Inhibition of Human Immunodeficiency Virus Type 1 Replication by Phosphonoformate- and Phosphonoacetate-2',3'-Dideoxy-3'-thiacytidine Conjugates

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The synthesis of potential "combined prodrugs" where phosphonoformic acid (PFA) or phosphonoacetic acid (PAA) was attached to the 5'-O or N^4 position of 2',3'-dideoxy-3'-thiacytidine (BCH-189) is described. The anti-HIV-1 activity of 11 analogues which included carboxylic ester or phosphoric ester linkages of PFA or PAA to BCH-189 was determined in MT-4 cells. Of these compounds, the IC_{50} of analogues **3**, **4**, **6**, and **7** ranged from 0.2 to 100 μM, while IC_{50} for BCH-189 in this system was 0.1 μM. *In vitro* hydrolysis of the various esters or amides in human plasma indicated that these agents were relatively stable in the presence of plasma esterases with $t_{1/2}$ values of up to 120 min. Moreover, lipophilicity of these compounds (partition coefficient) was determined in order to establish correlation between lipophilicity and diffusion of BCH-189 analogues into the cells. The active compounds may exert their effects by extracellular or intracellular hydrolysis to the corresponding antiviral agent BCH-189, but intrinsic anti-HIV-1 activity of some of PAA and PFA adducts, themselves, may also be involved.

Introduction

Despite the remarkable efforts provided on biology, virology, and drug research during the first decade following the initial discovery and analysis of AIDS in 1981, only three drugs, 3'-azido-3'-deoxythymidine[1] (AZT, Zidovudine, Retrovir), 2',3'-dideoxyadenosine[2] (Didanosine, Videx), and 2',3'-dideoxycytidine[3] (ddC, Zalcitabine), were approved for the treatment of AIDS patients. These drugs inhibit reverse transcriptase[4] (RT), the enzyme responsible for the production of DNA copies of the viral genome, a required first step in the infectious lifecycle of all retroviruses including HIV-1. Although FDA-approved drugs extend the life of AIDS patients, bone marrow toxicity and side effects such as peripheral neuropathy and pancreatis limit the long-term use of these drugs. Therefore, it is imperative to search for new anti-HIV drugs with improved selectivity and efficiency. Since 1989, several unusual classes of nucleosides such as (±)-dioxolane-T[5-9] and 2',3'-dideoxy-3'-thiacytidine (BCH-189)[5,10-15] have been reported to be active against HIV-1 *in vitro*. BCH-189 was first reported as a potent anti-HIV agent *in vitro* by Belleau *et al.*[5] It showed potent anti-HIV activity (EC_{50} = 0.73 μM) in MT-2 cells and no cross resistance to AZT resistant strains. BCH-189 was 10 times less toxic than AZT in the same cell system,[10] and it also exhibited potent anti-HIV activity (EC_{50} = 0.02-0.06 μM) in human PMB cells.[11] More recently, our team have reported the anti-HIV activities of new BCH-189 analogues carrying various functional groups such as the retinoyl group,[16] the *N*-formylmethionyl peptide,[17] and the phosphoryl group,[18] which were found less potent than BCH-189 itself. We became aware that an AZT-phosphonoformic acid (PFA) conjugate, consisting of a molecule of PFA, was stably linked to the 5'-OH group of AZT and inhibit RT more effectively than separated molecules of triphosphorylated AZT and PFA.[19-21] We began a program of synthesis and biological evaluation of the 2',3'-dideoxy-3'-thiacytidine conjugate to phosphonoformic acid (PFA) and phosphonoacetic acid (PAA). There are several factors which have resulted in the design of these new nucleoside analogues. Depending on the nature of the linkage, a 2',3'-dideoxy-3'-thiacytidine-PFA or -PAA conjugate could perhaps divide into BCH-189 and PFA or PAA inside the cell, thereby serving as a prodrug. Regardless of whether the PFA was linked to BCH-189 through the COOH or $PO(OH)_2$ group, a decreased negative charge on this molecule relative to PFA ought to facilitate movement across the cell membrane. 2',3'-Dideoxy-3'-thiacytidine (**1**) permits amide formation between PAA and the N^4 nitrogen atom of **5** and **6**, leaving free the 5'-OH functionality of the oxathiolane ring. This structural feature could be of interest since RT inhibition required enzymatic phosphorylation at the 5'-OH position by cellular kinase.[4,22,23] Moreover conjugates which consisted of PFA linked directly to BCH-189 through the $PO(OH)_2$ group could serve as a 5'-monophosphate of BCH-189 prodrug via an oxidative decarboxylation reaction.

Chemistry

As preamble to this topic, it should be mentioned that resonance and field effects of the functional groups like $(RO)_2PO$- and $(RO)_2POCH_2$- in PFA and PAA affect the reactivity of COOH in two distinct ways. The electron-withdrawing groups increase acidity by stabilizing $RCOO^-$ by charge dispersal and also affect the entropy by lowering the charge on the COO^- group and changing the electron density distribution in the COOH group. As an example, the acid-strengthening effect on the $(RO)_2PO$- in $(RO)_2POCOOH$ is stronger than the $(RO)_2PO$- in $(RO)_2POCH_2COOH$. Also the entropic effect by lowering the charge on the COO^- group is stronger in PFA than in PAA. In general, entropy effects are the most important.[24] These general remarks should be taken into account in order to explain the observed lower reactivity of phosphonoformic derivatives in comparison of the corresponding phosphonoacetic acid ones.

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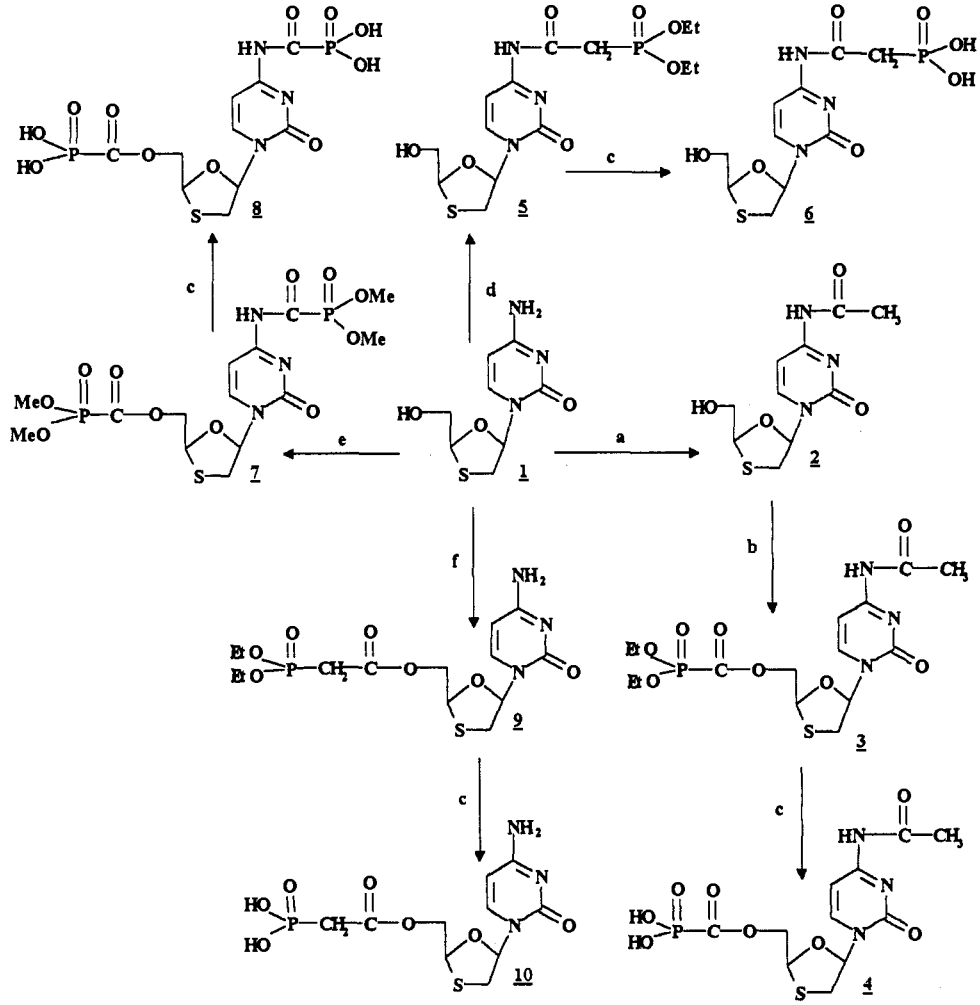
Scheme 1[a]



[a] (a) Ac_2O, DMF, room temperature; (b) $(EtO)_2POCOCl$, DMAP, Et_3N, CH_2Cl_2, room temperature; (c) TMSBr, CH_3CN, room temperature; (d) BOP, DMF, $(EtO)_2POCH_2COOH$, Et_3N, room temperature; (e) $(CH_3O)_2POCOCl$, pyridine, reflux; (f) $(EtO)_2POCH_2COOH$, DMF, DCC, HOBT, CH_2Cl_2, room temperature.

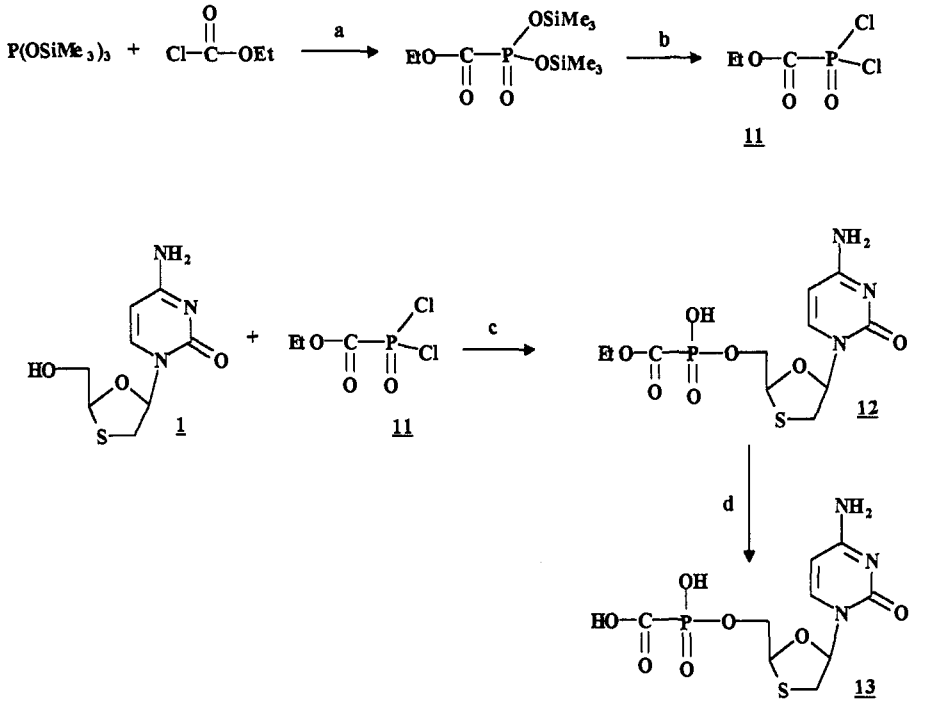
Phosphonoformates **3**, **4**, **7**, **8** and phosphonoacetates **5**, **6**, **9**, **10** were prepared by the reaction of nucleoside or nucleoside intermediates with the corresponding phosphonoformic acid chloride, or phosphonoacetic acid, as shown in Scheme 1. Linkage by a carboxylic ester bond was accomplished by condensation of unprotected nucleoside **1** with (diethylphosphono)acetic acid. This was performed in the presence of *N*,*N*-dicyclohexylcarbodiimide (DCC) to give the corresponding phosphonates **9** and **10**. However a similar condensation attempted in the same experimental conditions with PFA was unsuccessful. Phosphonoformate esters **3** and **4** were obtained by condensation of the corresponding (diethylphosphono)formic acid chloride[25] with N^4-acetyl-2′,3′-dideoxy-3′-thiacytidine (**2**) in the presence of Et_3N and 4-(dimethylamino)pyridine (DMAP). When the same condensation was accomplished starting with the nonprotected BCH-189 (**1**), only the disubstituted analogue **7** was isolated. It should be pointed out that attempts to deacetylate compounds **3** and **4** failed whatever the experimental conditions: In each case, subsequent hydrolysis of the carboxylic ester bond occurred, leading to the starting material **1**.

Interestingly, when the DCC method was used for the coupling between BCH-189 (**1**) and phosphonoacetic acid, the carboxylic ester derivative **9** was selectively obtained. When the same coupling reaction was performed in the presence of (benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate (BOP)[26] as coupling reagent, only the N^4-(diethylphosphono)acetyl derivative **5** was formed, and no 5′-O esterification was observed.

The free phosphonic acids **4**, **6**, **8**, **10** were obtained from the corresponding diethylphosphono esters (**3**, **5**, **7**, **9**) by treatment with bromotrimethylsilane and subsequent methanolysis.[27,28]

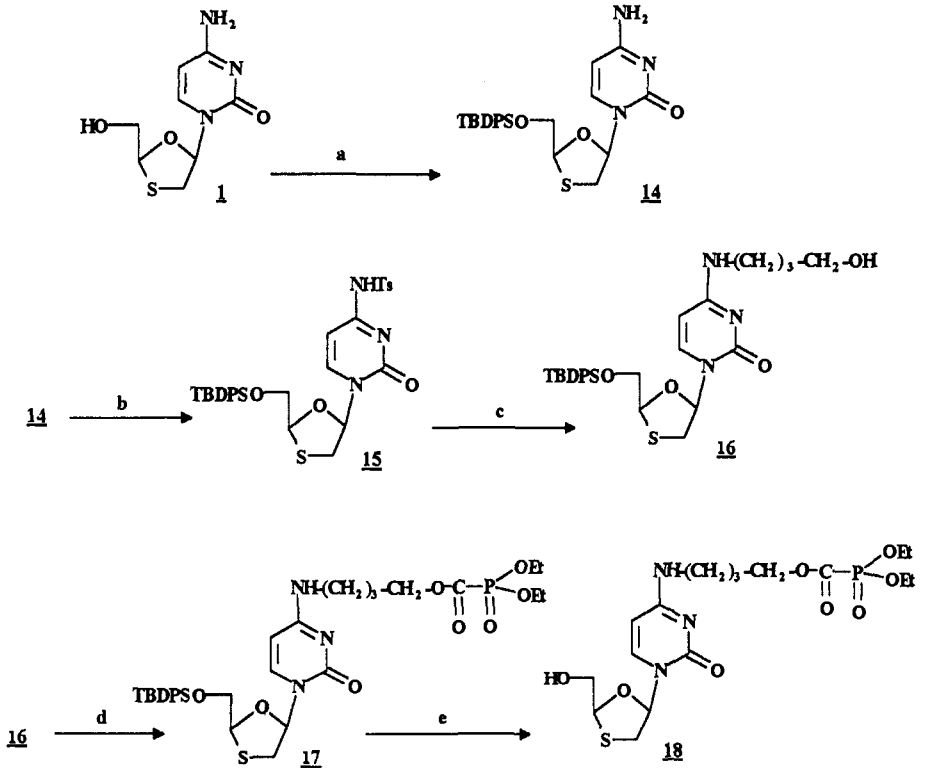
Linkage by a phosphoric ester bond was achieved according to a synthetic strategy summarized in Scheme 2. Tris(trimethylsilyl) phosphite[29] was treated with ethyl chloroformate to yield bis(trimethylsilyl) (ethoxycarbonyl)phosphonate with a good yield.[30] (Ethoxycarbonyl)-phosphonic dichloride (**11**) was synthesized by treatment of the bis(trimethylsilyl) (ethoxycarbonyl)phosphonate derivative with thionyl chloride according to a procedure described by Vaghefi *et al.*[31] Coupling between BCH-189 (**1**) and **11** in DMF at 0 °C leads to the formation of 3′-thiacytidine 5′-(ethoxycarbonyl)phosphonate (**12**) in 60% yield. The corresponding free carboxylic acid **13** was obtained by saponification with 1 N sodium hydroxide. Nucleoside analogue **18**, in which the (diethylphosphono)-formic moiety is linked to the N^4-amino group of the cytosine through a spacer, has been synthesized according to a specific strategy shown in Scheme 3. After protection of the 5′-OH position of the nucleoside **1** by a *tert*-butyldiphenylsilyl group, the resulting compound **14** was N^4-tosylated (compound **15**) and was converted into the

Scheme 2[a]



[a] (a) 0 °C, and overnight at room temperature; (b) $SOCl_2$, benzene Δ; (c) (1) DMF, 0 °C, room temperature; (2) H_2O; (d) NaOH, H_2O, room temperature.

Scheme 3[a]



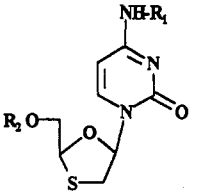
[a] (a) TBDPSCl, pyridine; (b) TsCl, pyridine, Δ; (c) $NH_2(CH_2)_4OH$, pyridine, Δ; (d) (1) triphosgene, CH_2Cl_2, 0 °C; (2) $(EtO)_3P$, CH_2Cl_2, room temperature; (e) TBAF, THF, room temperature.

corresponding N^4-(4-hydroxybutyl) derivative 16 following a procedure reported by Markiewicz *et al.*[32] By reacting 16 with triphosgene[33] in pyridine followed by addition of triethyl phosphite, the PFA adduct 17 was isolated in low yield (11%). Treatment overnight of 17 with tetrabutylammonium fluoride produced the analogue 18. All of the new anti-HIV candidates synthesized are presented in Table 1.

Biological Results

The inhibition of HIV-1 replication is measured by the formation of syncytia in HIV-1 infected MT-4 cells. We observed for all the tested compounds (1, 3–10, 12, 13, and 18) a dose-dependent relationship of this inhibition. Their IC_{50} (concentration required to produce 50% inhibition of syncytia formation) values are presented in Table 1.

Table 1. Anti-HIV-1 Activity and Biophysical Properties of Various Phosphorylated BCH-189 Analogues



no.	R_1	R_2	P^a	$t_{1/2}$,[b] h	IC_{50},[c] μM	TI[e] (ID_{50}[d]/IC_{50})
1	H	H	0.92	-	0.1 ± 0.05	1000
3	$COCH_3$	$(EtO)_2P(O)C(O)$	4.5	-	0.3 ± 0.05	350
4	$COCH_3$	$(HO)_2P(O)C(O)$	0.12	2	0.2 ± 0.05	500
5	$(EtO)_2P(O)CH_2C(O)$	H	9.8	2.5	5 ± 2	100
6	$(HO)_2P(O)CH_2C(O)$	H	0.15	-	100 ± 50	10
7	$(MeO)_2P(O)C(O)$	$(MeO)_2P(O)C(O)$	5.2	7.5	inactive/toxic	-
8	$(HO)_2P(O)C(O)$	$(HO)_2P(O)C(O)$	0.06	-	inactive/toxic	-
9	H	$(EtO)_2P(O)CH_2C(O)$	3.0	>4	10 ± 5	50
10	H	$(HO)_2P(O)CH_2C(O)$	0.8	-	1 ± 0.5	300
12	H	EtOC(O)P(O)(OH)	0.56	>4	inactive/toxic	-
13	H	HOC(O)P(O)(OH)	0.03	-	100 ± 50	10
18	$(EtO)_2P(O)C(O)(CH_2)_4$	H	16.2	-	inactive	-

[a] Partition coefficient. [b] Half-life time. [c] IC_{50}: concentration required to inhibit syncitia formation by 50%. [d] ID_{50}: concentration required to cause 50% death of uninfected MT-4 cells. [e] TI: therapeutic index.

Generally speaking, the results of the experiments presented on Table 1 showed that among the 11 phosphorylated BCH-189 analogues, the four (**3**, **4**, **5**, **9**) that inhibit HIV-1 replication had potencies (IC_{50}) ranging between 0.2 and 10 μM. Under these experimental conditions, the ID_{50} value for BCH-189 was 0.1 μM. PFA–BCH-189 analogues were found to be the most active compounds (**3** and **4**) compared to the corresponding PAA–BCH-189 analogues (**6**, **5**, **9**, **10**). At first this observation appeared not surprising since PFA was reported active against a wide variety of viruses, including HIV-1,[21] while PAA was mostly active on HSV-1 and HSV-2.[34] In contrast, the nonactivity of compounds **12** and **13**, with which PFA is linked through the $PO(OH)_2$ group, was more intriguing. On the one hand the length of the side chain in compound **12** was approximately the same as that of the triphosphate moiety in triphosphorylated BCH-189 (BCH-189TP), which was the active metabolite involved in the RT inhibition.[35] On the other hand, phosphonoformate esters of AZT[19] were found active on HIV-1 replication. Based on this, one could foresee that these analogues would be efficient. In view of these results, a BCH-189–PFA conjugate, consisting of a molecule of PFA that is stably linked to the 5′-OH of BCH-189 through the $PO(OH)_2$ group, did not produce any anti-HIV activity. This result excluded the possibility of these compounds serving as a 5′-monophosphate of BCH-189 prodrug via an oxidative decarboxylation reaction.

Regarding the total antiviral inactivity of compound **18**, it can be concluded that introduction of a phosphonoformic diethyl ester moiety, through a carbon chain spacer, by direct N^4-alkylation, abolished any antiviral activity. Moreover, the diphosphonoformic analogues **7** and **8** were found toxic at 100 μM and totally inactive at lower concentrations. Previous studies have reported that nucleosides such as AZT cross the cell membranes by nonfacilitated diffusion. Additionally their uptake was insensitive to the inhibitors of nucleoside transport,[36] indicating that the partition coefficient of nucleoside analogues might have a significant role in their diffusion. According to these reports, the partition coefficients of the newly synthesized analogues were determined by equilibrating their solutions in 1-octanol with a phosphate buffer (0.2 mol, pH 7.4) at room temperature, according to a method reported by Fujita *et al.*[37] The data in Table 1 indicate that the partition coefficient of **18** was 16.2, highest in this series, while compound **8**, with a partition coefficient of 0.06, appears to be less lipophilic than BCH-189.

The usefulness of the prodrugs of BCH-189 should not solely rely on the stability of the prodrug for its transport across the cell membrane, but also upon its intracellularly reversion to the parent compound in the virally infected cells. The half-lives ($t_{1/2}$) of hydrolysis of compounds **12**, **5**, **9**, **7**, and **4** were determined in human plasma and are reported in Table 1. All of the tested various phosphorylated analogues of BCH-189 were found to be relatively stable in contact with plasma esterases with a $t_{1/2}$ of up to 120 min. These results suggest that compounds like **4** may have increased plasma $t_{1/2}$ under the *in vivo* conditions. Indeed, active antiviral compounds which have a half-life in the body of up to 30 min are considered as potential *in vivo* candidates.[38]

Discussion

Most of the PAA and PFA derivatives tested showed *in vitro* anti-HIV-1 activity less than that of the active antiviral agent BCH-189. One explanation is that these compounds may be acting as prodrugs, thereby releasing the active antiviral agent by hydrolysis. Our results have also shown that the reduction in activity of the PAA or PFA adducts, compared to the component pieces, is not in a fixed ratio. Therefore it is possible that some of the PAA and PFA adducts themselves have antiviral activities. Indeed if the observed anti-HIV activity was only due to the release of BCH-189 in the hydrolysis process, all of the prodrug analogues should be active in the experimental testing conditions. Our present results and previous observations[16-18] have clearly shown that some of N^4-substituted BCH-189 conjugates are not active. However, delivery seems to be the most likely reason for the observed differences in the *in vitro* activity. Clearly, more detailed biochemical studies are necessary before the mode of action of the compounds can be explained.

In summary, we have developed the synthesis of new PFA–BCH-189 and PAA–BCH-189 conjugate analogues. Among them, analogues like **3** and **4** may be viewed as

prototypes of a new class of antiviral dideoxyheteronucleosides in which the conjugate moiety PFA itself has antiviral activity. If these analogues were not susceptible to plasma esterases, and like compound 3 had a greater lipophilicity than BCH-189 itself, such compounds may offer clinical interest.

Experimental Section

Chemistry. Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker AMX-200 (^{1}H NMR, ^{31}P NMR, ^{13}C NMR) spectrometer. Chemical shifts were expressed in δ values (part per million) relative to tetramethylsilane as an internal standard for ^{1}H and relative to H_3PO_4 for ^{31}P. FAB$^+$ mass spectra were obtained on a JEOL DX-100 mass spectrometer (Laboratoire de Mesures Physiques-RMN, Dr. Astier, USTL, Montpellier, France) using a cesium ion source and a glycerol/HCl matrix. Ultraviolet spectra were obtained using a UVIKON 930 (Kontron Instruments) spectrophotometer. Infrared spectra were obtained using a 1605 FT-IR (Perkin-Elmer) spectrophotometer. Elemental microanalysis were determined by Service Central d'Analyse CNRS Vernaison-Lyon France and gave combustion values for C, H, N within 0.4% of the theoretical values. Preparative flash column chromatographies[39] were performed using silica gel Merck G60 230-240 mesh. Analytical thin-layer chromatographies were performed on silica gel 60F 254 aluminum plates (Merck, Darmstadt) of 0.2-mm thickness. The synthesis of the key intermediate 2′,3′-dideoxy-3′-thiacytidine (1, BCH-189) has been performed according to the procedure reported previously.[5,10]

Cis Isomers of 2-(Hydroxymethyl)-5-(N^4-acetylcytosin-1′-yl)-1,3-oxathiolane (2). Dry DMF (2 mL) was added to BCH-189 (1) (100 mg, 0.43 mmol). The mixture was stirred for 5 min under nitrogen atmosphere at room temperature. Acetic anhydride (45 μL, 0.48 mmol) was added dropwise, and the mixture was stirred overnight. The solvent was removed under reduced pressure, and the residue was azeotroped with toluene to give 124 mg of a white solid in a quantitative yield. TLC (EtOAc/MeOH, 4/1): R_f 0.54. ^{1}H NMR ($CDCl_3$): δ 2.25 (s, 3 H, Ac), 3.26 (dd, 2H, CH_2-4), 3.66 (dd, 2H, C2-CH_2O), 5.35 (t, 1H, CH-2), 6.33 (t, 1H, CH-5), 7.47 (d, 1H, CH-5′), 8.48 (d, 1H, CH-6′), 9.60 (s, 1H, NH).

(Diethylphosphono)formic Chloride. Under nitrogen atmosphere, triphosgene (1.19 g, 4 mmol, 1/3 equiv) was cooled to 0 °C. Triethyl phosphite (2.06 mL, 12 mmol) was carefully added dropwise. Then 30 μL of DMF was added, and the mixture was gently stirred and heated at 60 °C overnight. Triethyl phosphite was removed under vacuum (10 mmHg) to give 1.32 g of colorless liquid in 65% yield. ^{1}H NMR ($CDCl_3$): δ 1.35 (m, 6H, 2 × CH_3), 4.24 (m, 4H, 2 × CH_2).

Cis Isomers of 2-[[[(Diethylphosphono)carbonyl]oxy]methyl]-5-(N^4-acetylcytosin-1′-yl)-1,3-oxathiolane (3). To a solution of 2 (50 mg, 0.18 mmol) in 3 mL of anhydrous dichloromethane were added (diethylphosphono)formic chloride (33 mg, 0.20 mmol) and triethylamine (75 μL, 0.54 mmol). The mixture was refluxed overnight under nitrogen atmosphere. The solvent was removed under reduced pressure. Water was added, and the mixture was extracted three times with EtOAc. Organic phases were dried over sodium sulfate and filtered, and the solvent was removed. The crude compound was purified by flash column chromatography (eluent: CH_2Cl_2/EtOAc, 3/2) to give a glassy solid (19 mg) in 27% yield. TLC (EtOAc/MeOH, 9/1): R_f 0.55. ^{1}H NMR ($CDCl_3$): δ 1.27–1.30 (m, 9H, $COCH_3$ and 2 × CH_3), 4.18–4.22 (m, 6H, C2-CH_2O and 2 × CH_2O), 5.33 (dd, 1H, CH-2), 6.26 (dd, 1H, CH-5), 7.40 (d, 1H, CH-5′), 8.11 (d, 1H, CH-6′). MS: 435 (M + H)$^+$. Anal. ($C_{15}H_{22}N_3O_8PS$) C, H, N.

Cis Isomers of 2-[[(Phosphonocarbonyl)oxy]methyl]-5-(N^4-acetylcytosin-1′-yl)-1,3-oxathiolane (4). Compound 3 (14 mg, 0.034 mmol) was dissolved in CH_3CN. Trimethylsilyl bromide was added (45 μL, 0.34 mmol), and the mixture was heated at 70 °C overnight, under a nitrogen atmosphere. Then the crude compound was purified by flash chromatography (eluent: EtOAc/MeOH, 9.5/0.5) to give 6 mg of the desired compound in 56% yield. TLC (EtOAc/MeOH, 9/1) R_f 0.09. ^{1}H NMR (CD_3OD): δ 2.08 (s, 3H, $COCH_3$), 3.29 (dd, 2H, CH_2-4), 3.52 (dd, 2H, C2-CH_2O), 5.43 (dd, 1H, CH-2), 5.97 (d, 1H, CH-5′), 6.34 (dd, 1H, CH-5), 7.92 (d, 1 H, CH-6′). MS: 380 (M + H)$^+$. Anal. ($C_{11}H_{14}N_3O_8PS$) C, H, N.

(Diethylphosphono)acetic Acid. Ethyl diethylphosphonoacetate (4.4 mL, 22.3 mmol) was dissolved in 50 mL of a mixture of THF and water (4:1). Sodium hydroxide (1 N, 50 mL) was added, and the mixture was stirred for 1 h. Then THF was removed, and the residue was filtered through a DOWEX (50W X8-100 mesh) column, previously washed with water. The column was then washed with 500 mL of water, and the residue obtained after evaporation was dissolved in EtOAc, dried over Na_2SO_4, and filtered. The solvent was removed to give a colorless oil (776 mg) in 84% yield. ^{1}H NMR ($CDCl_3$): δ 1.29 (m, 6H, 2 × CH_3), 4.15 (m, 4H, 2 × [CH_2OP]), 10.79 (s, 1H, COOH). ^{31}P NMR ($CDCl_3$): δ 21.00.

Cis Isomers of 2-(Hydroxymethyl)-5-[N^4-[(diethylphosphono)acetyl]cytosin-1′-yl]-1,3-oxathiolane (5). In 10 mL of dry DMF were dissolved (diethylphosphono)acetic acid (107 mg, 0.55 mmol), BCH-189 (1) (125 mg, 0.55 mmol), BOP (241 mg, 0.55 mmol), and NEt_3 (152 μL, 1.1 mmol). The mixture was stirred under a nitrogen atmosphere at room temperature. After 3 h, 4-(dimethylamino)pyridine (3.0 equiv) was added, and the reaction was stirred overnight. The solvent was removed under reduced pressure, and the crude residual compound was purified by flash chromatography (eluent: toluene/MeOH, 23:2), to give 45 mg of product in 20% yield. TLC (EtOAc/MeOH, 4:1): R_f 0.39. ^{1}H NMR (DMSO-d_6): δ 1.22 (t, 6H, 2 × CH_3), 3.14 (d, 2H, PCH_2), 3.11 and 3.36 (dd, 2H, CH_2-4), 4.02 (m, 4H, [CH_2OP] × 2), 4.37 (d, 2H, C2-CH_2O), 5.35 (t, 1H, CH-2), 5.9 (d, 1H, CH-5′), 6.23 (t, 1H, CH-5), 7.21 (d, 1H, CH-6′). ^{31}P NMR (DMSO-d_6): δ 20.29. ^{13}C NMR (DMSO-d_6): δ 15.85 (d, J^3_{CP} = 5.7 Hz, 2 × CH_3), 33.24 (d, J^1_{CP} = 131.6 Hz, $OPCH_2$), 35.22 (C4), 61.90 (d, J^2_{CP} = –6 Hz, 2 × CH_2OP), 65.42 (C2-CH_2O), 80.33 (C2), 86.83 (C5), 94.29 (C5′), 140.49 (C6′), 154.43 (C2′), 165.40 (d, J^2_{CP} = –6 Hz, CO), 165.46 (C4′). MS: 408 (M + H)$^+$. Anal. ($C_{14}H_{22}N_3O_7PS$) C, H, N.

Cis Isomers of 2-(Hydroxymethyl)-5-[N^4-(phosphonoacetyl)cytosin-1′-yl]-1,3-oxathiolane (6). Compound 5 (30 mg, 0.07 mmol) was dissolved in 10 mL of dry acetonitrile, and 150 μL of trimethylsilylbromide was added. The mixture was stirred overnight under a nitrogen atmosphere at room temperature. The reaction mixture was then cooled to 0 °C, and 3 mL of MeOH was added. The solvent was removed to give an orange oily solid, which was washed three times with ether and acetone to give a white solid (20 mg) in 85% yield. TLC (EtOAc/MeOH, 4:1): R_f 0.03. MS: 352 (M + H)$^+$. Anal. ($C_{10}H_{14}N_3O_7PS$) C, H, N.

(Dimethylphosphono)formic Chloride. (Dimethylphosphono)formic acid (1.18 g, 7.65 mmol) was stirred with thionyl chloride (0.6 mL, 7.65 mmol) and then heated at reflux overnight. The solvent was azeotroped with toluene under reduced pressure. The residue was distilled under vacuum (10 mmHg). The acid chloride was collected at 92–98 °C (0.612 g) in 46% yield as a colorless liquid. ^{1}H NMR ($CDCl_3$): δ 4.01 (m, 6H, 2 × CH_3).

Cis Isomers of 2-[[[(Dimethylphosphono)carbonyl]oxy]methyl]-5-[N^4-[(dimethylphosphono)formyl]cytosin-1′-yl]-1,3-oxathiolane (7). BCH-189 (1) (50 mg, 0.218 mmol) was dissolved in 5 mL of dry pyridine, and (dimethylphosphono)-formic chloride (112 mg, 0.65 mmol) was added. The mixture was stirred under a nitrogen atmosphere and refluxed for 3 h. The solvent was evaporated to give an orange solid which was washed with methanol (3 × 3 mL). The methanolic phases were evaporated to give a yellow liquid. This crude compound was purified by flash column chromatography (eluent: EtOAc/MeOH, 90/10 to 50/50) yielded 7 (15 mg) in 14% yield. TLC (*n*-BuOH/H_2O/acetic acid, 5/2.5/2.5): R_f 0.72. ^{1}H NMR (CD_3OD): δ 3.1 (m, 2H, CH_2-4), 3.29 (dd, 2H, C2-CH_2O), 3.53 (m, 12H, 4 × CH_3O), 5.33 (dd, 1H, CH-2), 5.20 (d, 1H, CH-5′), 6.29 (dd, 1H, CH-5), 7.52 (d, 1H, CH-6′). MS: 502 (M + H)$^+$. Anal. ($C_{14}H_{21}N_3O_{11}P_2S$) C, H, N.

Cis Isomers of 2-[[(Phosphonocarbonyl)oxy]methyl]-5-[N^4-(phosphonoformyl)cytosin-1′-yl]-1,3-oxathiolane (8). Compound 7 (7 mg, 0.013 mmol) was dissolved in dry acetonitrile, and trimethylsilyl bromide (13 μL, 0.13 mmol) was added. The mixture was stirred for 3 h under a nitrogen atmosphere, the solvent was then evaporated, and the residue partially dissolved in ether was left stirring overnight. Ether was removed, and the residue was washed twice again with ether. Then the crude

compound was flash chromatographed with EtOAc/MeOH (4:1) as eluent. A very hydroscopic solid (5 mg) was obtained in 85% yield. TLC (EtOAc/MeOH, 4/1): R_f 0.30. ^{1}H NMR (CD_3OD): δ 3.2 (dd, 2H, CH_2-4), 3.4 (dd, 2H, C2-CH_2O), 5.5 (dd, 1H, CH-2), 5.9 (d, 1H, CH-5′), 6.5 (dd, 1H, CH-5), 7.7 (d, 1H, CH-6′). MS: 446 $(M + H)^+$. Anal. ($C_{10}H_{13}N_3O_{11}P_2S$) C, H, N.

Cis Isomers of 2-[[[(Diethylphosphono)acetyl]oxy]methyl]-5-(cytosin-1′-yl)-1,3-oxathiolane (9). (Diethylphosphono)acetic acid (146 mg, 0.75 mmol) was dissolved in dry CH_2Cl_2; DCC (166 mg, 0.82 mmol) and HOBT (110 mg, 0.82 mmol) were added, and the solution was stirred under a nitrogen atmosphere at room temperature for 2 h. When a white precipitate of DCU was observed, BCH-189 (1) (100 mg, 0.43 mmol) was dissolved in a minimum of DMF and added by portions to the reaction mixture, which was left stirring overnight. The solvent was removed under reduced pressure and the crude residual compound purified by flash chromatography (eluent: EtOAc/MeOH, 98:2). A glassy solid (64 mg) was obtained in 37% yield. TLC (toluene/MeOH, 3/2): R_f 0.30. ^{1}H NMR (DMSO-d_6): δ 1.24 (t, 6H, 2 × CH_3), 3.27 (d, 2H, CH_2CO), 3.57 (dd, 2H, CH_2-4), 3.83 (m, 2H, C2-CH_2O), 4.02 (m, 4H, [CH_2OP] × 2), 5.45 (dd, 1H, CH-2), 6.21 (t, 1H, CH-5), 7.17 (d, 1H, CH-5′), 8.43 (d, 1H, CH-6′), 10.99 (s, 2H, NH_2). ^{31}P NMR (DMSO-d_6): δ 21.38. ^{13}C NMR (DMSO-d_6): δ 15.97 (CH_3), 34.59 (d, J^1_{CP} = 133 Hz, $OPCH_2$), 37.24 (C4), 61.89 (C2-CH_2O and 2 × CH_2O), 87.12 (C2), 87.68 (C5), 94.77 (C5′), 145.44 (C6′), 153.94 (C2′), 162.09 (C4′), 165.4 (CO). MS: 408 $(M + H)^+$. Anal. ($C_{14}H_{22}N_3O_7PS$) C, H, N.

Cis Isomers of 2-[[(Phosphonoacetyl)oxy]methyl]-5-(cytosin-1′-yl)-1,3-oxathiolane (10). Compound **9** (37 mg, 0.09 mmol) was dissolved in 10 mL of dry acetonitrile, and trimethylsilyl bromide (150 μL) was added. The mixture was stirred overnight under a nitrogen atmosphere at room temperature. The solvent was removed to give an oily orange solid, which was dissolved and washed with acetone to give a white solid (28 mg) in 89% yield. TLC (toluene/MeOH, 7.3): R_f 0.07. MS: 352 $(M + H)^+$. Anal. ($C_{10}H_{14}N_3O_7PS$) C, H, N.

Tris(trimethylsilyl) Phosphite. In a 500-mL three-necked round-bottomed flask, equipped with a condenser and a $CaCl_2$ trap, was dissolved phosphorous acid (10 g, 0.121 mol) in 100 mL of anhydrous pyridine and 55.6 mL (0.4 mol) of NEt_3. The mixture was cooled to 0 °C, and trimethylsilyl chloride (51.4 mL, 0.4 mol) was carefully added dropwise, during 90 min. The reaction was then left stirring for 2 h at room temperature and overnight at 70 °C. The mixture was directly distilled under vacuum (10 mmHg). Pyridine distilled first, and tris(trimethylsilyl) phosphite (26.16 g) was then obtained as a colorless liquid (bp_{10} 73 °C) in 80% yield. ^{1}H NMR ($CDCl_3$): δ 0.11 (s, 24H, [$(CH_3)_3$]). ^{31}P NMR ($CDCl_3$): δ −13.73.

Bis(trimethylsilyl) (Ethylphosphono)formate. A 50-mL three-necked round-bottomed flask containing ethyl chloroformate (9.35 mL, 97.9 mmol) was cooled to 0 °C. Tris(trimethylsilyl) phosphite (26.16 g, 97.9 mmol) was added dropwise. The mixture was left stirring overnight at room temperature and distilled under 10 mmHg vacuum. Trimethylsilyl chloride distilled first, then bis(trimethylsilyl) phosphorous acid, and finally bis(trimethylsilyl) (ethylphosphono)formate (15.03 g, bp_{10} 68–70 °C) was obtained in 57% yield. ^{1}H NMR ($CDCl_3$): δ 0.25 (s, 18H, ($OSiMe_3$) × 2), 1.25 (m, 3H, CH_3), 4.21 (m, 2H, OCH_2). ^{31}P NMR ($CDCl_3$): δ −23.19. IR: 1720.4 (C=O), 1256.4 (P=O), 1054.9 (Si–O), 848.5 (Si–C).

Ethyl (Dichlorophosphonyl)formate (11). Ethyl [bis(trimethylsilyl)phosphono]formate (3 g, 11.1 mmol) was dissolved in 10 mL of benzene, under a nitrogen atmosphere. Thionyl chloride (2.43 mL, 33.3 mmol) was added, and the mixture was refluxed for 2 h. Benzene and thionyl chloride were first distilled and ethyl (dichlorophosphono)formate was distilled at 64 °C under vacuum (1 mmHg) to give 530 mg of colorless liquid in 25% yield. ^{1}H NMR ($CDCl_3$): δ 1.36 (m, 3H, CH_3), 4.44 (m, 2H, CH_2O). ^{31}P NMR ($CDCl_3$): δ 12.65. ^{13}C NMR ($CDCl_3$): δ 13.63 (CH_3), 66.04 (CH_2O), 159.25 and 164.24 (d, J^1_{CP} = 351 Hz, O=C–P=O).

Cis Isomers of 2-[[[(Ethoxycarbonyl)hydroxyphosphonyl]oxy]methyl]-5-(cytosin-1′-yl)-1,3-oxathiolane (12). Ethyl (dichlorophosphonyl)formate (110 mg, 0.57 mmol) was dissolved in 1 mL of anhydrous DMF. The mixture was cooled to 0 °C, and BCH-189 (1) (100 mg, 0.43 mmol) was added. The mixture was stirred for 1 h at 0 °C under nitrogen and then for 1 h at room temperature. The solvent was removed under reduced pressure. Water was added, and the mixture was extracted three times with EtOAc in order to remove the organic soluble materials. Water was evaporated, and the crude compound was purified by flash chromatography (eluent: *n*-BuOH/H_2O, 8:1) to give 90.6 mg of a pure white solid, in 58% yield. TLC (*n*-BuOH/H_2O/formic acid, 7/1/1.5): R_f 0.19. ^{1}H NMR (D_2O): δ 1.11 (m, 3H, CH_3), 3.22 (dd, 2H, CH_2-4), 4.20 (m, 4H, CH_2OP and C2-CH_2O), 5.30 (t, 1H, CH-2), 5.90 (d, 1H, CH-5′), 6.17 (t, 1H, CH-5), 7.89 (d, 1H, CH-6′). ^{31}P NMR (D_2O): δ −4.80. ^{13}C NMR (D_2O): δ 16.03 (CH_3), 39.37 (C-4), 64.57 (CH_2O), 69.03 (CH_2-C2), 86.58 (C-2), 89.29 (C-5), 98.62 (C-5′), 144.57 (C-6′), 153 (C-2′), 166.42 (C-4′). MS: 366 $(M + H)^+$. Anal. ($C_{11}H_{16}N_3O_7PS$) C, H, N.

Cis Isomers of 2-[[[(Hydroxycarbonyl)hydroxyphosphonyl]oxy]methyl]-5-(cytosin-1′-yl)-1,3-oxathiolane (13). Compound **12** (18.8 mg, 0.05 mmol) was dissolved in 2 mL of water, and 4.5 equiv of 0.4 N sodium hydroxide was added. The pH was 10, and the mixture was neutralized with DOWEX (50W X8-100 mesh, previously washed with distilled water), filtered, and lyophilized to give 10 mg of white solid in 51% yield. TLC (*n*-BuOH/H_2O/formic acid, 7/1/1.5): R_f 0.03. ^{1}H NMR (D_2O): δ 3.37 (dd, 2H, CH_2-4), 4.16 (dd, 2H, C2-CH_2O), 5.38 (t, 1H, CH-2), 6.10 (d, 1H, CH-5′), 6.25 (t, 1H, CH-5), 8.18 (d, 1H, CH-6′). ^{31}P NMR (D_2O): δ 6.57. ^{13}C NMR (D_2O): δ 37.14 (C4), 63.92 (CH_2-C2), 85.47 (C2), 87.53 (C5), 95.24 (C5′), 143.77 (C6′), 198.42 (C2′), 178.34 (C4′), 196.68 (O=C–P=O). MS: 338 $(M + H)^+$. Anal. ($C_9H_{12}N_3O_7PS$) C, H, N.

Cis Isomers of 2-[[(*tert*-Butyldiphenylsilyl)oxy]methyl]-5-(cytosin-1′-yl)-1,3-oxathiolane (14). BCH-189 (1) (100 mg, 0.43 mmol) was dissolved, under a nitrogen atmosphere, in 6 mL of anhydrous pyridine. After the addition of *tert*-butylchlorodiphenylsilane (135 μL, 0.52 mmol), the mixture became clear and was stirred overnight. The solvent was removed under reduced pressure, and the residue was hydrolyzed and extracted with EtOAc (3 × 5 mL). The organic phase was dried over Na_2SO_4 and filtered, and the solvent was evaporated to give a solid which was recrystallized in EtOAc to give a white solid (159 mg) in 80% yield. TLC (EtOAc/MeOH, 2/1): R_f 0.62. ^{1}H NMR ($CDCl_3$): δ 1.09 (s, 9 H, tBu), 3.39 (dd, 2H, CH_2-4), 4.08 (dd, 2H, C2-CH_2O), 5.25 (t, 1H, CH-2), 5.51 (d, 1H, CH-5′), 6.37 (t, 1H, CH-5), 7.44 (m, 5H, arom), 7.69 (m, 5H, arom), 8.04 (d, 1H, CH-6′).

Cis Isomers of 2-[[(*tert*-Butyldiphenylsilyl)oxy]methyl]-5-[N^4-(*p*-tolylsulfonyl)cytosin-1′-yl]-1,3-oxathiolane (15). Compound 14 (352 mg, 0.75 mmol) and *p*-toluenesulfonyl chloride (287.5 mg, 1.50 mmol) were dissolved in 10 mL of anhydrous pyridine to give an orange mixture, which was heated for 30 h at 60 °C under a nitrogen atmosphere. Solvent was removed under reduced pressure. The resulting mixture was hydrolyzed and extracted with EtOAc, washed with a 5% citric acid aqueous solution, dried over Na_2SO_4, and filtered, and the solvent was evaporated to give 419 mg of a solid in 89% yield. TLC (toluene/MeOH, 4:1): R_f 0.46. ^{1}H NMR ($CDCl_3$): δ 1.07 (s, 9H, tBu), 2.42 (s, 3H, CH_3), 3.34 (dd, 2H, C2-CH_2O), 4.04 (dd, 2H, CH_2-4), 5.22 (t, 1H, CH-2), 6.27 (t, 1H, CH-5), 7.35 (d, 1H, CH-5′), 7.43 (m, 5H, arom), 7.65 (m, 5H, arom), 7.80 (m, 2H, arom), 7.85 (m, 2H, arom), 8.04 (d, 1H, CH-6′).

Cis Isomers of 2-[[(*tert*-Butyldiphenylsilyl)oxy]methyl-5-[N^4-(4-hydroxybutyl)cytosin-1′-yl]-1,3-oxathiolane (16). Compound **15** (420 mg, 0.67 mmol) was dissolved in anhydrous pyridine (10 mL), 4-aminobutanol was added (308 μL, 3.35 mmol), and the solution was heated for 3 days at 80 °C under nitrogen atmosphere. Pyridine was evaporated under reduced pressure, and the crude resulting compound was hydrolyzed and extracted with EtOAc (2 × 10 mL). The organic phase was washed with 5% citric acid aqueous solution (2 × 10 mL), dried over Na_2SO_4, and filtered, and the solvent was evaporated to give a yellow solid, which was purified by flash chromatography (eluent: EtOAc/MeOH, 95:5) to yield 209 mg (57%) of an off-white solid. TLC (toluene/MeOH, 4/1): R_f 0.40. ^{1}H NMR ($CDCl_3$): δ 1.08 (s, 9 H, tBu), 1.63 (m, 2H, CH_2), 1.85 (m, 2H, CH_2), 2.60 (dd, 2H, C2-CH_2O), 3.57 (m, 2H, CH_2), 3.69 (m, 2H, CH_2), 4.03 (dd, 2H, CH_2-4), 5.30 (t, 1H, CH-2), 5.34 (d, 1H, CH-5′), 6.39 (t, 1H, CH-5), 7.42 (m, 5H, arom), 7.69 (m, 5H, arom), 7.87 (d, 1H, CH-6′).

Cis Isomers of 2-[[(*tert*-Butyldiphenylsilyl)oxy]methyl]-5-[N^4-[4-[[(diethylphosphono)carbonyl]oxy]butyl]cytosin-1'-yl]-1,3-oxathiolane (17). Under a nitrogen atmosphere, triphosgene (39 mg, 0.13 mmol, $^1/_3$ equiv) was dissolved in distilled CH_2Cl_2. The solution was cooled to 0 °C, and 210 mg of 16 (0.39 mmol) was added. The reaction was stirred for 30 min at 0 °C, and triethyl phosphite (67 μL, 0.39 mmol) dissolved in CH_2Cl_2 was added. The resulting solution was left stirring overnight at room temperature. Solvent was removed under vacuum, and the obtained residue was hydrolyzed and extracted with CH_2Cl_2 (3 × 10 mL). The organic phase was dried over Na_2SO_4 and filtered, and the solvent was evaporated. The crude compound was purified by flash chromatography (eluent: EtOAc/CH_2Cl_2, 1/1) to give 29 mg of a glassy solid in 11% yield. (A mixture of the desired compound and the chloroformate intermediate was also separated.) TLC (toluene/MeOH, 4/1): R_f 0.31. ^{1}H NMR ($CDCl_3$): δ 1.09 (s, 9H, tBu), 1.34 (m, 6H, 2 × CH_3), 1.83 (m, 4H, CH_2CH_2), 3.54 (dd, 2H, C2-CH_2O), 3.74 (m, 4H, CH_2OCO and NHCH_2), 4.09 (m, 4H, 2 × CH_2OP), 4.20 (dd, 2H, CH_2-4), 5.27 (t, 1H, CH-2), 6.37 (dd, 1H, CH-5), 6.60 (d, 1H, CH-5'), 7.43 (m, 5H, arom), 7.69 (m, 5H, arom), 8.19 (d, 1H, CH-6').

Cis Isomers of 2-(Hydroxymethyl)-5-[N^4-[4-[[(diethylphosphono)carbonyl]oxy]butyl]cytosin-1'-yl]-1,3-oxathiolane (18). Compound 17 (29 mg, 0.04 mmol) was dissolved in THF under a nitrogen atmosphere, and tetrabutylammonium fluoride (356 μL) was added dropwise. The solution was stirred for 1 h at room temperature. The solvent was evaporated, and the crude compound was purified by flash chromatography (eluent: EtOAc/MeOH, 90:10) to give 7 mg of 18 (foam) in 40% yield. TLC (toluene/MeOH, 4/1): R_f 0.25. ^{1}H NMR ($CDCl_3$): δ 1.32 (m, 6H, 2 × CH_3), 1.84 (m, 4H, CH_2CH_2), 3.31 (dd, 2H, C2-CH_2O), 3.73–4.13 (m, 8H, CH_2OCO and NHCH_2 and 2 × CH_2-OP), 4.27 (dd, 2H, CH_2-4), 5.30 (t, 1H, CH-2), 5.62 (d, 1H, CH-5'), 6.38 (t, 1H, CH-5), 7.73 (d, 1H, CH-6'). Anal. ($C_{17}H_{28}N_3O_8PS$) C, H, N.

Biological Methods. Hydrolysis of the New Analogues in Human Plasma. To 100 μL of human plasma was added 10 μL of a solution of one of the analogues (10 mg/mL, in DMSO or H_2O), and the mixture was incubated at 37 °C in a water bath. At various intervals of time, 10 μL of the samples was withdrawn and transferred to a quartz cuvet containing 500 μL of H_2O. UV spectra of the solution between 200 and 400 nm were recorded. The reference cuvet contained 10 μL of human plasma in 500 μL of H_2O. From the observed optical density changes, at various wavelengths, the half-lives of the analogues in human plasma were calculated.

Partition Coefficients. Partition coefficients of the new tested analogues were determined according to a well-known procedure described in the ref 37.

***In Vitro* Inhibition of HIV-1 Replication in MT-4 Blood Lymphocytes.** Representative compounds were tested for their ability to inhibit HIV-1 infection in cell culture. The fusogenic effect of HIV in the MT-4 cell line[40] was determined as described by Rey *et al.*[41,42] A total of 3 × 10^5 MT-4 cells was infected with 100 μL of diluted virus for 1 h at 37 °C. After three washes, the infected cells were cultured in 24-well cell culture plates in the presence of the inhibitor. The appearance of syncytia was measured with an inverted optical microscope 5 days after infection. The inhibitory concentration was expressed as the concentration of the tested compound which causes 50% inhibition of syncytia formation (IC_{50}) but was not toxic for the cells.

For toxicity testing, three replication cultures of each uninfected MT-4 cells (2 × 10^5 cells) were incubated with various concentrations of 2',3'-dideoxy-3'-thiacytidine analogues. Cell viability was determined 6 days from drug addition by trypan blue exclusion.

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