Supplementary Information

Non-Enzymatic Biomimetic Remodeling of Phospholipids in Synthetic Liposomes

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Materials and Methods

General Considerations

Commercially available 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso C_{16} PC-OH), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso C_{18} PC-OH), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (Lyso C_{18} PG-OH) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were used as obtained from Avanti® Polar Lipids. N-Boc-*L*-Cys(Trt)-OH, iodomethane (IMe), sodium hydride (NaH), tetrahydrofuran (THF), *N,N'*-diisopropylcarbodiimide (DIC), 4-dimethylaminopyridine (DMAP), trifluoroacetic acid (TFA), triethylsilane (TES), 2,2-dimethoxypropane (DMP), 2,4,6-trichlorobenzoyl chloride (TCBC), oleic acid, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC.HCl), sodium 2-mercaptoethanesulfonate (MESNA), sodium phosphate monobasic monohydrate ($NaH_2PO_4.H_2O$), dithiothreitol (DTT), 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), 1,6-diphenyl-1,3,5-hexatriene (DPH) and cholesterol were obtained from Sigma-Aldrich. Texas Red® 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red® DHPE) and Bodipy® 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Bodipy® FL DHPE) were obtained from Life Technologies. Deuterated chloroform ($CDCl_3$), methanol (CD_3OD) and dimethyl sulfoxide (d_6-DMSO) were obtained from Cambridge Isotope Laboratories. All reagents obtained from commercial suppliers were used without further purification unless otherwise noted. Analytical thin-layer chromatography was performed on E. Merck silica gel 60 F_{254} plates. Compounds, which were not UV active, were visualized by dipping the plates in a ninhydrin or potassium permanganate solution and heating. Silica gel flash chromatography was performed using E. Merck silica gel (type 60SDS, 230-400 mesh). Solvent mixtures for chromatography are reported as v/v ratios. HPLC analysis was carried out on an Eclipse Plus C8 analytical column with *Phase A*/*Phase B* gradients [*Phase A*: H_2O with 0.1% formic acid; *Phase B*: MeOH with 0.1% formic acid]. HPLC purification was carried out on Zorbax SB-C18 semipreparative column with *Phase A*/*Phase B* gradients [*Phase A*: H_2O with 0.1% formic acid; *Phase B*: MeOH with 0.1% formic acid]. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian VX-500 MHz or Jeol Delta ECA-500 MHz spectrometers, and were referenced relative to residual proton resonances in $CDCl_3$ (at 7.24 ppm), CD_3OD (at 4.87 or 3.31 ppm) or d_6-DMSO (at 2.50 ppm). Chemical shifts were reported in parts per million (ppm, δ) relative to tetramethylsilane (δ 0.00). ^{1}H NMR splitting patterns are assigned as singlet (s), doublet (d), triplet (t), quartet (q) or pentuplet (p). All first-order splitting patterns were designated on the basis of the appearance of the multiplet. Splitting patterns that could not be readily interpreted are designated as multiplet (m) or broad (br). Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Varian VX-500 MHz or Jeol Delta ECA-500 MHz spectrometers, and were referenced relative to residual proton resonances in $CDCl_3$ (at 77.23 ppm), CD_3OD (at 49.15 ppm) or d_6-DMSO (at 39.51 ppm). Electrospray Ionization-Time of Flight (ESI-TOF) spectra were obtained on an Agilent 6230 Accurate-Mass TOFMS mass spectrometer. Anisotropy measurements were obtained on a SPEX FluoroMax-3 spectrofluorometer. Transmission electron microscopy images were recorded on a FEI Tecnai™ Sphera 200 kV microscope equipped with a LaB_6 electron gun, using the standard cryotransfer

holders developed by Gatan, Inc. Giant unilamellar vesicle (GUV) electroformation was carried out on a Vesicle Prep Pro® device developed by Nanion Technologies.

Synthesis of N-methylated Cysteine

N-(*tert*-butoxycarbonyl)-N-methyl-*S*-trityl-L-cysteine [N-Boc-^{Me}N-*L*-Cys(Trt)-OH, 17]. A solution of N-Boc-*L*-Cys(Trt)-OH (1.00 g, 2.16 mmol) in dry THF (16 mL) was treated with NaH (60% in mineral oil, 0.26 g, 6.48 mmol) and stirred at 0 ºC for 30 min. Then, iodomethane (404.0 μL, 6.48 mmol) was added and the resulting mixture was stirred at rt for 3 h. After quenching with water, the THF was removed and the resulting aqueous solution was washed with Et_2O (3 × 10 mL), acidified to pH 3 by addition of HCl (10%), and finally extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure, giving a pale yellow foam. The corresponding crude was purified by flash chromatography (0-5% MeOH in CH_2Cl_2), affording 0.91 g of **17** as a white foam [89%, R_f = 0.28 (5% MeOH in CH_2Cl_2)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 7.41 (d, *J* = 7.9 Hz, 6H, 6 × CH_{Ar}), 7.30-7.24 (m, 6H, 6 × CH_{Ar}), 7.22-7.16 (m, 3H, 3 × CH_{Ar}), 3.85 (m, 0.5H, 0.5 × CH), 3.59 (m, 0.5H, 0.5 × CH), 2.83-2.75 (m, 1H, 0.5 × CH_2), 2.70-2.55 (m, 4H, 0.5 × CH_2 + 1 × N-CH_3), 1.42 (s, 4.5H, 1.5 × CH_3), 1.35 (s, 4.5H, 1.5 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 176.2 and 175.3, 153.3 and 154.9, 144.6, 129.8, 128.2, 127.0, 81.3 and 81.0, 67.2 and 67.1, 60.6 and 59.7, 34.4 and 33.8, 31.7 and 31.0, 28.5 and 28.4. MS (ESI-TOF) [m/z (%)]: 500 ([M +Na]$^+$, 100). HRMS (ESI-TOF) calculated for $C_{28}H_{31}NO_4SNa$ ([M +Na]$^+$) 500.1866, found 500.1868.

Synthesis of Lysolipids

1-oleoyl-2-[N-Boc-^{Me}N-*L*-Cys(Trt)]-*sn*-glycero-3-phosphocholine (18). A solution of N-Boc-^{Me}N-*L*-Cys(Trt)-OH (**17**, 91.5 mg, 191.7 μmol) in CH_2Cl_2 (7.5 mL) was stirred at rt for 10 min, and then DIC (45.0 μL, 287.5 μmol) and DMAP (11.7 mg, 95.8 μmol) were successively added. After 10 min stirring at rt, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (**Lyso C_{18} PC-OH**, 25.0 mg, 47.9 μmol) was added. After 12 h stirring at rt, the solvent was removed under reduced pressure, and the crude was purified by HPLC, affording 43.1 mg of **18** as a colorless foam [92%, R_t = 9.2 min (Zorbax SB-C18 semipreparative column, 100% *Phase B*, 15.5 min)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 7.41-7.33 (d, *J* = 7.9 Hz, 6H, 6 × CH_{Ar}), 7.30-7.23 (m, 6H, 6 × CH_{Ar}), 7.22-7.16 (m, 3H, 3 × CH_{Ar}), 5.39-5.25 (m, 2H, 2 × CH), 5.14-4.97 (m, 1H, 1 × CH), 4.38-4.16 (m, 3H, 1.5 × CH_2), 4.05-3.80 (m, 4H, 1 × CH + 1.5 × CH_2), 3.75-3.61 (m, 2H, 1 × CH_2), 3.24 (s, 9H, 3 × CH_3), 2.77-2.68 (m, 1H, 0.5 × CH_2), 2.62 (s, 3H, 1 × N-CH_3), 2.46-2.56 (m, 1H, 0.5 × CH_2), 2.23-2.07 (m, 2H, 1 × CH_2), 2.04-1.92 (m, 4H, 2 × CH_2), 1.56-1.43 (m, 2H, 1 × CH_2), 1.39 (s, 4.5H, 1.5 × CH_3), 1.36 (s, 4.5H, 1.5 × CH_3), 1.34-1.16 (m, 20H, 10 × CH_2), 0.85 (t, *J* = 7.1 Hz, 3H, 1 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 173.3, 169.7, 163.9, 155.7, 144.4, 130.0, 129.7, 129.5, 128.0, 126.8, 80.8 and 80.3, 71.8, 67.0, 66.4, 63.1, 62.4, 59.1, 54.5, 33.9, 32.9, 31.9, 31.5, 31.2, 29.8, 29.8, 29.5, 29.3, 29.3, 29.2, 29.2, 29.1, 28.4, 27.2, 27.2, 24.8, 22.7, 14.2. MS (ESI-TOF) [m/z (%)]: 981 ([MH]$^+$, 100). HRMS (ESI-TOF) calculated for $C_{54}H_{82}N_2O_{10}PS$ ([MH]$^+$) 981.5422, found 981.5420.

1-oleoyl-2-(^{Me}N-*L*-Cys)-*sn*-glycero-3-phosphocholine (1). A solution of 1-oleoyl-2-[N-Boc-^{Me}N-*L*-Cys(Trt)]-*sn*-glycero-3-phosphocholine (**18**, 20.0 mg, 20.4 μmol) in

4 mL of TFA/CH_2Cl_2/TES (1.9:1.9:0.2) was stirred at rt for 30 min. After removal of the solvent, the residue was dried under high vacuum for 3 h. Then, the corresponding residue was diluted in MeOH (1 mL), filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 11.7 mg of the lysolipid **1** as a colorless oil [78%, Rt = 8.7 min (Zorbax SB-C18 semipreparative column, 50% *Phase A* in *Phase B*, 5 min, and then 5% *Phase A* in *Phase B*, 10 min)]. ^{1}H NMR (CD_3OD, 500.13 MHz, δ): 5.47-5.24 (m, 3H, 3 × CH), 4.50-4.37 (m, 1H, 0.5 × CH_2), 4.33-4.20 (m, 3H, 1.5 × CH_2), 4.16-4.00 (m, 2H, 1 × CH_2), 3.98-3.80 (m, 1H, 1 × CH), 3.72-3.59 (m, 2H, 1 × CH_2), 3.45-3.18 (m, 2H, 1 × CH_2), 3.23 (s, 9H, 3 × CH_3), 2.55 (s, 3H, 1 × N-CH_3), 2.35 (t, *J* = 7.6 Hz, 2H, 1 × CH_2), 2.12-1.92 (m, 4H, 2 × CH_2), 1.73-1.52 (m, 2H, 1 × CH_2), 1.42-1.20 (m, 20H, 12 × CH_2), 0.91 (t, *J* = 7.0 Hz, 3H, 1 × CH_3). ^{13}C NMR (CD_3OD, 125.77 MHz, δ): 175.0, 171.6, 130.9, 73.9, 67.6, 65.0, 63.5, 62.9, 60.7, 54.8, 35.0, 34.1, 33.2, 31.0, 31.0, 30.8, 30.8, 30.6, 30.6, 30.5, 30.5, 30.4, 28.3, 28.3, 26.1, 23.9, 14.7. MS (ESI-TOF) [m/z (%)]: 639 ([MH]$^+$, 100). HRMS (ESI-TOF) calculated for $C_{30}H_{40}N_2O_8PS$ ([MH]$^+$) 639.3803, found 639.3804.

1-palmitoyl-2-[N-Boc-^{Me}N-*L*-Cys(Trt)]-*sn*-glycero-3-phosphocholine (19). A solution of N-Boc-^{Me}N-*L*-Cys(Trt)-OH (**17**, 77.1 mg, 161.6 μmol) in CH_2Cl_2 (6 mL) was stirred at rt for 10 min, and then DIC (37.0 μL, 242.4 μmol) and DMAP (9.9 mg, 80.8 μmol) were successively added. After 10 min stirring at rt, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (**Lyso C_{16} PC-OH**, 20.0 mg, 40.4 μmol) was added. After 12 h stirring at rt, the solvent was removed under reduced pressure, and the crude was purified by HPLC, affording 29.7 mg of **19** as a colorless foam [77%, R_t = 8.8 min (Zorbax SB-C18 semipreparative column, 100% *Phase B*, 15.5 min)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 7.38 (d, *J* = 7.8 Hz, 6H, 6 × CH_{Ar}), 7.29-7.23 (m, 6H, 6 × CH_{Ar}), 7.22-7.16 (m, 3H, 3 × CH_{Ar}), 5.18-5.01 (m, 1H, 1 × CH), 4.36-4.17 (m, 3H, 1.5 × CH_2), 4.08-3.99 (m, 1H, 1 × CH), 3.99-3.81 (m, 3H, 1.5 × CH_2), 3.74-3.60 (m, 2H, 1 × CH_2), 3.22 (s, 9H, 3 × CH_3), 2.77-2.69 (m, 1H, 0.5 × CH_2), 2.62 (s, 3H, 1 × N-CH_3), 2.56-2.48 (m, 1H, 0.5 × CH_2), 2.29-2.08 (m, 2H, 1 × CH_2), 1.57-1.43 (m, 2H, 1 × CH_2), 1.39 (s, 4.5H, 1.5 × CH_3), 1.36 (s, 4.5H, 1.5 × CH_3), 1.30-1.17 (m, 24H, 12 × CH_2), 0.85 (t, *J* = 7.0 Hz, 3H, 1 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 173.5, 169.9, 164.0, 155.9, 144.6, 129.7, 128.2, 127.0, 81.1 and 80.6, 71.8, 67.2, 66.6, 63.4, 62.4, 59.4, 54.7, 34.1, 33.2, 32.1, 31.7, 31.3, 29.9, 29.9, 29.9, 29.7, 29.6, 29.5, 29.4, 28.6, 28.5, 25.0, 22.9, 14.4. MS (ESI-TOF) [m/z (%)]: 977 ([M +Na]$^+$, 21), 955 ([MH]$^+$, 100). HRMS (ESI-TOF) calculated for $C_{52}H_{80}N_2O_{10}PS$ ([MH]$^+$) 955.5266, found 955.5255.

1-palmitoyl-2-(^{Me}N-*L*-Cys)-*sn*-glycero-3-phosphocholine (4). A solution of 1-palmitoyl-2-[N-Boc-^{Me}N-*L*-Cys(Trt)]-*sn*-glycero-3-phosphocholine (**19**, 20.0 mg, 20.9 μmol) in 4 mL of TFA/CH_2Cl_2/TES (1.9:1.9:0.2) was stirred at rt for 30 min. After removal of the solvent, the residue was dried under high vacuum for 3 h. Then, the corresponding residue was diluted in MeOH (1 mL), filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 13.7 mg of the lysolipid **4** as a colorless oil [92%, Rt = 8.5 min (Zorbax SB-C18 semipreparative column, 50% *Phase A* in *Phase B*, 5 min, and then 5% *Phase A* in *Phase B*, 10 min)]. ^{1}H NMR (CD_3OD, 500.13 MHz, δ): 5.37 (m, 1H, 1 × CH), 4.51-4.42 (m, 1H, 0.5 × CH_2), 4.32-4.22 (m, 3H, 1.5 × CH_2), 4.18-4.01 (m, 3H, 1 × CH + 1 × CH_2), 3.69-3.61 (m, 2H, 1 × CH_2), 3.24 (s, 9H, 3 × CH_3), 3.18-3.02 (m, 2H, 1 × CH_2), 2.68 (s, 3H, 1 × N-CH_3), 2.35 (t, *J* = 7.5 Hz, 2H, 1 × CH_2), 1.68-1.54 (m, 2H, 1 × CH_2),

1.39-1.21 (m, 24H, 12 × CH_2), 0.90 (t, J = 6.9 Hz, 3H, 1 × CH_3). ^{13}C NMR (CD_3OD, 125.77 MHz, δ): 175.0, 166.7, 74.7, 67.5, 65.1, 63.9, 63.4, 60.7, 54.8, 35.0, 33.3, 31.0, 31.0, 30.9, 30.9, 30.8, 30.8, 30.7, 30.7, 30.6, 30.5, 30.4, 26.1, 26.1, 23.9, 14.6. MS (ESI-TOF) [m/z (%)]: 613 ([MH]$^+$, 100). HRMS (ESI-TOF) calculated for $C_{28}H_{58}N_2O_8PS$ ([MH]$^+$) 613.3646, found 613.3647.

1-palmitoyl-2-[N-Boc-*L*-Cys(Trt)]-*sn*-glycero-3-phosphocholine (20).[2] A solution of N-Boc-*L*-Cys(Trt)-OH (93.7 mg, 201.6 μmol) in CH_2Cl_2 (7.5 mL) was stirred at rt for 10 min, and then DIC (47.0 μL, 302.4 μmol) and DMAP (12.3 mg, 100.8 μmol) were successively added. After 10 min stirring at rt, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (**Lyso C_{16} PC-OH**, 25.0 mg, 50.4 μmol) was added. After 12 h stirring at rt, the solvent was removed under reduced pressure, and the crude was purified by HPLC, affording 29.6 mg of **20** as a colorless foam [88%, R_t = 12.5 min (Zorbax SB-C18 semipreparative column, 5% *Phase A* in *Phase B*, 15.5 min)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 7.38-7.31 (d, J = 8.0 Hz, 6H, 6 × CH_{Ar}), 7.30-7.23 (m, 6H, 6 × CH_{Ar}), 7.22-7.16 (m, 3H, 3 × CH_{Ar}), 5.25-5.13 (m, 1H, 1 × CH), 5.10 (d, J = 7.8 Hz, 0.3H, 1 × NH), 5.01 (d, J = 7.8 Hz, 0.7H, 1 × NH), 4.38-4.21 (m, 3H, 1.5 × CH_2), 4.20-4.11 (m, 1H, 1 × CH), 4.10-3.87 (m, 3H, 1.5 × CH_2), 3.86-3.66 (m, 2H, 1 × CH_2), 3.25 (s, 9H, 3 × CH_3), 2.73-2.45 (m, 2H, 1 × CH_2), 2.33-2.06 (m, 2H, 1 × CH_2), 1.62-1.43 (m, 2H, 1 × CH_2), 1.38 (s, 9H, 3 × CH_3), 1.31-1.16 (m, 24H, 12 × CH_2), 0.85 (t, J = 7.0 Hz, 3H, 1 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 173.6, 170.4, 163.9, 155.3, 144.5, 129.7, 128.3, 121.2, 80.2, 72.2, 67.3, 66.4, 64.1, 62.6, 59.9, 54.7, 52.8, 34.2, 34.1, 32.1, 29.9, 29.9, 29.9, 29.7, 29.6, 29.5, 29.4, 29.3, 28.6, 24.9, 22.9, 14.3. MS (ESI-TOF) [m/z (%)]: 941 ([MH]$^+$, 100). HRMS (ESI-TOF) calculated for $C_{51}H_{78}N_2O_{10}PS$ ([MH]$^+$) 941.5109, found 941.5111.

1-palmitoyl-2-(*L*-Cys)-*sn*-glycero-3-phosphocholine (6).[2] A solution of 1-palmitoyl-2-[N-Boc-*L*-Cys(Trt)]-*sn*-glycero-3-phosphocholine (**20**, 30.0 mg, 31.9 μmol) in 6 mL of TFA/CH_2Cl_2/TES (2.85:2.85:0.3) was stirred at rt for 30 min. After removal of the solvent, the residue was dried under high vacuum for 3 h. Then, the corresponding residue was diluted in MeOH (1 mL), filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 14.2 mg of the lysolipid **6** as a colorless foam [64%, Rt = 9.0 min (Zorbax SB-C18 semipreparative column, 50% *Phase A* in *Phase B*, 5 min, and then 5% *Phase A* in *Phase B*, 10 min)]. ^{1}H NMR (CD_3OD, 500.13 MHz, δ): 5.37 (m, 1H, 1 × CH), 4.43-4.36 (m, 1H, 0.5 × CH_2), 4.35-4.28 (m, 1H, 1 × CH), 4.27-4.14 (m, 3H, 1.5× CH_2), 4.13-3.98 (m, 2H, 1 × CH_2), 3.63-3.56 (m, 2H, 1 × CH_2), 3.17 (s, 9H, 3 × CH_3), 3.16-2.99 (m, 2H, 1 × CH_2), 2.33-2.25 (m, 2H, 1 × CH_2), 1.60-1.49 (m, 2H, 1 × CH_2), 1.31-1.19 (m, 24H, 12 × CH_2), 0.84 (t, J = 6.9 Hz, 3H, 1 × CH_3). ^{13}C NMR (CD_3OD, 125.77 MHz, δ): 175.0, 168.5, 75.0, 67.5, 65.1, 63.3, 60.7, 55.9, 54.8, 34.9, 33.2, 31.0, 31.0, 30.9, 30.9, 30.9, 30.8, 30.7, 30.6, 30.4, 26.1, 25.5, 25.3, 23.9, 14.6. MS (ESI-TOF) [m/z (%)]: 599 ([MH]$^+$, 100). HRMS (ESI-TOF) calculated for $C_{27}H_{56}N_2O_8PS$ ([MH]$^+$) 599.3490, found 599.3484.

1-oleoyl-2-[N-Boc-*L*-Cys(Trt)]-*sn*-glycero-3-phosphocholine (21). A solution of N-Boc-*L*-Cys(Trt)-OH (88.9 mg, 191.7 μmol) in CH_2Cl_2 (7.5 mL) was stirred at rt for 10 min, and then DIC (45.0 μL, 287.5 μmol) and DMAP (11.7 mg, 95.8 μmol) were successively added. After 10 min stirring at rt, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (**Lyso C_{18} PC-OH**, 25.0 mg, 47.9 μmol) was added. After 12 h stirring at rt, the solvent was removed under reduced pressure, and the crude was purified by

HPLC, affording 34.6 mg of **21** as a colorless foam [75%, R_t = 7.8 min (Zorbax SB-C18 semipreparative column, 100% *Phase B*, 15.5 min)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 7.38 (d, *J* = 7.5 Hz, 6H, 6 × CH_{Ar}), 7.33-7.26 (m, 6H, 6 × CH_{Ar}), 7.25-7.19 (m, 3H, 3 × CH_{Ar}), 5.40-5.29 (m, 2H, 2 × CH), 5.27-5.13 (m, 1H, 1 × CH), 5.07 (d, *J* = 9.0 Hz, 1H, 1 × NH), 4.41-3.88 (m, 7H, 3 × CH_2 + 1 × CH), 3.77-3.57 (m, 2H, 1 × CH_2), 3.25 (s, 9H, 3 × CH_3), 2.74-2.48 (m, 2H, 1 × CH_2), 2.31-2.09 (m, 2H, 1 × CH_2), 2.07-1.93 (m, 4H, 2 × CH_2), 1.61-1.43 (m, 2H, 1 × CH_2), 1.42 (s, 9H, 3 × CH_3), 1.31-1.17 (m, 20H, 10 × CH_2), 0.88 (t, *J* = 7.0 Hz, 3H, 1 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 173.5, 170.4, 163.9, 155.3, 144.4, 130.1, 129.9, 129.7, 129.6, 128.2, 128.2, 127.1, 80.1, 72.3, 67.2, 66.5, 63.8, 62.7, 59.4, 54.6, 52.7, 34.1, 34.1, 34.0, 32.0, 29.9, 29.9, 29.7, 29.5, 29.5, 29.4, 29.4, 29.3, 29.2, 29.2, 28.5, 28.5, 27.4, 27.3, 24.9, 24.8, 22.8, 14.3. MS (ESI-TOF) [m/z (%)]: 967 ($[MH]^+$, 100), 989 ($[M+Na]^+$, 20). HRMS (ESI-TOF) calculated for $C_{53}H_{80}N_2O_{10}PS$ ($[MH]^+$) 967.5266, found 967.5269.

1-oleoyl-2-(*L*-Cys)-*sn*-glycero-3-phosphocholine (12). A solution of 1-oleoyl-2-[N-Boc-*L*-Cys(Trt)]-*sn*-glycero-3-phosphocholine (**21**, 20.0 mg, 20.7 μmol) in 4 mL of TFA/CH_2Cl_2/TES (1.9:1.9:0.2) was stirred at rt for 30 min. After removal of the solvent, the residue was dried under high vacuum for 3 h. Then, the corresponding residue was diluted in MeOH (1 mL), filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 11.2 mg of the lysolipid **12** as a colorless foam [75%, Rt = 8.1 min (Zorbax SB-C18 semipreparative column, 50% *Phase A* in *Phase B*, 5 min, and then 5% *Phase A* in *Phase B*, 10 min)]. MS (ESI-TOF) [m/z (%)]: 625 ($[MH]^+$, 100). HRMS (ESI-TOF) calculated for $C_{29}H_{58}N_2O_8PS$ ($[MH]^+$) 625.3651, found 625.3647.

Acetonide-protected 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoglycerol (22). A suspension of 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (**Lyso C_{18} PG-OH**, 75.0 mg, 141.0 μmol) in acetone (1 mL) was subsequently treated with 2,2-dimethoxypropane (DMP, 65 mL) and a catalytic amount of TFA (40 μL). After 5 min stirring at rt, $CHCl_3$ (65 mL) was added until the lysolipid was fully dissolved. The reaction mixture was stirred for 60 h at rt. After evaporation of solvent under reduced pressure, the residue was dried *in vacuo* for 30 min to remove the TFA. The product was then re-dissolved in dry $CHCl_3$ (10 mL) (do not use wet $CHCl_3$ as this will react with the acetonide moiety), and the solvent was removed by rotary evaporation. This was repeated a total of three times to remove trace TFA in the oily residue. The corresponding residue was dried *in vacuo* for 5 h to give a pale yellow oil, which was used without further purification. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 8.28 (br s, 1H, 1 × OH), 5.45-5.20 (m, 2H, 2 × CH), 4.33-4.21 (m, 1H, 1 × CH), 4.19-4.10 (m, 1H, 0.5 × CH_2), 4.09-3.96 (m, 4H, 2 × CH_2), 3.95-3.83 (m, 3H, 1.5 × CH_2), 3.77-3.66 (m, 1H, 1 × CH), 2.29 (t, *J* = 7.5 Hz, 2H, 1 × CH_2), 2.08-1.90 (m, 4H, 2 × CH_2), 1.67-1.49 (m, 2H, 1 × CH_2), 1.38 (s, 3H, 1 × CH_3), 1.32 (s, 3H, 1 × CH_3), 1.30-1.17 (m, 20H, 10 × CH_2), 0.85 (t, *J* = 6.9 Hz, 3H, 1 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 174.3, 130.2, 129.9, 110.1, 74.5, 69.0, 67.8, 67.2, 66.1, 64.4, 34.2, 32.1, 30.0, 29.9, 29.7, 29.5, 29.5, 29.5, 29.4, 29.4, 27.4, 27.4, 26.8, 25.3, 25.0, 22.9, 14.3. MS (ESI-TOF) [m/z (%)]: 549 ($[M]^-$, 100). HRMS (ESI-TOF) calculated for $C_{27}H_{50}O_9P$ ($[M]^-$) 549.3198, found 549.3193.

Acetonide-protected 1-oleoyl-2-[N-Boc-*L*-Cys(Trt)]-*sn*-glycero-3-phosphoglycerol (23). A solution of acetonide-protected 1-oleoyl-2-hydroxy-*sn*-glycero-3-

phosphoglycerol (**22**, 40.0 mg, 69.9 μmol), N-Boc-*L*-Cys(Trt)-OH (81.0 mg, 174.7 μmol), DMAP (51.2 mg, 419.4 μmol) and Et_3N (34 μL) in $CDCl_3$ (2.5 mL) was stirred for 10 min at rt. Then, 2,4,6-trichlorobenzoyl chloride (TCBC, 71 μL) was added and the reaction was stirred for 12 h at rt. Afterwards, H_2O (75 μL) was added to the reaction mixture to quench the acid chloride, and the solvent was removed by rotary evaporation to give a yellow oil. The crude was dissolved in 500 μL of $CHCl_3$ and purified by flash column chromatography (0-20% MeOH in $CHCl_3$), affording 55.8 mg of **23** as a pale yellow oil [78%, R_f = 0.57 (20% MeOH in $CHCl_3$)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 7.45-7.31 (m, 6H, 6 × CH_{Ar}), 7.30-7.22 (m, 6H, 6 × CH_{Ar}), 7.21-7.13 (m, 3H, 3 × CH_{Ar}), 5.45-5.4 (m, 2H, 2 × CH), 5.26-5.07 (m, 1H, 1 × NH), 5.09-4.90 (m, 1H, 1 × CH), 4.64-3.38 (m, 10H, 4 × CH_2 + 2 × CH), 3.38-3.07 (m, 1H, 0.5 × CH_2), 2.75-2.44 (m, 1H, 0.5 × CH_2), 2.36-2.07 (m, 2H, 1 × CH_2), 2.08-1.84 (m, 4H, 2 × CH_2), 1.64-1.44 (m, 2H, 1 × CH_2), 1.43-1.17 (m, 35H, 10 × CH_2 + 5 × CH_3), 0.86 (t, J = 6.4 Hz, 3H, 1 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 173.2, 169.7, 158.1, 144.9, 130.2, 130.2, 129.9, 129.8, 128.2, 127.0, 82.2, 74.8, 72.0, 71.4, 68.0, 67.1, 66.4, 54.8, 34.1, 32.1, 30.0, 30.0, 29.9, 29.8, 29.6, 29.5, 29.5, 29.4, 29.4, 28.6, 27.5, 27.4, 27.0, 25.6, 24.9, 22.9, 14.3. MS (ESI-TOF) [m/z (%)]: 994 ([M]$^-$, 100). HRMS (ESI-TOF) calculated for $C_{54}H_{77}NO_{12}PS$ ([M]$^-$) 994.4910, found 994.4907.

1-oleoyl-2-(*L*-Cys)-*sn*-glycero-3-phosphoglycerol (15). A solution of acetonide-protected 1-oleoyl-2-[N-Boc-*L*-Cys(Trt)]-*sn*-glycero-3-phosphoglycerol (**23**, 10.0 mg, 9.80 μmol) in 2 mL of TFA/CH_2Cl_2/TES (0.95:0.95:0.1) was stirred at rt for 30 min. After removal of the solvent, the residue was dried under high vacuum for 3 h. Then, the corresponding residue was diluted in MeOH (500 μL), filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 4.7 mg of the lysolipid **15** as a colorless oil [65%, Rt = 9.9 min (Zorbax SB-C18 semipreparative column, 50% *Phase A* in *Phase B*, 5 min, and then 5% *Phase A* in *Phase B*, 10 min)]. ^{1}H NMR (MeOD, 500.13 MHz, δ): 5.43-5.25 (m, 3H, 3 × CH), 4.49-4.38 (m, 1H, 1 × CH), 4.39-4.21 (m, 2H, 1 × CH_2), 4.19-4.01 (m, 2H, 1 × CH_2), 3.98-3.81 (m, 2H, 1 × CH_2), 3.80-3.73 (m, 1H, 1 × CH), 3.67-3.52 (m, 2H, 1 × CH_2), 3.29-2.99 (m, 2H, 1 × CH_2), 2.35 (t, J = 7.6 Hz, 2H, 1 × CH_2), 2.10-1.94 (m, 4H, 2 × CH_2), 1.72-1.52 (m, 2H, 1 × CH_2), 1.45-1.16 (m, 20H, 10 × CH_2), 0.90 (t, J = 6.8 Hz, 3H, 1 × CH_3). ^{13}C NMR (MeOD, 125.77 MHz, δ): 175.0, 169.9, 131.1, 130.9, 74.9, 72.7, 72.7, 68.0, 65.0, 64.0, 63.3, 35.0, 33.8, 33.2, 33.2, 31.0, 30.9, 30.8, 30.6, 30.5, 30.5, 30.4, 28.3, 28.3, 26.1, 23.9, 14.6. MS (ESI-TOF) [m/z (%)]: 612 ([M]$^-$, 100). HRMS (ESI-TOF) calculated for $C_{27}H_{51}NO_{10}PS$ ([M]$^-$) 612.2977, found 612.2974.

Synthesis of Thioesters

MESNA thiooleate (2).[2] A solution of oleic acid (189.2 mg, 670.0 μmol) in CH_2Cl_2 (5 mL) was stirred at 0 ºC for 10 min, and then DMAP (7.4 mg, 60.9 μmol) and EDC.HCl (128.4 mg, 670.0 μmol) were successively added. After 10 min stirring at 0 ºC, sodium 2-mercaptoethanesulfonate[38] (MESNA, 100.0 mg, 609.1 μmol) was added. After 5 h stirring at rt, the mixture was extracted with H_2O (2 × 3 mL) and the combined aqueous phases were washed with EtOAc (3 mL). After evaporation of H_2O under reduced pressure, the residue was washed with CH_3CN (5 mL), and then filtered to yield 194.7 mg of **2** as a white solid [75%]. ^{1}H NMR (d_6-DMSO, 500.13 MHz, δ): 5.36-5.27 (m, 2H, 2 × CH), 3.05-2.99 (m, 2H, 1 × CH_2), 2.60-2.51 (m, 4H, 2 × CH_2), 2.02-1.92 (m,

4H, 2 × CH_2), 1.58-1.49 (m, 2H, 1 × CH_2), 1.34-1.18 (m, 20H, 10 × CH_2), 0.85 (t, J = 6.9 Hz, 3H, 1 × CH_3). ^{13}C NMR (d_6-DMSO, 125.77 MHz, δ): 198.7, 129.8, 129.7, 51.0, 43.4, 31.4, 29.2, 29.1, 28.9, 28.8, 28.7, 28.6, 28.5, 28.3, 26.7, 26.6, 25.1, 24.4, 22.2, 14.1. MS (ESI-TOF) [m/z (%)]: 429 ([MH]$^+$, 100). HRMS (ESI-TOF) calculated for $C_{20}H_{38}NaO_4S_2$ ([MH]$^+$) 429.2104, found 429.2105.

MESNA thiopalmitate (8). A solution of palmitic acid (171.8 mg, 670.0 μmol) in CH_2Cl_2 (5 mL) was stirred at 0 ºC for 10 min, and then DMAP (7.4 mg, 60.9 μmol) and EDC.HCl (128.4 mg, 670.0 μmol) were successively added. After 10 min stirring at 0 ºC, sodium 2-mercaptoethanesulfonate[4] (MESNA, 100.0 mg, 609.1 μmol) was added. After 5 h stirring at rt, the mixture was extracted with H_2O (2 × 3 mL) and the combined aqueous phases were washed with EtOAc (3 mL). After evaporation of H_2O under reduced pressure, the residue was washed with CH_3CN (5 mL), and then filtered to yield 189.3 mg of **8** as a white solid [77%]. ^{1}H NMR (d_6-DMSO, 500.13 MHz, δ): 3.08-2.96 (m, 2H, 1 × CH_2), 2.62-2.50 (m, 4H, 2 × CH_2), 1.62-1.45 (m, 2H, 1 × CH_2), 1.34-1.14 (m, 24H, 12 × CH_2), 0.85 (t, J = 7.0 Hz, 3H, 1 × CH_3). ^{13}C NMR (d_6-DMSO, 125.77 MHz, δ): 198.7, 50.9, 43.3, 31.3, 29.1, 29.1, 29.1, 29.0, 29.0, 29.0, 28.9, 28.8, 28.7, 28.2, 25.1, 24.3, 22.1, 14.0. MS (ESI-TOF) [m/z (%)]: 379 ([M -Na]$^-$, 100). HRMS (ESI-TOF) calculated for $C_{18}H_{35}O_4S_2$ ([M -Na]$^-$) 379.1971, found 379.1973.

Synthesis of Phospholipids

1-oleoyl-2-[^{Me}N-*L*-Cys-(oleoyl)]-*sn*-glycero-3-phosphocholine (3). 1-oleoyl-2-(^{Me}N-*L*-Cys)-*sn*-glycero-3-phosphocholine (**1**, 7.50 mg, 11.75 μmol) and MESNA-thiooleate (**2**, 5.03 mg, 11.75 μmol) were dissolved in 1.18 mL of 50 mM DTT in 200 mM NaH_2PO_4 pH 7.1 buffer and stirred under N_2 at rt. After 30 min, the corresponding mixture was filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 7.5 mg of the amidophospholipid **3** as a colorless oil [71%, R_t = 15.6 min (Zorbax SB-C18 semipreparative column, 100 % *Phase B*, 20.5 min)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 5.38-5.27 (m, 4H, 4 × CH), 5.25-5.13 (m, 1H, 1 × CH), 5.14-4.95 (m, 1H, 1 × CH), 4.45-4.22 (m, 3H, 1.5 × CH_2), 4.17-3.91 (m, 3H, 1.5 × CH_2), 3.86-3.65 (m, 2H, 1 × CH_2), 3.31 (s, 9H, 3 × CH_3), 3.20-3.06 (m, 1H, 0.5 × CH_2), 2.98 (s, 3H, 1 × N-CH_3), 2.86-2.68 (m, 1H, 0.5 × CH_2), 2.35 (t, J = 7.5 Hz, 2H, 1 × CH_2), 2.26 (t, J = 7.5 Hz, 2H, 1 × CH_2), 2.10-1.85 (m, 8H, 4 × CH_2), 1.69-1.45 (m, 4H, 2 × CH_2), 1.39-1.12 (m, 41H, 20 × CH_2 + 1 × SH), 0.86 (t, J = 7.0 Hz, 6H, 2 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 174.8, 173.7, 169.4, 130.7 and 130.4, 130.2 and 129.9, 72.7, 66.7, 63.9, 62.6, 59.9, 59.5, 54.8 and 54.7, 34.3, 33.8, 33.0, 32.9, 32.8, 32.1, 30.0, 30.0, 29.9, 29.9, 29.8, 29.7, 29.7, 29.6, 29.6, 29.6, 29.5, 29.5, 29.4, 29.4, 29.4, 29.3, 27.5, 27.4, 25.9, 25.3, 25.2, 25.1, 23.7, 22.9, 14.7. MS (ESI-TOF) [m/z (%)]: 925 ([M +Na]$^+$, 100), 903 ([MH]$^+$, 80). HRMS (ESI-TOF) calculated for $C_{48}H_{91}N_2O_9PSNa$ ([M +Na]$^+$) 925.6075, found 925.6073.

1-palmitoyl-2-[^{Me}N-*L*-Cys-(oleoyl)]-*sn*-glycero-3-phosphocholine (5). 1-palmitoyl-2-(^{Me}N-*L*-Cys)-*sn*-glycero-3-phosphocholine (**4**, 7.50 mg, 12.25 μmol) and MESNA-thiooleate (**2**, 5.24 mg, 12.25 μmol) were dissolved in 1.23 mL of 50 mM DTT in 200 mM NaH_2PO_4 pH 7.1 buffer and stirred under N_2 at rt. After 30 min, the corresponding mixture was filtered using a 0.2 μm syringe-driven filter, and the crude

solution was purified by HPLC, affording 8.2 mg of the amidophospholipid **5** as a colorless oil [76%, R_t = 14.9 min (Zorbax SB-C18 semipreparative column, 100 % *Phase B*, 20.5 min)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 5.38-5.28 (m, 2H, 2 × CH), 5.27-5.17 (m, 1H, 1 × CH), 5.09-5.02 (m, 1H, 1 × CH), 4.47-4.22 (m, 3H, 1.5 × CH_2), 4.18-3.92 (m, 3H, 1.5 × CH_2), 3.85-3.68 (m, 2H, 1 × CH_2), 3.30 (s, 9H, 3 × CH_3), 3.21-3.08 (m, 1H, 0.5 × CH_2), 2.98 (s, 3H, 1 × N-CH_3), 2.83-2.73 (m, 1H, 0.5 × CH_2), 2.35 (t, J = 8.3 Hz, 2H, 1 × CH_2), 2.26 (t, J = 8.3 Hz, 2H, 1 × CH_2), 2.06-1.85 (m, 4H, 2 × CH_2), 1.68-1.44 (m, 4H, 2 × CH_2), 1.38-1.17 (m, 45H, 22 × CH_2 + 1 × SH), 0.85 (t, J = 6.6 Hz, 6H, 2 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 174.8, 173.7, 169.4, 130.7 and 130.4, 130.2 and 129.9, 72.6, 66.7, 63.9, 62.4, 59.9, 59.6, 54.8, 34.3, 33.8, 33.0, 32.9, 32.9, 32.1, 32.1, 30.0, 30.0, 29.9, 29.9, 29.9, 29.8, 29.8, 29.7, 29.6, 29.6, 29.6, 29.6, 29.5, 29.5, 29.5, 29.4, 29.4, 27.5, 27.4, 25.3, 25.0, 23.7, 22.9, 14.4. MS (ESI-TOF) [m/z (%)]: 899 ([M +Na]$^+$, 100), 877 ([MH]$^+$, 75). HRMS (ESI-TOF) calculated for $C_{46}H_{89}N_2O_9PSNa$ ([M +Na]$^+$) 899.5919, found 899.5917.

1-palmitoyl-2-[*L*-Cys-(oleoyl)]-*sn*-glycero-3-phosphocholine (7).[2] 1-palmitoyl-2-(*L*-Cys)-*sn*-glycero-3-phosphocholine (**6**, 5.00 mg, 7.18 μmol) and MESNA-thiooleate (**2**, 3.07 mg, 7.18 μmol) were dissolved in 1.44 mL of 25 mM DTT in 200 mM NaH_2PO_4 pH 7.1 buffer and stirred under N_2 at rt. After 30 min, the corresponding mixture was filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 4.2 mg of the amidophospholipid **7** as a white solid [68%, R_t = 12.7 min (Zorbax SB-C18 semipreparative column, 100% *Phase B*, 15.5 min)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 6.87 (d, 1H, 1 × NH, J = 7.5 Hz), 5.37-5.29 (m, 2H, 2 × CH), 5.28-5.18 (m, 1H, 1 × CH), 4.88-4.77 (m, 1H, 1 × CH), 4.46-4.24 (m, 3H, 1.5 × CH_2), 4.23-3.99 (m, 3H, 1.5 × CH_2), 3.98-3.80 (m, 2H, 1 × CH_2), 3.36 (s, 9H, 3 × CH_3), 3.08-2.81 (m, 2H, 1 × CH_2), 2.34-2.17 (m, 4H, 2 × CH_2), 2.04-1.87 (m, 4H, 2 × CH_2), 1.67-1.48 (m, 4H, 2 × CH_2), 1.35-1.16 (m, 45H, 22 × CH_2 + 1 × SH), 0.85 (t, J = 6.8 Hz, 6H, 2 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 173.7, 169.9, 163.5, 130.7 and 130.4, 130.2 and 129.9, 72.3, 66.3, 64.6, 62.4, 60.2, 54.7, 53.7, 36.5, 34.2, 32.9, 32.1, 30.0, 29.9, 29.9, 29.9, 29.8, 29.7, 29.6, 29.6, 29.6, 29.5, 29.4, 29.4, 29.3, 27.4, 26.9, 25.9, 25.0, 22.9, 14.4. MS (ESI-TOF) [m/z (%)]: 885 ([M +Na]$^+$, 60), 863 ([MH]$^+$, 100). HRMS (ESI-TOF) calculated for $C_{45}H_{88}N_2O_9PS$ ([MH]$^+$) 863.5943, found 863.5948.

1-palmitoyl-2-[^{Me}N-*L*-Cys-(palmitoyl)]-*sn*-glycero-3-phosphocholine (9). 1-palmitoyl-2-(^{Me}N-*L*-Cys)-*sn*-glycero-3-phosphocholine (**4**, 5.00 mg, 8.17 μmol) and MESNA-thiopalmitate (**8**, 3.28 mg, 8.17 μmol) were dissolved in 1.63 mL of 25 mM DTT in 200 mM NaH_2PO_4 pH 7.1 buffer and stirred under N_2 at rt. After 30 min, the corresponding mixture was filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 4.0 mg of the amidophospholipid **9** as a colorless oil [57%, R_t = 15.8 min (Zorbax SB-C18 semipreparative column, 100 % *Phase B*, 20.5 min)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 5.32-5.14 (m, 1H, 1 × CH), 5.11-5.00 (m, 1H, 1 × CH), 4.50-4.23 (m, 3H, 1.5 × CH_2), 4.21-3.96 (m, 3H, 1.5 × CH_2), 3.94-3.78 (m, 2H, 1 × CH_2), 3.36 (s, 9H, 3 × CH_3), 3.22-3.07 (m, 1H, 0.5 × CH_2), 2.98 (s, 3H, 1 × N-CH_3), 2.86-2.72 (m, 1H, 0.5 × CH_2), 2.35 (t, J = 7.2 Hz, 2H, 1 × CH_2), 2.27 (t, J = 7.2 Hz, 2H, 1 × CH_2), 1.69-1.45 (m, 4H, 2 × CH_2), 1.34-1.17 (m, 49H, 24 × CH_2 + 1 × SH), 0.85 (t, J = 7.2 Hz, 6H, 2 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 174.9, 173.8, 165.6, 72.3, 66.5, 66.3, 64.2, 62.3, 59.8, 54.8 and 54.7, 34.2, 33.8, 33.0, 32.1, 29.9, 29.9, 29.9, 29.9, 29.9, 29.8, 29.7, 29.7, 29.6, 29.6, 29.6, 29.4, 29.4, 25.3, 25.2, 25.1, 25.0,

23.7 22.9, 14.4. MS (ESI-TOF) [m/z (%)]: 873 ([M +Na]$^+$, 100), 851 ([MH]$^+$, 67). HRMS (ESI-TOF) calculated for $C_{44}H_{88}N_2O_9PS$ ([MH]$^+$) 851.5943, found 851.5939.

1-oleoyl-2-[^{Me}N-*L*-Cys-(palmitoyl)]-*sn*-glycero-3-phosphocholine (10). 1-oleoyl-2-(^{Me}N-*L*-Cys)-*sn*-glycero-3-phosphocholine (**1**, 2.00 mg, 3.14 μmol) and MESNA-thiopalmitate (**8**, 1.26 mg, 3.14 μmol) were dissolved in 625 μL of 25 mM DTT in 200 mM NaH_2PO_4 pH 7.1 buffer and stirred under N_2 at rt. After 30 min, the corresponding mixture was filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 1.5 mg of the amidophospholipid **10** as a colorless oil [68%, R_t = 16.9 min (Zorbax SB-C18 semipreparative column, 100 % *Phase B*, 20.5 min)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 5.43-5.28 (m, 2H, 2 × CH), 5.27-5.14 (m, 1H, 1 × CH), 5.13-5.00 (m, 1H, 1 × CH), 4.50-4.24 (m, 3H, 1.5 × CH_2), 4.20-3.97 (m, 3H, 1.5 × CH_2), 3.89-3.71 (m, 2H, 1 × CH_2), 3.33 (s, 9H, 3 × CH_3), 3.21-3.09 (m, 1H, 0.5 × CH_2), 2.98 (s, 3H, 1 × N-CH_3), 2.86-2.70 (m, 1H, 0.5 × CH_2), 2.38-2.22 (m, 4H, 2 × CH_2), 2.03-1.89 (m, 4H, 2 × CH_2), 1.67-1.43 (m, 4H, 2 × CH_2), 1.38-1.13 (m, 45H, 22 × CH_2 + 1 × SH), 0.85 (t, *J* = 7.1 Hz, 6H, 2 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 174.7, 173.7, 169.4, 130.7 and 130.4, 130.2 and 129.9, 72.5, 66.7, 66.3, 64.1, 62.4, 59.9, 54.9 and 54.7, 34.3, 34.2, 33.8, 32.9, 32.8, 32.2, 32.1, 30.0, 30.0, 29.9, 29.9, 29.9, 29.9, 29.8, 29.7, 29.6, 29.6, 29.5, 29.4, 29.4, 29.4, 29.4, 29.3, 27.5, 27.4, 25.3, 25.2, 25.1, 23.7, 22.9, 14.4. MS (ESI-TOF) [m/z (%)]: 899 ([M +Na]$^+$, 100), 877 ([MH]$^+$, 81). HRMS (ESI-TOF) calculated for $C_{46}H_{90}N_2O_9PS$ ([MH]$^+$) 877.6098, found 877.6099.

1-palmitoyl-2-[*L*-Cys-(palmitoyl)]-*sn*-glycero-3-phosphocholine (11). 1-palmitoyl-2-(*L*-Cys)-*sn*-glycero-3-phosphocholine (**6**, 3.00 mg, 5.01 μmol) and MESNA-thiopalmitate (**8**, 2.02 mg, 5.01 μmol) were dissolved in 1.00 mL of 25 mM DTT in 200 mM NaH_2PO_4 pH 7.1 buffer and stirred under N_2 at rt. After 30 min, the corresponding mixture was filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 2.4 mg of the amidophospholipid **11** as a colorless oil [57%, R_t = 14.9 min (Zorbax SB-C18 semipreparative column, 100 % *Phase B*, 20.5 min)]. MS (ESI-TOF) [m/z (%)]: 859 ([M +Na]$^+$, 46), 837 ([MH]$^+$, 100). HRMS (ESI-TOF) calculated for $C_{43}H_{86}N_2O_9PS$ ([MH]$^+$) 837.5792, found 837.5795.

1-oleoyl-2-[*L*-Cys-(oleoyl)]-*sn*-glycero-3-phosphocholine (13). 1-oleoyl-2-(*L*-Cys)-*sn*-glycero-3-phosphocholine (**12**, 9.00 mg, 14.41 μmol) and MESNA-thiooleate (**2**, 6.20 mg, 14.41 μmol) were dissolved in 1.44 mL of 50 mM DTT in 200 mM $NaH_2PO_4.H_2O$ pH 7.1 buffer and stirred under N_2 at rt. After 30 min, the corresponding mixture was filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 8.7 mg of the amidophospholipid **13** as a white foam [68%, R_t = 14.4 min (Zorbax SB-C18 semipreparative column, 100% *Phase B*, 20.5 min)]. ^{1}H NMR ($CDCl_3$, 500.13 MHz, δ): 7.02 (d, 1H, 1 × NH, *J* = 7.9 Hz), 5.42-5.30 (m, 4H, 4 × CH), 5.29-5.22 (m, 1H, 1 × CH), 4.98-4.76 (m, 1H, 1 × CH), 4.44-3.92 (m, 6H, 3 × CH_2), 3.89-3.68 (m, 2H, 1 × CH_2), 3.33 (s, 9H, 3 × CH_3), 3.23-2.80 (m, 2H, 1 × CH_2), 2.38-2.17 (m, 4H, 2 × CH_2), 2.10-1.84 (m, 8H, 4 × CH_2), 1.71-1.45 (m, 4H, 2 × CH_2), 1.44-1.13 (m, 41H, 20 × CH_2 + 1 × SH), 0.85 (t, *J* = 6.8 Hz, 6H, 2 × CH_3). ^{13}C NMR ($CDCl_3$, 125.77 MHz, δ): 173.7, 173.6, 170.0, 130.6, 130.3, 130.2, 129.8, 72.8, 66.6, 63.9, 62.6, 59.4, 54.8, 53.6, 36.5, 34.2, 34.1, 32.8, 32.7, 32.1, 30.0, 29.9, 29.8, 29.7, 29.7, 29.6, 29.5, 29.5, 29.4, 29.4, 29.4, 29.4, 29.3, 29.3, 27.4, 27.4, 27.3, 27.3, 26.9, 25.8,

25.8, 25.0, 22.9, 14.3. MS (ESI-TOF) [m/z (%)]: 889 ($[MH]^+$, 100), 911 ($[M+Na]^+$, 25). HRMS (ESI-TOF) calculated for $C_{47}H_{90}N_2O_9PS$ ($[MH]^+$) 889.6105, found 889.6102.

1-oleoyl-2-[*L*-Cys-(palmitoyl)]-*sn*-glycero-3-phosphocholine (14). 1-oleoyl-2-(*L*-Cys)-*sn*-glycero-3-phosphocholine (**12**, 5.00 mg, 8.01 μmol) and MESNA-thiopalmitate (**8**, 3.22 mg, 8.01 μmol) were dissolved in 0.80 mL of 50 mM DTT in 200 mM $NaH_2PO_4.H_2O$ pH 7.1 buffer and stirred under N_2 at rt. After 30 min, the corresponding mixture was filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 3.9 mg of the amidophospholipid **14** as a white foam [56%, R_t = 13.8 min (Zorbax SB-C18 semipreparative column, 100% *Phase B*, 20.5 min)]. MS (ESI-TOF) [m/z (%)]: 863 ($[M]^-$, 100). HRMS (ESI-TOF) calculated for $C_{45}H_{88}N_2O_9PS$ ($[MH]^+$) 863.5948, found 863.5947.

1-oleoyl-2-[*L*-Cys-(oleoyl)]-*sn*-glycero-3-phosphoglycerol (16). 1-oleoyl-2-(*L*-Cys)-*sn*-glycero-3-phosphoglycerol (**15**, 3.00 mg, 4.90 μmol) and MESNA-thiooleate (**2**, 2.10 mg, 4.90 μmol) were dissolved in 1.00 mL of 25 mM DTT in 200 mM NaH_2PO_4 pH 7.1 buffer and stirred under N_2 at rt. After 30 min, the corresponding mixture was filtered using a 0.2 μm syringe-driven filter, and the crude solution was purified by HPLC, affording 2.7 mg of the amidophospholipid **16** as a colorless oil [63%, R_t = 17.2 min (Zorbax SB-C18 semipreparative column, 100 % *Phase B*, 20.5 min)]. MS (ESI-TOF) [m/z (%)]: 877 ($[M]^-$, 100). HRMS (ESI-TOF) calculated for $C_{45}H_{83}NO_{11}PS$ ($[M]^-$) 876.5430, found 876.5435.

Micelle Sizes: Critical Micelle Concentrations (cmc's)

100.0 μL of aqueous solutions (10 mM, 1 mM, 100 μM, 10 μM and 1 μM) of the control (lysolipid 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, **Lyso C_{16} PC-OH**) and the precursors (**1**, **2**, **4**, **6** or **8**) were analyzed by Dynamic Light Scattering (DLS) in order to determine the micelle sizes and the critical micelle concentrations (cmc's).

De novo phospholipid synthesis

General procedure. N-methylated cysteine-modified lysolipid **1** (3.20 mg, 5.01 μmol) and MESNA thiooleate **2** (2.14 mg, 5.01 μmol) were dissolved in 1.00 mL of 25 mM DTT in 200 mM NaH_2PO_4 pH 7.1 buffer and stirred under N_2 for 30 min at rt, affording the phospholipid **3**. Phospholipids **5**, **7**, **9**, **10** and **11** were also prepared in the same way, using the corresponding lysolipids **1**, **4** or **6**, and MESNA thioesters **2** or **8**.

De novo phospholipid synthesis: *in situ* vesicle formation

5.0 μL of a 25 mM DTT solution in 200 mM NaH_2PO_4 pH 7.1 buffer were added to 2.5 μL of a 10 mM solution of N-methylated cysteine-based lysolipid **1** in 200 mM NaH_2PO_4 pH 7.1 buffer. Afterward, 2.5 μL of a 10 mM solution of MESNA thiooleate **2** in NaH_2PO_4 pH 7.1 buffer were added, and the mixture was briefly agitated. The resulting solution was added to a glass microscope slide and covered with a glass coverslip supported with vacuum grease. The sample was then monitored by phase contrast microscopy in order to analyze the *in situ* phospholipid **3** vesicle formation.

Remodeling: lipid chain RNCL exchange

General procedure. 100.0 μL of a previously *de novo* formed phospholipid **3** solution were added to cysteine-modified lysolipid (N-methylated **4** or un-N-methylated **6**; 0.50 μmol), and stirred under N_2 for 30 min at rt, affording the corresponding remodeled phospholipids (equimolar mixture of N-methylated phospholipids **3** and **5** or un-N-methylated phospholipid **7**). Phospholipids **10** and **11** were also prepared in the same way, using the corresponding *de novo* formed phospholipid **9**, and lysolipids **1** or **6**.

Remodeling: head group RNCL exchange

General procedure. 100.0 μL of a previously *de novo* formed N-methylated phosphatidylcholine phospholipid **3** solution were added to cysteine-modified phosphatidylglycerol lysolipid **15** (0.50 μmol), and stirred under N_2 for 30 min at rt, affording the corresponding remodeled phosphatidylglycerol phospholipid **16**.

LC/MS Analysis

De novo formation (NCL reaction) and remodeling (RNCL reaction) of phospholipid membranes were performed in the appropriate buffer (200 mM NaH_2PO_4 pH 7.1 buffer containing 25 mM DTT) as described above. Aliquots of 1.5 μL of sample were taken at various time points, diluted with 50.0 μL of MeOH and analyzed using an Eclipse Plus C8 analytical column (5% *Phase A* in *Phase B*, 5.5 min) with an Evaporative Light Scattering Detector (ELSD) at a flow of 1.0 mL/min. For all LC/MS runs, solvent *Phase A* consisted of H_2O with 0.1% formic acid and solvent *Phase B* of MeOH with 0.1% formic acid.

Fluorescence Microscopy of Phospholipid Membrane Vesicles: Texas Red® DHPE

***In situ* vesicle formation.** 5.0 μL of a 25 mM DTT solution in 200 mM NaH_2PO_4 pH 7.1 buffer were added to 2.5 μL of a 10 mM solution of N-methylated cysteine-based lysolipid **1** in 200 mM NaH_2PO_4 pH 7.1 buffer. Afterward, 2.5 μL of a 10 mM solution of MESNA thiooleate **2** in NaH_2PO_4 pH 7.1 buffer were added, and the mixture was briefly agitated. Then, 5.0 μL of the resulting solution were mixed with 0.1 μL of a 100 μM Texas Red® DPHE dye solution in EtOH, and the resulting solution was added to a glass microscope slide and covered with a glass coverslip supported with vacuum grease. The corresponding sample was then monitored by fluorescence and phase contrast microscopy in order to analyze the *in situ* vesicle formation and determine the corresponding vesicular structure.

Hydration method. 10.0 μL of a 20 mM solution of phospholipid **3** in $CHCl_3$ were added to a 1 mL vial, placed under N_2 and dried for 15 min to prepare a lipid film. Then, 200.0 μL of H_2O were added and the solution was tumbled at 25 ºC for 1 h. Afterward, to 10.0 μL of this 1 mM aqueous solution of phospholipid **3** were added 0.1 μL of a 100 μM Texas Red® DPHE dye solution in EtOH, and the mixture was briefly agitated. The corresponding sample was finally monitored by fluorescence and phase contrast microscopy in order to determine the vesicle structure.

Sonication method. 10.0 μL of a 20 mM solution of phospholipid **3** in $CHCl_3$ were added to a 1 mL vial, placed under N_2 and dried for 15 min to prepare a lipid film. Then, 200.0 μL of H_2O were added, and the resulting mixture was sonicated with heat (≈55ºC) for 1 h. Afterward, to 10.0 μL of this 1 mM aqueous solution of phospholipid **3** were

added 0.1 μL of a 100 μM Texas Red® DPHE dye solution in EtOH, and the mixture was briefly agitated. The corresponding sample was finally monitored by fluorescence and phase contrast microscopy in order to determine the vesicle structure.

Transmission Electron Microscopy (TEM) Studies

General. A deposition System Balzers Med010 was used to evaporate a homogeneous layer of carbon. The samples were collected over 400 mesh Cu grids. The grids were then negatively stained with a solution of 1% (w/w) uranyl acetate. Micrographs were recorded on a FEI Tecnai™ Sphera microscope operating at 200 kV and equipped with a LaB_6 electron gun, using the standard cryotransfer holders developed by Gatan, Inc. For image processing, micrographs were digitized in a Zess SCAI scanner with different sampling windows.

TEM measurements. Copper grids (formvar/carbon-coated, 400 mesh copper) were prepared by glow discharging the surface at 20 mA for 1.5 min. Once the surface for vesicle adhesion is ready, 3.5 μL of a 5 mM solution of phospholipid **3** in H_2O (previously sonicated at ≈55 ºC for 1 h) was deposited on the grid surface. This solution was allowed to sit for 10 seconds before being washed away with 10 drops of glass distilled H_2O and subsequent staining with 3 drops of 1% w/w uranyl acetate. The stain was allowed to sit for 10 seconds before wicking away with filter paper. All grid treatments and simple depositions were on the dark/shiny/glossy formvar-coated face of the grid (this side face up during glow discharge). Samples were then imaged via TEM, revealing the presence of several populations of spherical compartments (50-950 nm in diameter), consistent with the vesicle architecture.

***In situ* TEM measurements.** Copper grids (formvar/carbon-coated, 400 mesh copper) were prepared by glow discharging the surface at 20 mA for 1.5 min. Once the surface for vesicle adhesion is ready, 3.5 μL of a previously *in situ* formed phospholipid **3** sample in 200 mM NaH_2PO_4 pH 7.1 buffer was deposited on the grid surface. This solution was allowed to sit for 10 seconds before being washed away with 10 drops of glass distilled H_2O and subsequent staining with 3 drops of 1% w/w uranyl acetate. The stain was allowed to sit for 10 seconds before wicking away with filter paper. All grid treatments and simple depositions were on the dark/shiny/glossy formvar-coated face of the grid (this side face up during glow discharge). Samples were then imaged via TEM, revealing the presence of several populations of spherical compartments (50-950 nm in diameter), consistent with the vesicle architecture.

Encapsulation Experiments

Encapsulation of HPTS (Standard method). 60.0 μL of a 20 mM solution of phospholipid **3** in $CHCl_3$ were added to a 1 mL vial, placed under N_2 and dried for 15 min to prepare a lipid film. Then, 240.0 μL of a 100 μM HPTS solution in H_2O were added and the mixture was tumbled at 25 ºC for 1 h. Afterwards, the solution was transferred to a 100K spin filter and centrifuged for 10 min at 9000-10000 rcf in order to remove the non-encapsulated HPTS. Then, the sample was washed and centrifuged with H_2O (5 × 250 μL). The lipid-containing solution was finally examined by fluorescence microscopy, observing vesicles containing the desired HPTS.

Encapsulation of HPTS (Inverse emulsion method).[5] 60.0 μL of a 20 mM solution of phospholipid **3** in $CHCl_3$ were added to a 1 mL vial, placed under N_2 and

dried for 15 min to prepare a lipid film. Then, 200.0 μL of mineral oil (Fisher Chemical, O122-1) were added and placed under N_2 to displace the air above the mineral oil. The resulting mixture was sonicated with heat (≈55ºC) for 1 h. Afterward, 100.0 μL of the amidophospholipid oil was added to a 1 mL eppendorf. Then, 10.0 μL of the *upper buffer* [50 μM HPTS + 200 mM sucrose in 100 mM HEPES buffer pH 7.5 solution] was added, and the resulting mixture was flicked and vortexed till it was a cloudy emulsion. The corresponding emulsion was added to a 1 mL eppendorf containing 100.0 μL of the *lower buffer* [200 mM glucose in 100 mM HEPES buffer pH 7.5 solution], so it floated on top. Then, we waited for 10 min. After this time, the sample was centrifuged for 10 min at 9000-10000 rcf. The sample was separated from the oil (either aspirating off the oil or using a syringe/needle to collect the sample from the bottom). The corresponding sample contained vesicles encapsulating HPTS that were observed using fluorescence microscopy.

***In situ* encapsulation of HPTS.** 1-oleoyl-2-(^{Me}N-*L*-Cys)-*sn*-glycero-3-phosphocholine (**1**, 0.50 mg, 0.78 μmol) was dissolved in 78.0 μL of a 100 μM solution of HPTS in 200 mM NaH_2PO_4 pH 7.1 buffer. Then, 78.0 μL of a 50 mM DTT solution in 200 mM NaH_2PO_4 pH 7.1 buffer was added. Finally, MESNA-thiooleate **2** (0.34 mg, 0.78 μmol) was added and the resulting mixture was stirred under N_2 at rt. After 30 min, the reaction was transferred to a 100K spin filter and centrifuged for 10 min at 9000-10000 rcf in order to remove the non-encapsulated HPTS. Then, the sample was washed and centrifuged with a 200 mM solution of NaH_2PO_4 pH 7.1 buffer (5 × 250 μL). The lipid-containing solution was finally examined by fluorescence microscopy, observing vesicles containing the desired HPTS.

Anisotropy Studies

Vesicle preparation and extrusion. 10.0 μL of a 100 mM solution of phospholipid (DOPC, POPC, **3**, **5** or **7**) in $CHCl_3$ were added to a 1 mL vial, placed under N_2 and dried for 15 min to prepare a lipid film. The dried film was hydrated in 500.0 μL of H_2O [Final concentration of phospholipid: 2 mM]. The lipid solution was briefly vortexed and then sonicated with heat (≈55ºC) for 45 min. Once the sample was fully hydrated, the vesicles were extruded through 100 nm membrane. Extruded vesicles were finally analyzed by Dynamic Light Scattering (DLS).

Anisotropy measurements. 5.0 μL of a 500 μM solution of DPH in EtOH were added to 495.0 μL of 100 nm phospholipid (DOPC, POPC, **3**, **5** or **7**) extruded vesicles [Final concentration of DPH: 5 μM, 1% v/v]. The solution was tumbled at 25 ºC overnight. Steady-state anisotropy was measured at different temperatures (from -10 to 40 ºC) on a Perkin spectrophotometer with a manual polarizer accessory and peltier temperature controller.

Fluorescence Microscopy of Giant Unilamellar Vesicles (GUVs): Bodipy® FL DHPE

GUVs preparation: electroformation method.[3] 20.0 μL of a 5 mM solution of phospholipid **3** containing 0.1 mol% Bodipy® FL DHPE in $CHCl_3$ were placed on the conductive side of an indium tin oxide (ITO) slide. The slide was then dried *in vacuo* for 1 h, after which a rubber O-ring was placed around the dried lipid film and filled with 260.0 μL of 150 mM sucrose solution. A second ITO slide was placed onto of the first

and everything assembled within a Vesicle Prep Pro® device. The giant unilamellar vesicles were electroformed with the following parameters: Frequency 5 Hz, Amplitude 3 V, Temperature 55 ºC, Rise time 10 min, Main time 120 min, Fall time 10 min. The corresponding sample was finally monitored by fluorescence and phase contrast microscopy in order to determine the giant unilamellar vesicle structure.

GUVs Remodeling: RNCL exchange

GUVs preparation: electroformation method.[3] 20.0 μL of a 5 mM solution of phospholipid **3** in $CHCl_3$ were placed on the conductive side of an indium tin oxide (ITO) slide. The slide was then dried *in vacuo* for 1 h, after which a rubber O-ring was placed around the dried lipid film and filled with 260.0 μL of 150 mM sucrose solution. A second ITO slide was placed onto of the first and everything assembled within a Vesicle Prep Pro® device. The giant unilamellar vesicles were electroformed with the following parameters: Frequency 5 Hz, Amplitude 3 V, Temperature 55 ºC, Rise time 10 min, Main time 120 min, Fall time 10 min.

RNCL exchange. 100.0 μL of previously electroformed **3** GUVs solution (Final concentration of phospholipid **3**: 400 μM) and 2.0 μL of 100 mM DTT in 150 mM NaH_2PO_4 pH 7.1 buffer were added to cysteine-modified lysolipid (N-methylated **4** or un-N-methylated **6**; 0.04 μmol), and stirred under N_2 for 30 min at rt, affording the corresponding remodeled GUVs (GUVs from equimolar mixture of N-methylated phospholipids **3** and **5** or GUVs from un-N-methylated phospholipid **7**).

LC/MS analysis. Remodeling (RNCL reaction) of phospholipid GUV membranes were performed in the appropriate buffer as described above. Aliquots of 50.0 μL were analyzed using an Eclipse Plus C8 analytical column (5% *Phase A* in *Phase B*, 5.5 min) with an Evaporative Light Scattering Detector (ELSD) at a flow of 1.0 mL/min. For all LC/MS runs, solvent *Phase A* consisted of H_2O with 0.1% formic acid and solvent *Phase B* of MeOH with 0.1% formic acid.

GUVs Remodeling: *In situ* Formation of Lipid Microdomains

GUVs preparation: electroformation method.[1] 20.0 μL of a solution of **3**:**10**:cholesterol [Final concentration of phospholipid mixture: 5 mM] in a 1:1:0.8 molar ratio containing 0.1 mol% Texas Red® DHPE in $CHCl_3$ were placed on the conductive side of an indium tin oxide (ITO) slide. The slide was then dried in vacuo for 1 h, after which a rubber O-ring was placed around the dried lipid film and filled with 260.0 μL of 150 mM sucrose solution. A second ITO slide was placed onto of the first and everything assembled within a Vesicle Prep Pro® device. The giant unilamellar vesicles were electroformed with the following parameters: Frequency 5 Hz, Amplitude 3 V, Temperature 55 ºC, Rise time 10 min, Main time 120 min, Fall time 10 min.

***In situ* formation of microdomains.** 9.0 μL of previously electroformed **3**:**10**:cholesterol GUVs (Final concentration of phospholipid mixture: 400 μM), 1.0 uL of 50 mM DTT in 150 mM NaH_2PO_4 pH 7.1 buffer and 9.0 μL of a 400 μM solution of lysolipid **6** in 150 mM NaH_2PO_4 pH 7.1 buffer were subsequently mixed. Then, 3.0 μL of the resulting solution were added to a glass microscope slide and covered with a glass coverslip supported with vacuum grease. Fluorescence microscope images of microdomain formation were acquired at different times after initial mixing on an Axio Observer Z1 inverted microscope (Carl Zeiss Microscopy GmbH, Germany) with a 60x,

1.4 NA oil immersion objective to an ORCA-Flash 4.0 camera using the manufacturers software (Hamamatsu, Japan). Texas Red® DHPE fluorophore was excited with a HXP 120 metal halide arc lamp (Carl Zeiss Microscopy GmbH, Germany).

GUVs Remodeling: Membrane curvature

Plasmid construction. Amphiphysin and epsin 1 were cloned from plasmids obtained from Addgene (Plasmid #22213)[6] and DNAsu (Plasmid ID: HsCD00403825) respectively, and inserted into SNAP-tag_EGFP_Hisx6[7] plasmid as N-terminal fusions. BL21 competent *E. coli* cells (Agilent Technologies, Santa Clara, CA) were transformed with each plasmid per the manufacturers protocol. Cells were grown in a 1 L culture at 37 ºC with constant shaking and induced with 1 mM IPTG once the culture reached 0.6 O.D. After induction, the culture was grown for 4 additional h after which *E. coli* were pelleted and stored at 4 ºC until purification. His-tag purification was performed by the manufacturer's specifications using Ni-NTA resin (Thermo, Rockford, IL). Purified protein was exchanged into 1×PBS using a 10 kDa molecular weight cutoff microcentrifuge filter and stored at -80 ºC. Final protein concentration was determined via UV-Vis analysis.

GUVs preparation: electroformation method.[1] 20.0 μL of a solution of phospholipid **3** [Final concentration of phospholipid **3**: 5 mM] containing 0.1 mol% Bodipy® FL DHPE in $CHCl_3$ were placed on the conductive side of an indium tin oxide (ITO) slide. The slide was then dried in vacuo for 1 h, after which a rubber O-ring was placed around the dried lipid film and filled with 265.0 μL of 150 mM sucrose solution. A second ITO slide was placed onto of the first and everything assembled within a Vesicle Prep Pro® device. The giant unilamellar vesicles were electroformed with the following parameters: Frequency 5 Hz, Amplitude 3 V, Temperature 55 ºC, Rise time 10 min, Main time 120 min, Fall time 10 min.

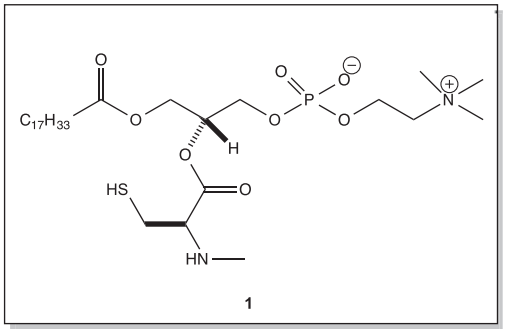
Membrane curvature experiments: remodeling with subsequent addition of protein. 9.0 μL of previously electroformed **3** GUVs (Final concentration of phospholipid **3**: 380 μM), 1.0 uL of 50 mM DTT in 150 mM NaH_2PO_4 pH 7.1 buffer and 9.0 μL of a 380 μM solution of lysolipid **15** in 150 mM NaH_2PO_4 pH 7.1 buffer were subsequently mixed. After incubation during 2 h, 9.0 μL of the resulting solution were added to 1.0 μL of protein (*Amphiphysin:* 728 μM solution, final concentration: 72.8 μM; *Epsin 1:* 27.7 μM solution, final concentration: 2.8 μM). Fluorescence microscope images of membrane curvature events were acquired at different times after initial mixing on an Axio Observer Z1 inverted microscope (Carl Zeiss Microscopy GmbH, Germany) with a 60x, 1.4 NA oil immersion objective to an ORCA-Flash 4.0 camera using the manufacturers software (Hamamatsu, Japan). Bodipy® FL DHPE fluorophore was excited with a HXP 120 metal halide arc lamp (Carl Zeiss Microscopy GmbH, Germany).

Membrane curvature experiments: without remodeling but with subsequent addition of protein. (*Control*). 9.0 μL of previously electroformed **3** GUVs (Final concentration of phospholipid **3**: 380 μM), 1.0 uL of 50 mM DTT in 150 mM NaH_2PO_4 pH 7.1 buffer and 9.0 μL of 150 mM NaH_2PO_4 pH 7.1 buffer were subsequently mixed. After incubation during 2 h, 9.0 μL of the resulting solution were added to 1.0 μL of protein (*Amphiphysin:* 728 μM solution, final concentration: 72.8 μM; *Epsin 1:* 27.7 μM solution, final concentration: 2.8 μM) or H_2O (*Control*). Fluorescence microscope images of membrane curvature events were acquired at different times after initial mixing on an

Axio Observer Z1 inverted microscope (Carl Zeiss Microscopy GmbH, Germany) with a 60x, 1.4 NA oil immersion objective to an ORCA-Flash 4.0 camera using the manufacturers software (Hamamatsu, Japan). Bodipy® FL DHPE fluorophore was excited with a HXP 120 metal halide arc lamp (Carl Zeiss Microscopy GmbH, Germany). No observable membrane curvature events were detected.

Membrane curvature experiments: remodeling without subsequent addition of protein (*Control*). 9.0 μL of previously electroformed **3** GUVs (Final concentration of phospholipid **3**: 380 μM), 1.0 uL of 50 mM DTT in 150 mM NaH_2PO_4 pH 7.1 buffer and 9.0 μL of a 380 μM solution of lysolipid **15** in 150 mM NaH_2PO_4 pH 7.1 buffer were subsequently mixed. After incubation during 2 h, 9.0 μL of the resulting solution were added to 1.0 μL of H_2O. Fluorescence microscope images were acquired at different times after initial mixing on an Axio Observer Z1 inverted microscope (Carl Zeiss Microscopy GmbH, Germany) with a 60x, 1.4 NA oil immersion objective to an ORCA-Flash 4.0 camera using the manufacturers software (Hamamatsu, Japan). Bodipy® FL DHPE fluorophore was excited with a HXP 120 metal halide arc lamp (Carl Zeiss Microscopy GmbH, Germany). No observable membrane curvature events were detected.

NMR SPECTRA



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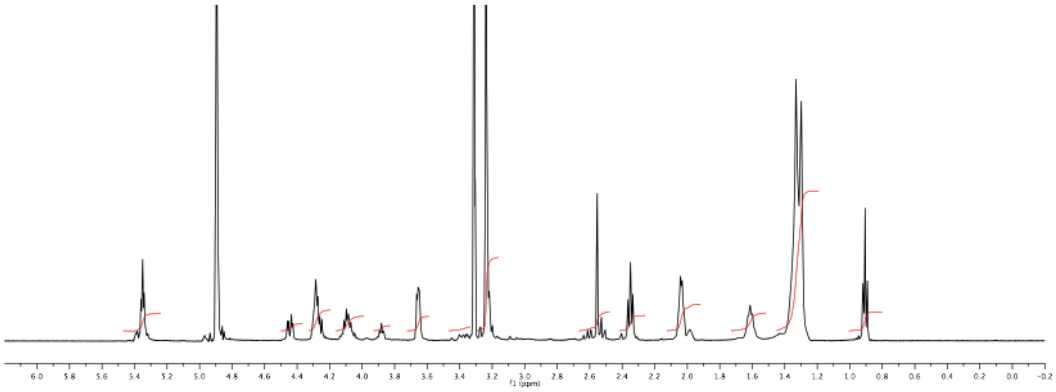

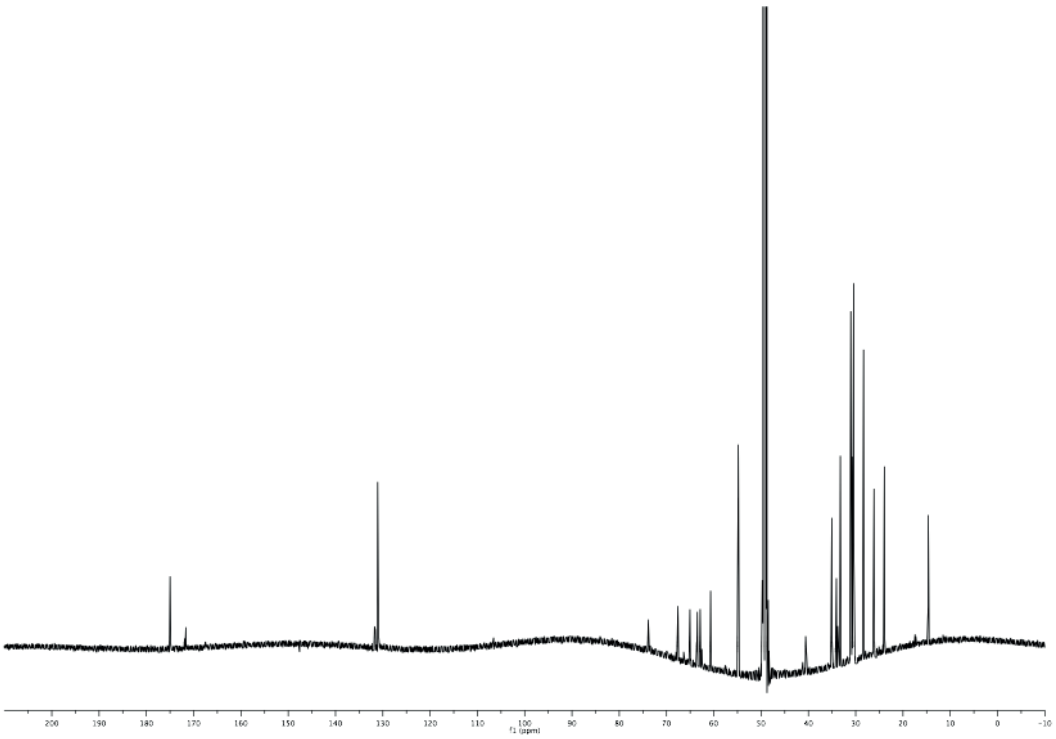

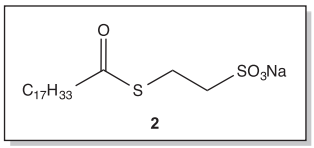

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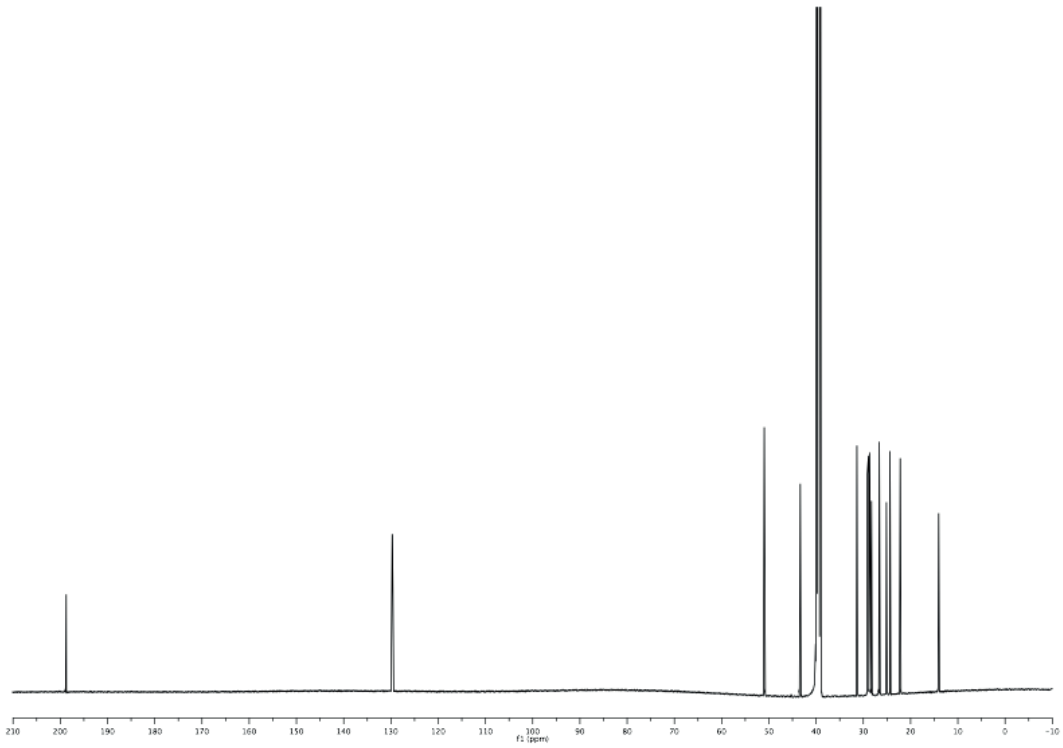

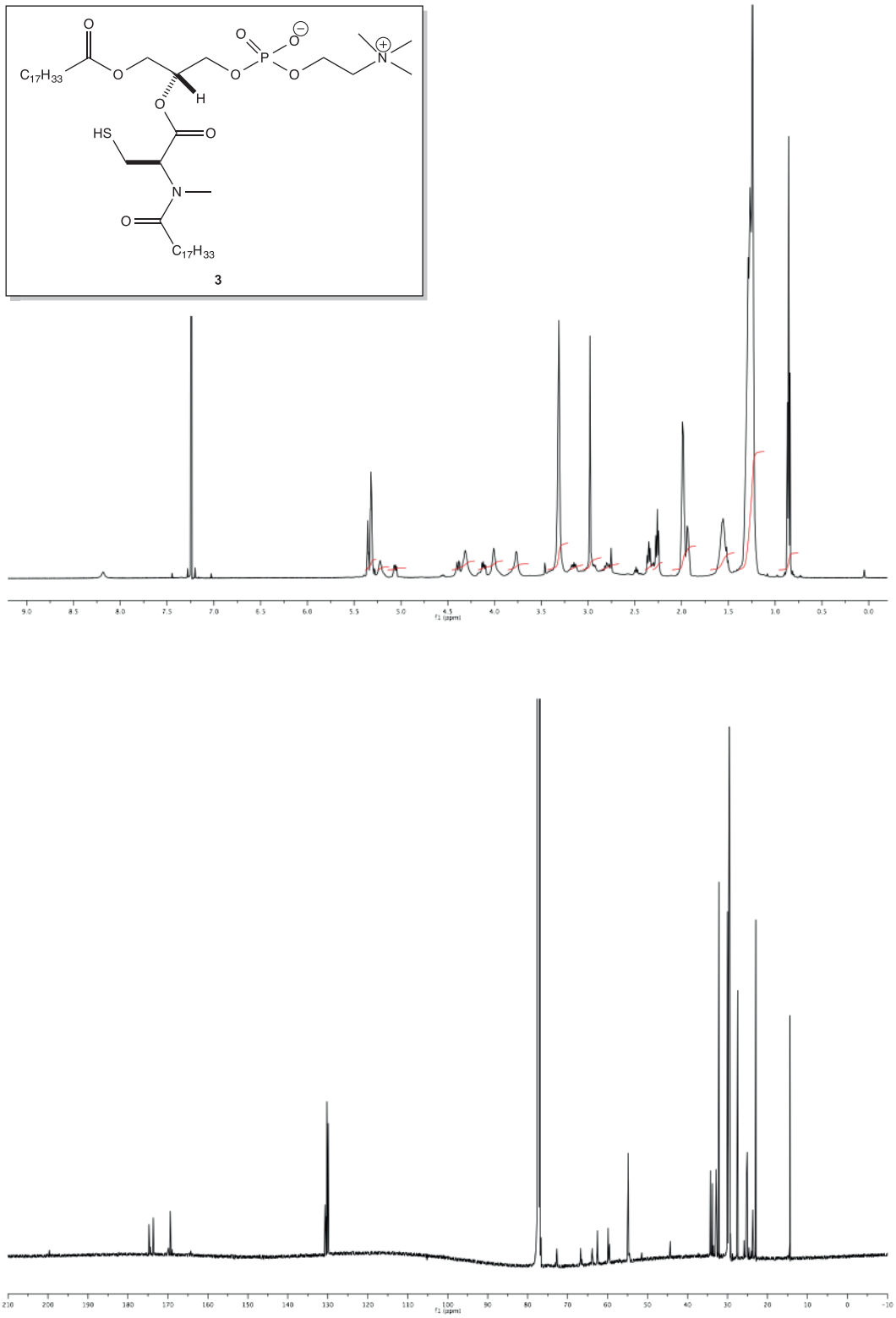
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C17H33
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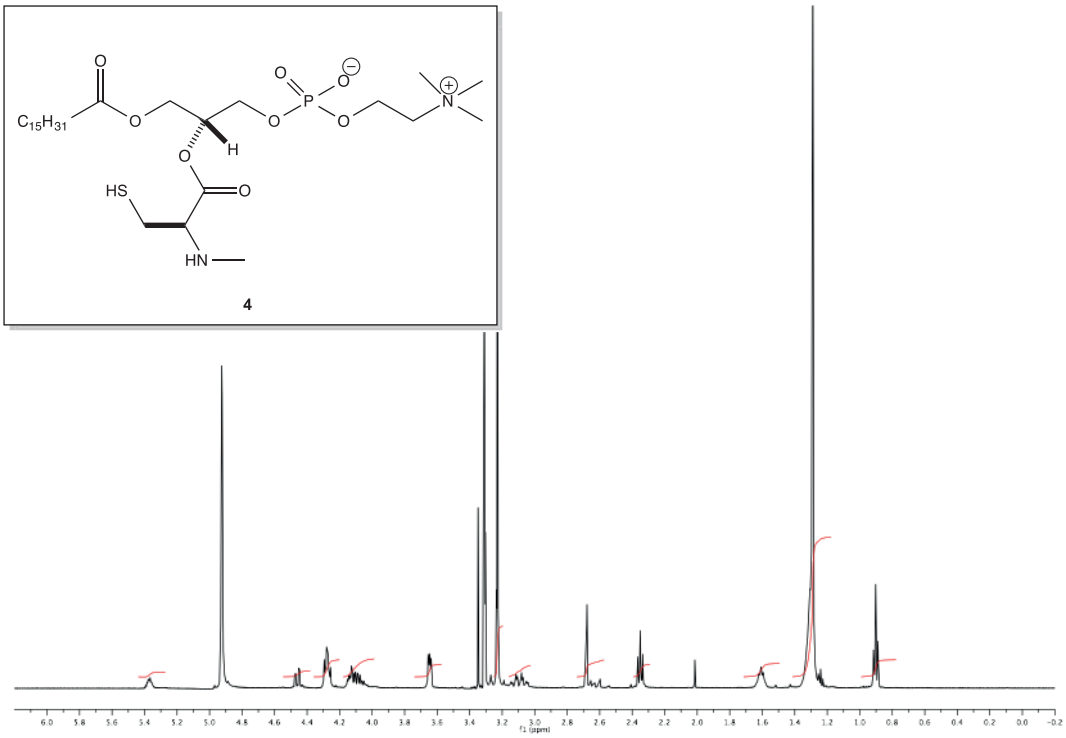
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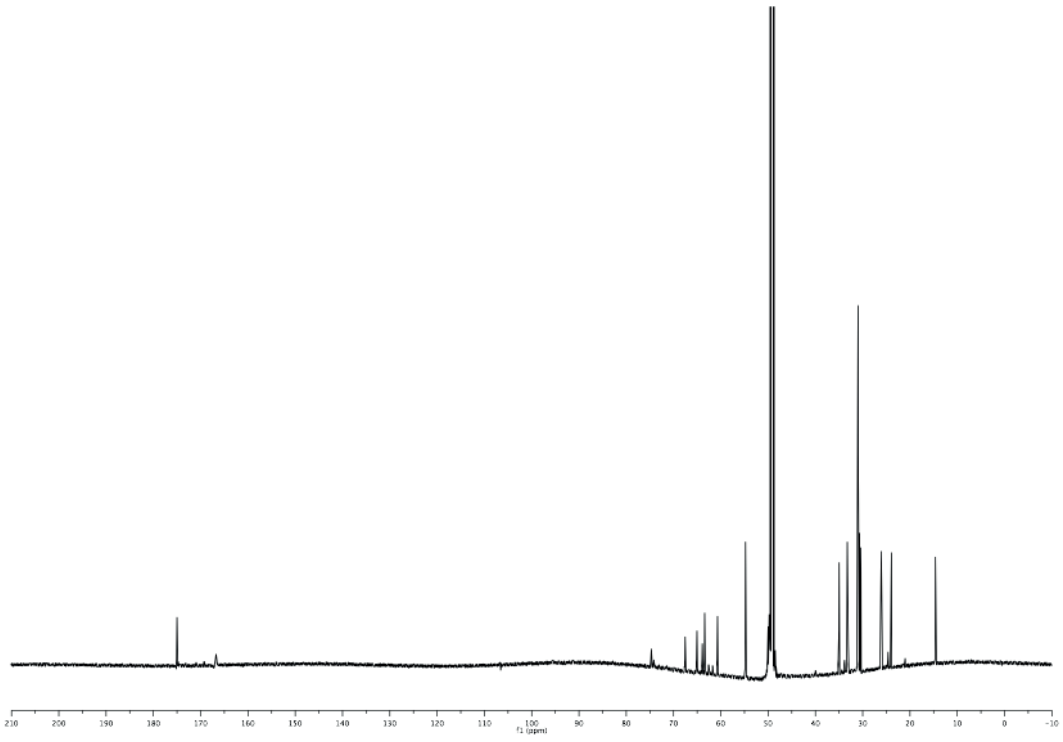

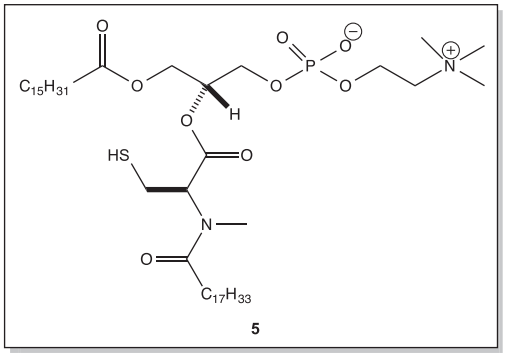

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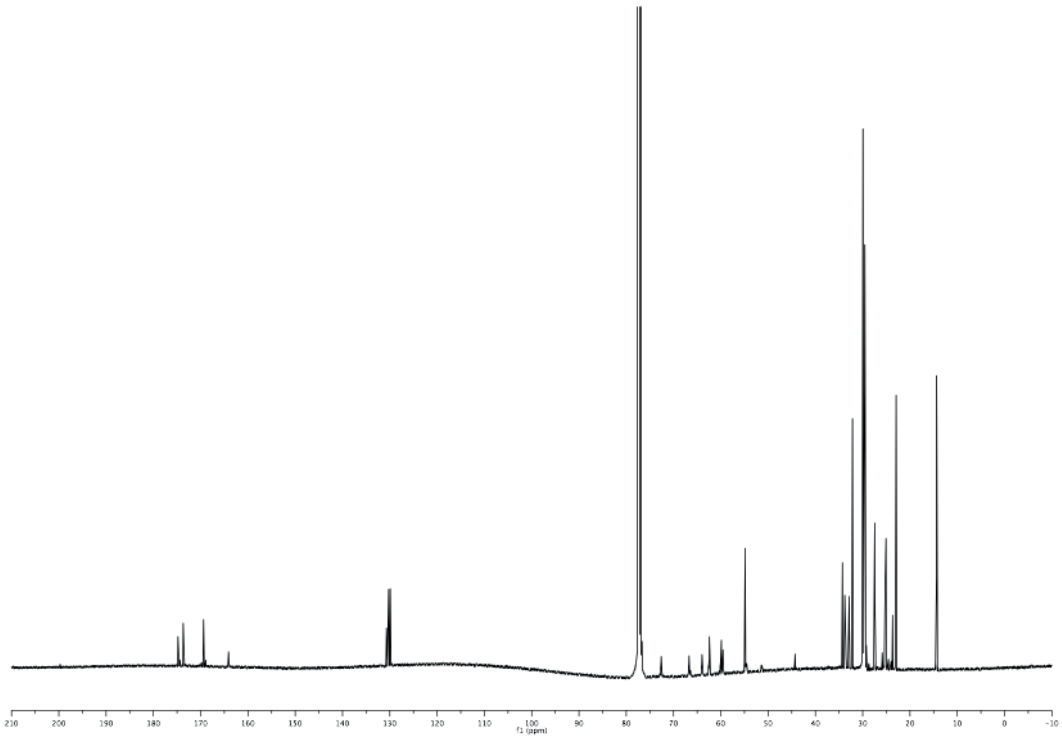

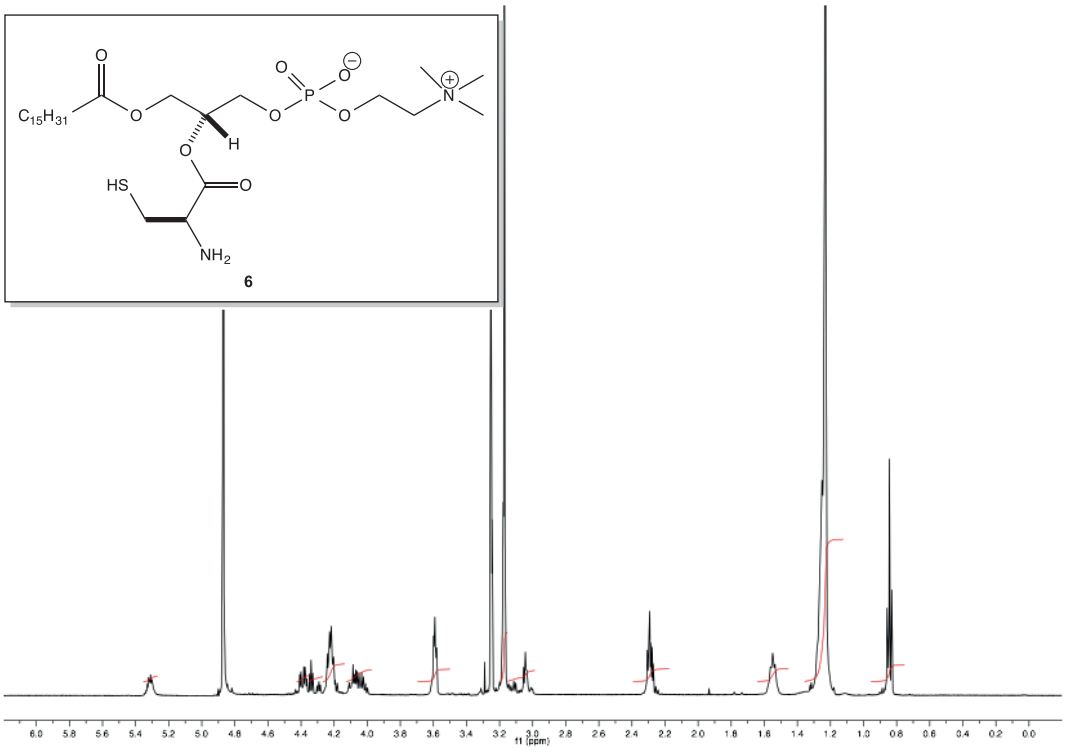
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H
HS
O
NH2
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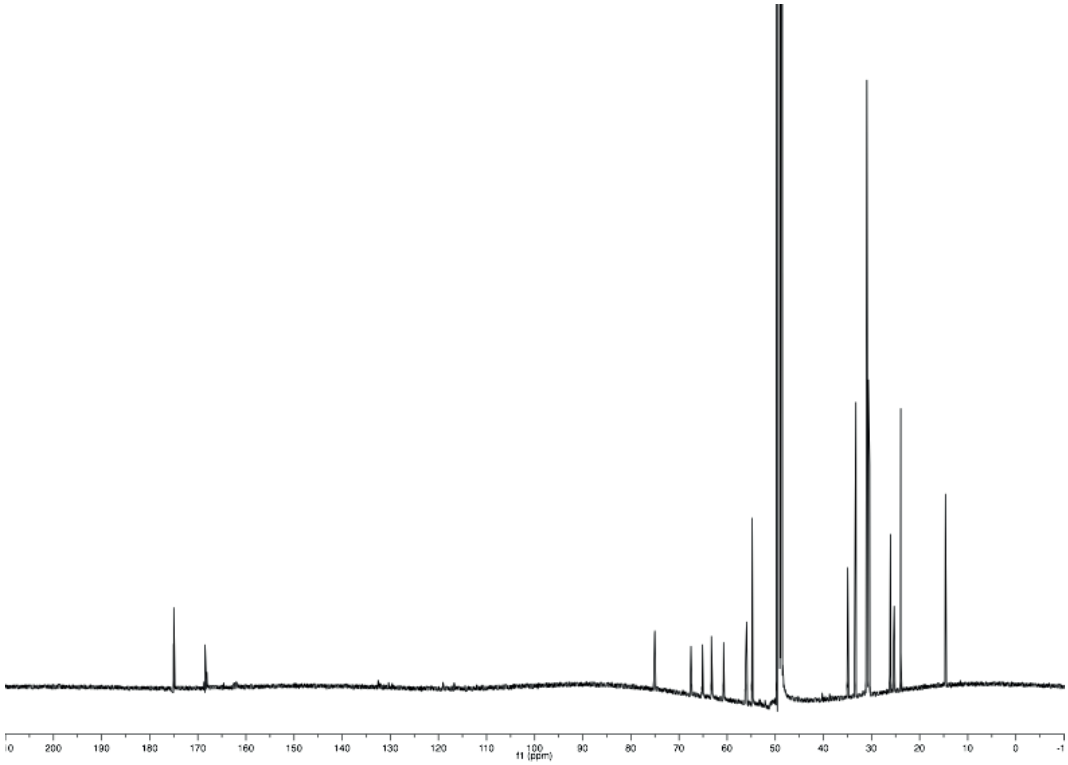
f1 (ppm)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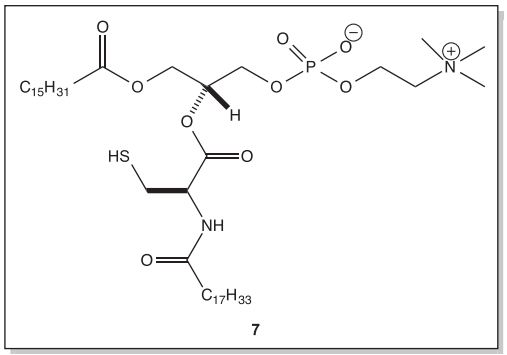
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HS
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NH
O
C17H33

7

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7.5
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5.5
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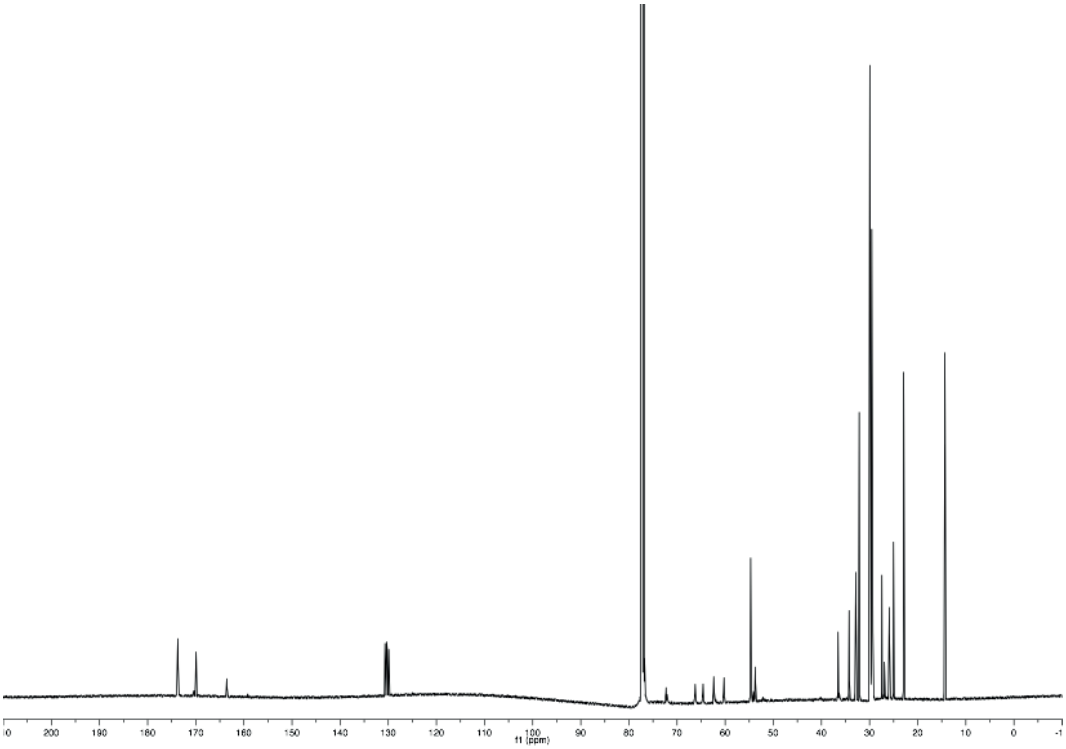
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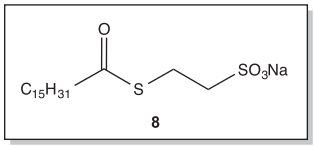

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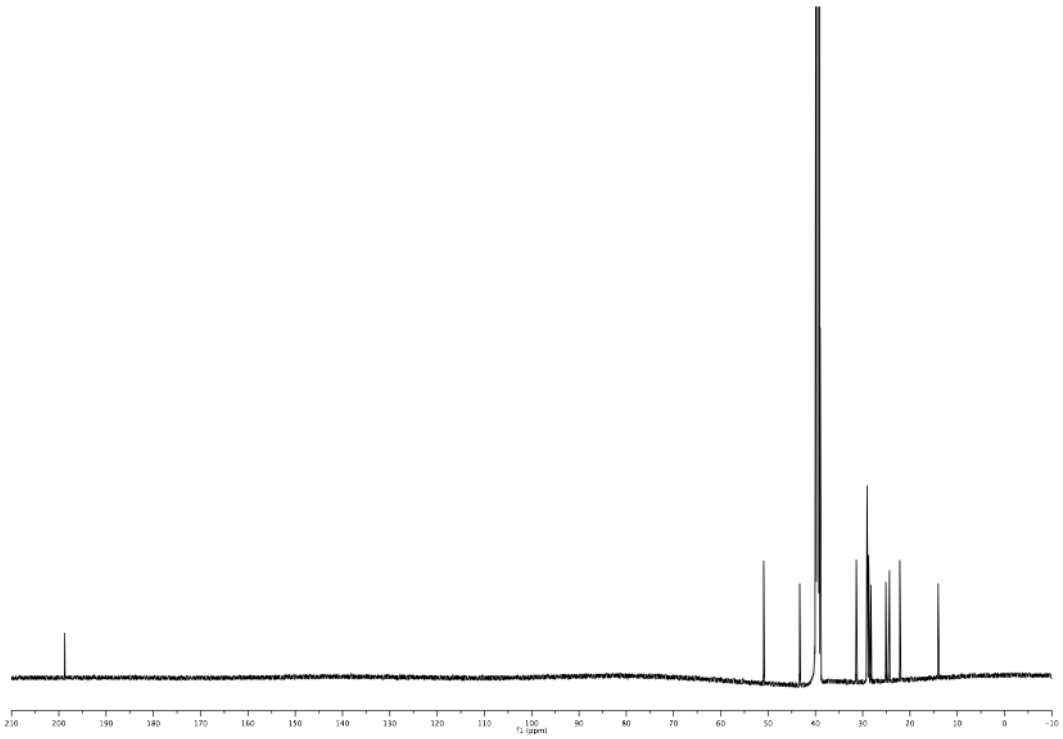

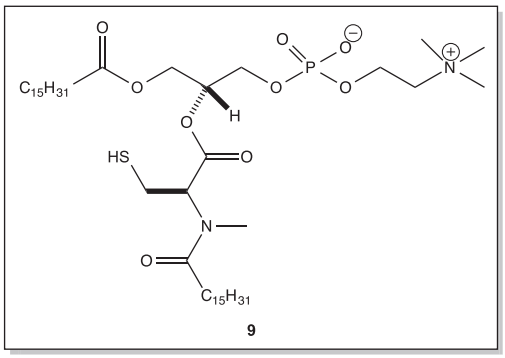
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C15H31

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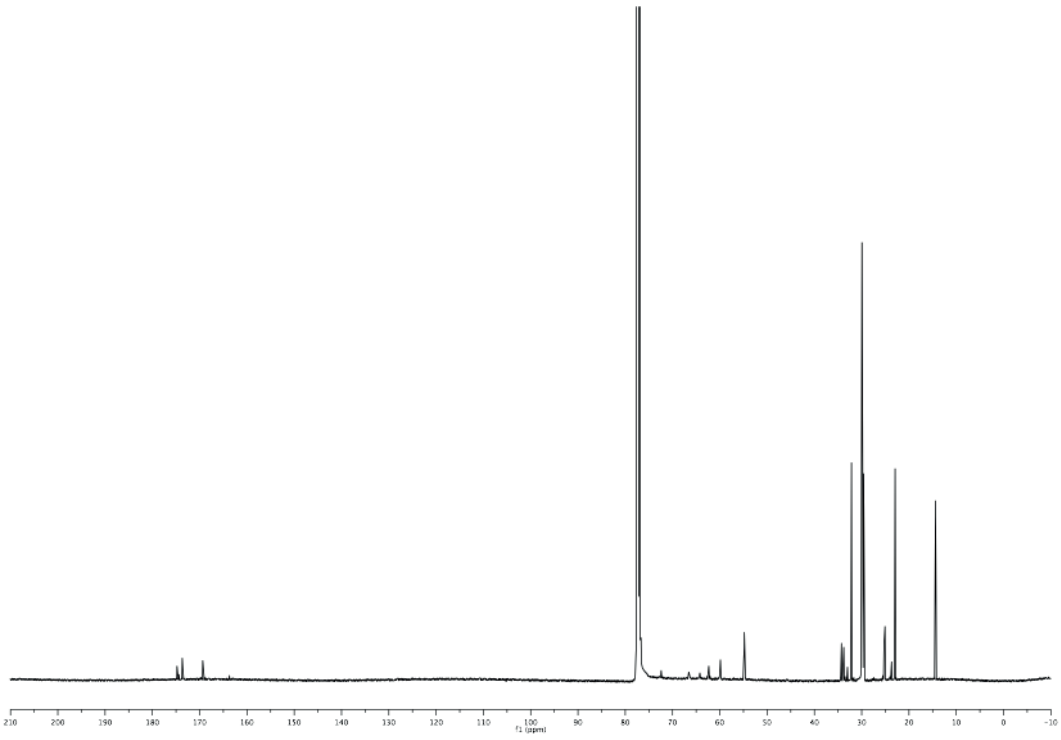
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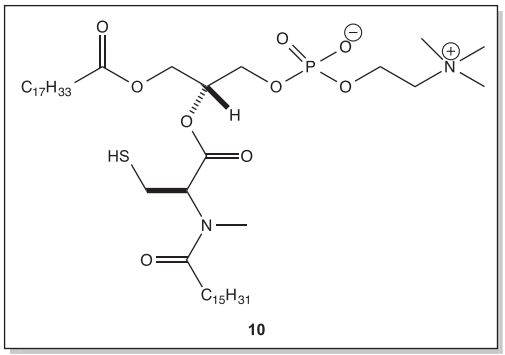

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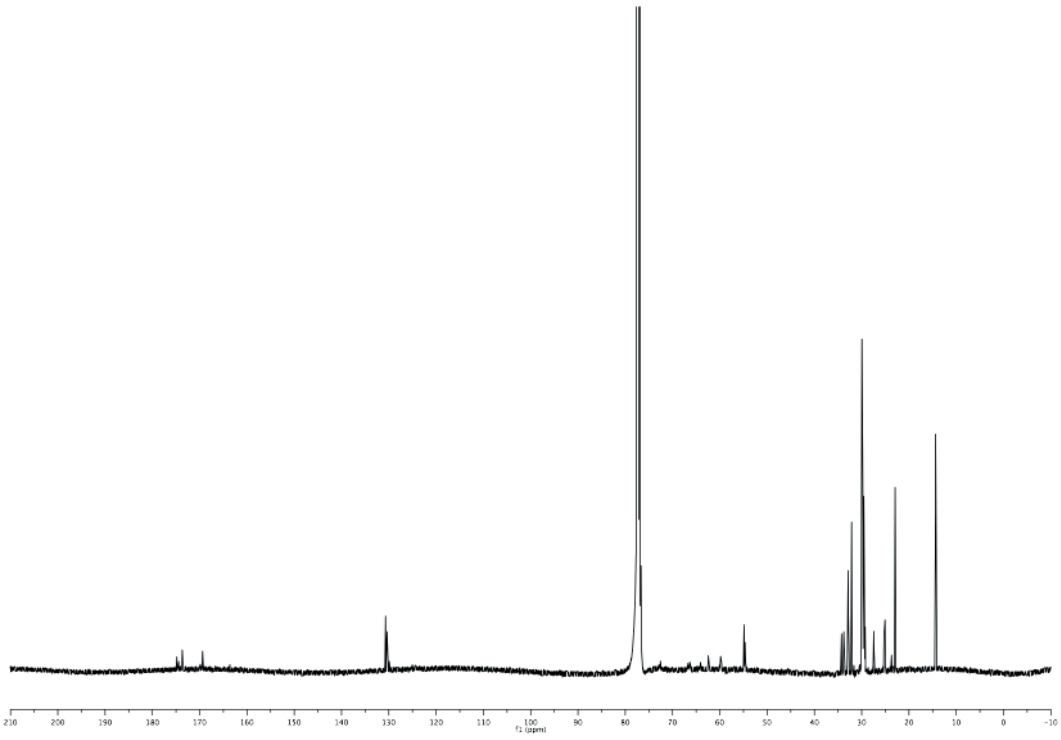

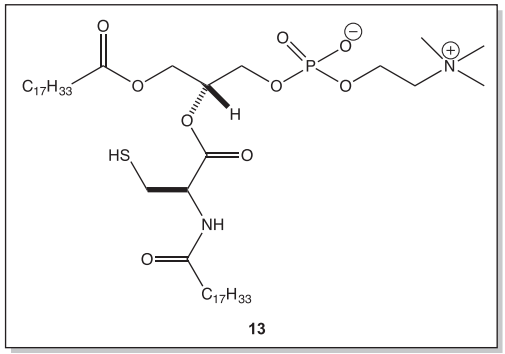
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HS
O
NH
O
C17H33

13

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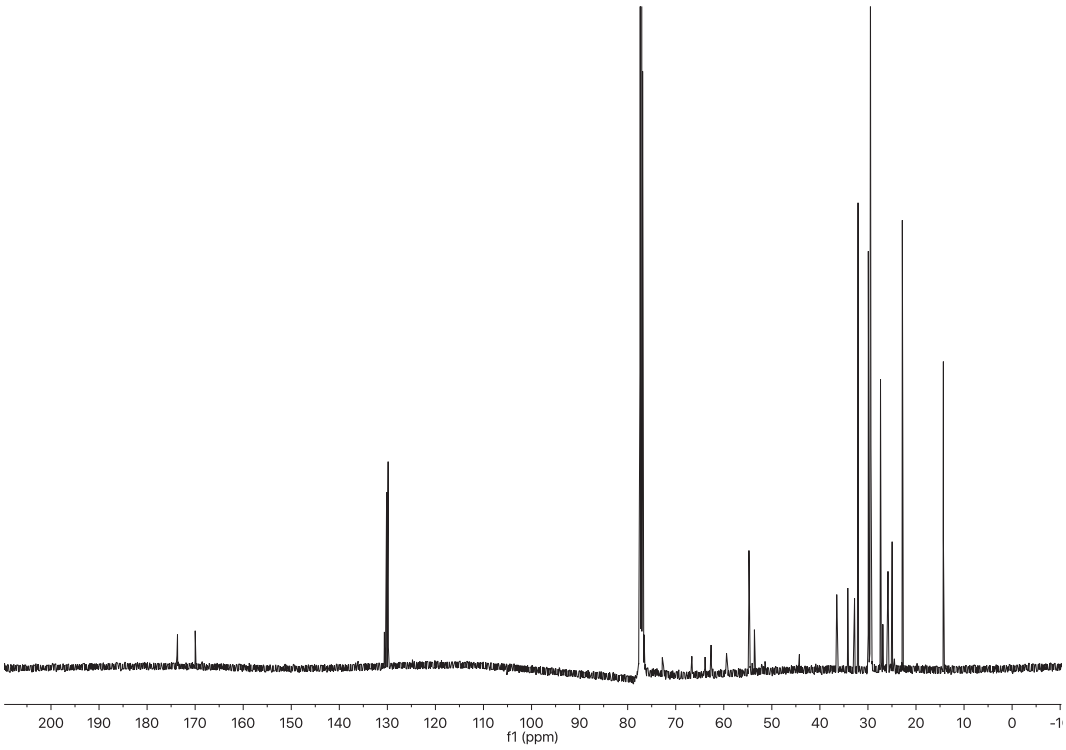
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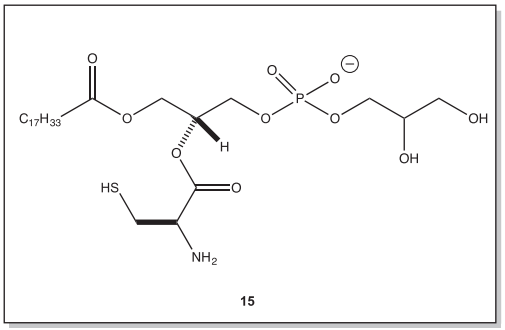

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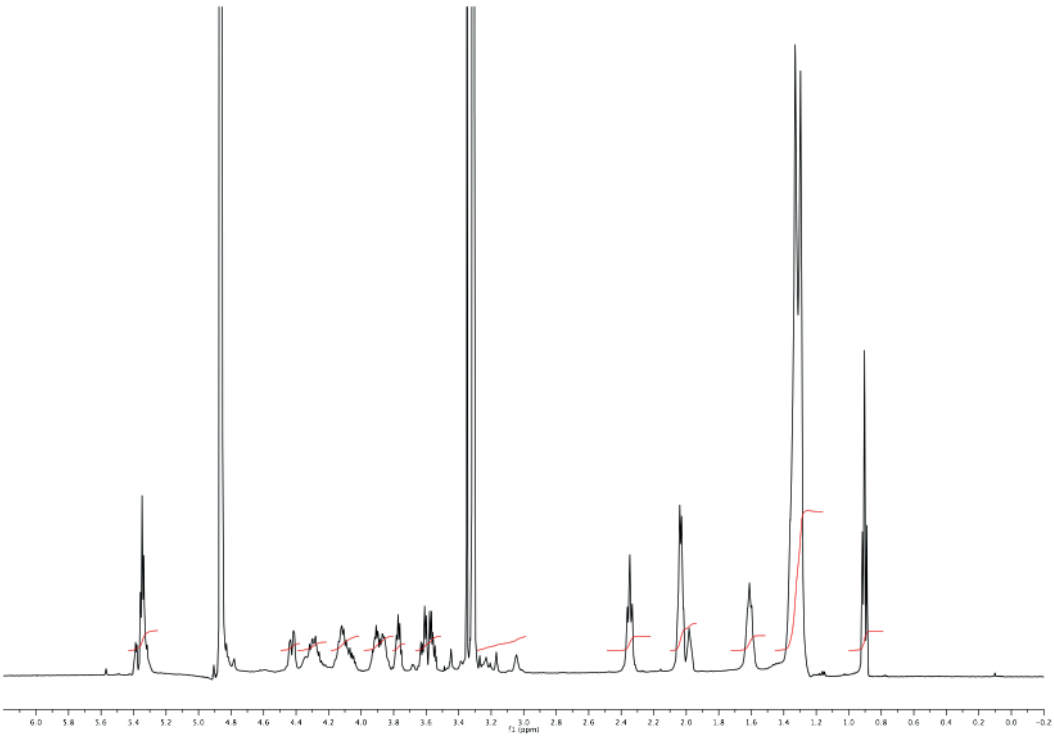

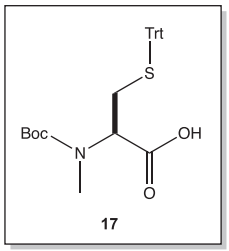

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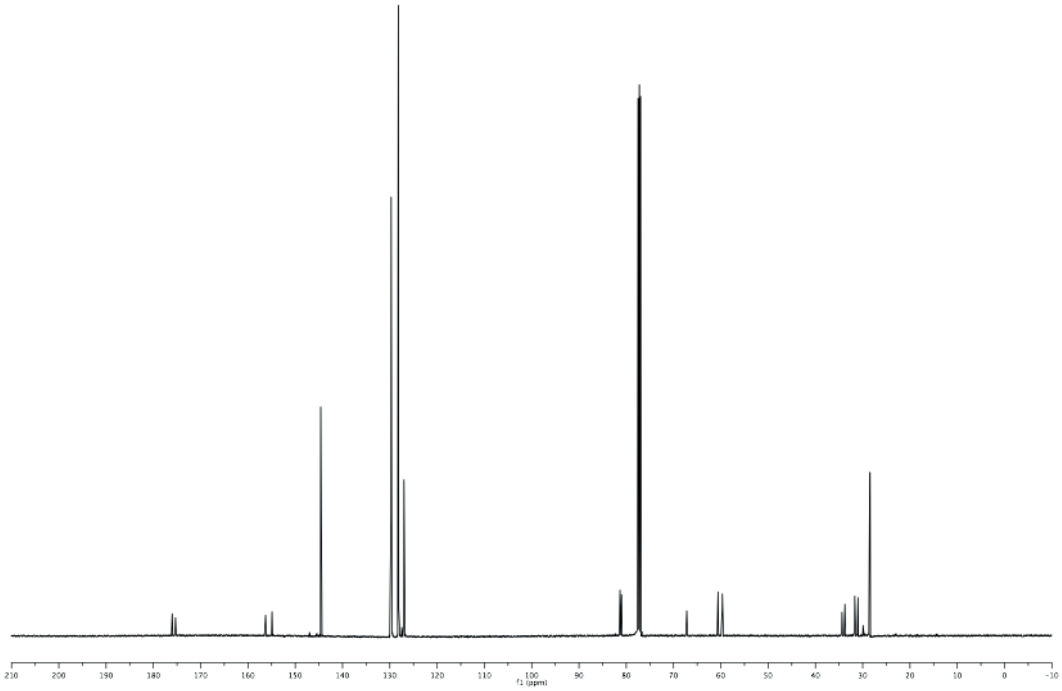

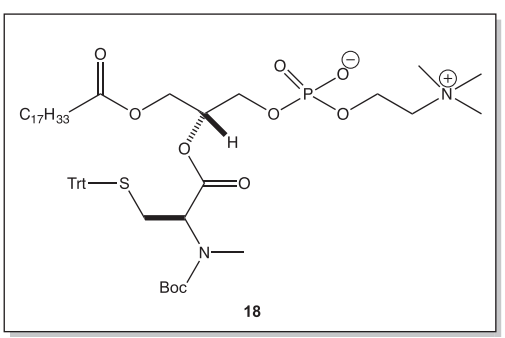

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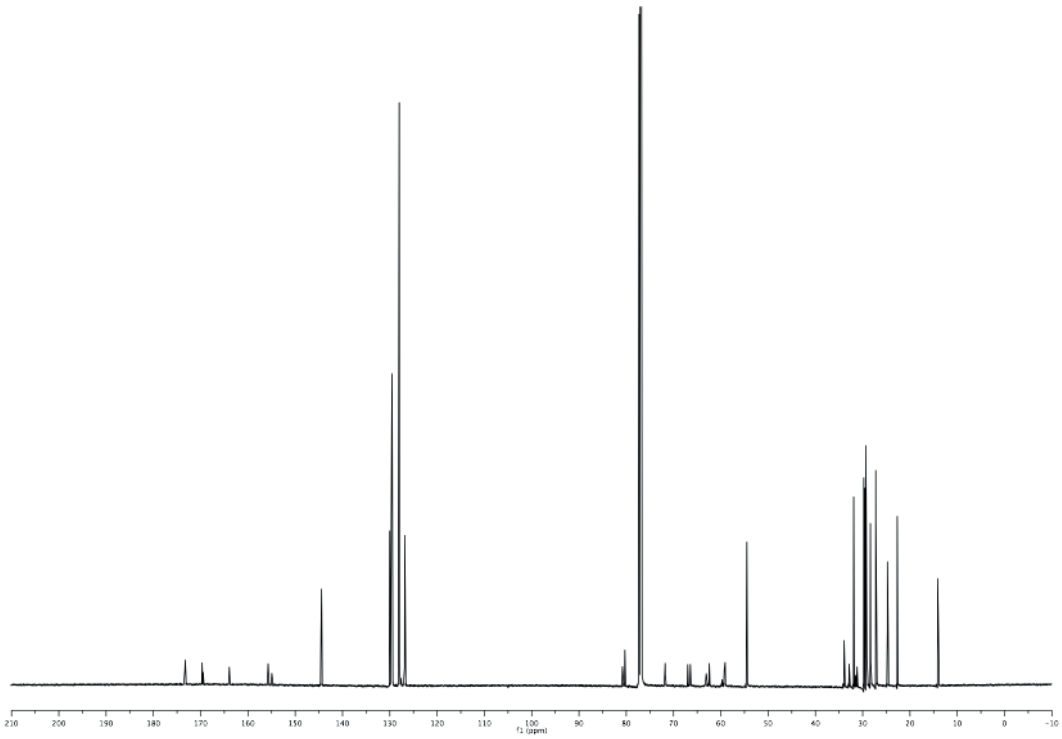

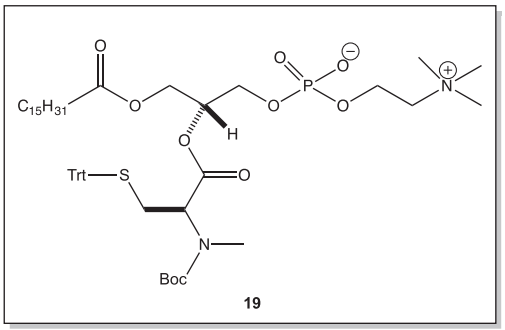

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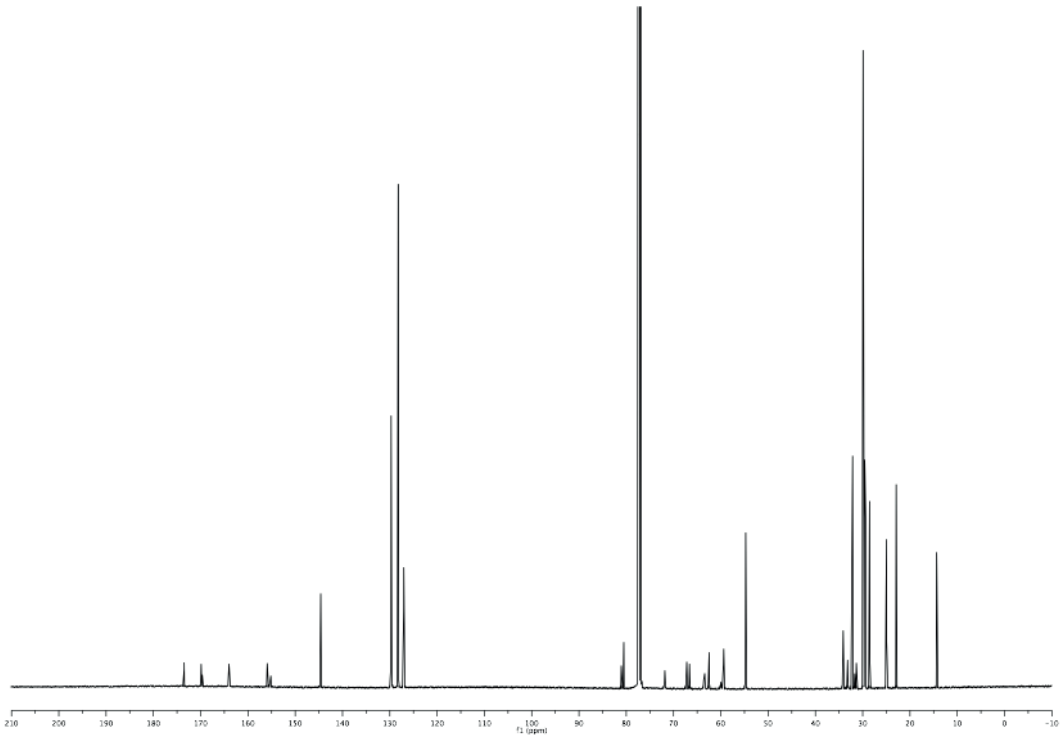

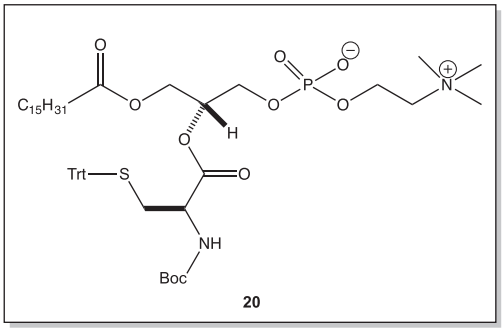
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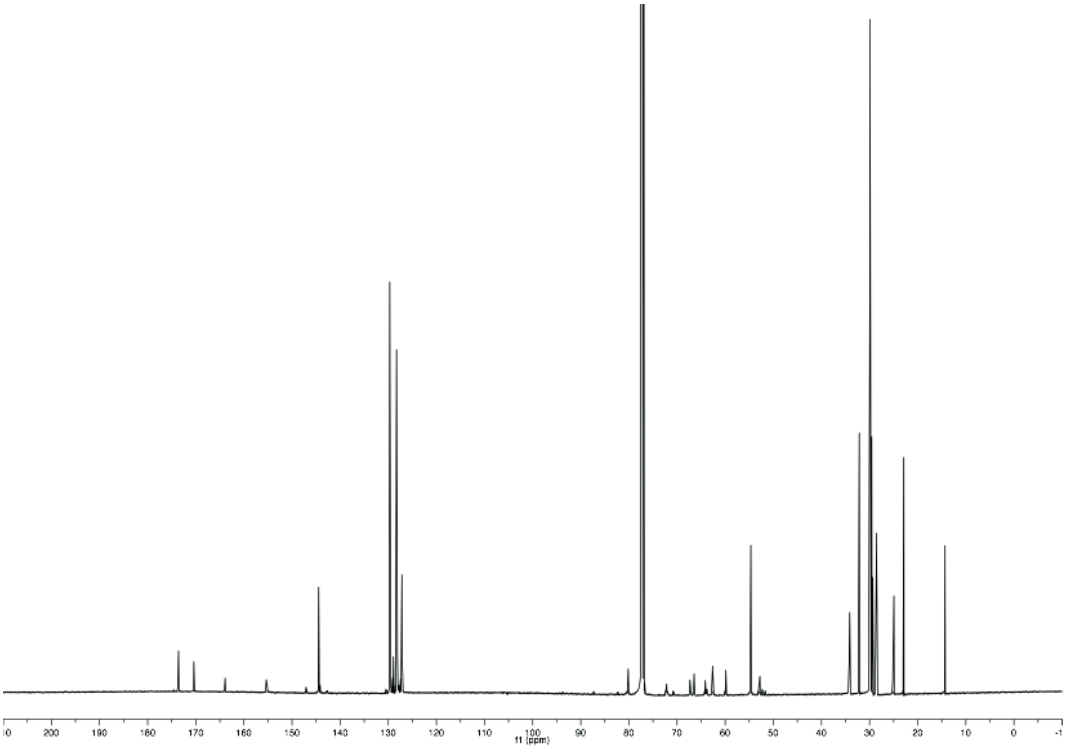
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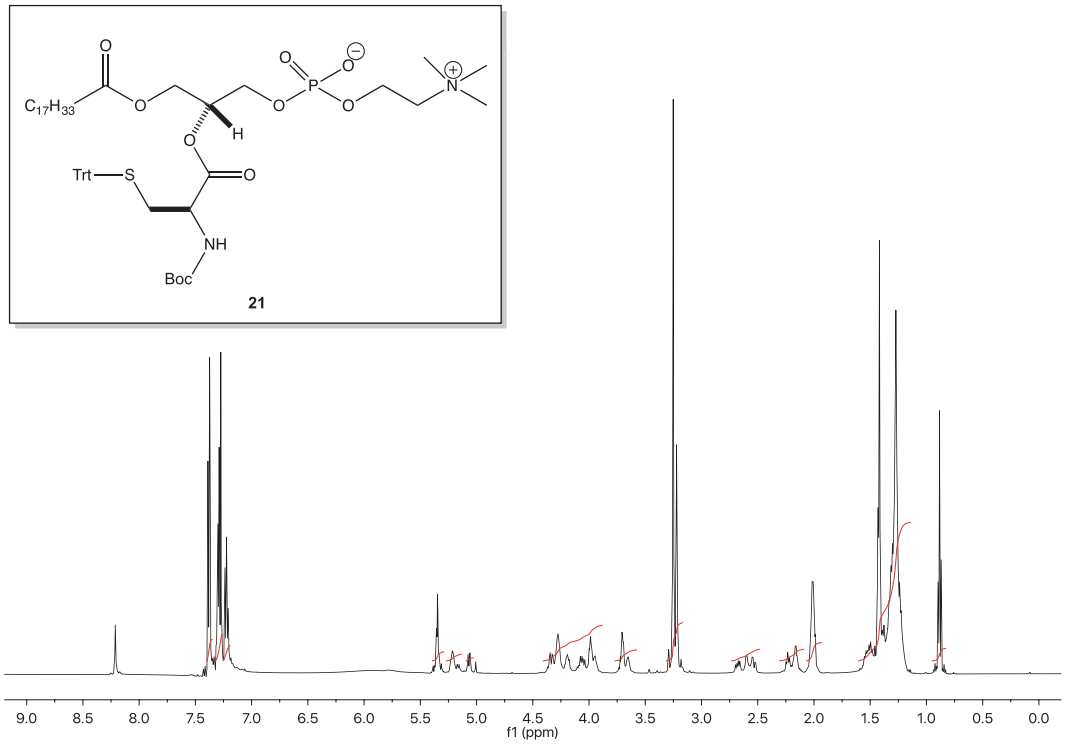
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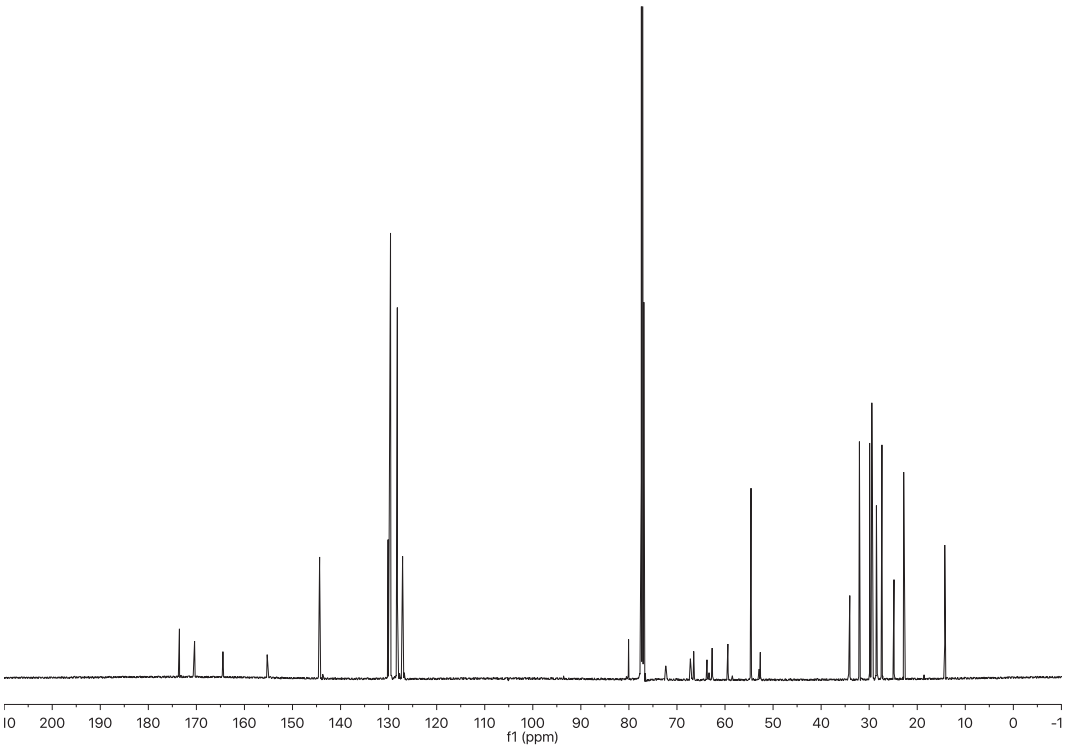
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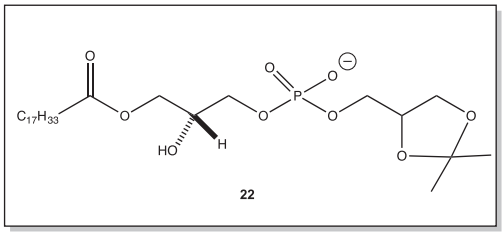
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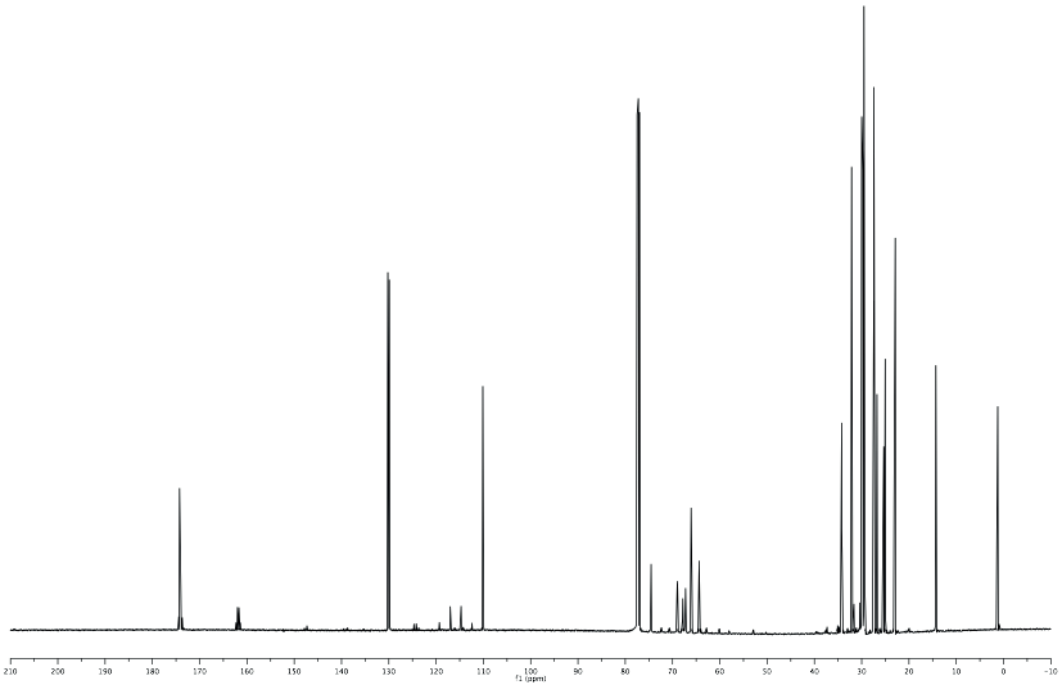
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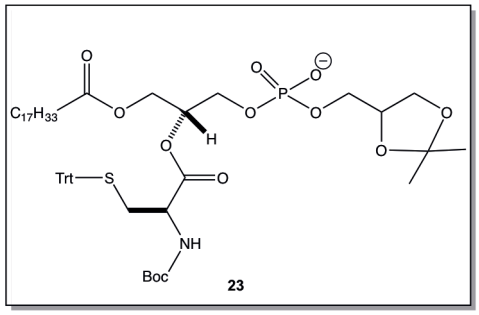

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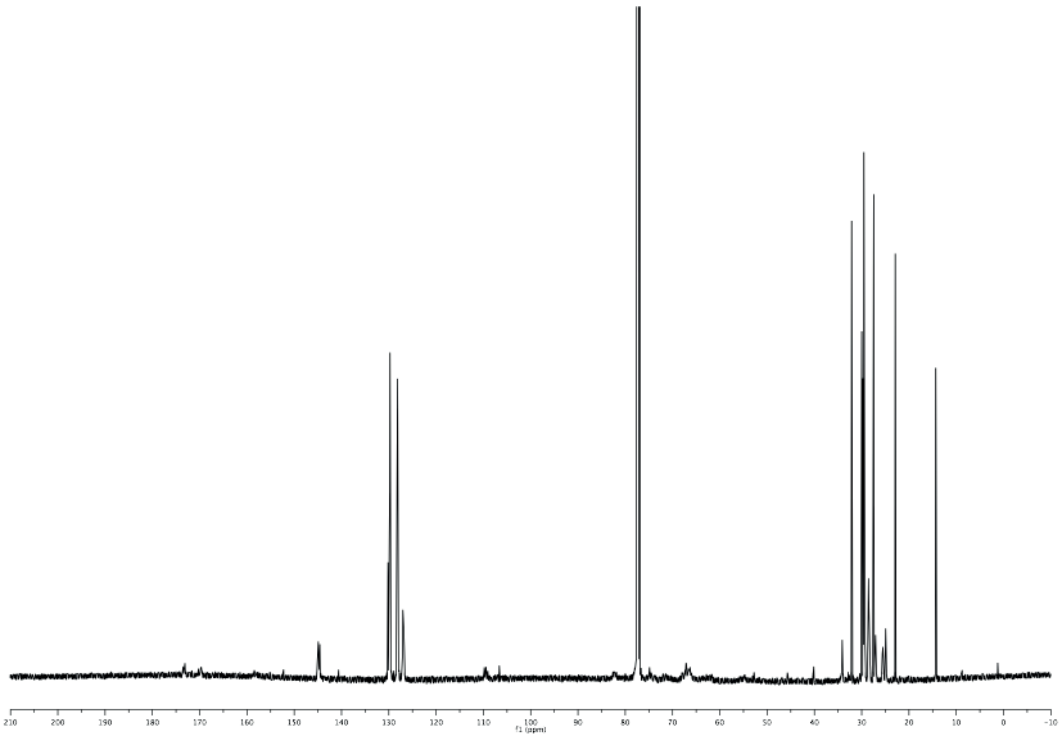


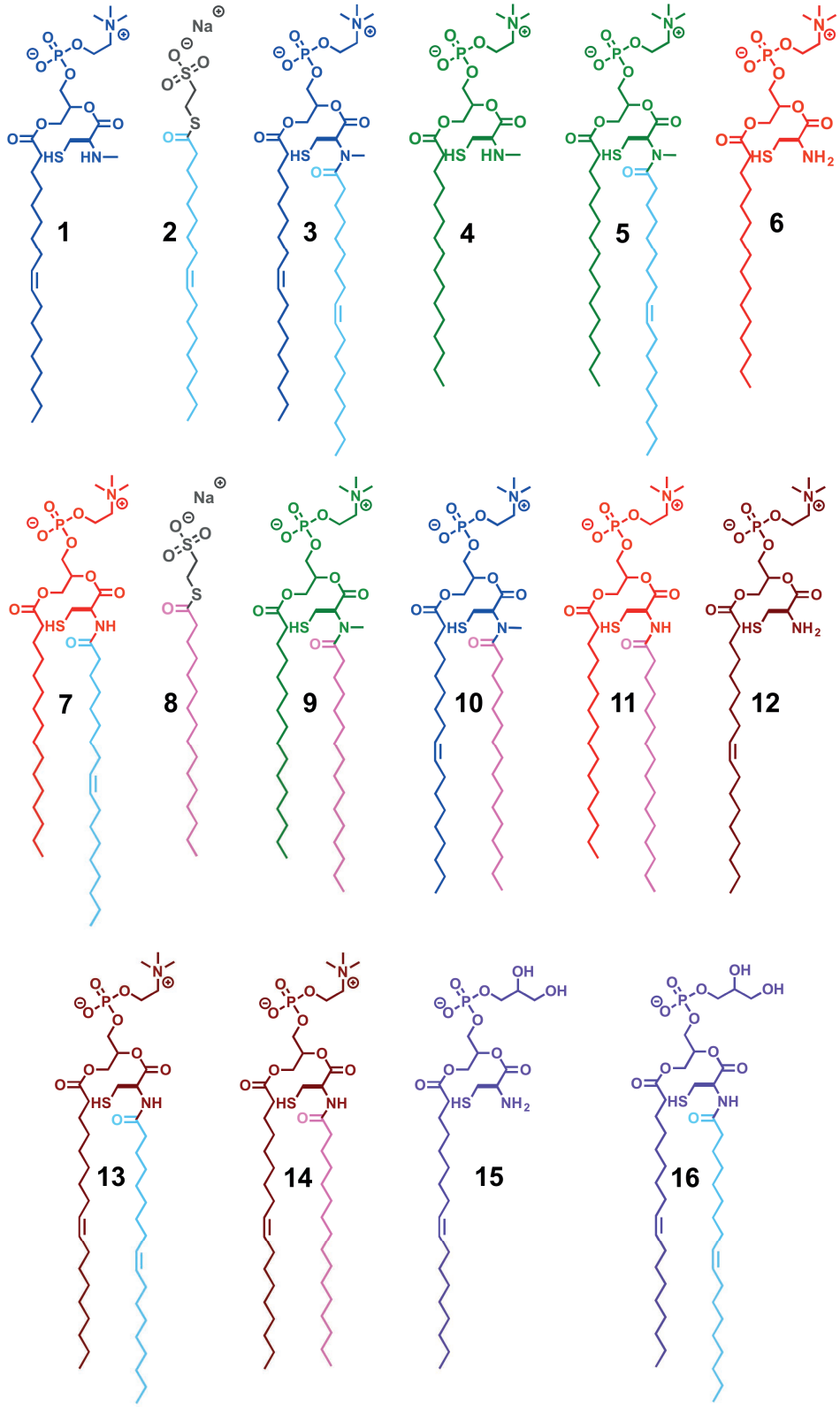


Fig. S1

Chemical structures of all the lipids used in this study.

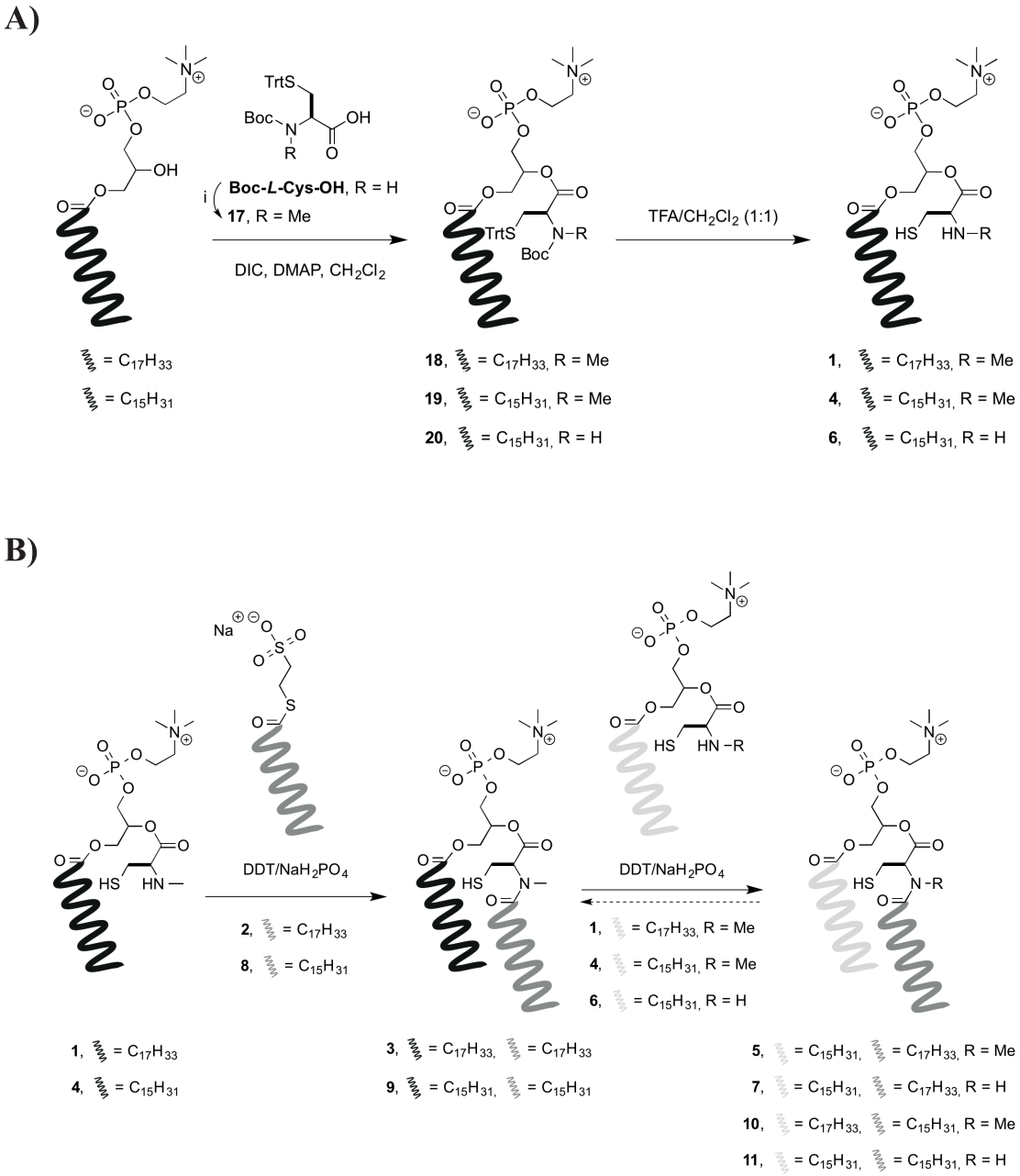


Fig. S2

De novo formation and remodeling of phospholipid membranes. **A)** Synthesis of N-methylated and un-N-methylated cysteine-functionalized lysolipids. **B)** *De novo* synthesis of phospholipids by NCL reaction of an N-methylated cysteine-functionalized lysolipid (**1** or **4**) and MESNA acyl thioester (oleoyl **2** or palmitoyl **8**), followed by subsequent remodeling of the corresponding phospholipids (**3** or **9**) by RNCL in the presence of another cysteine-functionalized lysolipid (N-methylated **4** or **1**; un-N-methylated **6**).

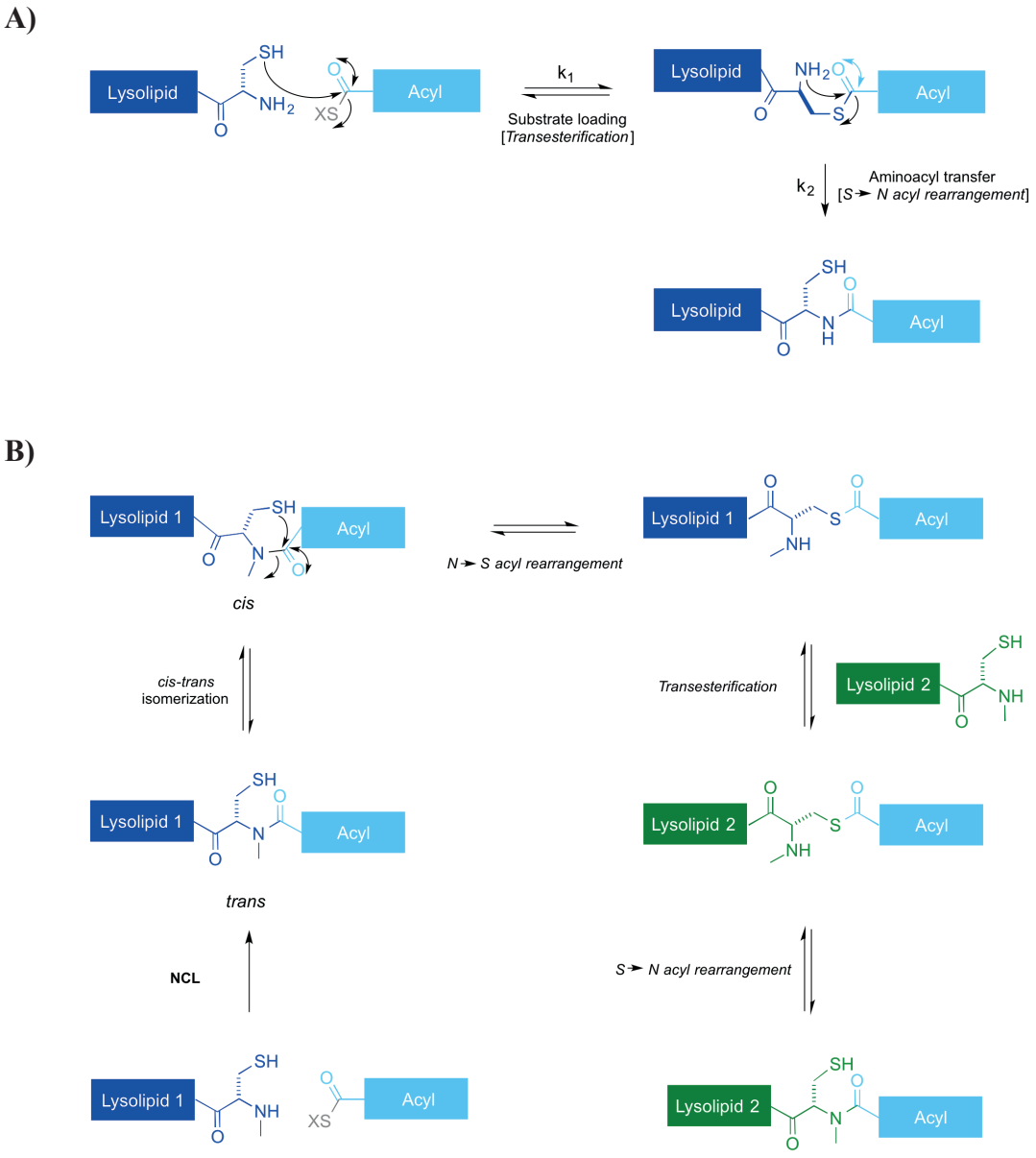


Fig. S3

Reaction mechanisms of NCL and RNCL. **A)** Mechanism of NCL. The mechanism involves a two-step process consisting of a thiol-exchange step between a C-terminal acyl thioester and the sulfhydryl moiety of an N-terminal cysteine residue in a lysolipid, which prompts an intramolecular nucleophilic attack by the α-amino group of the cysteine (S→N acyl rearrangement) to form the final amide bond. **B)** Mechanism of RNCL. The mechanism involves a sequence of *cis-trans* isomerization, N→S acyl rearrangement, transthioesterification, and S→N acyl shift.

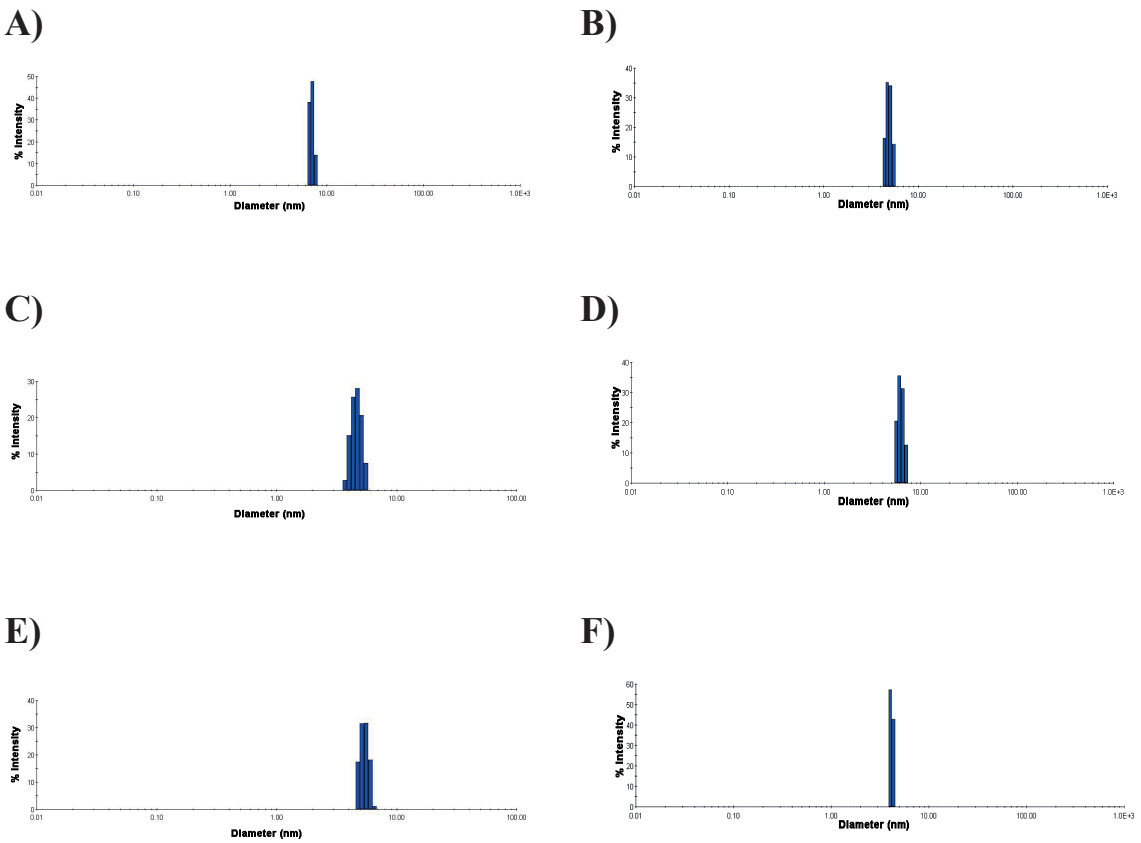


Fig. S4

Dynamic Light Scattering corresponding to 1.0 mM aqueous solution of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine **Lyso C_{16} PC-OH** (**A**), 1-oleoyl-2-(*L*-^{Me}N-Cys)-*sn*-glycero-3-phosphocholine **1** (**B**), MESNA thiooleate **2** (**C**), 1-palmitoyl-2-(*L*-^{Me}N-Cys)-*sn*-glycero-3-phosphocholine **4** (**D**), 1-palmitoyl-2-(*L*-Cys)-*sn*-glycero-3-phosphocholine **6** (**E**), and MESNA thiopalmitate **8** (**F**). Lysolipids show critical micelle concentrations (cmc's) below 100 μM, whereas MESNA thiooesters present cmc's below 10 μM.[8]

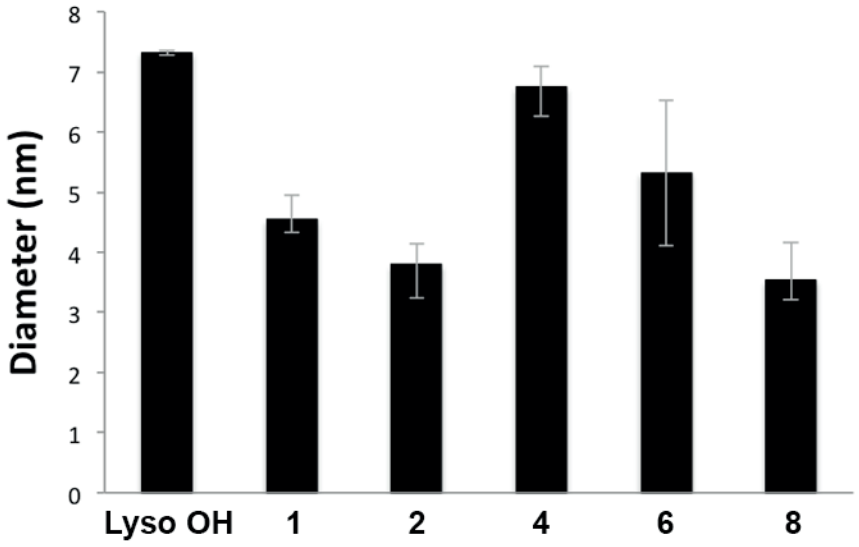


Fig. S5

Micelle sizes estimated from DLS studies corresponding to 1.0 mM aqueous solution of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine **Lyso C_{16} PC-OH** (7.32 nm in diameter), 1-oleoyl-2-(^{Me}N- *L*-Cys)-*sn*-glycero-3-phosphocholine **1** (4.54 in diameter), MESNA thiooleate **2** (3.79 nm in diameter), 1-palmitoyl-2-(*L*-^{Me}N-Cys)-*sn*-glycero-3-phosphocholine **4** (6.74 nm), 1-palmitoyl-2-(*L*-Cys)-*sn*-glycero-3-phosphocholine **6** (5.32 nm), and MESNA thiopalmitate **8** (3.53 nm in diameter).

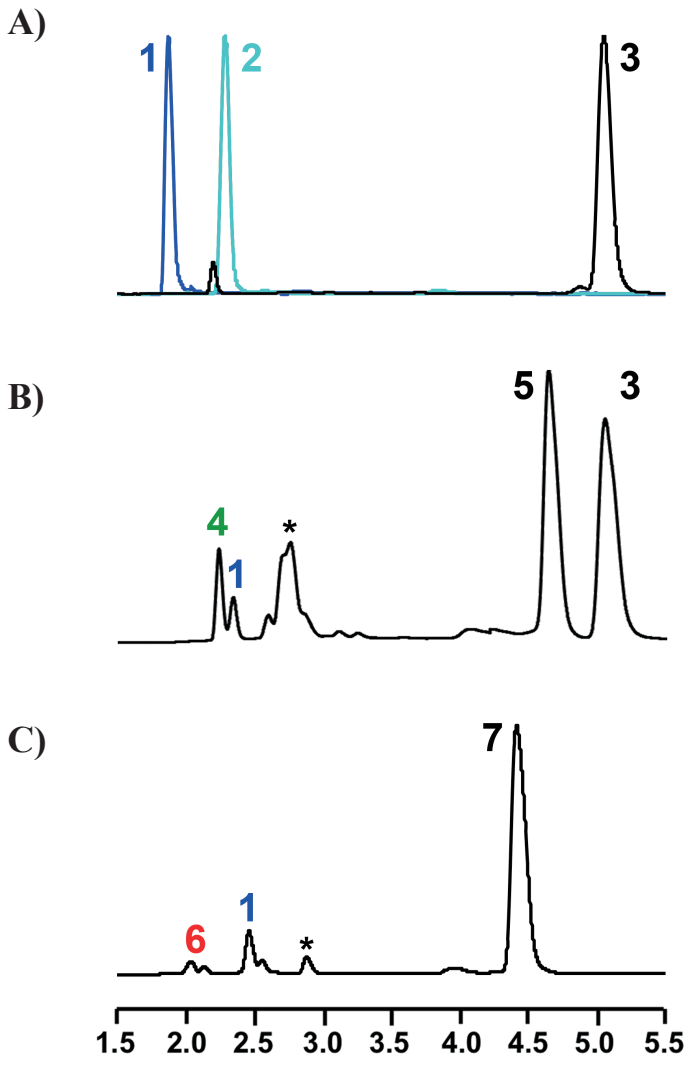


Fig. S6

HPLC/ELSD spectra monitoring the *de novo* phospholipid membrane formation and subsequent remodeling driven by non-enzymatic reactions. **A)** ELSD spectra showing the progress of the *in situ* phospholipid formation of **3** by NCL reaction. Addition of MESNA thioester **2** to the cysteine-based lysolipid **1** in 25 mM DTT in 200 mM NaH_2PO_4 pH 7.1 buffer immediately led to amidophospholipid formation, and this process progressed to near completion over a period of 30 min using millimolar concentrations of reactants. **B)** ELSD spectra corresponding to the RNCL exchange reaction between the phospholipid **3** and the N-methylated cysteine-functionalized lysolipid **4**, leading to an equimolar mixture of **3** and **5**. **C)** ELSD spectra corresponding to the RNCL exchange reaction between the phospholipid **3** and cysteine-functionalized lysolipid **6**, leading to the selective formation of **7**. Retention times (R_t's) for lysolipids (**1**, **4** and **6**), MESNA oleoyl thioester (**2**) and phospholipids (**3, 5** and **7**) were verified by mass spectrometry. Extra peaks (*) correspond with oleoyl-DTT intermediate and/or side products resulting from lysolipid hydrolisys.

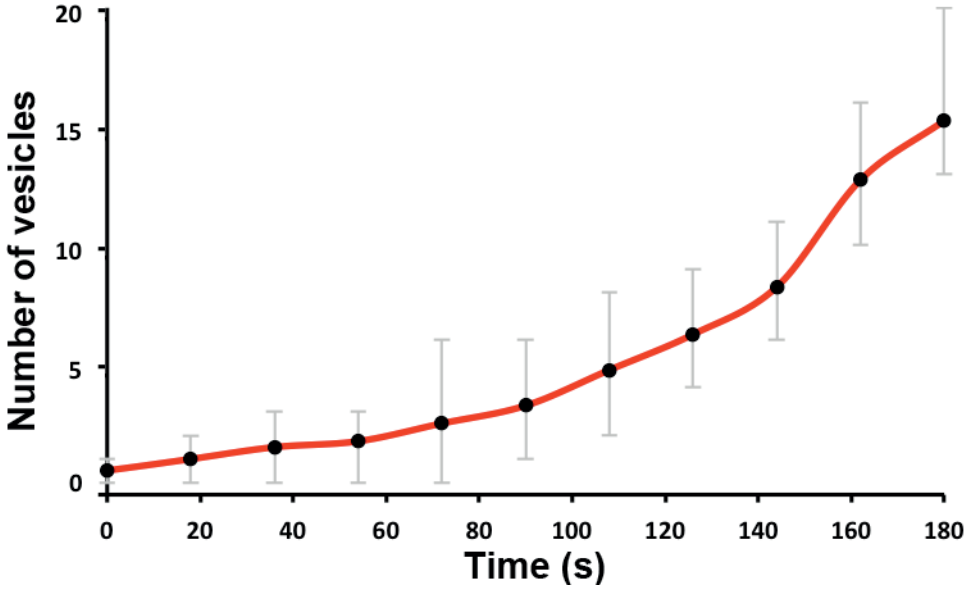


Fig. S7

Kinetics of *in situ* vesicle formation. An aqueous solution of MESNA thiooleate **2** (4 mM) was added to another aqueous solution of N-methylated cysteine-based lysolipid **1** (4 mM) and DTT (20 mM in 200 mM NaH_2PO_4 buffer pH 7.1). The population of phospholipid **3** vesicles larger than 1 μm in diameter was counted every 18 s over a period of 3 min in four different 26×26 μm regions.

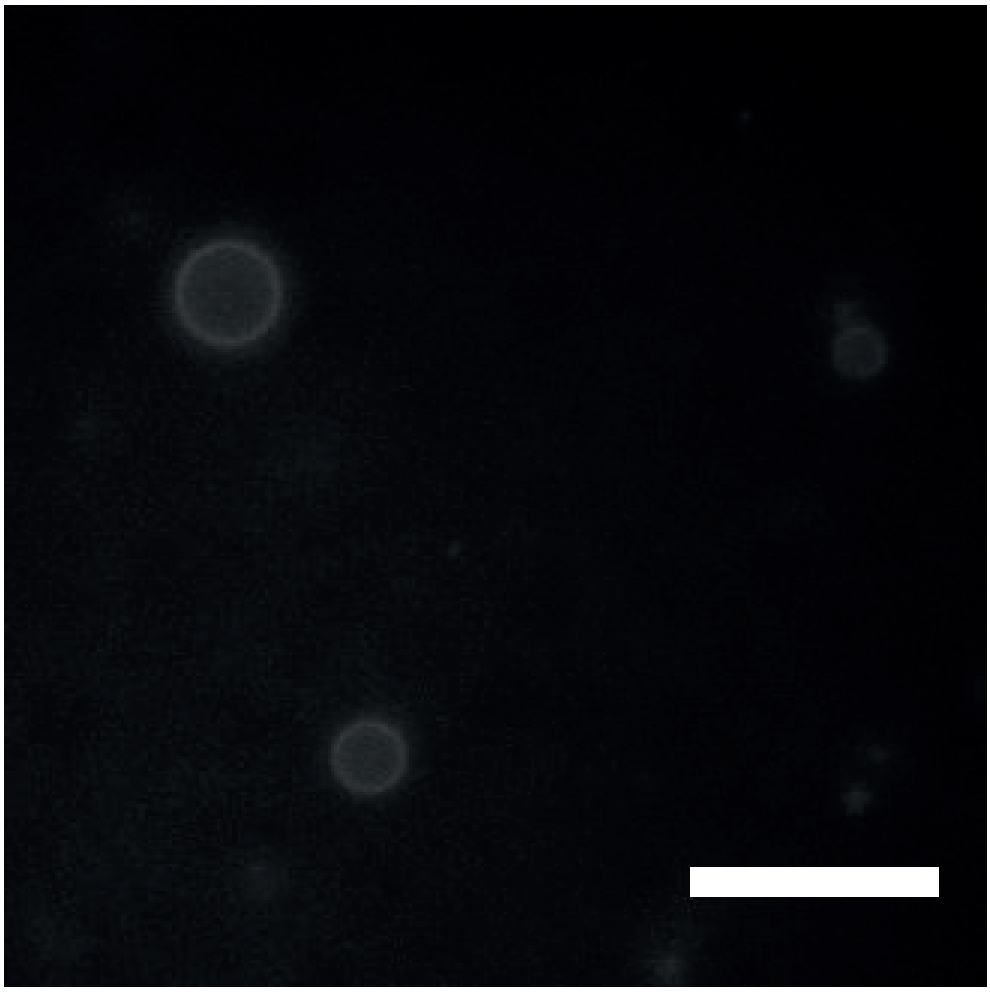


Fig. S8

Fluorescence microscopy image of membrane-containing vesicles formed by hydration of a thin film of phospholipid **3**. Membranes were stained with 100 μM of Texas Red® DPHE. Scale bar denotes 10 μm.

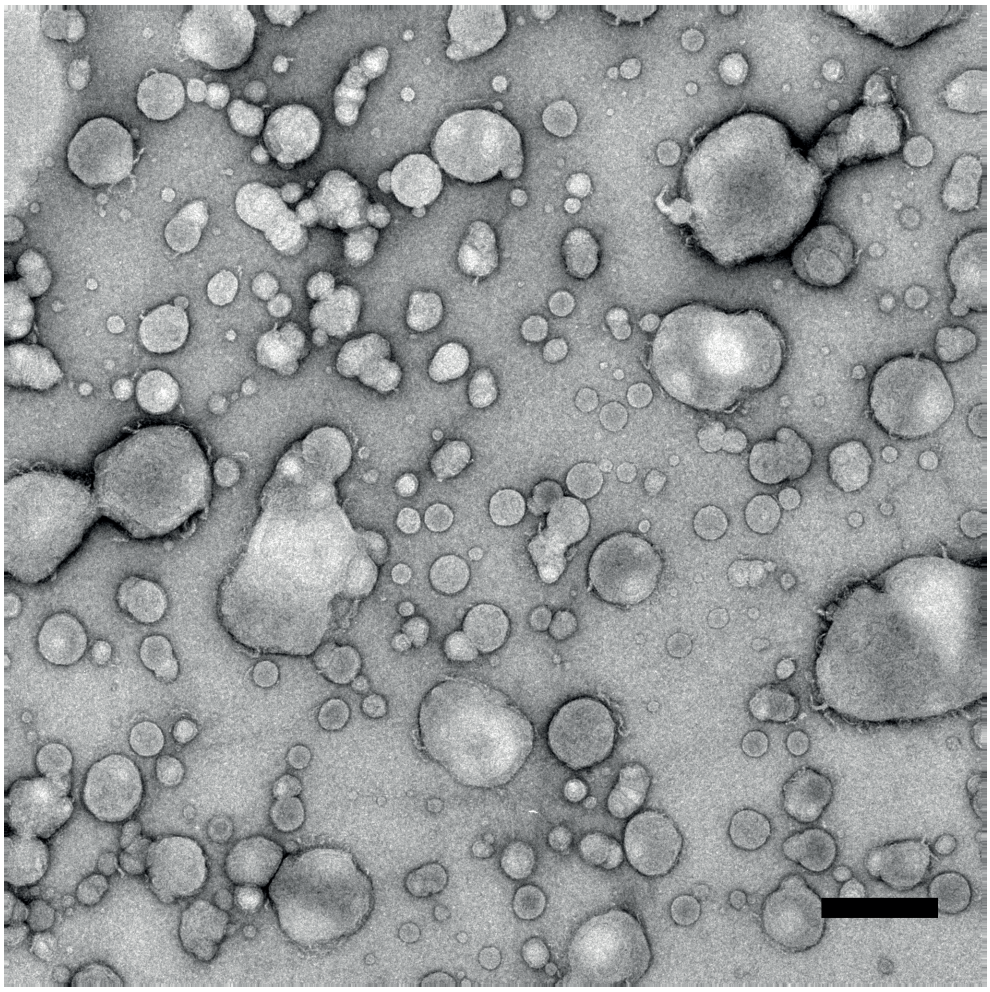


Fig. S9

TEM images of *in situ* formed phospholipid **3** sample, showing the presence of vesicles. Scale bar denotes 500 nm.

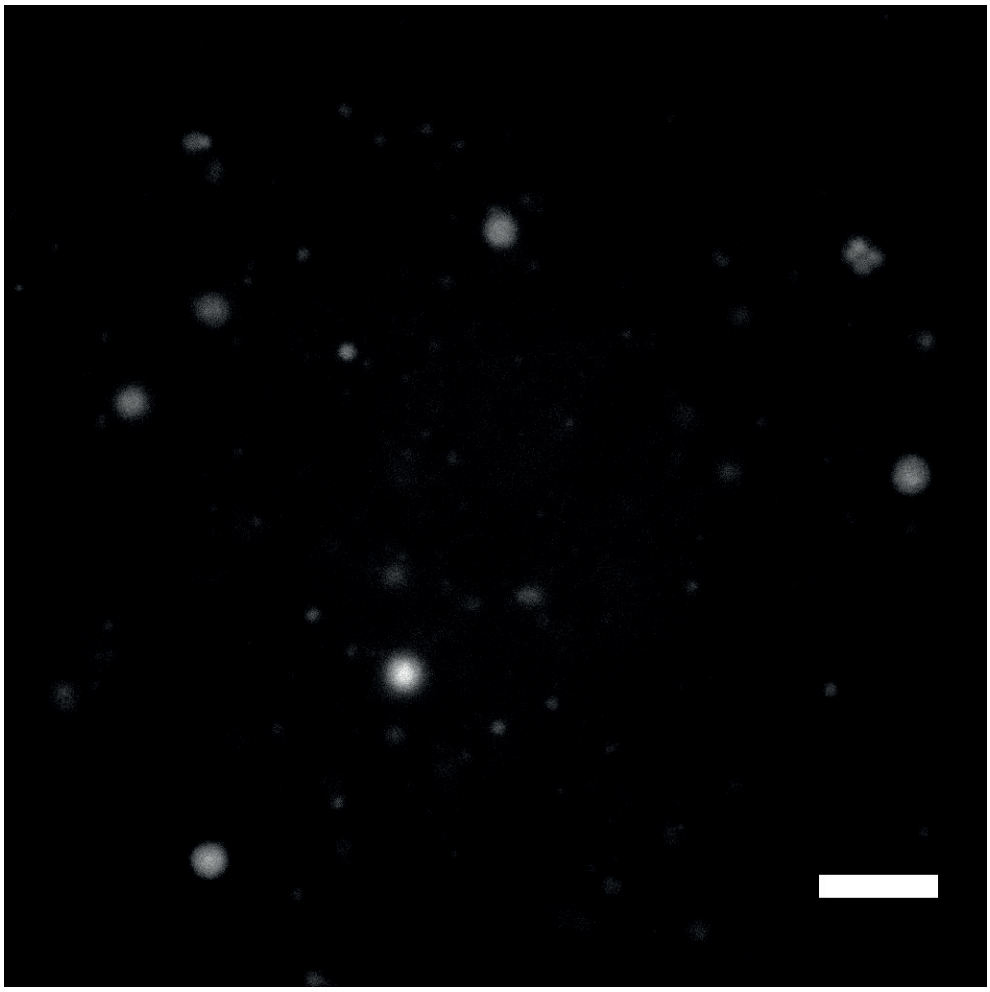
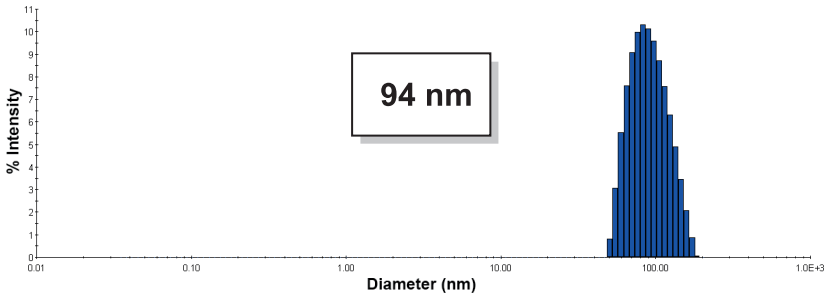


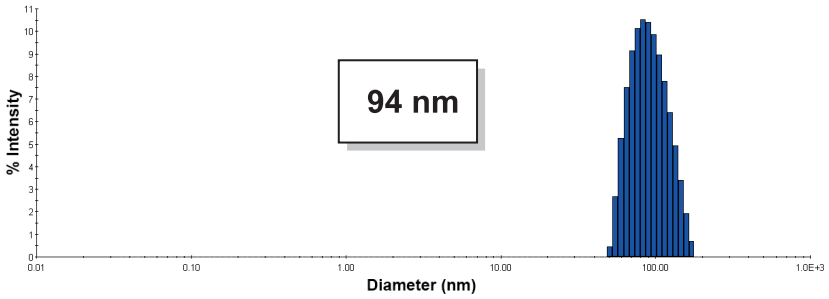
Fig. S10

Fluorescence microscopy images corresponding to the *in situ* encapsulation of polar fluorescent dye 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) in phospholipid **3** membrane vesicles. Scale bar denotes 25 μm.

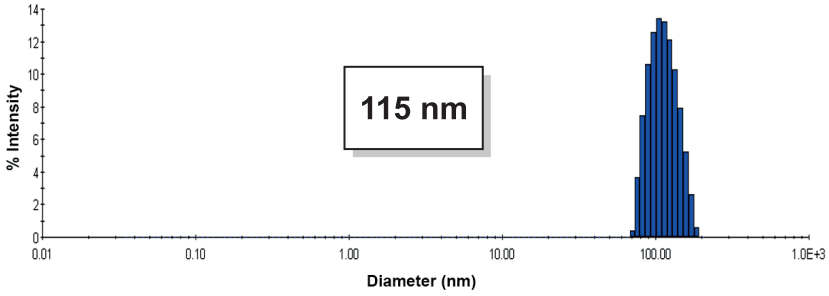
A)



B)



C)



D)

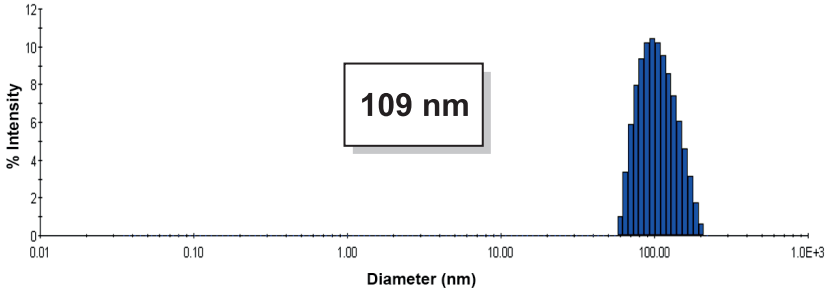


Fig. S11

Dynamic Light Scattering corresponding to the 100 nm extruded DOPC (**A**), POPC (**B**), phospholipid **3** (**C**) and phospholipid **5** (**D**) membrane vesicles.

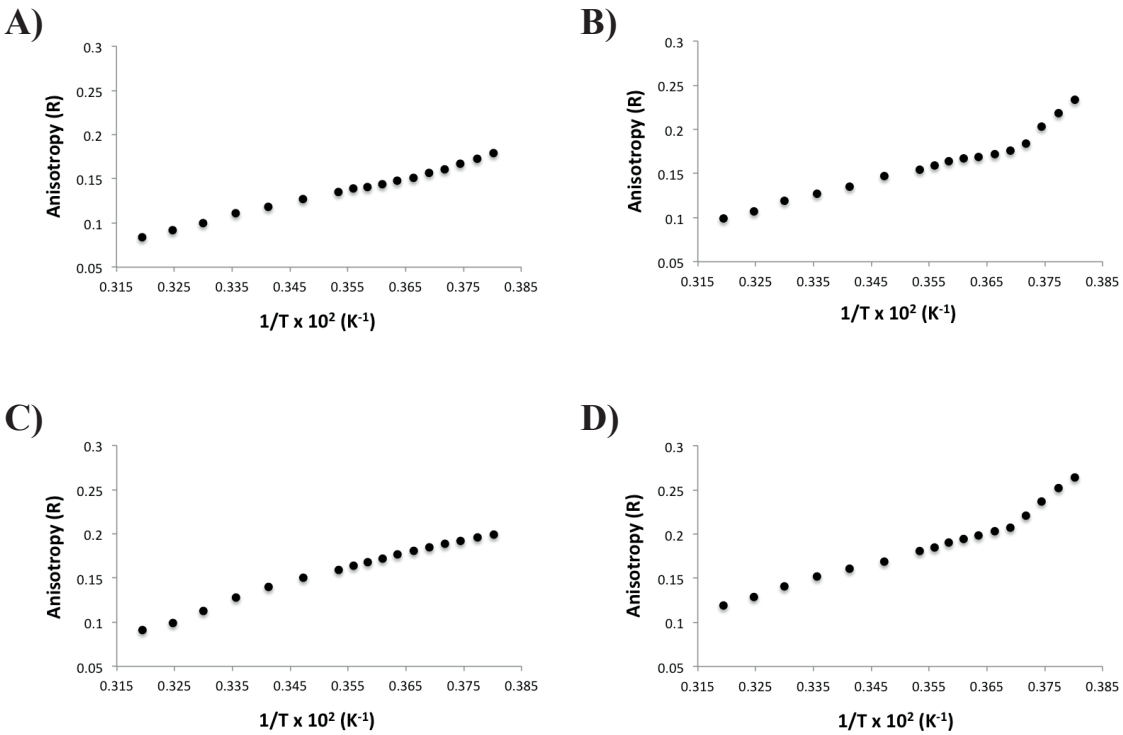


Fig. S12

Chain melting temperatures of different phospholipids. 1,6-diphenyl-1,3,5-hexatriene (DPH) anisotropy was used as a function of temperature to measure phase transitions of the lipid chains.[9] A sudden change in the slope of the anisotropy indicates a transition from a gel to liquid-crystalline phase. **A)** DPH anisotropy graph corresponding to the sample of DOPC vesicles. Within the temperatures tested (263-313 K), a phase transition was not detected in membranes from DOPC phospholipid, consistent with previous measurements.[10] **B)** DPH anisotropy graph corresponding to the sample of POPC vesicles. The melting temperature of the lipid chains in POPC membranes was detected at 270 K, consistent with previous measurements.[10] **C)** DPH anisotropy graph corresponding to the sample of phospholipid **3** vesicles. Within the temperatures tested (263-313 K), a phase transition was not detected in membranes formed from **3**, as predicted by comparing to analogous DOPC membranes. **D)** DPH anisotropy graph corresponding to the sample of phospholipid **5** vesicles. The melting temperature of the lipid chains in **5** membranes was detected at 270 K, comparable to the transition temperature of analogous POPC membranes.

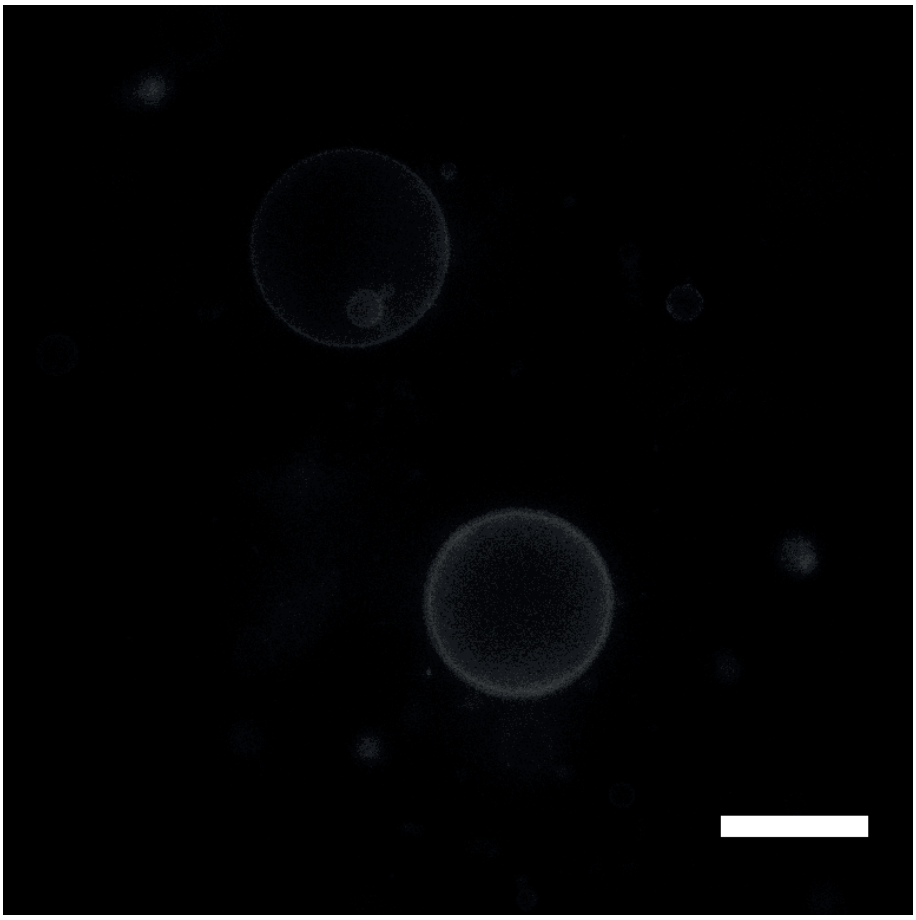


Fig. S13

Characterization of the giant unilamellar vesicular structure. Fluorescence microscopy image of membrane-containing giant unilamellar vesicles (GUVs) formed by electroformation of phospholipid **3**. Membranes were stained using 0.1 mol% of Bodipy® FL DHPE. Scale bar denotes 25 μm.

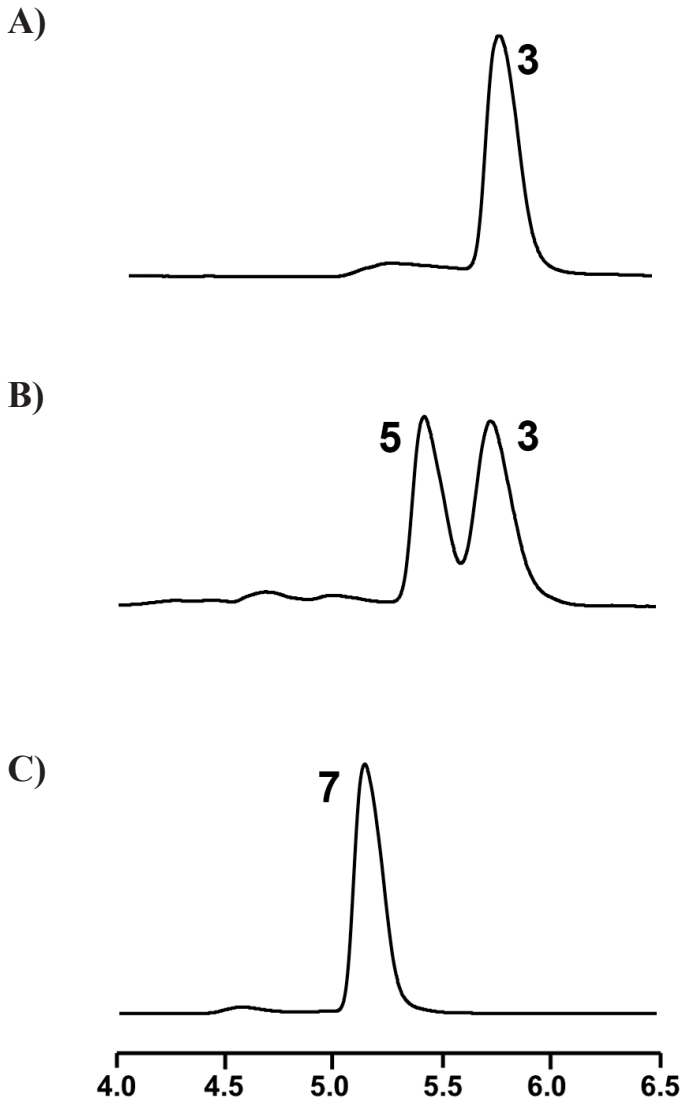


Fig. S14

HPLC/ELSD spectra monitoring the remodeling of phospholipid membrane GUVs driven by RNCL. **A)** ELSD spectra showing the GUVs from phospholipid **3**. **B)** ELSD spectra corresponding to the RNCL exchange reaction between the GUVs from phospholipid **3** and the N-methylated cysteine-functionalized lysolipid **4**, leading to an equimolar mixture of **3** and **5**. **C)** ELSD spectra corresponding to the RNCL exchange reaction between the GUVs from phospholipid **3** and cysteine-functionalized lysolipid **6**, leading to the selective formation of **7**. Retention times (R_t's) for phospholipids (**3, 5** and **7**) were verified by mass spectrometry.

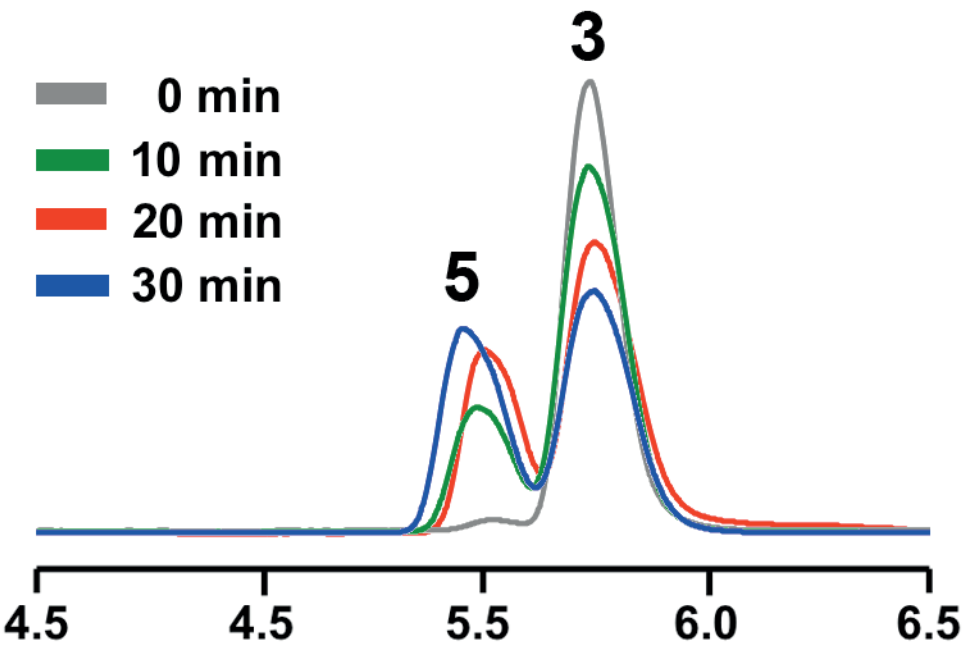


Fig. S15

HPLC/ELSD traces monitoring the progress of the remodeling. ELSD spectra corresponding to the RNCL exchange reaction between phospholipid **3** and the N-methylated cysteine-functionalized lysolipid **4**, leading to an equimolar mixture of **3** and **5**. The retention times for all the species were verified by mass spectrometry and the use of known standards.

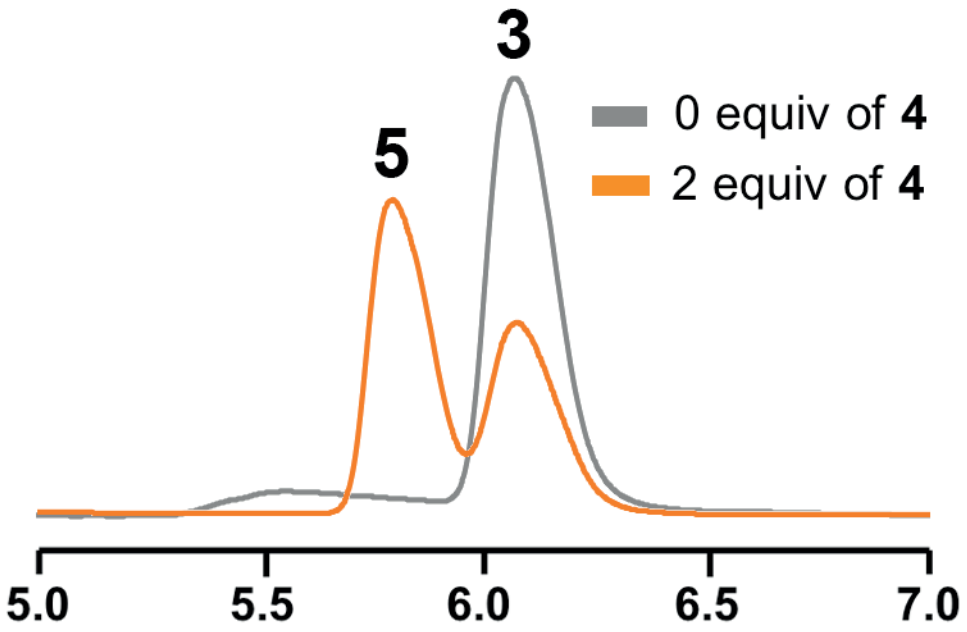


Fig. S16

ELSD spectra corresponding to the RNCL exchange reaction between phospholipid **3** and 2.0 equivalents of the N-methylated cysteine-functionalized lysolipid **4**, leading to an approximately 1:2 mixture of **3** and **5**. The retention times for all the species were verified by mass spectrometry and the use of known standards.

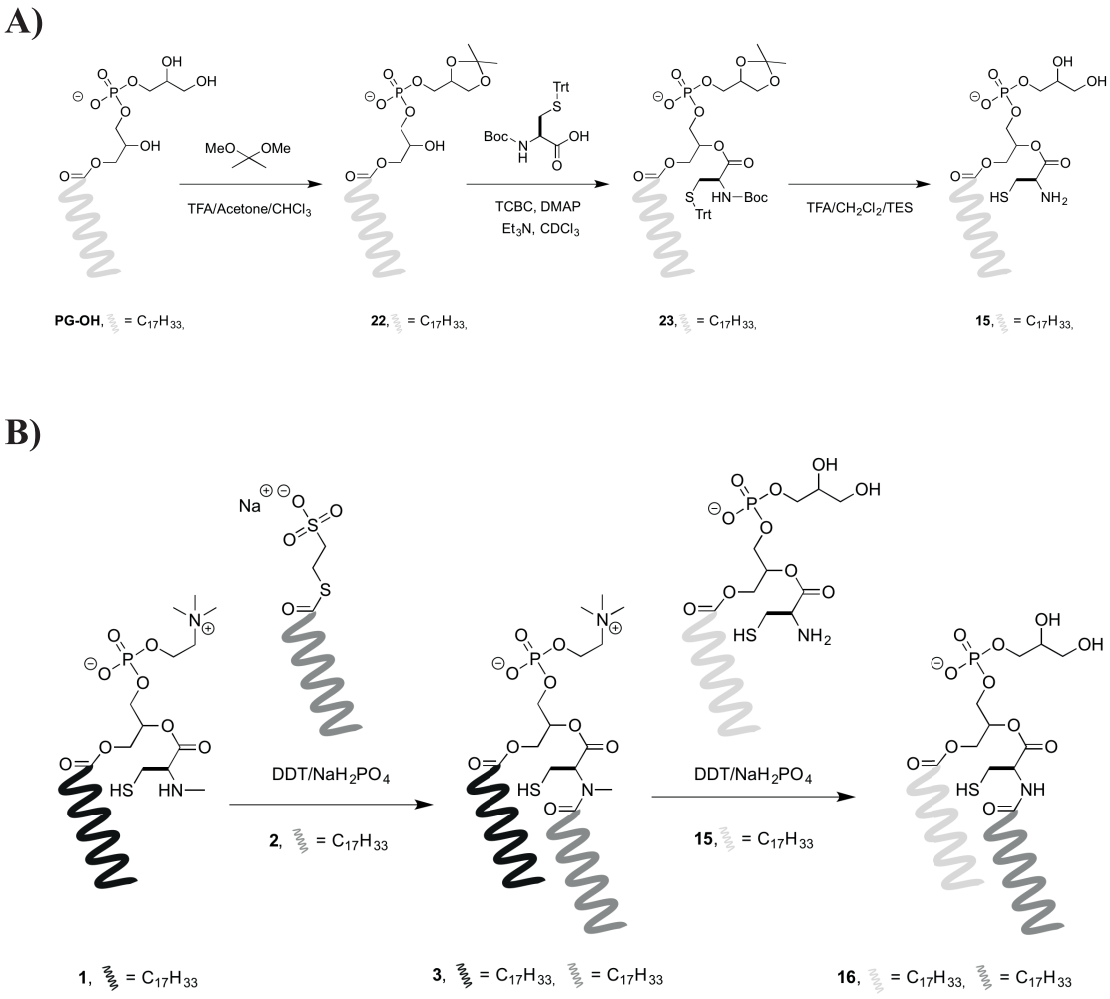


Fig. S17

De novo formation and head group remodeling of phospholipid membranes. **A)** Synthesis of negatively charged cysteine-functionalized phosphatidylglycerol lysolipid **15**. **B)** *De novo* synthesis of phospholipids by NCL reaction of the N-methylated cysteine-functionalized phosphatidylcholine lysolipid **1** and MESNA oleoyl thioester **2**, followed by subsequent head group remodeling of the corresponding neutral phospholipid **3** by RNCL in the presence of the cysteine-functionalized phosphatidylglycerol lysolipid **15**.

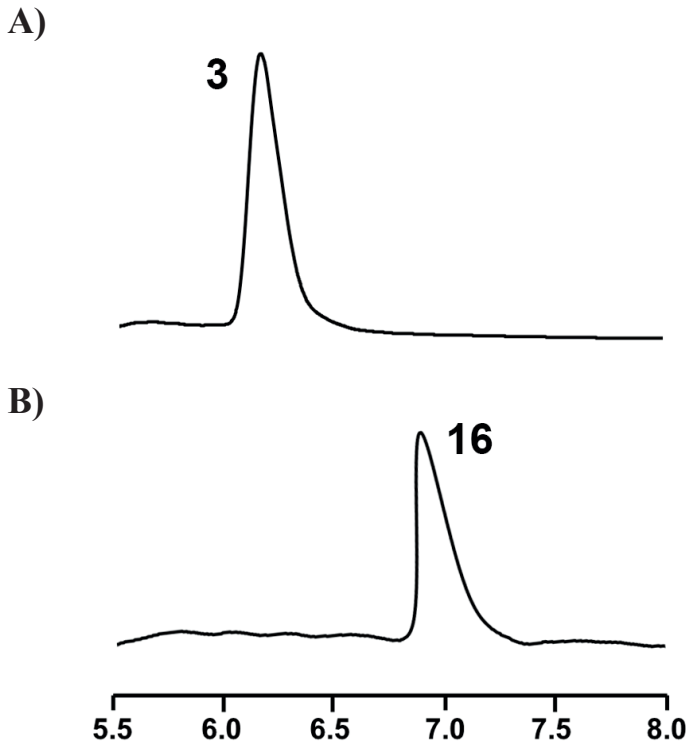
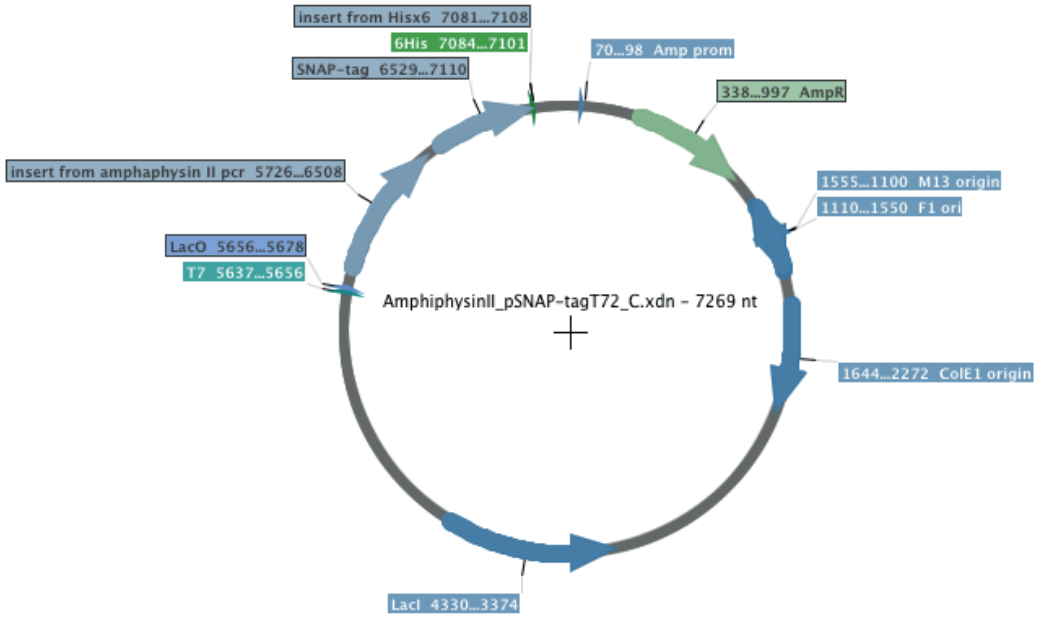


Fig. S18

HPLC/ELSD spectra monitoring the head group remodeling of phospholipid membrane GUVs driven by RNCL. **A)** ELSD spectra showing the GUVs from phospholipid **3**. **B)** ELSD spectra corresponding to the RNCL exchange reaction between the GUVs from phospholipid **3** and the cysteine-functionalized lysolipid **15**, leading to the selective formation of **16**. Retention times (R_t's) for phospholipids (**3** and **16**) were verified by mass spectrometry.

A)



B)

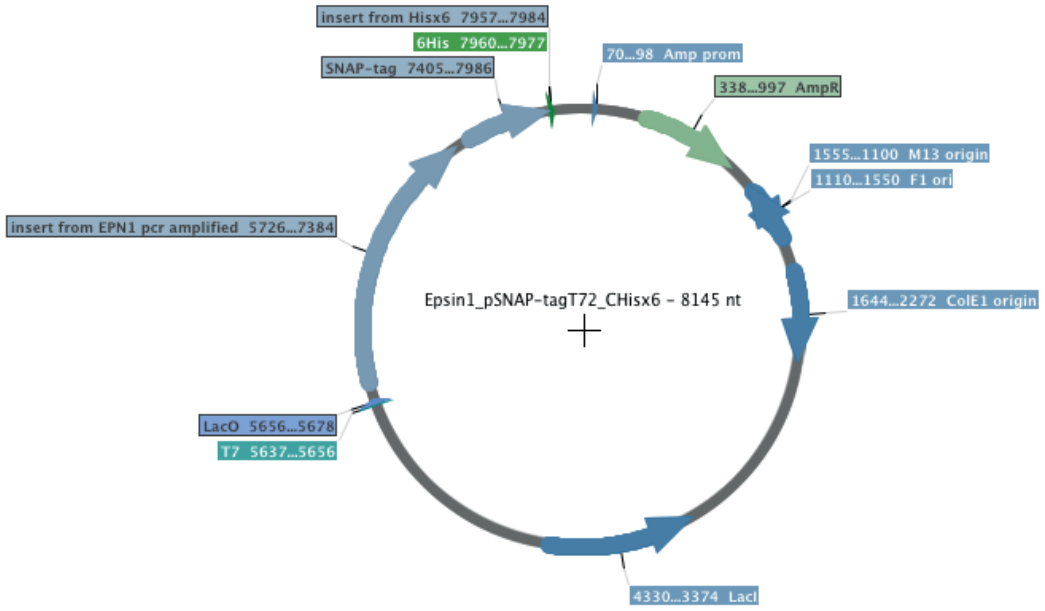


Fig. S19

Plasmid maps corresponding to amphiphysinII_pSNAP-tagT72_C.xdn - 7269 nt (**A**) and epsin1_pSNAP-tagT72_CHisx6 - 8145 nt (**B**).

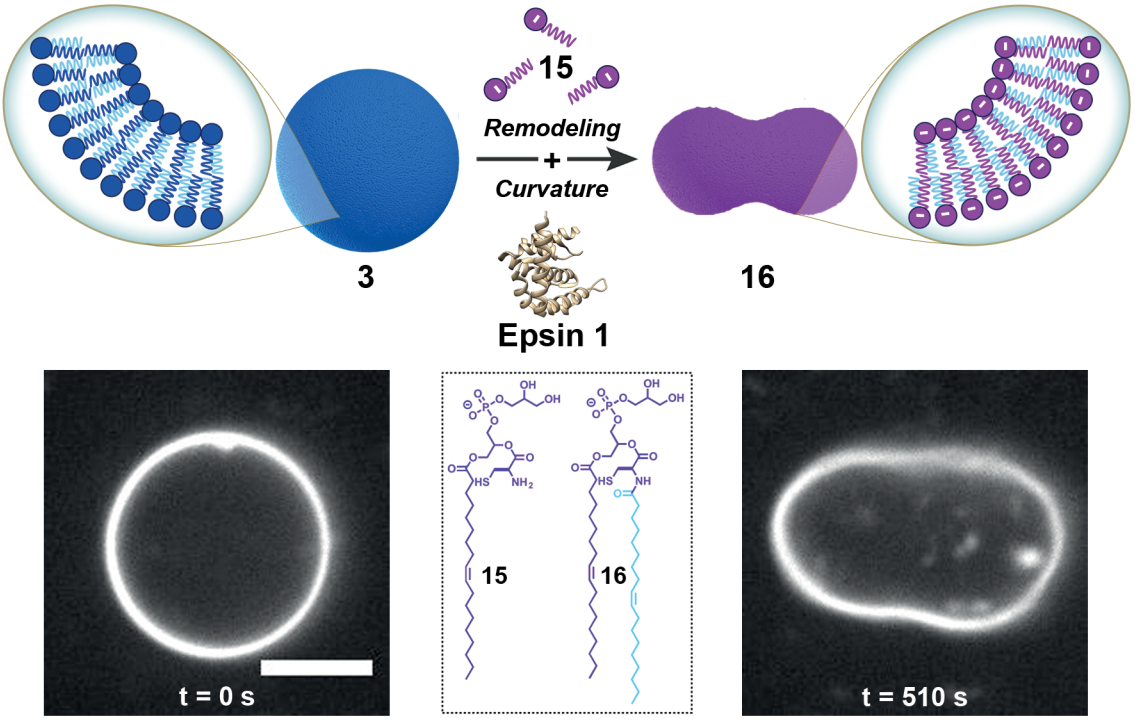


Fig. S20

Membrane curvature induced by RNCL-based remodeling. GUVs composed of neutral phospholipid **3** containing 0.1 mol % of Bodipy® FL DHPE dye are remodeled by RNCL exchange reactions with negatively charged lysolipid **15**, leading to the formation of a new class of GUVs composed of **16**. After addition of epsin 1 to the mixture, dramatic membrane curvature events in the negatively charged GUVs **16** were observed as a result of the protein binding. Scale bar denotes 10 μm.

Table S1

Characteristics of all the lipids used in this study.

Compound	Head[a]	Lipid chain	N-R'	Transition temperature	Cmc's	Micelle size
Lysolipid **1**	PC	R = Oleoyl	N-Me		<100 μM	4.54 nm
Thioester **2**		R = Oleoyl			<10 μM	3.79 nm
Phospholipid **3**	PC	R_1 = Oleoyl R_2 = Oleoyl	N-Me	<263 K		
Lysolipid **4**	PC	R = Palmitoyl	N-Me		<100 μM	6.74 nm
Phospholipid **5**	PC	R_1 = Palmitoyl R_2 = Oleoyl	N-Me	270 K		
Lysolipid **6**	PC	R = Palmitoyl	N-H		<100 μM	5.32 nm
Phospholipid **7**	PC	R_1 = Palmitoyl R_2 = Oleoyl	N-H	270 K		
Thioester **8**		R = Palmitoyl			<10 μM	3.53 nm
Phospholipid **9**	PC	R_1 = Palmitoyl R_2 = Palmitoyl	N-Me			
Phospholipid **10**	PC	R_1 = Oleoyl R_2 = Palmitoyl	N-Me			
Phospholipid **11**	PC	R_1 = Palmitoyl R_2 = Palmitoyl	N-H			
Lysolipid **12**	PC	R = Oleoyl	N-H			
Phospholipid **13**	PC	R_1 = Oleoyl R_2 = Oleoyl	N-H	<263 K		
Phospholipid **14**	PC	R_1 = Oleoyl R_2 = Palmitoyl	N-H			
Lysolipid **15**	PG	R = Oleoyl	N-H			
Phospholipid **16**	PG	R_1 = Oleoyl R_2 = Oleoyl	N-H			

[a] PC: phosphocholine, PG: phosphoglycerol

Movie S1

Time lapse phase contrast microscopy of the *in situ* vesicle formation. An aqueous solution of MESNA thiooleate **2** (4 mM) was added to another aqueous solution of N-methylated cysteine-based lysolipid **1** (4 mM) and DTT (20 mM in 200 mM NaH_2PO_4 buffer pH 7.1). Sequential phase contrast images were taken over a period of 3 min. Scale bar denotes 5 μm.

Movie S2

Time lapse fluorescence microscopy corresponding to the microdomain formation induced by RNCL-based membrane remodeling. An aqueous buffer solution of **3**:**10**:cholesterol GUVs (molar ratio of 1:1:0.8; 400 μM) containing 0.1 mol % of Texas Red® DHPE dye was added to another aqueous solution of cysteine-functionalized lysolipid **6** (400 μM) in the presence of DTT (2 mM). Initially, lipid microdomains were not present. However, shortly after the species had been mixed, the microdomain formation was observed. Sequential fluorescence images were taken over a period of 135 s. Frames are 100 ms apart. Movie frame rate is 30 fps. Scale bar denotes 20 μm.

Movie S3

Time lapse fluorescence microscopy corresponding to the addition of **3**:**10**:cholesterol GUVs to the non-reactive (under RNCL conditions) lysolipid **Lyso C_{16} PC-OH** (*Control*). An aqueous buffer solution of **3**:**10**:cholesterol GUVs (molar ratio of 1:1:0.8; 400 μM) containing 0.1 mol % of Texas Red® DHPE dye was added to another aqueous solution of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine **Lyso C_{16} PC-OH** (400 μM) in the presence of DTT (2 mM). Microdomain formation was not observed. Sequential fluorescence images were taken over a period of 135 s. Frames are 100 ms apart. Movie frame rate is 30 fps. Scale bar denotes 20 μm.

Movie S4

Time lapse fluorescence microscopy corresponding to the addition of **3**:**10**:cholesterol GUVs to the reactive (under RNCL conditions) lysolipid **12** (*Control*). An aqueous buffer solution of **3**:**10**:cholesterol GUVs (molar ratio of 1:1:0.8; 400 μM) containing 0.1 mol % of Texas Red® DHPE dye was added to another aqueous solution of cysteine-functionalized lysolipid **12** (400 μM) in the presence of DTT (2 mM). Microdomain formation was not observed. Sequential fluorescence images were taken over a period of 900 s. Frames are 60 s apart. Movie frame rate is 5 fps. Scale bar denotes 15 μm.

Movie S5

Time lapse fluorescence microscopy corresponding to the membrane curvature induced by RNCL-based membrane remodeling. An aqueous buffer solution of **3** GUVs (380 μM) containing 0.1 mol % of Bodipy FL DHPE dye was added to another aqueous solution of cysteine-functionalized lysolipid **15** (380 μM) in the presence of DTT (2.6 mM), leading to the formation of GUVs composed of phospholipid **16**. After 2 h of reaction, amphiphysin-His (728 μM; final concentration: 72.8 μM) was added, observing dramatic membrane curvature events as a result of the protein binding. Sequential fluorescence images were taken over a period of 15 min. Frames are 30 s apart. Movie frame rate is 5 fps. Scale bar denotes 10 μm.

Movie S6

Time lapse fluorescence microscopy corresponding to the addition of amphiphysin to **3** GUVs (under RNCL conditions) (*Control*). An aqueous buffer solution of amphiphysin-His (728 μM; final concentration: 72.8 μM) was added to another aqueous solution of **3** GUVs (380 μM) containing 0.1 mol % of Bodipy® FL DHPE dye in the presence of DTT (2.6 mM). Membrane curvature events were not observed. Sequential fluorescence images were taken over a period of 25 min. Frames are 30 s apart. Movie frame rate is 5 fps. Scale bar denotes 10 μm.

Movie S7

Time lapse fluorescence microscopy corresponding to the RNCL-based membrane remodeling without the subsequent addition of protein (amphiphysin or epsin 1) (*Control*). An aqueous buffer solution of **3** GUVs (380 μM) containing 0.1 mol % of Bodipy FL DHPE dye was added to another aqueous solution of cysteine-functionalized lysolipid **15** (380 μM) in the presence of DTT (2.6 mM), leading to the formation of GUVs composed of phospholipid **16**. After 2 h of reaction, H_2O was added (instead of amphiphysin or epsin 1). Membrane curvature events were not observed. Sequential fluorescence images were taken over a period of 20 min. Frames are 30 s apart. Movie frame rate is 5 fps. Scale bar denotes 5 μm.

Movie S8

Time lapse fluorescence microscopy corresponding to the membrane curvature induced by RNCL-based membrane remodeling. An aqueous buffer solution of **3** GUVs (380 μM) containing 0.1 mol % of Bodipy FL DHPE dye was added to another aqueous solution of cysteine-functionalized lysolipid **15** (380 μM) in the presence of DTT (2.6 mM), leading to the formation of GUVs composed of phospholipid **16**. After 2 h of reaction, epsin 1-His (27.7 μM; final concentration: 2.8 μM) was added, observing dramatic membrane curvature events as a result of the protein binding. Sequential fluorescence images were taken over a period of 10 min. Frames are 30 s apart. Movie frame rate is 5 fps. Scale bar denotes 5 μm.

Movie S9

Time lapse fluorescence microscopy corresponding to the addition of epsin 1 to **3** GUVs (under RNCL conditions) (*Control*). An aqueous buffer solution of epsin 1-His (27.7 μM; final concentration: 2.8 μM) was added to another aqueous solution of **3** GUVs (380 μM) containing 0.1 mol % of Bodipy® FL DHPE dye in the presence of DTT (2.6 mM). Membrane curvature events were not observed. Sequential fluorescence images were taken over a period of 22.5 min. Frames are 30 s apart. Movie frame rate is 5 fps. Scale bar denotes 5 μm.

Movie S10

Time lapse fluorescence microscopy corresponding to the absence of membrane curvature in the presence of RNCL-based membrane remodeling and amphiphysin (*Control*). An aqueous buffer solution of **3** GUVs (380 μM) containing 0.1 mol % of Texas Red® DHPE dye was added to another aqueous solution of cysteine-functionalized lysolipid **12** (380 μM) in the presence of DTT (2.6 mM), leading to the formation of GUVs composed of phospholipid **13**. After 2 h of reaction, amphiphysin-His (728 μM; final concentration: 72.8 μM) was added, observing no membrane curvature events. Sequential fluorescence images were taken over a period of 15 min. Frames are 30 s apart. Movie frame rate is 5 fps. Scale bar denotes 15 μm.

Movie S11

Time lapse fluorescence microscopy corresponding to the absence of membrane curvature in the presence of RNCL-based membrane remodeling and epsin 1 (*Control*). An aqueous buffer solution of **3** GUVs (380 μM) containing 0.1 mol % of Texas Red® DHPE dye was added to another aqueous solution of cysteine-functionalized lysolipid **12** (380 μM) in the presence of DTT (2.6 mM), leading to the formation of GUVs composed of phospholipid **13**. After 2 h of reaction, epsin 1-His (27.7 μM; final concentration: 2.8 μM) was added, observing no membrane curvature events. Sequential fluorescence images were taken over a period of 15 min. Frames are 30 s apart. Movie frame rate is 5 fps. Scale bar denotes 15 μm.

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