Synthesis of Phospholipid–Inhibitor Conjugates by Enzymatic Transphosphatidylation with Phospholipase D

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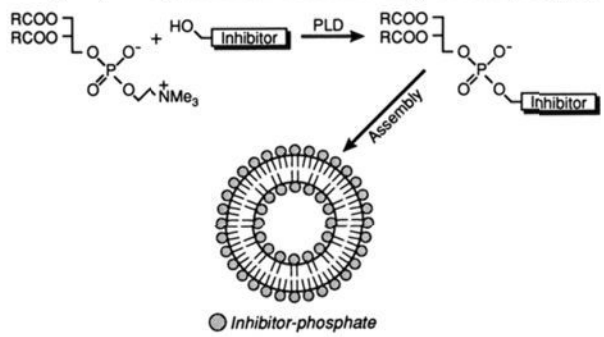
Abstract: This paper describes an efficient enzymatic procedure for the synthesis of phospholipid–inhibitor conjugates. The chemoselectivity, regioselectivity, and stereoselectivity of phospholipase-D-catalyzed phosphatidylations were investigated, and phospholipids containing inhibitors such as azasugars, nucleosides, and peptides were synthesized. These phospholipid conjugates in aqueous solution generally form liposome bilayers with multivalent inhibitors (the head groups) displayed on the surface and may find use in drug delivery and targeting. They also exhibit interesting structural features in different solvent systems as indicated in the NMR spectra.

Liposome technology has provided a powerful tool for efficient drug delivery and targeting,[1] as a number of pharmaceuticals have been encapsulated in liposome forms[2] or attached onto the surface of liposomes by a labile bond. Liposomes containing 2,3-dipalmitoyl-*sn*-glycerol-1-phospho-5′-azidothymidine, for example, showed a greatly enhanced inhibition of human immunodeficiency virus (HIV) replication *in vitro*.[3,4] The liposomes with inhibitors displayed on the surface could either directly interact with the targeted surface receptors via a multivalent contact or serve as prodrugs for a sustained release of the inhibitor upon enzymatic hydrolysis *in vivo* (e.g., by cellular lipases and phospholipases).[5] As part of our interest in the development of new methods for the preparation of phospholipids, we describe here the study of phospholipase D (PLD, EC 3.1.4.4) for the synthesis of phospholipid–inhibitor conjugates.

PLD catalyzes the hydrolysis of the terminal phosphate diester bond of glycerophospholipids to release phosphatidic acid.[6] Previous studies showed that PLD catalyzed the transfer of the phosphatidyl group from phosphatidylcholine to primary alcohols,[7–9] but little was known regarding its synthetic utility.

Scheme I. Phospholipase-D-Catalyzed Transphosphatidylation and Self-Assembly of Phospholipids



In the present work, we investigated the specificity of PLD from *Streptomyces* sp. (Sigma, P-4912) and carried out the transphosphatidylation in a chloroform/aqueous buffer two-phase system with various organic molecules as acceptors (Scheme I). We have investigated the chemoselectivity, regioselectivity, and stereoselectivity of the enzymatic reactions and prepared phospholipids containing azasugars, nucleosides, and peptides. These phospholipid conjugates exhibit unusual NMR patterns in different solvent systems.

Results and Discussion

We first examined the PLD-mediated coupling reaction with both (*S*)- and (*R*)-prolinol. Typically, the transphosphatidylation was performed as follows: To 0.7 mL of 1.6 M (*S*)-prolinol in 100 mM NaOAc and 50 mM $CaCl_2$ buffer (pH 6.5) was added PLD (100 units) and dimyristoyl-L-α-phosphatidylcholine (180 mg, 0.26 mmol, $17/g, from Sigma) in 5 mL of chloroform. The mixture was shaken at 30 °C for 4 h. The organic layer was then separated and washed twice with water and evaporated to dryness. Purification by silica gel chromatography ($CHCl_3$/MeOH/NH_3·H_2O = 120/30/1) afforded the desired product **1** (84% yield) as a white solid. Using (*R*)-prolinol under the same reaction conditions, we obtained product **2** in 85% yield. When lower concentrations of the alcohol were used, phospholipase D showed a slight preference to the *R* enantiomer but the reaction resulted in a lower yield. For example, when (*S*)- or (*R*)-prolinol was used at 0.4 M for the transphosphatidylation, **1** was obtained in 52% and **2** in 68% yields, respectively. In a reaction with serinol, the enzyme prefers the pro-*R* hydroxyl group, as indicated in a

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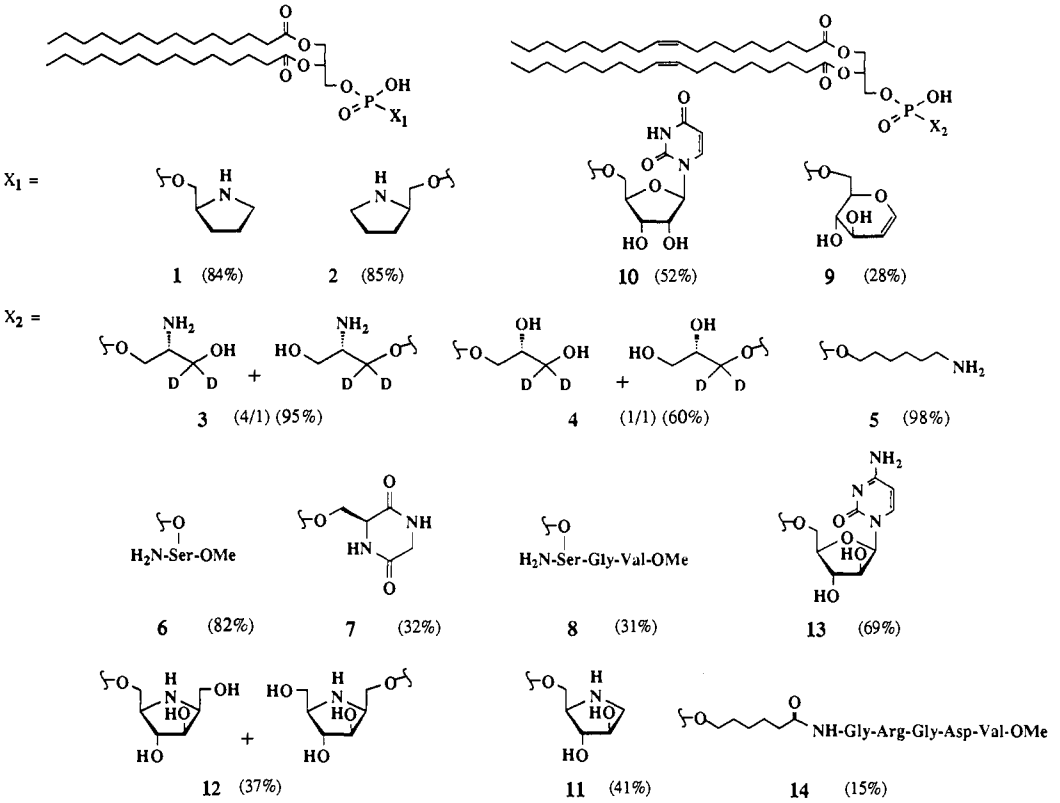
Chart I. Enzymatic Transphosphatidylation Products



separate reaction with (*S*)-1,1-dideuteroserinol (prepared by reduction of *N*-Cbz-L-serine with $LiAlD_4$) to give a 4:1 mixture of **3**. The ratio was obtained from the intensity of the chemical shift at the C_3-H of the serinol moiety. In contrast, when (*S*)-1,1-dideuteroglycerol (prepared from the reduction of isopropylidene-protected L-glyceric acid) was used, no preference was found for either the *R* or *S* hydroxyl group.[10]

It is worth noting that PLD is chemoselective for primary hydroxy groups but not reactive with amine and thiol groups. For example, when 6-amino-1-hexanol is used as a substrate, **5** is formed exclusively. Compound **6** was obtained from L-serine methyl ester in good yield, while no product was detected when L-cysteine methyl ester was used.

Dipeptides and tripeptides with an *N*-terminal serine residue are good substrates. The phosphatidylated Ser-Gly-OMe product was easily cyclized in basic solution during purification (chromatographed on silica gel with $CHCl_3/MeOH/NH_3{\cdot}H_2O = 6/2/0.1$) to afford the phospholipid–cyclic peptide conjugate **7**. Transphosphatidylation of Ser-Gly-Val-OMe gave **8**. However, Gly-Ser-Gly-OMe is not a substrate, indicating that the serine residue must be at the *N*-terminus. Sugars and nucleosides can also be selectively phosphatidylated at the primary hydroxy group to produce phospholipid conjugates such as compounds **9**, **10**, and **13**. PLD from other sources (e.g., from cabbage, peanut, and *Streptomyces chromofuscus*) gave insignificant yields of transphosphatidylation products under identical conditions.

We next utilized PLD to synthesize phospholipid–azasugar conjugates. Azasugars are inhibitors of glycosidase enzymes and have potential utility in the treatment of metabolic disorders such as diabetes[11] or as antiviral[12] and anticancer[13] agents. The azasugar moiety in **11**[14] was reacted with dioleoyl-L-α-phosphatidylcholine in the presence of PLD to give **11** in 41% yield. Similarly, **12** was prepared in 37% yield as a 1:1 mixture of diastereomers. The enzyme effectively transforms cytosine β-D-arabinofuranoside (Ara-C, an effective chemotherapeutic agent for the treatment of cancers[16]) to its phospholipid conjugate **13**. Furthermore, we extended the reaction to peptide-based inhibitors, especially those containing Arg-Gly-Asp (RGD) as the ligand for the integrin superfamily of adhesion receptors.[17] It was found that Ser-Gly-Arg-Gly-Asp-Val-OMe was not a substrate, probably due to the steric hindrance of the peptide fragment after the serine in this sequence. However, when a six-carbon spacer group was linked to the *N*-terminus of a Gly-Arg-Gly-Asp-Val-OMe sequence, the resulting α-*N*-6-hydroxyhexanoate derivative of Gly-Arg-Gly-Asp-Val-OMe was a good substrate for PLD and product **14** was synthesized in 15% yield. We then prepared liposomes containing the phospholipid–inhibitor conjugate.[18] A representative formulation is a mixture of dioleoylphosphatidylcholine (62%, weight percent), cholesterol (26%), and the phospholipid–inhibitor conjugate (12%). The resulting mixture was sonicated for 40 min. Electron microscopy studies showed that the resulting liposomes are small unilamellar vesicles with an average diameter of 7–25 nm (Figure 1). The liposome containing compound **14** (12%) exhibited excellent inhibitory

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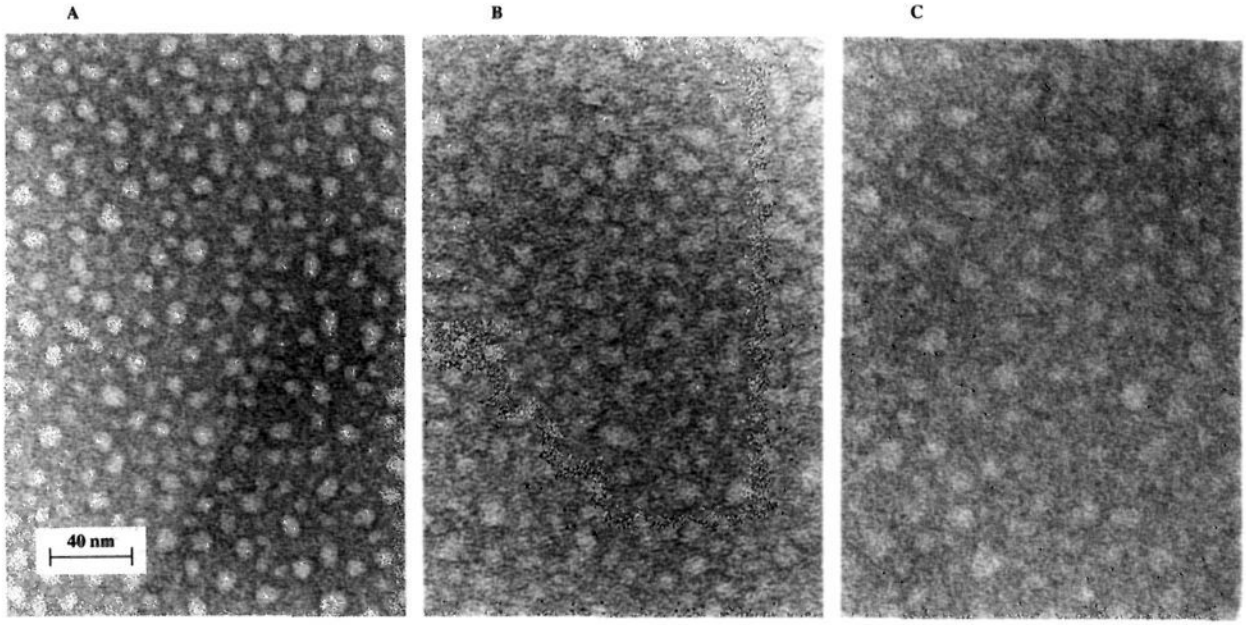

Figure 1. *Negative stain electron micrographs of liposomes. All three liposomes contain 10 mg of dioleoylphosphatidylcholine, 4 mg of cholesterol, and either 2 mg of dioleoylphosphatidylethanolamine or 2 mg of the phospholipid–inhibitor conjugate in 2 mL of 20 mM sodium phosphate buffer, pH 7.0: A contains dioleoylphosphatidylethanolamine (12%), B contains compound* **13** *(12%), and C contains compound* **14** *(12%).*

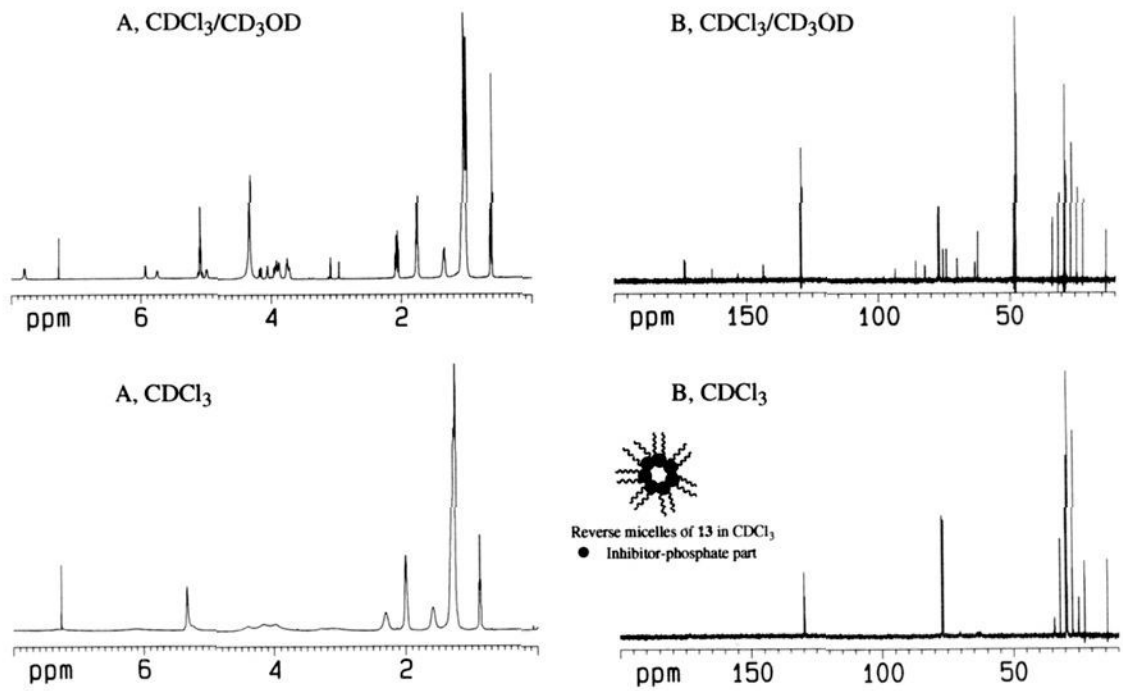

Figure 2. ^{1}H NMR (A) and ^{13}C NMR (B) spectra of **13** in $CDCl_3/CD_3OD$ (2/1, v/v) and in $CDCl_3$.

activities against fibronectin adhesion to integrins. Detailed biological studies will be published later.

During the course of this study, we have observed interesting NMR patterns of phospholipid–inhibitor conjugates (Figure 2). In $CDCl_3/CD_3OD$ (2/1), all the products gave "normal" ^{1}H and ^{13}C NMR spectra (i.e., all H and C peaks were observed). However, in $CDCl_3$, the hydrophilic portion (including the inhibitor and the polar end of the phospholipid) of some compounds (e.g., **10**, **11**, **12**, and **13**) showed peak broadening in the ^{1}H NMR spectra and peak reduction or disappearance in the ^{13}C NMR spectra. In $CDCl_3$, the ^{1}H peaks for Ara-C (e.g., 7.79, 5.93, and 5.74 ppm) disappeared and those for the glycerol portion were broadened. Similarly, the ^{13}C peaks for Ara-C and the glycerol portions (e.g., 173, 163, 153, 143, and 93–62 ppm) are missing. In contrast, phosphatidylcholines, phosphatidic acids, and compounds **1** and **2** exhibit normal NMR spectra in both types of solvents. A possible explanation is that in $CDCl_3$, compounds **11–13** form aggregates[19] with the inhibitor and the polar end of the phospholipid buried inside.[20] Compounds **1** and **2**, on the other hand, are less polar and have less tendency to form aggregates in $CDCl_3$. The aggregates could be reverse liposomes or reverse micelles. A full understanding of this observation requires further physical studies. In conclusion, we have described

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a new enzymatic procedure for the synthesis of various phospholipid–inhibitor conjugates which may have improved activities *in vitro* and *in vivo*.

Experimental Section

General. All chemicals were purchased from commercial sources as reagent grade. ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX-500 spectrometer. High-resolution mass spectra (HRMS) were recorded on a VG ZAB-ZSE mass spectrometer under fast atom bombardment (FAB) conditions. Electron microscopy was conducted with a PHILIPS, CM12 instrument. Thin-layer chromatography was conducted on Baker Si250F silica gel TLC plates with fluorescent indicator. Column chromatography was conducted with silica gel, grade 62, 60–200 mesh, 150 Å. Phospholipase D was purchased from Sigma Co.

Typical Enzymatic Transphosphatidylation Procedure. Synthesis of Compound 1. (*S*)-Prolinol (160 mg, 1.58 mmol) was dissolved in 0.6 mL of 100 mM NaOAc and 50 mM $CaCl_2$ buffer, acetic acid (about 120 μL) was added to adjust the pH to 6.5. Then, PLD from *Streptomyces* species (Sigma, P-4912) (100 units in 100 μL of 100 mM NaOAc buffer) was added, and the solution was mixed with dimyristoyl-L-α-phosphatidylcholine (180 mg, 0.26 mmol) in 5 mL of chloroform. The mixture was shaken at 30 °C for 4 h. The organic layer was then separated, washed twice with water, and evaporated to dryness. Purification by silica gel chromatography ($CHCl_3$/MeOH/NH_3·H_2O = 120/30/1) afforded the desired product **1** (150 mg, 84%) as a white solid. ^1H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.670 (t, *J* = 6.5 Hz, 6H, 2 CH_3 of dimyristoyl), 1.048 (m, 40H, 20 CH_2 of dimyristoyl), 1.392 (m, 4H, β-CH_2 of dimyristoyl), 1.600 (m, 1H, pyrrolidine), 1.791 (m, 1H, pyrrolidine), 1.916 (m, 1H, pyrrolidine), 2.109 (m, 4H, α-CH_2 of dimyristoyl), 3.062 (m, 2H), 3.133 (m, 1H), 3.405 (m, 1H), 3.562 (m, 1H), 3.682 (m, 1H), 3.795 (m, 2H), 3.811 (m, 2H), 4.152 (m, 1H), 4.982 (m, 1H, H-C_2 of glycerol). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.5, 22.2, 23.9, 24.4, 24.5, 25.6, 28.7, 28.8, 28.9, 29.1, 29.2, 31.5, 33.6, 33.8, 44.9, 59.8, 62.1, 63.2, 63.4, 63.5, 69.8, 69.9, 173.2, 173.5. HRMS calcd for $C_{36}H_{70}NO_8P + H^+$: 676.4917, found 676.4932.

2. ^1H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.594 (t, *J* = 7.0 Hz, 6H, 2 CH_3 of dimyristoyl), 0.979 (m, 40H, 20 CH_2 of dimyristoyl), 1.322 (q, 4H, 2 CH_2 of dimyristoyl), 1.539 (m, 1H, pyrrolidine), 1.759 (m, 1H, pyrrolidine), 1.849 (m, 2H, pyrrolidine), 2.037 (q, *J* = 8.0 Hz, 4H, α-CH_2 of dimyristoyl), 3.006 (t, *J* = 7.0 Hz, 2H, CH_2O of pyrrolidine), 3.543 (m, 1H), 3.676 (m, 1H), 3.712 (t, *J* = 6.7 Hz, 2H), 3.830 (m, 1H), 3.880 (m, 1H), 4.120 (dd, *J* = 3.5, 12 Hz, 1H), 4.951 (m, 1H, H-C_2 of glycerol). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.3, 22.1, 23.6, 24.3, 24.4, 25.5, 28.6, 28.8, 29.0, 29.1, 31.4, 33.5, 33.6, 44.8, 59.5, 59.5, 61.9, 63.3, 69.7, 69.8, 172.9, 173.3. HRMS calcd for $C_{36}H_{70}NO_8P + H^+$: 676.4917, found 676.4910.

(*S*)-1,1-Dideuteroglycerol. The compound was obtained by $LiAlD_4$ reduction of 1,2-dimethoxypropyl-(*S*)-glyceric acid in THF and subsequent deprotection using Dowex 50-H^+ in methanol/water in 30% overall yield. ^1H NMR (300 MHz, D_2O): δ 3.39 (dd, *J* = 6.5, 11.8 Hz, 1H), 3.49 (dd, *J* = 4.3, 11.8 Hz, 1H), 3.58–3.63 (m, 1H). ^{13}C NMR (D_2O): δ 46.2, 55.5.

(*S*)-1,1-Dideuteroserinol. The compound was prepared by reduction of L-Cbz-Ser(OBu^t)-OH with $LiAlD_4$ and subsequent deprotection with $TiCl_4$ (removing the OBu^t group) and catalytic hydrogenation (removing the Cbz group). ^1H NMR (500 MHz, D_2O): δ 2.73–2.76 (m, 1H), 3.32 (dd, *J* = 6.0, 11.0 Hz), 3.43 (dd, *J* = 5.0, 11.0 Hz, 1H). ^{13}C NMR (D_2O): δ 36.4, 46.5. HRMS calcd for $C_3H_7D_2NO_2 + Cs^+$: 225.9813, found 225.9810.

Using the standard procedure (see synthesis of **1**), we phosphatidylated serinol and glycerol as well as (*S*)-1,1-dideuteroserinol and (*S*)-1,1-dideuteroglycerol. The comparison of the integral of the CH_2OH–methylene signals around 3.7 ppm of each pair of products allows for the calculation of the enantioselectivity of the reaction.

Phosphatidylserinol. ^1H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.891 (m, 6H, 2 CH_3 of dioleyl), 1.30 (m, 40H, 20 CH_2 of dioleyl), 1.61 (m, 4H, 2 CH_2 of dioleyl), 2.03 (m, 8H, 4 CH_2CH=CH of dioleyl), 2.32 (q, *J* = 8.0 Hz, 4H, 2 α-CH_2 of dioleyl), 3.34 (m, 1H, CH of serinol), 3.67 (dd, *J* = 6.5, 13.0 Hz, 1H, CH_2OH of serinol), 3.74 (dd, *J* = 4.5, 13.0 Hz, 1H, CH_2OH of serinol), 4.0 (m, 4H), 4.18 (dd, *J* = 6.5, 12.0 Hz, 1H), 4.42 (dd, *J* = 3.0, 12.0 Hz, 1H), 5.23 (m, 1H, CH of glycerol), 5.35 (m, 4H, 2 CH=CH of dioleyl). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.3, 22.1, 24.3, 24.3, 26.6, 28.6, 28.7, 28.8, 29.0, 29.2, 31.4, 33.7, 33.8, 52.8, 58.5, 58.8, 62.1, 62.4, 63.2, 69.9, 129.1, 129.4, 173.0, 173.4. HRMS calcd for $C_{42}H_{80}NO_9P + Cs^+$: 906.4625, found 906.4635.

3. ^1H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.890 (t, *J* = 7.0 Hz, 6H, 2 CH_3 of dioleyl), 1.30 (m, 40H, 20 CH_2 of dioleyl), 1.62 (m, 4H, 2 CH_2 of dioleyl), 2.02 (m, 8H, 4 CH_2CH=CH of dioleyl), 2.33 (q, *J* = 7.5 Hz, 4H, 2 CH_2 of dioleyl), 3.31 (m, 1H, CH of serinol), 3.65 (dd, *J* = 6.5, 13.0 Hz, 0.23H, CH_2OH of serinol), 3.73 (dd, *J* = 4.5, 13.0 Hz, 0.22H, CH_2OH of serinol), 4.00 (m, 3–4H), 4.18 (dd, *J* = 7.0, 12.0 Hz, 1H), 4.42 (dd, *J* = 3.0, 12.0 Hz), 5.24 (m, 1H, CH of glycerol), 5.34 (m, 4H, 2 CH=CH of dioleyl). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.4, 22.2, 24.4. 24.4, 26.7, 28.6, 28.8, 28.8, 29.0, 29.2, 31.4, 33.5, 33.7, 52.5, 62.1, 63.1, 63.2, 69.8, 69.9, 129.2, 129.5, 173.2, 173.5. MS calcd for $C_{42}H_{78}D_2NO_9P + H^+$: 777, found 777 (799 for M + Na^+).

Phosphatidylglycerol. ^1H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.892 (t, *J* = 7.0 Hz, 6H, 2 CH_3 of dioleyl), 1.30 (m, 40H, 20 CH_2 of dioleyl), 1.62 (m, 4H, 2 CH_2 of dioleyl), 2.03 (m, 8H, 4 CH_2CH=CH of dioleyl), 2.33 (q, *J* = 7.5 Hz, 4H, 2 α-CH_2 of dioleyl), 3.64 (m, 2H, CH_2OH of glycerol), 3.83 (m, 1H, CH of glycerol), 3.90–4.05 (m, 4–5H), 4.18 (dd, *J* = 7.0, 12.0 Hz, 1H), 4.42 (dd, *J* = 2.5, 12.0 Hz), 5.24 (m, 1H), 5.36 (m, 4H). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.3, 22.1, 24.3, 24.4, 26.6, 28.6, 28.6, 28.7, 28.7, 28.8, 29.0, 29.2, 31.4, 33.5, 33.6, 61.7, 62.1, 63.1, 65.8, 69.8, 69.9, 70.8, 129.1, 129.4, 173.4, 173.8. HRMS calcd for $C_{42}H_{79}O_{10}P + Na^+$: 797.5309, found 797.5300.

4. ^1H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.885 (t, *J* = 6.5 Hz, 6H, 2 CH_3 of dioleyl), 1.30 (m, 40H, 20 CH_2 of dioleyl), 1.62 (m, 4H, 2 CH_2 of dioleyl), 2.03 (m, 8H, 4 CH_2CH=CH of dioleyl), 2.33 (m, 4H, 2 CH_2 of dioleyl), 3.62 (m, 0.82 H, CH_2OH of glycerol), 3.90–4.05 (m, 4H), 4.18 (m, 2H), 4.41 (m, 1H), 5.24 (m, 1H), 5.34 (m, 4H). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.3, 22.2, 24.4, 24.4, 26.7, 28.7, 28.8, 29.0, 29.3, 31.4, 33.6, 33.7, 61.8, 62.2, 65.9, 69.9, 70.0, 70.5, 129.2, 173.1, 173.5.

5. ^1H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.696 (t, *J* = 7.0 Hz, 6H, 2 CH_3 of dioleyl), 1.131 (m, 40H, 20 CH_2 of dioleyl), 1.293 (m, 4H, 2 CH_2 of dioleyl), 1.42–1.47 (m, 8H, hexanol), 1.846 (m, 8H, 4 CH_2C=C of dioleyl), 2.136 (t, *J* = 7.0 Hz, 4H, α-CH_2 of dioleyl), 2.742 (d, *J* = 7.0 Hz, hexanol), 3.712 (t, *J* = 6.5 Hz, 1H, hexanol), 3.782 (d, *J* = 5.5 Hz, 1H, hexanol), 4.009 (m, 1H, glycerol), 4.131 (m, 2H, glycerol), 4.131 (m, 1H, glycerol), 5.012 (m, 1H, CH of glycerol), 5.160 (m, 4H, 2 CH=CH of dioleyl). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.6, 22.3, 23.7, 24.5, 26.4, 26.8, 28.8, 28.9, 29.0, 29.2, 29.3, 29.4, 31.3, 33.7, 33.8, 38.3 (hexanol, CH_2NH_2), 62.3 (glycerol), 63.0 (glycerol), 64.4 (hexanol, OCH_2), 70.0 (glycerol), 129.3, 129.6, 173.1, 173.5. HRMS calcd for $C_{45}H_{86}NO_8P + H^+$: 800.8169, found 800.8165.

6. ^1H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.605 (t, *J* = 6.5 Hz, 6H, 2 CH_3 of dioleyl), 1.040 (m, 40 H, 20 CH_2 of dioleyl), 1.330 (m, 4H, 2 CH_2 of dioleyl), 1.743 (m, 8H, 4 CH_2C=C of dioleyl), 2.050 (q, *J* = 7.0 Hz, 4H, α-CH_2 of dioleyl), 3.506 (m, 4H), 3.642 (m, 2H), 3.825 (m, 3H), 4.325 (m, 1H), 4.952 (m, 1H, H-C_2 of glycerol), 5.063 (m, 4H, 2 CH=CH of dioleyl. ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.4, 22.1, 24.3, 24.4, 26.6, 26.7, 28.6, 28.7, 28.8, 29.0, 29.2, 31.4, 33.5, 33.6, 51.9, 62.2, 63.3, 69.9, 129.0, 129.1, 129.2, 129.3, 129.4, 129.5, 172.1, 173.0, 173.4. Ion-spray MS: calcd for $C_{43}H_{80}NO_{10}P + H^+$ 802, found 802; calcd for $C_{43}H_{80}NO_{10}P + Na^+$ 824, found 824.

7. ^1H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.594 (t, *J* = 7.0 Hz, 6H, 2 CH_3 of dioleyl), 1.006 (m, 40H, 20 CH_2 of dioleyl), 1.315 (m, 4H, 2 CH_2 of dioleyl), 1.732 (m, 8H, 4 CH_2C=C of dioleyl), 2.040 (q, *J* = 6.5 Hz, 4H, α-CH_2 of dioleyl), 3.557 (s, 1H), 3.662 (t, *J* = 5.5 Hz, 2H), 3.77–3.81 (m, 3H), 3.85–3.92 (m, 4H), 4.123 (dd, *J* = 3.5, 12.0 Hz, 1H, glycerol), 4.938 (m, 1H, H-C_2 of glycerol), 5.056 (m, 4H, 2 CH=CH of dioleyl). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.4, 22.1, 24.3, 24.4, 26.6, 28.6, 28.7, 28.8, 29.0, 29.2, 31.4, 33.5, 33.7, 43.9, 62.1, 63.1, 66.2, 66.3, 69.9, 129.1, 129.2, 129.4, 166.6, 167.3, 173.2, 173.6. HRMS calcd for $C_{45}H_{83}N_2O_{11}P + Na^+$: 881.5632, found 881.5664.

8. ^1H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.618 (t, *J* = 6.5 Hz, 6H, 2 CH_3 of dioleyl), 1.040 (m, 40H, 20 CH_2 of dioleyl), 1.156 (d, *J* = 7.5 Hz, 3H, Val), 1.350 (m, 4H, 2 CH_2 of dioleyl), 1.756 (m, 8H, 4 CH_2C=C of dioleyl), 2.053 (m, 4H, α-CH_2 of dioleyl), 3.471 (s, 3H, Val OCH_3), 3.725 (m, 2H), 3.82–3.90 (m, 4H), 3.92 (m, 1H), 4.145 (dd, *J* = 3.0, 12.0 Hz, 1H, glycerol), 4.202 (m, 1H), 4.964 (m, 1H, H-C_2 of glycerol), 5.076 (m, 4H, 2 CH=CH of dioleyl). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.4, 16.4, 22.2, 24.4, 26.7, 28.6, 28.7, 28.8, 29.0, 29.2, 31.4, 33.5, 33.7, 51.8, 62.2, 63.3, 63.4, 69.9, 129.1, 129.2, 129.3, 129.4, 129.5, 166.6, 168.7, 173.0, 173.2, 173.5. HRMS calcd for $C_{48}H_{88}N_3O_{12}P + Na^+$: 952.6603, found 952.5962.

9. ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.602 (t, J = 7.0 Hz, 6H, 2 CH_3 of dimyristoyl), 1.023 (m, 40H, 20 CH_2 of dimyristoyl), 1.323 (m, 4H, 2 CH_2 of dimyristoyl), 2.050 (m, 4H, 2 CH_2 of dimyristoyl), 3.061 (m, 1H), 3.471 (m, 1H), 3.733 (t, J = 5.5 Hz, 2H), 3.885 (m, 1H), 3.909–3.963 (m, 3H), 4.142 (dd, J = 3.0, 12 Hz, 1H, glycerol), 4.424 (m, 1H, glucal), 6.025 (dd, J = 1.5, 6.0 Hz, 1H, glucal). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.3, 22.1, 24.4, 24.5, 28.6, 28.8, 29.0, 29.1, 31.4, 33.6, 33.7, 62.1 (glycerol), 63.2 (glycerol), 63.5 (glycerol), 68.3 (glycerol), 68.5 (glycerol), 69.9 (glycerol), 103.1, 143.3, 173.2, 173.6. HRMS calcd for $C_{69}O_{11}P + H^+$: 721.4656, found 721.4670.

10. ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.772 (t, J = 7.0 Hz, 6H, 2 CH_3 of dimyristoyl), 1.162 (m, 40H, 20 CH_2 of dimyristoyl), 1.507 (m, 4H, 2 CH_2 of dimyristoyl), 2.226 (q, J = 6.5 Hz, 4H, α-CH_2 of dimyristoyl), 3.281 (m, 1H), 3.500 (m, 2H), 3.879 (m, 1H), 4.022 (m, 4H), 4.285 (m, 1H), 5.141 (m, 1H, H-C_2 of glycerol), 5.696 (s, 1H), 5.743 (s, 1H), 7.739 (s, 1H). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.9, 22.5, 24.6, 24.7, 28.9, 29.1, 29.2, 29.3, 29.5, 31.7, 33.9, 34.0, 63.0, 89.6, 102.1, 140.9, 173.4, 173.8. HRMS calcd for $C_{40}H_{71}N_2O_{13}P + Cs^+$: 951.3748, found 951.3788.

11. ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.607 (t, J = 6.5 Hz, 6H, 2 CH_3 of dioleyl), 1.038 (m, 40H, 20 CH_2 of dioleyl), 1.330 (m, 4H, 2 CH_2 of dioleyl), 1.738 (m, 8H, 4 $CH_2C{=}C$ of dioleyl), 2.053 (q, J = 8.0 Hz, 4H, α-CH_2 of dioleyl), 3.071 (m, 2H), 3.289 (m, 1H), 3.714 (m, 3H), 3.901 (m, 3H), 4.152 (m, 1H), 4.961 (m, 1H, H-C_2 of glycerol), 5.064 (m, 4H, 2 CH=CH of dioleyl). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.3, 22.1, 24.3, 24.4, 26.6, 28.6, 28.7, 28.8, 29.0, 29.2, 31.4, 33.5, 33.7, 62.1, 63.3, 69.8, 69.9, 129.1, 129.3, 129.4, 129.5, 173.1, 173.5. HRMS calcd for $C_{44}H_{82}NO_{10}P + Cs^+$: 948.4731, found 948.4720.

12. ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.879 (t, J = 6.5 Hz, 6H, 2 CH_3 of dioleyl), 0.950 (m, 40H, 20 CH_2 of dioleyl), 1.602 (m, 4H, 2 CH_2 of dioleyl), 2.009 (m, 8H, 4 $CH_2C{=}C$ of dioleyl), 2.322 (m, 4H, α-CH_2 of dioleyl), 3.201 (m), 3.340 (m), 3.495 (m), 3.964 (m), 4.005 (m), 4.103 (m), 4.162 (m), 4.185 (m), 4.402 (m), 5.203 (m, 1H, CH of glycerol), 5.334 (m, 4H, 2 CH=CH of dioleyl). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.0, 21.9, 24.1, 26.4, 28.3, 28.4, 28.5, 28.7, 28.8, 28.9, 31.1, 33.2, 33.4, 59.6, 61.8, 63.0, 69.7, 74.5, 128.8, 129.1, 172.8, 173.2. HRMS calcd for $C_{45}H_{83}NO_{11}P + H^+$: 847.5860, found 847.5860.

13. ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.626 (t, J = 6.5 Hz, 6H, 2 CH_3 of dioleyl), 1.050 (m, 40H, 20 CH_2 of dioleyl), 1.344 (q, J = 6.5 Hz, 4H, 2 CH_2 of dioleyl), 1.764 (m, 8H, 4 $CH_2C{=}C$ of dioleyl), 2.062 (m, 4H, α-CH_2 of dioleyl), 3.722 (m, 3H), 3.876 (m, 4H), 4.157 (m, 1H), 4.327 (m, 1H), 5.020 (m, 1H, H-C_2 of glycerol), 5.082 (m, 4H, 2 CH=CH of dioleyl), 5.743 (d, J = 7.0 Hz, 1H, H-C_5 of pyrimidine), 5.928 (d, J = 5.0 Hz, 1H, H-$C_{1'}$ of cytosine), 7.788 (d, J = 7.0 Hz, 1H, H-C_6 of pyrimidine). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 13.4, 22.2, 24.4, 26.7, 28.6, 28.7, 28.8, 29.0, 29.3, 31.4, 33.6, 33.7, 62.1, 63.1, 69.9, 70.0, 74.0, 75.3, 82.2, 82.3, 85.7, 93.6, 129.2, 129.5, 143.8, 153.2, 163.0, 173.2, 173.5. HRMS calcd for $C_{48}H_{84}N_3O_{12}P + Cs^+$: 1058.4847, found 1058.4847.

14. ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ = 2/1, v/v): δ 0.613 (t, J = 7.0 Hz, 6H, 2 CH_3 of dioleyl), 0.663 (dd, J = 3.0, 7.0 Hz, 6H, Val), 1.024 (m, 40 H, 20 CH_2 of dioleyl), 1.172 (m, 3H), 1.34–1.40 (m, 10H), 1.53 (m, 1H), 1.731 (m, 8H, 4 $CH_2C{=}C$ of dioleyl), 1.902 (m, 1H), 2.053 (m, 4 H, α-CH_2 of dioleyl), 2.335 (dd, J = 5.0, 16.0 Hz, 1H, Asp β-CH_2), 2.507 (dd, J = 5.0, 16.0 Hz, 1H, Asp β-CH_2), 2.91 (m, 2H), 3.4557 (s, 3H, ValOMe), 3.57–3.61 (m, 4H), 3.696 (m, 2H), 3.915 (dd, J = 7.0, 12.0 Hz, 1H), 4.088 (d, J = 5.5 Hz, 1H), 4.14 (m, 1H), 4.163 (d, J = 3.0 Hz, 1H). ^{13}C NMR ($CDCl_3/CD_3OD$ = 2/1, v/v): δ 17.2, 18.2, 22.1, 24.4, 26.7, 28.6, 28.8, 29.0, 29.2, 31.4, 33.6, 33.7, 51.4, 57.4, 62.2, 64.8, 70.0, 129.2, 129.5, 156.8, 169.5, 170.3, 171.8, 172.8, 173.1, 173.5, 175.1, 176.2. HRMS calcd for $C_{65}H_{117}N_8O_{17}P + H^+$: 1313.8353, found 1313.8363.

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