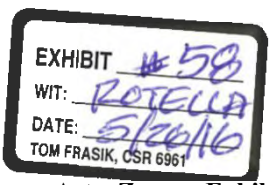
IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

ASTRAZENECA AB, Plaintiff, v. AUROBINDO PHARMA LTD. and AUROBINDO PHARMA U.S.A., INS., Defendants.	C.A. No. 14-664-GMS (consolidated)
ASTRAZENECA AB, Plaintiff, v. MYLAN PHARMACEUTICALS INC., Defendants.	C.A. No. 14-696-GMS **CONFIDENTIAL INFORMATION – SUBJECT TO PROTECTIVE ORDER**

REPLY EXPERT REPORT OF DAVID P. ROTELLA, PH.D.



I. INTRODUCTION

1. I, David P. Rotella, have been retained by counsel for Mylan Pharmaceuticals Inc. in connection with the above-captioned litigation matter to provide expert testimony concerning U.S. Patent No. RE 44,186 ("the '186 patent") by Robl *et al.*, and entitled "Cyclopropyl-fused pyrrolidine-based inhibition of dipeptidyl peptidase IV and method." This report responds to various statements and issues raised in the March 4, 2016 report of Dr. Ann Weber concerning the invalidity of the '186 patent. This report sets forth further opinions as to which, if asked, I will testify at trial with respect to the '186 patent.

2. I am the same David P. Rotella that previously submitted an expert report served on January 29, 2016 ("Opening Report"). My Opening Report set forth my credentials, qualifications, publications, compensation, and prior testimony. That report also laid out the education and experience that a POSA would possess. That information all remains the same.

3. In forming my opinions and preparing this report, I reviewed and considered the materials cited in this report and those materials listed in Exhibit A to this report. I have further relied on my knowledge, education, and training, as reflected in my qualifications and credentials set forth in my *curriculum vitae*, which is attached to my Opening Report.

4. I reserve the right to provide further background regarding the relevant technology by way of tutorial at trial and any trial exhibits that would assist in communicating my opinions. I also reserve the right to supplement this report if I become aware of additional pertinent information. I may also testify or provide an opinion in rebuttal to testimony or opinions offered.

5. For the reasons detailed below, it is my opinion that the supposed objective indicia of non-obviousness raised in Dr. Weber's report are not sufficient to support a finding

that the claimed invention would not have been obvious. I have reveiwed Dr. Weber's report, and nothing contained therein alters my previously presented opinion that the asserted claims of the '186 patent would have been obvious in view of the prior art to a person of ordinary skill in the art at the time of the claimed invention. As an initial matter, Dr. Weber failed to establish a nexus between any of the supposed objective indicia of non-obviousness and anything novel in the purported invention.

6. While I have confined this report to the topic of objective indicia of nonobviousness, I reserve the right to respond to other aspects of Dr. Weber's report.

II. APPLICABLE LEGAL STANDARDS

7. My understanding of the applicable legal standards, as explained by counsel, is set forth in my Opening Report.

8. Additionally, I understand from counsel that the Court can consider what are known as objective indicia of non-obviousness presented by the patentee to rebut a showing of obviousness. I understand that the patentee has the burden of providing evidence of any alleged objective indicia, including evidence of a connection, or "nexus," between each alleged objective indicia and the invention of the asserted claims.

9. I understand that unexpected results is one such objective indicia. I further understand when evaluating the objective indicia of unexpected results, the purported unexpected results should be evaluated based on what a POSA would or would not have expected at the time of the alleged invention. I further understand that that in order to allege unexpected results, the results of the claimed invention must be compared to the results of the closest prior art. I also understand that for an alleged unexpected result to be probative of non-obviousness, there must

be evidence showing show a difference in kind, not just in degree, as compared to the closest prior art.

III. NONE OF THE OBJECTIVE INDICIA IDENTIFIED BY DR. WEBER PROVIDE EVIDENCE OF NON-OBVIOUSNESS

A. Dr. Weber Provides No Evidence Of Unexpected Results That Is Probative Of Non-Obviousness

10. As an initial matter, Dr. Weber overwhelmingly focuses her discussion of unexpected results on differences between saxagliptin and vildagliptin. Weber Report at ¶¶235-256. As discussed in my Opening Report (¶¶107-113), it is my opinion that a POSA would have been motivated to select Ashworth compound 25 as a lead compound. It is my understanding from counsel that, in order to provide evidence of an unexpected result that is probative of non-obviousness, there must be a comparison between the claimed invention and the closest relevant prior art. Dr. Weber has not provided any evidence with respect to the differences between Ashworth compound 25 and saxagliptin in terms of unexpected results. Despite this lack of evidence, I will briefly address certain assertions made by Dr. Weber regarding unexpected results.

11. As discussed in my Opening Report (¶¶139-151), a POSA would have been motivated to improve upon the prior art Ashworth compound 25 by fusing a cyclopropane ring to the pyrrolidine ring, in part because there was a reasonable expectation that the resulting conformational restriction of the pyrrolidine ring would prevent intramolecular cyclization and thus increase stability. So motivated, the POSA would only have three choices as to the position for fusing the cyclopropyl ring, and two choices as to the spatial configuration of the cyclopropyl ring in each position. Opening Report at ¶145. Thus, the *cis*-4,5- configuration seen in saxagliptin is one of only six possible choices for cyclopropanation of the pyrrolidine ring. A

POSA at the time of the invention would have had a reasonable expectation that at least one of these six possibilities would result in a configuration that would improve the stability of the molecule. Selecting the *cis*-4,5- orientation from such a small, discrete set of compounds would be a matter of routine optimization for a POSA. Thus, it is not unexpected that a POSA would be able to improve stability of the resulting compound by cyclopropanation of the pyrrolidine ring.

12. Dr. Weber also alleges the "slow, tight binding kinetics" of saxagliptin constitutes an unexpected result. While Dr. Weber only compares the binding properties of saxagliptin to those of vildagliptin, DPP-4 inhibitors that demonstrated slow, tight binding were known in the prior art before the alleged invention of saxagliptin. For example, it was already known that "NVP-DPP728 inhibits DPP-IV by a slow-binding mechanism" (Hughes, *et al.* "NVP-DPP728 (1-[[[2-[(5-Cyanopyridin-2-yl)amino]ethyl]amino]acetyl]-2-cyano-(*S*)-pyrrolidine), a Slow-Binding Inhibitor of Dipeptidyl Peptidase IV," Biochem., 38:11597-11603, at 11598 (1999)), and that boroproline-based DPP4 inhibitors exhibited slow, tight binding inhibition of DPP-4. (Gutheil, W.G. & W.W. Bachovchin, "Separation of $_{L}$-Pro-$_{DL}$-boroPro into Its Component Diastereomers and Kinetic Analysis of Their Inhibition of Dipeptidyl Peptidase IV. A New Method for the Analysis of Slow, Tight-Binding Inhibition," Biochem., 32(34):8723-8731 (Aug. 31, 1993); Kelly, *et al.* "Immunosuppressive Boronic Acid Dipeptides: Correlation between Conformation and Activity," J. Am. Chem. Soc., 115:12637-12638 (1993)) As such, at least the existence of compounds capable of slow, tight binding inhibition of DPP-4 is not unexpected.

B. The Purported Failure Of Others Does Not Indicate Non-Obviousness

13. I disagree with Dr. Weber's assertion that the presence of "only four FDA-approved DPP-4 inhibitors on the market" indicates a failure of others that would support the non-obviousness of saxagliptin. Weber Report at ¶¶257-261.

14. The fact that other DPP-4 inhibitor candidates did not ultimately receive FDA approval is not indicative of saxagliptin's non-obviousness, but simply a reflection of the risks inherent in the drug discovery process. Any person of ordinary skill in the field of drug discovery is well aware that most drug candidates ultimately fail to produce FDA-approved drugs, and that failure at the clinical trial stage is very common.

15. Drug discovery, from lead compound identification to market entry, is a lengthy process which requires a substantial commitment of resources. It is not uncommon for drug discovery decisions to be driven as much by market and competitive concerns as by technical considerations rooted in chemistry and medicine. For example, a researcher might be instructed not to pursue a promising drug candidate because, even if the candidate were to lead to an approved product, that product would be a late entrant into a market where patients already have access to treatment options that act by the same mechanism. The existence of four different FDA-approved DPP-4 inhibitor products merely indicates that four different companies were willing to enter that particular sub-section of the diabetes treatment market; it is not indicative of any particular scientific difficulty in the underlying drug development process. Thus the existence of "only four" DPP-4 inhibitors on the market cannot reasonably be taken as evidence that saxagliptin was non-obvious.

C. Saxagliptin Did Not Meet Any Long-Felt Unmet Need

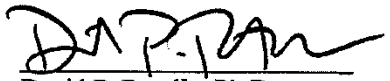
16. I disagree with Dr. Weber's assertion that saxagliptin met a long-felt need for alternative therapies for type-2 diabetes. Weber Report at ¶¶262-264. As discussed above, saxagliptin was one of several DPP-4 inhibitor drugs developed to treat type-2 diabetes. But, as Dr. Weber herself admits, saxagliptin was not the first DPP-4 inhibitor approved by the FDA to treat type-2 diabetes. *Id.* at ¶264. The first DPP-4 inhibitor therapy for treat type-2 diabetes was sitagliptin, which received FDA-approval three years before saxagliptin was approved by the FDA. (*See* JANUVIA® Prescribing Information (Aug. 2015), at 1; ONGLYZA® Prescribing Information (Apr. 2016), at 1)

17. Nothing in Dr. Weber's report suggests that saxagliptin met any need for a DPP-4 therapy that was left unmet by sitagliptin. Indeed, I am unaware of any evidence that or indication in saxagliptin's FDA-approved drug information label suggesting any material benefits compared to sitagliptin. (*Id.*) I am thus unaware of any unmet need which was met by saxagliptin.

IV. CONCLUSION

18. For the foregoing reasons it is my opinion that Dr. Weber has not identified any evidence of objective indicia that would support the non-obviousness of the asserted claims. I maintain my opinion, as laid out in my Opening Report, that claims 8, 9, 25, and 26 of the '186 patent are obvious.

Executed on this 8th day of April, 2016.



David P. Rotella, Ph.D.

EXHIBIT A

EXHIBIT A

Materials Considered by David P. Rotella, Ph.D.

DESCRIPTION	BATES
Hughes, et al., "NVP-DPP728 (1-[[[2-[(5-Cyanopyridin-2-yl)amino]ethyl]amino]acetyl]-2-cyano-(*S*)-pyrrolidine), a Slow-Binding Inhibitor of Dipeptidyl Peptidase IV," Biochem., 38:11597-11603 (1999)	MYL_SAX0298717 – MYL_SAX0298725
Gutheil, W.G. & W.W. Bachovchin, "Separation of L-Pro-DL-boroPro into Its Component Diastereomers and Kinetic Analysis of Their Inhibition of Dipeptidyl Peptidase IV. A New Method for the Analysis of Slow, Tight-Binding Inhibition," Biochem., 32(34):8723-8731 (Aug. 31, 1993)	MYL_SAX0298726 – MYL_SAX0298732
Kelly, et al., "Immunosuppressive Boronic Acid Dipeptides: Correlation between Conformation and Activity," J. Am. Chem. Soc., 115:12637-12638 (1993)	MYL_SAX0298733 – MYL_SAX0298734
JANUVIA® Prescribing Information (Aug. 2015)	MYL_SAX0298735 – MYL_SAX0298757
ONGLYZA® Prescribing Information (Apr. 2016)	MYL_SAX0298758 – MYL_SAX0298790

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Summary of Accomplishments:

- **Montclair State University**-Obtained $2.5MM, 5 year drug discovery research grant from Defense Threat Reduction Agency and an additional $300K in pharmaceutical industry research funding in three years.
- **Wyeth Research**-led chemistry teams in CNS drug discovery projects and key leader for collaboration with Solvay Pharmaceuticals. Delivered a clinical candidate, managed chemists in group that delivered another.
- **Lexicon Pharmaceuticals**- Beginning from a screening hit, in less than one year, led project team that identified potent, selective, orally bioavailable inhibitors of PDE7A.
- **Bristol-Myers Squibb**-First to publish the discovery of novel phosphodiesterase type 5 inhibitors with better *in vitro* potency and selectivity compared to sildenafil. Contributed to discovery of 2 clinical candidates (PDE5 inhibitor, DPP4 inhibitor).
- **Cephalon**-Responsible for initial conception and development of several programs. Key leader in collaborations with Kyowa Hakko and Schering Plough. Discovered CEP 1347, which advanced to phase III trials for Parkinson's Disease.

Experience:

- **Montclair State University July 2011-present**
  Margaret and Herman Sokol Professor of Chemistry, Department of Chemistry and Biochemistry; joint appointment in Sokol Institute of Pharmaceutical Life Sciences
- **Independent Consultant, February 2010-present**
  Established consulting agreements with pharmaceutical companies and law firms to advance drug discovery programs and provide expert information on selected topics in drug development
- **Wyeth Research/Pfizer, 2005-February 2010**
  Principal Research Scientist III, chemistry team leader. Directed up to 20 chemists. Member of Princeton Chemical Science leadership team.
- **Lexicon Pharmaceuticals, 2003-2005**
  Senior Group Leader, responsible for multiple drug discovery programs. Directed up to 18 FTEs with 4 direct reports. Member of department leadership team.
- **Bristol-Myers Squibb PRI, 1997-2003**
  Principal Scientist, cardiovascular and metabolic disease drug discovery
- **Cephalon, Incorporated, 1991-1997**
  Group Leader, CNS and cancer drug discovery.
- **School of Pharmacy, University of Mississippi**

Assistant Professor, Department of Pharmacognosy 1987-1991
Adjunct Professor, Department of Medicinal Chemistry, 2009-present

- **School of Pharmacy, University of Pittsburgh**, 2010-present
  Adjunct Professor, Department of Pharmaceutical Sciences
- **Center for Drug Discovery, Northeastern University**, 2010-present
  Adjunct Professor
- Registered pharmacist, Pennsylvania, 1981-1991, 2010-present

Edueation:

- Postdoctoral Scholar, Department of Chemistry, The Pennsylvania State University, 1985-1987, under the direction of Prof. K. S. Feldman.
- Ph.D. Medicinal Chemistry, The Ohio State University, 1985, under the direction of Prof. D. T. Witiak.
- B.S. Pharm., Magna cum laude, School of Pharmacy, University of Pittsburgh, April 1981.

Professional Service:

American Chemical Society, Organic and Medicinal Chemistry Divisions
Fellow, Royal Society of Chemistry

Division of Medicinal Chemistry, American Chemical Society:

- Five year term as Vice Chair/Long Range Planning Committee chair, Program Chair, Chair and past Chair (2004-2008). These roles required leadership and collaborative interactions nationally and internationally.
- Three year term as academic councilor (2012-2014)
- Treasurer, 2015-2017

Gordon Research Conference on Medicinal Chemistry

- 2012 vice chair elect
- 2013 vice chair
- 2014 chair

Co-editor, 3rd edition, Comprehensive Medicinal Chemistry 2014-present

Co-editor, 7th edition, Burger's Medicinal Chemistry 2007-present

Senior Editor, Royal Society of Chemistry series on Drug Discovery, 2008-present

Co-editor, "Successful Drug Discovery", (2014), Wiley VCH

Co-editor, "Analogue-Based Drug Discovery", volume 3, (2012), Wiley VCH

Program co-chair, National Medicinal Chemistry Symposium (2010)

Scientific Advisory Board National Medicinal Chemistry Symposium (2014)

Scientific Advisory Board Frontiers in Medicinal Chemistry 2014-2015

Organizer and conference co-chair for "Frontiers in CNS and Oncology Medicinal Chemistry", Siena, Italy, October 7-9, 2007, jointly organized with European Federation for Medicinal Chemistry.

Current Research Funding:

- Discovery of Novel Botulinum Toxin Protease A Inhibitors, 9/29/14-9/28/19, $2.5MM, Defense Threat Reduction Agency
- Protein Kinase Inhibitors for Parasitic Diseases, 3/1/14-2/28/15, $90,000, Celgene Corporation
- Research Support, 9/1/11-8/31/16, $50,000 annually, Margaret and Herman Sokol Endowment

Past Research Funding:

- Protein Kinase Inhibitors for Parasitic Diseases, 3/1/13-2/28/14, $115,000, Celgene Corporation
- Protein Kinase Inhibitors for Parasitic Diseases, 3/1/12-2/28/13, $90,000, Celgene Corporation
- Purchase of LCMS, 10/1/13, $70,000, Shimadzu Corporation
- Lactam Inhibitors of Phospholipase A2, 7/1/88-6/30/90, direct costs $25,000, Mississippi Affiliate, American Heart Association
- Novel Calmodulin Inhibitors, 7/1/89-6/30/91, direct costs $35,000, Elsa U Pardee Foundation
- Phospholipase A2 Inhibitors as Novel Anti-inflammatory Agents, 7/1/89-6/30/91, direct costs $200,000, American Lung Association

Publications:

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2. "Synthesis and P-388 Antitumor Properties of the Four Diastereomeric Dichloro 1-Hydroxy-3,4-diaminocyclohexane Pt(II) Complexes" , Donald T. Witiak, David P. Rotella, Yong Wei, Joyce A. Filppi and Judith C. Gallucci *J. Med. Chem.* **32**, 214 (1989).

3. "Mechanistic and Preparative Studies of the Intramolecular Photocyclization of Methylated 2-(4-Pentenyl)tropones", Ken S. Feldman, Jon H. Come, Benedict J. Kosmider, Pamela M. Smith, David P. Rotella and Ming-Jung Wu, *J. Org. Chem.* **54**, 592 (1989).

4. "Homoallylically Controlled Epoxidation of Δ^4-*cis*-I,2-Disubstituted Cyclohexenes", David P. Rotella, *Tetrahedron Letters*, 1913 (1989).

5. "Application of an Intramolecular Tropone-Alkene Photocyclization to the Total Synthesis of (±) Dactylol", Ken S. Feldman, Ming-Jung Wu and David P. Rotella, *J. Am. Chem. Soc.* **111**, 6457 (1989).

6. "Chloroperoxidase Mediated Halogenation of Phenols", Cheryl F. Wannstedt, David P. Rotella and Jerome F. Siuda, *Bull. Contamin. Environ. Toxicol.* **44**, 282 (1990).

7. "Stereocontrolled Iodolactonization of *Erythro* and *Threo* Tertiary Amides", David P. Rotella and Xun Li, *Heterocycles* **31**, 1205 (1990).

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9. "Synthesis and Structural Analysis of Stereospecific 3,4,5-Trisubstituted γ-Butyrolactone Phospholipids", Xun Li and David P. Rotella, *Lipids* **29**, 211-224 (1994).

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14. "Facile Lewis Acid-Mediated Ring Opening of 4-Hydroxypyrrolidin-2-ones by Amino Acid Esters", David P. Rotella, *Synlett*, 479-480 (1996).

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16. "Neurotrophic 3,9-Bis[(alkylthio)methyl]- and -Bis(alkoxymethyl)-K-252a Derivatives", Masami Kaneko, Yutaka Saito, Hiromitsu Saito, Tadashi Matsumoto, Yuzuru Matsuda, Jeffry L. Vaught, Craig A. Dionne, Thelma S. Angeles, Marcie A. Glicksman, Nicola T. Neff, David P. Rotella, James C. Kauer, John P. Mallamo, Robert L. Hudkins, Chikara Murakata, *J. Med. Chem.* **40**, 1863-1869 (1997).

17. "An Update on COX-2 and Farnesyltransferase Inhibitor Development", David P. Rotella, *Curr. Opin. Drug Discovery and Development*, **1**, 165-174 (1998).

18. "Rank-Order of Potencies for Inhibition of the Secretion of Aβ40 and Aβ42 Suggests that Both are Generated by a Single γ-Secretase", John T. Durkin, Seetha Murthy, E. Jean Husten, Stephen P. Trusko, Mary J. Savage, David P. Rotella, Barry D. Greenberg and Robert Siman, *J. Biol. Chem.* **274**, 20499-20504 (1999).

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21. "Phosphodiesterase 5 Inhibitors: Discovery and Therapeutic Utility", David P. Rotella, *Drugs of the Future* **26**, 153-162 (2001).

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37. "The Spectral Properties of (-)-Epigallocatechin-3-O-Gallate (EGCG) Fluorescence in Different Solvents: Dependence on Solvent Polarity", Vladislav Snitsarev, Michael N. Young, Ross M. S. Miller, David P. Rotella, PLoS One, **2013**, 8(11) e79834.

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40. "Progress in the Discovery and Development of Hsp90 Inhibitors", Rohit Bhat, Sreedhar Reddy Tummalapalli, David P. Rotella, invited perspective *J. Med. Chem.* **2014**, 57, 8718-8728.

Patents:

1. "Diastereomeric Mono- and Di-Substituted Diaminocyclohexane Compounds and Methods of Preparation Thereof" Donald T. Witiak and David P. Rotella, US 5,206,400.

2. "K252a Functional Derivatives Potentiate Neurotrophin-3 Activity for the Treatment of Neurological Disorders" Marcie A. Glicksman, Robert L. Hudkins, David P. Rotella, Nicola Neff and Chikara Murakata, US 5,468,872.

3. "K252 Derivatives Which Enhance Neurotrophin-Induced Activity" Marcie A. Glicksman, Robert L. Hudkins, David P. Rotella, Nicola Neff and Chikara Murakata, US 5,516,772.

4. "Quinazolinone Inhibitors of cGMP Phosphodiesterase", David P. Rotella, John E. Macor, David Cushman, Joseph Yevich, US 6,087,368

5. "Quinoline Inhibitors of cGMP Phosphodiesterase", Yingzhi Bi, David P. Rotella, Guixue Yu, John E. Macor, US 7,378,430 .

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"Design, Synthesis and Use of Novel Diamine Templates in Medicinal Chemistry" Eli Lilly Research Laboratories, May 2013.

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Journal of
Medicinal
Chemistry

Volume 47, Number 17 August 12, 2004

Miniperspectives: Advances in Type 2 Diabetes Therapy

Novel "Second-Generation" Approaches for the Control of Type 2 Diabetes

David P. Rotella[†]

Lexicon Pharmaceuticals, 350 Carter Road, Princeton, New Jersey 08540

Received December 24, 2003

Numerous reports have documented the sharply increasing incidence of type 2 diabetes in the industrialized Western world. Recent estimates project that the number of patients diagnosed with type 2 diabetes will more than double to 300 million before 2025. Once found in primarily in middle-aged adults (hence the terminology "adult onset" diabetes), the disease is now being observed with increasing frequency in young children and adolescents. This group of patients has been reported to suffer from an increased risk of cardiovascular disease, similar to that observed in adults. Clearly type 2 diabetes is a serious chronic disease, a major health risk, and a major cause of blindness, kidney failure, amputation, and cardiovascular disease.

Current treatment approaches for type 2 diabetes include diet, exercise, and a variety of pharmacologic agents including insulin, biguanides, sulfonylureas, and thiazolidinediones (TZDs). These agents act by different mechanisms to attempt to normalize blood glucose levels and avoid the well-recognized, serious complications of diabetes that affect the kidneys, cardiovascular, ophthalmic, and nervous systems. These sequelae are directly related to the substantially increased morbidity and mortality associated with the disease. Adverse effects of these "first-generation" therapies include hypoglycemia, weight gain, and edema. Each of these can be serious, and none of the mechanisms by which these compounds act offer the potential to preserve the function of insulin-producing β-cells in the pancreas and thereby attempt to preserve endogenous glucose homeostasis and endocrine function. In many cases, monotherapy gradually fails to improve blood glucose control, and combination therapy is employed. The long-term success of this treatment paradigm varies substantially and can often be further complicated by other medications taken to help control blood pressure and to lower plasma cholesterol. Thus, there is an urgent need for novel therapeutic approaches for blood sugar control that can complement existing agents, offer new choices and combinations to physicians and patients, and possibly attempt to preserve normal endocrine responses to food intake.

The Miniperspectives that make up this series focus clearly on this issue and provide excellent surveys of some of the most active areas of diabetes research: PPARα/γ receptor agonists, GLP-1 analogues, dipeptidyl peptidase 4 (DPP4) and protein tyrosine phosphatase 1B (PTP1B) inhibitors. The following Miniperspective, written by Jay S. Skyler of the University of Miami, succinctly summarizes the serious nature of type 2 diabetes and the status and effectiveness of currently available treatments and identifies desirable characteristics of new agents.

Several pharmaceutical companies have PPARα/γ agonists in advanced stages of clinical evaluation, and Brad R. Henke of GlaxoSmithKline (an organization recognized as a leader in the field of orphan nuclear receptor research) reviews the contributions these nuclear hormone receptors play in diabetes, recent advances in the design of new agents with a range of activity at each receptor, and X-ray crystallographic information useful in the development of ligand design. The issues facing development of dual-acting agonists include a determination of the appropriate balance between α and γ

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activity and how to model this in animals and then effectively to translate this information in human use.

GLP-1 is an incretin hormone that is the most potent stimulator of endogenous insulin release known. In addition, GLP-1 is known to have beneficial effects on β-cell function, can restore insulin sensitivity, and can reduce gastric emptying and food intake, all without inducing hypoglycemia. This combination of favorable activity is tempered by the rapid metabolic degradation of the peptide. One approach to make use of the GLP-1 axis for blood sugar control is outlined by Lotte Bjerre Knudsen of Novo Nordisk, who details work being undertaken at her company to develop a metabolically stable, fatty acid modified GLP-1 analogue, liraglutide, presently in phase III trials, and exendin-4, a GLP-1 analogue, also in late-stage clinical evaluation by Eli Lilly and Amylin. Preliminary clinical evidence suggests that these compounds are safe and effective treatments for the reduction of blood sugar in type 2 diabetic patients. The primary drawback to GLP-1-based therapy is mechanism-based nausea due to delayed gastric emptying. This appears to be manageable in small-scale trials but must be carefully evaluated in larger patient populations.

An alternative approach to enhance GLP-1 activity is to inhibit its degradation by the enzyme dipeptidyl peptidase 4 (DPP4), a nonclassical serine protease. Several DPP4 inhibitors are in phase III evaluation, and Ann E. Weber of Merck Research Laboratories describes the variety of structures known to be effective DPP4 inhibitors and summarizes the early-stage clinical results that have been released by Novartis, which indicate that DPP4 inhibitors effectively and safely lower blood glucose in diabetic patients. A key question facing researchers in this field is the potential effect in humans of inhibition of DPP4 function on other systems, such as the immune system, and its role as a cellular adhesion molecule. Like dual PPAR agonists, these questions will be addressed in large-scale clinical trials. An additional benefit associated with both of these GLP-1-based therapies is weight loss, which may contribute to their ultimate clinical effectiveness and stands in contrast to the weight gain associated with both insulin and TZD use.

The final Miniperspective written by Rob Hooft van Huijsduijnen et al. of Serono Pharmaceutical Research Institute addresses a very new and exciting but challenging target, the enzyme protein tyrosine phosphatase 1B (PTP1B). This enzyme, whose potential role in the treatment of diabetes, was discovered by analysis of mice lacking the PTP1B gene in 1999. PTP1B knockout mice have increased ability to clear glucose in a glucose tolerance test, have decreased plasma insulin levels, and are resistant to weight gain when placed on a high-fat diet. The challenge facing medicinal chemists is the development and optimization of cell-permeable, selective inhibitors of PTP1B with sufficient potency to have a beneficial physiological effect. The degree of phosphatase selectivity needed to achieve a measurable effect on blood glucose while minimizing off-target effects remains to be determined. The highly charged polyanionic nature of the active site, which is very similar in phosphatase enzymes, is a primary contributor to the hurdles medicinal chemists in the field currently face. Antisense inhibition of PTP1B has shown sufficient promise in animal studies such that clinical evaluation is now underway.

David J. Triggle, the Perspective Editor for the *Journal of Medicinal Chemistry*, conceived the idea for this timely set of Miniperspectives following a session sponsored by the Division of Medicinal Chemistry at the 224th National Meeting of the American Chemical Society in New Orleans in the spring of 2003. He encouraged me to identify topics reflecting the state of the art in the field and authors who could speak authoritatively on the subject, and as readers will discover, the contributors have done an outstanding job, and we thank them for their manuscripts.

Biography

David P. Rotella earned a Ph.D. in Medicinal Chemistry at The Ohio State University under the direction of Donald T. Witiak. After postdoctoral studies at Pennsylvania State University in Ken Feldman's laboratory, he held a faculty position at the University of Mississippi School of Pharmacy for 4 years before moving to Cephalon. At Cephalon, he led the group that discovered CEP1347, a kinase inhibitor in clinical trials for Parkinson's disease. At Bristol-Myers Squibb, his team was the first to publish on PDE5 inhibitors more potent and selective than sildenafil. Currently, he is a senior group leader at Lexicon Pharmaceuticals in Princeton, NJ, where he is responsible for a drug discovery program in obesity. He has published over 20 peer-reviewed papers, is a co-inventor on six patents, and has written several invited reviews.

JM030626A

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

ASTRAZENECA AB, Plaintiff, v. AUROBINDO PHARMA LTD. and AUROBINDO PHARMA U.S.A., INC., Defendants.	C.A. No. 14-664-GMS (CONSOLIDATED) **CONFIDENTIAL INFORMATION - SUBJECT TO PROTECTIVE ORDER**

REBUTTAL EXPERT REPORT OF ANN E. WEBER, PH.D.

DATED: March 2, 2016

Ann E. Weber

Ann E. Weber, Ph.D.

EXHIBIT #61
WIT: ROTELLA
DATE: 5/26/16
TOM FRASIK, CSR 6961

TABLE OF CONTENTS

I. ACADEMIC AND PROFESSIONAL QUALIFICATIONS

A. Current Position

1. I am a chemist with more than 28 years of professional experience in organic chemistry and medicinal chemistry.

2. I am currently an independent consultant on drug discovery, clinical candidate nomination, and early development. A copy of my *curriculum vitae* is attached as Exhibit A.

B. Education and Experience

3. I obtained a Bachelor of Science degree in chemistry from the University of Notre Dame in 1982, graduating *summa cum laude*. I received my Ph.D., as a NSF pre-doctoral fellow, in organic chemistry from Harvard University in 1987.

4. Immediately upon receiving my Ph.D., I joined Merck & Co. as a Senior Research Chemist, and stayed with the company until retiring as Vice President in November 2015. I was promoted to Research Fellow in December 1991, and later to Associate Director of Medicinal Chemistry in November 1994 and Director of Medicinal Chemistry in November 1997. From 1987 to 2000, my research focused on the design and synthesis of conformationally-restricted renin inhibitors for the treatment of hypertension, as well as human selective β3 adrenergic receptor agonists for the treatment of obesity.

5. In January 2000, I assumed responsibility for the chemistry effort and co-led the discovery program on Dipeptidyl Peptidase 4 ("DPP-4") inhibitors. This program led to the identification of sitagliptin (JANUVIA®), the first marketed DPP-4 inhibitor for the treatment of patients with type 2 diabetes. Following the discovery of sitagliptin, I continued to lead Merck's DPP-4 inhibitor development program as Senior Director of Medicinal Chemistry. Through this program, omarigliptin (MARIZEV®) was identified as a once-weekly DPP-4 inhibitor, which has recently been approved in Japan for the treatment of type 2 diabetes.

6. I was promoted to Executive Director of Medicinal Chemistry in July 2005, and to Vice President in February 2010. Between July 2005 and my retirement from Merck & Co. in November 2015, I oversaw medicinal chemistry discovery programs in the fields of diabetes, urinary incontinence, pain, cardiovascular disease, infectious diseases, oncology, asthma, and neurological disorders. Over the course of my career, these efforts led to the identification of over 40 clinical candidates, including one late stage clinical candidate, vibegron, for urinary incontinence and two marketed drugs (JANUVIA® and MARIZEV®) for diabetes.

7. I have published extensively in the scientific literature and have authored or co-authored over 80 scientific articles and book chapters relating to organic synthesis and medicinal chemistry. I have authored or co-authored 35 articles specifically on the synthesis and evaluation of DPP-4 inhibitors. I am also a named inventor on over 30 issued U.S. patents involving a variety of small molecule drugs, including 24 patents specifically drawn to DPP-4 inhibitors and their use for the treatment of diabetes. A list of my publications and patents is included in Exhibit A.

8. I have been invited to deliver more than 50 presentations at national and international scientific conferences and universities. I served as session chairs for "Med Chem Lunch & Learn. From Bench to Bedside: JANUVIA® (sitagliptin), a Selective DPP-4 Inhibitor for the Treatment of Diabetes" during the 232nd American Chemical Society National Meeting in 2008; "New Approaches to the Treatment of Type 2 Diabetes" during the 30th Medicinal Chemistry Symposium in 2006; and "DPP-IV inhibitors" during the 228th American Chemical Society National Meeting in 2004.

9. I served on the editorial advisory board of *ACS Medicinal Chemistry Letters* from 2009 to 2014. I was also a member of Industrial Advisory Board of the Department of

Chemistry & Chemical Biology of Rutgers, The State University of New Jersey from 2009 to 2015.

10. I have received various professional honors and awards throughout my career. For example, I was a 2013 Liberty Science Center Women in STEM honoree and the recipient of the 2015 Gift of Mentoring Award from Metro Women Chemists Committee of the American Chemical Society, the 2011 Science and Technology Medal from the Research and Development Council of New Jersey, the 2011 Industrial Award from Philadelphia Organic Chemists' Club, the 2011 Discoverer's Award from Pharmaceutical Research and Manufacturers of America, the 2010 Heroes of Chemistry Award from the American Chemical Society, the 2010 Robert M. Scarborough Award for Excellence in Medicinal Chemistry from American Chemical Society, the 2008 Outstanding Women in Science from New Jersey Association for Biomedical Research, the 2007 Thomas Alva Edison Patent Award from Research and Development Council of New Jersey, the 2007 Merck Directors' Award, and the 2002 Women at the Forefront Award from the American Chemical Society Women Chemists Committee.

II. INSTRUCTIONS FROM COUNSEL

11. I have relied on the lawyers representing Plaintiff AstraZeneca AB ("AstraZeneca") in this case for the applicable legal standards governing my analyses and opinions. The legal standards that were provided to me are set forth below.

12. I understand that the patent law requires that a patent applicant is entitled to a patent unless the claimed invention would have been obvious to a person of ordinary skill at the time of the invention. I understand that in considering this issue, I must consider 1) the level of skill in the art, 2) scope and content of the prior art, 3) differences between the claimed invention and prior art, and 4) objective evidence of non-obviousness. I have been told by AstraZeneca's counsel to assume the "time of the invention" mentioned above to be no later than February 16,

2001. None of the art cited by Dr. Powers or Dr. Rotella falls between October 2000 and the filing date of February 16, 2001. Therefore, my opinions would not differ depending on which date applies. I reserve the right to rely on the October 2000 date should it become important.

13. I understand that for a *prima facie* case of obviousness, structural similarity between the claimed compound and the prior art compound is not enough, the prior art must also have suggested making the specific modifications necessary to achieve the claimed invention.

14. I understand that a "lead" compound is one that is most promising to modify and that selection of a lead compound is guided by all of the compound's pertinent properties.

15. I understand that the analysis includes whether the prior art supplied a person of skill in the art with a reason or motivation to select a lead compound and a reason or motivation to modify the lead compound to make the claimed compounds with a reasonable expectation of success.

16. I further understand that I must not use hindsight in my analysis, and that I must consider the claimed invention as a whole to determine whether or not it would have been obvious in light of the prior art.

17. I also understand that various other factors, or "objective evidence," should be considered in connection with the obviousness analysis. Some of these factors include: 1) a long-felt but unsolved need for the claimed invention; 2) the failure of others in the prior art to fill this need; 3) unexpected or surprising results of the claimed invention; 4) skepticism as to the inventor's chances for success; 5) industry praise for the invention; and 6) commercial success of the claimed invention.

18. Finally, I understand a party challenging the validity of a patent for alleged obviousness must present clear and convincing evidence of invalidity, which I understand

requires evidence which produces in the mind of the trier of fact an abiding conviction that the truth of the factual contentions are highly probable.

III. ANTICIPATED TESTIMONY

19. I expect to testify at the request of AstraZeneca as an expert in the field of medicinal chemistry. I have been asked to provide my opinion regarding the training and qualifications of a person of ordinary skill in the art to whom AstraZeneca's U.S. Patent No. RE44,186 ("the RE'186 patent") is addressed. I have also been asked to provide my opinion on a variety of issues, as set forth fully below.

20. I have been asked to respond to the Expert Report of James C. Powers, Ph.D., submitted in this litigation on behalf of Defendants. (*See* Expert Report of James C. Powers dated January 28, 2016 ("Powers Report").)

21. I have also been asked to respond to the Expert Report of David P. Rotella, Ph.D., submitted in this litigation on behalf of Defendant Mylan Pharmaceuticals Inc. (*See* Expert Report of David P. Rotella dated January 29, 2016 ("Rotella Report").)

22. More specifically, I have considered Dr. Powers' and Dr. Rotella's opinions regarding whether claims 8, 9, 25, and 26 of the RE'186 patent are invalid as obvious. As explained below, I disagree with Dr. Powers' and Dr. Rotella's opinions that claims 8, 9, 25, and 26 of the RE'186 would have been obvious and certainly do not view those opinions as clear and convincing evidence of invalidity. Rather, it is my opinion that the inventions of claims 8, 9, 25, and 26 would not have been obvious to a person of ordinary skill in the art on or before October 2000.

23. I have also been asked to provide the bases and reasons supporting my opinions.

24. In reaching these opinions, I have considered the Expert Reports of Dr. Powers and Dr. Rotella, and the materials listed in the Appendices of those reports. I have also

considered Defendants' Invalidity Contentions and the materials referenced therein. I considered the RE'186 patent and its prosecution history. A list of the materials that I considered in developing this report is attached as Exhibit B.

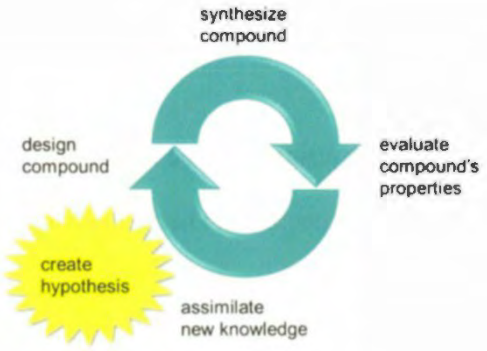
IV. BACKGROUND

A. Medicinal Chemistry

25. Medicinal chemistry is inherently a multidisciplinary subject that deals with the design, synthesis, evaluation, and development of chemical compounds which exert beneficial effects upon living systems. Medicinal chemists must have a firm understanding of organic and synthetic chemistry as well as knowledge of other disciplines such as biological, medical, and pharmaceutical sciences.

26. Medicinal chemists study relationships between the structure of a particular compound or group of compounds and their properties, including their interactions with biological systems. These relationships are called structure-activity relationships ("SAR"). The rationale behind SAR is that the structure of a chemical implicitly determines its physical and biological properties.

27. SAR studies involve an iterative process of compound design, compound synthesis, compound evaluation (physiochemical and biological properties), knowledge assimilation, and hypothesis generation.

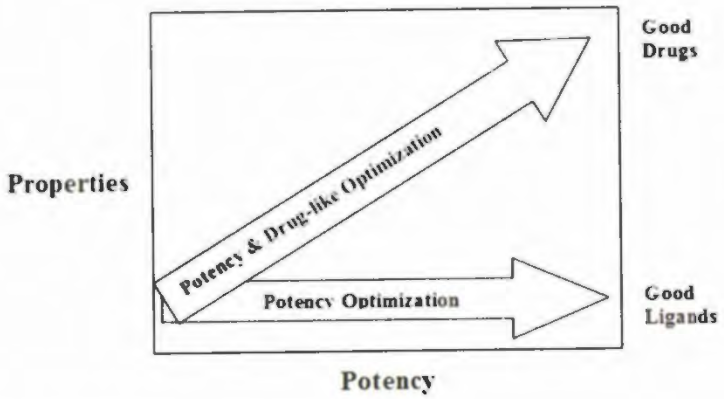


28. One of the properties typically evaluated first is a compound's potency. Potency is the molar concentration required to produce a given response, *e.g.*, for an inhibitor to block the ability of an enzyme like DPP-4 to cleave a substrate or pseudo-substrate such as Gly-Pro-*para*-nitroanilide. For enzyme inhibitors, it is typically expressed as K_i or IC_{50}. K_i is the inhibition constant, which is a measure of the strength of the enzyme-inhibitor complex, E•I. The lower the K_i, the more favorable is the formation of the complex relative to the free enzyme "E" and free inhibitor "I," and hence the more potent the inhibitor. (*Burger's Medicinal Chemistry and Drug Discovery*, 113-128; 738-761, 738 (Wolff ed., John Wiley & Sons 1995).) The IC_{50} is the concentration of inhibitor that blocks half of the enzyme's activity and is typically measured by keeping the concentration of the substrate and enzyme constant and varying the concentration of the inhibitor. IC_{50} is directly related to K_i. (*Id.* at 745.) Like K_i, the lower the IC_{50}, the more potent the inhibitor.

29. The goal of the medicinal chemist is to optimize not only the pharmacological properties, such as potency, but also the drug-like properties of the molecules in order to identify a compound suitable for therapeutic use. Drug-like properties of interest include *e.g.*, hydrogen bonding, lipophilicity, molecular weight, pK_a, polar surface area, shape, reactivity, solubility, permeability, chemical stability, metabolism, protein and tissue binding, transport, clearance, half-life, bioavailability, drug-drug interaction, and acute toxicity (LD_{50}). Importantly, the SAR controlling potency at the target of interest need not be in any way related to the SAR for, *e.g.*, safety or pharmacokinetic properties.

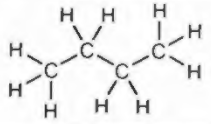
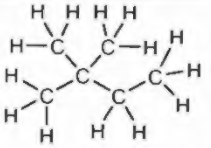
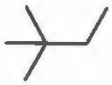
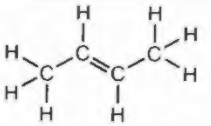
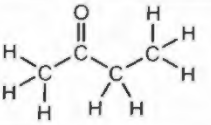
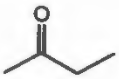
30. A focus on potency may result in a good ligand (binder), but poor drug-like properties. In contrast, a less potent compound with better drug-like properties may produce a

better drug. The best development candidates typically balance potency with other drug-like properties.

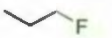


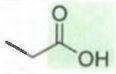
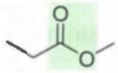
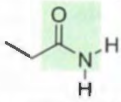
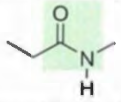
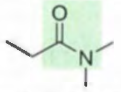
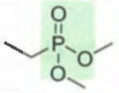
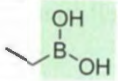
B. Basic Chemistry Nomenclature

31. Medicinal chemists communicate using structural formulas. A structural formula shows the arrangement of atoms in the molecule of a compound. In the classical Kekulé structures, all atoms within a compound are shown, and each line between atoms represents the two electrons in a covalent bond. As shorthand, chemists often do not show carbon and hydrogen atoms unless for emphasis or clarity, but all other atoms other than carbon and hydrogen are indicated. Further, chemists often use condensed formulas to communicate structural information. A condensed formula is made up of the elemental symbols, where the order of the atoms suggests the connectivity. Brackets or parentheses may be used to reduce ambiguity. When used, these show that the polyatomic group within a formula is attached to the nearest non-hydrogen atom on the left. Kekulé and shorthand structures for several exemplary compounds along with the condensed formulas for each are shown below.

Kekulé Structure	Shorthand Structure	Condensed Formula
		$CH_3CH_2CH_2CH_3$
		$CH_3C(CH_3)_2CH_2CH_3$
		$CH_3CHCHCH_3$
		$CH_3C(O)CH_2CH_3$

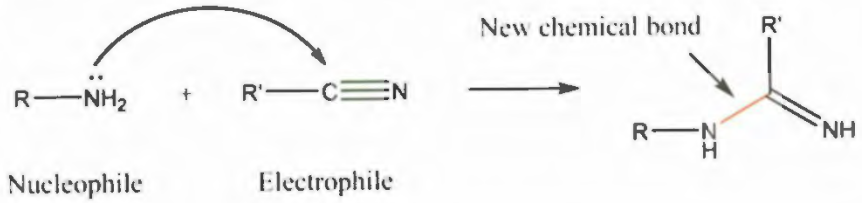
32. The compounds studied by medicinal chemists often have one or more functional groups. A functional group is a small structural unit within a larger molecule and is composed of an atom or group of atoms whose bonds have a characteristic chemical behavior. Functional groups contribute to a compound's properties. Examples of functional groups are shown below.

Family Name	Functional Group Example
Alcohol (Hydoxyl)	
Halide (Halo)	
Nitrile (Cyano)	
Ether	
Sulfide	
Amino	 Primary Amine

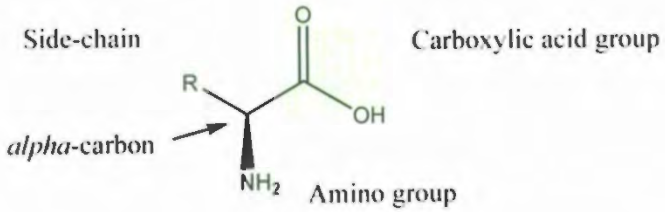
Family Name	Functional Group Example
	 Secondary Amine
	 Tertiary Amine
Carbonyl	
Carboxylic Acid	
Ester	
Amide	 Primary Amide
	 Secondary Amide
	 Tertiary Amide
Phosphonate	
Boronic acid	

33. Some functional groups can serve as nucleophiles and others as electrophiles. Nucleophiles are electron rich; electrophiles are electron deficient. A nucleophile, such as an amine or a hydroxyl group, donates electrons to an electrophile, such as a cyano group or a carbonyl group, to form a chemical bond. The ability to form such a chemical bond is dependent on the properties of the nucleophile and electrophile, including the electronic and steric

properties of each. For example, the more sterically hindered these groups are, the less likely they are to react.

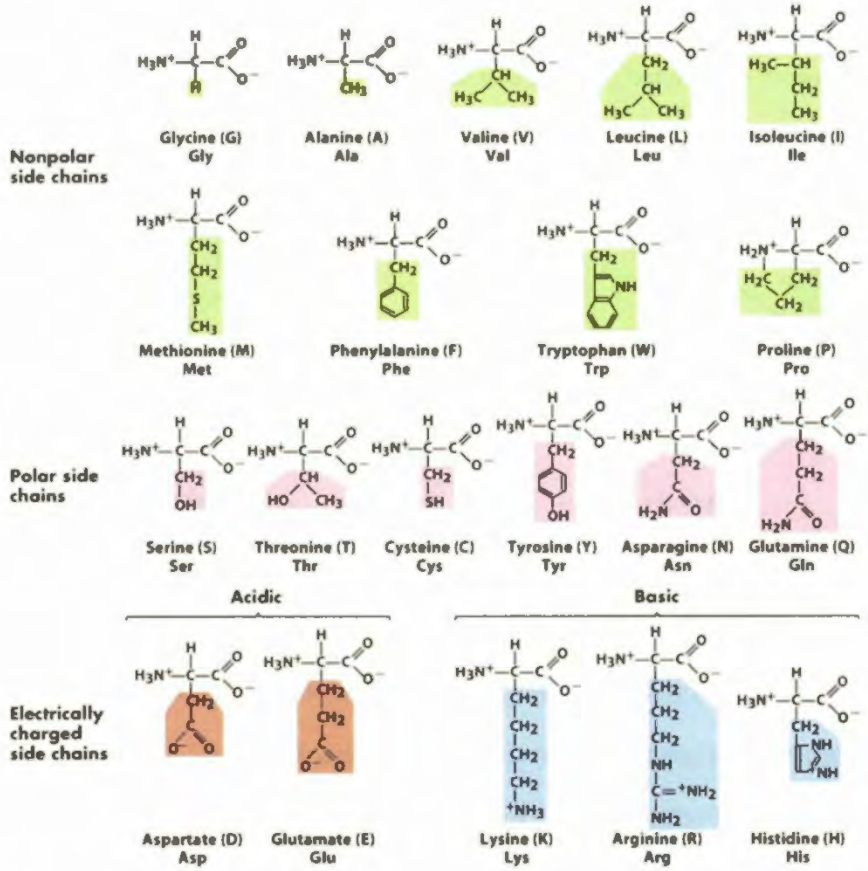


34. Functional groups may be combined to form more complex compounds. Amino acids (a combination of a basic amino group, an acidic carboxy group, and a side-chain) are representative of such compounds.

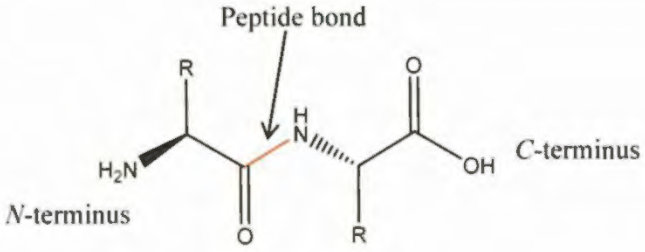


35. Amino acids are the building blocks of proteins. The structure of the R-group attached to the central, or *alpha*-carbon, atom determines the chemical properties of the amino acid and, in-turn, the protein. The change of a single amino acid can alter the function of a protein. For example, sickle cell anemia is caused by a change of one amino acid from glutamine to valine.

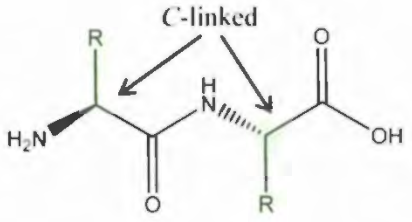
36. The 20 amino acids that are commonly found within proteins, their abbreviations, and some of the chemical properties of each are shown below.



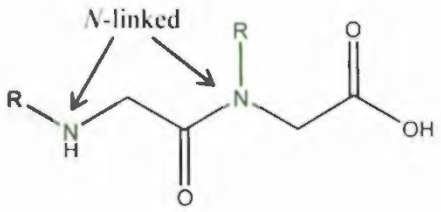
37. When the amino group of one amino acid is joined with the carboxylic acid group of a second amino acid, the resultant compound is referred to as a peptide. The bond formed is called a peptide bond, and the resultant peptide has two ends, a *C*-terminus (carboxylic acid end) and an *N*-terminus (amino end). Peptides containing more than about fifty amino acids are generally referred to as proteins.



38. In addition to modifying the R-group, medicinal chemists have investigated peptoids, or poly-*N*-substituted glycines as a way to alter structure and function. The side chain is connected to the nitrogen of the peptoid backbone instead of the *alpha*-carbon as in peptides.

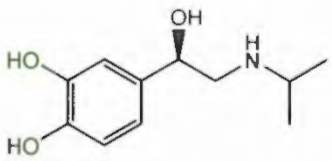


Peptide

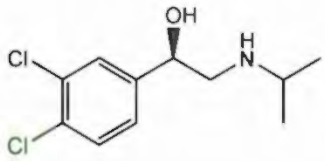


Peptoid

39. Alterations in a functional group, such as, *e.g.*, replacing a hydrogen atom with a hydroxyl group, replacing a hydroxyl group with a halo group, changing the length, location, or nature of an R group, and changing ring size, can have profound effects on not only the physico-chemical properties of a compound, but also on its pharmacologic activity. This important point is illustrated by Powell's classic finding that isoproterenol is a β-adrenergic receptor ***agonist*** (*i.e.*, activates the β-receptor), whereas changing the 2 hydroxyl groups to chloro groups results in the dichloro analog, dichloroisoproterenol, a β-adrenergic receptor ***antagonist*** (*i.e.*, blocks the responses of agonists on the β-receptor). (*See* Powell and Slater, *Blocking of inhibitory adrenergic receptors by a dichloro analog of isoproterenol*, J. Pharmacol. Exp. Ther., 122:480-488, 487-488 (1958).)



β-Agonist
(activates receptor)



β-Antagonist
(inhibits receptor activation)

C. Stereoisomers

40. The properties of a compound are dependent not only on what functional groups are present in the molecule but also on the spatial arrangement of these groups. "[T]he ensemble of steric and electronic features that is necessary to ensure the optimal supra-molecular interactions with a specific biological target and to trigger (or block) its biological response" is referred to as a pharmacophore. (Wermuth et al., *Glossary of terms used in medicinal chemistry*, Pure & Appl. Chem., 70:1129-1143, 1140 (1998).) These steric and electronic features include *e.g.* electrostatic interactions, H-bonding, aromatic interactions, hydrophobic regions, and coordination to metal ions.

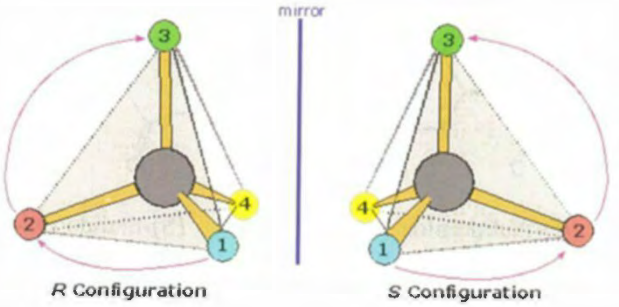
41. The spatial arrangement of the groups within a pharmacophore is especially important when a compound is to be used as a medicament, where the compound is likely to be subjected to an asymmetric environment because proteins and other biologic targets exist in a particular three-dimensional arrangement in the human body. Productive binding interactions with the biological target (*e.g.*, receptor) will not be possible if crucial functional groups do not occupy the proper spatial region. The absence of productive binding interactions can potentially negate the desired pharmacologic effect.

42. The wedge and dash notation is used to represent the spatial arrangement of a compound using a two-dimensional surface. In a wedge and dash notation, solid lines represent chemical bonds in the plane of the surface, block wedges represent chemical bonds coming out of the plane toward you, and dashed lines represent bonds that extend out of the plane away from you.

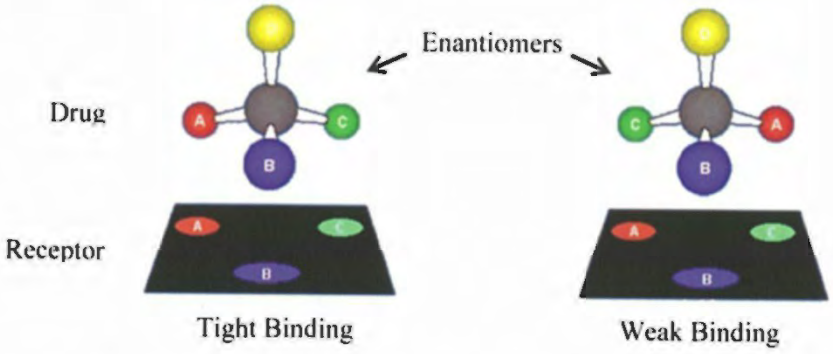
comes *forward* out of the plane of the page
goes *backward* out of the plane of the page

43. Stereoisomers are compounds containing the same number and kind of atoms and the same arrangement of bonds, but in a different spatial arrangement (three-dimensional structures). Stereoisomers can be divided into two subtypes: enantiomers and diastereomers. Enantiomers are stereoisomeric compounds where the three-dimensional arrangement of atoms is such that the compounds are non-superimposable mirror images. Diastereomers include all other stereoisomeric compounds.

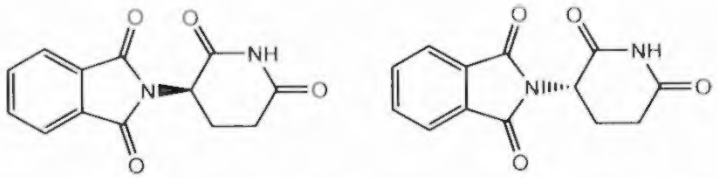
44. In order for a molecule to exist as an enantiomer, it must have a chiral center. A chiral center is characterized by an atom (usually carbon) that has four different groups bonded to it – provided it is not superimposable on its mirror image. The Cahn-Ingold-Prelog (CIP) descriptors *R* and *S* are assigned to each chiral center according to whether its configuration is right- or left-handed. To assign these descriptors, the substituent atoms are prioritized in the order of decreasing atomic number. If two substituents have the same immediate substituent atom, the next attached atoms in those substituents are evaluated until a difference is found. Further, an atom that is multiply bonded is treated as an equivalent set of single-bonded atoms. Once the relative priorities have been determined, the molecule is viewed in an orientation which places the lowest priority group behind the viewing surface. The remaining substituents, when counted from highest to lowest priority, will be arranged in either a clockwise or counterclockwise manner. If the turn is clockwise, the configuration is classified as *R* (for Latin rectus, right-handed). If the turn is counterclockwise, the configuration is classified as *S* (for Latin sinister, left-handed).



45. Enantiomers have identical chemical and physical properties in an achiral environment; however, because the biological environment is mostly chiral, enantiomers will generally display different properties when administered. This is because the molecular configuration of the drug must fit in the receptor site, and typically only one of the enantiomers will do so. (*See* illustration below.)



46. Frequently, only one enantiomer is active, and sometimes the other one is toxic. The thalidomide tragedy of 1961 illustrates this point. Thalidomide exists as two enantiomers, shown below. The only difference between the two enantiomers is the positioning of the functional groups. While the R isomer is an effective sedative, the S enantiomer exerts an embryotoxic and teratogenic effect. (Muller et al., *A concise two-step synthesis of thalidomide*, Org. Process Res. Dev., 3:139-140, 139 (1999).)



(R)-thalidomide
(Sedative)

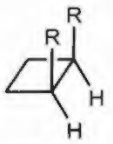
(S)-thalidomide
(Teratogen)

47. Diastereomers also have chiral centers. The specification of the configuration of a diastereomer is done in the same manner as described above. Each chiral center is designated with the CIP descriptors, *R* or *S*, by application of the sequence rules. Generally, a structure will have 2^n possible combinations of stereoisomers where n is the number of chiral centers in the molecule. Diastereomers can have quite different physical and chemical properties and, for the reasons discussed above, usually display different properties when administered.

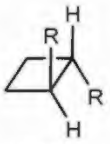
48. The same CIP precedence rules apply to describe stereoisomeric olefins (*i.e.*, double bonds) having two, three, or four substituents. If both higher precedence substituents are on the same side of the double bond, the configuration is *Z* (for German zusammen, together). If the higher precedence substituents are on opposite sides, the configuration is *E* (for German entgegen, opposite). Chemists often refer to the *Z* configuration as the *cis*-isomer and the *E* configuration as the *trans*-isomer. The *cis-trans* naming convention is also used to describe the relative position for atoms relative to a reference plane in cyclic systems. In the *cis*-isomer, the atoms are on the same side. In the *trans*-isomer, they are on opposite sides.

(Z) or *cis*

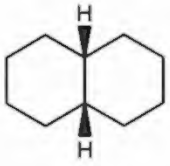
(*E*) or *trans*



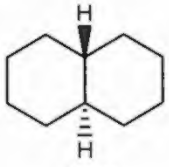
cis



trans



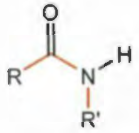
cis



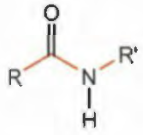
trans

49. Interconversion between *cis* and *trans* stereoisomers, like the above stereoisomers, can only be done by breaking and reforming chemical bonds. Unlike stereoisomers, other isomers, called conformational isomers, conformers, or rotamers, can be interconverted exclusively by rotations about single bonds. The interconversion of conformers is dependent upon the rotational energy barrier, taking into account the through-space steric interactions of substituents, which must be small enough to interconvert one conformer to another.

50. The secondary and tertiary amide groups can take either the *cis* or *trans* conformation or be a mixture of both, which is dependent, in part, on both steric and electronic factors. In the *trans* conformation, the two carbon atoms (represented by R and R' below) are on the opposite sides of the central C-N bond. In the *cis* configuration, the two carbon atoms are on the same side of the C-N bond. When the amide nitrogen has two carbon atoms, the CIP rules apply to determine which *alpha*-carbon has the highest priority for determining *cis* or *trans*.



cis conformer

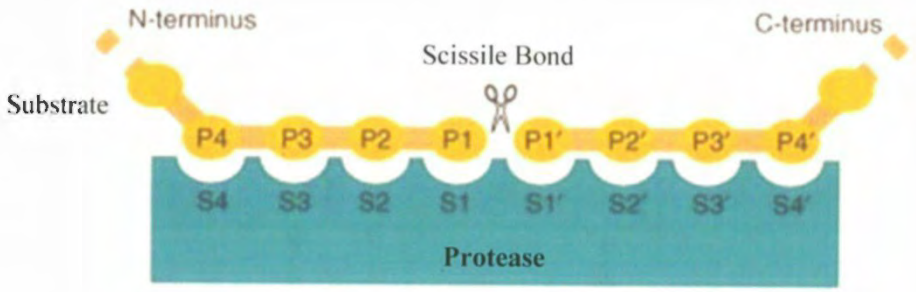


trans conformer

D. Proteases

51. A protease is an enzyme that catalyzes (speeds up) the cleavage of peptide bonds in a protein or peptide (*i.e.*, substrate). Proteases with peptide substrates are typically referred to as peptidases.

52. There is a special nomenclature that is commonly used to describe the interactions of the peptide substrate and the protease active (catalytic) site. The active site of the protease is composed of sub-sites on either side of the scissile (cleaved) bond. Each sub-site accommodates an amino acid of the peptide substrate. The sub-sites in the protease (S) are numbered S1, S2, *etc.*, from the active site towards the *N*-terminus of the substrate, and S1', S2', *etc.*, from the active site towards the *C*-terminus of the substrate. The amino acid residues within the peptide (P) substrate are similarly numbered P1, P2, etc. from the scissile bond towards the *N*-terminus and P1', P2', *etc.*, from the scissile bond towards the *C*-terminus. (Schechter and Berger, *On the size of the active site in proteases. I. Papain*, Biochemical and Biophysical Research Communications, 27:157-162, 157, 159 (1967).)



53. The lock-and-key hypothesis is a simplified model for understanding the interaction of a protease with its peptide substrate. (Voet and Voet, *Biochemistry* (John Wiley & Sons, 2nd ed. 1995) at 333-334.) It holds that "*[t]he specificity of an enzyme (the lock) for its substrate (the key) arises from their geometrically complementary shapes.*" The binding

interactions between the complementary shapes may include electrostatic forces, dispersion (van der Waals) forces, hydrophobic interactions, and hydrogen bonds (described below). These interactions are responsible for the substrate specificity of the protease. The amino acid residues of the protease are ideally arranged to complement the amino acid residues of the protease's substrate(s). Peptides that are not complementary to a protease's binding site do not bind and are thus not cleaved. These same interactions govern the binding of a protease with an inhibitor. (Burger's at 739-740; Voet and Voet at 333-334.)

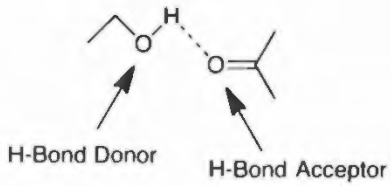
54. Electrostatic forces are interactions between charged atoms of opposite polarity. (Burger's at 739.) One example of an electrostatic interaction is the interaction between a protonated amine (a positively charged moiety) in a protease inhibitor and a carboxylate anion (a negatively charged moiety) on a glutamic acid residue of a protease.

55. Dispersion forces, often referred to as van der Waals interactions, are interactions that result from induced dipoles (temporary polarization) between non-polar moieties which causes an attraction between them. These forces are considered weak, but additive. (Burger's at 739.)

56. Hydrophobic interactions arise from a non-polar, lipophilic moiety in an inhibitor preferring to be in a non-aqueous environment. A complementary lipophilic binding site in a protease serves as such an environment, and this interaction is energetically more favorable than the moiety's interaction with water in an unbound state. (*Id.*)

57. Hydrogen bonding interactions arise from the sharing of a proton (hydrogen cation) between two electronegative atoms. Generally, the distances between the hydrogen and the electronegative atoms are not the same, and the shorter one is more of a typical covalent bond

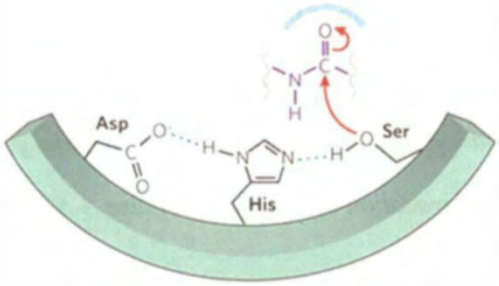
(the hydrogen bond donor) and is represented by a straight line. The longer one (to the hydrogen bond acceptor) is represented as a dashed line. (*Id.*)



58. Proteases can be divided into classes based on whether they cleave internal peptide bonds (endopeptidases) or terminal peptide bonds (exopeptidases). (Hooper, *Proteases: a primer*, Essays in Biochem. 38:1-8, 4 (2002).) Within exopeptidases, there are those that cleave *C*-terminal peptide bonds (carboxypeptidases) and those that cleave *N*-terminal peptide bonds (aminopeptidases). (*Id.*)

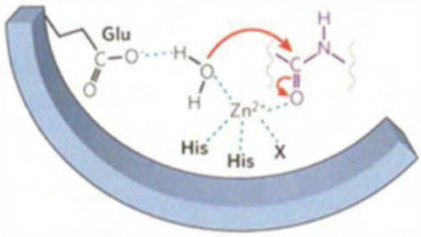
59. Proteases can also be classified based on the mechanism of cleaving the peptide bond, with each type having its own unique set of chemical groups responsible for catalysis. Proteases fall into four main mechanistic classes: serine, cysteine, aspartyl, and metalloproteases. (Rawlings and Barrett, *Evolutionary families of peptidases*, Biochem. J., 290: 205-218 (1993).)

60. For serine proteases, the chemical groups responsible for peptide bond cleavage are the side-chains of three amino-acids: Ser, His, and Asp. (De Meester et al., *CD26, let it cut or cut it down*, Immunol. Today, 20:367-375, 367 (1999).) The hydroxyl on the side-chain of the Ser serves as the nucleophile in the cleavage reaction. (*Id.*)



Serine Protease (*e.g.*, DPP-4)

61. For metalloproteases, the chemical groups include a metal, such as zinc, at the active site that is bound to two His side-chains and one Glu side-chain. In contrast to serine proteases, a water molecule, rather than a side-chain functional group on the protease, serves as the nucleophile in the cleavage reaction.

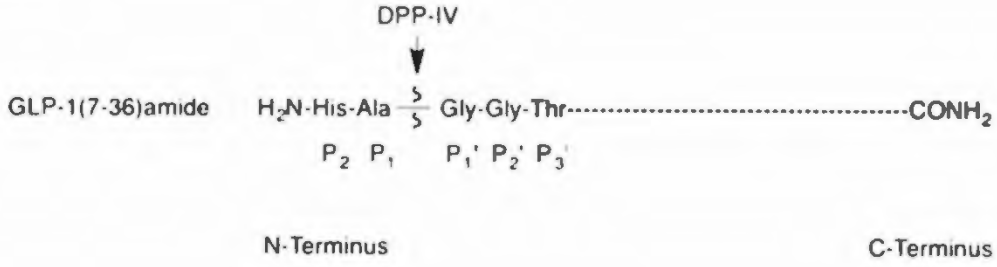


Metalloproteases (*e.g.*, ACE)

62. In serine proteases, the S1 site is generally responsible for substrate specificity. In contrast, the S1' site in metalloproteases is generally responsible for the substrate specificity. (Cushman and Ondetti, *Design of angiotensin converting enzyme inhibitors*, Nat. Med. 5:1110-1112, 1110-1111 (1999).)

63. The differences between serine proteases and metalloproteases are illustrated by the differences between DPP-4 and Angiotensin Converting Enzyme (ACE). DPP-4 is a serine protease classified as an aminodipeptidase because it cleaves the *N*-terminal two amino acids of its substrates (*e.g.*, GLP-1(7-36)amide). DPP-4 shows a strong preference for cleavage of

peptides containing a proline amino acid in the "P1" position, though its natural substrates may also contain alanine ("Ala") at this position. (Mentlein et al., *Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum*, Eur. J. Biochem., 214: 829-835 (1993).)



64. In contrast, ACE, a carboxypeptidase, is a zinc metalloprotease that cleaves the C-terminal two amino acids of its substrates (*e.g.*, angiotensin I and bradykinin). (Cushman and Ondetti at 1110.)

E. DPP-4 Was an Emerging Medicinal Drug Target in the Late 1990s

1. DPP-4 in Type 2 Diabetes

65. DPP-4 was one of a number of "emerging" drug targets for treating type 2 diabetes in the late '90s. Over the years, other such targets included, *e.g.*, GPR119, GPR40, TGR5, SGLT2, SIRT1, Glycokinase, DGAT1, PTP1B, 11b-HSD1, SCD1, FBPase, Glycogen Phosphorylase, Glucagon Receptor, and ACC. (Kemp, *Chapter 1: Type 2 diabetes: disease*

overview, 1-14, 8 (Table 1.2) (Jones ed., Royal Society of Chemistry 2012).) Of the targets for type 2 diabetes that medicinal chemists have explored and which entered clinical testing over the last 15 years, only 2 classes of oral agents have proven successful and ultimately achieved FDA-approval (DPP-4 inhibitors and later SGLT-2 inhibitors), demonstrating the general unpredictability in drug discovery in this area. (*Id.*)

66. DPP-4 is an enzyme that regulates glucose homeostasis by inactivating incretin peptide hormones that control insulin release. When blood glucose levels are high, *e.g.*, after a meal, the incretin hormone glucagon-like peptide 1 (GLP-1[7-36]amide or "GLP-1") stimulates insulin secretion in the gut and inhibits glucagon release. (Weber, *Dipeptidyl peptidase IV inhibitors for the treatment of diabetes*, J. Med. Chem., 47:4135-4141, 4135 (2004).) In the late 1990s, GLP-1 was considered a potential therapeutic agent for type 2 diabetes; however, its short half-life when administered *in vivo* limited the potential of directly administering GLP-1 itself as a therapy. (*See* Holst and Deacon, *Inhibition of the activity of dipeptidyl-peptidase IV as a treatment for type 2 diabetes*, Diabetes, 47:1663-1670, 1663-1664 (1998).)

67. DPP-4 acts on GLP-1 and rapidly degrades it to an inactive form (GLP1[9-36]amide). (*Id.* at 1663.) Therefore, inhibition of DPP-4, which leads to an increase in circulating levels of endogenous GLP-1, was thought to be a potential alternative target for treating patients with type 2 diabetes. (*Id.* at 1663-1664.) DPP-4 has effects beyond GLP-1, and potentiation of another incretin hormone, glucose-dependent insulinotropic peptide (GIP), was also thought to contribute to the activity of DPP-4 inhibitors. A schematic of DPP-4 inactivation of GLP-1 and other potential substrates is shown below. (Weber at 4315, Figure 1 reproduced below.)

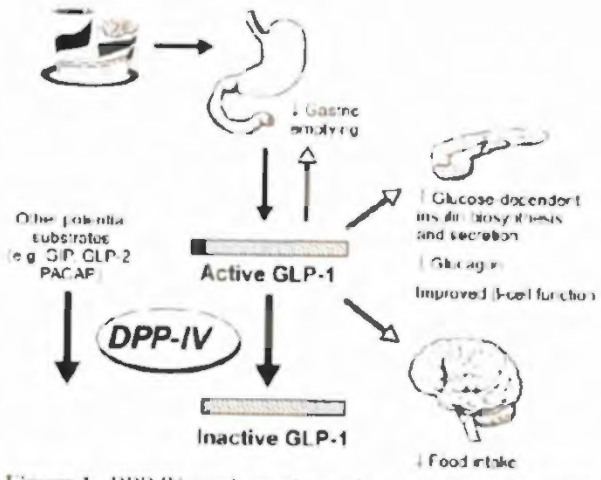


Figure 1. DPP-IV regulates glucose homeostasis via inactivation of GLP-1 and other incretin hormones

68. In the late 1990s, there were still many unknowns with respect to safety and efficacy of DPP-4 inhibitors in type 2 diabetes and "many questions [remained] unanswered." (Holst and Deacon at 1669.) Unknowns included side effects due to mechanism-based toxicities, side effects due to non-mechanism-based toxicities (off-target or compound-specific toxicities), and long-term safety and efficacy with DPP-4 inhibition and protection of endogenous GLP-1 levels. (*Id.* at 1664-1668.)

69. DPP-4 itself was known to be ubiquitously expressed in the body. (De Meester at 367-368.) DPP-4 is identical to the cell-membrane associated protein called "CD26" found on activated T cells of the immune system. (*Id.* at 367.) CD26 (or DPP-4) is strongly expressed on epithelial cells (kidney, intestine, and bile duct), and on several types of endothelial cells, fibroblasts, and leukocyte subsets. (*Id.* at 368.) In addition to the cell-associated form, a soluble form of DPP-4 is found in the circulation. (*Id.*) Thus, the potential impact of DPP-4 inhibition on its active participation in the immune, nerve, and endocrine networks in human physiology

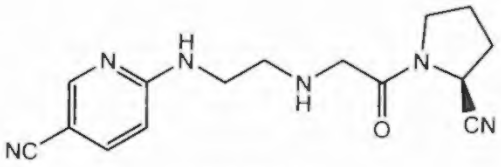
raised serious concerns. Such concerns and unknowns about DPP-4's many activities were reflected in the literature at the time:

> The presentation of the multifaceted function of DPP IV, with potential involvement in regulatory circuits, immune function, nutrition, cell adhesion and peptide hormone metabolism, as a "guardian angel" is, given the actual state of knowledge, to be understood only as a working hypothesis. Although not validated, it is intended as a cautious comment on attempts to use specific inhibitors of DPP IV enzymic [sic] activity for therapeutic purposes, e.g. Type II diabetes [32,96], in view of the broad implications that this may have for circuits that are only partly understood. To date, no human disease has been reported to be specifically associated with a loss of DPP IV expression. Given the highly conserved structure of DPP IV in evolution and, perhaps, redundancy of the specific post-proline cleavage activity provided by very few other enzymes, DPP IV activity may be too important as a "guardian angel" to be lost or abrogated by the use of selective inhibitors.

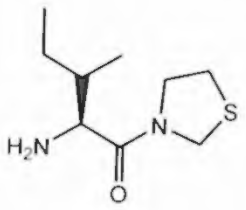
(Hildebrandt et al., *A guardian angel: the involvement of dipeptidyl peptidase IV in psychoneuroendocrine function, nutrition and immune defence*, Clinical Science, 99:93-104, 100 (2000).)

70. In 2000, only two DPP-4 inhibitors had entered the clinic as candidate therapies for type 2 diabetes. Novartis's compound NVP-DPP728 was reported in a phase I clinical study to increase prandial active GLP-1 levels and reduce prandial glucose excursion without causing hypoglycemia or causing significant adverse events in humans at a single dose of 100 mg. (Rothenberg et al., *Treatment with a DPP-IV inhibitor, NVP-DPP728, increases prandial intact GLP-1 levels and reduces glucose exposure in humans*, Diabetes, 49(supp.1):A39 (160-OR) (2000).) Rothenberg concluded that these data "support the investigation of the glucose-lowering potential of NVP-DPP728 for the treatment of type 2 diabetes." (*Id.*) A compound from Probiodrug, P32/98 (Ile-Thia), was reported in a phase I clinical study to enhance the insulin response in human volunteers and improve glucose tolerance after an oral glucose

tolerance test in patients with a single dose of 60 mg. (Demuth et al., *Single dose treatment of diabetic patients by the DP IV inhibitor P32/98*, Diabetes 49(supp.1):A102 (413-P) (2000) (AZ-SAXA-8023508 to AZ-SAXA-8023510) ("Demuth 2000").) These early reports, while promising, were limited to acute responses after single doses of the compounds to patients and included minimal safety data. Thus, many of the longer-term safety and efficacy concerns remained.



NVP-DPP728



P32/98

2. DPP-4 Structure and Mechanism of Action

71. In the late 1990's, the x-ray crystal structure of DPP-4 was not known. The first DPP-4 structure was reported in 2003, well after the time of the invention here. (Rasmussen et al., *Crystal structure of human dipeptidyl peptidase IV/CD26 in complex with a substrate analog*, Nat. Struc. Biol. 10:19-25 (2003); *see also* De Meester at 373.) Prior to this, it was understood that only the high-resolution crystal structure would "permit significant progress in the detailed understanding of the selective ligand-binding and substrate hydrolysis by CD26." (De Meester at 367.) Without an x-ray crystal structure, scientists could not accurately model the active site of DPP-4, and as a result, inhibitor design was unguided by intimate knowledge of the enzyme-inhibitor complex.

72. At the time of the invention here, what was known about DPP-4's structure was based largely upon studies evaluating its binding requirements for substrates and inhibitors. For example, in 1993, Mentlein reported that purified human DPP-4 enzyme cleaved the *N*-terminus

of peptide GLP-1 and glucose-dependent insulinotropic peptide ("GIP") *in vitro*. (Mentlein at 830.) These DPP-4 substrates contained amino acids His-Ala and Tyr-Ala at their *N*-termini, respectively. (*Id.*)

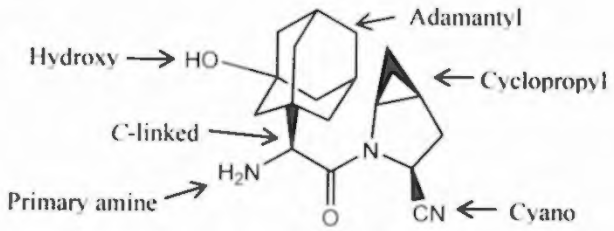
73. Mentlein's report was consistent with the understanding at the time of the "preferential specificity of DPP IV for penultimate proline or alanine residues." (Mentlein at 833.) Based on these results, Mentlein proposed a description of the substrate-binding site of DPP-4 in which: 1) proline or alanine fit into the P1 pocket, "whereas serine appears to be too hydrophilic to yield appreciable binding;" 2) "bulky amino acids [Tyr or His] with an obligate free amino group" fit into the P2 pocket; and 3) the preferential amino acids for the P1' position remained unknown. (*Id.* at 833, Figure 4.)

74. By the late 1990s, a large number of peptides were known to be cleaved by DPP-4 *in vitro*, but only a few had been confirmed as endogenous substrates. (De Meester at Table 1; Hildebrandt at Tables 1, 2, 4, and 5.) Three substrates had been confirmed with *in vivo* data: neuropeptide Substance P, and two glucagon family members GIP and GLP-1. (De Meester at Table 1.) Of these, Substance P contains a proline in the P1 position (GIP and GLP-1 contain alanine). (*Id.*) In addition, due to the presence of a soluble form of DPP-4 in the serum, it was thought that "any peptide circulating in the blood carrying a proline in the penultimate N-terminal position is a candidate substrate for DPP IV and will be metabolized within minutes, resulting in activation, inactivation or modulation of its biological effect." (Hildebrandt at 98.) Given the many potential substrates for DPP-4, it was understood at the time that DPP-4 inhibition could have "broad implications." (*Id.*)

3. Saxagliptin

75. In 1999, scientists at Bristol Myers Squibb ("BMS") discovered a new class of compounds useful for the treatment of patients with type 2 diabetes as a result of their in-house DPP-4 research efforts.

76. Saxagliptin, having the structure shown below, is one of the compounds resulting from BMS's efforts and is now marketed by AstraZeneca in the FDA approved drugs Onglyza® and Kombiglyze™ XR.



77. Saxagliptin's unique structure includes a *cis*-4,5 cyclopropyl group fused to a cyanopyrrolidine ring. The resulting *cis* 4,5-methano-cyanopyrrolidine moiety represents the P1 group. Saxagliptin also contains a 3-hydroxyl substituted adamantyl group C-linked to the peptide backbone. The C-linked hydroxyadamantyl glycine represents the P2 amino acid moiety.

78. These structures form particularly favorable and unexpected binding interactions in the DPP-4 active site. As discussed further in ¶¶ 179, 249-251 below, the hydroxyl group of saxagliptin is uniquely positioned to make a favorable hydrogen bond with Tyr547 of DPP-4 in the P2 site. Additionally, the cyclopropyl group is buried in the hydrophobic S1 pocket, next to the catalytic serine, where it forms van der Waals interactions with the side-chain residues that form the pocket. (Metzler et al., *Involvement of DPP-IV catalytic residues in enzyme-saxagliptin complex formation*, Protein Science 17:240-250, 241 (2008) (AZ-SAXA-8031266 to AZ-SAXA-8031276).) These structural features contribute to the slow, tight binding kinetics in the DPP-4

active site. In other words, the DPP-4 saxagliptin complex is slow to form, but once formed is long lived (slow off rate).

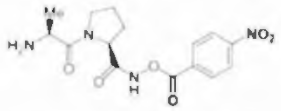
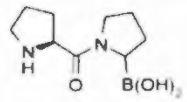
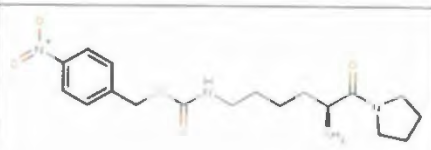
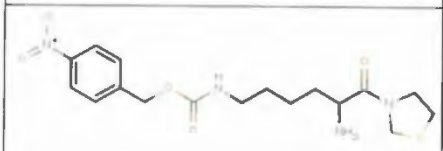
79. The combination of saxagliptin's structural features also yields a series of unexpected properties. As discussed further in ¶¶ 169, 235-236 below, the cyclopropyl group, when positioned in the particular *cis*-4,5-orientation as in saxagliptin, imparts increased solution stability at physiological temperature. (Magnin et al., *Synthesis of novel potent dipeptidyl peptidase IV inhibitors with enhanced chemical stability: interplay between the N-terminal amino acid alkyl side chain and the cyclopropyl group of α-aminoacyl-L-cis-4,5-methanoprolinenitrile-based inhibitors*, J. Med. Chem., 47:2587-2598 (2004) (AZ-SAXA-8046946 to AZ-SAXA-8046957).) Saxagliptin also has good potency and a long residence time in the DPP-4 binding site. (Wang et al., *Potency, selectivity and prolonged binding of saxagliptin to DPP4: maintenance of DPP4 inhibition by saxagliptin in vitro and ex vivo when compared to a rapidly-dissociating DPP4 inhibitor*, BMC Pharmacology, 12:1-11 (2012) (AZ-SAXA-8043605 to AZ-SAXA-8043615).) Further, saxagliptin is metabolized to an active metabolite, which also extends *in vivo* efficacy. (Fura et al., *Pharmacokinetics of the dipeptidyl peptidase 4 inhibitor saxagliptin in rats, dogs, and monkeys and clinical projections*, Drug Metabolism and Disposition, 37:1164-1171 (2009) (AZ-SAXA-5121790); Su et al., *Characterization of the in vitro and in vivo metabolism and disposition and cyctochrome P450 inhibition/induction profile in saxagliptin in human*, Drug Metabolism and Disposition, 40:1345-1356 (2012).) In addition, saxagliptin shows good bioavailability and low cell permeability with beneficial effects on selectivity. (*See* AZ-SAXA- 8007427.) Saxagliptin's properties allow for the use of a low dose in patients to treat type 2 diabetes with the added significant benefit of once

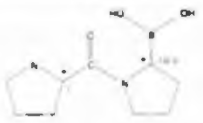
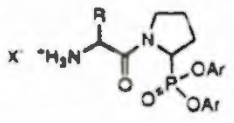
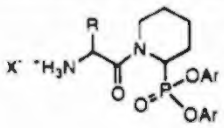
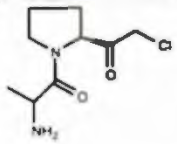
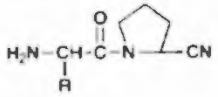
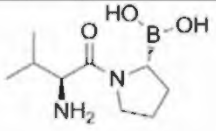
daily dosing. (Onglyza® Prescribing Info., (2015) (AZ-SAXA-8073638 to AZ-SAXA-8073679); Kombiglyze™ XR Prescribing Info., (2015) (AZ-SAXA-8073607 to AZ-SAXA-8073637).)

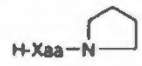
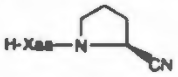
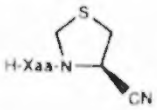
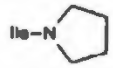
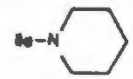
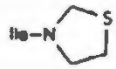
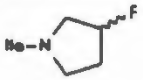
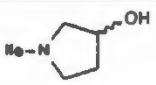
4. Prior Art DPP-4 Inhibitors Varied Greatly

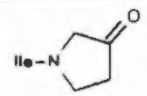
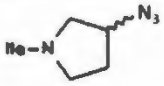
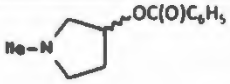
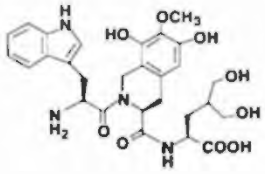
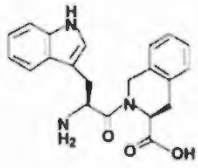
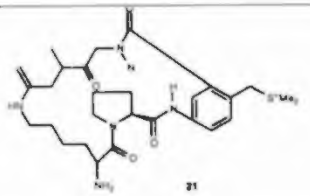
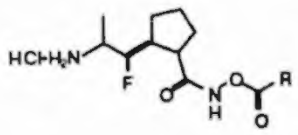
80. Uncertainty in DPP-4 inhibitor structure and properties for a putative clinical candidate led to a wide variety of proposed structural motifs among DPP-4 inhibitors. Indeed, a wide variation in molecular structures existed among the DPP-4 inhibitors known as of the effective filing date of the RE'186 patent (February 16, 2001). In the chart below, I have listed some of these DPP-4 inhibitors, but there were many others.

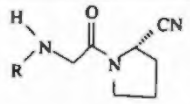
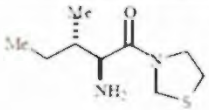
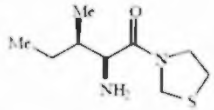
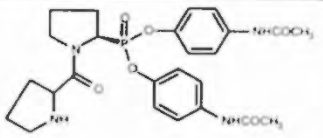
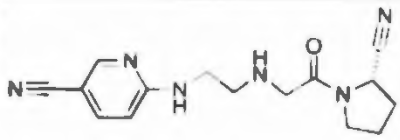
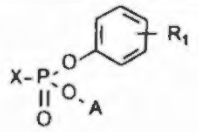
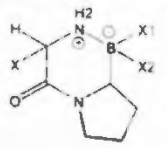
Examples of DPP-4 Inhibitors Disclosed Before February 16, 2001

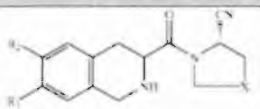
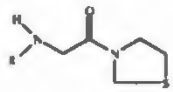
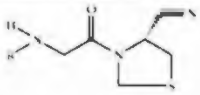
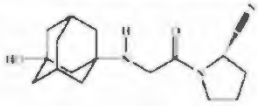
Compound Structure	Ultimately Approved by FDA?	Reference
	No	Demuth et al., *Dipeptidylpeptidase IV - inactivation with N-peptidyl-o-aroyl hydroxylamines*, J. Enzyme Inhib., 2:129-42 (1988).
	No	Flentke et al., *Inhibition of dipeptidyl aminopeptidase IV (DP-IV) by Xaa-boroPro dipeptides and use of these inhibitors to examine the role of DP-IV T cell function*, Proc. Natl. Acad. Sci., 88:1556–59 (1991); US 4,935,493.
	No	Schon et al., *Dipeptidyl peptidase IV in the immune system*, Biol. Chem. Hoppe-Seyler, 372:305-311 (1991).
	No	Schon.

Compound Structure	Ultimately Approved by FDA?	Reference
	No	Gutheil and Bachovchin, *Separation of L-Pro-DL-boroPro into its component diastereomers and kinetic analysis of their inhibition of dipeptidyl peptidase IV. A new method for the analysis of slow, tight-binding interaction*, Biochemistry, 32:8723-31 (1993).
	No	Boduszek et al., *Dipeptide phosphonates as inhibitors of dipeptidyl peptidase IV*, J. Med. Chem., 37:3969-3976 (1994); WO 95/29691.
	No	Boduszek; WO 95/29691.
	No	Demuth and Heins, *Catalytic mechanism of dipeptidyl peptidase IV in Dipeptidyl peptidase IV (CD26) in metabolism and the immune response*, Fleischer ed. Springer-Verlag: Heidelberg, 1-35 (1995).
 Arg(PMC)-Pyrr-2-CN Arg-Pyrr-2-CN Ala-Pyrr-2-CN Phe-Pyrr-2-CN	No	Li et al., *Aminoacylpyrrolidine-2-nitriles: potent and stable inhibitors of dipeptidyl-peptidase IV (CD 26)*, Arch. Biochem. Biophys., 323:148-52 (1995).
 PT-100 (Talabostat)	No	Snow and Bachovchin, *Boronic acid inhibitors of dipeptidyl peptidase IV: a new class of immunosuppressive agents*, Adv. Med. Chem., 3:149-177 (1995).

Compound Structure	Ultimately Approved by FDA?	Reference
	No	Ashworth et al., *2-cyanopyrrolidines as potent, stable inhibitors of dipeptidyl peptidase IV*, Bioorg. Med. Chem. Lett., 6:1163-1166 (1996) ("Ashworth I").
	No	Ashworth I.
	No	Ashworth et al., *4-cyanothiazolidides as very potent, stable inhibitors of dipeptidyl peptidase IV*, Bioorg. Med. Chem. Lett., 6:2745-2748 (1996) ("Ashworth II").
	No	Augustyns et al., *Pyrrolidides: synthesis and structure-activity relationship as inhibitors of dipeptidyl peptidase IV*, Eur. J. Med. Chem., 32:301-309 (1997) (AZ-SAXA-8023465 to AZ-SAXA-8023473) ("Augustyns 1997").
	No	Augustyns 1997.
	No	Augustyns 1997.
	No	Augustyns 1997.
	No	Augustyns 1997.
	No	Augustyns 1997.

Compound Structure	Ultimately Approved by FDA?	Reference
	No	Augustyns 1997.
	No	Augustyns 1997.
	No	Augustyns 1997.
	No	Tanaka et al., *Anti-arthritic effects of the novel dipeptidyl peptidase IV inhibitors TMC-2A and TSL-225*, Immunopharmacology, 40:21-26 (1998) (AZ-SAXA-8023749 to AZ-SAXA-8023754).
	No	Tanaka.
	No	Nguyen et al., *Specific and irreversible cyclopeptide inhibitors of dipeptidyl peptidase IV activity of the T-cell activation antigen CD26*, J. Med. Chem., 41:2100-2110 (1998).
	No	Lin et al., *Inhibition of dipeptidyl peptidase IV by fluoroolefin-containing N-peptidyl-O-hydroxylamine peptidomimetics*, Proc. Natl. Acad. Sci., 95:14020-14024 (1998) (AZ-SAXA-8023697).

Compound Structure	Ultimately Approved by FDA?	Reference
	No	WO 98/19998 ("Villhauer-998"); US 6,011,155 ("Villhauer-155")
	No	Pauly et al., *Improved glucose tolerance in rats treated with the dipeptidyl peptidase IV (CD26) inhibitor Ile-thiazolidide*, Metab. Clin. Exp., 48:385-89 (1999).
	No	WO 99/61431.
	No	Augustyns et al., *The unique properties of dipeptidyl-peptidase IV (DPP IV / CD26) and the therapeutic potential of DPP-IV inhibitors*, Current Med. Chem., 6:311-327 (1999) ("Augustyns 1999").
	No	Hughes et al., *NVP-DPP728 (1-[[[2-[(5-Cyanopyridin-2-yl)amino]ethyl]amino]acetyl]-2-cyano-(S)-pyrrolidine), a slow-binding inhibitor of dipeptidyl peptidase IV*, Biochemistry, 38:11597-11603 (1999) (AZ-SAXA-8023676 to AZ-SAXA-8023682).
	No	WO 99/46272.
	No	WO 99/62914.

Compound Structure	Ultimately Approved by FDA?	Reference
 X is CH_2, S, O, or $C(CH_3)_2$	No	US 6,172,081.
	No	US 6,107,317.
	No	US 6,110,949 ("Villhauer-949").
 Vildagliptin	No	US 6,166,063 ("Villhauer-063").

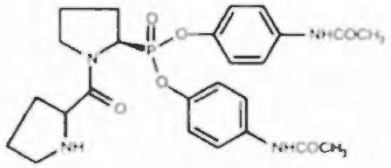
81. While many of these prior art compounds demonstrated good potency *in vitro*, almost all failed to make it to the clinic. As reflected in the chart above, none of these DPP-4 inhibitors became an FDA-approved drug.

82. In the late 1990s, several academic and industrial research groups were developing DPP-4 inhibitors using different approaches. These different approaches are reflected in the large variability of the structures in the chart above. It was recognized at the time that many of the inhibitors being developed "significantly differ" in chemical structure and mechanism of action. (De Meester at 368.)

83. By the 1990s, two mechanistically distinct classes of DPP-4 inhibitors, reversible and irreversible, had been reported. (Augustyns 1999 at 314-317.) Irreversible inhibitors inactivate DPP-4 by forming a permanent covalent bond with the enzyme. (*Id.* at 316.) By contrast, reversible inhibitors can dissociate from the enzyme after interaction with the active site

of the enzyme. (*Id.* at 314-316.) Both reversible and irreversible DPP-4 inhibitors were actively investigated at the time, and it was unknown whether DPP-4 inhibition should be permanent or transient. (Holtz and Deacon at 1668.)

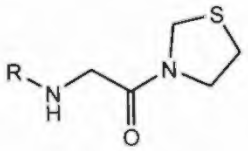
84. An example of an irreversible DPP-4 inhibitor is shown below. The compound was developed by a group at the University of Antwerp and was reported to have "high potency," "high stability," as well as selectivity for DPP-4. Additionally, the compound was shown to have "favorable properties *in vivo*." (Augustyns 1999 at 317.)



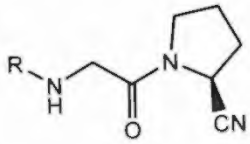
85. Within reversible DPP-4 inhibitors, boronic acid compounds were reported among the most potent inhibitors with Ki values in the nanomolar range. (Augustyns 1999 at 316.) These compounds are referred to as transition-state analogs because the empty P-orbital at boron interacts with the catalytic serine in the active site of DPP-4 to form a complex which mimics the transition state of amide hydrolysis. (*Id.*) Boronic acid compounds exhibited slow, tight-binding kinetics with DPP-4. (*Id.*) Before 2000, at least one boronic acid compound (Pro-boro-Pro) was shown to "clearly lower blood sugar based upon results from an oral glucose challenge in mice." (WO 99/38501 at 50, lines 25-27.) In addition, Val-boro-Pro (also called PT-100 or Talabostat) was developed based on DPP-4's immunological roles and was later taken to clinical trials by Point Therapeutics for the treatment of cancer. (Snow and Bachovchin; Nemunaitis et al., *A phase 1 trial of PT-100 in patients receiving myelosuppressive chemotherapy*, J. Clin. Oncol., 22(14S):2572 (2004).)

86. In 1996, a group at Ferring published two articles reporting reversible DPP-4 inhibitors with various P1 cores (pyrrolidines, cyanopyrrolidines, and cyanothiazolidines). (Ashworth I; Ashworth II.) Ashworth I explored the SAR of different substituents in the P2 position of compounds with either pyrrolidine or cyanopyrrolidine as the P1 core. (Ashworth I at 1164-1166, Tables 1 and 2.) The later article, Ashworth II, explored the SAR associated with the P1 position. Various five- and six-membered saturated heterocycles containing a combination of nitrogen, sulfur and oxygen were evaluated. (Ashworth II at 2747-2748, Tables 1 and 2.) The authors concluded that cyanothiazolidine was the "optimum" P1 core. (Ashworth II at 2746.)

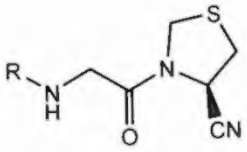
87. Novartis also explored reversible DPP-4 inhibitors with various P1 cores (cyanopyrrolidines, thiazolidines, and cyanothiazolidines) but on a different backbone (*N*-substituted glycine derivatives; or *N*-linked) as shown below.



US 6,107,317

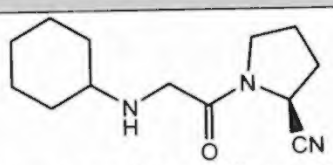


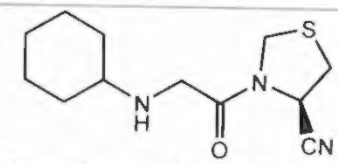
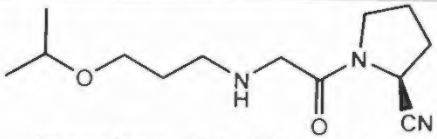
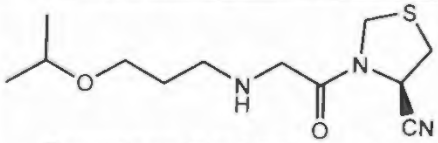
US 6,011,155



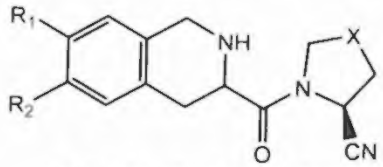
US 6,110,949

88. Comparing the disclosure in Novartis's U.S. Patent Nos. 6,011,155 and 6,110,949, the cyanothiazolidine derivatives have much higher potency (as reflected in lower IC_{50} values) than their counterparts with cyanopyrrolidine in the P1 position. (Villhauer-155 and Villhauer-949.)

Compounds	IC_{50} (nM) Caco-2	IC_{50} (nM) Human plasma	IC_{50}(nM) Rat plasma
 Example no. 28 in Villhauer-155	90	180	60

 Example no. 1 in Villhauer-949	7	10	10
 Example no. 12 in Villhauer-155	279	95	38
 Example no. 2 in Villhauer-949	7	5	1

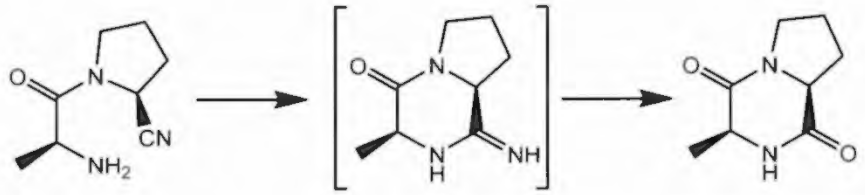
89. Around the same time, Novartis also developed a series of tetrahydroisoquinoline-3-carbonylcyanopyrrolidine derivatives and also evaluated various P1 cores. Some of these compounds were reported to have good DPP-4 inhibitory activity. (US 6,172,081.)



X is CH_2, S, O, or $C(CH_3)_2$

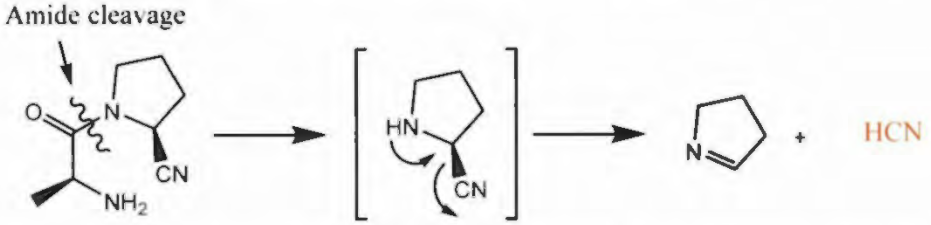
90. By 2000, among the Novartis compounds, only one compound (NVP-DPP728) was tested in human subjects. (*See* ¶ 70 above.)

91. When we initiated medicinal chemistry on DPP-4 inhibitors at Merck, we were concerned about the presence of the cyano group in the P1 position because of the potential for intramolecular cyclization of DPP-4 inhibitors leading to inactive diketopiperazines. This occurs from the nucleophilic attack of the *N*-terminal nitrogen of the P2 group with the electrophilic nitrile on the P1 group while in the *cis*-conformation.



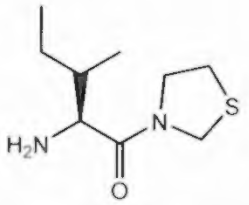
cis-conformer cyclic amidine diketopiperazine

92. We also had safety concerns about nitrile-containing DPP-4 compounds because of the potential for toxic cyanide release should amide bond cleavage occur. Therefore, we elected to investigate compounds that did not contain a nitrile group.

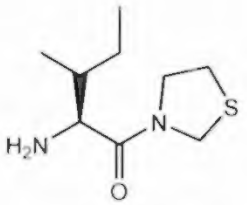


93. In order to jump start Merck's internal program, we in-licensed L-*threo*-isoleucyl thiazolidide (Ile-Thia also referred to as "P32/98") and its *allo* stereoisomer (L-*allo*-isoleucyl thiazolidide) from Probiodrug. By 2000, P32/98 was one of only two DPP-4 inhibitors that had been tested in human subjects. (*See* ¶ 70 above.) P32/98 had been shown to be well tolerated, increase active GLP-1, and reduce glycemic excursion following food or glucose intake in normal volunteers. (Glund et al., *Single dose-escalation study to investigate the safety and tolerability of the DP IV inhibitor P32/98 in healthy volunteers*, Exp. Clin. Endocronol. Diabetes, 108:S159 (pFr105) (2000).) In addition, Probiodrug reported enhanced insulin secretion and improved glucose tolerance in single dose studies in a small number of diabetic patients. (Demuth 2000.) The development of these compounds was discontinued in 2001 due to unforeseen toxicity issues that were later attributed to inhibition of the related enzymes DPP-8 and/or DPP-9. (Thornberry and Weber, *Discovery of JANUVIA™ (sitagliptin), a selective*

dipeptidyl peptidase IV inhibitor for the treatment of type 2 diabetes, Curr. Top. in Med. Chem. 7:557-568, 558-560 (2007).)

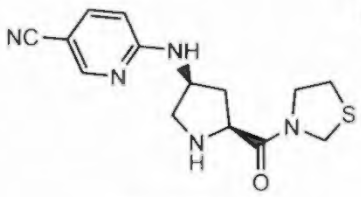


Ile-Thiazolidide (P32/98)



allo-Ile-Thiazolidide

94. Merck was not alone in choosing to develop thiazolidine derivatives as DPP-4 inhibitors. In the early 2000s, at least three pharmaceutical companies developed DPP-4 inhibitors with thiazolidine as the P1 core. (Wiedeman, *DPPIV inhibition: promising therapy for the treatment of type 2 diabetes, Progress in Medicinal Chemistry*, 45:63-109, 77-78, 89 (2007).)



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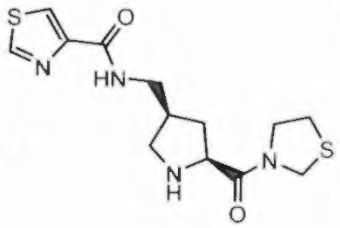
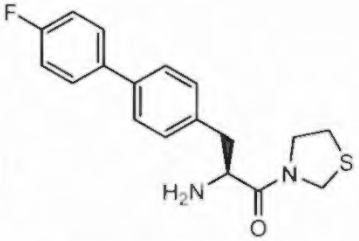


Abbott



Johnson and Johnson

5. Additional Unpredictable Hurdles Beyond Potency

95. Researchers faced a variety of unpredictable hurdles beyond potency that had to be overcome in order to identify a DPP-4 inhibitor suitable for use as a therapeutic agent to treat patients with type 2 diabetes.

96. Potency is only one of many properties to be optimized in designing a DPP-4 inhibitor for therapeutic use. Additional, and often more difficult, properties to optimize include selectivity, physico-chemical properties, pharmacokinetic and ADME (absorption, distribution,

metabolism, elimination) properties, and pharmacodynamic properties including efficacy and safety.

97. These properties bear directly on the ability of the DPP-4 inhibitor to be progressed into development and ultimately to be successfully marketed as a therapeutic agent to treat patients with type 2 diabetes. Like potency, they are often unpredictable. The importance of each is outlined below.

98. Selectivity of the inhibitor for DPP-4 over, *e.g.*, other enzymes, receptors, ion channels, and transporters is important to avoid unwanted side-effects due to activity at these other targets ("off-target activity"). For instance, it was selectivity issues that ultimately led to Merck's discontinuation of P32/98. (Thornberry and Weber at 558-560.) Typically, to assess such issues, a compound of high interest would be assayed in a screen of over 100 to 200 different targets, either in-house and/or outsourced to a contract research organization.

99. Physico-chemical properties are important for several reasons. The DPP-4 inhibitor must be sufficiently chemically stable both in the drug product (*e.g.*, a pill) and *in vivo* so that it is not extensively degraded either before or after it is delivered to the patient. Chemical stability may be measured by half-life in solution, either under physiologic conditions, *e.g.*, about 37°C, or at room temperature. For example, as described above, many DPP-4 inhibitors known in the art were susceptible to intramolecular cyclization due the presence of a potency-enhancing electrophile and the strict requirement for a basic amine, which could serve as a nucleophile. This caused many compounds to be abandoned in favor of more stable alternatives. (*See e.g.*, Lin at 14020-14021, 14023.)

100. Various researchers published different structural solutions to the problem of intramolecular stabilization:

- Villhauer used a backbone with a secondary amine and reported less than 1% cyclization. (Hughes at 11599.)
- Lin developed a fluorine containing structure. (Lin at 14020-14021, 14023.)
- Nguyen reported a complex cyclic structure that prevented decomposition by cyclization. (Nguyen at 2106.)

101. In addition to stability, aqueous solubility and lipophilicity are also important physico-chemical properties that would typically be explored in the drug discovery process. For a DPP-4 inhibitor to be orally bioavailable, it must be absorbed in solution. Hence, a highly insoluble inhibitor would be expected to have poor oral bioavailability. Oral bioavailability also depends on the compound's lipophilicity, which is generally measured as logP, where P is the partition coefficient between octanol and water. The higher the logP, the more lipophilic the compound. A logP of greater than 5 has been associated with poor absorption. (Lipinski et al., *Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings*, Adv. Drug Deliv. Rev. 23:3-25, 3 (1997).).

102. Pharmacokinetic (PK) properties such as oral bioavailability and half-life describe the body's affect on the drug. (Burger's at 114-15.) They are important to explore in part because they directly affect the dose and dosing interval of the DPP-4 inhibitor. A low dose is typically desirable for both safety and cost reasons. A lower dose means lower cost of goods. And safety would be expected to increase with decreasing body burden of drug. For example, idiosyncratic reactions would typically not be seen in drugs that are dosed at ~10 mg or less. (Uetrech, *New concepts in immunology relevant to idiosyncratic drug reactions: the "danger hypothesis" and innate immune system*, Chem. Res. Toxicol., 12:387-395, 388 (1999); *see also*

Lammert et. al., *Relationship between daily dose and oral medications and idiosyncratic drug-induced liver injury: search for signals*, Hepatology, 47:2003-2009 (2008).)

103. A dosing interval of once daily over, *e.g.*, twice daily, would be preferred due to increased patient compliance. A DPP-4 inhibitor would have to have an appropriate *in vivo* half-life to support once daily dosing to avoid the necessity of "dosing up" in order to cover the dosing interval.

104. Pharmacokinetics are used to quantitate the absorption, distribution, metabolism and elimination (ADME) properties of a DPP-4 inhibitor. Absorption, metabolism and elimination all affect bioavailability (*e.g.*, oral bioavailability) of the DPP-4 inhibitor. An understanding and appropriate selection of these properties would also be necessary to avoid unwanted drug-drug interactions. Distribution of the DPP-4 inhibitor to the site of action, in this case believed to be the DPP-4 enzyme lining the capillaries of the gut, would be important for *in vivo* efficacy. It would also be necessary to understand whether any metabolites form and, if so, what the properties of such metabolites are (*e.g.*, whether they have activity at the target).

105. Pharmacodynamics (PD) refers to a DPP-4 inhibitor's affects on the body. (Burger's at 115.) These would include measures of efficacy, *e.g.*, inhibition of the DPP-4 enzyme in plasma, stabilization of the *in vivo* substrates GLP-1 and GIP, and glucose lowering, as well as any toxic effects. In many cases the PD effects of the inhibitor are directly related to the inhibitor's plasma concentration (PK); however, in some cases, *e.g.*, when the DPP-4 enzyme-inhibitor complex has a very long half-life, there may be a PK/PD disconnect, where the PD effect may last longer than expected based on the inhibitor's plasma concentration. Identification of compounds with such a longer than expected PD effect is desirable in that the lower plasma level required for efficacy would be expected to minimize any potential off-target

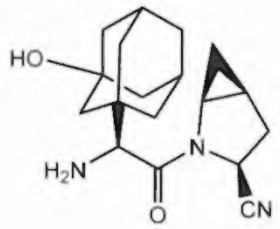
toxic effects. In addition, a PK/PD disconnect may be caused by the formation of an active metabolite.

106. Safety of a DPP-4 inhibitor in both rodents, *e.g.*, rats or mice, and non-rodents, *e.g.*, dogs or monkeys, would be of particular importance to understand. Safety studies would be designed to assess the DPP-4 inhibitor's affects on ancillary pharmacology, *e.g.*, effects on cardiac parameters, respiration, CNS, as well as acute and chronic toxicity (*e.g.*, liver toxicity), carcinogencity, and genotoxicity. Any safety issues that would prevent a compound from advancing would need to be identified and addressed through structural modification.

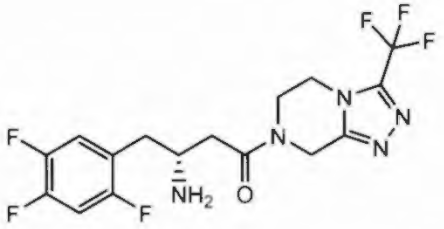
107. In addition to these parameters, there were also biological unknowns, including potential mechanism-based side effects. (*See* ¶¶ 68-69 above.) For example, the literature showed an immunological role for DPP-4 in the activation of T cells. (De Meester at 368-369.) Should DPP-4 inhibition inhibit T cell activation *in vivo*, this could lead to undesired immunosuppression in patients. (*See id.* at 370.) Furthermore, DPP-4 had been reported to cleave a large number of substrates *in vitro*. (*Id.* at 371, Table 1.) It was not clear whether there would be *in vivo* biological consequences as a result. (*Id.* at 373.) And the expression levels of DPP-4 were known to be up-regulated and down-regulated in a variety of disease states. (*Id.* at 371, Box 2.) The possibility of mechanism-based side effects was a significant concern.

F. FDA Approved DPP-4 Inhibitors Have No Successful Standard Motifs

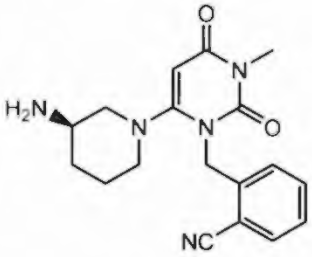
108. Today, there are only four FDA-approved DPP-4 inhibitors. The structures of these four inhibitors (saxagliptin, sitagliptin, alogliptin, and linagliptin) are shown below. (Onglyza® Prescribing Info., at 9 (2015); Januvia® Prescribing Info., at 8 (2015); Nesina® Prescribing Info., at 9 (2015); Tradjenta® Prescribing Info., at 4 (2015).)



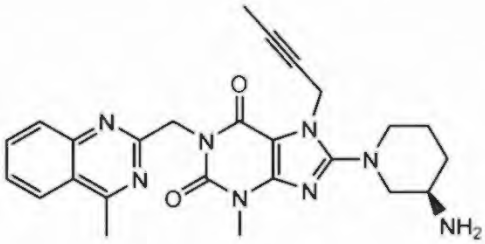
Saxagliptin



Sitagliptin



Alogliptin



Linagliptin

109. As reflected by their diverse structures, there is no recipe or common structural motif that provides a successful DPP-4 inhibitor. Saxagliptin is the only FDA-approved DPP-4 inhibitor to contain: 1) a cyclopropyl group; 2) pyrrolidine ring in the P1 position; 3) a nitrile group that forms an adduct with the active site serine; and 4) a hydroxyadmantyl group in the P2 position.

110. Each of the companies that developed an FDA-approved DPP-4 inhibitor approached the problem in a unique way resulting in a different structural solution.

111. None of the FDA approved DPP-4 inhibitors were in the prior art, each being discovered after saxaglitpin's invention.

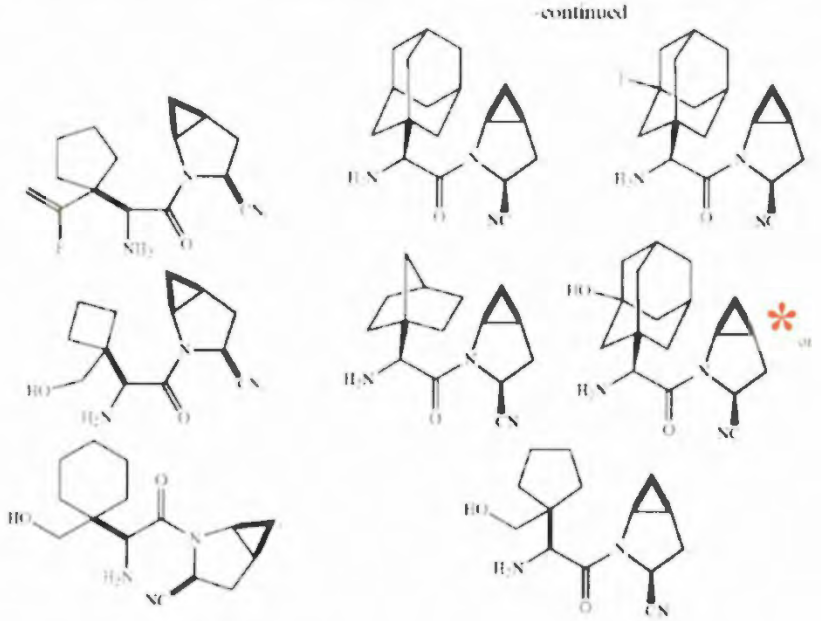
V. THE RE44,186 PATENT

112. The RE'186 patent titled "Cyclopropyl-fused pyrrolidine-based inhibitors of dipeptidyl peptidase IV and method" issued on April 30, 2013 from application 13/308,658. I understand this patent is a reissue of U.S. Patent No. 6,395,767 ("the '767 patent"), which

originally issued on May 28, 2002. The '767 patent was filed on February 16, 2001 and claims priority to provisional application 60/188,155 ("the '155 application) filed on March 10, 2000.

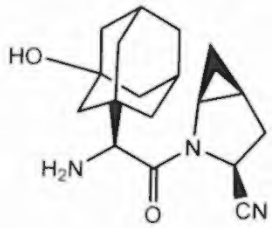
113. I understand that the claims at issue in this litigation are claims 8, 9, 25, 26 of the RE'186 patent. All of these claims require a cyclopropyl group in the *cis*-4,5- orientation on the pyrrolidine ring in the P1 position and a quaternary carbon (a carbon with four non-hydrogen groups attached to it) *C*-linked to the amino acid moiety in the P2 position.

114. More specifically, claim 8 recites "[a] compound having the structure:



or a pharmaceutically acceptable salt thereof." The compound indicated with an asterisk is saxagliptin.

115. Claim 25 recites the compound saxagliptin. Specifically, "[a] compound that is



or a pharmaceutically acceptable salt thereof."

116. Claims 9 and 26 depend from claims 8 and 25, respectively, and recite the hydrochloride salt.

VI. STATEMENT OF OPINIONS AND THEIR BASES

A. The Person of Ordinary Skill in the Art

117. A person of ordinary skill in the art relevant to the RE'186 patent is a medicinal chemist with a Ph.D. in chemistry and several years of practical experience working with pharmaceutical chemical compounds for potential and eventual clinical use in patients. A person of ordinary skill in the art may also have a B.S. or M.S. degree in chemistry with significantly more experience. The person of ordinary skill in the art also has familiarity with the spectrum of properties needed for a successful drug, the potential difficulties associated with attaining them, and the potential effects of pharmaceuticals in the human body.

B. A Safe and Effective Therapeutic DPP-4 Inhibitor for Type 2 Diabetes Was Unobvious

1. DPP-4 Was Not an Obvious Target

118. At the time of the invention, DPP-4 was not an obvious viable target for treating patients with type 2 diabetes. While DPP-4 was believed to play a role in glucose homeostasis through its regulation of the incretin hormones GLP-1 and GIP, there were also data in the literature showing an immunological role for DPP-4 in the activation of T cells. (De Meester at

368-369.) Early studies on DPP-4 reported its role in the activation and proliferation of T lymphocytes and proposed inhibitors to modulate this function. (Schon at 305-311.) Indeed, Ashworth's compounds were being investigated as potential immunomodulators. (Ashworth I at 1163-1164, 1166.) Additional potential therapeutic areas that were being considered for DPP-4 inhibitors were AIDS, autoimmune disease, rheumatoid arthritis, multiple sclerosis, psoriasis, graft rejection, wound healing, anxiety, type 2 diabetes, and cancer. (*Id.*; *see also*, Hoffman and Demuth, *Therapeutic strategies exploiting DP IV inhibition, target disease: type 2 diabetes, in Ectopeptidases* 259, 260 (Table 1) (Langner and Ansorge eds., Springer Science and Business Media, New York, 2002).)

119. In addition, DPP-4 had been reported to cleave a large number of substrates *in vitro*. (*See* ¶107 above.) It was not clear whether there would be *in vivo* biological consequences as a result. And the expression levels of DPP-4 were known to be up-regulated and down-regulated in a variety of disease states. (De Meester at 371, Box 2.) Thus, the possibility of mechanism-based side effects was a significant concern and would likely have limited the number of scientists working on the target. It also was not clear at the time whether 24 hour coverage was desirable based on the potential for these mechanism-based side effects. (*See* Hughes at 11603.)

120. In 2000, it remained to be established whether DPP-4 inhibition was a feasible approach for treatment of type 2 diabetes in humans. (*See* ¶70 above.) It was not until Ahrens et al. reported a 4-week study with NVP-DPP728 in June 2001 showing that it was effective in lowering fasting, fed, and 24-hour glucose levels in patients with type 2 diabetes upon chronic administration that the field accepted DPP-4 as a good target for type 2 diabetes and many others began working on it. (Ahren et al., *Inhibition of DPPIV by NVP DPP728 improves metabolic*

control over a 4 week period in type 2 diabetes, Diabetes, 50:A104 (416-P) (2001).) Clinical data in patients prior to this report were limited to single dose studies. (*See* ¶ 70 above; Demuth; Rothenberg.)

121. At the time, it was known that DPP-4 was a member of a family of DASH (DPP activity structure homologs) proteins, and the biological functions of the other proteins in this family were largely unknown. These included for example, FAP (which has the highest sequence homology to DPP-4), DPP-7 (also known as "QPP" and "DPP-2"), inhibition of which induces apoptosis in quiescent lymphocytes but not activated lymphocytes, and DPP-8. (*See e.g.*, Abbott et al., *Cloning, expression and chromosomal localization of a novel human dipeptidyl peptidase (DPP) IV homolog, DPP8*, Eur. J. Biochem. 267:6140-6150 (2000) (AZ-SAXA-8006639 to AZ-SAXA-8006649); Underwood et al., *Sequence, purification, and cloning of an intracellular serine protease, quiescent cell proline dipeptidase*, J. Biol. Chem., 274:34053-34058 (1999); Weber at 4136-4137.) It was not known whether a DPP-4 selective compound was needed, and if so, what the structural requirements for selectivity were.

2. The Structural Features Required for A Successful Therapeutic DPP-4 Inhibitor Were Unobvious

122. In my opinion it would have been unobvious to a person of ordinary skill in the art how to obtain a safe and effective DPP-4 inhibitor at the time of the invention.

123. A variety of structural series were being explored. These included, *e.g.*, irreversible inhibitors containing diphenyl phosphonate esters and *O*-acyl hydroxamic acids; covalent reversible inhibitors, including boronic acids, *alpha*-substituted amino acid nitriles, and *N*-substituted glycine nitriles; product-like reversible inhibitors, including thiazolidines and pyrrolidines; and fluoro olefin inhibitors. (*See* discussion at ¶¶ 80-83 above.)

124. Among these different series, potent DPP-4 inhibitors were known, but the structural requirements for obtaining other necessary properties for a safe and effective therapeutic DPP-4 inhibitor were largely unknown. These properties included effective selectivity for DPP-4 versus *e.g.*, other DASH family members, ion-channels, and P450 enzymes; physico-chemical properties, including *e.g.*, chemical stability, solubility, and logP; pharmacokinetic and ADME properties, including *e.g.*, *in vivo* half-life and oral bioavailability; pharmacodynamic properties including efficacy and safety (*e.g.*, ancillary pharmacology, including effects on cardiac parameters, respiration, CNS, and acute and chronic toxicity (*e.g.*, liver toxicity), carcinogencity, and genotoxicity).

125. Data for these other properties was largely lacking in the prior art. With the exception of the two most advanced compounds in the prior art, NVP-DPP728 and P32/98, the scope of data that were available for other compounds included *in vitro* potency, some solution stability data, and limited *in vivo* activity in animals, *e.g.*, insulin response after a single dose in rats. (*See* above discussion at ¶¶ 85-91.)

126. For NVP-DPP728, additional data were available. These included information about binding kinetics, additional preclinical data in animal models, and phase I clinical data in humans. (Hughes; Rothenberg; Balkin et al., *Inhibition of dipeptidyl peptidase IV with NVP-DPP728 increases plasma GLP-1(7-36 amide) concentrations and improves oral glucose tolerance in obese Zucker rats*, Diabetologia, 42:1324-1331 (1999).)

127. For P32/98, additional data included effects on T cell activity, pre-clinical animal data, and phase I clinical data in humans. (Schon; Pauly; Pederson et al., *Improved glucose tolerance in Zucker fatty rats by oral administration of the dipeptidyl peptidase IV inhibitor isoleucine thiazolidide*, Diabetes, 47:1253-1258 (1998); Demuth 2000.)

128. More complete characterizations of even these two most advanced compounds were not available. In general, data were lacking concerning selectivity, detailed pharmacokinetic and ADME properties, and chronic safety and efficacy. Such data would be important for informing on the viability of DPP-4 as a type 2 diabetes target and the compounds as potential therapeutic agents.

129. Ultimately, like all of the other compounds in the prior art, NVP-DPP728 and P32/98 failed and were not approved for treatment of patients with diabetes in the U.S. These compounds failed for a variety of reasons. Development of NVP-DPP728 was discontinued because of its short half-life. (Weber at 4138.) P32/98 failed due to pre-clinical safety findings that were attributed to its lack of selectivity. (Thornberry and Weber at 558-560.)

130. Because there were no DPP-4 inhibitors approved for any indication, it was unclear which structural series, if any, would lead to a safe and effective agent. It was further unclear what structural modifications within any given series would be required to achieve all the necessary properties ***within one molecule*** to result in an FDA approved drug. The teachings from any given series would not necessarily be applicable to another series.

131. In fact, none of the structures in the prior art has led to an FDA approved drug, which highlights the difficulty and unpredictability associated with drug discovery. Further, none of the four subsequently FDA-approved compounds arose from structural series that were known in the art at the time.

3. Vildagliptin Was Not a Lead Compound

132. Dr. Powers argues that a person of ordinary skill in the art would have selected vildagliptin as a lead compound for further optimization. (Powers Report at ¶¶ 91, 104, and 139.) I disagree.

133. Vildagliptin would not have been selected as the lead in light of the available data on the two most advanced clinical candidates NVP-DPP728 and P32/98. (*See* Hoffman and Demuth at 272.) Based on the limited state of the art in February 2001, a person of ordinary skill in the art would, in my opinion, have selected either NVP-DPP728 or P32/98 as a lead compound, each of which is fundamentally, structurally different from the compounds claimed in RE'186 claim 8 in general and saxagliptin in particular.

134. A person of ordinary skill in the art would have selected NVP-DPP728 over vildagliptin because of the additional data that were available for NVP-DPP728 and the lack of data available for vildagliptin. Specifically, NVP-DPP728 had been shown in a phase I clinical study to increase prandial active GLP-1 levels and reduce prandial glucose excursion without causing hypoglycemia or causing significant adverse events in humans at a single dose of 100 mg. (Rothenberg.) Rothenberg concluded that these data "support the investigation of the glucose-lowering potential of NVP-DPP728 for the treatment of type 2 diabetes." (*Id.*) A person of ordinary skill in the art would have understood that NVP-DPP728: 1) appeared safe and effective in initial studies in humans; and 2) held promise for the treatment of type 2 diabetes. This information would have been highly valuable to a medicinal chemist starting a DPP-4 inhibitor program and would have influenced his or her consideration of a lead compound.

135. In contrast, there were no human data available for vildagliptin, which like NVP-DPP728 is a member of the *N*-linked glycine nitrile class of inhibitors. The only data reported for vildagliptin were its *in vitro* potency and its ability to induce insulin release after a single dose to rats. (Villhauer-063 at col. 5, lines 1-8, 38-45 and col. 6, lines 26-33.) Even the latter data were not the best reported among the class of *N*-linked glycine nitrile inhibitors disclosed by

Novartis. (*See, e.g.*, Villhauer-998 at 19, Example 8 (reporting 144% increase in insulin response).) In addition, the 64% increase in insulin response reported for vildaglipin was not an improvement over the clinical candidate NVP-DPP728 (66% increase in insulin response). (Villhauer-063 at col. 6, lines 26-33; Villhauer-998 at 19, Example 3.)

136. A person of ordinary skill in the art would also have selected P32/98 as a lead compound over vildagliptin. P32/98 has the advantage of eliminating the concern of chemical stability associated with nitrile-based inhibitors. Specifically, P32/98 is incapable of cyclizing to form the diketopiperazine discussed above. (*See* ¶ 91 above.) Another advantage of P32/98 is that, because it lacks a nitrile group, there is no possibility for toxic cyanide release *in vivo*. Such release could result from cleavage of the amide bond and subsequent amine-assisted elimination. (*See* ¶ 92 above.)

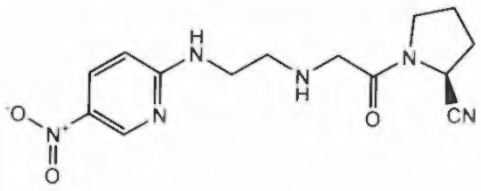
137. While P32/98 was not the most potent compound in the prior art, it had been shown to enhance the insulin response in human volunteers and improve glucose tolerance after an oral glucose tolerance test in patients with a single dose of 60 mg. (Demuth 2000.) These clinical data would teach the person of ordinary skill in the art that: 1) this compound had sufficient safety to enter the clinic; and 2) while moderately potent, its potency was sufficient to lower glucose acutely in patients at a reasonable dose. This information would have been highly valuable to a medicinal chemist starting a DPP-4 inhibitor program and would have influenced his or her consideration of a lead compound.

138. In addition, the choice of thiazolidine P32/98 is consistent with its identification by BMS as a "literature standard" when starting its DPP-4 inhibitor program. (AZ-SAXA-8026740.)

139. Dr. Powers, in support of his selection of vildaglipin as a lead compound, argues that "an appropriate design strategy for constructing an effective DPP-4 inhibitor would be the synthesis of AA-proline-warhead." (Powers Report at ¶ 93.) I disagree with the presumption that a "warhead" (*e.g.*, an electrophile such as a cyano group that reacts with a nucleophilic serine hydroxyl group in a serine protease active site) is required. For example, the clinical candidate P32/98 did not contain such a "warhead," yet improved glucose tolerance after an oral glucose tolerance test in patients with type 2 diabetes. (Demuth 2000.)

140. While there were no FDA-approved DPP-4 inhibitors at the time, other than saxagliptin, none of the four subsequently approved compounds contains a "warhead." (*See* ¶ 108 above showing the structures of sitagliptin, linagliptin, and alogliptin.)

141. Dr. Powers argues that "[v]ildagliptin is an appropriate lead compound because in an animal model it gave at least a 64% increase in insulin response at 10 [µ]mol/kg." (Powers Report at ¶ 91.) As discussed above, the significance of animal data would have been superseded by the data in diabetic patients with the two most advanced clinical candidates P32/98 and NVP-DPP728. (*See* ¶ 70 above.) And the 64% increase in insulin would not have been a reason to select vildagliptin as other Villhauer compounds from the Villhauer-998 publication had greater % increases, *e.g.*, Example 8 (144% increase). (Villhauer-063 at col. 6, lines 26-33; Villlhauer-998 at 19, Example 8.)



Example 8
144% increase of insulin response

142. In selecting vildagliptin as a lead compound for further modification, Dr. Powers argues "[i]t would be useful to have an improved version of vildagliptin, which could be given fewer times per day and at a lower dose." (Powers Report at ¶ 92.) The Villhauer-063 patent provides ranges for dose and dosing interval, but does not indicate what the actual dose and interval would be when administered as a safe and effective therapy to humans. (Villhauer-063 at col 6, lines 34-54.) Thus, it is not clear from the available information that one skilled in the art would be motivated to optimize the structure of vildagliptin for this reason.

143. In my opinion, the only reason to select vildagliptin as a lead compound would be hindsight based on the success of saxagliptin, which I understand is improper in assessing obviousness.

4. No Suggestion to Add and No Reasonable Expectation of Success in Adding Cyclopropyl to P1 Moiety

a. Potency Was Not A Reason to Add Cyclopropyl

144. Dr. Powers argues one of ordinary skill in the art "would add a cyclopropyl group to the proline ring of vildagliptin to produce a proline analog inhibitor of vildaglipin in order to increase the potency of the molecule." (Powers Report at ¶ 115.)

145. I disagree. First, potency was not an issue with the vildagliptin compound. (*See* Villhauer-063 at col. 5, lines 38-45 (reporting an IC_{50} of 2.7 nM in human plasma).) Based on the potencies of the most advanced clinical candidates, NVP-DPP728 and P32/98, both of which had demonstrated acute glucose lowering effects in humans, the potency of vildagliptin would have been considered sufficient by one of skill in the art. (*See* Villhauer-998 at 21 (reporting an IC_{50} of 7 nM in human plasma for NVP-DPP728); Schon at 308 (reporting an IC_{50} of 2.8 μM in mononuclear cells of human blood for P32/98); Augustyn 1997 at 304 (reporting a K_i for P32/98 of 1.8 μM in human plasma).)

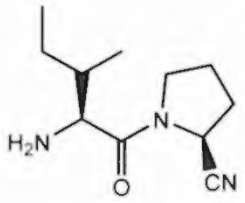
146. Second, even if a person skilled in the art wanted to increase potency of vildagliptin, one would not add a cyclopropyl to its pyrrolidine ring in order to affect such an increase in potency with a reasonable expectation of success.

147. In support of Dr. Powers' position that one of skill in the art would add a cyclopropyl to the pyrrolidine in vildagliptin to increase potency, he argues that "Boduszek *et al.* (1994) teaches that DPP-IV prefers a larger ring than the 5-membered pyrrolidine ring in the P1 proline residue of inhibitors." (Powers Report at ¶ 115.) Dr. Powers states that "[i]n all cases, inhibitors with the piperidine ring were more potent inhibitors than the corresponding inhibitors with a proline ring." (Powers Report at ¶ 62.) This disregards the body of more relevant evidence that was available to one skilled in the art at the time that teaches away from increasing the size of the P1 moiety. (See ¶¶ 149-151 below.)

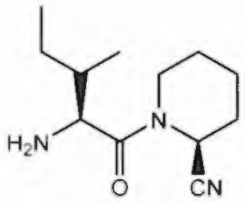
148. The Boduszek compounds are irreversible phosphonate inhibitors that inactivate the DPP-4 enzyme upon binding by forming a stable phosphonylated derivative of DPP-4, while vildagliptin is a reversible inhibitor that does not permanently modify the enzyme. (Boduzek at 3972.) Irreversible phosphonate inhibitors would not have been considered by one of skill in the art to be a viable approach to identifying a therapeutic agent because of concerns of toxicity (for example, due to their resemblance to nerve agents and insecticides). Even Dr. Powers acknowledges that medicinal chemists "do not consider phosphonates a viable warhead" and they "fear that this would introduce toxicity and no phosphonate warheads have yet been used in drugs as protease inhibitors." (Powers Report at ¶ 60.)

149. Other, more relevant data in the prior art demonstrated that increasing the size of the pyrrolidine ring in reversible inhibitors was detrimental for potency. Specifically:

- Increasing the ring size of Ashworth's 5-membered pyrrolidine ring to a 6-membered piperidine ring resulted in a 100-fold decrease in potency. (Ashworth II at Table I (*compare* compound 5, *with* compound 8).)

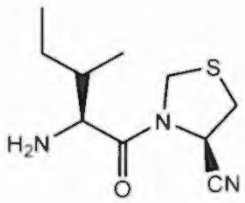


Compound 5
K_i = 2.2 nM

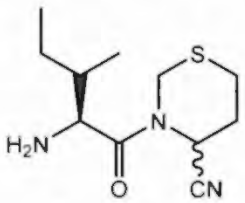


Compound 8
K_i = 260 nM

- Increasing the ring size of Ashworth's 5-membered thiazolidine ring to a 6-membered thiazinane ring resulted in greater than a 1000-fold decrease in potency. (Ashworth II at Table I (*compare* compound 3, *with* compound 12).)

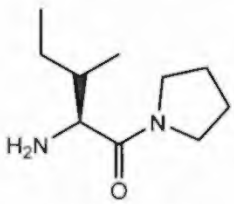


Compound 3
K_i = 0.41 nM

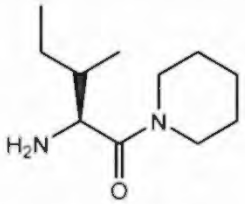


Compound 12
K_i = 6000 nM

- Increasing the ring size of Augustyn's 5-membered pyrrolidine ring to a 6-membered piperidine ring resulted in a 24-fold decrease in potency (Augustyn 1997 at Table I (*compare* compound 3, *with* compound 6b).)

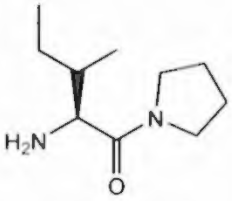


Compound 3
IC_{50} = 21 μM

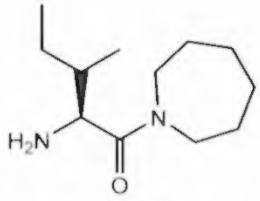


Compound 6b
IC_{50} = 510 μM

- Further increasing the ring size of Augustyn's 5-membered pyrrolidine ring to a 7-membered azepane ring resulted in a greater than 100-fold decrease in potency (Augustyn 1997 at Table I (*compare* compound 3, *with* compound 7b).)



Compound 3
IC_{50} = 21 µM

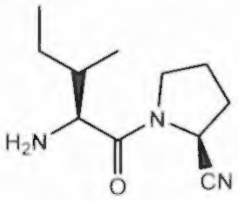


Compound 7b
IC_{50} = 2700 µM

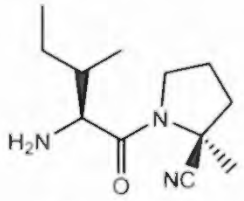
150. As Dr. Powers states, "the cyclopropyl ring converts the 5-membered pyrrolidine ring into a fairly rigid [6-membered] piperidine ring." (Powers Report at ¶ 131.) The art as a whole taught away from making a 6-membered piperidine ring, and thus away from making a fused-cyclopropyl derivative of the vildagliptin compound.

151. In addition, data in the prior art also showed that increasing the size of the P1 pyrrolidine moiety by adding a substituent was not well-tolerated.

- Adding a methyl to Ashworth's pyrrolidine ring resulted in a 1900-fold decrease in potency. (Ashworth II at Table I (*compare* compound 5, *with* compound 11).)

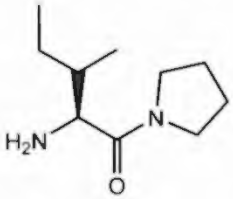


Compound 5
K_i = 2.2 nM

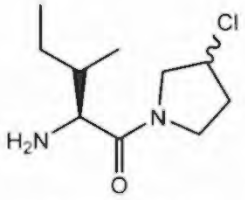


Compound 11
K_i = 4200 nM

- Adding a chlorine (which is similar in size to a methyl group) to Augustyn's pyrrolidine ring resulted in a 29-fold decrease in potency. (Augustyn 1997 at Table I (*compare* compound 3, *with* compound 19b).)

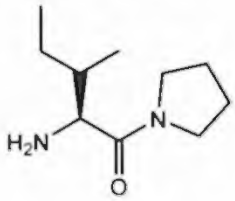


Compound 3
IC_{50} = 21 μM

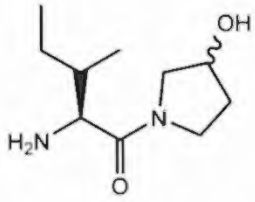


Compound 19b
IC_{50} = 610 μM

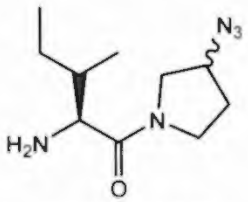
- Other substituents, including hydroxyl, azido, methoxy, and oxo also result in a decrease in potency ranging from a 21-fold to greater than 475-fold decrease. (Augustyn 1997 at Table I (*compare* compound 3, *with* compounds 16b, 20b, 22b, 23b).)



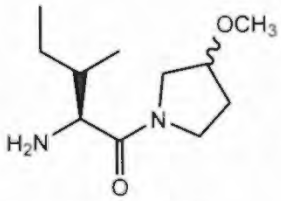
Compound 3
IC_{50} = 21 μM



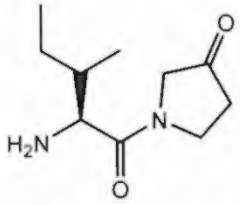
Compound 16b
IC_{50} = 740 μM



Compound 20b
IC_{50} = 1070 μM



Compound 22b
IC_{50} = >10,000 μM

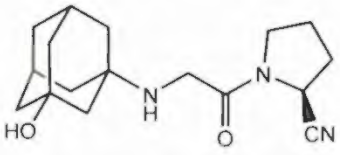


Compound 23b
IC_{50} = 320 μM

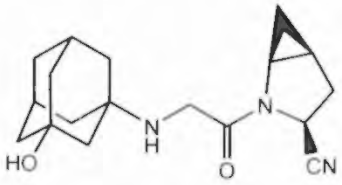
152. The addition of fluorine to Augustyn's pyrrolidine ring was tolerated; however, one skilled in the art would understand that fluorine is closer in size to a hydrogen atom and therefore would have a minimal effect on the size of the P1 moiety. (Augustyn 1997 at Table I.)

153. Thus, one skilled in the art would have expected that increasing the size of the P1 moiety of vildagliptin, whether by increasing the ring size or by addition of a substituent other than fluorine, would have been detrimental to potency.

154. In fact if one increases the size of vildagliptin's pyrrolidine ring by adding a cyclopropyl as Dr. Powers suggests, the potency of the resultant vildagliptin analog decreases. (AZ-SAXA-8031408 (*compare* compound 471211 (vildagliptin; K_i = 135 nM), *with* compound 471216 (K_i = 362 nM)).)

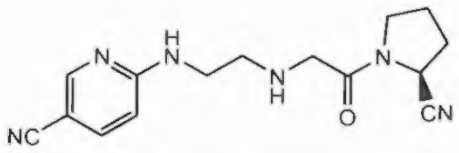


BMS-471211 (Vildagliptin)
K_i = 135 nM

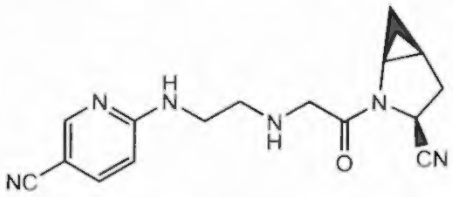


Compound 16b
K_i = 362 nM

155. Similarly, adding a cyclopropyl ring to the clinical candidate NVP-DPP728 results in an analog with decreased potency. (AZ-SAXA-8031449 (*compare* BMS-428245 (NVP-DPP728; K_i = 7 nM), *with* cyclopropyl analog BMS-436952 (K_i = 160 nM)).)



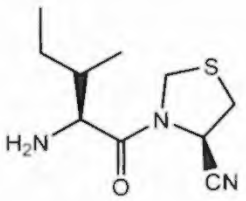
BMS-428245 (NVP-DPP728)
K_i = 7 nM



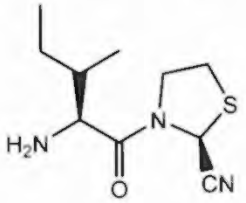
BMS-436952
K_i = 160 nM

156. Dr. Powers argues that a "sulfur atom in the heterocyclic ring makes the ring slightly larger and more hydrophobic" and that such modifications "lead to increased potency." (Powers Report at ¶ 126.) I disagree with Dr. Powers' implication that the increase in potency for the sulfur containing thiazolidine compounds compared to the pyrrolidine compounds is a result of an increase in ring size. In Ashworth II, compounds 3 and 4, both of which contain sulfur in the ring and thus would be expected to have the same size and hydrophobicity, differ in activity by 4-fold. (Ashworth II at 2747, Table I.) In light of these results, one of skill in the art

would have understood that other properties of sulfur (*e.g.*, electronic properties), not its size, were responsible for the increased potency. Even if true, that observation does not suggest increasing the size of cyanopyrrolidines by adding additional carbons, which had already been rejected as inferior (*see* ¶¶ 149-153 above), but instead selecting the sulfur containing compounds of Ashworth II, which is exactly what Ashworth II suggests.



Compound 3
K_i = 0.41 nM



Compound 4
K_i = 1.7 nM

157. Dr. Powers argues that Hanessian teaches "that replacing proline with proline analogs in protease inhibitors increases the potency of the inhibitor." (Powers Report at ¶ 115.) In support of this argument, Dr. Powers presumes that a skilled artisan would have looked to the ACE inhibitor literature in designing a DPP-4 inhibitor. (Powers Report at ¶¶ 116, 118.)

158. I disagree. Inhibitors of ACE do not meaningfully inform the design of DPP-4 inhibitors because the enzymes are fundamentally different. For example:

- ACE and DPP-4 have different mechanisms of action. ACE is a zinc metalloprotease and DPP-4 is a serine protease. (*See* ¶¶ 60-64 above.) Because of these mechanistic differences, known ACE inhibitors contained a nucleophile at P1' such as a thiol (captopril) or carboxylate (enalipril, ramipril, and perindopril) that bound to the electrophilic zinc in the active site, while known DPP-4 inhibitors contained no such nucleophile. (*See* Cushman and Ondetti at 1111) In fact, many DPP-4 inhibitors contained electrophiles at P1 such as a boronic acid or a cyano group which react with the nucleophilic serine in the active site. (*See* Augustyns 1999.)

- ACE and DPP-4 cut at opposite ends of their respective peptide substrates. ACE cleaves a dipeptide at the *C*-terminus, whereas DPP-4 cuts at the *N*-terminus. (*See* ¶ 64 above.) Thus, the design of inhibitors would have been understood to be fundamentally different. This is reflected in the requirement

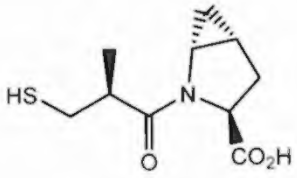
for a carboxylic acid in an ACE inhibitor at the P2' position and an amino group in a DPP-4 inhibitor at the P2 position.

- While both ACE and DPP-4 cleave proline-containing peptides, the position of the proline in the peptide substrate is different. In ACE, the proline is typically at the P2' position, while in DPP-4 it is at the P1 position. Thus, the design of inhibitors would have been understood to be fundamentally different. (Yoshioka et al., *Role of rat intestinal brush-border membrane angiotensin-converting enzyme in dietary protein digestion*, Am. J. Physiol. 253:G781-G786 (1987); Mentlein.)

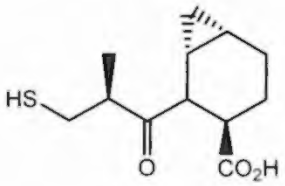
159. Thus, one skilled in the art would not have looked to ACE inhibitors such as captopril, enalipril, ramipril, and perindopril for the design of a DPP-4 inhibitor. Further, one would not have looked to analogs of these compounds such as those described by Hanessian for guidance on the design of DPP-4 inhibitors. As with Dr. Powers selection of vildagliptin as a lead compound, Dr. Powers' focus on Hanessian is supported only by hindsight, specifically, hindsight knowledge from the publications of the inventors of saxagliptin that they used the chemistry disclosed in Hanessian to make some of their cyclopropanated compounds. I understand that the inventors' thought processes are not part of the prior art and cannot be used to establish obviousness.

160. Even if one were to look at Hanessian's work on ACE inhibitors for motivation to add a cyclopropyl to vildagliptin to increase potency, one skilled in the art would see that the SAR for ACE inhibitors was fundamentally different from what was known for DPP-4 inhibitors. For example, Hanessian teaches that 5- and 6-membered rings were similarly potent for ACE inhibition. (Hanessian et al., *Probing the importance of spacial and conformational domains in captopril analogs for angiotensin converting enzyme activity*, Bioorg. & Med. Chem. Letts., 8:2123-2128, 2127, Table 1 (1998) ("Hanessian 1998") (compound 4 (7.6 nM) and compound 8 (5.3 nM), respectively).) This is in contrast to the body of DPP-4 literature

described above that teaches away from a 6-membered ring in the P1 position of DPP-4 inhibitors. (*See* ¶¶ 149-153 above.)

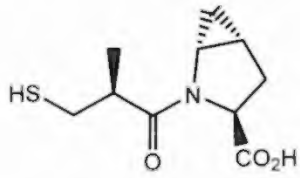


Compound 4
IC_{50} = 7.6 nM

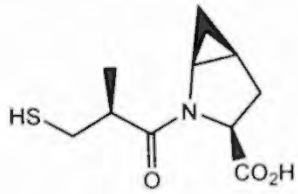


Compound 8
IC_{50} = 5.3 nM

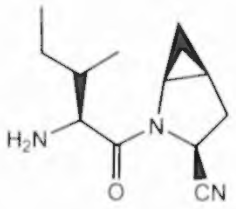
161. Consistent with the anticipated differences in SAR, Hanessian shows that the addition of cyclopropyl increases potency in the context of ACE inhibitors (*see* captopril (13 nM)), whereas the addition of cyclopropyl decreases potency in the context of DPP-4 inhibitors (Hanessian 1998 at 2127, Table 1; *see* ¶¶ 153-154 above). Further, Hanessian shows that the *cis* and *trans* cyclopropyl isomers have similar potencies in the context of ACE inhibitors (*see* Hannesian 1998 at 2127, Table 1 (compound 4 (IC_{50} = 7.6 nM) and compound 6 (IC_{50} = 6.6 nM) respectively)), whereas the *cis* and *trans* cyclopropyl isomers in the context of DPP-4 inhibitors have different potencies (*see e.g.*, Magnin at 2589, Table 1 (compound 24 (K_i = 25 nM) and compound 22 (K_i = 1620 nM) respectively).)



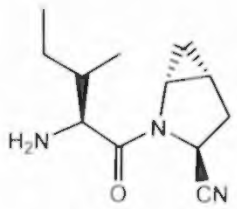
Compound 4
IC_{50} = 7.6 nM



Compound 6
IC_{50} = 6.6 nM



Compound 24
K_i = 25 nM

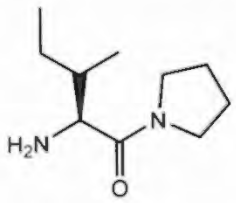


Compound 22
K_i = 1620 nM

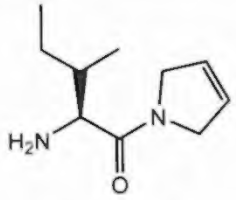
162. Dr. Powers also argues that adding the cyclopropyl would make the ring "more rigid, resulting in a beneficial entropic effect upon binding to the enzyme." (Powers Report at ¶ 130.) As Dr. Powers acknowledges, "[o]f course the rigid molecule must be designed to fit into the active site of the enzyme." (Powers Report at ¶ 129.) A person skilled in the art would have had no motivation to increase rigidity with a reasonable expectation of a beneficial entropic effect on potency, because as described above, the potency of vildagliptin was already seemingly sufficient. Further, one would be unable to rationally design an appropriately rigid molecule in the absence of a DPP-4 x-ray crystal structure, which was not disclosed until 2003.

163. Even Hanessian demonstrates the failure of adding cyclopropyl in the absence of structural data. In his work on glutamate receptors, Hanessian reported that "no significant binding affinity was found" with cyclopropanated inhibitors. (Hanessian et al., *The synthesis of 4,5-methano congeners of α-kainic and α-allo-kainic acids as probes for glutamate receptors*, Tetrahedron Letts., 37:8971-8974, 8973 (1996) ("Hanessian 1996").) He stated that "[c]learly, the structural requirements for effective binding to these receptors have not been satisfied by our methano [(cyclopropanated)] analogs in spite of their novel structures." (*Id.*) He goes on to state that the lack of activities are "reflective of the lack of our understanding for specific spatial requirements and hydrophobic interactions of the appended cyclopropane…." (*Id.*) The available knowledge regarding the effect of cyclopropanation of DPP-4 inhibitors was no better.

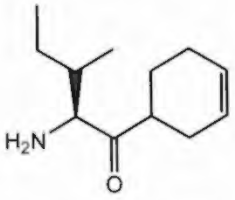
164. Dr. Powers argues that Hanessian showed that the cyclopropyl ring "flattens" the 5-membered ring and "makes a structure resembling a conformationally-fixed six membered ring." (Powers Report at ¶ 131.) One skilled in the art would understand that a partially unsaturated pyrrolidine ring or piperidine ring would also result in flattening and conformational rigidity. One of skill in the art would have further understood that flattening the ring or making the ring resemble a conformationally-fixed 6-member ring had previously resulted in a decrease in potency. (Augustyn 1997 at 304, Table I (showing that such structural modifications resulted in a ~5-fold decrease in potency (*compare* compound 3, *with* compound 9b) and ~15-fold decrease in potency (*compare* compound 3, *with* compound 10b) respectively).)



Compound 3
IC_{50} = 21 μM



Compound 9b
IC_{50} = 100 μM



Compound 10b
IC_{50} = 310 μM

b. Stability Was Not A Reason to Add Cyclopropyl

165. Dr. Powers further argues that addition of cyclopropyl would make the resultant vildagliptin analog "much less likely to cyclize to form the cyclization product" and "more stable than vildagliptin itself" because of the increased ring strain in the diketopiperazine product as a result of the flattening from the cyclopropyl group. (Powers Report at ¶¶ 134-135.)

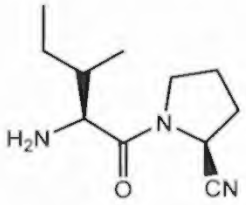
166. One of skill in the art would not have had a motivation to make a more stable vildagliptin molecule when there were no data available for vildagliptin indicating stability was an issue. The available data for NVP-DPP728, also an *N*-linked glycine nitrile, showed good stability. (Hughes at 11599 (reporting a solution stability half-life of approximately 72 hours for NVP-DPP728).) In addition, this stability of NVP-DPP728 was apparently sufficient for

Novartis to proceed with clinical development. Indeed, given the reports of cyclization stability issues with *C*-linked, primary amine-containing cyanopyrrolidine compounds like Ashworth's, that never proceeded to clinical trials, a person of ordinary skill in the art would have concluded that Novartis solved any stability problem by employing an *N*-linked secondary amine in its compounds.

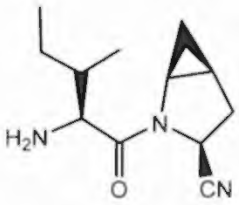
167. Dr. Powers' argument is further problematic because it assumes any flattening and increasing of rigidity in the ring would have a beneficial effect on stability. Dr. Powers provides no support from the literature for this assertion. The effect of a cyclopropyl group on the reactivity of the resultant methano pyrrolidine nitrile toward cyclization had never been explored and was entirely unpredictable.

168. Had one skilled in the art wanted to improve stability, one would have taken other factors into account, other than "flattening." Such other factors would have included *e.g.*, the steric hindrance around the amine nucleophile or the cyano electrophile.

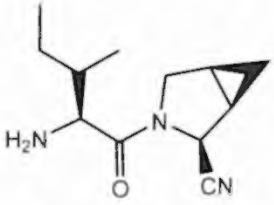
169. The importance of these other factors is born out in the data later published by BMS. Magnin showed that the surprising increase in solution stability with the *cis*-4,5-cyclopropyl addition to the pyrrolidine ring was specific to this particular attachment of the cyclopropyl group. For example, while the *cis*-4,5-cyclopropyl gave an improvement in stability half-life as compared to the pyrrolidine nitrile without a cyclopropyl group, the *cis*-3,4-cyclopropyl showed no such increase. (Magnin at 2589, Table 1 (*compare* compound 24 ($t_{½}$ = 22 hours), *with* compound 21 ($t_{½}$ = 5 hours) and *compare* compound 25 ($t_{½}$ = 4 hours), *with* compound 21 ($t_{½}$ = 5 hours), respectively).)



Compound 21
$t_{½}$ = 5 h



Compound 24
$t_{½}$ = 22 h



Compound 25
$t_{½}$ = 4 h

170. Thus, there would have been no reasonable expectation that modifying the pyrrolidine ring with a cyclopropyl group would lead to an improvement in potency and/or stability.

5. No Motivation to Move from *N*-Linkage to *C*-Linkage and No Reasonable Expectation of Success

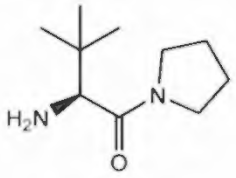
171. Dr. Powers argues that a person skilled in the art would be motivated to modify vildagliptin to a primary amine "in order to increase the potency of the molecule and increase the substrate-like character of the inhibitor." (Powers Report at ¶ 105.)

172. I disagree. As stated above, based on the available prior art, one skilled in the art would not have considered the potency of vildagliptin to be problematic. (*See* ¶ 145 above.)

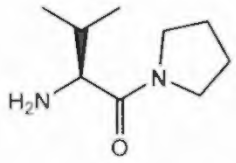
173. Further a skilled artisan would not have been motivated to make vildagliptin more like a peptide substrate for at least three reasons. First, peptide-like inhibitors tend to have issues with oral bioavailability. Second, peptide-like inhibitors are prone to rapid metabolism resulting in a short half-life. Third, if one wanted to make vildagliptin more substrate-like, one would replace the proline with the alanine found in the endogenous substrates GLP-1 and GIP. (*See* Mentlein at 830, Figure 1.)

174. Dr. Powers points to Ashworth I as teaching that a skilled artisan "should move the hydroxyadamantyl group from the nitrogen of vildagliptin to the alpha-carbon [ ] because dipeptide nitrile inhibitors with *beta*-branching at the P2 position are the most potent." (Powers

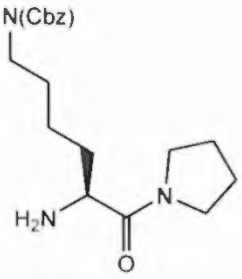
Report at ¶ 109.) While Ashworth I states that for the non-nitrile series "β-branched α-amino acid derivatives were the most potent compounds," those data are limited and show that a quaternary carbon (which is most relevant to the adamantyl group) is *less* potent. (*See* Ashworth I at 1165.) Specifically, a direct comparison between *t*-butyl glycine compound 11 (880 nM) and valine compound 9 (470 nM) shows decreased potency moving from the tertiary isopropyl to the quaternary t-butyl group. (*Id.* at 1164, Table 1.) In fact, the *t*-butyl compound 11 is less potent than the Lys(Cbz) compound 10 (520 nM), which has no branching at all. (*Id.*) Thus, Ashworth I would not have provided motivation to one skilled in the art to place a quaternary carbon like that found in the hydroxyadamantyl group of vildagliptin on the *alpha*-carbon of the P2 amino acid moiety.



Compound 11
K_i = 880 nM



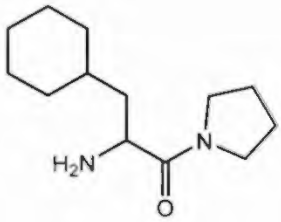
Compound 9
K_i = 470 nM



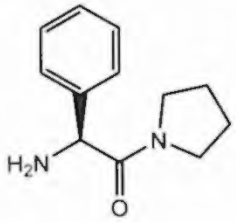
Compound 10
K_i = 520 nM

175. Dr. Powers also argues that "since aminoacyl pyrrolidine inhibitors with large bulky group are among the more potent inhibitors of DPP-IV without a warhead, a person skilled in the art would have been motivated to move the hydroxyadamantyl to the *alpha*-carbon of the dipeptide inhibitor to improve interaction between the inhibitor and the S2 subsite of the enzyme." (Powers Report at ¶ 112.) As discussed at ¶ 145 above, a skilled artisan would have considered vildagliptin already sufficiently potent and would have had no motivation to move the hydroxyadamantyl group. Dr. Powers points to two examples of bulky side chains, but their

activity differs by about 10-fold (cyclohexylglyine compound 5 has a potency of 64 nM and Lyz(Cbz) compound 10 has a potency of 520 nM), suggesting no uniform rule for potency based on "bulk." (Powers Report at ¶ 112; Ashworth I at 1164, Table 1.) Furthermore, Dr. Powers ignores the remaining examples with bulky groups in Table 1 of Ashworth I that are even less potent than compound 10. (Ashworth I at 1164, Table 1.) For example, compound 17 (cyclohexylalanine) has a potency of 2150 nM and compound 20 (phenylglycine) has a potency of 5300 nM. (*Id.*) This teaches that having a bulky group *per se* is not advantageous. One skilled in the art would have understood that the interactions between the inhibitor and the enzyme would depend on the specific substituent, and further would be different depending on whether that P2 substituent was attached to the nitrogen or the *alpha*-carbon.



Compound 17
K_i = 2150 nM



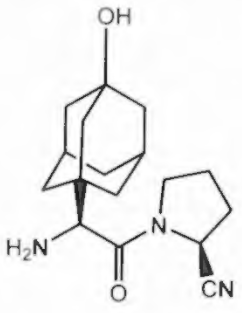
Compound 20
K_i = 5300 nM

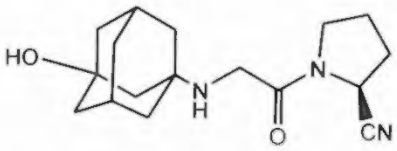
176. Furthermore, moving the "bulky" group, whatever it may be, to the *alpha*-carbon causes the group to be located differently in space in relation to the enzyme S2 sub-site, and whether that change would be beneficial or detrimental to potency was entirely unpredictable. Again, Dr. Powers' contention appears to be driven by hindsight recognition that the inventors found that *C*-linkage of the hydroxyadamantyl in a cyclopropanated cyanopyrrolidine like saxagliptin does in fact improve potency in relation to *N*-linkage (like vildagliptin) in the same molecule. (AZ-SAXA-8031405 *(compare* BMS-477118 (K_{is} = 38 nM), *with* BMS-471216 (K_{is} = 362 nM)).) That information, however, was unknown in the prior art. Whether or not the

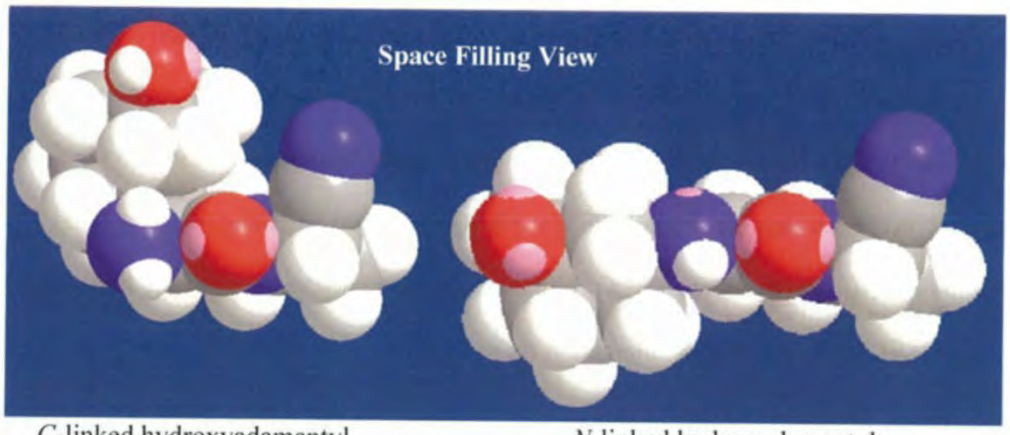
interactions arising from *N*- or *C*- linkage were better or worse would depend on the nature of the group and could not have been predicted, particularly in the absence of an x-ray crystal structure.

177. Specifically, had one skilled in the art even considered the change proposed by Dr. Powers, he or she would have recognized that a large, rigid *N*-linked hydroxyadamantyl group could not bind in the same region of the P2 pocket (S2 sub-site) as a *C*-linked hydroxyadamantyl group, and thus would expect that the SAR for the two series would be different. While a structure of the DPP-4 enzyme-inhibitor complex was unknown, one skilled in the art would understand that such an inhibitor would have been anchored in the enzyme active site in at least two places. (Hughes at 11602 (reporting that "structure-activity relationships support the involvement of several key interactions.") Specifically, the pyrrolidine ring would bind in a proline-binding pocket, and the required amino group would bind in an amino binding site. This would anchor the inhibitor such that the position of the hydroxyadamantyl group would be restricted by its point of linkage (*C*- versus *N*-linkage).

178. This would have been readily apparent by examining molecular models that would have been available to one of skill in the art at the time. One skilled in the art at the time would have concluded that these linkages were not interchangeable, especially for large bulky, rigid groups such as hydroxyadamantyl.

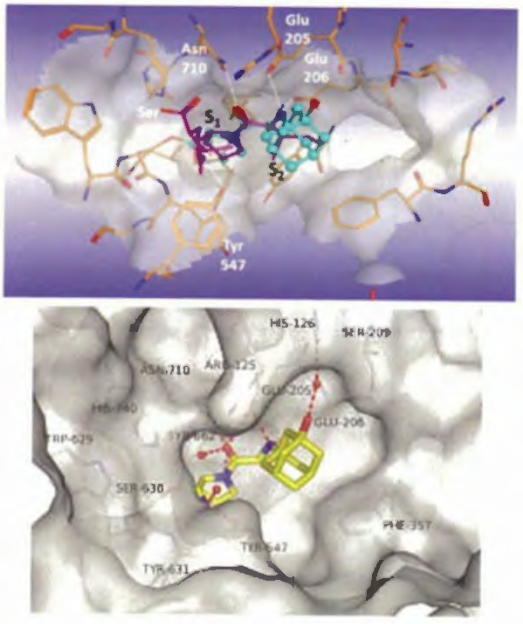




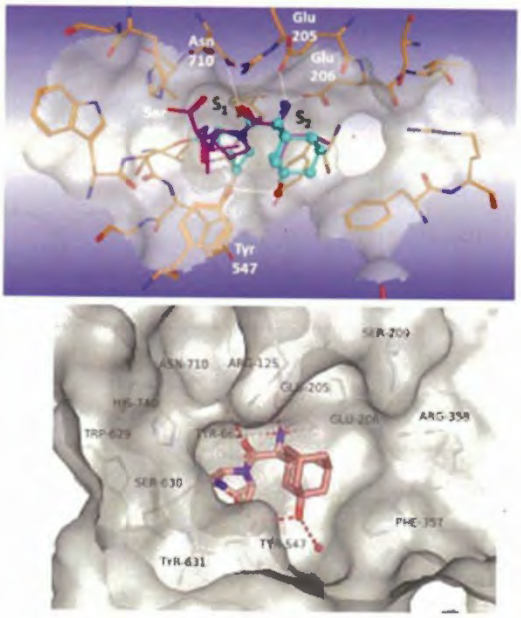


C-linked hydroxyadamantyl *N*-linked hydoxyadamantyl

179. The fact that *N*-linked hydroxyadamantyl and *C*-linked hydroxyadamantyl could not have been predicted to bind in the same place in the binding pocket is confirmed by subsequent data showing they do not. (Nabeno et al., *A comparative study of the binding modes of recently launched dipeptidyl peptidase IV inhibitors in the active site*, Biochem. Biophys. Res. Comm. 434:191-196, 194 (2013); Merck public presentation at 28 ("Merck presentation").).



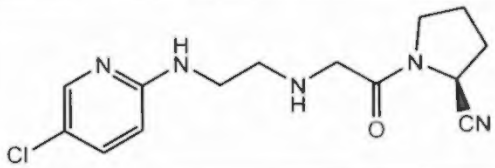
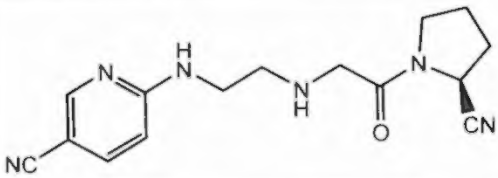
Vildagliptin

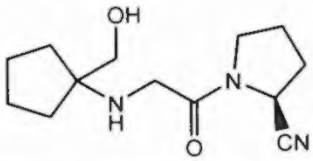
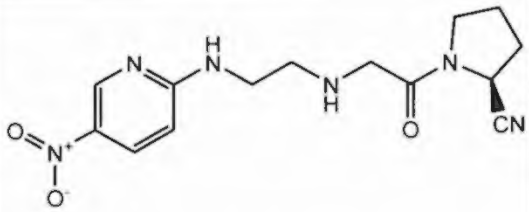
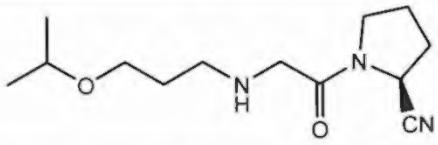


Saxagliptin

180. The hydroxyadamantyl group of vildagliptin binds in the top part of the S2 sub-site. In contrast, the hydroxyadamantyl group of saxagliptin binds in the bottom of part of the S2 sub-site and forms a favorable hydrogen-bonding interaction between the adamantyl hydroxyl group and Tyr547, which is not available to the hydroxyl group on vildagliptin due to its different orientation in the pocket. (*Id.*) Without a crystal structure, there would have been no way to predict that this favorable interaction for saxagliptin would exist.

181. Dr. Powers points to Villhauer-998 and argues that the "N-substituted derivatives were poorer [(less potent)] than the Ashworth I compounds that have no substitution on the *alpha*-amino P2 amino acid residue." (Powers Report at ¶ 111.) Dr. Powers interprets Villhauer as teaching "that introduction of a bulky group on the N-terminus of a Gly-Pro-CN inhibitor decreases potency." (Powers Report at ¶ 111.) In my opinion, Dr. Powers misinterprets and misrepresents the data in Villhauer-998. The structures for the 5 compounds and the reported data are shown below.

Example	Structure	Increase of Insulin Response at 10 mmol/kg	IC_{50} (nM)
Ex. 1		61%	27
Ex. 3		66%	7

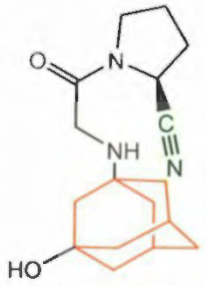
Example	Structure	Increase of Insulin Response at 10 mmol/kg	IC_{50} (nM)
Ex. 5		108%	37
Ex. 8		144%	12
Ex. 12		59%	95

182. The structure Dr. Powers presents at ¶ 110 of his report for Example 3 is incorrect. Example 3 is NVP-DPP728, as shown above. Examples 1 and 8 are analogs of NVP-DPP728 where the cyano group on the pyridine ring has been replaced by chloro and nitro, respectively. (Villhauer-998 at 9-11.) Example 5 is an *N*-((1-hydroxymethyl)cyclopent-1-yl) derivative and Example 12 is an *N*-(3-(isopropoxy)propyl) derivative. (*Id.* at 11.)

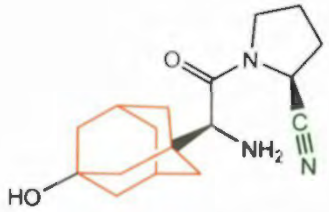
183. Dr. Powers argues that a bulky group on the *N*-terminus of a glycyl inhibitor decreases potency. Contrary to his characterization in ¶ 110, of the five examples presented in Villhauer-998, the compound with the least bulky group off of the nitrogen (branching limited to one methyl group five atoms away) is the least potent (Example 12). (*Id.* at 21.) Further, one skilled in the art would likely consider vildagliptin itself as more bulky than any of these compounds, and its potency is not inferior to the Examples reported in Villhauer-998.

184. Dr. Powers acknowledges the concerns of intramolecular cyclization but then surmises that "[m]oving the hydroxyadamantyl group to the *alpha*-carbon of the P2 residue of vildagliptin would likely introduce steric bulk nearer to the nitrile warhead" that is "likely to further diminish the potential for cyclization reaction." (Powers Report at ¶ 113.)

185. I disagree. By moving the hydroxyadamantyl group to the *alpha*-carbon, steric bulk around the electrophile, in the conformation required for cyclization, is not increased. As depicted below, moving the hydroxyadamantyl group from nitrogen to carbon moves it ***away*** from the electrophile and would therefore be expected to ***increase*** cyclization, not decrease it.

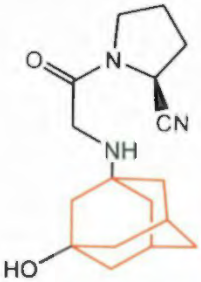


Adamantyl Close to Cyano

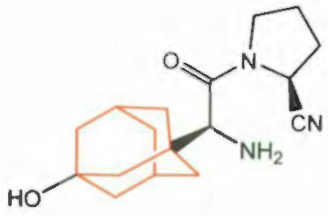


Adamantyl Away from Cyano

186. Furthermore, a person skilled in the art would have understood that steric bulk around both the electrophile and the nucleophile affects nucleophilic attack. Moving the hydroxyadamantyl to the *alpha*-carbon would also decrease steric bulk around the nucleophile, which would potentially increase its propensity for nucleophilic attack.

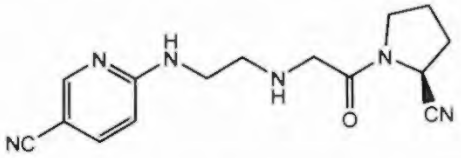


Sterically "***Hindered***" Amine

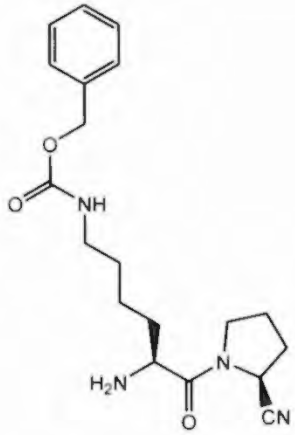


Sterically "***Unhindered***" Amine

187. Comparing the solution stabilities of NVP-DPP728 ($t_{1/2}$ = 72 hours) with the closest analogous Ashworth compound, compound 28 ($t_{1/2}$ = 24 hours), suggests to the skilled artisan that moving a substituent from nitrogen to carbon would not necessarily increase stability. (Hughes at 11599; Ashworth I at 1166, Table 2.)



NVP-DPP728
$t_{1/2}$ = 72 h



Compound 28
$t_{1/2}$ = 24 h

188. Thus, a skilled artisan would not expect that moving the hydroxyadamantyl group from the nitrogen to the carbon would improve stability. Indeed, as noted above, a person of ordinary skill in the art would have viewed the *N*-linkage of the P2 group in Novartis' clinical candidate NVP-DPP728, and even in vildagliptin, as Novartis' solution to any stability problem, not as creating a stability problem that needed to be fixed.

189. Dr. Powers relies on data from Li on an Ala-Pro-nitrile compound to support his argument that *C*-linked inhibitors would have been viewed as stable. (*See* Powers Report at ¶ 114.) The Li data are inconsistent with both the Ashworth I data and Dr. Powers' argument that greater steric bulk for *C*-linked nitrile compounds leads to better stability. Li shows half-life stability of greater than 72 hours with alanine (non-bulky and non-beta-branched) in the P2 position. (Li at 152-153.) By comparison, Ashworth shows half-life stability of only 48 hours

with the bulkier *beta*-branched cyclopentyl glycine and isoleucine compounds. (Ashworth I at 1166, Table 2.) One skilled in the art relying on Li would have no motivation to put a *C*-linked bulky *beta*-branched group in the P2 position of a DPP-4 inhibitor to improve stability.

190. While the stability of vildagliptin was not reported in the literature, if the skilled artisan had made vildaglipin and measured its stability, one would have found it was highly stable. Later data confirmed that 98.4% of vildagliptin remains after 3 days in solution at room temperature, which equates to a half-life solution stability of greater than 1000 hours. (US 2008/279932 at [0282].) These data show that vildagliptin was even more stable than the NVP-DPP728 compound that had gone into the clinic with half-life solution stability of 72 hours. (*See* Hughes at 11599.) Thus, there would have been no reason to modify vildagliptin to increase stability.

191. In summary, a skilled artisan would not have selected vildagliptin as a lead compound in light of available data on the two most advanced clinical candidates (NVP-DPP728 and P32/98). (*See* ¶¶ 70, 133-138 above.) Furthermore, if selected as a lead compound, there would have been no motivation to improve either potency or stability. Further, if one wanted to improve potency or stability, there would have been no motivation and no reasonable expectation of success that this could be accomplished by adding the cyclopropyl group to the cyanopyrrolidine in vildagliptin or moving the hydroxyadamantyl group from *N*- to *C*-linkage.

6. Ashworth I Compound 25 Was Not a Lead Compound

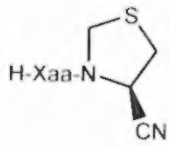
192. In contrast to Dr. Powers, Dr. Rotella argues that a skilled artisan would have been motivated to select compound 25 from the Ashworth I reference as a lead compound for further modification. (Rotella Report at ¶ 107.)

193. Compound 25 of Ashworth I would not have been selected as a lead compound in view of the two most advanced clinical candidates NVP-DPP28 and P32/98. (*See* discussion at ¶¶ 70, 133-138 above; Demuth 2000; Rothenberg.)

194. In support of his selection of compound 25 as a lead, Dr. Rotella argues that "Ashworth provides motivation to select the 2-cyanopyrrolidine analogues" in part because they "possessed activity comparable to the boroprolines" but "were more synthetically accessible than the boroprolines and metabolic considerations (boronic acid vs. cyano group) would make them more desirable." (Rotella Report at ¶ 109.)

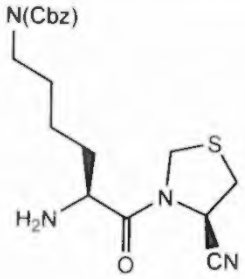
195. In my opinion, one of skill in the art would have recognized that one of the most advanced compounds in the clinic, P32/98, did not contain Ashworth I's cyano group and yet P32/98 showed acute glucose lowering in patients with type 2 diabetes. (Demuth 2000.) P32/98 has the advantage of eliminating the concern of chemical stability associated with nitrile-based inhibitors. Specifically, P32/98 is incapable of cyclizing to form the diketopiperazine discussed above. (*See* ¶ 91 above.) P32/98 also has the advantage of being more synthetically accessible than even the cyanopyrrolidines and resolves any metabolic considerations due to the boronic acid or nitrile groups. Thus, a skilled artisan concerned about these issues would have selected P32/98 over Ashworth I compound 25.

196. Dr. Rotella argues that compound 25 would have been selected as a lead because it "exhibits high potency" and "good solution stability compared to other analogs lacking a β-branched moiety at C2." (Rotella Report at ¶ 110.) Dr. Rotella disregards Ashworth's second publication teaching "4-cyanothiazolidide as an optimum C-terminal residue." (Ashworth II at 2746.)

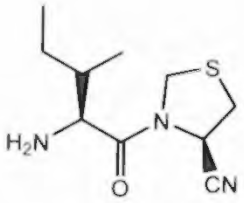


197. In this later publication, Ashworth II states that the "increase in activity was accompanied by a slight decrease in stability," but that these compounds nonetheless "were prepared with sub-nanomolar activity against DP-IV and good stability in aqueous buffer." (Ashworth II at 2746.) Thus, stability differences would not have dissuaded a person of ordinary skill in the art from following the clear teaching of Ashworth II that the cyanothiazolidines were the optimal P1 groups.

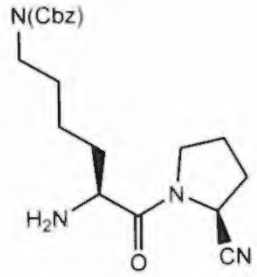
198. Furthermore, Ashworth I did not disclose a clear stability benefit in use of cyanopyrrolidine over cyanothiazolidine. Rather, the data showed that sometimes the cyanopyrrolidine was more stable and sometimes the cyanothiazolidine was more stable, as shown by the Lys(Cbz) and isoleucine examples in Ashworth I and II. (*Compare* compound 15 (>48 hours) and compound 3 (27 hours) of Ashworth II, *with* compound 28 (24 hours) and compound 26 (48 hours) of Ashworth I.) Thus, one skilled in the art would not a priori select compound 25 over the cyanothiazolidine series.



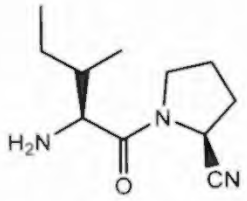
Compound 15
$t_{1/2}$ = > 48 h



Compound 3
$t_{1/2}$ = 27 h

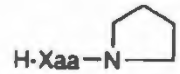


Compound 28
$t_{1/2}$ = 24 h



Compound 26
$t_{1/2}$ = 48 h

199. Dr. Rotella argues the comparison of Ashworth's pyrrolidide compound 5 and cyanopyrrolidine compound 25 "illustrates the beneficial effect on potency of the cyano moiety in DP-IV inhibitors." (Rotella Report at ¶ 112.) He argues this "provides motivation to optimize this to further improve potency." (Rotella Report at ¶ 112.) I disagree. As noted above, both the pyrrolidine and cyanopyrrolidine P1 residues of Ashworth I were superseded by the later expressed preference for cyanothiazolidine of Ashworth II. A person skilled in the art would have appreciated that the most advanced clinical candidates, both of which had shown acute glucose lowering in patients at reasonable doses, were already sufficiently potent. Ashworth I's pyrrolidide compound 5 (and in fact, also compounds 6-14) had greater reported *in vitro* potency than the clinical candidate P32/98 (K_i = 1.8 μM). (Ashworth I at 1164, Table 1; Augustyn 1997 at 304.) This highlights that one skilled in the art would not have been motivated to select and optimize compound 25 for potency reasons.



Compound N°	Xaa	K_i (μM)[13]
5	Cyclohexylglycine [Chg]	0.064 ± 0.01
6	(R,S)-Cyclopentylglycine [Cpg]	0.21 ± 0.04
7	Ile	0.41 ± 0.01
8	*allo*-Ile	0.44 ± 0.04
9	Val	0.47 ± 0.02
10	Lys(Cbz)	0.52 ± 0.07
11	tert-Butylglycine [Tbg]	0.88 ± 0.20
12	Thr(Me)	0.90 ± 0.15
13	Orn(Cbz)	0.91 ± 0.20
14	2-Aminohexanoic acid [Aha]	1.20 ± 0.20

200. Dr. Rotella argues that "[t]aken as a whole, the Ashworth reference contains ample and explicit motivation for one of skill in the art to select compound 25 as a lead." (Rotella Report at ¶ 113.) However, a variety of structural series were being explored at this time. Among these, potent inhibitors such as reported by Ashworth II were known, but the structural requirements for designing a safe and effective therapeutic DPP-4 inhibitor were largely unknown. Ashworth I provides data largely limited to *in vitro* potency and room temperature stability for a limited number of compounds, and the Ashworth I compounds were never developed into clinically useful inhibitors. In contrast, more complete data on other pertinent properties were available for the most advanced compounds which had in fact entered the clinic. (*See* ¶¶ 70, 93, 126-127 above.) This information would have been highly valuable to a medicinal chemist starting a DPP-4 inhibitor program and would have influenced their consideration of a lead compound away from Ashworth I, compound 25.

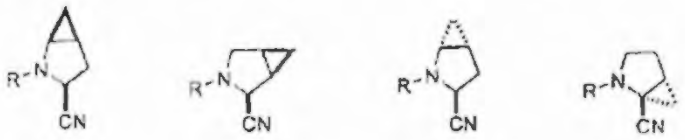
7. Even Accepting Ashworth Compound 25 as a Lead, A Skilled Artisan Would Not Make the Modifications Dr. Rotella Suggests

a. No Reason to Add Cyclopropyl and No Expectation of Success in Doing So

201. Dr. Rotella argues that a person of skill in the art would have "sought to modulate the orientation of the 2-cyano moiety on the pyrrolidine ring in order to optimize interaction with and inhibition of the DP-IV enzyme." (Rotella Report at ¶ 141.) I disagree. As stated above, compound 25 of Ashworth I is already sufficiently potent. (*See* ¶ 199 above.)

202. One of skill in the art would not look to the P1 pyrrolidine ring as a place for optimizing the interaction with DPP-4 given the body of SAR evidence showing limited tolerability for changes at that position. As discussed in ¶¶ 149-153 above, even small changes in size or substitution had adverse effects on potency. Further, there would have been no reasonable expectation of success that changing the orientation of the nitrile would lead to an improvement in interactions with DPP-4 given that highly potent, subnanomolar nitrile-containing compounds were already known.

203. One of skill in the art would expect that fusing a cyclopropyl ring to the pyrrolidine would have had an effect on the orientation of the nitrile, but one would have no way of predicting whether the effect would increase potency or be positive in any way. In fact, if one of skill in the art had fused a cyclopropyl ring as Dr. Rotella suggests to the Ashworth pyrrolidine compounds, regardless of the location and position, one would have found potency to be decreased. For example, Magnin reported a series of Ile cyclopropyl cyanopyrrolidines with a 7.5-fold to greater than 3500-fold decrease in potency compared to Ile cyanopyrrolidine compound 21. (Magnin at 2589, Table 1.)



cis-4,5 *cis*-3,4 *trans*-4,5 *trans*-2,3

compd	N-terminal amino acid[a]	substituted prolinenitrile	K_i (nM)[b]	stability, $t_{1/2}$ (h)[c]
21	Ile	prolinenitrile	2 ± 0.5	5
22	Ile	*trans*-4,5-	1620 ± 80	
23	Ile	*trans*-2,3-	7500 ± 200	
24	Ile	*cis*-4,5-	25 ± 1	22
25	Ile	*cis*-3,4-	15 ± 1	4

204. Further, if one had fused the cyclopropyl group in the *cis*-4,5- position, as in saxagliptin, to compound 25 of Ashworth I, one would have found a 10-fold ***decrease*** in potency. (*Compare* Magnin at 2589, Table 1, compound 39 (K_i = 15 nM), *with* Ashworth I at 1166, Table 2, compound 25 (K_i = 1.4 nM).)

205. Dr. Rotella argues that one of skill in the art would have been motivated to optimize compound 25 "by making small, incremental changes to its size and rigidity near the active 2-cyano group." (Rotella Report at ¶ 143.) I disagree. A skilled artisan would have been aware of data showing that small, incremental changes on the pyrrolidine ring resulted in a loss of activity. (*See* ¶¶ 149-153 above.) Thus, there would have been no motivation to modify the pyrrolidine ring and if one had modified it with a cyclopropyl group, one would have found a decrease in activity as described in ¶¶ 154-155 above.

206. Dr. Rotella argues that cyclopropanation "would have been one of the first modifications a person of ordinary skill would have chosen for its optimization." (Rotella Report at ¶ 144.) I disagree. Based on the discussion above, it would not have been obvious even to try this modification. In fact, with the exception of the BMS scientists, I am not aware of any evidence of cyclopropyl modifications being made by anyone else working in the DPP-4 field.

207. Dr. Rotella argues the 4,5-methanopyrrolidine "would have been one of only a small set of compounds that would have been investigated by one of ordinary skill in the art." (Rotella Report at ¶ 146.) This contention, like others, appears to have been driven by hindsight. Such hindsight, I understand, is forbidden in the obviousness analysis. While there was no motivation to make such compounds, if one had made all possible regio- and stereoisomers in order to optimize for potency, one would have observed a decrease in all cases. Further, if forced to choose a compound to optimize based on potency, one would have selected the *cis*-3,4, not the *cis*-4,5 isomer. While potency decreased for compounds with cyclopropyl addition, the potency was least diminished with addition of cyclopropyl in the *cis*-3,4- position. (Magnin at 2589, Table 1.)

208. Dr. Rotella argues that "Hanessian provides motivation for producing a fused pyrrolidine-cyclopropyl ring system as found in saxagliptin." (Rotella Report at ¶ 147.) I disagree. As stated above, Hanessian deals with ACE inhibitors and one skilled in the art would recognize that ACE inhibitors do not meaningfully inform the design of DPP-4 inhibitors. (*See* ¶ 158 above.)

209. Even if one skilled in the art were to look at Hanessian's work on ACE inhibitors, one would have recognized that ACE inhibitors and DPP-4 inhibitors have fundamentally different SAR. (*See* ¶ 158 above.)

210. Dr. Rotella argues one of skill in the art would have had a reason to "modify the 2-cyano substituted proline portion of the Ashworth lead compound 25 to a 4,5-methanoproline ring system in order to enhance compound stability." (Rotella Report at ¶ 149.) While this is now known in hindsight, there was no guidance leading in this direction in the prior art. And as noted in ¶ 203, there was no teaching and no way to predict the effect of cyclopropanation on

Ashworth I-like compounds. Moreover, the lesson learned from NVP-DPP728 was that the way to increase stability was to utilize an *N*-linked P2 group rather than a C-linked P2 group as proposed by Dr. Rotella.

211. If one had wanted to increase stability, I disagree that one would have reason to modify the pyrrolidine ring with a cyclopropyl group to accomplish this. Dr. Rotella does not provide any support for such modification. Dr. Rotella suggests such modification would result in flattening of the ring, "thereby adjusting the orientation of the cyano substituent to the proline ring and minimizing or preventing intramolecular cyclization." (Rotella Report at ¶ 149.) As discussed above in ¶¶ 164-168, one skilled in the art would have had no expectation of success by taking this approach.

212. Thus, in my opinion, a skilled artisan would not have been motivated to try adding cyclopropyl to compound 25 of Ashworth I, and further would have had no reasonable expectation of success that doing so would be beneficial to potency and/or stability.

b. No Reason to Substitute the Cyclohexyl of Compound 25 with an Adamantyl and No Expectation of Success in Doing So

213. Dr. Rotella also argues that a skilled artisan would have been motivated to substitute the cyclohexyl group in Ashworth I compound 25 with an adamantyl group because "adamantyl [is] an obvious alternative to cyclohexyl." (Rotella Report at ¶ 125.) I disagree. I am unaware of any teaching in the art of the interchangeability of these groups, and without hindsight, in my opinion, one of skill in the art would not have considered replacing a cyclohexyl group with an adamantyl group.

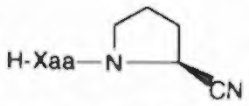
214. Dr. Rotella argues that Villhauer-998 performed a "comparative analysis between cyclohexyl and adamantyl" and thus Villhauer "provides motivation for selecting adamantyl as an alternative to cycloalkyl in a DP-IV inhibitor." (Rotella Report at ¶ 125.) The Villhauer-998

publication discloses no such comparative analysis. Furthermore, as discussed previously, Villhauer's DPP-4 work was on a fundamentally different backbone (*N*-linked) than Ashworth I compound 25 (*C*-linked). One skilled in the art would have recognized that a large, rigid *N*-linked adamantyl group could not bind in the same region of the S2 sub-site as a *C*-linked adamantyl group, and thus could not have predicted that the SAR for the two series would be the same. (*See* ¶¶ 179 above, and 249-251 below.) Hence, there would be no expectation of success.

215. Assuming, one of skill in the art was to consider the teachings of Villhauer-998, which they would not, in my opinion, one would look to "[t]he agents of Examples 1, 3, 5, 8, and 12" over examples 28 (cyclohexyl) and 47 (adamantyl), because examples 1, 3, 5, 8, and 12 have data whereas there are no reported data for examples 28 and 47. (Villhauer-998 at 21; *see also* ¶¶ 181-182 above including the table.) Of the 5 examples with data, Villhauer teaches that example 3 is "especially" preferred. (Villhauer-998 at 21) Example 3 is NVP-DPP728. Given these data, one of skill in the art would not have chosen to replace the cyclohexyl of Ashworth I compound 25 with the adamantyl from Villhauer-998.

216. Dr. Rotella argues that "[o]ne of ordinary skill in the art would have expected the modification to improve the characteristics of the compound, and particularly, to increase the potency and stability of the compound." (Rotella Report at ¶ 127.) I disagree. A skilled person would have considered Ashworth I compound 25 sufficiently potent, and Ashworth provides no motivation for replacing the cyclohexyl of Ashworth compound 25 with the adamantyl from Villhauer to increase potency or stability. (*See* ¶ 199 above.) Furthermore, there was no basis in the prior art to conclude that adamantyl substitution on those molecules would result in a DPP-4 inhibitor, and that such an inhibitor would have superior properties.

217. Dr. Rotella states that Ashworth I provides the motivation to substitute the C-linked cyclohexyl group with a large adamantyl group because Ashworth "showed increased stability for compounds having larger substituents at the 2-position." (Rotella Report at ¶ 119.) Yet, Ashworth I made no such showing. As reproduced below, compound 24-27 each have stability half-lives of 48 hours or longer. (Ashworth I at 1166, Table 2.) The largest group, Lys(Z) of compound 28, resulted in a decrease in half-life ($t_{1/2}$ = 24 hours). (*Id.*) Therefore, there would be no motivation to use a larger group to increase stability and no expectation of success.



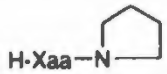
Compound N°	Xaa	K_i (nM)[13]	$t_{1/2}$ (h)[19]
24	Cpg	1.1 ± 0.2	48
25	Chg	1.4 ± 0.5	>48
26	Ile	2.2 ± 0.5	48
27	Tbg	3.8 ± 0.8	>48
28	Lys(Z)	5.2 ± 1.0	24

218. Dr. Rotella relies on Pal to support his contention that intramolecular cyclization could be reduced by "the addition of a large, steric group to the compound at C2 [(the *alpha*-carbon)]." (Rotella Report at ¶ 118.) Rotella argues that such a group would reduce intramolecular cyclization "by selecting against a conformation that favors intramolecular cyclization (i.e., selecting against the cis conformation)." (*Id.*) Dr. Rotella's reliance on Pal is misguided. Pal describes the preference of *cis*-peptide bonds adjacent to a proline residue in the context of large proteins rather than adjacent to a cyanopyrrolidine in a dipeptide-like structure. In addition, it is clear from Pal that sterics are not the only factor contributing to the *cis* conformation. (*See, e.g.*, Pal et al., *Cis peptide bonds in proteins: residues involved, their conformations, interactions and location*, J. Mol. Biol., 294:271-288, 272-73 and Figure 2

(1999).) Therefore, in my opinion, one of skill in the art would not have been motivated by the teachings of Pal to place a large, steric group in *alpha*-position of the P2 residue to improve stability of cyanopyrrolidine analogs.

219. Dr. Rotella also argues that one of skill in the art would recognize that there are regulatory guidelines for chemical stability that must be met and therefore a person of skill in the art would be motivated to develop compounds with good chemical stability. (Rotella Report at ¶ 120.) Dr. Rotella's reliance on the then current FDA stability guidelines is misplaced. The FDA guidelines provide nonbinding recommendations for stability testing the drug substance under conditions sufficient to "cover storage, shipment, and subsequent use" – NOT testing of aqueous solutions (pH 7.4 buffer) as reported in Ashworth. (International Conference on Harmonization; stability testing of new drug substances and products, 59 Fed. Reg. 48754-01 (Sept. 22, 1994).) More importantly, the FDA guidelines may identify a generally desired stability result, but they provide no guidance as to what specific modifications should be made to Ashworth I compounds to obtain it. If anything, the prior art as a whole suggests only that the stability of Ashworth's compounds might be improved by adapting the *N*-linkage strategy of Villhauer. Hence, there would be no motivation to modify Ashworth I compound 25 based on the FDA guidelines to arrive at saxagliptin.

220. Dr. Rotella argues that one of ordinary skill in the art would have also been motivated to substitute the cyclohexyl group at the 2-position of Ashworth compound 25 with an adamantyl group to improve potency. Specifically, Dr. Rotella states that "compound 5 (having a cyclohexyl group) of Table 1 shows at least a 100-fold increase in potency compared to compound 7 (having a 1-methyl propyl group) or compound 9 (having an isopropyl group)." (Rotella Report at ¶ 123.) As shown below, Dr. Rotella's comparison is incorrect.



Compound N°	Xaa	K_i (μM)[13]
5	Cyclohexylglycine [Chg]	0.064 ± 0.01
6	(R,S)-Cyclopentylglycine [Cpg]	0.21 ± 0.04
7	Ile	0.41 ± 0.01
8	*allo*-Ile	0.44 ± 0.04
9	Val	0.47 ± 0.02
10	Lys(Cbz)	0.52 ± 0.07
11	tert-Butylglycine [Tbg]	0.88 ± 0.20

221. While the cyclohexyl compound 5 is more potent than compounds 7 and 9 (by a factor of about 7, not 100) in the pyrrolidine series, as discussed in ¶ 174, Ashworth I teaches that a compound with a quaternary carbon (which is most relevant to the adamantyl group) is ***less*** potent than compounds 5, 7, and 9 and provides no teaching related to cyclic quaternary alkyl moieties. (*See* compound 11 in Ashworth I at 1164, Table 1.) Therefore, one of skill in the art would have no motivation to replace the cyclohexyl of compound 5 with an adamantyl and no expectation of success if such a compound were made.

222. Therefore, one of skill in the art would not be motivated to change the tertiary cyclohexyl group of Ashworth I compound 25 to a much larger group with a quaternary carbon, such as an adamantyl. Further, there would be no expectation that such a modification would increase potency.

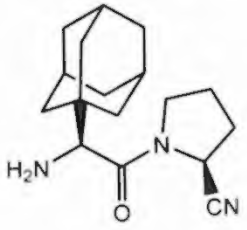
c. No Reason to Add a Hydroxyl to the Admantyl and No Expectation of Success in Doing So

223. Dr. Rotella argues that a skilled artisan would have been motivated to add a hydroxyl group at the 3-position of the adamantyl "to potentially increase compound solubility and possibly increase absorption of the compound without diminishing its activity." (Rotella Report at ¶ 129.)

224. I disagree. There would have been no motivation for one of skill in the art to increase compound solubility. As relatively low molecular weight amines, these compounds would have been expected to have excellent solubility. Further, there would have been no reason to think there would have been solubility-limited absorption issues. Thus, increasing solubility would not have been a reason to add a hydroxyl group given that doing so would likely have been unnecessary.

225. Dr. Rotella argues that "having a hydroxyl group at the 3-position would also improve the Lipinski parameters." (Rotella Report at ¶ 136.) I disagree. One skilled in the art would have understood that the Lipinski parameters include limits on the number of hydrogen bond donors and acceptors and that adding a hydroxyl group actually adds one hydrogen bond donor and one hydrogen bond acceptor to the compound. In my opinion, addition of a hydroxyl group as Dr. Rotella suggests would be a move in the opposite direction of Lipinski's teaching that "[a]n excessive number of hydrogen bond donor groups impairs permeability." (Lipinski at 8.)

226. Furthermore, Dr. Rotella presupposes that one skilled in the art would be motivated to lower the log P. However, if one would calculate the log P for the compound without a hydroxyl group, one would find that it is already well below Lipinski's cutoff of 5. (*See* Lipinski at 3.) Specifically, the calculated log P is 1.31.



clogP = 1.31

227. Dr. Rotella argues that Raag would have provided additional motivation to incorporate the hydroxyl group to the adamantyl to "block metabolism of a substituted adamantyl ring at the 3-position." (Rotella Report at ¶ 133.)

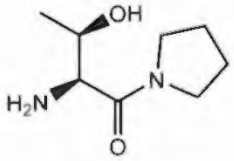
228. It was unknown at the time whether or not the adamantyl analog of Ashworth I compound 25 would metabolize and, if it did, whether or not the metabolism would occur on the adamantyl group. One skilled in the art would have understood that metabolism can be unpredictable. Even if a group was known to have a propensity to be metabolized in a specific way, whether or not it would undergo such metabolism would depend on the specific compound in which it was found. This is highlighted by the comparison of the metabolites of saxagliptin and vildagliptin. In saxagliptin, the hydroxyadmantyl group is metabolized to form a dihydroxyadamantyl active metabolite, and no such metabolite is seen for vildagliptin. (Su at 1346; He et al., *Absorption, metabolism and excretion of [^{14}C]vildagliptin, a novel dipeptidyl peptidase, in humans. Drug Metabo. and Dispos.*, 37:536-544, 543 (2009).)

229. In addition, if metabolism was a concern, a person skilled in the art would typically block the site of metabolism with a fluoro substituent, which would be a minimal change from hydrogen and as such less likely to affect the permeability and potency of the molecule. (*See, e.g.*, Augeri et al., *Discovery and preclinical profile of saxagliptin (BMS-477118): a highly potent, long-acting, orally active dipeptidyl peptidase IV inhibitor for the treatment of type 2 diabetes*, J. Med. Chem., 48:5025-5037 (2005) (AZ-SAXA-5201874) (Table 1, Fluoro analog of saxagliptin, compound 30 (K_i = 1.8 nM)).)

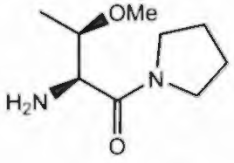
230. Furthermore, one of skill in the art would have recognized that a large lipophilic group like adamantyl would bind in a hydrophobic pocket and that the addition of a hydroxyl group would likely disrupt this favorable binding interaction. Thus, a priori and in the absence

of structural data, one skilled in the art would have no reason to expect that adding a hydroxyl group would lead to the unexpected hydrogen bond with Tyr547 found with saxagliptin and not negatively impact the potency of the compound. (*See* ¶¶ 178, 249-251.)

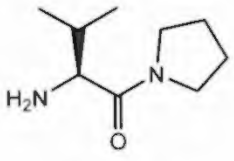
231. In addition, Ashworth I taught that compound 19 (Thr) with a hydroxyl group at the P2 position was 5-fold less potent than compound 12 (Thr(Me)) with a methoxy instead of hydroxyl, and was 10-fold less potent than compound 9 (Val) with a methyl instead of hydroxyl. (Ashworth I at 1164, Table I.)



Compound 19
K_i = 4.90 μM



Compound 12
K_i = 0.90 μM



Compound 9
K_i = 0.47 μM

232. Thus, in my opinion, a person skilled in the art would have had no motivation to add a hydroxyl group and no reasonable expectation that doing so would be a successful modification.

233. In summary, a skilled artisan would not have selected Ashworth compound 25 as a lead compound in light of available data on the two most advanced clinical candidates (NVP-DPP728 and P32/98). (*See* ¶¶ 70, 133-138 above.) Furthermore, if selected as a lead compound, there would have been no motivation to improve potency. Further, if one wanted to improve potency or stability, there would have been no motivation and no reasonable expectation of success that this could be accomplished by adding the cyclopropyl group, substituting the cyclohexyl with an adamantyl group, and adding a hydroxyl group to the adamantyl.

234. In constructing his three-step obviousness allegation starting with Ashworth I compound 25 (cyclopropanate, add adamantyl, hydroxylate adamantyl), Dr. Rotella avoids the

fact that there was already a hydroxyadamantyl containing DPP-4 inhibitor in the prior art: vildagliptin. (*See* Villhauer-063.) By *N*-linking the hydroxyadamantyl group and skipping the cyclopropanation step altogether, vildagliptin continued the conventional wisdom from the two actual clinical candidates, P32/98 and NVP-DPP728, either to avoid cyanopyrrolidines altogether or to use an *N*-linked P2 group to improve stability. The modifications alleged by Dr. Rotella to have been obvious from earlier prior art would have been perceived as a great step backward from P32/98, NVP-DPP728, and vildagliptin.

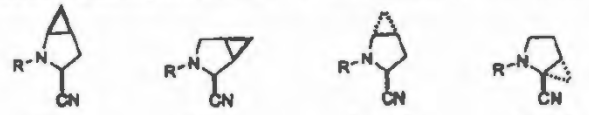
C. Objective Evidence of Non-Obviousness Supports the Patentability of the Claims

1. The Combination of C-Linkage and the 4,5-*Cis*-Cyclopropyl Yields Unexpected Results

235. In my opinion, the combination of saxagliptin's structural features resulted in a constellation of properties that would have been surprising and unexpected to the person of ordinary skill in the art.

236. The cyclopropyl group, when positioned in the particular *cis*-4,5- orientation as in saxagliptin, imparts increased solution stability at elevated temperature on the saxagliptin family of compounds that could not have been predicted. Magnin reported this finding from the BMS series in compounds containing either isoleucine or *t*-butyl glycine (*tert*-Leu) at the P2 position. (Magnin at Table 1, reproduced below.)

Table 1. In Vitro Inhibition Constants for Porcine DPP-IV and Solution Stability Half-Lives for Prolinenitrile DPP-4 Inhibitors



cis-4,5 *cis*-3,4 *trans*-4,5 *trans*-2,3

compd	N-terminal amino acid[a]	substituted prolinenitrile	K_i (nM)[b]	stability, $t_{1/2}$ (h)[c]
21	Ile	prolinenitrile	2 ± 0.5	5
22	Ile	*trans*-4,5-	1620 ± 80	
23	Ile	*trans*-2,3-	7500 ± 200	
24	Ile	*cis*-4,5-	25 ± 1	22
25	Ile	*cis*-3,4-	15 ± 1	4
26	Val	*cis*-4,5-	29 ± 1	28
27	Val	*cis*-3,4-	12 ± 1	2
28	*tert*-Leu	prolinenitrile	8 ± 0.5	27
29	*tert*-Leu	*cis*-4,5-	7 ± 0.5	42
30	*tert*-Leu	*cis*-3,4-	14 ± 1	4

237. Table 1 from Magnin shows increased solution stability when cyclopropyl was added in the *cis*-4,5 position (compound 24 with a half-life of 22 hours) as compared to the *cis*-3,4 position (compound 25 with a half-life of 4 hours) or as compared to no cyclopropyl addition (compound 21 with a half-life of 5 hours) in the isoleucine series. Table 1 also shows increased solution stability when cyclopropyl was added in the *cis*-4,5 position (compound 29 with a half-life of 42 hours) as compared to the *cis*-3,4 position (compound 30 with a half-life of 4 hours) or as compared to no cyclopropyl addition (compound 28 with a half-life of 27 hours) in the t-butyl series. In the case of the t-butyl compounds containing a quaternary carbon as required for all the compounds in the asserted claims, the addition of cyclopropyl in the *cis*-4,5 orientation not only increased solution stability, but did not result in decreased potency (*compare* compound 29 (*cis*-4,5-cyclopropyl) with a potency of 7 nM, *with* compound 28 (no cyclopropyl) with a potency of 8 nM).

238. The unique properties afforded by the combination of saxagliptin's *cis*-4,5-cyclopropyl group and *C*-linked hydroxyadamantyl group are highlighted by comparison to

vildagliptin, which does not contain a cyclopropyl group in the P1 position and has a different substituent in the P2 position (*N*-linked hydroxyadamantyl).

239. Saxagliptin is approximately "10-fold more potent than vildaglipin." (Wang at 2, Table 2 (*compare* the potency of saxagliptin (1.3 nM), *with* vildagliptin (13 nM).)

240. Saxagliptin displays slow, tight binding kinetics with a longer residence time in the binding pocket of DPP-4 as compared to vildagliptin. This slow tight binding provides a longer pharmacodynamic half-life. Wang reported that saxagliptin shows "slow binding" when tested at 37 degrees Celsius with a half-life of the enzyme-inhibitor complex of 50 minutes and a slow off rate of 23 $x10^{-5}$ s^{-1}. (*Id.* at 3, Table 4.) By comparison, vildagliptin has a half-life of 3.5 minutes in addition to a faster off rate of 330 x 10^{-5} s^{-1}. (*Id.*)

Table 4 On and off rates of DPP4 inhibitors at 37°C

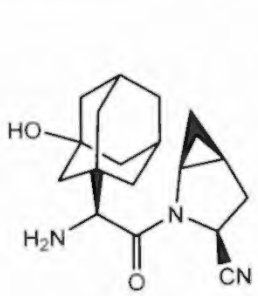
Compound	kon, 10^5 M^{-1} s^{-1}	koff, 10^{-5} s^{-1}	$t_{1/2}$ (min.)
	37°C		
Saxagliptin	4.6 ± 0.6	23 ± 1	50
5 hydroxysaxagliptin	0.7 ± 0.1	50 ± 2	23
Vildagliptin	1.2 ± 0.2	330 ± 30	3.5
Sitagliptin	> 100	> 580	< 2

mean ± standard error. Standard errors for kon were calculated from equations (2), (3) and (4), and for koff are from the fits to equation (5)

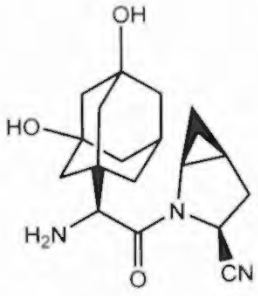
241. Further, saxagliptin is metabolized to form an active metabolite, referred to as 5-hydroxysaxagliptin. As shown in Table 4 of Wang 5-hydroxysaxagliptin displays "slow binding" with a half-life of the enzyme-inhibitor complex of 23 minutes and an off rate of 50 $10^{-5}s^{-1}$. (*Id.* at 3, Table 4.) Thus, the binding properties of saxagliptin's active metabolite are also slower and tighter as compared to vildagliptin.

242. Saxagliptin's active metabolite contains an additional hydroxyl group on the adamantyl in the P2 position. (Fura; Su.) The structure of saxagliptin's active metabolite "M2"

is shown below. (Fura at Figure 1, reproduced below; *see also* Su at Figure 1.) Vildagliptin forms no such metabolite.



Saxagliptin



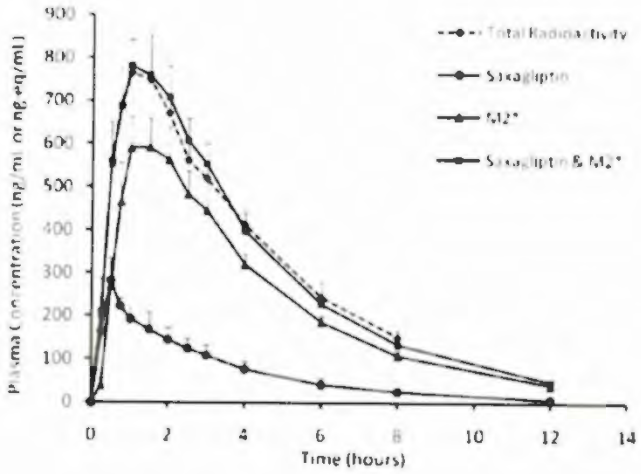
M2

243. The formation of saxagliptin's active metabolite contributes to the favorable properties of saxagliptin observed *in vivo*. Fura reported that

> [f]irst, there is likely to be a contribution from the active metabolite, M2, to the overall efficacy of the compound. This metabolite, which is also a potent and specific inhibitor of DPP4, circulates in significant concentrations in human plasma (Table 3). Second, both saxagliptin and M2 display prolonged binding to the catalytic site of DPP4. The extended rate of dissociation of saxagliptin and M2 from the DPP4 active site would be expected to give hysteresis between plasma concentration and DPP4 inhibition, which has been observed in animal models and humans, providing a longer pharmacodynamic half-life than pharmacokinetic half-life for the compound.

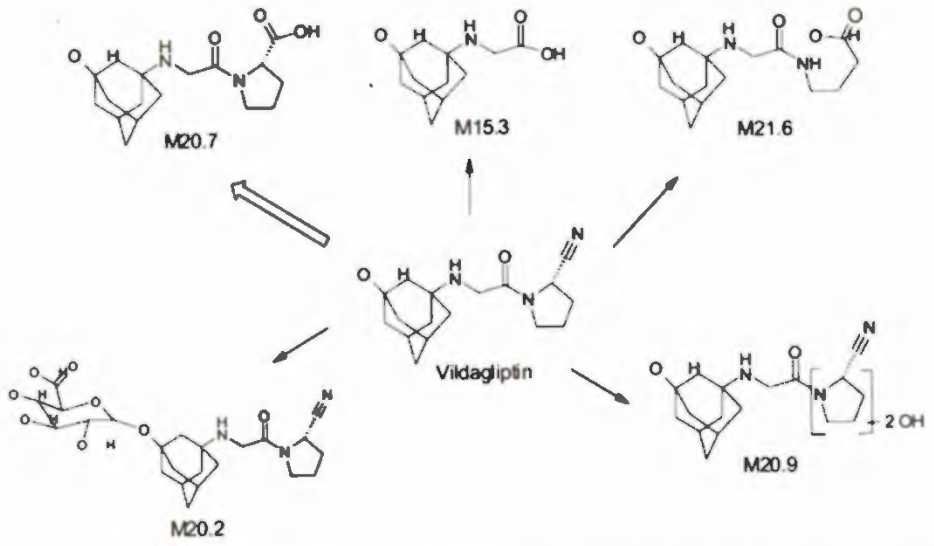
(Fura at 1170 (internal citations omitted).) Further, the extravascular distribution of saxagliptin and M2 to the intestinal tissues "could also play a significant role in its extended therapeutic effect." (*Id.* at 1171.)

244. Su reported concentration versus time profiles for saxagliptin and its active metabolite M2 in humans and showed the extended plasma concentration of saxagliptin's metabolite over time. (Su at Figure 2, reproduced below.)



245. Saxagliptin's extended pharmacodynamic profile and the contribution of the active 5-hydroxysaxagliptin metabolite to saxagliptin's effect *in vivo* was a surprising result that could not have been predicted *a priori*.

246. In contrast, vildagliptin does not have an extended pharmacodynamic profile like that of saxagliptin. Vildagliptin does not metabolize to form an active metabolite, and it does not metabolize to the form a dihydoxyadamantyl metabolite like that found with saxagliptin. (He at 539, Figure 2.) Rather, four metabolic pathways are involved in the *in vivo* biotransformation of vildagliptin: 1) the cyano group hydrolysis leads to formation of the major metabolite, a carboxylic metabolite (M20.7 shown below); 2) amide bond hydrolysis leads to the formation of another carboxylic metabolite (M15.3); 3) glucuronidation leads to formation of a glucuronic acid conjugate of vildagliptin (M20.2); and 4) oxidation on the pyrrolidine leads to formation of additional carboxylic metabolites (M21.6 and M20.9). (He at 540, 542-43, Figure 5, reproduced below.)



247. The differences between saxagliptin and vildagliptin are further apparent by comparison of the chemical contacts each inhibitor makes with the DPP-4 binding pocket, as evidenced by later-obtained x-ray crystal structures.

248. Saxagliptin forms unique and favorable binding interactions with DPP-4. The x-ray crystal structure showed a covalent attachment between the serine in the active site S630 of DPP-4 and the nitrile of saxagliptin, and histidine H740 of DPP-4 plays an essential role in the formation of this covalent bond. (Metzler at 240.) Thus, saxagliptin binds with a "histidine-assisted covalent bond formation." (*Id.*) Saxagliptin's cyclopropyl group is buried in the hydrophobic S1 pocket, next to the catalytic serine, where it forms van der Waals interactions with the side-chain residues that form the pocket. (*Id.* at 241.)

249. The crystal structure of saxagliptin also revealed a unique interaction in the S2 pocket of DPP-4. Specifically the adamantyl extends into the S2 pocket, enabling a favorable hydrogen bonding interaction between the hydroxyl group of saxagliptin and Tyr547 of DPP-4. (Metzler at 241-42.)

250. In contrast to saxagliptin, the hydroxyl on the adamantyl group of vildagliptin does not form the favorable hydrogen bond with Tyr547. Rather, the hydroxyl group of vildagliptin forms hydrogen bonds with His126 and Ser209 through water molecules. (Nabeno at 193.)

251. The x-ray crystal structure of saxagliptin's metabolite 5-hydroxysaxaglitpin, which has two hydroxyl groups, shows hydrogen bonds are formed with both Tyr547 and His126. (Merck presentation at 28.) The ability of saxagliptin's metabolite to reach both Tyr547 and His126 for hydrogen bonding indicates that saxagliptin's preference for bonding to Tyr547 is the more favorable interaction. (*Id.*) Because the hydroxyadamantyl group of vildaglipin binds to the S2 pocket in a different way from saxagliptin, it does not show formation of this favorable interaction.

252. The unique structural properties of saxagliptin and its binding interaction to DPP-4 translate into properties that are beneficial *in vivo*. For example, saxagliptin has increased bioavailability as compared to deshydroxy saxagliptin by addition of the hydroxyl group. (AZ-SAXA-8073054.) This feature of saxagliptin, imparted by the hydroxyl group, was discovered at BMS as a result of pharmacokinetic and metabolic studies that "revealed a similar profile to that exhibited previously with vinyl-containing analogues" from within the BMS discovery program. (*Id.*) As discussed above, bioavailability is an important property for a therapeutic candidate and one that can be difficult to achieve.

253. In addition, saxagliptin demonstrated surprisingly low cell permeability. Specifically, in Caco-2 cells, saxagliptin showed a permeability of 18 nm/s. (AZ-SAXA-8007427.) The low cell permeability of saxagliptin provides better selectivity for DPP-4 over DPP-8 and DPP-9, as the latter two are intracellular proteins. (Abbott; Olsen and Wagtmann,

Identification and characterization of human DPP9, a novel homologue of dipeptidyl peptidase IV, Gene, 299:185-193 (2002).)

254. The combination of saxagliptin's properties, including its potency and slow tight binding features, provided the ability to use a low, once daily dosing regimen. Saxagliptin (Onglyza®) is approved for treatment of patients with type 2 diabetes given at the doses of either 2.5 or 5 mg once per day. (Onglyza® Prescribing Info. (2015).) In contrast, vildagliptin did not receive FDA-approval, but is approved in Europe at a recommended daily dose of 50 mg twice per day due to a dose-dependent liver toxicity signal that was observed in clinical trials. (AZ-SAXA-8073534.) As a result of this liver signal, vildagliptin's European label requires liver toxicity screening, which is not required for saxagliptin nor for any of the other FDA-approved DPP-4 inhibitors. (AZ-SAXA-8073571.)

255. Low doses are considered a desirable property to medicinal chemists. In general, lower doses are advantageous for toxicity reasons. (Nakayama et al., *A zone of classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding*, Drug Metabolism and Disposition, 37:1970-1977, 1970 (2009).)

256. Once daily dosing is also considered a desirable property for treatment of type 2 diabetes because of compliance issues. (Guillausseau, *Influence of Oral Antidiabetic Drugs Compliance on Metabolic Control in Type 2 Diabetes. A Survey in General Practice*, Diabetes Metabolism, 29:79-81, 80 (2003).)

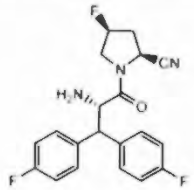
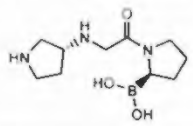
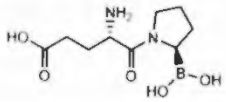
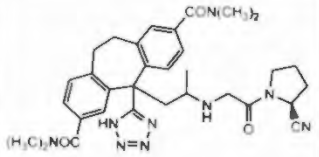
2. Failure of Others to Develop an FDA-Approved DPP-4 Inhibitor

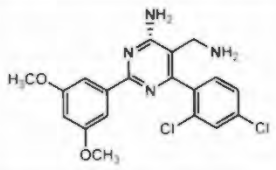
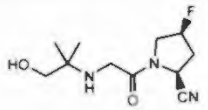
257. Today, there are only four FDA-approved DPP-4 inhibitors on the market, despite considerable efforts and expenditures to develop many others. As discussed in ¶¶ 80-81

(including table) above, not a single prior art compound to saxagliptin has achieved FDA-approval, highlighting the many failures in this field. This includes vildagliptin.

258. Other prior art compounds that entered the clinic also failed. Clinical trials with NVP-DPPIV728 were discontinued because it had a short half-life. (Weber at 4138.) Clinical trials with P32/98 were discontinued because of toxicities that were thought to result from selectivity issues against DPP-8 and DPP-9. (Thornberry and Weber at 558-560.)

259. Following saxagliptin, many other DPP-4 inhibitors also failed when they were brought into the clinic. (*See e.g.*, Adisinsight.springer.com/drugs.) These include, for example:

DPP-4 Inhibitor	Developer	Structure
GW823093C (denagliptin)	GlaxoSmithKline PLC	
PHX1149T (dutogliptin)	Phenomix Corp. and Forrest Laboratories, Inc.	
PT-630	Point Therapeutics, Inc.	
AMG-222	Amgen Inc.	
PSN-9301	Priobiodrug AG, Prosidion Ltd., and OSI Pharmaceuticals, Inc.	

R-1438	F. Hoffmann-La Roche Ltd.	
TA-6666	Mitsubishi Tanabe Pharma Corp.	
TS-021	Taisho Pharmaceutical Co., Ltd. and Eli Lilly and Co.	
SSR-162339	Sanofi-Aventis U.S. LLC	
SYR-619	Takeda Pharmaceutical Co. Ltd.	

260. The structures of the numerous failed DPP-4 inhibitors, as shown in the table above, demonstrate that there has been no common structural motif that defines failure. Similarly, there has been no common structural motif that provides for a successful therapeutic DPP-4 inhibitor.

261. Thus, in my opinion, the many failures in this field further support the unobviousness of the invention and serve to highlight the significant unpredictability in this field.

3. Long Felt Need for Alternative Therapies for Type 2 Diabetes

262. I understand evidence of a long felt need is objective evidence of the nonobviousness of an invention.

263. At the time of the invention, there was a long felt need for alternative treatments for diabetes, particularly an incretin-based therapy. The available treatment options which had safety and tolerability issues did not include such a therapy. (Thornberry and Weber at 557-

558.) Because of this need, a number of companies at the time launched efforts to discover a DPP-4 inhibitor that could be used safely and effectively to treat patients with type 2 diabetes.

264. DPP-4 proved successful as an alternative target for treating patients with type 2 diabetes, despite the many concerns around mechanism-based toxicities discussed in ¶¶ 68-69, 107, and 119 above. While saxagliptin was ultimately the second FDA-approved DPP-4 inhibitor, it was the first invented, meeting what scientists at the time considered to be an important long felt need for patients with type 2 diabetes.

VII. OTHER TESTIMONY

265. I may testify further as to subject matter within my area of expertise which will be useful to inform the Court as to the bases for my opinions. I may also testify concerning additional subject matter properly raised at trial that is within my area of expertise. In addition, I may testify as to subject matter responsive or in rebuttal to contentions advanced at trial by the Defendants or their experts. I also reserve the right to supplement this report in consideration of any further documents, interrogatory responses, admissions, deposition testimony, expert opinions, or other information that the Defendants may provide or other additional relevant information in any form.

VIII. MATERIALS CONSIDERED IN FORMING OPINIONS

266. I considered the documents listed in Exhibit B in forming the opinions expressed above. I may rely upon any or all of these materials at trial.

267. In addition to these documents, I may consider additional documents and information in forming any necessary rebuttal opinions. These documents and information may include deposition testimony or exhibits from this case, any documents produced in this case or other materials or information that I consider relevant.

IX. EXHIBITS TO BE USED AS SUPPORT FOR A SUMMARY OF MY OPINIONS

268. In addition to the documents cited herein, I may develop demonstrative and summary exhibits. Counsel for AstraZeneca will provide these materials to counsel for Defendants as required by the Court.

X. COMPENSATION

269. I am being compensation for my work on this case at my customary rate of $600 per hour. My compensation does not depend in any way on the outcome of this litigation.

Exhibit A

ANN E WEBER, PHD

103 Chestnut Street, Apt. 217, Cranford, NJ 07016 | (732) 771-4704 | weberan8@gmail.com

PROFESSIONAL SUMMARY

Accomplished independent consultant and former pharmaceutical executive with a passion for discovering innovative therapeutics to address unmet medical needs. Over 28 years of industrial experience focused on small molecule and peptide drug discovery across therapeutic areas leading to over 40 development candidates, including JANUVIA® (sitagliptin), a treatment for patients with Type 2 diabetes (T2DM), and MARIZEV® (omarigliptin), a once-weekly treatment for T2DM recently approved in Japan; vibegron for the treatment of overactive bladder is in late stage clinical trials. Highly collaborative scientific leader in drug discovery and early development, recognized for building strong teams, setting strategy and managing change. Noted for strong interpersonal skills, talent development, and commitment to advancing women in chemistry.

EXPERIENCE

ANN WEBER PHARMA CONSULTING

December 2015 – present

Independent consultant to biotech and pharma for all aspects of drug discovery including target and lead identification, lead optimization, and development candidate nomination

MERCK & CO

August 1987 – November 2015

Vice President – Lead Optimization Chemistry, Kenilworth, NJ and Boston, MA

November 2013 – November 2015

Responsible for delivering the lead optimization pipeline to the clinic, particularly in the areas of cardiometabolic diseases, infectious diseases, neurological disorders, oncology and asthma; talent recruitment, management and development for department of ~100 lead optimization chemists in Kenilworth and Boston, working in small-molecule and peptide modalities; Cubist integration team co-lead for Discovery Research

Vice President – Kenilworth Discovery Chemistry Site Head, Kenilworth, NJ

September 2011 – October 2013

Discovery of innovative therapeutic agents to treat patients with cardiovascular disease, diabetes, infectious diseases, and neurological disorders; leadership of chemists at Kenilworth site working in Lead Identification, Lead Optimization, and Automated Synthesis & Purification; Joint Steering Committee for Theravance collaboration; leadership and executive sponsorship of strategic initiatives; Six Sigma Executive Black Belt

Vice President – Rahway Discovery Chemistry Site Head, Rahway, NJ

February 2010 – August 2011

Primary focus on the development of new therapies for cardiovascular disease and metabolic disorders; provided leadership for department of ~200 medicinal chemists during re-organization activities following Schering Plough merger; established Rahway Women in Chemistry Lunch, providing networking and leadership opportunities for emerging women leaders across the internal chemistry organization, sponsored the first Merck Women in Chemistry Symposium

Executive Director, Medicinal Chemistry, Rahway, NJ

July 2005 – January 2010

Scientific oversight for teams that identified clinical candidates in the fields of obesity, diabetes, urinary incontinence, and pain; discovery of omarigliptin (approved in Japan Sept 2015), a once weekly agent for diabetes, and vibegron (licensed to Kyorin at Phase III) for urinary incontinence; Diabetes & Obesity Research Licensing Committee; Joint Research Committees for Metabasis and Neuromed collaborations; leadership of Early

Development Teams in diabetes and pain; basic research representative on Urinary Incontinence Product Development Team; chair of the Lead Optimization Work Stream tasked with implementing the new Basic Research Global Operating Strategy in the lead optimization space; Merck Women's Global Constituency Group member

Senior Director, Medicinal Chemistry, Rahway, NJ
March 2002 – June 2005
Leadership of a group of 24 medicinal chemists; identification of clinical candidates in the areas of obesity, diabetes, and pain; co-chair of DPP-4 Back-Up Early Development Team; Diabetes & Obesity Research Licensing Committee

Director, Medicinal Chemistry, Rahway, NJ
November 1997 – February 2002
Leadership for emerging programs in obesity and transplant therapy; initiation of chemistry effort on the DPP-4 inhibitor program for diabetes; co-lead of program core team that identified JANUVIA® (sitagliptin), the first marketed DPP-4 inhibitor for the treatment of patients with type2 diabetes; basic research representative on DPP-4 Early Development Team

Associate Director, Medicinal Chemistry, Rahway, NJ
November 1994 – October 1997
Chemistry group leader of the β3 adrenergic receptor agonist program, leading to the identification of two compounds that entered clinical development; medicinal chemistry representative on the Product Development Team

Research Fellow, Medicinal Chemistry, Rahway, NJ
December 1991 – October 1994
Initiation of the β3 adrenergic receptor agonist program and identification of the first truly human selective β3 adrenergic receptor agonists for the treatment of obesity

Senior Research Chemist, Medicinal Chemistry, Rahway, NJ
August 1987 – November 1990
Design and synthesis of conformationally-restricted renin inhibitors for hypertension

Research Assistant, Harvard University, Cambridge, MA
September 1983 – July 1987
Oxazolidinethiones, active ester analogs of oxazolidinone chiral auxiliaries in the asymmetric aldol reaction; asymmetric synthesis of beta-hydroxy amino acids including the total synthesis of Echinocandin D

Research Assistant, California Institute of Technology, Pasadena, CA
September 1982 – August 1987
Initiated graduate work; assisted in laboratory move to Cambridge, MA

Research Assistant / Summer Intern, Monsanto Company, St. Louis, MO
June 1982 – August 1982
Synthesis of novel herbicides for crop protection

EDUCATION

Harvard University
1987 - Ph.D. in Organic Chemistry
Thesis Advisor: David A. Evans

University of Notre Dame
1982 - B.S. in Chemistry, Summa cum Laude
Research Advisor: Conrad J. Kowlaski

AWARDS

1981 Chicago Drug and Chemical Association Undergraduate Scholarship
1981 American Chemical Society Division of Analytical Chemistry Undergraduate Award
1981 National Science Foundation Summer Undergraduate Research Program Participant
1982 Atlantic Richfield Academic Excellence Award
1982 American Institute of Chemists Student Award Certificate
1982 Phi Beta Kappa
1982 Valedictorian, University of Notre Dame
1982 – 1985 National Science Foundation Pre-doctoral Fellowship
2002 American Chemical Society Women Chemists Committee, Women at the Forefront of Chemistry
2007 Merck Directors' Award
2007 Thomas Alva Edison Patent Award (Research & Development Council of New Jersey)
2007 Prix Galien USA for JANUVIA® (team member)
2008 Outstanding Women in Science (New Jersey Association for Biomedical Research)
2009 Award for Drug Discovery (Society for Medicines Research, London) for JANUVIA® (team member)
2010 Robert M. Scarborough Award for Excellence in Medicinal Chemistry (Medicinal Chemistry Division of the American Chemical Society)
2010 Heroes of Chemistry Award for JANUVIA® (American Chemical Society)
2011 Discoverer's Award (Pharmaceutical Research and Manufacturers of America)
2011 Industrial Award (Philadelphia Organic Chemists' Club)
2011 Science and Technology Medal (Research & Development Council of NJ)
2013 Liberty Science Center Women in STEM Honoree (LSC Women's Leadership Council and Board of Trustees)
2015 Gift of Mentoring Award (Metro Women Chemists Committee of the American Chemical Society)

ADVISORY BOARDS

Industrial Advisory Board, Department of Chemistry & Chemical Biology, Rutgers, The State University of NJ
2009 – 2015

Editorial Advisory Board, ACS Medicinal Chemistry Letters
2009 – 2014

PUBLICATIONS

C. J. Kowalski, A. E. Weber, and K. W. Fields; "α-Keto Dianion Precursors *via* Conjugate Additions to Cyclic α-Bromo Enones." *J. Org. Chem.* **1982**, *47*, 5088.

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Exhibit B

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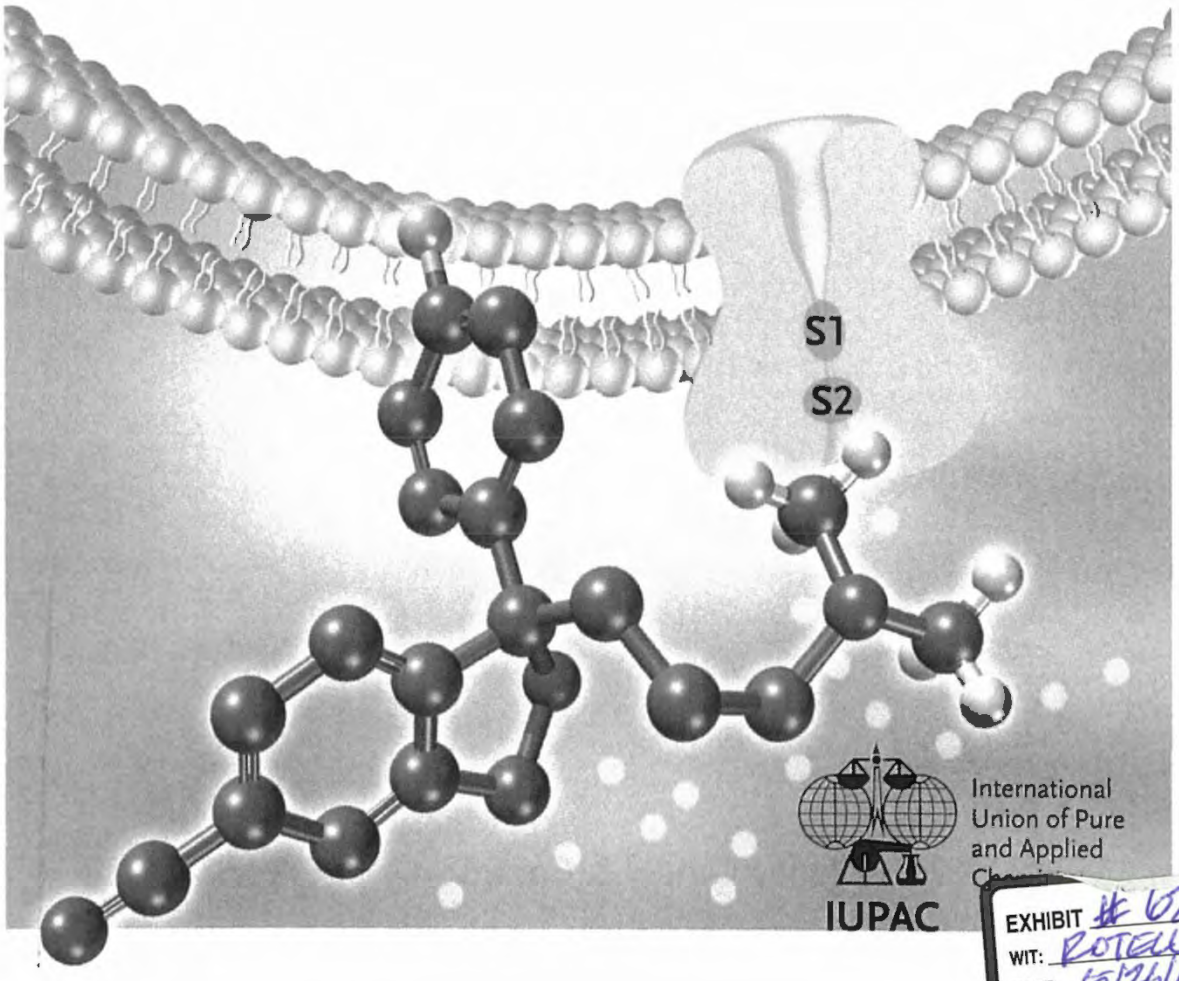

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Analogue-based Drug Discovery III

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1
Pioneer and Analogue Drugs

János Fischer, C. Robin Ganellin, and David P. Rotella

A *pioneer drug* ("first in class") represents a breakthrough invention that affords a marketed drug where no structurally and/or pharmacologically similar drug was known before its introduction. The majority of drugs, however, are *analogue drugs*, which have structural and/or pharmacological similarities to a pioneer drug or, as in some cases, to other analogue drugs.

The aim of this chapter is to discuss these two drug types [1].

The term "pioneer drug" is not used very often, because only a small fraction of drugs belongs to this type and in many cases the pioneer drugs lose their importance when similar but better drugs are discovered. A pioneer drug and its analogues form a drug class in which subsequent optimization may be observed. Analogue drugs typically offer benefits such as improved efficacy and/or side effect profiles or dose frequency than a pioneer drug to be successful on the market.

The discovery of both *pioneer* and *analogue drugs* needs some serendipity. A pioneer drug must clinically validate the safety and efficacy of a new molecular target and mechanism of action based on a novel chemical structure. In the case of an analogue drug, it is helpful that a pioneer or an analogue exists; nevertheless, some serendipity is needed to discover a new and better drug analogue, because there are no general guidelines on how such molecules can be identified preclinically. The analogue approach is very fruitful in new drug research, because there is a higher probability of finding a better drug than to discover a pioneer one. A significant risk with this approach is based on the potential for one of the many competitors in the drug discovery area to succeed prior to others.

The similarity between two drugs cannot be simply defined. Even a minor modification of a drug structure can completely modify the properties of a molecule. Levodopa (1) and methyldopa (2) are applied in different therapeutic fields; however, their structures differ only in a methyl group. Both molecules have the same stereochemistry as derivatives of L-tyrosine. Levodopa [2] is used for the treatment of Parkinson's disease as a dopamine precursor, whereas methyldopa [3] was an important antihypertensive agent before safer and more efficacious molecules (e.g., ACE inhibitors) appeared on the market.

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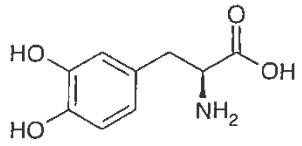
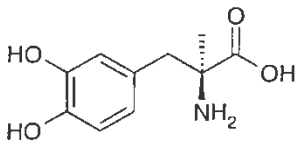
Methyldopa (first synthesized at Merck Sharp & Dohme) has a dual mechanism of action: it is a competitive inhibitor of the enzyme DOPA decarboxylase and its metabolite acts as an α-adrenergic agonist.



levodopa

1

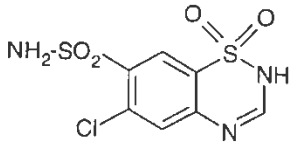


methyldopa

2

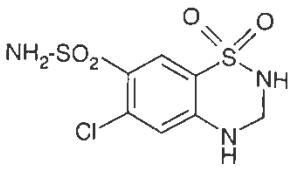
Levodopa and methyldopa are not analogues from the viewpoint of medicinal chemistry. Both are pioneer drugs in their respective therapeutic fields and can be considered as stand-alone drugs, because they have no successful analogues.

There are several examples, and it is a usual case that a minor modification of a drug molecule affords a much more active drug in the same therapeutic field. The pioneer drug chlorothiazide (3) and its analogue hydrochlorothiazide (4) from Merck Sharp & Dohme differ only by two hydrogen atoms; however, the diuretic effect of hydrochlorothiazide [4] is 10 times higher than that of the original drug. The pioneer drug chlorothiazide is rarely used, but its analogue, hydrochlorothiazide, is an important first-line component in current antihypertensive therapy as a single agent and in combination with other compounds.



chlorothiazide

3



hydrochlorothiazide

4

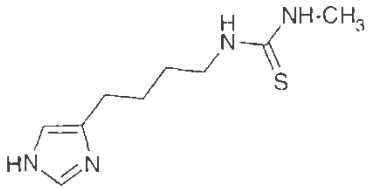
Chlorothiazide and hydrochlorothiazide are *direct analogues*, which term emphasizes their close relationship.

The terms "pioneer drugs" and "analogue drugs" will be discussed in the following sections.

1.1
Monotarget Drugs

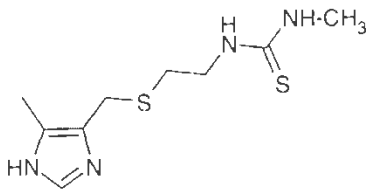
1.1.1
H_2 Receptor Histamine Antagonists

Before the launch of cimetidine (1976), only short-acting neutralization of gastric acid was possible by administration of various antacids (e.g., sodium bicarbonate, magnesium hydroxide, aluminum hydroxide, etc.) that did not affect gastric acid secretion. Cimetidine [5], the first successful H_2 receptor histamine antagonist, a pioneer drug for the treatment of gastric hyperacidity and peptic ulcer disease, was discovered by researchers at Smith, Kline & French. The inhibition of histamine-stimulated gastric acid secretion was first studied in rats. Burimamide (5) was the first lead compound, a prototype drug, that also served as a proof of concept for inhibition of acid secretion in human subjects when administered intravenously, but its oral activity was insufficient. Its analogue, metiamide (6), was orally active, but its clinical studies had to be discontinued because of a low incidence of granulocytopenia. Replacing the thiourea moiety in metiamide with a cyanoguanidino moiety afforded cimetidine (7). Its use provided clinical proof for inhibition of gastric acid secretion and ulcer healing and was a great commercial and clinical success in the treatment of peptic ulcer disease.



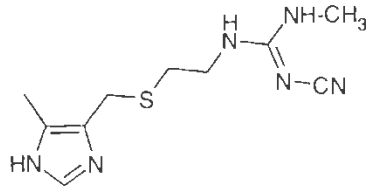
burimamide

5



metiamide

6

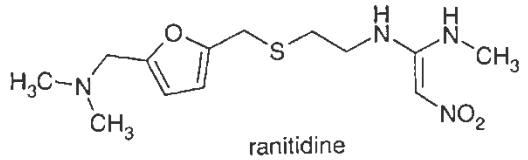


cimetidine

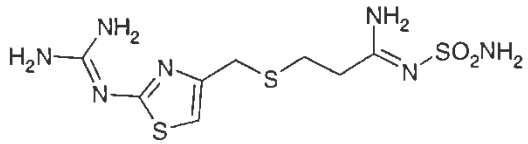
7

Although cimetidine was very effective for the treatment of peptic ulcer disease and related problems of acid hypersecretion, there were some side effects associated with its use, albeit at a very low level. A low incidence of gynecomastia in men can occur at high doses of cimetidine due to its antiandrogen effect. Cimetidine also inhibits cytochrome P450, an important drug metabolizing enzyme. It is therefore advisable to avoid coadministration of cimetidine with certain drugs such as propranolol, warfarin, diazepam, and theophylline.

Cimetidine led to the initiation of analogue-based drug research affording more potent analogue drugs such as ranitidine (8) and famotidine (9) that lack the above side effects of cimetidine.



ranitidine
8



famotidine
9

Ranitidine [6] also has a pioneer character, because ranitidine is the first H_2 receptor histamine antagonist that has no antiandrogen adverse effect and does not inhibit the cytochrome CYP450 enzymes. Famotidine is the most potent member of this drug class, which has been discussed in Volume I of this series [7].

Summary:

Pioneer H_2 receptor histamine antagonist: cimetidine.
First H_2 receptor histamine antagonist with no antiandrogen adverse effects and without inhibition of P450 enzymes: ranitidine.

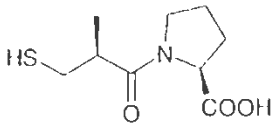
1.1.2 ACE Inhibitors

A natural product, the nonapeptide teprotide (10), was the pioneer drug for angiotensin-converting enzyme (ACE) inhibitors. Teprotide [8] was used as an active antihypertensive drug in patients with essential hypertension. It could only be administered parenterally, which is a great drawback for chronic use of a drug. A breakthrough occurred with the approval of the first orally active ACE inhibitor captopril (11) in 1980 by Squibb. Captopril [9] has a short onset time (0.5–1 h), and its duration of action is also relatively short (6–12 h); as a result, two to three daily doses are necessary. Captopril can be regarded as a pharmacological analogue of teprotide, but it is also the pioneer orally active ACE inhibitor. Captopril's discovery

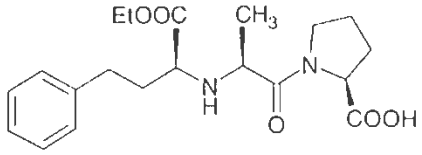
initiated intensive research by several other drug companies to discover longer acting ACE inhibitors. Enalapril (12) was introduced by Merck in 1984. Enalapril [10] can be regarded as the first long-acting oral ACE inhibitor. The long-acting ACE inhibitors are once-daily antihypertensive drugs. There are several long-acting ACE inhibitors, whose differences have been discussed in the first volume of this book series [11].

pyro-Glu - Trp - Pro - Arg - Pro - Gln - Ile - Pro - Pro -OH

teprotide
10



captopril
11



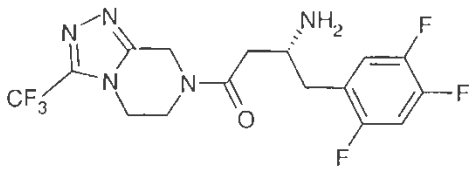
enalapril
12

Summary:

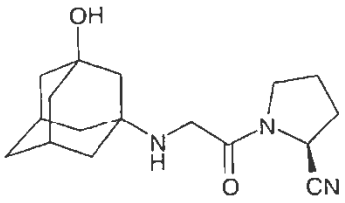
Pioneer ACE inhibitor drug: teprotide.
First orally active ACE inhibitor drug: captopril.
First orally long-acting ACE inhibitor drug: enalapril.

1.1.3 DPP IV Inhibitors

Sitagliptin (13) [12], a pioneer dipeptidyl peptidase IV (DPP IV) inhibitor, was launched in 2006 by Merck for the treatment of type 2 diabetes. The medicinal chemistry team began its research in 1999 when some DPP IV inhibitor molecules were known as substrate-based analogues. The lead molecule derived from this research was vildagliptin (14) [13]; discovered at Novartis in 1998, it was the second compound to be introduced to the market.



sitagliptin
13



vildagliptin

14

The pioneer drug sitagliptin is a commercial success with 2010 sales greater than USD 3 billion. Vildagliptin was the first successful discovery in this drug class, but its development time was longer and it was introduced in 2007, after sitagliptin. Vildagliptin is only moderately selective over DPP-8 and DPP-9 compared to sitagliptin that is a highly selective DPP IV inhibitor. Based on long-term safety studies, these selectivity differences do not influence the toxicity of vildagliptin. Sitagliptin and vildagliptin show similar clinical efficacies. Vildagliptin has a short half-life (3 h) and its dosing regimen is twice a day, whereas sitagliptin has a long half-life (12 h) and once-daily dosing is used. DPP IV inhibitors are typical early-phase analogues that result from a highly competitive industry, and not the first candidate (vildagliptin) but a follow-on drug (sitagliptin) became the pioneer drug on the market ("first-in-class drug"). Further DPP IV inhibitors are available (alogliptin, saxagliptin, and linagliptin) and the individual compounds differ significantly in their mode of metabolism and excretion and these differences help the treatment of patients with type 2 diabetes in an individual way [14] (see Chapter 5 of Volume II of this book series).

Summary:

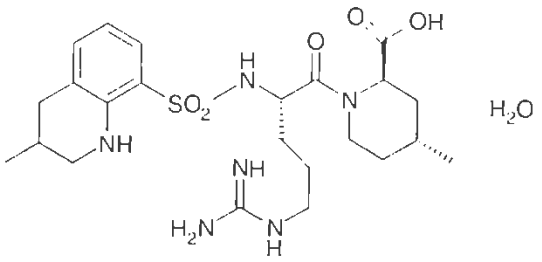
Pioneer DPP IV inhibitor drug: sitagliptin (long-acting inhibitor).
First DPP IV inhibitor analogue drug: vildagliptin (short-acting inhibitor).

1.1.4 Univalent Direct Thrombin Inhibitors

Thrombin is a serine protease enzyme whose inhibition plays an important role in the mechanism of several anticoagulants. Univalent direct thrombin inhibitors bind only to the active site of the enzyme, whereas bivalent direct thrombin inhibitors (e.g., hirudin and bivalirudin) block thrombin at both the active site and exosite 1.

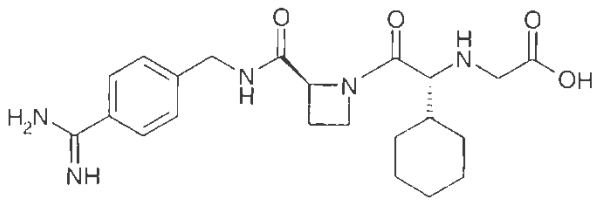
The pioneer univalent direct thrombin inhibitor is argatroban monohydrate (15) [15] that was launched by Daiichi Pharmaceutical and Mitsubishi Pharma in 1990. Argatroban was approved by the FDA for prophylactic anticoagulation in the treatment of thrombosis in patients with heparin-induced thrombocytopenia. Argatroban is a rather selective reversible inhibitor for human thrombin. Despite its low molecular weight, argatroban is administered parenterally due to the presence of the highly basic guanidine moiety that prevents absorption from the

gastrointestinal tract. This characteristic limits the clinical use of the compound. The first oral direct thrombin inhibitor was ximelagatran (17) [16], which was introduced in 2004 by AstraZeneca. Ximelagatran is a double prodrug derivative of melagatran (16) with a bioavailability of about 20%, a measurable improvement compared to melagatran with oral bioavailability of 5.8%. Ximelagatran was withdrawn from the market in 2006 because of unacceptable hepatic side effects (alanine aminotransferase increased threefold and bilirubin level increased twofold above the normal upper limit) [17]. In this drug class, dabigatran etexilate (18) [18] was discovered by Boehringer Ingelheim as a new direct thrombin inhibitor without adverse liver effects [19] (see Chapter 10)



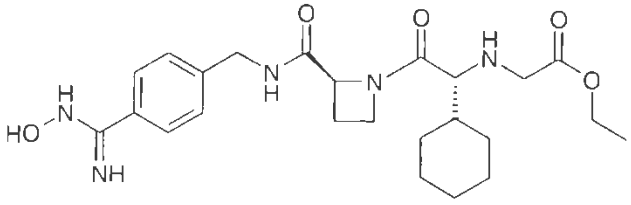
argatroban monohydrate

15



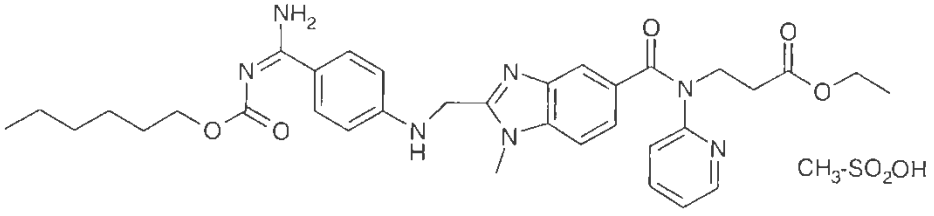
melagatran

16



ximelagatran

17



dabigatran etexilate mesylate

18

Summary:

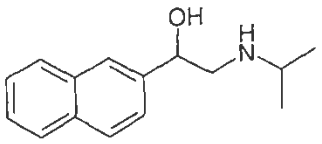
Pioneer univalent direct thrombin inhibitor drug: argatroban.
First orally active univalent thrombin inhibitor: ximelagatran.
First orally active univalent thrombin inhibitor without adverse liver affects: dabigatran etexilate.

1.2
Dual-Acting Drugs

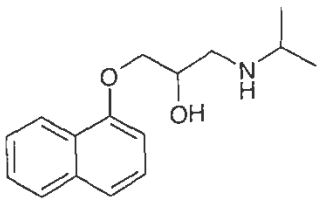
1.2.1
Monotarget Drugs from Dual-Acting Drugs

1.2.1.1 Optimization of Beta-Adrenergic Receptor Blockers

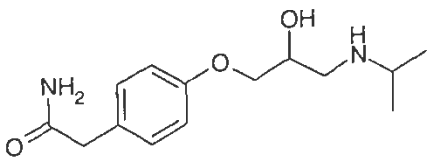
James W. Black and coworkers at ICI invented propranolol as a product of analogue-based drug discovery (ABDD) using their prototype drug, pronethalol (**19**), as a lead compound. Pronethalol [20] was an active drug for the treatment of angina pectoris in humans, but its development was discontinued because it proved to be carcinogenic in mice in long-term toxicology studies. Continuation of the analogue-based drug discovery afforded propranolol (**20**), where an oxymethylene link was inserted between the 1-napthyl group and the secondary alcohol moiety of pronethalol. Propranolol [21] was more potent than pronethalol. Propranolol became the pioneer nonselective β-adrenergic receptor antagonist, a true antagonist without partial agonist properties (intrinsic sympathomimetic activity). It was a breakthrough discovery for the treatment of arrhythmias, angina pectoris, and hypertension.



pronethalol
19



propranolol
20



atenolol
21

The pioneer drug propranolol has equal antagonist affinity for β_1 and β_2 adrenergic receptors; however, β_1 receptors are located only in the heart and the nonselective propranolol also blocks β_2 receptors in bronchial smooth muscle. Therefore, propranolol is not used in patients with bronchial asthma. Several analogues have been tested in a battery of *in vivo* pharmacological tests resulting in the discovery of atenolol (21) [22]. A guinea pig bronchospasm test served for investigation and demonstration of β_1 selectivity. Atenolol had no intrinsic sympathomimetic effect (partial agonism), similar to propranolol (see Chapter 8 of Volume I (Part II) of this book series).

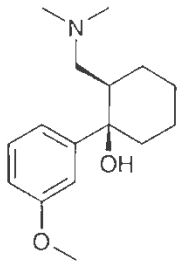
Summary:

Pioneer dual-acting (β_1 and β_2) beta-adrenergic receptor antagonist: propranolol. First β_1 selective antagonist drug without intrinsic sympathomimetic activity: atenolol.

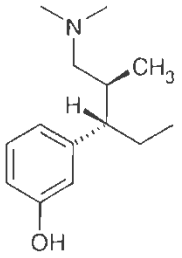
1.2.2 Dual-Acting Drugs from Monotarget Drugs

1.2.2.1 Dual-Acting Opioid Drugs

Most ligands designed from the morphine template are mu (μ) opioid receptor (MOP) agonists. A simplified version of the morphine skeleton afforded tramadol that is marketed in its racemic form. The (+)-isomer is a weak MOP agonist, whereas the (−)-isomer inhibits neurotransmitter reuptake. Tramadol (22) [23] was discovered by Grünenthal and was introduced in 1977 for the treatment of moderate to severe pain.



tramadol
22



tapentadol
23

Grünenthal continued analogue-based drug research using tramadol as a starting compound, and out of several analogues, tapentadol (23) [24] was selected and developed. It was introduced in 2009 to the market as a new opioid analgesic drug with dual activity: a MOP agonist and an inhibitor of norepinephrine reuptake. Tramadol is thousands of times less potent than morphine on the mu opioid receptor, whereas tapentadol's analgesic activity is comparable to that of oxycodone with reduced constipation and respiratory depression (see Chapter 12).

Summary:

Pioneer dual-acting (MOP agonist and norepinephrine reuptake inhibitor) opioid drug racemate: tramadol.
First dual-acting (MOP agonist and norepinephrine reuptake inhibitor) opioid drug in a single molecule: tapentadol.

1.3 Multitarget Drugs

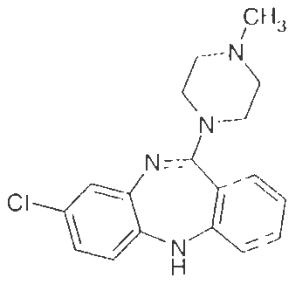
1.3.1 Multitarget Drug Analogue to Eliminate a Side Effect

1.3.1.1 Clozapine and Olanzapine

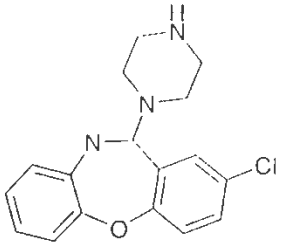
Clozapine (24) [25] is the pioneer drug in the class of atypical antipsychotic agents. It was a serendipitous discovery by researchers at Wander in Switzerland in 1960 from the structural analogue antidepressant amoxapine (25). Its discovery was unexpected from the structurally very close analogue and therapeutically it had a great advantage over the typical antipsychotic drugs such as chlorpromazine and haloperidol because clozapine produced no extrapyramidal side effects (EPS). Clozapine causes agranulocytosis in about 1% of the patients, and this side effect limited its application. Analogue-based drug design afforded quetiapine (26) [26], a clozapine analogue without this side effect. It was discovered at ICI in 1986 and it became one of the main products of AstraZeneca. Instead of the dibenzodiazepine nucleus of clozapine, the analogue quetiapine has a dibenzothiazepine scaffold. Both clozapine and quetiapine have affinity for a number of receptors. The antipsychotic activity is believed to be associated primarily by virtue of affinity for D_2 and 5-HT_{2A} receptors. Chapter 3 discusses metabolic aspects that may contribute to the distinct adverse event profiles of these two drugs (see the chapter of Volume I on clozapine analogues).

Summary:

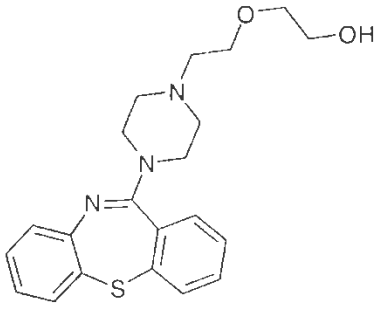
Pioneer atypical antipsychotic drug: clozapine.
First atypical clozapine-like antipsychotic drug without the side effect of agranulocytosis: quetiapine.



clozapine
24



amoxapine
25

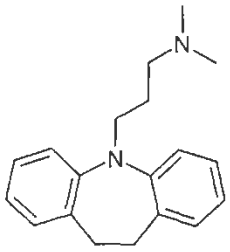


quetiapine
26

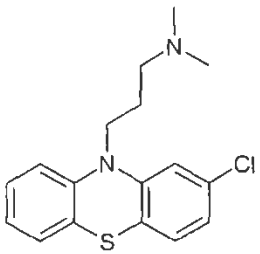
1.3.2
Selective Drug Analogue from a Pioneer Multitarget Drug

1.3.2.1 **Selective Serotonin Reuptake Inhibitors**

From a retrospective viewpoint, imipramine (27) was the pioneer antidepressant drug with a multitarget receptor profile, where serotonin and norepinephrine reuptake inhibition played an important role, but no *in vitro* activities were known at the time of its serendipitous discovery. Researchers at Geigy first synthesized the molecule in 1948. It was an analogue of the antipsychotic chlorpromazine (28), but the Swiss psychiatrist Roland Kuhn [27] found imipramine to be an effective antidepressant drug. It was launched by Geigy in 1959.

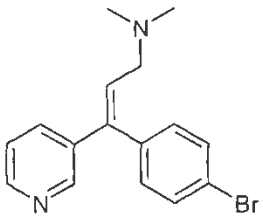


imipramine
antidepressant
27



chlorpromazine
antipsychotic
28

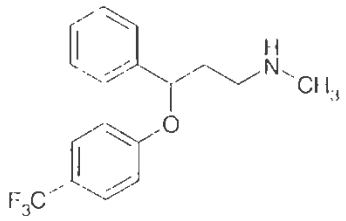
Imipramine and the analogue tricyclic antidepressants inhibit the reuptake of serotonin and norepinephrine but they also exhibit a variety of side effects. The anticholinergic side effects include dry mouth, blurred vision, and sinus tachycardia. The histamine H_1 receptor antagonist activity likely contributes to the sedative effects associated with the compound.



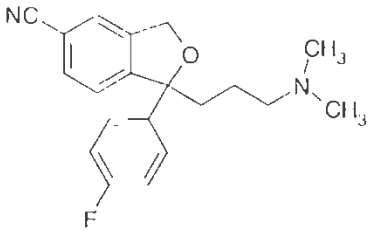
zimelidine
29

Arvid Carlsson initiated research on selective serotonin reuptake inhibitors (SSRIs) in order to get new antidepressants with less side effects. The first SSRI was zimelidine (**29**) [28]. It was launched by Astra in 1982, but it had to be withdrawn shortly afterward because of serious peripheral nerve side effects. Further research was continued at several pharmaceutical companies. The first successful SSRIs were fluoxetine (**30**) [29] (Lilly, 1988) and citalopram (**31**) [30] (Lundbeck, 1989). The antihistamine diphenhydramine (**32**) served as a lead compound for fluoxetine, whereas talopram (**33**) was the lead structure for citalopram. The

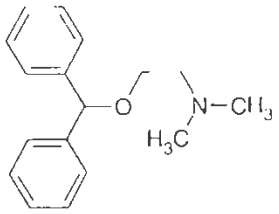
discovery of citalopram and its refinement to escitalopram is discussed in Chapter 11.



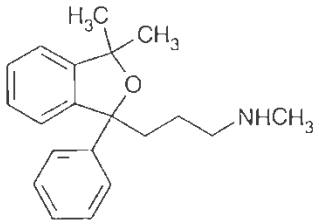
fluoxetine
30



citalopram
31



diphenhydramine
(lead for fluoxetine)
32



talopram
(lead for citalopram)
33

Summary:

Pioneer multitarget nonselective serotonin/norepinephrine reuptake inhibitor antidepressant drug: imipramine.
First (but unsuccessful) selective serotonin reuptake inhibitor: zimelidine.
First selective serotonin reuptake inhibitors: fluoxetine and citalopram.

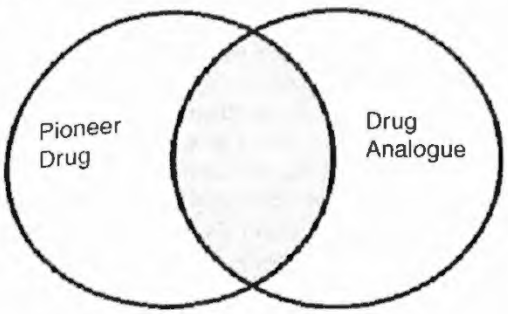


Figure 1.1 Pioneer drugs and drug analogues have overlapping properties.

1.4 Summary

Pioneer drugs open up new therapeutic treatments. They are also called "first-in-class" drugs.

Analogue-based drug discovery is a very important part of medicinal chemistry, because the analogues frequently are intended to optimize drug therapy. There is a continuous development in a drug class and in several cases the pioneer drugs disappear from the market and analogue drugs achieve a dominant role. It is the main reason why so many successful drugs are among the analogue drugs. The above examples focused on some cases where these drugs have a unique character in a drug class.

For early-phase analogues, it is possible that a pioneer drug derives from the analogue-based drug discovery for different reasons; for example, a more convenient lead compound or a more successful optimization can strongly influence which drug will be introduced to the market as a pioneer drug.

There are several examples where a new prototype drug candidate was discontinued at the late phase of drug research and then an analogue was introduced to the market as a successful pioneer drug.

The properties of pioneer and analogue drugs overlap (Figure 1.1). The drug analogues preserve some properties of the pioneer drug and they have to achieve some new and better properties in order to be successful on the market.

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5
Dipeptidyl Peptidase IV Inhibitors for the Treatment of Type 2 Diabetes

Jens-Uwe Peters and Patrizio Mattei

5.1
Introduction

The symptoms of diabetes mellitus, a metabolic disorder characterized by hyperglycemia (abnormally high blood glucose) due to inadequate insulin levels, have been described since antiquity. The introduction of insulin replacement therapy for diabetes in 1922 was a major feat in the history of medicine and was awarded with the Nobel Prize in medicine in the following year. Later in the 1920s, the first oral antidiabetic drugs (OADs) were introduced. Although they were imperfect and later withdrawn, they led to the recognition that two types of diabetics exist – the juvenile type, requiring insulin therapy, and the late-onset type, which also benefits from OAD treatment [1, 2]. The late-onset form, today known as type 2 diabetes, accounts for more than 90% of all diabetic patients and affects about 4% of the world population [3].

The treatment of type 2 diabetes aims to normalize blood glucose levels by diet, exercise, and medication, and is monitored by measuring glycosylated hemoglobin (HbA_{1c}) as a long-term marker of elevated blood glucose. The amount of HbA_{1c} reflects the average glucose level over the last 120 days (the life span of red blood cells) and should be maintained below 7% [4]. Each percentage reduction in HbA_{1c} leads to a 21% reduction of the risk for any diabetes-related end point [5]. Poorly controlled, chronic hyperglycemia causes microvascular damage, which affects organs with delicate capillary systems such as the eyes and kidneys, and can lead to blindness and renal failure. In addition, hyperglycemia leads to atherosclerosis of larger vessels, which increases the risk of myocardial infarction and stroke. An important complication resulting from micro- and macroangiopathy are lesions of the lower limbs ("diabetic foot") that may ultimately require amputation. Unfortunately, the majority of diabetic patients do not reach recommended HbA_{1c} levels and are therefore at risk of developing these disabling comorbidities. Furthermore, the prevalence of type 2 diabetes has increased over recent years, mainly due to higher life expectancies and an increasing prevalence of obesity [3]. Several classes of OADs have been introduced into clinical practice since the 1950s and are widely prescribed. However, they all come along with side effects such as hypoglycemia, weight gain, or gastrointestinal

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problems. Moreover, they often fail to achieve sustained glycemic control. Thus, there is a critical unmet need for OADs with novel modes of action.

In the late 1980s, several research groups could show that the peptidic hormone GLP-1 (glucagon-like peptide 1), which is secreted by the L-cells of the intestinal epithelium in response to food ingestion, is a potent stimulator of glucose-dependent insulin release. This finding raised hopes that exogenous GLP-1 might be used to stimulate the impaired insulin secretion in type 2 diabetic patients. Disappointingly, single subcutaneous injections of GLP-1 were ineffective in normalizing blood glucose [6]. A few years later, it was discovered that DPP-IV (dipeptidyl peptidase IV), a serine protease first isolated in 1966, rapidly cleaves and inactivates GLP-1 [7]. Several research groups recognized the implications of this finding:

- Inhibition of DPP-IV should prevent the rapid degradation of GLP-1 and should thus increase circulating GLP-1 levels.
- Increased GLP-1 levels should enhance glucose-dependent insulin secretion, leading to lower blood glucose levels.
- Consequently, DPP-IV inhibitors should have an antidiabetic effect.

The glucose-lowering/antidiabetic effect of DPP-IV inhibitors was soon demonstrated in animals and humans and triggered enormous research activities throughout the pharmaceutical industry in the first decade of the new millennium [8].

5.2
In Vitro Assays and Animal Models for the Assessment of DPP-IV Inhibitors

The discovery of DPP-IV inhibitors was facilitated by the availability of robust and high-throughput *in vitro* assays, which often rely on a simple chromogenic or fluorogenic readout. For instance, DPP-IV cleaves Ala-Pro-AFC, a peptidyl derivative of 7-amino-4-trifluoromethylcoumarin (AFC), and the green fluorescence of the cleavage product, AFC, can be distinguished from the violet-blue fluorescence of the substrate (Figure 5.1). The cleavage of Ala-Pro-AFC serves as a measure of DPP-IV activity in an *in vitro* assay, in which the candidate inhibitor is evaluated by its ability to suppress the formation of fluorescent AFC. Furthermore, animal models with high relevance to the human disease state were available. For instance, the oral glucose tolerance test (OGTT) in diabetic rats measures the glucose excursion, or the insulin response, after an oral ingestion of a standardized amount of glucose, and is equivalent to the OGTT used in the diagnosis of diabetes in humans. The efficaciousness of DPP-IV inhibitors can be evaluated in such an animal model by their ability to reduce the glucose excursion after their administration prior to the glucose challenge.

5.3
Substrate-Based DPP-IV Inhibitors

Speculations about the relevance of DPP-IV in the processing of bioactive peptides, and its potential role in diseases such as cancer and AIDS, might have provided much

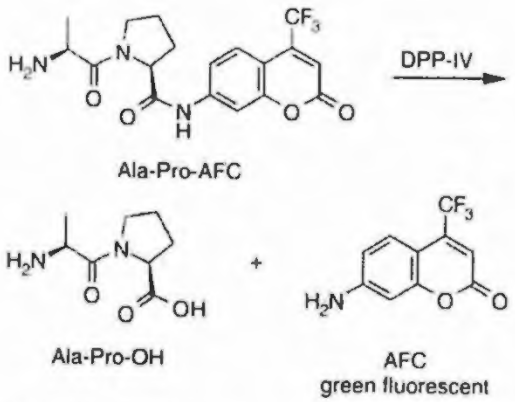


Figure 5.1 DPP-IV liberates AFC from its dipeptidyl derivative, Ala-Pro-AFC. The green fluorescence of the product is used as a readout in a DPP-IV inhibition assay.

of the impetus for DPP-IV inhibitor research in the 1980s [9]. At this time, the ACE inhibitor success story had just proven that substrate-based design is a viable approach to drug discovery, and it seems natural that this concept was also pursued in DPP-IV research. DPP-IV is an endopeptidase that releases dipeptides from the N-terminus of a wide variety of peptidic hormones, with a preference for proline at the penultimate position. This proline preference is pronounced in small substrates (such as Ala-Pro-AFC, Figure 5.1), even if the larger peptide GLP-1 (30 amino acids) is cleaved after an alanine (Figure 5.2).

In the early 1990s, several academic research groups disclosed dipeptide-like DPP-IV inhibitors, in which a pyrrolidine or a thiazolidine replaces the proline, and an attached amino acid with a free amino group mimics the N-terminus of a substrate peptide (Figure 5.3). The scissile peptide bond was either omitted, as in the prototypical DPP-IV inhibitor P32/98 [10], or replaced by a functional group designed to mimic the proteolytic transition state or to covalently bind to the enzyme's active site serine. For instance, prolineboronic acids such as 1 have been designed as transition-state analogues and are reversible, slow-binding inhibitors with activities in the low nanomolar range [11]. Phosphonates such as 2 are irreversible inhibitors, which form stable esters with DPP-IV's catalytically active serine. However, these early types of serine-interacting inhibitors did initially not provide clear advantages over the noncovalent inhibitors, as they were too unstable, too unselective, or did not show a substantially improved activity. Nevertheless, the boronic acid dutogliptin, a DPP-IV inhibitor discovered by Phenomix, has

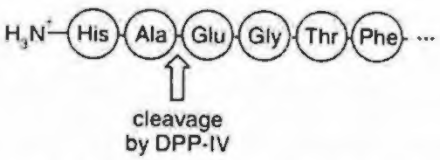


Figure 5.2 DPP-IV cleaves GLP-1 at the penultimate position from the N-terminus.

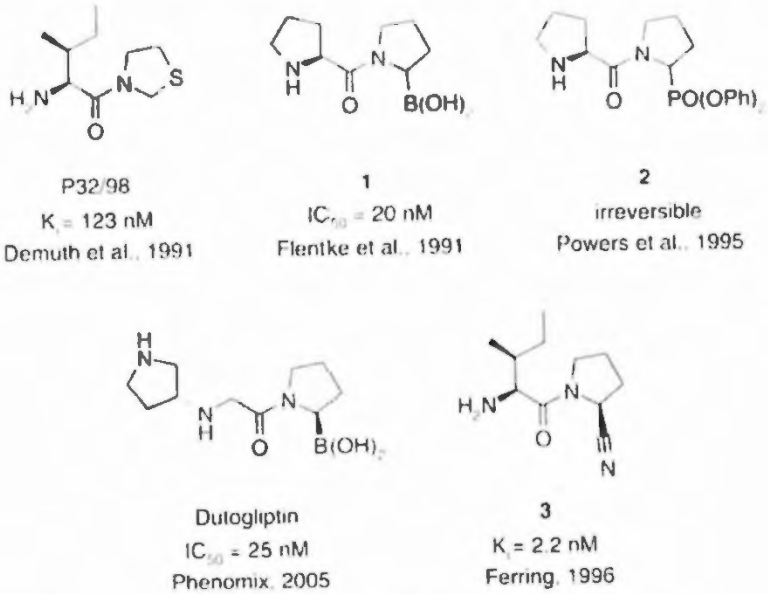


Figure 5.3 Early substrate-based DPP-IV inhibitors and dutogliptin.

apparently overcome these limitations and entered phase 3 clinical development in 2008 [12, 13].

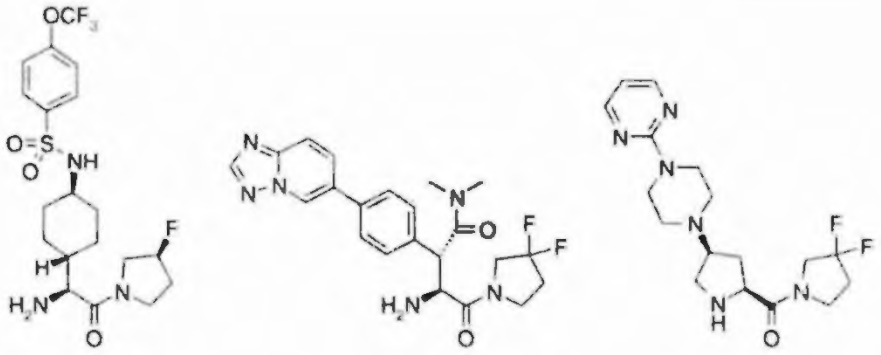
In 1994, a publication demonstrated that nitriles could be used as serine-interacting motifs in inhibitors of prolyl endopeptidase (PEP), a serine protease related to DPP-IV [14]. So far, nitriles had only been known to be cysteine protease inhibitors, but were regarded unreactive to typical serine proteases. This surprising finding prompted Sherwin Wilk's research group at the City University of New York, and researchers working with Paul D. Jenkins at Ferring Pharmaceuticals, to introduce nitriles into their substrate-based DPP-IV inhibitors [15–17]. These new cyanopyrrolidine-type DPP-IV inhibitors, for example, 3 (Figure 5.3), turned out to have an approximately 100-fold improved inhibitory potency, and additionally both a good selectivity profile and an acceptable chemical stability.

Up to this point, the role of DPP-IV in glucose homeostasis was not fully recognized. Rolf Mentlein *et al.* from the University of Kiel had already demonstrated in 1993 that GLP-1 is a substrate of DPP-IV *in vitro* [7], but this did not necessarily mean that DPP-IV would be the main metabolic enzyme of GLP-1 *in vivo*. Actually, 3 was proposed as a potential immunomodulator, as DPP-IV is identical to CD26, a component of the T-cell receptor complex. In 1995, Jens Holst and coworkers from the University of Copenhagen concluded from their studies that DPP-IV is responsible, at least in part, for the observed rapid degradation of GLP-1 in humans and proposed that inhibition of DPP-IV could be a useful adjunct in the management of type 2 diabetes [19]. Shortly thereafter, a collaborating team of scientists working with Hans-Ulrich Demuth from the University of Halle and Christopher H.S. McIntosh and Ray A. Pederson from the University of British Columbia patented DPP-IV

inhibition as a method to lower blood glucose [20]. The patent application was disclosed in 1997 and demonstrated that DPP-IV inhibition with P32/98 did indeed improve glucose tolerance in rats. Demuth, who had spent most of his academic career working on DPP-IV, would later start the biotech company, Probiodrug, to exploit this invention and to bring P32/98 into the clinic. The improvement in glucose tolerance by P32/98 was then reproduced in human healthy volunteers and diabetic patients. P32/98 and the epimeric allo-isoleucyl-thiazolidide were licensed to Merck in late 2000. However, development of both compounds was discontinued in February 2001, after Merck had identified unacceptable toxicity profiles for both compounds. Later, insufficient selectivity over the related dipeptidases DPP-8 and/or DPP-9 was postulated to be the reason for the observed toxicities [21]. At that time, Merck had already identified fluoropyrrolidine 4 (Figure 5.4) as a potential development compound. Because the rationale for subtype selectivity was compelling, 4 was rejected on the basis of a selectivity of only 50-fold over DPP-8 and DPP-9, and medicinal chemistry focused on HTS-based DPP-IV inhibitors, which culminated in the discovery of sitagliptin (see Section 5.4). Further exploration of the substrate analogue series provided 5, with a selectivity of >10 000-fold over DPP-8/9 [22]. This compound was brought forward as a backup for sitagliptin [23].

Another potent and selective difluoropyrrolidine derivative, PF-00734200, has been discovered by Pfizer. This compound was reported to be in phase 2 clinical studies in September 2008 [24, 25].

During this time, the cyanopyrrolidines originally discovered by Sherwin Wilk and the group at Ferring had become the most popular class of DPP-IV inhibitors, as judged by the number of patent applications [18]. While the SAR around the cyanopyrrolidine ring was rather limited, a wide variety of attached amino acids with lipophilic or polar, negatively or positively charged, side chains were tolerated, which provided ample room for proprietary structures.



4
IC_{50} = 35 nM
Merck, 2004

5
K_i = 8.8 nM
Merck, 2004

PF-00734200
IC_{50} = 13 nM
Pfizer, 2005

Figure 5.4 Pyrrolidides without a serine-interacting motif.

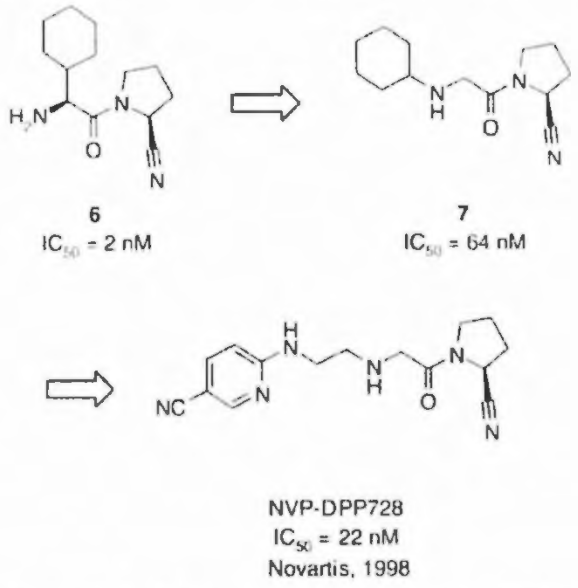


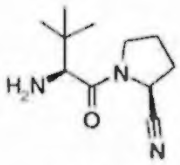
Figure 5.5 Scaffold change leading to *N*-alkylglycine DPP-IV inhibitors. NVP-DPP728 was efficacious in a proof-of-concept trial.

An important extension of this SAR was made already in 1996 by scientists at Novartis. Edwin B. Villhauer, a chemist with a long-standing interest in diabetes, was looking for a new project when Jens Holst's paper was published in 1995. Within a few days, he and his colleagues had a DPP-IV project running. Cells that happened to express DPP-IV were just available and provided an *in vitro* assay. A paper from 1988, describing a DPP-IV substrate with sarcosine (*N*-methylglycine) as an N-terminal amino acid [26], caught Villhauer's attention and led him to explore *N*-alkylglycine cyanopyrrolidines, in which the side chain of the pyrrolidine-attached amino acid is, formally, shifted to the nitrogen atom (e.g., 6 → 7, Figure 5.5) [27]. The novel *N*-alkylglycine cyanopyrrolidines were amenable to resin-based chemistry, which was a very popular technology in those years, enabling the preparation of 1300 diverse compounds within 7 months. Only a few inhibitors with low nanomolar activities were identified in this campaign, one of them carrying a (5-nitro-pyridin-2-yl)-aminoethyl substituent. Replacement of the nitro functionality by a nitrile then led to NVP-DPP728 (Figure 5.5) with an improved selectivity over DPP-II and PPCE (postproline cleaving enzyme), which were then standard enzymes in DPP-IV selectivity studies. Within only 9 months, the Novartis project team had identified a development compound. Clinical trials with NVP-DPP728 began in 1998. A first phase 2 trial based on the then widely held paradigm that any type 2 diabetes patient treated with a DPP-IV inhibitor should experience an immediate benefit, gave disappointing results and almost stopped the project. A detailed data analysis suggested that patients with a certain level of pancreatic beta cell activity might benefit over a longer time frame. A second trial designed with the hindsight from this analysis was a huge success: after 4 weeks of treatment, NVP-DPP728 reduced

postmeal glucose excursion, fasting glucose, and 24 h mean glucose. For the first time, it was shown that chronic DPP-IV inhibition in diabetic patients was safe and also led to a reduction in HbA_{1c} levels [28].

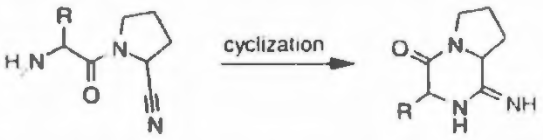
NVP-DPP728's relatively short half-life of 0.85 h was initially not seen as a disadvantage. On the contrary, the many possible physiological roles of DPP-IV made it desirable for a proof-of-concept compound that any potential adverse effects would abate quickly after a discontinuation of administration. DPP-IV cleaves, at least *in vitro*, not only GLP-1 but also several peptidic hormones, neurotransmitters, and chemokines. Of particular concern was initially the fact that DPP-IV is identical to CD26, a surface protein on activated T-cells, which mediates stimulatory signals; fortunately, it was found that NVP-DPP728 had no immunosuppressant effect. (Later on it was shown that the enzymatic activity of DPP-IV is not required for T-cell function.) It might have been envisioned that NVP-DPP728 could be a short-acting, meal-dependently administered drug to reduce postprandial glucose excursion. Such a treatment would allow an intermittent recovery of DPP-IV activity, and the normal regulation of other potential DPP-IV substrates, thus minimizing side effects. However, a team of Novo Nordisk researchers, collaborating with the Miami School of Medicine, demonstrated in 2001 that a 24 h infusion of GLP-1 over 7 days gave a much better outcome for diabetic patients than a 16 h infusion, indicating that a 24-h blockade of DPP-IV was needed to maximize the therapeutic effect [29]. In 2002, Ferring researchers published their results with the long-acting DPP-IV inhibitor FE 999011 (Figure 5.6), which clearly showed that full inhibition of DPP-IV over 24 h gave the best results in animal models of diabetes [30]. In the following years, most companies therefore focused on inhibitors with high metabolic stability, and today all clinically proven inhibitors show >50% plasma DPP-IV inhibition over 24 h.

Apart from the demonstrated clinical efficacy and the facile synthetic access, there might be yet another reason why the *N*-alkylglycine inhibitors became very popular throughout the industry in the following years: it was generally perceived that they had a superior chemical stability. As already mentioned, cyanopyrrolidine DPP-IV inhibitors, and other substrate-based inhibitors with an electrophilic serine-interacting motif, are chemically unstable in solution. This solution instability is due to an intramolecular reaction between the amino function and the electrophilic motif, as depicted in Scheme 5.1. The short solution half-life typically of a few hours was



FE 999011
K_i = 3.8 nM
Ferring, 1996

Figure 5.6 Studies with FE 999011 showed that sustained inhibition of DPP-IV leads to best results in animal models of diabetes.



Scheme 5.1 The limited solution stability of cyanopyrrolidine DPP-IV inhibitors is due to an intramolecular reaction between the mandatory amino and cyano functionalities.

causing problems for formulation and was made responsible for the short *in vivo* half-life of some compounds.

To overcome this limitation, many research groups explored *N*-alkylglycines with sterically hindered amines, which would undergo cyclization less readily. Early on, Novartis scientists had identified an adamantyl derivative **8** (Figure 5.7), which was one of the most potent inhibitors discovered in their program. Also, the primary metabolites of this compound were found to be highly active. Already in 1998, Villhauer synthesized one of the putative metabolites, LAF-237, which turned out to have an excellent solution stability, potent inhibitory activity, and good selectivity over related enzymes [31]. The improved pharmacokinetic profile and longer lasting pharmacodynamic effect of LAF-237 led to a replacement of Novartis' front-runner NVP-DPP728. LAF-237 was later named vildagliptin, in reference to Villhauer, its inventor [32]. Vildagliptin has been, after sitagliptin, the second compound to obtain market approval in the European Union and other countries. In the United States, Novartis has paused its efforts to seek regulatory approval after the FDA had requested additional data to address concerns about the tolerability in patients with renal impairment and skin lesions in nonhuman primates [33] (although no skin

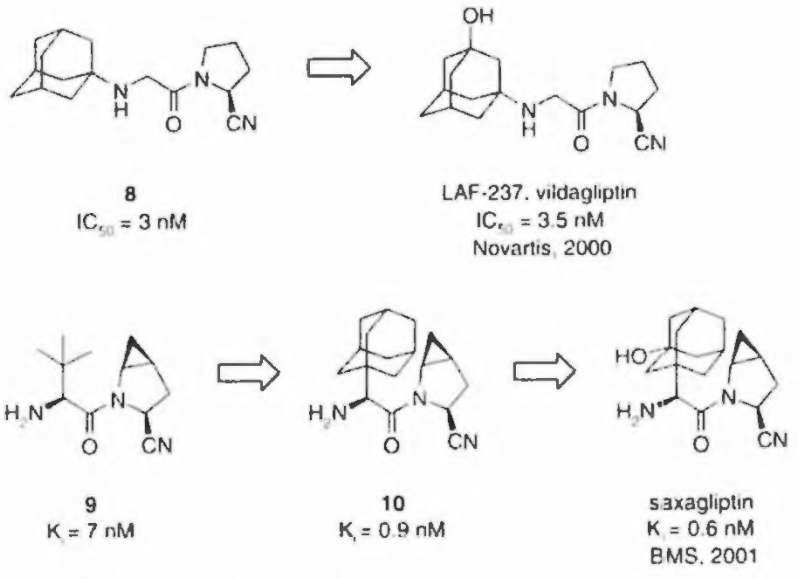


Figure 5.7 Discovery of vildagliptin and saxagliptin.

Table 5.1 Chemical stabilities of primary amine inhibitors.

Compound	Half-life[a]
3 (Figure 5.3)	5 h
FE 999011 (Figure 5.6)	27 h
9 (Figure 5.7)	42 h

a) In aqueous buffer at pH 7.2; 39.5 °C.

lesions have been observed in humans during clinical trials [67]). Vildagliptin is only moderately selective over DPP-8 and DPP-9. Following the highly publicized Merck study on the potential toxicities associated with DPP-8/9 inhibition [21], Novartis undertook long-term rodent toxicity studies with vildagliptin at exposures that are high enough for complete inhibition of DPP-IV, DPP-8, and DPP-9. As vildagliptin did not display any of the toxicities observed with P32/98 and structurally related molecules, the toxicity of the compounds studied by Merck is more likely the result of unidentified off-target effects that are independent of DPP-8/9, and the relevance of isoform selectivity remains unclear [34].

Researchers at Bristol-Myers Squibb found that converting a tertiary (3, Figure 5.3) to a quaternary alpha-carbon (FE 999011, Figure 5.6) improves the solution half-life by fivefold (Table 5.1). The long-lasting pharmacodynamic effect of FE 999011 might, at least in part, be attributed to this improved solution stability. Also, the introduction of a methylene bridge into the cyanopyrrolidine ring leads to steric bulk that similarly improves the chemical stability (compare FE 999011 and 9, Table 5.1). Molecular modeling demonstrated that these effects are, in both cases, due to intramolecular van der Waals interactions. These interactions disfavor a *cis* conformation of the amide, which is a prerequisite for cyclization, and thereby increase stability [35]. These findings led the Bristol-Myers Squibb scientists, in striking analogy to the efforts at Novartis, to 10 with an adamantyl substituent. This compound showed an excellent plasma-DPP-IV inhibition after oral dosing in rats, despite a low bioavailability (2%). This seemed to indicate that 10 is converted into an active metabolite *in vivo*, which prompted the synthesis of a hydroxy analogue as a putative metabolite. Quite similar to the vildagliptin story, it was found that this metabolite, later named saxagliptin (Figure 5.7), was highly potent and had an excellent solution stability [36]. This high solution stability, together with a relatively high distribution volume, makes saxagliptin a long-acting DPP-IV inhibitor. Bristol-Myers Squibb and AstraZeneca have shared the clinical development and filed a New Drug Application in 2008 [37].

Other companies also came up quickly with *N*-alkylglycines with a wide variety of quaternary N-substituents. TS-021, 11, and ABT-279 (Figure 5.8) are examples of *N*-alkylglycines that were evaluated in clinical trials. Taisho scientists identified TS-021, which had a much higher solution stability than a previously explored primary amine and an alkylglycine analogue without a quaternary N-substituent [38]. This improved stability translated into markedly higher plasma concentrations in rats, as measured 6 h after oral administration. An oral dose of TS-021 of 0.3 mg/kg in rats almost completely inhibited plasma DPP-IV activity for 120 min and exhibited a significant

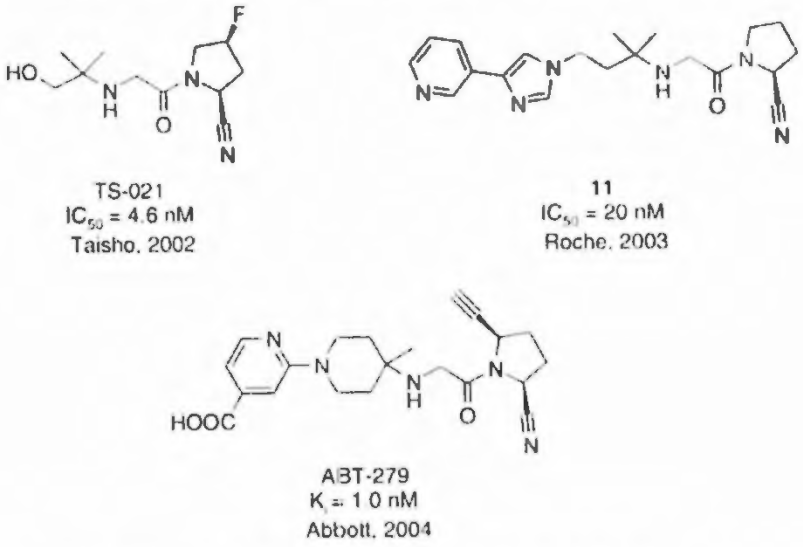


Figure 5.8 Various *N*-alkylglycine compounds in clinical development.

antihyperglycemic effect. The compound underwent phase I clinical studies in 2004, and was licensed to Eli Lilly in 2005; however, no further development was reported. Roche's clinical compound, **11**, was well tolerated in healthy volunteers up to doses of 2 g. In a multiple-dose study, the oral administration of 400 mg of **11** twice daily achieved >50% inhibition of plasma DPP-IV activity over the 12 h dose interval [39]. ABT-279 features a 5-ethynyl substituent on the cyanopyrrolidine ring, which had been demonstrated to improve selectivity over DPP-8/9 [40]. Indeed, the compound has an excellent selectivity over these enzymes as well as related peptidases and other safety-relevant targets. In healthy volunteers, ABT-279 was well tolerated up to doses of 1 g.

A primary amine inhibitor with a bulky side-chain, GSK-23A (Figure 5.9), was discovered at GlaxoSmithKline [41]. A combination of steric and electronic effects

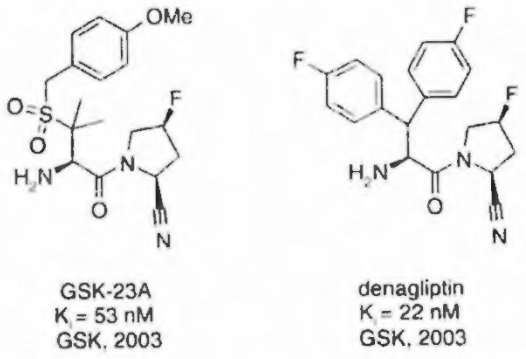


Figure 5.9 GSK-23A and denagliptin.

might be responsible for a reduced nucleophilicity of the free amine function, which leads to an extraordinarily long half-life of 1733 h in aqueous buffer at pH 7.2 and 37 °C. Denagliptin, another compound from the same company, was developed up to phase 3, but was finally put on hold in 2006 due to unfavorable data from preclinical long-term toxicology experiments [42, 43].

Today, we can look back on more than two decades of research on substrate-based DPP-IV inhibitors. These dipeptide-like compounds provided the first tools to elucidate the function of DPP-IV *in vivo*. Especially, P32/98 and NVP-DPP728 have played a pivotal role in establishing DPP-IV's role in glucose homeostasis and in establishing DPP-IV as a therapeutic target for type 2 diabetes. The exciting results obtained with these and other compounds triggered a race in the pharmaceutical industry toward DPP-IV inhibitors as a novel class of antidiabetic medicines, and many companies embarked on fast-follower projects with similar substrate-based compounds. This research culminated in the discovery of vildagliptin, which has obtained market approval in several countries, and other advanced compounds undergoing clinical development. However, other important classes of DPP-IV inhibitors have also emerged more recently, as will be shown in the next sections.

5.4
Sitagliptin and Analogues

Sitagliptin has been the first DPP-IV inhibitor to be approved as a treatment for type 2 diabetes. Launched by Merck in 2006, the annual sales for 2008 have already exceeded US$ 1000 million. The medicinal chemistry team led by Ann E. Weber started in 1999 and initially focused on substrate analogue inhibitors (see Section 5.3). After the identification of unwanted off-target activity as possible reason for multiorgan toxicity, the objective became to achieve a high (>1000-fold) selectivity over related proline peptidases, especially DPP-8 and DPP-9 [23, 44, 45]. The link between activity at DPP-8/9 and toxicity remains a matter of debate, but the goal per se has successfully guided the team toward the discovery of sitagliptin.

A high-throughput screening of the Merck sample library was performed in parallel with the medicinal chemistry work on substrate analogues. The screening produced only very few hits, among which the legacy compounds **12** and **13** (Figure 5.10) were followed up. At that time, no structural information of DPP-IV was available, and it was (wrongly) assumed that the pyrrolidine subunit of **13** might reside in the S1 substrate specificity pocket. As a consequence, the pyrrolidine was replaced with a thiazolidine, in analogy with substrate analogues such as P32/98. The truncated molecule **14**, with a much reduced molecular weight, was roughly equipotent to **13** but left little room for structural variations. The trifluorophenyl derivative, **15**, had a respectable potency but poor pharmacokinetic properties and an insufficient selectivity over DPP-8 [46].

In the meantime, the weakly active HTS hit **12** was combined with the 3-amino-4-phenylbutyryl side chain of **13**. The resulting hybrid molecule **16** was more than 100-fold more potent. A fluorine substituent at C(2) (**17**) led to an additional fourfold

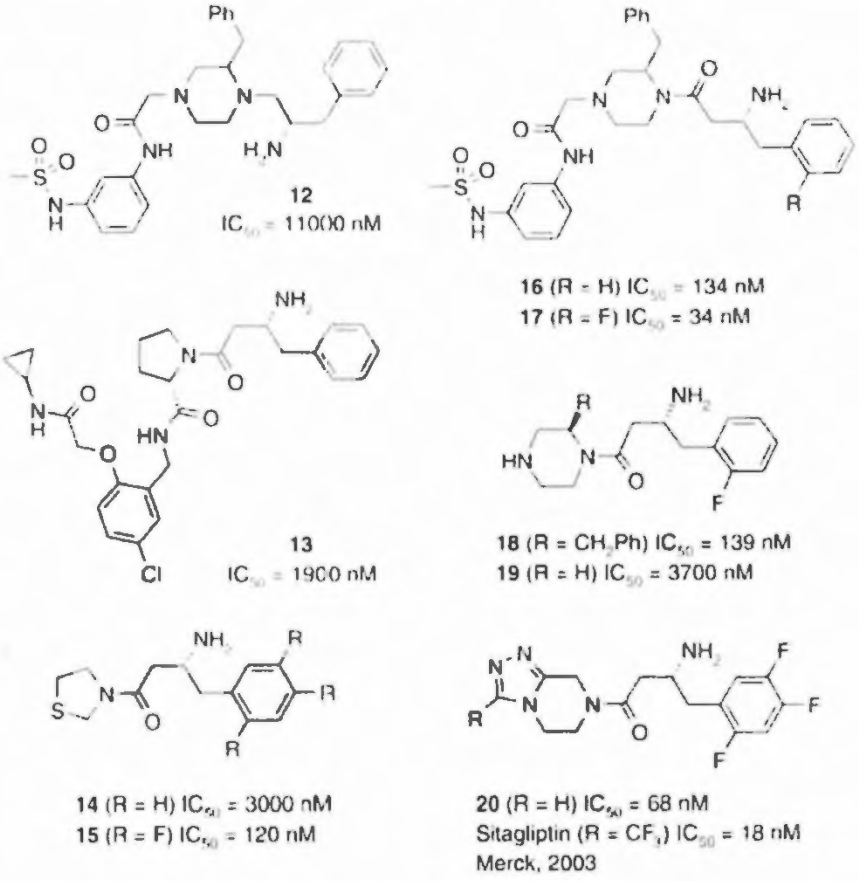


Figure 5.10 Evolution of sitagliptin from screening hits 12 and 13.

potency improvement. By removing the decoration of the piperazine, **18** and **19** were obtained. Molecule **18** was reasonably potent and selective but displayed a poor pharmacokinetic profile, which was attributed to the metabolic instability of the piperazine ring [47]. Unsubstituted piperazine **19** was only marginally active but had a low molecular weight and set the stage for further refinement.

Incorporation of the 2,4,5-trifluoro substitution pattern on the phenethylamine and replacement of the piperazine by a triazolopiperazine led to a significant improvement in potency. The poor bioavailability of **20** was improved to excellent values by installation of a trifluoromethyl group in the triazole ring, resulting in sitagliptin [48].

Interestingly, triazolopiperazines (systematic name: 5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-*a*]pyrazine) have only recently found widespread use. The parent compound was first disclosed by Merck, as late as 2001, as an intermediate for $GABA_A$ ligands as cognition enhancers [49] and soon became a fashionable building block in various Merck projects [50, 51]. Since the public disclosure of sitagliptin as development

compound in 2004, the trifluoromethyl-substituted triazolopiperazine has become a frequently used amine subunit across the medicinal chemistry community.

Sitagliptin was discovered in the absence of biostructural information. However, as soon as Merck had determined the cocrystal structure of sitagliptin within DPP-IV, the rational design of sitagliptin analogues became feasible. The cocrystal structure shows that the trifluorophenyl group occupies the S1 pocket of the enzyme; this pocket is a central recognition motif and normally accommodates the penultimate amino acid of the substrate (Figure 5.11). The fluorine atoms at C(4) and C(5) optimally fit the hydrophobic niche in the back of the S1 pocket, whereas the fluorine at the *ortho* position makes a favorable electrostatic interaction with the side chains of Asn710 and Arg125 [52]. Like the class of substrate-based inhibitors, which use a pyrrolidine or thiazolidine derivative to fill the S1 pocket, this class has a rather limited SAR around the trifluorophenyl group. Accordingly, a number of sitagliptin analogues have been made, which use the 2,4,5-trifluorophenethylamine subunit for selective recognition of DPP-IV but differ in the remaining part of the molecule for additional interactions with the target and refinement of the pharmacokinetic properties. For instance, Merck has designed the cyclic analogue 21 (Figure 5.12), in which the butyryl moiety of sitagliptin is replaced by a cyclohexane. Like sitagliptin, 21 is potent and selective over DPP-8/9 but has improved pharmacokinetic properties, with lower clearance and longer half-lives across species [53].

Researchers at Abbott have adapted the major fragments of the Merck inhibitors sitagliptin and 21 to create their own DPP-IV inhibitor, ABT-341. This compound is a potent DPP-IV inhibitor, is selective over DPP-8/9, and has excellent pharmacokinetic properties, comparable to 21 [54]. Despite the similarity to sitagliptin, the binding mode of ABT-341 is different from that of sitagliptin, in that the triazolopyrazinecarbonyl subunit occupies a different part of the binding pocket and induces some conformational change at the target [55]. The compound was selected as

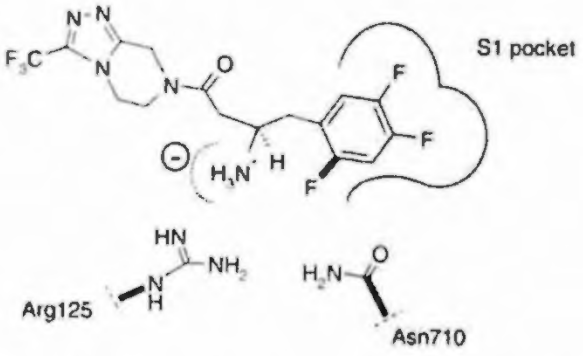
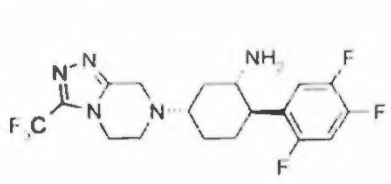
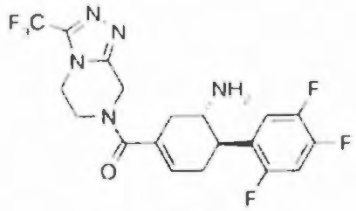


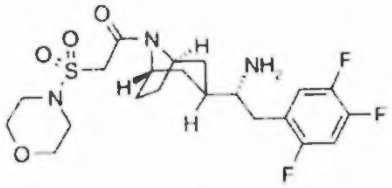
Figure 5.11 Schematic illustration of key interactions of sitagliptin with DPP-IV: the trifluorophenyl substituent resides in the lipophilic S1 pocket. The *ortho*-F makes favorable electrostatic interactions with Arg125 and Asn710. The protonated amine binds to a negatively charged surface of the protein (comprised of Glu205, Glu206, and Tyr662, not shown for clarity).



21
IC_{50} = 21 nM
Merck, 2006



ABT-341
K_i = 1.3 nM
Abbott, 2006



22
Novartis, 2007

Figure 5.12 Sitagliptin analogues with a 2,4,5-trifluorophenethylamine motif.

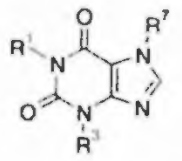
development candidate, but no clinical development has been reported as of December 2008.

Several years after the discovery of vildagliptin, Novartis has also embarked on a DPP-IV follow-on project, using sitagliptin as seed structure. As a late entrant to the phenethylamine class, compound **22**, with a bicyclic subunit, has been identified as a potent DPP-IV inhibitor [56].

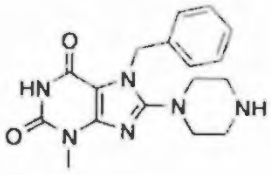
5.5
Xanthines and Analogues

The natural products theophylline, theobromine, and caffeine are known as xanthine alkaloids. They are among the oldest drugs, mainly exhibiting vasodilatory and stimulating effects, which can be rationalized through their actions as (nonselective) phosphodiesterase inhibitors and adenosine receptor antagonists [57]. Owing to their rich pharmacology and chemical tractability, xanthine derivatives are well represented in corporate screening libraries. After DPP-IV had emerged as an attractive target for type 2 diabetes, several companies performed a high-throughput screening to identify novel classes DPP-IV inhibitors.

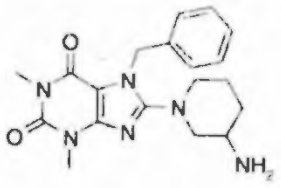
Compound **23** (Figure 5.13) is a commercially available "lead-like" xanthine derivative that inhibits DPP-IV in the low micromolar range. As a consequence, **23** has been discovered as a screening hit by a number of research teams. For instance, Merck invested some limited resources on substituent alterations of **23** with little success but then focused on more promising activities (see preceding sections) [44]. On the other hand, Novo Nordisk and Boehringer Ingelheim have identified



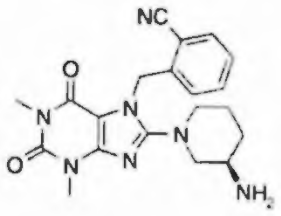
Xanthine $R^1 = R^3 = R^7 = H$
Theophylline $R^1 = R^3 = Me$, $R^7 = H$
Theobromine $R^1 = H$, $R^3 = R^7 = Me$
Caffeine $R^1 = R^3 = R^7 = Me$



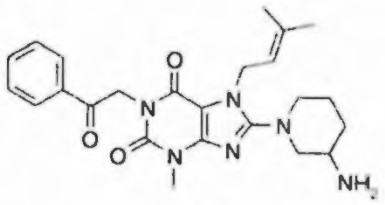
23
IC_{50} = 3900 nM



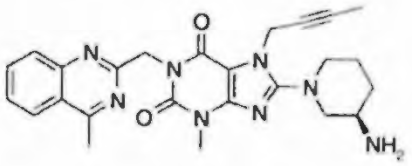
24
IC_{50} = 82 nM
Boehringer Ingelheim, 2002



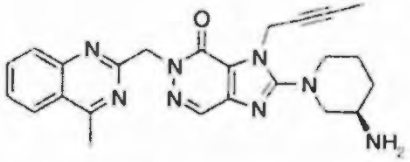
25
Novo Nordisk, 2003



26
IC_{50} = 6 nM



Linagliptin
IC_{50} = 1 nM
Boehringer Ingelheim, 2004



27
IC_{50} = 1 nM
Boehringer Ingelheim, 2004

Figure 5.13 Linagliptin and other DPP-IV inhibitors originating from a commercially available screening compound, 23.

a 3-aminopiperidine subunit to be a superior replacement for the piperazine moiety (compounds **24** and **25**) and filed patent applications, which overlap to a significant degree [58]. Boehringer Ingelheim has best succeeded in elaborating the xanthine series: modification of the substituents at N(1) and N(7) led to **26**, which was very potent on DPP-IV but had unacceptable off-target activities at the hERG channel and the muscarinic receptor M_1. Replacement of the substituent at N(7) by a 2-butynyl group and installation of a quinazolylmethyl substituent in lieu of the phenacyl group gave linagliptin, in which the hERG interaction was greatly reduced and the selectivity over the M_1 receptor was increased to 300-fold [59]. Comparative preclinical *in vivo* characterization with vildagliptin, saxagliptin, sitagliptin, and alogliptin shows that linagliptin has a superior potency and longer duration of action [60]. Linagliptin has entered phase 3 clinical trials in 2008. The X-ray crystal structure of linagliptin within DPP-IV reveals that the 2-butynyl group resides in

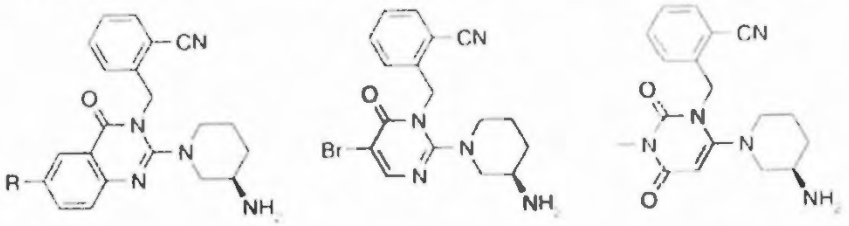


Figure 5.14 Structural insight led to a successful core replacement of xanthine 25, and finally to alogliptin.

the S1 pocket. The 4-methylquinazolinone group stacks on top of a tryptophan residue of the protein (Trp629); this π–π interaction [61] is not exploited by other classes of DPP-IV inhibitors and contributes to the very high affinity of linagliptin. The main binding contribution of the xanthine moiety comes from another π–π interaction, a stacking of the central uracil ring with a tyrosine side chain (Tyr547). Comparable aromatic–aromatic interactions can also be affected by a wide variety of other heterocycles [58]. For instance, Boehringer Ingelheim has reported analogue 27, in which the xanthine core has been replaced by an imidazopyridazinone. This compound is equipotent to linagliptin but has a superior selectivity over M_1 (>1000-fold) and a different pharmacokinetic profile [62].

Researchers at Syrrx (now Takeda San Diego) have performed a remarkable scaffold hopping exercise, which provided interesting new classes of patentable DPP-IV inhibitors. Supported by high-throughput structural biology and molecular modeling as the company's core expertise, they started from seed structures such as Novo Nordisk's xanthine derivative 25 (Figure 5.14). In 25, the cyanobenzyl substituent fills the cavity of the S1 pocket. The cyano group does not engage in a covalent interaction with the enzyme (in contrast to the cyano group in the cyanopyrrolidine series) but makes a favorable electrostatic interaction with the side chains of Asn710 and Arg125, similar to that of the *ortho*-fluorine of sitagliptin [52]. In search for central scaffolds that could take advantage of the π–π interaction with Tyr547 like the xanthine core of 25, they identified 4-quinazolinone as a suitable heterocyclic replacement. Indeed, compound 28 was very potent. Pharmacokinetic shortcomings were amended by introducing a fluorine at the metabolically vulnerable position of the quinazolinone. Compound 29 had attractive pharmacological and pharmacokinetic properties but showed unacceptable levels of CYP3A4 and hERG inhibition. To minimize the interaction at these off-targets, more polar heterocycles were explored as quinazolinone replacements. Pyrimidinone 30 and the analogous uracil compound, later named alogliptin, retained the potency, and greatly improved the selectivity over the off-targets. Alogliptin, which is the least lipophilic in this series, showed the most favorable pharmacological profile and no evident safety issues [63, 64]. Alogliptin has progressed through clinical development very rapidly, and a New Drug Application has been filed in December 2007.

5.6
Pharmacological Comparison of DPP-IV Inhibitors

DPP-IV is a chemically very tractable target, and several DPP-IV inhibitors have progressed into clinical trials as medicines to treat type 2 diabetes. In this highly competitive field, the structural diversity is remarkable, with a primary or secondary amino group as the sole recurring motif. Nevertheless, a comparison of phase 3 clinical data at therapeutic doses shows that vildagliptin, sitagliptin, and alogliptin (as representative compounds from each structural class) have similar clinical efficacies. Thus, the average reduction of glycosylated hemoglobin (HbA_{1c}) is 0.5–0.8% after 24 or 26 weeks of treatment at therapeutic doses (Table 5.2). It should be noted that the magnitude of the HbA_{1c} reduction depends on the severity of the disease. For instance, vildagliptin (50 mg twice a day) achieves an HbA_{1c} reduction of 0.6% from a baseline-HbA_{1c} of $\leq$8% but a reduction of 1.6% from a baseline of $\geq$10% (similar patterns for HbA_{1c} changes are reported for other classes of OADs).

While the determination of meaningful changes in HbA_{1c} requires long-term treatment of diabetic patients and a correct estimation of the therapeutic dose, DPP-IV inhibition has the benefit of offering an instant-readout biomarker that can forecast the efficacy of the drug in an exploratory setting: DPP-IV activity can be easily determined in blood plasma by measuring the turnover rate of a peptidic substrate using UV spectroscopy. Thus, the notion that sustained inhibition of DPP-IV activity leads to a maximal therapeutic effect [29, 30] has been exploited by Merck in designing phase 1 clinical studies. In healthy volunteers, near-maximal ($\geq$80%) DPP-IV inhibition was achieved at daily doses of $\geq$100 mg (Figure 5.15). The dose of 100 mg/day was confirmed in phase 2 studies to be therapeutically adequate in type 2 diabetic patients and later taken on to phase 3. The successful implementation of a simple pharmacodynamic readout as biomarker enabled Merck to progress sitagliptin from entry into human to phase 3 in only 2.1 years [65].

For vildagliptin, the DPP-IV inhibition after administration of 50 mg is greater than 80% over 12 h but reduced to about 20% after 24 h [66]. Accordingly, the recommended dosing regimen for vildagliptin in the majority of settings is 50 mg

Table 5.2 HbA_{1c} changes after chronic administration of DPP-IV inhibitors (phase 3 data).

	Vildagliptin [67]	Sitagliptin [68]	Alogliptin [74]
Number of subjects	90	229	131
Duration of treatment	24 weeks	24 weeks	26 weeks
Dose	50 mg	100 mg	25 mg
Dosing regimen	Twice daily	Once daily	Once daily
HbA_{1c} baseline	8.6%	8.0%	7.90%
Mean change from baseline HbA_{1c}	−0.8%	−0.6%	−0.59%
HbA_{1c} change from placebo	−0.5%[a]	−0.8%[b]	−0.57%[c]

a) 95% confidence interval: (−0.8; −0.1); $p < 0.05$ compared to placebo.
b) 95% confidence interval: (−1.0; −0.6); $p < 0.001$ compared to placebo.
c) $p < 0.001$ compared to placebo.

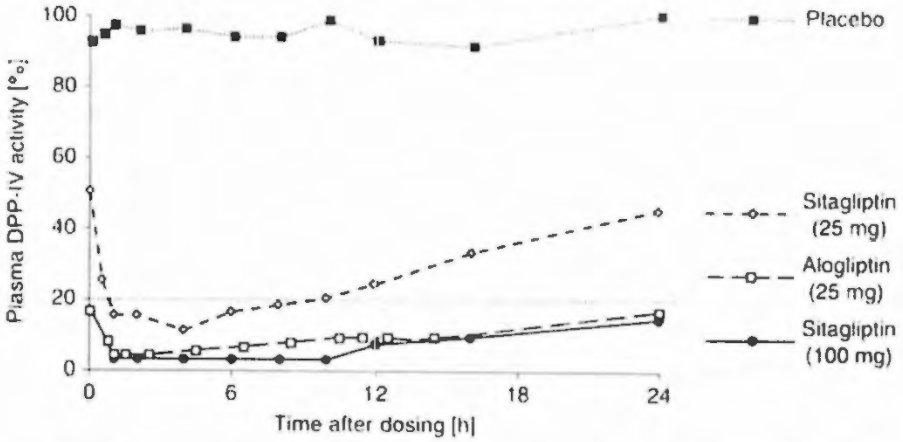


Figure 5.15 Time course of inhibition of plasma DPP-IV activity after administration of placebo [75], and multiple daily oral doses of sitagliptin (after 10 days, healthy volunteers) [75], and alogliptin (after 14 days, type 2 diabetic patients) [69]. Adapted with permission from Excerpta Medica, Inc.: Clinical Therapeutics, copyright 2006, 2008.

twice a day [67]. Alogliptin achieves near-maximal DPP-IV inhibition over 24 h already at much lower doses – a 25 mg dose has approximately the same effect as a 100 mg dose of sitagliptin (Figure 5.15).

DPP-IV inhibitors are typically hydrophilic compounds that are rapidly absorbed. Otherwise, the pharmacokinetic properties of the individual DPP-IV inhibitors are quite distinct (Table 5.3): sitagliptin has a relatively low clearance and a large volume of distribution. This translates into a long terminal half-life. Protein binding is low. Sitagliptin is predominantly excreted unchanged through the kidneys, with limited metabolic contribution through CYP3A4 and CYP2C8. Accordingly, patients with renal impairment should use lower doses [68].

In comparison, vildagliptin has a higher clearance and lower volume of distribution, which is reflected in a relatively short half-life. Protein binding is very low. CYP-dependent metabolism does not occur. The major elimination pathway is hydrolytic

Table 5.3 Pharmacokinetic data of DPP-IV inhibitors.

	Sitagliptin [68]	Vildagliptin [67]
Dose	100 mg	50 mg
t_{max}	1–4 h	1.7–2.5 h
Clearance	350 ml/min	680 ml/min
Volume of distribution	198 l	71 l
Half-life	12.4 h	2 h (intravenous), 3 h (oral)
Bioavailability	87%	85%
Protein binding	38%	9.3%
Renal excretion of parent	79%	23%

metabolism at the cyano group, followed by renal excretion of the inactive metabolite; renal excretion of parent drug accounts only for a minor fraction. Vildagliptin is not recommended for renally impaired patients due to insufficient data. Additional safety concerns are related to elevated levels of liver aminotransferases and skin lesions; therefore, monitoring for liver function and skin disorders is recommended [67].

For the less advanced DPP-IV inhibitors, only limited pharmacokinetic information is available. Alogliptin has pharmacokinetic properties similar to sitagliptin, with an apparent half-life of about 20 h and mainly renal excretion of unmetabolized drug [69].

Saxagliptin is also renally excreted, as parent and active metabolite, both of which have apparent half-lives of about 3 and 5 h, respectively [70]. The conversion of saxagliptin to its active metabolite is mediated by CYP3A4/5, a clear difference from its close structural analogue, vildagliptin [71].

Finally, linagliptin has a completely different pharmacokinetic profile in that renal excretion is only a minor elimination route. The compound is largely bound to plasma proteins, has a very long apparent terminal half-life of about 3 days, and has a bioavailability of 30% [72].

Taken together, DPP-IV inhibitors achieve an average HbA_{1c} reduction of 0.5–0.8% after 6 months, independent of the structural class. Inhibition of DPP-IV activity is a relevant biomarker for antihyperglycemic efficacy, and near-maximal inhibition over 24 h is required for an optimal effect. Besides, the individual compounds differ significantly in their mode of metabolism and excretion, which may be an important consideration for the individual patient.

5.7 Concluding Remarks

DPP-IV inhibitors represent only one of the many classes of drugs to treat patients with type 2 diabetes. The main goal of management of type 2 diabetes is to achieve glycemic levels as close to the nondiabetic range (HbA_{1c} at 4–6%) as practicable, in order to reduce the risk of late-stage complications. A consensus algorithm of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) released in 2008 calls for a therapeutic intervention in cases where HbA_{1c} exceeds 7%. In principle, most patients diagnosed with type 2 diabetes would massively benefit from weight loss and increased physical activity, but only a minority is willing and able to adhere to lifestyle changes in the long term. Therefore, medical management is the common practice, with metformin as first-line treatment. In cases where the HbA_{1c} goal of 7% is not met with metformin alone, either insulin or a sulfonylurea should be added. Alternatively, when hypoglycemia (as frequent side effect of insulin and sulfonylureas) is particularly undesirable, pioglitazone or a GLP-1 agonist can be used as an add-on to metformin. Other approved classes of drugs including DPP-IV inhibitors are not within the list of preferred agents, in part due to their limited clinical data [73].

Sitagliptin, launched in 2006, is often used in combination with metformin. Its rapid rise in popularity is due to the favorable safety profile (no hypoglycemia, no weight gain, and no gastrointestinal side effects). The absence of competition from other DPP-IV inhibitors has also contributed to a highly successful start for this drug. Vildagliptin has been approved in several countries, and other DPP-IV inhibitors are expected to be introduced in the near future. They all lower HbA_{1c} to a similar extent but have quite diverse pharmacokinetic properties. The result of ongoing studies, with focus on long-term benefits and safety, will determine the future role of DPP-IV inhibitors among the options to treat type 2 diabetes.

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PREDICTION OF HUMAN CLEARANCE OF TWENTY-NINE DRUGS FROM HEPATIC MICROSOMAL INTRINSIC CLEARANCE DATA: AN EXAMINATION OF IN VITRO HALF-LIFE APPROACH AND NONSPECIFIC BINDING TO MICROSOMES

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ABSTRACT:

Twenty-nine drugs of disparate structures and physicochemical properties were used in an examination of the capability of human liver microsomal lability data ("in vitro $T_{1/2}$" approach) to be useful in the prediction of human clearance. Additionally, the potential importance of nonspecific binding to microsomes in the in vitro incubation milieu for the accurate prediction of human clearance was investigated. The compounds examined demonstrated a wide range of microsomal metabolic labilities with scaled intrinsic clearance values ranging from less than 0.5 ml/min/kg to 189 ml/min/kg. Microsomal binding was determined at microsomal protein concentrations used in the lability incubations. For the 29 compounds studied, unbound fractions in microsomes ranged from 0.11 to 1.0. Generally, basic compounds demonstrated the greatest extent of binding and neutral and acidic compounds the least extent of binding. In the projection of human clearance values, basic and neutral compounds were well predicted when all binding considerations (blood and microsome) were disregarded, however, including both binding considerations also yielded reasonable predictions. Including only blood binding yielded very poor projections of human clearance for these two types of compounds. However, for acidic compounds, disregarding all binding considerations yielded poor predictions of human clearance. It was generally most difficult to accurately predict clearance for this class of compounds; however the accuracy was best when all binding considerations were included. Overall, inclusion of both blood and microsome binding values gave the best agreement between in vivo clearance values and clearance values projected from in vitro intrinsic clearance data.

The use of in vitro drug metabolism data in the understanding of in vivo pharmacokinetic data has recently become an area of scientific interest (Houston, 1994; Houston and Carlile, 1997; Iwatsubo et al., 1997). This has partially stemmed from a trend in the pharmaceutical industry to use in vitro drug metabolism data, using human-derived reagents, as a criterion to select compounds for further development (Rodrigues, 1997). Thus, in vitro metabolism data is used in a prospective manner to choose those compounds for further development that are expected to possess commercially acceptable pharmacokinetic properties (e.g., half-life permitting once-per-day administration regimens, low oral clearance to reduce dose, etc.). Several investigators have recently described methods whereby preclinical drug metabolism and pharmacokinetic data can be used to predict human pharmacokinetic parameters (Obach et al., 1997; Lave et al., 1997a,b; Mahmood, 1998a,b).

The first demonstration of the correlation between in vivo clearance values and clearance values calculated from liver microsomal metabolism intrinsic clearance data was made by Rane et al. (1977) for the rat. Intrinsic clearance data were obtained by determination of the enzyme kinetic parameters (V'_{max} and K_M). In our work, we described two related methods whereby human clearance could be predicted from in vitro metabolism data (Obach et al., 1997). In one method, the enzyme kinetic parameters V'_{max} and K_M were determined and converted to intrinsic clearance (CL'_{int})[1], which is similar to that described by Rane et al. (1977). In the other method, referred to as the "in vitro $T_{1/2}$ method", CL'_{int} was determined by measuring the first-order rate constant for consumption of the substrate at a low concentration. Interestingly, for both of these methods, a better correlation was observed between the actual and predicted clearance values if the free fraction in blood was disregarded in the well-stirred or parallel-tube equations describing hepatic extraction.

One possible reason for the observation that a better prediction of human clearance was made when disregarding plasma protein binding was that the substrates were bound in the microsomal incubations, and that the extent of this binding could be great enough so as to almost cancel out the plasma protein binding term in the well-stirred and parallel-tube equations (Obach, 1996). This possibility was further substantiated in an examination of probe substrates propranolol, imipramine, and warfarin (Obach, 1997). In this report, it was demonstrated that the lipophilic amines propranolol and imipramine were bound to microsomes, and that incorporation of this binding term aided in the accurate prediction of human clearance from in vitro intrinsic clearance data. The acidic drug, warfarin, exhibited this phenomenon to a much lesser extent. However, for all three drugs overall, incorporation of both plasma protein and microsome binding

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[1] Abbreviations used are: CL'_{int}, intrinsic clearance; $f_{u(mic)}$, unbound fraction in microsomal incubation mixtures; $f_{u(blood)}$, unbound fraction in blood; Q, hepatic blood flow; ISTD, internal standard.

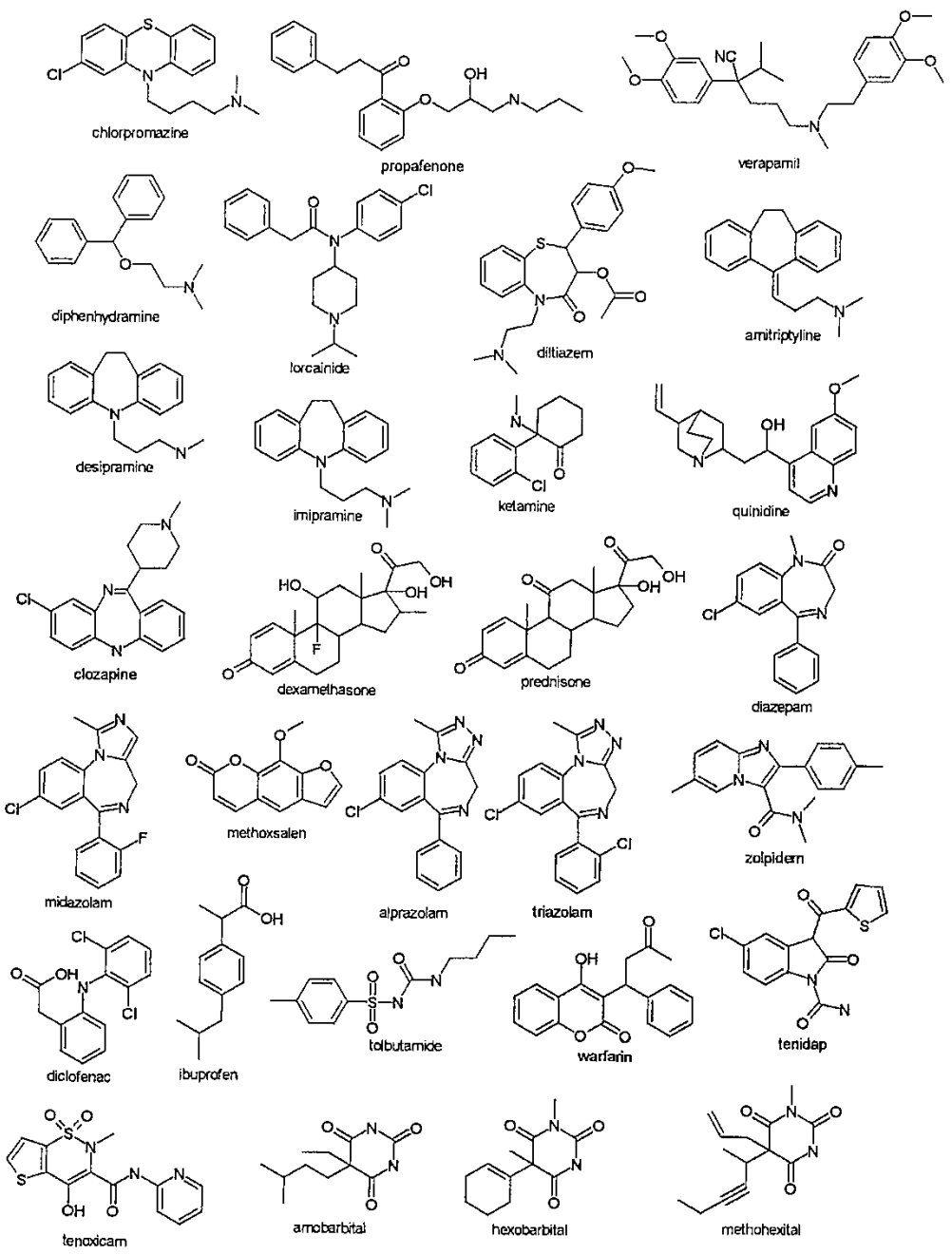

FIG. 1. *Chemical structures of the 29 drugs examined in this study.*

terms generally yielded more accurate predictions of human clearance.

The objective of the experiments described herein is to more exhaustively test the hypothesis that microsomal binding is an important phenomenon in the prediction of in vivo pharmacokinetics from in vitro drug metabolism data. To this end, human hepatic microsomal metabolism data were gathered for 29 drugs, using the in vitro $T_{1/2}$ approach. Additionally, the extent of nonspecific binding to microsomes in the in vitro matrix was measured for each drug. The drugs used in these experiments span a broad range of structural types (Fig. 1) and include basic compounds (positively charged at pH 7.5), acidic compounds (negatively charged at pH 7.5), and neutral compounds (no charge at pH 7.5). The data set was used to project human clearance from the in vitro intrinsic clearance data to determine

TABLE 1

Sample processing and HPLC-MS conditions for 29 drugs used in this analysis

Drug	Internal Standard	Incubation Termination	Mobile Phase System	CH_3CN	MS Polarity	*m/z*	R_t
				%			*min*
Basic compounds							
Chlorpromazine	Amitriptyline	NaOH	1	36.5	+	318.8	1.2
Propafenone	Verapamil	NaOH	1	32.0	+	341.9	1.4
Verapamil	Propafenone	NaOH	1	32.0	+	455.1	1.6
Diphenhydramine	Propafenone	NaOH	1	32.0	+	256.0	0.8
Lorcainide	Propafenone	NaOH	1	32.0	+	371.0	1.4
Diltiazem	Propafenone	NaOH	1	32.0	+	415.0	1.0
Amitriptyline	Imipramine	NaOH	1	36.5	+	278.0	1.0
Desipramine	Amitriptyline	NaOH	1	36.5	+	266.5	0.8
Imipramine	Amitriptyline	NaOH	1	36.5	+	281.0	1.0
Ketamine	Metoprolol	NaOH	1	18.5	+	237.8	0.8
Quinidine	Ondansetron	NaOH	1	18.5	+	325.0	1.5
Clozapine	Diltiazem	NaOH	1	27.5	+	326.9	1.2
Neutral compounds							
Dexamethasone	Prednisone	NaOH	1	32.0	+	393.1	1.8
Prednisone	Dexamethasone	NaOH	1	32.0	+	359.1	1.1
Diazepam	Midazolam	NaOH	1	50.0	+	284.9	1.4
Midazolam	Diazepam	NaOH	1	50.0	+	325.8	0.8
Methoxsalen	Diazepam	CH_3CN	1	50.0	+	217.0	1.0
Alprazolam	Triazolam	NaOH	1	41.0	+	309.0	0.9
Triazolam	Alprazolam	NaOH	1	41.0	+	342.9	1.0
Zolpidem	Quinine	NaOH	1	23.0	+	308.0	1.5
Acidic compounds							
Diclofenac	Ibuprofen	HCl	2	32.0	−	294.0	1.1
Ibuprofen	Diclofenac	HCl	2	32.0	−	205.1	1.3
Tolbutamide	Warfarin	HCl	2	27.5	−	269.0	1.2
Warfarin	Tolbutamide	HCl	2	27.5	−	307.3	1.2
Tenidap	Warfarin	HCl	2	32.0	−	319.1	1.3
Tenoxicam	Piroxicam	HCl	2	27.5	−	336.1	0.8
Amobarbital	Methohexital	HCl	2	45.5	−	225.2	0.8
Hexobarbital	Methohexital	HCl	2	45.5	−	235.1	0.8
Methohexital	Amobarbital	HCl	2	45.5	−	261.1	1.5

whether the most accurate projections are made by disregarding all binding data, including only blood binding values, or including both blood and microsomal binding values.

Experimental Procedures

Materials. The 29 drugs examined in these experiments were obtained from Sigma Chemical Co. (St. Louis, MO) with the exception of lorcainide (obtained from ICN, Aurora, OH), methoxsalen (obtained from Aldrich Chemical, Milwaukee, WI), zolpidem (obtained from Research Biochemicals International, Natick, MA), and methohexital (obtained from Radian Inc., Dallas, TX). NADPH was obtained from Sigma. Solvents and other reagents were from common sources and were of HPLC grade or better. Human liver microsomes were from an in-house bank of liver microsomes maintained at Pfizer Central Research (Groton, CT). A pool was prepared from six liver microsomal preparations from six individual donors that were selected on the basis of having average activities for five of the major drug metabolizing cytochrome P-450 (CYP) enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A) normalized per microsomal protein content. Microsomes from putative CYP2D6 and CYP2C19 poor metabolizers were excluded. The P-450 content of this pool, as determined by spectral means (Omura and Sato, 1964) was 0.26 nmol/mg microsomal protein. CYP isoform specific marker substrate activities were as follows: CYP1A2, phenacetin *O*-deethylase of 0.147 nmol/min/mg protein (at 50 μM phenacetin); CYP2C9, tolbutamide 4-hydroxylase of 0.23 nmol/min/mg protein (at 1.0 mM tolbutamide); CYP2C19, *S*-mephenytoin 4′-hydroxylase of 0.093 nmol/min/mg protein (at 1.0 mM *S*-mephenytoin); CYP2D6, bufuralol 1′-hydroxylase of 0.075 nmol/min/mg protein (at 10 μM bufuralol); and CYP3A4, testosterone 6β-hydroxylase of 2.7 nmol/min/mg protein (at 250 μM testosterone). All glassware was subjected to gas phase silylation before use.

Metabolic Incubations. Human liver microsomal incubations were conducted in triplicate. General conditions are described as follows with details specific to each drug listed in Table 1. Incubation mixtures consisted of liver microsomes (0.3–10 mg microsomal protein/ml), substrates (1.0 μM), $MgCl_2$ (3.3 mM), and NADPH (1.3 mM) in a total volume of 0.5 ml potassium phosphate buffer (25 mM, pH 7.5). Reactions were commenced with the addition of NADPH and shaken in a water bath open to the air at 37°C. At $T = 0$ and at five time points ranging to 40 min, aliquots (50 μl) were removed and added to termination mixtures containing internal standards as listed in Table 1. The samples were processed by extraction into methy *t*-butyl ether (3 ml), the aqueous layer was frozen in a dry ice-acetone bath, the organic solvent was decanted and evaporated under N_2 at 30°C. The residue was reconstituted in 50 μl HPLC mobile phase A (see below). For methoxsalen samples, the work-up procedure consisted of precipitation of protein with CH_3CN (100 μl), removal of precipitated materials by centrifugation, and analysis of the supernatant by HPLC-mass spectrometry (MS).

Equilibrium Dialysis. Drugs (1.0 μM) were mixed with human liver microsomes (at protein concentrations used for the respective metabolic incubations), $MgCl_2$ (3.3 mM) and potassium phosphate buffer (25 mM; pH 7.5). The mixtures were subjected to equilibrium dialysis versus buffer/$MgCl_2$ at 37°C using a Spectrum apparatus (Spectrum Industries, Los Angeles, CA) as per instructions of the manufacturer. Spectra-Por no. 4 membranes, with molecular mass cutoff of 12 to 14 kDa, were used and the cells were rotated at 20 rpm for 5 h. (These dialysis conditions had been previously shown to give equilibrium for this dialysis apparatus; Obach, 1997). Dialysis experiments were done in triplicate. On completion of the dialysis period, the microsome and buffer samples were removed, processed as described above, and analyzed by HPLC-MS. Microsome samples (50 μl) were mixed with control buffer (100 μl), and buffer samples (100 μl) were mixed with control microsomes (50 μl) to yield an identical matrix before sample work-up. Drug recovery through the dialysis procedure was determined by analyzing samples of the mixtures that were not subjected to dialysis, and recovery values were 86% or greater.

HPLC-MS Analysis. The HPLC-MS system consisted of a Hewlett-Packard 1100 quaternary gradient HPLC pump with membrane degasser (Hewlett-Packard, Palo Alto, CA), a CTC PAL autoinjector (Leap Technologies, Carrboro, NC), and a PE-Sciex API 100 single quadrupole mass spec-

TABLE 2

Values for systemic clearance, fraction unbound in plasma, and blood-to-plasma ratio for 29 drugs examined in this analysis

Drug	Fraction Unbound in Plasma (f_u)	Blood-to-Plasma Ratio	Nonrenal Clearance[a]		References
			Plasma	Blood	
			ml/min/kg		
Basic compounds					
Chlorpromazine	0.05	0.78	8.6[b]	11	Dahl and Strandjard, 1974; Maxwell et al., 1972; Lund, 1980
Propafenone	0.04	0.70	13	19	Bryson et al., 1993
Verapamil	0.10	0.77[c]	15	19	Eichelbaum et al., 1984
Diphenhydramine	0.22	0.65[c]	6.2	9.5	Blyden et al., 1986
Lorcainide	0.15	0.77	14	18	Somani et al., 1987; Klotz et al., 1978
Diltiazem	0.22	1.0	12	12	Echizen and Eichelbaum, 1986; Smith et al., 1983
Amitriptyline	0.05	0.86	10	12	Schulz et al., 1983
Desipramine	0.18	0.96	12	12	Brosen and Gram, 1988
Imipramine	0.10	1.1	13	12	Sallee and Pollack, 1990; Abernathy et al., 1985
Ketamine	0.88	0.82[c]	16	20	White et al., 1985
Quinidine	0.13	0.92	2.5	2.7	Greenblatt et al., 1977; Rakhit et al., 1984; Hughes et al., 1975
Clozapine	0.05	0.87	2.5	2.9	Cheng et al., 1988
Neutral compounds					
Dexamethasone	0.32	0.93	3.5	3.8	Tsuei et al., 1979; Peterson et al., 1983
Prednisone	0.25	0.83[c]	4.1	4.9	Schalm et al., 1977
Diazepam	0.013	0.71	0.4	0.6	Greenblatt et al., 1980; Maguire et al., 1980
Midazolam	0.05	0.53	4.6	8.7	Heizmann et al., 1983
Methoxsalen	0.09	0.67	12	18	Billard et al., 1995; Pibouin et al., 1987
Alprazolam	0.32	0.78[c]	0.59	0.76	Smith et al., 1984
Triazolam	0.10	0.62[c]	2.9	4.7	Smith et al., 1987
Zolpidem	0.08	0.76[c]	4.3	5.7	Durand et al., 1992
Acidic compounds					
Diclofenac	0.005	0.55[c]	4.2	7.6	Willis et al., 1979; Chan et al., 1987
Ibuprofen	0.01	0.55[c]	0.8	1.5	Martin et al., 1990
Tolbutamide	0.04	0.55[c]	0.2	0.36	Balant, 1981; Scott and Poffenbarger, 1979
Warfarin	0.01	0.55	0.045	0.081	O'Reilly, 1972
Tenidap	0.0007	0.56	0.058	0.10	Gardner et al., 1995
Tenoxicam	0.009	0.67	0.02	0.03	Heintz et al., 1984
Amobarbital	0.39	1.5	0.53	0.35	Bachmann, 1987; Sawada et al., 1985
Hexobarbital	0.53	1.0	3.6	3.6	Breimer et al., 1975; Sawada et al., 1985
Methohexital	0.27	0.70[c]	11	16	Breimer, 1976; Gillis et al., 1976

[a] All clearance values from the literature were from i.v. dosing. In the case of dependence of clearance on genetic polymorphism of drug-metabolizing enzymes, data from poor metabolizers was excluded. Nonrenal clearance values were calculated by: $Cl_{non\text{-}renal} = Cl_{total} \cdot (1 -$ fraction of the dose excreted unchanged in urine).

[b] Chlorpromazine clearance values from i.m. dose; assumes complete absorption from i.m. route.

[c] Denotes blood-to-plasma ratios that were unavailable in the scientific literature. Values were determined in duplicate after incubation of drug at 1.0 μg/mL in whole blood at ambient temperature for 45 min.

trometer (Sciex, Thornhill, Ontario, Canada) with a turbo ionspray interface. There were various mobile phases used for the different drugs as listed in Table 1. Mobile phase system 1 consisted of 20 mM acetic acid (adjusted to pH 4 with NH_4OH) and CH_3CN used at various percentages of organic solvent (as listed in Table 1). System 2 consisted of 5 mM NH_4OAc (pH unadjusted) and CH_3CN at various percentages as listed in Table 1. The column used was a Phenomenex Luna C18 narrow bore column (2.5 × 50 mm) with a 3-μm particle size (Phenomenex, Torrance, CA). The flow rate was 0.5 ml/min and the mobile phase composition was held isocratically for each analyte. The injection volume was 25 μl.

The effluent was split with approximately 0.25 ml/min introduced into the turbo ionspray source of the mass spectrometer. Source parameters (e.g., orifice voltage, temperature, gas flow rates, etc.) were individually optimized for each drug, and the molecular ion (either $M + H^+$ or $M - H^-$, depending on the orifice polarity) was followed for each compound and internal standard in the selected ion monitoring mode.

Calculations. In the determination of the in vitro $t_{1/2}$, the analyte/ISTD peak height ratios were converted to percentage drug remaining, using the $T = 0$ peak height ratio values as 100%. The slope of the linear regression from log percentage remaining versus incubation time relationships (−k) was used in the conversion to in vitro $T_{1/2}$, values by in vitro $T_{1/2} = -0.693/k$. Conversion to in vitro CL'_{int} (in units of ml/min/kg) was done using the following formula (Obach et al., 1997):

$$CL'_{int} = \frac{0.693}{\text{in vitro } T_{1/2}} \cdot \frac{\text{ml incubation}}{\text{mg microsomes}} \cdot \frac{\text{45 mg microsomes}}{\text{gm liver}} \cdot \frac{\text{20 gm liver}}{\text{kg b.w.}}$$

For microsomal binding, the fraction unbound in the incubation mixture was calculated by:

$$f_{u(mic)} = \frac{\text{drug/ISTD peak height ratio in buffer sample}}{2 \cdot \text{drug/ISTD peak height ratio in microsome sample}}$$

with the factor of 2 in the denominator because the aliquot volume of buffer samples analyzed was twice that analyzed for the microsome samples (see above).

The overall accuracies of clearance prediction methods were determined by (Obach et al., 1997):

$$\text{average fold error} = 10^{\left|\frac{\Sigma \log\left(\frac{\text{predicted}}{\text{actual}}\right)}{N}\right|}$$

Literature values for i.v. clearance, plasma binding, and blood-to-plasma ratio for the 29 compounds are listed in Table 2. For those compounds in which renal excretion of unchanged drug represents a significant component of total clearance, clearance values were corrected to nonrenal clearance values by:

$$CL_{nonrenal} = CL_{total} \cdot (1 - \text{fraction of dose excreted unchanged in urine})$$

Results

The use of HPLC-atmospheric pressure ionization-MS was an important tool in the gathering of these metabolic lability and microsomal binding data. The selectivity and sensitivity of this instrumentation permitted facile quantitation of a wide variety of drug structures. Chromatographic methods were developed for each compound

TABLE 3

In vitro intrinsic clearance values and fraction unbound in the incubation conditions for 29 drugs examined

Each in vitro $T_{1/2}$ and microsomal binding value represents mean ± S.D. for triplicate determinations. Intrinsic clearance values were calculated from in vitro $T_{1/2}$ data as described in *Experimental Procedures*.

Drug	Microsomal Concentration	In Vitro $T_{1/2}$	CL'_{int}	$f_{u(mic)}$
	mg/ml	*min*	*ml/min/kg*	
Basic compounds				
Chlorpromazine	1.0	25 ± 6	25 ± 6	0.11 ± 0.02
Propafenone	0.5	8.0 ± 0.4	166 ± 8	0.26 ± 0.04
Verapamil	0.5	10 ± 0.2	122 ± 2	0.43 ± 0.10
Diphenhydramine	6.0	49 ± 24	2.1 ± 0.9	0.29 ± 0.02
Lorcainide	1.0	13 ± 2	50 ± 6	0.52 ± 0.03
Diltiazem	2.0	21 ± 3	15 ± 2	0.76 ± 0.10
Amitriptyline	0.5	92 ± 13	14 ± 2	0.15 ± 0.04
Desipramine	0.5	74 ± 24	17 ± 7	0.21 ± 0.01
Imipramine	0.5	66 ± 5	19 ± 2	0.18 ± 0.04
Ketamine	1.0	23 ± 3	27 ± 4	0.49 ± 0.02
Quinidine	5.0	37 ± 5	3.4 ± 0.5	0.32 ± 0.17
Clozapine	5.0	27 ± 5	4.6 ± 0.9	0.13 ± 0.01
Neutral compounds				
Dexamethasone	5.0	42 ± 3	3.0 ± 0.2	1.00 ± 0.07
Prednisone	5.0	47 ± 1	2.7 ± 0.0	0.20 ± 0.02
Diazepam	5.0	54 ± 19	2.3 ± 0.7	0.28 ± 0.05
Midazolam	1.0	3.9 ± 0.1	160 ± 3	0.88 ± 0.12
Methoxsalen	0.5	31 ± 3	40 ± 3	0.94 ± 0.11
Alprazolam	5.0	105 ± 66	1.6 ± 1.0	0.66 ± 0.04
Triazolam	1.0	33 ± 2	19 ± 1	0.78 ± 0.09
Zolpidem	5.0	44 ± 5	2.8 ± 0.3	0.58 ± 0.10
Acidic compounds				
Diclofenac	0.3	11 ± 3	189 ± 39	1.00 ± 0.13
Ibuprofen	2.0	36 ± 4	8.8 ± 0.9	0.84 ± 0.13
Tolbutamide	10	71 ± 12	0.90 ± 0.15	0.95 ± 0.03
Warfarin	10	>120	<0.52	0.47 ± 0.05
Tenidap	3.0	26 ± 2	8.3 ± 0.7	0.32 ± 0.01
Tenoxicam	10	38 ± 11	1.7 ± 0.4	0.78 ± 0.03
Amobarbital	10	66 ± 5	0.94 ± 0.07	0.76 ± 0.08
Hexobarbital	5.0	48 ± 6	2.3 ± 0.3	0.81 ± 0.05
Methohexital	1.0	13 ± 2	49 ± 8	0.86 ± 0.13

using the same column and only two types of mobile phases, with virtually the only customization required for each compound being determination of an optimal percentage of organic modifier (CH_3CN) to effect elution of drug and internal standard within a reasonable run time.

In vitro $T_{1/2}$ data in pooled human liver microsomes for the 29 compounds examined are listed in Table 3. Metabolic lability of this set of compounds spanned a wide range, the most stable compound being warfarin (in vitro $T_{1/2}$ was immeasurably long at a microsomal protein concentration of 10 mg/ml), and the most labile being diclofenac, propafenone, and midazolam (scaled CL'_{int} values of 160 ml/min/kg or greater). Within each general class of compounds (weak bases, weak acids, and neutral compounds), intrinsic clearance values spanned a broad range. Bases ranged from low intrinsic clearance values of 3.4 and 4.6 ml/min/kg for quinidine and clozapine, respectively, to high intrinsic clearance values of 122 and 166 ml/min/kg for verapamil and propafenone, respectively. Intrinsic clearance values for acids ranged from less than 0.52 ml/min/kg for warfarin and 0.90 and 0.94 ml/min/kg for tolbutamide and amobarbital, respectively, up to 189 ml/min/kg for diclofenac. Intrinsic clearance values for the neutral compounds ranged from 1.6 ml/min/kg for alprazolam to 160 ml/min/kg for midazolam.

The extent of microsomal binding was determined for each compound using a microsomal protein concentration equal to that used in the metabolic incubations (Table 3). Because different protein concentrations were used, the compounds cannot be rank ordered with regard to extent of binding to microsomes. The values ranged from no binding to approximately 90% bound. Furthermore, those compounds that exhibited the greatest extent of binding were not necessarily those in which the microsomal protein concentration was highest. In general, the weak bases demonstrated greater binding to microsomes, despite the fact that microsomal concentrations used for the bases were, on average, lower than those used for the neutral and acidic compounds.

A summary of human blood clearance predictions from the in vitro data is presented in Table 4 and predicted clearance values are plotted versus actual clearance values in Fig. 2. Equations for both the well-stirred and the parallel-tube models of hepatic extraction were applied under three variations: disregarding all binding values (Table 4, eqs. 1 and 4), including only blood binding (Table 4, eqs. 2 and 5), and including both blood and in vitro microsome binding (Table 4, eqs. 3 and 6). Overall accuracy values, determined as described in *Experimental Procedures*, are listed in Table 5. For all compounds examined ($n = 29$), average fold error values were just over 2-fold in the cases in which either no binding values were considered or all binding values were considered. The most accurate method was the use of the parallel-tube model with both blood and microsome binding incorporated (average fold error of 2.13). Using only the blood binding value in either model of hepatic extraction yielded very poor predictions of human clearance. When subsets of compounds were considered, some differences as to which were the most accurate methods were observed. For weak bases and neutral compounds, disregarding all binding in either model of hepatic extraction yielded the best agreement between actual human clearance values and those projected from in vitro intrinsic clearance data. However, for the acidic compounds, the most accurate clearance prediction methods

TABLE 4

Summary of human clearance values predicted from in vitro intrinsic clearance, protein binding, and microsome binding values using well-stirred and parallel-tube models of hepatic extraction

Drug	Actual Human CL_{blood}	Predicted Human CL_{blood}[a]					
		Well-Stirred			Parallel-Tube		
		No Binding (eq. 1)	$f_{u(bl)}$ Only (eq. 2)	$f_{u(bl)}$ and $f_{u(mic)}$ (eq. 3)	No binding (eq. 4)	$f_{u(bl)}$ Only (eq. 5)	$f_{u(bl)}$ and $f_{u(mic)}$ (eq. 6)
	mL/min/kg						
Basic compounds							
Chlorpromazine	11	11	1.5	8.6	15	1.5	11
Propafenone	19	19	6.5	13	21	7.6	17
Verapamil	19	18	9.0	13	21	11	17
Diphenhydramine	9.5	1.9	0.7	2.2	2.0	0.7	2.3
Lorcainide	18	15	6.7	9.9	19	7.8	12
Diltiazem	12	8.7	2.9	3.6	11	3.0	3.9
Amitriptyline	12	8.2	0.8	4.2	10	0.8	4.6
Desipramine	12	9.4	2.8	8.8	12	2.9	11
Imipramine	12	10	1.6	6.6	12	1.6	7.7
Ketamine	20	12	12	15	15	15	20
Quinidine	2.7	2.9	0.5	1.4	3.1	0.5	1.4
Clozapine	2.9	3.8	0.3	1.9	4.1	0.3	1.9
Neutral compounds							
Dexamethasone	3.8	2.6	1.0	1.0	2.8	1.0	1.0
Prednisone	4.9	2.4	0.8	3.4	2.5	0.8	3.6
Diazepam	0.6	2.1	0.04	0.2	2.2	0.04	0.2
Midazolam	8.7	19	8.8	9.4	21	11	12
Methoxsalen	18	14	4.3	4.5	18	4.7	5.0
Alprazolam	0.76	1.5	0.64	0.95	1.5	0.64	0.97
Triazolam	4.7	10	2.7	3.3	12	2.8	3.6
Zolpidem	5.7	2.5	0.3	0.5	2.6	0.3	0.5
Acidic compounds							
Diclofenac	7.6	19	1.6	1.6	21	1.6	1.6
Ibuprofen	1.5	6.2	0.2	0.2	7.2	0.2	0.2
Tolbutamide	0.36	0.86	0.07	0.07	0.88	0.07	0.07
Warfarin	0.08	0.46	0.01	0.02	0.46	0.01	0.02
Tenidap	0.10	5.9	0.01	0.03	6.8	0.01	0.03
Tenoxicam	0.03	1.6	0.02	0.03	1.6	0.02	0.03
Amobarbital	0.35	0.90	0.24	0.32	0.92	0.24	0.32
Hexobarbital	3.6	2.1	1.2	1.40	2.2	1.2	1.45
Methohexital	16	15	9.9	11	19	12	14

Equations used are as follows:

$$CL_{blood} = \frac{Q \cdot CL'_{int}}{Q + CL'_{int}} \quad (1)$$

$$CL_{blood} = \frac{Q \cdot f_{u(blood)} \cdot CL'_{int}}{Q + f_{u(blood)} \cdot CL'_{int}} \quad (2)$$

$$CL_{blood} = \frac{Q \cdot f_{u(blood)} \cdot \frac{CL'_{int}}{f_{u(mic)}}}{Q + f_{u(blood)} \cdot \frac{CL'_{int}}{f_{u(mic)}}} \quad (3)$$

$$CL_{blood} = Q \cdot (1 - e^{\left(\frac{-CL_{int}}{Q}\right)}) \quad (4)$$

$$CL_{blood} = Q \cdot (1 - e^{\left(\frac{-f_{u(blood)} \cdot CL_{int}}{Q}\right)}) \quad (5)$$

$$CL_{blood} = Q \cdot (1 - e^{\left(\frac{-f_{u(blood)} \cdot CL_{int}}{Q \cdot f_{u(mic)}}\right)}) \quad (6)$$

[a] A value of 21 ml/min/kg was used for human hepatic blood flow (Q).

incorporated both blood and microsome binding. Figure 3 contains plots of accuracy of predicted clearance values using the six equations versus the respective human clearance values.

Discussion

The prediction of human pharmacokinetic parameters for new chemical entities has become an important approach in the drug discovery process. For any given drug discovery approach, hundreds of compounds will satisfy potency objectives, however few can be examined in humans. New chemical entities require extensive investigation and investment of resources prior to administration to humans, and therefore it is desirable to be able to exclude compounds from this process that would be expected to exhibit unsatisfactory human pharmacokinetic properties. Recently, several investigators have described various methods and approaches whereby human pharmacokinetic parameters can be predicted from in vitro and/or preclinical pharmacokinetic data (Hoener, 1994; Gobburu and Shelver, 1995; Lave et al., 1997a,b; Obach et al.,

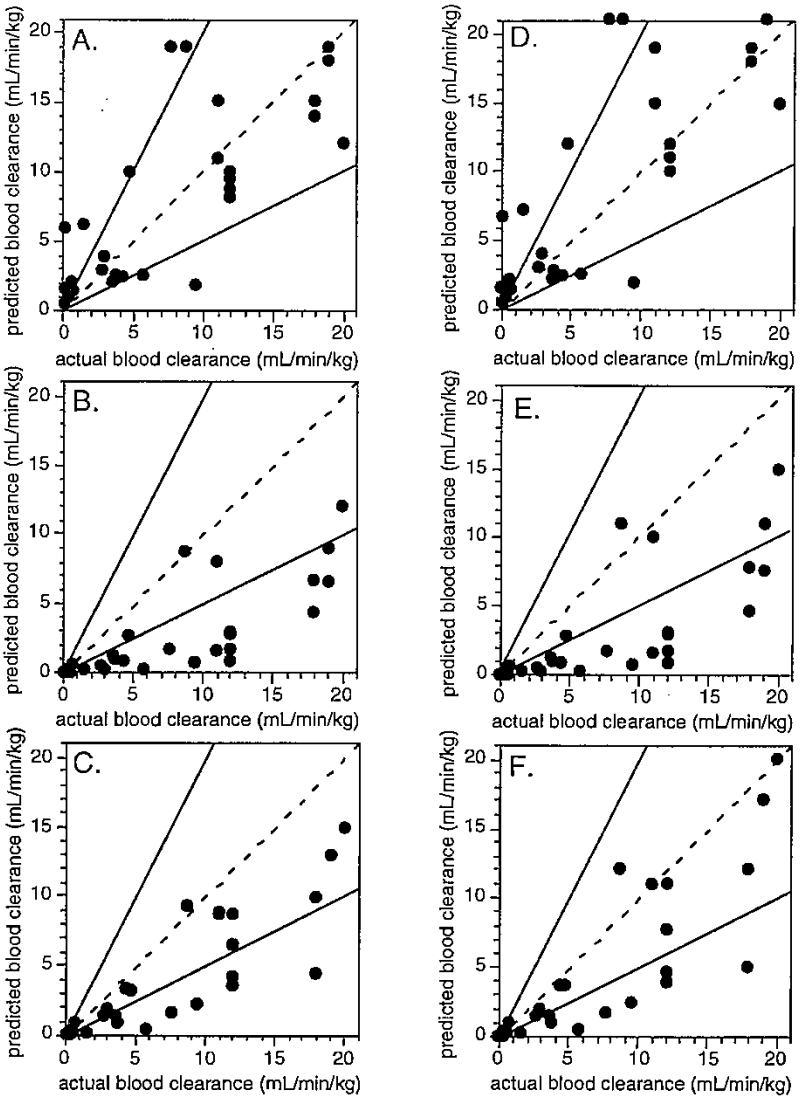

FIG. 2. *Plots of human clearance values predicted from in vitro intrinsic clearance data versus actual human clearance values.*

A, well-stirred model disregarding all binding parameters. B, well-stirred model incorporating only plasma protein binding. C, well-stirred model incorporating both plasma protein and microsome binding. D, parallel-tube model disregarding all binding parameters. E, parallel-tube model incorporating only plasma protein binding. F, parallel-tube model incorporating both plasma protein and microsome binding.

1997; Kuhnz and Gieschen, 1998; Sarver et al., 1997; Mahmood, 1998a,b). These methods vary in complexity and the amount of data required for accurate predictions.

One of the simplest methods described to predict human clearance is the use of human hepatic microsomal lability data, termed the in vitro $T_{1/2}$ approach (Obach et al., 1997). In this method, one incubates the test compound with human liver microsomes in the presence of appropriate cofactors (NADPH for CYP catalyzed reactions) and measures the first-order rate of consumption of the test compound. This rate is converted to an in vitro CL'_{int} value, scaled-up to reflect CL'_{int} in vivo, and inserted into a model of hepatic extraction. A high degree of success was previously reported for this particular approach, however the number of test compounds was low, and a majority of the compounds were of a similar physicochemical class (lipophilic amines). One of the objectives of the present experimentation was to further test the in vitro $T_{1/2}$ approach with more compounds and greater structural diversity.

The use of hepatic microsomes in the prediction of clearance requires acceptance of several assumptions and caveats: 1) metabolic clearance is the major mechanism of clearance (i.e., $CL_{metabolism} \gg CL_{renal} + CL_{biliary} + CL_{other}$); 2) the liver is the major organ of clearance (i.e., $CL_{hepatic} \gg \Sigma CL_{all\ other\ organs}$); 3) oxidative metabolism predominates over other metabolic routes such as direct conjugative metabolism, reduction, hydrolysis, etc.; 4) rates of metabolism and enzyme activities in vitro are truly reflective of those that exist in vivo. Additionally, a tenet of the well-stirred and parallel-tube models

TABLE 5

Accuracy of clearance predictions from liver microsomal in vitro $T_{1/2}$ values including and excluding values for blood binding and microsome binding

	Average Fold Error (predicted CL_{blood}/actual CL_{blood})			
	Basic compounds only (n = 12)	Neutral compounds only (n = 8)	Acidic compounds only (n = 9)	All compounds (n = 29)
Well-stirred model				
No binding considered (eq. 1)	1.37	1.99	5.05	2.28
Including $f_{u(blood)}$ (eq. 2)	5.17	3.90	3.91	4.39
Including $f_{u(blood)}$ and $f_{u(mic)}$ (eq. 3)	1.86	2.55	2.83	2.31
Parallel-tube model				
No binding considered (eq. 4)	1.31	1.99	5.35	2.28
Including $f_{u(blood)}$ (eq. 5)	4.75	3.90	3.79	4.20
Including $f_{u(blood)}$ and $f_{u(mic)}$ (eq. 6)	1.60	2.53	2.74	2.14

of hepatic extraction is that the unbound concentration of drug in the plasma is equal to the unbound concentration in the hepatocyte. Therefore, facilitated transport processes that could possibly be responsible for drug uptake or drug extrusion from hepatocytes are not accounted for in these models. The in vitro $T_{1/2}$ approach has two additional inherent assumptions that are not required if intrinsic clearance were determined using the more rigorous approach of calculating intrinsic clearance from enzyme kinetic data (i.e., V_{max}/K_M). These are: 1) the substrate concentration employed (1.0 μM in the case of this report) is well below the apparent K_M for substrate turnover, and 2) there is no significant product inhibition, nor is there any mechanism based inactivation of enzyme. Overall, clearance for these 29 compounds was generally underestimated using any of the six approaches, giving credence to the notion that the aforementioned assumptions are not completely valid in many cases. It should be noted that the 29 compounds chosen for this examination represent a set for which other clearance mechanisms (e.g., renal clearance, nonoxidative clearance, etc.) are known to be less important than hepatic oxidative metabolic clearance, but some are known to fall outside the scope of the aforementioned assumptions, e.g., nonhepatic metabolism of dexamethasone (Diederich et al., 1996; Tomlinson et al., 1997), nonoxidative components of metabolic clearance such as the reductive metabolism of warfarin (Moreland and Hewick, 1975) or glucuronidation of ibuprofen (Rudy et al., 1991), and product inhibition of diltiazem (Sutton et al., 1997).

A second objective of this work was to further explore the potential importance of nonspecific reversible binding of substrate in microsomal incubations in the prediction of human clearance from in vitro intrinsic clearance data. Earlier work suggested that this phenomenon was important in the projection of clearance of imipramine and propranolol from hepatic microsomal CL'_{int} (Obach, 1997). The present experimentation sought to examine this possibility for a larger number of compounds of wider physicochemical properties.

Two models of hepatic clearance were examined (well-stirred and parallel-tube models) under three different variations each: 1) assuming that no binding parameter has impact on clearance (Table 4, eqs. 1 and 4); 2) incorporating binding to blood constituents only (Table 4, eqs. 2 and 5); and 3) incorporating both blood and microsome binding (Table 4, eqs. 3 and 6). When considering all 29 compounds, either disregarding all binding or including both blood and microsome binding yielded the most accurate projections of human clearance (Table 5). However, when the three different classes of compounds were examined separately, it appeared that disregarding all binding yields the most accurate projections of human clearance for basic and neutral compounds but not for the acidic compounds. For the acidic compounds, human clearance projections were most accurate when including all binding parameters.

For the basic compounds, disregarding any potential impact of blood and microsome binding yielded accurate projections of human clearance. However, when the free-fraction in blood was accounted for, clearance of many of the basic compounds, especially those highly bound to blood proteins, were markedly underpredicted (e.g., chlorpromazine, amitriptyline, desipramine, imipramine, diltiazem, quinidine, and clozapine). Many of these compounds were substantially bound to microsomes; thus incorporation of both blood and in vitro unbound fractions again yielded more accurate projections of human clearance. However, clearance for some of the basic compounds were still underprojected (e.g., amitriptyline and diltizem). Diphenhydramine was substantially underprojected in all cases; the reason for this is not known. Also, an interesting case was that of ketamine, in which the extent of binding to microsomes was greater than that to blood proteins.

For neutral compounds, disregarding all binding in the projection of clearance from in vitro intrinsic clearance yielded some overprojections (e.g., diazepam, midazolam, and triazolam), however disregarding all binding yielded the greatest degree of accuracy. As with the basic compounds, inclusion of blood protein binding led to underprojections of clearance (e.g., steroids, diazepam, methoxsalen, and zolpidem), whereas including both blood and microsome binding yielded some improvements in clearance projections (e.g., triazolam, diazepam, and prednisone).

Disregarding all binding yielded marked overprojections of human clearance for many of the acidic drugs (e.g., diclofenac, ibuprofen, tenidap, tenoxicam, and warfarin), whereas including only the unbound fraction in blood yielded underprojections (e.g., diclofenac, warfarin, tenidap, and ibuprofen). Binding to microsomes was not substantial in the cases of many of the acidic drugs, so that improvements in the projection of clearance that were observed when including both blood and microsome binding for basic and neutral compounds, were not observed for all of the acidic drugs (e.g., diclofenac, tolbutamide, and ibuprofen). However, some of the acidic drugs demonstrated significant microsome binding and clearance projections were improved with the inclusion of this parameter (e.g., tenoxicam, tenidap, and amobarbital).

It should be recognized that the phenomenon of nonspecific binding to microsomes in in vitro metabolic incubations is not necessary reflective of the in vivo situation. The inclusion of the unbound fraction of drug in the in vitro incubation matrix is necessary so that the in vitro and in vivo situations can be directly reconciled via a common parameter: unbound intrinsic clearance. Drug that is sequestered in microsomes in vitro is presumed to be unavailable for direct interaction with metabolizing enzymes, just as drug that is bound to plasma proteins and tissue macromolecules in vivo is presumed to be unable to be directly acted on by drug metabolizing enzymes. In both cases, drug molecules must first dissociate from the nonspecific

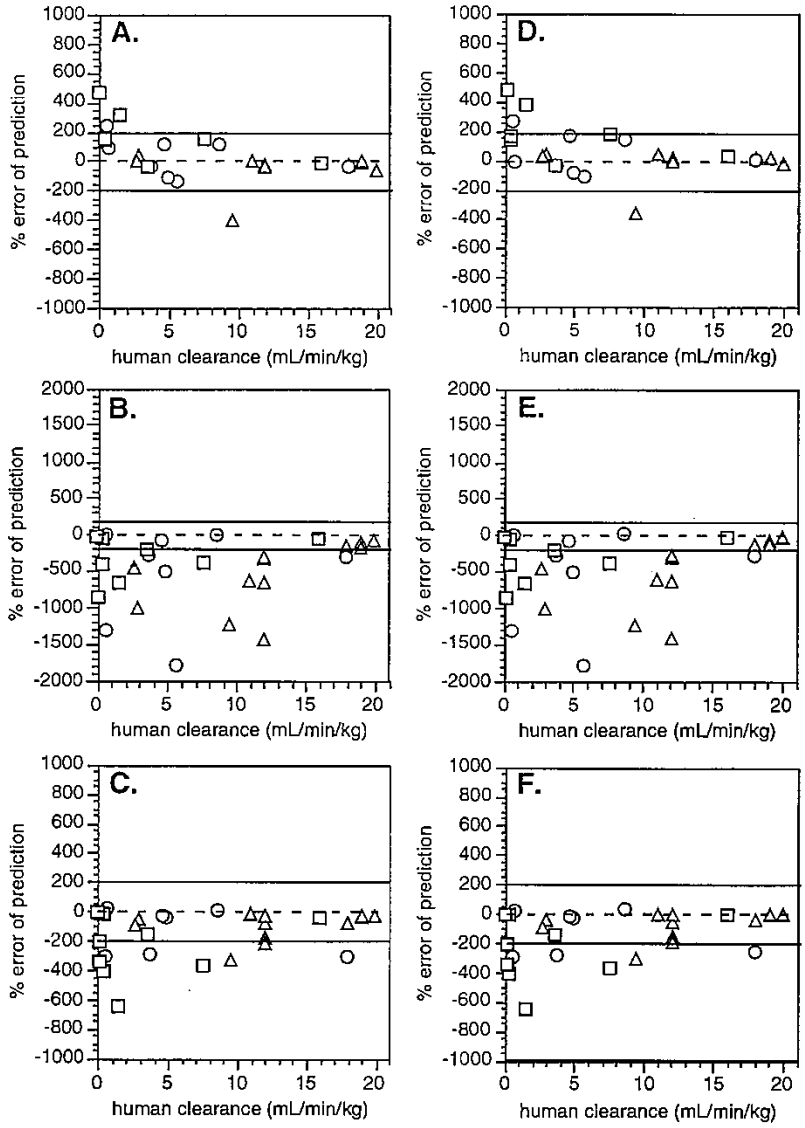

FIG. 3. *Accuracy of human clearance values predicted from in vitro intrinsic clearance data versus actual human clearance values.*

A, well-stirred model disregarding all binding parameters. B, well-stirred model incorporating only plasma protein binding. C, well-stirred model incorporating both plasma protein and microsome binding. D, parallel-tube model disregarding all binding parameters. E, parallel-tube model incorporating only plasma protein binding. F, parallel-tube model incorporating both plasma protein and microsome binding. Symbols are as follows: basic compounds (△); neutral compounds (○); acidic compounds (□). Lines signify 2-fold errors between predicted and actual clearance values. Note the different scales for B and E. Errors for tenidap and tenoxicam in A and D are well off scale (5000–6000% error) and are not depicted.

binding sites before they can bind to, and be metabolized by, drug metabolizing enzymes.

In summary, these data support two conclusions. First, the liver microsomal in vitro $T_{1/2}$ approach can be a suitable approach to measure in vitro CL'_{int}, which can be scaled up to the in vivo situation and used in the prediction of human clearance. This is provided that several caveats, outlined above, are taken into consideration. Second, the measurement of nonspecific binding to microsomes under conditions used in the measurement of in vitro $T_{1/2}$, and inclusion of these unbound fraction values in the prediction of clearance from in vitro intrinsic clearance data appears to be a more broadly applicable approach than either disregarding all binding or including only blood binding parameters. However, it may be the case for some classes of compounds (especially basic compounds), that disregarding all binding values yields more accurate predictions of human clearance.

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Synthesis of Novel Potent Dipeptidyl Peptidase IV Inhibitors with Enhanced Chemical Stability: Interplay between the N-Terminal Amino Acid Alkyl Side Chain and the Cyclopropyl Group of α-Aminoacyl-L-*cis*-4,5-methanoprolinenitrile-Based Inhibitors

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A series of methanoprolinenitrile-containing dipeptide mimetics were synthesized and assayed as inhibitors of the N-terminal sequence-specific serine protease dipeptidyl peptidase IV (DPP-IV). The catalytic action of DPP-IV is the principle means of degradation of glucagon-like peptide-1, a key mediator of glucose-stimulated insulin secretion, and DPP-IV inhibition shows clinical benefit as a novel mechanism for treatment of type 2 diabetes. However, many of the reversible inhibitors to date suffer from chemical instability stemming from an amine to nitrile intramolecular cyclization. Installation of a cyclopropyl moiety at either the 3,4- or 4,5-position of traditional 2-cyanopyrrolidide proline mimetics led to compounds with potent inhibitory activity against the enzyme. Additionally, *cis*-4,5-methanoprolinenitriles with β-branching in the N-terminal amino acid provided enhanced chemical stability and high inhibitory potency. This class of inhibitors also exhibited the ability to suppress prandial glucose elevations after an oral glucose challenge in male Zucker rats.

Introduction

With the spread of Western lifestyles, the prevalence of type 2 diabetes in the world's population is rising.[1] Current treatment strategies include reducing insulin resistance using glitazones,[2] supplementing insulin supplies with exogenous insulin,[3] increasing insulin secretion with sulfonylureas,[4] reducing hepatic glucose output with biguanides,[5] and limiting glucose absorption with glucosidase inhibitors.[6] Promising new targets for drug development are also emerging. Of particular interest is the pharmacology surrounding the incretin hormone glucagon-like peptide 1 (GLP-1).[7] GLP-1 is known to function as a mediator of glucose-stimulated insulin secretion, and several clinical studies have shown that administration of the peptide or its analogues results in antidiabetic action in subjects with type 2 diabetes.[8] Although GLP-1 is secreted as GLP-1 (7−36) amide from the small and large intestines in response to dietary signals, it is rapidly truncated to GLP-1 (9−36) by cleavage of the N-terminal dipeptide residues. The truncated metabolite has antagonist activity against the GLP-1 receptor both in vitro and in vivo.[9] The principle enzyme responsible for the cleavage of GLP-1 (7−36) amide to GLP-1 (9−36) amide is dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5), a nonclassical, sequence-specific serine protease that catalyzes the cleavage of dipeptides from the N-terminus of proteins with the sequence H-X-Pro-Y or H-X-Ala-Y (where X, Y = any amino acid; Y ≠ Pro).[10] Inhibition of DPP-IV has been shown to be effective at sustaining circulating levels of GLP-1 (7−36) and therefore offers a new therapeutic approach for the treatment of type 2 diabetes.[11]

Early reports of DPP-IV inhibitors included proline-based dipeptide mimics bearing boronic acid[12] (1) or diphenyl phosphonate substituents (2).[13] These compounds were irreversible inhibitors of DPP-IV or were slow to dissociate from the enzyme. Several first-generation dipeptide surrogates have been disclosed as reversible inhibitors of DPP-IV, including both C-substituted (3) and N-substituted (4) glycinylprolinenitrile dipeptide analogues.[14,15] These compound classes include many potent inhibitors of the enzyme, but all suffer from chemical instability whereby the N-terminal amine intramolecularly cyclizes onto the nitrile, forming inactive cyclic imidates and/or their diketopiperazine hydrolysis products. However, more recent publications have disclosed a series of more hindered *N*-alkylamines (5) that have much greater chemical stability.[16] Thia-

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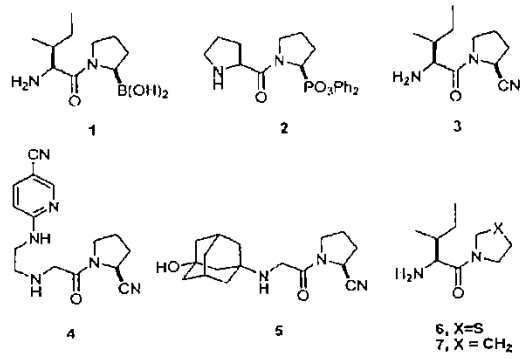


Figure 1. Known proline-derived dipeptidyl peptidase IV inhibitors.

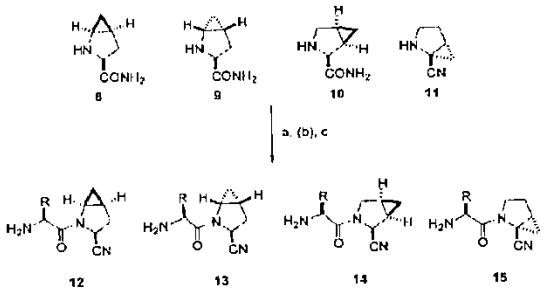
zolidide (**6**) and pyrrolidide (**7**) based inhibitors have also been disclosed that lack the nitrile moiety and thus possess greater stability, though at the cost of reduced potency[17d–f] with a few very recent notable exceptions.[17d–f] These compounds are shown in Figure 1.

We endeavored to synthesize novel dipeptide surrogates containing a proline mimic linked to an N-terminal amino acid that would act as reversible inhibitors of DPP-IV with maximum potency and enhanced solution stability. Hanessian has reported that installation of a 4,5-methano moiety into a proline residue has the effect of flattening the five-membered ring,[18] and one might extend a similar conformational argument to other cyclopropanated prolines as well. By inference, the cyclopropane bridge may occupy the space-filling region that would normally be given to the methylene group in the puckered or "envelope" conformation of a typical five-member ring. With this precedent we sought to establish whether a methanoproline derivative could serve as a viable proline surrogate in a DPP-IV inhibitor. Our strategy was to prepare dipeptide surrogates containing a cyclopropanated prolinenitrile derived from L-proline. The regio- and stereochemical disposition of the cyclopropane bridge was varied in order to identify a compound with maximal stability and potency. Herein, we report the discovery of L-*cis*-4,5-methanoprolinenitrile dipeptides as potent inhibitors of DPP-IV with increased chemical stability and high potency. In addition, we also present data demonstrating that these novel DPP-IV inhibitors effectively lower plasma glucose after a glucose challenge in rodent models.

Chemistry

Dipeptides (**12**–**15**) composed of N-terminal isoleucine appended to cyclopropylprolineamides or nitriles derived from L-proline (**8**–**11**) were targeted to probe the potential for this type of inhibitor scaffold. Isoleucine was selected as the N-terminal residue because it was the most potent natural amino acid reported in the 2-cyanopyrrolidide series.[14a] These inhibitors were expected to provide a dependable inhibitory benchmark for the differing methanoproline structures. The synthetic routes used to prepare the dipeptides derived from L-*cis*- and L-*trans*-4,5-methanoproline,[18b] L-*cis*-3,4-methanoproline,[19] and L-2,3-methanoproline[20] are shown in Scheme 1.

Scheme 1[a]



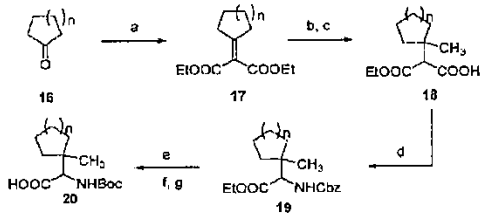
[a] (a) *N*-Boc-amino acid, EDAC, DMAP or PyBop, 50–90%; (b) (for intermediates **8**–**10**) $POCl_3$, pyridine, imidazole, 70–90%; (c) TFA, CH_2Cl_2, TFA or HCl in Et_2O or EtOAc, 70–90%.

Standard conditions were employed to couple the enantiomerically pure L-*cis*-4,5-cyclopropylprolineamide nucleus (**8**) to give the corresponding Boc-protected dipeptides in good to excellent yield.[21] Dehydration[14b,22] ($POCl_3$, pyridine, imidazole) of the amide and removal[23] of the N-terminal Boc protecting group (TFA or HCl) gave the proline dipeptide (**12**) in high yield. Similar protocols were used for the L-*cis*-3,4-methanoproline fragment **10** and the L-4,5-*trans*-methanoproline compound **9**. The known (−)-(2*S*,3*R*)-methanoprolinenitrile (**11**) was prepared according to the method of Hercout.[20a] In this instance, the dehydration reaction was performed prior to introduction of the isoleucine fragment.

Preparation of a library was undertaken to probe structure–activity relationships between the N-terminal amino acid residue and the *cis*-4,5-methanoprolinenitriles. These inhibitors were generated in a three-step sequence in parallel array format in a manner similar to that described in Scheme 1. Initial reaction of the Boc-protected amino acid with methanoprolineamide, EDAC, and DMAP in dichloromethane, and subsequent purification through an SCX ion exchange cartridge, gave good to excellent yields of coupled dipeptides. Dehydration and acid-promoted deprotection (TFA in dichloromethane) yielded the inhibitors as TFA salts. Further purification of the final products was easily accomplished by preparative reverse-phase HPLC or by trituration with Et_2O.

Holmberg has prepared *tert*-alkylmalonic acid derivatives through a $TiCl_4$-mediated Knoevenagel process and subsequent copper-assisted conjugate addition of a Grignard reagent or conjugate reduction. Following this method (Scheme 2), diesters can be converted to the protected amino acids in three steps.[24] This methodology provided an approach to the cycloalkylglycine amino acid derivatives **20**. First, the malonic acid diesters **17** were subjected to conjugate addition of a methyl group or hydride and then hydrolyzed to the corresponding monoacids **18**. Subsequent Curtius rearrangement of **18** by treatment with diphenylphosphoryl azide, followed by trapping of the intermediate isocyanate with benzyl alcohol, provided the Cbz adducts **19**. Finally, the esters were hydrolyzed to give the amino acids **20** in racemic form. The racemic Boc-protected acids **20** were then coupled to the L-*cis*-4,5-methanoprolineamide group.

Scheme 2[a]



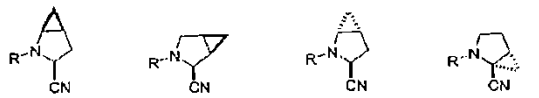
[a] (a) $TiCl_4$, THF, CCl_4, diethyl malonate, 0 °C, then pyridine, 0 °C to room temp, 68%; (b) MeMgI, CuCl, Et_2O, 0 °C, 69%; (c) NaOH, THF, EtOH, 78%; (d) (i) diphenylphosphorylazide, NEt_3, PhH, reflux; (ii) BnOH, reflux, 18 h, 100%; (e) H_2, $Pd(OH)_2$, EtOAc, 100%; (f) $(Boc)_2O$, K_2CO_3, THF, H_2O, 92%, two steps; (g) NaOH, MeOH, THF, room temp, then aqueous HCl, 95%.

Processing as previously described (Scheme 1) gave a mixture of diastereomers that were separated at the nitrile stage by silica gel flash chromatography. The later-eluting isomer in each case was consistently identified as the desired L,L-dipeptide.[25]

Results and Discussion

All compounds were tested in vitro against purified porcine DPP-IV. Inhibition was determined against the substrate H-Ala-Pro-*p*NA. Production of *p*-nitroaniline (*p*NA) was measured at 405 nm over 15 min. A comparison of methanoproline-derived isoleucine N-terminal dipeptides shows that the enzyme inhibitory activity is critically dependent on both the position and the orientation of the methano bridge (Table 1). The presence of the methano bridge on the trans side of the prolinenitrile (e.g., **22**, **23**) resulted in a significant loss of activity relative to the unsubstituted prolinenitrile **21**. This result was consistent with early reports where insertion of a methyl group at the 2-postion of 2-cyanopyrrolidides resulted in a 2000-fold decrease in activity.[29] In contrast, when the methano bridge was oriented cis to the nitrile at either the 4,5- or 3,4-position, inhibitory activity was only slightly diminished. This is clearly illustrated with *cis*-4,5-methano inhibitors **24** and **26** and *cis*-3,4-methano inhibitors **25** and **27**, where inhibitory potency resides in the 20 nM range. A more complete exploration of N-terminal amino acids reveals that increasing the degree of β-branching to that of *tert*-Leu (e.g., **29**) further increases potency in the 4,5-methano series to that of the prolinenitrile version (**28**). Replacement of these alkyl substituents with aromatic residues such as Phe (**31**) or Trp (**32**) in the N-terminal position significantly eroded potency. Alkyl substitution on the terminal amine was generally ill-tolerated in this series (e.g., **33**–**35**), though it would appear that the relatively more accessible N-terminus found in proline derivative **34** is capable of restoring a modest degree of inhibitory activity. Replacement of Leu and *tert*-Leu side chains with medium-ring cycloalkyl and methylcycloalkyl (**37**–**42**) generally led to inhibitors with comparable or slightly improved activity, though potency dropped steadily with increasing larger ring size (data not shown). The Cp-Gly and Me-Cp-Gly derived analogues **38** and **41** were among the most potent compound prepared in this series, exhibiting K_i values of 4 and 7 nM, respectively.

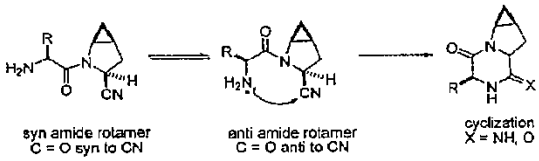
Table 1. In Vitro Inhibition Constants for Porcine DPP-IV and Solution Stability Half-Lives for Prolinenitrile DPP-4 Inhibitors



compd	N-terminal amino acid[a]	substituted prolinenitrile	K_i (nM)[b]	stability, $t_{1/2}$ (h)[c]
21	Ile	prolinenitrile	2 ± 0.5	5
22	Ile	*trans*-4,5-	1620 ± 80	
23	Ile	*trans*-2,3-	7500 ± 200	
24	Ile	*cis*-4,5-	25 ± 1	22
25	Ile	*cis*-3,4-	15 ± 1	4
26	Val	*cis*-4,5-	29 ± 1	28
27	Val	*cis*-3,4-	12 ± 1	2
28	*tert*-Leu	prolinenitrile	8 ± 0.5	27
29	*tert*-Leu	*cis*-4,5-	7 ± 0.5	42
30	*tert*-Leu	*cis*-3,4-	14 ± 1	4
31	Phe	*cis*-4,5-	65 ⊥ 3	
32	Trp	*cis*-4,5-	230 ± 10	
33	*N*-Me-Val	*cis*-4,5-	1940 ± 80	
34	Pro	*cis*-4,5-	107 ± 5	
35	Pip	*cis*-4,5-	10000	
36	Met	*cis*-4,5-	135 ± 10	
37	Cb-Gly	*cis*-4,5-	12 ± 0.5	
38	Cp-Gly	*cis*-4,5-	4 ± 0.5	
39	Ch-Gly	*cis*-4,5-	15 ± 1	19
40	(1-Me-Cb-1-yl)-Gly	*cis*-4,5-	11 ± 0.5	
41	(1-Me-Cp-1-yl)-Gly	*cis*-4,5-	7 ± 0.5	24
42	(1-Me-Ch-1-yl)-Gly	*cis*-4,5-	8 ± 0.5	

[a] Cb = cyclobutyl; Cp = cyclopentyl; Ch = cyclohexyl. [b] Values represent the mean ± SEM and are at least triplicate determinations. [c] Solution stability data are measured at 39.5 °C and pH 7.2 in phosphate buffer.

Scheme 3



Solution Stability. After the discovery that the L-*cis*-4,5- and L-*cis*-3,4-methanoprolinenitriles were potent inhibitors of DPP-IV in vitro, a comparative study was initiated to investigate aqueous solution stability of these analogues. The N-terminal valine, isoleucine, and *tert*-leucine dipeptide nitriles were selected in order to make direct comparisons between methanoprolinenitrile and unsubstituted prolinenitrile compounds with respect to solution stability. Reaction rates were monitored by following the disappearance of starting material on reverse-phase HPLC at pH 7.2 and 39.5 °C in phosphate buffer. HPLC mass spectral analysis revealed that the two major products that formed during the stability experiments had either an identical mass or an M + 1 mass to the parent starting material. These data are consistent with the formation of intramolecular cyclization products, with the initial cyclic imidate (X = NH) surrendering to the diketopiperazine (X = O) upon hydrolysis (Scheme 3).

Two interesting aspects of the solution half-life data should be noted. The first is that there is increased solution stability associated with the *cis*-4,5-methanoprolinenitrile derivatives when compared to either the

cis-3,4-methanoprolinenitrile or the unadorned prolinenitrile compounds. For instance, in the case where the N-terminal amino acid is isoleucine, the isomeric 4,5-methano-substituted compound **24** has a solution half-life of 22 h, which is 5.5-fold longer than the analogous 3,4-methano-substituted compound **25** and approximately 4.5-fold longer than the unadorned prolinenitrile analogue **21**. The second feature is that there is a strong correlation between steric size at the β-position of the N-terminal amino acid and relative solution stability. Increasing the degree of branching at the β-position of the alkylglycine substituent increases solution stability. The most stable compounds within their respective prolinenitrile series have the *tert*-Leu N-terminal amino acid fragment in common. For example, unadorned prolinenitrile **28** ($t_{1/2}$ = 27 h) is more stable than its less branched isomer **21** ($t_{1/2}$ = 5 h). Similarly, *cis*-4,5-methanoprolinenitrile **29** ($t_{1/2}$ = 42 h) is more stable than the less branched dipeptidenitriles **24** and **39** ($t_{1/2}$ = 22 and 19 h, respectively), though the magnitude of the effect of increased β-branching in this series appears to be blunted by the inherent baseline stability imparted by the methano bridge. This observation is in agreement with data reported by Coutes[12a] and Snow[27] where the rates of cyclization for Xaa-boroproline dipeptides were shown to be Gly-BoroPro > Ala-BoroPro > Val-BoroPro.

Computational Analysis. Computational analysis was undertaken to more fully understand the relative stabilities of the methanoprolinenitriles. Ground-state conformations were generated for methanoprolinenitrile and prolinenitrile forms of the N-terminal *tert*-leucine dipeptide compounds. The calculated ground-state structure for the *tert*-leucine dipeptidenitrile is identical to the conformation observed through single-crystal X-ray structural analysis[28] of the TFA salt of **29** (rms = 0.1 Å for calculated and observed heavy atoms; see Figure 2, lower structure). Both have the same syn conformation around the amide bond, characterized by a small C(2)−N−C(8)=O torsional angle (−5° and +2° for the TFA structure). It is of additional interest that a similar conformation has been observed in several recently disclosed cocrystal structures of DPP-IV/inhibitor complexes.[29]

In addition to the syn conformation, there is a calculated local low-energy minimum where the reactive amine and nitrile are close to each other; in this anti conformation (Figure 2, upper structure), the C(2)−N−C(8)=O torsional angle is 180°. Moreover, the angle between the amine N and the C≡N group is 109° ± 1° and the distance between the these reactive partners is 2.95 Å. It is therefore reasonable to assume that the observed intramolecular cyclization is initiated from this conformation. The value of 109° between the amine group and the nitrile is in close agreement with the hypothetical angle of attack of at least 108° reported by Baxter and Connor.[30] It was envisioned that the relative energetic differences between the global minimum and the reactive local minimum would represent a means to evaluate the relative stabilities of compounds in solution.

The calculated conformations and their relative energies can be used to examine the basis for two aspects of the experimental compound stability: the increase in

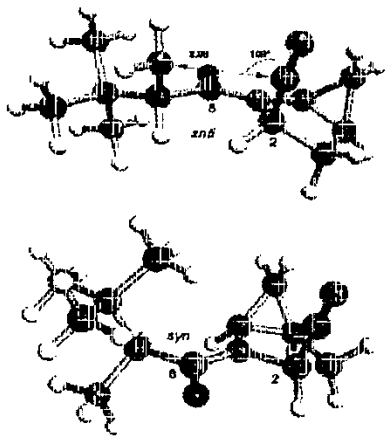


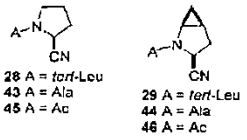
Figure 2. The upper structure depicts the local low-energy minimum for **29** where the reactive amine is close to the nitrile (anti conformation). The lower structure is the solid-state conformation observed in the X-ray crystallographic structure of the TFA salt of compound **29** (syn conformation). Two independent cations were observed in the crystal structure, though because these have identical conformations, only one is shown for purposes of clarity. Carbon atoms C(2) and C(8) are labeled.

stability due to side chain bulk (specifically β-branching) and the increase in stability upon conversion from prolinenitrile to *cis*-4,5-methanoprolinenitrile. Calculated relative energies between the ground-state conformation and the local low-energy minimum conformation that brings the reactive amine and nitrile in proximity are presented in Table 2 for the methanoprolinenitrile and prolinenitrile forms of N-terminal *tert*-leucine and alanine dipeptidenitriles, as well as for the same prolinenitriles with a simple acetamide cap.

Conformational Stability Due to Side Chain Bulk. The values in the first column of Table 2 compare the conformational energy differences between the ground state and the geometry where internal cyclization could occur for the unsubstituted prolinenitrile series. The ab initio (G98) results are expected to be considerably more accurate than the force field values and indicate that the energy required to assume the anti conformation increases with side chain bulk (e.g., 0.3, 1.9, 2.8 kcal/mol for no side chain, the alanine side chain, and the *tert*-leucine side chain, respectively). The force field energy results agree qualitatively with the ab initio values and suggest that the primary contribution is due to van der Waals interactions. Examination of the structures reveals extremely close contacts between two of the side chain methyl groups and the carbonyl oxygen in the anti conformation (approximately 3 Å each) that would increase the energy barrier for internal cyclization and thus lead to greater stability. A similar trend is observed for increasing side chain bulk in the methano-substituted series (column 2), and these values are in good agreement with the solution data where increased β-branching enhances stability.

Conformational Stability Due to Methano Substitution. As shown in the third column of Table 2, the ab initio (G98) energy calculations indicate that the

Table 2. Calculated Energy[a] Differences between the Calculated Ground State and the Local Low-Energy Minimum Required for Cyclization



28 (Prolinenitrile) and 29 (Methanoprolinenitrile) (A = *tert*-Leu)			
method interaction	ΔH(28) [b]	ΔH(29) [b]	$\Delta\Delta H$(29 − 28)
G98 ab initio	2.8	3.4	0.6
Insight CFF force field			
total nonbond	3.5	4.2	0.7
van der Waals	3.3	3.9	0.6
electrostatic	0.2	0.3	0.1

43 (Prolinenitrile) and 44 (Methanoprolinenitrile) (A = Ala)			
method interaction	ΔH(43) [b]	ΔH(44) [b]	$\Delta\Delta H$ (44 − 43)
G98 ab initio	1.9	2.6	0.6
Insight CFF force field			
total nonbond	3.3	3.9	0.6
van der Waals	2.9	3.4	0.6
electrostatic	0.4	0.4	0.0

45 (Prolinenitrile) and 46 (Methanoprolinenitrile) (A = Ac)			
method interaction	ΔH(45) [b]	ΔH(46) [b]	$\Delta\Delta H$ (46 − 45)
G98 ab initio	0.3	0.9	0.6
Insight CFF force field			
total nonbond	−0.2	0.0	0.2
van der Waals	−0.4	−0.2	0.2
electrostatic	0.2	0.3	0.1

[a] Energies are for the *cis*-4,5-methanoprolinenitrile and prolinenitrile versions of the compounds and are given in kcal/mol. [b] Energies are for the anti conformation (Scheme 3 and Figure 2 upper structure) relative to the global minimum conformation, which corresponds to the syn geometry in Scheme 3 and Figure 2 lower structure.

addition of the methano bridge increases the energy barrier toward adopting the conformation required for cyclization by approximately 0.6 kcal/mol for the N-terminal *tert*-leucine dipeptide compound as well as for the alanine dipeptide and acetamide compounds. The lack of dependence on the nature of the side chain suggests that the observed increase in stability of the *cis*-4,5-methano compounds is due to a local interaction between the methano bridge and the N-terminal amino acid that favors the ground state relative to the anti conformation where cyclization is initiated. The force field energy analysis (Insight CFF) results also agree qualitatively, implying that van der Waals interactions are primarily responsible for the increase in energy. These conclusions are supported by the cyclization rate differences between the isomeric isoleucine derivatives **24** ($t_{1/2}$ = 22 h) and **21** ($t_{1/2}$ = 5 h), where a 4.5-fold increase in stability is observed for the methanoprolinenitrile. This increase in stability is in fair agreement with the calculated value of 0.6 kcal/mol. The calculated $\Delta\Delta H$ and observed solution half-life results support contributions to stability from both β-branching and the presence of the 4,5-methano bridge on the pyrrolidine ring.

In Vivo Activity. In vivo evaluation of DPP-IV inhibitors has supported the connection between DPP-IV inhibition, increases in plasma insulin levels, and an improvement in glucose tolerance.[31] Compounds **29** and **41** were potent inhibitors of DPP-IV in vitro and demonstrated excellent solution stability. As such, these inhibitors were selected to determine the effects of DPP-IV inhibition in vivo on glucose tolerance and insulin secretion in Zucker$^{fa/fa}$ rats. An oral glucose tolerance test (oGTT) in the Zucker$^{fa/fa}$ rat is a frequently used model of hyperglycemia in type 2 diabetes and obesity research. Zucker$^{fa/fa}$ rats are severely hyperphagic, extremely obese, markedly insulin-resistant, and mildly hyperglycemic because of a mutation and loss of function of the leptin receptor gene.[32,33] Fasted male Zucker$^{fa/fa}$ rats were dosed orally with water or with inhibitors **29** and **41** (3 μmol/kg), and an oGTT was conducted 0.5 h after the dosing. Plasma DPP-IV activity, glucose, and insulin levels were then monitored over a 2 h period. Figures 3−5 show the ex vivo plasma DPP-IV activity, insulin response, and glucose excursion curves in response to an oral glucose challenge (2 g/kg). Animals in the control group reached peak plasma glucose levels 60 min after glucose administration, at which point the drug-treated animals exhibited a 30−35% decrease in glucose levels compared to controls (control animals, 356 mg/dL; compound **29** treated animals, 226 mg/dL; compound **41** treated animals, 245 mg/dL). Glucose levels were significantly reduced in the drug-treated animals from 30 min onward, with maximal reductions in glucose observed at 90 min (−34% to −38%). Plasma DPP-IV activity was maximally suppressed (60%) 30 min after dosing (Figure 3), and the effects of these inhibitors were sustained throughout the course of the experiment (60−35%). The insulin response to oral glucose was also enhanced by treatment with DPP-IV inhibitors (Figure 4), demonstrating the link between the glucose-lowering effects and DPP-IV inhibition of these compounds.

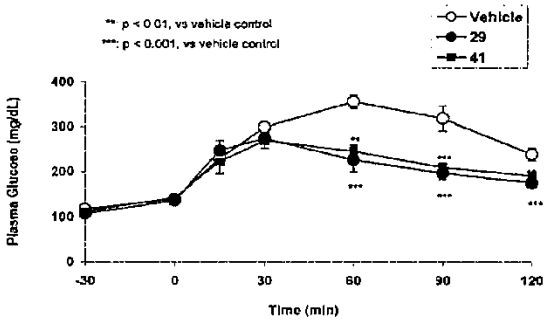


Figure 3. Effects of inhibitors **29** (●) and **41** (■) dosed at 3 μmol/kg po versus vehicle control (○) on plasma DPP-IV activity in Zucker$^{fa/fa}$ rats.

Interestingly, a significant decrease in plasma glucose levels occurred when DPP-IV activity in plasma was inhibited only by 35−60%. This finding suggests that it may not be necessary to completely suppress plasma DPP-IV activity in order to achieve antihyperglycemic efficacy in type 2 diabetics. However, differences may exist in the inhibition of the turnover of native substrates such as GLP-1 compared with that of the pseudosubstrate used in this assay, and potential assay

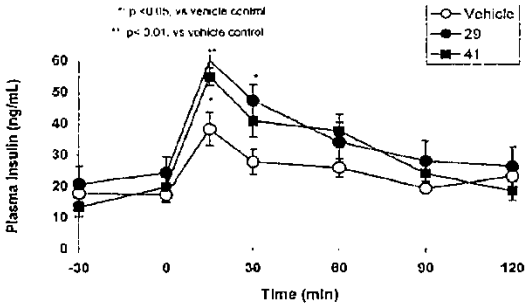


Figure 4. Effects of inhibitors **29** (●) and **41** (■) dosed at 3 μmol/kg po versus vehicle control (○) on plasma glucose after an oGTT in Zucker$^{fa/fa}$ rats.

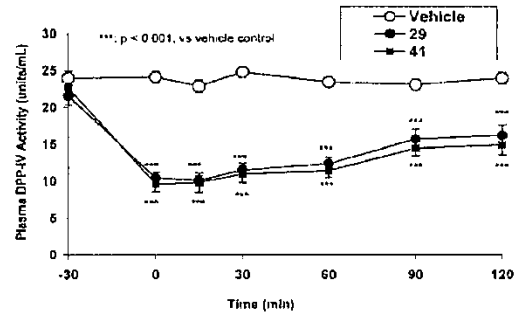


Figure 5. Effects of inhibitors **29** (●) and **41** (■) dosed at 3 μmol/kg po versus vehicle control (○) on plasma insulin after an oGTT in Zucker$^{fa/fa}$ rats.

artifacts relating to the kinetic aspects of certain inhibitors cannot be ruled out at present.

Conclusion

We have demonstrated that the prolinenitrile fragment of previously reported DPP-IV inhibitors can be replaced with either a *cis*-3,4-methano- or a *cis*-4,5-methanoprolinenitrile ring system to provide novel and highly potent DPP-IV inhibitors. Solution stability studies demonstrate that introduction of either a sterically bulky amino acid side chain on the N-terminal amino acid or a *cis*-4,5-methano bridge to the prolinenitrile moiety significantly enhances solution stability, minimizing a known intramolecular cyclization pathway. The greatest improvement in stability is observed when both of these structural features are present and at work in concert. This added stability has the potential to beneficially impact the chemical and formulation stability of cyanopyrrolidide-based pharmaceuticals. In many cases, the presence of a β-branched amino acid also provides increased inhibitory potency as well as solution stability. This class of inhibitors has been shown to be effective in suppression of prandial glucose elevations after an oral glucose challenge. These initial findings are being used as the basis for the development of DPP-IV inhibitors with even greater stability and in vivo efficacy for the treatment of diabetes and impaired glucose homeostasis through potentiation of meal-induced levels of GLP-1 (7−36) and insulin.

Experimental Section

General Chemical Procedures. All reactions were carried out using oven-dried or flame-dried round-bottomed (rb) flasks and glassware under a static atmosphere of argon or nitrogen, and the mixtures were stirred magnetically unless otherwise noted. All reagents used were of commercial quality and were obtained from Aldrich Chemical Co., Sigma Chemical Co., Lancaster Chemical Co., or Acros Chemical Co. All reactions were carried out using commercially available anhydrous solvents from Aldrich Chemical Co. or EM Science Chemical Co. unless otherwise noted. "Brine" refers to a saturated aqueous solution of NaCl. Unless otherwise specified, solutions of common inorganic salts used in workups are aqueous solutions. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a JEOL JNM-ECP500 spectrometer, and ^{1}H (400 MHz) and ^{13}C (100 MHz) spectra were recorded on a JEOL GSX400 spectrometer. Chemical shifts are given in parts per million (ppm) downfield from internal reference tetramethylsilane in δ units, and coupling constants (*J* values) are given in hertz (Hz). Selected data are reported in the following manner: chemical shift; multiplicity; coupling constants; assignment. Elemental analyses were performed by the Analytical Chemistry department at Bristol-Myers Squibb. Melting points were taken on a Hoover Uni-melt melting point apparatus and are uncorrected. Boiling points are reported uncorrected. Kügelrohr distillations were performed using a Büchi GKR-51 apparatus, and reported boiling points correspond to uncorrected oven air bath temperatures. Optical rotations were obtained on a Perkin-Elmer 241 polarimeter using a cell 1 dm in length and are reported as follows: $[\alpha]^{temp}_{wavelength}$ (concentration in g/100 mL, solvent). All flash chromatographic separations were performed using E. Merck silica gel (particle size, 0.040−0.063 mm). Reactions were monitored by TLC using 0.25 mm E. Merck silica gel plates (60 F_{254}) and were visualized using UV light and 5% phosphomolybdic acid in 95% EtOH, or by sequential treatment with 1 N HCl in CH_3OH followed by ninhydrin staining. LC/MS data were recorded on a Shimadzu LC-10AT equipped with an SIL-10A injector, an SPD-10AV detector normally operating at 220 nm and interfaced to a Micromass ZMD mass spectrometer. LC/MS or HPLC retention times are reported using a Phenomenex Luna C-18 4.6 mm × 50 mm column, with elution with a 4 min gradient of 0 100% solvent B, where solvent A is 10:90:0.1 CH_3OH−H_2O−TFA and solvent B is 90:10:0.1 CH_3OH−H_2O−TFA (HPLC, method 1). Other HPLC methods include the following: method 2, YMC S-5 C 18 4.6 mm × 50 mm, 0−100% B, elution with a 4 min gradient of 0−100% solvent B, where solvent A is 10:90:0.1 CH_3CN−H_2O−TFA and solvent B is 90:10:0.1 CH_3CN−H_2O−TFA; method 3, Zorbax SB C-18 4.6 mm × 75 mm column, elution with an 8 min gradient of 0−100% solvent B, where solvent A is 10:90:0.1 CH_3OH−H_2O−H_3PO_4 and solvent B is 90:10:0.1 CH_3OH−H_2O−H_3PO_4. All solvents were removed by rotary evaporation under vacuum using a standard rotary evaporator equipped with a dry ice condenser. All filtrations were performed using vacuum unless otherwise noted.

Representative Example of Preparation: General Method A. *N*-Boc-L-*cis*-4,5-methanoprollneamide. To a solution of *N*-Boc-4,5-methanoproline[18b] (1.20 g, 5.28 mmol) in THF (20 mL) at −15 °C was added 4-methylmorpholine (0.71 mL, 6.50 mmol) and then isobutyl chloroformate (0.78 mL, 6.00 mmol) over 5 min. The reaction mixture was stirred at −15 °C for 30 min, cooled to −30 °C, and then treated with a solution of NH_3 in dioxane (50 mL, 25 mmol). The reaction mixture was stirred at −30 °C for 0.5 h, warmed to room temperature, and stirred overnight. The reaction mixture was quenched with citric acid solution (pH 4) and extracted with Et_2O (3 × 50 mL). The combined organic fractions were washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by flash column chromatography on silica gel with EtOAc to give 1.00 g (84%) of the title compound. ^{1}H NMR and ^{13}C NMR signals were very broad and poorly defined as a result of carbamate rotamers. Anal. ($C_{11}H_{18}N_2O\cdot0.4H_2O$) C, H, N.

L-*cis*-4,5-Methanoprolineamide TFA Salt (8). To a solution of *N*-Boc-4,5-methanoprolineamide (0.90 g, 4.00 mmol) in CH_2Cl_2 (3 mL) at 0 °C was added TFA (3 mL). The reaction mixture was stirred at 0 °C for 18 h and concentrated under reduced pressure to give 0.98 g (100%) of the title compound as an oil that solidified upon prolonged standing. Alternatively, the protected material can be treated with HCl(g) in EtOAc to give a white powder. Data for HCl salt: ^{1}H NMR (CD_3OD, 400 MHz) δ 0.78 (m, 1H), 0.95 (q, J = 8.0, 1H), 1.85 (m, 1H), 2.27 (dd, J = 14.1, 3.0, 1H), 2.75 (m, 1H), 3.42 (m, 1H), 4.57 (dd, J = 11.1, 3.0, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 10.7, 18.8, 33.3, 38.3, 62.2, 170.3. Anal. ($C_6H_{10}N_2O\cdot1.03HCl\cdot0.10H_2O$) C, H, N, Cl.

Amides Prepared from Known Carboxylic Acids by Method A. L-*trans*-4,5-Methanoprolineamide TFA Salt (9). ^{1}H NMR (CD_3OD, 400 MHz) δ 0.89 (q, J = 6.0, 1H), 0.95 (m, 1H), 1.90 (m, 1H), 2.18 (td, J = 5.0, 11.4, 1H), 2.59 (dd, J = 8.1, 13.2, 1H), 3.47 (td, J = 2.5, 6.6, 1H), 4.01 (dd, J = 8.1, 10.8, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 7.1, 17.0, 32.0, 36.1, 58.3, 118.6 (q, J = 263.5), 163.0 (q, J = 35.4), 172.0. Anal. ($C_6H_{10}N_2O\cdot1.00TFA$) C, H, N, F.

L-*cis*-3,4-Methanoprolineamide TFA Salt (10). ^{1}H NMR (CD_3OD, 400 MHz) δ 0.60 (m, 1H), 0.75 (q, J = 7.5, 1H), 1.85 (m, 1H), 2.10 (m, 1H), 3.45 (d, J = 10.9, 1H), 3.49 (dd, J = 11.0, 3.5, 1H), 4.44 (d, J = 4.3, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 5.3, 17.5, 20.4, 62.5, 170.4 (TFA not observed). Anal. ($C_6H_{10}N_2O\cdot1.30TFA$) C, H, N, F.

L-2,3-Methanoprolinenitrile HCl Salt (11). ^{1}H NMR (CD_3OD, 400 MHz) δ 1.55 (m, 1H), 1.70 (m, 1H), 2.10 (m, 1H), 2.25 (m, 1H), 2.37 (m, 1H), 2.67 (m, 1H), 3.15 (m, 1H).

Representative Example of Preparation: General Method B. l-*tert*-Leucinyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (29). An oven-dried 15 mL test tube was charged with the L-*cis*-4,5-methanoprolineamide TFA salt (56 mg, 0.22 mmol), *N*-*tert*-butyloxycarbonyl-L-*tert*-leucine (53 mg, 0.23 mmol), 4-(dimethylamino)pyridine (0.11 g, 0.88 mmol), and CH_2Cl_2 (4 mL). The mixture was treated with 1-[(3-(dimethyl)amino)propyl]-3-ethylcarbodiimide (84 mg, 0.44 mmol), and the tube was sealed under a nitrogen atmosphere. The tube was placed in a shaker and shaken overnight. The product was purified by solid-phase extraction using a United Technology SCX column (2 g of sorbent in a 6 mL column) by loading the material on an SCX ion exchange column and successively washing with CH_2Cl_2 (5 mL), 1:3 $CH_3OH-CH_2Cl_2$ (5 mL), 1:2 $CH_3OH-CH_2Cl_2$ (5 mL), and 1:1 $CH_3OH-CH_2Cl_2$ (10 mL). The product-containing fractions were concentrated under reduced pressure to give the desired amide. Further purification by reverse-phase preparative HPLC on a YMC S5 ODS 20 mm × 250 mm column gave the desired amide (50 mg, 68% yield). Purification conditions were the following: gradient elution from 30:70:0.1 CH_3OH-H_2O-TFA to 90:10:0.1 CH_3OH-H_2O-TFA over 15 min; flow rate, 20 mL/min; detection wavelength, 220 nm; retention time, 14 min. ^{1}H NMR (CD_3OD, 500 MHz) δ 0.87 (m, 1H), 1.08 (s + m, 10H), 1.44 (s, 9H), 1.75 (m, 1H), 2.01 (dd, J = 13.7, 3.5, 1H), 2.58 (m, 1H), 3.87 (m, 1H), 4.53 (s, 1H), 4.77 (dd, J = 11.8, 3.2, 1H); ^{13}C NMR (CD_3OD, 125 MHz) δ 14.6, 18.4, 27.1, 28.7, 31.4, 36.4, 39.9, 60.4, 62.7, 80.7, 157.9, 172.4, 176.7.

An oven-dried 15 mL test tube was charged with the *N*-Boc-*tert*-leucine-*cis*-4,5-methanoprolineamide (50 mg, 0.15 mmol), imidazole (31 mg, 0.46 mmol), and pyridine (1 mL). The tube was sealed under nitrogen atmosphere and cooled to −30 °C. Slow addition of $POCl_3$ (141 mg, 88 μL, 0.92 mmol) gave after mixing a thick slurry. The tube was mixed at −30 °C for 1 h, and the volatiles were evaporated. The product was purified by solid-phase extraction using a United Technology silica extraction column (2 g of sorbent in a 6 mL column) by loading the material on a silica column and successively washing with CH_2Cl_2 (5 mL), 5:95 $CH_3OH-CH_2Cl_2$ (5 mL), 7:93 $CH_3OH-CH_2Cl_2$ (5 mL), and 12:88 $CH_3OH-CH_2Cl_2$ (10 mL). The product-containing fractions were pooled and concentrated under reduced pressure to give the *N*-Boc-protected nitrile compound (46 mg, 96%). ^{1}H NMR (CD_3OD, 500 MHz) δ 0.88 (m, 1H), 1.08 (s + m, 10H), 1.43 (s, 9H), 1.94 (m, 1H), 2.32 (dd, J = 11.5, 2.5, 1H), 2.65 (m, 1H), 3.95 (m, 1H), 4.51 (s, 1H), 5.09 (dd, J = 11.2, 2.2, 1H).

An oven-dried 15 mL test tube was charged with the *N*-Boc-*tert*-leucine-4,5-methanoprolinenitrile (45 mg, 0.14 mmol), CH_2Cl_2 (1 mL), and TFA (1 mL). The reaction mixture was vortexed for 40 min at room temperature, diluted with toluene (4 mL), and concentrated under reduced pressure to a thick oil. The product was purified by reverse-phase preparative HPLC on a YMC S5 ODS 20 mm × 250 mm column to give 14 mg (35%) of the title compound. Purification conditions were the following: gradient elution from 10:90:0.1 CH_3OH-H_2O-TFA to 90:10:0.1 CH_3OH-H_2O-TFA over 18 min; flow rate, 20 mL/min; detection wavelength, 220 nm; retention time, 10 min. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 0.73 (m, 1H), 1.05 (s + m, 10H), 1.99 (m, 1H), 2.28 (dd, J = 13.5, 2.2, 1H), 2.50 (m, 1H), 4.11 (m, 1H), 4.37 (s, 1H), 5.26 (dd, J = 10.5, 2.2, 1H), 8.20 (s, 2H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 12.7, 17.3, 25.9, 29.8, 34.2, 37.6, 45.0, 57.6, 118.0 (q, J = 280), 119.5, 157.5 (q, J = 38), 166.7.

L-*tert*-Leucinyl-L-*cis*-4,5-methanoprolinenitrile HCl Salt (29). An oven-dried 15 mL test tube was charged with *N*-Boc-*tert*-leucine-4,5-methanoprolinenitrile (0.45 mg, 0.14 mmol) and Et_2O (1 mL) and was treated with a 2 N HCl solution in Et_2O (1 mL). The reaction mixture was vortexed for 15 min at room temperature and concentrated under reduced pressure to a white powder. The product was purified by trituration with Et_2O, collected, and dried under vacuum to give the title compound (25 mg, 85%). ^{1}H NMR (CD_3OD, 400 MHz) δ 0.91 (m, 1H), 1.13 (s + m, 10H), 2.00 (m, 1H), 2.32 (dd, J = 14.0, 2.2, 1H), 2.65 (m, 1H), 3.97 (m, 1H), 4.37 (s, 1H), 5.28 (dd, J = 10.5, 2.2, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.1, 19.1, 26.7, 31.4, 35.7, 39.4, 46.9, 60.1, 120.4, 168.1. Anal. ($C_{12}H_{20}ClN_3O\cdot0.25H_2O$) C, H, N.

Dipeptide Nitriles Prepared by General Method B. L-Isoleucine-L-prolinenitrile HCl Salt (21). ^{1}H NMR (400 MHz, CD_3OD) δ 0.99 (t, J = 7.2, 3H), 1.10 (d, J = 6.6, 3H), 1.20 (m, 2H), 1.70 (m, 1H), 1.90−2.45 (m, 4H), 3.70 (m, 2H), 4.11 (s, 1H), 4.81 (d, J = 8.0, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 11.7, 15.3, 25.1, 26.4, 30.9, 37.8, 48.0, 57.4, 119.2, 169.1. Anal. ($C_{11}H_{19}N_3O\cdot1.00HCl\cdot0.5H_2O$) C, H, N.

L-Isoleucinyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (24). ^{1}H NMR (D_2O, 500 MHz) δ 0.90 (m, 1H), 0.95 (t, J = 7.3, 3H), 1.12 (d, J = 7.3, 3H), 1.17 (m, 1H), 1.25 (m, 1H), 1.47 (m, 1H), 2.05 (m, 1H), 2.32 (m, 1H), 2.55 (dd, J = 13.8, 2.8, 1H), 2.78 (m, 1H), 3.73 (td, J = 6.4, 2.8, 1H), 4.46 (d, J = 9.1, 1H), 5.32 (dd, J = 11, 2.8, 1H); ^{13}C NMR (D_2O, 125 MHz) δ 10.9, 13.7, 14.9, 18.5, 23.6, 30.1, 35.5, 38.0, 47.0, 56.9, 116.6 (q, J = 290), 119.8, 163.2 (q, J = 35), 165.4. HPLC: method 3: t_R = 2.49 3.03 min. HRMS (FAB) *m/z*: $[M + H]^+$ calcd for $C_{12}H_{20}N_3O$, 222.1606; found, 222.1606.

L-Valinyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (26). ^{1}H NMR (CD_3OD, 500 MHz) δ 0.93 (m, 1H), 1.07 (d, J = 7.1, 3H), 1.09 (m, 1H), 1.17 (d, J = 7.1, 3H), 2.01 (m, 1H), 2.35 (dd, J = 13.8, 3.0, 1H), 2.50 (m, 1H), 2.65 (m, 1H), 3.82 (td, J = 6.0, 2.5, 1H), 4.39 (d, J = 5.0, 1H), 5.16 (dd, J = 11.0, 2.2, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.3, 17.4, 19.4, 30.9, 31.8, 38.7, 47.6, 58.5, 120.8, 168.9. HPLC: method 2; t_R = 0.89 min. HRMS (FAB) *m/z*: $[M + H]^+$ calcd for $C_{11}H_{19}N_3O$, 208.1450; found, 208.1447.

L-*tert*-Leucinyl-L-prolinenitrile TFA Salt (28). ^{1}H NMR (CD_3OD, 400 MHz) δ 1.13 (s, 9H), 1.90−2.45 (m, 4H), 3.72 (t, J = 6.3, 1H), 4.00 (s, 1H), 4.81 (d, J = 8.0, 1H); ^{13}C NMR (CD_3OD, 100 MHz) 26.7, 26.8, 31.2, 35.9, 48.2, 60.5, 119.4 (q, J = 248), 119.5, 163.2 (q, J = 46), 169.1. Anal. ($C_{11}H_{19}N_3O\cdot1.00TFA$) C, H, N, F.

L-Phenylalaninyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (31). ^{1}H NMR (CD_3OD, 500 MHz) δ 0.31 (m, 1H), 0.66 (dd, J = 8.5, 6.5, 1H), 1.84 (m, 1H), 2.25 (dd, J = 13.8, 2.5, 1H), 2.57 (m, 1H), 3.25 (m, 1H), 3.30 (s, 2H), 3.65 (m, 1H), 4.73 (t, J = 6.6, 1H), 5.10 (dd, J = 10.2, 2.2, 1H), 7.32 (m, 5H); ^{13}C NMR (CD_3OD, 100 MHz) δ 13.7, 18.7, 31.9, 38.4, 38.7, 47.6, 54.3, 118.0 (q, J = 280), 120.0, 129.4, 130.7, 131.3, 135.1, 164.0 (q, J = 43), 168.0. HPLC: method 2; t_R = 1.33 min. HRMS (FAB) *m/z*: $[M + H]^+$ calcd for $C_{16}H_{17}N_3O$, 256.1450; found, 256.1445.

L-Tryptophanyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (32). ^{1}H NMR (CD_3OD, 500 MHz) δ 0.00 (m, 2H), 1.64 (m, 1H), 2.11 (dd, J = 13.8, 2.2 Hz, 1H), 2.44 (m, 1H), 3.23 (s, 2H), 3.35 (m, 1H), 4.71 (t, J = 6.6, 1H), 5.00 (dd, J = 10.5, 2.2, 1H), 7.00 (t, J = 7.2, 1H), 7.07 (t, J = 7.7, 1H), 7.10 (s, 1H), 7.32 (d, J = 8.2, 1H), 7.56 (d, J = 7.7, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 13.4, 18.6, 28.8, 31.8, 38.9, 47.4, 53.4, 107.7, 113.1, 119.2, 120.5, 120.9, 123.3, 126.2, 128.9, 138.9, 169.5. HPLC: method 2; t_R = 1.39 min. HRMS (FAB) m/z $[M + H]^+$ calcd for $C_{17}H_{19}N_4O$, 295.1559; found, 295.1558.

L-*N*-Methylvalinyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (33). ^{1}H NMR (CD_3OD, 500 MHz) δ 0.94 (m, 1H), 1.11 (m + d, J = 7.2, 4H), 1.18 (d, J = 7.2, 3H), 2.02 (m, 1H), 2.35 (dd, J = 13.8, 2.3, 1H), 2.50 (m, 1H), 2.65 (m, 1H), 2.70 (s, 3H), 3.85 (td, J = 6.0, 2.7, 1H), 4.42 (d, J = 5.0, 1H), 5.20 (dd, J = 10.5, 2.2, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.4, 18.3, 19.0, 19.5, 31.4, 31.8, 33.8, 38.8, 47.6, 66.8, 120.7, 167.5. HPLC: method 2; t_R = 0.87 min. HRMS (FAB) m/z $[M + H]^+$ calcd for $C_{12}H_{19}N_3O$, 222.1606; found, 222.1604.

L-Prolinyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (34). ^{1}H NMR (CD_3OD, 400 MHz) δ 0.93 (m, 1H), 1.10 (m, 1H), 2.00 (m, 1H), 2.11 (m, 3H), 2.38 (dd, J = 13.8, 2.2, 1H), 2.66 (m, 2H), 3.43 (m, 2H), 3.75 (td, J = 6.0, 2.7, 1H), 4.85 (t, J = 8.8, 1H), 5.11 (dd, J = 12.6, 2.2, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.1, 19.2, 25.4, 29.8, 30.0, 32.0, 38.3, 47.8, 61.1, 120.7, 168.4. HPLC: method 2; t_R = 0.48 min. HRMS (FAB) m/z $[M + H]^+$ calcd for $C_{11}H_{15}N_3O$, 206.1296; found, 206.1293.

L-Pipecolinyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (35). ^{1}H NMR (CD_3OD, 500 MHz) δ 0.93 (m, 1H), 1.12 (q, J = 6.5, 1H), 1.20 (m, 1H), 1.60–2.10 (m, 3H), 2.35 (dd, J = 14.0, 2.5, 1H), 2.65 (m, 1H), 3.10 (m, 1H), 2.66 (m, 1H), 3.45 (d, J = 11.0, 1H), 3.56 (t, J = 5.0, 1H), 3.68 (t, J = 4.5, 1H), 3.76 (m, 1H), 4.48 (dd, J = 12.5, 3.5, 1H), 5.06 (dd, J = 10.5, 2.5, 1H); ^{13}C NMR (CD_3OD, 125 MHz) δ 13.5, 16.5, 21.0, 21.7, 25.5, 29.1, 34.2, 40.5, 47.8, 61.4, 118.5 (q, J = 289), 120.7, 166.3 (q, J = 36), 171.1. HPLC: method 1; t_R = 0.85 min. MS m/z 220 $[M + H]^+$.

L-Methioninyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (36). ^{1}H NMR (CD_3OD, 500 MHz) δ 0.93 (m, 1H), 1.13 (q, J = 7.1, 1H), 2.03 (m, 1H), 2.25 (s, 3H), 2.29 (m, 2H), 2.36 (dd, J = 13.8, 2.0, 1H), 2.66 (m, 3H), 3.79 (td, J = 6.0, 3.0, 1H), 4.68 (dd, J = 7.0, 4.5, 1H), 5.15 (dd, J = 10.5, 2.0, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 13.9, 15.2, 19.0, 29.6, 30.3, 31.4, 38.1, 47.4, 52.4, 120.4, 168.4. HPLC: method 1; t_R = 1.18 min. MS m/z 240 $[M + H]^+$.

(*S*)-Cyclobutylglycinyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (37). ^{1}H NMR (D_2O, 400 MHz) δ 0.85 (m, 1H), 1.05 (m, 1H), 1.73 (m, 1H), 1.92 (m, 7H), 2.30 (dd, J = 14.1, 2.2, 1H), 2.56 (m, 1H), 2.89 (m, 1H), 3.70 (m, 1H), 4.45 (d, J = 8.4, 1H), 5.06 (dd, J = 10.8, 2.4, 1H); ^{13}C NMR (D_2O, 400 MHz) δ 13.8, 17.8, 18.3, 24.5, 24.7, 30.1, 38.0, 46.8, 55.1, 119.9, 168.3. HRMS (ESI) m/z: $[M + H]^+$ calcd for $C_{12}H_{17}N_3O$, 220.145; found, 220.144. HPLC: method 1: t_R = 1.26 min. Anal. ($C_{12}H_{17}N_3O$·1.00TFA·0.67H_2O) C, H, N, F.

(*S*)-Cyclopentylglycinyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (38). ^{1}H NMR (D_2O, 400 MHz) δ 0.86 (m, 1H), 1.04 (q, J = 7.1, 1H), 1.25–1.80 (m, 8H), 1.93 (m, 1H), 2.32 (dd, J = 11.5, 2.6, 1H), 2.40 (m, 1H), 2.59 (m, 1H), 3.70 (td, J = 6.1, 2.6, 1H), 4.43 (d, J = 7.5, 1H), 5.09 (dd, J = 11.0, 2.6, 1H); ^{13}C NMR (D_2O, 100 MHz) δ 13.5, 18.2, 24.3, 24.7, 27.9, 28.4, 30.2, 37.9, 41.3, 46.8, 55.1, 119.6, 169.1. HRMS (FAB) m/z: $[M + H]^+$ calcd for $C_{13}H_{19}N_3O$, 234.1602; found, 234.1606. Anal. ($C_{13}H_{19}N_3O$·1.23TFA) C, H, N.

(*S*)-Cyclohexylglycinyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (39). ^{1}H NMR (400 MHz, CD_3OD) δ 0.90 (m, 1H), 1.05–1.35 (m, 6H), 1.70–1.90 (m, 5H), 2.00 (m, 1H), 2.15 (m, 1H), 2.37 (dd, J = 13.6, 1.8, 1H), 2.63 (m, 1H), 3.83 (m, 1H), 4.35 (d, J = 5.7, 1H), 5.17 (dd, J = 10.6, 2.2, 1H); ^{13}C NMR (D_2O, 100 MHz) δ 13.9, 19.1, 26.7, 26.9, 27.1, 28.5, 30.0, 31.4, 38.4, 40.3, 47.1, 57.2, 120.5, 168.5. HRMS (FAB) m/z: $[M + H]^+$ calcd for $C_{14}H_{21}N_3O$, 248.1768; found, 248.1763. Anal. ($C_{14}H_{21}N_3O$·1.00TFA) C, H, N.

(*S*)-1-Methylcyclobut-1-ylglycinyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (40). ^{1}H NMR (D_2O, 400 MHz) δ 0.99 (m, 1H), 1.19 (q, J = 8.8, 6.6, 1H), 1.37 (s, 3H), 1.89 (m, 3H), 2.12 (m, 2H), 2.24 (m, 1H), 2.47 (dd, J = 13.6, 2.6, 1H), 2.54 (m, 1H), 2.72 (m, 1H), 3.90 (m, 1H), 4.82 (s, 1H), 5.27 (dd, J = 10.8, 2.4, 1H); ^{13}C NMR (D_2O, 400 MHz) δ 14.1, 15.1, 18.5, 21.1, 30.0, 30.9, 31.0, 38.6, 40.1, 46.8, 59.0, 120.0, 167.8. HRMS (ESI) m/z: $[M + H]^+$ calcd for $C_{13}H_{19}N_3O$, 234.1607; found, 234.1592. HPLC: method 3; t_R = 3.55 min. Anal. ($C_{13}H_{19}N_3O$·1.00TFA) C, H, N, F.

(*S*)-1-Methylcyclopent-1-ylglycinyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (41). ^{1}H NMR (D_2O, 400 MHz) δ 0.82 (m, 1H), 0.95 (s, 3H), 1.00 (q, J = 7.1, 1H), 1.25–1.75 (m, 8H), 1.90 (m, 1H), 2.28 (dd, J = 14.0, 2.7, 1H), 2.54 (m, 1H), 3.70 (td, J = 6.1, 3.1, 1H), 4.35 (s, 1H), 5.08 (dd, J = 8.5, 2.6, 1H); ^{13}C NMR (D_2O, 100 MHz) δ 13.8, 14.5, 21.3, 23.2, 23.4, 30.0, 36.2, 36.7, 45.7, 46.7, 58.8, 119.8, 168.2. HRMS (FAB) m/z: $[M + H]^+$ calcd for $C_{14}H_{20}N_3O$, 248.1756; found, 248.1749. Anal. ($C_{14}H_{21}N_3O$·1.00TFA·0.89H_2O) C, H, N.

(*S*)-1-Methylcyclohex-1-ylglycinyl-L-*cis*-4,5-methanoprolinenitrile TFA Salt (42). ^{1}H NMR (D_2O, 400 MHz) δ 0.86 (m, 1H), 1.03 (s, 3H), 1.07 (m, 2H), 1.39 (m, 9H), 1.95 (m, 1H), 2.31 (dd, J = 13.6, 2.6, 1H), 2.58 (m, 1H), 3.76 (m, 1H), 4.26 (s, 1H), 5.12 (dd, J = 11, 2.6, 1H); ^{13}C NMR (D_2O, 400 MHz) δ 13.8, 18.2, 18.5, 20.9, 21.0, 25.3, 30.0, 33.5, 34.1, 37.9, 38.9, 46.7, 60.0, 119.8, 168.0. HRMS (ESI) m/z $[M + H]^+$ calcd for $C_{15}H_{23}N_3O$, 262.1920; found, 262.1904. HPLC: method 3; t_R = 4.77 min. Anal. ($C_{15}H_{24}N_3O$·1.00TFA·0.50H_2O) C, H, N, F.

Representative Example of Preparation: General Method C, 1-*tert*-Leucinyl-L-*cis*-3,4-methanoprolinenitrile TFA Salt (30). An oven-dried rb flask was charged with *cis*-3,4-methanoprolineamide TFA salt (50 mg, 0.21 mmol), *N*-*tert*-butyloxycarbonyl-L-*tert*-leucine (48 mg, 0.21 mmol), benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (164 mg, 0.32 mmol), 4-methylmorpholine (70 μL, 0.63 mmol), and CH_2Cl_2 (4 mL). The flask was sealed under nitrogen atmosphere and stirred overnight. The product was purified by solid-phase extraction using a United Technology silica extraction column (2 g of sorbent in a 6 mL column) by loading the material on a silica column and successively washing with $CHCl_2Cl_2$ (5 mL), 5:95 CH_3OH–$CHCl_2Cl_2$ (5 mL), 7:93 CH_3OH–CH_2Cl_2 (5 mL), and 12:88 CH_3OH–CH_2Cl_2 (10 mL). The product-containing fractions were pooled and concentrated under reduced pressure to give the desired amide compound (64 mg, 90%). ^{1}H NMR (CD_3OD, 400 MHz) δ 0.69 (m, 1H), 0.78 (q, J = 4.7, 1H), 1.00 (s, 9H), 1.42 (s, 9H), 1.74 (m, 1H), 1.93 (m, 1H), 3.82 (d, J = 9.2, 1H), 3.98 (m, 1H), 4.16 (s, 1H), 4.61 (d, J = 5.2, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 8.9, 17.7, 21.0, 26.8, 28.7, 36.1, 52.5, 60.2, 62.5, 80.7, 156.0, 174.0, 175.0. MS m/z 340 $[M + H]^+$.

An oven-dried flask was charged with *N*-Boc-*tert*-leucinyl-*cis*-4,5-methanoprolineamide (65 mg, 0.19 mmol), imidazole (31 mg, 0.46 mmol), and pyridine (2 mL). The flask was sealed under nitrogen atmosphere and cooled to −30 °C. Slow addition of $POCl_3$ (132 mg, 81 μL, 0.86 mmol) gave, after mixing, a thick slurry that was mixed at −30 °C for 1 h and concentrated. The product was purified by silica gel column chromatography using 1:5 EtOAc–CH_2Cl_2. The product-containing fractions were pooled and concentrated under reduced pressure to give the desired *N*-Boc-protected title compound (50 mg, 80%). ^{1}H NMR ($CDCl_3$ 400 MHz) δ 0.65 (q, J = 4.7, 1H), 0.99 (s, 9H), 1.00 (m, 1H), 1.42 (s, 9H), 1.89 (m, 1H), 2.00 (m, 1H), 3.80 (d, J = 9.2, 1H), 3.99 (m, 1H), 4.15 (d, J = 9.2, 1H), 4.72 (d, J = 4.9, 1H), 5.18 (d, J = 9.6, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 11.1, 18.5, 19.1, 26.2, 28.3, 35.3, 50.0, 50.7, 58.6, 79.9, 116.9, 156.0, 173.0. MS m/z 322 $[M + H]^+$.

An oven-dried flask was charged with *N*-Boc-*tert*-leucinyl-3,4-methanoprolinenitrile (0.45 mg, 0.14 mmol), CH_2Cl_2 (1 mL), and TFA (1 mL). The reaction mixture was stirred for 1 h at room temperature, diluted with toluene (4 mL) and concentrated under reduced pressure. The product was purified by reverse-phase preparative HPLC on a YMC S5 ODS 20 mm × 250 mm column to give the title compound (20 mg, 45%). Purification conditions were the following: gradient

elution from 10:90:0.1 CH_3OH-H_2O-TFA to 90:10:0.1 CH_3OH-H_2O-TFA over 18 min; flow rate, 20 mL/min; detection wavelength, 220 nm; t_R, 10 min. ^1H NMR (CD_3OD, 400 MHz) δ 0.55 (q, J = 5.7, 1H), 1.10 (s + m, 10H), 1.95 (m, 1H), 2.13 (m, 1H), 3.80 (m, 2H), 3.93 (s, 1H), 4.84 (d, J = 5.0, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 11.3, 19.7, 20.1, 26.8, 35.8, 52.1, 52.3, 60.5, 117.3 (q, J = 289), 119.2, 161.6 (q, J = 38), 170.5. Anal. ($C_{12}H_{19}N_3O$·1.00TFA) C, H, N, F.

Dipeptide Nitriles Prepared by General Method C.

l-Isoleucinyl-L-*trans*-4,5-methanoprolinenitrile TFA Salt (22). ^1H NMR (D_2O, 500 MHz) δ 0.72 (m, 1H), 0.92 (t, J = 7.3, 3H), 1.07 (d+m, J = 7.3, 4H), 1.25 (m, 1H), 1.55 (m, 1H), 2.19 (m, 2H), 2.43 (m, 1H), 2.60 (m, 1H), 3.63 (m, 1H), 4.39 (d, J = 5.5, 1H), 4.82 (q, J = 9.1, 1H); ^{13}C NMR (D_2O, 125 MHz) δ 10.7, 14.6, 18.2, 18.8, 23.7, 31.6, 36.4, 37.1, 50.9, 56.6, 116.6 (q, J = 290), 118.5, 163.2 (q, J = 35), 170.2; HPLC: method 3; t_R = 2.89 min. HRMS (FAB) m/z: $[M - H]^+$ calcd for $C_{12}H_{19}N_3O$, 222.1606; found, 222.1601.

L-Isoleucinyl-L-*trans*-2,3-methanoprolinenitrile TFA Salt (23). ^1H NMR (D_2O, 500 MHz) δ 0.84 (t, J = 7.3, 3H), 1.05 (d, J = 7.3, 3H), 1.14–1.30 (m 2H), 1.64 (m, 1H), 1.76 (m, 1H), 2.10 (m, 2H), 2.27 (m, 1H), 2.75 (m, 1H), 3.08 (m, 1H), 3.90 (m, 1H), 4.30 (d, J = 2.3, 1H); ^{13}C NMR (D_2O, 125 MHz) δ 11.5, 15.0, 22.4, 23.6, 24.0, 28.1, 36.5, 42.0, 44.8, 60.7, 116.6 (q, J = 290), 117.7, 163.2 (q, J = 35), 165.6, HPLC: method 3: t_R = 2.49 min. HRMS (FAB) m/z: $[M - H]^+$ calcd for $C_{12}H_{19}N_3O$, 222.1606; found, 222.1611.

L-Isoleucinyl-L-*cis*-3,4-methanoprolinenitrile TFA Salt (25). ^1H NMR (500 MHz, D_2O) δ 0.53 (m, 1H), 0.90 (t, J = 7.3, 3H), 1.00 (d, J = 6.9, 3H), 1.08 (m, 1H), 1.18 (m, 1H), 1.48 (m, 1H), 2.00 (m, 2H), 2.20 (m, 1H), 3.83 (s, 2H), 4.10 (d, J = 5.5, 1H), 4.89 (d, J = 5.0, 1H); ^{13}C NMR (D_2O, 125 MHz) δ 10.1, 10.8, 14.6, 18.5, 23.8, 36.4, 50.7, 51.2, 56.4, 116.6 (q, J = 290), 118.1, 163.2 (q, J = 35), 170.4. HPLC: method 3; t_R = 3.15 min. HRMS (FAB) m/z: $[M + H]^+$ calcd for $C_{12}H_{19}N_3O$, 222.1606; found, 222.1605.

L-Valinyl-L-*cis*-3,4-methanoprolinenitrile TFA Salt (27). ^1H NMR (CD_3OD, 400 MHz) δ 0.55 (q, J = 5.7, 1H), 1.03 (d, J = 6.5, 3H), 1.08 (d + m, J = 7.0, 4H), 1.95 (m, 1H), 2.10 (m, 1H), 2.20 (m, 1H), 3.80 (d, J = 9.5, 1H), 3.86 (dd, J = 9.5, 4.8, 1H), 4.00 (d, J = 5.7, 1H), 4.84 (d, J = 4.8, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 11.5, 17.8, 19.2, 19.8, 20.2, 31.7, 51.6, 52.1, 58.2, 118.4, 118.8 (q, J = 289), 162.0 (q, J = 37), 171.0. Anal. ($C_{11}H_{17}N_3O$·1.30TFA) C, H, N, F.

Representative Example of Preparation of Intermediates 19 and 20: General Method D. 2-Cyclopentylidenemalonic Acid Diethyl Ester (17b). A mixture of dry THF (200 mL) and dry CCl_4 (25 mL) was cooled to 0 °C and treated with $TiCl_4$ (11.0 mL, 0.1 mol). The resulting yellow suspension was stirred at 0 °C for 5 min, treated sequentially with cyclopentanone (4.4 mL, 0.05 mol) and freshly distilled diethyl malonate (7.6 mL, 0.05 mol), and then stirred at 0 °C for 0.5 h. The reaction mixture was then treated with a solution of dry pyridine (16 mL, 0.20 mol) in dry THF (30 mL) and stirred at 0 °C for 1.0 h and then at room temperature for 72 h. The reaction mixture was quenched with water and extracted with Et_2O (2 × 100 mL). The combined organic extracts were washed with brine and saturated $NaHCO_3$, dried ($MgSO_4$), and concentrated under reduced pressure. The residue (12.1 g) was purified by flash column chromatography (EtOAc–hexanes, 5:95) to give the title compound (5.25 g, 46%). ^1H NMR ($CDCl_3$, 400 MHz) δ 1.30 (m, 6H), 1.73 (m, 4H), 2.68 (m, 4H), 4.24 (m, 4H). LC/MS m/z: 227 $[M + H]^-$.

2-(1-Methylcyclopent-1-yl)malonic Acid Monoethyl Ester (18b). A mixture of 3.0 M methylmagnesium iodide (3.1 mL, 9.4 mmol) and cuprous chloride (10.6 mg) was stirred at 0 °C, treated with a solution of 2-cyclopentylidenemalonic acid diethyl ester (**17b**) (1.41 g, 6.24 mmol) in dry Et_2O (2 mL) over 5 min, and stirred at 0 °C for 1 h and then at room temperature for 1 h. The reaction was then quenched by the dropwise addition of ice–water (15 mL) followed by 3 M HCl (3.7 mL). The mixture was then extracted with EtOAc (3 × 25 mL). The combined organic extracts were washed with 1% $Na_2S_2O_3$ and brine, dried ($MgSO_4$), and evaporated (1.57 g). The residue was purified by flash column chromatography on silica gel (5:95 Et_2O–hexanes) to give the diethyl ester compound (1.09 g, 68.7%). ^1H NMR ($CDCl_3$, 400 MHz) δ 1.15 (s, 3H), 1.26 (t, J = 7.0, 6H), 1.57 (m, 2H), 1.55–1.70 (m, 6H), 3.34 (s, 1H), 4.20 (q, J = 7.0, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.1, 23.7, 24.0, 38.6, 43.9, 60.7, 60.8, 168.7. LC/MS m/z: 243 $[M + H]^+$. A solution of 2-(1-methylcyclopentyl)malonic acid diethyl ester (736 mg, 3.04 mmol) in a mixture of EtOH (12 mL) and THF (6 mL) was treated with 1.0 M NaOH (3.04 mL, 3.04 mmol) and stirred at room temperature for 24 h. The reaction mixture was evaporated to a syrup, dissolved in water (10 mL), and extracted with Et_2O (15 mL). The aqueous phase was acidified with 1 M HCl to pH 2.0 and extracted with EtOAc (3 × 25 mL). The combined organic extracts were washed with brine, dried ($MgSO_4$), and evaporated to give the title compound (0.87 g, 95%). R_f = 0.56 ($CH_2Cl_2-CH_3OH$, 9:1; PMA). ^1H NMR ($CDCl_3$, 400 MHz) δ 1.15 (s, 3H), 1.28 (t, J = 7.0, 3H), 1.55–1.70 (m, 8H), 3.75 (s, 1H), 4.20 (q, J = 7.0, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.0, 23.2, 23.9, 38.5, 44.5, 60.4, 61.4, 169.7, 173.5. LC/MS m/z: 215 $[M + H]^+$.

Benzyloxycarbonylamino(1-methylcyclopent-1-yl)acetic Acid Ethyl Ester (19b). A solution of 2-(1-methylcyclopentyl)malonic acid monoethyl ester (0.48 g, 2.26 mmol) in dry benzene (2.85 mL) was treated with triethylamine (0.3 mL, 2.26 mmol) and diphenylphosphoryl azide (0.5 mL, 2.26 mmol), refluxed for 1 h, and cooled to room temperature. The solution was treated with benzyl alcohol (0.35 mL, 3.39 mmol), refluxed for 17 h, cooled, then partitioned between aqueous 10% citric acid solution (3.8 mL) and EtOAc (2 × 25 mL). The combined organic extracts were washed with 5% $NaHCO_3$ solution, dried ($MgSO_4$), and evaporated. The remainder was purified by flash column chromatography (1:9 EtOAc–hexanes) to give the title compound (1.20 g, 100%). ^1H NMR ($CDCl_3$, 400 MHz) δ 0.92 (s + t, J = 7.0, 6H), 1.20–1.80 (m, 8H), 4.20 (m, 3H), 5.11 (s, 2H), 5.38 (m, 1H), 7.30–7.50 (m, 5H). LC/MS m/z: 342 $[M + Na]^+$.

***tert*-Butyloxycarbonylamino(1-methylcyclopent-1-yl)acetic Acid (20b).** A solution of benzyloxycarbonylamino(1-methylcyclopentyl)acetic acid ethyl ester (0.798 g, 2.26 mmol) in EtOAc (35 mL) was treated with 10% palladium hydroxide on carbon (190 mg) under an atmosphere of H_2 at room temperature for 20 h. The mixture was diluted with EtOAc and filtered through a Celite pad, washing the pad well with EtOAc (2 × 35 mL). The filtrate was evaporated to dryness to give the crude amine (0.42 g, 100%). The crude amine (0.42 g, 2.26 mmol) in a mixture of THF (9 mL) and water (9 mL) was treated with di-*tert*-butyl dicarbonate (0.73 g, 3.35 mmol) and K_2CO_3 (0.68 g, 4.54 mmol) and stirred at room temperature for 36 h. The reaction mixture was partitioned between water and Et_2O. The aqueous fraction was then extracted with Et_2O (3 × 30 mL), and the combined organic extracts were washed with brine, dried ($MgSO_4$), and evaporated. The remainder was purified by flash column chromatography (1:9 EtOAc–hexane) to give the desired *N*-Boc-protected ester (0.57 g, 92%). ^1H NMR ($CDCl_3$, 400 MHz) δ 0.95 (s, 3H), 1.28 (t, J = 7.0, 3H), 1.30 (m, 2H), 1.44 (s, 9H), 1.55–1.80 (m, 6H), 4.20 (q, J = 7.0, 2H), 5.11 (d, J = 6.0, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 22.5, 23.9, 24.3, 28.3, 36.8, 45.8, 60.8, 80.0, 155.7, 172.5. LC/MS m/z: 286 $[M + H]^+$. A solution of *tert*-butyloxycarbonylamino(1-methylcyclopentyl)acetic acid ethyl ester (0.55 g, 1.97 mmol) in MeOH (6.9 mL) and THF (6.9 mL) was treated with 1.0 M NaOH (2.9 mL, 2.9 mmol) and stirred at room temperature for 24 h. The reaction mixture was evaporated, dissolved in water (10 mL), and extracted with Et_2O. The aqueous phase was acidified to pH 2 with 1.0 M HCl (2.9 mL) and extracted with EtOAc (3 × 30 mL). The combined organic extracts were washed with brine, dried ($MgSO_4$), and evaporated to give the title compound (0.81 g, 96%): mp = 151–152 °C. ^1H NMR ($CDCl_3$, 400 MHz) δ 0.98 (s, 3H), 1.30 (m, 2H), 1.44 (s + m, 11H), 1.55–1.80 (m, 6H), 4.20 (d, J = 7.0, 2H), 5.11 (d, J = 7.0, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 22.5, 23.9, 24.3, 28.3, 36.9, 45.6, 60.8, 80.0, 155.7, 176.8. LC/MS m/z: 258 $[M + H]^+$.

The amino acids prepared according to this method were converted to the corresponding dipeptide nitriles using general method B.

Computational Assessment Methods. Conformations were generated for both methanoprolinenitrile and prolinenitrile forms of the N-terminal *tert*-leucine dipeptide and alanine dipeptides using Macromodel with OPLSAA parameters and GB/SA solvation model.[34] Two conformations were selected for energetic comparisons: the global minimum, which has the syn amide rotamer (Figure 2, lower structure), and an anti conformation, which brings the reactive amine and nitrile close to each other (Figure 2, upper structure). These conformations were reminimized using ab initio quantum mechanics with the B3LYP DFT method and the 6-31+G** basis set in Gaussian 98.0.[35] To approximate van der Waals and electrostatic contributions, relative energies of the conformations were also calculated using Insight's CFF force field.[36] Ab initio energetic minimizations and force field calculations were also performed on the proline and methanoproline compounds with a simple acetamide cap in both conformations.

In Vitro Assays. All compounds were tested in vitro against purified porcine DPP-IV as previously published.[37] Inhibition was determined using the substrate H-Gly-Pro-PNA. Production of *p*-nitroaniline was measured at 405 nm at 9 s intervals over 15 min. Reactions were initiated by addition of the enzyme, and data collection was started immediately. The reactions were run at 11 substrate concentrations (ranging from 60 to 3000 μM) and 7 inhibitor concentrations (ranging from 1 to 1000 nM). Enzyme reactions contained a final volume of 100 μL, ATE buffer (100 mM Aces, 52 mM Tris, 52 mM ethanolamine, pH 7.4), 4.5 nM porcine DPP-IV, and 1% DMSO.

In Vivo Assay Methods. Male Zucker$^{fa/fa}$ rats (Harlan) weighing between 400 and 450 g were housed in a room that was maintained on a 12 h light−dark cycle and were allowed free access to normal rodent chow and tap water. The day before the experiment, the rats were weighed and divided into control and treated groups of six. Rats were fasted 17 h prior to the start of the study. On the day of the experiment, animals were dosed orally with vehicle (water) or DPP-IV inhibitors (3 μmol/kg) at −30 min. Two blood samples were collected at −30 and 0 min by tail bleed. Glucose (2 g/kg) was administered orally at 0 min. Additional blood samples were collected at 15, 30, 60, 90, and 120 min. Blood samples were collected into EDTA containing tubes from Starstedt. Plasma glucose was determined by Cobas Mira (Roche Diagnostics) by the glucose oxidation method.

Rat plasma insulin was assayed using an ELISA kit from Crystal Chem Inc. Properly diluted plasma samples were added to the ELISA microplate. Insulin was then detected using guinea pig anti insulin serum. DPP-IV activity in rat plasma was assayed ex vivo using Ala-Pro-AFC·TFA, a fluorescence-generating substrate from Enzyme Systems Products. A 20 μL plasma sample was mixed with 200 μL of reaction buffer, 50 mM Hepes, and 140 mM NaCl. The buffer contained 0.1 mM Ala-Pro-AFC-TFA. Fluorescence was then read for 20 min on a Perseptive Biosystem Cytofluor-II at 360 nm excitation wavelength and 530 nm emission wavelength. The initial rate of DPP-IV enzyme activity was calculated over the first 20 min of the reaction, with units/mL defined as the rate of increase of fluorescence intensity (arbitrary units) per milliliter of plasma. All in vivo data presented are the mean ± SE (n = 6). Data analysis was performed using ANOVA followed by Fisher Post-hoc.

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Supporting Information Available: Crystallographic data and atomic coordinate information for **29**. This material is available free of charge via the Internet at http://pubs.acs.org.

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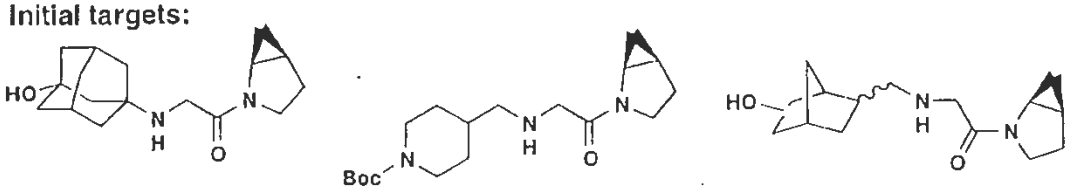
JM049924D

DPP4 Update 22 Oct 01 Dave Rotella

Initial targets:



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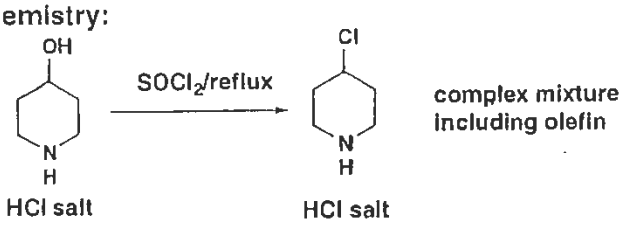
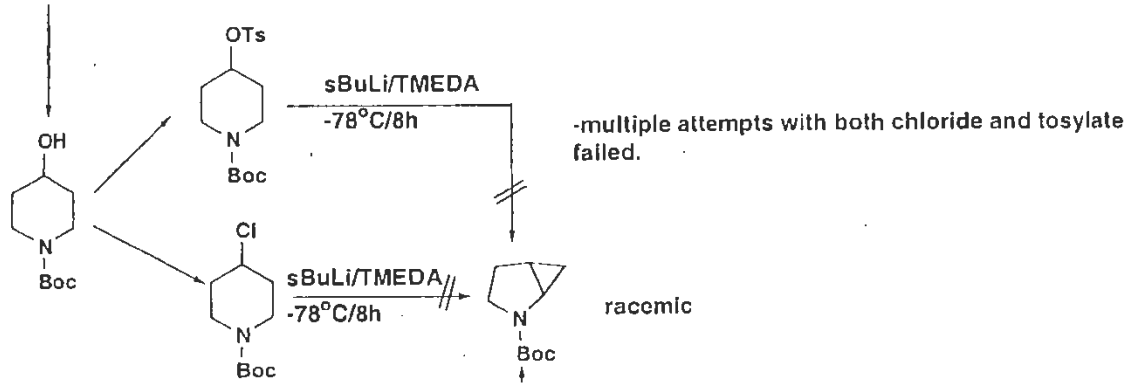
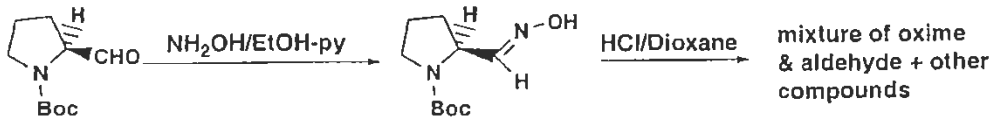






ca 7:1 mixture
of E/Z oxime isomers

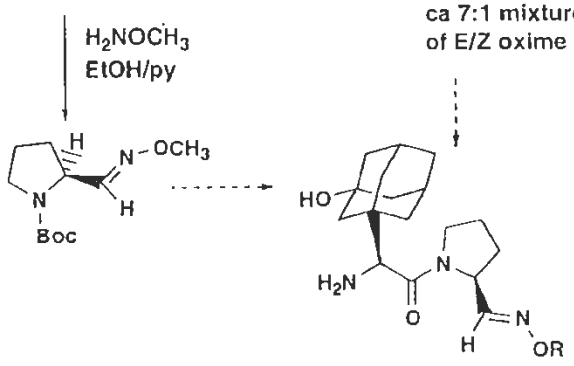
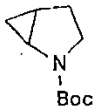
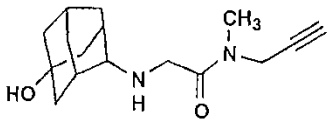


EXHIBIT #66
WIT: Rotella
DATE: 5-26-16
TOM FRASIK, CSR 6961

Plans:



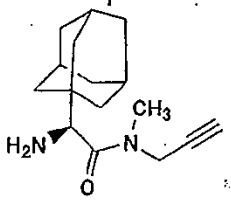
this week!



finish oxime-based compounds

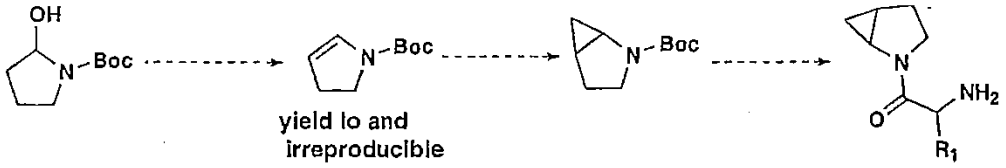
begin synthesis of [2.2.1]-bicycloheptane amines

DPP4 Update 19 Nov 01 Dave Rotella



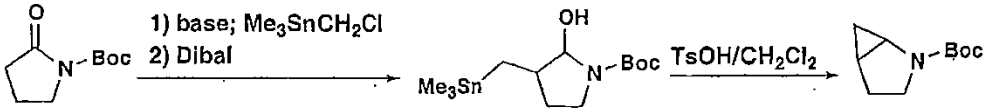
BMS-554158
inactive

Chemistry update

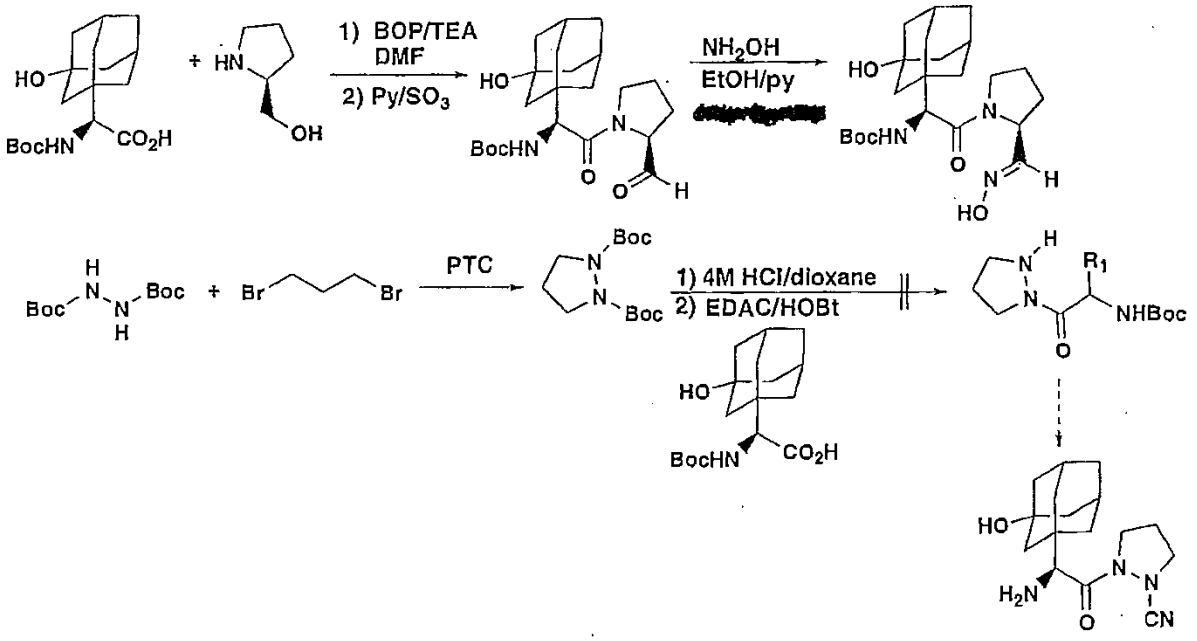


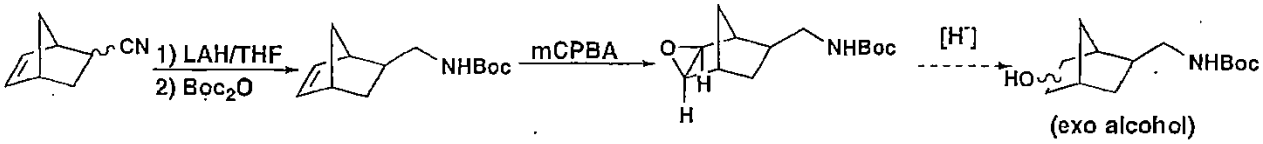
yield lo and
irreproducible

potential solution:

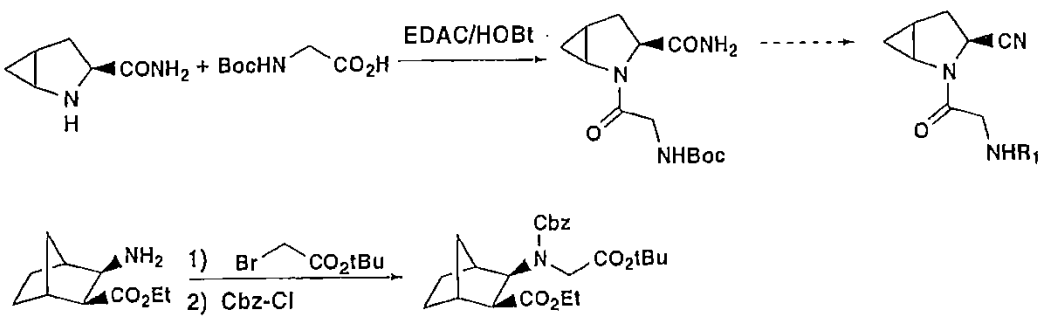


see e.g. Hannessian et al. BMCL 1998 (8) 2123 & Angew Chem Int Ed Eng 1997 (36) 1881





(exo alcohol)



Plans

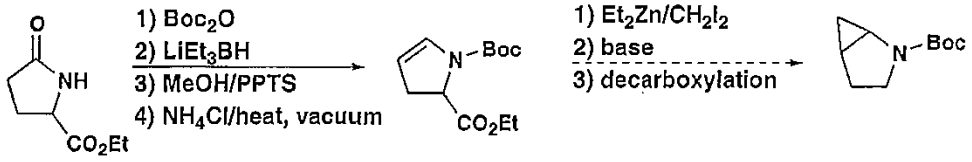
1) finish oxime & O-CH_3 oxime

2) explore R_1 in cyclopropyl cyanopyrrolidine above

3) prepare N-CN-N-acyl pyrazolines

DPP4 Update 17 Dec 01 Dave Rotella

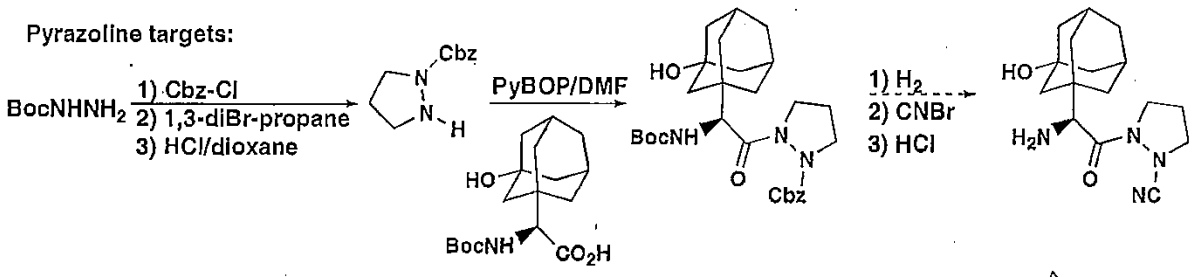
Des-cyanopyrrolidine targets:

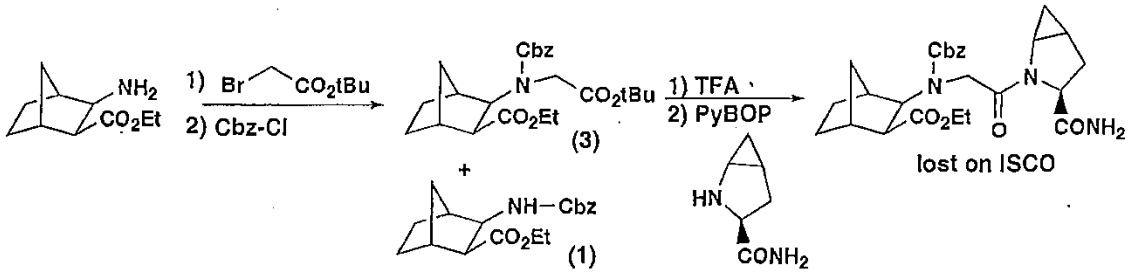


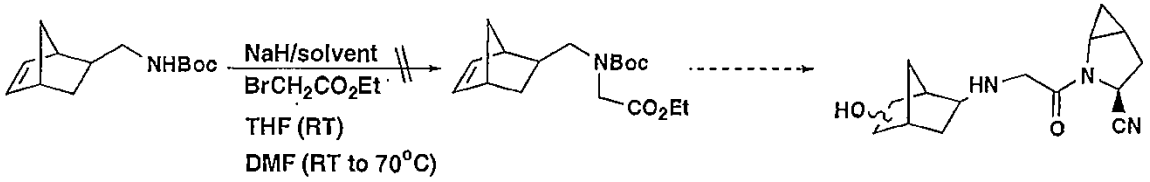
1.39 g
(15 g of Boc intermediate in hand)

800 mg

Pyrazoline targets:





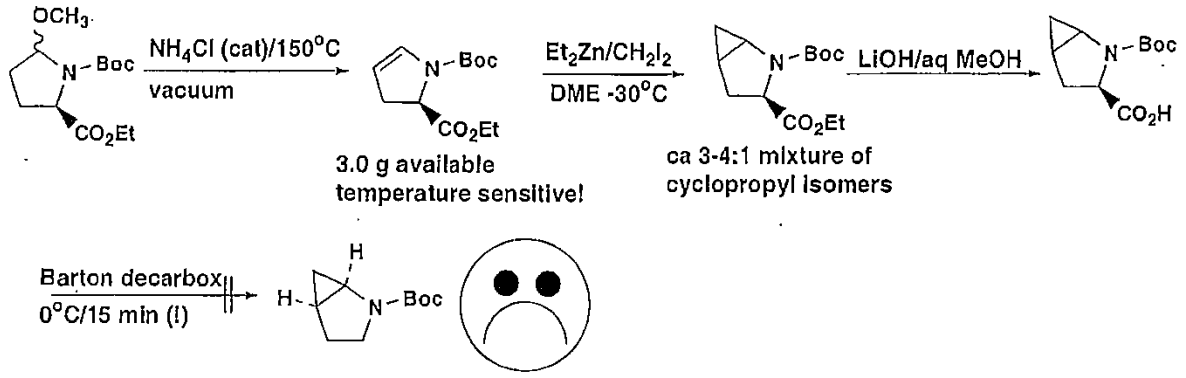


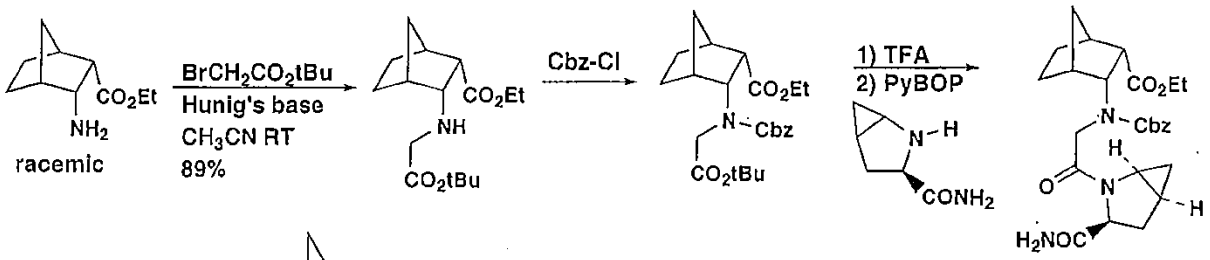
Plans:

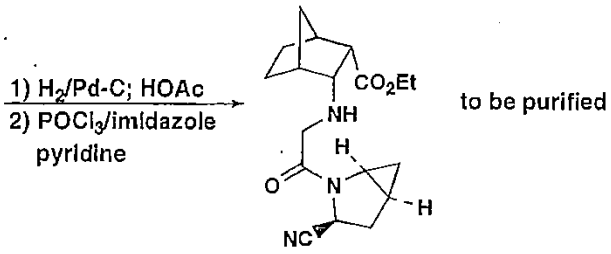
-cyclopropyl pyrrolidines

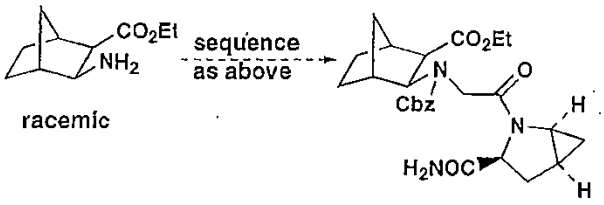
-pyrazolidine derivatives

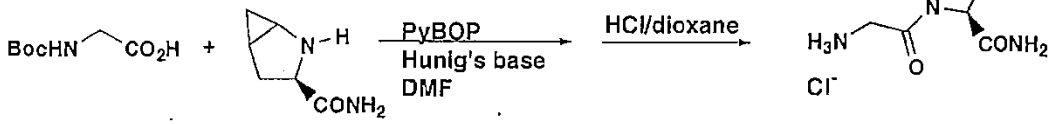
DPP4 Chemistry Update Dave Rotella 1 Feb 02







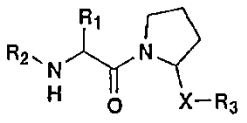




Plans

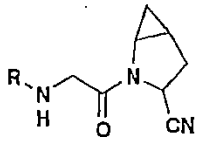
1) des cyano pyrroline analogs

2) "prime side" extended inhibitors:

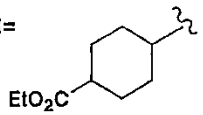


X=CH_2O and others
R_1=tBu initially, with R_2=H, R_3 based on DPP4 substrate preferences
other X to be evaluated: olefin, amides
may also consider replacing proline with 2-substituted piperidine

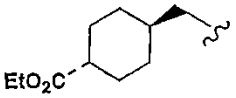
3) N-substituted analogs:

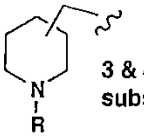


R=

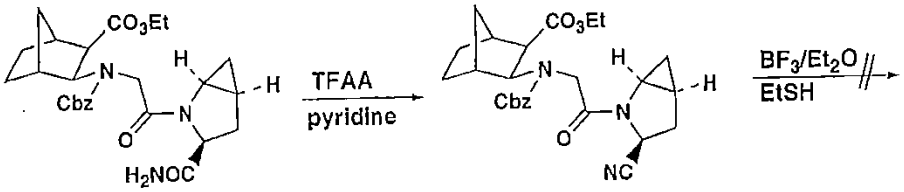


cis & trans

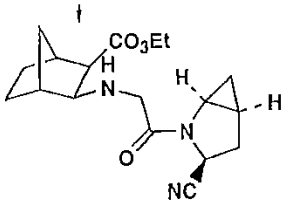




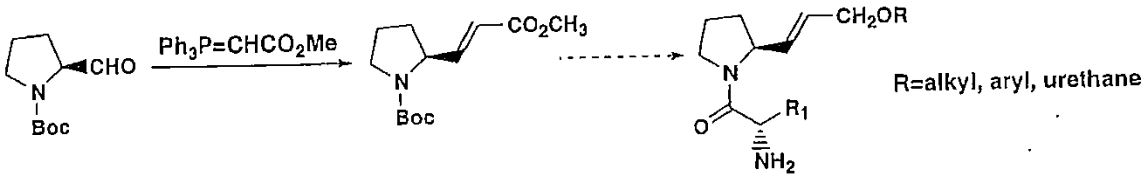
3 & 4-substituted



1) H_2
2) dehydration



(separate diastereomers If possible)

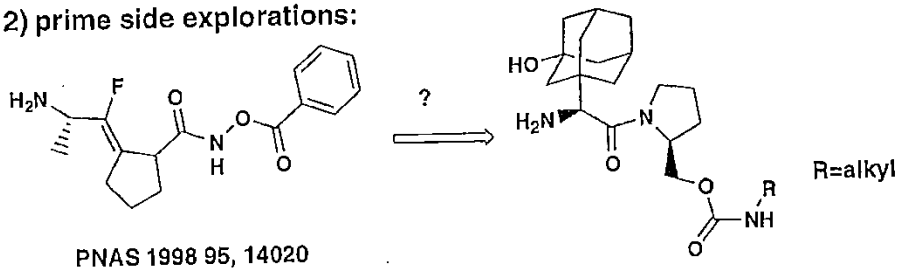


R=alkyl, aryl, urethane

Plans

1) cyclopropyl pyrrolidines

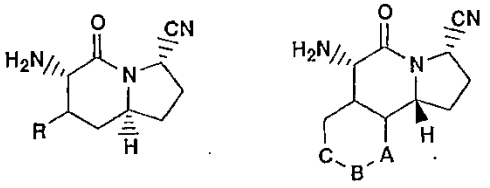
2) prime side explorations:



R=alkyl

PNAS 1998 95, 14020
188 nM DPP4 inhibitor

3)

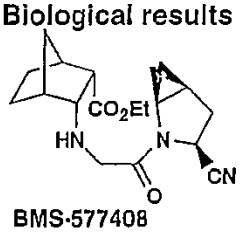


Prevents intramolecular cyclization of amine and nitrile

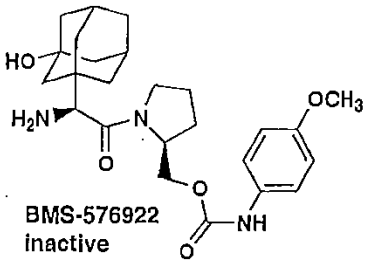
A, B, C=CH_2 or N-R

DPP4 Update Dave Rotella 12 Mar 02

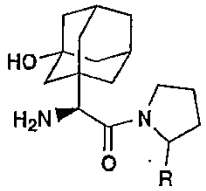
Biological results



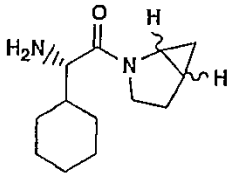
BMS-577408
Inactive
(mixture of diastereomers)



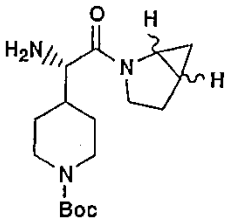
BMS-576922
inactive



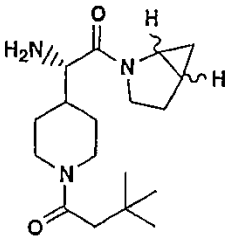
BMS-582879 R=benzyl
BMS-582833 R=CH_2OCH_3
BMS-582887 R=CH_3
inactive

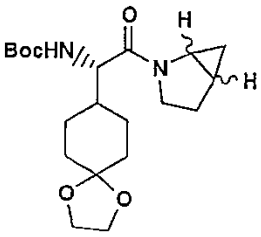


BMS-584226
pending



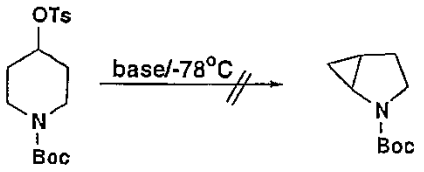
ready for submission





submission after PG cleavage

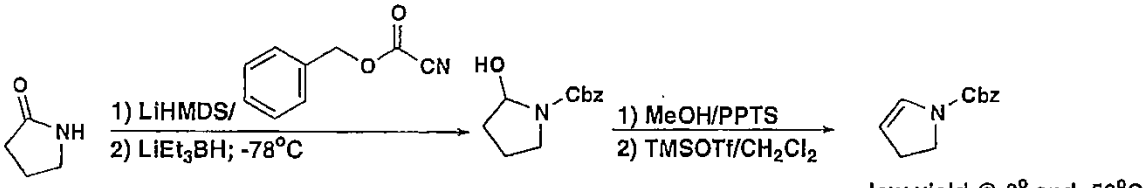
Chemistry



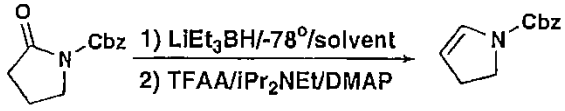
Selected Conditions:
1) 1.1 eq t-BuLi +/- TMEDA
2) 2.2 eq t-BuLi +/- TMEDA
3) 2.2 eq s-BuLi + TMEDA

reactions tried by adding tosylate to base/TMEDA. Base + TMEDA complex "aged" for 15 or 30 minutes prior to adding tosylate. In addition, base was added to tosylate-TMEDA complex. THF contained either no peroxide stablizer or 25 ppm BHT (s-BuLi). Sure seal ether from Aldrich (fresh bottle for each reaction). TMEDA redistilled from CaH_2 and stored over activated sieves.

in every reaction, starting material remains. Crude NMR of several reactions shows no evidence for cyclopropane formation



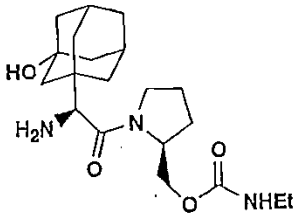
low yield @ 0° and -50°C
(ca. 5-10%)
NOT VOLATILE



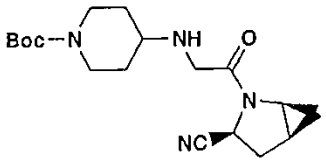
-78 to RT 15-25%
solvents: THF & toluene

DPP4 Update Dave Rotella 6 May 02

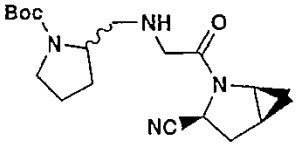
Biology update



BMS-586480 inactive

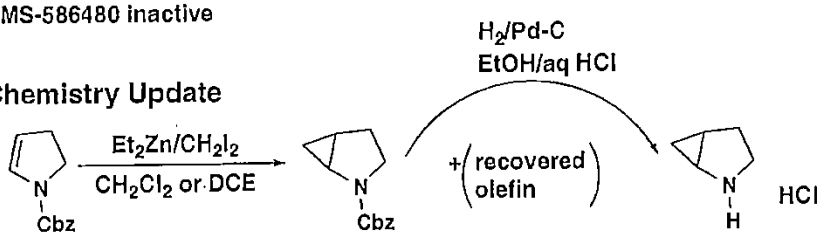


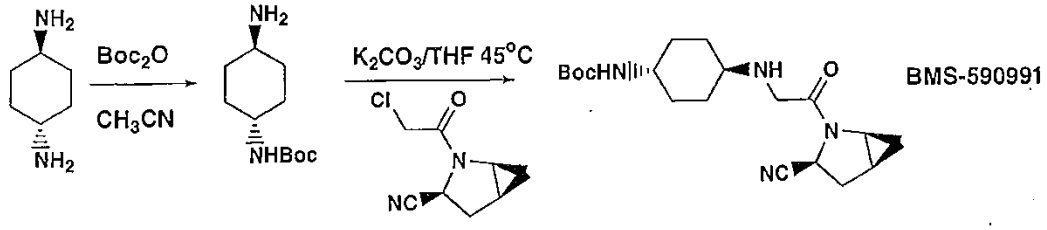
BMS-585981 1 μM

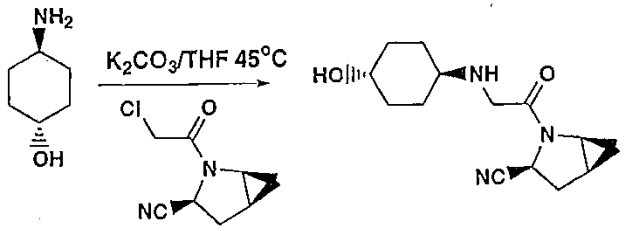


BMS-585980 ([R] Isomer)
BMS-585979 ([S] isomer) } inactive

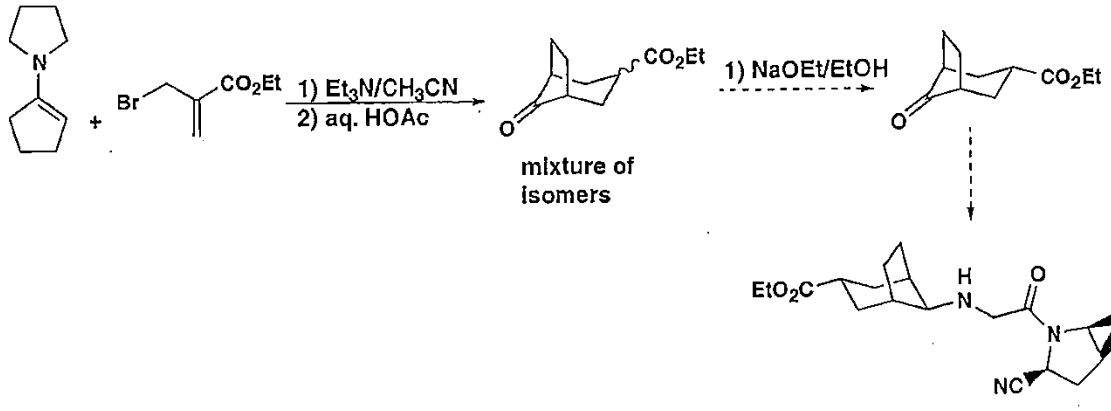
Chemistry Update



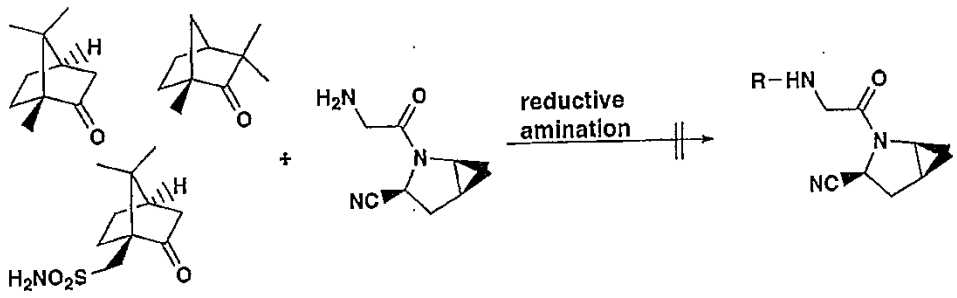


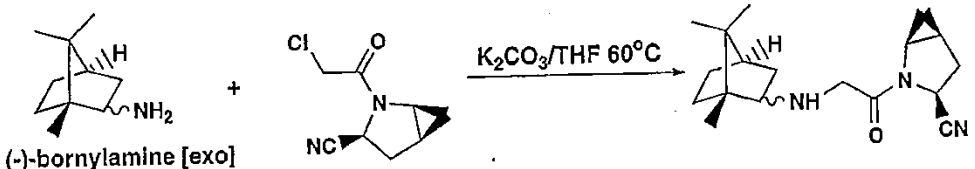


compound not stable-decomposes upon attempted purification



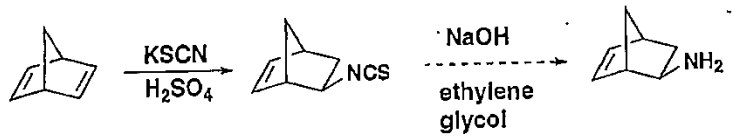
mixture of isomers



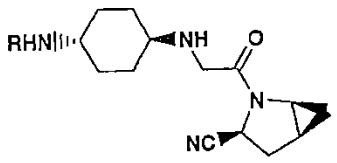


(-)-bornylamine [exo]
(-)-isobornylamine [endo]

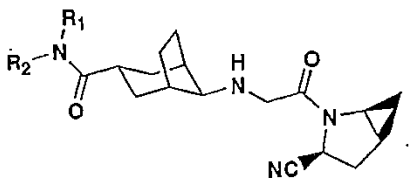
BMS-591268 [exo]
BMS-591269 [endo]

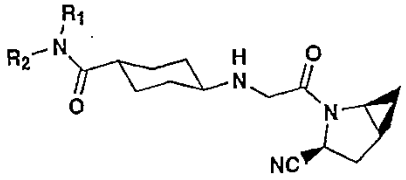


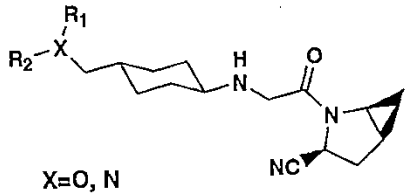
Plans



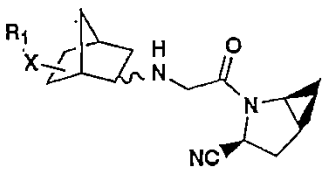
R=amides, sulfonamides (6-10 of each, diverse set)





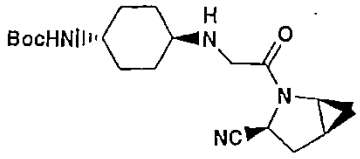


X=O, N

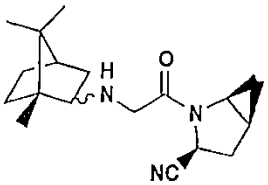


DPP4 Update 10 June 02 Dave Rotella

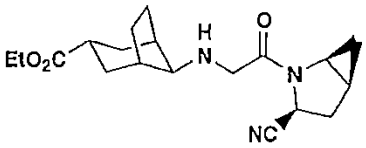
Biology Update



BMS-590991
74 nM

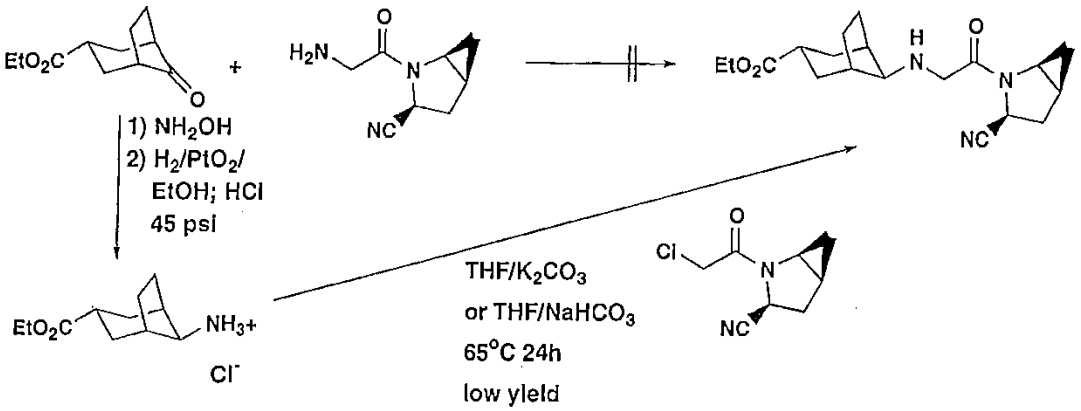


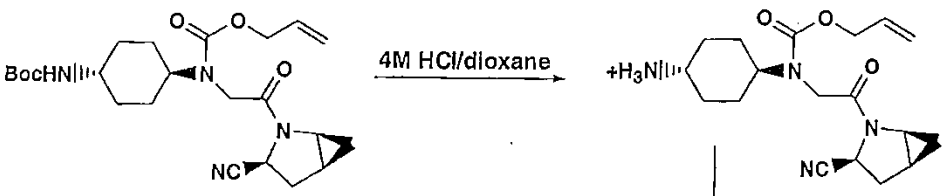
BMS-591268 [exo] 1.1 μM
BMS-591269 [endo] 170 nM

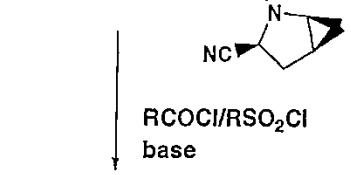


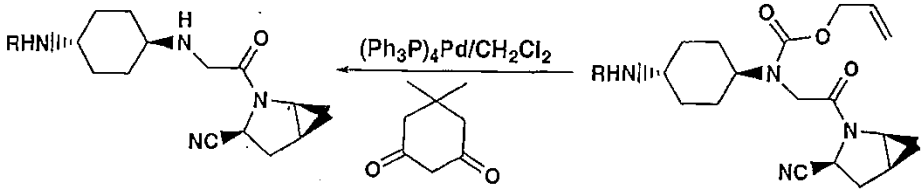
BMS-593907
600 nM

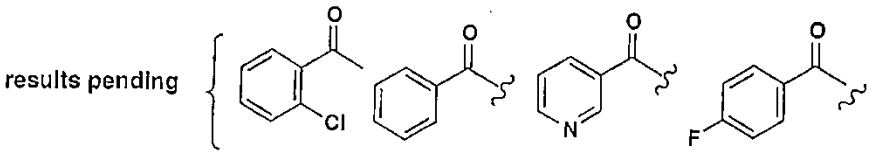
Chemistry Update:



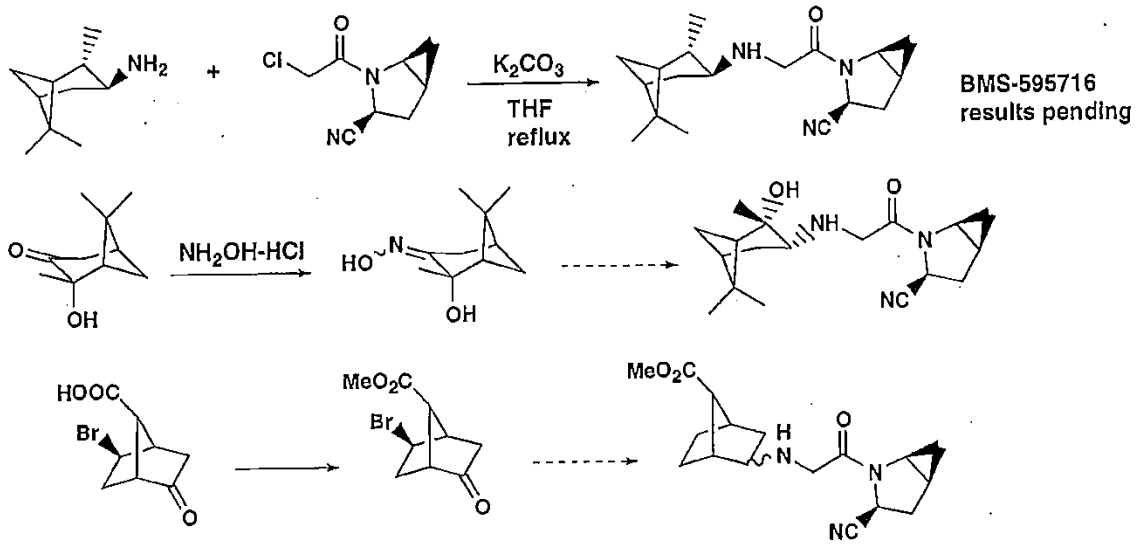




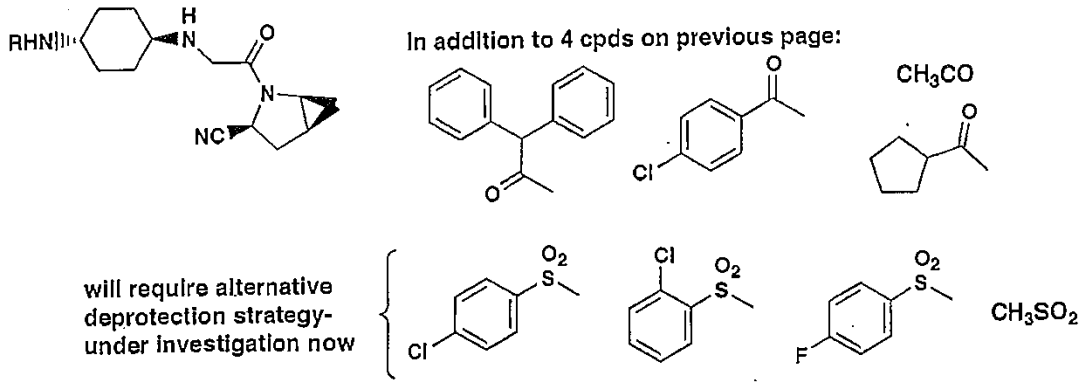




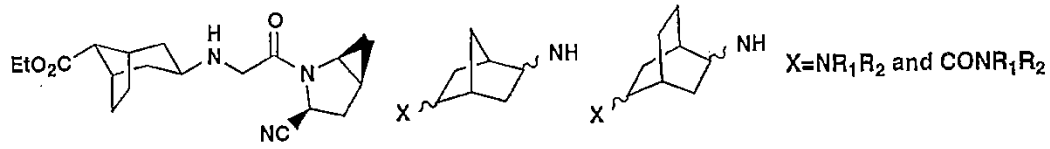
⟹ DEPROTECTION FAILS WITH SULFONAMIDES



Plans:

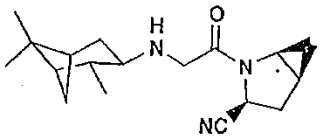


Explore other bicylic frameworks, e.g.:

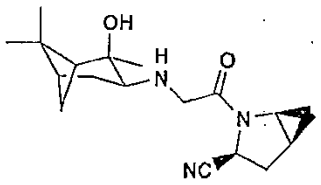


DPP4 Update Dave Rotella 5 August 02

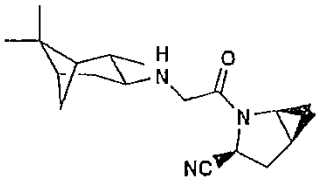
Biology Update



BMS-599749 Ki=76 nM

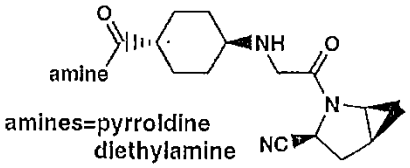


BMS-602531 Ki=3200 nM

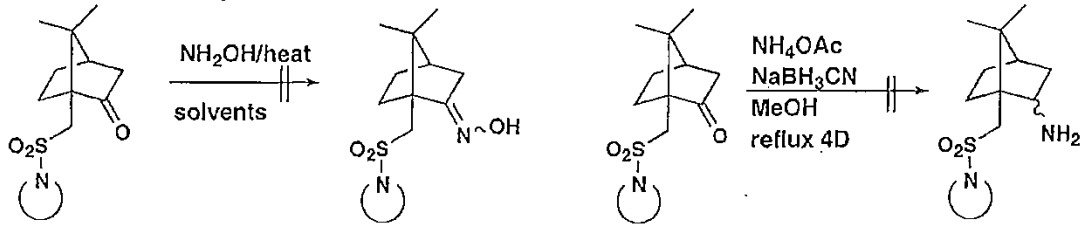


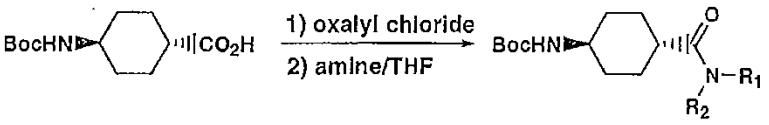
BMS-595716 Ki=65 nM
(linear binding kinetics)
ex vivo DPP4I 67% inhib.
effect on glucose, following
oGTT: 85% of control (=to
BMS-477118 @ 3 μM/kg)
solution stability 6.2 days

Ready to submit:

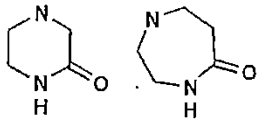


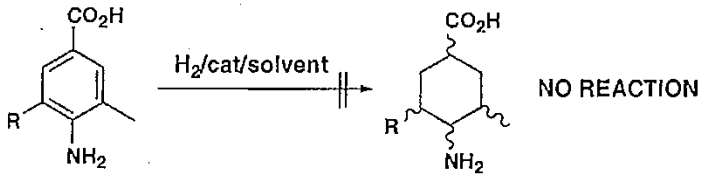
Chemistry update





amines=pyrrolidine
diethylamine
t-Butyl ethylamine
2-OH-ethyl-ethylamine
2-OMe-ethyl-ethylamine

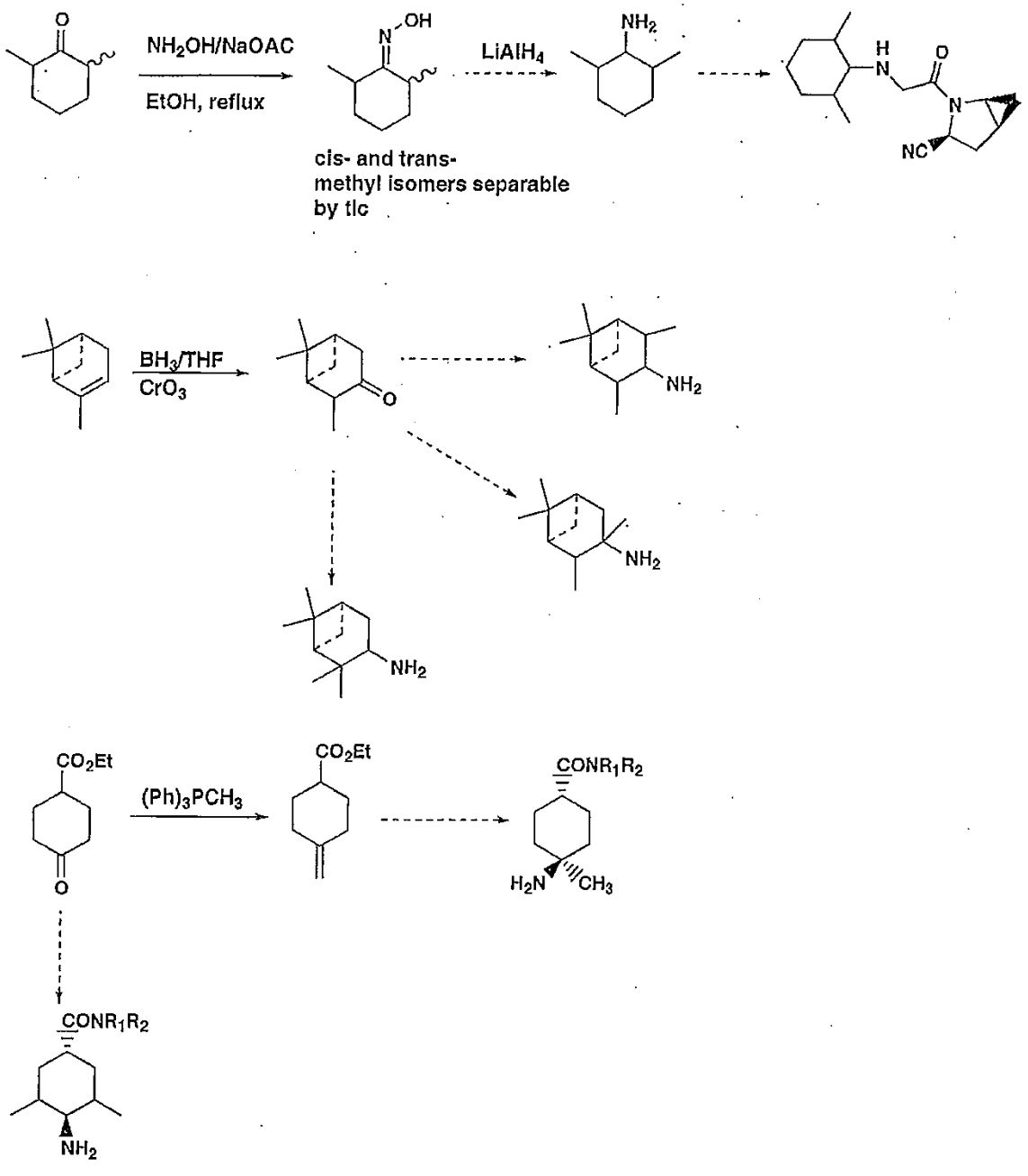




NO REACTION

R=H, CH_3

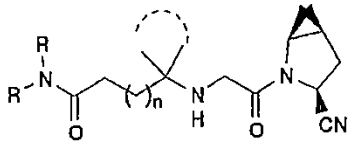
catalysts: Rh/C, Rh/Al, Pt/C
solvents: EtOH, EtOH/HCl, HOAc
60 psi H_2 in all reactions


NH2OH/NaOAC
EtOH, reflux
LiAlH4
cis- and trans-
methyl isomers separable
by tlc
BH3/THF
CrO3
(Ph)3PCH3
CO2Et
CONR1R2
NH2
H2N
CH3
NC

Plans:

1) examine amides:

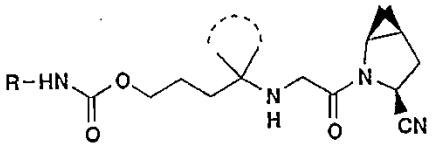
R=cyclic, acyclic



n=0, 1

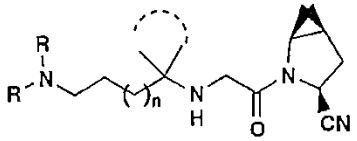
2) examine alcohol derivatives:

R=aromatic, alkyl



3) examine amine derivatives:

R=acyl, sulfonyl, alkyl

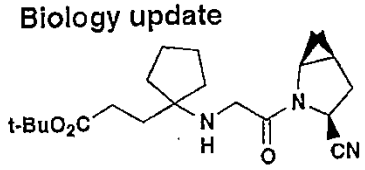


n=0, 1

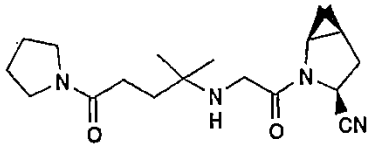
ALL OF THE ABOVE DEPEND ON POSITIVE DATA FROM SOLUTION STABILITY TESTING. DATA ANTICIPATED LATE THIS WEEK/EARLY NEXT WEEK.

DPP4 Update 21 Oct 02 Dave Rotella

Biology update

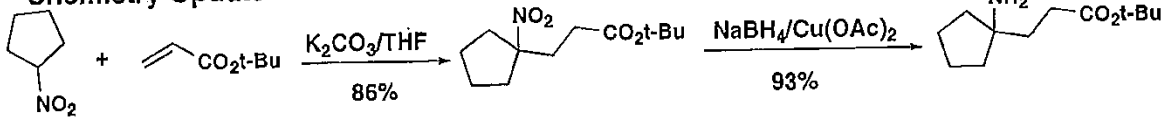


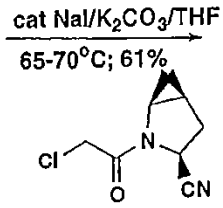
BMS-632394 submitted



ready for submission

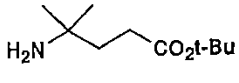
Chemistry Update

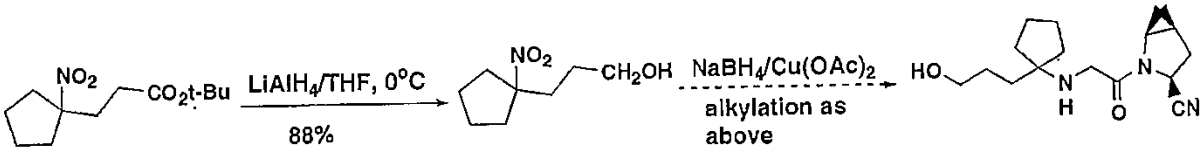


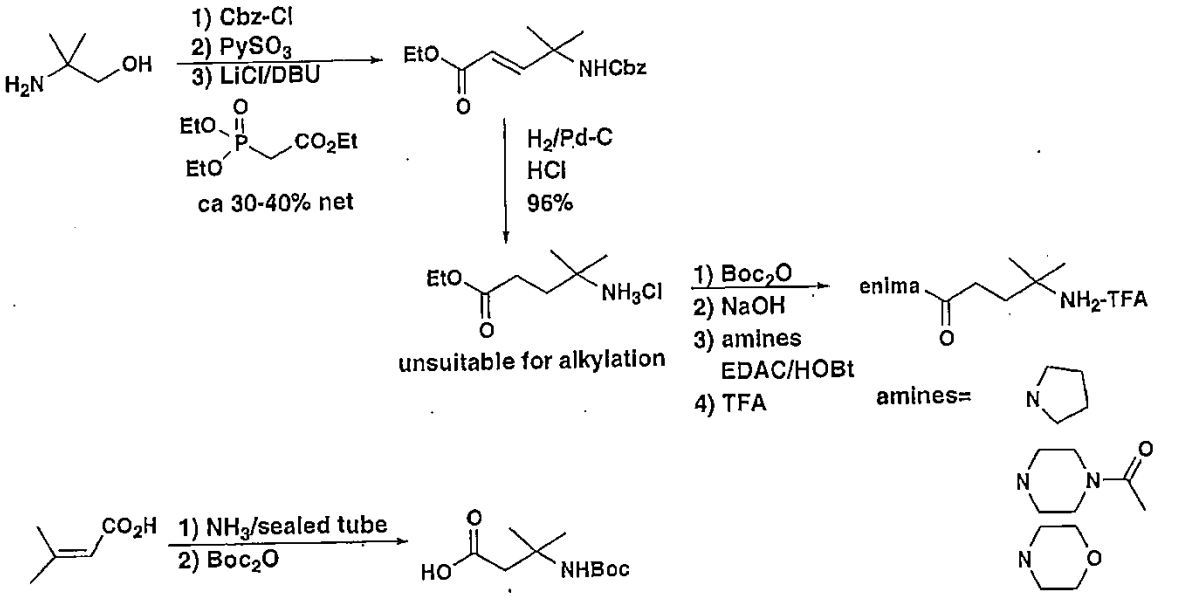


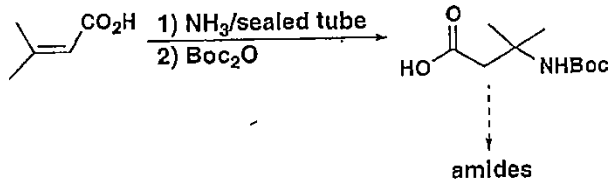
BMS-632394

identical route to:

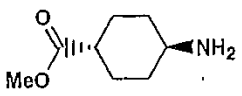




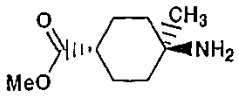




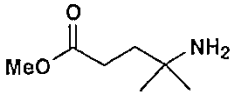
Modeling Studies:



A



B



C

compared conformationally by Doree Sitkoff

A has a more limited number of low energy conformations available to it than B

C has a larger number of low energy conformations because it is more flexible

In order to achieve optimal DPP4 inhibition, it may be necessary to limit conformational flexibility of chain extending from gem-dimethyl moiety, in combination with correct (e.g. amide) funtional group at end of chain.

What makes a good DPP4 inhibitor with slow binding properties?

-slow binding is important to achieve prolonged pharmacodynamic effect associated with qd/bid dosing

-the only des-cyano slow binders identified to date contain adamantyl moieties

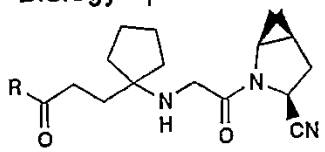
-SAR associated with slow binding remains complex & difficult to predict in nonadamantyl containing DPP4 inhibitors

-slow binding can be achieved with both C- and N-substituted compounds-and now we have N-substituted slow binders with excellent solution stability

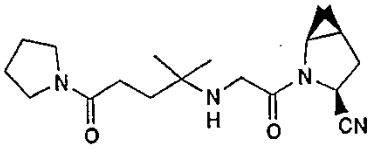
-BMS-471211 (Novartis 2nd generation cpd) has K_i=80 nM, K_i^*=8 nM; best **stable** N-subst. cpd with slow binding properties to date has K_i=220 nM, K_i^* TBD. Several N-substituted cpds with K_i^* ca 15-30 nM have shown good *ex vivo* DPP4 inhibition and glucose lowering in Zucker rat model.

DPP4 Update Dave Rotella 18 Nov 02

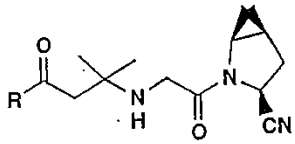
Biology Update



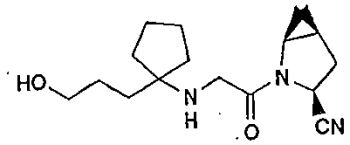
BMS-632394 R=CO_2t-Bu K_i=540 nM
BMS-639391 R=NMe_2 K_i=520 nM
BMS-639542 R=$NHCH_2Ph$ K_i=260 nM



BMS-633105 K_i=310 nM

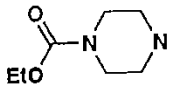


R=NMe_2 BMS-639389 K_i=530 nM
R=pyrrolidinyl BMS-635218 K_i=440 nM
R=morpholinyl BMS-635219 K_i=590 nM

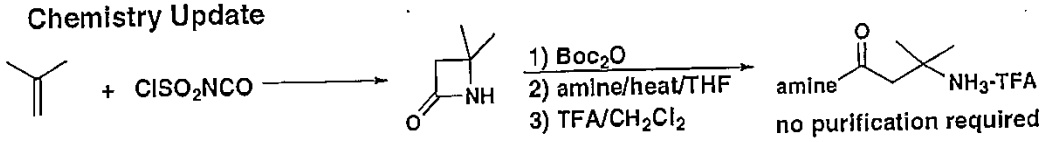


BMS-634593 K_i=390 nM

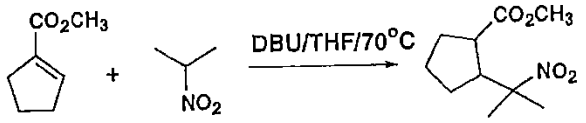
Ready for submission:
R=4-pyridylmethylamino, cyclopentylamino, benzylamino, isopropylamino



Chemistry Update



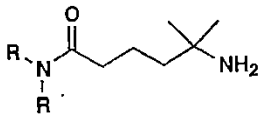
no purification required



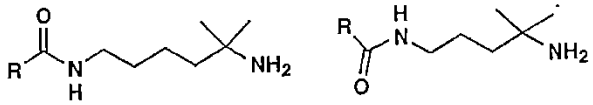
$NaBH_4/Cu(OAc)_2$ and $NaBH_4$/Pd-C failed- former complex mix, latter no reaction

Plans:

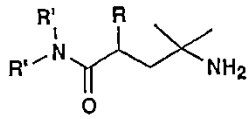
1) explore longer chain analogs:



2) explore amino analogs: e.g.



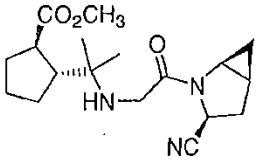
3) explore substitutions on alkyl chain: e.g.



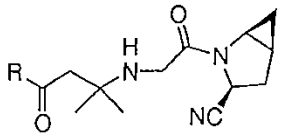
R=CH_3, Ph, IC_3H_7

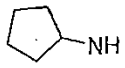
DPP4 Update 17 December 02 Dave Rotella

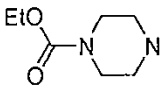
Biology Update:



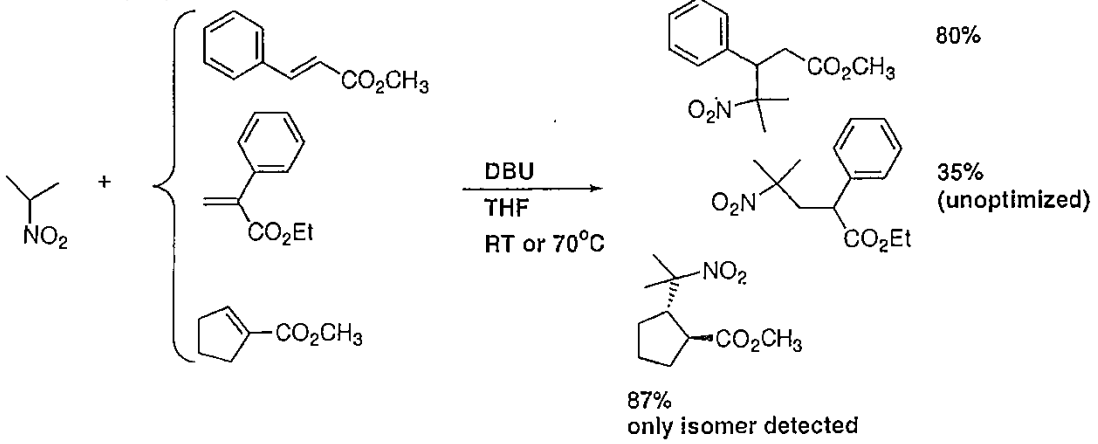
BMS-641032 Inactive



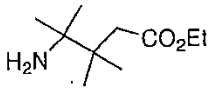
R=  BMS-640210 K_i=365 nM

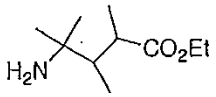
R=  BMS-640211 Inactive

Chemistry update:

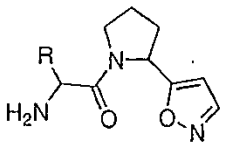


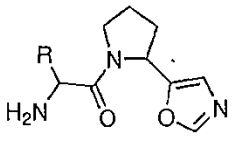
Plans



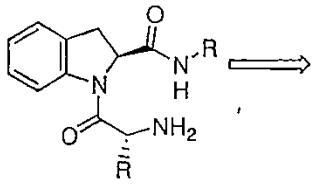


to probe other possible steric/conformational effects on DPP4 inhibition

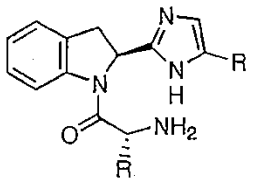




based on possible analogy with tripeptidyl peptidase II-see *J. Med. Chem.* 2002, *45*, 5303:



7 nM TPPII inhibitor prone to intramolecular cyclization



3 nM TPPII inhibitor with in vivo efficacy

Specific and Irreversible Cyclopeptide Inhibitors of Dipeptidyl Peptidase IV Activity of the T-Cell Activation Antigen CD26

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The dipeptidyl peptidase IV (DPP IV) activity of CD26 is characterized by its post-proline-cleaving capacity that plays an important but not yet understood role in biological processes. Here we describe a new family of specific and irreversible inhibitors of this enzyme. Taking into account the substrate specificity of DPP IV for P_2-P_1> <-P_1' cleavage, we have designed and synthesized cyclopeptides c[($^{\alpha}H_2N^+$)-Lys-Pro-Aba-(6-CH_2-S^+R_2)-Gly_n] $2TFA^-$ (Aba = 3-aminobenzoic acid, R = alkyl) possessing a proline at the P_1 position and a lysine in the P_2 position, which allows the closing of the cycle on its side chain. These molecules show a free N-terminus, necessary for binding to the CD26 catalytic site, and a latent quinoniminium methide electrophile, responsible for inactivation. Treatment of c[$^{\alpha}$Z-Lys-Pro-Aba-(6-CH_2-OC_6H_5)-Gly_n], obtained by peptide synthesis in solution, with R_2S/TFA simutaneously cleaved the Z protecting group and the phenyl ether function and led to a series of cyclopeptide sulfonium salts. These cyclopeptides inhibited rapidly and irreversibly the DPP IV activity of CD26, with IC_{50} values in the nanomolar range. Further studies were carried out to investigate the effect of the modification of the ring size (n = 2 or 4) and the nature of the sulfur substituents (R = Me, Bu, Oct). Cycle enlargement improved the inhibitory activity of the methylsulfonio cyclopeptide, whereas the increase of the alkyl chain length on the sulfur atom had no apparent effect. Other aminopeptidases were not inhibited, and a much weaker activity was observed on a novel isoform of DPP IV referred to as DPP IV-β. Thus, this new family of irreversible inhibitors of DPP IV is highly specific to the peptidase activity of CD26.

Introduction

Dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5), a membrane-bound exopeptidase, has been classically associated to the T-cell activation antigen CD26, a multifunctional sialoglycoprotein expressed on a variety of different epithelia and also by different hematopoietic cell types (for some recent reviews, see Yaron and Naider,[1] and Fleischer et al.[2]). Dipeptidyl peptidase IV (CD26) is a serine protease which has the unique specificity to cleave dipeptides from the N-terminus of polypeptides provided that proline is the penultimate residue.[1] In HIV infection, CD26 has been implicated in the viral entry process and its cytopathic effect.[3] Furthermore, DPP IV activity inhibition by HIV-1 Tat protein has been proposed as the mechanism of the lack of response to recall antigens observed in early stages of HIV infection;[4] however, the relevance of this inhibition in physiological conditions is unclear.[5] Whatever is the case, the addition of soluble CD26 can restore response to recall antigens of HIV-infected individuals in vitro.[6]

Irrespective of its peptidase activity, CD26 is associated with other molecules on the cell surface. It has been shown to be the main receptor of adenosine deaminase,[7] and on T-lymphocytes, CD26 is associated with CD45,[8] a cell-surface-expressed phosphotyrosine phosphatase involved in signal transduction. Both features of CD26, its signaling capacity and its peptidase activity, contribute to the costimulatory function of CD26 in T-cell activation events. However, the role of DPP IV activity of CD26 in these events is unclear. Some authors have described that small synthetic inhibitors of DPP IV impair mitogen and antigen stimulation of PBMC and other lymphocytic cell types.[9] In contrast, others have found no effect of DPP IV inhibitors on stimulated T-cells.[10] Similarly, contradictory results on the function of the DPP IV activity of CD26 have been reported by using cell lines expressing a mutated, catalytically inactive form of CD26.[11,12] The failure to understand the role of DPP IV activity is, in part, due to the fact that no physiological substrates have been identified. However, a broad spectrum of bioactive peptides, including some interleukines, chemokines, neuropeptides, and growth factors, can be potentially cleaved by DPP IV.

The availability of stable specific irreversible inhibitors or highly potent reversible inhibitors of DPP IV should be useful in studies for the determination of the physiological and pathological role(s) of this enzyme. Several competitive, tight-binding, or irreversible inhibitors of the enzyme are already known: oligopeptides with the N-terminal X-Pro sequence (X = various amino acid residues) such as the diprotins A and B (Ile-Pro-Ile or Val-Pro-Leu),[13,14] X-pyrrolidides and X-thiazolidides,[14−16] X-cyanoPro and X-cyanoThia,[17−19] X-phosphonoPro or Pip aryl esters,[20,21] X-boroPro,[22−25] X-ProCH_2N^+-

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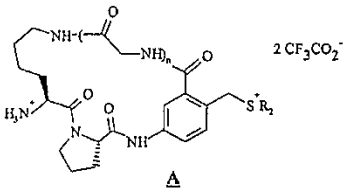
Chart 1. General Structure of the Cyclopeptides A Designed for Irreversible Inhibition of the DPP IV Activity of CD26



A

Me$_3$,[26] X-azaPro derivatives,[27] X-Pro-*N*-(arylcarbonyloxy)-amides,[28,29] and one of its ψ(CF=C) fluoro olefin isosteres.[30,31] Owing to the presence of a free amino N-terminus and the flexibility of the imino peptide bond, several of these inhibitors easily cyclize and are not very stable in solution.[32] The diacylated hydroxylamines are mechanism-based irreversible inhibitors of the protease: a demasked acylnitrene can react directly with an active site nucleophile or can lead, through a Lossen rearrangement, to an electrophilic isocyanate.[33]

We have previously designed and studied functionalized cyclopeptides containing a latent quinoniminium methide electrophile as suicide substrates for serine proteases.[34] Taking into account the substrate specificity of the DPP IV enzyme for P_2-Pro> <-P'_1 cleavages (P_1 = Pro; Schechter and Berger notation[35]), we have now designed, synthesized, and studied cyclopeptides **A** (Chart 1): c[($^{\alpha}H_2N^+$)-Lys-Pro-Aba-(6-CH_2-S^+R_2)-Gly$_n$] 2TFA$^-$ (Aba = 3-aminobenzoic acid, R = alkyl), possessing the same latent electrophile, as selective suicide substrates for this exoprotease. These cyclopeptides were able to induce the complete, rapid, and irreversible inhibition of the DPP IV activity of CD26 with IC_{50} in the nanomolar range. Their specificity was demonstrated by the lack of its effect on the activity of other peptidases, including the cell-surface-expressed DPP IV-β, the recently described protein with typical DPP IV activity.[36]

Results

From the relative weak importance of the nature of the P_2 side chain on the rate of hydrolysis of the enzyme substrates (vide infra), we hypothesized that the cyclization to the ϵ-amino function of a P_2 lysine residue would result in new molecules able to bind the catalytic site of the DPP IV activity in CD26. Moreover, this cyclization would leave the N-terminal α-amino group free and protonated, a necessary condition for the recognition by the enzyme (Chart 1). In macrocycles **A**, the substituted P'_1 aminobenzoic acid residue Aba-(6-CH_2-S^+R_2) is a precursor of the latent electrophilic quinoniminium methide cation. The presence of different numbers of glycine residues (n) in the cyclopeptides **A** allows the variation of the ring size, whereas the nature of the sulfur substituents (R) will modify the bulk and the lipophilicity in this part of the molecule. The influence of the leaving group ability on the inhibition efficiency and selectivity has been also examined by replacing the benzylic sulfonium substituent by an acetate group.

Chemistry. Cyclopeptides **A** were prepared according to the reaction sequence shown in Scheme 1. Peptide synthesis in solution, using DCC/HOBt for the formation of the amide bonds, was applied for the synthesis of the linear precursors of these macrocycles. The strategy involved the use of a nitro substituent as a latent amino group.[37] Coupling of the 2-(phenoxymethyl)-5-nitrobenzoic acid (Nba-[CH_2OPh], **5**[38]) with either ethyl diglycinate or ethyl tetraglycinate gave the substituted ethyl nitrobenzoyl polyglycinates **6a,b**. Selective catalytic hydrogenation of the nitro substituent, without hydrogenolysis of the benzyl ether function, occurred in the presence of platinium oxide in MeOH/DMF. The unstable aminobenzoyl polyglycine derivatives were rapidly acylated with *N*-Boc-L-proline to generate compounds Boc-Pro-Aba-[CH_2OPh]-Gly$_n$-OEt **7a,b**. Cleavage of the N-protecting group occurred in trifluoroacetic acid. In the following coupling step, the orthogonally diprotected Z-L-Lys(Boc)-OH derivative was used. The linear peptides **8a,b** were obtained in good yields. Cyclization of these precursors was achieved by the azide method: hydrazinolysis of the ester function leading to protected hydrazides **9a,b**, selective cleavage of the N$^{\epsilon}$-Boc group of the lysine residue in the presence of the N$^{\alpha}$-Z protecting group, treatment of the resulting hydrazides with an alkyl nitrite, and dilution in DMF in the presence of a tertiary amine. The cyclization yields were 45% and 44% for **10a,b**, respectively.

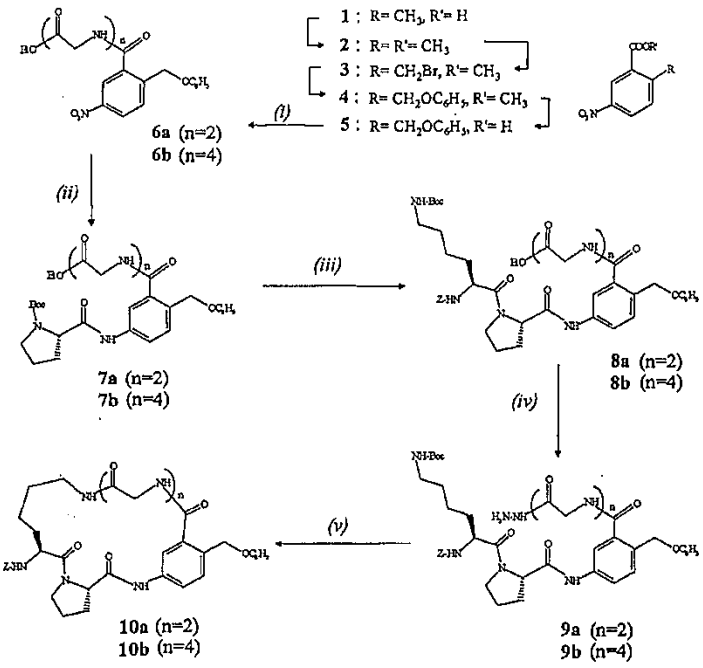
Organic sulfides, such as thioanisole, in trifluoroacetic acid are known to deprotect *O*-benzyltyrosine without the formation of O-to-C rearrangement products[39] and also to cleave the *N*-benzyloxycarbonyl protecting group.[40] The reactions occur by a "push−pull mechanism": nucleophilic attack of the sulfide lone pair on the benzylic carbon of a protonated ether or carbamate function. The byproducts in these deprotection reactions are sulfonium salts. We reasoned that treatment of the c[$^{\alpha}$Z-Lys-Pro-Aba-(6-CH_2-OC_6H_5)-Gly$_n$] cyclopeptides with various dialkyl sulfides in trifluoroacetic acid should cleave both the Z protecting group and the phenyl ether function (Scheme 2), thus leading to sulfonium salts having a protonated N-terminus. Effectively, such a treatment gave the bis trifluoroacetate salts **A**: **11a** (n = 2, R = Me, 62% yield), **11b** (n = 4, R = Me, 60% yield), **12a** (n = 2, R = Bu, 74.5% yield), **13a** (n = 2, R = Oct, 71% yield).

For the preparation of the cyclopeptide having a benzylic acetoxy substituent, the corresponding dimethylsulfonium salt **11a** was treated with potassium acetate in dry DMF. The resulting acetate was not very stable and decomposed during purification. The crude product was therefore reacted with di-*tert*-butyl dicarbonate to give the stable N-protected derivative which was easily purified by chromatography. Treatment with trifluoroacetic acid cleaved the Boc protecting group and led to the expected acetate salt **15a** in 76% yield.

Finally, selective hydrogenolysis of the Z protecting group of the cyclopeptide in the presence of the benzylic phenyl ether function was achieved by using a palladium on carbon catalyst in aqueous methanol and gave the cyclopeptide **16a** possessing a phenoxy substituent (Scheme 2).

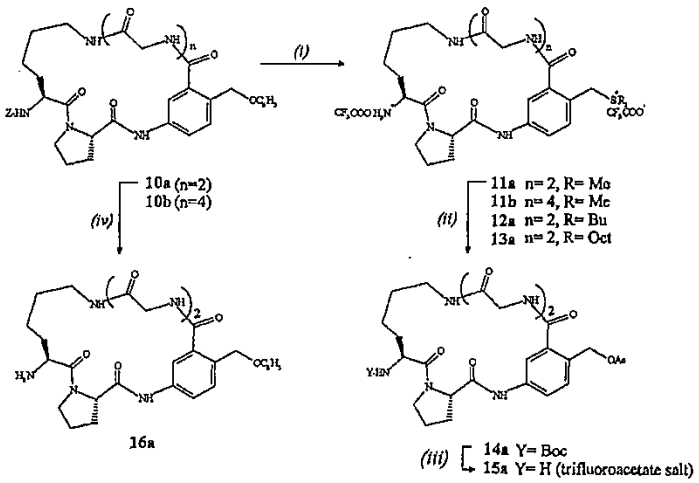
For comparison, simplified linear analogues of the cyclopeptides **A** were studied. The trifluoroacetate salts H_2^+-Ala-Pro-Aba(6-CH_2-S^+Me_2)-OMe and H_2^+-Lys(H^+)-

Scheme 1. Synthesis of the Cyclopeptides **10a,b** Having a Benzylic Phenoxy Substituent and a Terminal N-Z Protecting Group[a]



[a] (i) Gly_nOEt, DCC/HOBt; (ii) 1. H_2/PtO_2, MeOH–DMF, 2. Boc-Pro-OH, DCC/HOBt; (iii) 1. CF_3CO_2H or HCl/CH_2Cl_2, 2. Z-Lys(Boc)-OH, DCC/HOBt; (iv) NH_2NH_2/MeOH; (v) 1. CF_3CO_2H/CH_2Cl_2, 2. HCl/DMF–THF, 3. *i*-$PrNEt_2$/DMF.

Scheme 2. Last Steps of the Synthesis of Cyclopeptides **11a–16a**[a]



[a] (i) R_2S/CF_3CO_2H; (ii) 1. KOAc/DMF, 2. Boc_2O; (iii) CF_3CO_2H; (*iv*) H_2/Pd/C, MeOH.

Pro-Aba(6-CH_2-S^+Me_2)-OMe (**18a,b**) were prepared as above from the corresponding benzylic ethers H_2^+-X-Pro-Aba(6-CH_2-OC_6H_5)-OMe (**17a,b**).

Biological Studies. In all the studies described here, the dipeptidyl peptidase activity of CD26 and DPP IV-β was investigated using the enzymes in their natural habitat, i.e., by using either crude cell extracts or intact cells[36] as described in the Experimental Section. Indeed, CD26 and DPP IV-β are expressed on the cell surface, and thus their enzymatic activity could be assayed by using intact cells.[36] As a source of CD26 and DPP IV-β, we used either human CEM cells overexpressing CD26 by transfection[49] or human C8166 cells which express only DPP IV-β.[36] In some experiments partially purified enzyme preparations were also used.[51] In the case of CD26, we also used immunoaffinity-purified preparations of CD26 as we have described previously.[36] For the measurement of the

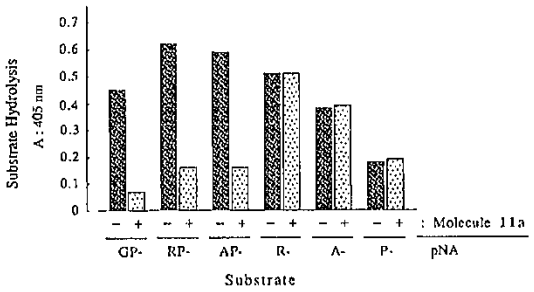


Figure 1. Specificity of molecule **11a**. Effect of inhibitor **11a** on different types of peptidase activities. Crude MOLT4 cell extracts[36] were assayed for the effect of 10 μM compound **11a** on different aminopeptidase activities. The peptidases tested were as follows: DPP IV by the cleavage of GP-pNA, RP-pNA, and AP-pNA; Arg-peptidase by the cleavage of R-pNA; Ala-peptidase by the cleavage of A-pNA; and Pro-peptidase by the cleavage of P-pNA. At 10 μM **11a**, the DPP IV activity against different substrates was inhibited by more than 75%, while no apparent effect on the other peptidases was observed. Abbreviations of the amino acids: G = glycine, P = proline, A = alanine, and R = arginine.

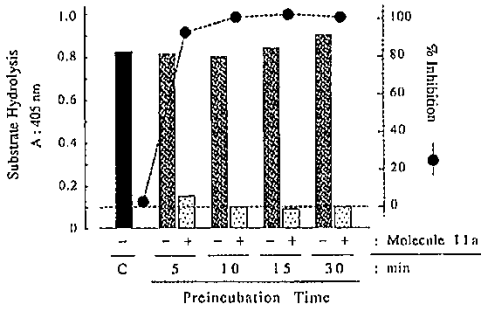


Figure 2. Kinetics of molecule **11a**-mediated inhibition of the cell-surface-expressed DPP IV activity of CD26. Intact CEM cells expressing high levels of CD26 (clone H01)[49] were incubated for 5, 10, 15, and 30 min in PBS with or without 5 μM compound **11a** at 37 °C and then washed twice with PBS. DPP IV activity of CD26 was then monitored by the cleavage of GP-pNA as described in the Experimental Section. The binding of the inhibitor **11a** to DPP IV active site is very rapid since 5 min is sufficient to obtain more than 75% inhibition.

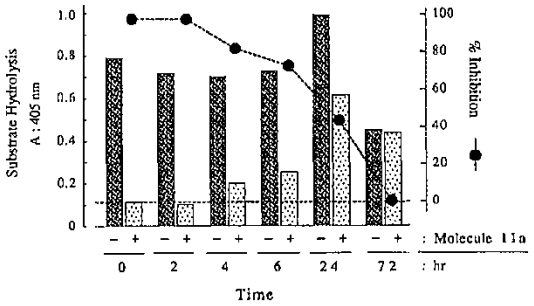


Figure 3. Irreversibility of DPP IV inhibition by molecule **11a** demonstrated by the cell-surface-expressed CD26. Intact CEM cells overexpressing CD26 (clone H01) were preincubated for 15 min in PBS in the absence or presence of 5 μM **11a** at 37 °C, then washed twice in PBS, and cultured in RPMI supplemented medium. Aliquots of cells were taken at the indicated times, washed twice with PBS, and then assayed for DPP IV activity by incubating cells with the substrate GP-pNA for 1 h at 37 °C. The samples at time 0 represent the DPP IV activity just after the 15 min of preincubation.

activity of different peptidases, extracts from human MOLT4 cells were used.[36]

Preliminary results pointed out that molecule **11a** was a potent inhibitor of DPP IV activity of CD26. Indeed, the IC_{50} value for the inhibition of the DPP IV activity of a purified preparation of CD26[36] was found to be 0.01 μM. For this reason, we first studied the specificity of this inhibitor by determining its effect on different aminopeptidases found in crude cell extracts. The peptidases tested were as follows: arginine-peptidase (EC 3.4.11.6) by the cleavage of Arg-pNA, alanine-peptidase (EC 3.4.11.2) by the cleavage of Ala-pNA, proline-peptidase (EC 3.4.11.5) by the cleavage of Pro-pNA, and DPP IV by the cleavage of different substrates (Gly-Pro-, Arg-Pro-, and Ala-Pro-pNA).[36] At 10 μM molecule **11a**, which is 3 orders of magnitude higher than its IC_{50}, the DPP IV activity monitored with the different substrates was inhibited by more than 80% (Figure 1). The remaining residual 20% activity was probably due to the background (i.e., a nonspecific and CD26-independent cleavage of the substrates) since it was observed even at 100 μM molecule **11a** (not shown). In contrast, molecule **11a** exerted no inhibitory effect on the activity of arginine-, alanine-, and proline-aminopeptidase (Figure 1). These results therefore demonstrated the specific nature of the DPP IV inhibition by molecule **11a**.

To investigate the irreversible nature of the inhibitory molecule **11a**, its effect on the DPP IV activity of CD26 expressed on the cell surface was studied. For this purpose, we established by transfection of CD26 cDNA, human CEM cells which express high levels of CD26, i.e., cells which express high levels of DPP IV activity on the cell surface. Consequently, intact cells (clone H01) could be assayed for DPP IV activity by incubation with an appropriate substrate, such as Gly-Pro-pNA.[36] Under these experimental conditions, molecule **11a** was found to be a potent inhibitor of cell-surface DPP IV activity, with maximum inhibition occurring at 2 μM. First, we investigated the kinetics of inhibition of cell-surface DPP IV activity in these CEM cells preincubated with 5 μM molecule **11a**. At times of 5, 10, 15, and 30 min, cells were washed extensively to remove unbound molecule **11a** before assay of the DPP IV activity. As shown in Figure 2, inhibition was almost maximal after 5 min of incubation, since the degree of inhibition was only slightly increased at 30 min. These results demonstrated that molecule **11a** has the capacity to bind CD26 rapidly and thus inhibit irreversibly its DPP IV activity. Second, to confirm the irreversible nature of molecule **11a**, cells were incubated with 5 μM inhibitor for 15 min before extensive washing and further incubation in the culture medium for up to 3 days. At different times during this period, cells were monitored for cell-surface-expressed DPP IV activity (Figure 3). The maximum inhibition observed following the 15-min incubation of cells with molecule **11a** (time 0 h) was found to last for several hours. At 6 h, there was a very slight difference on the maximum inhibition, whereas at 24 h, there was about 50% inhibition. Interestingly, this 50% reduction of the inhibition at 24 h coincided with the doubling time of cells, which results in the

Table 1. Inhibition of DPP IV Activity by the Cyclopeptide Compounds[a]

cyclopeptides A			inhibition of Gly-Pro-pNa hydrolysis (IC_{50}, μM)	
ref	glycine (n)	leaving group	CD26	DPP IV-β
11a	2	S^+Me_2	0.012	1
11b	4	S^+Me_2	0.003	0.61
12a	2	S^+Bu_2	0.02	0.57
13a	2	S^+Oct_2	0.03	0.63
15a	2	OAc	1.5	3.5
16a	2	OPh	10	25

[a] The DPP IV activity was monitored by the cleavage of Gly-Pro-pNA in purified preparations of CD26 and DPP IV-β (Experimental Section). CD26 was purified using extracts of CEM H01 cells expressing very high levels of CD26,[49] DPP IV-β was purified using extracts of CD26-negative C8166 cells.[36] Both purification procedures were as described elsewhere.[51] Such purified preparations of CD26 and DPP IV-β were free of any significant contamination by other peptidases. CD26 and DPP IV-β preparations were preincubated for 15 min with different concentrations of each inhibitor ranging from 1 nM to 10 μM before adding the substrate Gly-Pro-pNA as indicated.

production of cells that express newly synthesized CD26 molecules. At 3 days, no inhibition of cell-surface DPP IV activity was observed. Taken together, these data indicate that the inhibitory effect of molecule **11a** is irreversible, since the DPP IV activity could be resumed only by the newly synthesized CD26 (Figure 3). CD26 being a cell-surface-expressed protein, its expression on the cell surface could be modified with respect to the different phases of the cell cycle. Consequently at 24 and 72 h, the DPP IV activity was variable in control cells (without preincubation with the inhibitor) (Figure 3). It should also be noted that the cell-surface expression of CD26 was not affected by coupling with molecule **11a**. Indeed, cells in the absence or presence of preincubation with molecule **11a** manifested similar levels of cell-surface-expressed CD26, as monitored by FACS analysis using anti-CD26-specific monoclonal antibodies (data not shown).

The other cyclopeptides, possessing different leaving groups (**12a**, **13a**, **15a**, **16a**) or a larger ring (**11b**) were also assayed for their capacity to inhibit the DPP IV activity of CD26 using crude extracts from CEM cells (clone H01). The IC_{50} values for the inhibition of Gly-Pro-pNA hydrolysis are given in Table 1. All of these cyclopeptides showed different IC_{50} values in the nanomolar or micromolar range. However, no correlation was observed between these IC_{50} values and the length or lipophilicity of the leaving group. Molecules **15a** and **16a** with leaving groups as acetate and phenoxy, respectively, manifested significantly reduced IC_{50} values. In contrast, increasing the ring size in a given molecule generated a compound with an increased inhibitory activity (Table 1, compare molecules **11a**,**b**).

By use of the CD26-negative T-lymphoblastoid cell line C8166, we have recently described a CD26-like cell-surface protein with typical DPP IV activity.[36] This novel form of DPP IV, referred to as DPP IV-β, was found to be distinct from CD26. However the pH optimum and the profiles for substrate molecules were found to be indistinguishable for both CD26 and DPP IV-β. Similarly, several previously described inhibitors of DPP IV exerted a very similar mode of action on both CD26 and DPP IV-β.[36] Consequently, it remained

Table 2. Characterization of the Inhibition Kinetics of CD26 and DPP IV-β by the Cyclopeptide **11a**

	inhibition constants[b]	
enzyme[a]	K_I (μM)	k_{inact} (s^{-1})
CD26	0.085	$11.\ 10^{-4}$
DPP IV-β	0.470	$22.\ 10^{-5}$

[a] The DPP IV activities of CD26 and DPP IV-β were assayed by the use of extracts from CEM H01 (cells expressing very high levels of recombinant CD26) and C8166 cells (cells expressing only DPP IV-β), respectively. The cleavage of the substrate Gly-Pro-pNA (0.5 mM) was monitored in the presence of increasing concentrations of inhibitor **11a** by measuring absorbance at 405 nm. Preparation of extracts and assay conditions were as described before[36] and as in the Experimental Section. [b] Calculations were done as indicated in the Experimental Section. K_I is the equilibrium constant of the inhibitor binding to the enzyme, whereas k_{inact} is the constant of the irreversible reaction that leads to the inactivation of the enzyme.

essential to assess the action of the cyclopeptide inhibitors on DPP IV-β. The results given in Table 1 show that the inhibitory effect of the different irreversible cyclopeptide inhibitors is much more pronounced for CD26 compared to DPP IV-β. For example, it is interesting to note that the inhibitory effect of molecules **11a**,**b** is 83- and 203-fold higher on the DPP IV activity of CD26 compared to that of DPP IV-β, respectively (Table 1). In contrast to such a significant selectivity, molecules **15a** and **16a** exerted only about 2-fold difference between the two enzymes. These data might suggest that the higher specificity of the inhibitors on the DPP IV activity of CD26 could be related to the reactivity of the cyclopeptide sulfonium salts.

To further investigate the significant differences in the effect of the mostly studied inhibitorory molecule**11a** on DPP IV activity of CD26 and DPP IV-β (Table 1), we studied the kinetics of inhibition of both enzymes, by using the approach previously described for irreversible inhibitors of trypsin-like proteases.[34] This approach considers two steps in the inhibition process: first, the reversible binding of the inhibitor to the enzyme and, second, the cleavage and subsequent irreversible covalent modification of the enzyme leading to the loss of catalytic activity. The inhibition kinetics of the DPP IV activities of CD26 and DPP IV-β fit well to this model, and the equilibrium constant of the first step (K_I) as well as the kinetic constant of the second step, was calculated. The results are summarized in Table 2 and show that the higher potency of inhibitor **11a** on the DPP IV activity of CD26 is the consequence of a higher affinity for this enzyme as demonstrated by the K_I values, along with a faster inactivation rate as pointed out by the k_{inact} values found for CD26.

The difference in the inhibitory effect of irreversible inhibitors was further emphasized by testing the cell-surface-expressed enzymes. For this purpose, we used CEM cells expressing high levels of CD26 (clone H01) as a source of CD26 and C8166 cells as a source of DPP IV-β. Figure 4 shows the effect of different concentrations of molecule **11a** and a previously described reversible inhibitor, Lys-[Z(NO_2)]-pyrrolidide. As we had reported previously,[36] Lys-[Z(NO_2)]-pyrrolidide inhibited to a similar extent the DPP IV activity of both CD26 and DPP IV-β, with comparable IC_{50} values. On the other hand, the molecule **11a** completely inhibited the DPP IV activity of cell-surface-expressed CD26 without

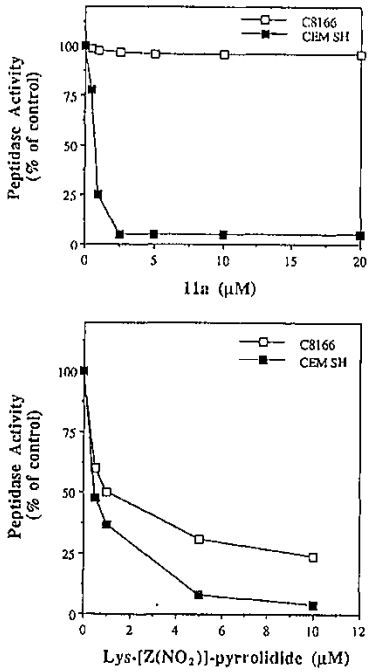


Figure 4. Molecule **11a** inhibits the DPP IV activity of the cell-surface-expressed CD26 but not that of DPP IV-β. Intact CEM cells overexpressing CD26 (clone H01) and C8166 cells expressing only DPP IV-β were preincubated for 15 min in PBS containing different concentrations of molecule **11a** (upper panel) or Lys-[Z(NO_2)]-pyrrolidide (lower panel), before adding GP-pNA to monitor residual DPP IV activity. Control activity (100%) was determined in the absence of inhibitor.

any apparent effect on cell-surface-expressed DPP IV-β, even when used at high concentrations such as 10−20 μM (Figure 4). It should be noted that molecule **11a** was active against the soluble form of DPP IV-β with an IC_{50} value of 1 μM. Thus, the inability of molecule **11a** to affect cell-surface-expressed DPP IV-β appears to be correlated with the structure of this protein when expressed on the cell surface. Whatever is the case, these results emphasize specific differences in the overall structure of DPP IV-β compared to that of CD26.

Discussion

The DPP IV enzyme has an absolute requirement for the L-configuration of the amino acid residues, both in the penultimate and in the N-terminal positions. The amide bond between the N-terminal P_2 residue and the P_1 proline residue must be in a trans conformation. A protonated amino end is necessary for enzymatic hydrolysis. Amino acids with aliphatic side chains at P_2 are slightly favored over aromatic ones, but the effect is not very large. On the basis of these literature data and the results of directed mutagenesis studies, Brandt et al. have recently proposed a model of the enzyme active site[41] and a new catalytic mechanism for this glycoprotein,[42] which has not yet been crystallized. Compared to usual serine proteases, the main difference is the stabilization of the first tetrahedral intermediate

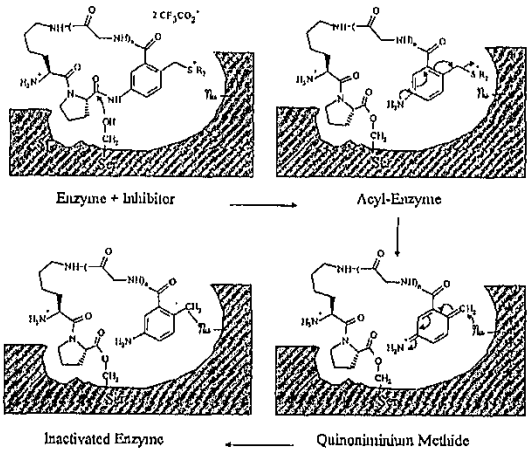


Figure 5. Postulated mechanism of enzyme inactivation. Two covalent bonds are formed between the inhibitor and the enzyme (double-hit mechanism). First, an acyl-enzyme is formed which should involve the catalytic serine 630 of CD26 and proline residue P_1 of the inhibitory molecule (step 1). Second, by means of the unmasked quinoniminium methide (step 2), a second covalent bond is formed implicating a nucleophile residue Nu in the vicinity of the catalytic serine (step 3). Consequently, this second bond is responsible for the irreversible blockade of the catalytic site of the enzyme.

IT_1 by formation of an oxazolidine ring (IT_2). Proton transfer from the terminal ammonium function leads to a third tetrahedral intermediate (IT_3) and then preferentially to a cis acyl-enzyme which isomerizes to the trans isomer.

For the herein described cyclopeptides A, the postulated mechanism of the observed enzyme inactivation is presented in Figure 5. Provided that the protease can accommodate the large molecule A, formation of an acyl-enzyme, by selective nucleophilic attack of the hydroxyl function of serine 630 on the P_1 proline carbonyl of the cyclopeptide (step 1), would unmask a P'_1-substituted aniline having a good benzylic leaving group. Owing to the strong electron-releasing property of the amino substituent ($\sigma^+_{p\text{-}NH_2} = -1.31$, compared to $\sigma^+_{p\text{-}NHCOR} = -0.69$[43] in the starting cyclopeptide), a fast elimination of a neutral dialkyl sulfide (or an anionic acetate) should give a substitued quinoniminium methide ion[34] (step 2) tethered in the active site during the lifetime of the acyl-enzyme by means of the peptide chain. A Michael-type addition of a second nucleophile Nu, in the vicinity of the active serine, on this demasked electrophile (step 3) would establish a second covalent enzyme−inhibitor bond (elimination−addition process).[44] Such a bridge between the active site serine and another nucleophilic residue active site leads to an irreversible loss of the enzymatic activity. Moreover, the inactivation is expected to be very selective as the inhibitory activity of suicide substrates (mechanism-based inhibitors) requires discrimination in the binding steps, catalytic activation by the target enzyme, and irreversible modification of the active center.[45]

It should be emphasized that the macrocyclic sulfonium salts A are obtained in just one step by reacting an alkyl sulfide in trifluoroacetic acid with the cyclopeptides having a benzylic phenoxy substituent and an

N^{α}-Z protecting group, themselves readily obtained by peptide synthesis in solution and cyclized by the azide method. These cyclopeptide sulfonium salts, as all compounds **11**–**16**, belonging to the A series of molecules, are stable and water-soluble, and their activity does not decrease even after several months in solution. Their cyclic structure prevents their decomposition by cyclization due to the attack of the free amine on the carbonyl of the proline. Moreover, the toxicity of these compounds on CEM cells was observed only at concentrations higher than 250 μM.

Similar inhibitory activities were observed among the compounds **11a**, **12a**, and **13a**, suggesting that the length of the alkyl chains on the sulfonium group has little or no influence on the inhibition of DPP IV activity of CD26. Interestingly, the size of the macrocycle appeared to play an important role, since a strong inhibition was observed with compound **11b** which contains four glycine residues in its cycle. The inhibitory activity of this compound is 4 times more effective than that of its homologue molecule **11a**, which possesses only two glycine residues. The weak inhibition observed with compounds **15a** and **16a** is not surprising, since the acetate and the phenoxy groups are not good leaving groups compared to the alkyl sulfide ones. For the linear analogues of the cyclopeptides, no inhibition was observed at 100 μM. In this case, a fast diffusion of the quinoniminium methide cation out of the active center may occur due to the lack of the peptide chain which tethers the electrophile in the active site. Taken together, our results show that large substituents on the sulfur atom did not significantly modify the inhibition reaction. In contrast the ring size enlargement improved it, probably by allowing more favorable interactions with the enzyme active center and/or facilitating the approach to the nucleophilic group Nu in the active site.

Comparative studies on the inhibition of the DPP IV activity of CD26 and DPP IV-β revealed that the two enzymes should be distinct from each other. In soluble enzyme preparations, the irreversible cyclopeptide inhibitors were less active on DPP IV-β compared to CD26. This latter is most probably due to both a different affinity and a different inactivation rate, as we have demonstrated to be the case with the cyclopeptide **11a** (Table 2). Consequently, it is plausible to suggest the presence of a less reactive active site nucleophile Nu–DPP IV-β compared to the corresponding Nu residue in CD26 and/or to a different localization of this group with respect to the active catalytic serine residue. These point out that there should be important structural differences between DPP IV-β and CD26. Such structural differences probably become accentuated with the cell-surface-expressed proteins. Indeed, cyclopeptide inhibitors active at nanomolar concentrations on the cell-surface-expressed CD26 manifest no apparent activity on the cell-surface-expressed DPP IV-β. Accordingly, the catalytic domain necessary for the DPP IV activity could be differentiated between CD26 and DPP IV-β. For the cell-surface-expressed DPP IV-β, the particular structure of its catalytic domain presumably cannot accommodate the inhibitor. On the other hand, the solubilized DPP IV-β can bind the functionalized cyclopeptide and therefore be inhibited.

We have previously reported that the irreversible inhibitor **11a** affects HIV-1 Lai infection of CEM cells in a dose-dependent manner, with an IC_{50} value of 10 μM.[46] In contrast, another potent but reversible inhibitor of the DPP IV activity, Lys-[Z(NO_2)]-pyrrolidide, has no apparent effect on HIV infection of CEM cells, even at 100 μM.[46] Interestingly, molecule **11a**, which has no inhibitory activity on the cell-surface-expressed DPP IV-β, manifests no effect on the HIV-1 infection of CD26-negative but DPP IV-β expressing C8166 cells.[46] These observations indicate that the irreversible inhibitors behave as specific compounds affecting the DPP IV activity of CD26, and consistent with previously published reports,[3,36,47–49] they demonstrate once again the implication of CD26 in the mechanism of HIV infection. Recently, Bekesi and collaborators have synthesized reversible inhibitors of the type X-Pyrr-2-CN and have shown that they inhibit HIV-1 infection with IC_{50} values of 2.4–5.3 μM.[17,50]

Taken together, our results describe for the first time potent irreversible inhibitors of DPP IV which could be used specifically on CD26 and could be used in different types of studies for better understanding the role of CD26 in biological processes.

Experimental Section

Synthetic Procedures. Melting points were recorded on a Tottoli (Büchi) or a Mettler FP61 and are uncorrected. The ^{1}H NMR measurements were performed on a Brüker AM 300 instrument. The chemical shifts are reported in ppm, the deuterated solvents being used as internal standards: CD_3OD (3.31 ppm) and CD_3COOD (2.10 ppm). The coupling constants are given in hertz. The optical rotations were obtained on a Perkin-Elmer 241 polarimeter. Analyses were performed by the Services of CNRS at ICSN (Gif sur Yvette). The mass spectrometry measurements were performed by Mr. Deroussent (Inst. G. Roussy, Villejuif) or Ms. Kargar (Ecole Polytechnique) and for the FAB HRMS data, Mr. Fabre-Bonvin (CNRS, Vernaison). Analytical thin-layer chromatography (TLC) and preparative column chromatography were performed on Kieselgel F 254 and Kieselgel 60, 0.063–0.5 and 0.040–00.063 mm (Merck), respectively, with the following solvent systems (by vol): I (*x*/*y*), MeOH *x*%–CH_2Cl_2 *y*%; II, AcOEt–*n*-BuOH–AcOH–H_2O (1/1/1/1). UV light (254 nm) allowed visualization of the spots after TLC runs for all compounds, even at low concentration.

Nba-[CH_2OPh]-Gly$_2$-OEt (6a). 2-(Phenoxymethyl)-5-nitrobenzoic acid (**5**) (2.5 g, 9.15 mmol), prepared as previously described[38] was dissolved in a 50-mL solution of CH_2Cl_2/THF (1/1). DCC (2.45 g, 11.89 mmol) and HOBt (1.36 g, 10.05 mmol) were added, and the solution was stirred at 0 °C for 15 min. Diglycine ethyl ester hydrochloride (1.98, 10.05 mmol) and a solution of triethylamine (3.9 g, 27.45 mmol) in CH_2Cl_2/THF (1/1) (40 mL) were then added. The reaction mixture was stirred for 1 h at 0 °C and for 24 h at room temperature. Solvents were evaporated under reduced pressure at 50 °C, the residue was dissolved in acetone, and the DCU was filtered. The filtrate was then evaporated, dissolved in AcOEt, and washed successively with H_2O (100 mL), 0.5 N aqueous HCl (100 mL), H_2O (100 mL), 5% aqueous $NaHCO_3$ (100 mL), and H_2O (100 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated. Chromatography on silica gel with eluent I (2/98) led to 3.23 g (85.5%) of pure peptide **6a** as a white powder. Mp: 132.4 °C. R_f (I: 2/98) = 0.5. ^{1}H NMR (CD_3OD): 8.56, d (2.3), 1H (ArH); 8.39, dd (2.3, 8.5), 1H (ArH); 7.97, d (8.5), 1H (H3); 7.32, m, 2H and 7.01, m, 3H (ArH); 5.45, s, 2H (CH_2OPh); 4.21, q (7.1), 2H (OEt); 1.30, t (7.1), 3H (OEt). Anal. Calcd for $C_{20}H_{21}N_3O_7 \cdot 0.5H_2O$: C, 56.59; H, 5.18; N, 9.90. Found: C, 56.61; H, 5.11; N, 10.21.

Boc-Pro-Aba-[CH_2OPh]-Gly$_2$-OEt (7a). The peptide **6a** (2.5 g, 6.02 mmol) was dissolved in hot DMF/MeOH (25/75;

60 mL), and platinium oxide (0.2 g) was added to the warm solution, which was hydrogenated in a Parr apparatus at room temperature for 2 h. The solution was filtered in order to remove the catalyst. Analytical TLC of the filtrate with eluent I (15/85) showed a new unique spot, highly fluorescent on UV light at 254 and 366 nm, corresponding to the intermediate aromatic amine. This compound was not isolated and was used directly in the next step, because of its instability in solution. Thus methanol and DMF were rapidly evaporated in vacuo at 40 °C. The residue was dissolved in CH_2Cl_2/THF (1/1; 30 mL). The clear solution was cooled at 0 °C, and a solution of Boc-L-proline (1.295 g, 6.02 mmol) and HOBt (8.94 g, 6.62 mmol) in CH_2Cl_2/THF (1/1; 70 mL) and then DCC (1.613 g, 7.82 mmol) were added. The reaction mixture was stirred from 0 °C during 2 h to room temperature overnight. The solvents were evaporated, ethyl acetate (300 mL) was added, and the dicyclohexylurea precipitate was removed by filtration. The filtrate was extracted successively with 0.5 M aqueous HCl (2 × 100 mL), H_2O (100 mL), 5% aqueous $NaHCO_3$ (2 × 100 mL), and H_2O (2 × 100 mL), dried over Na_2SO_4, filtered, and evapored. The residue was chromatographed on a column of silica gel. Elution with eluent system I (2/98) led to 2.05 g (58%) of the pure peptide **7a**. R_f (I: 10/90) = 0.5. ^{1}H NMR (CD_3OD): 7.84, d (1.8), 1H (ArH); 7.68, dd (1.8, 8.4), 1H (ArH); 7.54, d (8.4), 1H (ArH); 7.24, m, 2H and 6.94, m, 3H (ArH); 5.23, s, 2H (CH_2OPh); 4.31, md, 1H (HαPro); 4.14, q (7.1), 2H (OEt,); 3.89 and 3.68, 2s, 4H (HαGly); 3.50, m, 2H (HδPro); 2.29, m, 1H (HβPro); 1.97, m, 3H (HβPro, HγPro); 1.47 and 1.36, 9H (Boc); 1.24, t (7.1), 3H (OEt). Anal. Calcd for $C_{30}H_{38}N_4O_8 \cdot 0.5H_2O$: C, 60.89; H, 6.64; H, 9.47. Found: C, 60.86; H, 6.57; N, 9.45.

Boc-Pro-Aba-[CH_2OPh]-Gly$_4$-OEt (7b): obtained as above from **6b**[32] (3.5 g, 6.6 mmol). Yield: 1.4 g (30%), white powder. R_f (I: 10/90) = 0.5. ^{1}H NMR (CD_3OD): 7.85, s, 1H (ArH); 7.65, m, 1H (ArH); 7.52, m, 1H (ArH); 7.25, m, 2H and 6.95, m, 3H (ArH); 5.16, s, 2H (CH_2OPh); 4.26, md, 1H, (HαPro); 4.10, q, 2H (OEt); 4.05, s, 2H (HαGly); 3.90, 3.89 and 3.88, m, 6H (HαGly); 3.53, m, 2H (HδPro); 2.29, m, 1H (HβPro); 2.03, m, 2H (HγPro); 1.89, m, 1H (HβPro); 1.47 and 1.36, 9H (Boc); 1.24, t, 3H (OEt). Anal. Calcd for $C_{34}H_{44}N_6O_{10} \cdot H_2O$: C, 57.12; H, 6.48; H, 11.75. Found: C, 57.11; H, 6.38; N, 11.44.

^{a}Z-Lys(^{t}Boc)-Pro-Aba-[CH_2OPh]-Gly$_2$-OEt (8a). The peptide **7a** (1.5 g, 2.57 mmol) was dissolved in CH_2Cl_2 (15 mL) at 0 °C, and TFA (15 mL) was added. The reaction mixture was stirred at 0 °C for 1.5 h. Excess of TFA was evaporated in vacuo. The residue was dissolved in CH_2Cl_2, and the solution was washed with 5% aqueous $NaHCO_3$, dried over Na_2SO_4, filtered, and evaporated. The residue was dissolved in CH_2Cl_2/THF (1/1; 15 mL) and added to a mixture of Z-Lys(Boc)-OH (0.98 g, 2.57 mmol), DCC (0.689 g, 3.34 mmol), and HOBt (0.382 g, 2.82 mmol) in CH_2Cl_2/THF (1/1; 30 mL) at 0 °C. The reaction mixture was stirred from 0 °C during 2 h to room temperature overnight. The solvents were evaporated, ethyl acetate (300 mL) was added, and the dicyclohexylurea precipitate was removed by filtration. The filtrate was extracted successively with 0.5 M aqueous HCl (2 × 100 mL), H_2O (100 mL), 5% aqueous $NaHCO_3$ (2 × 100 mL), and H_2O (2 × 100 mL), dried over Na_2SO_4, filtered, and evapored. The residue was chromatographed on a column of silica gel. Elution with eluent system I (6/94) led to 1.4 g (65%) of the pure peptide **8a**. Mp: 78.7 °C. R_f (I: 5/95) = 0.5. ^{1}H NMR (CD_3OD): 7.74, d (2.0), 1H (ArH); 7.60, dd (2.0, 8.4), 1H (ArH); 7.45, d (8.4), 1H (ArH); 7.26, m, 7H (ArH); 6.92, m, (ArH); 5.19, s, 2H (CH_2-OPh); 5.06, s, 2H (CH_2Z); 4.57, m, 1H (HαPro); 4.41, m, 1H (HαLys); 4.13, q (7.1), 2H (OEt); 4.03, s and 3.89, s, 4H (HαGly); 3.86 and 3.69, m, 2H (HδPro); 3.03, m, 2H (HεLys); 2.19−1.48, m, 10H (HβPro, HγPro, HδLys, HβLys, HγLys); 1.41, s, 9H (Boc); 1.23, t (7.1), 3H (OEt). Anal. Calcd for $C_{44}H_{56}N_6O_{11}$: C, 62.54; H, 6.68; N, 9.94. Found: C, 62.35; H, 6.63; N, 9.53.

^{a}Z-Lys(^{t}Boc)-Pro-Aba-[CH_2OPh]-Gly$_4$-OEt (8b). The peptide **7b** (0.25 g, 0.35 mmol) was dissolved in ethanol (5 mL), and a solution of HCl (3 N) in CH_2Cl_2 (20 mL) was added. The reaction mixture was stirred at 0 °C for 1.5 h and evaporated in vacuo. The residue was dissolved in diethyl ether, triturated, and evaporated. After this operation was repeated three times, the residue was dissolved in THF (20 mL) and *N*-methylmorpholine (40 μL, 0.35 mmol) was added. This solution **a** was stirred at 0 °C for 30 min. To a solution of Z-Lys(Boc)-OH (136 mg, 0.35 mmol) and *N*-methylmorpholine (40 μL, 0.35 mmol) in THF (20 mL) cooled at ~ 10 °C was added ethyl chloroformate (34 μL, 0.35 mmol). This mixture **b** containing the mixed anhydride was stirred at −10 °C for 15 min and the former solution **a** was added. The reaction mixture was stirred for 1 h at −10 °C and at room temperature overnight. After evaporation of the solvents, the residue was chromatographed on a column of silica gel. Elution with eluent system I (10/90) led to the pure peptide **8b** (0.14 g, 36%). Mp: 149.5 °C. R_f (I: 10/90) = 0.4. ^{1}H NMR (CD_3OD): 7.75, s, 1H (ArH); 7.56, m, 1H (ArH); 7.40, m, 1H (ArH); 7.28, m, 7H (ArH); 6.88, m, 3H (ArH); 5.15, s, 2H (CH_2OPh); 5.01, s, 2H (CH_2Z); 4.36, m, 1H (HαPro); 4.05, m, 5H (HαLys, OEt, HαGly); 3.85, ~s, 6H (HαGly); 3.54, m, 2H (HδPro); 2.99, m, 2H (HεLys); 2.21−1.58, m, 10H (HβPro, HγPro, HβLys, HγLys, HδLys); 1.43, s, 9H (Boc); 1.16, t, 3H (OEt). Anal. Calcd for $C_{48}H_{62}N_8O_{13}$: C, 60.11; H, 6.52; N, 11.68. Found: C, 59.42; H, 6.57; N, 10.51.

^{a}Z-Lys(^{t}Boc)-Pro-Aba-[CH_2OPh]-Gly$_2$-NH-NH$_2$ (9a). The peptide **8a** (1 g, 1.18 mmol) was dissolved in methanol (50 mL). A large excess of hydrazine monohydrate (6 mL, 118 mmol) was added, and the solution was stirred at room temperature for 24 h. Methanol and the excess of hydrazine were evaporated in vacuo. The residue was evacuated at 40 °C/0.1 mmHg for 1 h, solubilized in methanol (20 mL), and precipitated by addition of ether (200 mL). The precipitate was filtered, washed with ether, and dried, leading to 0.69 g (70%) of hydrazide **9a** as a white powder. R_f (I: 10/90) = 0.5. ^{1}H NMR (CD_3OD): 7.83, s, 1H (ArH); 7.63, d (8.4), 1H (ArH); 7.51, d (8.4), 1H (ArH); 7.28, m, 7H (ArH); 6.76, m, 3H (ArH); 5.22, s, 2H (CH_2OPh); 5.07, s, 2H (CH_2Z); 4.57, m, 1H (HαPro); 4.41, m, 1H (HαLys); 4.23, 4.07, 4.04, and 3.94, 2sd, 4H (HαGly); 3.89 and 3.71, md, 2H (HδPro); 3.04, m, 2H (HεLys); 1.99−1.46, m, 10H (HβPro, HγPro, HγLys, HγLys, HδLys); 1.41, s, 9H (Boc). Anal. Calcd for $C_{42}H_{54}N_8O_{10} \cdot 0.5H_2O$: C, 60.05; H, 6.60; N, 13.34. Found: C, 60.32; H, 6.75; N, 12.76.

^{a}Z-Lys(^{t}Boc)-Pro-Aba-[CH_2OPh]-Gly$_4$-NH-NH$_2$ (9b): obtained as above from the peptide **8b** (0.058 g, 0.6 mmol), white powder (0.041 g, 72%). R_f (I: 8/92) = 0.1. ^{1}H NMR (CD_3OD): 7.77, s, 1H (ArH); 7.61, d (8.4), 1H (ArH); 7.45, m, 1H (ArH); 7.32, m, 7H (ArH); 6.92, m, 3H (ArH); 5.18, dd~s, 2H (CH_2-OPh); 5.05, dd~s, 2H (CH_2Z); 4.55, m, 1H (HαPro); 4.40, m, 1H (HαLys); 4.04, s, 2H (HαGly); 3.87, s, 3.86, s and 3.80, s, 6H (HαGly); 3.68, m, 2H (HδPro); 3.03, m, 2H (HεLys); 2.21−1.62, m, 10H (HβPro, HγPro, HβLys, HγLys, HδLys); 1.40, s, 9H (Boc). Anal. Calcd for $C_{46}H_{60}N_{10}O_{12} \cdot 2H_2O$: C, 56.31; H, 6.57; N, 14.27. Found: C, 56.15; H, 6.55; N, 14.87.

Cyclo(-Lys(^{a}Z)-Pro-Aba-[CH_2OPh]-Gly$_2$-) (10a). The peptide **9a** (0.640 g, 0.77 mmol) was dissolved in CH_2Cl_2 (10 mL) at 0 °C, and TFA (15 mL) was added. The solution was stirred for 2 h. Then CH_2Cl_2 and excess of TFA were evaporated in vacuo. The residue was evacuated at 40 °C/0.1 mmHg for 1 h and triturated several times in diethyl ether to give a white powder. This compound was dissolved in DMF (17 mL). After cooling at −40 °C, a solution of 5.5 N HCl in THF (1.5 mL, 8.47 mmol) and isoamyl nitrite (0.145 mL, 1.08 mmol) were successively added. The reaction mixture was stirred at −40 °C for 0.5 h. The resulting peptide azide was diluted with cold DMF (200 mL). The solution was then brought to pH 9 by addition of diisopropylethylamine (2 mL, 11.55 mmol) and kept in a refrigerator for 24 h. DMF was evaporated, and distilled water was added to the residue. The resulting solid was filtered, dissolved in MeOH, and chromatographed on a column of silica gel. Elution with eluent system I (10/90) led to 0.242 g (45%) of the pure cyclopeptide **10a**. Mp: 144.6 °C. R_f (I: 10/90) = 0.6. ^{1}H NMR (DMSO-d_6): 9.86, s, 1H (NHAr); 8.85, ~t (5.5), 1H (NHGly1); 8.33, ~t (5.5), 1H (NHGly2); 8.09, s, 1H (ArH); 7.62, ~t (5.4), 1H (NHεLys); 7.50, m, 3H (2 ArH and NHαLys); 7.32, m, 7H (ArH); 6.91, m, 3H (ArH); 5.23 and

5.16, 2d (12.3), 2H (CH_2OPh); 5.01 and 5.00, 2d (12.7), 2H (CH_2Z); 4.35, m, 2H (HαPro, HαLys); 3.72, m, 5H (HαGly1, HαGly2, HδPro); 3.56, ~dd (15.9, 5), 1H (HαGly2); 3.09, m, 2H (HεLys); 2.19, m, 1H (HβPro); 3.79, m, 3H (HβPro, HγPro); 1.39, m, 5H (H_2βLys, HδLys, HγLys); 0.85, m, 1H (HβLys). ^{1}H NMR (CD_3OD): 8.44, d, 1H (ArH); 7.62, d (8.3), 1H (ArH); 7.34, m, 5H (ArH); 7.32, m, 3H (ArH); 6.93, m, 3H (ArH); 5.28 and 5.23, 2d (12.7), 2H (CH_2OPh); 5.07, s, 2H (CH_2Z); 4.51, m, 2H (HαPro, HαLys); 4.11 and 3.97, 2d (16.6), 2H (HαGly1); 3.93 and 3.86, 2d (16.8), 2H (HαGly2); 3.72 and 3.54, 2m, 2H (HδPro); 3.25, m, 2H (HεLys); 2.34 and 2.10, 2m, 2H (HβPro); 2.04, m, 2H (HγPro); 1.81, m, 2H (HβLys); 1.54, m, 2H (HδLys); 1.35, m, 2H (HγLys). Anal. Calcd for $C_{37}H_{42}N_8O_8 \cdot H_2O$: C, 61.90; H, 6.18; N, 11.70. Found: C, 61.53; H, 6.07; N, 11.31.

Cyclo(-Lys($^{\alpha}$Z)-Pro-Aba-[CH_2OPh]-Gly$_4$-) (10b): obtained as above from the peptide **9b** (0.05 g, 0.053 mmol). Yield: 0.019 g (44%), white powder. R_f (I: 10/90) = 0.4. ^{1}H (CD_3OD): 7.66, s, 1H (ArH); 7.54, d, 1H (ArH); 7.34, m, 8H (ArH); 6.92, m, 3H (ArH); 5.21, s, 2H (CH_2OPh); 5.06, s, 2H (CH_2Z); 4.56, m, 1H (HαPro); 4.43, m, 1H (HαLys); 4.10–3.80, m, 10H (HαGly1,HαGly2, HαGly3, HαGly4, HδPro); 3.13, m, 2H (HεLys); 2.20–1.50, m, 10H (HβPro, HγPro, HβLys, HγLys, HδLys). MS (FAB^+): 813 (MH^+), 835 (MNa^+), 851(MK^+).

Cyclo(-Lys($^{\alpha}H_2^+$)-Pro-Aba-[$CH_2SMe_2^+$]-Gly$_2$-), $2CF_3COO^-$ (11a). To a mixture of cyclopeptide **10a** (0.05 g, 71 μmol) and dimethyl sulfide (0.52 mL, 71 mmol) was added TFA (2.7 mL). The solution was stirred at room temperature for 48 h. Addition of a large excess of ether led to precipitation of a white solid. The precipitate was collected by centrifugation, repeatedly washed with ether, and then centrifuged, dried, and chromatographed on a column of silica gel. Elution with eluent system II, followed by complete evaporation of the solvents in vacuo at 25–30 °C, dissolution of the residue in ca. 2 mL of methanol, precipitation with ether, repeated washings of the precipitate with ether, centrifugation, and drying in vacuo at 25 °C, led to 0.025 g of the title product **11a** (62%). Mp: 124.8 °C. R_f (II) = 0.05. ^{1}H (CD_3COOD): 8.95, s, 1H (ArH); 7.64, d (8.2), 1H (ArH); 7.45, m~s (8.2), 1H (ArH); 4.83, s, 2H ($ArCH_2S^+$); 4.76, t, 1H (HαPro); 4.61, t, 1H (HαLys); 4.33, 2d (16.9), 2H (HαGly1); 4.09, s, 2H (HαGly2); 3.91 and 3.71, 2m, 2H (HδPro); 3.34, m, 2H (HεLys); 2.97, s, 6H (S^+Me_2); 2.44, m, 2H (HβPro); 2.08, m, 4H (HβLys,HγPro); 1.52, m, 4H (HδLys, HγLys). ^{1}H NMR (CD_3OD): 8.33, 1H (ArH); 7.57, d (8.2), 1H (ArH); 7.31, m~s (8.2), 1H (ArH); 4.72, s, 2H ($ArCH_2S^+$); 4.59, m, 1H (HαPro); 4.50, m, 1H (HαLys); 4.26, 2d (16.9), 2H (HαGly1); 3.72, s, 2H (HαGly2); 3.74, m, 2H (HδPro); 3.25, m, 2H (HεLys); 2.91, s, 6H (S^+Me_2); 2.41, m, 2H (HβPro); 1.98, m, 4H (HβLys,HγPro); 1.55, m, 4H (HδLys, HδγLys). HRMS (FAB^+): calcd for $C_{25}H_{37}N_6O_5S^+$ (M^+), 533.2546; found, 533.2567; calcd for $C_{23}H_{31}N_6O_5$ ($M^+ - Me_2S$), 471.2356; found, 471.2386.

Cyclo(-Lys($^{\alpha}H_2^+$)-Pro-Aba-[$CH_2S^+Me_2$]-Gly$_4$-), $2CF_3COO^-$ (11b): obtained as above from the peptide **10b** (0.01 g, 0.012 mmol). Yield: 6.5 mg (60%), white powder. R_f (II) = 0.5. ^{1}H NMR (CD_3OD): 8.57, s, 1H (ArH); 7.57, m, 1H (ArH); 7.34, m, 1H (ArH); 4.72, s, 2H ($ArCH_2S^+$); 4.21–3.92, m, 12H (HαPro, HαLys, HαGly, HδPro); 3.19, m, 2H (HεLys); 2.91, s, 6H (S^+Me_2); 2.36 to 1.28, m, 10H (HβPro, HγPro, HβLys, HγLys, HδLys). MS (FAB^+): 647 (M^+), 585 ($M^+ - Me_2S$). HRMS (FAB^+): calcd for $C_{29}H_{43}N_8O_7S^+$ (M^+), 647.2975; found, 647.2997.

Cyclo(-Lys($^{\alpha}H_2^+$)-Pro-Aba-[$CH_2S^+Bu_2$]-Gly$_2$-), $2CF_3COO^-$ (12a): obtained as above from the peptide **10a** (0.05 g, 71 μmol) and dibutyl sulfide (0.52 mL, 71 mmol) in TFA (2.7 mL). Yield: 0.045 g (74,5%), white powder. Mp: 176 °C dec. R_f (II) = 0.1. ^{1}H NMR (CD_3OD): 8.28, sd (2.2), 1H (ArH); 7.22, d (8.4), 1H (ArH); 7.08, dd (2.2; 8.2), 1H (ArH); 4.57, m, 1H (HαPro); 4.35, t~m, 1H (HαLys); 4.09, s, 2H ($ArCH_2S^+$); 4.1–3.8, m, 4H (HαGly); 3.79 and 3.67, 2m, 2H (HδPro); 3.54, t (6.4), 4H ($2CH_2S^+$); 3.20, m, 2H (HεLys); 2.36, m, 2H (HβPro); 2.05, m, 3H (HβLys, HγPro); 1.87, m, 1H (HγPro); 1.60–1.30, m, 12H (HδLys, HδγLys, $4CH_2Bu$); 0.93, t, 6H ($2CH_3Bu$). MS (FAB^+): 617 (M^+), 471 ($M^+ - Bu_2S$). HRMS (FAB^+): calcd for $C_{31}H_{49}N_6O_5S^+$ (M^+), 617.3485; found, 617.3498.

Cyclo(-Lys($^{\alpha}H_2^+$)-Pro-Aba-[$CH_2S^+Oct_2$]-Gly$_2$-), $2CF_3COO^-$ (13a): obtained as above from the peptide **10a** (0.035 g, 50 μmol) and dioctyl sulfide (0.5 mL, 50 mmol) in TFA (2 mL). Yield: 0.034 g (71%), white powder. Mp: 174 °C dec. R_f (II) = 0.6. ^{1}H NMR (CD_3OD): 8.29, s, 1H (ArH); 7.60, d (8.8), 1H (ArH); 7.28, m, 1H (ArH); 4.58, m, 1H (HαPro); 4.22, m, 1H (HαLys); 4.09, m, 2H (CH_2S^+); 3.98–3.80, m, 4H (HαGly); 3.67, m, 2H (HδPro); 3.54, t (6.2), 4H ($2CH_2S^+$); 3.32 and 3.20, 2m, 2H (HεLys); 2.38, m, 2H (HβPro); 2.04, m, 8H (HβLys, HγPro, $2CH_2Oct$); 1.80, m, 4H (HδLys, HδγLys); 1.50, m, 8H (CH_2-Oct); 1.29, s, 12H ($6CH_2Oct$); 0.90, t, 6H ($2CH_3Oct$). MS (FAB^+): 729 (M^+), 471 ($M^+ - Oct_2S$). HRMS (FAB^+): calcd for $C_{39}H_{65}N_6O_5S^+$ (M^+), 729.4737; found, 729.4758.

Cyclo(-Lys($^{\alpha}$Boc)-Pro-Aba-[CH_2OAc]-Gly$_2$-) (14a). A mixture of peptide **11a** (0.02 g, 0.031 mmol) and dry potassium acetate (0.05 g, 0.63 mmol) in 5 mL of DMF was stirred at room temperature for 1.5 h. The solvent was evaporated in vacuo. The residue was dissolved in dioxane (1 mL) and aqueous NaOH (1.5 mg, 0.046 mmol) (1 mL). Boc_2O (8.5 mg, 0.046 mmol) was added, and the reaction mixture was stirred from 0 °C during 2 h to room temperature overnight. Solvents were evaporated. Column chromatography of the residue (silica gel, eluent I: 8/92), followed by evaporation of the product containing pooled fractions, dissolution of the residue in methanol, filtration, concentration to ca. 3 mL, precipitation with ether, and treatment of the precipitate as before (centrifugation, repeated washings with ether, and drying), yielded 0.011 g (70%) of acetate 14a as a white powder. R_f (I: 8/92) = 0.4. ^{1}H NMR (CD_3OD): 8.41, d (2.5), 1H (ArH); 7.57, d (8.0), 1H (ArH); 7.31, dd (2.5, 8.0), 1H (ArH); 5.28, dd~q, 2H (CH_2-OAc); 4.53, m, 1H (HαPro); 4.42, m, 1H (HαLys); 4.08, m, 2H (HαGly1); 3.89, s, 2H (HαGly2); 3.71, m, 2H (HδPro); 3.22, m, 2H (HεLys); 2.34 and 2.10, 2m, 2H (HβPro); 2.06, s, 3H (CH_3-Ac); 1.94, m, 2H (HβLys); 1.62, m, 6H (HγPro, HδLys, HγLys); 1.43, s, 9H (Boc). MS (FAB^+): 631 (MH^+), 653 (MNa^+), 669 (MK^+). HRMS (FAB^+): calcd for $C_{30}H_{43}N_6O_9$ (M^+), 631.3091; found, 631.3109.

Cyclo(-Lys($^{\alpha}H_2^+$)-Pro-Aba-[CH_2OAc]-Gly$_2$-), CF_3COO^- (15a). The peptide **14a** (0.010 g, 0.016 mmol) was dissolved in CH_2Cl_2 (10 mL) at 0 °C, and TFA (15 mL) was added. The reaction mixture was stirred for 2 h. CH_2Cl_2 and excess of TFA were evaporated in vacuo. The residue was evacuated at 40 °C/0.1 mmHg for 1 h and triturated several times in diethyl ether to give a white powder (7.7 mg, 76%). R_f (II) = 0.7. ^{1}H NMR (CD_3OD): 8.40, s, 1H (ArH); 7.60, d (8.0), 1H (ArH); 7.30, d (8.0), 1H, (ArH); 5.30, dd~q, 2H (CH_2OAc); 4.55, m, 1H (HαPro); 4.30, m, 1H (HαLys); 4.10, m, 2H (HαGly1); 3.90, s, 2H (HαGly2); 3.70, m, 2H (HδPro); 3.25, m, 2H (HεLys); 2.40 and 2.10, 2m, 2H (HβPro); 2.10, s, 3H (OAc); 1.95, m, 2H (HβLys); 1.65, m, 6H (HγPro, HδLys, HγLys). MS (FAB^+): 531 (MH^+), 471 ($M^+ - OAc$). HRMS (FAB^+): calcd for $C_{25}H_{35}N_6O_7$ (MH^+), 531.2567; found, 531.2591.

Cyclo(H-Lys-Pro-Aba-[CH_2OPh]-Gly$_2$-) (16a). The peptide **10a** (0.055 g, 0.078 mmol) was dissolved in a mixture of MeOH (5 mL) and H_2O (1 mL). Palladium on charcoal (0.2 g) was added, and the solution was hydrogenated in a Parr apparatus at room temperature for 1 h. The solution was filtered in order to remove the catalyst, and the solvent was evaporated. The residue was chromatographed on a column of silica gel. Elution with solvent system I (20/80), followed by evaporation of the product containing pooled fractions, dissolution of the residue in methanol, filtration, concentration to ca. 3 mL, precipitation with ether, and treatment of the precipitate as before (centrifugation, repeated washings with ether, and drying), yielded 38.2 mg (80%) of pure peptide 16a. Mp: 140.1 °C. R_f (I: 20/80) = 0.1. ^{1}H NMR (CD_3OD): 8.43, d, 1H (ArH); 7.53, d (8.1), 1H (ArH); 7.22, m, 3H (ArH); 6.89, m, 3H (ArH); 5.22, dd~q (12.5), 2H (CH_2OPh); 4.57, m, 2H (HαPro, HαLys); 4.10–3.71, m, 6H (2HαGly, HδPro); 3.25, m, 2H (HεLys); 2.36–1.99, m, 4H (HβPro, HγPro); 1.54–1.23, m,

6H (HβLys, HδLys, HγLys). MS (FAB+): 565 (MH^+), 471 ($MH^+ - OPh$). HRMS (FAB+): calcd for $C_{29}H_{37}N_6O_6$ (MH^+), 565.2775.

Enzymatic Studies. Cells and Preparation of Extracts: MOLT4 cells are lymphoblastoid $CD4^+$ T-cells routinely used in our laboratory for the assay of different peptidases.[36] Cells (from Dr. G. Farrar) were generously provided by the Medical Research Council AIDS Directed Programm Reagent Project, U.K. CEM clones expressing enhanced levels of human CD26 were obtained in the laboratory by transfection of CEM cells as described previously;[49] clones were selected by their very high level of CD26 expression determined by FACS analysis and by DPP IV activity measurement. All cells were cultured in suspension medium RPMI-1640 (BioWhittaker, Verviers, Belgium) containing heat-inactivated (56 °C, 30 min) 10% (by vol) fetal calf serum. For preparation of cell extracts, cells were first washed extensively with phosphate-bufferd saline (PBS) before lysis in buffer E (20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 0.2 mM phenylmethanesulfonyl fluoride ($PhMeSO_2F$), 5 mM 2-mercaptoethanol, aprotinin (100 U/mL), and 0.5% Triton X-100) (75 μL/10^7 cells), and the nuclei were pelleted by centrifugation (1000*g* for 8 min). The supernatant was diluted with 1 volume of buffer I (20 mM Tris/HCl, pH 7.6, 400 mM NaCl, 50 mM KCl, 1 mM EDTA, 0.2 mM $PhMeSO_2F$, aprotinin (100 U/mL), 5 mM 2-mercaptoethanol, 1% Triton X-100, and 20% glycerol) and centrifugated at 12000*g* for 10 min. The supernatants (cell extracts) were stored at −80 °C. Under these extraction conditions, extracts from CEM cells (clone H01) showed more than 95% of DPP IV activity associated to CD26; thus no further purification was necessary. Extracts from C8166 cells showed important levels of DPP II contamination; for this reason DPP IV-β was purified by gel filtration and ion-exchange chromatography using a FPLC system (Pharmacia, Uppsala, Sweden) as described elsewhere.[51]

The DPP IV peptidase activity was also measured on the surface of intact cells (2×10^6) in a total reaction volume of 0.5 mL of peptidase buffer PB (100 mM Hepes, pH 7.6, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose, and 1% BSA). Cells were incubated with 0.55 mM dipeptide-pNa at 37 °C for 90 min. The reactions were stopped by addition of 1 mM sodium acetate, pH 4.5 (1 mL). After centrifugation at 12000*g*, the production of *p*-nitroaniline in the supernatant was assessed by measurement of the absorbance at 405 nm. In this case, DPP II contamination of C8166 cells was not detectable, due to the intracellular localization of this enzyme. For determination of DPP IV peptidase activity in extracts from CEM cells or partially purified preparations of DPP IV-β, 50 μL of appropriately diluted samples was incubated in a total volume of 200 μL of buffer PB with 1 mM Gly-Pro-pNA at 37 °C for 120 min in flat-bottom 96-well microplates; activity was determined by measuring absorption at 405 nm.

Inhibitory effect upon DPP IV in soluble preparations was assayed in 96-well microplates, by preincubating CD26 and DPP IV-β preparations with different concentrations of each inhibitor ranging from 1 nM to 10 μM, for 15 min, before adding the substrate as indicated. The inhibitory effect on cell-surface-expressed cells was performed similarly; however, cells were extensively washed after preincubation with the inhibitor. The DPP IV activity-reversible inhibitor Lys-[$Z(NO_2)$]-pyrrolidide was kindly provided by Dr. A. Barth (Halle, Germany). When its inhibitory effect was studied, no washes were performed, due to the reversibility of this inhibitor. IC_{50} values were calculated by nonlinear regression as described.[5] The values of K_I and k_{inact} were calculated using the simplified approach described previously,[34] which ignores the inactivation of the inhibitor by the enzyme. Inhibition kinetics of the DPP IV activities of both enzymes tested (CD26 and DPP IV-β) fit well to this model. K_m values were calculated as decribed.[51] Briefly, in 96-well microtiter plates, aliquots of each enzyme preparation were added to wells containing 0.5 nM Gly-Pro-pNA and increasing concentrations of inhibitor (ranging from 0.3- to 3-fold IC_{50} values) in a total volume of 200 μL. Plates were incubated at 37 °C, and absorbance at 405 nM was determined every 15 min. Data were plotted semilogarithmically as ln(absorbance increase/time increase) against time, to obtain a straight line with slope $-\pi$. Plotting this slope against $1/[I(S/K_m + S)]$, where I = absorbance increase/time increase and S = substrate concentration, yields another straight line with an *x*-intercept of $-K_I$ and a *y*-intercept of k_{inact}.[34]

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Crystal Structures of Cytochrome P-450$_{CAM}$ Complexed with Camphane, Thiocamphor, and Adamantane: Factors Controlling P-450 Substrate Hydroxylation[†,‡]

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ABSTRACT: X-ray crystal structures have been determined for complexes of cytochrome P-450$_{CAM}$ with the substrates camphane, adamantane, and thiocamphor. Unlike the natural substrate camphor, which hydrogen bonds to Tyr96 and is metabolized to a single product, camphane, adamantane and thiocamphor do not hydrogen bond to the enzyme and all are hydroxylated at multiple positions. Evidently the lack of a substrate–enzyme hydrogen bond allows substrates greater mobility in the active site, explaining this lower regiospecificity of metabolism as well as the inability of these substrates to displace the distal ligand to the heme iron. Tyr96 is a ligand, via its carbonyl oxygen atom, to a cation that is thought to stabilize the camphor–P-450$_{CAM}$ complex [Poulos, T. L., Finzel, B. C., & Howard, A. J. (1987) *J. Mol. Biol. 195*, 687–700]. The occupancy and temperature factor of the cationic site are lower and higher, respectively, in the presence of the non-hydrogen-bonding substrates investigated here than in the presence of camphor, underscoring the relationship between cation and substrate binding. Thiocamphor gave the most unexpected orientation in the active site of any of the substrates we have investigated to date. The orientation of thiocamphor is quite different from that of camphor. That is, carbons 5 and 6, at which thiocamphor is primarily hydroxylated [Atkins, W. M., & Sligar, S. G. (1988) *J. Biol. Chem. 263*, 18842–18849], are positioned near Tyr96 rather than near the heme iron. Therefore, the crystallographically observed thiocamphor–P-450$_{CAM}$ structure may correspond to a nonproductive complex. Disordered solvent has been identified in the active site in the presence of uncoupling substrates that channel reducing equivalents away from substrate hydroxylation toward hydrogen peroxide and/or "excess" water production. A buried solvent molecule has also been identified, which may promote uncoupling by moving from an internal location to the active site in the presence of highly mobile substrates.

The cytochrome P-450 superfamily of enzymes catalyzes many different types of oxidative reactions involved in steroid hormone biosynthesis, fatty acid metabolism, and detoxification of foreign compounds (Nebert et al., 1981; Nebert & Gonzalez, 1987; Anders, 1985). Xenobiotic-metabolizing P-450s generally oxidize substrates to more soluble forms, facilitating their excretion. Occasionally these products linger in the cytoplasm as "activated" electrophilic compounds, many of which are mutagens and/or carcinogens (Heidelberger, 1975; Sato & Omura, 1978; Anders, 1985; Ortiz de Montellano, 1986; Wolf, 1986). Due to the broad substrate specificity of this superfamily, and its ability to catalyze multiple types of reactions, there is much interest in structure–function relationships of P-450s. Ultimate goals include designing compounds to selectively inhibit individual P-450s and engineering novel P-450s to facilitate detoxification of specific environmental contaminants.

The best characterized P-450, and the only one for which a crystal structure is known, is the bacterial camphor hydroxylase P-450$_{CAM}$ (Gunsalus et al., 1974; Debrunner et al., 1978; Gunsalus & Sligar, 1978; Ullrich, 1979; Wagner & Gunsalus, 1982; Poulos et al., 1985, 1987). The reaction cycle of cytochrome P-450$_{CAM}$ is shown in Figure 1. Besides hydroxylating camphor, P-450$_{CAM}$ will also hydroxylate various other compounds. We have determined the X-ray crystal structures of ferric cytochrome P-450$_{CAM}$ complexed with different substrates and inhibitors, as well as in the ferrous carbon monoxide and camphor bound form (Raag & Poulos, 1989a,b, 1990; Raag et al., 1990). These structures, together with data on substrate-dependent parameters and site-directed mutagenesis of P-450s (White et al., 1984; Fisher & Sligar, 1985; Atkins & Sligar, 1988a,b), have enabled us to better understand factors that influence regiospecificity and efficiency of P-450 reactions. Here we extend these studies to include three additional substrates. All substrate–P-450$_{CAM}$ coordinates have been submitted to the Brookhaven Protein Data Bank (Bernstein et al., 1977).

Materials and Methods

Thiocamphor synthesis was according to Scheeren et al. (1973) with the exception that P_2S_5 (FLUKA, Ronkonkoma, NY) was used in place of P_4S_{10}. Samples were analyzed (Galbraith Laboratories, Knoxville, TN) for C, H, O, and S to confirm that the correct compound had been prepared. P-450$_{CAM}$ was crystallized according to our earlier procedure (Poulos et al, 1982). To prepare the various substrate–P-450$_{CAM}$ complexes, crystals were soaked in a mother liquor

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[‡] Crystallographic coordinates have been submitted to the Brookhaven Protein Data Bank under the following file names: 4CPP, cytochrome P-450$_{CAM}$–adamantane; 6CPP, cytochrome P-450$_{CAM}$–camphane; 8CPP, cytochrome P-450$_{CAM}$–thiocamphor.

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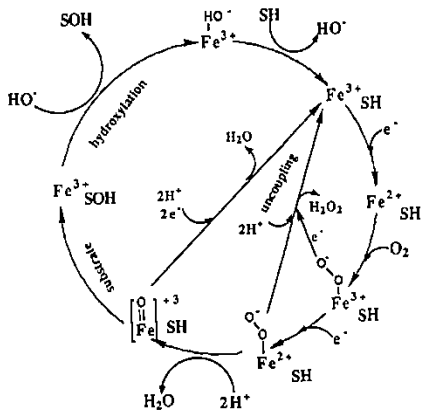

FIGURE 1: P-450 reaction cycle [modified from Atkins and Sligar (1988a)]. SH and SOH represent substrate and oxidized substrate, respectively. "Uncoupling" reactions compete with substrate hydroxylation. "Efficiency" refers to the percentage of reducing equivalents utilized toward substrate oxidation, as opposed to hydrogen peroxide/"excess" water production.

Table I: Summary of Substrate–P-450 Data Collection

substrate	camphane	adamantane	thiocamphor
max resolutn	1.91 Å	2.11 Å	2.09 Å
total observatns	108 560	144 737	133 336
R_{sym}[a]	0.067	0.079	0.058
% data collected to	3.47 Å, 100%	3.82 Å, 100%	3.79 Å, 100%
	2.76 Å, 100%	3.04 Å, 100%	3.01 Å, 100%
	2.41 Å, 100%	2.65 Å, 100%	2.63 Å, 100%
	2.19 Å, 100%	2.41 Å, 100%	2.39 Å, 100%
	2.03 Å, 72%	2.24 Å, 100%	2.22 Å, 100%
	1.91 Å, 45%	2.11 Å, 73%	2.09 Å, 54%
$I/\sigma(I)$	2.19 Å, 1.91	2.41 Å, 2.50	2.13 Å, 2.15
	2.03 Å, 1.08	2.24 Å, 1.46	2.11 Å, 2.13
	1.91 Å, 0.55	2.11 Å, 0.72	2.09 Å, 0.24

[a] $R_{sym} = \sum|I_i - \langle I_i\rangle|/\sum I_i$ where I_i = intensity of the ith observation and $\langle I_i\rangle$ = mean intensity.

consisting of 40% saturated ammonium sulfate, 0.05 M potassium phosphate, and 0.25 M KCl at pH 7.0, with saturating amounts of camphane, adamantane, or thiocamphor. Soak times were about three to four days. X-ray diffraction data were collected from single crystals of the various substrate–P-450$_{CAM}$ complexes by using a Siemens area detector/Rigaku rotating anode and processed by using the XENGEN program package (Howard et al., 1987) on a Digital Equipment Corporation Microvax II. Data collection statistics are presented in Table I.

Substrates were initially sketched by using the Chemnote two-dimensional molecular construction facility in the molecular modeling package QUANTA (Polygen Corp., Waltham, MA), installed on a Silicon Graphics IRIS workstation. Following two-dimensional model building, substrate coordinates were energy minimized, again through QUANTA, using CHARMm steepest descents and Newton-Raphson energy minimization procedures. Substrate van der Waals volumes were calculated with QUANTA as well. Thiocamphor was modeled by substituting sulfur for oxygen in camphor, with the sulfur–carbon bond length maintained at the corresponding value for the oxygen–carbon bond. Such a model for thiocamphor should be adequate since the bond orders of C=S and C=O bonds are similar (Demarco et al., 1969).

Crystallographic refinement was carried out by using the restrained parameters–least squares package of programs

Table II: Summary of Substrate–P-450 Crystallographic Refinement

substrate	camphane	adamantane	thiocamphor
resolutn range (Å)	10.0–1.9	10.0–2.1	10.0–2.1
reflectns measured	27 786	20 548	22 650
reflectns used[a]	20 585	15 174	19 565
R factor[b]	0.190	0.184	0.175
rms deviation of bond dist (Å)	0.020	0.019	0.020
bond angles (Å)	0.032	0.033	0.033
dihedral angles (Å)	0.036	0.037	0.036

[a] Reflections with $I > 2\sigma(I)$; I = intensity. [b] $R = \sum|F_o - F_c|/\sum F_o$.

(Hendrickson & Konnert, 1980) and is summarized in Table II. Initial $F_o - F_c$[1] and $2F_o - F_c$ difference Fourier maps were based on structure factor calculations using coordinates from the 1.7-Å refined camphor–P-450$_{CAM}$ structure (Poulos et al., 1987) and diffraction data obtained from the substrate–P-450$_{CAM}$ complexes. Camphor coordinates were not included in the initial structure factor calculations. $F_o - F_c$ maps were contoured at $\pm 3\sigma$ and $2F_o - F_c$ maps were contoured at +0.5 and $+1\sigma$ (σ is the standard deviation calculated over an entire asymmetric unit of the electron density map). Substrates were positioned into the $F_o - F_c$ maps and refined together with the protein, with substrate temperature factors initially starting at 19–20 Å^2, or near the mean temperature factor for all protein and heme atoms in the camphor–P-450$_{CAM}$ structure. Structures were judged to have refined sufficiently once $F_o - F_c$ maps showed little or no interpretable density when contoured at 3σ. Initial $F_o - F_c$ and final $2F_o - F_c$ maps are shown in Figures 2 and 3. Refined models were subjected to additional refinement without bond, angle, or nonbonded contact distance restraints to better estimate active site distances. Comparison of both coordinate and temperature factor shifts was carried out as described elsewhere (Poulos & Howard, 1987).

RESULTS

Figures 2 and 3 show the initial $F_o - F_c$ and final $2F_o - F_c$ maps of the camphane–, adamantane–, and thiocamphor–P-450$_{CAM}$ complexes. Modeling of camphane and adamantane bound to the enzyme was relatively straightforward. However, neither adamantane nor camphane is able to hydrogen bond to P-450$_{CAM}$ and so we cannot be sure that these substrates do not occupy multiple orientations. Since models with single orientations for these substrates were successful in eliminating most of the difference electron density from the active site region, we take this as evidence that at least the *major* binding orientations of these substrates have been identified.

Camphane. Despite the similarity in structure and binding orientation between camphane and camphor, the atomic temperature factors of camphane refined to values (30 Å^2) about twice those of camphor (16 Å^2). This indicates that camphane is highly mobile when bound to P-450$_{CAM}$. One factor that could artificially raise temperature factors is an inaccurate model. We modeled the observed active site electron density with a single, fully occupied camphane molecule, but such a model would be inaccurate if the camphane occupancy in the crystal was incomplete. This is feasible since neither camphane nor adamantane was especially soluble in the crystallization mother liquor. However, since we were able to successfully model these substrates with full occupancy, the contribution

[1] Abbreviations: F_c, calculated structure factors; F_o, observed structure factors; L6, sixth or distal ligand to heme iron; R factor, $\sum|F_o - F_c|/\sum F_o$.

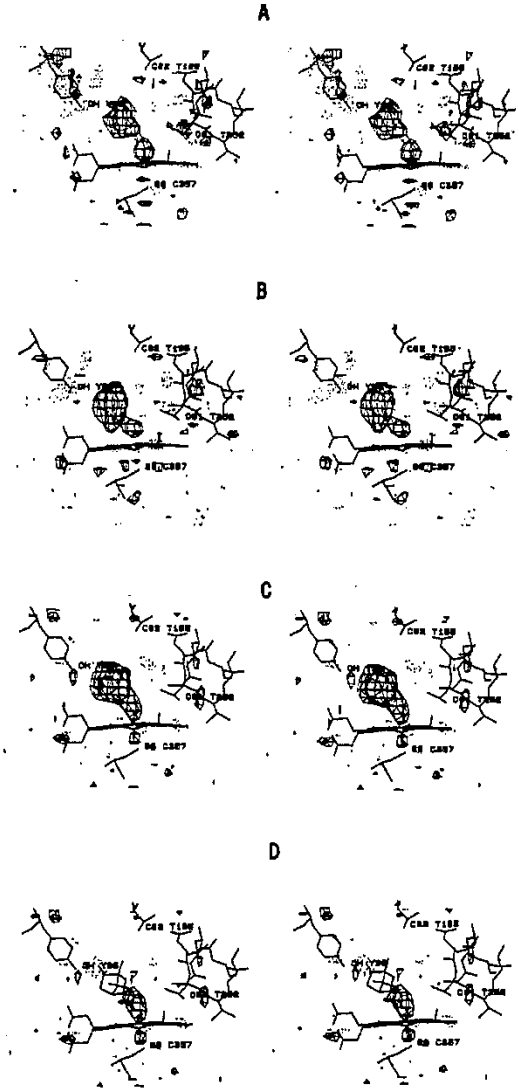

FIGURE 2: Initial $F_o - F_c$ difference electron density maps for P-450$_{CAM}$ complexed with camphane (A), adamantane (B), and thiocamphor (C, D). Maps were calculated with diffraction amplitudes from substrate–P-450$_{CAM}$ complexes and phases from camphor–P-450$_{CAM}$ coordinates (Poulos et al., 1987). Maps are contoured at $\pm 3\sigma$ with negative and positive density depicted as dotted and solid lines, respectively. Substrate coordinates were not included in phase calculations for maps A, B, and C, but camphor coordinates were included in the calculation of map D. Map D, with the camphor carbonyl oxygen in negative density and with positive density between the substrate and heme, indicates that thiocamphor binds "upside down" in the active site, compared with camphor. Note the electron density corresponding to the distal ligand to iron in all maps.

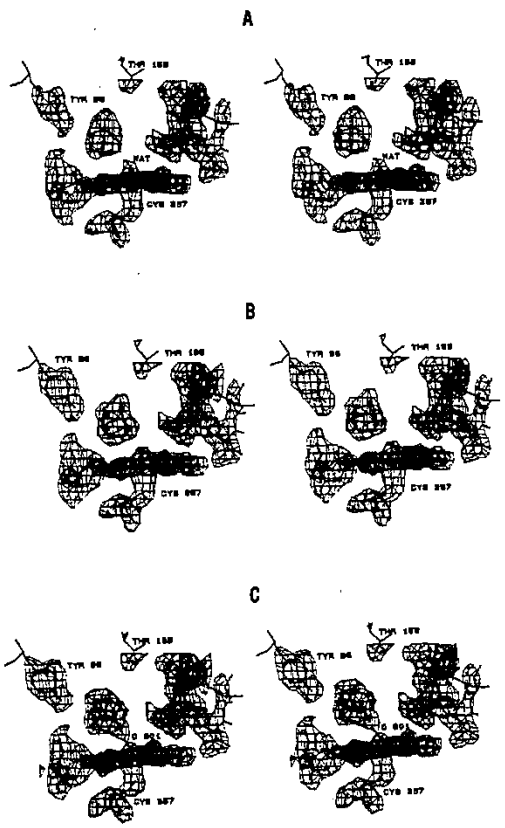

FIGURE 3: Final $2F_o - F_c$ electron density maps for P-450$_{CAM}$ complexed with camphane (A), adamantane (B), and thiocamphor (C). Substrate and distal ligand coordinates were omitted from phase calculations for the maps shown. Note the electron density corresponding to the distal ligand to iron in all maps.

from the substrate-free structure is probably minimal (less than 20%). The presence of minor, unmodeled, binding orientations due to the lack of an enzyme–substrate hydrogen bond also could artificially raise substrate temperature factors.

With these caveats in mind, we still believe the high temperature factor of camphane is genuine. One reason is that the temperature factors of Tyr96 side-chain atoms in the camphane–P-450$_{CAM}$ structure are in the vicinity of 28 Å^2 and only about 13 Å^2 in the camphor–P-450$_{CAM}$ complex, suggesting that the high camphane atomic temperature factors are real.

Adamantane. Temperature factors of Tyr96 side-chain atoms were about 19 Å^2 in adamantane-bound and 10 Å^2 in adamantanone-bound P-450$_{CAM}$ structures. These values are consistent with the lower average temperature factor of adamantane (24 Å^2) than camphane and indicate that adamantane is less mobile than camphane when bound by P-450$_{CAM}$. We were concerned about this implication, considering that adamantane is highly symmetric and smaller than camphane and that neither substrate is able to hydrogen bond to the enzyme. Therefore we performed several refinement experiments.

Using the refined coordinates for adamantane–P-450$_{CAM}$, with an R factor of 18.1%, we repositioned adamantane in the substrate electron density in two different ways: (1) by approximately switching the locations of secondary and tertiary carbons and (2) by approximately switching the locations of atoms and bonds. Both of these repositionings resulted in a somewhat poorer fit of the substrate to the electron density. Next, 10 cycles of refinement were conducted in which tem-

perature factors of all atoms and occupancies of solvent atoms were refined alternately. In both experiments, the average temperature factor of adamantane increased by about 2.5 Å^2 (to 26.1 and 25.9 Å^2, respectively). Ten control refinement cycles increased the temperature factor of the best-fit adamantane model by approximately 1.0 Å^2 to 24.7 Å^2. In all three cases, the R factor dropped only 0.1% to 18.0%. However, electron density maps calculated with both sets of repositioned coordinates contained substantial amounts of $\pm 3\sigma$ $F_o - F_c$ difference electron density, indicating that the substrate was incorrectly positioned.

Our next two experiments involved fixing the temperature factors of all adamantane atoms arbitrarily at 16.0 and 32.0 Å^2 and calculating electron density maps to determine if there would be any observable effects. No differences were found either in R factor or in electron density maps when refined adamantane temperature factors (23.6 Å^2 average) or arbitrary ones of 32.0 Å^2 were used. However, when adamantane atomic temperature factors were set to 16.0 Å^2, although the R factor remained at 18.0%, positive 3σ difference electron density appeared around the substrate in the $F_o - F_c$ map, suggesting that the new temperature factors were incorrect.

In the final adamantane refinement experiment, the adamantane model considered best fit to the electron density was again used but all substrate atomic temperature factors were started at 32.0 Å^2. After 10 refinement cycles, the average atomic temperature factor for adamantane rose insignificantly to 32.1 Å^2, rather than dropping toward the previously determined value of 23.6 Å^2. Once again, the R factor remained at 18.0% and difference electron density maps showed no indication that substrate temperature factors might be incorrect. On the basis of these refinement experiments, we cannot give a definitive value for the adamantane temperature factor, but we regard it as being somewhere in the neighborhood of 25–32 Å^2.

Thiocamphor. In the initial thiocamphor $F_o - F_c$ map, which was based on camphor–P-450$_{CAM}$ coordinates without camphor included, the substrate appeared to be binding "upside down" with respect to camphor (Figure 2C). Thus a second $F_o - F_c$ map was calculated, again based on the camphor–P-450$_{CAM}$ coordinates, but this time including camphor coordinates, in their original orientation, in the phase calculation. When this map was contoured at $\pm 3\sigma$, the camphor carbonyl oxygen was found to occupy a region of negative difference electron density and a large region of positive difference electron density remained between the substrate and heme (Figure 2D). Although it seemed apparent that thiocamphor and camphor do not bind to P-450$_{CAM}$ in the same orientation, another map was calculated, based on coordinates in which thiocamphor occupied the same orientation as camphor and in which an additional water molecule was included to occupy the positive density near the distal heme ligation site. As expected, the thiocamphor sulfur atom was surrounded by negative density and the water ligand was insufficient to account fully for the positive density between thiocamphor and the heme. Next, the distal water ligand was removed and thiocamphor was rotated (by approximately 180° in the plane of Figures 2 and 3) so that the thiocarbonyl was no longer directed toward Tyr96 but was directed toward the heme iron. Maps based on this model contained much less difference electron density but some positive difference density still remained between the sulfur and the heme iron. After including the distal ligand again, with thiocamphor in the new "upside down" orientation (Figure 4A), only a small amount of positive and negative difference density remained to either side of the sulfur atom: positive between the substrate and porphyrin ring; negative between the substrate and distal helix. Since a rotation of the sulfur to better accommodate this residual density resulted in a much deteriorated fit of the thiocamphor methyl groups to the electron density, we attempted to fit thiocamphor to the density in yet a third orientation. This orientation again had the sulfur directed toward the heme, but now the six-membered ring of the substrate was essentially parallel to the porphyrin plane (Figure 4B), rather than perpendicular as it had been previously (and as it is when camphor binds). The new $F_o - F_c$ map based on this thiocamphor orientation had considerable difference electron density, indicating that this was not the major binding orientation of thiocamphor.

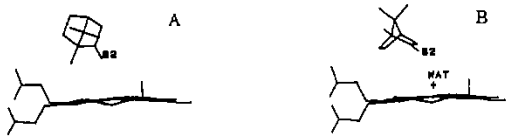

FIGURE 4: Comparison of the two different binding orientations determined crystallographically for thiocamphor. The presence of the distal ligand is only compatible sterically with the minor (30%) orientation shown on the right.

Finally, we were best able to minimize the difference electron density by including thiocamphor in both orientations (Figure 4), neither corresponding to that of camphor. The occupancy of the first orientation was estimated and fixed at 70%. In this position the sulfur atom approaches to within 2.35 Å of the distal ligand and most likely displaces it. The occupancy of the second orientation was fixed at 30%, and in this position the sulfur atom is 3.80 Å from the distal ligand. Curiously enough, when the occupancy of the distal ligand was fixed at 0.30 (to correspond with the second thiocamphor orientation) and only its temperature factor allowed to refine, although the temperature factor dropped to quite a low value (10 Å^2), positive difference density still remained around this ligand in $F_o - F_c$ maps. Nor did subsequent release of the fixed occupancy of the distal ligand, during refinement, remove the difference density, despite the fact that the occupancy climbed to 0.51 (after 20 refinement cycles; the temperature factor also dropped to 8.3 Å^2 during this time). These results suggest that occupancies and temperature factors are unable to recover, in a reasonable number of refinement cycles, from initial poor estimates of these values. They also confirm that though highly correlated, occupancies and temperature factors are not entirely interchangeable as modeling parameters.

We were only able to eliminate the difference electron density from around the distal ligand by including it initially with full occupancy and a temperature factor of 20.0 Å^2, near the mean temperature factor for all protein and heme atoms, and allowing both occupancy and temperature factor of this ligand to refine. In the final model, the distal ligand has a temperature factor of 19.6 Å^2 and an occupancy of 0.90 and $F_o - F_c$ maps calculated with these coordinates show no residual difference density around the sixth ligation position. The temperature factor of thiocamphor itself refined to about 23.5 Å^2, or about 50% higher than that of camphor bound in the active site. This higher mobility for thiocamphor, a larger substrate than camphor, suggests that thiocamphor may occupy additional minor orientations, possibly the one corresponding to camphor bound to the enzyme. Minor thiocamphor orientations which we have not modeled could potentially account also for the discrepancy between the occupancies of the distal ligand and of thiocamphor orientation 2 (Figure 4B). Another influential feature could be the fact that

Table III: Various Substrate-Dependent Parameters[g]

	 camphor	 adamantanone	 adamantane	 thiocamphor	 camphor/Y96F	 norcamphor	 camphane
molec vol	315 Å^3	300 Å^3	293 Å^3	322 Å^3	315 Å^3	236 Å^3	309 Å^3
hydrogen bond to Y96	yes[a]	yes[b]	no	no	no	yes[b]	no
no. of iron ligands	5[a]	5[b]	6	6		6[b]	6
redox pot. Fe^{3+}/Fe^{2+}	−170 mV[c]	−175 mV[c]				−206 mV[c]	
high-spin %	94–97%[c–e]	96–98%[c,d]	99%[d]	65%[e]	59%[e]	46%[c]	46%[e]
regiospecif of substr hydroxylatn	5-exo (100%)[d–f]	5 (100%)[d]	1 (100%)[d]	5-exo (64%)[e] 6-exo (34%) 3-exo (2%)	5-exo (92%)[e,f] 4 (1%) 6-exo (2–4%) 3-exo (0–4%) 9 (<1%)	5-exo (45%)[f] 6-exo (47%) 3-exo (8%)	5-exo (90%)[e] 6-exo (10%)
substr temp factor (Fe^{3+})	16.2 Å^2[a]	16.5 Å^2[b]	24.7 Å^2	23.5 Å^2		33.5 Å^2[b]	30.1 Å^2
substr hydrophilic groups	yes	yes	no	yes	yes	yes	no
hydroxylatn "efficiency"	100%[e,f]			98%[e]	100%[e]	12%[f]	8%[e]
L6–substr dist	NA	NA	2.63 Å	2.35 Å (70%) 3.35 Å (30%)		3.0 Å[b]	2.88 Å
L6–iron dist	NA	NA	1.95 Å	1.35 Å		1.73 Å[b]	1.67 Å
L6 occupancy	NA	NA	1.00	0.90		0.97[b]	1.00
L6 temp factor	NA	NA	14.3 Å^2	19.6 Å^2		3.8 Å^2[b]	7.7 Å^2
cation occupancy	1.00[a]	1.00[b]	0.89	0.91		1.00[b]	0.72
cation temp factor	12.1 Å^2[a]	10.0 Å^2[b]	15.5 Å^2	14.2 Å^2		7.9 Å^2[b]	21.7 Å^2

[a] Poulos et al. (1985, 1987). [b] Raag and Poulos (1989a). [c] Fisher and Sligar (1985). [d] White et al. (1984). [e] Atkins and Sligar (1988b). [f] Atkins and Sligar (1989). [g] Carbon numbering for each substrate begins with C-1 at the top of the six-membered ring which is in the plane of the table. Numbering proceeds counterclockwise such that the carbonyl carbon is C-2 and C-5 is in the lower right-hand portion of the ring. Note that C-5 is a secondary carbon in some substances and a tertiary carbon in others.

the distal ligand is located between the heme iron and the substrate sulfur atom. These two neighbors could conceivably interact via the distal ligand and increase electron density at this location, which could be reflected in an anomalously high ligand occupancy.

Although initial occupancy estimates for the two thiocamphor orientations were successful in eliminating substrate-associated difference electron density, we decided to explore other occupancy combinations because of the discrepancy between the refined occupancy of the distal ligand (0.90) and the estimated occupancy of the thiocamphor orientation (0.30), which would be sterically compatible with the presence of the ligand. After calculating and examining maps based on occupancy combinations ranging from 0.30/0.70 to 0.80/0.20 in increments of 0.10, we concluded that the relative occupancies of thiocamphor orientations 1 and 2 (parts A and B of Figure 4, respectively) are probably around 65% and 35%, respectively, with an error of roughly 10%.

Discussion

Substrate Hydroxylation Profiles

Camphane. Although camphane is incapable of hydrogen bonding with Tyr96, its similarity to camphor in overall shape and size causes it to be bound in a nearly identical position in the P-450$_{CAM}$ active site. The methyl groups of camphane and camphor interact with the same active site features. As with camphor, the 5-carbon atom of camphane is the nearest to the heme iron atom, explaining the observed preference (90% of products) for 5-exo hydroxylation of this substrate (Atkins & Sligar, 1988b). That 10% of the products are 6-exo hydroxylated (Atkins & Sligar, 1988b) can be attributed to the enhanced mobility of camphane in the P-450$_{CAM}$ active site (Table III).

Hydroxylation profiles and crystallographic data on norcamphor– and camphane–P-450$_{CAM}$ complexes, in comparison with camphor complexes, demonstrate that two features, a hydrogen bond to the enzyme and complementary van der Waals interactions, are both necessary to lower the mobility of a substrate. Low substrate mobility, as revealed by crystallographic temperature factors, appears to be critical for high regiospecificity of substrate metabolism (Table III).

Adamantane. Adamantane is the only substrate we have investigated, in this study, that is metabolized to a single product despite having a relatively high active site mobility. The single product can be attributed to the existence of only two types of unique carbon atoms in adamantane, together with the greater reactivity of tertiary versus secondary carbons (White et al., 1984).

Thiocamphor. Thiocamphor binds to P-450$_{CAM}$ in two orientations, both of which are different from that preferred by camphor and both of which have sulfur as the substrate atom nearest to iron. A priori, the proximity of the sulfur atom to the heme suggests that the thiocamphor hydroxylation mechanism might involve an initial single electron transfer from sulfur to heme instead of, or in competition with, initial hydrogen abstraction, as is thought to occur with camphor (Ortiz de Montellano, 1986). However, the major products of thiocamphor metabolism are 5- and 6-exo hydroxylated, and these substrate atoms are among the farthest substrate atoms from the active oxygen location in our thiocamphor–P-450$_{CAM}$ model. Modeling of thiocamphor in the orientation preferred by camphor (with full occupancy) resulted in difference electron density maps strongly suggesting that such a model was incorrect. Nevertheless, the products of thiocamphor hydroxylation imply that this substrate is only metabolized when it adopts a camphor-like orientation in the active site. These data lead us to conclude that the conformers seen in the crystal structure are nonproductive and that the camphor-like conformer is fractionally occupied and crystallographically unobservable. Although thiocamphor appears to make a snug van der Waals fit with P-450$_{CAM}$, it may be possible for it to occassionally rotate within the active site to yield a camphor-like complex, as suggested by molecular dynamics simulations of the Tyr96Phe mutant–camphor complex

(Richard Ornstein and Mark Paulsen, personal communication).

Distal Aqua Ligand (L6)

Camphor and adamantanone both have low temperature factors and displace the distal ligand, while all of the other substrates investigated here are more loosely bound and do not displace the ligand. As with norcamphor (Raag & Poulos, 1989a), the distal ligand in the presence of camphane has full occupancy and a very low temperature factor (7.7 Å^2), indicating that the 46% high-spin percentage of the camphane-bound enzyme is probably not due to partial occupancy of the distal ligation site. Rather, by displacing most of the active site solvent of the substrate-free enzyme (Poulos et al., 1986), camphane increases the active site hydrophobicity, thereby shifting the OH^-/H_2O equilibrium toward H_2O and yielding an increase in redox potential. Partial protonation of the distal ligand decreases its ligand field strength, which is responsible for the increase in high-spin percentage. This argument is supported by the observation that only high-spin P-450$_{CAM}$ is protonated (Sligar & Gunsalus, 1979).

The adamantane–P-450$_{CAM}$ complex is especially interesting because, although the small size and relatively high mobility of this substrate allow the heme to remain hexacoordinate, this complex is fully high-spin like the pentacoordinate camphor– and adamantanone–P-450$_{CAM}$ complexes (Table III). There are two factors that could account for the high-spin nature of the adamantane complex: a relatively long Fe–L6 distance and a high distal ligand mobility (Table III). Of all the substrate–P-450$_{CAM}$ structures we have determined, the iron–sixth ligand distance is at its longest (1.96 Å) in the presence of adamantane. Both the long bond length and greater ligand mobility could result from the relatively close approach of adamantane to the distal ligand: approximately 0.4 Å closer than norcamphor or camphane. This short substrate–ligand distance may also promote protonation of the ligand, resulting in the observed high-spin hexacoordinate complex.

Thiocamphor does not fit the shorter iron–L6 distance/lower spin pattern. It induces a spin state (65% high spin) intermediate between those induced by norcamphor and camphane (46%) and adamantane (100%), but in the presence of thiocamphor the iron–L6 distance is only 1.35 Å, the shortest we have seen. However, the thiocamphor complex is not directly comparable to the other substrate complexes since at least two orientations are observable. It may be that a population in which thiocamphor bound exclusively in the ligand-allowing orientation (Figure 4b) would have a greater low-spin component than the 65% quoted in Table III, which presumably arises from a population with mixed binding modes. In addition, in the presence of thiocamphor the distal ligand appears to be linking iron and the substrate sulfur atom and its ligand field strength may thus be different from that of a simple OH^-/H_2O ligand.

A final point regarding the almost unbelievably short iron–L6 distance in the presence of thiocamphor should be made. Recall that only the minor crystallographically observed thiocamphor orientation is sterically compatible with the presence of a distal ligand. However, the electron density against which our model is refined represents contributions from all thiocamphor orientations in all protein molecules in the crystal lattice. This averaged electron density, which for the most part lacks a distal ligand contribution, probably obscures the real distal ligand position in the minor orientation, resulting in the anomalously short iron–L6 distance observed.

Distal ligand movement, not iron movement, appears responsible for differences in the iron–ligand distance. The ligand location changes by about 0.6 Å in the presence of different substrates. Iron and proximal cysteine positions are quite static, despite the large changes in spin equilibrium associated with these three substrates. Although iron–distal ligand and iron–cysteine sulfur (proximal ligand) distances changed very little (0.03–0.05 Å) as a result of unrestrained refinement, multiple substrate orientations as well as incomplete substrate occupancies may have resulted in somewhat artifactual distal ligand positions and distal ligand related distances.

Cation Site and Non-Hydrogen-Bonding Substrates

In considering the various parameters interrelating substrate binding, spin state, and redox potential, the role of cations is important. Cations stabilize high-spin, substrate-bound P-450$_{CAM}$ (Peterson, 1971; Lange & Debey, 1979; Lange et al., 1979; Hui Bon Hoa & Marden, 1982; Fisher et al., 1985; Hui Bon Hoa et al., 1989). Poulos et al. (1987) identified a potential cation binding site most likely responsible for these effects. The presumed cation is octahedrally coordinated by two solvent molecules and by four carbonyl oxygens, including that of Tyr96 (Poulos, 1987; Poulos et al., 1987). The cation appears to stabilize a rather unusual conformation for the tyrosine, which occurs at the end of a helix. Further support for the role of this cation site as the one responsible for stabilizing the high-spin state stems from recent mutagenesis studies (Di Primo et al., 1990). Conversion of Tyr96 to phenylalanine significantly decreases the ability of potassium ions to convert P-450$_{CAM}$ to the high-spin state.

An interesting feature (Table III) of all of the structures we have determined in which the substrate does not hydrogen bond with Tyr96 (camphane, adamantane, and thiocamphor) is that the temperature factor and occupancy of the cation are higher and lower, respectively, than in structures with hydrogen-bonding substrates (camphor, adamantanone, and norcamphor). In the presence of camphane, the occupancy of the cation (0.72) is even lower than it is in the substrate-free enzyme (0.91; Poulos et al., 1986). The temperature factors of the six cationic ligands also are higher in the absence of an enzyme–substrate hydrogen bond. These results, together with the known cation stabilization of P-450$_{CAM}$, suggest that the enzyme is generally more stable in the presence of hydrogen-bonding than non-hydrogen-bonding substrates. Thus by hydrogen bonding to Tyr96 (or by forming nonbonded interactions near Tyr96), substrates may enhance enzyme stability via cation occupancy and mobility, which may indirectly induce subtle global conformational changes in the enzyme.

Uncoupling

In camphor hydroxylation by P-450$_{CAM}$, 100% of NADH consumed is channeled toward 5-*exo*-hydroxycamphor production. That is, camphor hydroxylation by P-450$_{CAM}$ proceeds with 100% efficiency. However, the efficiency drops to only 12% and 8% when norcamphor and camphane, respectively, are metabolized (Table III). Norcamphor and camphane are "uncouplers" of P-450$_{CAM}$ metabolism (Figure 1) and promote the enzyme to produce hydrogen peroxide and/or water rather than hydroxylated substrate (Staudt et al., 1974; Zhukov & Archakov, 1982; Gorsky et al., 1984; Atkins & Sligar, 1987, 1988a). Substrates may also induce spontaneous decay of ferrous O_2–P-450$_{CAM}$, leading to enzyme autoxidation (Eisenstein et al., 1977).

Clearly, uncoupling cannot be a simple function of the absence of either complementary enzyme–substrate interactions or of an enzyme–substrate hydrogen bond, as both norcamphor

and camphane uncouple P-450$_{CAM}$ metabolism. Uncoupling may occur even when the cation site has a low temperature factor and full occupancy, as seen with norcamphor–P-450$_{CAM}$. Uncoupling is not strictly related to high or low regiospecificity of substrate metabolism. Nor does it appear to be related solely to the fraction of low-spin heme or presence of the distal ligand since thiocamphor hydroxylation occurs with 98% efficiency (Table III). However, since the thiocamphor–P-450$_{CAM}$ complex may be nonproductive, it would be interesting to know if thiocamphor in a camphor-like orientation displaces the distal ligand.

Substrate Mobility and Uncoupling. Norcamphor and camphane do have common features, which may account for their inefficient hydroxylation by P-450$_{CAM}$. One of these is that both substrates exhibit high mobility in the active site, with similar average atomic temperature factors of 33.5 and 30.1 Å^2, respectively. These high mobilities are attributable to the same factors that cause loss of hydroxylation regiospecificity, the lack of a complementary fit between enzyme and substrate in one case and the lack of an enzyme–substrate hydrogen bond in the other, suggesting that uncoupling could have multiple proximal causes. Although substrate mobility may be somehow correlated with uncoupling, the relationship cannot be linear. Judging from even the small number of substrates with known hydroxylation efficiencies (Table III), it is rather curious that there are none that are metabolized with intermediate efficiencies; they are all either "good" or "bad" substrates. The relative mobilities of substrates bound to P-450$_{CAM}$ suggest that there may exist some kind of mobility threshold above which uncoupling occurs.

Substrate Hydrophilic Groups, Uncoupling and Catalysis. Besides orienting the substrate and helping to lower substrate mobility, the Tyr96–substrate hydrogen bond may have another role. In the CO–camphor–P-450$_{CAM}$ structure (Raag & Poulos, 1989b), we observed that carbon monoxide pushes camphor by nearly a full angstrom toward the putative substrate access channel (Poulos et al., 1986), although the camphor carbonyl oxygen–Tyr96 hydrogen bond is preserved. On the basis of the ferrous CO–camphor–P-450$_{CAM}$ structure, we expect that when O_2 binds, it will also push substrates "up" away from the heme. Following cleavage of the dioxygen bond and departure of the terminal oxygen, the substrate should have room to return toward the now activated and single, iron-linked oxygen atom. This process may depend on the existence of a hydrogen bond between Tyr96 and the substrate. We thus propose that beyond orienting a substrate and reducing its mobility, the active site tyrosine may act as a "restoring force" to facilitate substrate return toward the hydroxylating species. If a substrate did not return toward the catalytic oxygen–iron species, one might anticipate uncoupling of the P-450 reaction, with reduction of the iron-linked oxygen to water or hydrogen peroxide rather than substrate oxidation. Camphane (and adamantane?) may be such an effective uncoupler because it gets "stuck" in or near the hydrophobic access channel and is unable to return toward the active oxygen intermediate following cleavage of the dioxygen bond.

Ultimate Cause of Uncoupling. Uncoupling may be related to substrate mobility (norcamphor, camphane, adamantane?), hydrophobicity (camphane, adamantane?), the distance of a substrate from the active oxygen location, or some combination of these factors. However, regardless of the initial cause in any specific case, the primary factor causing the switch from monooxygenase to oxidase activity is probably the presence of extra solvent, in addition to the distal ligand, around the dioxygen bond undergoing cleavage. Solvent in the active site during catalysis would provide a source of protons that could facilitate dioxygen dissociation as hydrogen peroxide and/or dioxygen cleavage and reduction to water (Atkins & Sligar, 1987). Thus, in cases in which a substrate is highly mobile, or is not close enough to the heme, uncoupling may be favored over productive metabolism.

Evidence for Active Site Solvent in the Presence of Uncouplers. That *discrete* "extra" water molecules are not evident in either camphane–P-450$_{CAM}$ or norcamphor–P-450$_{CAM}$ electron density maps is not overly troublesome as both substrates are highly mobile. Active site solvent not strongly hydrogen bonded to protein or substrate may be severely disordered and unobservable. In fact, randomly ordered, mobile active site solvent in the presence of norcamphor and camphane could itself be one factor driving the temperature factors of these substrates to such high values during refinement.

We do, however, have evidence that *disordered* solvent may be in the active site together with some substrates. In electron density maps of both camphane– and adamantane–P-450$_{CAM}$ complexes, an internal solvent molecule (water 687) that is normally located "behind" the distal helix on the side away from the active site is missing (in $2F_o - F_c$ maps when contoured above approximately 0.25–0.30σ) (Figure 5A,B). Solvent 687 is present in all other structures we have determined, including thiocamphor–P-450$_{CAM}$, which also lacks an enzyme–substrate hydrogen bond (Figure 5C). As shown schematically in Figure 6, water 687 is part of an internal solvent channel connecting Thr252 with the buried residue Glu366. In the presence of camphor, this internal solvent pocket contains three crystallographically ordered solvent molecules. Glu366 is found in 42 of 53 sequences aligned by Nelson and Strobel (1989) and is part of the proximal helix (helix L), which is one of the most highly conserved segments in all P-450s. Therefore, this unusual structural feature with ordered solvent situated between a key active site residue (Thr252) and a buried glutamate side chain may be a structural feature shared by many P-450s. Unlike peroxidases where suitably positioned charged and acid-base side chains serve as catalytic groups for heterolytic O–O bond cleavage (Poulos & Finzel, 1984), P-450$_{CAM}$ has no similar set of residues, leaving water as the main candidate for a proton source. The solvent pocket linking Thr252 and Glu366 may serve this function.

Water 687 is missing not only in the presence of camphane and adamantane but also in the substrate-free P-450$_{CAM}$ structure. This suggests that, as the natural substrate camphor enters through the proposed access channel (Poulos et al., 1986), it may push at least one active site solvent molecule farther into the active site, toward and over the distal helix and into the water 687 location. Since the water 687 site is unoccupied in the presence of camphane and adamantane, one might expect electron density corresponding to an active site solvent molecule somewhere between these substrates and the water 687 site, near the distal helix.

This is an interesting possibility because in camphane–P-450$_{CAM}$, adamantane–P-450$_{CAM}$, and substrate-free P-450$_{CAM}$ maps, there is positive $F_o - F_c$ difference electron density at 3σ near Thr252 and Gly248, the highly conserved (Nelson & Strobel, 1989) distal helix residues that form the putative dioxygen binding groove (Figure 5D,E). The difference density is too close to the helix to be accounted for by hydrogen-bonded water. There is also no corresponding negative density to indicate that major side-chain rearrangements have occurred.

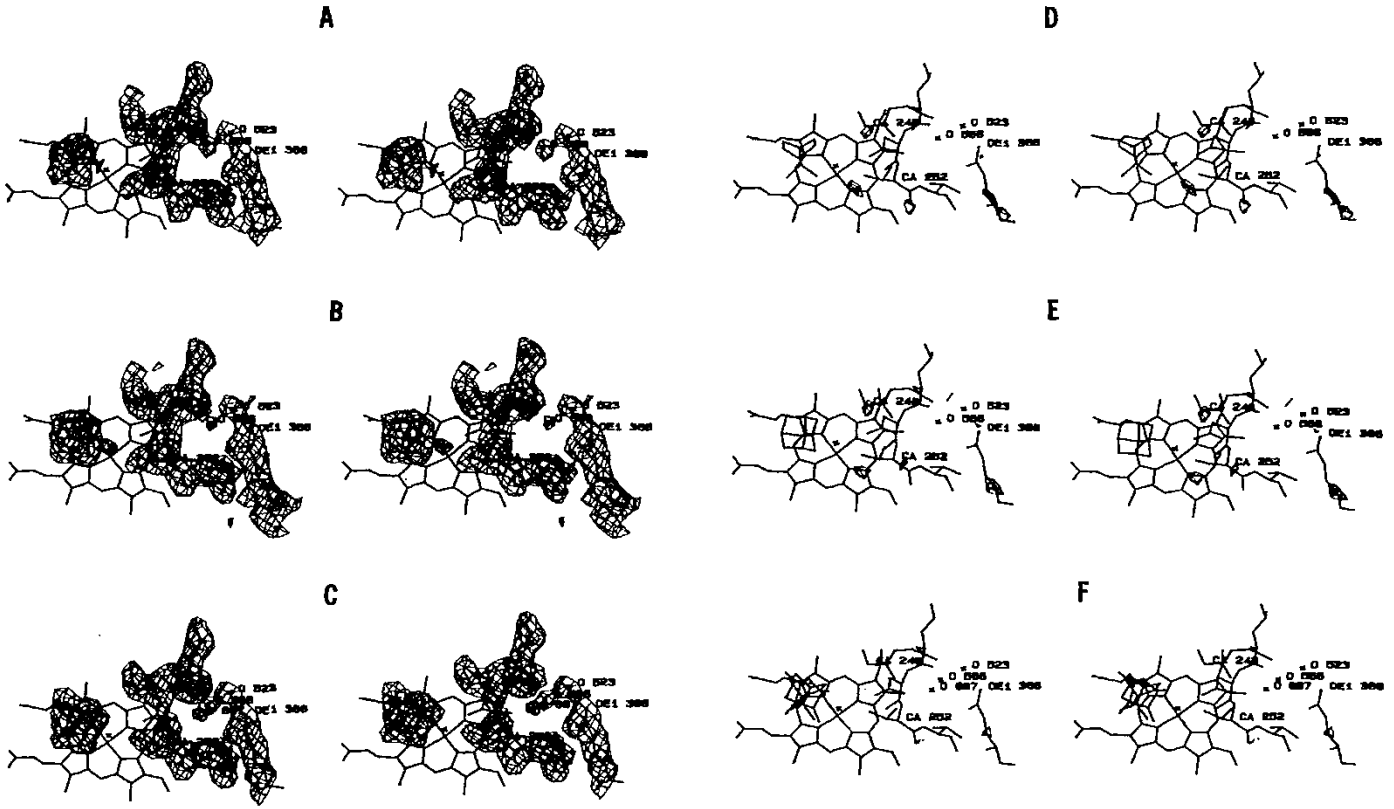

FIGURE 5: Final $2F_o - F_c$ (A–C, contoured at $+1\sigma$) and $F_o - F_c$ (D–F, contoured at $+3\sigma$) electron density maps for camphane– (A, D), adamantane– (B, E), and thiocamphor– (C,F) P-450$_{CAM}$ complexes. There is no significant negative (-3σ) $F_o - F_c$ density in the regions contoured. The view is looking along the heme normal or rotated by about 90° around the horizontal axis from the view used in other figures. Substrates are on the left of the figure, the distal ligand is denoted by a cross near the heme iron, and the dioxygen binding region of the distal helix appears on the right. On the non active site side of the distal helix is the buried solvent channel shown schematically in Figure 6. This solvent channel leads from the distal helix and Thr252 to Glu366 via three water molecules (numbered 687, 566, and 523). Note the presence of $F_o - F_c$ density near Gly248 and Thr252 (top and bottom of the dioxygen binding groove, respectively) in camphane (D) and adamantane (E) $F_o - F_c$ maps but not in the thiocamphor (F) $F_o - F_c$ map. We attribute this positive $F_o - F_c$ density to disordered solvent in the active site in the presence of camphane and adamantane. Note also the $2F_o - F_c$ density corresponding to Wat687 (on the nonactive site side of distal helix) in the thiocamphor (C) map but missing in camphane (A) an adamantane (B) $2F_o - F_c$ maps. Water 687 may play a role in uncoupling P-450$_{CAM}$ metabolism by moving over the distal helix to provide a proton source for hydrogen peroxide and water production. Val253 is located on the "back" of the distal helix near the water 687 location. Mutation of Val253 to threonine or serine in P-450$_{CAM}$ might protect this enzyme against uncoupling.

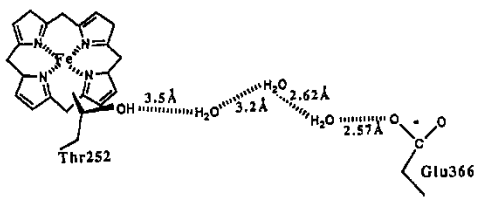

FIGURE 6: Schematic depiction of the solvent channel between Thr252 and Glu366 containing internal solvent molecules 523, 566, and 687 (Poulos et al., 1987). These solvent molecules, as well as Glu366, are completely enclosed by protein from the active site as well as from bulk solvent.

Thus *disordered* water may exist near the dioxygen binding groove in adamantane–P-450$_{CAM}$, camphane–P-450$_{CAM}$, and substrate-free P-450$_{CAM}$. Moreover, in most structures in which water 687 is present, residual positive difference density is *not* observed or is essentially negligible around Gly248 and Thr252 (Figure 5F).

That water 687 is present "behind" the distal helix in the norcamphor–P-450$_{CAM}$ structure but not in camphane-bound P-450$_{CAM}$ might suggest that its presence or absence is not related to uncoupling. However, temperature factors of Gly248 and Thr252 atoms are on the order of 40% higher (5–6 Å^2) in camphane-, adamantane-, and norcamphor-bound P-450$_{CAM}$ than they are in other substrate complexes. (In the substrate-free enzyme, the temperature factors of Gly248 atoms are even higher.) Thus, disordered active site solvent also may be present with norcamphor. The fact that the atomic temperature factors are so much higher near this proposed disordered solvent supports its existence. Temperature factors of protein atoms could refine to higher values to account for neighboring density, which has not been included in an atomic model.

Water 687 may be involved in uncoupling of P-450$_{CAM}$ substrate metabolism. Substrates with high mobility in the active site such as norcamphor and camphane may jostle iron-bound dioxygen, causing it to adopt alternate orientations. This hypothesis is supported by multiple infrared CO stretching modes in P-450$_{CAM}$ (Jung & Marlow, 1987) as well as by the ferrous CO–camphor–P-450$_{CAM}$ structure (Raag & Poulos, 1989b). When we allowed occupancies of carbon monoxide atoms to refine, the lower occupancy of oxygen (0.8) than of carbon (1.0) suggested that even in the presence of camphor, diatomic ligands may not be confined to a single orientation. If and when dioxygen is not directed toward the Gly248–Thr252 helical groove, a solvent molecule may bind there, facilitating uncoupling. Note that in its internal binding site, water 687 forms about 10 contacts with neighbors under 3.5 Å distant, including ones with three amide nitrogens, two carbonyl oxygens, the side-chain hydroxyl of Thr252, and another internal water molecule (water 566). We do not know the energy barrier for displacement of water 687 but the groove on the "back side" of the distal helix in which water 687 is bound appears to shrink slightly when water 687 is absent. Subtle global conformational changes of the protein, associated with binding of uncoupling substrates, could force the water 687 binding site to contract, effectively ejecting this molecule into the active site, resulting in uncoupling. Of course, the disordered active site solvent does not necessarily have to be water 687, although the solvent channel between Thr252 and Glu366 containing water 687 is the nearest solvent source to the active site.

Mutation-Induced Uncoupling of P-450s. Finally, not only do some metabolizable compounds uncouple P-450 substrate oxygenation, but certain mutations of the enzyme have similar effects. Several amino acids that are predicted to occur in all P-450s in the active site region, based on the structure of P-450$_{CAM}$ (Poulos et al., 1985, 1986), are highly conserved (Nelson & Strobel, 1988, 1989; Gotoh & Fujii-Kuriyama, 1989). Two of these, Gly249 and Thr252 (numbering according to P-450$_{CAM}$), are both invariant among at least 51 P-450 sequences (Nelson & Strobel, 1989). In P-450$_{CAM}$ they are located in the distal helix and may influence substrate specificity and/or oxygen activation during catalysis (Poulos et al., 1985, 1987). The highly conserved threonine is in contact with carbon monoxide in ferrous CO–camphor–P-450$_{CAM}$ (Raag & Poulos, 1989b) and due to its polar character probably stabilizes iron-bound dioxygen (Collman et al., 1980). Mutations of the active site Thr residue can cause uncoupling of P-450 reactions (Imai et al., 1989; Martinis et al., 1989). Imai et al. (1989) have suggested that Thr252 of P-450$_{CAM}$ might serve as a proton donor to the iron-linked oxygen to facilitate O–O bond cleavage. However, we doubt that a side-chain OH group with such a high pK would operate as an acid catalyst. The finding that the Thr252Val mutant allows O_2 cleavage to occur, producing more water than observed with any other mutant, casts further doubt on Thr252 as a proton donor (Imai et al., 1989). Water production, unlike the generation of hydrogen peroxide, depends on the cleavage of the O–O bond. Thus, it appears more likely that the role of Thr252 is structural.

Mutation-induced uncoupling of P-450 substrate oxygenation could be caused by distortions in the oxygen binding site that destabilize oxygen, causing it to dissociate, and/or by increasing accessibility of the active site to solvent, perhaps via the buried solvent channel between Thr252 and Glu366 (Figure 6). We propose that the way in which mutations of the invariant threonine to valine or alanine promote uncoupling is as follows. In wild-type P-450$_{CAM}$, dioxygen is electrostatically stabilized by Thr252 (Collman et al., 1980; Raag & Poulos, 1989b), one of the few hydrophilic active site groups. Mutation of this residue to a hydrophobic one destabilizes the major dioxygen binding orientation and allows the ligand more freedom of movement in the active site. Destabilization also occurs because the hydrogen bond between the Thr252 side-chain OH group and the carbonyl oxygen atom of Gly248, which helps to form part of the O_2 site, is disrupted. That hydrogen bonding is important in stabilizing the oxy complex is evidenced by the Thr252Ser mutant, which has nearly full wild-type efficiency (Imai et al., 1989) presumably because serine is capable of the same hydrogen bonding interactions as threonine. With the distal helix groove between Gly248 and Thr252 no longer dominated by dioxygen, there may be room for water 687 or another solvent molecule to bind in this region. On the active site side of the distal helix, there are few potential interactions that could strongly bind a solvent molecule. Thus its binding location may encompass the entire dioxygen binding groove region, from Gly248 to the residue at the 252 location and perhaps beyond, accounting for the disordered quality of the density we attribute to this water molecule. If dioxygen spends less time directed toward the distal helix, not only may it allow solvent to enter the dioxygen binding groove but it may itself sterically interfere with the approach of substrate toward the heme, facilitating uncoupling.

Whether dioxygen dissociates as peroxide or is cleaved and reduced to water appears to depend on the specific mutation (Imai et al., 1989). That different P-450 mutants have different high- and low-spin fractions (Imai & Nakamura, 1989) supports the idea that these mutants have varying active site

solvent accessibilities. Some substrates also appear to be more sensitive than others to P-450 active site mutations (Furuya et al., 1989a,b), a phenomenon that appears to be related to conformational flexibility of the substrate.

A Residue That May Protect against Uncoupling. In 36 of 52 P-450 sequences that have been aligned (Nelson & Strobel, 1989), the sequence Thr-Thr replaces Thr-Val (252–253) in P-450$_{CAM}$. Residue 253 of P-450$_{CAM}$, immediately downstream of the invariant threonine, is located on the "back" of the distal helix in a position in which it should be able to hydrogen bond to water 687, if it were a threonine or serine. A residue capable of hydrogen bonding to water 687 on the back of the distal helix could protect P-450s against uncoupling, at least when and if uncoupling is mediated via this water molecule. In mutagenesis experiments with different P-450s, hydrogen peroxide and water production (resulting from uncoupling) should be monitored in addition to catalytic activities to explore this issue. If water 687 plays a role in uncoupling it is likely that (1) a Thr (corresponding to 253 in P-450$_{CAM}$) may yield an enzyme more resistant to uncoupling induced by *mutations* of the immediately preceding, invariant threonine and (2) a Thr-Thr sequence (corresponding to 252–253 in P-450$_{CAM}$) may be more resistant to *substrate*-induced uncoupling than a Thr-Val sequence in this location.

In this light, a report from the 1990 Microsomes and Drug Oxidations VIII meeting in Stockholm, Sweden, is very intriguing. Imai and colleagues observed lower laurate and caprate hydroxylase activities with a Thr-Val sequence than with Thr-Ser or Thr-Thr sequences in chimeric P450s, although they did not monitor peroxide or "excess" water production (Imai et al., 1990).

Conclusions

Hydroxylation profiles of norcamphor and camphane by P-450$_{CAM}$ demonstrate that both a complementary fit between substrate and enzyme and a hydrogen bonding "anchor" between substrate and enzyme are essential to orient and bind the substrate tightly in order to obtain the absolute regio- and stereospecificity observed with camphor hydroxylation (Atkins & Sligar, 1988b, 1989). Our crystallographic results show that high mobility and loss of specificity are strongly correlated.

Distal ligand displacement by a substrate may also depend on the mobility-lowering effect of an enzyme–substrate hydrogen bond, as demonstrated by comparison of camphor/camphane and adamantanone/adamantane temperature factors (Table III). Hydrogen bonding between enzyme and substrate, in turn, lowers the mobility and increases the occupancy of the cation site, which results in enhanced stability of the enzyme in the presence of hydrogen-bonding substrates (Hui Bon Hoa et al., 1989; Di Primo et al., 1990).

Whether or not the distal aqua ligand remains in a substrate–P-450 complex is controlled by steric crowding between the substrate and ligand, and is influenced by both substrate size and mobility. Moreover, the high/low spin equilibrium is controlled not only by the presence or absence of the aqua ligand but also by the state of distal ligand protonation (Sligar & Gunsalus, 1979). Based on the adamantane complex, P-450$_{CAM}$ can remain hexacoordinate yet be fully high-spin due to the high mobility of the aqua ligand and the long Fe–L6 distance. High L6 mobility and long Fe–L6 distance result from the close approach of adamantane to the aqua ligand, which may also increase the proton affinity of the ligand.

Disordered solvent is present in the active site in the presence of highly mobile substrates. This active site water(s) may be directly responsible for uncoupling, the diversion of reducing equivalents away from substrate hydroxylation and toward hydrogen peroxide and/or water production. We have identified an internal solvent molecule, water 687, located on the opposite side of the distal helix from the dioxygen-binding groove, which does not appear to be present in all substrate–P-450$_{CAM}$ complexes and which may be associated with uncoupling. We propose that P-450s in which the residue immediately downstream of the invariant active site threonine is also a threonine, or a serine, may resist uncoupling by providing an additional hydrogen bond to water 687 and thereby stabilizing it in its nonactive site location.

Water 687 is part of an internal solvent pocket between two highly conserved residues, Thr252 in the active site and the buried side chain of Glu366. This solvent channel may serve as the source of protons required in cleaving the O–O bond during the P-450 catalytic cycle.

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Regioselectivity in the Cytochromes *P*-450: Control by Protein Constraints and by Chemical Reactivities[1]

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Three alicyclic compounds (D-camphor, adamantanone, adamantane) were found to be hydroxylated by the cytochrome *P*-450 isoenzymes *P*-450$_{cam}$ and *P*-450$_{LM2}$. With *P*-450$_{cam}$ as the catalyst only one product was formed from each of the substrates: 5-*exo*-hydroxycamphor, 5-hydroxyadamantanone, and 1-adamantanol. With *P*-450$_{LM2}$ as the catalyst, two or more isomeric products were formed from each substrate: 3-*endo*-, 5-*exo*-, and 5-*endo*-hydroxycamphor; 4-*anti*- and 5-hydroxyadamantanone; and 1- and 2-adamantanol. The products from *P*-450$_{cam}$ hydroxylations were found to be isosteric with one another, suggesting that each of them was attacked at a topologically congruent position within a rigid enzyme–substrate complex. The distribution of products from *P*-450$_{LM2}$ hydroxylations, on the other hand, were similar to the distributions expected during solution-phase hydroxylations. Thus, it would appear that the complex which *P*-450$_{LM2}$ forms with its substrate allows considerable movement of the substrate molecule, such that most of the hydrogens in the substrate are exposed to the enzymatic hydrogen abstractor. Under these conditions, the distribution of products more nearly reflects the rank order of chemical reactivities of the various hydroxylatable positions, with only a moderate protein-based steric constraint being expressed. These suggestions were also evident in the tightness of binding of the substrates to the two enzymes and in the magnitude of coupling between the substrate binding and the spin-state equilibria. Thus, the product from *P*-450$_{cam}$-catalyzed hydroxylation may be predicted by a consideration of the relation of the topology of the prospective substrate to that of D-camphor. The products from *P*-450$_{LM2}$-catalyzed hydroxylations, on the other hand, may be approximately predicted from the chemical reactivities of the various abstractable hydrogens in the prospective substrate.

The cytochromes *P*-450[2] comprise a family of ubiquitous enzymes whose function is to introduce a hydroxyl group into an organic compound. The reasons for such functionalization of the organic molecule are as diverse as the biological locations of the enzymes themselves. For instance, *P*-450$_{cam}$ hydroxylates D-camphor as the first step in the catabolism of this terpene when *Pseudomonas putida* is grown on camphor as the sole carbon source (9). On the other hand, the mammalian adrenal cytochromes *P*-450 are involved in several hydroxylations of the steroid nucleus during steroidogenesis (9). Still another example is the hydroxylation of various lipophilic xenobiotics by mammalian liver

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[2] Abbreviations used: *P*-450$_{cam}$, soluble, camphor-hydroxylating cytochrome *P*-450 isolated from *Pseudomonas putida*; *P*-450$_{LM2}$, rabbit liver microsomal cytochrome *P*-450 inducible by *in vivo* pretreatment with phenobarbital; *P*-450$_{LM4}$, rabbit liver microsomal cytochrome *P*-450 inducible by *in vivo* pretreatment with β-naphthoflavone; *P*-450, generic term encompassing all forms of cytochrome *P*-450; GC-MS, gas chromatography–mass spectrometry.

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microsomal cytochromes for the purpose of providing a suitable nucleophilic functional group for subsequent hydrophilic derivatization by conjugating enzymes such as UDP-glucuronyl transferase.

As we would expect, the substrate specificities of the various *P*-450 enzymes vary widely. However, since the basic chemical transformation is the same among them all, we can inquire as to the relative roles of the substrate-binding site on the protein and the intrinsic chemistries of the substrate carbon skeleton and of the *P*-450 reactive oxygen intermediate in determining which molecules and, just as importantly, which positions on those molecules are hydroxylated. In this paper, we have examined the hydroxylation of three substrates by two *P*-450 isozymes. The substrates (camphor, adamantanone, and adamantane) were chosen such that both enzymes exhibited hydroxylase activity toward them. We report two near extremes of substrate specificity and regioselectivity. The selection of substrates and hydroxylation sites by $P\text{-}450_{cam}$ is strongly controlled by the substrate-binding site, while $P\text{-}450_{LM2}$ shows little selection among substrates and a regioselectivity suggestive of chemical rather than protein control.

MATERIALS AND METHODS

Chemicals. Three of the hydroxycamphors, the 5-*exo*- and 5-*endo* epimers, and 6-*endo* were a gift from Dr. I. C. Gunsalus. 3-*exo*-Hydroxycamphor was prepared by zinc dust reduction of 3-ketocamphor (camphoroquinone) as described by Kreiser (11). 5-Ketocamphor and adamantane-2,4-dione were prepared by Jones oxidation of 5-*exo*-hydroxycamphor and of 4-hydroxyadamantanone. Authentic samples of 4-*syn*-, 4-*anti*-, and 5-hydroxyadamantanones were the generous gift of Dr. James Henkel, Department of Medicinal Chemistry, University of Connecticut. Dilauroyl glyceryl-3-phosphorylcholine, isocitric acid, and $NADP^+$ were purchased from Sigma Chemical Company. All other chemicals were purchased from Aldrich Chemical Company.

Enzymes. Previously described procedures were followed for the preparation of electrophoretically homogeneous $P\text{-}450_{cam}$, putidaredoxin, and putidaredoxin reductase (10); $P\text{-}450_{LM2}$ and $P\text{-}450_{LM4}$ (1); and NADPH-cytochrome *P*-450 oxidoreductase (3). Catalase and isocitric dehydrogenase were purchased from Sigma.

Enzyme-substrate titrations. With $P\text{-}450_{LM2}$, a solution of the protein (1 nmol) and dilauroyl glyceryl-3-phosphorylcholine (50 μg) in 1.0 ml of potassium phosphate buffer (0.1 M, pH 7.4) at 25°C was titrated with small aliquots of a methanolic solution of the substrate. Optical spectra in the region 350 to 500 nm were collected with a Varian-Cary 219 spectrophotometer interfaced to an Apple II Plus microcomputer. Difference spectra were calculated by subtracting the original spectrum from the subsequent substrate-perturbed spectra, using 405 nm as an isosbestic point. Plots of the reciprocal of the magnitude of the difference spectrum versus the reciprocal of the substrate concentration were linear, with correlation coefficients from 0.991 to 0.998. From such plots apparent dissociation constants for the enzyme-substrate complexes and maximal mole fractions of high-spin heme were calculated. In the case of tight binding substrates, corrections for substrate depletion effects were applied by computer iteration. Substrate titrations of $P\text{-}450_{cam}$ were performed as previously described (6).

Enzymatic hydroxylation reactions. Hydroxylation reactions involving $P\text{-}450_{LM2}$ or $P\text{-}450_{LM4}$ utilized an NADPH-generating system consisting of isocitric dehydrogenase (0.4 units), $NADP^+$ (100 μM), isocitric acid (5 mM), and magnesium chloride (10 mM). Catalase (6000 units) was included to prevent the accumulation of hydrogen peroxide. Reaction mixtures contained $P\text{-}450_{LM2}$ or $P\text{-}450_{LM4}$ (1.0 nmol), NADPH-cytochrome *P*-450 oxidoreductase (3.0 nmol), dilauroyl glyceryl-3-phosphorylcholine (50 μg, added as a sonicated suspension in water), potassium phosphate (100 μmol, pH 7.4), one of the substrates (added as a concentrated solution in methanol), catalase, and the NADPH-generating system in a total volume of 1.0 ml. Reactions were initiated by the addition of isocitric dehydrogenase and were allowed to proceed for 3 to 6 h at 25°C. Accumulation of product in such reactions was linear with time for at least 3 h. The generating system prevents the accumulation of $NADP^+$, which inhibits reductase, and the catalase removes H_2O_2, which can destroy *P*-450. In these circumstances, rates will be constant until one of the substrates becomes depleted or until product inhibition becomes important. These effects do not occur for hours with the rates observed here. The observed regioselectivity was invariant with the time period of reaction. Thus, product yields were maximized by allowing the reaction to run to completion. Rate measurements were, of course, made within linear reaction times. Control reactions were also run in which the cytochrome *P*-450 reductase was omitted. No background levels of the reported products were present in these controls. Substrate concentrations were: D-camphor, 5 mM; adamantanone, 8 mM; and adamantane, 0.3 mM.

Hydroxylation reaction mixtures involving $P\text{-}450_{cam}$ contained $P\text{-}450_{cam}$ (0.5 nmol), putidaredoxin (2.2

nmol), putidaredoxin reductase (0.5 nmol), and potassium phosphate (100 μmol, pH 7.0) in a total volume of 1.0 ml. Reactions were initiated by the addition of NADH (200 nmol) and were conducted at 20°C. Substrate concentrations were all 1 mM with $P\text{-}450_{cam}$.

At the end of the reaction periods an appropriate amount of a suitable internal standard was added and the products were extracted with 1 ml of chloroform. The standards used were *p*-chlorobenzyl alcohol with camphor and adamantanone, and 1-phenylethanol with adamantane. The chloroform solutions were concentrated by evaporation under nitrogen and subjected to gas chromatographic analysis on both a polar and a nonpolar column, using flame ionization detection. Column A (polar) was 10% Carbowax 20 M on Supelcoport (80/100). Column B (nonpolar) was 3% OV-17 on Supelcoport (80/100). Both were 1/8 in. by 6 ft stainless-steel columns. Gas chromatographic peaks were quantitated with a Hewlett-Packard 3390A Integrator. Gas chromatography–mass spectrometry was performed on a Hewlett-Packard 5992 instrument, using 3-ft versions of Columns A or B.

Oxidation of secondary alcohols to ketones for structural identification purposes was accomplished by use of the Jones reagent (0.1 M sodium dichromate in 2.5 M sulfuric acid). The alcohol sample in chloroform (0.1 ml) was shaken with Jones reagent (0.2 ml) for a few minutes at room temperature until the orange color had changed to green. The sample was centrifuged and the aqueous layer was removed. The chloroform layer was washed once with water and then subjected to gas chromatography.

RESULTS

Hydroxylation of D-camphor. Ten isomeric hydroxycamphors could in principle result from enzymatic hydroxylation of D-camphor. These are 3-*exo*-, 3-*endo*-, 4-, 5-*exo*-, 5-*endo*, 6-*exo*, 6-*endo*-, 8-, 9-, and 10-hydroxycamphor. Exposure of D-camphor to the reconstituted $P\text{-}450_{cam}$ enzyme system leads to the accumulation of a single product which has been rigorously shown to be 5-*exo*-hydroxycamphor (5). Under the conditions described here, this product is produced as a catalytic rate of 60 mol/mol $P\text{-}450_{cam}$/min. Greater rates may be obtained by manipulation of putidaredoxin concentrations and extrapolation of measured values to infinite redoxin concentration. However, moderate concentrations of the redoxin were used here in the interests of conservation of enzyme preparations. When a reconstituted $P\text{-}450_{LM2}$ enzyme system is substituted for the $P\text{-}450_{cam}$ system, three products accumulate which GC–MS demonstrates to be hydroxycamphors (designated A, B, and C; see Fig. 1). Gas chromatography on both polar and nonpolar columns (Table I) as well as their mass spectra (Table II) demonstrated hydroxycamphors B and C to be 5-*exo*- and 5-*endo*-hydroxycamphor, respectively. The evidence strongly indicates hydroxycamphor A to be 3-*endo*-hydroxycamphor. The mass spectrum of A showed a substantial $M^+ - 73$ peak (*m/z* 95) due to loss of hydroxyketene followed by methyl, indicating the hydroxyl group to be in the 3 position, adjacent to the carbonyl. An appreciable $M^+ - 18$ peak (*m/z* 150) due to loss of water would indicate a 3-*endo*-hydroxyl stereochemistry since a 3-*exo*-hydroxyl has no readily abstractable hydrogen atoms nearby. Furthermore, oxidation of the mixture of A, B, and C by the Jones reagent gave two ketocamphors D and E, which were shown by gas chromatography (Table I) and GC–MS (Table II) to be 3-keto- and 5-ketocamphor, respectively, with the aid of the authentic compounds. Also, the mass spectrum of authentic 3-*exo*-hydroxycamphor was distinct from that of C. However, in the absence of an authentic sample of 3-*endo*-hydroxycamphor one cannot be absolutely certain of our assignment. As seen in Table

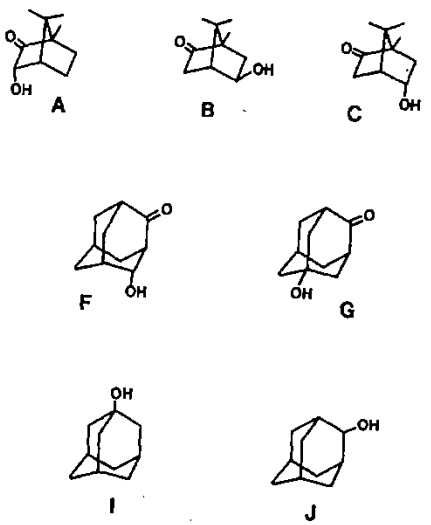

FIG. 1. Hydroxylation products with $P\text{-}450_{LM2}$.

TABLE I

GAS CHROMATOGRAPHIC DATA

Compound	Retention times, min (at °C)	
	Carbowax 20 M	OV-17
3-*exo*-Hydroxycamphor	2.90 (180)	1.70 (150)
5-*exo*-Hydroxycamphor	5.64 (180)	2.77 (150)
5-*endo*-Hydroxycamphor	7.28 (180)	3.18 (150)
6-*endo*-Hydroxycamphor	—	3.5 (150)
Hydroxycamphor A	2.90 (180)	1.79 (150)
Hydroxycamphor B	5.68 (180)	2.75 (150)
Hydroxycamphor C	7.28 (180)	3.17 (150)
3-Ketocamphor	2.15 (180)	2.18 (150)
5-Ketocamphor	1.96 (180)	1.90 (150)
Ketocamphor D	2.15 (180)	2.18 (150)
Ketocamphor E	1.93 (180)	1.82 (150)
4-*syn*-Hydroxyadamantanone	10.30 (200)	7.84 (150)
4-*anti*-Hydroxyadamantanone	9.32 (200)	6.98 (150)
5-Hydroxyadamantanone	6.18 (200)	5.94 (150)
Hydroxyadamantanone F	9.24 (200)	8.09 (150)
Hydroxyadamantanone G	6.14 (200)	5.72 (150)
Adamantane-2,4-dione	4.99 (200)	7.39 (150)
Adamantanedione H	4.98 (200)	7.41 (150)
1-Adamantanol	6.44 (140)	2.12 (130)
2-Adamantanol	9.69 (140)	2.75 (130)
Adamantanol I	6.44 (140)	2.11 (130)
Adamantanol J	9.68 (140)	2.74 (130)

III, 5-*endo*-hydroxycamphor was by far the predominant product, with the 3-*endo*- and 5-*exo*-isomers being about equally formed. Small amounts of two or three compounds not present in no-enzyme controls were detected. Their total amount was only about 7% of the total, but because of their low yields they could not be identified. None of the product peaks had a gas chromatographic retention time similar to that of 6-*endo*-hydroxycamphor (Table I). The overall catalytic rate of camphor hydroxylation by $P\text{-}450_{LM2}$ was 1.3 mol/mol $P\text{-}450_{LM2}$/min.

A third P-450 isozyme, $P\text{-}450_{LM4}$, was allowed to oxidize D-camphor as well. In this case, the rate was exceedingly low, only 0.006 mol/mol $P\text{-}450_{LM4}$/min. The only product detected was 5-*exo*-hydroxycamphor, although, in view of the amount of product, small amounts (10–20%) of other isomeric hydroxycamphors could have escaped notice.

Hydroxylation of adamantanone. Because of the symmetry of the adamantanone skeleton (C_{2v} symmetry), only five isomeric hydroxyadamantanones are possible, neglecting enantiomers. These are 1-, 4-*syn*-, 4-*anti*-, 5-, and 6-hydroxyadamantanone. The 4-*syn*- and 4-*anti*-hydroxyadamantanones may exhibit enantiomerism, but the present analytical methods would not distinguish enantiomers. Adamantanone was hydroxylated by $P\text{-}450_{cam}$ to a single product, hydroxyadamantanone G, at a rate of 52 mol/mol $P\text{-}450_{cam}$/min. $P\text{-}450_{LM2}$, on the other hand, produced two hydroxyadamantanones F and G, in a 3:2 ratio (Table III). The rate was 1.9 mol/mol $P\text{-}450_{LM2}$/min. Fortunately, authentic samples were available to us for three of the five possible hydroxyadamantanones. Gas chromatographic retention times (Table I) and mass spectra (Table II) indicated that F and G were 4-*anti*- and 5-hydroxyadamantanone, respectively. Deconvolution of the mass spectra in order to rule out the possibility that F and G were other isomers such as the 1- or 6-isomers was not feasible due to the complexity of fragmentation pathways inherent in the symmetric tricyclic skeleton. However, Jones oxidation of the mixture of F and G produced an adamantanedione H and left G unchanged. H was identified as adamantane-2,4-dione by gas chromatography (Table I). This proved that G is a tertiary alcohol while F is a 4-hydroxyadamantanone. Since the gas chromatographic behavior and mass spectra of 4-*syn*- and 4-*anti*-hydroxyadamantanone were similar (cf. Tables I and II), we cannot be absolutely sure of our assignment that F is 4-*anti*-hydroxyadamantanone as opposed to the *syn* stereoisomer. However, the assignment that G is the 5-hydroxy isomer is certain.

Hydroxylation of adamantane. The adamantane skeleton has an even higher symmetry than does adamantanone (T_d symmetry). As a consequence, only two isomeric adamantanols (1- and 2-adamantanol) are possible, neither of which may exhibit enantiomerism. As before, $P\text{-}450_{cam}$ produced a single product, I, identified as 1-adamantanol. The gas chromatographic technique used would have detected the

TABLE II

MASS SPECTROMETRIC DATA

Compound	Prominent Ions, *m/z* (% Abundance)
3-*exo*-Hydroxycamphor	168 (26%), 125 (36%), 95 (20%), 84 (75%), 83 (91%), 70 (52%), 41 (100%)
5-*exo*-Hydroxycamphor	168 (32%), 153 (13%), 125 (41%), 124 (25%), 123 (23%), 111 (100%), 109 (25%), 107 (27%)
5-*endo*-Hydroxycamphor	168 (23%), 153 (69%), 125 (29%), 124 (42%), 123 (41%), 111 (88%), 109 (75%), 108 (100%), 107 (98%), 93 (67%)
Hydroxycamphor A	168 (29%), 150 (14%), 135 (58%), 125 (46%), 95 (31%), 84 (93%), 83 (100%)
Hydroxycamphor B	168 (35%), 153 (4%), 125 (50%), 124 (27%), 123 (21%), 111 (100%), 109 (15%), 107 (27%)
Hydroxycamphor C	168 (13%), 153 (36%), 125 (8%), 124 (10%), 123 (10%), 111 (31%), 109 (37%), 108 (100%), 107 (54%), 93 (59%)
3-Ketocamphor	166 (12%), 138 (19%), 123 (19%), 110 (11%), 95 (100%), 83 (51%)
5-Ketocamphor	166 (87%), 151 (11%), 138 (11%), 123 (71%), 109 (81%), 107 (25%), 95 (41%), 69 (100%)
Ketocamphor D	166 (50%), 138 (32%), 123 (49%), 110 (26%), 95 (100%), 83 (76%)
Ketocamphor E	166 (99%), 151 (21%), 138 (20%), 123 (80%), 109 (96%), 95 (27%), 69 (100%)
4-*syn*-Hydroxyadamantanone	166 (20%), 148 (18%), 138 (31%), 120 (12%), 109 (10%), 96 (40%), 79 (100%)
4-*anti*-Hydroxyadamantanone	166 (24%), 148 (14%), 138 (46%), 120 (13%), 109 (18%), 96 (73%), 79 (100%)
5-Hydroxyadamantanone	166 (26%), 148 (10%), 108 (22%), 95 (100%), 79 (14%)
Hydroxyadamantanone F	166 (21%), 148 (14%), 138 (47%), 120 (15%), 109 (19%), 96 (70%), 79 (100%)
Hydroxyadamantanone G	166 (20%), 148 (9%), 108 (19%), 95 (100%)

Note. Electron-impact mass spectra were recorded on various authentic standards and on cytochrome *P*-450 reaction products using a Hewlett-Packard 5992 gas chromatograph-mass spectrometer. The ionization energy was 14 eV.

presence of less than 1% of 2-adamantanol because of the good separation (see Table I). However, none was detectable. The catalytic rate was 43 mol/mol $P\text{-}450_{cam}$/min. Also, as before, $P\text{-}450_{LM2}$ gave more than one product, specifically I and J, which were identified by gas chromatography (Table I) as 1- and 2-adamantanol. Jones oxidation of the mixture of I and J gave 2-adamantanone and unchanged I. The catalytic rate was 1.6 mol/mol $P\text{-}450_{LM2}$/min.

Substrate-binding phenomena. Dissociation constants for the various enzyme-substrate complexes were measured by monitoring the substrate-induced spectral change as the enzyme was titrated with substrate (14). The spectral change arises from a perturbation of the ligand field experienced by the heme iron, resulting in a change in the equilibrium constant between the high-spin and low-spin states (15). The maximal spectral change (ΔA_{max}) at saturating substrate concentration was extrapolated from iterative double-reciprocal plots of substrate concentration versus absorbance change. The maximum change in the mole fraction of high-spin heme was calculated by dividing ΔA_{max} by the differential extinction coefficient ($\Delta\epsilon$) and the total heme concentration. The total mole fraction of high-spin heme (X^{HS}_{max}) is given by the sum of this change and the initial high-spin mole fraction (X^{HS}_0). Thus, the new, substrate-perturbed equilibrium constant between high- and low-spin heme can be calculated from X^{HS}_{max}.

$$X^{HS}_{max} = [\Delta A_{max}/(\Delta\epsilon/[\text{heme}])] + X^{HS}_0$$

$$K_{eq} = \frac{[\text{high-spin}]}{[\text{low-spin}]} = \frac{X^{HS}_{max}}{1 - X^{HS}_{max}}$$

$P\text{-}450_{cam}$ bound its ketonic substrates tightly with dissociation constants in the low micromolar region, while the hydrocarbon adamantane bound less tightly. Camphor, the natural substrate, is well known to cause nearly a complete shift of

TABLE III

HYDROXYLATION OF ALICYCLIC SUBSTRATES BY VARIOUS CYTOCHROMES *P*-450

Enzyme	Substrate	K_d	X^{HS}_{max}	K_{eq}	Rate	Regioselectivity (%)
P-450$_{cam}$	D-Camphor	2.9	0.94	16	60	5-*exo* (100)
P-450$_{cam}$	Adamantanone	3.5	0.98	49	52	5- (100)
P-450$_{cam}$	Adamantane	50	0.99	99	43	1- (100)
P-450$_{LM2}$	D-Camphor	44	0.37	0.59	1.3	3-*endo* (16) 5-*exo* (14) 5-*endo* (63) other (7)
P-450$_{LM2}$	Adamantanone	65	0.25	0.33	1.9	4-*anti* (57) 5- (43)
P-450$_{LM2}$	Adamantane	2.0	0.54	1.2	1.6	1- (91) 2- (9)
P-450$_{LM4}$	D-Camphor	—[a]	—[a]	—	0.006	5-*exo* (100)

Note. Hydroxylation reactions were conducted as described under Materials and Methods. Rates are expressed as total mole product per mole enzyme per minute, while regioselectivity is expressed as the positions of hydroxylation of that substrate, with the corresponding percentage of the total product in parentheses. Dissociation constants (K_d) and mole fractions of high-spin heme were determined from titrations of the ferric cytochromes with the respective substrates as described under Materials and Methods. Dissociation constants are expressed in micromolar values.

[a] Dash indicates that this quantity was not measured.

resting P-450$_{cam}$ to the high-spin form (14). We were therefore surprised to find that adamantanone pushed the enzyme to a higher mole fraction of high-spin form than did camphor (Table III). Adamantane pushed the equilibrium even more toward high-spin. The binding of the ketonic substrates to P-450$_{LM2}$ was about 10–20 times weaker than to P-450$_{cam}$, but adamantane actually bound much more tightly to P-450$_{LM2}$ than to P-450$_{cam}$. Adamantane bound some 30 times tighter than did adamantanone to P-450$_{LM2}$, in agreement with the general phenomenon of more polar substrates displaying higher K_d values with this enzyme. In general, P-450$_{LM2}$ does not exhibit spin-state changes as large as does P-450$_{cam}$. With P-450$_{LM2}$, camphor produced a larger spin shift than did adamantanone, in contrast to the observation with P-450$_{cam}$. However, the biggest change with P-450$_{LM2}$, as with P-450$_{cam}$, was observed with adamantane.

DISCUSSION

In a purely chemical, nonenzymatic, solution-phase hydroxylation reaction, every molecule dissolved in the medium will be attacked by the hydroxylating reagent during the frequent bimolecular collisions. If the molecule contains C–H bonds in various positions, then the potential exists for several isomeric hydroxylated products to result. The factors which determine the percentage composition of this product mixture include (a) the ground-state electron density of the various C–H bonds, (b) the efficiency of resonance dispersal of the unpaired spin of each potential carbon-centered radical, (c) the ability of each carbon center to adopt a planar geometry after hydrogen abstraction, (d) the degree of steric hindrance of approach of the hydrogen abstractor to the several C–H bonds, (e) the steric requirements of the hydrogen abstractor, and (f) the electro-

philic reactivity of the hydrogen abstractor.

A hydroxylase enzyme must also act according to these factors; control of which molecules will be hydroxylated in which positions can only be exerted at four levels. First, at a level prior to (a) through (f), the topography of the active site may deny access to the substrate, so that collisions between the hydrogen abstractor and the substrate never occur. In addition, the enzyme may allow the substrate to bind, but only in a fashion which restricts collisions to selected loci on the substrate (i.e., a special case of (e) above). With a flexible molecule, the enzyme might bind only a special conformation which removes some of the steric hindrance referred to in (d). Of course, this is not possible with rigid substrates like camphor or adamantane. Finally, the enzyme may modulate the electrophilicity of the hydrogen abstractor so that some of the substrate positions are no longer reactive enough to be successfully attacked (i.e., a special case of (f) above). However, an enzyme may do little about factors (a), (b), and (c). It is, of course, conceivable that some substrate-binding energy might be used to deform the substrate and thereby modulate reactivity of the various C–H bonds, but such effects must necessarily be small, except in large substrate molecules with one or more polar functional groups. For instance, steroid hydroxylases might owe some of their high catalytic specificity to deformation effects. Nonetheless, on a given molecule, a poorly reactive position cannot be efficiently hydroxylated by any enzyme without making the hydrogen abstractor hyperreactive. Conversely, if it is necessary to avoid hydroxylating very reactive positions on a substrate, the enzyme must set in place difficult stereochemical barriers to "fence off" the undesired regions. In particular, it is not possible to use effects such as hydrogen-bonding, ion-pairing, and general acid-base catalysis to specifically activate a particular C–H bond, as is possible with other reactions by enzymes such as proteases. In short, the *P*-450 hydroxylases must rely primarily on steric effects of the protein envelope at the active site to produce high regiospecificity in their hydroxylation reactions.

In the following discussion, we make the assumption that $P\text{-}450_{LM2}$ and $P\text{-}450_{cam}$ utilize the same reactive oxygen intermediate, although the species produced by the two isozymes may show small differences in their reactivities. We will present arguments that the hydroxylation products of $P\text{-}450_{LM2}$ are approximately predicted by simple chemical considerations of the substrate molecules (i.e., factors (a)–(f) above). Ullrich earlier made a similar suggestion with respect to hydroxylations carried out by liver microsomal suspensions (4). On the other hand, the products of $P\text{-}450_{cam}$ are not predicted by chemical principles, but can be rationalized on stereochemical grounds by *a priori* knowledge of the regiospecificity of any one of its substrates. We make note of the general phenomenon observed in the present data that $P\text{-}450_{cam}$ always makes a single isomer, while $P\text{-}450_{LM2}$ characteristically makes two or more isomers. Figure 1 shows the seven alcohols produced by $P\text{-}450_{LM2}$ from the three substrates examined here. The three alcohols produced by $P\text{-}450_{cam}$ are depicted in Fig. 2.

One potential alicyclic substrate for $P\text{-}450_{cam}$, norcamphor, has the same bicyclic skeleton as camphor but lacks the three bulky methyl groups; it is not attacked (12). Apparently, the steric effects associated with the methyl groups are essential for a productive association of norcamphor. 5-Bromocamphor was previously shown to be hydroxylated at the 5-position and subsequently to undergo loss of HBr to yield only 5-ketocamphor as the observed product (6). As shown under Results, the three substrates tested here each yield a single product as well. It is obvious that both camphor and 5-bromocamphor are attacked by the enzyme in isosteric locations (i.e., the 5-position). However, as seen in

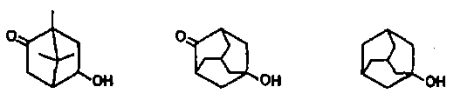

FIG. 2. Views of $P\text{-}450_{cam}$ hydroxylation products perpendicular to the cyclohexanone ring.

Fig. 2, adamantanone is also attacked at a position isosteric with the 5-position of camphor. This position is directly opposite the carbonyl group in a cyclohexanone ring. It will be noticed that in each case the cyclohexanone ring is capped by a bulky bridge containing three or four carbons. Thus, it would appear that the binding pocket of $P\text{-}450_{cam}$ holds both camphor derivatives and adamantanone rigidly in the same steric configuration, essentially allowing the hydrogen abstractor access to only one position on the molecule. The hydrocarbon adamantane, of course, contains no carbonyl group and so we cannot indicate the position of hydroxylation with respect to the cyclohexanone ring. However, inspection of Fig. 2 makes it clear that adamantane is hydroxylated with exactly the same topology as with adamantanone and therefore the same as with camphor. With camphor, adamantanone, and adamantane, the position of hydroxylation corresponds to one of the most reactive positions, but with 5-bromocamphor the 3- and 6-positions are probably similar in reactivity to the 5-position. Nonetheless, attack is limited to the 5-carbon. Thus, the position of attack by $P\text{-}450_{cam}$ is selected by the imposition of a strong steric effect on the normal reactivity profile of the substrate molecule. In fact, the product from hydroxylation by $P\text{-}450_{cam}$ is in all cases one of the products from $P\text{-}450_{LM2}$.

$P\text{-}450_{LM2}$ appears to define its regioselectivity somewhat differently. Steric effects due to the binding pocket are still present, but they do not overwhelm the chemical reactivity profile of the substrate molecule. We find that $P\text{-}450_{LM2}$ is much less selective about the molecules which can be hydroxylated. For instance, while $P\text{-}450_{cam}$ will not attack norcamphor, $P\text{-}450_{LM2}$ will readily hydroxylate norbornane, which, like norcamphor, lacks the three methyl groups (7). We also find that $P\text{-}450_{LM2}$ is not regiospecific, giving instead a mixture of isomers in all cases. As shown below, the distribution of isomers in the product mixture can be largely rationalized by applying the reactivity factors outlined above.

$P\text{-}450_{LM2}$ hydroxylates camphor only at the secondary positions on the molecule. This may be rationalized by reference to factors (a) through (f) above. Primary positions (methyl groups) are expected to be much less reactive toward hydrogen abstraction, and the tertiary positions (bridgeheads) cannot achieve planarity following hydrogen abstraction, making them much less prone to hydroxylation. Of those left, the 3-position is α to a deactivating carbonyl, while the 6-position is sterically blocked by the 10-methyl. Thus, the 5-carbon is the most reactive. Furthermore, examination of molecular models shows that the 5-*exo*-position is more sterically hindered (by the 9-methyl) than is the 5-*endo*-position. In fact, we predict the following rank order of reactivity toward a selective hydrogen abstractor by the various hydrogen atoms on the camphor molecule: most reactive, 5-*endo;* next, 5-*exo;* next, 3-*exo,endo* and 6-*exo,endo;* poorly reactive, 8-, 9-, 10-; not reactive, 4-. This is indeed the order of product yield from the $P\text{-}450_{LM2}$-catalyzed hydroxylation of camphor, if we allow for a moderate steric effect to be operating as well. For instance, we found no 3-*exo* or 6-*exo,endo* alcohols even though we predict roughly equal reactivities of those three and 3-*endo.* This indicates that the enzyme does not allow truly free and random access of the hydrogen abstractor to all parts of the molecule, but neither does it severely restrict substrate movements.

The present observation that the major product from $P\text{-}450_{LM2}$ is the 5-*endo* isomer demonstrates that oxygen delivery to the *endo* position is perfectly feasible. Previously Gelb *et al.* observed that oxygen delivery always occurred to the *exo* face even when hydrogen was removed from the *endo* position (5). They suggested that the hydrogen-abstracting and oxygen-delivering species were separate and distinct entities. However, if there were a chemical barrier to delivery of oxygen to the *endo* face, the suggestion would be invalidated. From the $P\text{-}450_{LM2}$ experiments, however, we are assured that there is no chemical barrier to formation of *endo* alcohol. Thus, the suggestion remains viable.

We may apply reactivity estimates to adamantanone as well. The 1-position is deactivated by the α-carbonyl. The 5-position is tertiary and so is more reactive than the secondary positions 4 and 6. The rank order of reactivity we predict for hydrogen abstraction from adamantanone is: most reactive, 5-; less reactive 4-*syn*,*anti*- and 6-; least reactive, 1-. Actually, the 4-*anti*-isomer predominates over the 5-(bridgehead)-isomer 57 to 43%. The 2°:3° ratio is 1.33:1. However, since there are twice as many 4-*anti*-hydrogens as 5-hydrogens, we must make a statistical correction to accurately reflect the per hydrogen reactivity. The corrected ratio is 0.67:1 (2°:3°). This is similar to the 2°:3° reactivity of 0.8:1 observed in *P*-450_{LM2} hydroxylation of methylcyclohexane (2). With the hydrocarbon adamantane the raw 2°:3° ratio with *P*-450_{LM2} is 0.10:1. After statistical correction (there are 12 2° hydrogens and only 4 3° hydrogens), the ratio becomes 0.033:1, which is very similar to the ratio of 0.028:1 observed in the solution-phase hydroxylation of adamantane by iron tetraphenylporphyrin (8). Thus, as with camphor, the hydroxylation of adamantanone and of adamantane occurs with a regioselectivity in reasonable agreement with our expectations of chemical reactivity.

However, the absence of hydroxylation of adamantanone at the 6-position (across the tricyclic cage opposite the carbonyl) provides a further indication of the presence of a moderate steric restraint of the substrate movement due to the protein environment. The only possible anchoring point of the adamantanone molecule by the protein would be the carbonyl oxygen (e.g., by hydrogen bonding, Coulombic attraction, or nucleophilic addition). In the first two cases, such anchoring would be unaffected by rotations of the molecule about an axis passing through and parallel to the carbonyl double bond (the C_2 axis). Any other rotations, on the other hand, would necessarily disrupt such anchoring. Therefore, rotations about the C_2 axis would be relatively unrestricted, but other rotations would be much less facile. As seen in Fig. 3, free rotation about the C_2 axis sweeps the 1-, 4-, and 5-carbons past any fixed point beside the tricyclic nucleus, but the 6-carbon, which lies directly on the C_2 axis, remains fixed in space. Thus, the 6-carbon is exposed to a hydrogen abstractor only if the hydrogen abstractor happens to lie on or near the C_2 axis, whereas all other carbons would tend to be exposed if the hydrogen abstractor lay anywhere else. Perhaps, then, the lack of 6-hydroxylation merely represents the inability due to protein-derived constraints of the substrate molecule to turn completely freely around all possible axes.

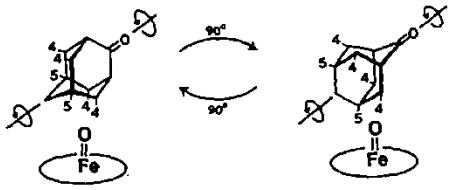

FIG. 3. Rotation of adamantanone about the C_2 axis in the vicinity of a hydrogen abstractor.

We also investigated the general interaction of the enzyme active site and the substrate by means of the thermodynamics of the substrate-binding reaction and the perturbation of the high-spin/low-spin equilibrium. We found no correlation between X^{HS}_{max} and K_d with *P*-450_{cam} but did find a correlation with *P*-450_{LM2}. Of course, a correlation of three points may have little significance. A rough correlation may be seen between the molecular volume and X^{HS}_{max} with *P*-450_{cam}, perhaps a reflection of the same steric factors which allow this enzyme's absolute regiospecificity. Two important new observations are that adamantane induces the largest spin-shift ever reported with *P*-450_{cam} and that it is hydroxylated essentially as rapidly as the normal substrate camphor. Recent reports from the Dus laboratory using substrate analogs for *P*-450_{cam} suggested a central and crucial role of the camphor carbonyl in substrate binding and subsequent processes through formation of a thioketal (13). Our results demonstrate that the carbonyl function is not an absolute requirement for substrate binding or for efficient hydroxylation. The binding of substrates to *P*-450_{LM2} was found to be less tight and

the coupling to the spin-state equilibrium much weaker than with $P\text{-}450_{cam}$. This could suggest a looser fit of the substrates in the binding site and consequently more mobility while associated with the enzyme. The differential substrate mobility between the two enzymes probably constitutes the principal reason for the striking difference in regioselectivity of hydroxylation of the same substrates by these two hemeprotein hydroxylases.

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Pharmacokinetics and Metabolism of Rimantadine Hydrochloride in Mice and Dogs

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We studied the pharmacokinetics and metabolism of rimantadine hydrochloride (rimantadine) following single-dose oral and intravenous administration in mice and dogs. Absorption of the compound in mice was rapid. Maximum concentrations in plasma occurred at less than 0.5 h after oral administration, and the elimination half-life was 1.5 h. Peak concentrations in plasma following oral administration were markedly disproportional to the dose (274 ng/ml at 10 mg/kg, but 2,013 ng/ml at 40 mg/kg). The bioavailability after an oral dose of 40 mg/kg was 58.6%. Clearance was 4.3 liters/h per kg, and the volume of distribution was 7.6 liters/kg at 40 mg/kg. In contrast to the results observed in mice, absorption of the compound in dogs was slow. Maximum concentrations in plasma occurred at 1.7 h after oral administration, and the elimination half-life was 3.3 h. A further difference was that peak concentrations in plasma were approximately proportional to the dose. Following administration of a single oral dose of 5, 10, or 20 mg/kg, maximum concentrations in plasma were 275, 800, and 1,950 ng/ml, respectively. The bioavailability after an oral dose of 5 mg/kg was 99.4%. The clearance was 3.7 liters/h per kg, and the volume of distribution was 13.8 liters/kg at 5 mg/kg. Mass balance studies in mice, using [*methyl*-^{14}C]rimantadine, indicated that 98.7% of the administered dose could be recovered in 96 h. Less than 5% of the dose was recovered as the parent drug in dog urine within 48 h. Finally, gas chromatography-mass spectrometry studies, done with mouse plasma, identified the presence of two rimantadine metabolites. These appeared to be ring-substituted isomers of hydroxyrimantadine.

Rimantadine hydrochloride (rimantadine), which is chemically related to the anti-influenza A drug amantadine hydrochloride (amantadine, Symmetrel), has been reported to be effective against influenza A in human studies and in mouse model systems (2, 7, 11). It is being used in the USSR for both prophylaxis and therapy of influenza A infections (12).

The pharmacokinetics and metabolism of rimantadine in humans have been reported (F. G. Hayden and H. E. Hoffman, Abstr. 14th Annu. UCLA Symp., J. Cell. Biochem., suppl. 96, p. 276, 1985; L. P. Van Voris, J. Bartram, H. E. Hoffman, L. M. Shalaby, J. C. Gaylord, L. S. Davis, and F. G. Hayden, Program Abstr. 23rd Intersci. Conf. Antimicrob. Agents Chemother, abstr. no. 684, 1983) for healthy subjects, but no animal studies have been reported. We have studied rimantadine kinetics and metabolism in mice (mice are used for most influenza model infections) and in dogs.

MATERIALS AND METHODS

Rimantadine assay. (i) Plasma. The rimantadine level in plasma was analyzed as previously described (3), with modifications for measuring the levels in urine, feces, and tissues.

A 1-ml sample of plasma was extracted by using cyano Bond Elut disposable extraction columns (Analytichem International). The extracted rimantadine was derivatized with pentafluorobenzoyl chloride (PFB) (Aldrich Chemical Co., Inc.), yielding the pentafluorobenzoyl derivative of rimantadine, which was then analyzed by gas chromatography (HP 5880A; Hewlett-Packard Co.) with an electron capture detector (6). PFB-amantadine was used as the internal standard and was prepared in house. The oven temperature was programmed for 240°C for 5 min, followed by a temperature gradient of 5°C/min for 6 min, to 270°C. The 6-ft (1.83-m) column was packed with 10% OV-1 (Ohio Valley Specialty Chemical, Inc.) on Chromasorb W (Hewlett-Packard Co.). The method was linear between 10 and 10,000 ng/ml in plasma-free solutions. The limit of detection was 5 ng/ml. However, linearity in biological solutions, such as plasma or tissue extracts, was restricted to a narrower range and was determined for each analysis. Samples were assayed only in the linear portion of the curve. Absolute recovery from plasma ranged from 54% at 50 ng/ml to 72% at 1,000 ng/ml. Reproducibility was 7% relative standard deviation. Rimantadine in plasma was stable when stored at −20°C for 5 weeks.

The resultant peaks on the chromatograms indicated that the method was highly specific. Retention times were reproducible regardless of the biological fluids, and there was no interference from other substances in the extracts. Retention times for principal peaks were as follows: internal standard, 4.3 min; rimantadine, 6.6 min; metabolite M-1, 9.9 min; and metabolite M-2, 12.2 min.

(ii) Mouse lung assay. Individual lungs were each homogenized in 3 ml of 5 N NaOH. A 1-ml sample of the resulting homogenate was added to 5 ml of 5 N NaOH–50 mg of NaCl–15 ml of hexane. After 30 min on a wrist action shaker and

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centrifugation to separate the phases, the hexane layer was removed and evaporated to dryness. The residue was dissolved in 1 ml of toluene and derivatized as with the plasma extracts. Recovery of rimantadine from mouse lungs was 42%.

(iii) Standard curves. Standards were prepared in each biological fluid analyzed, i.e., mouse, rat, and dog plasma; lung extracts; and dog and mouse urine. A standard curve was prepared from the peak height ratios by linear regression analysis, and concentrations were computed from the regression equation. Four to six points were used in each regression analysis.

(iv) β-Glucuronidase hydrolysis. Glucuronide conjugation was determined by using β-glucuronidase supplied by Sigma Chemical Co. (kit 325). The enzyme, at a final concentration of 10 U/ml of urine, was incubated with dog urine for 2 h at 37°C. β-Glucuronidase activity was monitored by hydrolyzing phenolphthalein glucuronic acid. After incubation, the urine samples were extracted as described above.

(v) Urine assay. A 1-ml sample of urine was placed in 5 ml of 5 N NaOH and extracted as described for the mouse lung assay.

[^{14}C]rimantadine methods. [^{14}C]rimantadine (specific activity, 5.34 mCi/mmol), prepared by Du Pont, NEN Research Products, was labeled at the methyl group. Its radiochemical purity was 98%.

Virus preparation. Influenza virus A/Bangkok/1/79 (H3N2) was prepared by serial passage in CD-1 mice inoculated intranasally with 50 μl of an appropriately diluted stock preparation. At about 36 h postinfection, lungs were surgically excised under aseptic conditions and homogenized in phosphate-buffered saline containing bovine serum albumin (0.2%), penicillin (100 IU/ml), and kanamycin (25 μg/ml). The homogenate was frozen and thawed three times and clarified by centrifugation. The supernatant was collected and stored frozen at −70°C in 1-ml aliquots.

CD-1 female mice (Sendai virus free), 20 to 30 days of age and weighing an average of 15 g, were each infected intranasally, under light anesthesia, with 50 μl of influenza virus A/Bangkok/1/79 (H3N2) at dilutions of $10^{-3.8}$, $10^{-4.8}$, and $10^{-5.8}$, respectively, with 12 mice per dilution level. Virus dilutions were made in phosphate-buffered saline (pH 7.2), as described above. The virus preparation yielded a 50% lethal dose of $10^{-5.24}$ and a 90% lethal dose of $10^{-4.25}$.

Sample preparation for liquid scintillation counting. (i) Radiometric assay. Samples were counted in a Packard Prias scintillation spectrometer with Atomlight (Du Pont, NEN) as the scintillation fluid.

(ii) Standards. Using the external standard system, we determined counting efficiencies with graded quenched standards programmed into the Prias counter. The radioactivity in the test sample was measured as counts per minute, corrected for quenching, and reported as disintegrations per minute.

(iii) Plasma, urine, and cage wash. A measured volume of plasma, urine, or liquid used for washing the cages was placed into a scintillation vial containing 7 ml of Atomlight and counted in the liquid scintillation counter.

(iv) Feces. Feces samples (50 to 500 mg) were oxidized in a Packard Tri-Carb sample oxidizer, and the $^{14}CO_2$ was trapped in Carbosorb (Packard) absorbent with Permafluor (Packard) as the scintillant. All samples were counted in triplicate when possible, and control and spiked [^{14}C]rimantadine standards were run concurrently with the experimental samples. The mean ^{14}C recoveries from feces spiked with known amounts of labeled rimantadine and subjected to combustion were 94.3%.

Animal treatments. (i) Mice. Groups of six nonfasted female mice (mean weight, 22 g) were dosed perorally with 10 or 40 mg of rimantadine per kg, formulated in saline at 1 mg/ml. An additional group received 40 mg/kg intravenously. Blood was drawn by intracardiac puncture into heparin-containing tubes and pooled, and plasma was prepared. Blood samples were taken at 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h after dosing.

To determine the effects of virus infection on the pharmacokinetics of rimantadine, mice (five per time interval) were infected intranasally with influenza virus A/Bangkok/1/79 (H3N2), as above, at a virus dilution of $10^{-4.8}$, and dosed orally with the drug (40 mg/kg) at 72 h postinfection. Uninfected control mice simultaneously received the same oral doses of rimantadine. At 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 16, 24, and 48 h after drug dosing, blood was collected, pooled, and then centrifuged to separate the plasma. The lungs were excised at the same time points, and all samples were frozen until assayed.

(ii) Dogs. Female beagles were dosed with rimantadine at 5, 10, and 20 mg/kg perorally and 5 mg/kg intravenously. Rimantadine was formulated in water at 5 mg/ml for peroral dosing and in sterile saline for intravenous dosing. The dogs were fasted overnight and treated with drug in the morning. Blood was collected from the jugular vein at 0.5, 1, 2, 4, 6, 8, 10, 12, 16, and 24 h in sodium heparin-containing tubes. All samples from dogs were assayed separately.

(iii) Material balance study in mice. [^{14}C]rimantadine was dissolved in water (1 mg/ml) at a specific activity of 3.2 μCi/mg. Three groups of two Charles River CD female mice each were given a single oral dose of 40 mg of [^{14}C]rimantadine per kg and placed into metabolism chambers. Groups 1 and 3 received a total dose of 4.86 μCi, and group 2 received 5 μCi. Urine and feces were collected at 24-h intervals during the study. Samples (1 ml) of the urine and cage-washing liquid were counted in triplicate. Fecal samples were oxidized prior to liquid scintillation counting.

Mass spectrometry. The gas chromatography separations were made on a 15-m J&W DB-1, 0.25-mm (inner diameter) capillary column which was directly interfaced to a Finnigan model 4500 mass spectrometer. The column temperature was programmed from 200 to 260°C at 10°C/min. Mouse plasma extract, containing rimantadine and its metabolites, was reacted with PFB and dissolved in toluene. The derivatized sample was initially analyzed by capillary gas chromatography-mass spectrometry in the electron impact mode, and to enhance the molecular (M^+) ion, the gas chromatography-mass spectrometry was rerun with chemical ionization.

Pharmacokinetic calculations. All pharmacokinetic parameters were determined with the RS-1 computer program (BBN Research Systems, Cambridge, Mass.). The area under the concentration-time curve from zero to infinity ($AUC_{0-\infty}$) was calculated by summing the area to the last measured time point (C_{last}) determined by the linear trapezoidal rule, and the extrapolated area was determined by C_{last}/k_{el}, where k_{el} is the elimination rate constant and was determined from the slope of the terminal portion of the ln concentration-time curve. The half-life ($t_{1/2}$) was $0.693/k_{el}$. The clearance (CL) was D/AUC, and the volume of distribution (V) was CL/k_{el}.

TABLE 1. Pharmacokinetics of rimantadine in mice[a]

Route	Dose (mg/kg)	C_{max} (ng/ml)	T_{max} (h)	AUC (ng · h/ml)	$t_{1/2}$ (h)	CL (liters/h per kg)	V (liters/kg)	F (%)
Oral	10	274	0.5	555	1.253			
	40	2,013	0.5	5,421	2.063			58.6
i.v.[b]	40		0.5	9,247	1.224	4.3	7.6	

[a] Plasma was pooled from six mice per period.
[b] i.v., Intravenous.

RESULTS

Mouse pharmacokinetics. Plasma pharmacokinetic data for mice are summarized in Table 1. Oral absorption was rapid, with the maximum concentration in plasma (C_{max}) occurring at 30 min, the earliest time point measured (Fig. 1). The elimination half-life ($t_{1/2}$) was between 1 and 2 h. V was 7.6 liters/kg, which suggests extensive distribution of the drug into tissues. CL was 4.3 liters/h per kg. The increase in AUC values was not proportional to the dose. The bioavailability of rimantadine in mice after oral administration of 40 mg/kg was 58.6%.

Influenza A virus-infected mice. In a separate study, normal and influenza virus-infected mice were compared (Table 2). Peak levels in both plasma and lungs decreased in infected mice, and the peak time in the lungs increased from 0.25 to 2.0 h, whereas it remained the same in plasma. Thus, infection both decreases and delays the uptake of rimantadine in the lungs. However, AUC values for lungs from infected and noninfected mice were not different (Table 2), nor were the AUC_{lung}/AUC_{plasma} ratios. The ratio for normal mice, 45.7, is similar to that for infected mice, 48.5, suggesting that the virus infection does not alter the total impact of rimantadine during the period studied.

TABLE 2. Pharmacokinetic parameters in mouse plasma and lungs from uninfected and influenza A virus-infected mice given a single oral dose of 40 mg/kg

Specimen	n[a]	k_{el} (h^{-1})	$t_{1/2}$ (h)	AUC (ng · h/ml)	C_{max} (ng/ml)	T_{max} (h)
Plasma						
Uninfected	6	0.324	2.1	7,631	2,169	0.25
Infected	5	0.299	2.3	6,667	1,436	0.25
Lung						
Uninfected	6	0.385	1.8	348,546	74,251	0.25 (2.0)[b]
Infected	5	0.156	4.4	323,457	45,436	2.00

[a] n, Number of mice per period.
[b] A second C_{max} occurred at 2 h.

Dog plasma pharmacokinetics. Plasma pharmacokinetic data for dogs are shown in Table 3. The $t_{1/2}$s were 2.9, 3.4, and 3.7 h for oral administration of 5, 10, and 20 mg/kg, respectively, and 2.6 h for a 5-mg/kg intravenous dose. As found for mice, the increase in AUC values for dogs was not proportional to the dose. The bioavailability after a 5-mg/kg oral dose was 99.5%, nearly twice that found in mice (58.6%) after a 10-mg/kg dose. The values for the time to maximum concentration of drug (T_{max}) were 1 h at 5 mg/kg and 2 h at 10 and 20 mg/kg. C_{max} increased with increasing dose, but the increase was not proportional to the dose. CL was 3.7 liters/h per kg, similar to that for mice, and V was 13.8 liters/kg.

Metabolism studies. Mouse plasma extracts contained two metabolites: M-1, with a retention time of 9.9 min, and M-2, with a retention time of 12.2 min. Both rimantadine and M-1 were observed at the earliest time sampled (0.5 h). M-1 and M-2 were also found in mouse urine.

Concentrations of M-1 and rimantadine in mouse lungs

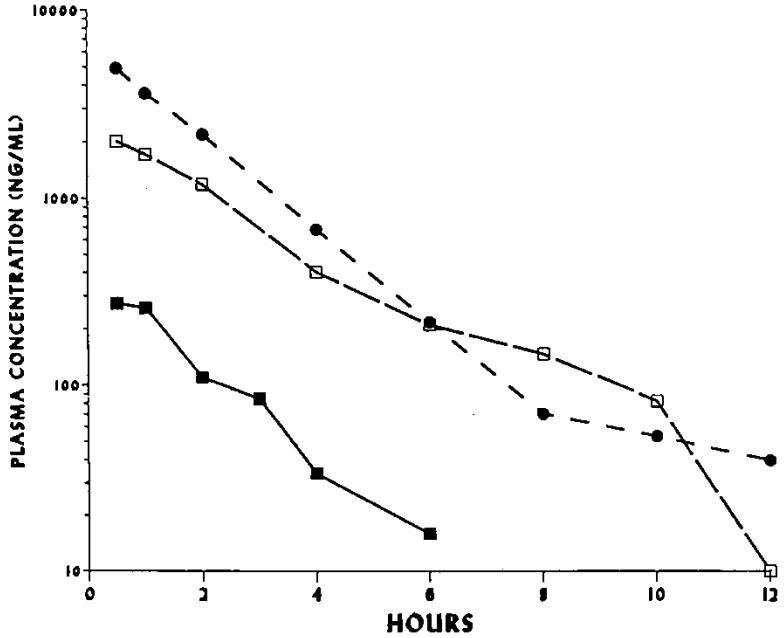

FIG. 1. Concentration of rimantadine in uninfected mouse plasma after a single dose. Groups of six nonfasted female mice, weighing 20 to 23 g, were dosed at 10 mg/kg perorally (■), 40 mg/kg perorally (□), and 40 mg/kg intravenously (●) and bled by cardiac puncture into heparinized tubes. The serum was pooled. The mean variation of the method for replicate samples was 7% (coefficient of variation).

TABLE 3. Pharmacokinetics of rimantadine in dogs

Route	Dose (mg/kg)	n^a	k_{el} (h^{-1})	$t_{1/2}$ (h)	AUC (ng · h/ml) (±SD)	C_{max} (ng/ml) (±SD)	T_{max} (h)	CL (liter/h per kg)	V (liter/kg)	F (%)
Oral	5	3	0.242	2.86	1,353 (236)	275 (47)	1.0			99.4
	10	2	0.202	3.43	4,066 (263)	788 (16)	2.0			
	20	1	0.190	3.65	11,520	1,950	2.0			
i.v.[b]	5	3	0.267	2.60	1,361 (94)			3.7	13.8	

[a] n, Number of animals per dose level used. Samples from each dog were assayed separately.
[b] i.v., Intravenous.

were measured after oral dosing. Peak concentrations of rimantadine were observed at 0.5 h, whereas M-1 concentrations peaked at 2 h after dosing. A plot of the concentrations of rimantadine and M-1 in lung tissues is shown in Fig. 2. Metabolite M-2 in mouse lungs was not determined quantitatively owing to poor chromatographic resolution but was found in mouse and rat plasma and human, mouse, and dog urine with a retention time of about 12 min.

Dog urine (0 to 24 h) (see Table 5) and plasma also contained an abundance of M-1 and M-2 after a single oral 10-mg/kg dose. The M-1 concentration in dog plasma peaked at 2 h and reached approximately one-half the C_{max} of rimantadine. The pharmacokinetics of the metabolites were not analyzed owing to the absence of pure compound and the resultant inability to develop suitable methods.

Structural elucidation of metabolites by gas chromatography-mass spectrometry. Rimantadine metabolites in mouse plasma were identified by mass spectrometry as rimantadine having a hydroxyl group on the adamantane ring. The metabolites were separated from other components on a capillary gas chromatograph, and the fragmentation patterns were investigated by both electron impact and chemical ionization mass spectrometry. Comparison of the chemical ionization mass spectra for both rimantadine and the metabolite indicated a gain of 16 mass units on the adamantane ring of the metabolite. This suggested the presence of a hydroxyl group. The spectral data suggested that the isolated rimantadine PFB metabolite had a molecular weight of 389 and made up of the structure shown in Fig. 3.

Preparation of the trimethylsilyl derivatives for both PFB rimantadine and the metabolite confirmed that the hydroxyl group is on the adamantane ring of the metabolite. Our studies of human urine extracts showed that there are three isomers of hydroxyrimantadine: the 1-, 2-, and 3-hydroxyrimantadines (Van Voris et al., 23rd ICAAC).

Material balance in mice and dogs. Most of the radioactivity (69.4%) excreted by mice after 24 h was found in the urine, with only 1.7% excreted in feces (Table 4). During the next 24 h, 13.8% was excreted in urine, while only 1.1% was

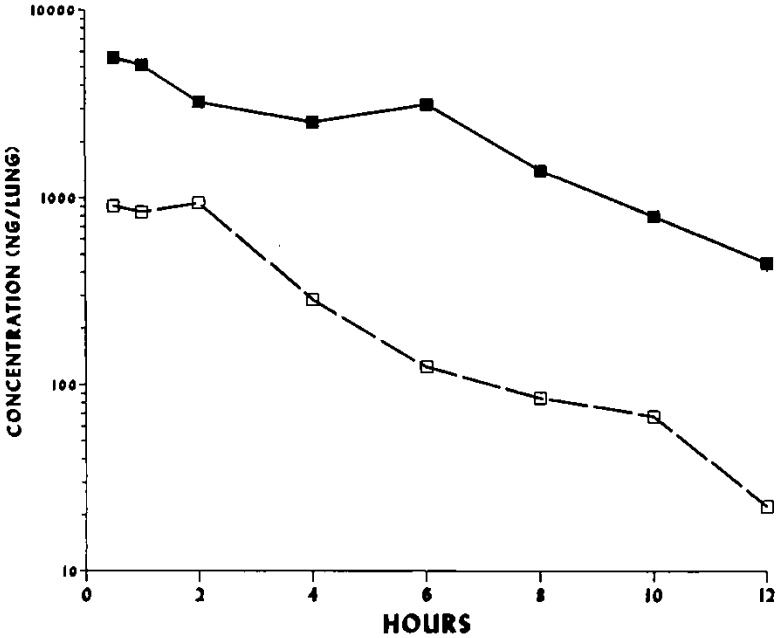

FIG. 2. Concentration of rimantadine and metabolite M-1 in mouse lungs after a single oral dose of 40 mg/kg. Each point represents the mean of rimantadine (■) or M-1 (□) levels in extracts from two lungs collected at 0.5, 1, 2, 4, 6, 8, 10, and 12 h after dosing. The rimantadine high-pressure liquid chromatography retention time was 7.0 min, and that of M-1 was 9.1 min.

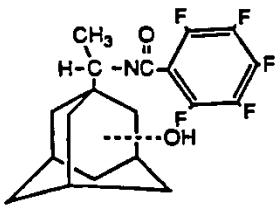

FIG. 3. Structure of isolated rimantadine PFB metabolite.

found in feces. The total percentages of the dose after 96 h were 89.4% in urine and 3.7% in feces.

Dog urine was analyzed for rimantadine and metabolites M-1 and M-2 (Table 5). High-pressure liquid chromatography analysis of the metabolites gives estimates only, based on the assumption that the absorption spectra were similar to those of rimantadine. The amounts of intact rimantadine were less than 2% in the first 24 h, regardless of dose, and less than 1% in the second 24 h. The major excretion product was M-1. The discrepancy between the dogs receiving 10 mg/kg is unexplainable. Approximately 50% of the recovered drug was M-1, while M-2 accounted for 10% or less. The total percentages of the 10-mg/kg dose recovered were 69.4% for dog 75 and 58% for dog 76. At 20 mg/kg, 61.6% was recovered. All values increased 20% following β-glucuronidase hydrolysis. The data in Table 5 were collected after enzyme hydrolysis.

DISCUSSION

In both mice and dogs, absorption of rimantadine was rapid. No significant differences in $t_{1/2}$ were noted. The differences observed in bioavailability between mice and dogs were not directly comparable owing to differences in dose. It is probable that bioavailability is not constant with dose; this should be studied.

Infection of mice with influenza A virus 72 h prior to oral administration of rimantadine significantly altered the drug disposition from that in uninfected mice. Reduction in the uptake of rimantadine by lung tissue from infected mice has been previously reported (4), and our results confirm this finding (Table 2). The rimantadine concentrations in plasma and lungs at the time of peak concentration in virus-infected mice were approximately one-half those in uninfected mice. The lung elimination half-life lengthened from 1.8 h in uninfected mice to 4.4 h in infected mice. The net effect of these changes, however, resulted in equivalent AUC values. Although rimantadine concentrations in lungs were not determined, Schulman demonstrated that doses of 25 mg of rimantadine per kg dramatically reduced lung lesions and virus titers in mice (8). Studies of virus titers in lungs versus drug concentrations in lungs and plasma would be of interest.

TABLE 4. [^{14}C]rimantadine mouse material balance study

Collection interval (h)	Mean % administered dose ± SD in:	
	Urine	Feces
0–24	69.4 ± 5.0	1.7 ± 0.5
24–48	13.8 ± 1.0	1.1 ± 0.2
48–72	4.3 ± 3.0	0.3 ± 0.2
72–96[a]	1.9 ± 1.0	0.6 ± 0.7

[a] At 96 h, the cage wash activity was 5.6 + 0.1%. Total recovery was 98.7%.

TABLE 5. Recovery of total rimantadine and metabolites in the urine[a] of dogs given single oral doses of rimantadine

Collection interval (h) and substance	% of dose in:		
	Dog 75[b]	Dog 76[b]	Dog 74[c]
0–24			
Rimantadine	1.6	1.2	1.8
M-1	15.9	43.7	46.6
M-2	2.4	10.0	6.5
24–48			
Rimantadine	0.8	0.3	0.1
M-1	39.9	2.3	5.3
M-2	8.8	0.5	1.3

[a] Urine was treated with β-glucuronidase for 2 h at 37°C before extraction (see text).
[b] Dose was 10 mg/kg.
[c] Dose was 20 mg/kg.

After administration of one oral dose of [^{14}C]rimantadine to mice, 89.4% of the radioactivity was found in the urine and 3.7% was found in the feces. Most of the radioactivity was excreted during the first 24 h, and only 1.9% of the dose was recovered during the period from 72 to 96 h.

Dogs receiving oral doses of 10 or 20 mg/kg excreted very little intact rimantadine. The main excretion product was M-1, which made up about half of the administered dose; M-2 was about 10%, and rimantadine was less than 5%. Only 58 to 69% of administered drug was recovered in 48 h in the three dogs. This may in part be due to further metabolism of M-1 and M-2 into smaller, as yet unidentified products. Further, until standards of M-1 and M-2 can be prepared and analyzed, quantification of these remains only an estimate.

The metabolites identified in mouse and dog urine, M-1 and M-2, are ring-hydroxylated derivatives of rimantadine. Other investigators have noted ring hydroxylation of adamantane derivatives in vivo. Wesemann et al. (10) demonstrated the presence of a ring-hydroxylated metabolite of 1-amino-3,5-dimethyladamantane in the rat. Spiers and Chatfield (9), studying a novel adamantane derivative, *N*-methyl-1-(2-phenyladamant-1-yl)-2-aminopropane hydrochloride, in humans, determined that two isomers of a ring-hydroxylated metabolite were excreted. The ring position of hydroxylation was not determined. Less than 1% of the dose was excreted unchanged. Approximately 30% of the dose was excreted as conjugated hydroxyl metabolites.

The metabolism of amantadine is less clear. Recent studies by Koppel and Denzer (5) have shown small quantities of eight metabolites recovered from a patient under a therapeutic dosing regimen. A major metabolic pathway was N acetylation, with several other unusual metabolic pathways observed. However, no metabolites were detected with a hydroxylated adamantane ring system.

Differences between the metabolism and kinetics of rimantadine and amantadine are noteworthy. For both, >90% is excreted in the urine, with trace amounts in the feces. However, the percentage of unchanged amantadine found in mouse urine was 63% (9), several times that found for intact rimantadine. In humans, amantadine is excreted largely unchanged in urine, whereas less than 10% of rimantadine is excreted intact (Van Voris et al., 23rd ICAAC) in urine.

Little has been reported about the concentration of either drug in lung tissue. Bleidner et al. (1) reported the C_{max} in mouse lungs at 0.25 h to be 59 μg/g following a single oral dose of 25 mg/kg. The concentration of amantadine in blood was 4 μg/ml. The ratio $C_{max,lung}/C_{max,blood}$ of 15 was half the

ratio reported here for rimantadine, owing to the lower concentrations of rimantadine than amantadine in plasma.

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M1363

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

Donald T. Witiak et al. Exr. P. Shaver
Serial No: 06/882,258 Art Unit: 124
Filed: July 7, 1986
For: Diastereomeric Mono- and Di-Substituted Diamino Cyclohexane Compounds and the Method of Preparation Thereof

Commissioner of Patents
and Trademarks
Washington, D.C. 20231

DECLARATION

I, David P. Rotella, Ph.D., do declare and state

1. Presently I am employed as a research chemist for Cephalon Inc., 145 Brandywine Parkway, West Chester, PA.

2. I am a co-inventor along with Donald T. Witiak in the above-identified patent application Serial No. 06,882,258, filed July 7, 1986.

3. I am familiar with the outstanding Patent Office Action dated June 20, 1991 for the above-identified application and the references cited therein including the Amundsen et al., Serial No. 835,584, filed March 3, 1986, now U.S. Patent No. 4,786,725 reference and Hlavka et al., Serial No. 824,479, filed January 31, 1986, now U.S. Patent No. 4,670,458 reference.

4. Dr. Witiak and I completed the present invention prior to the filing dates of the Amundsen et al. '725 and Hlavka et al. '458 references.

I received my Doctor of Philosophy from the graduate school of The Ohio State University. As part of my requirements for the Doctor of Philosophy degree I presented a dissertation entitled the "Synthon Concept in Medicinal Chemistry: Synthesis and Applications of Cyclohexane Diol-diamines.

Attached hereto as Exhibit A is a copy of the Ohio State University Invention Disclosure Form which indicates that the present invention was (1) conceived at least as early as July 7, 1983 and recorded in the Research Notebook I-David Rotella, page 204; (2) successfully reduced to practice at least as early as January 8, 1985 and recorded in Research Notebook II, page

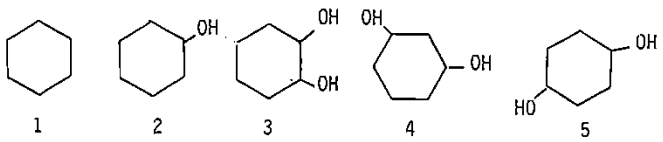
101; and (3) disclosed to others at least as early as November 13-15, 1985.

Attached hereto as Exhibit B is a copy of an Abstract published in the Biomedical High Technology 1985 International Conference held in Columbus, Ohio, November 13-15, 1985 wherein Dr. Witiak and I disclose the synthesis and biological evaluation of novel hydroxy-cyclohexane Diamine Pt(II) complexes as potential antitumor agents.

5. I have further reviewed the outstanding Patent Office Action dated June 20, 1991 and the Examiner's comments with respect to the Amundsen et al., U.S. Patent No. 4,500,465 reference cited therein. In my review of the Amundsen et al. '465 reference I have found that it merely discusses dibromide dihydroxylated amines. The Amundsen et al. '465 reference does not discuss monohydroxylated compounds.

(a) It is well-known that the presence of one (or more) hydroxyl group(s) in an organic molecule can have a profound effect on the physical properties of that compound. The degree and exact nature of the influence are not obvious and depend on a variety of "molecule specific" factors. For example, examine the physical properties of a series of compounds related to those disclosed in the present invention, cyclohexane (1), cyclohexanol (2) and the isomeric cyclohexanediols (3-5) shown in Figure 1 below and described in the Exhibits C, D and E attached hereto from the 1990-1991 Aldrich Catalog.

Figure 1:



(b) The boiling point of these compounds increases substantially upon addition of the first OH group (i.e., 1--->2) from ca. 81°C to 160°C. Further, 2 is more soluble in water (3.6% w/w, 11th Merck Index) than 1, which is essentially insoluble in water. Note however, that the effects of

addition of a second hydroxyl group depend on the position of the new substituent. The 1,2-substituted compound 3 is a solid with a melting point of ca. 100°C, indicative of relatively stronger inter and intramolecular hydrogen bonding (vide infra), compared to 2. Positional isomer 3 is a high boiling liquid (bp 246°C), while 4 is again a solid (mp 100°C).

(c) The effect of polyhydroxyl substitution in a compound exerts unusual changes in the water solubility of a compound. Ethyl alcohol is freely miscible with both water and a wide range of organic solvents, including benzene, chloroform and diethyl ether. However, ethylene glycol, a 1,2-dihydroxy analogue of ethanol, is insoluble in benzene, chloroform and diethyl ether (See Exhibits F and G attached hereto from the Merck Index, p. 594 and 599, respectively). The basis for this difference is related, in part, to the differences in hydrogen bonding potential of the two-compounds. It is known that hydrogen bonds are important because of the effects they have on properties of compounds. Two important principles at work here are (1) hydrogen bonding causes lack of ideality in gas and solution laws; and (2) hydrogen bonding changes many chemical properties. These principles are discussed in Advanced Organic Chemistry, 3rd ed. by Jerry March at page 73, attached hereto as Exhibit H.

(d) Given these effects which are, a priori, unpredictable and most importantly, unknown, on the addition of hydroxyl groups to the 1,2-diaminocyclohexane Pt (II) nucleus. I believe that the most effective and logical scientific approach to study this structural change on physical and biological properties is to prepare both mono and dihydroxy compounds.

(e) Until our invention, no one had prepared diastereomeric mono- and di- hydroxylated diaminocyclohexanes in a sterocontrolled manner.

Therefore, inferring that mono- and di- hydroxy compounds both possess the same physical and biological properties is not consistent with the realities of medicinal chemistry.

6. An additional important point in this regard is the biological/pharmacological consequence of the added OH group. The inclusion or deletion of this moiety dramatically alters pharmacodynamic properties (absorption, distribution, metabolism), as well as biological activity (e.g. addition of OH groups to the betaphenethylamine nucleus). The human body will react in a substantially different manner, based on the presence (and number if relevant) of OH groups. In effect, the two species, i.e., mono vs. dihydroxy complexes, are considered by the body as separate and possibly unrelated drugs.

I further declare that I do not know and do not believe that the invention has been in public use or on sale in this country, or patented or described in a printed publication in this or any foreign country for more than one year prior to my invention, and have never abandoned my invention.

I further declare that I do not know and do not believe that the invention has been in public use or on sale in this country, or patented or described in a printed publication in this or any foreign country for more than one year prior to my invention, and have never abandoned my invention.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

David P. Rotella

Date: 12.NOV.91

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Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings

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Abstract

Experimental and computational approaches to estimate solubility and permeability in discovery and development settings are described. In the discovery setting 'the rule of 5' predicts that poor absorption or permeation is more likely when there are more than 5 H-bond donors, 10 H-bond acceptors, the molecular weight (MWT) is greater than 500 and the calculated Log P (CLogP) is greater than 5 (or MlogP > 4.15). Computational methodology for the rule-based Moriguchi Log P (MLogP) calculation is described. Turbidimetric solubility measurement is described and applied to known drugs. High throughput screening (HTS) leads tend to have higher MWT and Log P and lower turbidimetric solubility than leads in the pre-HTS era. In the development setting, solubility calculations focus on exact value prediction and are difficult because of polymorphism. Recent work on linear free energy relationships and Log P approaches are critically reviewed. Useful predictions are possible in closely related analog series when coupled with experimental thermodynamic solubility measurements.

Keywords: Rule of 5; Computational alert; Poor absorption or permeation; MWT; MLogP; H-Bond donors and acceptors; Turbidimetric solubility; Thermodynamic solubility; Solubility calculation

Contents

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1. Introduction

This review presents distinctly different but complementary experimental and computational approaches to estimate solubility and permeability in drug discovery and drug development settings. In the discovery setting, we describe an experimental approach to turbidimetric solubility measurement as well as computational approaches to absorption and permeability. The absence of discovery experimental approaches to permeation measurements reflects the authors' experience at Pfizer Central Research. Accordingly, the balance of poor solubility and poor permeation as a cause of absorption problems may be significantly different at other drug discovery locations, especially if chemistry focuses on peptidic-like compounds. This review deals only with solubility and permeability as barriers to absorption. Intestinal wall active transporters and intestinal wall metabolic events that influence the measurement of drug bioavailability are beyond the scope of this review. We hope to spark lively debate with our hypothesis that changes in recent years in medicinal chemistry physical property profiles may be the result of leads generated through high throughput screening. In the development setting, computational approaches to estimate solubility are critically reviewed based on current computational solubility research and experimental solubility measurements.

2. The drug discovery setting

2.1. Changes in drug leads and physico-chemical properties

In recent years, the sources of drug leads in the pharmaceutical industry have changed significantly. From about 1970 on, what were considered at that time to be large empirically-based screening programs became less and less important in the drug industry as the knowledge base grew for rational drug design [1]. Leads in this era were discovered using both in vitro and primary in vivo screening assays and came from sources other than massive primary in vitro screens. Lead sources were varied coming from natural products; clinical observations of drug side effects [1]; published unexamined patents; presentations and posters at scientific meetings; published reports in scientific journals and collaborations with academic investigators. Most of these lead sources had the common theme that the 'chemical lead' already had undergone considerable scientific investigation prior to being identified as a drug lead. From a physical property viewpoint, the most poorly behaved compounds in an analogue series were eliminated and most often the starting lead was in a range of physical properties consistent with the previous historical record of discovering orally active compounds.

This situation changed dramatically about 1989–1991. Prior to 1989, it was technically unfeasible to screen for in vitro activity across hundreds of thousands of compounds, the volume of random screening required to efficiently discover new leads. With the advent of high throughput screening in the 1989–1991 time period, it became technically feasible to screen hundreds of thousands of compounds across in vitro assays [2–4]. Combinatorial chemistry soon began[1] and allowed automated synthesis of massive numbers of compounds for screening in the new HTS screens. The process was accelerated by the rapid progress in molecular genetics which made possible the expression of animal and human receptor subtypes in cells lacking receptors that might interfere with an assay and by the construction of receptor constructs to facilitate signal detection. The screening of very large numbers of compounds necessitated a radical departure from the traditional method of drug solubilization. Compounds were no longer solubilized in aqueous media under thermodynamic equilibrating conditions. Rather, compounds were dissolved in dimethyl sulfoxide (DMSO) as stock solutions, typically at about 20–30 mmol and then were serially diluted into 96-well plates for assays (perhaps with some non ionic surfactant to improve solubility). In this paradigm, even very insoluble drugs could be tested because the kinetics of compound crystallization determined the apparent 'solubility' level. Moreover, compounds could partition into assay components such as membrane particulate material or cells or could bind to protein attached to the walls of the wells in the assay plate. The net effect was a screening technology for compounds in the μM concentration range that was largely divorced from the compounds true aqueous thermodynamic solubility. The apparent 'solubility' in the HTS screen is always higher, sometimes dramatically so, than the true thermodynamic solubility achieved by equilibration of a well characterized solid with aqueous media. The in vitro HTS testing process is quite reproducible and potential problems related to poor compound solubility are often compensated for by the follow-up to the primary screen. This is typically a more careful, more labor-intensive process of in vitro retesting to determine IC50s from dose response curves with more attention paid to solubilization. The net result of all these testing changes is that in vitro activity is reliably detected in compounds with very poor thermodynamic solubility properties. A corollary result is that the measurement of the true thermodynamic aqueous solubility is not very relevant to the screening manner in which leads are detected.

2.2. Factors affecting physico-chemical lead profiles

The physico-chemical profile of current leads i.e. the 'hits' in HTS screens now no longer depends on compound solubility sufficient for in vivo activity but depends on: (1) the medicinal chemistry principles relating structure to in vitro activity; (2) the nature of the HTS screen; (3) the physico-chemical profile of the compound set being screened and (4) to human decision making, both overt and hidden as to the acceptability of compounds as starting points for medicinal chemistry structure activity relationship (SAR) studies.

One of the most reliable methods in medicinal chemistry to improve in vitro activity is to incorporate properly positioned lipophilic groups. For example, addition of a single methyl group that can occupy a receptor 'pocket' improves binding by about 0.7 kcal/mol [6]. By way of contrast, it is generally difficult to improve in vitro potency by manipulation of the polar groups that are involved in ionic receptor interactions. The interaction of a polar group in a drug with solvent versus interaction with the target receptor is a 'wash' unless positioning of the polar group in the drug is precise. The traditional lore is that the lead has the polar groups in the correct (or almost correct) position and that in vitro potency is improved by correctly positioned lipophilic groups that occupy receptor pockets. Polar groups in the drug that are not required for binding can be tolerated if they occupy solvent space but they do not add to receptor binding. The net effect of these simple medicinal chemistry principles is that, other factors being equal, compounds with correctly positioned polar functionality will be more readily

[1] A search through SciSearch and Chemical Abstracts for references to combinatorial chemistry in titles or descriptors using the truncated terms COMBIN? and CHEMISTR? gave the following number of references respectively: 1990, 0 and 0; 1991, 2 and 1; 1993, 8 and 8; 1994, 12 and 11; 1995, 46 and 45.

detectable in HTS screens if they are larger and more lipophilic.

The nature of the screen determines the physico-chemical profile of the resultant 'hits'. The larger the number of hits that are detected, the more the physico-chemical profile of the 'hits' resembles the overall compound set being screened. Technical factors such as the design of the screen and human cultural factors such as the stringency of the evaluation as to what is a suitable lead worth are major determinants of the physico-chemical profiles of the eventual leads. Screens designed with very high specificity, for example many receptor based assays, generate small numbers of hits in the μM range. In these types of screens the signal is easy to detect against background noise, the hits are few or can be made few by altering potency criteria and the physico-chemical profiles tend towards more lipophilic, larger, less soluble compounds. Tight control of the criteria for activity detection in the initial HTS screen minimizes labor-intensive secondary evaluation and minimizes the effect of human biases. The downside is that lower potency hits with more favorable physico-chemical property profiles may be discarded.

Cell-based assays, by their very nature tend to produce more 'hits' than receptor-based screens. These types of assays monitor a functional event, for example a change in the level of a signaling intermediate or the expression level of M-RNA or protein. Multiple mechanisms may lead to the measured end point and only a few of these mechanisms may be desirable. This leads to a larger number of hits and therefore their physico-chemical profile will more closely resemble that of the compound set being screened. Perhaps, equally importantly, a larger volume of secondary evaluation allows for a greater expression of human bias. Bias is especially difficult to quantify in the chemists perception of a desirable lead structure.

The physico-chemical profile of the compound set being screened is the first filter in the physico-chemical profile of an HTS 'hit'. Obviously high molecular weight, high lipophilicity compounds will not be detected by a screen if they are not present in the library. In the real world, trade-offs occur in the choice of profiles for compound sets. An exclusively low molecular weight, low lipophilicity library likely increases the difficulty of detecting 'hits' but simplifies the process of discovering an orally active drug once the lead is identified. The converse is true of a high molecular weight high lipophilicity library. In our experience, commercially available (non combinatorial) compounds like those available from chemical supply houses tend towards lower molecular weights and lipophilicities.

Human decision making, both overt and hidden can play a large part in the profile of HTS 'hits'. For example, a requirement that 'hits' possess an acceptable range of measured or calculated physico-chemical properties will obviously affect the starting compound profiles for medicinal chemistry SAR. Less obvious are hidden biases. Are the criteria for a 'hit' changing to higher potency (lower IC50) as the HTS screen runs? Labor-intensive secondary follow-up is decreased but less potent, perhaps physico-chemically more attractive leads, may be eliminated. How do chemists react to potential lead structures? In an interesting experiment, we presented a panel of our most experienced medicinal chemists with a group of theoretical lead structures — all containing literature 'toxic' moieties. Our chemists split into two very divergent groups; those who saw the toxic moieties as a bar to lead pursuit and those who recognized the toxic moiety but thought they might be able to replace the offending moiety. An easy way to illustrate the complexity of the chemists perception of lead attractiveness is to examine the remarkably diverse structures of the new chemical entities (NCEs) introduced to market that appear at the back of recent volumes of *Annual Reports in Medicinal Chemistry*. No single pharmaceutical company can conduct research in all therapeutic areas and so some of these compounds, which are all marketed drugs, will inevitably be less familiar and potentially less desirable to the medicinal chemist at one research location, but may be familiar and desirable to a chemist at another research site.

2.3. Identifying a library with favorable physico-chemical properties

The idea in selecting a library with good absorption properties is to use the clinical Phase II selection process as a filter. Drug development is expensive and the most poorly behaved compounds are weeded out early. Our hypothesis was that poorer physico-chemical properties would predominate in the many

compounds that enter into and fail to survive preclinical stages and Phase I safety evaluation. We expected that the most insoluble and poorly permeable compounds would have been eliminated in those compounds that survived to enter Phase II efficacy studies. We could use the presence of United States Adopted Name (USAN) or International Non-proprietary Name (INN) names to identify compounds entering Phase II since most drug companies (including Pfizer) apply for these names at entry to Phase II.

The (WDI) World Drug Index is a very large computerized database of about 50 000 drugs from the Derwent Co. The process used to select a subset of 2245 compounds from this database that are likely to have superior physico-chemical properties is as follows: From the 50 427 compounds in the WDI File, 7894 with a data field for a USAN name were selected as were 6320 with a data field for an INN. From the two lists, 8548 compounds had one or both USAN or INN names. These were searched for a data field 'indications and usage' suggesting clinical exposure, resulting in 3704 entries. From the 3704 using a substructure data field we eliminated 1176 compounds with the text string 'POLY', 87 with the text string 'PEPTIDE' and 101 with the text string 'QUAT'. Also eliminated were 53 compounds containing the fragment O = P-O. We coined the term 'USAN' library for this collection of drugs.

2.4. The target audience — medicinal chemists

Having identified a library of drugs selected by the economics of entry to the Phase II process we sought to identify calculable parameters for that library that were likely related to absorption or permeability. Our approach and choice of parameters was dictated by very pragmatic considerations. We wanted to set up an absorption–permeability alert procedure to guide our medicinal chemists. Keeping in mind our target audience of organic chemists we wanted to focus on the chemists very strong pattern recognition and chemical structure recognition skills. If our target audience had been pharmaceutical scientists we would not have deliberately excluded equations or regression coefficients. Experience had taught us that a focus on the chemists very strong skills in pattern recognition and their outstanding chemistry structural recognition skills was likely to enhance information transfer. In effect, we deliberately emphasized enhanced educational effectiveness towards a well defined target audience at the expense of a loss of detail. Tailoring the message to the audience is a basic communications principle. One has only to look at the popular chemistry abstracting booklets with their page after page of chemistry structures and minimal text to appreciate the chemists structural recognition skills. We believe that our chemists have accepted our calculations at least in part because the calculated parameters are very readily visualized structurally and are presented in a pattern recognition format.

2.5. Calculated properties of the 'USAN' library

Molecular weight (formula weight in the case of a salt) is an obvious choice because of the literature relating poorer intestinal and blood brain barrier permeability to increasing molecular weight [7,8] and the more rapid decline in permeation time as a function of molecular weight in lipid bi-layers as opposed to aqueous media [9]. The molecular weights of compounds in the 2245 USANs were lower than those in the whole 50 427 WDI data set. In the USAN set 11% had MWTs > 500 compared to 22% in the entire data set. Compounds with MWT > 600 were present at 8% in the USAN set compared to 14% in the entire data set. This difference is not explainable by the elimination of the very high MWTs in the USAN selection process. Rather it reflects the fact that higher MWT compounds are in general less likely to be orally active than lower MWTs.

Lipophilicity expressed as a ratio of octanol solubility to aqueous solubility appears in some form in almost every analysis of physico-chemical properties related to absorption [10]. The computational problem is that an operationally useful computational alert to possible absorption–permeability problems must have a no fail log P calculation. In our experience, the widely used and accurate Pomona College Medicinal Chemistry program applied to our compound file failed to provide a calculated log P (CLogP) value because of missing fragments for at least 25% of compounds. The problem is not an inordinate number of 'strange fragments' in our chemistry libraries but rather lies in the direction of the trade off between accuracy and ability to calculate all compounds adopted by the Pomona College

team. The CLogP calculation emphasizes high accuracy over breadth of calculation coverage. The fragmental CLogP value is defined with reference to five types of intervening isolating carbons between the polar fragments. As common a polar fragment as a sulfide (-S-) linkage generates missing fragments when flanked by rare combinations of the isolating carbon types. Polar fragments as defined by the CLogP calculation can be very large and are not calculated as the sum of smaller, more common, polar fragments. This approach enhances accuracy but increases the number of missing fragments.

We implemented the log P calculation (MLogP) as described by Moriguchi et al. [11] within the Molecular Design Limited MACCS and ISIS base programs to avoid the missing fragment problem. As a rule-based system, the Moriguchi calculation always gives an answer. The pros and cons of the Moriguchi algorithm have been debated in the literature [12,13]. We recommend that, within analog series, our medicinal chemists use the more accurate Pomona CLogP calculation if possible. For calculation or tracking of library properties the less accurate MLogP program is used.

Only about 10% of USAN compounds have a CLogP over 5. The CLogP value of 5 calculated on the USAN data set corresponds to an MLogP of 4.15. The slope of CLogP (x axis) versus MLogP (y axis) is less than unity. At the high log P end, the Moriguchi MLogP is somewhat lower than the MedChem CLogP. In the middle log P range at about 2, the two scales are similar. Experimentally there is almost certainly a lower (hydrophilic) log P limit to absorption and permeation. Operationally, we have ignored a lower limit because of the errors in the MLogP calculation and because excessively hydrophilic compounds are not a problem in compounds originating in our medicinal chemistry laboratories.

An excessive number of hydrogen bond donor groups impairs permeability across a membrane bilayer [14,15]. Hydrogen donor ability can be measured indirectly by the partition coefficient between strongly hydrogen bonding solvents like water or ethylene glycol and a non hydrogen bond accepting solvent like a hydrocarbon [15] or as the log of the ratio of octanol to hydrocarbon partitioning. In vitro systems for studying intestinal drug absorption have been recently reviewed [16]. Computationally, hydrogen donor ability differences can be expressed by the solvatochromic α parameter of a donor group with perhaps a steric modifier to allow for the interactions between donor and acceptor moieties. Experimental α values for hydrogen bond donors and β values for acceptor groups [17] have been compiled by Professor Abraham in the UK and by the Raevsky group in Russia [18,19]. Both research groups currently express the hydrogen bond donor and acceptor properties of a moiety on a thermodynamic free energy scale. In the Raevsky C scale, donors range from about -4.0 for a very strong donor to -0.5 for a very weak donor. Acceptors values in the Raevsky C scale are all positive and range from about 4.0 for a strong acceptor to about 0.5 for a weak acceptor. In the Abraham scale both donors and acceptors have positive values that are about one-quarter of the absolute C values in the Raevsky scale.

We found that simply adding the number of NH bonds and OH bonds does remarkably well as an index of H bond donor character. Importantly, this parameter has direct structural relevance to the chemist. When one looks at the USAN library there is a sharp cutoff in the number of compounds containing more than 5 OHs and NHs. Only 8% have more than 5. So 92% of compounds have five or fewer H bond donors and it is the smaller number of donors that the literature links with better permeability.

Too many hydrogen bond acceptor groups also hinder permeability across a membrane bi-layer. The sum of Ns and Os is a rough measure of H bond accepting ability. This very simple calculation is not nearly as good as the OH and NH count (as a model for donor ability) because there is far more variation in hydrogen bond acceptor than donor ability across atom types. For example, a pyrrole and pyridine nitrogen count equally as acceptors in the simple N O sum calculation even though a pyridine nitrogen is a very good acceptor (2.72 on the C scale) and the pyrrole nitrogen is an far poorer acceptor (1.33 on the C scale). The more accurate solvatochromic β parameter which measures acceptor ability varies far more on a per nitrogen or oxygen atom basis than the corresponding α parameter. When we examined the USAN library we found a fairly sharp cutoff in profiles with only about 12% of compounds having more than 10 Ns and Os.

2.6. The 'rule of 5' and its implementation

At this point we had four parameters that we

thought should be globally associated with solubility and permeability; namely molecular weight; Log P; the number of H-bond donors and the number of H-bond acceptors. In a manner similar to setting the confidence level of an assay at 90 or 95% we asked how these four parameters needed to be set so that about 90% of the USAN compounds had parameters in a calculated range associated with better solubility or permeability. This analysis led to a simple mnemonic which we called the 'rule of 5' [20] because the cutoffs for each of the four parameters were all close to 5 or a multiple of 5. In the USAN set we found that the sum of Ns and Os in the molecular formula was greater than 10 in 12% of the compounds. Eleven percent of compounds had a MWT of over 500. Ten percent of compounds had a CLogP larger than 5 (or an MLogP larger than 4.15) and in 8% of compounds the sum of OHs and NHs in the chemical structure was larger than 5. The 'rule of 5' states that: poor absorption or permeation are more likely when:

There are more than 5 H-bond donors (expressed as the sum of OHs and NHs);
The MWT is over 500;
The Log P is over 5 (or MLogP is over 4.15);
There are more than 10 H-bond acceptors (expressed as the sum of Ns and Os)
Compound classes that are substrates for biological transporters are exceptions to the rule.

When we examined combinations of any two of the four parameters in the USAN data set, we found that combinations of two parameters outside the desirable range did not exceed 10%. The exact values from the USAN set are: sum of N and O + sum of NH and OH — 10%; sum of N and O + MWT — 7%; sum of NH and OH + MWT — 4% and sum of MWT + Log P — 1%. The rarity (1%) among USAN drugs of the combination of high MWT and high log P was striking because this particular combination of physico-chemical properties in the USAN list is enhanced in the leads resulting from high throughput screening.

The rule of 5 is now implemented in our registration system for new compounds synthesized in our medicinal chemistry laboratories and the calculation program runs automatically as the chemist registers a new compound. If two parameters are out of range, a 'poor absorption or permeability is possible' alert appears on the registration screen. All new compounds are registered and so the alert is a very visible educational tool for the chemist and serves as a tracking tool for the research organization. No chemist is prevented from registering a compound because of the alert calculation.

2.7. Orally active drugs outside the 'rule of 5' mnemonic and biologic transporters

The 'rule of 5' is based on a distribution of calculated properties among several thousand drugs. Therefore by definition, some drugs will lie outside the parameter cutoffs in the rule. Interestingly, only a small number of therapeutic categories account for most of the USAN drugs with properties falling outside our parameter cutoffs. These orally active therapeutic classes outside the 'rule of 5' are: antibiotics, antifungals, vitamins and cardiac glycosides. We suggest that these few therapeutic classes contain orally active drugs that violate the 'rule of 5' because members of these classes have structural features that allow the drugs to act as substrates for naturally occurring transporters. When the 'rule of 5' is modified to exclude these few drug categories only a very few exceptions can be found. For example, among the NCEs between 1990 and 1993 falling outside the double cutoffs in 'the rule of 5', there were nine non-orally active drugs and the only orally active compounds outside the double cutoffs were seven antibiotics. Fungicides–protoazocides–antiseptics also fall outside the rule. For example, among the 41 USAN drugs with MWT > 500 and MLogP > 4.15 there were nine drugs in this class. Vitamins are another orally active class drug with parameter values outside the double cutoffs. Close to 100 vitamins fell into this category. Cardiac glycosides, an orally active drug class also fall outside the parameter limits of the rule of 5. For example among 90 USANs with high MWT and low MLogP there were two cardiac glycosides.

2.8. High MWT USANs and the trend in MLogP

In our USAN data set we plotted MLogP against MWT and examined the compound distributions as defined by the 50 and 90% probability ellipses. A large number of USAN compounds had MLogP more negative than −0.5. Among the USAN compounds there was a trend for higher MWT to correlate with lower MLogP. This type of trend is distinctly different from the positive correlation between MLogP and MWT found in most SAR data sets. Usually as MWT increases, compound lipophilicity increases and MLogP becomes larger (more positive). From among the 2641 USANs, we selected the 405 with MLogP more negative than −0.5 and from among these selected those with MWT in excess of 500 and mapped the resulting 90 against therapeutic activity fields in the MACCS WDI database. About one half (44 of 90) of these high MWT, low MLogP USANs were orally inactive consisting of 26 peptide agonists or antagonists, 11 quaternary ammonium salts and seven miscellaneous non-orally active agents.

Among the USAN compounds in our list fewer than 10% of compounds had either high MLogP or high MWT. The combination of both these properties in the same compound was even rarer. Among 2641 USANs there were only 41 drugs with MWT > 500 and MLogP > 4.15, about one-half (21) were orally inactive. Among the remainder there were only six orally active compounds not in the fungicide and vitamin classes.

2.9. New chemical entities, calculations

New chemical entities introduced between 1990 and 1993 were identified from a summary listing in vol. 29 of *Annual Reports in Medicinal Chemistry*. All our computer programs for calculating physicochemical properties require that the compound be described in computer-readable format. We mapped compound names and used structural searches to identify 133 of the NCEs in the Derwent World Drug to give us the computer-readable formats to calculate the rule of 5. The means of calculated properties were well within the acceptable range. The average Moriguchi log P was 1.80, the sum of H-bond donors was 2.53, the molecular weight was 408 and the sum of Ns and Os was 6.95. The incidence of alerts for possible poor absorption or permeation was 12%.

2.10. Drugs in absorption and permeability studies, calculations

Very biased data sets are encountered in the types of drugs that are reported in the absorption or permeability literature. Calculated properties are quite favorable when compared to the profiles of compounds detected by high throughput screening. Compounds that are studied are usually orally active marketed drugs and therefore by definition have properties within the acceptable range. What is generally not appreciated is that absorption and permeability are mostly reported for the older drugs. For example, our list of compounds with published literature on absorption or permeability, studied internally for validation purposes, is highly biased against NCEs. Only one drug in our list of 73 was introduced in the period 1990 to date. In part this reflects drug availability, since drugs under patent are not sold by third parties. Drugs studied in absorption or permeability models tend to be those with value for assay validation purposes, i.e. those with considerable pre-existing literature. In addition, some of the newer studies are driven by a regulatory agency interest in the permeability properties of generic drugs. In our listing of 73 drugs in absorption or permeability studies there are 33 generic drugs whose properties the FDA is currently profiling. Our list includes an additional 23 drugs with CACO-2 cell permeation data. Most of these are from the speakers' handouts at a recent meeting on permeation prediction [21]; a few are from internal Pfizer CACO-2 studies. A final 12 drugs are those with zwitterionic or very hydrophilic properties for which there are either literature citations or internal Pfizer data. The means of calculated properties for compounds in this list are well within the acceptable range. The average Moriguchi log P was 1.60, the sum of H-bond donors was 2.49, the molecular weight was 361 and the sum of Ns and Os was 6.27. The incidence of alerts for possible poor absorption or permeation was 12% (Table 1).

2.11. Validating the computational alert

Validating a computational alert for poor absorption or permeation in a discovery setting is quite different than validating a quantitative prediction calculation in a developmental setting. In effect, a discovery alert is a very coarse filter that identifies

Table 1
Partial list of drugs in absorption and permeability studies

Drug name	MLogP	OH+NH[c]	MWT	N+O[d]	Alert[e]
Aciclovir[a,b]	−0.09	4	225.21	8	0
Alprazolam[a]	4.74	0	308.77	4	0
Aspirin[b]	1.70	1	180.16	4	0
Atenolol[a,b]	0.92	4	266.34	5	0
Azithromycin[b]	0.14	5	749.00	14	1
AZT[a]	−4.38	2	267.25	9	0
Benzyl-penicillin[b]	1.82	2	334.40	6	0
Caffeine[b]	0.20	0	194.19	6	0
Candoxatril[b]	3.03	2	515.65	8	0
Captopril[a]	0.64	1	217.29	4	0
Carbamazepine[a]	3.53	2	236.28	3	0
Chloramphenicol[b]	1.23	3	323.14	7	0
Cimetidine[a,b]	0.82	3	252.34	6	0
Clonidine[b]	3.47	2	230.10	3	0
Cyclosporine[b]	−0.32	5	1202.64	23	1
Desipramine[a,b]	3.64	1	266.39	2	0
Dexamethasone[b]	1.85	3	392.47	5	0
Diazepam[b]	3.36	0	284.75	3	0
Diclofenac[a]	3.99	2	296.15	3	0
Diltiazem-HCl[a]	2.67	0	414.53	6	0
Doxorubicin[b]	−1.33	7	543.53	12	1
Enalapril-maleate[a]	1.64	2	376.46	7	0
Erythromycin[b]	−0.14	5	733.95	14	1
Famotidine[a]	−0.18	8	337.45	9	0
Felodipine[a,b]	3.22	1	384.26	5	0
Fluorouracil[b]	−0.63	2	130.08	4	0
Flurbiprofen[a]	3.90	1	244.27	2	0
Furosemide[a]	0.95	4	330.75	7	0
Glycine[b]	−3.44	3	75.07	3	0
Hydrochlorthiazide[a]	−1.08	4	297.74	7	0
Ibuprofen[b]	3.23	1	206.29	2	0
Imipramine[b]	3.88	0	280.42	2	0
Itraconazole[a]	5.53	0	705.65	12	1
Ketaconazole[a]	4.45	0	380.92	1	0
Ketoprofen[a]	3.37	1	254.29	3	0
Labetalol-HCl[a]	2.67	5	328.42	5	0
Lisinopril[a]	1.11	5	405.50	8	0
Mannitol[b]	−2.50	6	182.18	6	0
Methotrexate[b]	1.60	7	454.45	13	1
Metoprolol-tartrate[a,b]	1.65	2	267.37	4	0
Nadolol[a]	0.97	4	309.41	5	0
Naloxone[b]	1.53	2	327.38	5	0
Naproxen-sodium[a,b]	2.76	1	230.27	3	0
Nortriptylene-HCl[a]	4.14	1	263.39	1	0
Omeprazole[a]	−4.38	2	267.25	9	0
Phenytoin[a]	2.20	2	451.49	10	0
Piroxicam[a]	0.00	2	331.35	7	0
Prazosin[b]	2.05	2	383.41	9	0
Propranolol-HCl[a,b]	2.53	2	259.35	3	0
Quinidine[b]	2.19	1	324.43	4	0
Ranitidine-HCl[a]	0.66	2	314.41	7	0
Scopolamine[b]	1.42	1	303.36	5	0
Tenidap[b]	1.95	2	320.76	5	0
Terfenadine[a]	4.94	2	471.69	3	0
Testosterone[b]	3.70	1	288.43	2	0
Trovafloxacin[b]	2.81	3	416.36	7	0
Valproic-acid[b]	2.06	1	144.22	2	0
Vinblastine[b]	2.96	3	811.00	13	1
Ziprasidone[b]	3.71	1	412.95	5	0

[a]Standard or drug in FDA bioequivalence study.
[b]Studied in CACO-2 permeation.
[c]Sum of OH and NH H-bond donors.
[d]Sum of N and O H-bond acceptors.
[e]Computational alert according to the rule of 5; 0, no problem detected; 1, poor absorption or permeation are more likely.

compounds lying in a region of property space where the probability of useful oral activity is very low. The goal is to move chemistry SAR towards the region of property space where oral activity is reasonably possible (but not assured) and where the more labor-intensive techniques of drug metabolism and the pharmaceutical sciences can be more efficiently employed. A compound that fails the computational alert will likely be poorly bio-available because of poor absorption or permeation and lies within that region of property space where good absorption or solubility is unlikely. We believe the alert has its primary value in identifying problem compounds. In our experience, most compounds failing the alert also will prove troublesome if they progress far enough to be studied experimentally. However, the converse is not true. Compounds passing the alert still can prove troublesome in experimental studies.

In this perspective, a useful computational alert correctly identifies drug projects with known absorption problems. Drugs in human therapy, whether poorly or well absorbed from the viewpoint of the pharmaceutical scientist, should profile as 'drugs', i.e. as having reasonable prospects for oral activity. The larger the computational and experimental difference between drugs in human therapy and those which are currently being made in medicinal chemistry laboratories, the greater the confidence that the differences are meaningful. We assert that absorption problems have recently become worse in the pharmaceutical industry as attested to by recent meetings and symposia on this subject [22] and by the informal but industry-wide concern of pharmaceutical scientists about drug candidates with less than optimal physical properties. If we are correct, within any drug organization, one should be able to quantify by calculation whether time-dependent changes that might impair absorption have occurred in medicinal chemistry. If these changes have occurred one can try to correlate these with changes in screening strategy.

2.12. Changes in calculated physical property profiles at Pfizer

How relevant is our experience at the Pfizer Central Research laboratories in Groton to what may be expected to be observed in other drug discovery organizations? The physical property profiles of drug leads discovered through HTS will be similar industry-wide to the extent that testing methodology, selection criteria and the compounds being screened are similar. Changes in physical property profiles of synthetic compounds, made in follow-up of HTS leads by medicinal laboratories, depend on the timing of a major change towards HTS screening. The Pfizer laboratories in Groton were one of the first to realize and implement the benefits of HTS in lead detection. As a consequence, we also have been one of the first to deal with the effects of this change in screening strategy on physico-chemical properties. In Groton, 1989 marked the beginning of a significant change towards HTS screening. This process was largely completed by 1992 and currently HTS is now the major, rich source of drug discovery leads and has largely supplanted the pre-1989 pattern of lead generation.

At the Pfizer Groton site, we have retrospectively examined the MWT distributions of compounds made in the pre-1989 era and since 1989. Since our registration systems unambiguously identify the source of each compound, we can identify any time-dependent change in physical properties and we can compare the profiles of internally synthesized compounds with the profiles of compounds purchased from external commercial sources.

Before 1989, the percentage of internally synthesized high MWT compounds oscillated in a range very similar to the USAN library (Table 2). Starting in 1989, there was an upward jump in the percentage of high MWT compounds and a further jump in 1992 to a new stable MWT plateau that is higher than in

Table 2
Percent of compounds with MWT (including salt) above 500

Year registered	Synthetic compounds	Commercial compounds
Pre-1984	16.0	5.4
1984	18.9	14.7
1985	12.1	15.5
1986	12.6	5.5
1987	13.4	5.8
1988	14.6	8.2
1989	23.4	4.1
1990	21.1	3.3
1991	25.4	1.8
1992	34.2	6.8
1993	33.2	8.4
1994	32.7	7.9

the USAN library and higher than any yearly oscillation in the pre-1989 era. By contrast, there was no change in the MWT profiles of commercially purchased compounds over the same time period. A comparison of the MWT and MLogP percentiles of synthetic compounds for a year before the advent of HTS and for 1994 in the post-HTS era shows a similar pattern (Table 3). The upper range percentiles for MWT and MLogP properties are skewed towards physical properties less favorable for oral absorption in the more recent time period.

The trend towards higher MWT and LogP is in the direction of the property mix that is least populated in the USAN library. There was no change over time in the population of compounds with high numbers of H-bond donors or acceptors.

2.13. The rationale for measuring drug solubility in a discovery setting

In recent years, we have been exploring experimental protocols in a discovery setting that measure drug solubility in a manner as close as possible to the actual solubilization process used in our biological laboratories. The rationale is that the physical forms of the compounds solubilized and the methods used to solubilize compounds in discovery are very different from those used by our pharmaceutical scientists and that mimicking the discovery process will lead to the best prediction of in vivo SAR.

In discovery, the focus is on keeping a drug solubilized for an assay rather than on determining the solubility limit. Moreover, there is no known automated methodology that can efficiently solubilize hundreds of thousands of sometimes very poorly soluble compounds under thermodynamic conditions. In our biological laboratories, compounds that are not obviously soluble in water or by pH adjustment are pre-dissolved in a water miscible solvent (most often DMSO) and then added to a well stirred aqueous medium. The equivalent of a thermodynamic solubilization, i.e. equilibrating a solid compound for 24–48 h, separating the phases, measuring the soluble aqueous concentration and then using the aqueous in an assay, is not done. When compounds are diluted into aqueous media from a DMSO stock solution, the apparent solubility is largely kinetically driven. The influence of crystal lattice energy and the effect of polymorphic forms on solubility is, of course, completely lost in the DMSO dissolution process. Drug added in DMSO solution to an aqueous medium is delivered in a very high energy state which enhances the apparent solubility. The appearance of precipitate (if any) from a thermodynamically supersaturated solution is kinetically determined and to our knowledge is not predictable by computational methods. Solubility may also be perturbed from the true thermodynamic value in purely aqueous media by the presence of a low level of residual DMSO.

Table 3
Synthetic compound properties in 1986 (pre-HTS) and 1994 (post-HTS)

Percentile	MLogP		MWT	
	1986	1994	1986	1994
90th	4.30	4.76	514	726
75th	3.48	3.90	415	535
50th	2.60	2.86	352	412

The physical form of the first experimental lot of a compound made in a medicinal chemistry lab can be very different from that seen by the pharmaceutical scientist at a later stage of development. Solution spectra, HPLC purity criteria and mass spectral analysis are quite adequate to support a structural assignment when the chemist's priority is on efficiently making as many well selected compounds as possible in sufficient quantity for in vitro and in vivo screening. All the measurements that support structural assignment are unaffected by the energy state (polymorphic form) of the solid. Indeed, depending on the therapeutic area, samples may not be crystalline and most compounds synthesized for the first time are unlikely to be in lower energy crystalline forms. Attempts to compute solubility using melting point information are not useful if samples do not have well defined melting points. Well characterized, low energy physical form (from a pharmaceutics viewpoint) reduces aqueous solubility and may actually be counter productive to the discovery chemists priority of detecting in vivo SAR.

In this setting, thermodynamic solubility data can be overly pessimistic and may mislead the chemist who is trying to relate chemical structural changes to absorption and oral activity in the primary in vivo assay. Our goal is to provide a relevant experimental

solubility measurement so that chemistry can move from the pool of poorly soluble, orally inactive compounds towards those with some degree of oral activity. For maximum relevance to the in vivo biological assay our solubility measurement protocol is as close as possible to the biological assay 'solubilization'. In this paradigm, any problems that might be related to the poor absorption of a low energy crystalline solid under thermodynamic conditions are postponed and not solved. The efficiency gain in an early discovery stage solubility assay lies in the SAR direction provided to chemistry and in the more efficient application of drug metabolism and pharmaceutical sciences resources once oral activity is detected. The value of this type of assay is very stage-dependent and the discovery type of assay is not a replacement for a thermodynamic solubility measurement at a later stage in the discovery process.

2.14. Drugs have high turbidimetric solubility

Measuring solubility by turbidimetry violates almost every precept taught in the pharmaceutical sciences about 'proper' thermodynamic solubility measurement. Accordingly, we have been profiling known marketed drugs since our initial presentation on turbidimetric solubility measurement [23] and have measured turbidimetric solubilities on over 350 drugs from among those listed in the Derwent World Drug Index. The calculated properties of these drugs are well within the favorable range for oral absorption. The average of the calculated properties are: MLogP, 1.79; the sum of OH and NH, 2.01: MWT, 295.4; the sum of N and O, 4.69. Without regard to the therapeutic class, only 4% of these drugs would have been flagged as having an increased probability of poor absorption or permeability in our computational alert. Of the 353 drugs, 305 (87%) had a turbidimetric solubility of greater than 65 μg/ml. There were only 20 drugs (7%) with a turbidimetric solubility of 20 μg/ml or less. If turbidimetric solubility values lie in this low range, we suggest to our chemists that the probability of useful oral activity is very low unless the compound is unusually potent (e.g. projected clinical dose of 0.1 mg/kg) or unusually permeable (top tenth percentile in absorption rate constant) or unless the compound is a member of a drug class that is a substrate for a biological transporter.

Our drug list was compiled without regard to literature thermodynamic solubilities but does contain many of the types of compounds studied in the absorption literature. Of the 353 drugs studied in the discovery solubility assay, 171 are drugs from four sources. There are 77 drugs from the compilation of 200 drugs by Andrews et al. [6]. This compilation is biased towards drugs with reliable measured in vitro receptor affinity and with interesting functionality and not necessarily towards drugs with good absorption or permeation characteristics. There are 23 drugs from a list of generics whose properties FDA is currently profiling for bio-equivalency standards. In addition, there are 42 NCEs introduced between 1983 and 1993 and 37 entries are for drugs with CACO-2 cell permeation data.

The profile of drug turbidimetric solubilities serves as a useful benchmark. Compounds that are drugs have a very low computational alert rate for absorption or permeability problems and a low measured incidence of poor turbidimetric solubility of about 10%. The calculated profiles and alert rates of compounds made in medicinal chemistry laboratories can be compared to those of drugs and the profiles can be compared on a project by project basis.

Within the physical property manifold of 'marketed drugs' we would expect a poor correlation of our turbidimetric solubility data with literature thermodynamic solubility data since the properties of 'drugs' occupy only a small region of property space relative to what is possible in synthetic compounds and HTS 'hits'. Our turbidimetric solubilities for drugs are almost entirely at the top end of a relatively narrow solubility range, whereas from a thermodynamic viewpoint the drugs in our list cover a wide spectrum of solubility. We caution that turbidimetric solubility measurements are most definitely not a substitute for careful thermodynamic solubility measurements on well characterized crystalline drugs and should not be used for decision making in a development setting.

2.15. High throughput screening hits, calculations and solubility measurements

Calculated properties and measured turbidimetric solubilities for the best compounds identified as

'hits' in our HTS screens are in accord with the hypothesis that the physico-chemical profiles of leads have changes from those in the pre-1989 time period. Nearly 100 of the most potent 'hits' from our high throughput screens were examined computationally and their turbidimetric solubilities were measured. The profiles are strikingly different from those of the 353 drugs we studied. The HTS hits are on average more lipophilic and less soluble than the drugs. The 96 compounds we measured were the end product of detection in HTS screens and secondary in vitro evaluation. These were the compounds highlighted in summaries and which captured the chemist's interest with many IC50s clustered in the 1 μM range. As such, they are the product of a biological testing process and a chemistry evaluation as to interesting subject matter. Average MLogP for the HTS hits was a full log unit higher than for the drugs and the average MWT was nearly 50 Da higher. By contrast, there was little difference in the number of hydrogen bond donors and acceptors. The distribution curves for MLogP and MWT are roughly the same shape for the HTS hits and drugs but the means are shifted upwards in the HTS hits with a higher distribution of compounds towards the unfavorable range of physico-chemical properties. The actual averages, HTS vs. Drug are: MLogP, 2.81 vs. 1.79; MWT, 366 vs. 295; sum of OH NH, 1.80 vs. 2.01; sum of N and O, 5.4 vs. 4.69.

2.16. The triad of potency, solubility and permeability

Acceptable drug absorption depends on the triad of dose, solubility and permeability. Our computational alert does not factor in dose, i.e. drug potency. It only addresses properties that are related to potential solubility and permeation problems and it does not allow for a very favorable value of one parameter to compensate for a less favorable value of another parameter. In a successful marketed drug, one parameter can compensate for another. For example, a computational alert is calculated for azithromycin, a successful marketed antibiotic. In azithromycin, which has excellent oral activity, a very high aqueous solubility of 50 mg/ml more than counterbalances a very low absorption rate in the rat intestinal loop of 0.001 min^{-1}. Poorer permeability in orally active peptidic-like drugs is usually compensated by very high solubility. Our solubility guidelines to our chemists suggest a minimum thermodynamic solubility of 50 μg/ml for a compound that has a mid-range permeability and an average potency of 1.0 mg/kg. These solubility guidelines would be markedly higher if the average compound had low permeability.

2.17. Protocols for measuring drug solubility in a discovery setting

The method and timing of introduction of the drug into the aqueous media are key elements in our discovery solubility protocol. Drug is dissolved in DMSO at a concentration of 10 μg/μl of DMSO which is close to the 30 mM DMSO stock concentration used in our own biology laboratories. This is added a microlitre at a time to a non-chloride containing pH 7 phosphate buffer at room temperature. The decision to avoid the presence of chloride was a tradeoff between two opposing considerations. Biology laboratories with requirements for iso-osmotic media use vehicles containing physiological levels of saline (e.g. Dulbecco's phosphate buffered saline) with the indirect result that the solubility of HCl salts (by far the most frequent amine salt from our chemistry laboratories) can be depressed by the common ion effect. Counter to this consideration, is the near 100% success rate of our pharmaceutical groups in replacing problematical HCl salts with other salts not subject to a chloride common ion effect. We chose the non-chloride containing medium to avoid pessimistic solubility values resulting from a historically very solvable problem.

The appearance of precipitate is kinetically driven and so we avoid a short time course experiment where we might miss precipitation that occurs on the type of time scale that would affect a biological experiment. The additions of DMSO are spaced a minute apart. A total of 14 additions are made. These correspond to solubility increments of < 5 μg/ml to a top value of > 65 μg/ml if the buffer volume is 2.5 ml (as in a UV cuvette). If it is clear that precipitation is occurring early in the addition sequence, we stop the addition so that we have two consecutive readings after the precipitate is first detected. Precipitation can be quantified by an absorbance increase due to light scattering by precipitated particulate material in a dedicated diode array

UV machine. The sensitivity to light scattering is a function of the placement of the diode array detector relative to the cuvette and differs among instruments. We found that the array placement in a Hewlett Packard HP8452A diode array gives high sensitivity to light scattering. Increased UV absorbance from light scattering is measured in the 600–820 nm range because most drugs have UV absorbance well below this range.

In its simplest implementation, the precipitation point is calculated from a bilinear curve fit to the Absorbance (y axis) vs. μl of DMSO (x axis) plot. The coordinates of the intersect point of the two line segments are termed X crit and Y crit. X crit is the microlitres of DMSO added when precipitation occurs and Y crit is the UV Absorbance at the precipitation point. The concentration of drug in DMSO (10 μg/ml) is known. The volume of aqueous buffer (typically 2.5 ml in a cuvette) is known so the drug concentration expressed as μg of drug per ml buffer at the precipitation point is readily calculated. The volume percent aqueous DMSO at the precipitation point is also reported. Under our assay conditions it does not exceed 0.67% for a turbidimetric solubility of >65 μg/ml. The upper solubility limit is based on the premise that for most projects permeability is not a major problem and that solubility assays will most often be requested for poorly soluble compounds. In the absence of poor permeability, solubilities above 65 μg/ml suggest that if bio-availability is poor, solubility is not the problem.

2.18. Technical considerations and signal processing

In our experience, most UV active compounds made in our Medicinal Chemistry labs have UV peak maxima below 400 nm. Approximation to a Gaussian form for absorbance peaks allows an estimate for the UV absorbance at long wavelength from the peak maximum and peak width at half height. A soluble compound with maximum absorbance at 400 nm and extinction coefficient of 10 000 and peak width at half height of 100 nm at a concentration of 400 μg/ml (well above the maximum for our assay) has calculated absorbance of 0.000151 at 600 nm.

The sensitivity of UV absorbance measurements to light scattering is largely a function of how closely the diode array is positioned to the UV cuvette and varies among manufacturers. The HP89532 DOS software detects a curve due to light scattering by fitting the absorbance over a wavelength range to a power curve of the form. $\text{Abs} = k \times \text{nm}^{-n}$, where k is a constant, nm = wavelength

Values for 'n' were examined in a total of 45 solubility experiments. The last scan in each solubility series was examined since precipitation is most likely at the highest drug concentration. In this 45 assay series precipitation was not observed in 10 assays (as assessed by values of $n > 0$). Positive values of n ranged as high as 5.054 in the 35 assays in which precipitation occurred. Once precipitation occurred, all scans in an assay sequence could be fit with a power curve. The overall absorbance increase due to light scattering can be quite low. In most of the 45 assays, the total absorbance increase at 690 nm (due to precipitate formation) was in the OD range 0–0.01. Half the absorbance increases were in the range 0–0.001. Measurements within these very small ranges quantitate the precipitation point.

Problems in determining the precipitation point occur when a compound is intensely colored since colored compounds may be miscalled as insoluble. In collaboration with Professor Chris Brown at the University of Rhode Island, we implemented a fast fourier transform (FFT) signal processing procedure to enhance assay sensitivity and to avoid false positive solubility values due to colored compounds [20]. The absorbance curve due to light scattering has an apparent peak width at half height which is much wider than the apparent peak width at half height for a typical UV absorption curve. An analysis procedure that is sensitive to the degree of curvature can be used to differentiate color from light scattering. The even wavelength spacing in our diode array UV means that the absorbance vs. wavelength matrix in each scan can be treated as if it were a time series (which it really is not). In a time series, the early terms in an FFT describe components of low curvature (low frequency). An FFT over a 256 nm range (566–820 nm) generates 128 absorbance values which in turn generates 128 FFT terms. FFT term 1 describes the baseline shift. By plotting the real component of FFT term 1 or term 2 vs. DMSO addition, the false positive rate from color is much reduced and we detect the onset of precipitation as if

we were plotting absorbance at a single wavelength vs. absorbance.

An alternative to the use of a dedicated diode array UV is to use one of a number of relatively inexpensive commercially available nephelometers. The solubility protocol using a nephelometer as the signal detector is identical to that using a UV machine. We have experience using a HACH AN2100 as a turbidity detector. A nephelometer has the advantage that colored impurities do not cause a false positive precipitation signal and so signal processing is avoided. The disadvantage is the larger volume requirement relative to a UV cuvette. The HACH unit uses inexpensive disposable glass test tubes that can be as small as 100 mm × 12 mm. The use of even smaller tubes and the resultant advantage of reduced volume is precluded by light scattering from the more sharply curved surface of a smaller diameter tube.

Using nephelometric turbidity unit (NTU) standards, the threshold for detection using a UV detector-based assay is 0.2 NTUs and a 0.4 NTU standard can be reliably detected vs. a water blank. Turbidity standards in the range 0.2–2 NTU units suffice to cover the scattering range likely to be detected in a solubility assay. Some type of signal detector is necessary if light scattering is the analytical signal used to detect precipitation. For example, a 1.0 NTU standard was our lower visual detection limit using a fiber optic illuminator to visualize Tyndall light scattering. The European Pharmacopoeia defines the lowest category of turbidity — 'slight opalescence' on the basis of measured optical density changes in the range 0.0005–0.0156 at 340–360 nm. These optical density readings correspond to NTU standards well below 1.0 (in the 0.2–0.4 range) in our equipment.

3. Calculation of absorption parameters

3.1. Overall approach

The four parameters used for the prediction of potential absorption problems can be easily calculated with any computer and a programming language that supports or facilitates the analysis of molecular topology. At Pfizer, we began our programming efforts using MDL's sequence and MEDIT languages for MACCS and have since successfully ported the algorithms to Tripos' SPL and MDL's ISIS PL languages without difficulty.

The parameters of molecular weight and sum of nitrogen and oxygen atoms are very simple to calculate and require no further discussion. Likewise, the calculation of the number of hydrogen-bond acceptors is simply the number of nitrogen and oxygen atoms attached to at least one hydrogen atom in their neutral state.

3.2. MLogP. Log P by the method of Moriguchi

The calculation of log P via the method of Moriguchi et al. [11] required us to make some assumptions that were not clear from the rules and examples in the two papers describing the method [11,12]. Therefore, more detailed discussion on how we implemented this method is necessary.

The method begins with a straightforward counting of lipophilic atoms (all carbons and halogens with a multiplier rule for normalizing their contributions) and hydrophilic atoms (all nitrogen and oxygen atoms). Using a collection of 1230 compounds, Moriguchi et al. found that these two parameters alone account for 73% of the variance in the experimental log Ps. When a 'saturation correction' is applied by raising the lipophilic parameter value to the 0.6 power and the hydrophilic parameter to the 0.9 power, the regression model accounted for 75% of the variance.

The Moriguchi method then applies 11 correction factors, four that increase the hydrophobicity and seven that increase the lipophilicity, and the final equation accounts for 91% of the variance in the experimental log Ps of the 1230 compounds. The correction factors that increase hydrophobicity are:

1. UB, the number of unsaturated bonds except for those in nitro groups. Aromatic compounds like benzene are analyzed as having alternating single and double bonds so a benzene ring has 3 double bonds for the UB correction factor, naphthalene has a value of 5;
2. AMP, the correction factor for amphoteric compounds where each occurrence of an alpha amino acid structure adds 1.0 to the AMP parameter, while each amino benzoic acid and each pyridine carboxylic acid occurrence adds 0.5;

3. RNG, a dummy variable which has the value of 1.0 if the compound has any rings other than benzene or benzene condensed with other aromatic, hetero-aromatic, or hydrocarbon rings;
4. QN, the number of quaternary nitrogen atoms (if the nitrogen is part of an N-oxide, only 0.5 is added);

The seven correction factors that increase lipophilicity are:

1. PRX, a proximity correction factor for nitrogen and oxygen atoms that are close to one another topologically. For each two atoms directly bonded to each other, add 2.0 and for each two atoms connected via a carbon, sulfur, or phosphorus atom, add 1.0 unless one of the two bonds connecting the two atoms is a double bond, in which case, according to some examples in the papers, you must add 2.0. In addition, for each carboxamide group, we add an extra 1.0 and for each sulfonamide group, we add 2.0;
2. HB, a dummy variable which is set to 1.0 if there are any structural features that will create an internal hydrogen bond. We limited our programs to search for just the examples given in the Moriguchi paper [11] as it is hard to determine how strong a hydrogen bond has to be to affect lipophilicity;
3. POL, the number of heteroatoms connected to an aromatic ring by just one bond or the number of carbon atoms attached to two or more heteroatoms which are also attached to an aromatic ring by just one bond;
4. ALK, a dummy parameter that is set to 1.0 if the molecule contains only carbon and hydrogen atoms and no more than one double bond;
5. NO2, the number of nitro groups in the molecule;
6. NCS, a variable that adds 1.0 for each isothiocyanate group and 0.5 for each thiocyanate group;
7. BLM, a dummy parameter whose value is 1.0 if there is a beta lactam ring in the molecule.

3.3. *MLogP calculations*

Log Ps, calculated by our Moriguchi-based computer program for a set of 235 compounds were less accurate than the calculated log Ps (CLogPs) from Hansch and Leo's Pomona College Medicinal Chemistry Project MedChem software distributed by Biobyte. The set of 235 was chosen so that the CLogP calculation would not fail because of missing fragments. Our implementation of the Moriguchi method accounts for 83% of the variance with a standard error of 0.6 whereas the Hansch values account for 96% of the variance with a standard error of 0.3. The advantages of the Moriguchi method are that it can be easily programmed in any language so that it can be integrated with other systems and it does not require a large database of parameter values.

4. The development setting: prediction of aqueous thermodynamic solubility

4.1. *General considerations*

The prediction of the aqueous solubility of drug candidates may not be a primary concern in early screening stages, but the knowledge of the thermodynamic solubility of drug candidates is of paramount importance in assisting the discovery, as well as the development, of new drug entities at later stages. A poor aqueous solubility is likely to result in absorption problems, since the flux of drug across the intestinal membrane is proportional to its concentration gradient between the intestinal lumen and the blood. Therefore even in the presence of a good permeation rate a low absorption is likely to be the result. Conversely, a compound with high aqueous solubility might be well absorbed, even if it possesses a moderate or low permeation rate.

Formulation efforts can help in addressing these problems, but there are severe limitations to the absorption enhancement that can be realistically achieved. Stability and manufacturing problems also have to be taken into account since it is likely that an insoluble drug candidate may not be formulated as a conventional tablet or capsule, and will require a less conventional approach such as, for example, a soft gel capsule. Low solubility may have an even greater impact if an i.v. dosage form is desired. Obviously, a method for predicting solubility of drug candidates at an early stage of discovery would have a great impact on the overall discovery and development process.

Unfortunately the aqueous solubility of a given

molecule is the result of a complex interplay of several factors ranging from the hydrogen-bond donor and acceptor properties of the molecule and of water, to the energetic cost of disrupting the crystal lattice of the solid in order to bring it into solution ('fluidization') [24].

In any given situation, not all the factors may play an important role and it is difficult to predict the solubility of a complex drug candidate, on the basis of the presence or absence of certain functional groups. Conformational effects in solution may play a major role in the outcome of the solubility and cannot be accounted for by a simple summation of 'contributing' groups.

Thus, any method which would aim at predicting the aqueous solubility of a given molecule would have to take into account a more comprehensive 'description' of the molecule as the outcome of the complex interplay of factors.

The brief discussion of the problem outlined above can be summarized by considering the three basic quantities governing the solubility (S) of a given solid solute:

$$S = f(\text{Crystal Packing Energy} + \text{CavitationEnergy} + \text{Solvation Energy})$$

In this equation, the crystal packing energy is a (endoergic) term which accounts for energy necessary to disrupt the crystal packing and to bring isolated molecules in gas phases, i.e. its enthalpy of sublimation. The cavitation energy is a (endoergic) term which accounts for the energy necessary to disrupt water (structured by its hydrogen bonds) and to create a cavity into which to host the solute molecule. Finally, the solvation energy might be defined as the sum (exoergic term) of favorable interactions between the solvent and the solute.

In dealing with the prediction of the solubility of crystalline solids[2], a first major hurdle to overcome is the determination or estimation of their melting point or, better, of their enthalpy of sublimation. At present no accurate and efficient method is available to predict these two quantities for the relatively complex molecules which are encountered in the pharmaceutical research. Gavezzotti[3] [26] has discussed this point in a review article on the predictability of crystal structures and he states that '...the melting point is one of the most difficult crystal properties to predict.' This author has pioneered the use of computational methods to predict crystal structures and polymorphs and, consequently, properties such as melting point and enthalpy of sublimation. A commercially available program has been recently developed [27] but the use of these approaches is still far from being routine and from being useful in a screening stage for a relatively large number of compounds, all of which possess a relatively high conformational flexibility.

Thus, although there are several approaches to estimating and predicting the solubility of organic compounds, the authors of this article are of the opinion that none of the presently available methods can truly be exploited for a relatively accurate prediction of solubility, if the target of the prediction is the solubility of complex pharmaceutical drug candidates. Although the judicious application of some these approaches might be useful for 'rank-ordering' of compounds and prioritization of their synthesis, we are not aware of any such systematic use of estimation methods.

The sections that follow will discuss available methods, taking into account the second and third terms of the above relationship and the feasibility of their assessment a priori, and they will be treated as one term since the available methods consider the interactions in solution as the (algebraic) sum of the two terms and their contributors. This discussion is by no means exhaustive but it is rather intended as an overview of the methods available as seen, in particular, from a pharmaceutical perspective.

4.2. LSERs and TLSER methods

Linear Solvation Energy Relationships (LSERs), based upon solvatochromic parameters, have the advantage of a good theoretical background and offer a correlation between several molecular properties, and a solute property, SP. Several LSERs have been developed over the past few years and they seem to work well for predicting a generalized SP for a series

[2]Since the vast majority of drug molecules and most substances of pharmaceutical interest are crystalline solids, this discussion will focus on the prediction of the solubility of crystalline solids.

[3]The program PROMET is available from Professor Gavezzotti, University of Milan, Italy.

of solutes in one or more (immiscible) phases. Most notably, the work of Abraham et al. [28] has generated an equation of the general type:

$$LogSP = c + rR_2 + a\Sigma\alpha_2^H + b\Sigma\beta_2^H + s\pi_2^H + nV_x$$

where c is a constant, R_2 is an excess molar refractivity, $\Sigma\alpha_2^H$ and $\Sigma\beta_2^H$ are the (summation or 'effective') solute hydrogen-bond acidity and basicity, respectively, π_2^H is the solute dipolarity-polarizability and V_x is McGowan's characteristic volume [29]. The main problem encountered when using parameterized equations is that such quantities (parameters or descriptors) cannot easily be estimated, from structures only, for complex multi-functional molecules such as drug candidates, especially if they are capable of intra-molecular hydrogen bonding, as is often the case. Nevertheless, the method was successfully applied to the correlation between the solvatochromic parameters described above and the aqueous solubility of relatively simple organic non-electrolytes [30].

More recently, Kamlet [31] has published equations describing the solubility of aromatic solutes including polycyclic and chlorinated aromatic hydrocarbons. In these equations a term accounting for the crystal packing energy was introduced, and the equation has the general form:

$$\log S_w(aromatics) = \frac{0.24 - 5.28V_I}{100} + 4.03\beta_m + 1.53\alpha_m - 0.0099(m.p. - 25)$$

where V_I is the intrinsic (van der Waals) molar volume of the solute, the other parameters are defined as above and the subscript m indicates a non self-associating solute monomer. It is interesting to note that the term 0.0099(m.p.−25) is used, in the words of the author, 'to account for the process of conversion of the solid solute to super-cooled liquid at 25°C.' This term is therefore related to the crystal packing energy mentioned earlier, albeit representing the conversion from a solid to a 'super-cooled' liquid, not to isolated molecules in gas phase. The author finds the above term 'robust' in its statistical significance and it should be noted that coefficient of 0.0099 implies that a variation of less than one order of magnitude will be observed for variations in melting points of less than 100°C.

This finding might be exploited in a series of close structural analogs where a large variation in melting points (>100°C) is not expected (as might often be the case) and the 'solution behavior' could be estimated by solvatochromic parameters. Thus, with some error, the prioritization of more soluble synthetic targets might be achieved, since the relative ('rank-order') solubility of structurally close analogs may be all that it is sought at an early stage. However this prioritization would rely on the assumption that variations in structural properties which bring about a (desired) lowering of the crystal packing energy, would not significantly and adversely alter the properties of a molecule with respect to its solvation in water. If the lower crystal packing energy is the result, for example, of a lower hydrogen-bond capability, a diminished solvation in water may offset the lowering of the crystal packing energy.

Even with the assumption described above, the estimation of a relatively good rank-ordering of aqueous solubilities would still require the determination of solvatochromic parameters which is generally achieved through the determination of several partition coefficients. On the other hand, descriptor values for several fragments (functional groups) are available and they may be used to calculate the 'summation' parameters for the molecules of interest. This process is not without caveats though, as a very judicious choice of the 'disconnection pattern' must be made to obtain reliable results. In a recent paper describing the partition of solutes across the blood-brain barrier, Abraham et al. [32] reported the calculation and use of these descriptors for compounds of pharmaceutical interest but he warned about the possibility of inter-molecular hydrogen bonding, which may be a source of error if not present in the 'reference' compounds, and pointed out the fact that these correlations are best used within the descriptors range used to generate them.

Some authors have reported the calculation of quantities related to those descriptors, via ab initio [33–35] or semi-empirical methods [36,37]. The equations stemming from computed values have been termed TLSERs (Theoretical Linear Solvation Energy Relationships) [36]. However, we are not aware of any application of this approach to a series

of complex multifunctional compounds, and these types of correlations are likely to be difficult for these compounds, due to the relatively high level of computation involved.

Ruelle and Kesselring and colleagues [38–40] reported a multi-parameter equation, qualitatively similar to the LSERs described above. This equation attempts to predict solubility by using terms which account for the quantities that play a role in the process. It does contain a solute 'fluidization' term (endoergic cost of destroying the crystal lattice of a solid) and other terms describing the hydrophobic effect, hydrogen bond formation between proton-acceptor solutes and proton-donor solvents, and the H-bond formation between amphiphilic solutes and proton acceptor and/or proton-donor solvents as well as the auto-association of the solute in solution.

Although this equation takes into account the free energy changes involved in the dissolution process, in our opinion its complexity prevents its use for multifunctional molecules. The examples reported address simple hydrocarbons or mono-functional molecules and much emphasis is placed on organic (associated and non-associated) solvents. In many such cases, approximations leading to the cancellation of some term, can be made but, if an attempt to predict the solubility of complex drug candidates in water is made, all those terms might be present at the same time and thus it would be very difficult to treat solubility within the framework of this equation.

4.3. LogP and AQUAFAC methods

Prominent in this area is the work of Yalkowski [41] who has published a series of papers describing the prediction of solubility using LogP (the logarithm of the octanol/water partition coefficient) and a term describing the energetic cost of the crystal lattice disruption. However Yalkowski's work is largely based on the prediction or estimation of the solubility of halogenated aromatic and polycyclic halogenated aromatic hydrocarbons [42], due to their great environmental importance. The general solubility equation, for organic non-electrolytes is reported below.

$$\log S_{pred} = -\frac{\Delta S_m(m.p. - 25)}{1364} - \log P + 0.80$$

In this equation, ΔS_m is the entropy of melting and m.p. is the melting point in °C. The signs of the two terms considered are physically reasonable, since an increase in either the first term (higher crystal packing energy) or in LogP (more lipophilic compound), would cause a decrease in the observed (molar) solubility S_m. In a recent paper [43], this author discusses the predictive use of the above equation and, in particular, the prediction of activity coefficients. The latter is a term which accounts for deviations from ideal solubility behavior due to differences in size and shape, but also in hydrogen bonding ability, between the solute and the solvent. The conclusion is that, among methods based upon solvatochromic parameters, or simply based on molecular volume, molecular weight or regular solution theory, the estimation of the activity coefficient is best achieved by using the LogP method.

Many computational methods are indeed available to address the prediction of LogP and the aqueous solubility of complex molecules. A well known and widely used program to predict LogP values is CLogP [44] which uses a group-contribution approach to yield a LogP value. Another method, developed by Moriguchi et al. [11], which uses atomic constants and correction factors to account for different atom types is discussed in detail in Section 3.2. We have observed that, in the daily practice of pharmaceutical sciences, both methods have their 'outliers' but methods based on fragmental constants tend to fail, in the not infrequent instances where appropriate constants are not available.

However, LogP prediction aside, the method reported by Yalkowski was developed on a data set largely based upon rigid, polycyclic and halogenated aromatic compounds and does not seem to easily yield itself to the prediction of complex pharmaceutical compounds. The basic difficulty is that while LogP could be estimated albeit with some error by computational approaches, the melting point and entropy of melting are still difficult to calculate or even simply to estimate. Yalkowski discusses this point in several papers [42,45,46] and shows the relationship between the entropy of fusion and the molecular rotational and translational entropies. Some rules are offered for the estimation of entropy, but the work is limited to relatively simple molecules. The melting point prediction is also discussed and a computational approach, based on molecular properties such as eccentricity (the ratio between the

maximum molecular length and the mean molecular diameter) is proposed. However, the calculation of such properties may be easy to perform on simple polychlorinated biphenyls, but would not easily be applicable for complex drug candidates.

A similar approach to solubility predictions using a group-contribution method has been implemented in the CHEMICALC-2 program [47], which calculates LogP and log $1/S$ where S is the molar aqueous solubility. This program uses several different algorithms to calculate log $1/S$ depending on the complexity and nature of the molecule, and requires knowledge of the melting point, T_m. If T_m is not available, the program calculates the solubility of the super-cooled liquid at 25°C. In the case of complex molecules, fragmental constants may be missing from its database and poor results are obtained. We have used this program to some extent and we are not encouraged by the correlation between 'predicted' and experimental solubility.

Yalkowski and colleagues [48] have more recently discussed an improvement of the AQUAFAC (AQUeous Functional group Activity Coefficients) fragmental constant method. In this work, the authors describe a correlation between the sum of fragmental constants of a given molecule and the activity coefficient, defined as a measure of the non-ideality of the solution. The knowledge or estimation of ΔS_m and m.p. is necessary, but the method seems to be somewhat better than the general solubility equation based on LogP values. Yalkowski explains this by pointing out that these group contribution constants were derived entirely from aqueous phase data and they should perform better than octanol-water partition coefficients. We concur with this explanation since it is known that the octanol-water partition coefficients are rather insensitive to the hydrogen-bond donor capability of the solute. Furthermore the authors point out the fact that molecules like small carboxylic acids are likely to dimerize in octanol, while in water they would not.

The solubility equation derived using the AQUAFAC coefficients is reported below.

$$\log S_{pred} = -\frac{\Delta S_m(m.p. - 25)}{1364} - \Sigma n_i q_i$$

where q_i is the group contribution of the ith group and n_i is the number of times the ith group appears in the molecule. The negative sign of the second term stems from the fact that the constant of polar groups (e.g. OH = −1.81) has a negative sign and a net negative sign of the summation of contributors would yield an overall positive contribution to solubility. However while this method might be of simple application, its scope seems limited to molecule containing relatively simple functional groups, and the objections to the use of group contribution methods, which do not consider conformational effects, remain.

4.4. Other calculation methods

Bodor and Huang [49] and Nelson and Jurs [50] have reported methods based entirely on calculated geometric, electronic and topological descriptors, for a series of relatively simple liquid and solid solutes.

We favor these methods as truly a priori predictions based on molecular structures only, but some questions arise when the compounds have conformational flexibility and multiple functional groups, and some of the descriptors will depend upon the particular conformation chosen. As it is generally true for many QSAR approaches, there is uncertainty about the actual predictive value of a test set which does not include a wide variety of compounds and, in Bodor's training set of 331 compounds we fail to recognize with few exceptions represented by rigid steroids, complex multifunctional molecules. Furthermore a large number of the compounds used are liquids or gases at ambient temperature.

Bodor's method involves the calculation of 18 descriptors, among which are the ovality of the molecule, the calculated dipole moment, and the square root of the sum of squared charges on oxygen atoms, but it does yield a good correlation for the 331-compound set. The predictive power of the model is illustrated by a table of 17 compounds, but most of them are rigid aromatics, although a reasonably good prediction is offered for dexamethasone. The latter however is an epimer of betamethasone which is present in the training set, and it is difficult to predict the robustness of the correlation with regard to its application to a truly diverse set of molecules. Similar considerations could be extended to the work by Nelson and Jurs, which is also based on calculated descriptors and it does not seem to involve any polyfunctional molecule or any solid compound at 25°C. Overall the correlation is good

but the caveats on its application to drug-like compounds remain, as well as our objections on the ease of calculation of the parameters for compounds of pharmaceutical interest.

Finally, Bodor et al. [25] and Yalkowski and colleagues [5] have reported the use of neural networks to develop correlations using the calculated parameters discussed above or the AQUAFAC coefficients, respectively. While we have no direct experience with the use of neural networks, we are of the opinion that it may not be a trivial task to set up and 'train' a neural network and the superiority of this approach in comparison to 'conventional' regression techniques may be more apparent than real. Indeed Bodor reports a similar standard deviation for the prediction using the neural network or regression analysis [49] on the same data set, and the use of a neural network does not appear to offer any advantage over the regression analysis.

5. Conclusion

Combinatorial chemistry and high throughput screening (HTS) techniques are used in drug research because they produce leads with an efficiency that compares favorably with 'rational' drug design and, perhaps more importantly, because these techniques expand the breadth of therapeutic opportunities and hence the leads for drug discovery. Established methodology allows the medicinal chemist, often in a relatively short time, to convert these novel leads to compounds with in vitro potency suitable to a potential drug candidate. This stage of the discovery process is highly predictable. However, the majority of drugs are intended for oral therapy and introducing oral activity is not predictable, is time and manning expensive and can easily consume more resources than the optimization of in vitro activity. The in vitro nature of HTS screening techniques on compound sets with no bias towards properties favorable for oral activity coupled with known medicinal chemistry principles tends to shift HTS leads towards more lipophilic and therefore generally less soluble profiles. This is the tradeoff in HTS screening. Efficiency of lead generation is high, and therapeutic opportunities are much expanded, but the physical profiles of the leads are worse and oral activity is more difficult. Obtaining oral activity can easily become a rate-limiting step and hence methods which allow physico-chemical predictions from molecular structure are badly needed in both early discovery and pharmaceutical development settings.

Computational methods in the early discovery setting need to deal with large numbers of compounds and serve as filters which direct chemistry SAR towards compounds with greater probability of oral activity. These computational methods become particularly important as experimental studies become more difficult because compounds are available for physico-chemical screening in only very small quantities and in non-traditional formats. Early discovery methods deal with probabilities and not exact value predictions. They enhance productivity by indicating which types of compounds are less likely to be absorbed and which are more likely to require above average manning expenditures to become orally active. Calculations, however imprecise, are better than none when choices must be made in the design or purchase of combinatorial libraries. Drug discovery requires a starting point — a lead. Hence the current literature correctly focuses on improving in vitro activity detection by optimizing chemical diversity so as to maximize coverage of three-dimensional receptor space. Assuming this goal is not compromised by physico-chemical calculations, we believe a competitive advantage accrues to the organization that can identify compound sets likely to give leads more easily converted to orally active drugs.

Methods in the pharmaceutical developmental setting deal with much smaller numbers of compounds. Here, a more accurate prediction is computationally complex because exact values rather than probabilities are important, and because the prediction of crystal packing energies is at present extremely difficult. The problem of polymorphism, common in pharmaceutical research, which may have been deferred in the discovery setting has to be addressed in the development setting. Currently, only approximate estimates of the solubility of multifunctional and conformationally flexible drug candidates are possible and these need to be supported by physical measurements which provide experimental 'feedback' on analogs in a particular class of compounds. In our view, a priori solubility estimation methods like Bodor's multi-parameter equation [49] are the

current best choice, but some of the required properties are not easily computed without a preliminary optimization of preferred conformations and good initial estimates. The accurate prediction of the solubility of complex multifunctional compounds at the moment still remains an elusive target. The requirements for high accuracy and the complexity of possible studies in the drug developmental setting means that even small changes towards poorer, but still acceptable, physico-chemical properties in compounds approaching candidacy can translate to higher developmental time and manning requirements. Moreover, there has not been the same level of efficiency improvement in many developmental assays as there has been in discovery screening. For example, there is not the same level of efficiency improvement in measuring accurate equilibrium solubility as there has been in the efficiency of detecting leads.

Medicinal chemists efficiently and predictably optimize in vitro activity, especially when the lead has no key fragments missing. This ability will likely be reinforced because the current focus on chemical diversity should produce fewer leads with missing fragments. Oral activity prospects are improved through increased potency, but improvements in solubility or permeability can also achieve the same goal. Despite increasingly sophisticated formulation approaches, deficiencies in physico-chemical properties may represent the difference between failure and the development of a successful oral drug produc..

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OPTIMALIZATION OF PHARMACOKINETICS - AN ESSENTIAL ASPECT OF DRUG DEVELOPMENT - BY "METABOLIC STABILIZATION"

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1) INTRODUCTION

Generally speaking, the chemical properties and hence the chemical structure of a compound definitely determine the way in which it participates in the various part-processes involved in biological action. A structure-action relationship (SAR), therefore, has to be a fundamental characteristic of bioactive agents. The apparent absence of such a relationship can only be due to deficient methods of investigation and to the multiplicity and complexity of the process as a whole. Although the various part-processes are biochemical and physicochemical in nature, they differ greatly. Totally different SAR patterns may be expected for, for instance, the rate of absorption, the mode of distribution, the renal excretion, the various types of metabolic conversion, and the capacity of the agent to activate the molecular sites of action (the receptors) in the target tissue - the structure-action relationship in a strict sense. Absorption, distribution and excretion, which are mainly based on passive diffusion processes, will largely depend on partition coefficients. Metabolic conversion will depend mainly on the presence in the molecule of particular groups that are open to attack by enzymes. These groups as a rule have little or nothing to do with the chemical characteristics which are essential for the induction of the effect. Whether an agent is hydrolysed by esterases, for instance, depends mainly on the presence of a suitable ester group in the molecule. The ester group, however, has little or nothing to do with the question whether the agent has a curariform, an anticholinergic, a local anesthetic action, is an insecticide, a herbicide, a plasticizer, or some toxon. Whether an agent is capable of inducing a particular type of biological effect is usually dependent on various specific chemical characteristics in the molecule.

SAR will emerge most clearly if it is studied for particular part-processes such as those involved in absorption, distribution or excretion, where passage of membranes is essential, those involved in drug metabolism, where SAR will depend on the particular enzyme, and SAR for the final step of action, the induction of the effect. In a comparison of the quantitative dose-effect relationship for a

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group of compounds the in vivo-SAR is the resultant of the integrated contribution of SAR for the various part-processes.

2) THE MAIN PHASES IN BIOLOGICAL ACTION

The complex of processes involved in biological action can be split up in three phases (Fig. 1) (ref. 1, 2).

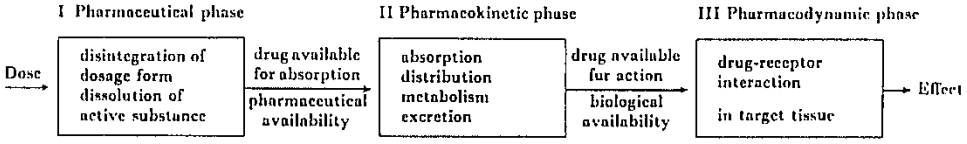

Fig. 1.

I. The pharmaceutical phase

This phase comprises the processes that are determinant for the efficacy of the application. Here the disintegration of the dosage form, tablets, capsules, etc., in such a way that the active agent becomes available in a molecular dispersed form suitable for absorption, and avoidance of chemical or enzymatic activation of the active agent before absorption, e.g. in the intestinal tract, count. In general, for the absorption - which implies passage of biological membranes - the lipid/water solubility and therewith the partition coefficient is determinant. For weak bases and acids also the degree of ionization and therewith the pK_A of the compound and the pH at the site of absorption count. The fraction of the dose available for absorption is indicated as the "pharmaceutical availability". The time course of the events has to be taken into account, too, and results in the "pharmaceutical availability profile".

II. The pharmacokinetic phase

This phase comprises the processes involved in absorption, distribution, excretion and metabolic conversion of the agent after absorption. The fraction of the dose that reaches the general circulation is indicated as the "biological (systemic) availability". Also here the time course represented in the "biological availability profile" is of particular significance. The concentration of the active agent in the target tissue as a function of time is represented by the "pharmacological availability profile". In the pharmacokinetic phase, besides the lipid/water solubility and degree of ionization of the agent, particularly its sensitivity to various enzymes counts. The presence in the molecule of vulnerable moieties accessible to enzymatic attack plays a predominant role. In this respect, like in the case of active, carrier-related transport, the charge distribution of the agent and its steric properties are determinant factors. The metabolic conversion of the agent applied may result in its bioinactivation (biodetoxification) or bioactivation

(biotoxification).

The involvement of various metabolites greatly complicates pharmacokinetics. Metabolic conversion usually increases hydrophilicity thus facilitating renal excretion.

III. The pharmacodynamic phase

This phase comprises the processes involved in the interaction between the bioactive compounds and their molecular sites of action, receptors, enzymes, etc. Pharmacon-receptor interaction results in the induction of a stimulus which initiates a sequence of biochemical and biophysical events which finally lead to the effect observed.

3) PHARMACON METABOLISM, A NATURAL DEFENCE AGAINST INTRUSION OF CHEMICALS (XENOBIOTICS), INCLUDING DRUGS

Although one might get the impression that the toxicological risks involved in the exposure to chemicals, including drugs, are generated by the evolvement of chemical and pharmaceutical industries and thus are of recent origin, this is definitely not the case. Already since the very beginning of evolution living systems have been exposed to chemicals. This especially holds true for the heterotrophic organisms (in general, animal life) which are to a large extent dependent on the consumption of autotrophic organisms (mainly plant material) and are exposed, therefore, to a great variety of potentially toxic chemicals of plant origin. These plant products are xenobiotic to the animal concerned. The term "biogenic xenobiotics" is appropriate here.

As long as life was limited to the oceans, the problems were relatively small, since there was a tremendous water compartment available for the disposal of undesired body-foreign chemicals, even if these were rather lipid-soluble. The "affinity" thereof for the relatively lipophilic biomass was counterbalanced by the tremendous volume of the disposal compartment. Photodegradation and oxidation in the surface layers of the waters largely took care of chemical degradation. By the time animal life switched from water to land, this opportunity got lost. Water became relatively scarce and only a small volume became available for disposal (for man about 1 liter a day). This increased the danger of accumulation of lipophilic, poorly water-soluble agents in the biomass. In the line of evolution an answer was found in the development of enzyme systems which take care of the conversion of relatively lipophilic compounds into highly water soluble end-products suitable for renal excretion (table 1). This conversion occurs in two steps: a first predominantly oxidative step and a second predominantly conjugational step (Fig. 2). Simultaneously, a strong increase in the concentration of plasma albumin took place (table 1), important for osmotic regulation but serving as well as a temporary sink, a kind of parking lot, for lipophilic xenobiotics. Such agents would easily pass the various membranes in the body and so enter tissues and cells where damage

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TABLE I. EVOLUTIONARY ASPECTS OF PLASMA PROTEIN AND DRUG METABOLISM (ref. 3).

Species	*Plasma protein* %	*Oxidative N-demethylation*[c]	*Phenol glucuronidation*[d]	*Species*
man	6.5	19 ± 2	21 ± 3	mouse
dog	6.1–6.7	15 ± 2	46 ± 13	rat
turtle	4.8	26 ± 8	85 ± 22	pigeon
crocodile	3.69	4 ± 0.6	8.9 ± 2.3	lizard
frog	1.5–4.3	1.6 ± 0.45	1.26 ± 0.47	frog
skate	2.4–3.1	1.1 ± 0.30	1.72 ± 0.25	trout
menhaden	0.72–2.9	0.71 ± 0.28	1.9 ± 0.33	goldenorfe
goosefish	1.4–2.2	0.86 ± 0.23	2.68 ± 0.65	carp

[c] µmoles formaldehyde formed per gram fresh liver tissue/hour.

[d] µmoles p-nitrophenol glucuronidation per gram fresh liver tissue/hour.

Note the increases at the switch from water to land animals.

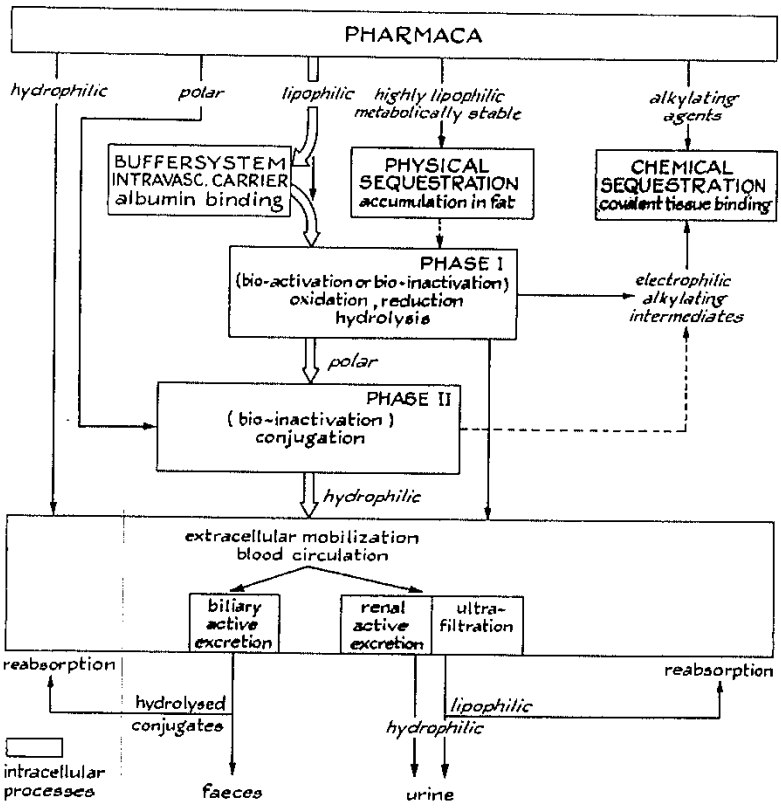

Fig. 2. Schematic representation of the main steps in drug metabolism and elimination.

might be done. Binding to albumin implies lowering of the free concentration in plasma and therewith lowering of the effective concentration to which cells and tissues are exposed. The agents involved are temporarily stored on the albumin in the circulation, where they are available for the enzymes, particularly in the liver, that especially take care of biochemical conversion to products that are suitable for renal excretion (ref. 3).

In short, by the time chemical and pharmaceutical industry came to development, animal and man were more or less prepared for dealing with - in fact for defence against - exposure to the products of these industries, "synthetic xenobiotics", including drugs, thanks to their experience with "biogenic xenobiotics".

4) DRUG METABOLISM - DETOXIFICATION AND TOXIFICATION

In the early days of studies on this subject, drug metabolism was put more or less synonymous with detoxification as indicated, for instance, by the classic book entitled "Detoxication Mechanisms" by R.T. Williams, 1959 (ref. 4). In the case the metabolic elimination of xenobiotics concerns the application of drugs as therapeutics, the action is considered, at least by the prescribing physician, as desirable, although some components in the action still may count as undesirable, i.e. as side-effects. In fact in drug metabolism two classes of undesirable aspects can be distinguished:

1) the generation of toxic metabolites, still xenobiotic in nature, biotoxification;
2) the untimely elimination of the drug and complication of pharmacokinetics with as a consequence blurring of the dose-effect relationship due to drug metabolism.

5) BIOTOXIFICATION

Drug metabolites may be biologically active and in some cases are fully responsible for the action of the drug, which then in fact must be considered as a prodrug. With regard to the bioactive metabolites, distinctions can be made between:

a) Stable metabolites, active in a pharmacological sense, producing effects mostly related to that of the mother compound. This type of bioactivation which depending on the circumstances may be considered positive or negative will not be discussed in further detail here.

b) Chemically highly reactive, electrophilic, biologically alkylating intermediate products with a very short half-life time, formed in the course of the metabolic conversion - particularly oxidation, but also conjugation reactions. These intermediates act under covalent binding with nucleophilic groups on biological macromolecules such as nucleic acids and proteins. The resulting "chemical lesions" may have serious consequences such as:

a) carcinogenesis, involving chemical lesions in chromosomal DNA
b) mutagenesis, also involving chemical lesions in chromosomal DNA
c) possibly accelerated aging, caused by an increase in the error frequency in-

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duced in chromosomal DNA,
d) teratogenesis, caused by disturbed cell proliferation due to chemical lesions during embryogenesis,
e) allergic sensitization, due to chemical lesions in proteins that cause them to act as allergens,
f) cell degeneration and necrosis, due to chemical damage to the membranes of lysosomes or to essential enzymes,
g) photosensitization, involving formation of reactive products by radiation of the drug or its metabolite(s), thus causing local chemical lesions, or formation of allergens.

As a matter of fact, toxic effects such as the ones just mentioned may also be induced by directly alkylating agents, such as some cytostatics used in the chemotherapy of cancer. Particularly troublesome with regard to the carcinogenesis and mutagenesis is the latency, the long lag-time between exposure to the agent and the appearance of the effect. This is partly due to the irreversible nature of the chemical lesions, which implies an accumulation of the effect. Like in the case of exposure to ionizing radiation, in fact each dose, how small it may be, counts and contributes to the effect. The total lifetime exposure constitutes the dose. Further, especially for lesions in chromosomal DNA, "syncarcinogenesis" due to various agents has to be taken into account. The chemical lesions in proteins are reversible to a certain extent on the basis of de novo synthesis of proteins. If the damage is limited, it may be largely reversible. This is not the case for protein damage resulting in allergic sensitization where the immunological memory of the lymphocytes is involved. In the case of damage to DNA, to a certain extent, especially short term, repair mechanisms may eliminate part of the chemical lesions.

Metabolic systems protecting against biochemical lesions

In the line of evolution, nature not only developed biochemical clearance systems for xenobiotics, but also systems to control the risks thereof, namely those involved in the formation of reactive intermediate metabolites. The major protecting systems are: the glutathione transferase system, coupling glutathione to the chemically reactive, biologically alkylating metabolic intermediate, under the formation of conjugation products that appear in the urine as water soluble mercapturic acid derivatives (Fig. 3) and methylthiolation which implies the coupling of a methylthio ($-SCH_3$) group to the chemically reactive, electrophilic group in the alkylating metabolic intermediate, which thus is detoxicated. Further there is the epoxide hydratase system taking care of the hydrolysis of alkylating epoxides under the formation of diols which appear in the urine mostly as water-soluble phenol sulphate conjugates (Fig. 3) (ref. 1, 2, 5, 6).

Conjugation products such as those formed by acetylation or sulphate conjugation as

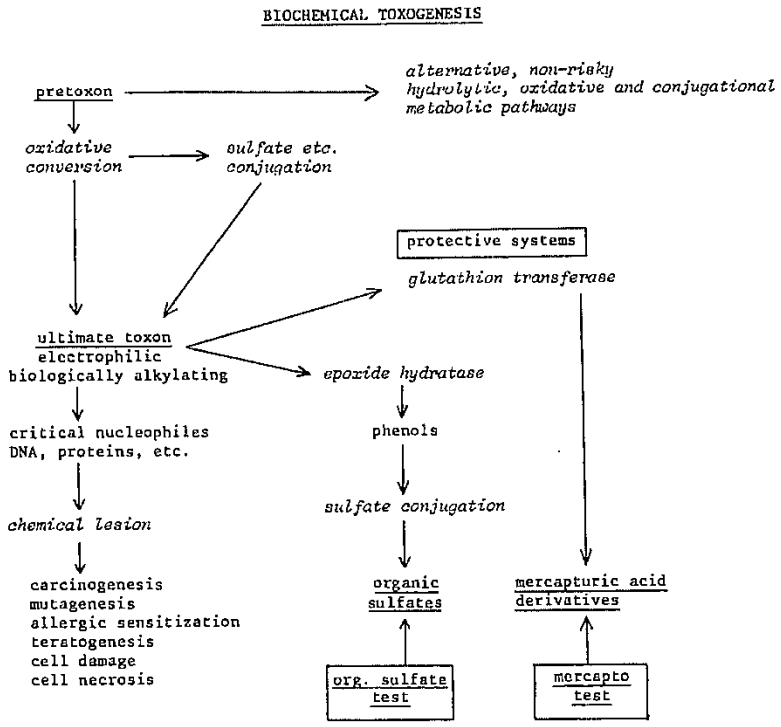

Fig. 3.

a rule are considered as harmless final detoxification products. However, although exceptionally, also such conjugation products, may be chemically reactive, biologically alkylating, and thus toxic. Also here the glutathione transferase system has a protective function.

Oxidation products formed in the course of drug metabolism may also lead to peroxides and oxidation products with a toxic character with regard to redox systems. An example of the damage caused is the formation of methemoglobin from hemoglobin. The latter type of action is well known for aniline derivatives. Again, glutathione has a protecting action since it contributes to the regeneration of hemoglobin from methemoglobin.

Early detection of biotoxification

It will be clear from the foregoing that considerations on drug metabolism and its consequences such as biotoxification are essential already in an early phase, if possible on the drawing table, of drug design. Reasoning on the relationship between chemical structure and action in this respect implies recognition in the

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structures of chemical groups potentially open to metabolic conversion. One has to differentiate between moieties leading to non-risky conversion - being particularly significant for the bioavailability and duration of action,i.e. the pharmacokinetics - and to risky conversion via reactive, alkylating intermediates particularly significant for toxicity. Moieties determining the lipophilicity of the compound are especially important in absorption, distribution and excretion and thus for pharmacokinetics (ref. 1, 2).

Remarkably, up to now, very little attention has been paid to the relationship between chemical structure and pharmacon metabolism. There is no doubt that such a relationship exists and even that in many cases, for instance in the case of particular enzymatic conversion, the relationships are relatively clear and simple. In vivo, however, often multiple metabolic pathways and a sequence of different metabolic steps are involved. Avoidance in the chemical structure of moieties potentially involved in biotoxification is advisable. Once compounds have been synthesized, a testing on mutagenic and carcinogenic action is advisable, in order to select or at least incorporate in the groups of compounds to be studied, the agents with a reduced risk with respect to the causation of chemical lesions. The use of the Ames-test, or better a properly chosen set of such in vitro tests, gives a reasonable indication of the risk for a mutagenic and carcinogenic action (ref. 7, 8, 9).By the way, one has to be well aware that even,although there is not a 100% correlation between mutagenic action as detected by such in vitro tests and the carcinogenic action in vivo, mutagenesis as such - for which the bill will be paid by future generations - , should be taken at least as serious as carcinogenesis.

A covalent binding of the toxon to biopolymers has as a consequence that the toxon cannot be extracted from the tissues anymore with hydrophilic or lipophilic solvents. A chemical sequestration is involved, to be distinguished from a physical sequestration where the compound, due to its metabolic stability combined with high lipophilicity, is kept back in the organism, predominantly by dissolution in the body fat. In balance studies, relating the dose to the quantity of the agent excreted, the fraction missing in that balance is important, even if it may be small, especially if chemical sequestration is involved (ref. 10).

The final inevitable step in the testing of a new drug, before its release for practical use, is the study of its carcinogenic potential in animal species. This still does not present a 100% safeguarding. Even after the agent has been released for application to the patient it has to be monitored in a toxicological sense. Introduction in a number of steps, comprising larger and larger groups of individuals, for drugs widely used for minor ailments numbering many thousands of individuals, is advisable.

6) BLURRING OF PHARMACOKINETICS AND THUS OF THE DOSE-EFFECT RELATIONSHIP BY DRUG METABOLISM

Therapeuticals usually are metabolized and eliminated at the time that the action is still wanted; thus sequential dosages have to be supplied. This in fact means a drug waste. Also other aspects of drug metabolism count as negative, e.g. the first-pass losses, due to metabolic conversion in the intestinal wall and the liver, the patient-to-patient and intra-patient variations in metabolic capacity with as a result a highly variable bioavailability, and the drug interactions related to drug metabolism. A highly variable relationship between dose and plasma level due to drug metabolism makes expensive therapeutic monitoring on basis of plasma level measurements, especially of drugs with a small therapeutic margin, necessary. Species differences, mostly related to differences in drug metabolism make extrapolation of animal data to the human situation difficult. An answer to the problems inherent in drug metabolism may be the development of drugs resisting drug metabolism, metabolic stabilization (ref. 1, 2, 11).

If short or ultrashort action is required or at local application, systemic action has to be avoided, introduction of suitable, safe, vulnerable moieties may be required. The same holds true in the case that the prodrug principle is to be applied. In general, however, avoidance of drug metabolism or reduction of it to the possible minimum will be advantageous.

For metabolically stable agents pharmacokinetics (absorption, distribution and excretion) are mainly determined by the balance between lipid and water solubility as expressed by the partition coefficient which in its turn is related to the pK_A-value. An exception has to be made for active transport processes. A modulation of pharmacokinetics on the basis of adaptation in the partition coefficient will usually be much simpler than adaptation in the metabolic pathways and the rates of conversion. Often various metabolic pathways and a sequence of different metabolic conversions are involved in the processing of one drug.

With regard to metabolic stabilization two aspects have to be taken into account:
1) Metabolic stabilization in general, predominantly aimed at the simplification and control of pharmacokinetics. In this case a reduction of the fraction of the dose metabolized counts.
2) Metabolic stabilization, particularly concerned with those moieties in the molecule that can be converted to electrophilic, alkylating groups. The aim is to control biotoxification. In this case a reduction in the absolute quantity of reactive intermediates counts.

7) METABOLIC STABILIZATION TO CONTROL PHARMACOKINETICS

Metabolic stabilization implies a longer half-life time and therewith less drug waste, less exposure to unnecessary quantities of the drug in repeated application, and simpler dosage regimens and therewith a better patient compliance.

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Metabolic stabilization contributes to a reduction in drug interactions which in many cases are generated on the drug metabolic level.

Metabolic stabilization reduces the patient-to-patient and the intra-patient variability in the relationship between dose and effect, since this variability is largely based on differences and variations in the drug metabolic capacity.

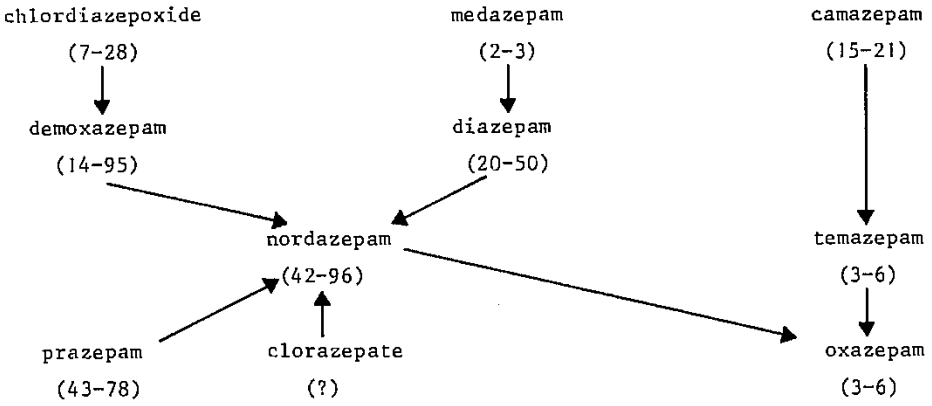
Metabolic stabilization will reduce the variability in the relationship between dose and plasma concentration. This will reduce or eliminate the need for expensive therapeutic monitoring via plasma drug concentration measurements for drugs with a relatively small therapeutic margin. The uncertainty in the dose-effect relationship which enforces plasma level monitoring is largely related to the variability in drug metabolism. Therapeutics that require therapeutic monitoring should be replaced as soon as possible by analogously acting new drugs which are pharmacokinetically better controlled, an aim which may be realized by metabolic stabilization. Such new drugs definitely cannot be regarded as "me-too" drugs, but in fact are badly needed revisions in the therapeutic arsenal (ref. 12, 13, 14).

Metabolic stabilization implies a reduction in species differences which are largely related to species differences in metabolic capacities. It will make the now highly uncertain transfer of animal data to man more reliable.

Metabolic stabilization will greatly reduce the number and significance of possibly active metabolites, which implies a fargoing reduction of elaborate and expensive studies on drug metabolites on both the preclinical and clinical level.

Metabolic stabilization will reduce the chance that the drug applied in fact is a prodrug or the situation that, besides the active agent applied, a number of more or less similarly active, but pharmacokinetically different metabolites complicate the picture. These situations which occur incidentally should in no way be regarded as advantageous. In the given circumstances it is advisable to consider (one of) the active metabolite(s) as a potential drug. Clearcut examples of this situation are found among the benzodiazepines (table 2). Various benzodiazepines on the market are in fact benzodiazepine metabolites. The use of the therapeutically active metabolites as such, especially the ones in the most advanced oxidized state, will automatically reduce the impact of metabolic conversion and thus reduce both the metabolic toxicological risks and the complexity of pharmacokinetics. If so required , the metabolite can be presented as a prodrug - e.g. to enhance absorption of the usually more hydrophilic metabolite. This has as a matter of fact to be based on a safe metabolic handle for bioactivation. As such,hydrolytic cleavage is to be preferred,but also oxidation of a saturated alkyl side-chain may be considered. Unsaturated alkyl side-chains may lead to risky epoxides. Similar reasonings hold true if a vulnerable moiety has to be introduced into the molecule in order to obtain an ultrashort or short action or to avoid systemic action after local application.

TABLE 2



Flow scheme of benzodiazepine metabolism.

All substances (half-life in hours) are in use as drugs.

8) METABOLIC STABILIZATION AND CONTROL OF BIOTOXIFICATION

The aim is a reduction in the absolute quantity of reactive intermediates formed. There are two approaches here:

a) reduction in the dose of the drug required;

b) metabolic stabilization.

A reduction in the quantity of reactive, potentially carcinogenic, mutagenic, etc. intermediate products is to a certain extent a natural consequence of the development of highly potent agents. Only low dosages are required then, which implies the reduction of the quantity of metabolites anyway and therewith a reduction in the risk of induction of chemical lesions.

An increase in potency, as far as related to the process in the pharmacodynamic phase - that is to the induction of the effect on specific sites of action, and not to, for instance, reduction in first pass loss - implies that lower plasma and tissue concentrations are needed for the induction of the effect desired. If the therapeutic effect and side-effect are induced on different target molecules (receptors, enzymes, etc.), an increase in the affinity to the sites involved in the therapeutic action only under particular circumstances will go hand in hand with a comparable increase in the affinity to the sites on which the side-effects are induced. An exception has to be made for those cases in which the higher therapeutic potency is related to accumulation of the agent in a phase (e.g. a lipophilic phase), in which both the sites for therapeutic effect and side-effect are located. In those cases that the increase in therapeutic potency is related to a higher degree of complementarity of the active agent to the molecular sites for therapeutic action, as a rule, this

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tends to enhance selectivity and thus to reduce side-effects. Besides this the lowering of the dose required also implies a smaller metabolic turnover and thus a reduction in the quantity of potentially toxic reactive intermediate products.

Metabolic stabilization aimed at a control of pharmacokinetics also implies a reduction of the dose required and therewith a reduction in the risky metabolic turnover as well as a reduction in the formation of metabolic products causing pharmacological side-effects. If stabilization of risky metabolic handles, chemical groups open to conversion to electrophilic, alkylating moieties, is involved, biotoxification is brought under control even more effectively. An alternative to metabolic stabilization of risky metabolic handles, is introduction into the molecule of safe metabolic handles offering a preferred alternative route of conversion. An example is toluene as compared to benzene (see fig. 4).

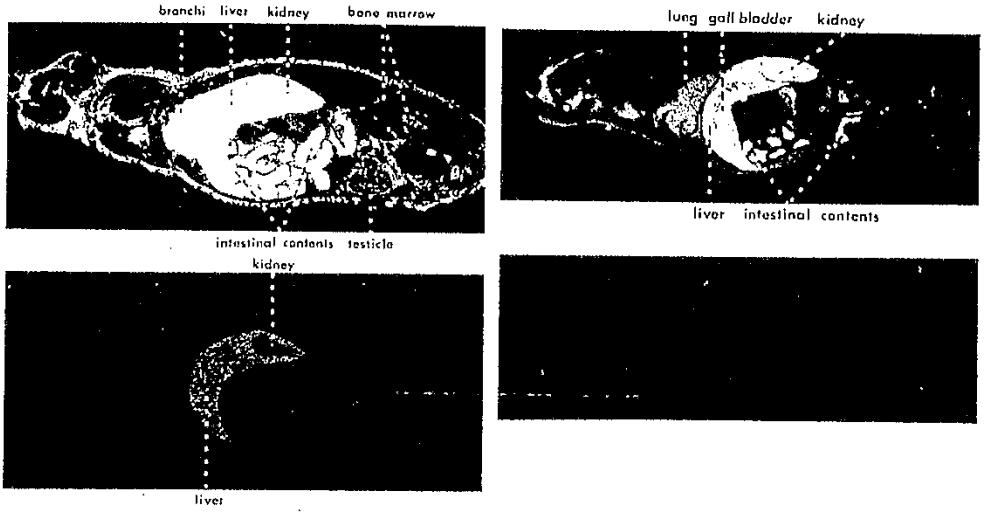
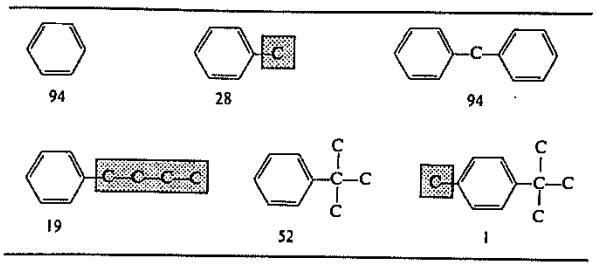


Fig. 4 a-b. Autoradiograms of mice 1 h after inhalation for 10 minutes of 5 µl ^{14}C-benzene (a) and 10 µl ^{14}C-toluene (b). Preparation: dried and evaporated (upper), additionally extracted (lower). Note: benzene metabolites are irreversibly bound in kidney cortex and liver (a); all toluene metabolites are completely extractable (b) After Bergman (ref.10).

The oxidative attack on the benzene ring leads to the formation of an epoxide as toxic reactive intermediate. In toluene the methyl group serves as a safe metabolic handle preferably attacked by the mixed function oxidases leading to benzoic acid as an end product. This principle is further elucidated in fig. 5.

The objection that metabolically stable agents, lipophilic enough to penetrate the central nervous system, would not be eliminated by renal excretion can be rejected for a number of reasons. Centrally active compounds excreted to a large extent unmetabolized exist. Examples are anorectic agents, such as phentermine and derivatives and phenphluramine, which still have relatively short half-life times

Fig. 5. AVOIDANCE OF RISKY AROMATIC RING OXIDATION (PHENOL FORMATION) BY INTRODUCTION OF ALTERNATIVE SAFER METABOLIC HANDLES.



The figures indicate the organic (phenolic) sulphate as a fraction of the total sulphate excretion in the urine (rat). High values imply ring oxidation, low values imply attack along safer metabolic pathway. The low value 1 for the last compound - which implies nearly complete ring oxidation - results from the combination of blockade in the ring (tertiary butyl group) and alternative pathway (methyl group). Based on data from H.W. Gerarde. "Toxicology and Biochemistry of Aromatic Hydrocarbons." Elsevier, Amsterdam 1960 (ref. 15).

in the order of 10 to 30 hours (ref. 16). Metabolically stable agents are not necessarily highly lipophilic. On the other hand, prolonged half-life times in the order of 48 hours or even longer may allow for simple dosage regimens. If so required, the drugs, as far as tertiary amines, mostly weak bases, or weak acids are involved, - many centrally acting drugs belong to these categories - can be driven out by acidifying or alkalinizing the urine by means of, for instance, ammonium chloride or sodium bicarbonate, respectively.

Although our insight in the relationship between structure and metabolic conversion is still scanty, a number of principles applicable in metabolic stabilization or, if required so, metabolic destabilization, have been worked out and proven effective. As such can be mentioned the principle of "packing" of the vulnerable moiety thus sterically or otherwise hindering the enzymatic attack on the group concerned and blocking of vulnerable positions in the drug molecule, for instance by substitution of hydrogen by fluorine or possibly deuterium. For examples the reader is referred to various reviews in the literature (1, 2, 11, 21, 22).

In conclusion, drug metabolism should be regarded as acceptable only if it has a particular, well defined purpose, such as: realization of short or ultrashort action; solely local action under the avoidance of systemic action; prodrug formation aimed at, for instance, facilitation of absorption; avoidance of local irritation; protection against first-pass loss; selective bioactivation, e.g. in the target tissue; increase in water solubility for intravenous application; increase in lipophilicity to obtain depot preparations, etc. These are areas for the "soft drug approach" (ref. 17, 18, 19, 20).

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CHAPTER

VI

Cluster Analysis and the Design of Congener Sets

VI-1 POSSIBILITIES OF MOLECULAR MODIFICATION

As the study of structure-activity relationships develops and especially as work in the biomedicinal chemical field gains momentum, the need to deal with many more variables becomes pressing. From an economic standpoint, the probelm of ruling out irrelevant parameters and focusing on the relevant ones early in a structure-activity study is one of extreme importance. This problem is both crucial and complex and deserves much more systematic attention than has been customarily allotted to it in the past.

It has been pointed out[1] that the number of derivatives that can be made from a set of N substituents where m is the number of nonsymmetric positions on the parent compound is N^m; for example, if one were making derivatives of quinoline using the 166 well-char-

acterized substituents of Table VI-1 in all possible combinations, this would amount to 166^7 or approximately 3.5×10^{15} molecules. Of course, 166 is a small fraction of the almost 2000 substituents in Appendix I. What constitutes a reasonable sample of 10^{15} congeners? Even if we made only one-billionth of the possibilities, it would amount to one million molecules; yet relatively few drug modification programs make as many as a thousand derivatives.

A general formula for calculating the possibilities is

$$X^k \cdot \frac{n!}{k!(n-k)!}$$

In this expression, X is the number of substituents to be considered, n is the total number of nonsymmetric positions on the parent molecule, and k is the number of substituents to be placed on the parent compound at one time. With this formula we can consider simpler and more varied cases. For example, using only 100 substituents from the 2000 of Appendix I and considering only three out of the seven possible positions on quinoline leads to 35,000,000 analogs. With 100 substituents and only two positions, one still has to face 210,000 possibilities. Even considering only 20 substituents, two at a time, means 8400 possibilities. No wonder that "me too" drugs are always being developed.

VI-2 THE COLLINEARITY PROBLEM

Since the cost of modifying a parent structure is so great and since the possibilities are so enormous, one wants to

Table VI-1 Well-Characterized[a] Aromatic Substituents[b]

	π	H-Accpt	H-Donor	MR	$\mathcal{F}$	$\mathcal{R}$	σ_m	σ_p
Br	0.86	0	0	8.88	0.44	−0.17	0.39	0.23
Cl	0.71	0	0	6.03	0.41	−0.15	0.37	0.23
F	0.14	0	0	0.92	0.43	−0.34	0.34	0.06
SO_2F	0.05	1	0	8.65	0.75	0.22	0.80	0.91
SF_5	1.23	0	0	9.89	0.57	0.15	0.61	0.68
I	1.12	0	0	13.94	0.40	−0.19	0.35	0.18
IO_2	−3.46	1	0	63.51	0.63	0.20	0.68	0.78
NO	−1.20	1	0	5.20	0.50	0.45	0.62	0.91
NO_2	−0.28	1	0	7.36	0.67	0.16	0.71	0.78
NNN	0.46	0	0	10.20	0.30	−0.13	0.27	0.15
H	0.00	0	0	1.03	0.00	0.00	0.00	0.00
OH	−0.67	1	1	2.85	0.29	−0.64	0.12	−0.37
SH	0.39	0	1	9.22	0.28	−0.11	0.25	0.15
$B(OH)_2$	−0.55	1	1	11.04	−0.07	0.18	−0.01	0.12
NH_2	−1.23	1	1	5.42	0.02	−0.68	−0.16	−0.66
NHOH	−1.34	1	1	7.22	0.06	−0.40	−0.04	−0.34
SO_2NH_2	−1.82	1	1	12.28	0.41	0.19	0.46	0.57
$NHNH_2$	−0.88	1	1	8.44	0.17	−0.71	−0.02	−0.55
5-Cl-1-Tetrazolyl	−0.65	1	0	23.16	0.58	0.07	0.60	0.61
$N{=}CCl_2$	0.41	0	0	18.35	0.23	−0.08	0.21	0.13
CF_3	0.88	0	0	5.02	0.38	0.19	0.43	0.54
OCF_3	1.04	1	0	7.86	0.38	0.00	0.38	0.35
SO_2CF_3	0.55	1	0	12.86	0.73	0.26	0.79	0.93
SCF_3	1.44	0	0	13.81	0.35	0.18	0.40	0.50
CN	−0.57	1	0	6.33	0.51	0.19	0.56	0.66
NCS	1.15	1	0	17.24	0.51	−0.09	0.48	0.38
SCN	0.41	1	0	13.40	0.36	0.19	0.41	0.52
CO_2^-	−4.36	1	0	6.05	−0.15	0.13	−0.10	0.00
1-Tetrazolyl	−1.04	1	0	18.33	0.52	0.02	0.52	0.50
NHCN	−0.26	1	1	10.14	0.26	−0.18	0.21	0.06
CHO	−0.65	1	0	6.88	0.31	0.13	0.35	0.42
CO_2H	−0.32	1	1	6.93	0.33	0.15	0.37	0.45
CH_2Br	0.79	0	0	13.39	0.10	0.05	0.12	0.14
CH_2Cl	0.17	0	0	10.49	0.10	0.03	0.11	0.12
CH_2I	1.50	0	0	18.60	0.09	0.03	0.10	0.11
NHCHO	−0.98	1	1	10.31	0.25	−0.23	0.19	0.00
$CONH_2$	−1.49	1	1	9.81	0.24	0.14	0.28	0.36
CH=NOH	−0.38	1	1	10.28	0.25	−0.13	0.22	0.10
CH_3	0.56	0	0	5.65	−0.04	−0.13	−0.07	−0.17
$NHCONH_2$	−1.30	1	1	13.72	0.04	−0.28	−0.03	−0.24
$NHC{=}S(NH_2)$	−1.40	1	1	22.19	0.23	−0.05	0.22	0.16
OCH_3	−0.02	1	0	7.87	0.26	−0.51	0.12	−0.27
CH_2OH	−1.03	1	1	7.19	0.00	0.00	0.00	0.00
$SOCH_3$	−1.58	1	0	13.70	0.52	0.01	0.52	0.49
SO_2CH_3	−1.63	1	0	13.49	0.54	0.22	0.60	0.72
OSO_2CH_3	−0.88	1	0	16.99	0.39	0.00	0.39	0.36
SCH_3	0.61	0	0	13.82	0.20	−0.18	0.15	0.00
$SeCH_3$	0.74	0	0	17.03	0.13	−0.12	0.10	0.00
$NHCH_3$	−0.47	1	1	10.33	−0.11	−0.74	−0.30	−0.84
$NHSO_2CH_3$	−1.18	1	1	18.17	0.25	−0.20	0.20	0.03
CF_2CF_3	1.68	0	0	9.23	0.44	0.11	0.47	0.52
C≡CH	0.40	0	0	9.55	0.19	0.05	0.21	0.23
$NHCOCF_3$	0.08	1	1	14.30	0.36	−0.21	0.30	0.12
CH_2CN	−0.57	1	0	10.11	0.21	−0.18	0.16	0.01

Table VI-1 Well-Characterized[a] Aromatic Substituents[b] (Continued)

	π	H-Accpt	H-Donor	MR	$\mathcal{F}$	$\mathcal{R}$	σ_m	σ_p
$CH{=}CHNO_2$-(trans)	0.11	1	0	16.42	0.33	−0.05	0.32	0.26
$CH{=}CH_2$	0.82	0	0	10.99	0.07	−0.08	0.05	−0.02
$NHC{=}O(CH_2Cl)$	−0.50	1	1	19.77	0.23	−0.25	0.17	−0.03
$COCH_3$	−0.55	1	0	11.18	0.32	0.20	0.38	0.50
$SCOCH_3$	0.10	1	0	18.42	0.36	0.11	0.39	0.44
$OCOCH_3$	−0.64	1	0	12.47	0.41	−0.07	0.39	0.31
CO_2CH_3	−0.01	1	0	12.87	0.33	0.15	0.37	0.45
$NHCOCH_3$	−0.97	1	1	14.93	0.28	−0.26	0.21	0.00
$NHCO_2CH_3$*	−0.37	1	1	16.53	0.14	−0.28	0.07	−0.15
$C{=}O(NHCH_3)$	−1.27	1	1	14.57	0.34	0.05	0.35	0.36
$CH{=}NOCH_3$	0.40	1	0	14.93	0.39	−0.06	0.37	0.30
$NHC{=}S(CH_3)$	−0.42	1	1	23.40	0.27	−0.13	0.24	0.12
$CH{=}NNHC{=}S(NH_2)$	−0.27	1	1	29.92	0.46	−0.02	0.45	0.40
CH_2CH_3	1.02	0	0	10.30	−0.05	−0.10	−0.07	−0.15
$CH{=}NNHCONHNH_2$	−1.32	1	1	24.86	0.23	−0.05	0.22	0.16
CH_2OCH_3	−0.78	1	0	12.07	0.01	0.02	0.02	0.03
OCH_2CH_3	0.38	1	0	12.47	0.22	−0.44	0.10	−0.24
SOC_2H_5*	−1.04	1	0	18.35	0.52	0.01	0.52	0.49
SC_2H_5	1.07	0	0	18.42	0.23	−0.18	0.18	0.03
SeC_2H_5*	1.28	0	0	21.68	0.13	−0.12	0.10	0.00
NHC_2H_5	0.08	1	1	14.98	−0.11	−0.51	−0.24	−0.61
$SO_2C_2H_5$*	−1.09	1	0	18.14	0.54	0.22	0.60	0.72
$N(CH_3)_2$	0.18	1	0	15.55	0.10	−0.92	−0.15	−0.83
$NHSO_2C_2H_5$*	−0.64	1	1	22.82	0.25	−0.20	0.20	0.03
$P(CH_3)_2$	0.44	0	0	21.19	−0.08	0.39	0.03	0.31
$PO(OCH_3)_2$	−1.18	1	0	21.87	0.37	0.19	0.42	0.53
$C(OH)(CF_3)_2$	1.28	1	1	15.18	0.28	0.05	0.29	0.30
$CH{=}CHCN$	−0.17	1	0	16.23	0.26	−0.07	0.24	0.17
Cyclopropyl	1.14	0	0	13.53	−0.03	−0.19	−0.07	−0.21
COC_2H_5*	0.06	1	0	15.83	0.32	0.20	0.38	0.50
$SCOC_2H_5$*	0.64	1	0	23.07	0.36	0.11	0.39	0.44
$CO_2C_2H_5$	0.51	1	0	17.47	0.33	0.15	0.37	0.45
$OCOC_2H_5$*	−0.10	1	0	17.12	0.41	−0.07	0.39	0.31
$CH_2CH_2CO_2H$	−0.29	1	1	16.52	−0.02	−0.05	−0.03	−0.07
$NHCO_2C_2H_5$	0.17	1	1	21.18	0.14	−0.28	0.07	−0.15
$CONHC_2H_5$*	−0.73	1	1	19.22	0.34	0.05	0.35	0.36
$NHCOC_2H_5$*	−0.43	1	1	19.58	0.28	−0.26	0.21	0.00
$CH{=}NOC_2H_5$*	0.94	1	0	19.58	0.39	−0.06	0.37	0.30
$NHC{=}S(C_2H_5)$*	0.12	1	1	28.05	0.27	−0.13	0.24	0.12
$CH(CH_3)_2$	1.53	0	0	14.96	−0.05	−0.10	−0.07	−0.15
C_3H_6	1.55	0	0	14.96	−0.06	−0.08	−0.07	−0.13
$NHC{=}S(NHC_2H_5)$	−0.71	1	1	31.66	0.38	−0.28	0.30	0.07
$OCH(CH_3)_2$	0.85	1	0	17.06	0.30	−0.72	0.10	−0.45
OC_3H_7	1.05	1	0	17.06	0.22	−0.45	0.10	−0.25
$CH_2OC_2H_5$*	−0.24	1	0	16.72	0.01	0.02	0.02	0.03
SOC_3H_7*	−0.50	1	0	23.00	0.52	0.01	0.52	0.49
$SO_2C_3H_7$*	−0.55	1	0	22.79	0.54	0.22	0.60	0.72
SC_3H_7*	1.61	0	0	23.07	0 23	−0.18	0.15	0.00
SeC_3H_7*	1.82	0	0	26.33	0.13	−0.12	0.10	0.00
NHC_3H_7*	0.62	1	1	19.63	−0.11	−0.51	−0.24	−0.61
$NHSO_2C_3H_7$*	−0.10	1	1	27.47	0.25	−0.20	0.20	0.03
$N(CH_3)_3$	−5.96	0	0	21.20	0.89	0.00	0.88	0.82
$Si(CH_3)_3$	2.59	0	0	24.96	−0.04	−0.04	−0.04	−0.07
$CH{=}C(CN)_2$	0.05	1	0	21.53	0.58	0.30	0.66	0.84

Table VI-1 Well-Characterized[a] Aromatic Substituents[b] (Continued)

	π	H-Accpt	H-Donor	MR	$\mathcal{F}$	$\mathcal{R}$	σ_m	σ_p
1-Pyrryl	0.95	1	0	21.85	0.50	-0.09	0.47	0.37
2-Thienyl	1.61	0	0	24.04	0.10	0.04	0.09	0.05
3-Thienyl	1.81	0	0	24.04	0.04	-0.06	0.03	-0.02
$CH{=}CHCOCH_3$	-0.06	1	0	21.10	0.28	-0.27	0.21	-0.01
$CH{=}CHCO_2CH_3$*	0.32	1	0	22.56	0.24	-0.19	0.19	0.03
COC_3H_7*	0.53	1	0	20.48	0.32	0.20	0.38	0.50
$SCOC_3H_7$*	1.18	1	0	27.72	0.36	0.11	0.39	0.44
$OCOC_3H_7$*	0.44	1	0	21.77	0.41	-0.07	0.39	0.31
$CO_2C_3H_7$*	1.07	1	0	22.17	0.33	0.15	0.37	0.45
$(CH_2)_3CO_2H$*	0.25	1	1	21.17	-0.02	-0.05	-0.03	-0.07
$CONHC_3H_7$*	-0.19	1	1	23.87	0.34	-0.05	0.35	0.36
$NHCOC_3H_7$*	0.11	1	1	24.23	0.28	-0.26	0.21	0.00
$NHC{=}OCH(CH_3)_2$	-0.18	1	1	24.23	0.18	-0.26	0.11	-0.10
$NHCO_2C_3H_7$*	0.71	1	1	25.83	0.14	-0.28	0.07	-0.15
$CH{=}NOC_3H_7$*	1.48	1	0	24.23	0.39	-0.06	0.37	0.30
$NHC{=}S(C_3H_7)$*	0.66	1	1	32.70	0.27	-0.13	0.24	0.12
C_4H_9	2.13	0	0	19.61	-0.06	-0.11	-0.08	-0.16
$C(CH_3)_3$	1.98	0	0	19.62	-0.07	-0.13	-0.10	-0.20
OC_4H_9	1.55	1	0	21.66	0.25	-0.55	0.10	-0.32
$CH_2OC_3H_7$*	0.30	1	0	21.37	0.01	0.02	0.02	0.03
$N(C_2H_5)_2$	1.18	1	0	24.85	0.01	-0.91	-0.23	-0.90
NHC_4H_9*	1.16	1	1	24.26	-0.28	-0.25	-0.34	-0.51
$P(C_2H_5)_2$*	1.52	0	0	30.49	-0.08	0.39	0.03	0.31
$PO(OC_2H_5)_2$*	-0.10	1	0	31.16	0.37	0.19	0.42	0.53
$CH_2Si(CH_3)_3$	2.00	0	0	29.61	-0.15	-0.07	-0.16	-0.21
$CH{=}CHCOC_2H_5$*	0.48	1	0	25.75	0.28	-0.27	0.21	-0.01
$CH{=}CHCO_2C_2H_5$	0.86	1	0	27.21	0.24	-0.19	0.19	0.03
$CH{=}NOC_4H_9$*	2.02	1	0	28.88	0.39	-0.06	0.37	0.30
C_5H_{11}*	2.67	0	0	24.26	-0.06	-0.08	-0.08	-0.16
$CH_2OC_4H_9$*	0.84	1	0	26.02	0.01	0.02	0.02	0.03
C_6H_5	1.96	0	0	25.36	0.08	-0.08	0.06	-0.01
$N{=}NC_6H_5$	1.69	0	0	31.31	0.28	0.13	0.32	0.39
OC_6H_5	2.08	1	0	27.68	0.34	-0.35	0.25	-0.03
$SO_2C_6H_5$	0.27	1	0	33.20	0.56	0.18	0.61	0.70
$OSO_2C_6H_5$	0.93	1	0	36.70	0.36	0.00	0.36	0.33
NHC_6H_5	1.37	1	1	30.04	-0.02	-0.38	-0.12	-0.40
$NHSO_2C_6H_5$	0.45	1	1	37.88	0.21	-0.18	0.16	0.01
2,5-di-Me-1-pyrryl	1.95	1	0	31.15	0.52	-0.10	0.49	0.38
$CH{=}CHCOC_3H_7$*	1.02	1	0	30.40	0.28	-0.27	0.21	-0.01
$CH{=}CHCO_2C_3H_7$*	1.40	1	0	31.86	0.24	-0.19	0.19	0.03
Cyclohexyl	2.51	0	0	26.69	-0.13	-0.10	-0.15	-0.22
2-Benzthiazolyl	2.13	1	0	38.88	0.25	0.06	0.27	0.29
COC_6H_5	1.05	1	0	30.33	0.30	0.16	0.34	0.43
$CO_2C_6H_5$	1.46	1	0	32.31	0.33	0.13	0.37	0.44
$OCOC_6H_5$	1.46	1	0	32.33	0.23	-0.08	0.21	0.13
$N{=}CHC_6H_5$	-0.29	1	0	33.01	0.09	-0.63	-0.08	-0.55
$CH{=}NC_6H_5$	-0.29	1	0	33.01	0.31	0.13	0.35	0.42
$NHCOC_6H_5$	0.49	1	1	34.64	0.09	-0.27	0.02	-0.19
$CH_2C_6H_5$	2.01	0	0	30.01	-0.08	-0.01	-0.08	-0.09
$CH_2OC_6H_5$	1.66	1	0	32.19	0.02	0.02	0.03	0.04
$C{\equiv}CC_6H_5$	2.65	0	0	33.21	0.12	0.05	0.14	0.16
$CH{=}NNHCOC_6H_5$	0.43	1	1	42.37	0.33	0.20	0.39	0.51
$CH_2Si(C_2H_5)_3$*	3.26	0	0	43.56	-0 15	-0.07	-0.16	-0.21
$CH{=}CHC_6H_5$-(trans)	2.68	0	0	34.17	0.06	-0.12	0.03	-0.07

Table VI-1 Well-Characterized[a] Aromatic Substituents[b] (Continued)

	π	H-Accpt	H-Donor	MR	$\mathcal{F}$	$\mathcal{R}$	σ_m	σ_p
$CH{=}CHCOC_6H_5$	0.95	1	0	40.25	0.22	−0.15	0.18	0.05
Ferrocenyl	2.46	0	0	48.24	−0.15	−0.04	−0.15	−0.18
$N(C_6H_5)_2$	3.61	1	0	54.96	0.07	−0.29	0.00	−0.22
$P{=}O(C_6H_5)_2$	0.70	1	0	59.29	0.31	0.24	0.38	0.53

[a]By well-characterized, we mean that the set of eight constants is known for each substituent; we do not mean to imply that all of the constants are of the highest accuracy.
[b]Substituents are ordered first by number of C, then by number of H, and the remaining elements alphabetically.

Table VI-2 Well-Characterized[a] Aliphatic Substituents

	Fr	H-Accpt	H-Donor	MR	$\mathcal{F}$
Br	0.20	0	0	8.80	0.44
Cl	0.06	0	0	5.93	0.41
F	−0.38	0	0	1.05	0.43
I	0.59	0	0	13.76	0.40
NO_2	−1.16	1	0	6.71	0.67
H	0.23	0	0	1.03	0.00
OH	−1.64	1	1	2.55	0.29
SH	−0.23	0	1	8.76	0.28
NH_2	−1.54	1	1	4.37	0.02
CBr_3	2.03	0	0	28.81	0.27
CCl_3	1.61	0	0	20.12	0.31
CF_3	0.29	0	0	5.02	0.38
CN	−1.27	1	0	5.39	0.51
SCN	−0.48	1	0	13.40	0.36
CO_2^-	−5.19	1	0	5.15	−0.15
CO_2H	−1.11	1	1	6.03	0.33
CH_2Br	0.74	0	0	13.39	0.10
CH_2Cl	0.60	0	0	10.49	0.10
CH_2I	1.13	0	0	18.60	0.09
$CONH_2$	−2.18	1	1	9.81	0.24
$CH{=}NOH$	−1.02	1	1	10.28	0.25
CH_3	0.77	0	0	5.65	−0.04
$NHCONH_2$	−2.90	1	1	13.72	0.04
OCH_3	−1.54	1	0	7.33	0.26
CH_2OH	−1.10	1	1	7.19	0.00
$SOCH_3$	−2.24	1	0	13.70	0.52
OSO_2CH_3	−1.34	1	0	16.99	0.39
SCH_3	−0.02	0	0	13.33	0.20
$NHCH_3$	−1.38	1	1	9.11	−0.11
CF_2CF_3	1.34	0	0	9.23	0.44
$C{\equiv}CH$	0.01	0	1	8.25	0.19
CH_2CN	−0.73	1	0	10.11	0.21
$CH{=}CHNO_2$-trans	−0.63	1	0	16.42	0.33
$CH{=}CH_2$	0.88	0	0	9.79	0.07
$COCH_3$	−1.13	1	0	10.29	0.32
$OCOCH_3$	−0.72	1	0	11.85	0.41
CO_2CH_3	−0.72	1	0	11.85	0.33
$NHCOCH_3$	−1.94	1	1	13.71	0.28
$C{=}O(NHCH_3)$	−1.94	1	1	13.39	0.34

Table VI-2 Well-Characterized[a] Aliphatic Substituents (Continued)

	Fr	H-Accpt	H-Donor	*MR*	π
CH_2CH_3	1.43	0	0	10.30	-0.05
OCH_2CH_3	-0.51	1	0	11.93	0.22
CH_2OCH_3	-0.23	1	0	12.07	0.01
SOC_2H_5*	-1.70	1	0	18.35	0.52
SC_2H_5	0.52	0	0	17.93	0.23
$CH_2Si(CH_3)_3$	3.62	0	0	29.61	-0.15
NHC_2H_5	-0.84	1	1	13.76	-0.11
$N(CH_3)_2$	-0.64	1	0	14.14	0.10
CH=CHCN	-0.74	1	0	15.33	0.26
Cyclopropyl	1.49	0	0	13.53	-0.03
COC_2H_5*	-0.59	1	0	14.65	0.32
$CO_2C_2H_5$	-0.18	1	0	16.76	0.33
$OCOC_2H_5$*	-0.18	1	0	17.12	0.41
$EtCO_2H$	-0.03	1	1	16.52	-0.02
$NHCO_2C_2H_5$	-1.40	1	1	19.96	0.14
$CONHC_2H_5$*	-1.40	1	1	18.04	0.34
$NHCOC_2H_5$*	-1.40	1	1	18.36	0.28
$CH(CH_3)_2$	1.84	0	0	14.96	-0.05
C_3H_7	1.97	0	0	14.96	-0.06
$OCH(CH_3)_2$	-0.10	1	0	16.52	0.30
OC_3H_7	0.03	1	0	16.52	0.22
$CH_2OC_2H_5$*	0.03	1	0	16.72	0.01
SOC_3H_7*	-1.16	1	0	23.00	0.52
SC_3H_7*	1.06	0	0	22.58	0.23
NHC_3H_7*	-0.30	1	1	18.41	-0.11
$Si(CH_3)_3$	2.96	0	0	24.96	-0.04
2-Thienyl	1.58	0	0	24.04	0.10
3-Thienyl	1.58	0	0	24.04	0.04
$CH=CHOCH_3$	-0.13	1	0	19.92	0.28
$CH=CHCO_2CH_3$*	0.28	1	0	21.85	0.24
COC_3H_7*	-0.05	1	0	19.30	0.32
$OCOC_3H_7$*	0.36	1	0	21.77	0.41
$CO_2C_3H_7$*	0.36	1	0	21.46	0.33
$(CH_2)_3CO_2H$*	0.51	1	1	21.17	-0.02
$NHCOC_3H_7$*	-0.86	1	1	23.01	0.28
$CONHC_3H_7$*	-0.86	1	1	22.69	0.34
C_4H_9	2.51	0	0	19.61	-0.06
$C(CH_3)_3$	2.22	0	0	19.62	-0.07
OC_4H_9	0.57	1	0	21.12	0.25
$CH_2OC_3H_7$*	0.57	1	0	21.37	0.01
NHC_4H_9*	0.24	1	1	23.06	-0.28
$N(C_2H_5)_2$	0.16	1	0	23.44	0.01
$CH=CHCOC_2H_5$*	0.41	1	0	24.57	0.28
$CH=CHCO_2C_2H_5$	0.82	1	0	26.03	0.24
C_5H_{11}*	3.10	0	0	24.26	-0.06
$CH_2OC_4H_9$*	1.11	1	0	26.02	0.01
C_6H_5	1.90	0	0	25.36	0.08
OC_6H_5	1.22	1	0	27.02	0.34
$SO_2C_6H_5$	-0.39	1	0	33.20	0.56
NHC_6H_5	0.75	1	1	28.50	-0.02
2-Benzthiazolyl	1.78	1	0	38.88	0.25
$CH=CHCOC_3H_7$*	0.95	1	0	29.22	0.28
$CH=CHCO_2C_3H_7$*	1.36	1	0	26.50	0.24
COC_6H_5	0.69	1	0	29.96	0.30
$CO_2C_6H_5$	0.60	1	0	31.60	0.33

Table VI-2 Well-Characterized[a] Aliphatic Substituents (Continued)

	Fr	H-Accpt	H-Donor	*MR*	$\mathcal{F}$
$OCOC_6H_5$	1.22	1	0	32.33	0.23
$NHCOC_6H_5$	-0.03	1	1	34.28	0.09
$CH_2C_6H_5$	2.44	0	0	30.01	-0.08
$CH_2OC_6H_5$	1.71	1	0	31.77	0.02
$CH_2Si(C_2H_5)_3$*	4.82	0	0	43.56	-0.15
$CH{=}CHC_6H_5$-(trans)	2.72	0	0	32.97	0.06
$CH{=}CHCOC_6H_5$	1.81	1	0	39.05	0.22
Ferrocenyl	2.43	0	0	48.24	-0.15
$N(C_6H_5)_2$	2.43	1	0	53.55	0.07

[a] See footnote *a*, Table VI-1.

gain the maximum amount of information possible from each derivative that is to be tested in some standard system. This means that at any given point in time, one wants to consider all of the known variables that cause change in activity of the parent molecule when a substituent change is made. For quantitative work, we are limited to those variables that can be defined in numerical terms. Four such parameters, π, *MR*, $\mathcal{F}$ and $\mathcal{R}$, are well characterized and have been shown to be relevant in many biomedicinal QSAR. The problem of selecting a set of substituents that would be independent with respect to these four variables is illustrated in Tables VI-3 and VI-4.[2]

Table VI-3 Two Sets of Substituents for Compound Modification

Set A		Set B	
CH_3	NHC_6H_5	CH_3	CH_2CH_3
NO_2	$OCH_2CH_2CH_3$	CF_3	$NHCOCH_3$
$COCH_3$	$SO_2CH_2CH_2CH_3$	F	$CONH_2$
$C{\equiv}CH$	I	CN	SO_2NH_2
SCH_3	CH_2Cl	NO_2	OCF_3

Table VI-4 Squared Correlation Matrices for Substituents of Table VI-3[a]

	Set A					Set B			
	π	$\mathcal{R}$	$\mathcal{F}$	*MR*		π	$\mathcal{R}$	$\mathcal{F}$	*MR*
π	1	0.94	0.30	0.32	π	1	0.09	0.08	0.26
$\mathcal{R}$		1	0.32	0.38	$\mathcal{R}$		1	0.15	0.03
$\mathcal{F}$			1	0.09	$\mathcal{F}$			1	0.03
MR				1	*MR*				1

[a] The figures are r^2 for the correlation between variables.

From an inspection of these two sets, even knowing the values of π, $\mathcal{F}$, $\mathcal{R}$, and *MR*, it is not possible to decide which is the better set as far as independence among the four variables is concerned. However, it can easily be done by formulating the correlation matrices for the two sets of substituents as in Table VI-4. One sees immediately from the correlation matrix that π and $\mathcal{R}$ are almost completely collinear in Set A; either of these two vectors would give almost the same result in a correlation equation. Hence, one cannot be sure whether the correct variable is π or $\mathcal{R}$ or if indeed both variables are involved. All of the other variables except $\mathcal{F}$ and *MR* show significant collinearity. Selecting data Set A would mean that one would have to make more derivatives if either π or $\mathcal{R}$ turned up in the correlation equation to resolve their relative importance. Only π and *MR* show significant collinearity in data Set B, and this is not serious for most purposes. Although 10 substituents would not be enough to work in a system where four variables are influencing a given process, they do illustrate the collinearity problem.

Since only a tiny fraction of the almost infinite number of possibilities can be studied in drug modification, we cannot afford redundancy. Testing two congeners that have essentially the same physicochemical properties is most likely to be less valuable than testing two with different properties. One often sees sets of congeners in the literature where all of the normal alkyl groups from methyl to decyl have been made and tested. While this *may* give one useful information on optimum lipophilicity, it also may not. The variables π and *MR* for such substituents are prefectly collinear so that hydrophobicity and bulk tolerance effects cannot be resolved with such a set of congeners. Of course, such a data set would reveal nothing about the electronic effect of substituents.

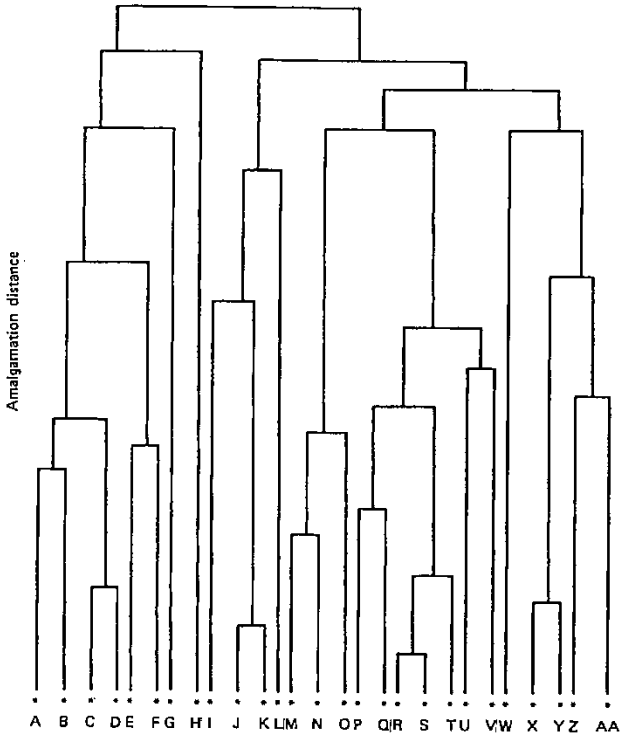

Figure VI-1

VI-3 CLUSTER ANALYSIS

The problem of selecting a set of substituents with independence among several parameters has concerned medicinal chemists for some time. Meyer and Hemmi[3] pointed out in 1935 that one could not gain information about the relative role of physicochemical properties of narcotics from the study of sets of homologous series. The alkyl groups are completely collinear with respect to *many* parameters.

Craig[4] first emphasized the importance of plotting π vs σ to obtain a set of substituents with minimal collinearity and, at the same time, good coverage of substituent space. Wooton et al.[5] have developed a sophisticated algorithm for substituent selection.

Hierarchical clustering can greatly assist in the proper selection of substituents from the ever-growing number available. Most computer centers have available the UCLA biomed BMDP2M, BMDP1M, or the Xerox Data Systems CLUSANL programs. The clustering in this chapter has been done with the Xerox program.

In this program, the parameters X' can be placed on the same scale via eq VI-1. $\bar{X}$ in this expression is the mean value of a given parameter and S_i is the standard

$$X_{ik} = \frac{X'_{ik} - \bar{X}_i}{S_i} \qquad \text{(VI-1)}$$

deviation. This equation defines the deviation of each point from the group average $\bar{X}_i$ in units of the standard deviation. If we have N substituents, each with K parameters, then the Euclidian distance between them is given by

$$d_{ij} = \left[\sum_{k=1}^{K} (X'_{ik} - X'_{jk})^2\right]^{1/2} \quad i, j = 1, 2 \ldots N \qquad \text{(VI-2)}$$

In hierarchical clustering, all interpoint distances in K space are calculated via eq VI-2, and the two closest points are clustered into a pseudo point (see Figure VI-1). In Figure VI-1, R and S are the two points closest to each other; the pseudo point is formed from these

two and is then clustered with T; this group of three is then clustered with P and Q. Note that H is so isolated in data space that it does not enter a cluster until the penultimate group. At the bottom of the graph all points are distinct units in data space; at the top, all have been forced into a single cluster.

The composition of the clusters that one obtains depends entirely on the parameters that are used in eq VI-2 to obtain the d_{ij}. If one uses parameters not pertinent to the data set for which substituents are being selected, this will not result in a well-balanced group of congeners on which to base the QSAR. For this reason, in the case of substituents for use in an aromatic system we have clustered only on $\pi, \mathcal{F}, \mathcal{R}, MR$, and H-bonding; these are established variables that can be shown to be reasonably independent. The parameters σ_m and σ_p have been shown to be strongly related.[2] This can be seen from the correlation matrix of Table VI-9 for all 166 aromatic substituents.

If steric effects are involved, it is assumed that MR will *approximate* the steric properties of substituents.[1] Since at the present time we do not have a set of hydrogen-bonding parameters, we have assigned hydrogen bond acceptors a value of 1, hydrogen bond donors a value of 1 (at present there are no substituents acting only as donors), and other substituents a value of 0. We believe that the inclusion of hydrogen bonding gives better balanced sets of clusters.

The aliphatic constants of Table VI-2 have been clustered on the same variables (Table VI-6), except that $\mathcal{R}$ has not been included. These constants refer to systems where resonance is not present.

The 166 "aromatic" substituents of Table VI-1 have been forced into three sets of clusters containing 20, 10, and 5 substituents, respectively. Note that in the "20" set the clusters vary in size from 2 to 16 members. Those substituents closest to each other in six-dimensional space are forced into clusters; hence, when choosing a set of substituents, one should, insofar as possible, select one substituent from each cluster. Some substituents turn out to be rather strange from the point of view of synthesis, metabolic stability, or chemical reactivity; for this reason, one may need to take two substituents from the same cluster.

When one forces 166 substituents into 20 clusters, it means of necessity that the substituents in a particular set may not appear similar from the traditional viewpoint of organic chemistry. For example, in the "5" cluster set of Table VI-5 we find H and cyclopropyl together. If, instead of forcing 166 substituents into five clusters, we had compressed them to a lesser degree into 60, then a single cluster with H and Me would result; the similarity is obviously much closer. At the "60" cluster level, 15 substituents are so far removed from the others in data space that they "cluster" alone [e.g., F, IO_2, CO_2^-, $\overset{+}{N}(CH_3)_3$].

One does not assume by selecting one substituent from each group that a set of substituents perfectly orthogonal with respect to each vector will be obtained. The next imperative step is to form a correlation matrix as in Table VI-4. The collinearity between two variables will often be unacceptably high; a plot of the values of one variable against the other helps one choose new substituents to break up this feature.

For example, a chemist selects a substituent from each cluster in the "10" cluster set of aromatic substituents giving the following groups: Br, NO_2, $\overset{+}{N}(CH_3)_3$, OH, SO_2NH_2, OCF_3, NCS, $NHCOC_6H_5$, OCH_3, C_4H_9. The correlation matrix is given in Table VI-7. This is not a satisfactory set because of the high collinearity between π and $\mathcal{F}$ and between H-acceptor and MR.

Discussing this set with medicinal chemists brings out a number of further objections. The NO_2 group is so readily reduced in biological systems that it would be more appropriate to substitute it with CN from the same cluster. The $\overset{+}{N}(CH_3)_3$ group bears a charge. It is well known from studies in physical organic chemistry that charged substituents behave poorly when mixed in sets of neutral groups. In the initial phases of study, one would not expect the charged group to behave in the same fashion as neutral substituents. Several such groups could be introduced later via an indicator variable. The only other substituent in this cluster is IO_2, which is not selected because little is known of its behavior in biologic systems. $\overset{+}{N}(CH_3)_3$ is replaced with H from the first and largest cluster. The NCS group is so highly active chemically that it might not behave as a true congener and is replaced with OC_6H_5. The new set is then: Br, H, CN, OH, SO_2NH_2, OCF_3, OC_6H_5, $NHCOC_6H_5$, OCH_3, C_4H_9. This affords the correlation matrix of Table VI-8.

Table VI-8 shows that although there is still some collinearity between certain variables, it has been reduced to a reasonable level. Also, the new set of substituents contains a good spread in values of the various parameters: π (−1.82 to 2.13), $\mathcal{F}$(−0.06 to 0.44, $\mathcal{R}$ (−0.64 to 0.19), and MR (0.10 to 3.46).

The hydrogen bonding parameters are of course the most poorly defined. Nevertheless, inspection of the clustering of the aromatic substituents at the 10 set level shows that they do cluster hydrogen-bonding groups well. Most of the nonhydrogen bonders are clustered into groups 1 and 8. The acceptors fall into clusters 2, 6, and 7. The substituents that are both donors and

Table VI-5a Aromatic Constants–Twenty Cluster Sets

1	10 members = Br	Cl	I	NNN	F
	SH	SF_5	CF_3	SCF_3	CF_2CF_3
2	17 members = SO_2F	NO_2	$SO_2(CF_3)$	NO	CN
	SO_2CH_3	5-Cl-1-tetrazolyl	SOC_3H_7*	$SO_2C_2H_5$*	$SO_2C_3H_7$*
	$CH{=}C(CN)_2$	$PO(OCH_3)_2$	1-Tetrazolyl	SOC_2H_5*	$SOCH_3$
	OSO_2CH_3	$OCOCH_3$			
3	16 members = H	CH_3	CH_2Br	$CH{=}CH_2$	CH_2Cl
	C≡CH	CH_2CH_3	Cyclopropyl	$HC(CH_3)_2$	C_3H_7
	$N{=}CCl_2$	SCH_3	$Se(CH_3)$	$S(C_2H_5)$	SC_3H_7*
	$Se(C_2H_5)$*				
4	3 members = OH	NH_2	$NHNH_2$		
5	4 members = $B(OH)_2$	CH_2OH	$EtCO_2H$	$(CH_2)_3CO_2H$*	
6	8 members = NH(OH)	$NHCONH_2$	NHCN	CH=NOH	$NHCOCF_3$
	NHCHO	$NHSO_2CH_3$	$NHCOCH_3$		
7	5 members = $SO_2(NH_2)$	$CONH_2$	$C{=}O(NHCH_3)$	$CONHC_2H_5$*	CO_2H
8	16 members = OCF_3	CH_2CN	$CH{=}CHNO_2$ (tr)	$CH{=}NOCH_3$	$OCOC_2H_5$*
	CH=CHCN	SCN	CO_2CH_3	COC_2H_5*	CHO
	$COCH_3$	$SCOCH_3$	$CO_2C_2H_5$	COC_3H_7*	$SCOC_2H_5$*
	$CO_2C_3H_7$*				
9	15 members = NCS	Pyrryl	$CH{=}NOC_2H_5$*	$OCOC_3H_7$*	$CH{=}NOC_3H_7$*
	$CH{=}NOC_4H_9$*	2,5-di-Me-Pyrryl*	$CH{=}CHCOCH_3$	$CH{=}CHCO_2CH_3$*	$CH{=}CHCOC_2H_5$*
	$CH{=}CHCO_2C_2H_5$	$CH{=}CHCOC_3H_7$*	$CH{=}CHCO_2C_3H_7$*	$OCOC_6H_5$	OC_6H_5
10	15 members = CH_2I	2-Thienyl	$Se(C_3H_7)$*	3-Thienyl	C_6H_5
	$N{=}NC_6H_5$	$C{\equiv}CC_6H_5$	$CH{=}CHC_6H_5$ (tr)	$Si(CH_3)_3$	C_5H_{11}*
	Cyclohexyl	$CH_2Si(CH_3)_3$	$CH_2C_6H_5$	C_4H_9	$C(CH_3)_3$
11	21 members = $NHC{=}S(NH_2)$	$CH{=}NNHCONHNH_2$	$CH{=}NNHC{=}S(NH_2)$	$CONHC_3H_7$*	$NHC{=}S(NHC_2H_5)$
	$NHC{=}O(CH_2Cl)$	$NHCOC_2H_5$*	$NHC{=}S(CH_3)$	$NHSO_2C_2H_5$*	$NHCO_2CH_3$*
	$NHCO_2C_2H_5$	$NHC{=}OCH(CH_3)_2$	$NHCO_2C_3H_7$*	$NHC{=}S(C_2H_5)$*	$NHSO_2C_3H_7$*
	$NHCOC_3H_7$*	$C(OH)(CF_3)_2$	$NHC{=}S(C_3H_7)$*	$NHSO_2C_6H_5$	$NHCOC_6H_5$
	$CH{=}NNHCOC_6H_5$				
12	8 members = OCH_3	OCH_2CH_3	$OCH(CH_3)_2$*	OC_3H_7	OC_4H_9*
	$N(CH_3)_2$	$N(C_2H_5)_2$	$N{=}CHC_6H_5$		
13	5 members = $NHCH_3$	$NH(C_2H_5)$	NHC_3H_7*	NHC_4H_9*	NHC_6H_5
14	5 members = CH_2OCH_3	$CH_2OC_2H_5$*	$CH_2OC_3H_7$*	$CH_2OC_4H_9$*	$CH_2OC_6H_5$
15	2 members = $P(CH_3)_2$	$P(C_2H_5)_2$*			
16	10 members = $SCOC_3H_7$*	COC_6H_5	$CO_2C_6H_5$	$OSO_2C_6H_5$	$CH{=}CHCOC_6H_5$
	2-Benzthiazolyl	$PO(OC_2H_5)_2$*	$CH{=}NC_6H_5$	$SO_2C_6H_5$	$P{=}O(C_6H_5)_2$
17	3 members = $CH_2Si(C_2H_5)_3$*	Ferrocenyl	$N(C_6H_5)_2$		
18	3 single-				
1	member = IO_2	CO_2^-	$\overset{+}{N}(CH_3)_3$		
20	groups				

Table VI-5b Aromatic Constants--Ten Cluster Sets

1	26 members = Br	Cl	I	NNN	F
	SH	SF_5	CF_3	SCF_3	CF_2CF_3
	H	CH_3	CH_2Br	$CH{=}CH_2$	CH_2Cl
	$C{\equiv}CH$	CH_2CH_3	Cyclopropyl	$CH(CH_3)_2$	C_3H_7
	$N{=}CCl_2$	SCH_3	$Se(CH_3)$	$S(C_2H_5)$	SC_3H_7*
	$Se(C_2H_5)$*				
2	17 members = SO_2F	NO_2	$SO_2(CF_3)$	NO	CN
	SO_2CH_3	5-Cl-1-tetrazolyl	SOC_3H_7*	$SO_2C_2H_5$*	$SO_2C_3H_7$*
	$CH{=}C(CN)_2$	$PO(OCH_3)_2$	1-Tetrazolyl	SOC_2H_5*	$SOCH_3$
	OSO_2CH_3	$\overset{-}{O}C\overset{+}{O}CH_3$			
3	2 members = IO_2	$\overset{+}{N}(CH_3)_3$			
4	8 members = OH	NH_2	$NHNH_2$	$NHCH_3$	$NH(C_2H_5)$
	NHC_3H_7*	NHC_4H_9*	NHC_6H_5		
5	18 members = $B(OH)_2$	CH_2OH	$EtCO_2H$	$(CH_2)_3CO_2H$*	NH(OH)
	$NHCONH_2$	NHCN	CH=NOH	$NHCOCF_3$	NHCHO
	$NHSO_2CH_3$	$NHCOCH_3$	$SO_2(NH_2)$	$CONH_2$	$C{=}O(NHCH_3)$
	$CONHC_2H_5$*	CO_2H	CO_2^-		
6	21 members = OCF_3	CH_2CN	$CH{=}CHNO_2$ (tr)	$CH{=}NOCH_3$	$OCOC_2H_5$*
	$CH{=}CHCN$	SCN	CO_2CH_3	COC_2H_5*	CHO
	$COCH_3$	$SCOCH_3$	$CO_2C_2H_5$	COC_3H_7*	$SCOC_2H_5$*
	$CO_2C_3H_7$*	CH_2OCH_3	$CH_2OC_2H_5$*	$CH_2OC_3H_7$	$CH_2OC_4H_9$*
	$CH_2OC_6H_5$				
7	25 members = NCS	Pyrryl	$CH{=}NOC_2H_5$*	$OCOC_3H_7$*	$CH{=}NOC_3H_7$*
	$CH{=}NOC_4H_9$*	2,5-di-Me-Pyrryl*	$CH{=}CHOCH_3$	$CH{=}CHO_2CH_3$*	$CH{=}CHCOC_2H_5$*
	$CH{=}CHCO_2C_2H_5$	$CH{=}CHCOC_3H_7$*	$CH{=}CHCO_2C_3H_7$*	$OCOC_6H_5$	OC_6H_5
	$SCOC_3H_7$*	COC_6H_5	$CO_2C_6H_5$	$OSO_2C_6H_5$	$CH{=}CHCOC_6H_5$
	2-Benzthiazolyl	$PO(OC_2H_5)_2$*	$CH{=}NC_6H_5$	$SO_2C_6H_5$	$P{=}O(C_6H_5)_2$
8	20 members = CH_2I	2-Thienyl	$Se(C_3H_7)$*	3-Thienyl	C_6H_5
	$N{=}NC_6H_5$	$C{\equiv}CC_6H_5$	$CH{=}CHC_6H_5$ (tr)	$Si(CH_3)_3$	C_5H_{11}*
	Cyclohexyl	$CH_2Si(CH_3)_3$	$CH_2C_6H_5$	C_4H_9	$C(CH_3)_3$
	$P(CH_3)_2$	$P(C_2H_5)_2$*	$CH_2Si(C_2H_5)_3$*	Ferrocenyl	$N(C_6H_5)_2$
9	21 members = $NHC{=}S(NH_2)$	$CH{=}NNHCONHNH_2$	$CN{=}NNHC{=}S(NH_2)$	$CONHC_3H_7$*	$NHC{=}S(NHC_2H_5)$
	$NHC{=}O(CH_2Cl)$	$NHCOC_2H_5$*	$NHC{=}S(CH_3)$	$NHSO_2C_2H_5$*	$NHCO_2CH_3$*
	$NHCO_2C_2H_5$	$NHC{=}OCH(CH_3)_2$	$NHCO_2C_3H_7$*	$NHC{=}S(C_2H_5)$*	$NHSO_2C_3H_7$*
	$NHCOC_3H_7$*	$C(OH)(CF_3)_2$	$NHC{=}S(C_3H_7)$*	$NHSO_2C_6H_5$	$NHCOC_6H_5$
	$CH{=}NNHCOC_6H_5$				
10	8 members = OCH_3	OCH_2CH_3	$OCH(CH_3)_2$*	OC_3H_7	OC_4H_9*
	$N(CH_3)_2$	$N(C_2H_5)_2$	$N{=}CHC_6H_5$		

Table VI-5c Aromatic Constants–Five Cluster Sets

1 26 members =	Br	Cl	I	NNN	F
	SH	SF_5	CF_3	SCF_3	CF_2CF_3
	H	CH_3	CH_2Br	$CH{=}CH_2$	CH_2Cl
	$C{\equiv}CH$	CH_2CH_3	Cyclopropyl	$CH(CH_3)_2$	C_3H_7
	$N{=}CCl_2$	SCH_3	$Se(CH_3)$	$S(C_2H_5)$	SC_3H_7*
	$Se(C_2H_5)$*				
2 65 members =	SO_2F	NO_2	$SO_2(CF_3)$	NO	CN
	SO_2CH_3	5-Cl-1-Tetrazolyl	SOC_3H_7*	$SO_2C_2H_5$*	$SO_2C_3H_7$*
	$CH{=}C(CN)_2$	$PO(OCH_3)_2$	1-Tetrazolyl	SOC_2H_5*	$SOCH_3$
	OSO_2CH_3	$OCOCH_3$	OCF_3	CH_2CN	$CH{=}CHNO_2$ (tr)
	$CH{=}NOCH_3$	$OCOC_2H_5$*	$CH{=}CHCN$	SCN	CO_2CH_3
	COC_2H_5*	CHO	$COCH_3$	$SCOCH_3$	$CO_2C_2H_5$
	COC_3H_7*	$SCOC_2H_5$*	$CO_2C_3H_7$*	CH_2OCH_3	$CH_2OC_2H_5$*
	$CH_2OC_3H_7$*	$CH_2OC_4H_9$*	$CH_2OC_6H_5$	IO_2	$N(CH_3)_3$
	NCS	Pyrryl	$CH{=}NOC_2H_5$*	$OCOC_3H_7$*	$CH{=}NOC_3H_7$*
	$CH{=}NOC_4H_9$*	2,5-di-Me-Pyrryl*	$CH{=}CHCOCH_3$	$CH{=}CHCO_2CH_3$*	$CH{=}CHCOC_2H_5$*
	$CH{=}CHCO_2C_2H_5$	$CH{=}CHCOC_3H_7$*	$CH{=}CHCO_2C_3H_7$*	$OCOC_6H_5$	OC_6H_5
	$SCOC_3H_7$*	COC_6H_5	$CO_2C_6H_5$	$OSO_2C_6H_5$	$CH{=}CHCOC_6H_5$
	2-Benzthiazolyl	$PO(OC_2H_5)_2$*	$CH{=}NC_6H_5$	$SO_2C_6H_5$	$P{=}O(C_6H_5)_2$
3 16 members =	OH	NH_2	$NHNH_2$	$NHCH_3$	$NH(C_2H_5)$
	NHC_3H_7*	NHC_4H_9*	NHC_6H_5	OCH_3	OCH_2CH_3
	$OCH(CH_3)_2$*	OC_3H_7	OC_4H_9*	$N(CH_3)_2$	$N(C_2H_5)_2$
	$N{=}CHC_6H_5$				
4 39 members =	$B(OH)_2$	CH_2OH	$EtCO_2H$	$(CH_2)_3CO_2H$*	NH(OH)
	$NHCONH_2$	NHCN	$CH{=}NOH$	$NHCOCF_3$	NHCHO
	$NHSO_2CH_3$	$NHCOCH_3$	$SO_2(NH_2)$	$CONH_2$	$C{=}O(NHCH_3)$
	$CONHC_2H_5$*	CO_2H	CO_2^-	$NHC{=}S(NH_2)$	$CH{=}NNHCONHNH_2$
	$CH{=}NNHC{=}S(NH_2)$	$CONHC_3H_7$*	$NHC{=}S(NHC_2H_5)$	$NHC{=}O(CH_2Cl)$	$NHCOC_2H_5$*
	$NHC{=}S(CH_3)$	$NHSO_2C_2H_5$*	$NHCO_2CH_3$*	$NHCO_2C_2H_5$	$NHC{=}OCH(CH_3)_2$
	$NHCO_2C_3H_7$*	$NHC{=}S(C_2H_5)$*	$NHSO_2C_3H_7$*	$NHCOC_3H_7$*	$C(OH)(CF_3)_2$
	$NHC{=}S(C_3H_7)$*	$NHSO_2C_6H_5$	$NHCOC_6H_5$	$CH{=}NNHCOC_6H_5$	
5 20 members =	CH_2I	2-Thienyl	$Se(C_3H_7)$*	3-Thienyl	C_6H_5
	$N{=}NC_6H_5$	$C{\equiv}CC_6H_5$	$CH{=}CHC_6H_5$ (tr)	$Si(CH_3)_3$	C_5H_{11}*
	Cyclohexyl	$CH_2Si(CH_3)_3$	$CH_2C_6H_5$	C_4H_9	$C(CH_3)_3$
	$P(CH_3)_2$	$P(C_2H_5)_2$*	$CH_2Si(C_2H_5)_3$*	Ferrocenyl	$N(C_6H_5)_2$

Table VI-6a Aliphatic Constants—Twenty Cluster Sets

1	6 members =	Br	Cl	CF_3	F	I
		CF_2CF_3				
2	5 members =	NO_2	CN	$SOCH_3$	SOC_2H_5*	SOC_3H_7*
3	6 members =	H	CH_3	CH_2Br	CH_2Cl	$CH{=}CH_2$
		SCH_3				
4	6 members =	OH	CO_2H	CH=NOH	$CONH_2$	$NHCOCH_3$
		$C{=}O(NHCH_3)$				
5	2 members =	SH	$C{\equiv}CH$			
6	5 members =	NH_2	CH_2OH	$NHCH_3$	$NH(C_2H_5)$	$NHCONH_2$
7	8 members =	CBr_3	CCl_2	$S(C_2H_5)$	SC_3H_7*	CH_2I
		2-Thienyl	C_6H_5	3-Thienyl		
8	11 members =	SCN	CO_2CH_3	$OCOCH_3$	$CH{=}CHNO_2$ (tr)	COC_2H_5*
		CH=CHCN	OSO_2CH_3	OCH_3	$COCH_3$	CH_2CN
		OCH_2CH_3				
9	6 members =	CH_2CH_3	Cyclopropyl	$CH(CH_3)_2$	C_3H_7	C_4H_9
		$C(CH_3)_3$				
10	3 members =	CH_2OCH_3	$CH_2OC_2H_5$*	$N(CH_3)_2$		
11	5 members =	$CH_2Si(CH_3)_3$	$Si(CH_3)_3$	C_5H_{11}*	$CH_2C_6H_5$	$CH{=}CHC_6H_5$ (tr)
12	11 members =	$CO_2C_2H_5$	$OCH(CH_3)_2$	$CH{=}CHCOCH_3$	COC_3H_7*	OC_3H_7
		$OCOC_2H_5$*	$OCOC_3H_7$*	$CH{=}CHCO_2CH_3$*	OC_4H_9	$CO_2C_3H_7$*
		$CH{=}CHCOC_2H_5$*				
13	6 members =	$EtCO_2H$	NHC_3H_7*	$(CH_2)_3CO_2H$*	NHC_4H_9*	NHC_6H_5
		$NHCOC_6H_5$				
14	5 members =	$NHCO_2C_2H_5$	$CONHC_2H_5$*	$NHCOC_2H_5$*	$NHCOC_3H_7$*	$CONHC_3H_7$*
15	4 members =	$CH_2OC_3H_7$*	$N(C_2H_5)_2$	$CH_2OC_4H_9$*	$CH_2OC_6H_5$	
16	7 members =	$CH{=}CHCO_2C_2H_5$	$CH{=}CHCO_2C_3H_7$*	$OCOC_6H_5$	OC_6H_5	$CH{=}CHCOC_3H_7$*
		COC_6H_5	$CO_2C_6H_5$			
17	3 members =	2-Benzthiazolyl	$CH{=}CHCOC_6H_5$	$N(C_6H_5)_2$		
18	2 members =	$CH_2Si(C_2H_5)_3$*	Ferrocenyl			
19	2 single-					
1	member =	CO_2^-	$SO_2C_6H_5$			
20	groups					

Table VI-6b Aliphatic Constants–Ten Cluster Sets

1	6 members = Br	Cl	CF_3	F	I
	CF_2CF_3				
2	27 members = NO_2	CN	$SOCH_3$	SOC_2H_5*	SOC_3H_7*
	SCN	CO_2CH_3	$OCOCH_3$	$CH{=}CHNO_2$ (tr)	COC_2H_5*
	$CH{=}CHCN$	OSO_2CH_3	OCH_3	$COCH_3$	CH_2CN
	OCH_2CH_3	$CO_2C_2H_5$	$OCH(CH_3)_2$	$CH{=}CHCOCH_3$	COC_3H_7*
	OC_3H_7	$OCOC_2H_5$*	$OCOC_3H_7$*	$CH{=}CHCO_2CH_3$*	OC_4H_9
	$CO_2C_3H_7$*	$CH{=}CHCOC_2H_5$*			
3	20 members = H	CH_3	CH_2Br	CH_2Cl	$CH{=}CH_2$
	SCH_3	CH_2CH_3	Cyclopropyl	$CH(CH_3)_2$	C_3H_7
	C_4H_9	$C(CH_3)_3$	CBr_3	CCl_3	$S(C_2H_5)$
	SC_3H_7*	CH_2I	2-Thienyl	C_6H_5	3-Thienyl
4	11 members = OH	CO_2H	$CH{=}NOH$	$CONH_2$	$NHCOCH_3$
	$C{=}O(NHCH_3)$	$NHCO_2C_2H_5$	$CONHC_2H_5$*	$NHCOC_2H_5$*	$NHCOC_3H_7$*
	$CONHC_3H_7$*				
5	2 members = SH	$C{\equiv}CH$			
6	11 members = NH_2	CH_2OH	$NHCH_3$	$NH(C_2H_5)$	$NHCONH_2$
	$EtCO_2H$	NHC_3H_7*	$(CH_2)_3CO_2H$*	NHC_4H_9*	NHC_6H_5
	$NHCOC_6H_5$				
7	7 members = CH_2OCH_3	$CH_2OC_2H_5$*	$N(CH_3)_2$	$CH_2OC_3H_7$*	$N(C_2H_5)_2$
	$CH_2OC_4H_9$*	$CH_2OC_6H_5$			
8	7 members = $CH_2Si(CH_3)_3$	$Si(CH_3)_3$	C_5H_{11}*	$CH_2C_6H_5$	$CH{=}CHC_6H_5$ (tr)
	$CH_2Si(C_2H_5)_3$*	Ferrocenyl			
9	11 members = $CH{=}CHCO_2C_2H_5$	$CH{=}CHCO_2C_3H_7$*	$OCOC_6H_5$	OC_6H_5	$CH{=}CHCOC_3H_7$*
	COC_6H_5	$CO_2C_6H_5$	$SO_2C_6H_5$	2-Benzthiazolyl	$CH{=}CHCOC_6H_5$
	$N(C_6H_5)_2$				
10	1 member = CO_2^-				

Table VI-6c Aliphatic Constants—Five Cluster Sets

1	26 members = Br	Cl	CF_3	F	I
	CF_2CF_3	H	CH_3	CH_2Br	CH_2Cl
	$CH=CH_2$	SCH_3	CH_2CH_3	Cyclopropyl	$CH(CH_3)_2$
	C_3H_7	C_4H_9	$C(CH_3)_3$	CBr_3	CCl_3
	$S(C_2H_5)$	SC_3H_7*	CH_2I	2-Thienyl	C_6H_5
	3-Thienyl				
2	35 members = NO_2	CN	$SOCH_3$	SOC_2H_5*	SOC_3H_7*
	SCN	CO_2CH_3	$OCOCH_3$	$CH=CHNO_2$ (tr)	COC_2H_5*
	$CH=CHCN$	OSO_2CH_3	OCH_3	$COCH_3$	CH_2CN
	OCH_2CH_3	$CO_2C_2H_5$	$OCH(CH_3)_2$	$CH=CHCOCH_3$	COC_3H_7*
	OC_3H_7	$OCOC_2H_5$*	$OCOC_3H_7$*	$CH=CHCO_2CH_3$*	OC_4H_9
	$CO_2C_3H_7$*	$CH=CHCOC_2H_5$*	CO_2^-	CH_2OCH_3	$CH_2OC_2H_5$*
	$N(CH_3)_2$	$CH_2OC_3H_7$*	$N(C_2H_5)_2$	$CH_2OC_4H_9$*	$CH_2OC_6H_5$
3	24 members = OH	CO_2H	CH=NOH	$CONH_2$	NHCOCH3
	$C=O(NHCH_3)$	$NHCO_2C_2H_5$	$CONHC_2H_5$*	$NHCOC_2H_5$*	$NHCOC_3H_7$*
	$CONHC_3H_7$*	SH	$C\equiv CH$	NH_2	CH_2OH
	$NHCH_3$	$NH(C_2H_5)$	$NHCONH_2$	$EtCO_2H$	NHC_3H_7*
	$(CH_2)_3CO_2H$*	NHC_4H_9*	NHC_6H_5	$NHCOC_6H_5$	
4	7 members = $CH_2Si(CH_3)_3$	$Si(CH_3)_3$	C_5H_{11}*	$CH_2C_6H_5$	$CH=CHC_6H_5$ (tr)
	$CH_2Si(C_2H_5)_3$*	Ferrocenyl			
5	11 members = $CH=CHCO_2C_2H_5$	$CH=CHCO_2C_3H_7$*	$OCOC_6H_5$	OC_6H_5	$CH=CHOC_3H_7$*
	COC_6H_5	$CO_2C_6H_5$	$SO_2C_6H_5$	2-Benzthiazolyl	$CH=CHCOC_6H_5$
	$N(C_6H_5)_2$				

Table VI-7 Squared Correlation Matrix

	π	MR	$\mathcal{F}$	$\mathcal{R}$	H-D	H-A
π	1.00	0.01	0.51	0.03	0.00	0.00
MR		1.00	0.05	0.02	0.06	0.51
$\mathcal{F}$			1.00	0.17	0.04	0.00
$\mathcal{R}$				1.00	0.04	0.01
H-D					1.00	0.00
H-A						1.00

Table VI-8 Squared Correlation Matrix

	π	MR	$\mathcal{F}$	$\mathcal{R}$	H-D	H-A
π	1.00	0.23	0.13	0.04	0.32	0.13
MR		1.00	0.07	0.02	0.05	0.04
$\mathcal{F}$			1.00	0.03	0.00	0.25
$\mathcal{R}$				1.00	0.04	0.04
H-D					1.00	0.18
H-A						1.00

Table VI-9 Squared Correlation Matrix for Substituents of Table VI-1

	π	H-A	H-D	MR	$\mathcal{F}$	$\mathcal{R}$
π	1.00	0.17	0.12	0.10	0.12	0.00
H-A		1.00	0.12	0.01	0.09	0.02
H-D			1.00	0.01	0.02	0.08
MR				1.00	0.01	0.01
$\mathcal{F}$					1.00	0.09
$\mathcal{R}$						1.00

acceptors are clustered in groups 4, 5, and 9. There are no groups classified only as donors.

At the initial stage of a drug modification program, there are two limiting cases for which separate strategies should be considered. If the biologic testing is about as fast as the organic synthesis, then by the time six or eight derivatives of the initial group (say, 10) have been made, it may be apparent from preliminary regression equations that certain physicochemical properties have no role in the QSAR. At this point one can use the developing QSAR as the guide in selecting new substituents for better exploration of data space.

In the second of the limiting cases, testing may be so slow that one can easily make 15 or 20 compounds before the first results come in. In such a situation one should start out by clustering a larger number of substituents using clusters from the "20" or even "60" level to design the initial set.

It must be strongly emphasized that the number of parameters that can be clustered using numerical constants is limited at present. There are all kinds of geometrical factors or distances between certain atoms, metabolic effects, etc., that cannot be delineated at the start of a drug modification study. The importance of cluster analysis, as it now stands, is that it enables the chemist to design a set of congeners in which the known parameters are reasonably orthogonal and hence allows one to draw some conclusions with a degree of certainty. This frees the chemist's mind from these problems and allows him to "play his hunches" and also exercise his subjective judgment on problems such as the cost of synthesis and qualitative factors of recognized importance such as known chemical behavior.

For example, in the aromatic set with five clusters (Table VI-5c) one finds CH_2Cl and CH_3 in cluster 1 and immediately recognizes the great difference in chemical reactivity between these two groups. Benzyl chloride would be a good choice if one were interested in alkylating action; CH_3 would not. At the "5" level of clustering one has many choices in each group and the mature medicinal chemist will draw on his experience to make the wisest choices. Clustering is only an aid, albeit an important one, at this stage in drug design development. In time, no doubt other parameters will also be included.

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Potent non-nitrile dipeptidic dipeptidyl peptidase IV inhibitors

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Abstract—The synthesis and structure–activity relationships of novel dipeptidyl peptidase IV inhibitors replacing the classical cyanopyrrolidine P1 group with other small nitrogen heterocycles are described. A unique potency enhancement was achieved with β-branched natural and unnatural amino acids, particularly adamantylglycines, linked to a (2*S*,3*R*)-2,3-methanopyrrolidine based scaffold.

Dipeptidyl peptidase IV (DPP-IV) is an exopeptidase ubiquitously expressed in mammalian tissues, specifically on epithelial and endothelial cells and lymphocytes, which specifically cleaves dipeptides from the amino terminus of peptide substrates with proline or alanine at the penultimate position.[1] DPP-IV is responsible for the degradation of several important incretin hormones, most notably the gut hormone glucagon-like peptide-1 (GLP-1) which is released post-prandially from the L-cells of the intestine, and acts to potentiate glucose-stimulated insulin secretion resulting in the lowering of plasma glucose.[2] Due to DPP-IV's actions, the circulating half-life of GLP-1 is <90 s. Several DPP-IV inhibitors have reached late stages of clinical development (Fig. 1), including the dipeptidic inhibitors vildagliptin[3] and saxagliptin,[4] and the non-peptidic, structurally novel sitagliptin[5] and alogliptin.[6] Robust antidiabetic efficacy has been demonstrated clinically with DPP-IV inhibitors, and the most advanced compound has recently gained FDA approval for treatment of type 2 diabetes.[7]

Although multiple distinct chemical classes of DPP-IV inhibitors have been disclosed spanning diverse structural types,[8] some of the most potent compounds to date are those containing a proline mimetic cyanopyrrolidine P1 group.[9] This enhanced potency is thought to be due in part to a transient covalent trapping of the active site Ser630 hydroxyl of DPP-IV by the nitrile group, resulting in delayed dissociation kinetics and slow-tight binding of certain inhibitors.[10]

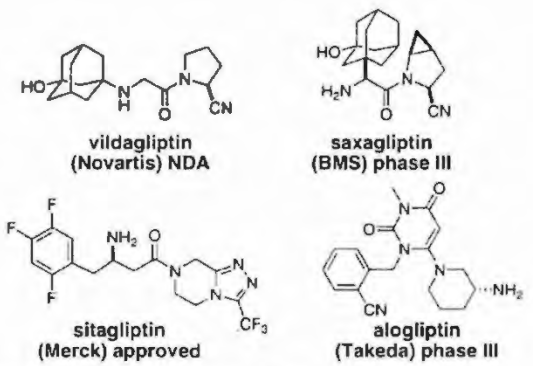

Figure 1. Clinically advanced DPP-IV inhibitors.

Along with this potency enhancement, chemical stability issues had been noted with early generations of nitrile-based inhibitors. While these issues were largely resolved

Keywords: DPP4; Serine protease; Diabetes; Non-nitrile; GLP-1.
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with more advanced molecules, we sought to understand whether adequate potency might be achieved in a dipeptidic inhibitor without a serine trap in order to obviate this potential chemical stability issue altogether. These efforts were initially both guided and tempered by two fundamental assumptions derived from extensive internal and external structure–activity relationship (SAR) analysis: (1) that very strict steric constraints exist around the pyrrolidine ring of cyanopyrrolidide-based inhibitors, with only hydrogen,[11] fluoro,[12] acetylene,[13] nitrile,[14] or methano[15] substitution permitted; and (2) that presence of a nitrile moiety on the pyrrolidine ring is critical to achieving potent activity. The overall strategy we pursued involved exploration of both P2 and P1 residues of the dipetide mimics lacking a prolinenitrile moiety.

The presumption that the presence of a nitrile group is critical to achieving potent DPP-IV inhibition in pyrrolidine-derived P1 containing inhibitors is based on known thiazolidine-based inhibitors such as isoleucine thiazolidide (P32/98, K_i = 126 nM)[16] and simple pyrrolidine-based inhibitors such as valine pyrrolidide (K_i = 470 nM)[17] and fluoropyrrolidides,[18] which exhibit considerably weaker (typically >10-fold) potency than their respective cyanopyrrolidine analogues. Furthermore, the des-cyano analogue of NVP-DPP728 was reported to have very weak potency (K_i = 15.6 μM).[19] These inhibitors also lack slow-binding kinetic properties, as an additional consequence of their reduced affinity. Nonetheless, valine pyrrolidide was found to potentiate plasma levels of active GLP-1 and insulin in response to glucose,[20] and isoleucine thiazolidide was shown to improve glucose tolerance in obese Zucker rats.[21] Studies with these DPP-IV inhibitor series showed a preference for β-branched L-amino acids for improved potency. We sought to probe the validity of this assumption regarding a requirement for a nitrile moiety for potent inhibition, and to evaluate whether DPP-IV inhibitors possessing bulkier P2 groups which more fully fill the S2 pocket of the enzyme (proven to result in potency enhancements in the nitrile series, such as with saxagliptin) might experience enhanced potency with simple P1 groups. A partial list of those non-nitrile containing proline surrogates evaluated in this study is shown in Figure 2.

Proline surrogates 1–5 are available commercially. Methanopyrrolidines 6–8 were prepared as follows. 3,4-Methanopyrrolidine 6 was prepared according to a known procedure (Scheme 1).[22] Thus, ethyl chloroacetate (9) and ethyl acrylate (10) were reacted in the presence of NaH to give the diethyl cyclopropanedicarboxylate 11. Saponification with NaOH afforded the corresponding

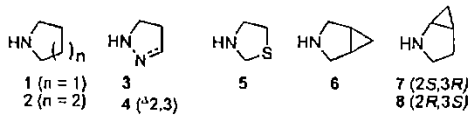
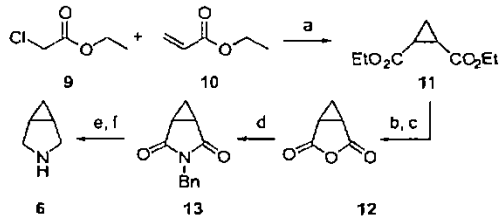


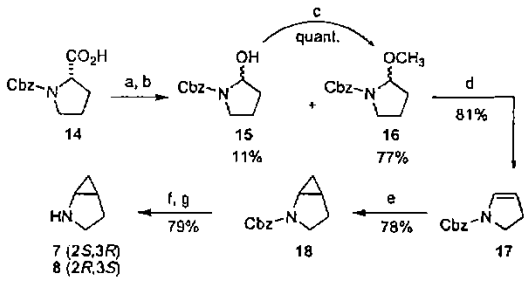
Figure 2. P1 Proline surrogates used in synthesis of non-nitrile DPP-IV inhibitors.



Scheme 1. Reagents and conditions: (a) NaH, toluene, rt, 2.5 h, 35 °C, 1.5 h; (b) NaOH, reflux, 8–10 h; (c) Ac_2O, reflux 40 min; (d) $BnNH_2$, toluene, 180 °C, 1.0 h, 150 °C, 20 h; (e) Red-Al, Et_2O, 0 °C, 70 min, reflux 3 h, then rt, o.n.; (f) i—10% Pd/C, CH_3OH, HOAc, 40 psi, 4 d, ii—4.0 N HCl/dioxane

diacid which was converted to the methanosuccinic anhydride 12 by heating in Ac_2O. Reaction of 12 with benzylamine in toluene gave the corresponding benzyl azabicyclohexane-2,4-dione 13. Finally, reduction of the imide with Red-Al followed by catalytic hydrogenation in the presence of 10% Pd/C effected N-debenzylation to give the desired 3,4-methanoproline 8 which was obtained as the HCl salt by filtering the methanolic solution directly into a solution of 4.0 N HCl in dioxane.

The initial syntheses of enantiomeric 2,3-methanopyrrolidines 7 and 8 utilized a stereorandom construction of a racemic 2,3-methanopyrrolidine, which was subsequently coupled to a suitably protected homochiral amino acid prior to resolution at the analogue stage (Scheme 2). Commercially available Cbz-protected L-proline (14) was oxidatively decarboxylated by treatment with iodobenzene diacetate and elemental iodine in CH_2Cl_2, followed by stirring in methanol to provide the racemic protected 2-methoxypyrrolidine 16 in 77% yield, along with the corresponding hydroxy product 15 (11%). The hydroxy product could be recycled by quantitative conversion to the desired methoxy compound 16 by treatment with pyridinium *p*-toluene sulfonate (PPTS) in MeOH. Dehydration of methoxy compound 16 was achieved by treatment with Hunig's base and TMSOTf to give protected dihydropyrrole 17 in 81% yield. Standard cyclopropanation conditions



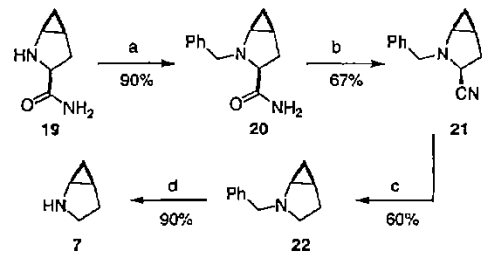
Scheme 2. Reagents and conditions: (a) iodobenzene diacetate, I_2, CH_2Cl_2, rt, (b) MeOH, rt; (c) PPTS, MeOH, rt, 20 h, (d) TMSOTf, *N,N*-diisopropylethylamine, ClH_2Cl_2, 0 °C; (e) diethylzinc, $ClCH_2I$, Et_2O, 0 °C to rt (f) H_2, 10% Pd/C, HCl, EtOH; (g) chiral HPLC resolution

(diethylzinc, chloroiodomethane) to give the methano product **18**, followed by deprotection of the Cbz group under acidic conditions, afforded the racemic 2,3-methanopyrrolidine **7/8** as the corresponding HCl salt in 62% overall yield for the two steps.

In a second generation synthesis, the desired 2*S*,3*R*-stereoisomer **7** could be obtained in optically pure form by a formal deamidation of a key intermediate used in the preparation of saxagliptin (Scheme 3). Beginning with L-4,5-methanoprolinamide (**19**),[15] protection of the proline nitrogen was accomplished using benzyl bromide and Hunig's base in CH_2Cl_2 to give intermediate **20** in 90% yield. Dehydration of the amide to the corresponding nitrile was achieved using TFAA and triethylamine in CH_2Cl_2 to give cyano compound **21** in 67% yield. Reductive removal of the cyano group by treatment with $NaBH_4$ in aqueous ethanol afforded benzyl protected methanopyrrolidine **22** in 60% yield. Removal of the benzyl protecting group was accomplished by treatment with α-chloroethyl acetyl chloride (ACE-Cl) in refluxing CH_2Cl_2 to give the desired (2*S*,3*R*)-2,3-methanopyrrolidine **7** in optically pure form as the HCl salt in 90% yield.

The series of dipeptides in the present study were then prepared via standard peptide coupling (PyBOP/NMO or EDAC/HOBT/DMAP) of the appropriate P1 proline surrogate with the various Boc-protected P2 L-amino acids. Subsequent removal of the Boc-protecting group with TFA in CH_2Cl_2 or HCl in dioxane afforded inhibitors **28–40** as their corresponding TFA or HCl salts.[23] All compounds were tested in vitro against purified human DPP-IV under steady state conditions with gly-pro-*p*-nitroanilide as substrate as previously described (Table 1).[4]

We systematically examined the influence of both P1 and P2 moiety contributions to DPP-IV inhibitory potency, beginning with a survey of both natural and unnatural amino acids in the P2 position, while fixing the P1 subunit as the homochiral des-cyano methanopyrrolidine (**7**) corresponding to saxagliptin. As shown previously for nitrile containing inhibitors, P2 amino acids with aryl (**23–28**) or polar (**29–36**) side-chains failed to exhibit any appreciable DPP-IV inhibition



Scheme 3. Reagents and conditions: (a) benzyl bromide, *N*,*N*-diisopropylethylamine, CH_2Cl_2, rt. (b) trifluoroacetic acid anhydride TEA, CH_2Cl_2, 0 °C. (c) $NaBH_4$, EtOH/H_2O, rt (d) 1-chloroethyl chloroformate, CH_2Cl_2, reflux.

Table 1. Inhibition constants versus human DPP-IV for compounds **23–60**

Compound	P1	P2-Xaa[a]	DPP4 K_i[b] (nM)
Saxagliptin	2*S*-CN-7	3-HO-Ad-Gly	0.6 ± 0.06
23	7	Ph-Gly	>10,000
24	7	Phe	3653 ± 206
25	7	4-Cl-Phe	877 ± 286
26	7	His	>10,000
27	7	Tyr	3007 ± 180
28	7	Trp	>10,000
29	7	Asn	>10,000
30	7	*N*-Ac-Lys	>10,000
31	7	Orn	>10,000
32	7	Ser	>10,000
33	7	*O*-Me-Ser	>10,000
34	7	*O*-*t*-Bu-Ser	>10,000
35	7	homo-Ser	>10,000
36	7	Thr	>10,000
37	7	*n*-Bu-Gly	3257 ± 453
38	7	Leu	>10,000
39	7	Neopentyl-Gly	1010 ± 146
40	7	Val	1065 ± 485
41	7	Ile	530 ± 36
42	7	Allo-Ile	731 ± 76
43	7	*tert*-Leu	356 ± 68
44	8	*tert* -Leu	>10,000
45	3	*tert*-Leu	>10,000
46	7	β,β-di-*i*-Pr-Ala	112 ± 9
47	7	3,3,5,5-tetra-Me-Ch-Gly	152 ± 21
48	7	3-HO-Ad-Gly	10 ± 3
49	7	3,5-di-HO-Ad-Gly	14 ± 7
50	8	3-HO-Ad-Gly	1944 ± 334
51	1	3-HO-Ad-Gly	49 ± 8
52	4	3-HO-Ad-Gly	3311 ± 563
53	5	3-HO-Ad-Gly	28 ± 3
54	(±)-2-Me-1	3-HO-Ad-Gly	>10,000
55	Isoindole	3-HO-Ad-Gly	1420 ± 196
56	Indoline	3-HO-Ad-Gly	>10,000
57	6	3,5-di-HO-Ad-Gly	270 ± 58
58	7	5,7-di-Me-3-HO-Ad-Gly	2.9 ± 0.5
59	2	5,7-di-Me-3-HO-Ad-Gly	607 ± 27
60	7	*N*-(3-HO-Ad)Gly	3081 ± 790

[a] All P2 amino acids bear the natural L-configuration at the α-stereocenter.
[b] All K_i values are mean ± SD of at least triplicate determinations.

(K_is ⩾ 1 μM). Also consistent with findings in the analogous nitrile series, a strong potency dependence on β-branching in the P2 side-chain was revealed in comparison of those simple alkyl side-chains with and without β-branching (compare **37–39** vs. **40–43**). Increasing the steric bulk of the β-branched substituents gave only modest incremental enhancement of potency (**46**, **47**). In all of these cases, it appeared that potency versus the corresponding nitrile series suffered an approximately 20- to 50-fold loss.

Combining our optimized P2 group present in saxagliptin (3-hydroxyadamantylglycine) with the 2*S*,3*R*-methanopyrrolidine **7** gave compound **48**, with potency equivalent to that of some of the most active nitrile-containing inhibitors in the clinic (K_i = 10 nM); similar

potency was observed in the dihydroxy compound **49** (K_i = 14 nM). As was noted for the nitrile-containing series, a strong stereochemical preference was maintained for the methano bridge bearing the 2*S*,3*R*-configuration (compare **43** with **44** and **48** with **50**). The apparently unique potency enhancing properties of this bulky P2 unit are further demonstrated with simple pyrrolidine (**51**) and thiazolidine (**53**) P1 groups, though the 3- to 5-fold diminished potency compared with **48** serve to validate the role of the methano bridge in this precise regio- and stereochemical orientation (compare with **57**) in favorably contributing to DPP-IV binding affinity. It is noteworthy that strict steric constraints exist in the S1 pocket, such that even simple methyl substitution as in **54** essentially destroys all activity. The most potent compound in the series was obtained by packing further bulk into the S2 pocket (**58**, K_i = 2.9 nM), though a change as subtle as opening the bicyclic methanopyrrolidine to a piperidine (**59**) results in a 200-fold drop in potency. Interestingly, methanopyrrolidine analogues with N-linked substitution analogous to vildagliptin (**60**) failed to demonstrate any significant DPP-IV inhibitory activity.

The unique structural features imparted to inhibitors by the hydroxyadamantylglycine P2 group appear to be capable of conferring significant potency to non-nitrile compounds, though limited by the same narrow steric and stereochemical requirements shown for nitrile-containing inhibitors. Interestingly, several of the more potent analogues in this series have retained some slow binding kinetic properties, despite the lack of a nitrile (dissociation rate increases from 4.6×10^{-5}/s for saxagliptin to 2.0×10^{-3}/s for compound **48** at 25 °C, unpublished results). Compound **48** maintains potent and fully efficacious antihyperglycemic effects in rodent models and mirrors the PK and safety profiles of clinical lead compound saxagliptin, yet is incapable of undergoing degradative cyclization. As previously discussed, this inhibitor also shows uniquely potent inhibition relative to other non-cyano compounds, suggesting that the summation of contributions to the binding energy of this compound in the active site is largely dominated by the P2 hydroxyadamantylglycine moiety. These studies have shown the development of chemically more stable and potent DPP-IV inhibitors in the low nM range, specifically compounds **48**, **49** and **58**. Further studies examining in vivo pharmacological effects as well as biochemical and biophysical aspects of the binding interactions for these potent compounds will be the subject of forthcoming disclosures from these laboratories.

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23. Data for compound **48** (HCl salt): HPLC (Phenominex Luna 3 μ C18 4.6 × 150 mm, 95% A to 95% B (A = H_2O + 0.05% TFA, B = CH_3CN + 0.05% TFA, flow rate 1 mL/min, linear gradient over 42 min) retention time 13.37 min (97.9%); Chiral analytical HPLC (Chiralpak AD 10 μ 4.6 × 250 mm, 80% heptane + 20% 1:1 EtOH–MeOH + 0.1% DEA, flow rate 1 mL/min, isocratic) retention time 10.56 min (98.2% ee); LC/MS *m/z* 291 $[M+H]^+$; ^1H NMR (D_2O, 400 MHz) δ 4.16 (s, 1H), 3.82 (ddd, 1H, *J* = 13.2, 10.3, 2.9 Hz), 3.48 (td, 1H, *J* = 6.2, 2.6 Hz), 2.94 (dt, 1H, *J* = 13.1, 8.7 Hz), 2.14 (bs, 2H), 1.94–2.05 (m, 1H), 1.88 (ddd, 1H, *J* = 12.4, 8.4, 3.3 Hz), 1.74 (ddd, 1H, *J* = 8.8, 11.4, 5.2), 1.3–1.73 (m, 12H), 0.74–0.85 (m, 1H), 0.65–0.71 (td, 1H, *J* = 5.7, 2.6); ^{13}C NMR (D_2O, 100 MHz) δ 167.3, 69.1, 59.6, 45.3, 45.1, 43.1, 39.7, 38.2, 37.1, 36.8, 36.6, 34.5, 30.2, 30.1, 24.4, 18.9, 12.8; Anal. Calcd for $C_{18}H_{25}N_3O_3 \cdot 1.64\ HCl \cdot 1.33\ H_2O$: C, 54.56; H, 8.16; N, 7.49; Cl, 15.57. Found: C, 54.42; H, 7.86; N, 7.35; Cl, 15.57. KF, 6.39.

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PROBING THE IMPORTANCE OF SPACIAL AND CONFORMATIONAL DOMAINS IN CAPTOPRIL ANALOGS FOR ANGIOTENSIN CONVERTING ENZYME ACTIVITY

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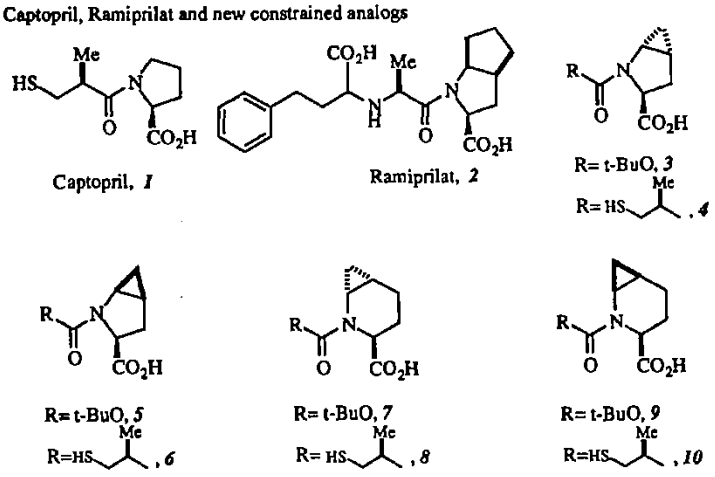
Abstract: A new synthesis of 4,5-methano-L-prolines and the enzymatic activity of the corresponding N-(3-mercapto-2-*R*-methyl-propionyl) analogs as inhibitors of angiotensin converting enzyme are described.

Target and chemistry-driven drug design based on molecular interactions with enzymes has emerged in recent years as a stimulating area of research endeavor.[1] Although there are a multitude of small-molecule enzyme inhibitors, even at nM concentrations in vitro, the process of their development into a marketable drug is arduous to say the least. One of the remarkably successful attempts at drug design based on molecular interactions with an enzyme is exemplified in captopril,[2] an inhibitor of angiotensin converting enzyme, and used for the treatment of hypertension (Figure 1)

Figure 1. Captopril, Ramiprilat and new constrained analogs



The discovery of captopril, an exquisitely simple molecule by today's standards of increasing molecular complexity, is a classic example of design based on a knowledge of possible interactions between the drug and

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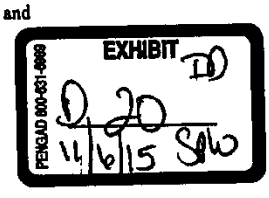

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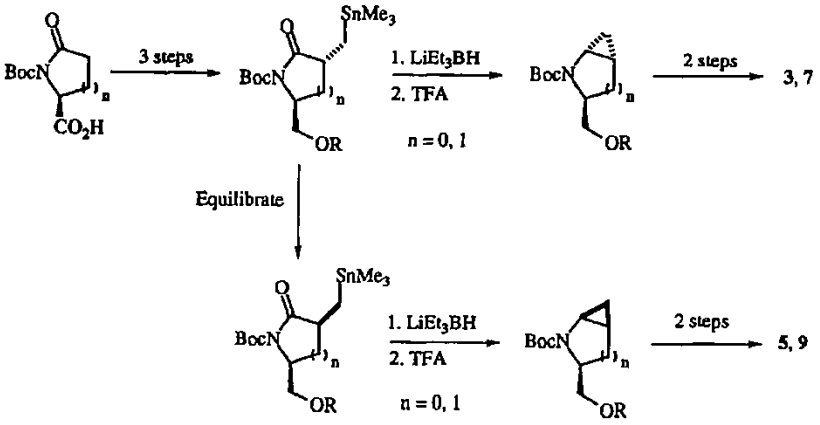
the active site of the enzyme. This was also aided by extensive analog synthesis of proline peptides, which led to the conclusion that captopril appears to offer effective interactions with the functional groups on the surface of the enzyme that involve charge, H-bonding and hydrophobic contact.[3]

The importance of such multiple interactions has been discussed in detail,[2] and was the basis of an intense effort directed at the discovery of analogs of captopril,[2a,b] such as enalapril,[2c] and of 2. Since the *cis*-orientation of the cyclopentane ring in ramiprilat is important for biological activity,[4] the relevance of the hydrophobic interaction of that region of the proline moiety with the enzyme can be appreciated.

Our interest in the synthesis of conformationally constrained heterocycles, coupled with the desire to further probe the role of substituents on the enzymatic activity of captopril, instigated the research work reported in this paper.

Previous work in our laboratory[5,6] (Scheme 1) has described methods for the synthesis of Boc derivatives of *trans*-4,5-methano-L-proline 3, *cis*-4,5-methano-L-proline 5, *trans*-5,6-methano-L-pipecolic acid, 7, and *cis*-5,6-methano-L-pipecolic acid, 9. It was further observed that the proline ring in the N-Boc derivative 3 was virtually flattened (rms, 0.003 Å) compared to 5 (rms, 0.013 Å) and Boc-L-proline (rms, 0.018 Å).[5] While the reasons for this structural effect were not evident, we anticipated that such constrained proline derivatives should be explored further as surrogates for L-proline in a selected group of medicinally relevant compounds. We thus set out to prepare the (2*R*)-methyl-3-mercapto N-propionyl amides of the above mentioned ω-methano-L-prolines and L-pipecolic acids as constrained analogs of captopril, and to study their inhibitory activity on ACE (angiotensin converting enzyme).

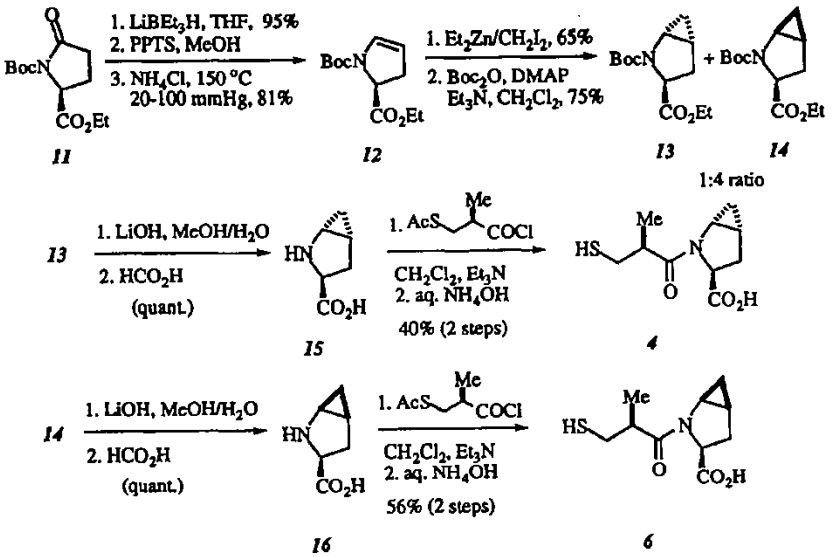
Scheme 1.



2,3-Methano-prolines have been previously reported by a number of groups.[7] *cis*- and *trans*-3,4-Methano-prolines were described by Witkop and coworkers[8] in 1971. We were aware of one synthesis of racemic 4,5-methano-proline amides[9] which was based on a Beckmann rearrangement of *cis*-bicyclo[3.1.0]hexan-2-one to a δ-lactam followed by chlorination and ring contraction.

Our synthesis of the analogs **3**, **5**, **7**, and **9**, relied on the intramolecular cyclopropanation of α-trimethylstannylmethyl lactams, via the corresponding iminium salts.[5,6] The *cis*- or *trans*-orientation of the cyclopropane ring could be controlled by the β- or α-disposition of the trimethylstannylmethyl group respectively vis-à-vis the stereocontrolling substituent in the corresponding derivatives (Scheme 1).

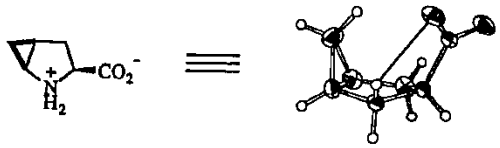
Scheme 2.



We have now devised an expedient route to 4,5-methano-prolines that affords the enantiopure derivatives **3** and **5** in essentially eight steps from commercially available L-pyroglutamic acid (Scheme 2). Thus reduction of the lactam carbonyl in **11** with lithium triethylborohydride followed by transformation to the methoxy hemiaminal carbamate and elimination of methanol in the presence of ammonium chloride[10] led to the enecarbamate analog **12** in good yield.[11] Originally, we had used other published methods[12] to effect the dehydration, but in our hands, yields were modest and reproducibility depended on the scale of the reaction. Application of the modified Simmons–Smith cyclopropanation[13] reaction to the enecarbamate **12** followed by protection of the amine with Boc anhydride, led to a mixture of the *trans*-4,5-methano-L-proline analog **13** and the corresponding *cis*-4,5-isomer **14** in a ratio of 1:4 in a combined yield of 75%.[14] This expedient method based on a well-known reaction,[13] proceeds in good overall yield and it is 3 steps shorter than our previous synthesis[5] which utilized an organotin reagent. It has allowed us to prepare gram quantities of both 4,5-methano-L-prolines which were easily separable by column chromatography as their N-Boc derivatives **13** and **14**. Hydrolysis with LiOH in aq. methanol gave the known respective 4,5-methano-N-Boc-L-prolines[5] as crystalline solids. Treatment of **13** with aq base followed by formic acid gave the free acid **15** as a white solid in quantitative yield. Analogous hydrolysis of **14** gave the isomeric free acid **16** as a crystalline solid. The

X-ray structure and solid state conformational characteristics of **16** revealed considerable flattening of the pyrrolidine ring (rms 0.09 Å) compared to L-proline (rms 0.181 Å). A H-bond was evident between the protonated amine and the carboxylate group. (Figure 2).

Figure 2.



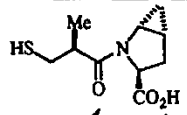
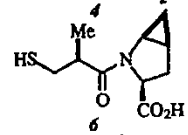
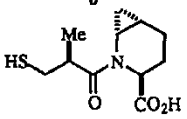
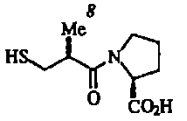
The predominance of the *cis*-4,5-methano isomer **14** in the cyclopropanation reaction is of interest, since the tin-mediated iminium ion cyclization protocol[5] (Scheme 1) favored the formation of the *trans*-isomer due to steric factors imposed by a bulky resident group. Most probably, the *cis*-cyclopropanation is the result of an anchoring effect of the zinc species with the ester group in **12**, thus delivering the nucleophile from the same side to give **14**.[15]

Acylation of the free amino acids with the readily available S-acetyl-2-(*R*)-methyl propionyl chloride and saponification afforded the captopril analogs **4** and **6**. The L-pipecolic acid analog **8** was similarly prepared from the precursor amino acid.[5]

Inhibition of ACE obtained from rabbit lung and partially purified, was studied using hippuryl-His-Leu as a substrate following the procedure of Cushman and Cheung.[16] The results shown in Table 1 indicate that the *cis*- and *trans*-5-methano analogs of L-proline **4**,**6**, and the *trans*-L-pipecolic acid analog **8** are highly potent inhibitors, even surpassing captopril. The *cis*-4,5-carbocyclic analog of captopril, ramiprilat **2**, is much more active than the corresponding *trans*-isomer. In this respect it is of interest that the *cis*-analog **6** is equally as active as the *trans*-analog **4**. Clearly, this study has shown that small rings can be tolerated at the 4,5-position of captopril with *cis*- or *trans*-orientations. It also appears that the degree of ring flattening in these derivatives relative to captopril is not having an adverse effect on enzyme inhibitory activity, and it could even be an advantage.

Analogs **4**, **6**, and **8**, were inactive against neutral endopeptidase 24.11 enzyme at 10 μM as well as against endothelin converting enzyme at 1 μM. The selectivity described herein exhibited by captopril and the methano analogs **4**, **6**, and **8** towards ACE is obviously of interest. Future work will focus on the incorporation of 4,5-methano prolines and 5,6-methano pipecolic acids into strategic positions as replacements of the corresponding natural amino acids in pharmacologically relevant molecules.

Table 1 Inhibition Tests on Angiotensin Converting Enzyme (ACE)[a]

Compound	IC_{50}
 *4*	7.6 (± 0.7 nM)
 *6*	6.6 (± 0.3 nM)
 *8*	5.3 (± 0.2 nM)
 *1*, Captopril	13.0 (± 1.0 nM)

a. For assay method, see ref 16

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11. Typical procedure: A mixture containing N-Boc-5-methoxy-(2*S*)-pyrrolidine-1-carboxylic acid ethyl ester (1.2 g, 4.4 mmol) and ammonium chloride (0.044 g, 0.15 equiv) was heated under reduced pressure (20–100 mm Hg) in a flask equipped with a reflux condenser. The reaction mixture was allowed to cool to room temperature, and the resultant oil was purified by flash chromatography on silica gel (hexanes-ethyl acetate, 9:1) to afford the product 12 (0.854 g, 81%) as a colorless oil.
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14. Typical procedure: To a cooled solution of 12 (0.5708 g, 2.366 mmol) in dry 1,2-dichloroethane under nitrogen was added dropwise 1.1 equiv of diethyl zinc (Et_2Zn, 1.0M solution in hexane, 2.603 mmol, 2.6 mL) and 1.5 equiv. of diiodomethane (3.55 mmol, 0.29 mL) at 0 °C. The reaction mixture was stirred for 30 min at 0 °C then 6 h at room temperature and quenched with dilute $NaHCO_3$ (7 mL). Water was added (40 mL), and the aqueous layer was extracted with dichloromethane (4 x 50 mL) and processed as usual. The residue after evaporation was purified by flash chromatography (methanol-hexanes-ethyl acetate, 1:5:13) to afford the isomeric mixture of products (0.332 g, 65%) as a pale-yellow oil that was transformed into the N-Boc derivatives (Boc_2O, DMAP, Et_3N, CH_2Cl_2), and separated by chromatograhy to give 13 and 14 (75% yield, 1:4 ratio).
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(19) **United States**

(12) **Reissued Patent**

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(45) **Date of Reissued Patent: Apr. 30, 2013**

(54) **CYCLOPROPYL-FUSED PYRROLIDINE-BASED INHIBITORS OF DIPEPTIDYL PEPTIDASE IV AND METHOD**

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(21) Appl. No.: **13/308,658**

(22) Filed: **Dec. 1, 2011**

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(51) **Int. Cl.**
C07D 209/02 (2006.01)
A61K 31/403 (2006.01)

(52) **U.S. Cl.**
USPC **514/412**; 548/452

(58) **Field of Classification Search** 514/412; 548/452
See application file for complete search history.

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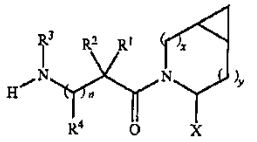
Primary Examiner — Gregg Polansky

(74) *Attorney, Agent, or Firm* — Woodcock Washburn LLP

(57) **ABSTRACT**

Dipeptidyl peptidase IV (DP 4) inhibiting compounds are provided having the formula



where

x is 0 or 1 and y is 0 or 1 (provided that x=1 when y=0 and x=0 when y=1);

n is 0 or 1; X is H or CN;

and wherein R^1, R^2, R^3 and R^4 are as described herein.

A method is also provided for treating diabetes and related diseases, especially Type II diabetes, and other diseases as set out herein, employing such DP 4 inhibitor *or a combination of such DP 4 inhibitor and one or more of another antidiabetic agent such as metformin, glyburide, troglitazone, pioglitazone, rosiglitazone and/or insulin and/or one or more of a hypolipidemic agent and/or anti-obesity agent and/or other therapeutic agent.

41 Claims, No Drawings

DX 18

EXHIBIT

PENGAD 800-631-6989

D 18 DD

11/6/15 SDW

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CYCLOPROPYL-FUSED PYRROLIDINE-BASED INHIBITORS OF DIPEPTIDYL PEPTIDASE IV AND METHOD

Matter enclosed in heavy brackets [ ] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue.

This application takes priority from U.S. provisional application No. 60/188,555, filed Mar. 10, 2000.

FIELD OF THE INVENTION

The present invention relates to cyclopropyl-fused pyrrolidine-based inhibitors of dipeptidyl peptidase IV (DP-4), and to a method for treating diabetes, especially Type II diabetes, as well as hyperglycemia, Syndrome X, diabetic complications, hyperinsulinemia, obesity, atherosclerosis and related diseases, as well as various immunomodulatory diseases and chronic inflammatory bowel disease, employing such cyclopropyl-fused pyrrolidines alone or in combination with another type antidiabetic agent and/or other type therapeutic agent.

BACKGROUND OF THE INVENTION

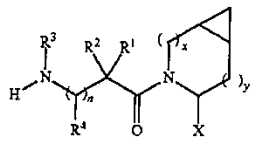
Depeptidyl peptidase IV (DP-4) is a membrane bound non-classical serine aminodipeptidase which is located in a variety of tissues (intestine, liver, lung, kidney) as well as on circulating T-lymphocytes (where the enzyme is known as CD-26). It is responsible for the metabolic cleavage of certain endogenous peptides (GLP-1(7-36), glucagon) in vivo and has demonstrated proteolytic activity against a variety of other peptides (GHRH, NPY, GLP-2, VIP) in vitro.

GLP-1(7-36) is a 29 amino-acid peptide derived by post-translational processing of proglucagon in the small intestine. GLP-1(7-36) has multiple actions in vivo including the stimulation of insulin secretion, inhibition of glucagon secretion, the promotion of satiety, and the slowing of gastric emptying. Based on its physiological profile, the actions of GLP-1(7-36) are expected to be beneficial in the prevention and treatment of type II diabetes and potentially obesity. To support this claim, exogenous administration of GLP-1(7-36) (continuous infusion) in diabetic patients has demonstrated efficacy in this patient population. Unfortunately GLP-1(7-36) is degraded rapidly in vivo and has been shown to have a short half-life in vivo (t1/2=1.5 min). Based on a study of genetically bred DP-4 KO mice and on in vivo/in vitro studies with selective DP-4 inhibitors, DP-4 has been shown to be the primary degrading enzyme of GLP-1(7-36) in vivo. GLP-1 (7-36) is degraded by DP-4 efficiently to GLP-1(9-36), which has been speculated to act as a physiological antagonist to GLP-1(7-36). Thus, inhibition of DP-4 in vivo should potentiate endogenous levels of GLP-1(7-36) and attenuate formation of its antagonist GLP-1(9-36) and thus serve to ameliorate the diabetic condition.

DESCRIPTION OF THE INVENTION

In accordance with the present invention, cyclopropyl-fused pyrrolidine-based compounds are provided which inhibit DP-4 and have the structure

I



wherein

x is 0 or 1 and y is 0 or 1 (provided that
x=1 when y=0 and
x=0 when y=1);

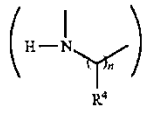
n is 0 or 1;

X is H or CN (that is cyano);

R^1, R^2, R^3 and R^4 are the same or different and are independently selected from H, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, bicycloalkyl, tricycloalkyl, alkylcycloalkyl, hydroxyalkyl, hydroxyalkylcycloalkyl, hydroxycycloalkyl, hydroxybicycloalkyl, hydroxytricycloalkyl, bicycloalkylalkyl, alkylthioalkyl, arylalkylthioalkyl, cycloalkenyl, aryl, aralkyl, heteroaryl, heteroarylalkyl, cycloheteroalkyl and cycloheteroalkylalkyl, all optionally substituted through available carbon atoms with 1, 2, 3, 4 or 5 groups selected from hydrogen, halo, alkyl, polyhaloalkyl, alkoxy, haloalkoxy, polyhaloalkoxy, alkoxycarbonyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, polycycloalkyl, heteroarylamino, arylamino, cycloheteroalkyl, cycloheteroalkylalkyl, hydroxy, hydroxyalkyl, nitro, cyano, amino, substituted amino, alkylamino, dialkylamino, thiol, alkylthio, alkylcarbonyl, acyl, alkoxycarbonyl, aminocarbonyl, alkynylaminocarbonyl, alkylaminocarbonyl, alkenylaminocarbonyl, alkylcarbonyloxy, alkylcarbonylamino, arylcarbonylamino, alkylsulfonylamino, alkylaminocarbonylamino, alkoxycarbonylamino, alkylsulfonyl, aminosulfonyl, alkylsulfinyl, sulfonamido or sulfonyl;

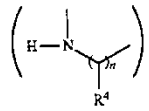
and R^1 and R^3 may optionally be taken together to form $-(CR^5R^6)_m-$ where m is 2 to 6, and R^5 and R^6 are the same or different and are independently selected from hydroxy, alkoxy, cyano, H, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, cycloalkenyl, aryl, arylalkyl, heteroaryl, heteroarylalkyl, cycloheteroalkyl, halo, amino, substituted amino, cycloheteroalkylalkyl, alkylcarbonylamino, arylcarbonylamino, alkoxycarbonylamino, aryloxycarbonylamino, alkoxycarbonyl, aryloxycarbonyl, or alkylaminocarbonylamino, or R^1 and R^4 may optionally be taken together to form $-(CR^7R^8)_p-$ where p is 2 to 6, and R^7 and R^8 are the same or different and are independently selected from hydroxy, alkoxy, cyano, H, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, cycloalkenyl, aryl, arylalkyl, heteroaryl, heteroarylalkyl, cycloheteroalkyl, halo, amino, substituted amino, cycloheteroalkylalkyl, alkylcarbonylamino, arylcarbonylamino, alkoxycarbonylamino, aryloxycarbonylamino, alkoxycarbonyl, ary-

loxycarbonyl, or alkylaminocarbonylamino, or optionally R^1 and R^3 together with



form a 5 to 7 membered ring containing a total of 2 to 4 heteroatoms selected from N, O, S, SO, or SO_2;

or optionally R^1 and R^3 together with

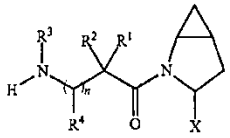


form a 4 to 8 membered cycloheteroalkyl ring wherein the cycloheteroalkyl ring has an optional aryl ring fused thereto or an optional 3 to 7 membered cycloalkyl ring fused thereto;

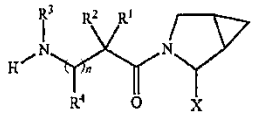
and including pharmaceutically acceptable salts thereof, and prodrug esters thereof, and all stereoisomers thereof.

Thus, the compounds of formula I of the invention include the following structures

IA



IB



In addition, in accordance with the present invention, a method is provided for treating diabetes, especially Type II diabetes, as well as impaired glucose homeostasis, impaired glucose tolerance, infertility, polycystic ovary syndrome, growth disorders, frailty, arthritis, allograft rejection in transplantation, autoimmune diseases (such as scleroderma and multiple sclerosis), various immunomodulatory diseases (such as lupus erythematosis or psoriasis), AIDS, intestinal diseases (such as necrotizing enteritis, microvillus inclusion disease or celiac disease), inflammatory bowel syndrome, chemotherapy-induced intestinal mucosal atrophy or injury, anorexia nervosa, osteoporosis, Syndrome X, dysmetabolic syndrome, diabetic complications, hyperinsulinemia, obesity, atherosclerosis and related diseases, as well as inflammatory bowel disease (such as Crohn's disease and ulcerative colitis), wherein a therapeutically effective amount of a compound of structure I (which inhibits DP 4) is administered to a human patient in need of treatment.

The conditions, diseases, and maladies collectively referenced to as "Syndrome X" or Metabolic Syndrome are detailed in Johannsson J. Clin. Endocrinol. Metab., 82, 727-734 (1997).

In addition, in accordance with the present invention, a method is provided for treating diabetes and related diseases as defined above and hereinafter as well as any of the other disease states mentioned above, wherein a therapeutically effective amount of a combination of a compound of structure I and one, two, three or more of other types of antidiabetic agent(s) (which may be employed to treat diabetes and related diseases) and/or one, two or three or more other types of therapeutic agent(s) is administered to a human patient in need of treatment.

The term "diabetes and related diseases" refers to Type II diabetes, Type I diabetes, impaired glucose tolerance, obesity, hyperglycemia, Syndrome X, dysmetabolic syndrome, diabetic complications, dysmetabolic syndrome, and hyperinsulinemia.

The conditions, diseases and maladies collectively referred to as "diabetic complications" include retinopathy, neuropathy and nephropathy, and other known complications of diabetes.

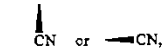
The term "other type(s) of therapeutic agents" as employed herein refers to one or more antidiabetic agents (other than DP4 inhibitors of formula I), one or more anti-obesity agents, and/or one or more lipid-modulating agents (including anti-atherosclerosis agents), and/or one or more infertility agents, one or more agents for treating polycystic ovary syndrome, one or more agents for treating growth disorders, one or more agents for treating frailty, one or more agents for treating arthritis, one or more agents for preventing allograft rejection in transplantation, one or more agents for treating autoimmune diseases, one or more anti-AIDS agents, one or more anti-osteoporosis agents, one or more agents for treating immunomodulatory diseases, one or more agents for treating chronic inflammatory bowel disease or syndrome and/or one or more agents for treating anorexia nervosa.

The term "lipid-modulating" agent as employed herein refers to agents which lower LDL and/or raise HDL and/or lower triglycerides and/or lower total cholesterol and/or other known mechanisms for therapeutically treating lipid disorders.

In the above methods of the invention, the compound of structure I will be employed in a weight ratio to the antidiabetic agent or other type therapeutic agent (depending upon its mode of operation) within the range from about 0.01:1 to about 500:1, preferably from about 0.1:1 to about 100:1, more preferably from about 0.2:1 to about 10:1.

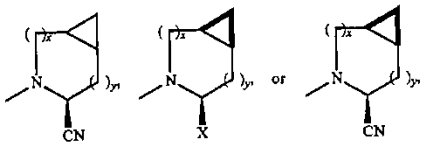
Preferred are compounds of formula I wherein R^3 is H or alkyl, R^1 is H, alkyl, cycloalkyl, bicycloalkyl, tricycloalkyl, alkylcyclo alkyl, hydroxyalkyl, hydroxytricyclo alkyl, hydroxycycloalkyl, hydroxybicycloalkyl, or hydroxyalkylcycloalkyl, R^2 is H or alkyl, n is 0, X is CN, x is 0 or 1 and y is 0 or 1.

Most preferred are preferred compounds of formula I as described above where X is



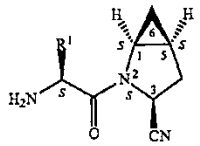
and/or wherein the fused cyclopropyl group is identified as

Thus, preferred compounds of formula I of the invention will include the moiety:



Particularly preferred are the following compounds:

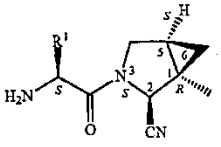
A)



[1S, 2(2S), 3S, 5S]

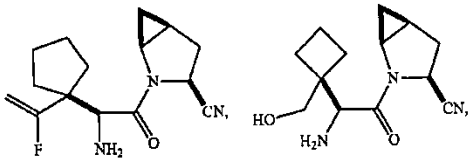
wherein R^1 is alkyl, cycloalkyl, bicycloalkyl, tricycloalkyl, alkylcycloalkyl, hydroxyalkyl, hydroxycycloalkyl, hydroxyalkylcycloalkyl, hydroxybicycloalkyl or hydroxytricycloalkyl;

B)



[1R, 2S, 3(2S), 5S]

wherein R^1 is alkyl, cycloalkyl, bicycloalkyl, tricycloalkyl, hydroxybicycloalkyl, hydroxytricycloalkyl, alkylcycloalkyl, hydroxyalkyl, hydroxycycloalkyl or hydroxyalkylcycloalkyl as well as the following:



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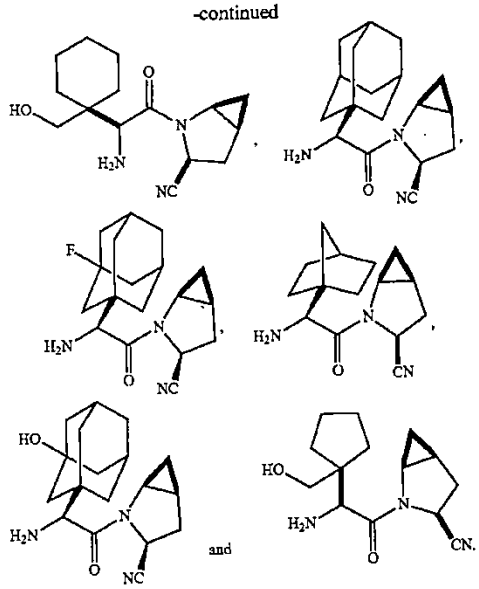


and

DETAILED DESCRIPTION OF THE INVENTION

Compounds of the structure I may be generated by the methods as shown in the following reaction schemes and the description thereof.

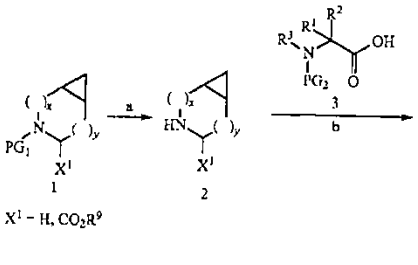
Referring to Reaction Scheme 1, compound 1, where PG_1 is a common amine protecting group such as Boc, Cbz, or FMOC and X^1 is H or CO_2R^9 as set out below, may be generated by methods as described herein or in the literature (for example see Sagnard et al, Tet-Lett., 1995, 36, pp. 3148-3152, Tverezovsky et al, Tetrahedron, 1997, 53, pp. 14773-14792, Hanessian et al, Bioorg. Med. Chem. Lett., 1998, 8, p. 2123-2128). Removal of the PG_1 group by conventional methods (e.g. (1) TFA or HCl when PG_1 is Boc, or (2) H_2/Pd/C, TMSI when PG_1 is Cbz, or (3) Et_2NH when PG_1 is (FMOC) affords the free amine 2 Amine 2 may be coupled to various protected amino acids such as 3 (where PG_2 can be any of the PG_1 protecting groups) using standard peptide coupling conditions (e.g. EDAC/HOAT, i-BuCOCOCl/TEA, PyBop/NMM) to afford the corresponding dipeptide 4. Removal of the amine protecting group PG_2 provides compound Ia of the invention where X=H.

In the case where X^1=CO_2R^9 (where R^9 is alkyl or aralkyl groups such as methyl, ethyl, t-butyl, or benzyl), the ester may be hydrolyzed under a variety of conditions, for example with aqueous NaOH in a suitable solvent such as methanol, THF, or dioxane, to provide the acid 5. Conversion of the acid group to the primary carboxamide, affording 6, may be effected by activation of the acid group (e.g. employing i-BuOCOCl/TEA or EDAC) followed by treatment with NH_3 or an ammonia equivalent in a solvent such as dioxane, ether, or methanol. The amide functionality may be converted to the nitrile group by a variety of standard conditions (e.g. $POCl_3$/pyridine/imidazole or cyanuric chloride/DMF or trifluoroacetic anhydride, THF, pyridine) to give 7. Finally, removal of the PG_2 protecting group similar to above provides compound of the invention Ib.

In a different sequence (Scheme 2), compound 1 where X^1 is CO_2R^9 may be saponified to the acid and subsequently amidated as described above to give amide 8. Removal of the PG_1 group followed by peptide coupling to 3 affords compound 6, an intermediate in the synthesis of Ib.

Alternately, the carboxamide group in 8 may be converted to the nitrile as described above to give compound 9. Deprotection of PG_1 affords 10 which may be subject to standard peptide coupling conditions to afford 7, an intermediate in the synthesis of Ib. Compound 10 may also be generated by oxidation of the amine 2 (e.g. NCS) followed by hydrolysis and subsequent cyanide treatment. Compound 10 may be obtained as a mixture of stereoisomers or a single isomer/diastereomer which may be epimerized (employing conventional procedures) to afford a mixture of stereoisomers.

Scheme 1



X^1 = H, CO_2R^9

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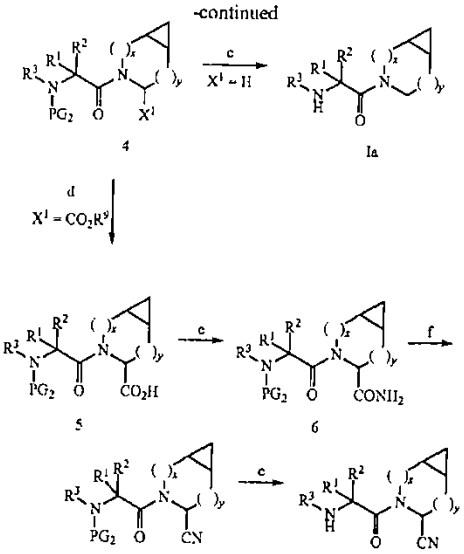
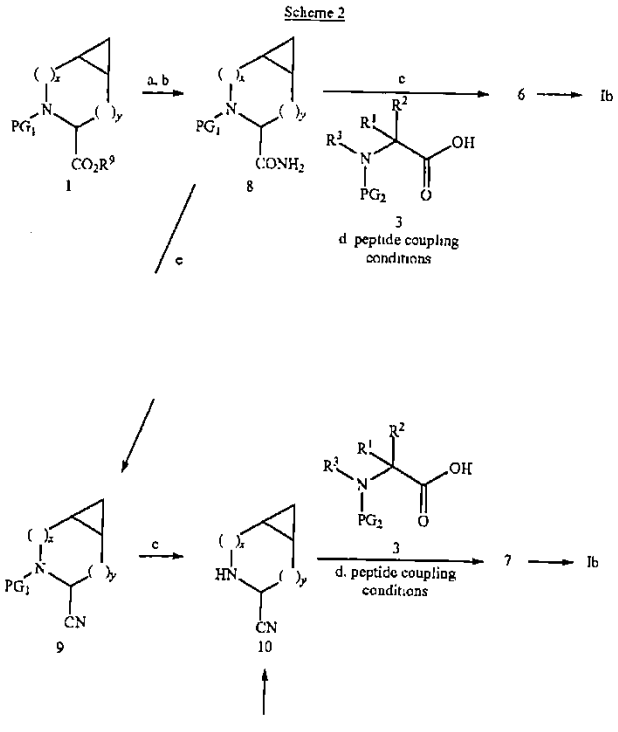


a. PG_1 = Boc, TFA or HCl, PG_1 = Cbz, H_2/Pd/C or TMSI, PG_1 = FMOC, Et_2NH
b EDAC, HOBT,DMF or i-BuOCOCl/TEA or PyBop, NMM
c. $PG_2 = PG_1$, (see conditions for a)
d. LiOH or NaOH MeOH or THF/H_2O or dioxane
e i-BuOCOCl/NMM or i-BuOCOCl/TEA or EDAC, then NH_3 in dioxane or Et_2O
f $POCl_3$, pyridine, imidazole or cyanuric chloride, DMF or TFAA, THF, pyridine

Scheme 2



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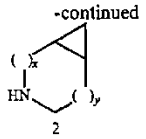
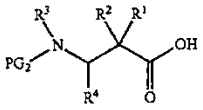


2

a. LiOH or NaOH in MeOH or THF/H_2O or dioxane
b. t-BuOCOCl/NMM or t-BuOCOCl/TEA or EDAC, then NH_3 in dioxane or Et_2O
c. PG_1 = Boc, TFA or HCl; PG_1 = Cbz, H_2/Pd/C or TMSI; PG_1 = FMOC, Et_2NH
d. EDAC, HOBT, DMF or t-BuOCOCl/TEA or PyBop, NMM
e. $POCl_3$, pyridine, imidazole or cyanuric chloride, DMF

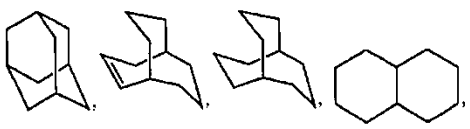
In a like manner, β-amino acids such as



may be coupled with 2, the free amine of 8, or 10 to give the corresponding amides which may be converted to the β-amino acid derivatives of compound Ia or Ib following the same chemistry.

Unless otherwise indicated, the term "lower alkyl", "alkyl" or "alk" as employed herein alone or as part of another group includes both straight and branched chain hydrocarbons, containing 1 to 20 carbons, preferably 1 to 10 carbons, more preferably 1 to 8 carbons, in the normal chain, such as methyl, ethyl, propyl, isopropyl, butyl, t-butyl, isobutyl, pentyl, hexyl, isohexyl, heptyl, 4,4-dimethylpentyl, octyl, 2,2,4-trimethyl-pentyl, nonyl, decyl, undecyl, dodecyl, the various branched chain isomers thereof, and the like as well as such groups including 1 to 4 substituents such as halo, for example F, Br, Cl or I or CF_3, alkyl, alkoxy, aryl, aryloxy, aryl(aryl) or diaryl, arylalkyl, arylalkyloxy, alkenyl, cycloalkyl, cycloalkylalkyl, cycloalkylalkyloxy, amino, hydroxy, hydroxyalkyl, acyl, heteroaryl, heteroaryloxy, heteroarylalkyl, heteroarylalkoxy, aryloxyalkyl, alkylthio, arylalkylthio, aryloxyaryl, alkylamido, alkanoylamino, arylcarbonylamino, nitro, cyano, thiol, haloalkyl, trihaloalkyl and/or alkylthio.

Unless otherwise indicated, the term "cycloalkyl" as employed herein alone or as part of another group includes saturated or partially unsaturated (containing 1 or 2 double bonds) cyclic hydrocarbon groups containing 1 to 3 rings, including monocyclic alkyl, bicyclic alkyl (or bicycloalkyl) and tricyclic alkyl (tricycloalkyl), containing a total of 3 to 20 carbons forming the ring, preferably 3 to 10 carbons, forming the ring and which may be fused to 1 or 2 aromatic rings as described for aryl, which includes cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclodecyl and cyclododecyl, cyclohexenyl, adamantyl,



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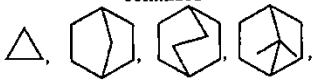
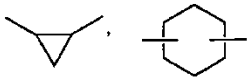


any of which groups may be optionally substituted with 1 to 4 substituents such as halogen, alkyl, alkoxy, hydroxy, aryl, aryloxy, arylalkyl, cycloalkyl, hydroxyalkyl, alkylamido, alkanoylamino, oxo, acyl, arylcarbonylamino, amino, nitro, cyano, thiol and/or alkylthio and/or any of the substituents for alkyl.

The term "cycloalkenyl" as employed herein alone or as part of another group refers to cyclic hydrocarbons containing 3 to 12 carbons, preferably 5 to 10 carbons and 1 or 2 double bonds. Exemplary cycloalkenyl groups include cyclopentenyl, cyclohexenyl, cycloheptenyl, cyclooctenyl, cyclohexadienyl, and cycloheptadienyl, which may be optionally substituted as defined for cycloalkyl.

The term "cycloalkylene" as employed herein refers to a "cycloalkyl" group which includes free bonds and thus is a linking group such as



and the like, and may optionally be substituted as defined above for "cycloalkyl".

The term "alkanoyl" as used herein alone or as part of another group refers to alkyl linked to a carbonyl group.

Unless otherwise indicated, the term "lower alkenyl" or "alkenyl" as used herein by itself or as part of another group refers to straight or branched chain radicals of 2 to 20 carbons, preferably 2 to 12 carbons, and more preferably 1 to 8 carbons in the normal chain, which include one to six double bonds in the normal chain, such as vinyl, 2-propenyl, 3-butenyl, 2-butenyl, 4-pentenyl, 3-pentenyl, 2-hexenyl, 3-hexenyl, 2-heptenyl, 3-heptenyl, 4-heptenyl, 3-octenyl, 3-nonenyl, 4-decenyl, 3-undecenyl, 4-dodecenyl, 4,8,12-tetradecatrienyl, and the like, and which may be optionally substituted with 1 to 4 substituents, namely, halogen, haloalkyl, alkyl, alkoxy, alkenyl, alkynyl, aryl, arylalkyl, cycloalkyl, amino, hydroxy, heteroaryl, cycloheteroalkyl, alkanoylamino, alkylamido, arylcarbonyl-amino, nitro, cyano, thiol, alkylthio and/or any of the alkyl substituents set out herein.

Unless otherwise indicated, the term "lower alkynyl" or "alkynyl" as used herein by itself or as part of another group refers to straight or branched chain radicals of 2 to 20 carbons, preferably 2 to 12 carbons and more preferably 2 to 8 carbons in the normal chain, which include one triple bond in the

normal chain, such as 2-propynyl, 3-butynyl, 2-butynyl, 4-pentynyl, 3-pentynyl, 2-hexynyl, 3-hexynyl, 2-heptynyl, 3-heptynyl, 4-heptynyl, 3-octenyl, 3-nonenyl, 4-decenyl,3-undecenyl, 4-dodecenyl and the like, and which may be optionally substituted with 1 to 4 substituents, namely, halogen, haloalkyl, alkyl, alkoxy, alkenyl, alkynyl, aryl, arylalkyl, cycloalkyl, amino, heteroaryl, cycloheteroalkyl, hydroxy, alkanoylamino, alkylamido, arylcarbonylamino, nitro, cyano, thiol, and/or alkylthio, and/or any of the alkyl substituents set out herein.

The terms "arylalkenyl" and "arylalkynyl" as used alone or as part of another group refer to alkenyl and alkynyl groups as described above having an aryl substituent.

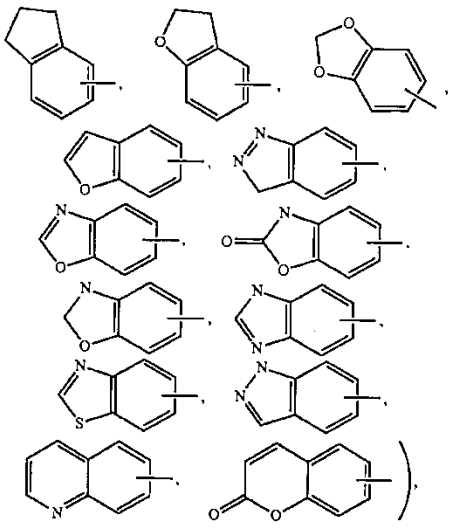
Where alkyl groups as defined above have single bonds for attachment to other groups at two different carbon atoms, they are termed "alkylene" groups and may optionally be suhstituted as defined above for "alkyl".

Where alkenyl groups as defined above and alkynyl groups as defined above, respectively, have single bonds for attachment at two different carbon atoms, they are termed "alkenylene groups" and "alkynylene groups", respectively, and may optionally be substituted as defined above for "alkenyl" and "alkynyl".

The term "halogen" or "halo" as used herein alone or as part of another group refers to chlorine, bromine, fluorine, and iodine as well as CF_3, with chlorine or fluorine being preferred.

The term "metal ion" refers to alkali metal ions such as sodium, potassium or lithium and alkaline earth metal ions such as magnesium and calcium, as well as zinc and aluminum.

Unless otherwise indicated, the term "aryl" as employed herein alone or as part of another group refers to monocyclic and bicyclic aromatic groups containing 6 to 10 carbons in the ring portion (such as phenyl or naphthyl including 1-naphthyl and 2-naphthyl) and may optionally include one to three additional rings fused to a carbocyclic ring or a heterocyclic ring (such as aryl, cycloalkyl, heteroaryl or cycloheteroalkyl rings for example



and may be optionally substituted through available carbon atoms with 1, 2, or 3 groups selected from hydrogen, halo, haloalkyl, alkyl, haloalkyl, alkoxy, haloalkoxy, alkenyl, trifluoromethyl, trifluoromethoxy, alkynyl, cycloalkylalkyl, cycloheteroalkyl, cycloheteroalkylalkyl, aryl, heteroaryl, arylalkyl, aryloxy, aryloxyalkyl, arylalkoxy, arylthio, arylazo, heteroarylalkyl, heteroarylalkenyl, heteroarylheteroaryl, heteroaryloxy, hydroxy, nitro, cyano, amino, substituted amino wherein the amino includes 1 or 2 substituents (which are alkyl, aryl or any of the other aryl compounds mentioned in the definitions), thiol, alkylthio, arylthio, heteroarylthio, arylthioalkyl, alkoxyarylthio, alkylcarbonyl, arylcarbonyl, alkylaminocarbonyl, arylaminocarbonyl, alkoxycarbonyl, aminocarbonyl, alkylcarbonyloxy, arylcarbonyloxy, alkylcarbonylamino, arylcarhonylamino, arylsulfinyl, arylsulfinylalkyl, arylsulfonylamino or arylsulfonaminocarhonyl and/or any of the alkyl substituents set out herein.

Unless otherwise indicated, the term "lower alkoxy", "alkoxy", "aryloxy" or "aralkoxy" as employed herein alone or as part of another group includes any of the above alkyl, aralkyl or aryl groups linked to an oxygen atom.

Unless otherwise indicated, the term "substituted amino" as employed herein alone or as part of another group refers to amino substituted with one or two substituents, which may be the same or different, such as alkyl, aryl, arylalkyl, heteroaryl, heteroarylalkyl, cycloheteroalkyl, cycloheteroalkylalkyl, cycloalkyl, cycloalkylalkyl haloalkyl, hydroxyalkyl, alkoxyalkyl or thioalkyl. These substituents may he further substituted with any of the R^1 groups or substituents for R^1 as set out above. In addition, the amino substituents may be taken together with the nitrogen atom to which they are attached to form 1-pyrrolidinyl, 1-piperidinyl, 1-azepinyl, 4-morpholinyl, 4-thiamorpholinyl, 1-piperazinyl, 4-alkyl-1-piperazinyl, 4-arylalkyl-1-piperazinyl, 4-diarylalkyl-1-piperazinyl, 1-pyrrolidinyl, 1-piperidinyl, or 1-azepinyl, optionally substituted with alkyl, alkoxy, alkylthio, halo, trifluoromethyl or hydroxy.

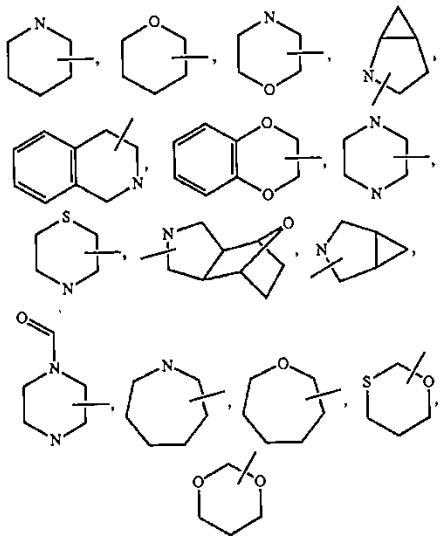
Unless otherwise indicated, the term "lower alkylthio", "alkylthio", "arylthio" or "aralkylthio" as employed herein alone or as part of another group includes any of the above alkyl, aralkyl or aryl groups linked to a sulfur atom.

Unless otherwise indicated, the term "lower alkylamino", "alkylamino", "arylamino", or "arylalkylamino" as employed herein alone or as part of another group includes any of the above alkyl, aryl or arylalkyl groups linked to a nitrogen atom.

Unless otherwise indicated, the term "acyl" as employed herein by itself or part of another group, as defined herein, refers to an organic radical linked to a carbonyl

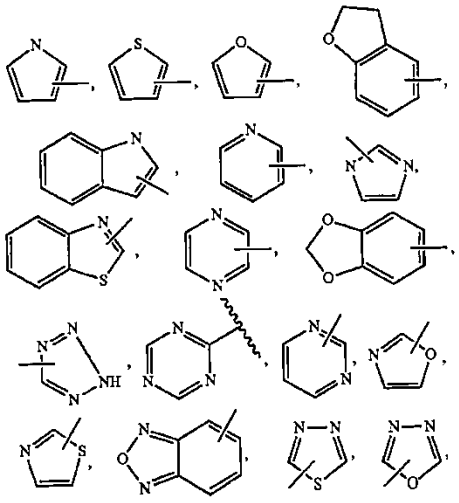
group; examples of acyl groups include any of the R^1 groups attached to a carbonyl, such as alkanoyl, alkenoyl, aroyl, aralkanoyl, heteroaroyl, cycloalkanoyl, cycloheteroalkanoyl and the like.

Unless otherwise indicated, the term "cycloheteroalkyl" as used herein alone or as part of another group refers to a 5-, 6- or 7-membered saturated or partially unsaturated ring which includes 1 to 2 hetero atoms such as nitrogen, oxygen and/or sulfur, linked through a carbon atom or a heteroatom, where possible, optionally via the linker $(CH_2)_r$ (where r is 1, 2 or 3), such as:



and the like. The above groups may include 1 to 4 substituents such as alkyl, halo, oxo and/or any of the alkyl substituents set out herein. In addition, any of the cycloheteroalkyl rings can be fused to a cycloalkyl, aryl, heteroaryl or cycloheteroalkyl ring.

Unless otherwise indicated, the term "heteroaryl" as used herein alone or as part of another group refers to a 5- or 6-membered aromatic ring which includes 1, 2, 3 or 4 hetero atoms such as nitrogen, oxygen or sulfur, and such rings fused to an aryl, cycloalkyl, heteroaryl or cycloheteroalkyl ring (e.g. benzothiophenyl, indolyl), and includes possible N-oxides. The heteroaryl group may optionally include 1 to 4 substituents such as any of the substituents set out above for alkyl. Examples of heteroaryl groups include the following:



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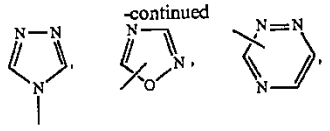


and the like.

The term "cycloheteroalkylalkyl" as used herein alone or as part of another group refers to cycloheteroalkyl groups as defined above linked through a C atom or heteroatom to a $(CH_2)_r$ chain.

The term "heteroarylalkyl" or "heteroarylalkenyl" as used herein alone or as part of another group refers to a heteroaryl group as defined above linked through a C atom or heteroatom to a —$(CH_2)_r$— chain, alkylene or alkenylene as defined above.

The term "polyhaloalkyl" as used herein refers to an "alkyl" group as defined above which includes from 2 to 9, preferably from 2 to 5, halo substituents, such as F or Cl, preferably F, such as CF_3CH_2, CF_3 or $CF_3CF_2CH_2$.

The term "polyhaloalkoxy" as used herein refers to an "alkoxy" or "alkyloxy" group as defined above which includes from 2 to 9, preferably from 2 to 5, halo substituents, such as F or Cl, preferably F, such as CF_3CH_2O, CF_3O or $CF_3CF_2CH_2O$.

All stereoisomers of the compounds of the instant invention are contemplated, either in admixture or in pure or substantially pure form. The compounds of the present invention can have asymmetric centers at any of the carbon atoms including any one or the R substituents. Consequently, compounds of formula I can exist in enantiomeric or diastereomeric forms or in mixtures thereof. The processes for preparation can utilize racemates, enantiomers or diastereomers as starting materials. When diastereomeric or enantiomeric products are prepared, they can be separated by conventional methods for example, chromatographic or fractional crystallization.

Where desired, the compounds of structure I may be used in combination with one or more other types of antidiabetic agents (employed to treat diabetes and related diseases) and/or one or more other types of therapeutic agents which may be administered orally in the same dosage form, in a separate oral dosage form or by injection.

The other type of antidiabetic agent which may be optionally employed in combination with the DP4 inhibitor of formula I may be 1,2,3 or more antidiabetic agents or antihyperglycemic agents including insulin secretagogues or insulin sensitizers, or other antidiabetic agents preferably having a mechanism of action different from DP4 inhibition and may include biguanides, sulfonyl ureas, glucosidase inhibitors, PPAR γ agonists, such as thiazolidinediones, SGLT2 inhibitors, PPAR α/γ dual agonists, aP2 inhibitors, glycogen phosphorylase inhibitors, advanced glycosylation end (AGE) products inhibitors, and/or meglitinides, as well as insulin, and/or glucagon-like peptide-1 (GLP-1) or mimetics thereof.

It is believed that the use of the compounds of structure I in combination with 1, 2, 3 or more other antidiabetic agents produces antihyperglycemic results greater than that possible from each of these medicaments alone and greater than the combined additive antihyperglycemic effects produced by these medicaments.

The other antidiabetic agent may be an oral antihyperglycemic agent preferably a biguanide such as metformin or phenformin or salts thereof, preferably metformin HCl.

Where the other antidiabetic agent is a biguanide, the compounds of structure I will be employed in a weight ratio to biguanide within the range from about 0.01:1 to about 100:1, preferably from about 0.1:1 to about 5:1.

The other antidiabetic agent may also preferably be a sulfonyl urea such as glyburide (also known as glibenclamide), glimepiride (disclosed in U.S. Pat. No. 4,379,785), glipizide, gliclazide or chlorpropamide, other known sulfonylureas or other antihyperglycemic agents which act on the ATP-dependent channel of the β-cells, with glyburide and glipizide being preferred, which may be administered in the same or in separate oral dosage forms.

The compounds of structure I will be employed in a weight ratio to the sulfonyl urea in the range from about 0.01:1 to about 100:1, preferably from about 0.05:1 to about 5:1.

The oral antidiabetic agent may also be a glucosidase inhibitor such as acarbose (disclosed in U.S. Pat. No. 4,904,769) or miglitol (disclosed in U.S. Pat. No. 4,639,436), which may be administered in the same or in a separate oral dosage forms.

The compounds of structure I will be employed in a weight ratio to the glucosidase inhibitor within the range from about 0.01:1 to about 100:1, preferably from about 0.2:1 to about 50:1.

The compounds of structure I may be employed in combination with a PPAR γ agonist such as a thiazolidinedione oral anti-diabetic agent or other insulin sensitizers (which has an insulin sensitivity effect in NIDDM patients) such as troglitazone (Warner-Lambert's Rezulin®, disclosed in U.S. Pat. No. 4,572,912), rosiglitazone (en), pioglitazone (Takeda), Mitsubishi MCC-555 (disclosed in U.S. Pat. No. 5,594,016), Glaxo-Wellcome's GL-262570, englitazone (CP-68722, Pfizer) or darglitazone (CP-86325, Pfizer, isaglitazone (MIT/J&J), JTT-501 (JPNT/P&U), L-895645 (Merck), R-119702 (Sankyo/WL), NN-2344 (Dr. Reddy/NN), or YM-440 (Yamanouchi), preferably rosiglitazone and pioglitazone.

The compounds of structure I will be employed in a weight ratio to the thiazolidinedione in an amount within the range from about 0.01:1 to about 100:1, preferably from about 0.1:1 to about 10:1.

The sulfonyl urea and thiazolidinedione in amounts of less than about 150 mg oral antidiabetic agent may be incorporated in a single tablet with the compounds of structure I.

The compounds of structure I may also be employed in combination with a antihyperglycemic agent such as insulin or with glucagon-like peptide-1 (GLP-1) such as GLP-1(1-36) amide, GLP-1(7-36) amide, GLP-1(7-36) (as disclosed in U.S. Pat. No. 5,614,492 to Habener, disclosure of which is incorporated herein by reference), or a GLP-1 mimic such as AC2993 or Exendin-4 (Amylin) and LY-315902 or LY-307167 (Lilly) and NN2211 (Novo-Nordisk), which may be administered via injection, intranasal, or by transdermal or buccal devices.

Where present, metformin, the sulfonyl ureas, such as glyburide, glimepiride, glipyride, glipizide, chlorpropamide and gliclazide and the glucosidase inhibitors acarbose or miglitol or insulin (injectable, pulmonary, buccal, or oral) may be employed in formulations as described above and in amounts and dosing as indicated in the Physician's Desk Reference (PDR).

Where present, metformin or salt thereof may be employed in amounts within the range from about 500 to about 2000 mg per day which may be administered in single or divided doses one to four times daily.

Where present, the thiazolidinedione anti-diabetic agent may be employed in amounts within the range from about 0.01 to about 2000 mg/day which may be administered in single or divided doses one to four times per day.

Where present insulin may be employed in formulations, amounts and dosing as indicated by the Physician's Desk Reference.

Where present GLP-1 peptides may be administered in oral buccal formulations, by nasal administration (for example inhalation spray) or parenterally as described in U.S. Pat. Nos. 5,346,701 (TheraTech), 5,614,492 and 5,631,224 which are incorporated herein by reference.

The other antidiabetic agent may also be a PPAR α/γ dual agonist such as AR-HO39242 (Astra/Zeneca), GW-409544 (Glaxo-Wellcome), KRP297 (Kyorin Merck) as well as those disclosed by Murakami et al, "A Novel Insulin Sensitizer Acts As a Coligand for Peroxisome Proliferation—Activated Receptor Alpha (PPAR alpha) and PPAR gamma. Effect on PPAR alpha Activation on Abnormal Lipid Metabolism in Liver of Zucker Fatty Rats", Diabetes 47, 1841-1847 (1998), and in U.S. application Ser. No. 09/664,598, filed Sep. 18, 2000, (attorney file LA29NP) the disclosure of which is incorporated herein by reference, employing dosages as set out therein, which compounds designated as preferred are preferred for use herein.

The other antidiabetic agent may be an SGLT2 inhibitor such as disclosed in U.S. application Ser. No. 09/679,027, filed Oct. 4, 2000 (attorney file LA49NP), which is incorporated herein by reference, employing dosages as set out herein. Preferred are the compounds designated as preferred in the above application.

The other antidiabetic agent which may be optionally employed in combination with the DP4 inhibitor of formula I may be an aP2 inhibitor such as disclosed in U.S. application Ser. No. 09/391,053, filed Sep. 7, 1999, and U.S. application Ser. No. 09/519,079, filed Mar. 6, 2000 (attorney file LA27NP), which is incorporated berein by reference, employing dosages as set out herein. Preferred are the compounds designated as preferred in the above application.

The other antidiabetic agent which may be optionally employed in combination with the DP4 inhibitor of formula I may be a glycogen phosphorylase inhibitor such as disclosed in WO 96/39384, WO 96/39385, EP 978279, WO 2000/47206, WO 99/43663, and U.S. Pat. Nos. 5,952,322 and 5,998,463, WO 99/26659 and EP 1041068.

The meglitinide which may optionally be employed in combination with the compound of formula I of the invention may be repaglinide, nateglinide (Novartis) or KAD1229 (PF/Kissei), with repaglinide being preferred.

The DP4 inhibitor of formula I will be employed in a weight ratio to the meglitinide, PPAR γ agonist, PPAR α/γ dual agonist, SGLT2 inhibitor, aP2 inhibitor, or glycogen phosphorylase inhibitor within the range from about 0.01:1 to about 100:1, preferably from about 0.1:1 to about 10:1.

The hypolipidemic agent or lipid-modulating agent which may be optionally employed in combination with the compounds of formula I of the invention may include 1,2,3 or more MTP inhibitors, HMG CoA reductase inhibitors, squalene synthetase inhibitors, fibric acid derivatives, ACAT inhibitors, lipoxygenase inhibitors, cholesterol absorption inhibitors, ileal Na^+/bile acid cotransporter inhibitors, upregulators of LDL receptor activity, ATP citrate lyase inhibitors, cholesteryl ester transfer protein inhibitors, bile acid sequestrants, and/or nicotinic acid and derivatives thereof.

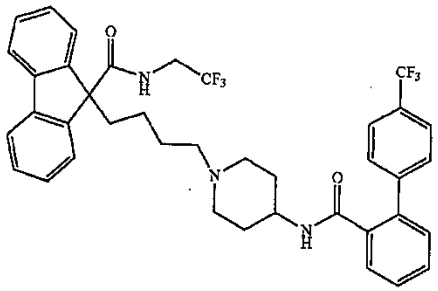
MTP inhibitors employed herein include MTP inhibitors disclosed in U.S. Pat. No. 5,595,872, U.S. Pat. No. 5,739,135, U.S. Pat. No. 5,712,279, U.S. Pat. No. 5,760,246, U.S. Pat. No. 5,827,875, U.S. Pat. No. 5,885,983 and U.S. application

Ser. No. 09/175,180 filed Oct. 20, 1998, now U.S. Pat. No. 5,962,440. Preferred are each of the preferred MTP inhibitors disclosed in each of the above patents and applications.

All of the above U.S. Patents and applications are incorporated herein by reference.

Most preferred MTP inhibitors to be employed in accordance with the present invention include preferred MTP inhibitors as set out in U.S. Pat. Nos. 5,739,135 and 5,712,279, and U.S. Pat. No. 5,760,246 as well as implitapide (Bayer).

The most preferred MTP inhibitor is 9-[4-[4-[[2-(2,2,2-Trifluoroethoxy)benzoyl]amino]-1-piperidinyl] butyl]-N-(2,2,2-trifluoroethyl)-9H-fluorene-9-carboxamide



The hypolipidemic agent may be an HMG CoA reductase inhibitor which includes, but is not limited to, mevastatin and related compounds as disclosed in U.S. Pat. No. 3,983,140, lovastatin (mevinolin) and related compounds as disclosed in U.S. Pat. No. 4,231,938, pravastatin and related compounds such as disclosed in U.S. Pat. No. 4,346,227, simvastatin and related compounds as disclosed in U.S. Pat. Nos. 4,448,784 and 4,450,171. Other HMG CoA reductase inhibitors which may be employed herein include, but are not limited to, fluvastatin, disclosed in U.S. Pat. No. 5,354,772, cerivastatin disclosed in U.S. Pat. Nos. 5,006,530 and 5,177,080, atorvastatin disclosed in U.S. Pat. Nos. 4,681,893, 5,273,995, 5,385,929 and 5,686,104, atavastatin (Nissan/Sankyo nisvastatin (NK-104)) disclosed in U.S. Pat. No. 5,011,930, Shionogi-Astra/Zeneca visastatin (ZD-4522) disclosed in U.S. Pat. No. 5,260,440.

The squalene synthetase inhibitors suitable for use herein include, but are not limited to, a-phosphono-sulfonates disclosed in U.S. Pat. No. 5,712,396, those disclosed by Biller et al, J. Med. Chem., 1988, Vol. 11, No. 10, pp 1869-1871, including isoprenoid (phosphinyl-methyl) phosphonates as well as other known squalene synthetase inhibitors, for example, as disclosed in U.S. Pat. Nos. 4,871.721 and 4,924,024 and in Biller, S. A., Neuenschwander, K., Ponpipom, M. M., and Poulter, C. D., Current Pharmaceutical Design, 2, 1-40 (1996).

In addition, other squalene synthetase inhibitors suitable for use herein include the terpenoid pyrophosphates disclosed by P. Ortiz de Montellano et al, J. Med. Chem., 1977, 20, 243-249, the farnesyl diphosphate analog A and presqualene pyrophosphate (PSQ-PP) analogs as disclosed by Corey and Volante, J. Am. Chem. Soc., 1976, 98, 1291-1293, phosphinylphosphonates reported by McClard, R. W. et al, J.A.C.S., 1987, 10, 5544 and cyclopropanes reported by Capson, T. L., PhD dissertation, June, 1987, Dept. Med. Chem. U of Utah, Abstracts Table of Contents, pp 16, 17, 40-43, 48-51, Summary.

Other hypolipidemic agents suitable for use herein include, but are not limited to, fibric acid derivatives, such as fenofibrate, gemfibrozil, clofibrate, bezafibrate, ciprofibrate, clinofibrate and the like, probucol, and related compounds as disclosed in U.S. Pat. No. 3,674,836, probucol and gemfibrozil being preferred, bile acid sequestrants such as cholestyramine, colestipol and DEAE-Sephadex (Secholex®, Policexide®), as well as lipostabil (Rhone-Poulenc), Eisai E-5050 (an N-substituted ethanolamine derivative), imanixil (HOE-402), tetrahydrolipstatin (THL), istigmastanylphos-phorylcholine (SPC, Roche), aminocyclodextrin (Tanabe Seiyoku), Ajinomoto AJ-814 (azulene derivative), melinamide (Sumitomo), Sandoz 58-035, American Cyanamid CL-277,082 and CL-283,546 (disubstituted urea derivatives), nicotinic acid, acipimox, acifran, neomycin, p-aminosalicylic acid, aspirin, poly (diallylmethylamine) derivatives such as disclosed in U.S. Pat. No. 4,759,923, quaternary amine poly (diallyldimethylammonium chloride) and ionenes such as disclosed in U.S. Pat. No. 4,027,009, and other known serum cholesterol lowering agents.

The other hypolipidemic agent may be an ACAT inhibitor such as disclosed in, Drugs of the Future 24, 9-15 (1999), (Avasimibe); "The ACAT inhibitor, Cl-1011 is effective in the prevention and regression of aortic fatty streak area in hamsters", Nicolosi et al, Atherosclerosis (Shannon, Irel). (1998), 137(1), 77-85; "The pharmacological profile of FCE 27677: a novel ACAT inhibitor with potent hypolipidemic activity mediated by selective suppression of the hepatic secretion of ApoB100-containing lipoprotein", Ghiselli, Giancarlo, Cardiovasc. Drug Rev. (1998), 16(1), 16-30; "RP 73163: a bioavailable alkylsulfinyl-diphenylimidazole ACAT inhibitor", Smith, C., et al, Bioorg. Med. Chem. Lett. (1996), 6(1), 47-50; "ACAT inhibitors: physiologic mechanisms for hypolipidemic and anti-atherosclerotic activities in experimental animals", Krause et al, Editor(s): Ruffolo, Robert R., Jr.; Hollinger, Mannfred A., Inflammation: Mediators Pathways (1995), 173-98, Publisher: CRC, Boca Raton, Fla.; "ACAT inhibitors: potential anti-atherosclerotic agents", Sliskovic et al, Curr. Med. Chem. (1994), 1(3), 204-25; "Inhibitors of acyl-CoA:cholesterol O-acyl transferase (ACAT) as hypocholesterolemic agents. 6. The first water-soluble ACAT inhibitor with lipid-regulating activity. Inhibitors of acyl-CoA:cholesterol acyltransferase (ACAT). 7. Development of a series of substituted N-phenyl-N'-[(1-phenylcyclopentyl) methyl]ureas with enhanced hypocholesterolemic activity", Stout et al, Chemtracts: Org. Chem. (1995), 8(6), 359-62, or TS-962 (Taisho Pharmaceutical Co. Ltd).

The hypolipidemic agent may be an upregulator of LD2 receptor activity such as MD-700 (Taisho Pharmaceutical Co. Ltd) and LY295427 (Eli Lilly).

The hypolipidemic agent may be a cholesterol absorption inhibitor preferably Schering-Plough's SCH48461 as well as those disclosed in Atherosclerosis 115, 45-63 (1995) and J. Med. Chem. 41, 973 (1998).

The hypolipidemic agent may be an ileal Na^+/bile acid cotransporter inhibitor such as disclosed in Drugs of the Future, 24, 425-430 (1999).

The lipid-modulating agent may be a cholesteryl ester transfer protein (CETP) inhibitor such as Pfizer's CP 529, 414 (WO/0038722 and EP 818448) and Pharmacia's SC-744 and SC-795.

The ATP citrate lyase inhibitor which may be employed in the combination of the invention may include, for example, those disclosed in U.S. Pat. No. 5,447,954.

Preferred hypolipidemic agents are pravastatin, lovastatin, simvastatin, atorvastatin, fluvastatin, cerivastatin, atavastatin and ZD-4522.

The above-mentioned U.S. patents are incorporated herein by reference. The amounts and dosages employed will be as indicated in the Physician's Desk Reference and/or in the patents set out above.

The compounds of formula I of the invention will be employed in a weight ratio to the hypolipidemic agent (were present), within the range from about 500:1 to about 1:500, preferably from about 100:1 to about 1:100.

The dose administered must be carefully adjusted according to age, weight and condition of the patient, as well as the route of administration, dosage form and regimen and the desired result.

The dosages and formulations for the hypolipidemic agent will be as disclosed in the various patents and applications discussed above.

The dosages and formulations for the other hypolipidemic agent to be employed, where applicable, will be as set out in the latest edition of the Physicians' Desk Reference.

For oral administration, a satisfactory result may be obtained employing the MTP inhibitor in an amount within the range of from about 0.01 mg/kg to about 500 mg and preferably from about 0.1 mg to about 100 mg, one to four times daily.

A preferred oral dosage form, such as tablets or capsules, will contain the MTP inhibitor in an amount of from about 1 to about 500 mg, preferably from about 2 to about 400 mg, and more preferably from about 5 to about 250 mg, one to four times daily.

For oral administration, a satisfactory result may be obtained employing an HMG CoA reductase inhibitor, for example, pravastatin, lovastatin, simvastatin, atorvastatin, fluvastatin or cerivastatin in dosages employed as indicated in the Physician's Desk Reference, such as in an amount within the range of from about 1 to 2000 mg, and preferably from about 4 to about 200 mg.

The squalene synthetase inhibitor may be employed in dosages in an amount within the range of from about 10 mg to about 2000 mg and preferably from about 25 mg to about 200 mg.

A preferred oral dosage form, such as tablets or capsules, will contain the HMG CoA reductase inhibitor in an amount from about 0.1 to about 100 mg, preferably from about 5 to about 80 mg, and more preferably from about 10 to about 40 mg.

A preferred oral dosage form, such as tablets or capsules will contain the squalene synthetase inhibitor in an amount of from about 10 to about 500 mg, preferably from about 25 to about 200 mg.

The other hypolipidemic agent may also be a lipoxygevase inhibitor including a 15-lipoxygenase (15-LO) inhibitor such as benzimidazole derivatives as disclosed in WO 97/12615, 15-LO inhibitors as disclosed in WO 97/12613, isothiazolones as disclosed in WO 96/38144, and 15-LO inhihitors as disclosed by Sendobry et al "Attenuation of diet-induced atherosclerosis in rabbits with a highly selective 15-lipoxygenase inhibitor lacking significant antioxidant properties", Brit. J. Pharmacology (1997) 120, 1199-1206, and Cornicelli et al, "15-Lipoxygenase and its Inhibition: A Novel Therapeutic Target for Vascular Disease", Current Pharmaceutical Design, 1999, 5, 11-20.

The compounds of formula I and the hypolipidemic agent may be employed together in the same oral dosage form or in separate oral dosage forms taken at the same time.

The compositions described above may be administered in the dosage forms as described above in single or divided doses of one to four times daily. It may be advisable to start a patient on a low dose combination and work up gradually to a high dose combination.

The preferred hypolipidemic agent is pravastatin, simvastatin, lovastatin, atorvastatin, fluvastatin or cerivastatin.

The other type of therapeutic agent which may be optionally employed with the DP4 inhibitor of formula I may be 1, 2, 3 or more of an anti-obesity agent including a beta 3 adrenergic agonist, a lipase inhibitor, a serotonin (and dopamine) reuptake inhibitor, a thyroid receptor beta drug, an anorectic agent and/or a fatty acid oxidation upregulator.

The beta 3 adrenergic agonist which may be optionally employed in combination with a compound of formula I may be AJ9677 (Takeda/Dainippon), L750355 (Merck), or CP331648 (Pfizer) or other known beta 3 agonists as disclosed in U.S. Pat. Nos. 5,541,204, 5,770,615, 5,491,134, 5,776,983 and 5,488,064, with AJ9677, L750,355 and CP331648 being preferred.

The lipase inhibitor which may be optionally employed in combination with a compound of formula I may be orlistat or ATL-962 (Alizyme), with orlistat being preferred.

The serotonin (and dopoamine) reuptake inhibitor which may be optionally employed in combination with a compound of formula I may be sibutramine, topiramate (Johnson & Johnson) or axokine (Regeneron), with sibutramine and topiramate being preferred.

The thyroid receptor beta compound which may be optionally employed in combination with a compound of formula I may be a thyroid receptor ligand as disclosed in WO97/21993 (U. Cal SF), WO099/00353 (KaroBio) and GB98/284425 (KaroBio), with compounds of the KaroBio applications being preferred.

The anorectic agent which may be optionally employed in combination with a compound of formula I may be dexamphetamine, phentermine, phenylpropanolamine or mazindol, with dexamphetamine being preferred.

The fatty acid oxidation upregulator which may be optionally employed in combination with the compound of formula I can be famoxin (Genset).

The varions anti-obesity agents described above may be employed in the same dosage form with the compound of formula I or in different dosage forms, in dosages and regimens as generally known in the art or in the PDR.

The infertility agent which may be optionally employed in combination with the DP4 inhibitor of the invention may be 1, 2, or more of clomiphene citrate (Clomid®, Aventis), bromocriptine mesylate (Parlodel®, Novartis),LHRH analogs, Lupron (TAP Pharm.), danazol, Danocrine (Sanofi), progestogens or glucocorticoids, which may be employed in amounts specified in the PDR.

The agent for polycystic ovary syndrome which may be optionally employed in combination with the DP4 inhibitor of the invention may be 1, 2, or more of gonadotropin releasing hormone (GnRH), leuprolide (Lupron®), Clomid®, Parlodel®, oral contraceptives or insulin sensitizers such as PPAR agonists, or other conventional agents for such use which may be employed in amounts specified in the PDR.

The agent for treating growth disorders and/or frailty which may be optionally employed in combination with the DP4 inhibitor of the invention may be 1, 2, or more of a growth hormone or growth hormone secretagogue such as MK-677 (Merck), CP-424,391 (Pfizer), and compounds disclosed in U.S. Ser. No. 09/506,749 filed Feb. 18, 2000 (attorney docket LA26), as well as selective androgen receptor modulators (SARMs), which is incorporated herein by reference, which may be employed in amounts specified in the PDR, where applicable.

The agent for treating arthritis which may be optionally employed in combination with the DP4 inhibitor of the invention may be 1, 2, or more of aspirin, indomethacin, ibuprofen, diclofenac sodium, naproxen, nabumetone (Relafen®, SmithKline Beecham), tolmetin sodium (Tolectin®, Ortho-McNeil), piroxicam (Feldene®, Pfizer), ketorolac tromethamine (Toradol®, Roche), celecoxib (Celebrex®, Searle), rofecoxib (Vioxx®, Merck) and the like, which may be employed in amounts specified in the PDR.

Conventional agents for preventing allograft rejection in transplantation such as cyclosporin, Sandimmune (Novartis), azathioprine, Immuran (Faro) or methotrexate may be optionally employed in combination with the DP4 inhibitor of the invention, which may be employed in amounts specified in the PDR.

Conventional agents for treating autoimmune diseases such as multiple sclerosis and immunomodulatory diseases such as lupus erythematosis, psoriasis, for example, azathioprine, Immuran, cyclophosphamide, NSAIDS such as ibuprofen, cox 2 inhibitors such as Vioxx and Celebrex, glucocorticoids and hydroxychloroquine, may be optionally employed in combination with the DP4 inhibitor of the invention, which may be employed in amounts specified in the PDR.

The AIDS agent which may be optionally employed in combination with the DP4 inhibitor of the invention may be a non-nucleoside reverse transcriptase inhibitor, a nucleoside reverse transcriptase inhibitor, a protease inhibitor and/or an AIDS adjunct anti-infective and may be 1, 2, or more of dronabinol (Marinol®, Roxane Labs), didanosine (Videx®, Bristol-Myers Squibb), megestrol acetate (Megace®, Bristol-Myers Squibb), stavudine (Zerit®, Bristol-Myers Squibb), delavirdine mesylate (Rescriptor®, Pharmacia), lamivudine/zidovudine (Combivir™, Glaxo), lamivudine (Epivir™, Glaxo), zalcitabine (Hivid®, Roche), zidovudine (Retrovir®, Glaxo), indinavir sulfate (Crixivan®, Merck), saquinavir (Fortovase™, Roche), saquinovir mesylate (Invirase®, Roche), ritonavir (Norvir®, Abbott), nelfinavir (Viracept®, Agouron).

The above anti-AIDS agents may be employed in amounts specified in the PDR.

The agent for treating inflammatory bowel disease or syndrome which may be optionally employed in combination with the DP4 inhibitor of the invention may be 1, 2, or more of sulfasalazine, salicylates, mesalamine (Asacol®, P&G) or Zelmac®, (Bristol-Myers Squibb), which may be employed in amounts specified in the PDR or otherwise known in the art.

The agent for treating osteoporosis which may be optionally employed in combination with the DP4 inhibitor of the invention may be 1, 2, or more of alendronate sodium (Fosamax®, Merck, tiludronate (Skelid®, Sanofi), etidronate disodium (Didronel®, P&G), raloxifene HCl (Evista®, Lilly), which may be employed in amounts specified in the PDR.

In carrying our the method of the invention, a pharmaceutical composition will be employed containing the compounds of structure I, with or without another antidiabetic agent and/or other type therapeutic agent, in association with a pharmaceutical vehicle or diluent. The pharmaceutical composition can be formulated employing conventional solid or liquid vehicles or diluents and pharmaceutical additives of a type appropriate to the mode of desired administration. The compounds can be administered to mammalian species including humans, monkeys, dogs, etc. by an oral route, for example, in the form of tablets, capsules, granules or powders, or they can be administered by a parenteral route in the form of injectable preparations. The dose for adults is preferably between 10 and 1,000 mg per day, which can be administered in a single dose or in the form of individual doses from 1-4 times per day.

A typical capsule for oral administration contains compounds of structure I (250 mg), lactose (75 mg) and magnesium stearate (15 mg). The mixture is passed through a 60 mesh sieve and packed into a No. 1 gelatin capsule.

A typical injectable preparation is produced by aseptically placing 250 mg of compounds of structure I into a vial, aseptically freeze-drying and sealing. For use, the contents of the vial are mixed with 2 mL of physiological saline, to produce an injectable preparation.

DP4 inhibitor activity of the compounds of the invention may be determined by use of an in vitro assay system which measures the potentiation of inhibition of DP4. Inhibition constants (Ki values) for the DP4 inhibitors of the invention may be determined by the method described below.

Purification of Porcine Dipeptidyl Peptidase IV

Porcine enzyme was purified as previously described (1), with several modifications. Kidneys from 15-20 animals were obtained, and the cortex was dissected away and frozen at −80° C. Frozen tissue (2000 -2500 g) was homogenized in 12 L of 0.25 M sucrose in a Waring blender. The homogenate then was left at 37° C. for 18 hours to facilitate cleavage of DP-4 from cell membranes. After the cleavage step, the homogenate was clarified by centrifugation at 7000×g for 20 min at 4° C., and the supernatant was collected. Solid ammonium sulfate was added to 60% saturation, and the precipitate was collected by centrifugation at 10,000×g and was discarded. Additional ammonium sulfate was added to the supernatant to 80% saturation, and the 80% pellet was collected and dissolved in 20 mM Na_2HPO_4, pH 7.4.

After dialysis against 20 mM Na_2HPO_4, pH 7.4, the preparation was clarified by centrifugation at 10,000×g. The clarified preparation then was applied to 300 mL of ConA Sepharose that had been equilibrated in the same buffer. After washing with buffer to a constant A_{280}, the column was eluted with 5% (w/v) methyl α-D-mannopyranoside. Active fractions were pooled, concentrated, and dialyzed against 5 mM sodium acetate, pH 5.0. Dialyzed material then was flowed through a 100 mL Pharmacia Resource S column equilibrated in the same buffer. The flow through material was collected and contained most of the enzyme activity. Active material again was concentrated and dialyzed into 20 mM Na_2HPO_4, pH 7.4. Lastly, the concentrated enzyme was chromatographed on a Pharmacia S-200 gel filtration column to removed low molecular weight contaminants. Purity of column fractions was analyzed by reducing SDS-PAGE, and the purest fractions were pooled and concentrated. Purified enzyme was stored in 20% glycerol at −80° C.

Assay of Porcine Dipeptidyl Peptidase IV

Enzyme was assayed under steady-state conditions as previously described (2) with gly-pro-p-nitroanilide as substrate, with the following modifications. Reactions contained, in a final volume of 100 μl, 100 mM Aces, 52 mM TRIS, 52 mM ethanolamine, 500 μM gly-pro-p-nitroanilide, 0.2 % DMSO, and 4.5 nM enzyme at 25° C., pH 7.4. For single assays at 10 μM test compound, buffer, compound, and enzyme were added to wells of a 96 well microtiter plate, and were incubated at room temperature for 5 min. Reactions were started by addition of substrate, The continuous production of p-nitroaniline was measured at 405 nM for 15 min using a Molecular Devices Tmax plate reader, with a read every 9

seconds. The linear rate of p-nitroaniline production was obtained over the linear portion of each progress curve. A standard curve for p-nitroaniline absorbance was obtained at the beginning of each experiment, and enzyme catalyzed p-nitroaniline production was quantitated from the standard curve. Compounds giving greater than 50% inhibition were selected for further analysis.

For analysis of positive compounds, steady-state kinetic inhibition constants were determined as a function of both substrate and inhibitor concentration. Substrate saturation curves were obtained at gly-pro-p-nitroanilide concentrations from 60 μM to 3600 μM. Additional saturation curves also were obtained in the presence of inhibitor. Complete inhibition experiments contained 11 substrate and 7 inhibitor concentrations, with triplicate determinations across plates. For tight binding inhibitors with K_is less than 20 nM, the enzyme concentration was reduced to 0.5 nM and reaction times were increased to 120 min. Pooled datasets from the three plates were fitted to the appropriate equation for either competitive, noncompetitive or uncompetitive inhibition.

(1) Rahfeld, J. Schutkowski, M., Faust, J., Neubert., Barth, A., and Heins, J. (1991) Biol. Chem. Hoppe-Seyler. 372, 313-318.

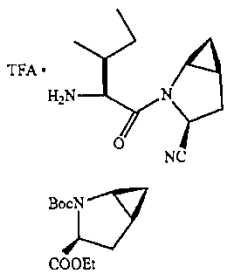
(2) Nagatsu, T., Hino, M., Fuyamada, H., Hayakawa, T., Sakakibara, S., Nakagawa, Y, and Takemoto, T. (1976) Anal. Biochem., 74, 466-476.

The following abbreviations are employed in the Examples and elsewhere herein:

Ph=phenyl
Bn=benzyl
i-Bu=iso-butyl
Me=methyl
Et=ethyl
Pr=propyl
Bu=butyl
TMS=trimethylsilyl
FMOC=fluorenylmethoxycarbonyl
Boc or BOC=tert-butoxycarbonyl
Cbz=carbobenzyloxy or carbobenzoxy or benzyloxycarbonyl
HOAc or AcOH=acetic acid
DMF=N,N-dimethylformamide
EtOAc=ethyl acetate
THF=tetrahydrofuran
TFA=trifluoroacetic acid
Et_2NH=diethylamine
NMM=N-methyl morpholine
n-BuLi=n-butyllithium
Pd/C=palladium on carbon
PtO_2=platinum oxide
TEA=triethylamine
EDAC=3-ethyl-3'-(dimethylamino)propyl-carbodiimide hydrochloride (or 1-[(3-(dimethyl)amino)propyl])-3-ethylcarbodiimide hydrochloride)
HOBT or HOBT.H_2O=1-hydroxybenzotriazole hydrate
HOAT=1-hydroxy-7-azabenzotriazole
PyBOP reagent=benzotriazol-1-yloxy-tripyrrolidino phosphonium hexafluorophosphate
min=minute(s)
h or hr=hour(s)
L=liter
mL=milliliter
μL=microliter
g=gram(s)
mg=milligram(s)
mol=mole(s)
mmol=millimole(s)
meq=milliequivalent
rt=room temperature
sat or sat'd=saturated
aq.=aqueous
TLC=thin layer chromatography
HPLC=high performance liquid chromatography
LC/MS=high performance liquid chromatography/mass spectrometry
MS or Mass Spec=mass spectrometry
NMR=nuclear magnetic resonance
mp=melting point

The following Examples represent preferred embodiments of the invention.

EXAMPLE 1



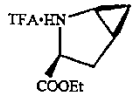
Step 1

Step 1 title compound was synthesized by following the literature procedure [Stephen Hanessian, Ulrich Reinhold, Michel Saulnier, and Stephen Claridge; Bioorganic & Medicinal Chemistry Letters 8 (1998) 2123-2128] or with the following modifications. L-pyroglutamic acid ethyl ester was N-protected as the t-butylcarbamate (Boc_2O, DMAP or NaH) and then dehydrated to the 4,5-dehydroproline ethyl ester in one pot by carbonyl reduction (triethylborohydride, toluene, −78° C.) followed by dehydration (TFAA, lutidine). The title compound was obtained by cyclopropanation of the 4,5-dehydroproline ethyl ester (Et_2Zn, $ClCH_2I$, 1,2-dichloroethane, −15° C.). A more detailed protocol is as follows;

Synthesis of 4,5-dehydro-L-proline ethyl ester: L-pyroglutamic acid ethyl ester (200 g, 1.27 mol) was dissolved in 1.2 liters of methylene chloride and treated sequentially with di-tert-butyldicarbonate (297 g, 1.36 mol) and a catalytic DMAP (1.55 g, 0.013 mol) at ambient temperature. After 6 h, the mixture was quenched with saturated brine and the organic phase was dried (Na_2SO_4) and filtered through a short silica gel column to give 323 g (100%) of N-Boc- L-pyroglutamic acid ethyl ester. N-Boc-L-pyroglutamic acid ethyl ester (160 g, 0.62 mol) was dissolved in 1 liter of toluene, cooled to −78° C. and treated with lithium triethylborohydride (666 mL of a 1.0 M soln in THF) and added dropwise over 90 minutes. After 3 h, 2,6-lutidine (423 mL, 3.73 mol) was added dropwise followed by DMAP (0.2 g, 0.0016 mol). To this mixture was added TFAA (157 g, 0.74 mol) and the reaction was allowed to come to ambient temperature over 2 h. The mixture was diluted with EtOAc and water and the organics were washed with 3 N HCl, water, aqueous bicarbonate and brine and dried (Na_2SO_4) and filtered through a silica gel plug to give 165 g of the crude 4,5-dehydroproline ethyl ester that was purified by flash column chromatography on silica gel with 1:5 ethyl acetate:hexanes to give 120 g, 75% of the olefin.

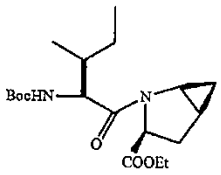
Cyclopropanation of 4,5-dehydro-L-proline ethyl ester: 4,5-Dehydro-L-proline ethyl ester (35.0 g, 0.145 mol) was added to a solution of neat Et_2Zn (35.8 g, 0.209 mol) in 1 liter of 1,2-dichloroethane at −15° C. To this mixture was added a dropwise addition of $ClCH_2I$ (102 g, 0.58 mol) over 1 h and the mixture stirred at −15° C. for 18 h. The reaction was quenched with saturated aqueous bicarbonate and the solvent was evaporated and the reaction was taken up in EtOAc, washed with brine and purified by silica gel chromatography using a stepwise gradient of from 20% EtOAc/hexanes to 50% EtOAc/hexanes to give 17.5 g (50%) of diastereomerically pure step 1 title compound.

Step 2



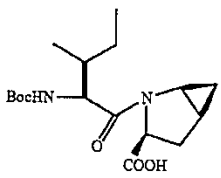
To a stirred solution of Step 1 compound (411 mg, 1.61 mmol) in CH_2Cl_2 (1.5 mL) at rt was added TFA (1.5 mL). The reaction mixture was stirred at rt for 2 h and evaporated. The residue was diluted with CH_2Cl_2 and then evaporated and re-evaporated three times to give the title compound as a colorless oil, 433 mg, 100% yield.

Step 3



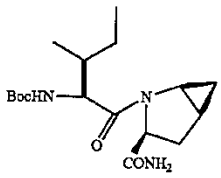
To a stirred solution of (S)-N-tert-butoxycarbonylisoleucine (372.6 mg, 1.61 mmol) and benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (1.25 g, 2.42 mmol) in CH_2Cl_2 (6 mL) under nitrogen at rt was added 4-methylmorpholine (NMM) (0.36 mL, 3.2 mmol). After 5 min, a solution of Step 2 compound (433 mg, 1.61 mmol) and NMM (0.27 mL, 2.4 mmol) in CH_2Cl_2 (1 mL) was added. After addition, the reaction mixture was stirred under nitrogen at room temperature overnight. The reaction mixture was diluted with CH_2Cl_2 (40 mL) and washed with 4% $KHSO_4$ (10 mL), aqueous $NaHCO_3$ (10 mL) and brine (10 mL), dried (Na_2SO_4) and evaporated. Purification by flash chromatography (1:4 EtOAc/hexane) gave the title compound as a colorless oil, 530 mg, 89% yield.

Step 4



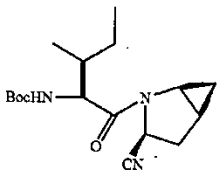
To a stirred solution of Step 3 compound (530 mg, 1.44 mmol) in MeOH (4 mL) and H_2O (4 mL) at rt was added LiOH—H_2O (91 mg, 2.16 mmol). The reaction mixture was stirred at rt overnight and evaporated. Water (10 mL) was added to the residue and extracted with Et_2O (2×10 mL). The aqueous layer was acidified to ~pH 4 by adding 4% $KHSO_4$ dropwise. The milky solution was extracted with EtOAc (15 mL×3). Combined EtOAc layers were washed with brine, dried over Na_2SO_4 and evaporated to give the title compound as a white solid, 440 mg, 90% yield.

Step 5



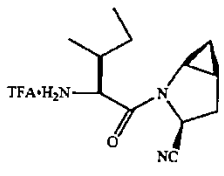
To a stirred solution of Step 4 compound (300 mg, 0.88 mmol) in THF (6 mL) at −15° C. under nitrogen, was added 4-methylmorpholine (0.12 mL, 1.06 mmol) and then isobutyl chloroformate (0.13 mL, 0.97 mmol) over 2 min. White precipitate was formed. The reaction mixture was stirred at −15° C. under nitrogen for 25 min and a solution of NH_3 in dioxane (8.8 mL, 4.4 mmol) was added. The reaction mixture was stirred at −15° C. for 30 min, warmed to rt and stirred at rt overnight. The reaction mixture was quenched by 4% $KHSO_4$ to ~pH 4 and extracted with EtOAc (20 mL×3). The extracts were combined, washed with brine (10 mL) dried (Na_2SO_4) and evaporated. Purification by flash column chromatography (1:1 EtOAc/hexane) gave the title compound as a white foam, 268 mg, 90% yield.

Step 6



To a stirred solution of Step 5 compound (248 mg, 1.38 mmol) and imidazole (94 mg, 1.38 mmol) in dry pyridine (12 mL) at −35° C. under nitrogen was added $POCl_3$ (0.26 mL, 2.76 mmol) dropwise. The reaction mixture was stirred between −35° C. to −20° C. for 1 h and evaporated. CH_2Cl_2 (10 mL) was added and white precipitates were formed. After filtration, the filtrate was concentrated and purified by flash chromatography (2:5 EtOAc/hexane) to give the title compound as a colorless oil, 196 mg, 88% yield.

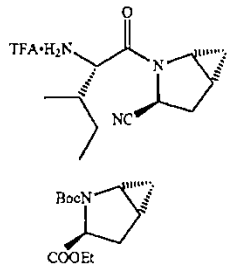
Step 7



To a stirred solution of Step 6 compound (130 mg, 0.4 mmol) in CH_2Cl_2 (2 mL) at rt was added TFA (2 mL). The

reaction mixture was stirred at rt for 2 h. The reaction mixture was added slowly to a pre-cooled slurry of $NaHCO_3$ (3.8 g) in H_2O (3 mL). The mixture was extracted with CH_2Cl_2 (6 mL×5), and the. combined CH_2Cl_2 layers were evaporated and purified by preparative HPLC to give the title compound as a white powder, 77 mg. 57% yield, mp=141-143° C. LC/MS gave the correct molecular ion [(M+H)$^+$=222] for the desired compound.

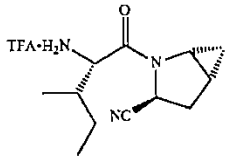
EXAMPLE 2



Step 1

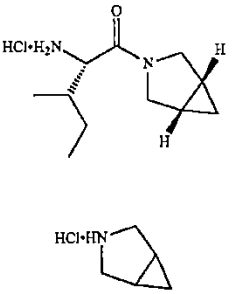
Step 1 title compound was synthesized by following the literature procedure. [Stephen Hanessian, Ulrich Reinhold, Michel Saulnier, and Stephen Claridge; Bioorganic & Medicinal Chemistry Letters 8 (1998) 2123-2128.]

Step 2



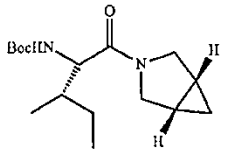
The title compound was prepared from Step 1 compound, employing the same procedure as that described for Example 1, Steps 2-6. LC/MS gave the correct molecular ion [(M+H)$^+$= 222] for the desired compound.

EXAMPLE 3



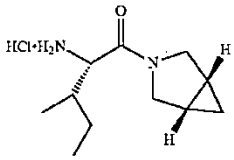
Step 1 title compound was prepared by following the literature procedure. [Willy D. Kollmeyer, U.S. Pat. No. 4,183,857.]

Step 2



To a stirred solution of (S)-N-tert-butoxycarbonylisoleucine (231 mg, 1 mmol) and benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (780 mg, 1.5 mmol) in CH_2Cl_2 (6 mL) under nitrogen at rt was added 4-methylmorpholine (0.33 mL, 3 mmol). After 5 min, Step 1 compound (120 mg, 1 mmol) was added in one portion. The reaction mixture was stirred under nitrogen at rt overnight and then diluted with CH_2Cl_2 (30 mL), washed with 4.1% $KHSO_4$ (10 mL)), aqueous $NaHCO_3$ (10 mL), brine (10 mL), dried (Na_2SO_4) and evaporated. Purification by flash chromatography on silica gel (2.4×20 cm column, 1:3 EtOAc/hexane) gave the title compound as a colorless oil, 290 mg, 90% yield. LC/MS gave the correct molecular ion [(M+H)$^+$=297] for the desired compound.

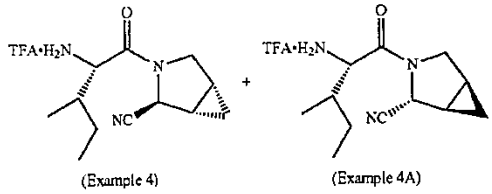
Step 3



The reaction mixture of Step 2 compound (220 mg, 0.74 mmol) and 4 M HCl in dioxane (1.5 mL, 6 mmol) was stirred at rt for 2 h and evaporated under reduced pressure. Et_2O was added to the residue and a precipitate was formed. Et_2O was decanted and this was done three times. The precipitate was dried in vacuo to give the title compound as a white powder, 130 mg (76% yield), mp 205-206° C. LC/MS gave the correct molecular ion [(M+H)$^+$=197] for the desired compound.

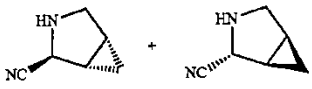
EXAMPLES 4-4A

Step 1



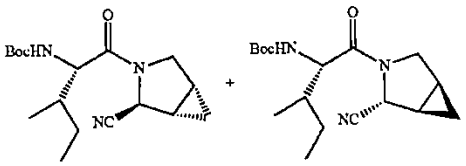
(Example 4) (Example 4A)

Step 1



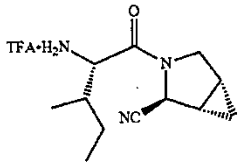
Step 1 title compound, as a 1:1 ratio of enantiomers, was prepared by following the literature procedure. [Willy D. Kollmeyer, U.S. Pat. No. 4,183,857.]

Step 2

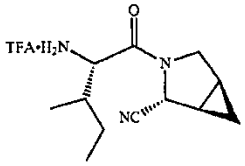


A slurry of (S)-N-tert-butoxycarbonyl-isoleucine (92.5 mg, 0.4 mmol), 1-[(3-(dimethyl)amino)propyl]-3-ethylcarbodiimide (77 mg, 0.4 mmol) and HOAT (54.4 mg, 0.4 mmol) in $ClCH_2CH_2Cl$ (0.3 mL) was stirred under nitrogen at rt for 1 h, then Step 1 compound (22 mg, 0.2 mmol) was added, followed by Et_3N (0.015 mL, 0.1 mmol). The reaction mixture was stirred under nitrogen at rt over night and then diluted with CH_2Cl_2 (3 mL), washed with H_2O (1 mL), aqueous $NaHCO_3$(1 mL) and brine (1 mL), dried (Na_2SO_4) and evaporated. Purification by flash chromatography on silica gel (2.4×12 cm column, 2:7 EtOAc/hexane) gave the title compound as a colorless oil, 33 mg, 51% yield. LC/MS gave the correct molecular ion [(M+H)$^+$322] for the desired compound.

Step 3



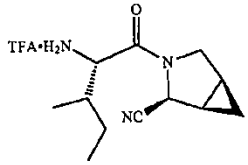
(Example 4)



(Example 4A)

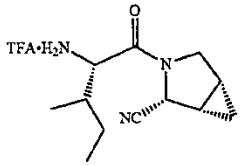
To a stirred solution of Step 2 compound (30 mg, 0.4 mmol) in CH_2Cl_2 (0.5 mL) at rt was added TFA (0.5 mL). The reaction mixture was stirred at rt for 2 h. The reaction mixture was added slowly to a precooled slurry of $NaHCO_3$ (0.8 g) in H_2O (1 mL). The mixture was extracted with CH_2Cl_2 (2 mL×5), and combined CH_2Cl_2 layers were evaporated and purified by preparative HPLC to give the title compounds as a 1:1 ratio of diastereomers, 22 mg, 73% yield. LC/MS gave the correct molecular ion [(M+H)$^+$=222] for the desired compounds.

EXAMPLES 5-5A



and

(Example 5)

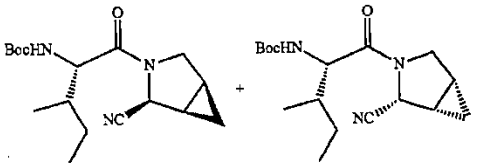


(Example 5A)

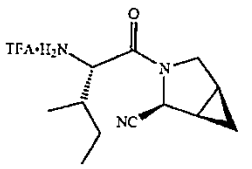
Step 1

To a solution of Example 4, Step 1 compound (150 mg, 1.39 mmol) in 2-propanol (0.8 mL), was added NaCN (40 mg, 1.0 mmol). The reaction mixture was heated to reflux for 3 h. After cooling to rt, the reaction mixture was evaporated and then slurried in Et_2O (5 mL). After filtration, the filtrate was evaporated to give Example 4 Step 1 compounds and Example 5 Step 1 compounds (140 mg, 93%) as a 2:1 mixture of diastereomers, each as a racemic mixture.

Step 2

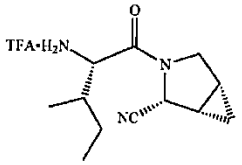


A slurry of (S)-N-tert-butoxycarbonyl-isoleucine (595 mg, 2.57 mmol), 1-[(3-(dimethyl)amino)propyl]-3-ethylcarbodiimide (493 mg, 2.57 mmol) and 1-hydroxy-7-azabenzotriazole (350 mg, 2.57 mmol) in $ClCH_2CH_2Cl$ (2 mL) was stirred under nitrogen at rt for 1 h, then Step 1 compound mixture (139 mg, 1.28 mmol) was added. The reaction mixture was stirred under nitrogen at rt overnight and then diluted with CH_2Cl_2 (30 mL), washed with H_2O (10 mL), saturated aqueous $NaHCO_3$ (10 mL) and brine (10 mL), dried (Na_2SO_4) and evaporated. Purification by flash chromatography on silica gel (2.4×20 cm column, 1:3 EtOAc/hexane) gave the Example 4, Step 2 compound (260 mg), and the title compounds (105 mg) as a ratio of 1:1 diastereomers. LC/MS gave the correct molecular ion [(M+H)$^+$=322] for the desired compounds.



and

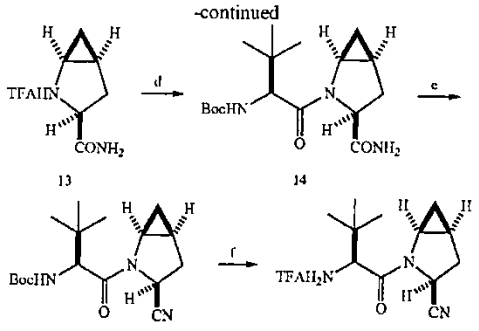
(Example 5)



(Example 5A)

-continued

Step 3



13 14 15 16

a LiOH in THF/H_2O or $MeOH/H_2O$

B i-BuOCOCl/NMM or i-BuOCOCl/TEA at -30 C or EDAC, then NH_3 in dioxane or Et_2O at RT

c TFA, CH_2Cl_2, RT

d Boc-t-butylglycine and PyBop/NMM or EDAC, DMAP, CH_2Cl_2

e. $POCl_3$, pyridine, imidazol, -20 C.

f TFA, CH_2Cl_2, RT

To a stirred solution of Step 2 compounds (104 mg, 0.32 mmol) in ClI_2Cl_2 (1 mL) at rt was added TFA (1 mL). The reaction mixture was stirred at rt for 2 h. The reaction mixture was added slowly to a precooled slurry of $NaHCO_3$ (2 g) in H_2O (2 mL). The mixture was extracted with CH_2Cl_2 (4 mL×4), and combined CH_2Cl_2 layers were evaporated and purified by preparative HPLC to give the title compound Example 5 (36 mg) and Example 5A (36 mg). LC/MS gave the correct molecular ion [(M+H)$^+$=222] for the desired compounds.

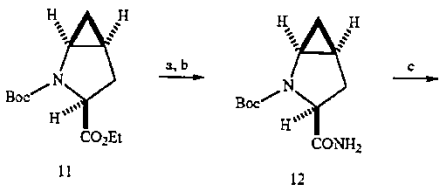
EXAMPLE 6

General Method A: Parallel array synthesis methods for preparation of inhibitors from commercially available amino acids. As shown in Scheme 3, the ester 11, described in Example 1 Step 1, was saponified to the acid with LiOH in THF/H_2O and converted to the amide 12 by treatment with isobutyl chloroformate/NMM followed by ammonia in dioxane. The Boc protecting group was removed under acidic conditions using TFA in methylene chloride to give 13. The TFA salt was coupled to Boc-t-butylglycine using either EDAC/HOBT/DMF or EDAC/DMAP/ClI_2Cl_2 to give 14. The amide was dehydrated to the nitrile 15 using $POCl_3$/imidazole in pyridine at ~20° C. and finally deprotected with TFA in CH_2Cl_2 at ambient temperature to afford the target 16.

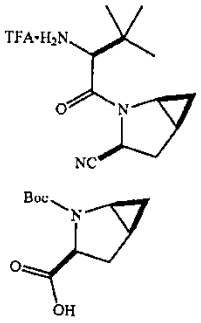
SCHEME 3, GENERAL METHOD A (EXAMPLES 6-27)

Scheme 3

General Method A (Examples 6-27)

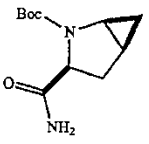


11 12



Step 1

To a stirred solution of Example 1 Step 1 compound (1.40 g, 5.49 mmol) in 40 mL of a 1:1 methanol:water solution at rt was added lithium hydroxide (0.20 g, 8.30 mmol). The reaction mixture was stirred at rt for 18 h and then heated to 50° C. for 2 h. The mixture was diluted with equal volumes of ether and water (50 mL) and then acidified with $KHSO_4$ to pH 3. The milky solution was extracted with ether (3×20 mL). The combined ether layers were dried over Na_2SO_4 and evaporated. The residue was stripped from toluene (2×10 mL) and dried under reduced pressure to give the title compound as a thick syrup, 1.20 g, 96%.

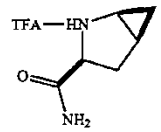


Step 2

To a stirred solution of Step 1 compound (1.20 g, 5.28 mmol) in THF (20 mL) at −15° C. under nitrogen was added 4-methylmorpholine (0.71 mL, 6.50 mmol) and then isobutyl chloroformate (0.78 mL, 6.00 mmol) over 5 min. The reaction

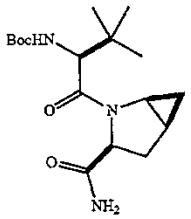
was stirred at −15° C. for 30 min, cooled to −30° C. and treated with a solution of NH_3 in dioxane (50 mL, 25 mmol). The reaction mixture was stirred at −30° C. for 30 min, warmed to rt and stirred overnight. The reaction mixture was quenched with citric acid solution (pH 4) and extracted with ether (3×50 mL). The combined organic fractions were washed with brine, dried over Na_2SO_4 and concentrated. Purification by flash column chromatography on silica gel with EtOAc gave the Step 2 compound, 1.00 g, 84%.

Step 3



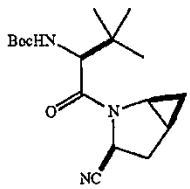
To a stirred solution of Step 2 compound (0.90 g, 4.00 mmol) in CH_2Cl_2 (3 mL) at 0° C. was added TFA (3 mL). The reaction mixture was stirred at 0° C. for 18 h. The reaction mixture was concentrated under reduced pressure to produce title compound in the form of a thick oil, 0.98 g, 100%. The oil gradually solidified upon prolonged standing.

Step 4



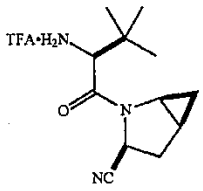
An oven-dried 15-mL test tube was charged with Step 3 compound (56 mg, 0.22 mmol), N-tert-butoxycarbonyl-(L)-tert-leucine (53 mg, 0.23 mmol), dimethylaminopyridine (0.11 g, 0.88 mmol), and CH_2Cl_2 (4 mL). The tube was sealed under nitrogen atmosphere and treated with 1-[(3-(dimethylpamino)propyl]-3-ethylcarbodiimide (84 mg. 0.44 mmol). The mixture was placed in a shaker and vortexed overnight. The product was purified by solid phase extraction using a United Technology SCX column (2 g of sorbent in a 6 mL column) by loading the material on a SCX ion exchange column and successively washing with CH_2Cl_2 (5 mL), 30% methanol in CH_2Cl_2 (5 mL), 50% methanol in CH_2Cl_2 (5 mL) and methanol (10 mL). The product containing fractions were concentrated under reduced pressure to give the desired amide. Further purification by reverse phase preparative column chromatography on a YMC S5 ODS 20×250 mm column gave the title compound, 50 mg (68% yield). Purification conditions: Gradient elution from 30% methanol/water/0.1 TFA to 90% methanol/water/0.1 TFA over 15 min. 5 min hold at 90% methanol/water/0.1 TFA. Flow rate: 20 mL/min Detection wavelength: 220. Retention Time: 14 min.

Step 5



An oven-dried 15-mL test tube was charged with Step 4 compound (50 mg, 0.15 mmol), imidazole (31 mg, 0.46 mmol), and pyridine (1 mL). The tube was sealed under nitrogen atmosphere and cooled to −30° C. Slow addition of $POCl_3$ (141 mg, 88 uL, 0.92 mmol) gave after mixing a thick slurry. The tube was mixed at −30° C. for 3 h and the volatiles evaporated. The product was purified by solid phase extraction using a United Technology silica extraction column (2 g of sorbent in a 6 mL column) by loading the material on a silica column and successively washing with CH_2Cl_2 (5 mL), 5% methanol in CH_2Cl_2 (5 mL), 7% methanol in CH_2Cl_2 (5 mL) and 12% methanol in CH_2Cl_2 (10 mL). The product containing fractions were pooled and concentrated under reduced pressure to give the title compound, 46 mg, 96%.

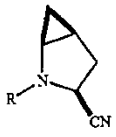
Step 6



An oven-dried 15-mL test tube was charged with Step 5 compound (0.45 mg, 0.14 mmol), CH_2Cl_2 (1 mL), and TFA (1 mL). The reaction mixture was vortexed for 40 min at rt, diluted with toluene (4 mL) and concentrated under reduced pressure to a thick oil. The product was purified by reverse phase preparative column chromatography on a YMC S5 ODS 20×250 mm column to give the Example 6 compound, 14 mg, 35%. Purification conditions: gradient elution from 10% methanol/water/0.1 TFA to 90% methanol/water/0.1 TFA over 18 min; 5 min hold at 90% methanol/water/0.1 TFA. Flow rate: 20 mL/min Detection wavelength: 220. Retention Time: 10 min.

Examples 7-27 were prepared from amino acids available from commercial sources according to the procedure in Example 6.

TABLE 1



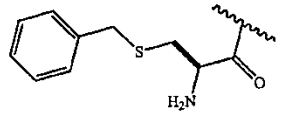
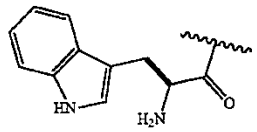
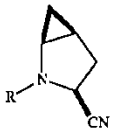
Example	R	[M + H]
7		302
8		295

TABLE 1-continued



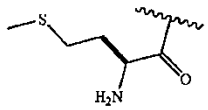
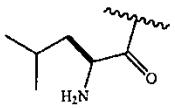
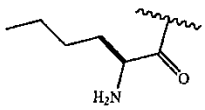
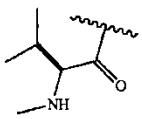
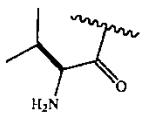
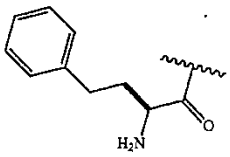
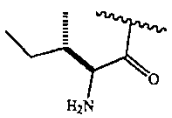
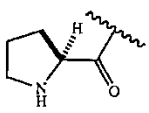
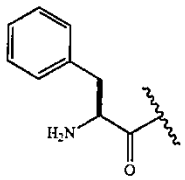
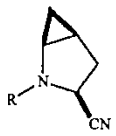
Example	R	[M + H]
9		240
10		222
11		222
12		222
13		208
14		270
15		222
16		206
17		256

TABLE 1-continued



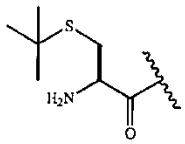
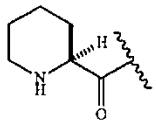
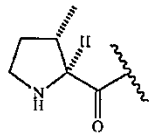
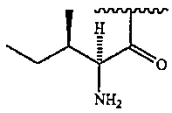
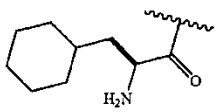
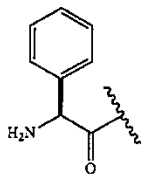
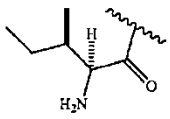
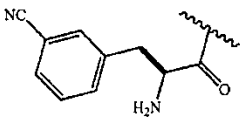
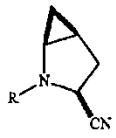
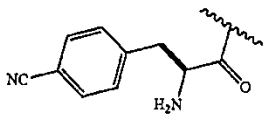
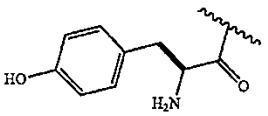
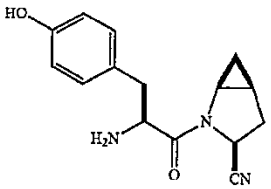
Example	R	[M + H]
18		268
19		220
20		220
21		210
22		262
23		242
24		210
25		281

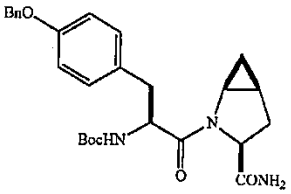
TABLE 1-continued



Example	R	[M + H]
26		281
27		272

EXAMPLE 27

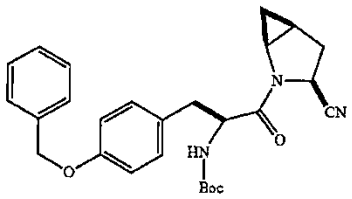




Step 1

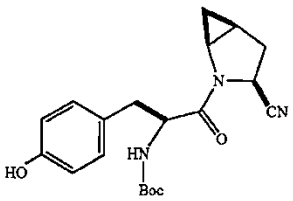
(2S,4S,5S)-4,5-methano-L-proline carboxylamide, TFA salt (53 mg, 0.22 mmol) was coupled to N-Boc-L-Tyrosine-benzyl ether (82 mg, 0.22 mmol) using PyBop (172 mg, 0.33 mmol) and N-methylmorpholine (67 mg, 0.66 mmol) in 4 mL CH_2Cl_2. The reaction stirred for 16 h, was taken up in EtOAc, washed with H_2O, 1N aqueous HCl, brine, then evaporated and purified by silica gel flash chromatography to give the coupled product (FAB MH+480).

Step 2



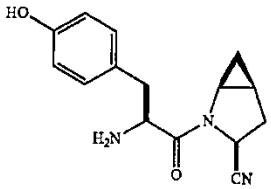
The Step 1 amide was dehydrated to the nitrile using the general method C (which follows Example 29) (FAB MH+462).

Step 3



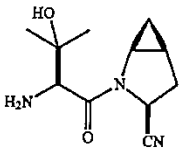
The Step 2 benzyl ether was cleaved by catalytic hydrogenolysis using 10% palladium on carbon and 1 atmosphere hydrogen gas in MeOH at rt for 1.5 h. The reaction was filtered through celite and concentrated to an oil and taken on without further purification (FAB MH+372).

Step 4



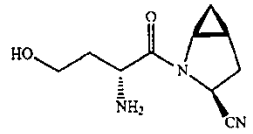
Step 3 N-[N-Boc-L-Tyrosine-]-(2S,4S,5S)-2-cyano-4,5-methano-L-prolylamide was dissolved in CH_2Cl_2 and TFA was added at rt. The reaction stirred for 1 h and was evaporated and purified by preparative HPLC as described in general method B (set out following Example 29) to afford the title compound (FAB MH+272).

EXAMPLE 28

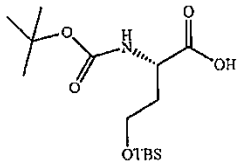


The title compound was prepared by coupling (2S,4S,5S)-4,5-methano-L-proline carboxylamide, TFA salt described in Example 6 Step 3 compound with N-(tert-butyloxy-carbonylhydroxyvaline. After hydroxyl protection with triethylsilyl chloride and dehydration of the amide with $POCl_3$/imidazole in pyridine and deprotection (N-terminal nitrogen and valine hydroxyl) with TFA using general method C (FAB MH+224), the title compound was obtained.

EXAMPLE 29

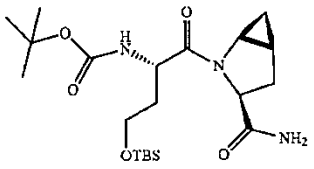


Step 1



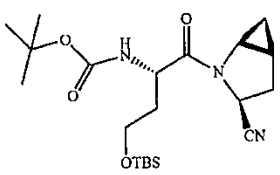
N-Boc-L-homoserine (1.20 g, 5.47 mmol) upon treatment with tert-butyldimethylsilyl chloride (1.67 g, 11.04 mmol) and imidazole (938 mg, 13.8 mmol) in THF (17 mL) was stirred as thick slurry for 48 h under N_2. The solvent was evaporated, and the crude material was dissolved in MeOH (10 mL). The resulting solution was stirred at rt for 2 h. The solvent was evaporated, and the crude material was diluted with CH_2Cl_2 (50 mL) and treated with 0.1N HCl (2×10 mL). The CH_2Cl_2layer was washed with brine and dried over $MgSO_4$. Removal of the volatiles gave title compound as an oil (1.8 g), which was used without further purification (LC/Mass, + ion): 334 (M+H).

Step 2



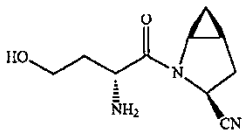
To a stirred solution of Step 1 compound (333 mg, 1.0 mmol) in 6 mL of CH_2Cl_2 was added 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (256 mg, 1.32 mmol). The solution was then stirred at rt for 30 min, followed by addition with Example 6 Step 3 amine TFA salt (160 mg, 0.66 mmol) and 4-(dimethylamino)pyridine (244 mg, 2.0 mmol). The solution was then stirred at rt overnight. The mixture was diluted with CH_2Cl_2 (5 mL) and washed sequentially with H_2O, 10% citric acid, brine, then dried over Na_2SO_4 and evaporated to give the title compound (350 mg) which was used without further purification (LC/Mass, + ion): 442 (M+H).

Step 3



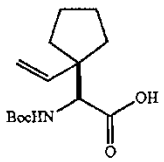
An oven-dried 10-mL round bottomed flask was charged with Step 2 compound (350 mg, 0.79 mmol), imidazole (108 mg, 1.58 mmol), pyridine (3 mL). The flask under argon was cooled to −30° C. Slow addition of $POCl_3$ (0.30 mL, 3.16 mmol) gave after mixing a thick slurry. The slurry was mixed at −30° C. for 3 h and the volatiles evaporated. Dichloromethane (5 mL) was then added and the insoluble solid was removed by filtration. The organic layer was washed with H_2O, 10% citric acid, brine and dried over Na_2SO_4. Removal of solvent gave crude desired nitrile (330 mg) (LC/Mass, + ion): 424 (M+H).

Step 4



Trifluoroacetic acid (3.3 mL) was added to a stirred solution of Step 3 compound (330 mg, 0.58 mmol) in 3.3 mL CH_2Cl_2. The solution was then stirred at rt for 30 min, a few drops of water were added and the mixture mixture stirred for 0.5 h. The mixture was diluted with CH_2Cl_2 (5 mL) and concentrated under reduced pressure to a thick oil. The product was purified by reverse phase preparative column chromatography on a YMC S5 ODS 20×100 mm column to give the title compound, 59 mg, 17%. Purification conditions: gradient elution from 10% methanol/water/0.1 TFA to 90% methanol/water/ 0.1 TFA over 15 min; 5 min hold at 90% methanol/water/0.1 TFA. Flow rate: 20 mL/min. Detection wavelength: 220. Retention Time 10 Min. (LC/Mass, + ion): 210 (M+H).

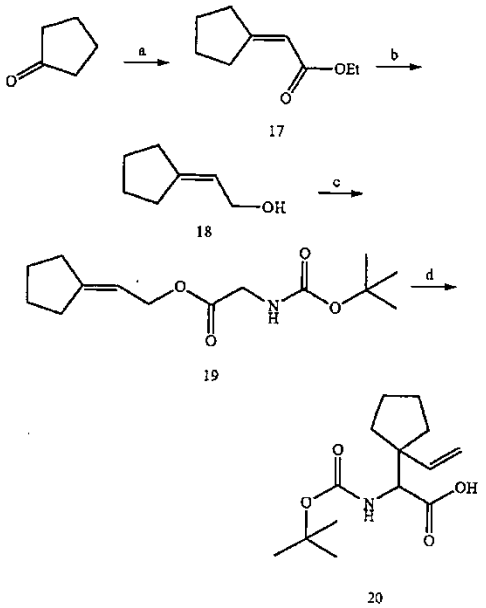
General Method B: Claisen rearrangement sequence to Boc-protected amino acids.



General method B affords the quaternary Boc-protected amino acids. Examples 30-47 contain the vinyl sidechain by coupling amino acids of which Scheme 4, compound 20 is representative. Cyclopentanone was olefinated under Horner-Emmons conditions to afford 17 which was reduced to the allylic alcohol 18 using DIBAL-H in toluene −78° C. to rt. Allylic alcohol 18 was esterified with N-Boc glycine using DCC/DMAP in CH_2Cl_2 to give 19. Glycine ester 19 was subjected to a Lewis acid mediated Claisen rearrangement hy

complexation with anhydrous zinc chloride and deprotonation at −78° C. with lithium diisopropylamide followed by warming to ambient temperature to afford 20.

Scheme 4
General Method B, Examples 30-47



a. Triethylphosphonoacetate, NaH, THF 0 C to RT
b. DIBAL-H, toluene, -78 C. to RT
c. N-Boc glycine, DCC, DMAP, CH_2Cl_2, RT
d. $ZnCl_2$, THF, LDA, -78 C. to RT

Step 1

Cyclopentylideneacetic Acid Ethyl Ester

To a flame-dried 500-mL round-bottomed flask containing NaH (5.10 g of a 60% dispersion in mineral oil, 128 mmol, 1.10 equiv) in 120 mL anhydrous THF at 0° C. under argon was added triethylphosphonoacetate (25.6 mL, 128 mmol, 1.10 equiv) dropwise through an addition funnel. The mixture was allowed to warm to rt, stirring for an additional 1 h. A solution of cyclopentanone (10.3 mL, 116 mmol) in 10 mL anhydrous THF was added dropwise over 20 min through an addition funnel, and the mixture was allowed to stir at rt for 2.5 h. Ether (200 mL) and water (100 mL) were then added, and the layers were separated. The organic phase was washed successively with water (100 mL) and brine (100 mL), dried (Na_2SO_4), and concentrated under reduced pressure, giving 17.5 g (98%) of the desired ester as a colorless oil.

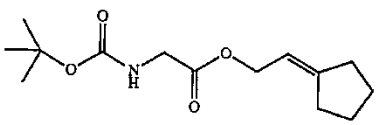
Step 2

2-Cyclopentylideneethanol

To a flame-dried 500-mL round-bottomed flask containing cyclopentylideneacetic acid ethyl ester (17.5 g, 113 mmol) in 100 mL anhydrous toluene at −78° C. under argon was added DIBAL-H (189 mL of a 1.5 M solution in toluene, 284 mmol, 2.50 equiv) dropwise over a 30 min period through an addition funnel, and the mixture was then allowed to warm to rt, stirring for 18 h. The reaction mixture was then recooled to −78° C., and quenched by the careful addition of 30 mL anhydrous MeOH. Upon warming to rt, 1 N Rochelle's salt (100 mL) was added, and the mixture was stirred 90 min. The biphasic reaction mixture was then diluted with Et_2O (200 mL) in a separatory funnel, and the layers were separated. The organic layer was then washed with brine (100 mL), dried (Na_2SO_4), and concentrated under reduced pressure. Purification by flash column chromatography (silica gel, CH_2Cl_2/EtOAc, 10:1) gave 11.6 g (92%) of the desired allylic alcohol as a colorless oil.

Step 3

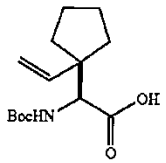
(2-Cyclopentylideneethyl)-N-(tert-Butyloxycarbonyl) glycinate



To a flame-dried 500-mL round-bottomed flask containing N-(tert-butyloxycarbonyl)glycine (13.45 g, 76.75 mmol) in 100 mL CH_2Cl_2 at rt was added Step 2 compound 8.61 g, 76.75 mmol, 1.00 equiv) in 20 mL CH_2Cl_2, followed by dicyclohexylcarbodiimide (16.63 g, mmol, 1.05 equiv) in 80 mL CH_2Cl_2. To this reaction mixture was then added 4-dimethylaminopyridine (0.94 mg, mmol, 0.10 equiv), and the mixture was allowed to stir overnight. The reaction mixture was then filtered through a medium sintered-glass funnel, rinsing with 100 mL CH_2Cl_2, and concentrated under reduced pressure. The crude product was then purified by flash chromatography (silica gel, hexanes/EtOAc, 20:1 to 1:1 gradient) to give 19.43 g (94%) of the desired glycinyl ester as a colorless oil.

Step 4

N-(tert-Butyloxycarbonyl)(1'vinylcyclopentyl)-glycine

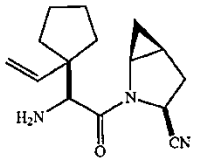


A flame-dried 500-mL round-bottomed flask under argon was charged with $ZnCl_2$ (11.8 g, mmol, 1.20 equiv) and 20 mL toluene. The mixture was heated under vacuum with vigorous stirring to azeotrope off any traces of moisture with the distilling toluene, repeating this process (2 ×). The flask was then cooled to rt under argon, (2-cyclopentylideneethyl) N-(tert-butyloxycarbonyl)glycinate (19.36 g, 71.88 mmol) was added via cannula as a solution in 180 mL THF, and the mixture was then cooled to −78° C. In a separate flame-dried 200-mL round-bottomed flask containing diisopropylamine (26.3 mL, mmol, 2.60 equiv) in 90 mL THF at −78° C. was added n-butyllithium (71.89 mL of a 2.5 M solution in hex-

anes, mmol, 2.5 equiv), and the mixture was allowed to warm to 0° C. for 30 min before recooling to −78° C. The lithium diisopropylamine thus generated was then added via cannula to the $ZnCl_2$ ester mixture dropwise at a steady rate over 40 min, and the resultant reaction mixture was allowed to slowly warm to rt and stir overnight. The yellow reaction mixture was then poured into a separatory funnel, diluted with 300 mL Et_2O, and the resultant organic solution was washed successively with 300 mL 1N HCl and 300 mL brine, dried (Na_2SO_4), and concentrated under reduced pressure. Purification by flash chromatography (silica gel, 3% MeOH in CH_2Cl_2 with 0.5% HOAc) gave 17.8 g (92%) of the desired amino acid product as a white solid. (FAB MH+270).

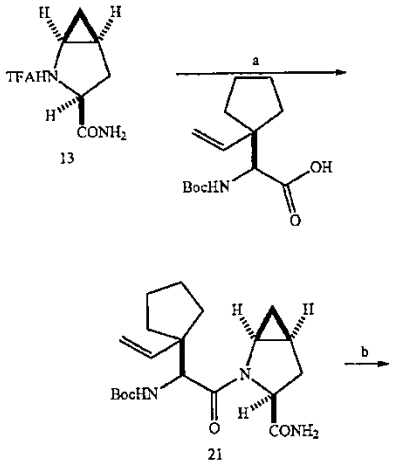
EXAMPLE 30

General Method C: Peptide coupling to 4,5-methanoprolinamide, amide dehydration and final deprotection.

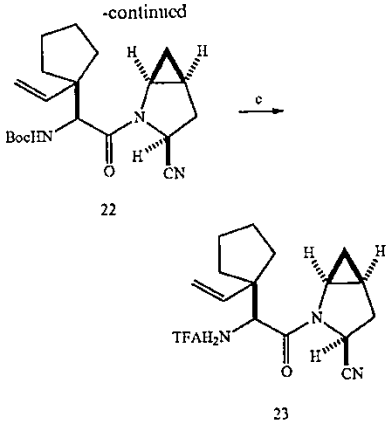


The TFA salt of amide 13 was coupled to a variety of racemic quaternary protected amino acids using HOBT/EDC in DMF at rt to give a D/L mixture of diastereomers at the N-terminal amino acid. The desired L diastereomer was chromatographically isolated either as the amide 21 or as the nitrile 22. Nitrile 22 was obtained by treatment of the amide with $POCl_3$/imidazole in pyridine at −20° C. The final target 23 was obtained by deprotection under acidic conditions using TFA in CH_2Cl_2.

Scheme 5
General Method C

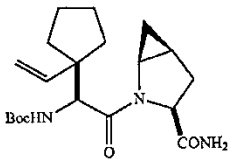


-continued



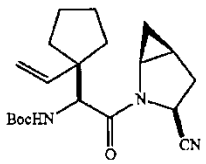
a. EDAC, HOBT, DMF
b. $POCl_3$, pyridine, imidazole, -20 C.
c. TFA, CH_2Cl_2, RT

Step 1



Example 6 Step 3 compound (877 mg, 3.65 mmol) and N-Boc cyclopentylvinylamino acid, described in Step 4 of general method B (1.13 g, 4.20 mmol) were dissolved in 20 mL anhydrous DMF, cooled to 0° C. and to this mixture was added EDAC (1.62 g, 8.4 mmol), HOBT hydrate (2.54 g, 12.6 mmol, and TEA (1.27 g, 12.6 mmol) and the reaction was allowed to warm to rt and stirred for 24 h. The reaction mixture was taken up in EtOAc (100 mL), washed with H_2O (3×20 mL), dried (Na_2SO_4), and purified by silica gel flash column chromatography (100% EtOAc) to give 1.38 g (86%) of Step 1 compound (MH+, 378).

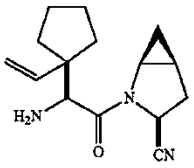
Step 2



Step 1 compound (1.38 g, 3.65 mmol) and imidazole (497 mg, 7.30 mmol) were dried by toluene azeotrope (5 mL×2), dissolved in 10 mL anhydrous pyridine, cooled to −30° C. under nitrogen gas and $POCl_3$ (2.23 g, 14.60 mmol) was added by syringe. The reaction was complete after 1 h and was evaporated to dryness and the remainder purified by two sequential flash column chromatographies over silica gel. The first column (100% EtOAc) was used to isolate the mixture of diastereomers (1.15 g, 88%) from the by-products of

the reaction. The second column (gradient of 25% EtOAC/hexanes to 50% EtOAc/hexanes) was run to resolve the mixture of diastereomers and provided 504 mg of the desired Step 2 nitrile (MH+360).

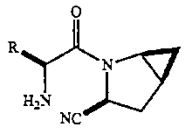
Step 3



Step 2 compound (32 mg, 0.09 mmol) was dissolved in 1 mL of CH_2Cl_2 and 1 mL of TFA was added and the reaction stirred for 30 min at rt and was evaporated to dryness. The product was purified by reverse phase preparative column chromatography on a YMC S5 ODS 20×250 mm column to give 12 mg of the TFA salt (lyophilized from water or isolated after evaporation of eluent and trituration with ether) the title compound. Purification conditions: gradient elution from 10% methanol/water/0.1 TFA to 90% methanol/water/0.1 TFA over 18 min; 5 min. hold at 90% water/0.1 trifluoroacetic acid. Flow rate: 20 mL/min. Detection wavelength: 220.

Examples 30-39 were prepared by the methods outlined in General Method B and General Method C starting from cyclopentanone, cyclobutanone, cyclohexanone, cycloheptanone, cyclooctanone, cis-3,4-dimethylcyclopentanone, and 4-pyranone, cyclopropaneethylhemiacetal, acetone, and 3-pentanone respectively.

TABLE 2



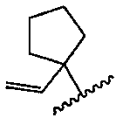
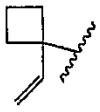
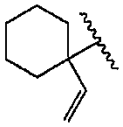
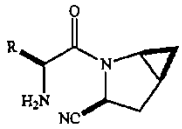
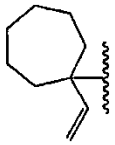
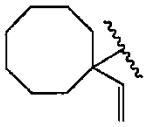
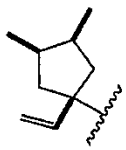
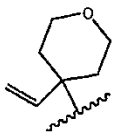
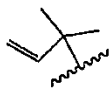
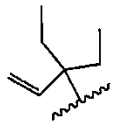
Example	R	MS [M + H]
30		260
31		246
32		274

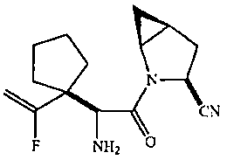
TABLE 2-continued



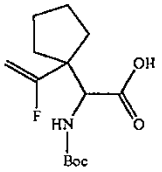
Example	R	MS [M + H]
33		288
34		302
35		288
36		276
37*		232
38		234
39		262

*Step 3 compound was prepared by the method described in Tetrahedron Letters 1986, 1281-1284.

EXAMPLE 40

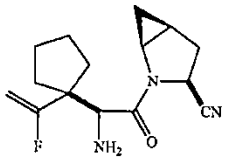


Step 1



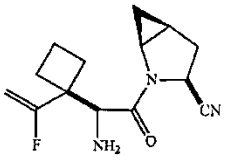
Step 1 compound was prepared employing general method B starting from cyclopentanone and 2-fluorotriethylphosphonoacetate instead of triethylphosphonoacetate.

Step 2

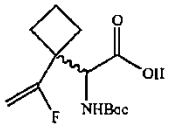


Title compound was prepared by the peptide coupling of Step 1 acid followed by dehydration and final deprotection as described in general method C [MS (M+H) 278].

EXAMPLE 41

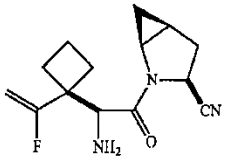


Step 1



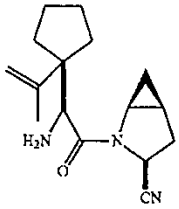
Step 1 compound was prepared employing general method B starting from cyclobutanone and 2-fluorotriethylphosphonoacetate instead of triethylphosphonoacetate.

Step 2

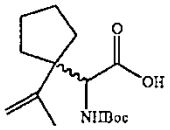


Title compound was prepared by the peptide coupling of Step 1 acid followed by dehydration and final deprotection as described in general method C. MS (M+H) 264.

EXAMPLE 42

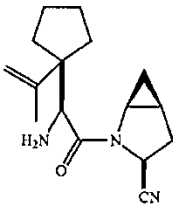


Step 1



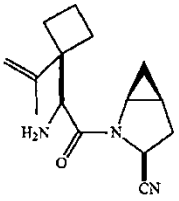
Step 1 compound was prepared employing general method B starting from cyclopentanone and triethylphosphono propionate instead of triethylphosphonoacetate.

Step 2

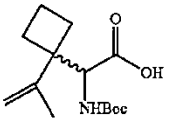


Title compound was prepared by the peptide coupling of Step 1 acid followed by dehydration and final deprotection as described in general method C. MS (M+H) 274

EXAMPLE 43

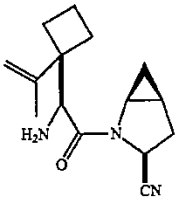


Step 1



Step 1 compound was prepared employing general method B starting from cyclobutanone and triethylphosphono propionate instead of triethylphosphonoacetate.

Step 2

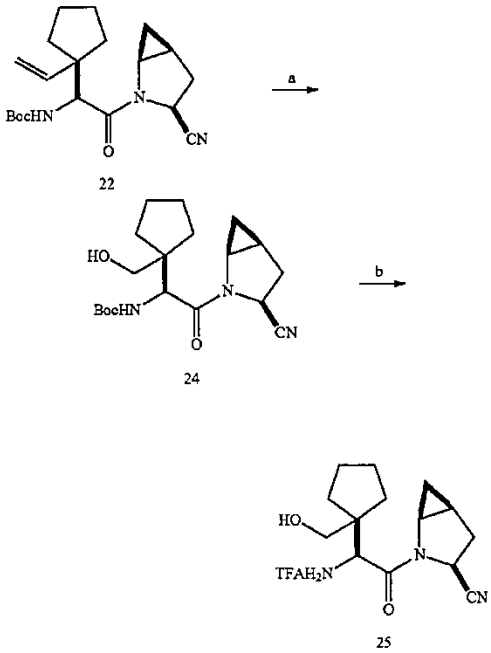


Title compound was prepared by the peptide coupling of Step 1 acid followed by dehydration and final deprotection as described in general method C. MS (M+H) 260.

EXAMPLE 44

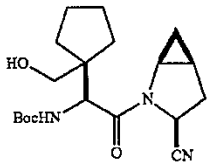
General Method D: Oxidative cleavage of vinyl substituent by ozonolysis. The protected cyclopentylvinyl nitrile 22 was treated with ozone for 6-8 min and subjected to a reductive quench with sodium borohydride to furnish the hydroxymethyl analog 24 directly. This compound was deprotected under acidic conditions with TFA in CH_2Cl_2 at 0° C. to give the target compound 25.

Scheme 6
General Method D, Examples 44, 46, 48



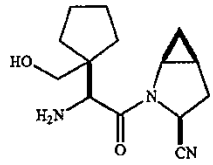
a O_3, MeOH CH_2Cl_2, 10:4, -78 C., then $NaBH_4$, -78 C to 0 C, 79%
b. TFA.CH_2Cl_2, 1:2, 0 degrees C.

Step 1



Cyclopentylvinyl compound prepared in Step 2 of general method C (1.28 g, 3.60 mmol) was dissolved in 56 mL of a 2:5 mixture of CH_2Cl_2:methanol, cooled to —78° C. and was treated with a stream of ozone until the reaction mixture took on a blue color, at which time, $NaBH_4$ (566 mg, 15.0 mmol, 4.2 equiv) was added and the reaction was warmed to 0° C. After 30 min, the reaction was quenched with 2 mL saturated aqueous $NaHCO_3$ and then warmed to rt. The reaction mixture was evaporated to dryness and taken up in EtOAc. A small amount of water was added to dissolve the inorganics and the layers separated. The EtOAc layer was dried (Na_2SO_4), filtered and evaporated to an oil that was purified by flash column chromatography on silica gel with EtOAc to give 922 mg (71%) of Step 1 compound. MS(M+H)364.

Step 2

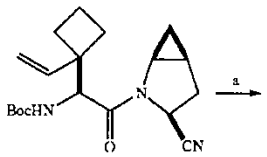


Step 1 compound (900 mg, 2.48 mmol) was dissolved in 60 mL of CH_2Cl_2, cooled to 0° C. and treated with 20 mL of freshly distilled TFA. The reaction was complete in 80 min and the mixture was evaporated to dryness and purified by preparative HPLC (YMC S5 ODS 30×100 mm, 18 minute gradient 80% Solv A:Solv B to 100% Solv B, Solvent A=10% MeOH-90%H_2O-0.1% TFA, Solvent B=90% MeOH-10% H_2O-0.1% TFA, collected product from 5.1-6.5 min) to give, after lyophillization from water, 660 mg (71%) of title compound, TFA salt as a white lyophillate. (MH+264).

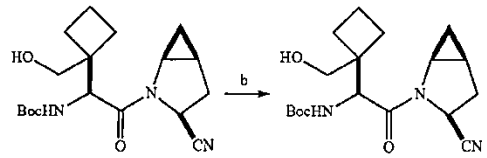
EXAMPLE 45

General Method E: Oxidative cleavage of vinyl substituent by osmium tetroxide-sodium periodate followed by sodium borohydride reduction to alcohol. The cyclobutylolefin 26 was treated with osmium tetroxide and sodium periodate in THF:water, 1:1, and the intermediate aldehyde was isolated crude and immediately reduced with sodium borohydride to give 27 in 56% yield. Standard deprotection conditions using TFA afforded the target compound 28.

Scheme 7
General Method E, Examples 45 47

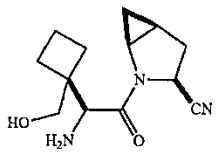


26

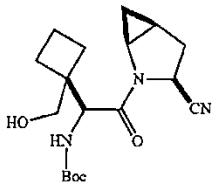


27 28

a OsO_4, THF H_2O, 1 1, $NaIO_4$, workup, then $NaBH_4$, MeOH, RT 56%
b TFA·CH_2Cl_2, 1 2, 0 degrees C to RT

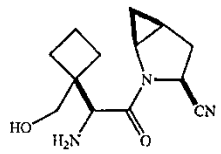


Step 1



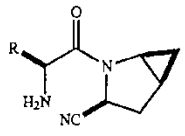
N-Boc protected cyclobutylvinyl compound (Example 31, prepared by general method C) (0.16 g, 0.46 mmol) was dissolved in 10 mL of a 1:1 mixture of THF:water and treated with OsO4 (12 mg, catalyst) and $NaIO_4$ (0.59 g, 2.76 mmol, 6 equiv). After 2 h, the reaction mixture was diluted with 50 mL of ether and 10 mL of water. The layers were equilibrated and the organic fraction was washed one time with $NaHCO_3$ solution, dried over $MgSO_4$ and concentrated to give a dark oil. The oil was diluted with 10 mL of methanol and treated with $NaBH_4$ (0.08 g, 2.0 mmol). The mixture turned very dark and after 30 min was diluted with ether and the reaction was quenched with aqueous $NaHCO_3$ solution. The mixture was equilibrated and layers separated. The organic fraction was washed with solutions of $NaHCO_3$ and 0.1 M HCl. The organics were dried ($MgSO_4$) and concentrated to give 90 mg (56%) of the Step 1 compound as a dark oil.

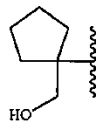
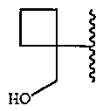
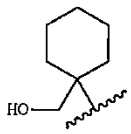
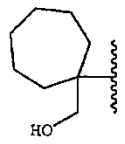
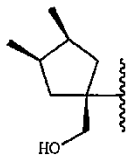
Step 2



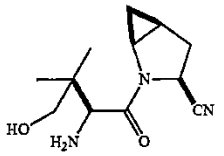
Step 1 compound (90 mg, 0.26 mmol) was dissolved in 3 mL of CH_2Cl_2, cooled to 0° C. and treated with 3 mL of freshly distilled TFA. The reaction was complete in 80 min and evaporated to dryness and purified by preparative HPLC (YMC S5 ODS 30x100 mm, 10 minute gradient 100%A to 100% B, Solvent A=10% MeOH-90%H20O-0.1% TFA, Solvent B=90% MeOH-10% H_2O-0.1% TFA, to give, after removal of water, 50 mg (60%) of title compound. (MH+ 250).

TABLE 3

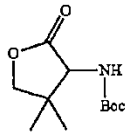


Example	R	Method of Preparation	(M + H)
44		Ozonolysis/borohydride	264
45		Osmium/periodate/ borohydride	250
46		Ozonolysis/borohydride	278
47		Osmium/periodate/ borohydride	292
48		Ozonolysis/borohydride	292

EXAMPLE 49



Step 1

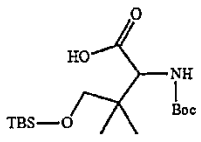


Part A. A 50-mL flask was charged with dihydro-4,4-dimethyl-2,3-furandione (5.0 g, 39.0 mmol), acetic acid (10 mL), sodium acetate (3.82 g, 39.0 mmol) and hydroxylamine hydrochloride (2.71 g, 39.0 mmol). The reaction mixture was stirred for 2 h at rt and concentrated under reduced pressure to remove most of the acetic acid. The remainder was poured into water (100 mL) and the aqueous phase extracted with EtOAc (3×40 mL). The organics were dried over Na_2SO_4 and concentrated to a colorless oil which solidified on standing.

Part B. A 200-mL round bottomed flask was charged with Part A solid (@ 39 mmol) and diluted with 80 mL of ethanol and 39 mL of 2N HCl (78 mmol). The mixture was treated with 1.0 g of 5% Pd/carbon and the mixture degassed. The flask was placed under an atmosphere of H_2 for 8 h. The mixture was filtered through celite and the filtrate concentrated to an off white solid.

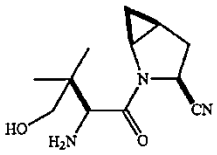
Part C. A 250-mL round bottomed flask was charged with Part B solid and diluted with THF (50 mL) and water (15 mL). The mixture was treated with di-tert-butyldicarbonate (12.7 g, 117 mmol) and sodium bicarbonate (10.0 g, 117 mmol). After 4 h of stirring the mixture was diluted with 50 mL of ether and 50 mL of water. The layers were separated and the organic fraction dried over $MgSO_4$ and concentrated. The residue was purified by flash column chromatography on silica gel with 30% EtOAc in hexanes to give 2.00 g (22% overall) of Step 1 compound as a white solid.

Step 2



To a stirred solution of Step 1 compound (1.00 g, 3.80 mmol) in THF (20 mL) at rt under nitrogen was added LiOH hydrate (0.16 g, 3.80 mmol) and then water (5 mL). The reaction was stirred at 40° C. for 0.5 h and then cooled to rt. The mixture was concentrated to dryness and the remainder was stripped from THF (2×), toluene (2×) and THF (1×). The remaining glass was diluted with 5 mL of THF and treated with imidazole (0.63 g, 9.19 mmol) followed by t-butyldimethylsilyl chloride (1.26 g, 8.36 mmol). The reaction was stirred overnight and quenched with 10 mL of methanol. After 1 h of stirring the mixture was concentrated. An additional portion of methanol was added and the mixture concentrated. The oil was diluted with ether and 0.1 N HCl (pH 2). The layers were equilibrated and aqueous drawn off. The organic fraction was dried over $MgSO_4$ and concentrated to give 1.25 g (83%) of Step 2 compound as a colorless glass.

Step 3

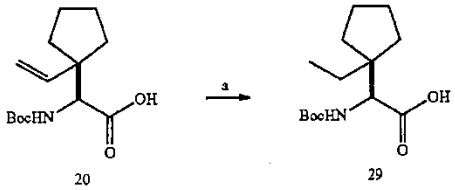


The Title compound was prepared by the peptide coupling of Step 2 carboxylic acid with Example 6 Step 3 amine, followed by dehydration and deprotection as outlined in General Method C. MS (M+H) 238.

General Method F: Catalytic Hydrogenation of vinyl substituent. As shown in Scheme 8, the protected vinyl substituted amino acid 20 was transformed to the corresponding saturated analog 29 by catalytic hydrogenation using 10% Pd/C and hydrogen at atmospheric pressure.

Scheme 8

General Method F, Examples 50-56



20 29

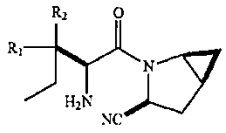
a 10% Pd/C, 1 atm H_2, MeOH, 12 h, 100%

Step 1.

The N-(tert-Butyloxycarbonyl)(1'vinylcyclopentyl) glycine (2.23 g, 8.30 mmol) was dissolved in 50 mL MeOH and placed in a hydrogenation vessel purged with argon. To this mixture was added 10% Pd-C (224 mg, 10% w/w) and the reaction stirred under 1 atm H_2 at rt for 12 h. The reaction was filtered through celite and concentrated and purified by flash column chromatography on silica gel with 1:9 methanol: CH_2Cl_2 to give the Step 1 compound as a glass. (FAB MH+272)

Examples 50-56 were prepared by the peptide coupling of amino acids (where the vinyl substituent has been hydrogenated according to general method F) followed by dehydration and deprotection as described in general method C.

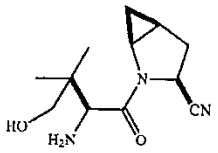
TABLE 4



Example	R1, R2	MS [M + H]
50	Cyclopentyl	262
51	cyclobutyl	248
52	cycloheptyl	290
53	4-pyranyl	278
54	methyl, methyl	236
55	ethyl, ethyl	264
56	methyl, ethyl	250

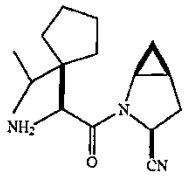
EXAMPLE 57

Step 3



The title compound in Example 57 was prepared by the peptide coupling of the isopropyl cyclobutane amino acid (where the olefin substituent has been hydrogenated according to general method F) followed by dehydration and deprotection as described in general method C.

EXAMPLE 58

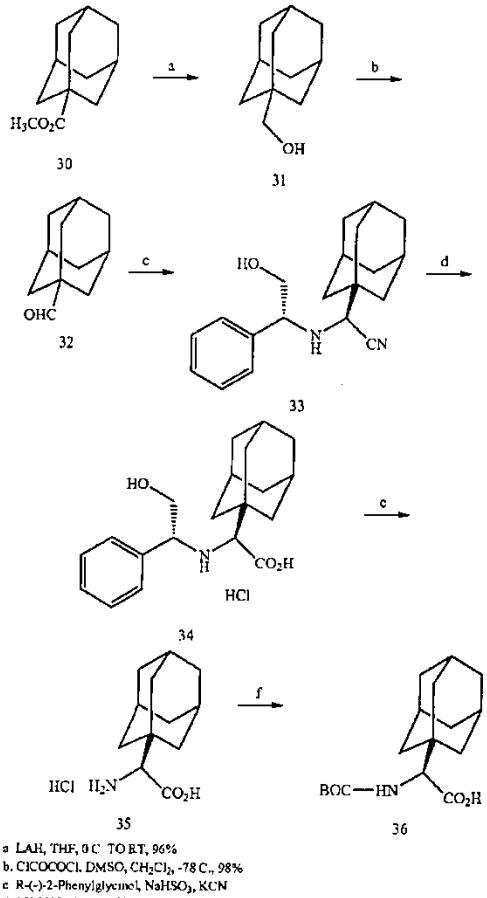


The title compound in Example 58 was prepared by the peptide coupling of the isopropyl cyclopentane amino acid (where the olefin substituent has been hydrogenated according to general method F) followed by dehydration and deprotection as described in general method C. MS (M+H) 276

General Method G: L-Amino acids synthesized by Asymmetric Strecker Reaction. Commercially available adamantyl carboxylic acid was esterified either in MeOH with HCl at reflux or using trimethylsilyldiazomethane in Et_2O/methanol to give 30. The ester was reduced to the alcohol 31 with LAH in THF and then subjected to a Swern oxidation to give aldehyde 32. Aldehyde 32 was transformed to 33 under asymmetric Strecker conditions with KCN, $NaHSO_3$ and R-(−)-2-phenylglycinol. The nitrile of 33 was hydrolyzed under strongly acidic conditions using 12M HCl in HOAc to give 34. The chiral auxiliary was removed by catalytic reduction using Pearlman's catalyst in acidic methanol under 50 psi hydrogen to give 35 and the resulting amino group was protected as the t-butylcarbamate to give 36.

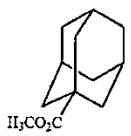
Scheme 9

General Method G, Examples 59-64



a LAH, THF, 0 C TO RT, 96%
b. ClCOCOCl, DMSO, CH_2Cl_2, -78 C., 98%
c R-(-)-2-Phenylglycinol, $NaHSO_3$, KCN
d. 12M HCl, HOAc, 80 C , 16 h, 78%
e 20% $Pd(OH)_2$, 50 psi H_2, MeOH HOAc, 5 1
f. $(Boc)_2O$, K_2CO_3, DMF, 92%, 2 steps

Step 1



Adamantane-1-carboxylic acid (10.0 g, 55 mmol, 1 equiv) was dissolved in a mixture of Et_2O (160 mL) and MeOH (40 mL), and was treated with trimethylsilyl diazomethane (2.0 M in hexane, 30 mL, 60 mmol, 1.1 equiv) and stirred at rt for

3 h. The volatiles were then removed by rotary evaporation and the product purified by flash column chromatography on silica gel (5×15 cm) with 40% CH_2Cl_2/hexanes to give the product as a white crystalline solid (10.7 g, 100%).

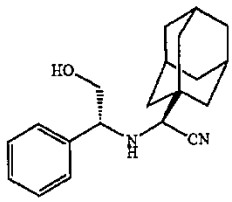
Step 2

Step 1 compound (10.7 g, 0.055 mmol, 1 equiv) was dissolved in anhydrous THF (150 mL) under argon and was treated with a solution of $LiAlH_4$ (1 M in THF, 69 mL, 69 mmol, 1.25 equiv). After stirring at rt for 1.5 h, the reaction was cooled to 0° C. and quenched sequentially with H_2O (5.1 mL), 15% aq NaOH (5.1 mL), and H_2O (10.2 mL). After stirring at rt for 15 min, the slurry was vacuum filtered, and the solids washed with EtOAc (2×100 mL). The filtrate was concentrated by rotary evaporation and the resulting solid purified by flash column chromatography on silica gel (5×15 cm) with 10% EtOAc/CH_2Cl_2. This afforded the Step 2 product as a white solid (8.74 g, 96%).

Step 3

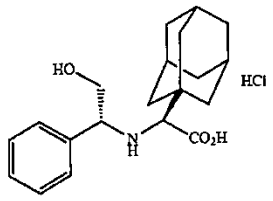
An oven-dried 3-neck flask equipped with 125-mL addition funnel was charged with anhydrous CH_2Cl_2 (150 mL) and anhydrous DMSO (10.3 mL, 0.145 mol, 2.5 equiv) under argon atmosphere and cooled to −78° C. Slow dropwise addition of oxalyl chloride (6.7 mL, 0.0768 mol, 1.32 equiv) followed by stirring for 15 min provided an activated DMSO adduct. This was treated with a solution of Step 2 compound (9.67 g, 58.2 mmol, 1 equiv) in dry CH_2Cl_2 (75 mL) and the reaction allowed to stir for 1 h. The resulting white mixture was then treated dropwise with triethylamine (40.5 mL, 0.291 mol, 5 equiv). After 30 min, the cooling bath was removed, and the reaction quenched sequentially with cold 20% aq KH_2PO_4 (25 mL) and cold H_2O (150 mL). After stirring at rt for 15 min the mixture was diluted with Et_2O (400 mL)and the layers were separated. The organics were washed organic with cold 10% aq KH_2PO_4 (3×150 mL) and satd aq NaCl (100 mL). The organics were dried (Na_2SO_4), filtered and concentrated. The residue was purified by flash column chromatography on silica gel (5×10 cm) with CH_2Cl_2 to give the Step 3 compound as a white solid (9.40 g, 98%).

Step 4



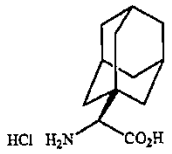
Step 3 compound (9.40 g, 57 mmol, 1 equiv) was suspended in H_2O (145 mL) and cooled to 0° C. The mixture was treated with $NaHSO_3$ (5.95 g, 57 mmol, 1 equiv), KCN (4.0 g, 59 mmol, 1.04 equiv), and a solution of (R)-(−)-phenylglycinol (8.01 g, 57 mmol, 1 equiv) in MeOH (55 mL). The resulting mixture was stirred at rt for 2 h, then refluxed for 16 h. The mixture was cooled to rt, and 200 mL of EtOAc added. After mixing for 15 min the layers were separated. The aqueous fraction was extracted with EtOAc. The combined EtOAc extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4, filtered and the filtrate concentrated. The product was purified by flash column chromatography on silica gel (6.4×20 cm) with 20% EtOAc/hexanes to give the desired (R,S) product as a white solid (11.6 g, 37.4 mmol, 65%): MS m/e 311 $(M+H)^+$.

Step 5



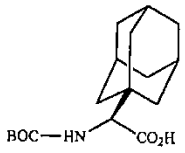
The Step 4 nitrile (5.65 g, 18 mmol) was heated in conc. HCl (120 mL) and HOAc (30 mL) at 80° C. for 18 h, at which time the reaction was cooled in an ice bath. Vacuum filtration of the resulting precipitate afforded the desired product as a white solid (5.21 g, 14 mmol, 78%). MS m/e 330 $(m+H)^+$.

Step 6



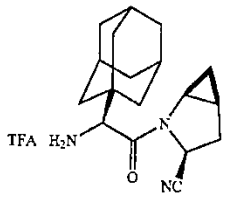
The Step 6 compound (5.21 g, 14 mmol) was dissolved in MeOH (50 mL) and HOAc (10 mL), and hydrogenated with H_2 (50 psi) and Pearlman's catalyst (20% $Pd(OH)_2$, 1.04 g, 20% w/w) for 18 h. The reaction was filtered through a PTFE membrane filter and the catalyst washed with MeOH (3×25 mL). The filtrate was concentrated by rotary evaporation to afford a white solid. The product was used in Step 7 without further purification.

Step 7



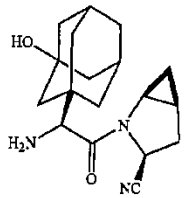
The crude Step 6 compound (@ 14 mmol) was dissolved in anhydrous DMF (50 mL) under argon and treated with K_2CO_3 (5.90 g, 42 mmol, 3 equiv) and di-tert-butyldicarbonate (3.14 g, 14 mmol, 1 equiv) under argon at rt. After 19 h, the DMF was removed by rotary evaporation (pump) and the residue dried further under reduced pressure. The residue was mixed with H_2O (100 mL) and Et_2O (100 mL), the layers separated, and the alkaline aqueous with Et_2O (2×100 mL) to remove the by-product from the hydrogenolysis step. The aqueous was cooled to 0° C., diluted with EtOAc (200 mL), and stirred vigorously while care fully acidifying the aqueous to pH 3 with 1N aq HCl. The layers separated and the aqueous extracted with EtOAc (100 mL). The combined EtOAc extracts were washed with brine (50 mL), dried (Na_2SO_4), filtered and the filtrate concentrated by rotary evaporation. The residue was purified by SiO_2 flash column (5×12 cm) with 5% MeOH/CH_2Cl_2+0.5% HOAc. The product was chased with hexanes to afford the product as a white foam (4.07 g, 13 mmol, 92%): MS m/e 310 $(m+H)^+$.

EXAMPLE 59



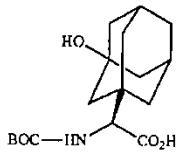
The title compound in Example 59 was prepared by the peptide coupling of the Step 7 compound in general method G followed by dehydration and deprotection as described in general method C. MS m/e 300 $(m+H)^+$.

EXAMPLE 60



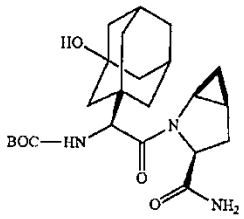
-continued

Step 1



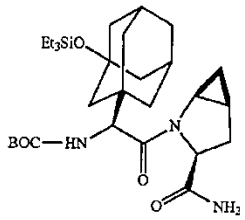
A solution of $KMnO_4$ (337 mg, 2.13 mmol, 1.1 equiv) in 2% aq KOH (6 mL) was heated to 60° C. and Step 7 compound in general method G (600 mg, 1.94 mmol, 1 equiv) was added in portions, and heating increased to 90° C. After 1.5 h, the reaction was cooled to 0° C., EtOAc (50 mL) was added, and the mixture was carefully acidified to pH 3 with 1N HCl. The layers were separated and the aqueous was extracted with EtOAc (50 mL). The combined organic extracts were washed with brine, dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash column chromatography on silica gel (3.8×15 cm) with 2% (200 mL), 3% (200 mL), 4% (200 mL), and 5% (500 mL) MeOH/CH_2Cl_2+0.5% HOAc. After isolation of the product, the material was chased with hexanes to afford a white solid (324 mg, 51%): MS m/e 326 $(m+H)^+$.

Step 2



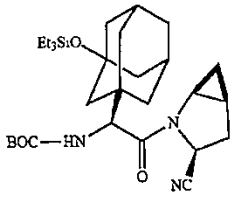
The Step 1 compound (404 mg, 1.24 mmol, 1 equiv) was dissolved in anhydrous DMF (10 mL) under argon and cooled to 0° C. The following were added in order: Example 6 Step 3 salt (328 mg, 1.37 mmol, 1.1 equiv), HOBT (520 mg, 3.85 mmol, 3.1 equiv), EDAC (510 mg, 2.61 mmol, 2.1 equiv), and TEA (0.54 mL, 3.85 mmol, 3.1 equiv). The reaction mixture was allowed to warm to rt overnight and the DMF removed by rotary evaporation (pump). The remainder was dried further under vacuum. The residue was dissolved in EtOAc (100 mL), washed with satd aq $NaHCO_3$ (50 mL) and satd aq NaCl (25 mL), dried over anhydrous Na_2SO_4, filtered and concentrated by rotary evaporation. The product was purified flash column chromatography on silica gel (3.8×15 cm) with a gradient of 6% (200 mL), 7% (200 mL), and 8% (500 mL) MeOH/CH_2Cl_2 to give the product as a white solid (460 mg, 1.06 mmol, 85%): MS m/e 434 $(m+H)^+$.

Step 3



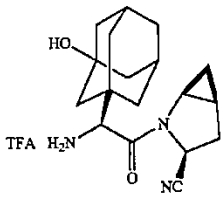
The Step 2 compound (95 mg, 0.22 mmol, 1 equiv) was dissolved in anhydrous CH_2Cl_2 (2.5 mL) under argon and cooled to −78° C. The mixture was treated with diisopropylethylamine (65 μL, 0.37 mmol, 1.7 equiv), and triethylsilyl triflate (75 μL, 0.33 mmol, 1.5 equiv), and stirred at 0° C. for 1.5 h. The reaction was mixed with MeOH (0.5 mL), silica gel (200 mg) and H_2O (2 drops) and stirred at rt for 18 h. The solvent was removed by rotary evaporation and the residue purified flash column chromatography on silica gel(2.5×10 cm) with 4% MeOH/CH_2Cl_2 to afford the product (92 mg, 0.17 mmol, 77%): Ms m/e 540 $(m+H)^+$.

Step 4



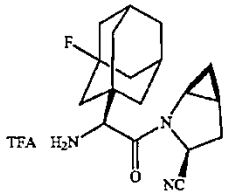
The Step 3 compound (90 mg, 0.16 mmol, 1 equiv) was dissolved in anhydrous pyridine (2 mL) under argon and cooled to −30° C. Treatment with imidazole (24 mg, 0.35 mmol, 2.1 equiv) and phosphorous oxychloride (66 μL, 0.67 mmol, 4.1 equiv), and continued stirring at −30° C. for 45 min gave a thick slurry. Volatiles were by rotary evaporation and the cake dried further under reduced pressure. The product was purified by flash column chromatography on silica gel (2.5×10 cm) with 7% EtOAc/CH_2Cl_2 to afford the product as a white foam (76 mg, 87%): MS m/e 530 $(m+H)^+$

Step 5

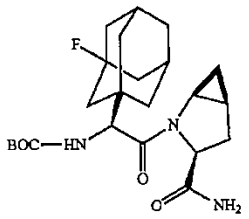


The Step 4 compound (76 mg, 0.14 mmol) was dissolved in anhydrous CH_2Cl_2 (1 mL) and cooled to 0° C. and treated with TFA (1 mL) and H_2O (2 drops) and stirred for 1.5 hr at 0° C. The solvents were removed by rotary evaporation and the residue was chased with toluene (5 mL) and dried under reduced pressure. Trituration with Et_2O afforded the title compound as a white solid (54 mg, 88%): MS m/e 316 $(m+H)^+$.

EXAMPLE 61

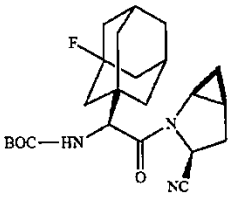


Step 1



An oven-dried flask purged with argon was charged with anhydrous CH_2Cl_2 (3 mL) and cooled to −78° C. Treatment with diethylaminosulfur trifluoride (DAST, 60 μL, 0.45 mmol, 1.5 equiv), followed by a solution of the Example 60 Step 2 compound (131 mg, 0.30 mmol, 1 equiv) in dry CH_2Cl_2 (3 mL). After 15 min, the reaction was poured into a separatory funnel containing satd aq $NaHCO_3$ (25 mL) and the layers were separated. The aqueous fraction was extracted with CH_2Cl_2 (25 mL), then the combined organic extracts were washed with brine (10 mL), dried (Na_2SO_4), filtered and concentrated. The product was purified by flash column chromatography on silica gel (2.5×10 cm) with 5% MeOH/CH_2Cl_2 to give Step 1 compound (124 mg, 0.29 mmol, 94%): MS m/e 436 $(m+H)^+$.

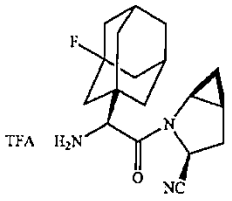
Step 2



The fluorinated amide from Step 1 (161 mg, 0.37 mmol, 1 equiv) was dissolved in anhydrous pyridine (4 mL) under argon and cooled to −30° C. The mixture was treated with imidazole (54 mg, 0.77 mmol, 2.1 equiv) and phosphorous oxychloride (143 μL, 1.52 mmol, 4.1 equiv) and stirred at −30° C. for 40 min. The solvent was removed by rotary evaporation and dried further under reduced pressure. The product was purified by flash column chromatography on silica gel (2.5×10 cm) with 5% EtOAc/CH_2Cl_2 to give the

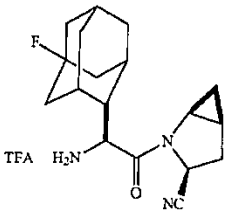
Step 2 compound as a white foam (126 mg, 82%): MS m/e 418 $(m+H)^+$.

Step 3

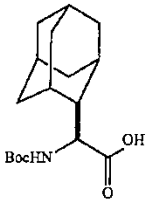


The Step 2 compound (125 mg, 0.30 mmol) was dissolved in TFA/CH_2Cl_2 (1:1 v/v, 2 mL), and stirred at rt. After 30 min, the solvents were removed by rotary evaporation, the remainder was chased with toluene (2×5 mL), and the solid dried under reduced pressure. Trituration with Et_2O afforded the title compound as a white solid (93 mg, 0.21 mmol, 72%): MS m/e 318 $(m+H)^+$.

EXAMPLE 62

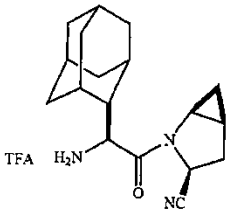


Step 1



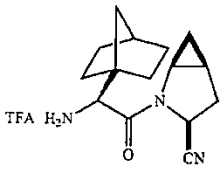
The Step 1 compound was prepared beginning with 2-adamantanal and elaborated to the homochiral Boc-amino acid by an asymmetric Strecker synthesis according to general method G.

Step 2

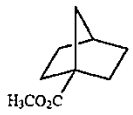


The title compound in Example 62 was prepared by the peptide coupling of the 2-adamantyl amino acid described in Step 1 followed by dehydration and deprotection as described in general method C.MS (M+H) 300.

EXAMPLE 63

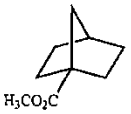


Step 1



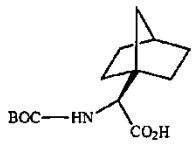
An oven-dried flask equipped with a condenser and drying tube was charged with norbornane-2-carboxylic acid (4.92 g, 35 mmol, 1 equiv) and treated with bromine (2.1 mL, 41 mmol, 1.15 equiv) and phosphorous trichloride (0.153 mL, 1.8 mmol, 0.05 equiv). The mixture was heated at 85° C. for 7 h protected from light. Additional bromine (0.4 mL, 7.8 mmol, 0.22 equiv) was added with continued heating for 1 h. The mixture was cooled to rt, and Et_2O (100 mL) was added. The mixture was washed with 10% aq $NaHSO_3$ (50 mL), H_2O (2×50 mL), and brine (25 mL). The ether fraction was dried (Na_2SO_4), filtered and concentrated by rotary evaporation. The product was purified by flash column chromatography on silica gel (5×15 cm) with 2% to 4% MeOH/CH_2Cl_2+0.5% HOAc. The product was chased with hexanes to remove residual HOAc. The isolated material consists of two inseparable materials (4.7 g), which was used without further purification in the next step.

The crude product from above, exo-2-bromonorbornane-1-carboxylic acid (4.7 g, impure) in Et_2O (80 mL) and MeOH (20 mL), was mixed with trimethylsilyldiazomethane (2.0 M in hexane, 11.8 mL, 23.6 mol), and stirred at rt for 1 h. Solvent was removed by rotary evaporation, and purification of the oil by flash column chromatography on silica gel (5×18 cm) with a gradient of CH_2Cl_2/hexanes (600 mL each of 20% and 30%) followed by CH_2Cl_2 afforded the product as a white solid (3.97 g, 0.017 mol, 79% for 2 steps): MS m/e 233/235 $(m+H)^+$.



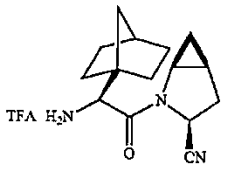
Methyl exo-2-bromonorbornane-1-carboxylate (2.0 g, 8.58 mmol, 1 equiv) was dissolved in anhydrous THF (50 mL) in an oven-dried 3-neck flask equipped with a condenser, and purged with argon. The mixture was treated with AIBN (288 mg, 1.71 mmol, 0.2 equiv) and tributyltin hydride (3.6 mL, 12.87 mmol, 1.5 equiv), and then heated to reflux for 2 h. The flask was cooled to rt, and the THF was removed by rotary evaporation to give the crude product. The product was purified by flash column chromatography on silica gel(5×10 cm) with 5% EtOAc/hexanes. The resulting material was used in the next step without further purification.

Step 2



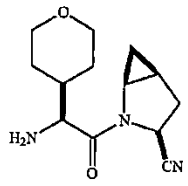
The Step 1 compound was prepared beginning with 1-norbonyl methyl carboxylate and elaborated to the homochiral Boc amino acid by an asymmetric Strecker synthesis according to general method G.

Step 3

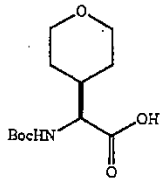


The title compound in Example 63 was prepared by the peptide coupling of the 1-norbonyl amino acid described in Step 2, followed by dehydration and deprotection as described in general method C. MS (M+H) 260.

EXAMPLE 64

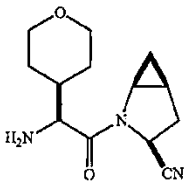


Step 1



The Step 1 compound was prepared beginning with 4-formylpyran and elaborated to the homochiral Boc amino acid by an asymmetric Strecker synthesis according to general method G.

Step 2

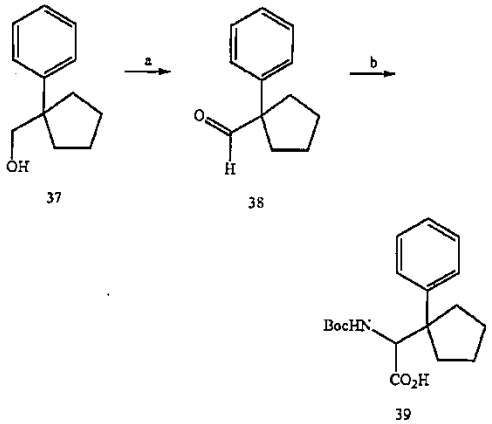


The title compound in Example 64 was prepared by the peptide coupling of the 4-pyranyl amino acid described in Step 2, followed by dehydration and deprotection as described in general method C. MS (M+H) 250.

General Method H: Strecker Synthesis of Racemic Amino Acids.

Scheme 10

General Method H, Examples 65-66

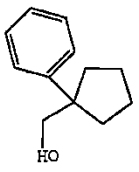


37 38 39

a celite, PCC, CH_2Cl_2, RT, 91%

b NH_4Cl, NaCN, MeOH, 12M HCl, HOAc, $(Boc)_2O$, TEA, DMF

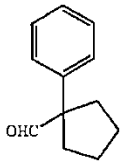
Step 1



To a stirred solution of 1-phenylcyclo-1-pentanecarboxylic acid (5.00 g, 26.3 mmol) in 25 mL of THF at 0° C. was added LAH (52 mL , 52 mmol, 1M) in THF. The reaction mixture was slowly warmed to rt and then refluxed for 18 h. The reaction was quenched according to the Fieser procedure: careful addition of 2 mL of water; 6 mL of 15% NaOH in water; and 2 mL of water. The biphasic mixture was diluted with 100 mL of ether and the granular white solid filtered off.

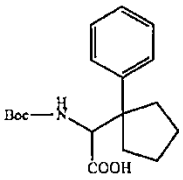
The ether fraction was dried over Na_2SO_4 and evaporated to give 4.30 g (93%) of the Step 1 compound.

Step 2



To a stirred solution of Step 1 compound (0.80 g, 4.50 mmol) in 15 mL of CH_2Cl_2 at rt was added celite (5 g) followed by PCC (1.95 g, 5.00 mmol). After stirring for 3 h the reaction mixture was diluted with 40 mL of CH_2Cl_2 and filtered through celite. The filtrate was filtered an additional time through silica gel resulting in a colorless filtrate. The CH_2Cl_2 fraction was evaporated to give 0.72 g (91%) of the aldehyde as a colorless oil.

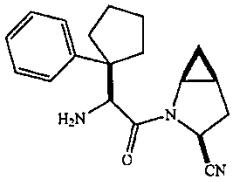
Step 3



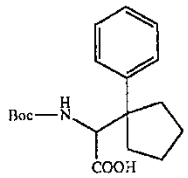
To a 50-mL round-bottomed flask containing Step 2 compound (0.72 g, 4.20 mmol) in 8 mL of water at rt was added NaCN (0.20 g, 4.20 mmol) followed by NH_4Cl (0.20 g, 5.00 mmol). To this reaction mixture was then added methanol (8 mL) and the mixture was allowed to stir overnight. The reaction mixture was then extracted with ether (2×15 mL), dried ($MgSO_4$) and concentrated under reduced pressure to give the crude Strecker product.

To a 100-mL round-bottomed flask containing the crude Strecker product was added 10 mL of HOAc and 10 mL of conc. HCl. The mixture was refluxed overnight. The mixture was concentrated under reduced pressure to give a yellow solid. The solid was triturated with 5 mL of 1:1 mixture of ether and hexanes. The white solid was treated with triethylamine (1.4 mL, 9.99 mmol) and di-tert-butyldicarbonate (1.00 g, 4.60 mmol) in 50 mL DMF. After 4 h the pH of the mixture was adjusted to 9 with saturated Na_2CO_3 soln. After an additional 3 h of stirring the mixture was extracted with 1:1 ether and hexanes and the aqueous fraction acidified to pH 2 with 5% $KHSO_4$ solution. The aqueous phase was washed with ether (2×40 mL), the organics dried ($MgSO_4$), and evaporated to an oil that was purified by silica gel flash chromatography with 8:92 methanol:CH_2Cl_2 to give 0.3 g (23%) of the Boc-protected amino acid as a light oil (M-H, 318).

EXAMPLE 65

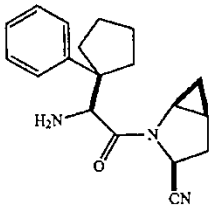


Step 1



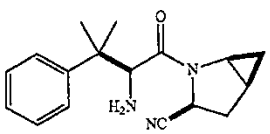
The synthesis of the Step 1 compound was described in general method H for the Strecker synthesis of racemic amino acids.

Step 2

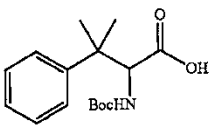


The title compound in Example 65 was prepared by the peptide coupling of the cyclopentylphenyl amino acid described in Step 1 and general method H followed by dehydration and deprotection as described in general method C. MS (M+H) 310.

EXAMPLE 66

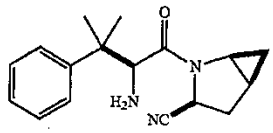


Step 1



Step 1 compound was prepared using racemic Strecker synthesis according to general method H starting from 2,2-dimethyl-phenylacetic acid.

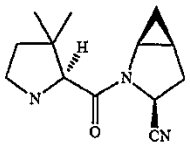
Step 2



The title compound in Example 66 was prepared by the peptide coupling of the dimethylphenyl amino acid described in step 1 followed by dehydration and deprotection as described in general method C. MS (M+H) 284.

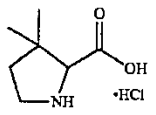
EXAMPLE 67

Step 1



N-(Benzyloxycarbonyl)succinimide (5.6 g, 22.4 mmol) was dissolved in CH_2Cl_2 (25 mL) and the solution was added to a cooled (0° C.) and stirred solution of diethyl aminomalonate hydrochloride (5.0 g, 23.6 mmol) and triethylamine (13.4 mL, 95 mmol) in CH_2Cl_2 (125 ml). The resulting solution was stirred at 0° C. for 10 min and then at rt for 1 h. The solution was washed with 10% citric acid (2×50 mL),10% sodium bydrogen carbonate (2×50 mL), and water (50 mL) and was then dried (Na_2SO_4) and evaporated to afford diethyl N-benzyloxycarbonylaminomalonate as a colorless oil, which crystallized upon standing at 0° C. (6.3 g) (LC/Mass + ion): 310 (M+H).

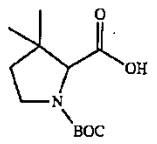
Step 2



Step 1 compound (6.18 g, 20 mmol) was dissolved in dry ethanol (30 mL) and added to a solution of sodium ethoxide (2.85 g, 8.8 m mol; 21% w/w solution in ethanol (6 mL). A solution of 3-methyl-2-butenal (1.68 g, 20 mmol) in ethanol (12 mL) was added, and the solution stirred at 25° C. for 24 h. Acetic acid (0.56 mL) was then added the solution hydrogenated at 50 psi for 24 h using 10% Pd/C (2.0 g) as catalyst. The solution was filtered, evaporated and the residue chromatographed on silica with CH_2Cl_2 /EtOAc (9:1) to give 2,2-dicarboethoxy-3,3-dimethyl-pyrrolidine (1.6 g) (LC/Mass, +ion): 244 (M+H).

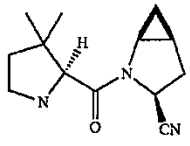
This diester (850 mg) was refluxed in 5 M hydrochloric acid (10 mL)/TFA (1 mL) for 8 h to give, after evaporation, a powdery white solid. Crystallization from methanol/ether gave 3,3-dimethyl-dl-proline hydrochloride (190 mg) as white crystals mp 110-112° C.

Step 3



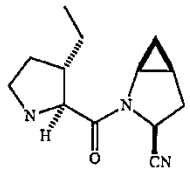
Step 2 compound (173 mg, 0.97 mmol) was dissolved in DMF (3 mL)/water (3 mL). To this clear solution was added triethylamine (0.46 mL, 3.18 mmol) and di-t-butyl dicarbonate (0.23 g, 1.06 mmol), and the reaction mixture was stirred at rt for 5 h. The solution was evaporated and the residue chromatographed on silica column using CH_2Cl_2/methanol (9:1) as eluent to yield t-butyloxy-carbonyl-3,3-dimethyl-dl-proline (200 mg) as an oil (LC/Mass, + ion): 244 (M+H).

Step 4

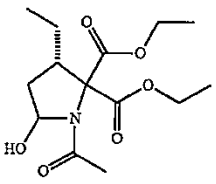


The title compound in Example 67 was prepared by the peptide coupling of the t-butyloxycarbonyl-3,3-dimethyl-dl-proline amino acid described in Step 3 followed by dehydration and deprotection as described in general method C. MS (M+H) 220.

EXAMPLE 68



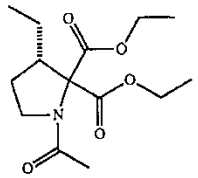
Step 1



Sodium ethoxide (940 mg of 21 wt % solution in ethanol, 2.9 mmol) in ethanol (2 mL) was added to a stirred solution of diethyl acetamidomalonate (4.31 g, 19,8 mmol) in EtOH (23 mL) at rt under argon. The reaction mixture was cooled to 0° C.; and trans-2-pentenal (1.51 g, 18.0 mmol) was added dropwise maintaining the reaction temperature at <5° C. After the addition, the reaction was allowed to warm to rt, stirred for 4 h, then quenched with acetic acid (460 μl). The solution was concentrated in vacuo, and the residue dissolved in EtOAc (25 mL), washed with 10% $NaHCO_3$ solution (2×5 mL), brine and dried ($MgSO_4$). The solution was filtered and con-

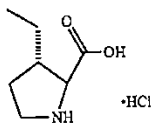
centrated to a 10 mL volume, then heated to reflux and diluted with hexane (20 mL). Upon cooling to rt, the title compound precipitated and was collected to give 3.0 g (50%) of the Step 1 compound (mp 106-109° C.; LC/Mass: + ions, 324 M+Na).

Step 2



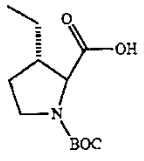
To a solution of Step 1 compound (2.87 g, 9.5 mmol) and triethylsilane (2.28 mL, 14.3 mmol) in CH_2Cl_2 (30 mL) under argon was added TFA (7.35 mL, 95.3 mmol) dropwise with stirring while maintaining the internal temperature at 25° C. by means of an ice bath. After stirring for 4 h at rt. the solution was concentrated. The residue was diluted with CH_2Cl_2 (100 mL), then treated with H_2O (50 mL) and solid Na_2CO_3 with vigorous stirring until the mixture was basic. The organic layer was separated, dried (Na_2SO_4), filtered, then concentrated to give the Step 2 compound as a yellow oil which was used without further purification (LC/Mass: + ions, 308 M+Na).

Step 3



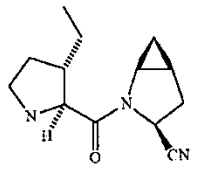
Step 2 compound (3.73 g, 9.5 mmol) was suspended in 6 N HCl (20 mL) and HOAc (5 mL) and heated at reflux for 20 h. The reaction mixture was then cooled, washed with EtOAc (20 mL), then concentrated to give an oil which crystallized upon trituration with ether to give the title compound (1.2 g, 70.6%) (LC/Mass, + ion): 144 (M+H).

Step 4



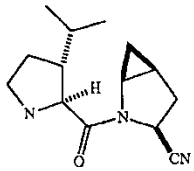
Step 3 compound (692 mg, 3.76 mmol) was dissolved in acetone (12 mL)/ water (12 mL). To this clear solution was added triethylamine (1.9 mL, 12.8 mmol) and di-t-butyl dicarbonate (928 mg, 4.24 mmol). The reaction mixture was stirred at rt for 18 h. The solvents were evaporated and the residue chromatographed on silica with 1:9 methanol: CH_2Cl_2 to give the Step 4 compound as an oil (LC/Mass: + ions, 266 M+Na).

Step 5

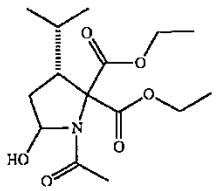


Example 68 compound was prepared by peptide coupling of Step 4 amino acid followed by dehydration and deprotection as described in general method C (MS (M+H) 234).

EXAMPLE 69

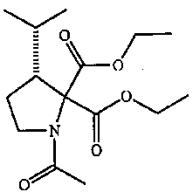


Step 1



Sodium ethoxide (940 mg, 2.9 mmol; 21% w/w solution in ethanol) in ethanol (2 mL) was added to a stirred solution of diethyl acetamidomalonate (4.31 g, 19.8 mmol) in EtOH (23 mL) at rt under argon. The reaction mixture was cooled to 0° C.; and 4-methyl-2-pentenal (1.77 g, 18.0 mmol)was added dropwise maintaining the reaction temperature at <5° C. After the addition, the reaction was allowed to warm to rt, stirred for 4 h, then quenched with acetic acid (460 μl). The solution was concentrated and the remainder dissolved in EtOAc (25 mL). The organics were washed with 10% $NaHCO_3$ solution (2×5 mL), brine and dried ($MgSO_4$). The solution was filtered and concentrated to 10 mL volume, then heated to reflux and treated with hexane (20 mL). On cooling, the Step 1 compound precipitated and was collected (3.3 g) (LC/Mass, + ion): 338 (M+Na).

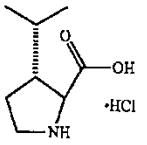
Step 2



To a solution of Step 1 compound (3.0g, 9.5 mmol) and triethylsilane (2.28 mL, 14.3 mmol) in CH_2Cl_2 (30 mL) under

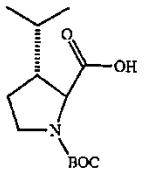
argon was added TFA (7.35 mL, 95.3 mmol) dropwise with stirring while maintaining the internal temperature at 25° C., by means of an ice bath. After stirring for 4 h at rt, the solution was concentrated, the residue diluted with CH_2Cl_2 (100 mL), then treated with H_2O (50 mL) and solid Na_2CO_3 with vigorous stirring until the mixture was basic. The organic layer was separated, dried (Na_2SO_4), filtered, then concentrated to give the title compound as an oil which was used without further purification (LC/Mass:+ ions, 300 M+H).

Step 3



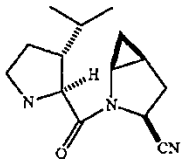
Step 2 compound (3.8 g, 9.5 mmol) was suspended in 6 N HCl (20 mL) and HOAc (5 mL) and heated at reflux for 20 h. The reaction mixture was cooled, washed with EtOAc (20 mL), then concentrated to give an oil which crystallized upon trituration with ether to give the step 3 compound (1.4 g, 76.0%). LC/Mass: + ions, 158 (M+H).

Step 4



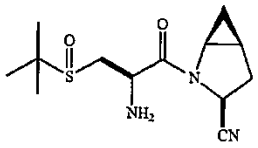
Step 3 compound (728 mg, 3.76 mmol) was dissolved in a 1:1 acetone/water solution (24 mL). To this clear solution was added triethylamine (1.9 mL, 12.8 mmol) and di-t-butyl dicarbonate (928 mg, 4.24 mmol). The reaction mixture was stirred at rt for 18 h. The solution was evaporated and the residue chromatographed on silica column using CH_2Cl_2/methanol (9:1) as eluent to give the title compound as an oil (LC/Mass, + ion): 258 (M+H).

Step 5



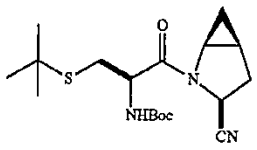
Example 69 compound was prepared by peptide coupling of Step 4 amino acid followed by dehydration and deprotection as described in general method C (MS (M+H) 248).

EXAMPLE 70



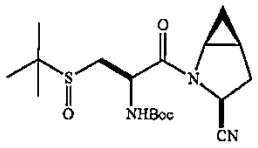
-continued

Step 1



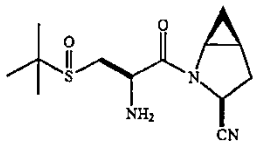
Step 1 compound was prepared by the procedure described in General Method C starting from N-Boc-S-t-butylcysteine.

Step 2



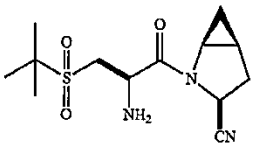
A 25-mL round-bottomed flask equipped with a magnetic stirring bar and N_2 inlet was charged with Step 1 compound (78 mg, 0.21 mmol) and chloroform (3 mL). The mixture was cooled to 0° C. and treated with m-chloroperoxybenzoic acid (85 mg, 0.44 mmol) in $CHCl_3$ (2 mL). After 3 h the solution was diluted with $CHCl_3$ (7 mL), washed with 5% $NaHCO_3$ (2×5 mL), H_2O and dried over Na_2SO_4. Removal of solvent gave crude sulfoxide (100 mg), which was used without further purification (LC/Mass, + ions): 384 (M+H).

Step 3

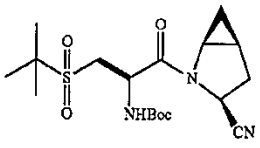


Trifluoroacetic acid (1.5 mL) was added to a cooled (0° C.) solution of Step 2 compound (100 mg, 0.26 mmol) in 5 mL CH_2Cl_2. The solution was then stirred at 0° C. for 1.5 h, diluted with CH_2Cl_2 (5 mL) and concentrated under reduced pressure to a thick oil. The product was purified by reverse phase preparative column chromatography on a YMC S5 ODS 20×100 mm column to give the title compound of Example 70 , 17 mg, 16%. Purification conditions: gradient elution from 10% methanol/water/0.1 TFA to 90% methanol/water/0.1 TFA over 15 min 5 min hold at 90% methanol/water/0.1 TFA. Flow rate: 20 mL/min. Detection wavelength: 220. Retention Time 10 Min (LC/Mass, + ion): 284 (M+H).

EXAMPLE 71

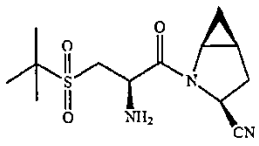


Step 1



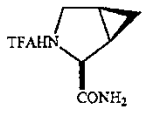
A 25-mL round-bottomed flask equipped with a magnetic stirring bar and N_2 inlet was charged with compound from Example 70, Step 1 (78 mg, 0.21 mmol) in chloroform (3 mL). The mixture was cooled to 0° C. and treated with m-chloroperoxybenzoic acid (144 mg, 0.84 mmol) in $CHCl_3$ (2 mL). After 30 min at rt, the solution was diluted with $CHCl_3$ (7 mL), washed with 5% $NaHCO_3$ (2×10 mL), H_2O and dried over Na_2SO_4. Removal of solvent gave the crude sulfone (100 mg), which was used without further purification (LC/Mass, + ion): 344 (M+H–Bu).

Step 2



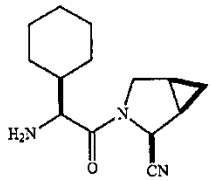
Trifluoroacetic acid (1.5 mL) was added to a cooled (0° C.) and stirred solution of Step 1 compound (100 mg, 0.26 mmol) in 5 mL CH_2Cl_2. The solution was stirred at 0° C. for 30 min, diluted with CH_2Cl_2 (5 mL) and concentrated under reduced pressure to a thick oil. The product was purified by reverse phase preparative column chromatography on a YMC S5 ODS 20×100 mm column to give the title compound, 14 mg, 17%. Purification conditions: gradient elution from 10% methanol/water/0.1 TFA to 90% methanol/water/0.1 TFA over 15 min 5 min hold at 90% methanol/water/0.1 TFA. Flow rate: 20 mL/min. Detection wavelength: 220. Retention Time 10 Min. (LC/Mass, + ion): 300 (M+H).

EXAMPLE 72



The title compound was prepared following a published procedure (Sasaki et al, Tetrahedron Lett. 1995, 36, 3149, Sasaki et al. Tetrahedron 1994, 50, 7093) used to synthesize (2S,3R,4S)-N-Boc-3,4-methano-L-proline carboxylate. The corresponding amide was prepared by general method A and deprotected with TFA to give the TFA salt also as described in general method A.

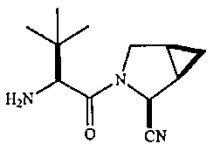
EXAMPLE 73



The title compound was prepared by coupling (2S,3R,4S)-3,4-methano-L-proline carboxamide-N-trifluoroacetate

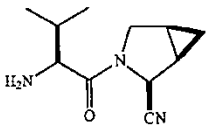
described in Example 72 with L-cyclohexylglycine and then dehydrated to the amide with $POCl_3$/imidazole and deprotected (N-terminal nitrogen) with TFA using general C (FAB MH+248).

EXAMPLE 74



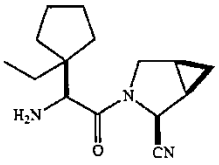
The title compound was prepared by coupling (2S,3R,4S)-3,4-methano-L-proline carboxamide-N-trifluoroacetate described in Example 72 with L-tert-butylglycine and then dehydrated to the amide with $POCl_3$/imidazole and deprotected (N-terminal nitrogen) with TFA using general C (FAB MH+222).

EXAMPLE 75



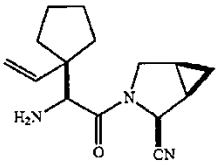
The title compound was prepared by coupling (2S,3R,4S)-3,4-methano-L-proline carboxamide-N-trifluoroacetate described in Example 72 with L-valine and then dehydrated to the amide with $POCl_3$/imidazole and deprotected (N-terminal nitrogen) with TFA using general C (FAB MH+207).

EXAMPLE 76



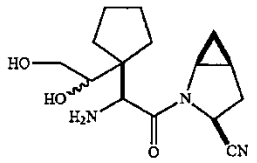
The title compound was prepared by coupling (2S,3R,4S)-3,4-methano-L-proline carboxamide-N-trifluoroacetate described in Example 72 with N-(tert-butyloxycarbonyl)-(1'ethylcyclopentyl)glycine described in General Method B and then dehydrated to the amide with $POCl_3$/imidazole and deprotected (N-terminal nitrogen) with TFA using general C (FAB MH+262).

EXAMPLE 77



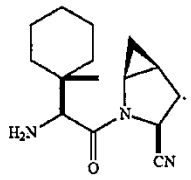
The title compound was prepared by coupling (2S,3R,4S)-3,4-methano-L-proline carboxamide-N-trifluoroacetate described in Example 72 with N-(tert-butyloxycarbonyl)-(1'vinylcyclopentyl)glycine described in General Method B and then dehydrated to the amide with $POCl_3$/imidazole and deprotected (N-terminal nitrogen) with TFA using General Method C (FAB MH+260).

EXAMPLE 78



N-[((S)-cyclopentylvinyl)-N-tert-butoxycarbonylglycinyl]-(2S,4S,5S)-2-cyano-4,5-methano-L-prolylamide (70 mg, 0.19 mmol) described in General Method C, Step 2 was dissolved in a mixture of 2 mL t-BuOH/3 mL THF and N-methylmorpholine-N-oxide (33mg, 0.28 mmol) was added followed by osmium tetroxide (0.1 mmol, 50 mol %). The reaction was quenched with 1 mL of 10% aqueous Na_2SO_3 and was taken up in EtOAc and washed with H_2O 5 mL, dried (Na_2SO_4), filtered, evaporated and purified by silica gel flash chromatography (5% MeOH/CH_2Cl_2) to give 41 mg (55%) of the protected diol as an oil. The title compound was obtained by deprotection of the amine functionality with TFA according to General Method C (FAB MH+294).

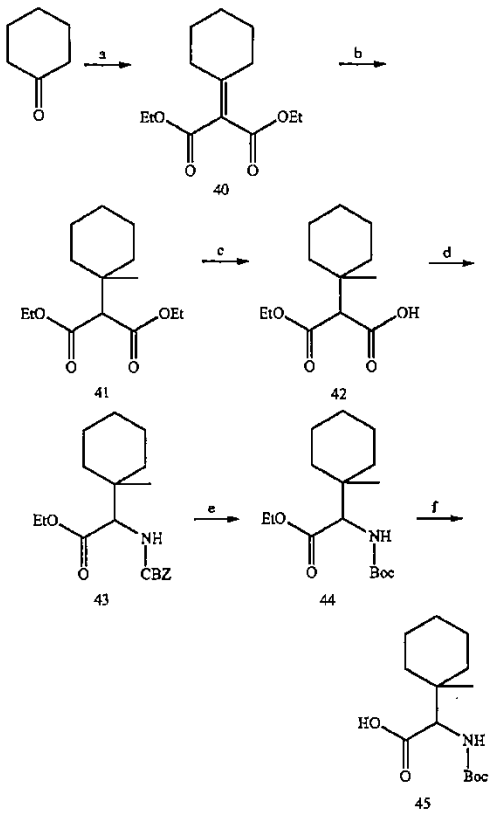
EXAMPLE 79



General Procedure I: Synthesis of Quaternary Amino Acids Via Michael Addition to Malonates followed by Selective Hydrolysis and Curtius Rearrangement. Examples 79-84.

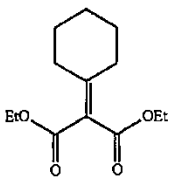
Cyclohexanone and diethylmalonate underwent Knoevenagel condensation mediated by titanium tetrachloride in THF and CCl_4 to give 40. Copper (I) mediated Grignard addition of methylmagnesium bromide gave 41 which was selectively saponified to 42. Curtius rearrangement with trapping by benzyl alcohol gave 43 which was converted to 44 by a standard deprotection-protection protocol. Ester 44 was saponified to give the quaternary amino acid 45.

Scheme 11
General Method I



a. THF, CCl_4, $TiCl_4$, diethylmalonate, 0 C., pyridine, THF, 0 to RT 72 h
b MeMgBr, CuI, Et_2O, 0 C
c 1N NaOH, EtOH, RT 6 days
d. Ph_2PON_3, TEA, RT to reflux to RT, BnOH
e 10% $Pd(OH)_2$/C, EtOAc, $(Boc)_2O$, K_2CO_3, THF
f. 1N NaOH, dioxane

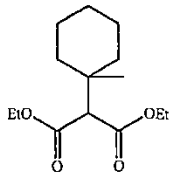
Step 1



According to literature procedure (Tetrahedron 1973, 29, 435), a mixture of dry tetrahydrofuran (400 mL) and dry carbon tetrachloride (50 mL) was cooled to 0° C. (ice-salt bath) and treated with titanium tetrachloride (22.0 mL, 0.2 mole). The resulting yellow suspension was stirred at 0° C. for 5 min, treated sequentially with cyclohexanone (10.3 mL, 0.1 mole) and distilled diethylmalonate (15.2 mL, 0.1 mole) then stirred at 0° C. for 30 min. The reaction mixture was then

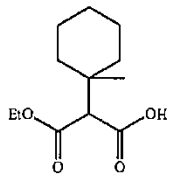
treated with a solution of dry pyridine (32 mL, 0.40 mole) in dry THF (60 mL), stirred at 0° C. for 1.0 h, then at rt for 72 h. The reaction mixture was quenched with water (100 mL), stirred for 5 min then extracted with ether (2×200 mL). The combined organic extracts were washed with saturated sodium chloride (100 mL), saturated sodium bicarbonate (100 mL) and brine (100 mL), dried over anhydrous magnesium sulfate, filtered and concentrated. Flash chromatography using 5% EtOAc in hexane gave step 1 compound as a light yellow oil. Yield: 5.25 g (22%). MS (M+Na) 263.

Step 2



According to literature (Org. Syn. VI, 442, 1988; Liebigs Ann. Chem. 1981, 748) a mixture of 3.0 M methylmagnesium iodide (3.1 mL, 9.36 mmol) and cuprous chloride (9.0 mg) was stirred at 0° C. (ice-salt water bath), treated with a solution of Step 1 compound (1.5 g, 6.24 mmol) in dry ether (1.8 mL) over 5 min and stirred at 0° C. for 1 h, then at rt for 40 min. The mixture was slowly added to a slurry of ice and water (15 mL), treated dropwise with 10% HCl (3.7 mL) then extracted with EtOAc (3×25 mL). The combined organic extracts were washed with 1% sodium thiosulfate (2.0 mL) and saturated sodium chloride (2.0 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated. Flash chromatography on a silica gel column using 5% ether in hexane (1.0 L) gave step 2 compound as a clear syrup. Yield: 1.09 g,(68%). MS (M+H)257.

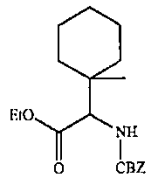
Step 3



A solution of Step 2 compound (1.09 g, 4.03 mmol) in a mixture of methanol (5.4 mL) and water (2.7 mL) was treated with 1N sodium hydroxide (4.84 mL, 4.84 mmol or 1.2 equiv) and stirred at rt for 6 days. The reaction mixture still showed the presence of starting material, so THF (4.0 mL) was added and the entire mixture stirred for another 2 days. The solution was evaporated to dryness and the resulting syrup partitioned between water (8.0 mL) and ether (15 mL). The aqueous phase was acidified with 1N hydrochloric acid (4.8 mL) to pH 2-3 and extracted with EtOAc (3 ×25 mL). The combined organic extracts were washed with brine (10.0 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated to give step 3 compound as a thick syrup. Yield: 875 mg, (95.1%). MS (M+H) 229.

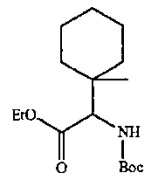
Or alternately: solutions of the diester in a mixture of ethanol, THF, dioxane and water or mixtures thereof may be hydrolyzed with sodium hydroxide.

Step 4



According to literature (J. Org. Chem 1994, 59, 8215), a solution of Step 3 compound (0.875 g, 3.83 mmol) in dry benzene (4.0 mL) was treated with triethylamine (0.52 mL, 3.83 mmol) and diphenylphosphoryl azide (0.85 mL, 3.83 mmol), refluxed under nitrogen for 1 h and cooled to rt. The solution was treated with benzyl alcohol (0.60 mL, 5.75 mmol or 1.5 equiv), refluxed for 17 h, cooled then diluted with ether (40 mL). The solution was washed with 10% aqueous citric acid (2×3 mL), back-extracting the citric acid wash with ether (40 mL). The combined organic extracts were washed with 5% sodium bicarbonate (2×3 mL), dried ($MgSO_4$), filtered, and concentrated. Flash chromatography on silica gel of the crude product with 10% EtOAc in hexane (1.0 L) gave step 4 compound as a clear thick syrup. Yield: 1.15 g (90%). MS(M+H) 334.

Step 5



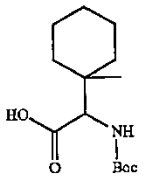
A solution of Step 4 compound (1.15 g, 3.46 mmol) in EtOAc (60 mL) was treated with palladium hydroxide on carbon (298 mg) and hydrogenated at rt for 20 h. The mixture was filtered through a celite pad and then washing the pad well with EtOAc (3×25 mL) then the filtrate was concentrated to give the free amine. A solution of the amine in tetrahydrofuran (12 mL) and water (12 mL) was treated with di-t-butyl dicarbonate (1.0 g, 4.58 mmol or 1.48 equiv) and potassium carbonate (854 mg, 6.18 mmol or 2.0 equiv), then stirred at rt for 20 h. The reaction mixture was partitioned between water (8 mL) and diethyl ether (3×40 mL) and the combined organic extracts were washed with brine (8 mL), dried ($MgSO_4$), filtered, and concentrated. Flash chromatography of the crude product with 10% EtOAc in hexane (1 L) gave step 5 compound as a clear thick syrup. Yield: 1.18 g (100%). MS:(M+H) 300.

Other methods can also be employed, for example:

According to Tetrahedron Lett. 1988, 29, 2983, where a solution of the benzylcarbamate in ethanol may be treated with triethylsilane (2 equiv), di-t-butyldicarbonate (1.1 equiv), catalytic palladium acetate and triethylamine (0.3 equiv) to give the BOC-protected amine in a "one-pot" manner.

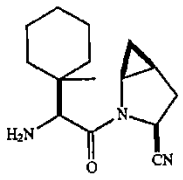
Or alternately: Solutions of the benzylcarbamate in methanol may be subjected to hydrogenolysis in the presence of di-t-butyldicarbonate to give the BOC-protected amine in a "one-pot" manner.

Step 6



A solution of Step 5 compound (1.18 g, 3.09 mmol) in dioxane (8.0 mL) was treated with 1N sodium hydroxide (9.1 mL, 9.1 mmol or 3.0 equiv) and stirred at 60° C. (oil bath) for 28 h. The reaction mixture was concentrated to a syrup which was dissolved in water (15 mL) and extracted with ether (25 mL). The aqueous phase was acidified to pH 2-3 with 1N hydrochloric acid (9.2 mL) then extracted with EtOAc (3×50 mL). The combined organic extracts were washed with saturated sodium chloride (10 mL), dried ($MgSO_4$), filtered, and concentrated to give Step 6 compound as an off-white solid. Yield: 808 mg (96%). MS (M+H) 272.

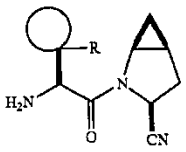
Step 7



The title compound was prepared from Step 6 compound according to the procedure in General Method C where the amino acid was coupled, the amide was dehydrated, and the protecting group removed to give the title compound. MS (M+H) 262.

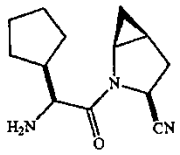
Compounds 90-100 were prepared by General Method I and General Method C starting from cyclohexanone, cyclopentanone and cyclobutanone, and employing methyl-, ethyl-, allyl- and propylmagnesium halides as Grignard reagents.

TABLE 5

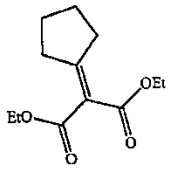


Example #	Cycloalkane	R	MS Data M + H
79	cyclohexane	Methyl	262
80	cyclohexane	Ethyl	276
81	cyclopentane	Methyl	248
82	cyclopentane	Allyl	274
83	cyclopentane	Propyl	276
84	cyclobutane	Methyl	234

EXAMPLE 85

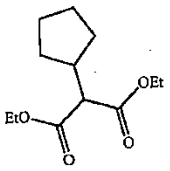


Step 1



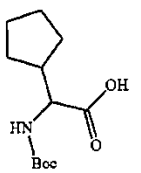
According to Example 79: A mixture of dry carbon tetrachloride (50 mL) was cooled to 0° C. (ice-salt bath) and treated with titanium tetrachloride (11.0 mL, 0.1 mol). The resulting yellow suspension was stirred at 0° C. for 5 min, treated sequentially with cyclopentanone (4.42 mL, 0.05 mol) and distilled diethylmalonate (7.6 mL, 0.05 mol) then stirred at 0° C. for 30 min The reaction mixture was then treated with a solution of dry pyridine (16 mL, 0.20 mol) in dry THF (30 mL), stirred at 0° C. for 1.0 h, then at rt for 20 h. The reaction mixture was quenched with water (50 mL), stirred for 5 min then extracted with ether (2×100 mL). The combined organic extracts were washed with saturated sodium chloride (50 mL), saturated sodium bicarbonate (50 mL) and brine (50 mL), dried ($MgSO_4$), filtered and concentrated. Flash chromatography using 5% EtOAc in hexane gave Step 1 compound as a light yellow oil. Yield: 7.67 g (68%). MS (M+H) 226.

Step 2



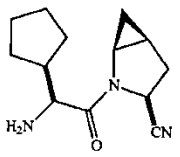
A solution of Step 1 compound (1.00 g 4.42 mmol) in methanol (50 mL) was treated with 10% Pd/C (0.20 g, 10 mol %) and hydrogenated (balloon pressure) at rt for 20 h. The mixture was diluted with methanol and filtered through a pad of celite. The filtrate was concentrated and purified by flash column chromatography on silica gel with 7% EtOAc in hexanes to give 0.84 g (91%) of Step 2 compound. MS (M+H) 229.

Step 3



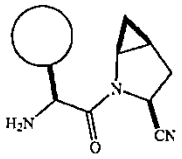
The Step 3 compound was prepared by the process outlined in General Method H, where the ester underwent hydrolysis, Curtius Rearrangement, protecting group exchange, and again final ester hydrolysis.

Step 4



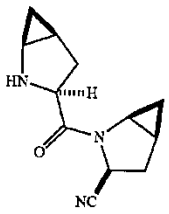
The title compound was prepared from Step 3 compound according to the procedure in General Method C where the amino acid was coupled, the amide was dehydrated, and the protecting group removed to give the title compound. MS (M+H) 234.

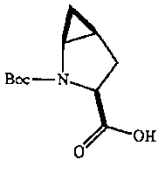
Examples 86 and 87 were prepared by the procedures used for Example 85 starting from cyclohexanone and cyclobutanone respectively



Example #	Cycloalkane	Mass Spec M + H
85	cyclopentyl	234
86	cyclohexyl	248
87	cyclobutyl	220

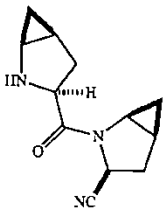
EXAMPLE 89





Step 1 compound was prepared in Example 6 Step 1.

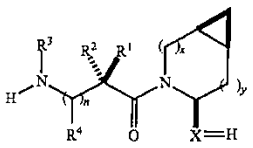
Step 2

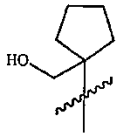
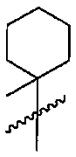
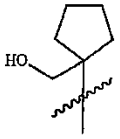


The title compound was prepared from Step 1 compound according to General Method C, where the carboxylic acid underwent a peptide coupling, the amide dehydration and protecting group removal. MS (M+H) 218.

EXAMPLES 90 TO 99

Examples of compounds where X=H include the following compounds which may be prepared employing procedures as described hereinbefore.



Ex. #	n	x	y	R^1	R^2	R^3	R^4
90	0	0	1	t-Bu	H	H	—
91	0	0	1	adamantyl	H	H	—
92	0	0	1		H	H	—
93	0	0	1		H	Me	—
94	0	1	0	t-Bu	H	H	—
95	0	1	0	adamantyl	H	H	—
96	0	1	0		H	H	—

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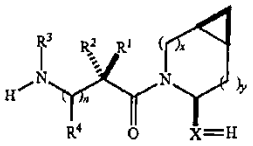
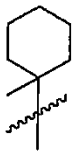
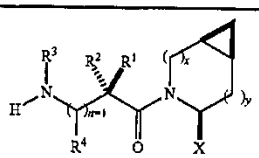
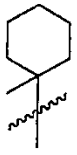


Ex. #	n	x	y	R^1	R^2	R^3	R^4
97	0	1	0		H	Me	—
98	1	0	1	H	H	H	t-Bu
99	1	1	0	Me	H	H	t-Bu

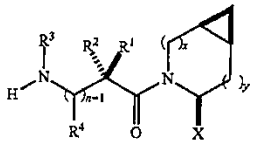
-continued

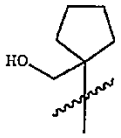
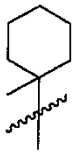
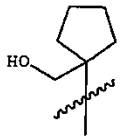


Ex. #	X	x	y	R^1	R^2	R^3	R^4
107	CN	1	0	H	H	Me	
108	H	0	1	t-Bu	H	H	H
109	H	1	0	Me	H	H	t-Bu

EXAMPLES 100 TO 109

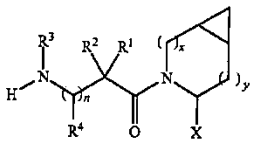
Examples of compounds where n=1 include the following compounds which may be prepared employing procedures as described hereinbefore.



Ex. #	X	x	y	R^1	R^2	R^3	R^4
100	CN	0	1	H	H	H	t-Bu
101	CN	0	1	H	H	H	adamantyl
102	CN	0	1	H	Me	H	
103	CN	0	1		H	Me	H
104	CN	1	0	t-Bu	H	H	H
105	CN	1	0	adamantyl	H	H	Me
106	CN	1	0		Et	H	H

What is claimed is:

1. A compound having the structure



wherein x is 0 or 1 and y is 0 or 1, provided that

x=1 when y=0 and

x=0 when y=1; and wherein

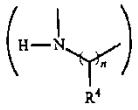
n is 0 or 1;

X is H or CN;

R^1, R^2, R^3 and R^4 are the same or different and are independently selected from hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, bicycloalkyl, tricycloalkyl, alkylcycloalkyl, hydroxyalkyl, hydroxyalkylcycloalkyl, hydroxycycloalkyl, hydroxybicycloalkyl, hydroxytricycloalkyl, bicycloalkylalkyl, alkylthioalkyl, arylalkylthioalkyl, cycloalkenyl, aryl, aralkyl, heteroaryl, heteroarylalkyl, cycloheteroalkyl or cycloheteroalkylalkyl; all optionally substituted through available carbon atoms with 1, 2, 3, 4 or 5 groups selected from hydrogen, halo, alkyl, polyhaloalkyl, alkoxy, haloalkoxy, polyhaloalkoxy, alkoxycarbonyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, polycycloalkyl, heteroarylamino, arylamino, cycloheteroalkyl, cycloheteroalkylalkyl, hydroxy, hydroxyalkyl, nitro, cyano, amino, substituted amino, alkylamino, dialkylamino, thiol, alkylthio, alkylcarbonyl, acyl, alkoxycarbonyl, aminocarbonyl, alkynylaminocarbonyl, alkylaminocarbonyl, alkenylaminocarbonyl, alkylcarbonyloxy, alkylcarbonylamino, arylcarbonylamino, alkylsulfonylamino, alkylaminocarbonylamino, alkoxycarbonylamino, alkylsulfonyl, aminosulfinyl, aminosulfonyl, alkylsulfinyl, sulfonamido or sulfonyl;

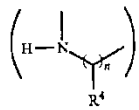
and R^1 and R^3 may optionally be taken together to form $-(CR^5R^6)_m-$ where m is 2 to 6, and R^5 and R^6 are the same or different and are independently selected from hydroxy, alkoxy, H, alkyl, alkenyl, alkynyl, cycloalkyl,

halo, amino, substituted amino, cycloalkylalkyl, cycloalkenyl, aryl, arylalkyl, heteroaryl, heteroarylalkyl, cycloheteroalkyl, cycloheteroalkylalkyl, alkylcarbonylamino, arylcarbonylamino, alkoxycarbonylamino, aryloxycarbonylamino, alkoxycarbonyl, aryloxycarbonyl, or alkylaminocarbonylamino, or R^1 and R4 may optionally be taken together to form $-(CR^7R^8)_p-$ wherein p is 2 to 6, and R^7 and R^8 are the same or different and are independently selected from hydroxy, alkoxy, cyano, H, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkylalkyl, cycloalkenyl, halo, amino, substituted amino, aryl, arylalkyl, heteroaryl, heteroarylalkyl, cycloheteroalkyl, cycloheteroalkylalkyl, alkylcarbonylamino, arylcarbonylamino, alkoxycarbonylamino, aryloxycarbonylamino, alkoxycarbonyl, aryloxycarbonyl, or alkylaminocarbonylamino, or optionally R^1 and R^3 together with



form a 5 to 7 membered ring containing a total of 2 to 4 heteroatoms selected from N, O, S, SO, or SO_2;

or optionally R^1 and R^3 together with



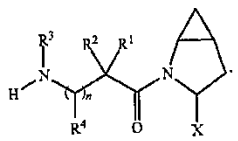
form a 4 to 8 membered cycloheteroalkyl ring wherein the cycloheteroalkyl ring has an optional aryl ring fused thereto or an optional 3 to 7 membered cycloalkyl ring fused thereto;

with the proviso that where x is 1 and y is 0, X is H, n is o, and one of R^1 and R^2 is H and the other is alkyl, then R^3 is other than pyridyl or substituted pyridyl;

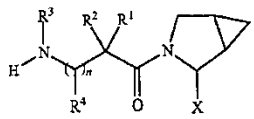
including all stereoisomers thereof;

[and] *or* a pharmaceutically acceptable salt thereof[, or a prodrug ester thereof], and all stereoisomers thereof.

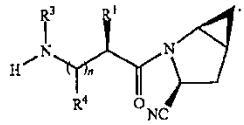
2. The compound as defined in claim 1 having the structure:



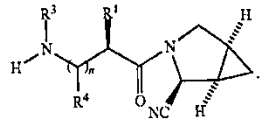
3. The compound as defined in claim 1 having the structure:



4. The compound as defined in claim 1 having the structure:



5. The compound as defined in claim 1 having the structure:



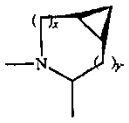
6. The compound as defined in claim 1 wherein:

R^3 is H, R^1 is H, alkyl, cycloalkyl, bicycloalkyl, tricycloalkyl, alkylcycloalkyl, hydroxyalkyl, hydroxyalkylcycloalkyl, hydroxycycloalkyl hydroxybicycloalkyl, or hydroxytricycloalkyl,

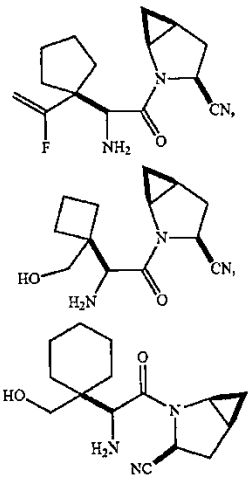
R^2 is H or alkyl, n is 0,

X is CN.

7. The compound as defined in claim 1 wherein the cyclopropyl fused to the pyrrolidine has the configuration:



8. A compound having the structure:



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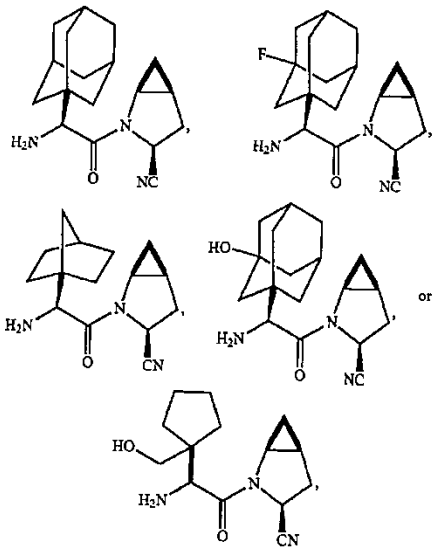
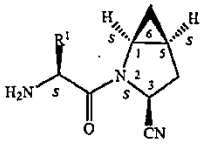


or a pharmaceutically acceptable salt thereof.

9. The compound as defined in claim 8 wherein the pharmaceutically acceptable salt is the hydrochloride salt or the trifluoroacetic acid salt.

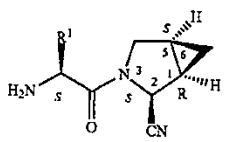
10. A compound which is



(1S, 2 (2S). 3S, 5S)

wherein R^1 is alkyl, cycloalkyl, bicycloalkyl, tricycloalkyl, alkylcycloalkyl, hydroxyalkyl, hydroxycycloalkyl, hydroxyalkylcycloalkyl, hydroxybicycloalkyl, or hydroxytricycloalkyl,

or



(1R, 2S, 3(2S), 5S)

wherein R^1 is alkyl, cycloalkyl, bicycloalkyl, tricycloalkyl, alkylcycloalkyl, hydroxyalkyl, hydroxycycloalkyl, hydroxyalkylcycloalkyl, hydroxybicycloalkyl, or hydroxytricycloalkyl.

11. A pharmaceutical composition comprising a compound as defined in claim 1 and a pharmaceutically acceptable carrier therefor.

12. A pharmaceutical combination comprising a [DP4 inhibitor] compound as defined in claim 1 and an antidiabetic agent other than a DP4 inhibitor for treating diabetes and related diseases, an anti-obesity agent and/or a lipid-modulating agent.

13. The pharmaceutical combination as defined in claim 12 comprising said [DP4 inhibitor] compound *as defined in claim 1* and [an] *the* antidiabetic agent *other than a DP4 inhibitor*.

14. The combination as defined in claim 13 wherein the antidiabetic agent is 1, 2, 3 or more of a biguanide, a sulfonyl urea, a glucosidase inhibitor, a PPAR γ agonist, a PPAR α/γ dual agonist, an SGLT2 inhibitor, an aP2 inhibitor, a glycogen phosphorylase inhibitor, an AGE inhibitor, an insulin sensitizer, a glucagon-like peptide-1 (GLP-1) or mimetic thereof, insulin and/or a meglitinide.

15. The combination as defined in claim 14 wherein the antidiabetic agent is 1, 2, 3 or more of metformin, glyburide, glimepiride, glipyride, glipizide, chlorpropamide, gliclazide, acarbose, miglitol, pioglitazone, troglitazone, rosiglitazone, insulin, G1 -262570, isaglitazone, JTT-501, NN-2344, L895645, YM-440, R-119702, AJ9677, repaglinide, nateglinide, KAD1129, AR-HO39242, GW-409544, KRP297, AC2993, Exendin-4, LY307161, NN2211, and/or LY315902.

16. The combination as defined in claim 13 wherein the compound *as defined in claim 1* is present in a weight ratio to the antidiabetic agent within the range from about 0.01 to about 100:1.

17. The combination as defined in claim 12 wherein the anti-obesity agent is a beta 3 adrenergic agonist, a lipase inhibitor, [a serotonin (and dopamine) reuptake inhibitor,] a thyroid receptor beta compound, an anorectic agent, and/or a fatty acid oxidation upregulator.

18. The combination as defined in claim 17 wherein the anti-obesity agent is orlistat, ATL-962, AJ9677, L750355, CP331648, sibutramine, topiramate, axokine, dexamphetamine, phentermine, phenylpropanolamine, famoxin, and/or mazindol.

19. The combination as defined in claim 12 wherein the lipid modulating agent is an MTP inhibitor, an HMG CoA reductase inhibitor, a squalene synthetase inhibitor, a fibric acid derivative, an upregulator of LDL receptor activity, a lipoxygenase inhibitor, an ACAT inhibitor, a cholesteryl ester transfer protein inhibitor, or an ATP citrate lyase inhibitor.

20. The combination as defined in claim 19 wherein the lipid modulating agent is pravastatin, lovastatin, simvastatin, atorvastatin, cerivastatin, fluvastatin, nisvastatin, visastatin, fenofibrate, gemfibrozil, clofibrate, implitapide, CP-529, 414, avasimibe, TS-962, MD-700, and/or LY295427.

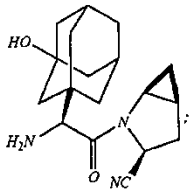
21. The combination as defined in claim 19 wherein the [DP4 inhibitor] *compound as defined in claim 1* is present in a weight ratio to the lipid-modulating agent within the range from about 0.01 to about 100:1.

22. A pharmaceutical combination comprising a [DP4 inhibitor] compound as defined in claim 1 and an agent for treating infertility, an agent for treating polycystic ovary syndrome, an agent for treating a growth disorder and/or frailty, an anti-arthritis agent, an agent for preventing *or* inhibiting allograft rejection in transplantation, an agent for treating autoimmune disease, an anti-AIDS agent, an agent for treating inflammatory bowel disease/syndrome, an agent for treating anorexia nervosa, an anti-osteoporosis agent and/or an anti-obesity agent.

[23. A method for treating diabetes, insulin resistance, hyperglycemia, hyperisulinemia, or elevated blood levels of free fatty acids or glycerol, obesity, Syndrome X, dysmetabolic syndrome, diabetic complications, hypertriglyceridemia, hyperinsulinemia, atherosclerosis, impaired glucose homeostasis, impaired glucose tolerance, infertility, polycystic ovary syndrome, growth disorders, frailty, arthritis, allograft rejection in transplantation, autoimmune diseases, AIDS, intestinal diseases, inflammatory bowel syndrome, nervosa, osteoporosis, or an immunomodulatory disease or a chronic inflammatory bowel disease, which comprises administering to a mammalian species in need of treatment a therapeutically effective amount of a compound as defined in claim 1.]

[24. The method as defined in claim 23 for treating type II diabetes and/or obesity.]

25. A compound that is



or a pharmaceutically acceptable salt thereof.

26. The compound as defined in claim 25, wherein the pharmaceutically acceptable salt is the hydrochloride salt.

27. A pharmaceutical composition comprising the compound of claim 25 and a pharmaceutically acceptable carrier therefor.

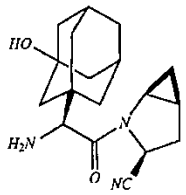
28. A pharmaceutical composition comprising the compound of claim 26 and a pharmaceutically acceptable carrier therefor.

29. The composition of claim 27 or 28 further comprising an antidiabetic agent other than a DP4 inhibitor.

30. The composition of claim 29 wherein the antidiabetic agent is metformin.

31. The composition of claim 29, wherein the antidiabetic agent is a SGLT2 inhibitor.

32. A method for treating diabetes, insulin resistance, hyperglycemia, hyperinsulinemia, impaired glucose homeostasis, or impaired glucose tolerance in a mammal comprising administering to the mammal a pharmaceutical composition comprising a compound that is



or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier therefor.

33. The method of claim 32, wherein the pharmaceutically acceptable salt is the hydrochloride salt.

34. The method of claim 32, for treating diabetes.

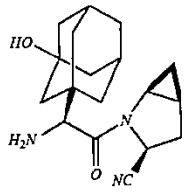
35. The method of claim 33, for treating diabetes.

36. The method of any one of claim 32, 33, 34, or 35 wherein the pharmaceutical composition further comprises an antidiabetic agent other than a DP4 inhibitor.

37. The method of claim 36, wherein the antidiabetic agent is metformin.

38. The method of claim 36, wherein the antidiabetic agent is a SGLT2 inhibitor.

39. A method for treating type II diabetes in a mammal comprising administering to the mammal a pharmaceutical composition comprising a compound that is



or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier therefor.

40. The method of claim 39, wherein the pharmaceutically acceptable salt is the hydrochloride salt.

41. The method of any one of claims 39 or 40, wherein the pharmaceutical composition further comprises an antidiabetic agent other than a DP4 inhibitor.

42. The method of claim 41, wherein the antidiabetic agent is metformin.

43. The method of claim 41, wherein the antidiabetic agent is a SGLT2 inhibitor.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO.	: RE44,186 E
APPLICATION NO.	: 13/308658
DATED	: April 30, 2013
INVENTOR(S)	: Jeffrey A. Robl et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Specifications:

Column 4,
Line 56, delete "alkylcyclo alkyl," and insert -- alkylcycloalkyl, --.
Line 56, delete "hydroxytricyclo alkyl," and insert
-- hydroxytricycloalkyl, --.

Column 17,
Line 48, delete "a-phosphono-sulfonates" and insert -- α-phosphono-sulfonates --.

Column 19,
Line 51, delete "lipoxygevase" and insert -- lipoxygenase --.

Column 28,
Lines 16-17, delete "butoxycarbonylisoleucine" and insert
-- butoxycarbonyl-isoleucine --.

Column 33,
Lines 38-39, delete "1-[(3-dimethypamino)propyl]" and insert
-- 1-[(3-dimethyl)amino)propyl] --.

Signed and Sealed this
Eighth Day of October, 2013

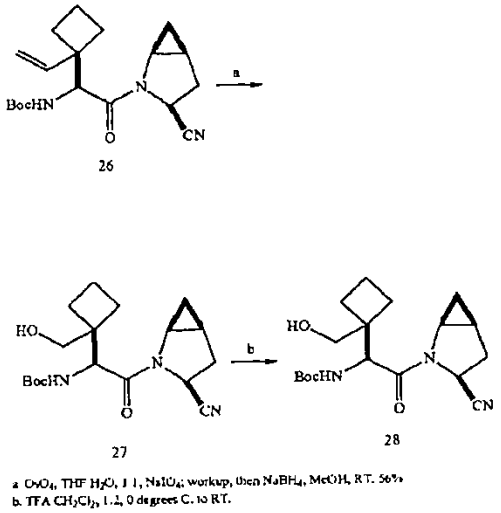
Teresa Stanek Rea
Deputy Director of the United States Patent and Trademark Office

CERTIFICATE OF CORRECTION (continued)
U.S. Pat. No. RE44,186 E

In the Specifications:

Column 51,

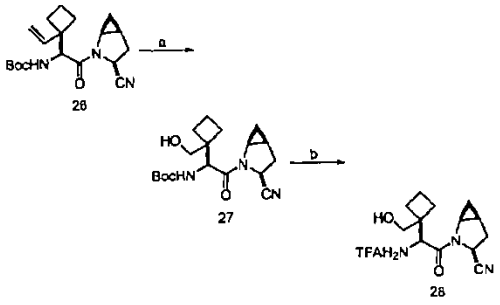
Scheme 7
General Method E, Examples 45 47



a. OsO_4, THF H_2O, 1:1, $NaIO_4$; workup, then $NaBH_4$, MeOH, RT. 56%
b. TFA CH_2Cl_2, 1:2, 0 degrees C. to RT.

Lines 1-30, delete " "

and insert --Scheme 7
General Method E, Examples 45 47



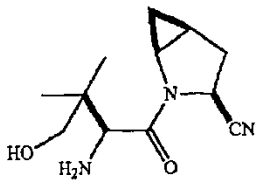
a.OsO_4, THF:H_2O, 1:1, $NaIO_4$, workup,then $NaBH_4$, MeOH, RT. 56%
b TFA:CH_2Cl_2, 1:2, 0 degrees C to RT. --.

Column 51,
Line 54, delete "OsO4" and insert -- OsO_4 --.

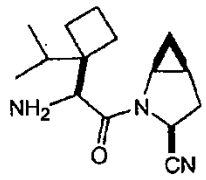
In the Specifications:

Column 55,

Step 3

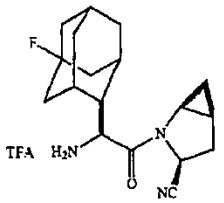


Lines 19-31, EXAMPLE 57, delete " " and

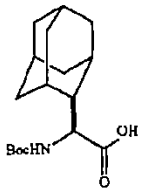


insert -- --.

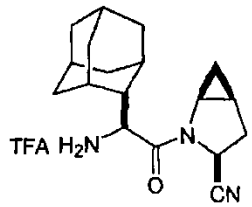
Column 63,



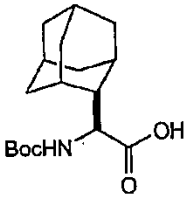
Step 1



Lines 25-46, EXAMPLE 62, delete " " and



Step 1



insert -- --.

In the Specifications:

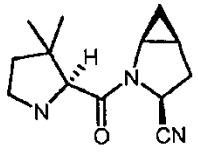
Column 64,
Line 31, delete "$NaHS0_3$" and insert -- $NaHSO_3$ --.

Column 69,

EXAMPLE 67

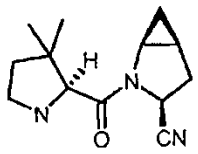
Step 1

Lines 20-32, delete "



" and

EXAMPLE 67



insert -- Step 1 --.

Column 70,
Line 59, delete "19,8 mmol" and insert -- 19.8 mmol --.

Column 82,
Line 27, after "30 min" insert -- . --.

In the Claims:

Column 87,
Line 7, Claim 1, delete "R4" and insert -- R^4 --.

Column 92,
Line 21, Claim 36, delete "any one of claim" and insert -- any one of claims --.