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Phosphoramidate and phosphate prodrugs of (−)-β-D-(2*R*,4*R*)-dioxolane-thymine: Synthesis, anti-HIV activity and stability studies

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Abstract—A series of phosphoramidate and phosphate prodrugs of DOT were synthesized via dichlorophosphate or H-phosphonate chemistry and evaluated for their anti-HIV activity against LAI M184V mutants in PBM cells as well as for their cytotoxicity. The antiviral and cytotoxic profiles of the prodrugs were compared with that of the parent compound (DOT), and it was found that four aryl phosphoramidates **5**, **18**, **20**, and **26** showed a significant enhancement (8- to 12-fold) in anti-HIV activity without cytotoxicity. Chemical stability of these prodrugs was evaluated in phosphate buffer at pH values of biological relevance (i.e., pH 2.0 and 7.4). Enzymatic hydrolysis was also studied in esterase or lipase in buffer solution. Chemical stability studies indicate that the phosphoramidates have good chemical stability at pH 2.0 and at pH 7.4 phosphate buffer. Phosphoramidate prodrugs were hydrolyzed in vitro by esterase or lipase and found to be better substrates for lipases than for esterases. 1,3-Diol cyclic phosphates showed potent anti-HIV activity without increasing the cytotoxicity compared with that of DOT and have good chemical and enzymatic stability. Long-chain lipid phosphates, although showed potent anti-HIV activity, exhibited increased cytotoxicity.

1. Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) have been widely used in the treatment of HIV-1 infection. Their antiviral activity depends on the cellular uptake and conversion to the corresponding nucleoside triphosphates (NTPs) by cellular kinases.[1,2] NTPs act either as inhibitors of viral DNA polymerase or as chain terminators following incorporation into a growing DNA of HIV. Unfortunately, nucleoside kinases often have narrow substrate specificity and are unable to catalyze the initial phosphorylation, that is, conversion of the nucleoside to the nucleoside 5′-monophosphates (NMPs), while the subsequent phosphorylations are not usually rate-limiting. Phosphoramidate triester of nucleoside is a prodrug methodology increasingly being used to bypass the required initial phosphorylation and to improve the pharmacological activity of existing nucleosides.[3,4a] In another potential phosphate prodrug strategy, the phosphate groups are masked with lipophilic groups in order to obtain lipophilic membrane-permeable derivatives and to be able to access intracellular target sites, wherein enzymatic or chemical hydrolysis yields the nucleoside monophosphates.[4b] 1,3-Diol cyclic phosphate prodrugs, known as HepDirect prodrugs, represent a new class of prodrugs for improving nucleoside-based drug therapies.[5]

(−)-β-D-(2*R*,4*R*)-Dioxolane-thymine (DOT) was reported from our laboratory and shows anti-HIV activity against most of the nucleoside resistant HIV-1 mutants.[6a] We carried out molecular modeling studies for DOT and found that dioxolane moiety plays a significant role in stabilizing the binding between the mutant HIV-1 RT and nucleoside triphosphate.[6b] Due to DOT's pronounced activity, we were interested in synthesizing prodrugs of DOT. Accordingly, herein we report aryl and long-chain lipid phosphoramidates, 1,3-diol cyclic phosphates, and long-chain lipid phosphates of DOT with the objective of further enhancing the anti-HIV activity of DOT. We also studied chemical and enzymatic hydrolysis of aryl phosphoramidates and 1,3-diol cyclic phosphates of DOT.

Keywords: Nucleoside; Prodrug; Dioxolane-thymine; Anti-HIV activity.
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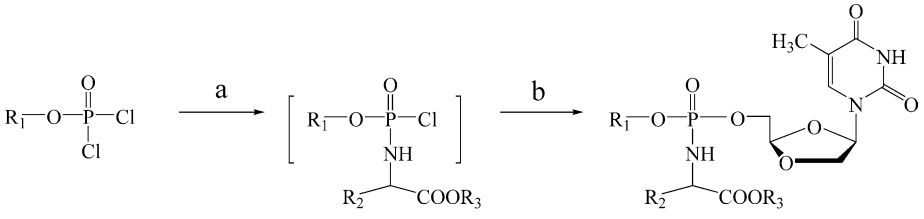
2. Results and discussion

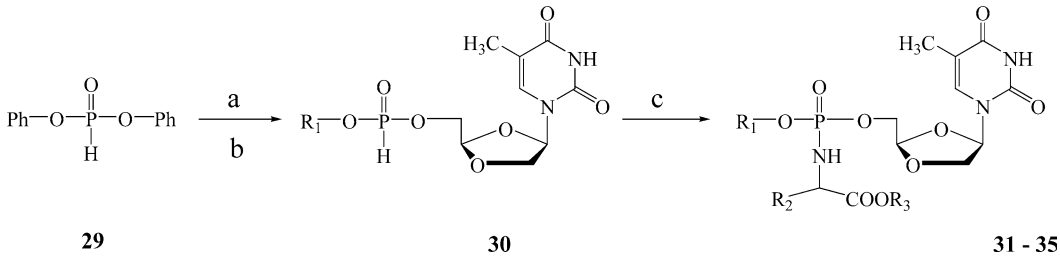
2.1. Chemistry

Aryl phosphoramidates of DOT (**3**–**28**) were synthesized according to the method described by McGuigan et al.[9] as shown in Scheme 1. Thus, phenol or naphthol-derivatives were first reacted with phosphoryl chloride in diethyl ether to give aryl dichlorophosphates (**1**), which were reacted with amino acid alkyl ester hydrochloride in dichloromethane in the presence of triethylamine at −78 °C to yield arylalkoxy-amino acid phosphorochloridates (**2**). These arylalkoxy-amino acid phosphorochloridates (**2**), without further purification, were reacted with DOT in THF in the presence of *N*-methylimidazole to provide the target compounds, aryl phosphoramidates of DOT, in various yields. The chirality at the phosphorus center results in the formation of two diastereomeric phosphoramidates in an approximately 1:1 ratio, which was inseparable by silica gel chromatography. They displayed two closely spaced signals in the ^{31}P NMR and two isomers were also apparent from ^{1}H NMR spectroscopy as well as by HPLC. Variation in the amino acid side chains was studied by the preparation of the glycine (**3**), 2-aminoisobutyric acid (**4**), L- and D-alanine (**5** and **6**), L- and D-valine (**7** and **8**), and L- and D-phenylalanine (**9** and **10**). Variation of the ester group in the amino acids was also studied by synthesizing L-alanine phosphoramidates of *tert*-butyl (**11**) and benzyl esters (**12**, **21**, **23**, and **28**). The structural modification with different substituents of phenyl group was also conducted (**13**–**25**). The long-chain lipid phosphoramidates of DOT (**31**–**35**) were synthesized using the H-phosphonate approach as shown in Scheme 2.[10] The long-chain lipid alcohols used were 3-hexadecyloxy-1-propanol (HDP), 3-octadecyloxy-1-ethanol (ODE), and oleyl alcohol. In order to obtain the phosphoramidates (**31**–**35**), one equivalent of DOT was added to two equivalents of diphenyl phosphite (**29**) in pyridine at 0 °C over a period of 1 h (by this method, the formation of by-product, di-DOT H-phosphonates, could be greatly reduced). Long-chain lipid alcohol was then added to yield the corresponding 5′-O-phospholipids of DOT (**30**). The 5′-O-phospholipids of DOT (**30**) were readily oxidized and produced the corresponding phosphoramidates (**31**–**35**) by reacting with amino acid methyl ester in a mixture of $Et_3N/CCl_4/H_2O/MeCN$ (1/1/1/10, v/v).[11]

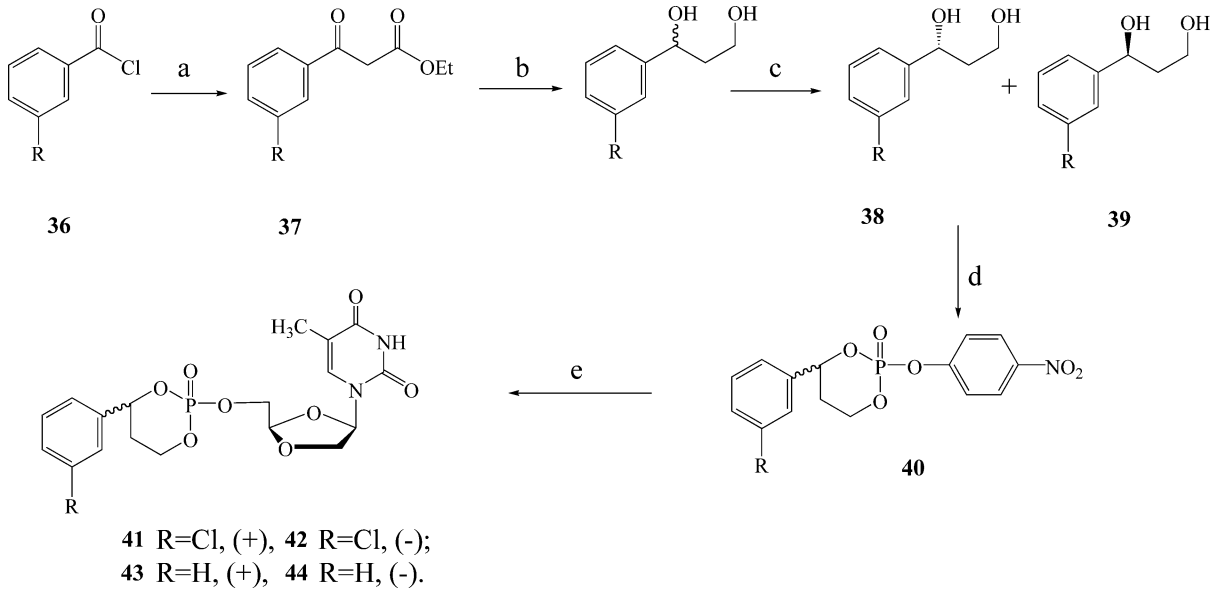
For the synthesis of aryl-1,3-propanediol-cyclic phosphates of DOT (Scheme 3),[12] the corresponding diol enantiomers were first prepared. The benzoyl chloride (**36**) was reacted with potassium ethyl malonate using $MgCl_2$–Et_3N base system and then treated with HCl to give a β-keto ester, 3-phenyl-2-oxo-propionate (**37**). The β-keto ester was reduced by $NaBH_4$ to provide racemic diols. In order to separate the mixture, the racemic diols first reacted with (−)-menthone to form diastereomeric (−)-menthone ketals. The ketals were separated by column chromatography on silica gel and the removal of (−)-menthone yielded the enantiomeric 1,3-propanediol with the high optical purity.[13] Reaction of 1,3-propanediol with *p*-nitrophenyl dichlorophosphate in the presence of 4 equiv of *p*-nitrophenol and Et_3N gave the cyclic phosphate intermediate (**40**). The cyclic phosphate intermediate (**40**) was then reacted with DOT in the presence of *tert*-BuMgCl to give cyclic phosphoramidates with high optical purity (**41**–**44**). Coupling of the phosphate intermediate (**40**) with nucleosides proved to be the most challenging reaction, resulting in poor yields in various reaction conditions. The magnesium salt of the 5′-hydroxyl DOT reacted with the



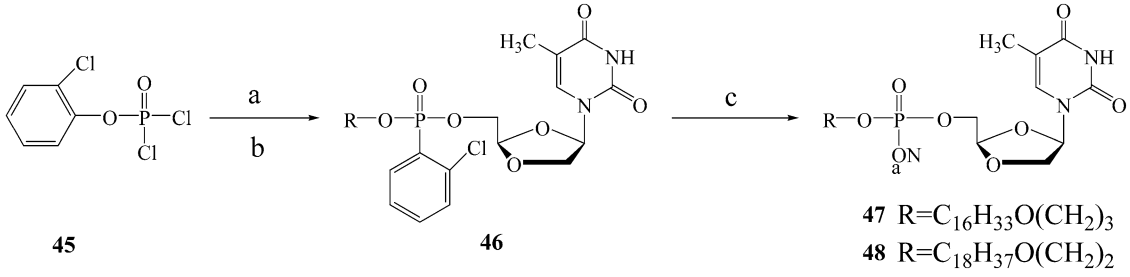
Scheme 1. Synthesis of aryl phosphoramidates. Reagents and conditions: (a) amino acid ester hydrochloride, Et_3N, −78 °C; (b) DOT, *N*-methylimidazole, THF, rt.



Scheme 2. Synthesis of long-chain lipid phosphoramidates. Reagents and conditions: (a) DOT, Py, 0 °C; (b) R_1OH, Py, rt; (c) amino acid methyl ester, $Et_3N/CCl_4/H_2O/MeCN$(1/1/1/10, v/v).



Scheme 3. Synthesis of cyclic phosphates. Reagents and conditions: (a) (i) potassium ethyl malonate, $MgCl_2$, Et_3N; (ii) HC1; (b) $NaBH_4$, MeOH, THF, reflux; (c) (i) HMDS, TMSOTf; (ii) (−)-menthone, TMSOTf; (iii) HC1, MeOH; (d) 4-nitrophenyloxy dichlorophosphate, Et_3N, *p*-nitrophenol; (e) DOT, *tert*-buty MgCl.



Scheme 4. Synthesis of long-chain lipid phosphates. Reagents and conditions: (a) DOT, 1,2,4-triazole; Et_3N, rt; (b) ROH, *N*-methylimidazole, THF, rt; (c) 0.5 N NaOH, THF/H_2O.

phosphate intermediate with inversion of configuration and afforded the prodrugs of DOT in ca. 50% yield.

The long-chain lipid phosphates of DOT (**47** and **48**) were synthesized using the phosphotriester approach as shown in Scheme 4.[14] DOT was reacted with 2-chlorophenyl dichlorophosphate (**45**) in the presence of 1,2,4-triazole and triethylamine to provide the intermediate **46**. Without further purification, the intermediate **46** was treated with long-chain lipid alcohol (3-hexadecyloxy-1-propanol or 2-octadecyloxy-1-ethanol) to give the fully protected phosphotriester, which was isolated by flash chromatography in good yield. The phosphotriester displayed two closely spaced signals in the ^{31}P NMR corresponding to the two diastereoisomers. The presence of diastereoisomers was also apparent from ^{1}H NMR spectroscopy. For the removal of the 2-chlorophenyl groups, the phosphotriester was dissolved in THF and treated with 0.5 N NaOH at 50 °C for 1.5 h to give the target prodrugs. The identity of all the prodrugs was confirmed by electron-spray ionization-mass spectrometry (ESI-MS), ^{1}H NMR, and elemental analysis.

2.2. Anti-HIV activity (SAR studies)

The anti-HIV activity of all prodrugs and the parent nucleoside DOT was evaluated against LAI M184V strain in PBM cells. Cytotoxicity was evaluated in PBM, CEM, and Vero cells. AZT was used as the positive control and the results are summarized in Table 1 and Table 2. The results of aryl phosphoramidates of DOT (**3**–**12**) with the different amino acid methyl esters indicate that changes in the amino acid lead to significant changes in activity (Table 1). Among the series, 2-amino-3-methyl-butyric acid **4** and alanine prodrugs **5** were the most potent, while the other prodrugs being less potent in comparison to DOT. Prodrug **5** was found to be approximately 7.5-fold more potent than DOT. The results are similar to the SAR studies of the phosphoramidates of D4T by McGuigan.[15] In general, the phoshoramidates with L-amino acid methyl ester were more active than those with D-amino acid methyl ester; the prodrug **5**, **7**, and **9** were more active than **6**, **8**, and **10**. The D-valine derivative **10** was the least active of the series, being approximately 12-fold less potent than that of DOT. There was some variation in activity with the

Table 1. Anti-HIV activity and cytotoxicity of phosphoramidates and long-chain lipid phosphoramidates

Compound	R_1	R_2	R_3	M184V(μM)[a]		Cytotoxicity (μM)		
				EC_{50}	EC_{90}	PBM	CEM	Vero
3	Phenyl	H	CH_3	2.2	14.2	>100	>100	>100
4	Phenyl	$(CH_3)_2$	CH_3	0.62	3.3	>100	>100	>100
5	Phenyl	(L)CH_3	CH_3	0.2	1.2	>100	39.2	>100
6	Phenyl	(D)CH_3	CH_3	4.5	38.6	>100	>100	>100
7	Phenyl	(L)$PhCH_2$	CH_3	1.9	8.4	>100	>100	>100
8	Phenyl	(D)$PhCH_2$	CH_3	13.7	49.9	40.1	49.2	59.3
9	Phenyl	(L)$(CH_3)_2CH$	CH_3	9.6	37.4	>100	>100	>100
10	Phenyl	(D)$(CH_3)_2CH$	CH_3	18.0	82.8	>100	>100	>100
11	Phenyl	(L)CH_3	$C(CH_3)_3$	24.8	>100	>100	>100	58.2
12	Phenyl	(L)CH_3	CH_2Ph	1.08	4.6	>100	>100	>100
13	4-Methylphenyl	(L)CH_3	CH_3	0.61	3.4	74.3	63.8	>100
14	4-Propylphenyl	(L)CH_3	CH_3	0.2	1.6	25.6	13.5	36.6
15	4-Neopentylphenyl	(L)CH_3	CH_3	0.99	4.7	15.4	43.5	91.5
16	4-Methoxyphenyl	(L)CH_3	CH_3	0.56	2.6	21.1	>100	>100
17	4-Cyanophenyl	(L)CH_3	CH_3	0.54	1.6	>100	>100	>100
18	4-Chlorophenyl	(L)CH_3	CH_3	0.18	0.62	78.4	>100	>100
19	4-Fluorophenyl	(L)CH_3	CH_3	1.7	7.3	>100	>100	>100
20	4-Bromophenyl	(L)CH_3	CH_3	0.15	1.4	32.0	>100	>100
21	4-Bromophenyl	(L)CH_3	CH_2Ph	1.9	8.9	>100	15.7	84.7
22	2-Chlorophenyl	(L)CH_3	CH_3	0.53	2.6	>100	>100	>100
23	2-Chlorophenyl	(L)CH_3	CH_2Ph	0.57	4.7	>100	77.1	>100
24	2-Allylphenyl	(L)CH_3	CH_3	3.3	12.9	>100	24.3	>100
25	2,4-Dichlorophenyl	(L)CH_3	CH_3	0.85	2.8	>100	>100	>100
26	Naphthyl	(L)CH_3	CH_3	0.14	0.64	>100	>100	>100
27	Naphthyl	$(CH_3)_2$	CH_3	0.38	2.6	>100	>100	>100
28	Naphthyl	(L)CH_3	CH_2Ph	2.2	11.6	>100	22.2	>100
31	$C_{16}H_{33}O(CH_2)_3$	H	CH_3	11.9	80.5	>100	9.31	13.71
32	$C_{16}H_{33}O(CH_2)_3$	(L)CH_3	CH_3	2.7	15.0	19.7	4.8	8.1
33	$C_{16}H_{33}O(CH_2)_3$	(L)$(CH_3)_2CH$	CH_3	2.8	13.6	10.7	2.42	4.96
34	$C_{18}H_{37}O(CH_2)_2$	(L)CH_3	CH_3	4.4	35.1	61.4	15.7	33.6
35	Oleyl	(L)CH_3	CH_3	4.9	23.7	31.3	3.7	11.4
DOT				1.5	8.8	>100	>100	>100

[a] LAI M184V mutants in PBM cells unless otherwise indicated.

Table 2. Anti-HIV activity and cytotoxicity of cyclic phosphates and long-chain lipid phosphates

Compound	M184V (μM)[a]		Cytotoxicity (μM)		
	EC_{50}	EC_{90}	PBM	CEM	Vero
41	1.8	55.9	>100	>100	>100
42	2.0	40	>100	>100	>100
43	0.48	10.1	>100	>100	>100
44	3.2	22.7	>100	>100	>100
47	2.0	15.4	66.19	9.83	23.63
48	0.3	3.6	15.04	3.91	10.08
DOT	1.5	8.8	>100	>100	>100

[a] LAI M184V mutants in PBM cells unless otherwise indicated.

benzyl or *tert*-butyl group instead of the methyl group for alanine prodrugs **11** and **12**. The alanyl-benzyl ester prodrug **12** exhibited increased activity with an IC_{50} value of 1.08 μM, while alanyl-*tert*-butyl ester prodrug **11** showed decreased activity with an IC_{50} value of 24.8 μM in comparison to DOT. These prodrugs, except for D-phenylalanine prodrug **8**, did not show any increased cytotoxicity. After having completed the alanyl-methyl- and alanyl-benzyl- and 2-amino-3-methylbutryic methyl ester of the phosphoramidates of DOT, the phosphoramidate series from **13** to **25** with varying substitutions in the aryloxy moiety were synthesized and their antiviral activity and cytotoxicity were evaluated. Similarly, there were some variations in activity and toxicity. Except for the 4-fluorophenyl-**19** and 2-allylphenyl-prodrug **24**, which were 2-fold less potent than DOT, all other prodrugs showed increased antiviral activity with an IC_{50} value ranging from 0.15 to 0.99 μM. 4-Bromophenyl prodrug **20** was found to be very active, approximately 10-fold more potent than DOT. 4-Chlorophenyl prodrug **18** was also found to be highly potent and showed an 8-fold increase in the EC_{50} and the 14-fold enhancement in the EC_{90} value in comparison to DOT. Introduction of the lipophilic substituent in the aryloxy moiety (**13**–**15**), although led to increased activity, resulted in enhanced cytotoxicity. However, introduction of the electron-withdrawing substituents in the aryloxy moiety (**16**–**25**) did not result in increased cytotoxicity.

It is reported that, naphthyl phosphoramidates of nucleosides can increase the anticancer activity. 1-Naphthyloxy L-alanine benzyl ester phosphoramidate of BVdU showed a 100-fold increase in activity against prostate cancer when compared to the parent drug BvdU.[16] Thus, three naphthyl phosphoramidates were synthesized (**26**, **27**, and **28**), and their anti-HIV activity and cytotoxicity were investigated (Table 1). All three prodrugs showed increased antiviral activity and did

not show any enhanced cytotoxicity in comparison to DOT. Prodrugs **26** and **28** showed 11-fold and **27** exhibited 4-fold enhanced activity when compared with DOT. Long-chain lipid phosphoramidates **31**–**35** exhibited the decreased activity and the increased cytotoxicity. Prodrug **31** showed the most decreased activity, being 8-fold less potent than DOT. Thus, the long-chain lipid phosphoramidates of DOT should not be considered as the prodrugs of DOT. Among the aryl phosphoramidates of DOT, compounds **5**, **18**, **20**, and **26** emerged as the most potent inhibitors of HIV in PBM cells. The structure–activity relationship studies suggest that the decreasing order of activity is phenyl = substituted phenyl = naphthyl > alkyl. For amino acids, L-alanine methyl ester seems to be the most active. The results are in consistent with the results of the phosphoramidates of AZT by McGuigan.[17]

Cyclic phosphate prodrugs are known to be highly stable in plasma and tissue.[18a] These prodrugs undergo an oxidative cleavage catalyzed reaction by a cytochrome P_{450} (CYP) expressed in the liver. They are capable of bypassing the rate-limiting step of nucleoside kinase in vivo and generating high levels of liver NTP.[18b] All synthesized cyclic phosphates of DOT exhibited potent anti-HIV activity (Table 2). Prodrug **43** showed the most increased activity, being 3-fold more potent than DOT, while others showed a slightly decreased activity. The anti-HIV activity and cytotoxicity of long-chain lipid phosphates of DOT (**47** and **48**) are shown in Table 2. According to the study by Hostetler and co-workers,[19] the addition of an alkoxyalkyl ester group to the phosphonate of cidofovir or cyclic cidofovir resulted in a remarkable increase in antiviral activity against CMV and HSV-1 in vitro. In the case of DOT, the cytotoxicity of the analogs was also increased. The long-chain lipid phosphates of DOT, however, were not so effective. Prodrug **47** showed decreased activity compared with that of DOT. Although the prodrug **48** showed increased antiviral activity as well as cytotoxicity thus, decreasing the selectivity. It was found that the introduction of the long-chain lipids led DOT to be more cytotoxic in vitro in its phosphoramidate or phosphate form.

2.3. Hydrolysis

Documented work strongly suggests that the carboxyl ester cleavage is a necessary prerequisite for the activation of the phosphoramidate prodrugs to parent nucleosides for antiviral activity.[20] The role of esterase in the activation step of the prodrugs was confirmed in this study. However, in vivo the prodrugs can reach the target cells only if they are resistant to hydrolysis by extracellular and intracellular carboxylesterases during the absorption and distribution process. Partial conversion of the prodrugs to the intermediates or free nucleosides would almost certainly result in a lower cell penetration and reduce antiviral potency. Thus, in vitro chemical and enzymatic hydrolysis of representative phosphoramidates and cyclic phosphates was investigated. Chemical hydrolysis was studied under experimental conditions of biological relevance, i.e., at pH 2.0 and pH 7.4 at 37 °C. Enzymatic hydrolysis was studied at pH 8.0, phosphate buffer at 37 °C (Table 3). The studies indicated that they are stable both in acidic and neutral media. The decomposition by hydrolysis was less than 8% over 24 h. These phosphoramidates were also stable to esterase. McGuigan et al.[21] demonstrated that phosphoramidates of AZT with electron-withdrawing substituents on the phenyl ring could increase the anti-HIV activity, being associated with the possibility that electron-withdrawing substituents can increase the rate of hydrolysis. For the phosphoramidates of DOT, however, no clear correlation was found between electron-donating (*p*-Me) or electron-withdrawing (*p*-Cl, *p*-Br) substituents on the phenyl ring. Uckun et al.[22] found that halogen-substituted stavudine phosphoramidates could be activated by lipase-mediated hydrolysis. In our studies, we noticed that the phosphoramidates of DOT were less stable in the presence of lipases than in esterases, suggesting that lipase promotes the activation of the parent drug DOT. The phosphoramidates of DOT were better substrates for lipases than for esterases. The hydrolysis by lipases was 15–70% over 24 h, and hydrolysis by esterases was 6–25% over 24 h. It was also observed that the anti-HIV activity of phosphoramadates of DOT does not correlate with the hydrolytic rate of lipase hydrolysis. Two cyclic phosphates (**41** and **42**) showed good chemical stability at pH 2.0 and at pH 7.4, and they also exhibited good enzymatic stability to esterase and lipase (Table 3). The decomposition was 0.8–4.6% over 24 h in chemical or enzymatic media.

Table 3. Chemical and enzymatic hydrolysis recovery (%) of phosphoramidates and 1,3-diol cyclic phosphates

Compound	Chemical hydrolysis[a]		Enzymatic hydrolysis[b]	
	pH 2.0	pH 7.4	Esterase	Lipase
5	95.1	98.8	87.7	77.8
7	96.5	98.3	75.3	29.6
11	96.3	99.4	86.2	84.5
13	93.9	98.4	94.1	32.4
16	97.4	99.7	94.2	45.6
18	98.7	99.4	77.5	37.8
20	97.3	99.8	79.1	40.5
21	96.3	98.8	86.4	36.7
22	95.7	98.4	86.3	36.1
26	92.1	97.2	87.4	68.4
27	94.3	98.1	88.7	47.8
28	96.5	98.6	77.3	66.7
41	98.6	98.3	96.5	95.4
42	99.2	98.5	97.6	96.4

[a] pH 2.0 and pH 7.4 phosphate buffer at 37 °C.
[b] pH 8.0 buffer solution of esterase or lipase at 37 °C.

In conclusion, the synthesized aryl phosphoramidate and cyclic phosphate prodrugs of DOT appear to have advantages in antiviral therapy over the parent nucleoside. In some cases, newly synthesized prodrugs exhibited a significantly increased anti-HIV activity with increased selectivity. They also exhibited good chemical and enzymatic stability. Thus, suggesting that both phosphoramidates and cyclic phosphates of DOT may have improved pharmacological activity in vivo.

3. Materials and methods

3.1. Chemistry

DOT was synthesized in our laboratory as described before.[8] Bis-dPEG$_8$ acid was purchased from Quanta BioDesign, Ltd (Powell, Ohio). Porcine esterases and lipases (from Candida rugosa) enzyme and other chemicals were obtained from Sigma Chemical Company (St. Louis, MO) and Advanced Chem. Tech. (Louisville, KY). Melting points were determined with a Mel-temp II apparatus and are uncorrected. TLC was performed on a Uniplate (silica gel) purchased from Analtech Co. Column chromatography was performed using either silica gel-60 (220–240 mesh) for flash chromatography or silica gel G (TLC grade, >440 mesh) for vacuum flash chromatography. NMR spectra were recorded on a Varian Inova 500 spectrometer at 500 MHz with software VNMRC6-1 with Me_4Si as the internal standard. Chemical shifts (δ) are reported as s (single), d (doublet), t (triplet), q (quartet), m (multiplet) or b (broad). UV spectra were recorded on a Beckman DU-650 spectrophotometer. Optical rotations were measured on a Jasco DIP-370 digital polarimeter. Mass spectra were measured on a Micromass Autospec mass spectrometer. Elemental analyses were performed by Atlantic Micro lab, Inc. (Norcross, GA). HPLCs were performed using Waters 2996 instrument manufactured by Waters Corporation with an analytical Symmetry C_{18} column (4.6×75 mm) and acetonitrile/water mixture as eluent.

3.1.1. General procedure for the synthesis of aryl phosphoramidates. A solution of triethylamine (9.48 mmol) in anhydrous dichloromethane (25 mL) was added dropwise with vigorous stirring to a solution of amino acid ester hydrochloride (4.74 mmol) and phenyl phosphorodichloride (4.74 mmol) in dichloromethane (20 mL) at −78 °C over a period of 2 h. The reaction temperature was slowly raised to room temperature and stirred for another 6 h. The solvent was removed under reduced pressure and solid residue was dissolved in diethyl ether (20 mL) and filtered. The filtrate was evaporated in vacuum at room temperature to yield the phosphorochloromidates as a colorless oil. Without further purification, the colorless oil was dissolved in 2 mL of anhydrous THF and added to the reaction mixture of DOT (4.74 mmol) in THF (2 mL) and *N*-methylimidazole (7.35 mmol) with vigorous stirring. The mixture was stirred at room temperature for 8 h. The solvent was removed under reduced pressure. The residue was dissolved in dichloromethane (15 mL) and washed with 1 N HCl solution (2×15 mL), saturated $NaHCO_3$ aqueous solution (2×10 mL), water (3×10 mL), and brine (2×20 mL), dried over Na_2SO_4, and evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel by elution with 2–12% methanol in dichloromethane to yield aryl phosphoramidates (**3**–**28**) as white solids.

3.1.2. Dioxolane-thymine 5′-(phenyl methoxy-glycinyl phosphate) (3). Yield 85%. Mp: 147.5 °C. ^{31}P NMR ($CDCl_3$): δ 2.1, 1.8 (1:1); ^{1}H NMR ($CDCl_3$): δ 1.91 (s, 3H, 5-Me), 3.72 (s, 3H, OCH_3), 3.77 (m, 2H, Gly-CH_2), 4.03 (m, 1H, NH), 4.22 (m, 2H, H5′), 4.40 (m, 2H, H2′), 5.18 (s, 1H, H4′), 6.41 (m, 1H, H1′), 7.14–7.35 (m, 5H, Ph), 7.46 (s, 1H, H6), 9.80 (s, 1H, H3). MS: *m*/*z* 469 $(M + 1)^+$. Anal. Calcd for $C_{19}H_{24}N_3O_9P$: C, 47.48; H, 4.87; N, 9.23. Found: C, 47.44; H, 5.10; N, 9.11.

3.2. Dioxolane-thymine 5′-(phenyl methoxy-2-aminoisobutyric phosphate) (4)

Yield 83%. Mp: 146.3 °C. ^{31}P NMR ($CDCl_3$): δ 3.0, 2.8 (1:0.6); ^{1}H NMR ($CDCl_3$): δ 1.51 and 1.57 (s, 6H, $(CH_3)_2C$), 1.91 (s, 3H, 5-Me), 3.70 (s, 3H, OCH_3), 4.08 (m, 1H, NH), 4.20 (m, 2H, H5′), 4.38 (m, 2H, H2′), 5.18 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.12–7.35 (m, 5H, Ph), 7.43 (s, 1H, H6), 8.24 (s, 1H, H3). MS: *m*/*z* 483 $(M + 1)^+$. Anal. Calcd for $C_{20}H_{26}N_3O_9P$ 0.3%H_2O: C, 49.14; H, 5.48; N, 8.60. Found: C, 48.77; H, 5.38; N, 8.33.

3.3. Dioxolane-thymine 5′-(phenyl methoxy-L-alanyl phosphate) (5)

Yield 81%. Mp: 145.2 °C. ^{31}P NMR ($CDCl_3$): δ 3.3, 3.0 (1:0.3); ^{1}H NMR ($CDCl_3$): δ 1.30–1.39 (d, 3H, Ala-CH_3), 1.91 (s, 3H, 5-Me), 3.66 (m, 1H, Ala-CH), 3.74 (s, 3H, OCH_3), 4.02 (m, 1H, NH), 4.20 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.18 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.12–7.35 (m, 5H, Ph), 7.43 (s, 1H, H6), 8.24 (s, 1H, H3). MS: *m*/*z* 469 $(M + 1)^+$. Anal. Calcd for $C_{19}H_{24}N_3O_9P$: C, 48.62; H, 5.15; N, 8.95. Found: C, 48.67; H, 5.24; N, 8.86.

3.4. Dioxolane-thymine 5′-(phenyl methoxy-D-alanyl phosphate) (6)

Yield 78%. Mp:145.3 °C. ^{31}P NMR ($CDCl_3$): δ 3.1, 3.0 (1:0.8); ^{1}H NMR ($CDCl_3$): δ 1.30–1.39 (d, 3H, Ala-CH_3), 1.91 (s, 3H, 5-Me), 3.66 (m, 1H, Ala-CH), 3.74 (s, 3H, OCH_3), 4.02 (m, 1H, NH), 4.20 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.18 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.12–7.35 (m, 5H, Ph), 7.43 (s, 1H, H6), 8.24 (s, 1H, H3). MS: *m*/*z* 469 $(M + 1)^+$. Anal. Calcd for $C_{19}H_{24}N_3O_9P$: C, 48.62; H, 5.15; N, 8.95. Found: C, 48.73; H, 5.18; N, 8.81.

3.5. Dioxolane-thymine 5′-(phenyl methoxy-L-phenylalanyl phosphate) (7)

Yield 84%. Mp: 147.1 °C. ^{31}P NMR ($CDCl_3$): δ 3.1, 3.0 (1:0.7); ^{1}H NMR ($CDCl_3$): δ 1.91 (s, 3H, 5-Me), 3.02 (m, 2H, Ph-CH_2), 3.46 (m, 1H, Ph-CH), 3.64 (s, 3H, OCH_3), 4.05 (m, 1H, NH), 4.37 (m, 4H, H5′ and H2′), 5.07 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.12–7.35 (m, 10H, Ph), 7.43 (s, 1H, H6), 8.24 (s, 1H, H3). MS: *m*/*z* 546 $(M + 1)^+$. Anal. Calcd for $C_{25}H_{28}N_3O_9P$: C, 55.05; H, 5.17; N, 7.70. Found: C, 55.27; H, 5.31; N, 7.82.

3.6. Dioxolane-thymine 5′-(phenyl methoxy-D-phenylalanyl phosphate) (8)

Yield 88%. Mp: 147.3 °C. ^{31}P NMR ($CDCl_3$): δ 3.4, 2.9 (1:0.9); ^{1}H NMR ($CDCl_3$): δ 1.91 (s, 3H, 5-Me), 3.02 (m, 2H, Ph-CH_2), 3.46 (m, 1H, Ph-CH), 3.64 (s, 3H, OCH_3), 4.05 (m, 1H, NH), 4.37 (m, 4H, H5′ and H2′), 5.07 (s,

1H, H4′), 6.39 (m, 1H, H1′), 7.12–7.35 (m, 10H, Ph), 7.41 (s, 1H, H6), 8.04 (s, 1H, H3). MS: *m/z* 546 $(M + 1)^+$. Anal. Calcd for $C_{25}H_{28}N_3O_9P$: C, 55.05; H, 5.17; N, 7.70. Found: C, 54.69; H, 5.29; N, 8.40.

3.7. Dioxolane-thymine 5′-(phenyl methoxy-L-valyl phosphate) (9)

Yield 87%. Mp: 141.2 °C. ^{31}P NMR ($CDCl_3$): δ 3.4, 3.1 (1:0.5); ^1H NMR ($CDCl_3$): δ 1.20–1.28 (d, 6H, Val-CH_3), 1.91 (s, 3H, 5-Me), 2.81 (m, 1H, Val-CH), 3.66 (m, 1H, Val-CH), 3.74 (s, 3H, OCH_3), 4.02 (m, 1H, NH), 4.20 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.18 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.12–7.35 (m, 5H, Ph), 7.43 (s, 1H, H6), 8.24 (s, 1H, H3). MS: *m/z* 497 $(M + 1)^+$. Anal. Calcd for $C_{21}H_{28}N_3O_9P$: C, 50.70; H, 5.67; N, 8.45. Found: C, 50.60; H, 5.85; N, 8.23.

3.8. Dioxolane-thymine 5′-(phenyl methoxy-D-valyl phosphate) (10)

Yield 85%. Mp: 141.5 °C. ^{31}P NMR ($CDCl_3$): δ 3.3, 2.8 (1:0.6); ^1H NMR ($CDCl_3$): δ 1.20–1.28 (d, 6H, Val-CH_3), 1.91 (s, 3H, 5-Me), 2.81 (m, 1H, Val-CH), 3.66 (m, 1H, Val-CH), 3.74 (s, 3H, OCH_3), 4.02 (m, 1H, NH), 4.20 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.18 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.12–7.35 (m, 5H, Ph), 7.43 (s, 1H, H6), 8.24 (s, 1H, H3). MS: *m/z* 497 $(M + 1)^+$. Anal. Calcd for $C_{21}H_{28}N_3O_9P$: C, 50.70; H, 5.67; N, 8.45. Found: C, 50.74; H, 5.75; N, 8.20.

3.9. Dioxolane-thymine 5′-(phenyl isobutyloxy-L-alayl phosphate) (11)

Yield 86%. Mp: 141. 3 °C. ^{31}P NMR ($CDCl_3$): δ 3.1, 2.9 (1:1); ^1H NMR ($CDCl_3$): δ 1.30–1.39 (d, 3H, Ala-CH_3), 1.41 (s, 9H, $C(CH_3)_3$), 1.91 (s, 3H, 5-Me), 3.66 (m, 1H, Ala-CH), 4.02 (m, 1H, NH), 4.20 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.18 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.12–7.35 (m, 5H, Ph), 7.43 (s, 1H, H6), 8.24 (s, 1H, H3). MS: *m/z* 511 $(M + 1)^+$. Anal. Calcd for $C_{22}H_{30}N_3O_9P$: C, 51.66; H, 5.91; N, 8.22. Found: C, 51.38; H, 5.75; N, 7.99.

3.10. Dioxolane-thymine 5′-(phenyl benzyloxy-L-alayl phosphate) (12)

Yield 84%. Mp: 128.2 °C. ^{31}P NMR ($CDCl_3$): δ 3.1, 2.7 (1:0.5); ^1H NMR ($CDCl_3$): δ 1.30–1.39 (d, 3H, Ala-CH_3), 1.91 (s, 3H, 5-Me), 3.66 (m, 1H, Ala-CH), 4.02 (m, 1H, NH), 4.20 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.12 (s, 1H, H4′), 5.17 (m, 2H, $PhCH_2O$), 6.39 (m, 1H, H1′), 7.12–7.35 (m, 10H, Ph), 7.43 (s, 1H, H6), 8.34 (s, 1H, H3). MS: *m/z* 545 $(M + 1)^+$. Anal. Calcd for $C_{25}H_{28}N_3O_9P$: C, 55.05; H, 5.17; N, 7.70. Found: C, 54.93; H, 5.21; N, 7.30.

3.11. Dioxolane-thymine 5′-(4-methylphenyl methoxy-L-alanyl phosphate) (13)

Yield 68%. Mp: 138.0 °C. ^{31}P NMR ($CDCl_3$): δ 3.2, 2.9 (1:0.6); ^1H NMR ($CDCl_3$): δ 1.30–1.42 (d, 3H, Ala-CH_3), 1.91 (s, 3H, 5-Me), 2.33 (m, 3H, CH_3Ph), 3.64 (s, 3H, OCH_3), 3.78 (m, 1H, Ala-CH), 4.04 (m, 1H, NH), 4.20 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.18 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.08–7.31 (m, 4H, Ph), 7.43 (s, 1H, H6), 8.84 (s, 1H, H3). MS: *m/z* 483 $(M + 1)^+$. Anal. Calcd for $C_{20}H_{26}N_3O_9P$: C, 49.69; H, 5.42; N, 8.69. Found: C, 49.59; H, 5.47; N, 8.64.

3.12. Dioxolane-thymine 5′-(4-propylphenyl methoxy-L-alanyl phosphate) (14)

Yield 61%. Mp: 135.5 °C. ^{31}P NMR ($CDCl_3$): δ 3.2, 2.9 (1:0.5); ^1H NMR ($CDCl_3$): δ 0.91 (m, 3H, CH_3), 1.30–1.42 (d, 3H, Ala-CH_3), 1.61 (m, 2H, CH_2), 1.91 (s, 3H, 5-Me), 3.69 (s, 3H, OCH_3), 3.78 (m, 1H, Ala-CH), 4.05 (m, 1H, NH), 4.20 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.18 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.08–7.31 (m, 4H, Ph), 7.43 (s, 1H, H6), 8.81 (s, 1H, H3). MS: *m/z* 511 $(M + 1)^+$. Anal. Calcd for $C_{22}H_{30}N_3O_9P$: C, 51.66; H, 5.91; N, 8.22. Found: C, 51.36; H, 5.99; N, 7.98.

3.13. Dioxolane-thymine 5′-(4-neopentylphenyl methoxy-L-alanyl phosphate) (15)

Yield 55%. Mp: 134.1 °C. ^{31}P NMR ($CDCl_3$): δ 3.2, 2.9 (1:0.5); ^1H NMR ($CDCl_3$): δ 0.86 and 1.45–1.64 (m, 11H, neopentyl-CH_2 and CH_3), 1.30–1.42 (d, 3H, Ala-CH_3), 1.91 (s, 3H, 5-Me), 3.69 (s, 3H, OCH_3), 3.78 (m, 1H, Ala-CH), 4.05 (m, 1H, NH), 4.20 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.18 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.08–7.28 (m, 4H, Ph), 7.43 (s, 1H, H6), 8.85 (s, 1H, H3). MS: *m/z* 539 $(M + 1)^+$. Anal. Calcd for $C_{24}H_{34}N_3O_9P$: C, 53.43; H, 6.35; N, 7.79. Found: C, 53.53; H, 6.30; N, 7.62.

3.14. Dioxolane-thymine 5′-(4-methyoxyphenyl methoxy-L-alanyl phosphate) (16)

Yield 62%. Mp: 142.5 °C. ^{31}P NMR ($CDCl_3$): δ 3.4, 13.2 (1:0.7); ^1H NMR ($CDCl_3$): δ 1.28–1.40 (d, 3H, Ala-CH_3), 1.91 (s, 3H, 5-Me), 3.64 (s, 3H, OCH_3), 3.71 (s, 3H, CH_3OPh), 3.78 (m, 1H, Ala-CH), 4.04 (m, 1H, NH), 4.20 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.08 (s, 1H, H4′), 6.41 (m, 1H, H1′), 6.83 and 7.15 (m, 4H, Ph), 7.45 (s, 1H, H6), 8.96 (s, 1H, H3). MS: *m/z* 499 $(M + 1)^+$. Anal. Calcd for $C_{20}H_{26}N_3O_{10}P$: C, 48.10; H, 5.25; N, 8.41. Found: C, 47.93; H, 5.34; N, 8.34.

3.15. Dioxolane-thymine 5′-(4-cyanophenyl methoxy-L-alanyl phosphate) (17)

Yield 56%. Mp: 178.9 °C. ^{31}P NMR ($CDCl_3$): δ 3.3, 3.0 (1:1.1); ^1H NMR ($CDCl_3$): δ 1.33–1.40 (d, 3H, Ala-CH_3), 1.90 (s, 3H, 5-Me), 3.61 (m, 1H, Ala-CH), 3.72 (s, 3H, OCH_3), 4.05 (m, 1H, NH), 4.20 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.18 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.01–7.19 (m, 4H, Ph), 7.43 (s, 1H, H6), 8.15 (s, 1H, H3). MS: *m/z* 494 $(M + 1)^+$. Anal. Calcd for $C_{20}H_{23}N_4O_9P$: C, 48.59; H, 4.69; N, 11.33. Found: C, 48.86; H, 4.52; N, 11.25.

3.16. Dioxolane-thymine 5′-(4-chlorophenyl methoxy-L-alanyl phosphate) (18)

Yield 58%. Mp: 153.5 °C. ^{31}P NMR ($CDCl_3$): δ 3.1, 2.8 (1:0.5); ^1H NMR ($CDCl_3$): δ 1.36–1.38 (d, 3H, Ala-

CH_3), 1.86 (s, 3H, 5-Me), 3.61 (m, 1H, Ala-CH), 3.73 (s, 3H, OCH_3), 4.05 (m, 1H, NH), 4.21 (m, 2H, H5′), 4.40 (m, 2H, H2′), 5.19 (s, 1H, H4′), 6.42 (m, 1H, H1′), 7.18–7.29 (m, 4H, Ph), 7.43 (s, 1H, H6), 8.35 (s, 1H, H3). MS: *m/z* 503 $(M + 1)^+$. Anal. Calcd for $C_{19}H_{23}ClN_3O_9P$: C, 45.29; H, 4.60; N, 8.34. Found: C, 45.55; H, 4.67; N, 8.30.

3.17. Dioxolane-thymine 5′-(4-fluorophenyl methoxy-L-alanyl phosphate) (19)

Yield 63%. Mp: 141.3 °C. ^{31}P NMR ($CDCl_3$): δ 2.9, 2.5 (1:1); ^1H NMR ($CDCl_3$): δ 1.36–1.38 (d, 3H, Ala-CH_3), 1.86 (s, 3H, 5-Me), 3.73 (s, 3H, OCH_3), 3.97 (m, 1H, Ala-CH), 4.05 (m, 1H, NH), 4.21 (m, 2H, H5′), 4.40 (m, 2H, H2′), 5.19 (s, 1H, H4′), 6.42 (m, 1H, H1′), 7.25–7.42 (m, 4H, Ph), 7.63 (s, 1H, H6), 9.08 (s, 1H, H3). MS: *m/z* 487 $(M + 1)^+$. Anal. Calcd for $C_{19}H_{23}FN_3O_9P$: C, 46.82; H, 4.76; N, 8.62. Found: C, 47.10; H, 4.52; N, 8.89.

3.18. Dioxolane-thymine 5′-(4-bromophenyl methoxy-L-alanyl phosphate) (20)

Yield 58%. Mp: 176.5 °C. ^{31}P NMR ($CDCl_3$): δ 3.0, 2.7 (1:0.5); ^1H NMR ($CDCl_3$): δ 1.36–1.38 (d, 3H, Ala-CH_3), 1.86 (s, 3H, 5-Me), 3.63 (m, 1H, Ala-CH), 3.73 (s, 3H, OCH_3), 4.05 (m, 1H, NH), 4.21 (m, 2H, H5′), 4.40 (m, 2H, H2′), 5.19 (s, 1H, H4′), 6.42 (m, 1H, H1′), 7.08–7.42 (m, 4H, Ph), 7.43 (s, 1H, H6), 7.96 (s, 1H, H3). MS: *m/z* 547 $(M + 1)^+$. Anal. Calcd for $C_{19}H_{23}BrN_3O_9P$: C, 41.62; H, 4.23; N, 7.66. Found: C, 41.86; H, 4.29; N, 7.59.

3.19. Dioxolane-thymine 5′-(4-bromophenyl benzyloxy-L-alanyl phosphate) (21)

Yield 58%. Mp: 142.1 °C. ^{31}P NMR ($CDCl_3$): δ 3.4, 2.9 (1:0.8); ^1H NMR ($CDCl_3$): δ 1.36–1.38 (d, 3H, Ala-CH_3), 1.88 (s, 3H, 5-Me), 3.68 (m, 1H, Ala-CH), 4.05 (m, 1H, NH), 4.21 (m, 2H, H5′), 4.40 (m, 2H, H2′), 5.15 (s, 1H, H4′), 5.19 (m, 2H, $PhCH_2$), 6.42 (m, 1H, H1′), 7.08–7.42 (m, 9H, Ph), 7.43 (s, 1H, H6), 7.96 (s, 1H, H3). MS: *m/z* 624 $(M + 1)^+$. Anal. Calcd for $C_{25}H_{27}BrN_3O_9P$: C, 48.09; H, 4.36; N, 6.73. Found: C, 48.33; H, 4.41; N, 6.55.

3.20. Dioxolane-thymine 5′-(2-chlorophenyl methoxy-L-alanyl phosphate) (22)

Yield 78%. Mp: 149.5 °C. ^{31}P NMR ($CDCl_3$): δ 3.4, 3.2 (1:0.9); ^1H NMR ($CDCl_3$): δ 1.32–1.41 (d, 3H, Ala-CH_3), 1.85 (s, 3H, 5-Me), 3.68 (m, 1H, Ala-CH), 3.73 (s, 3H, OCH_3), 4.09 (m, 1H, NH), 4.21 (m, 2H, H5′), 4.43 (m, 2H, H2′), 5.19 (s, 1H, H4′), 6.41 (m, 1H, H1′), 7.12–7.42 (m, 4H, Ph), 7.44 (s, 1H, H6), 7.86 (s, 1H, H3). MS: *m/z* 503 $(M + 1)^+$. Anal. Calcd for $C_{19}H_{23}ClN_3O_9P$: C, 45.29; H, 4.60; N, 8.34. Found: C, 45.28; H, 4.71; N, 8.816.

3.21. Dioxolane-thymine 5′-(2-chlorophenyl benzyloxy-L-alanyl phosphate) (23)

Yield 77%. Mp: 137.1 °C. ^{31}P NMR ($CDCl_3$): δ 3.3, 3.1 (1:0.8); ^1H NMR ($CDCl_3$): δ 1.33–1.42 (d, 3H, Ala-CH_3), 1.88 (s, 3H, 5-Me), 3.68 (m, 1H, Ala-CH), 4.05 (m, 1H, NH), 4.21 (m, 2H, H5′), 4.40 (m, 2H, H2′), 5.15 (s, 1H, H4′), 5.19 (m, 2H, $PhCH_2$), 6.42 (m, 1H, H1′), 7.08–7.42 (m, 9H, Ph), 7.43 (s, 1H, H6), 7.96 (s, 1H, H3). MS: *m/z* 580 $(M + 1)^+$. Anal. Calcd for $C_{25}H_{27}ClN_3O_9P$: C, 51.78; H, 4.69; N, 7.25. Found: C, 52.09; H, 4.85 ; N, 7.05.

3.22. Dioxolane-thymine 5′-(2-allylphenyl methoxy-L-alanyl phosphate) (24)

Yield 45%. Mp: 127.0 °C. ^{31}P NMR ($CDCl_3$): δ 2.8, 2.7 (1:1); ^1H NMR ($CDCl_3$): δ 1.32–1.41 (d, 3H, Ala-CH_3), 1.85 (s, 3H, 5-Me), 3.40 (m, 2H, All-CH_2), 3.45 (m, 1H, Ala-CH), 3.77 (s, 3H, OCH_3), 4.07 (m, 1H, NH), 4.21 (m, 2H, H5′), 4.43 (m, 2H, H2′), 5.17 (s, 1H, H4′), 5.19 and 5.98 (m, 3H, CH═CH_2), 6.41 (m, 1H, H1′), 7.12–7.42 (m, 4H, Ph), 7.47 (s, 1H, H6), 8.76 (s, 1H, H3). MS: *m/z* 509 $(M + 1)^+$. Anal. Calcd for $C_{22}H_{28}N_3O_9P$ 0.4%H_2O: C, 51.14; H, 5.62; N, 8.13. Found: C, 50.56; H 5.32; N, 7.91.

3.23. Dioxolane-thymine 5′-(2,4-dichlorophenyl methoxy-L-alanyl phosphate) (25)

Yield 52%. Mp: 171.5 °C. ^{31}P NMR ($CDCl_3$): δ 2.9, 2.8 (1:1); ^1H NMR ($CDCl_3$): δ 1.34–1.42 (d, 3H, Ala-CH_3), 1.86 (s, 3H, 5-Me), 3.68 (m, 1H, Ala-CH), 3.73 (s, 3H, OCH_3), 4.09 (m, 1H, NH), 4.21 (m, 2H, H5′), 4.43 (m, 2H, H2′), 5.19 (s, 1H, H4′), 6.41 (m, 1H, H1′), 7.22–7.42 (m, 3H, Ph), 7.54 (s, 1H, H6), 8.89 (s, 1H, H3). MS: *m/z* 537 $(M + 1)^+$. Anal. Calcd for $C_{19}H_{22}N_3O_9P$: C, 42.40; H, 4.12; N, 7.81. Found: C, 42.69; H, 4.19; N, 7.58.

3.24. Dioxolane-thymine 5′-(naphthyl methoxy-L-alanyl phosphate) (26)

Yield 45%. Mp: 142.7 °C. ^{31}P NMR ($CDCl_3$): δ 3.3, 3.1 (1:0.7); ^1H NMR ($CDCl_3$): δ 1.34–1.42 (d, 3H, Ala-CH_3), 1.86 (s, 3H, 5-Me), 3.68 (m, 1H, Ala-CH), 3.73 (s, 3H, OCH_3), 4.09 (m, 1H, NH), 4.21 (m, 2H, H5′), 4.43 (m, 2H, H2′), 5.19 (s, 1H, H4′), 6.41 (m, 1H, H1′), 7.23–7.82 (m, 7H, naphthyl), 7.96 (s, 1H, H6), 8.87 (s, 1H, H3). MS: *m/z* 2519 $(M + 1)^+$. Anal. Calcd for $C_{23}H_{26}N_3O_9P$: C, 53.18; H, 5.05; N, 8.09. Found: C, 52.97; H, 5.17; N, 8.06.

3.25. Dioxolane-thymine 5′-(naphthyl methoxy-2-amino isobutyric phosphate) (27)

Yield 43%. Mp: 140.5 °C. ^{31}P NMR ($CDCl_3$): δ 3.4, 3.1 (1:0.7); ^1H NMR ($CDCl_3$): δ 1.44–1.53 (d, 6H, Isobut-CH_3), 1.86 (s, 3H, 5-Me), 3.75 (s, 3H, OCH_3), 4.08 (m, 1H, NH), 4.21 (m, 2H, H5′), 4.43 (m, 2H, H2′), 5.19 (s, 1H, H4′), 6.41 (m, 1H, H1′), 7.23–7.82 (m, 7H, naphthyl), 8.06 (s, 1H, H6), 8.57 (s, 1H, H3). MS: *m/z* 533 $(M + 1)^+$. Anal. Calcd for $C_{24}H_{28}N_3O_9P$: C, 54.03; H, 5.29; N, 7.88. Found: C, 52.89; H, 5.16; N, 7.68.

3.26. Dioxolane-thymine 5′-(naphthyl benzyloxy-L-alanyl phosphate) (28)

Yield 48%. Mp: 132.1 °C. ^{31}P NMR ($CDCl_3$): δ 3.3, 3.2 (1:0.8); ^1H NMR ($CDCl_3$): δ 1.34–1.47 (d, 6H, Ala-

CH_3), 1.88 (s, 3H, 5-Me), 3.68 (m, 1H, Ala-CH), 4.08 (m, 1H, NH), 4.21 (m, 2H, H5′), 4.43 (m, 2H, H2′), 5.16 (s, 1H, H4′), 5.20 (m, 2H, $PhCH_2$), 6.41 (m, 1H, H1′), 7.23–7.74 (m, 12H, Ph and naphthyl), 8.01 (s, 1H, H6), 8.77 (s, 1H, H3). MS: *m/z* 596 $(M + 1)^+$. Anal. Calcd for $C_{29}H_{30}N_3O_9P$: C, 58.49; H, 5.08; N, 7.06. Found: C, 58.45; H, 5.27; N, 6.77.

3.27. Dioxolane-thymine 5′-(3-hexadecyloxypropyl methoxy-L-alanyl phosphate) (32)

To a solution of diphenyl phosphite (234 mg, 1.0 mmol) in pyridine (10 mL) was added DOT (114 mg, 0.5 mmol) in pyridine (10 mL) over a period of 1 h under nitrogen atmosphere at 0 °C, and the reaction mixture was stirred for 30 min. Anhydrous long-chain alcohol 3-hexadecyloxypropyl-1-ol (450 mg, 1.5 mmol) was added to the above mixture and stirred for an additional 2 h at room temperature. The solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel (2% MeOH in CH_2Cl_2) to provide dioxolane thymine 5′-(3-hexadecyloxypropyl phosphite) in good yield. (130 mg, 70%). ^{31}P NMR ($CDCl_3$): δ 4.5; ^1H NMR ($CDCl_3$): δ 0.89–1.95 (m, 33H, HDP-H), 1.96 (s, 3H, 5-Me), 3.38, 3.48 and 4.21 (m, 6H, OCH_2), 4.18 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.15 (s, 1H, H4′), 6.39 (m, 1H, H1′), 6.18 and 7.62 (d, 1H, P-H), 7.48 (s, 1H, H6), 8.20 (s, 1H, H3). The above H-phosphonate (1 mmol) in CH_2Cl_2 (2 mL) was added dropwise to a solution of the corresponding L-amino acid methyl ester hydrochloride (1.2 mmol) in Et_3N (0.5 mL)-CCl_4 (0.5 mL)-H_2O (0.5 mL)-MeCN (5 mL) at 0 °C. The mixture was stirred at room temperature for 20 min. The solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel (5% MeOH in CH_2Cl_2) to afford **32**. Yield 98%. ^{31}P NMR ($CDCl_3$): δ 3.7, 3.4 (1:1); ^1H NMR ($CDCl_3$): δ 0.89–1.95 (m, 33H, HDP-H), 1.35 (d, 3H, Ala-CH_3), 1.96 (s, 3H, 5-Me), 3.34 (m, 1H, Ala-CH), 3.38, 3.48, and 4.21 (m, 6H, OCH_2), 3.74 (m, 1H, NH), 3.78 (s, 3H, OCH_3), 4.18 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.15 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.48 (s, 1H, H6), 8.17 (s, 1H, H3). MS: *m/z* 675 $(M + 1)^+$. Anal. Calcd for $C_{32}H_{58}N_3O_{10}P$: C, 56.87; H, 8.65; N, 6.22. Found: C, 57.04; H, 8.86; N, 6.18.

3.28. Dioxolane-thymine 5′-(3-hexadecyloxypropyl methoxy-glycinyl phosphate) (31)

Overall yield 67%. ^{31}P NMR ($CDCl_3$): δ 4.1, 3.8 (1:1); ^1H NMR ($CDCl_3$): δ 0.89–1.95 (m, 33H, HDP-H),1.96 (s, 3H, 5-Me), 3.36 (m, 2H, Gly-CH_2), 3.38, 3.48, and 4.21 (m, 6H, OCH_2), 3.76 (m, 1H, NH), 3.79 (s, 3H, OCH_3), 4.18 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.15 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.49 (s, 1H, H6), 8.18 (s, 1H, H3). MS: *m/z* 662 $(M + 1)^+$. Anal. Calcd for $C_{31}H_{56}N_3O_{10}P$ 0.1%H_2O: C, 56.11; H, 8.54; N, 6.33. Found: C 56.16; H, 8.59; N, 6.17.

3.29. Dioxolane-thymine 5′-(3-hexadecyloxypropyl methoxy-L-valyl phosphate) (33)

Overall yield 68%. ^{31}P NMR ($CDCl_3$): δ 3.7, 3.6 (1:1); ^1H NMR ($CDCl_3$): δ 0.85 (dd, 6H, Val-CH_3), 0.97-1.95 (m, 33H, HDP-H), 1.96 (s, 3H, 5-Me), 2.05 (m, 1H, Val-CH), 3.34 and 3.75 (m, 2H, Val-CHNH), 3.38, 3.48, and 4.21 (m, 6H, OCH_2), 3.78 (s, 3H, OCH_3), 4.18 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.15 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.48 (s, 1H, H6), 8.17 (s, 1H, H3). MS: *m/z* 704 $(M + 1)^+$. Anal. Calcd for $C_{34}H_{62}N_3O_{10}P$ 0.1%H_2O: C, 57.87; H, 8.88; N, 5.95. Found: C, 57.99; H, 8.95; N, 5.94.

3.30. Dioxolane-thymine 5′-(2-octadecyloxyethyl methoxy-L-alanyl phosphate) (34)

Overall yield 68%. ^{31}P NMR ($CDCl_3$): δ 3.7, 3.6 (1:1); ^1H NMR ($CDCl_3$): δ 0.93–1.90 (m, 35H, ODE-H), 1.33 (d, 3H, Ala-CH_3), 1.95 (s, 3H, 5-Me), 3.34 (m, 1H, Ala-CH), 3.38, 3.48, and 4.21 (m, 6H, OCH_2), 3.74 (m, 1H, NH), 3.78 (s, 3H, OCH_3), 4.18 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.15 (s, 1H, H4′), 6.39 (m, 1H, H1′), 7.48 (s, 1H, H6), 8.17 (s, 1H, H3); MS: *m/z* 689 $(M + 1)^+$. Anal. Calcd for $C_{33}H_{60}N_3O_9P$: C, 57.46; H, 8.77; N, 6.09. Found: C, 57.58; H, 9.05; N, 5.95.

3.31. Dioxolane-thymine 5′-(oleyl methoxy-L-alanyl phosphate) (35)

Overall yield 67%. ^{31}P NMR ($CDCl_3$): δ 3.7, 3.6 (1:1); ^1H NMR ($CDCl_3$): δ 0.90–1.80 (m, 27H, Oleyl-H), 1.35 (d, 3H, Ala-CH_3), 1.95 (s, 3H, 5-Me), 2.03 (m, 4H, CH_2C=), 3.34 (m, 1H, Ala-CH), 3.66 (m, 2H, OCH_2), 3.74 (m, 1H, NH), 3.78 (s, 3H, OCH_3), 4.18 (m, 2H, H5′), 4.39 (m, 2H, H2′), 5.15 (s, 1H, H4′), 5.38 (m, 2H, CH=CH), 6.39 (m, 1H, H1′), 7.48 (s, 1H, H6), 8.17 (s, 1H, H3). MS: *m/z* 643 $(M + 1)^+$. Anal. Calcd for $C_{32}H_{58}N_3O_9P$: C, 57.84; H, 8.45; N, 6.53. Found: C, 57.80; H, 8.39; N, 6.38.

3.32. (+)-*cis*-5′-O-[4-(*S*)-(3-Chlorophenyl)-2-oxo-1,3,2-dioxaphosphorinan-2-yl] dioxolane-thymine (41)

A solution of diol (120 mg, 0.79 mmol) and triethylamine (0.44 mL, 3.17 mmol) in THF (10 mL) was added dropwise to a solution of 4-nitrophenoxyphosphorodichloridate (405 mg, 1.58 mmol) in THF (2 mL) at 0 °C. After 2h, additional triethylamine (0.44 mL, 3.17 mmol) and 4-nitrophenol (1.76 g, 14.68 mmol) were added and the reaction mixture was stirred overnight. The solvent was evaporated, and the residue was partitioned between EtOAc and water. The organic phase was washed with 0.2 M NaOH (1 × 10 mL), water (2 × 10 mL), brine (2 × 10 mL), and dried over $MgSO_4$. The mixture was concentrated under reduced pressure and purified by flash chromatography (50% EtOAc in hexanes) to yield 5′-O-[4-(*S*)-(3-chlorophenyl)-2-oxo-1,3,2-dioxaphosphorinan-2-yl]-4-nitrobenzene (143 mg, 88%). ^{31}P NMR ($CDCl_3$): δ −4.8, −4.2 (1:0.1); ^1H NMR ($CDCl_3$): δ 2.09–2.48 (m, 2H, CH_2), 4.61 (m, 2H, CH_2O), 5.60 (m, 1H, PhCH), 7.37–8.22 (m, 9H, Ph).

A solution of dioxolane-thymine (45 mg, 0.18 mmol) in DMF (5 mL) was treated with a THF solution of 1 M *tert*-BuMgCl (20 mg, 0.23 mmol) and stirred at room temperature. After 30 min., 5′-O-[4-(*S*)-(3-chlorophenyl)-2-oxo-1,3,2-dioxaphosphorinan-2-yl]-4-nitrobenzene

(100 mg, 0.31 mmol) was added. The reaction mixture was stirred for 20 h at room temperature and then quenched with saturated NH_4Cl (10 mL) and extracted with EtOAc. The organic extract was washed with 1 N NaOH (2×20 mL), water (2×20 mL), brine (2×20 mL), and dried (Na_2SO_4), concentrated to a residue, and purified by flash chromatography (5% MeOH in CH_2Cl_2) to give **41**. Yield 52%. Mp: 128.7 °C. $[\alpha]_D^{22}$ +25.13 (*c* 0.99, $CHCl_3$). ^{31}P NMR ($CDCl_3$): δ −4.2, −4.1 (1:0.1); ^1H NMR ($CDCl_3$): δ 1.90 (s, 3H, 5-Me), 2.05–2.35 (m, 2H, CH_2), 4.19 (m, 2H, H5′), 4.43 (m, 2H, H2′), 4.41 and 4.71 (m, 2H, CH_2O), 5.16 (s, 1H, H4′), 5.68 (m, 2H, PhCH), 6.41 (m, 1H, H1′), 7.29–7.44 (m, 4H, Ph), 7.50 (s, 1H, H6), 8.21 (s, 1H, H3). MS: *m/z* 458 $(M + 1)^+$. Anal. Calcd for $C_{18}H_{20}ClN_2O_8P$ 0.1%H_2O: C, 46.94; H, 4.42; N, 6.08. Found: C46.70; H, 4.45; N, 5.96.

3.33. (−)-*cis*-5′-O-[4-(*R*)-(3-Chlorophenyl)-2-oxo-1,3,2-dioxaphosphorinan-2- yl]dioxolane-thymine (42)

Yield 61%. Mp: 126.8 °C. $[\alpha]_D^{22}$ −26.75 (*c* 1.01, $CHCl_3$). ^{31}P NMR ($CDCl_3$): δ −4.4, −4.2(1:0.1); ^1H NMR ($CDCl_3$): δ 1.90 (s, 3H, 5-Me), 2.07–2.40 (m, 2H, CH_2), 4.20 (m, 2H, H5′), 4.43 (m, 2H, H2′), 4.46 and 4.71 (m, 2H, CH_2O), 5.18 (s, 1H, H4′), 5.65 (m, 2H, PhCH), 6.41 (m, 1H, H1′), 7.29–7.43 (m, 4H, Ph), 7.50 (s, 1H, H6), 8.20 (s, 1H, H3). MS: *m/z* 458 $(M + 1)^+$. Anal. Calcd for $C_{18}H_{20}ClN_2O_8P$: C, 47.12; H, 4.39; N, 6.11. Found: C, 47.06; H, 4.54; N, 5.96.

3.34. (+)-*cis*-5′-O-[4-(*S*)-(3-Phenyl)-2-oxo-1,3,2-dioxaphosphorinan-2-yl]dioxolane -thymine (43)

Yield 57%. Mp: 121.5 °C. $[\alpha]_D^{22}$ +32.70 (*c* 0.99, $CHCl_3$). ^{31}P NMR ($CDCl_3$): δ −3.8, −3.7(1:0.1); ^1H NMR ($CDCl_3$): δ 1.90 (s, 3H, 5-Me), 2.07-2.40 (m, 2H, CH_2), 4.20 (m, 2H, H5′), 4.41 (m, 2H, H2′), 4.44 and 4.71 (m, 2H, CH_2O), 5.16 (s, 1H, H4′), 5.67 (m, 2H, PhCH), 6.41 (m, 1H, H1′), 7.29–7.43 (m, 5H, Ph), 7.50 (s, 1H, H6), 8.20 (s, 1H, H3). MS: *m/z* 424 $(M + 1)^+$. Anal. Calcd for $C_{18}H_{21}N_2O_8P$: C, 50.95; H, 4.99; N, 6.60. Found: C, 50.68; H, 5.34; N, 6.32.

3.35. (−)-*cis*-5′-O-[4-(*R*)-(3-Phenyl)-2-oxo-1,3,2-dioxaphosphorinan-2-yl]dioxolane-thymine (44)

Yield 56%. Mp: 121.3 °C. $[\alpha]_D^{22}$ −28.52 (*c* 0.98, $CHCl_3$). ^{31}P NMR ($CDCl_3$): δ −3.9, −3.8 (1:0.1); ^1H NMR ($CDCl_3$): δ 1.90 (s, 3H, 5-Me), 2.07–2.40 (m, 2H, CH_2), 4.20 (m, 2H, H5′), 4.41 (m, 2H, H2′), 4.44 and 4.71 (m, 2H, CH_2O), 5.18 (s, 1H, H4′), 5.67 (m, 2H, PhCH), 6.40 (m, 1H, H1′), 7.29–7.45 (m, 5H, Ph), 7.50 (s, 1H, H6), 8.20 (s, 1H, H3). MS: *m/z* 424 $(M + 1)^+$. Anal. Calcd for $C_{18}H_{22}N_2O_8P$: C, 50.95; H, 4.99; N, 6.60. Found: C, 50.45; H, 5.27; N, 6.77.

3.36. Dioxolane-thymine 5′-[(3-hexadecyloxypropyl) phosphate] (47)

To a solution of 1,2,4-trizole (1.1 g, 16 mmol) and triethylamine (91.62 g, 16 mmol) in anhydrous THF (10 mL) was added a solution of 2-chlorophenyl dichlorophosphate (1.96 g, 8 mmol) in THF (10 mL). The mixture was stirred for 30 min. and then filtered. To the filtrate were added sequentially, additional 30 mL of THF, dioxolane thymine (1.37 g, 6 mmol), and 1-methylimidazole (0.66 g, 8 mmol). After 1 h, 3-hexadecyl-1-propanol (HDP, 1.8 g, 6 mmol) was added to the mixture and stirred overnight at room temperature. The solvent was evaporated and the residue was purified by flash chromatography on silica gel (4% MeOH in CH_2Cl_2) to afford dioxolane thymine 5′-[(2-chlorophenyl 3-hexadecyloxypropyl) phosphate] (1.88 g, 60%). ^{31}P NMR ($CDCl_3$): δ 6.7, 6.5 (1:0.9); ^1H NMR ($CDCl_3$): δ 0.88–1.87 (m, 33H, HDP-H), 1.90 (s, 3H, 5-Me), 3.35, 3.47, and 4.35 (m, 6H, OCH_2), 4.20 (m, 2H, H5′), 4.45 (m, 2H, H2′), 5.18 (s, 1H, H4′), 6.40 (m, 1H, H1′), 7.11–7.40 (m, 4H, Ph), 7.46 (s, 1H, H6), 8.65 (s, 1H, H3). Then it was dissolved in THF and 0.5N NaOH was added at 0 °C. The mixture was stirred at 50 ± 3 °C for 1.5 h and neutralized with HCl at 0 °C. The solvent was evaporated and the residue was purified by flash chromatography on silica gel (10% MeOH in CH_2Cl_2) to provide **47**. Yield 93%. ^{31}P NMR ($CDCl_3$): δ 5.4; ^1H NMR ($CDCl_3$): δ 0.88–1.88 (m, 33H, HDP-H), 1.96 (s, 3H, 5-Me), 3.33, 3.45, and 4.21 (m, 6H, OCH_2), 3.87 (m, 2H, H5′), 4.15 (m, 2H, H2′), 5.16 (s, 1H, H4′), 6.32 (m, 1H, H1′), 7.78 (s, 1H, H6). MS: *m/z* 591 $(M + 1)^+$. Anal. Calcd for $C_{28}H_{51}N_2O_9P$ 1.2% H_2O: C, 54.92; H, 8.69; N, 4.58. Found: C, 54.52; H, 8.32; N, 4.45.

3.37. Dioxolane-thymine 5′-[(2-octadecyloxyethyl) phosphate] (48)

Yield 94%. ^{31}P NMR ($CDCl_3$): δ 5.3; ^1H NMR ($CDCl_3$): δ 0.88–1.92 (m, 35H, HDP-H), 1.96 (s, 3H, 5-Me), 3.43, 3.60, and 4.01 (m, 6H, OCH_2), 4.17 (m, 2H, H5′), 4.19 (m, 2H, H2′), 5.15 (s, 1H, H4′), 6.34 (m, 1H, H1′), 7.68 (s, 1H, H6). MS: *m/z* 619 $(M + 1)^+$. Anal. Calcd for $C_{30}H_{55}N_2O_9P$ 0.7% H_2O: C, 57.07; H, 9.00; N, 4.44. Found: C, 56.69; H, 8.81; N, 4.44.

3.38. Virology

3.38.1. Antiviral assays. The procedures for the antiviral assays in human peripheral blood mononuclear (PBM) cells have been published previously.[7a,7b] PBM cells (obtained from Atlanta Red Cross) were isolated by Ficoll–Hypaque discontinuous gradient centrifugation from healthy seronegative donors. Cells were stimulated with phytohemagglutinin A (Difco, Sparks, MD) for 2–3 days prior to use. HIV-1 LAI obtained from the Center for Disease Control and Prevention (Atlanta, GA) was used as the standard reference virus for the antiviral assays. The molecular infectious clones HIV-1 M184 V pitt were obtained from Dr. John Mellors (University of Pittsburgh). Infections were done in bulk for 1 h, either with 100 $TCID_{50}/1 \times 10^7$ cells for a flask (T25) assay or with 200 $TCID_{50}/6 \times 10^7$ cells/well for the 24-well plate assay. Cells were added to a plate or a flask containing a 10-fold serial dilution of the test compound. Assay medium was RPMI-1640 supplemented with heat-inactivated 16% fetal bovine serum. 1.6 mM L-glutamine, 80 IU/mL penicillin, 80 μg /mL streptomycin, 0.0008% DEAE–Dextran, 0.045% sodium bicarbonate,

and 26 IU/mL recombinant interleukin-2 (Chiron Corp., Emeryville, CA). AZT was used as a positive control for the assay. Uninfected PBM cells were grown in parallel at equivalent cell concentrations as control. The cell cultures were maintained in a humidified 5% CO_2-air at 37 °C for 5 days, and supernatants were collected for reverse transcriptase activity. Supernatants were centrifuged at 12,000 rpm for 2 h to pellet the virus. The pellet was solubilized with vortexing in 100 μL of virus solubilization buffer (0.5% Triton X-100, 0.8 M NaCl, 0.5 mM phenylmethylsulfonyl chloride, 20% glycerol, and 0.05 M Tris, pH 7.8). 10 μL of each sample was added to 70 μL RT reaction mixture [0.06 M Tris, pH 7.8, 0.012 M $MgCl_2$, 0.006 M dithiothreitol, 0.006 mg/mL poly(rA)$_n$ oligo(dT)$_{12-18}$, 96 μg/mL dATP, and 1 μM of 0.08 mCi/mL [^{3}H]thymidine triphosphate (Moravek Biochemicals, Brea, CA) and incubated at 37 °C for 2 h. The reaction was stopped by the addition of 100 μL of 10% trichloroacetic acid containing 0.05% sodium perophosphate. The acid-insoluble product was harvested onto filter paper using a Packard Harvester (Meriden, CT), and RT activity was read on a Packard Direct Beta Counter (Meriden, CT). RT results were expressed in counts per minute (CPM) per milliliter. The antiviral 50% effective concentration (EC_{50}) and 90% effective concentration (EC_{90}) were determined from the concentration–response curve using the median effect method.

3.38.2. Cytotoxicity assays. The compounds were evaluated for their potential toxic effects on uninfected PHA-stimulated human PBM cell, in CEM (T-lymphoblastoid cell line obtained from American Type Culture Collection, Rockville, MD) and Vero (African green monkey kidney) cells. Log-phase Vero, CEM, and PHA-stimulated human PBM cells were seeded at density at 5×10^3, 2.5×10^3, and 5×10^4 cells/well, respectively. All cells were planted in 96-well cell culture plates containing 10-fold serial dilutions of the test drug. The cultures were incubated for 2, 3, and 4 days for Vero, CEM, and PBM cells, respectively, in a humidified 5% CO_2-air at 37 °C. At the end of incubation, MTT tetrazolium dye solution (cell titer 96, Promega, Madison, WI) was added to each well and incubated overnight. The reaction was stopped with stop solubilization solution (Promega, Madison, WI). The plates were incubated for 5 h to ensure that the formazan crystals were dissolved. The plates were read at a wavelength of 570 nm using an ELISA plate reader (Bio-Tek Instruments, Inc., Winooski, VT, Model EL 312 e). The 50% inhibition concentration (IC_{50}) was determined from the concentration–response curve using the median effect method.[7c]

3.39. Hydrolysis of prodrugs

3.39.1. Aqueous buffer kinetics. Hydrolysis of prodrugs was studied in pH 2.0 and 7.4. A 3 mL aliquot of a 1 mM solution of prodrugs in DMSO was diluted to 10 mL using phosphate buffer (10 mM). A constant ionic strength (μ) of 0.1 was maintained by the addition of an appropriate quantity of NaCl to the solutions. Buffer solutions containing ester prodrugs were maintained at 37 ± 0.5 °C in screw-capped vials in a water bath. The samples were withdrawn at appropriate time intervals and filtered. The filtration was analyzed by HPLC.

3.39.2. Enzyme study. Prodrugs (1.4 mM) were incubated at 37 °C in pH 8.0 phosphate buffer containing 20 μg/mL porcine esterase. Aliquots were removed at intervals, quenched with ice-cold methanol/acetonitrile 1/1 (v/v), filtered, and analyzed by HPLC. The enzyme activity was determined using ethyl butyrate as substrate with HPLC to monitor substrate disappearance.

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