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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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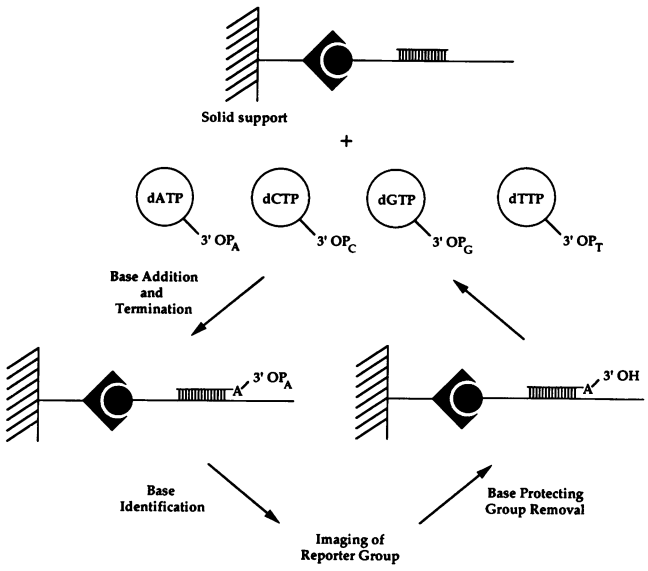


Figure 1. Cartoon of the Base Addition Sequencing Scheme (BASS). A primer is annealed to a biotinylated-labeled template bound to a solid support. Four deoxynucleotides triphosphates that have spectroscopically unique blocking groups attached to the 3'-position are added. Polymerase extension is terminated 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.

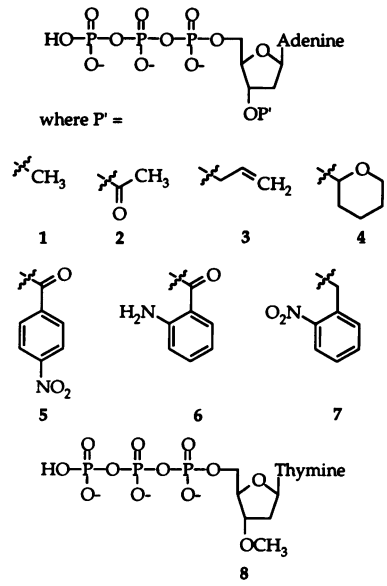


Figure 2. Chemical structures of the 3'-modified-nucleotides. Details of the chemical syntheses are described in the Materials and Methods section.

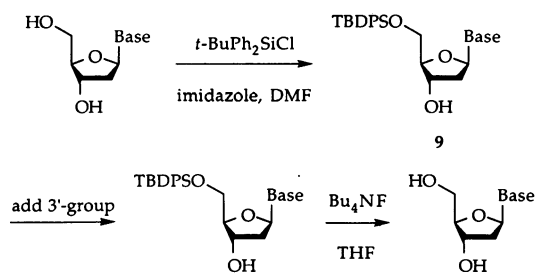
MATERIALS AND METHODS

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$_{R^{\circledR}}$ (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 .

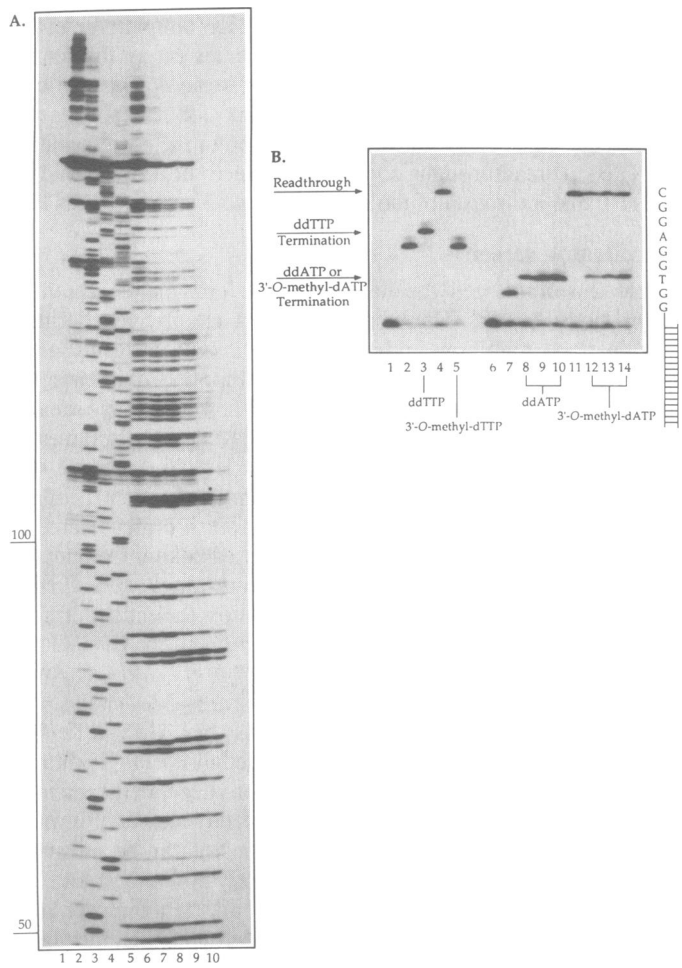


Figure 3. Incorporation of 3′-*O*-methyl-dATP by AMV-RT. (A) M13mp19-template assay: In addition to the conditions specified in Table 1, lane 1 contained no ddNTPs, and lanes 2–5 contained ddTTP, ddGTP, ddCTP, and ddATP, respectively. Lanes 6 and 7 contained 5 μM and 10 μM of ddATP, respectively. Lanes 8–10 contained 1 mM, 5 mM, or 10 mM of 3′-*O*-methyl-dATP, respectively. (B) Conditions for the AMV-RT Oligo-template assay were used where lanes 1 and 6 contained no dNTPs or ddNTPs. Lanes 2–5 contained dATP, dCTP and ddGTP. In addition, lane 3 contained ddTTP, lane 4 contained dTTP, and lane 5 contained 500 μM of 3′-*O*-methyl-dTTP. Lanes 7–14 contained dCTP, dTTP and ddGTP. In addition, lane 8–10 contained 0.1 μM, 0.5 μM, and 2.5 μM of ddATP; lane 11 contained dATP; and lanes 12–14 contained 10 μM, 50 μM, and 250 μM of 3′-*O*-methyl-dATP.

DNA polymerases. In the M-MuLV-RT Oligo-template assay, we observed a batch-to-batch difference in assaying both 3′-*O*-methyl-dATP [**1**] and 3′-*O*-acyl-dATP [**2**], (data not shown). While, the ddNTP controls gave similar results in both M-MuLV-RT batch assays, 3′-*O*-methyl-dATP [**1**] showed minor termination, and both 3′-*O*-dATP analogs ([**1**] and [**2**]) showed partial inhibition of DNA synthesis in M-MuLV-RT batch 1. In contrast, 3′-*O*-methyl-dATP [**1**] showed significant termination of DNA synthesis, and both 3′-*O*-dATP analogs ([**1**] and [**2**]) showed significant readthrough in M-MuLV-RT batch 2. This observation illustrates the importance of assaying candidate compounds with multiple enzyme batches.

3′-*O*-methyl-dTTP [8] incorporation

Unlike 3′-*O*-methyl-dATP [**1**], 3′-*O*-methyl-dTTP [**8**] was not incorporated by AMV-RT, (Figure 3B). A similar result was obtained by using the AMV-RT M13mp19-template assay (data not shown). However, 3′-*O*-methyl-dTTP [**8**] was efficiently incorporated by AmpliTaq® DNA polymerase in the M13mp19-template assay, and the termination pattern mimicked the ddTTP DNA ladders in a concentration dependent manner, (Figure 4A). Additional bands, however, were observed in the 3′-*O*-methyl-dTTP [**8**] ladders that were generated by both AmpliTaq® DNA polymerase (see arrows) and *Bst* DNA polymerase (data not shown) assays. The position of these additional bands corresponded to ddATP termination bands suggesting misincorporation of the thymidine analog in place of the deoxyadenosine analog. The efficiency of incorporation relative to ddTTP was, therefore, not determined in the M13mp19-template assays because of the additional bands.

Figure 4B shows the results of challenging 3′-*O*-methyl-dTTP [**8**] against four thermostable DNA polymerases in the Oligo-template assay. 3′-*O*-Methyl-dTTP [**8**] terminates all four polymerases in a concentration dependent manner. From the comparison of band intensities of 3′-*O*-methyl-dTTP [**8**] to ddTTP termination products, it can be estimated that 3′-*O*-methyl-dTTP [**8**] is approximately 50-fold less efficiently incorporated by *Bst* DNA polymerase than ddTTP, 20-fold less efficiently incorporated by AmpliTaq® DNA polymerase than ddTTP, 2-fold less efficiently incorporated by r*Tth* DNA polymerase than ddTTP, and 10-fold less efficiently incorporated by $Vent_R$® (exo$^-$) DNA polymerase than ddTTP.

Further investigation of a different nucleotide composition in the Oligo-template assay revealed altered base specificity of 3′-*O*-methyl-dTTP [**8**]. In the absence of dATP, 3′-*O*-methyl-dTTP [**8**] was incorporated by both *Bst* and AmpliTaq® DNA polymerases, and the termination bands mimicked the ddATP controls, (Figure 4C). It is noteworthy that in addition to 3′-*O*-methyl-dTTP [**8**] incorporation in Figure 4C, significant levels of readthrough were observed. Since the Oligo-template assay was performed in the absence of a deoxyadenosine analog, the readthrough must reflect the misincorporation of dNTPs in the noncomplement base position. This result suggests that 3′-*O*-methyl-dTTP [**8**] alters the base specific properties of DNA polymerases, not only in its incorporation, but in the incorporation of other natural nucleotides present in the reaction. This result also highlights the importance of the precise conditions of incorporation assays.

3′-*O*-(2-nitrobenzyl)-dATP [7] incorporation by *Bst* DNA polymerase

3′-*O*-(2-Nitrobenzyl)-dATP [**7**] terminated *Bst* DNA synthesis in a base specific manner. In the *Bst* M13mp19-template assay, however, the termination did not correspond to any of the ddNTP controls, (Figure 5A). This result made it difficult to assign a specific mode of action to 3′-*O*-(2-nitrobenzyl)-dATP [**7**]. The assignment was resolved using the biotinylated Oligo-template assay that gave strong evidence for termination. As shown in Figure 5B, 3′-*O*-(2-nitrobenzyl)-dATP [**7**] terminates *Bst* DNA synthesis where the DNA fragment migrates slower than the ddATP control, (compare lanes 3 and 7). 3′-*O*-(2-Nitrobenzyl)-dATP [**7**] is a UV sensitive compound that undergoes an intramolecular rearrangement by irradiation to give dATP and nitrosobenzaldehyde (22). Following UV exposure, the DNA termination fragment migrated at the same rate as the ddATP control, (compare lanes 3 and 8). This observation suggests that the 2-nitrobenzyl group can significantly alter the mobility of DNA in the gel and may provide a possible reason for the

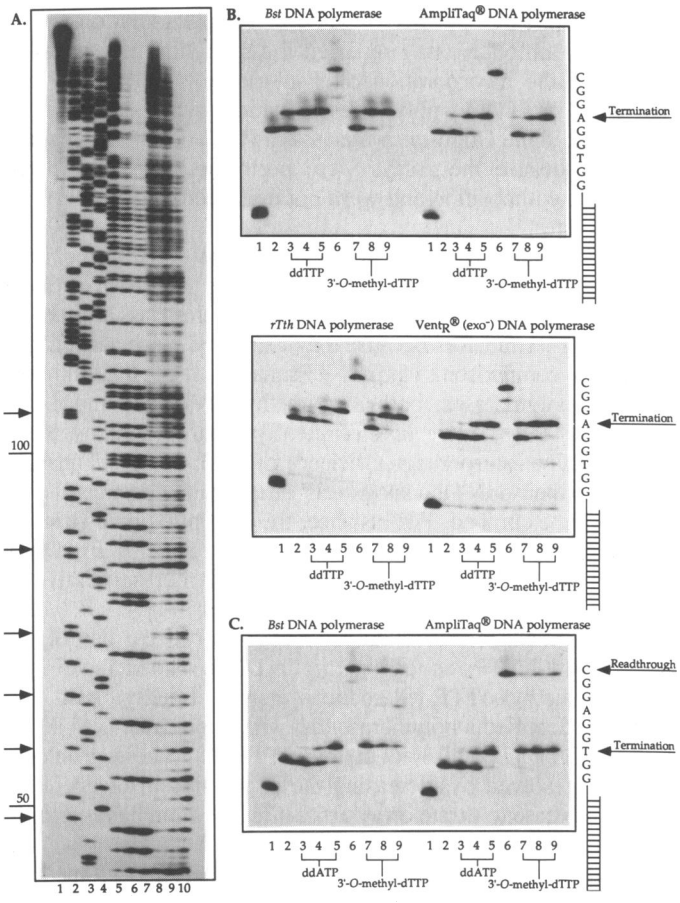


Figure 4. Incorporation of 3′-*O*-methyl-dTTP by *Bst*, AmpliTaq® , r*Tth* , and Vent$_R$® (exo$^-$) DNA polymerases. (A) M13mp19-template assay (AmpliTaq®): Enzymatic conditions in Table 1 were used where lane 1 contained no ddNTPs, and lanes 2−5 contained ddATP, ddCTP, ddGTP, and ddTTP, respectively. Lanes 6 and 7 contained 450 μM and 750 of μM ddTTP, respectively. Lanes 8−10 contained 1 mM, 2.5 mM, or 5 mM of 3′-*O*-methyl-dTTP, respectively. Arrows correspond to termination bands that are observed in the ddATP control (compare lanes 2 with 8−10). (B) Conditions for the Oligo-template assay were used for *Bst*, AmpliTaq® , r*Tth* , and Vent$_R$® (exo$^-$) DNA polymerases. Lane 1 contained no dNTPs or ddNTPs. Lanes 2−7 contained dATP, dCTP and ddGTP. In addition, lanes 3−5 contained (*Bst*) 0.1 μM, 0.5 μM and 2.5 μM ddTTP; (AmpliTaq®) 1.0 μM, 5.0 μM and 25 μM ddTTP; (r*Tth*) and (Vent$_R$® (exo$^-$)) 4 μM, 20 μM, and 100 μM ddTTP, respectively; lane 6 contained dTTP; and lanes 7−9 contained (*Bst*) 4 μM, 20 μM, and 100 μM of 3′-*O*-methyl-dTTP; (AmpliTaq®), (r*Tth*), and (Vent$_R$® (exo$^-$)) 20 μM, 100 μM, and 500 μM of 3′-*O*-methyl-dTTP, respectively. (C) Oligo-template assay (*Bst*, and AmpliTaq® DNA polymerases): Lane 1 contained no dNTPs or ddNTPs. Lanes 2−9 contained dCTP, dTTP and ddGTP. In addition, lanes 3−5 contained (*Bst*) 0.01 μM, 0.05 μM, and 0.25 μM of ddATP and (AmpliTaq®) 0.1 μM, 0.5 μM, and 2.5 μM of ddATP, respectively; lane 6 contained dATP; and lanes 7−9 contained (*Bst*) and (AmpliTaq®) 20 μM, 100 μM, and 500 μM of 3′-*O*-methyl-dTTP, respectively.

complicated pattern observed in the M13mp19-template assay. However, after exposing the DNA fragments to UV, a complex pattern was still observed in the M13mp19-template assay (data not shown).

The biotinylated Oligo-template assay was used to examine the reinitiation of DNA synthesis after 3′-*O*-(2-nitrobenzyl)-dATP [7] incorporation and UV deprotection. Following 3′-*O*-(2-nitrobenzyl)-dATP [7] termination, the solid-phase bound template was thoroughly washed and exposed to UV for 10 min. All the nucleotides required for the reinitiation of *Bst* DNA synthesis were then added in the absence of a deoxyadenosine analog. DNA synthesis clearly continues subsequent to 3′-*O*-(2-nitrobenzyl)-dATP [7] incorporation and UV deprotection, (Figure 5C).

DISCUSSION

In this study, the behavior of a series of novel nucleotide analogs was evaluated. Two different template tests were developed using various nucleotide compositions to assay the 3′-modified-dNTPs for incorporation by a variety of polymerases. Three compounds, 3′-*O*-methyl-dATP [**1**], 3′-*O*-methyl-dTTP [**8**], and 3′-*O*-(2-nitrobenzyl)-dATP [**7**], proved to be interesting DNA synthesis terminators. In Sanger sequencing, the 3′-*O*-methyl analogs generated clean terminating ladders, thus demonstrating their possible role as alternative terminators to ddNTPs. One of the labile nucleotide analogs, 3′-*O*-(2-nitrobenzyl)-dATP [**7**] was incorporated by *Bst* DNA polymerase. Subsequent removal of the 2-nitrobenzyl group and the reinitiation of DNA synthesis were used to demonstrate one complete cycle in BASS DNA sequencing.

Some notable correlations were observed between the structure of the 3′-modified-dNTPs and the specificity of enzymatic incorporation. First, the protecting groups containing ether linkages at the 3′-position, compounds [**1**], [**3**], [**4**], [**7**], and [**8**] were incorporated by some of the polymerases. With the exception of inhibition of Vent$_R$® (exo$^-$) DNA synthesis by 3′-*O*-acyl-dATP [**2**], those substrates containing ester linkages at the 3′-position, i.e., compounds [**2**], [**5**], and [**6**], were not accepted for any of the polymerases examined here. A comparison of compounds [**5**] and [**6**] with [**7**] illustrates this point. Both 3′-*O*-benzoyl-dATPs [**5**] and [**6**] were determined to be inactive in all the polymerase assays, whereas the 3′-*O*-(2-nitrobenzyl)-dATP [**7**] was incorporated by *Bst*, AmpliTaq® , and Vent$_R$® exo$^-$) DNA polymerases and inhibited Sequenase® DNA synthesis. Second, no consistent activity pattern between the polymerases and the analogs could be discerned. However, the incorporation of 3′-*O*-methyl-dATP [**1**] and of 3′-*O*-methyl-dTTP [**8**] was favored by reverse transcriptases and by thermostable DNA polymerases, respectively.

The data presented here are consistent with previous reports (1,3−9,11). A number of other 3′-modified-dNTPs have been synthesized and tested by various incorporation assays. Some examples from this nucleotide set include the 3′-*O*-methyl (5−7), 3′-amino (3), 3′-azido (1,6), 3′-chloro (8), 3′-fluoro (4), and 3′-mercapto (11) nucleoside-5′-triphosphates. The compounds in this study, with the exception of 3′-*O*-methyl-dATP [**1**], represent novel 3′-modified-dNTPs. The activity of 3′-*O*-methyl-dATP [**1**] has previously been shown to be incorporated by AMV-RT (4,6) and was shown to be inactive using Klenow fragment (5). We have expanded the list of polymerases to include those enzymes developed for DNA sequencing purposes (23−26).

The assay conditions are important determinants for evaluating the combined behavior of enzymes and 3′-modified-dNTPs. Here, two different template assays were used to enhance the chances of detecting enzymatic activity. Typically in the past, either a 'short' or a 'long' template assay has been used to monitor nucleotide incorporation (23−27) and misincorporation (28−30), and it is not clear that either template assay mimics the natural environment of DNA replication more than the other (31). In addition to patterns of termination, the sensitivity of these assays

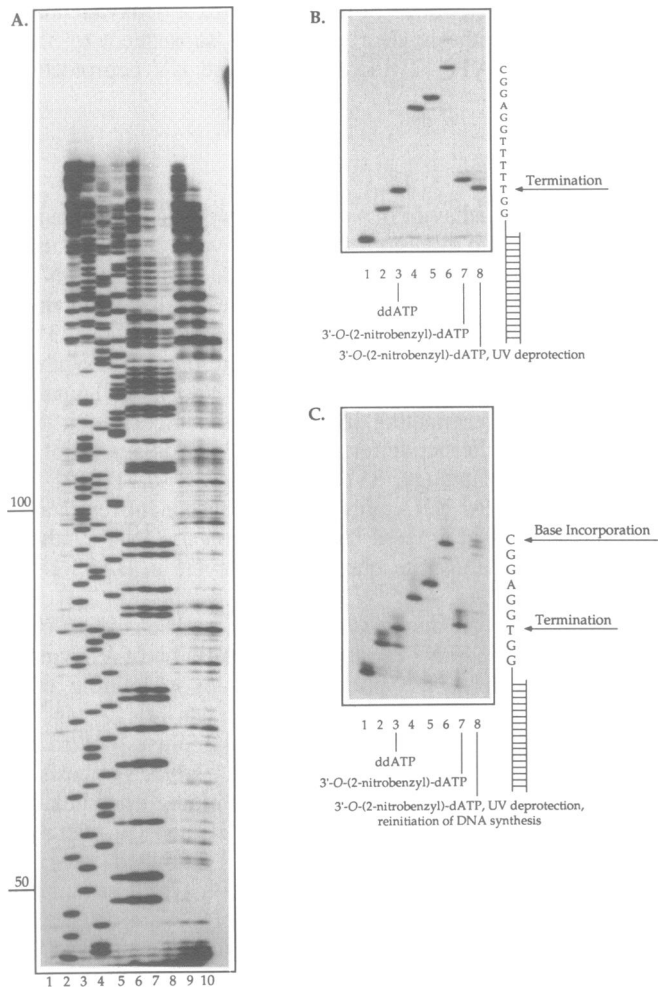


Figure 5. Incorporation of 3′-*O*-(2-nitrobenzyl)-dATP by *Bst* DNA polymerase. (**A**) M13mp19-template: In addition to the conditions specified in Table 1, lane 1 contained no ddNTPs, and lanes 2−5 contained ddTTP, ddGTP, ddCTP, and, ddATP, respectively. Lanes 6 and 7 contained 25 μM and 50 μM of ddATP, respectively. Lanes 8−10 contained 0.25 mM, 0.5 mM, or 1 mM of 3′-*O*-(2-nitrobenzyl)-dATP, respectively. (**B**) *Bst* Biotinylated Oligo-template assay conditions specified in Table 1 were used. Lane 1 contained no dNTPs or ddNTPs. Lanes 2−8 contained 0.05 μM of dCTP and 2.5 μM ddGTP. In addition, lane 3 contained 0.05 μM of dTTP and 5 μM of ddATP, lane 4 contained 0.05 μM of dATP, lanes 5 contained 0.05 μM of dATP and 50 μM of ddTTP, lane 6 contained 0.05 μM of dATP and dTTP. Lanes 7 and 8 contained 0.05 μM of dTTP and 250 μM of 3′-*O*-(2-nitrobenzyl)-dATP. Lane 8 was exposed to UV for 10 minutes. (**C**) The reactions conditions for lanes 1−8 are equivalent to (B) except for lane 8. Following UV deprotection, the reinitiation reaction were performed under the conditions specified in Table 1 and 0.05 μM of dCTP and dTTP and 2.5 μM ddGTP.

revealed isolated cases of DNA synthesis inhibition. The mode of inhibition is potentially of biological significance and is worthy of further investigation.

The short and long template assays employed in this study were tested either in the absence or in the presence of its competing dNTP, respectively. Each template assay, therefore, generated additional information not provided by the other. The Oligo-template assay was particularly useful for examining the incorporation of 3′-modified-dNTPs at reduced concentrations and revealed the presence of natural nucleotide contamination in the RP-HPLC purified 3′-modified-dNTP solutions. The M13mp19-template assay revealed the reduction of enzymatic fidelity in the incorporation of 3′-*O*-methyl-dTTP [**8**] by the thermostable DNA polymerases which was subsequently confirmed in the Oligo-template assay. These observations were possible because the assays were performed by varying the nucleotide composition and were not dependent of the length of the template.

The 3′-modified-dNTPs synthesized here were tested in a Sanger-type DNA sequencing scheme as alternative chain terminators. The 3′-*O*-methyl analogs best fit this class of dideoxy sequencing terminators because they generated clean sequencing ladders. In comparison to the ddNTPs that terminate the syntheses of DNA polymerases, both 3′-*O*-methyl-dATP [**1**] and 3′-*O*-methyl-dTTP [**8**] were less efficiently incorporated by these enzymes. The incorporation efficiency of the 3′-*O*-methyl analogs relative to their ddNTP analogs was variable and dependent upon the enzyme examined. For instance, the incorporation efficiency 3′-*O*-methyl-dTTP [**8**] ranged from 50-fold less for *Bst* DNA polymerase to 2-fold less for r*Tth* DNA polymerase relative to ddTTP.

Several unexpected results were observed in the use of the 3′-*O*-methyl-dNTPs as terminators in DNA sequencing. First, the 3′-*O*-methyl-dTTP [**8**] reduced enzyme fidelity. Also, we have not identified a polymerase that will incorporate both 3′-*O*-methyl-dATP [**1**] and 3′-*O*-methyl-dTTP [**8**]. These problems could be resolved by increasing the concentration of dATP in the former case to titrate away artifact termination bands and by testing more polymerases in the latter case. This issue, however, could also be resolved by substituting 2′-deoxy-3′-*O*-methyl-uracil-5′-triphosphate (3′-*O*-methyl-dUTP) for 3′-*O*-methyl-dTTP [**8**]. Kutateladze *et al.* have shown the incorporation of 3′-*O*-methyl-dATP, 3′-*O*-methyl-dCTP, 3′-*O*-methyl-dGTP, and 3′-*O*-methyl-dUTP by AMV-RT in a base specific manner (7). The use of these 3′-*O*-methyl terminators, however, has not been exploited in DNA sequencing strategies. We have synthesized the 3′-*O*-methyl-dUTP, and we are currently evaluating its activity with AMV-RT in the M13mp19-template assay. The eventual utility of the 3′-*O*-methyl terminators in Sanger sequencing and whether these novel terminators can eliminate artifacts such as gel compression remains to be determined.

The base specific incorporation of a labile terminator, the efficient removal of its 3′-protecting group, and the reinitiation of DNA synthesis demonstrate a one-cycle example of BASS. 3′-*O*-(2-Nitrobenzyl)-dATP [**7**] is a UV sensitive compound that decomposes to dATP, and both the incorporation and deprotection characteristics of this nucleotide analog made it an ideal compound to test the feasibility of this system. Here, it has been shown that 3′-*O*-(2-nitrobenzyl)-dATP [**7**] terminates *Bst* DNA synthesis in a base specific manner, and that following incorporation into DNA, the 2-nitrobenzyl group can be efficiently removed by UV. This UV deprotection was demonstrated by a band shifting pattern of the terminated reaction that mimicked the ddATP control and by the reinitiation of DNA synthesis in the Oligo-template assay, (Figures 5B and 5C, respectively). The band shifting experiment was crucial in demonstrating base specificity in that it discriminates between true incorporation of the nucleotide analog and nonspecific termination followed by incorporation of the natural, contaminating dNTP that we and others (7) have observed in the synthesis of 3′-modified-dNTPs.

Identification of a labile terminator sets the stage in the development of BASS. Investigations into the efficiencies of each step are required to further develop this strategy into a working model. We are currently evaluating both different base analogs containing the 2-nitrobenzyl protecting group and 3′-*O*-fluorescently labeled dNTPs for enzymatic activity. Efforts into high-density solid support technologies and quantitative imaging systems are also being pursued to integrate BASS into a feasible alternative to Sanger sequencing.

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