Phosphate bioisostere containing amphiphiles: a novel class of squaramide-based Lipids

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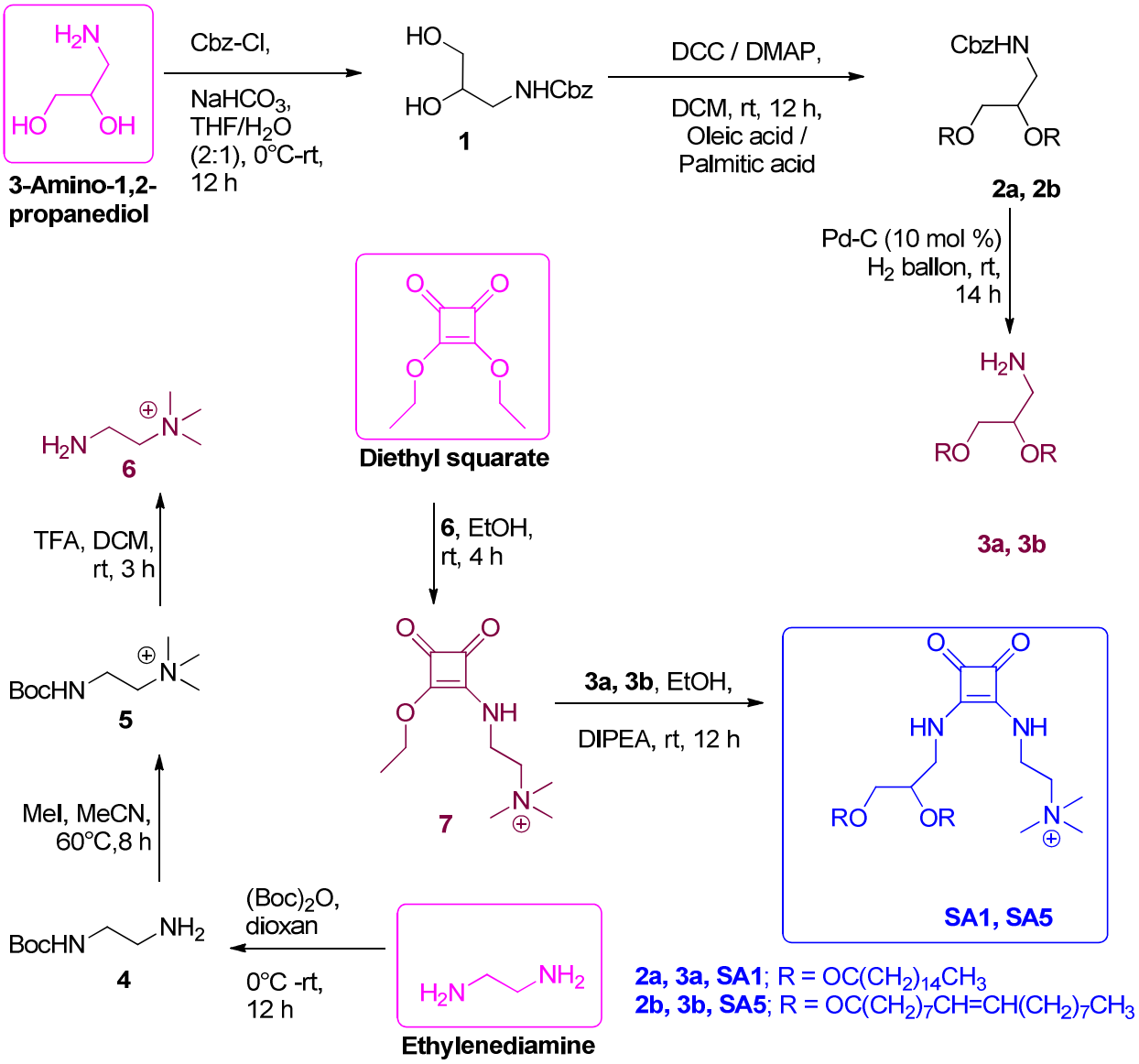
Materials and methods

All reagents were purchased from Sigma (St. Louis, MO), Merck (Mumbai, India), Himedia (Mumbai, India) and used directly without further purification. Dry solvents were obtained according to the reported procedures. Column chromatography was performed using 60–120 mesh silica gel. Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F254 (0.25 mm). ^{1}H NMR and ^{13}C NMR spectra were recorded at 600, 400, 151 and 100, respectively using Bruker-600 and Varian AS400 spectrometer. Coupling constants (J values) are reported in Hertz, and chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane using residual chloroform (d = 7.24 for ^{1}H NMR, d = 77.23 for ^{13}C NMR) as an internal standard. Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet), and br (broadened). Mass spectra were recorded using a Waters Q-TOF Premier mass spectrometry system, and data were analyzed using the built-in software. 1,2-dipalmitoylsn-glycero-3-phospho-L-serine (DPPS), 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPPA) and 1,2-dipalmitoyl-sn-glycero-3-phospho-(10-rac-glycerol) (DPPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Ultrapure water (Milli-Q system, Millipore, Billerica, MA) was used for the preparation of buffers.

Synthesis and characterization of the compounds:

Scheme 1. Synthetic routes to compounds **SA1** and **SA5** —



Synthesis of benzyl (2,3-dihydroxypropyl) carbamate (1): Benzylchloroformate (1.0 equiv.) was added dropwise to a suspension of 3-amino-1,2-propandiol (1.0 equiv.) and $NaHCO_3$ (2.0 equiv.) in THF/H_2O (2:1, 75 mL) at 0 °C. The resulting solution was then warmed to room temperature and stirred for 12 h. The reaction mixture was then acidified to pH ≤ 2 with HCl (1 M aq.) and extracted with EtOAc (3 × 100 mL). Combined organic layer was further washed

with brine and dried over anhydrous Na_2SO_4. Removal of the organic solvent under reduced pressure yielded a crude product which was purified by recrystallization (yield 2.39 g, 90%). The compound was characterized by ^1H and ^{13}C NMR and in accordance with the literature.[1]

Synthesis of 3-(((benzyloxy) carbonyl) amino) propane-1,2-diyl dipalmitate (2a): Palmitic acid (2.0 equiv.), dicyclohexylcarbodiimide (2.2 equiv.) and *N,N*-dimethylaminopyridine (0.1 equiv.) were added to a solution of *N*-Cbz protected diol (1.0 equiv.) in anhydrous dichloromethane (30 mL) under the N_2 atmosphere. The reaction mixture was then allowed to stir for 12 h at room temperature. After completion of the reaction, the reaction mixture was filtered and washed (3 × 20 mL) with dichloromethane. The filtrate was concentrated under reduced pressure and column chromatography with silica gel and a gradient solvent system of 0–10% ethyl acetate to hexane yielded the corresponding esters.[2] **Characterization of the compound 2a:** white solid (85% yield); ^1H NMR (600MHz, $CDCl_3$) δ_{ppm} 7.32-7.28 (m, 5H), 5.06 (s, 2H), 5.01-4.98 (m, 1H), 4.26-4.22 (m, 1H), 4.12-4.05 (m, 1H), 3.48-3.35 (m, 2H), 2.32-2.24 (m, 4H), 1.57-1.55 (m, 4H), 1.27-1.14 (m, 48H), 0.86-0.83 (m, 6H) ; ^{13}C NMR (151 MHz, $CDCl_3$) δ_{ppm} 173.7, 173.4, 156.6, 136.5, 128.8, 128.4, 128.3, 70.5, 67.2, 62.7, 41.8, 34.4, 34.3, 32.2, 29.92, 29.89, 29.86, 29.7, 29.6, 29.5, 29.34, 29.31, 25.1, 24.9, 22.9, 14.4; HRMS (ESI) calcd. for $C_{43}H_{75}NO_6$ $[M + H]^+$: 702.5594, found: 702.5590.

Synthesis of 3-(((benzyloxy)carbonyl)amino)propane-1,2-diyl (9E,9'E)-bis(octadec-9-enoate) (2b): Oleic acid (2.0 equiv.), dicyclohexylcarbodiimide (2.2 equiv.) and *N,N*-dimethylaminopyridine (0.1 equiv.) were added to a solution of *N*-Cbz protected diol (1.0 equiv.) in anhydrous dichloromethane (30 mL) under the N_2 atmosphere. The reaction mixture was then

allowed to stir for 12 h at room temperature. After completion of the reaction, the reaction mixture was filtered and washed (3 × 20 mL) with dichloromethane. The filtrate was concentrated under reduced pressure and column chromatography with silica gel and a gradient solvent system of 0–10% ethyl acetate to hexane yielded the corresponding esters.[2] **Characterization of the compound 2b:** colorless oil (80% yield); ^{1}H NMR (600MHz, $CDCl_3$) δ_{ppm} 7.35-7.31 (m, 5H), 5.37-5.34 (m, 4H), 5.09 (s, 2H), 5.05-5.04 (m, 1H), 4.28-4.26 (m, 1H), 4.14-4.11 (m, 1H), 3.48-3.39 (m, 2H), 2.35-2.28 (m, 4H), 2.06-2.00 (m, 8H), 1.64-1.60 (m, 4H), 1.29-1.26 (m, 44H), 0.89-0.86 (m, 6H) ; ^{13}C NMR (151 MHz, $CDCl_3$) δ_{ppm} 173.7, 173.4, 156.6, 136.5, 130.4, 130.2, 129.9, 128.4, 128.3, 70.5, 67.2, 62.7, 41.5, 34.4, 34.2, 34.1, 32.1, 31.7, 30.0, 29.9, 29.8, 29.7, 29.5, 29.4, 29.32, 29.29, 29.26, 27.4, 27.36, 25.8, 25.0, 24.9, 22.9, 22.8, 14.3; HRMS (ESI) calcd. for $C_{47}H_{79}NO_6$ $[M + H]^+$: 754.5907, found: 754.5923.

Synthesis of 3-aminopropane-1,2-diyl dipalmitate (3a) : 3-Aminopropane-1,2-diyl dipalmitate compound was synthesized by the hydrogenation of compound **2a** in the presence of 10 mol % Pd/charcoal (stirred for 14 hours at room temperature) according to the literature procedure and used without further purification.[1]

Synthesis of 3-aminopropane-1,2-diyl (9E,9'E)-bis(octadec-9-enoate) (3b) : 3-Aminopropane-1,2-diyl (9E,9'E)-bis(octadec-9-enoate) compound was synthesized by the hydrogenation of compound **2b** in the presence of 10 mol % Pd/charcoal (stirred for 14 hours at room temperature) according to the literature procedure and used without further purification.[1]

Synthesis of tert-butyl (2-aminoethyl)carbamate (4): To a solution of di-tert-butyl dicarbonate (1.0 equiv.) in 1,4-dioxane (10 mL) a solution of ethylenediamine (3.5 equiv.) in 30 mL 1,4-dioxane was added dropwise at 0 °C. After that the solution was warmed up to room temperature and allowed to stir for 12 hours. The white precipitate was removed by filtration and clear oil was obtained by removing the organic solvent under reduced pressure (yield, 90 %). This mono-Boc protected ethylene diamine was used without further purification.[3] The compound was characterized by ^{1}H and ^{13}C NMR and HRMS and in accordance with the literature.

Synthesis of 2-((tert-butoxycarbonyl)amino)-N,N,N-trimethylethan-1-aminium (5): To a solution of mono-boc protected ethylene diamine (1.0 equiv.) in MeCN (30 mL) and K_2CO_3 (4.0 equiv.) was added and mixture was continued to stir for 30 min at room temperature. After that methyl iodide (3.3 equiv.) was added and the reaction mixture was heated under reflux condition for 20 hours. After that the reaction mixture was cooled down to room temperature and K_2CO_3 was removed by filtration. Removal of organic solvent under reduced pressure yielded an oily crude product. Addition of diethyl ether to this oily crude product yielded the target product as colorless solid, which was filtered (yield, 80 %).[3] The compound was characterized by ^{1}H and ^{13}C NMR and HRMS and in accordance with the literature.

Synthesis of 2-amino-*N,N,N*-trimethylethan-1-aminium (6): To a solution of compound **5** (1.10 g, 1 equiv.) in dichloromethane (9 mL) TFA (1 mL) was added dropwise at 0 °C (using an ice-salt bath) and stirred for 30 min. After that the reaction mixture was warmed up to room temperature and stirring was continued for another 3 hours. The solvent was removed under reduced pressure and then diethyl ether (10 mL) was added into the oily liquid and the solid was

filtered out to get the target product (yield, 80%).[3] The compound was characterized by ^{1}H and ^{13}C NMR and HRMS and in accordance with the literature.

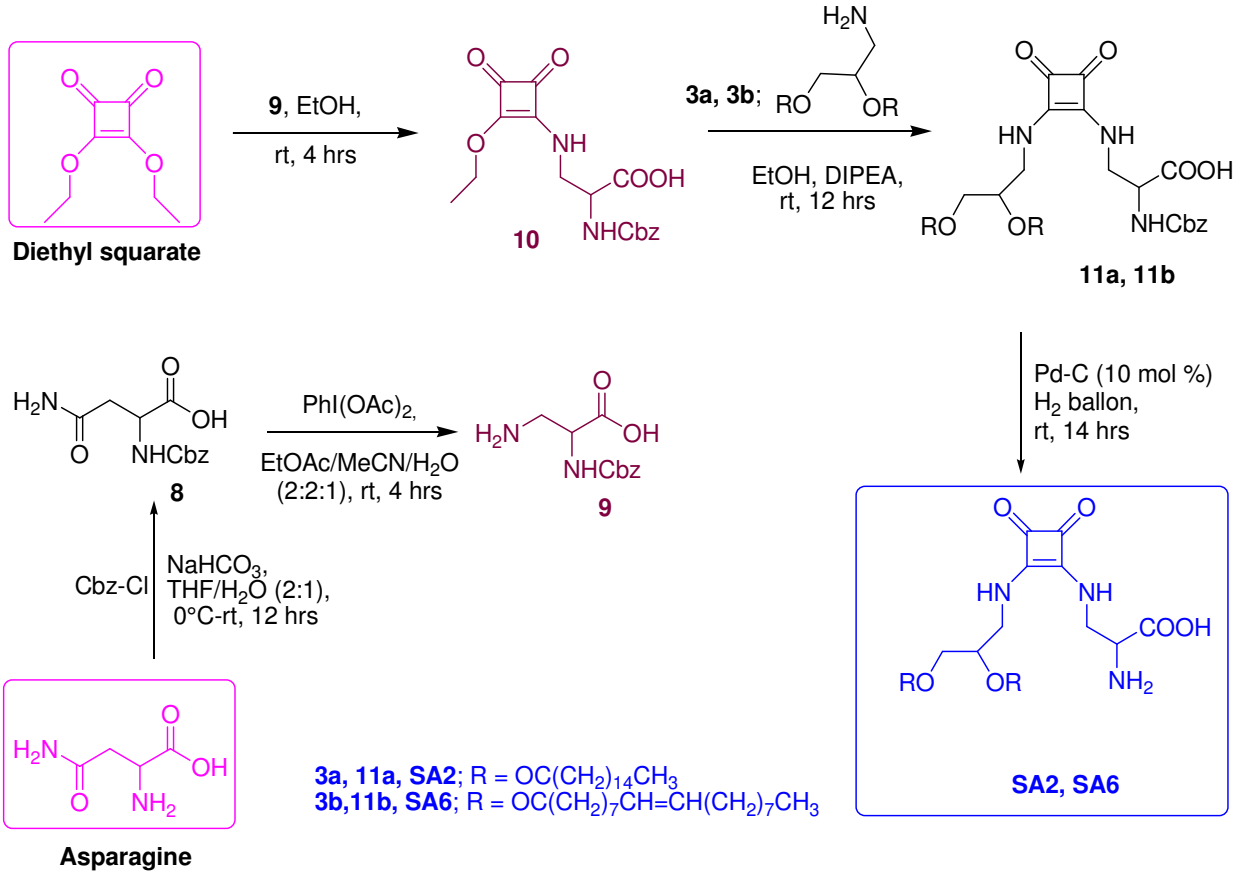
Synthesis of 2-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)-N,N,N-trimethylethan-1-aminium (7): In an oven dried round bottom flask 2-amino-N,N,N-trimethylethan-1-aminium (1.0 equiv.) was first added and then it was dissolved in 4 ml of ethanol. Diethyl squarate (1.1 equiv.) was then added drop wise into the reaction mixture and allowed to stir at room temperature for 4 hours. After completion of the reaction, the mixture was washed with diethyl ether and then it was dried under reduced pressure, which yielded a white solid compound.[4] **Characterization of compound 7:** white solid (yield, 80%); ^{1}H NMR (600MHz, DMSO-d_6) δ_{ppm} 9.78 (br s, 1H), 4.56-4.48 (m, 4H), 3.22-3.08 (m, 2H), 1.20-1.18 (m, 12H); ^{13}C NMR (151 MHz, DMSO-d_6) δ_{ppm} 194.5, 53.0, 51.7, 45.9, 8.7. HRMS (ESI) calcd. for $C_{11}H_{19}N_2O_3$ $[M]^+$: 227.1396, found: 227.1325.

Synthesis of 2-((2-((2,3-bis(palmitoyloxy)propyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)-N,N,N-trimethylethanaminium (SA1) : In an oven dried round bottom flask compound **7** (1.0 equiv.) and compound **3a (**1.1 equiv.**)** were dissolve in 3.5 ml of ethanol. Then DIPEA (5.5 equiv.) was added to the reaction mixture and the reaction mixture was allowed to stir at room temperature for 12 hours. After completion of the reaction, the solvent was removed under reduced pressure. Then the reaction mixture was washed with a mixture of diethyl ether and dichloromethane (for several times) and the solid residue was dried under reduced pressure. **Characterization of the compound SA1:** white solid (yield, 70%); ^{1}H NMR (400MHz, $CDCl_3$) δ_{ppm} 6.24 (br s, 1H),4.16-4.12 (m, 1H), 4.08-4.03 (m, 1H), 3.96-3.93 (m, 1H), 3.77 (br s, 1H),

3.57-3.51 (m, 1H), 3.25-3.16 (m, 3H), 2.35-2.19 (m, 6H), 1.65-1.57 (m, 4H), 1.43 (s, 9H), 1.29-1.22 (m, 48H), 0.89-0.86 (t, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) δ_{ppm} 199.2, 175.2, 69.5, 65.9, 53.7, 42.9, 42.1, 36.8, 34.4, 32.1, 29.9, 29.8, 29.7, 29.6, 25.9, 22.9, 14.3. MS (ESI) calcd. for $C_{44}H_{82}N_3O_6$ $[M + H]^+$: 748.6198, found: 748.5309.

Synthesis of 2-((2-((2,3-bis(((E)-octadec-9-enoyl)oxy)propyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)-N,N,N-trimethylethan-1-aminium (SA5) : In an oven dried round bottom flask compound **7** (1.0 equiv.) and compound **3b (**1.1 equiv.**)** were dissolve in 3.5 ml of ethanol. Then DIPEA (5.5 equiv.) was added to the reaction mixture and the reaction mixture was allowed to stir at room temperature for 12 hours. After completion of the reaction, the solvent was removed under reduced pressure. Then the reaction mixture was washed with a mixture of diethyl ether and dichloromethane (for several times) and the solid residue was dried under reduced pressure. **Characterization of the compound SA5:** white solid (yield, 60%); ^{1}H NMR (600 MHz, $CDCl_3$ + MeOD-d_4 + DMSO-d_6) δ_{ppm} 7.84 (br s, 1H),5.38 (br s, 1H), 5.12-5.08 (m, 4H), 4.09-4.02 (m, 1H), 3.70-3.62 (m, 1H), 3.39-3.31 (m, 2H), 3.23-3.19 (m, 2H), 2.64-2.62 (m, 2H), 2.34-2.30 (m, 4H), 1.62-1.58 (m, 4H), 1.39-1.22 (m, 61H), 0.91-0.87 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$ + MeOD-d_4 + DMSO-d_6) δ_{ppm} 197.0, 173.6, 173.3, 137.2, 128.7, 128.1, 70.6, 66.5, 63.1, 54.5, 42.7, 34.3, 34.2, 32.1, 29.8, 29.6, 29.5, 29.2, 25.0, 22.8, 14.2. MS (ESI) calcd. for $C_{48}H_{86}N_3O_6$ $[M + 3]^+$: 803.6558, found: 803.3310.

Scheme 2. Synthetic routes to compounds **SA2** and **SA6 —**



Synthesis of (benzyloxy) carbonyl) asparagine (8): To a stirred and ice-cooled suspension of L-asparagine (1.0 equiv.) in THF/H_2O (2:1, 75 mL) at 0 °C, $NaHCO_3$ (2.0 equiv.) and benzylchloroformate (1.1 equiv.; added dropwise). The resulting solution was then warmed to room temperature and stirred for 12 h. The reaction mixture was then acidified to pH ≤ 2 with HCl (1 M aq.) and extracted with EtOAc (3 × 100 mL). Combined organic layer was further washed with brine and dried over anhydrous Na_2SO_4. Removal of the organic solvent under reduced pressure yielded the solid product which and crystallized from methanol (yield, 80 %).[5] This product was used for next reaction without further purification.

Synthesis of 3-amino-2-(((benzyloxy) carbonyl) amino) propanoic acid (9): To the suspension of (benzyloxy) carbonyl) asparagine (1.0 equiv.) in a solvent mixture of EtOAc/CH_3CN/H_2O (12:12:6 mL) at 16 °C, iodobenzene diacetate (1.2 equiv.) was added and stirred for 30 min. Then the reaction mixture was warmed to room temperature and stirring was continued for another 4 hours. After completion of the reaction the mixture was cooled to 5 °C and the solid product was filtered, washed with ethyl acetate (10 mL), and dried under reduced pressure to give the target compounds with 85 % yield.[5] The compound was characterized by ^{1}H and ^{13}C NMR and in accordance with the literature.

Synthesis of 2-(((benzyloxy)carbonyl)amino)-3-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino) propanoic acid (10): To an oven dried round bottom flask compound 9 (1.0 equiv.) was first added and then it was dissolve in 5 ml ethanol. After that diethyl squarate (1.1 equiv.) was added drop wise to the reaction mixture allowed to stir at room temperature for 4 hours. After completion of the reaction the mixture was washed with diethyl ether and then dried under reduced pressure to give a white semisolid compound. **Characterization of the compound 10:** yellowish-white semi solid (yield, 70%); ^{1}H NMR (600MHz, DMSO-d_6) δ_{ppm} 7.36-7.32 (m, 5H), 6.55 (s, 1H), 5.04 (s, 2H), 4.67-4.63 (m, 4H), 4.47-4.46 (m, 1H), 1.38-1.36 (m, 3H); ^{13}C NMR (151 MHz, DMSO-d_6) δ_{ppm} 189.8, 184.3, 129.0, 128.4, 128.37, 70.8, 40.7, 16.0. HRMS (ESI) calcd. for $C_{17}H_{18}N_2O_7$ $[M + H]^+$: 363.1145, found: 363.1160.

Synthesis of 2-(((benzyloxy) carbonyl) amino)-3-((2-((2,3-bis (palmitoyloxy) propyl) amino) -3,4-dioxocyclobut-1-en-1-yl) amino) propanoic acid (11a): In an oven dried round bottom flask, compound **9** (1.0 equiv.), and compound **3a** (1.1 equiv.), were dissolved in 2.5 ml of

ethanol. Then DIPEA (5.5 equiv.) was added to the reaction mixture and allowed to stir at room temperature for 12 hours. After completion of the reaction, the solvent was removed under reduced pressure. Then the solid residue was washed with diethyl ether and dichloromethane (1:1) for several times. After that the remaining solid residue was dried under reduced pressure and the target product was obtained. **Characterization of the compound 11a:** white solid (yield, 65 %); ^{1}H NMR (600MHz, $CDCl_3$) δ_{ppm} 7.33-7.31 (m, 5H), 5.08 (s, 2H), 4.74-4.68 (m, 1H), 4.27-4.26 (m, 1H), 4.12-4.10 (m, 1H), 3.46-3.48 (m, 1H), 3.16-3.14 (m, 4H), 2.29-2.28 (m, 4H), 1.62-1.57 (m, 4H), 1.29-1.20 (m, 52H), 0.87-0.85 (m, 6H) ; ^{13}C NMR (151 MHz, $CDCl_3$) δ_{ppm} 189.6, 173.7, 173.4, 156.5, 136.4, 128.7, 128.4, 128.3, 70.4, 67.1, 62.7, 46.0, 41.5, 34.4, 34.2, 32.1, 29.9, 29.8, 29.7, 29.53, 29.45, 29.3, 26.5, 25.0, 22.9, 14.3. MS (ESI) calcd. for $C_{50}H_{81}N_3O_{10}$ $[M + H]^+$: 884.5968, found: 884.5309.

Synthesis of 2-(((benzyloxy)carbonyl) amino) -3- ((2-((2,3-bis (((E) - octadec-9-enoyl)oxy)propyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)propanoic acid (11b): In an oven dried round bottom flask, compound **9** (1.0 equiv.), and compound **3b** (1.1 equiv.), were dissolved in 2.5 ml of ethanol. Then DIPEA (5.5 equiv.) was added to the reaction mixture and allowed to stir at room temperature for 12 hours. After completion of the reaction, the solvent was removed under reduced pressure. Then the solid residue was washed with diethyl ether and dichloromethane (1:1) for several times. After that the remaining solid residue was dried under reduced pressure and the target product was obtained. **Characterization of the compound 11b:** As a white solid with 55% yield; ^{1}H NMR (600 MHz, $CDCl_3$ + MeOD-d_4) δ_{ppm} 7.32-7.27 (m, 5H), 5.06 (s, 2H), 4.68-4.61 (m, 4H), 4.32-4.31 (m, 1H), 4.09-4.01 (m, 1H), 3.90-3.83 (m, 1H), 3.72-3.64 (m, 2H), 3.42-3.38 (m, 1H), 3.19-3.15 (m, 2H), 2.34-2.27 (m, 4H), 1.60-1.57 (m, 4H),

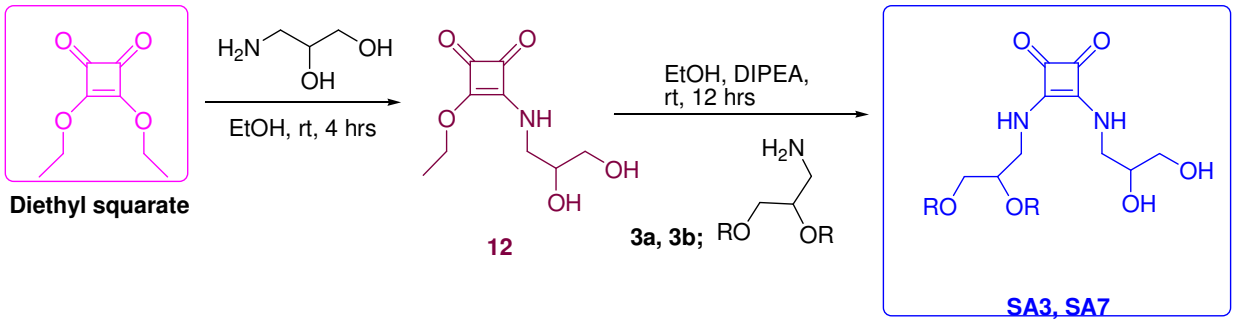
1.40-1.35 (m, 8H), 1.29-1.26 (m, 44H), 0.88-0.86 (m, 6H) ; ^{13}C NMR (151 MHz, $CDCl_3$ + MeOD-*d*$_4$) δ_{ppm} 188.9, 183.2, 176.9, 173.4, 156.0, 136.7, 128.44, 128.38, 128.0, 127.9, 127.7, 127.68, 69.7, 66.6, 66.57, 53.7, 42.0, 31.9, 29.7, 29.6, 29.5, 29.3, 22.7, 15.8, 15.3, 14.3; MS (ESI) calcd. for $C_{54}H_{85}N_3O_{10}$ $[M + H]^+$: 936.6235, found: 936.6438.

Synthesis of 2-amino-3-((2-((2,3-bis(palmitoyloxy)propyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)propanoic acid (SA2) : Compound **SA2** was synthesized by the hydrogenation of compound **11a** in the presence of 10 mol % Pd/charcoal (stirred for 14 hours at room temperature) according to the literature procedure.[1] **Characterization of the compound SA2:** white solid (yield, 70 %); ^{1}H NMR (600MHz, $CDCl_3$) δ_{ppm} 12.10 (br, s, 1H), 5.25 (br, s, 2H), 4.63-4.59 (m, 1H), 4.06-3.99 (m, 1H), 3.63-3.55 (m, 1H), 2.96-2.93 (m, 4H), 2.48-2.25 (m, 4H), 1.58-1.46 (m, 4H), 1.28-1.19 (m, 56H), 0.87-0.77 (m, 6H) ; ^{13}C NMR (151 MHz, $CDCl_3$) δ_{ppm} 194.9, 188.9, 177.2, 174.4, 69.9, 65.2, 46.6, 34.3, 34.0, 31.8, 29.58, 29.55, 29.4, 29.3, 29.2, 29.1, 24.7, 22.6, 13.9.; MS (ESI) calcd. for $C_{42}H_{75}N_3O_8$ $[M]^+$: 749.5554, found: 749.5329.

Synthesis of 2-amino-3-((2-((2,3-bis (((E)-octadec-9-enoyl)oxy)propyl) amino)-3,4-dioxocyclobut-1-en-1-yl)amino)propanoic acid (SA6): Compound **SA6** was synthesized by the hydrogenation of compound **11b** in the presence of 10 mol % Pd/charcoal (stirred for 14 hours at room temperature) according to the literature procedure.[1] **Characterization of the compound SA6:** As a white solid with 50% yield; ^{1}H NMR (600 MHz, $CDCl_3$) δ_{ppm} 10.38 (br s, 1H), 5.11-5.01 (m, 4H), 4.70-4.55 (m, 2H), 4.30-4.27 (m, 1H), 3.84-3.80 (m, 1H), 3.66-3.60 (m, 2H), 3.48-3.33 (m, 2H), 3.08-3.02 (m, 4H), 1.45-1.32 (m, 12H), 1.29-1.16 (44H), 0.87-0.83 (6H); ^{13}C NMR (151 MHz, $CDCl_3$ + MeOD-*d*$_4$) δ_{ppm} 191.8, 187.8, 183.0, 173.4, 172.2, 128.7, 128.1, 70.1, 67.0,

54.9, 43.0, 32.3, 30.0, 29.7, 25.2, 23.0, 18.2, 15.6, 13.9, 12.5. MS (ESI) calcd. for $C_{46}H_{79}N_3O_8$ $[M+ HCOO^-]^+$: 845.5554, found: 845.5591.

Scheme 3. Synthetic routes to compounds **SA3** and **SA7** —



3a, SA3; R = $OC(CH_2)_{14}CH_3$
3b, SA7; R = $OC(CH_2)_7CH{=}CH(CH_2)_7CH_3$

Synthesis of 3-((2,3-dihydroxypropyl)amino)-4-ethoxycyclobut-3-ene-1,2-dione (12): To an oven dried round bottom flask 3-aminopropane-1,2-diol (1 equiv.) and 5 mL of ethanol were added sequentially. Then diethyl squarate (1.1 equiv.) was added dropwise to the reaction mixture and allowed to stir at room temperature for 4 hrs. After completion of the reaction, the reaction mixture was successively washed with diethyl ether and then dried under reduced pressure, which produced a yellow sticky compound. **Characterization of the compound 12:** white semi solid (yield, 75%); ^{1}H NMR (600MHz, CDCl3 + MeOD-*d*4 + DMSO-d_6) δ_{ppm} 8.24 (br s, 1H), 3.77-3.64 (m, 1H), 3.48-3.39 (m, 2H), 3.11-3.06 (m, 4H), 1.19-1.15 (m, 3H); ^{13}C NMR (151 MHz, CDCl3 + MeOD-*d*4 + DMSO-d_6) δ_{ppm} 204.3, 75.7, 72.8, 72.2, 68.5, 67.9, 50.8, 13.2; MS (ESI) calcd. for $C_9H_{13}NO_5$ $[M+H]^+$: 215.0794, found: 215.0766.

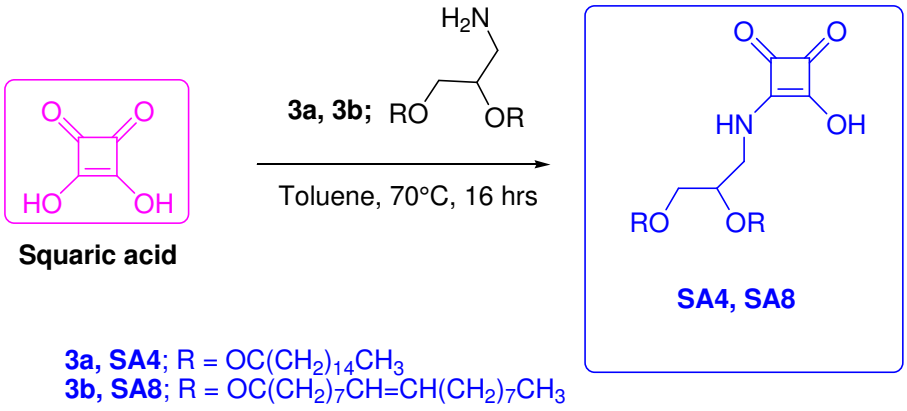
Synthesis of 3-((2-((2,3-dihydroxypropyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)propane-1,2-diyl dipalmitate (SA3): In an oven dried round bottom flask, compound

12 (1.0 equiv.**)**, and compound **3a (**1.1 equiv.**)**, were dissolved in 2.5 ml of ethanol. Then DIPEA (5.5 equiv.) was added to the reaction mixture and allowed to stir at room temperature for 12 hours. After completion of the reaction, the solvent was removed under reduced pressure. Then the solid residue was washed with diethyl ether and dichloromethane (1:1) for several times. After that the remaining solid residue was dried under reduced pressure and the target product was obtained. **Characterization of the compound SA3:** As a white solid with 55 % yield; ^{1}H NMR (600MHz, $CDCl_3$ + DMSO-*d*$_6$) δ_{ppm} 6.74 (br s, 1H), 4.92 (br s, 1H), 4.06-3.99 (m, 1H), 3.63-3.55 (m, 1H), 2.96-2.93 (m, 4H), 2.48-2.25 (m, 4H), 1.58-1.46 (m, 4H), 1.28-1.19 (m, 56H), 0.87-0.77 (m, 6H) ; ^{13}C NMR (151 MHz, $CDCl_3$ + DMSO-*d*$_6$) δ_{ppm} 197.4, 174.6, 174.0, 68.9, 65.9, 53.6, 42.8, 42.1, 36.6, 34.2, 32.0, 29.73, 29.69, 29.6, 29.5, 29.46, 29.39, 29.3, 29.2, 25.8, 25.0, 22.7, 18.3, 14.2; MS (ESI) calcd. for $C_{42}H_{76}N_2O_8$ $[M+H]^+$: 737.5635, found: 737.2932.

Synthesis of 3-((2-((2,3-dihydroxypropyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)propane-1,2-diyl (9E,9'E)-bis(octadec-9-enoate) (SA7): In an oven dried round bottom flask, compound **12 (**1.0 equiv. **)**, and compound **3b (**1.1 equiv.**)**, were dissolved in 2.5 ml of ethanol. Then DIPEA (5.5 equiv.) was added to the reaction mixture and allowed to stir at room temperature for 12 hours. After completion of the reaction, the solvent was removed under reduced pressure. Then the solid residue was washed with diethyl ether and dichloromethane (1:1) for several times. After that the remaining solid residue was dried under reduced pressure and the target product was obtained. **Characterization of the compound SA7:** As a white solid with 50% yield; ^{1}H NMR (600 MHz, $CDCl_3$ + MeOD-*d*$_4$) δ_{ppm} 7.50 (br, s, 1H), 5.28 (br, s, 1H), 5.04-5.00 (m, 4H), 4.24-4.22 (m, 1H), 4.02-4.01 (m, 1H), 3.66-3.64 (m, 2H), 3.51-3.49 (m, 1H), 3.35-3.33 (m, 1H), 3.10-3.08 (m, 2H), 2.24-2.21 (m, 4H), 1.54-1.51 (m, 4H), 1.32-1.30 (m, 8H),

1.25-1.17 (m, 44H), 0.80-0.78 (m, 6H); ^{13}C NMR (151 MHz, $CDCl_3$ + MeOD-d_4) δ_{ppm} 199.9, 173.6, 173.4, 136.2, 128.0, 127.4, 70.1, 66.3, 62.5, 53.8, 45.7, 42.0, 40.6, 33.8, 31.5, 29.3, 29.1, 28.9, 28.7, 24.5, 22.2, 18.4, 13.4; MS (ESI) calcd. for $C_{46}H_{80}N_2O_8$ $[M+4]^+$: 792.5915, found: 792.6092.

Scheme 4. Synthetic routes to compounds **SA4** and **SA8 —**

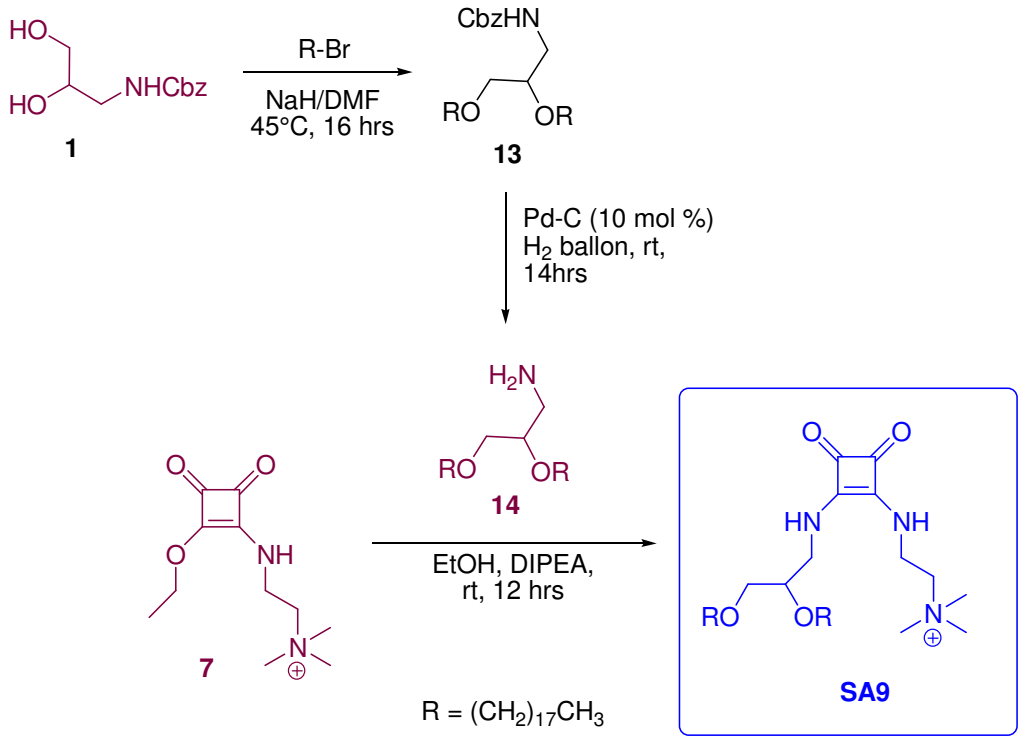


Synthesis of 3-((2-hydroxy-3,4-dioxocyclobut-1-en-1-yl) amino) propane-1,2-diyl dipalmitate (SA4): Squaric acid (0.40 mmol) and compound **3a** (0.44 mmol) were placed in an oven dried round bottom flask. Then, the reaction mixture was dissolved in 3.5 ml of toluene and allowed to reflux at 70 °C for 16 hrs. The progress of the reaction was monitored by thin layer chromatography. After completion of the reaction the organic solvent was removed under reduced pressure. The obtained white solid product was washed with diethyl ether and further column chromatography with silica gel and a gradient solvent system of 0–10% ethyl acetate to hexane yielded the target product. The isolated product was characterized by ^{1}H and ^{13}C NMR and LCMS analysis. **Characterization of the compound SA4:** white solid (75% yield); ^{1}H NMR (400 MHz DMSO-d_6) δ_{ppm} 3.92-3.87 (m, 2H), 3.71-3.68 (m, 1H), 3.19-3.07 (m, 2H), 2.25-

2.23 (m, 2H), 2.07-2.03 (m, 2H), 1.50-1.47 (m, 4H), 1.31-1.12 (m, 56H) 0.83-0.80 (m, 6H) ; ^{13}C NMR (100 MHz, $CDCl_3$ + MeOD-d_4) δ_{ppm} 194.4, 187.2, 180.5, 174.4, 167.3, 62.2, 52.3, 46.0, 45.9, 38.0, 34.2, 31.7, 29.7, 29.3, 29.1, 29.0, 24.8, 22.4, 20.8, 13.7; MS (ESI) calcd. for $C_{39}H_{69}NO_7$ $[M]^+$: 663.5074, found: 663.4414.

Synthesis of 3-((2-hydroxy-3,4-dioxocyclobut-1-en-1-yl)amino)propane-1,2-diyl (9E,9'E)-bis(octadec-9-enoate) (SA 8): Squaric acid (0.40 mmol) and compound **3b (**0.44 mmol**)** were placed in an oven dried round bottom flask. Then, the reaction mixture was dissolved in 3.5 ml of toluene and allowed to reflux at 70 °C for 16 hrs. The progress of the reaction was monitored by thin layer chromatography. After completion of the reaction the organic solvent was removed under reduced pressure. The obtained white solid product was washed with diethyl ether and further column chromatography with silica gel and a gradient solvent system of 0–10% ethyl acetate to hexane yielded the target product. The isolated product was characterized by ^{1}H and ^{13}C NMR and LCMS analysis. **Characterization of the compound SA8:** white solid (70% yield); ^{1}H NMR (600 MHz, $CDCl_3$) δ_{ppm} 5.37 (br s, 1H), 5.12-5.09 (m, 4H), 5.02-4.98 (m, 1H), 4.29-4.25 (m, 1H), 4.15-4.10 (m, 1H), 3.49-3.47 (m, 2H), 2.32-2.27 (m, 4H), 1.98-1.94 (m, 4H), 1.83-1.59 (m, 8H), 1.30-1.18 (m, 44H), 0.89-0.86 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_{ppm} 175.8, 175.5, 162.4, 117.9, 116.0, 71.3, 63.6, 63.0, 52.8, 45.8, 42.2, 41.0, 36.6, 32.0, 29.8, 29.7, 29.5, 29.4, 25.9, 22.8, 22.6, 14.2; MS (ESI) calcd. for $C_{43}H_{73}NO_7$ $[M^{+3}+NH_4]^+$: 736.5731, found: 736.4954.

Scheme 5. Synthetic routes to compound **SA9 —**



Synthesis of benzyl (2,3-bis (hexadecyloxy) propyl) carbamate (13) : To a solution of NaH (5 equiv.) in DMF, 1-bromo hexadecane (4 equiv.) was added and stirred for 30 min at room temperature under N_2 atmosphere. After that compound **1** (1 equiv.) was added to the reaction mixture and stirred for 16 hours at 45 °C. After completion of the reaction the reaction mixture was quenched with water and the compound was extracted with EtOAc (3 × 50 mL) and dried over anhydrous Na_2SO_4. The organic layer was concentrated under reduced pressure and column chromatography with silica gel and a gradient solvent system of 0–10% ethyl acetate to hexane yielded the corresponding ether. **Characterization of the compound 13:** colorless oil (yield, 65

%); ^{1}H NMR (600MHz, $CDCl_3$) δ_{ppm} 8.07 (br s, 1H), 7.36-7.26 (m, 5H), 4.52 (s, 2H), 4.19-4.15 (m, 1H), 4.08-4.05 (m, 1H), 4.00-3.96 (m, 1H), 3.50-3.46 (m, 6H), 1.69-1.59 (m, 4H), 1.37-1.27 (m, 52H), 0.92-0.88 (m, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) δ_{ppm} 138.7, 128.3 127.6, 127.5, 74.2, 72.9, 70.5, 32.0, 29.8, 29.7, 29.7, 29.7, 29.5, 29.4, 26.2, 22.7, 14.2; HRMS (ESI) calcd. for $C_{43}H_{79}NO_4$ $[M + H]^+$: 673.6009, found: 673.6009.

Synthesis of 2,3-bis(hexadecyloxy)propan-1-amine (14) : 2,3-bis(hexadecyloxy)propan-1-amine was synthesized by the hydrogenation of compound **13** in the presence of 10 mol % Pd/charcoal (stirred for 14 hours at room temperature) according to the literature procedure and used without further purification.

Synthesis of 2-((2-((2,3-bis(hexadecyloxy)propyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)-N,N,N-trimethylethanaminium (SA9): In an oven dried round bottom flask 1.0 equiv. of compound **7,** 1.1 equiv. of compound **14** were dissolve in 3.5 ml of ethanol. Then, 5.5 equiv. of anhydrous DIPEA was added to the reaction mixture and the reaction mixture was allowed to stir at room temperature for 12 hrs. After completion of the reaction, the solvent was removed under reduced pressure. The reaction mixture was then washed for several times with a 1:1 mixture of diethyl ether and dichloromethane solvents. Then the left over solid residue was dried under reduced pressure and characterized by ^{1}H and ^{13}C NMR and LCMS analysis. The product yield was 65 % for this reaction. **Characterization of the compound SA9:** white solid (yield, 65 %); ^{1}H NMR (400 MHz, $CDCl_3$ + MeOD-*d*$_4$) δ_{ppm} 6.25 (br s, 1H), 3.65-3.62 (m, 1H), 3.35-3.38 (m, 2H), 2.93-2.75 (m, 6H), 1.01-0.97 (m, 78H), 0.93-0.88 (m, 6H); ^{13}C NMR (151 MHz, $CDCl_3$ + MeOD-*d*$_4$) δ_{ppm} 199.3, 65.8, 54.3, 51.9, 46.3, 42.4, 37.4, 32.3, 31.7, 29.4, 29.4,

29.4, 29.3, 29.1, 25.6, 22.4, 11.9; MS (ESI) calcd. for $C_{44}H_{86}N_3O_4$ $[M+3]^+$: 723.6613, found: 723.3215.

Liposome preparation

Liposome was prepared by thin film hydration method in 25 mM HEPES buffer, pH 7.2, containing 100 mM KCl. First 40-50 μL solution of the lipid was taken from 10 mM lipid stock and dried for 3h to prepare the lipid film. Then 500 μL of buffer was added into the lipid film and heated to 60-70 °C for 10 min (final concentration of the liposome ~1 mM). After that the solution was vortexed well until the dry lipid film was disappeared. Finally, the solution was sonicated for 10 times (30 s of sonication followed by 30 s of cooling on ice). A hand-held mini-extruder (Avanti Polar Lipids, Alabaster, AL) with polycarbonate membrane (diameter of 200 nm) was used to prepare small unilamellar vesicles (at room temperature) whenever required.[2] We observed that hydration of the dried film of the SA lipids over 60-70 °C only resulted in the formation of bilayer membranes.

Transmission electron microscopy

For transmission electron microscope (TEM) imaging liposome was prepared by the above mention method in 25 mM HEPES buffer, pH 7.2, containing 100 mM KCl (no extrusion method was used). Identity of the unsaturated lipids under this experimental condition was confirmed by ^{1}H NMR spectroscopy (spectra not shown here). First, 10 μL solution of liposome was placed onto a carbon-coated copper grid and allowed to absorb for 1 minute. Then the grid was carefully blotted with filter paper. After that uranyl acetate (1% solution in water) was added on the grid and allowed to wait for another 1 minute. The excess uranyl acetate solution was then

removed and the grid was washed with water and excess water was removed, and the grid was allowed to dry at 37 °C for overnight. The images of the liposomes formed on the carbon-coated copper grid were collected using a JEOL JEM 2100 transmission electron microscope (operated at a maximum accelerating voltage of 200 kV).[2]

Anisotropy measurements

Temperature dependent steady-state anisotropy measurement was used to determine the gel to liquid-disordered phase transition temperature (T_m) values of the lipid bilayers. Environment sensitive fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was used for this study. For liposome preparation, first 35-50 μL of the lipid from its stock solution (15 mM solution in chloroform) was added to a 3 mL sample vial and dried for 3h to prepare the lipid film.[6] Then 800 μL of 25 mM HEPES buffer, pH 7.2, containing 100 mM KCl was added into the lipid film and heated to 60-70 °C for 10 min (final concentration of the liposome 2 mM). After that the solution was vortexed well until the dry lipid film was disappeared. Finally, the solution was sonicated for 10 times (30 s of sonication followed by 30 s of cooling on ice). A hand-held mini-extruder with polycarbonate membrane (diameter of 200 nm) was used to prepare large unilamellar vesicles (at room temperature). Then 7 μL of 1 mM DPH solution in THF was added to the extruded vesicles final concentration of DPH ~ 8.5 μM was maintained < 1% v/v]. This Liposomal solution was kept under shaking or tumbling condition at room temperature for overnight for maximum incorporation of the DPH into the membrane. Steady-state anisotropy measurements were performed on a fluoromax-4 spectrofluorometer (Horiba Scientific, Singapore) with a refrigerated system for temperature control. The temperature was regulated using a peltier temperature controller (without removing the cuvette throughout the experiment).

All anisotropy values of the DPH probe are the mean values of three individual determinations. The degree (r) of anisotropy in the DPH fluorescence (λ_{ex} = 350 nm; λ_{em} = 429 nm) was calculated using eq 1 at the peak of the fluorescence spectrum, where I_{VV} and I_{VH} are the fluorescence intensities of the emitted light polarized parallel and perpendicular to the excited light, respectively, and $G = I_{VH}/I_{HH}$ is the instrumental grating factor.

$$r = \frac{(I_{VV} - GI_{VH})}{(I_{VV} + 2GI_{VH})} \quad (1)$$

Anisotropy values were collected at every 3-4 °C temperature differences, apart from near to the T_m value of the lipid; in that case the anisotropy values were collected at every 1 or 2 °C. Plot of degree (r) of anisotropy values of the DPH probe as a function of temperature was used to determine the T_m value of the lipid. The stability of lipid was examined by TLC measurements before and after the anisotropy measurements. However, the T_m values of SA5-8 lipids with dioleic-alkyl chains could not be measured because of the lowest temperature setup for our steady-state fluorimeter was 5 °C. The anisotropy values decreased continuously for these lipids within the range of 5-90 °C, without any sharp change in the anisotropy value within this temperature range (data not shown here).

Microviscosity measurements

Microviscosity of DPH molecule (a polarity probe) in the vicinity of lipid headgroup was calculated from steady state fluorescence anisotropy and fluorescence lifetimes measurements. The microviscosity, η can be calculated using Perrin's equation.[7, 8]

$$\frac{r_0}{r_s} = 1 + C(r)\frac{T}{\eta}\,\tau \quad (1)$$

Where r_0 is the limiting steady state fluorescence anisotropy of DPH in the absence of any depolarizing methods such as diffusion or energy transfer, r_{ss} is the observed steady state fluorescence anisotropy of DPH at any temperature, T is the temperature in Kelvin, τ is the fluorescence lifetime of DPH in seconds and C(r) is a molecular shape parameter having a precalibrated value for each value of r. C(r) is also related with the location of the transition dipoles of the rotating DPH molecule. C(r) takes care of the non-spherical nature of the molecule. C(r) = k/v; where k is Boltzman constant (1.38×10^{-23} m^2 kg s^{-2} K^{-1}) and v is the volume (m^3) of the DPH molecule. Obtaining a precise value of C(r) in the heterogeneous microenvironments (such as gels, micelles, emulsions and others) is often intricate for fluorescent molecules including DPH. Hence equation (1) can also be written as-

$$\eta = \frac{r_{ss} C_{(r)T\tau}}{\delta r}; \delta r = r_0 - r_{ss} \quad (2)$$

$$\eta = \frac{r_{ss} k\tau}{\delta r} \quad ; k = C(r)T \quad (3)$$

$$\eta \propto \frac{r_{ss}\tau}{\delta r} \quad (4)$$

The term $\frac{r_{ss}\tau}{\delta r}$ is called the microviscosity parameter (τ_η).[7, 8] This τ_η also reflects the microviscosity of the environment around the fluorescent molecule. The measurements of τ_η values are advantageous since precise and difficult measurement of C(r) is crucial for the exact value of η. In this regards, we calculated the τ_η values of the DPH molecules under the liposomal environment at different temperatures. The limiting anisotropy (r_o) of DPH is 0.362.[9] Liposomes were prepared according to the mentioned procedure. DPH solution (14 µL of 1 mM solution) in THF was added to the extruded vesicles final concentration of DPH ~ 8.5 µM was maintained.

Temperature was varied from 5 to 90 °C. Microviscosity parameter, ($\tau_\eta = \frac{r_{ss}\tau}{\delta r}$) was used to compare the microviscosities of DPH molecule through the liposome of **SA1**, **SA9** and DPPC lipids.

pH dependent zeta potential measurements

For zeta potential measurements liposomes were prepared according to the previous method in 5 mM Tris buffer, pH 8.6, containing 5 mM NaCl. The liposomes were then extruded through a polycarbonate membrane (200 nm) using a handheld mini-extruder. Isosmotic buffers consisting of 10 mM buffering agent and 10 mM salt at different pH values were newly prepared and used for the measurements. Depending on the pK_a values of the buffering agent buffer solutions at different pH were prepared. Citric acid and trisodium-citrate was used for pH 3.0–6.5; 3-(N-morpholino)propanesulfonic acid (MOPS) was used for pH 7.0, tris(hydroxymethyl)aminomethane (Tris)–HCl was used for pH 7.5–8.5. Zeta potential measurements of the liposomes in buffer at different pH were carried out using a Zetasizer Nano ZS90 (Malvern, Westborough, MA) instrument. Liposomal solution was diluted into 1 mL of isosmotic buffer (final lipid concentration was 150 nM) in a disposable capillary cell (DTS1061) for zeta potential measurements. All the measurements were performed three times per sample.[2]

Calcium-induced zeta potential measurements

Liposome preparation used in the pH dependent zeta potential measurements were also used for the calcium-induced zeta potential measurements in 20 mM of 2-(N-morpholino)-ethanesulfonic acid (MES) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.2. Liposomal solution was added into 1 mL of 10 mM HEPES-MES buffer, pH 7.2 (final lipid

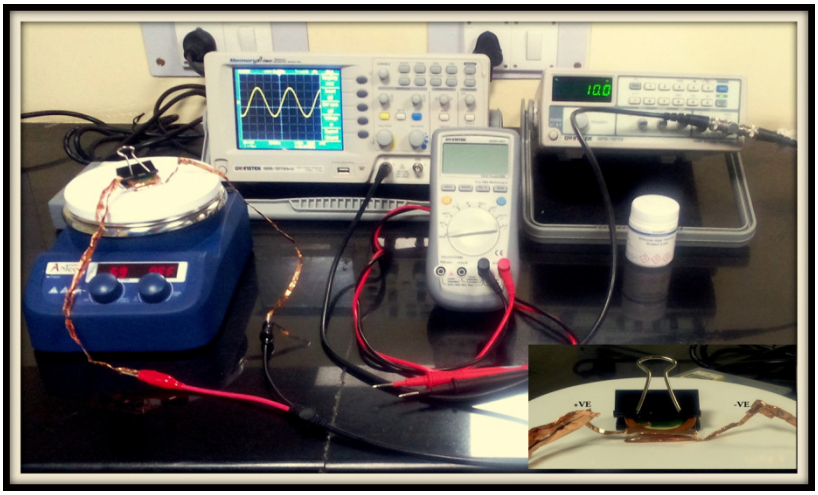
concentration was 100 nM) with various concentration of $CaCl_2$ and NaCl. NaCl was added along with $CaCl_2$ to maintain a constant ionic strength across all Ca^{2+} ion concentrations. Ionic strengths of the solutions were calculated according to the Debye–Huckel model, where the ionic strength (I) = $0.5(4[Ca^{2+}] + [Cl^-] + [Na^+])$. Zeta potential measurements of liposomes were performed using a Zetasizer Nano ZS90 (Malvern, Westborough, MA) instrument.[2, 10]

Vesicle leakage study

Liposomes were prepared according to the previous method (without passing through the extruder) in 10 mM HEPES buffer, pH 7.2, in the presence of 2 mM 5(6)-carboxyfluorescein (CF) or 4 mM rhodamine 6G (R6G).[2, 10] Free CF or R6G was removed by size exclusion chromatography on a PD-10 sephadex column (Sigma, St. Louis, MO) by eluting with 10 mM HEPES buffer, pH 7.2, containing 92 mM NaCl and 8 mM NaN_3. Then, purified liposome solution was added into 1 mL of the elution buffer (final lipid concentration was 1.7 mM). The release of CF and R6G was measured by monitoring the emission signals at 516 nm (λ_{ex} = 485 nm) and 554 nm (λ_{ex} = 530 nm), respectively. Steady state fluorescence measurements were performed using Fluoromax-4 spectrofluorometer (Horiba Scientific, Singapore) at room temperature. Finally liposomes were lysed using TRITON X-100 surfactant (final concentration was 2 mM) to measure the total fluorescence of CF/R6G. Percent release was calculated using the following relation: percentage release at time, (t) = (measured fluorescence at time, (t)) / (total fluorescence from lysed liposomes) × 100. All measurements were performed at 37 °C.

Preparation of giant unilamellar vesicles (GUVs) by electroformation method

Giant unilamellar vesicles (GUVs) were prepared using a home-made electroformation unit on indium tin oxide coated (ITO) glass slide (surface resistivity 70-100 Ω/sq).[11, 12] For the preparation of GUV of the lipids, chloroform/methanol (8:2) stock solutions containing 1.0 mM of the lipid (i.e., SA1 and SA9) were prepared. Then, 10 µL of 2 mM 8-hydroxypyrene-1,3,6-



trisulfonic acid (HPTS) in methanol solution was added into 50 µL of chloroform-methanol stock solutions of the lipids and mixed thoroughly. After that 10 µL of this lipid mixture was spread in a snakelike pattern without overlap over a 2.5 × 2.5 cm^2 area using a 10 µL Hamilton syringe (on the conductive surface of both the ITO-coated glass plates). Following the deposition of the lipid film onto the ITO-coated glass, the solvent was evaporated by passing a stream of nitrogen gas over the glass for 2 hours and then the electroformation chamber was assembled. Using two ITO-coated plates (both ITO-coated surfaces were facing each other) the chamber was constructed and the plates were connected using copper tapes. The ITO coated plates were separated by a silicone 'O'-ring (20 mm × 3 mm). The resulting electroformation chamber was then slowly filled with 500 µL of 25 mM HEPES buffer, pH 7.2 containing 100 mM glucose. Silicone high vacuum grease was used to seal the chamber. Both ITO-coated cover-slips were then connected with copper tapes and a sinusoidal AC electric field of 10 Hz and 2.0 V (rms) was then applied to

the system. This electroformation process was continued for 12h to generate GUVs at temperature greater than that of the phase transition temperature (T_m) of the lipid using a temperature regulated hotplate (60-70 °C for both the lipids). For membrane labeling, 0.1 µL of 100 µM Texas Red DHPE (Invitrogen, CA) solution in EtOH was added to 10 µL of GUV solution prior to microscope observation. Finally, the GUV solution was spread on a glass slide and covered with a 17 mm cover slip and fluorescence microscopic images were collected using Olympus BX51y fluorescence microscope. In general, large amounts of the GUVs with a diameter > 10 µm was obtained under this condition. The GUVs were prepared around 60-70 °C because of relatively high T_m value of the **SA1** and **SA9** lipid. The efficiency of GUV preparations at lower temperature was extremely poor.

HPLC Analysis of SA lipids stability against PLA_2 and PLC enzymes

2 µg of lipid (DPPC or **SA1** or **SA9**) was taken in a glass vial and dried for 2 hours under continuous nitrogen gas flow. Dried lipid was then dissolve with 50 µL 100 mM Tris-base at pH 8.5 containing 10 mM $CaCl_2$ buffer solution which was vortexed for 1 min and sonicated (for 3 times interval of 1min).[6, 13] For PLA_2 activity assay 1 Unit of bovine pancreatic PLA_2 enzyme was added to the liposome containing buffer solution (50 µL) and the reaction mixture incubated at 37 ºC for overnight. For PLC activity assay 0.2 unit of PLC enzyme (from Clostridium Perfringens) was added to the liposome containing buffer solution (50 µL) and the reaction incubated at 25 ºC for 2 hours. After that the reaction mixtures were diluted with methanol and subject to HPLC/LCMS analyses. UV signals were simultaneously monitored to confirm the identity of the compounds. HRMS analyses of the peaks were also performed for further characterization of the compounds. HPLC analysis was carried out on a Thermo-Fisher C18

analytical column in Thermo-Fisher UHPLC ultimate 3000 machine. Both **SA1** and **SA9** lipids have a strong absorption peak at 270 nm. In this regard HPLC analysis was performed using an UV-detector at 270 nm. Because of the unavailability of the ELSD detector hydrolysis of DPPC lipid by PLA_2 and PLC enzymes were investigated by TLC and LCMS analyses.

HPLC gradient： 0 min – 2 min 50% Phase A in Phase B to 5% Phase A in Phase B, 2 min – 12 min, 5% Phase A in Phase B, 12 min – 14 min, 5% Phase A in Phase B to 50% Phase A in Phase B. (Phase A: H_2O with 0.1% formic acid, Phase B: MeOH with 0.1% formic acid).[6]

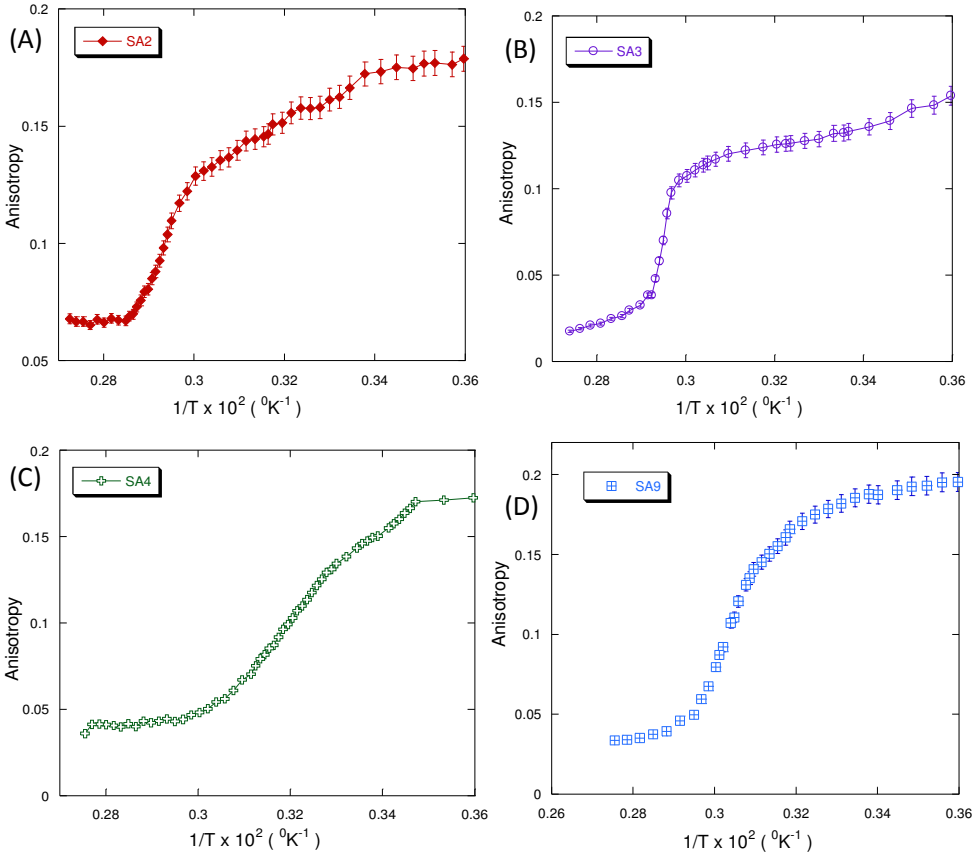


Fig. S1 (A-D) Anisotropic curve of **SA2**, **SA3**, **SA4** and **SA9** lipids within the temperature range from 5 to 90 °C.

Table S1. Temperature dependent fluorescence lifetime data of DPH in the liposomal solution and their microviscosity parameter values.

Lipid	Temperature (K)	τ_1 (ns) (α1)	τ_2 (ns) (α2)	τ_3 (ns) (α3)	τ_{avg} (ns)	χ^2	r_{ss}	δr	Microviscosity parameter (τ_η) (ns)
DPPC	288	4.2 (0.08)	9.6 (0.92)	-	9.21	1.029	0.33910	0.02290	136.4396
	298	4.2 (0.07)	9.8 (0.93)	-	9.44	1.007	0.33615	0.02585	122.7565
	303	4.8 (0.09)	10.0 (0.91)	-	9.51	1.039	0.32664	0.03536	87.84916
	313	5.0 (0.07)	10.1 (0.93)	-	9.71	1.019	0.17410	0.18790	8.996865
	318	3.2 (0.06)	7.6 (0.94)	-	7.34	0.995	0.09897	0.26302	2.761929
	323	2.4 (0.09)	6.9 (0,91)	-	6.50	1.003	0.08510	0.27689	1.997867
	338	2.5 (0.13)	5.2 (0.87)	-	4.86	1.010	0.05916	0.30283	0.949481
	348	2.5 (0.30)	4.2 (0.70)	-	3.70	1.001	0.04339	0.31860	0.503952
	358	2.2 (0.32)	3.6 (0.68)	-	3.15	1.008	0.03893	0.32306	0.379608
SA1	288	1.4 (0.47)	5.5 (0.53)	-	3.57	1.005	0.26553	0.09647	9.826289
	298	1.4 (0.49)	5.7 (0.51)	-	3.60	1.006	0.25298	0.10902	8.353770
	308	1.6 (0.48)	5.8 (0.52)	-	3.78	1.014	0.24194	0.12006	7.617301
	318	1.6 (0.50)	6.0 (0.50)	-	3.80	1.019	0.23060	0.13140	6.668798
	323	1.3 (0.40)	5.1 (0.60)	-	3.58	1.032	0.14235	0.21965	2.320114
	328	1.4 (0.42)	4.9 (0.58)	-	3.43	1.015	0.08418	0.27782	1.039297
	333	1.3 (0.32)	4.5 (0.68)	-	3.48	0.995	0.04768	0.31432	0.527890
	338	1.2 (0.30)	4.2 (0.70)	-	3.30	1.032	0.04499	0.31701	0.468335
	343	1.1 (0.25)	3.7 (0.75)	-	3.04	0.981	0.04111	0.32089	0.389462
	348	0.9 (0.20)	3.5 (0.80)	-	2.97	1.030	0.03756	0.32444	0.343833
	358	0.9 (0.20)	3.0 (0.80)	-	2.58	1.000	0.03276	0.32924	0.256715
SA9	288	0.7 (0.80)	3.3 (0.20)	-	1.22	1.006	0.19252	0.16948	1.385853
	298	0.7 (0.80)	3.8 (0.20)	-	1.32	1.072	0.18540	0.17660	1.385776
	308	0.7 (0.80)	4.3 (0.20)	-	1.43	0.991	0.17490	0.18710	1.336756
	318	0.7 (0.76)	4.4 (0.24)	-	1.60	0.999	0.15514	0.20686	1.199961
	323	0.8 (0.77)	4.5 (0.23)	-	1.61	0.989	0.14067	0.22133	1.023263
	328	0.8 (0.44)	2.8 (0.36)	0.1 (0.20)	1.36	1.006	0.11056	0.25144	0.598002
	338	0.7 (0.44)	2.4 (0.36)	0.1 (0.20)	1.21	1.107	0.05940	0.30260	0.236933
	348	0.7 (0.50)	2.3 (0.25)	0.1 (0.25)	1.03	1.095	0.03925	0.32275	0.125259
	358	0.7 (0.55)	2.6 (0.20)	0.1 (0.25)	0.91	1.019	0.03401	0.32799	0.094049

All experiments were carried out in 25 mM HEPES buffer, pH 7.2, containing 100 mM KCl. r_0 of DPH = 0.362.

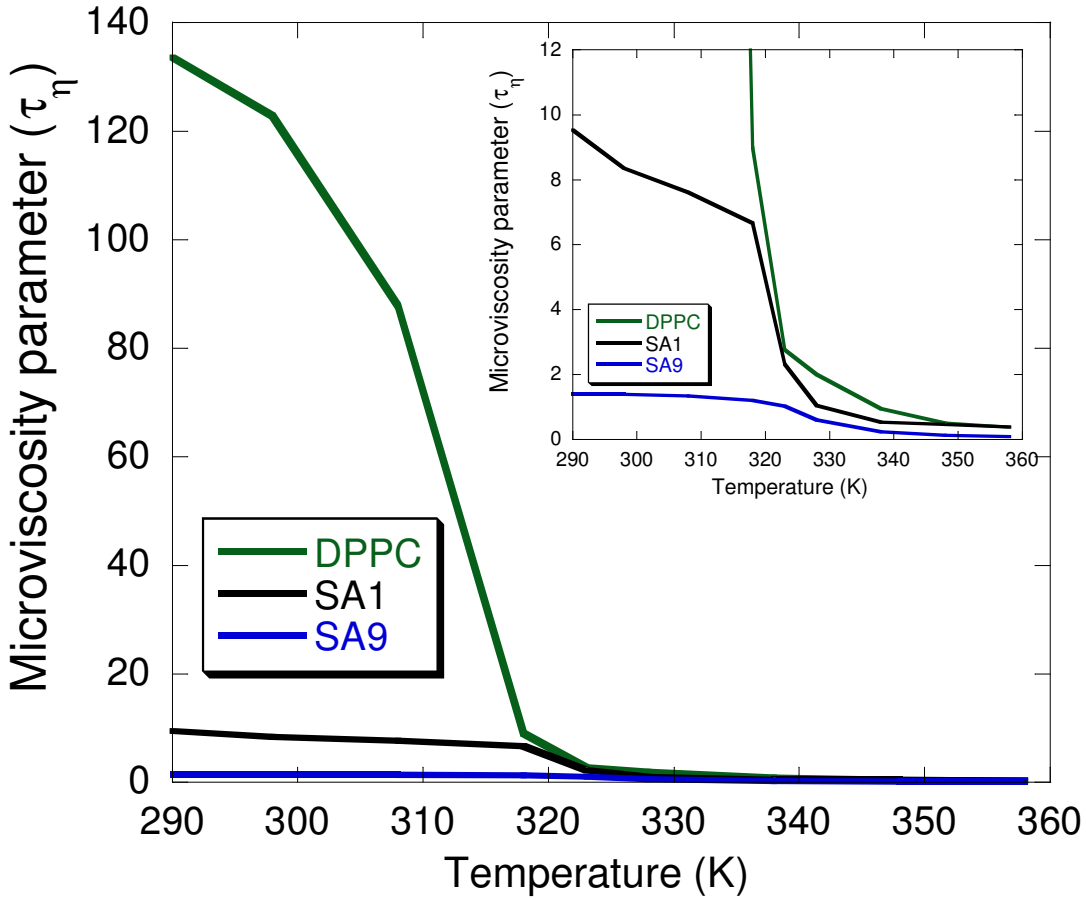


Fig. S2: Microviscosity parameter (τ_η) of DPH under the liposomal environment at different temperatures. The DPH concentration for all the experiment sets was ~ 8.5 μM. All experiments were carried out in 25 mM HEPES buffer, pH 7.2, containing 100 mM KCl. r_0 of DPH = 0.362.

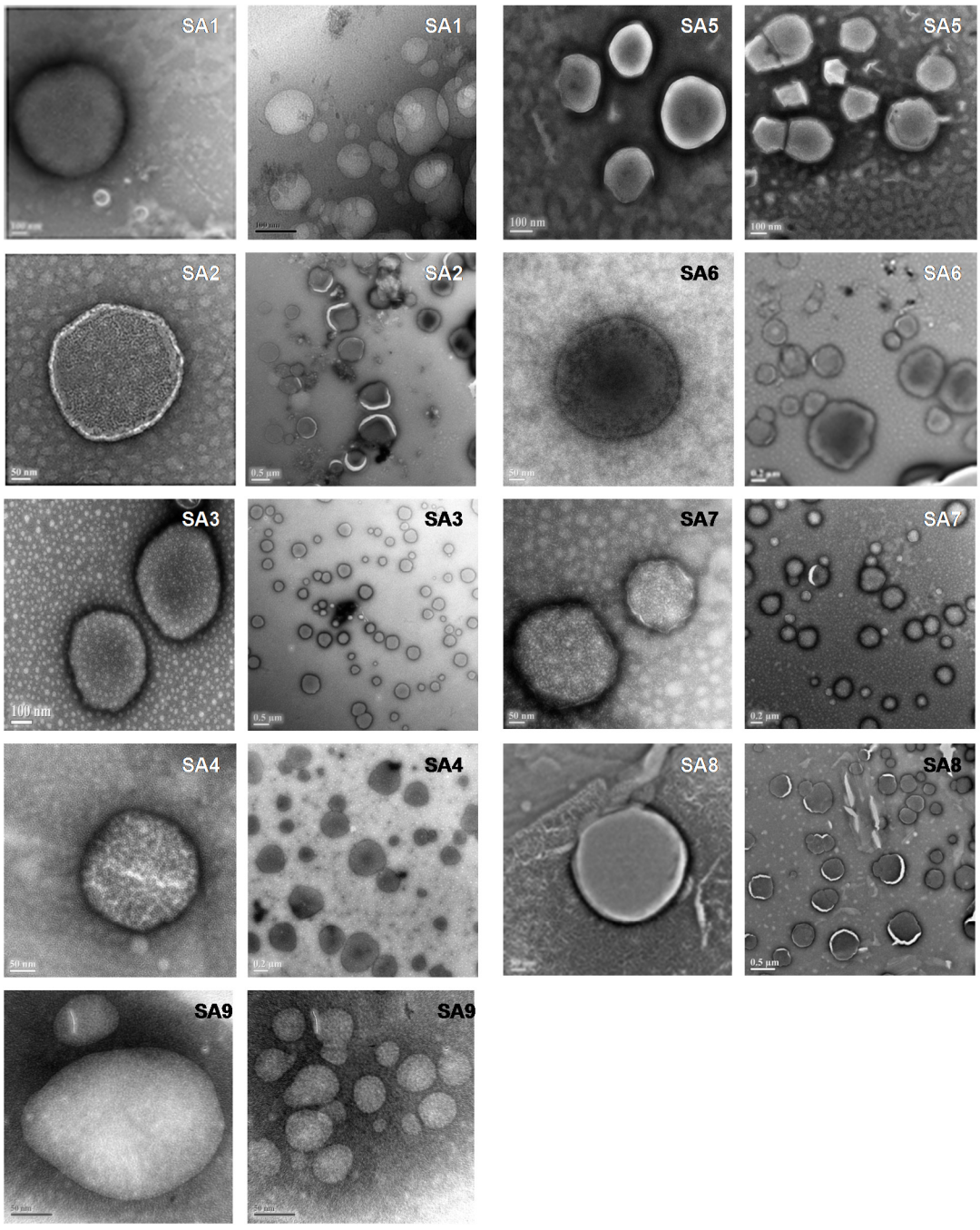
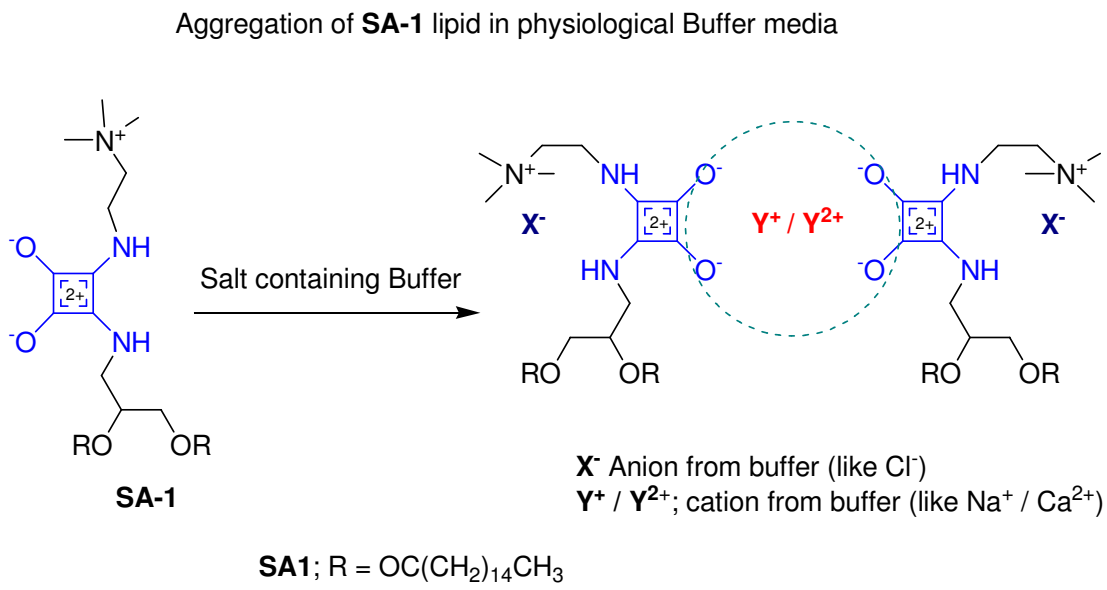


Fig. S3 TEM image of the liposomes generated from 100% SA lipids.



SA1; R = $OC(CH_2)_{14}CH_3$

Fig. S4 Schematic representation for the proposed partial aggregation mechanism of **SA1** lipid in salt containing buffer. A similar mechanism can be proposed for **SA5** and **SA9** lipids under the similar experimental conditions.

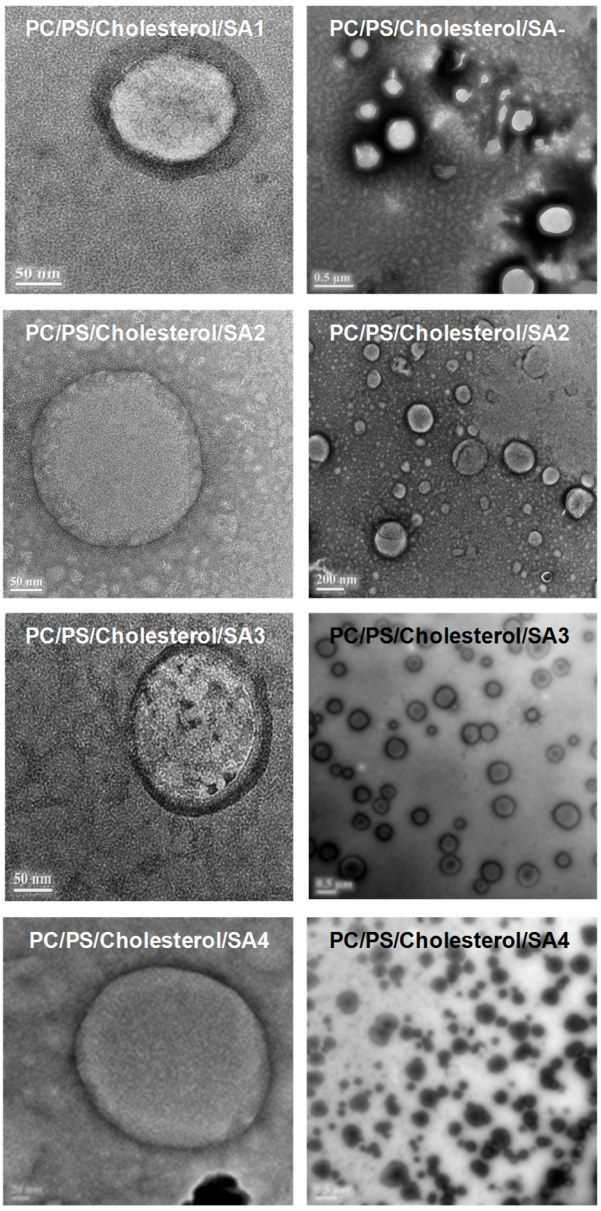


Fig. S5 TEM image of the liposomes generated from the lipid mixtures of SA lipids/DPPC/DPPS/cholesterol (in the molar ratio of 2:4:2:2).

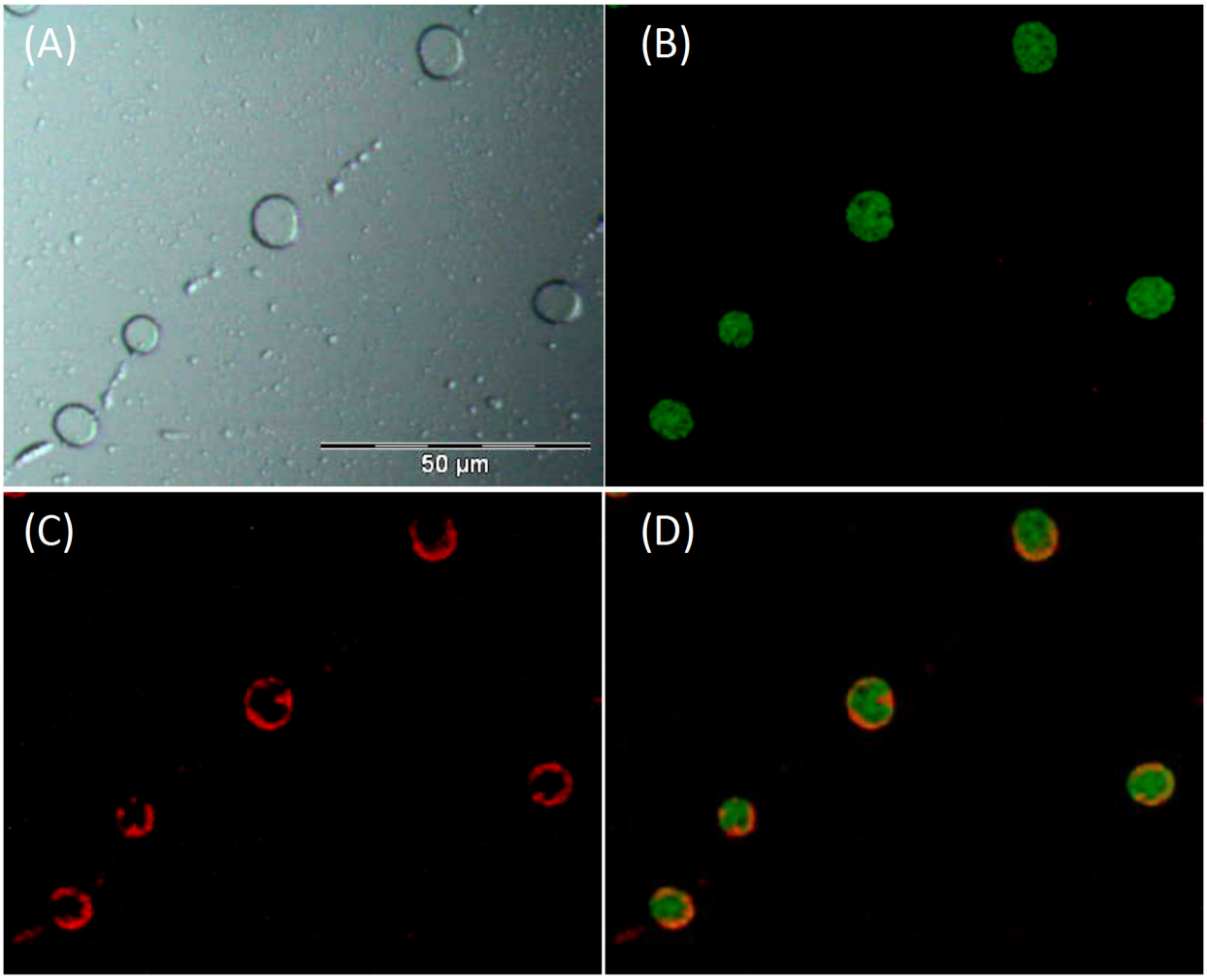
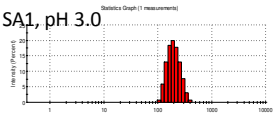
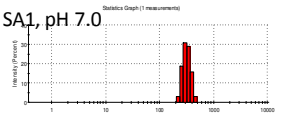


Fig. S6 Fluorescence microscopic images of GUVs generated from pure **SA9** lipid. (A) Bright field. (B) Green channel illustrating HPTS encapsulation. (C) Red channel illustrating the staining of the lipid membrane with Texas Red DHPE. (D) Merge of Green and Red channels.

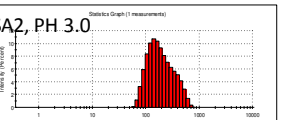
SA1, pH 3.0



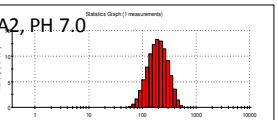
SA1, pH 7.0



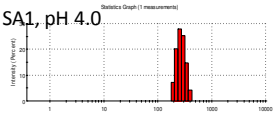
SA2, PH 3.0



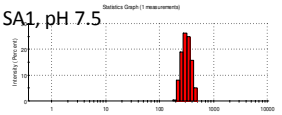
SA2, PH 7.0



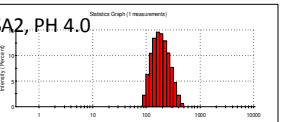
SA1, pH 4.0



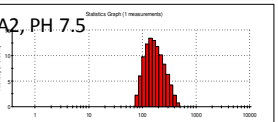
SA1, pH 7.5



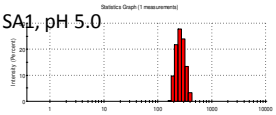
SA2, PH 4.0



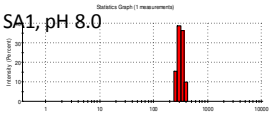
SA2, PH 7.5



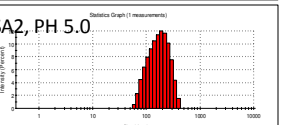
SA1, pH 5.0



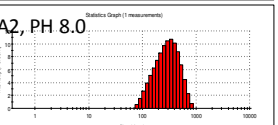
SA1, pH 8.0



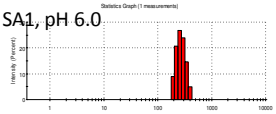
SA2, PH 5.0



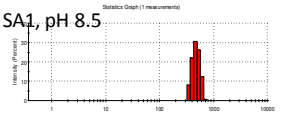
SA2, PH 8.0



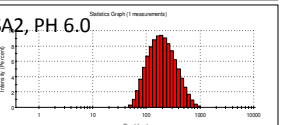
SA1, pH 6.0



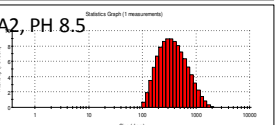
SA1, pH 8.5



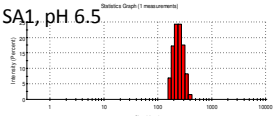
SA2, PH 6.0



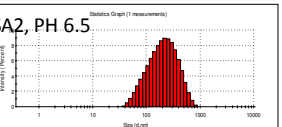
SA2, PH 8.5



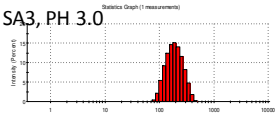
SA1, pH 6.5



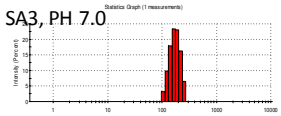
SA2, PH 6.5



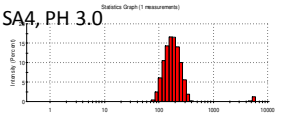
SA3, PH 3.0



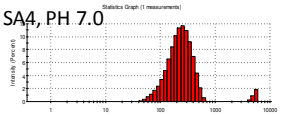
SA3, PH 7.0



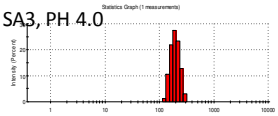
SA4, PH 3.0



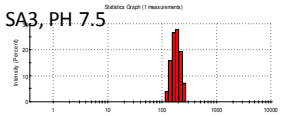
SA4, PH 7.0



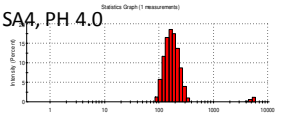
SA3, PH 4.0



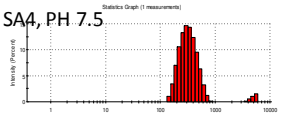
SA3, PH 7.5



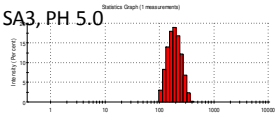
SA4, PH 4.0



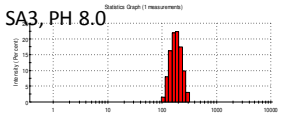
SA4, PH 7.5



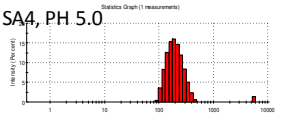
SA3, PH 5.0



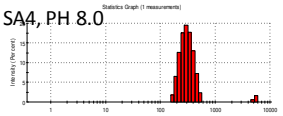
SA3, PH 8.0



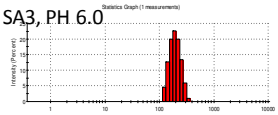
SA4, PH 5.0



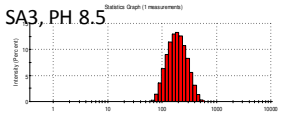
SA4, PH 8.0



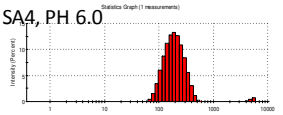
SA3, PH 6.0



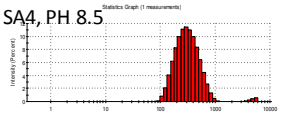
SA3, PH 8.5



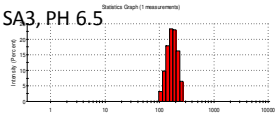
SA4, PH 6.0



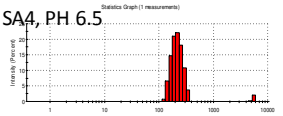
SA4, PH 8.5



SA3, PH 6.5



SA4, PH 6.5



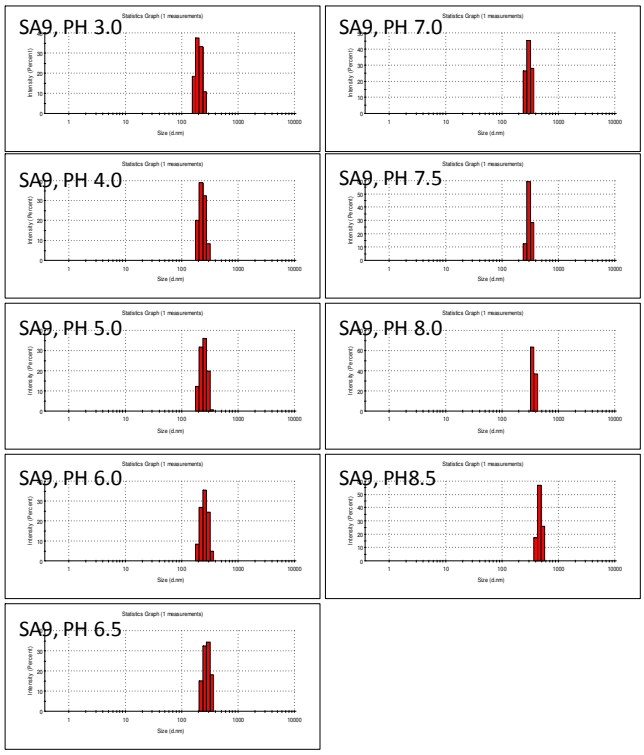
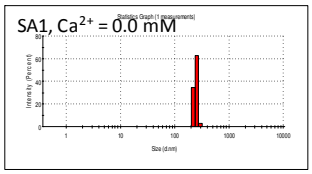
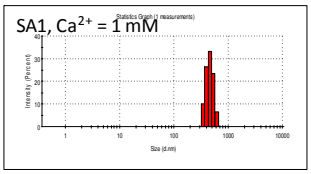
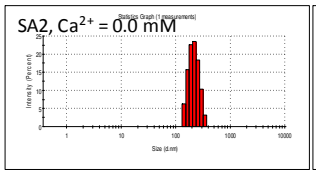
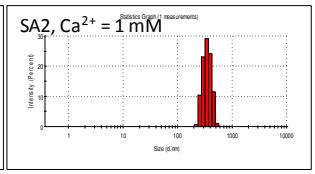


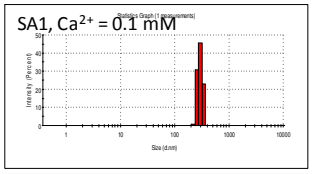
Fig. S7 Dynamic light scattering (DLS) measurements. Size distribution of extruded liposomes (200 nm) formed from **SA1-4** and **SA9** lipids measured at 25 °C. Content is given in percentage distribution of different sized liposome at different pH.

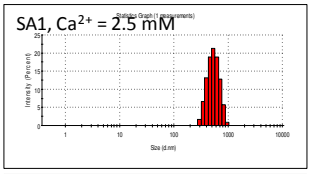

SA1, Ca2+ = 0.0 mM

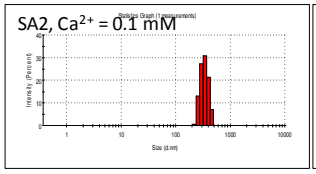

SA1, Ca2+ = 1 mM

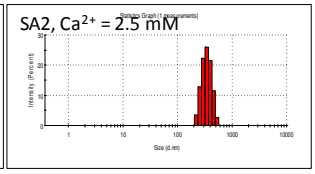

SA2, Ca2+ = 0.0 mM

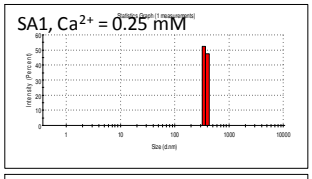

SA2, Ca2+ = 1 mM

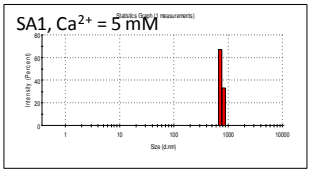

SA1, Ca2+ = 0.1 mM

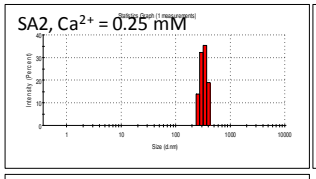

SA1, Ca2+ = 2.5 mM

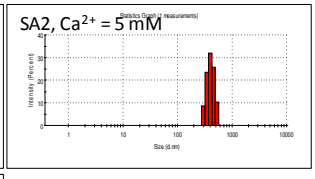

SA2, Ca2+ = 0.1 mM

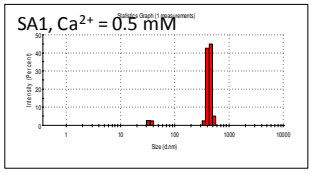

SA2, Ca2+ = 2.5 mM

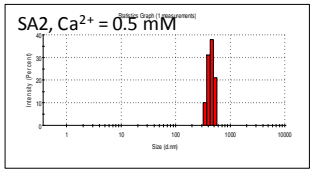

SA1, Ca2+ = 0.25 mM

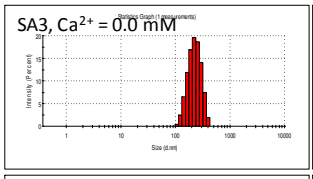

SA1, Ca2+ = 5 mM


SA2, Ca2+ = 0.25 mM


SA2, Ca2+ = 5 mM


SA1, Ca2+ = 0.5 mM


SA2, Ca2+ = 0.5 mM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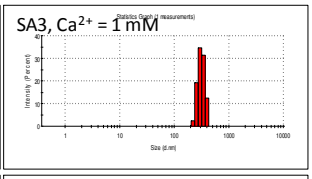
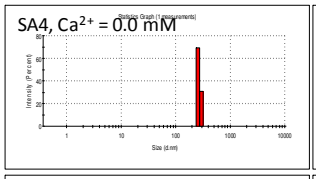
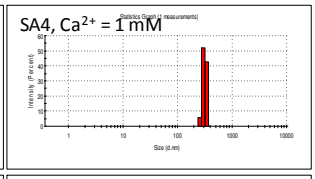
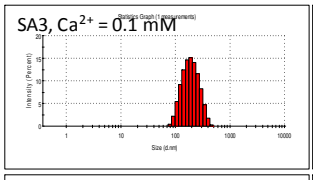
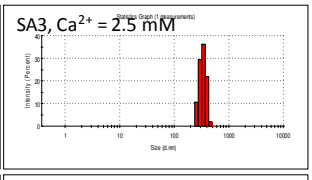
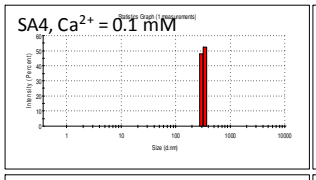

SA3, Ca2+ = 1 mM

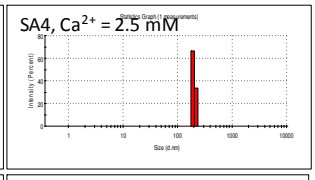

SA4, Ca2+ = 0.0 mM

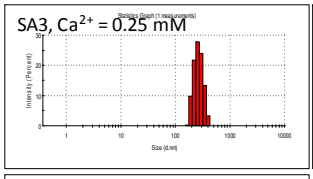

SA4, Ca2+ = 1 mM

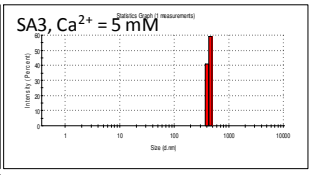

SA3, Ca2+ = 0.1 mM

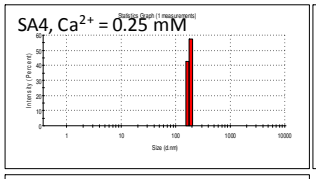

SA3, Ca2+ = 2.5 mM

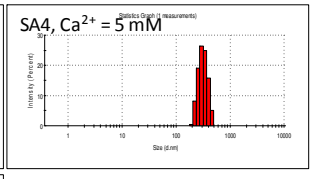

SA4, Ca2+ = 0.1 mM

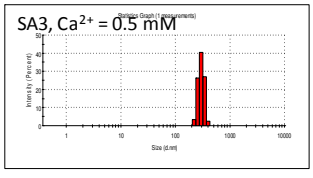

SA4, Ca2+ = 2.5 mM

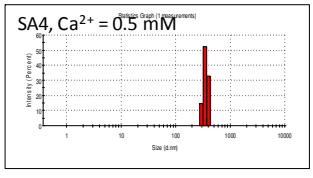

SA3, Ca2+ = 0.25 mM


SA3, Ca2+ = 5 mM


SA4, Ca2+ = 0.25 mM


SA4, Ca2+ = 5 mM


SA3, Ca2+ = 0.5 mM


SA4, Ca2+ = 0.5 mM

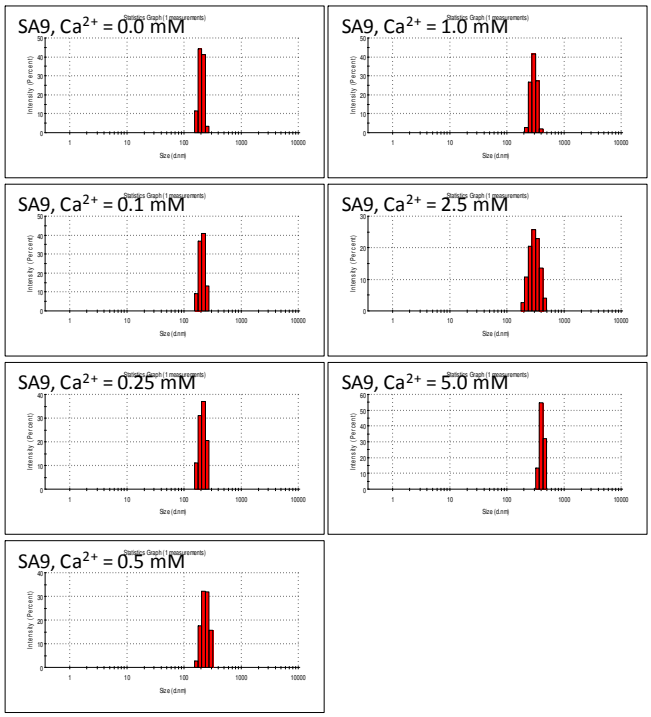


Fig. S8 Dynamic light scattering (DLS) measurements. Size distribution of extruded liposomes (200 nm) formed from **SA1-4** and **SA9** lipids measured at 25 °C. Content is given in percentage distribution of different sized liposomes at pH 7.2 in the presence of different concentration of Ca^{2+} ion.

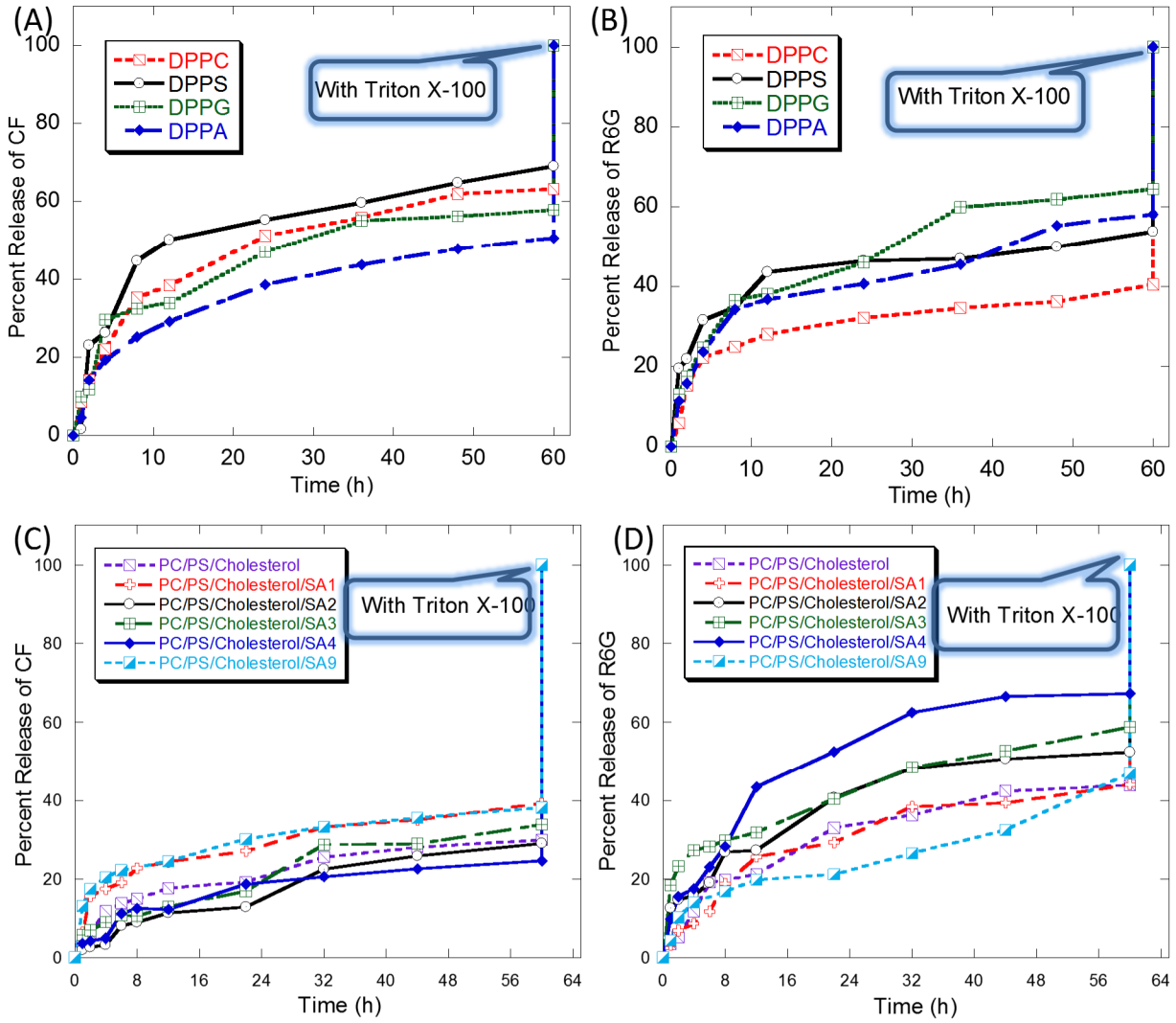


Fig S9 (A) CF and (B) R6G release profiles from the liposomes of 100 % phospholipids. (C) CF and (D) R6G release profiles from the liposomes of mixed lipids DPPC/DPPS/cholesterol/**SA1-4** and **SA9** (in the molar ratio of 2:4:2:2).

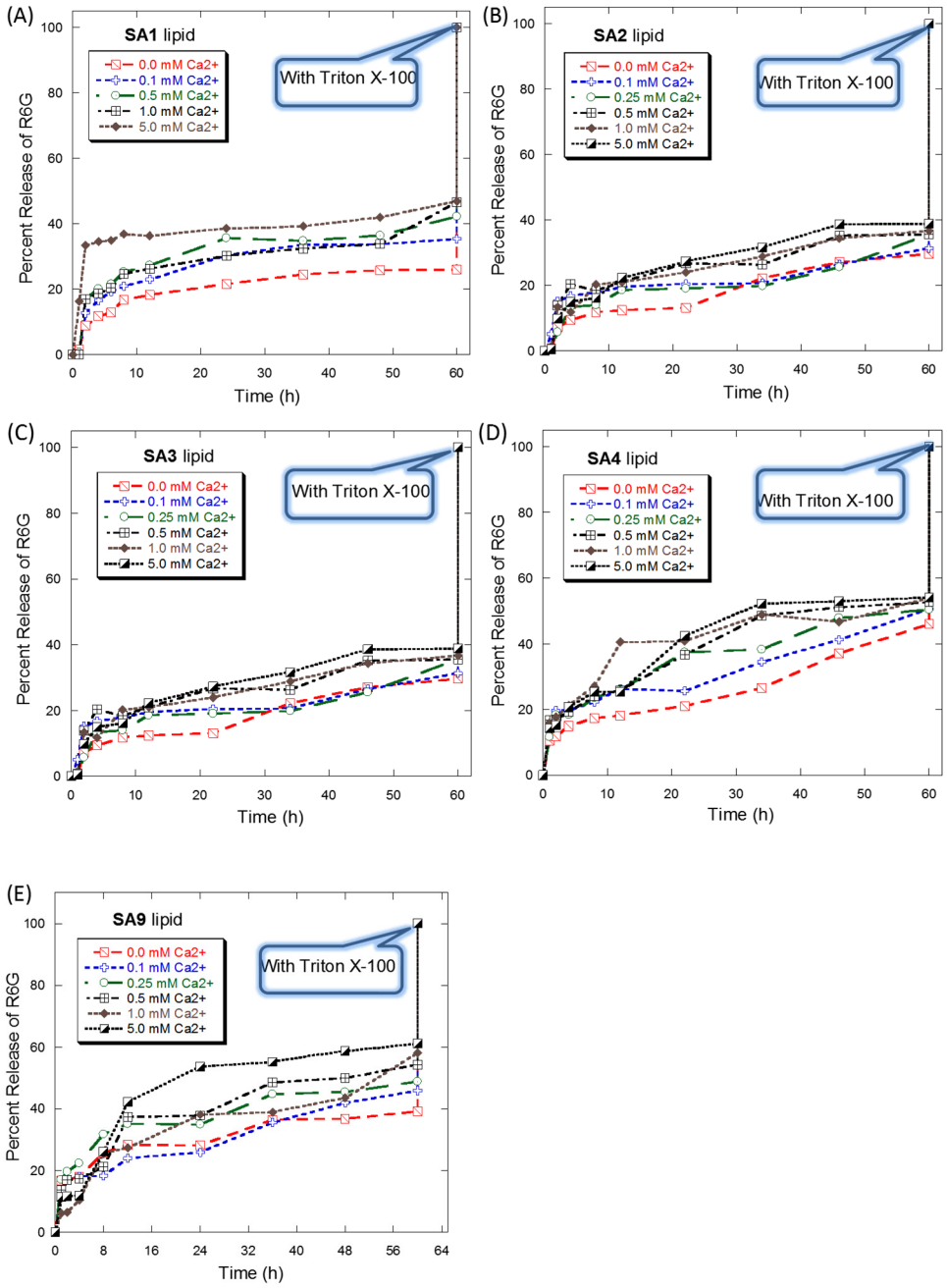


Fig S10 R6G release profile from the liposomes of 100 % SA lipids in the absence and presence of different Ca^{2+} concentrations (0-5 mM). (A) R6G release profile of **SA1** lipid. (B) R6G release profile of **SA2** lipid. (C) R6G release profile of **SA3** lipid. (D) R6G release profile of **SA4** lipid. (E) R6G release profile of **SA9** lipid.

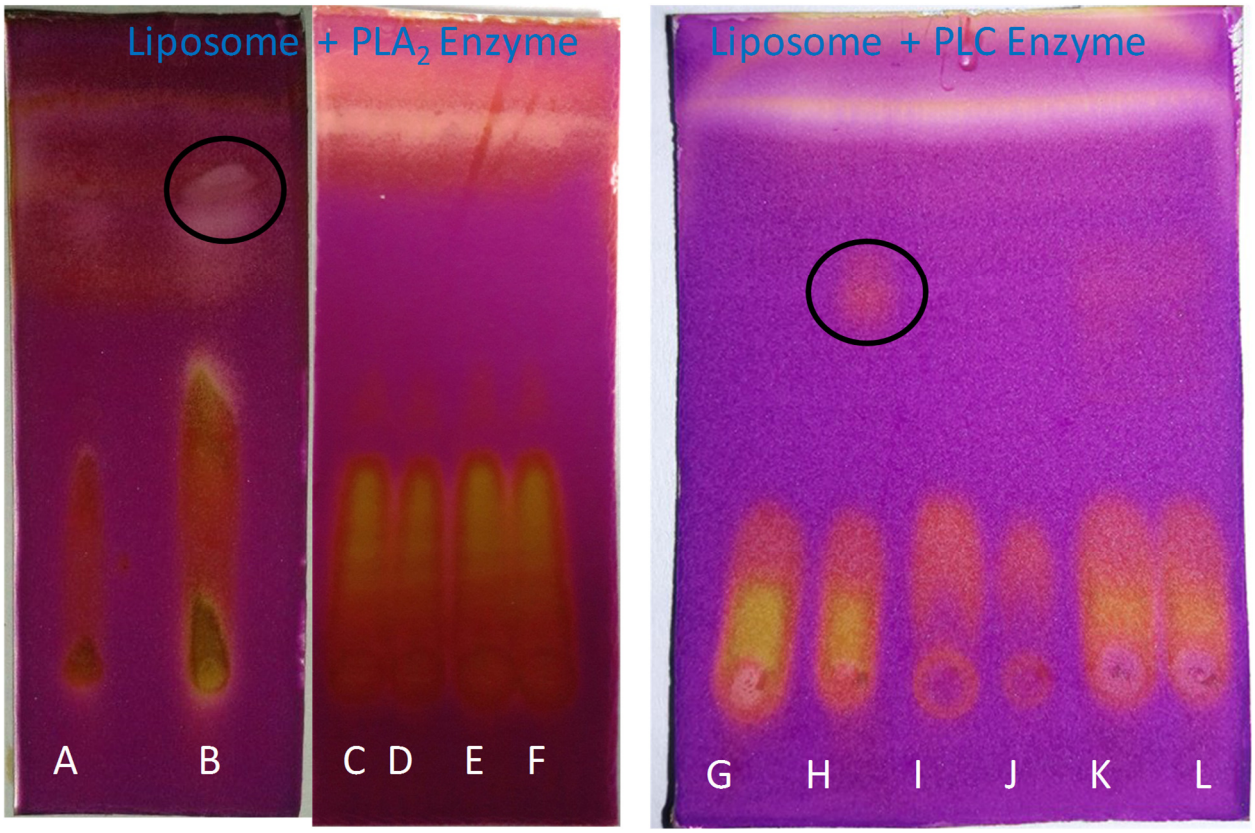


Fig. S11 TLC images ($KMnO_4$ stained) of the lipids in the absence and presence of PLA_2 and PLC enzymes. (A) only DPPC liposome. (B) DPPC liposome + PLA_2 enzyme. (C) only **SA1** liposome. (D) **SA1** liposome + PLA_2 enzyme. (E) only **SA9** liposome. (F) **SA9** liposome + PLA_2 enzyme. (G) only DPPC liposome. (H) DPPC liposome + PLC enzyme. (I) only **SA1** liposome. (J) **SA1** liposome + PLC enzyme. (K) only **SA9** liposome. (L) **SA9** liposome + PLC enzyme. Aluminum sheet TLC silica gel 60 F_{254} plate was used for TLC checking. Solvent system for PLA_2 and PLC assays (TLC) was 10 % MeOH / Dichloromethane. Marked circles (black color) are the hydrolyzed product of DPPC in the presence of PLA_2 and PLC enzymes.

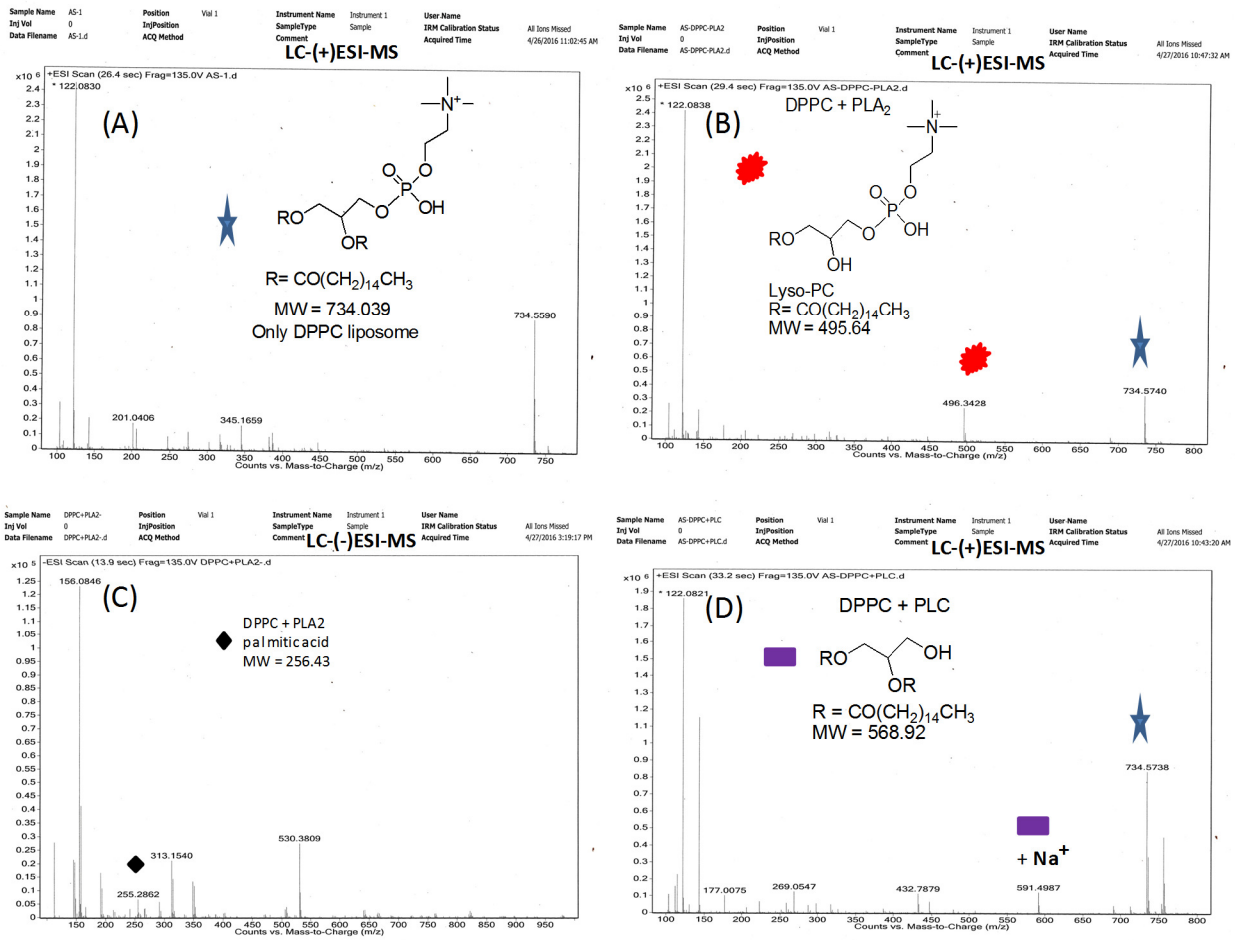


Fig. S12 LC-MS analyses of the PLA_2 and PLC treated DPPC lipid. (A) LC-(+)ESI-MS of only DPPC liposome in methanol. (B) LC-(+)ESI-MS of PLA_2 treated DPPC liposome in methanol. (C) LC-(+)ESI-MS of PLA_2 treated DPPC liposome in methanol. (D) LC-(+)ESI-MS of PLC treated DPPC liposome in methanol.

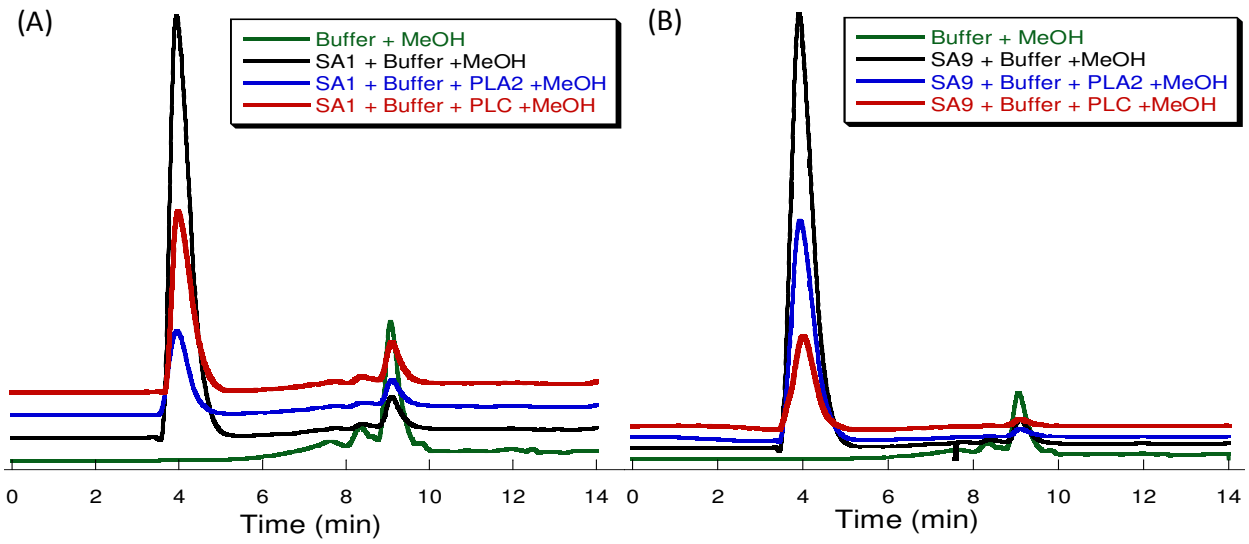


Fig. S13 HPLC-UV (270 nm) signals for PLA_2 and PLC treatment of **SA1** and **SA9** lipids. **SA1/SA9** before (black), after the treatment of PLA_2 (blue) and after the treatment of PLC (red). No hydrolysis was observed. Retention time for **SA1/SA9** lipids is 3.9 min. The HPLC-UV signals at R_t = 3.9 min were further confirmed by LCMS analyses, which indicates its stability under the enzymatic assay conditions. The HPLC-UV signals at R_t = 9.5 min may come from buffer, which cannot be properly characterized by MS analyses.

NMR and Mass spectra of the compounds:

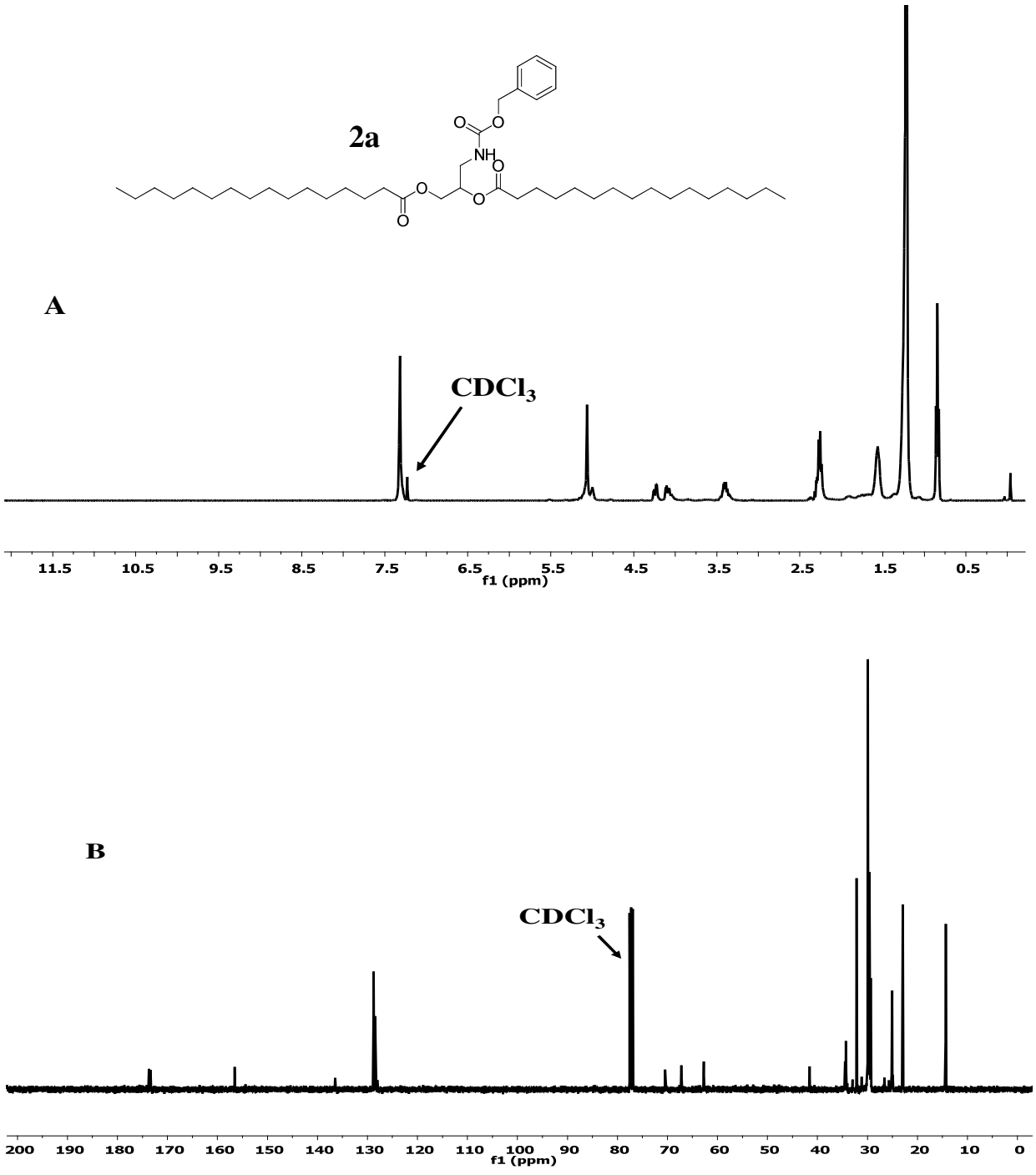


Figure S14: ^{1}H & ^{13}C NMR of compound **2a.**

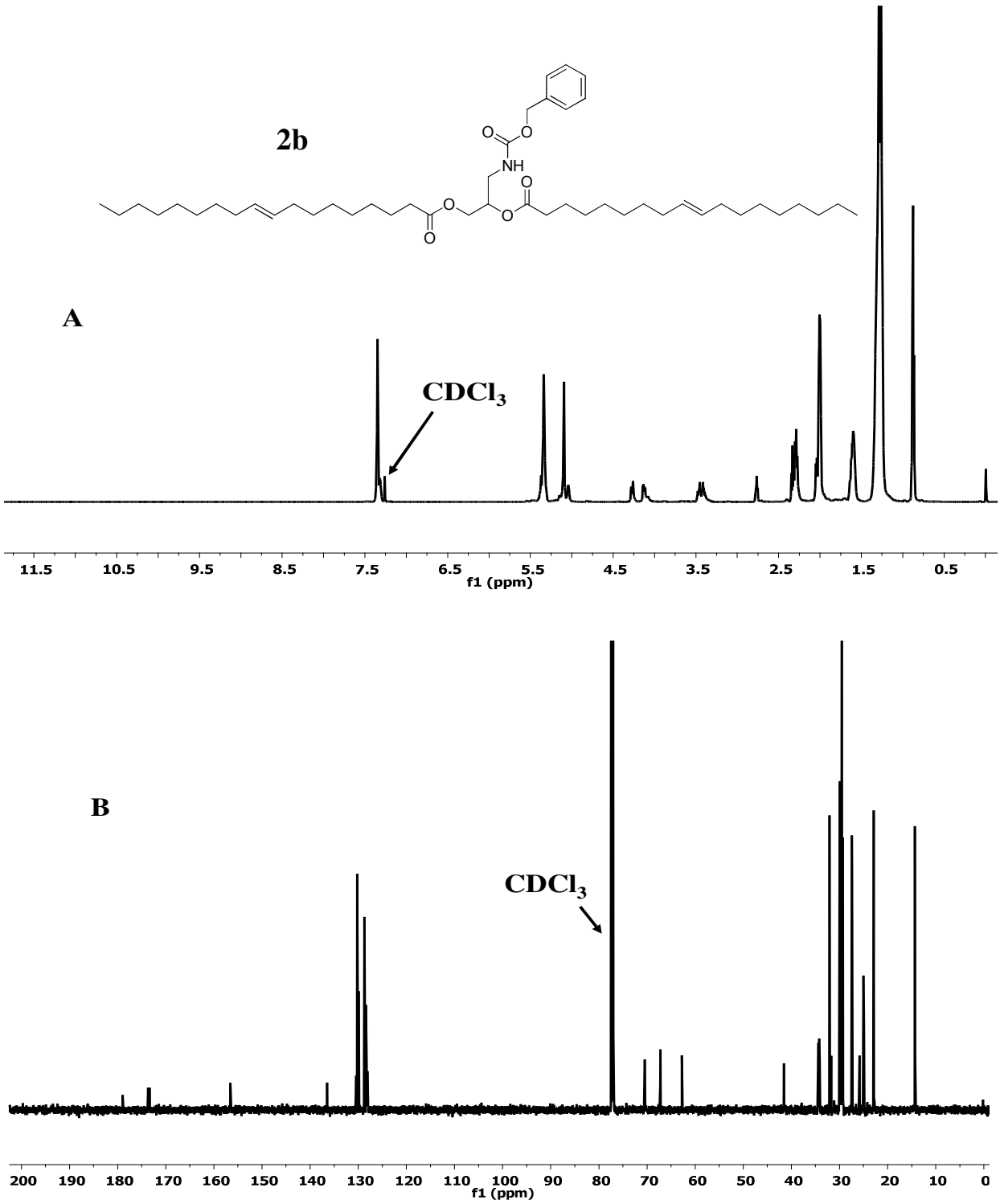


Figure S15: ^{1}H & ^{13}C NMR of compound **2b.**

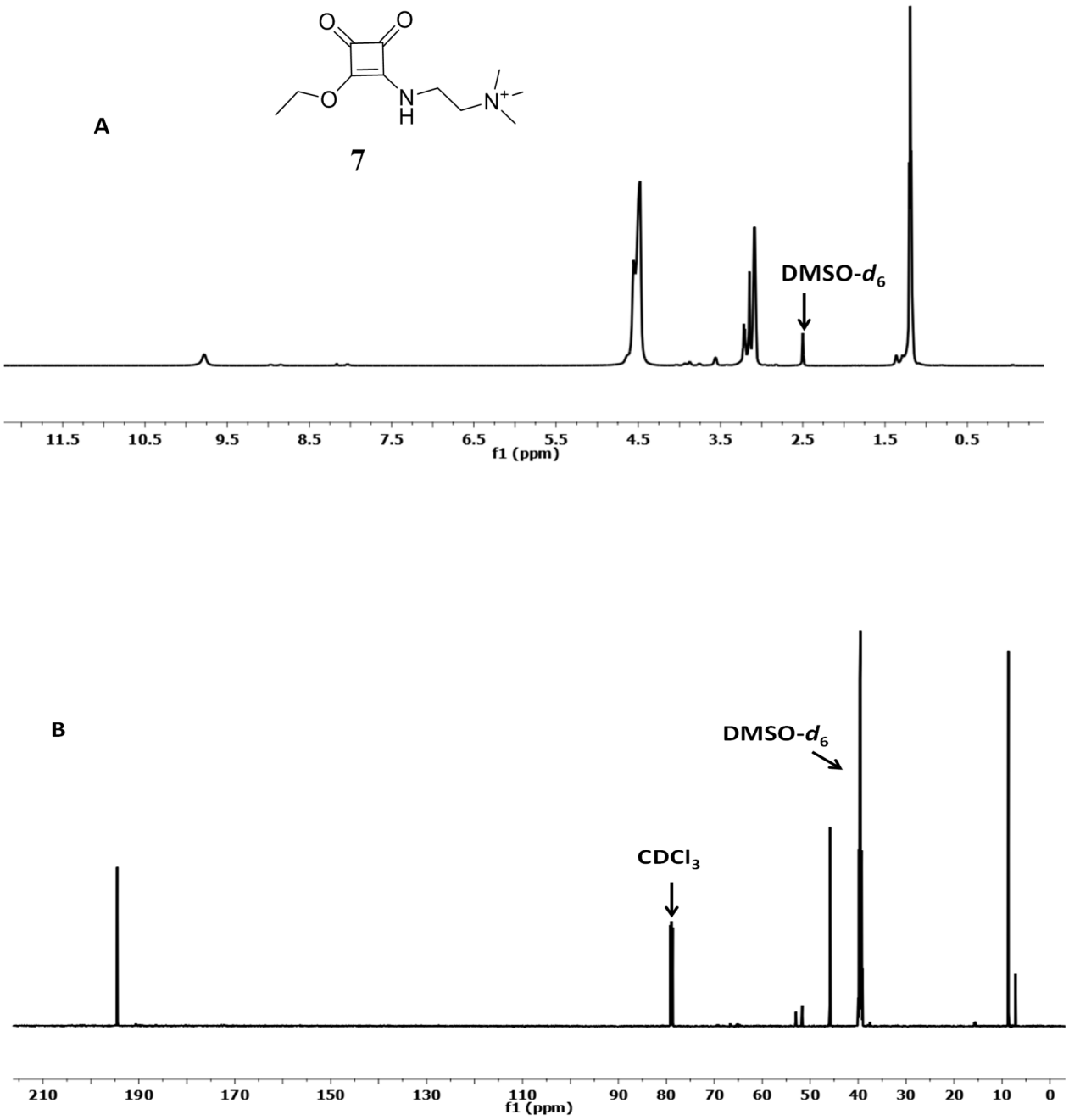


Figure S16: ^{1}H & ^{13}C NMR of compound **7**.

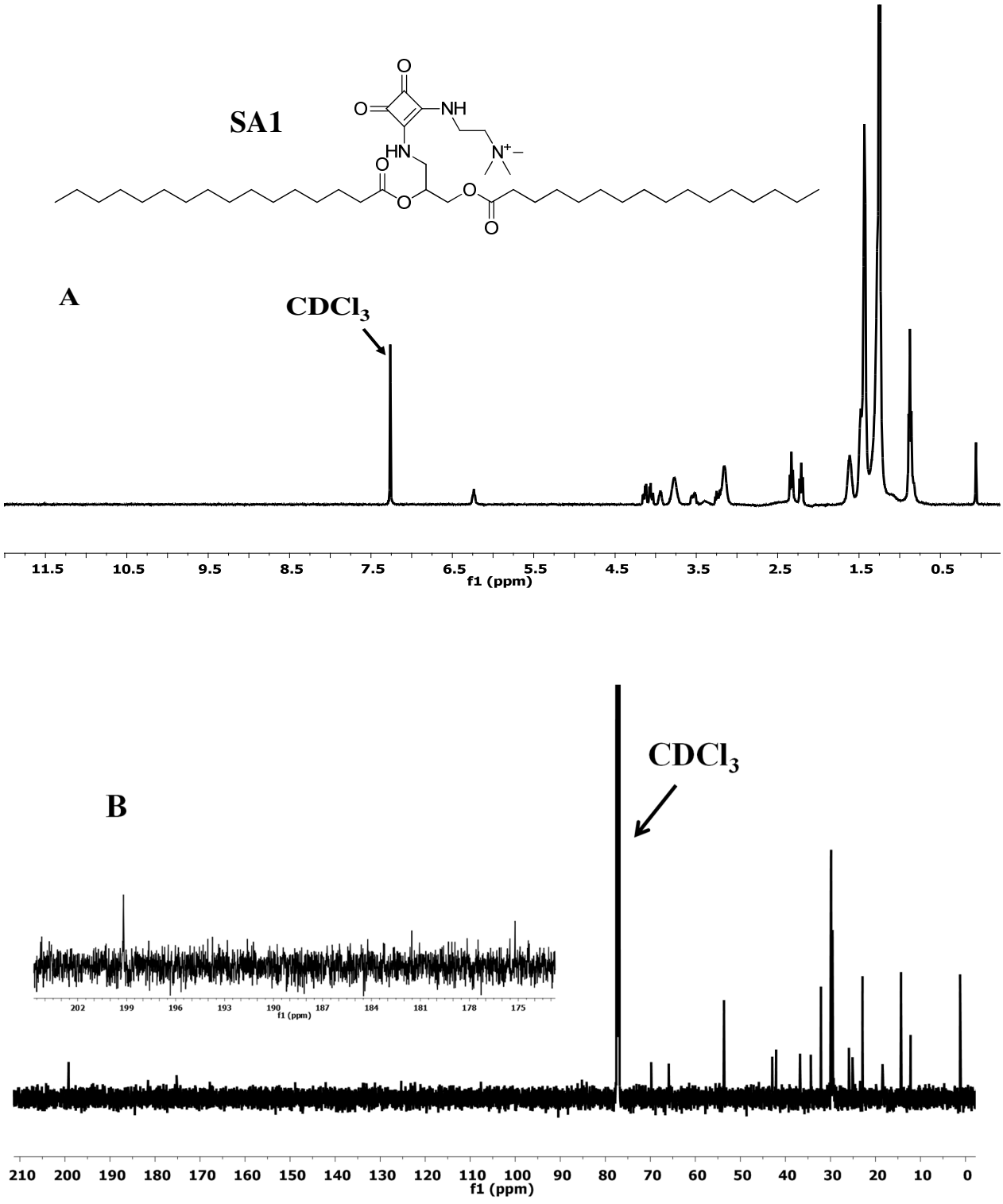


Figure S17: ^{1}H & ^{13}C NMR of compound **SA1.**

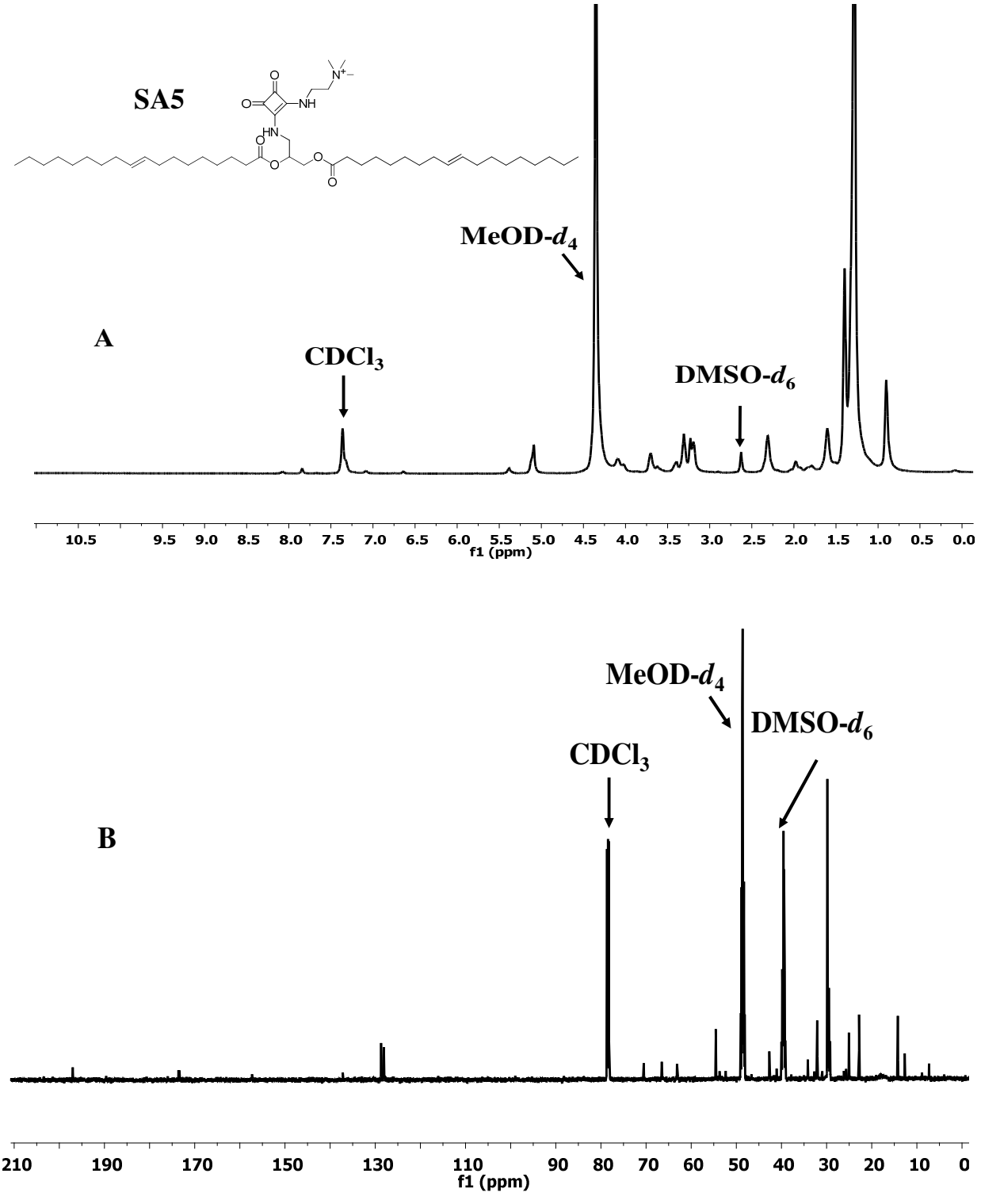


Figure S18: ^{1}H & ^{13}C NMR of compound **SA5.**

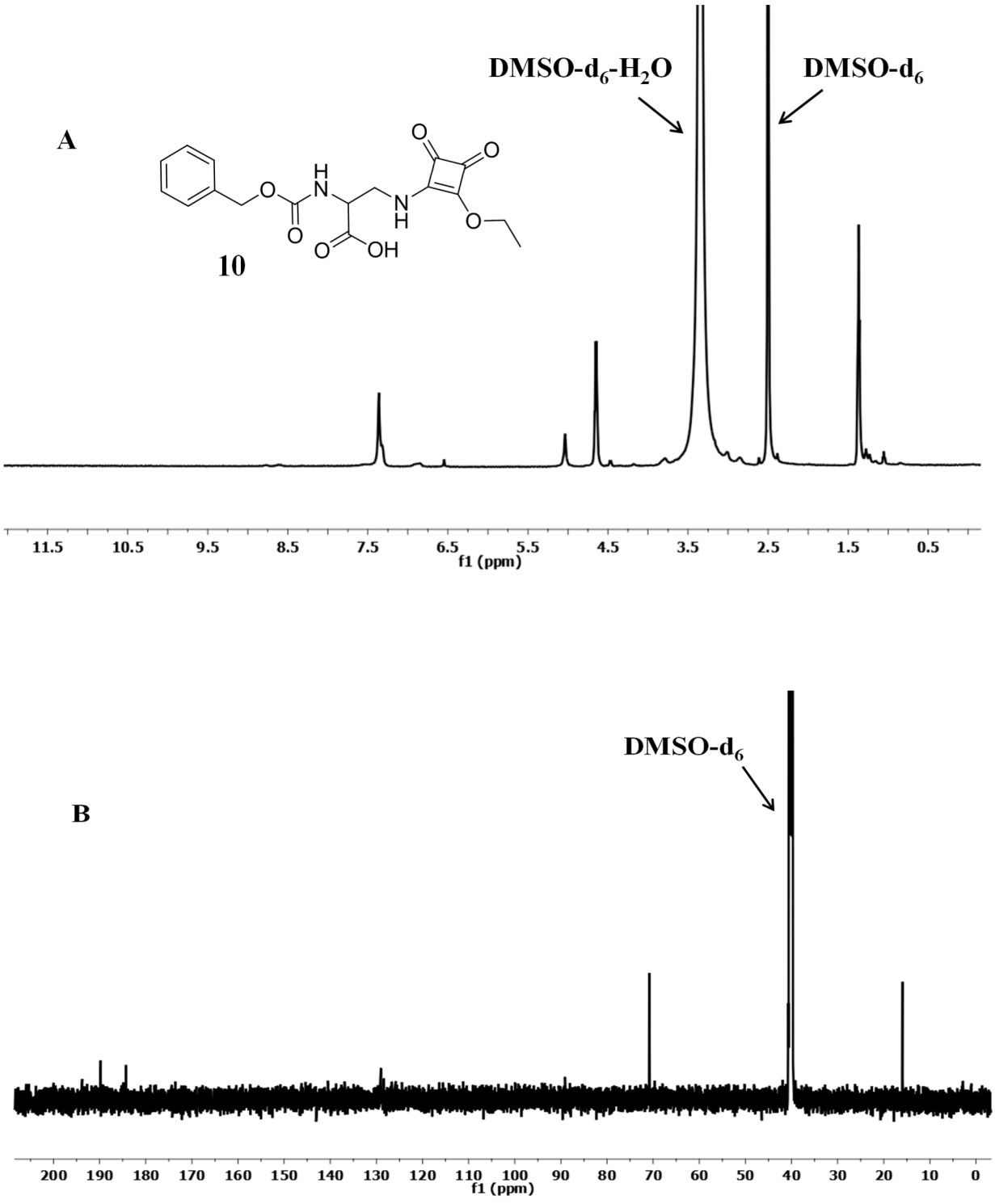


Figure S19: ^{1}H & ^{13}C NMR of compound **10.**

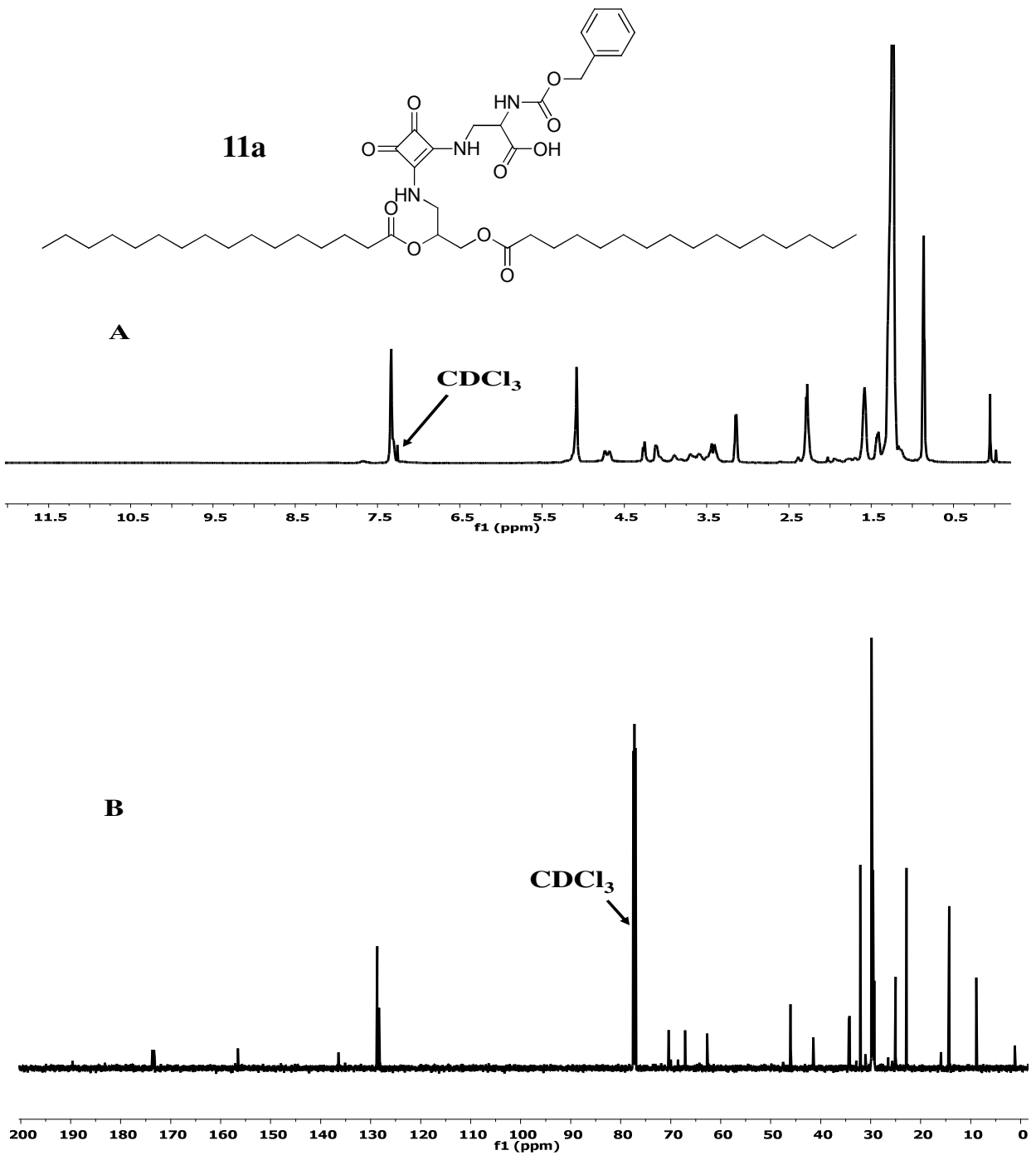


Figure S20: ^{1}H & ^{13}C NMR of compound **11a.**

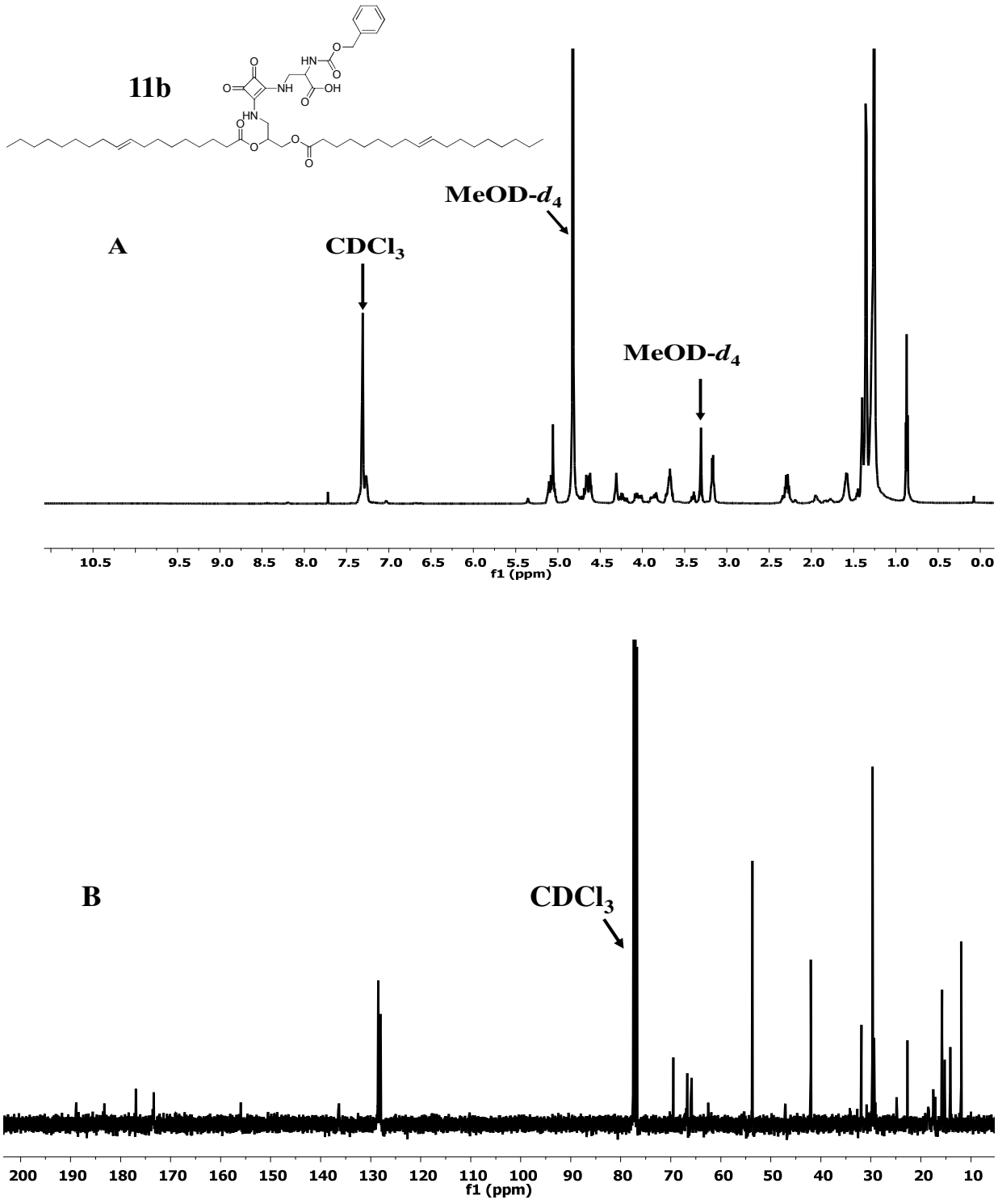


Figure S21: ^{1}H & ^{13}C NMR of compound **11b.**

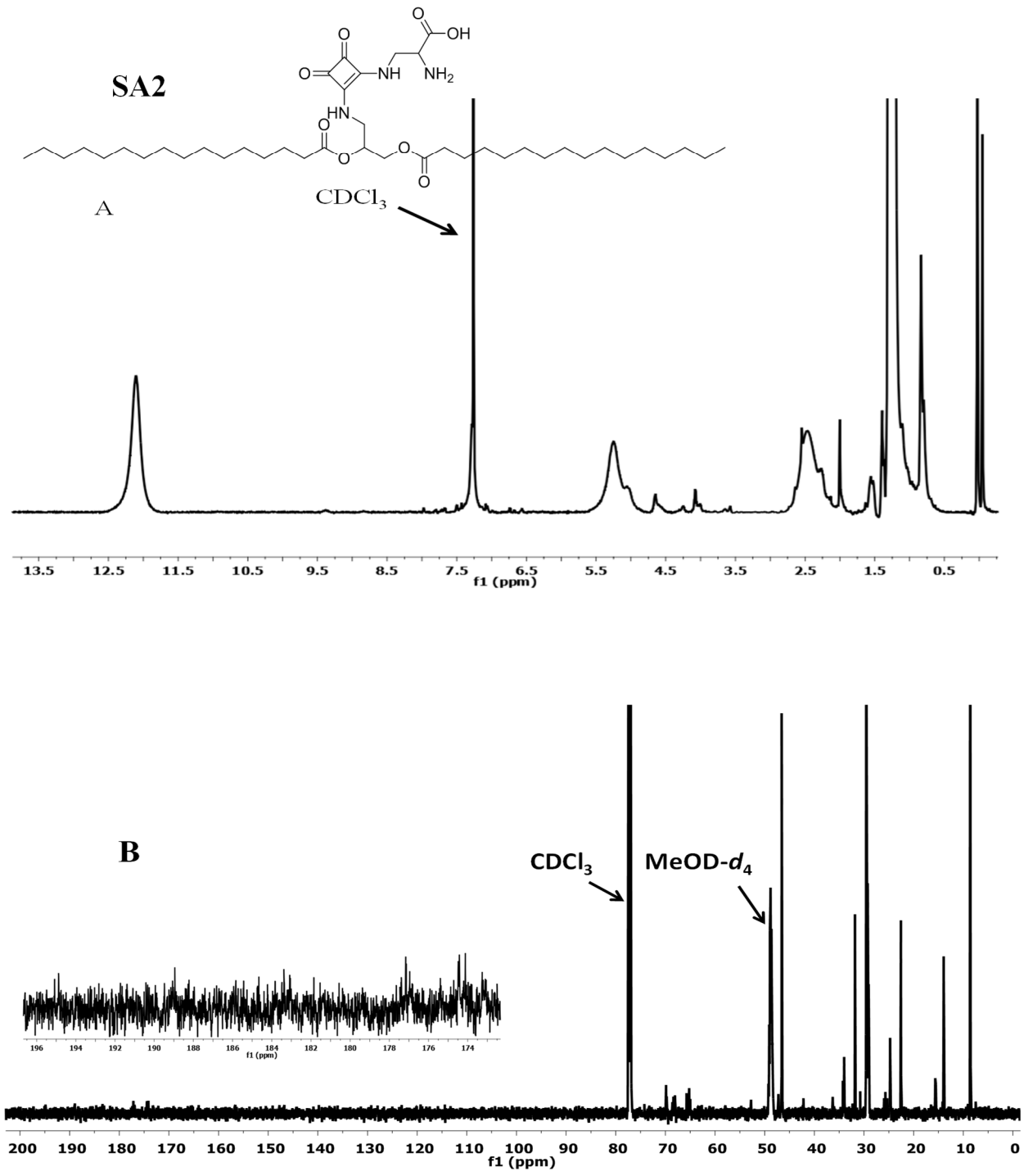


Figure S22: ^{1}H & ^{13}C NMR of compound **SA2.**

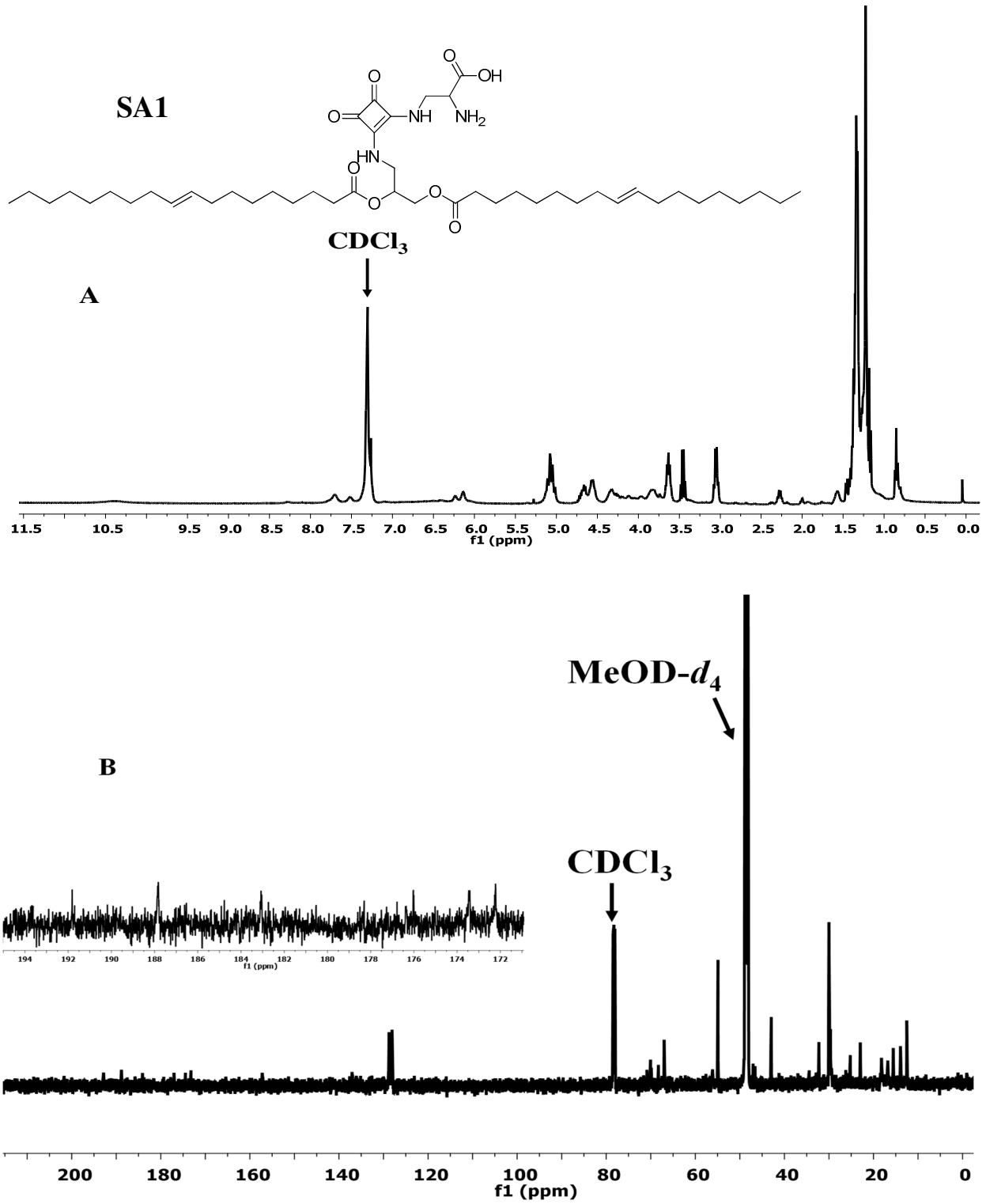


Figure S23: ^{1}H & ^{13}C NMR of compound **SA6.**

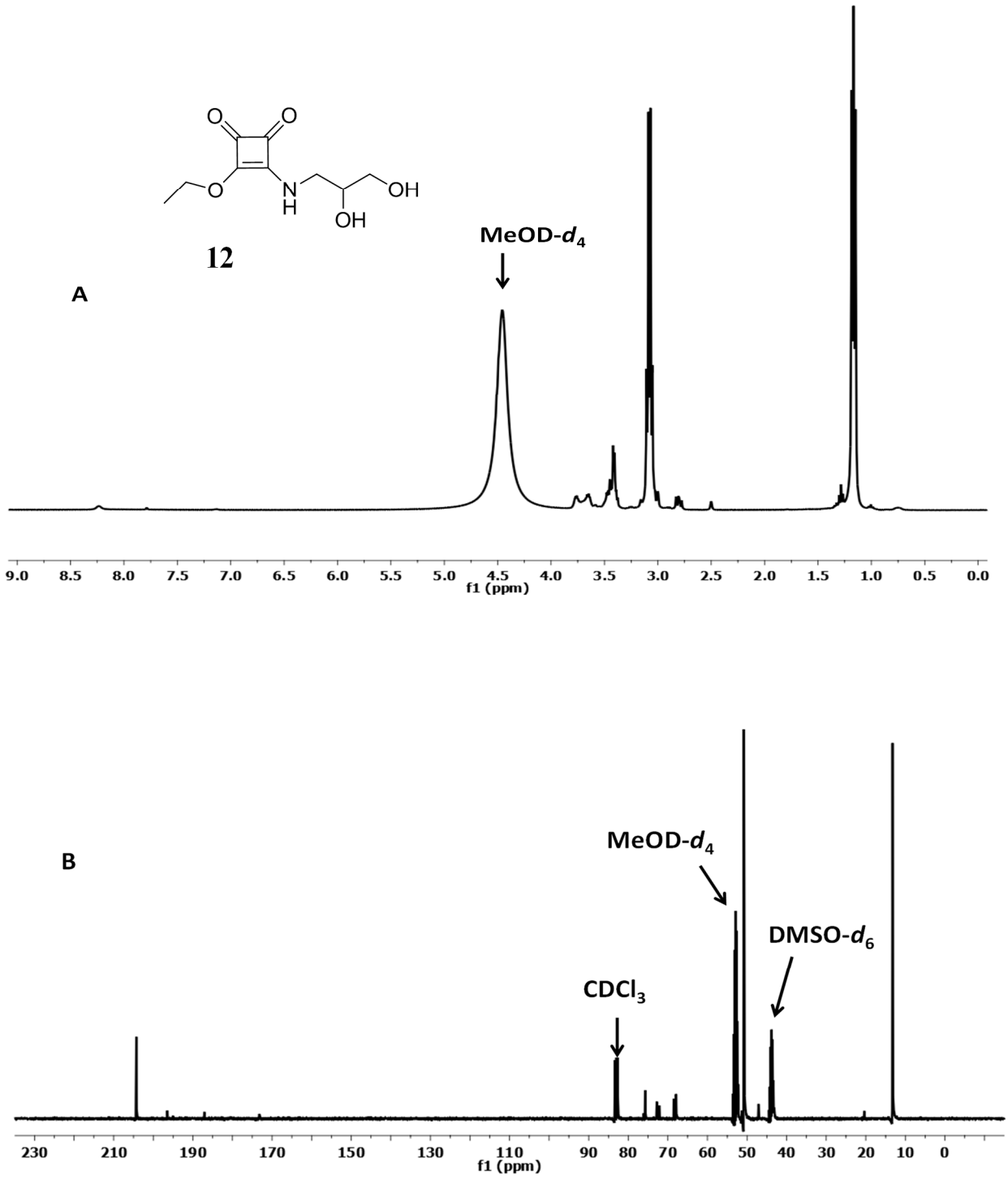


Figure S24: ^{1}H & ^{13}C NMR of compound **12.**

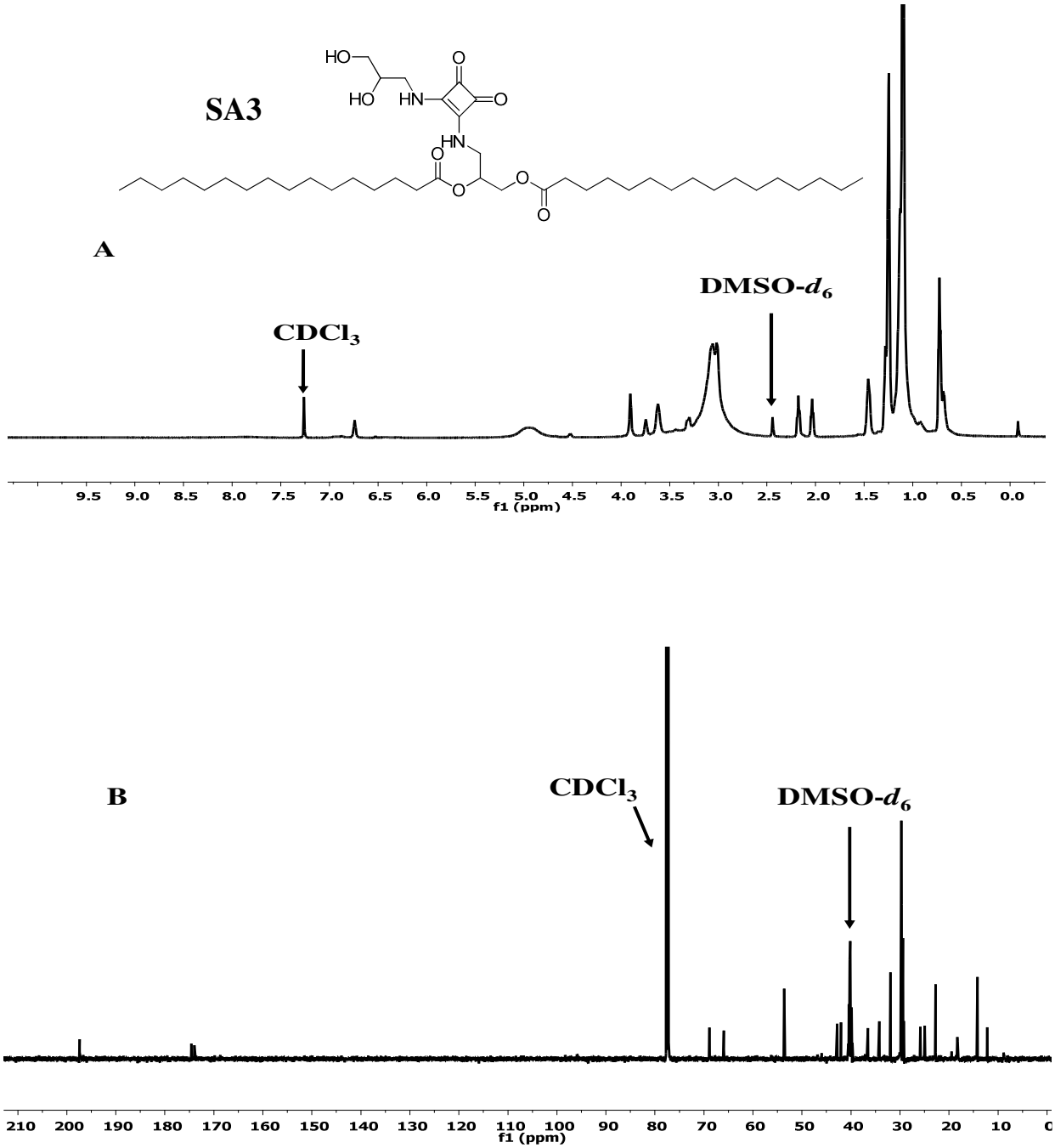


Figure S25: ^{1}H & ^{13}C NMR of compound **SA3.**

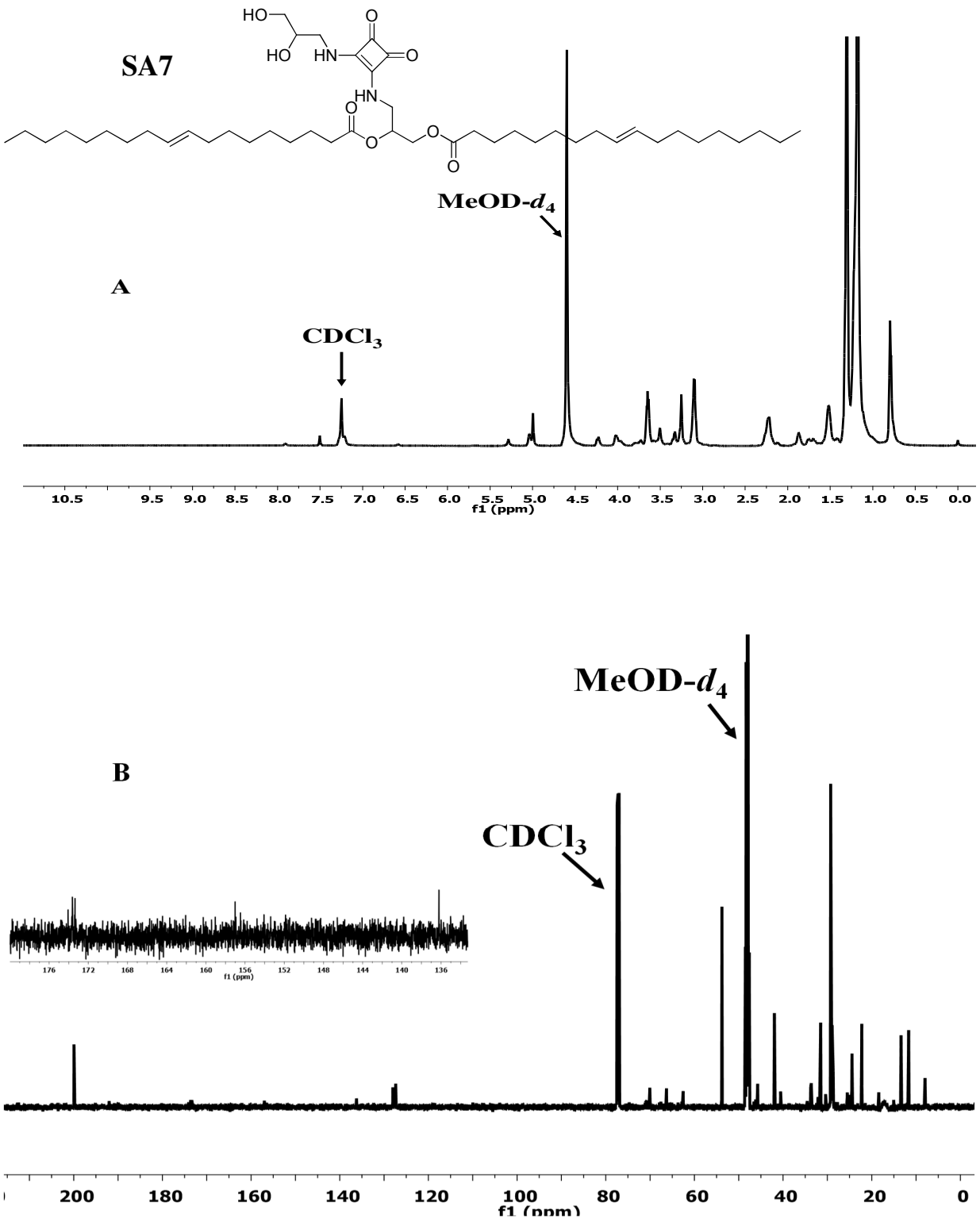


Figure S26: ^{1}H & ^{13}C NMR of compound **SA7.**

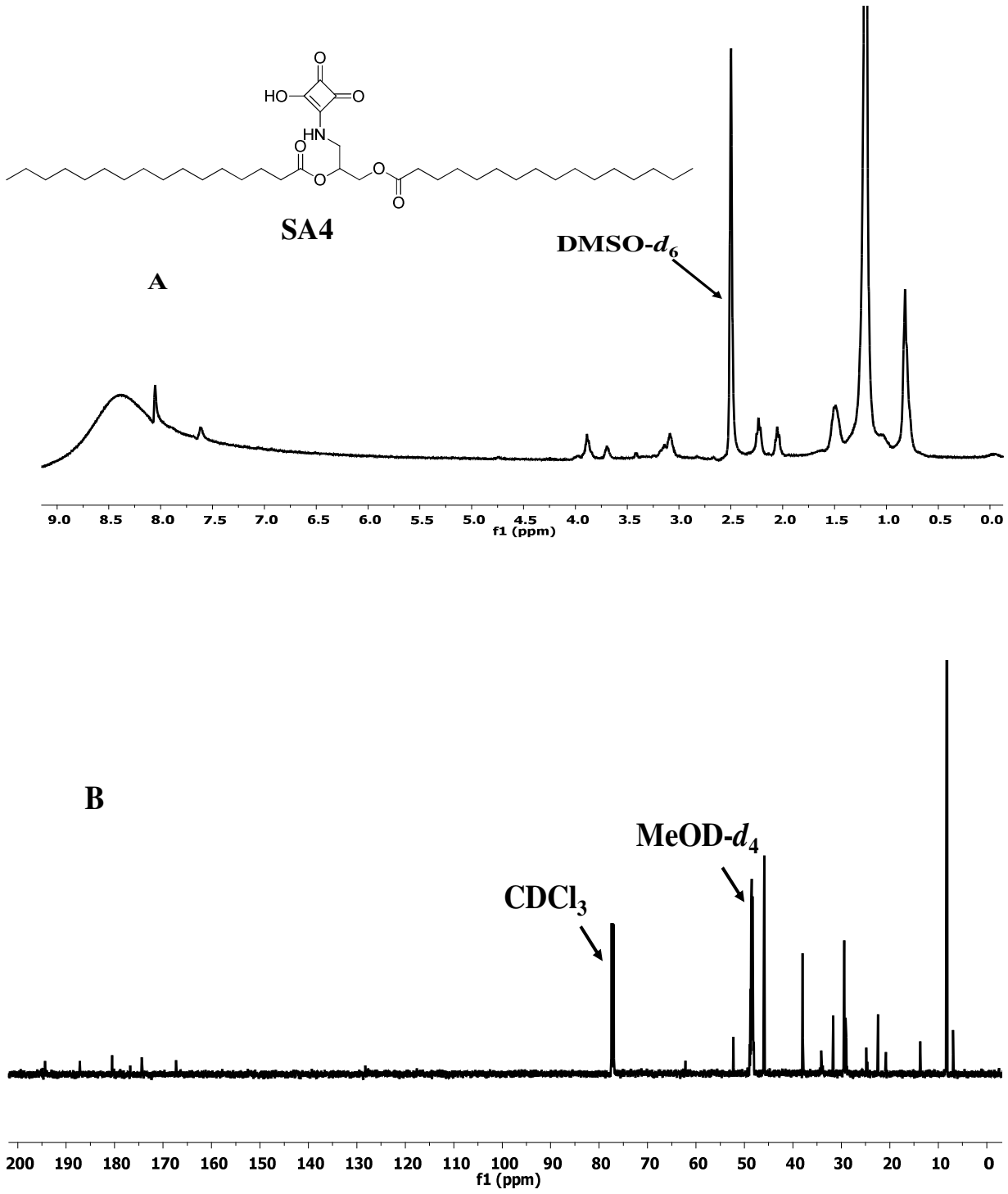


Figure S27: ^{1}H & ^{13}C NMR of compound **SA4.**

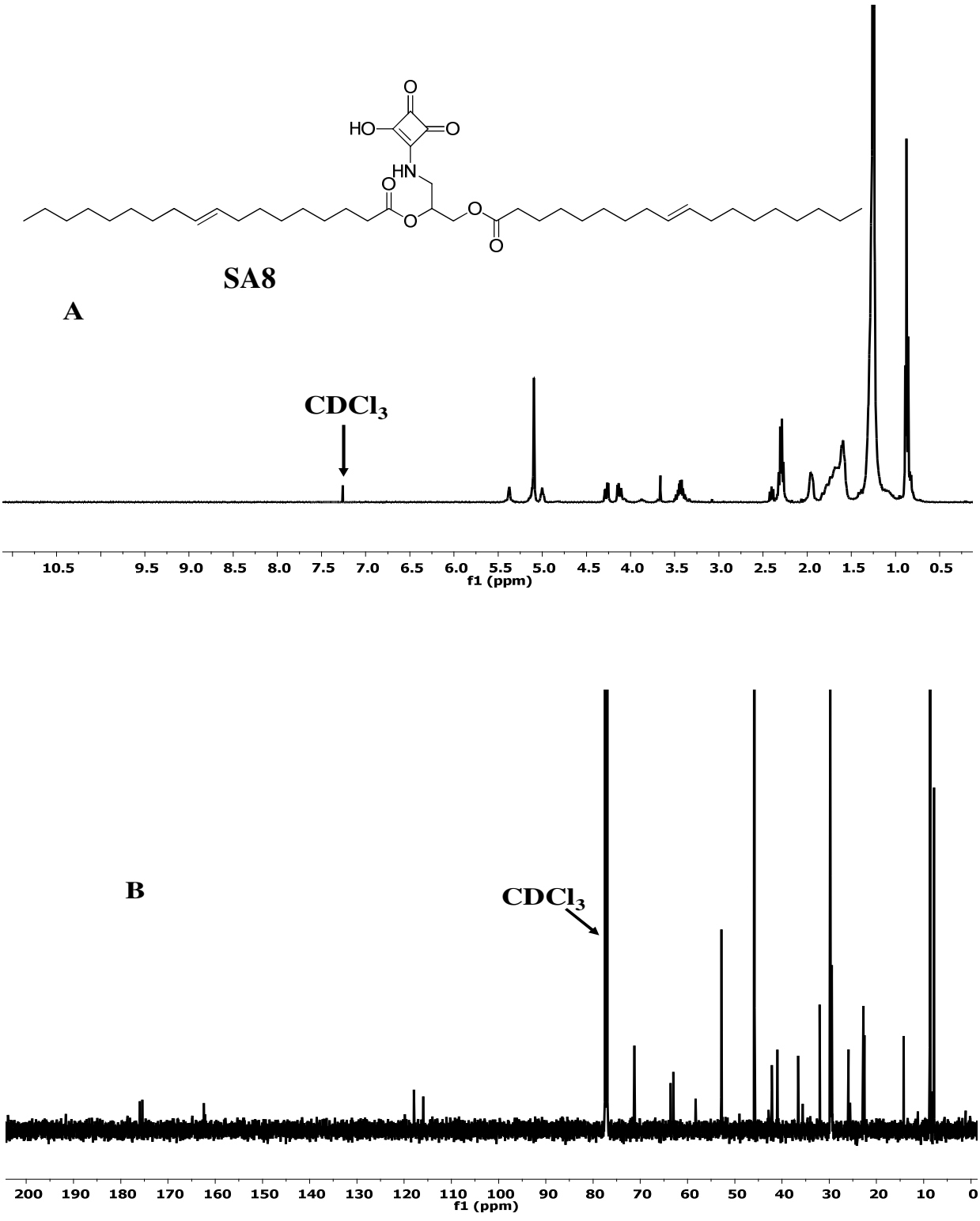


Figure S28: ^{1}H & ^{13}C NMR of compound **SA8.**

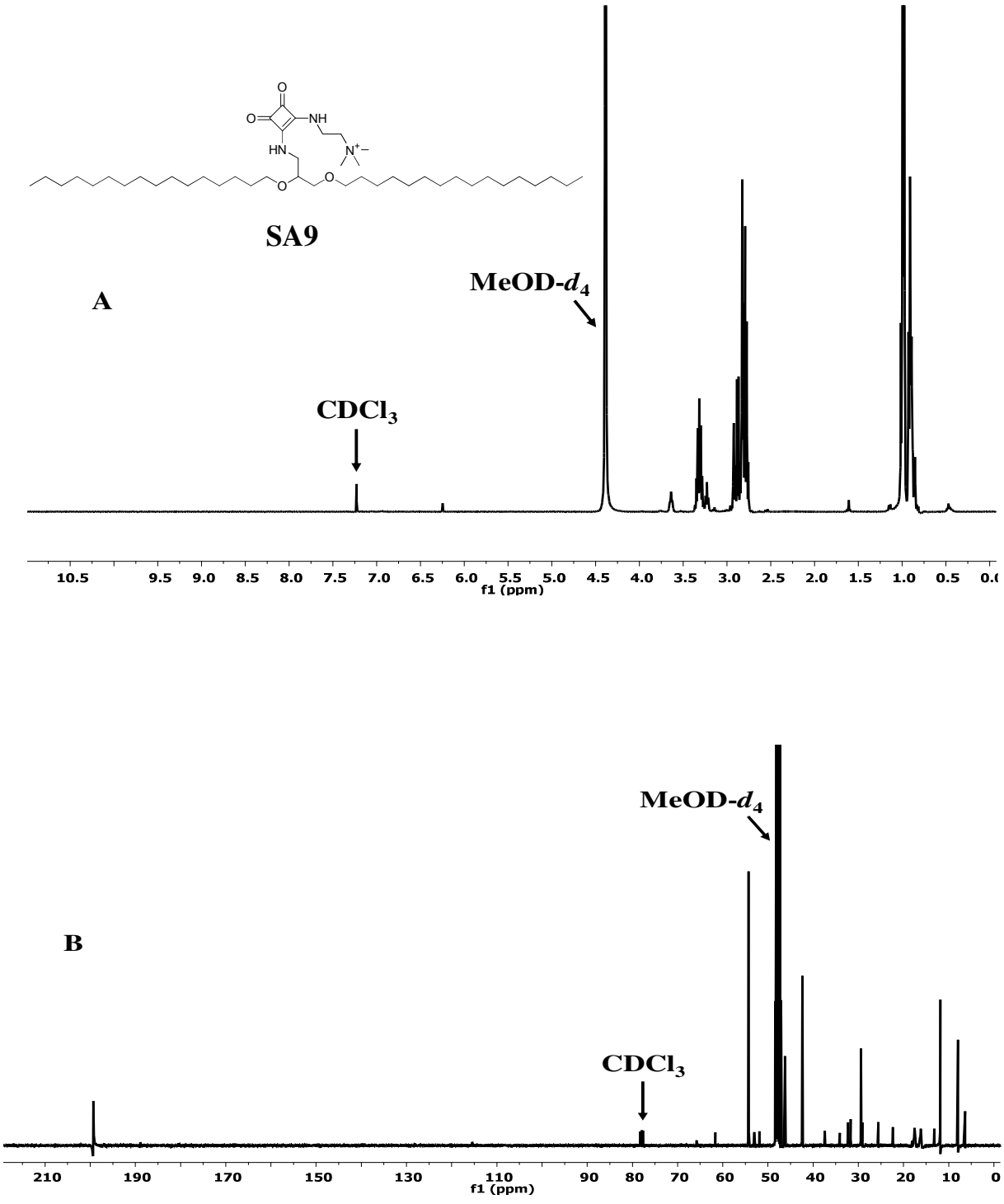


Figure S29: ^{1}H & ^{13}C NMR of compound **SA9.**

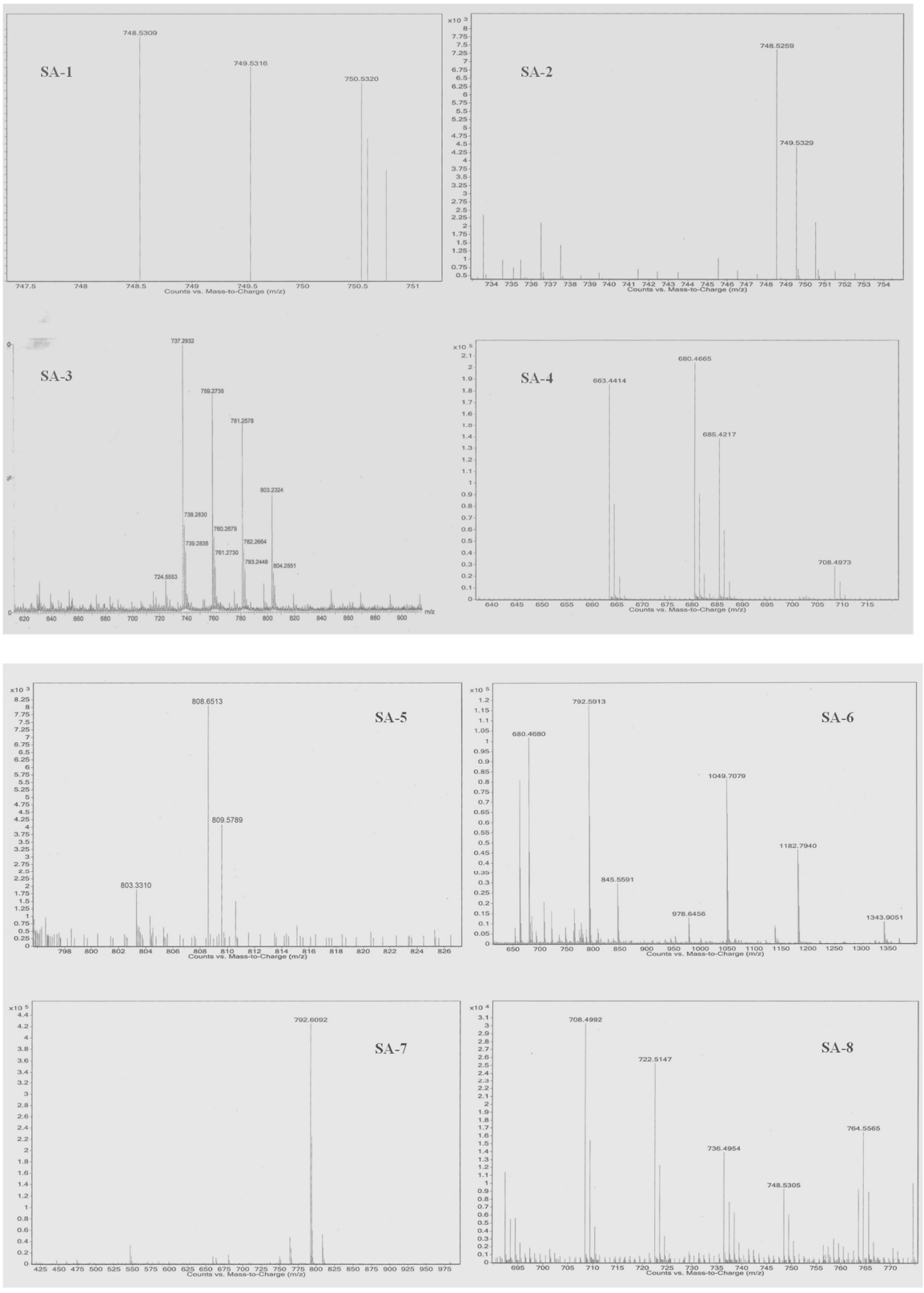


Figure S30. MS (+ESI) data of compounds **SA1-8**.

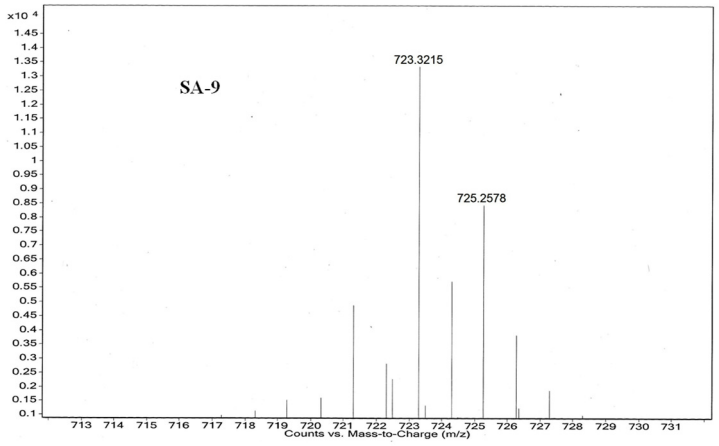


Figure S31. MS (+ESI) data of compound **SA9**.

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