Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Characterization of Drug Encapsulation and Retention in Archaea-Inspired Tetraether Liposomes

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1. General Information

List of abbreviations: Ethyl acetate (EtOAc), Methanol (MeOH), Tetrahydrofuran (THF), Diethyl ether (Et_2O), Sodium sulfate (Na_2SO_4), Dichloromethane (DCM), Hydrochloric acid (HCl), Ethanol (EtOH), Palladium hydroxide ($Pd(OH)_2$), Dimethylsulfoxide (DMSO), Acetonitrile (ACN), Ammonium chloride (NH_4Cl).

All reagents were purchased from commercial sources and used without further purification. EggPC lipids (Avanti Polar Lipids, catalog number 840051) were stored under Argon at -20°C, and used within 3 months of purchase. 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Avanti Polar Lipids, catalog number 810158) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000] (ammonium salt) (Avanti Polar Lipids, catalog number 880124) were stored under Argon at -20°C, and used within one year 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, Dynamic Adsorbents, Inc). Reactions were monitored by thin-layer chromatography (TLC) using 0.25 mm silica gel plates (60F-254, Dynamic Adsorbents, Inc). Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. ^{1}H, ^{13}C, ^{31}P NMR spectra were recorded 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.

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 = 5 μL. Detection: 214, 254, 280 and 300

nm. Internal standards (4-nitrophenol and 4-hydroxybenzophenone) were used to ensure correct quantification. Mobile phase: water/acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA).

Method A: Isocratic conditions at 3% of ACN for 3 minutes followed by a linear gradient from 3% to 95% of ACN in 7 minutes and 2 minutes of re-equilibration at 3% of ACN.

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

Absorbance measurements were taken on a Perkin Elmer Enspire© multimode plate reader. Corning™ clear polystyrene 96-well microplates were used (ref Corning™ 3370).

2. Synthesis of GMGTPC

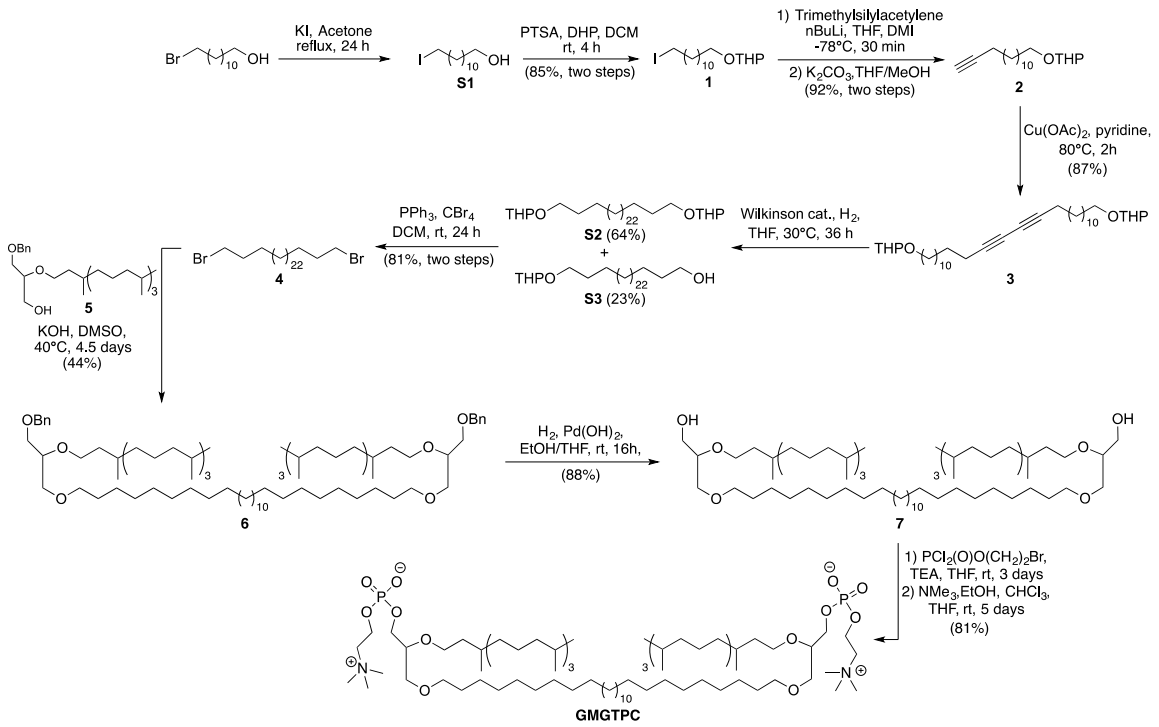
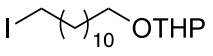


Figure S1. General synthesis of GMGTPC

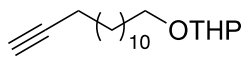
2-((12-iododododecyl)oxy)tetrahydro-2H-pyran (**1**)



Compound **1** (2.7 g, 85% for two steps) was prepared from 12-bromododecan-1-ol (2.1 g, 8.0 mmol) following a reported protocol published by Lee and coworkers[1] and purified by silica gel column chromatography using hexane/EtOAc (98:2 to 95:5).

^{1}H NMR (500 MHz, $CDCl_3$-d_1) δ 4.55 (dd, J = 2.9, 4.4 Hz, 1H), 3.86-3.82 (m, 1H), 3.70 (td, J = 6.9, 9.6 Hz, 1H), 3.49-3.25 (m, 1H), 3.35 (td, J = 6.8, 10.0 Hz, 1H), 3.16 (t, J = 7.1 Hz, 2H), 1.82-1.76 (m, 3H), 1.71-1.66 (m, 1H), 1.59-1.47 (m, 6H), 1.37-1.24 (m, 16H).

2-(tetradec-13-yn-1-yloxy)tetrahydro-2H-pyran (**2**)

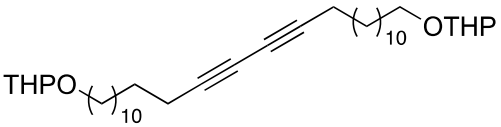


To a solution of trimethylsilylacetylene (0.44 g, 4.5 mmol) in dry THF (12 mL) at -78 °C was slowly added n-BuLi (2.5 M in hexanes, 1.8 mL, 4.5 mmol) over 10 min. After 30 min of stirring at -78 °C, 1,3-Dimethyl-2-imidazolidinone (3 mL) was added. Then, a solution of **1** (1.18 g, 3.0 mmol) in dry THF (3 mL) was added dropwise, and the reaction mixture was stirred at room temperature for 16 hours. The reaction was quenched with saturated NH_4Cl solution (25 mL) and extracted with EtOAc (3x10

mL). The combined organic layers were washed with brine, dried over Na_2SO_4, evaporated and purified by silica gel column chromatography using hexane/EtOAc (98:2 to 95:5) as eluent. To a solution of the TMS-protected product obtained above in MeOH (15 mL) potassium carbonate (3.0 g) was added and the reaction mixture was stirred at room temperature for 16 hours. Solvent was evaporated and the resulting residue was extracted with Et_2O (50 mL). The organic solution was washed with water (2x30 mL), brine (50 mL) and dried over Na_2SO_4. Compound **2** (0.81 g, 92%) was obtained as a colorless oil without further purification.

^{1}H NMR (500 MHz, $CDCl_3$-d_1) δ 4.53 (dd, J = 2.9, 4.4 Hz, 1H), 3.82 (ddd, J = 2.9, 7.8, 11.2 Hz, 1H), 3.71-3.66 (m, 1H), 3.47-3.43 (m, 1H), 3.33 (td, J = 6.8, 10.0 Hz, 1H), 2.13 (dt, J = 2.6, 7.1 Hz, 2H), 1.89 (t, J = 2.6 Hz, 1H), 1.82-1.65 (m, 2H), 1.57-1.44 (m, 8H), 1.35-1.33 (m, 16H); ^{13}C NMR (126 MHz, $CDCl_3$-d_1) δ 99.0, 84.9, 68.2, 67.8, 62.5, 30.9, 29.9, 29.7, 29.6, 29.3, 28.9, 28.6, 26.4, 25.7, 19.8, 18.5.

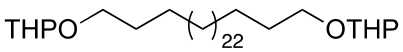
1,28-bis((tetrahydro-2H-pyran-2-yl)oxy)octacosa-13,15-diyne (**3**)



To a solution of **2** (0.80 g, 2.73 mmol) in dry pyridine (9 mL), Copper(II) acetate monohydrate (1.10 g, 5.47 mmol) was added and the reaction was stirred at 80 °C for 2 hours. The reaction mixture was then cooled to room temperature, and acidified to pH 2 using a 1 M HCl (aq) solution. The mixture was extracted with EtOAc (3x30 mL) and combined organic layers were washed with 1% HCl solution (50 mL), water (50 mL), brine (50 mL) and dried over Na_2SO_4. Diyne **3** (0.69 g, 87%) was obtained as a colorless oil after purification by silica gel column chromatography using hexane/EtOAc (100:0 to 90:10) as eluent.

Rf: 0.29 (hexane/EtOAc 95:5); ^{1}H NMR (500 MHz, $CDCl_3$-d_1) δ 4.52 (dd, J = 2.9, 4.4 Hz, 2H), 3.82 (ddd, J = 2.9, 7.8, 11.2 Hz, 2H), 3.70-3.65 (m, 2H), 3.47-3.43 (m, 2H), 3.32 (td, J = 6.8, 10.0 Hz, 2H), 2.18 (t, J = 7.1 Hz, 4H), 1.81-1.74 (m, 2H), 1.69-1.63 (m, 2H), 1.57-1.42 (m, 16H), 1.33-1.21 (m, 32H); ^{13}C NMR (126 MHz, $CDCl_3$-d_1) δ 98.9, 77.6, 67.8, 65.4, 62.4, 30.9, 29.9, 29.7, 29.6, 29.2, 29.0, 28.5, 26.4, 25.6, 19.8, 19.3.

1,28-bis((tetrahydro-2H-pyran-2-yl)oxy)octacosane (**S2**)

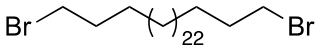


Diyne **3** (0.69 g, 1.18 mmol) was dissolved in degassed THF (12 mL) and Wilkinson catalyst (0.22 g, 0.24 mmol) was added. The reaction was stirred under hydrogen atmosphere at 30 °C for 36 hours. Solvent was evaporated and the resulting residue was purified by silica gel column chromatography using hexane/EtOAc (100:0 to 95:5) as eluent. **S2** (0.45 g, 64%) was obtained as a white solid while

28-((tetrahydro-2*H*-pyran-2-yl)oxy)octacosan-1-ol **S3** (0.14 g, 23%) was also isolated as a white solid using hexane/EtOAc (80:20) as eluent.

Rf: 0.38 (hexane/EtOAc 95:5); ^{1}H NMR (400 MHz, $CDCl_3$-d_1) δ 4.55 (dd, J = 2.9, 4.4 Hz, 2H), 3.87-3.83 (m, 2H), 3.70 (td, J = 6.9, 9.6 Hz, 2H), 3.49-3.44 (m, 2H), 3.35 (td, J = 6.8, 10.0 Hz, 2H), 1.84-1.76 (m, 2H), 1.72-1.66 (m, 2H), 1.58-1.46 (12H), 1.33-1.16 (m, 48H); ^{13}C NMR (100 MHz, $CDCl_3$-d_1) δ 99.0, 67.9, 62.5, 31.0, 29.9, 29.8, 29.7, 26.4, 25.7, 19.9; ESI-MS: 617.5 $[M+Na]^+$; HRMS 617.5479 calcd for $[C_{38}H_{74}O_4Na]^+$, found 617.5480.

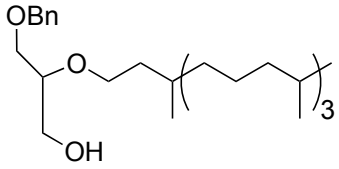
1,28-dibromooctacosane (**4**)



Carbon tetrabromide (1.02 g, 3.08 mmol) was added to a solution of **S2** (0.45 g, 0.76 mmol) and **S3** (0.14 g, 0.27 mmol) in dry DCM (17 mL). After stirring for 10 min, the solution was cooled down and triphenylphosphine (1.62 g, 6.15 mmol) was added. The mixture was stirred 24 hours at room temperature and purified by silica gel column chromatography using DCM/hexane (1:1) as the eluent. 1,28-dibromooctacosane **4** (0.53 g, 81% two steps from **3**) was obtained as a white solid.

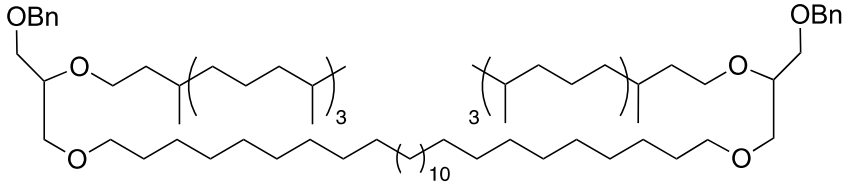
Rf: 0.90 (DCM/hexane 1:1); ^{1}H NMR (500 MHz, $CDCl_3$-d_1) δ 3.39 (t, J = 6.9 Hz, 4H), 1.86-1.80 (m, 4H), 1.42-1.37 (m, 4H), 1.29-1.23 (m, 44H); ^{13}C NMR (126 MHz, $CDCl_3$-d_1) δ 34.3, 33.0, 29.9, 29.8, 29.8, 29.7, 29.0, 28.4.

3-(benzyloxy)-2-((3,7,11,15-tetramethylhexadecyl)oxy)propan-1-ol (**5**)



Compound **5** was synthesized following a reported protocol.[2]

18,51-bis((benzyloxy)methyl)-2,6,10,14,55,59,63,67-octamethyl-17,20,49,52-tetraoxaoctahexacontane (**6**)

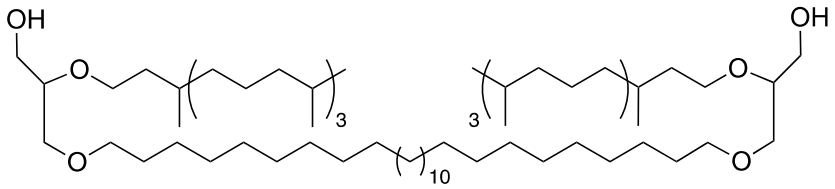


A suspension of KOH (192 mg, 3.44 mmol) in dry DMSO (4 mL) was stirred at room temperature for 30 minutes. The mixture was cooled with ice water and a solution of **4** (238 mg, 0.43 mmol) and **5** (600 mg, 1.30 mmol) in dry DMSO (4 mL) was added. The mixture was then stirred at room temperature for 16 hours and then at 40 °C for 3 days. Water (250 mL) was added and the mixture was extracted with EtOAc (5x50 mL). The combined organic layers were washed with water (2x100 mL), brine (100

mL) and dried over Na_2SO_4. Purification by silica gel column chromatography using hexane/EtOAc (95:5) as the eluent yielded **6** (246 mg, 44%) as a colorless oil.

Rf: 0.26 (hexane/EtOAc 95:5); ^{1}H NMR (500 MHz, $CDCl_3$-d_1) δ 7.32-7.25 (m, 10H), 4.54 (s, 4H), 3.64-3.45 (m, 14H), 3.41 (t, J = 6.9 Hz, 4H), 1.67-1.48 (m, 10H), 1.36-1.02 (m, 90H), 0.86-0.82 (m, 30H); ^{13}C NMR (126 MHz, $CDCl_3$-d_1) δ 138.6, 128.5, 127.8, 127.7, 78.1, 73.5, 71.9, 70.9, 70.5, 69.1, 39.6, 37.7, 37.7, 37.6, 37.6, 37.5, 37.4, 37.3, 33.0, 30.0, 29.9, 29.9, 29.7, 28.2, 26.3, 25.0, 24.7, 24.6, 22.9, 22.8, 20.0, 19.9, 19.8.

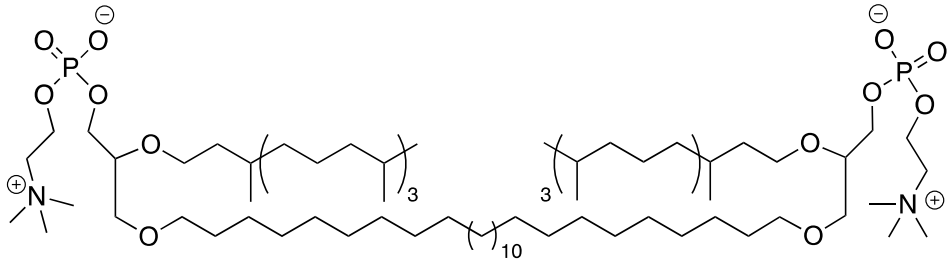
3-((28-(3-hydroxy-2-((3,7,11,15-tetramethylhexadecyl)oxy)propoxy)octacosyl) oxy)-2-((3,7,11,15-tetramethylhexadecyl) oxy)propan-1-ol (**7**)



Compound **6** (246 mg, 0.19 mmol) was dissolved in a degassed mixture of EtOH/THF (1:1, 13 mL) and 20% $Pd(OH)_2$ (25 mg, 10% w/w) was added. The reaction was stirred under hydrogen atmosphere at room temperature for 6 hours. The catalyst was removed by filtration through a pad of celite, and the resulting residue was purified by column chromatography on silica gel using hexane/EtOAc (9:1 to 8:2) as the eluent. Diol **7** (187 mg, 88%) was obtained as a white solid.

Rf: 0.52 (hexane/EtOAc 8:2); ^{1}H NMR (500 MHz, $CDCl_3$-d_1) δ 3.71-3.39 (m, 18H), 2.16 (brs, 2H), 1.62-1.45 (m, 10H), 1.39-1.02 (m, 90H), 0.85-0.81 (m, 30H); ^{13}C NMR (126 MHz, $CDCl_3$-d_1) δ 78.5, 72.1, 72.1, 68.9, 63.3, 39.6, 37.7, 37.7, 37.6, 37.5, 37.5, 37.3, 37.2, 33.0, 30.0, 29.9, 29.8, 29.7, 28.2, 26.3, 25.0, 24.7, 24.6, 22.9, 22.8, 20.0, 19.9,19.8; ESI-MS: 1135.8 $[M+H]^+$.

(octacosane-1,28-diylbis(oxy))bis(2-((3,7,11,15-tetramethylhexadecyl)oxy) propane-3, 1-diyl) bis(2-(trimethylammonio)ethyl) bis(phosphate) (**GMGTPC**)



First, bromoethyldichlorophosphate was prepared following a reported protocol.[3] To a solution of bromoethyldichlorophosphate (610 mg, 2.53 mmol) in dry THF (11 mL), a solution of **7** (359 mg, 0.32 mmol) and Et_3N (0.48 mL, 3.42 mmol) in dry THF (11 mL) was added dropwise. After stirring the mixture for 3 days in the

dark at room temperature, toluene (100 mL) was added to precipitate triethylammonium chloride. Then, the solution was filtered through a small pad of celite and the filtrate concentrated. The resulting residue was dissolved in a mixture of THF/$NaHCO_3$ (sat) (1:1, 100 mL) and the reaction was stirred for 16 hours at room temperature. Solvents were evaporated under vacuum and the resulting aqueous solution was acidified to pH 1 using a dilution solution of HCl (1M) and extracted using several portions of DCM/MeOH (8:2) (5x30 mL). The organic layers were combined, dried over Na_2SO_4 and concentrated under reduced pressure. The resulting residue was used in the next step without further purification. To a solution of the previous crude intermediate in a mixture of THF/$CHCl_3$ (2:1) (7.5 mL), Me_3N (33% in EtOH) (10 mL) 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/H_2O (70:30:5) as the eluent. Lipid **GMGTPC** (380 mg, 81%) was obtained as a white gum.

Rf: 0.33 (DCM/MeOH/H_2O 70:30:5); ^{1}H NMR (500 MHz, MeOD-d_4/$CDCl_3$-d_1 1:1) δ 4.00-3.96 (m, 4H), 3.63 (t, *J* = 5.5 Hz, 4H), 3.40-3.31 (m, 12H), 3.23-3.17 (m, 6H), 2.95 (s, 18H), 1.39-1.22 (m, 10H), 1.14-0.79 (m, 90H), 0.62-0.58 (30H); ^{13}C NMR (126 MHz, MeOD-d_4/$CDCl_3$-d_1 1:1) δ 77.8, 77.7, 77.7, 77.6, 71.3, 70.2, 68.6, 68.3, 66.0, 64.7, 64.6, 58.6, 58.5, 53.5, 39.0, 37.3, 37.1, 37.0, 36.8, 36.8, 36.7, 32.4, 29.5, 29.3, 29.1, 27.5, 25.6, 24.3, 24.0, 22.0, 21.9, 19.1, 19.0, 19.0, 18.9, 18.9; ^{31}P NMR (202 MHz, MeOD-d_4/$CDCl_3$-d_1 1:1) δ 0.16; ESI-MS: 1466.0 $[M+H]^+$; HRMS calcd 1466.2615 for $[C_{84}H_{175}N_2O_{12}P_2]^+$, found 1466.2597.

3. Liposome preparation

N.B. HBS buffer refers to a buffer solution made of 10 mM HEPES and 150 mM NaCl, at pH 7.4.

20 mg/mL liposome solutions were prepared by first dissolving 10 mg of lipid of interest (EPC or GMGTPC) 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 high vacuum pump (Welch 1402) for 4 hours.

Passive encapsulation of Cytarabine (Ara-C) and Methotrexate (MTX): The thin lipid films were hydrated, in solutions of either Ara-C (200 mM) or MTX (10 mM) in HBS buffer by vortexing the solutions for 30 seconds followed by sonication in a water bath sonicator (Branson 2510) for 30 minutes. After sonication, the lipid mixtures underwent 5 freeze thaw cycles that consisted of 2 minutes at -78°C followed by 2 minutes at 50 °C. The lipid solutions were then extruded (Avanti mini-extruder) through 100 nm polycarbonate membrane 25 times followed by another extrusion with a 50 nm polycarbonate membrane 25 times and 11 times for GMGTPC and EPC, respectively. Non-encapsulated drugs were removed by passing the liposomes (100 µL of extruded solutions) through two successive PD

miniTrap™ G-25 Sephadex™ columns (GE Healthcare). Lipid concentrations were measured using the Bartlett assay[4] and liposome radii were measured using DLS (Figure S2). Drug concentrations were determined from titration curves (1 to 100 μM) using HPLC. Purified liposomes were used immediately after preparation.

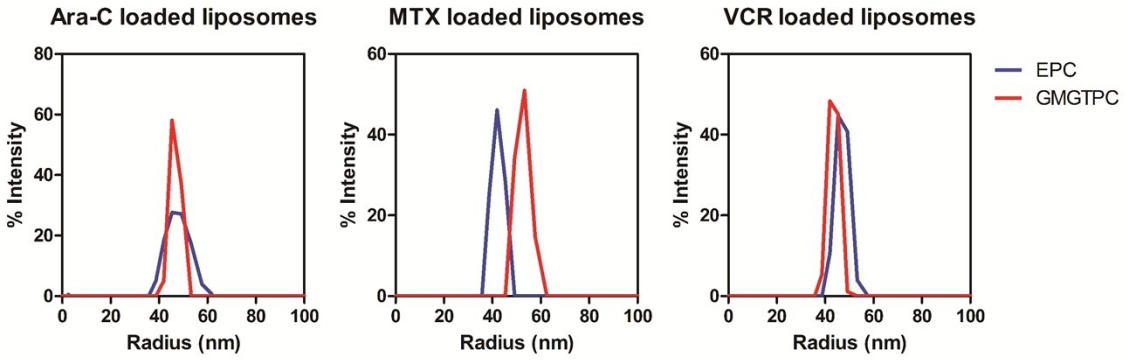


Figure S2. DLS hydrodynamic radius of EggPC and GMGTPC liposomes (blue and red lines respectively) loaded with Ara-C, MTX and VCR.

	Drug to lipid ratio *(μmol of drug/mg of lipid)*	
Drug	**GMGTPC**	**EPC**
Ara-C	0.32	0.29
MTX	0.016	0.012

Figure S3. Drug to lipid ratio measured after passive loading of Ara-C and MTX in EPC and GMGTPC liposomes.

Active encapsulation of Vincristine (VCR): The thin lipid films were hydrated in a citrate buffer (300 mM, pH 4) buffer by vortexing the solutions for 30 seconds followed by sonication in a water bath sonicator (Branson 2510) for 30 minutes. The lipid solutions were then extruded (Avanti mini-extruder) through 100 nm polycarbonate membrane 25 times followed by another extrusion with a 50 nm polycarbonate membrane 25 times and 11 times for GMGTPC and EPC, respectively. Lipid concentrations were measured using the Bartlett assay.[4] Extruded solutions (100 μL) were then subjected to a buffer exchange by gel filtration through PD miniTrap™ G-25 Sephadex™ columns equilibrated with HBS buffer. VCR was added as an aqueous solution of its sulfate salt to the preformed liposomes (drug/lipid ratio 0.1:1). The resulting solution were then incubated for 10 minutes at 60° C. Non-encapsulated VCR was removed by gel filtration on a PD miniTrap™ G-25 Sephadex™ columns. Encapsulation efficiency was then determined by HPLC and liposome radii were measured using DLS (Figure S2). Purified liposomes were used immediately after preparation.

4. Cryo-EM imaging of GMGTPC liposomes

5 µL of GMGTPC liposomes in PBS (2 mg/mL, prepared by extrusion) was applied to a holey grid, which had been previously treated by glow discharged in an oxygen plasma chamber. The Cryo-EM sample was vitrified using liquid ethane as the cryogen. The frozen sample was transferred into a precooled cryo-transfer holder to maintain low temperature. The image was acquired on a FEI Tecnai G2 Sphera operated at 200 keV using a Gatan Ultrascan 1000 UHS 4 MP CCD camera.

5. Drug leakage experiments

The *Ex vitro* leakage of the drugs from liposomes was measured using a dialysis method. Purified liposomal suspensions were diluted to 0.3 mg/mL (1.2 mL) in HBS buffer and placed in a dialysis device (Slide-A-Lyzer® Mini dialysis, ref 88405, Thermo Scientific) with a molecular weight cutoff of 20 kDa. Liposomes were dialyzed against 45 mL of buffer at 37 °C. 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, containing 5 µL of internal standard (1 mM in HBS) and 35 µL of a Triton solution (2% w/v in HBS) was added 40 µL of liposomal suspension. To avoid coelution between the drug and the internal standard, two different internal standards were used:

- 4-nitrophenol for the titration of Ara-C and MTX

- 4-hydroxybenzophenone for the titration of VCR

Samples were vortexed and analyzed by HPLC using method A for the detection of Ara-C and method B for the detection of MTX and VCR (see General information above). Data were analyzed using Agilent analysis software (Chemstation®). Drug signals were normalized according to the area of the peak of the internal standard. For each time point, the percentage of drug remaining in the liposomes was calculated using the following equation (1):

$$Retained\ drug\ (\%) = 100 \times \frac{Area(t)}{Area\ (t0)} \quad (1)$$

The observed rate of leakage of VCR and Ara-C was then calculated using equation (2) by combining individual measurements (n=3) using GraphPad Prism 5 software.

$$ln\ (Retained\ Drug) = -kt \quad (2)$$

The rate of drug diffusion across the dialysis membrane was also measured in the same conditions at 50 µM, 40 µM and 40 µM for Ara-C, MTX and VCR, respectively.

6. Cellular uptake of GMGTPC liposomes

6.1. Cell toxicity study of GMGTPC liposomes

Suspended GMGTPC liposomes (≈100 nm diameter) in HBS buffer were prepared as described in section 3 (Liposome Preparation).

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 GMGTPC liposomes, in triplicate. The cells were incubated with the liposomes for 24 hours, and then washed to remove unbound liposomes. After incubation with the liposomes, the cells were washed three times with 200 µL phosphate buffered saline (PBS) buffer and fixed with a solution of 200 µL PBS and 50 µL 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. Then, 100 µL of a 0.4 % sulforhodamine B (SRB, Sigma Aldrich, S1402) solution in 1% acetic acid was added to each well and incubated for 30 minutes at room temperature while shaking using an orbital shaker. The SRB-treated cells were then washed five times with 1 % acetic acid and allowed to dry. Tris base solution (100 mM, 200 µL) was then added to each well and incubated for 30 minutes at room temperature while shaking. Absorbance of each well was then read using a microplate reader at 515 nm. Liposomes showed no toxicity up to 100 µM, as shown in Figure S4.

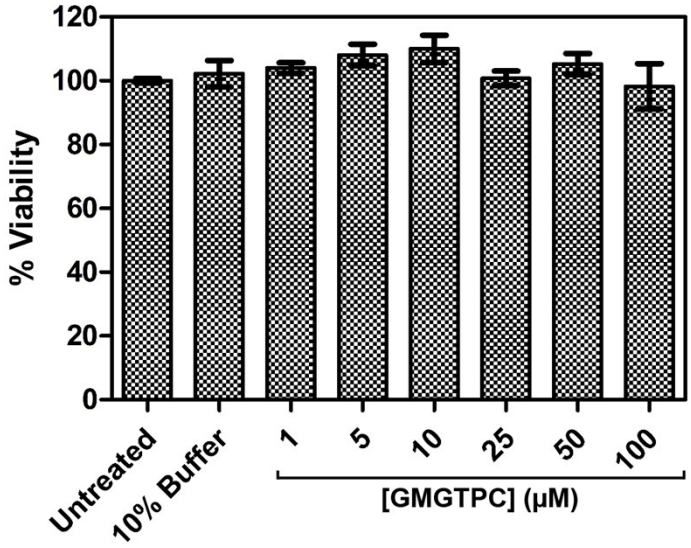


Figure S4. Cytotoxicity of GMGTPC liposomes on KB cells after 24 hours of incubation.

6.2. GMGTPC liposome uptake

Two dry lipid films were prepared using GMGTPC/Rh-PE/FA-PEG_{2000}-DSPE (ratio 98.5/1/0.5 mol%) for targeted liposomes and GMGTPC/Rh-PE (ratio 99/1 mol%) for untargeted liposomes. Both thin lipid films were then hydrated in HBS buffer by vortexing the solutions for 30 seconds followed by sonication in a water bath sonicator (Branson 2510) for 30 minutes. After sonication, the lipid mixtures underwent 5 freeze thaw cycles that consisted of 2 minutes at -78°C followed by 2 minutes at 50°C. The lipid solutions were then extruded (Avanti mini-extruder) through 100 nm polycarbonate membrane 25 times followed by another extrusion with a 50 nm polycarbonate membrane 25 times. Lipid concentrations were measured using the Bartlett assay[4] and liposome radius were measured using DLS.

KB cells were plated with folate deficient RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) on Lab-Tek®II Coverglass system (Nunc, catalog number 155409) previously coated with collagen type IV. Cells were incubated with either HBS buffer (control) or rhodamine labeled liposomes (10 μM) for 5 hours. Cells were then rinsed with cold PBS once, and a fresh media containing 2 μg/mL Hoescht nuclear stain was added for 10 min. After three more washings with cold PBS, the living cells were imaged with an Olympus FluoView FV1000 deconvolution IX81 inverted confocal microscope equipped with a 405, 488, and 543 laser line.

6.3. Cytoplasmic delivery of calcein mediated by GMGTPC liposomes

Two dry lipid films were prepared using GMGTPC/FA-PEG_{2000}-DSPE (ratio 99.5/0.5 mol%) for targeted liposomes and GMGTPC only for untargeted liposomes. Both thin lipid films were then hydrated in a 10 mM calcein solution in HBS buffer (pH 7.4), by vortexing the solutions for 30 seconds followed by sonication in a water bath sonicator (Branson 2510) for 30 minutes. After sonication, the lipid mixtures underwent 5 freeze thaw cycles that consisted of 2 minutes at -78°C followed by 2 minutes at 50°C. The lipid solutions were then extruded (Avanti mini-extruder) through 100 nm polycarbonate membrane 25 times followed by another extrusion with a 50 nm polycarbonate membrane 25 times. Free calcein was removed by passing the liposomes through a Sephadex G-100 column equilibrated in HBS buffer. Lipid concentrations were measured using the Bartlett assay[4] and liposome radius were measured using DLS. Calcein encapsulated concentration was measured by absorbance (at 490 nm) using a titration curve.

KB cells were plated with folate deficient RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) on Lab-Tek®II Coverglass system (Nunc, catalog number 155409) previously coated with collagen type IV. Cells were incubated with either HBS buffer (control) or calcein encapsulated liposomes (100 μM) or

free calcein (5 μM) for 6 hours. Cells were then rinsed with cold PBS once, and a fresh media containing 2 μg/mL Hoescht nuclear stain was added for 10 min. After three more washings with cold PBS, the living cells were imaged with an Olympus FluoView FV1000 deconvolution IX81 inverted confocal microscope equipped with a 405, 488, and 543 laser line.

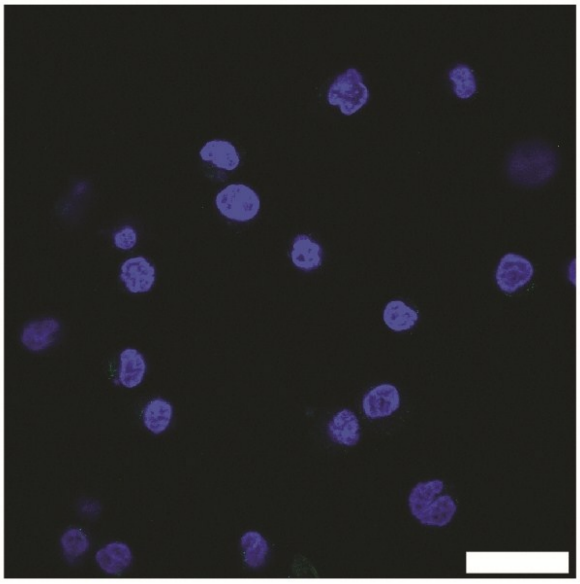


Figure S5. Incubation of free calcein (5 μM, 6 h) on KB-cells. Scale bar: 20 μm.

6.4. Cytoplasmic delivery of cytarabine and vincristine mediated by GMGTPC liposomes

Liposomes-encapsulated cytarabine (Ara-C) and vincristine (VCR) were prepared as described in section 3 (Liposome Preparation).

An MTT cell proliferation assay was performed to assess the drug delivery capacity of loaded GMGTPC liposomes. Briefly, KB cells were plated in 96-well plates at a density of 5,000 cells/well in 100 μL of folate deficient RPMI-1640 medium supplemented with 10% FBS (complete medium). After adhering overnight, the medium was replaced with 100 μL of fresh serum-free medium containing various concentrations of either free drug, drug-loaded GMGTPC liposomes with (+) and without (-) FA-PEG_{2000}-DSPE, or empty GMGTPC liposomes. Final concentrations of drugs ranged from 0-600 μM and final concentrations of lipids ranged from 0-4 mM depending on the experiment. Cells were exposed to these solutions for 2 hours at 37 °C, following which all solutions were removed, cells were rinsed (3x cold PBS) and fresh medium was added (200 μL of complete medium). After 48 hours of incubation, the MTT reagent (10 μL of the solution from the commercially available kit, ATCC # 30-1010K) was then added and cells were incubated at 37 °C for 3 additional hours. The solutions were subsequently removed and the formazan crystals were solubilized with DMSO (100 μL). The cell viability was determined by measuring

the absorbance at 570 nm using a Spectramax 190 microplate reader (Molecular Devices). All results were expressed as percent reduction of MTT relative to untreated controls (defined as 100% cell viability) and the average absorbance value for each treatment was blanked with the absorbance reading of medium control wells.

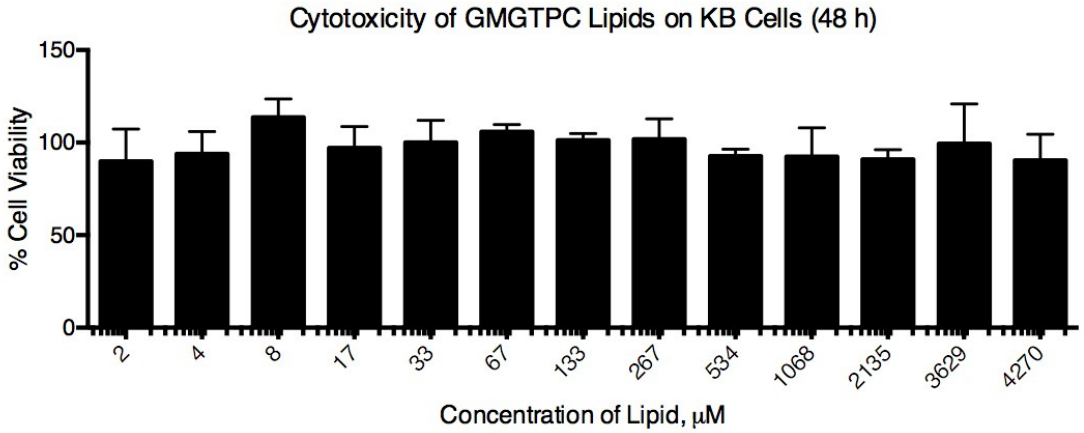


Figure S6. Cell viability of empty GMGTPC liposomes on KB cells after 2 h treatment, followed by 48 hours of incubation after removal of the liposomes. Data expressed as mean values ± SD, n ≥ 3 for each concentration.

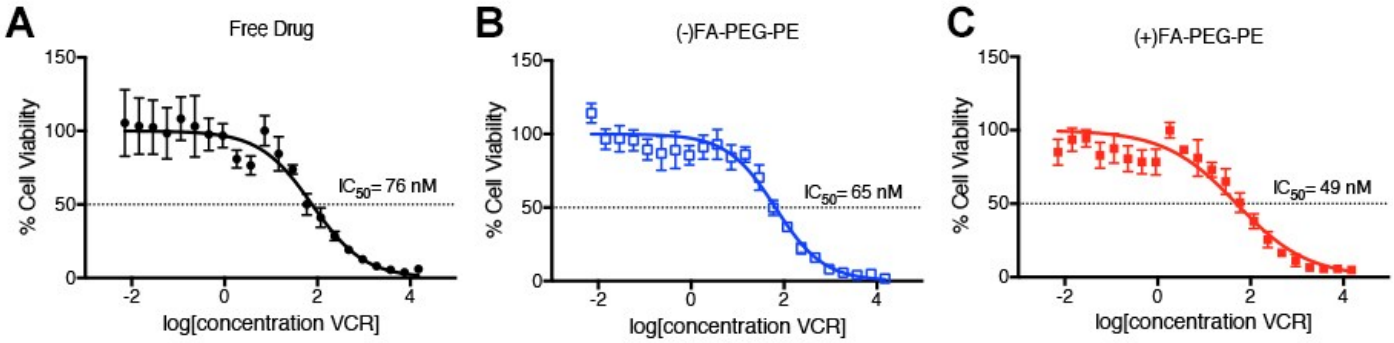


Figure S7. Liposomal delivery of vincristine (VCR) to KB cells. Cytotoxicity of KB cells after 2 h treatment with either the free drug (A), VCR loaded GMGTPC liposomes with (+ FA-PEG-PE) (C) or without (- FA-PEG-PE) folate targeting (B), followed by 48 h of additional incubation after drug removal. Data expressed as mean values ± SD, n ≥ 3 for each concentration.

7. References

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(2) Febo-Ayala, W.; Morera-Félix, S. L.; Hrycyna, C. A.; Thompson, D. H. *Biochemistry* **2006**, *45* (49), 14683–14694.

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(4) Bartlett, G. R. *J. Biol. Chem.* **1959**, *234* (3), 466–468.

8. NMR Spectra

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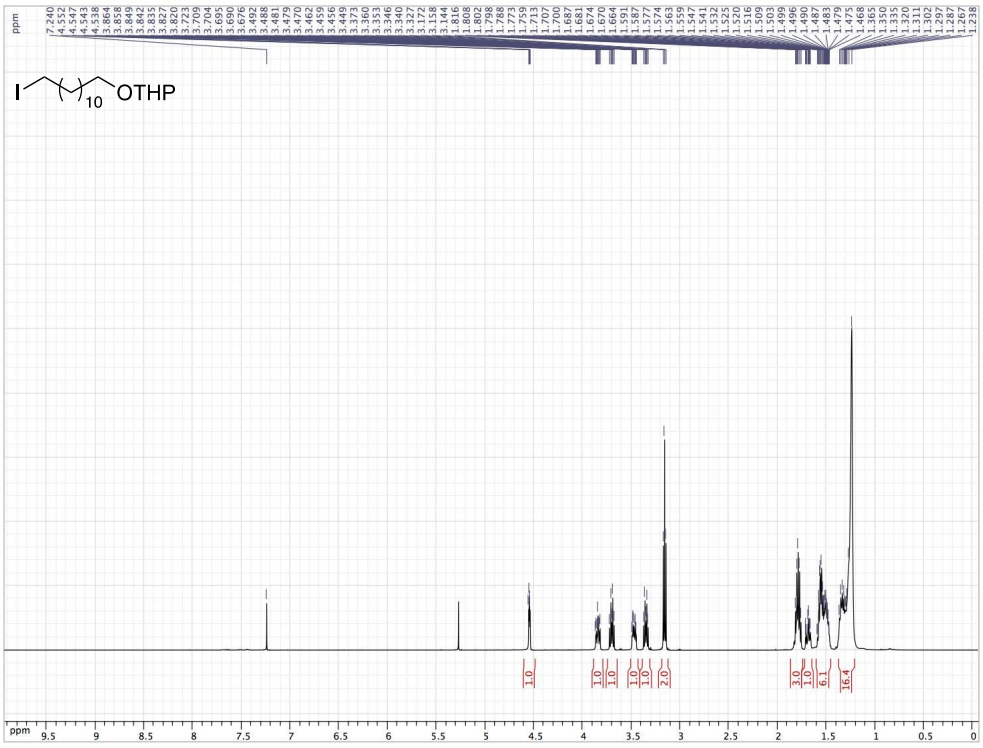

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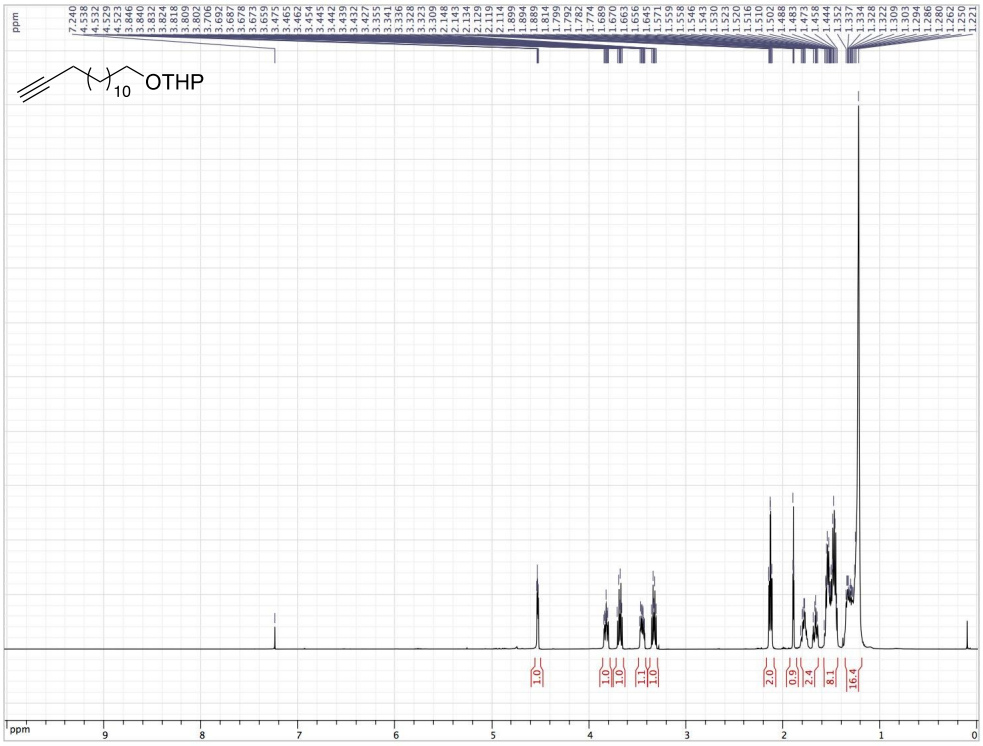

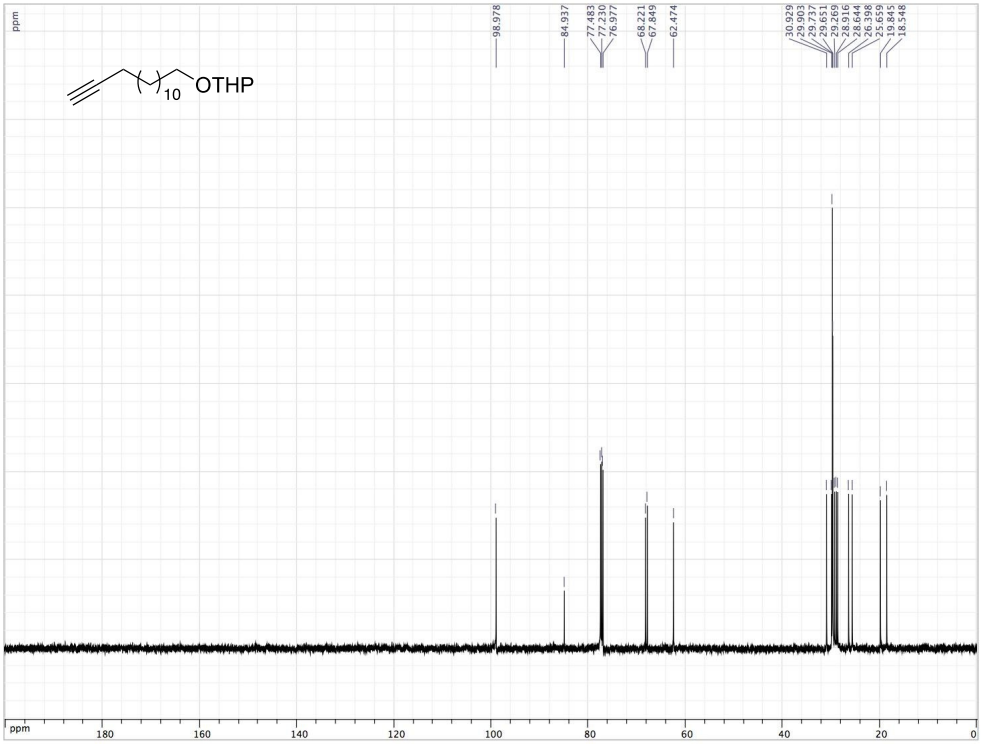

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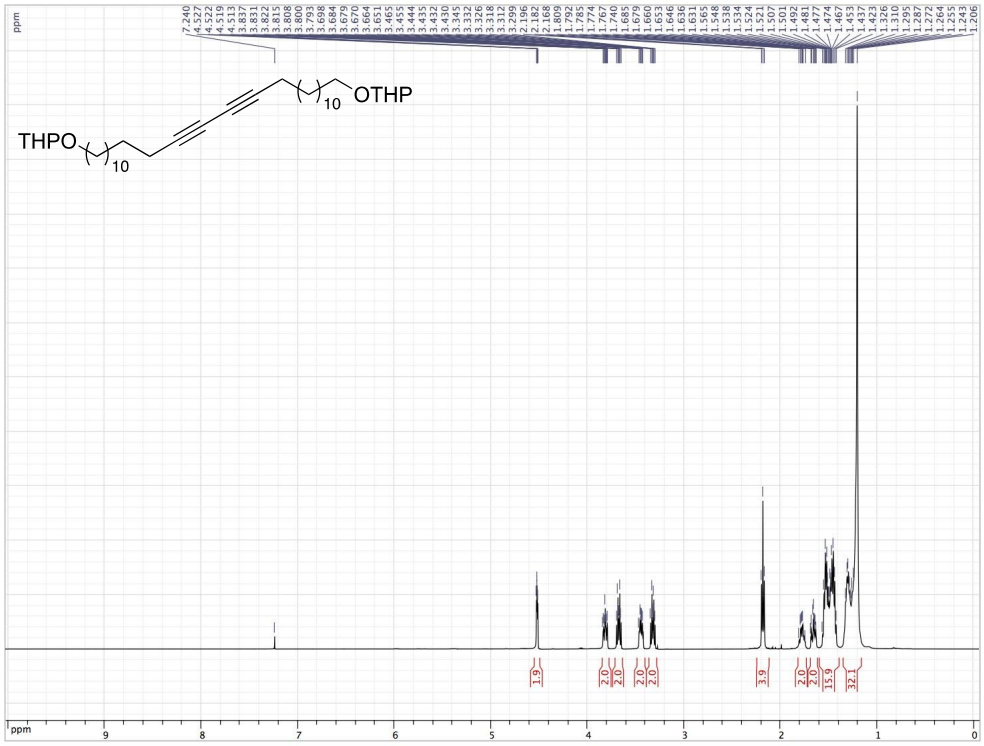

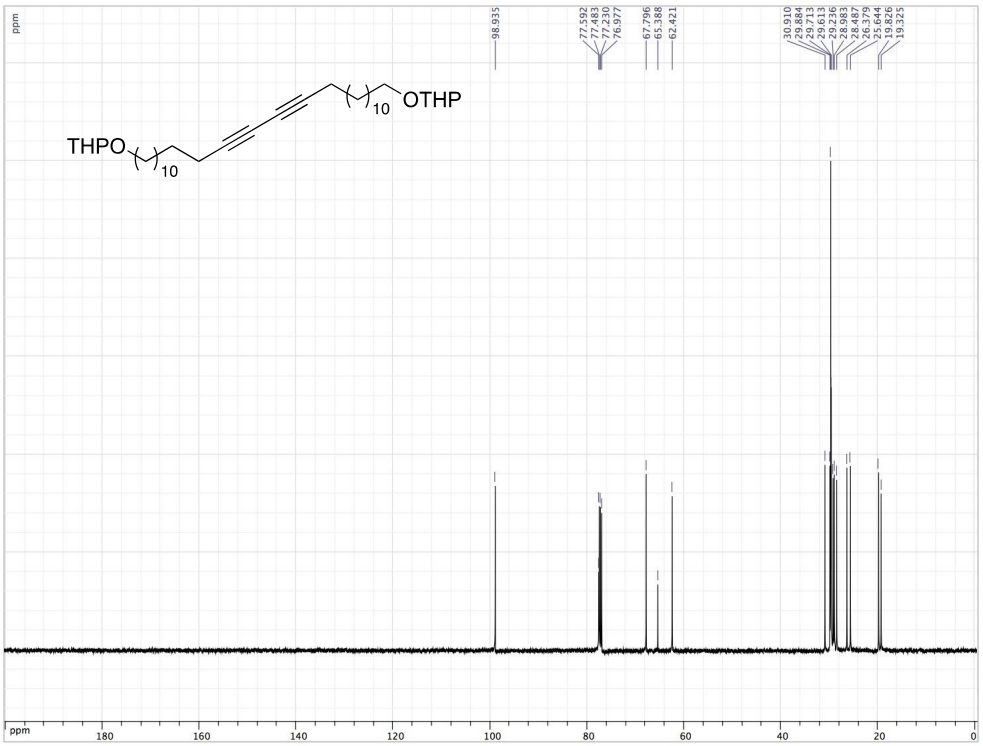

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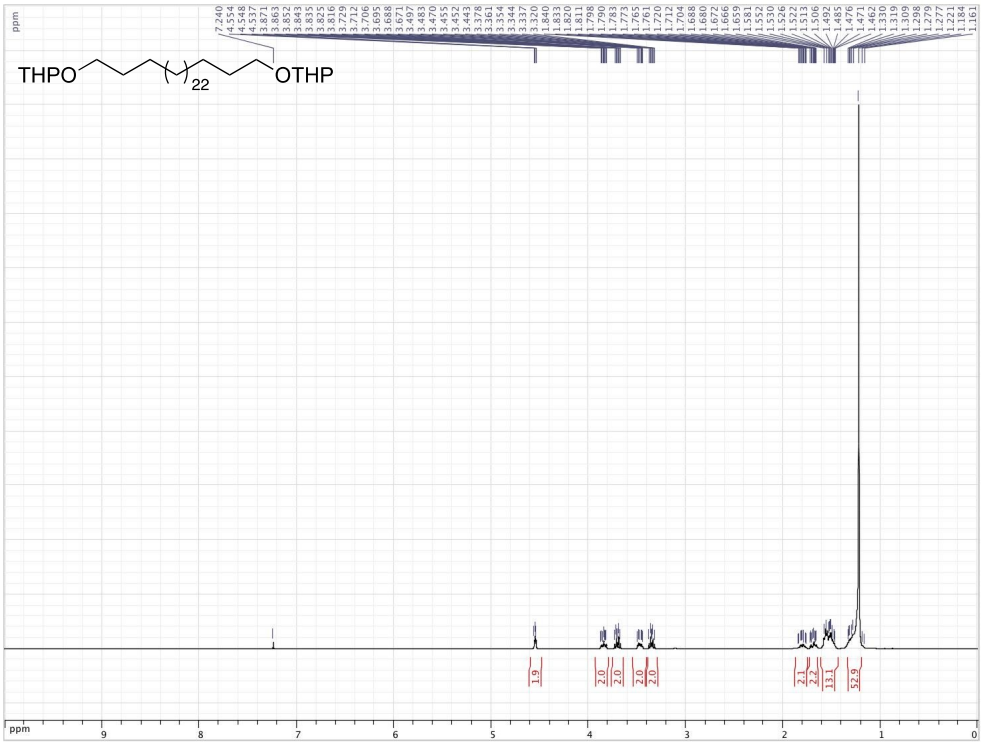

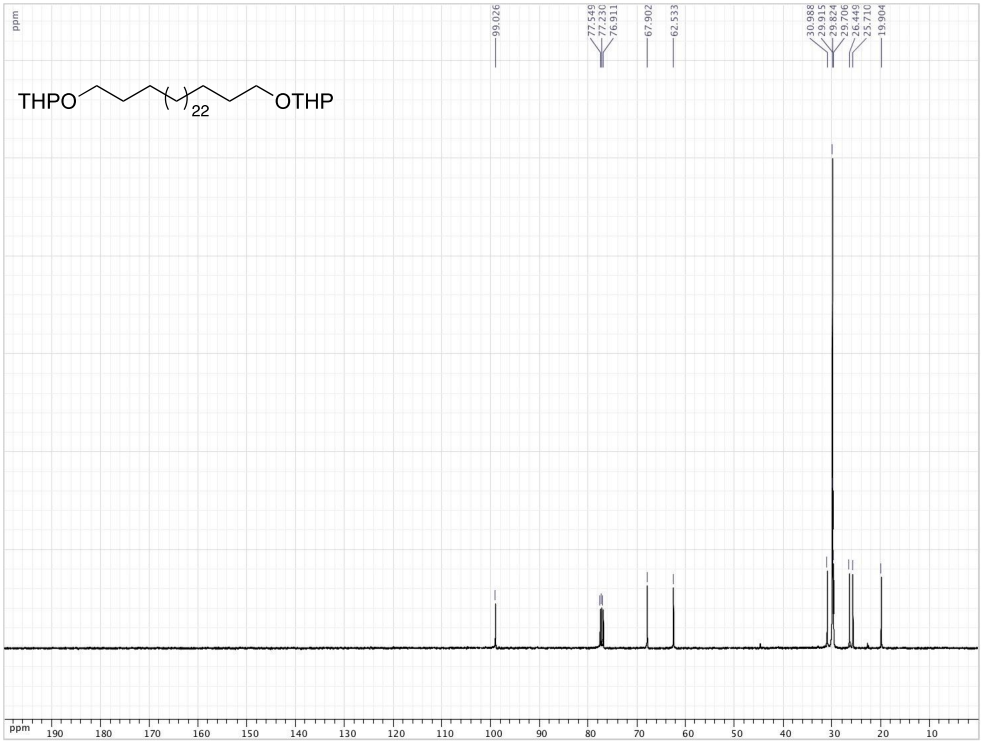

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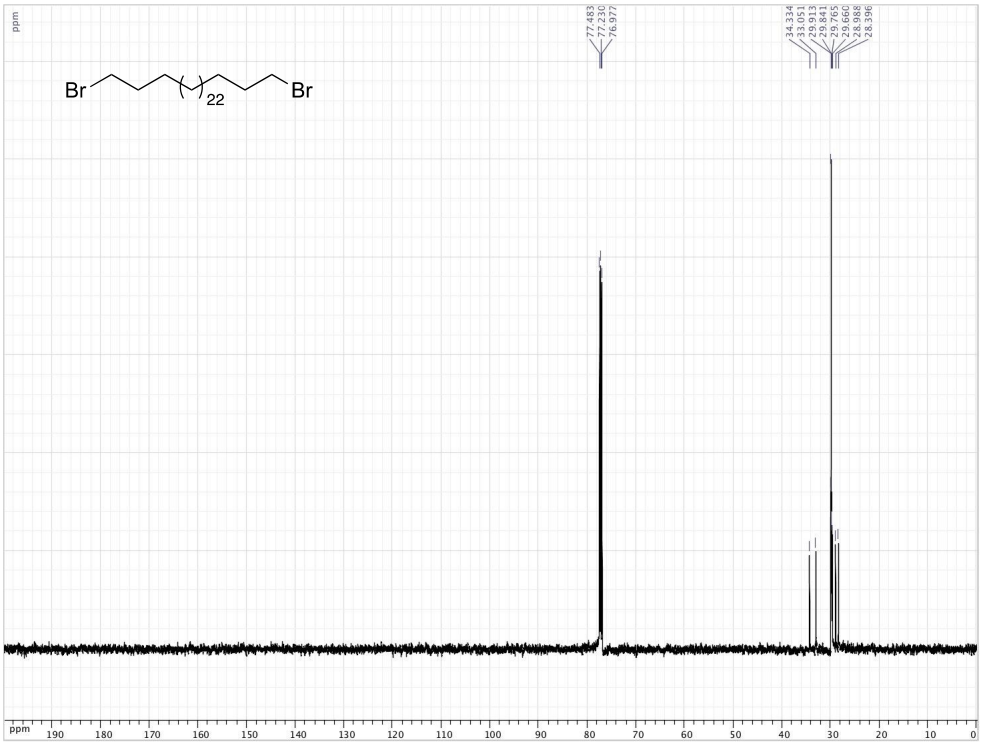
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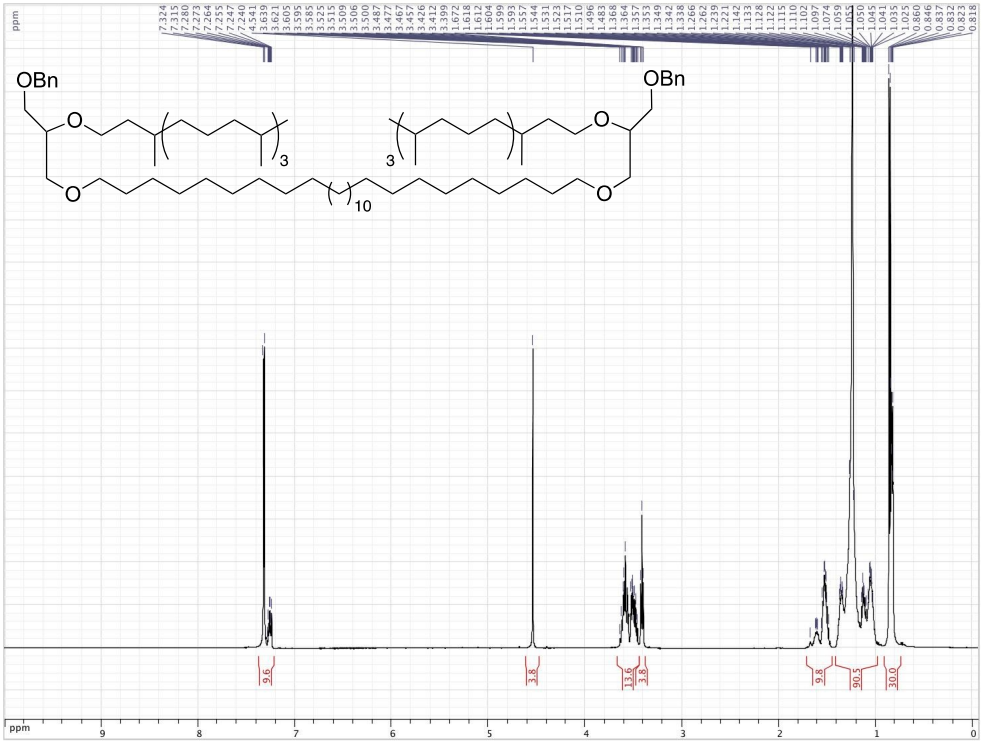
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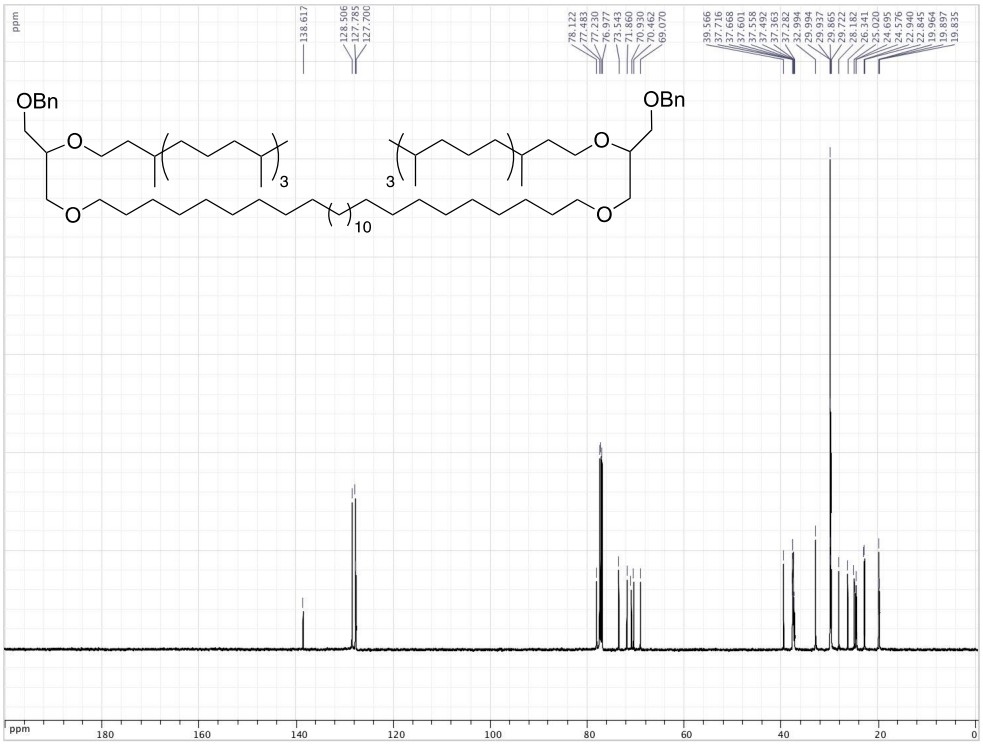
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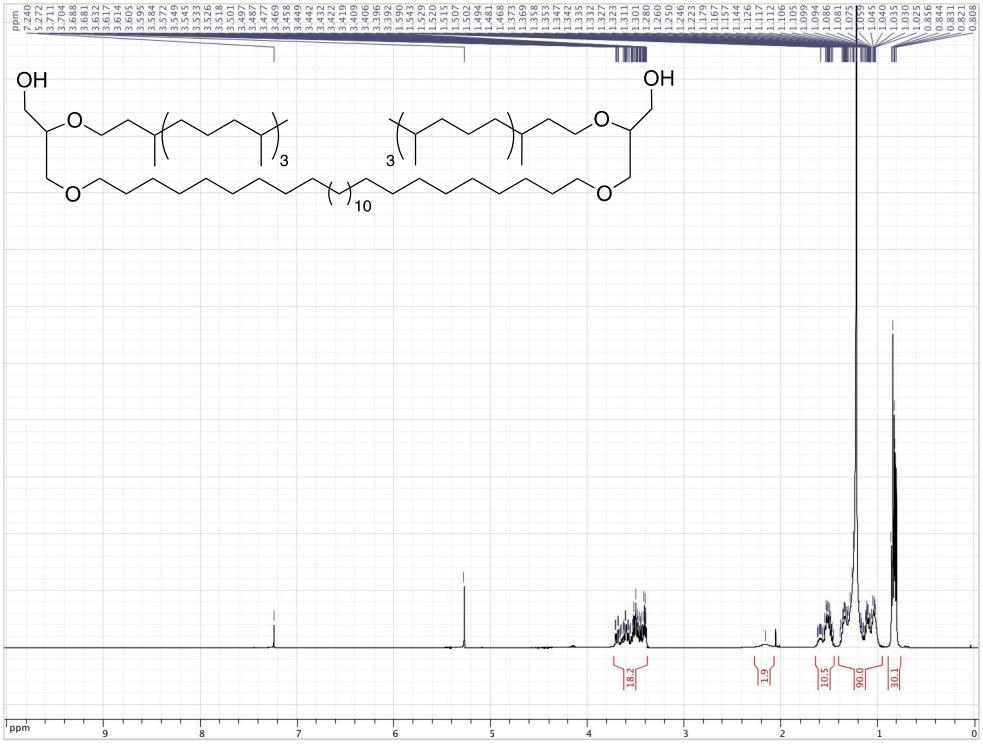
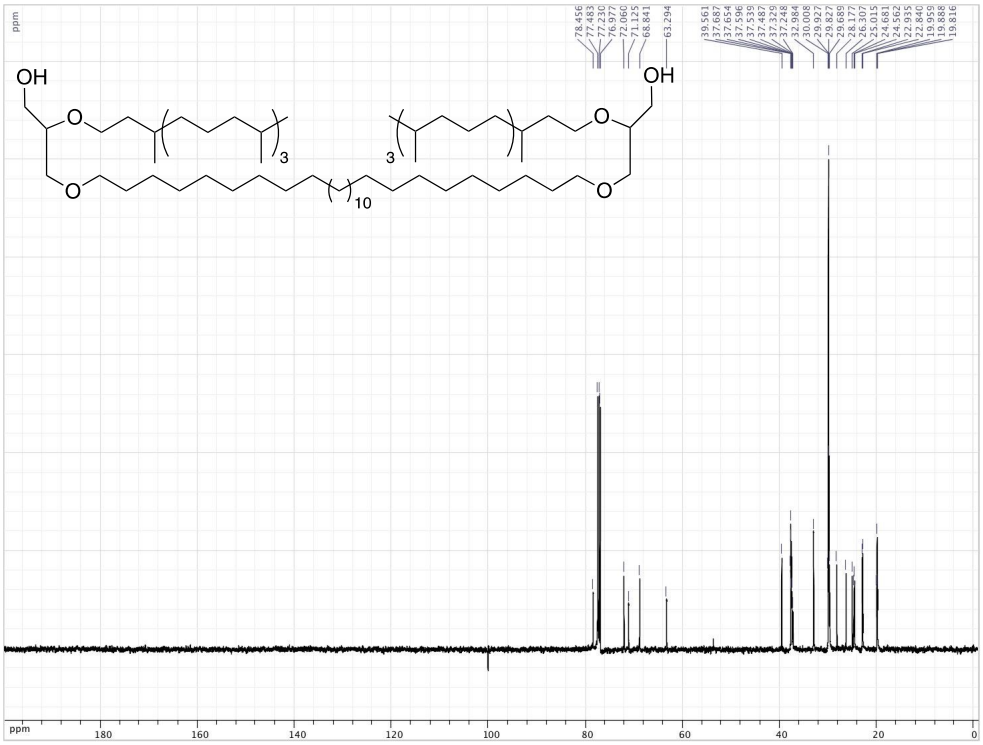
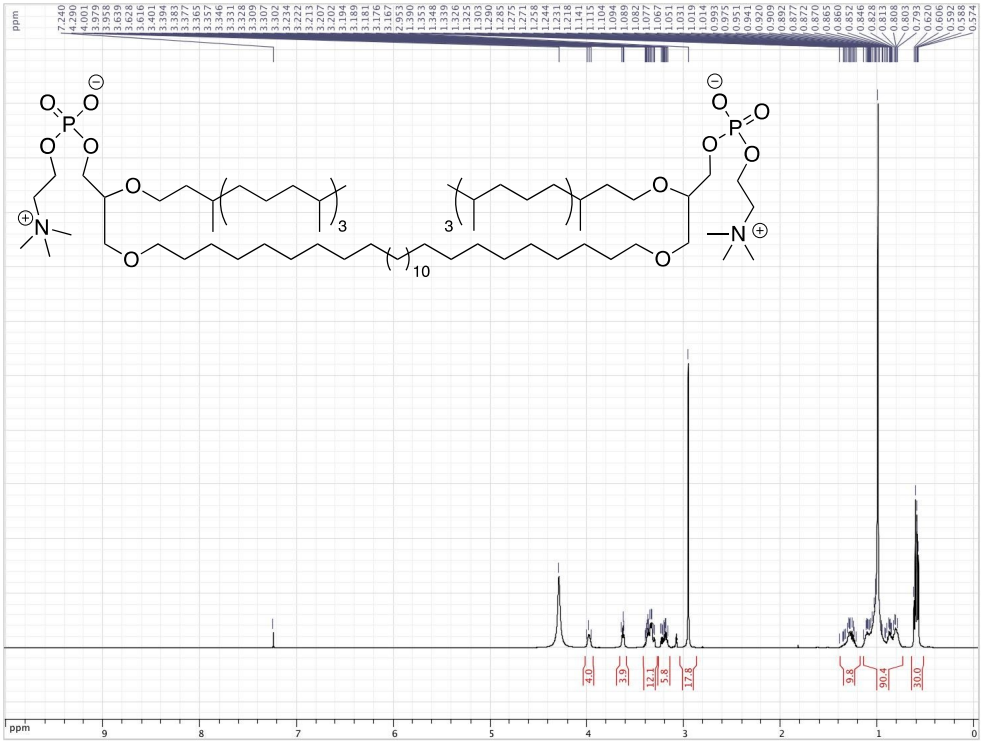
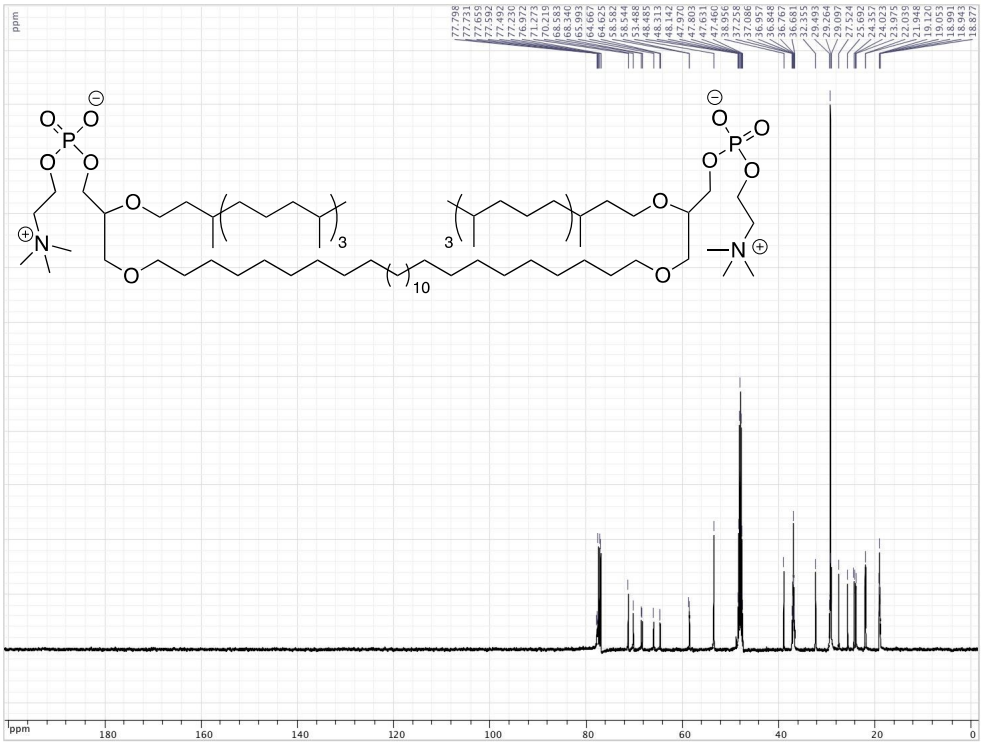
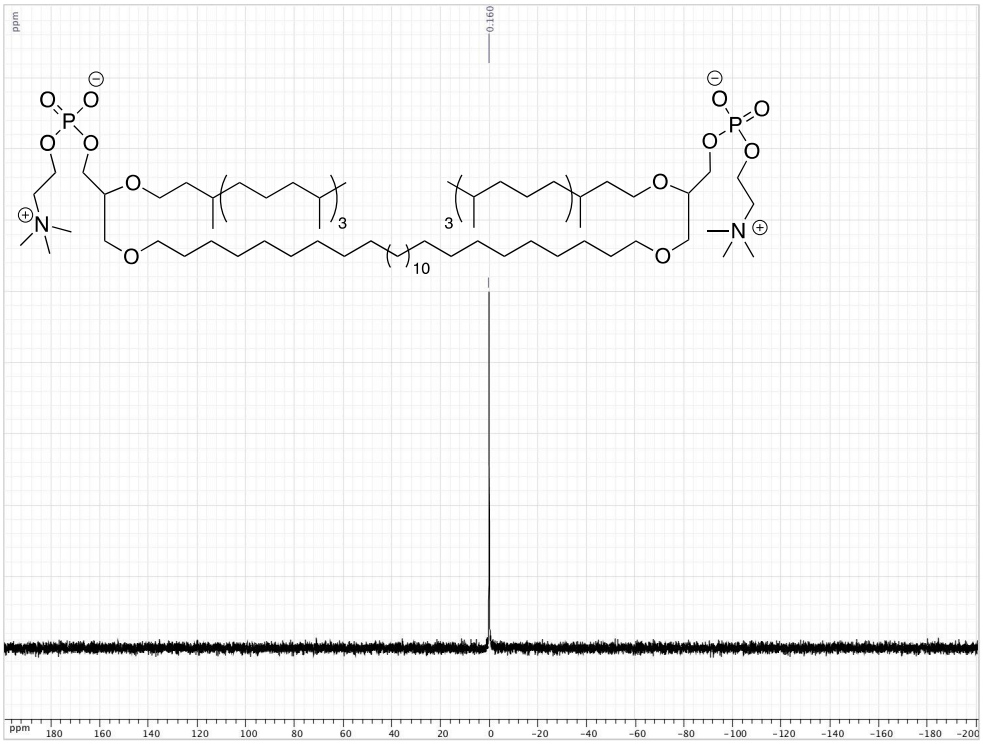




Lipid **GMGTPC**



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