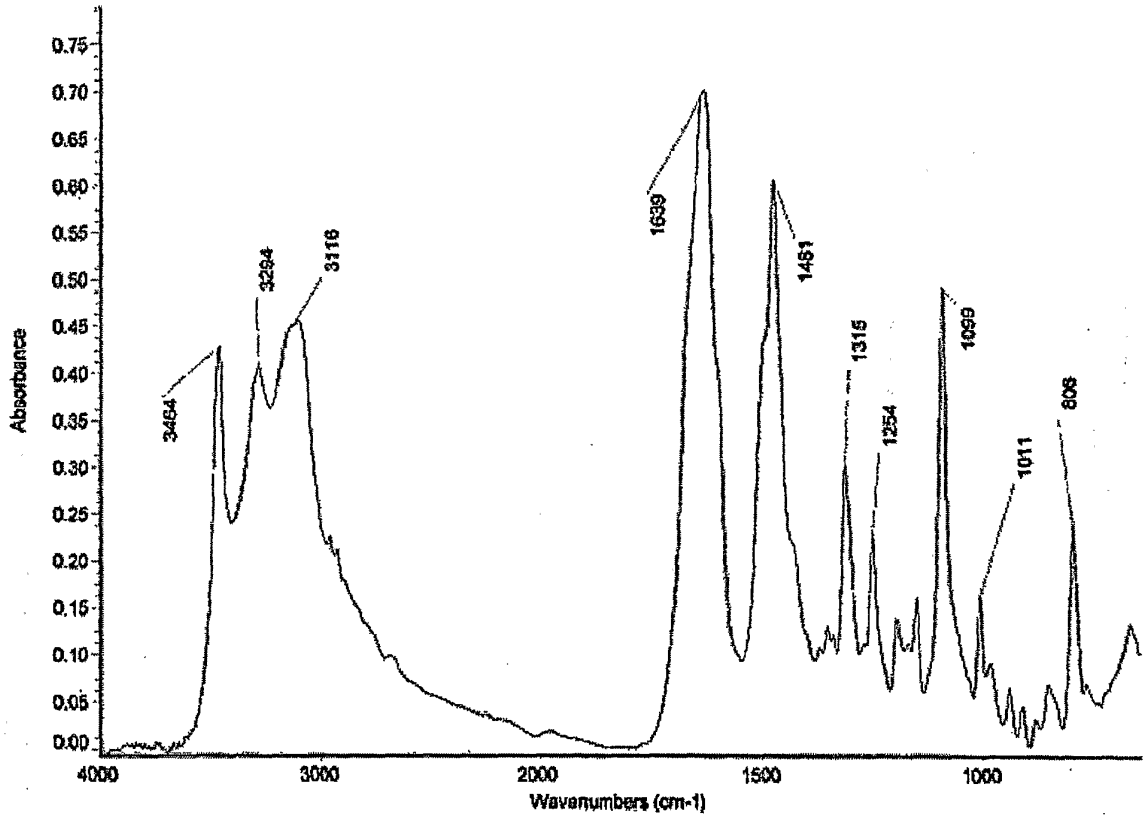

Figure 66

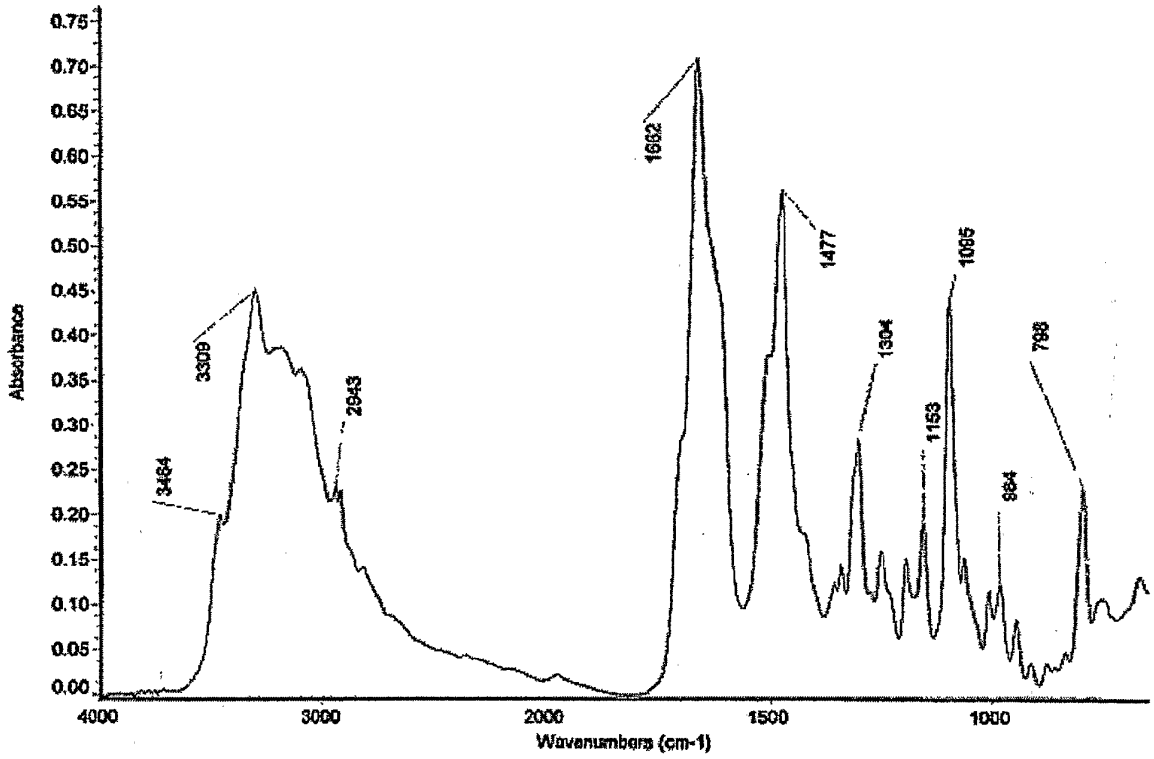

Figure 67

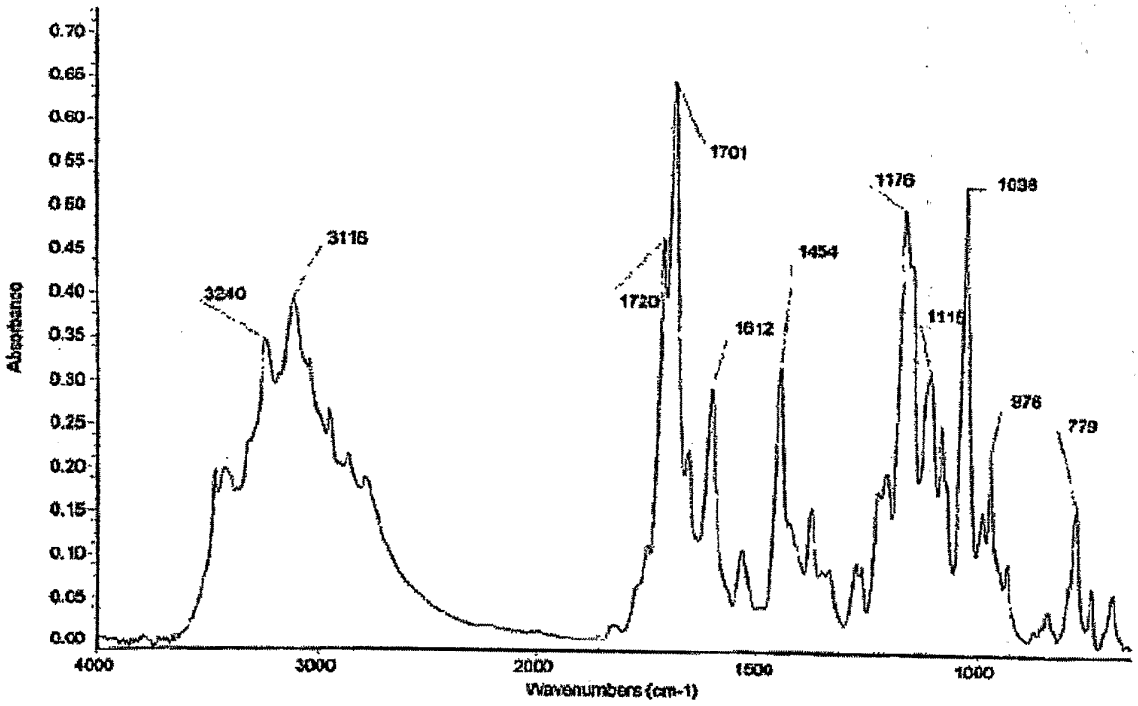

Figure 68

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(54) Title: SOLID STATE FORMS OF 5-AZACYTIDINE AND PROCESSES FOR PREPARATION THEREOF

(57) Abstract: The present invention provides novel crystalline forms of 5-deazacytidine, and pharmaceutical compositions comprising these novel forms. The invention also provides methods for the preparation of the novel forms and compositions.

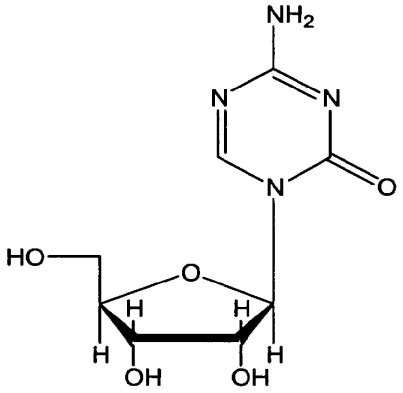
SOLID STATE FORMS OF 5-AZACYTIDINE AND PROCESSES FOR PREPARATION THEREOF

FIELD OF THE INVENTION

The present invention encompasses solid state forms of 5-azacytidine as well as processes for preparation thereof and pharmaceutical composition thereof.

BACKGROUND OF THE INVENTION

5-Azacytidine, 4-amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1H)-one, a compound having the chemical structure,



is an antineoplastic drug exhibiting activity against, e.g., leukemia, lymphoma and various solid tumours. 5-Azacytidine acts also as an inhibitor of DNA methyltransferase and was approved for the treatment of myelodispactic syndromes, a family of bone-marrow disorders. It is being marketed under the name Vidaza by Pharmion.

Crystallization of 5-azacytidine providing a methanol solvate of 5-azacytidine and crystallization of 5-azacytidine hydrate were described by Pískala and Šorm (Nucleic acid chemistry, Improved and new synthetic procedures, methods and techniques, Part one, L.B. Townsend and R.S. Tipson, Eds., Wiley Inc., New York, 1978, pp. 435-441).

US patent No. 6,943,249 ("'249") claims in claim 1 preparation of form I by recrystallization of 5-azacytidine from a solvent mixture comprising at least one primary solvent and at least one co-solvent selected from the group consisting C_2-C_5 alcohols, aliphatic ketones, and alkyl cyanides, by cooling said solvent mixture from a temperature

selected to allow said 5-azacytidine to dissolve completely to about ambient temperature, and isolating the recrystallized 5-azacytidine. The US '249 patent also claims in claim 11 a method for preparing Form I comprising recrystallizing 5-azacytidine from a solvent mixture comprising at least one primary solvent and at least one co-solvent selected from the group consisting C_3-C_5 alcohols and alkyl cyanides, by cooling said solvent mixture from a temperature selected to allow said 5-azacytidine to dissolve completely to about – 20 °C, and isolating the recrystallized 5-azacytidine" also leads to form I of 5-azacytidine.. All of the examples of the '249 patent use DMSO as a solvent to which a co-solvent is added. The '249 patent also describes form I having the most prominent 2 theta angles at 12.182, 13.024, 14.399, 16.470, 18.627, 19.049, 20.182, 21.329, 23.033, 23.872, 26.863, 27.1735, 29.277, 29.591, 30.369, and 32.072.

US patent No. 6,887,855 discloses eight polymorphic forms of 5-azacytidine, denominated Forms I-VIII, for which Forms I-III are reported to be in the prior art. The characterization of each of these forms in US patent No. 6,887,855 is incorporated herein by reference.

US patent No. 6,887,855 ("'855") discloses the synthesis and isolation of Form I , where the obtained form I is reported to be characterized by the same most prominent two theta angles as described in the '249 patent and by figure 1. The '855 patent discloses also a mixture of 5-azacytidine form I and a form identified by the most prominent two theta angles at 13.5, 17.6, and 22.3 degrees two-theta, denominated form II.

U.S. 6,887,855 also reports additional crystalline forms of 5-azacytidine, denominated Form IV having the most prominent 2 theta angles at 5.704, 11.571, 12.563, 14.070, 15.943, 16.993, 18.066, 20.377, 20.729, 21.484, 21.803, 22.452, 22.709, 23.646, 24.068, 25.346, 25.346, 26.900, 27.991, 28.527, 28.723, 30.124, 30.673, 31.059, 35.059, 38.195 and 38.403; Form V having the most prominent 2 theta angles at 11.018, 12.351, 13.176, 13.747, 14.548, 15.542, 16.556, 17.978, 18.549, 19.202, 19.819, 20.329, 21.518, 21.970, 22.521, 23.179, 24.018, 24.569, 27.224, 28.469, 29.041, 29.429, 30.924, 31.133 and 37.938; Form VI, a mixture of form I and a crystalline form which exhibits distinctive peaks at 5.8, 11.5, 12.8, 22.4, and 26.6 degrees two-theta, denominated Form VII; a crystalline form having the most prominent two theta angles at 6.599, 10.660,

12.600, 13.358, 15.849, 17.275, 20.243, 20.851, 21.770, 22.649, 25.554, 25.740, 29.293, 32.148, 35.074, and 38.306 degrees two-theta, denominated Form VIII; as well as an amorphous form, processes for preparation thereof, and conversion of form I to the crystalline form having most prominent diffractions on PXRD at two theta values at 6.566, 11.983, 13.089, 15.138, 17.446, 20.762, 21.049, 22.776, 24.363, 25.743, 26.305, 28.741, 31.393, 32.806, 33.043, 33.536, 36.371, 39.157, and 41.643 degrees two-theta, denominated Form III, and to amorphous form.

According to U.S. patent and 7,078,518 (a divisional of the '855 patent), 5-azacytidine Forms IV, V, VI, and mixtures of form I and VII, are prepared by recrystallization processes that include dissolving 5-azacytidine in dimethylsulfoxide, and at least one co solvent is added to the solution of 5-azacytidine facilitating the crystallization; wherein the co solvents is toluene, methanol or chloroform.

The present invention relates to the solid-state physical properties of 5-azacytidine as well as to processes for preparation thereof.

These properties can be influenced by controlling the conditions under which 5-azacytidine is obtained in solid form. Solid-state physical properties include, for example, the flowability of the milled solid. Flowability affects the ease with which the material is handled during processing into a pharmaceutical product. When particles of the powdered compound do not flow past each other easily, a formulation specialist must take that fact into account in developing a tablet or capsule formulation, which may necessitate the use of glidants such as colloidal silicon dioxide, talc, starch or tribasic calcium phosphate.

Another important solid-state property of a pharmaceutical compound is its rate of dissolution in aqueous fluid. The rate of dissolution of an active ingredient in a patient's stomach fluid can have therapeutic consequences since it imposes an upper limit on the rate at which an orally-administered active ingredient can reach the patient's bloodstream. The rate of dissolution is also a consideration in formulating syrups, elixirs and other liquid medicaments. The solid-state form of a compound may also affect its behavior on compaction and its storage stability.

These practical physical characteristics are influenced by the conformation and orientation of molecules in the unit cell, which defines a particular polymorphic form of a substance that can be identified unequivocally by X-ray spectroscopy. The polymorphic

form may give rise to thermal behavior different from that of the amorphous material or another polymorphic form. Thermal behavior is measured in the laboratory by such techniques as capillary melting point, thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) and can be used to distinguish some polymorphic forms from others. A particular polymorphic form may also give rise to distinct spectroscopic properties that may be detectable by solid-state ^{13}C NMR spectrometry and infrared spectroscopy.

The present invention also relates to solvates of 5-azacytidine. When a substance crystallizes out of solution, it may trap molecules of solvent at regular intervals in the crystal lattice. Solvation also affects utilitarian physical properties of the solid-state like flowability and dissolution rate.

One of the most important physical properties of a pharmaceutical compound, which can form polymorphs or solvates, is its solubility in aqueous solution, particularly the solubility in gastric juices of a patient. Other important properties relate to the ease of processing the form into pharmaceutical dosages, as the tendency of a powdered or granulated form to flow and the surface properties that determine whether crystals of the form will adhere to each other when compacted into a tablet.

The discovery of new polymorphic forms and solvates of a pharmaceutically useful compound provides a new opportunity to improve the performance characteristics of a pharmaceutical product. It enlarges the repertoire of materials that a formulation scientist has available for designing, for example, a pharmaceutical dosage form of a drug with a targeted release profile or other desired characteristic.

SUMMARY OF THE INVENTION

In one embodiment, the present invention provides crystalline 5-azacytidine characterized by data selected from a group consisting of: a PXRD pattern with peaks at about 12.2, 13.1, 14.4, 16.2, and 23.1 ± 0.2 degrees two-theta, a PXRD pattern as depicted in Figure 11, and combination thereof containing less than about 5% by weight of a crystalline form having most prominent diffractions at PXRD at two theta values at 6.566, 11.983, 13.089, 15.138, 17.446, 20.762, 21.049, 22.776, 24.363, 25.743, 26.305,

28.741, 31.393, 32.806, 33.043, 33.536, 36.371, 39.157, and 41.643 degrees two-theta and less than about 5% by weight of a crystalline form with having most prominent PXRD diffractions at 13.4, 17.6, and 22.1 degrees two-theta..

In another embodiment, the present invention encompasses 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents. Preferably, the 5-azacytidine is crystalline 5-azacytidine characterized by data selected from a group consisting of: a PXRD pattern with peaks at about 12.2, 13.1, 14.4, 16.2, and 23.1 ± 0.2 degrees two-theta, a PXRD pattern as depicted in Figure 11, and combination thereof, having less than about 5% by weight of a crystalline form having most prominent diffractions at PXRD at two theta values at 6.566, 11.983, 13.089, 15.138, 17.446, 20.762, 21.049, 22.776, 24.363, 25.743, 26.305, 28.741, 31.393, 32.806, 33.043, 33.536, 36.371, 39.157, and 41.643 degrees two-theta and less than about 5% by weight of a crystalline 5-azacytidine having most prominent PXRD diffractions at 13.4, 17.6, and 22.1 degrees two-theta.

In yet another embodiment, the present invention provides a process for preparing 5-azacytidine containing about 10 ppm to about 2000 ppm of non volatile solvents comprising heating a suspension of 5-azacytidine in a single solvent selected from a group consisting of: aliphatic alcohol, nitrile, ether, nitromethane, and pyridine, or in a mixture of solvents comprising the above single solvent and a non-polar organic solvent selected from a group consisting of ketone and ester, and recovering the obtained 5-azacytidine containing about 10 ppm to about 2000 ppm of non volatile solvents; wherein the single polar solvent or its mixture with a non-polar solvent has boiling point of less than 140°C. Preferably, the obtained 5-azacytidine is crystalline 5-azacyitidine characterized by data selected from a group consisting of: a PXRD pattern with peaks at about 12.2, 13.1, 14.4, 16.2, and 23.1 ± 0.2 degrees two-theta, a PXRD pattern as depicted in Figure 11, and combination thereof, containing less than about 5% by weight of a crystalline 5-azacytidine characterized having most prominent diffractions at PXRD at two theta values at 6.566, 11.983, 13.089, 15.138, 17.446, 20.762, 21.049, 22.776, 24.363, 25.743, 26.305, 28.741, 31.393, 32.806, 33.043, 33.536, 36.371, 39.157, and 41.643 degrees two-theta and less than about 5% by weight of a crystalline 5-azacytidine having most prominent PXRD diffractions at t 13.4, 17.6, and 22.1 degrees two-theta.

In one embodiment, the present invention provides crystalline 5-azacytidine characterized by data selected from a group consisting of: a powder XRD pattern with peaks at about 8.7, 9.5, 12.1, 14.4, and 17.3 ± 0.2 degrees two-theta, a PXRD pattern as depicted in Figure 1; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.2, 155.9, and 154.2 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 2; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and others in the chemical shift range of 90 to 180 ppm of about 60.5, 62.2, and 72.5 ± 0.1 ppm, and combination thereof.

In another embodiment, the present invention provides a process for the preparation of crystalline 5-azacytidine characterized by data selected from a group consisting of: a powder XRD pattern with peaks at about 8.7, 9.5, 12.1, 14.4, and 17.3 ± 0.2 degrees two-theta, a PXRD pattern as depicted in Figure 1; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.2, 155.9, and 154.2 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 2; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and others in the chemical shift range of 90 to 180 ppm of about 60.5, 62.2, and 72.5 ± 0.1 ppm, and combination thereof, comprising providing a solution of 5-azacytidine in N-methylpyrrolidone (referred to as NMP), and precipitating the said crystalline by cooling to a temperature of about 20°C to about 0°C to obtain a suspension comprising of the said crystalline form.

In one embodiment, the present invention provides solvated forms of 5-azacytidine selected from a group consisting of: 1,3-dimethyl-2-imidazolidinone solvate; and 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone solvate.

In another embodiment, the present invention provides crystalline 5-azacytidine characterized by data selected from the group consisting of: a powder XRD pattern with peaks at about 5.8, 11.6, 12.8, 16.2, and 17.4 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 3; a single crystal XRD with the following data: monoclinic crystal system; unit cell parameters: a, b, c : a = 5.14 Å, b = 7.78 Å, c = 15.40 Å, alpha = 90 °, beta = 99.60 °, and gamma = 90 °, respectively; an ORTEP view of a single crystal as

depicted in figure 5; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 167.3, 156.2, and 93.4 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 4; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 62.8 and 73.9 ± 0.1 ppm, and combination thereof containing less than about 20% by weight of crystalline 5-azacytidine having the most prominent 2 theta angles at 12.182, 13.024, 14.399, 16.470, 18.627, 19.049, 20.182, 21.329, 23.033, 23.872, 26.863, 27.1735, 29.277, 29.591, 30.369, and 32.072.

In yet another embodiment, the present invention provides crystalline 5-azacytidine characterized by data selected from a group consisting of: a powder XRD pattern with peaks at about 8.5, 9.4, 12.0, 14.4, 17.1 and 31.3 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 7; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.0, 153.9, and 93.4 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 8; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 60.5 and 72.6 ± 0.1 ppm, and combination thereof.

In one embodiment, the present invention provides crystalline 5-azacytidine characterized data selected from a group consisting of: a powder XRD pattern with peaks at about 9.4, 11.8, 12.1, 14.3 and 16.5 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 9; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.6, 154.3, and 93.9 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.6, 155.8, and 93.9 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 10; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 60.4 and 72.7 ± 0.1 ppm, a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 61.9 and 72.7 ± 0.1 ppm, and combination thereof.

In another embodiment, the present invention provides crystalline 5-azacytidine characterized by data selected from the group consisting of: a powder XRD pattern with peaks at about 11.0, 12.4, 13.7, 16.5, and 18.5 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 11, and combination thereof containing less than about 20% by weight of crystalline 5-azacytidine having the most prominent 2 theta angles at 12.182, 13.024, 14.399, 16.470, 18.627, 19.049, 20.182, 21.329, 23.033, 23.872, 26.863, 27.1735, 29.277, 29.591, 30.369, and 32.072.

In yet another embodiment, the present invention encompasses a pharmaceutical composition comprising any one of the above forms of 5-azacytidine, and at least one pharmaceutically acceptable excipient; wherein the starting 5-azacytidine includes also 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents.

In one embodiment, the present invention encompasses a pharmaceutical composition comprising any one of the above forms of 5-azacytidine prepared according to the processes of the present invention, and at least one pharmaceutically acceptable excipient; wherein the starting 5-azacytidine includes also 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents.

In another embodiment, the present invention encompasses a process for preparing a pharmaceutical formulation comprising combining any one of the above forms of 5-azacytidine with at least one pharmaceutically acceptable excipient; wherein the 5-azacytidine includes also 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents.

In yet another embodiment, the present invention encompasses a process for preparing a pharmaceutical composition comprising any one of the above forms of 5-azacytidine, prepared according to the processes of the present invention, and at least one pharmaceutically acceptable excipient; wherein the 5-azacytidine includes also 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents.

In one embodiment, the present invention encompasses the use of any one of the above forms of 5-azacytidine, for the manufacture of a pharmaceutical composition;

wherein the 5-azacytidine includes also 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates a powder X-ray diffraction pattern of crystalline 5-azacytidine Form IX.

Figure 2 illustrates a solid-state ^{13}C NMR spectrum of crystalline 5-azacytidine form IX.

Figure 3 illustrates a powder X-ray diffraction pattern for pure crystalline 5-azacytidine Form VII.

Figure 4 illustrates a solid-state ^{13}C NMR spectrum of pure crystalline 5-azacytidine Form VII.

Figure 5 illustrates microscopic views of pure crystalline 5-azacytidine form VII (Fig. 5a), and of pure crystalline form I (Fig. 5b).

Figure 6 illustrates the ORTEP view of a single crystal of pure crystalline 5-azacytidine Form VII..

Figure 7 illustrates a powder X-ray diffraction pattern of crystalline 5-azacytidine Form XI.

Figure 8 illustrates a solid-state ^{13}C NMR spectrum of the above crystalline 5-azacytidine Form XI.

Figure 9 illustrates a powder X-ray diffraction pattern of crystalline 5-azacytidine Form XII.

Figure 10 illustrates a solid-state ^{13}C NMR spectrum of crystalline 5-azacytidine Form XII..

Figure 11 illustrates a powder X-ray diffraction pattern of pure crystalline 5-azacytidine Form V.

Figure 12 illustrates a powder X-ray diffraction pattern for crude commercial 5-azacytidine containing about 80 % of form II and 20 % of form I.

Figure 13 illustrates a powder X-ray diffraction pattern for pure crystalline 5-azacytidine form I

DETAILED DESCRIPTION OF THE INVETION

The present invention provides different solid state forms of 5-azacytidine, as well as 5-azacytidine having low residual solvent level of non-volatile solvents, methods for preparation thereof, and pharmaceutical compositions comprising thereof.

The presence of residual non-volatile solvents in the forms of 5-azacytidine in the prior art, particularly in Form I of 5-azacytidine, is despite a final step of vacuum drying. This contamination with non-volatile solvent is a particular problem for 5-azacytidine because it is sparingly soluble in most solvents other than non-volatile solvents, requiring use of these non-volatile solvents for production. Further, many of the polymorphic forms present in the prior art occur as a multi-phase of multiple crystal forms, which is not suitable for pharmaceutical formulation.

As used herein, the term "solvate" refers to a crystalline substance that includes any solvent other than water at levels of more than 1 % by weight, as determined by GC or NMR.

As used herein, the term chemical shift difference refers to the difference in chemical shifts between a reference signal and another signal in the same solid-state ^{13}C NMR spectrum. In the present patent application the chemical shift differences were calculated by subtracting the chemical shift value of the signal exhibiting the lowest chemical shift (reference signal) in the solid-state ^{13}C NMR spectrum in the range of 90 to 180 ppm from chemical shift values of another (observed) signals in the same solid-state NMR spectrum in the range of 90 to 180 ppm. These chemical shift differences are to provide a measurement for a substance, for example 5-azacytidine, of the present invention compensating for a phenomenon in NMR spectroscopy wherein, depending on the instrumentation, temperature, and calibration method used, a shift in the solid-state NMR "fingerprint" is observed. This shift in the solid-state NMR "fingerprint", having signals at certain positions, is such that although the individual chemical shifts of signals

have altered, the difference between chemical shifts of each signal and another is retained.

The PXRD diffractogram of form I disclosed in Figure 1 of US 6,887,855 shows the presence of peaks of form III at approximately 6.6, 15.1 and 17.4 degrees two-theta. Thus, the disclosed form I is actually a mixture of form I and III. Form I contains about 5% by weight of form III. The content of form III can be measured by PXRD using the peak at 15.1 degrees two-theta.

The present invention provides crystalline 5-azacytidine characterized by data selected from a group consisting of: a PXRD pattern with peaks at about 12.2, 13.1, 14.4, 16.2, and 23.1 ± 0.2 degrees two-theta, a PXRD pattern as depicted in Figure 11, and combination thereof , designated pure form I, containing less than about 5% by weight of a crystalline form having most prominent diffractions at PXRD at two theta values at 6.566, 11.983, 13.089, 15.138, 17.446, 20.762, 21.049, 22.776, 24.363, 25.743, 26.305, 28.741, 31.393, 32.806, 33.043, 33.536, 36.371, 39.157, and 41.643 degrees two-theta , designated form III, and less than about 5% by weight of a crystalline 5-azacytidine with having most prominent PXRD diffractions at 13.4, 17.6, and 22.1 ± 0.2 degrees two-theta, designated form II. This form can be identified as "pure form I" or "pure crystalline form I".

Preferably, pure form I contains less than about 4%, 3%, 2%, or preferably less than about 1% by weight of form III, and less than about 4%, 3%, 2%, or preferably less than about 1% by weight of form II. The content of form III in pure form I can be measured by PXRD using any one of the peaks at 6.6, 15.1 and 17.4 ± 0.2 degrees two-theta. For example, Figure 1 of US Patent No. 6,887,855 shows peaks at 15.1 and 17.4 degrees two-theta. The content of form II in pure form I can be measured by PXRD using any one of the peaks at 13.4, 17.6, and 22.1 ± 0.2 degrees two-theta.

The pure form I of the present invention can be further characterized by a powder XRD pattern with peaks at about 19.1, 20.2, 27.1, 29.3, and 32.1 ± 0.2 degrees two-theta.

The above pure crystalline form I is also characterized by polymorphic homogeneity and crystal uniformity.

The present invention also provides 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents, more preferably about 10 to about 500 ppm. Preferably, the 5-azacytidine is pure crystalline form I of 5-azacytidine.

As used herein, the term "non-volatile solvents" refers to organic solvents having a boiling point of at least 140°C. Examples for such solvents include but not limited to DMSO, formamide, DMF, DMA, NMP, and others.

The present invention also relates to a method of preparing 5-azacytidine containing about 10 ppm to about 2000 ppm, more preferably about 10 to about 500 ppm of non-volatile solvents. The method comprises heating a suspension of 5-azacytidine in a single polar organic solvent selected from a group consisting of: aliphatic alcohol, nitrile, ether, nitromethane, pyridine, or in a mixture of solvents comprising the said polar organic solvent and a non-polar organic solvent selected from a group consisting of: ketone, a hydrocarbon, or ester, and recovering 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents; wherein the single polar solvent or its mixture with a non-polar solvent has boiling point of less than 140°C. Preferably, the obtained 5-azacytidine is pure crystalline form I of 5-azacytidine.

The suspension of 5-azacytidine is provided by combining 5-azacytidine and a single polar organic solvent or a mixture of solvents comprising the said polar organic solvent and a non-polar organic solvent.

Preferably, the aliphatic alcohol is a C_{2-6} aliphatic alcohol, more preferably, methanol, ethanol, 2-propanol, 1-propanol, 1-butanol, 2-butanol, i-butanol, amylalcohol, methoxyethanol, ethoxyethanol or mixtures thereof, most preferably, either 1-butanol or ethanol. Preferably, the nitrile is C_{2-4} nitrile, more preferably, acetonitrile. Preferably, the ether is a C_{3-8} ether, including penta or hexa - cyclic ether, more preferably, dimethoxyethane, tert-butylmethylether, dioxolane, tetrahydrofurane, methyl-tetrahydrofurane, or dioxane. Most preferably, the ether is 1,4-dioxane. Preferably, the single polar organic solvent is either ethanol or 1-butanol.

Preferably, the ketone is C_{3-6} ketone, more preferably, acetone, methylethylketone, or methylbutylketone, most preferably, either methylethyl ketone or methylisobutyl ketone. Preferably, the ester is C_{2-6} ester, more preferably, ethylacetate,

propyl acetate, isopropyl acetate, butylacetate, isobutylacetate, most preferably ethylacetate. Preferably the hydrocarbon is a C_{6-10} hydrocarbon, more preferably hexane, heptane, cyclohexane, methylcyclohexane, toluene, m-xylene, p-xylene, or chlorobenzene, most preferably toluene.

Preferably, the solvent used to prepare the suspension is a single polar organic solvent, more preferably, either ethanol or 1-butanol.

The suspension is, preferably, heated to a temperature of about 30°C to about 130°C, more preferably to a temperature of about 60°C to about 120°C. The heating is done, preferably, under stirring. Heating the suspension of 5-azacytidine may be done under inert atmosphere. Preferably, the inert atmosphere is obtained by using nitrogen.

Optionally the hot suspension may be seeded with 5-azacytidine crystalline form I to facilitate the rate of crystallization of 5-azacytidine crystalline form I.

The recovery of the obtained 5-azacytidine may be accomplished for example by cooling the heated suspension; filtering the cooled suspension; washing the filtered precipitate and drying. Preferably, the heated suspension is cooled to a temperature of about 30°C to about 20°C. Drying may be carried out at any suitable temperature, such as about 20°C to about 50°C.

The present invention provides crystalline 5-azacytidine characterized by data selected from a group consisting of: a powder XRD pattern with peaks at about 8.7, 9.5, 12.1, 14.4 and 17.3 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 1; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.2, 155.9, and 154.2 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 2; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 60.5, 62.2, and 72.5 ± 0.1 ppm, and combination thereof. The signal exhibiting the lowest chemical shift in the chemical shift range of 90 to 180 ppm is typically at about 93.7 ± 1 ppm. This form can be designated "Form IX" or crystalline form IX"..

The crystalline Form IX may be further characterized by a powder XRD pattern with peaks at about 19.4, 21.3, and 28.6 ± 0.2 degrees two-theta. In addition, the

crystalline may be further characterized by a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 86.9, and 73.5 ± 0.2 ppm.

Furthermore, the crystalline Form IX may be additionally characterized by a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 174.6 and 28.7 ± 0.2 ppm.

The above crystalline Form IX is a solvated form of 5-azacytidine, preferably an N-methylpyrrolidone solvate, more preferably, a mono- N-methylpyrrolidone solvate. Preferably, the ratio of NMP to 5-azacytidine, as determined by solution ^{1}H NMR analysis is 1:1 (molecular ratio)..

Furthermore, the crystalline Form IX can be characterized by any other method known to a skilled artisan, such as, for example, FTIR, and Raman Spectroscopy.

The above form crystallizes in well developed crystals and thus, can be easily recovered by filtration. The well defined crystals also contribute to a smaller surface area and thus, to lower absorption of impurities from the mother liquor when precipitated.

The above crystalline form IX has less than about 10%, preferably less than about 5%, more preferably less than about 1% by weight of crystalline 5-azacytidine form III or crystalline 5-azacytidine having the most prominent two theta angles at 6.599, 10.660, 12.600, 13.358, 15.849, 17.275, 20.243, 20.851, 21.770, 22.649, 25.554, 25.740, 29.293, 32.148, 35.074, and 38.306 degrees two-theta, denominated Form VIII. The content of form III can be measured by PXRD using any one of the peaks of form III at 6.6, 13.1, 22.8 and 31.4 degrees two-theta. The content of form VIII can be measured by PXRD using any one of the peaks at 6.6, 12.6, 21.8 and 22.7 ± 0.2 degrees two-theta.

The process for preparation of Form IX comprises providing a solution of 5-azacytidine in N-methylpyrrolidone (referred to as NMP), and precipitating the crystalline 5-azacytidine by cooling to a temperature of about 20°C to about 0°C to obtain a suspension comprising the crystalline form IX.

Preferably, the solution is provided by combining 5-azacytidine and NMP and heating the combination. Heating can be carried out to a temperature of about 50°C to

reflux temperature, more preferably to about 70°C to about 100°C, and most preferably to about 90°C.

The ratio of 5-azacytidine to NMP is preferably from about 1:8 to about 1:20, more preferably, from about 1:8 to about1:12 w/v, respectively.

Optionally, the solution of 5-azacytidine in NMP can include a second solvent. The second solvent can be selected from, but not limited to a C_3-C_8 ketone such as methylethyketone or a C_5-C_{12} aromatic or saturated hydrocarbon such as toluene.

Preferably, the precipitation is done by cooling the solution to a temperature of about 10 °C to about 0°C.

The process for preparing the above crystalline form IX of 5-azacytidine can further comprise a recovery process. Preferably, the recovery is done by filtering the suspension, washing the filtered crystalline and drying it. Drying may be carried out at any suitable temperature, such as about 20°C to about 50°C.

The present invention also provides solvated forms of 5-azacytidine selected from a group consisting of: 1,3-dimethyl-2-imidazolidinone solvate and 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone solvate.

The PXRD diffractogram of form VII disclosed in Figure 7 of US 7078518 shows that the mixture of form I and form VII has about 60 % by weight of form I. The content of form I can be measured by PXRD using the peak at about 20.2 ± 0.2 degrees two-theta.

The present invention provides crystalline 5-azacytidine characterized by data selected from the group consisting of: a powder XRD pattern with peaks at about 5.8, 11.6, 12.8, 16.2, and 17.4 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 3; a single crystal XRD with the following data: monoclinic crystal system; unit cell parameters: a, b, c : a = 5.14 Å, b = 7.78 Å, c = 15.40 Å, alpha = 90 °, beta = 99.60 °, and gamma = 90 °, respectively; an ORTEP view of a single crystal as depicted in figure 5; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 167.3, 156.2, and 93.4 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 4; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the

lowest chemical shift and others in the chemical shift range of 90 to 180 ppm of about 62.8 and 73.9 ± 0.1 ppm, and combinations thereof, containing less than about 20% by weight of crystalline 5-azacytidine having the most prominent 2 theta angles at 12.182, 13.024, 14.399, 16.470, 18.627, 19.049, 20.182, 21.329, 23.033, 23.872, 26.863, 27.1735, 29.277, 29.591, 30.369, and 32.072, designated "form I" or "crystalline form I". This form can be identified as "pure form VII" or "pure crystalline form VII". Preferably, pure crystalline 5-azacytidine form VII contains less than about 10%, more preferably, less than about 5%, most preferably, less than about 1% by weight of crystalline 5-azacytidine form I. Preferably, the content of form I in pure form VII is measured by PXRD using the peak at about 20.2 ± 0.2 degrees two-theta.

The signal exhibiting the lowest chemical shift in the chemical shift range of 90 to 180 ppm is typically at about 93.4 ± 1 ppm.

The pure crystalline Form VII may be further characterized by a powder XRD pattern with peaks at about 20.8, 22.4, 25.9, 26.6, 29.2, 31.4, and 38.4 ± 0.2 degrees two-theta. In addition, said crystalline form may be further characterized by a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 84.5 and 75.3 ± 0.2 ppm.

Furthermore, the pure crystalline Form VII may be further characterized by a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 50.4 and 62.0 ± 0.2 ppm.

The above pure crystalline Form VII is a solvated form of 5-azacytidine, preferably a methanol solvate, more preferably, a mono-methanol solvate. Preferably, the ratio of methanol to 5-azacytidine, as determined by solution ^{1}H NMR analysis, is of about 1:1 (molecular ratio).. The methanol solvate form may be also substantially identified by the spatial arrangement of 5-azacytidine and methanol molecules depicted in Figure 6.

The pure crystalline form VII can be characterized by any other method known to a skilled artisan, such as, for example, FTIR and Raman spectroscopy.

The pure crystalline form VII crystallizes in well-developed crystals having an arrow-shaped morphology, and thus can be easily recovered by filtration. Preferably, the arrow-shaped crystals have a length of more than 10 μm. The advantage of having such

crystal size is that the surface area is smaller and the crystalline material absorbs fewer impurities from the solution when it precipitates. Furthermore, the crystals of this crystalline 5-azacytidine are not as prone to electrostatic charging as the microcrystalline forms of 5-azacytidine that are present in prior-art, and thus can be easily manipulated without being scattered and lost, and without contamination of the working area. The environmental factor is especially important when working with substances such as 5-azacytidine.

The above pure crystalline form VII can be prepared by a process comprising crystallizing 5-azacytidine from a solvent mixture comprising methanol and a polar solvent selected from the group consisting of: a cyclic urea, a cyclic amide, and mixtures thereof.

A ratio of about1 to about 50, preferably, about 1 to about 30 of methanol to the polar solvent by volume can be used.

The crystallization can comprise providing a solution of 5-azacytidine in a solvent mixture comprising methanol and a polar solvent selected from the group consisting of: cyclic urea, cyclic amide and mixtures thereof, and precipitating the said crystalline to obtain a suspension.

The crystallization can be performed by combining 5-azacytidine and the polar solvent; heating the combination to obtain a solution; cooling the solution; and admixing with methanol to facilitate the crystallization. Preferably, the heating is to a temperature of about 30 °C to about 130 °C., more preferably, to a temperature 60 °C to about 90 °C.

Preferably, the cyclic amide is a C_{5-7} cyclic amide, and the cyclic urea is a C_{3-6} cyclic urea. More preferably C_{5-7} cyclic amides and the C_{3-6} cyclic urea are selected from a group consisting of: N-methylpyrrolidone, 1,3-dimethyl-2-imidazolidinone, and 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone.

Preferably, the solution is cooled to a temperature of about 50°C to about 0°C, more preferably to about 30°C to about 20°C, prior to the addition of methanol.

Preferably, methanol is added to the solution.

After the addition of methanol, the obtained mixture is cooled to a temperature of about 20°C to about -30°C, more preferably about 10 °C to about -10 °C.

The process for preparing the above crystalline 5-azacytidine can further comprise a recovery process. The recovery can be performed by filtering the suspension, washing the filtered crystalline and drying it. Drying may be carried out at any suitable temperature, such as about 20°C to about 50°C.

The present invention provides a crystalline 5-azacytidine characterized by data selected from a group consisting of: a powder XRD pattern with peaks at about 8.5, 9.4, 12.0, 14.4, 17.1 and 31.3 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 7; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.0, 153.9, and 93.4 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 8; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and others in the chemical shift range of 90 to 180 ppm of about 60.5 and 72.6 ± 0.1 ppm, and combinations thereof. The signal exhibiting the lowest chemical shift in the chemical shift range of 90 to 180 ppm is typically at about 93.4 ± 1 ppm. This form can be designated Form XI.

The crystalline Form XI may be further characterized by a powder XRD pattern with peaks at about 19.3, 21.2, 25.8, and 28.6 ± 0.2 degrees two-theta. Also, the said crystalline may be further characterized by a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 86.6 and 73.9 ± 0.2 ppm.

Furthermore, the crystalline Form XI may be further characterized by a solid-state ^{13}C NMR having signals with chemical shifts at about 161.0, 43.3, and 30.5 ± 0.2 ppm.

The above crystalline Form XI is a solvated form of 5-azacytidine, preferably a 1,3-dimethyl-2-imidazolidinone solvate, more preferably, a mono-1,3-dimethyl-2-imidazolidinone solvate. Preferably, the ratio of 1,3-dimethyl-2-imidazolidinone to 5-azacytidine, as determined by solution ^{1}H NMR analysis, is about 1:1 (molecular ratio).

The crystalline form can be characterized by any other method known to a skilled artisan, such as, for example, FTIR, and Raman spectroscopy.

The above crystalline Form XI has less than about 10%, preferably less than 5 %, more preferably less than 1% by weight of 5-azacytidine forms I or III. The content of form I can be measured by PXRD using any one of the peaks at 12.2, 20.2 and 23.9 degrees two-theta. The content of form III can be measured by PXRD using any one of the peaks at 6.6, 17.4 and 22.7 ± 0.2 degrees two-theta.

The above crystalline form XI of 5-azacytidine is prepared by a process comprising crystallizing 5-azacytidine from 1,3-dimethyl-2-imidazolidinone.

The crystallization is done by a process comprising providing a solution of 5-azacytidine in 1,3-dimethyl-2-imidazolidinone, and precipitating the said crystalline to obtain a suspension.

Preferably, the solution is provided by combining 5-azacytidine and 1,3-dimethyl-2-imidazolidinone and heating the combination. Preferably, the heating is to a temperature of about 50 °C to about 130 °C, more preferably to about 60°C to about 100°C, most preferably, to about 90 °C.

Optionally, the solution of 5-azacytidine in 1,3-dimethyl-2-imidazolidinone can include a second solvent. The second solvent can be selected from, but not limited to a C_3-C_8 ketone such as methylethyketone, a C_5-C_{12} aromatic or saturated hydrocarbon such as toluene, C_2-C_8 carboxylic acid ester such as ethylacetate, or C_4-C_{10} ether such as t-butyl methyl ether

Preferably, the precipitation is done by cooling the solution to a temperature of about 20 °C to about 0 °C, more preferably to about 0°C to about 10 °C.

The process for preparing the above crystalline form XI of 5-azacytidine can further comprise a recovery process. The recovery may be performed by filtering the suspension, washing the filtered crystalline and drying it. Drying may be carried out at any suitable temperature, such as about 20°C to about 50°C.

The present invention provides crystalline 5-azacytidine characterized data selected from a group consisting of: a powder XRD pattern with peaks at about 9.4, 11.8, 12.1, 14.3 and 16.5 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 9; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.6, 154.3, and 93.9 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum having signals with chemical shifts

at about 166.6, 155.8, and 93.9 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 10; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 60.4 and 72.7 ± 0.1 ppm, a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and others in the chemical shift range of 90 to 180 ppm of about 61.9 and 72.7 ± 0.1 ppm, and combinations thereof. The signal exhibiting the lowest chemical shift in the chemical shift range of 90 to 180 ppm is typically at about 93.9 ± 1 ppm. This form can be designated form XII.

The crystalline Form XII may be further characterized by a powder XRD pattern with peaks at about 18.9, 20.5, 21.1, 26.0, and 28.6 ± 0.2 degrees two-theta. In addition, the said crystalline may be further characterized by a solid-state ^{13}C NMR spectrum having chemical shifts at about 87.2 and 74.0 ± 0.2 ppm.

Furthermore, the crystalline form XII may be further characterized by a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 22.6, 35.7, and 47.6 ± 0.2 ppm.

The above crystalline form XII is a solvated form of 5-azacytidine, preferably a 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone solvate, more preferably, a mono-1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone solvate. Preferably, the molecular ratio of 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone to 5-azacytidine, as determined by solution ^{1}H NMR analysis, is about1:1.

The crystalline form XII can be characterized by any other method known to a skilled artisan, such as, for example, FTIR, and Raman spectroscopy.

The above crystalline Form XII has less than about 10%, preferably less than 5 %, and more preferably less than 1% by weight of 5-azacytidine form III. The content of form III can be measured by PXRD using any of the peaks at 6.6, 15.1 and 22.3 ± 0.2 degrees two-theta.

The above crystalline form XII of 5-azacytidine is prepared by a process comprising crystallizing 5-azacytidine from 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone.

The crystallization is done by a process comprising providing a solution of 5-azacytidine in 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone, and precipitating the said crystalline to obtain a suspension.

Preferably, the solution is provided by combining 5-azacytidine and 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone and heating the combination. Preferably, the heating is to a temperature of about 50 °C to about 130 °C, more preferably to about 60°C to about 100°C, most preferably, to about 90 °C.

Optionally, the solution of 5-azacytidine in 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone can include a second solvent. The second solvent can be selected from, but not limited to a C_3-C_8 ketone such as methylethylketone ("MEK", 2-butanone), a C_5-C_{12} aromatic or saturated hydrocarbon such as toluene, a C_2-C_8 carboxylic acid ester such as ethylacetate, or a C_4-C_{10} ether such as t-butyl methyl ether.

Preferably, the precipitation is done by cooling the solution to a temperature of about 20°C to about -30°C, more preferably to about 0°C to about 10°C.

The process for preparing the above crystalline form XII of 5-azacytidine can further comprise a recovery process. The recovery may be performed by filtering the suspension, washing the filtered crystalline and drying it. Drying may be carried out at any suitable temperature, such as about 20°C to about 50°C.

The PXRD diffractogram of form V disclosed in Figure 5 of US Patent 7078518 shows the presence of peaks of form I at approximately 13.176, 14.548, 19.202 and 20.329 degrees two-theta. Thus, the disclosed form V is a mixture of form V and about 30% by weight of form I. The content of form I can be measured by PXRD using any one of the peaks at 13.2, 14.5, 20.2, 23.0 and 23.8 ± 0.2 degrees two-theta.

The present invention provides crystalline 5-azacytidine characterized by data selected from the group consisting of: a powder XRD pattern with peaks at about 11.0, 12.4, 13.7, 16.5, and 18.5 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure

11, and a combination thereof, containing less than about 20% by weight of crystalline 5-azacytidine form I. This form can be identified as "pure form V" or "pure crystalline form V". Preferably, pure crystalline 5-azacytidine form V contains less than about 10%, more preferably less than about 5%, and most preferably less than about 1% by weight of crystalline 5-azacytidine form I. Preferably, the content of form I in the pure form V is measured by PXRD using any one of the peaks at about 13.2, 14.5, 20.2, 23.0 and 23.8 ± 0.2 degrees two-theta.

The pure crystalline form V may be further characterized by a powder XRD pattern with peaks at about 15.5, 24.5, 27.1, 28.4, and 29.0 ± 0.2 degrees two-theta.

The pure crystalline form V can be characterized by any other method known to a skilled artisan, such as, for example, FTIR, and Raman spectroscopy.

The above pure crystalline form V of 5-azacytidine is prepared by a process comprising lyophilizing a solution of 5-azacytidine in dimethylsulfoxide. Lyophilization is also known as freeze-drying.

In the lyophilization process, a solution of 5-azacytidine in dimethylsulfoxide is frozen, and then the frozen mass is subjected to a pressure of less than one atmosphere, to remove the solvent.

Preferably, the solution is provided by a process comprising combining 5-azacytidine and dimethylsulfoxide and heating the combination. Preferably, the heating is to a temperature of about 50 °C to about 130 °C, more preferably at about 70°C to about 80 °C.

Preferably, freezing the solution is done gradually. First, cooling to a temperature of about 30 °C is done, and then cooling to a temperature of about 18°C to about -30°C, is performed, providing a frozen solution. Typically, the evaporation of the solvents is done at about 18°C to about -30°C. Preferably, evaporation of the solvent is done under reduced pressure (less than one atmosphere). Preferably, the reduced pressure is used in the range of about 0.01 to 100 mBar, more preferably at about 0.1 to about 3 mBar.

5-azacytidine used as a starting material in the above processes of the present invention (including processes that proceed through a solution or slurry) may be prepared

according to known to a skilled artisan, including those disclosed in U.S. Patent Nos. 6,887,885, 6,943,249, 7,078,518, which are incorporated herein by reference. Preferably, the starting 5-azacytidine in the process for preparing 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents; wherein the obtained 5-azacytidine is also pure crystalline 5-azacytidine form I, can be selected from a group consisting of: crude 5-azacytidine, crystalline 5-azacytidine having the most prominent 2 theta angles at 5.704, 11.571, 12.563, 14.070, 15.943, 16.993, 18.066, 20.377, 20.729, 21.484, 21.803, 22.452, 22.709, 23.646, 24.068, 25.346, 25.346, 26.900, 27.991, 28.527, 28.723, 30.124, 30.673, 31.059, 35.059, 38.195 and 38.403, designated form IV, crystalline 5-azacytidine form IX, crystalline 5-azacytidine form VII, and crystalline 5-azacytidine form III. Crude 5-azacytidine can be 5-azacytidine anhydrate, hydrate, solvate or mixtures thereof. Preferably, crude 5-azacytidine is a mixture of forms I and II.

The crystalline forms of the present invention can be used to prepare formulations for treating myelodysplactic syndromes.

The present invention encompasses a pharmaceutical composition comprising any one of the above forms of 5-azacytidine, and at least one pharmaceutically acceptable excipient; wherein the 5-azacytidine includes also 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents.

The present invention also encompasses a pharmaceutical composition comprising anyone of the above forms of 5-azacytidine prepared according to the processes of the present invention, and at least one pharmaceutically acceptable excipient; wherein the 5-azacytidine includes also 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents.

The present invention further encompasses a process for preparing a pharmaceutical formulation comprising combining anyone of the above forms of 5-azacytidine with at least one pharmaceutically acceptable excipient; wherein the 5-azacytidine includes also 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents.

The present invention encompasses a process for preparing a pharmaceutical composition comprising anyone of the above forms of 5-azacytidine, prepared according to the processes of the present invention, and at least one pharmaceutically acceptable excipient; wherein the 5-azacytidine includes also 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents.

The present invention further encompasses the use of any one of the above forms of 5-azacytidine, for the manufacture of a pharmaceutical composition; wherein the starting 5-azacytidine includes also 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents.

The present invention further encompasses the use of any one of the 5-azacytidine crystals forms provided by the invention, for the manufacture of a pharmaceutical composition.

Methods of administration of a pharmaceutical composition of the present invention may comprise administration in various preparations depending on the age, sex, and symptoms of the patient. The pharmaceutical compositions can be administered, for example, as tablets, pills, powders, suspensions, emulsions, granules, capsules, suppositories, injection preparations, and the like. When the pharmaceutical composition is a liquid pharmaceutical composition, it will be in the form of a suspension or emulsion wherein the 5-deazacytidine retains its crystalline form.

Pharmaceutical compositions of the present invention can optionally be mixed with other forms of 5-deazacytidine and/or other active ingredients. In addition, pharmaceutical compositions of the present invention can contain inactive ingredients such as diluents, carriers, fillers, bulking agents, binders, disintegrants, disintegration inhibitors, absorption accelerators, wetting agents, lubricants, glidants, surface active agents, flavoring agents, and the like.

Diluents increase the bulk of a solid pharmaceutical composition and can make a pharmaceutical dosage form containing the composition easier for the patient and care giver to handle. Diluents for solid compositions include, for example, microcrystalline cellulose (e.g., Avicel®), microfine cellulose, lactose, starch, pregelitinized starch, calcium carbonate, calcium sulfate, sugar, dextrates, dextrin, dextrose, dibasic calcium

phosphate dihydrate, tribasic calcium phosphate, kaolin, magnesium carbonate, magnesium oxide, maltodextrin, mannitol, polymethacrylates (e.g., Eudragit®), potassium chloride, powdered cellulose, sodium chloride, sorbitol, or talc.

Carriers for use in the pharmaceutical compositions may include, but are not limited to, lactose, white sugar, sodium chloride, glucose, urea, starch, calcium carbonate, kaolin, crystalline cellulose, or silicic acid.

Binders help bind the active ingredient and other excipients together after compression. Binders for solid pharmaceutical compositions include for example acacia, alginic acid, carbomer (e.g. carbopol), carboxymethylcellulose sodium, dextrin, ethyl cellulose, gelatin, guar gum, hydrogenated vegetable oil, hydroxyethyl cellulose, hydroxypropyl cellulose (e.g. Klucel®), hydroxypropyl methyl cellulose (e.g. Methocel®), liquid glucose, magnesium aluminum silicate, maltodextrin, methylcellulose, polymethacrylates, povidone (e.g. Kollidon®, Plasdone®), pregelatinized starch, sodium alginate, or starch.

Disintegrants can increase dissolution. Disintegrants include, for example, alginic acid, carboxymethylcellulose calcium, carboxymethylcellulose sodium (e.g. Ac-Di-Sol®, Primellose®), colloidal silicon dioxide, croscarmellose sodium, crospovidone (e.g. Kollidon®, Polyplasdone®), guar gum, magnesium aluminum silicate, methyl cellulose, microcrystalline cellulose, polacrilin potassium, powdered cellulose, pregelatinized starch, sodium alginate, sodium starch glycolate (e.g. Explotab®) and starch.

Disintegration inhibitors may include, but are not limited to, white sugar, stearin, coconut butter, hydrogenated oils, and the like.

Absorption accelerators may include, but are not limited to, quaternary ammonium base, sodium laurylsulfate, and the like.

Wetting agents may include, but are not limited to, glycerin, starch, and the like. Adsorbing agents may include, but are not limited to, starch, lactose, kaolin, bentonite, colloidal silicic acid, and the like.

A lubricant can be added to the composition to reduce adhesion and ease release of the product from a punch or dye during tableting. Lubricants include for example magnesium stearate, calcium stearate, glyceryl monostearate, glyceryl palmitostearate, hydrogenated castor oil, hydrogenated vegetable oil, mineral oil, polyethylene glycol, sodium benzoate, sodium lauryl sulfate, sodium stearyl fumarate, stearic acid, talc and zinc stearate.

Glidants can be added to improve the flowability of non-compacted solid composition and improve the accuracy of dosing. Excipients that can function as glidants include for example colloidal silicon dioxide, magnesium trisilicate, powdered cellulose, starch, talc and tribasic calcium phosphate.

Flavoring agents and flavor enhancers make the dosage form more palatable to the patient. Common flavoring agents and flavor enhancers for pharmaceutical products that can be included in the composition of the present invention include for example maltol, vanillin, ethyl vanillin, menthol, citric acid, fumaric acid, ethyl maltol, and tartaric acid.

Tablets can be further coated with commonly known coating materials such as sugar coated tablets, gelatin film coated tablets, tablets coated with enteric coatings, tablets coated with films, double layered tablets, and multi-layered tablets. Capsules can be coated with shell made, for example, from gelatin and optionally contain a plasticizer such as glycerin and sorbitol, and an opacifying agent or colorant.

Solid and liquid compositions can also be dyed using any pharmaceutically acceptable colorant to improve their appearance and/or facilitate patient identification of the product and unit dosage level.

In liquid pharmaceutical compositions of the present invention, the 5-deazacytidine is suspended or otherwise dispersed in a liquid carrier, retaining its crystalline form. A dispersant, such as for example sodium lauryl sulfate, may optionally be employed to stabilize the preparation; suitable dispersants are known to those skilled in the art. Suitable liquid carriers include, but are not limited to, water, vegetable oils, alcohol, polyethylene glycol, propylene glycol and glycerin, and combinations thereof.

Other solid ingredients, which may optionally be present, can be dissolved or suspended in the carrier.

Liquid pharmaceutical compositions can contain emulsifying agents to disperse uniformly throughout the composition an active ingredient or other excipient that is not soluble in the liquid carrier. Emulsifying agents that can be useful in liquid compositions of the present invention include, for example, gelatin, egg yolk, casein, cholesterol, acacia, tragacanth, chondrus, pectin, methyl cellulose, carbomer, cetostearyl alcohol and cetyl alcohol.

Liquid pharmaceutical compositions of the present invention can also contain viscosity enhancing agents to improve the mouth-feel of the product and/or coat the lining of the gastrointestinal tract. Such agents include for example acacia, alginic acid bentonite, carbomer, carboxymethylcellulose calcium or sodium, cetostearyl alcohol, methyl cellulose, ethylcellulose, gelatin guar gum, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, maltodextrin, polyvinyl alcohol, povidone, propylene carbonate, propylene glycol alginate, sodium alginate, sodium starch glycolate, starch tragacanth and xanthan gum.

Sweetening agents such as sorbitol, saccharin, sodium saccharin, sucrose, aspartame, fructose, mannitol and invert sugar can be added to improve the taste.

Preservatives and chelating agents such as alcohol, sodium benzoate, butylated hydroxy toluene, butylated hydroxyanisole and ethylenediamine tetraacetic acid can be added at safe levels to improve storage stability.

A liquid pharmaceutical composition according to the present invention can also contain a buffer, such as for example gluconic acid, lactic acid, citric acid, acetic acid, phosphoric acid, and pharmaceutically acceptable salts thereof.

Selection of excipients and the amounts to use can be readily determined by an experienced formulation scientist in view of standard procedures and reference works known in the art.

A composition for tableting or capsule filing can be prepared by wet granulation. In wet granulation some or all of the active ingredients and excipients in powder form are

blended and then further mixed in the presence of a liquid, typically water, which causes the powders to clump up into granules. The granulate is screened and/or milled, dried and then screened and/or milled to the desired particle size. The granulate can then be tableted or other excipients can be added prior to tableting, such as a glidant and/or a lubricant.

A tableting composition can also be prepared conventionally by dry blending. For instance, the blended composition of the actives and excipients can be compacted into a slug or a sheet and then comminuted into compacted granules. The compacted granules can be compressed subsequently into a tablet.

As an alternative to dry granulation, a blended composition can be compressed directly into a compacted dosage form using direct compression techniques. Direct compression produces a more uniform tablet without granules. Excipients that are particularly well-suited to direct compression tableting include microcrystalline cellulose, spray dried lactose, dicalcium phosphate dihydrate and colloidal silica. The proper use of these and other excipients in direct compression tableting is known to those in the art with experience and skill in particular formulation challenges of direct compression tableting.

A capsule filling of the present invention can comprise any of the aforementioned blends and granulates that were described with reference to tableting, only they are not subjected to a final tableting step.

When shaping the pharmaceutical composition into pill form, any commonly known excipient used in the art can be used. For example, carriers include, but are not limited to, lactose, starch, coconut butter, hardened vegetable oils, kaolin, talc, and the like. Binders used include, but are not limited to, gum arabic powder, tragacanth gum powder, gelatin, ethanol, and the like. Disintegrating agents used include, but are not limited to, agar, laminalia, and the like.

For the purpose of shaping the pharmaceutical composition in the form of suppositories, any commonly known excipient used in the art can be used. For example, excipients include, but are not limited to, polyethylene glycols, coconut butter, higher alcohols, esters of higher alcohols, gelatin, semisynthesized glycerides, and the like.

When preparing injectable pharmaceutical compositions, solutions and suspensions are sterilized and are preferably made isotonic to blood. Injection preparations may use carriers commonly known in the art. For example, carriers for injectable preparations include, but are not limited to, water, ethyl alcohol, propylene glycol, ethoxylated isostearyl alcohol, polyoxylated isostearyl alcohol, and fatty acid esters of polyoxyethylene sorbitan. One of ordinary skill in the art can easily determine with little or no experimentation the amount of sodium chloride, glucose, or glycerin necessary to make the injectable preparation isotonic. Additional ingredients, such as dissolving agents, buffer agents, and analgesic agents may be added.

The amount of a 5-azacytidine crystal form of the present invention contained in a pharmaceutical composition is not specifically restricted, and an effective dose may be divided among two or more individual dose units (e.g., tablets or capsules). In general, the total dose should be sufficient to treat, ameliorate, or reduce the myelodysplastic syndrome for which treatment is intended.

Having described the invention with reference to certain preferred embodiments, other embodiments will become apparent to one skilled in the art from consideration of the specification. The invention is further defined by reference to the following examples describing in detail the process and compositions of the invention. It will be apparent to those skilled in the art that many modifications, both to materials and methods, may be practiced without departing from the scope of the invention.

EXAMPLES

Instruments

PXRD

XRD diffraction was performed on X-Ray powder diffractometer: Philips X'pert Pro powder diffractometer, CuK_α radiation, $\lambda = 1.5418$ Å. X'Celerator detector active length (2 theta) = 2.122°, laboratory temperature 22-25°C.

Single crystal XRD method

Data were collected on Xcalibur PX, Cu Kα using combined φ and ω scans. All non-hydrogen atoms were refined anisotropically, hydrogen atoms were refined riding in expected geometric positions, OH hydrogen atoms were located from fourier maps. Data collection: *CrysAlis RED* (Oxford Diffraction, 2002); cell refinement: *CrysAlis RED*; data reduction: *CrysAlis RED*; program used to solve structure: SIR92 (Altomare et al., 1994); program used to refine structure: *CRYSTALS* (Betteridge et al., 2003)

DSC

DSC measurements were performed on Differential Scanning Calorimeter DSC823e (Mettler Toledo). Al crucibles 40 µl with PIN were used for sample preparation. Usual weight of sample was 1 – 2.5 mg.

Program: temperature range 50°C - 300°C, 10°C/min.

NMR spectroscopy in solution

NMR spectra of solutions in deuterated dimethylsulfoxide were measure on Varian INOVA-400 spectrometer using 399.87 MHz for ^{1}H and 100.55 MHz for ^{13}C at 30 °C.

Solid-state ^{13}C NMR

The CP/MAS ^{13}C NMR measurements were made at Bruker Avance 500 NMR US/WB spectrometer in 4-mm ZrO_2 rotor. Magic angle spinning (MAS) speed was 10 kHz. As used herein, the term "^{13}C NMR chemical shifts" refers to the shifts measured under above specified conditions, however, these shifts can slightly differ instrument to instrument and can be shifted either upfield or downfield due to the different instrumental setup and calibration used. Nevertheless the sequence of individual peaks remains identical.

GC

Residual solvents were determined by gas chromatography using head-space sampling. Headspace instrument HP7694 together with Gas chromatograph A6890 equipped with FID detector (Agilent technologies).

Example 1: Preparation of crystalline Form IX of 5-azacytidine

5-Azacytidine (500 mg) was dissolved in N-methylpyrrolidone (5 ml) at 90 °C. Then, the solution was allowed to crystallize at 15 °C without stirring for overnight.. The white solid was filtered, washed subsequently with toluene (20 ml) and n-hexane (20 ml) and dried in a stream of nitrogen for 1 h to obtain the crystalline form. (594 mg).

Example 2: Preparation of pure crystalline Form VII of 5-azacytidine

5-azacytidine (1 g, 97.3 % by HPLC) was dissolved in N-methylpyrrolidone (5 ml) at 115 °C. Solution was allowed to cool to 40 °C and methanol was added with stirring (90 ml). The solution was allowed to crystallise overnight at 20 °C in a form of large, arrow-shaped crystals having length above 10 μm. The crystalline form was recovered by filtration, washed with diethyl ether (20 ml), n-hexane (10 ml), and dried under a stream of nitrogen at 25 °C (807 mg, 98.5 % by HPLC).

Example 3: Preparation of pure crystalline Form VII of 5-azacytidine

5-azacytidine (1 g, 97.3 % by HPLC) was dissolved in 1,3-dimethyl-2-imidazolidinone (9 ml) at 90 °C. Solution was allowed to cool to 40 °C and methanol was added with stirring (90 ml). The solution was allowed to crystallize overnight at 20 °C in a form large, arrow-shaped crystals having length above 10 μm. the crystalline form was recovered by filtration, washed with diethyl ether (20 ml), n-hexane (10 ml), and dried in a stream of nitrogen at 25 °C (930 mg, 98.1 % by HPLC).

Example 4: Preparation of pure crystalline Form VII of 5-azacytidine

5-azacytidine (1 g, 97.3 % by HPLC) was dissolved in 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone (5 ml) at 115 °C. Solution was allowed to cool to 40 °C and methanol was added with stirring (90 ml). The solution was allowed to crystallize overnight at 20 °C in a form large, arrow-shaped crystals having length above 10 μm. The crystalline form was recovered by filtration, washed with diethyl ether (20 ml), n-hexane (10 ml), and dried in a stream of nitrogen at 25 °C (960 mg, 99.0 % by HPLC).

Example 5: Preparation of crystalline Form XI of 5-azacytidine

5-Azacytidine (900 mg) was dissolved in 1,3-dimethyl-2-imidazolidinone (9 ml) at 90 °C. Then the solution was allowed to crystallize at 15°C, without stirring for overnight The

white solid was filtered, washed subsequently with diethyl ether (50 ml) and n-hexane (50 ml) and dried in a stream of nitrogen for 1 h to obtain the crystalline form. (1146 mg).

Example 6: Preparation of crystalline Form XII of 5-azacytidine

5-Azacytidine (900 mg) was dissolved in 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone (5 ml) at 90 °C and 2-butanone (10 ml) was added to the solution. Then the solution was allowed to crystallize without stirring at –30 °C overnight. The white solid was filtered, washed subsequently with diethyl ether (50 ml) and n-hexane (50 ml) and dried in a stream of nitrogen for 1 h to obtain the crystalline form. (990 mg).

Example 7: Preparation of pure crystalline Form V of 5-azacytidine

5-Azacytidine (350 mg) was dissolved in dimethylsulfoxide (7 ml) at 80 °C. Then the solution was allowed to cool to 30 °C and put to the refrigerator set at –30 °C. The frozen liquid was put the lyophiliser and dimethylsulfoxide was evaporated within 24 h at 1.5 mBar and 15 °C.

Example 8: Preparation of pure crystalline form I of 5-azacytidine from a mixture of crystalline form I and crystalline 5-azacytidine Form II in n-butanol

Crude 5-azacytidine (1 g) containing about 80 % of form II and 20 % of form I, was suspended in n-butanol (50 ml). The suspension was heated to 117 °C for 15 min. the suspension was allowed to cool to 25 °C and 5-azacytidine form I was recovered by filtration, washed with acetone (10 ml), petrolether (10 ml), and dried in a stream of nitrogen at 25 °C (0.83 g, yield: 83 %).

Example 9: Preparation of pure crystalline form I of 5-azacytidine from a mixture of crystalline form I and crystalline 5-azacytidine Form II in ethanol

Crude 5-azacytidine (1 g) containing about 80 % of form II and 20 % of form I was suspended in ethanol (50 ml). The suspension was heated to 78 °C for 15 min. The suspension was allowed to cool to 25 °C and 5-azacytidine form I was recovered by filtration, washed with acetone (10 ml), petrolether (10 ml), and dried in a stream of nitrogen at 25 °C (0.82 g, yield: 82 %).

Example 10: Preparation of pure crystalline form I of 5-azacytidine from a mixture of crystalline form I and crystalline 5-azacytidine form II in 1,4-dioxane

Crude 5-azacytidine (1 g) containing about 80 % of form II and 20 % of form I was suspended in 1,4-dioxane (50 ml). The suspension was heated to 101 °C for 15 min. The suspension was allowed to cool to 25 °C and 5-azacytidine form I was recovered by filtration, washed with acetone (10 ml), petrolether (10 ml), and dried in a stream of nitrogen at 25 °C (0.83 g, yield: 83 %).

Example 11: Preparation of pure crystalline form I of 5-azacytidine from crystalline form IX of 5-azacytidine

Crystalline form of IX of 5-azacytidine (500 mg) was suspended in n-butanol (20 ml). The suspension was heated to 117 °C for 15 min. The suspension was allowed to cool to 25 °C and 5-azacytidine form I was recovered by filtration, washed with diethyl ether (20 ml), and n-hexane (20 ml), and dried in a stream of nitrogen at 25 °C (277 mg, yield: 78 %).

Example 12: Preparation of pure crystalline form I of 5-azacytidine from pure crystalline form VII of 5-azacytidine

Crystalline form of VII 5-azacytidine (400 mg) was suspended in n-butanol (15 ml). The suspension was heated to 117 °C for 15 min. The suspension was allowed to cool to 25 °C and 5-azacytidine form I was recovered by filtration, washed with diethyl ether (20 ml), and n-hexane (20 ml), and dried in a stream of nitrogen at 25 °C (223 mg, yield: 82 %).

Example 13: Preparation of pure crystalline form I of 5-azacytidine from crystalline 5-azacytidine form III in ethanol

5-Azacytidine (1 g) form III was suspended in ethanol (20 ml). The suspension was heated to 78 °C for 15 min. The suspension was allowed to cool to 25 °C and azacytidine form I was recovered by filtration, washed with acetone (15 ml), and n-hexane (20 ml), and dried in a stream of nitrogen at 25 °C (856 mg, yield: 92 %).

Comparative Example 14: crystallization of 5-azacytidine according to US '855 example 1

5-Azacytidine (1.14 g) was dissolved in dimethylsulfoxide (25 ml) by heating to 90 °C for 10 min. The solution was cooled to about 40 °C and methanol (250 ml) was added with stirring. The solution was placed into refrigenerator set at –20 °C for 24 hours. The crystalline material was recovered by filtration, washed with methanol (50 ml) and dried at 35 °C, 1 mBar for 2 h (1.07 g). The measurement of powder diffraction pattern revealed that the solid is a mixture of 5-azacytidine form I and form VII containing about 40 % of form I. The mixture was further characterized by the content of residual solvents as measured by GC: methanol 62 370 ppm and DMSO 5570 ppm.

Example 15: Preparation of pure crystalline form I having low residual solvents

5-Azacytidine (350 mg) prepared according to Example 14 was suspended in n-butanol (10 ml). The suspension was heated to 117 °C for 15 min. The suspension was allowed to cool to 25 °C and 5-azacytidine was recovered by filtration, washed with n-butanol (10 ml) and dried at 35 °C, 1 mBar for 2 h (305 mg). The measurement of powder diffraction pattern revealed that the solid is pure 5-azacytidine form I. 5-Azacytidine form I was further characterized by the content of residual solvents as measured by GC: methanol 244 ppm, DMSO 262 ppm, and n-butanol 1161 ppm.

Example 16: Preparation of pure crystalline form I having low residual solvents

5-Azacytidine (350 mg) prepared according to Example 14 was suspended in iso-amyl alcohol (10 ml). The suspension was heated to 131 °C for 15 min. The suspension was allowed to cool to 25 °C and 5-azacytidine was recovered by filtration, washed with iso-amyl alcohol (10 ml) and dried at 35 °C, 1 mBar for 2 h (310 mg). The measurement of powder diffraction pattern revealed that the solid is pure 5-azacytidine form I. 5-Azacytidine form I was further characterized by the content of residual solvents as measured by GC: methanol 213 ppm, DMSO 197 ppm, and iso-amyl alcohol 1519 ppm.

Example 17: Preparation of crystalline form IX of 5-azacytidine

5-Azacytidine (1.25 g) was dissolved in N-methylpyrrolidone (12.5 ml) at 120 °C, cooled to 25 °C and 2-butanone (90 ml) was added with stirring and the mixture was

allowed to stand 3h at 20 °C. 5-Azacytidine form IX was recovered by filtration, washed with 2-butanone (10 ml), petroleum ether (10 ml), and dried in air (1.48 g).

Example 18: Preparation of crystalline Form XII of 5-azacytidine

5-Azacytidine (900 mg) was dissolved in 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone (6 ml) at 90 °C, cooled to 25 °C and t-butyl methyl ether (10 ml) was added to the solution. Then the solution was allowed to crystallize without stirring at –30 °C overnight. The white solid was filtered, washed subsequently with diethyl ether (50 ml) and n-hexane (50 ml) and dried in a stream of nitrogen for 1 h to obtain the 5-azacytidine form XII. (990 mg).

Example 19: Preparation of pure crystalline form I of 5-azacytidine from crystalline form IV in ethanol

5-Azacytidine form IV (1 g), was suspended in ethanol (25 ml). The suspension was heated to 78 °C for 15 min. the suspension was allowed to cool to 25 °C and 5-azacytidine form I was recovered by filtration, washed with acetone (10 ml), hexane (10 ml), and dried in a stream of nitrogen at 25 °C (0.76 g, yield: 76 %).

Example 20: Preparation of pure crystalline form I of 5-azacytidine from a mixture of crystalline form I and crystalline 5-azacytidine Form II in pyridine

5-Azacytidine (1 g) containing about 80 % of form II and 20 % of form I, was suspended in pyridine (50 ml). The suspension was heated to 115 °C for 15 min. the suspension was allowed to cool to 25 °C and 5-azacytidine form I was recovered by filtration, washed with acetone (10 ml), petrolether (10 ml), and dried in a stream of nitrogen at 25 °C (0.59 g, yield: 59 %).

CLAIMS

What is claimed is:

1. Crystalline 5-azacytidine characterized by data selected from a group consisting of: a PXRD pattern with peaks at about 12.2, 13.1, 14.4, 16.2, and 23.1 ± 0.2 degrees two-theta, a PXRD pattern as depicted in Figure 13, and combination thereof containing less than about 5% by weight of crystalline 5-azacytidine having most prominent diffractions at PXRD at two theta values at 6.566, 11.983, 13.089, 15.138, 17.446, 20.762, 21.049, 22.776, 24.363, 25.743, 26.305, 28.741, 31.393, 32.806, 33.043, 33.536, 36.371, 39.157, and 41.643 degrees two-theta , and less than about 5% by weight of a crystalline 5-azacytidine with having most prominent PXRD diffractions at 13.4, 17.6, and 22.1 degrees two-theta.
2. The crystalline 5-azacytidine of claim 1, characterized by a PXRD pattern with peaks at about 12.2, 13.1, 14.4, 16.2, and 23.1 ± 0.2 degrees two-theta.
3. The crystalline 5-azacytidine of claim 1 characterized by a PXRD pattern as depicted in Figure 13.
4. The crystalline 5-azacytidine of claim 2 further characterized by a powder XRD pattern with peaks at about 19.1, 20.2, 27.1, 29.3, and 32.1 ± 0.2 degrees two-theta.
5. 5-Azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents.
6. 5-azacytidine of claim 5 containing about 10 ppm to about 500 ppm of non-volatile solvents
7. The 5-azacytidine of claim 5, wherein it is crystalline 5-azacytidine characterized by data selected from a group consisting of: a PXRD pattern with peaks at about 12.2, 13.1, 14.4, 16.2, and 23.1 ± 0.2 degrees two-theta, a PXRD pattern as depicted in Figure 13, and combination thereof containing less than about 5% by weight of crystalline 5-azacytidine having most prominent diffractions at PXRD at two theta values at 6.566, 11.983, 13.089, 15.138, 17.446, 20.762, 21.049, 22.776, 24.363, 25.743, 26.305, 28.741, 31.393, 32.806, 33.043, 33.536, 36.371, 39.157, and 41.643 degrees two-theta , and less than about 5% by weight of a crystalline 5-azacytidine

with having most prominent PXRD diffractions at 13.4, 17.6, and 22.1 degrees two-theta

8. A method for preparing the 5-azacytidine of claim 5 comprising heating a suspension of 5-azacytidine in a single polar organic solvent selected from a group consisting of: aliphatic alcohol, nitrile, ether, nitromethane, pyridine, or in a mixture of solvents comprising the said polar organic solvent and a non-polar organic solvent selected from a group consisting of: ketone, a hydrocarbon, or ester, and recovering 5-azacytidine containing about 10 ppm to about 2000 ppm of non-volatile solvents; wherein the single polar solvent or its mixture with a non-polar solvent has boiling point of less than 140°C.

9. The method of claim 8, wherein the obtained 5-azacytidine is crystalline 5-azacytidine characterized by data selected from a group consisting of: a PXRD pattern with peaks at about 12.2, 13.1, 14.4, 16.2, and 23.1 ± 0.2 degrees two-theta, a PXRD pattern as depicted in Figure 13, and combination thereof containing less than about 5% by weight of crystalline 5-azacytidine having most prominent diffractions at PXRD at two theta values at 6.566, 11.983, 13.089, 15.138, 17.446, 20.762, 21.049, 22.776, 24.363, 25.743, 26.305, 28.741, 31.393, 32.806, 33.043, 33.536, 36.371, 39.157, and 41.643 degrees two-theta , and less than about 5% by weight of a crystalline 5-azacytidine with having most prominent PXRD diffractions at 13.4, 17.6, and 22.1 degrees two-theta

10. The method of claim 8, wherein the starting 5-azacytidine is selected from a group consisting of: crude 5-azacytidine, crystalline 5-azacytidine having the most prominent 2 theta angles at 5.704, 11.571, 12.563, 14.070, 15.943, 16.993, 18.066, 20.377, 20.729, 21.484, 21.803, 22.452, 22.709, 23.646, 24.068, 25.346, 25.346, 26.900, 27.991, 28.527, 28.723, 30.124, 30.673, 31.059, 35.059, 38.195 and 38.403, crystalline 5-azacytidine characterized by data selected from a group consisting of: a powder XRD pattern with peaks at about 8.7, 9.5, 12.1, 14.4 and 17.3 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 1; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.2, 155.9, and 154.2 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 2; a solid-state ^{13}C NMR

spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 60.5, 62.2, and 72.5 ± 0.1 ppm, and combination thereof., crystalline 5-azacytidine which exhibits distinctive peaks at 5.8, 11.5, 12.8, 22.4, and 26.6 degrees two-theta, and crystalline 5-azacytidine having most prominent diffractions on PXRD at two theta values at 6.566, 11.983, 13.089, 15.138, 17.446, 20.762, 21.049, 22.776, 24.363, 25.743, 26.305, 28.741, 31.393, 32.806, 33.043, 33.536, 36.371, 39.157, and 41.643 degrees two-theta.

11. The method of claim 8, wherein the suspension of 5-azacytidine is provided by combining 5-azacytidine and a single polar organic solvent or a mixture of solvents comprising the said polar organic solvent and a non-polar organic solvent.

12. The method of claim 11, wherein the aliphatic alcohol is a C_{2-6} aliphatic alcohol, the nitrile is C_{2-4} nitrile, and the ether is a C_{3-8} ether including penta or hexa-cyclic ether.

13. The method of claim 12, wherein the C_{2-6} aliphatic alcohol is methanol, ethanol, 2-propanol, 1-propanol, 1-butanol, 2-butanol, i-butanol, amylalcohol, methoxyethanol, ethoxyethanol or mixtures thereof, the C_{2-4} nitrile is acetonitrile, and the C_{3-8} ether including penta or hexa-cyclic ether is dimethoxyethane, tert-butylmethylether, dioxolane, tetrahydrofurane, methyl-tetrahydrofurane, or dioxane.

14. The method of claim 13, wherein the C_{2-6} aliphatic alcohol is either 1-butanol or ethanol.

15. The method of claim 13, wherein the C_{3-8} ether including penta or hexa-cyclic ether is 1,4-dioxane.

16. The method of claim 8, wherein the ketone is C_{3-6} ketone, the ester is C_{2-6} ester, and the hydrocarbon is a C_{6-10} hydrocarbon.

17. The method of claim 16, wherein the C_{3-6} ketone is acetone, methylethylketone, or methylbutylketone, the C_{2-6} ester is ethylacetate, propyl acetate, isopropyl acetate, butylacetate, isobutylacetate, and the C_{6-10} hydrocarbon is hexane, heptane, cyclohexane, methylcyclohexane, toluene, m-xylene, p-xylene, or chlorbenzene.

18. The method of claim 17, wherein the C_{3-6} ketone is either methylethyl ketone or methylisobutyl ketone, the C_{2-6} ester is ethylacetate, and the C_{6-10} hydrocarbon is toluene.

19. The method of claim 8, wherein the solvent used to prepare the suspension is a single polar organic solvent, either ethanol or 1-butanol.

20. The method of claim 8, wherein the suspension is heated to a temperature of about 30°C to about 130°C.

21. The method of claim 8, further comprising recovering the said 5-azacytidine.

22. Crystalline 5-azacytidine characterized by data selected from a group consisting of: a powder XRD pattern with peaks at about 8.7, 9.5, 12.1, 14.4, and 17.3 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 1; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.2, 155.9, and 154.2 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 2; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 60.5, 62.2, and 72.5 ± 0.1 ppm, and combination thereof.

23. The crystalline 5-azacytidine of claim 22, characterized by a powder XRD pattern with peaks at about 8.7, 9.5, 12.1, 14.4, and 17.3 ± 0.2 degrees two-theta.

24. The crystalline 5-azacytidine of claim 22, characterized by a PXRD pattern as depicted in Figure 1.

25. The crystalline 5-azacytidine of claim 22, characterized by a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.2, 155.9, and 154.2 ± 0.2 ppm.

26. The crystalline 5-azacytidine of claim 22, characterized by a solid-state ^{13}C NMR spectrum as depicted in Figure 2.

27. The crystalline 5-azacytidine of claim 22 characterized by a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 60.5, 62.2, and 72.5 ± 0.1 ppm.

28. The crystalline 5-azacytidine of claim 23, further characterized by a powder XRD pattern with peaks at about 19.4, 21.3, and 28.6 ± 0.2 degrees two-theta.

29. The crystalline 5-azacytidine of claim 25, further characterized by a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 86.9, and 73.5 ± 0.2 ppm.

30. The crystalline 5-azacytidine of claim 25 or 29, further characterized by a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 174.6 and 28.7 ± 0.2 ppm.

31. The crystalline 5-azacytidine of claim 22, wherein the crystalline form is an N-methylpyrrolidone solvate.

32. The crystalline 5-azacytidine of claim 22, wherein the crystalline form has less than about 10% by weight of crystalline 5-azacytidine having most prominent diffractions at PXRD at two theta values at 6.566, 11.983, 13.089, 15.138, 17.446, 20.762, 21.049, 22.776, 24.363, 25.743, 26.305, 28.741, 31.393, 32.806, 33.043, 33.536, 36.371, 39.157, and 41.643 degrees two-thetaor crystalline 5-azacytidine having the most prominent two theta angles at 6.599, 10.660, 12.600, 13.358, 15.849, 17.275, 20.243, 20.851, 21.770, 22.649, 25.554, 25.740, 29.293, 32.148, 35.074, and 38.306 degrees two-theta. .

33. A process for preparing the crystalline 5- azacytidine of claim 22 comprising providing a solution of 5-azacytidine in N-methylpyrrolidone (referred to as NMP), and precipitating the said crystalline by cooling to a temperature of about 20°C to about 0°C to obtain a suspension comprising of the said crystalline form.

34. The process of claim 33, wherein the solution is provided by combining 5-azacytidine and NMP and heating the combination.

35. The process of claim 34, wherein the heating is carried out to a temperature of about 50°C to reflux temperature.

36. The process of claim 33, wherein the solution of 5-azacytidine in NMP includes a second solvent.

37. The process of claim 36, wherein the second solvent is selected from a C_3-C_8 ketone or a C_5-C_{12} aromatic or saturated hydrocarbon.

38. The process of claim 37, wherein the C_3-C_8 ketone is methylethylketone and the C_5-C_{12} aromatic or saturated hydrocarbon is toluene.

39. The process of claim 33, wherein the precipitation is done by cooling the solution to a temperature of about 10°C to about 0°C.

40. The process of claim 33, further comprising recovering the precipitated crystalline form.

41. Crystalline 5-azacytidine solvate selected from a group consisting of: 1,3-dimethyl-2-imidazolidinone solvate; and 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone solvate.

42. Crystalline 5-azacytidine solvate of claim 41, wherein the solvate is 1,3-dimethyl-2-imidazolidinone solvate.

43. Crystalline 5-azacytidine solvate of claim 41, wherein the solvate is 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone solvate.

44. Crystalline 5-azacytidine selected from a group consisting of:

(a) Crystalline 5-azacytidine characterized by data selected from a group consisting of: a powder XRD pattern with peaks at about 5.8, 11.6, 12.8, 16.2, and 17.4 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 3; a single crystal XRD with the following data: monoclinic crystal system; unit cell parameters: a, b, c : a = 5.14 Å, b = 7.78 Å, c = 15.40 Å, alpha = 90 °, beta = 99.60 °, and gamma = 90 °, respectively; an ORTEP view of a single crystal as depicted in figure 5; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 167.3, 156.2, and 93.4 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 4; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 62.8 and 73.9 ± 0.1 ppm, and combination thereof containing less than about 20% by weight of crystalline 5-azacytidine having the most prominent 2 theta angles

at 12.182, 13.024, 14.399, 16.470, 18.627, 19.049, 20.182, 21.329, 23.033, 23.872, 26.863, 27.1735, 29.277, 29.591, 30.369, and 32.072;

(b) Crystalline 5-azacytidine characterized by data selected from a group consisting of: a powder XRD pattern with peaks at about 8.5, 9.4, 12.0, 14.4, 17.1 and 31.3 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 7; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.0, 153.9, and 93.4 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 8; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 60.5 and 72.6 ± 0.1 ppm, and combination thereof;

(c) Crystalline 5-azacytidine characterized data selected from a group consisting of: a powder XRD pattern with peaks at about 9.4, 11.8, 12.1, 14.3 and 16.5 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 9; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.6, 154.3, and 93.9 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.6, 155.8, and 93.9 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 10; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 60.4 and 72.7 ± 0.1 ppm, a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 61.9 and 72.7 ± 0.1 ppm, and combination thereof; and

(d) Crystalline 5-azacytidine characterized by data selected from a group consisting of: a powder XRD pattern with peaks at about 11.0, 12.4, 13.7, 16.5, and 18.5 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 11, and combination thereof containing less than about 20% by weight of crystalline 5-azacytidine having the most prominent 2 theta angles at 12.182, 13.024, 14.399, 16.470, 18.627, 19.049, 20.182, 21.329, 23.033, 23.872, 26.863, 27.1735, 29.277, 29.591, 30.369, and 32.072;

45. The crystalline 5-azacytidine of claim 44, wherein the crystalline 5-azacytidine is crystalline 5-azacytidine characterized by data selected from a group consisting of: a

powder XRD pattern with peaks at about 5.8, 11.6, 12.8, 16.2, and 17.4 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 3; a single crystal XRD with the following data: monoclinic crystal system; unit cell parameters: a, b, c : a = 5.14 Å, b = 7.78 Å, c = 15.40 Å, alpha = 90 °, beta = 99.60 °, and gamma = 90 °, respectively; an ORTEP view of a single crystal as depicted in figure 5; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 167.3, 156.2, and 93.4 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 4; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 62.8 and 73.9 ± 0.1 ppm, and combination thereof containing less than about 20% by weight of crystalline 5-azacytidine having the most prominent 2 theta angles at 12.182, 13.024, 14.399, 16.470, 18.627, 19.049, 20.182, 21.329, 23.033, 23.872, 26.863, 27.1735, 29.277, 29.591, 30.369, and 32.072.

46. The crystalline 5-azacytidine of claim 44, characterized by data selected from a group consisting of: a powder XRD pattern with peaks at about 8.5, 9.4, 12.0, 14.4, 17.1 and 31.3 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 7; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.0, 153.9, and 93.4 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 8; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 60.5 and 72.6 ± 0.1 ppm, and combination thereof.

47. The crystalline 5-azacytidine of claim 44, characterized data selected from a group consisting of: a powder XRD pattern with peaks at about 9.4, 11.8, 12.1, 14.3 and 16.5 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 9; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.6, 154.3, and 93.9 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum having signals with chemical shifts at about 166.6, 155.8, and 93.9 ± 0.2 ppm; a solid-state ^{13}C NMR spectrum as depicted in Figure 10; a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 60.4 and 72.7 ± 0.1 ppm, a solid-state ^{13}C NMR spectrum having chemical shift differences between the signal exhibiting

the lowest chemical shift and another in the chemical shift range of 90 to 180 ppm of about 61.9 and 72.7 ± 0.1

48. The crystalline 5-azacytidine of claim 44, wherein the crystalline 5-azacytidine is crystalline 5-azacytidine characterized by data selected from a group consisting of: a powder XRD pattern with peaks at about 11.0, 12.4, 13.7, 16.5, and 18.5 ± 0.2 degrees two-theta; a PXRD pattern as depicted in Figure 11, and combination thereof containing less than about 20% by weight of crystalline 5-azacytidine having the most prominent 2 theta angles at 12.182, 13.024, 14.399, 16.470, 18.627, 19.049, 20.182, 21.329, 23.033, 23.872, 26.863, 27.1735, 29.277, 29.591, 30.369, and 32.072.

48. A pharmaceutical composition comprising any one of the forms of 5-azacytidine of claims 1, 5, 22, 41 and 44, and at least one pharmaceutically acceptable excipient.

49. A pharmaceutical composition comprising a crystalline form of 5-azacytidine prepared by the method of any one of claims 8-21 and 33-40, and at least one pharmaceutically acceptable excipient.

50. A process for preparing a pharmaceutical formulation, comprising combining any one of the forms of 5-azacytidine of claims 1, 5, 22, 41 and 44 with at least one pharmaceutically acceptable excipient.

51. Use of a crystalline form of 5-azacytidine according to any one of claims 1, 5, 22, 41 and 44 for the preparation of a pharmaceutical composition.

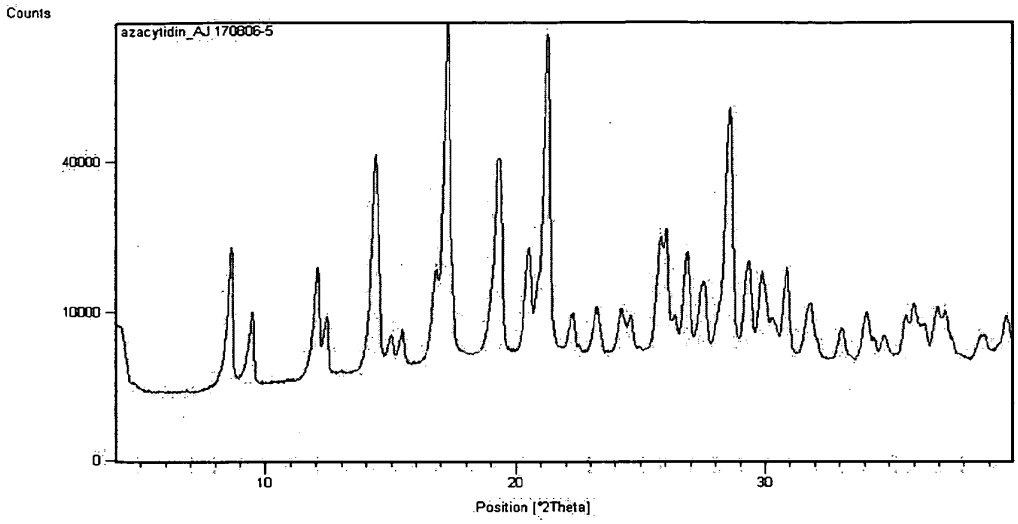


Figure 1

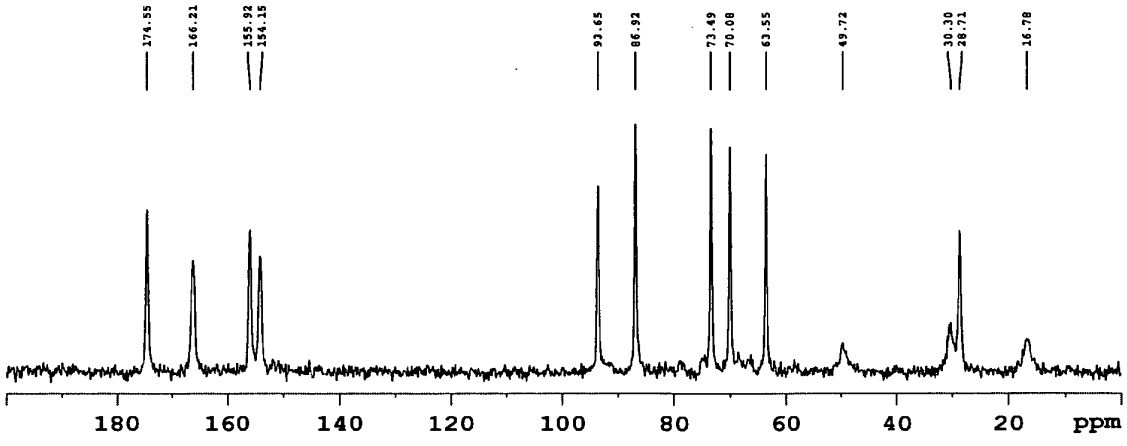


Figure 2

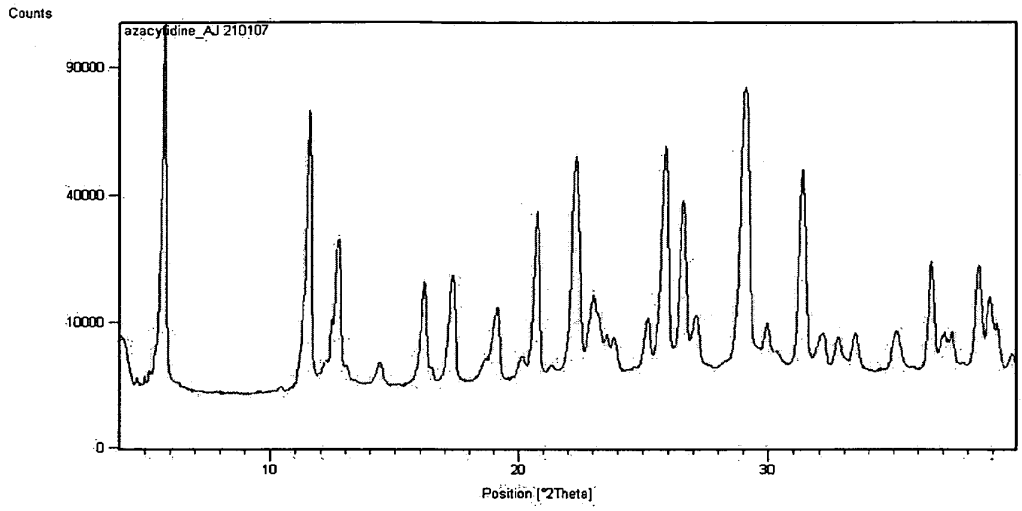


Figure 3

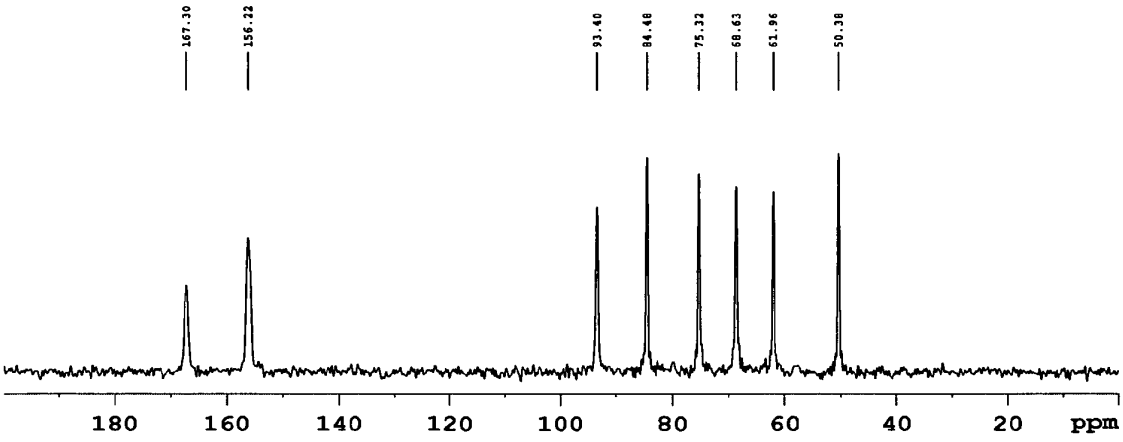


Figure 4

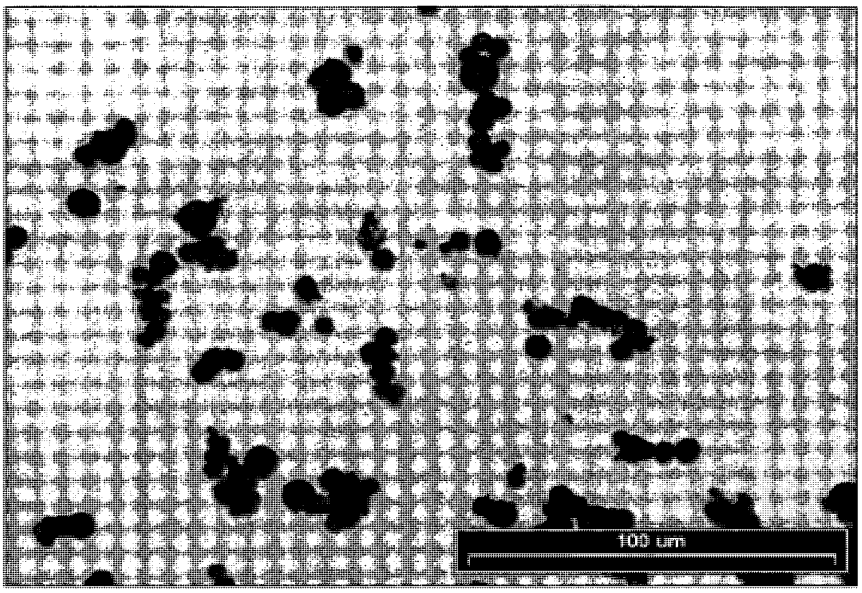


Figure 5A

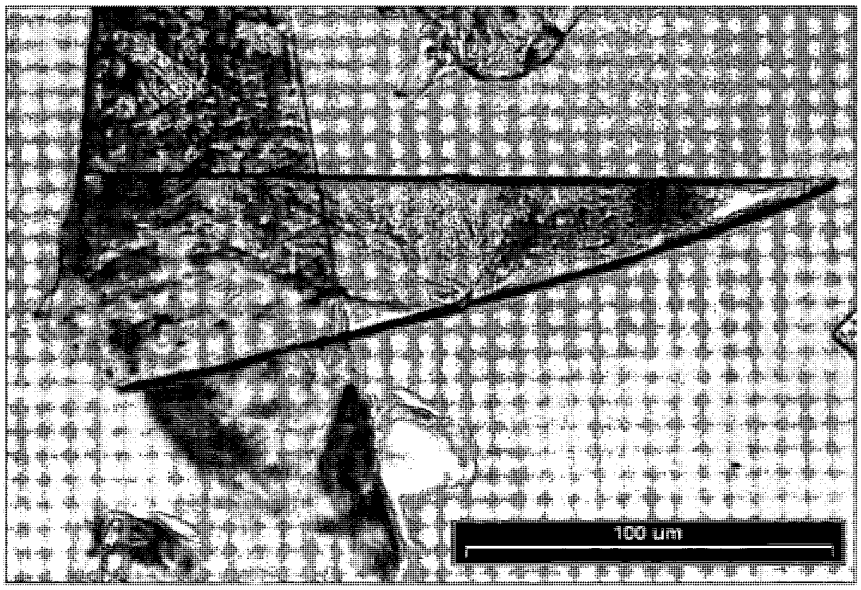


Figure 5B

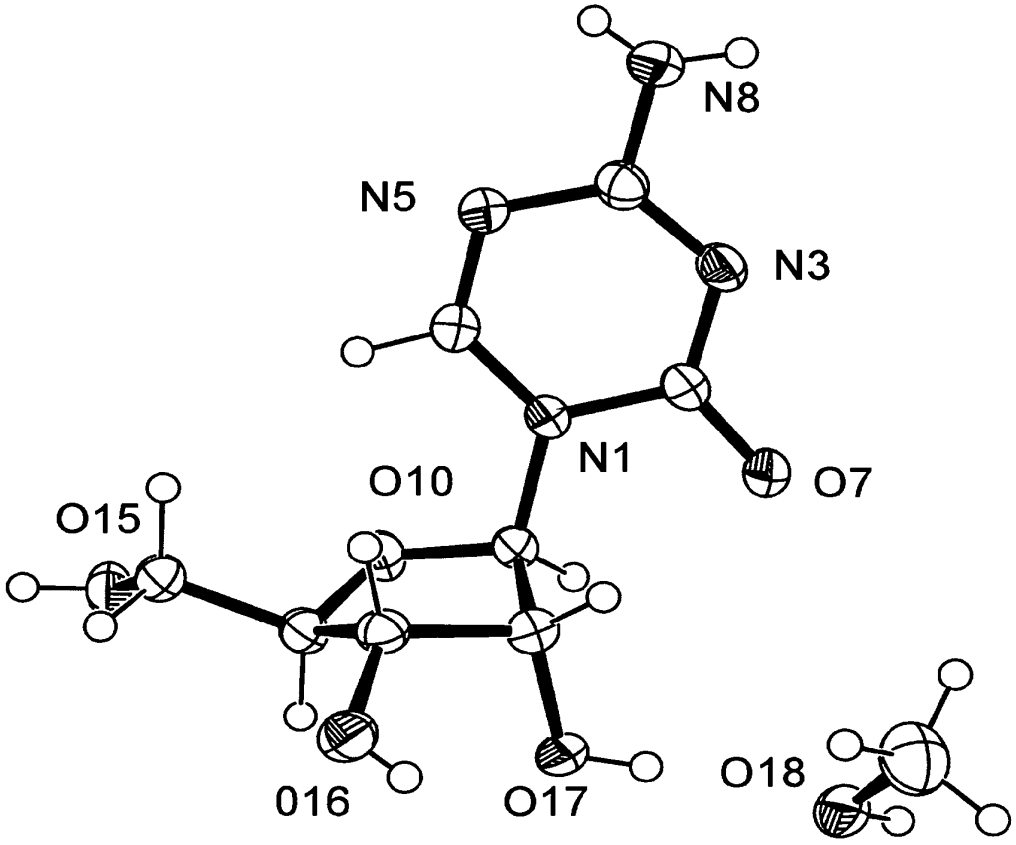


Figure 6

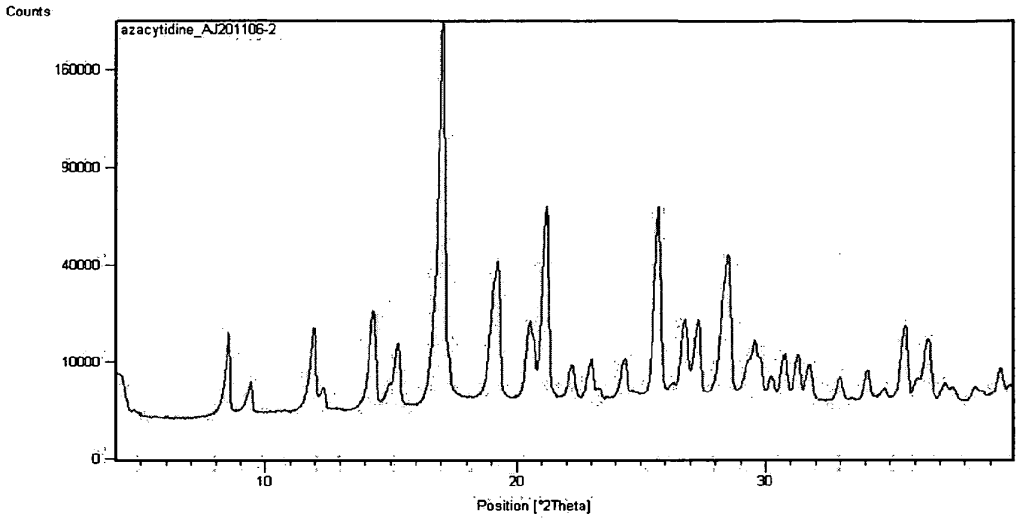


Figure 7

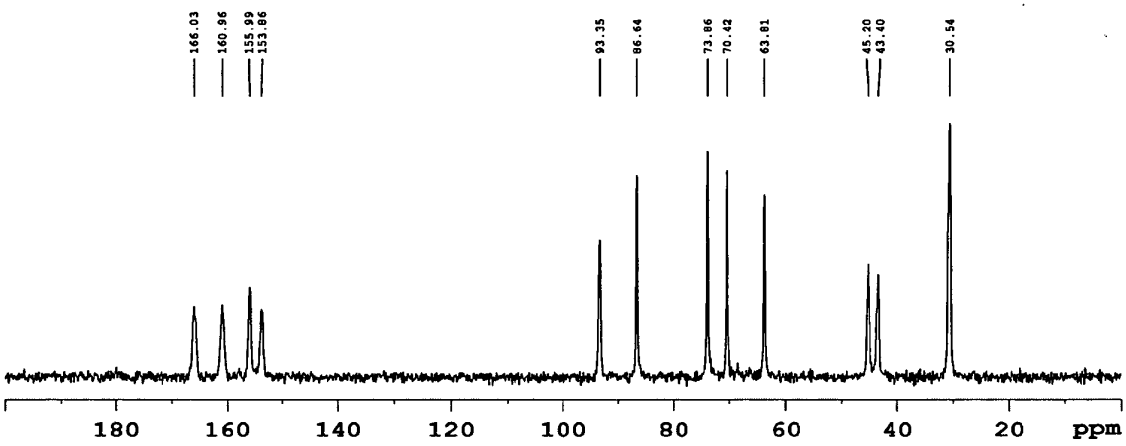


Figure 8

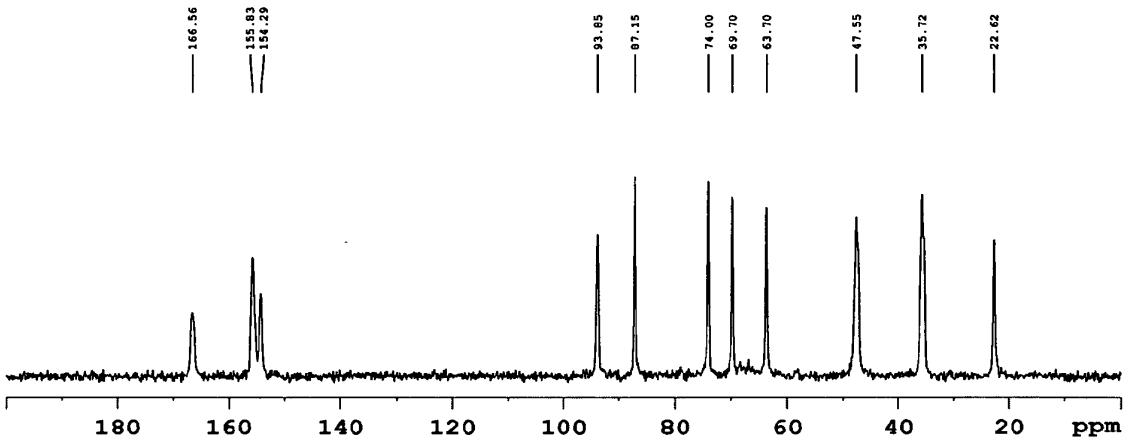


Figure 9

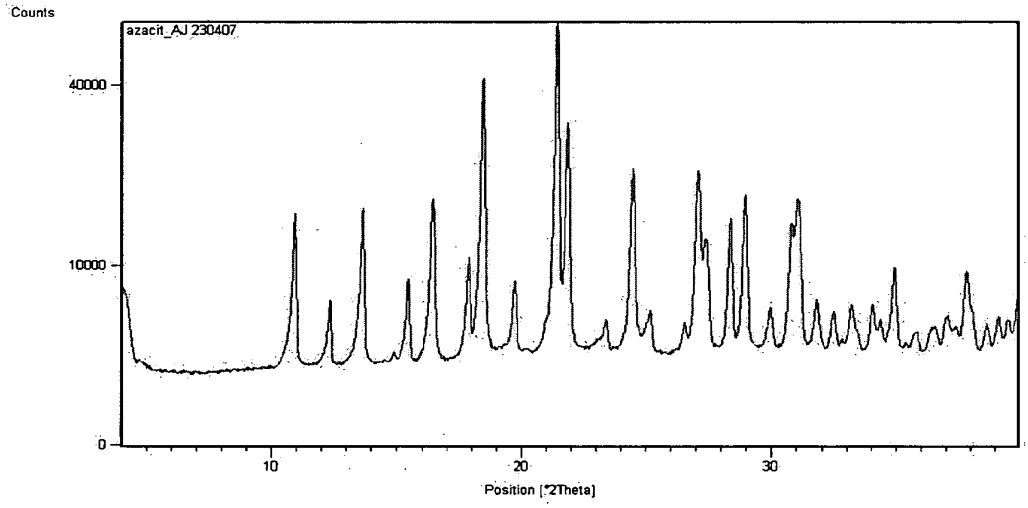
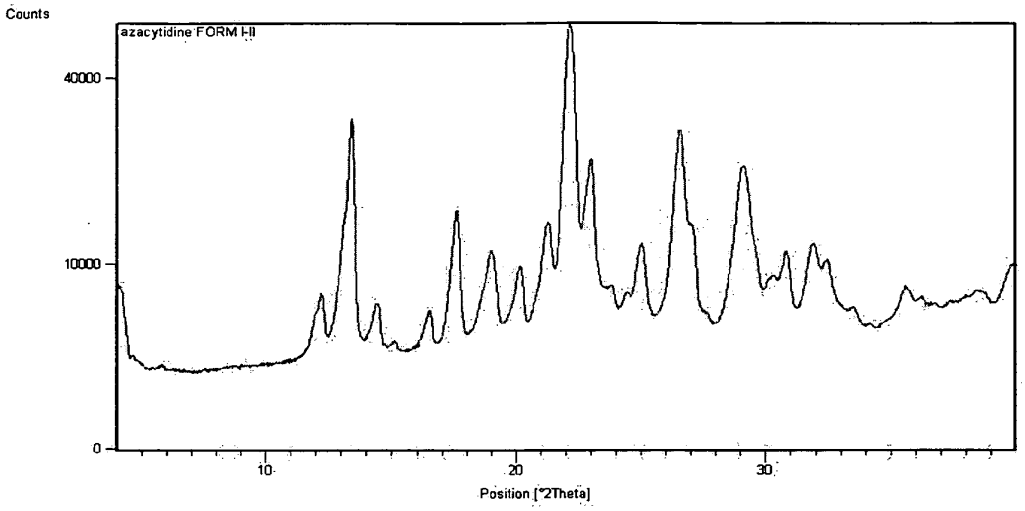


Figure 10



Fivgure 11

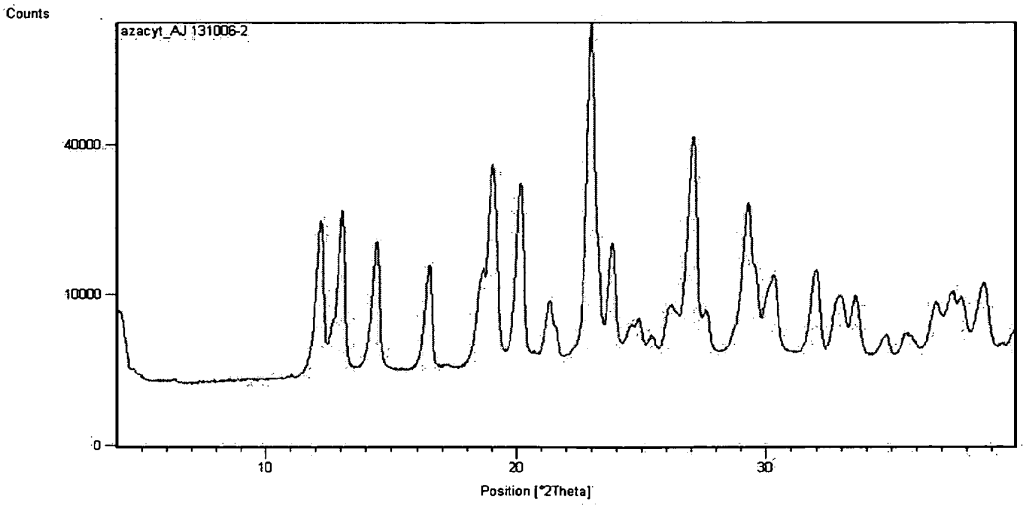


Figure 12

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NOTICE OF ALLOWANCE AND FEE(S) DUE

84802 7590 09/20/2011

JONES DAY
222 E. 41ST. STREET
NEW YORK, NY 10017

EXAMINER	
CHU, YONG LIANG	
ART UNIT	PAPER NUMBER
1626	

DATE MAILED: 09/20/2011

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/729,116	03/22/2010	Dumitru Ionescu	9516-846-999 (CAM 501872-	6614

TITLE OF INVENTION: PHARMACEUTICAL COMPOSITIONS COMPRISING CRYSTAL FORM I OF 5-AZACYTIDINE

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1510	$300	$0	$1810	12/20/2011

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

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If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

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III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

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(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/729,116	03/22/2010	Dumitru Ionescu	9516-846-999 (CAM 501872-	6614

TITLE OF INVENTION: PHARMACEUTICAL COMPOSITIONS COMPRISING CRYSTAL FORM I OF 5-AZACYTIDINE

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1510	$300	$0	$1810	12/20/2011

EXAMINER	ART UNIT	CLASS-SUBCLASS
CHU, YONG LIANG	1626	514-043000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

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(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☐ Corporation or other private group entity ☐ Government

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☐ Advance Order - # of Copies _____

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☐ A check is enclosed.

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☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/729,116	03/22/2010	Dumitru Ionescu	9516-846-999 (CAM 501872-	6614

84802 7590 09/20/2011

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EXAMINER	
CHU, YONG LIANG	
ART UNIT	PAPER NUMBER
1626	

DATE MAILED: 09/20/2011

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 91 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 91 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No.	Applicant(s)	
	12/729,116	IONESCU ET AL.	
	Examiner	Art Unit	
	YONG CHU	1626	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *08/10/2010*.

2. ☒ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are *22-61 (renumbered as 1-40)*.

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☐ All b) ☐ Some* c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.

6. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

7. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☒ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☐ Examiner's Amendment/Comment
8. ☒ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

DETAILED ACTION

Claims 22-61 are currently pending in this Application, and are under examination on the merits.

Priority

This application is a continuation of U.S. Patent Application Serial No. 11/198,550, filed August 5, 2005, entitled Methods for Isolating Crystalline Form I of 5-Azacytidine; which is a divisional of U.S. Patent Application Serial No. 10/390,530, filed March 17, 2003, entitled Methods for Isolating Crystalline Form I of 5-Azacytidine (now U.S. Patent No. 6,943,249)

Reasons for Allowance

The present invention is directed to a pharmaceutical composition for **oral** administration comprising (crystalline) **Form I** of 5-azacytidine substantially free of other forms of 5-azacytidine. The methods of making **Form I** of 5-azacytidine have been patented in the parent patents. The XRPD characterization of (crystalline) **Form I** of 5-azacytidine is disclosed in the Example 4 of the instant specification on page 3. In addition, a pharmaceutical formulation into the form of dosage units for oral administration is also disclosed on page 9 of the specification. Since crystalline **Form I** of 5-azacytidine substantially free of other forms of 5-azacytidine is patentable, a pharmaceutical composition comprising said **Form I** of 5-azacytidine is also patentable. See *SmithKline Beecham Corp. v. Apotex Corp., 74 USPQ2d 1398,* (Fed. Appl. Cir. 2005).

The closest prior art of record is a reference by *Dover et al., Blood, (1985), Vol. 66(3), p.527-532.*

Dover et al. teaches a pharmaceutical composition comprising 5-azacytidine for oral administration. However, Dover **does not** teach a pharmaceutical composition comprising crystalline **Form I** of 5-azacytidine substantially free of other forms of 5-azacytidine. Therefore, claims 22-61 are allowed.

Conclusions

- Claims 22-61 are allowed.

Telephone Inquiry

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Yong Chu, Ph.D., whose telephone number is 571-272-5759. The examiner can normally be reached on 7:00 am - 3:30 pm EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Joseph K. McKane can be reached on 571-272-0699. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Status Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

/Yong Chu/
Primary Examiner
Art Unit 1626

Electronic Acknowledgement Receipt

EFS ID:	13661086
Application Number:	12466213
International Application Number:	
Confirmation Number:	5370
Title of Invention:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF
First Named Inventor/Applicant Name:	Jeffrey B. Etter
Customer Number:	84802
Filer:	Jihong Lou/Eric Baclig
Filer Authorized By:	Jihong Lou
Attorney Docket Number:	9516-847-999
Receipt Date:	05-SEP-2012
Filing Date:	14-MAY-2009
Time Stamp:	17:40:30
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		9516-847-999_IDS.PDF	37469 cca966f70e320f2550e1069d036d733bd0013a48	yes	2

	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Transmittal Letter		1	1	
	Information Disclosure Statement (IDS) Form (SB08)		2	2	
Warnings:					
Information:					
2	Foreign Reference	WO2004082619.pdf	1422214 9e519860448c7b2749c3741cbc92379485901527	no	36
Warnings:					
Information:					
3	Foreign Reference	WO06-034154.pdf	3422227 7d312b6e3fa4ff223878a3dbcc2671e242c13e43	no	105
Warnings:					
Information:					
4	Foreign Reference	WO2008088779.pdf	2690842 c56634d61a4fdadc8065af6aadbc3ac5f4cce787	no	52
Warnings:					
Information:					
5	Non Patent Literature	12729116_NOA_05-04-12.PDF	192681 f8542ef8569ee44c7545835c611c94cae11ca350	no	8
Warnings:					
Information:					
		Total Files Size (in bytes):	7765433		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Jeffrey B. Etter et al.	**Confirmation No.:**	5370
Application No.:	12/466,213	**Group Art Unit:**	1623
Filed:	May 14, 2009	**Examiner:**	Lawrence E. Crane
For:	ORAL FORMULATIONS OF CYTIDINE ANALOGS AND METHODS OF USE THEREOF	**Attorney Docket No.:**	9516-847-999 (501872-999847)

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

E-FILE
Commissioner for Patents
P.O. Box 1450
Washington, D.C. 22313-1450

Sir:

Pursuant to Applicants' duty of disclosure under 37 C.F.R. §§ 1.56 and 1.97, enclosed is a list of five (5) references for the Examiner's review and consideration. These references are listed on the enclosed form entitled "List of References Cited by Applicant." Copies of B10–B12 and C71 are also enclosed herewith.

Identification of the foregoing references is not to be construed as an admission of Applicants or Attorneys/Agents for Applicants that such references are available as "prior art" against the subject application. Applicants respectfully request that the Examiner review the foregoing references and make them of record by completing and returning the enclosed List of References.

No fee is believed to be due for the submission of this statement, because it is submitted after a Request for Continued Examination. However, the Director is authorized to charge any required fees to Jones Day Deposit Account No. 50-3013 (referencing CAM: 501872-999847).

Respectfully submitted,

Date September 5, 2012

/Jihong Lou/

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SDI-136024v1