Articles

New Thymidine Triphosphate Analogue Inhibitors of Human Immunodeficiency Virus-1 Reverse Transcriptase†

Qi-Feng Ma,‡ Ian C. Bathurst,§ Philip J. Barr,§ and George L. Kenyon*,‡

Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143, and Chiron Corporation, 4560 Horton Street, Emeryville, California 94608. *Received January 13, 1992*

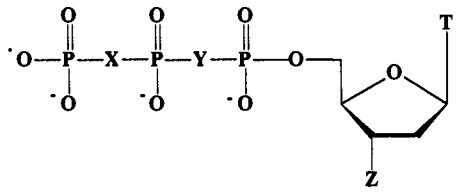
Several novel imidotriphosphate analogues of thymidine have been synthesized and have been shown to be effective inhibitors of human immunodeficiency virus-1 reverse transcriptase (HIV-1 RT). When the α,β-bridging oxygens of thymidine triphosphate (TTP) and 3′-azido-3′-deoxythymidine 5′-triphosphate (AZTTP) were replaced by a nitrogen, the resulting analogues were no longer substrates but instead became competitive inhibitors of HIV-1 RT. The most potent of the α,β-imidotriphosphate derivatives tested was thymidine 5′-[α,β-imido]triphosphate (TMPNPP, **1a**). This analogue has a K_i value of 2.4 μM, inhibiting HIV-1 RT 400-fold more potently than it inhibits DNA polymerase I large fragment (Klenow). 3′-Azido-3′-deoxythymidine 5′-[α,β-imido]triphosphate (AZTMPNPP, **1b**) gave a K_i value about 10-fold greater than that for TMPNPP, indicating that a 3′-azido substituent decreases the affinity of AZTTP to HIV-1 RT relative to the normal 3′-OH substituent. Dideoxythymidine 5′-[α,β-imido]triphosphate (ddTMPNPP, **1c**) was intermediate in potency, giving a K_i value of 15 μM. In contrast, substitution at the β,γ-bridging oxygen by nitrogen did not block the enzymatic cleavage of the adjacent α,β-phosphate linkage, and 3′-azidothymidine 5′-[β,γ-imido]triphosphate (AZTMPPNP, **1e**), the 5′-[β,γ-imido]triphosphate analogue of AZTTP, is therefore both a substrate for and a potent inhibitor of HIV-1 RT with an observed K_i value of 87 nM. Further nitrogen substitution of the bridging oxygens in the phosphate chain decreases the inhibitory potency by approximately 10-fold, as in the case of thymidine 5′-[α,β:β,γ-diimido]triphosphate (TMPNPNP, **1d**).

Introduction

Human immunodeficiency virus-1 reverse transcriptase (HIV-1 RT) plays an important role in the life cycle of the virus and has been a major target for the design of drugs to combat acquired immune deficiency syndrome (AIDS).[1-6] An important class of HIV-1 RT inhibitors is that of nucleoside analogues. Foremost among these analogues are 3′-azido-3′-deoxythymidine (AZT, **3b**), 2′,3′-dideoxycytidine (ddC), and 2′,3′-dideoxyinosine (ddI), which are considered to be prodrugs for the treatment of HIV infection.[7-9] These analogues are converted into their triphosphate forms by cellular enzymes, the triphosphate forms are then recognized by HIV-1 RT as substrates and the corresponding nucleoside monophosphate moieties are incorporated into DNA chains. Since these analogues lack the 3′-hydroxyl group, their incorporation leads to DNA chain termination.

Although many nucleotide-based HIV-1 RT inhibitors have been discovered and studied, little has been reported concerning the influence of polyphosphate chain modification on the inhibitory potency of the nucleotide analogues against HIV-1 RT. Since the enzyme catalyzes the cleavage of the α,β-P–O–P bond of 2′-deoxynucleoside triphosphates (dNTPs), we are interested in the substitution of bridging oxygens in the polyphosphate chain of nucleotides. We rationalized that if the α,β-bridging oxygen were replaced appropriately, the resulting compounds would still be recognized by HIV-1 RT but could not be converted into products. In this way typical competitive inhibitors would be generated. Previous work[10,11] showed that suitable substitution of the bridging oxygen *adjacent* to the cleaving site of a nucleoside triphosphate leads to analogues that retain activities as substrates for kinases.

Chart I



	Name	X	Y	Z
1a	TMPNPP	O	N	OH
1b	AZTMPNPP	O	N	N_3
1c	ddTMPNPP	O	N	H
1d	TMPNPNP	N	N	OH
1e	AZTMPPNP	N	O	N_3

We reasoned by analogy that if we replaced the β,γ-bridging oxygen atom with a suitable group, the modified

* To whom correspondence should be addressed.

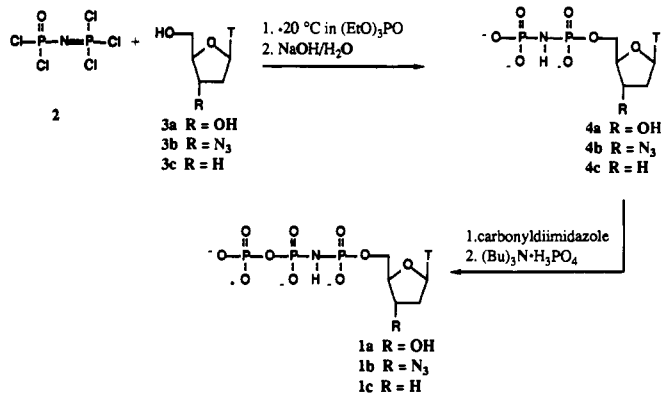
† This work was supported by Public Health Service Grant GM 39552 from the National Institutes of Health.

‡ University of California.
§ Chiron Corp.

(1) Hirsch, M. S. Chemotherapy of Human Immunodeficiency Virus Infections: Current Practice and Future Prospects. *J. Infect. Dis.* **1990**, *161*, 845–857.
(2) Mitsuya, H.; Broder, S. Inhibition of the *in vitro* Infectivity and Cytopathic Effect of Human T-lymphotrophic Virus Type III/Lymphadenopathy-Associated Virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1911–1915.
(3) Sandstrom, E. G.; Kaplan, J. C.; Byington, R. E.; Hirsch, M. S. Inhibition of Human T-Cell Lymphotropic Virus Type III in vitro by Phosphonoformate. *Lancet* **1985**, 1480–1481.
(4) Pauwels, R.; Andries, K.; Desmyter, J.; Schols, D.; Kukla, M. J.; Breslin, H. J.; Raeymaeckers, A.; Gelder, J. V.; Woestenborghs, R.; Heykants, J.; Schellekens, K.; Janssen, M. A. C.; Clercq, E. D.; Janssen, P. A. J. Potent and Selective Inhibition of HIV-1 Replication *in vitro* by a Novel Series of TIBO Derivatives. *Nature* **1990**, *343*, 470–474.
(5) Nakane, H.; Ono, K. Differential Inhibitory Effects of Some Catechin Derivatives on the Activities of Human Immunodeficiency Virus Reverse Transcriptase and Cellular Deoxyribonucleic and Ribonucleic Acid Polymerases. *Biochemistry* **1990**, *29*, 2841–2845.

0022-2623/92/1835-1938$03.00/0

Scheme I



analogues might still be substrates for HIV-1 RT. By comparison of the substrate specificities of the analogues toward HIV-1 RT vs cellular DNA polymerases, we can gain more insight into designing better, more selective DNA chain terminators for HIV-1 RT. Since the P–N–P bond resembles the P–O–P bond in bond angles, bond lengths, and other properties but is resistant to enzymatic cleavage in most cases,[12,13] we reasoned that NH groups would be the best replacements for the bridging oxygens of dNTPs, and we have therefore designed and synthesized a series of imidotriphosphate analogues of deoxynucleosides (Chart I). In this paper we describe their syntheses, characterizations, and biochemical activities.

Chemistry

Previous syntheses of 5′-[α,β-imido]triphosphates of nucleosides involved the reactions of nucleoside 5′-[α,β-imido]diphosphates with phosphocreatine catalyzed by creatine kinase.[10,11,14] Although thymidine 5′-diphosphate is a good substrate for several kinases, such as creatine kinase, pyruvate kinase, and arginine kinase (data not shown), TMPNP (**4a**) is not a substrate for these kinases. We therefore utilized activation of the pyrophosphate moiety of nucleoside diphosphates (NMPNPs) by carbonyldiimidazole as an alternative way to incorporate the terminal phosphate (Scheme I). Although we also tried other chemical methods such as activation of inorganic phosphate with carbonyldiimidazole, only the procedures described here gave detectable products.

Scheme II

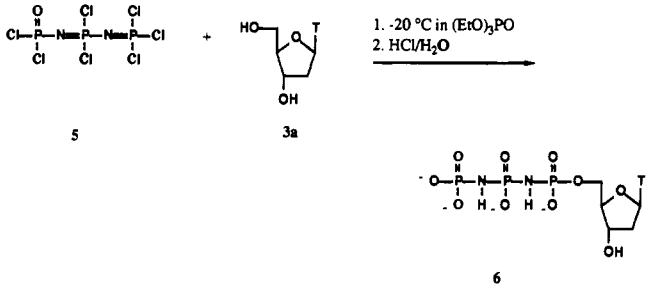


Table I. Inhibition of HIV-1 RT by TTP Analogues[a]

compounds	inhibition type	K_i (μM)
TMPNPP	competitive	2.4 ± 0.1
ddTMPNPP	competitive	15 ± 0.9
AZTMPNPP	competitive	22 ± 1.3
AZTMPPNP	competitive	0.087 ± 0.005
TMPNPNP	competitive	19.1 ± 1.0
AZTTP	competitive	0.13 ± 0.012[b]
		0.0036 ± 0.0004[b]

[a] The assay mixture (50 μL) for the kinetic study contained the following: 50 mM Tris-HCl (pH 8.0), 6 mM MgCl, 8 mM DTT, 80 mM KCl, 0.4 μM poly(rA)·oligo(dT), 60 ng/mL of HIV-1 RT (P66), 0.4 μM poly(rA)·oligo(dT), different inhibitors, and variable amounts of [^{3}H]TTP (4–20 μM, 0.2–1 μCi/20 μL). After incubation at 37 °C for 30 min, the mixtures were quenched by 15 μL of 0.5 M EDTA, and the template primers were collected on 2-(diethylamino)ethylcellulose (DE-81) paper.[9] The incorporation rate of TMP moieties was measured by counting ^{3}H on DE-81 paper, and the K_i values were calculated from double-reciprocal plots. [b] The inhibitory potency of AZTTP for HIV-1 RT is template-dependent. With a template of defined sequence, AZTTP gave a K_i value of 0.13 μM. With poly(rA)·oligo(dT) as a template primer, its K_i value dropped to 0.0036 μM.[15]

The only existing nucleoside 5′-[α,β:β,γ-diimido]triphosphate (NMPNPNP) is adenosine 5′-[α,β:β,γ-diimido]triphosphate (AMPNPNP), which was synthesized by reaction of 5′-tosyladenosine with tetrabutylammonium salts of diimidotriphosphate.[14] The reaction also generated another isomeric product, adenosine 5′-[α,β:α,β′-diimido]triphosphate (AMP(NP)$_2$), which is very difficult to separate from AMPNPNP. We have successfully synthesized TMPNPNP (**1d**) by the reaction of thymidine with [*P,P*-dichloro-*N*-(dichlorophosphinyl)phosphinimyl]phosphorimidic trichloride (**5**) in triethyl phosphate followed by acidic hydrolysis (Scheme II). No branched products were detected from the reaction mixture in this case, and the products were readily purified. The unique step of this reaction is the acidic hydrolysis. Hydrolyses of phosphazine derivatives of nucleosides are classically carried out in basic solutions which did not work for the synthesis of TMPNPNP (**1d**).

AZTMPPNP (**1e**) was synthesized by methods similar to those of Vrang et al.[15] and is a potent inhibitor of HIV-1

(6) Merluzzi, V. J.; Hargrave, K. D.; Labadia, M.; Grozinger, K.; Skoog, M.; Wu, J. C.; Shih, C. K.; Eckner, K.; Hattox, S.; Adams, J.; Rosehthal, A. S.; Faanes, R.; Eckner, J.; Koup, R. A.; Sullivan, J. L. Inhibition of HIV-1 Replication by a Non-nucleoside Reverse Transcriptase Inhibitor. *Science* **1990**, *250*, 1411–1413.

(7) Yarchoan, R.; Mitsuya, H.; Thomas, R. V.; Pluda, J. M.; Hartman, N. R.; Perno, C. F.; Marczyk, K. S.; Allain, J. P.; Johns, D. G.; Broder, S. *In vitro* Activity Against HIV and Favorable Toxicity Profile of 2′,3′-Dideoxyinosine. *Science* **1989**, *245*, 412–415.

(8) Mitsuya, H.; Weinhold, K. J.; Furman, P. A.; St. Clair, M. H.; Lehrman, S. N.; Gallo, R. C.; Bolognesi, D.; Barry, D. W.; Broder, S. 3′-Azido-3′-deoxythymidine (BW A509U): An Antiviral Agent that Inhibits the Infectivity and Cytopathic Effect of Human T-Lymphotropic Virus Type III/Lymphadenopathy-Associated Virus *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7096–7100.

(9) Cooney, D. A.; Dalal, M.; Mitsuya, H.; McMahon, J. B.; Nadkarni, M.; Balzarini, J.; Broder, S.; Johns, D. G. Initial Studies on the Cellular Pharmacology of 2′,3′-dideoxycytidine, an inhibitor of HTLV-III Infectivity. *Biochem. Pharmacol.* **1986**, *35*, 2065–2068.

(10) Ma, Q.; Babbitt, P. C.; Kenyon, G. L. Adenosine 5′-[α,β-Imido]triphosphate, a Substrate for T7 RNA Polymerase and Rabbit Muscle Creatine Kinase. *J. Am. Chem. Soc.* **1988**, *110*, 4060–4061; 8267.

(11) Ma, Q.; Reynolds, M. A.; Kenyon, G. L. Syntheses and Characterizations of Adenosine 5′-(α,β-N-Methylimido)di and Triphosphates and Adenosine 5′-(β,γ-N-Methylimido)triphosphate—Comparisons with Their Nonmethylated Analogs. *Bioorg. Chem.* **1989**, *17*, 194–206.

(12) Larsen, M.; Willet, R.; Yount, R. G. Imidodiphosphate and Pyrophosphate: Possible Biological Significance of Similar Structures. *Science* **1969**, *166*, 1510–1511.

(13) Yount, R. G.; Babcock, D.; Ballantyne, W.; Ojala, D. Adenylyl Imidodiphosphate, an Adenosine Triphosphate Analog Containing a P–N–P Linkage. *Biochemistry* **1971**, *10*, 2484–2489.

(14) Ma, Q.; Kenyon, G. L.; Markham, G. D. Specificity of S-Adenosylmethionine Synthetase for ATP Analogues Mono- and Disubstituted in Bridging Positions of the Polyphosphate Chain. *Biochemistry* **1990**, *29*, 1412–1416.

RT (Table I). Since this compound is similar to AZTTP, it is possible that the inhibition could result from AZTTP-contamination during synthesis. We have therefore developed a novel method to eliminate possible contamination by AZTTP. We found that AZTTP is a good substrate for hexokinase whereas AZTMPPNP (1e) is not (data not shown). Using this enzymatic procedure, AZTTP can be quantitatively converted into 3′-azido-3′-deoxythymidine 5′-diphosphate (AZTDP). The inhibitory potency of AZTMPPNP (1e) was tested before and after hexokinase treatment and showed no significant difference.

Biochemical Activities

All of the α,β-imidotriphosphates of thymidine and thymidine analogues are good competitive inhibitors of HIV-1 RT (Table I). Their K_i values range from 2.4 to 23 μM. Since these analogues are noncleavable in the HIV-1 RT reaction and showed typical competitive inhibition patterns, one can expect that their K_i values should be close to their dissociation constants (K_d's). We note that the K_d value for TTP is 4 times higher than the corresponding K_i value for TMPNPP (1a) (K_d value for TTP is 10 μM[16]). Since the true substrates for these enzymes are Mg^{2+}–nucleotide complexes, the enhanced affinity for the α,β-imidotriphosphates of nucleosides over their corresponding triphosphates may reflect an altered distribution among the numerous metal–chelate complexes present in the solution.

Further substitution of the bridging oxygens of TMPNPP (1a) by nitrogen decreases the binding to HIV-1 RT by 1 order of magnitude (Table I). Similar results were also found for adenosine [α,β-imido]triphosphate (AMPNPP) and AMPNPNP in their interactions with *S*-adenosylmethionine synthetase.[14] In the case of *S*-adenosylmethionine synthetase, AMPNPP binds to the enzyme 60-fold more tightly than does AMPNPNP.[14] Thus, diimido nucleotide analogues appear generally to bind to enzymes less well than monoimido analogues.

Several nucleotide triphosphate analogues have proven to be inhibitors for HIV-1 RT. Among these are AZTTP and dideoxythymidine triphosphate (ddTTP), whose observed K_i values are comparable to each other.[17] Since they are DNA chain terminators, the fact that they have the same observed K_i values does not mean that they necessarily have the same affinities for the enzyme.[18] Since nucleoside α,β-imidotriphosphates can bind to but cannot be cleaved by HIV-1 RT, their observed K_i values can be used to compare the relative affinities of these analogues for the enzyme. As shown in Table I, TMPNPP (1a), AZTMPNPP (1b), and ddTMPNPP (1c) are typical competitive inhibitors with K_i values of 2.4, 23, 15 μM, respectively. These results, along with previous reports,[19] suggest that a 3′-azido substituent does not increase the affinity of AZTTP to HIV-1 RT.

Table II. Specificity of AZTMPPNP and TMPNPP[a]

enzyme	IC_{50} (μM)	
	TMPNPP	AZTMPPNP
HIV-1 RT	10	0.1
Klenow	4000[b]	2000[b]

[a] The assays were performed in 50 μL mixtures containing the following: 50 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 8 mM DTT, 80 mM KCl, 20 μM [^{3}H]TTP (1 μCi/50 μL), 0.4 μM poly(rA)·oligo(dT) (or 0.4 μM poly(dA)·oligo(dT) in DNA polymerase I assay), 1 unit/mL DNA polymerase I large fragment (Klenow) or 60 ng/mL HIV-1 RT, and variable amounts of either TMPNPP or AZTMPPNP. The experimental procedures are the same as those described in footnote *a* of Table I. [b] Only ~25% *inhibition* was observed at this concentration.

AZTMPPNP (1e), the 5′-[β,γ-imido]triphosphate analogue of AZTTP, turned out to be a surprisingly potent competitive inhibitor of HIV-1 RT with a K_i value of 87 nM (Table I). Since 1e is also a substrate for HIV-1 RT (data not shown), and the kinetic parameter was obtained using a homopolynucleotide as template, we tentatively conclude that the inhibitory potency of 1e at least partially results from DNA chain termination.[18]

As shown in Table II, both TMPNPP (1a) and AZTMPPNP (1e) are much better inhibitors of HIV-1 RT than of DNA polymerase I large fragment (Klenow). In the case of 1a, the 50% inhibition concentration for HIV-1 RT is at least 400-fold lower than that for DNA polymerase I (Klenow). Since 1a is a noncleavable nucleotide analogue inhibitor of HIV-1 RT, this result suggests that 1a binds to the active site of HIV-1 RT much more tightly than to that of DNA polymerase I. Since the reported K_m values for TTP are close to the same for HIV-1 RT and DNA polymerase (K_m = 3.9 μM for DNA polymerase I;[20] K_m = 5.9 μM for HIV-1 RT[21]), the selective inhibition of HIV-1 RT by TMPNPP (1a) apparently arises solely from the substitution of the imido group. Further investigation, including cocrystallization of 1a with HIV-1 RT and determining the three-dimensional structure of the resulting complex, should give clearer insights regarding these observations.

Experimental Section

^{31}P NMR spectra were obtained at 79.5 MHz on a Nicolet NTCFT-1180 NMR spectrometer. A sweep width of 4000 Hz, a probe temperature of 25 °C, and ^{1}H broadband decoupling were used in ^{31}P NMR measurements. Chemical shifts were determined relative to 85% H_3PO_4, with positive shifts being downfield from the reference. High- and low-resolution liquid secondary ion mass spectra (LSIMS) were recorded on a Kratos MS-50 mass spectrometer, and a negative ion probe was used to measure the mass of the M − 1 peak.

Polyethylenimine (PEI)-cellulose plates were obtained from J. T. Baker Co. Other chemicals were from Aldrich. Poly(rA)·oligo(dT)$_{12-18}$, poly(dA)·oligo(dT)$_{12-18}$, DNA polymerase I large fragment (Klenow), and deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, dTTP) were from Pharmacia. HIV-1 reverse transcriptase-p66 was prepared as described previously.[22]

(15) Vrang, L.; Bazin, H.; Remaud, G.; Chattopadhyaya, J.; Öberg, B. Inhibition of the Reverse Transcriptase from HIV by 3′-Azido-3′-deoxythymidine Triphosphate and Its *threo* Analogue. *Antiviral Res.* **1987**, *7*, 139–149.

(16) Müller, B.; Restle, T.; Reinstein, J.; Goody, R. S. Interaction of Fluorescently Labeled Dideoxynucleotides with HIV-1 Reverse Transcriptase. *Biochemistry* **1991**, *30*, 3709–3715.

(17) Cheng, Y. C.; Dutschman, G. E.; Bastow, K. F.; Sarngadharan, M. G.; Ting, R. Y. C. Human Immunodeficiency Virus Reverse Transcriptase. *J. Biol. Chem.* **1987**, *262*, 2187–2189.

(18) Ma, Q.; Bathurst, I. C.; Barr, P. J.; Kenyon, G. L. The Observed Inhibitory Potency of 3′-Azido-3′-deoxythymidine 5′-Triphosphate for HIV-1 Reverse Transcriptase Depends on the Length of the Poly(rA) Region of the Template. *Biochemistry* **1992**, *31*, 1375–1379.

(19) Reardon, J. E.; Miller, W. H. Human Immunodeficiency Virus Reverse Transcriptase. *J. Biol. Chem.* **1990**, *265*, 20302–20307.

(20) Slater, J. P.; Tamir, I.; Loeb, L. A.; Mildvan, A. S. The Mechanism of *Escherichia Coli* Deoxyribonucleic Acid Polymerase I. *J. Biol. Chem.* **1972**, *247*, 6784–6794.

(21) Hizi, A.; Tal, R.; Shaharabany, M.; Loya, S. Catalytic Properties of the Reverse Transcriptases of Human Immunodeficiency Viruses Type 1 and Type 2. *J. Biol. Chem.* **1991**, *266*, 6230–6239.

(22) Bathurst, I. C.; Moen, L. K.; Lujan, M. A.; Gibson, H. L.; Feucht, P. H.; Pichuantes, S.; Craik, C. S.; Santi, D. V.; Barr, P. J. Characterization of the Human Immunodeficiency Virus Type-1 Reverse Transcriptase Enzyme Produced in Yeast. *Biochem. Biophys. Res. Commun.* **1990**, *171*, 589–595.

Tetrasodium imidodiphosphate, trichloro[(dichlorophosphoryl)imino]phosphorane (2), and [*P,P*-dichloro-*N*-(dichlorophosphinyl)phosphinimyl]phosphorimidic trichloride (5) were prepared as described previously.[10,11,14] Dideoxythymidine was synthesized from thymidine by the method reported previously.[23] They were used as the precursors for the synthesis of thymidine triphosphate analogues.

5′-Imidodiphosphates of the Thymidine Analogues. 5′-Imidodiphosphates of the thymidine analogues were prepared from the corresponding thymidine analogues (**3a–c**) by methods similar to our earlier published procedures.[14] The thymidine analogues (**3a–c**, 1 mmol) were dried and stirred in anhydrous triethyl phosphate (5 mL) at −15 °C, and 1.5 equiv of [(dichlorophosphoryl)imino]trichlorophosphorane (2, 1.5 mmol) in 0.5 mL of anhydrous triethyl phosphate was added dropwise. The mixture was stirred at −15 °C for 1 h and then 25 mL of 0.1 N NaOH was quickly added to the mixture under vigorous stirring at 0 °C. The mixture was extracted with three aliquots of 25 mL of ethyl acetate, and the water layer was evaporated to about 10 mL. The remaining water solution was applied to a AG^{R}MP-1 (analytical grade macroporous anion resin) column (Bio-Rad, 2.5 × 30 cm), which was subsequently eluted with a linear triethylammonium bicarbonate (TEAB) buffer (pH 8.5) gradient of 0–0.5 M at 4 °C. The appropriate fractions were collected and evaporated to dryness. The 5′-diphosphates of the analogues were converted into their sodium salts using sodium iodide in acetone. The yield was 50% of the theoretical value for TMPNP (**4a**), 47% for AZTMPNP (**4b**), and 35% for ddTMPNP (**4c**). All have similar UV spectra with absorption maxima at 266 nm and show a single spot on PEI-TLC with an R_f value comparable to that of TDP (0.3 M TEAB buffer as a eluent, R_f = 0.55). Their ^{1}H NMR spectra are consistent with the proposed structures. The analogues were further characterized by LSIMS and ^{31}P NMR. For **4a**: ^{31}P NMR (D_2O, pD = 10.3, ^{1}H broadband decoupling on) δ 2.18 (1 P, s, β-P), 5.61 (1 P, s, α-P); liquid secondary ion mass spectrum (LSIMS) gave the expected molecular weight (M − 1 = 400). For **4b**: ^{31}P NMR (D_2O, pD = 10.5, ^{1}H broadband decoupling on) δ 2.32 (1 P, s, β-P), 5.49 (1 P, s, α-P); the molecular weight from LSIMS further confirmed the proposed structure (M − 1 = 425). For **4c**: LSIMS gave the expected molecular weight (M − 1 = 384).

5′-Imidotriphosphates of Thymidine Analogues (TMPNPP, 1a; AZTMPNPP, 1b; and ddTMPNPP, 1c). Compounds **1a–c** were synthesized from their corresponding nucleoside imidodiphosphates (**4a–c**). Nucleoside imidodiphosphates (**4a–c**, Na^+ salts, 0.5 mmol) were converted into mono(tributylammonium) salts by passing through an Sp-Sephadex column (H^+ form, 2.0 × 10 cm) followed by adding 1 equiv of tributylamine (0.5 mmol). The resulting solution was evaporated to dryness and dissolved in 5 mL of anhydrous DMF. The mixture was then cooled to 0 °C, and 5 equiv of carbonyldiimidazole (2.5 mmol) in 5 mL of DMF was added to the mixture with stirring under an argon atmosphere. The reaction was allowed to remain at 0 °C for 30 min and at room temperature for 3 h. Dry methanol (4 equiv) was added, and the resulting solution was stirred for 30 min. Mono(tributylammonium) phosphate (1.5 equiv) in 5 mL of DMF was added to the mixture, and the solution was stirred for another 7 h. The reaction was quenched by adding 30 mL of 0.1 N NaOH, and the mixture was then separated as described above for the imidodiphosphate analogues. The yield was 41% of the theoretical value for **1a**, 38% for **1b**, and 25% for **1c**. All showed behavior similar to that of TTP on PEI-TLC (single spot with R_f = 0.4, 0.4 M TEAB buffer as eluent), and the structures were elucidated by ^{31}P NMR and LSIMS. For **1a**: ^{31}P NMR (D_2O, pD = 7.5, ^{1}H broadband decoupling on) δ 2.79 (1 P, s, α-P), −5.67 (1 P, d, J = 20 Hz, γ-P), −8.77 (1 P, d, J = 20 Hz, β-P); LSIMS gave the expected molecular weight (M − 1 = 480). For **1b**: ^{31}P NMR (D_2O, pD = 9.9, ^{1}H broadband decoupling on) δ 0.93 (1 P, d, J = 7 Hz, α-P), −4.19 (1 P, d, J = 22 Hz, γ-P), −10.45 (1 P, dd, J = 7, 22 Hz, β-P); LSIMS gave the expected molecular weight (M − 1 = 505). For **1c**: ^{31}P NMR (D_2O, pD = 7.5, ^{1}H broadband decoupling on) δ 2.69 (1 P, s, α-P), −5.7 (1 P, d, J = 19.8 Hz, γ-P), −8.52 (1 P, d, J = 20 Hz, β-P); accurately mass-measured parent ion of its sodium form (M − 1), calcd for $C_{10}H_{16}N_3O_{12}P_3Na$ 485.9845, found 485.9845.

Thymidine 5′-[α,β:β,γ-Diimido]triphosphate (TMPNPNP, 1d). Compound **1d** was synthesized using a different method from that used for the AMPNPNP synthesis.[14] Dried thymidine (**3a**, 3 mmol) was dissolved in 3 mL of triethyl phosphate, and the solution was cooled to −15 °C. [*P,P*-Dichloro-*N*-(dichlorophosphinyl)phosphinimyl]phosphorimidic trichloride (**5**, 3 mmol) in triethyl phosphate (3 mL) was added to the mixture dropwise with stirring. After 1 h, the reaction was quenched with 15 mL of 0.1 N HCl and kept at 0 °C for 20 min. The mixture was then neutralized by 1 N NaOH and extracted with three aliquots of 25 mL ethyl acetate. The water layer was evaporated under vacuum until 5 mL remained, and the residue was loaded onto a AG^{R}MP-1 column. **1d** was purified and converted to sodium salts in the same way as described for the TMPNPP synthesis. A white powder was obtained (0.8 mmol, 26% yield). The UV spectrum and TLC behavior were the same as for TMPNPP (**1a**): ^{31}P NMR (D_2O, pD = 10, ^{1}H broadband decoupling on) δ 3.93 (1 P, d, J = 7 Hz), 2.85 (1 P, d, J = 4 Hz), 1.01 (1 P, d, J = 4 Hz); accurately mass-measured parent ion of its disodium form (M − 1), calcd for $C_{10}H_{16}N_4O_{12}P_3Na_2$ 522.9774, found: 522.9766.

3′-Azido-3′-deoxythymidine 5′-Triphosphate (AZTTP) and 3′-Azido-3′-deoxythymidine 5′-[β,γ-Imido]triphosphate (AZTMPPNP, 1e). AZTTP and **1e** were prepared by methods similar to those of Vrang et al.[15] AZT (**3b**, 3 mmol) was dissolved in 5 mL of triethyl phosphate and cooled to 0 °C. Oxyphosphoryl trichloride (3 mmol) was added to the mixture with stirring, and the reaction was kept at 0 °C for 1 h. Mono(tributylammonium) salts of diphosphate analogues (pyrophosphate for AZTTP and imidodiphosphate for **1e**, 6 mmol) and 6 mmol of tributylamine were added to the mixture, and the solution was kept at 0 °C for another 10 min. The reaction was quenched with 20 mL of 0.1 N NaOH, and the residue was further treated as described for the **1a** synthesis. The yields are 47% for AZTTP and 25% for **1e**, respectively. Both compounds showed the same behavior on PEI-TLC and gave the same UV spectra as **1a**. AZTTP was characterized by ^{31}P NMR and LSIMS, and the data were fully consistent with the structure.[15] **1e** was characterized in the same manner: ^{31}P NMR (D_2O, pD = 10, ^{1}H broadband decoupling on) δ −8.21 (1 P, d, J = 20 Hz, α-P), −4.95 (1 P, dd, J = 20 Hz, 4 Hz, β-P), 2.25 (1 P, d, J = 20 Hz, γ-P); accurately mass-measured parent ion of its monosodium form (M − 1), calcd for $C_{10}H_{15}N_8O_{12}P_3Na$ 526.9877, found 526.9859.

1e was further treated with hexokinase to remove possible AZTTP contamination. (This procedure is based on our findings that AZTTP is a substrate for hexokinase but that **1e** is not.) The sodium salt of **1e** (0.5 μmol) was dissolved in 2 mL of buffer (pH 8) containing 200 mM glycine, 12 mM $MgCl_2$, 15 mM NADP, 100 mM glucose, 100 units of glucose-6-phosphate dehydrogenase and 200 units of hexokinase. The mixture was incubated at 25 °C for 12 h and then loaded onto a DEAE-Sephadex A-25 column (1 × 20 cm) and eluted with 100 mL of 0–0.5 M linear gradient of TEA-HCO_3^- (pH 8.5). The appropriate fractions were pooled, and the TEAB buffer was removed under high vacuum. **1e** was then converted to its sodium salt by passing the residue through an Sp-Sephadex column (Na^+ form), and the desired fractions were lyophilized to dryness. The AZTMPPNP (**1e**) obtained was ready for kinetic studies.

Inhibition Assays. Details of the enzymatic assays and biochemical evaluations are given as footnotes to Tables I and II.

(23) Robins, M. J.; Robins, R. K. The Synthesis of 2′,3′-Dideoxyadenosine from 2′-Deoxyadenosine. *J. Am. Chem. Soc.* **1964**, *86*, 3585–3586.