Uncharged AZT and D4T Derivatives of Phosphonoformic and Phosphonoacetic Acids as Anti-HIV Pronucleosides

Elena A. Shirokova,*,† Maxim V. Jasko,† Anastasiya L. Khandazhinskaya,† Alexander V. Ivanov,† Dmitry V. Yanvarev,† Yury S. Skoblov,† Vladimir A. Mitkevich,† Eduard V. Bocharov,‡ Tatyana R. Pronyaeva,§ Nina V. Fedyuk,§ Marina K. Kukhanova,† and Andrey G. Pokrovsky§

Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32 Vavilov str., Moscow 119991, Russian Federation; Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 16/10 Miklukho-Maklaya str., Moscow 117997, Russian Federation; and State Research Center of Virology & Biotechnology "Vector", Koltsovo, Novosibirsk region 633159, Russian Federation

Received September 2, 2003

Two series of new lipophilic phosphonoformate and phosphonoacetate derivatives of AZT and d4T were synthesized and evaluated as anti-HIV agents. The efficacy of some of the synthesized compounds in cell cultures infected with HIV-1 was higher than that of the parent nucleosides and only slightly correlated to their stability in the phosphate buffer and human blood serum. The synthesized phosphonates are most probably prodrug forms of the corresponding nucleosides.

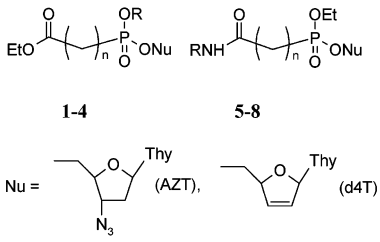
Introduction

Modified nucleosides used in medical practice for AIDS therapy are targeted at the inhibition of the proviral DNA synthesis catalyzed by HIV reverse transcriptase. To become active, these nucleosides must be phosphorylated by cellular kinases to give successively the corresponding 5′-mono-, -di-, and -triphosphates. The efficacy of this process is extremely low (for example, 0.3% for AZT). Therefore, many efforts have been made to improve therapeutic properties by shortening this cascade and bypassing at least the first phosphorylation step. This approach has resulted in the synthesis of numerous nucleotide analogues.[1−8] Another way of increasing the efficacy of the currently used drugs involves altering the bioavailability of the drug itself. For example, different modifications have been performed in the AZT molecule with the goal to improve its pharmacological properties (refs 9, 10, and references therein).

On the basis of our ongoing studies of antiviral nucleoside analogues, we describe herein the synthesis and chemical and enzymatic stability of several new AZT and d4T derivatives containing uncharged 5′-(alkyl)phosphonoformate and 5′-(alkyl)phosphonoacetate residues (**1**−**8**) as well as their evaluation as potential anti-HIV agents in MT-4 cell cultures infected with HIV-1.

Results

Chemistry. The target triesters **1**, **2**, **3a**, and **4a** were synthesized by the reaction of the corresponding nucleoside with ethyl ethoxycarbonylphosphonyl chloride or ethyl ethoxycarbonylmethylphosphonyl chloride, respectively, obtained from commercially available triesters.[11,12] The product yields did not exceed 60−70% (in particular, because of the formation of 5′-chloro-5′-deoxy derivatives).



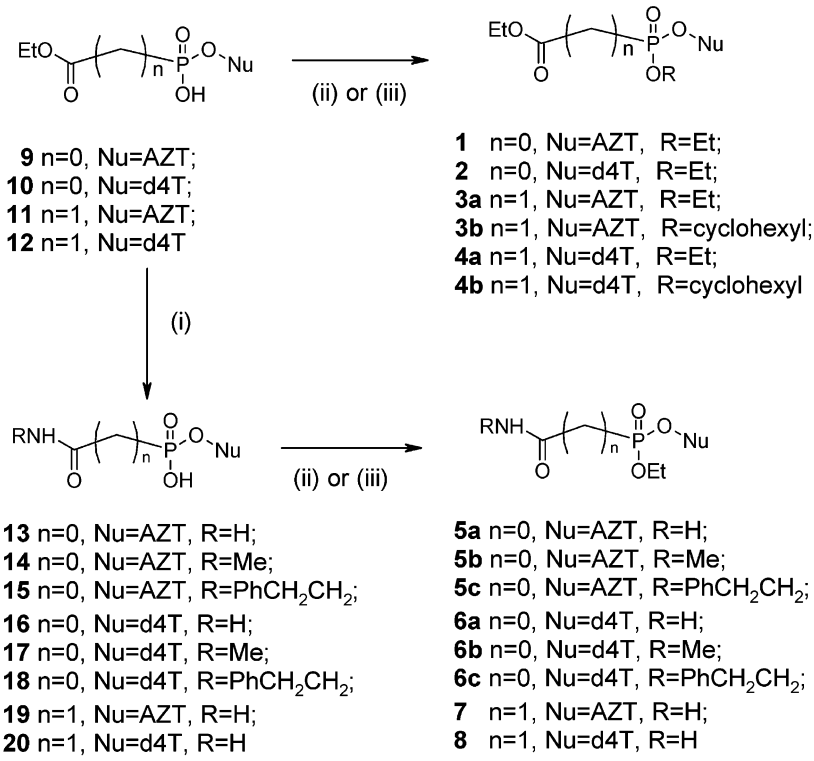
Comp	n	Nu	R
1	0	AZT	Et
2	0	d4T	Et
3a	1	AZT	Et
3b	1	AZT	cyclohexyl
4a	1	d4T	Et
4b	1	d4T	cyclohexyl
5a	0	AZT	H
5b	0	AZT	Me
5c	0	AZT	CH_2CH_2Ph
6a	0	d4T	H
6b	0	d4T	Me
6c	0	d4T	CH_2CH_2Ph
7	1	AZT	H
8	1	d4T	H

A general scheme of synthesis for the target triesters and amidodiesters of types **1**−**8** involved the preparation of the corresponding charged derivatives **9**−**20** and their

* To whom correspondence should be addressed. Phone: 7 (095) 135-2255; fax: 7 (095) 135-1405; e-mail: shirokova-elena@list.ru.
† Engelhardt Institute of Molecular Biology, Russian Academy of Sciences.
‡ Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences.
§ State Research Center of Virology & Biotechnology "Vector".

10.1021/jm0310176 CCC: $27.50
Published on Web 05/29/2004

Scheme 1



9 n=0, Nu=AZT;
10 n=0, Nu=d4T;
11 n=1, Nu=AZT;
12 n=1, Nu=d4T

1 n=0, Nu=AZT, R=Et;
2 n=0, Nu=d4T, R=Et;
3a n=1, Nu=AZT, R=Et;
3b n=1, Nu=AZT, R=cyclohexyl;
4a n=1, Nu=d4T, R=Et;
4b n=1, Nu=d4T, R=cyclohexyl

13 n=0, Nu=AZT, R=H;
14 n=0, Nu=AZT, R=Me;
15 n=0, Nu=AZT, R=$PhCH_2CH_2$;
16 n=0, Nu=d4T, R=H;
17 n=0, Nu=d4T, R=Me;
18 n=0, Nu=d4T, R=$PhCH_2CH_2$;
19 n=1, Nu=AZT, R=H;
20 n=1, Nu=d4T, R=H

5a n=0, Nu=AZT, R=H;
5b n=0, Nu=AZT, R=Me;
5c n=0, Nu=AZT, R=$PhCH_2CH_2$;
6a n=0, Nu=d4T, R=H;
6b n=0, Nu=d4T, R=Me;
6c n=0, Nu=d4T, R=$PhCH_2CH_2$;
7 n=1, Nu=AZT, R=H;
8 n=1, Nu=d4T, R=H

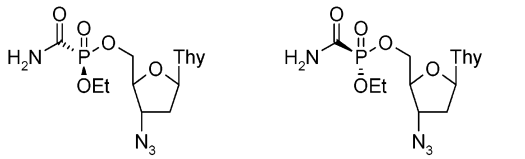
[a] (i) RNH_2; (ii) TPSCl, Py, DMF, ROH; (iii) DEAD, PPh_3, ROH.

subsequent esterification (Scheme 1). Phosphonoformates **9** and **10** and phosphonoacetates **11** and **12** were obtained by the coupling of the corresponding nucleoside with ethoxycarbonylphosphonic or ethoxycarbonylmethylphosphonic acids in the presence of 1,3-dicyclohexylcarbodiimide (DCC).[13] The yields of products achieved 70−80%. Phosphonates **9**−**12** were converted to the corresponding amides **13**−**20** by treatment with the corresponding amines. Phosphonates **9**−**20** were esterified by the coupling with the corresponding alcohols either in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl)[14] or under the Mitsunobu reaction conditions.[15] One disadvantage to the first procedure is the requisite of use of a large excess of the condensing reagent (2−3 equiv) because of the side formation of the corresponding TPSOR esters. In contrast, however, the Mitsunobu reaction is attractive for the preparation of pH-sensitive compounds, since it proceeds under neutral conditions, although its efficacy noticeably falls in the case of application of secondary alcohols.[15]

The newly formed chiral center at the phosphorus atom in compounds **1**−**8** results in the formation of two diastereoisomers. Indeed, two similar ^{31}P-signals in the ratio of approximately 1:1 were observed for each of these phosphonates in their ^{31}P NMR spectra acquired with ^{1}H broad-band decoupling (see Experimental Section). The 1D ^{1}H NMR spectrum of compound **5a** dissolved in H_2O also supported the existence of two diastereoisomeric forms. The resonances of the ethyl and amino groups were doubled with the same intensity as the resonances in the ^{31}P spectral pattern (data are not shown).

It is noteworthy that splitting patterns for the protons of the NH_2CO group of compound **5a** observed in ^{1}H NMR spectral patterns were different: one, a singlet at 7.24 ppm, while the other was a doublet at 6.83 ppm with $^3J_{P-H}$ 35 Hz. Using HPLC on a reverse-phase Nucleosil C-18 column in the elution system (a) (see the Experimental Section) we could easily separate the diastereomers of **5a** (their retention times differed by more than 2 min and were 13.3 and 15.4 min, respectively). The ^{31}P NMR spectra supported the hypothesis of an interaction between the phosphorus atom and one of the amide protons (see below).

According to the spatial model of compound **5a** (constructed with the HYPERCHEM program), the ribose ring of the *R*-isomer is in close proximity to the



S-isomer *R*-isomer

ethyl group of the chiral phosphorus atom, while in the *S*-isomer it is neighbored to the amide group. As a result, we then tried to establish both an absolute configuration for the **5a**-isomers and to elucidate the exact nature of the factors affecting their chromatographic properties.

The CD spectra of AZT, A and B isomers of amide **5a**, and phosphonate **13** in the wavelength range of 220−370 nm are presented in Figure 1a. A positive maximum on CD curves in the region of 275−285 nm demonstrates the prevalence of the anti-conformation of the thymine base in these compounds.[16] The differences in their CD spectra can be explained by a different ratio of syn and anti conformations of the pyrimidine

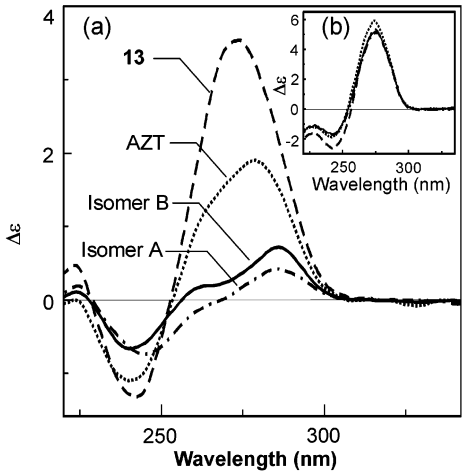


Figure 1. (a) CD spectra of AZT (dotted line), A (solid line), and B (dot-and-dashed line) isomers ofamide **5a** and aminocarbonylphosphonate **13** (dashed line) in the far UV region at pH 6.5 (5 mM NaH_2PO_4, 25 °C). (b) CD spectra in ethanol.

base. These conformations are dependent on the various conformations of the furanose ring, which result from the presence of different substituents at the 5′ C atom.

To further explore our hypothesis regarding the effect of substituents at the phosphorus atom on the conformation of the compounds under study, we compared their CD spectra in ethanol in order to disturb the interactions that would occur in water. Figure 1b shows that all four curves were nearly identical. This allowed us to postulate that the difference between phosphonates A and B was based on the difference in the configuration of the substituents at the phosphorus atom.

The 1D ^{1}H NMR spectrum of compound **5a** dissolved in H_2O (Figure 2a) also supported the existence of stereoisomers [the resonances of the protons that display the NOE interaction with the thymine H-6 proton owing to their spatial proximity (<5 Å) are shown]. The analysis of the distances between the H-6 proton and the 1′−5′ protons of the ribose ring confirmed the prevalence of the base anti-conformation in the phosphonate **5a** and implied the absence of hydrophobic interactions between the thymine ring and the ethoxy group at the phosphorus atom.

Analysis of the 1D section of 2D ^{1}H−^{1}H ROESY spectrum of compound **5a** showed a weak NOE interaction between the ribose protons and the substituents at the phosphorus atom (Figure 2b). Moreover, for isomer A, in contrast to B, we observed NOE contacts between the ethyl CH_2 protons and the 3′- and 4′-protons of the ribose ring. This suggested a spatial proximity of the ethyl group and the ribose ring in isomer A. On the basis of all evidence obtained, the *R* configuration can be assigned to isomer A and the *S* configuration, to isomer B.

Additionally, the ^{1}H−^{1}H ROESY spectral pattern showed that a large $^3J_{H-P}$ coupling constant of 35 Hz for one of the protons of amide group can result from the NH_{trans} proton located in the trans position relative to the phosphorus atom. Hence, the NH_{trans} is located farther from the ribose ring than the amide cis proton NH_{cis}. It follows, therefore, that the ethyl group in isomer A is located closer to the thymine base and favors

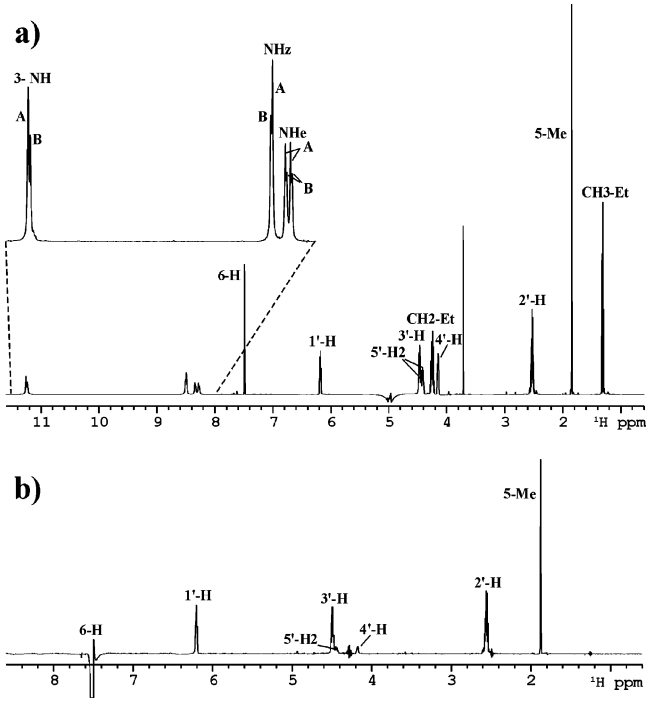


Figure 2. ^{1}H NMR spectra of compound **5a**. (a) ^{1}H NMR spectrum in 90% H_2O and 10% D_2O at 5 °C; (b) 1D section of 2D ^{1}H−^{1}H ROESY spectrum (mixing time 200 ms, 99.9% D_2O, 5 °C) cut on proton H-6 frequency. The peak assignments are marked. Resonances of the A and B isomers are labeled as A and B, respectively.

Table 1. Hydrolysis of the Synthesized Phosphonates in Phosphate Buffer (pH 7.2, 20 °C)

AZT derivative	half-life	d4T derivative	half-life
1	10 min ± 2	**2**	30 min ± 3
3b	≫8 h	**4a**	≫8 h
5a	2.5 h ± 0.25	**6a**	7 h ± 0.30
5b	6 h ± 0.30	**6b**	15 h ± 0.33
5c	10 h ± 0.30	**6c**	>20 h
7	≫20 h	**8**	≫24 h

a more perfectly packed hydrophobic “core”. This may explain the difference in the chromatographic behavior of the diastereoisomers A and B.

Chemical Stability. Chemical stability of the synthesized compounds was studied in the phosphate buffer (pH 7.2) at 20 °C and the concentration of the compound under study of 0.5 mM (Table 1). The composition of the reaction mixture was analyzed by HPLC on an RP-18 column.

In the case of phosphonoformate triesters **1** and **2** we observed the formation of two products, a nucleoside and the corresponding 5′-ethyl phosphite of AZT or d4T, respectively, (ROP(H)(O)O−nucleoside), although the nucleoside was formed considerably faster. The nucleoside was a major product; indeed, the amount of the minor product at the point of 50% hydrolysis of the starting triester did not exceed 10−12% of the total amount of products. Conversely, the phosphonoacetate derivatives of both AZT and d4T (**3**, **4**, **7**, and **8**) were very stable: after 8 h under the same conditions we observed more than 95% of the phosphonates under study and only 3−4% of the corresponding nucleoside.

In contrast, the hydrolysis of amides **5** and **6** each yielded one major product, which was identified as the corresponding nucleoside AZT or d4T. We performed a

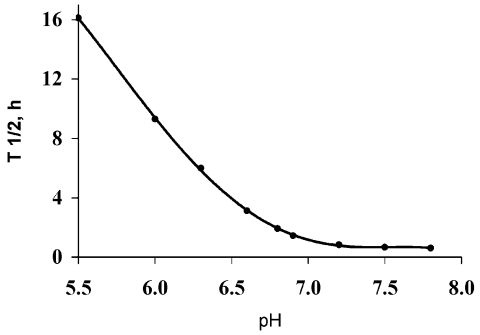


Figure 3. The time-pH dependence of AZT 5′-(ethyl)aminocarbonylphosphonate **5a** hydrolysis.

more detailed study of the composition of the reaction mixture at the hydrolysis of compound **5a** at 37 °C and pH 7.2. In the course of the analysis we observed two products, one of them being formed much more quickly. AZT and phosphonate **13** were found to be major and minor products, respectively, and the amount of the latter did not exceed 10% of the total amount of all the components. To identify the hydrolysis products, authentic AZT and AZT 5′-aminocarbonylphosphonate **13** were used for comparison.

We also studied the chemical stability of 0.45 mM amide **5a** in the 0.05 M phosphate buffer at 37 °C in the pH range of 5.5−7.8. The time dependence of its hydrolysis relative to pH is presented in Figure 3; the stability of compound **5a** is obviously strongly affected by the pH. For example, the half-life at pH 5.5 was about 16 h, whereas at pH 7.8 it was reduced to 34 min. This implies that the hydrolysis rate of amide **5a** was grew dramatically with a pH increase and achieved maximum values at pH > 7.5. The plotted kinetic hydrolysis curve is inherent for first-order reactions. The half-life of the amide at pH 7.2 and 37 °C was about 50 min.

Similar experiments were carried out with A and B isomers of amide **5a**. The hydrolytic properties of individual isomers completely coincided with those of the diastereomeric mixture **5a** (data not shown).

Enzymatic Hydrolysis. We also studied enzymatic stability of amides **5**−**8**. In experiments with human blood serum, which is enriched with hydrolyzing enzymes, our results showed that phosphonoformate derivatives **5** and **6** were readily cleaved to give the corresponding nucleosides (Figure 4), whereas phosphonoacetate derivatives **7** and **8** proved to be stable under the same conditions (Table 2).

The enzymatic hydrolysis of amide **5a** in the presence of hydrolases, such as snake venom phosphodiesterase, calf spleen phosphodiesterase, and P1 endonuclease, was not observed (data not shown). On the contrary, in the presence of *Crotalus atrox* snake venom preparation, amide **5a** was rapidly cleaved to give AZT, with a half-life less than 5 min. The properties of homogeneous (*R*)-**5a** and (*S*)-**5a** diastereomers in these experiments did not differ from those of the diastereomeric mixture (data not shown).

Antiviral Activity. Antiviral activity of the synthesized compounds was studied in MT-4 cell cultures infected with HIV-1, strain BIII (Table 3). The activity of the compounds under study was evaluated according

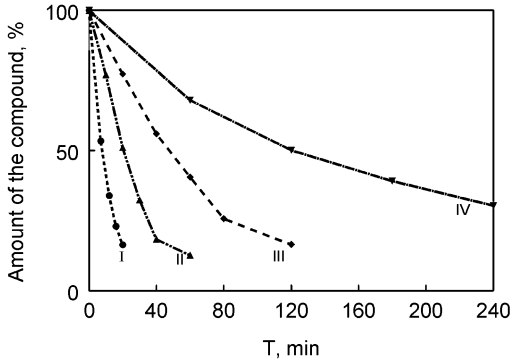


Figure 4. Hydrolysis of AZT and d4T 5′-(ethyl)aminocarbonylphosphonates **5a** and **6a** at 37 °C. Curves I and II, **5a** and **6a**, respectively, in human blood serum; III and IV, **5a** and **6a**, respectively, in phosphate buffer (pH 7.2).

Table 2. Hydrolysis of the Phosphonates in Human Blood Serum (37 °C)

AZT derivative	half-life	d4T derivative	half-life
5a	7 min ± 2	**6a**	21 min ± 3
7	>20 h	**8**	>24 h

to their ability to suppress viral replication by measuring the amount of viral antigen p24.

The phosphonoacetate derivatives of both series (**3**, **4**, **7**, and **8**) were inactive; their toxicities were comparable with those of parent nucleosides, except triesters **4**, which were twice as toxic as compared with d4T. The aminocarbonyl derivatives of both series displayed a high anti-HIV effect. This effect was more pronounced in the case of AZT compounds (**5a**−**c**). For the corresponding d4T counterparts, the gain was more moderate, since a higher activity was accompanied by a higher toxicity. It is also worth mentioning that AZT phosphonates **1** and **5a**−**c** inhibited the virus reprlication by 90% at the concentrations, which were considerably lower than those for AZT. Anti-HIV properties of homogeneous (*R*)-**5a** and (*S*)-**5a** coincided with those for the diastereomeric mixture (data not shown).

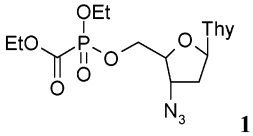
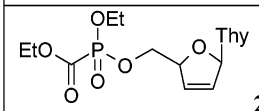
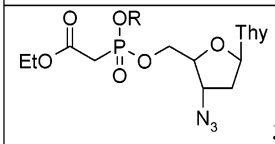
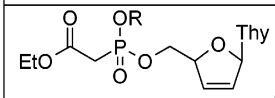
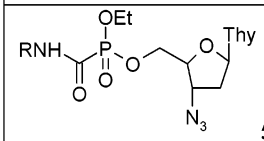
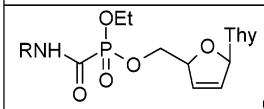
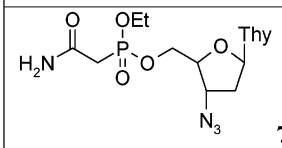
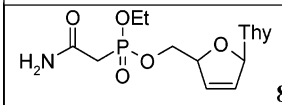
Discussion

The insight into the nature of viral diseases and the discovery of valid targets for the antiviral agents have enabled the development of a number of drugs for the treatment of HIV, herpes simplex viruses, cytomegalovirus, and other infections. One focus for further studies has been to increase the efficacy of the approved drugs. A chemical approach has involved structural modifications that could potentially improve the bioavailability of these molecules by affecting their penetration through cell membranes, as well as the rate of transformation into the active form (i.e. metabolism).

In the early 1990s, AZT-phosphonoformic acid conjugates of type **21** were prepared with the expectation that both fragments being biologically active can display synergism.[11,17] These diesters showed some advantages in the cultures resistant to both phosphonoformic acid and AZT, although they exhibited lower anti-HIV activities and (as a rule) selectivity indexes than AZT against the wild-type strain.[17]

Later Meier et al. reported the synthesis and antiviral activity of compounds of type **22**, which are highly

Table 3. Antiviral Activity of the Compounds under Study

Compound		CD_{50}[a], μM	ID_{50}[b], μM	ID_{90}[c], μM	IS[d]
 **1**		116	0.0071	0.016	16338
 **2**		136	0.23	21.7	591
 **3**	**3a** R = Et	>224	0.23	-	>974
	3b R = cyclohexyl	280	0.4	1.4	700
 **4**	**4a** R = Et	136	>10	-	<13
	4b R = cyclohexyl	131.4	>10	-	<13
 **5**	**5a** R = H	248.6	0.002	0.019	124300
	5b R = Me	112.9	0.00024	0.0072	470416
	5c R = $PhCH_2CH_2$	177.4	0.009	0.002	19711
 **6**	**6a** R = H	214.3	0.03	7.21	7143
	6b R = Me	85.7	0.013	0.27	6592
	6c R = $PhCH_2CH_2$	66.9	0.043	-	1555
 **7**		456.3	0.29	1.68	1573
 **8**		267.9	5.4	18.8	49.6
AZT		187.5	0.017	0.75	11029
d4T		314	0.24	23.8	1308

[a] Compound concentration required to cause a 50% inhibition of cell proliferation. [b] Compound concentration required to cause a 50% inhibition of HIV replication. [c] Compound concentration required to cause a 90% inhibition of HIV replication. [d] CD_{50}/ID_{50}.

lipophilic.[18] Triesters of type **22** showed rather good activity in cell cultures infected with HIV-1 but were inactive in the thymidine kinase-deficient cells. In addition, the stability of these compounds was low. We believed that a more detailed investigation of the structures of this type with varied substituents both at the phosphorus atom and carbonyl group could prove interesting.

21 R = eicosyl, docosyl, octadecyl, etc.

22 R = C_8H_{17}, $(CH_3)_3CC(O)S(CH_2)_2$-

It is worth mentioning that the synthesized d4T derivatives were more stable in comparison with the corresponding AZT phosphonates (Tables 1 and 2). All the prepared aminocarbonylphosphonates **5**−**8** exhibited higher stability than the corresponding triesters **1**−**4** both in the buffer and in human blood serum. In both series phosphonoacetate derivatives **3**, **4**, **7**, and **8** were considerably more stable than phosphonoformates **1**, **2**, **5**, and **6**.

It should be noted that phosphonoformate and phosphonoacetate derivatives were hydrolyzed differently. Particularly, in the case of amides **5** and **6**, the major mechanism involved the degradation of the phosphodiester bond while releasing nucleoside. In the case of triesters **1** and **2**, however, we observed the formation of P-esters of AZT or d4T 5′-H-phosphonates in addition to the corresponding nucleoside. Similar transformations of phosphonoformate esters into H-phosphonate esters were reported in ref 19.

According to the hydrolysis studies, the resulting phosphonoformates are depot forms of the corresponding nucleosides that are actual anti-HIV drugs. To elucidate potential enzymatic pathways of the conversion of the synthesized phosphonates into antiviral nucleosides, we tested amide **5a** in several enzymatic systems. Exonucleases (snake venom and calf spleen phosphodiesterases) as well as *Penicillinium citrum* endonuclease P1 did not digest amide **5a**. At the same time, *Crotalus atrox* snake venom preparation efficiently hydrolyzed compound **5a** to give AZT. Most probably, this effect can be attributed to the action of 5′-nucleotidase, which is capable of cleaving nucleoside 5′-phosphonates.[20] We also observed hydrolysis of amides **5a** and **6a** in human blood serum, which, is likely carried out by phosphatases.

Modifications of the nucleosides with a phosphonoformate residue contributed to the anti-HIV efficacy more noticeably for AZT-containing compounds by affecting to a greater degree level of the activity rather than the toxicity (Table 3). In the d4T series, most of the prepared phosphonates were more toxic than d4T. Therefore, although amides **6a**,**b** showed higher activity than the parent nucleoside, the increase in toxicity lowered their antiviral effect. We believe that an increase in antiviral activity can be attributed to a higher intracellular concentration of the derivatives under study if compared with that of the parent nucleosides.

Although we could not observe a direct correlation between the stability and antiviral activity of the synthesized compounds, it is obvious that the analogues with too high as well as with too low levels of stability cannot be regarded as promising antivirals. It is likely that the synthesized phosphonoacetates did not show a noticeable antiviral effect in cell culture because of the lack of the enzymes, which could hydrolyze these structures. To conclude, AZT and d4T modified at 5′-position with an aminocarbonylphosphonyl fragment displayed reasonable stability and good anti-HIV potency in vitro and are worth further investigation.

Experimental Section

Bromotrimethylsilane, diethyl azodicarboxylate, triphenylphosphine, triethylphosphonoformate, and triethylphosphonoacetate were from Fluka; TPSCl, pyridine, and DMF were from Aldrich. AZT was a kind gift of "AZT Association" Ltd (Moscow, Russia).

Column chromatography was performed on silica gel 60 (40−63 μm), reverse-phase chromatography was carried out on LiChroprep RP-8 and LiChroprep RP-18 (25−40 μm) (Merck), ion-exchange column chromatography was performed on DEAE-Toyopearl (HCO_3^-) (Toyosoda, Japan), and HPLC was performed on a Gilson chromatograph (France) supplied with an LKB 2220 integrator. A Nucleosil 100 C-18 (5 μm) column (4 × 150 mm) and two eluting systems were used. (a) Buffer A: 0.065 M NaH_2PO_4; buffer B: 72% ethanol; gradient: 0−5 min, 0% B; 5−10 min, 0−15% B; 10−30 min, 15−25% B; 30−35 min, 25−100% B. The flow rate was 0.5 mL/min. (b) Buffer A: 50 mM TEAB; buffer B: 70% ethanol; gradient: 0−5 min, 0% B; 5−30 min, 0−30% B; 30−40 min, 30−32% B; 40−45 min, 32−100% B. The flow rate was 0.5 mL/min.

NMR spectra were registered on an AMX III-400 spectrometer (Bruker) with the working frequency of 400 MHz for ^{1}H (Me_4Si as an internal standard for organic solvents and sodium 3-(trimethylsilyl)-1-propanesulfonate for D_2O) and 162 MHz for ^{31}P NMR (with phosphorus−proton interaction decoupling, 85% H_3PO_4 as an external standard). 1D (^{1}H and ^{31}P) and 2D (^{1}H−^{1}H) NMR experiments were performed on a Varian Unity-600 spectrometer with 0.6 mL of 5 mM water solution (99.9% D_2O or 90% H_2O + 10% D_2O) of diastereomeric mixture **5a** at 5 °C and 30 °C. The homonuclear 2D NMR spectra were acquired at 5 °C using pulsed-field-gradient watergate[21] scheme for water signal suppression: TOCSY (2) with isotropic mixing period of 80 ms and ROESY[22] with mixing time 200 ms. The 1D ^{1}H spectra of 0.5 mM water solution (99.9% D_2O) of individual A and B isomers of compound **5a** were obtained at 30 °C. Mass spectra were registered on a COMPACT MALDI-4 mass spectrometer (Kratos Analytical). UV spectra were recorded on a Shimadzu UV-1201 spectrophotometer (Japan) in methanol at pH 7.0. The λ_{max} values were 266−267 nm and ϵ 9100−9700 for all the synthesized compounds. CD spectra were recorded on a J-715 spectropolarimeter (Jasco, Japan) in a 1 cm cell. The nucleotide concentration was 0.1−0.2 mM. The results were expressed in the molar circular-dichroic absorption $\Delta\epsilon = \theta/(32980Cl)$ cm^2/mol, where θ was the measured ellipticity in degrees, C was molar concentration (mol/L), and l was optical path in centimeters.

Human blood serum was a kind gift of Dr. S. A. Grachev (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences). Calf spleen phosphodiesterase (17 U/mg) was from Worthington, and crystalline *Crotalus atrox* snake venom and *Penicillum citrum* P1 nuclease (1200 U/mg) were from Sigma.

3′-Azido-3′-deoxythymidine 5′-(ethyl)(ethoxycarbonyl) phosphonate 1 was prepared from AZT and triethylphosphonoformate according to[11] (method A) in a yield of 68%. ^{1}H NMR (CD_3CN; δ, ppm; J, Hz): 8.98 (1H, s, NH), 7.43 (1H, m, H6), 6.27 (1H, t, J 7.5, H1′), 4.40 (1H, m, H3′), 4.31 (4H, m, 2CH_2-CH_3), 4.20 (2H, m, H5′), 4.07 (1H, m, H4′), 2.25 (2H, m, H2′), 1.88 (3H, s, CH_3-Thy), 1.57 and 1.52 (6H, 2m, 2CH_2CH_3). ^{31}P NMR (CD_3CN; δ, ppm; J, Hz): −4.84 s, −4.37 s. Mass: m/e [M^+] 431.1.

Method B. A solution of phosphonate **9** (80 mg, 0.2 mmol) in pyridine (3 mL), ethanol (0.5 mL) and TPSCl (120 mg, 0.4 mmol) were stirred for 16 h at room temperature and evaporated, and the residue was purified by silica gel column chromatography in a chloroform−methanol (96:4) system to give 58 mg (67%) of **1**.

2′,3′-Dideoxy-2′,3′-didehydrothymidine 5′-(ethyl)(ethoxycarbonyl)phosphonate 2 was prepared from d4T similarly to triester **1** by method A in a yield of 65%. ^{1}H NMR ($CDCl_3$):

8.50 (1H, s, NH), 7.29 and 7.28 (1H, 2s, H6), 7.01 (1H, m, H1′), 6.33 (1H, m, H2′), 5.90 (1H, m, H3′), 5.00 (1H, m, H4′), 4.43 (2H, m, H5′), 4.31 (4H, m, $2CH_2CH_3$), 1.89 (3H, s, CH_3-Thy), 1.38 and 1.33 (6H, 2t, *J* 7.2, $2CH_2CH_3$). ^{31}P NMR ($CDCl_3$): −3.56 s, −3.85 s. Mass: *m/e* [M^+] 388.1.

3′-Azido-3′-deoxythymidine 5′-(ethyl)(ethoxycarbonylmethyl)phosphonate 3a was prepared from AZT and triethylphosphonoacetate under conditions of method A in a yield of 72%. ^{1}H NMR ($CDCl_3$): 9.52 and 9.50 (1H, s, NH), 7.45 and 7.37 (1H, 2s, H6), 6.21 (1H, t, *J* 6.5, H1′), 4.33 (3H, m, H3′ + H5′), 4.17 (4H, m, $2CH_2CH_3$), 4.01 (1H, m, H4′), 3.01 (2H, d, *J* 21.2, CH_2P), 2.37 (2H, m, H2′), 1.91 and 1.90 (3H, 2s, CH_3-Thy), 1.34 and 1.26 (6H, 2t, *J* 7.2, $2CH_2CH_3$). ^{31}P NMR ($CDCl_3$): 21.44 s, 20.82 s. Mass: *m/e* [M^+] 445.1.

3′-Azido-3′-deoxythymidine 5′-(cyclohexyl)(ethoxycarbonylmethyl)phosphonate 3b was prepared from phosphonate **11** and cyclohexanol under conditions of method **B** in a yield of 46%. ^{1}H NMR (CD_3CN): 9.41 (1H, s, NH), 7.45 and 7.38 (1H, 2q, *J* 1.2, H6), 6.16 and 6.15 (1H, 2t, *J* 6.8, H1′), 4.46 (1H, m, CH (cyclohexyl)) 4.37 (1H, m, H3′), 4.25 (2H, m, H5′), 4.14 (2H, m, CH_2CH_3), 4.03 (1H, m, H4′), 3.03 and 3.02 (2H, 2d, *J* 21.5, CH_2P), 2.38 (2H, m, H2′), 1.90 (2H, m, cyclohexyl), 1.86 (3H, br.s, CH_3-Thy), 1.70, 1.50, 1.32 (8H, 3m, cyclohexyl), 1.24 and 1.23 (3H, 2t, *J* 7.2, CH_2CH_3). ^{31}P NMR (CD_3CN): 20.83 s, 20.52 s. Mass: *m/e* [M^+] 499.2.

2′,3′-Dideoxy-2′,3′-didehydrothymidine 5′-(ethyl)(ethoxycarbonylmethyl)phosphonate 4a was prepared from d4T and triethylphosphonoacetate by method A in a yield of 67%. ^{1}H NMR ($CDCl_3$): 8.98 (1H, s, NH), 7.34 and 7.27 (1H, 2s, H6), 7.00 (1H, m, H1′), 6.32 (1H, m, H3′), 5.88 (1H, m, H2′), 4.99 (1H, m, H4′), 4.49−4.28 (2H, m, H5′), 4.17−4.12 (4H, m, $2CH_2CH_3$), 2.97−2.90 (2H, m, CH_2P), 1.90 (3H, s, CH_3-Thy), 1.30 and 1.25 (6H, 2t, *J* 7.2, $2CH_2CH_3$). ^{31}P NMR ($CDCl_3$): 21.21 s, 21.03 s. Mass: *m/e* [M^+] 402.1.

2′,3′-Dideoxy-2′,3′-didehydrothymidine 5′-(cyclohexyl)(ethoxycarbonylmethyl)phosphonate 4b. Phosphonate **10** was treated with cyclohexanol under the conditions described in method B to give target **4b** in a yield of 39%. ^{1}H NMR ($CDCl_3$): 8.98 (1H, s, NH), 7.34 and 7.27 (1H, 2s, H6), 7.00 (1H, m, H1′), 6.32 (1H, m, H3′), 5.88 (1H, m, H2′), 4.99 (1H, m, H4′), 4.49−4.28 (2H, m, H5′), 4.22 (1H, m, CH (cyclohexyl)), 4.17−4.12 (2H, m, CH_2CH_3), 2.97−2.90 (2H, m, CH_2P), 1.90 (3H, s, CH_3-Thy), 1.70, 1.58−1.48, 1.33, 1.28 (10H, 4m, $5CH_2$ (cyclohexyl)), 1.25 (3H, m, CH_2CH_3). ^{31}P NMR ($CDCl_3$): 19.91 s, 19.74 s. Mass: *m/e* [M^+] 456.16.

3′-Azido-3′-deoxythymidine 5′-(ethyl)(aminocarbonyl)-phosphonate 5a was prepared from compound **13** and ethanol by method B in a yield of 72%. ^{1}H NMR (CD_3CN): 9.43 (1H, s, NH-Thy), 7.41 and 7.39 (1H, 2q, *J* 1, H6), 7.24 (1H, br.s, Ha (NH_2)), 6.83 (1H, d, $^3J_{H-P}$ 34.9, Hb (NH_2)), 6.14 (1H, t, *J* 6.5, H1′), 4.41 (1H, m, H3′), 4.34 (2H, m, H5′), 4.21 (2H, m, $2CH_2CH_3$), 4.03 (1H, m, H4′), 2.39 (2H, m, H2′), 1.85 (3H, m, CH_3-Thy), 1.33 and 1.32 (3H, 2t, *J* 7, $2CH_2CH_3$). ^{31}P NMR (CD_3CN): −0.08 s, −0.35 s. Mass: *m/e* [M^+] 402.1.

3′-Azido-3′-deoxythymidine 5′-(ethyl)(methylaminocarbonyl)phosphonate 5b was prepared from compound **14** and ethanol by method B in a yield of 64%. ^{1}H NMR (CD_3CN): 9.47 (1H, s, NH-Thy), 7.72 (1H, m, CH_3NH), 7.43 (1H, s, H6), 6.22 (1H, t, *J* 6.8, H1′), 4.40 (3H, m, H3′ + H5′), 4.22 (2H, m, CH_2-CH_3), 4.01 (1H, m, H4′), 2.91 and 2.89 (3H, 2s, CH_3N), 2.39 (2H, m, H2′), 1.94 and 1.93 (3H, 2s, CH_3-Thy), 1.37 (3H, m, CH_2CH_3). ^{31}P NMR (CD_3CN): −0.10 s, −0.54 s. Mass: *m/e* [M^+] 416.1.

3′-Azido-3′-deoxythymidine 5′-(ethyl)[(2-phenylethyl)-aminocarbonyl]phosphonate 5c was prepared from compound **15** and ethanol by method B in a yield of 70%. ^{1}H NMR (CD_3CN): 9.43 (1H, s, NH-Thy), 7.62 (1H, m, CH_2NH), 7.41 and 7.40 (1H, 2s, H6), 7.25 (5H, m, Ph), 6.15 (1H, t, *J* 6.8, H1′), 4.33 (1H, m, H3′), 4.27 (2H, m, H5′), 4.12 (2H, m, CH_2-CH_3), 4.00 (1H, m, H4′), 3.53 (2H, s, CH_2NH), 2.83 (2H, t, *J* 7, CH_2Ph), 2.36 (2H, m, H2′), 1.86 and 1.84 (3H, 2s, CH_3-Thy), 1.28 (3H, m, CH_2CH_3). ^{31}P NMR (CD_3CN): 0.04 s, −0.40 s. Mass: *m/e* [M^+] 506.2.

2′,3′-Dideoxy-2′,3′-didehydrothymidine 5′-(Ethyl)(aminocarbonyl)phosphonate 6a. Method C. Triphenylphosphine (130 mg, 0.5 mmol) and ethanol (100 μL) were added to a solution of phosphonate **16** (170 mg, 0.5 mmol) in DMF (2 mL), the solution was cooled to 0 °C, and DEAD (0.5 mmol, 77 μL) solution in THF (1 mL) was added. The reaction mixture was kept at room temperature for 5 h and evaporated; the residue was dissolved in water (5 mL) and extracted with chloroform (3 mL × 3). The aqueous layer was chromatographed on a LiChroprep RP-8 column eluting in a gradient of MeOH in water (0−10%) to give 124 mg (69%) of phosphonate **6a**. ^{1}H NMR (CD_3OD): 7.35 (1H, s, H6), 6.96 (1H, m, H1′), 6.37 (1H, m, H2′), 5.94 (1H, m, H3′), 5.02 (1H, m, H4′), 4.37 (2H, m, H5′), 4.19 (2H, m, CH_2CH_3), 1.88 (3H, s, CH_3-Thy), 1.35 (3H, m, CH_2CH_3). ^{31}P NMR (CD_3OD): 0.15 s, −0.12 s. Mass: *m/e* [M^+] 359.1.

2′,3′-Dideoxy-2′,3′-didehydrothymidine 5′-(ethyl)(methylaminocarbonyl)phosphonate 6b was obtained from 2′,3′-dideoxy-2′,3′-didehydrothymidine 5′-(methylaminocarbonyl)-phosphonate **17** and ethanol by method C and was isolated by chromatography on a silica gel column (2.5 × 30 cm) eluting in a gradient of MeOH in chloroform (0−10%) to give 48% of phosphonate **6b**. ^{1}H NMR (CD_3CN): 8.92 (1H, s, NH-Thy), 7.53 (1H, m, CH_3NH), 7.33 and 7.31 (1H, 2s, H6), 7.00 (1H, m, H1′), 6.28 (1H, m, H2′), 5.88 (1H, m, H3′), 4.97 (1H, m, H4′), 4.41 (2H, m, H5′), 4.19 (2H, m, CH_2CH_3), 2.87 and 2.86 (3H, s, CH_3N), 1.91 (3H, s, CH_3-Thy), 1.34 (3H, m, CH_2CH_3). ^{31}P NMR (CD_3CN): −0.27 s, −0.76 s. Mass: *m/e* [M^+] 373.1.

2′,3′-Dideoxy-2′,3′-didehydrothymidine 5′-(ethyl)[(2-phenylethyl)aminocarbonyl]phosphonate 6c was prepared from 2′,3′-dideoxy-2′,3′-didehydrothymidine 5′-[(2-phenylethyl)aminocarbonyl]phosphonate **18** and ethanol in a yield of 51% by method C and 72% by method D. ^{1}H NMR ($CDCl_3$): 9.17 (1H, s, NH-Thy), 7.45 (1H, s, CH_2NH), 7.33 and 7.31 (1H, 2s, H6), 7.45−7.17 (5H, m, Ph), 6.99 (1H, br.s, H1′), 6.27 and 6.17 (1H, 2m, H2′), 5.84 (1H, m, H3′), 4.91 (1H, m, H4′), 4.29 (2H, m, H5′), 4.09 (2H, m, CH_2CH_3), 3.62 (2H, m, CH_2N), 2.85 (2H, t, *J* 7, CH_2Ph), 1.90 and 1.91 (3H, 2s, CH_3-Thy), 1.29 (3H, m, CH_2CH_3). Mass: *m/e* [M^+] 463.2.

3′-Azido-3′-deoxythymidine 5′-(ethyl)(aminocarbonylmethyl)phosphonate 7 was prepared from phosphonate **19** and ethanol by method C in a yield of 77%. ^{1}H NMR (CD_3CN): 10.03 (1H, s, NH-Thy), 7.48 and 7.44 (1H, 2q, *J* 1, H6), 6.68 (1H, s, Ha (NH_2)), 6.44 and 6.43 (1H, 2s, Hb (NH_2)), 6.12 and 6.13 (1H, 2t, *J* 6.5, H1′), 4.47 and 4.42 (1H, 2m, H3′), 4.31−4.23 (2H, m H5′), 4.15 (2H, m, CH_2CH_3), 4.03 (1H, m, H4′), 2.94 and 2.93 (2H, 2d, *J* 21.5, CH_2P), 2.39 (2H, m, H2′), 1.84 (3H, br.s, CH_3-Thy), 1.30 (3H, t, *J* 7, CH_2CH_3). ^{31}P NMR (CD_3CN): 24.36s, 24.03 s. Mass: *m/e* [M^+] 416.1.

2′,3′-Dideoxy-2′,3′-didehydrothymidine 5′-(ethyl)(aminocarbonylmethyl)phosphonate 8 was prepared from 2′,3′-dideoxy-2′,3′-didehydrothymidine 5′-(aminocarbonylmethyl)-phosphonate **20** and ethanol by method C in a yield of 67%. ^{1}H NMR (CD_3CN): 10.02 (1H, s, NH-Thy), 8.78 (1H, s, NH), 7.32 and 7.29 (1H, 2s, H6), 6.89 (1H, m, H1′), 6.37 (1H, m, H3′), 5.92 (1H, m, H2′), 4.98 (1H, m, H4′), 4.28−4.18 (2H, m, H5′), 4.17−4.02 (2H, m, CH_2CH_3), 2.85−2.79 (2H, m, CH_2P), 1.85 and 1.84 (3H, 2s, CH_3-Thy), 1.25 (6H, m, CH_2CH_3). ^{31}P NMR (CD_3CN): 25.36 s, 25.16 s. Mass: *m/e* [M^+] 373.1.

3′-Azido-3′-deoxythymidine 5′-(ethoxycarbonyl)phosphonate 9. To a solution of AZT (133 mg, 0.5 mmol) and pyridinium salt of ethoxycarbonylphosphonic acid (151 mg, 0.65 mmol) in pyridine (4 mL) was added DCC (436 mg, 2 mmol), and the suspension was stirred for 18 h at room temperature. The mixture was diluted with water (10 mL), the precipitate was filtered, and the filtrate was purified on a DEAE-Toyopearl column in a linear gradient of NH_4HCO_3 (0−0.15 M). Compound **9** was obtained in a yield of 169 mg (81%). ^{1}H NMR (D_2O): 7.58 (1H, s, H6), 6.15 (1H, t, *J* 7.5, H1′), 4.40 (1H, m, H3′), 4.10 (2H, m, CH_2CH_3), 4.07 (3H, m, H4′ + H5′), 2.38 (2H, m, H2′), 1.78 (3H, s, CH_3-Thy), 1.11 (3H, t, *J* 7.1, CH_2CH_3). ^{31}P NMR (D_2O): −4.93 s.

2′,3′-Dideoxy-2′,3′-didehydrothymidine 5′-ethoxycarbonylphosphonate 10 was obtained from d4T and pyridinium salt of ethoxycarbonylphosphonic acid under the

conditions described for **9** in a yield of 79%. ^{1}H NMR (D_2O): 7.54 (1H, q, *J* 1, H6), 6.78 (1H, m, H1′), 6.31 (1H, m, H2′), 5.79 (1H, m, H3′), 4.91 (1H, m, H4′), 3.98 (4H, m, H5′ + CH_2-CH_3), 1.75 (3H, d, CH_3-Thy), 1.04 (3H, t, *J* 7.2, CH_2CH_3). ^{31}P NMR (D_2O): −5.16 s.

3′-Azido-3′-deoxythymidine 5′-(ethoxycarbonylmethyl)phosphonate 11 was prepared from AZT, pyridinium salt of ethoxycarbonylmethylphosphonic acid, and DCC similarly to **9** in a yield of 69%. ^{1}H NMR (D_2O): 7.77 (1H, s, H6), 6.32 (1H, t, *J* 6.6, H1′), 4.55 (1H, m, H3′), 4.27−4.19 (5H, m, H4′ + H5′ + CH_2CH_3), 2.96 (2H, d, *J* 20.6, CH_2P), 2.57 (2H, m, H2′), 1.99 (3H, br.s, CH_3-Thy), 1.31 (3H, t, *J* 7.2, CH_2CH_3). ^{31}P NMR (D_2O): 15.09 s.

2′,3′-Dideoxy-2′,3′-didehydrothymidine 5′-(ethoxycarbonylmethyl)phosphonate 12 was obtained from d4T and pyridinium salt of ethoxycarbonylmethylphosphonic acid under the conditions described for **9** in a yield of 78%. ^{1}H NMR (D_2O): 7.45 (1H, q, *J* 1, H6), 6.78 (1H, m, H1′), 6.31 (1H, m, H2′), 5.79 (1H, m, H3′), 4.91 (1H, m, H4′), 3.93 (4H, m, H5′ + CH_2CH_3), 2.63 (2H, d, *J* 20.4, CH_2P), 1.72 (3H, d, CH_3-Thy), 1.04 (3H, t, *J* 7.2, CH_2CH_3). ^{31}P NMR (D_2O): 14.86 s.

3′-Azido-3′-deoxythymidine 5′-(aminocarbonyl)phosphonate 13. A solution of phosphonate **9** (80 mg, 0.2 mmol) in 25% aqueous ammonia (3 mL) was kept for 18 h at 20 °C and evaporated. The residue was dissolved in water and purified on a DEAE-Toyopearl column in a linear gradient of NH_4HCO_3 (0−0.1 M) to give 70 mg (94%) of phosphonate **13**. ^{1}H NMR (DMSO-d_6): 7.82 (1H, m, H6), 7.17 and 7.13 (2H, 2s, NH_2), 6.12 (1H, t, *J* 6.9, H1′), 4.50 (1H, m, H3′), 3.95 (3H, m, H4′ + H5′), 2.30 (2H, m, H2′), 1.81 (3H, br.s, CH_3-Thy). ^{31}P NMR (DMSO-d_6): −1.56 s.

3′-Azido-3′-deoxythymidine 5′-(methylaminocarbonyl)phosphonate 14. A solution of phosphonate **9** (80 mg, 0.2 mmol) in 25% aqueous methylamine (3 mL) was kept for 1 h at 20 °C and evaporated. The residue was dissolved in water and purified on a DEAE-Toyopearl column in a linear gradient of NH_4HCO_3 (0−0.2 M) to give 71 mg (91%) of phosphonate **14**. ^{1}H NMR (D_2O): 7.52 (1H, s, H6), 6.06 (1H, t, *J* 6.7, H1′), 4.32 (1H, m, H3′), 4.01 (3H, m, H4′ + H5′), 2.61 (3H, s, CH_3N), 2.32 (2H, m, H2′), 1.72 (3H, s, CH_3-Thy). ^{31}P NMR (D_2O): −1.40 s.

3′-Azido-3′-deoxythymidine 5′-[(2-Phenylethyl)aminocarbonyl]phosphonate 15. A solution of **9** (80 mg, 0.2 mmol) in 2-phenylethylamine (1 mL) was stirred for 4 h at 20 °C and evaporated. The residue was dissolved in water and purified on a DEAE-Toyopearl column in a linear gradient of NH_4HCO_3 (0−0.15 M) to give 82 mg (86%) of phosphonate **15**. ^{1}H NMR (D_2O): 7.42 (1H, s, H6), 7.17−7.05 (5H, m, Ph), 6.02 (1H, t, *J* 6.5, H1′), 4.17 (1H, m, H3′), 3.90 (1H, m, H4′), 3.81 (2H, m, H5′), 3.39 (2H, m, CH_2N), 2.69 (2H, t, *J* 6.5, CH_2Ph), 2.24 (2H, m, H2′), 1.68 (3H, s, CH_3). ^{31}P NMR (D_2O): −1.57 s.

2′,3′-Dideoxy-2′,3′-didehydrothymidine 5′-(aminocarbonyl)phosphonate 16. Treatment of 2′,3′-dideoxy-2′,3′-didehydrothymidine 5′-(ethoxycarbonyl)phosphonate **10** with 25% ammonia analogously to the preparation of **13** yielded 96% of the title phosphonate. ^{1}H NMR (D_2O): 7.51 (1H, s, H6), 6.88 (1H, m, H1′), 6.41 (1H, m, H2′), 5.91 (1H, m, H3′), 5.04 (1H, m, H4′), 4.09 (2H, m, H5′), 1.81 (3H, br.s, CH_3-Thy). ^{31}P NMR (D_2O): −1.62 s.

2′,3′-Dideoxy-2′,3′-didehydrothymidine 5′-(Methylaminocarbonyl)phosphonate 17. Treatment of 2′,3′-dideoxy-2′,3′-didehydrothymidine 5′-(ethoxycarbonyl)phosphonate **10** with 25% aqueous $MeNH_2$ yielded 92% of 2′,3′-dideoxy-2′,3′-didehydrothymidine 5′-(methylaminocarbonyl)phosphonate **17**, which was further used without purification. ^{1}H NMR (D_2O): 7.43 (1H, s, H6), 6.85 (1H, m, H1′), 6.35 (1H, m, H2′), 5.86 (1H, m, H3′), 4.97 (1H, m, H4′), 4.03 (2H, m, H5′), 2.61 (3H, s, CH_3N), 1.76 (3H, s, CH_3-Thy). ^{31}P NMR (D_2O): −1.53 s.

2′,3′-Dideoxy-2′,3′-didehydrothymidine 5′-[(2-Phenylethyl)aminocarbonyl]phosphonate 18. Treatment of 2′,3′-dideoxy-2′,3′-didehydrothymidine 5′-(ethoxycarbonyl)phosphonate **10** with 2-phenylethylamine yielded crude 2′,3′-dideoxy-2′,3′-didehydrothymidine 5′-[(2-phenylethyl)aminocarbonyl]-phosphonate **18**, which was purified on a DEAE-Toyopearl column in a linear gradient of NH_4HCO_3 (0−0.2 M). The yield was 86%. ^{1}H NMR (D_2O): 7.43 (1H, s, H6), 7.40−7.17 (5H, m, Ph), 6.89 (1H, m, H1′), 6.25 (1H, m, H2′), 5.86 (1H, m, H3′), 4.95 (1H, m, H4′), 4.10 (2H, m, H5′), 3.60 (2H, m, CH_2N), 2.61 (2H, t, *J* 7, CH_2Ph), 1.86 (3H, s, CH_3). ^{31}P NMR (D_2O): −1.47 s.

3′-Azido-3′-deoxythymidine 5′-(aminocarbonylmethyl)phosphonate 19. A solution of phosphonate **11** (85 mg, 0.2 mmol) in 25% aqueous ammonia (3 mL) was kept for 18 h at 20 °C and evaporated. The residue was dissolved in water and purified on a DEAE-Toyopearl column in a linear gradient of NH_4HCO_3 (0−0.1 M) to give 54 mg (68%) of phosphonate **19**. ^{1}H NMR (D_2O): 7.56 (1H, s, H6), 6.10 (1H, t, *J* 6.6, H1′), 4.35 (1H, m, H3′), 4.03−4.00 (3H, m, H4′ + H5′), 2.63 (2H, d, *J* 20.6, CH_2P), 2.34 (2H, m, H2′), 1.76 (3H, s, CH_3). ^{31}P NMR (D_2O): 16.26 s.

2′,3′-Dideoxy-2′,3′-didehydrothymidine 5′-(Aminocarbonylmethyl)phosphonate 20. Treatment of 2′,3′-dideoxy-2′,3′-didehydrothymidine 5′-(ethoxycarbonylmethyl)phosphonate **12** with 25% ammonia yielded 94% of 2′,3′-dideoxy-2′,3′-didehydrothymidine 5′-(aminocarbonylmethyl)phosphonate **20**. ^{1}H NMR (D_2O): 7.45 (1H, s, H6), 6.78 (1H, m, H1′), 6.31 (1H, m, H2′), 5.79 (1H, m, H3′), 4.91 (1H, m, H4′), 3.93 (2H, m, H5′), 2.63 (2H, d, *J* 20.4, CH_2P), 1.72 (3H, br.s, CH_3-Thy). ^{31}P NMR (D_2O): 16.86 s.

Stability Studies. Hydrolysis was carried out in the 0.05 M phosphate buffer at pH 7.2 and 20 °C at the concentrations of the compounds under study of 0.5 mM. The aliquots (10 μL) were taken out after certain intervals and frozen in liquid nitrogen, and the products were analyzed by HPLC.

The chemical stability of amide **5a** was also studied in 0.05 M sodium phosphate buffer at 37 °C and pH in the range of 5.5−7.8 (the concentration of amide **5a** was 0.45 mM). The reaction mixture was treated and analyzed by HPLC under the conditions described above.

The enzymatic hydrolysis was studied in the mixture (200 μL) containing 0.1 M Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, and 0.45 mM amide **5a**, incubated at 37 °C in the presence of either snake venom phosphodiesterase (0.1 mg/mL) or *Crotalus atrox* snake venom (1 mg/mL). The reaction mixture was treated and analyzed by HPLC under the conditions described above.

The reaction mixture (200 μL) containing 0.1 M acetate buffer (pH 6.0), 0.45 mM amide **5a**, and calf spleen phosphodiesterase (0.5 mg/mL) was incubated at 37 °C. The reaction mixture was treated and analyzed by HPLC as described above.

The reaction mixture (200 μL) containing 0.02 M Tris-HCl buffer (pH 7.2), 0.45 mM amide **5a**, and P1 nuclease (0.1 mg/mL) was incubated at 37 °C. The reaction mixture was treated and analyzed by HPLC as described above.

Antiviral Activity. MT-4 cells were infected with HIV-1 strain (GKV-4046). The multiplicity of infection was 0.2−0.5 units per cell. MT-4 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 300 μg/mL L-glutamine, 80 μg/mL gentamycin, and 30 μg/mL lincomycin at 37 °C in 5% CO_2 atmosphere. For infecting, the cells at the concentration 2×10^6 cell/mL and viability exceeding 90% were used.

Cytotoxicity. MT-4 cells were cultured in the presence of various doses of the tested compounds (0.001−200 μg/mL, three replicates for each dose), on a 96-well cultural plate for 3 days. The concentration and viability of MT-4 cells were measured by the trypan blue-dye exclusion colorimetric assay, and the CD_{50} for each compound was calculated.

Anti-HIV Assay. The infected cells were cultured in the presence of various doses of the tested compounds (0.001−100 μg/mL, three replicates for each dose) on a 96-well cultural plate for 3 days. Anti-HIV activity of the tested compounds was assessed by the measurement of p24 antigen amount using immunoassay.[23] The cell concentration and viability were estimated using the colorimetric assay as described above.

Acknowledgment. The work was supported by the Russian Foundation for Basic Research, projects 02-04-

49009 and 03-04-06712; Russian State AIDS program, project 31; and the program of Presidium of Russian Academy of Sciences (Physicochemical Biology).

References

(1) De Clercq, E. New development in anti-HIV chemotherapy. *Biochim. Biophys. Acta* **2002**, *1587*, 258−275.
(2) Zemlicka, J. Lipophilic phosphoramidates as antiviral pronucleotides. *Biochim. Biophys. Acta* **2002**, *1587*, 276−286.
(3) Perigaud C.; Gosselin G.; Imbach J.-L. Minireview: from the pronucleotide concept to the SATE phosphate protecting groups. *Curr. Top. Med. Chem*. **1997**, *2*, 15−29.
(4) Siddiqui, A. Q.; McGuigan, C.; Ballatore, C.; Zuccotto, F.; Gilbert, I. H.; De Clercq, E.; Balzarini, J. Design and synthesis of lipophilic phosphoramidate d4T-MP prodrugs expressing high potency against HIV in cell culture: structural determinants for in vitro activity and QSAR. *J. Med. Chem*. **1999**, *42*, 4122−4128.
(5) Wagner, C. R.; Iyer V. V.; McIntee, E. J. Pronucleotides: toward the in vivo delivery of antiviral and anticancer nucleotides. *Med. Res. Rev*. **2000**, *20*, 417−451
(6) Mavromoustakos, T.; Calogeropoulou, T.; Koufaki, M.; Kolocouris, A.; Daliani, I.; Demetzos, C.; Meng, Z.; Makriyannis, A.; Balzarini, J.; De Clercq, E. Ether phospholipid-AZT conjugates possessing anti-HIV and antitumor cell activity. Synthesis, conformational analysis, and study of their thermal effects on membrane bilayers. *J. Med. Chem*. **2001**, *44*, 1702−1709.
(7) Balzarini, J.; Aquaro, S.; Knispel, T.; Rampazzo, C.; Bianchi, V.; Perno, C. F.; De Clercq, E.; Meier, C. Cyclosaligenyl-2′,3′-didehydro-2′,3′-dideoxythymidine monophosphate: efficient intracellular delivery of d4TMP. *Mol. Pharmacol*. **2000**, *58*, 928−35.
(8) Cardona, V. M.; Ayi, A. I.; Aubertin, A. M.; Guedj, R. Synthesis and anti-HIV activity of some novel aryl phosphate and H-phosphonate derivatives of 3′-azido-2′,3′-dideoxythymidine and 2′,3′-didehydro-2′,3′-dideoxythymidine. *Antiviral Res*. **1999**, *42*, 189−196.
(9) Machado J.; Salomon H.; Oliveira M.; Tsoukas C.; Krayevsky A. A.; Wainberg M. A. Antiviral activity and resistance profile of phosphazid—a novel prodrug of AZT. *Nucleosides Nucleotides* **1999**, *18*, p 901−6.
(10) Parang, K.; Wiebe, L. I.; Knaus, E. E. Novel approaches for designing 5′−O-ester prodrugs of 3′-azido-2′,3′-dideoxythymidine (AZT). *Curr. Med. Chem*. **2000**, *7*, 995−1039.
(11) Rozowsky, A.; Saha, J.; Fazely, F.; Koch, J.; Ruprecht, R. M. Inhibition of human immunodeficiency virus type 1 replication by phosphonoformate esters of 3′-azido-3′-deoxythymidine. *Biochem. Biophys. Res. Commun*. **1990**, *172*, 288−294.
(12) Lambert, R. W.; Martin, J. A.; Thomas, G. J.; Duncan, I. B.; Hall, M. J.; Heimer, E. P. Synthesis and antiviral activity of phosphonoacetic and phosphonoformic acid esters of 5-bromo-2′-deoxyuridine and related pyrimidine nucleosides and acyclonucleosides. *J. Med. Chem*. **1989**, *32*, 367−374.
(13) Jasko, M.; Shipitsin, A.; Shirokova, E.; Kraevsky, A.; Polsky, B.; Baron, P.; MacLow, C.; Ostrander, M.; O'Hara, B. 5′-Phosphonates of ribonucleosides and 2′-deoxyribonucleosides: synthesis and antiviral activity. *Nucleosides Nucleotides* **1993**, *12*, 879−893.
(14) Karpenko, I. L.; Jasko, M. V.; Andronova, V. L.; Ivanov, A. V.; Kukhanova, M. K.; Galegov, G. A.; Skoblov, Y. S. Synthesis and antiherpetic activity of acyclovir phosphonates. *Nucleosides, Nucleotides Nucleic Acids* **2003**, *22*, 319−328.
(15) Campbell, D. A. The synthesis of phosphonate esters, an extension of the Mitsunobu reaction. *J. Org. Chem*. **1992**, *57*, 6331−6335.
(16) Miles, D. W.; Robins, M. J.; Robins, R. K.; Winkley, M. W.; Eyring, H. Circular dichroism of nucleoside derivatives. V. Cytosine derivatives. *J. Am. Chem. Soc*. **1969**, *91*, 831−838.
(17) Rozowsky, A.; Fu, H.; Pai, N.; Mellors, J.; Richman, D. D.; Hostetler, K. Y. Synthesis and in vitro activity of long-chain 5′-O−((alkoxycarbonyl)phosphinyl)-3′-azido-3′-deoxythymidines against wild-type and AZT- and Foscarnet-resistant strains of HIV-1. *J. Med. Chem*. **1997**, *40*, 2482−2490.
(18) Meier, C.; Aubertin, A. M.; de Monte, M.; Faraj, A.; Sommadossi, J. P.; Perigaud, C.; Imbach, J. L.; Gosselin, G. Synthesis and antiviral evaluation of SATE-foscarnet prodrugs and new foscarnet-AZT conjugates. *Antivir. Chem. Chemother*. **1998**, *9*, 41−52.
(19) Noren, J. O.; Helgstrand, E.; Johansson, N. G.; Misiorny, A.; Stening G. Synthesis of esters of phosphonoformic acid and their antiherpes activity. *J. Med. Chem*. **1983**, *26*, 264−270.
(20) Kukhanova, M. K.; Tarussova, N. B.; Jasko, M. V.; Arzumanov, A. A.; Gudima, S. O.; Kraevsky, A. A.; Chidgevadze, Z. G.; Beabealashvilli, R. Sh. Hydrolysis of 5′-phosphonates by phoshatases of different origin and human or calf blood sera. *Molekulyarnaya Biol. (Russian)* **1992**, *26*, 1148−1159.
(21) Plotto, M.; Saudek, V.; Sklenar V. Gradient tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *J. Biomol. NMR* **1992**, *2*, 661−665.
(22) Cavanagh, J.; Fairbrother, W. J.; Palmer, A. G.; Skelton, N. J. *Protein NMR spectroscopy: principles and practice*; Academic Press: San Diego, 1996.
(23) Svinarchik, F. P.; Konevetz, D. A.; Plyasunova, O. A.; Pokrovsky, A. G.; Vlassov, V. V. Inhibition of HIV proliferation by antisense oligonucleotide conjugated to lipophilic groups. *Biochimie* **1993**, *75*, 49−54.

JM0310176