Syntheses of Racemic and Nearly Optically Pure Ether Lipids and Evaluation of *in Vitro* Antineoplastic Activities

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In addition to *rac*-2-*O*-methyl-1-*O*-octadecylglycero-3-phosphocholine (*rac*-ET-18-OCH_3, *rac*-Edelfosine, **1**), three racemic ether lipid analogs, **4**, **5**, and **6**, were synthesized where *N*,*N*-dimethylamino, *N*-methylpyrrolidino, and *N*-methylmorpholino groups, respectively, have been substituted for the trimethylammonio group. The two enantiomers, (*R*)-ET-18-OCH_3 (**2**) and (*S*)-ET-18-OCH_3 (**3**), and all four possible chiral methylcholine analogs, **7**, **8**, **9**, and **10**, of (*R*)-ET-18-OCH_3 (**2**) were also synthesized. Three human leukemic cell lines (CEM, HUT 78, and Namalwa) were used to assess the *in vitro* antineoplastic properties of these 10 ether lipid analogs. At ether lipid concentrations of 5–50 μg/mL, dose- and time-dependent cytotoxicities were demonstrated up to 24 h. CEM and HUT 78, both T cell derived, were more sensitive to the ether lipids than Namalwa, which is B cell derived. *rac*-ET-18-OCH_3 (**1**) with its *R* and *S* enantiomeric forms, **2** and **3**, respectively, exhibited modest stereoselectivity in HUT 78 and Namalwa with **1** and **2** slightly more cytotoxic than **3**. Ether lipid (EL) analogs **4**, **5**, and **6** demonstrated significantly greater cytotoxicity in normal peripheral lymphocytes, **4** and **6** exhibited a modest increase in cytotoxicity in HUT 78 and Namalwa ($P < 0.05$), and **5** demonstrated greater cytotoxicity ($P < 0.05$) in Namalwa than the parent EL **1**. The calculated 24 h ID_{50} values suggest that the β-methyl analogs, **9** and **10**, were more cytotoxic than the α-methyl analogs, **7** and **8**, in all the tested cancer cell lines.

Introduction

Ether lipids (EL, alkyl lysophospholipid, ALP) have received considerable attention due to their antineoplastic and immunomodulatory activities, which have been the subjects of several recent reviews.[1] The most studied of the ether lipids include *rac*-2-*O*-methyl-1-*O*-octadecylglycero-3-phosphocholine (*rac*-ET-18-OCH_3, *rac*-Edelfosine, **1**),[2,3] BM 41.440 (Ilmofosine),[4] hexadecylphosphocholine (Miltefosine),[5] SRI 62-834,[6] and others.[7] *rac*-ET-18-OCH_3 (**1**) has a long *O*-alkyl tail at C1, a short nonpolar *O*-alkyl group at C2, and a polar phosphocholine head group at C3 and is the parent compound of all antineoplastic and immunomodulatory ether lipids. The unique molecular mechanism(s) of action of *rac*-ET-18-OCH_3 (**1**) and related ether lipids seems to be due to its interaction with the plasma membrane.[8] In spite of the somewhat similar structures of antineoplastic ether lipids to platelet activating factor, its known agonists, and antagonists, the binding of antineoplastic ether lipids to the platelet activating factor receptor does not seem to be essential for neoplastic cytotoxicity.[4b,6b,7f] No molecular mechanism has yet emerged to unambiguously explain the antineoplastic and immunomodulatory activities of ether lipids.

rac-ET-18-OCH_3 (**1**) was first synthesized as a racemic mixture.[3] However, recently, both enantiomers, ((*R*)-ET-18-OCH_3, **2**)[9a–n,10] and ((*S*)-ET-18-OCH_3, **3**),[9h–o] became available for biological studies. In addition to the optically pure enantiomers of the ether lipid ET-18-OCH_3, **2** and **3**, we were particularly interested in the antineoplastic properties and other biological activities of head group analogs where other polar groups have been substituted for choline. A number of such head group analogs which are derivatives of *rac*-2-*O*-methyl-1-*O*-octadecylglycerol have already been reported in the literature including the 3-phosphoethanolamine,[3c,9j,11,12] 3-phospho-*N*-methylethanolamine,[9j] 3-phospho-*N*,*N*-dimethylethanolamine (**4**),[9j] 3-phospho-β-pyridinioethanol,[3c] 3-phospho-β-thiazoloethanol,[4b] 3-phospho-ω-(trimethylammonio)decanol,[13] 3-phosphate,[12,14] 3-phosphoserine,[12] 3-*O*-β-D-glucopyranose,[15,16] 3-*O*-β-D-maltose,[15] 3-phospho-*myo*-inositol,[17] the 3-diphospho-1-β-D-arabinofuranosylcytosine derivative,[14,18] and other glycolipid derivatives.[19] The syntheses of a number of optically active ether lipids have also included 2-*O*-methyl-1-*O*-octadecyl-*sn*-glycero-3-phosphoserine[20,21] and the corresponding (2*S*)-diastereomer,[20] 2-*O*-methyl-1-*O*-octadecyl-*sn*-glycero-3-phospho-*sn*-1′-glycerol sodium salt[20] and the corresponding (2*S*)-diastereomer,[20] 2-*O*-methyl-1-*O*-octadecyl-*sn*-glycero-3-*O*-β-D-glucopyranose[15] and the corresponding (2*S*)-isomer,[15] as well as 2-*O*-methyl-1-*O*-octadecyl-*sn*-glycero-3-phosphatidic acid ((*R*)-**13**),[9f,1,21–23] and other glycolipid derivatives[24] of 2-*O*-methyl-1-*O*-octadecyl-*sn*-glycerol ((*S*)-**11**). Racemic methylcholine analogs of *rac*-1,2-di-*O*-palmitoylglycero-3-phosphocholine,[25] 1,2-di-*O*-palmitoyl-*sn*-glycero-3-phosphocholine,[26] racemic lyso-platelet activating factor,[27] racemic platelet activating factor,[27] and the synthetic intermediate *rac*-2-*O*-benzyl-1-*O*-hexadecylglycero-3-phosphocholine[27] had previously been synthesized, but only two derivatives of optically active methylcholine, (2*R*,a*R*)-1,2-di-*O*-palmitoyl-*sn*-glycero-3-phospho-α-methylcholine[26] and the corresponding (α*S*)-diastereomer,[26] were synthesized. The racemic 2-

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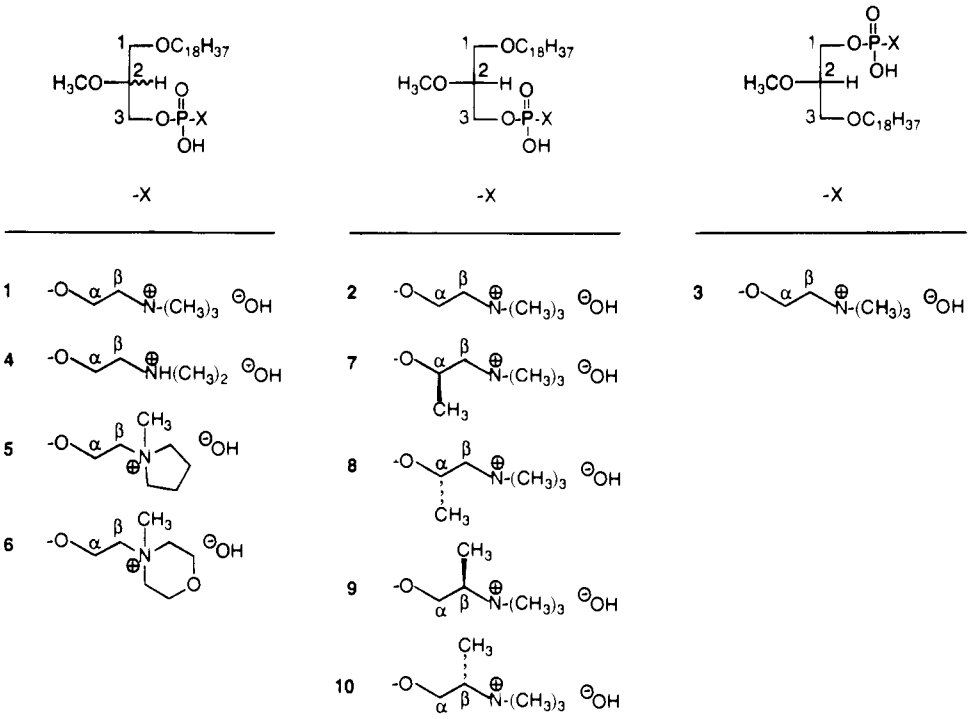


Figure 1. Structures of ether lipids.

methyl analog of *rac*-ET-16-OCH_3, which has the additional methyl group in the glycerol portion of the ether lipid molecule, was reported to have the same *in vitro* cytotoxic activity as ET-16-OCH_3 itself against HL-60 cells.[28]

In addition to the two enantiomers **2** and **3** of *rac*-ET-18-OCH_3 (**1**),[9,10] there are six other enantiomeric pairs of ether lipids including the two pairs of enantiomeric 1,4-dioxanyl analogs of ET-18-OCH_3,[7j,29] the phytanyl analogs,[30a] the hexadecyl analogs (ET-16-OCH_3),[30] as well as the corresponding phosphonate ether lipid analogs,[31] and the phosphonate thioether lipid analogs.[31] Chiral ether lipids for which both enantiomers are available are necessary to investigate stereochemical and structural characteristics and their correlated antineoplastic or immunomodulatory activities which would be characteristic of more specific receptor-mediated mechanisms of activities. All seven pairs of enantiomers have been evaluated for neoplastic cytotoxicities. The (*R*)-ET-18-OCH_3 (**2**) and (*S*)-ET-18-OCH_3 (**3**) enantiomers have also been evaluated for macrophage activation[9i,j] and inhibition of protein-mediated glucose transport.[9m] Enantiomeric ether lipids have generally exhibited very similar activities *in vitro* and somewhat less similar activities *in vivo*.

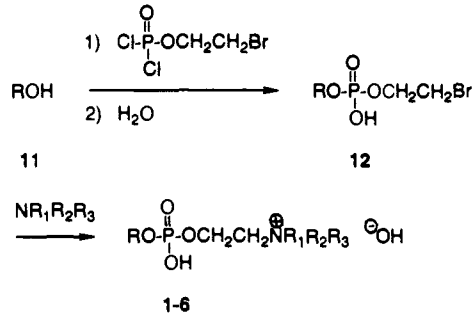
We have been interested in the structural and stereochemical characteristics of amphipathic ether lipids, which result in their preferential accumulation in neoplastic cells, and their selective effects on these membranes, both of which are properties which may be responsible for their selective cytotoxicity and/or immunomodulation. We selected a few literature compounds (**1**, **4**) to be resynthesized, and prepared some new analogs (**5**, **6**) to probe the effects on *in vitro* direct antineoplastic activity of various polar head group analogs of *rac*-ET-18-OCH_3 (**1**) which have variations in steric bulk and polarity (see Figure 1). The two optical antipodes **2** and **3** were also resynthesized and tested to evaluate the effect of stereochemistry on neoplastic cytotoxicity. Also, the four possible methylcholine head group analogs, **7**, **8**, **9**, and **10**, of (*R*)-ET-18-OCH_3 (**2**) were synthesized, and these four novel chiral methylcholine analogs were tested to probe small stereochemical and steric changes in the parent ether lipid structure for possible differences in efficacy of neoplastic cytotoxicity.

We used three leukemic cell lines (CEM, HUT 78, and Namalwa) to assess the *in vitro* antineoplastic properties of these 10 ether lipids.[32] The cell lines were incubated with various concentrations of ether lipid (5–50 μg/mL) up to 24 h. Cytotoxic properties were estimated by quantifying lactic dehydrogenase (LDH) leakage into the media.[8a,30a,32–34] In general, the ether lipids tested demonstrated dose- and time-dependent cytotoxic effects. Variations in sensitivity among the three leukemic cell lines and the ether lipids tested were noted. CEM and HUT 78, both derived from T lymphocytes, were more sensitive to the ether lipids than Namalwa, derived from B lymphocytes. Among the ether lipids tested, there were differences in the rates of cytolysis, the overall cytotoxicity, and the selective toxicity to the leukemic cell lines relative to normal peripheral lymphocytes.

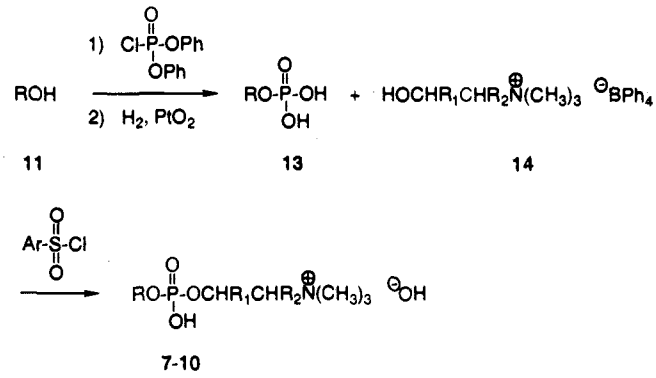
Chemistry

The ether lipid analogs **1**–**6** were synthesized via β-bromoethyl phosphodiesters **12** according to Scheme 1. *rac*-ET-18-OCH_3 (**1**)[3,12] was synthesized via *rac*-2-*O*-methyl-1-*O*-octadecylglycero-3-phospho-β-bromoethanol (*rac*-**12**)[3] from *rac*-2-*O*-methyl-1-*O*-octadecylglycerol (*rac*-**11**).[3,9h,12,14] (*R*)-ET-18-OCH_3 (**2**)[9a–n] was prepared via (*R*)-**12**[9c,l] from (*S*)-**11**.[9c,h,l,n,23,24,35] The enantiomeric (*S*)-ET-18-OCH_3 (**3**)[9h–o] was prepared via the corresponding (*S*)-**12**[9l] from (*R*)-**11**.[9h,l,n,35] The *rac*-**11**, (*S*)-**11** (99.0 ± 0.5% ee), and (*R*)-**11** (99.5 ± 0.5% ee) were prepared from *rac*-1,2-*O*-isopropylideneglycerol, 2,3-*O*-isopropylidene-*sn*-glycerol, and 1,2-*O*-isopropylidene-*sn*-glycerol, respectively, by the method reported for the

Scheme 1. Synthetic Methodology for Ether Lipids **1–6** via β-Bromoethyl Phosphodiester **12** (R = $H_{37}C_{18}OCH_2CHOCH_3CH_2$)



Scheme 2. Synthetic Methodology for Ether Lipids **7–10** via Phosphate (*R*)-**13** (R = $H_{37}C_{18}OCH_2CHOCH_3CH_2$, Ar = 2,4,6-Triisopropylphenyl)



preparation of 1-*O*-alkyl-2-*O*-alkyl'-*sn*-glycerols and chiral purities were determined by the reported NMR method.[36] Alkylation of dimethylamine with *rac*-**12** gave **4**.[9j] Quaternizations of *N*-methylpyrrolidine and *N*-methylmorpholine with *rac*-**12** gave the two new EL analogs **5** and **6**, respectively.

We synthesized each of the four novel methylcholine analogs, **7**, **8**, **9**, and **10**, of (*R*)-ET-18-OCH_3 (**2**) according to Scheme 2. Each isomeric EL analog was nearly optically pure (highly scalemic). The 2-*O*-methyl-1-*O*-octadecyl-*sn*-glycero-3-phosphatidic acid ((*R*)-**13**),[9f,l,21–23] was prepared from 2-*O*-methyl-1-*O*-octadecyl-*sn*-glycerol ((*S*)-**11**) (99.0 ± 0.5% ee) by phosphorylation with chloro diphenyl phosphate followed by hydrogenolysis. We then quaternized[25,37] each of the four commercially available (Aldrich, Milwaukee, WI) optically active amino alcohols, (*R*)-(−)-1-amino-2-propanol, (*S*)-(+)-1-amino-2-propanol, (*R*)-(−)-2-amino-1-propanol, and (*S*)-(+)-2-amino-1-propanol, to give the corresponding optically active methylcholine tetraphenyl borates. Condensation of each of the methylcholine tetraphenyl borates with (*R*)-**13** in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride gave (2*R*,α*R*)-2-*O*-methyl-1-*O*-octadecyl-*sn*-glycero-3-phospho-α-methylcholine (**7**), the (α*S*)-diasteromer **8**, (2*R*,β*R*)-2-*O*-methyl-1-*O*-octadecyl-*sn*-glycero-3-phospho-β-methylcholine (**9**), and the (β*S*)-diasteriomer **10**, respectively.

Results and Discussion

The cytotoxicity of the EL analogs with regard to the three leukemic cell lines, CEM, HUT 78, and Namalwa, as well as to normal peripheral lymphocytes, was assessed by measurement of lactic dehydrogenase (LDH) leakage.[8a,11a,30–32] The cell lines were incubated with various concentrations of EL (5, 10, 20, 40, and 50 mg/mL final concentrations) and sampled at 2, 4, 8, and 24 h. A clear time dependency was demonstrated in all cell lines with all EL analogs (representative curves for EL analogs **8** and **10** shown, Figure 2). A dose dependency was also noted in all cell lines at all incubation time periods (representative data for EL analogs **8** and **10** with HUT 78 at 24 h, Figure 3).

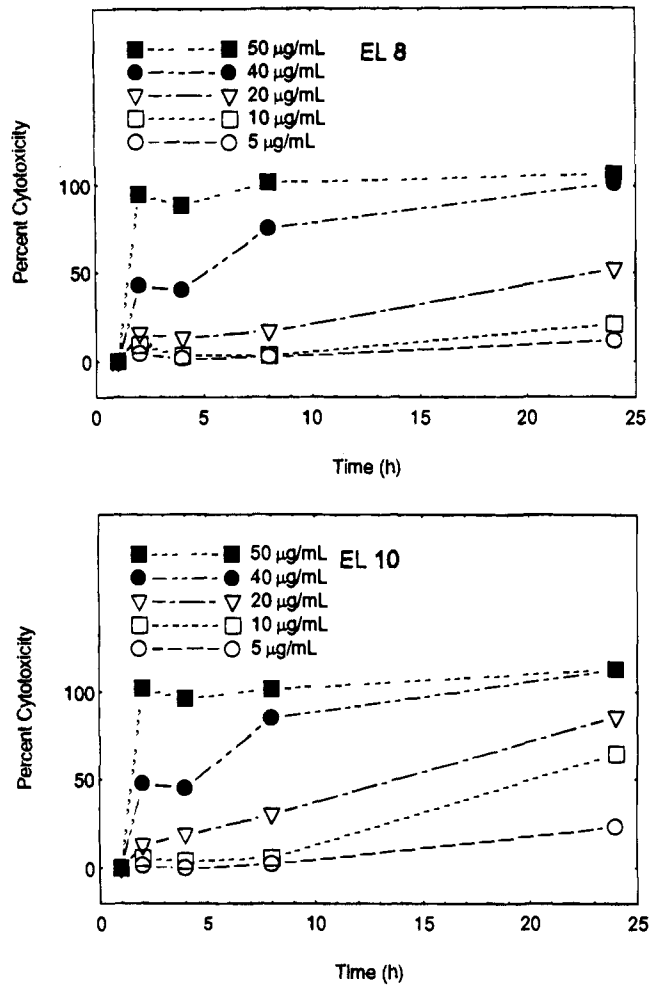


Figure 2. Time-dependent effects of EL analogs 8 and **10** on percent cytotoxicity[38] in HUT 78 cell line. HUT 78 cells (10^5/well) were incubated in 0.2 mL of RPMI medium supplemented with 10% FCS in microtiter plates with EL analog 8 or **10** at 5, 10, 20, 40, and 50 µg/mL. After 2, 4, 8, and 24 h incubation, release of cytosolic lactic dehydrogenase (LDH) into the media was assayed by a spectrophotometric method. Each data point represents the mean (n = 3).

A convenient manner for describing the cytotoxic effects of the different EL analogs was to calculate the dose of the EL required to produce a cytotoxicity of 50% (ID_{50}).[38] The calculated 24 h ID_{50} values (mean ± SD) for the EL analogs for all cell lines are listed in Table 1. The comparison of ID_{50} values of the three leukemic cell lines to the normal peripheral lymphocytes within the 24 h time period was intended only to demonstrate the selective nature of the cytotoxicity of the various EL analogs. The three cell lines differed considerably in their susceptibility to the EL analogs. CEM and HUT 78, both T cell derived, were more sensitive to the tested EL analogs than Namalwa, a B cell derived line, with CEM the most susceptible to all EL analogs tested as reflected in the ID_{50} values after 24 h incubation (Table

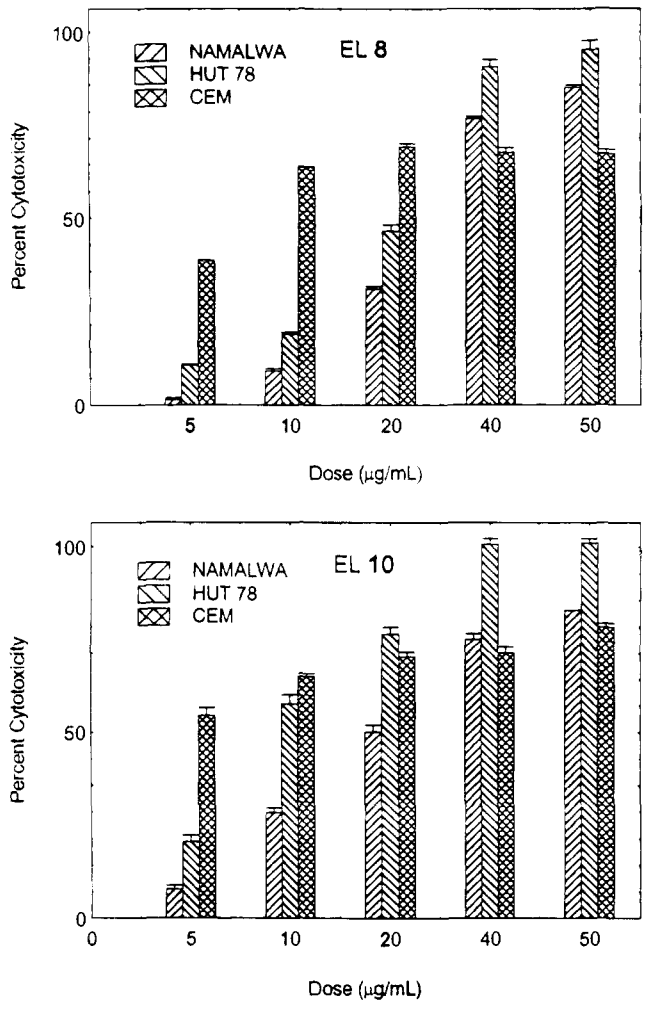


Figure 3. Concentration-dependent effect of EL analogs 8 and 10 on LDH release from CEM, HUT 78, and Namalwa after 24 h incubation. CEM, HUT 78, and Namalwa cells (10^5/well) were incubated in 0.2 mL supplemented with 10% FCS in microtiter plates with EL 8 and 10 at 5, 10, 20, 40, and 50 μg/mL. After 24 h incubation, release of cytosolic lactate dehydrogenase (LDH) activity into the media was assayed by a spectrophotometric method. All bars represent mean ± SD (n = 3). Data are reported as percent cytotoxicity.[38]

1). Unlike the other two cell lines, Namalwa showed greater sensitivity to most of the new analogs when compared to EL 1. In general, the differences in antineoplastic potencies among the tested analogs were relatively modest and did not vary by more than 3-fold from each other. Below we describe those differences which were found to be statistically significant (P < 0.05).

Our data show that the two enantiomers of *rac*-ET-18-OCH_3 (1), namely **2** (*R*) and **3** (*S*), exhibit very modest stereoselectivity with the *R* isomer (**2**) being consistently slightly more cytotoxic. Replacement of the trimethylammonio group of 1 with *N,N*-dimethylammino, *N*-methylpyrrolidino, and *N*-methylmorpholino groups produced analogs **4**, **5**, and **6**, respectively, with significantly greater cytotoxicity toward normal lymphocytes to that of the parent EL 1 while as a group only modestly increasing cytotoxicity in HUT 78 (**4** and **6**, P < 0.05) and in Namalwa (**4**, **5**, and **6**, P < 0.05).

All four possible methylcholine analogs **7**, **8**, **9**, and **10** derived from (*R*)-ET-18-OCH_3 (**2**) were cytotoxic to the cancer cell lines examined with the extent of specific

Table 1. ID_{50} Values (μg/mL) of Various Ether Lipids after 24 h Incubation with Three Human Leukemic Cell Lines and with Human Normal Peripheral Lymphocytes

ether lipid	ID_{50}[e]			
	CEM[a]	Hut 78[b]	Namalwa[c]	normal lymphocytes[d]
1	1.5 ± 0.1	4.5 ± 0.3	15.3 ± 0.5	>100
2	1.9 ± 0.1	4.1 ± 0.2	16.3 ± 0.2	>100
3	2.0 ± 0.1	6.6 ± 0.2	20.4 ± 0.5	>100
4	4.6 ± 0.3	3.5 ± 0.1	9.6 ± 0.1	24.6 ± 3.1
5	2.7 ± 0.2	9.3 ± 0.6	13.2 ± 0.2	22.7 ± 2.4
6	2.8 ± 0.2	3.4 ± 0.3	8.4 ± 0.3	52.1 ± 2.7
7	2.7 ± 0.2	9.1 ± 0.3	10.1 ± 0.1	>100
8	2.3 ± 0.1	9.3 ± 0.5	9.8 ± 0.1	>100
9	2.0 ± 0.1	6.9 ± 0.2	9.9 ± 0.2	>100
10	2.1 ± 0.1	5.9 ± 0.2	8.9 ± 0.2	>100

[a] Human T lymphoblastic leukemic cell line. [b] Human cutaneous T lymphoma cell line. [c] Human B lymphoblastic leukemic cell line. [d] Freshly isolated human peripheral lymphocytes (see methods for details[39]). [e] ID_{50}, the drug concentration (μg/mL) required to produce a cytotoxicity of 50% as determined by cytosolic lactic dehydrogenase (LDH) leakage (see text for definition of percent cytotoxicity which has been previously described[38]), was determined from a nonlinear regression fit of cytotoxicity versus dose plot, reported as the mean ± SD.

toxicities dependent upon the cell line tested and on whether the methyl group was in the α or β position. The calculated ID_{50} values reported in Table 1 suggest that the β-methyl analogs, **9** and **10**, are more effective than the α-methyl analogs, **7** and **8**, in all the tested cancer cell lines; however, only in HUT 78 were the 24 h ID_{50} values for **9** and **10** significantly different from **7** and **8**. Data from the dose response studies (Figure 3 for **8** and **10**; data for **7** and **9** not shown) indicate that at lower doses (5, 10, 15, 20 μg/mL) β-methylcholine analogs (**9**, **10**) have greater cytotoxicity in HUT 78 and Namalwa than the α-methylcholine analogs (**7**, **8**). While both these α and β-methylcholine EL analogs, **7**, **8** and **9**, **10**, respectively, demonstrated high *in vitro* cytoxicities to each of the three leukemic cell lines tested, only in Namalwa were the four analogs significantly more toxic than the parent EL **2**.

Conclusions

This study evaluated how changes in the choline head group of *rac*-ET-18-OCH_3 (**1**) altered the *in vitro* cytotoxic efficacy of the ether lipids in three cancer cell lines. In general, the EL analogs tested demonstrated dose- and time-dependent cytotoxic effects with variations in sensitivity among the three leukemic cell lines. In general, the cell lines varied in sensitivity to the EL analogs following the order CEM > HUT 78 > Namalwa ≫ normal peripheral lymphocytes. Even though none of the EL analogs tested demonstrated consistently greater selective toxicity to each of the three leukemic cell lines relative to normal lymphocytes than *rac*-ET-18-OCH_3 (1) and its stereoisomers **2** and **3**, some interesting observations were noted. *N,N*-Dimethylammino, *N*-methylpyrrolidino, and *N*-methylmorpholino head groups are more toxic to normal cells and may thus be less attractive head group modifications when designing novel antineoplastic ether lipids. Conversely, of all the compounds tested the β-methylcholine analogs had the most favorable profile, indicating that the β-methyl group may be a desirable substitution in EL head groups.

There is still no clearly accepted molecular mechanism of action which would explain the selective tox-

icities of ether lipid analogs toward cancer cells when compared to normal cells. An attractive explanation may be based on the ability of the ether lipids to selectively accumulate in higher concentrations in cancer cell membranes, thus resulting in the destruction of these cells. This selective accumulation may, in turn, be due to differences in membrane organization and/or composition between cancer and normal cells. The observed differences in selective toxicity for cancer cells by the different EL analogs described in this report as well as the observed variations in sensitivity among the three cell lines used are consistent with such an explanation.

Experimental Section

Cell Cultures and Cell Preparation. CCRF CEM (ATCC No. CCL 119), a human T lymphoblastic leukemic cell line, Namalwa (ATCC No. CRL 1432), a human B lymphoblastic leukemic cell line, and HUT 78 (ATCC No. TIB 161), a human cutaneous T lymphoma cell line, were obtained from the American Tissue Culture Collection (Rockville, MD). Cells were grown in T-75 culture flasks with RPMI 1640 medium in a Napco water jacketed incubator (Portland, OR) in an atmosphere of 5% carbon dioxide, 95% air, and 100% relative humidity at 37 °C. Cells in logarithmic growth were removed from the culture growth flask, placed into 50 mL conical centrifuge tubes, and spun at 500 g in an IEC Centra-7R centrifuge (Needham Hts, MA) for 10 min. The resultant pellet was washed with fresh RPMI 1640 without phenol red, counted using the trypan blue exclusion test, repelleted, and finally resuspended at a concentration of 1×10^6 in RPMI 1640 without phenol red. Aliquots of 1.2 mL were used in the LDH assay. The source of the normal human peripheral lymphocytes was a pool of two human donors who had received no medication the previous 2 weeks. Lymphocytes were isolated according to the method of Boyum,[39] cell viability was measured by trypan blue exclusion, and cell counts were measured in a hemocytometer. Final cell preparations contained more than 85% lymphocytes. The untreated peripheral lymphocytes remained viable during the course of the 24 h period used for the *in vitro* cytotoxicity studies.

Ether Lipid Solubilization. EL analogs were completely dissolved in 100% ethanol (1 mg of EL/100 μL). This ethanolic EL solution was diluted with RPMI 1640 medium (without phenol red and with 20% calf serum, supplemented with 20 mM Hepes buffer, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol) to 100 μg/mL as a stock drug solution. A stock solution for the ethanol control was prepared by adding 1 mL of 100% ethanol to 99 mL of the above media mixture. Stock solutions were mixed at room temperature using a magnetic stirring bar for 1 h and then filtered by a MILLEX-GV 0.22 μm filter unit (Sigma no. SLGV025LS, St. Louis, MO) that had been previously rinsed with 0.6 mL of RPMI 1640 without phenol red. The sterile stock solutions were placed in sterile 30 mL glass pyrogen-free vials (Solopak Laboratories, Franklin Park, IL), wrapped in amber bags, and placed on a rocker platform (Bellco rocking platform, Vineland, NJ, speed of 4) at 4 °C in a cold room overnight. Prior to use, the EL stock solutions were shaken for 30 min at 25 °C. The stock solutions were diluted with sterile media mixture (described above) at 25 °C to effect EL concentrations of 10, 20, 40, and 50 μg/mL.

Lactic Dehydrogenase Assay. Lactic dehydrogenase (LDH) leakage has been routinely used as a cell viability assay.[8a,30a,32-34] The microtiter plate ELISA (enzyme linked immunosorbent assay) method used was essentially that of Korzeniewski[38] modified slightly in our laboratory for application to cytotoxicity assays of EL analogs in leukemic cell lines. Briefly, cultured cells were harvested, washed, and resuspended as described above. A 1.2 mL aliquot of each cell suspension was mixed with a 1.2 mL aliquot of each EL solution to effect a cell concentration of 5×10^5 cells/mL and final EL concentrations of 0, 5, 10, 20, 40, and 50 μg/mL with a final ethanol concentration of 0.5%. To determine if ethanol had an effect on release of cytosolic LDH into the media, an ethanol control was used containing 0.5% ethanol, cells at a density of 5×10^5 cells/mL, and no EL. For a positive control group, cell suspensions of 5×10^5 cells/mL in RPMI 1640 medium (with 10% FCS) were stored frozen at −70 °C overnight. The EL and cell suspensions were seeded in triplicate into sets of Linbro/Titertek (Flow Laboratories no. 76-23205, McLean, VA) microtiter 96-well flat bottom plates (one set of plates for each time period assayed). The plates incubated in a Napco water jacketed incubator with an atmosphere of 5% carbon dioxide, 95% air, and 100% relative humidity at 37 °C and assayed at various time points. At each time interval a plate was removed and spun in an IEC Centra-7R centrifuge at 500*g* for 10 min. An aliquot of 0.1 mL of the supernatant was transferred to another microtiter plate. LDH substrate mixture (0.1 mL) [5.4×10^{-2} M L-(+)-lactate, 6.6×10^{-4} M 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), 2.8×10^{-4} M phenazine methosulfate (PMS), and 1.3×10^{-3} M NAD in 0.2 M Tris buffer at pH 8.2] was added to each well at 1 s intervals. The plate was read at 490 nm on a Dynascan Microtiter plate reader (Model MR-700, Dynatech Laboratories, Inc., Vineland, NJ) after 3 min. The ethanol control group was compared to the medium control group for CEM, HUT 78, and Namalwa at 2, 4, 8, and 24 h. There were no significant differences ($P < 0.05$) at any of the times between the ethanol control and the media control for any of the three leukemic cell lines. Moreover, there was no significant difference ($P < 0.05$) between the ethanol control and the media control for normal peripheral lymphocytes at 8 and 24 h incubation (data not shown).

Percent Cytotoxicity. Percent cytotoxicity was calculated using the following formula:[38] Percent cytotoxicity = $[(E - S)/M - S]*100$, where E is the experimental release of LDH activity from the target cells, S is the spontaneous release of LDH activity from target cells, and M is the maximal release of LDH from target cells determined by freezing aliquots of cells at various concentrations at −70 °C overnight, then flash thawing and determining the postcentrifugation supernatant LDH activity.

ID_{50} (μg/mL). The ID_{50}, the concentration at which the EL produced 50% cytotoxicity, was determined from a nonlinear regression fit of cytotoxicity versus dose plot.[40]

Statistics. Values have been expressed as the mean of at least three separate experiments with duplicate determinations. Where noted, statistical comparisons were performed using analysis of variance (ANOVA) and Duncan's multiple range test or student's t as appropriate $P < 0.05$ was considered to be significantly different.

General Synthetic Methods. Tetrahydrofuran was distilled from benzophenone ketyl. Reactions were carried out under a N_2 atmosphere with magnetic stirring, and temperatures were reported as bath temperatures (bT). Organic phases were dried over Na_2SO_4 and evaporated under reduced pressure. Silica gel (grade 60, 230–400 mesh, E. Merck, Germany) was used for column chromatography. All compounds were demonstrated to be homogeneous by analytical TLC on precoated silica gel TLC plates (grade 60, F254, E. Merck, Germany), and chromatograms were visualized by iodine staining. Phosphorus-containing products were also checked by staining duplicate chromatograms with molybdic acid reagent.[41] Melting points of all solids were determined in open Pyrex capillaries. All ^1H NMR spectra of all compounds were recorded in $CDCl_3$ at 200 MHz with TMS as an internal reference, and exchangeable resonances are not reported. In addition, the spectra of the phosphocholines were run in $CDCl_3/CD_3OD$ (2:1). All reported solvent ratios were by volume. Specific rotations were determined using a 1.00 dm cell.

***rac*-2-*O*-Methyl-1-*O*-octadecylglycero-3-phosphocholine (1).** To a stirred solution of 0.70 g (1.3 mmol) of *rac*-**12**[3] in 9 mL of $CHCl_3$/*i*-PrOH/DMF (2:2:1) in a glass pressure bottle was added excess trimethylamine, the reaction bottle was sealed with a Teflon screw top, and the reaction mixture was heated at 50 °C (bT) for 48 h. After cooling, the excess trimethylamine was evaporated with a stream of N_2, and the reaction mixture was then concentrated. Chromatography

($CHCl_3$/MeOH/H_2O (60:30:0) increased stepwise to $CHCl_3$/MeOH/H_2O (60:30:4)), and evaporation followed by filtration (0.5 μm pore Teflon membrane, Alltech, Deerfield, IL) of a $CHCl_3$ solution of the product gave 0.56 g (1.0 mmol, 77%) of **1**.[3,12] An analytical sample was prepared by precipitation from a $CHCl_3$ solution with acetone to give a white solid: mp 249–251 °C dec; TLC ($CHCl_3$/MeOH/H_2O (60:30:4)) R_f 0.12; ^1H NMR ($CDCl_3$) δ 4.15–4.35 (m, 2 H), 3.65–4.00 (m, 4 H), 3.20–3.60 (m, 5 H), 3.42 (s, 3 H), 3.33 (s, 9 H), 1.45–1.65 (m, 2 H), 1.26 (br s, 30 H), 0.88 (t, 3 H, J = 6.6 Hz); FAB mass spectrum, m/z 524 $(M + H)^+$, 224, 184, 166; FAB HRMS calcd for $C_{27}H_{59}NO_6P$ $(M + H)^-$ m/z 524.4080, found m/z 524.4092.

2-*O*-Methyl-1-*O*-octadecyl-*sn*-glycero-3-phosphocholine (2). The quaternization of excess trimethylamine with (*R*)-**12**[9c,l] gave **2**[9a–n] as a white solid: mp 90–92 °C (soften), 215–218 °C dec (lit.[9c] mp 80 °C (soften), 230 °C dec; lit.[9h] mp 270 °C); $[\alpha]^{25}_D$ −2.47° (*c* 1.82, $CHCl_3$) (lit.[9c] $[\alpha]^{20}_D$ +2.8° (10% in $CHCl_3$), lit.[9h] $[\alpha]^{25}_D$ −0.78 ± 0.06° (*c* 5.1, $CHCl_3$/MeOH (1:1)), lit.[9i] $[\alpha]^{?}_D$ −2.3° (*c* 1, $CHCl_3$)); FAB HRMS calcd for $C_{27}H_{59}NO_6P$ $(M + H)^-$ m/z 524.4080, found m/z 524.4063.

2-*O*-Methyl-3-*O*-octadecyl-*sn*-glycero-1-phosphocholine (3). The quaternization of excess trimethylamine with (*S*)-**12**[9l] gave **3**[9h–o] as a white solid: mp 86–89 °C (soften), 218–221 °C dec (lit.[9h] mp 270 °C); $[\alpha]^{25}_D$ +3.52° (*c* 1.82, $CHCl_3$) (lit.[9h] $[\alpha]^{25}_D$ +0.80 ± 0.06° (*c* 5, $CHCl_3$/MeOH (1:1)), lit.[9i] $[\alpha]^{?}_D$ +1.7° (*c* 1, $CHCl_3$)); FAB HRMS calcd for $C_{27}H_{59}NO_6P$ $(M + H)^-$ m/z 524.4080, found m/z 524.4078.

***rac*-2-*O*-Methyl-1-*O*-octadecylglycero-3-phospho-β-(dimethylamino)ethanol (4).** The monoalkylation of excess aqueous dimethylamine with *rac*-**12**[3] gave **4**[9j] as a white solid: mp 136–138 °C; TLC ($CHCl_3$/MeOH/H_2O (60:30:4)) R_f 0.22; ^1H NMR ($CDCl_3$) δ 4.15–4.40 (m, 2 H), 3.80–4.10 (m, 2 H), 3.30–3.75 (m, 8 H), 3.10–3.30 (m, 2 H), 2.83 (s, 6 H), 1.45–1.65 (m, 2 H), 1.26 (br s, 30 H), 0.88 (t, 3 H, J = 6.6 Hz); FAB mass spectrum, m/z 510 $(M + H)^-$, 210, 170, 152; FAB HRMS calcd for $C_{26}H_{57}NO_6P$ $(M + H)^+$ m/z 510.3924, found m/z 510.3942. Anal. ($C_{26}H_{56}NO_6P \cdot H_2O$) C, H, N.

***rac*-2-*O*-Methyl-1-*O*-octadecylglycero-3-phospho-β-(*N*-methylpyrrolidino)ethanol (5).** The quaternization of excess *N*-methylpyrrolidine with *rac*-**12**[3] gave **5** as a white solid: mp 248 °C; TLC ($CHCl_3$/MeOH/H_2O (60:30:4)) R_f 0.15; ^1H NMR ($CDCl_3$) δ 4.15–4.35 (m, 2 H), 3.55–4.10 (m, 8 H), 3.10–3.50 (m, 5 H), 3.44 (s, 3 H), 3.29 (s, 3 H), 2.10–2.35 (m, 4 H), 1.45–1.65 (m, 2 H), 1.26 (br s, 30 H), 0.88 (t, 3 H, J = 6.6 Hz); FAB mass spectrum, m/z 550 $(M + H)^+$, 250, 210, 192; FAB HRMS calcd for $C_{29}H_{61}NO_6P$ $(M + H)^-$ m/z 550.4237, found m/z 550.4239. Anal. ($C_{29}H_{60}NO_6P \cdot H_2O$) C, H, N.

***rac*-2-*O*-Methyl-1-*O*-octadecylglycero-3-phospho-β-(*N*-methylmorpholino)ethanol (6).** The quaternization of excess *N*-methylmorpholine with *rac*-**12**[3] gave **6** as a white solid: mp 235–236 °C; TLC ($CHCl_3$/MeOH/H_2O (60:30:4)) R_f 0.14; ^1H NMR ($CDCl_3$) δ 4.25–4.50 (m, 2 H), 3.60–4.25 (m, 12 H), 3.30–3.60 (m, 5 H), 3.52 (s, 3 H), 3.44 (s, 3 H), 1.45–1.65 (m, 2 H), 1.26 (br s, 30 H), 0.88 (t, 3 H, J = 6.6 Hz); FAB mass spectrum, m/z 566 $(M + H)^+$, 280, 226, 208; FAB HRMS calcd for $C_{29}H_{61}NO_7P$ $(M + H)^+$ m/z 566.4186, found m/z 566.4180. Anal. ($C_{29}H_{60}NO_7P \cdot H_2O$) C, H, N.

2-*O*-Methyl-1-*O*-octadecyl-*sn*-glycero-3-phosphatidic Acid Diphenyl Ester. To a solution of 5.60 g (15.6 mmol) of (*S*)-**11**[9c,h,l,n,23,24,35] in 100 mL of pyridine was added a solution of 6.81 g (25.4 mmol) of chloro diphenyl phosphate in 35 mL of pyridine, and the reaction mixture was stirred for 20 h. Then 10 mL of H_2O was added, and the mixture was stirred for 20 min. The mixture was concentrated and then partitioned between 80 mL of H_2O and 80 mL of Et_2O. The organic phase was washed with 0.5 N HCl (30 mL), H_2O (25 mL), 0.5 M $NaHCO_3$ (25 mL), and H_2O (until neutral) and then dried and evaporated. Chromatography (EtOAc/$CHCl_3$ (7:93)) gave 8.58 g (14.5 mmol, 93%) of 2-*O*-methyl-1-*O*-octadecyl-*sn*-glycero-3-phosphatidic acid phenyl ester[91] as a clear colorless liquid: TLC (EtOAc/$CHCl_3$ (1:9)) R_f 0.64; ^1H NMR ($CDCl_3$) δ 7.25–7.40 (m, 5 H), 7.15–7.25 (m, 5 H), 4.25–4.40 (m, 2 H), 3.35–3.55 (m, 5 H), 3.40 (s, 3 H), 1.40–1.60 (m, 2 H), 1.26 (br s, 30 H), 0.88 (t, 3 H, J = 6.6 Hz); $[\alpha]^{25}_D$ −1.4° (*c* 3.78, $CHCl_3$); FAB mass spectrum, m/z 591 $(M + H)^+$; FAB HRMS calcd for $C_{34}H_{56}O_6P$ $(M + H)^-$ m/z 591.3815, found m/z 591.3823. Anal. ($C_{34}H_{55}O_6P$) C, H.

2-*O*-Methyl-1-*O*-octadecyl-*sn*-glycero-3-phosphatidic Acid ((*R*)-13). A mixture of 8.23 g (13.9 mmol) of 2-*O*-methyl-1-*O*-octadecyl-*sn*-glycero-3-phosphatidic acid diphenyl ester and 0.79 g (3.5 mmol) of PtO_2 in 50 mL of $CHCl_3$/EtOH (1:3) was mechanically shaken under 25 psi of H_2 on a Parr apparatus for 10 h. The catalyst was removed by filtration (0.5 μm pore Teflon membrane), and the solvent was evaporated. The residue was dissolved in 80 mL of $CHCl_3$/MeOH (1:1), washed with 35 mL of 0.1 N HCl and then with 10 mL of H_2O/MeOH (9:1), dried, evaporated, and stored in a desiccator over P_2O_5 for 3 days at 0.1 mm to give 6.01 g (13.7 mmol, 98%) of (*R*)-**13**[9f,l,21–23] as a white solid: mp 59–60 °C; TLC ($CHCl_3$/MeOH/HOAc/H_2O (80:13:8:0.3)) R_f 0.20; ^1H NMR ($CDCl_3$) δ 4.00–4.25 (m, 2 H), 3.40–3.65 (m, 5 H), 3.51 (s, 3 H), 1.40–1.60 (m, 2 H), 1.26 (br s, 30 H), 0.88 (t, 3 H, J = 6.6 Hz); $[\alpha]^{25}_D$ +1.0° (*c* 0.49, $CHCl_3$); FAB mass spectrum, m/z 439 $(M + H)^+$; FAB HRMS calcd for $C_{22}H_{48}O_6P$ $(M + H)^-$ m/z 439.3189, found m/z 439.3198. Anal. ($C_{22}H_{47}O_6P$) C, H.

(2*R*,α*R*)-2-*O*-Methyl-1-*O*-octadecyl-*sn*-glycero-3-phospho-α-methylcholine (7). The condensation of (*R*)-13 with (*R*)-2-methylcholine tetraphenyl borate (prepared[25,37] from commercially available (*R*)-(−)-1-amino-2-propanol) in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride gave **7**[9l] as a white solid: mp 83–86 °C (soften), 180–183 °C dec; TLC ($CHCl_3$/MeOH/H_2O (60:30:4)) R_f 0.26; ^1H NMR ($CDCl_3$/CD_3OD (2:1)) δ 4.80–4.85 (m, 1 H), 3.95–4.10 (m, 2 H), 3.30–3.60 (m, 19 H), 1.40–1.60 (m, 5 H), 1.26 (br s, 30 H), 0.88 (t, 3 H, J = 6.6 Hz); $[\alpha]^{25}_D$ +2.2° (*c* 2.0, $CHCl_3$); FAB mass spectrum, m/z 538 $(M + H)^+$, 238, 198, 180; FAB HRMS calcd for $C_{28}H_{61}NO_6P$ $(M + H)^+$ m/z 538.4237, found m/z 538.4245. Anal. ($C_{28}H_{60}NO_6P \cdot 1.25H_2O$) C, H, N.

(2*R*,α*S*)-2-*O*-Methyl-1-*O*-octadecyl-*sn*-glycero-3-phospho-α-methylcholine (8). The condensation of (*R*)-13 with (*S*)-2-methylcholine tetraphenyl borate (from commercially available (*S*)-(+)-1-amino-2-propanol) gave **8**[9l] as a white solid: mp 91–94 °C (soften), 188–191 °C dec; TLC ($CHCl_3$/MeOH/H_2O (60:30:4)) R_f 0.21; ^1H NMR ($CDCl_3$/CD_3OD (2:1)) δ 4.75–4.85 (m, 1 H), 3.80–4.00 (m, 2 H), 3.30–3.60 (m, 19 H), 1.40–1.60 (m, 5 H), 1.26 (br s, 30 H), 0.88 (t, 3 H, J = 6.6 Hz); $[\alpha]^{25}_D$ −1.1° (*c* 2.0, $CHCl_3$); FAB mass spectrum, m/z 538 $(M + H)^+$, 238, 198, 180; FAB HRMS calcd for $C_{28}H_{61}NO_6P$ $(M + H)^+$ m/z 538.4237, found m/z 538.4255. Anal. ($C_{28}H_{60}NO_6P \cdot 0.5H_2O$) C, H, N.

(2*R*,β*R*)-2-*O*-Methyl-1-*O*-octadecyl-*sn*-glycero-3-phospho-β-methylcholine (9). The condensation of (*R*)-13 with (*R*)-1-methylcholine tetraphenyl borate (from commercially available (*R*)-(−)-2-amino-1-propanol) gave **9**[9l] as a white solid: mp 85–88 °C (soften), 195–197 °C dec; TLC ($CHCl_3$/MeOH/H_2O (60:30:4)) R_f 0.25; ^1H NMR ($CDCl_3$/CD_3OD (2:1)) δ 4.25–4.35 (m, 2 H), 3.90–4.05 (m, 2 H), 3.35–3.70 (m, 18 H), 1.40–1.60 (m, 5 H), 1.26 (br s, 30 H), 0.88 (t, 3 H, J = 6.6 Hz); $[\alpha]^{25}_D$ −6.8° (*c* 2.0, $CHCl_3$); FAB mass spectrum, m/z 538 $(M + H)^+$, 238, 198, 180; FAB HRMS calcd for $C_{28}H_{61}NO_6P$ $(M + H)^-$ m/z 538.4237, found m/z 538.4245. Anal. ($C_{28}H_{60}NO_6P \cdot H_2O$) C, H, N.

(2*R*,β*S*)-2-*O*-Methyl-1-*O*-octadecyl-*sn*-glycero-3-phospho-β-methylcholine (10). The condensation of (*R*)-13 with (*S*)-1-methylcholine tetraphenyl borate (from commercially available (*S*)-(+)-2-amino-1-propanol gave **10**[9l] as a white solid: mp 100–102 °C (soften), 200–203 °C dec; TLC ($CHCl_3$/MeOH/H_2O (60:30:4)) R_f 0.20; ^1H NMR ($CDCl_3$/CD_3OD (2:1)) δ 4.25–4.35 (m, 2 H), 3.85–4.10 (m, 2 H), 3.35–3.70 (m, 18 H), 1.40–1.60 (m, 5 H), 1.26 (br s, 30 H), 0.88 (t, 3 H, J = 6.6 Hz); $[\alpha]^{25}_D$ +5.0° (*c* 2.0, $CHCl_3$); FAB mass spectrum, m/z 538 $(M + H)^-$, 238, 198, 180; FAB HRMS calcd for $C_{28}H_{61}NO_6P$ $(M + H)^+$ m/z 538.4237, found m/z 538.4255. Anal. ($C_{28}H_{60}NO_6P \cdot H_2O$) C, H, N.

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