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Synthesis of novel phosphatidylcholine lipids with fatty acid chains bearing aromatic units. Generation of oxidatively stable, fluid phospholipid membranes

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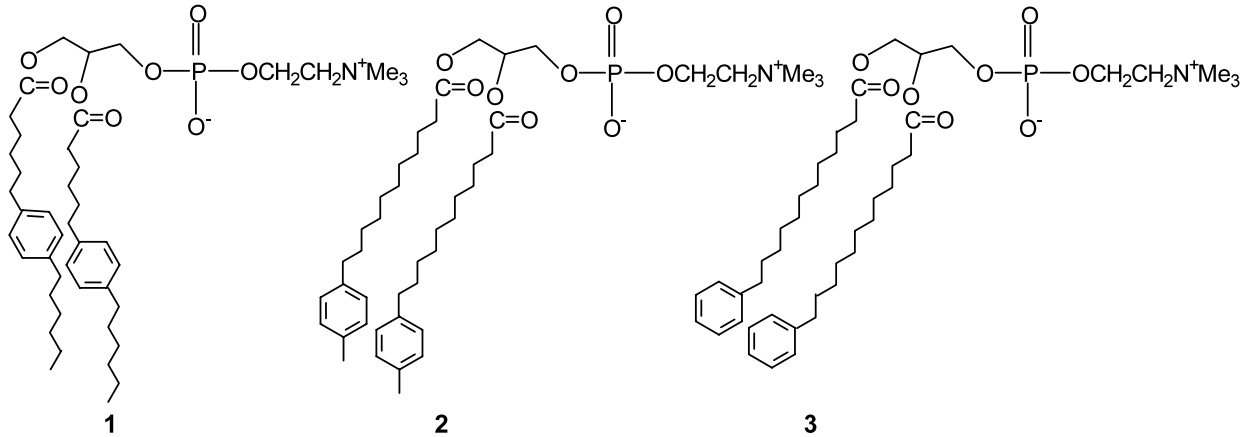
Abstract—The first examples of diacylphospholipid analogs, which contain aromatic units at various depths of their hydrocarbon chains, have been synthesized. The membranes produced from the sonicated aqueous suspensions of these newly synthesized lipids are oxidatively stable and possess long shelf life. These membranes maintain fluid character at ambient temperature making them ideal for membrane protein reconstitution studies. © 2002 Elsevier Science Ltd. All rights reserved.

Glycerophospholipids are important structural components of the bilayer membranes that constitute the cellular organelles.[1] Their lipophilic parts are typically composed of fatty acid chains, which are linked to the glycerol backbones via ester or ether functionalities.[2] Due to their importance in the investigations of the structure and function of biological membranes, a number of phospholipid analogues have been synthesized incorporating modified lipophilic chains. These include ones containing sulfur-substituted fatty acids,[3a] vicinal-chain tethered macrocyclic phospholipids,[3b–d] chains incorporating fluorenyl residues,[3e] or chains with monoacetylenic residues,[3f] or with terminal acryloyl groups,[3g] and phospholipids with methyl-substituted chains,[3h,i] among others. In addition, some phospholipid analogues exhibit significant antifungal,[4a] antitumor,[4b] antihypertensive,[4c] anti-inflammatory[4d] properties. Consequently, there have been many attempts to find novel routes to the synthesis of various phospholipid derivatives.[5]

Phospholipids bearing unsaturated fatty acid chains are particularly useful in the reconstitution of membrane proteins in vitro.[6] However, in order to keep the protein functional, it is necessary to maintain the membrane in its functionally fluid state with significant dynamic disorder. Lipophilic chains with olefinic unsaturation help maintain the membrane in a fluidized state, which is necessary for optimal membrane bound protein activity and its function of signal transduction



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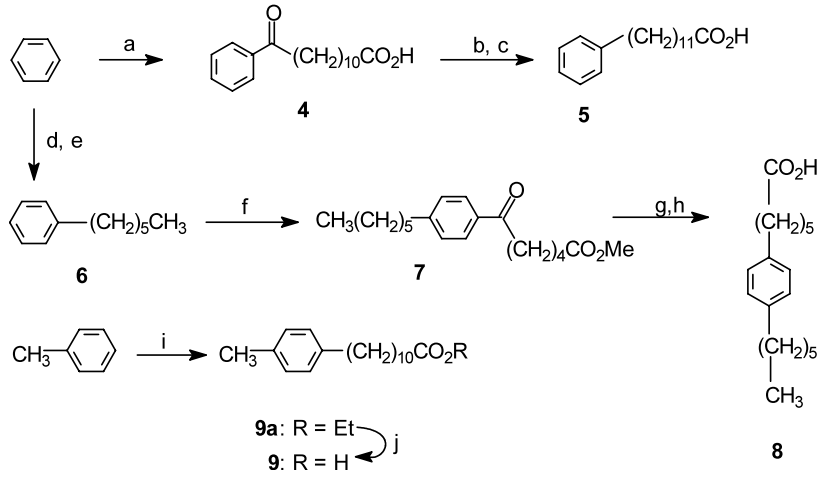
across bilayers. Phospholipids containing naturally occurring unsaturated fatty acids bearing *cis*-double bonds such as oleic acid (*cis*-9-octadecenoic acid), linoleic acid (*cis*,*cis*-9,12-octadecadienoic acid), or arachidonic acid (*cis*,*cis*,*cis*,*cis*-5,8,11,14-eicosatetraenoic acid) are generally used for membrane protein reconstitution. However, the vesicular membranes generated from such phospholipids, suffer from easy lipid peroxidation[7] limiting their potential for protein reconstitution studies. In addition, these lipids are photochemically sensitive, restricting the shelf life and applicability of the resulting vesicular formulations even further.

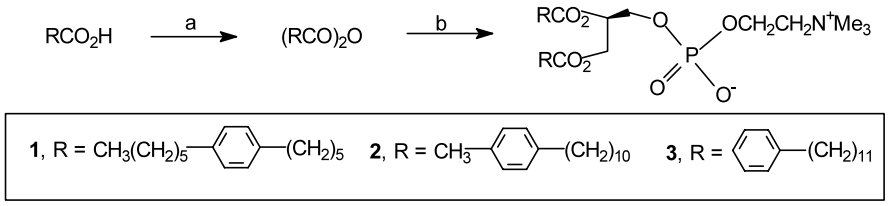
One way to alleviate such difficulties is to design phospholipids with chains bearing oxidatively stable unsaturation. Unlike their olefinic counterparts, aromatic systems possess far greater chemical stability, especially *para*-substituted aromatic rings (e.g. in **1** and **2**) which are generally metabolically stable to biological oxidation. As a part of our ongoing chemical biology program[8] focused on optimizing lipid lead structures that would offer suitable membranes for reconstitution of membrane proteins at ambient conditions, we became interested in achieving syntheses of a series of phospholipids which incorporates aromatic units in their hydrophobic segments. Incorporation of such aromatic units into hydrocarbon chains leads to spontaneous fluidization of the resulting membranes. In this paper we describe a convenient synthesis of three novel phospholipid analogues (**1**–**3**), in which unsaturation in the form of aromatic units forms part of the acyl chains at various locations along the chains.

The synthesis of the target phospholipids began with the preparation of fatty acids with aromatic units incorporated at specified locations of the hydrocarbon chain (Scheme 1). Friedel–Crafts acylation of benzene with *n*-hexanoyl chloride in the presence of anhydrous $AlCl_3$, under refluxing conditions for ~10 h afforded hexanophenone in 58% yield. This upon reduction under Huang–Minlon conditions gave *n*-hexyl benzene, **6**, which was isolated in ~66% yield as a colorless liquid, upon distillation under reduced pressure. The *n*-hexyl benzene was then subjected to acylation with methyl adipoyl chloride in the presence of anhydrous $AlCl_3$ in dry carbon disulfide under refluxing conditions to afford **7** in ~55% yield. The ketoester, **7** was then converted to 6-(4-*n*-hexylphenyl)hexanoic acid, **8** in ~60% yield first upon reduction (under reflux) with NH_2NH_2/KOH in ethylene glycol followed by workup and acidification. The synthesis of 12-phenyl-dodecanoic acid, **5** also began with acylation of benzene with methyl 12-chloro-12-oxo dodecanoate in the presence of anhydrous $AlCl_3$. Work up afforded the keto acid, **4** in 58% yield. Then the keto group in **4** was selectively reduced again under Huang–Minlon conditions to furnish **5** in ~70% yield. For the synthesis of 11-(4-methylphenyl)undecanoic acid, **9**, toluene was subjected to alkylation using ethyl 11-bromoundecanoate under Friedel–Crafts conditions. Workup followed by saponification and acidification afforded **9** in ~57% isolated yield.

The key starting material for the synthesis of phospholipid, free *sn*-glycerophosphocholine was obtained[9] from natural lecithin, which was first extracted from egg yolk and then subjected to hydrolysis using 0.1 M $Bu_4N^+{\cdot}OH^-$ in MeOH to afford free *sn*-glycerophosphocholine [(*R*)-GPC] after chromatographic purification. The resulting material was then converted to a complex with $CdCl_2$ (GPC·$CdCl_2$) to render it soluble in $CHCl_3$. Finally the syntheses of the desired phospholipids from the newly synthesized fatty acids were accomplished by full acylation of GPC·$CdCl_2$ with 2.2 mol of the respective anhydride of the appropriate fatty acid containing aromatic moieties in ethanol-free dry $CHCl_3$ in the presence of 4-dimethylaminopyridine (DMAP). This is summarized in Scheme 2. All the



Scheme 1. *Reagents*, *conditions and yields*: (a) $MeO_2C(CH_2)_{10}COCl$, anhyd. $AlCl_3$, in benzene, 4°C (1 h)→rt (12 h) workup in dil. ice-cold HCl, 58%; (b) NH_2NH_2–KOH, ethylene glycol, reflux, 4 h; (c) H_3O^+, ice-water, 70%; (d) caproyl chloride, anhyd. $AlCl_3$, reflux, 10 h, 58%; (e) NH_2NH_2–KOH, ethylene glycol, reflux, 4 h, 66%; (f) $MeO_2C(CH_2)_4COCl$, anhyd. $AlCl_3$, CS_2, reflux, 12 h, 55%; (g) NH_2NH_2–KOH, ethylene glycol, reflux, 4 h, 66%; (h) H_3O^+, ice-water, 90%; (i) ethyl 11-bromoundecanoate, anhyd. $AlCl_3$, rt, 4 h; HCl-ice water, 60%; (j) 10% KOH–MeOH, reflux, 6 h; H_3O^+–ice water, 95%.



Scheme 2. *Reagents, conditions and yields*: (a) i. RCO_2H (1 equiv.) Et_3N, −20°C, 1 h; add $ClCO_2Et$–THF, stir at −20°C, 2 h, then at rt, 1 h, ii cool to −20°C, add a solution of RCO_2H (1 equiv.)/Et_3N in THF −20°C, 1 h; stir overnight at rt, (isolated yields: 90–95%); (b) DMAP, GPC·$CdCl_2$, $CHCl_3$, stir at rt, 48 h (isolated yields: 72, 55, 63% for **1**–**3**, respectively).

numbered intermediates and final products were characterized by IR, ^1H, ^{13}C NMR spectroscopy and elemental analysis.[10]

Brief sonic dispersal, or vortex mixing (10 min) above 50°C, of the thin film prepared from each of the above phospholipid in HEPES (5 mM) buffer containing 1 mM EDTA at pH 7.4 afforded stable, translucent aqueous suspensions. The existence of multi-walled vesicular microstructures (MLVs) was evident from negative stain transmission electron microscopy studies (not shown) with each of these samples. Differential scanning calorimetric examination of these membranes spanning 5–90°C did not show the presence of any peak due to main-chain melting transition. Inclusion of any of these lipids with dipalmitoyl phosphatidylcholine (DPPC), however, reduced the main chain melting transition of the DPPC by several degrees depending on the mol% of the synthetic phospholipids incorporated. Taken together it is evident that these newly synthesized phospholipids retain a fluid like melted state (liquid-crystalline like state) at ambient temperatures in their membranes.

In summary, a convenient, general and adaptable method has been developed for the synthesis of three novel phospholipid analogues bearing acyl chains with unsaturations, which form the first examples of this class of compounds that are oxidatively stable. Such synthetic phospholipids will become valuable tools for both standard and innovative experiments in the fields of membrane receptor research, enzymology and bioseparation techniques. Work is now underway in our laboratory toward the protein reconstitution studies with these stable phospholipid membranes.

Acknowledgements

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10. All the intermediates and final compounds were characterized by IR, ^1H, ^{13}C NMR and elemental analysis. Selected spectral data for compounds **1**–**3**: (**1**) ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 0.88 (t, 6H, 2×-C$\underline{H}_3$), 1.29 (br m, 16H, 2×(-C$\underline{H}_2$)$_4$), 1.58 (br m, 12H, 2×(-C$\underline{H}_2$)$_3$), 2.29 (m, 4H, 2×-OC(O)C$\underline{H}_2$) 2.54 (m, 8H, 2×(-C$\underline{H}_2$-C$_6$H$_4$C$\underline{H}_2$-) 3.32 (s, 9H, 3×N$^+$(C$\underline{H}_3$)$_3$), 3.74 (t, 2H, -C$\underline{H}_2$N$^+$(CH$_3$)$_3$), 3.92 (m, 2H, -CH-C$\underline{H}_2$OC(O)), 4.11 (m,

1H, -CH-C$\underline{H}_a\underline{H}_b$OP(O)), 4.28 (m, 3H, OC$\underline{H}_2CH_2N^+Me_3$-CH-CH$_a\underline{H}_b$OP(O)), 5.19 (m, 1H, -C$\underline{H}$-CH$_2$O), 7.05 (m, 8H, 2×-C$_6\underline{H}_4$-). ^{13}C NMR (67.5 MHz, $CDCl_3$) δ (ppm) 13.85, 22.38, 24.55, 26.67, 30.96, 31.31, 31.53, 33.86, 34.03, 35.13, 35.37, 54.19, 59.22, 62.83, 63.39, 66.23, 70.29, 70.43, 127.98, 128.05, 139.34, 139.99, 172.97, 173.26. IR (cm^{-1}): 1735 (ester C=O), 1460, 1230 (P=O), 820 (P-O). Anal. calcd for $C_{44}H_{72}NO_8P.H_2O$: C, 66.72, H, 9.42, N, 1.77. Found: C, 66.4, H, 9.72, N, 1.54. (**2**) ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.18 (br m, 24H, 2×(-C$\underline{H}_2$)$_6$), 1.5 (br m, 8H, 2×(-C$\underline{H}_2$)$_2$), 2.4 (mixture of br m, 14H, 2×-OC(O)C$\underline{H}_2$) and 2×(-C$\underline{H}_2$-C$_6$H$_4$-C$\underline{H}_3$), 3.22 (s, 9H, 3×N$^+$(C$\underline{H}_3$)$_3$), 3.65 (t, 2H, -C$\underline{H}_2$N$^+$(CH$_3$)$_3$), 3.83 (m, 2H, -CH-C$\underline{H}_2$OC(O)), 4.05 (m, 1H, -CH-C$\underline{H}_a\underline{H}_b$OP(O)), 4.29 (m, 1H, -CH-CH$_a\underline{H}_b$OP(O)), 4.31 (m, 2H, OC$\underline{H}_2$CH$_2$N$^+$Me$_3$), 5.1 (m, 1H, -C$\underline{H}$-CH$_2$O), 6.98 (m, 8H, 2×-C$_6\underline{H}_4$-). ^{13}C NMR (67.5 MHz, $CDCl_3$) δ (ppm) 20.96, 21.47, 22.28, 24.88, 26.19, 27.63, 29.31, 29.49, 31.53, 34.11, 34.29, 35.5, 35.91, 36.89, 38.41, 50.5, 53.86, 54.46, 59.25, 62.96, 63.44, 66.48, 70.57, 115.23, 124.67, 125.4, 126.32, 126.51, 128.12, 128.92, 129.22, 173.21, 173.57. IR (cm^{-1}): 1730 (ester C=O), 1230 (P=O), 810 (P-O). Anal. calcd for $C_{44}H_{72}NO_8P$: C, 68.27, H, 9.38, N, 1.81. Found: C, 67.9, H, 9.71, N, 1.6. (**3**) ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.25 (br m, 28H, 2×(-C$\underline{H}_2$)$_7$), 1.59 (m, 8H, 2×(-C$\underline{H}_2$)$_2$), 2.29 (m, 4H, 2×-OC(O)C$\underline{H}_2$), 2.58 (m, 4H, 2×-C$\underline{H}_2$-C$_6$H$_5$), 3.33 (s, 9H, 3×N$^+$(C$\underline{H}_3$)$_3$), 3.76 (br t, 2H, -C$\underline{H}_2$N$^+$(CH$_3$)$_3$), 3.93 (m, 2H, -CH-C$\underline{H}_2$OC(O)), 4.12 (m, 1H, -CH-C$\underline{H}_a\underline{H}_b$OP(O)), 4.30 (m, 1H, -CH-CH$_a\underline{H}_b$OP(O)), 4.39 (m, 2H, OC$\underline{H}_2$CH$_2$N$^+$Me$_3$), 5.19 (m, 1H, -C$\underline{H}$-CH$_2$O) 7.17–7.25 (mixture of m, 10H, aromatic protons). ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm) 24.9, 24.99, 29.24, 29.37, 29.64, 31.47, 34.16, 34.31, 35.97, 50.29, 54.31, 59.43, 63.02, 63.56, 63.62, 65.32, 66.37, 70.44, 70.56, 125.56, 128.21, 128.35, 142.86, 173.46, 173.76. IR (cm^{-1}): 1740 (ester C=O), 1400, 1220 (P=O), 820 (P-O). Anal. calcd for $C_{44}H_{72}NO_8P\cdot0.5H_2O$: C, 67.49; H, 9.4; N, 1.79. Found: C, 67.23; H, 9.76; N, 1.49%.