Synthesis and Anti-HIV Evaluation of D4T and D4T 5'-Monophosphate Prodrugs

C. Sergheraert,*,† C. Pierlot,† A. Tartar,† Y. Henin,‡ and M. Lemaitre‡,§

Faculté de Pharmacie, Institut Pasteur de Lille, URA CNRS 1309, 1 rue Calmette, 59019 Lille, France, and Rhône-Poulenc Rorer, CRVA, Dept. Biologie, 13 quai J. Guesde, BP 14, 94403 Vitry Cédex, France

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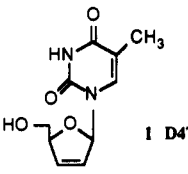
Several 5-monophosphate D4T derivatives and their analogues were synthesized as potential lipophilic prodrugs of D4T. Cholesteryl D4T phosphate diester and bis-5'-D4T phosphate inhibited HIV replication in CEM-Cl13 cells more efficiently than D4T itself as measured by the inhibition of cytopathic effect based on MTT assay or reverse transcriptase activity. The two compounds were devoid of toxicity on CEM-Cl13 cells at doses equal to 50 and 100 μM, respectively, which brought the selectivity index into the same range as AZT.

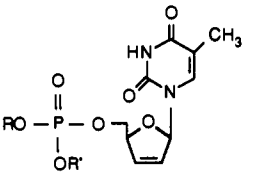
Introduction

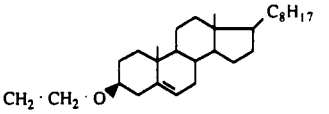
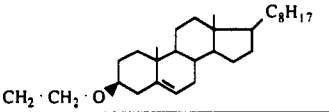
Despite the remarkable efforts provided in biology, virology, and drug research during the first decade after the initial description of AIDS in 1981, only two drugs, 3'-azido-3'-deoxythymidine[1] (AZT, zidovudine, Retrovir) and 2',3'-dideoxyadenosine[2] (didanosine, Videx), are approved for the treatment of AIDS patients. Both compounds are 2',3'-dideoxynucleosides (ddN) analogues. They must be phosphorylated intracellularly to their 5'-triphosphate derivatives to interact with their target enzyme, the HIV-associated reverse transcriptase (RT). At the RT level, the dideoxynucleoside triphosphate (ddNTPs) may act as either competitive inhibitors preventing the incorporation of the natural substrates (dNTPs) or alternate substrates incorporated in the growing DNA chain. Their incorporation leads to termination of the DNA chain since they do not possess the 3'-hydroxy necessary for chain elongation. The rate and extent to which the ddN analogues are converted to their active (triphosphate) forms may be at least as important as the affinity of the triphosphates for the target enzyme.[3] One of the possibilities to improve the efficiency of ddN should be to bypass at least in part these phosphorylation steps. Unfortunately phosphorylated derivatives, due to their increased hydrophilicity, are not likely to cross membranes efficiently and thus to reach their intracellular targets. We have thus decided to undertake the preparation of dideoxynucleoside monophosphate (ddNMP) prodrugs in which the phosphate groups were modified by hydrophobic moieties. The efficiency of such a prodrug also depends on the rate at which it will be metabolized intracellularly to the corresponding free ddNMP available to be further transformed to the triphosphate derivative active on RT. In order to allow this transformation, the hydrophobic groups were introduced as phosphate esters of the corresponding alcohols.

The other point was to select the most relevant ddN to test this strategy. Among the well-documented ddN analogues active as HIV inhibitors, AZT and D4T[4] (2',3'-didehydro-3'-deoxythymidine (1)) were the most prominent candidates for such modifications. Furman et al.[5] have demonstrated that AZT-MP accumulation causes a severe inhibition of cellular dTMP kinase, resulting in a blockade of further phosphorylation steps, while on the contrary, D4T appeared unable to induce the same effect[6] and is clearly unable to inhibit its own phosphorylation.[7,8] For these reasons, it was clear that increasing the concentration of AZT-MP was of little value while by contrast, as D4T-MP does not accumulate, being readily converted to the triphosphate derivative, D4T was a much better candidate. Moreover, D4T has been found less toxic than AZT for bone marrow stem cells[8,9] and less inhibitory to mitochondrial DNA replication.[10] With the aim of testing this hypothesis, we describe here the synthesis and the biological evaluation of 5'-monophosphate D4T prodrugs. Two potential phosphate diester prodrugs **2a,b** have been synthesized with a cholesterol or a hexadecyl moiety chosen to increase the lipophilicity and improve the biotransport through plasma membranes or lipid barriers. The cholesterol moiety was introduced as 3β-(2'-hydroxyethoxy)-cholest-5-ene,[11] i.e. cholesterol-3β-OR' (with R' = CH_2-CH_2OH); the palmityl moiety was introduced as a 1-hexadecanol ($CH_3(CH_2)_{15}OH$) ester.



1 D4T



compd	R	R'
2a	CH_2-$(CH_2)_{14}$-CH_3	H
3a	CH_2-$(CH_2)_{14}$-CH_3	CH_2-CH_2-CN
2b		H
3b		CH_2-CH_2-CN

The corresponding phosphate triesters **3a** and **3b** in which a cyanoethoxy group eliminates the remaining charge were also prepared. We have also synthesized the

* To whom correspondence should be addressed.
† Institut Pasteur de Lille.
‡ Rhône-Poulenc Rorer.
§ Present address: Eurogentec, B4102 Seraing, Belgium.

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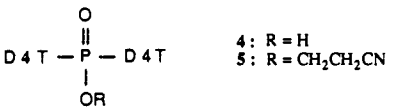
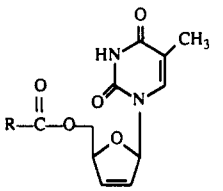
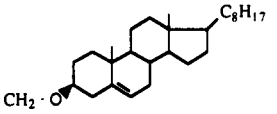
bis-5'-D4T-phosphate which is less hydrophobic than the previous compounds but, due to its symmetrical structure, releases upon cleavage by phosphatase one molecule of D4T and one molecule of its monophosphate derivative. In this case also, a cyanoethoxy ester **5** was prepared.[12]



4: R = H
5: R = CH_2CH_2CN

To assess the advantage of incorporating the phosphate moiety in the above compounds the corresponding palmityl and cholesteryl D4T derivatives **6** and **7** were prepared. The palmityl moiety was introduced as the palmitic ester of D4T, the cholesterol moiety as 3β-(2'-carboxymethoxy)-cholest-5-ene[11] ester of D4T. In this case, only the hydrophobicity of the prodrug was maintained as their hydrolysis releases D4T which will have to be phosphorylated. The importance of the structure of the hydrophobic moiety was studied by the synthesis of the linoleic and linolenic esters **10** and **11**. Finally, in order to modulate the release of D4T from its prodrug, two additional linkages of D4T to the palmityl moiety were tested: a carbonate in compound **8** and a carbamate in compound **9**.

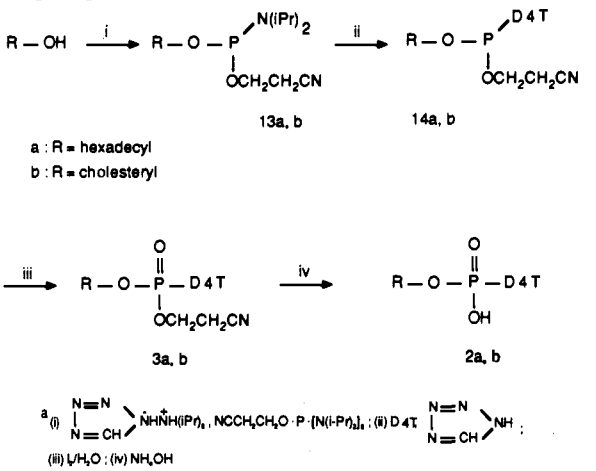


compd	R
6	CH_2-$(CH_2)_{13}$-CH_3
7	
8	O-CH_2-$(CH_2)_{14}$-CH_3
9	NH-CH_2-$(CH_2)_{14}$-CH_3
10	CH_2-$(CH_2)_6$-$(CH_2$-CH=CH$)_2$-$(CH_2)_3$-CH_3
11	CH_2-$(CH_2)_6$-$(CH_2$-CH=CH$)_3$-CH_3
12	

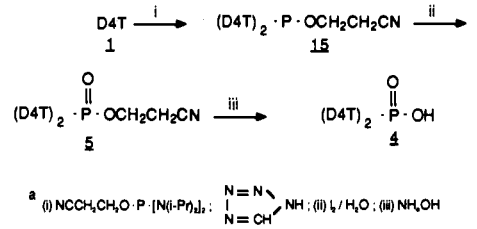
Chemistry

The esterification reactions of D4T in the conditions described for AZT[13] (use of 1.5 equiv of 4-(dimethylamino)-pyridine in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC)) remained incomplete even after a long period of time. Therefore we used an excess of DCC (2.4 equiv) and acid (2.2 equiv) to obtain the esters **6**, **7**, **10**, and **11** in approximately 40–60% yields. The carbonate **8** and carbamate **9** derivatives of D4T were obtained from the nucleoside by forming its activated alcohol from **12** by using carbonyldiimidazole[14] (5 equiv). The imidazolyl moiety was then displaced by an excess of nucleophilic compound (hexadecanol or hexadecylamine) in acetonitrile under reflux to give **8** or **9**. The palmityl and cholesteryl derivatives of D4T monophosphate **2a** and **2b** were synthesized in four steps as shown in Scheme I starting from the phosphite diesters **13a** and **13b** obtained by action of 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphoramidite on the corresponding alcohols in the presence of bis-

Scheme I.[a] Synthesis of Palmityl and Cholesteryl D4T Monophosphate



Scheme II.[a] Synthesis of Bis-5'-D4T Phosphate



(diisopropyl)ammoniumtetrazolide.[15] The phosphite triesters **14a** and **14b** obtained by reaction of the phosphite diesters with D4T and tetrazole[16] were transformed into the corresponding phosphates **3a** and **3b** using iodine as oxidizing agent. The cyanoethoxy protecting group was then removed by treatment with NH_4OH to give **2a** and **2b**. The symmetrical phosphate diester of D4T, **4**, was prepared by reaction of D4T with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphoramidite (0.47 equiv) and tetrazole (1 equiv), followed by treatment of the intermediate protected triester **5** with ammonia (Scheme II).

Antiviral Activity of the D4T Derivatives

The antiviral activities of the compounds measured on lymphocytic cell line (CEM-Cl13) infected with HIV-1 cell free supernatants are reported in Table I. Two parameters were studied to evaluate the antiviral activity: the inhibition of HIV-1-induced cytopathic effect using the MTT cell viability assay and the inhibition of the reverse transcriptase production in culture supernatants. Under the assay conditions described in the Experimental Section, the reference compound AZT displayed a IC_{50} of 20 μM and a EC_{50} of 0.006 μM or 0.01 μM at day 7 postinfection in the MTT assay and the reverse transcriptase production, respectively. These values were comparable with previously reported activities.[17] Compounds **2b**, **4**, **7**, and **10** were able to significantly inhibit the HIV-1-induced cytopathic effect in CEM-Cl13 cells; they were more active than D4T but less active or comparable to AZT. The inhibiting capacity was much weaker for compounds **3a**, **5**, **6**, and **8** while the carbamate **9** was inactive.

The second parameter which was determined was the toxicity for uninfected cells (Table I): in our series, compounds **2b** and especially **4** exhibited low toxicity for CEM-Cl13 cells with IC_{50} concentrations, respectively, equal to 50 and 100 μM. This decreased toxicity compared

Table I. Activities of Products 2a–11 against HIV-1 in CEM Cells[a]

compd	IC_{50}[b]	EC_{50}[c] (MTT)	EC_{50}[c] (RT)	SI[d] (MTT)	SI[d] (RT)
AZT	20	0.006	0.01	3333	2000
D4T	10	0.08	0.05	125	200
2a	10	0.12	0.20	83	50
2b	50	0.02	0.05	2500	1000
3a	17	4.30	nd	17	nd
4	100	0.02	0.05	5000	2000
5	17	1	nd	17	nd
6	4	0.10	0.08	40	50
7	4	0.009	0.02	440	200
8	>20	0.30	nd	>66	nd
9	>20	>20	nd	inactive	nd
10	30	0.04	0.02	750	1500
11	30	0.10	0.04	300	750

[a] All data represented average values for at least two separate experiments. The variation of these results under standard operating procedures is below ±10%. See the Experimental Section (Antiviral Assay on Cells). [b] IC_{50} (50% cytotoxic dose): concentration (in μM) required to reduce the viability of uninfected cells by 50% at 7 days of incubation in the presence of the compound. [c] EC_{50} (50% antiviral effective dose): concentration (in μM) that reduced by 50% the HIV induced cytopathic effect (based on MTT assay or reverse transcriptase activity). [d] SI = selectivity index: ratio of IC_{50} to EC_{50}.

to D4T brought the selectivity index of these compounds into the same range as that of AZT, 10-fold higher than D4T. The inhibiting potency of the most promising compound, **4**, was also evaluated on monocytic cell lines (U937) where the selectivity index was also similar to that for AZT.

In order to determine their stability in physiological conditions, the half-lives ($t_{1/2}$) of the compounds were estimated in human plasma after separation by TLC. Phosphodiesters (**2a**, **2b**, **4**), carbonate **8**, carbamate **9**, and ester **7** show the largest half-lives (>24 h). Phosphotriesters (**3a**, **3b**, **5**) were more susceptible to the action of plasma enzymes with $t_{1/2}$ in the range of 12 h. As it has been shown by Pauwels et al.[18] these ddN analogs have no effect on the HIV replication when added more than 6 h after cell infection, and as these prodrugs display half-lives largely over this 6-h limit, that suggests that the compounds are internalized into infected cells rapidly and intracellularly metabolized to yield active compounds. Esters (**6**, **10**, **11**) were the most rapidly hydrolyzed with respective half-lives of 4, 2, and 1 h. As a control 3′-azido-3′-deoxy-5′ *O*-phenylalanylthymidine was also studied and was stable as expected.[13]

Several informations can be drawn from these results. Among the five different phosphate derivatives which were tested, the two fully protected triesters **3a** and **5** exhibited low activities. On the contrary, the two phosphodiesters, **2a** and **2b**, exhibited activities in the same range as D4T itself, the cholesteryl derivative, **2b**, being both more active and less toxic than the corresponding palmityl derivative **2a**. The symmetrical phosphodiester of D4T, **4**, has a very low toxicity on CEM-Cl13 cells which was confirmed on a monocytic cell line (U937). This decreased toxicity compared to D4T brings the selectivity index of **4** into the same range as AZT, 10-fold higher than D4T itself.

Among the other derivatives, which are devoid of phosphate groups, the hydrophobic residues being directly linked to the OH group of D4T, the cholesteryl derivative **7** again appears to be more active than the other linear chain derivatives, **6**, **8**, **9**, **10**, and **11**. However, due to its toxicity, its selectivity index remains comparable to D4T. An additional observation was the importance of the linker between the 5′-OH group of D4T and the hydrophobic moiety reflected by the different activities of the three palmityl derivatives, **6**, **8**, and **9**. Although the three compounds have large plasma half-lives, only the ester derivative **6** displays a good antiviral activity.

Conclusion

We have synthesized and tested on CEM-Cl13 cells several 5′-monophosphate D4T derivatives devised as potential lipophilic prodrugs of D4T. Because phosphate ester hydrolysis can liberate the nucleoside instead of the desired nucleoside monophosphate, bis-5′-D4T-phosphate and its cyanoethoxy derivative were also prepared. As a control of the advantage of incorporating the phosphate moiety in the above compounds two corresponding palmityl and cholesteryl D4T esters were synthesized. In this case the influence of the lipophilic moiety was only studied and compared to the linoleic and linolenic groups. Finally two other different linkages of the palmitic group to D4T were tested. The in vitro antiviral results clearly show that the D4T phosphotriesters were notably less active than the corresponding phosphodiesters. Among these latter derivatives, two compounds present a clear advantage over D4T: cholesteryl D4T phosphate (**2b**) and bis-5′-D4T phosphate (**4**). The observed EC_{50} indicate that they were correctly hydrolyzed in the cell and could be converted into triphosphate RT inhibitors. A better inhibiting potency was also obtained with the cholesteryl D4T ester **7** while other esters displayed weaker activities. In contrast to this cholesteryl ester, cholesteryl D4T phosphate (**2b**) and bis-5′-D4T phosphate (**4**) appeared much less toxic. The strong toxicity of the cholesteryl ester may be explained by an increased intracellular D4T concentration due to improved transmembrane transport. Possible superior bioavailability or pharmacokinetics of these compounds versus D4T must be now verified by further investigation in animal models.

Experimental Section

Melting points were determined on a Gallenkamp apparatus in open capillary tubes. Silica gel plates (Merck F254) were used for thin-layer chromatography; solvent A: CH_2Cl_2–CH_3OH (9:1), solvent B: 2-PrOH–NH_4OH 25%–H_2O (85:5:10). Elemental analyses were performed by the Service de Microanalyse du CNRS (Vernaison, France) and are within ±0.4% of the calculated values when specified by symbols. ^1H and ^{31}P NMR spectra were recorded at 400 MHz on a Bruker spectrometer using tetramethylsilane as the internal reference. Mass spectra were recorded on a four-sector Kratos concept II HH. The FAB ionization was obtained with a FAB field source operated with Xenon at 8 kV and 1 mA. Cesium iodide was used for calibration; accelerating voltage was 6 kV.

General Procedure for the Esterification of D4T. DCC (2.4 equiv) was added to a stirred solution consisting of D4T (1 equiv), 4-(dimethylamino)pyridine (1.5 equiv), and the appropriate acid (2.2 equiv) in ethyl acetate (100 mL/g of D4T). The progress of the reaction was monitored by TLC (solvent A). After completion (1–2 days), the DCU was filtered, and the filtrate was concentrated under reduced pressure and then purified by thick-layer chromatography: CH_2Cl_2–MeOH (95:5), yield (40–60%).

1-(2,3-Dideoxy-5-palmitoyl-β-D-*glycero*-pent-2-enofuranosyl)thymidine (6): R_f A 0.7; mp 75 °C; ^1H NMR ($CDCl_3$) δ 0.85 (t, 3 H, CH_3, aliphatic), 1.2 (m, 24 H, $(CH_2)_{12}$), 1.6 (m, 2 H, $CH_2CH_2C(O)O$), 1.95 (s, 3 H, 5-CH_3), 2.3 (t, 2 H, $CH_2C(O)O$), 4.15–4.4 (d, 2 H, 5′-H), 5.05 (m, 1 H, 4′-H), 5.9 (m, 1 H, 3′-H, vinyl), 6.25 (m, 1 H, 2′-H, vinyl), 6.95 (m, 1 H, 1′-H), 7.25 (s, 1 H, 6-H), 8.2 (s, 1 H, 3-H); FAB MS *m/e* 463 $(M+H)^+$. Anal. ($C_{26}H_{42}N_2O_5$) C, H, N.

1-(2,3-Dideoxy-5-linoleoyl-β-D-*glycero*-pent-2-enofuranosyl)thymidine (10): R_f A 0.7; mp 82 °C, ^1H NMR ($CDCl_3$) δ 0.85 (t, 3 H, CH_3, aliphatic), 1.25 (m, 14 H, $(CH_2)_3$, $(CH_2)_4$), 1.65 (m,

2 H, $CH_2CH_2C(O)O$), 1.9 (s, 3 H, 5-CH_3), 2.05 (m, 4 H, CH_2=CH=CH), 2.3 (t, 2 H, $CH_2C(O)O$), 2.75 (t, 2 H, CH=CHCH_2CH=CH), 4.15–4.4 (2 d, 2 H, 5'-H), 5.05 (m, 1 H, 4'-H), 5.35 (m, 4 H, CH=CH), 5.9 (m, 1 H, 3'-H, vinyl), 6.25 (m, 1 H, 2'-H, vinyl), 7.0 (m, 1 H, 1'-H), 7.25 (s, 1 H, 6-H), 8.55 (s, 1 H, 3-H); FAB MS *m/e* 487 $(M + H)^+$. Anal. ($C_{28}H_{42}N_2O_5$) C, H, N.

1-(2,3-Dideoxy-5-linolenoyl-β-D-*glycero*-pent-2-enofuranosyl)thymidine (11). R_f A 0.7; mp 70–120 °C; ^{1}H NMR ($CDCl_3$) δ 0.95 (t, 3 H, CH_3, aliphatic), 1.3 (m, 6 H, $(CH_2)_3$), 1.6 (m, 2 H, $CH_2CH_2C(O)O$), 1.95 (s, 3 H, 5-CH_3), 2.05 (m, 4 H, CH_2CH=CH), 2.3 (t, 2 H, $CH_2C(O)O$), 2.8 (m, 4 H, CH=CHCH_2CH=CH), 4.2–4.4 (d, 2 H, 5'-H), 5.05 (m, 1 H, 4'-H), 5.35 (m, 6 H, CH=CH), 5.9 (m, 1 H, 3'-H, vinyl), 6.25 (m, 1 H, 2'-H, vinyl), 7.0 (m, 1 H, 1'-H), 7.25 (s, 1 H, 6-H), 8.5 (s, 1 H, 3-H); FAB MS *m/e* 485 $(M + H)^+$. Anal. ($C_{28}H_{40}N_2O_5$) C, H, N.

1-[2,3-Dideoxy-5-[[(cholest-5-en-3β-yloxy)methyl]carbonyl]-β-D-*glycero*-pent-2-enofuranosyl]thymidine (7). 7 was prepared from 3β-(carboxymethoxy)cholestene[12] and D4T according to the general procedure for esterification: R_f A 0.7; ^{1}H NMR ($CDCl_3$) δ 0.65 (s, 3 H, 18''-H), 0.85 (m, 6 H, 26''-H, 27''-H), 0.9 (d, 3 H, 21''-H), 1.0 (s, 3 H, 19''-H), 1.95 (s, 3 H, 5-CH_3), 4.15 (s, 2 H, $OCH_2C(O)O$), 4.3–4.45 (d, 2 H, 5'-H), 5.05 (m, 1 H, 3'-H, vinyl), 5.35 (m, 1 H, 6''-H, vinyl), 5.9 (m, 1 H, 3'-H, vinyl), 6.35 (m, 1 H, 2'-H, vinyl), 6.95 (m, 1 H, 1'-H), 7.2 (s, 1 H, 6-H), 8.25 (s, 1 H, 3-H); FAB MS *m/e* 673 $(M + Na)^+$, 689 $(M + K)^+$. Anal. ($C_{39}H_{58}N_2O_6$) C, H, N.

1-[2,3-dideoxy-5-[(hexadecyloxy)carbonyl]-β-D-*glycero*-pent-2-enofuranosyl]thymidine (8). Carbonyldiimidazole (356 mg, 2.2 mmol) was added to a stirred solution of D4T (100 mg, 0.4 mmol) in 5 mL of anhydrous acetonitrile. Transformation of D4T to ester was complete after 30 min. The solvent was then evaporated and the crude residue was triturated in ethyl acetate to give 126 mg of crystals 12. The totality of 12 and 1-hexadecanol (943 mg, 3.9 mmol) were dissolved in acetonitrile (5 mL), and the solution was refluxed overnight. Purification of the reaction mixture by thick-layer chromatography CH_2Cl_2–MeOH (95:5) gave 20 mg of 11 (9%): R_f A 0.7; mp 70–90 °C; ^{1}H NMR ($CDCl_3$) δ 0.9 (t, 3 H, CH_3-aliphatic), 1.25 (m, 26 H, $(CH_2)_{13}$), 1.6 (m, 2 H, $CH_2CH_2OC(O)$), 1.95 (s, 3 H, 5-CH_3), 3.65 (t, 2 H, $CH_2OC(O)$), 4.25–4.45 (d, 2 H, 5'-H), 5.0 (m, 1 H, 4'-H), 5.85 (m, 1 H, 3'-H, vinyl), 6.35 (m, 1 H, 2'-H, vinyl), 7.05 (m, 1 H, 1'-H), 7.4 (s, 1 H, 6-H), 8.15 (s, 1 H, 3-H); FAB MS *m/e* 493 $(M + H)^+$. Anal. ($C_{27}H_{44}N_2O_6$) C, H, N.

1-[2,3-Dideoxy-5-[(hexadecylamino)carbonyl]-β-D-*glycero*-pent-2-enofuranosyl]thymidine (9). 12 (110 mg, 0.34 mmol) and hexadecylamine (823 mg, 3.4 mmol) were dissolved in 5 mL of acetonitrile and the solution was refluxed overnight. Purification by thick-layer chromatography CH_2Cl_2–MeOH (95:5) gave 15 mg of 9 (9%): R_f A 0.6; mp 140–145 °C; ^{1}H NMR δ 0.85 (t, 3 H, CH_3-aliphatic), 1.25 (m, 26 H, $(CH_2)_{13}$), 1.45 (m, 2 H, CH_2CH_2NH), 1.9 (s, 3 H, 5-CH_3), 3.15 (m, 2 H, CH_2NH), 4.25–4.7 (d, 2 H, 5'-H), 5.0 (m, 1 H, 4'-H), 5.35 (m, 1 H, 3'-H, vinyl), 6.3 (m, 1 H, 2'-H, vinyl), 6.95 (m, 1 H, 1'-H), 7.2 (s, 1 H, 6-H), 8.4 (s, 1 H, 3-H). FAB MS *m/e* 492 $(M + H)^+$. Anal. ($C_{27}H_{45}N_3O_5$) C, H, N.

3'-Deoxy-2',3'-didehydrothymidine 5'-(2-Cyanoethyl hexadecyl phosphate) (3a). 1-Hexadecanol (106 mg, 0.44 mmol) was dissolved in anhydrous chloroform (3 mL), and then bis-(diisopropyl)ammoniumtetrazolide (38 mg, 0.22 mmol) and 2-cyanoethyl tetraisopropylphosphorodiamidite (172 μL, 0.54 mmol) were added. After continuous stirring for 4 h, the reaction mixture was poured into a $NaHCO_3$ saturated solution (10 mL) and extracted with chloroform for several times. The combined organic layers were dried over Na_2SO_4 and evaporated to dryness. The residue was dried overnight under P_2O_5 to give crude 13a. To a solution of 13a in anhydrous THF (3 mL) were added D4T (100 mg, 0.44 mmol) and tetrazole (31 mg, 0.44 mmol). The reaction mixture was stirred at room temperature for 4 h under N_2 and then a solution of I_2 (111 mg, 0.44 mmol) in 3 mL THF–H_2O (2:1) was added. Thick-layer chromatography CH_2Cl_2–MeOH (95:5), and extraction from silica gel (acetone, CH_2Cl_2) gave 28 mg (11%); of an oily residue: R_f A 0.6; ^{1}H NMR ($CDCl_3$) δ 0.9 (t, 3 H, CH_3, aliphatic), 1.25 (m, 26 H, $(CH_2)_{13}$), 1.7 (m, 2 H, CH_2OP), 1.95 (s, 3 H, 5-CH_3), 2.75 (t, 2 H, CN-CH_2), 4.1–4.3 (d, 2 H, 5'-H), 4.35 (m, 2 H, $CNCH_2CH_2$), 5.9 (m, 1 H, 3'-H, vinyl), 6.35 (m, 1 H, 2'-H, vinyl), 7.0 (m, 1 H, 1'-H), 7.3 (s, 1 H, 6-H), 8.5 (s, 1 H, 3-H); FAB MS *m/e* 580 $(M - H)^-$.

Ammonium Salt of 3'-Deoxy-2',3'-didehydrothymidine 5'-(Hexadecyl phosphate) (2a). Treatment of 3a (28 mg, 0.048 mmol) with concentrated aqueous ammonia (5 mL) overnight and purification by thick-layer chromatography *i*-PrOH–NH_4OH–H_2O (85:5:10) gave 2a: 7 mg (27%); R_f B 0.4; ^{1}H NMR ($(CD_3)_2$SO) δ 0.9 (t, 3 H, CH_3-aliphatic), 1.25 (m, 28 H, $(CH_2)_{14}$), 1.8 (s, 3 H, 5-CH_3), 3.6 (m, 2 H, CH_2OP), 3.75–3.85 (2 m, 2 H, 5'-H), 4.9 (m, 1 H, 4'-H), 5.85 (m, 1 H, 3'-H, vinyl), 6.45 (m, 1 H, 2'-H, vinyl), 6.8 (m, 1 H, 14-H), 7.75 (s, 1 H, 6-H); ^{31}P NMR ($(CD_3)_2$SO) δ −0.35; FAB MS *m/e* 327 $(M - H)^-$.

Bis(3'-deoxy-2',3'-didehydrothymidin-5'-*O*-yl) 2-Cyanoethyl Phosphate (5). D4T (200 mg, 0.88 mmol) was dissolved in anhydrous acetonitrile, and then 2-cyanoethyl tetraisopropylphosphorodiamidite (133 μL, 0.42 mmol) and tetrazole (62 mg, 0.88 mmol) were added. After stirring for 30 min, a solution of I_2 (222 mg, 0.88 mmol) in 3 mL of THF–H_2O was added. Final purification by thick-layer chromatography CH_2Cl_2–MeOH (95:5) gave 82 mg of 5 (17%): R_f A 0.4; ^{1}H NMR ($(CD_3)_2$SO) δ 1.9 (s, 6 H, 5-CH_3), 2.8 (t, 2 H, $CNCH_2$), 4.2 (m, 6 H, $CNCH_2CH_2$, 5'-H), 4.9 (m, 2 H, 4'-H), 5.9 (m, 2 H, 3'-H, vinyl), 6.35 (m, 2 H, 2'-H, vinyl), 6.85 (m, 2 H, 1'-H), 7.25 (s, 2 H, 6-H), 9.0 (s, 2 H, 3-H); FAB MS *m/e* 564 $(M + H)^+$, 586 $(M + Na)^+$.

Ammonium Salt of Bis(3'-deoxy-2',3'-didehydrothymidin-5'-*O*-yl) Monophosphate (4). Treatment of 5 (50 mg, 0.09 mmol) with concentrated aqueous ammonia (3 mL) overnight and final purification by thick-layer chromatography *i*-PrOH–NH_4OH–H_2O (85:5:10) gave 12 mg of 4 (25%): R_f B 0.4; ^{1}H NMR ($(CD_3)_2$SO) δ 1.85 (s, 6 H, 5-CH_3), 3.7–3.85 (2 n, 4 H, 5'-H), 4.8 (m, 2 H, 4'-H), 5.85 (m, 2 H, 3'-H, vinyl), 6.35 (m, 2 H, 2'-H, vinyl), 6.80 (m, 2 H, 1'-H), 7.65 (s, 2 H, 6-H); ^{31}P NMR ($(CD_3)_2$SO) δ −0.74; FAB MS *m/e* 509 $(M - H)^-$.

Ammonium Salt of 3'-Deoxy-2',3'-didehydrothymidine 5-[(Cholest-5-en-3β-yloxy)ethyl phosphate] (2b). 3β-(2'-Hydroxyethoxy)cholest-5-ene[11] (192 mg, 0.44 mmol) was dissolved in anhydrous chloroform (3 mL), and then bis(diisopropyl)-ammoniumtetrazolide (38 mg, 0.22 mmol) and 2-cyanoethyl tetraisopropylphosphoramidite (172 μL, 0.54 mmol) were added. After continuous stirring for 4 h, the reaction mixture was poured into a $NaHCO_3$ saturated solution (10 mL) and extracted several times with chloroform. The combinated chloroform layers were dried over Na_2SO_4, and evaporated to dryness to produce crude 13b. To the solution of 13b in $CHCl_3$ (3 mL) were added D4T (100 mg, 0.44 mmol) and tetrazole (31 mg, 0.44 mmol). The reaction mixture was stirred at room temperature for 4 h under N_2. A solution of I_2 (11 mg, 0.44 mmol) in 3 mL THF–H_2O (2:1) was added. The solvent was evaporated and the residue was treated with concentrated aqueous ammonia (5 mL) overnight. Thick-layer chromatography *i*-PrOH–NH_4OH–H_2O (85:5:10) and extraction from silica gel (acetone) gave 11 mg of 2b (4%): R_f B 0.4; ^{1}H NMR ($(CD_3)_2$SO) δ 0.65 (s, 3 H, 18''-H), 0.85 (m, 6 H, 26''-H, 27''-H), 0.9 (d, 3 H, 21''-H), 1.0 (s, 3 H, 19''-H), 1.85 (s, 3 H, 5-CH_3), 3.65 (m, 2 H, CH_2OP), 3.75–3.85 (m, 2 H, 5'-H), 4.85 (m, 1 H, 4'-H), 5.3 (m, 1 H, 6''-H, vinyl), 5.9 (m, 1 H, 3'-H, vinyl), 6.4 (m, 1 H, 2'-H, vinyl), 6.8 (m, 1 H, 1'-H), 7.75 (s, 1 H, 6-H); ^{31}P NMR ($(CD_3)_2$SO) δ −0.31; FAB MS *m/e* 739 $(M + Na)^+$.

Cells. The CEM-Cl13, subclone enriched in CD4 receptors was obtained from CEM T-lymphoblastoid tumor cell line,[19] originally isolated from a child with acute lymphocytic leukemia.[20] Cells were grown at 37 °C in a CO_2 incubator (5%) in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL) (complete medium).

Virus. The LAV-Bru strain of HIV-1 was isolated at the Pasteur Institute from lymphocytes of a patient with lymphadenopathy.[21] For infection studies, cell-free supernatants from virus-producer cultures were filtered through a 0.45-μm (pore size) filter (Millipore). The infectious titer of the virus pool was determined by MTT and RT activities by making 10-fold dilutions of the virus stock in growth medium. The titer was 2000 $TCID_{50}$/mL viral supernatant or 5000 $TCID_{50}/10^6$ cells. Then each dilution was mixed with 100 μL of medium containing 1×10^4 CEM C113 in 96-well plates. Fifty percent cell culture infective dose (CCID 50/mL) was standardized by using an MTT assay[17,22] and by reverse transcriptase activity (1×10^6 cpm/mL).[22]

Antiviral Assay on Cells. Compounds were tested and compared to AZT (obtained from Sigma) and D4T (synthesized in our laboratory using a modification of the procedure described in reference 4a) for cytotoxicity and for their ability to inhibit HIV replication. Compounds were first dissolved in dimethylformamide (DMF) (1 mM) and then diluted (H_2O) just before use. The maximum final concentration of DMF added to the cell cultures was 0.1% at the highest concentration of compound. We have determined that at this concentration DMF does not interfere with cell growth. The HIV-1-induced CPE was monitored by the MTT viability assay.[17,22] Reverse transcriptase activity in supernatants was routinely measured to follow HIV replication (see the next section). In the microplate tests (96-well), 50 μL of each compound dilution or PBS alone were distributed in triplicate. The cells were adjusted to 1×10^5 cells/mL and then were plated in each well at the rate of 100 μL per well; in all experiments we started at the day of infection with 10000 cells/well. Virus suspension (100 μL) was added to cells with or without drugs and cultured for 7 days. The virus titer was always determined in the cells used in the experiments, in this case in CEM-Cl13 cells. The cultures were then infected by the addition of 100 μL of HIV-1 containing 50–250 times the minimal dose necessary to infect a CEM cell culture. Mock-infected cultures were carried out in parallel to determine the cytotoxicity of the compound. Briefly, 100 μL of cell suspension were collected and mixed with 10 μL of a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 7 μg/mL in PBS.[22] After a 3-h incubation at 37 °C, most of the supernatant was removed and the formazan precipitate dissolved in 100 μL of 0.04 N HCl in 2-propanol. The absorbance at 540 nm was measured. The percentage of toxicity was defined with noninfected and untreated control cells. The 50% cytotoxic dose (IC_{50}) was defined as the concentration of compound that reduced the absorbance of the mock-infected control sample by 50%. The dose achieving 50% protection was defined as the 50% effective dose (EC_{50}); the selectivity index is the ratio IC_{50}/EC_{50}.

In Vitro RT Assay. The determination of RT activity in cell supernatants as marker of HIV replication was performed as described previously.[22] Briefly, 50 μL of supernatant were incubated for 1 h at 37 °C with 50 μL buffer containing 50 mM Tris·HCl (pH 7.8), 50 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 0.05% Triton X-100, 0.5 mM EGTA, poly(rA)-oligo(dT) (0.5 OD_{260}/mL), and [^{3}H]TTP (0.11 MBq−1 μM). The polynucleotides were then precipitated with 20 μL of 60% TCA in 120 mM $Na_4P_2O_7$ and the samples were filtered on glass fiber filters using a Skatron cell harvester. The filters were dried and counted in a 1240 rack-β-scintillation counter (LKB, Uppsala, Sweden).

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References

(1) Mitsuya, H.; Weinhold, K. J.; Furman, P. A.; St-Clair, M. H.; Nusinoff-Lehrman, S.; Gallo, R. C.; Bolognesi, D. P.; Barry, W.; Broder, S. 3′-azido-3′-deoxythymidine (BW A509U) an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/Lymphoadenopathy associated virus in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7096–7100.

(2) Mitsuya, H.; Broder, S. Inhibition of the in vitro infectivity and cytopathic effect of HTLV-III/LAV by 2′,3′-dideoxynucleosides. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1911–1915.

(3) Hao, Z.; Cooney, D. A.; Hartman, N. R.; Perno, C. F.; Fridland, A.; DeVico, 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.

(4) (a) Horwitz, J. P.; Chua, J.; Da Rooge, M. A.; Noel, M.; Klundt, I. L. The formation of 2′,3′-unsaturated pyrimidine nucleosides via a novel β-elimination reaction. *J. Org. Chem.* **1966**, *31*, 205–211. (b) Baba, M.; Pauwels, R.; Herdewijn, P.; De Clercq, E.; Desmyter, J.; Vandeputte, M. 2′,3′-Dideoxythymidine and its 2′,3′-unsaturated derivatives are potent and selective inhibitors of HIV replication in vitro. *Biochem. Biophys. Res. Commun.* **1987**, *142*, 128–134. (c) Hitchcock, M. J. M. 2′,3′-Didehydro-2′,3′-dideoxythymidine (D4T), an anti-HIV agent. *Antiviral Chem. Chemother.* **1991**, *2* (3), 125–132 and references therein.

(5) Furman, P. A.; Fyfe, J. A.; St Clair, M. H.; Weinhold, K.; Rideout, J. L.; Freeman, G. A.; Nusinoff Lehrman, S.; Bolognesi, D. P.; Broder, S.; Mitsuya, H.; Barry, D. W. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 8333–8337.

(6) Balzarini, J.; Herdewijn, P.; DeClercq, E. Differential patterns of intracellular metabolism of 2′,3′-didehydro-3′-dideoxythymidine (D4T) and 3′-azido-3′-dideoxythymidine (AZT), two potent anti-HIV compounds. *J. Biol. Chem.* **1989**, *264*, 6127–6133.

(7) Ho, H.-T.; Hitchcock, M. J. M. Cellular Pharmacology of 3′-dideoxy-2′,3′-didehydrothymidine, a nucleoside analog active against HIV. *Antimicrob. Agents Chemother.* **1989**, *33*, 844–849.

(8) Mansuri, M. M.; Hitchcock, M. J. M.; Buroker, R. A.; Bregman, C. L.; Ghazzouli, I.; Desiderio, J. V.; Starrett, J. E., Jr.; Sterzycki, R. Z.; Martin, J. C. Comparison of in vitro biological properties and mouse toxicities of three thymidine analogs active against HIV. *Antimicrob. Agents Chemother.* **1990**, *34*, 637–641.

(9) Mansuri, M. M.; Starrett, J. E., Jr.; Ghazzouli, I.; Hitchcock, M. J. M.; Sterzycki, R. Z.; Brankovan, V.; Lin, T. S.; August, E. M.; Prusoff, W. H.; Sommadossi, J. P.; Martin, J. C. 1-(2,3-dideoxy-β-*glycero*-pent-2-enofuranosyl)thymidine. A highly potent and selective anti-HIV agent. *J. Med. Chem.* **1989**, *32*, 461–466.

(10) Simpson, M. V.; Chin, C. D.; Keilbaugh, S. A.; Lin, T. S.; Prusoff, W. H. Studies in the inhibition of mitochondrial DNA replication by 3′-azido-3′-deoxythymidine and other dideoxynucleosides analogues which inhibit HIV-1 replication. *Biochem. Pharmacol.* **1989**, *38*, 1033–1036.

(11) Ahmad, M. S.; Logani, S. C. Lithium aluminum hydride-aluminum chloride reduction of steroidal cyclic acetals. *Aust. J. Chem.* **1971**, *24*, 143–151.

(12) Farrow, S. N.; Jones, A. S.; Kumar, A.; Walker, R. T.; Balzarini, J.; De Clercq, E. Synthesis and biological properties of novel phosphotriesters: a new approach to the induction of biologically active nucleotides into cells. *J. Med. Chem.* **1990**, *33*, 1400–1406.

(13) Aggarwal, S. K.; Sudhir, R. G.; Rangan, S. R. S.; Agrawal, K. C. Synthesis and biological evaluation of prodrugs of zidovudine. *J. Med. Chem.* **1990**, *33*, 1505.

(14) Pochet, S.; Kansal, V.; Destouesse, F.; Sarfati, S. R. Alkylglycoside carbonates of AZT. *Tetrahedron Lett.* **1990**, *31*, 6021–6024.

(15) Yu-Hua, J. I.; Bannarth, W.; Bang, L. Application of the phosphoramidite-phosphate triester approach for the synthesis of combinations between oxygenated sterols and nucleoside analogues linked by phosphodiesters bonds. *Tetrahedron* **1990**, *2*, 487–502.

(16) Nielsen, J.; Taagaard, M.; Marugg, J. E.; Van Boom, J. H.; Dahl, O. Application of 2-cyanoethyl N,N,N′,N′-tetraisopropyl phosphoro-diamidite for in situ preparation of deoxyribonucleoside phosphoramidites. *Nucleic Acids Res.* **1986**, 7391–7403.

(17) 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.

(18) Pauwels, A.; Andries, K.; Desmyter, J.; Schols, D.; Kukla, M. J.; Breslin, H. J.; Raeymaeckers, A.; Van Gelder, J.; Woestenborghs, R.; Heykants, J.; Schellekens, K.; Janssen, M. A. C.; De Clercq, E.; Janssen, P. A. J. Potent and selective inhibition of HIV-1 replication in vitro by a novel series of TIBO derivatives. *Nature* **1990**, *343*, 470–474.

(19) Montagnier, L.; Clavel, F.; Krust, B.; Chamarest, S.; Rey, F.; Barre-Sinoussi, F.; Chermann, J. C. Identification and antigenicity of the major envelope glycoprotein of lymphadenopathy-associated virus. *Virology* **1985**, *144*, 283–290.

(20) Folley, G. E.; Lazarus, H.; Faber, S.; Uzman, B. G.; Boone, B. A.; McCarthy, R. E. Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer* **1965**, *18*, 522–529.

(21) Barre-Sinoussi, F.; Chermann, J. C.; Rey, F.; Nugeyre, M. T.; Chamaret, S.; Gruest, J.; Dauguet, C.; Axler-Blin, C.; Vezinet-Brun, F.; Rouzioux, C.; Rozenbaum, W.; Montagnier, L. Isolation of a T-lymphotropic retrovirus from a patient at risk for AIDS. *Science* **1983**, *220*, 668–874.

(22) Schwartz, O.; Henin, Y.; Marechal, V.; Montagnier, L. A rapid and simple colorimetric test for the study of anti-HIV agents. *AIDS Res. Hum. Retroviruses* **1988**, *4*, 441–448.