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Intracellular delivery of nucleoside monophosphates through a reductase-mediated activation process

Frédéric Puech[a], Gilles Gosselin[a], Isabelle Lefebvre[a], Alain Pompon[a], Anne-Marie Aubertin[b], André Kirn[b] and Jean-Louis Imbach[a]

[a]*Laboratoire de Chimie Bioorganique, U.R.A. C.N.R.S. n° 488, Université de Montpellier II, Sciences et Techniques du Languedoc, 34095 Montpellier, France and* [b]*Institut de Virologie de la Faculté de Médecine de Strasbourg, Unité I.N.S.E.R.M. 74, 3 rue Koeberlé, 67000 Strasbourg, France*

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Summary

On the basis of three different models (namely: ddU, AZT and PMEA), mononucleotide phosphotriester derivatives were designed to be able to liberate the corresponding monophosphate (or phosphonate) inside the cell through a reductase-mediated activation process. It was demonstrated that the use of bis[*S*-(2-hydroxyethylsulfidyl)-2-thioethyl] esters of ddUMP (**11**), AZTMP (**12**) and PMEA (**17**) resulted in intracellular delivery of the parent monophosphate (or phosphonate). This point was corroborated by observation of an anti-HIV effect of, **11** in various cell lines, for **12** in CEM TK$^-$ cells and by the enhanced activity observed for **17**. Furthermore, the reported decomposition data in cell extracts fully confirm the validity of this approach and show unambiguously the potential for intracellular reductase-mediated activation of the starting drug.

Prodrugs; Nucleoside phosphotriesters; Reductase-mediated activation; Anti-HIV nucleoside derivatives

Introduction

In the search for effective agents against human immunodeficiency virus

Correspondence to: G. Gosselin or J.-L. Imbach, Laboratoire de Chimie Bioorganique, Case Courrier 008, Université de Montpellier II, Sciences et Techniques du Languedoc, Place Eugène Bataillon, 34095 Montpellier Cédex 5, France. Fax: (33) 67 04 20 29.

(HIV), a large number of dideoxynucleosides have emerged as efficient drugs (De Clercq, 1992). Their antiretroviral effects involve their conversion, through cellular enzymes, to the corresponding triphosphate metabolites, which competitively inhibit HIV reverse transcriptase or terminate the newly synthesized viral DNA chain. These nucleoside analogues suffer from a dependence on kinase-mediated phosphorylations which explains the low activity of some of them. Therefore, attempts have been made to circumvent this dependence through the use of prodrugs of the bioactive nucleotide forms (Mullah et al., 1992). With such a strategy, anti-HIV nucleoside analogues may show better biological responses. In the case of compounds such as 2′ 3′-dideoyuridine (ddU) which are inactive due to their inability to be phosphorylated by cellular kinases (Hao et al., 1990), an antiviral activity can be observed if the mononucleotide is delivered inside the cell (Sastry et al., 1992; Zelphati et al., 1993). In addition, this approach appears to be an interesting way to improve the cellular uptake and bioavailability of charged active nucleotidic phosphonate analogues such as 9-(2-phosphonylmethoxyethyl)adenine (PMEA) (Starrett et al., 1992).

This report describes the synthesis, biological evaluation and preliminary pharmacokinetic studies of derivatives of modified nucleotides designed to release the corresponding free monophosphate, once inside the cell, through a reductase-mediated activation process.

Materials and Methods

Cell cultures an viruses.

The human T-cell lines CEM-SS (Nara et al., 1987), MT-4 (Harada et al., 1985), CEM-X 174 fusion of a B-cell line and CEM (Salter et al., 1985), and CEM-TK$^-$ (provided by Farquhar and Plunkett, University of Texas) were maintained in RPMI 1640 medium with 2 mM glutamine and 10% foetal calf serum (FCS, heated 30 min at 56°C) in a CO_2 incubator at 37°C. Human peripheral blood mononuclear cells (PBMC) from HIV-1 seronegative donors, separated on ficoll, were cultured in RPMI 1640 containing 10% FCS in the presence of 4 μg/ml phytohaemagglutinin (PHA). After 3 days, PHA was removed, cells were washed with medium and reincubated, after infection, in medium containing interleukin-2 (20 U/ml). The virus isolates used were HIV-1-LAI, formerly HIV-1-BRU (Barré-Sinoussi et al., 1983; Wain-Hobson et al., 1991) and HTLV-III B (Popovic et al., 1984). The following reagents were obtained through the AIDS Research and Reference Reagent Program-Division of AIDS, NIAID, NIH: CEM-SS from Nara, P.L. and CEM-X 174 from Cresswell, P.

Cytotoxicity assay on MT-4 cells.

Replication of HIV-1 in MT-4 was measured by the reduction in viability of the cells, resulting from the infection (Génu-Dellac et al., 1991). 50% effective

concentration (EC_{50}) was defined as the concentration of the compound required to achieve 50% protection of the infected cells.

Inhibition of virus production.

Production of virus particles was evaluated by measurement of virion-associated reverse transcriptase activity in the culture supernatant as described (Jonassen, T.O., Skatron Application Notes, April 1986).

CEM-SS,CEM-TK$^-$, CEM-X 174 and PBMC cells were infected with HIV-1-LAI. After 0.5 h absorption, the residual free virus was removed by centrifugation, the cultures were resuspended, respectively, at the concentration of 40 $\times$ 10^3, 80 $\times$ 10^3, and 160 $\times$ 10^3 cells/ml in the medium indicated above and distributed into microtitration plates (100 μl/well) containing 0.1 ml/well of different dilutions of the antiviral drugs. Reverse transcriptase activity was measured after 5 days for CEM-SS cultures, 6 days for CEM-TK$^-$, 7 days for CEM-X 174 and PBMC cultures. In the PBMC cultures, 100 μl of medium was removed after 5 days and replaced with fresh medium containing the same concentration of drug. 50% effective concentration (EC_{50}) was defined as the concentration of drug that reduced the reverse transcriptase activity by 50%. 50% cytotoxic concentration (CC_{50}) was determined by the MTT dye reduction assay as for MT-4 cells.

Preparation of cell extracts.

Exponentially growing CEM-SS cells were collected by centrifugation, washed three times in phosphate-buffered saline and resuspended in 10 mM Tris-HCl, 140 mM KCl (pH 7.4), at the concentration of 24 $\times$ 10^6 cells/ml. Cells were lyzed by ultrasonic treatment (15 s) and cellular debris were removed by centrifugation at 100 000 $\times$ *g*, 1 h at 4°C. The soluble proteins were recovered in the supernatant (about 5.5 mg/ml) and analyzed for their enzymatic activities.

Starting materials and chemical synthesis.

2',3'-Dideoxycytidine (ddC) and ddU were obtained from Synthélabo-Recherche (France). 3'-Azido-2',3'-dideoxythymidine (AZT) was purchased from Intsel Marsing France. PMEA was synthesized as described by Holý and Rosenberg (1987). General procedures and instrumentation used have been described previously (Périgaud et al., 1992). ^{31}P-NMR spectra were recorded at ambient temperature on a Bruker WP 200 SY spectrometer with proton decoupling.

O-[N^4-(4-Monomethoxytrityl)-2',3'-dideoxycytidin-5'-yl]-O'-(2-chlorophenyl)-phosphate (**2**).

A solution of 1,2,4-triazole (1.21 g, 17.5 mmol), 2-chlorophenyl phosphorodichloridate (1.10 ml, 6.7 mmol) and triethylamine (1.90 ml, 13.6 mmol) in anhydrous acetonitrile (15 ml) was stirred for 0.5 h at room temperature. This mixture was added to a solution of N^4-(4-monomethoxytrityl)-2',3'-dideox-

ycytidine (**1**) (Puech et al., 1990) (1.10 g, 2.27 mmol) in pyridine (20 ml). The reaction mixture was stirred for 0.5 h, and a solution of triethylamine (0.75 ml) and water (0.25 ml) in pyridine (1.5 ml) was then added. After 15 min the mixture was diluted with saturated aqueous sodium hydrogen carbonate and repeatedly extracted with methylene chloride. The combined extracts were washed with water, dried over sodium sulfate, evaporated, and co-evaporated with toluene. The resulting gum was precipitated from hexane to afford **2** as the triethylammonium salt (1.43 g, 85% after exhaustive drying in vacuo), which was chromatographically pure. This material was used directly without further purification: UV (ethanol) λ_{max} 278 nm (ε, 15700), λ_{min} 251 nm (ε 12200); ^{1}H-NMR ($CDCl_3$) δ ppm 7.6 (br s, 1H, NH), 7.3–6.6 (*m*, 19H; trityl, phenyl and H-6), 5.87 (*m*, 1H, H-1'), 5.08 (*d*, 1H, H-5; $J_{5,6}$ = 7.7 Hz), 4.20 (*m*, 1H, H-4'), 4.1–3.9 (*m*, 2H, H-5',5''), 3.73 (*s*, 3H, OCH_3), 2.98 [*m*, 6H, $(CH_2CH_3)_3\,NH^+$], 2.35 (*m*, 1H, H-2'), 2.05–1.70 (*m*, 3H, H-2'', 3', 3''), 1.24 [*t*, 9H, $(CH_2CH_3)_3NH^+$; J = 7.3 Hz]; ^{31}P-NMR ($CDCl_3$) – 4.96 ppm; mass spectrum (FAB < 0, matrix = glycerol/thioglycerol, 1:1, v/v): 672 $[M - (CH_2CH_3)_3NH]^-$, 382 $[B]^-$.

O,O'-Bis[N^4-(4-monomethoxytrityl)-2',3'-dideoxycytidin-5'-yl]-O''-(2-chlorophenyl)phosphate (**3**).

Compounds **1** (0.87 g, 1.80 mmol) and **2** (1.40 g, 1.89 mmol) were first co-evaporated three times with anhydrous pyridine and then dissolved in pyridine (25 ml) and 1-mesitylene-2-sulfonyl-3-nitro-1,2,4-triazole (Jones et al., 1980) (MSNT, 1.4 g, 4.7 mmol) was added to the stirred solution at room temperature. After 2 h, saturated aqueous sodium hydrogen carbonate (2 ml) was added and the stirring continued for 15 min. The reaction mixture was poured into saturated aqueous sodium hydrogen carbonate and repeatedly extracted with methylene chloride. The combined extracts were dried over sodium sulfate, filtered, and evaporated to dryness. The. residue was reevaporated three times in toluene solvent and then chromatographed on a silica-gel column [eluent: stepwise gradient of methanol (0–4%) in methylene chloride] to afford 1.48 g (72%) of pure **3**: UV (ethanol) λ_{max} 278 nm (ε 27000), λ_{min} 251 nm (ε, 12200); ^{1}H-NMR ($CDCl_3$) δ ppm 7.6–6.7 (*m*, 36H, 2 trityl, phenyl, 2 NH and 2 H-6), 5.96 (*m*, 2H, 2 H-1'), 4.99 and 4.98 (2*d*, 1H each, 2 H-5; $J_{5,6}$ = 7.6 Hz each), 4.25–4.00 (*m*, 6H, 2 H-4', 2 H-5' and 2 H-5''), 3.77 and 3.76 (2*s*, 3H each, 2 OCH_3), 2.40 (*m*, 2H, 2 H-2') 2.05–1.50 (*m*, 6H, 2 H-2'', 2 H-3' and 2 H-3''); ^{31}P-NMR ($CDCl_3$) –5.25 ppm; mass spectrum (FAB < 0, matrix = glycerol/thioglycerol, 1:1, v/v): 1137 $[M - H]^-$, 382 $[B]^-$.

O,O'-Bis[N^4-(4-monomethoxytrityl)-2',3'-dideoxycytidin-5'-yl]-O''-[S-(2-hydroxyethylsulfidyl)-2-thioethyl]phosphate (**4**).

A solution of 2,2'-dithiodiethanol (5.5 ml), compound **3** (0.63 g, 0.55 mmol) and cesium fluoride (0.84 g, 5.5 mmol) in methylene chloride (5.5 ml) was stirred for 26 h. Water was added and the reaction mixture was extracted with methylene chloride. The organic phase was dried over sodium sulfate, and evaporated to dryness. Chromatography of the residue on a silica-gel column

[eluent: stepwise gradient of methanol (0–6%) in methylene chloride] afforded pure **4** (0.40 g, 62%): UV (ethanol) λ_{max} 280 nm (ε 25200), λ_{min} 251 nm (ε 19900); ^{1}H-NMR ($CDCl_3$) δ ppm 8.3 (br *s*, 2H, 2 NH), 7.42 (*d*, 2H, 2 H-6; J = 7.2 Hz), 7.35–6.80 (*m*, 28 H, 2 trityl), 5.94 (*m*, 2H, 2 H-1'), 5.11 and 5.09 (2*d*, 1H each, 2 H_5; $J_{5,6}$ = 7.2 Hz each), 4.30–4.00 (*m*, 10H, 2 H-4', 2 H-5', 2 H-5'' and CH_2-CH_2-S-S-CH_2-CH_2), 3.79 (*s*, 6H, 2 OCH_3), 3.43 (*m*, 4H, CH_2-CH_2-S-S-CH_2-CH_2), 2.82 (*m*, 2H, 2 H-2'), 2.15–1.85 (*mn*, 4H, 2 H-2'' and 2 H-3'), 1.70 (*m*, 2H, 2 H-3''); ^{31}P-NMR (Me_2SO-d_6) – 0.66 ppm.

O,O'-Bis(2',3'-dideoxycytidin-5'-yl)-O''-[S-(2-hydroxyethylsulfidyl)-2-thioethyl]phosphate (**5**).

Compound **4** (0.20 g, 0.172 mmol) was dissolved in a solution of 4% trifluoroacetic acid in methylene chloride (5 ml), and the reaction mixture was stirred at room temperature for 18 h. The reaction mixture was neutralized by addition of cold methanolic ammonia (previously saturated at -10°C), and then the solvents were quickly removed by evaporation under reduced pressure. The residue was partitioned between water and methylene chloride, and the aqueous layer was evaporated. The resulting crude material was twice chromatographed on silanized silica-gel column [RP-2 Merck No 7719; 1.4 × 20 cm; eluent: linear gradient of methanol (0–100%) in water] to give pure **5** (40 mg, 38%): UV (H_2O) λ_{max} 271 nm (ε, 18000), λ_{min} 249 nm (ε 12000); ^{1}H-NMR (Me_2SO-d_6) δ ppm 7.62 (*d*, 2H, 2 H-6; $J_{5,6}$ = 7.4 Hz), 7.1 (*br*, *s*, 4H, 2 NH_2), 6.01 and 5.99 (2*dd*, 1H each, 2 H-1'; J = 2.3 and 4.0 Hz, and J = 2.3 and 4.3 Hz, respectively), 5.71 (*d*, 2H, 2 H-5; $J_{5,6}$ = 7.4 Hz), 4.87 (*t*, 1H, OH; J = 5.4 Hz), 4.20–4.10 (*m*, 8H, 2 H-4', 2 H-5', 2 H-5'' and P-O-CH_2-CH_2), 3.61 (*m*, 2H, CH_2-CH_2OH), 2.99 (*t*, 2H, P-O-CH_2-CH_2), 2.79 (*t*, 2H, CH_2-CH_2OH), 2.28 (*m*, 2H, 2 H-2'), 2.00 (*m*, 2H, 2 H-3'), 1.90–1.75 (*m*, 4H, 2 H-2'' and 2 H-3''); ^{31}P-NMR (Me_2SO-d_6 + D_2O) – 0.60 ppm; mass spectrum (FAB > 0, matrix = glycerol): 621 $[M + H]^+$, 510 $[M - B]^+$, 112 $[BH_2]^+$.

O,O'-Bis[N^4-(4-monomethoxytrityl)-2',3'-dideoxycytidin-5'-yl]phosphate (**6**).

A solution of *syn*-4-nitrobenzaldoxime (Jones et al., 1980) (0.73 g, 4.39 mmol) and tetramethylguanidine (0.55 ml, 4.38 mmol) in dioxane/water (2:1, v/v; 10 ml) was added to dimer **3** (1.0 g, 0.88 mmol), and the reaction mixture was stirred for 6 h. The solution was partitioned between water and methylene chloride, the organic layer was separated, dried over sodium sulfate and evaporated. The residue was first precipitated three times from hexane, and then purified by silica-gel column chromatography (eluent: stepwise gradient of methanol (0–25%) in methylene chloride). The fractions containing the pure compound were pooled, evaporated, dissolved in methylene chloride, filtered (HV-4 Millipore) and evaporated to give **6** (0.91 g, 90%) as the tetramethylguanidinium salt: UV (ethanol) λ_{max} 280 nm (ε, 25200), λ_{min} 253 nm (ε, 19600); ^{1}H-NMR (Me_2SO-d_6) δ ppm 8.36 (*br s*, 2H, 2 NH), 7.84 [*br s*, 2H, $(CH_3)_2N$-C (= NH_2^+)-$N(CH_3)_2$], 7.79 (*d*, 2H, 2 H-6; $J_{5,6} \approx$ 6.4 Hz), 7.35–6.75 (*m*, 28H, 2 trityl), 6.19 (*d*, 2H, 2 H-5; $J_{5,6} \approx$ 6.8 Hz), 5.79 (*m*, 2H, 2 H-1'),

4.02 (*m*, 4H, 2 H-5′ and 2 H-5″), 3.70 (*s*, 6H, 2 OCH_3), 2.86 [*s*, 12H $(CH_3)_2N$-C(= NH_2^+)-N$(CH_3)_2$] 2.25–2.00 (*m*, 2H, 2 H-2′), 1.90–1.60 (*m*, 6H, 2 H-2″, 2 H-3′ and 2 H-3″); ^{31}P-NMR ($CDCl_3$) + 0.97 ppm; mass spectrum (FAB<0, matrix = glycerol/thioglycerol, 1:1, v/v): 1027 [M –$(CH_3)_2N$-C(= NH_2^+)-$N(CH_3)_2]^-$, 382 $[B]^-$.

O,O′-Bis(2′,3′-dideoxycytidin-5′-yl)phosphate (**7**).

This compound was prepared by detritylation of **6** (0.19 g, 0.17 mmol) with a solution of 4% trifluoroacetic acid in methylene chloride (5 ml) as described above for the synthesis of **5**. After work-up, the resulting crude material was chromatographed on a RP-2 silanized silica-gel column (1.5 × 38 cm; eluent/water). The fractions containing the pure compound were pooled, evaporated, dissolved in water, filtered (HV-4 Millipore), and lyophilized to give **7** (45 mg, 54%): UV (H_2O) λ_{max} 271 nm (ε, 17000), λ_{min} 248 nm (ε, 10000); ^{1}H-NMR (Me_2SO-d_6) δ ppm 7.92 (*d*, 2H, 2 H-6; $J_{5,6}$ = 7.4 Hz), 7.1 (*br s*, 4H, 2 NH_2), 5.94 (*dd*, 2H, 2 -H-1′; *J* = 3.1 and 6.5 Hz), 5.71 (*d*, 2H, 2 H-5; $J_{5,6}$ = 7.4 Hz), 4.10 (*m*, 2H, 2 H-4′), 3.86 (*m*, 2H, 2 H-5′), 3.78 (*m*, 2H, 2 H-5″), 2.27–2.20 (*m*, 2H, 2 H-2′), 1.95–1.75 (*m*, 6H, 2 H-2″, 2 H-3′ and 2 H-3″); ^{31}P-NMR (Me_2SO-d_6 + D_2O) – 0.42 ppm; mass spectrum (FAB<0, matrix = glycerol): 483 [M – H]$^-$

O,O′-Mono(4-monomethoxytrityl)dithiodiethanol (**9**).

To a solution of dithiodiethanol (9.4 ml, 78 mmol) and diisopropylethylamine (6.8 ml, 39 mmol) in dry methylene chloride (78 ml) was added 4-monomethoxytrityl chloride (mMTrCl; 12 g, 39 mmol), and the reaction mixture was stirred at room temperature for 24 h. Saturated aqueous sodium hydrogen carbonate was added, and the resulting mixture was extracted with methylene chloride. The combined organic layers were washed with water, dried over sodium sulfate, and evaporated. The residue was chromatographed on a silica-gel column (eluent: methylene chloride) to afford pure **9** (11.5 g, 69%) as an oil. ^{1}H-NMR (Me_2SO-d_6) δ ppm 6.5–6.0 (*m* 14H trityl), 3.98 (*t*, 1H, OH; *J* = 5.4 Hz), 2.84 (*s*, 3H, OCH_3), 2.69 (*m*, 2H, S-CH_2-CH_2-OH), 2.35 and 2.01 (2*t*, 2H each, S-CH_2-CH_2-OmMTr; *J* = 6.2 Hz each), 1.80 (*t*, 2H, *S*-CH_2-CH_2-OH; *J* = 6.5 Hz); mass spectrum (FAB<0, matrix = glycerol/thioglycerol, 1:1, v/v): 425 [M – H]$^-$.

O,O′-Bis(S-[O-(4-monomethoxytrityl)-2-oxethylsulfidyl]-2-thioethyl)-phosphate (**10**).

To a solution of imidazole (0.91 g, 13.4 mmol) in anhydrous pyridine (18 ml) was added, with stirring and cooling in an ice bath, phosphorus oxychloride (0.41 ml, 4.45 mmol). The mixture was stirred for 0.5 h at room temperature, and then compound **9** (3.80 g, 8.91 mmol) was added. The reaction mixture was stirred at room temperature for 18 h and a 1 M aqueous solution of triethylammonium acetate was added. The resulting mixture was extracted with methylene chloride, the organic layers were dried over sodium sulfate,

evaporated to dryness, and co-evaporated with toluene. The residue was chromatographed on a silica-gel column (eluent: stepwise gradient of methanol (0–10%) in methylene chloride) to afford pure **10** (2.2 g, 48%) as its triethylammonium salt: ^{1}H-NMR (Me_2SO-d_6) δ ppm 7.45–6.80 (*m*, 28H, 2 trityl), 3.87 (*m*, 4H, 2 S-CH_2-CH_2-O-P), 3.71 (*s*, 6H, 2 OCH_3), 3.21 (*t*, 4H, 2 S-CH_2-CH_2-OmMTr), 2.99 [*q*, 6H, $(CH_3$-$CH_2)_3NH^+$; J = 7.3 Hz], 2.86 (*t*, 4H, 2 S-CH_2-CH_2-OmMTr), 2.78 (*t*, 4H, 2 S-CH_2-CH_2-O-P), 1.14 [*t*, 9H, $(CH_3$-$CH_2)_3NH^+$; J= 7.3 Hz]; ^{31}P-NMR (Me_2SO-d_6) – 2.70 ppm; mass spectrum (FAB<0, matrix = NBA): 913 [M – $(CH_3$-$CH_2)_3NH^+]^-$.

O,O'-Bis[S-(2-hydroxyethylsulfidyl)-2-thioethyl-O''-(2',3'-dideoxyuridin-5'-yl)phosphate (**11**).

Compound **10** (0.67 g, 0.66 mmol) and 2',3'-dideoxyuridine (0.14 g, 0.66 mmol) were dissolved in dry pyridine (5 ml) and MSNT (0.49 g 1.64 mmol) was added to the stirred solution at room temperature. After 30 h, the reaction mixture was diluted with methylene chloride. washed first with a 1 M aqueous solution of triethylammonium acetate, and then with water. The organic layer was dried over sodium sulfate, concentrated under reduced pressure, co-evaporated with toluene, and then chromatographed on a silica-gel column (eluent: stepwise gradient of methanol (0–4%) in methylene chloride). The fractions containing the tritylated phosphotriester were pooled, evaporated, dissolved in an acetic acid/water/methanol mixture (8:1:1, v/v; 5 ml), and stirred for 24 h at room temperature. The solvents were removed by evaporation under reduced pressure, and the resulting oil was co-evaporated with toluene. The residue was twice chromatographed, first on a silica-gel column (eluent: stepwise gradient of methanol (0–6%) in methylene chloride) and then on a RP-2 silanized silica-gel column (eluent: linear gradient of ethanol (0–40%) in water) to afford pure **11** (52 mg, 14%) which was lyophilized from dioxane: UV (ethanol) λ_{max} 261 nm (ε, 9900) λ_{min} 231 nm (ε, 3100); ^{1}H-NMR (Me_2SO-d_6) δ ppm 11.3 (*br s*, 1H, NH-3), 7.65 (*d*, 1H, H-6; $J_{5,6} \approx 8.0$ Hz), 6.00 (*dd*, 1H, H-1'; J = 4.1 and 7.9 Hz), 5.60 (*d*, 1H, H-5; $J_{5,6} \approx 8.1$ Hz), 4.89 (*t*, 2H, 2 *HO*-CH_2-CH_2-S; J = 4.9 Hz), 4.30–4.05 (*m*, 7H, H-4',5',5'' and 2 S-CH_2-CH_2-O-P), 3.61 (*m*, 4H, 2 HO-CH_2-CH_2-S), 3.00 (*t*, 4H, 2 S-CH_2-CH_2-O-P; J = 6.3 Hz), 2.81 (*t*, 4H, 2 HO-CH_2-CH_2-S; J = 6.4 Hz), 2.40–2.33 (*m*, 1H, H-2'), 2.10–1.9 (*m*, 2H, H-2'',3'), 1.9–1.63 (*m*, 1H, H-3''); ^{31}P-NMR (Me_2SO-d_6) – 0.88 ppm; mass spectrum (FAB>0, matrix = glycerol/thioglycerol, 1:1, v/v): 565 $[M + H]^+$, 489 $[M - SCH_2{}^-CH_2OH + 2H]^+$, 429 $[M - CH_2CH_2SSCH_2CH_2OH + 2H]^+$.

O,O'-Bis[S-(2-hydroxyethylsulfidyl-2-thioethyl-O''-(3'-azido-2',3'-dideoxythymidin-5'-yl)phosphate (**12**).

This compound was prepared from 3'-azido-2',3'-dideoxythymidine (AZT; 0.19 g, 0.72 mmol) as described above for the synthesis of **11**. After work-up, the residue was chromatographed on a silica-gel column (eluent: stepwise gradient of methanol (0–6%) in methylene chloride) to afford pure **12** (0.13 g,

29%) which was lyophilized from dioxane: UV (ethanol) λ_{max} 264 nm (ε, 9600), λ_{min} 234 nm (ε 2100); ^{1}H-NMR (Me_2SO-d_6) ppm 11.4 (*br s*, 1H, NH-3), 7.49 (*s*, 1H, H-6), 6.14 (*t*, 1H, H-1'; J = 6.6 Hz), 4.90 (*t*, 2H, 2 *HO*-CH_2-CH_2-S, J = 5.3 Hz), 4.48 (*m*, 1H, H-3'), 4.45–4.10 (*m*, 6H, H-5',5'' and 2 S-CH_2-*CH_2*-O-P), 4.02 (*m*, 1H, H-4'), 3.61 (*m*, 4H, 2 HO-*CH_2*-CH_2-S), 2.99 (*t*, 4H, 2 S-*CH_2*-CH_2-O-P), 2.80 (*t*, 4H, 2 HO-CH_2-*CH_2*-S), 2.5–2.25 (*m*, 2H, H-2',2''), 1.80 (*s*, 3H, CH_3); ^{31}P-NMR (Me_2SO-d_6) – 0.95 ppm; mass spectrum (FAB>0, matrix = glycerol/thioglycerol, 1:1, v/v): 620 $[M + H]^+$, 544 $[M - SCH_2CH_2OH + 2H]^+$.

N^6-(4-Monomethoxytrityl)-9-(2-diethoxyphosphonylmethoxyethyl)adenine (**14**).

To a solution of 9-(2-diethoxyphosphonylmethoxyethyl)adenine **13** (Holý and Rosenberg, 1987) (3.93 g, 11.9 mmol) and 4-dimethylaminopyridine (146 mg, 1.19 mmol) in dry methylene chloride (50 ml) were added mMTrCl (7.35 g, 23.8 mmol) and triethylamine (3.31 ml, 23.8 mmol), and the reaction mixture was stirred at room temperature for 4 h. Saturated aqueous sodium hydrogen carbonate was added, and the resulting mixture was extracted with methylene chloride. The combined organic layers were washed with water, dried over sodium sulfate and evaporated. The residue was chromatographed on a silica-gel column (eluent: stepwise gradient of methanol (0–3%) in methylene chloride) to afford pure **14** (5.43 g, 84%): UV (ethanol) λ_{max} 275 nm (ε, 27 200), λ_{min} 246 nm (ε, 11 200); ^{1}H-NMR (Me_2SO-d_6) δ ppm 8.18 and 7.91 (2*s*, 1H each, H-2 and H-8), 7.4–6.8 (*m*, 14H, trityl), 4.33 (*t*, 2H, *CH_2*-CH_2; J = 4.8 Hz), 3.88 (*q*, 4H, 2 CH_3-*CH_2*-O; J = 8 Hz), 4.0–3.8 (*m*, 4H, CH_2-*CH_2*-O-*CH_2*-P), 3.71 (*s*, 3H, OCH_3), 1.10 (*t*, 6H, 2 *CH_3*-CH_2-O; J = 7.0 Hz); ^{31}P-NMR (Me_2SO-d_6) 21.35 ppm; mass spectrum (FAB<0, matrix = glycerol/thioglycerol, 1:1, v/v): 601 $[M - H]^-$, 406 $[B]^-$, 328 $[M - mMTr]^-$.

N^6-(4-Monomethoxytrityl)-9-(2-phosphonylmethoxyethyl)adenine (**15**).

Bromotrimethylsilane (3.29 ml, 24.9 mmol) was added to a solution of **14** (5.0 g, 8.31 ml) in acetonitrile (29 ml) and the reaction mixture was stirred at room temperature for 14 h. The excess of reagent and the solvent were removed by evaporation under reduced pressure and the oily residue was triturated with an aqueous solution of triethylammonium hydrogen carbonate. After evaporation under reduced pressure, purification was achieved by chromatography on a silica-gel column (eluent: stepwise gradient of methanol (0–50%) in methylene chloride). The fractions containing the pure compound were pooled, evaporated, dissolved in methylene chloride, filtered (HV-4 Millipore) and evaporated to give **15** (3.4 g, 63%) as a 1:1 mixture of the acidic and triethylammonium forms: ^{1}H-NMR (Me_2SO-d_6) δ ppm 8.31 and 7.83 (2*s*, 1H each, H-2 and H-8), 7.35–6.65 (*m*, 14H, trityl), 4.27 (*t*, 2H, *CH_2*-CH_2; J = 4.5 Hz), 3.8 (*m*, 2H, CH_2-*CH_2*), 3.68 (*s*, 3H, OCH_3), 3.34 (*d*, 2H, CH_2-P; J = 8.4 Hz), 2.96 [*q*, $(CH_3$-*CH_2*$)_3NH^+$; J = 7.3 Hz], 1.11 [*t*, $(*CH_3*$-$CH_2)_3NH^+$; J = 7.3 Hz]; ^{31}P-NMR (Me_2SO-d_6) 11.40 ppm; mass spectrum (FAB<0, matrix =

glycerol/thioglycerol, 1:1, v/v): 544 $[M - H]^-$, 272 $[M - mMTr]^-$.

N^6-(4-Monomethoxytrityl)-9-(2-[O,O'-bis(S-(O-(4-monomethoxytrityl)-2-oxethylsulfidyl)-2-thioethyl)]phosphonylmethoxyethyl)adenine (**16**).

Compound **15** (0.30 g, 0.46 mmol) and compound **9** (0.98 g, 2.29 mmol) were dissolved in dry pyridine (5 ml) and MSNT (0.34 g, 1.15 mmol) was added to the stirred solution at room temperature. After 3 days, the reaction mixture was diluted with methylene chloride, washed first with a saturated aqueous solution of sodium hydrogen carbonate, and then with water. The organic layer was dried over sodium sulfate, concentrated under reduced pressure and co-evaporated with toluene. The residue was chromatographed on a silica-gel column (eluent: stepwise gradient of methanol (0–5%) in methylene chloride) to afford pure **16** (0.33 g, 53%): UV (ethanol) λ_{max} 275 nm (ε, 28 200), λ_{min} 253 nm (ε, 18 300); ^{1}H-NMR (Me_2SO-d_6) δ ppm 8.12 and 7.88 (2*s*, 1H each, H-2 and H-8), 7.45–6.85 (*m*, 42 H, 3 trityl), 4.28 (*t*, 2H, CH_2-CH_2-O-CH_2-P; J = 4.6 Hz), 4.05 (*m*, 4H, 2 S-CH_2-CH_2-O-P), 3.83 (*m*, 4H, CH_2-CH_2-O-CH_2-P), 3.69 and 3.68 (2*s*, 6 and 3H, respectively, 3 OCH_3), 3.19 (*t*, 4H, 2 mMTrO-CH_2-CH_2-S; J = 6.0 Hz), 2.86 (*t*, 4H, 2 mMTrO-CH_2-CH_2-S; J = 5.9 Hz), 2.75 (*t*, 4H, 2 S-CH_2-CH_2-O-P; J = 6.3 Hz); ^{31}P-NMR (Me_2SO-d_6) 22.09 ppm; mass spectrum (FAB < 0, matrix = NBA): 1360 $[M - H]^-$, 952 $[M-CH_2CH_2SSCH_2CH_2OmMTr]^-$.

9-(2-(O,O'-Bis[S-(2-hydroxyethylsulfidyl)-2-thioethyl])phosphonylmethoxyethyl)adenine (**17**).

Compound **16** (0.29 g, 0.213 mmol) was dissolved in an acetic acid/water/methanol mixture (8:1:1, v/v; 15 ml), and stirred for 15 h at room temperature. The solvents were removed by evaporation under reduced pressure, and the resulting oil was co-evaporated with toluene. The residue was chromatographed on a silica-gel column (eluent: stepwise gradient of methanol (0–8%) in methylene chloride) to give pure **17** (116 mg, 90%) which was lyophilized from a water-dioxane mixture: UV (ethanol) λ_{max} 260 nm (ε, 14 700), λ_{min} 228 nm (ε, 3600). ^{1}H-NMR (Me_2SO-d_6) δ ppm 8.14 and 8.08 (2*s*, 1H each, H-2 and H-8), 7.20 (*s*, 2H, NH_2), 4.32 (*t*, 2H, CH_2-CH_2-O-CH_2-P; J = 5.0 Hz), 4.15 (*m*, 4H, 2 S-CH_2-CH_2-O-P), 3.95 (*d*, 2H, CH_2-CH_2-O-CH_2-P; J = 8.2 Hz), 3.91 (*t*, 2H, CH_2-CH_2-O-CH_2-P; J = 5.1 Hz), 3.61 (*m*, 4H, 2 HO-CH_2-CH_2-S), 2.91 (*t*, 4H, 2 HO-CH_2-CH_2-S; J = 6.4 Hz), 2.80 (*t*, 4H, 2 S-CH_2-CH_2-O-P; J = 6.4 Hz); ^{31}P-NMR (Me_2SO-d_6) 22.24 ppm; mass spectrum (FAB > 0, matrix = glycerol/thiglycerol, 1:1, v/v): 545 $[M + H]^+$.

Decomposition studies of compounds **5**, **11** *and* **12**.

The method for determination of kinetics of decomposition of nucleotide prodrugs has recently been described (Pompon et al., 1993). Briefly, HPLC was performed on a Waters-Millipore instrument equipped with two Model 510 solvent delivery systems, a model 680 solvent programmer, a model 712 autosampler, and a model 990 diode-array UV-detector. The column assembly

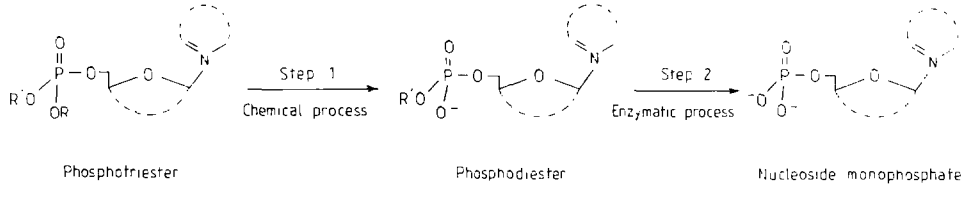
consisted of an Internal-Surface Reversed-Phase (ISRP) precolumn (Ultrabiosep C_8, 10 × 4.6 mm id, 10 μm particle size) linked in series to a Reversed Phase (RP) analytical column (Nucleosil, C_{18}, 100 × 4.6 mm, 3 μm), both columns purchased from SFCC/Shandon (Eragny, France). The analytical column was thermostated at 30°C, and a six-port 7000 Reodyne valve allowed to modify the flow path.

Results and Discussion

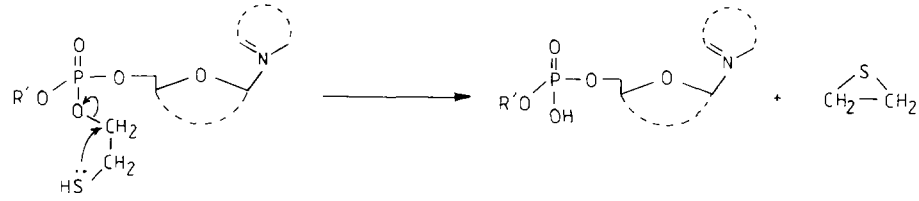
A large number of nucleotide derivatives have previously been envisaged as ways to deliver nucleoside monophosphates inside the cells. Such a masked nucleotide approach may be ideally rationalised as follows: (i) one has to use a neutral species, e.g., a phosphotriester, which would be expected to cross the cell membrane by passive diffusion; (ii) such a nucleoside phosphotriester must be stable under the experimental cell culture conditions currently used, i.e., RPMI containing 10% heat-inactivated foetal calf serum (FCS) at 37°C; otherwise, extracellular decomposition to the parent nucleoside could lead to mistaken biological interpretation (this point is of prime importance and may often have been neglected in previous work); (iii) once inside the cells, the nucleoside phosphotriester must be selectively transformed to the nucleoside monophosphate during the time period when virus replication is sensitive to the compound tested. In other words, the nucleoside phosphotriester has to be intracellularly transformed to the nucleoside monophosphate through a two-step mechanism as shown in Scheme 1.

The first step must involve a chemical process as no evident phosphotriesterase activity has been reported to be present inside cells. Once transformed into a phosphodiester, one can expect an enzymatic mediated release of the nucleoside monophosphate. This two-step transformation must be consistent with the experimental screening conditions, i.e., enough stability in culture medium and reliable cellular uptake of the neutral phosphotriester, followed by its conversion into the nucleoside monophosphate. This last point may be expected if intracellular enzymes are able to promote the first step as well as the second one.

At this stage it is necessary to consider the chemical hydrolysis mechanism of



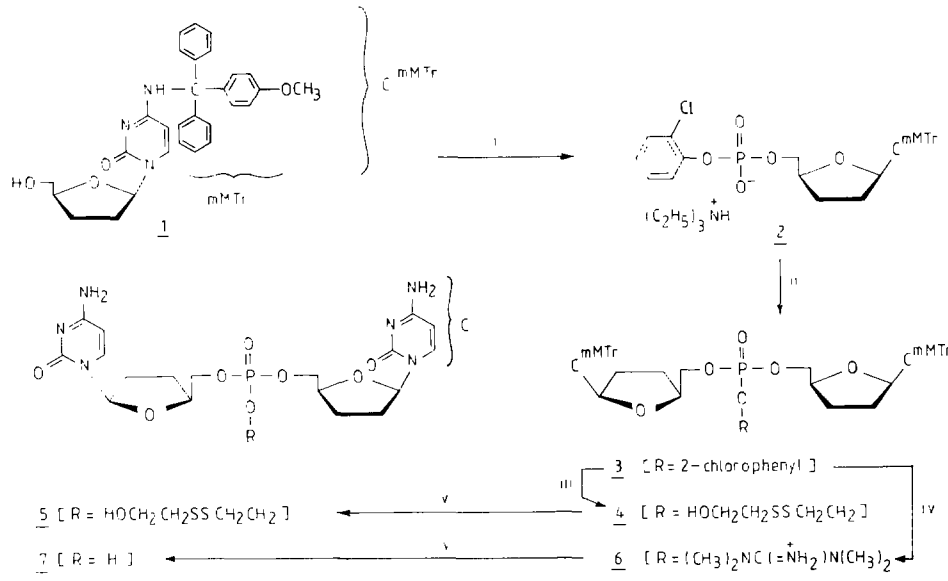
Scheme 1. Transformation pathway of a nucleoside phosphotriester into its corresponding nucleoside monophosphate.



Scheme 2. Intramolecular nucleophilic mechanism involving selective C-O bond breakage.

phosphotriesters at pH $\approx$ 7.0 in order to define the structural requirements of the starting masked nucleotide compounds. Considering trimethylphosphate in neutral solution as a model in which both carbon and phosphorus atoms are vulnerable to nucleophilic attack by water, it has been shown that this compound is hydrolyzed via C-O bond fission (Bruice and Benkovic, 1966). This process is very slow as further demonstrated by our previous work on a 5',5'-dinucleoside methylphosphotriester where only 6% of hydrolysis of the methyl group was observed after 5 days of incubation both in the culture medium and in cell extracts (Puech et al., 1992). However, the observed selectivity induced by such a C-O bond fission mechanism was found encouraging and led us to envisage another kind of nucleophilic attack on the carbon atom but through an intramolecular mechanism as a means to increase the reaction rate as shown in Scheme 2.

Since soft nucleophiles dealkylate phosphotriesters with C-O cleavage, it seemed worthwhile to consider a sulfur atom as the nucleophile (Scheme 2) in order to avoid competitive attack on the phosphorus atom. Such a mechanism involving episulfide elimination is well established in the literature, as exemplified by Eckstein's DNA and RNA sequence determination method (Eckstein and Gish, 1989). However, one can assume from literature data that the rate of transformation of a thioethyl phosphotriester may be too fast, and hence unsuitable for a prodrug approach. Therefore we decided to temporarily mask the thiol function with an enzymatically labile protecting group which could be selectively cleaved inside the cells. This led us to synthesize, as a first model, the dimeric phosphotriester **5** and to study the kinetics of its decomposition. Compound **5**, which has the thiol group masked with a disulfide bond in the expectation that a reductase-dependent intracellular activation mechanism might ensue (Ziegler, 1985; Cashman and Liu, 1991), was synthesized as shown in Scheme 3. The full-protected dimer **3** was prepared from N^4-tritylated 2',3'-dideoxycytidine **1** (Puech et al., 1990) by an established procedure (Puech et al., 1988). Transesterification with dithiodiethanol in the presence of fluoride ions (Weinfeld and Livingston, 1986) followed by an acidic treatment afforded the desired phosphotriester **5**. On the other hand, the reference phosphodiester **7** was also obtained in two steps from the full-protected dimer **3**.



Scheme 3. Synthesis of the dimeric phosphotriester 5 and phosphodiester 1 of 2',3'-dideoxycytidine. Reaction conditions: (i) $ClC_6H_4OP(O)Cl_2$, triazole, $(C_2H_5)_3N$/C_5H_5N; (ii) 1, MSNT/C_5H_5N; (iii) $(HOCH_2CH_2)_2S_2$, CsF/CH_2Cl_2; (iv) $O_2NC_6H_4CH{=}NOH$, $(CH_3)_2NC(=NH)N(CH_3)_2$/dioxane-H_2O; (v) CF_3COOH/CH_2Cl_2.

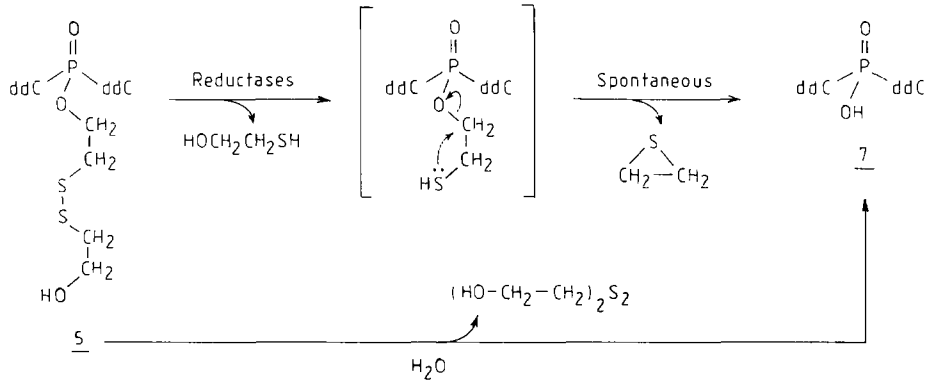
By using an adapted 'on-line ISRP cleaning' HPLC methodology previously described for the degradation studies of oligonucleotides (Pompon et al., 1992), we determined the kinetics of decomposition of 5 under three experimental conditions: (i) incubation at 37°C in a phosphate buffer (pH 7.2); (ii) RPMI containing 10% heat-inactivated foetal calf serum (culture medium); (iii) CEM cell extracts. The observed pathway and the kinetic data for the decomposition of 5 into the corresponding phosphodiester 7 are reported in Scheme 4 and Table 1.

From these results, the following comments can be made: (i) in buffered water, the starting phosphotriester 5 was quite stable; the observed slow decomposition was selective and may have been related to a nucleophilic attack of water on the C atom as only the corresponding phosphodiester 7 was detected; (ii) in RPMI containing 10% heat-inactivated FCS, a slight increase

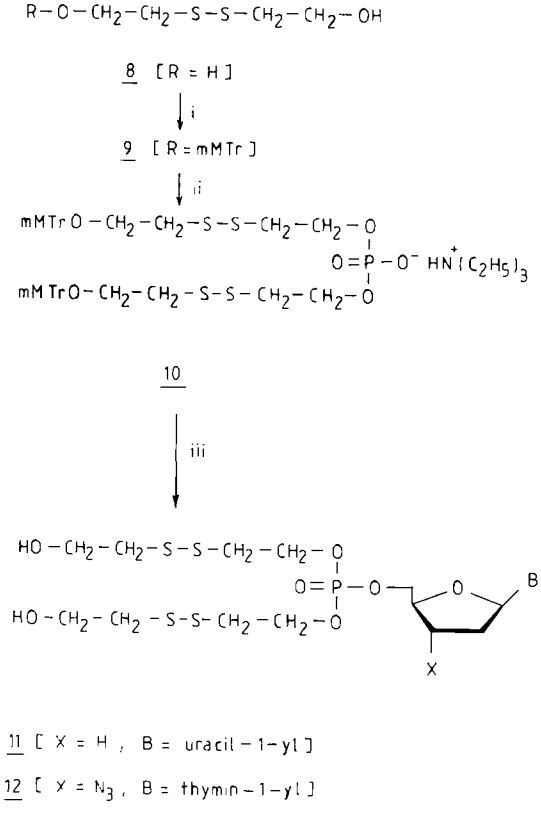
TABLE 1

Rate constants (k) and half-life times ($T_{1/2}$) of the decomposition of the phosphotriester 5 into the phosphodiester 7 in different media.

	Phosphate buffer (pH 7.2)	RPMI + 10% heat-inactivated FCS	CEM cell extract
$T_{1/2}$	56 h	50 h	0.5 h
k (min^{-1})	$1.9\ 10^{-4}$	$2.5\ 10^{-4}$	$1.0\ 10^{-2}$



Scheme 4. Decomposition pathway of the dimeric phosphotriester **5** into the phosphodiester **7** (ddC = 2',3'-dideoxycytidin-5'-yl).



Scheme 5. Synthesis of the mononucleoside phosphotriester derivatives of ddU and AZT.
Reaction conditions: (i) mMTrCl, $[(CH_3)_2CH]_2NC_2H_5/CH_2Cl_2$; (ii) $P(O)Cl_3$, imidazole/C_5H_5N; (iii) ddU or AZT, MSNT/C_5H_5N, then CH_3COOH/CH_3OH-H_2O.

in the kinetic of decomposition was observed, but again appeared to involve the above mentioned selective hydrolysis process as only 7 was formed; the increase may have been related to the presence of some reductase activity in the heat-inactivated FCS as noted in preliminary experiments; (iii) in the cell extract, compound 5 was rapidly and selectively converted into the corresponding phosphodiester 7; this point may be interpreted in terms of reductase-dependent activation, and as expected the intramolecular nucleophilic attack of the thiol function on the carbon atom was very fast since no intermediate was detected. In addition, in buffered water and in cell extract no further transformation of 7 into ddC and its monophosphate was observed, confirming our previous results that symmetrical unnatural dinucleotidyl-5',5' phosphodiesters do not seem to be subject to intracellular chemical or enzymatic hydrolysis (Puech et al., 1992).

In order to verify the potential of such a reductase-activated, temporary protecting group in the masked-phosphate prodrug approach, we decided to synthesize (Scheme 5) and to study the mononucleotide derivatives of ddU and AZT.

The mononucleotide derivatives 11 and 12 were evaluated for their in vitro inhibitory effects on the replication of HIV in several cells (Table 2).

These biological data deserve the following comments. On the one hand, compound 11 produced an anti-HIV effect in various cell lines, proving unequivocally that ddUMP was released inside the cells since: (i) ddU itself was inactive in the same experiment; (ii) its monophosphate, which is also inactive (data not shown), is rapidly hydrolysed by phosphatases in culture medium; and (iii) when introduced into infected cells following a prodrug approach (Sastry et al., 1992) or using targeted liposomes (Zelphati et al., 1993), ddUMP

TABLE 2

Antiviral activity of the phosphotriester derivatives 11, 12 and their nucleoside units ddU and AZT, respectively, in various cells infected with HIV-1

	MT-4		CEM-SS		CEM-X 174		CEM-TK		PBMC	
	EC_{50}[a]	CC_{50}[b]	EC_{50}[a]	CC_{50}[b]	EC_{50}[a]	CC_{50}[b]	EC_{50}[a]	CC_{50}[b]	EC_{50}[a]	CC_{50}[b]
11	$> 10^{-4}$ (15%)[c]	$> 10^{-4}$ (0%)[d]	$6\ 10^{-5}$	$> 10^{-4}$ (41%)[c]	ND[e]	ND[e]	$8\ 10^{-6}$	$8\ 10^{-5}$	$7\ 10^{-5}$	$> 10^{-4}$ (49%)[d]
ddU	$> 10^{-4}$ (0%)[c]	$> 10^{-4}$ (25%)[d]	$> 10^{-4}$ (18%)[c]	$> 10^{-4}$ (41%)[d]	ND[e]	ND[e]	$> 10^{-4}$ (0%)[c]	$> 10^{-4}$ (18%)[d]	10^{-4}	$> 10^{-4}$ (4%)[d]
12	10^{-9}	$> 5\ 10^{-7}$ (0%)[d]	$7\ 10^{-10}$	$> 5\ 10^{-7}$ (0%)[d]	$4\ 10^{-8}$	$> 10^{-4}$ (32%)[d]	$7\ 10^{-6}$	$8\ 10^{-5}$	ND[e]	ND[e]
AZT	$2.5\ 10^{-9}$	$>5\ 10^{-5}$ (0%)[d]	$5\ 10^{-9}$	$> 5\ 10^{-7}$ (0%)[d]	10^{-8}	$> 10^{-4}$ (0%)[d]	$> 10^{-4}$ (30%)[c]	$> 10^{-4}$ (0%)[d]	ND[e]	ND[e]

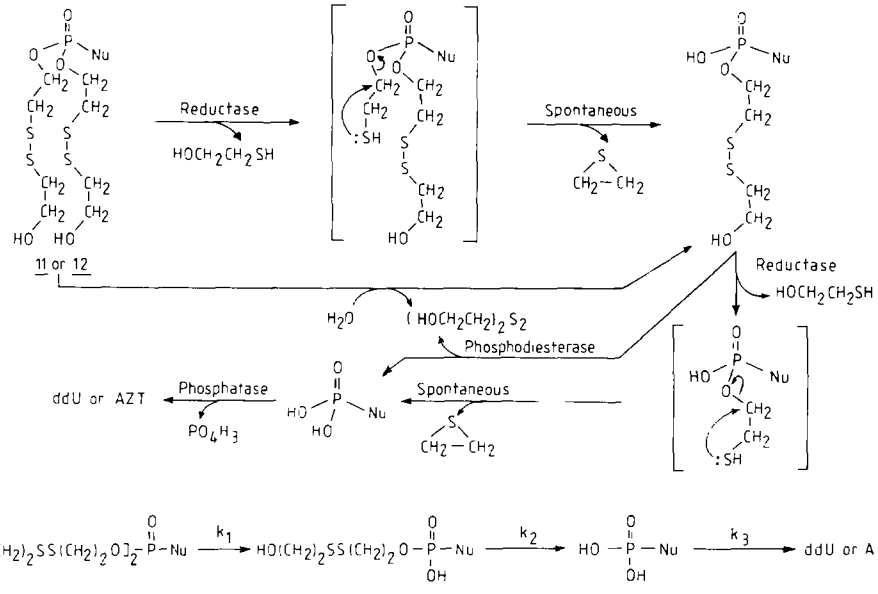
[a]50% effective molar concentration or molar concentration required to inhibit the replication of HIV by 50%.

[b]50% cytotoxic molar concentration or molar concentration required to reduce the viability of the cells by 50%.

[c]Percent inhibition of HIV replication at the indicated highest concentration tested.

[d]Percent reduction of viable cells at the indicated highest concentration tested.

[e]Not determined.



Scheme 6. Decomposition pathway of the monomeric phosphotriesters **11** and **12**. (Nu = 2',3'-dideoxyuridin-5'-yl or 2',3'-dideoxy-3'-azido-thymidinyl-5'-yl).

exerts an antiviral effect, probably owing to its enzymatic conversion into ddUTP. On the other hand, the AZT phosphotriester derivative **12** exhibited antiviral activity in the CEM-TK$^-$ cell line where AZT was inactive. Furthermore, in three cell lines (MT-4, CEM-SS and CEM-X 174) which are not deficient in thymidine kinase, no striking differences were observed between **12** and AZT. So, in these cells, the antiviral activity of **12** may have been due to intracellular release of AZTMP but also to its extracellular decomposition into the parent nucleoside AZT. The release of AZT in culture medium was

TABLE 3

Rate constants (k) and half-life times ($T_{1/2}$) of the decomposition of the phosphotriesters **11** and **12** at a concentration of $5\ 10^{-5}$ M and 37°C in culture medium (RPMI + 10% heat-inactivated FCS) and in a CEM cell extract.

		Culture medium		CEM cell extract	
11	k_1[a] (min^{-1}) [$T_{1/2}$]	$3.5\ 10^{-4}$	[33 h]	$1.1\ 10^{-2}$	[1.1 h]
	k_2[a] (min^{-1}) [$T_{1/2}$]	$7.3\ 10^{-3}$	[1.6 h]	$3.4\ 10^{-3}$	[3.5 h]
	k_3[a] (min^{-1}) [$T_{1/2}$]	[b]		[b]	
12	k_1[a] (min^{-1}) [$T_{1/2}$]	$3.7\ 10^{-4}$	[31 h]	$8.8\ 10^{-3}$	[1.3 h]
	k_2[a] (min^{-1}) [$T_{1/2}$]	$2.1\ 10^{-3}$	[5.5 h]	$6.9\ 10^{-4}$	[17 h]
	k_3[a] (min^{-1}) [$T_{1/2}$]	$4.4\ 10^{-3}$	[2.6 h]	$1.2\ 10^{-3}$	[9.6 h]

[a]The constants k_1, k_2 and k_3 are related to the three steps depicted at the bottom of Scheme 6.
[b]The value cannot be determined.

demonstrated in our decomposition studies (see below) and that is the reason why all the previously reported works on masked nucleotide derivatives of AZT need to be confirmed using TK^- cell lines.

We then examined the decomposition pathways of **11** and **12** in culture medium and in a CEM cell extract using the 'on-line ISRP cleaning' HPLC approach. The decomposition pattern is presented in Scheme 6 and the corresponding kinetic data are shown in Table 3.

The obtained results deserve the following comments: (i) for compound **11** and **12**, the same overall decomposition pattern was observed in culture medium and cell extract, but the kinetics of transformation strongly differed according to the medium; (ii) as the formation of ddUMP and ddU could not be monitored in our HPLC experiments due to their high polarity, only the rate constants k_1 (disappearance of the triester) and k_2 (disappearance of the intermediate phosphodiester) were determined. On the other hand, AZT and its monophosphate were easily detected under the experimental conditions of our HPLC analysis; (iii) both compounds **11** and **12** were only slightly hydrolysed to their corresponding phosphodiester in culture medium but this transformation was about 30 times faster in cell extract. These data corroborated our previous observations on the dimeric phosphotriester **5** and were in accordance with a preferential reductase-mediated activation process in the cell extract; (iv) once formed, the intermediate phosphodiester was transformed into its 5'-nucleotide. This process occurred more readily in culture medium than in cell extract and may have been phosphodiesterase-mediated since in the first step the reductase activities were more important in cell extract than in culture medium. Some recent pharmacokinetic studies from our group on the bispivaloyloxymethyl ester of AZTMP seem also to indicate that a phosphodiesterase is involved in the formation of AZTMP in culture medium (Pompon et al., 1993); (v) in both media AZTMP is further degraded to the parent nucleoside (rate constant k_3) presumably through a phosphatase catalyzed reaction.

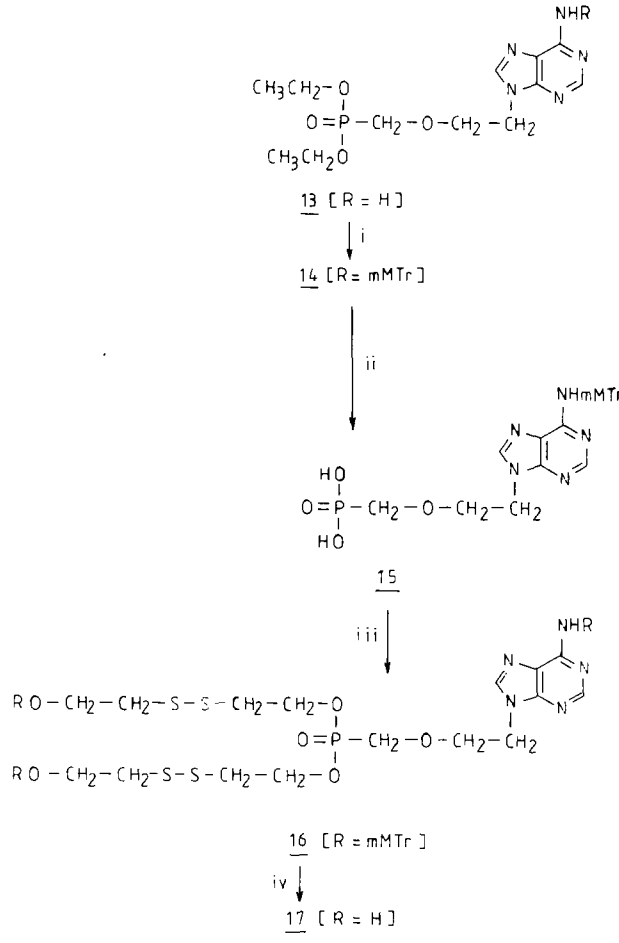
Having demonstrated the efficacy of our strategy using ddU and AZT as models we extended our approach to PMEA, a phosphonate derivative. This compound is only slowly taken up by the cells and, therefore, its derivatisation

TABLE 4

Antiviral activity of the phosphonotriester derivative **17** and of PMEA in various cell lines against HIV-1.

	MT-4		CEM-SS		CEM-X 174		CEM TK^-	
	EC_{50}[a]	CC_{50}[b]	EC_{50}[a]	CC_{50}[b]	EC_{50}[a]	CC_{50}[b]	EC_{50}[a]	CC_{50}[b]
17	$8\ 10^{-7}$	$2\ 10^{-5}$	$3\ 10^{-6}$	$> 10^{-4}$ (33%)[d]	$8\ 10^{-8}$	$> 10^{-4}$ (26%)[d]	$8\ 10^{-8}$	$4\ 10^{-5}$
PMEA	$> 10^{-5}$ (10%)[c]	$> 10^{-5}$ (27%)[d]	10^{-5}	$> 10^{-5}$ (9%)[d]	$2\ 10^{-6}$	$> 10^{-5}$ (13%)[d]	$6\ 10^{-6}$	$> 10^{-5}$ (0%)[d]

[a–d]see the footnote to Table 2.



Scheme 7. Synthesis of the phosphonotriester derivative **17** of PMEA.
Reaction conditions: (i) mMTrCl, DMAP, $(C_2H_5)_3N/CH_2Cl_2$; (ii) $(CH_3)_3SiBr/CH_3CN$; (iii) **9**, MSNT/C_5H_5N; (iv) CH_3COOH/H_2O-CH_3OH.

into the neutral phosphonotriester **17** was undertaken in order to enhance its transport across the cell membrane. Accordingly, the phosphonotriester of PMEA, **17** was synthesized as shown in Scheme 7. Starting from 9-(2-diethoxyphosphonylmethoxyethyl)adenine (**13**) (Holý and Rosenberg, 1987), a monomethoxytrityl group was introduced on the base in order to increase the solubility of the phosphonate in organic solvents during the step of condensation with the monotritylated dithiodiethanol **9**.

When evaluated for its antiviral activity in various cell lines (Table 4), compound **17** exhibited a higher anti-HIV effect than the parent phosphonate PMEA. This increase of activity is in accordance with our rational and may

result from an enhanced cellular uptake of **17** followed by intracellular release of PMEA.

Conclusion

The present results demonstrate that nucleoside dithioethanol phosphotriesters allow the intracellular delivery of their parent monophosphate (or phosphonate). There is a reasonable chance that the use of various other disulphide ethanol substitutions (i.e., R-S-S-CH_2-CH_2-O-) may also permit modulation of the bioavailability of the corresponding nucleotide and additional data will be reported in due course. The use of intracellular enzyme to activate nucleotide prodrugs in situ is obviously a very promising approach which has been recently discussed for nucleotide derivatives activated through a carboxylic esterase mediated process (Sastry et al., 1992; Starrett et al., 1992). In addition, as it is now well established that some nucleoside triphosphates are potent in vitro inhibitors of the DNA polymerase activity of the HIV reverse transcriptase, whereas the corresponding nucleosides are not substrates for the cellular phosphorylation enzymes and hence are inactive in vivo (e.g. (+)carbovir (Miller et al., 1992); L-ddT and L-d_4T (Van Draanen et al., 1992)) one can expect that such an approach, by-passing the more specific first kinase, could be of great help in the design of new anti-HIV agents. The overall strategy we present opens a wide field of study for in situ delivery of nucleotide derivatives and further work is in progress along these lines.

Acknowledgements

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