Supporting Information

Dual Enzyme Responsive Mannose-6-Phosphate Based Vesicle for Controlled Lysosomal Delivery

Basudeb Mondal,* Tahiti Dutta, and Sayam Sen Gupta*

Instrumentation:

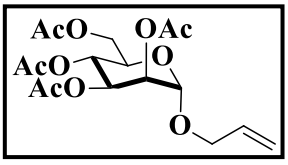
^{1}H NMR spectra were recorded on JEOL (200 MHz and 400 MHz) or Bruker (500 MHz) Spectrometers.^{13}C NMR spectra were recorded on JEOL (50 MHz and 400 MHz) or Bruker (500 MHz) Spectrometers and reported relative signals according to the deuterated solvent used. ^{31}P NMR spectra recorded on JEOL (200 MHz and 400 MHz) or Bruker (500 MHz) Spectrometers using an internal 85% aqueous H_3PO_4 as a reference. Hydrodynamic size (particle size) distribution measurements were performed on Malvern Zetasizer instrument. All data were recorded for an average of three scans, and the data were reported as a function of intensity versus hydrodynamic diameter (d, nm). Transmission electron microscopy (TEM) measurements were performed at 200 kV on an FEI Technai F20 and F30 instrument. Atomic force microscopy (AFM) analysis was performed on the Asylum Research, USA, MFP-3D BIO instrument (Operating Mode: Contact mode AFM under dry and wet conditions; System Specification: Closed-loop sensors on all three axes: X & Y range 90 μm, X & Y sensors <0.5 nm noise, <0.5% non-linearity; Z range >15 μm, Z sensor <0.25 nm noise; Optional Extended Z Head with range >40 μm; DC height noise <50 pm). Fluorescence experiments were carried out on fluorimeter QM40 from Photon Technology International. UV-vis spectra were recorded on an Agilent UV-Vis spectrophotometer. Fluorescence spectra were recorded on Agilent Fluorometer.

Materials and Method:

Other chemicals and solvents such as petroleum ether, ethyl acetate, diethyl ether, tetrahydrofuran, methanol, dioxane, and dichloromethane were obtained from Merck, India. They are dried by using conventional drying methods and finally stored in the glove-box prior to use. Unless otherwise reported, all reactions were performed under argon atmosphere. Removal of the solvent *in vacuum* using a rotary evaporator attached to an efficient vacuum pump and products obtained as solids or syrups were dried under a high vacuum. Analytical thin-layer chromatography was performed on pre-coated silica plates (F254, 0.25 mm thickness); compounds were visualized by UV light or by staining with anisaldehyde spray.

Rhodamine B octadecyl ester perchlorate (RBOE), BF_3-diethyl ether, Pd/C, di-butyl tin oxide (Bu_2SnO), doxorubicin hydrochloride (DOX), bovine serum albumin (BSA), calcein, fluorescein iso-thiocyanate (FITC), fluorescein-NHS, esterase enzyme from horse liver, alkaline phosphatase (ALP) enzyme, dialysis tubing (12 kDa) were purchased from Sigma-Aldrich Chemical. Dialysis tubing (MWCO 3.5 kDa, 7 kDa, and 12 kDa) was purchased from Thermo-Fisher Scientific. Trityl chloride (TrCl), benzyl alcohol, glycidol, benzyl bromide, sodium methoxide (NaOMe), and sodium hydride (NaH) were purchased from TCI Chemicals, India. Stearic acid, palmitic acid, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC-HCl), 1-hydroxy benzotriazole (HOBT), N-hydroxy succinimide (NHS), allyl alcohol, formic acid, phosphorus trichloride (PCl_3), and diisopropylamine (DIPA) were purchased from Spectrochem.

Preparation of (allyl)-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (1a)



To a suspension of D-mannose (10g, 55.56 mmol) in glacial acetic acid (100 mL) and acetic anhydride (39.35 mL, 416.7 mmol) was added conc. H_2SO_4 (2 mL) dropwise, and the reaction mixture was stirred at

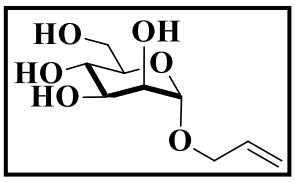
room temperature. After half an hour, the reaction mixture became clear, indicating the completion of the reaction. The reaction mixture was dissolved in 200 mL of DCM and washed with cold water (3×300 mL) and then saturated bicarbonate solution to afford penta-*O*-acetyl-α-D-mannopyranoside without any acid impurities. The DCM solution was washed with brine followed by drying over sodium sulfate and removal of the solvent by rotary evaporator to obtain completely pure penta-*O*-acetyl-α-D-mannopyranoside (Yield 21.5g; ~99%).

Penta-*O*-acetyl-α-D-mannopyranoside (21.5g, 25.6 mmol) and allyl alcohol (2.09 mL, 30.7 mmol) in dry dichloromethane (50 mL) was placed in 250 mL round bottom flask which was covered by aluminium foil and fitted with a dropping funnel. The solution was cooled to 0˚C, after which $BF_3.Et_2O$ (31.1 mL, 219.2 mmol) was added dropwise over a period of 30 minutes. The reaction mixture was then stirred for an additional 2 hrs at 0˚C and then subsequently at room temperature for 12 hrs. The completion of the reaction was monitored by TLC. The reaction mixture was diluted by dichloromethane and poured onto ice water while stirring. The organic layer was separated and washed successively with water, saturated sodium bicarbonate, and brine. The organic layer was dried on Na_2SO_4, concentrated on a rotary evaporator, and the resulting residue was purified by column chromatography on silica gel using ethyl acetate-petroleum ether as eluent to get allyl-penta-*O*-acetyl-α-D-mannopyranoside (13.8g, 65%) as a crystalline solid.

^{1}H NMR (200 MHz, $CDCl_3$): δ(ppm) 2.00(s, 3H), 2.02(s, 3H), 2.05(s, 3H), 2.09(s, 3H), 3.65-3.72(m, 1H), 4.11-4.29 (m, 4H), 4.54-4.58(d, 1H, *J*=1.52 Hz), 5.03-5.32(m, 5H), 5.78-5.92(m, 1H).

^{13}C NMR (50 MHz, $CDCl_3$): δ(ppm) 20.54 (3C), 20.73, 62.33, 66.05, 68.42, 68.50, 68.94, 69.49, 96.45 (anomeric), 118.29, 132.81, 169.58, 169.71, 169.88, 170.46.

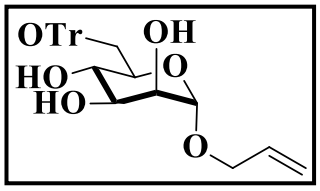
Preparation of (allyl)-2,3,4,6-tetra-hydroxy-α-D-mannopyranoside (1b)



To a solution of allyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (13.8g, 35.7 mmol) in anhydrous methanol (25 mL) was added freshly prepared solution NaOMe in MeOH (1 M, 3mL) at 0°C. The reaction mixture was stirred for 0.5 hrs, and the progress of the reaction was monitored by TLC. After complete conversion, the reaction mixture was neutralized with amberlite IR 120 H^+ resin and filtered. The crude reaction product was obtained by removal of the solvent on the rotary evaporator to get the desired tetraol of allyl mannopyranoside, which was directly used for the next step.

^{1}H NMR (200 MHz, $CDCl_3$): δ(ppm) 3.22-3.48(m, 4H), 3.68-3.71(m, 1H), 3.87-3.92(m, 1H), 4.22-4.25(m, 1H), 4.34-4.50(m, 2H), 5.24-5.41(m, 2H), 5.90-6.03(m, 1H).

Preparation of (allyl)-6-trityl-2,3,4,-tri-hydroxy-α-D-mannopyranoside (1c)

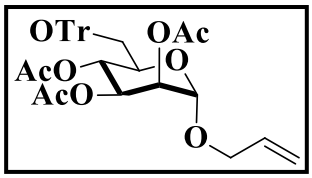


The above tetraol (7.8g, 35.34 mmol) was dissolved in dry pyridine (150mL) and to this, trityl chloride (49.1g, 176.7 mmol) and 4-dimethylaminopyridine (431mg, 3.53 mmol) was added under argon. The reaction mixture was then heated to 55ºC and stirred for 10 hrs. After completion of the reaction, the solvent was evaporated by applying a vacuum. The residue obtained was purified by silica gel column chromatography using ethyl acetate and 0.5% MeOH as eluent to get 6-*O*-trityl-allylmannoside as a yellowish oil (12.68g, 78%). The starting material was also recovered (20%) and used again in this trityl protection reaction.

^{1}H NMR (200 MHz, $CDCl_3$): δ(ppm) 2.97(bs, 1H), 3.17(bs, 1H), 3.31(bs, 1H), 3.47-3.49(d, 2H), 3.74-4.12(m, 5H), 4.24-4.34(m, 1H), 4.92-4.93(d, 1H, *J*=1.26), 5.31-5.42(m, 2H), 5.90-6.09(m, 1H, allylic), 7.31-7.55(m, 15H).

^{13}C NMR (50 MHz, $CDCl_3$): δ(ppm) 63.84, 64.70, 68.00, 70.16, 70.36, 71.64, 87.25, 98.62, 117.63, 127.17-128.59(15C), 133.64, 143.58(3C).

Preparation of (allyl)-6-trityl-2,3,4,-tri-*O*-acetyl-α-D-mannopyranoside (1d)

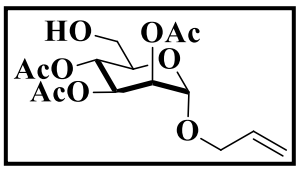


The unprotected 6-*O*-trityl derivative of ally mannopyranoside (12.68g, 27.56 mmol) was dissolved in dry pyridine (100 mL), and the solution was cooled to 0ºC. To this ice-cold solution, acetic anhydride (15.5 mL, 165.36 mmol) was added slowly while stirring. The reaction mixture was kept at r.t for 4 hrs and then concentrated under vacuum. The residue was diluted with DCM (200 mL) and washed successively with ice-cold 1M HCl (2×200 mL), saturated sodium bicarbonate (2×200 mL), and brine. The organic layer was dried over Na_2SO_4 and then concentrated on a rotary evaporator. The crude oily product allyl-6-*O*-trityl-2,3,4,-tri-*O*-acetyl-α-D-mannopyranoside (15.98g, 99%) was used without any further purification for the next reaction.

^{1}H NMR (200 MHz, $CDCl_3$): δ(ppm) 2.05(s, 3H), 2.26(s, 3H), 2.31(s, 3H), 3.26-3.28(d, 2H), 4.00-4.05(m, 1H), 4.14-4.23(m, 1H), 4.34-4.41 (m,1H), 5.00(d, 1H, *J*=1.01 Hz), 5.31-5.48(m, 5H), 5.97-6.14(m, 1H), 7.31-7.57(m, 15H).

^{13}C NMR (50 MHz, $CDCl_3$): δ(ppm) 20.52, 20.70, 20.90, 62.49, 66.67, 68.28, 69.39, 69.89, 70.26, 86.58, 96.27(anomeric), 118.13, 126.96-128.69(15C), 133.28, 143.75, 169.39, 169.98, 170.13.

Preparation of (allyl)-6-hydroxy-2,3,4,-tri-*O*-acetyl-α-D-mannopyranoside (1e)

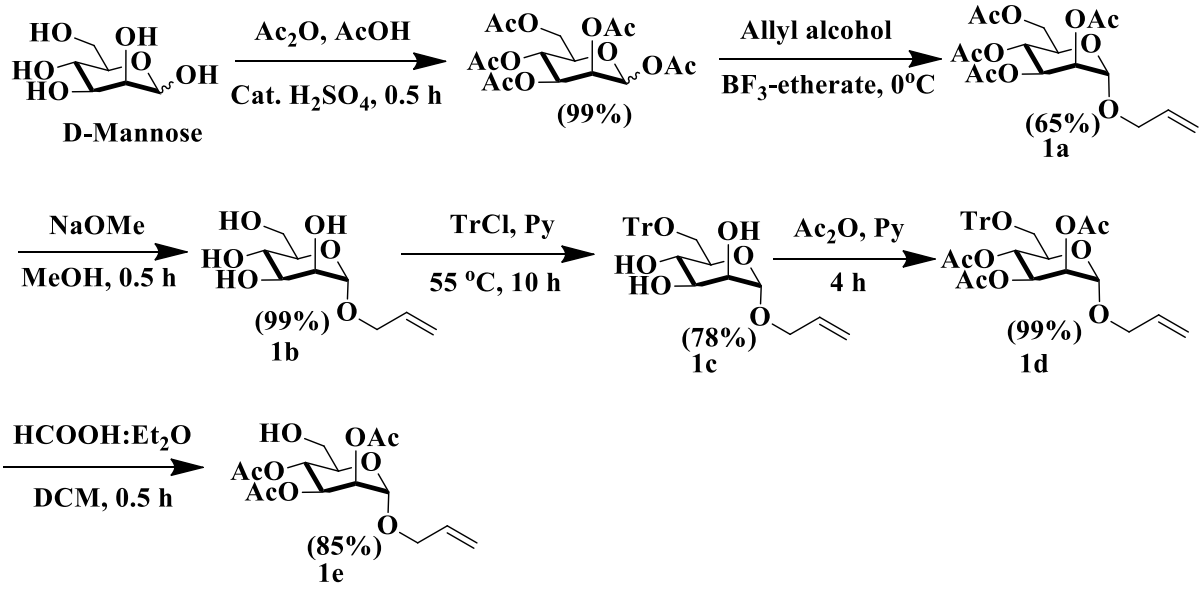


To a solution of allyl-6-*O*-trityl-2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (15.98g, 27.28 mmol) in DCM (50 mL) was added 180 mL of HCOOH: Et_2O (1:1) mixture and the reaction mixture was

stirred at r.t for 1 hrs. It was then diluted with DCM (150 mL) and washed successively with water (2×200 mL) to remove HCOOH and brine. The organic layer was dried on Na_2SO_4 and concentrated to obtain a crude product which was further purified by silica gel column chromatography using ethyl acetate-petroleum ether as eluent to get allyl-6-hydroxy-2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (8.05g, 85%).

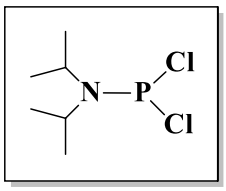
^{1}H NMR (200 MHz, $CDCl_3$): δ(ppm) 2.00(s, 3H), 2.07(s, 3H), 2.14(s, 3H), 2.43-2.49(t, 1H), 3.55-3.81(m, 3H), 4.02-4.06(m, 1H), 4.14-4.17(m, 1H), 4.87(d, 1H, *J*=1.64 Hz), 5.18-5.44(m, 5H), 5.82-5.90(m, 1H).

^{13}C NMR (50 MHz, $CDCl_3$): δ(ppm) 20.65, 20.68, 20.83, 61.17, 66.45, 68.50, 68.81, 69.63, 70.61, 96.57(anomeric), 118.24, 132.96, 169.84, 170.07, 170.83.



Scheme S1: Synthesis of (allyl)-6-hydroxy-2,3,4,-tri-*O*-acetyl-α-D-mannopyranoside (**1e**) starting from D-mannose.

Preparation of 1,1-dichloro-N,N-diisopropylphosphinamine (2a)

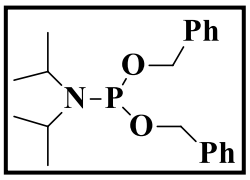


Phosphorous trichloride (5 mL, 57.3 mmol) was dissolved in 50 ml of dry hexane under N_2, and the mixture was cooled to -78°C. A solution of freshly distilled di-isopropylamine (16 mL, 114.6 mmol) in 50 mL of dry hexane added to the reaction mixture over 1 hrs using an addition funnel. Following the addition, the reaction was stirred at -70ºC for an additional hour. The cooling bath was then removed and the reaction was stirred at room temperature for another 4 hrs. The reaction was then filtered through a glass frit under positive N_2 pressure, and the solvent with excess phosphorus trichloride was removed by short-path distillation at 40ºC with a high vacuum. The product was obtained as a clear, colorless liquid and was stored at -22ºC (10g, 88%), which transformed into a crystalline solid at low temperature.

^{1}H NMR (200 MHz, $CDCl_3$): δ(ppm) 1.27-1.30(d, 12H), 3.84-4.07(m, 2H).

^{31}P NMR (202 MHz, $CDCl_3$): δ(ppm) 169.57

Preparation of dibenzyl diisopropyl phosphoramidite (2b)

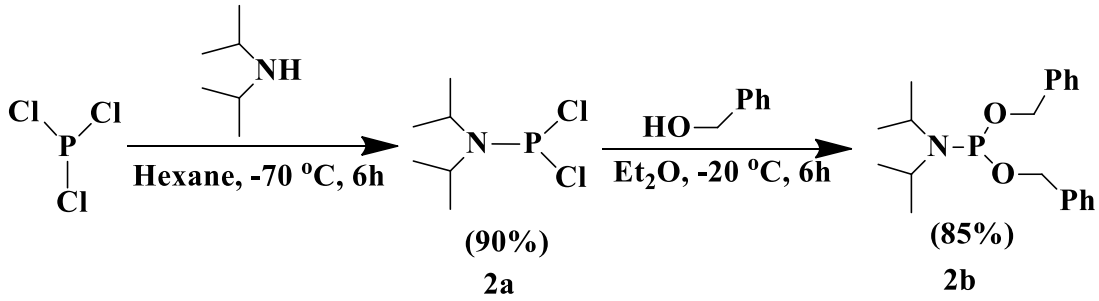


1,1-dichloro-N,N-diisopropylphosphinamine (10g, 50.1 mmol) was dissolved in 100 ml of dry diethyl ether under N_2, and the mixture was cooled to -10ºC. A solution of benzyl alcohol (11.45 mL, 110.2 mmol), triethylamine (15.35 ml, 110.2 mmol), and 100 ml of dry diethyl ether was added to the reaction vessel through an addition funnel over the course of 1.5 hrs. After completion of the addition, the cooling bath was then removed, and the reaction was stirred at room temperature for 6 hrs. The reaction was filtered through a glass frit, and the solvent was removed under reduced pressure yielding yellowish oil. The crude oil was then purified by column chromatography. The column was packed with silica gel (100-200 mesh) using2.5% triethylamine in hexane until the smell of triethylamine persisted. After loading the crude reaction mixture, the desired product was eluted using 2% ethyl acetate-2.5% triethylamine in hexane as the eluent. The

fractions containing the product were combined and concentrated under reduced pressure, during which the volatiles was removed under vacuum, yielding clear and colorless oil (15.5g, 90%).

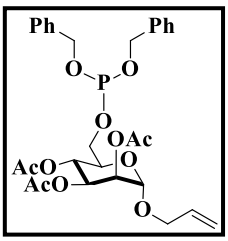
^{1}H NMR (200 MHz, $CDCl_3$): δ(ppm) 1.24-1.27 (d, 12H), 3.69-3.85(m, 2H), 4.69-4.94(m, 4H), 7.29-7.43(m, 10H)

^{31}P NMR (202 MHz, $CDCl_3$): δ(ppm) 146.68



Scheme S2: Synthesis of dibenzyl diisopropyl phosphoramidite (**2b**) starting from phosphorous trichloride.

Preparation of (allyl)-6-dibenzyl phosphite-2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (3)



Allyl-6-hydroxy-2,3,4-tri-*O*-acetyl-α-D-mannopyranoside (8.05g, 23.19 mmol) and dibenzyl diisopropyl phosphoramidite (14.4g, 41.74 mmol) were evaporated twice with dry MeCN (35 mL) and then dissolved in dry MeCN (100 mL). In another RB, 2.9 g (41.74 mmol) of ^{1}H-tetrazole (dried by twofold evaporation with MeCN) was dissolved in 25 mL of MeCN. The ^{1}H-tetrazole solution was then added dropwise to the reaction mixture on an ice bath with continuous stirring. After addition, the ice bath was removed, and the reaction mixture was stirred at room temperature for 30 mins upon which precipitation of white solid was observed. Precipitated white crystalline side product was filtered, discarded, and the filtrate was concentrated on a rotary

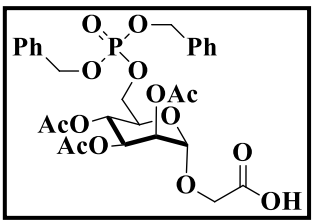
evaporator. The crude product obtained after removal of the solvent was purified by silica gel column chromatography. The column was packed with silica gel (100-200 mesh) using 2.5% triethylamine in hexane until the smell of triethylamine persisted. After loading the crude reaction mixture, the desired product was eluted using 20% ethyl acetate-2.5% triethylamine in hexane as the eluent. The fractions containing the product were combined and concentrated under reduced pressure, and the volatiles were removed under vacuum, yielding a clear and colorless oil (allyl)-6-dibenzyl phosphite-2,3,4-tri-O-acetyl-α-D-mannopyranoside (13.55g, 99%).

^{1}H NMR (200 MHz, $CDCl_3$): δ(ppm) 1.98(s, 3H), 2.00(s, 3H), 2.09(s, 3H), 3.86-4.03(m, 4H), 4.13-4.23(m, 1H), 4.85-4.91(m, 5H), 5.18-5.42(m, 5H), 5.77-5.96(m, 1H), 7.28-7.36(m, 10H).

^{13}C NMR (50 MHz, $CDCl_3$): δ(ppm) 20.65(2C), 20.78, 61.32, 64.23, 65.23, 66.57, 68.30, 69.20, 69.68, 69.93, 96.31(anomeric), 118.10, 126.90-128.47(10C), 132.99, 138.03, 138.14, 169.69, 169.91, 170.08.

^{31}P NMR (202 MHz, $CDCl_3$): δ(ppm) 139.24.

Preparation of 6-dibenzyl phosphate-2,3,4-tri-*O*-acetyl-α-D-mannopyranoside ethyl carboxylic acid (4)



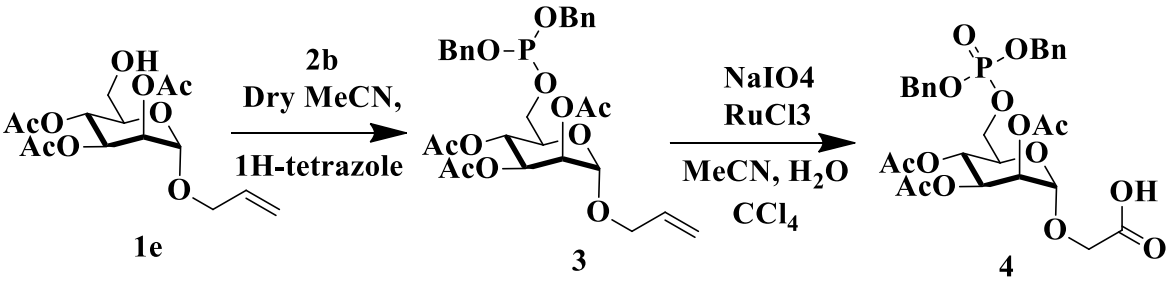
To a stirring solution of (allyl)-6-dibenzyl phosphite-2,3,4-tri-O-acetyl-α-D-mannopyranoside (13.55 g, 22.96 mmol) in CCl_4 (97 mL), CH_3CN (97 mL) and H_2O (133 mL) at 0°C in a round bottom flask was added $NaIO_4$ (24.57 g, 114.8 mmol) and $RuCl_3.H_2O$ (151 mg, 0.46 mmol). After 10 min of vigorous stirring of the suspension at 0ºC, the reaction mixture was allowed to attain room temperature and further stirred for an additional 1 hrs. The reaction mixture was then concentrated on a rotary evaporator and diluted with DCM (200 mL) and

washed with 1M HCl (2×200 mL). Then the organic layer was washed with brine and dried extensively over Na_2SO_4. The solvent was removed by rotary evaporation, and the crude oily product was purified by silica gel column chromatography using ethyl acetate-petroleum ether (50%) and then 2% MeOH in ethyl acetate as eluent to yield6-dibenzyl phosphate-2,3,4-tri-O-acetyl-α-D-mannopyranoside ethyl carboxylic acid (11.75 g, 82%).

^{1}H NMR (200 MHz, $CDCl_3$): δ(ppm) 1.98-2.09(m, 9H), 4.07-4.22(m, 4H), 4.52(bs, 1H), 4.82-4.88(m, 1H), 5.04-5.08(m, 4H), 5.25-5.40(m, 3H), 7.34-7.35(m, 10H), 9.59(s, 1H, acid proton).

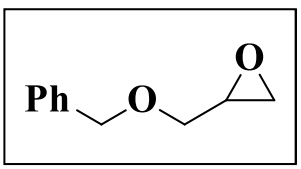
^{13}C NMR (50 MHz, $CDCl_3$): δ(ppm) 20.57, 20.65, 20.96, 60.36, 64.36, 65.69, 66.06, 68.09, 69.16, 69.51, 69.62, 97.72 (anomeric), 127.89-128.53(10C), 135.41, 135.56, 169.70, 169.79(2C), 171.40 (acid carbonyl).

^{31}P NMR (202 MHz, $CDCl_3$): δ(ppm) -1.47



Scheme S3: Synthesis of 6-dibenzyl phosphate-2,3,4-tri-O-acetyl-α-D-mannopyranoside ethyl carboxylic acid (4) starting from (allyl)-6-hydroxy-2,3,4,-tri-*O*-acetyl-α-D-mannopyranoside (1e).

Synthesis of Benzyl Glycidyl Ether (5):



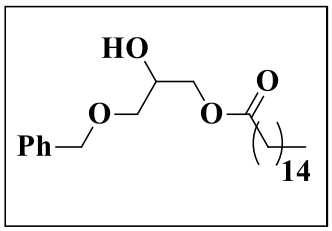
Glycidol (5 g, 4.5 ml, 67.5 mmoL) was dissolved in dry DMF, and then benzyl bromide (10.3 ml, 87 mmoL) was added to the solution, and the mixture was cooled to 0°C. In the cooled reaction mixture

NaH (60% dispersion in oil, 1eq) was mixed slowly under argon atmosphere, and the reaction mixture was warm to room temperature under stirring condition.[1] Then this reaction mixture was allowed to stirrer overnight at room temperature. After completion of the reaction, the crude reaction mixture was slowly added to water and extracted with CH_2Cl_2 (DCM). The organic part was washed with water and brine solution. Then all the organic portion was combined and dried over sodium sulfate. The organic layer was concentrated, and the crude product was purified by silica gel column chromatography using ethyl acetate and hexane as eluent. Yield (9.8 g, 88 %).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 2.58-2.60 (dd, 1H), 2.76-2.79 (dd, 1H), 3.15-3.19 (m, 1H), 3.39-3.44 (dd, 1H), 3.73-3.77 (dd, 1H), 4.52-4.61 (q, 2H), 7.26-7.34 (m, 5H)

^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) 44.16 (1C), 50.76 (1C), 70.68 (1C), 70.71 (1C), 73.20 (1C), 127.66 (2C), 128.32 (2C), 137.79 (1C)

Synthesis of Palmitic Benzyl Glycidyl Ether (6) (3-(Benzyloxy)-2-hydroxypropyl hexadecanoate):

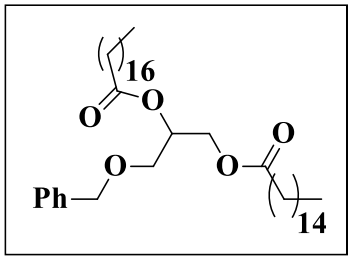


Benzyl glycidyl ether (5) (9.8 g, 60 mmoL), palmitic acid (15.4 g, 60 mmoL), and tetraethylammonium bromide (0.3 g, 1.2 mmoL) were mixed together and stirred at 100°C for 3-4 hrs.[1] After completion of reaction the crude reaction mixture was purified by silica gel column chromatography using hexane and ethyl acetate mixture as eluent to afford compound 6 as a white wax. Yield (15 g, 64%).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.87-0.91 (t, 1H), 1.27 (s, 24H), 1.60-1.63 (t, 2H), 2.30-2.35 (m, 2H), 3.48-3.58 (m, 2H), 4.02-4.05 (m,1H), 4.12-4.21 (m, 2H), 4.57-4.58 (t, 2H), 7.27-7.38 (m, 5H)

^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) 14.09 (1C), 22.66 (1C), 24.88 (1C), 29.10 (1C), 29.23 (1C), 29.34 (1C), 29.43 (1C), 29.23 (1C), 29.62 (1C), 29.66 (2C), 31.89 (1C), 34.13 (1C), 65.31 (1C), 68.91 (1C), 70.85 (1C), 73.49 (1C), 127.73 (2C), 17.87 (1C), 128.46 (2C), 137.63 (1C), 173.96 (1C).

Synthesis of Palmitic Stearic Benzyl Glycidyl Ether (7) (3-(Benzyloxy)-2-(octaadeca-2-yloxy) propyl hexadecanoate):

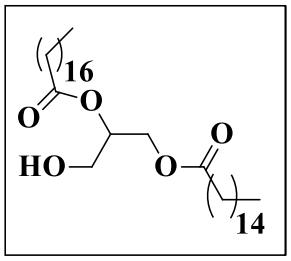


3-(Benzyloxy)-2-hydroxypropyl hexadecanoate (6) (15 g, 38 mmoL), stearic acid (14 g, 49.4 mmoL), DMAP (6 g, 49.4 mmoL), and EDCI (14.7 g, 95 mmoL) was mixed and dissolved in dry CH_2Cl_2 (150 ml). The mixed solution was stirred for overnight at room temperature under argon atmosphere.[1] After completion of the reaction, the reaction mixture was filtered through a celite bed and washed with CH_2Cl_2. The organic layer was concentrated, and the crude residue was purified by silica gel chromatography using ethyl acetate and hexane as eluent to afford the compound 3-(Benzyloxy)-2-(octaadeca-2-yloxy) propyl hexadecanoate as a white wax 7. Yield (21 g, 89%).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.87-0.91 (t, 6H), 1.22-1.31 (s, 53H), 1.58-1.64 (m, 5H), 2.26-2.34 (m, 4H), 3.57-3.61 (dd, 2H), 4.18-4.22 (dd, 1H), 4.33-4.37 (dd, 1H), 4.51-4.58 (dd, 2H), 5.22-5.30 (m, 1H), 7.27-7.35 (m, 5H)

^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) 14.10 (1C), 22.67 (1C), 24.86 (1C), 24.94 (1C), 29.98 (1C), 29.11 (1C), 29.28 (1C), 29.35 (1C), 29.48 (1C), 29.65 (1C), 29.69 (1C), 31.91 (1C), 34.10 (1C), 34.32 (1C), 62.64 (1C), 68.25 (1C), 69.99 (1C), 73.29 (1C), 127.61 (2C), 127.76 (1C), 128.40 (2C), 137.69 (1C), 173.12 (1C), 173.41 (1C).

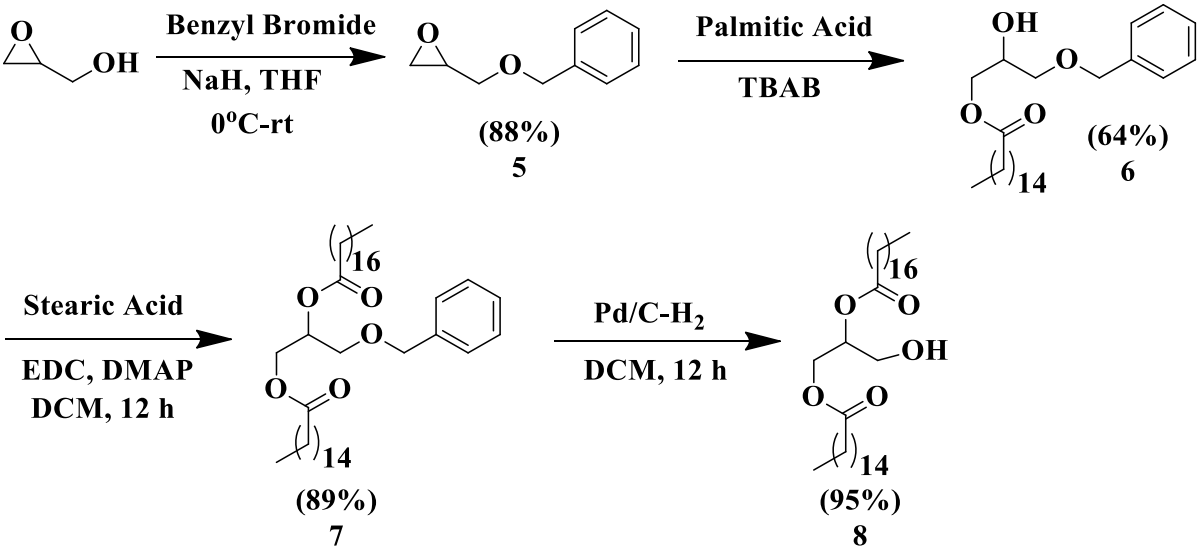
Synthesis of 3-Hydroxy-2-(octadeca-2-yloxy) propyl hexadecanoate (8):



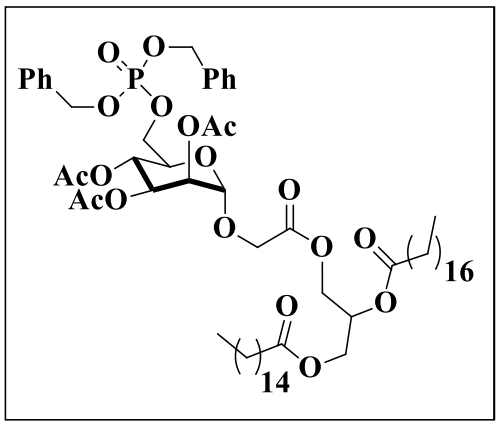
A mixture of 3-(benzyloxy)-2-(octadeca-2-yloxy) propyl hexadecanoate (7) (8 g, 19.65 mmoL) was dissolved in dry methanol, and then Pd/C was added to it under argon atmosphere. Then the reaction mixture was stirred for 12 hrs at room temperature under hydrogen balloon pressure. After completion of the reaction, the reaction mixture was filtered through a celite bed, and the filtrate was concentrated to get the compound 3-Hydroxy-2-(octadeca-2-yloxy) propyl hexadecanoate 8 as a colorless oil. Yield (5 g, 18.83 mmol, 95 %).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.87-0.90 (t, 6H), 1.26-1.29 (52H), 1.60-1.65 (dd, 5H), 2.31-2.37 (m, 4H), 3.73-3.74 (d, 2H), 4.22-4.26 (m, 1H), 4.31-4.35 (dd, 1H), 5.07-5.10 (dd, 1H)

^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) 14.10 (1C), 22.67 (1C), 24.87 (1C), 24.92 (1C), 29.08 (1C), 29.11 (1C), 29.26 (1C), 29.36 (1C), 29.46 (1C), 29.61 (1C), 29.64 (1C), 29.68 (1C), 31.91 (1C), 34.09 (1C), 34.28 (1C), 61.51 (1C), 62.00 (1C), 72.08 (1C), 173.44 (1C), 173.80 (1C).



Synthesis of M6P Derivative (9):



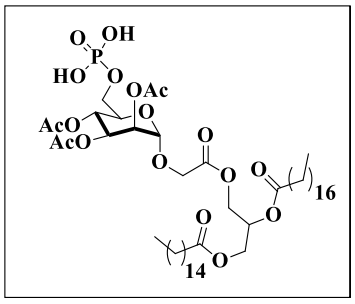
To a solution of 6-dibenzyl phosphate-2,3,4-tri-O-acetyl-α-D-mannopyranoside ethyl carboxylic acid (11.75 g, 18.83 mmol) and 3-Hydroxy-2-(octadeca-2-yloxy) propyl hexadecanoate (5 g, 18.83 mmol) in dry DCM (70 mL) was added 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (3.56 g, 22.6 mmol), N-hydroxy succinimide and 4-Dimethylaminopyridine (226 mg, 1.88 mmol). The reaction mixture was stirred overnight, and the progress of the reaction was monitored by TLC. After completion of the reaction, the reaction mixture was diluted with DCM and washed with water. The organic layer was washed with brine and dried over sodium sulfate. The dried DCM part was concentrated under a rotary evaporator. The crude product was purified by silica gel column chromatography using ethyl acetate/hexane as eluent to afford compound 9. Yield (12.31 g, 75%).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.87-0.90 (t, 6H), 1.26-1.43 (52H), 1.60-1.68 (m, 4H), 1.99-2.02 (m, 7H), 2.03-2.12 (m, 5H), 2.29-2.34 (m, 2H), 4.09-4.17 (m, 5H), 4.20-4.31 (m, 2H), 4.91-4.97 (m, 2H), 5.03-5.07 (m, 4H), 5.13-5.37 (m, 4H), 7.35 (10H)

^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) 14.07 (1C), 14.16 (1C), 20.59 (1C), 20.70 (1C), 21.00 (1C), 22.65 (1C), 24.83 (1C), 24.86 (1C), 24.92 (1C), 29.09 (1C), 29.24 (1C), 29.33 (1C), 29.42 (1C), 29.44 (1C), 29.56 (1C), 29.62 (1C), 29.67 (1C), 31.89 (1C), 34.08 (1C), 61.50 (1C), 62.02 (1C), 65.01 (1C), 65.75 (1C), 65.80 (1C), 68.33 (1C), 68.72 (1C), 69.11 (1C), 69.36 (1C), 69.40 (1C), 69.45 (1C),72.10 (1C), 72.68 (1C), 97.84 (1C), 127.86 (1C), 127.90 (1C), 127.95 (1C), 128.36 (1C), 128.56 (1C), 128.59 (1C), 130.04 (1C), 135.65 (1C), 135.71 (1C), 169.63 (1C), 169.79 (1C), 173.89 (1C), 197.90 (1C).

^{31}P NMR (400 MHz, $CDCl_3$): δ (ppm) -1.52

Deprotection of Benzyl from M6P Based Lipid Derivative (10):

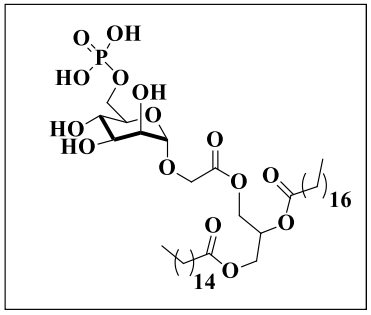


Deprotection of benzyl groups from the phosphate group, present in M6P moiety, was performed by hydrogenation. Hydrogenolysis of M6P based lipid derivative was carried out using 10% Pd/C in dry DCM at H_2 balloon pressure for 12 hrs. After completion of the reaction, the reaction mixture was filtered through celite bed and concentrated under reduced pressure to afford benzyl deprotected M6P-based lipid derivative (10) in almost quantitative yield. The resulting compounds were directly used for the next step without any further purification. (yield 65 %)

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.87-0.90 (t, 6H), 1.26-1.43 (m, 56H), 1.61-1.68 (m, 3H), 1.99-2.19 (m, 7H), 2.33-2.37 (m, 2H), 2.82-2.92 (m, 1H), 3.14-3.89 (m, 2H), 4.08-4.34 (m, 3H), 4.81-5.33 (m, 2H)

^{31}P NMR (400 MHz, $CDCl_3$): δ (ppm) -14.11, -20.62

Deprotection of Acetyl group from M6P Based Lipid Derivative (11): Benzyl deprotected

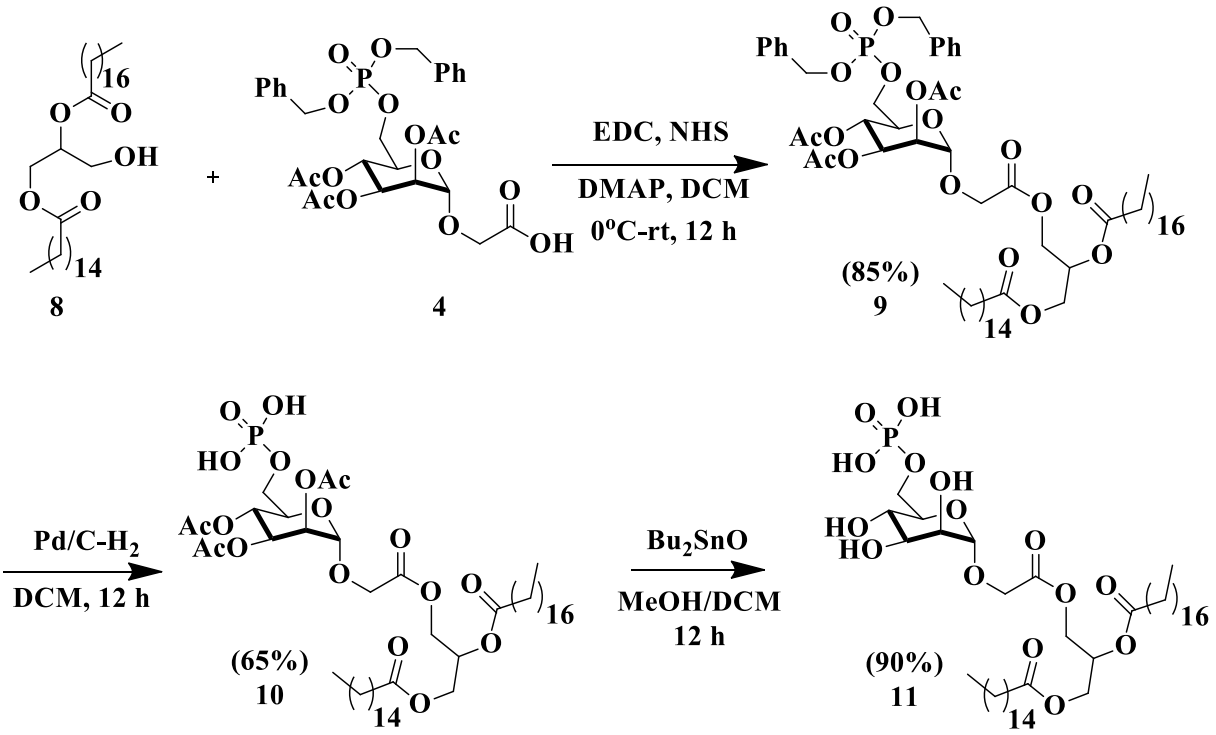


M6P-based lipid derivative (10) was dissolved in a mixture of dry MeOH and DCM. The di-butyl tin (IV) oxide was added to the solution of compound 10, and the reaction mixture was stirred for 12 hrs at room temperature. The progress of the reaction was monitored by TLC. After completion of the reaction, the solvent was removed under reduced pressure. The crude product was dissolved in DCM and washed with water and brine. The combined organic layer was dried over sodium

sulfate and concentrated under a rotary evaporator to afford completely deprotected final compound 11. Yield (90% yield)

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.87-0.90 (9H), 1.26-1.34 (52H), 1.62-1.63 (5H), 2.03-2.11 (2H), 2.29-2.37 (2H), 3.29-3.35 (2H), 3.93-4.06 (5H), 4.10-4.27 (1H), 4.82 (1H).

^{31}P NMR (400 MHz, $CDCl_3$): δ (ppm) -13.75, -20.26



8 4 9 10 11

Fabrication of Vesicles

The synthesized M6PL small molecule amphiphile, 5 mg (6.2×10^{-3} mmol), was dissolved in dichloromethane in a round bottom flask or in a glass vial. The solvent was evaporated using nitrogen flow to make a thin film on the surface. The thin film was

dried under a high vacuum for 6-8 hrs. Then 5 ml of water or buffer was added to the dried film and agitated for overnight followed by sonication. The solution was filtered through 0.45 µm filter paper to afford monodisperse vesicles. The assembly solution was used directly for characterization (TEM, DLS, AFM).

TEM Sample Preparation

10 µL of vesicle solutions were cast on a carbon-coated 400 mesh Cu grid and kept for 15-20 min. The excess solvent was removed by Whatman filter paper, and the sample cast grids were negatively stained by 0.2 wt% uranyl-acetate for 10 seconds, and excess solvent was soaked by Whatman filter paper. After that, the unbound uranyl-acetate was removed from the grids by washing with fresh deionized water for 2-3 times. Finally, the grids were vacuum dried in a desiccator to remove the moisture completely. Then, the dry state transmission electron microscopy (TEM) was performed to analyze the samples.

DLS Sample Preparation

The vesicles solution was diluted five times and then filtered using 0.45 µm filtered paper. Then the filtered solution was directly used to determine the hydrodynamic diameter of the vesicles using the dynamic light scattering (DLS) technique.

AFM Sample Preparation

10 µL of vesicle solutions were dropped cast on a silicon wafer and kept it for 20-30 min to evaporate the solvent. Finally, the grids were vacuum dried in a desiccator to remove the solvent completely. Then, the dry state atomic force microscopy (AFM) was performed to analyze the samples.

Dye Encapsulation into Vesicles

The synthesized M6PL small molecule amphiphile, 5 mg (6.2×10^{-3} mmol), was dissolved in dichloromethane in a round bottom flask or in a glass vial. The solvent was evaporated using nitrogen flow to make a thin film on the surface. The thin film was dried under a high vacuum for 6-8 hrs. Then 5 ml of water or buffer with 50 µL of Calcein (stock 1 mg/mL) was added to the dried film and agitated for overnight followed by sonication. The solution mixture was placed into a dialysis membrane (3.5 kDa molecular weight cutoff) and then dialyzed against deionized water for 24 hrs while changing deionized water at 4 hrs interval. Then, it was dialyzed against phosphate buffer (pH 8.0) containing 50 mM phosphate, 50 mM NaCl, and 0.2 mM disodium salt of EDTA. Within 16 hrs, all unencapsulated Calcein came out from inside the dialysis membrane. After the removal of free Calcein, the mixture was dialyzed against deionized water for 24 hrs with the water being changed three times. The calcein loaded vesicles are named as CLV.

Similarly, hydrophilic lysosomotropic agent chloroquine diphosphate (CQ) has been encapsulated in the vesicle (data not shown).

The synthesized M6PL small molecule amphiphile, 5 mg (6.2×10-3 mmol), was dissolved in dichloromethane in a round bottom flask or in a glass vial. The solvent was evaporated using nitrogen flow to make a thin film on the surface. The thin film was dried under a high vacuum for 6-8 hrs. Then 5 ml of water or buffer was added to the dried film and agitated for overnight followed by sonication. Then, 50 µL of the dye Rhodamine B octadecyl ester (RBOE) (1 mg/mL in acetone) or doxorubicin (DOX) (1 mg/mL in acetone) was added to the vesicular solution and kept overnight for encapsulation. This solution was filtered through 0.45 µm filter paper before analysis. RBOE loaded vesicles are named as RLV and DOX loaded vesicles are named as DLV. The dual dye calcein and RBOE loaded vesicles are named as CRLV.

Dye/Drug loading efficiency (DLE) was determined by using the following formula:

DLE % = (Weight of encapsulated Dye/Drug)/(Weight of Dye/Drug in Feed) x 100

RBOE Loading Efficiency of RLV = ~42 %

DOX Loading Efficiency of DLV = ~32%

Enzyme/Protein Encapsulation into Vesicles

The synthesized M6PL small molecule amphiphile, 5 mg (6.2×10^{-3} mmol), was dissolved in dichloromethane in a round bottom flask or in a glass vial. The solvent was evaporated using nitrogen flow to make a thin film on the surface. The thin film was dried under a high vacuum for 6-8 hrs. Then 5 ml of water or buffer with 50 µL of β-galactosidase/BSA (stock 1 mg/mL) was added to the dried film and agitated for overnight followed by sonication. The solution mixture was poured into a Sephadex column, and free β-galactosidase/BSA was separated from the encapsulated β-galactosidase/BSA in vesicles. The loading of β-galactosidase/BSA was confirmed from absorption spectroscopy and fluorescence spectroscopy. The β-galactosidase loaded vesicles are named as GLV and BSA loaded vesicles are named as BLV.

Enzyme loading efficiency (ELE) was determined by using the following formula:

ELE % = (Weight of encapsulated Enzyme)/(Weight of Enzyme in Feed) x 100

ELE for BSA loaded vesicles (BLV) = 30 %

ELE for β-galactosidase loaded vesicles (BLV) = 22 %

Enzyme-Responsive Dye/Drug Release Studies of Vesicles

The release of Calcein was studied in the presence of the ALP enzyme using the dialysis method. Calcein-loaded vesicle solution (CLV: 1 mL) was incubated with 10 μM of ALP enzyme, transferred to a 3.5 kDa MWCO dialysis membrane, and placed in PBS, pH = 7.4, 10 mM released media that was shaken at 100 rpm at 37 °C. At a pre-determined time interval, the amounts of released Calcein were monitored by fluorescence spectroscopy at 515 nm. For the dye release study at pH = 5.0, the pH was adjusted by adding 1 M HCl to the vesicle solution.

The in vitro enzyme-responsive RBOE/DOX release study from the vesicle was carried out at pH = 7.4 in 10 mM PBS buffer. Briefly, 1 mL (1 mg/mL) solution of RBOE/DOX encapsulated vesicles (RLV/DLV) in 10 mM PBS buffer solution (pH = 7.4) was placed in an eppendorf tube and vortexed slowly in the presence of 10 μM esterase at 37°C. At pre-determined time interval, the amounts of released RBOE/DOX were monitored by fluorescence spectroscopy at 575 nm/590 nm. For the dye release study at pH = 5.0, the pH was adjusted by adding 1 M HCl to the vesicle solution. The amounts of released RBOE/DOX were monitored by fluorescence spectroscopy at 575 nm/590 nm, at the pre-determined time interval.

Dual enzyme-responsive dye/drug release study from the calcein and RBOE encapsulated vesicle (CRLV) was carried out in a similar way using ALP and esterase enzyme together.

Enzyme-Responsive Enzyme/Protein Release Studies of Vesicles

The release of β-galactosidase/BSA was studied in the presence of ALP and esterase enzyme using the dialysis method. β-galactosidase/BSA loaded vesicle solution (GLV/BLV: 1 mL) was incubated with 10 μM of ALP enzyme and placed in PBS, pH 7.4, 10 mM released media that was shaken at 100 rpm at 37°C. At pre-determined time interval, the released β-galactosidase/BSA were separated from the vesicles by Sephadex column chromatography,

and the amount of released β-galactosidase/BSA was calculated from fluorescence spectroscopy at 515 nm.

In Vitro Cytotoxicity Assay

In a flat bottomed 96-well adherent plate, HEK-293 cells were seeded at a density of 1×10^4 cells/well in 100 µL of MEM containing 10% FBS. The plates were incubated at 37°C with 5% CO_2 for 24 hrs. Vesicular solution was added to make a final concentration of 5, 10, 20, 40, 50, 75, 100, 150, and 200 µg/mL respectively in MEM containing 10% FBS. Cells were incubated for 48 hrs at 37°C with 5% CO_2. At the end of incubation, media was removed, and 110 µL solution of MEM containing 10% FBS with filter-sterilized MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.45 mg/mL) was added into each well and further incubated at 37°C with 5% CO_2 for 4 hrs. At the end of incubation, media was aspirated from the wells, and 100 µL DMSO was added to dissolve insoluble formazan crystals formed. Resultant absorbance was measured at 550 nm using a microtitre plate reader. The relative % cell viability was calculated from the following equation: Relative % cell viability = $(A_{test}/A_{control}) \times 100\%$ (A_{test} is the absorbance of the sample treated cells and $A_{control}$ is the absorbance of the untreated cells. Each absorbance was taken to be the mean of triplicate measurements). The cell viability was represented as a percentage relative to untreated cells as a control.

In Vitro Cell Growth Kinetics

In flat bottomed glass coverslip in 24 well non-adherent plates, HEK-293 cells were seeded at a density of 2×10^5 cells/well in 1 mL of MEM containing 10% FBS. The plates were incubated at 37°C with 5% CO_2 for 24 hrs. The vesicular solution was added to make a final concentration of 200 µg/mL in MEM containing 10% FBS. Cells were incubated for 12, 24,

and 36 hrs, respectively, at 37°C with 5% CO_2. The cells were counted at 12, 24, and 36 hrs, respectively, to know the effect of vesicles on cell growth.

Cellular Uptake Study Using Epifluorescence Microscopy

HEK-293 cells were seeded on a glass coverslip in 24 well non-adherent plates at a density of 3 x 10^5 cells/well in 1 mL of MEM containing 10% FBS. The plate was incubated at 37°C with 5% CO_2 for 24 hrs. Vesicles loaded with RBOE/Calcein/ β-galactosidase (CLV/RLV/CRLV/GLV) were prepared at the concentration of 1 mg/mL in serum-free MEM media. After UV sterilization for half an hour, all the samples were added on the cell-seeded glass coverslips at the concentration of 200 µg/mL (0.2 mg/mL) in complete media independently in respective wells. As a control free RBOE at the concentration of 20 µg/mL was added in control well and incubated at 37°C with 5% CO_2 for 4 hrs. After incubation, the media was replaced with 200 µg/mL of RBOE/Calcein/ β-galactosidase loaded vesicular solution and then further incubated for an additional 4 hrs at 37°C with 5% CO_2. At the end of incubation, cells were washed 3 times with PBS followed by the 15 min fixation with 4 % paraformaldehyde at room temperature. Cells were washed 3 times with PBS for the complete removal of paraformaldehyde. For lysosome staining, cells were treated with 50 nM Lysotracker green/red DND-26 (Molecular probes by Life Technologies) for 30 minutes at 37°C with 5% CO_2 followed by 3 times washings with PBS. The nucleus of the cell was stained with DAPI at a concentration of 600 nm for 5 minutes, followed by 3 times washing with PBS. Images were acquired using an epifluorescence microscope by Carl Zeiss (Model: Axio Observer.Z1, Oil emersion objective, 63X). (Filters set 49 DAPI shift free EX G 365, BS FT 395, EM BP 445/50, Filter set 20 Rhodamine shift-free EX BP 546/12, BS FT 560, EM BP 575-640, Filter set 10 shift free EX BP 450-490, BS FT 510, EM BP 515-565).

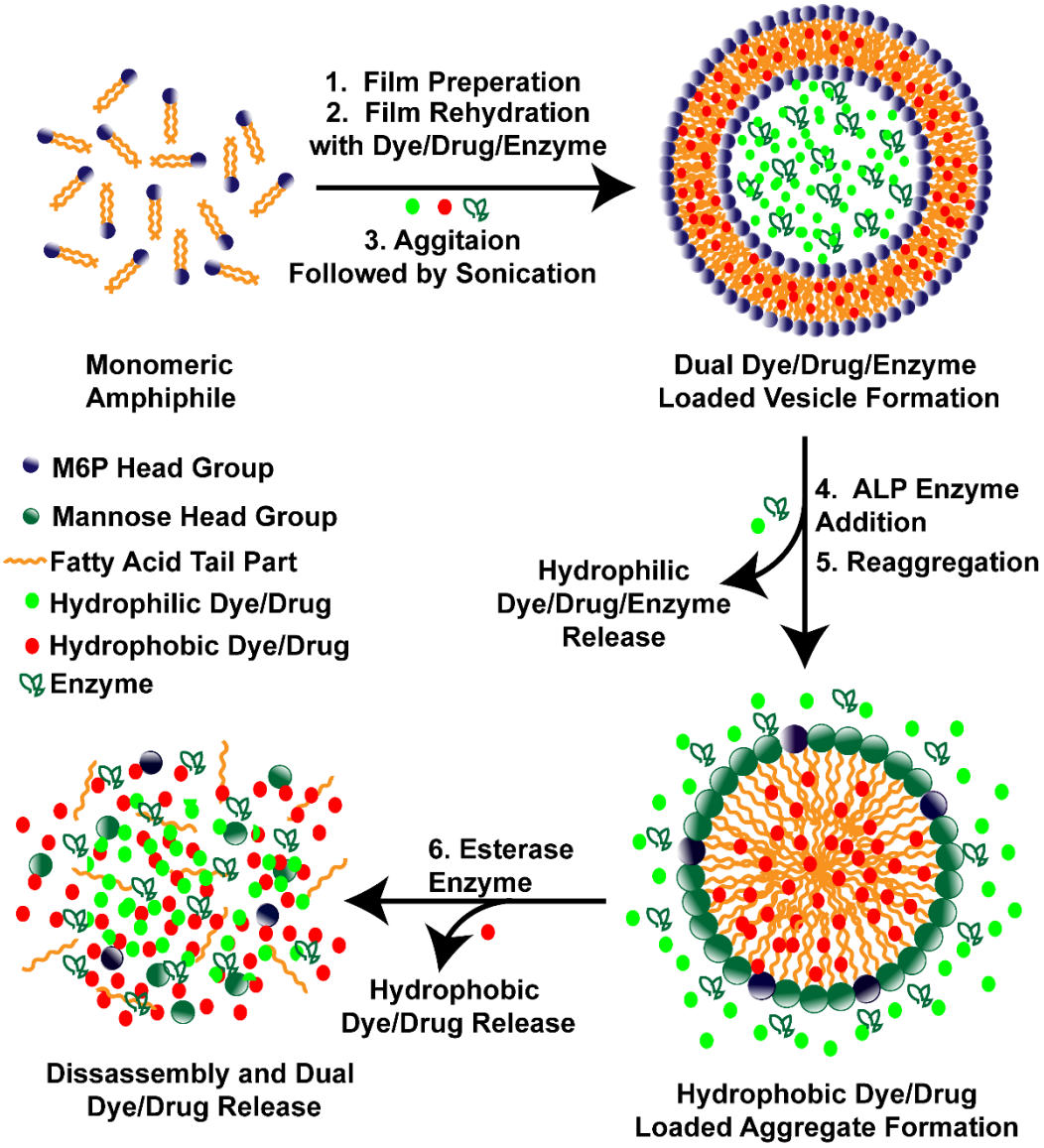


Figure S1: Schematic representation of vesicle formation from M6PL, cargo loading in vesicles, and dual enzyme responsive cargo release.

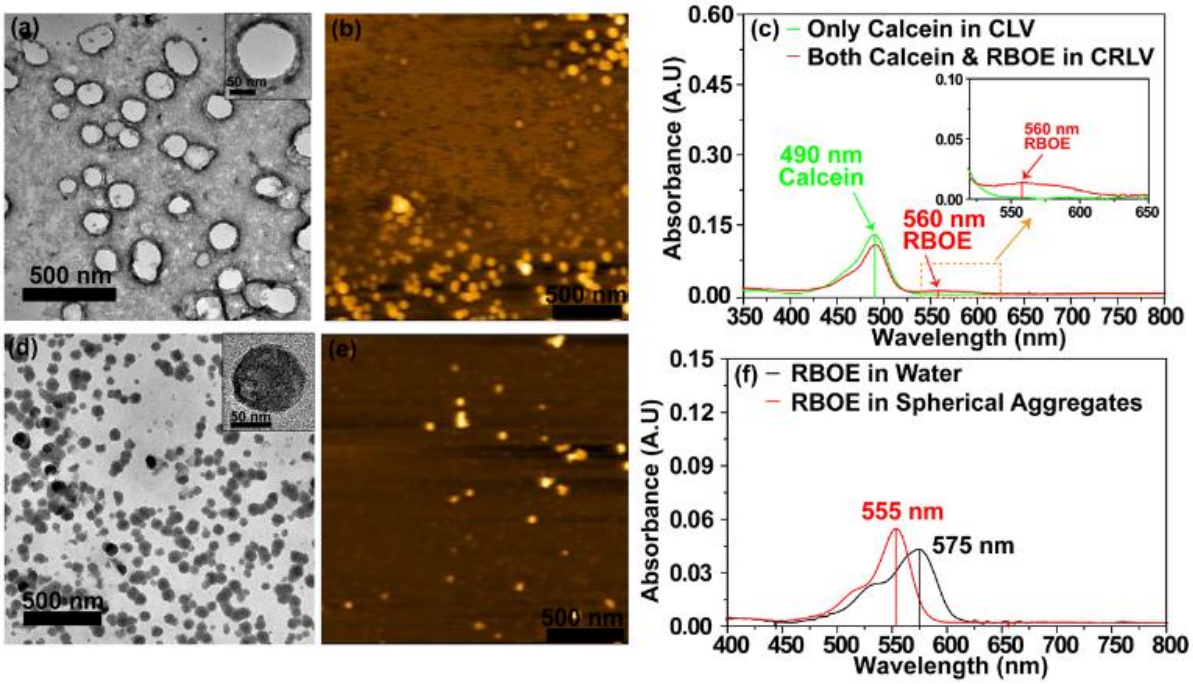


Figure S2: (a) TEM image of vesicle (scale bar 500 nm); (b) AFM image of vesicle (scale bar 500 nm); (c) UV-vis spectra of dual dye (calcein and RBOE) loaded vesicle (CRLV) in aqueous medium (inset: zoom absorption spectra from 520 to 650 nm); (d) TEM image of spherical aggregates (Scale bar 500 nm); (e) AFM of spherical aggregated (Scale bar 500 nm); (f) UV-vis spectra of RBOE loaded spherical aggregates in aqueous medium. TEM and AFM imaging has been performed in dry state.

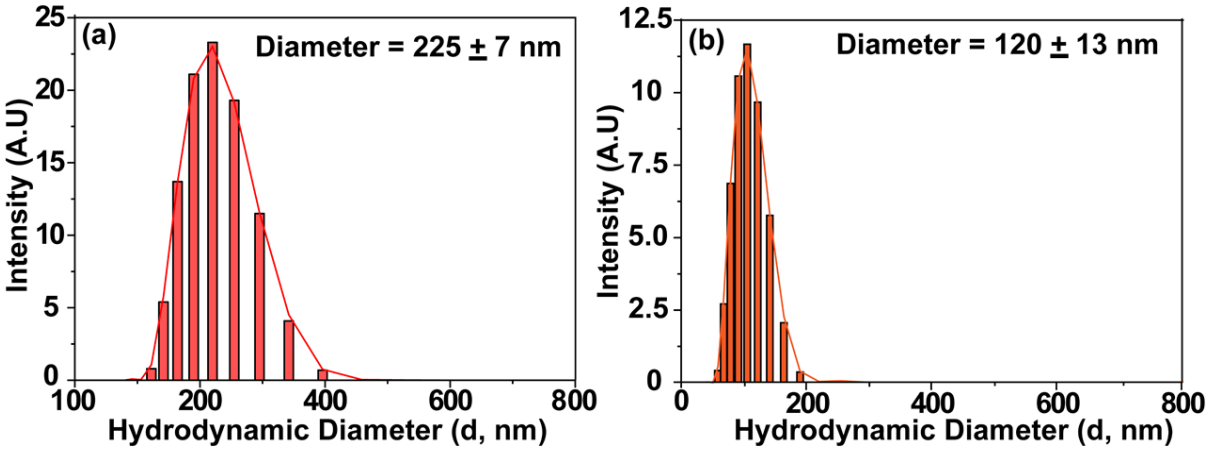


Figure S3: Hydrodynamic diameter of (a) vesicles vs (b) spherical aggregates in aqueous medium.

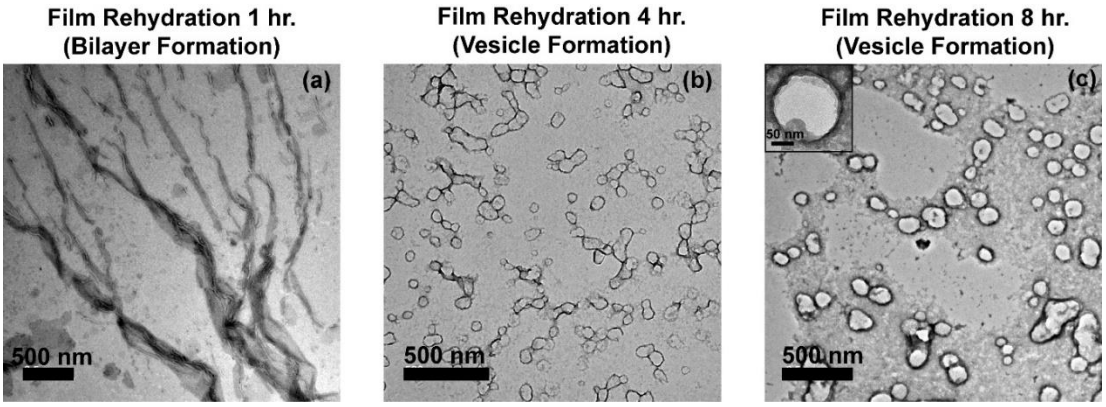


Figure S4: Vesicle formation of M6PL small molecule amphiphile using membrane hydration technique in an aqueous medium. (a) Dry state TEM image for bilayer formation of lipid membrane (rehydration for 1 hrs); (b) Dry state TEM image for vesicle formation during agitation (agitation for 4 hrs); and (c) Dry state TEM image for complete vesicle formation after sonication followed by agitation (8 hrs).

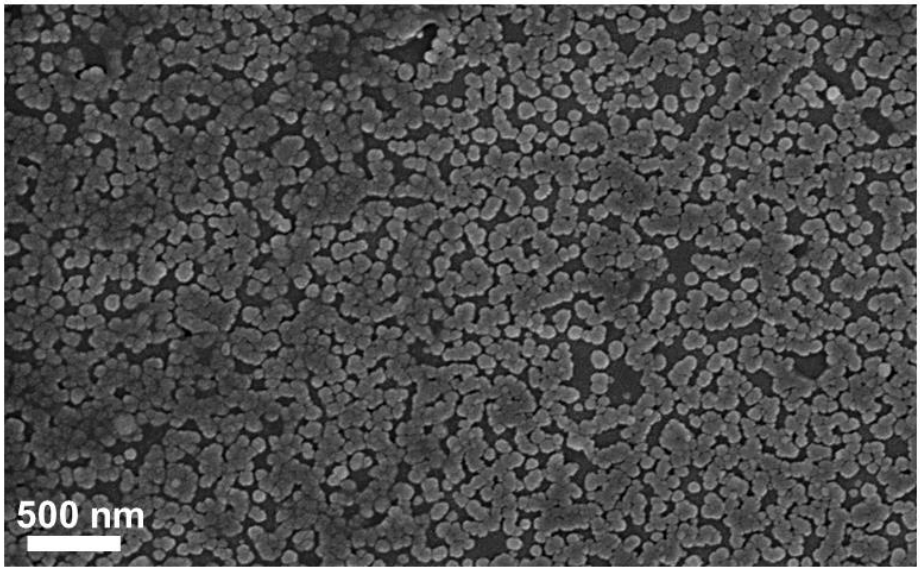


Figure S5: Vesicle formation of lipid membrane in an aqueous medium. Dry state SEM image of M6PL Vesicle.

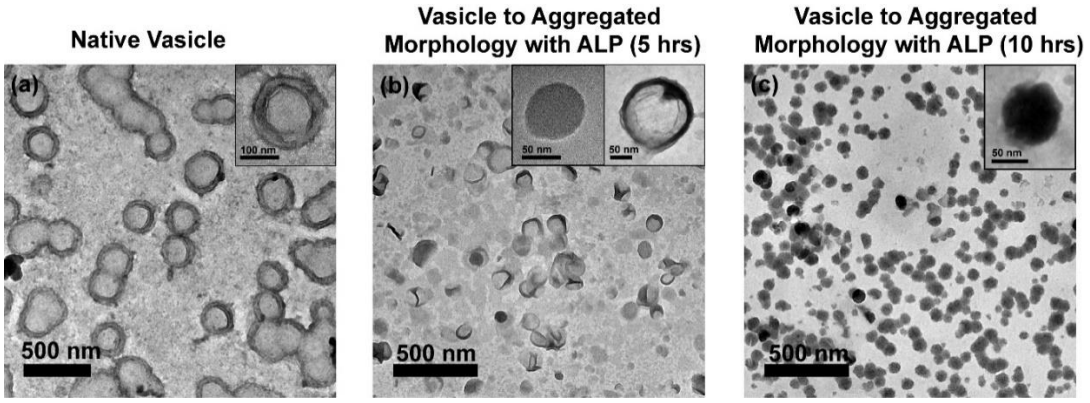


Figure S6: M6PL Vesicle to spherical aggregated structure transformation in the presence of ALP enzyme in an aqueous medium. (a) Dry state TEM image of native vesicle in the absence of ALP; (b) Dry state TEM image vesicle to aggregated structure formation during ALP treatment (both vesicle and aggregated structure present after ALP treatment for 5 hrs); and (c) Dry state TEM image vesicle to aggregated structure formation during ALP treatment (treatment for 10 hrs)

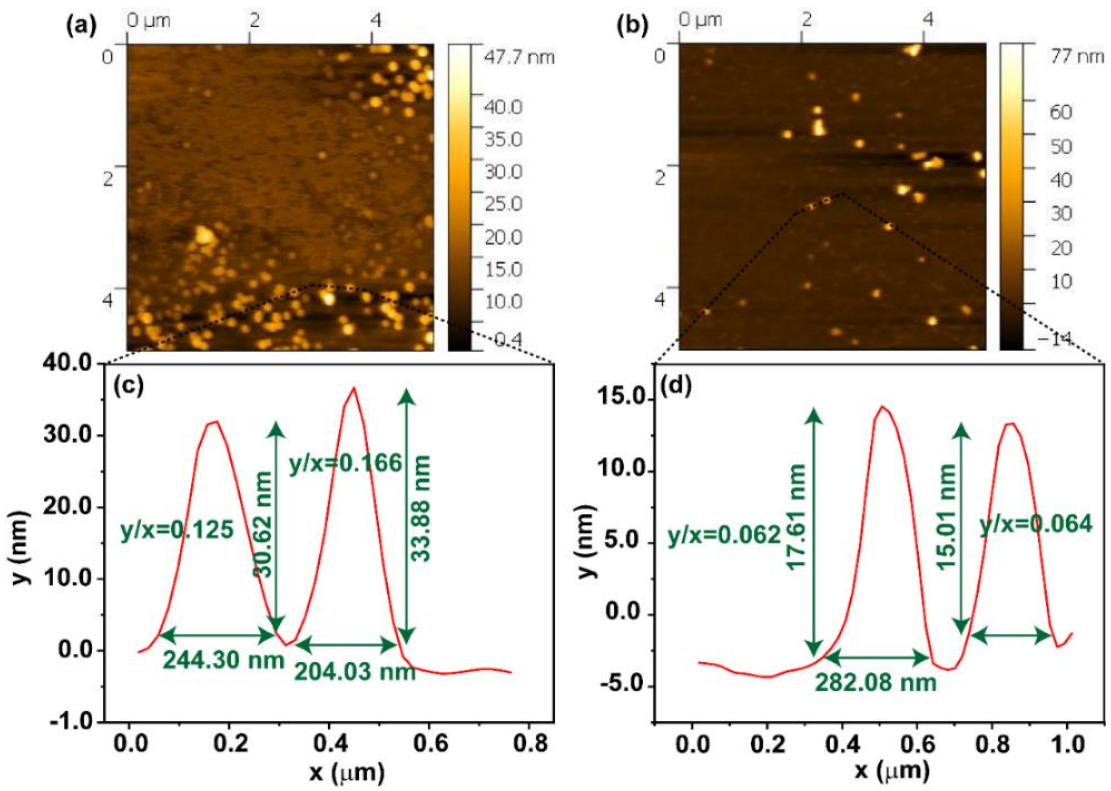


Figure S7: AFM Images and corresponding Height profile. (a) Dry state AFM image of M6PL vesicle; (b) Dry state AFM image of spherical aggregated structure; (c) height/width profile of M6PL vesicle; and (d) height/width profile of the aggregated structure.

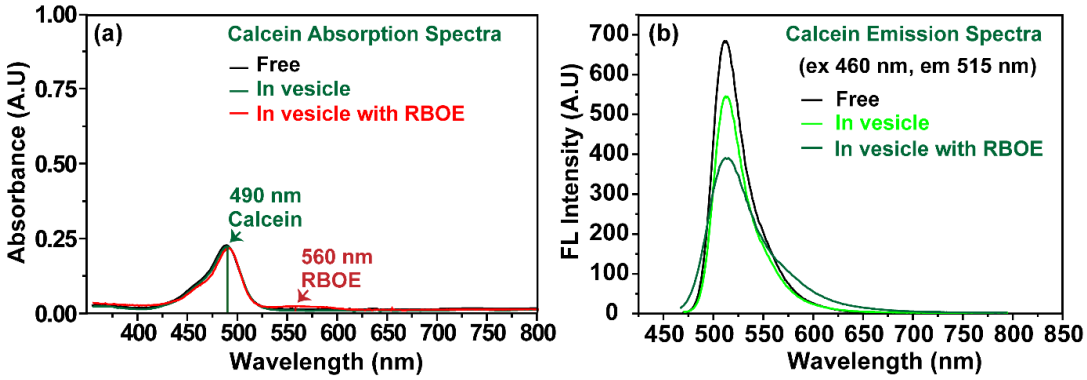


Figure S8: Dual dye loading in vesicle in an aqueous medium. (a) UV-Vis Spectroscopy; black: free Calcein; green: Calcein loaded vesicle (CLV); red: Calcein and RBOE loaded vesicle (CRLV); (b) fluorescence spectroscopy; black: free Calcein; green: Calcein loaded vesicle (CLV); red: Calcein and RBOE loaded vesicle (CRLV).

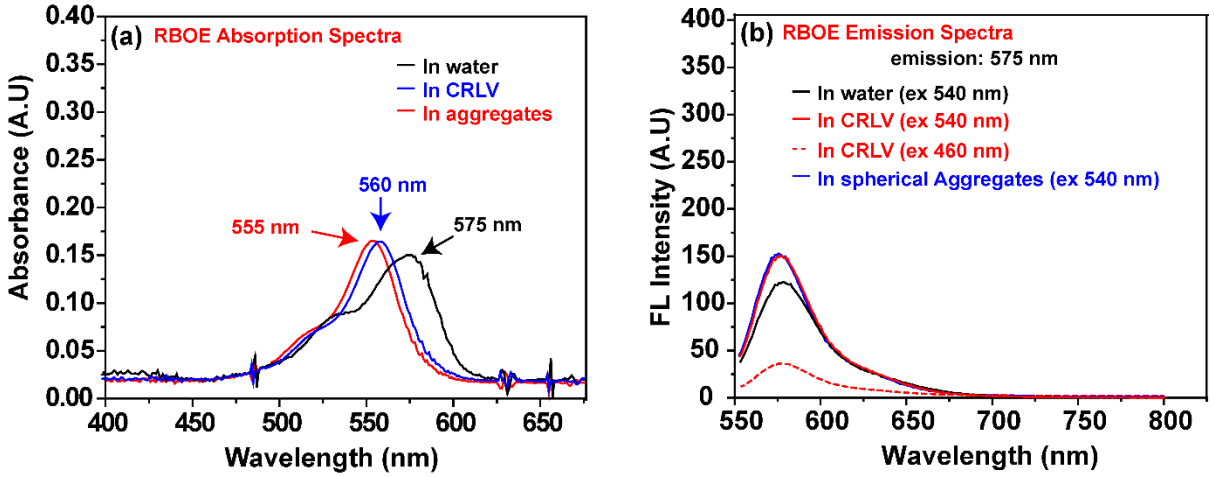


Figure S9: RBOE dye loading in the vesicle (dye loading efficiency = 42 %) and in an aggregated form in an aqueous medium; (a) UV-Vis spectroscopy of RBOE (red: in aggregates; blue: in vesicle (CRLV); black: in water); and (b) Fluorescence spectroscopy of RBOE (black: in water; blue: in aggregates; red: in vesicle (CRLV)).

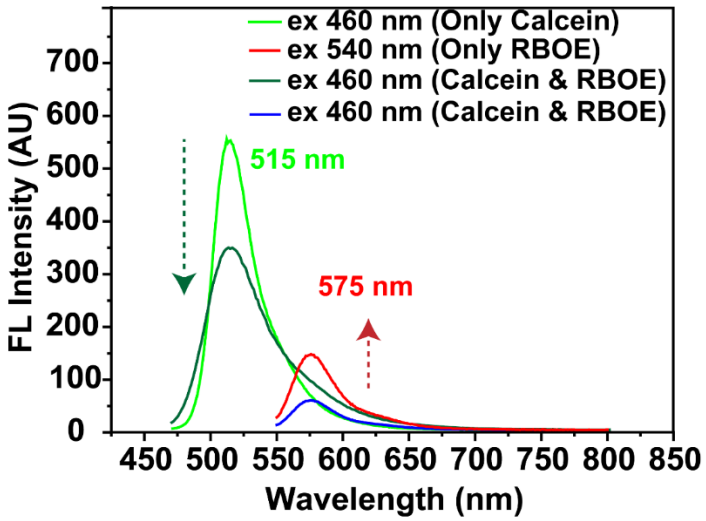


Figure S10: Dye loading in vesicle in an aqueous medium. Fluorescence spectroscopy: only Calcein: CLV; only RBOE: RLV; Calcein & RBOE: CRLV.

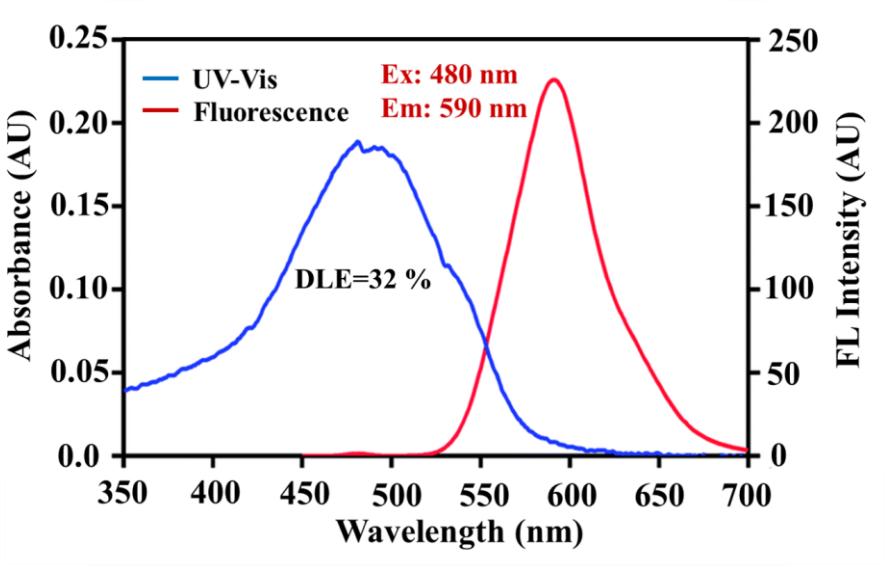


Figure S11: DOX loading (drug loading efficiency = 32%) in vesicle in an aqueous medium (DLV); UV-Vis: blue and Fluorescence Spectroscopy: red.

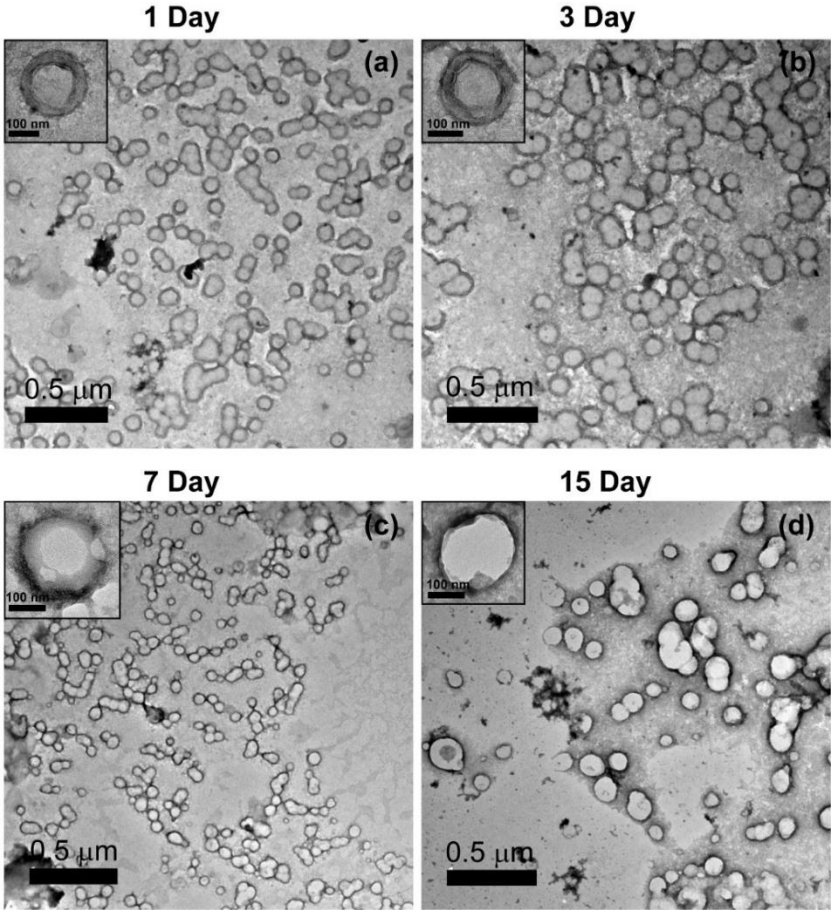


Figure S12: Stability of M6PL vesicle over time; (a) Dry state TEM image of vesicle on day 1, (b) Dry state TEM image of vesicle on day 3, (c) Dry state TEM image of vesicle on day 7, and (d) Dry state TEM image of vesicle on day 15.

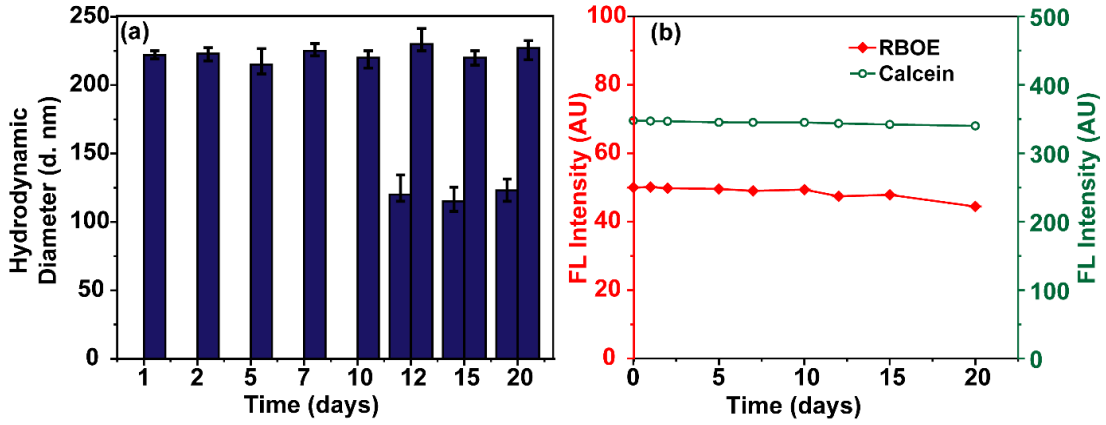


Figure S13: Stability of vesicle over time (a) DLS stability in aqueous medium and (b) Fluorescence of Calcein and RBOE loaded vesicle in aqueous medium (CRLV).

Discussion: The stability of dual cargo loaded M6PL vesicles were evaluated by monitoring the change in their hydrodynamic size and their ability to retain encapsulated dye molecules over time. DLS experiment of cargo loaded M6PL vesicles indicated no change in size up to 10 days, after which some small particles were observed over time (Figure S9a). Which may be due to some disruption of vesicles structures to small vesicles. The DLS measurement indicated that the vesicles are stable upon storage for 5-7 days without any disruption to small particles. Fluorescence spectra of the Calcein and RBOE loaded vesicles (CRLV) indicated more than 90% dye remains in the vesicle (Figure S9b). The fluorescence intensity over 20 days (Figure S9b), indicating that the both calcein and RBOE dye were reasonably stable in the vesicular environment.

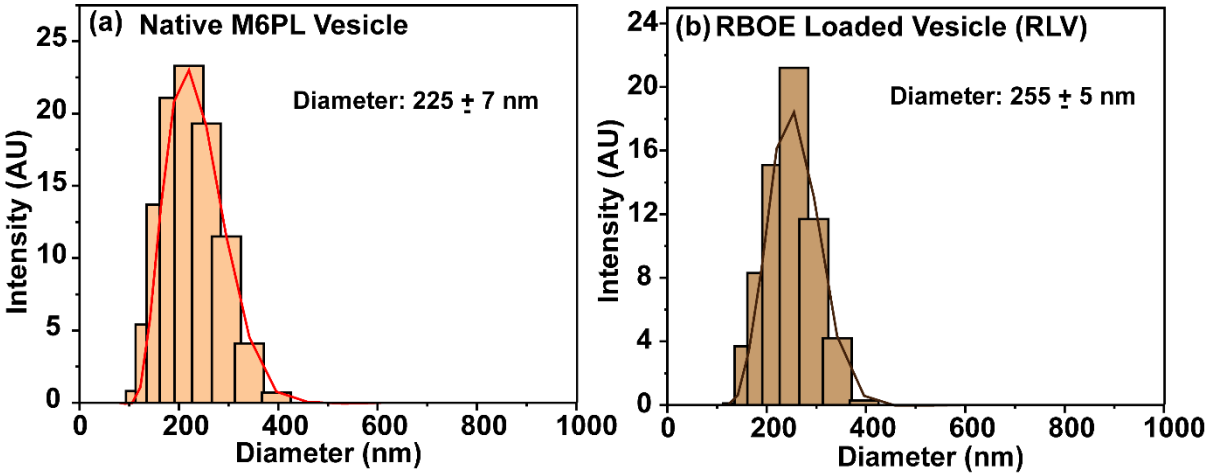


Figure S14: Hydrodynamic diameter of (a) native vesicles vs (b) RBOE loaded vesicle (RLV) in aqueous medium.

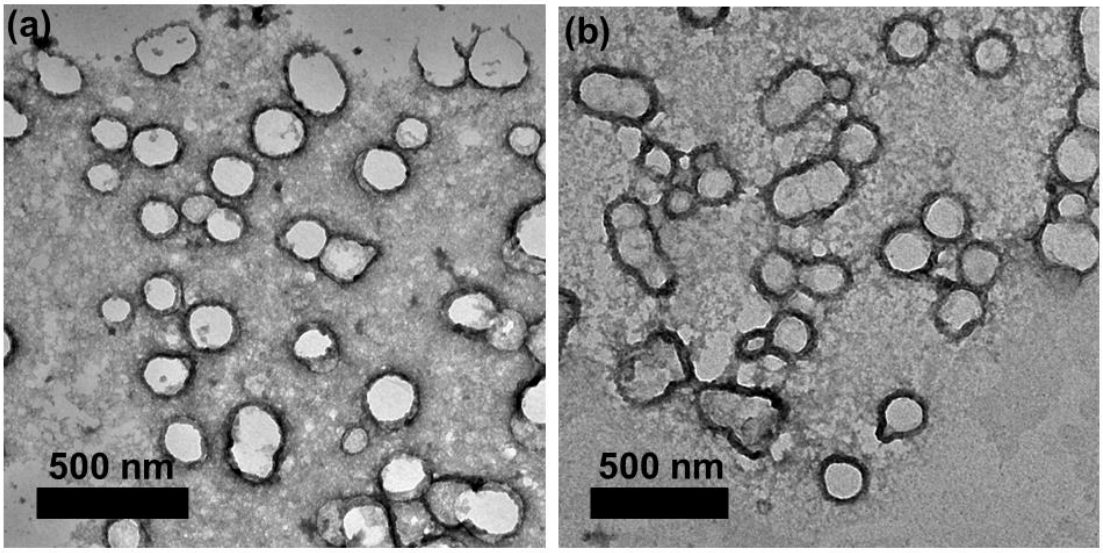


Figure S15: Dry state TEM images of (a) native vesicles vs (b) RBOE loaded vesicles (RLV).

Discussion: The effect of RBOE in the vesicle has been examined by DLS analysis and TEM imaging. The DLS analysis indicated that the RBOE remains relatively in hydrophobic microenvironment thus the hydrodynamic size is increased by 20 nm (Figure S13; native vesicle: 225 nm and RBOE loaded vesicle: 255 nm). In TEM images we get negligible change in diameter as the TEM imaging has been performed in dry state (Figure S14).

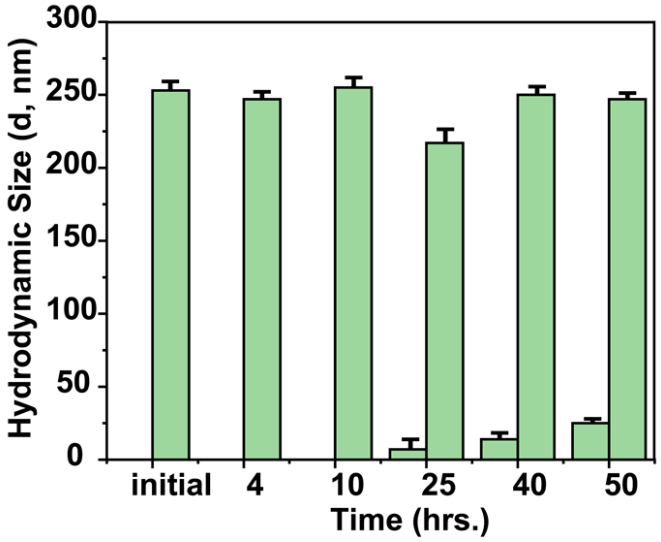


Figure S16: Stability of RBOE loaded vesicle (RLV) in human plasma for 50 hrs time interval. Bar diagram of hydrodynamic diameter in DLS measurement (Large bar: Intact vesicle; Small bar: fragments of vesicle).

Extraction of human plasma: Blood samples were collected from a group of healthy male voulentier (age group 22-30) in a collection tubes containing 3.8% sodium citrate solution (the ratio of blood : sodium citrate is 9 : 1 v/v). After that the citrated blood samples were centrifuged for 15-20 mins at 2500 rpm at 4 °C. The yellowish supernatants (platelet free citrated plasma) were then collected and used for experiments. The plasma was stored at -20 °C.

Discussion: The stability of RBOE loaded M6PL vesicles (RLV) were evaluated by monitoring the change in their hydrodynamic size over 50 hrs time period in human plasma. DLS experiment of RLV vesicles in human plasma indicated that no change in size up to 50 hrs time period (very little change: some small particles were observed over time) (Figure S15). Which may be due to the presence of esterase in blood plasma some disruption of vesicles structures to small particles.

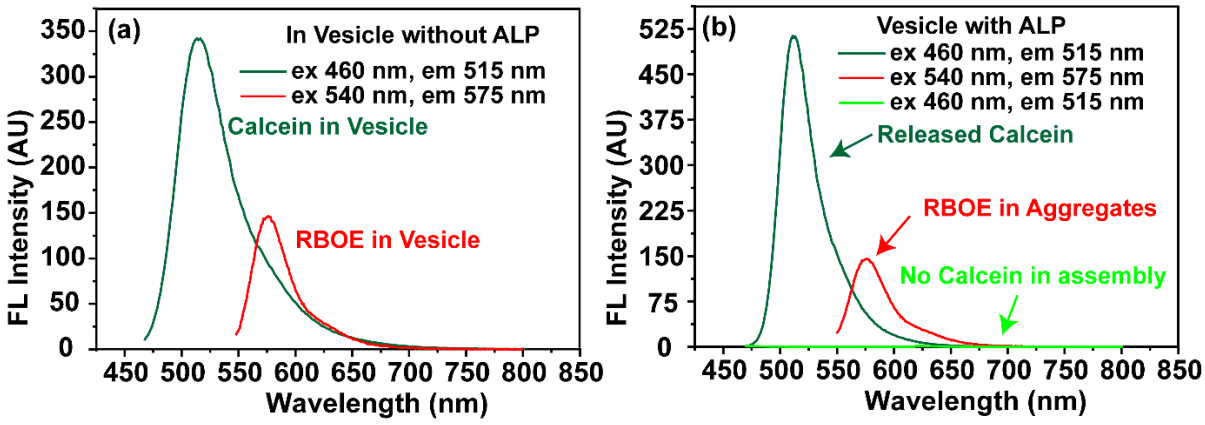


Figure S17: ALP enzyme effect on dye release from dual dye Calcein and RBOE loaded vesicle (CRLV) in an aqueous medium; (a) Fluorescence spectroscopy in the absence of ALP; (b) Fluorescence spectroscopy in the presence of ALP (10 hrs treatment).

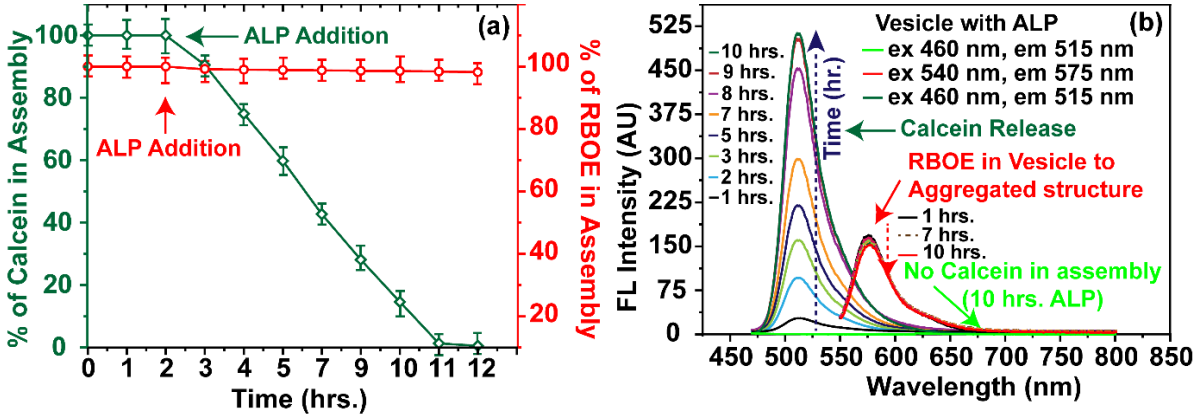


Figure S18: Effect of ALP enzyme on dual dye release of Calcein and RBOE loaded vesicle in aqueous medium (CRLV): (a) dye release kinetics over time in the presence of ALP; and (b) fluorescence spectroscopy of dye loaded assembly over time in the presence of ALP.

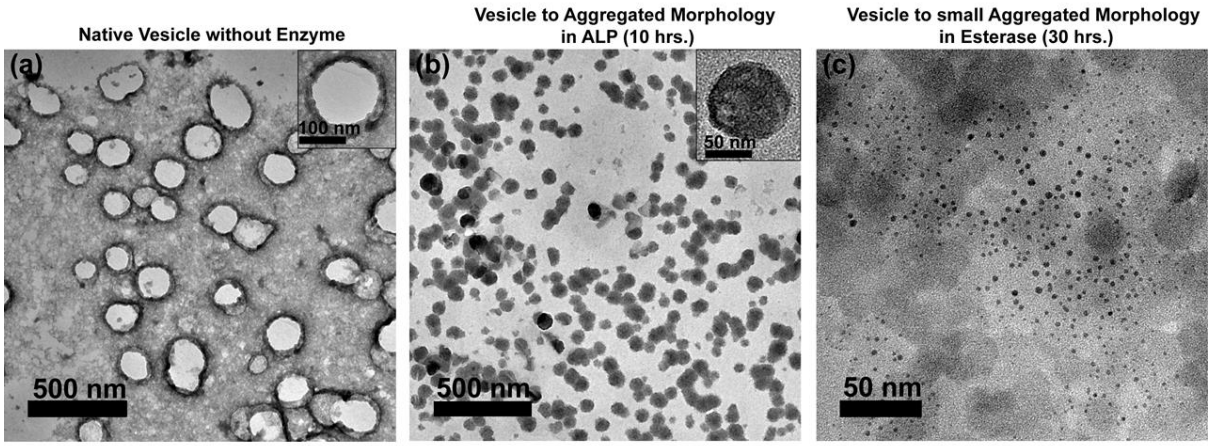


Figure S19: Change of M6PL vesiclular morphology in the presence of ALP and Esterase enzyme, respectively in the aqueous medium. (a) Dry state TEM image of native vesicle in the absence of both ALP and Esterase; (b) Dry state TEM image of vesicle to aggregated structure formation during ALP treatment (treatment for 10 hrs); and (c) Dry state TEM image of large

aggregate to small aggregate structure (particle) formation during Esterase treatment (treatment for 30 hrs).

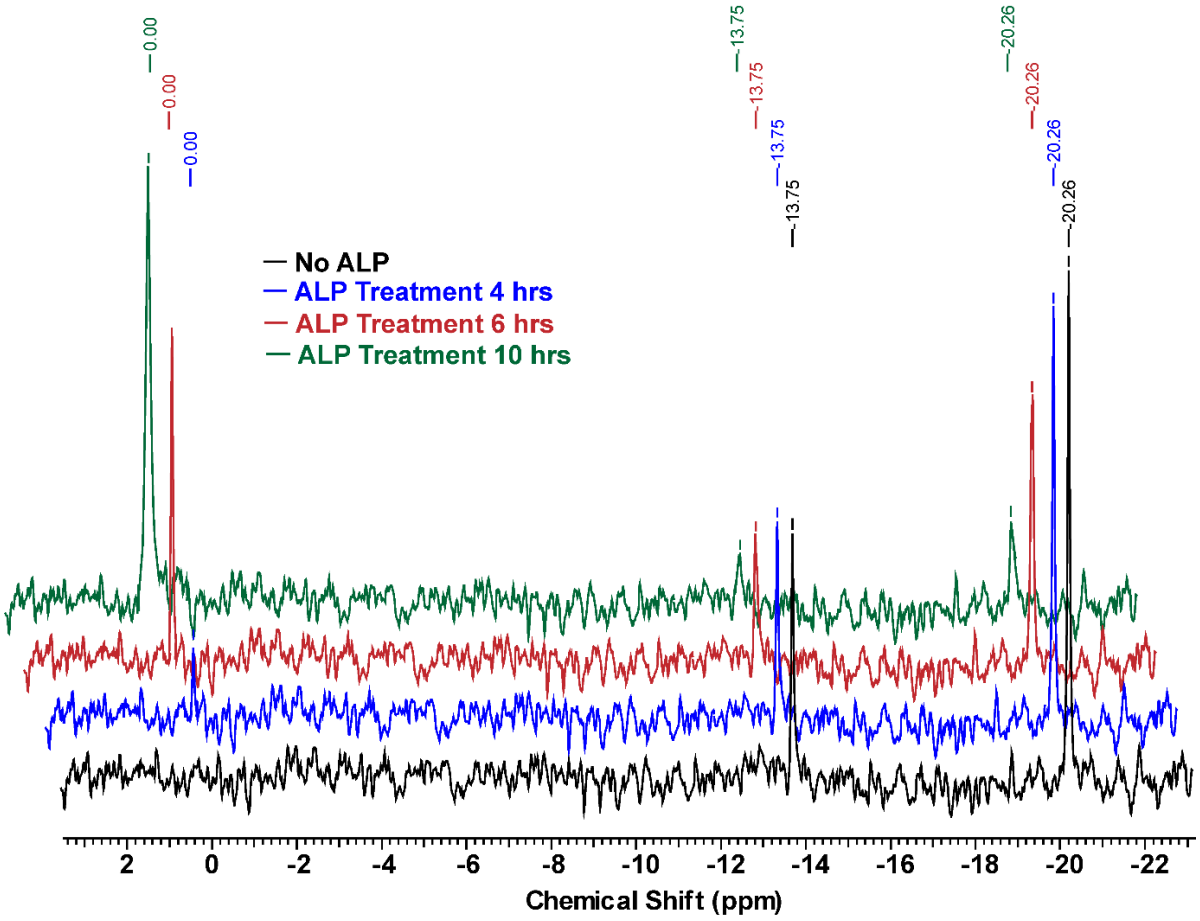


Figure S20: Time dependent ^{31}P NMR of small molecule amphiphile in presence of ALP enzyme in aqueous medium (effect of ALP on M6PL lipid molecule)

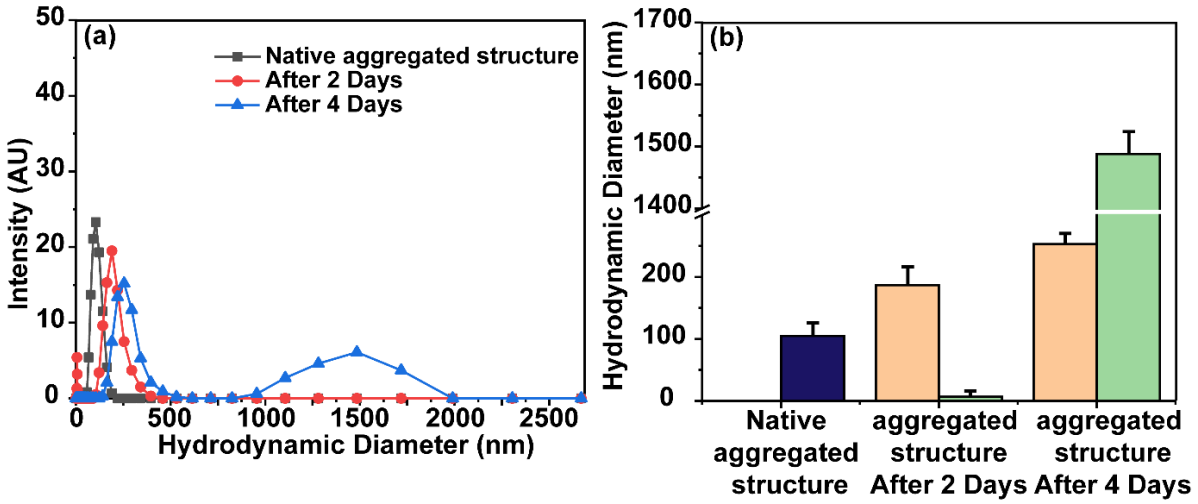


Figure 21: Stability of aggregated structure over time in aqueous medium (a) DLS stability and (b) bar diagram of DLS spectra.

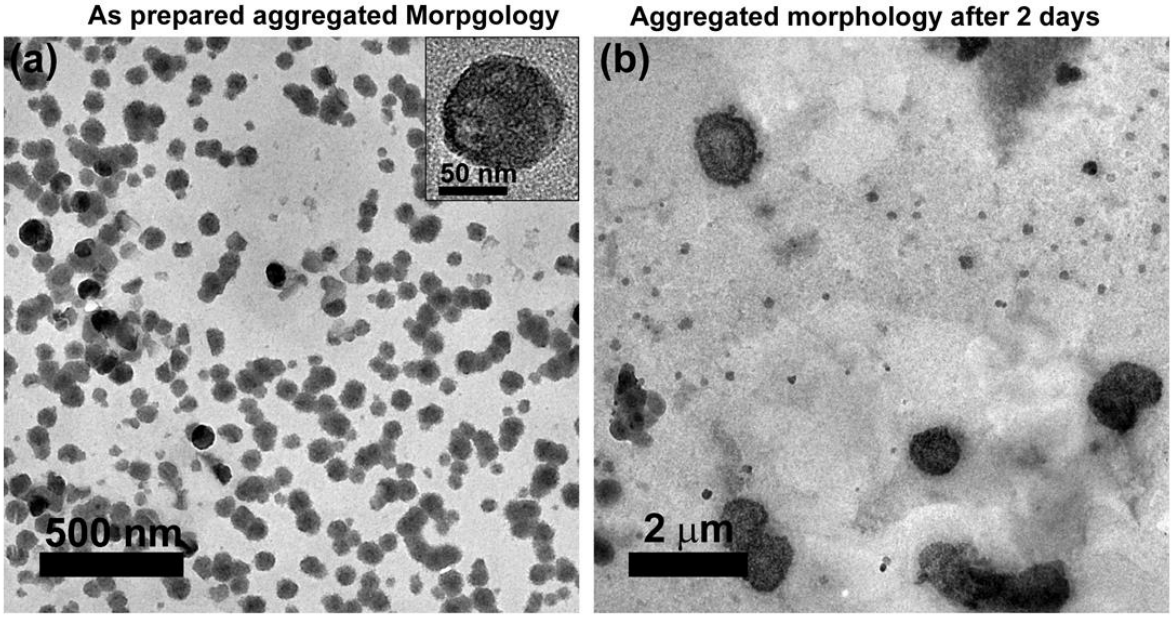


Figure S22: Stability of aggregated structure over time (a) Dry state TEM image of as prepared aggregated structure and (b) Dry state TEM image of aggregated structure after 2 days.

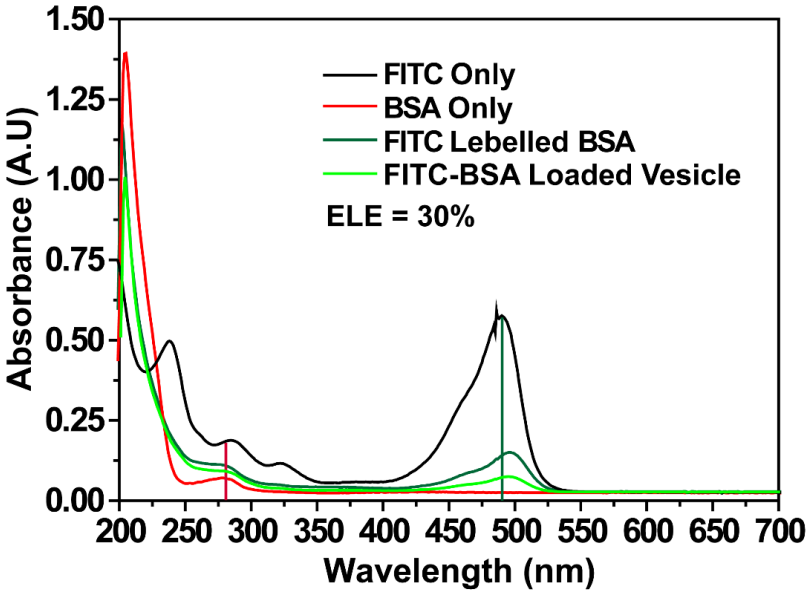


Figure S23: UV-Vis spectroscopy of FITC labeled BSA loading (protein loading efficiency = 30%) in vesicle (BLV in aqueous medium).

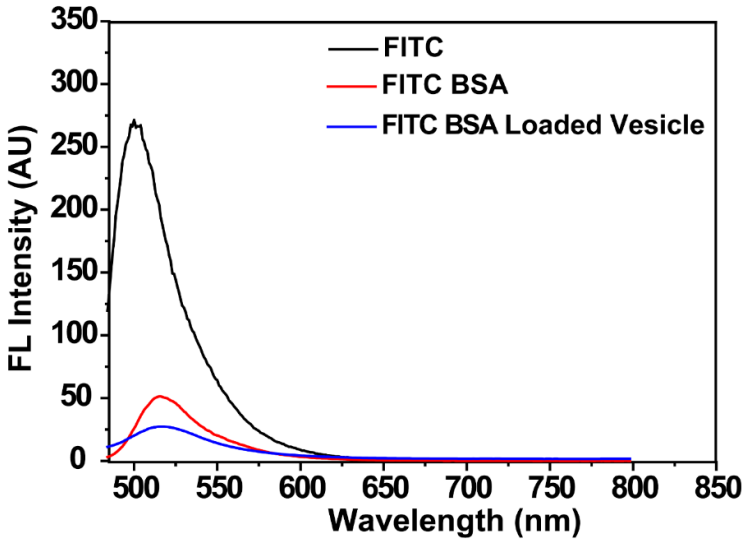


Figure S24: Fluorescence spectroscopy of FITC labeled BSA loading in vesicle (BLV in aqueous medium)

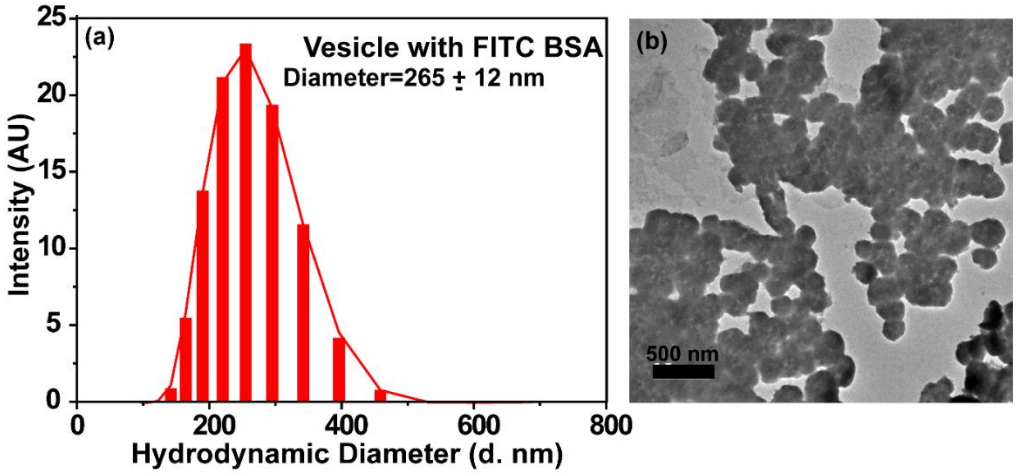


Figure S25: BSA loading in the vesicle (BLV). (a) DLS of BSA loaded vesicle in aqueous medium, and (b) Dry state TEM image of BSA loaded vesicle.

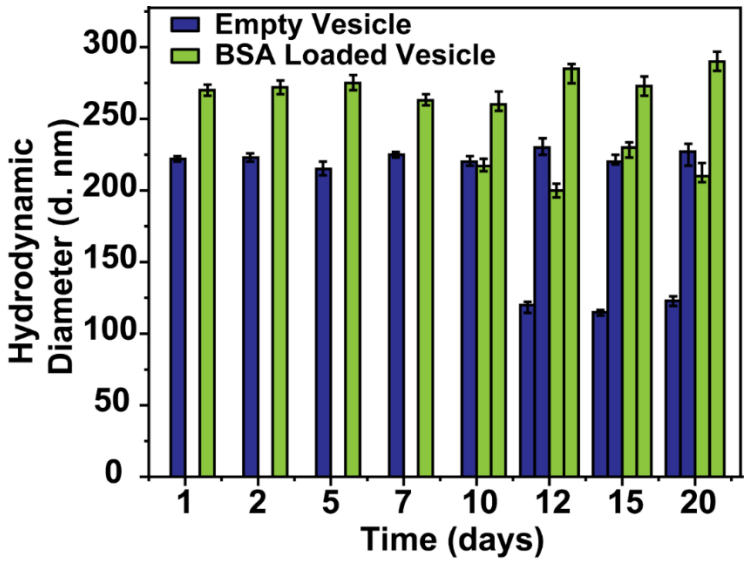


Figure S26: Stability of empty vesicle and BSA loaded vesicle (BLV) over time (blue: empty vesicle, and green: BSA loaded vesicle (BLV)). DLS analysis in the aqueous medium.

Discussion: The stability of BSA loaded M6PL vesicles (BLV) were evaluated by monitoring the change in their hydrodynamic size over 20 days time period. DLS experiment of BLV vesicles indicated that no change in size up to 7 days.

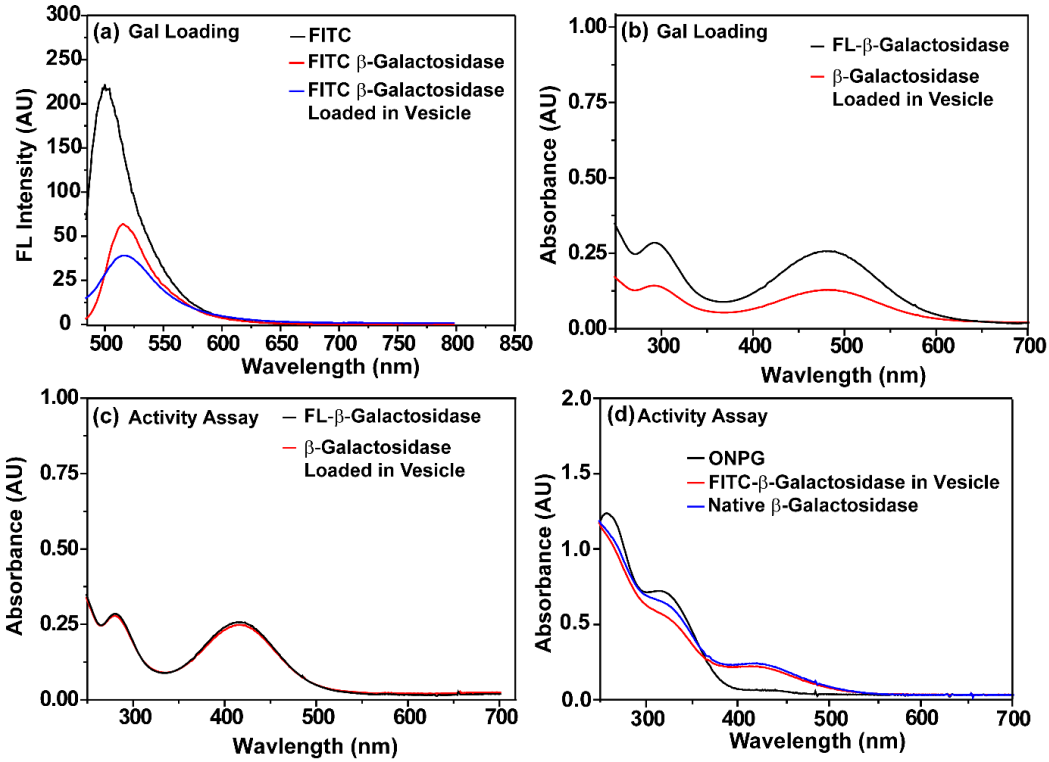


Figure S27: β-galactosidase encapsulation in M6PL vesicle in aqueous medium (a) β-galactosidase encapsulation; Fluorescence spectroscopy: FITC (black), FITC β-galactosidase (red), and FITC β-galactosidase encapsulated vesicle (GLV: blue); (b) β-galactosidase encapsulation; UV-Vis spectroscopy: FITC labeled β-galactosidase (black), and FITC β-galactosidase encapsulated vesicle (GLV: red). The activity assay of encapsulated β-galactosidase in aqueous medium: (c) UV-Vis spectroscopy: FITC β-galactosidase with ortho-nitrophenyl galactopyranoside (β-gal with ONPG: black), packaged (encapsulated) β-galactosidase with ortho-nitrophenyl galactopyranoside (GLV with ONPG: red); (d) UV-Vis spectroscopy: only ortho-nitrophenyl galactopyranoside (ONPG: black), packaged (encapsulated) β-galactosidase with ortho-nitrophenyl galactopyranoside (GLV with ONPG: red); and native β-galactosidase with with ortho-nitrophenyl galactopyranoside ((β-gal with ONPG: blue).

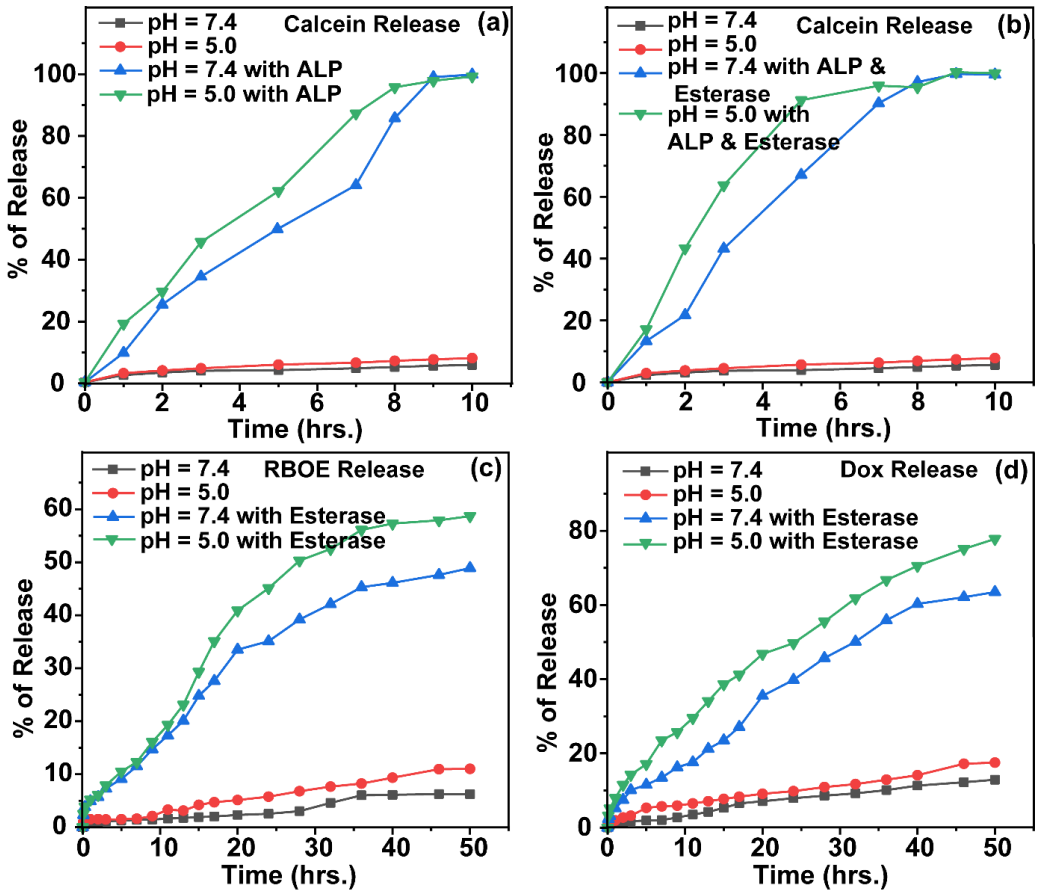


Figure S28: Dye and drug release from vesicle in PBS buffer (pH = 7.4 and 5.0); (a) calcein release from vesicle (CRLV) in the presence of ALP enzyme, (b) calcein release from vesicle (CRLV) in the presence of both ALP and Esterase enzyme, (c) RBOE release from vesicle (CRLV) in the presence of Esterase enzyme, and (d) DOX release from vesicle (DLV) in the presence of Esterase enzyme.

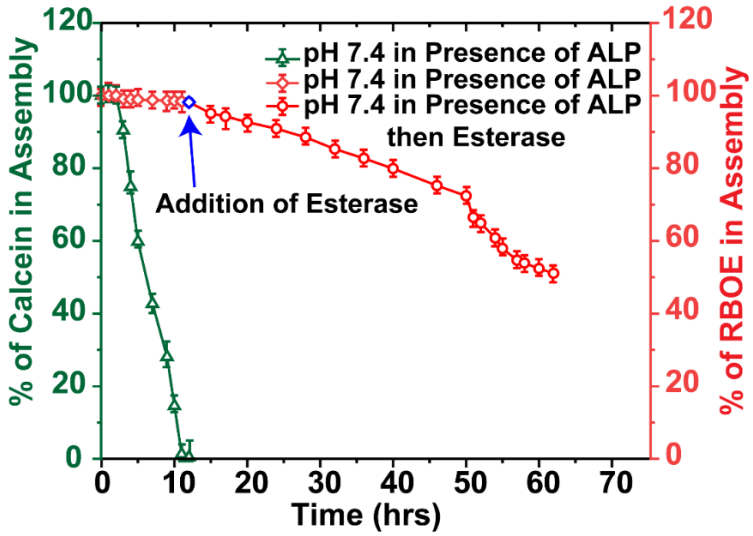


Figure S29: Dye release kinetics from vesicle (CRLV) over time in PBS buffer (green: calcein release in the presence of ALP; red: RBOE release in the presence of ALP and Esterase respectively; and blue: addition of Esterase).

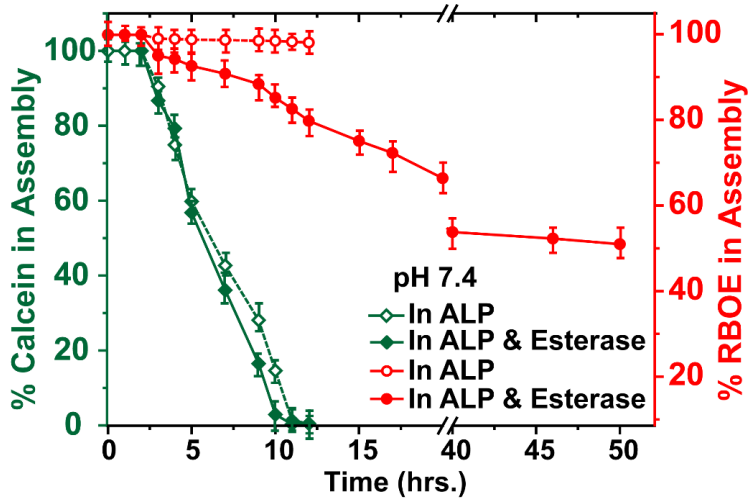


Figure S30: Dye release kinetics from vesicle (CRLV) over time in PBS buffer. Green line: calcein release in the presence of only ALP; and green dotted line: calcein release in the presence of both ALP & esterase. Red line: RBOE release in the presence of both ALP & Esterase; and red dotted line: in the presence of only ALP.

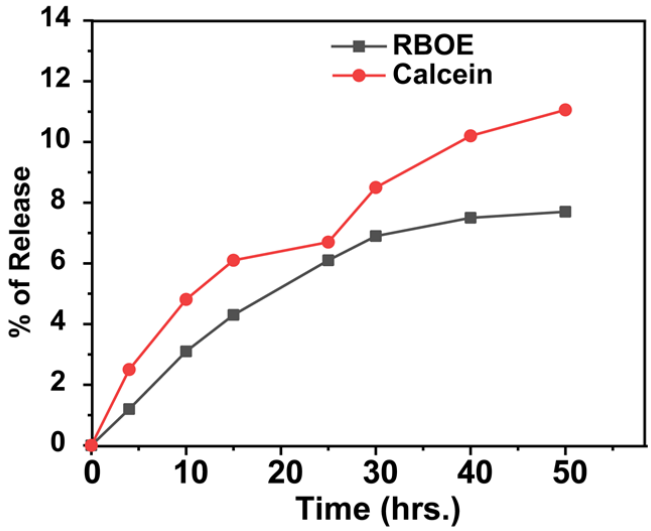


Figure S31: Dye release kinetics from CRLV vesicle in human plasma

Discussion: The dual dye release study from dual dye (calcein and RBOE) loaded M6PL vesicles (CRLV) were evaluated by monitoring the change in the fluorescence intensity over 50 hrs time period in human plasma. The dye release kinetics indicated that there is very little release of both dyes in human plasma (~7 % for RBOE and ~12 % for Calcein). The release profile indicates that the cargo loaded vesicles are highly stable in blood plasma.

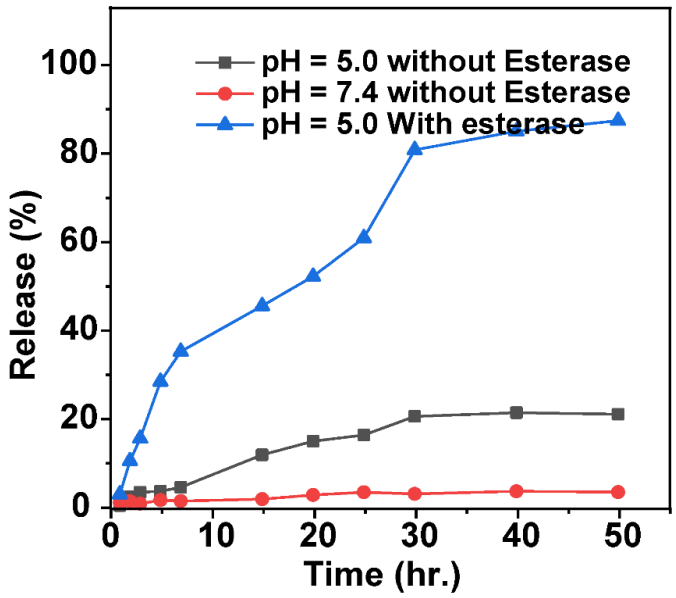


Figure S32: BSA release kinetics from BLV vesicle at different pH (PBS buffer) in the presence of esterase enzyme and in the absence of esterase enzyme.

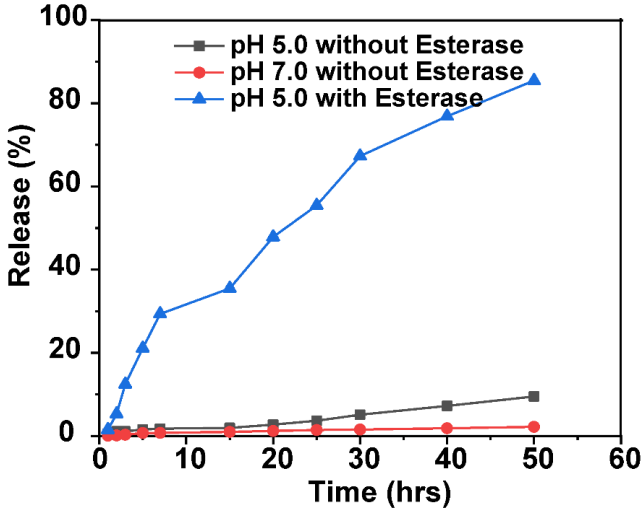


Figure S33: β-galactosidase release from GLV vesicle at different pH (PBS buffer) in the presence of esterase enzyme and in the absence of esterase enzyme.

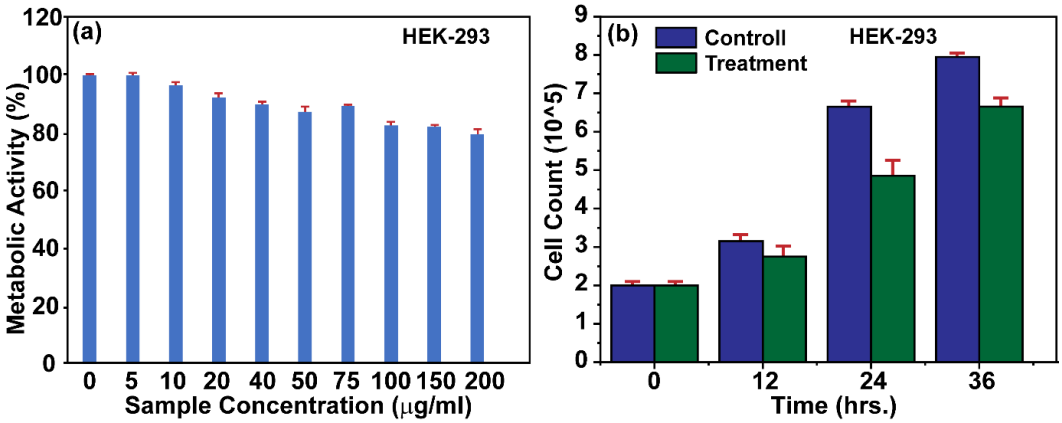


Figure S34: Cell viability test and cell growth kinetics of HEK-293 cell line. (a) MTT assay of M6PL vesicle; (b) cell growth kinetics in presence of M6PL vesicle.

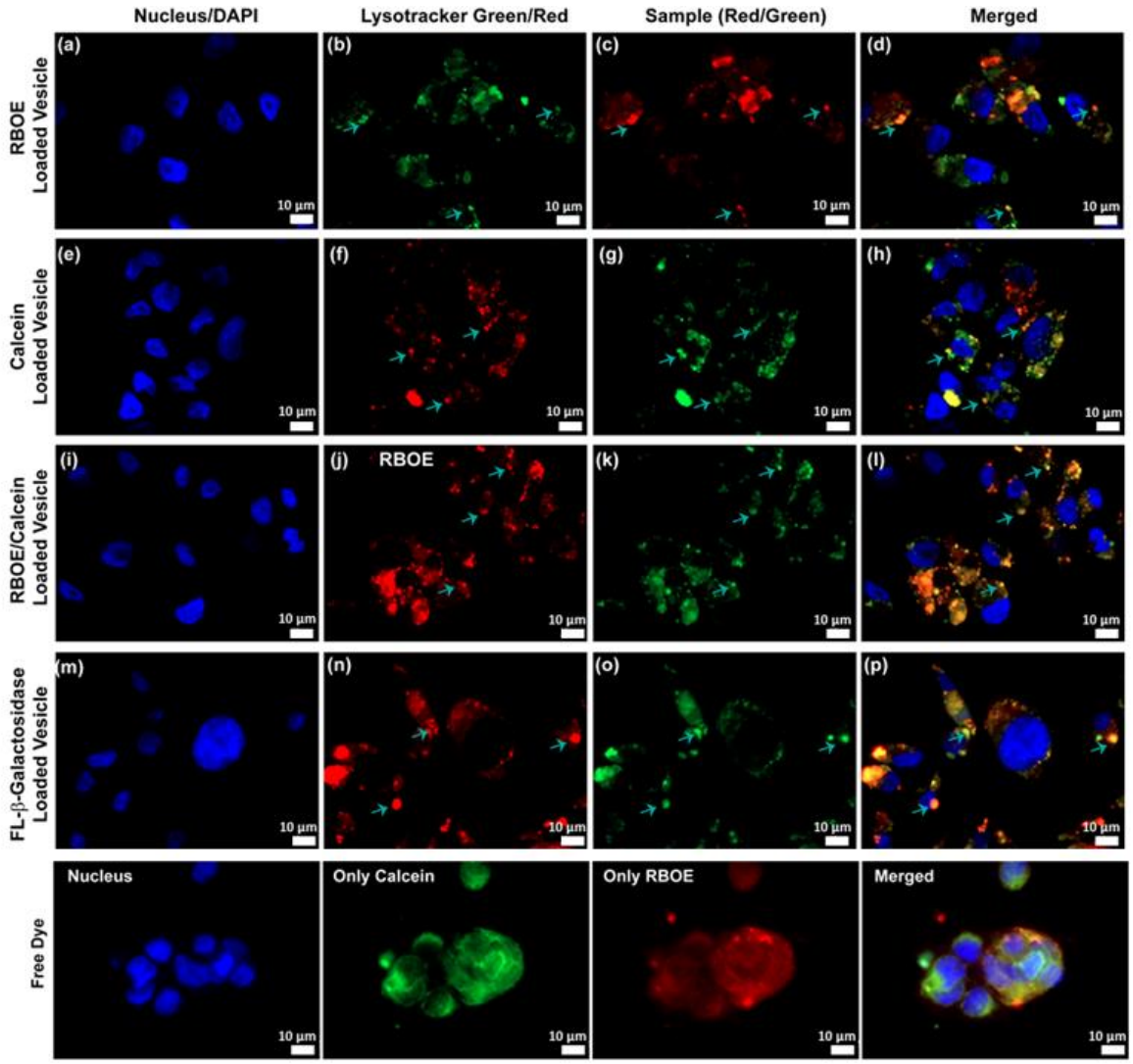


Figure S35: Lysosome delivery of encapsulated dual cargo molecule using M6PL vesicle (a-d) RBOE loaded Vesicle (RLV); (e-h) calcein loaded Vesicle (CLV); (i-l) both calcein and RBOE loaded vesicle (CRLV); (i-l) FL-β-galactosidase loaded vesicle (GLV); and free dye (non maked): HEK-293 cells were cultured for 4 hrs with RBOE loaded Vesicle (RLV: 0.2 mg/mL); calcein loaded Vesicle (CLV: 0.2 mg/mL); both calcein and RBOE loaded vesicle (CRLV: 0.2 mg/mL); FL-β-galactosidase loaded vesicle (GLV: 200 µg/mL); and free calcein & RBOE dye (20 µg/mL each) respectively in MEM containing 10% FBS, and then stained with LysoTracker Green/Red (50 nM) for 30 min. The cells were probed by epifluorescence microscopy. Merging of the RBOE/Calcein signal (shown in red for RBOE and green for Calcein) and that of LysoTracker Green/Red (shown in green/Red) revealed colocalization as

indicated by the orange-yellow spots/areas (bars, 10 μm); cyan colour arrow indicates punctate-like vesicles.

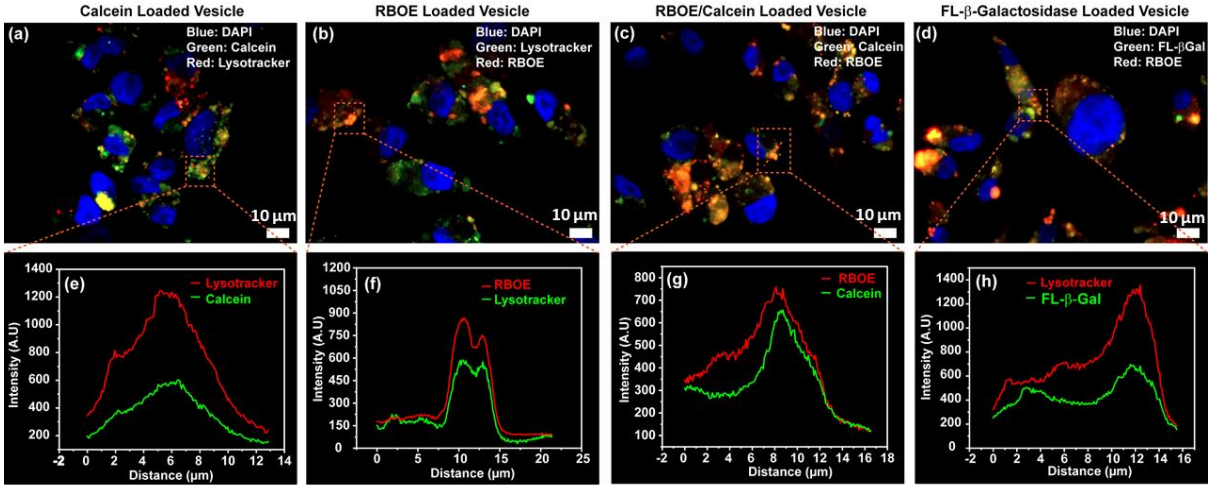


Figure S36: Colour intensity profile in HEK-293 cells representing the variation of colour (both green and red) intensity with distance for a small sub-section of the merged image. HEK-293 cells were incubated (a & e) calcein loaded Vesicle (CLV); (b & f) RBOE loaded vesicle (RLV); (c & g) calcein and RBOE loaded vesicle (CRLV); (d & h) FL-β-galactosidase loaded vesicle (GLV). Orange-yellowish spherical indicating co-localization of both dyes as is also observed object in the intensity profile.

NMR of All Synthesized Compounds

(* Indicates residual solvent peak)

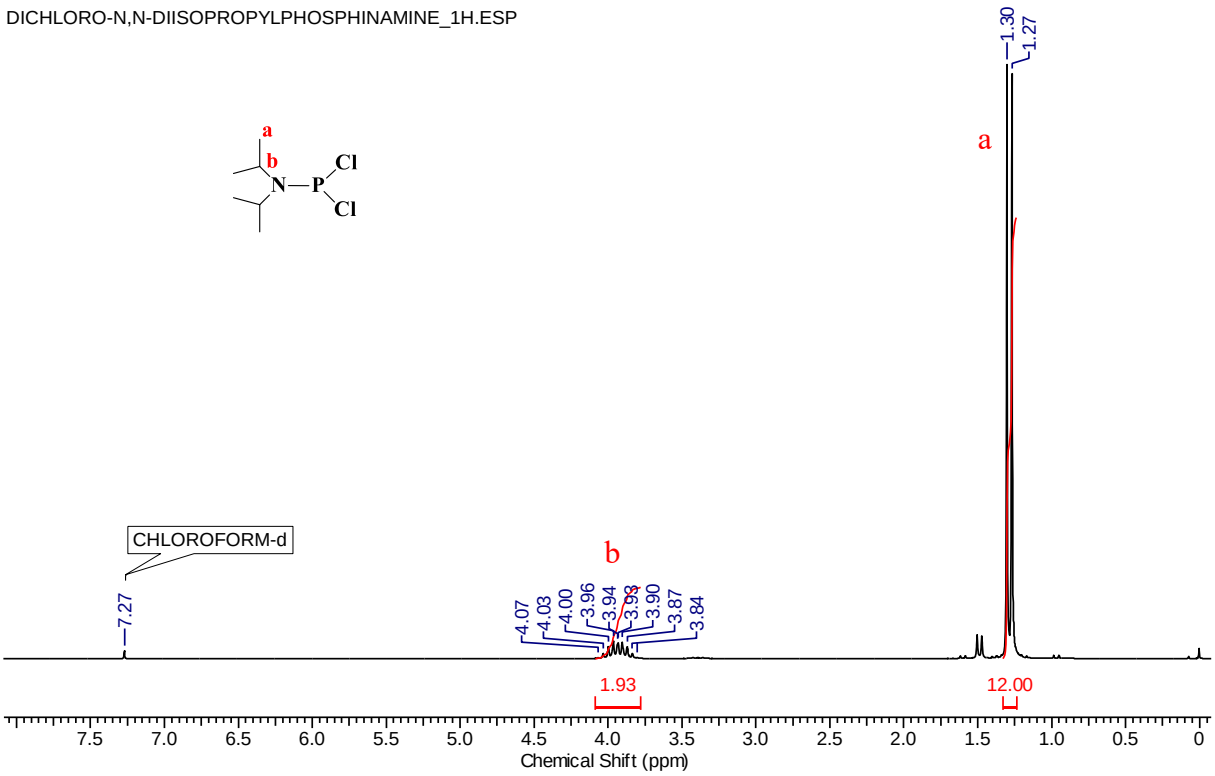


Figure S37: ^{1}H NMR ($CDCl_3$, 200 MHz) Spectrum of compound **2a**

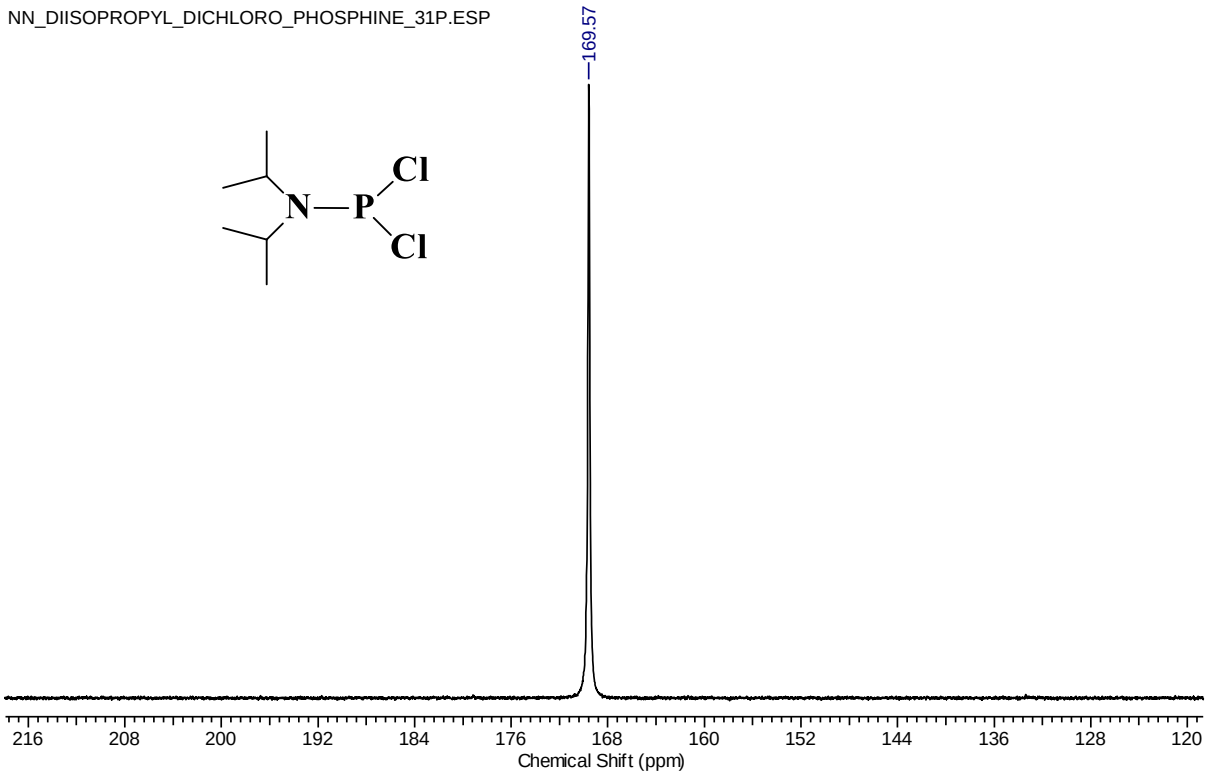


Figure S38: ^{31}P NMR ($CDCl_3$, 202 MHz) Spectrum of compound **2a**

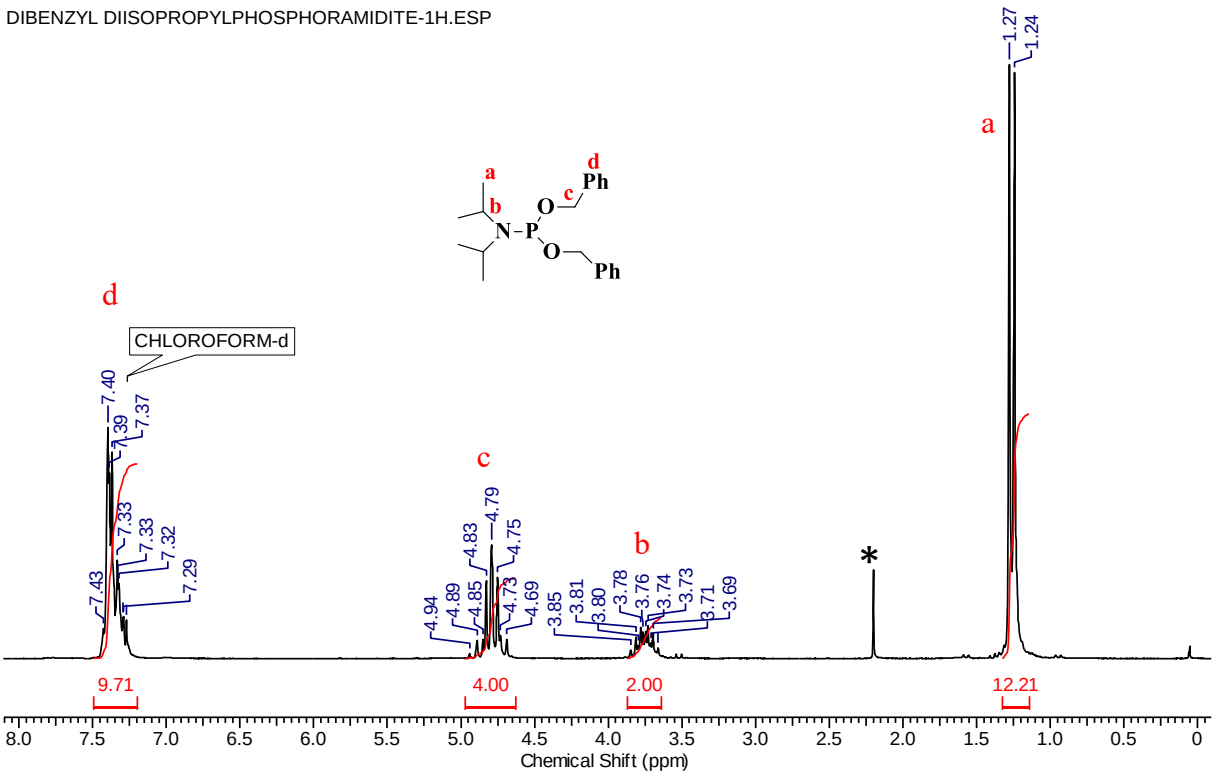


Figure S39: ^{1}H NMR ($CDCl_3$, 20 MHz) Spectrum of compound **2b**

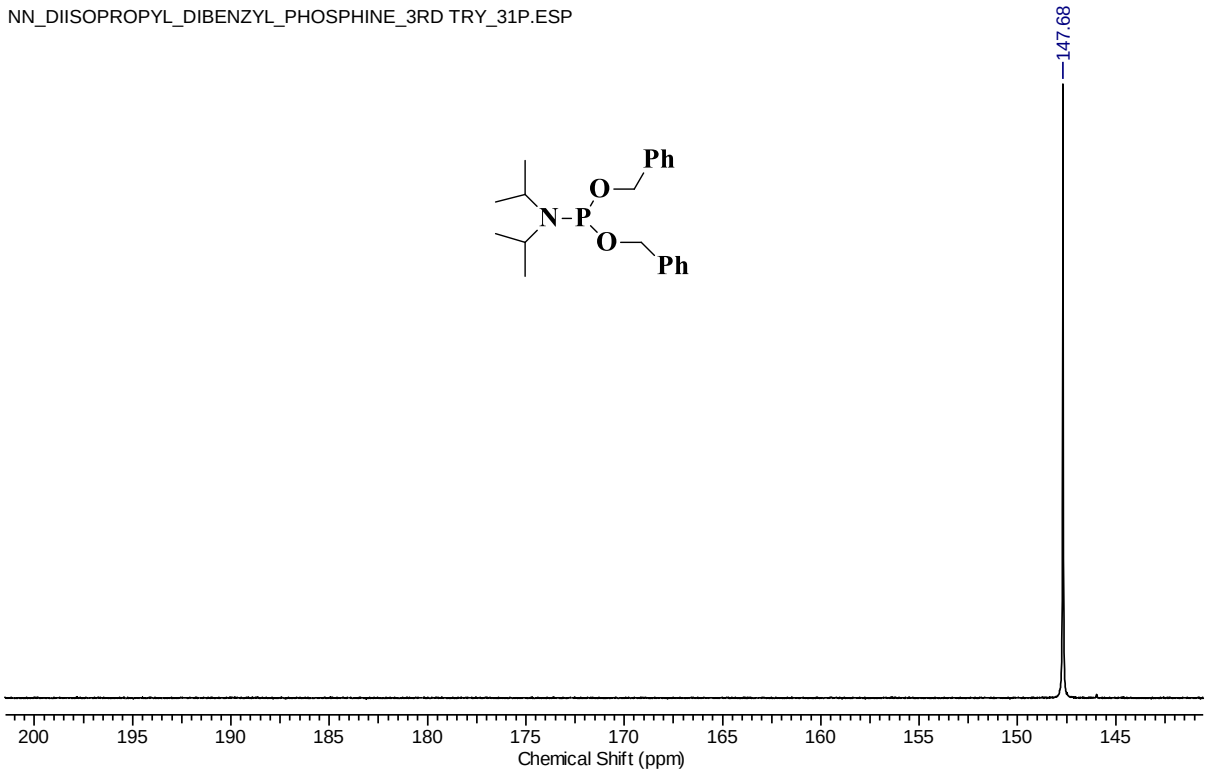


Figure S40: ^{31}P NMR ($CDCl_3$, 200 MHz) Spectrum of compound **2b**

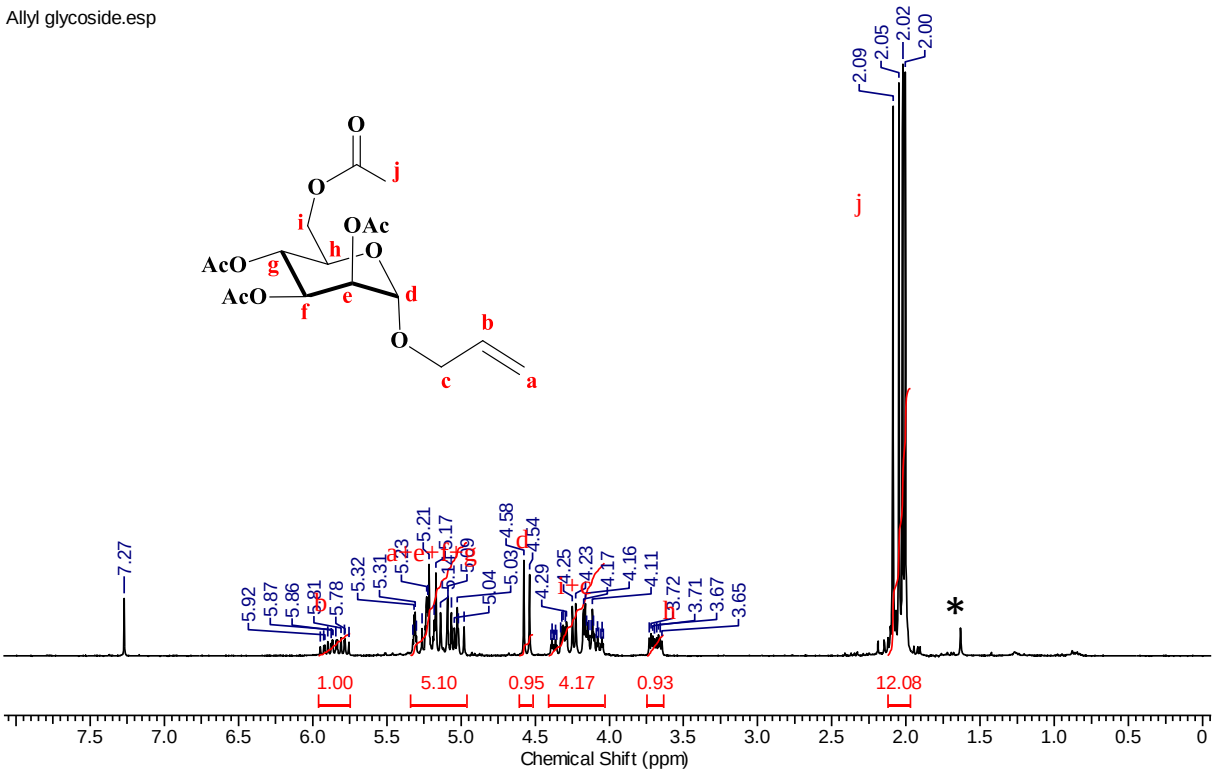


Figure S41: ^{1}H NMR ($CDCl_3$, 200 MHz) Spectrum of compound **1a**

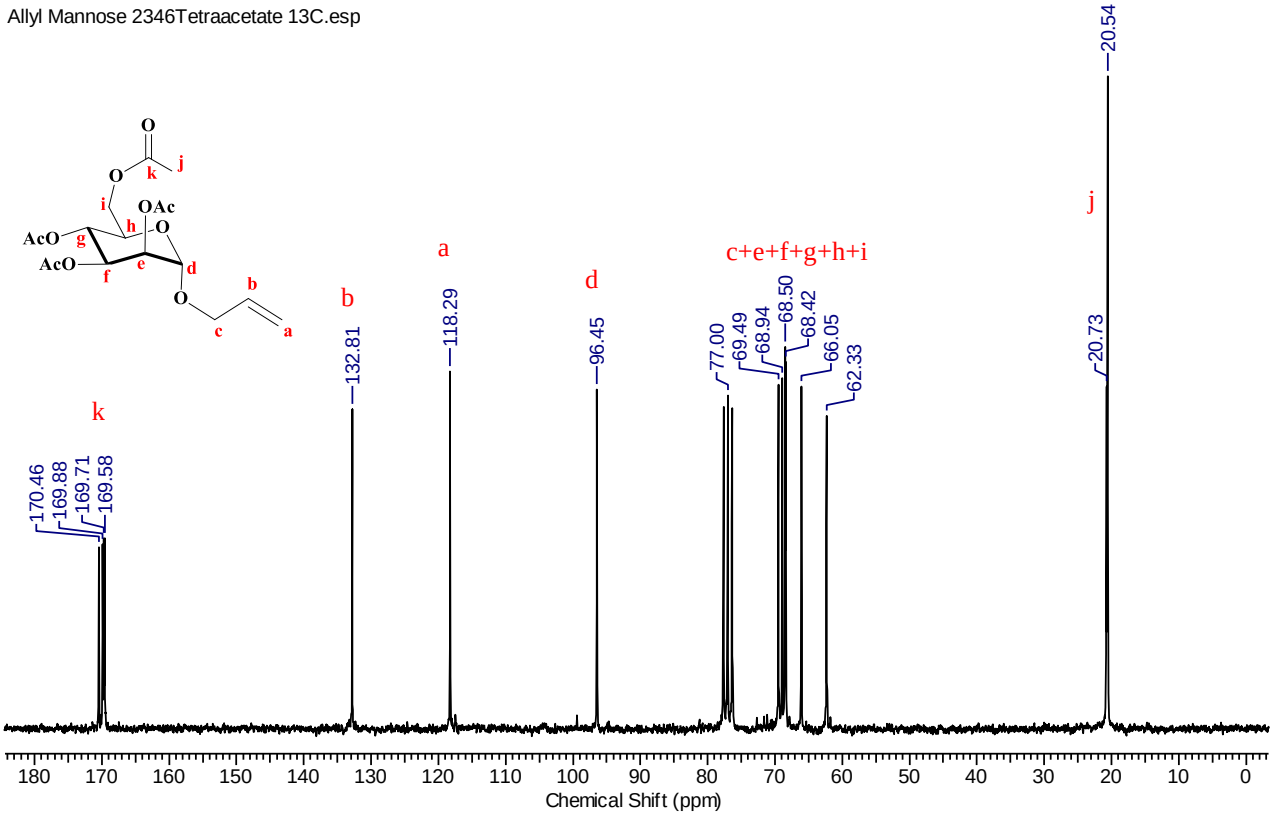


Figure S42: ^{13}C NMR ($CDCl_3$, 50 MHz) Spectrum of compound **1a**

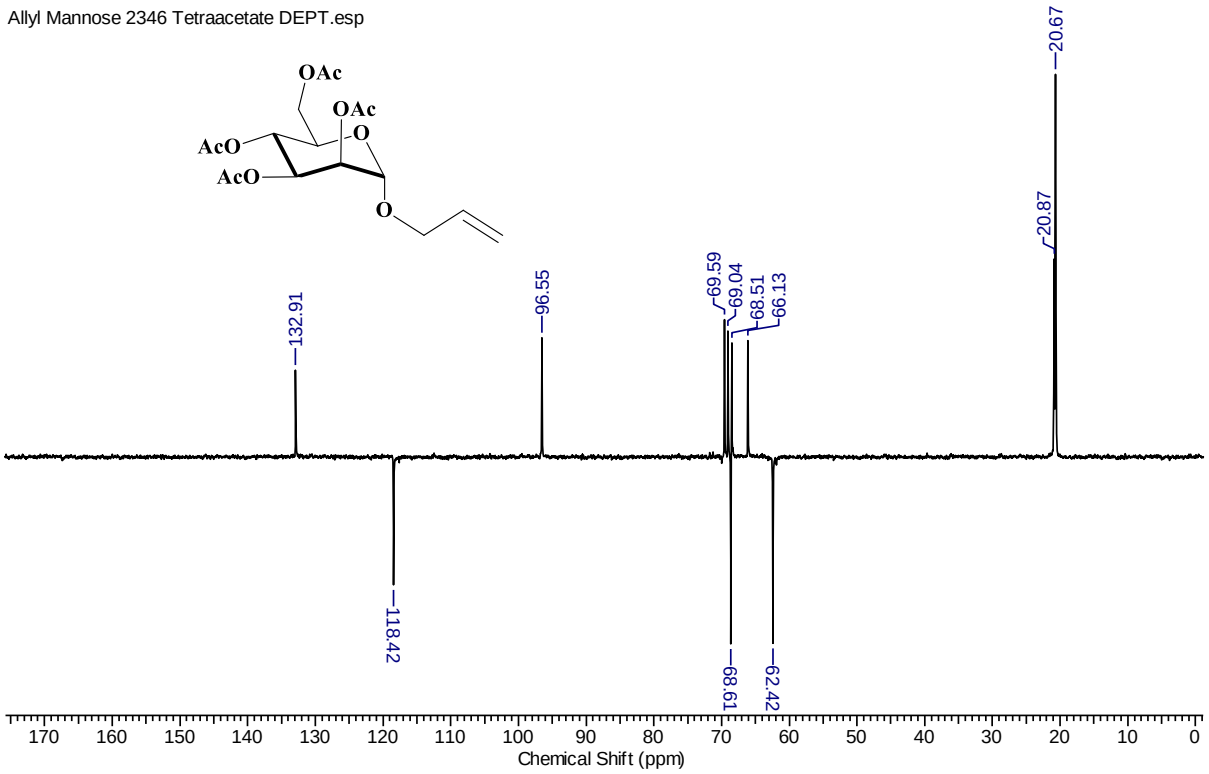


Figure S43: DEPT ($CDCl_3$**,** 50 MHz) Spectrum of compound **1a**

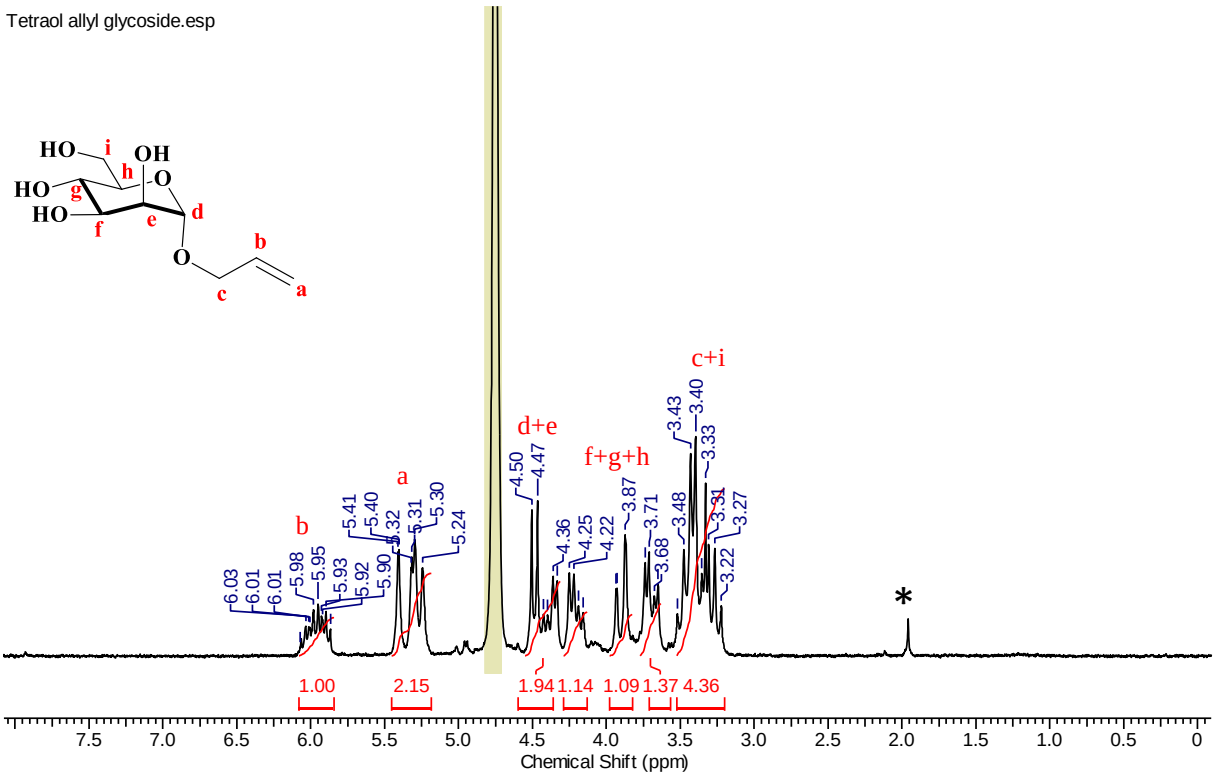


Figure S44: ^1H NMR (D_2O**,** 200 MHz) Spectrum of compound **1b**

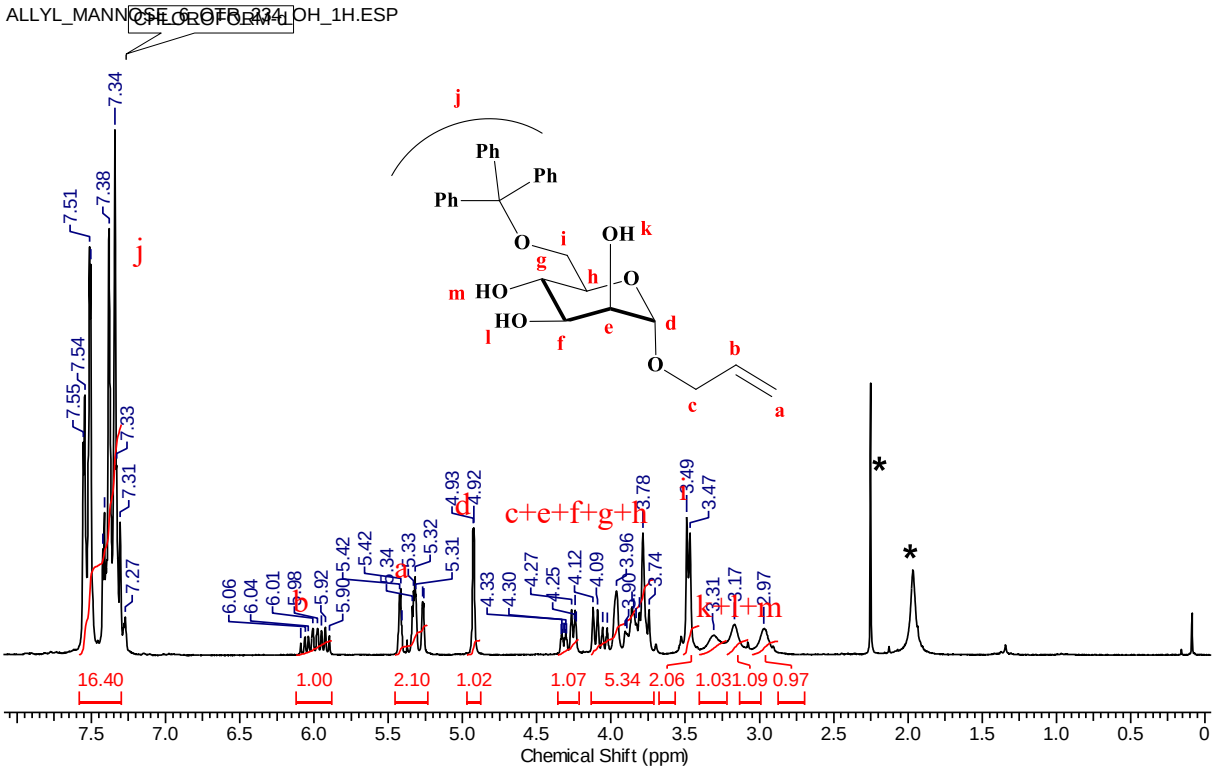


Figure S45: ^{1}H NMR ($CDCl_3$, 200 MHz) Spectrum of compound **1c**

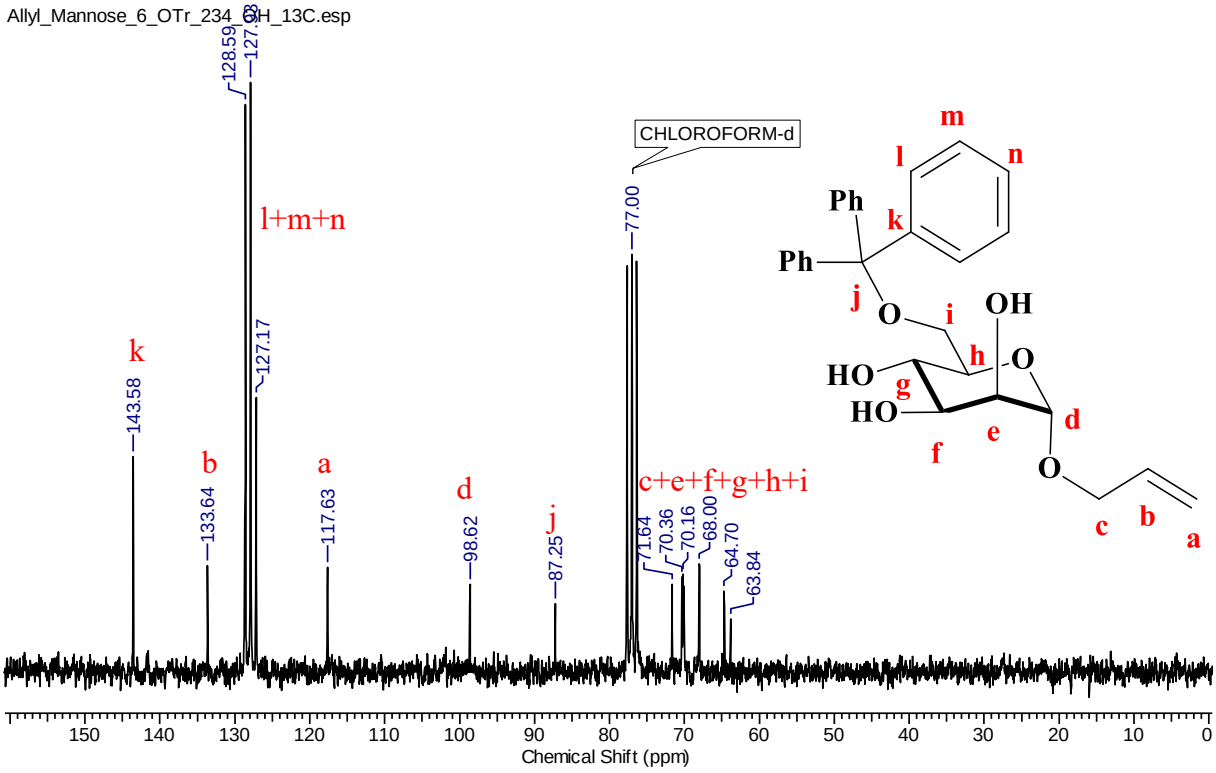


Figure S46: ^{13}C NMR ($CDCl_3$, 50 MHz) Spectrum of compound **1c**

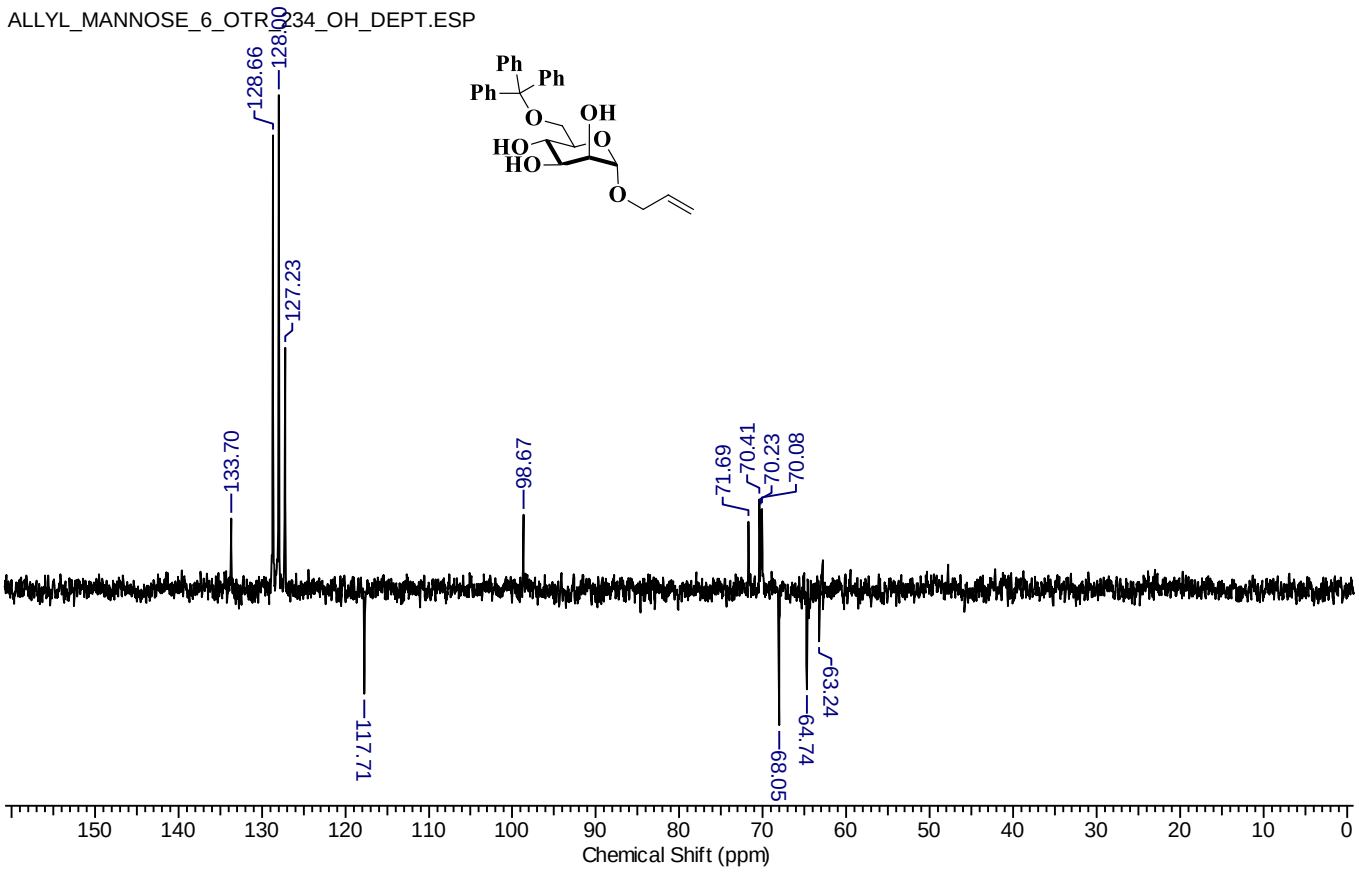


Figure S47: DEPT-NMR ($CDCl_3$, 50 MHz) Spectrum of Compound **1c**

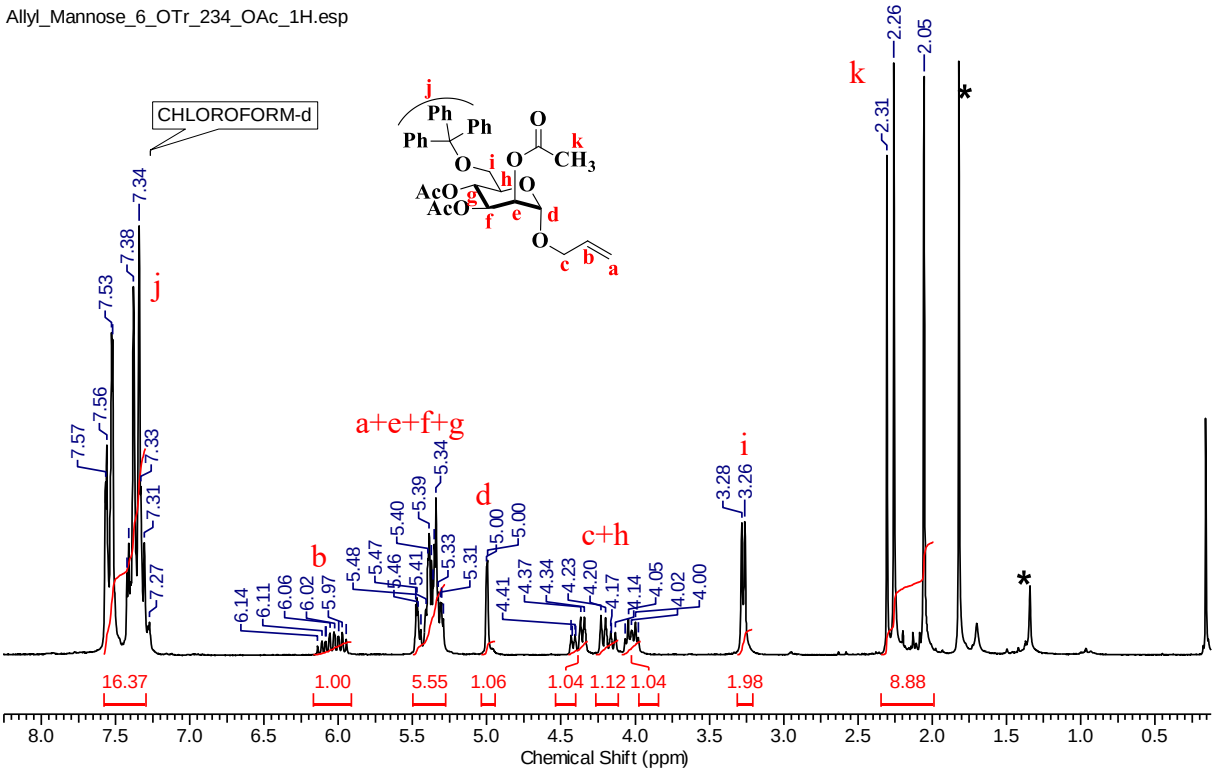


Figure S48: ^1H NMR ($CDCl_3$, 200 MHz) Spectrum of compound **1d**

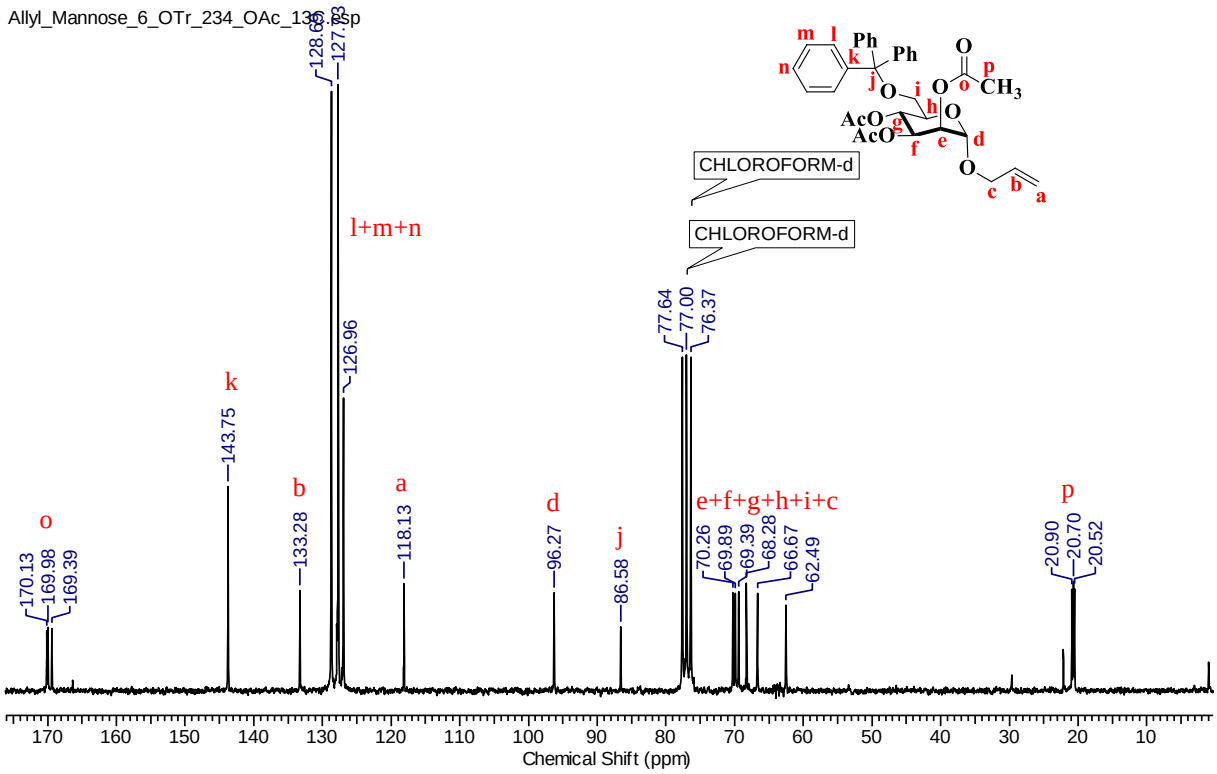


Figure S49: ^{13}C NMR ($CDCl_3$**,** 50 MHz) Spectrum of compound **1d**

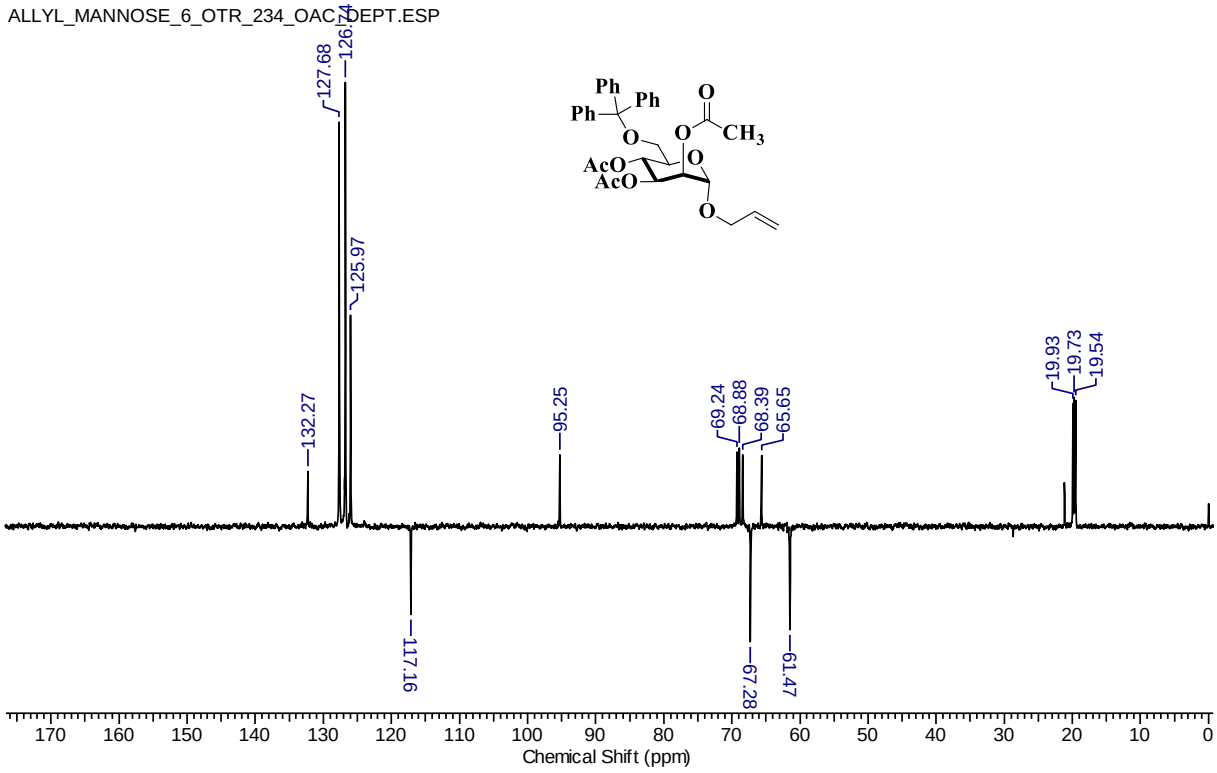


Figure S50: DEPT NMR ($CDCl_3$**,** 50 MHz) Spectrum of Compound **1d**

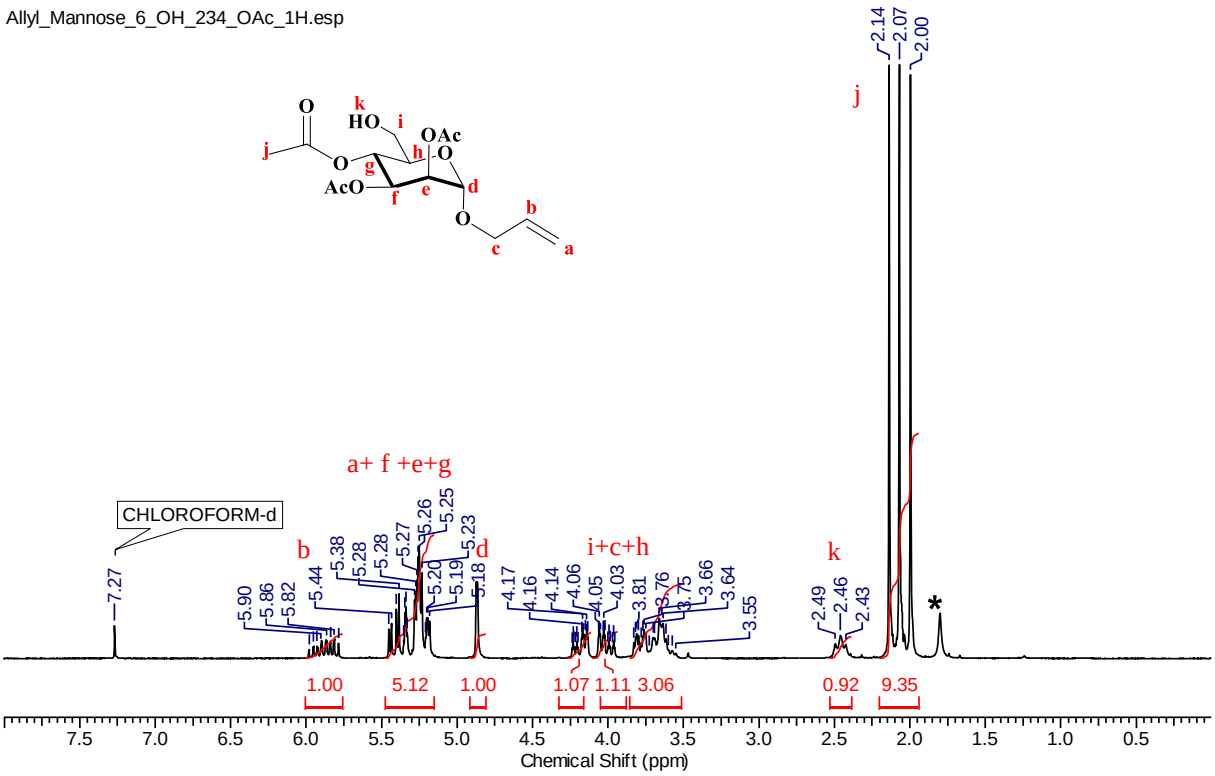


Figure S51: ^{1}H NMR ($CDCl_3$, 200 MHz) Spectrum of Compound **1e**

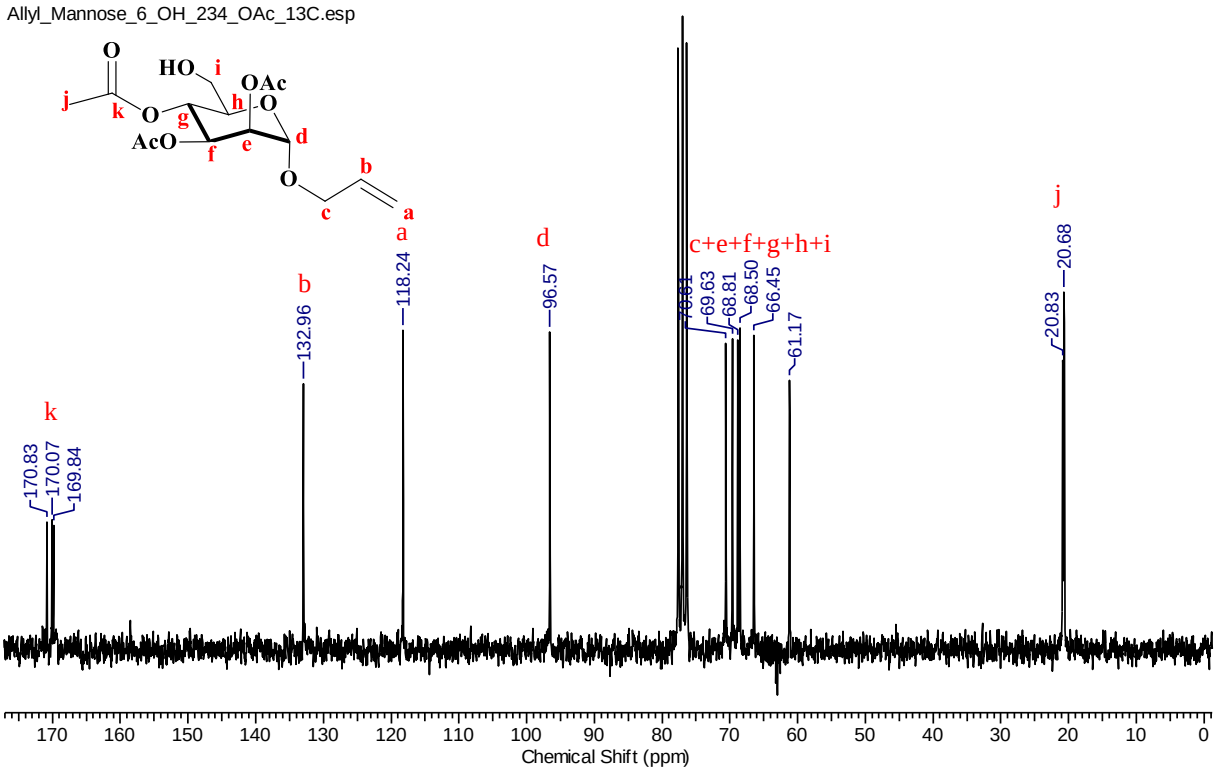


Figure S52: ^{13}C NMR ($CDCl_3$, 50 MHz) Spectrum of compound **1e**

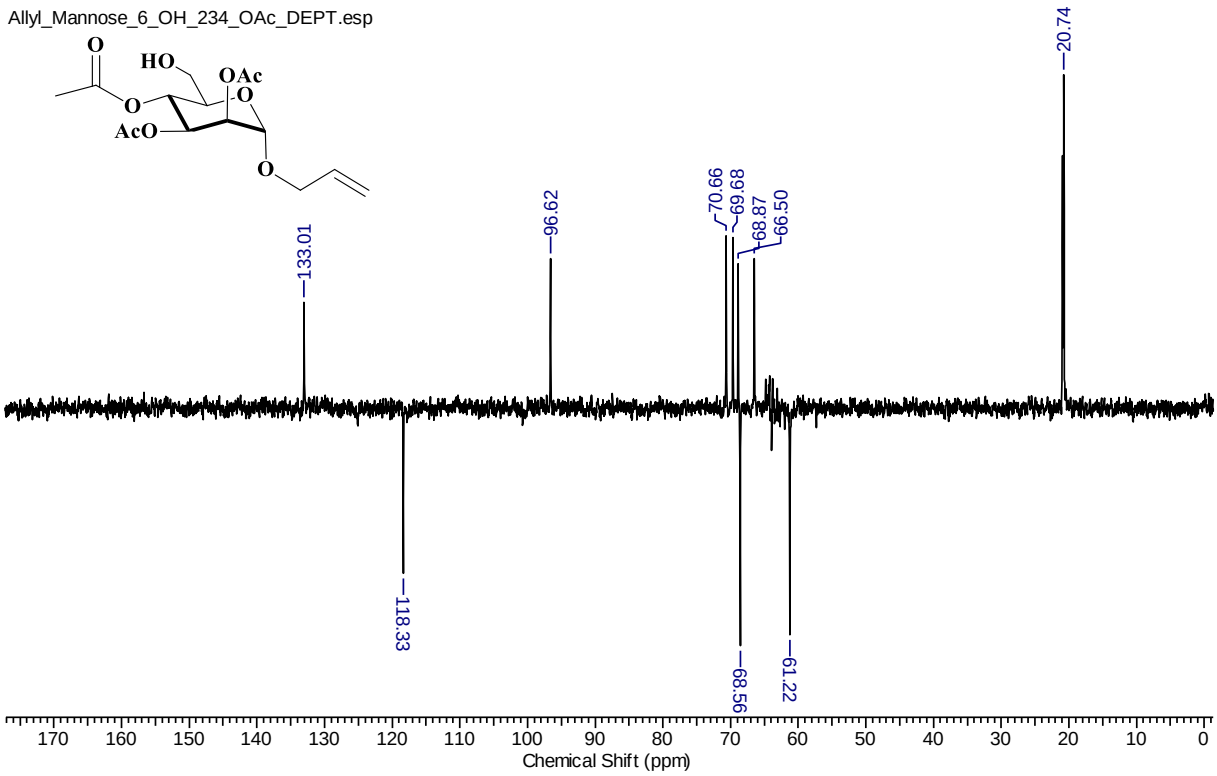


Figure S53: DEPT-NMR ($CDCl_3$**,** 50 MHz) Spectrum of compound **1e**

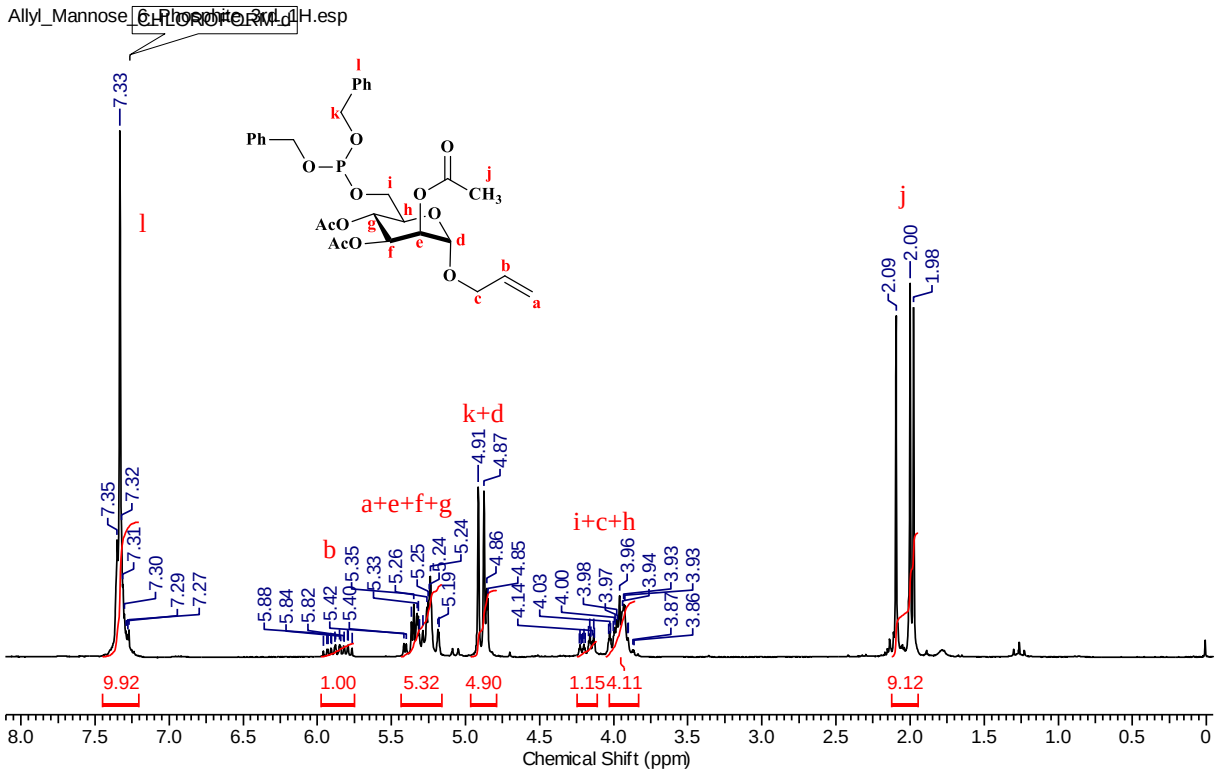


Figure S54: ^1H NMR ($CDCl_3$**,** 200 MHz) Spectrum of Compound **3**

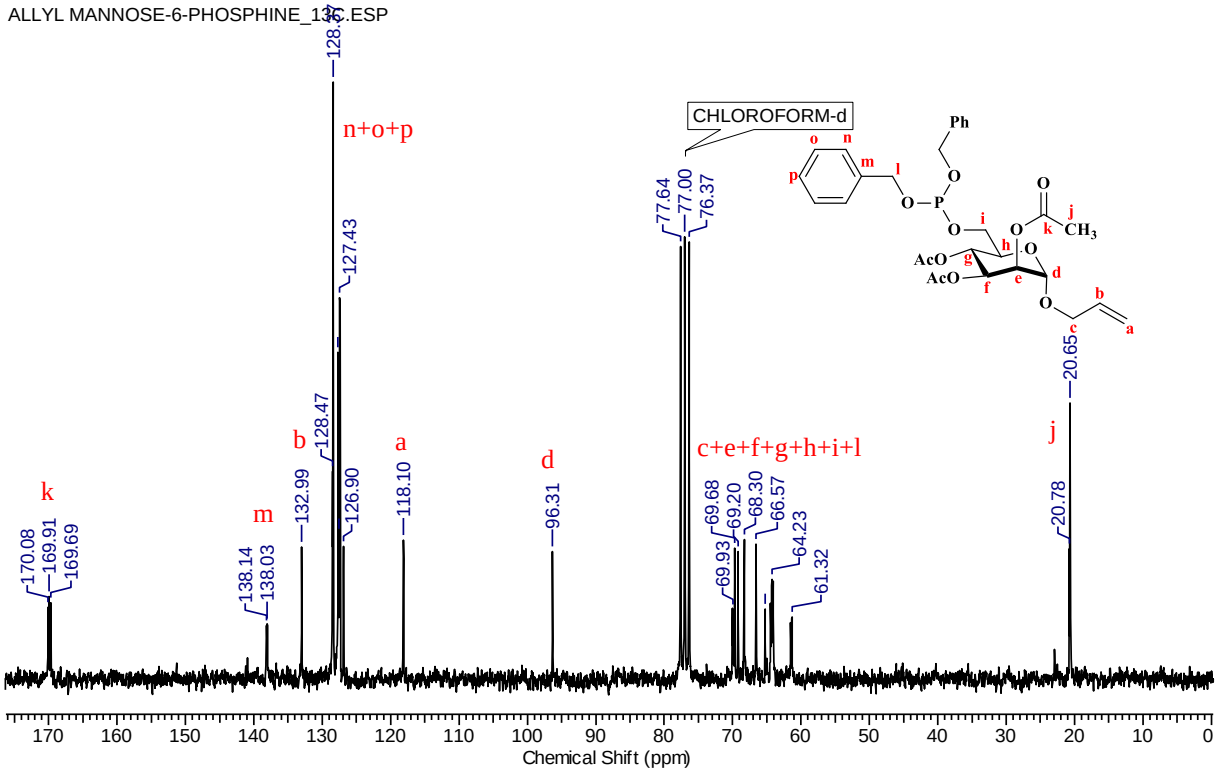


Figure S55: ^{13}C NMR ($CDCl_3$, 50 MHz) Spectrum of compound **3**

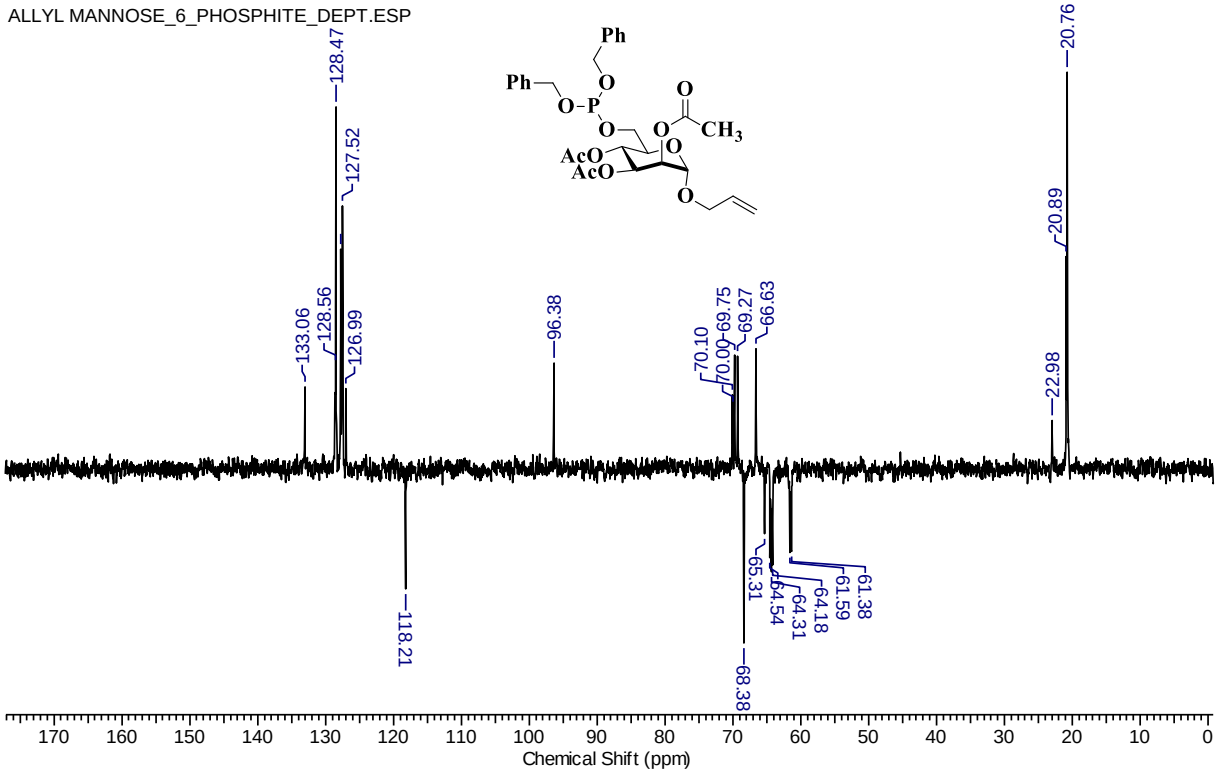


Figure S56: DEPT-NMR ($CDCl_3$, 50 MHz) Spectrum of compound **3**

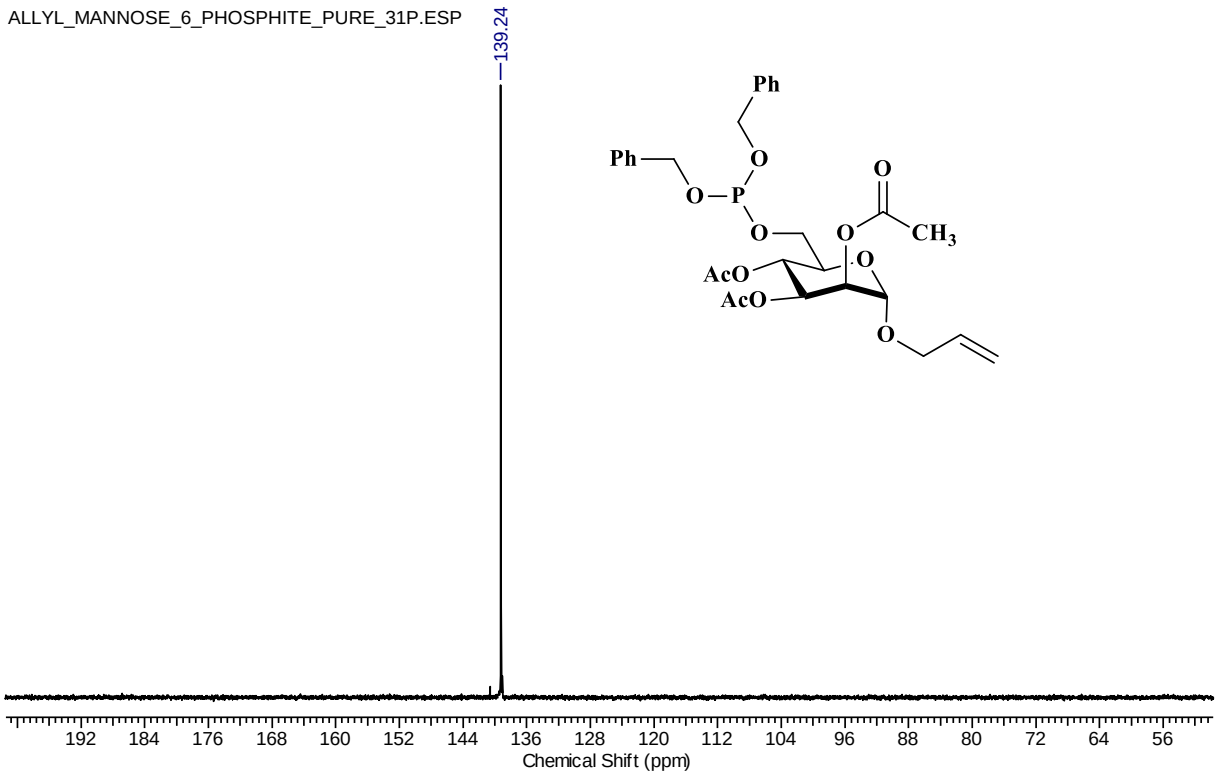


Figure S57: ^{31}P NMR ($CDCl_3$, 202 MHz) Spectrum of compound **3**

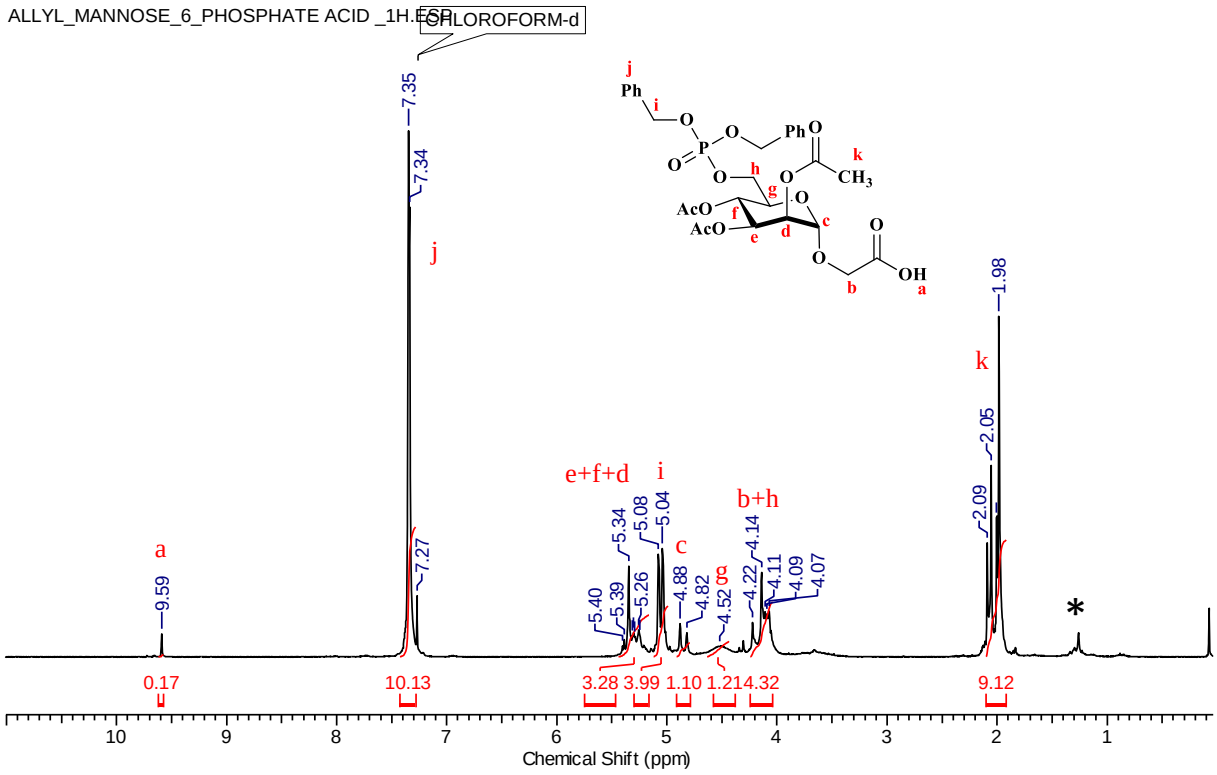


Figure S58: ^{1}H NMR ($CDCl_3$, 200 MHz) Spectrum of Compound **4**

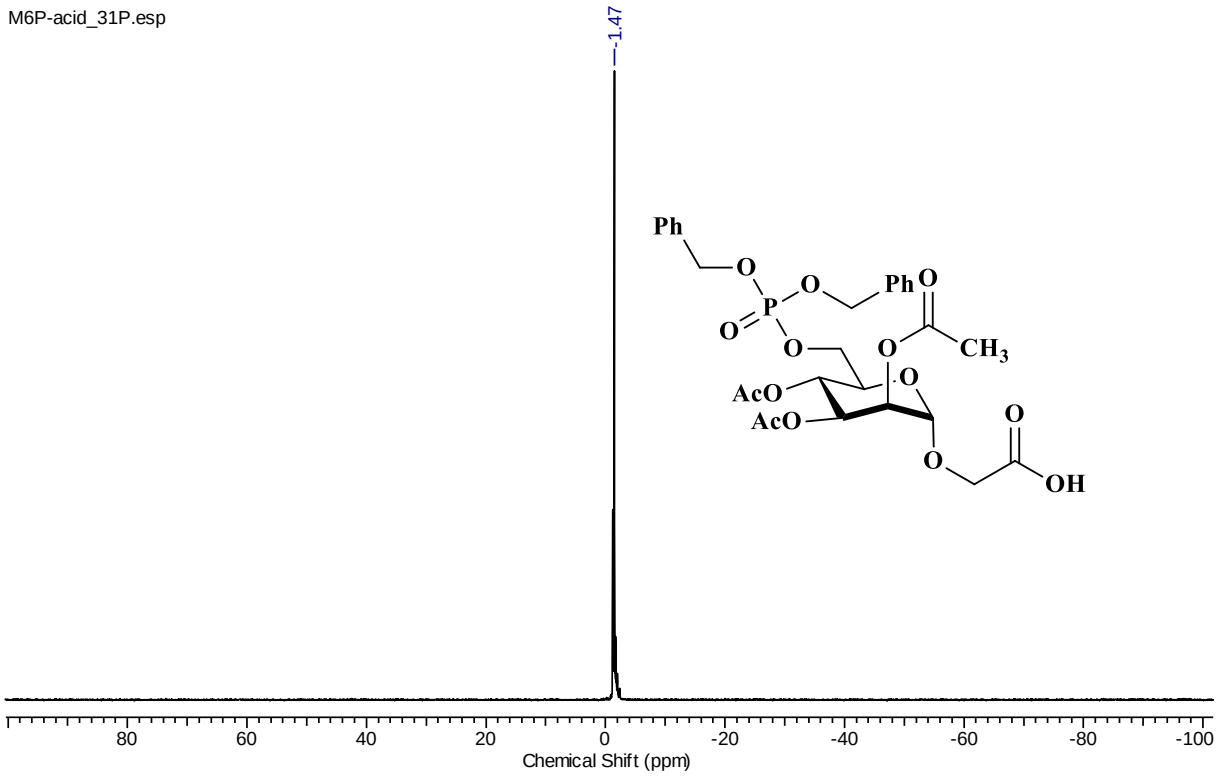


Figure S59: ^{31}P NMR ($CDCl_3$**,** 202 MHz) Spectrum of compound **4**

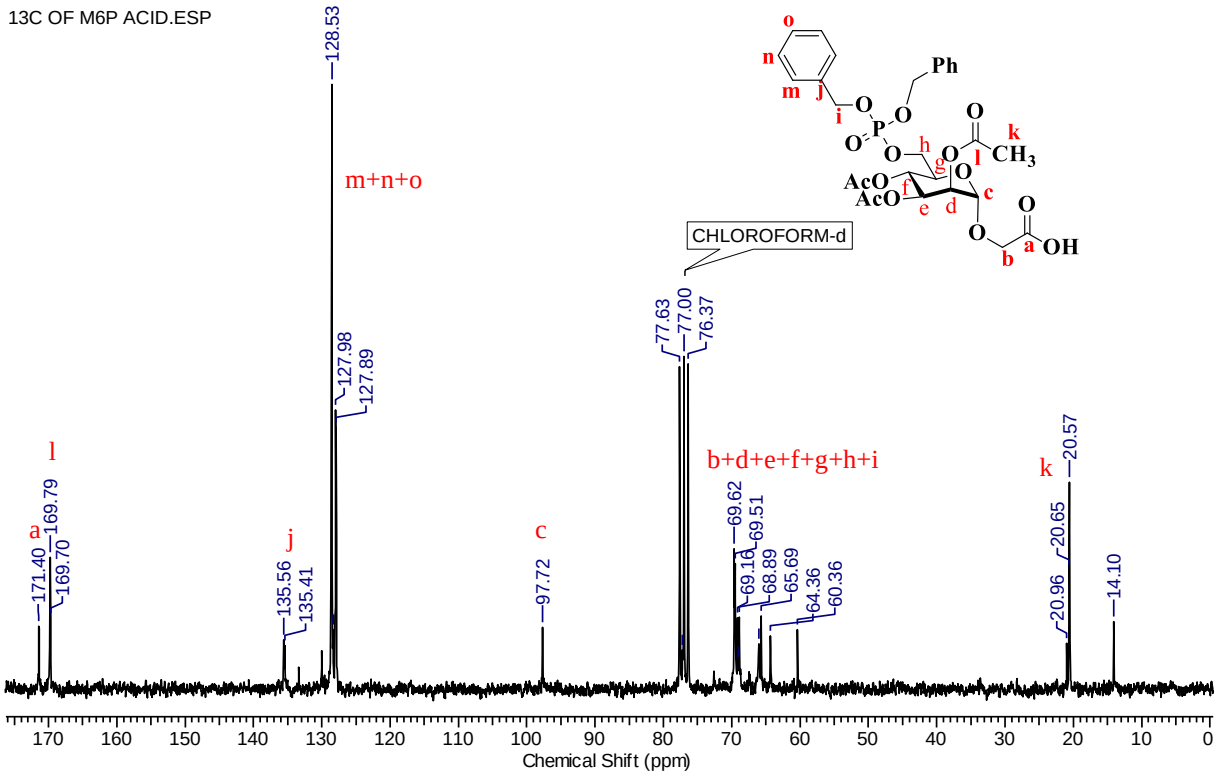


Figure S60: ^{13}C NMR ($CDCl_3$**,** 50 MHz) Spectrum of compound **4**

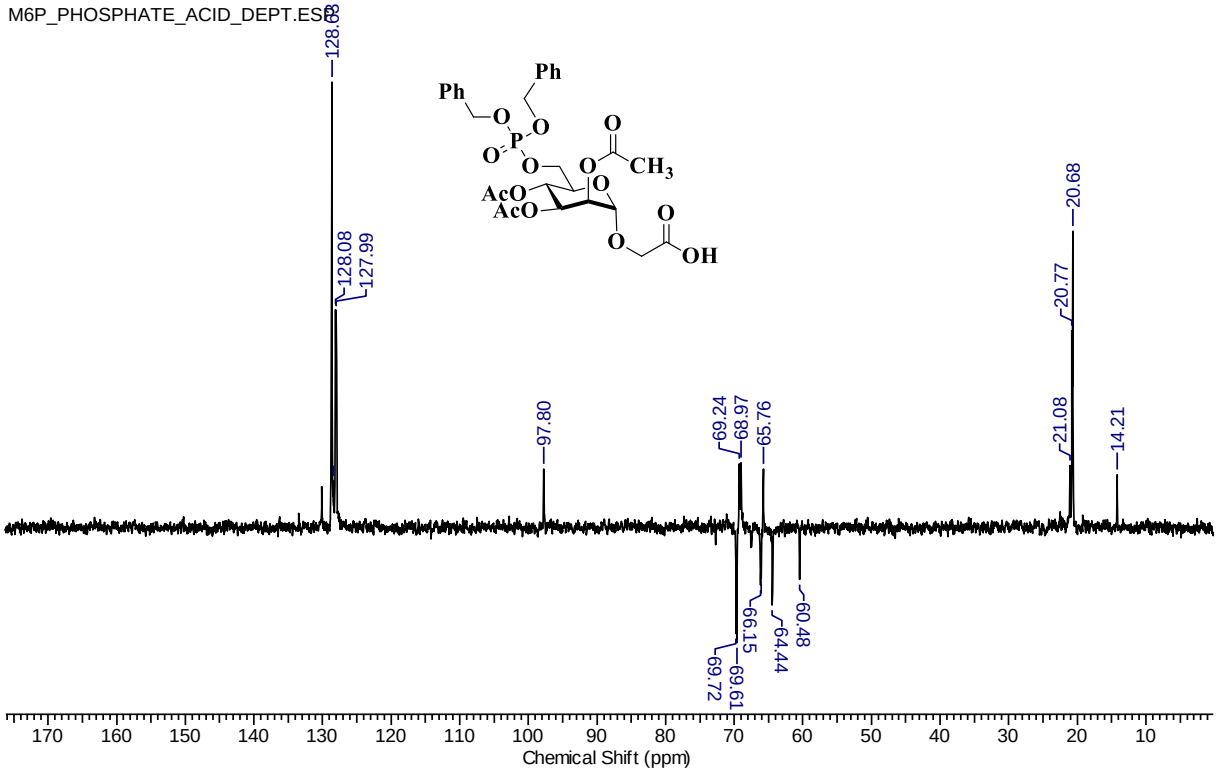


Figure S61: DEPT-NMR ($CDCl_3$**,** 50 MHz) Spectrum of compound **4**

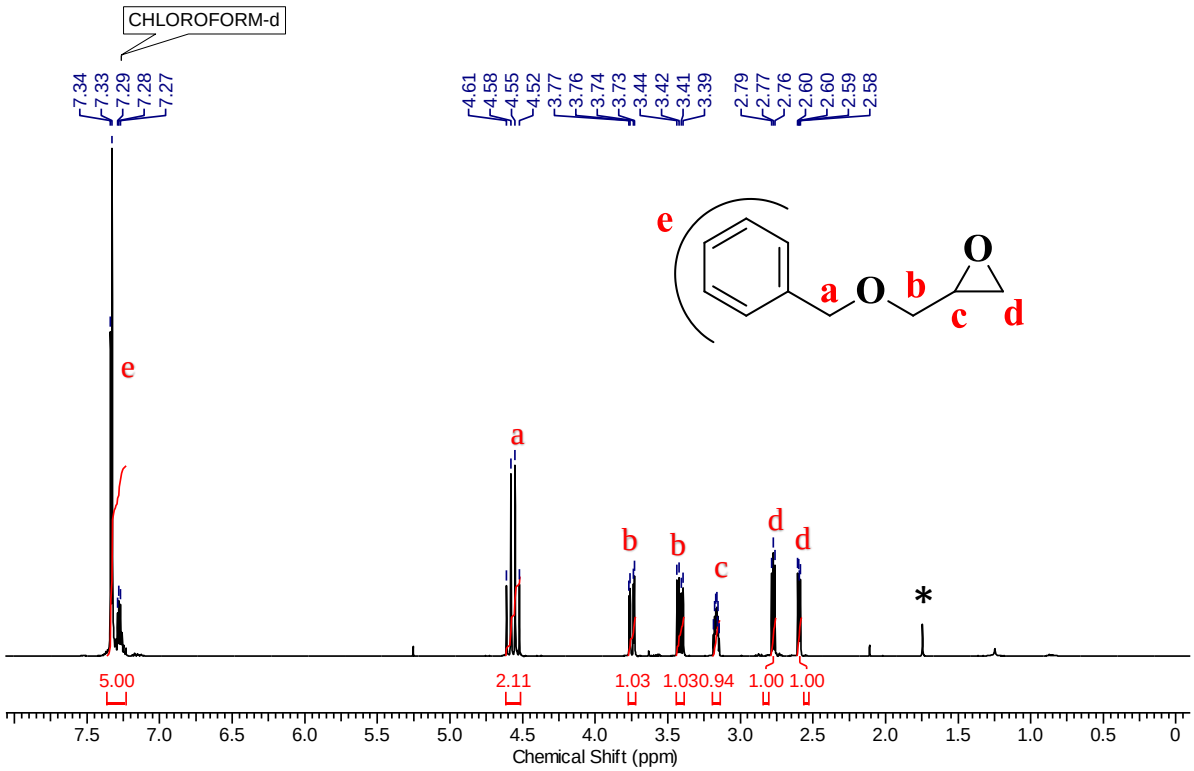


Figure S62: ^{1}H NMR ($CDCl_3$**,** 400 MHz MHz) Benzyl Glycidyl Ether

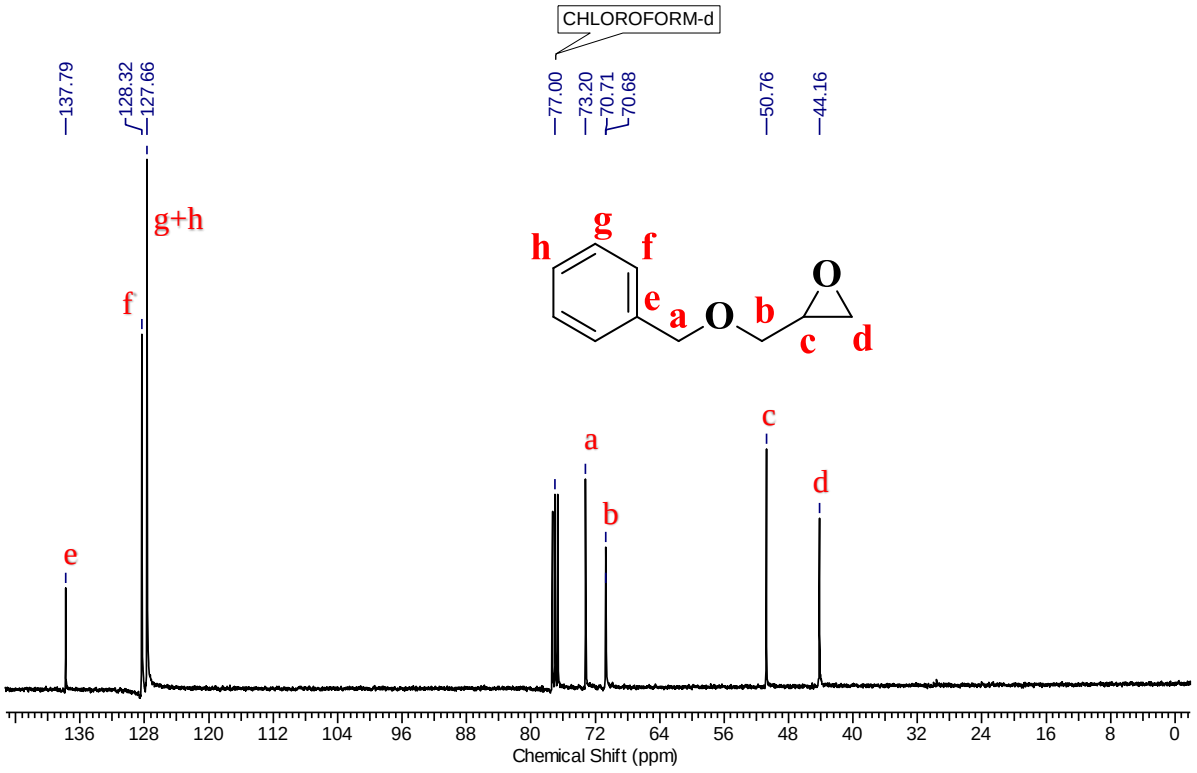


Figure S63: ^{13}C NMR ($CDCl_3$, 400 MHz) Benzyl Glycidyl Ether

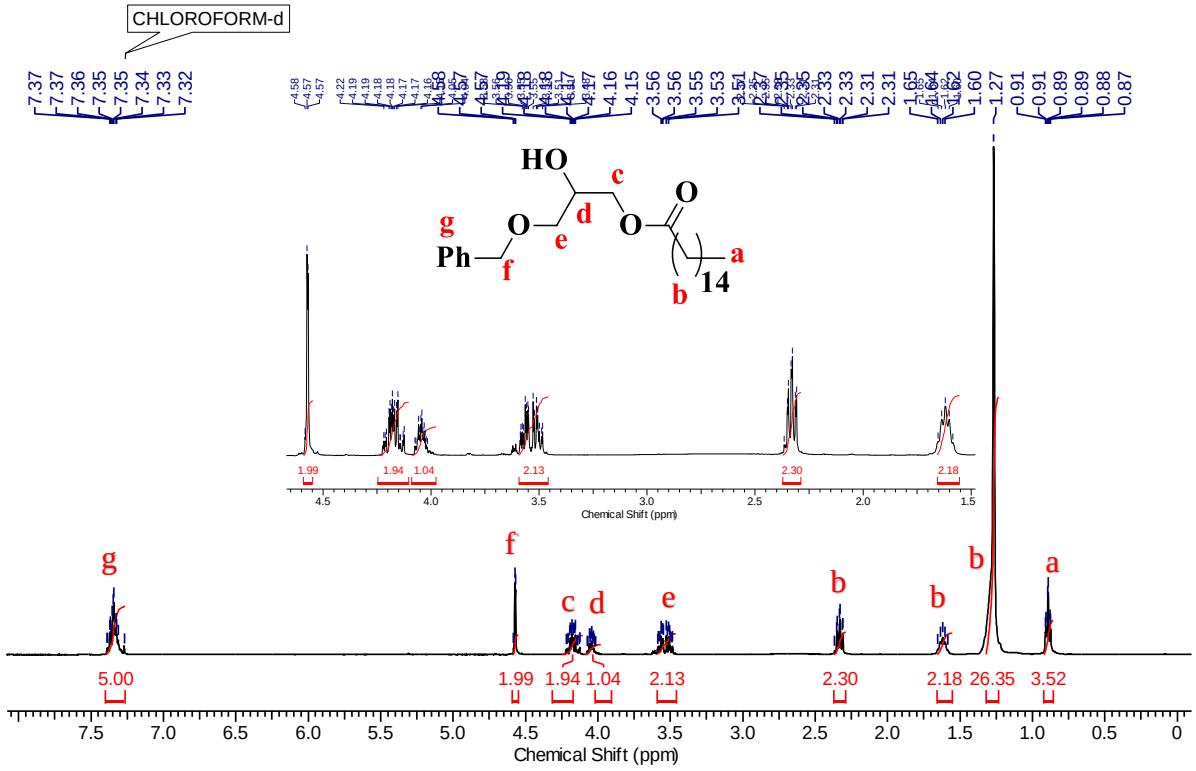


Figure S64: ^{1}H NMR ($CDCl_3$, 400 MHz) Benzyl Glycidyl Ether Palmitic

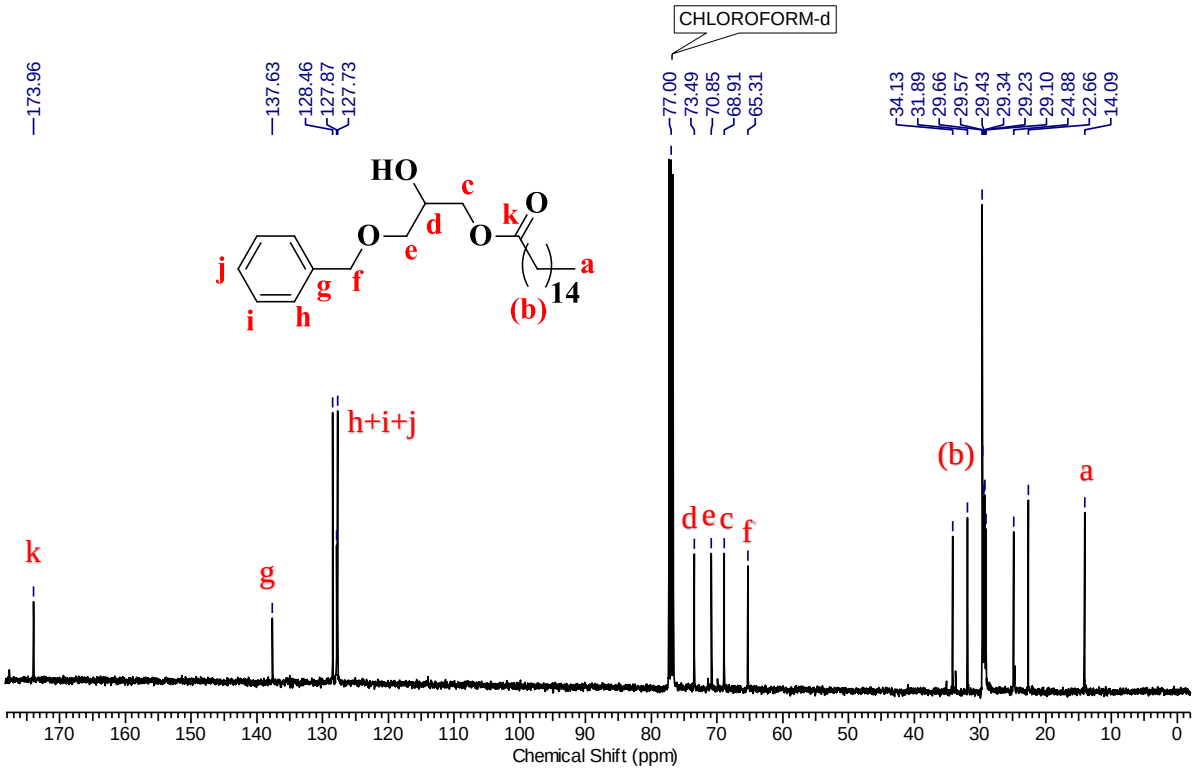


Figure S65: ^{13}C NMR ($CDCl_3$, 400 MHz) Benzyl Glycidyl Ether Palmitic

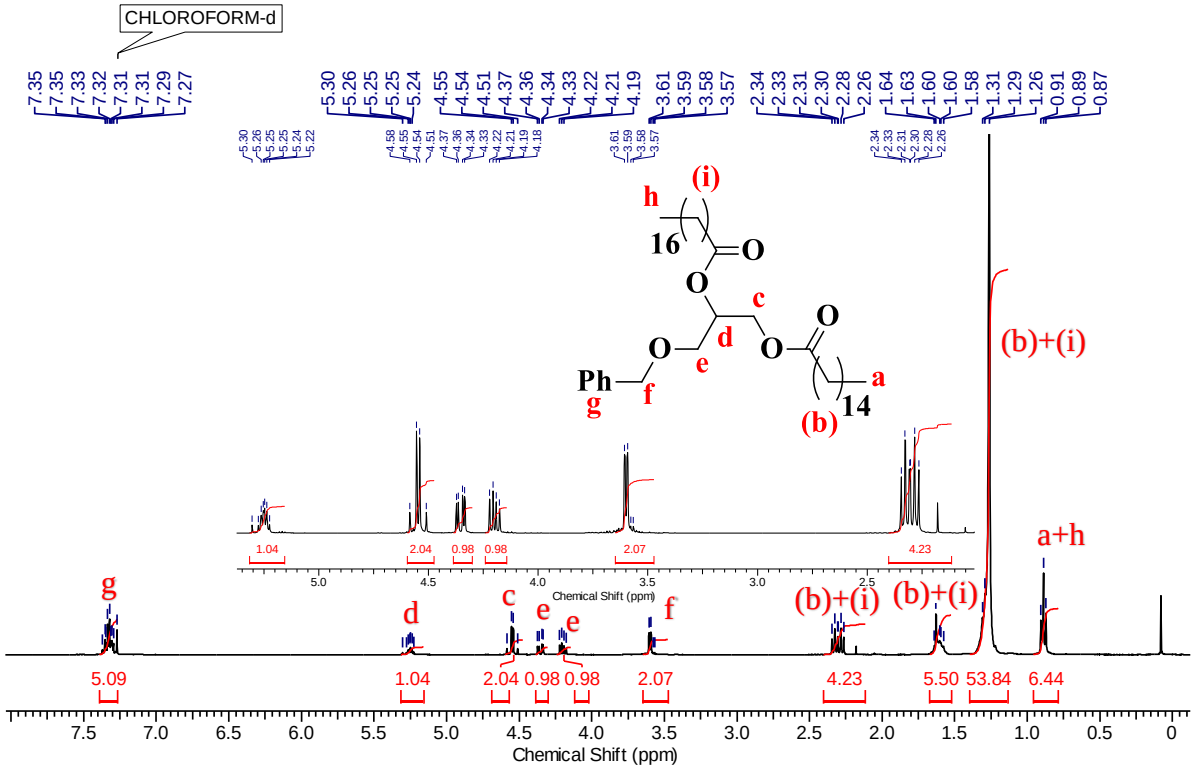


Figure S66: ^{1}H NMR ($CDCl_3$, 400 MHz) Benzyl Glycidyl Ether Palmitic Stearic

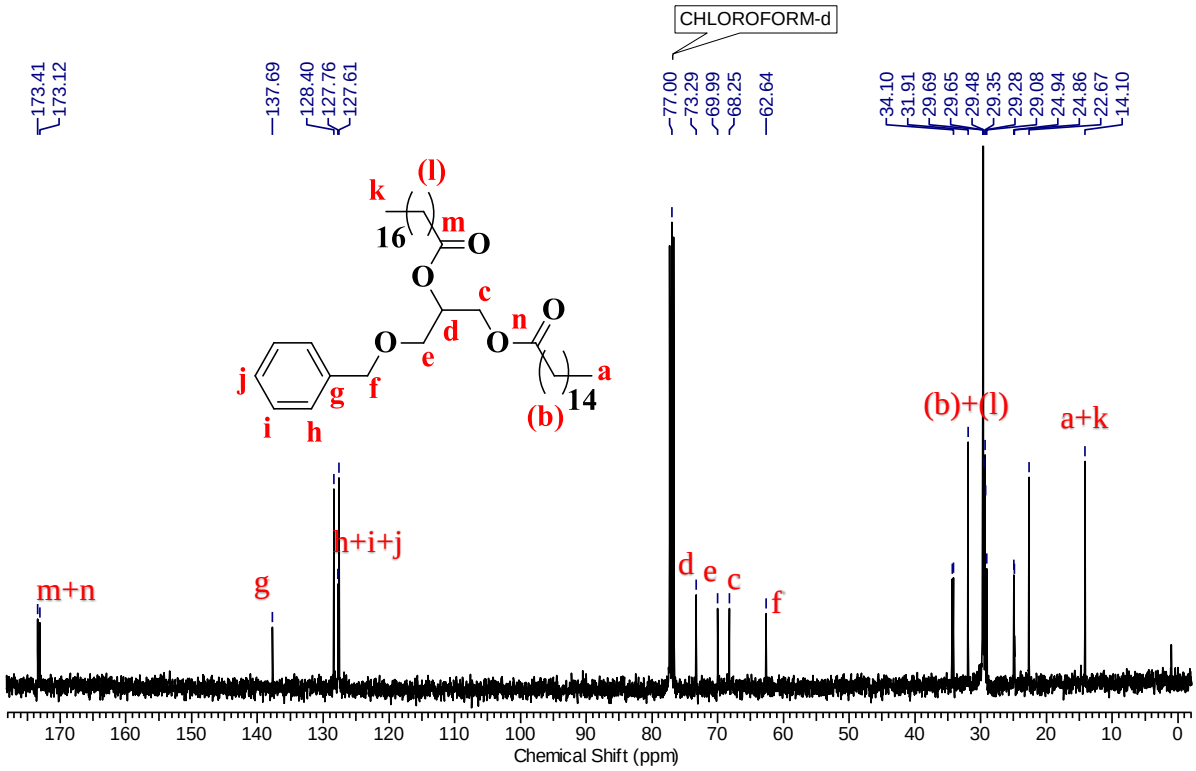


Figure S67: ^{13}C NMR ($CDCl_3$**,** 400 MHz) Benzyl Glycidyl Ether Palmitic Stearic

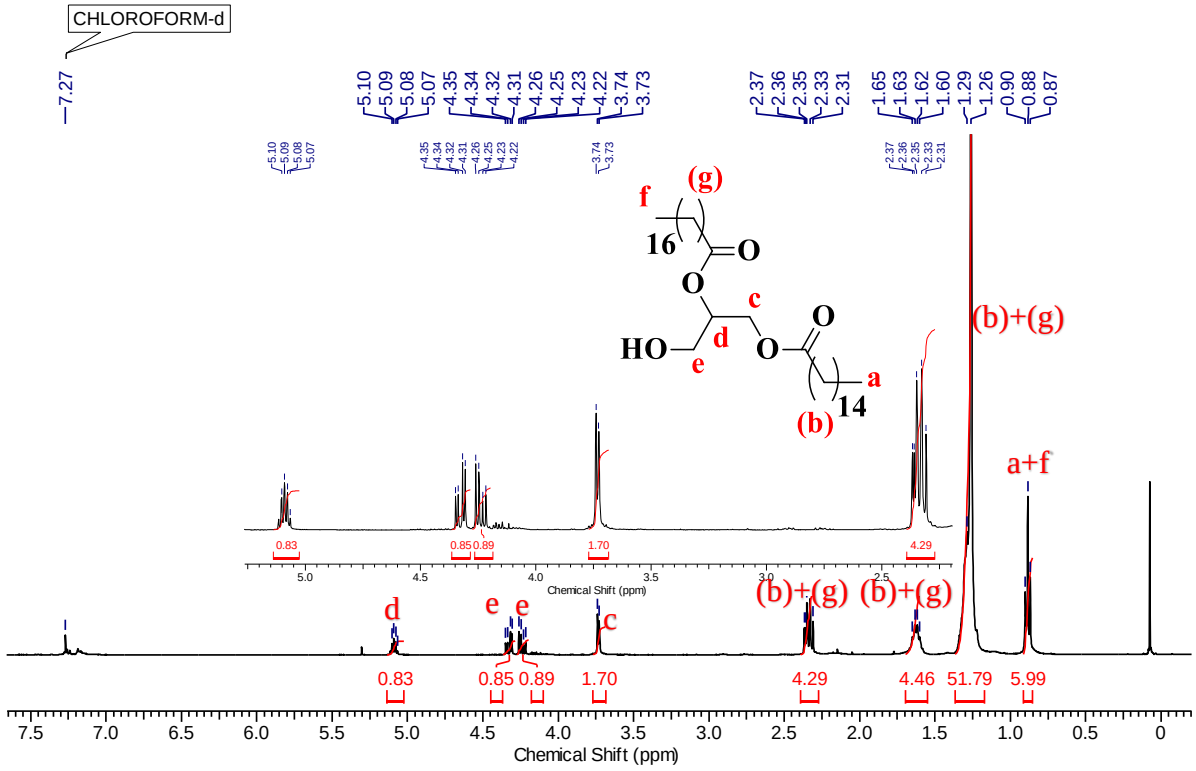


Figure S68: ^{1}H NMR ($CDCl_3$**,** 400 MHz) Benzyl deprotected Glycidyl Ether Palmitic Stearic

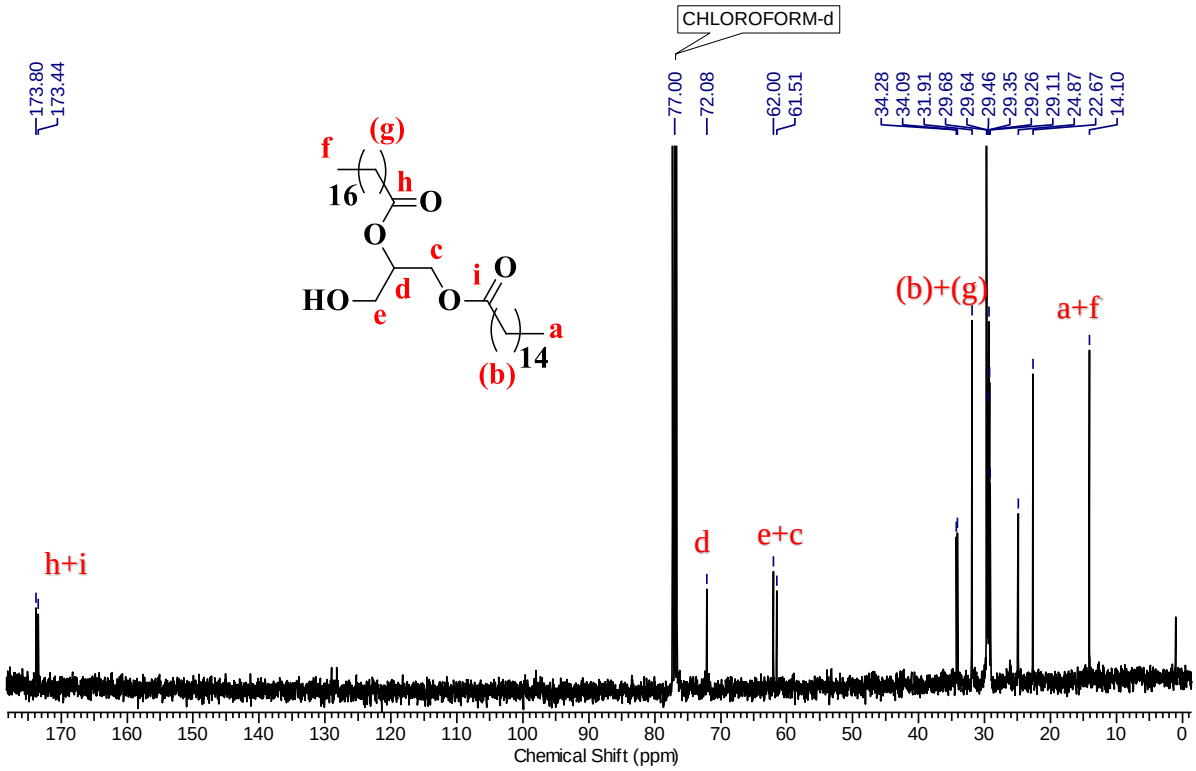


Figure S69: ^{13}C NMR ($CDCl_3$, 400 MHz) Benzyl deprotected Glycidyl Ether Palmitic Stearic

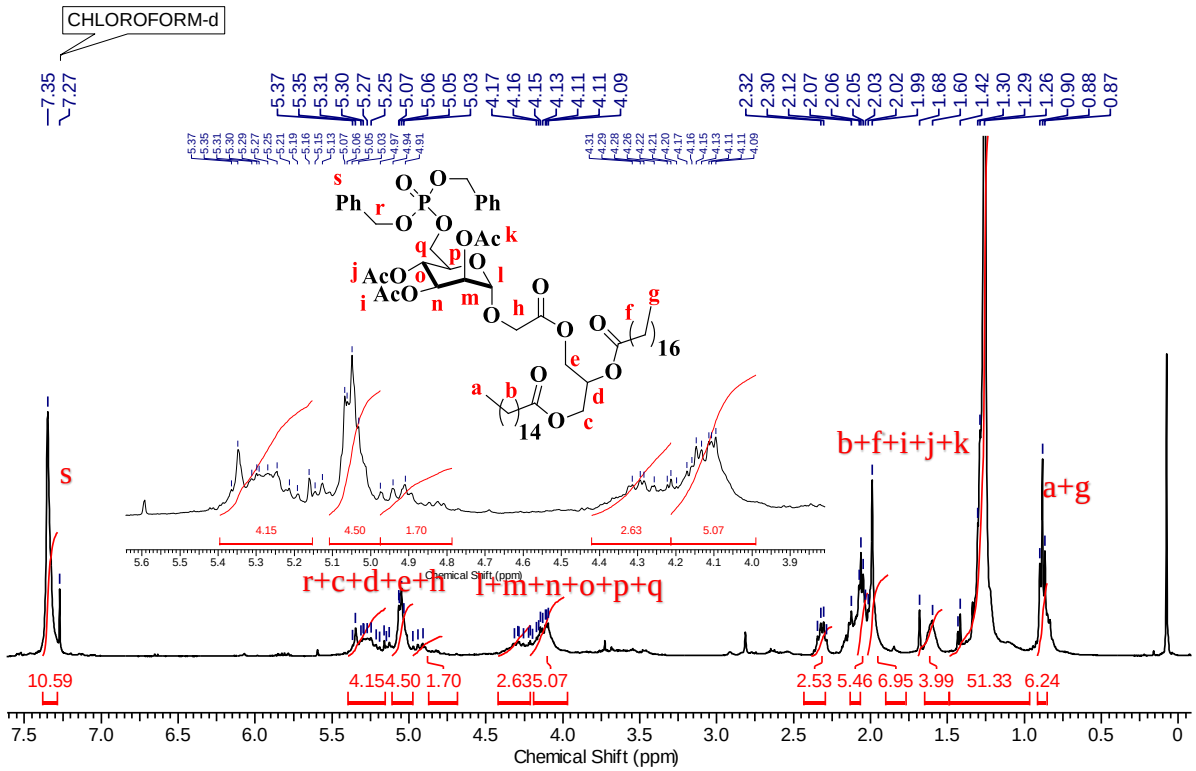


Figure S70: ^{1}H NMR ($CDCl_3$, 400 MHz) M6P Glycidyl Ether Palmitic Stearic Protected

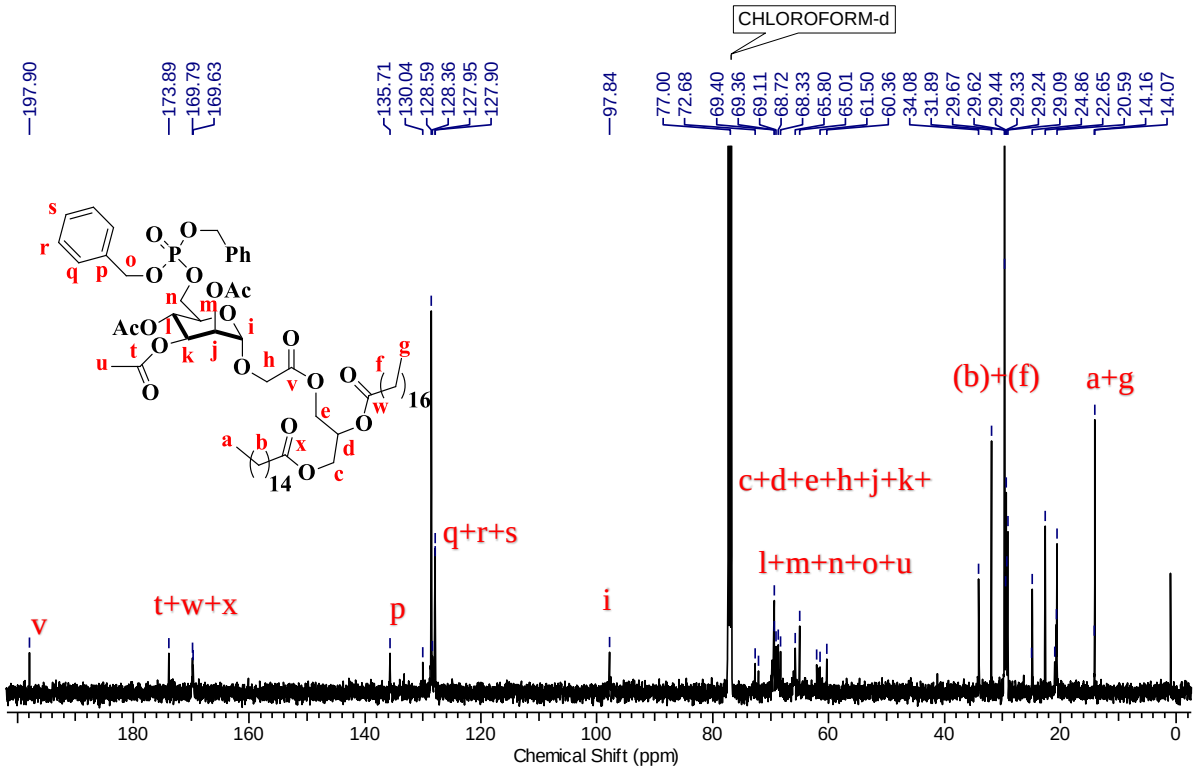


Figure S71: ^{13}C NMR ($CDCl_3$, 400 MHz) M6P Glycidyl Ether Palmitic Stearic Protected

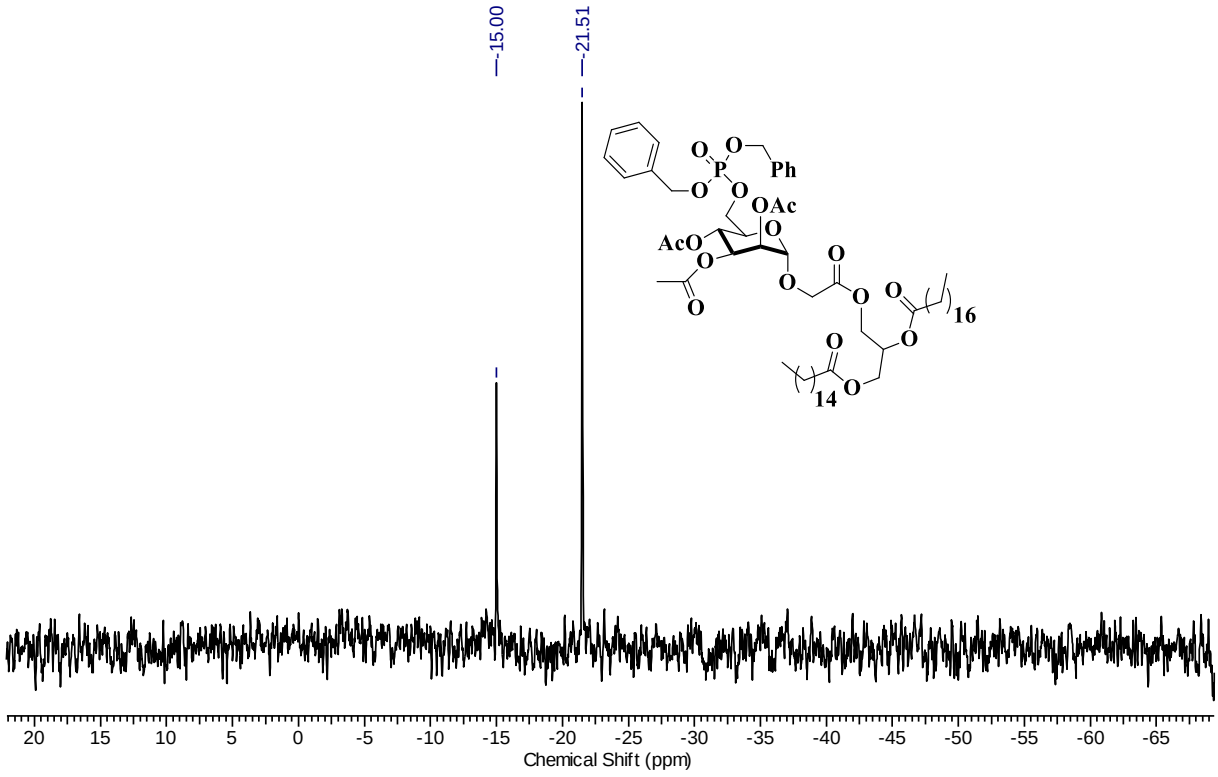


Figure S72: ^{31}P NMR ($CDCl_3$, 400 MHz) M6P Glycidyl Ether Palmitic Stearic Protected

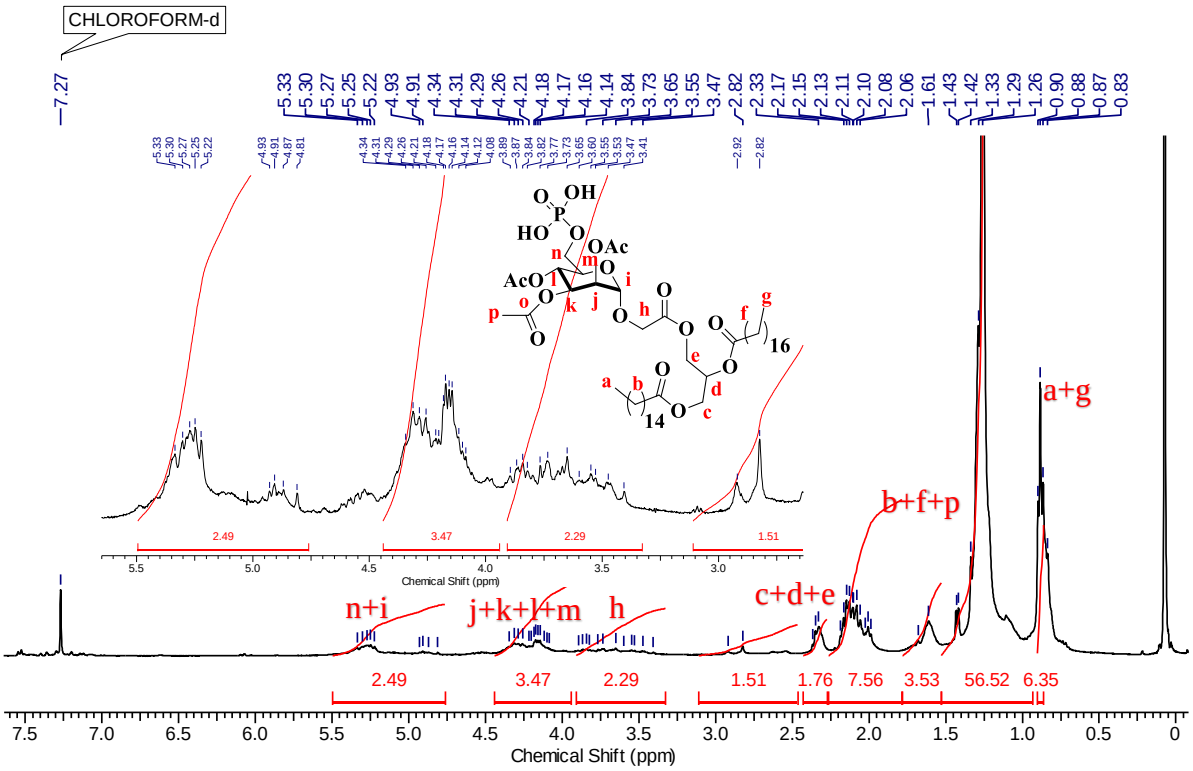


Figure S73: ^{1}H NMR ($CDCl_3$, 400 MHz) M6P Glycidyl Ether Palmitic Stearic Benzyl Deprotected

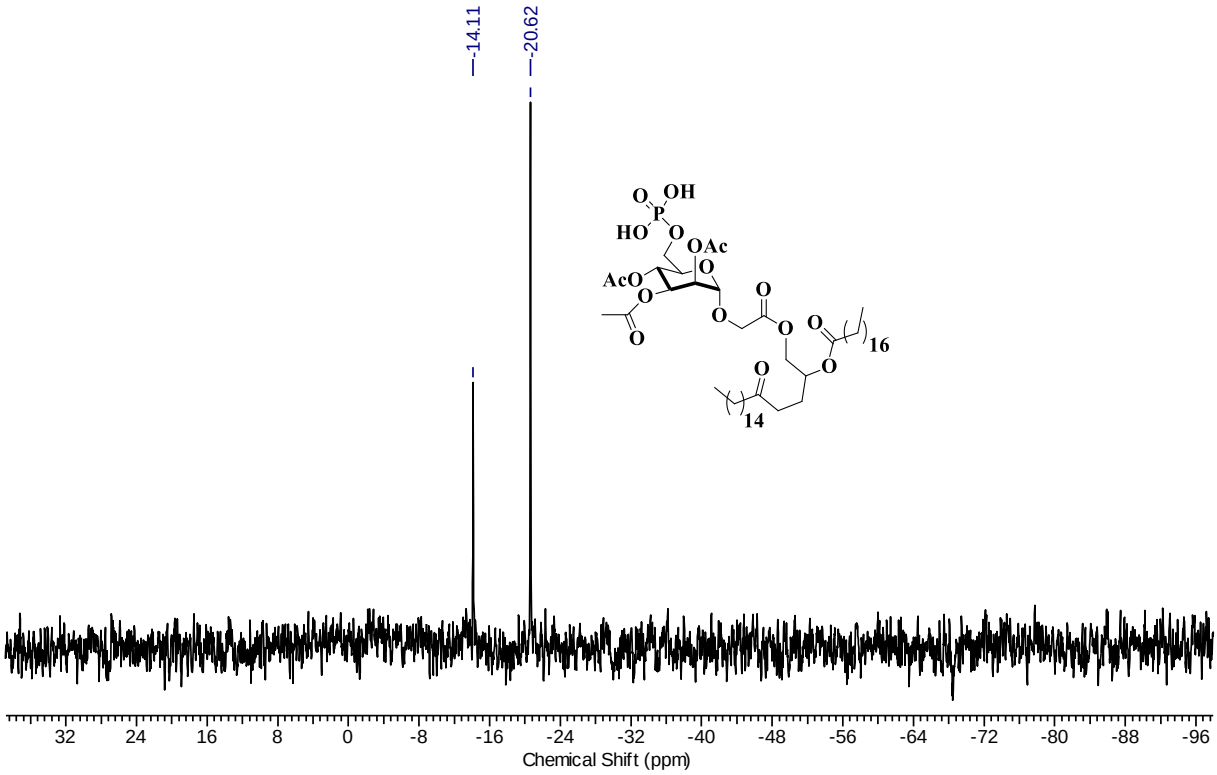


Figure S74: ^{31}P NMR ($CDCl_3$, 400 MHz) M6P Glycidyl Ether Palmitic Stearic Benzyl Deprotected

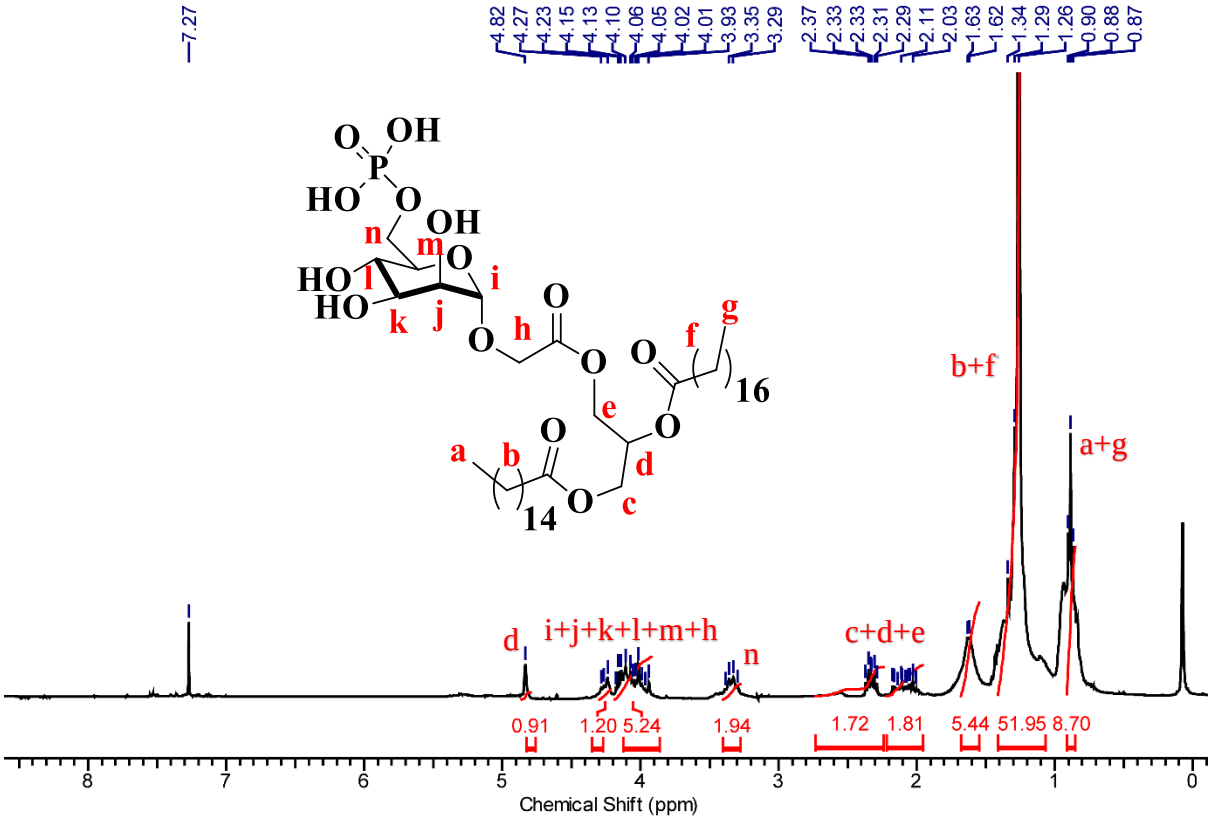


Figure S75: ^{1}H NMR ($CDCl_3$, 400 MHz) M6P Glycidyl Ether Palmitic all Deprotected

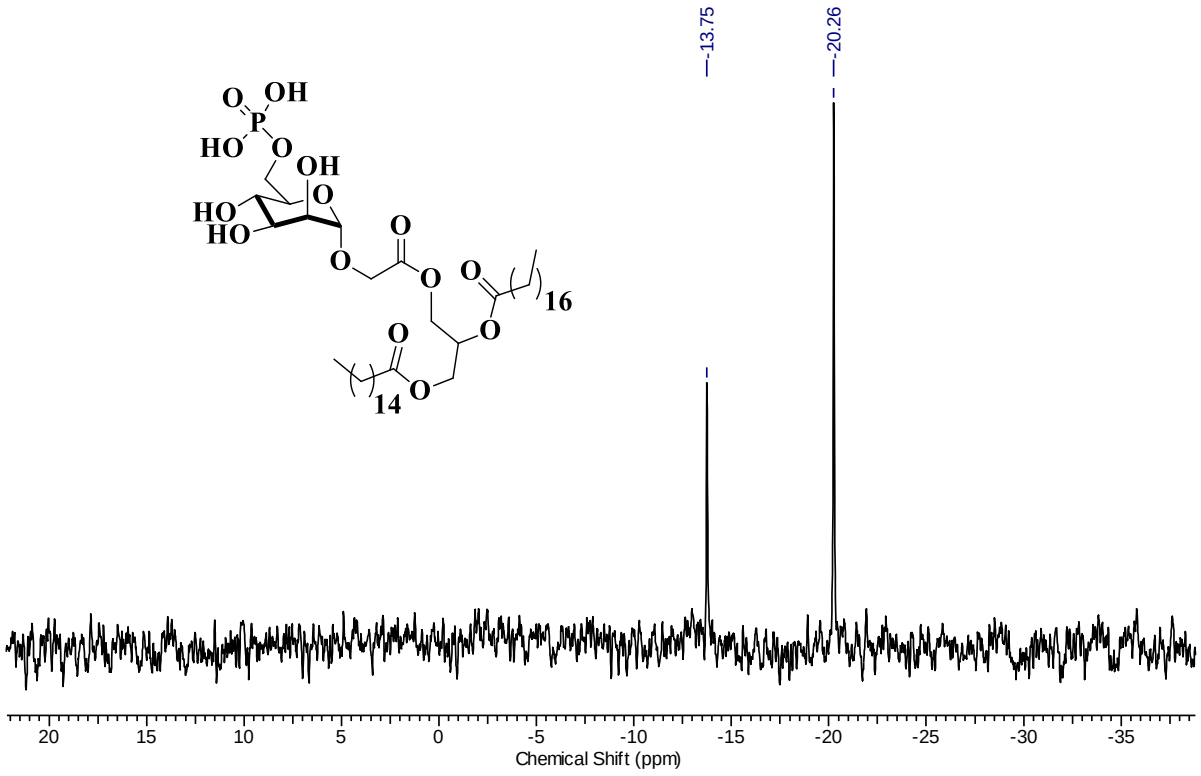


Figure S76: ^{31}P NMR ($CDCl_3$**,** 400 MHz) M6P Glycidyl Ether Palmitic Stearic All Deprotected

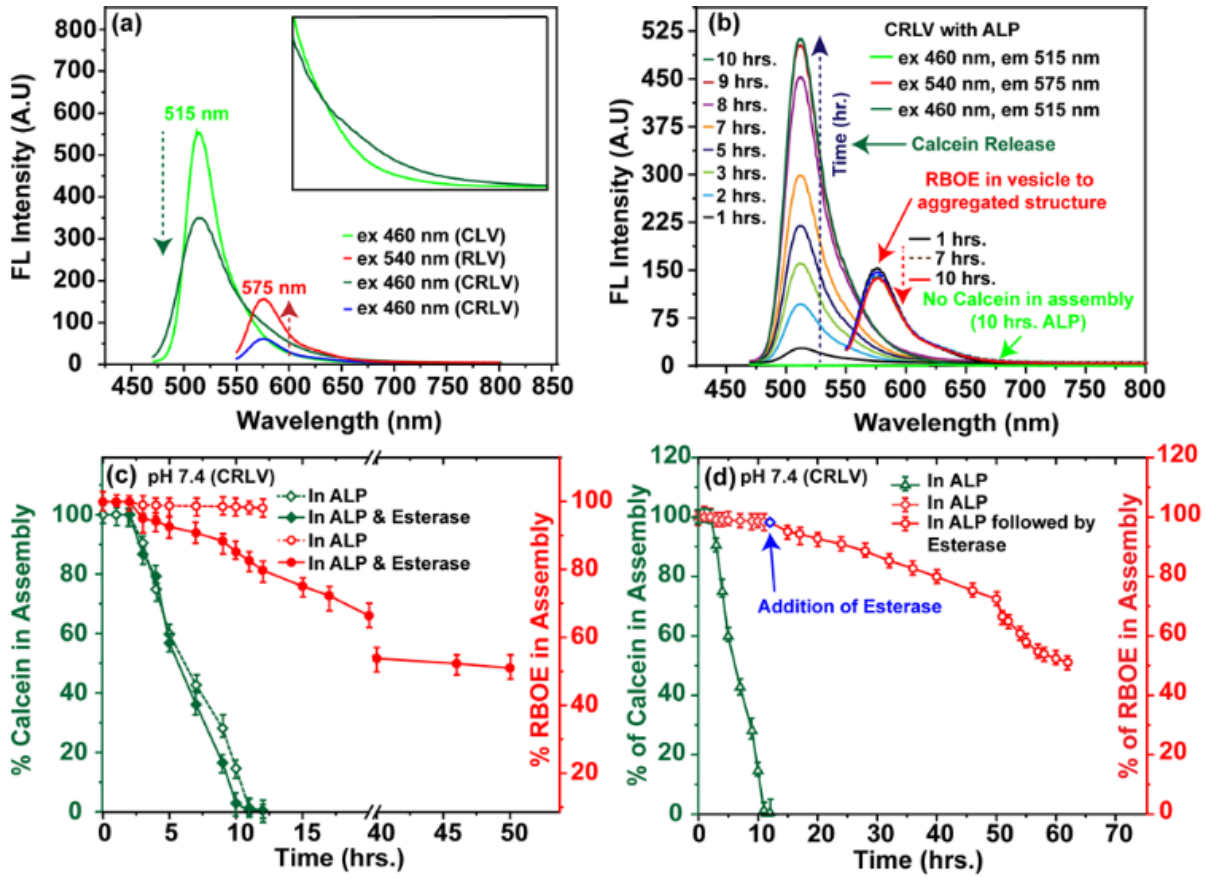


Figure S77: Fluorescence spectroscopy in aqueous medium: (a) dual dye encapsulated in CRLV (inset: the emission spectra from 525 to 650 nm of CLV and CRLV at 460 nm excitation); (b) time dependent ALP effect on CRLV. Dye release kinetics from CRLV: (c) in presence dual enzyme ALP and Esterase (red: RBOE; green: calcein); (d) sequential addition of ALP followed by esterase (green: calcein; red: RBOE; blue: esterase addition) in buffer medium.

Discussion: The fluorescence emission spectra of the dual dye encapsulated vesicles (termed as CRLV) upon excitation at 460 nm (excitation wavelength for calcein) show emission λmax at 515 nm (for calcein; Figure 4a: green) together with a shoulder at the λmax 575 nm (for RBOE; Figure 4a: green). The emission intensity at 515 nm for CRLV (Figure 4a, S8b: green) is much lower as compared to CLV (only calcein loaded vesicle; Figure 4a, S8b: Fluorescent) for the same amount of encapsulated calcein (Figure 4a and Figure S8b, S10). In contrast, a

much higher intensity was observed at the emission maximum (λmax) at 575 nm for RBOE in CRLV upon excitation at wavelength 540 nm (Figure 4a, Figure S9b, S10: red) than what was observed upon excitation at 460 nm (Figure 4a: blue and Figure S9b: red dotted, Figure S10: blue). Both calcein and rhodamine dyes can act as donor−acceptor FRET pairs because of the considerable overlap of calcein emission with RBOE absorption (Figure 4a and Figure S8b, S10). If both the dyes are encapsulated within the vesicles (as in CRLV) then there is a possibility to observe energy transfer between the donor and the acceptor, which should result in a decrease in the emission intensity of calcein and a simultaneous enhancement in the emission intensity of RBOE[2]. The noticeable shoulder at 575 nm (Figure 4a, S8b, S10: green) in the emission spectra upon excitation at 460 nm indicates some amount of energy transfer between the FRET pairs[2, 3] (Figure 4a and Figure S8b, S10). This is also corroborated by the reduction in the emission intensity of the donor (calcein) at 515 nm in the presence of the acceptor (RBOE), which again suggests energy transfer between the donor calcein and the acceptor RBOE (Figure 4a and Figure S8b, S10). These experiments prove the self-assembly of the M6PL amphiphile into vesicles and subsequent encapsulation of both dyes inside the vesicles.

References:

1. O. Y. Jeon and E. M. Carreira, *Org. Lett.* 2010, **12** (8), 1772–1775.
2. L. B. A. Johansson and A. Niemi, J. Phys. Chem. 1987, 91 (11), 3020–3023.
3. S. Das, D. K. Sharma, S. Chakrabarty, A. Chowdhury and S. S Gupta, Langmuir 2015, 31, 3402–3412.