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ACYCLIC NUCLEOSIDE TRIPHOSPHATE ANALOGS AS TERMINATORS IN BIOCATALYTIC DNA REPLICATION

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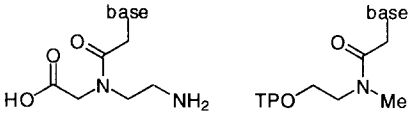
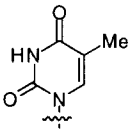
Abstract: The acyclic nucleoside triphosphates **1** and **2** were prepared and tested as substrates for several DNA replicating enzymes; AmpliTaq® FS and Taquenase® accepted these compounds as substrates leading to chain termination. © 1997 Elsevier Science Ltd.

Terminators of enzyme-directed DNA synthesis are central to the Sanger-based methods for DNA sequencing[1] used in The Human Genome Project and most other projects involving unraveling of DNA codes. It therefore surprising that the terminators still used are exactly those originally introduced. The human genome is approximately 3×10^9 bases hence the cost of the terminators becomes an important issue. A 1 cent saving per base is equivalent to $30 million dollars for the whole genome sequence. Attempts to find cheaper alternatives to the 2′,3′-dideoxyribonucleoside-5′-triphosphates (ddNTPs) that are generally used as chain terminators therefore are timely.[2] The extensive literature on peptide nucleic acids (PNAs)[3] indicates that the ribose framework of ddNTPs is not essential for high binding affinity and specificity. We therefore supposed that triphosphates like **1** and **2** might be effective terminators for biocatalytic DNA replication. This communication reports syntheses of compounds **1** and **2**, and gel assays to test their efficacy as ddNTP surrogates.

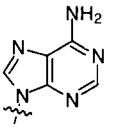


PNA

base
1 T
2 A



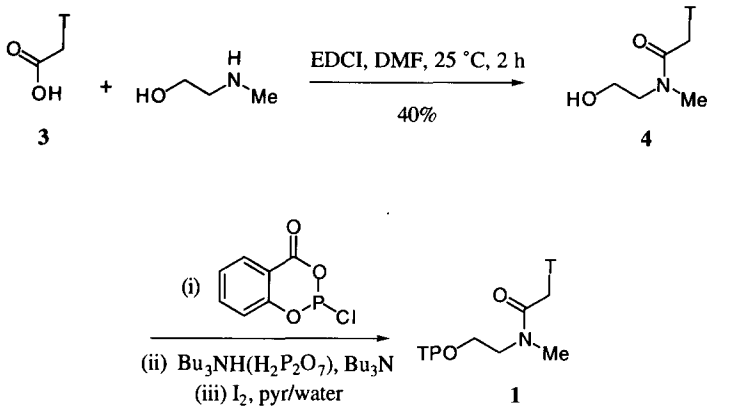
T



A

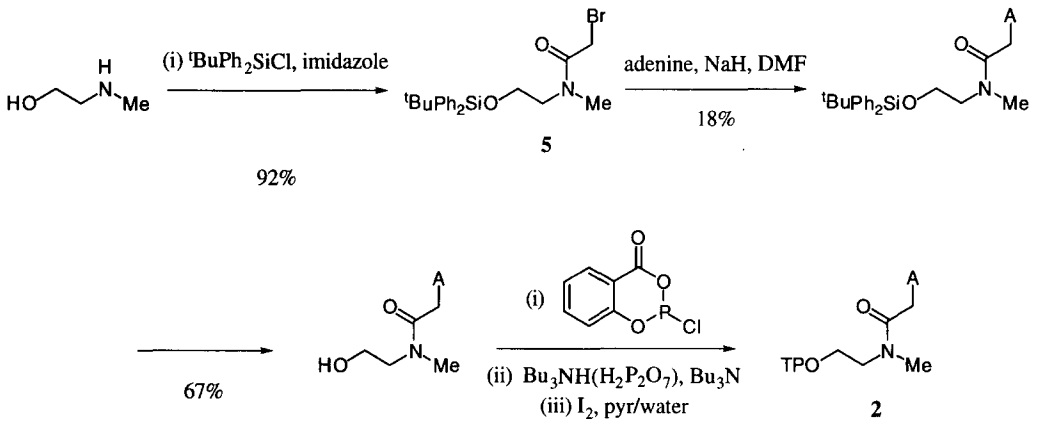
TP = $P_3O_9H_4$, *ie* triphosphate

Compound **1** was formed by coupling the known[4] thymidine acetate **3** with *N*-methylethanolamine as indicated below. Triphosphorylation of alcohol **4** using the conditions described by Eckstein and coworkers[5] proceeded to give the triphosphate **1** more efficiently than triphosphorylation of many other substrates that we have attempted. Thus, HPLC, ^{31}P NMR, and MALDI-MS analyses of the material recovered after ion exchange column chromatography indicated that it was predominantly triphosphate. A small sample of this material was purified by RP-HPLC for use in the experiments with DNA polymerases.



3 4 1

A slightly different approach was used to prepare the adenine derivative **2** (i.e., the bromoacetate intermediate **5** was prepared first) then coupled with adenine. Desilylation and triphosphorylation reactions were then used to complete the synthesis. This approach is more divergent than the synthesis of compound **1** insofar as many different base analogs could be prepared from bromoacetate **5**. In this particular case, the coupling of **5** to the heterocycle gave low yields under the conditions indicated. This yield was not improved when another base (K_2CO_3) and solvent (DMSO) were used. A possible explanation for these inefficient reactions is that competing reactions at the exocyclic amine of adenine may have occurred. RP-HPLC, ^{31}P NMR, and MALDI MS after ion exchange chromatography indicated the efficiency of the phosphorylation.



5 2

The gel assay used to test for incorporation of compounds **1** and **2** was adapted from that previously reported from these laboratories.[6] Thus a short synthetic template having the sequence 3′-ACATTTTGCTGCCGGTCAGGTGGAGGC was primed with a 5′-^{32}P-labeled primer with the sequence 3′-ACATTTTGCTGCCGGTCA. The double-strand DNA so produced was subjected to biocatalytic replication

using reaction mixtures containing either the thymidine analog **1** and dATP and dCTP, or the adenosine analog **2** and dCTP. Chain termination was characterized by comparison with control lanes using ddTTP and ddATP.[7]

The following DNA polymerases were tested in the assay outlined above: r*Tth* DNA Pol (Perkin–Elmer), HIV-1 RT, AmpliTaq® DNA Pol (Perkin Elmer), Taquenase® (ScienTech), AmpliTaq® FS DNA Pol (Perkin–Elmer), and *Bst* DNA Pol (Bio-Rad). The results demonstrated that incorporation/termination was observed for both analogs when AmpliTaq® FS and Taquenase® were used (Table 1). At a concentration of 100 μM, the adenosine analog **2** was incorporated by both enzymes, whereas a 1 mM concentration of **1** caused only partial termination under similar conditions (Figure 1). The adenine derivative *N*-MeA **2** was therefore incorporated more efficiently than the thymidine counterpart **1**.

The mutants Taquenase® and AmpliTaq® FS are known to have a relatively broad spectrum of substrate tolerance[8] so it was unsurprising that these two enzymes should show the desired activities whereas several others did not. In fact, amongst the wild type enzymes, only HIV-1 RT gave incorporation/termination, and in that case, substrate concentrations of 1 mM were required for both **1** and **2**.

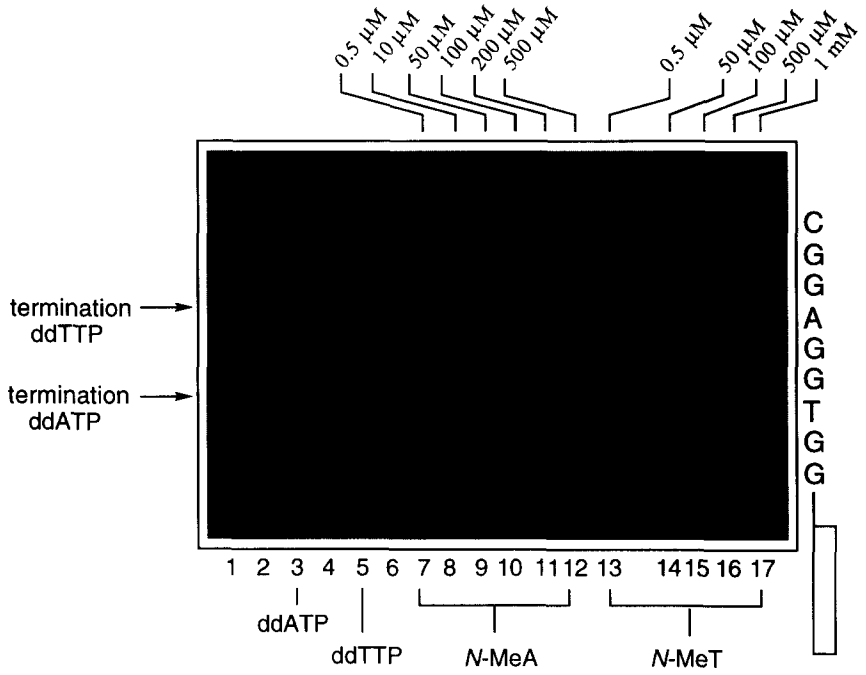


Figure 1. Incorporation of *N*-MeT **1** and of *N*-MeA **2** by AmpliTaq FS. All dNTPs were used at a concentration of 0.1 μM and ddNTPs at 0.5 μM. Substrates used in the control lanes 1–6 were as follows: lane 1 contains no ddNTPs or dNTPs; lane 2 dCTP; lane 3 dCTP and ddATP; lane 4 dCTP and dATP; lane 5 dCTP, dATP and ddTTP; lane 6 dCTP, dATP, dTTP and ddGTP. Lanes 7–12 and lanes 13–17 featured progressively greater concentrations of triphosphates **1** and **2** in combination with dCTP (lanes 7–12) or dCTP and dATP (lanes 13–17), respectively (Table 1). Note 7 gives further details.

Table 1. Termination assays for *N*-MeA **1** and *N*-MeT **2**.

enzymes	*N*-MeT **1**	*N*-MeA **2**
r*Tth* DNA Polymerase	no incorporation[a]	no incorporation
HIV-1 RT	termination (1 mM)[b]	termination (1 mM)
AmpliTaq® DNA Pol.	no incorporation	--[c]
Taquenase®	termination (1 mM)	termination (100 μM)
AmpliTaq® FS	termination (1 mM)	termination (100 μM)
Bst DNA Polymerase	no incorporation	no incorporation

[a]No incorporation observed. [b]Full termination observed at the concentration in parenthesis. [c]Not assayed.

In conclusion, the thymidine and adenosine analogs **1** and **2** are substrates for some DNA replicating enzymes with high tolerance for variation in substrate structure. They can be incorporated to give near complete termination of enzymatic extension of a short DNA-template. For the *N*-MeA analog **2**, concentrations as low as 100 μM were sufficient, whereas for *N*-MeT **1** termination only occurred when millimolar concentrations of were used. Analogs **1** and **2** are incorporated less readily than ddTTP and ddATP and they are not practical substitutes under the conditions described here. They are, however, easily prepared via facile triphosphorylation reactions, and may be incorporated more efficiently under other conditions.

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References and Notes

1. Sanger, F.; Nicklen, S.; Coulson, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463.
2. Hobbs, F. W.; Cocuzza, A. J., European, 1988, 87305844.0.
3. Hyrup, B.; Nielsen, P. E. *Bioorg. Med. Chem.* **1996**, *4*, 5.
4. Kosynkina, L.; Wang, W.; Liang, T. C. *Tetrahedron Lett.* **1994**, *35*, 5173.
5. Ludwig, J.; Eckstein, F. *J. Org. Chem.* **1989**, *54*, 631.
6. Metzker, M. L.; Raghavachari, R.; Richards, S.; Jacutin, S. E.; Civitello, A.; Burgess, K.; Gibbs, R. A. *Nucl. Acids Res.* **1994**, *22*, 4259.
7. For three of the enzymes (HIV-1 RT, AmpliTaq® FS, and Taquenase®) it was necessary to determine the minimum concentrations of dNTPs for efficient incorporation without misincorporation (i.e., mismatch formation), then repeat the procedure to ascertain the ddNTP concentrations. The protocol for these experiments has been published.[6] These concentrations were determined to be: 0.5 μM dNTPs and 0.05 μM ddNTPs for HIV-1 RT; 0.1 μM dNTPs and 0.5 μM for AmpliTaq® FS and Taquenase®. Optimal assay concentrations/conditions for *Bst* and r*Tth* DNA polymerases were those as reported previously.[6]

   Details of the assay were as follows. The synthetic template was primed with the 5′-^{32}P-labeled primer, melted at 80 °C, then allowed to anneal by slow cooling to room temperature. The double-strand DNA so produced was mixed with the analog (**1** or **2**) and the various dNTP and ddNTP's at the concentrations given above. After incubation for 15 min at the temperature prescribed for each enzyme, the reactions were quenched by addition of 5 μL of stop solution containing 98% diisopropyl formamide, 10 mM EDTA, pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanol. The samples were heated to 85 °C for 5 min, chilled in ice, and 3 μL were loaded into 20% acrylamide gels. Following electrophoresis the gel was fixed in aqueous 10% acetic acid, 10% methanolic solution (v/v), dried, and autoradiographed on Hyperfilm TM-MP (Amersham).
8. Tabor, S.; Richardson, C. C. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 6339.

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