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Supporting Information

Hybrid Lipids Inspired by Extremophiles and Eukaryotes Afford Serum-Stable Membranes with Low Leakage

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Supporting Information

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1. General Information

All reagents were purchased from commercial sources and used without further purification. POPC lipid was purchased from Avanti Polar Lipids. The POPC lipid was stored under Argon at -20°C and used within 3 months of purchase. Glassware was dried at 115°C overnight. Air and moisture-sensitive reagents were transferred using a syringe or stainless steel cannula. Intermediates were purified over silica (60Å, particle size 40-63 μm) purchased from Dynamic Adsorbents, Inc. Reactions were monitored by thin-layer chromatography (TLC) using 0.25 mm silica gel plates (60F-254) from Dynamic Adsorbents, Inc. Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. ^{1}H, ^{13}C, ^{31}P NMR spectra were obtained on either JEOL ECA 500 spectrometer or Varian 400 MHz/500MHz spectrometer. Chemical shifts are reported in ppm relative to residual solvent. The FID file was analyzed using NMRnotebook version 2.70 build 0.10 by NMRTEC.

Dynamic Light Scattering (DLS) measurements were performed on a Wyatt DynaPro NanoStar (Wyatt Technology, Santa Barbara, CA) instrument using a disposable cuvette (Eppendorf UVette 220 nm – 1,600 nm) and data processed using Wyatt DYNAMICS V7 software. Each analysis involved an average of 10 measurements. The data was exported for final plotting using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

Low resolution MS analysis was performed on a Micromass Quattro Ultima triple quadrupole mass spectrometer with an electrospray ionization (ESI) source. High resolution MS analysis was performed using Agilent 6230 Accurate-Mass TOFMS with an electrospray ionization (ESI) source by Molecular Mass Spectrometry Facility (MMSF) in the department of chemistry and biochemistry at University of California, San Diego.

Fluorescence decay measurements were taken on a Perkin Elmer Enspire© multimode plate reader (excitation 485 nm, emission 517 nm and 75 flashes); each measurement was taken with a 10 sec delay with 125 repeat (total acquisition time = 21 min). Costar EIA/RIA plates were used (96 well half area, no lid, flat bottom, non-treated black polystyrene). The data were exported for final plotting using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

HPLC analyses were performed using an Agilent 1100 Series HPLC and an analytical reverse-phase column (Eclipse XDB-C18 Agilent, 5 μm, 150 x 4.6 mm). Flow: 1 mL/min. Injection volume = 50 μL. Detection: 254 and 280 nm. Mobile phase: water/acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA).

Method A: Linear gradient from 5% to 95% of ACN in 10 minutes followed by 2 minutes of re-equilibration at 5% of ACN.

2. Synthesis of GcGTPC-CH lipid

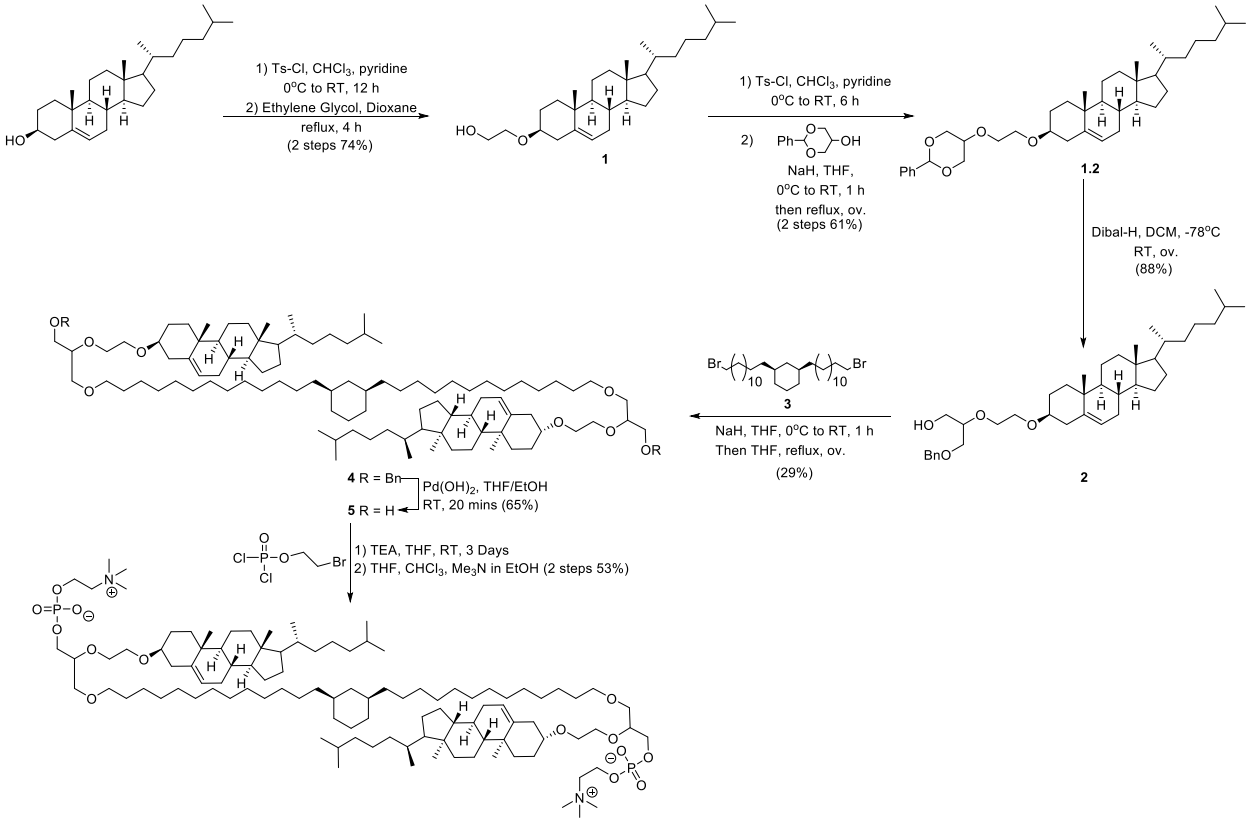
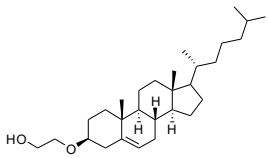


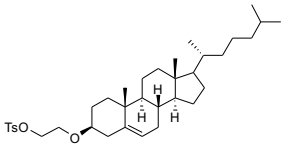
FIgure S1. Synthetic scheme to generate GcGTPC-CH

2-(((3S,8S,9S,10R,13R,14S)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethan-1-ol **(1)**



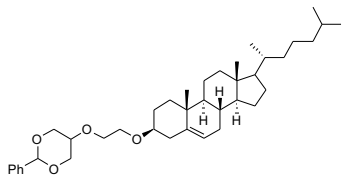
Compound **1** was prepared following a previously reported protocol.[1]

2-(((3S,8S,9S,10R,13R,14S)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethyl 4-methylbenzenesulfonate (**1.1**)



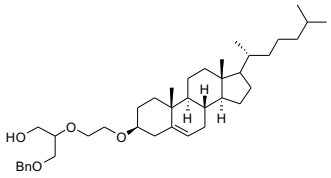
Compound **1.1** was prepared following a previously reported protocol.[1]

5-(2-(((3S,8S,9S,10R,13R,14S)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethoxy)-2-phenyl-1,3-dioxane **(1.2)**



To a cooled suspension of 0.27 g (11 mmol) of NaH in 5 mL of dry tetrahydrofuran (THF), 0.67 g (3.7 mmol) of cis-1,3-O-Benzylideneglycerol was added drop wise and reacted for 1 hour at room temperature. Next, 1.97 g (4.1 mmol) molecule **1.1** was added and then heated at reflux for 16 hours. The reaction was quenched with water and the solvent was removed under vacuum. The residue was extracted with DCM, washed successively with water and brine, then dried over $MgSO_4$ and compound **1.2** was obtained as a white solid and then engaged to the next step.

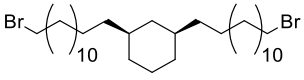
3-(benzyloxy)-2-(2-(((3S,8S,9S,10R,13R,14S)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethoxy)propan-1-ol **(2)**



1.57 g (2.7 mmol) of **1.2** was dissolved in 7.5 mL of dry dichloromethane (DCM) then cooled to -78 °C. 7.7 mL of DIBAL-H in DCM (1 M) was added dropwise and the reaction was slowly brought back to room temperature and reacted for 16 hours. The reaction was quenched with methanol and 27 mL of (5 M) NaOH was added. The solution was then extracted with diethyl ether, washed successively with water and brine, then dried over $MgSO_4$ and purified by column chromatography on silica gel using Hexane/EtOAc (1:1) as the eluent. Compound **2** was obtained as a white oily solid (1.39 g, 88%).

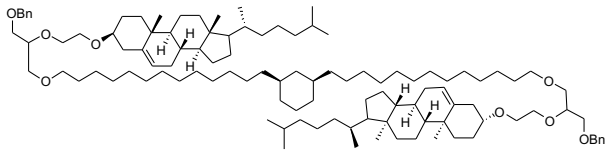
Rf: 0.59 (Hexane/EtOAc 1:1); ^{1}H NMR (500 MHz, $CDCl_3$-d_1) δ 7.28-7.17 (m, 5H), 5.27-5.26 (m, 1H), 4.46 (s, 2H), 3.82-3.80 (m, 1H), 3.63-3.38 (m, 9H), 3.18-3.08 (m, 1H), 2.34-2.27 (m, 1H), 2.17-2.11 (m, 1H), 1.96-1.72 (m, 5H), 1.51-1.18 (m, 11H), 1.09-0.78 (m, 22H), 0.61 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$-d_1) δ 140.5, 138.1, 128.3, 127.6, 127.6, 121.7, 80.2, 80.2, 79.5, 73.4, 70.3, 70.2, 70.1, 67.6, 67.5, 62.7, 56.7, 56.2, 50.1, 42.3, 39.8, 39.5, 38.9, 37.1, 36.8, 36.2, 35.8, 31.9, 31.8, 28.3, 28.0, 24.3, 23.9, 22.9, 22.6, 21.1, 19.4, 18.8, 11.9; ESI-MS: 617.53 $[M+Na]^+$; HRMS 617.4540 calcd for $[C_{39}H_{62}O_4Na]^+$, found 617.4534.

(1R,3S)-1,3-bis(14-bromotetradecyl)cyclohexane **(3)**



Compound **3** was prepared following a previously reported protocol.[2]

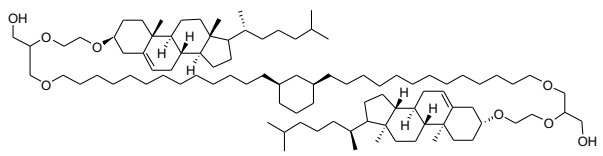
(3R,8R,9R,10S,13S,14R)-3-(2-((1-(benzyloxy)-3-((12-((1S,3R)-3-(13-(3-(benzyloxy)-2-(2-(((3S,8S,9S,10R,13R,14S)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethoxy)propoxy)tridecyl)cyclohexyl)dodecyl)oxy)propan-2-yl)oxy)ethoxy)-10,13-dimethyl-17-((S)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene **(4)**



To a cooled suspension of 0.08 g (3.2 mmol) of NaH in 34 mL of dry THF, 1.39 g (2.3 mmol) of **2** was added drop wise and reacted for 1 hour at room temperature. Next, 0.64 g (1.1 mmol) molecule **3** was added and then heated at reflux for 16 hours. The reaction was quenched with water and the solvent was removed under vacuum. The residue was extracted with DCM, washed successively with water and brine, then dried over $MgSO_4$ and purified by column chromatography on silica gel using Hexane/EtOAc (9:1) as the eluent. Compound **4** was obtained as a clear oil (0.441 g, 29%).

Rf: 0.15 (Hexane/EtOAc 9:1); ^{1}H NMR (500 MHz, $CDCl_3$-d_1) δ 7.33-7.25 (m, 10H), 5.32-5.31 (m, 2H), 4.55 (s, 4H), 3.76-3.74 (m, 4H), 3.723.58 (m, 2H), 3.63-3.49 (m, 12H), 3.42 (t, *J* = 6.7 Hz, 4H), 3.22-3.14 (m, 2H), 2.38-2.35 (m, 2H), 2.21-2.16 (m, 2H), 2.03-1.81 (m, 10H), 1.72-1.68 (m, 4H), 1.55-1.42 (m, 14H), 1.31-0.74 (m, 105 H), 0.67 (s, 6H), 0.51-0.44 (m, 1H); ^{13}C NMR (126 MHz, $CDCl_3$-d_1) δ 141.2, 138.6, 128.5, 127.8, 127.7, 121.7, 79.6, 78.7, 73.6, 71.9, 70.9, 70.5, 70.2, 68.2, 67.7, 57.0, 56.4, 50.4, 42.5, 40.8, 40.0, 39.7, 39.3, 38.0, 37.9, 37.4, 37.1, 36.4, 36.0, 33.6, 32.1, 32.1, 30.3, 30.0, 29.9, 29.7, 28.6, 28.4, 28.2, 27.2, 26.6, 26.3, 25.8, 24.5, 24.0, 23.0 ,22.8, 21.3, 19.6, 18.9, 12.1; ESI-MS: HRMS 1656.3883 calcd for $[C_{110}H_{184}O_8Na]^+$, found 1656.3879.

2-(2-(((3S,8S,9S,10R,13R,14S)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethoxy)-3-((13-((1R,3S)-3-(13-(2-(2-(((3R,8R,9R,10S,13S,14R)-10,13-dimethyl-17-((S)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethoxy)-3-hydroxypropoxy)tridecyl)cyclohexyl)tridecyl)oxy)propan-1-ol **(5)**

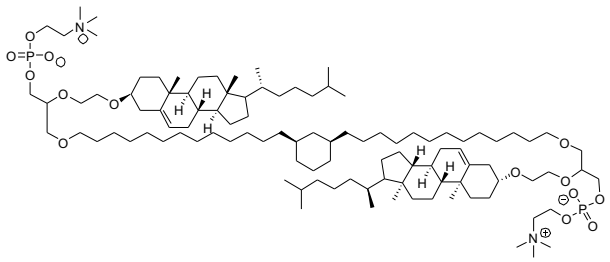


35 mg (0.022 mmol) of **4** and 1.8 mg (5 % w/w) of $Pd(OH)_2$ was added to 4 mL of solvent mixture THF/EtOH (1:1). The suspension was purged with N_2 gas for 10 seconds and repeated 3 times. The suspension was then purged using H_2 for 10

seconds and repeated 3 times. The reaction was stirred at room temperature for 20 mins. The suspension was then immediately filtered over celite and purified using column chromatography on silica gel using Hexane/EtOAc (7:3) as the eluent. Compound **5** was obtained as a white viscous oil (20.3 mg g, 65%).

Rf: 0.13 (Hexane/EtOAc 7:3); ^{1}H NMR (500 MHz, $CDCl_3$-d_1) δ 5.27-5.26 (m, 2H), 3.86-3.81 (m, 2H), 3.64-3.34 (m, 20H), 3.18-3.11 (m, 2H), 2.32-2.27 (m, 2H), 2.18-2.11 (m, 2H), 1.96-1.73 (m, 10H), 1.65-1.59 (m, 4H), 1.50-1.38 (m, 14H), 1.27-0.65 (m, 105H), 0.59 (s, 6H), 0.044-0.38 (m, 1H); ^{13}C NMR (126 MHz, $CDCl_3$-d_1) δ 140.9, 122.0, 80.3, 80.3, 79.8, 72.0, 71.2, 70.4, 70.4, 67.8, 67.7, 63.3, 57.0, 56.4, 50.4, 42.5, 40.8, 40.0, 39.7, 39.1, 39.1, 38.0, 38.0, 37,4, 37.1, 36.4, 36.0, 33.7, 32.1, 32.1, 30.3, 30.0, 29.9, 29.8, 29.7, 28.5, 28.5, 28.4, 28.2, 27.2, 26.6, 26.3, 24.5, 24.0, 23.0 22.8, 21.3, 19.6, 18.9, 12.1; ESI-MS: 1476.28 $[M+Na]^+$; HRMS 1476.2944 calcd for $[C_{96}H_{172}O_8Na]^+$, found 1476.2952.

2-(2-(((3S,8S,9S,10R,13R,14S)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethoxy)-3-((13-((1R,3S)-3-(13-(2-(2-(((3R,8R,9R,10S,13S,14R)-10,13-dimethyl-17-((S)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethoxy)-3-((oxido(2-(trimethylammonio)ethoxy)phosphoryl)oxy)propoxy)tridecyl)cyclohexyl)tridecyl)oxy)propyl (2-(trimethylammonio)ethyl) phosphate **(GcGTPC-CH)**



To a solution of 81 mg (0.34 mmol) of bromoethyldichlorophosphate in 1.5 mL of dry THF, a solution of 61 mg (0.04 mmol) of **5** and 64 µL of Et_3N (0.46 mmol) in 1.5 mL of dry THF was added dropwise. After stirring the mixture for 3 days in the dark at room temperature, toluene was added to precipitate triethylammonium chloride. Then, the solution was filtered through a small pad of celite and the filtrate was concentrated. The resulting residue was dissolved in a mixture of THF/$NaHCO_3$ (sat) (2.8 mM) and the reaction was stirred for 16 hours at room temperature. THF was evaporated under vacuum and the resulting aqueous solution was acidified to pH 1 using a dilution solution of hydrochloric acid (1M) and extracted using several portions of DCM/Methanol (MeOH) (8:2). The organic layers were combined, dried over Na_2SO_4 and concentrated under reduced pressure.

To a solution of the previous crude in a mixture of 3.5 mL of chloroform ($CHCl_3$) and 4 mL of Me_3N (33% in EtOH) was added and the reaction was stirred in a sealed tube at room temperature for 5 days. The reaction mixture was concentrated to dryness, purified on sephadex LH-20 using DCM/MeOH (1:1) as eluent and purified by column chromatography on silica gel using DCM/MeOH/Water (70:30:5) as the eluent. GcGTPC-CH was obtained as a white (viscous oil) (40 mg, 53%).

Rf: 0.15 (Hexane/EtOAc 9:1); ^{1}H NMR (500 MHz, MeOD-d_4/$CDCl_3$-d_1 1:1) δ 4.99-4.95 (m, 2H), 3.92-3.85 (m, 4H), 3.57-3.52 (m, 4H), 3.43-3.33 (m, 6H), 3.29-3.05 (m, 16H), 2.97-2.94 (m, 2H), 2.85-2.84 (m, 18H), 2.00-1.77 (m, 4H), 1.67-1.44 (m, 10H), 1.34-1.32 (m, 4H), 1.19-1.07 (m, 15H), 0.98-0.32 (m, 110H), 0.16-0.08 (m, 1H); ^{13}C NMR (126 MHz, $CDCl_3$-d_1) δ 140.2, 121.4, 79.1, 78.3, 78.2, 71.3, 70.3, 69.6, 67.1, 66.0, 64.8, 64.8, 58.6, 58.6, 56.5, 55.9, 53.5, 49.9, 41.9, 40.2, 39.5, 39.1, 38.7, 37.4, 36.8, 36.4, 35.8, 35.5, 33.1, 31.6, 31.5, 29.7, 29.4, 29.3, 29.2, 28.0, 27.8, 27.6, 26.6, 26.0, 25.8, 23.9, 23.4, 22.1, 21.9, 20.7, 18.8, 18.1, 11.3; ^{31}P NMR (202 MHz, MeOD-d_4/$CDCl_3$-d_1 1:1) δ 0.12; HRMS 1784.4235 calcd for $[C_{106}H_{197}N_2O_{14}P_2]^+$, found 1784.4264.

3. General Procedure for Liposome Extrusion

10 mg/mL liposome solution was prepared by first dissolving 5 mg of lipid of interest into a 5 mL round bottom flask in a DCM/MeOH (7/3) solution. A thin lipid film was achieved by evaporating the solvent using a rotary evaporator (BUCHI RE111) then dried further over a hi-vacuum pump (Welch 1402) for 4 hrs. The thin lipid film was then hydrated, in either 100 mM/4 mM Carboxyfluorescein (CF) or 10 mM calcein solution prepared in PBS or buffer A/C or (see section 5, general buffer preparation procedure) respectively, by vortexing the solution for 30 seconds followed by sonication in a water bath sonicator (Branson 2510) for 30 mins. After sonication, the lipid mixture underwent 5 freeze thaw cycles that consisted of 2 mins at -78°C followed by 2 mins at 50°C. The lipid solution was then extruded (Avanti mini-extruder) through 200 nm polycarbonate membrane 25 times followed by another extrusion with a 100 nm polycarbonate membrane 51 times. The lipid solution was then stored at 4°C in Protein Lo-Bind Eppendorf tube.

4. General Procedure for Differential Scanning Calorimetry (DSC) Measurement of GcGTPC-CH lipid

DSC experiments were performed in duplicate using a Thermal Analysis Q2000 DSC. Each experiment involved a 5 °C/min ramp from 0 °C to 67 °C under high purity N_2 at 50 mL/min. Samples were ~0.3 – 1.0 mg of liposomes dissolved in water at ~5% by weight. TA Universal Analysis was used to extract T_m for these samples. Both of the synthetic lipids did not exhibit a phase transition from 5 - 65 °C (Figure S2). DMPC was used as a positive control in the DSC measurements, which showed the expected phase transition at 24 °C. Commercially available diacyl lipid, POPC, was reported to have a phase transition from solid to liquid at -2 ºC.[3]

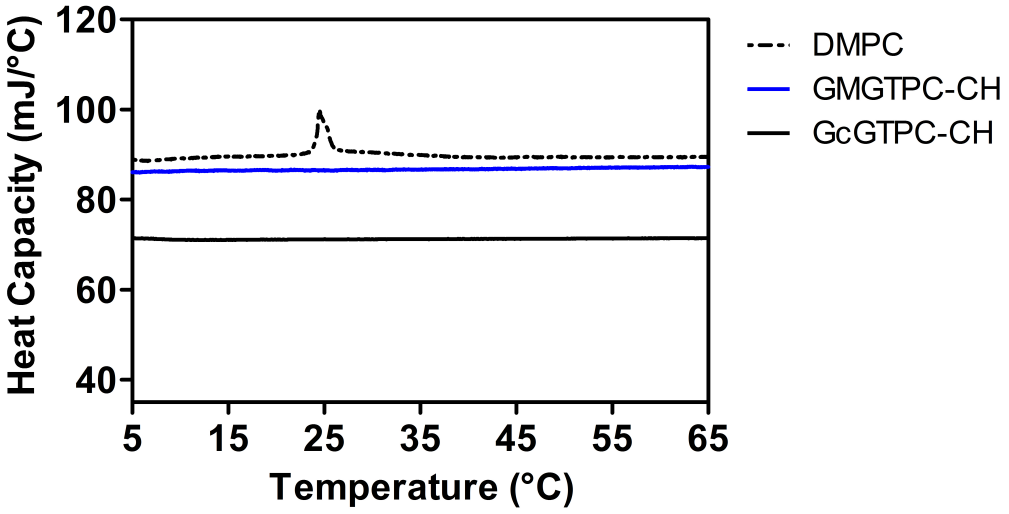


Figure S2. Differential scanning calorimetry traces of lipids

5. General Buffer Preparation Procedure

Preparation of Buffer A- 4.18 g of Bis Tris (10 mM) and 11.68 g of NaCl (100 mM) was dissolved in 2 L of Milli-Q filtered deionized water. The pH was then adjusted to 7.2 by minimal addition of 2 M HCl.

Preparation of Buffer B - 4.18 g of Bis Tris (10 mM) and 11.68 g of NaCl (100 mM) was dissolved in 2 L of Milli-Q filtered deionized water. The pH was then adjusted to 5.8 by minimal addition of 2 M HCl.

Preparation of Buffer C – 5.2 g of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) (10 mM) and 17.5 g of NaCl (150 mM), were dissolved in 2 L of Milli-Q filtered deionized water. The pH was then adjusted to 7.4.

Preparation of Buffer D – 0.46 g of 2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid, N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) (2 mM), 5.84 g of NaCl (100 mM), 0.029 g of ethylenediaminetetraacetic acid (EDTA) (0.1 mM), and 0.31 g of histidine (2 mM) was dissolved in 1 L of Milli-Q filtered deionized water. The pH was then adjusted to 7.4.

Preparation of Buffer E – 0.23 g of TES (2 mM), 2.11 g of NaCl (72.23 mM), 0.62 g of $CaCl_2$ (11.11 mM), 0.015 g of EDTA (0.1 mM), and 0.16 g of histidine (2 mM) was dissolved in 0.5 L of Milli-Q filtered deionized water. The pH was then adjusted to 7.4.

6. General Procedure to Measure pH Equilibrium of CF

CF was used as the reporter dye because CF is a pH responsive fluorophore and is known to exhibit fluorescence quenching upon acidification. We chose the pH values of 7.2 to 5.8 as internal and external liposomal pH values, respectively, because CF exhibits a linear correlation between its fluorescence intensity and environmental pH value with in this pH range.[4]

To estimate the initial rate of pH equilibration from liposomes, the decrease in fluorescence of CF was followed using Perkin Elmer Enspire© multimode plate reader. Before each assay, 10 µL of the stock extruded lipid solution was diluted in 500 µL of buffer A. Free CF was removed using a PD miniTrap™ G-25 Sephadex™ column from GE Healthcare ending in 100 times dilution from the stock extruded solution (0.1 mg/mL). 45 µL of purified liposome solution was next added into three 0.5 mL Protein Lo-Bind tubes for each lipid solution. In one tube, 405 µL of Buffer A was added. In the second tube, 400 µL of Buffer B was added with 5 µL of 100 µM solution of Nigericin in Ethanol. Nigericin, a polyether ionophore known to form pores in membranes, was used as our positive control of complete pH exchange between the internal/external buffer systems. In the third tube, right before starting the measurement, 405 µL of Buffer B was added. 125 µL was added to each well of the plate three times for each tubes resulting in three measurements with three replicates with a total of 9 measurements per lipid solution. No significant morphology change was observed after 5 hours (shown in Figure S3).

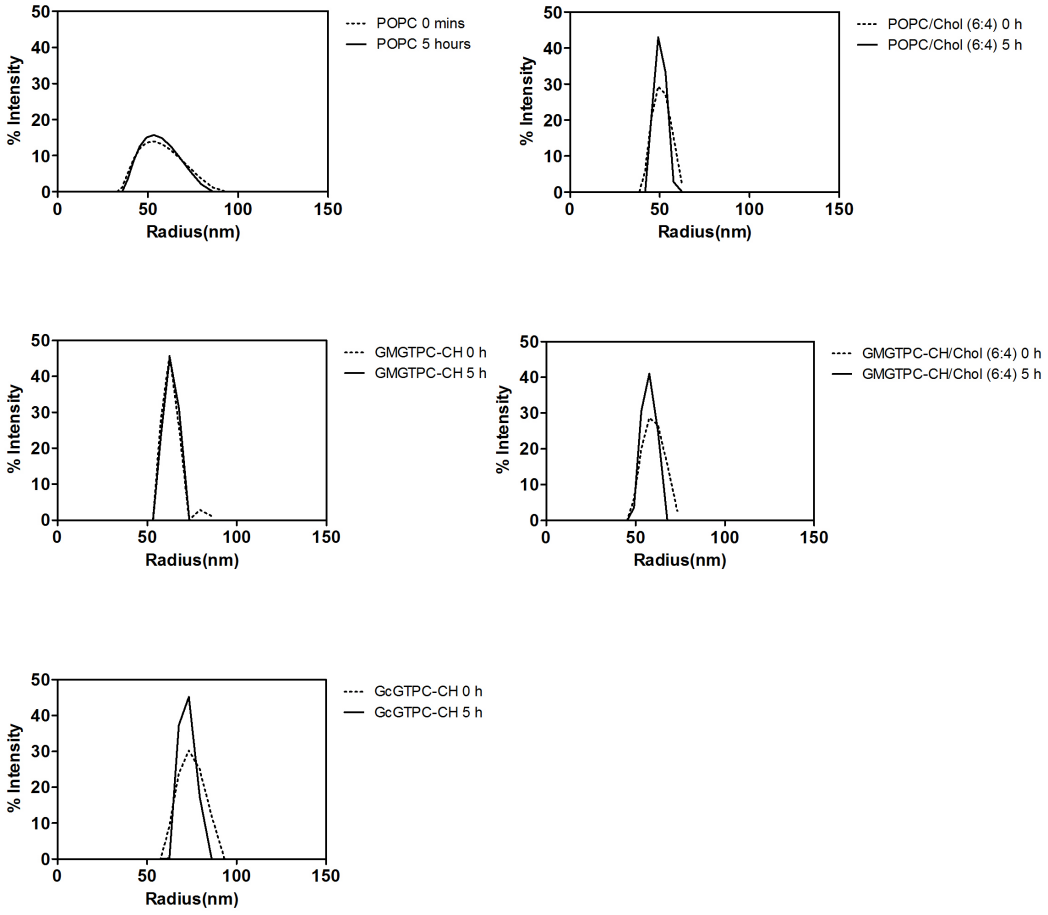


Figure S3. Dynamic light scattering (DLS) measurements of liposomes

7. General Calculation Procedure for Kinetic Analysis

For the pH equilibration leakage assay, the relative fluorescence (F_{rel}) of CF was normalized using equation (1). F_0 represents fluorescence at time 0, F_A represents fluorescence measurements at different times, and F_{Nig} is the fluorescence measurement of the liposome solution including Nigericin at 600 second in buffer B. After the data was normalized, equation (2) was used to determine the rate of decrease in CF fluorescence by combining individual measurements using GraphPad Prism 5 software. Additional experiments that collected more data points (up to 0.35 hours) were performed for liposomes made with pure POPC to obtain a more accurate calculated observed rate using initial rates. (shown in Figure S4).

$$F_{rel} = \left(1 - \frac{(F_0 - F_A)}{(F_0 - F_{Nig})}\right) X\ 100 \qquad (1)$$

$$ln\ (F_{rel}) = -kt \qquad (2)$$

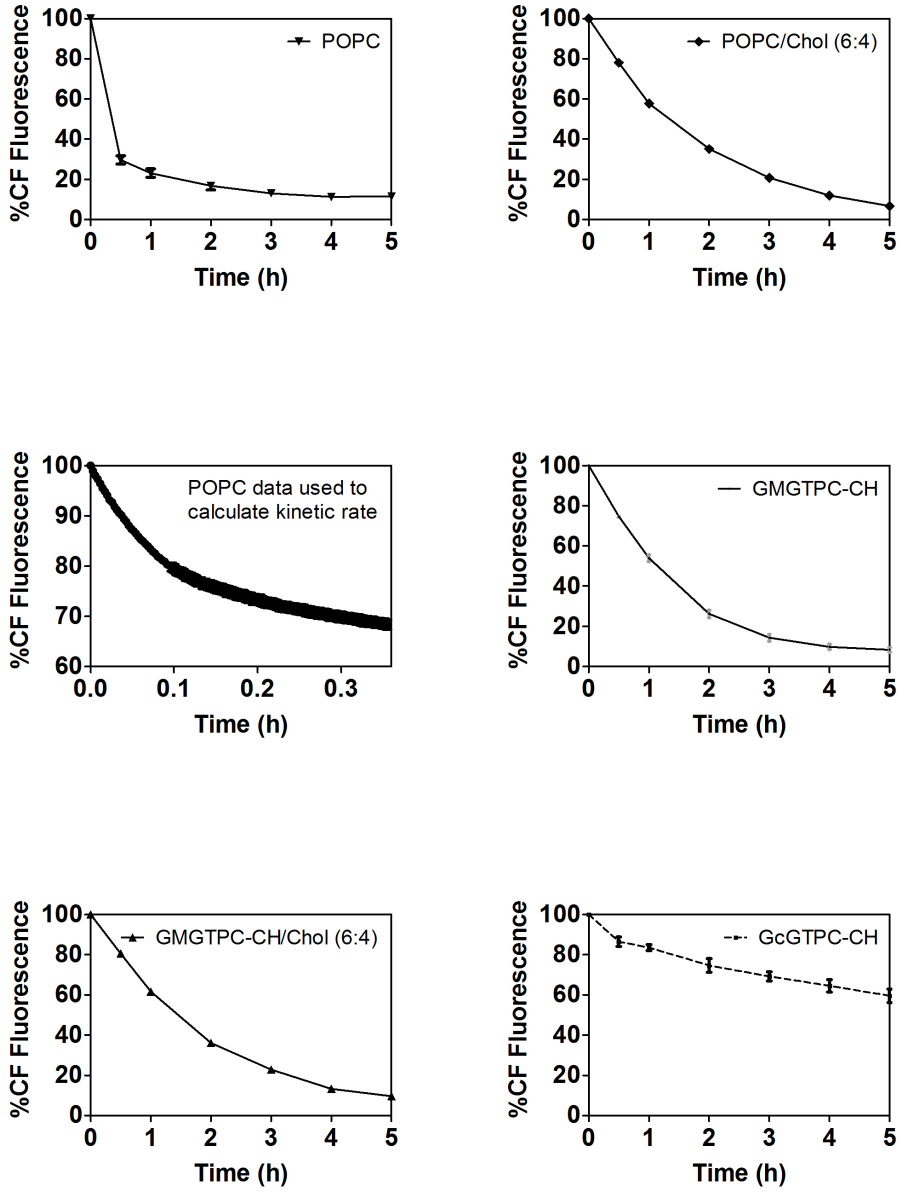


Figure S4. Average plot of 9 technical repeat experiments of % CF fluorescence vs. time (h)

8. Gemcitabine leakage experiment

The *in vitro* leakage of the gemcitabine (GEM) from liposomes was measured using a dialysis assay. Briefly, GEM encapsulated liposomes were made by weighing 10 mg of lipid and 40 mol% of cholesterol (only for POPC and GMGTPC-CH) into a 5 mL round bottom flask and dissolved in 1:1 chloroform/methanol. The solvent was evaporated *via vacuo* to form a lipid film and hydrated with 500 µL of 60 mM GEM in Buffer C. 50 µL of

the stock liposomal formulation (20 mg/mL) was diluted with buffer C to 500 µL and purified using sephadex G-25 to prepare a 1 mL working solution. The purified liposomal suspensions was placed in a dialysis device (Slide-A-Lyzer® Mini dialysis, ref 88405, Thermo Scientific) with a molecular weight cutoff of 20 kDa and dialyzed against 42.5 mL of buffer C at 37 °C and shaken on an orbital shaker (50 rpm). At various time points, aliquots (40 µL) were withdrawn from the dialysis compartment and retention of encapsulated drug was measured by HPLC.

To a vial, 40 µL of a Triton solution (0.5 % w/v in HBS) was added to 40 µL of liposomal suspension. The samples were vortexed and analyzed by HPLC using method A for the detection of GEM. Data was analyzed using Agilent analysis software (Chemstation®). For each time point, the percentage of drug remaining in the liposomes was calculated using the following equation (3):

$$Retained\ drug\ (\%) = 100 \times \frac{Area(t)}{Area\ (t0)} \qquad (3)$$

9. Molecular Dynamics Simulations and Analysis

Lipids were first constructed using Maestro (Schrödinger LLC, New York, NY). Membranes containing 729 lipids were then built and solvated using TIP3P water using VMD[5]. Each system was minimized and heated in 75 K steps, reaching a final temperature of 300 K. Following about 20 ns of equilibration, MD simulations for all membrane systems were performed for 100 ns under isothermal-isobaric (NpT) conditions. The temperature was maintained by using the Nose-Hoover chain method and the pressure was maintained at 1 atm. The CHARMM36 force field was used with a 10 Å cut-off for van der Waals with an 8.5 Å switching distance, and Particle Mesh Ewald for long-range electrostatics. All the simulations were carried out using NAMD[6], and post-simulation trajectory analysis was carried out using both VMD, R (http://www.r-project.org), and the Bio3D package[7,8].

To identify the number of water molecules in the core of the membrane, we used methods described previously[9]. In short, we identified water molecules that penetrated past the head groups and into the carbon-rich region of the membrane, i.e. between the ether or ester oxygens of the head groups on either side of the membrane. The calculation was performed in a grid fashion across the full membrane and the results reported were the total number of waters within the membrane for each snapshot over the final 50 ns of the trajectory shown in Figure S5.

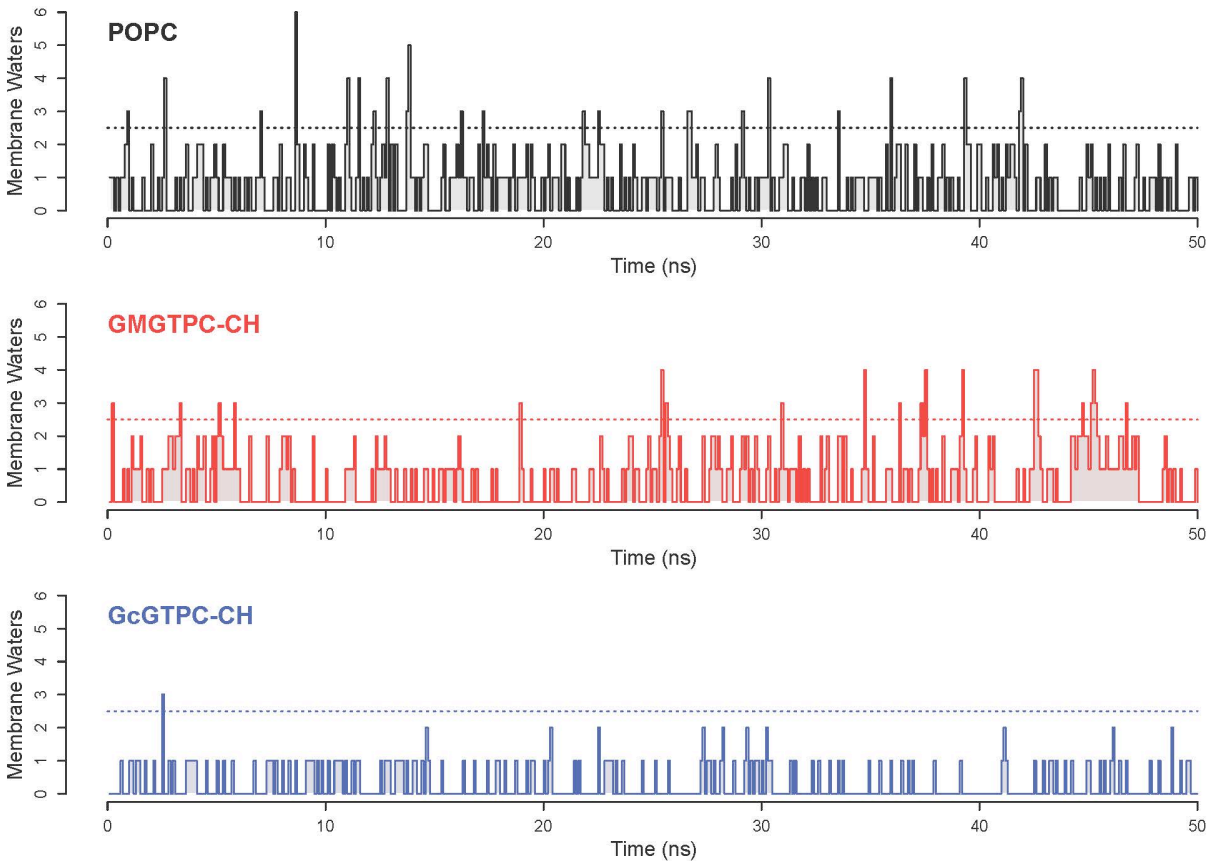


Figure S5. Total number of waters within the membrane for each snapshot over the final 50 ns of the trajectory

10. General Procedure for Monitoring Gramicidin A Activity In Liposomes

Liposomes were prepared as described in general procedures for liposome extrusion. The general procedure to measure pH equilibrium of CF was used with a small modification. After the liposomes were prepared, a solution of gA was prepared 225 μM in DMSO. To each tube (i.e. pH 7.2, pH 5.8, pH 5.8 + nigericin) 2 μL of buffer A was added to control the volume. Additional solution containing 2 μL of DMSO (vehicle) or 2 μL gA (final concentration of 1 μM) was incubated at 37 ºC with liposomes for 30 minutes and CF fluorescence was measured and analyzed. As shown in Figure S6A, no significant effect was observed when 2 μL of DMSO was added. The decrease in percent fluorescence of CF shown in Figure S5A suggests that gA was able to form a channel on the membrane to allow ions to rush inside. DLS measurements were taken to confirm the presence of liposomes after the addition of DMSO and gA, as shown in Figure S6B.

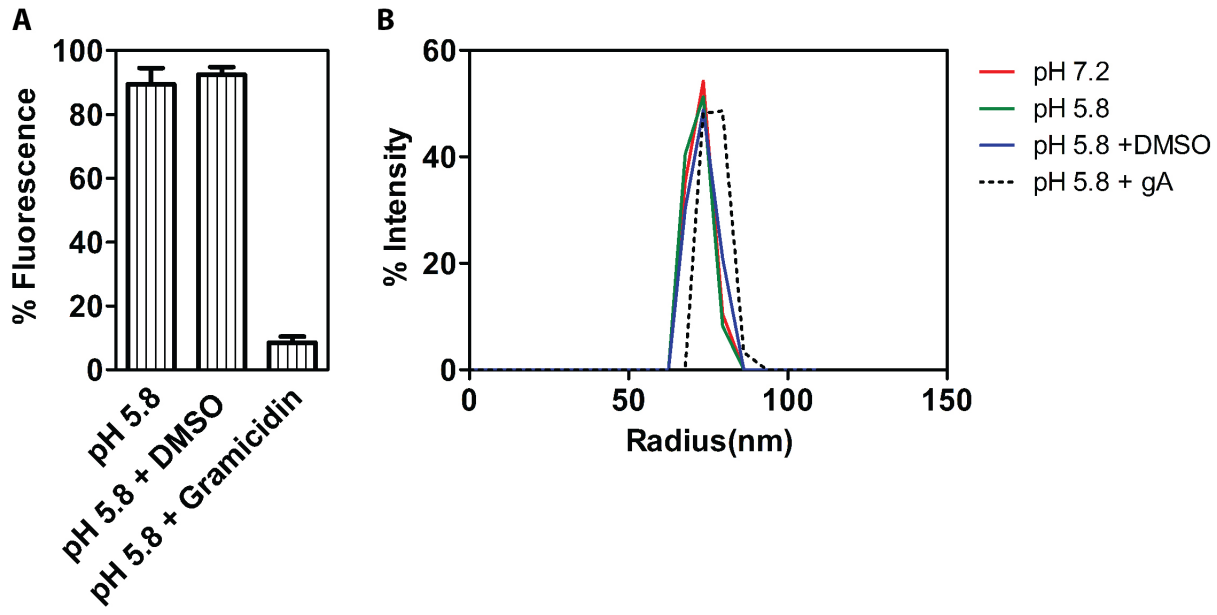


Figure S6. Effect of gramicidin A on GcGTPC-CH liposomes. A) Percent fluorescence of CF after incubation with or without gA at 37 ºC for 30 minutes. B) DLS measurements of GcGTPC-CH liposomes under different conditions.

11. General Procedure for phospholipase-D induced cleavage of choline

Liposomes were prepared as described in general procedures for liposome extrusion. The liposomes were first hydrated in buffer D, then the liposome formulation was incubated in buffer E with or without 5 units of PLD from cabbage (Sigma Aldrich) at 37 ºC for 30 minutes. After incubation, the liposome size was measured using dynamic light scattering (DLS) to study whether a morphology change occurred. As shown in Figure S7A, GcGTPC-CH liposomes without added PLD show a consistent size measured using DLS for 10 acquisitions after 30 mins at 37 ºC. However, as shown in Figure S7B, the size of GcGTPC-CH liposomes with added PLD after 30 mins 37 ºC shows a large variability in the liposomal diameter among the 10 acquisitions. The greater degree of variability is most likely caused by the inability of the DLS instrument to size particles with a great degree of multimodal size distributions caused by the destabilization of the liposomes through aggregation/fusion events.

Additionally, PLD activity was confirmed using a commercially available phosphorus assay kit (Sigma, MAK122) according to the manufacturers protocol. We estimated the percent choline released, which is directly correlated with the phosphorus concentration, by normalization to the phosphorus concentration obtained by the Bartlett assay.

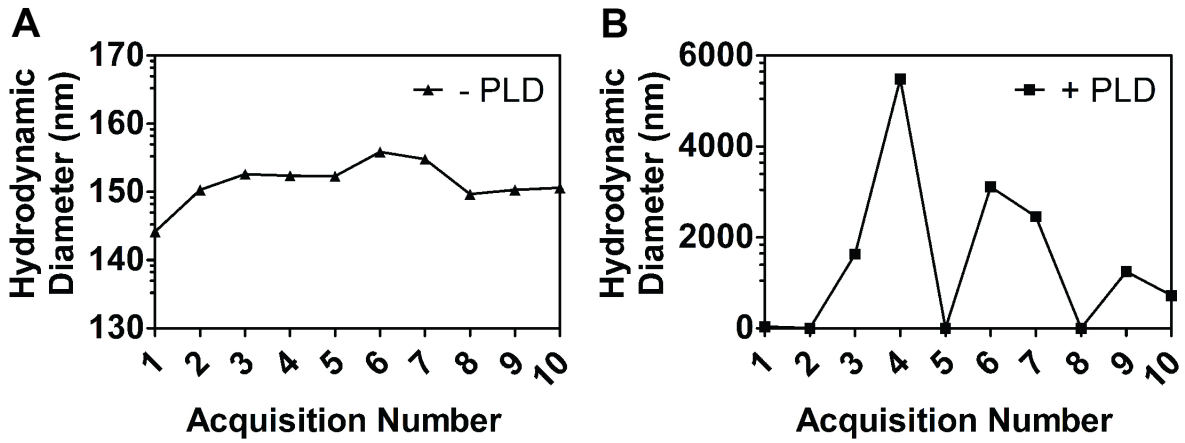


Figure S7. 10 DLS acquisition measurements. A) 10 DLS acquisition measurements without added PLD of GcGTPC-CH liposomes. B) 10 DLS acquisition measurements with added PLD of GcGTPC-CH liposomes.

12. General Procedure for self-quenched CF liposomal release assay in serum

Liposomes prepared as described in general procedures for liposome extrusion. Using the method of self-quenched CF loaded (100 mM in PBS) leakage assay[10], liposomes comprised of GcGTPC-CH (using general procedure for liposome extrusion) were incubated in PBS with 30 % fetal bovine serum (FBS) at 37 ºC (final liposome dilution of 1000 times from stock extruded solution) and CF fluorescence was monitored (Ex 485 nm/Em 517) nm for up to 5 days.

13. General procedure for cellular uptake of small molecule entrapped in GcGTPC-CH liposomes

13.1. Cell toxicity studies of GcGTPC-CH liposomes

KB cells were plated onto a fibronectin-treated 96 well plate at 5000 cells/well in folate deficient Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen). The cells were incubated for 24 hours under a humidified atmosphere of 95% air and 5% CO_2 at 37°C. After 24 hours of incubation, the cells were dosed with various concentrations of the liposomes, in triplicate. The cells were incubated with the liposomes for 24 hours, and then washed to remove unbound compound. After incubation with the liposomes, the cells were carefully washed three times with 200 mL phosphate buffered saline (PBS) buffer and fixed with a solution of 200 µL PBS and 50 mL of 50% trichloroacetic acid. The cells were allowed to fix at 4°C for 1 hr. After fixation, the cells were washed five times with water and allowed to dry. After the plates are dried, 100 mL of a 0.4 % sulforhodamine B (SRB, Sigma Aldrich, S1402) solution in 1% acetic acid was added to each of the wells and incubated for 30 minutes at room temperature on a shaker. The SRB-treated cells were then washed five times with 1 % acetic acid and allowed to dry. Tris base solution (100 mM, 200 mL) was then added to each well and the plates were placed on an oribital shaker for 30 minutes. The plates were then read on a microplate

reader at 515 nm. The absorbance values were used to create cell-toxicity curves. Liposomes showed no toxicity up to 100 μM, as shown in Figure S8.

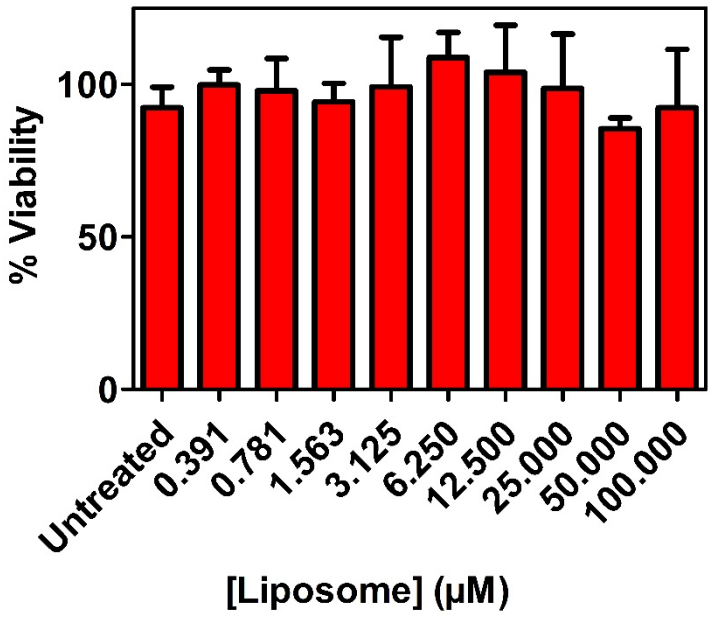


Figure S8. KB cell viability with varying concentration of liposome.

13.2. Fluorescence microscopy of GcGTPC-CH liposome uptake

To a dry lipid film of GcGTPC-CH lipid with added 0.5 mol % of DSPE-PEG2000-folate lipid, 10 mM calcein in buffer A was then used to hydrate the lipid film. Liposomes were then prepared as described in general procedures for liposomes extrusion.
KB cells were plated with folate deficient RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) on 10mm glass bottom plates and incubated for 12 hours. Media was then removed and a solution containing 10 μM of liposomes, free calcein, or PBS control was added to the cells and incubated for 6 hours. Cells were then rinsed with media once, and a fresh media containing 2 μg/mL Hoescht nuclear stain was added. The living cells where then immediately imaged with an Olympus FluoView FV1000 deconvolution IX81 inverted confocal microscope equipped with a 405, 488, and 543 laser line. Fluorescence images were processed with ImageJ shown in Figure S9.

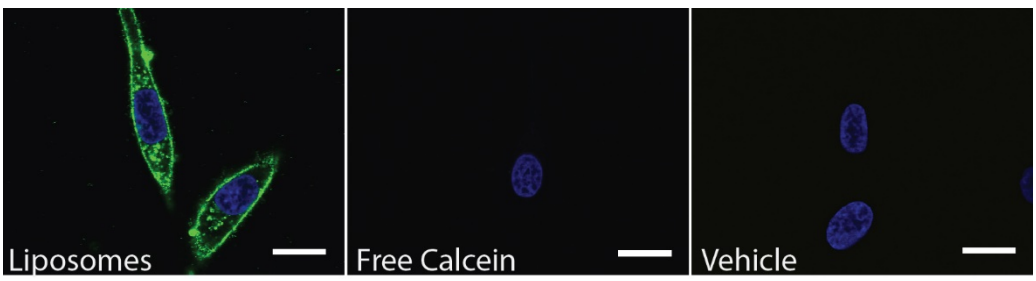
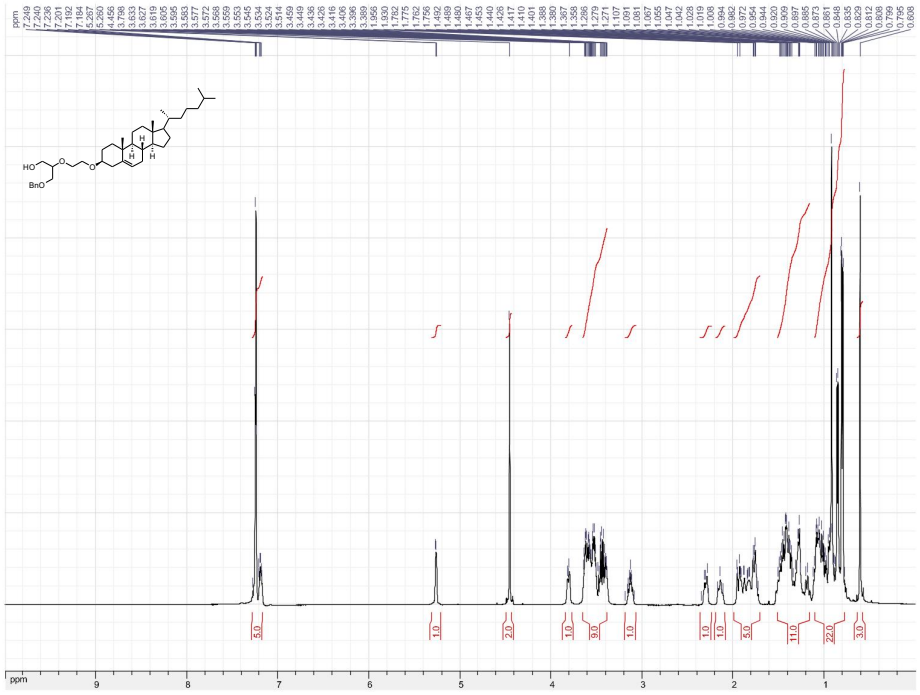
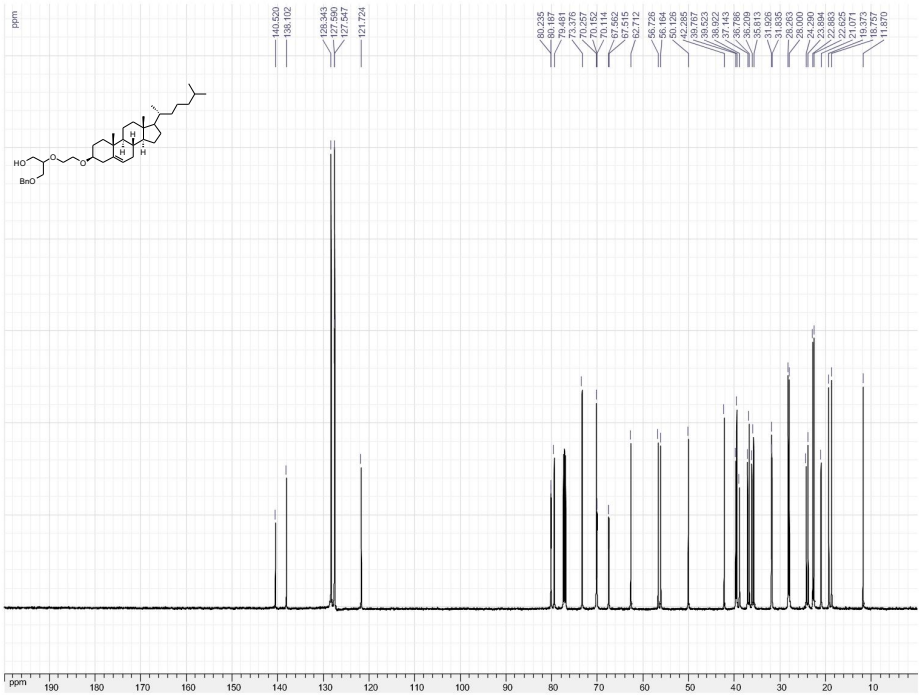


Figure S9. Cell images after cells were incubated with liposomes, free calcein, and vehicle. Scale bar = 20 μm

14. NMR Spectra

Compound 2





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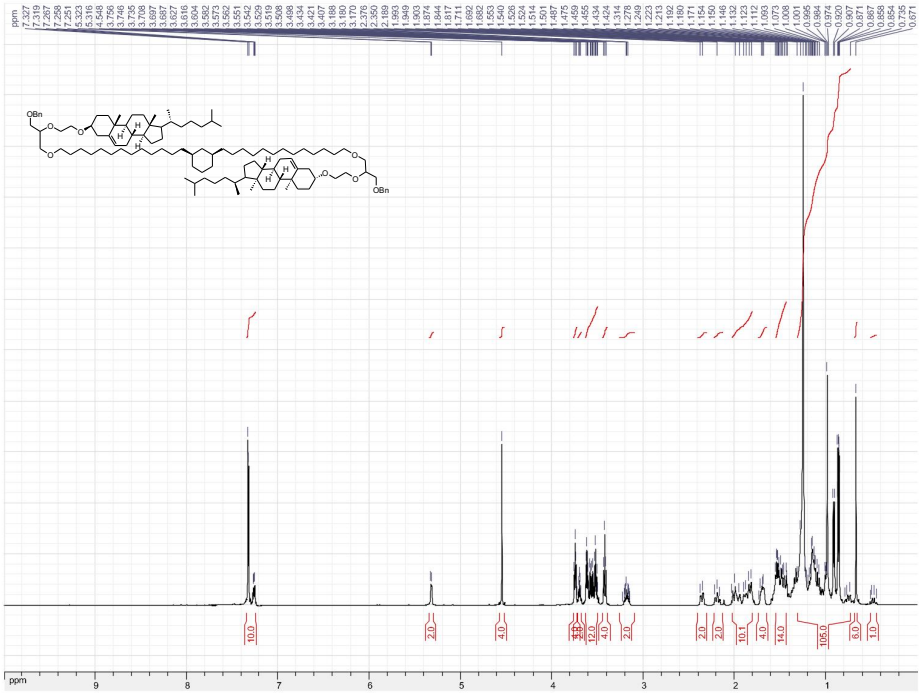

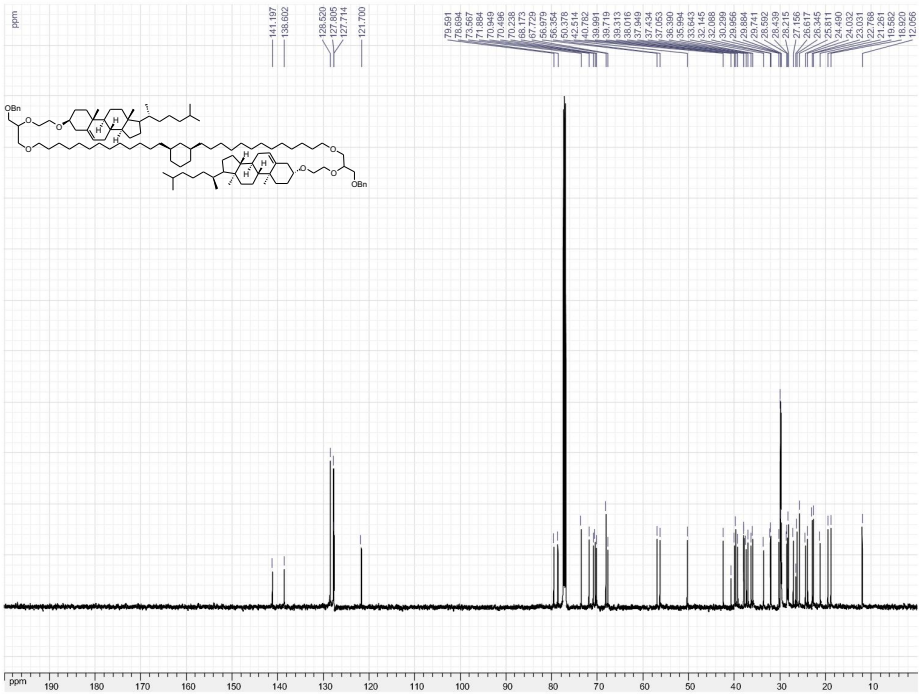

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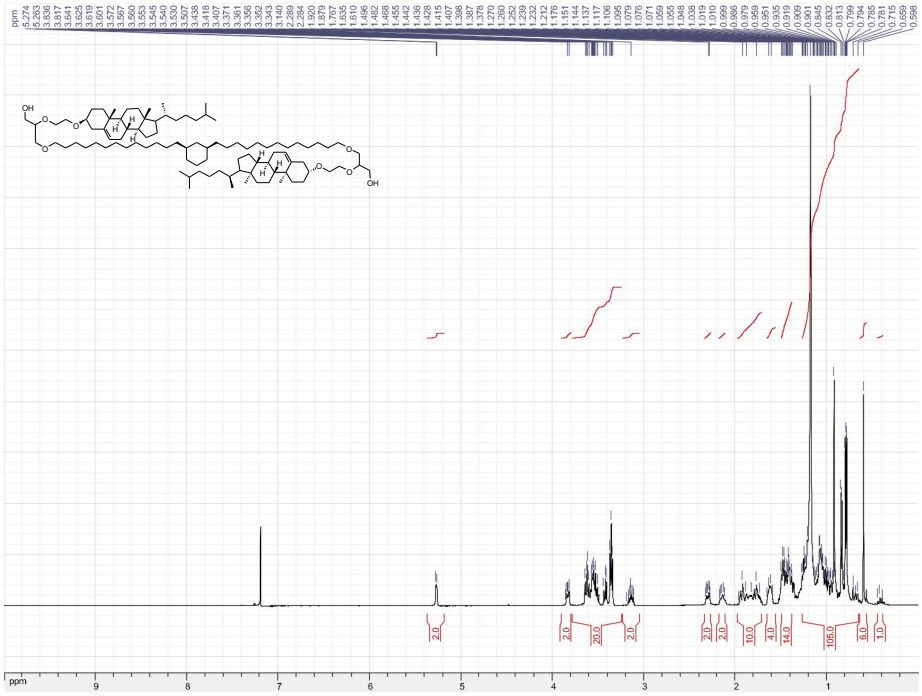
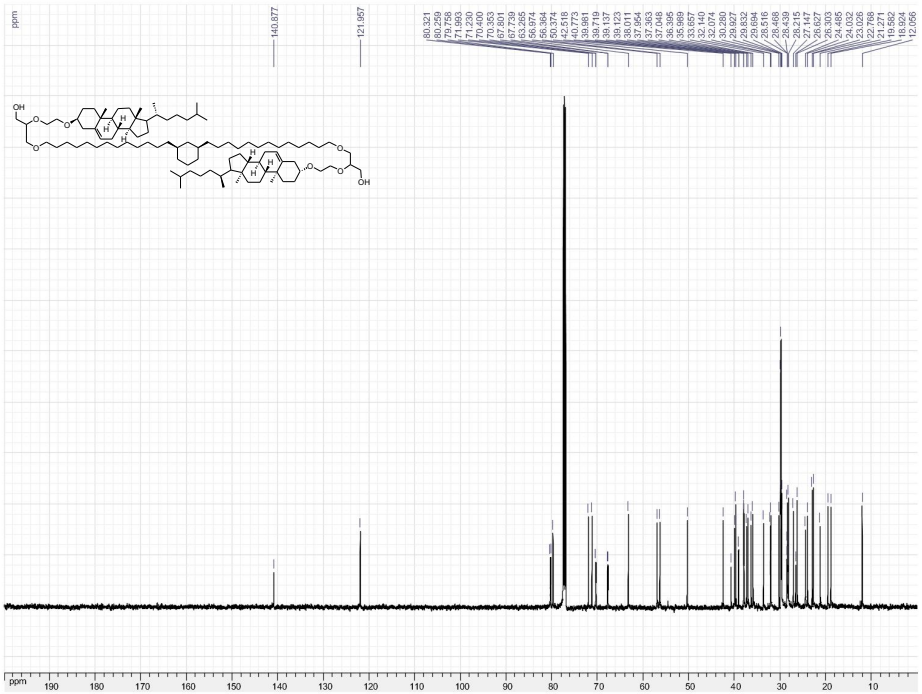
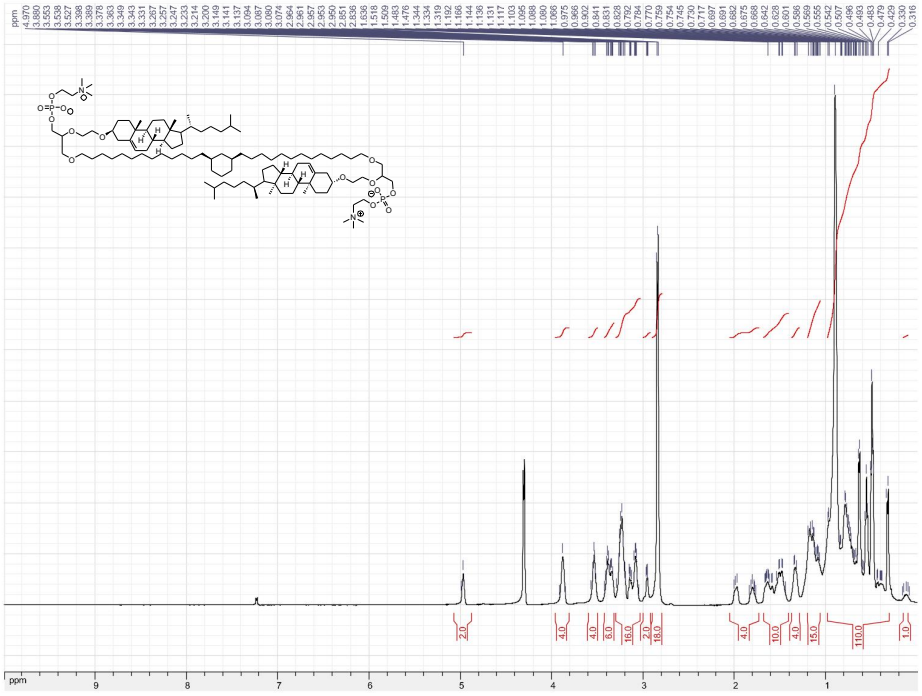
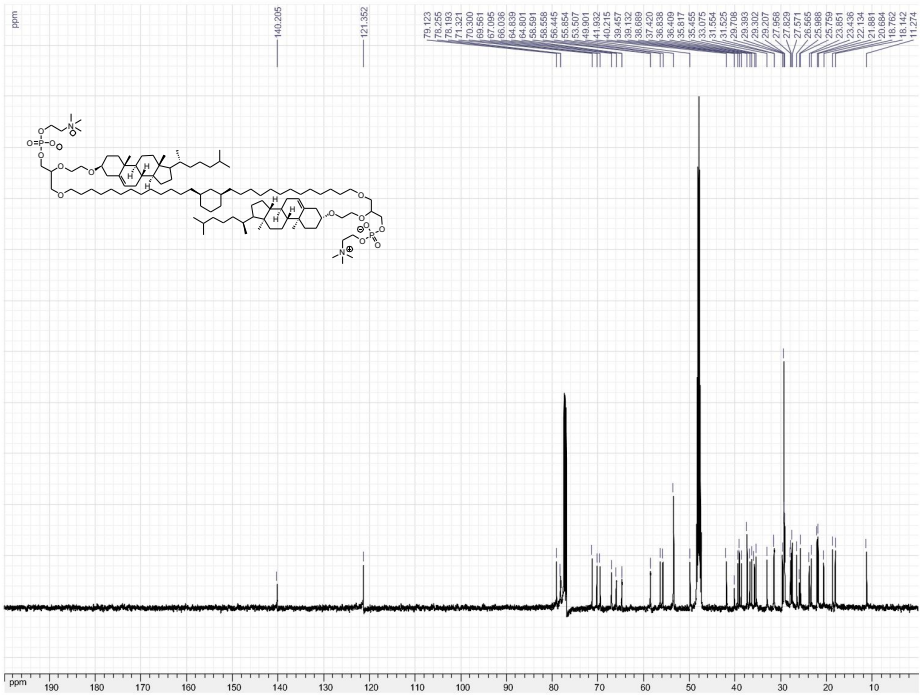
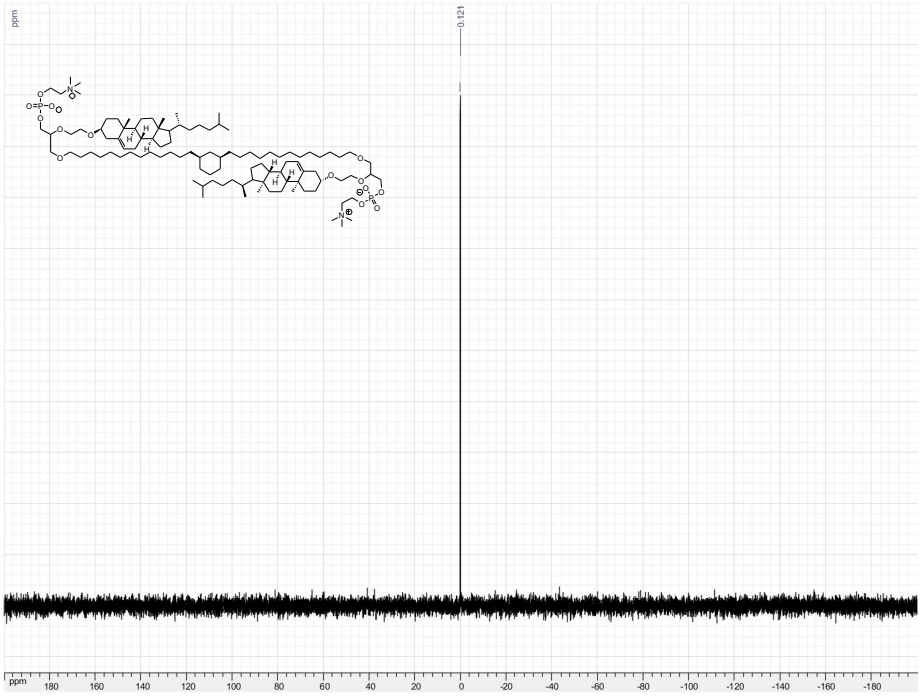




GcGTPC-CH



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