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Termination of DNA synthesis by novel 3′-modified-deoxyribonucleoside 5′-triphosphates

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ABSTRACT

Eight 3′-modified-dNTPs were synthesized and tested in two different DNA template assays for incorporation activity. From this enzymatic screen, two 3′-*O*-methyl-dNTPs were shown to terminate DNA syntheses mediated by a number of polymerases and may be used as alternative terminators in Sanger sequencing. 3′-*O*-(2-Nitrobenzyl)-dATP is a UV sensitive nucleotide and was shown to be incorporated by several thermostable DNA polymerases. Base specific termination and efficient photolytic removal of the 3′-protecting group was demonstrated. Following deprotection, DNA synthesis was reinitiated by the incorporation of natural nucleotides into DNA. The identification of this labile terminator and the demonstration of a one cycle stop-start DNA synthesis are initial steps in the development of a novel sequencing strategy.

INTRODUCTION

2′-Deoxyribonucleoside-5′-triphosphates (dNTPs) modified at their 3′-hydroxyl position can act as terminators of enzyme-directed DNA synthesis (1–12). These nucleotide analogs are useful as DNA sequencing tools, mechanistic probes, antimetabolites, and as antiviral agents. Consequently, such compounds have been used for analytical and therapeutic studies (2). Overall, however, the number of compounds that are well characterized is small, and there is considerable scope for new combinations of terminators and polymerases to be identified.

Among the most familiar terminators of DNA synthesis are the 2′, 3′-dideoxyribonucleoside-5′-triphosphates (ddNTPs) that are the basis for Sanger DNA sequencing (13). In that method oligonucleotide-primed DNA or RNA templates are enzymatically extended in a 5′ → 3′ direction in the presence of a mixture of dNTPs and ddNTPs to generate a population of molecules that are terminated at specific base positions. DNA fragments of different lengths are resolved by denaturing polyacrylamide gel electrophoresis and detected either by radioactive or fluorescent labels to reveal the underlying base sequence. Despite the obvious limitations of gel electrophoresis for sequencing long DNA strands, this method has been the favored approach for more than ten years (13,14).

Improvements to the Sanger protocols are being sought to meet the increasing demands of large scale sequencing of whole genomes (14). We and others (15–18) have independently conceived a radically different, gel-free alternative to the Sanger scheme for DNA sequencing. This method, called the Base Addition Sequencing Scheme (BASS), is based on novel nucleotide analogs that terminate DNA synthesis. BASS involves repetitive cycles of incorporation of each successive nucleotide, *in situ* monitoring to identify the incorporated base, and deprotection to allow the next cycle of DNA synthesis, (Figure 1). Compared to Sanger sequencing, BASS has two major advantages: base resolution would not require gel electrophoresis and there is a tremendous capacity for simultaneous analyses of multiple samples. The complete scheme demands nucleotide analogs that are tolerated by polymerases, spectroscopically distinct for each base, stable during the polymerization phase, and deprotected efficiently under mild conditions in aqueous solution. These stringent requirements are formidable obstacles for the design and synthesis of the requisite analogs.

The investigation of the interactive patterns between various terminating analogs and different enzymes is an important preliminary phase in the development of the BASS method. Consequently, eight 3′-modified-dNTPs were synthesized and examined for their ability to terminate DNA synthesis mediated by a variety of polymerases. The majority of 3′-modified analogs have labile protecting groups that have the potential to be incorporated into BASS. Active combinations of terminators and enzymes were identified using two different primer-template gel assays. One of these compounds, 3′-*O*-(2-nitrobenzyl)-dATP [7], was used to demonstrate one complete cycle of termination, deprotection, and reinitiation of DNA synthesis.

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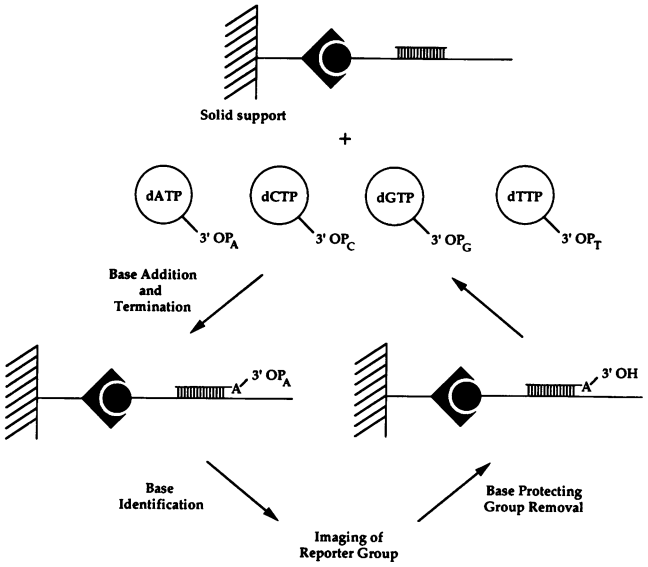


Figure 1. Cartoon of the Base Addition Sequencing Scheme (BASS). A primer is annealed to a biotinylated-labeled template bound to a solid support. Four deoxynucleotides triphosphates that have spectroscopically unique blocking groups attached to the 3′-position are added. Polymerase extension is terminated after the addition of one base. Upon imaging of the reporter group, the protecting group is removed resulting in a 'free' 3′-OH group, allowing the addition of the next complement base.

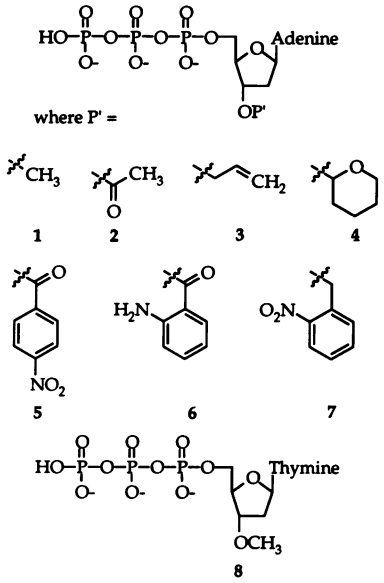


Figure 2. Chemical structures of the 3′-modified-nucleotides. Details of the chemical syntheses are described in the Materials and Methods section.

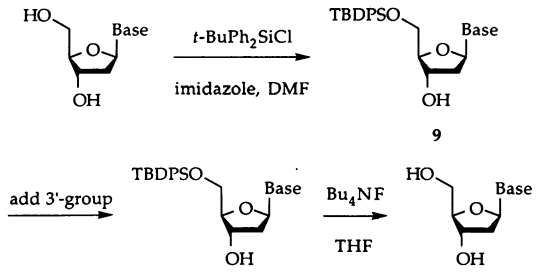
MATERIALS AND METHODS

General

High field Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker AC250 (^{1}H at 250 MHz, ^{13}C at 62.9 MHz, ^{31}P at 101.26 MHz) or a Varian XL 200 (^{1}H at 200 MHz, ^{13}C at 50 MHz, ^{31}P at 81 MHz). Ultraviolet (UV) spectra were recorded on a Hewlett-Parkard model 8452A diode array spectrophotometer. Thin layer chromatography was performed on Whatman silica gel 60 A F_{254} plates. Flash chromatography was performed on SP silica gel 60 (230–600 mesh ASTM). Ion-exchange chromatography was performed on Fluka DEAE cellulose C451 (HCO_3^- form). Photodecomposition of 3′-*O*-(2-nitrobenzyl)-dATP [**7**] was performed using a FisherBiotech transilluminator.

Organic syntheses

The chemical structures of compounds [**1**]–[**8**] are shown in Figure 2. Compounds [**1**]–[**4**], [**6**], [**8**] were prepared according to the general scheme:



The 5′-hydroxyl was protected with a *tert*-butyldiphenylsilyl (TBDPS) group, and the specific addition of the 3′-protecting groups (P') are described below. Desilylations were performed by the addition of 1.0 equiv. of tetrabutylammonium fluoride (Bu_4NF) to the 3′-protected-5′-silyl-adenosine or thymidine derivatives. The reactions were monitored by TLC; after completion (ca. 15 min.), the reactions were quenched with 1.0 equiv. of glacial acetic acid. The solvent was removed, and the residues were purified by silica column chromatography (10% methanol/ethyl acetate).

2′-Deoxy-3′-O-methyladenosine [1]. To 2′-deoxy-5′-*tert*-butyldiphenylsilyladenosine [**9**] (200 mg, 0.4 mmol) in benzene (5 mL), methyl iodide (568 mg, 4.0 mmol, 10 equiv.), tetrabutylammonium hydroxide (TBAH) (40% solution, 325 μL), and 1 M NaOH (5 mL) were vigorously stirred at 25°C for 16 h. The organic layer was extracted with ethyl acetate and washed with deionized (D.I.) water, saturated NaCl, dried over Na_2SO_4 and purified by flash chromatography using a stepwise gradient (0% methanol/ethyl acetate to 5% methanol/ethyl acetate in 2% intervals) (180 mg, 89%) (19).

The *O*-methyl derivative from the above procedure (80 mg, 0.16 mmol), after desilylation and flash chromatography gave compound [**1**] as colorless crystals (30 mg, 70%). High resolution mass spectrometry (HRMS) *m/e* calculated for $C_{11}H_{15}N_5O_3$: 265.1172, observed 265.1154.

2′-Deoxy-3′-O-acyladenosine [2]. 2′-Deoxy-5′-*tert*-butyldiphenylsilyladenosine [**9**] (100 mg, 0.2 mmol), acetic anhydride (28 mg, 0.27 mmol), and 4-dimethylaminopyridine (DMAP) (5 mg, 0.05 mmol) in dry pyridine were stirred at 25°C for 6 h. After removing pyridine under vacuum, the residue was dissolved in D.I. water, extracted in chloroform, washed with D.I. water, 10% HCl, saturated $NaHCO_3$, saturated NaCl, dried over Na_2SO_4 and flash chromatographed (96 mg, 90%).

The 3′-*O*-acyl derivative (100 mg, 0.19 mmol) following desilylation and flash chromatography afforded compoud [**2**] (44

mg, 80%). HRMS *m/e* calculated for $C_{12}H_{15}N_5O_4$: 293.1121, observed 293.1107.

2'-Deoxy-3'-O-allyladenosine [3]. 2'-Deoxy-5'-*tert*-butyldiphenylsilyladenosine [**9**] (200 mg, 0.4 mmol) in benzene (5 mL), allyl bromide (484 mg, 4.0 mmol, 10 equiv.), TBAH (40% solution, 390 μL), and 1 M NaOH (5 mL) were stirred at 25°C for 15 h. Following ethyl acetate extraction, the organic phase was washed with D.I. water, saturated NaCl, dried over Na_2SO_4, and flash chromatographed (157 mg, 74%).

The *O*-allyl derivative (198 mg, 0.37 mmol) following desilylation and flash chromatography gave compound [**3**] (106 mg, 98.5%). HRMS *m/e* calculated for $C_{13}H_{17}N_5O_3$: 291.1328, observed 291.1318.

2'-Deoxy-3'-O-tetrahydropyranyladenosine [4]. 2'-Deoxy-5'-*tert*-butyldiphenylsilyladenosine [**9**] (2.90 g, 5.92 mmol), dihydropyran (4.89 g, 59.2 mmol, 10 equiv.) and pyridinium nitrobenzenesulfonate (1.67 g, 5.92 mmol) were dissolved in methylene chloride (20 mL) and stirred at 40°C for 20 h. The reaction mixture was washed with D.I. water, saturated NaCl, dried over Na_2SO_4 and flash chromatographed to give a diastereomeric mixture of 2'-deoxy-3'-O-tetrahydropyranyl-5'-*tert*-butyldiphenylsilyladenosine (0.4 g, 12%).

The tetrahydropyran derivative formed above, after desilylation and flash chromatography yielded compound [**4**] (147 mg, 84%) as a mixture of diastereomers. HRMS *m/e* calculated for $C_{15}H_{21}N_5O_4$: 335.1589, observed 335.1581.

2'-Deoxy-3'-O-(2-aminobenzoyl)adenosine-5'-triphosphate [5]. This compound was prepared according to the procedure of Hiratsuka *et al.* (20) directly from the 2'-deoxyadenosine-5'-triphosphate sodium salt.

2'-Deoxy-3'-O-(4-nitrobenzoyl)adenosine [6]. 2'-Deoxy-5'-*tert*-butyldiphenylsilyladenosine [**9**] (100 mg, 0.2 mmol), 4-nitrobenzoyl chloride (89 mg, 0.48 mmol), DMAP (5 mg, 0.04 mmol) were dissolved in pyridine and stirred for 8 h. Following solvent removal, the residue was dissolved in chloroform and was washed D.I. water, saturated $NaHCO_3$, D.I. water, saturated NaCl, dried over Na_2SO_4, and flash chromatographed giving a colorless solid (73 mg, 57%).

The 4-nitrobenzoyl derivative (66 mg, 0.1 mmol) following desilylation and flash chromatography gave compound [**6**] (22 mg, 57%). HRMS *m/e* calculated for $C_{17}H_{16}N_6O_6$: 400.1128, observed 400.1140.

2'-Deoxy-3'-O-(2-nitrobenzyl)adenosine [7]. 2'-Deoxyadenosine (100 mg, 0.4 mmol) [dried by repeated coevaporation with pyridine] was dissolved in hot DMF and cooled to 0°C in an ice bath. To the above solution, NaH (26 mg, 0.52 mmol [50% in mineral oil] in DMF after washing with dry benzene was added and stirred for 45 min. 2-Nitrobenzyl bromide (95 mg, 0.44 mmol) in DMF was added, and the reaction stirred for 3 h. The reaction was quenched with cold D.I. water and stirred overnight. The solid obtained was filtered, dried, and recrystalized in ethanol (122 mg, 79%). HRMS *m/e* calculated for $C_{17}H_{18}N_6O_5$: 386.1335, No *m/e* was observed. Fast atom bombardment MS, nitrobenzyl alcohol (NBA) *m/e* 387.1 (M+1).

2'-Deoxy-3'-O-methylthymidine [8]. 2'-Deoxy-5'-*tert*-butyldiphenylsilylthymidine [**9**] (100 mg, 0.21 mmol) in benzene (5 mL), methyl iodide (43 mg, 0.3 mmol), TBAH (40% solution, 325 μL), and 1 M NaOH (5 mL) were vigorously stirred at 25°C for 6 h. The organic layer was extracted with ethyl acetate and washed with D.I. water, saturated NaCl, and dried over Na_2SO_4 (100 mg, 98%).

The above sample, following desilylation and purification by flash chromatography, gave compound [**8**] (36 mg, 88%). HRMS *m/e* calculated for $C_{11}H_{16}N_2O_5$: 256.1059, observed 256.1082.

Syntheses of nucleoside 5'-triphosphates

In general, the 3'-modified nucleoside (1.0 equiv.) was dissolved in trimethylphosphate under nitrogen atmosphere. Phosphorus oxychloride ($POCl_3$) (3.0 equiv.) was added, and the reaction stirred at −10°C for 4 h. The reaction was quenched with a solution of tributylammonium pyrophosphate (5.0 equiv.) in DMF and tributylamine (0.2 mL) (21). After stirring vigorously for 10 min., the reaction was quenched with 2 mL of 2 M TEAB, pH 7.5. The solution was concentrated, and the triphosphate derivative was isolated by linear gradient (0.01 M to 0.5 M TEAB) using a DEAE cellulose (HCO_3^- form) column.

Reverse-phase high performance liquid chromatography (RP-HPLC)

The RP-HPLC hardware system consisted of a Beckman controller and model 100A pumps, a Rheodyne model 7125 injector, an Applied Biosystems (ABI) model 759A absorbance detector, and a Spectra-Physics model SP4600 DataJet integrator. Gradient RP-HPLC was performed using an ABI aquapore OD-300 column (4.6 mm×250 mm) where 'Buffer A' is 100 mM triethylammonium acetate (TEAA), pH 7.0 and Buffer B' is 100 mM TEAA, 70 % (v/v) acetonitrile. Compounds [**1**]−[**6**] and [8] were purified using the following gradient conditions: 0% B, 5 min.; 0% B − 40% B, 60 min.; 40% B − 100% B, 18 min.; 100% B, 5 min. at a flow rate of 0.5 mL per min. Compound [**7**] was purified using the following gradient conditions: 30% B, 5 min.; 30% B − 70% B, 60 min.; 70% B − 100% B, 15 min.; 100% B, 5 min. The gradient conditions used to analyze individual nucleotides were: 0% B, 5 min.; 0% B − 40% B, 30 min.; 40% B − 100% B, 18 min.; 100% B, 5 min.

Polymerases

Avian Myeloblastosis Virus (AMV) and Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptases, Klenow fragment of DNA polymerase I, and T4 polynucleotide kinase were purchased from Pharmacia. *Bst* DNA polymerase was purchased from Bio-Rad Laboratories. AmpliTaq® DNA polymerase and r*Tth* DNA polymerase were purchased from Perkin Elmer. Sequenase® was purchased from United States Biochemical. $Vent_R$® (exo$^-$) DNA polymerase was kindly provided by New England Biolabs. *Pfu* (exo$^-$) DNA polymerase was purchased from Stratagene.

DNA templates

M13mp19 DNA was obtained from a 250 mL culture by polyethylene glycol precipitation and purified by a QIAGEN-tip 100 column according to the manufacture's protocol. Universal primer (5'-TGTAAAACGACGGCCAGT), biotinylated and unbiotinylated oligonucleotide template (5'-TACGGAGGTGG-ACTGGCCGTCGTTTTACA) and biotinylated oligonucleotide template (5'-TACGGAGGTTTTTGGACTGGCCGTCGTTT-

ACA) were synthesized using an ABI model 380B DNA synthesizer and purified by trityl-on RP-HPLC. All nonradioactive nucleotides were purchased from Pharmacia, and [γ-^{32}P]ATP was purchased from Amersham.

Polymerase incorporation assays

Two different template assays were used to test for 3′-modified nucleotide incorporation. In the first, designated the 'M13mp19-template assay', [^{32}P]-labeled universal primer was annealed to single-stranded M13mp19 DNA (0.1 pmol to 0.45 μg respectively, per 5 μL) in the specific enzyme buffer by heating to 80°C for 5 min. and cooling slowly to 25°C. The subsequent enzymatic extension of the primer−template complex was performed under conditions that are analogous to Sanger sequencing, where the natural nucleotides were mixed with either a dideoxynucleotide or 3′-modified nucleotide terminator to generate a sequencing ladder. For the second assay, designated the 'Oligo-template assay', [^{32}P]-labeled universal primer was annealed to an oligonucleotide template (0.05 pmol to 0.1 pmol respectively, per 5 μL) in the same fashion. Subsequent extensions were performed in the absence of the natural nucleotide when either a dideoxynucleotide or 3′-modified nucleotide was tested.

For each reaction, 5 μL aliquots of the annealed primer-template samples were dispensed into separate tubes containing 5 μL mixtures of each enzyme and nucleotides in their specific buffers. The final buffer conditions, concentrations of nucleotides, enzymatic units, and incubation temperatures are given in Table 1. The reactions were incubated for 10 min. and then stopped by the addition of 5 μL of stop solution containing 98% D.I. formamide, 10 mM EDTA, pH 8.0, 0.025% bromophenol blue, and 0.025% xylene cyanol. The samples were heated to 85°C for 3 min., chilled on ice, and either 4 μL (M13mp19-template Assay) or 3 μL (Oligo-template assay) were loaded on a 10% or 20% polyacrylamide gel, respectively. Following electrophoresis, the gel was fixed in an aqueous 10% acetic acid, 10 % methanolic solution (v/v), dried, and autoradiographed on HyperfilmTM-MP (Amersham).

Biotinylated Oligo-template assay

The conditions of this assay are similar to the Oligo-template assay except prior to primer annealing, 2.0 pmol of biotinylated template was captured on 10 mL streptavidin coated magnetic beads (Dynal Dynabeads® M-280) in 1 M NaCl for 15 min. After washing the bound template in the specific enzyme buffer, 0.1 pmol of [^{32}P]-labeled universal primer was annealed to an oligonucleotide template and extension reactions were performed as described in the Oligo-template assay.

RESULTS

Syntheses and purification

Compounds [**1**] through [**8**] were synthesized, desilylated, phosphorylated and purified as described above. In general, the yields of 3′-protection reactions ranged from 57% to 98% with the exception of 3′-*O*-THP-dATP [**4**]. This low yield (12%) is believed to be due to the acidic properties of the silica gel hydrolyzing the linkage to the THP group. For the desilylation reactions, the yields ranged from 70% to 98%, except for 3′-*O*-

Table 1. Specific enzymatic conditions for both the M13mp19-template and Oligo-template assays

Enzymatic Conditions	AMV-RT	M-MuLV-RT	Klenow fragment	Sequenase®	*Bst* DNA polymerase	AmpliTaq® DNA polymerase	*Pfu*(exo⁻) DNA polymerase	r*Tth* DNA polymerase	Vent(exo⁻)® DNA polymerase
Buffers									
[Tris-HCl]	50 mM, pH 8.3	50 mM, pH 8.3	10 mM, pH 8.5	40 mM, pH 7.5	10 mM, pH 8.5	10 mM, pH 8.5	20 mM, pH 8.75	50 mM, pH 8.3	20 mM, pH 8.8
[$MgCl_2$]	8 mM	8 mM	5 mM	5 mM	10 mM	10 mM	2 mM $MgSO_4$	8 mM	2 mM $MgSO_4$
	30 mM KCl	10 mM DTT		50 mM NaCl		50 mM NaCl	10 mM KCl	10 mM DTT	10 mM KCl
							10 mM $(NH_4)_2SO_4$		10 mM $(NH_4)_2SO_4$
							0.1% Triton X-100		0.1% Triton X-100
							0.1 mg/mL BSA		
Incubation Temperature (°C)	37	37	37	37	65	68	75	74	72
Units	1.3	1.9	1.0	1.3	0.1	0.3	0.1	0.3	0.1
M13mp19-Templ.									
[dATP] (μM)	40	N/D	N/D	10	1	2.5	N/D	N/D	N/D
[dCTP] (μM)	40	N/D	N/D	10	1	2.5	N/D	N/D	N/D
[dGTP] (μM)	40	N/D	N/D	10	1	2.5	N/D	N/D	N/D
[dTTP] (μM)	40	N/D	N/D	20	1	2.5	N/D	N/D	N/D
[ddATP] (μM)	2	N/D	N/D	0.25	10	150	N/D	N/D	N/D
[ddCTP] (μM)	2	N/D	N/D	0.25	10	75	N/D	N/D	N/D
[ddGTP] (μM)	2	N/D	N/D	0.25	10	15	N/D	N/D	N/D
[ddTTP] (μM)	2	N/D	N/D	0.5	10	150	N/D	N/D	N/D
Oligo-Templ.									
[dATP] (μM)	0.5	2.5	0.5	2.5	0.025	0.25	2.5	0.5	0.5
[dCTP] (μM)	0.5	2.5	0.5	2.5	0.025	0.25	2.5	2.5	0.5
[dTTP] (μM)	2.5	2.5	0.5	2.5	0.025	0.25	2.5	0.5	0.5
[ddATP] (μM)	2.5	250	0.05	2.5	0.25	2.5	I/T	125	125
[ddGTP] (μM)	0.5	250	0.05	2.5	0.25	0.25	I/T	25	25
[ddTTP] (μM)	5	5	0.5	2.5	2.5	25	I/T	100	100

N/D means the assay conditions were not determined and I/T means ddNTP termination was incomplete.

(4-nitrobenzoyl)-dATP [6]. The yield was reduced in that case to 57% due probably to a rearrangement of the 4-nitrobenzoyl group from the 3′-position to the 5′-position (data not shown). The phosphorylation yields of compounds [1]–[4] and [6]–[8] ranged from 25% to 40%. Thymidine analogs including 3′-*O*-methyl-dTTP [8], however, had to be handled more cautiously since they were more rapidly degraded by tributylammonium pyrophosphate than were the adenosine analogs.

The 3′-modified-dNTPs were further purified by RP-HPLC to ≥ 99% prior to the polymerase assay. Each nucleotide synthesis initially contained several major peaks that were individually tested in the Oligo-template assay to determine the active species. In general, the adenosine analogs contained both the natural dATP and 3′-modified-dATP.

Termination assays

A series of polymerases were chosen to test the candidate 3′-modified terminators, based on their broad template specificities and their commercial availability. The conditions for screening compounds [1]–[8] using each enzyme were first defined by a series of control polymerization experiments. For the M13mp19-template assay, a range of dNTP and ddNTP concentrations was identified that gave a clear sequencing ladder. Each test gel subsequently contained constant dNTP/ddNTP ratios in control lanes for three bases, while the concentrations of the test compound and its corresponding ddNTP were varied.

The Oligo-template assay was also standardized before testing each 3′-modified-dNTP for termination. The synthetic template contained all four bases to allow for the incorporation of the remaining natural nucleotides, so that other aspects of the enzyme performance could be identified. We found that all the polymerases misincorporated other dNTPs in the absence of the complement dNTP, and this nucleotide readthrough was concentration dependent. Thus, minimum dNTP concentrations that gave efficient incorporation, but no apparent misincorporation were first defined in this assay. These dNTP concentrations were then used to determine the minimum ddNTP concentration that yielded complete termination. *Pfu* (exo$^-$) DNA polymerase was excluded from the Oligo-template assay since a ddNTP concentration that yielded complete termination for this enzyme could not be identified. In each of the Oligo-template gels, the reactions contained all the required nucleotides except the natural nucleotide corresponding to the analog tested. The samples routinely used in this assay were a blank control (absence of corresponding dNTP or ddNTP), a titration of the corresponding ddNTP, a readthrough control (presence of corresponding dNTP), and a titration of the corresponding 3′-modified-dNTP.

Terminator screen

Table 2 summarizes the data from the enzymatic screen of compounds [1]–[8]. Three main classes of activity were defined: termination, inhibition, and inactive. Termination was apparent when the reaction containing the test compound mimicked the migration pattern of the ddNTP control. Inhibition was revealed when the presence of the test compound prevented the polymerase from incorporating the natural nucleotides. No activity was recorded when the 3′-modified-dNTPs mimicked either the blank or readthrough controls. In addition, a fourth effect related to an alteration in enzymatic fidelity is discussed below. Compounds [1], [8], and [7] showed specific termination and were further evaluated with respect to their concentration dependent effects.

3′-*O*-methyl-dATP [1] incorporation

The M13mp19-template assay in Figure 3A shows the incorporation of 3′-*O*-methyl-dATP [1] by AMV-RT. The termination of DNA synthesis by 3′-*O*-methyl-dATP [1] mimics the ddATP controls in a concentration dependent manner, although each band appears to migrate slightly slower. From the comparison of termination band intensities, it can be estimated that 3′-*O*-methyl-dATP [1] is approximately 200 to 250-fold less efficiently incorporated by AMV-RT than ddATP (compare lanes 6 and 8: 5 mM and 1 mM, respectively). In Figure 3B, the Oligo-template gel also shows the incorporation of 3′-*O*-methyl-dATP [1] by AMV-RT. In addition to termination, some readthrough was also observed due to the presence of contaminating dATP. All RP-HPLC purified 3′-modified-dATPs (compounds [1]–[7]) showed approximately 1% dATP contamination, and these trace levels could not be removed by subsequent RP-HPLC.

3′-*O*-Methyl-dATP [1] was also incorporated by M-MuLV-RT and inhibited DNA syntheses by r*Tth* and Vent$_{R}$® (exo$^-$)

Table 2. Activity matrix of RP-HPLC purified 3′-protecting dNTPs challenged against commercially available polymerases

3′-modified-dATP (except compound [8])	AMV-RT	M-MuLV-RT	Klenow fragment	Sequenase®	*Bst* DNA polymerase	AmpliTaq® DNA polymerase	Vent$_{R}$(exo$^-$)® DNA polymerase	r*Tth* DNA polymerase
[1] *O*-methyl	Termination	Termination*	-	-	-	-	Inhibition	Inhibition*
[2] *O*-acyl	-	-	-	-	-	-	Inhibition	-
[3] *O*-allyl	-	-	-	-	-	-	Termination*	-
[4] *O*-tetrahydropyran	-	-	-	-	-	-	-	-
[5] *O*-(4-nitrobenzoyl)	-	-	-	-	-	-	-	-
[6] *O*-(2-aminobenzoyl)	-	-	-	-	-	-	-	-
[7] *O*-(2-nitrobenzyl)	-	-	-	Inhibition	Termination	Termination*	Termination*	-
[8] 3′-*O*-methyl-dTTP	-	Inhibition	-	Inhibition	Termination	Termination	Termination	Termination

All compounds were assayed at a final concentration of 250 μM according to the conditions specified in Table 1. '–' means no activity was detected, 'Termination' means that the termination bands mimic ddNTP termination bands, and 'Inhibition' means the rate of DNA synthesis is reduced in a nonspecific manner. '*' means the activity was incomplete at a final concentration of 250 μM .

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