Synthesis and Evaluation of Neoplastic Cell Growth Inhibition of 1-*N*-Alkylamide Analogues of Glycero-3-phosphocholine†

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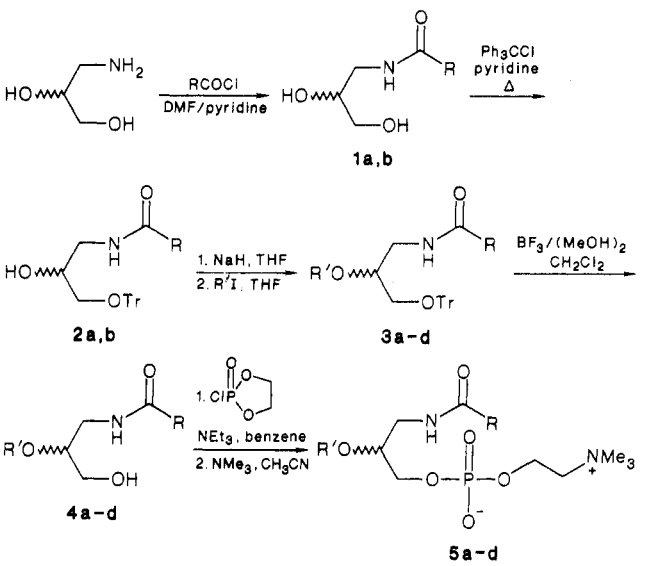
Previously unreported analogues of the synthetic antitumor phospholipid ET-18-OMe (1-octadecyl-2-methoxy-*rac*-glycero-3-phosphocholine), in which the 1-ether oxygen has been replaced by an amido group, have been prepared and evaluated for in vitro cytotoxic effects and for inhibition of protein kinase C. The title compounds exhibit cytotoxic effects against several tumor cell lines and are approximately equipotent to ET-18-OMe. The compounds were also found to inhibit protein kinase C in an in vitro assay. This work is a continuation of our previous structure–activity studies on thio-substituted derivatives of ET-18-OMe.

Certain alkyl ether phospholipids (1-alkyl-2-methoxy- and 1-alkyl-2-ethoxy-*rac*-glycero-3-phosphocholines, Figure 1) are potent, biologically active molecules.[1] One of the more interesting properties these ether lipids exhibit is the ability to inhibit neoplastic cell growth both in vivo and in vitro.[2,3] The mechanistic aspects involved in this activity are still to be fully understood. It is possible that the observed cytotoxic properties may result via interference with phospholipid metabolism[4-8] and/or inhibition of protein kinase C activity.[7-9] Induction of tumor cell differentiation and/or macrophage activation have also been suggested to contribute to the mechanism of action.[3,10] Interestingly, ether phospholipids do not affect nuclear DNA[2] and are not genotoxic.[11] Their major target site appears to lie in the plasma membrane of neoplastic cells, as evidenced by accumulation in the membrane after treatment with the reference compound ET-18-OMe (1-octadecyl-2-methoxy-*rac*-glycero-3-phosphocholine, Figure 1).[12] Their apparent nonclassical mechanism of action, together with their high antineoplastic potential in vitro, strongly suggest that ether phospholipids may represent a new chemotherapeutic approach to cancer treatment.

For these reasons, we have developed novel analogues of alkyl ether phospholipids.[13-16] The identification of new and more potent derivatives through structure–activity studies may contribute to a better understanding of the mechanism(s) of action and to elucidating the structural features responsible for the observed cytotoxicity. We began these studies with an investigation of thio-substituted derivatives[18] (Figure 1). These were of special interest because of the known increase in lipophilicity imparted by sulfur and evidence that ether lipids accumulate in the plasma membranes.[12] Detailed results of this earlier work have recently been published.[17]

Phospholipids containing amino substituents at position 1 or 2 have been synthesized by several groups. The 3- and 2-(*N*-palmitoylamino)propylphosphocholines are inhibitors of platelet-activating factor (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF) biosynthesis,[19] while 1-*O*-(*N*-octadecylcarbamoyl)-2-*O*-methylglycero-3-phospho-[2-(2-hydroxyethyl)thiazolium] (CV3988) is an antagonist of PAF.[20,21] Both 1-octadecyl-2-*O*-(*N*,*N*-dimethylcarbamoyl)glycero-3-phosphocholine[21] and its 2-acetamido analogue[22] exhibit macrophage activating properties. The 2-acetamido compound was reported to be more active

Scheme I



† The compounds can be named as derivatives of aminoglycerol in a manner analogous to the nomenclature accorded to thioglycerol derivatives: **1a**, *N*-(2,3-dihydroxypropyl)hexadecanamide, 1-*N*-hexadecanoyl-*rac*-aminoglycerol; **2a**, 1-*N*-hexadecanoyl-3-*O*-trityl-*rac*-aminoglycerol; **3a**, 1-*N*-hexadecanoyl-2-*O*-methyl-3-*O*-trityl-*rac*-aminoglycerol; **4a**, 1-*N*-hexadecanoyl-2-*O*-methyl-*rac*-aminoglycerol; **5a**, 1-*N*-hexadecanoyl-2-*O*-methyl-*rac*-amino-glycero-3-phosphocholine.

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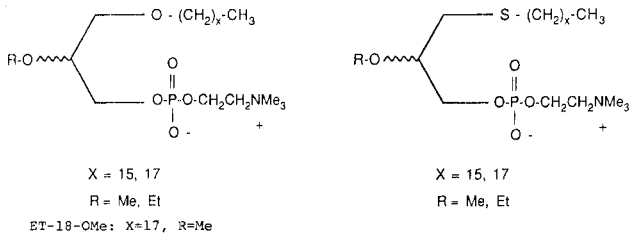


Figure 1.

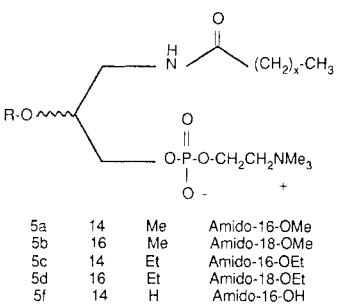


Figure 2.

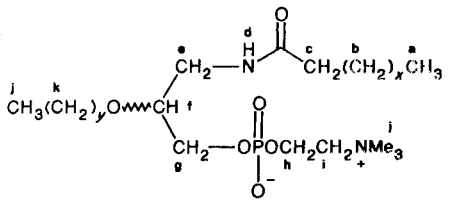
than the reference analogue ET-18-OMe as an inhibitor of HL60 leukemic cell growth in vitro via the trypan blue dye exclusion test.[23] However, we find this compound to be approximately as active as ET-18-OMe in the same assay system,[24] a result that is in accordance with other recent findings with freshly explanted human leukemic cells.[25]

These observations prompted our interest in 1-amido-substituted compounds, not only as simple analogues but also as hydrolysis-resistant bioisosteres of the carbamoyl and acyl derivatives. This paper describes the synthesis[26] and biological evaluation of **5a–d,f**, novel 1-amido derivatives (Figure 2).

Chemistry

The amidoglycerophosphocholines **5a–d** were prepared as shown in Scheme I. This sequence was selected because of the ready accessibility of intermediates **1a,b** and appeared to provide a flexible route to the desired phospholipids. Amidopropanediols **1a,b** were prepared directly from (±)-3-amino-1,2-propanediol in good yield (>90%) by reaction with the appropriate acyl chloride. By comparison, Bergman prepared **1b** in six steps from 3-chloro-2-hydroxypropylamine,[27] while Milks[28] and van Loo,[29] respectively, prepared **1b** in two steps (>90%) via the glycidyl ester with an excess of ammonia. Selective protection of the primary hydroxyl group with triphenylmethyl chloride in pyridine[29] at 70 °C was accompanied by large amounts of what appeared by IR and NMR to be 3-*N*-trityl-*N*-(alkanamido)-1-*O*-trityl-2-propanol. Lowering the reaction temperature to 50 °C (necessary to keep the diols in solution) resulted in **2a,b** of higher yield and purity, eliminating the need for chromatographic purification. Alternatively, the primary hydroxyl group was protected with the *tert*-butyldimethylsilyl group, resulting in mono-*O*-silyl product only. Alkylation of the secondary hydroxyl group was often accompanied by competing side reactions, giving little of the desired product (**3a–d**) and usually mostly trityl alcohol and the diol **1a** or **1b**. The alkylation was successful only when 1 equiv of base was used and the reaction was carried out at ambient temperature. The formation of **1a,b** may be due to an intramolecular displacement of the trityl group by the amido moiety. Such an intramolecular functional group interaction has been documented previously,[31,32] and the amido functionality is a known intramolecular, nucleophilic catalyst involved in many hydrolytic reactions.[33] The trityl protecting group was removed by using BF_3–

Table I. NMR Data for **5a–d**



5a: X = 13; Y = 0
b: X = 15; Y = 0
c: X = 13; Y = 1
d: X = 15; Y = 1

compd assign.	mult[a]	chemical shift, ppm (number of protons) 5a	5b	5c	5d
a	t	0.80 (3)	0.85 (3)	0.88 (3)	0.85 (3)
b	s	1.26 (26)	1.25 (30)	1.25 (26)	1.24 (30)
c	m	2.25 (2)	2.18 (2)	2.16 (2)	2.18 (2)
d	m	7.30 (1)	7.20 (1)	7.10 (1)	7.35 (1)
e	m	3.54 (2)	3.20 (2)	3.35 (2)	3.55 (2)
f	m	4.00 (1)	4.34 (1)	3.98 (1)	4.20 (1)
g	m	3.90 (2)	3.70 (2)	3.80 (2)	3.60 (2)
h	m	4.50 (2)	4.20 (2)	4.30 (2)	4.30 (2)
i	m	3.70 (2)	3.80 (2)	3.60 (2)	3.85 (2)
j	s	3.28 (9)	3.38 (9)	3.36 (9)	3.35 (9)
k	q			3.60 (2)	3.65 (2)
l	s or t	3.42 (s, 3)	3.40 (s, 3)	1.16 (t, 3)	1.15 (t, 3)

[a] s = singlet, t = triplet, q = quartet, m = multiplet.

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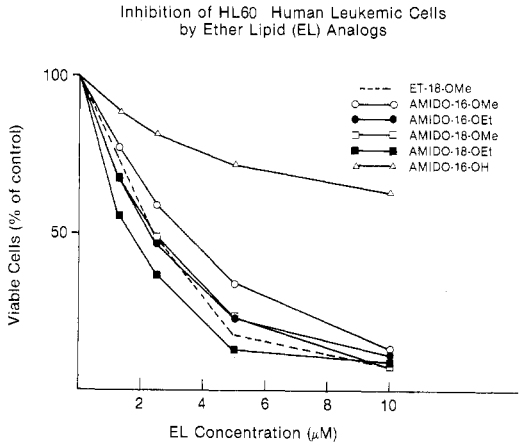


Figure 3. Evaluation of the cytotoxic activity of amido alkyl lipids against HL60 human leukemic cells.

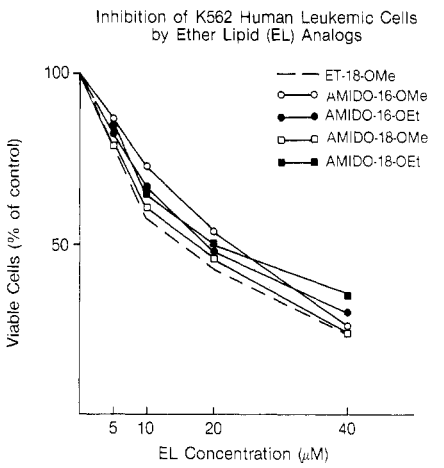
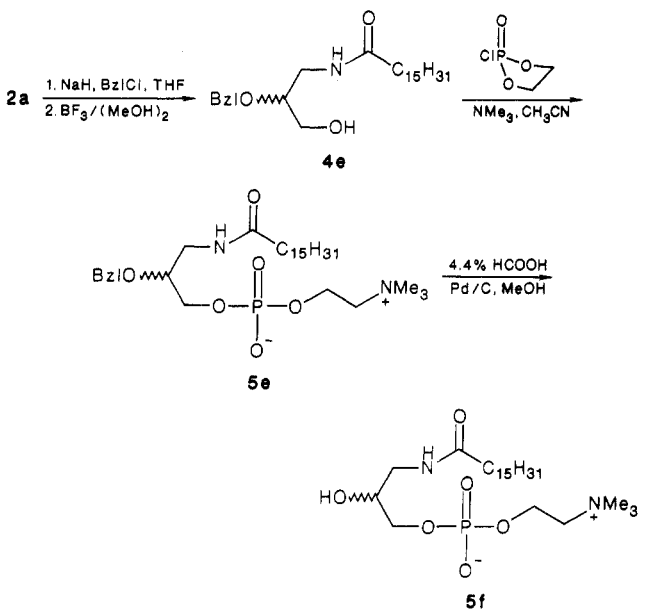


Figure 4. Evaluation of the cytotoxic activity of amido alkyl lipids against K562 human leukemic cells.

Scheme II



methanol[34] to give the amido alkyl alcohols **4a–d**, which were then phosphorylated. Many of the usual phosphorylation techniques failed to provide any useful amount of products, resulting instead in complex mixtures. This was especially true of procedures utilizing $POCl_3$[35,36] from which not even starting material could be recovered. Reaction with 2-(bromoethyl)phosphorodichloridate[37] also failed to give the desired product. This has been documented previously by Hajdu who noted that this reagent is a poor choice for the phosphorylation of β-amido alcohols.[22] The cyclic phosphorylating agent 2-chloro-2-oxo-1,3,2-dioxaphospholane,[38] which is much less reactive toward the amido functionality, gave by far the best results. Thus, the sequential reaction of **4a–d** with the cyclic reagent and trimethylamine, afforded the phosphorylcholines **5a–d** in reasonable yields. Treatment with Dowex 50W-X8 (OH^-) gave analytically pure products. NMR data for the final products are listed in Table I.

The lyso derivative **5f** was prepared via the same synthetic sequence as for **5a–d**, except for the utilization of a benzyl protecting group to provide the 2-*O*-benzyl intermediate **3e** (Scheme II). This group was subsequently removed via catalytic transfer hydrogenation as described by Means et al.,[39] resisting deprotection via more standard methods. A urea derivative, **10**, was also prepared. The synthetic pathway used is the same as for the amido analogues **5a–d,f**.

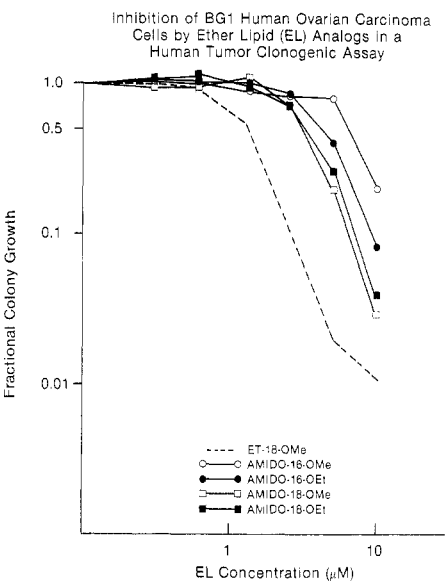


Figure 5. Evaluation of the antiproliferative effect of amido alkyl lipids against BG1 human ovarian adenocarcinoma.

Table II. ID_{50} Values of Amido Alkyl Lipids vs HL60 Human Leukemic Cells after Incubation for 48 h[a]

analogue	ID_{50}, μM	analogue	ID_{50}, μM
ET-18-OMe	2.47 ± 0.64	amido-18-OMe	2.45 ± 0.04
amido-16-OMe	3.32 ± 1.04	amido-18-OEt	1.64 ± 0.19**
amido-16-OEt	2.32 ± 0.32	amido-16-OH	>10

[a] Each value was derived from the dose–response curves (Figure 3) obtained from four different assays and was analyzed for statistical significance vs the ID_{50} for ET-18-OMe by using unpaired *t*-test. (**) $p < 0.01$.

Results and Discussion

Inhibition of Malignant Cells. The amido alkyl ether lipid analogues were found to be active in vitro against several experimental tumors: two human leukemic (HL60 promyelocytic leukemia and K562 chronic myelogenous

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leukemia) and a human ovarian adenocarcinoma (BG1) cell line. Figures 3 and 4 show the cytotoxic activity of this new group of ether phospholipid analogues against HL60 and K562 with use of a trypan blue dye exclusion assay. The amido alkyl analogue ID_{50} values against HL60 are listed in Table II. Figure 5 shows the inhibition of colony formation of BG1 ovarian adenocarcinoma after incubation with the amido analogues, with a semisoft agarose clonogenic assay. All the assays were conducted in comparison with ET-18-OMe, which, being the most studied analogue, has been used as a reference compound.

The amido analogues proved to be very promising compounds since activity was found in all the systems tested within the range of doses used. Their activity is particularly pronounced against HL60 (Figure 3, Table II) where the amido-18-OEt analogue exhibits a statistically significant increase in activity as can be seen by the difference in ID_{50} versus ET-18-OMe. The lyso amido analogue amido-16-OH (**5f**) exhibited lower toxicity when incubated with HL60 up to 10 μM, while very preliminary experiments indicate that the ureido derivative **10** is very active against this cell line (ID_{50} = 2.6 μM). The toxicity of the amido analogues against the K562 leukemic cell line was comparable to that of ET-18-OMe (Figure 4). It should be noted that K562 cells are less sensitive to the action of this group of agents,[17,40] and higher doses are required to exert a toxic effect.

The reference compound, however, has stronger antiproliferative activity against gynecologic tumors in the clonogenic assay (Figure 5). Despite this difference in behavior, an inhibition of colony formation greater than 90% is reached by amido-18-OMe (**5b**), amido-16-OEt (**5c**) and amido-18-OEt (**5d**) at the highest dose when compared to ET-18-OMe.

In summary, it can be concluded that (1) amido substitution at position 1 provides analogues with a strong inhibitory action against neoplastic cell growth in vitro; (2) in one case, an amido analogue (amido-18-OEt, **5d**) is significantly more active than the reference compound; (3) analogues with an ethyl group at position 2 exhibit better cytotoxic activity than those with a methyl group in the same position; and (4) a compound unsubstituted at position 2 has weaker cytotoxic effects at the doses used. These findings are in good agreement with previous structure–activity studies by us[12,13,17] and other investigators.[41] These results indicate that the amido dialkyl glycerophosphocholine analogues are promising compounds that warrant extended in vivo investigation.

Protein Kinase C Inhibition. The amido alkyl analogues were also tested for inhibition of protein kinase C. We found that all the analogues that had antineoplastic activity also inhibit protein kinase C in a concentration-dependent manner (Figure 6). Amido-16-OH is the least effective analogue in the enzyme-inhibition assay as well as in inhibiting neoplastic cell growth. The C_{16} analogues (amido-16-OMe and amido-16-OEt) were also tested (data not shown) and found to have comparable activity to the C_{18} analogues (Figure 6).

The structure–activity relationships observed with the protein kinase C and the cell growth assays were not identical. This may be because the enzyme inhibition assay was performed on an in vitro reconstitution system instead of intact cells. In intact cells, the activity of the compounds is probably based on a number of parameters, including uptake and metabolism by the cells.

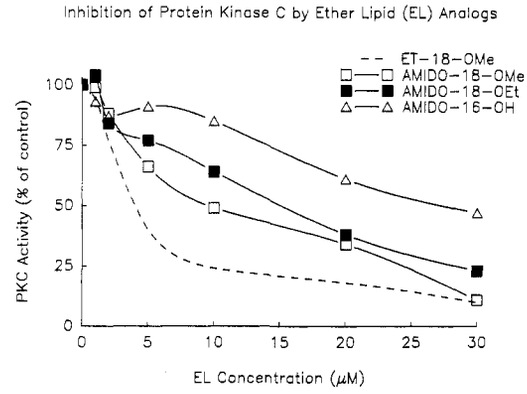

Figure 6.

Experimental Section

Chemical Synthesis. Proton nuclear magnetic resonance spectra were recorded in $CDCl_3$ on either a JEOLCO 60-MHz or a Bruker 250-MHz spectrometer and chemical shifts are reported in parts per million relative to internal tetramethylsilane. Infrared spectra were recorded on a Perkin-Elmer 1320 spectrometer as thin films (oils) or Nujol mulls (solids). Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Chemical-ionization mass spectra (CIMS) were recorded on a Ribermag R10-10 mass spectrometer. Microanalyses were performed by M-H-W Laboratories and Atlantic Microlab, Inc. All reactions requiring anhydrous conditions and/or an inert atmosphere were performed under a positive pressure of dry nitrogen with dry solvents and reagents. Tetrahydrofuran (THF) was distilled from LAH, benzene and acetonitrile (CH_3CN) from CaH_2, *N,N*-dimethylformamide (DMF) from CaO, pyridine and triethylamine (NEt_3) from KOH. Triphenylmethyl chloride (trityl chloride) was recrystallized from benzene/petroleum ether (1:2); 2-chloro-2-oxo-1,3,2-dioxaphospholane was prepared by literature procedure[44] and distilled before use. Chromatographic purification was achieved by using silica gel 60 (230–400 mesh). The phospholipids were detected with a 1% cobalt/acetone spray.[45]

(±)-3-Hexadecanamido-1,2-propanediol (1a). To a mechanically stirred solution of (±)-3-amino-1,2-propanediol (33.2 g, 0.36 mol) in 100 mL of pyridine was added a solution of palmitoyl chloride (100.0 g, 0.36 mol) in 25 mL of DMF. After 2 h the gelatinous mass was filtered and air-dried. The solid was recrystallized successively from hot EtOH and hot 2-propanol to give 109.4 g of **1a** (91%) as a white powder (mp 96–98 °C). ^1H NMR: δ 0.86 (t, 3 H, CH_3), 1.25 (s, 26 H, aliphatic CH_2), 2.20 (t, 2 H, $NCOCH_2$), 3.3–3.5 (m, 4 H, NCH_2CHCH_2OH), 4.10 (m, 1 H, $CHOH$). IR: 3500, 3320, 2960, 1640 (C=O), 1550 cm^{-1}. Anal. for $C_{19}H_{39}NO_3$.

(±)-3-Octadecanamido-1,2-propanediol (1b). This product was obtained as described for **1a** from (±)-3-amino-1,2-propanediol (10.1 g, 0.11 mol) and stearoyl chloride (33.6 g, 0.11 mol) to give 34.8 g of **1b** (95%) as a white powder, mp 104–106 °C (lit.[26] mp 105 °C, lit.[27] mp 92–97 °C).

(±)-3-Hexadecanamido-1-(triphenylmethoxy)-2-propanol (2a). Trityl chloride (13.7 g, 0.049 mol) was added to a stirring solution of **1a** (13.0 g, 0.040 mol) in 100 mL of pyridine. This was stirred for 10 h at 50 °C. After the pyridine was removed under reduced pressure, the residue was diluted with 100 mL of water and extracted with 3 × 50 mL of $CHCl_3$. The combined extracts were washed with 50 mL each of cold 5% HCl, and saturated NaCl, dried (Na_2SO_4), filtered, and evaporated to dryness. Part of the product (5.0 g, 22%) was obtained in pure form (mp 76–77 °C) by precipitation from hot petroleum ether.

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The filtrate was concentrated and purified by chromatography on silica gel (hexane/ethyl acetate, 4:1) to recover another 11.1 g (49%) of **2a**. ^{1}H NMR: δ 0.90 (t, 3 H, CH_3), 1.25 (s, 26 H, aliphatic CH_2), 2.10 (t, 2 H, $NCOCH_2$), 3.35–4.10 (m, 5 H, propyl backbone H), 7.35 (m, 15 H, aromatic H). IR: 3500, 3350, 3050, 2910, 1640 (C=O), 1550 cm^{-1}.

(±)-3-Octadecanamido-1-(triphenylmethoxy)-2-propanol (2b). This product was obtained as described for **2a** from **1b** (7.5 g, 0.023 mol) and trityl chloride (8.0 g, 0.029 mol) to give 8.6 g of **2b** (63%) as a white powder (mp 81–82 °C).

(±)-3-Hexadecanamido-2-methoxy-1-(triphenylmethoxy)propane (3a). A solution of **2a** (5.0 g, 0.0087 mol) in 35 mL of THF was added to a slurry of 80% NaH (0.31 g, 0.0087 mol) in 10 mL of THF. After the mixture was stirred for 30 min at 45 °C, methyl iodide (1.2 g, 0.0087 mol) was added, and the mixture was left to stir overnight at ambient temperature. Water (25 mL) was added slowly to decompose any residual NaH. Diethyl ether (25 mL) was added, and the layers were separated. The aqueous layer was extracted with 3 × 20 mL of ether. The combined organic extracts were washed with saturated NaCl solution, dried (Na_2SO_4), filtered, and evaporated to dryness. The product was precipitated from hot petroleum ether to give 4.8 g of **3a** (94%) as a fine white powder (mp 73–76 °C). ^{1}H NMR: δ 0.80 (t, 3 H, CH_3), 1.25 (s, 26 H, aliphatic CH_2), 2.15 (t, 2 H, $NCOCH_2$), 3.10 (s, 3 H, OCH_3), 3.30–4.15 (m, 5 H, propyl backbone H), 7.25 (m, 15 H, aromatic H). IR: 3260, 3080, 2940, 1650 (C=O), 1550 cm^{-1}.

(±)-3-Octadecanamido-2-methoxy-1-(triphenylmethoxy)propane (3b). This product was obtained as described for **3a** from **2b** (5.5 g, 0.0092 mol), NaH (0.28 g, 0.0092 mol), and methyl iodide (2.1 g, 0.015 mol) to give 5.2 g of **3b** (92%) as a white powder (mp 91–93 °C).

(±)-3-Hexadecanamido-2-ethoxy-1-(triphenylmethoxy)propane (3c). This product was obtained as described for **3a** from **2a** (2.1 g, 0.0037 mol), NaH (0.11 g, 0.0037 mol), and ethyl iodide (0.69 g, 0.0044 mol) to give 2.1 g of **3c** (95%). This was a brown oil, which could not be crystallized and was used without further purification.

(±)-3-Octadecanamido-2-ethoxy-1-(triphenylmethoxy)propane (3d). This product was obtained as described for **3a** from **2b** (4.3 g, 0.0072 mol), NaH (0.22 g, 0.0072 mol), and ethyl iodide (1.7 g, 0.011 mol) to give 3.1 g of **3d** (69%). This was a brown oil, which could not be crystallized and was used without further purification.

(±)-3-Hexadecanamido-2-(benzyloxy)-1-(triphenylmethoxy)propane (3e). This product was obtained as described for **3a** from **2a** (8.0 g, 0.014 mol), NaH (0.42 g, 0.014 mol), and benzyl chloride (2.13 g, 0.017 mol) to give 7.7 g of **3e** (86%). This was an orange/brown oil, which could not be crystallized and was used without further purification.

(±)-3-Hexadecanamido-2-methoxy-1-propanol (4a). A cooled (ice bath) solution of **3a** (4.8 g, 0.0082 mol) in 100 mL of CH_2Cl_2 was treated with a boron trifluoride/methanol complex (2.2 g, 0.016 mol) and left to stir for 4 h at ambient temperature. Water (50 mL) was added, and the layers were separated. The CH_2Cl_2 layer was washed with 2 × 25 mL of water and 25 mL of saturated NaCl solution, dried (Na_2SO_4), filtered, and evaporated to dryness. Crude product was obtained by precipitation from hot petroleum ether. This was purified by chromatography on silica gel with hexane/ethyl acetate (2:1) to give 1.4 g of **4a** (50%) as a fine white powder (mp 61–62 °C). ^{1}H NMR: δ 0.85 (t, 3 H, CH_3), 1.25 (s, 26 H, aliphatic CH_2), 2.20 (t, 2 H, $NCOCH_2$), 3.10 (s, 3 H, OCH_3), 3.30–4.25 (m, 5 H, propyl backbone H). IR: 3500, 3350, 2960, 1640 (C=O), 1550 cm^{-1}. Anal. for $C_{20}H_{41}NO_3$.

(±)-3-Octadecanamido-2-methoxy-1-propanol (4b). This product was obtained as described for **4a** from **3b** (6.3 g, 0.010 mol) and BF_3/MeOH (4.1 g, 0.031 mol) to give 2.9 g of **4b** (76%) as a white powder (mp 69–70 °C). Anal. for $C_{22}H_{45}NO_3$.

(±)-3-Hexadecanamido-2-ethoxy-1-propanol (4c). This product was obtained as described for **4a** from **3c** (2.2 g, 0.0037 mol) and BF_3/MeOH (1.2 g, 0.0092 mol) to give 0.60 g of **4c** (48%) as a white powder (mp 75–76 °C). Anal. for $C_{21}H_{43}NO_3$.

(±)-3-Octadecanamido-2-ethoxy-1-propanol (4d). This product was obtained as described for **4a** from **3d** (3.0 g, 0.0048 mol) and BF_3/MeOH (1.90 g, 0.014 mol) to give 1.0 g of **4d** (54%) as a white powder (mp 79–80 °C). Anal. for $C_{23}H_{47}NO_3$.

(±)-3-Hexadecanamido-2-(benzyloxy)-1-propanol (4e). This product was obtained as described for **4a** from **3e** (6.2 g, 0.0093 mol) and BF_3/MeOH (3.7 g, 0.028 mol) to give 2.8 g of **4e** (68%) as a white powder (mp 68–69 °C). Anal. for $C_{26}H_{45}NO_3$.

(±)-3-Hexadecanamido-2-methoxypropan-1-ol Phosphocholine (5a). To a cooled (ice bath) stirring solution of **4a** (0.60 g, 0.0018 mol) and NEt_3 (0.21 g, 0.0021 mol) in 25 mL of dry benzene was added 2-chloro-2-oxo-1,3,2-dioxaphospholane (0.30 g, 0.0021 mol) in 2 mL of benzene. The mixture was stirred at ambient temperature for 4 h. The precipitated amine salt was filtered off and washed with 10 mL of benzene. The filtrate was evaporated under reduced pressure to give the intermediate phosphotriester (single, phosphate-positive spot on TLC) as a white solid, which was used without further purification. This was transferred to a dry pressure flask containing 25 mL of dry CH_3CN and cooled with a dry ice/acetone bath. After the addition of 2 mL of anhydrous trimethylamine, the flask was sealed, and the mixture stirred at 65 °C overnight. Upon cooling, a white solid separated, which was collected by filtration and precipitated from $CHCl_3$/acetone (1:10). Purification by chromatography on silica gel with $CHCl_3$/MeOH/NH_4OH (75:25:5) followed by a final reprecipitation from acetone gave 0.40 g of **5a** (45%) as a hygroscopic white solid (decomposes at 240 °C). CIMS (NH_3): 509 (MH^+). Anal. for $C_{25}H_{53}N_2O_6P{\cdot}3H_2O$.

(±)-3-Octadecanamido-2-methoxypropan-1-ol Phosphocholine (5b). This product was obtained as described for **5a** from **4b** (1.0 g, 0.0027 mol), NEt_3 (0.30 g, 0.0030 mol), and the phosphorylating agent (0.42 g, 0.0030 mol) to give 0.90 g of **5b** (62%) as a hygroscopic white powder (decomposes at 230 °C). CIMS (NH_3): 537 (MH^+). Anal. for $C_{27}H_{57}N_2O_6P{\cdot}1.5H_2O$.

(±)-3-Hexadecanamido-2-ethoxypropan-1-ol Phosphocholine (5c). This product was obtained as described for **5a** from **4c** (0.60 g, 0.0017 mol), NEt_3 (0.20 g, 0.0020 mol), and the phosphorylating agent (0.26 g, 0.0020 mol) to give 0.35 g of **5c** (39%) as a hygroscopic white powder (decomposes at 250 °C). CIMS (NH_3): 523 (MH^+). Anal. for $C_{26}H_{55}N_2O_6P{\cdot}1H_2O$.

(±)-3-Octadecanamido-2-ethoxypropan-1-ol Phosphocholine (5d). This product was obtained as described for **5a** from **4d** (1.0 g, 0.0026 mol), NEt_3 (0.29 g, 0.0029 mol), and the phosphorylating agent (0.41 g, 0.0029 mol) to give 0.60 g of **5d** (42%) as a hygroscopic white powder (decomposes at 245 °C). CIMS (NH_3): 551 (MH^+). Anal. for $C_{28}H_{59}N_2O_6P{\cdot}1H_2O$.

(±)-3-Hexadecanamido-2-(benzyloxy)propan-1-ol Phosphocholine (5e). This product was obtained as described for **5a** from **4e** (1.50 g, 0.0036 mol), NEt_3 (0.40 g, 0.0040 mol), and the phosphorylating agent (0.56 g, 0.0040 mol) to give 0.50 g of **5e** (24%) as a hygroscopic white powder (decomposes at 250 °C). Also obtained was 0.50 g of the intermediate cyclic phosphotriester, which was recycled and aminated to give more of the choline. ^{1}H NMR: δ 0.90 (t, 3 H, CH_3), 1.28 (s, 26 H, aliphatic CH_2), 2.28 (t, 2 H, $NCOCH_2$), 3.10 (s, 3 H, OCH_3), 3.25 (s, 9 H, NCH_3), 3.30–4.25 (m, 5 H, propyl backbone H), 4.69 (s, 2 H, Ar CH_2), 7.36 (s, 5 H, Ar H). Anal. for $C_{31}H_{57}N_2O_6P{\cdot}1H_2O$.

(±)-3-Hexadecanamido-2-hydroxypropan-1-ol Phosphocholine (5f). A solution of **5e** (0.50 g, 0.00085 mol) in 10 mL of 4.4% formic acid/methanol was added to a 25-mL round-bottom flask containing 100 mg of 5% palladium black catalyst and 5 mL of 4.4% formic acid/methanol. After the mixture was stirred for 10 h at room temperature under an atmosphere of nitrogen, the catalyst was removed by filtration over Celite and washed with 2 × 10 mL of methanol. The combined filtrate and washings were evaporated to dryness, and the residue was precipitated from $CHCl_3$/acetone. Purification by chromatography on silica gel with $CHCl_3$/MeOH/NH_4OH (75:25:5) followed by a final reprecipitation from acetone gave 0.20 g of **5f** (48%) as a hygroscopic white solid (decomposes at 235 °C). ^{1}H NMR: δ 0.90 (t, 3 H, CH_3), 1.28 (s, 26 H, aliphatic CH_2), 2.28 (t, 2 H, $NCOCH_2$), 3.10 (s, 3 H, OCH_3), 3.25 (s, 9 H, NCH_3), 3.30–4.25 (m, 5 H, propyl backbone H). CIMS: 495 (MH^+). Anal. for $C_{24}H_{51}N_2O_6P{\cdot}1H_2O$.

(±)-3-(3-Octadecylureido)-1,2-propanediol (6). To a mechanically stirred solution of (±)-3-amino-1,2-propanediol (5.0 g, 0.055 mol) in a solution of 50 mL of pyridine and 25 mL of DMF was added a solution of octadecyl isocyanate (16.2 g, 0.055 mol) in 25 mL of DMF. After 3 h, the precipitated white product was filtered, washed with 100 mL of water, and air-dried. The solid was recrystallized successively from hot EtOH and hot 2-propanol

to give 17.8 g of **6** (84%) as a white powder (mp 121–123 °C). ^{1}H NMR (DMSO): δ 0.85 (t, 3 H, CH_3), 1.24 (s, 32 H, aliphatic CH_2), 3.10 (m, 4 H, NCH_2), 3.50 (m, 5 H, OH, propyl backbone H). IR: 3400–3200 (br), 3320, 2920, 2810, 1610 (C=O), 1580, 1450 cm^{-1}. Anal. for $C_{22}H_{46}N_2O_3$.

(±)-3-(3-Octadecylureido)-1-(triphenylmethoxy)-2-propanol (7). This product was obtained as described for **2a** from **6** (15.0 g, 0.038 mol) and trityl chloride (10.8 g, 0.038 mol) to give 13.0 g of **7** (53%) as a tan powder (mp 93–94 °C). ^{1}H NMR: δ 0.88 (t, 3 H, CH_3), 1.22 (s, 32 H, aliphatic CH_2), 3.0–3.4 (m, 4 H, NCH_2), 3.8–4.80 (m, 4 H, OH, propyl backbone H), 7.30 (m, 15 H, aromatic H). IR: 3340, 3050, 2920, 1610 (C=O), 1580, 1450 cm^{-1}.

(±)-3-(3-Octadecylureido)-2-ethoxy-1-(triphenylmethoxy)propane (8). This product was obtained as described for **3a** from **7** (2.0 g, 0.0032 mol), NaH (0.084 g, 0.0033 mol), and ethyl iodide (0.55 g, 0.0035 mol) to give 2.0 g of **8** (95%). This was a golden oil, which could not be crystallized and was used without further purification. ^{1}H NMR: δ 0.80 (t, 3 H, CH_3), 1.15 (t, 3 H, CH_3CH_2), 1.18 (s, 32 H, aliphatic CH_2), 2.90–3.55 (m, 7 H, NCH_2, propyl backbone H), 3.65 (m, 2 H, CH_3CH_2O), 7.20 (m, 15 H, aromatic H). IR: 3350, 3060, 3020, 2920, 1620 (C=O), 1560, 1440 cm^{-1}.

(±)-3-(3-Octadecylureido)-2-ethoxy-1-propanol (9). To a solution of **8** (2.0 g, 0.0030 mol) in a mixture of 50 mL of THF and 10 mL of wet MeOH was added 200 mg of *p*-toluenesulfonic acid. After the mixture was stirred for 16 h at ambient temperature, 5 mL of water was added, and the mixture was stirred for another 30 min. Ether (50 mL) was added, and the layers were separated. The organic phase was washed with 2 × 20 mL of saturated $NaHCO_3$ solution, dried (Na_2SO_4), filtered, and evaporated to dryness. The residue was precipitated twice from hot hexane to give 0.60 g of **9** (48%) as a fine white powder (mp 72–74 °C). ^{1}H NMR: δ 0.85 (t, 3 H, CH_3), 1.22 (s, 32 H, aliphatic CH_2), 1.18 (t, 3 H, CH_3CH_2O), 3.12 (t, 2 H, $NC(=O)NCH_2$), 3.55 (q, 2 H, CH_3CH_2), 3.20–3.50 (m, 5 H, propyl backbone H). IR: 3320, 2900, 1620 (C=O), 1560, 1450 cm^{-1}.

(±)-3-(3-Octadecylureido)-2-ethoxy-1-propanol Phosphocholine (10). This product was obtained as described for **5a** from **9** (0.55 g, 0.0013 mol), NEt_3 (0.16 g, 0.0016 mol), and the phosphorylating agent (0.23 g, 0.0016 mol) to give 0.30 g of **10** (39%) as a hygroscopic white powder (decomposes at 250 °C). ^{1}H NMR: δ 0.88 (t, 3 H, CH_3), 1.15 (t, 3 H, CH_3CH_2O), 1.25 (s, 32 H, aliphatic CH_2), 3.05 (t, 2 H, $NC(=O)NCH_2$), 3.34 (s, 9 H, NCH_3), 3.55–3.65 (m, 2 H, CH_3CH_2O), 3.80–3.90 (m, 5 H, propyl backbone H), 4.1–4.30 (m, 4 H, choline H). CIMS (NH_3): 580 (MH^+). Anal. for $C_{29}H_{62}N_3O_6P\cdot 2H_2O$.

Biological Evaluation. Growth Inhibition of Malignant Cells. Cell lines of human leukemias, HL60 and K562 (American Type Culture Collection, Bethesda, MD), were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/mL penicillin and 100 g/mL streptomycin (all from Gibco, Grand Island, NY). Human ovarian carcinoma cell line BG1[42] was propagated as a monolayer in McCoy's 5A culture media (Gibco) with 10% fetal calf serum, 0.1% penicillin, and 100 g/mL streptomycin. Serial passage following mild trypsinization was done weekly in culture flasks (Costar, Cambridge, MA).

Two techniques were used to evaluate the biological activity of the compounds. The cytotoxic potential against HL60 and K562 was measured by a vital stain, trypan blue, at different concentrations of compound incubated with 5 × 10^5 cells/mL in 16 culture dishes. Following incubation for 48 h at 37 °C in an atmosphere of 5% CO_2 and 95% humidified air, viable cells were enumerated as those that retained a cell membrane permeability barrier against trypan blue dye intrusion. A hemocytometer was used to count the cells. Changes in the clonogenicity of the tumor cells as a result of treatment with the test compounds were determined with a modified two-layer semisoft agarose assay as described previously.[16,42,43]

Preparation of Protein Kinase C. HL60 cells were grown in 75-mL flasks, harvested, and washed with ice-cold normal saline. After centrifugation (600*g*, 5 min, 4 °C), the cell pellet was resuspended (20 mM Tris·HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 50 mM mercaptoethanol, 2 mM phenylmethanesulfonyl fluoride) and sonicated for 20 s with a stepped microprobe. Unbroken cells were removed by centrifugation as above, and the supernatant was centrifuged (120000*g*, 90 min, 4 °C). The supernatant from this step (cytosol) was then fractionated on a 1 × 8 cm DEAE-Sephacel column after addition of sucrose to a final concentration of 10%. After the column was equilibrated (20 mM Tris, pH 7.5, 0.2 mM EDTA, 0.2 mM EGTA, 50 mM mercaptoethanol, 10% sucrose), the sample was loaded, and unbound material was washed through with 40 mL of the equilibration buffer. Then protein kinase C was eluted by a gradient from 0 to 0.5 M NaCl in the buffer. Fractions of 1 mL were collected at 25 mL/h, and 0.05-mL aliquots were assayed for protein kinase C as below. The fractions with the highest activity were pooled and used in further experiments.

Assay of Protein Kinase C Activity. The assays were done at pH 7.5 in a total volume of 0.25 mL, and all tubes contained 25 mM Tris, 10 mM $MgCl_2$, 40 μg of histone, 10 μM ATP (including 1 μCi of [γ-^{32}P]ATP), 0.1 μM $CaCl_2$, 20 μg/mL phosphatidylserine, and 2 μg/mL diolein plus 0.05 mL of protein kinase C. Enzymic activity was determined as the incorporation of ^{32}P from [γ-^{32}P]ATP into histone in the presence of Ca^{2+}, phosphatidylserine, and diolein minus the incorporation in the absence of these activators. Reactions were initiated by the addition of protein kinase C and halted after 20 min at 30 °C by the addition of 0.05 mL of bovine serum albumin (10 mg/mL and 1 mL of 25% ice-cold trichloroacetic acid. The tubes were kept on ice and then filtered in a Millipore vacuum box with Millipore HA filters and washed with 25% trichloroacetic acid. The radioactivity bound to the filters was determined by scintillation counting in 5 mL of Budgel Solve. The amount of enzyme used was shown to result in linear activity for at least 20 min, and the assay was linearly dependent on the amount of enzyme used.

ET-18-OMe or the analogue to be tested was added directly to the reaction mixture before the addition of protein kinase C. As a control for the small amount of ethanol from the stock solution of ET-18-OMe, 0.1% ethanol was included in the samples with the enzyme but no ET-18-OMe. In addition to the standard assay components, 0.01% Triton X-100 was included in the experiments.

Acknowledgment. We thank Dr. Zia Sajadi for his assistance in preparing some of the intermediates and Dr. Michael Thomas of the Bowman Gray School of Medicine for performing the mass spectral analyses. Thanks are due to J. Gaines White and George W. Small for performing the biological evaluations. The research was supported in part by National Institutes of Health research grant HL 28491 (to University of North Carolina) and National Cancer Institute research grants CA 41314 and CA 43297 (to Bowman Gray School of Medicine, Wake Forest University). A research fellowship to Dr. Alessandro Noseda was kindly provided by Dr. Luciano Berti, Champion Industries, Winston-Salem, NC.

Registry No. **1a**, 112988-88-2; **1b**, 112988-89-3; **2a**, 112988-90-6; **2b**, 112988-91-7; **3a**, 112988-92-8; **3b**, 113008-34-7; **3c**, 112988-93-9; **3d**, 112988-94-0; **3e**, 112988-95-1; **4a**, 112988-96-2; **4b**, 88876-05-5; **4c**, 112988-97-3; **4d**, 112988-98-4; **4e**, 112988-99-5; **5a**, 112989-00-1; **5b**, 88876-07-7; **5c**, 112989-01-2; **5d**, 112989-02-3; **5e**, 112989-03-4; **5f**, 112989-04-5; **6**, 112989-05-6; **7**, 112989-06-7; **8**, 112989-07-8; **9**, 112989-08-9; **10**, 112989-09-0; $H_3C(CH_2)_{17}NCO$, 112-96-9; (±)-3-amino-1,2-propanediol, 112988-87-1; palmitoyl chloride, 112-67-4; stearoyl chloride, 112-76-5; 2-chloro-2-oxo-1,3,2-dioxaphospholane, 6609-64-9; protein kinase C, 9026-43-1.