In Vitro Evaluation of Phosphocholine and Quaternary Ammonium Containing Lipids as Novel Anti-HIV Agents

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A series of synthetic lipids containing a two- or three-carbon backbone substituted with a thio, oxy, or amidoalkyl functionality and either a phosphocholine or quaternary ammonium moiety was evaluated as potential anti-HIV-1 agents. Several analogues were identified as possessing activity with the most promising compound being *rac*-3-octadecanamido-2-ethoxypropylphosphocholine (8). Compound 8 exhibited an IC_{50} for the inhibiton of plaque formation of 0.16 μM which was 84-fold lower than the IC_{50} value determined for CEM-SS cell growth inhibition. Initial mechanistic studies have indicated that these compounds, unlike AZT, are not reverse transcriptase (RT) inhibitors, but instead appear to inhibit a late step in HIV replication involving virus assembly and infectious virus production. Since these lipids are acting via a different mechanism, they represent an alternative approach to the chemotherapeutic treatment of AIDS as well as candidates for combination therapy with AZT.

Introduction

Acquired immunodeficiency syndrome (AIDS) is a degenerative disease of the immune and central nervous systems for which no known cure exists. Although the discovery of the human immunodeficiency virus type 1 (HIV-1) as the causative agent and the subsequent elucidation of the viral life cycle have identified many potential targets for the therapeutic intervention of this disease,[1] 3′-azido-3′-deoxythymidine (AZT; zidovudine) still remains the only clinically approved treatment. However, AZT which acts as a reverse transcriptase (RT) inhibitor[2] has several toxic side effects (e.g., bone marrow depression) associated with its use.[3,4] In addition, recent evidence suggests that extended exposure of HIV to AZT may lead to reduced sensitivity.[5,6] Finally, AZT does not eradicate the virus but serves only to delay the progression of the disease. Therefore, the development of new antiviral agents whose modes of action and side effects are different from that of AZT is paramount. Such agents could prove beneficial either alone or in combination therapy with AZT in the treatment of this disease. Benefits of combination therapy include an enhancement in activity (i.e., synergistic action), a reduction in toxicity, as well as the possible prevention of drug resistance. Glycerol ether lipids (EL) represent such a potential class of novel anti-AIDS compounds.

Ether lipids such as *rac*-1-*O*-octadecyl-2-*O*-methylglycero-3-phosphocholine (ET-18-OMe; Figure 1) are known to possess antineoplastic activity.[7,8] Although the exact mechanism(s) for this activity is not well understood, these agents are membrane-interactive and may function by fluidization of the neoplastic cell membrane.[9] Also, inhibition of the phospholipid and calcium-dependent protein kinase C (PKC) by EL analogues has been linked to the antineoplastic activity.[10,11] The development of new EL which possess improved antineoplastic activity and PKC inhibition has been a major concern of our group.

Recently, three studies were reported which led to the further evaluation of EL analogues as antiviral agents. First, AL721, a mixture of hen yolk lipids in a ratio of neutral lipid/phosphatidylcholine/phosphatidylethanolamine of 7:2:1 was reported to exhibit anti-HIV activity in vitro[12] possibly by fluidization of cell membranes. Second, phosphorylation of the CD4 receptor on the cell surface appears to be required for viral entry into susceptible cells via receptor-mediated endocytosis.[13] Preliminary studies suggest that PKC is the kinase involved in this process. Therefore, inhibition of this enzyme has the potential to prevent viral entry. Third, our laboratories recently reported that representative synthetic lipids (thio, oxy, and amidoalkyl) containing either a phosphocholine or a quaternary ammonium moiety exhibit activity against infectious HIV-1 multiplication.[14]

On the basis of this information, a more complete series of complex lipids has been evaluated for anti-HIV-1 activity by using a syncytial plaque assay. In addition, assessment of the cellular toxicity of these compounds was performed. These in vitro studies have been initiated in

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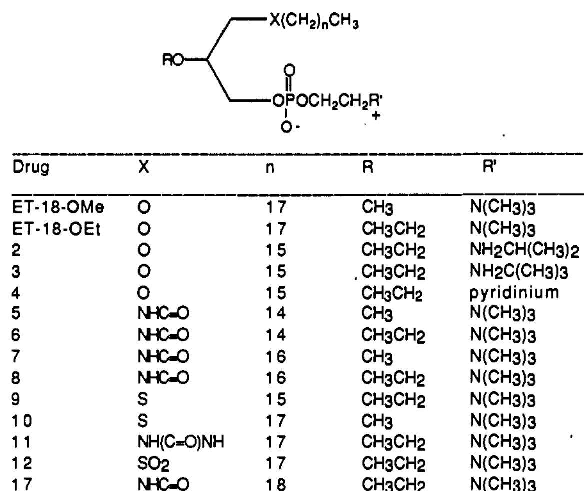
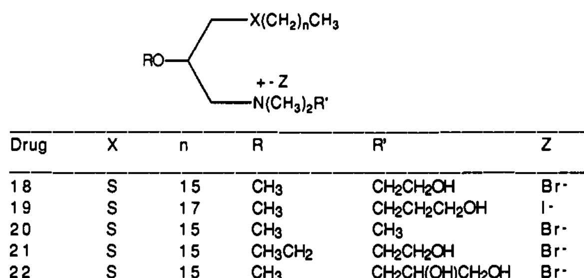


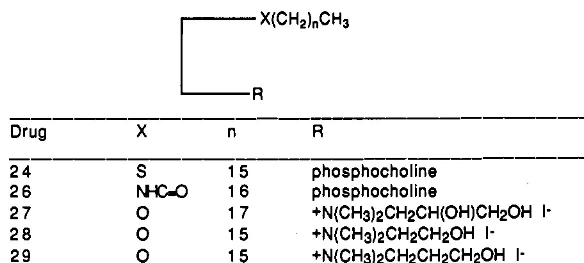
Drug	X	n	R	R'
ET-18-OMe	O	17	CH3	N(CH3)3
ET-18-OEt	O	17	CH3CH2	N(CH3)3
2	O	15	CH3CH2	NH2CH(CH3)2
3	O	15	CH3CH2	NH2C(CH3)3
4	O	15	CH3CH2	pyridinium
5	NHC=O	14	CH3	N(CH3)3
6	NHC=O	14	CH3CH2	N(CH3)3
7	NHC=O	16	CH3	N(CH3)3
8	NHC=O	16	CH3CH2	N(CH3)3
9	S	15	CH3CH2	N(CH3)3
10	S	17	CH3	N(CH3)3
11	NH(C=O)NH	17	CH3CH2	N(CH3)3
12	SO2	17	CH3CH2	N(CH3)3
17	NHC=O	18	CH3CH2	N(CH3)3

Figure 1. Phosphorus-containing lipids.



Drug	X	n	R	R'	Z
18	S	15	CH3	CH2CH2OH	Br-
19	S	17	CH3	CH2CH2CH2OH	I-
20	S	15	CH3	CH3	Br-
21	S	15	CH3CH2	CH2CH2OH	Br-
22	S	15	CH3	CH2CH(OH)CH2OH	Br-

Figure 2. Nonphosphorus-containing thioalkyl lipids.



Drug	X	n	R
24	S	15	phosphocholine
26	NHC=O	16	phosphocholine
27	O	17	+N(CH3)2CH2CH(OH)CH2OH I-
28	O	15	+N(CH3)2CH2CH2OH I-
29	O	15	+N(CH3)2CH2CH2CH2OH I-

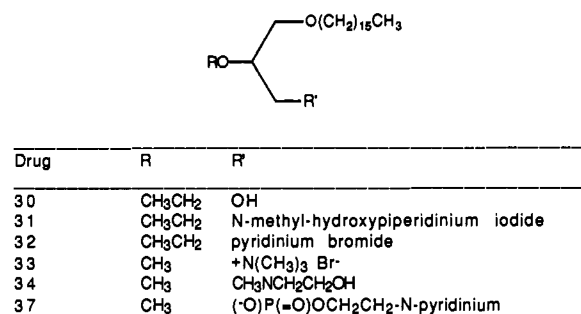
Figure 3. Phosphorus- and nonphosphorus-containing two-carbon backbone analogues.

order to evaluate the structure–activity relationships of the lipids which provide optimal antiviral activity.

Chemistry

The analogues under evaluation comprise two series (Figures 1–4). Series 1 includes the phospholipid analogues (i.e., phosphocholine and phosphoethanolamine derivatives). The syntheses of several of these phospholipids have been previously reported.[15-18] New analogues include compounds **2**, **3**, **17**, **24**, **26**, and **37**.

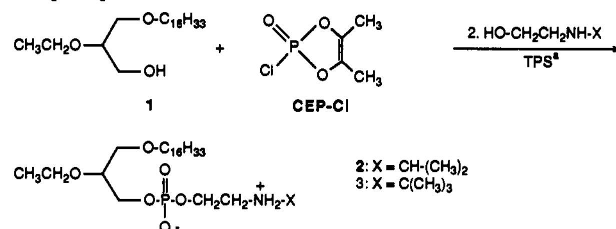
Preparation of the phosphoethanolamine analogues **2** and **3** is shown in Scheme I part A. Intermediate **1**, *rac*-1-*O*-hexadecyl-2-*O*-ethylglycerol was phosphorylated with 1,2-dimethylethylene phosphorochloridate (CEP-Cl, prepared according to ref 19) followed by immediate reaction of the intermediate with either 2-(isopropylamino)ethanol or 2-(*tert*-butylamino)ethanol (Fluka) to give the expected products **2** and **3**, respectively. Com-



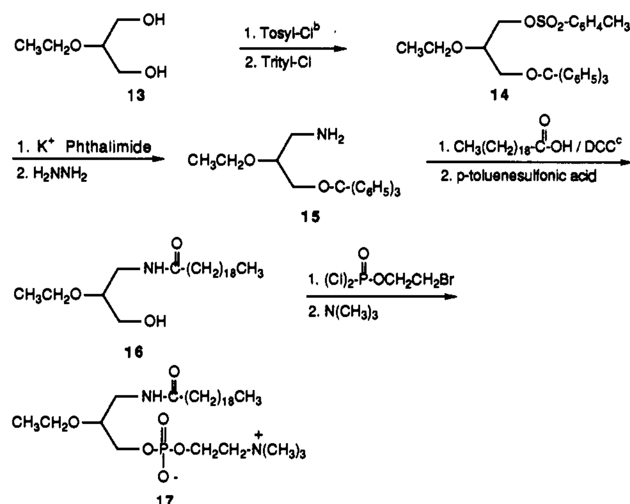
Drug	R	R'
30	CH3CH2	OH
31	CH3CH2	N-methyl-hydroxypiperidinium iodide
32	CH3CH2	pyridinium bromide
33	CH3	+N(CH3)3 Br-
34	CH3	CH3NCH2CH2OH
37	CH3	(-O)P(=O)OCH2CH2-N-pyridinium

Figure 4. Miscellaneous analogues.

Scheme I.[a] Part A. Synthesis of Isopropyl and *tert*-Butyl Phospholipids 2 and 3

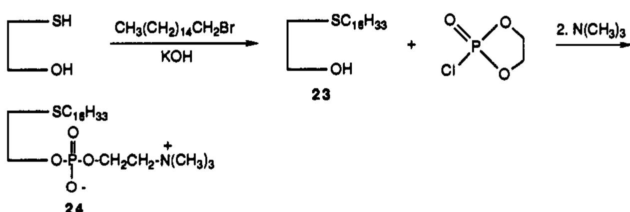


Part B. Synthesis of Eicosanamido Phosphocholine 17

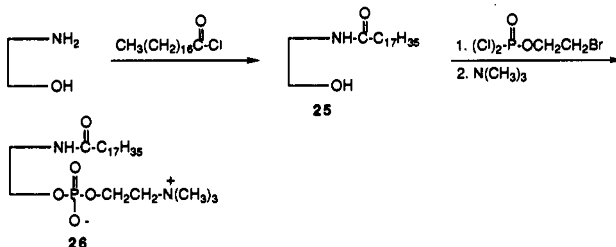


[a] (a) TPS = 2,4,6-triisopropylbenzenesulfonyl chloride. (b) Tosyl-Cl = *p*-toluenesulfonyl chloride. (c) DCC = dicyclohexylcarbodiimide.

Scheme II. Part A. Synthesis of Thioethanol Phosphocholine 24



Part B. Synthesis of Amidoethanol Phosphocholine 26



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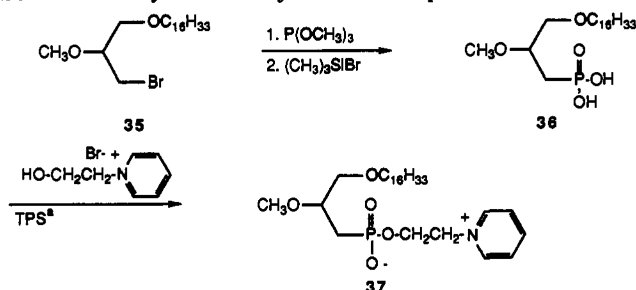
Scheme III. Synthesis of Pyridinium Phosphonate 37



[a] (a) TPS = 2,4,6-triisopropylbenzenesulfonyl chloride.

pound **17** was synthesized in eight steps as shown in Scheme I part B. First, 2-*O*-ethylglycerol[20] (**13**) was monotosylated and the remaining primary alcohol protected with triphenylmethyl (trityl) chloride in pyridine to give **14**. The sulfonic ester was displaced with potassium phthalimide followed by reaction with hydrazine hydrate to give the free amine **15**. Condensation with eicosanoic acid in the presence of 1,3-dicyclohexylcarbodiimide (DCC) followed by detritylation with *p*-toluenesulfonic acid yielded the desired alcohol **16**. Phosphorylation with 2-bromoethyl dichlorophosphate (prepared according to ref 21) followed by displacement of the bromide with trimethylamine provided the phosphocholine **17**.

Scheme II part A details the three-step synthesis of compound **24**. Alkylation of 2-mercaptoethanol with hexadecyl bromide in alcoholic KOH gave the resulting hexadecylthioethanol **23**. The alcohol was phosphorylated with 2-chloro-2-oxo-1,2,3-dioxaphospholane (Fluka) and the intermediate was reacted without purification with trimethylamine in a sealed vessel to obtain the phosphocholine **24**. Scheme II part B shows the synthesis of compound **26**. Amidation of 2-aminoethanol with octadecanoyl chloride gave intermediate **25** which was converted to the phosphocholine **26** following the procedure described above for compound **17**.

The phosphonate derivative **37** was prepared as shown in Scheme III from intermediate **35**, 1-(hexadecyloxy)-2-methoxy-3-bromopropane.[22] The bromopropane was reacted with trimethyl phosphite to obtain the dimethyl phosphonate derivative;[23] deprotection with bromotrimethylsilane gave the phosphonic acid **36**.[24] This intermediate was condensed with *N*-(β-hydroxyethyl)-pyridinium bromide in the presence of triisopropylbenzenesulfonyl chloride (TPSCl) to yield the final product **37**.[25]

Series 2 consists of the nonphosphorus analogues. These include both the inverse cholines [i.e., *N*-(3-alkoxy-2-alkoxypropyl)-*N*,*N*-dimethyl-*N*-(ω-hydroxyalkyl)ammonium bromide] and other quaternary ammonium analogues. Previous reports on the syntheses of these analogues have appeared in the literature.[11,22,26] The polyhydroxylated analogues **22** and **27** were prepared by reaction of the appropriate bromide with *N*,*N*-dimethyl-2,3-dihydroxypropylamine in dimethylformamide.

Table I. Effect of Phosphorus-Containing Lipids on HIV-1 Plaque Formation and CEM-SS Cell Growth[a]

drug	HIV plaque formation: IC_{50}, μM	CEM-SS cell growth: IC_{50}, μM	differential selectivity[b]
AZT	0.004 ± 0.001	5.6 ± 0.8	1400
ET-18-OMe	0.92 ± 0.39	4.0 ± 0.7	4.3
ET-18-OEt	0.27 ± 0.14	3.0 ± 1.4	11.1
2 (CP-44)	>5.0	7.0	ND[c]
3 (CP-45)	>5.0	10.0 ± 5.7	ND
4 (CP-31)	4.7 ± 0.52	8.1 ± 0.1	1.7
5 (CP-48)	0.93 ± 0.39	24.7 ± 7.4	26.6
6 (CP-49)	0.46 ± 0.01	19.0 ± 4.2	41.3
7 (CP-50)	0.30 ± 0.29	14.2 ± 5.3	47.3
8 (CP-51)	0.16 ± 0.06	13.5 ± 2.8	84.4
9 (CP-53)	0.70 ± 0.14	4.1 ± 0.4	5.9
10 (CP-60)	0.49 ± 0.16	3.8 ± 0.2	7.8
11 (CP-65)	0.57 ± 0.14	7.7 ± 0.8	13.5
12 (CP-59)	1.20 ± 0.57	9.0 ± 0.3	7.5
17 (CP-86)	0.16 ± 0.08	4.7 ± 0.5	29.4
24 (CP-9)	0.28 ± 0.00	7.6 ± 0.6	27.1
26 (CP-87)	0.28	19.3 ± 1.8	68.7
37 (CP-77)	2.20 ± 1.63	7.7 ± 1.2	3.5

[a] IC_{50} values are mean ± standard deviation ($n \geq 3$). Values without standard deviations represent single experiments. [b] Differential selectivity is the ratio of CEM-SS cell growth IC_{50} to HIV plaque formation IC_{50}. [c] ND = not determined.

Table II. Effect of Nonphosphorus-Containing Lipids on HIV-1 Plaque Formation and CEM-SS Cell Growth[a]

drug	HIV plaque formation: IC_{50}, μM	CEM-SS cell growth: IC_{50}, μM	differential selectivity[b]
18 (CP-7)	0.37 ± 0.01	2.1 ± 1.0	5.7
19 (CP-10)	0.41 ± 0.29	4.0 ± 2.7	9.8
20 (CP-19)	0.39 ± 0.24	5.3 ± 0.1	13.4
21 (CP-66)	0.63 ± 0.18	5.1 ± 0.4	8.1
22 (CP-80)	0.49 ± 0.23	2.0 ± 0.1	4.1
27 (CP-81)	0.20 ± 0.05	2.9 ± 0.5	14.5
28 (IP-1)	0.38 ± 0.29	6.0 ± 0.2	15.6
29 (IP-2)	0.24 ± 0.06	1.5 ± 0.1	6.1
30 (CP-0)	>5.0	>20.0	ND[c]
31 (CP-20)	0.30 ± 0.06	2.4 ± 1.1	8.0
32 (CP-23)	0.21 ± 0.01	1.1	5.2
33 (CP-26)	0.52 ± 0.06	1.1 ± 0.3	2.1
34 (CP-73)	2.10 ± 1.56	3.2 ± 0.4	1.5

[a] IC_{50} values are mean ± standard deviation ($n \geq 3$). Values without standard deviations represent single experiments. [b] Differential selectivity is the ratio of CEM-SS cell growth IC_{50} to HIV plaque formation IC_{50}. [c] ND = not determined.

Biological Results and Discussion

Evaluation of Anti-HIV-1 Activity. The in vitro antiviral activity of the lipid analogues was determined by using a syncytial plaque assay,[14,27] in which HIV-1 infected CEM-SS cells, a syncytial sensitive clone, were treated with various concentrations of the compounds. The results shown in Tables I and II are expressed as the concentration required to inhibit plaque formation and cell growth by 50% (inhibitory concentration$_{50}$ = IC_{50}). The differential selectivity, defined as the CEM-SS cell growth IC_{50} divided by the HIV-1 plaque formation IC_{50}, was used to identify potential anti-HIV-1 compounds. AZT was evaluated in the in vitro assays as an internal positive control.

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Among the analogues examined, the most selective compounds were in the phosphorus-containing series with compound **8**, an amido analogue being the optimum antiviral compound tested. In general, the order of selectivity based on functionalization of the carbon-1 position of the phospholipids followed the trend (Table I): amide (**8**) > urea (**11**) > oxygen ether (ET-18-OEt) ≥ thioether (**10**) ≥ sulfone (**12**). Preliminary results indicate that the chain length at this position of the three-carbon backbone may affect antiviral activity and toxicity. In the amido phospholipids, a comparison of compounds **6**, **8**, and **17** with carbon-1 moieties of 16, 18 and 20 carbons, respectively, identified the octadecanamido functionality as optimal for antiviral selectivity. Interestingly, **17** and **8** had identical IC_{50} values for the inhibition of plaque formation, however, the eicosanamido analogue was less selective. In the nonphosphorus-containing series (Table II), variations in the functionalization at the carbon-1 position have been less extensive and therefore no conclusions can as yet be proposed. However, initial results showed that the thioalkyl analogue **20** had an enhanced selectivity over the oxygen derivative **33**.

A definitive trend could not be established for the functionalization of the carbon-2 position in the phosphorus-containing series (Table I). Both methoxy and ethoxy moieties provided active compounds. In addition, replacement of the three-carbon backbone with two-carbon derivatives yielded analogues which retained selectivity. The thioethanol derivative **24** exhibited improved antiviral activity in the HIV plaque formation assay and reduced toxicity for cell growth over the thioglycerol analogue **9** resulting in a greater than 4-fold higher differential selectivity. The amidoethanol analogue **26** was more selective than the three-carbon hexadecanamido derivatives **5** and **6**, and the eicosanamido derivative **17**, yet was less selective than the octadecanamido analogue **8**. In the nonphosphorus-containing series (Table II), both a two carbon and three-carbon backbone provided active antiviral analogues. However, no definitive trend in selectivity could be established.

In both series, functionalization at the carbon-3 position appears to require the presence of a formally charged nitrogen in order to maintain anti-HIV activity. The phosphoethanolamine analogues **2** and **3** (Table I) were both inactive as antiviral compounds as was the dialkylglyceride **30** (Table II). The tertiary amine **34** displayed a differential selectivity of approximately 1 which suggests that the antiviral activity exhibited by this compound was due primarily to its cellular toxicity (Table II). Limited structural modifications of the carbon-3 position have been performed in the phospholipid series. In general, replacement of the trimethylamine of the phosphocholine moiety with pyridine resulted in a decrease in the inhibition of plaque formation (ET-18-OEt vs **4**), whereas, substitution of the phosphate with a phosphonate moiety had little or no effect (**4** vs **37**) as seen in Table I. Structural modifications of the quaternary ammonium moiety in the nonphosphorus-containing series have not yet established a preferred substitution. The incorporation of a trimethylamine, dimethylamino alcohol or (dimethylamino)propanediol yielded active analogues (e.g., **20**, **19**, and **22**, respectively, Table II).

In this study, AZT was chosen as a reference compound based on the fact that it is currently the only approved therapeutic agent for the treatment for AIDS. A comparison of the assay results observed for **8**, the most selective analogue identified, and AZT indicated that AZT was more active in the HIV-1 plaque formation inhibition assay while **8** exhibited a lower cytotoxic activity in the CEM-SS cell growth assay (Table I). The differential selectivity index determined for AZT was approximately 17-fold greater than that of **8** suggesting that AZT possesses a greater anti-HIV selectivity. However, it should be noted that AZT does not represent an ideal standard for the lipid analogues. Initial mechanistic studies have indicated that these compounds are not RT inhibitors, but they appear to inhibit a late step in HIV replication involving virus assembly and infectious virus production.[14] Therefore, the observed antiviral activity results from a different mechanism of action from that of AZT. Due to this apparent difference, combination studies with AZT and analogue **8** have been conducted. This combination has been shown to be synergistic in inhibiting HIV-1 replication.[28]

Conclusions

By using a syncytial plaque assay, several EL analogues have been identified which exhibit a dose-dependent anti-HIV-1 activity at concentrations below that required to produce CEM-SS cell cytotoxicity. For example, the most active analogue **8** possessed an IC_{50} value of 0.16 μM for the inhibition of HIV plaque formation which was 84-fold lower than the IC_{50} value determined for cell growth inhibition. Structural moieties which appear to favor antiviral activity include an octadecanamido moiety at the carbon-1 position, and a formally charged nitrogen at the carbon-3 position preferentially incorporated into a phosphocholine functionality.

Although in this study AZT exhibited an enhanced activity compared to the synthetic analogues, it is important to note the apparent differences in the mechanism of action between these compounds. Preliminary results indicate that lipid analogues inhibit infectious HIV-1 production and induce defective virus formation.[14] In the presence of these analogues, virus assembly is shifted from the plasma membrane to intracytoplasmic vacuoles. The mechanism of action of these lipid analogues may involve posttranslational processing of HIV-1 proteins (e.g., proteolytic cleavage, phosphorylation and myristoylation). Since these analogues target a late step in virus replication (i.e., assembly), this information suggests a potential for combination therapy of the lipid analogues with AZT which is known to target reverse transcriptase activity and viral DNA synthesis (i.e., early steps in HIV-1 replication).

Experimental Section

Virus Syncytial Plaque Assay.[14] A modified procedure developed from a previously reported HIV plaque assay was used.[27] Individual wells of a 96-well plate were coated with 100 μL of poly-L-lysine (50 μg/mL, mol wt 90 000), incubated for 30 min at room temperature, and washed three times with 0.01 M phosphate buffered 0.85% (w/v) sodium chloride (PBS). Aliquots of CEM-SS cells in log-phase growth were washed three times with PBS, suspended at a density of 50 000 CEM-SS cells per 50 μL of RPMI-1640 medium without serum, transferred to the precoated wells, and incubated for 30 min at 37 °C in a humidified atmosphere containing 5% CO_2. The cell monolayer was then inoculated with 50 μL of HIV-1 (60–90 plaque forming units) diluted in growth medium. After 60 min, the cell monolayer was overlaid with 100 μL of growth medium with or without test analogues and incubated as before at 37 °C. Serial twofold dilutions of the test compounds (0.03–20.0 μM) were used. After 3 days, each well received a second 100 μL of overlay of growth medium with or without compound(s) and incubation was continued for an additional 2 days. On day 5, the syncytial plaques were counted and the concentration required to inhibit 50% of the plaque formation (IC_{50}) was determined (Tables I and II).

CEM-SS Cell Growth Inhibition Assay.[14] A suspension of CEM-SS cells was treated with test analogues for 48 h at 37 °C. Serial twofold dilutions of compound (0.03–20.0 μM) were used.

Tritiated thymidine (1 μCi per well, specific activity = 20 Ci/mmol) was added to the suspension, and the cells were incubated for 6 h at 37 °C before DNA synthesis was measured and the concentration required to inhibit 50% of cell growth was determined (Tables I and II). In preliminary experiments uninfected CEM-SS cells were seeded in monolayer culture as described for the virus syncytial plaque assay and treated with serial twofold dilutions of the test compound ET-18-OMe starting at 0 h. On day 3, the cultures received a second overlay containing the same concentration of ET-18-OMe plus 1 μCi [^{3}H]-TdR (SA = 20 Ci/mmol) and incubation was continued. The cells were harvested on day 5 to measure radioactivity incorporated into cellular DNA by scintillation spectrometry. The results indicated that for ET-18-OMe the IC_{50} for cell toxicity using monolayer and suspension cell culture conditions was 4.7 and 3.9 μM, respectively. These data indicate no significant difference in IC_{50} values for ET-18-OMe measured by either monolayer or suspension culture conditions. Therefore, all test compounds used in this study were evaluated for cell growth inhibition (IC_{50}) by using the suspension cell culture condition. IC_{50} values for cell growth inhibition were derived prior to IC_{50} values for virus syncytial plaque assay.

Synthetic Methods. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded either on a Bruker 300-MHz or a Varian 400-MHz spectrometer. Chemical shifts were reported in parts per million (δ) relative to tetramethylsilane. Elemental analyses of final products were performed by Atlantic Microlabs, Inc. of Atlanta, GA. FAB mass spectrum was run on a VG 70S mass spectrometer. Column chromatography was performed with the use of silica gel 60 (230–400 mesh). Starting materials were generally purchased from Aldrich Chemical Co., unless otherwise indicated, and were used without further purification.

***rac*-1-*O*-Hexadecyl-2-*O*-ethylglycero-3-phospho-*N*-isopropylethanolamine (2).** A solution of 1,2-dimethylethylene phosphorochloridate, CEP-Cl[19] (0.49 g, 2.9 mmol) in anhydrous Et_2O (10 mL) was cooled to 0 °C. To this was added a solution of 1-*O*-hexadecyl-2-*O*-ethylglycerol[22] (1.0 g, 2.9 mmol) and Et_3N (0.29 g, 2.9 mmol) in anhydrous Et_2O (15 mL). The reaction mixture was allowed to stir at room temperature for 4 h and was then filtered. The solvent was evaporated and the residue (1.2 g) was dissolved in CH_3CN (50 ml). 2-(Isopropylamino)ethanol (0.30 g, 2.9 mmol, Fluka) and Et_3N (0.8 mL, 5.9 mmol) were added and the mixture was stirred at room temperature for 36 h. Water (15 mL) and Et_3N (0.8 g, 5.9 mmol) were then added, and the mixture was heated at 65 °C for 8 h. The solvents were removed. The residue was dissolved in $CHCl_3$, extracted with H_2O and saturated NaCl, dried (Na_2SO_4), and filtered. The filtrate was reduced, and the residue was recrystallized in $CHCl_3$/acetone at 0 °C. Further purification was obtained by column chromatography (gradient of $CHCl_3/MeOH/NH_4OH$, 70:35:1 to 70:35:5) to yield a white solid, **2**: 300 mg, 20.3%; mp 114–120 °C; NMR (400 MHz, $CDCl_3$) δ 0.89 (t, 3 H, CH_3), 1.18 (t, 3 H, OCH_2CH_3), 1.25 (s, 26 H, $(CH_2)_{13}$), 1.38 (d, 6 H, $2CH_3$), 1.55 (m, 2 H, CH_2CH_2O), 3.12 (bs, 2 H, CH_2N), 3.24 (m, 1 H, NCH), 3.35–3.70 (m, 7 H, CH_2OCH_2 and CH_2OCH), 3.95 (bs, 2 H, $POCH_2$), 4.26 (m, 2 H, CH_2OP). Anal. ($C_{26}H_{56}NO_6P\cdot0.5H_2O$) C, H, N.

***rac*-1-*O*-Hexadecyl-2-*O*-ethylglycero-3-phospho-*N*-*tert*-butylethanolamine (3).** Compound **3** was prepared from CEP-Cl[19] (0.49 g, 2.9 mmol), 1-*O*-hexadecyl-2-*O*-ethylglycerol[22] (1.0 g, 2.9 mmol), and 2-(*tert*-butylamino)ethanol (0.30 g, 2.9 mmol, Fluka) by following the identical reaction conditions described above for **2**: 700 mg, 46.1%; mp 180 °C; NMR (400 MHz, $CDCl_3$) δ 0.77 (t, 3 H, CH_3), 1.07 (t, 3 H, CH_3CH_2O), 1.16 (s, 26 H, $(CH_2)_{13}$), 1.28 (s, 9 H, $C(CH_3)_3$), 1.44 (m, 2 H, CH_2CH_2O), 1.67 (bs, 2 H, NH_2), 3.0 (bs, 2 H, CH_2N), 3.2–3.6 (m, 7 H, $CH_2OCHCH_2OCH_2$), 3.8 (t, 2 H, $POCH_2$), 4.1 (m, 2 H, CH_2OP). Anal. ($C_{27}H_{58}NO_6P$) C, H, N.

***rac*-1-*O*-Tosyl-2-*O*-ethylglycerol.** Pyridine (8.0 mL, 99 mmol) was added to a solution of 2-*O*-ethylglycerol[20] (4.0 g, 33 mmol) in CH_2Cl_2 (100 mL). The reaction mixture was cooled to 0 °C prior to the addition of *p*-toluenesulfonyl chloride (3.8 g, 20 mmol). The reaction was allowed to stir at room temperature overnight and was then extracted with ice cold H_2O. The CH_2Cl_2 layer was removed, and the solvent was evaporated. The product was purified by column chromatography (gradient of hexane/EtOAc 5:1 to 2:1) to yield a clear oil: 5.48 g, 63.9%; NMR (300 MHz, $CDCl_3$) δ 1.15 (t, 3 H, CH_3), 2.40 (s, 3 H, $SO_2C_6H_4CH_3$), 3.48–3.69 (m, 5 H, CH_2OCHCH_2OH), 4.07 (d, 2 H, CH_2OS), 7.25 (d, 2 H, ArH), 7.75 (d, 2 H, ArH).

***rac*-1-*O*-Tosyl-2-*O*-ethyl-3-*O*-tritylglycerol (14).** *rac*-1-*O*-Tosyl-2-*O*-ethylglycerol (1.0 g, 3.6 mmol) was dissolved in CH_2Cl_2 (15 mL). Trityl chloride (1.15 g, 4.12 mmol), Et_3N (1 ml, 8.0 mmol), and 4-(dimethylamino)pyridine, DMAP (100 mg, 0.82 mmol), were added, and the mixture was allowed to stir at room temperature overnight. CH_2Cl_2 (20 mL) was added, and the mixture was extracted with H_2O. The organic layer was removed and dried ($MgSO_4$), and the solvent was evaporated. The residue was purified by column chromatography (gradient of hexane/EtOAc, 10:1 to 7:1) to provide the trityl ether as an oil: 1.3 g, 68.4%; NMR (300 MHz, $CDCl_3$) δ 1.10 (t, 3 H, CH_3), 2.43 (s, 3 $SO_2C_6H_4CH_3$), $SO_2C_6H_4CH_3$), 3.19 (d, 2 H, CH_2OTr), 3.43–3.59 (m, 3 H, CH_2OCH), 4.10–4.20 (m, 2 H, CH_2OS), 7.19–7.40 (m, 17 H, ArH), 7.78 (d, 2 H, ArH).

***rac*-1-Amino-2-ethoxy-3-(trityloxy)propane (15).** *rac*-1-*O*-Tosyl-2-*O*-ethyl-3-*O*-tritylglycerol (1.3 g, 2.5 mmol), potassium phthalimide (0.61 g, 3.3 mmol), and DMSO (15 mL) were combined and heated at 55 °C for several hours. DMSO was removed, and the residue was partially purified by chromatography (gradient of hexane/EtOAc, 15:1 to 7:1). The resulting phthalimide (1.3 g) was combined with hydrazine hydrate (0.20 mL, 4.1 mmol) in MeOH (15 mL). The reaction mixture was heated at reflux for 3 h. The solvent was removed. $CHCl_3$ was added to the residue, and the mixture was filtered. The filtrate was reduced, and the residue was purified by column chromatography (gradient of $CHCl_3$/MeOH, 100:0 to 20:1) to provide the amine as an oil: 0.50 g, 55%; NMR (300 MHz, $CDCl_3$) δ 1.15 (t, 3 H, CH_3), 2.82 (m, 2 H, CH_2N), 3.03–3.25 (m, 2 H, CH_2OTr), 3.38–3.70 (m, 3 H, CH_2OCH), 7.21–7.50 (m, 15 H, ArH).

***rac*-1-Eicosanamido-2-ethoxy-3-(trityloxy)propane.** Eicosanoic acid (0.43 g, 1.4 mmol), dicyclohexylcarbodiimide, DCC (0.34 g, 1.7 mmol), and DMAP (0.10 g, 0.82 mmol) were added to a solution of *rac*-1-amino-2-ethoxy-3-(trityloxy)propane (0.50 g, 1.4 mmol) in CH_2Cl_2 (15 mL). The reaction mixture was allowed to stir at room temperature overnight. Additional CH_2Cl_2 was added and the mixture was filtered. The filtrate was extracted with H_2O, dried ($MgSO_4$), and concentrated. The residue was purified by column chromatography (gradient of hexane/EtOAc, 9:1 to 4:1) to yield the amide: 0.65 g, 71.1%; mp 77–79 °C; NMR (300 MHz, $CDCl_3$) δ 0.89 (t, 3 H, CH_3), 1.17–1.30 (m, 35 H, $(CH_2)_{16}$ and CH_3), 1.53 (m, 2 H, $(C{=}O)CH_2CH_2$), 2.07 (t, 2 H, $(C{=}O)CH_2$), 3.10–3.60 (m, 7 H, CH_2OCHCH_2OTr, CH_2N), 5.73 (m, 1 H, NH), 7.21–7.45 (m, 15 H, ArH).

***rac*-3-Eicosanamido-2-ethoxy-1-propanol (16).** *p*-Toluenesulfonic acid (0.10 g, 0.58 mmol) was added to a solution of *rac*-1-eicosanamido-2-ethoxy-3-(trityloxy)propane (0.65 g, 0.99 mmol) in $CHCl_3$/MeOH (3:1, 20 mL). The reaction mixture was allowed to stir at room temperature for 2 days. Saturated $NaHCO_3$ (1 mL) was added, and the mixture was stirred for 15 min. The mixture was then extracted with H_2O, and the organic layer was removed, dried ($MgSO_4$), and concentrated. Column chromatography (gradient of $CHCl_3$/MeOH, 9:1 to 5:1) was used to purify the alcohol: 0.38 g, 94.0%; mp 87–88 °C; NMR (300 MHz, $CDCl_3$) δ 0.90 (t, 3 H, CH_3), 1.15–1.38 (m, 35 H, $(CH_2)_{16}$ and CH_3), 1.60 (m, 2 H, $(C{=}O)CH_2CH_2$), 2.26 (t, 2 H, $(C{=}O)CH_2$), 3.28–3.72 (m, 7 H, CH_2OCHCH_2OH, CH_2N), 5.80 (m, 1 H, NH).

***rac*-3-Eicosanamido-2-ethoxypropyl 2′-Bromoethyl Phosphate.** To a solution of *rac*-3-eicosanamido-2-ethoxy-1-propanol (0.20 g, 0.48 mmol) in anhydrous Et_2O/THF (2:1, 12 mL) at 0 °C was added anhydrous pyridine (0.59 mL, 7.3 mmol) followed by 2-bromoethyl dichlorophosphate[21] (0.26 mL, 1.9 mmol). The reaction mixture was maintained at 0 °C for 30 min and then was heated at a gentle reflux for 4 h. The heat was removed, H_2O was added (2 mL), and the mixture was stirred an additional 30 min at room temperature. The solvent was removed. The residue was dissolved in $CHCl_3$/MeOH (2:1) and extracted with H_2O. Following the removal of the solvent, the residual oil was purified by column chromatography (gradient of $CHCl_3$/MeOH, 100:0 to 3:1) to obtain the phosphate ester: 150 mg, 51.7%; NMR (300 MHz, $CDCl_3$) δ 0.90 (t, 3 H, CH_3), 1.12–1.40 (m, 35 H, $(CH_2)_{16}$ and CH_3), 1.60 (m, 2 H, $(C{=}O)CH_2CH_2$), 2.28 (m, 2 H, $(C{=}O)CH_2$), 3.31–3.70 (m, 7 H, CH_2OCH, CH_2N, CH_2Br), 3.90 (m, 2 H, CH_2OP), 4.19 (m, 2 H, $POCH_2$), 6.65 (bs, 1 H, NH).

rac-3-Eicosanamido-2-ethoxypropylphosphocholine (17). Trimethylamine (6.4 M aqueous solution, 2 mL, 12.5 mmol, Fluka) was added at room temperature to a solution of *rac*-3-eicosanamido-2-ethoxypropyl 2′-bromoethyl phosphate (0.15 g, 0.25 mmol) in 2-propanol/$CHCl_3$/DMF (5:3:5, 13 mL). The reaction mixture was heated at 65 °C for 5 h and then allowed to cool to room temperature before Ag_2CO_3 (90 mg, 0.33 mmol) was added. Heat was reapplied for 1 h. The reaction mixture was allowed to cool to room temperature and then was filtered. The filtrate was reduced. The phospholipid was purified by column chromatography (gradient of $CHCl_3$ to $CHCl_3$/MeOH/NH_4OH, 70:35:7) to yield **17**: 68 mg, 47.2%; dec > 200 °C; NMR (300 MHz, $CDCl_3$) δ 0.89 (t, 3 H, CH_3), 1.10–1.33 (m, 35 H, $(CH_2)_{16}$ and CH_3), 1.60 (m, 2 H, $(C{=}O)CH_2CH_2$), 2.10 (t, 2 H, $(C{=}O)CH_2$), 3.30–3.66 (m, 15 H, CH_2O, CH_2N, $CH_2N(CH_3)_3$), 3.72–4.08 (m, 3 H, $CHCH_2OP$), 4.35 (m, 2 H, $POCH_2$), 6.97 (m, 1 H, NH). Anal. ($C_{30}H_{63}N_2O_6P{\cdot}H_2O$) C, H, N.

rac-N,N-Dimethyl-N-[3-(hexadecylthio)-2-methoxypropyl]-N-(2,3-dihydroxypropyl)ammonium Bromide (22). *rac*-3-(Hexadecylthio)-2-ethoxy-1-bromopropane[11] (2.0 g, 4.9 mmol) and *N,N*-dimethyl-*N*-(2,3-dihydroxypropyl)amine (0.60 ml, 5.0 mmol) were combined in CH_3CN (30 mL). The reaction mixture was heated at 60 °C for 4 days. Et_2O was added to precipitate the product. The solid was recrystallized with Et_2O and $CHCl_3$ to provide **22**: 120 mg, 4.6%; mp 81–85 °C; NMR (300 MHz, $CDCl_3$) δ 0.8 (t, 3 H, CH_3), 1.00–1.35 (m, 26 H, $(CH_2)_{13}$), 1.50 (m, 2 H, SCH_2CH_2), 2.50 (t, 2 H, SCH_2), 2.75 (m, 2 H, $CHCH_2S$), 3.35 (m, 9 H, OCH_3 and $N(CH_3)_2$), 3.40–3.90 (m, 4 H, $CHCH_2OH$ and $CHOCH_3$), 4.00–4.40 (m, 4 H, CH_2NCH_2). Anal. ($C_{25}H_{54}NO_3SBr$) C, H, N.

2-(Hexadecylthio)ethanol (23). Thioethanol (5.0 g, 64 mmol), hexadecyl bromide (25.0 g, 82 mmol) and KOH (4.5 g, 80 mmol) were combined in 95% EtOH (150 mL). The reaction mixture was stirred at room temperature overnight and then diluted with H_2O. The precipitate was collected and recrystallized from MeOH to provide the thioether: 19.0 g, 96%; mp 50 °C; NMR (300 MHz, $CDCl_3$) δ 0.89 (t, 3 H, CH_3), 1.30 (m, 26 H, $(CH_2)_{13}$), 1.60 (m, 2 H, CH_2CH_2S), 2.52 (t, 2 H, SCH_2), 2.72 (m, 2 H, CH_2S), 3.72 (m, 2 H, CH_2OH).

2-(Hexadecylthio)ethylphosphocholine (24). 2-(Hexadecylthio)ethanol (1.0 g, 3.0 mmol) and Et_3N (0.40 g, 4.0 mmol) were dissolved in anhydrous benzene (75 mL). The solution was cooled to 0 °C before a solution of 2-chloro-2-oxo-1,3,2-dioxaphospholane (0.65 g, 4.6 mmol, Fluka) in anhydrous benzene was slowly added. The reaction mixture was stirred overnight at room temperature and then filtered. The filtrate was reduced, and the residue was dissolved in CH_3CN (50 ml) and transferred to a glass bomb. Condensed $N(CH_3)_3$ (2.0 g, 34 mmol) was added, and the mixture was heated at a gentle reflux for 24 h. Upon cooling of the reaction mixture, a white precipitate formed. The solid was removed and recrystallized with Et_2O to provide **24**: 980 mg, 70%; dec > 200 °C; NMR (400 MHz, $CDCl_3$) δ 0.86 (t, 3 H, CH_3), 1.21 (s, 26 H, $(CH_2)_{13}$), 1.53 (m, 2 H, CH_2CH_2S), 2.51 (t, 2 H, SCH_2), 2.72 (t, 2 H, CH_2S), 3.37 (s, 9 H, $N(CH_3)_3$), 3.81 (m, 2 H, CH_2N), 3.91 (m, 2 H, CH_2OP), 4.31 (m, 2 H, $POCH_2$); FAB MS *m/e* 468 (MH^+).

2-Octadecanamidoethanol (25). 2-Aminoethanol (6.0 g, 0.10 mol) and pyridine (35 mL, 0.43 mol) were combined in DMF (80 mL). A solution of octadecanoyl chloride (21.0 g, 70 mmol) in DMF (35 mL) was added, and the mixture was stirred at room temperature for 2 h. A precipitate formed which was filtered, washed with H_2O and recrystallized first with EtOH and then $CHCl_3$ to obtain the amide: 10.0 g, 43.6%; mp 102–103 °C; NMR (300 MHz, $CDCl_3$) δ 0.90 (t, 3 H, CH_3), 1.30 (m, 28 H, $(CH_2)_{14}$), 1.50–1.70 (m, 2 H, $C({=}O)CH_2CH_2$), 2.25 (t, 2 H, $(C{=}O)CH_2$), 3.45 (m, 2 H, CH_2N), 3.75 (t, 2 H, CH_2OH), 5.9 (1 H, NH).

2-Octadecanamidoethyl 2′-Bromoethyl Phosphate. The compound was prepared in an analogous manner to that of *rac*-3-eicosanamido-2-ethoxypropyl 2′-bromoethyl phosphate with 2-octadecanamidoethanol (2.0 g, 6.1 mmol), 2-bromoethyl dichlorophosphate[21] (5.0 g, 21 mmol), anhydrous pyridine (7.3 ml, 91 mmol), anhydrous Et_2O (150 mL), and H_2O (6 mL). Purification by column chromatography (gradient of $CHCl_3$/petroleum ether, 5:2 to $CHCl_3$/MeOH, 10:1) provided bromide: 1.2 g, 40.0%; NMR (300 MHz, $CDCl_3$) δ 0.90 (t, 3 H, CH_3), 1.30 (m, 28 H, $(CH_2)_{14}$), 1.50–1.70 (m, 2 H, $C({=}O)CH_2CH_2$), 2.25 (t, 2 H, $(C{=}O)CH_2$), 3.50 (m, 4 H, CH_2Br and CH_2N), 4.00 (m, 2 H, CH_2OP), 4.2 (m, 2 H, $POCH_2$).

2-Octadecanamidoethylphosphocholine (26). By following a procedure analogous to the method described for **17**, **26** was prepared with 2-octadecanamidoethyl 2′-bromoethyl phosphate (500 mg, 0.97 mmol), $(CH_3)_3N$ (6.4 M aqueous solution, 6 mL, 38 mmol), Ag_2CO_3 (300 mg, 1.1 mmol), and $CHCl_3$/2-propanol/DMF (3:5:5, 39 mL). The pure phospholipid was obtained by column chromatography (gradient of $CHCl_3$/MeOH, 10:1 to 2:1): 300 mg, 62.6%; dec > 200 °C; NMR (300 MHz, $CDCl_3$) δ 0.90 (t, 3 H, CH_3), 1.30 (m, 28 H, $(CH_2)_{14}$), 1.60 (m, 2 H, $C({=}O)CH_2CH_2$), 2.20 (t, 2 H, $(C{=}O)CH_2$), 3.30 (s, 9 H, $N(CH_3)_3$), 3.35–3.40 (m, 2 H, CH_2N), 3.80 (m, 2H, $CH_2N(CH_3)_3$), 3.90 (m, 2 H, CH_2OP), 4.30 (m, 2 H, $POCH_2$), 7.70 (m, 1 H, NH). Anal. ($C_{25}H_{53}N_2O_5P{\cdot}0.75H_2O$) C, H, N.

N,N-Dimethyl-N-[2-(octadecyloxy)ethyl]-N-(2,3-dihydroxypropyl)ammonium Iodide (27). *rac*-3-(Dimethylamino)-1,2-propanediol (0.56 g, 4.7 mmol) was added to a solution of 1-(octadecyloxy)-2-iodoethane[26] (2.0 g, 4.7 mmol) in DMF (30 mL). The reaction mixture was maintained at a temperature of 45–50 °C for 72 h with continuous stirring. The reaction mixture was then cooled to room temperature before Et_2O (40 mL) was added. The resulting precipitate was filtered and washed with Et_2O to provide a white solid (2.5 g, 83% crude yield). An analytical sample was purified by column chromatography (gradient of $CHCl_3$/MeOH, 100:0 to 4:1) to yield **27**: 120 mg; mp 96–98 °C; NMR (300 MHz, $CDCl_3$) δ 0.90 (t, 3 H, CH_3), 1.30 (m, 30 H, $(CH_2)_{15}$), 1.60 (m, 2 H, OCH_2CH_2), 3.45 (s, 6 H, $N(CH_3)_2$), 3.50 (t, 2 H, OCH_2), 3.60–4.00 (m, 7 H, $OCH_2CH_2NCH_2CHCH_2OH$), 4.30 (m, 1 H, NCHH′), 4.60 (m, 1 H, NCHH′). Anal. ($C_{25}H_{54}NO_3I$) C, H, N.

N-(β-Hydroxyethyl)pyridinium Bromide. 2-Bromoethanol (6.6 g, 53.0 mmol) was dissolved in pyridine (5.2 g, 65.0 mmol). The solution was heated at 110 °C for 7 h. A precipitate formed which was recrystallized with a mixture of EtOH/MeOH/Et_2O (3:5:6) to yield the pyridinium salt: 8.0 g, 74%; mp 97–102 °C.

rac-Dimethyl 3-(Hexadecyloxy)-2-methoxypropylphosphonate. A mixture of *rac*-3-(hexadecyloxy)-2-methoxy-1-bromopropane[22] (454 mg, 2.4 mmol) and trimethylphosphite (4.9 g, 30 mmol) was warmed at 120–130 °C for 90 h. The low boiling point material was removed, and the residue was purified by column chromatography (gradient of petroleum ether/Et_2O, 10:1 to 1:1) to obtain the phosphonate as a viscous oil: (741 mg, 80%); NMR (400 MHz, $CDCl_3$) δ 0.87 (t, 3 H, CH_3), 1.25 (m, 26 H, $(CH_2)_{13}$), 1.57 (m, 2 H, CH_2CH_2O), 2.08 (m, 2 H, CH_2P), 3.43 (s, 3 H, CH_3O), 3.45 (t, 2 H, CH_2CH_2O), 3.56 (d, 2 H, $CHCH_2O$), 3.75 (m, 7 H, CH_3OCH and $P(OCH_3)_2$).

rac-3-(Hexadecyloxy)-2-methoxypropylphosphonic Acid (36). To a solution of *rac*-3-(hexadecyloxy)-2-methoxypropyl dimethyl phosphonate (3.2 g, 7.5 mmol) and alcohol-free $CHCl_3$ (8 ml) was added dropwise bromotrimethylsilane (7.0 g, 46 mmol). The reaction mixture was allowed to stir at room temperature for 1 h. The reaction mixture was reduced, and the residue was dissolved in a mixture of THF/H_2O (8:2, 10 mL). The mixture was allowed to stir at room temperature overnight. The solvent was then evaporated and the residue was recrystallized with Et_2O/CH_3CN to provide the deprotected phosphonic acid: 2.5 g, 84%; mp 59–61 °C; NMR (400 MHz, $CDCl_3$) δ 0.86 (t, 3 H, CH_3), 1.26 (s, 26 H, $(CH_2)_{13}$), 1.57 (m, 2 H, CH_2CH_2O), 2.12 (m, 2 H, CH_2P), 3.44 (s, 3 H, CH_3O), 3.46 (t, 2 H, CH_2CH_2O), 3.58 (m, 2 H, $CHCH_2O$), 3.74 (m, 1 H, CH_3OCH).

rac-2′-Pyridylethyl 3-(Hexadecyloxy)-2-methoxypropylphosphonate (37). The phosphonic acid **36** was initially dissolved in 15 mL of pyridine, reduced in vacuo, and dried under vaccum for 8 h prior to use. *N*-(β-hydroxyethyl)pyridinium bromide (270 mg, 1.3 mmol), *rac*-3-(hexadecyloxy)-2-methoxypropylphosphonate (560 mg, 1.0 mmol) and triisopropylbenzenesulfonyl chloride, TPSCl (533 mg, 1.77 mmol) were combined in anhydrous pyridine (10 mL). The reaction mixture was stirred at room temperature overnight before H_2O (1 mL) and 2-propanol (4 mL) were added. The solvents were removed, and the residue dissolved in a mixture of $CHCl_3$/CH_3CN/petroleum ether (3:8:4) and cooled to 0 °C. After a precipitate formed, the mixture was filtered, the filtrate was reduced, and the residue was purified by preparative TLC ($CHCl_3$/MeOH/H_2O, 45:40:5) to provide **37**: 410 mg, 52%; mp 83–87 °C dec; NMR (400 MHz, $CDCl_3$) δ 0.83 (t, 3 H, CH_3), 1.28

(s, 26 H, $(CH_2)_{13}$), 1.57 (m, 2 H, CH_2CH_2O), 2.8 (m, 2 H, CH_2P), 3.38–3.78 (m, 8 H, $CH_3OCHCH_2OCH_2$), 4.38 (bs, 2 H, $POCH_2$), 5.09 (m, 2 H, CH_2N), 8.03 (m, 2 H, pyridine), 8.40 (m, 1 H, pyridine), 9.49 (d, 2 H, pyridine). Anal. ($C_{27}H_{50}NO_5P{\cdot}1H_2O$) C, H, N.

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Registry No. **2**, 131973-30-3; **3**, 131933-48-7; **4**, 127642-24-4; **5**, 112989-00-1; **6**, 112989-01-2; **7**, 88876-07-7; **8**, 112989-02-3; **9**, 103304-64-9; **10**, 103304-65-0; **11**, 112989-09-0; **12**, 131933-49-8; **13**, 22598-16-9; **14**, 131973-31-4; **15**, 131933-50-1; **16**, 131933-51-2; **16** trityl derivative, 131933-60-3; **16** 2-bromoethyl phosphate derivative, 131933-61-4; **17**, 131933-52-3; **18**, 124581-78-8; **19**, 124581-94-8; **20**, 124581-81-3; **21**, 124581-79-9; **22**, 131933-53-4; **23**, 23248-47-7; **24**, 131933-54-5; **25**, 111-57-9; **26**, 82755-92-8; **27**, 131933-56-7; **28**, 119980-18-6; **29**, 119980-19-7; **30**, 92758-87-7; **31**, 131933-57-8; **32**, 126614-08-2; **33**, 126614-06-0; **34**, 131933-58-9; **35**, 126614-21-9; **36**, 131933-59-0; **36** dimethyl ester, 131933-63-6; **37**, 131933-64-7; Et-18-OMe, 70641-51-9; Et-18-OEt, 78858-43-2; AZT, 30516-87-1; 1-*O*-hexadecyl-2-*O*-ethylglycerol, 92758-87-7; *rac*-1-*O*-tosyl-2-*O*-ethylglycerol, 131973-32-5; *rac*-3-(hexadecylthio)-2-ethoxy-1-bromopropane, 124581-76-6; *N*,*N*-dimethyl-*N*-(2,3-dihydroxypropyl)amine, 98923-15-0; 2-(octadecanamido)ethyl 2′-bromoethyl phosphate, 131933-62-5; 1-(octadecyloxy)-2-iodoethane, 90339-56-3; *N*-(β-hydroxyethyl)pyridinium bromide, 31678-16-7; reverse transcriptase, 9068-38-6.

Receptor-Based Design of Novel Dihydrofolate Reductase Inhibitors: Benzimidazole and Indole Derivatives

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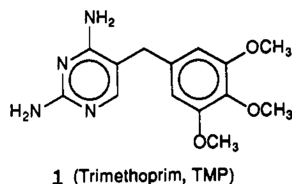
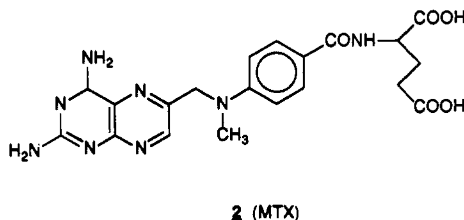
Although many thousands of inhibitors of the enzyme dihydrofolate reductase (DHFR) have been synthesized, all of the very active compounds have been 2,4-diaminopyrimidines or very close analogues. This paper describes 2,4-diamino-6-benzylbenzimidazole (**3b**) and the corresponding indole (**4**), as well as more complex tri- and tetracyclic derivatives (**5** and **6**). These were designed on the basis of molecular modeling to the known X-ray structure of *Escherichia coli* DHFR, in an effort to determine whether one could drastically alter the diamino configuration by placing one amino substituent in a 5-membered nitrogen-containing ring and the second in the ortho position of a fused ring and still inhibit DHFR significantly. Although the electronics and bond angles are quite different from that of a 2,4-diaminopyrimidine, the pK_a values are in an appropriate range, and hydrogen-bond distances appear to be quite reasonable. The most active compound, **4**, was very unstable and active only in the 10^{-4} M range. Dihydroindenoimidazole derivatives such as **6** showed quite a good fit to the enzyme by modeling studies, but had low activity. Since the most active compound made was 2 orders of magnitude weaker as an inhibitor of bacterial DHFR than the unsubstituted 5-benzyl-2,4-diaminopyrimidine, we concluded that such a ring system was unlikely to produce the high inhibitory potency of trimethoprim (**1**), even with greatly improved hydrophobic contacts. Thus the 2,4-diaminopyrimidine system remains unparalleled to date for the competitive inhibition of this enzyme.

Successful inhibitors of dihydrofolate reductase (DHFR, EC 1.5.1.3), such as trimethoprim (**1**) and methotrexate (**2**), have in almost every case been based on the 2,4-diaminopyrimidine skeleton or on closely allied 1,2,4-triazine or 1,3,5-dihydrotriazine analogues.[1] Prior to elucidation



1 (Trimethoprim, TMP)



2 (MTX)

of the 3-dimensional structure of this enzyme many other substituent patterns, as well as other ring systems, were examined for their inhibitory properties, but none possessed the apparent unique properties of this original prototype.

The 3-dimensional structures of DHFR from *Escherichia coli, Lactobacillus casei*, chicken liver, mouse liver lymphoma, and human DHFR have been solved and refined in the presence of several ligands,[2–7] and it is now known that a very complex hydrogen-bonding pattern exists between a protonated diaminopyrimidine and the protein, involving all of the available hydrogen atoms.[5] In

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