height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after exposure to the agonist. IC_{50} estimates and relative potency estimates were determined by fitting the mean data to logistic equation by using a computerized nonlinear least-squares method.[41] The number of replications done was two to four experiments for each compound in duplicate. In some cases, the weak μ agonist actions of these analogues did not permit completion of dose-response curves in the GPI.

Radioreceptor Assay. Adult male Sprague-Dawley rats (200-250 g) were sacrificed and brains immediately removed and placed on ice. Whole brain minus cerebellum was homogenized with a Polytron homogenizer (Brinkman, setting 5, 15 s). The homogenate was preincubated at 25 °C for 30 min three times (three washes with 20 volumes of fresh 50 mM Tris buffer) to remove endogenous opioids and then centrifuged two times at 43000g for 10 min before use in the radioreceptor assay.

Binding affinities vs [^{3}H]DPDPE, [^{3}H][p-ClPhe^4]DPDPE, and [^{3}H]CTOP (all from New England Nuclear, Boston, MA) were measured by rapid filtration methods as described for [p-ClPhe^4]DPDPE[26] and CTOP.[27] A 100-μL aliquot of rat brain homogenate (0.5% final) was incubated with either 1.0 nM [^{3}H]DPDPE or 0.8 nM [^{3}H][p-ClPhe^4]DPDPE or 0.5 nM [^{3}H]-CTOP in a total volume of 1 mL of 50 mM Tris-HCl, pH 7.4, at ~5 °C containing 5 mM $MgCl_2$, bovine serum albumin (1 mg/mL), and phenylmethanesulfonyl fluoride (100 μL). Steady-state binding experiments were carried out at 25 °C for 120 min. All binding measurements were done in duplicate and two to four duplicate experiments were done for each compound. The radioligand displaced by 1 μM naltrexone hydrochloride was defined as specific tissue binding. The binding reaction was terminated by rapid filtration of samples through GF/B Whatman glass-fiber filter strips pretreated with 0.1% polyethylenimine solution with a Brandel cell harvester; this was followed immediately by three rapid washes with 4-mL aliquots of ice-cold saline solution. Filters were removed and allowed to dry before assaying filter-bound radioactivity by liquid scintillation spectrophotometry (45% efficiency). Binding data were analyzed by nonlinear regression methods.[26,27] IC_{50} affinity constants were converted to K_i values by the Cheng and Prusoff[42] equation using K_D values for DPDPE, [p-ClPhe^4]DPDPE, and CTOP of 4.05, 0.328, and 0.160, respectively.

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Registry No. 1, 88373-73-3; 2, 133098-42-7; 3, 133098-43-8; 4, 133098-44-9; 5, 133098-45-0; 6, 129073-55-8; 7, 129063-25-8; 8, 129073-56-9; 9, 129048-79-9; DL-*erythro*-β-methyl-Phe, 25488-24-8; DL-*threo*-β-methyl-Phe, 80372-54-9; L-*erythro*-β-methyl-Phe, 25488-25-9; D-*erythro*-β-methyl-Phe, 25488-26-0; DL-*erythro*-β-methyl-*p*-nitro-Phe, 133009-51-5; DL-*threo*-β-methyl-*p*-nitro-Phe, 133009-52-6; L-*erythro*-β-methyl-*p*-nitro-Phe, 128502-51-2; D-*erythro*-β-methyl-*p*-nitro-Phe, 128502-68-1; BOC-DL-*erythro*-β-methyl-Phe, 115132-19-9; BOC-DL-*threo*-β-methyl-Phe, 133009-53-7; BOC-DL-*erythro*-β-methyl-*p*-nitro-Phe, 133009-54-8; BOC-DL-*threo*-β-methyl-*p*-nitro-Phe, 133009-55-9.

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Lipophilic Glycosyl Phosphotriester Derivatives of AZT: Synthesis, NMR Transmembrane Transport Study, and Antiviral Activity

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Phosphate derivatives of AZT esterified with a carbohydrate (D-glucose, D-mannose, and ethyl D-mannopyranoside) and a hexadecyl chain were prepared from glucose 6-phosphate and D-mannose precursors. The ^{31}P NMR study of the mannosyl phosphotriester series in the presence of large unilamellar vesicles demonstrated either an interaction with the external lipid layer or a transmembrane transport into the intravesicular interface. The antiviral activity, measured by the inhibition of cytopathogenicity on different infected cells and of reverse transcriptase activity in the supernatant of cultures, appeared to be comparable to that of AZT, in the micromolar range.

Among the numerous approaches for the control of acquired immunodeficiency syndrome (AIDS) caused by the retrovirus HIV, so far only 3′-azido-3′-deoxythymidine, AZT,[1] has been identified as a clinically useful drug and approved specifically for treatment.[2-4] AZT acts as an inhibitor of the viral reverse transcriptase after its conversion into 5′-triphosphate by host cell thymidine and thymidilate kinases.[5] In contrast with other nucleoside transport, AZT permeates the membrane by passive diffusion,[6] crosses over the blood-brain barriers,[3-7] and partially reverses the neurological dysfunction due to HIV in some patients.[8] However the therapeutic potential of AZT is limited by serious adverse reactions, particularly bone marrow suppression,[9] and intensive efforts are still needed

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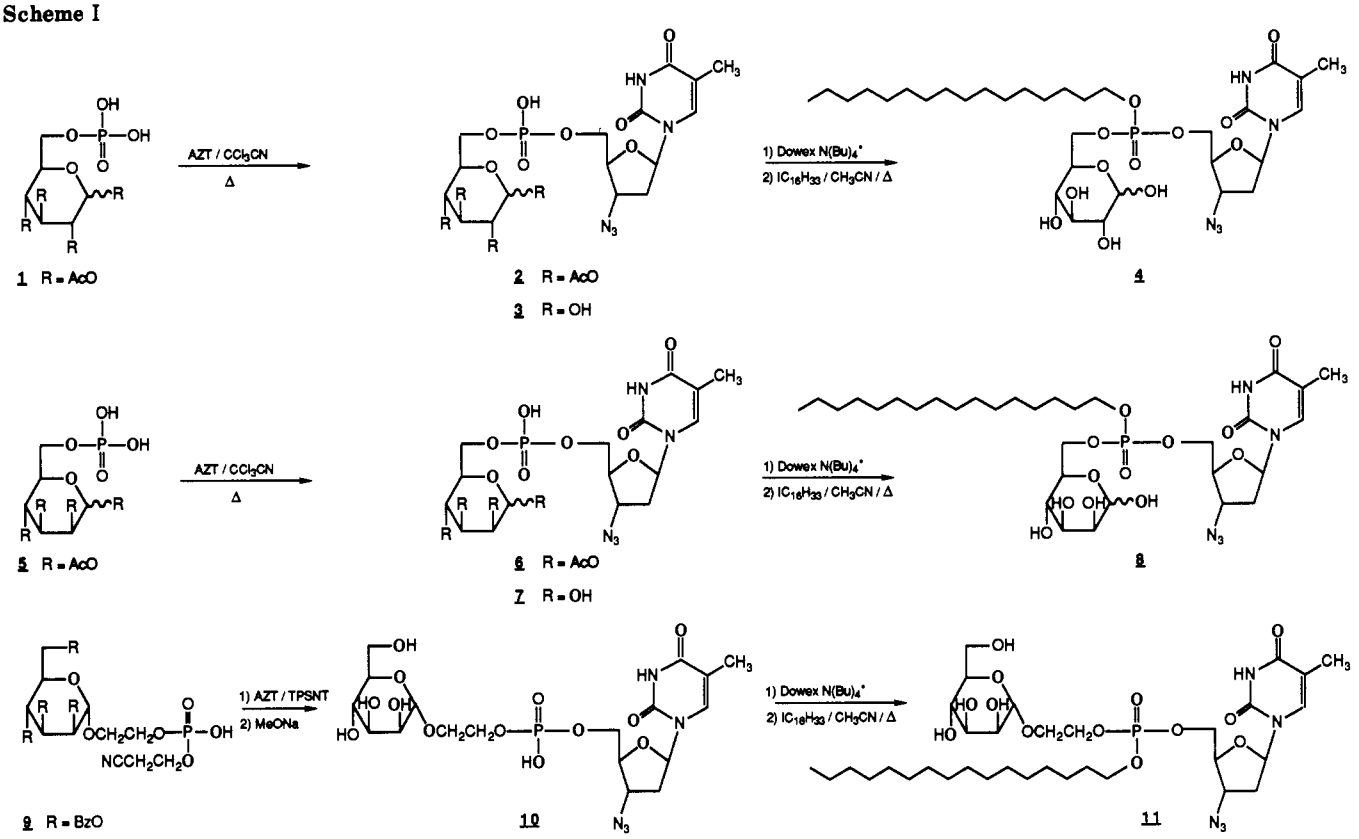
Scheme I



for the development of novel, more efficacious, and more selective nucleoside derivatives.

We have recently described (i) the synthesis of a lipophilic phosphotriester of thymidine (ii) its transmembrane transport across unilamellar model vesicles,[10] and (iii) demonstrated the importance of the alkyl chain length for the drug–membrane interaction.[11] As a development of this work, we present here the synthesis and the properties of phosphotriesters of AZT containing a polar carbohydrate and a hydrophobic hexadecyl chain. These compounds were synthesized in order to (1) verify if the phosphotriesters of AZT possess the transmembrane transport characteristics shown on the model with thymidine (The confirmation of a membrane permeation should confer to these derivatives a better bioavailability than for the nucleoside itself.) and (2) determine the antiviral effects of these lipophilic carriers of AZT (Their hydrolysis in situ will give, among other derivatives, the AZT 5′-monophosphate which will be further phosphorylated to an active triphosphate moiety.). A similar concept of phosphotriester derivatives bearing two alkyl chains[12,13] or an alkyl chain associated with two nucleosides[14] was recently described for antiviral nucleosides.

Chemistry

Three hexadecyl phosphotriesters of AZT were synthesized from 6-D-glucopyranosyl, 6-D-mannopyranosyl, and (hydroxyethyl)mannosidyl phosphates (Scheme I). The glucose series was the direct development of our previous work with the thymidine model.[10] The phosphorylation of AZT by 1,2,3,4-tetra-*O*-acetyl-D-glucose 6-phosphate gave after alkaline hydrolysis phosphodiester **3**, which was alkylated into hexadecyl 6-D-glucopyranosyl 5′-(3′-azido-3′-deoxythymidinyl) phosphate (4) (57%). The phosphotriester was isolated as an hygroscopic fluffy solid after silica gel column chromatography and, like the other phosphotriesters, its analytical purity was based mainly on NMR (Table I), mass spectral, and HPLC data. The four stereoisomers (α, β, *R*, and *S*) formed could not be separated by preparative HPLC.

The mannose 6-phosphate series was undertaken because, in addition to its higher water solubility, this carbohydrate could be used as a site-directing moiety toward mannosyl-binding proteins. This ligand–cell recognition is essential in the case of macrophages which are known to be the target of numerous pathogens which replicate in these cells and to present mannose-specific receptors responsible for the pathogenic binding.[15,16] Moreover, some

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HIV strains can replicate in monocytes/macrophages for a considerable time without inducing substantial cytopathic effects and it was suggested that these cells may be the reservoirs of the virus in vivo.[17,18] It is therefore hoped that compounds which have a deleterious effect on hematopoiesis will not act on bone marrow cells since most of mannosylated drugs will be preferentially endocytosed by liver and spleen macrophages; in these conditions a higher therapeutic index could be obtained, together with lower side effects and toxicity. 6-Mannosyl 5′-(3′-azido-3′-deoxythymidinyl) phosphate (7) and phosphotriester 8 were obtained with the same protocol as in the glucose series from commercial D-mannose 6-phosphate (5). The α anomer was predominant.

The third series with α-(hydroxyethyl)-D-mannoside was more closely related to the lipidic carrier dolichyl glucose 1-phosphate. As the pyranosyl sugars are converted into 6-phosphates during their active transport inside cells, we wanted to obtain glycosyl phosphates where the 6-OH was free in order to be taken up by the phosphotransferase system. The hydroxyethyl chain at the anomeric position was added to prevent the instability of phosphotriesters at the anomeric carbon observed in preliminary work. In this series, the phosphorylation of AZT with the cyanoethyl phosphate 9[20] was activated by TPSNT[19] often used in oligonucleotide chemistry. The protective groups were cleaved with sodium methylate, affording (α-D-mannopyranosidyl)ethyl 5′-(3′-azido-3′-deoxythymidinyl) phosphate (10), which was further alkylated into the corresponding hexadecyl phosphotriester 11.

^{31}P NMR Study of the Interactions between Phosphotriesters of AZT and Model Membranes

In previous studies,[10,11] we have shown that the transmembrane transport of phosphotriester compounds can be satisfactorily investigated by using large unilamellar vesicles (LUV) as model membranes and ^{31}P NMR spectroscopy. The well-known method used in these studies consists of recording a ^{31}P spectrum of a phosphotriester derivative in aqueous solution, then in the presence of LUV, and lastly after addition of Mn^{2+} ions. These ions do not cross the LUV membranes and their paramagnetic properties induce such a dramatic broadening for the resonances of molecules located in the extravesicular milieu that the corresponding signals are broadened beyond detection. Therefore, only the resonances of molecules located in the intravesicular milieu are observed.

In the present study, we first investigated the interaction of AZT itself with LUV membranes. Because of the lack of phosphorus nuclei, this interaction was monitored by

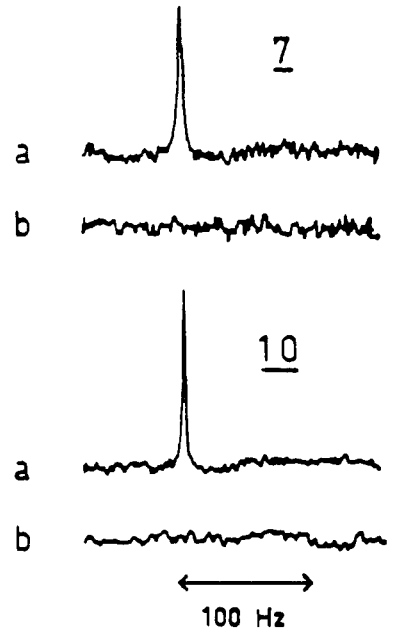

Figure 1. Phosphorus spectra (36.5 MHz) of phosphodiesters 7 and 10 in the presence of LUV (a) and after addition of Mn^{2+} ions (b).

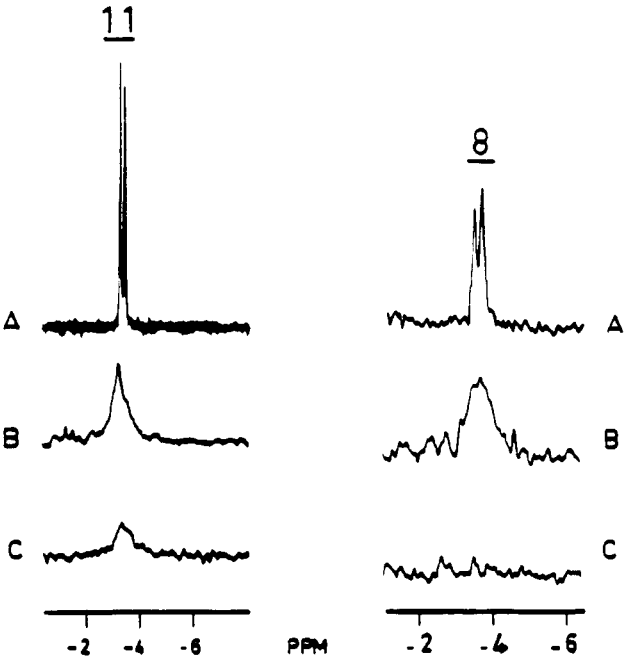

Figure 2. Phosphorus spectra (121 MHz) of phosphotriesters 8 and 11 in the absence (A) and the presence (B) of LUV and after addition of Mn^{2+} ions (C).

using the H6 base proton resonance as a probe and ^{1}H NMR experiments. Our data (not shown) indicated that the AZT molecules did not cross the LUV membrane. The phosphodiester derivatives of AZT, 7 and 10, were also tested and Figure 1 shows that both AZT derivatives were not transported through the membrane bilayer: in the presence of LUV, no phosphorus signal is detected after addition of Mn^{2+} ions.

As far as the phosphotriester derivatives of AZT are concerned, the first phosphotriester 4 obtained from glucose 6-phosphate was poorly soluble and its low hydrosolubility precluded any study of transmembrane transport. However, our data[11] suggested that because of the equilibrium between the hydrophobic and hydrophilic forces, the physicochemical properties of such molecules

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Table I. ^1H and ^{31}P NMR Spectral Data

	3		4		7:	8		10:	11:
	α (J_{12}, Hz)	β (J_{12}, Hz)	α (J_{12}, Hz)	β (J_{12}, Hz)	α	α (J_{12}, Hz)	β (J_{12}, Hz)	α	α
Aliphatic Chain									
CH_3			0.85			0.87			0.85
$(CH_2)_n$			1.25			1.25			1.25
CH_2CH_2OP			1.59			1.63			1.59
CH_2CH_2OP			4.00			4.01			4.00
Carbohydrate									
H_1	4.89 (4)	4.27 (7.6)	4.92 (4.1)	4.34 (7.7)	4.88 (1.9)	4.90 (2.0)	4.6 (1.5)	4.62 (2.1)	4.69 (2.0)
H_2	3.16	2.92	3.15	2.93	3.85	3.58		3.80	3.63
H_3	3.42	3.16	3.46	3.17	3.60	3.57		3.60	3.48
H_4	3.22	3.22	3.08	3.08	3.40	3.45		3.40	3.35
H_5	3.56	3.54	3.76	3.34	3.90	3.72		3.5 ± 0.5	3.42
H_6	3.90	3.84	4.14	4.23	3.90	4.23		3.5 ± 0.5	3.50
$H_{6'}$	3.94	3.88	4.14	4.00	3.90	4.09		3.5 ± 0.5	3.50
CH_2CH_2OP								3.58	3.59, 3.74
CH_2CH_2OP								3.92	4.18
AZT									
H_6	7.82		7.49 (44)	7.47 (56)	7.80	7.51 (41)	7.49 (59)	7.79	7.46
CH_3	1.80		1.80		1.80	1.82		1.80	1.80
$H_{1'}$	6.15		6.13, 6.12		6.15	6.14		6.16	6.13
$H_{2'}$	2.24		2.38		2.24–2.41	2.44		2.24–2.40	2.40
$H_{2''}$	2.39		2.38		2.24–2.41	2.36		2.24–2.40	2.40
$H_{3'}$	4.51		4.44		4.52	4.46		4.49	4.46
$H_{4'}$	3.96		4.02		3.97	4.02		3.92	4.02
$H_{5'}$	3.68		4.19		3.90	4.20		3.92	4.19
$H_{5''}$	3.77		4.19		3.90	4.20		3.92	4.19
Phosphate									
	−2.44	−2.38	−4.0 (br)		−2.62	−3.65 (50)	−3.84 (50)	−2.83	−3.65 (52)
									−3.82 (48)

could be changed by a minor modification of their structure. Since the nucleoside moiety as well as the hexadecyl chain cannot be altered because of the antiviral activity and the transport properties, one can expect a change of the physicochemical features of the phosphotriester analogues induced by a chemical modification of the sugar moiety. Prior NMR experiments, performed with the 5-fluoro-2′-deoxyuridine as the nucleoside moiety, showed that phosphotriesters derived from mannose 6-phosphate and mannopyranosidylethyl phosphate were more soluble and were transported through model membranes (unpublished data).

Figure 2A shows the phosphorus spectra of 6-substituted mannosyl phosphotriester of AZT 8 and 1-substituted phosphotriester 11 in aqueous solution at room temperature. As in the case of all the phosphotriester analogues already studied,[11] both AZT derivatives exhibit two phosphorus resonances corresponding to the two diastereoisomers. The chemical shifts of 8 and 11 are similar (Table I) and close to those of all the phosphotriesters previously investigated. In contrast, the signal line width of 8 is two times greater than that of 11. The latter value is close to the line width observed for the glycosyl phosphotriesters of thymidine or of 5-fluoro-2′-deoxyuridine and indicates that in aqueous solution, these molecules form similar small micelles. By comparison, 6-mannosyl derivative molecules 8 form larger aggregates. In the presence of model membranes (Figure 2B), the resonances of both AZT derivatives were broadened: the spectrum of 8 displayed a single signal, the line width of which was 10 times greater than in the absence of membranes, whereas the two diastereoisomeric resonances were still resolved. In the presence of LUV, the line width of the two resonances of phosphotriester 11 were different: 30 and 70 Hz for the low-field and high-field resonances, respectively (measured after deconvolution). After addition of paramagnetic ions, the signal of 8 was broadened beyond detection (Figure 2C) whereas the resonances of phosphotriester 11 were still observed: the intensity of the low-field resonance was reduced by a factor of 3 and that of the high-field signal by a factor of 2. Moreover, the line widths of the corresponding signals were not affected by the presence of Mn^{2+} ions. One can explain these paramagnetic effects as follows: (i) the disappearance of the signal of phosphotriester 8 indicates that it only interacts with the external lipid layer. (ii) the detection of the resonances of phosphotriester 11, with a reduced intensity but exhibiting the same line width as in the absence of Mn^{2+} ions, shows the presence of these molecules in the intravesicular water–membrane interface. As mentioned in our previous papers,[10,11] the difference observed between the two diastereoisomeric resonances of phosphotriester 11—line width and reduction factor intensity—can be explained in terms of difference of water–membrane exchange kinetics. Hence, the transmembrane transport of 11 inside the LUV showed the same diastereoisomeric effect observed previously with other pyrimidine nucleosides.

Antiviral Activity of Phosphotriester Derivatives of AZT

The antiviral activity of the compounds was measured (i) on lymphocytic (CEM-Cl13) and monocytic cell lines (U937) and (ii) on peripheral blood lymphocytes (PBL's), infected with HIV-1 cell-free supernatants. Two parameters were studied to evaluate the antiviral activity of the compounds: inhibition of HIV-1-induced cytopathic effect using the MTT cell viability assay (Table II) and the inhibition of the reverse transcriptase production in culture supernatants (Tables III–V).

Under the assay conditions described in the Experimental Section, the reference compound AZT displayed a CD_{50} of 22 μM and an ED_{50} (on inhibition of HIV-1-induced cytopathic effect) of 0.45 μM at day 22 postinfection (Table II). This ED_{50} value was quite high compared to previously published concentrations, in the nanomolar

Table II. Activity of the Phosphodi- and Triesters on Cytopathic Effect in CEM-Cl13 Cells Infected with HIV-1[a]

	7 days[b]			11 days[b]		
no.	CD_{50}	ED_{50}	SI	CD_{50}	ED_{50}	SI
3	30	0.2	150	35	0.16	219
4	11	0.18	61	3	0.18	17
7	27	0.13	208	28	0.2	140
8	2.6	0.14	18.5	2.6	0.3	9
10	25	0.16	156	28	0.15	187
11	20	0.18	111	28	1	28
AZT	26	0.12	217	23	0.12	192
	19 days			22 days		
no.	CD_{50}	ED_{50}	SI	CD_{50}	ED_{50}	SI
3	38	0.7	54	35	0.9	39
4	3.2	1.5	2	3.2	0.8	4
7	24	0.2	120	24	0.6	40
8	2.5	0.5	5	2.5	0.4	6.2
10	30	0.14	214	28	0.6	47
11	3.4	1.7	2	3.4	1.3	2.6
AZT	26	0.18	144	22	0.45	49

[a] See the Experimental Section (Antiviral Assay on Cells). All data represented average values for at least two separate experiments. [b] SI = selectivity index = ratio of CD_{50} to ED_{50} (based on MTT assay). CD_{50} (50% cytotoxic dose) = concentration required to reduce the viability of uninfected cells by 50% from 7 to 22 days of incubation in the presence of the compound. Results were expressed in μM. ED_{50} (50% antiviral effective dose) = concentration (in μM) that reduced by 50% the HIV induced CPE (cytopathic effect).

Table III. Effect on the HIV Production of CEM-Cl13 Cells

		reverse transcriptase activity,[a] cpm/50 μL			
compd	concn, μM	7 days	15 days	22 days	26 days
control virus	-	21000	19000	5000	15000
control cells	-	600	500	600	700
3	25	800	400	400	1100
	5	1100	400	1500	6000
	0.2	1600	750	2200	65000
4	1	2600	750	33000	72000
	0.2	4500	1600	45000	64000
7	25	1100	900	900	900
	5	1500	1300	12000	103000
	0.2	3400	1700	27000	85000
8	1	2000	500	3900	20000
	0.2	4000	1700	24000	73000
10	25	1204	304	500	1700
	5	1670	852	5500	42000
	0.2	4400	2600	27000	70000
11	1	6000	5500	48000	64000
	0.2	3900	2400	52000	61000
AZT	25	600	400	500	900
	5	900	500	500	1300
	1	100	600	1700	12000
	0.2	1500	1100	6400	28000

[a] Effects of viral replication were followed at the time indicated in the table. At these times, aliquots (50 μL) of supernatant were removed to measure RT activity. The results were expressed in [^{3}H] (cpm of 50 μL of supernatant) and each value was the mean of three independent samples.

range,[25] but these values correspond to inhibitory effects measured between day 4 and day 14 postinfection. Our present figure is a typical value in long-term experiments, characterized by HIV resumption in culture treated with low doses of AZT. All compounds presented in Table II presented the same feature.

In our series of derivatives, compounds **3**, **7**, and **10** were totally devoid of toxicity for CEM-Cl13 cells at doses equal or up to 25 μM. AZT displayed a toxicity equivalent to these three compounds after 19 days, but showed a weaker value of CD_{50} after 22 days of treatment. The other tested compounds (**4**, **8**, **11**) were more toxic than AZT with CD_{50} of 3.2, 2.5, and 3.4 μM, respectively. These values correspond to a constant treatment of the culture for 22 days. The compounds were renewed in the culture medium as described in the Experimental Section. The most striking observation was that in all cases, the phosphotriesters were notably more toxic for CEM-Cl13 cells than their phosphodiester precursors.

As reported in Table II, all compounds were able to significantly inhibit the HIV-1-induced cytopathic effect in CEM-Cl13 cells and all compounds were equally potent, in the same range as AZT (i.e. ED_{50} on day 22 postinfection was ca. 0.4 μM for compound **8** and 0.9 μM for compound **3**). As a consequence of virus resumption at the end of the incubation time, the selectivity indexes were 49 for AZT, 47 for ethyl mannoside phosphate **10**, 40 for mannose 6-phosphate **7**, and 39 for glucose 6-phosphate **3**.

We also evaluated the efficacy of the compounds by their ability to inhibit the production of reverse transcriptase (RT) in culture supernatant of CEM-Cl13 and U937 cell lines. We monitored the kinetics of RT production throughout the 26 and 14 days of culture, respectively (Tables III and IV). Under the experimental conditions of CEM-Cl13 cells (Table III) the reference AZT totally inhibited the RT production from 5 μM; at lower doses AZT only produced a delay in the RT activity appearance. The phosphotriester derivatives (**4**, **8**, **11**) were unable to consistently inhibit the RT production at nontoxic concentrations after day 15 postinfection. The phosphodiester compound **10** totally suppressed the RT production in cell culture supernatants until day 26 postinfection and its analogues **3** and **7** did the same all over the incubation time at the concentration of 25 μM. At lower concentrations, these compounds only induced delayed RT activity appearance.

In the experiment with monocytic U937 cells (Table IV), AZT and all derivatives except phosphotriester **11** showed at noncytotoxic doses a significant inhibition of the RT activity. After 11 days of culture only compound **4** at the concentration of 1 μM gave an inhibition (53%) of the RT activity throughout 14 days of culture. Thus, this phosphotriester was more active than AZT used at the same concentration.

In order to test the anti-HIV activity of our AZT derivatives in a more physiological experimental system, we analyzed their effect on HIV-1-preinfected PBL's (see the Experimental Section). In this model (Table V) phosphotriesters were confirmed as being unable to inhibit HIV-1 replication at nontoxic doses. Phosphodiesters displayed almost the same efficacy as in the CEM-Cl13 cell line. Compound **3** (glucose 6-phosphate) was as active as AZT from 5 μM and proved to be the most active compound in our series.

Finally, we verified (Table VI) that the AZT derivatives (and specially the alkyl derivatives) were devoid of direct anti-HIV (virucidal) or anti-RT activity. Clearly, none of the derivatives was able to inhibit RT activity after 1-h incubation at 37 °C. Further, after this 1 h preincubation with the drugs, HIV samples were added on CEM-Cl13 cells without addition of test compounds. None of these treatments induced a consistent and persistent direct inhibition.

Conclusion

We have synthesized phosphotriester compounds devised as potential lipophilic prodrugs of AZT. The NMR

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Table IV. Antiviral Activity on the Monocytic U937 Cells

		reverse transcriptase activity[a]					
		7 days		11 days		14 days	
compd	concn, μM	cpm/50 μL	% inhibn	cpm/50 μL	% inhibn	cpm/50 μL	% inhibn
control virus	-	247 516	-	144 333	-	18 666	-
3	25	68 000	73	209 000	0	42 000	0
	5	142 000	43	211 000	0	31 362	0
	1	158 000	36	145 000	0	17 000	9
4	1	66 000	73	68 000	53	4 000	79
	0.2	237 000	0	178 000	0	16 000	14
7	25	35 000	86	142 000	2	39 000	0
	5	133 000	46	193 000	0	24 000	0
	1	425 000	0	137 000	5	22 500	0
8	1	122 000	51	225 000	0	35 000	0
	0.2	251 000	0	227 000	0	27 000	0
10	25	56 000	77	115 000	20	11 000	41
	5	200 000	20	192 000	0	23 000	0
	1	306 000	0	174 000	0	25 000	0
11	1	401 000	0	243 000	0	55 000	0
	0.2	343 000	0	131 000	9	30 000	0
AZT	25	11 000	96	108 000	25	54 000	0
	5	35 000	86	118 000	18	33 000	0
	1	36 000	85	134 000	7	37 000	0
	0.2	23 000	91	152 000	0	60 000	0

[a] See legend of Table III. The results were expressed in [^{3}H] (cpm/50 μL of supernatant). Percentage of inhibition was calculated by the following formula:

$$\% \text{ inhibition} = 100 - 100 \times \frac{\text{cpm/50 } \mu\text{L of treated samples}}{\text{cpm/50 } \mu\text{L of control virus}}$$

Table V. Effect on the HIV Production in Preinfected PBL[a]

		reverse transcriptase activity, cpm/50 μL			
compd	concn, μM	7 days	10 days	14 days	21 days
control virus	-	17546	12118	30070	94120
control DMSO	-	11958	30219	14467	81198
3	25	761	1105	370	574
	5	890	798	732	373
	1	1011	771	408	16103
4	25	1636	7821	59993	76008
	5	8211	36437	157066	150234
	1	3490	26289	143741	71087
7	25	1112	950	351	343
	5	967	936	4131	19501
	1	943	3132	46089	33084
8	25	1164	594	toxic	-
	5	3135	27972	58328	22430
	1	9324	71458	113645	nd[b]
10	25	927	818	563	1486
	5	855	1101	471	3382
	1	923	4404	39708	21268
11	1	30227	60197	nd	nd
AZT	25	913	576	337	620
	5	965	984	302	542
	1	972	757	2018	44421

[a] Preinfected PBL cells were infected with HIV-1 by incubation for 1 h at 37 °C then washed twice and resuspended with the complete medium supplemented with 10% interleukin-2 and anti-human α-interferon. The cells suspension was diluted and distributed in microplate (1×10^5 cells/wells) with or without compounds. Effects of viral replication were followed at the time indicated in the table. At these times, aliquots (50 μL) of supernatant were removed to measure RT activity. [b] nd = not determined.

study demonstrated that their interaction with unilamellar vesicles was determined by the carbohydrate moiety. A phosphotriester could be detected in the intravesicular water membrane interface, implying the crossing of the membrane bilayer by a flip-flop process.

The in vitro antiviral results revealed that all phosphotriester derivatives were less active than AZT; moreover, they were found more toxic on CEM-Cl13 cells. These two facts could be related to a delayed hydrolysis of these compounds in the cells, and experiments have been undertaken to measure their degradation in different conditions. Phosphodiester derivatives were active in the same order of magnitude (in the micromolar range on day 22 postinfection) as AZT with less toxicity for compounds **3** and **10**, mannoside phosphodiester **10** presenting the same selectivity index as AZT. All compounds revealed a similar activity in different cells except phosphotriester **4**, which showed a clear advantage on monocytic U937 cells compared to AZT, at 1 μM.

In addition, treatment with phosphodiesters **3** and **10** appeared less toxic than with AZT for CEM-Cl13 cells throughout 22 days of culture; indeed, when AZT and compounds **3** and **10** were used at the concentration of 50 μM, we observed a higher cellular mortality with AZT (55%) than with phosphodiesters derivatives (from 5 to 14%) (data not shown). These in vitro models were certainly not the most suitable to prove the eventual advantages of our prodrug approach; however, the equivalent observed ED_{50} indicated that all the glycosyl derivatives were correctly hydrolyzed in the cell and could be converted into triphosphate RT inhibitors. This approach may thus merit further investigation in order to verify eventual superior bioavailability or pharmacokinetics in animal models.

Experimental Section

Dichloromethane was distilled from sodium carbonate; pyridine was twice redistilled from calcium hydride and p-toluenesulfonyl chloride; acetonitrile was distilled from phosphorus pentoxide; trichloroacetonitrile was redistilled just prior to use. Merck silica gel plates (60 F_{254}) were used for analytical TLC, and the spots were examined with UV light or anisaldehyde–sulfuric acid spray.

Mass spectra were obtained with a VG70-250 instrument. Analytical HPLC was performed on a Hewlett-Packard 1090 M series, equipped with a diode-array detector and a Nucleosil C18 (5 μm) column, with a 20-min gradient of 0–25% acetonitrile in 0.01 M triethylammonium acetate (pH 7.0).

For the NMR structural analysis, the phosphotriester molecules were dissolved in DMSO at a concentration of 4 mM. Proton spectra (500 MHz) were recorded on a Bruker WM 500 spectrometer and referenced to internal tetramethylsilane. Structural analysis was achieved by recording two-dimensional COSY and relayed coherence transfer COSY and by using the procedure

Table VI. Study of Direct Anti Reverse Transcriptase Activity

compd	days of culture	MTT dosage[a] % cell/ viability	MTT dosage[a] % protection	RT activity[b] cpm/ 50 μL	RT activity[b] % inhibn
control virus	0	-	-	17500	-
	7	23	-	63000	-
	9	18	-	31000	-
	13	19	-	nd[c]	-
3	0	-	-	32000	0
	7	50	36	19000	70
	9	34	13	28000	10
	13	17	0	nd	-
4	0	-	-	16000	9
	7	85	81	8000	87
	9	62	52	13000	58
	13	39	28	nd	-
7	0	-	-	25000	0
	7	54	38	24000	62
	9	36	22	34000	0
	13	18	3	nd	-
8	0	-	-	17000	30
	7	77	68	18000	71
	9	32	18	10000	68
	13	25	10	nd	-
10	0	-	-	30000	0
	7	39	20	47000	25
	9	28	12	39000	0
	13	20	7	nd	-
11	0	-	-	13000	26
	7	41	21	81000	0
	9	25	4	44000	0
	13	12	0	nd	-
AZT	0	-	-	19000	-
	7	100	100	5000	92
	9	79	76	7500	76
	13	34	26	nd	-

[a] The results were expressed in percentage according to the formula:

$$\% \text{ protection} = 100 \times \frac{\text{OD compounds}}{\text{OD HIV compounds}}$$

[b] The results were expressed in [^{3}H] (cpm/50 μL of supernatant) and in percentage according to the formula:

$$\% \text{ inhibition} = 100 - 100 \times \frac{\text{cpm/50 μL of treated samples}}{\text{cpm/50 μL of control virus}}$$

[c] nd = not determined.

described in a previous paper.[10]

6-α- and -β-D-Glucopyranosyl 3'-Azido-3'-deoxy-5'-thymidinyl Phosphate (3). 1,2,3,4-tetra-*O*-acetyl-6-D-glucopyranosyl 3'-azido-3'-deoxy-5'-thymidinyl phosphate (2) was prepared according to published procedures[10] from 400 mg (1.5 mmol) of AZT and 1312 mg (1.5 molar excess) of 1,2,3,4-tetra-*O*-acetyl-6-D-glucose phosphate (pyridinium form) in a trichloroacetonitrile–pyridine solution. After evaporation, the residue was chromatographed on a silica gel column with dichloromethane–methanol (90:10) to give 1150 mg (99%) of the protected phosphodiester 2 (R_f 0.54 CH_2Cl_2–MeOH–H_2O, 13:5:1), which was deacetylated by treatment with 1% sodium methoxide in methanol. After 10 min at room temperature, the solution was neutralized with Dowex 50WX8 (H^+); the resin was filtered off and washed well with methanol, and the filtrates were evaporated to dryness. The residue was dissolved in water and chromatographed first on a column of Biogel P2 (200/400 mesh) eluted with water and then on a column of reverse-phase C18 silica gel eluted with water to give 490 mg (55%) of 3: R_f 0.65 (isopropanol–NH_4OH–H_2O, 7:1:2); HPLC 9.48 min (60%) and 9.64 min (40%); MS FAB$^-$ *m/e* 508.00 (M - 1). UV (H_2O) 266 nm (9500). Anal. ($C_{16}H_{24}O_{12}N_5P^-N(C_4H_9)_4^+ \cdot 2H_2O$) C, H, N; P: calcd, 48.79, 8.13, 10.67, 3.94; found, 49.00, 8.03, 10.40, 4.08.

Hexadecyl 6-α- and -β-D-Glucopyranosyl 3'-Azido-3'-deoxy-5'-thymidinyl Phosphate (4). The tetrabutylammonium salt of the previous phosphodiester 3 (280 mg, 0.373 mmol) was alkylated with 1-iodohexadecane in acetonitrile overnight at 80 °C[10] to yield, after purification on a column of silica gel, 155 mg (57%) of phosphotriester 4: R_f 0.70 (CH_2Cl_2–MeOH–H_2O 13:5:1); HPLC 12.79 min (75%), 13.12 min (25%, shoulder); MS FAB$^-$ *m/e* 732.12 (M - 1), FAB$^+$ *m/e* 734.54 (M + 1); UV (H_2O) 266 nm (9000). Anal. ($C_{32}H_{56}O_{12}N_5P$) C, H, N; P: calcd, 52.39, 7.64, 9.55, 4.23; found, 51.78, 7.67, 9.36, 4.00.

6-α-D-Mannopyranosyl 3'-Azido-3'-deoxy-5'-thymidinyl Phosphate (7). The same procedure as for 3 using 500 mg (1.87 mmol) of AZT and 1650 mg (1.5 molar excess) of 1,2,3,4-tetra-*O*-acetyl-6-D-mannose phosphate (pyridinium form) gave, after purification, 934 mg (66%) of the protected phosphodiester 6 (R_f 0.62, CH_2Cl_2–MeOH–H_2O 13:5:1), which was deacylated to give 466 mg (74%) of 7: R_f 0.54 (2-propanol–NH_4OH–H_2O, 7:1:2); HPLC 9.65 min; MS FAB$^-$ *m/e* 508.07 (M - 1); UV (H_2O) 266 nm (9300). Anal. ($C_{16}H_{24}O_{12}N_5PNaH_2O$) C, H, N; P: calcd, 34.97, 4.56, 12.75, 5.64; found, 35.01, 5.00, 12.45, 5.66.

Hexadecyl 6-α-D-Mannopyranosyl 3'-Azido-3'-deoxy-5'-thymidinyl Phosphate (8). The alkylation of 303 mg of the tetrabutylammonium salt of 7 gave 177 mg (60%) of phosphotriester 8: R_f 0.67 (2-propanol–NH_4OH–H_2O, 7:1:2), 0.71 (CH_2Cl_2–CH_3OH–H_2O, 13:5:1); HPLC 13.27 min; MS FAB$^-$ *m/e* 732.54 (M - 1), FAB$^+$ *m/e* 734.00 (M + 1); UV (H_2O) 266 nm (8700). Anal. ($C_{32}H_{56}O_{12}N_5P$) C, H, N; P: calcd, 52.39, 7.64, 9.55, 4.23; found, 52.31, 7.83, 9.53, 4.27.

2-(α-D-Mannopyranosidyl)ethyl 3'-Azido-3'-deoxy-5'-thymidinyl Phosphate (10). A solution of cyanoethyl 2-(2,3,4,6-tetra-*O*-benzoyl α-D-mannopyranosidyl)ethyl phosphate (9)[20] (2382 mg, 2.80 mmol) and AZT (500 mg, 1.872 mmol) in anhydrous pyridine (50 mL) and 1725 mg (2.40 mmol) of TPSNT was stirred for 2 h at room temperature. The solvent was diluted with 500 mL of CH_2Cl_2, washed with a saturated solution of sodium hydrogen carbonate and water, dried, and evaporated to dryness. The residue was dissolved in CH_2Cl_2 and precipitated with petroleum ether; the solid was chromatographed on a silica gel column eluted with first CH_2Cl_2 then with CH_2Cl_2–MeOH 90:10 to yield 934 mg (66%) of phosphodiester (R_f 0.40 (CH_2Cl_2–CH_3OH 90:10) which was deprotected with 100 mL of sodium methoxide (1%) in CH_3OH. After purification on a column of reverse-phase C18 silica gel, phosphodiester 10 was isolated as a white solid (466 mg, 74%): R_f 0.53 (2-propanol NH_4OH–H_2O 7:1:2); HPLC 10.55 min; MS FAB$^-$ *m/e* 552.08 (M - 1); UV (H_2O) 267 nm (10300). Anal. ($C_{18}H_{27}O_{13}N_5PNa2H_2O$) C, H, N; P: calcd, 35.35, 5.07, 11.45, 5.07; found, 35.13, 5.29, 11.24, 5.29.

Hexadecyl 2-(α-D-Mannopyranosidyl)ethyl 3'-Azido-3'-deoxy-5'-thymidinyl Phosphate (11). Phosphotriester 11 was synthesized from compound 10 in 72% yield after purification: R_f 0.69 (2-propanol–NH_4OH–H_2O, 7:1:2); HPLC 13.11 min (54%), 13.49 min (46%); MS FAB$^-$ *m/e* 776.51 (M - 1); UV (H_2O) 266 nm (9000). Anal. ($C_{34}H_{60}O_{13}N_5P$) C, H, N; P: calcd, 52.51, 7.42, 3.99; found, 52.53, 7.62, 8.75, 3.78.

Cells. The CEM-Cl13, a subclone enriched in CD4 receptors, was obtained from the CEM T-lymphoblastoid tumor cell line,[21] originally isolated from a child with acute lymphocytic leukemia.[22] Peripheral blood lymphocytes (PBL), stored in liquid nitrogen, were isolated from heparinized blood of healthy volunteers by Ficoll-gradient centrifugation. 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), streptomycin (100 μg/mL), and polybrene (2 μg/mL) (complete medium). For growth of PBL, complete medium was supplemented with 10% interleukin-2 (Lymphocult-T-LF 20 units/mL) and anti-human α-interferon (1000 units/mL).

Virus. The LAV-Bru strain of HIV-1 was isolated at the Pasteur Institute from lymphocytes of a patient with lymphadenopathy.[23] 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. Then each dilution was mixed with 100 μL of medium containing 1×10^4 CEM Cl13 in 96-well plates. The titers of HIV-1 preparations ranged between 5×10^4 and 5×10^3. Fifty percent cell culture infective dose ($CCID_{50}$/mL) were standardized by using an MTT assay[24,25] and by reverse transcriptase activity (1×10^6 cpm/mL).[24]

Antiviral Assay on Cells. Compounds were tested and compared to AZT (obtained from Sigma) for cytotoxicity and for their ability to inhibit HIV replication. Compounds were first

dissolved at 5 mM in either a DMSO–H_2O (1:99) mixture (4, 8, 11) or H_2O (3, 7, 10). All stock solutions were made in phosphate-buffered saline (PBS) and kept at −30 °C until use. Solutions were thawed and diluted in PBS just before use. The HIV-1-induced CPE was monitored by the MTT viability assay.[24,25] 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 in each well. The cells were adjusted to 1×10^5 cells/mL for CEM-Cl13 or to 1×10^6 cells/mL for PBL and then were plated in each well at the rate of 100 μL per well. Virus suspension (100 μL) was added to cells with or without drugs and cultured for at least 7 days. 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.[24,25] 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 (CD_{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 (ED_{50}). The selectivity index is the ratio CD_{50}/ED_{50}. Longer (>7 days) incubations were carried out by adding 100 μL/well of fresh medium to the remaining cell suspension. From day 7 and every 3 or 4 days, 50 μL of supernatant was removed for an RT microassay. Cells were then resuspended, and 100 μL of cell suspension was removed for the MTT method. Fresh medium, plus or minus drug, was added to obtain a final volume of 250 μL per well. All assays were carried out in triplicate.

Reverse Transcriptase Assays. The effects of compounds on reverse transcriptase activity in vitro were evaluated with HIV-1 as sources of the enzyme. This reaction was performed at 37 °C for 60 min with 100 μL of 12 500 $CCID_{50}$ HIV-1 and 50 μL of compound solution (containing a final concentration of 25 μM of the compounds). After treatment, treated virus aliquots were pelleted by ultracentrifugation (100K for 7 min TL100 Beckman). The pellets were resuspended in 900 μL of complete medium. The reverse transcriptase activity was immediately evaluated on 50 μL of each sample mixed with a 50 μL of reaction mixture (50 mM, KCl, 5 mM dithiothreitol, 0.05% Triton-X100, 0.5 mM EDTA, 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 1 μCi of (methyl-^{3}H)dTTP, and 0.5 OD/mL poly(rA)-oligo(dT_{10}). The plates were incubated for 1 h at 37 °C. The reaction was stopped by the addition of 20 μL of ice-cold 120 mM $Na_4P_2O_7$ in 60% trichloroacetic acid. Radioactivity was collected on glass-fiber filters using a cell harvester apparatus (Skatron). RT activity was expressed in counts/min (cpm).

The resuspended pellet (150 μL) was transferred to microtiter tray wells containing 100 μL of CEM-Cl13 cell suspension (1×10^5 cells/mL). The cultures were incubated at 37 °C for 60 min and centrifugated (800*g* for 10 min; Sorvall IEC). Then 200 μL of supernatant was removed and fresh medium without compounds was added to obtain a final volume of 200 μL. After 7 and 9 days at 37 °C, the viral replication was determined by using (i) the RT microassay on 50 μL of cellular supernatants as described above and (ii) the MTT viability assay (100 μL of cell suspension).

NMR Study in the Presence of Model Membranes (Large Unilamellar Vesicles). (i) Vesicle Preparation. Large unilamellar vesicles (LUV) of defined size were prepared by reverse-phase evaporation[26] using a mixture of egg phosphatidylcholine and phosphatidic acid (mole ratio 9:1). Phosphatidylcholine was extracted from egg yolk according to the method of Singleton et al.[27] Phosphatidic acid was prepared from the former as described.[28] The buffer used was 50 mM pipes–KOH, pH 7.2, supplemented with 100 mM potassium sulfate. The vesicle suspension was sequentially extruded through 200-nm polycarbonate membranes (Nucleopore) in order to obtain a uniform size distribution.[29] The final lipid concentration was about 25 mM. For each experiment, LUV integrity was analyzed by recording a phosphorus NMR spectrum of a blank sample of vesicles.

(ii) NMR Spectra. Small amounts of 20 mM stock solution of each AZT derivative were injected into NMR tubes containing a buffered aqueous solution with or without LUV. The final concentration of the AZT derivatives was 1.1 mM. Phosphorus (36.5 and 121 MHz) NMR spectra were recorded on a Bruker WH 90 and a Bruker MSL 300 by using a Hahn spin-echo sequence with a refocusing delay of 5 (at 36.5 MHz) and 1 ms (at 121 MHz) in order to cancel the signal of membrane phospholipids, the transverse relaxation time of which is less than 2 (at 36.5 MHz) and 0.5 ms (at 121 MHz).

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Registry No. 1·C_5H_5N, 133101-33-4; α-**2**, 133163-36-7; β-**2**, 133163-41-4; α-**3**, 133101-34-5; β-**3**, 133163-42-5; α-(*R*)-**4**, 133101-35-6; α-(*S*)-**4**, 133163-46-9; β-(*R*)-**4**, 133163-43-6; β-(*S*)-**4**, 133163-44-7; **5**·C_5H_5N, 133101-36-7; **6**, 127306-76-7; **7**·Na, 133163-37-8; **7**·Bu_4N, 133267-41-1; (*R*)-**8**, 133163-38-9; (*S*)-**8**, 133163-47-0; **9**, 133163-39-0; **10**, 127306-80-3; **10** tetra-*O*-benzoyl derivative, 133163-45-8; (*R*)-**11**, 133163-40-3; (*S*)-**11**, 133163-48-1; $IC_{16}H_{33}$, 40474-98-4; reverse transcriptase, 9068-38-6.

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