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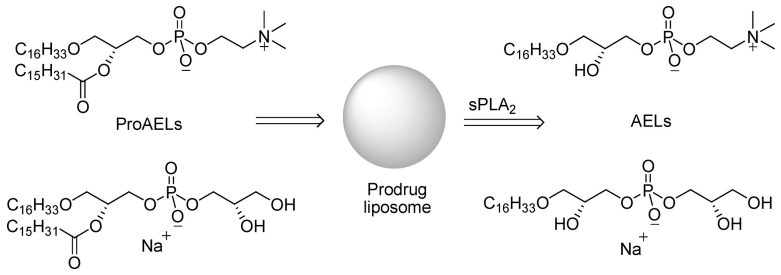

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Synthesis and Biological Activity of Anticancer Ether Lipids That Are Specifically Released by Phospholipase A_2 in Tumor Tissue

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The clinical use of anticancer lipids is severely limited by their ability to cause lysis of red blood cells prohibiting intravenous injection. Novel delivery systems are therefore required in order to develop anticancer ether lipids (AELs) into clinically useful anticancer drugs. In a recent article (*J. Med. Chem.* **2004**, *47*, 1694) we showed that it is possible to construct liposome systems composed of masked AELs that are activated by secretory phospholipase A_2 in cancerous tissue. We present here the synthesis of six AELs and evaluate the biological activity of these bioactive lipids. The synthesized AEL **1**−**6** were tested against three different cancer cell lines. It was found that the stereochemistry of the glycerol headgroup in AEL-**2** and **3** has a dramatic effect on the cytotoxicity of the lipids. AEL **1**−**4** were furthermore evaluated for their ability to prevent phosphorylation of the apoptosis regulating kinase Akt, and a correlation was found between their cytotoxic activity and their ability to inhibit Akt phosphorylation.

Introduction

The clinical use of many conventional chemotherapeutics is often limited due to inadequate delivery of therapeutic concentrations to the tumor target tissue or due to severe and harmful toxic effects on normal organs. It is therefore of importance to develop novel microcarrier technologies that can be used for targeted drug delivery and tumor specific activation.[1] The Doxil formulation is an example hereof.[2,3] However, the great stability of the Doxil formulation both during circulation and in the tumor tissue has been argued to present a paradoxical problem.[4−6] Therefore, it has been suggested that site-specific triggered drug release can be achieved by the design of liposomes that are sensitive to local hyperthermia,[7−10] by pH-sensitive liposomes,[11−14] by light sensitive,[15−17] or by liposomes that are destabilized by enzymes that are overexpressed in diseased tissue.[18,19] We have reported a new principle for targeted and triggered liposomal prodrug delivery of anticancer drugs to cancerous tissue.[6,20−23] By exploiting important physicochemical properties of the prodrug liposomes and certain pathophysiological characteristics of cancerous tissue, we have suggested that it is possible to obtain a triggered release and activation of anticancer ether lipids (AEL) specifically in tumor tissue. This principle is based on polymer-covered prodrug liposomes constituted of masked anticancer ether lipids (proAELs).[22] The proAELs are converted to active anticancer drugs by elevated levels of secretory phospholipase A_2 (sPLA$_2$) in the cancer tissue.[24,25]

Secretory PLA$_2$, a subgroup of the PLA$_2$ superfamily, can be devided into a large number of subtypes named IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII which have been identified in humans at gene or transcript level.[26]

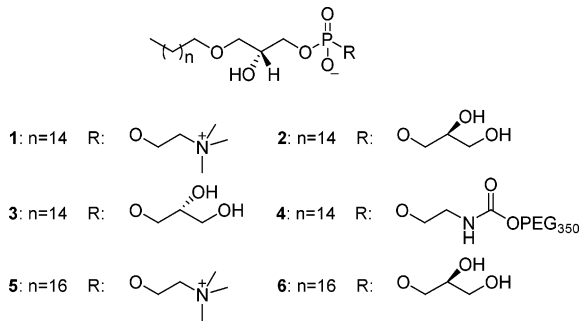

Figure 1. 1-*O*-hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine (AEL-**1**), two stereoisomers of 1-*O*-hexadecyl-2-lyso-*sn*-glycero-3-phosphoglycerol (AEL-**2** *(S)*-isomer, and AEL-**3** *(R)*-isomer), 1-*O*-hexadecyl-2-lyso-*sn*-glycero-3-phosphoethanolamine poly(ethylene glycol)$_{350}$ (AEL-**4**), 1-*O*-octadecyl-2-lyso-*sn*-glycero-3-phosphocholine (AEL-**5**), 1-*O*-octadecyl-2-lyso-*sn*-glycero-3-phospho-*(S)*-glycerol (AEL-**6**).

The sPLA$_2$ subtypes have different lipid substrate specificity, and it has been shown that sPLA$_2$ type IIA mainly acts on anionic lipid substrates whereas sPLA$_2$ type V and X hydrolyze both anionic and zwitterionic lipid membranes.[26] Type IIA and X sPLA$_2$ have been shown to be of particular interest in relation to human cancer, and increased expression of sPLA$_2$ IIA has been identified in several human tumors including breast,[27] stomach,[28,29] colorectal,[30−32] pancreatic,[33] prostate,[25,34] and liver cancer.[35]

The aim of the present work has been to synthesize and evaluate the biological activity of a series of anticancer ether lipids (Figure 1). These lipids can all be released specifically in cancer tissue by the action of sPLA$_2$ and their respective prodrugs (proAELs) are able to form stable liposome systems (Figure 2). The proAEL liposomes can furthermore be used for targeted transport and delivery of water-soluble drugs to tumors,[6,21,22] e.g. cisplatin and doxorubicin. A large variety of AELs have been synthesized and tested in many in vitro and in vivo models.[36] Many are very potent molecules, but

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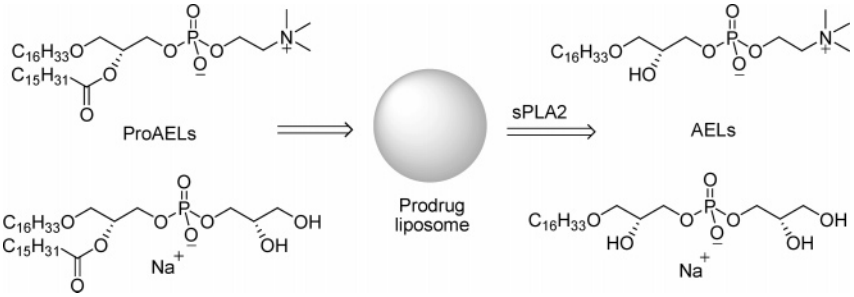


Figure 2. Schematic overview of the proAEL lipids that can be formulated as liposomes. After $sPLA_2$-mediated hydrolysis the proAEL lipids are converted into anticancer ether lipids (AELs) giving a free hydroxy group in the *sn-2* position. Because the AELs do not support a liposomal structure, the liposomes collapse and the ether lipids are released site specifically in the cancer tissue.[22]

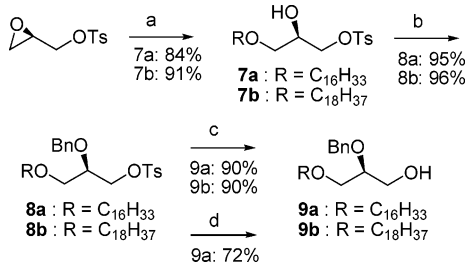
a general problem has been their toxicity toward red blood cells.[37] The generation of ether lipid prodrugs formulated as liposomes, which can be hydrolyzed and activated specifically by $sPLA_2$ in the tumor, circumvents the hemolytic limitations of using AELs as anticancer drugs for intravenous administration.[22,23] Anticancer ether lipids (e.g. edelfosine) and anticancer lipids (e.g. miltefosine) are known to achieve their cytotoxic activity through multiple mechanisms.[36] Some of these mechanisms involve direct membrane perturbation, induction of differentiation, and activation of macrophages resulting in cell cycle arrest and apoptosis possibly through inhibition of PKC, PLC, and Akt, which are known to be important cell growth regulating enzymes.[36,38−40] High levels of active Akt have been found in many types of human tumors[40] and a correlation has been found between high levels of Akt and defects in the regulatory phosphatase PTEN.[41] To obtain a better understanding of Akt in cancer biology, it is of interest to develop inhibitors directed against Akt. This can furthermore lead to new insight in the development of novel anticancer drugs. Not all lysophospholipids inhibit Akt phosphorylation. For example, the mitogenic compound lysophosphatidic acid (LPA) has been found to induce rather than inhibit Akt phosphorylation,[38] indicating that the phospho-linked headgroup is of crucial importance for the ability of these lipids to inhibit Akt and cause growth inhibition.

We report herein the synthesis of AEL **1**−**6** (Figure 1) and the biological activity of these ether lipids by exploring the cytotoxic activity in vitro and their ability to inhibit Akt phosphorylation. These studies are required in order to obtain knowledge about which lipids that are suitable candidates for the preparation of $sPLA_2$ degradable prodrug liposomes when progressing to in vivo experiments. Finally, we show that proAEL liposomes can be hydrolyzed in an in vitro cell culture experiment by $sPLA_2$ secreted from KATO III gastric cancer cells, generating the cytotoxic AELs that cause cancer cell death.

Results and Discussion

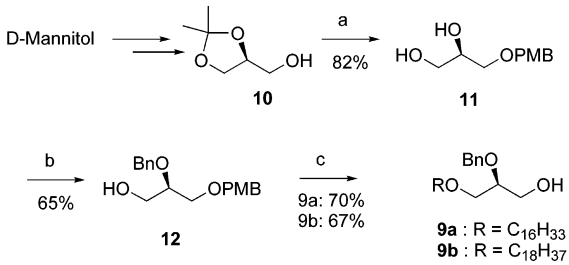
Synthesis of AEL 1−6 (Figure 1). The synthesis of various analogues of the target AELs has earlier been accomplished from D-mannitol,[42−46] the corresponding protected glycerols,[47−50] and by ring-opening of glycidols.[47,51−53] Synthesis of glycerol **9** was achieved in few steps on a small scale by using (*R*)-glycidol tosylate as the starting material (Scheme 1). By using BF_3 as a Lewis acid,[51,52] it was possible to obtain **7a** in high yield as a 9:1 mixture of regioisomers which was conveniently purified by recrystallization from hexane to obtain **7a** in 84% yield. The analogous ether **7b** was obtained in 91% yield after recrystallization as a higher regioselectivity was observed. By acid-catalyzed benzylation using benzyl trichloroacetimidate, it was possible to obtain the benzyl-protected glycerol **8** in good yield, which was converted into **9** by the use of cesium acetate followed by a reduction[52] or alternatively by a very convenient hydrolysis using sodium nitrite. D-mannitol is a cheap optically pure starting material that can be converted into glycerol **10** on large scale (Scheme 2). The synthetic transformations are simple and we decided to use this method for a large-scale synthesis of **9** starting from ∼50 g of D-mannitol. The synthesis of the isopropylidene-protected glycerol **10** was conducted as described by Schmid et al.[46] By optimizing the purification sequence,

Scheme 1. Synthesis of 1-*O*-Hexadecyl-2-*O*-benzyl-*sn*-glycerol and 1-*O*-Octadecyl-2-*O*-benzyl-*sn*-glycerol from *(R)*-Glycidyl Tosylate[a]



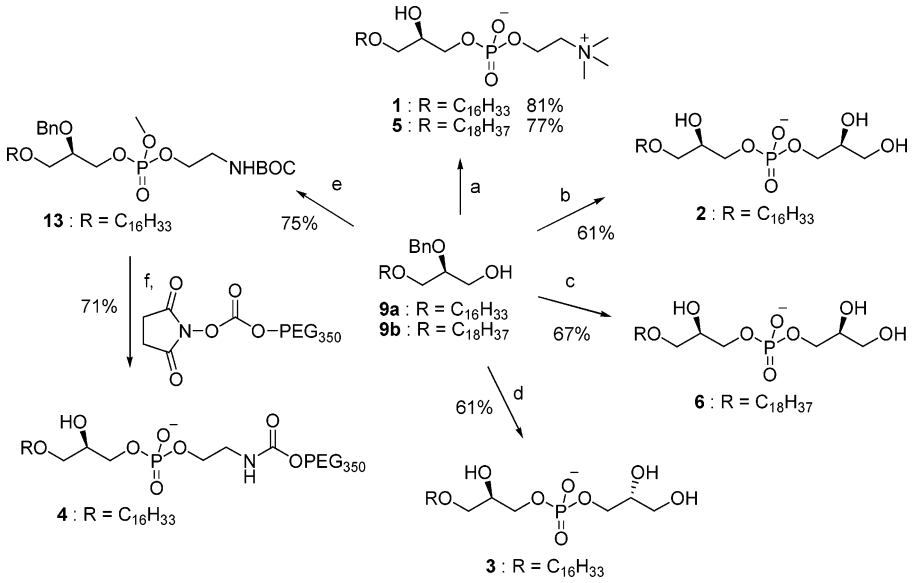
[a] (a) $C_{16}H_{33}OH$ or $C_{18}H_{37}OH$, $BF_3{\cdot}OEt_2$, DCM; (b) BnOC(NH-)CCl_3, TfOH, dioxane; (c) i. AcOCs, DMF, DMSO; ii. $LiAlH_4$, Et_2O; (d) $NaNO_2$, DMF.

Scheme 2. Synthesis of 1-*O*-Hexadecyl-2-*O*-benzyl-*sn*-glycerol and 1-*O*-Octadecyl-2-*O*-benzyl-*sn*-glycerol from D-Mannitol[a]



[a] (a) i. NaH, PMBCl, Bu_4NI, THF, DMF; ii. TsOH, MeOH (b) i. TrCl, DMAP, pyridine, CH_2Cl_2; ii. NaH, BnBr, THF, DMF; iii. TsOH, MeOH; (c) i. NaH, $C_{16}H_{33}Br$ or $C_{18}H_{37}Br$, THF, DMF; ii. DDQ, H_2O, CH_2Cl_2.

Scheme 3. Synthesis of AEL **1-6**[a]



[a] (a) ia. $POCl_3$, Et_3N, DCM; ib. pyridine, choline tosylate; ii. H_2, 10% Pd/C, MeOH (b) ia. $MeOPOCl_2$, TMP, toluene; ib. (*R*)-isopropylidene glycerol, TMP; iia. Me_3N, CH_3CN, isopropyl alcohol, CH_2Cl_2; iib. 0.5 M HCl, MeOH, CH_2Cl_2; iii. H_2, Pd/C, MeOH; (c) ia. $(i\text{Pr})_2NPClOMe$, TMP, CH_2Cl_2; ib. 5-phenyl-1*H*-tetrazole, (*R*)-isopropylidene glycerol; ic. *t*BuOOH; iia. Me_3N, CH_3CN, isopropyl alcohol, CH_2Cl_2; iib. 0.5 M HCl, MeOH, CH_2Cl_2; iii. H_2, Pd/C, MeOH; (d) as b using (*S*)-isopropylidene glycerol; (e) ia. $MeOPOCl_2$, TMP, toluene; ib. *N*-BOC-ethanol amine, TMP; (f) i. CF_3COOH, CH_2Cl_2; ii. Et_3N, activated PEG_{350}, $CHCl_3$; iii. Me_3N, CH_3CN, isopropyl alcohol, CH_2Cl_2; iv. H_2, Pd/C, MeOH.

it was possible to synthesize **9** from D-mannitol with a limited number of purification steps. Glycerol **10** was converted into **11** in 82% yield. By using a trityl ether to protect the primary alcohol,[49] it was possible to obtain glycerol **12** without purification of the intermediates. Alkyl ether **9** was easily obtained from **12** in acceptable yield, again without purification of the intermediate. We found that the large-scale synthesis of **9** from D-mannitol was easy to perform and a convenient method after optimizing the purification procedure. However, due to the limited number of steps the expensive (*R*)-glycidol tosylate is our preferred starting material for small-scale synthesis of **9**. The experimental details for the synthesis of **9** from D-mannitol are provided as Supporting Information.

The coupling with the choline headgroup to form **1** and **5** (Scheme 3) was performed by the use of phosphorus oxychloride and choline tosylate under standard conditions.[47,54] In the workup, it was not possible to remove the salts by washing with water due to the amphiphilic properties of the molecule. Instead, the crude product was passed through a MB-3 ion-exchange column prior to purification by column chromatography. The use of dichloromethane instead of chloroform as solvent[22] in the coupling gave consistently high yields around 80%.

Preparation of the protected phosphatidylethanolamine **13** from **9a** was performed with commercially available methyl dichlorophosphate[22] and tetramethyl piperidine (TMP) as a sterically hindered base. This resulted in the desired compound, easily separated from the byproducts, and in good yield. Toluene was superior to chloroform as solvent and furthermore has the advantage that salts precipitate and can be filtered off prior to column chromatography. We have found this new phosphorylation method convenient when using cheap headgroups that can be used in excess. The method gave **13** in 75% yield. The deprotection of **13** was performed with TFA[50] in quantitative yield. The resulting amine was, without purification, coupled to activated poly(ethylene glycol) (Scheme 3).[22,55] An attempt to improve the polymer coupling procedure by using carbonyldimidazole[56] in a one-pot reaction resulted in a 23% yield and was not investigated further. The deprotection of the phosphate was initially performed with NaI[57,58] in 95% yield. However, due to difficulties with removal of I_2, we found that it was more convenient to use Me_3N,[59] which also proceeded in excellent yield. Thiophenol[60] and *n*-butylamine[61] were also investigated as nucleophiles for the deprotection of the phosphate. Thiophenol did not work well in our hands while *n*-butylamine gave comparable yields to Me_3N.

AEL-**2** and **3** were synthesized with methyl dichlorophosphate as the phosphorylation reagent, and Me_3N was used to deprotect the phosphate. However, a general problem was observed when trying to couple **9b** with methyl dichlorophosphate and isopropylidene glycerol, which gave a very low yield (25−35%). It seemed that the extra two carbon atoms in the aliphatic chain altered the reactivity possibly due to the formation of secondary structures. Several attempts to overcome this problem were not successful, and a different phosphorylation procedure had to be applied. We investigated the use of the less sterically hindered phosphorylation reagents $(i\text{-Pr})_2NPClOMe$[61,62] and $((i\text{-Pr})_2N)_2POMe$.[58] In our hands, $(i\text{-Pr})_2NPClOMe$ gave higher yields. It was found that the commercially available 5-phenyl-1*H*-tetrazole worked as well as 1*H*-tetrazole and was more convenient to use.[63] Furthermore, *m*-CPBA was found to result in some decomposition when oxidizing the phosphite and the milder oxidizing agent *t*-BuOOH gave higher yields. We originally used TFA[58] to remove the isopropylidene protection group from the glycerol headgroup. However, it was found that this method gave inconsistent yields due to the relatively harsh conditions, which were necessary to remove the acid on a rotor evaporator in the workup. Instead, we washed the crude product with 1 M HCl and with MeOH as

Table 1. IC_{50} Values (μM) for Three Different Cancer Cell Lines Determined by the MTT Assay[a]

	AEL-**1** PC	AEL-**2** (*S*)-PG	AEL-**3** (*R*)-PG	AEL-**4** PE-PEG350
HT-29	32 ± 8	20 ± 4[b]	105 ± 41[b]	63 ± 23
KATO III	39 ± 10	49 ± 11	nd	33 ± 6
Caco-2	68 ± 12	75 ± 7	nd	90 ± 14

[a] Cytotoxicity of AEL **1**−**4** was measured using the MTT assay and showed by mean ± SD (n = 3−9); nd: not determined. [b] The two stereoisomers showed significantly different IC_{50} values in the HT-29 colon cancer cell line, evaluated by one-way ANOVA analysis ($P < 0.001$).

cosolvent to increase the product solubility, giving the protonated phosphate, which apparently was able to function as a good intramolecular proton donor and participate in the isopropylidene deprotection when leaving the product in a homogeneous mixture of DCM, MeOH, H_2O overnight. However, it was sometimes necessary to add a little aq HCl to secure complete deprotection overnight. AEL-**6** was hereby obtained in 67% yield from **9b**.

Evaluation of Cytotoxic Activity. The synthesized AEL compounds were tested for cytotoxic activity using the MTT assay[64] in two colon cancer cell lines (HT-29 and Caco-2) and one gastric cancer cell line (KATO III). The concentration of AELs causing a 50% reduction in cell number (IC_{50} value) was determined after exposing the cells to AEL **1**−**4** for 72 h (Table 1). AEL-**1** and AEL-**2** showed similar cytotoxicity in the 20−30 μM range in HT-29 cells, 40−50 μM in the KATO III cell line, and 70−75 μM in the Caco-2 cells. The (*R*)-stereoisomer of the AEL-PG lipids (AEL-**3**) showed a significantly weaker cytotoxicity at 105 μM ($P < 0.001$) than the (*S*)-stereoisomer (AEL-**2**) in HT-29 cells (Table 1). This 5-fold difference in activity is interesting bearing in mind that the (*S*)-isomer is the major isomer in "natural" phosphatidylglycerol lipids. The reason for this may be that (*S*)-phosphatidylglycerol interacts with growth regulating receptors in a stereospecific manner, which should be investigated further. In addition, there may be metabolizing enzymes that are more active toward AEL-**3** than AEL-**2**, e.g. reacylating enzymes may not be able to acylate the 2-alcohol in the headgroup of AEL-**2**. Interestingly, a similar observation was found for lysoplatelet activating factor (lyso-PAF) by Lohmeyer and Workman,[65] who showed a 5-fold increase in IC_{50} value between the synthetic *S*-isomer of lyso-PAF-C16 compared to the "natural" *R*-isomer, which could be due to stereoselective activity of reacylating enzymes. Due to the weaker cytotoxicity of AEL-**3** in HT-29 cells, it was not taken into further evaluation in other cell lines as AEL-**2** was found to be the preferred drug for the proAEL liposome formulations. AEL-**4** with the PEG_{350} polymer-linked headgroup was also evaluated in the cell lines and showed a cytotoxic activity 2−3-fold higher in HT-29 cells, but similar activity in KATO III and Caco-2 cells (Table 1). A representative cytotoxic assay of the four AEL compounds tested in the HT-29 cell line can be seen in Figure 3.

Increasing the chain length from C16 to C18 was expected to show slightly increased cytotoxicity, as earlier observed for lyso-PAF-C16 versus lyso-PAF-C18 and ET-16-OCH_3 versus ET-18-OCH_3.[65] The cytotoxicity of AEL-**5** and **6** was tested in the HT-29 cell line and

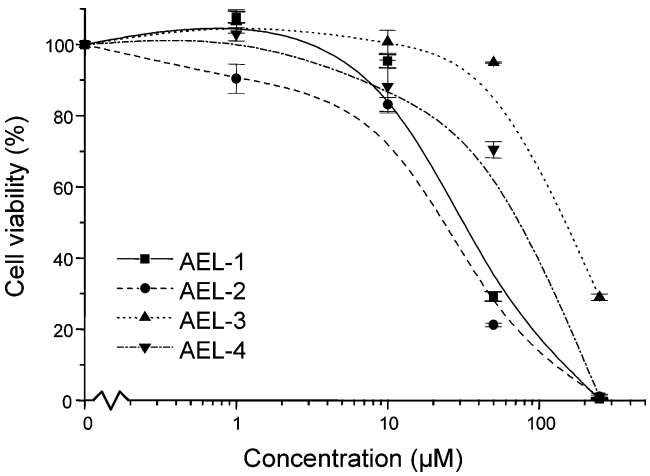


Figure 3. Representative graph showing the cytotoxic activity of AEL **1**−**4** in the HT-29 colon cancer cell line. Cells were exposed to the compounds for 72 h and the cytotoxic activity was evaluated by the MTT method.

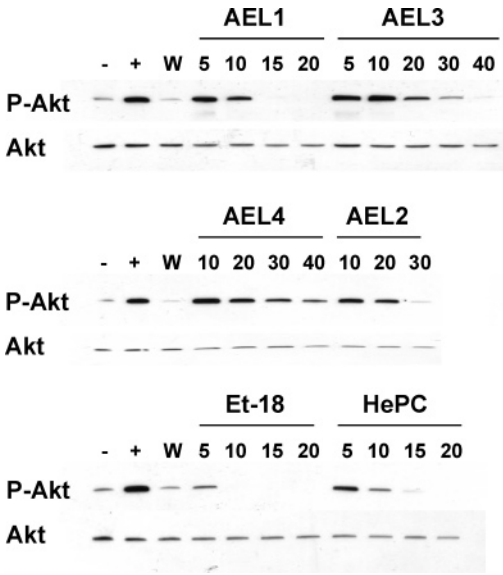


Figure 4. Ability of AEL **1**−**4**, edelfosine (ET-18-OCH_3), and miltefosine (HePC) to prevent Akt phosphorylation. MDA-MB-435 cells were serum starved for 18 h and treated with anticancer lipids (in μM concentrations) or the PI3K inhibitor wortmannin (W) (2 μM) for 2 h. Insulin was added for 30 min to all samples except (−) at a concentration of 10 μg/mL. Total cell lysates were analyzed for Ser-473-phosphorylated Akt (P-Akt) and total level of Akt (Akt) served as a control for equal loading of lysates.

compared to their corresponding C16 lipids (AEL-**1** and **2**). AEL-**5** and **6** showed a cytotoxic activity very similar to their C16-analogues (data not shown).

AEL Drugs Prevent Akt Phosphorylation. Recently, it was shown that several anticancer lipids are able to prevent phosphorylation and activation of the apoptosis regulating protein Akt in in vitro models.[38] ET-18-OCH_3, hexadecylphosphocholine (HePC), and D-21266 (perifosine) prevented insulin-induced activation of Akt after cell incubation for 2 h.[38] We have analyzed the ability of AEL **1**−**4** to prevent Akt activation (Figure 4). We chose the MDA-MB-435 breast cancer cell line containing high levels of Akt[66] to investigate the ability of AEL **1**−**4** to inhibit Akt and thereby induce apoptosis. After 18 h of serum starvation, the cells were treated for 2 h with increasing

Table 2. IC_{50} Values (μM) for AEL **1**−**4**, ET-18-OCH_3 (edelfosine), and HePC (miltefosine) for Inhibition of Akt Phosphorylation, and for Cytotoxic Activity in MDA-MD 435 Breast-cancer Cells, Measured under Serum Free Conditions for Comparison to Akt Inhibitory Properties[a]

	AEL-**1** PC	AEL-**2** (*S*)-PG	AEL-**3** (*R*)-PG	AEL-**4** PE-PEG350	ET-18-OCH_3	HePC
IC_{50}-Akt	9−12	24−25	24−37	26−33	3−4	5−7
IC_{50}-MDA-MB 435	31 ± 6	47 ± 2.5[b]	73 ± 3.0[b]	43 ± 25.7	12 ± 1.5	23 ± 4.2

[a] AEL-**2** showed a significantly higher cytotoxicity than AEL-**3**, evaluated by students *t*-test. [b] $p < 0.001$.

concentration of each AEL compound, after which Akt phosphorylation was stimulated for 30 min with insulin, known to cause a robust Akt phosphorylation.[38] All AEL compounds were able to prevent Akt phosphorylation although at different concentrations (Figure 4, Table 2). AEL-**1** was the most potent showing an IC_{50} value for inhibition of Akt at approximately 10 μM. AEL-**2** and AEL-**3** had a comparable ability to inhibit Akt phosphorylation with IC_{50} values at 24−37 μM, respectively. It could not be determined from these data that the Akt inhibition follows the same trend as for the cytotoxic activity (the *S*-isomer being the more potent).

The polymer-linked ether lipid AEL-**4** showed an IC_{50} value of 26−33 μM, which is at approximately the same level as for AEL-**2** and AEL-**3**. For comparison, ET-18-OCH_3 and HePC were included in these assays and both showed IC_{50} values below 10 μM (3 and 6 μM, respectively). To determine to what extent the ability to inhibit Akt phosphorylation can be correlated to the cytotoxic activity, all six compounds were tested for cytotoxicity in the MDA-MB-435 cell line (Table 2) under equal serum free conditions and evaluated by the MTT method after 3 days of treatment. It should be noted that serum free conditions results in slightly lower IC_{50} values. The order of potency was similar to the Akt inhibitory data, with ET-18-OCH_3 being slightly more potent than HePC followed by AEL-**1** whereas AEL-**2** was significantly more cytotoxic than AEL-**3**. Overall, our data suggest that the ability of ether lipids to prevent Akt phosphorylation is correlated to their cytotoxic activity, and that the Akt inhibitory properties are likely to be an initial process in the molecular mechanisms behind ether lipid mediated cytotoxic activity and apoptosis.

ProAEL Liposomes Are Hydrolyzed in Vitro and Generates Cytotoxic Ether Lipids. After confirming the cytotoxic activity of the AEL compounds, we tested the prodrug counterparts of AEL-**2** and **4** (proAEL-2 and proAEL-4). We formulated proAEL liposomes consisting of 80% proAEL-2[23] and 20% proAEL-4[22] by extrusion with an average diameter of 100 nm as described earlier,[22] and the liposome bilayer structure was characterized by differential scanning calorimetry. The liposomes were added to the KATO III gastric cancer cell line, secreting $sPLA_2$ into the cell media.[67] Addition of the proAEL liposomes caused a dose-dependent cytotoxic activity, whereas addition of the $sPLA_2$ specific inhibitor LY313727[68] 15 min prior to addition of the liposomes completely abolished the cytotoxicity. This shows that the cytotoxic activity, a concomitant of AEL generation, was solely dependent on $sPLA_2$ activity (Figure 5). When additional $sPLA_2$ was incubated together with 200 μM of liposomes, there was no further increase in cytotoxic activity, indicating that the amount of $sPLA_2$ secreted by the KATO III cells was sufficient for efficient liposomal hydrolysis (approximately 3−6 ng/mL is secreted over 3 days).[23]

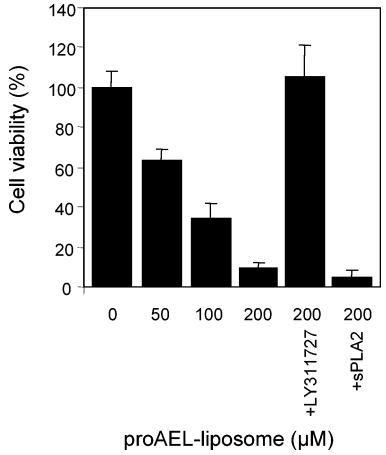


Figure 5. ProAEL liposomes (proAEL-2/proAEL-4 80:20) were hydrolyzed by $sPLA_2$, secreted from KATO III cells, causing a dose dependent cytotoxic activity on the cultured cancer cells. ProAEL liposomes were added to the KATO III cells and incubated for 72 h. The $sPLA_2$ specific inhibitor LY311727 (25μM) was added to the cells 15 min prior to addition of liposomes to allow optimal $sPLA_2$ interaction and inhibition. Cytotoxic activity was evaluated by the MTT method and each measurement point was determined in triplicate.

Conclusion. In a recent article (*J. Med. Chem,* 2004),[22] we showed that it is possible to construct liposome systems composed of masked anticancer ether lipids through the synthesis of proAELs and biophysical investigations of the formulated liposomes. The results we have presented herein continue this work and contribute to our understanding of how proAEL liposomes can be constructed to function as an "inactivated" drug delivery system that is specifically triggered by $sPLA_2$ and become cytotoxic in malignant tissue. We have carried out the synthesis of AEL **1**−**6** and evaluated these lipids for their cytotoxic activity toward HT-29, Caco-2, MDA-MB-435, and KATO III cancer cells, to establish which AELs that are the preferred candidates for constituting the proAEL liposomal drug delivery system. It is well-known that the cytotoxic activity of anticancer ether lipids is strongly dependent on the phospholipid headgroup. However, of particular interest, it was found that the stereochemistry of the glycerol headgroup (AEL-**2** and AEL-**3**) results in a 5-fold difference in activity. This is an interesting result as most lipid manufacturers currently sell phosphatidylglycerol lipids as headgroup racemates. The possible presence of lyso-phosphatidylglycerol specific receptors that are sensitive to the stereochemistry of the headgroup should be investigated further. In addition, we have investigated their ability to prevent the activation of the apoptosis regulating kinase Akt in MDA-MB-435 breast cancer cells. We found that there is a correlation between the cytotoxic activity exerted by anticancer ether lipids and their ability to inhibit Akt phosphorylation. Finally, we have shown that proAEL liposomes

can be activated specifically by $sPLA_2$ with concomitant release of the cytotoxic ether lipids causing cell death, pointing to this liposomal prodrug delivery strategy as a promising approach for tumor specific delivery of lipid-based drugs[23] as well as for delivery of encapsulated known chemotherapeutics.[6]

Experimental Section

Cell Culture and Measurement of Cytotoxicity. The KATO III human gastric carcinoma cell line was purchased from the Japan Health Sciences Foundation (Tokyo, Japan). The MDA-MB-435 breast cancer and HT-29 and Caco-2 human colon carcinoma cell lines were purchased from ATCC (Manassas, VA). KATO III were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate in a humidified 5% CO_2 atmosphere at 37 °C. HT-29 were grown in McCOY's 5A medium. MDA-MB 435 and Caco-2 in Dulbeccos were grown in modified eagles medium. Both were supplemented with 10% fetal calf serum and 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA). Cells were plated in 96-well plates at a density of 1×10^4 cells per well, 24 h prior to addition of AELs. AELs solubilized in PBS with 5−30% ethanol were added at indicated concentrations. After 72 h of incubation, cytotoxic activity was assessed using a standard 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay.[64] The MDA-MB-435 studies were performed without serum for the incubation period of 3 days, to be able to compare to the Akt inhibition studies. Values for cell survival are expressed as the percentage reduction in incorporated MTT. All studies were performed in triplicate. Cytotoxic activity measured in response to proAEL liposomes were performed by adding the extruded 100 nm liposomes in PBS to the cell culture media. The LY311727 inhibitor (kindly provided by Eli Lilly & Co, Indianapolis, IN) was dissolved in ethanol and added to a final concentration of 25 μM 15 min before the addition of liposomes in order to allow for optimal inhibition of the lipase.

Determination of Akt Inhibition. MDA-MB-435 cells were grown to 70% confluency and serum starved for 18 h. Each compound was added at the indicated concentration for 2 h after which Akt phosphorylation was stimulated by adding insulin at 10 μg/mL for 30 min. As negative control no insulin was added, and as positive control insulin was added for 30 min. For control of an active Akt pathway, the PI3-kinase inhibitor wortmannin (Sigma, Poole, United Kingdom) was added at 2 μM for 2 h prior to addition of insulin and showed in all cases lack of Akt phosphorylation. After 30 min stimulation with insulin cells were washed in ice-cold PBS and harvested in Laemmli sample buffer (LSB). Western blot was performed as recommended in manufacturers instructions (Invitrogen). The phosphospecific antibody against Ser473 was a rabbit monoclonal antibody, and the antibody recognizing total Akt was a rabbit polyclonal antibody (both were from Cell Signaling Technology, Beverly, MA). Immunodetection was accomplished using horseradish peroxidase-labeled goat anti rabbit IgG (H+L) (Vector Laboratories Inc., Burlingame, CA) diluted 1:4000, SuperSignalWest Pico enhanced chemiluminiscence (ECL) reagents (Pierce Biotech, Rockford, IL), and Hyperfilm-ECL (Amersham Pharmacia, Piscataway, NJ). Quantitation of phosphorylated Akt was performed using the Quantity One software from Biorad, Hercules, CA. Signal intensity was correlated to the total level of Akt, and the concentration capable of inhibiting Akt phosphorylation by 50% (IC_{50}) was calculated.

Synthesis. Reactions involving air-sensitive reagents were carried out under N_2 using syringe−septum technique. THF was freshly distilled over sodium/benzophenone ketyl. DMF, DMSO, CH_2Cl_2, $CHCl_3$, toluene, pyridine, TMP, and Et_3N were dried over 3 Å molecular sieves. Ether refers to diethyl ether. Reagents were purchased from Sigma-Aldrich Chemical Co. and used without further purification. Column chromatography was performed using silica gel (35−70 μm, 230−400 mesh). HPLC analysis was performed using a 5 μL diol column and an evaporative light scattering detector.[69] ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively.

(*R*)-1-*O*-Hexadecyl-3-(*p*-toluenesulfonyl)-glycerol (7a). (*R*)-Glycidyl tosylate (1.0 g, 4.39 mmol) and hexadecanol (1.5 g, 6.20 mmol) was dissolved in dry CH_2Cl_2 (20 mL) under N_2 and $BF_3{\cdot}OEt_2$ (3 drops) was added. The solution was stirred overnight and concentrated. A white solid formed that was recrystallized from hexane (10 mL) to give 1.74 g (84%) of **7**. $R_f = 0.59$ (ether/CH_2Cl_2 1:9); mp = 68−69 °C (lit.[47] 68−69 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.80 (d, J = 8.1 Hz, 2H), 7.38 (d, J = 8.1 Hz, 2H), 4.05−3.95 (m, 3H), 3.45−3.35 (m, 4H), 2.44 (s, 3H), 2.00 (br.s, 1H, *OH*), 1.50 (m, 2H), 1.29 (br.s, 26H), 0.89 (t, J = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 145.1, 133.2, 130.0, 128.2, 71.9, 70.8, 70.6, 68.6, 32.1, 29.9, 29.8, 29.8, 29.7, 29.6, 29.6, 26.2, 22.9, 21.8, 14.3. Anal. Calcd. $C_{26}H_{46}O_5S$: C 66.34, H 9.85, S 6.81; Found: C 66.24, H 9.98, S 6.80.

(*R*)-1-*O*-Octadecyl-3-(*p*-toluenesulfonyl)-glycerol (7b). Performed as for **7a** using octadecanol to afford 1.99 g (91%) of **7b** as white crystals. $R_f = 0.56$ (EtOAc/Hexane 3:7);^{1}H NMR (300 MHz, $CDCl_3$): δ 7.80 (d, J = 8.1 Hz, 2H), 7.38 (d, J = 8.1 Hz, 2H), 4.05−3.95 (m, 3H), 3.45−3.35 (m, 4H), 2.43 (s, 3H), 2.10 (br.s, 1H, *OH*), 1.51 (m, 2H), 1.29 (br.s, 30H), 0.90 (t, J = 6.7 Hz, 3H).

(*R*)-1-*O*-Hexadecyl-2-*O*-benzyl-3-(*p*-toluenesulfonyl)-glycerol (8a). (*R*)-1-*O*-hexadecyl-3-(*p*-toluenesulfonyl)-glycerol (**7a**) (1.70 g, 3.61 mmol) was dissolved in dioxane (35 mL) under N_2, and benzyl-2,2,2-trichloro-acetimidate (1.35 mL, 7.22 mmol) was added. Trifluoromethanesulfonic acid was added until the solution turned strongly acidic (approximately 15 drops). The reaction was stirred for 40 min after which CH_2Cl_2 (100 mL) was added, and the solution was washed with sat. $NaHCO_3$ (2 × 30 mL) and H_2O (2 × 20 mL). The organic phase was dried with $MgSO_4$ and concentrated. The yellow reminance was purified by column chromatography (EtOAc/hexane 1:6) to give 1.92 g (95%) of **8a** as clear oil. $R_f = 0.20$ (EtOAc/heptane 1:9). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.79 (d, J = 8.4 Hz, 2H), 7.39−7.22 (m, 7H), 4.59 (s, 2H), 4.20 (dd, J = 10.4, 4.3 Hz, 1H), 4.10 (dd, J = 10.4, 5.7 Hz, 1H), 3.78 (quintet, J = 5.2 Hz, 1H), 3.44 (m, 2H), 3.37 (t, J = 6.6 Hz, 2H), 2.42 (s, 3H), 1.50 (m, 2H), 1.29 (br.s, 26H), 0.90 (t, J = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 145.1, 138.0, 133.3, 129.9, 128.4, 128.1, 127.8, 75.6, 72.5, 71.9, 69.8, 69.6, 32.1, 29.9, 29.8, 29.8, 29.7, 29.6, 29.5, 26.2, 22.8, 21.5, 14.3. Anal. Calcd. $C_{33}H_{52}O_5S$: C 70.67, H 9.35, S 5.72; Found: C 70.60, H 9.39, S 5.70.

(*R*)-1-*O*-Octadecyl-2-*O*-benzyl-3-(*p*-toluenesulfonyl)-glycerol (8b). Performed as described for **8a** giving 2.27 g (96%) of **8b** as clear oil. R_f = 0.19 (EtOAc/heptane 1:9). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.80 (d, J = 8.4 Hz, 2H), 7.39−7.22 (m, 7H), 4.59 (s, 2H), 4.21 (dd, J = 10.1, 4.2 Hz, 1H), 4.10 (dd, J = 10.2, 5.4 Hz, 1H), 3.78 (m, 1H), 3.44 (m, 2H), 3.37 (t, J = 6.6 Hz, 2H), 2.41 (s, 3H), 1.51 (m, 2H), 1.29 (br.s, 30H), 0.90 (t, J = 6.3 Hz, 3H).

(*S*)-1-*O*-Hexadecyl-2-*O*-benzyl-glycerol (9a). (*R*)-1-*O*-Hexadecyl-2-*O*-benzyl-3-(*p*-toluenesulfonyl)-glycerol (**8a**) (1.90 g, 3.38 mmol) was dissolved in dry DMF (7 mL) and dry DMSO (28 mL) under N_2. CsOAc (1.37 g, 7.11 mmol) was added, and the reaction was heated to 60 °C and stirred at this temperature overnight. The mixture was quenched by addition of water (30 mL) and ether (80 mL), and the phases were separated. The organic phase was washed with water (3 × 30 mL), dried with Na_2SO_4, concentrated, and coevaporated with toluene (2 × 30 mL). The crude product was redissolved in dry ether (30 mL), the solution cooled to 0 °C, and $LiAlH_4$ (256 mg, 6.76 mmol) was added. The reaction was stirred for 30 min at 0 °C and then for 3 h at room temperature. TLC (EtOAc/heptane 1:4) indicated complete conversion, and the reaction was quenched by very slow addition of water (30 mL). The mixture was filtered through Celite, the phases were separated, and the aqueous phase was extracted with CH_2Cl_2 (3 × 15 mL). The combined organic phases were dried with Na_2SO_4 and concentrated, and the product was purified by column chromatography (EtOAc/heptane 1:4) to give 1.24 g (90%) of **9a** as a greasy solid. $R_f = 0.23$ (EtOAc/heptane 1:4).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40−7.24 (m, 5H), 4.68 (AB, J = 11.9 Hz, 2H), 3.81−3.49 (m, 5H), 3.44 (t, J = 6.6 Hz, 2H), 2.10 (br.s, 1H, *OH*), 1.60 (m, 2H), 1.29 (br.s, 26H), 0.90 (t, J = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 138.4, 128.6, 128.0, 77.9, 72.2, 72.0, 71.3, 63.2, 32.1, 29.9, 29.8, 29.6, 29.5, 26.3, 22.8, 14.3. Anal. Calcd. $C_{26}H_{46}O_3 \cdot H_2O$: C 73.54, H 11.39; Found: C 73.34, H 11.30.

(*S*)-1-*O*-Octadecyl-2-*O*-benzyl-glycerol (9b). Carried out as described above for **9a** giving 1.18 g (90%) of **9b** as a greasy white solid. R_f = 0.22 (EtOAc/heptane 1:4). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40−7.21 (m, 5H), 4.67 (AB, J = 11.8 Hz, 2H), 3.79−3.43 (m, 5H), 3.43 (t, J = 6.7 Hz, 2H), 2.23 (br.s, 1H, *OH*), 1.57 (m, 2H), 1.29 (br.s, 30H), 0.89 (t, J = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 138.4, 128.5, 127.9, 127.8 77.9, 72.1, 71.9, 71.2, 63.1, 32.0, 29.8, 29.7, 29.5, 29.4, 26.2, 22.8, 14.2. Anal. Calcd. $C_{28}H_{50}O_3 \cdot H_2O$: C 74.29, H 11.58; Found: C 74.11, H 11.50.

(*S*)-1-*O*-Hexadecyl-2-*O*-benzyl-glycerol (9a) using $NaNO_2$. (*R*)-1-*O*-Hexadecyl-2-*O*-benzyl-3-(*p*-toluenesulfonyl)-glycerol (**8a**) (1.0 g, 1.72 mmol) was dissolved in DMSO (35 mL) under N_2. $NaNO_2$ (3.56 g, 51.7 mmol) was added, and the reaction was heated to 40 °C and stirred overnight. Water (40 mL) was added and the solution stirred for 45 min after which it was extracted with CH_2Cl_2 (3 × 40 mL), dried with $MgSO_4$, and concentrated. The reaction was purified by column chromatography (EtOAc/hexane 1:3) giving 504 mg (72%) of **9a**.

1-*O*-Hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine (1). To a solution of $POCl_3$ (41 μL, 0.44 mmol) in dry CH_2Cl_2 (1.5 mL) at 0 °C was added a solution of **9a** (200 mg, 0.35 mmol) and dry Et_3N (48 μL, 0.47 mmol) in dry CH_2Cl_2 (3.5 mL) dropwise over 20 min. The reaction was stirred 30 min under N_2 at room temperature, after which dry pyridine (225 μL, 2.80 mmol) and choline tosylate (193 mg, 0.70 mmol) were added. The reaction was stirred overnight at room temperature. Water (0.2 mL) was added and the reaction stirred for 40 min. Continuous concentration with ethanol/toluene 1:1 (3 × 40 mL) gave the crude product as white foam. The residue was dissolved in THF/H_2O 9:1 and slowly passed through a MB-3 column (5 cm), and the solvent was removed by continuous concentration with ethanol/toluene 1:1 (3 × 40 mL). The crude product was purified by column chromatography (CH_2Cl_2/MeOH/H_2O 65:25:1) giving 162 mg (81%) of the benzyl-protected title compound as a white solid. R_f = 0.30 ($CHCl_3$/MeOH/H_2O 65:25:4). The product was redissolved in ethyl acetate (2 mL) and methanol (2 mL), and Pd/C 10% (20 mg) was added. The mixture was stirred under an H_2 atmosphere for 2.5 h after which TLC indicated that the reaction was complete ($CHCl_3$/MeOH/H_2O 65:25:4). The reaction was filtered giving the title compound in quantitative yield. R_f = 0.25 ($CHCl_3$/MeOH/H_2O 65:25:4). > 97% pure by HPLC. ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD 4:1): δ 4.21 (m, 2H), 3.95−3.70 (m, 3H), 3.57 (m, 2H), 3.43−3.32 (m, 4H), 3.15 (s, 9H), 1.53 (quintet, 2H), 1.28 (br.s, 26H); 0.86 (t, J = 6.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD): 71.6, 71.4, 69.6 (d, J = 6.3 Hz), 67.4 (d, J = 6.2 Hz), 66.3 (d, J = 6.5 Hz), 59.2 (d, J = 5.1 Hz), 54.0, 31.6, 29.6, 29.6, 29.5, 29.3, 25.8, 22.6, 13.8. Anal. Calcd. $C_{24}H_{52}NO_6P \cdot 1.5H_2O$: C 56.67, H 10.90, N 2.75; Found: C 56.95, H 10.51, N 2.77.

1-*O*-Octadecyl-2-lyso-*sn*-glycero-3-phosphocholine (5). Synthesized as described for **1** starting from 200 mg of **9b** giving 180 mg (77%) of **5** as a white solid. R_f = 0.17 ($CHCl_3$/MeOH/H_2O 65:25:4). >97% pure by HPLC. ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD/D_2O 65:25:4): δ 4.25 (m, 2H), 3.90 (m, 2H), 3.80 (m, 1H), 3.60 (m, 2H), 3.48−3.38 (m, 4H), 3.19 (s, 9H), 1.57 (quintet, 2H), 1.29 (br.s, 30H), 0.88 (t, J = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD/D_2O 65:25:4): δ 71.7, 71.5, 69.7, 67.4, 66.4, 59.3, 54.1, 31.8, 29.7, 29.7, 29.6, 29.4, 26.0, 22.7, 13.9. Anal. Calcd. $C_{26}H_{56}NO_6P \cdot 1.5H_2O$: C 58.18, H 11.08, N 2.61; Found: C 57.95, H 10.91, N 2.57.

General Procedure for the Deprotection of the Methyl Phosphate and the Isopropylidene Group. The crude product (scale 0.46 mmol) was redissolved in CH_3CN (4.2 mL), 2-propanol (5.7 mL), Me_3N (40% in aq. 5.25 mL), and CH_2Cl_2 (4.2 mL) followed by stirring overnight at room temperature. Concentration in vacuo and subsequent continuous concentration with toluene gave the desired product, which was dried at 0.1 mmHg for 1 h and used without further purification in the next step. The crude product was dissolved in CH_2Cl_2/MeOH (70 mL, 7:1) was washed with 1 M HCl (4 × 50 mL). 0.5 M HCl (4 mL) and MeOH (25 mL) were added to the organic phase resulting in a homogeneous solution that was stirred overnight after which TLC indicated that the isopropylidene deprotection was complete. The solution was neutralized by addition of $NaHCO_3$ (4.5 g) and stirred overnight. The reaction mixture was dried over Na_2SO_4, filtered, and concentrated in vacuo.

1-*O*-Hexadecyl-2-lyso-*sn*-glycero-3-phospho-(*S*)-glycerol (2). To a solution of Cl_2POOMe (0.256 μL, 2.56 mmol) in dry toluene (5 mL) at −25 °C was dropwise added a solution of **9a** (520 mg, 1.28 mmol) and TMP (497 μL, 2.95 mmol) in dry toluene (15 mL). The reaction was stirred for 6.5 h at room temperature after which TMP (650 μL, 3.84 mmol) and *(R)*-isopropylideneglycerol (960 μL, 7.68 mmol) were added. The mixture was stirred overnight. The solution was filtered through Celite after which CH_2Cl_2 (50 mL) was added. The solution was washed with brine (3 × 20 mL), dried with $MgSO_4$, and concentrated. The crude product was deprotected without further purification. However, purification by column chromatography (EtOAc/CH_2Cl_2 1:9) gives 75% yield. Anal. Calcd. for $C_{33}H_{59}O_8P$: C 64.47, H 9.67; Found: C 64.23, H 9.80. R_f = 0.33 (EtOAc/CH_2Cl_2 3:17). The deprotection of the methyl and the isopropylidene group was carried out using the deprotection procedure described above. Purification by column chromatography ($CHCl_3$/MeOH/H_2O 85:15:1) gave 454 mg (61%) of benzyl-protected **2** as a white solid. R_f = 0.27 ($CHCl_3$/MeOH/H_2O 80:20:2). The obtained product was redissolved in methanol (50 mL), Pd/C 10% (50 mg) was added, and the reaction was stirred under an H_2 atmosphere for 2.5 h. The mixture was filtered, and the title compound was obtained in quantative yield after removing the solvent in vacuo. R_f = 0.29 (CH_2Cl_2/MeOH/H_2O 60:20:2). >97% pure by HPLC. ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD 9:1): δ 3.98−3.73 (m, 6H), 3.71−3.41 (m, 6H), 1.62−1.50 (m, 2H), 1.27 (br.s, 26H), 0.87 (t, J = 6.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD 9:1): δ 71.6, 71.2, 70.6 (d, J = 5.2 Hz), 69.4 (d, J = 6.2 Hz), 67.2 (d, J = 6.2 Hz), 66.5 (d, J = 6.2 Hz), 62.3, 31.7, 29.5, 29.4, 29.3, 29.1, 25.8, 22.4, 13.8.

1-*O*-Hexadecyl-2-lyso-*sn*-glycero-3-phospho-(*R*)-glycerol (3). The synthesis of **3** was carried out as described for the synthesis of **2** to afford 234 mg (61%). NMR identical to **2**.

1-*O*-Octadecyl-2-lyso-*sn*-glycero-3-phospho-(*S*)-glycerol (6). 9b (200 mg, 0.46 mmol) was dissolved in dry CH_2Cl_2 (7 mL) in a flame-dried flask under N_2. To this solution were added TMP (156 μL, 0.92 mmol) and (i-Pr)$_2$NP(OMe)Cl (133 μL, 0.69 mmol). The solution was stirred for 2.5 h after which the reaction had gone to completion indicated by TLC (hexane/EtOAc/Et_3N 75:20:5). To the solution was added sat. $NaHCO_3$ (20 mL), the phases were separated, and the aqueous phase was extracted with CH_2Cl_2 (3 × 15 mL). The combined organic phases were washed with sat. $NaHCO_3$ (3 × 15 mL), dried over Na_2SO_4, and concentrated. The crude product was dried at 0.1 mmHg for 1 h after which it was redissolved in dry CH_2Cl_2 (7 mL) under N_2. 4 Å molecular sieves (~0.5 g) and (*R*)-isopropylideneglycerol (90 μL, 0.71 mmol) were added, and the solution was stirred for 1 h. The solution was cooled to 0 °C and 5-phenyl-1*H*-tetrazole (136 mg, 0.93 mmol) was added. The reaction was stirred for 30 min after which it was allowed to warm to room temperature. TLC (hexane/EtOAc/Et_3N 75:20:5) indicated complete conversion after 2.5 h, and the reaction mixture was cooled to 0 °C after which *t*-BuOOH (5.5 M in decane) (125 μL, 0.69 mmol) was added dropwise over 2 min. The reaction was stirred for 2 h and then quenched by addition of sat. $NaHCO_3$ (10 mL) and 1 M Na_2SO_3 (10 mL). The mixture was stirred for 20 min after which the phases were separated and the aqueous phase was extracted with CH_2Cl_2 (3 × 25 mL). The combined organic phases were washed with brine (3 × 25 mL), dried over $MgSO_4$, and concentrated to give the protected title compound. The crude

product was used directly in the next step without further purification. However, purification by column chromatography gives 84% yield. Anal. Calcd. for $C_{35}H_{63}O_8P$: C 65.39, H 9.88; Found: C 65.28, H 9.97, $R_f = 0.22$ (hexane/EtOAc 8:2).

The deprotection of the methyl and the isopropylidene group was carried out using the deprotection procedure described above. Purification by flash chromatography ($CHCl_3$/MeOH/H_2O 85:15:1) gave 183 mg (67%) of benzyl-protected **6** as a white solid. $R_f = 0.21$ ($CHCl_3$/MeOH/H_2O 80:20:2). The obtained product was redissolved in ethyl acetate (5 mL) and methanol (5 mL), Pd/C 10% (20 mg) was added, and the reaction was stirred under an H_2 atmosphere for 2.5 h. The mixture was filtered and the title compound was obtained in quantative yield. $R_f = 0.26$ (CH_2Cl_2/MeOH/H_2O 60:20:2). >97% pure by HPLC. ^{1}H NMR (300 MHz, CD_3OD): 3.98−3.82 (m, 5H), 3.80 (quintet, $J = 6.2$ Hz, 1H), 3.65−3.42 (m, 6H), 1.57 (m, 2H), 1.29 (br.s, 30H), 0.89 (t, $J = 6.7$ Hz, 3H). ^{13}C NMR (75 MHz, CD_3OD): δ 71.7, 71.5, 71.3 (d, $J = 5.1$ Hz), 69.8 (d, $J = 6.1$ Hz), 67.2 (d, $J = 6.2$ Hz), 66.5 (d, $J = 6.2$ Hz), 62.6, 31.9, 29.6, 29.6, 29.5, 29.3, 26.0, 22.6, 13.5.

1-*O*-Hexadecyl-2-*O*-benzyl-*sn*-glycero-3-[phospho-*N*-BOC-ethanolamine] methyl ester (13). To a solution of Cl_2-POOMe (0.163 μL, 1.63 mmol) in dry toluene (2 mL) at −25 °C was dropwise added a solution of **9a** (300 mg, 0.74 mmol) and TMP (287 μL, 1.70 mmol) in dry toluene (8 mL). The reaction was stirred for 6 h at room temperature after which TMP (375 μL, 2.22 mmol) and *N*-BOC-ethanolamine (687 μL, 4.44 mmol) were added. The reaction was stirred overnight. The solution was filtered through Celite and concentrated. The crude product was redissolved in CH_2Cl_2 (50 mL), washed with brine (3 × 20 mL), dried over $MgSO_4$, and concentrated. Purification by column chromatography (EtOAc/CH_2Cl_2 1:4) gave 357 mg (75%) **13** as a clear oil. $R_f = 0.22$ (EtOAc/CH_2Cl_2 1:4). ^{1}H NMR (300 MHz, $CDCl_3$, two diastereomers): δ 7.40−7.25 (m, 5H), 5.02 (br.s, 1H, *NH*), 4.68 (s, 2H), 4.29−4.00 (m, 4H), 3.81−3.75 (m, 1H), 3.75 (dd, $J = 11.3$, 1.1 Hz, 3H), 3.53 (dd, $J = 5.1$, 1.7 Hz, 2H), 3.43 (t, $J = 6.6$ Hz, 2H), 3.40−3.30 (m, 2H), 1.61−1.50 (m, 2H), 1.44 (s, 9H), 1.26 (br.s, 26H), 0.88 (t, $J = 6.6$ Hz, 3H). Anal. Calcd. for $C_{34}H_{62}NO_8P$: C 63.43, H 9.71, N 2.18; Found: C 62.93, H 9.88, N, 2.28.

1-*O*-Hexadecyl-2-lyso-*sn*-glycero-3-phospho-ethanolamine poly(ethylene-glycol)$_{350}$ (4). 13 (214 mg, 0.37 mmol) was dissolved in CH_2Cl_2 (6 mL). The solution was cooled to 0 °C, and TFA (6 mL) was added slowly. The solution was stirred for 1 h at 0 °C after which it was concentrated and coevaporated twice with toluene. Activated polymer (364 mg, 0.37 mmol) in $CHCl_3$ (4 mL) was added to a solution of the crude product and Et_3N (220 μL, 1.55 mmol) in $CHCl_3$ (10 mL). The solution was heated to 40 °C and stirred for 2.5 h. The solvent was removed under reduced pressure, and the crude product was redissolved in toluene (75 mL), washed with H_2O (5 × 30 mL), and concentrated. The product was redissolved in CH_2Cl_2 (3 mL), CH_3CN (6 mL), 2-propanol (6 mL), and Me_3N (40% aqueous, 7.5 mL) and then stirred overnight at room temperature. The solvent was removed under reduced pressure, and the residue was redissolved in chloroform (80 mL), and washed with 1 M HCl (2 × 20 mL) and gently with water (20 mL). The organic phase was dried over Na_2SO_4 and concentrated. Purification by column chromatography (17% MeOH in CH_2Cl_2) gave 238 mg (71%) of 1-*O*-hexadecyl-2-*O*-benzyl-*sn*-glycero-3-phospho-*O*-ethanolamine poly(ethylene-glycol)$_{350}$ as a clear oil. The product was redissolved in MeOH (30 mL), Pd/C 10% (25 mg) was added, and the reaction was stirred under an H_2 atmosphere overnight. The solution was filtered through Celite and concentrated giving **4** in quantitative yield as an amorphous white powder. $R_f = 0.29$ (CH_2Cl_2/EtOH/MeOH 7:3:1). >97% pure by HPLC.^{1}H NMR (300 MHz, $CDCl_3$): δ 4.29−4.10 (m, 2H), 4.05−3.79 (m, 5H), 3.72−3.58 (m, 24H), 3.57−3.51 (m, 4H), 3.35−3.44 (m, 4H), 3.37 (s, 3H), 1.59−1.47 (m, 2H), 1.26 (br.s, 26H), 0.88 (t, $J = 6.6$ Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.9, 72.2, 71.9, 71.8, 71.6, 70.6, 70,5, 70.2, 69.6, 67.7, 64.7, 63.6, 59.1, 42.0, 32.1, 29.9, 29.8, 29.8, 29.5, 26.2, 22.8, 14.2.

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Supporting Information Available: The experimental procedures for the synthesis of 1-*O*-hexadecyl-2-*O*-benzyl-*sn*-glycerol (**9a**) and 1-*O*-octadecyl-2-*O*-benzyl-*sn*-glycerol (**9b**) from D-mannitol are provided as Supporting Information. The preparation of the activated poly(ethylene glycol) (*N*-succinimide-poly(ethylene-glycol)$_{350}$-carbonate) is also provided as Supporting Information, as we have described this synthesis in a recent article.[22] This information is available free of charge via the Internet at http://pubs.acs.org.

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