2′,3′-Dideoxy-3′-thionucleoside Triphosphates: Syntheses and Polymerase Substrate Activities
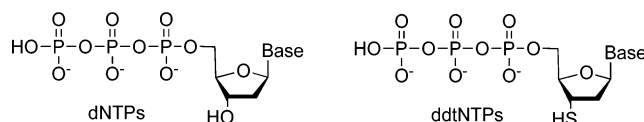
Meena,[†] Mui Sam,[‡] Kathryn Pierce,[†] Jack W. Szostak,[‡] and Larry W. McLaughlin*,[†]

Department of Chemistry, Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts 02467, and Howard Hughes Medical Institute and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114

mclaughl@bc.edu

ABSTRACT

All four 2′,3′-dideoxy-3′-thio-nucleosides (ddtNTPs) function as substrates for the Y410F mutant of Deep Vent (exo⁻) DNA polymerase. Not only are the ddtNTPs incorporated to form the N + 1 product, but further elongations are observed in which the key step is attack of the 3′-thiol on the 5′-triphosphate. Although other polymerases are likely to differ in their use of the ddtNTPs, there does not appear to be a fundamental prohibition against using a thiol nucleophile on a phosphate anhydride electrophile. The syntheses of four ddtNTPs (C, T, A, G) are described.

Template-directed polymerization of DNA catalyzed by polymerases uses dNTP building blocks in which the attacking nucleophile is a 3′-OH. Hydroxyl is not the only functionality that could be successful in such reactions. An amino group or thiol group might well be an effective nucleophile when placed on the 3′-carbon and positioned to attack a 5′-triphosphate.

The 3′-amino-2′,3′-dideoxynucleoside triphosphates or 3′-amino-3′-deoxynucleoside triphosphates place an amino group on the dNTP or NTP 3′-carbon. These derivatives are reported to function as inhibitors of DNA[1] or RNA[2] polymerases, with the former acting largely as chain terminators after the initial nucleotide elongation event.

Although an aliphatic thiol should be partially deprotonated at pH 7 and function better as a nucleophile than hydroxyl, that expectation does not hold with respect to attack on phosphates. For example, the intramolecular attack of a 2′-thiol on a nucleoside 3′-p-nitrophenolphosphodiester was very slow.[3] In the only study[4] we could locate where selected ddtNTPs have been examined as polymerase substrates, they appear to function better as chain terminators than as effective elongation substrates for Pol 1 and HIV reverse transcriptase.

Other examples would seem to support the contention that thiol is a poor nucleophile for phosphates. Topoisomerase and site-specific recombinase substrates containing a 5′-phosphorothiolate[5] are cleaved readily to generate the tyrosyl-3′-phosphodiester and a 5′-terminal thiol. To complete the reaction, it is necessary that the liberated thiol attack the phosphorylated tyrosine and regenerate the phosphorothiolate diester. In this[5] and a similar case[6] the thiol is an incompetent substrate relative to the native 5′-OH; the thiolate linkage functions as a suicide substrate. In a related example, the

† Boston College.
‡ Massachusetts General Hospital.
(1) Kutateladze, T.; Bebelashvili, R.; Azhaev, A.; Kraevskii, A. *FEBS Lett.* **1983**, *153*, 420−426.
(2) Armstrong, V. W.; Eckstein, F. *Eur. J. Biochem.* **1976**, *70*, 33−38.

(3) Dantzman, C. L.; Kiessling, L. L. *J. Am. Chem. Soc.* **1996**, *118*, 11715−11719.
(4) Yuzhakov, A. A.; Chidgevadze, Z. G.; Bibilashvilli, R. S. *FEBS Lett.* **1992**, *306*, 185−188.
(5) Burgin, A. B.; Huizenga, B. N.; Nash, H. A. *Nucleic Acids Res.* **1995**, *23*, 2973−2979.
(6) Nunes-Duby, S. E.; Radman-Livaja, M.; Kuimelis, R. G.; Pearline, R. V.; McLaughlin, L. W.; Landy, A. *J. Bacteriol.* **2002**, *184*, 1385−1394.

hammerhead ribozyme uses a 2′-OH of the active-site cytidine optimally positioned to transesterify the adjacent 3′-phosphodiester. Replacement of this functional group with a 2′-SH results not in transesterification of the neighboring phosphodiester, but rather in deglycosylation (attack at C1′) of the active-site cytidine.[7]

Previous reports suggest that ddtNTPs are unlikely to be effective polymerase substrates (at least after the incorporation of a single chain-terminating monomer),[4] and that thiols in general are poor nucleophiles for phosphates.[2-7] Although some synthetic efforts have been described for these derivatives,[8,9] we decided to prepare samples of all four ddtNTPs and provide some preliminary data for the activities of these derivatives as potential polymerase substrates or inhibitors.

The 2′,3′-dideoxy-3′-thiopyrimidine nucleosides were prepared from the relevant O2−C3′ anhydronucleosides essentially as described[10] (Scheme 1a). After removal of the
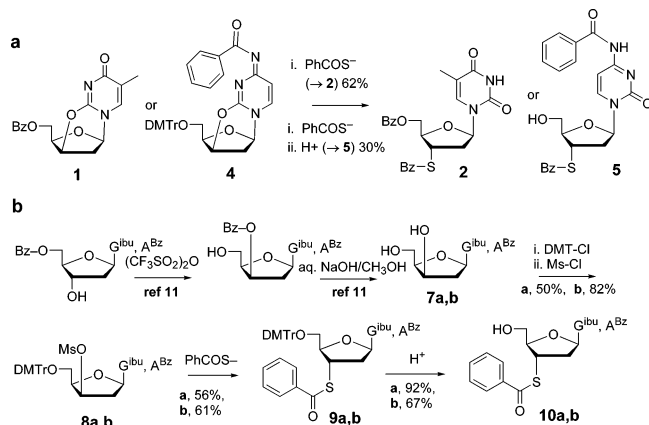
S-benzoyl protecting group the nucleosides could be oxidized to the disulfides and stored in this state (see Scheme 2a). The disulfides also functioned to protect the thiol group during subsequent formation of the bis(triphosphates). The purine derivatives were not so easily obtained since formation of the corresponding anhydro derivatives cannot occur. The most efficient procedure to epimerize the C3′ carbon and prepare it for a simple displacement reaction with thiol is that described by Herdewijn[11] in which formation of the 5′-benzoate and 3′-triflate results in a displacement of the triflate ester and migration of the benzoate ester to generate the 2′-deoxy-*xylo*-nucleoside (Scheme 1b). After removal of the benzoate ester and formation of the 5′-DMT ether and 3′-mesyl ester, the sodium salt of thiobenzoic acid could be

(7) Hamm, M. L.; Schwans, J. P.; Piccirilli, J. A. *J. Am. Chem. Soc.* **2000**, *122*, 4223−4224.

(8) Yuzhakov, A. A.; Chidzhavadze, Z. G.; Bibilashvilli, R. Sh.; Kraevskil, A. A.; Galegov, G. A.; Dorneeva, M. N.; Nosik, D. N.; Kilesso, T. Yu. *Bioorg. Khim.* **1991**, *17*, 504−509.

(9) El-Babary, A. A.; Khodair, A. I.; Pedersen, E. B.; Nielsen, C. *Monatsh. Chem.* **1994**, *125*, 1017−1025.

(10) Sabbagh, G.; Fettes, K. J.; Gosain, R.; O'Neil, I. A.; Cosstick, R. *Nucleic Acids Res.* **2004**, *32*, 495−501.

(11) Herdewijn, P. *J. Org. Chem.* **1988**, *53*, 5050−5053.
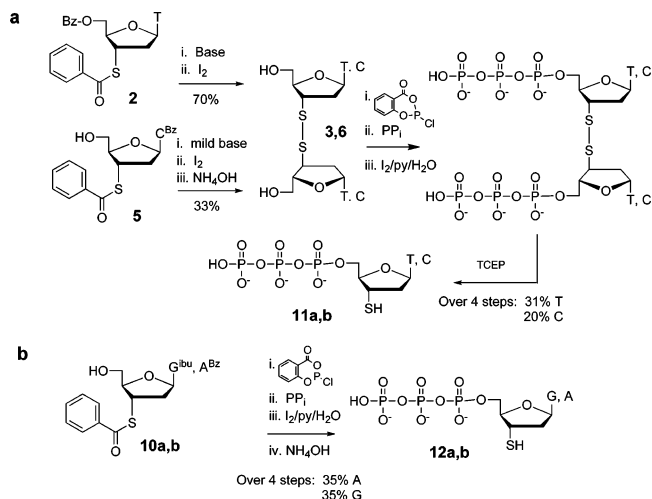
used in a second epimerization to form the 2′,3′-dideoxy-3′-thiopurine nucleosides (Scheme 1b). These derivatives were stored as the thioesters (disulfide formation was less efficient for the purine derivatives).

Formation of the corresponding nucleoside triphosphates used the procedure described by Eckstein[12] and either the dinucleoside disulfides (Scheme 2a) or the corresponding thiobenzoates (Scheme 2b). We were attracted to the possibility of long-term storage of the triphosphates as the disulfide dimers, and for 2′,3′-dideoxy-3′-thiothymidine as well as 2′,3′-dideoxy-3′-thiocytidine, formation of the bis(triphosphate) occurred with high yield (Scheme 2a). Although the bis(triphosphates) were not pure since triphosphate formation was not quantitative at both O5′-oxygens, they could be stored in this state. The desired 3′-thio ddNTPs were obtained after treatment of the bis(triphosphates) with TCEP and HPLC isolation. For the thiopurines it was more effective to convert the thiobenzoates into the desired triphosphates followed by treatment with ammonium hydroxide to generate the 3′-thio NTPs.

It seemed likely that DNA polymerases would elongate a DNA primer and form the *N* + 1 product with ddtNTP substrates, since the reaction chemistry is unchanged in the first incorporation. However, formation of the *N* + 2 and longer extension products requires that the thiol take part as the substrate nucleophile in the formation of internucleotide phosphorothiolate diesters (Figure 1). To test these possibilities, we initially screened a number of DNA polymerases (Pol I KF (*exo*-), Taq, Bst, Sequenase, Superscript reverse transcriptase, Therminator and wt and several mutants of Deep Vent (*exo*-) polymerase) for primer-extension with ddtTTP. Of these, the Y410F mutant of Deep Vent *exo*-polymerase appeared the best and was chosen for further study. We then examined all four 2′,3′-dideoxy-3′-thio-nucleoside triphosphates for substrate activity using a DNA primer/template complex with each template containing a stretch of four identical residues (T, C, A, or G) at its 5′-

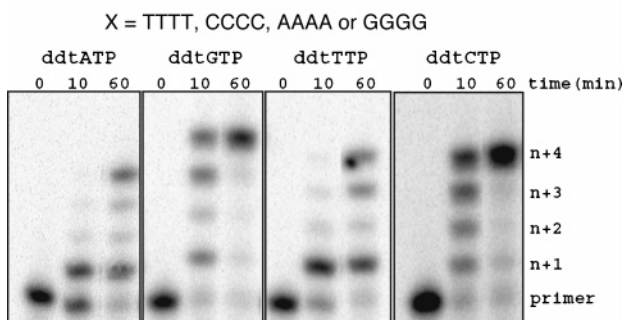(12) Ludwig, J.; Eckstein, F. *J. Org. Chem.* **1989**, *54*, 631−635.
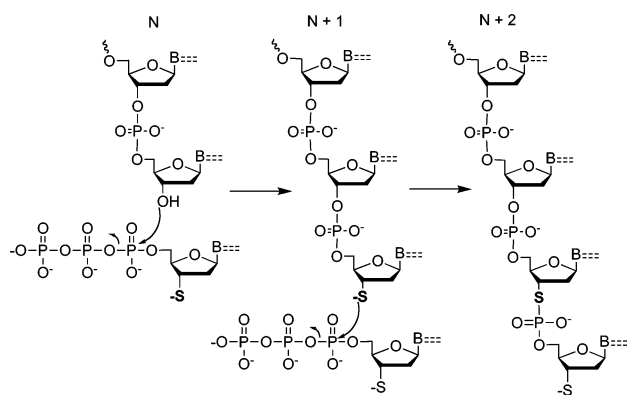
Figure 1. Elongation of a primer/template complex with ddtNTPs. Formaton of the $N + 1$ product generates a conventional phosphodiester with a 3′-terminal thiol. Formation of the $N + 2$ product and the 3′-bridging phosphorothiolate requires nucleophilic attack by the terminal 3′-thiol on the incoming triphosphate. "B" represents the nucleobases and the two dotted lines represent base pairing to the template residues.

5′-d(CGG AGC CAC CCT TAG GAC GTG ACG)
3′-d(GCC TCG GTG GGA ATC CTG CAC TGC XXXX)

X = TTTT, CCCC, AAAA or GGGG

Figure 2. Nucleotide primer/template extension reactions with varying ddtNTPs. Primer-extension reactions were performed by using 20 nM (for A, G and T) or 40 nM (for C) Y410F mutant of Deep Vent (*exo-*), 3 nM labeled primer/template duplex, and 200 µM ddtNTP in thermopol buffer at 50 °C. Extension reactions were monitored at various time points and analyzed by 20% denaturing PAGE.

end (Figure 2). The primer was labeled with radioactive phosphate and phosphorimaging was used to visualize the elongation bands. In all cases the ddtNTPs functioned as polymerase substrates and could extend the primer by four residues. In two cases (ddtGTP and ddtCTP) the majority of the primer had been elongated to the +4 product after a 60 min incubation. For ddtATP and ddtTTP there were distributions of elongation products with the +4 band in each case representing one of the major extension products.

This study illustrates that a thiol functional group can be used as the nucleophile in the formation of a phosphorothiolate linkage when primer extension is catalyzed by the Y410F mutant of the Deep Vent polymerase. On the basis of previous reports,[4] Pol I and HIV appear to be less accommodating in their use of such substrates. Although different polymerases are likely to vary in the details of substrate recognition, there does not appear to be any fundamental prohibition of using a thiol nucleophile on a phosphate anhydride electrophile, while corresponding catalyzed reactions on phosphodiesters[5,6] appear to be much less effective. The successful elongations illustrated in Figure 2 suggest that it may be possible to synthesize longer phosphorothiolate sequences in enzymatic reactions by using these monomers. If sufficiently long and accurate elongations can be achieved with ddtNTPs, it might be possible to use in vitro selection methods to generate functional thiolate-DNA molecules such as aptamers and DNA thio-enzymes.

DNA linkages containing 3′-phosphorothiolates have been used to stabilize structural motifs,[13] to probe enzymatic processes,[14] and to enhance antisense complexes.[15] The phosphorothiolate DNAs used in those previous studies were prepared by chemical syntheses.[16] The work reported here suggests that enzymatic syntheses may provide an alternate pathway for the preparation of similar materials.

Supporting Information Available: Experimental procedures and characterization of all compounds along with NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

OL070147W

(13) Brazier, J. A.; Fisher, J.; Cosstick, R. *Angew. Chem., Int. Ed.* **2006**, *45*, 114−117.
(14) Elliott, S. L.; Brazier, J.; Cosstick, R.; Connolly, B. A. *J. Mol. Biol.* **2005**, *353*, 692−703.
(15) Beevers, A. P. G.; Fettes, K. J.; Sabbagh, G.; Murad, F. K.; Arnold, R. P.; Cosstick, R.; Fisher, J *Org. Biomol. Chem.* **2004**, *2*, 114−119.
(16) Fettes, K. J.; O'Neil, I.; Roberts, S. M.; Cosstick, R. *Nucleosides, Nucleotides & Nucleic Acids* **2001**, *20*, 1351−1354.