Synthesis of AZT 5′-Triphosphate Mimics and Their Inhibitory Effects on HIV-1 Reverse Transcriptase

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Received June 18, 2004

In search of active nucleoside 5′-triphosphate mimics, we have synthesized a series of AZT triphosphate mimics (AZT P3Ms) and evaluated their inhibitory effects on HIV-1 reverse transcriptase as well as their stability in fetal calf serum and in CEM cell extracts. Reaction of AZT with 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, followed by treatment of the phosphite intermediate **2** with pyrophosphate analogues, yielded the cyclic triphosphate intermediates **4b**−**4f**, which were subjected to boronation and subsequent hydrolysis to give AZT 5′-α-borano-*β*,*γ*-bridge-modified triphosphates **6b**−**6f** in moderate to good yields. Reaction of the cyclic intermediate **4d** with iodine, followed by treatment with a series of nucleophiles, afforded the AZT 5′-*β*,*γ*-difluoromethylene-*γ*-substituted triphosphates (**7b**−**7i**). Several different types of AZT P3Ms containing α-*P*-thio (or dithio) and *β*,*γ*-difluoromethylene (**13**,**14**), α,*β*-difluoromethylene and *γ*-*P*-methyl(or phenyl) (**15**,**16**), and α-borano-*β*,*γ*-difluoromethylene and *γ*-*O*-methyl/phenyl (**11**,**12**) were also synthesized. The effectiveness of the compounds as inhibitors of HIV-1 reverse transcriptase was determined using a fluorometric assay and a poly(A) homopolymer as a template. A number of AZT P3Ms exhibited very potent inhibition of HIV-1 reverse transcriptase. Modifications at the *β*,*γ*-bridge of triphosphate rendered the AZT P3Ms **6b**−**6f** with varied activities (K_i from 9.5 to ≫500 nM) while modification at the α,*β*-bridge of triphosphate led to weak AZT P3M inhibitors. The results imply that the AZT P3Ms were substrate inhibitors, as is AZT triphosphate. The most active compound, AZT 5′-α-R_p-borano-*β*,*γ*-(difluoromethylene)triphosphate (AZT 5′-αB-*βγ*CF_2TP) (**6d**-I), is as potent as AZT triphosphate with a K_i value of 9.5 nM and at least 20-fold more stable than AZT triphosphate in the serum and cell extracts. Therefore, for the first time, a highly active and stable nucleoside triphosphate mimic has been identified, which is potentially useful as a new type of antiviral drug. The promising triphosphate mimic, 5′-α-borano-*β*,*γ*-(difluoromethylene)-triphosphate, is expected to be valuable to the discovery of nucleotide mimic antiviral drugs.

Introduction

Dideoxynucleoside (ddN) antiviral drugs are successively phosphorylated in cells to ddN 5′-monophosphates (ddNMPs), ddN 5′-diphosphates (ddNDPs), and ddN 5′-triphosphates (ddNTPs).[1] The triphosphates of the antiviral nucleosides are the active chemical species that inhibit viral DNA synthesis. Although ddNTP binding in the catalytic site of a viral polymerase exerts a competitive inhibitory effect, overall viral DNA synthesis is inhibited predominantly through incorporation of the ddNMP into the elongating DNA chain and subsequent chain termination.[1−4] Therefore, a prospective antiviral nucleoside should meet two basic requirements: (1) ready phosphorylation to NTP by cellular enzymes and (2) ready incorporation into a viral DNA sequence and subsequent termination of the viral DNA chain. Many nucleosides that could not be phosphorylated by cellular enzymes failed to show antiviral activities and were eliminated in the initial drug screening, although the triphosphates of these nucleosides might be potent inhibitors of viral polymerases.[5] Cellular phosphorylation of an antiviral nucleoside by kinases is a process leading to an active NTP, but not necessarily a prerequisite for antiviral activities. Therefore, any alternative route that can lead to sufficient cellular concentration of a desired NTP may be highly useful. A conceivable alternative is to directly use a nucleotide as a drug entity, which can bypass cellular phosphorylation. To bypass the first cellular phosphorylation, NMP prodrugs have been explored as an alternative form of nucleoside antiviral drugs. Several types of NMP prodrugs have been intensively studied and have shown promising in vitro activities.[6−10] However, a major obstacle to success is the inherent instability of NMPs' natural 5′-monophosphate to cellular dephosphorylating enzymes such as 5′-nucleotidases and phosphodiesterases. In the case of a starting nucleoside that cannot be phosphorylated by cellular enzymes, the NMP prodrug, most likely, will be degraded to the inactive, parent nucleoside. In most cases, a mixture of nucleotides at all possible phosphorylation stages is likely formed after the NMP is released from an NMP prodrug. As a result, administration of NMP prodrugs may not offer many advantages over current nucleoside drugs themselves. It seems that the NMP prodrug approach is more suitable to stable NMPs or NMP mimics. In fact, the NMP prodrug approach has been successfully applied to nonhydrolyzable acyclic

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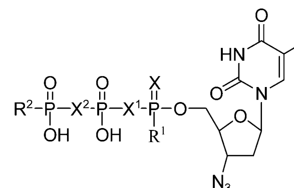
10.1021/jm040116w CCC: $27.50 © 2004 American Chemical Society
Published on Web 12/03/2004

nucleoside phosphonates, a type of NMP mimic. Currently, tenofovir disoproxil fumarate (a bis-POC prodrug of PMPA)[11] and adefovir dipivoxil (a bis-POM prodrug of PMEA)[12] are used clinically as anti-HIV and anti-HBV drugs, respectively. After it is liberated from an NMP mimic prodrug, the NMP mimic still needs further cellular phosphorylation to become an active species. Since NTPs are labile to cellular dephosphorylating enzymes, it is hardly imaginable to use NTPs as potential drugs. However, a NTP mimic (NP3M) that can entirely bypass cellular phosphorylation and possesses satisfactory cellular stability may be useful as an antiviral agent. If such an NP3M is identified, it is expected to offer certain advantages over nucleoside drugs concerning toxicity and drug resistance. Use of stable NP3Ms can avoid cellular buildup of undesired NMPs and NDPs, thus preventing unintended cellular interactions involving NMPs and NDPs. Drug resistance toward some antiviral nucleosides results from viral DNA repair arising from the deletion of incorporated ddNMP.[13] When an NP3M is employed, the viral repair activity is expected to be greatly reduced, or even diminished. Furthermore, an active NP3M may be obtained simply by attaching a favorable triphosphate mimic (P3M) to a variety of nucleoside analogues, regardless of their properties toward cellular phosphorylations.

Now the task is to identify active and stable P3Ms. There are previously known NP3Ms, in which a bridging oxygen of triphosphate is replaced by methylene,[14] halomethylene,[15,16] or imido[17,18] or in which a nonbridging oxygen is replaced by other functional groups.[19−23] A few NP3Ms containing a single β,γ-bridge modification have shown potent inhibition of HIV reverse transcriptase.[18,24] As expected, a single bridge modification on a triphosphate moiety can enhance the stability of a P3M to dephosphorylating enzymes, but it does not render the P3M sufficient stability for human therapeutics.[25] A couple of NP3Ms comprising two bridge modifications such as dimethylene and diimido were also reported, but unfortunately, no significant biological activities were observed.[18,26] Research in the field of NP3Ms has spanned more than 2 decades, but exploration of NP3Ms as human therapeutics remains in its infancy owing to challenges in chemical synthesis, purification, and drug delivery. To address the challenges, our first task was to identify useful P3Ms that can lead to very potent NP3M antiviral agents with satisfactory stability. In this paper, we report the synthesis of a new generation of AZT P3Ms as well as their inhibitory properties against HIV-1 reverse transcriptase and their stability in serum and cell extracts.

Considerations in Design of NP3Ms

Previously reported data have shown that AZT 5′-β,γ-imidotriphosphate was a much more potent inhibitor of HIV-1 reverse transcriptase than AZT 5′-α,β-imidotriphosphate, with K_i values of 0.087 vs 22 μM.[18] As the data have revealed, the mechanism of action mostly likely involves incorporation of the NMP mimic and subsequent DNA chain termination. Therefore, our initial work on NP3M antiviral agents was focused on incorporable NP3Ms having sufficient cellular stability. AZT and D4T 5′-α-R_P-boranotriphosphates were reported to be very potent inhibitors of both wild type and mutant (R72A, K65A, K65R) HIV-1 reverse transcriptases.[24] For the wild type, both compounds were more potent than AZT triphosphate (AZTTP), with K_i being 2.6- and 2.4-fold lower. For all three mutant enzymes, both compounds exhibited comparable potency to that of the wild-type enzyme, while AZTTP and d4TTP were less effective inhibitors of the mutant enzymes. Activity enhancement against mutant enzymes by the α-R_P-borano analogues was attributed to a decreased excision rate of α-R_P-borano-incorporated DNA chain by the mutant reverse transcriptases.[24] The work also demonstrated that the nucleoside 5′-α-R_P-boranotriphosphates had comparable binding affinity to HIV-1 RT as the NTPs, as measured by their K_D values. It appears that this P3M possesses desirable properties and can serve as a starting point in our search for superior P3Ms, although its stability to dephosphorylating enzymes is still of concern. Since our initial goal was to identify those P3Ms that are chemically and biologically stable enough to achieve their anticipated biological activities, stabilized analogues of active NP3Ms such as AZT 5′-α-R_P-boranotriphosphate naturally became our first priority. An easily conceivable approach to enhance the stability of 5′-α-R_P-boranotriphosphate without deteriorating its ability to be incorporated into DNA may be to modify the β,γ-bridge of the P3M by replacement of oxygen with methylene, halomethylene, or imido. In addition, the γ-P position of the P3M may be attached to a relatively small substituent in order to further enhance enzyme stability.[20] Besides 5′-α-R_P-borano, 5′-α-*P*-thiotriphosphate was also expected to provide adequate binding and incorporative properties.[19] For comparison purposes, it was appropriate to synthesize a few α,β-bridge-modified NP3Ms. Our first group of AZT P3Ms selected from variations as shown in Figure 1 has been synthesized and evaluated.



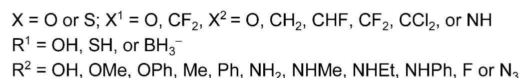
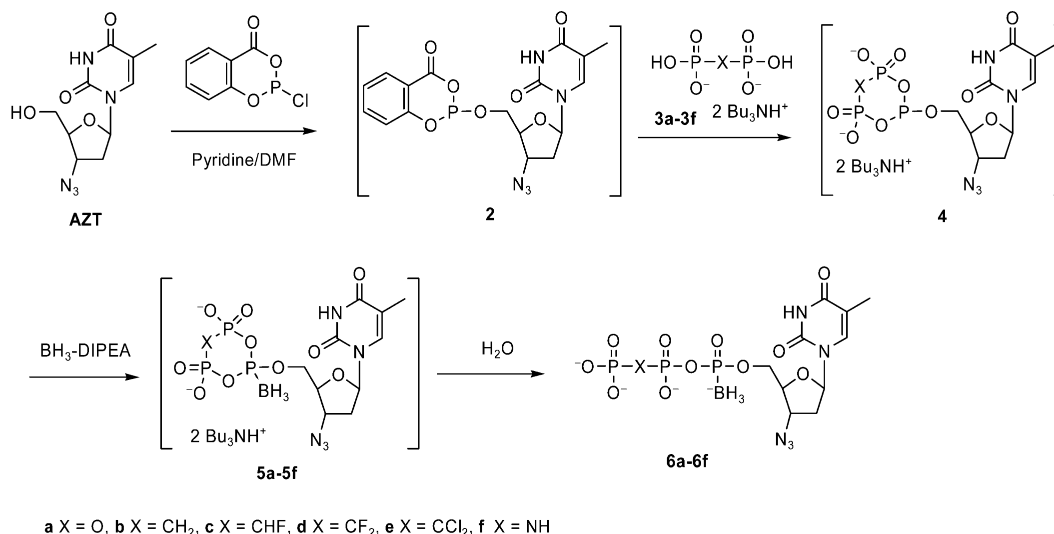


Figure 1. AZT P3Ms.

Chemistry

In this work, a very efficient synthetic procedure via an activated phosphite and a cyclized triphosphate intermediate[21] was adopted for preparation of AZT P3Ms **6b**−**6f** as shown in Scheme 1. The difference from the literature[21] was that methylenediphosphonate, halomethylenediphosphonates, and imidodiphosphate, instead of pyrophosphate, were employed as a coupling reagent. It was quickly concluded that the pyrophosphate analogues behaved similarly as pyrophosphate in this one-pot, multi-intermediate reaction after a few desired AZT P3Ms were successfully prepared. In the same manner as commercial pyrophosphate tributylammonium reagent, methylenediphosphonic acid (forming tributylammonium salt **3b** in the reaction mixture)

Scheme 1. Synthesis of AZT 5′-α-*P*-Borano-β,γ-bridge-modified Triphosphates



was used directly without further treatment. Commercially available dichloromethylenediphosphonic acid disodium salt was converted to its bis(tributylammonium) salt (**3e**) before the coupling reaction. Owing to its sensitivity to acid, imidodiphosphate sodium salt was converted to its tetrakis(tributylammonium) salt (**3f**). Fluoromethylenediphosphonic acid and difluoromethylenediphosphonic acid were prepared, respectively, by treatment with trimethylbromosilane of tetraisopropyl fluoromethylenediphosphonate and tetraisopropyl difluoromethylenediphosphonate, which were obtained by reaction of tetraisopropyl methylenediphosphonate with *N*-fluorobenzenesulfonimide in the presence of sodium bis(trimethylsilyl)amide. Initial preparations of tetraisopropyl difluoromethylenediphosphonate were conducted at low temperature and achieved only moderate yields. An improved procedure, including multiple, alternate addition of sodium bis(trimethylsilyl)amide and *N*-fluorobenzenesulfonimide at ambient temperature, gave tetraisopropyl difluoromethylenediphosphonate in more than 80% yield. Both fluoromethylenediphosphonic acid and difluoromethylenediphosphonic acid were converted to their bis(tributylammonium) salts (**3c** and **3d**), respectively.

Synthesis of the AZT P3Ms **6a**−**6f** is depicted in Scheme 1. Reaction of AZT (**1**) with 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one yielded the activated phosphite **2**, which was condensed with pyrophosphate analogues **3** to form the cyclic triphosphate intermediates **4**. It was critical to use the tributylammonium salt of pyrophosphate analogues free of water, since the intermediates **4** were very labile to moisture, leading to formation of undesired AZT 5′-*H*-phosphonate (not shown). Treatment of **4** with borane-diisopropylethylamine complex yielded, after hydrolysis, the AZT 5′-α-*P*-borano-β,γ-bridged-modified triphosphates **6b**−**6f**. Compound **6a** was prepared according to a known procedure.[21] We observed that in most cases a slight excess of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (1.2 equiv) and pyrophosphate analogue tributylammonium salts (1.3 equiv) would ensure the complete formation of the intermediates **2** and possibly **4**. The AZT P3Ms **6a**−**6e** were formed, respectively, in good yields (LCMS ~70−90% before purification). However, **6f** containing the β,γ-imido bridge was obtained in a lower yield, presumably owing to its lability to hydrolysis during purification.

Compounds **6a**, **6b**, **6d**, **6e**, and **6f** all consist of two diastereomers in approximately 1:1 ratio, which were purified first by anion-exchange HPLC and then separated on a reverse-phase HPLC, except for **6b** and **6f**, which were not separable on the reverse-phase HPLC. The separated diastereomers were designated as diastereomer I and diastereomer II, according to whether they were eluted earlier (I) or later (II) from the C18 reverse-phase HPLC. Determination of the stereochemistry of the diastereomers was not attempted. The diastereomers I tend to have much stronger inhibition of HIV-1 reverse transcriptase (Table 1) than the diastereomers II. Since the diastereomer I of **6a** was previously assigned as the α-R_P-borano diastereomer[24] and was a much more potent inhibitor than the other diastereomer, we tentatively designate the diastereomers I of **6a**, **6c**, **6d** and **6e** as the α-R_P-borano diastereomers. Unless specified, the diastereomer I means the R_p-isomer in this context. Compound **6c** consisted of four stereoisomers, and an HPLC separation yielded four fractions: the pure diastereomer I, the diastereomer II contaminated by other diastereomers, the diastereomer III contaminated by other diastereomers, and the pure diastereomer IV. The diastereomer I was first eluted and the diastereomer IV was last eluted.

Scheme 2 shows synthesis of AZT 5′-γ-*P*-substituted β,γ-(difluoromethylene)triphosphates **7a**−**i**. As in oligonucleotide synthesis,[27] the phosphite intermediate **4d** could be oxidized with iodine, and a subsequent treatment with a nucleophile yielded **7a**−**7i**. In the case of water as a nucleophile, the nucleophilic attack on any of the three phosphorus atoms of **4d** would result in the same product **7a**. When the nucleophile was not a hydroxyl anion or water, its attack on the different phosphorus atoms would result in the formation of two different products, α- or γ-substituted. Initially, we expected 7**b**−**7i** were either the α-*P*-substituted AZT P3Ms **10** (shown in Scheme 3) or a mixture of **10** and **7**. Surprisingly, only one diastereoisomer was obtained for each reaction, as indicated by ^{1}H, ^{19}F, and ^{31}P NMR.

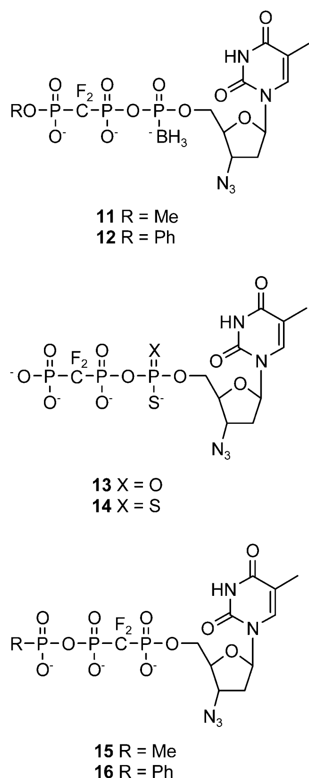
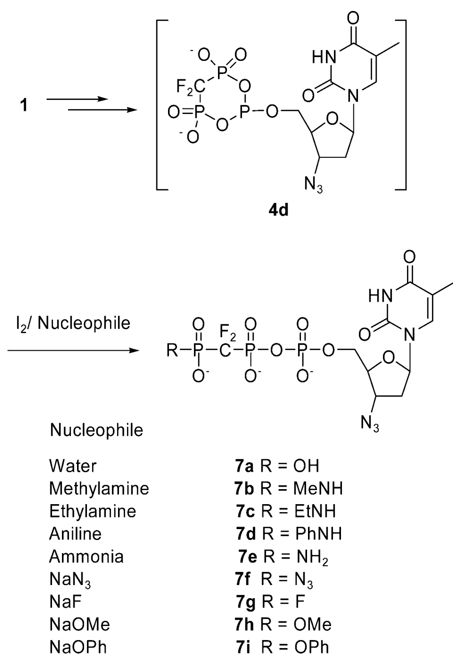


Figure 2. AZT P3Ms having α- and/or *γ-P*-modifications.

Scheme 2. Synthesis of AZT 5′-*γ*-*P*-Substituted *β*,*γ*-(Difluoromethylene)triphosphates



This largely excluded the possibility of the α-*P*-substitution that would yield two α-*P*-substituted diastereomers. A careful examination of ^{31}P NMR spectra revealed that compared to unsubstituted **7a**, compounds **7b**−**7i** maintained similar chemical shifts and the identical splitting pattern at the α-P, whereas their chemical shifts and/or splitting patterns of the *γ*-P are significantly different from that of **7a**. The results clearly support a *γ*-P substitution. Further evidence was obtained from ^{31}P NMR data of 7**g**, a *P*-fluoro AZT P3M. If fluorine was attached to the α-P, the coupling pattern would have been simple for the *γ*-P, most likely a sextet, while the α-P would be a double-double splitting. In fact, the α-P of **7g** displays a doublet coupling pattern, identical to that of **7a**, while the *γ*-P has a complicated, multiple splitting pattern (double−double−triple), instead of a sextet, indicating that fluorine is attached to the *γ*-P. From the above discussion, **7b**−**7i** can be clearly assigned as the *γ*-*P*-substituted AZT P3Ms.

To our knowledge, the formation of the *γ*-*P*-substitution described above has not been reported in the literature. After the structural determination of **7b**−**7i**, a possible reaction path was postulated as shown in Scheme 3. A nucleophile attacks on either the *β* or *β*′ of **8**, an oxidized intermediate of **4d**, and the resulting intermediate **9a** was subsequently hydrolyzed to give **7**. If a nucleophile attacked the α-P of **8**, an intermediate **9b** would be formed, which might be hydrolyzed to **10**. However, the absence of a detectable amount of **10** in the products suggests that a barrier to the formation of either **9b** or **10** exist. The observed regional selectivity can be ascribed to a favorable formation of **9a** over **9b**. The stronger electronegativity of difluoromethylene over oxygen may also contribute to the attack on the *β*-P or *β*′-P of **8**. The reaction mechanism remains to be further investigated.

Several other AZT P3Ms having diverse modifications on the triphosphate moiety were also synthesized, as shown in Figure 2. Compound **11** was prepared by reaction of **6d**-I/II with the corresponding methyl trifluoromethanesulfonate. For this reaction, only the monomethylated **11** was isolated, although dimethyl and trimethyl derivatives were also observed on LCMS. The results revealed that possible substitution at the α-P or *β*-P position of **6d** might have resulted in degradation of the triphosphate mimic moiety upon quenching reactions with water. This postulation was supported by fragments observed on LCMS. Thus, significant amounts of fragments 225, 239, and 345, corresponding to methyl (difluoromethylene)diphosphonate, dimethyl (difluoromethylene)diphosphonate, and AZT 5′-*P*-(OMe)-*P*-boranophosphinic acid (or N^3-methyl-AZT 5′-*P*-boranophosphinic acid), were observed. Compound **12** was synthesized in good yield from **6d**-I/II through a metaphosphate intermediate (most likely **5d**), which was reacted with phenol in the presence of triethylamine. Reaction of **4d** ($X = CF_2$) with elemental sulfur and subsequent treatment with water[28] yielded AZT 5′-α-*P*-thiotriphosphate (**13**). Compound **14** was obtained by treatment of **4d** first with sulfur and then with lithium sulfide.[28] Compounds **15** and **16** were prepared, respectively, by reactions of the tris(tributylammonium) salt of AZT 5′-(difluoromethylene)diphosphonate with methylphosphonic dichloride and phenylphosphonic dichloride by a similar procedure as that described for the preparation of nucleoside *γ*-substituted triphosphates.[29] AZT 5′-(difluoromethylene)diphosphonate was obtained by reaction of AZT 5′-*O*-tosylate[30] with the tetrabutylammonium salt of (difluoromethylene)diphosphonic acid. An excess of the diphosphonic acid salt (3−5 equiv) was necessary to suppress formation of a di-AZT diphosphonate (not shown).

Biology

Inhibition of HIV-1 Reverse Transcriptase. The effectiveness of the compounds as inhibitors of HIV-1

Scheme 3. Nucleophilic Reaction Pathways of the Oxidized Cyclic Triphosphate Intermediate **8**

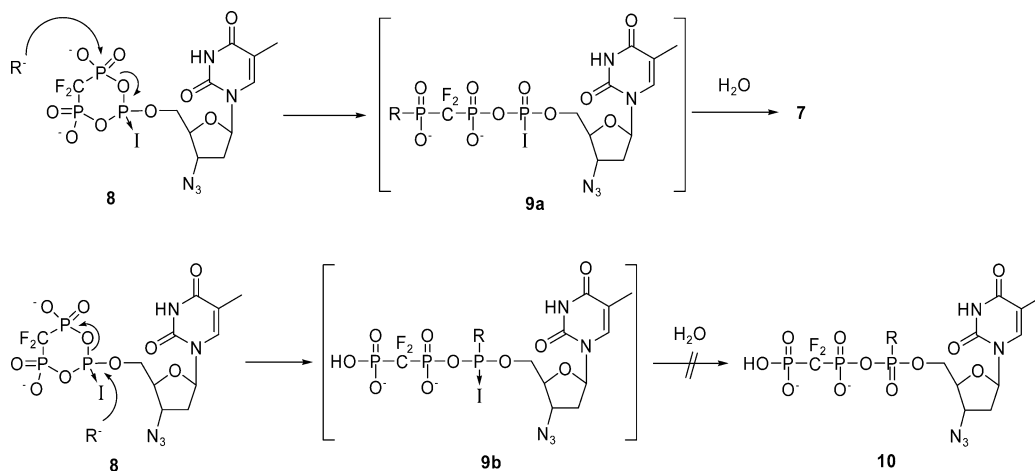


Table 1. Inhibition of HIV-1 Reverse Transcriptase by AZT P3Ms

compd[a]	R^1	R^2	X	X^1	X^2	K_i (μM)	rel inhibn[c]
AZT TP	OH	OH	O	O	O	0.0084	1
6a-I	BH_3	OH	O	O	O	0.0096	1.1
6d-I	BH_3	OH	O	O	CF_2	0.0095	1.1
6d-II	BH_3	OH	O	O	CF_2	4.56	543
6e-I	BH_3	OH	O	O	CCl2	0.093	11
6e-II	BH_3	OH	O	O	CCl2	0.074	8.8
6c-I	BH_3	OH	O	O	CHF	0.027	3.2
6c-IV	BH_3	OH	O	O	CHF	49%[b]	
6f-I/II	BH_3	OH	O	O	NH	0.105	12.5
6b-I/II	BH_3	OH	O	O	CH_2	17%[b]	4.94
7a	OH	OH	O	O	CF_2	0.041	4.88
7b	OH	NH_2	O	O	CF_2	0.234	5.56
7c	OH	NHMe	O	O	CF_2	0.124	14.8
7d	OH	NHEt	O	O	CF_2	0.415	49.4
7e	OH	NHPh	O	O	CF_2	0.254	30.2
7f	OH	N_3	O	O	CF_2	24%[b]	
7g	OH	F	O	O	CF_2	0.852	18.8
7h	OH	OMe	O	O	CF_2	0.597	71.1
7i	OH	OPh	O	O	CF_2	0.303	36.1
11-I/II	BH_3	OMe	O	O	CF_2	0.069	8.2
12-I/II	BH_3	OPh	O	O	CF_2	0.113	13.5
13-I/II	SH	OH	O	O	CF_2	0.090	10.7
14	SH	OH	S	O	CF_2	19%[b]	
15	OH	Me	O	CF_2	O	14.2%[b]	
16	OH	Ph	O	CF_2	O	11.5%[b]	

[a] Diastereomer I assigned to the AZT P3M having shorter HPLC retention time; Diastereomer II assigned to the AZT P3M having longer HPLC retention time; I/II refers to a mixture [b] Percentage inhibition at 0.5 μM of AZT P3M. [c] The inhibitory effect of AZTTP is designated as 1. Relative inhibition indicates the inhibitory effect of a compound relative to AZTTP. More potent, smaller the numbers are.

reverse transcriptase (RT) was determined using a fluorometric assay[31] and a poly(A) homopolymer as a template. Several compounds exhibited very potent inhibition, as shown in Table 1. Compound **6d**-I was equipotent to two of the most potent inhibitors of HIV-1 RT, AZTTP, and **6a**-I, with K_i values of 0.0095, 0.0084, and 0.0096 μM, respectively. Compounds **6c**-I, **6e**-I, and **6f**-I/II were also very active, with K_i values of 0.027, 0.093, and 0.105 μM, respectively. These AZT P3Ms have or contain an α-R_p-borano and a β,γ-bridge modification on the triphosphate moiety. Activities of the diastereomers II (containing an α-S_p-borano) including **6d**-II and **6c**-IV were varied but mostly decreased compared to those of the diastereomers I (containing an α-R_p-borano). An exception is **6e**-II, which had a similar activity as **6e**-I. Even with two modifications on the triphosphate moiety, a few AZT P3Ms still maintained the same level of inhibition as the triphosphates of clinically used nucleoside reverse transcriptase inhibitors (NRTIs). However, compound **6b**-I/II, a mixture of diastereomers I and II, was among the least active AZT P3Ms of this work, with 17% inhibition at 0.5 μM, indicating a striking effect of β,γ-bridge modifications.

AZT 5′-β,γ-(difluoromethylene)triphosphate (**7a**), the 5′-γ-*P*-substituted AZT P3Ms **7b**−**7i**, **11**, and **12** exhibited varied inhibitory effects. Except for **7f**, which was a weak inhibitor, **7a** and the other γ-substituted AZT P3Ms **7b-e**, **7g**-I containing γ-*P*-F, γ-*P*-NH_2, γ-*P*-OMe, γ-*P*-OPh, γ-*P*-NHMe, γ-*P*-NHEt, and γ-*P*-NHPh all showed significant activities with K_i values in a range of 0.04−0.85 μM. Positive effects of the γ-*P*-substituents on the inhibition were not observed, but certain tolerance of the reverse transcriptase to the γ-*P*-substituents was clearly demonstrated. γ-*P*-Substituted compounds **11** and **12** containing α-R_p/S_p-borano exhibited significantly stronger inhibitory effects than **7h** and **7i**, which clearly indicates a positive effect of the α-*P*-borano group. Compounds **13**, containing α-R_p/S_p-thio showed slightly higher K_i than **7a** (0.090 vs 0.041 μM), was still a potent inhibitor, whereas **14**, containing the α-*P*-dithio, was a much weaker inhibitor. Compounds **15** and **16**, which contain an α,β-difluoromethylene bridge in the triphosphate moiety, were the least active inhibitors of this work.

Serum and Cell Extract Stability. Serum stability of selected AZT P3Ms was assessed in fetal calf serum at 37 °C following a published procedure.[20] The most active AZT P3M **6d**-I of this work also demonstrated satisfactory serum stability (Table 2), having a half-life of more than 48 h relative to a half-life of 2 h that AZTTP and TTP had under the assay conditions used. Several other AZT P3Ms containing α-*P*-borano and β,γ-bridge modifications all showed satisfactory stability in serum, with half-lives in the range 36 h to more than 48 h. As expected, AZT 5′-α-*P*-boranotriphosphate (**6a-I** and **6a-**II) had only moderately higher half-life (6 h)

Table 2. Serum and Cell Extract Stability of AZT P3Ms

compd	half-life (h)	
	serum	cell extract
6d-I	>48	>48
6d-II	>48	>48
6e-I	45	>48
6e-II	>48	>48
6f-I/II	36	>48
6a-I	6	NT
6a-II	6	NT
AZTTP	2	NT
TTP	2	NT
ATP	2	0.25

than the NTPs. Compounds **6d**, **6e**, and **6f** were also subjected to a cell extract stability assay using CEM cells. The results show that all the AZT P3Ms had half-lives of more than 48 h relative to a half-life of 0.25 h for ATP.

Discussion

Substitution of borano for hydroxyl at the α-P position of triphosphate is crucial in maintaining and enhancing HIV-1 RT inhibition, leading to the AZT P3M **6d**-I, which is more potent than its α-*P*-hydroxyl analogue **7a** and equipotent to AZTTP. A borane attached to the α-phosphorus of a triphosphate maintains a negative charge but is not able to complex with metal ions, since it lacks a lone pair of electrons. A recent publication[24] suggested that only one coordinating oxygen at the α-P position be required for formation of a magnesium−triphosphate complex in the catalytic site of HIV-1 RT, and the other nonbridging oxygen may be replaced by a suitable substituent. Consistent with this, the substitution of a thiol for hydroxyl at the α-P position yielded the active AZT P3M **13**. Sulfur has properties more similar to oxygen than does borane, and consequently, **13** has a similar activity as **7a**. It is very interesting to note that **6d**-I is several fold more active than **7a** and **13**, suggesting an unusual role for the α-R_p-borano in the inhibition of the viral DNA synthesis mediated by HIV-1 RT. Under physiological conditions, P−OH, P−SH, and P−BH_3 all are negatively charged, and the negative charge is likely polarized toward sulfur in the case of α-P(=O)S^-, whereas the charge is polarized to oxygen in the case of α-P(=O)BH_3^-.[32] It seems that the electron distribution is one of factors governing the activity, since the least electronegative borano effects the strongest inhibition, while more electronegative oxygen and sulfur led to less active **7a** and **13**. A previous work[24] has shown that AZT 5′-α-R_p-boranotriphosphate had a similar binding affinity as AZTTP in the HIV-1 RT catalytic site, but its incorporation efficiency increased 3−9-fold. According to the results from the previous work[24] and this work, we surmise that a complex involving the α-nonbridging oxygen of P(=O)BH_3^- and a magnesium ion is stronger than the complex involving the α-nonbridging oxygen of P(=O)O^- and a magnesium ion, which facilitates the breakage of the P_α−O−P_β bond in the α-*P*-BH_3 analogue, resulting in more efficient incorporation for **6d**-I compared to **7a**. The effects of the α-*P*-borano on the binding in the catalytic site and on the P_α−O−P_β bond cleavage remain to be further explained. Recent work on suppression of dideoxynucleotide resistance by K65R HIV RT and Q151M HIV RT gives useful insight into the binding and incorporation of ddN 5′-α-*P*-boranotriphosphates.[33,34] Introduction of the second sulfur at the α-P position led to a weakly active compound **14**, which may be explained by a weakened magnesium−triphosphate complex. A few nucleoside α-*P*-alkyl triphosphates have been synthesized previously in other laboratories and found to be only moderately active.[35] It seems that for potent inhibition of HIV-1 RT, a preferred substitute for the α-*P*-hydroxyl should be ionizable to an anion under physiological conditions as is α-*P*-borane or α-*P*-thio.

As shown in Table 1, compounds **6a**−**6f** exhibited varied activities from very potent to almost inactive. The only structural difference among them is the β,γ-bridge modification. Several factors including size, polarity, and electronegativity may affect the activity. Since all these bridge modifications are relatively small, size does not appear to be a predominant factor. In fact, the bulkier bridge CCl_2 led to a much more potent inhibition than the smaller CH_2. It seems that the inhibitory effects correlate well with the electronegativity of the bridge modifications. It appears that the more electronegative atom at the β,γ-bridge position leads to the lower K_i with an inhibitory order O ∼ CF_2 > CHF > CCl_2 ∼ NH ≫ CH_2. This order is roughly the order of the last ionizable proton's pK_a values of adenosine 5′-β,γ-bridge-modified triphosphates: CF_2 (6.7) < CCl_2 (7.0) ≈ O (7.1) < CHF (7.4) < NH (7.7) < CH_2 (8.4).[14] It seems that size of the bridge modifications becomes more important when the bridge modifications have similar electronegativity. Dichloromethylene and oxygen have similar electronegativities, as reflected by the pK_a values shown above, but the larger size of dichloromethylene may have made **6e** less active than **6a**-I. Less electronegative fluoromethylene led to a more active inhibitor **6c**-I than **6e** containing the more electronegative dichloromethylene. A likely postulation is that the electronegativity and size of the bridge modifications may affect the ability of a P3M to form a complex with a magnesium ion, thereby influencing the binding affinity of the P3M in the catalytic site and subsequently the substrate property of the corresponding AZT P3Ms. In addition, more electronegative bridge modifications may also facilitate the departure of the bridge-modified diphosphate.

Conclusion

A series of novel AZT P3Ms have been synthesized, and practical synthetic and purification procedures have been developed. A number of AZT P3Ms exhibited very potent inhibition of HIV-1 RT and satisfactory stability to serum and cell extracts. The SAR presented here provides the guidance for future NP3M design. As can be seen, the most promising P3M of this work is 5′-α-R_p-borano-β,γ-(difluoromethylene)triphosphate (αB-β,γ-CF_2TP). The importance of this P3M is not only the high potency it leads to when attached to AZT, but also its favorable biological stability. This P3M is also anticipated to be useful when attached to other prospective nucleosides toward antiviral drugs. Compared to all the P3Ms known to date, αB-$\beta,\gamma CF_2$TP is the only one having two modifications that can render NP3Ms with both high potency of inhibition and dramatically enhanced biological stability relative to NTPs and NP3Ms

containing one modification. The most potent compound, **6d**-I, of this work is equipotent to AZTTP in the HIV-1 RT assay and at least 20-fold more stable than AZTTP in serum and cell extract assays. Therefore, for the first time, a highly active and stable NP3M has been identified. Further biological evaluation of **6d**-I clearly is justified and is underway. Considering the physical properties related to polarity and negative charges, an effective drug delivery approach may be required for the AZT P3Ms to be useful anti-HIV agents. Significant progress toward AZT P3M prodrugs has been made in these laboratories and will be reported in due time.

Experimental Section

^{1}H NMR spectra were recorded on a Varian Mercury 300 NMR spectrometer. Tetramethylsilane was used as internal reference for ^{1}H NMR, 85% phosphoric acid as external reference for ^{31}P NMR, and CF_3Cl as external reference for ^{19}F NMR. Pyrophosphate tributylammonium salt, dichloromethylenediphosphonate disodium salt, imidodiphosphate sodium salt, and methylenediphosphonic acid were purchased from Sigma and used with or without further treatment as indicated in the corresponding experiments. Anhydrous solvents purchased from Aldrich were used directly in the reactions without further treatment.

Purification of AZT P3Ms. AZT P3Ms were purified by anion exchange (AX) chromatography using a 10 × 160 mm Mono Q column (Pharmacia). Initial conditions were typically 0−35 mM NaCl. A linear elution gradient was typically initiated at 0−35 mM NaCl and terminated at 350 mM to 1 M NaCl in two to three column volumes at 6.5 mL/min. A constant concentration of 50 mM Tris, pH 8, was maintained throughout the purification. Fractions containing the target compounds were collected and desalted by reversed-phase HPLC (RP-HPLC) using a Luna C18 250 × 21 mm column (Phenomenex) with a flow rate of 10 mL/min. Elution gradients were generally from 0−20% to 95% methanol in 20−60 min at a constant concentration of triethylammonium acetate (50 mM). The AZT P3Ms of this work, unless specified, were purified using both the anion-exchange HPLC and then RP-HPLC. Fractions containing the desired AZT P3Ms were collected and lyophilized to give AZT P3M as mixed sodium and triethylammonium salts. Compounds that did not require anion exchange-HPLC purification were purified by RP-HPLC only, using the same conditions as described above. In these cases, the final AZT P3M products were triethylammonium salts. Yields of all AZT P3M products of this work were calculated on the basis of UV absorbance.

LCMS and HPLC Analysis of AZT P3Ms. Mass spectra and purity of the AZT P3Ms were obtained using on-line HPLC−mass spectrometry on a ThermoFinnigan (San Jose, CA) Deca XP plus. A Phenomenex Luna C18(2) or C5, 75 × 2 mm, 3-μm particle size was used for RP-HPLC. A 0−50% linear gradient (15 min) of acetonitrile in 10 mM *N*,*N*′-dimethyl-*n*-hexylammonium acetate, pH 7, was performed in series with mass spectra detection in the negative ionization mode. Nitrogen gas and a pneumatic nebulizer were used to generate the electrospray. The mass range of 150−1500 was typically sampled. All the purified AZT P3Ms of this work were subjected to the LCMS analysis as described above.

Preparation of Tetraisopropyl (Difluoromethylene)-diphosphonate and Tetraisopropyl (Fluoromethylene)-diphosphonate. Sodium bis(trimethylsilyl)amide (NaHMDS, 1.0 M in THF, 28.7 mL) was added to a stirred solution of tetraisopropyl methylenediphosphonate (4.5 g, 13.07 mmol) and *N*-fluorobenzenesulfonimide (NFSi, 9.89 g, 31.36 mmol) in anhydrous THF (20 mL) at −78 °C under argon. The reaction mixture was stirred at −78 °C for 1 h, quenched with saturated aqueous ammonium chloride (20 mL), warmed to room temperature, and diluted with ether (60 mL). The organic layer was separated and the aqueous layer was extracted with ether (60 mL). Combined organic layer was washed with 10% aqueous sodium bicarbonate and then with brine, dried over sodium sulfate, and concentrated. Chromatography on silica gel with 5−30% ethyl acetate in hexanes gave 2.18 g of tetraisopropyl (difluoromethylene)diphosphonate and 0.32 g of tetraisopropyl (fluoromethylene)diphosphonate. CF_2-product: ^{1}H NMR ($CDCl_3$) δ 1.39 (2d, J = 3.3 Hz, 4Me, 12H), 1.41 (2d, J = 3.3 Hz, 4Me, 12H), 4.92 (m, Me_2CH, 4H); ^{31}P NMR δ 3.41 (t, J = 86.9 Hz); ^{19}F NMR δ −122.10 (t, J = 87.2 Hz). CF-product: ^{1}H NMR ($CDCl_3$) δ 1.37 (m, 8Me, 24H), 4.85 (m, Me_2CH, 4H); ^{31}P NMR δ 10.72 (t, J = 63.6 Hz). ^{19}F NMR ($CDCl_3$) δ −225.9 (dt, $^{2}J_{F-H}$ = 43.6 Hz, $^{2}J_{F-P}$ = 63.0 Hz, CHF).

Improved Procedure for Preparation of Tetraisopropyl (Difluoromethylene)diphosphonate. NaHMDS (1.0 M, 235 mL) and anhydrous THF (250 mL solution) in an addition funnel and NFSi (82 g, 260 mmol) in THF (250 mL solution) in another addition funnel were added alternately and in 10 portions each, starting with NaHMDS, to stirred tetraisopropyl methylenediphosphonate (27 g, 76 mmol) in a three necked, 1 L flask under argon. After each addition a 1-min interval was allowed. A heavy precipitate occurred after the second and latter round of additions. A pale-amber color developed and substantial heat was given off, so the reaction mixture was near reflux during the additions. After the additions, the mixture was stirred until it reached room temperature, cooled to −78 °C, quenched with saturated aqueous ammonium chloride (100 mL), and diluted with 200 mL of diethyl ether. The mixture was warmed to room temperature and the organic layer was separated. The aqueous layer was extracted with ether (2 × 100 mL). Combined organics were washed with 5% citric acid/brine, bicarbonate, and brine; dried over magnesium sulfate; and concentrated. The resulting syrup was loaded on a silica gel column and eluted with 10% ether in hexanes (1 L), 20% ether in hexanes (1 L), and finally 50% ether/hexanes. Fractions containing pure product (TLC: eluted with ether/hexanes 3:1, stained with $KMnO_4$) were collected and concentrated to give 24.0 g (81%) of (difluoromethylene)diphosphonate as a colorless liquid. ^{1}H, ^{31}P, and ^{19}F NMR data are identical to those shown above.

Preparation of Difluoromethylenediphosphonic Acid Bis(tributylammonium) Salt (3d). Trimethylbromosilane (4.17 mL, 31.58 mmol) was added dropwise to a stirred solution of tetraisopropyl (difluoromethylene)diphosphonate (2.0 g, 5.26 mmol) in anhydrous acetonitrile (30 mL). The resulting solution was stirred at 40−42 °C for 24 h under argon, concentrated to dryness, and coevaporated with anhydrous acetonitrile. The residue was redissolved in an acetonitrile/water mixture and then coevaporated with DMF. The residue was dissolved in a solution of tributylamine (1.93 g, 2.48 mL, 10.43 mmol) in DMF (20 mL), concentrated to dryness, and coevaporated with anhydrous DMF three times. The residue was dried at 40 °C under vacuum for 4 h to give a slightly amber-colored residue (3.35 g): ^{1}H NMR (D_2O) δ 0.779 (t, J = 7.5 Hz, Me), 1.23 (m, $-CH_2Me$), 1.52 (m, $C-CH_2-CMe$), 2.97 (t, J = 8.1 Hz, NCH_2-)); ^{31}P NMR δ 3.41 (t, J = 83.5 Hz); ^{19}F NMR δ −121.47 (t, J = 83.3).

3′-Azido-3′-deoxythymidine 5′-α-P_I-Borano-β,γ-(difluoromethylene)triphosphates (6d). 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (2.18 g, 10.8 mmol) in DMF (9 mL) was added to a stirred solution of 3′-azido-3′-deoxythymidine (2.41 g, 9.0 mmol) in anhydrous DMF (18 mL) and pyridine (4.5 mL) at 0 °C under argon. The reaction mixture was stirred at room temperature for 2 h and an additional amount of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (0.37 g, 1.8 mmol) was added. The reaction mixture was stirred for 5 h at room temperature and then cooled with ice. Tributylamine (5.4 mL, 49.5 mmol) was added, followed by addition of (difluoromethylene)diphosphonic acid bis(tributylammonium) salt (6.5 g, 11.1 mmol) in DMF (20 mL). The reaction mixture was stirred at room temperature for 3 h and cooled with ice. Borane−diisopropylethylamine complex (24 mL) was added, and the resulting mixture was stirred at room temperature overnight, cooled with ice, and quenched by slow addition of water (90 mL). The mixture was stirred at room temperature for 2 h, concentrated at room temperature under reduced pressure to

a small volume, diluted with water (300 mL), and extracted with ethyl acetate three times. HPLC purification as described in the general section yielded 4.82 mmol (53.6%) of **6d**-I/II (two diastereomers) as a colorless foam: ^{1}H NMR (D_2O) δ −0.3 to 0.9 (br, BH_3, 3H), 1.13 (t, J = 7.2 Hz, CH_3 of TEA, 6H), 1.81 (s, 5-Me, 3H), 2.33 (t, J = 5.7 Hz, H-2′, 2H), 3.04 (q, J = 7.2 Hz, CH_2 of TEA, 4H), 4.08 (m, H-4′, H-5′, 3H), 4.40 (m, H-3′, 0.5H), 4.48 (m, H-3′, 0.5H), 6.12, 6.13 (2t, J = 6.9 Hz, H-1′, 1H), 7.59 (s, H-6, 1H); ^{31}P NMR δ −4.09 to −1.86 (m, P_β), 4.20 (t, J = 74.2 Hz, 1/2P_γ), 4.68 (t, J = 74.2 Hz, 1/2P_γ), 83.3 (br, P_α).

The two diastereoisomers (α-P_{I} and α-P_{II}) were separated on reverse-phase HPLC under the conditions as described in the general section to give 3′-azido-3′-deoxythymidine α-P_{I}-borano-β,γ-(difluoromethylene)triphosphate (**6d**-I, the isomer that has shorter retention time is designated as diastereomer I) and 3′-azido-3′-deoxythymidine α-P_{II}-borano-β,γ-(difluoromethylene)triphosphate (**6d**-II, the isomer that has longer retention time is designated as diastereomer II). **6d**-I: ^{1}H NMR (D_2O) δ 0.14−0.56 (br, BH_3, 3H), 1.81 (d, J = 0.9 Hz, 5-CH_3, 3H), 2.32 (t, J = 5.4 Hz, H-2′, 2H), 4.02−4.11(m, H-4′, H-5′, 3H), 4.48−4.49 (m, H-3′, 1H), 6.13 (t, J = 7.2 Hz, H-1′, 1H), 7.60 (d, J = 1.2 Hz, H-6, 1H); ^{31}P NMR (D_2O) δ − 4.47 to − 3.99 (m, P_β), 4.37 (ddt, $^2J_{P-P}$ = 59.1 Hz, $^2J_{P-F}$ = 81.6 Hz, P_γ), 84.02 (br, P_α); ^{19}F NMR (D_2O) δ −119.86 (m, CF_2); MS *m/z* 538 (M − H)$^-$; HPLC analysis, 97.8% purity. **6d**-II: ^{1}H NMR (D_2O) δ 0.14−0.48 (m, BH_3, 3H), 1.81 (s, 5-CH_3, 3H), 2.31−2.35 (t, J = 4.8 Hz, H-2′, 2H), 4.03−4.12 (m, H-4′, H-5′, 3H), 4.40−4.41 (m, H-3′, 1H), 6.12 (t, J = 6.9 Hz, H-1′, 1H), 7.61 (d, J = 1.2 Hz, H-6, 1H); ^{31}P NMR (D_2O) δ − 5.52 to − 3.31 (m, P_β), 4.40 (ddt, $^2J_{P-P}$ = 59.6 Hz, $^2J_{P-F}$ = 81.6 Hz, P_γ), 85.73 (br, P_α); ^{19}F NMR (D_2O) δ −119.44 (ddt, $^2J_{F-P}$ = 81.3 Hz, $^2J_{F-P}$ = 81.3 Hz, $^2J_{F-P}$ 69.5 Hz, CF_2); MS *m/z* 538 (M − H)$^-$; HPLC analysis, 98.4% purity.

3′-Azido-3′-deoxythymidine 5′-α-*P*-Borano-*β*,*γ*-methylenetriphosphate (6b). 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (101 mg, 0.495 mmol) in anhydrous DMF (0.5 mL) was added via syringe to a stirred solution of 3′-azido-3′-deoxythymidine (134 mg, 0.5 mmol) in 1 mL of anhydrous DMF and 0.25 mL of anhydrous pyridine under argon. After stirring at room temperature for 1 h, tributylamine (0.3 mL) was added, followed by a mixture of methylenediphosphonic acid (88 mg, 0.495 mmol) and tributylamine (0.35 mL) in 0.5 mL of anhydrous DMF. The reaction mixture was stirred for 1 h, and 2 mL of borane−diisopropylethylamine complex was added. After stirring at room temperature for 6 h, the reaction mixture was cooled, quenched with water (5 mL), and stirred at room temperature for 3 h. Purification by HPLC yielded 91.45 μmol (18.3%) of **6b**-I/II (two diastereomers): ^{1}H NMR (D_2O) δ 1.76, 1.80 (2s, 2 isomers, 5-CH_3, 3H), 2.10−2.23 (m, H-2′, 2H), 2.30−2.34 (m, 2H, β,γ-CH_2, 2 isomers) 4.01−4.10 (m, H-4′, H-5′, H-5″, 3H), 4.39−4.46 (m, H-3′, 1H), 6.12 (apparent q, J = 7.1 Hz, H-1′, 1H), 7.59−7.60 (2s, 2 isomers, H-6, 1H); ^{31}P NMR (D_2O) δ 9.10−9.40 (dd, $^2J_{P-P}$ = 90 Hz, $^2J_{P-H}$, 15 Hz, P_β), 15.93 (dt, $^2J_{P-P}$ = 59.3 Hz, P_γ), 84.50 (m, P_α); MS *m/z* 502 (M − H)$^-$; HPLC analysis, 99.9% purity.

3′-Azido-3′-deoxythymidine 5′-(α-*P*-Borano-*β*,*γ*-fluoromethylene)triphosphate (6c). Trimethylbromosilane (0.70 mL, 5.28 mmol) was added dropwise to a stirred solution of tetraisopropyl (fluoromethylene)diphosphonate (320 mg, 0.88 mmol) in 1,2-dichloroethane (5 mL). The resulting solution was stirred at 40−42 °C for 24 h, and 3 mL of anhydrous toluene was added. The solution was concentrated to dryness and coevaporated with toluene once. The residue was redissolved in DMF (3 mL)/water (2 mL) and concentrated. The residue was dissolved in a mixture of DMF (2 mL) and tributylamine (0.42 mL, 1.76 mmol) and then concentrated to dryness. The residue was coevaporated with anhydrous DMF twice. The resulting residue was dried in a vacuum oven at 30 °C overnight to give (fluoromethylene)diphosphonic acid bis(tributylammonium) salts as a slightly amber residue (460 mg).

To a stirred solution of 3′-azido-3′-deoxythymidine (78 mg. 0.29 mmol) in anhydrous DMF (1 mL) and pyridine (0.2 mL) at 0 °C under argon was added a solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (77 mg, 0.38 mmol). The reaction mixture was stirred at room temperature for 1 h and cooled with ice. Tributylamine (0.18 mL) was added, followed by addition of (fluoromethylene)diphosphonic acid bis(tributylammonium) salts (250 mg, 0.43 mmol) in DMF (0.6 mL). The reaction mixture was stirred at room temperature for 1 h and cooled with ice. Borane−diisopropylethylamine complex (1.20 mL) was added, and the resulting mixture was stirred at room temperature for 6 h, cooled with ice, and quenched by slow addition of water (3 mL). The mixture was stirred at room-temperature overnight, diluted with water (10 mL), and extracted with chloroform three times. HPLC purification gave **6c** in four portions: (a) the pure isomer **6c**-I, 7.20 μmol; (b) the isomer **6c**-II (containing other isomers), 15.82 μmol; (c) the isomer **6c**-III (containing other isomers), 11.06 μmol; (d) the pure isomer **6c**-IV, 4.89 μmol. The total yield was 13.4%. **6c**-I: MS *m/z* 520 (M − H)$^-$; HPLC analysis, 99.4% purity. **6c**-IV: MS *m/z* 520 (M − H)$^-$; HPLC analysis, 99.6% purity. **6c**-II: ^{1}H NMR (D_2O) δ 0.1−0.6 (br, BH_3, 3H), 1.78 (s, 5-CH_3, 3H), 2.30−2.38 (m, H-2′, 2H), 3.98−4.17 (m, H-4′, H-5′, 3H), 4.36−4.45 (m, H-3′, 1H), 6.12 (apparent q, J = 7.0 Hz, H-1′, 1H), 7.60 (s, H-6, 1H); ^{31}P NMR (D_2O) δ 1.79 (m, P_β), 9.65 (dq, $^2J_{P-P}$ = 14.3 Hz, $^2J_{P-F}$ = 61.2 Hz, P_γ), 83.80 (m, P_α); ^{19}F NMR (D_2O) δ −211.3 (m, CHF).

3′-Azido-3′-deoxythymidine 5′-(α-*P*-Borano-*β*,*γ*-dichloromethylene)triphosphate (6e). An aqueous solution of (dichloromethylene)diphosphonic acid disodium salt (1.0 g, 3.46 mmol) was loaded on a column of DOWEX 50WX8−100 ion-exchange resin and eluted with water. Tributylamine (1.65 mL, 6.92 mmol) was added and the mixture was shaken vigorously. The resulting solution was concentrated to dryness and coevaporated with anhydrous DMF three times. The residue was dried under vacuum overnight to give (dichloromethylene)diphosphonic acid bis(tributylammonium) salt as a colorless semisolid.

To a stirred solution of 3′-azido-3′-deoxythymidine (89 mg. 0.33 mmol) in anhydrous DMF (1 mL) and pyridine (0.2 mL) at 0 °C under argon was added a solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (83 mg, 0.41 mmol). The reaction mixture was stirred at room temperature for 1 h and cooled with ice. Tributylamine (0.2 mL) was added, followed by addition of (dichloromethylene)diphosphonic acid bis(tributylammonium) salt (430 mg, 0.43 mmol) in DMF (1 mL). The reaction mixture was stirred at room temperature for 1 h and cooled with ice. Borane−diisopropylethylamine complex (1.32 mL) was added and the resulting mixture was stirred at room temperature for 6 h, cooled with ice, and quenched by slow addition of water (3 mL). The mixture was stirred at room temperature overnight, diluted with water (10 mL), and extracted with chloroform three times. HPLC purification gave 14.51 μmol (4.4%) of **6e**-I and 19.76 μmol (6.0%) of **6e**-II. **6e**-I: ^{1}H NMR (D_2O) δ −0.1 to 0.7 (br, BH_3, 3H), 1.80 (s, 5-CH_3, 3H), 2.25−2.43 (m, H-2′, 2H), 4.01−4.26 (m, H-4′, H-5′, 3H), 4.49−4.58 (m, H-3′, 1H), 6.11 (t, J = 7.0 Hz, H-1′, 1H), 7.61 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −2.11 (apparent q, $^2J_{P-P}$ = 19.2 Hz, P_β), 9.33 (d, $^2J_{P-P}$ = 18.4 Hz, P_γ), 83.68 (m, P_α); MS *m/z* 570 (M − H)$^-$; HPLC analysis, 96.8% purity. **6e**-II: MS *m/z* 571 (M − H)$^-$; HPLC analysis, 89.0% purity.

3′-Azido-3′-deoxythymidine 5′-α-*P*-Borano-*β*,*γ*-imidotriphosphate (6f). Commercially available imidodiphosphate sodium salt (1.0 g) in 10 mL of water was loaded on a column which was filled with 20 g of DOWEX 50W ×8−100 acidic resin that was thoroughly washed with water/pyridine and then water. The column was eluted with water. Fractions containing imidodiphosphate were collected and made alkaline with tributylamine (3.6 mL). The solution was concentrated at room temperature under reduced pressure, coevaporated with anhydrous DMF three times, and dried under vacuum overnight to give 2.80 g of imidodiphosphate tetrakis(tributylammonium) salt (**3f**) as a semisolid.

2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (93 mg, 0.46 mmol) in anhydrous DMF (1 mL) was added to a stirred solution of 3′-azido-3′-deoxythymidine (93 mg, 0.35 mmol) in anhydrous DMF (1 mL) and anhydrous pyridine (0.2 mL) at 0 °C under argon. The reaction mixture was stirred at room temperature for 1 h and then cooled with ice. Tributylamine (0.2 mL) was added, followed by addition of imidodiphosphonic acid tetrakis(tributylammonium) salt (440 mg, 0.48 mmol) in DMF (1 mL). The reaction mixture was stirred at room temperature for 1 h and cooled with ice. Borane−diisopropylethylamine complex (1.4 mL) was added, and the resulting mixture was stirred at room temperature for 6 h, cooled with ice, and quenched by slow addition of water (3 mL). The mixture was stirred at room temperature overnight, concentrated at room temperature under reduced pressure to a small volume, diluted with water, and extracted with ethyl acetate three times. HPLC purification as described in the general section yielded 5.75 μmol (1.6%) of **6f**-I/II (two diastereomers): ^{1}H NMR (D_2O, TEA^+ form) δ 1.80 (s, 5-CH_3, 3H), 2.29−2.38 (m, H-2′, 2H), 3.99−4.13 (m, H-4′, H-5′, 3H), 4.36−4.46 (m, H-3′, 1H), 6.13 (t, J = 6.9 Hz, H-1′, 1H), 7.59 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −9.73 (m, P_β), −0.55, −0.42 (2d, 2 isomers, $^2J_{P-P}$ = 9.8 Hz, P_γ), 83.80 (m, P_α); MS *m/z* 503 (M − H)$^-$; HPLC analysis, 90.1% purity.

A General Procedure for Preparation of AZT 5′-γ-Substituted-β,γ-(difluoromethylene)triphosphates 7a−7i. A freshly prepared solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (0.165−0.5 mmol, 1.0−1.2 equiv) in anhydrous DMF (0.5−1.0 mL) was added to a stirred solution of 3′-azido-3′-deoxythymidine (40−134 mg, 0.15−0.5 mmol) in anhydrous DMF (0.5−1.0 mL) and anhydrous pyridine (0.1−0.25 mL) at 0 °C under argon. After stirring at room temperature for 1 h, tributylamine (0.21 to 0.3 mL) was added, followed by addition of difluoromethylenediphosphonic acid bis(tributylammonium) salt (0.18−0.65 mmol, 1.2−1.3 equiv) in anhydrous DMF (0.5−1.0 mL). The resulting mixture was stirred for 1 h and cooled with ice, and iodine (2 equiv, 0.3−1.0 mmol) was added. After stirring at room temperature for 1 h, a nucleophilic reagent (neat or in 0.5−1.0 mL of DMF) was added. The reaction mixture was stirred at room temperature for 1−2 h, cooled with ice, quenched with water, and stirred at room temperature for 30 min. HPLC purification yielded **7a**−**7i**, respectively. The products were purified on anion-exchange and reverse-phase HPLC as described in the general section.

3′-Azido-3′-deoxythymidine 5-(β,γ-difluoromethylene)triphosphate (7a). A freshly prepared solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (101.27 mg, 0.495 mmol) in anhydrous DMF (0.5 mL) was added via syringe to a stirred solution of 3′-azido-3′-deoxythymidine (133.63 mg, 0.495 mmol) and anhydrous pyridine (0.25 mL) in 1 mL of anhydrous DMF under argon. After stirring at room temperature for 1 h, tributylamine (0.3 mL) was added, followed by a solution of bis(tributylammonium) difluoromethylenediphosphate (coevaporated with anhydrous pyridine two times) in 0.5 mL of anhydrous DMF. The mixture was stirred for 1 h and 253.8 mg (1 mmol) of iodine was added. After stirring at room temperature for 3.5 h, the reaction was quenched with 5 mL of water and stirred at room temperature for 4 h. HPLC purification afforded 22.55 μmol (4.6%) of **7a**: ^{1}H NMR (D_2O) δ 1.34 (t, CH_3, 3H), 1.98 (s, 5-CH_3, 3H), 2.32−2.37 (m, H-2′, 1H), 2.80−2.82 (m, H-2′, 1H), 3.28−3.30 (m, 5′CH_2, 1H), 4.06−4.08 (m, H-4′, H-5″ 2H), 4.40−4.43 (m, H-3′, 1H), 6.10−6.15 (dd, J = 6.9, 13.8 Hz, H-1′, 1H), 7.59 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −10.77 (d, $^2J_{P-P}$ = 75.3 Hz, P_α), −3.95 (m, P_β), 4.16 (dt, J_{P-F} = 144 Hz, 2J_{P-P} = 75 Hz, P_γ); ^{19}F NMR (D_2O) −119.92 (ddd, $^2J_{F-P}$ = 84 Hz, $^2J_{F-P}$ = 92 Hz, $^3J_{F-F}$ = 8 Hz, CF_2); MS *m/z* 540 (M − H)$^-$; HPLC analysis, 98.3% purity.

3′-Azido-3′-deoxythymidine 5′-γ-*P*-Amino-β, γ-(difluoromethylene)triphosphate (7b). 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (66.8 mg, 0.33 mmol) in anhydrous DMF (1.0 mL) was added via syringe to a solution of 3′-azido-3′-deoxythymidine (80.2 mg, 0.30 mmol) in 1.0 mL of anhydrous DMF and 0.10 mL of anhydrous pyridine at 0 °C under argon. After stirring at room temperature for 1 h, tributylamine (0.42 mL) was added, followed by a solution of (difluoromethylene)diphosphonic acid bis(tributylammonium) salt (209.6 mg, 0.36 mmol) in anhydrous DMF (1.0 mL). The mixture was stirred at room temperature for 1 h and cooled to 0 °C, and iodine (152.2 mg, 0.60 mmol) was added. After stirring at room temperature for 1 h, ammonia (5 mL, freshly condensed) was added, and stirring was continued at room temperature for 1 h. Excess ammonia was evaporated and the solution was then diluted with water. HPLC purification gave 79.07 μmol (26.4%) of **7b**: ^{1}H NMR (D_2O) δ 1.78 (s, 5-CH_3, 3H), 2.32−2.36 (m, H-2′, 2H), 4.05−4.08 (m, H-4′, H-5′, 3H), 4.41−4.45 (m, H-3′, 1H), 6.13 (t, J = 7.0 Hz, H-1′, 1H), 7.61 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −10.40 (d, $^2J_{P-P}$ = 30.0 Hz, P_α), −4.04 (m, P_β), 9.50 (dt, $^2J_{P-P}$ = 59.2 Hz, $^2J_{P-F}$ = 79.3 Hz, P_γ); ^{19}F NMR (D_2O) δ −121.2 (apparent q, $^2J_{F-P}$ = 82.3 Hz, CF_2); MS *m/z* 539 (M − H)$^-$; HPLC analysis, 100% purity.

3′-Azido-3′-deoxythymidine 5′-β,γ-Difluoromethylene-γ-*P*-methylaminotriphosphate (7c). Reaction using 133.6 mg (0.5 mmol) of 3′-azido-3′-deoxythymidine and 2.0 M methylamine in THF (5 mL) as nucleophilic reagent yielded, after HPLC purification, 0.126 mmol (25.3%) of **7c**: ^{1}H NMR (D_2O) δ 1.97 (s, 5-CH_3, 3H), 2.32−2.42 (m, H-2′, 2H), 2.74−2.85 (m, H-5′, 1H), 3.22−3.25 (m, H-5″, 1H), 3.55 (s, CH_3, 3H), 4.04−4.06 (m, H-4′, 1H), 4.40−4.44 (m, H-3′, 1H), 6.09−6.14 (dd, J = 6.9, 13.8 Hz, H-1′, 1H), 7.56 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −10.45 (d, $^2J_{P-P}$ = 78 Hz, P_α), −3.89 (m, P_β), 8.88 (dt, J_{P-F} = 189 Hz, $^2J_{P-P}$ = 78 Hz, P_γ); ^{19}F NMR (D_2O) −119.22 (ddd, $^2J_{F-P}$ = 82 Hz, $^2J_{F-P}$ = 90 Hz, $^3J_{F-F}$ = 8 Hz, CF_2); MS *m/z* 553 (M − H)$^-$; HPLC analysis, 92.6% purity.

3′-Azido-3′-deoxythymidine 5′-β,γ-Difluoromethylene-γ-*P*-ethylaminotriphosphate (7d). Reaction using 133.6 mg (0.5 mmol) of 3′-azido-3′-deoxythymidine and 2.0 M ethylamine in THF (2 mL) as nucleophilic reagent yielded, after HPLC purification, 95.42 μmol (19.1%) of **7d**: ^{1}H NMR (D_2O) δ 1.05 (t, CH_3, 3H), 1.76, (s, 5-CH_3, 3H), 2.19−2.35 (m, H-2′, 2H), 2.77−2.88 (m, CH_2), 4.04−4.06 (m, H-4′, H-5′, H-5″, 3H), 4.40−4.44 (m, H-3′, 1H), 6.09−6.14 (dd, J = 6, 15 Hz, H-1′, 1H), 7.58 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −10.45 (d, $^2J_{P-P}$ = 78 Hz, P_α), −3.89 (m, P_β), 8.88 (dt, J_{P-F} = 189 Hz, $^2J_{P-P}$ = 78 Hz, P_γ); ^{19}F NMR (D_2O) −119.39 (ddd, $^2J_{F-P}$ = 81 Hz, $^2J_{F-P}$ = 81 Hz, $^3J_{F-F}$ = 12 Hz, CF_2); MS *m/z* 567 (M − H)$^-$; HPLC analysis, 96.4% purity.

3′-Azido-3′-deoxythymidine β,γ-Difluoromethylene-5′-γ-*P*-phenylaminotriphosphate (7e). Reaction using 103.8 mg (0.388 mmol) of 3′-azido-3′-deoxythymidine and aniline (0.55 mL, 3.88 mmol) as nucleophilic reagent yielded, after HPLC purification, 26.30 μmol (6.8%) of **7e**: ^{1}H NMR (D_2O) δ 1.72 (s, 5-CH_3, 3H), 2.18−2.22 (m, H-2′, 2H), 3.82−4.01 (m, H-4′, H-5′, 3H), 4.22−4.32 (m, H-3′, 1H), 6.03 (t, J = 6.3 Hz, H-1′, 1H), 6.7−6.78 (m, Ph, 1H), 6.97−7.03 (m, Ph, 4H), 7.45 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −10.10 (d, $^2J_{P-P}$ = 30.0 Hz, P_α), −4.14 (m, P_β), 2.56 (dt, $^2J_{P-P}$ = 58.0 Hz, $^2J_{P-F}$ = 80.0 Hz, P_γ); ^{19}F NMR (D_2O) δ −119.29 (t, $^2J_{F-P}$ = 83.0 Hz, CF_2); MS *m/z* 615 (M − H)$^-$; HPLC analysis, 92.5% purity.

3′-Azido-3′-deoxythymidine 5′-γ-*P*-Azido-β,γ-(difluoromethylene)triphosphate (7f). Reaction using 40.09 mg (0.15 mmol) of 3′-azido-3′-deoxythymidine and sodium azide (97.5 mg, 1.5 mmol) as nucleophilic reagent yielded, after HPLC purification, 29.50 μmol (19.7%) of **7f**: ^{1}H NMR (D_2O) δ 1.74 (s, 5-CH_3, 3H), 2.31−2.35 (m, H-2′, 2H), 4.01−4.10 (m, H-4′, H-5′, 3H), 4.41−4.46 (m, H-3′, 1H), 6.11 (t, J = 6.8 Hz, H-1′, 1H), 7.59 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −10.40 (d, $^2J_{P-P}$ = 30.1 Hz, P_α), −6.14 (m, P_β), 4.08 (dt, $^2J_{P-P}$ = 63.3 Hz, $^2J_{P-F}$ = 86.1 Hz, P_γ); ^{19}F NMR (D_2O) δ −120.2 (t, $^2J_{F-P}$ = 84.2 Hz, CF_2); MS *m/z* 565 (M − H)$^-$; HPLC analysis, 89.3% purity.

3′-Azido-3′-deoxythymidine 5′-β,γ-Difluoromethylene-γ-*P*-fluorotriphosphate (7g). Reaction using 80.2 mg (0.30 mmol) of 3′-azido-3′-deoxythymidine and sodium azide (174.2 mg, 3.0 mmol) as nucleophilic reagent yielded, after HPLC purification, 73.35 μmol (24.4%) of **7g**: ^{1}H NMR (D_2O) δ 1.78 (s, 5-CH_3, 3H), 2.30−2.36 (m, H-2′, 2H), 4.03−4.10 (m, H-4′, H-5′, 3H), 4.42−4.46 (m, H-3′, 1H), 6.13 (t, J = 7.0 Hz, H-1′, 1H), 7.60 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −10.50 (d, $^2J_{P-P}$ =

29.8 Hz, P_α), −6.43 (ddt, $^2J_{P-F}$ = 82.6 Hz, $^2J_{P-P}$ = 62.7 Hz, $^2J_{P-P}$ = 29.3 Hz, P_β), 5.23 (ddt, $^1J_{P-F}$ = 1024.9 Hz, $^2J_{P-F}$ = 91.3 Hz, $^2J_{P-P}$ = 63.2 Hz, P_γ); ^{19}F NMR (D_2O) δ −121.40 (ddd, $^2J_{F-P}$ = 91.1 Hz, $^2J_{F-P}$ = 83.2 Hz, $^3J_{F-F}$ = 4.0 Hz, CF_2), −68.75 (dt, $^1J_{F-P}$ = 1021.0 Hz, $^3J_{F-F}$ = 4.0 Hz, F−P_γ); MS *m/z* 543 (M − H)$^-$; HPLC analysis, 99.8% purity.

3′-Azido-3′-deoxythymidine 5′-*β*,*γ*-Difluoromethylene-*γ*-*O*-methyltriphosphate (7h). Reaction using 40.09 mg (0.15 mmol) of 3′-azido-3′-deoxythymidine and sodium methoxide (1.5 mmol in methanol) as nucleophilic reagent yielded, after HPLC purification, 18.20 μmol (12.1%) of **7h**: ^{1}H NMR (D_2O) δ 1.90 (s, 5-CH_3, 3H), 2.30−2.38 (m, H-2′, 2H), 4.02−4.07 (m, H-4′, H-5′, 3H), 4.41−4.46 (m, H-3′, 1H), 6.11 (t, *J* = 6.8 Hz, H-1′, 1H), 7.58 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −10.32 (d, $^2J_{P-P}$ = 30.6 Hz, P_α), −5.05 (m, P_β), 6.15 (dt, $^2J_{P-P}$ = 59.6 Hz, $^2J_{P-F}$ = 81.6 Hz, P_γ); ^{19}F NMR (D_2O) δ −119.4 (t, $^2J_{F-P}$ = 83.2 Hz, CF_2); MS *m/z* 554 (M − H)$^-$; HPLC analysis, >95% purity.

3′-Azido-3′-deoxythymidine 5′-*β*,*γ*-Difluoromethylene-*γ*-*O*-phenyltriphosphate (7i). Reaction using 59.0 mg (0.22 mmol) of 3′-azido-3′-deoxythymidine and sodium phenoxide (2.2 mmol in DMF) as nucleophilic reagent yielded, after HPLC purification, 3.65 μmol (1.7%) of **7I**: MS *m/z* 616 (M − H)$^-$; HPLC analysis, 95.4% purity.

3′-Azido-3′-deoxythymidine 5′-α-*P*-Borano-*β*,*γ*-difluoromethylene-*γ*-*O*-methyltriphosphate (11). A solution of the bis(tetrabutylammonium) salts of **6d**-I/II (0.12 mmol), tributylamine (85 μL, 0.36 mmol), and methyl trifluoromethanesulfonate (54 μL, 0.48 mmol) in anhydrous acetonitrile (2 mL) stood at room temperature overnight. The reaction was quenched with water (2 mL) at 0 °C and then most of the acetonitrile was evaporated. The aqueous solution was subject to reverse-phase HPLC purification to give 2.74 μmol (2.3%) of **11**: MS *m/z* 551 (M − H)$^-$; HPLC analysis, 94.0% purity.

3′-Azido-3′-deoxythymidine 5′-α-*P*-Borano-*β*,*γ*-difluoromethylene-*γ*-*O*-phenyltriphosphate (12). A slurry of sodium hydride (320 mg, 8.0 mmol) in anhydrous DMF (10 mL) was treated with phenol (0.9 g, 10.0 mmol) dissolved in anhydrous DMF (2 mL) under an argon atmosphere. After stirring for 1 h at room temperature, gas evolution had ceased. 15-Crown-5 (1.59 mL, 8.0 mmol) was added and stirring continued for 1 h. This mixture was used immediately.

The triethylammonium salt of **6d**-I/II (0.21 mmol) was coevaporated with anhydrous DMF (3 × 5 mL). For the third coevaporation, approximately half the volume of DMF was removed and anhydrous methanol (0.3 mL) was added, followed by addition of *N*,*N*′-dicyclohexylcarbodiimide (DCC, 200 mg, 0.97 mmol). After stirring at room temperature under argon for 3 h, the mixture was concentrated under reduced presure. The above procedure was repeated once more using DMF (3 × 5 mL), methanol (0.3 mL), and DCC. After removal of solvents, the residue was resuspended in anhydrous DMF (3 mL) and treated dropwise with the phenoxide solution from step 1. After stirring at room temperature under argon for 4 h, the reaction mixture was treated with water (20 mL), adjusted to pH 6 with hydrochloric acid (1.0 M), and extracted with diethyl ether (3 × 20 mL). The aqueous portion was purified on reverse-phase HPLC to give 67.15 μmol (32.0%) of **12**: ^{1}H NMR (D_2O) δ 0.1−0.7 (br, BH_3, 3H), 1.75, 1.76 (2s, 2 isomers, 5-CH_3, 3H), 2.18−2.29 (m, H-2′, 2H), 3.92−4.11 (m, H-4′, H-5′, 3H), 4.28−4.38 (m, H-3′, 1H), 6.07 (apparent q, *J* = 7.1 Hz, H-1′, 1H), 6.97−7.08 (m, Ph-meta, para, 3H), 7.15−7.21 (m, Ph-ortho, 2H), 7.52, 7.54 (2s, 2 isomers, H-6, 1H); ^{31}P NMR (D_2O) δ −5.42 (m, P_β), 2.20 (dt, $^2J_{P-P}$ = 59.3 Hz, $^2J_{P-F}$ = 87.0 Hz, P_γ), 84.70 (m, P_α); ^{19}F NMR (D_2O) δ −119.45, −119.50 (2t, 2 isomers, $^2J_{F-P}$ = 86.0 Hz, CF_2); MS *m/z* 614 (M − H)$^-$; HPLC analysis, 84.6% purity.

3′-Azido-3′-deoxythymidine 5′-*β*,*γ*-Difluoromethylene-α-*P*-thiotriphosphate (13). 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (101.2 mg, 0.495 mmol) in anhydrous DMF (1 mL) was added via syringe to a stirred solution of 3′-azido-3′-deoxythymidine (103.8 mg, 0.388 mmol) in 1 mL of anhydrous DMF and 0.20 mL of anhydrous pyridine at 0 °C under argon. After stirring at room temperature for 1 h, tributylamine (0.3 mL) was added, followed by (difluoromethylene)diphosphonic acid bis(tributylammonium) salt (294 mg, 0.504 mmol) in anhydrous DMF (1 mL). The mixture was stirred at room temperature for 1 h and cooled with ice, and sulfur (24.8 mg, 0.776 mmol) was added. After stirring at room temperature for 2 h, the reaction mixture was quenched with water (10 mL) and stirred at room temperature for 30 min. Purification by HPLC yielded 0.108 mmol (27.7%) of **13**: ^{1}H NMR (D_2O) δ 1.84 (s, 5-CH_3, 3H), 2.29−2.35 (m, H-2′, 2H), 4.02−4.12 (m, H-4′, H-5′, 3H), 4.39−4.43 (m, H-3′, 1H), 6.09 (t, *J* = 6.9 Hz, H-1′, 1H), 7.60 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −1.83 (m, P_β), 6.87 (dt, $^2J_{P-P}$ = 60.0 Hz, $^2J_{P-F}$ = 80.4 Hz, P_γ), 100.61 (apparent q, $^2J_{P-P}$ = 28.2 Hz, P_α); ^{19}F NMR (D_2O) δ −121.1 (t, $^2J_{F-P}$ = 81.3 Hz, CF_2); MS *m/z* 556 (M − H)$^-$; HPLC analysis, 100% purity.

3′-Deoxythymidine 5′-*β*,*γ*-Difluoromethylene-α-*P*-dithiotriphosphate (14). 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (101.2 mg, 0.495 mmol) in anhydrous DMF (1 mL) was added via syringe to a stirred solution of 3′-azido-3′-deoxythymidine (103.8 mg, 0.388 mmol) in 1 mL of anhydrous DMF and 0.20 mL of anhydrous pyridine at 0 °C under argon. After stirring at room temperature for 1 h, tributylamine (0.3 mL) was added, followed by (difluoromethylene)diphosphonic acid bis(tributylammonium) salt (294 mg, 0.504 mmol) in anhydrous DMF (1 mL). The mixture was stirred at room temperature for 1 h and cooled with ice, and sulfur (24.8 mg, 0.776 mmol) was added. After stirring at room temperature for 2 h, lithium sulfide (89.1 mg, 1.94 mmol) was added and stirring was continued for 1 h. Water (10 mL) was added, and the mixture was stirred at room temperature for 30 min. Purification on HPLC yielded 12.34 μmol (3.2%) of **14**: ^{1}H NMR (D_2O) δ 1.83 (s, 5-CH_3, 3H), 2.31−2.40 (m, H-2′, 2H), 4.12−4.22 (m, H-4′, H-5′, 3H), 4.50−4.71 (m, H-3′, 1H), 6.14 (t, *J* = 6.8 Hz, H-1′, 1H), 7.70 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −4.75 (m, P_β), 4.25 (dt, $^2J_{P-P}$ = 61.2 Hz, $^2J_{P-F}$ = 81.6 Hz, P_γ), 100.61 (d, $^2J_{P-P}$ = 43.2 Hz, P_α); ^{19}F NMR (D_2O) δ −120.6 (apparent q, $^2J_{F-P}$ = 81.3 Hz, CF_2); MS *m/z* 572 (M − H)$^-$; HPLC analysis, 97.3% purity.

Preparation of 3′-Azido-3′-deoxythymidine 5′-(Difluoromethylene)diphosphate. Trimethylbromosilane (4.3 mL, 33.3 mmol) was added to a stirred solution of tetraisopropyl (difluoromethylene)diphosphonate (2.10 g, 5.51 mmol) in 1,2-dichloroethane (20 mL) under argon. The reaction mixture was continued with magnetic stirring at 40 °C for 24 h. Volatiles were evaporated under reduced pressure, and the residue was coevaporated with toluene twice. The resulting residue was dissolved in dichloromethane, cooled with ice, and quenched with a DMF and water mixture (3:2). After removal of solvent, the residue was dissolved in water and cooled to 0 °C. Tetrabutylammonium hydroxide aqueous solution (40 wt %, 10.72 g, 16.52 mmol) was added, and the resulting solution was concentrated. The residue was coevaporated with DMF three times and dried at room temperature under vacuum to give 5.36 g of (difluoromethylene)diphosphonic acid tris(tetrabutylammonium) salt as a semisolid, which was coevaporated with pyridine two times and dried under vacuum: ^{31}P NMR (DMSO) δ 5.59 (t, J_{P-F} = 70.4 Hz); ^{19}F NMR (DMSO) δ −118.90 (t, J_{F-P} = 69.4 Hz).

To a flask containing the (difluoromethylene)diphosphonic acid tetrabutylammonium salt prepared above (3.86 g, 4.12 mmol) in acetonitrile (4 mL) under argon was added dropwise a solution of AZT 5′-tosylate[29] (358 mg, 0.85 mmol) in acetonitrile (2.5 mL). The resulting solution was stirred for 27 h at room temperature, quenched by adding 10 mL of water, and stirred for 5 min. HPLC purification yielded 160 mg (0.177 mmol) of 3′-azido-3′-deoxythymidine 5′-(difluoromethylene)diphosphate.

3′-Azido-3′-deoxythymidine 5′-*β*,*γ*-Difluoromethylene-*γ*-*P*-methyltriphosphate (15). Triethylamine (37 μL, 0.265 mmol) was added to a stirred solution of 1,2,4-1*H*-triazole (18.3 mg, 0.265 mmol) in anhydrous acetonitrile (0.5 mL). The solution was cooled to 0 °C, and a solution of methylphosphonic dichloride (17.6 mg, 0.133 mmol) in acetonitrile (0.5 mL) was added dropwise. The reaction mixture was stirred at room temperature for 40 min and then centrifuged. The supernatant

was added to a solution of the tributylammonium salt of 3′-azido-3′-deoxythymidine 5′-(difluoromethylene)diphosphonate (89.8 mg, 0.088 mmol) in DMF (1 mL). The reaction mixture was stirred for 1.5 h and then quenched with water (2 mL). Purification by reverse-phase HPLC gave 24.30 μmol (27.6%) of **15**: ^{1}H NMR (D_2O) δ 1.30 (d, P_γ-CH_3, 3H, J_{CH3-P} = 17.6 Hz), 1.76 (s, 5-CH_3, 3H), 2.29−2.33 (m, H-2′, 2H), 4.01 (m, H-4′, 1H), 4.10 (m, H-5′, 2H), 4.38−4.42 (m, H-3′, 1H), 6.06−6.11 (t, H-1′, 1H), 7.56 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −5.53 (m, P_β, $J_{P\beta-\gamma}$ = 31.7 Hz, $J_{P\beta-\alpha}$ = 61.0 Hz, $J_{P\beta-F}$ = 86.3 Hz), 4.43 (dt, Pα, J_{P-P} = 59.2 Hz, J_{P-F} = 81.4 Hz), 19.47 (d, P_γ, J_{P-P} = 31.3 Hz); ^{19}F NMR (D_2O) δ −119.58 (t, J_{P-F} = 83.2 Hz); MS *m/z* 538 (M − H)$^-$; HPLC analysis, 100% purity.

3′-Azido-3′-deoxythymidine 5′-α,*β*-Difluoromethylene-*γ*-*P*-phenyltriphosphate (16). Triethylamine (37.2 μL, 0.265 mmol) was added to a stirred solution of 1,2,4-1*H*-triazole (18.4 mg, 0.267 mmol) in anhydrous acetonitrile (0.5 mL). The solution was cooled to 0 °C, and a solution of phenylphosphonic dichloride (26 mg, 0.134 mmol) in acetonitrile (0.5 mL) was added dropwise. The reaction mixture was stirred at 4 °C for 40 min and then centrifuged. The supernatant was added to a solution of the tributylammonium salt of 3′-azido-3′-deoxythymidine 5′-(difluoromethylene)diphosphonate (90.3 mg, 0.089 mmol) in DMF (1 mL). Similar workup and purification as described for **15** gave 21.29 μmol (23.9%) of **16**: ^{1}H NMR (D_2O) δ 1.71 (s, 5-CH_3, 3H), 2.23 (m, H-2′, 2H), 3.93 (m, H-4′, 1H), 4.00 (m, H-5′, 2H), 4.27 (m, H-3′, 1H), 6.03−6.08 (t, H-1′, 1H), 7.27−7.33 and 7.61−7.68 (2m, Ph, 5H), 7.48 (s, H-6, 1H); ^{31}P NMR (D_2O) δ −5.24 (m, P_β, $J_{P\beta-\gamma}$ = 33.1 Hz, $J_{P\beta-\alpha}$ = 60.3 Hz, $J_{P\beta-F}$ = 85.3 Hz), 4.64 (dt, Pα, J_{P-P} = 59.9 Hz, J_{P-F} = 82.8 Hz), 17.61 (d, P_γ, J_{P-P} = 32.8 Hz); ^{19}F NMR (D_2O) δ −119.4 (t, J_{P-F} = 83.2 Hz); MS *m/z* 600 (M − H)$^-$; HPLC analysis, 100% purity.

HIV Reverse Transcriptase Inhibition Assays. This assay was used to measure the ability of the AZT P3Ms to inhibit the enzymatic synthesis of complimentary strand DNA from a DNA-primed template of homopolymeric RNA. This assay is a modification of a published procedure.[31] Assay buffer conditions (50 μL total/reaction): 50 mM Tris-HCl, pH 8.1, 6.5 mM $MgCl_2$, 100 mM, NaCl, 10 mM DTT, 5 μM dTTP (thymidine triphosphate), 1 μg/mL primed-poly(A) RNA, 2 nM purified HIV reverse transcriptase (type B, 66 kDa subunit). The compounds were tested at various concentrations up to 500 μM final concentration. DNA polymerase activity was measured in a reaction buffer containing primed-RNA template and dTTP diluted to appropriate concentrations in assay buffer. The AZT P3Ms were diluted in buffer and pipeted into the wells of a 96-well plate. The reaction was initiated by addition of enzyme and allowed to proceed at 37 °C for 10 min and then quenched by addition of 5 μL of 0.2 M EDTA, pH 8.0. Blank reactions were prepared in parallel with the test reactions in which either enzyme or dTTP was omitted from the reactions, substituted by an appropriate volume of enzyme diluent or assay buffer, respectively. Then 200 μL of diluted PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Inc, Eugene, OR) was added to each well of a 96-well plate and incubated at room temperature for 5 min. Plate wells were read on a microplate fluorometer (Molecular Devices Corp., Sunnyvale, CA). The wells were excited at 480 nm and the fluorescence emission intensity (RFU) was measured at 538 nm. The percentage of inhibition was calculated according to the equation: % nhibition = [1 − (RFU in test reaction − RFU in blank)/(RFU in control reaction − RFU in blank)] × 100. Inhibition constants (K_i) were determined for compounds that exhibited ≥50% inhibition at 10 μM. Each inhibitor was titrated over an appropriate range of concentrations, and inhibition constants were determined using a competitive inhibition (Synergy Software).

Serum Stability Assessment Using Serum. The stability of nucleotide mimics was assessed in fetal calf serum, generally following a published procedure.[20] Fetal calf serum purchased from HyClone Corporation was mixed 1:1 with each compound containing Tris-HCl buffer and $MgCl_2$. The final concentrations of the reaction components were as follows: 50 mM Tris-HCl, pH 7.4, 0.1 mM $MgCl_2$, 500 μM nucleotide mimic, 10% (v/v) fetal calf serum. The reaction mixtures were incubated at 37 °C. At appropriate times, aliquots of 25 μL were removed and added to 65 μL of ice-cold methanol. These solutions were incubated for at least 1 h at −20°C. After incubation, samples were centrifuged for 20 min at high speed in a microcentrifuge. The supernatant was transferred to a clean tube and the extract was dried under vacuum in a LabConco Centrivap Concentrator. The dried extracts were resuspended in deionized water and filtered to remove particulates and then analyzed on reverse phase HPLC. The reverse phase HPLC column used for the analysis was a Phenomenex C18 Aqua column (3 × 150 mm). The HPLC flow rate was 0.5 mL/min with the following buffer system: 5 mM tetrabutylammonium acetate, 50 mM ammonium phosphate, and an acetonitrile gradient running from 5% up to as high as 60%. The amount of remaining parent compound at each time point was used to determine the half-life of the compound. Time points were only taken through 48 h so that if greater than 50% of a compound was still intact after 48 h incubation the half-life was expressed as >48 h. Unmodified nucleoside triphosphates were used as positive controls.

Stability Assessment Using Cell Extracts. Cell lysis buffer was added to cell pellets, and the cells were frozen and thawed three times using dry ice. The lysis buffer (LB) was composed of the following: 50 mM Tris-HCl, pH 7.4 (100 μL/mL 10× stock), 20% glycerol (200 μL/mL), and 0.5% Triton X-100 (5μL/mL). LB (100 μL) was added to each microfuge tube containing 10^7 frozen CEM cells. After the cells were lysed, the extracts were centrifuged at high speed in a microcentrifuge for 5 min, and the clarified cell extract was transferred to a new tube. The cell stability reaction mixtures contained concentrations of buffer, magnesium, nucleotide, and cell extract as shown below: 50 mM Tris-HCl, pH 7.4, 0.1 mM, $MgCl_2$, 500 μM nucleotide mimic (or control nucleotide), 50% cell extract (v/v). The reaction mixtures were incubated at 37 °C. At each time point, including at time zero, 12.5 μL aliquots were added to 40 μL of ice-cold methanol. Typically, time points were taken after 30 and 60 min and 2, 3, 8, 24, and 48 h. The samples were incubated in methanol for at least 60 min and typically overnight at −20°C prior to further processing. The cell extracts were centrifuged at high speed in a refrigerated microcentrifuge for 20 min, and the supernatant was transferred to a new tube. The extract was then dried under vacuum in a LabConco Centrivap Concentrator. The samples were then resuspended in 40 or 50 μL of deionized water, filtered to remove any particulate, and analyzed by reverse phase HPLC.

The reverse phase HPLC columns used for the analysis were either a Phenomenex C18 Aqua column (2 × 100 mm) or the Phenomenex C18 Aqua column (3 × 150 mm) used with the appropriate guard column. The HPLC flow rate was either 0.2 mL/min (for the 2 × 100 mm column) or 0.5 mL/min (for the 3 × 150 mm column) with the following buffer system: 5 mM tetrabutylammonium acetate, 50 mM ammonium phosphate, and an acetonitrile gradient running from 5% up to as high as 60%. The amount of remaining parent compound at each time point was used to determine the half-life of the compound. Time points were only taken through 48 h so that if greater than 50% of a compound was still intact after 48 h incubation the half-life was expressed as >48 h. Unmodified nucleoside triphosphates were used as positive controls.

Acknowledgment. The authors wish to thank Drs. Kandasamy Sakthivel and Gregory J. Ewing, for their helpful suggestions and discussions of chemistry, and Dr. John Lambert, for his critical reading and suggestions in the preparation of the manuscript.

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JM040116W