Mononucleoside Phosphotriester Derivatives with S-Acyl-2-thioethyl Bioreversible Phosphate-Protecting Groups: Intracellular Delivery of 3′-Azido-2′,3′-dideoxythymidine 5′-Monophosphate

Isabelle Lefebvre,† Christian Périgaud,† Alain Pompon,† Anne-Marie Aubertin,‡ Jean-Luc Girardet,† André Kirn,‡ Gilles Gosselin,† and Jean-Louis Imbach*,†

Laboratoire de Chimie Bioorganique, URA CNRS 488, Université Montpellier II, Sciences et Techniques du Languedoc, 34095 Montpellier Cedex 5, France, and Institut de Virologie de la Faculté de Médecine de Strasbourg, Unité INSERM 74, 3 rue Koeberlé, 67000 Strasbourg, France

Received May 16, 1995®

The synthesis, *in vitro* anti-HIV-1 activity, and decomposition pathways of several mononucleoside phosphotriester derivatives of 3′-azido-2′,3′-dideoxythymidine (AZT) incorporating a new kind of carboxylate esterase-labile transient phosphate-protecting group, namely, *S*-acyl-2-thioethyl, are reported. All the described compounds showed marked antiviral activity in thymidine kinase-deficient CEM cells in which AZT was virtually inactive. The results strongly support the hypothesis that such pronucleotides exert their biological effects via intracellular delivery of the 5′-mononucleotide of AZT. This point was corroborated by decomposition studies in cell extracts and culture medium.

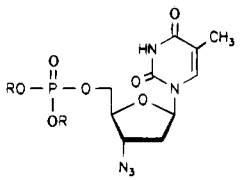
Introduction

In the search for effective agents against human immunodeficiency virus (HIV), a large number of dideoxynucleosides (ddNs) have emerged as efficient drugs.[1] The anti-retroviral effects of these compounds involve their conversion, through cellular enzymes, to the corresponding 5′-triphosphates (ddNTPs) which interact with HIV-associated reverse transcriptase (RT). At the RT level, ddNTPs may act as competitive inhibitors, preventing the incorporation of the natural substrates (dNTPs), or as alternate substrates incorporated in the growing DNA chain, leading to termination of the newly synthesized viral nucleic acid. The factors influencing the efficiency of ddNTP formation may be of equal or even greater importance than the differences in the relative abilities of ddNTPs to inhibit the RT (abilities which fall within a narrow range).[2] In many cases, ddNs have a poor affinity for nucleoside kinases or 5′-nucleotidases.[3] Moreover, the presence and activity of the intracellular enzymes necessary for the phosphorylation of ddNs are highly dependent on the host species, the cell type, and the stage in the cell cycle.[4] The dependence on phosphorylation for the activation of ddNs may explain the low activity of some of them and may thus be a problem in cells where the activity of phosphorylating enzymes is known to be low or even lacking.[4] One possibility to improve the efficiency of ddNs could be to bypass the phosphorylation steps. Unfortunately, nucleotides, due to their polar nature, are not able to cross the cell membrane efficiently.[5] Moreover, they are readily dephosphorylated in extracellular fluids and on cell surfaces by nonspecific phosphohydrolases.[6] Hence, the idea of temporarily masking or reducing the phosphate negative charges of nucleoside 5′-monophosphates (NMPs) with neutral substituents, thereby forming more lipophilic derivatives which would be expected to revert back to the corresponding NMPs once inside the cell, emerged. A large number of prodrug derivatives of NMPs (pronucleotides) have been prepared.[7] In this respect, recent examples of pronucleotides that display *in vitro* anti-HIV activity include mononucleoside phosphotriesters incorporating enzyme-labile transient phosphate-protecting groups. For instance, (acyloxy)alkyl or (acyloxy)aryl groups have been already studied as carboxylate esterase-mediated bioreversible phosphate protection,[8] and among them the (pivaloyloxy)methyl (POM) has been proposed as an efficient transient protecting group for several bioactive nucleoside monophosphate or phosphonate analogues.[9] We have previously reported the study of neutral mononucleoside phosphotriesters which incorporate the *S*-[(2-hydroxyethyl)sulfidyl]-2-thioethyl (DTE) group as another kind of enzyme-labile phosphate protection.[10] On the basis of several nucleoside models, we have demonstrated that mononucleoside bis(DTE) phosphotriester derivatives were able to liberate the NMPs inside the cell through a reductase-mediated activation process.

In this paper, we report the HIV-1 activity in wild-type [thymidine kinase positive (TK^+)] cells and thymidine kinase-deficient (TK^-) CEM cells of mononucleoside phosphotriester derivatives of 3′-azido-2′,3′-dideoxythymidine (AZT) incorporating a new kind of carboxylate esterase-labile transient phosphate-protecting group, namely, *S*-acyl-2-thioethyl (SATE).[11] In marked contrast to the parent nucleoside AZT, the novel pronucleotides **1a–d** showed potent anti-HIV activity in the TK^- cells. In TK^- cells, **1a** proved to be superior to the corresponding bis(DTE) ester (**2**) and bis(POM) ester (**3**) derivatives with regard to the antiviral selectivity. Furthermore, decomposition studies in culture medium and cell extracts of the bis(SATE) phosphotriesters **1a–c** corroborated their intracellular conversion to AZTMP.

* Address for correspondence: Laboratoire de Chimie Bioorganique, URA CNRS 488, case courrier 008, Université Montpellier II, Sciences et Techniques du Languedoc, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France. Tel: (33) 67 54 58 73. Fax: (33) 67 04 20 29.
† Université Montpellier II.
‡ Unité INSERM 74.
® Abstract published in *Advance ACS Abstracts*, August 15, 1995.

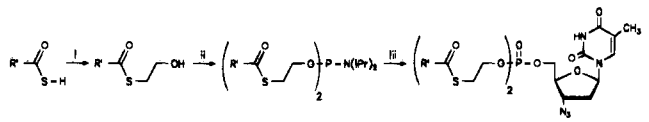
0022-2623/95/1838-3941$09.00/0 © 1995 American Chemical Society



compd	R	
1a	Me(SATE)	CH_3-C(O)-S-CH_2-CH_2
1b	iPr(SATE)	$(CH_3)_2$CH-C(O)-S-CH_2-CH_2
1c	tBu(SATE)	$(CH_3)_3$C-C(O)-S-CH_2-CH_2
1d	Ph(SATE)	C_6H_5-C(O)-S-CH_2-CH_2
2	DTE	HO-CH_2-CH_2-S-S-CH_2-CH_2
3	POM	$(CH_3)_3$C-C(O)-O-CH_2

Figure 1. Structure of the studied phosphotriester derivatives of AZT.

Scheme 1. Synthesis of Bis(SATE) Phosphotriester Derivatives (**1a–d**) of AZT[a]



4a (R'=Me)	**5a** (R'=Me)	**6a** (R'=Me)	**1a** (R'=Me)
4b (R'=iPr)	**5b** (R'=iPr)	**6b** (R'=iPr)	**1b** (R'=iPr)
4c (R'=tBu)	**5c** (R'=tBu)	**6c** (R'=tBu)	**1c** (R'=tBu)
4d (R'=Ph)	**5d** (R'=Ph)	**6d** (R'=Ph)	**1d** (R'=Ph)

[a] (i) ICH_2CH_2OH, DBU/$C_6H_5CH_3$; (ii) $Cl_2PN(iPr)_2$, NEt_3/THF; (iii) AZT, 1*H*-tetrazole/THF, then $ClC_6H_4CO_3H/CH_2Cl_2$.

Results

Chemistry. The synthesis of the mononucleoside bis(SATE), bis(DTE), and bis(POM) phosphotriesters described in Figure 1 involved several strategies imposed by the stability and the reactivity of the different enzyme-labile phosphate-protecting groups.

Phosphites are sensitive compounds and react with electrophiles and oxidizing agents; their high reactivity is due to the lone pair of electrons on the trivalent phosphorus atom. Largely used in oligodeoxynucleotide synthesis,[12] the P(III) chemistry using phosphoramidite intermediates is the most efficient method to prepare phosphate derivatives which are obtained in a single rapid step under milder conditions and with better yields than with P(V) chemistry. Recently, this approach has been used successfully for the synthesis of lipophilic phosphotriester derivatives of some nucleoside analogues.[13] In this work, we prepared the bis(SATE) phosphotriesters **1a–d** (Scheme 1) by a P(III) approach using appropriate phosphoramidite derivatives. The thioester precursors **5a–d** were synthesized by the reaction of 2-iodoethanol with the corresponding thioacid **4a–d**, commercially available except **4b,c** which were prepared according to a published procedure.[14] The P(III)-phosphorylating agents **6a–d** were obtained in yields of 40–74% by treatment of *N,N*-diisopropylphosphorodichloridite[15] with 2 equiv of the corresponding precursors **5a–d** in tetrahydrofuran in the presence of triethylamine followed by purification by flash column chromatography. Coupling of AZT with **6a–d** in the presence of 1*H*-tetrazole followed by *in situ* oxidation with 3-chloroperoxybenzoic acid gave the pronucleotides **1a–d** in yields of 58–75% after purification by silica gel column chromatography.

Regarding the pronucleotides **2** and **3** (Figure 1) which incorporate respectively the DTE and POM groups, these compounds were synthesized using P(V) chemistry. As reported, the bis(DTE) phosphotriester derivative of AZT **2** was prepared according to a phosphodiester method.[10] Concerning the bis(POM) phosphotriester derivative of AZT **3**, the physicochemical properties of this compound have not yet been described in detail.[16,17] The previously published approach for the synthesis of this pronucleotide was to react AZT with bis(POM) phosphate[18] using a Mitsunobu reaction.[17] However, the synthesis of bis(POM) phosphate, prepared analogously to a literature procedure for the 4-hydroxybut-2-enyl phosphate, required numerous steps with a low overall yield.[8b] Consequently, in this work, we preferred to synthesize the phosphotriester derivative **3** by direct alkylation of the free AZT 5′-monophosphate (AZTMP) with iodomethyl pivaloate[19] in dry acetonitrile in the presence of *N,N*-diisopropylethylamine.

Table 1. Antiviral Activity of the Phosphotriester Derivatives **1a–d**, **2**, and 3 Compared to AZT in Two Cell Lines Infected with HIV-1[a]

	CEM-SS			MT-4		
compd	EC_{50}[b]	CC_{50}[c]	SI[d]	EC_{50}[b]	CC_{50}[c]	SI[d]
1a	0.022	93	4300	0.050	67	1300
1b	0.046	>10[e]	>200	0.048	>10[e]	>200
1c	0.015	>10[e]	>670	0.81	>10[e]	>12
1d	0.125	>1[e]	>8	0.076	>1[e]	>13
2	0.023	>100	>4300	0.054	>100	>1850
3	0.077	67	860	0.052	59	1100
AZT	0.006	>100	>17300	0.017	75	4300

[a] All data represent average values for at least three separate experiments. The variation of these results under standard operating procedures is below ±10%. [b] EC_{50}, 50% effective concentration (μM) or concentration required to inhibit the replication of HIV-1 by 50%. [c] CC_{50}, 50% cytotoxic concentration (μM) or concentration required to reduce the viability of uninfected cells by 50%. [d] SI selectivity index, ratio CC_{50}/EC_{50}. [e] Highest concentration tested due to the relative low solubility of the mononucleoside phosphotriester derivatives **1b–d**.

Antiviral Activity. AZT and the phosphotriester derivatives **1a–d**, **2**, and **3** were evaluated for their inhibitory effects on the replication of HIV-1 in two cell culture systems (Table 1). Under the assay conditions, all compounds significantly inhibited the multiplication of HIV-1 in human T_4-lymphoblastoid cells, CEM-SS, and MT-4.

Striking differences were found in the antiviral activities of the test compounds in HIV-1-infected CEM/TK$^-$ cells (Table 2). This cell line is highly deficient in cytosol thymidine kinase and should be considered as an ideal target to investigate the antiviral activities of nucleotide analogues that may release the corresponding 5′-monophosphate derivative into the cells.[20] As expected, AZT proved to be completely inactive against HIV-1 replication in CEM/TK$^-$ cells at concentrations up to 100 μM. In contrast, the pronucleotides **1a–d** proved to be markedly inhibitory to HIV-1 replication in CEM/TK$^-$ cells. Compound **1a**, the bis(*S*-acetyl-2-thioethyl) ester of AZTMP, emerged as the most potent inhibitor with a 50% effective concentration (EC_{50}) value at 0.049 μM, which was in the same range as the EC_{50} values

Table 2. Antiviral Activity of the Phosphotriester Derivatives 1a–**d**, **2**, and 3 Compared to AZT in CEM/TK$^-$ Cells Infected with HIV-1[a]

compd	CEM/TK$^-$ EC_{50}[b]	CEM/TK$^-$ CC_{50}[c]	CEM/TK$^-$ SI[d]
1a	0.049	>100	>2050
1b	0.52	>10[e]	>17
1c	0.45	>10[e]	>22
1d	0.32	>1[e]	>4
2	0.55	>100	>180
3	0.255	65.5	250
AZT	>100	>100	ND[f]

[a–e] See the corresponding footnotes of Table 1. [f] ND, not determinable.

observed for this pronucleotide in wild-type TK$^+$ cell lines. Thus, compound **1a** was between 5- and 10-fold more effective than the previously described phosphotriesters **2** and **3**, incorporating respectively the DTE and POM enzyme-labile groups, in inhibiting HIV-1 replication in CEM-TK$^-$ cells. The inhibiting capacity was less for the other bis(SATE) phosphotriesters, **1b–d**, since their EC_{50} values were in the same range as those observed for the pronucleotides **2** and **3**.

Due to their low aqueous solubility in high concentration, the toxicity of the most lipophilic bis(SATE) derivatives, **1b–d**, in uninfected cells could not be determined accurately (Table 1). However, compound **1a** exhibited low cytotoxicity, with a 50% cytotoxic concentration (CC_{50}) higher than 100 μM for CEM/TK$^-$ cells (Table 2).

Stability and Decomposition Studies. The decomposition pathways and kinetic data of compounds **1a–c** (initial concentration 5×10^{-5} M) were studied at 37 °C in (i) RPMI 1640 containing 10% heat-inactivated fetal calf serum (culture medium, CM), in order to evaluate the stabilities of the three compounds in the extracellular medium used for antiviral evaluation in cell culture systems, and (ii) total cell extract (TCE) from CEM-SS cells, in order to mimic the behavior of the pronucleotides inside the cells. Additionally, **1a** was investigated in RPMI 1640 in order to differentiate between chemical and enzyme-controlled hydrolysis. Compound **1d** could not be examined, due to its poor solubility in aqueous media. The decomposition of AZTMP was also investigated in the cell extract.

Crude aliquots of incubates were HPLC analyzed by improving the "on-line ISRP cleaning" method previously described for the study of the pronucleotides **2**[10] and **3**[17] of AZT. In this on-line HPLC technique, a crude biological sample was directly injected by means of a buffer in a short precolumn of internal surface reverse-phase (ISRP) materials which did not retain proteins but retained the analytes of interest. Then, the precolumn was connected to an analytical column. When increasing the strength of the eluent (acetonitrile gradient), the analytes were transferred into the analytical column and chromatographed. In the present work, the ISRP materials of the cleaning precolumn were replaced with new reverse-phase (RP) materials (Guard-Pak, δ-pak C18, Waters). According to numerous assays, this phase was selected because it did not retain proteins in aqueous buffer, contrary to other RP materials. This property is likely to be due to the high hydrophobicity of this phase, free of residual silanols. The high stability of these materials allowed the technique to be used in the presence of ion-pairing reagents. Therefore, two

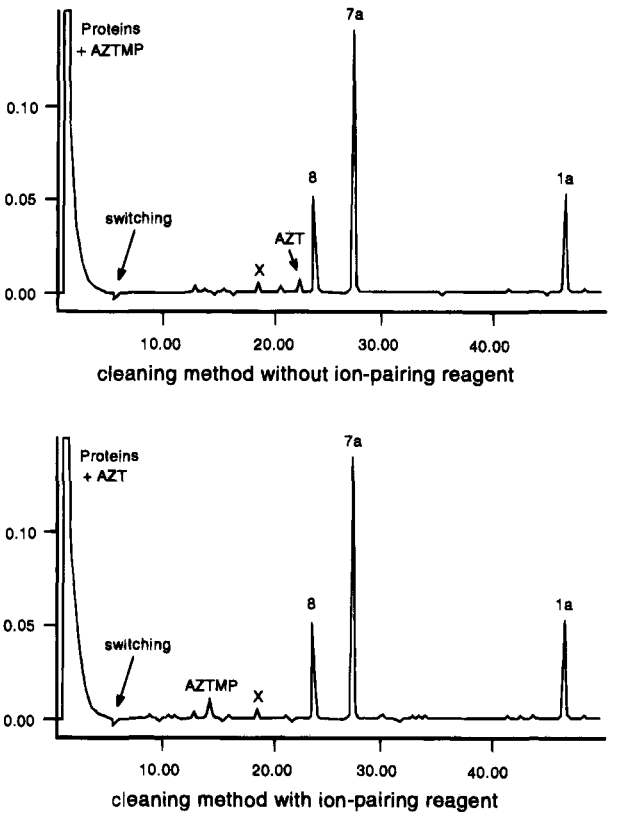
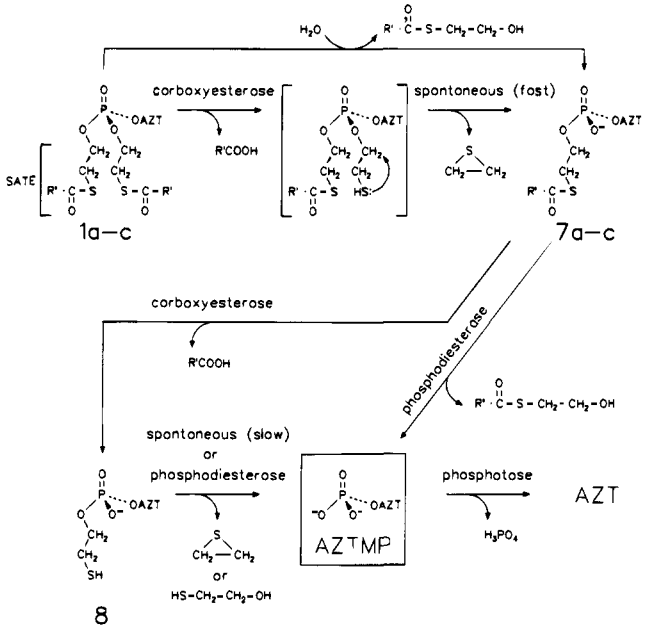


Figure 2. Examples of chromatograms obtained from the same sample by the two cleaning HPLC methods used. Both methods were needed for a complete analysis. In this case, **1a** was incubated during 20 min in total CEM-SS cell extract (TCE, initial concentration 25 μM, injection 100 μL; UV-diode array detection, absorbance at 266 nm). The abcissa shows the retention time (min) and the ordinate the absorbance unit. According to the method, AZTMP or AZT was eliminated during the cleaning step (5 min).

complementary techniques were used to purify the crude samples of proteins: (i) when injected in aqueous buffer, all metabolites, except AZTMP, were retained on the precolumn, and (ii) in an aqueous solution of the ion-pairing reagent (tetrabutylammonium sulfate), all metabolites (except AZT) were retained on the precolumn. For both techniques, the cleaned sample was transferred by means of a six-port rheodyne switching valve into an RP (C_{18}) analytical column and analyzed (acetonitrile gradient). When combining the results obtained with both techniques, all the degradation products could be easily detected and accurately quantified (Figure 2). These experiments allowed the proposal of coherent mechanisms for the decomposition of pronucleotides **1a–c** according to the medium (Scheme 2). As all the observed signals had the same UV spectra (λ_{max} = 266 nm), the direct calculation of the relative concentration of each species was related from the measured areas (the sum of areas at 266 nm was constant, and thus there was no loss of material during the cleaning step). The computation of the kinetic data of each pronucleotide decomposition was consistent with mechanisms which differed according to the medium (kinetic models 1–3). The rate constants (Table 3) were optimized by mono- or polyexponential regressions according to integrated equations (Scheme 3). These simple kinetic models fit the experimental data very well ($R > 0.99$ for $N = 10$) which substantiates the reliability of the method. Best-fit computed curves are shown in Figure

Scheme 2. Proposed Mechanisms for the Decomposition of Pronucleotides **1a** (R′ = CH_3), **1b** [R′ = $(CH_3)_2CH$], and **1c** [R′ = $(CH_3)_3C$][a]



[a] In culture medium (CM), the main pathway (99–100%) was 1 → **7** → AZTMP → AZT. In total cell extract (TCE) from CEM-SS cells, the main pathway (90–100%) was 1 → **7** → **8** → AZTMP → AZT. In nonenzymatic medium (RPMI 1640), the unique decomposition was 1 → **7**. Only in the case of **1a** were detected traces of an unknown product, X.

Table 3. Optimized Pseudo-first-order Rate Constants (10^{-3} min^{-1}) Related to the Decomposition Kinetic Models of the Phosphotriester Derivatives **1a–c** in Nonenzymatic Medium (RPMI), Culture Medium (CM), and Total Cell Extract (TCE)

rate constant	compd **1a**	**1b**	**1c**
	RPMI		
a	0.85 ± 0.04	ND[a]	ND[a]
d	0.30 ± 0.03	ND[a]	ND[a]
	CM		
a	1.28 ± 0.06	1.28 ± 0.07	0.11 ± 0.01
c	1.30 ± 0.09	0.94 ± 0.08	0.54 ± 0.04
j	0.23 ± 0.05	0.16 ± 0.04	0.17 ± 0.04
	TCE		
a	>140	15.4 ± 1.0	11.0 ± 0.8
b	>140	1.63 ± 0.10	1.6 ± 0.10
f	5.0 ± 0.9	1.2 ± 0.2	1.1 ± 0.2
j	2.9 ± 0.6	2.9 ± 0.6	2.9 ± 0.6

[a] ND, not determined.

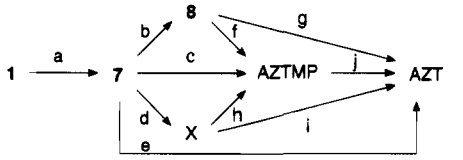
3, and the deduced half-lives of the starting materials and intermediates in the different media are summarized in Table 4.

In nonenzymatic medium (RPMI), the data were treated according to model 1:

$$\mathbf{1a} \xrightarrow{a} \mathbf{7a} \xrightarrow{d} X \qquad \text{(model 1)}$$

In this medium, **1a** is slowly hydrolyzed ($t_{1/2}$ = 13.6 ± 0.5 h), either by nucleophilic attack on the phosphorus atom with elimination of one SATE residue or (and) by hydrolysis of one acyl moiety quickly followed by the spontaneous elimination of episulfide. Both mechanisms give rise to the formation of diester **7a**, which, in turn, decomposes but very slowly ($t_{1/2}$ = 39 ± 4 h) to an unknown more hydrophilic product, X. Studies are in progress to determine the structure of this compound. No other signal was detected.

Scheme 3. Integrated Equations According to the Overall Competitive Consecutive Pseudo-first-order Kinetic Scheme of Decomposition of the Pronucleotides **1a–c**[a]



Rate constants: a, b, c, d, e, f, g, h, i, j; k = b + c + d + e; l = f + g; m = h + i

Functions: $\alpha = \exp(-a.t)$ $\beta = \exp(-k.t)$ $\gamma = \exp(-l.t)$ $\delta = \exp(-m.t)$ $\varepsilon = \exp(-j.t)$

Parameters:
$r = a.b/[(k-a).(l-a)]$ $s = a.b/[(a-k).(l-k)]$
$u = a.d/[(k-a).(m-a)]$ $v = a.d/[(a-k).(m-k)]$
$w = a.\{[b.f/(l-a)] + [d.h/(m-a)] + c\}/[(k-a).(j-a)]$
$x = a.\{[b.f/(l-k)] + [d.h/(m-k)] + c\}/[(a-k).(j-k)]$
$y = a.b.f/[(a-l).(k-l).(j-l)]$ $z = a.d.h/[(a-m).(k-m).(j-m)]$

Relative concentrations of metabolites *versus* incubation time:
$[1]/[1]_0 = \alpha$
$[7]/[1]_0 = a.(\alpha-\beta)/(k-a)$
$[8]/[1]_0 = r.\alpha + s.\beta - (r+s).\gamma$
$[x]/[1]_0 = u.\alpha + v.\beta - (u+v).\delta$
$[AZTMP]/[1]_0 = w.\alpha + x.\beta + y.\gamma + z.\delta - (w+x+y+z).\varepsilon$
$[AZT]/[1]_0 = 1 - \{[1] + [7] + [8] + [x] + [AZTMP]\}/[1]_0$

[a] Best-fit analysis of HPLC data allowed to determine the rate constant of the various possible steps. According to the medium, preferential pathways were found corresponding to the kinetic models 1–3.

Table 4. Calculated Half-lives of the Phosphotriester Derivatives **1a–c** and Their Metabolites in Culture Medium (CM) and Total Cell Extract (TCE)[a]

		compd			
		1a	**7a**	**8**	AZTMP
$t_{1/2}$	CM	9.0 h	8.9 h		50 h
	TCE	<5 min	<5 min	2.3 h	4.0 h
		1b	**7b**	**8**	AZTMP
$t_{1/2}$	CM	9.0 h	12.3 h		72 h
	TCE	45 min	7.1 h	9.0 h	4.0 h
		1c	**7c**	**8**	AZTMP
$t_{1/2}$	CM	4.3 day	21.3 h		67 h
	TCE	63 min	7.2 h	10.5 h	4.0 h

[a] Compound **8** was undetected in CM. The accuracy was better than 10% for 1 and **7** and better than 20% for **8** and AZTMP. The half-life in TCE of an authentic sample of AZTMP was 3.4 ± 0.2 h (data not shown).

In culture medium, the data were treated according to the "consecutive pseudo-first-order" model 2:

$$\mathbf{1} \xrightarrow{a} \mathbf{7} \xrightarrow{c} \text{AZTMP} \xrightarrow{j} \text{AZT} \qquad \text{(model 2)}$$

For each compound **1a–c**, four signals could be quantified. Three corresponded respectively to the parent pronucleotide **1**, AZT, and AZTMP (as ascertained by coinjection with authentic samples). The fourth compound was presumed to correspond to the intermediate phosphodiester which lacked one SATE residue (respectively **7a–c**, Scheme 2). The half-life of **1a** was shorter than in RPMI alone; this points to the existence of remaining carboxyesterase activity in the heat-inactivated serum. The formation of AZTMP from **7a–c** was likely to be phosphodiesterase-mediated (carboxyesterase activity would give rise to phosphodiester intermediate **8** which would be stable enough (see later) to be observed). Finally, AZTMP was very slowly dephos-

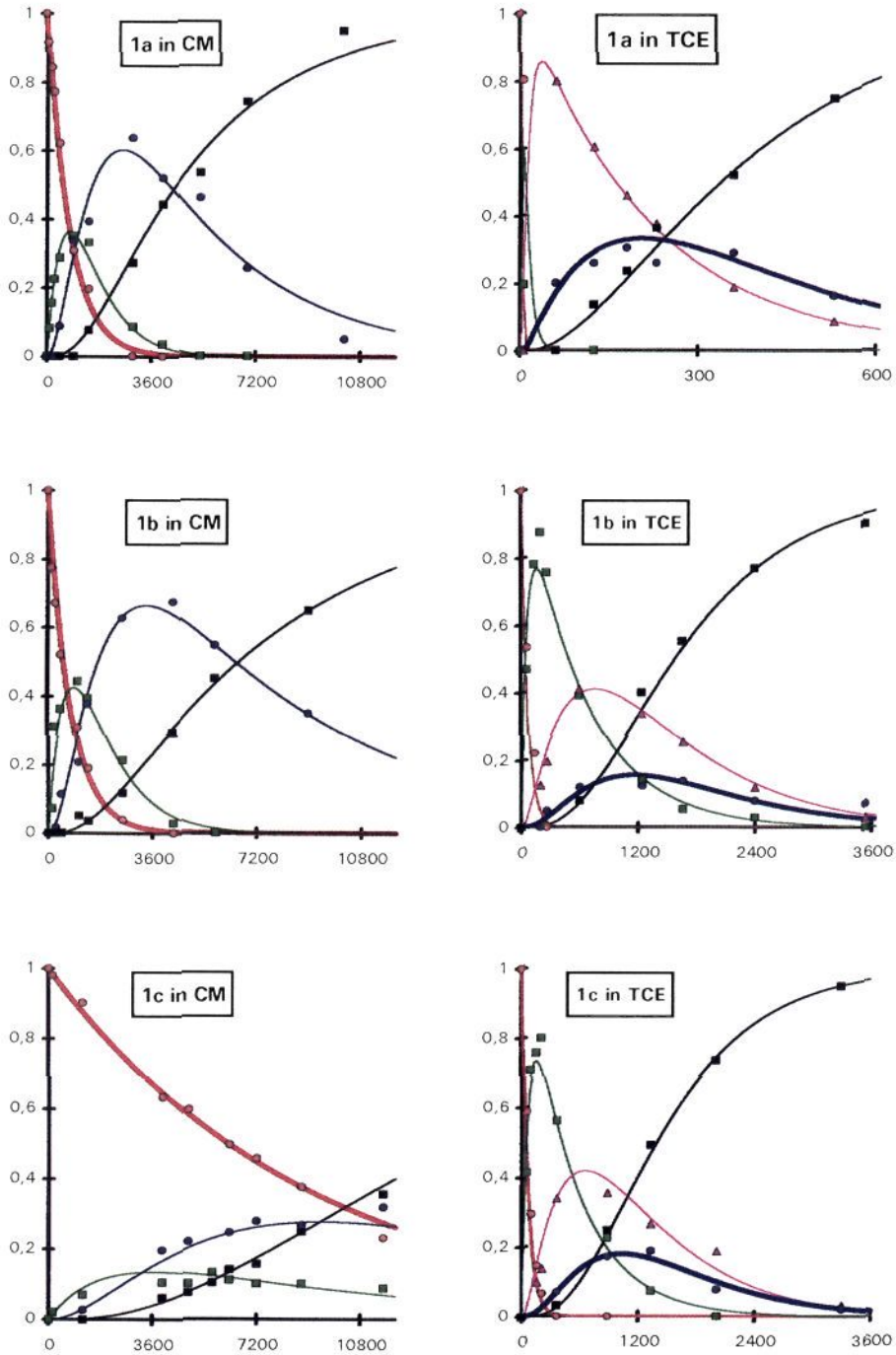
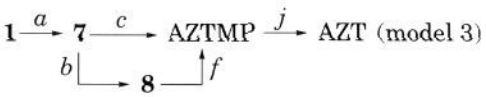


Figure 3. Decomposition kinetics of the pronucleotides **1a**–**c** (red circles) at 5×10^{-5} M initial concentration and their degradation products: **7a**–**c** (green squares), **8** (magenta triangles), AZTMP (blue circles), and AZT (black squares), in cell culture medium (CM) and total CEM-SS cell extract (TCE) at 37 °C. The computed kinetic curves related to the stability of the pronucleotides in CM and the release of AZTMP in TCE are bold-lined. The abcissa shows the incubation time (min) and the ordinate the relative concentration of each species.

phorylated to AZT, due to a remaining phosphatase activity. The degradation pattern and kinetics of **1b** were very similar to those of **1a**. Compound **1c** was metabolized according to the same mechanism, but it was much more stable than **1a**,**b**.

In cell extract, the data were treated according to the "consecutive competitive pseudo-first-order" model 3:



Apart from the signals observed in culture medium, an additional signal was observed whatever the starting pronucleotide. This signal was presumed to be the phosphodiester **8** (Scheme 2) produced from the enzymatic degradation of diesters **7a**–**c** due to the high activity of carboxyesterases in cell extracts. In the case of the pronucleotide **1a**, calculations of the rate constants showed that the main decomposition pathway (90%) of the corresponding phosphodiester **7a** produced the deacylated phosphodiester **8** (carboxyesterase activity), which, in turn, was relatively slowly converted to

AZTMP (phosphodiesterase activity or episulfide elimination) and finally to AZT (phosphatase activity). Other minor pathways gave rise to the formation of product X (less than 5% of the total amount) and the direct formation of AZTMP (5%, phosphodiesterase activity). The degradation pattern and kinetics of **1b,c** were very similar and differed from that of **1a**; the first (**1** → **7**) and second (**7** → **8**) decomposition steps were significantly slower, giving rise (100%) to **8**. The direct hydrolysis of **7b,c** to AZTMP or to other products was not observed. As expected, the calculated rate constants j (AZTMP → AZT) were the same whatever the starting pronucleotide and in accordance with the degradation rate of an authentic sample of AZTMP. The calculated rate constants f (**8** → AZTMP) were similar when starting from **1b,c** (as expected) but very different to the value found when starting from **1a** in the same cell extract. Experiments were reconducted in other cell extracts (three assays for **1a**, one for **1c**), giving similar results. We have no explanation for this unexpected difference.

These stability studies shown that the main decomposition pathways of the pronucleotides **1a−c** are different in culture medium and cell extract. It appears that the kinetics of decomposition of **1a,b** are similar in culture medium, **1c** being much more stable. On the contrary, **1b,c** behave similarly in cell extract, **1a** giving rise to a faster release of AZTMP. Furthermore, since AZT is formed upon incubation of the phosphotriester derivatives **1a−c** in culture medium, this justifies the need to test the antiviral activity of such pronucleotides using thymidine kinase-deficient cell lines, in order to prove unambiguously the intracellular NuMP delivery.

Discussion

Many strategies have been envisaged to mask or reduce the phosphate negative charges of 5′-mononucleotide analogues with neutral substituents, thereby giving rise to more lipophilic derivatives which would be expected to revert back to the corresponding 5′-mononucleotide inside the cell. To be effective, such a pronucleotide approach should incorporate the following features:[20] (i) utilization of a mononucleoside phosphotriester (neutral species), which would be expected to cross the cell membrane by passive diffusion, and (ii) a phosphotriester that must be stable under the usual experimental cell culture conditions (*i.e.*, RPMI containing 10% heat-inactivated fetal calf serum at 37 °C); otherwise, extracellular decomposition to the parent nucleoside could lead to misleading biological interpretations.

Consequently, to prove unambigously the validity of such an approach for the intracellular delivery of 5′-mononucleotide analogues, two strategies could be considered. In the first one, an inactive nucleoside can be used as a model, wherein the corresponding 5′-monosphosphate is further anabolized to its 5′-triphosphate, the latter interfering with the viral polymerase. In the second strategy, the phosphotriester must be evaluated for its inhibitory effect on the replication of the virus in cell lines deficient in the cellular nucleoside kinase responsible for the monophosphorylation of the corresponding nucleoside analogue. In these two cases, when a biological response is detected in cell cultures for such a pronucleotide, one can assume that it may be due to the intracellular release of the 5′-mononucleotide.

In this respect, we have previously reported the preliminary anti-HIV-1 activity of the mononucleoside phosphotriester derivative of 2′,3′-dideoxyuridine (ddU) which incorporates an *S*-acetyl-2-thioethyl group as a new carboxylate esterase-labile transient phosphate-protecting group.[21] Selected as a reference compound, ddU appeared to be an appropriate model as (i) it is inactive against HIV in cell cultures; (ii) the corresponding 5′-triphosphate is a potent RT inhibitor;[3e] and (iii) it has been shown in cell cultures that the delivery of ddUMP by liposomes results in an anti-HIV activity.[22] Actually, the bis(*S*-acetyl-2-thioethyl) phosphotriester of ddU inhibited the HIV-1 replication with an EC_{50} value that was *ca.* 5 μM in the two human T-cell lines studied, whereas ddU proved to be completely inactive at a concentration as high as 100 μM.[21] The moderate activity observed for this mononucleotide derivative may be related to an inhibition of cellular thymidylate kinase by ddUMP[33] or by the activity of dUTPase (an enzyme which normally hydrolyses dUTP back to dUMP).[24] In this regard, ddU does not seem to be the best nucleoside model as part of a pronucleotide approach. In order to evaluate the potential of the *S*-acetyl- and other *S*-acyl-2-thioethyl groups as enzymolabile-mediated bioreversible phosphate protection, we decided to synthesize the phosphotriester derivatives **1a−d** of AZT. AZT was selected as a model since its anti-HIV activity is strictly dependent on cellular thymidine kinase-mediated activation. Consequently, this nucleoside analogue proved to be completely inactive in HIV-1-infected CEM/TK$^-$ cells. Furthermore, due to the lipophilicity of AZT, it is easy to follow the decomposition of the corresponding neutral mononucleoside phosphotriesters in several media by HPLC.

The phosphotriester derivatives **1a−d** were obtained using P(III) chemistry (Scheme 1). Their anti-HIV-1 activity has been evaluated in two human lymphoblastoid cells (Table 1) and in a CEM/TK$^-$ cell line (Table 2). In this cell line, the pronucleotides **1a−d** showed potent anti-HIV activity, and **1a** proved to be superior to the previously described bis(DTE) ester (**2**)[10] and bis(POM) ester (**3**)[16,17] derivatives, with regard to the antiviral efficiency. These data clearly demonstrate that the test pronucleotides, particularly the bis(*S*-acetyl-2-thioethyl) phosphotriester of AZT, **1a**, are efficient prodrugs of AZTMP, circumventing the first activation step by cytosol thymidine kinase. Furthermore, it is noteworthy that the introduction of the SATE group as carboxylate esterase-labile transient phosphate protection of 5′-mononucleotide does not induce any additive toxicity compared to the parent nucleoside (manuscript in preparation).

In order to determine the mechanism by which these pronucleotides were converted to AZTMP (Scheme 2), the kinetics of decomposition of the mononucleotide derivatives **1a−c** were determined by a modified on-line cleaning HPLC method,[17] under two experimental conditions: (i) RPMI containing 10% heat-inactivated fetal calf serum (culture medium) and (ii) CEM cell extracts. For each compound, the kinetics of transformation differed according to the medium (Scheme 3). All bis(SATE) phosphotriesters **1a−c** were slightly

hydrolyzed to their corresponding mono(SATE) phosphodiesters **7a–c** in culture medium, but this transformation was faster in cell extract (Tables 3 and 4). Once formed, the phosphodiesters **7a–c** were essentially transformed into AZTMP, and the mechanism of this transformation differed according to the medium. In cell extracts, another phosphodiester intermediate, **8**, resulting from carboxylate esterase activation was observed. This intermediate **8** was not observed in the culture medium, suggesting that the formation of AZTMP in this case resulted from phosphodiesterase activity on the phosphodiesters **7a–c**. These results can explain the anti-HIV effect observed in the CEM/TK$^-$ cell line for the pronucleotides **1a–c**. Furthermore, the variation of the acyl chain in the immediate vicinity of the thiol ester functionality (*i.e.*, iPr, tBu) resulted in decreased hydrolysis rates by carboxylate esterase (compared to the Me) in both media. Thus, by varying the acyl portion of the *S*-acyl-2-thioethyl group, it was possible to control the rate of generation of the bioactive mononucleotide (Figure 2).

Conclusion

The present results demonstrate that nucleoside bis(SATE) phosphotriesters allow the efficient intracellular delivery of their parent 5′-monophosphate, circumventing the first phosphorylation step in the cells. It would now seem important to apply this SATE pronucleotide approach to other nucleoside analogues in which the corresponding 5′-triphosphates selectively inhibit viral polymerases but which may be hampered at the first phosphorylation step. Such studies are currently in progress in our laboratory. In this regard, we have recently reported that the potential of 2′,3′-dideoxyadenosine can be enhanced when such an approach is used.[25]

Experimental Section

Chemical Synthesis. Evaporation of solvents was carried out on a rotary evaporator under reduced pressure. ^{1}H NMR were run at ambient temperature in $(CD_3)_2SO$ or $CDCl_3$ with a Bruker AC 250 spectrometer. Chemical shifts are given in δ-values, $(CD_3)(CD_2H)SO$ being set at δ_H 2.49 as a reference; for $CDCl_3$, tetramethylsilane was used as the internal reference. Deuterium exchange and decoupling experiments were performed in order to confirm proton assignments. ^{31}P NMR spectra were recorded at ambient temperature on a Bruker AC 250 spectrometer with proton decoupling. Chemical shifts are reported relative to external H_3PO_4. FAB mass spectra were recorded in the positive-ion or negative-ion mode on a JEOL DX 300 mass spectrometer operating with a JMA-DA 5000 mass data system. Xe atoms were used for the gun at 3 kV with a total discharge current of 20 mA. The matrix used was 3-nitrobenzyl alcohol (NBA) or a mixture (50:50, v/v) of glycerol and thioglycerol (G–T). IR spectra were run in CCl_4 with an Acculab 4 (Beckman) spectrometer. UV spectra were recorded on an Uvikon 810 (Kontron) spectrometer. TLC was performed on precoated aluminum sheets of silica gel 60 F_{254} (Merck, article 5554), visualization of products being accomplished by UV absorbance followed by charring with 10% ethanolic sulfuric acid with heating; phosphorus-containing compounds were detected by spraying with Hanes molybdate reagent.[26] Column chromatography was carried out on silica gel 60 (Merck, article 9385) at atmospheric pressure. High-performance liquid chromatography (HPLC) studies were carried out on a Waters Assoc. unit equipped with a model 600E multisolvent delivery system, a model 600E system controller, a model U6K sample injector, a 486 tunable absorbance detector, and a base line 810 data workstation. The column was a reverse-phase analytical column (Hypersil, C_{18}, 100 × 4.6 mm, 3 μm) protected by a prefilter and a precolumn (Guard Pak, C_{18}). The pronucleotide to be analyzed was eluted using a linear gradient of 0.1 M ammonium acetate buffer (pH 5.9) to 50% acetonitrile programed over a 50 min period, with a flow rate of 1 mL/min and detection at 260 nm. 3′-Azido-2′,3′-dideoxythymidine (AZT) was purchased from Intsel Marsing France.

The test compounds were isolated as oils and found to be pure by rigorous HPLC analysis, high-field multinuclear NMR spectroscopy, and high-resolution mass spectroscopy.

General Procedure for the Preparation of Thioesters 5a–d. To a solution of the appropriate thioacid **4a–d** (11.5 mmol) in toluene (4.0 mL) at 0 °C were added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU; 1.71 mL, 11.5 mmol) and 2-iodoethanol (0.78 mL, 10.0 mmol). The reaction mixture was stirred at room temperature for 3 h and diluted with dichloromethane (40 mL) and water (5 mL). The organic layer was separated, washed with water (2 × 5 mL), dried over sodium sulfate, filtered, and evaporated to dryness under reduced pressure. Column chromatography of the residue on silica gel afforded the title compounds **5a–d** as colored oils which can be used directly or subjected to vacuum distillation to give the products as colorless oils.

***S*-(2-Hydroxyethyl) thioacetate (5a):** 0.78 g, 65%, after chromatography [eluent, stepwise gradient of ethyl acetate (0–5%) in dichloromethane]; bp 90–91 °C (10 mmHg) [lit.[27] bp 95–96 °C (8 mmHg), lit.[28] bp 95 °C (15 mmHg), lit.[29] bp 116–119 °C (26 mmHg)]; ^{1}H NMR (DMSO-d_6) δ 4.94 (t, 1H, OH, J = 5.5 Hz), 3.49–3.41 (m, 2H, CH_2OH), 2.90 (t, 2H, SCH_2CH$_2$, J = 6.6 Hz), 2.31 (s, 3H, CH$_3$); IR ν_{max} 1690 (C=O) cm^{-1}; n_D^{20} = 1.5092 (lit.[27] n_D^{20} = 1.4963, lit.[28] n_D^{20} = 1.4720).

***S*-(2-Hydroxyethyl) thioisobutyrate (5b):** 1.25 g, 84%, after chromatography [eluent, stepwise gradient of methanol (0–4%) in dichloromethane]; bp 79–80 °C (0.11 mmHg); ^{1}H NMR (DMSO-d_6) δ 4.95 (t, 1H, OH, J = 5.5 Hz), 3.48–3.41 (m, 2H, CH_2OH), 2.91 (t, 2H, SCH_2CH$_2$, J = 6.6 Hz), 2.79–2.68 (m, 1H, CHMe$_2$), 1.09 (d, 6H, CHMe_2, J = 6.8 Hz); IR ν_{max} 1670 (C=O) cm^{-1}; n_D^{20} = 1.4852; FAB MS (>0, G–T) *m/e* 255 (M + G + H)$^+$, 163 (M + H)$^+$.

***S*-(2-Hydroxyethyl) thiopivaloate (5c):** 1.44 g, 89%, after chromatography [eluent, stepwise gradient of ethyl acetate (0–10%) in dichloromethane]; bp 73 °C (0.15 mmHg); ^{1}H NMR (DMSO-d_6) δ 4.94 (t, 1H, OH, J = 5.5 Hz), 3.47–3.40 (m, 2H, CH_2OH), 2.88 (t, 2H, SCH_2CH$_2$, J = 6.6 Hz), 1.16 (s, 9H, tBu); IR ν_{max} 1670 (C=O) cm^{-1}; n_D^{20} = 1.4654.

***S*-(2-Hydroxyethyl) thiobenzoate (5d):** 1.49 g, 82%, after chromatography [eluent, stepwise gradient of ethyl acetate (0–10%) in toluene]; bp 138–139 °C (0.11 mmHg); ^{1}H NMR (DMSO-d_6) δ 7.9–7.5 (m, 5H, Ph), 5.05 (t, 1H, OH, J = 5.4 Hz), 3.60–3.53 (m, 2H, CH_2OH), 3.14 (t, 2H, SCH_2CH$_2$, J = 6.5 Hz); ^{1}H NMR ($CDCl_3$) δ 8.0–7.4 (m, 5H, Ph), 3.90–3.83 (m, 2H, CH_2OH), 3.29 (t, 2H, SCH_2CH$_2$, J = 6.1 Hz), 2.45 (t, 1H, OH, J = 5.2 Hz) [lit.[30] ^{1}H NMR ($CDCl_3$) δ 8.00–7.65 (m, 2H), 7.50–7.05 (m, 3H), 3.80 (t, 2H, J = 6.0 Hz), 3.22 (t, 2H, J = 6.0 Hz), 2.95 (bs, 1H, OH)]; IR ν_{max} 1660 (C=O) cm^{-1} (in accordance with literature data[30]); n_D^{20} = 1.5918; FAB MS (>0, G–T) *m/e* 365 (2M + H)$^+$, 183 (M + H)$^-$.

General Procedure for the Preparation of Phosphoramidites 6a–d. A solution of the appropriate thioester **5a–d** (10.0 mmol) and triethylamine (3.07 mL, 22.0 mmol) in tetrahydrofuran (25 mL) was added dropwise over 1 h to a stirred solution of *N,N*-diisopropylphosphorodichloridite[15] (1.01 g, 5.0 mmol) in tetrahydrofuran (35 mL), under argon at −78 °C. The reaction mixture was stirred at room temperature for 2 h, after which the resulting triethylammonium hydrochloride was filtered off. The filtrate was evaporated under reduced pressure to give a pale yellow oil. Purification by flash column chromatography[31] gave the title compounds **6a–d** as colorless oils.

Bis(*S*-acetyl-2-thioethyl) *N,N*-diisopropylphosphoramidite (6a): 1.33 g, 72%, after flash column chromatography [eluent, stepwise gradient of ethyl acetate (0–2%) in cyclohexane containing 1% triethylamine]; ^{1}H NMR (DMSO-d_6) δ

3.70–3.47 (m, 6H, Me_2CHN, CH_2CH_2O), 3.04 (t, 4H, SCH_2CH_2, J = 6.4 Hz), 2.34 (s, 6H, CH_3CO), 1.10 (d, 12H, Me_2CHN, J = 6.8 Hz); ^{31}P NMR (DMSO-d_6) δ 147.9 (s); FAB MS (>0, NBA) m/e 386 (M + O + H)$^+$, 370 (M + H)$^+$.

Bis(*S*-isobutyryl-2-thioethyl) *N,N*-diisopropylphosphoramidite (6b): 1.40 g, 66%, after flash column chromatography [eluent, stepwise gradient of ethyl acetate (0–2%) in cyclohexane containing 1% triethylamine]; ^1H NMR (DMSO-d_6) δ 3.70–3.47 (m, 6H, Me_2CHN, CH_2CH_2O), 3.03 (t, 4H, SCH_2CH_2, J = 6.3 Hz), 2.79–2.68 (m, 2H, Me_2CHCO), 1.10 (d, 12H, Me_2CHN or Me_2CHCO, J = 6.7 Hz), 1.09 (d, 12H, Me_2CHCO or Me_2CHN, J = 6.9 Hz); ^{31}P NMR (DMSO-d_6) δ 147.9 (s); FAB MS (>0, G–T) m/e 426 (M + H)$^+$.

Bis(*S*-pivaloyl-2-thioethyl) *N,N*-diisopropylphosphoramidite (6c): 1.68 g, 74%, after flash column chromatography [eluent, stepwise gradient of ethyl acetate (0–1%) in cyclohexane containing 1% triethylamine]; ^1H NMR (DMSO-d_6) δ 3.70–3.47 (m, 6H, Me_2CHN, CH_2CH_2O), 3.01 (t, 4H, SCH_2CH_2, J = 6.3 Hz), 1.16 (s, 18H, tBu), 1.10 (d, 12H, Me_2CHN, J = 6.8 Hz); ^{31}P NMR (DMSO-d_6) δ 147.9 (s); FAB MS (>0, G–T) m/e 470 (M + O + H)$^+$, 454 (M + H)$^+$.

Bis(*S*-benzoyl-2-thioethyl) *N,N*-diisopropylphosphoramidite (6d): 0.99 g, 40%, after flash column chromatography [eluent, stepwise gradient of ethyl acetate (0–1%) in cyclohexane containing 1% triethylamine]; ^1H NMR (DMSO-d_6) δ 7.9–7.5 (m, 10H, Ph), 3.69–3.46 (m, 6H, Me_2CHN, CH_2CH_2O), 3.27 (t, 4H, SCH_2CH_2, J = 6.3 Hz), 1.10 (d, 12H, Me_2CHN, J = 6.8 Hz); ^{31}P NMR (DMSO-d_6) δ 147.9 (s); FAB MS (>0, G–T) m/e 510 (M + O + H)$^+$, 494 (M + H)$^+$.

General Procedure for the Preparation of Phosphotriesters 1a–d. By adaption of a published procedure,[32] 1*H*-tetrazole (0.21 g, 3.0 mmol) was added to a stirred solution of AZT (0.27 g, 1.0 mmol) and the appropriate phosphoramidite **6a–d** (1.2 mmol) in tetrahydrofuran (2 mL) at room temperature. After 30 min, the reaction mixture was cooled to −40 °C, and a solution of 3-chloroperoxybenzoic acid (0.23 g, 1.3 mmol) in dichloromethane (2.5 mL) was added; the mixture was then allowed to warm to room temperature over 1 h. Sodium sulfite (10% solution, 1.3 mL) was added to the mixture to destroy the excess 3-chloroperoxybenzoic acid, after which the organic layer was separated and the aqeuous layer washed with dichloromethane (2 × 10 mL). The combined organic layers were washed with saturated aqueous sodium hydrogen carbonate (5 mL) and then water (3 × 5 mL), dried over sodium sulfate, filtered, and evaporated to dryness under reduced pressure. Column chromatography of the residue on silica gel afforded the title compounds **1a–d** as pale yellow oils.

3′-Azido-3′-deoxythymidin-5′-yl bis(*S*-acetyl-2-thioethyl) phosphate (1a): 0.42 g, 75%, after chromatography [eluent, stepwise gradient of methanol (0–3%) in dichloromethane]; ^1H NMR (DMSO-d_6) δ 11.4 (bs, 1H, 3-NH), 7.46 (s, 1H, 6-H), 6.12 (t, 1H, 1′-H, J = 6.7 Hz), 4.50–4.43 (m, 1H, 3′-H), 4.23–4.17 (m, 2H, 5′-H), 4.09–3.99 (m, 5H, 4′-H, CH_2CH_2O), 3.12 (t, 4H, SCH_2CH_2, J = 6.3 Hz), 2.44–2.33 (m, 8H, 2′-H, CH_3CO), 1.78 (s, 3H, 5-CH_3); ^{31}P NMR (DMSO-d_6) δ −0.72 (s); FAB MS (>0, G–T) m/e 644 (M + G + H)$^+$, 552 (M + H)$^+$; FAB MS (<0, G–T) m/e 1101 (2M − H)$^-$, 550 (M − H)$^-$; UV (ethanol) λ_{max} 264 nm (ϵ 9800); HPLC t_R 29.8 min.

3′-Azido-3′-deoxythymidin-5′-yl bis(*S*-isobutyryl-2-thioethyl) phosphate (1b): 0.41 g, 67%, after chromatography [eluent, stepwise gradient of methanol (0–2.5%) in dichloromethane]; ^1H NMR (DMSO-d_6) δ 11.4 (bs, 1H, 3-NH), 7.46 (s, 1H, 6-H), 6.12 (t, 1H, 1′-H, J = 6.6 Hz), 4.50–4.45 (m, 1H, 3′-H), 4.22–4.17 (m, 2H, 5′-H), 4.09–3.98 (m, 5H, 4′-H, CH_2CH_2O), 3.12 (t, 4H, SCH_2CH_2, J = 6.3 Hz), 2.80–2.69 (m, 2H, Me_2CHCO), 2.41–2.32 (m, 2H, 2′-H), 1.78 (s, 3H, 5-CH_3), 1.10 (d, 12H, Me_2CHCO, J = 7.0 Hz); ^{31}P NMR (DMSO-d_6) δ −0.72 (s); FAB MS (>0, NBA) m/e 608 (M + H)$^+$; FAB MS (<0, NBA) m/e 1213 (2M − H)$^-$, 606 (M − H)$^-$; UV (ethanol) λ_{max} 264 nm (ϵ 9600); HPLC t_R 38.6 min.

3′-Azido-3′-deoxythymidin-5′-yl bis(*S*-pivaloyl-2-thioethyl) phosphate (1c): 0.42 g, 65%, after chromatography [eluent, stepwise gradient of methanol (0–3%) in dichloromethane]; ^1H NMR (DMSO-d_6) δ 11.4 (bs, 1H, 3-NH), 7.47 (s, 1H, 6-H), 6.12 (t, 1H, 1′-H, J = 6.6 Hz), 4.50–4.43 (m, 1H, 3′-H), 4.20–4.17 (m, 2H, 5′-H), 4.08–3.98 (m, 5H, 4′-H, CH_2CH_2O), 3.10 (t, 4H, SCH_2CH_2, J = 6.3 Hz), 2.43–2.32 (m, 2H, 2′-H), 1.78 (s, 3H, 5-CH_3), 1.16 (s, 18H, tBu); ^{31}P NMR (DMSO-d_6) δ −0.70 (s); FAB MS (>0, G–T) m/e 636 (M + H)$^+$; FAB MS (<0, G–T) m/e 634 (M − H)$^-$; UV (ethanol) λ_{max} 264 nm (ϵ 9600); HPLC t_R 42.3 min.

3′-Azido-3′-deoxythymidin-5′-yl bis(*S*-benzoyl-2-thioethyl) phosphate (1d): 0.40 g, 58%, after chromatography [eluent, stepwise gradient of methanol (0–2%) in dichloromethane]; ^1H NMR (DMSO-d_6) δ 11.3 (bs, 1H, 3-NH), 7.9–7.5 (m, 10H, Ph), 7.45 (s, 1H, 6-H), 6.09 (t, 1H, 1′-H, J = 6.7 Hz), 4.48–4.41 (m, 1H, 3′-H), 4.25–4.16 (m, 6H, 5′-H, CH_2CH_2O), 4.01–3.98 (m, 1H, 4′-H), 3.38–3.35 (m, 4H, SCH_2CH_2), 2.42–2.22 (m, 2H, 2′-H), 1.75 (s, 3H, 5-CH_3); ^{31}P NMR (DMSO-d_6) δ −0.63 (s); FAB MS m/e (>0, G–T) m/e 676 (M + H)$^+$; FAB MS (<0, NBA) m/e 1349 (2M − H)$^-$, 674 (M − H)$^-$; UV (ethanol) λ_{max} 264 (ϵ 18 000), 244 (sh, ϵ 16 900), 240 nm (ϵ 17 100); HPLC t_R 40.4 min.

3′-Azido-3′-deoxythymidin-5′-yl Bis[(pivaloyloxy)methyl] Phosphate (3). The sodium salt of AZT 5′-monophosphate (0.40 g, 1.0 mmol), prepared according to a general procedure,[33] was dissolved in the minimum amount of a mixture of methanol–water, passed through a Dowex 50W X 2 (H^+ form) column (2 × 19 cm), and eluted with water. The free acid was collected, lyophilized, and suspended in dry acetonitrile (40 mL), and *N,N*-diisopropylethylamine (0.71 mL, 4.0 mmol) was added. After a few minutes, the mixture became clear, and iodomethyl pivaloate[19] (1.70 g, 7.0 mmol) was added. After stirring at room temperature for 4 days, the reaction mixture was evaporated to dryness. The residue was dissolved in dichloromethane (150 mL), and the organic layer was washed with 1 M triethylammonium bicarbonate buffer (pH 7.5, 30 mL) and then water (3 × 30 mL), dried over sodium sulfate, filtered, and evaporated under reduced pressure. Column chromatography of the residue on silica gel with a stepwise gradient of ethyl acetate (0–30%) in toluene afforded the title compound **3** as a colorless oil (0.13 g, 22%): ^1H NMR (DMSO-d_6) δ 11.4 (bs, 1H, 3-NH), 7.45 (s, 1H, 6-H), 6.12 (t, 1H, 1′-H, J = 6.6 Hz), 5.60 (d, 4H, CH_2O, J = 13.8 Hz), 4.47–4.42 (m, 1H, 3′-H), 4.26–4.20 (m, 2H, 5′-H), 4.00–3.97 (m, 1H, 4′-H), 2.42–2.29 (m, 2H, 2′-H), 1.77 (s, 3H, 5-CH_3), 1.15 and 1.14 (2s, 2 × 9H, 2 × tBu); ^{31}P NMR (DMSO-d_6) δ −2.97 (s); FAB MS (>0, G–T) m/e 576 (M + H)$^+$; FAB MS (<0, G–T) m/e 574 (M − H)$^-$; UV (ethanol) λ_{max} 264 nm (ϵ 8300); HPLC t_R 37.8 min.

Biological Methods. Assays on Cell Culture. The origin of the viruses and the techniques used for measuring inhibition of virus multiplication have previously been described.[10] Briefly, in MT-4 cells, the determination of the antiviral activity of the pronucleotides was based on a reduction of HIV-1-IIIB-induced cytopathogenicity, the metabolic activity of the cells being measured by the property of mitochondrial dehydrogenases to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into formazan.[10,34,35] For CEM cells, the production of virus HIV-LAI was measured by quantification of the reverse transcriptase activity associated with the virus particle released in the culture supernatant.[10,35] Cells, MT-4 and CEM, were respectively incubated with 50 or 100 $TCID_{50}$ of viruses during 30 min; after virus adsorption, unbound particles were eliminated by two washes, and cells were cultured in the presence of different concentrations of test compounds for 5 days, or 6 days in the case of CEM/TK$^-$ cells, before virus production determination. The 50% effective concentration (EC_{50}) was derived from the computer-generated median effect plot of the dose–effect data.[36] In parallel experiments, cytotoxicity of the test compounds was measured after an incubation of 5 days in their presence using the colorimetric MTT test.[37] The 50% cytotoxic concentration (CC_{50}) is the concentration at which OD_{540} was reduced by one-half and was calculated using the program mentioned above.

Stability and Decomposition Studies. The previously described HPLC procedure[17] was slightly modified. Firstly, the cleaning precolumn was replaced by a Guard-Pak insert (Delta-Pak C_{18}, 100 Å) hold in a Guard-Pak holder (Waters, Saint Quentin, France). Secondly, the analytical column used

was a Hypersil C_{18}, 3 μm, 120 Å, 4.6 × 100 mm (Shandon, Eragny, France). Thirdly, ammonium acetate buffer was used instead of phosphate buffer: stock solution S, 1.0 M, pH 6.0, from ammonium acetate and acetic acid *pro analysi* (Merck, Darmstadt, Germany); eluent A (v/v), S 10, water 90; eluent B (v/v/v), S 10, acetonitrile 50, water 40. Fourthly, when using the cleaning method with ion-pairing reagent, eluent A was replaced by eluent A′: 2.5 mM buffered tetrabutylammonium sulfate [one bottle of PIC A reagent (Waters) in 2.0 L of water]. Whatever the cleaning method (with or without ion-pairing agent), the crude sample (80 μL, initial concentration of **1a–c**, **2**, or **3** of 5×10^{-5} M) was injected onto the precolumn and eluted (eluent A or A′) during 5 min. Then, the switching valve for connecting the precolumn to the column was activated, and a linear gradient from A or A′ or B programmed over 50 min was used. When using method without ion-pairing reagent, retention times (min) of analytes were **1a**, 46.5; **1b**, 48.0; **1c**, 49.6; **7a**, 27.3; **7b**, 27.9; **7c**, 28.7; **8**, 24.4; AZT, 23.1; and AZTMP, <4. When using the method with ion-pairing reagent, all the retention times were close to the preceeding values, except for AZT (<4 min) and AZTMP (14.6 min).

Acknowledgment. These investigations were supported by grants from the CNRS, Agence Nationale de Recherches sur le SIDA (ANRS, France), and Association pour la Recherche sur le Cancer (ARC, France). Three of us were particularly grateful to ARC (I.L., J.-L.G.) and ANRS (C.P.) for a fellowship. We are indebted to Dr. R. Johnson for critical reading of the manuscript.

References

(1) (a) De Clercq, E. HIV inhibitors targeted at the reverse transcriptase. *AIDS Res. Hum. Retroviruses* **1992**, *8*, 119–134. (b) Sandström, E.; Oberg, B. Antiviral therapy in human immunodeficiency virus infections. *Drugs* **1993**, *45*, 488–508.

(2) Hao, Z.; Conney, D. A.; Hartman, N. R.; Perno, C. F.; Fridland, A.; De Vico, A. L.; Sarngadharan, M. G.; Broder, S.; Johns, D. G. Factors determining the activity of 2′,3′-dideoxynucleosides in suppressing human immunodeficiency virus in vitro. *Mol. Pharmacol.* **1988**, *34*, 431–435.

(3) (a) Balzarini, J.; Cooney, D. A.; Dalal, M.; Kang, G.-J.; Cupp, J. E.; De Clercq, E.; Broder, S.; Johns, D. G. 2′,3′-Dideoxycytidine: regulation of its metabolism and anti-retroviral potency by natural pyrimidine nucleosides and by inhibitors of pyrimidine nucleotide synthesis. *Mol. Pharmacol.* **1987**, *32*, 798–806. (b) Starnes, M. C.; Cheng, Y.-C. Cellular metabolism of 2′,3′-dideoxycytidine, a compound active against human immunodeficiency virus in vitro. *J. Biol. Chem.* **1987**, *262*, 988–991. (c) Johnson, M. A.; Ahluwalia, G.; Connelly, M. C.; Cooney, D. A.; Broder, S.; Johns, D. G.; Fridland, A. Metabolic pathways for the activation of the antiretroviral agent 2′,3′-dideoxyadenosine in human lymphoid cells. *J. Biol. Chem.* **1988**, *263*, 15354–15357. (d) Johnson, M. A.; Fridland, A. Phosphorylation of 2′,3′-dideoxyinosine by cytosolic 5′-nucleotidase of human lymphoid cells. *Mol. Pharmacol.* **1989**, *36*, 291–295. (e) Hao, Z.; Cooney, D. A.; Farquhar, D.; Perno, C. F.; Zhang, K.; Masood, R.; Wilson, Y.; Hartman, N. R.; Balzarini, J.; Johns, D. G. Potent DNA chain termination activity and selective inhibition of human immunodeficiency virus reverse transcriptase by 2′,3′-dideoxyuridine-5′-triphosphate. *Mol. Pharmacol.* **1990**, *37*, 157–163.

(4) (a) Sommadossi, J.-P. Nucleoside analogs: similarities and differences. *Clin. Infect. Dis.* **1993**, *16* (Suppl. 1), S7–S15. (b) Perno, C. F.; Yarchoan, R.; Cooney, D. A.; Hartman, N. R.; Webb, D. S. A.; Hao, Z.; Mitsuya, H.; Johns, D. G.; Broder, S. Replication of HIV in monocytes: granulocyte-macrophage colony stimulating factor (GM-CSF) potentiates viral production yet enhances the antiviral effect mediated by AZT and other dideoxynucleoside congeners of thymidine. *J. Exp. Med.* **1989**, *169*, 933–951. (c) Gao, W.-Y.; Shirasaka, T.; Johns, D. G.; Broder, S.; Mitsuya, H. Differential phosphorylation of azidothymidine, dideoxycytidine, and dideoxyinosine in resting and activated peripheral blood mononuclear cells. *J. Clin. Invest.* **1993**, *91*, 2326–2333. (d) Gao, W.-Y.; Agbaria, R.; Driscoll, J. S.; Mitsuya, H. Divergent anti-human immunodeficiency virus activity and anabolic phosphorylation of 2′,3′-dideoxynucleoside analogs in resting and activated human cells. *J. Biol. Chem.* **1994**, *259*, 12633–12638.

(5) (a) Leibman, K. C.; Heideberg, C. The metabolism of ^{32}P-labeled ribonucleotides in tissue slices and cell suspensions. *J. Biol. Chem.* **1955**, *216*, 823–830. (b) Roll, P. M.; Weinfeld, H.; Caroll, E.; Brown, G. B. The utilisation of nucleotides by the mammal. IV. Triply labeled purine nucleotides. *J. Biol. Chem.* **1956**, *220*, 439–454. (c) Lichtenstein, J.; Barner, H. D.; Cohen, S. S. The metabolism of exogenously supplied nucleotides by Escherichia coli. *J. Biol. Chem.* **1960**, *235*, 457–465.

(6) (a) Ho, D. H. W. Metabolism of 6-methylthiopurine ribonucleoside 5′-phosphate. *Biochem. Pharmacol.* **1971**, *20*, 3538–3539. (b) Schrecher, A. W.; Goldin, A. Antitumor effect and mode of action of 1-β-D-arabinosyl cytosine 5′-monophosphate in leukemia L1210. *Cancer Res.* **1968**, *28*, 802–803.

(7) (a) Alexander, P.; Holy, A. Prodrugs of analogs of nucleic acid components. *Collect. Czech. Chem. Commun.* **1994**, *59*, 2127–2165. (b) Périgaud, C.; Girardet, J.-L.; Gosselin, G.; Imbach, J.-L. Comments on nucleotide delivery forms. In *Advances in Antiviral Drug Design*; De Clercq, E., Ed.; 1995; Vol. 2, in press.

(8) (a) Farquhar, D.; Srivastava, D. N.; Kuttech, N. J.; Saunders, P. P. Biologically reversible phosphate protecting groups. *J. Pharm. Sci.* **1983**, *72*, 324–325. (b) Srivastava, D. N.; Farquhar, D. Bioreversible phosphate protecting groups: synthesis and stability of model acyloxymethyl phosphates. *Bioorg. Chem.* **1984**, *12*, 118–119. (c) Thomson, W.; Nicholls, D.; Irwin, W. J.; Al-Mushadani, J. S.; Freeman, S.; Karpas, A.; Petrik, J.; Mahmood, N.; Hay, A. J. Synthesis, bioactivation and anti-HIV activity of the bis(4-acyloxybenzyl) and mono(4-acyloxybenzyl) esters of the 5′-monophosphate of AZT. *J. Chem. Soc., Perkin Trans. 1* **1993**, 1239–1245. (d) Thomson, W.; Nicholls, D.; Mitchell, A. G.; Corner, J. A.; Irwin, W. J.; Freeman, S. Synthesis and bioactivation of bis(aroyloxymethyl) and mono(aroyloxymethyl) esters of benzylphosphonate and phosphonoacetate. *J. Chem. Soc., Perkin Trans. 1* 1993, 2303–2308.

(9) For recent examples, see: (a) Srinivas, R. V.; Robbins, B. L.; Connelly, M. C.; Gong, Y.-F.; Bischofberger, N.; Fridland, A. Metabolism and in vitro antiretroviral activities of bis(pivaloyloxymethyl) prodrugs of acyclic nucleoside phosphonates. *Antimicrob. Agents Chemother.* **1993**, *37*, 2247–2250. (b) Farquhar, D.; Khan, S.; Srivastava, D. N.; Saunders, P. P. Synthesis and antitumor evaluation of bis[(pivaloyloxy)methyl] 2′-deoxy-5-fluorouridine 5′-monophosphate (FdUMP): a strategy to introduce nucleotides into cells. *J. Med. Chem.* **1994**, *37*, 3902–3909. (c) Starett, J. E.; Tortolani, D. R.; Russel, J.; Hitchcock, M. J. M.; Whiterock, V.; Martin, J. C.; Mansuri, M. M. Synthesis, oral biovailability determination, and in vitro evaluation of prodrugs of the antiviral agent 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA). *J. Med. Chem.* **1994**, *37*, 1857–1864.

(10) Puech, F.; Gosselin, G.; Lefebvre, I.; Pompon, A.; Aubertin, A.-M.; Kirn, A.; Imbach, J.-L. Intracellular delivery of nucleoside monophosphates through a reductase-mediated activation process. *Antiviral Res.* **1993**, *22*, 155–174.

(11) A preliminary account of this work was presented at the Seventh International Conference on Antiviral Research, Charleston, SC, February 27–March 4, 1994. Imbach, J.-L.; Gosselin, G.; Périgaud, C.; Lefèbvre, I.; Girardet, J.-L.; Benzaria, S. Cytosolic delivery of anti-HIV nucleoside monophosphates having enzymelabile transient phosphate protecting groups. *Antiviral Res.* **1994**, *23* (Suppl I), 47.

(12) Beaucage, S. L.; Caruthers, M. H. Deoxynucleoside phosphoramidites. A new class of key intermediates for deoxypolynucleotide synthesis. *Tetrahedron Lett.* **1981**, *22*, 1859–1862.

(13) For recent examples using the phosphite triester method for the synthesis of pronucleotides, see: (a) Sergheraert, C.; Pierlot, C.; Tartar, A.; Henin, Y.; Lemaitre, M. Synthesis and anti-HIV evaluation of D4T and D4T 5′-monophosphate prodrugs. *J. Med. Chem.* **1993**, *36*, 826–830. (b) Namane, A.; Gouyette, C.; Filion, M.-P.; Filion, G.; Huynh-Dinh, T. Improved brain delivery of AZT using a glycosyl phosphotriester prodrug. *J. Med. Chem.* **1992**, *35*, 3039–3044.

(14) Fredga, A.; Bauer, H. Some homologues of tetraethenyl hexasulfide. *Ark. Kemi* **1951**, *2*, 113–118; *Chem. Abstr.* **1951**, *45*, 541e.

(15) Tanaka, T.; Tamatsukuri, S.; Ikehara, M. New approach to the synthesis of deoxynucleoside-phosphoramidite derivatives. *Tetrahedron Lett.* **1986**, *27*, 199–202.

(16) Farquhar, D.; Nowak, B.; Khan, S.; Plunkett, W. Masked nucleotides: a strategy to introduce therapeutic nucleoside 5′-monophosphates into cells. *Antiviral Res.* **1991**, *15* (Suppl. I), 143.

(17) Pompon, A.; Lefebvre, I.; Imbach, J.-L.; Kahn, S.; Farquhar, D. Decomposition pathways of the mono- and bis(pivaloyloxymethyl) esters of azidothymidine 5′-monophosphate in cell extract and in tissue culture medium: an application of the "online ISRP-cleaning" HPLC technique. *Antiviral Chem. Chemother.* **1994**, *5*, 91–98.

(18) Farquhar, D. Biologically reversible phosphate and phosphonate protective groups. U.S. Patent 4,816,570, March 28, 1989; *Chem. Abstr.* **1989**, *111*, 134697b.

(19) Miyake, A.; Yamaoka, M. Method for producing 1-iodoalkyl acylates. Eur. Patent Appl. EP 143 601, June 5, 1985; *Chem. Abstr.* **1986**, *104*, 5589z.

(20) Gosselin, G.; Imbach, J.-L. The anti-HIV nucleoside phosphotriester approach: controversial comments and complementary data. *Int. Antiviral News* **1993**, *1*, 100–102.

(21) Périgaud, C.; Gosselin, G.; Lefebvre, I.; Girardet, J.-L.; Benzaria, S.; Barber, I.; Imbach, J.-L. Rational design for cytosolic delivery of nucleoside monophosphates: ≪ SATE ≫ and ≪ DTE ≫ as enzyme-labile transient phosphate protecting groups. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 2521–2526.
(22) Zelphati, O.; Degols, G.; Loughrey, H.; Leserman, L.; Pompon, A.; Puech, F.; Maggio, A.-F.; Imbach, J.-L.; Gosselin, G. Inhibition of HIV-1 replication in cultured cells with phosphorylated dideoxyuridine derivatives encapsulated in immunoliposomes. *Antiviral Res.* **1993**, *21*, 181–195.
(23) Shimizu, S. I.; Balzarini, J.; De Clercq, E.; Walker, R. T. The synthesis and biological properties of some aryl bis(nucleosid-5′-yl) phosphates using nucleosides with proven anti-HIV activity. *Nucleosides Nucleotides* **1992**, *11*, 583–594.
(24) Nilsson, S.; Reichard, P.; Skoog, L. Deoxyuridine triphosphate pools after polyoma virus infection. *J. Biol. Chem.* **1980**, *255*, 9552–9555.
(25) (a) Périgaud, C.; Aubertin, A.-M.; Benzaria, S.; Pelicano, H.; Girardet, J.-L.; Maury, G.; Gosselin, G.; Kirn, A.; Imbach, J.-L. Equal inhibition of the replication of human immunodeficiency virus in human T-cell culture by ddA bis(SATE)phosphotriester and 3′-azido-2′,3′-dideoxythymidine. *Biochem. Pharmacol.* **1994**, *48*, 11–14. (b) Benzaria, S.; Girardet, J.-L.; Périgaud, C.; Aubertin, A.-M.; Pelicano, H.; Maury, G.; Gosselin, G.; Kirn, A.; Imbach, J.-L. The SATE pronucleotide derivative of ddA: a more potent HIV inhibitor than AZT. *Nucleic Acids Symp. Series* **1994**, *31*, 129–130.
(26) Hanes, C. S.; Isherwood, F. A. Separation of the phosphoric esters on the filter paper chromatogram. *Nature* **1949**, *154*, 1107–1112.
(27) Nylén, P.; Olsen, A. Thiol acids added to ethylene oxide. *Sven. Kem. Tidski.* **1941**, *53*, 274–281; *Chem. Abstr.* **1942**, *36*, 753b.
(28) Harding, J. S.; Owen, L. N. The alkaline hydrolysis of acetylated vicinal hydroxy-thiols. *J. Chem. Soc.* **1954**, 1528–1536.
(29) Martin, R. B.; Hedrick, R. I. Intramolecular S-O and S-N acetyl transfer reactions. *J. Am. Chem. Soc.* **1962**, *84*, 106–110.
(30) Kunieda, T.; Higuchi, T.; Abe, Y.; Hirobe, M. Activation of carbonyl groups by diphenyl 2-oxo-3-oxazolinylphosphonate. *Tetrahedron* **1983**, *39*, 3253–3260.
(31) Still, W. C.; Khan, M.; Mitra, A. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* **1978**, *43*, 2923–2925.
(32) Perich, J. W.; Johns, R. B. Di-*tert*-butyl *N,N*-diethylphosphoramidite. A new phosphitylating agent for efficient phosphorylation of alcohols. *Synthesis* **1988**, 142–144.
(33) Yoshikawa, M.; Kato, T.; Takenishi, T. A novel method for phosphorylation of nucleosides to 5′-nucleotides. *Tetrahedron Lett.* **1967**, 5065–5068.
(34) Pauwels, R.; Balzarini, J.; Baba, M.; Snoeck, R.; Schols, D.; Herdewijn, P.; Desmyter, J.; De Clercq, E. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J. Virol. Methods* **1988**, *20*, 309–321.
(35) Moog, C.; Wick, A.; Le Ber, P.; Kirn, A.; Aubertin, A.-M. Bicyclic imidazo derivatives, a new class of highly selective inhibitors for the human immunodeficiency virus type 1. *Antiviral Res.* **1994**, *24*, 275–288.
(36) Chou, J.; Chou, T.-C. Dose-effect analysis with microcomputers: quantitation of ED_{50}, LD_{50}, synergism, antagonism, low-dose risk, receptor binding and enzyme kinetics. *Computer software for Apple II series and IBM-PC and instruction manual*; Elsevier-Biosoft, Elsevier Science Publishers: Cambridge, U.K., 1985; pp 19–28.
(37) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *J. Immunol. Methods* **1983**, *65*, 55–63.

JM950360A