Dicationic Amphiphiles Bearing an Amino Acid Head Group With Long-Chain Hydrophobic Tail For *In Vitro* Gene Delivery Applications

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Supplementary data

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Experimental procedure:

Transfection biology

A general transfection approach was used for this study. Cells (HEK-293, CAL-27, U87) were grown 18–24 hours before transfection at a density of 10^4 cells per well in a 96-well plate. In serum-free DMEM (maximum volume should be 100 µL), the supercoiled pCMV-*β*-gal plasmid DNA (0.5 µg) was complexed with various amounts of desired liposomes and incubated for 30 minutes. Cationic liposome N/P ratios with pDNA ranged from 1:1 to 8:1 (+/-). The cells were subsequently treated with the liposome/plasmid complexes. After 4 hours of incubation, the cells were given 100 µL of Dulbecco's modified Eagle medium supplemented with 10% FBS. After 24 hours, the serum medium was changed to 10% complete medium, and the reporter gene activity was measured after 48 hours. The cells were lysed in 50 µL of lysis buffer [0.25 M Tris–HCl pH 8.0, 0.5 percent NP40] after being washed twice with PBS (100 µL each time). The process was carefully monitored to achieve complete lysis. In 96-well plate, the activity of *β*-galactosidase was determined by adding 50 µL of 2X-substrate solutions [1.33 mg mL^{-1} ONPG, 0.2 M sodium phosphate (pH 7.3), and 2 mM magnesium chloride] to the lysate. Using a calibration curve built with the commercial *β*-galactosidase enzyme, the 420 nm adsorption was converted to *β*-galactosidase units. The differences in *β*-galactosidase units across three trials conducted on the same day were less than 10%. Reported transfection efficiency figures are the average of the three experiments.

pEGFP expression study

50,000 cells per well were grown in a 24-well plate 18–24 hours before transfection for the cellular pEGFP expression experiment. Then, in serum-free DMEM (the total volume should be 100 µL), 0.9 µg of pEGFP plasmid DNA encoding the green fluorescent protein was complexed with liposomes of lipids C14-P, C14-M, and C14-S at N/P ratios (lipid/pDNA complexes) of 2:1 and 4:1(HEK-293) and 2:1 (CAL-27 and U87) for 30 minutes. Cells grown in the 24-well plate were rinsed with PBS (2-100 µL) just before transfection, then lipoplexes were added. After 4 hours of incubation, 400 µL of DMEM+10% FBS were introduced to the cells. The DMEM+10% FBS was withdrawn from each well after 48 hours, and the total cells were washed with PBS (2-200 µL). Finally, 200 µL of PBS was added to each well and the cells expressing the green fluorescent protein were observed using an inverted fluorescent microscope and the cells expressing pEGFP were quantified using a FACS Calibur flow cytometer (BD Biosciences, FACSAria III Cell Sorter)

equipped with an argon ion laser at 488 nm for excitation and detection at 530 nm for detection. Briefly, by the addition of 100 µL of 0.1% trypsin/EDTA leads to detach the cells, then added 400 µL of complete medium and allowed to centrifuge at 4 °C to form cell pellet. Then, cell pellet was re-suspended in 500 µL of cold PBS, without delay 500 µL of PBS contain cells were analyzed for each sample.

Cytotoxicity assay

A cell-based MTT experiment was done employing the pCMV-*β*-gal generated lipoplexes prepared from the enhanced pattern of N/P ratios from 1:1 to 8:1 to screen the toxicity profiles associated with these unique co-liposomal formulations towards the examined cell lines (HEK-293, CAL-27, U87). The transfection was performed by performing the methods for *β*-gal activity assay, and the complexes were allowed to incubate for 4 hours after being added. The complex medium was replaced with a new 10% FBS+DMEM, and the incubation was continued in an incubator for another 44 hours. Then incubation was maintained for 3 hours with MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] (0.5 mg μL^{-1}) solution in serum less DMEM. Cell lysis was performed using MeOH:DMSO (v/v, 1:1, 100 µL per well). The purple-coloured solutions were scanned at 570 nm in a Multiskan Spectrum multiplate reader, with untreated cells functioning as controls. The percentage viability of the results was calculated= [A (570) treated cells − background/A (570) untreated cells − background] × 100.

Intracellular uptake

Cells (HEK-293 and CAL-27) were sown in 6-well plate at a density of 2×10^5 cells/well 18-24 hours before to the treatment. At 2:1 lipid/pDNA N/P ratio, pCMV-*β*-gal DNA (1.8 µg of DNA diluted to 200 µL with serum free DMEM medium) was complexed with rhodamine-PE labelled cationic liposomes (diluted to 200 µL with DMEM). The cells were rinse with PBS (500 µL), then treated with prepared lipoplexes and incubated in 5% CO_2 at 37 ℃. After 6 hours of incubation, the cells were washed twice with PBS (0.5 mL) and fixed for 10 minutes at room temperature with 4% paraformaldehyde, followed by applied another PBS (0.5 mL) wash. The matching cells were counterstained with DAPI (300 nM in PBS) for 10 minutes at room temperature. After being cleaned with PBS wash, the coverslips were mounted on glass slides with 10 µL of vectashield (0.5 mL). Fluorescence was visualized under a confocal laser scanning microscope (Leica TCS S52) equipped with a 63×oil immersion objective. The mission collection wavelengths were set at

373–422 nm for DAPI and 568–583 nm for rhodamine-PE labelled lipoplexes. For the quantification of uptake analysis, cells were seeded without cover slips and after treatment of Rh-PE labelled lipoplexes allowed to incubate for 6 hours. Then, procedure followed as green fluorescence quantification analysis. The cells were analyzed using a FACS caliber system equipped with an argon ion laser at 561 nm for excitation and at 633 nm for detection by BD Biosciences, FACSAria III Cell Sorter.

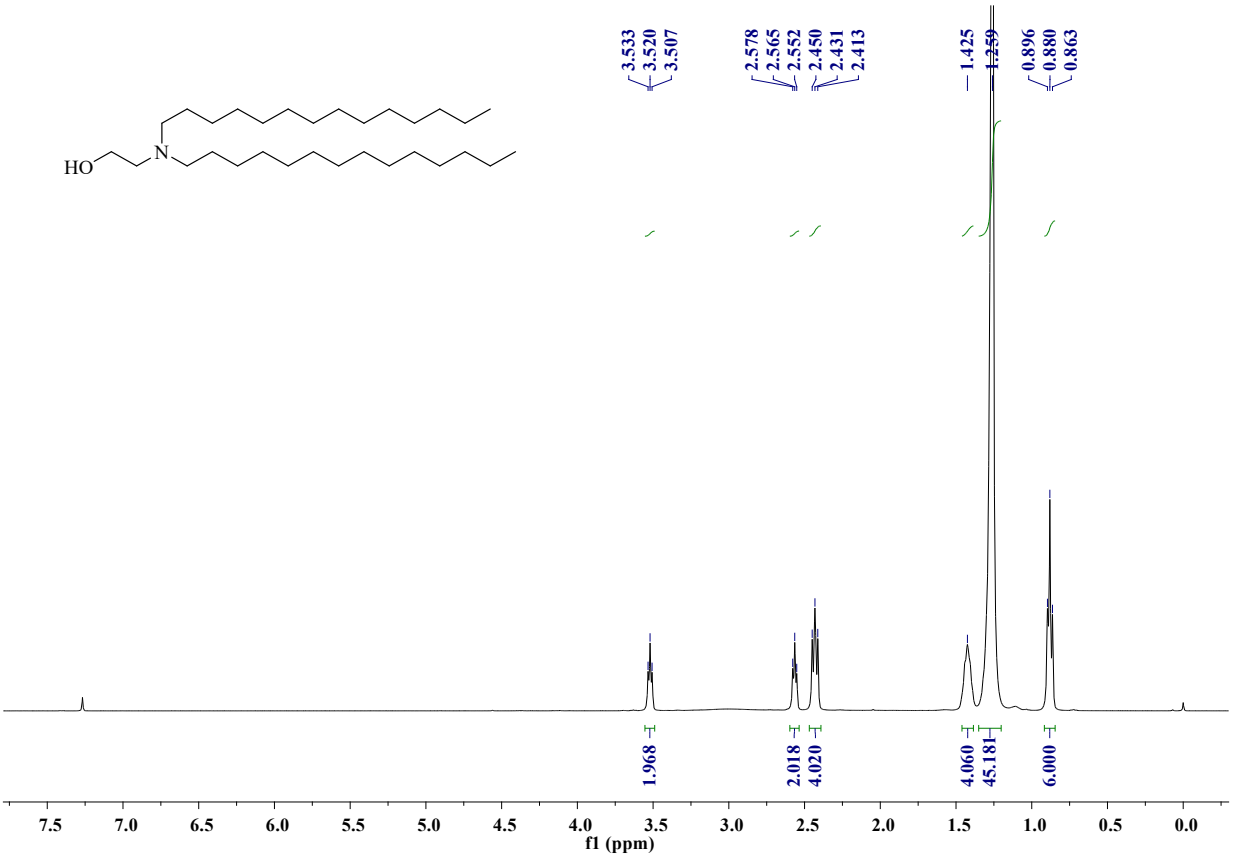


Figure S1: ^{1}H NMR Spectrum of **$(C14)_2NEOH$**

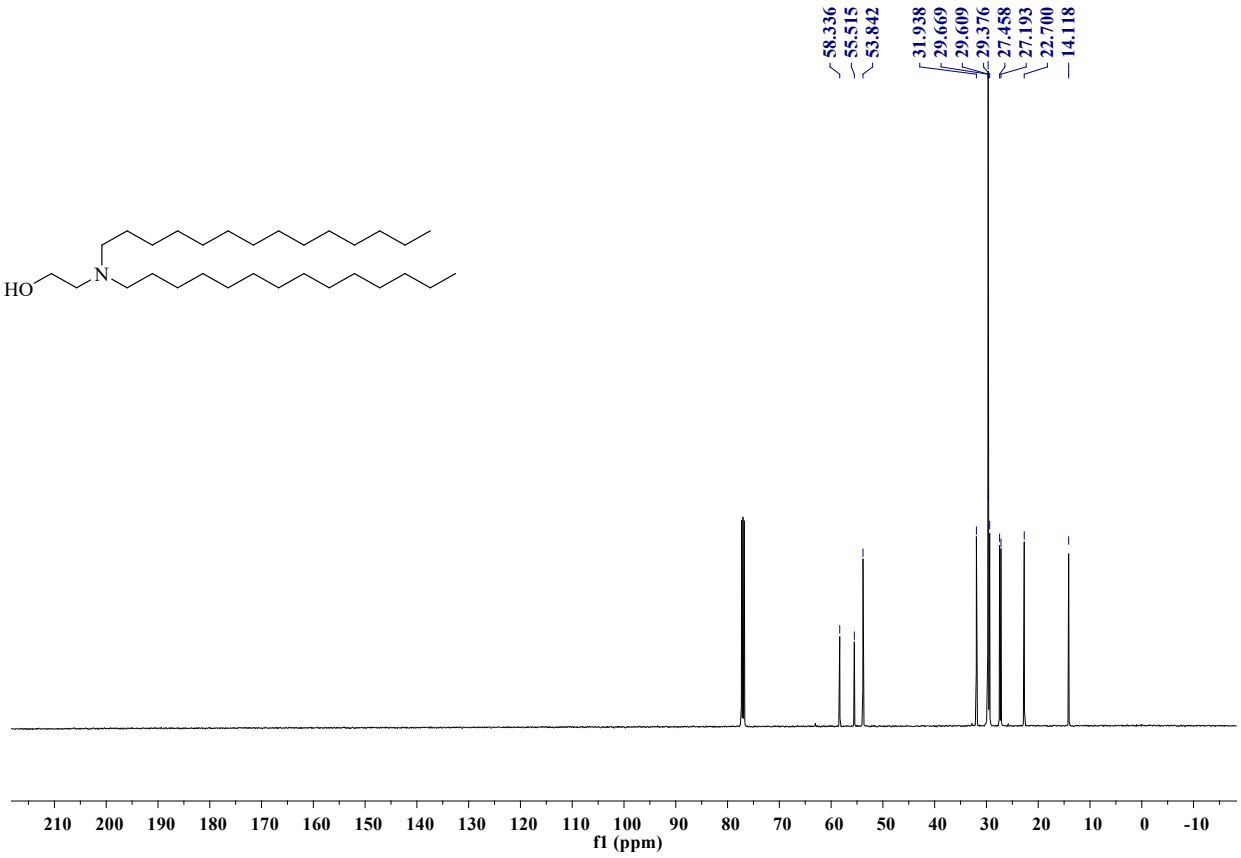


Figure S2: ^{13}C NMR Spectrum of **$(C14)_2$NEOH**

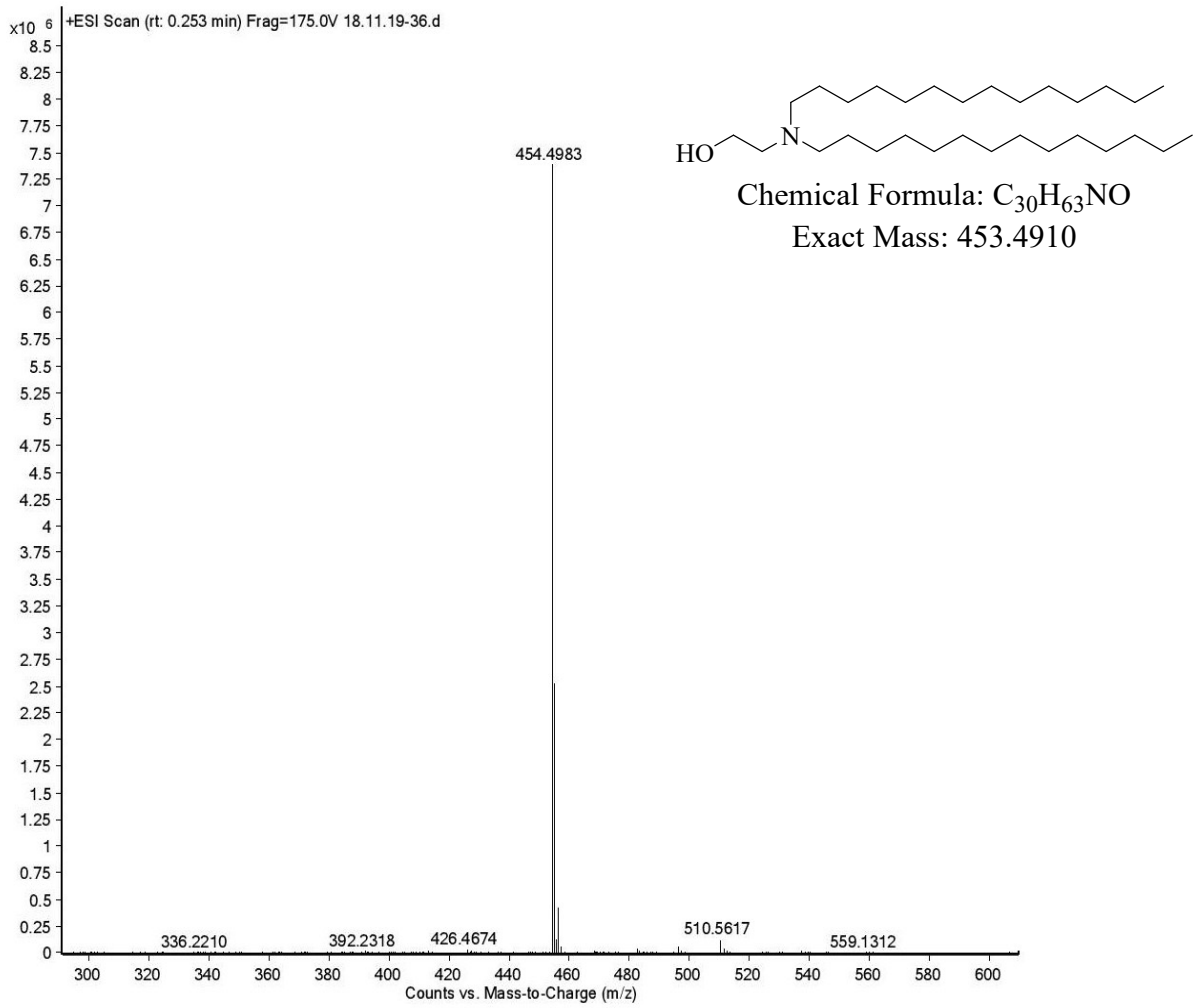


Figure S3: ESI-HRMS Spectrum of **$(C14)_2NEOH$**

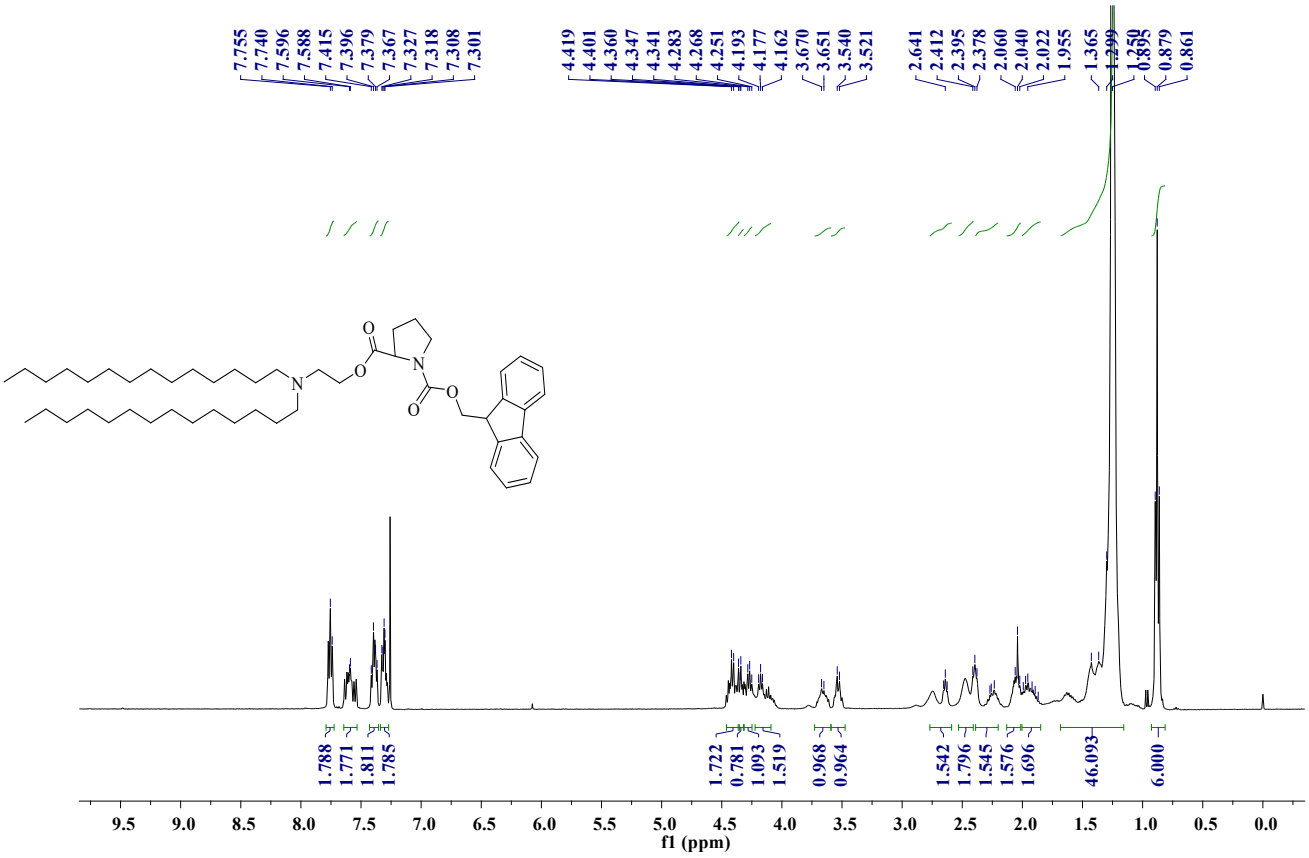


Figure S4: ^{1}H NMR Spectrum of compound **1a**

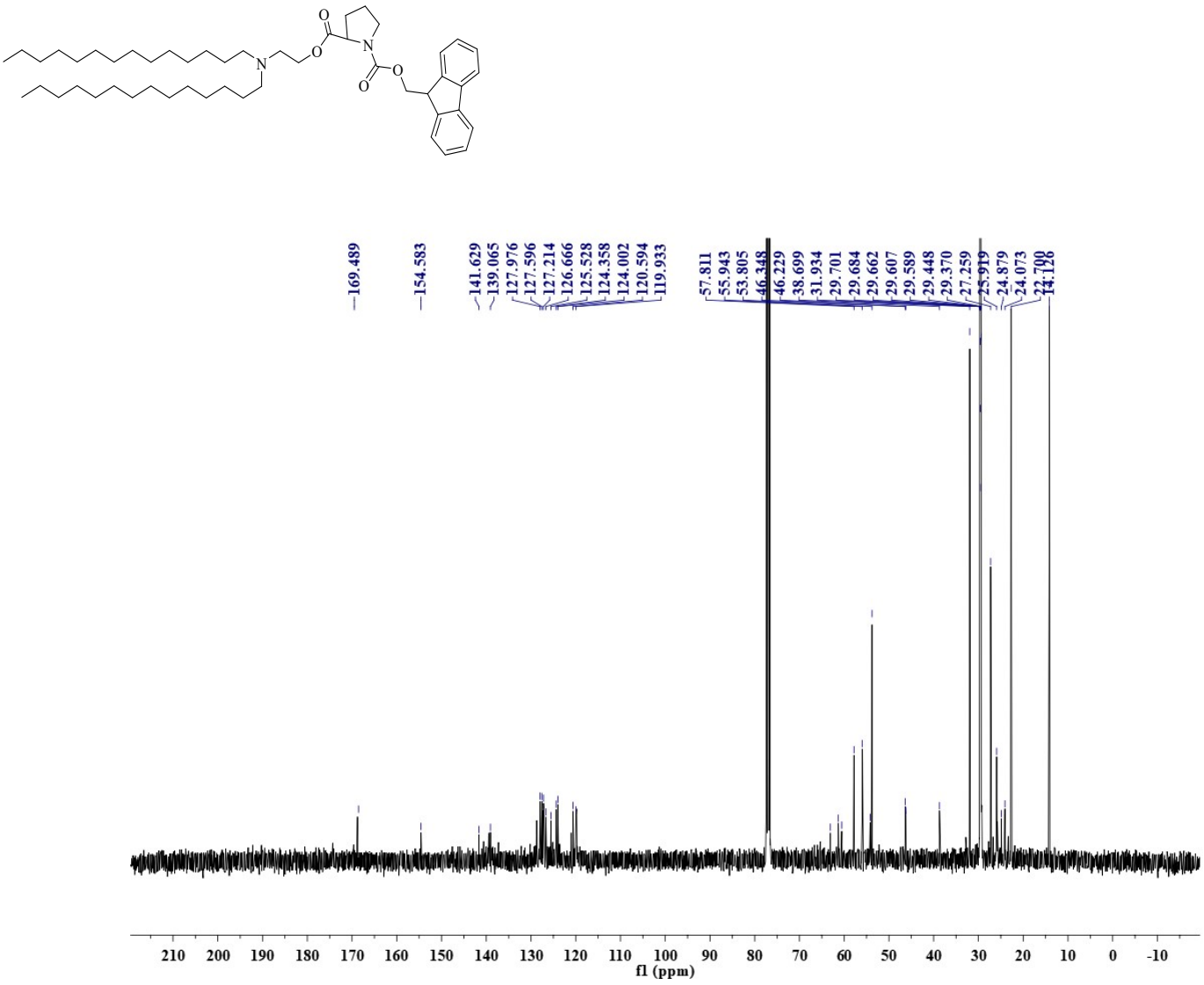


Figure S5: ^{13}C NMR Spectrum of compound **1a**

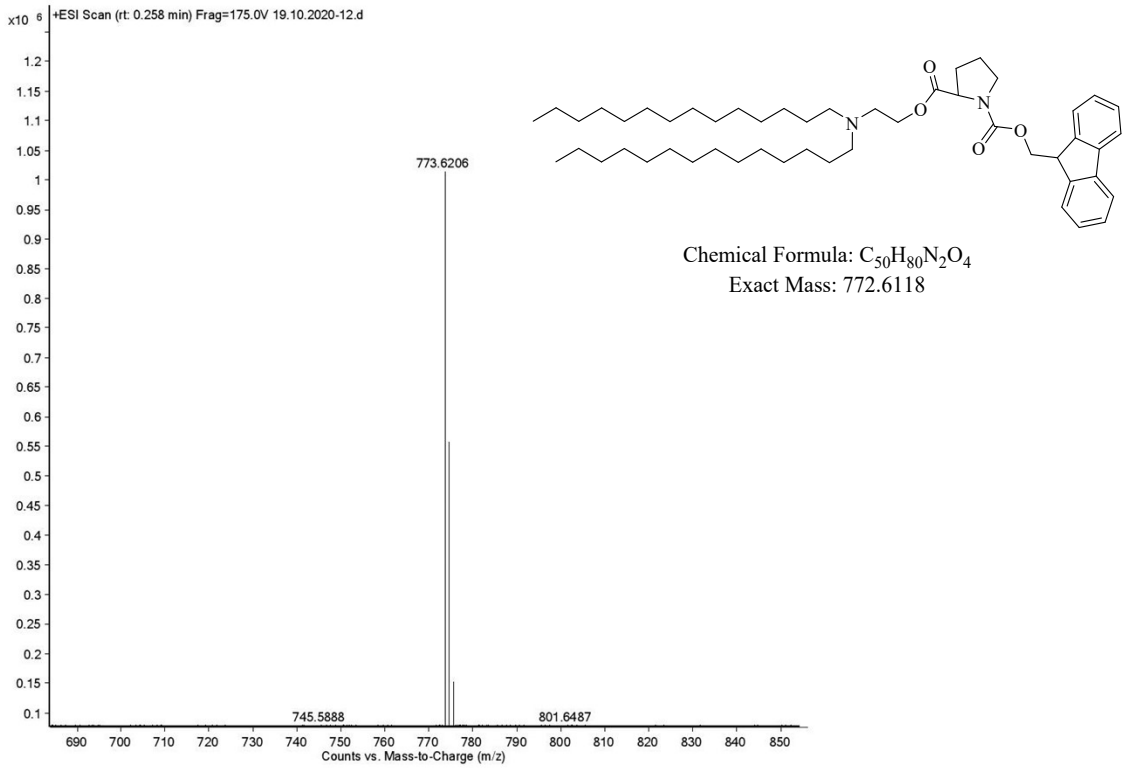


Figure S6: ESI-HRMS Spectrum of compound **1a**

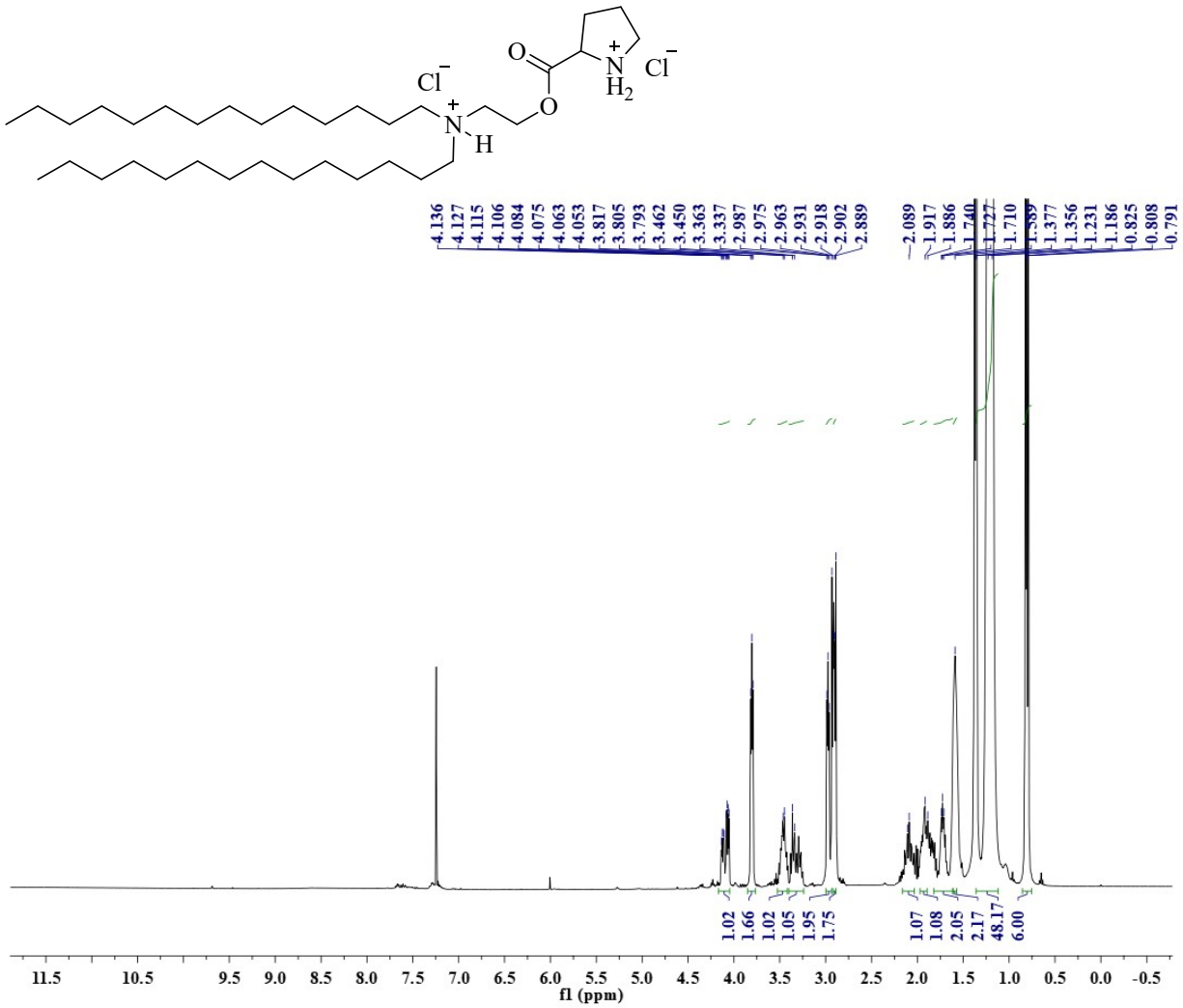


Figure S7: ^{1}H NMR Spectrum of **C14-P**

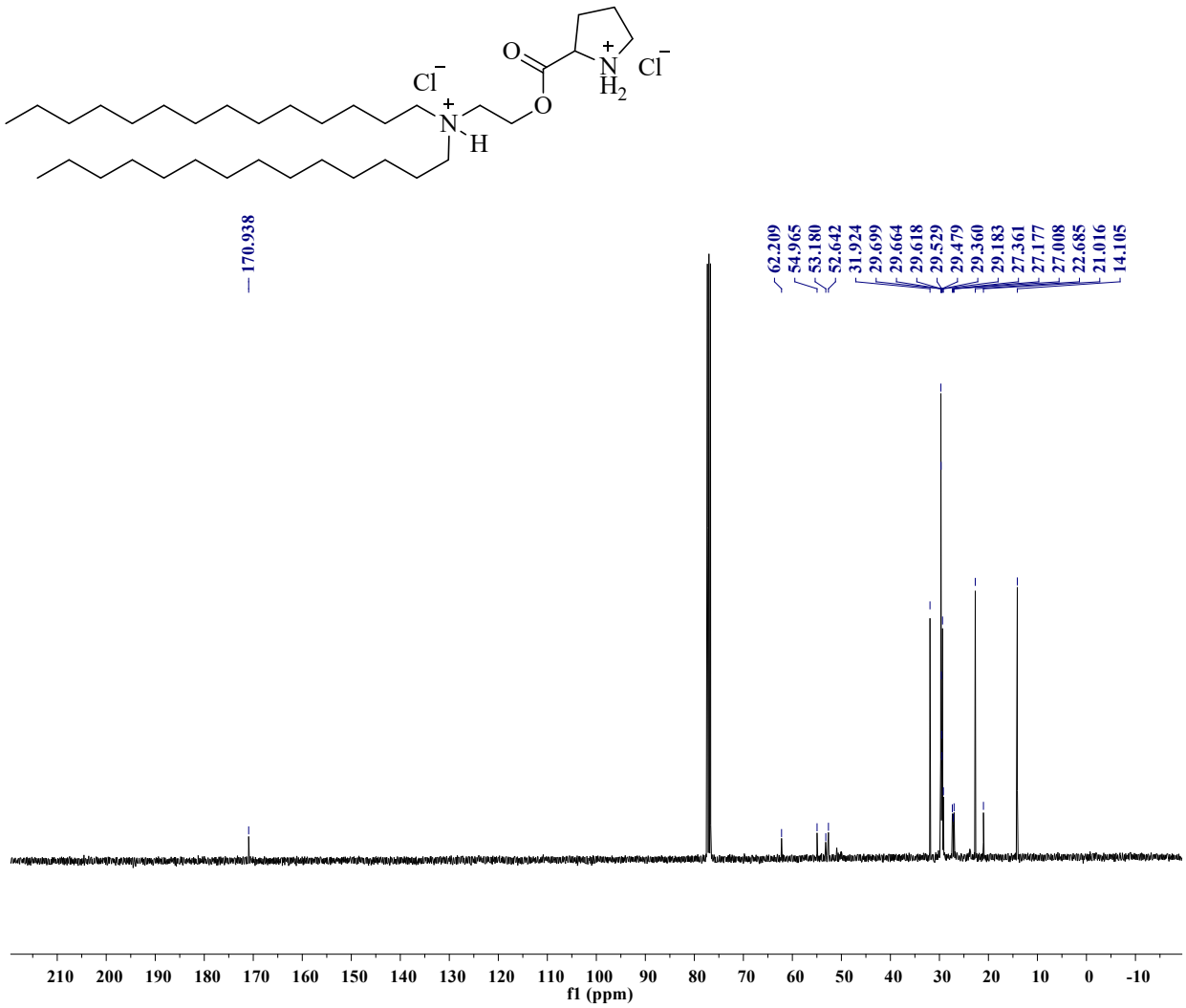
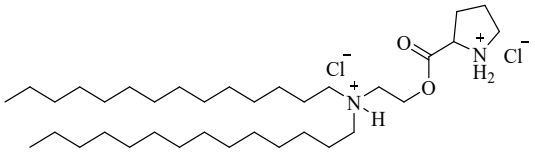


Figure S8: ^{13}C NMR Spectrum of **C14-P**



Chemical Formula: $C_{35}H_{72}N_2O_2^{2+}$
Exact Mass: 552.5583

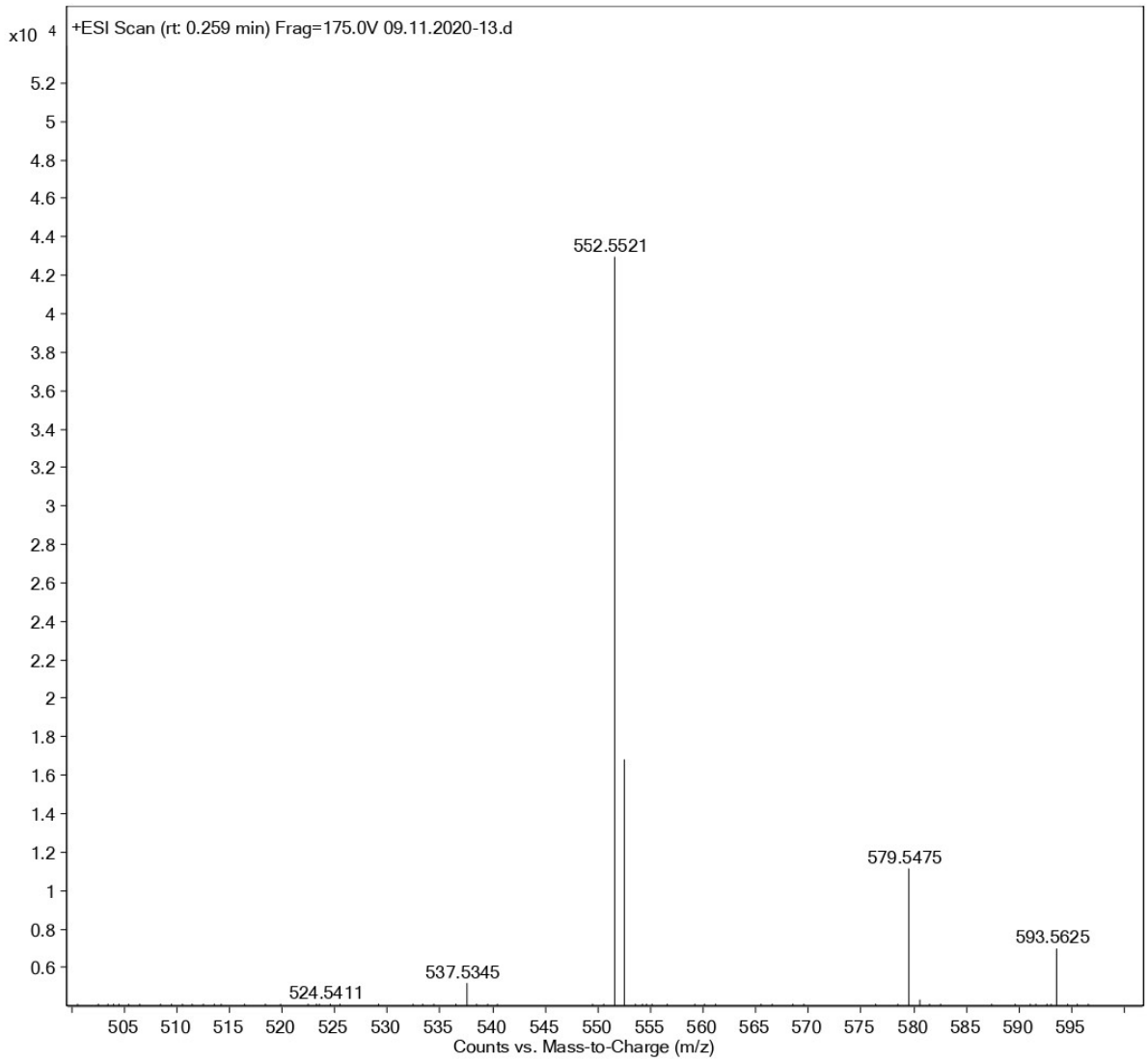


Figure S9: HRMS Spectrum of **C14-P**

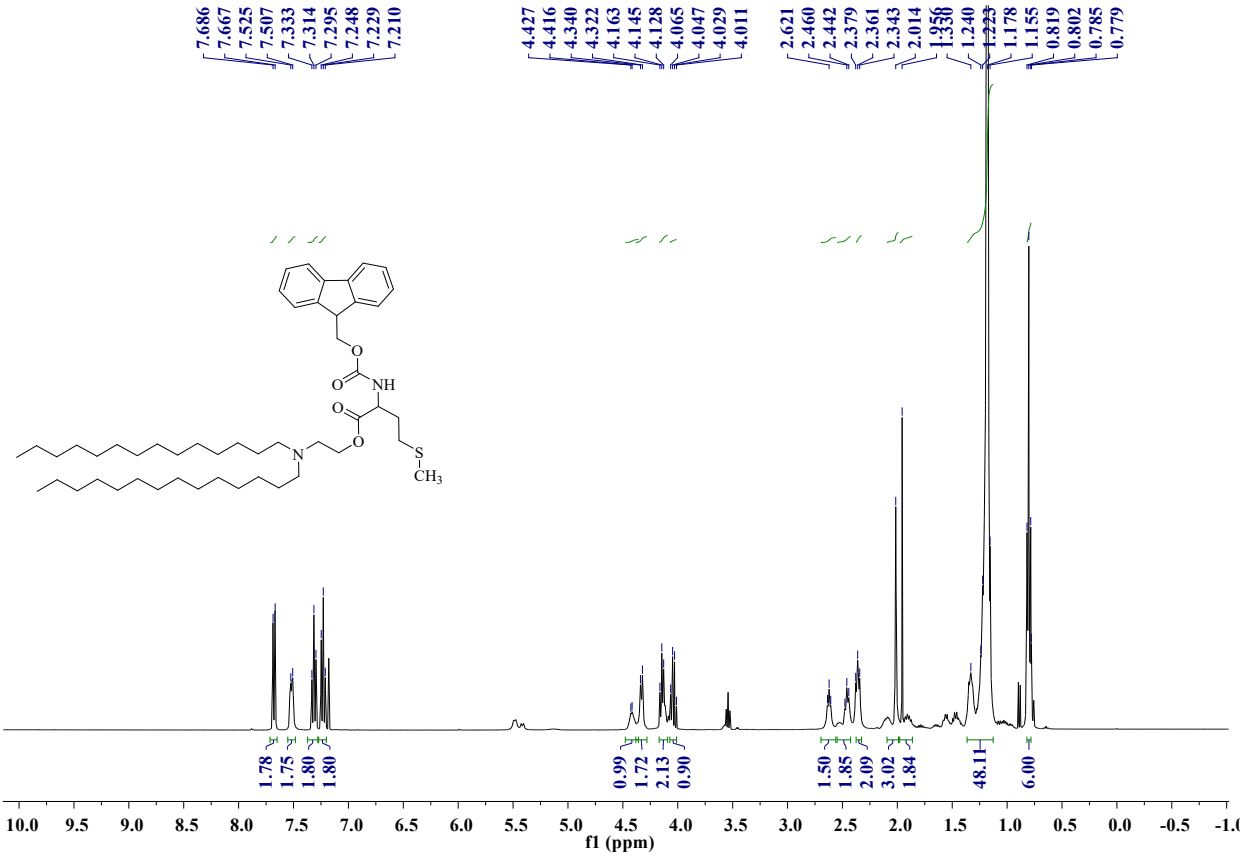


Figure S10: ^{1}H NMR Spectrum of compound **2a**

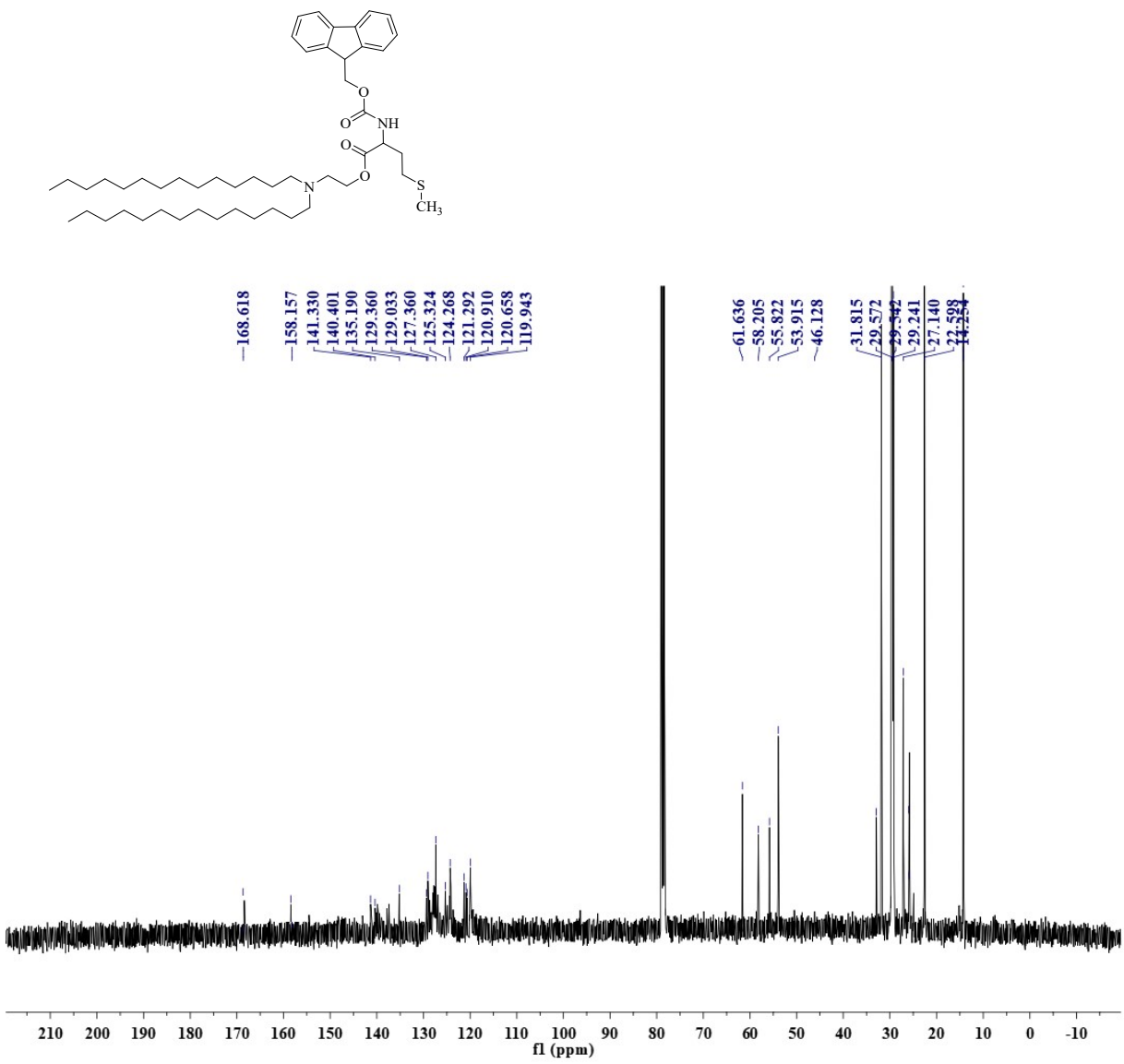


Figure S11: ^{13}C NMR Spectrum of compound **2a**

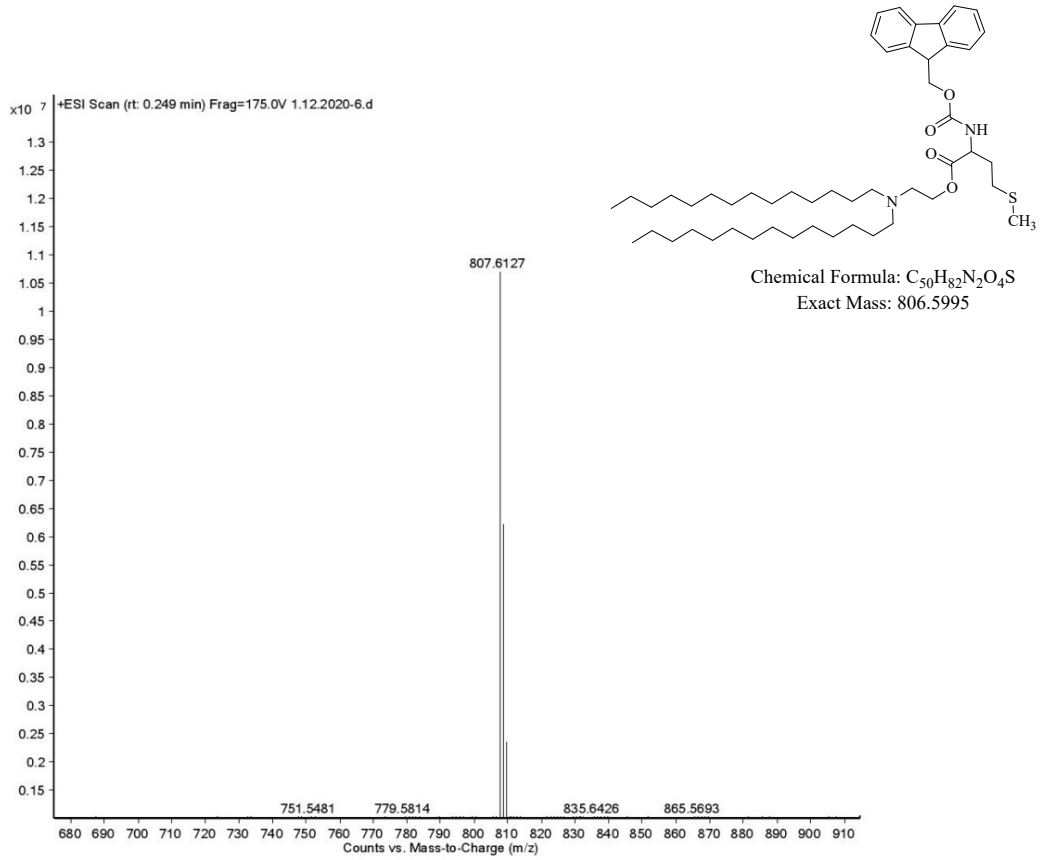


Figure S12: ESI-HRMS Spectrum of compound **2a**

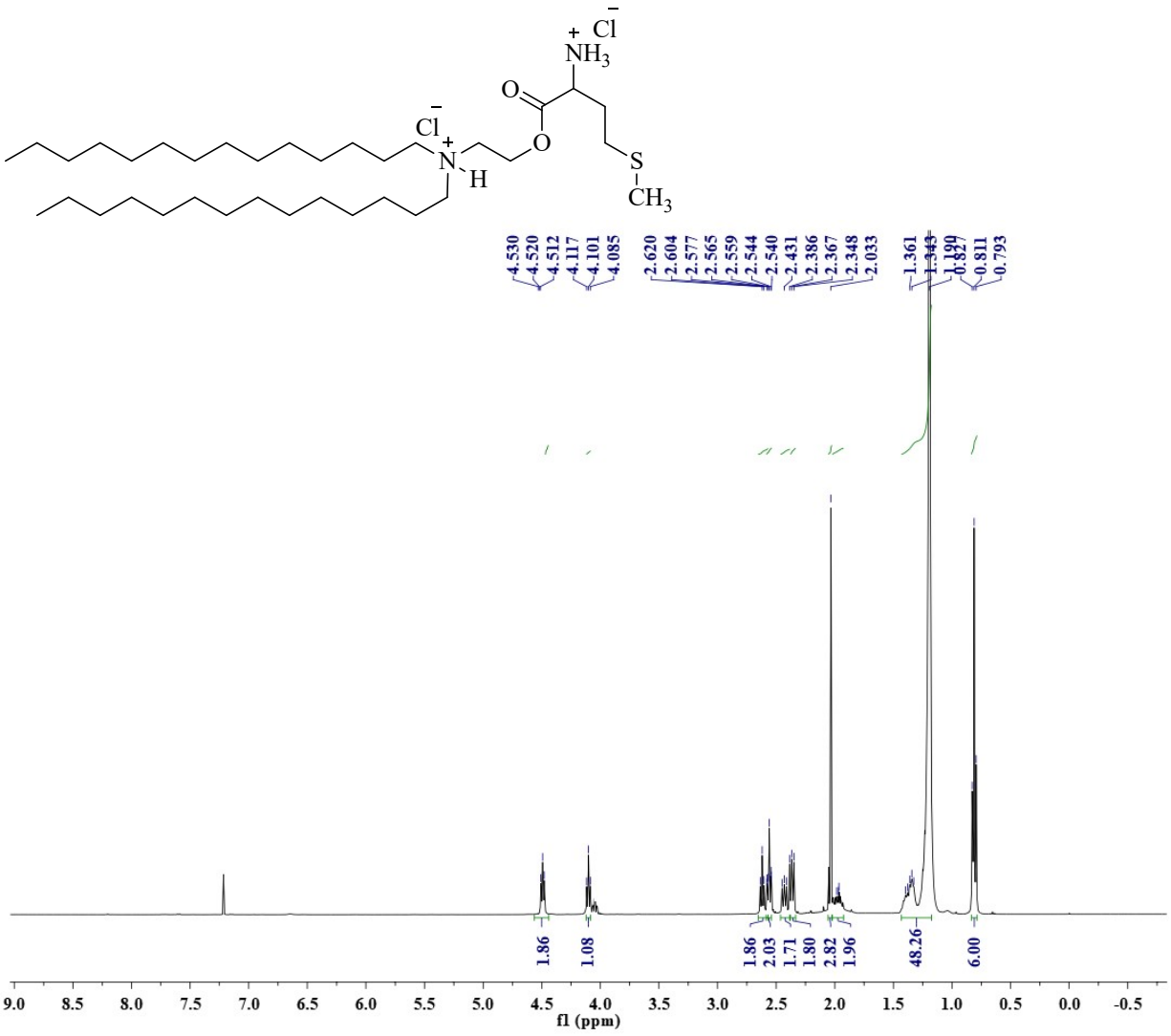


Figure S13: ^{1}H NMR Spectrum of **C14-M**

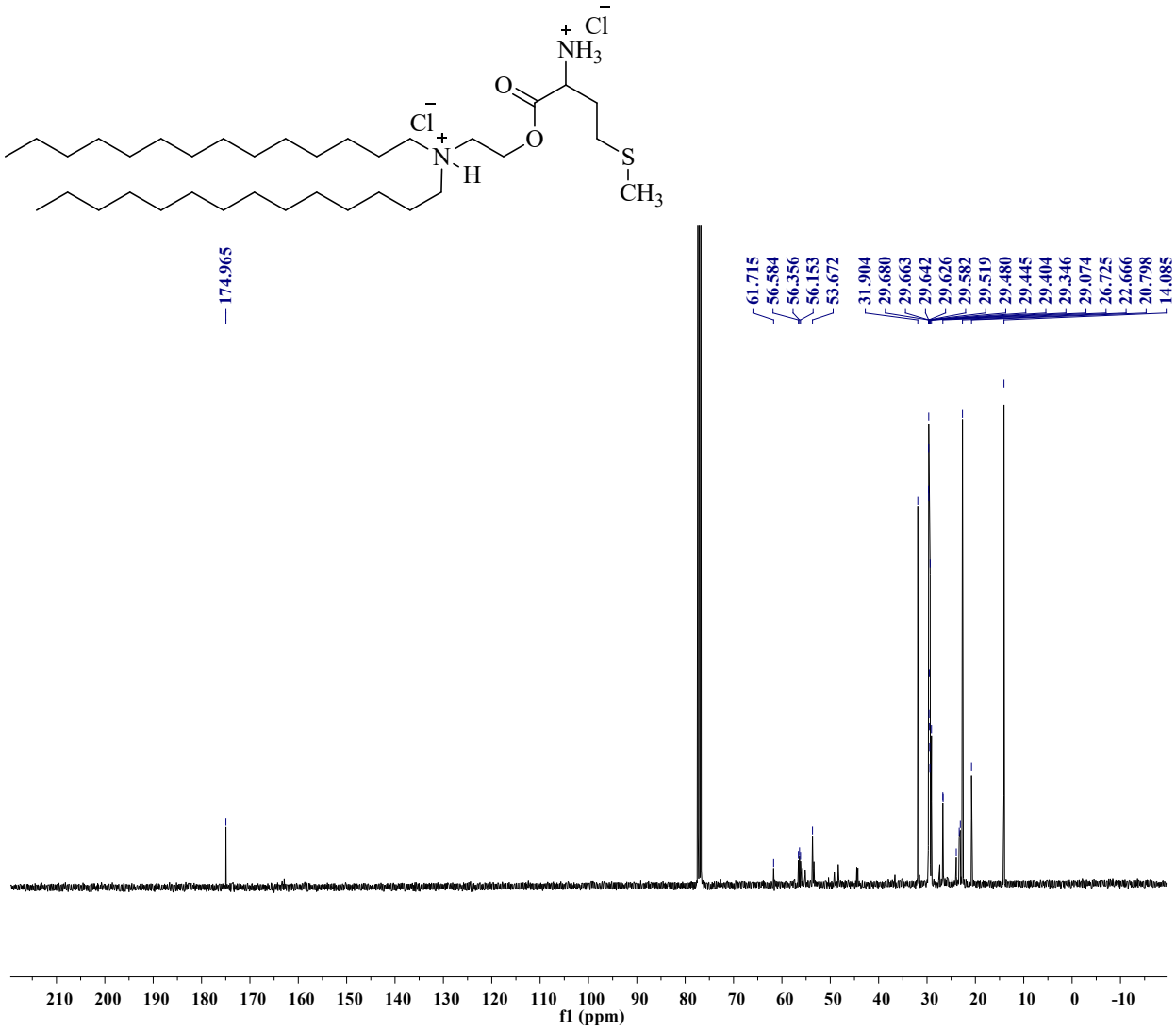
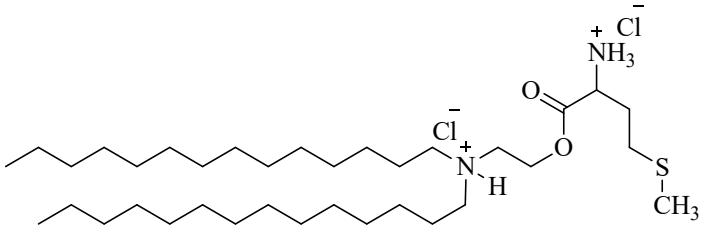


Figure S14: ^{13}C NMR Spectrum of **C14-M**



Chemical Formula: $C_{35}H_{74}N_2O_2S^{2+}$

Exact Mass: 586.5460

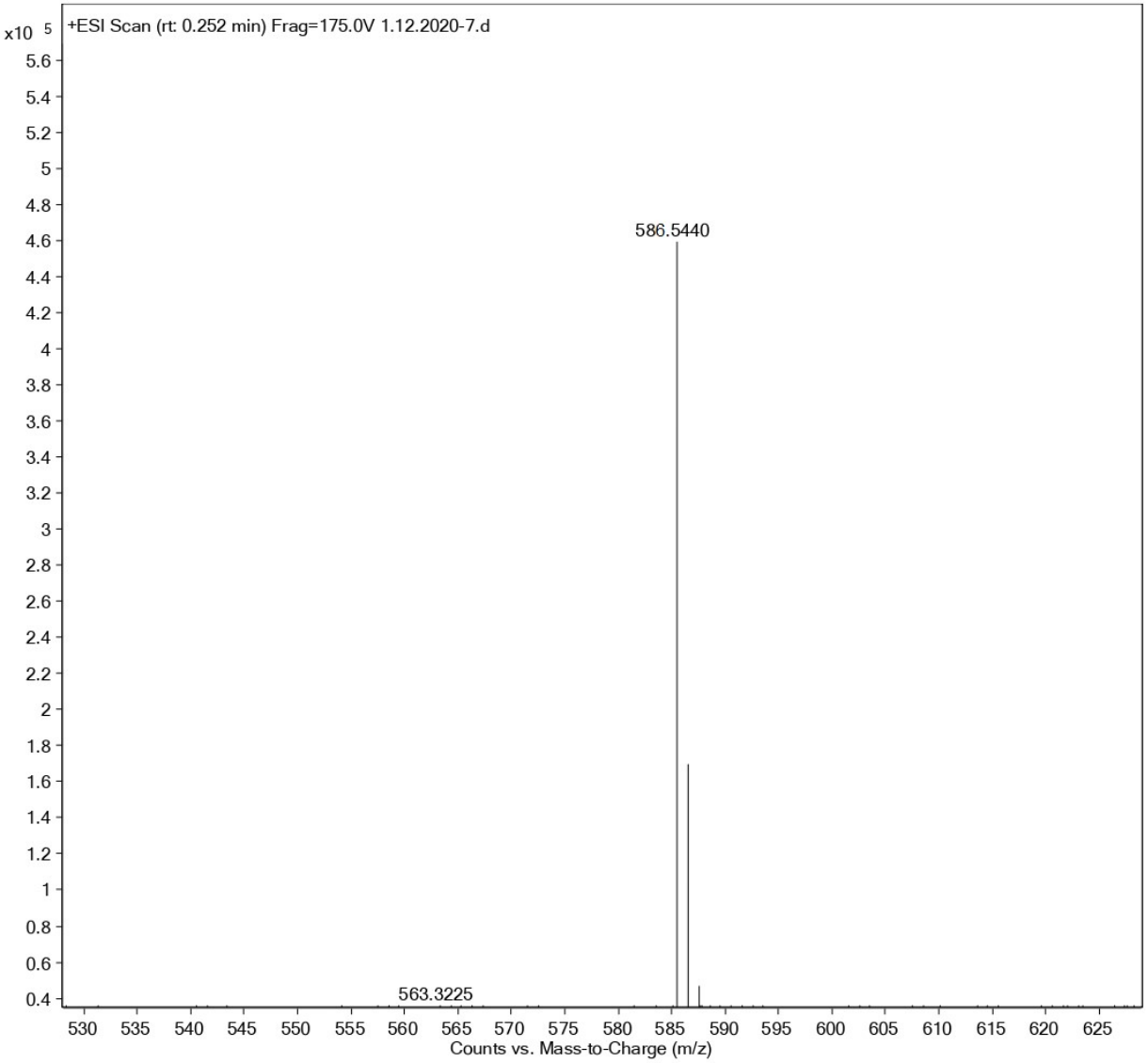


Figure S15: HRMS Spectrum of **C14-M**

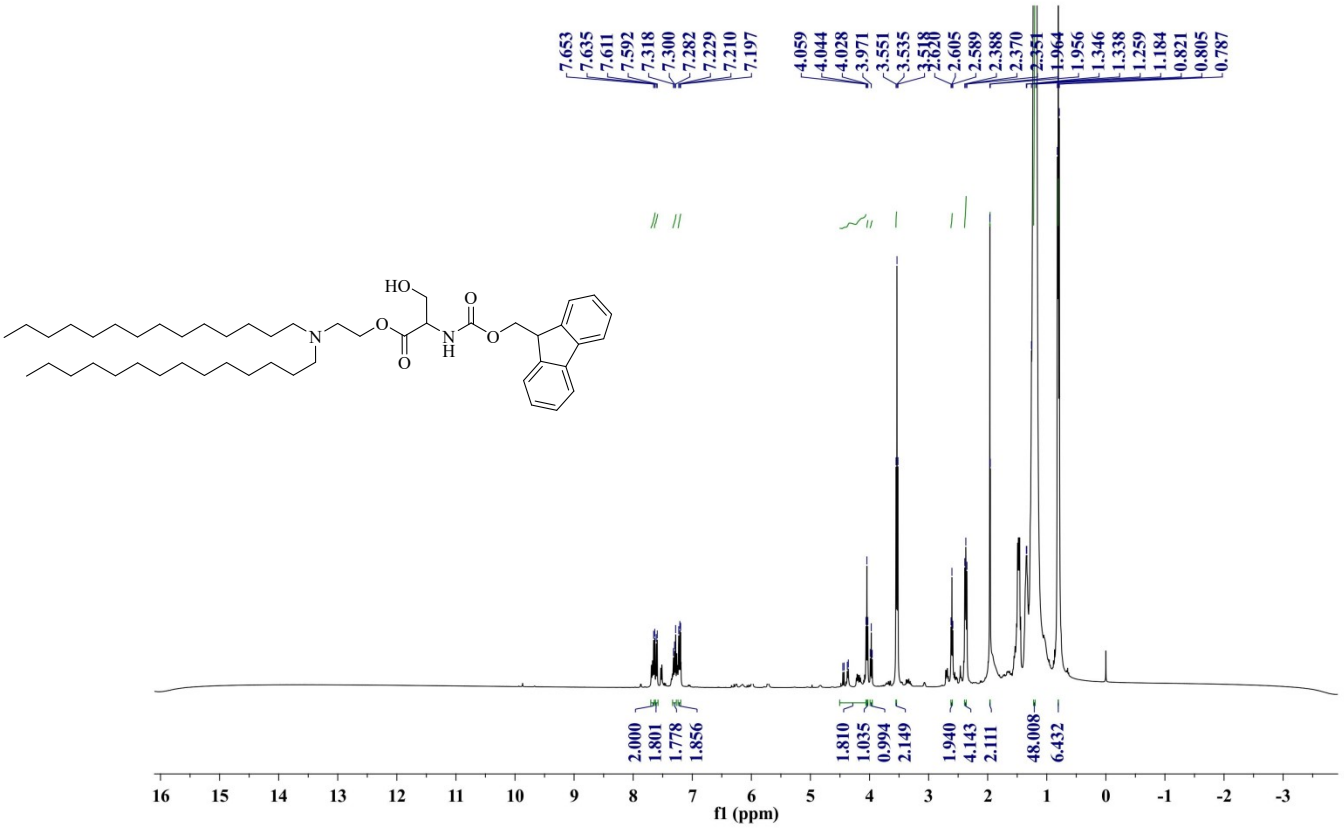


Figure S16: ^{1}H NMR Spectrum of compound **3a**

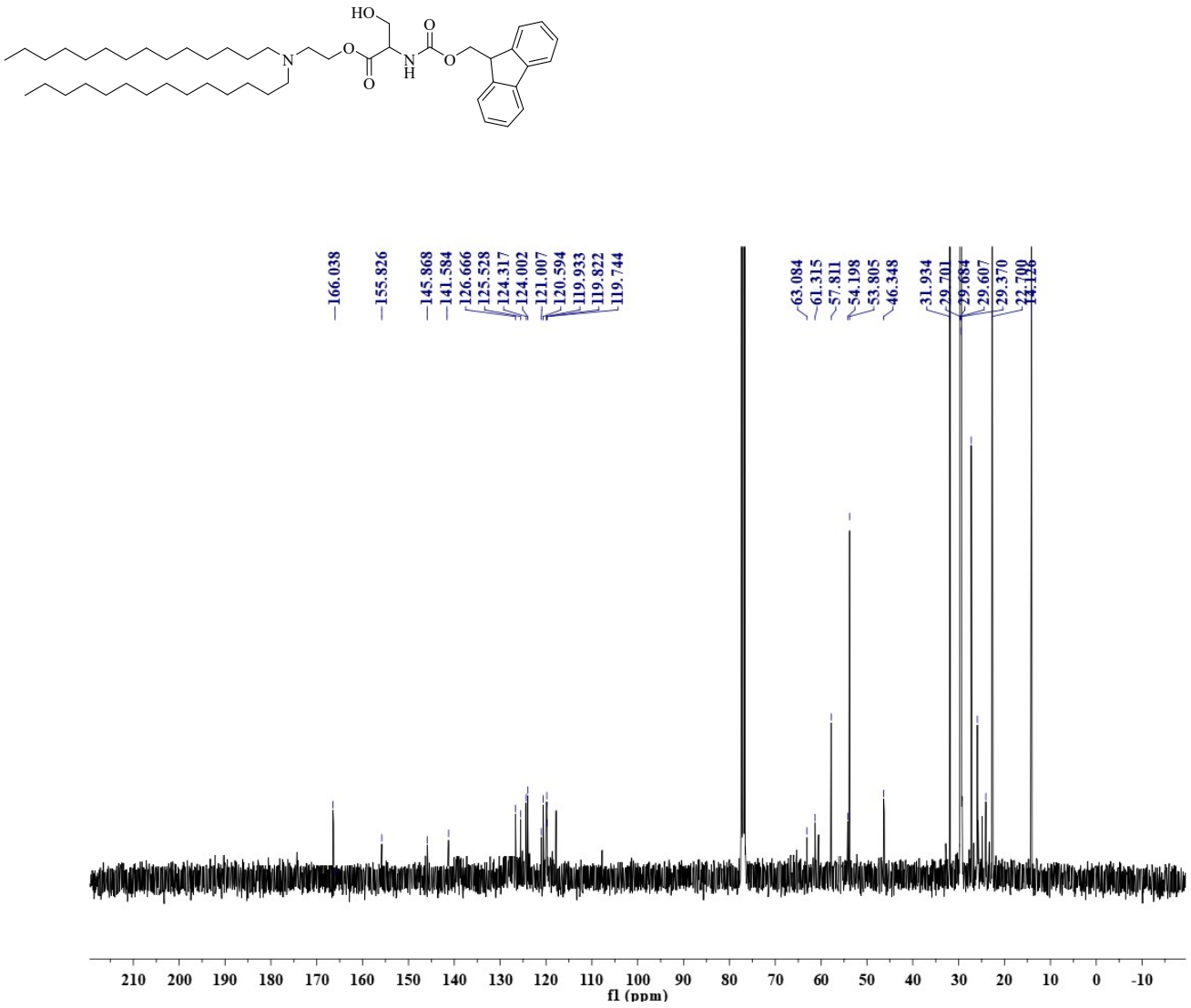


Figure S17: ^{13}C NMR Spectrum of compound **3a**

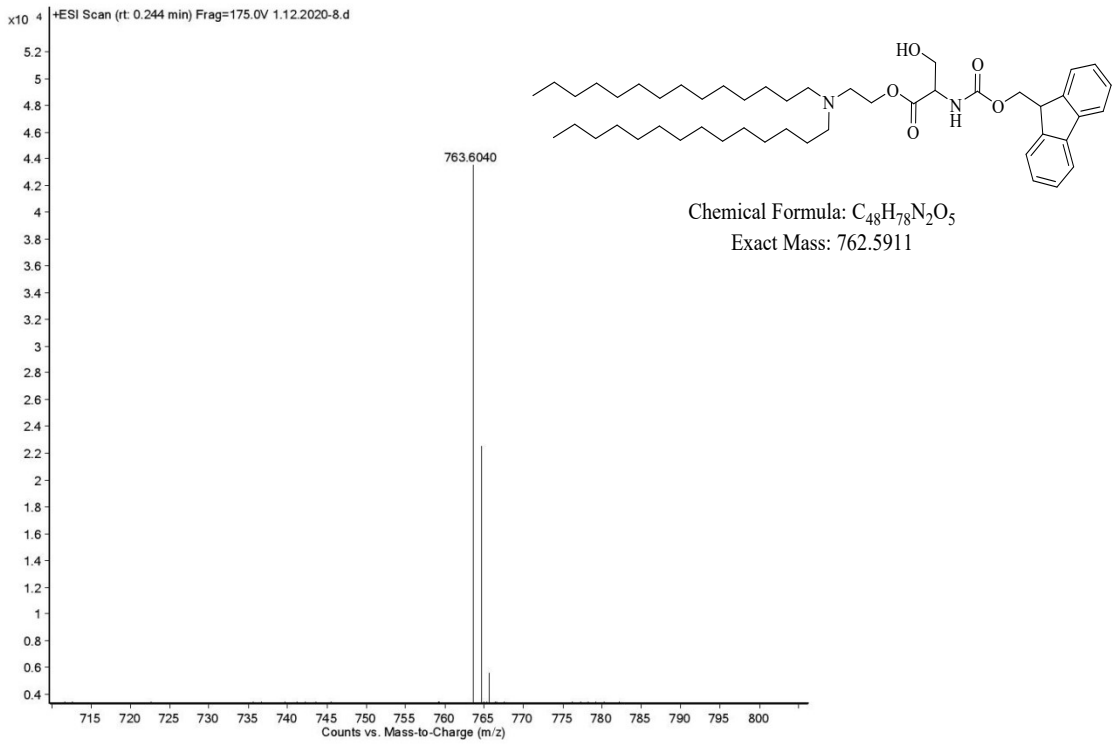


Figure S18: ESI-HRMS Spectrum of compound **3a**

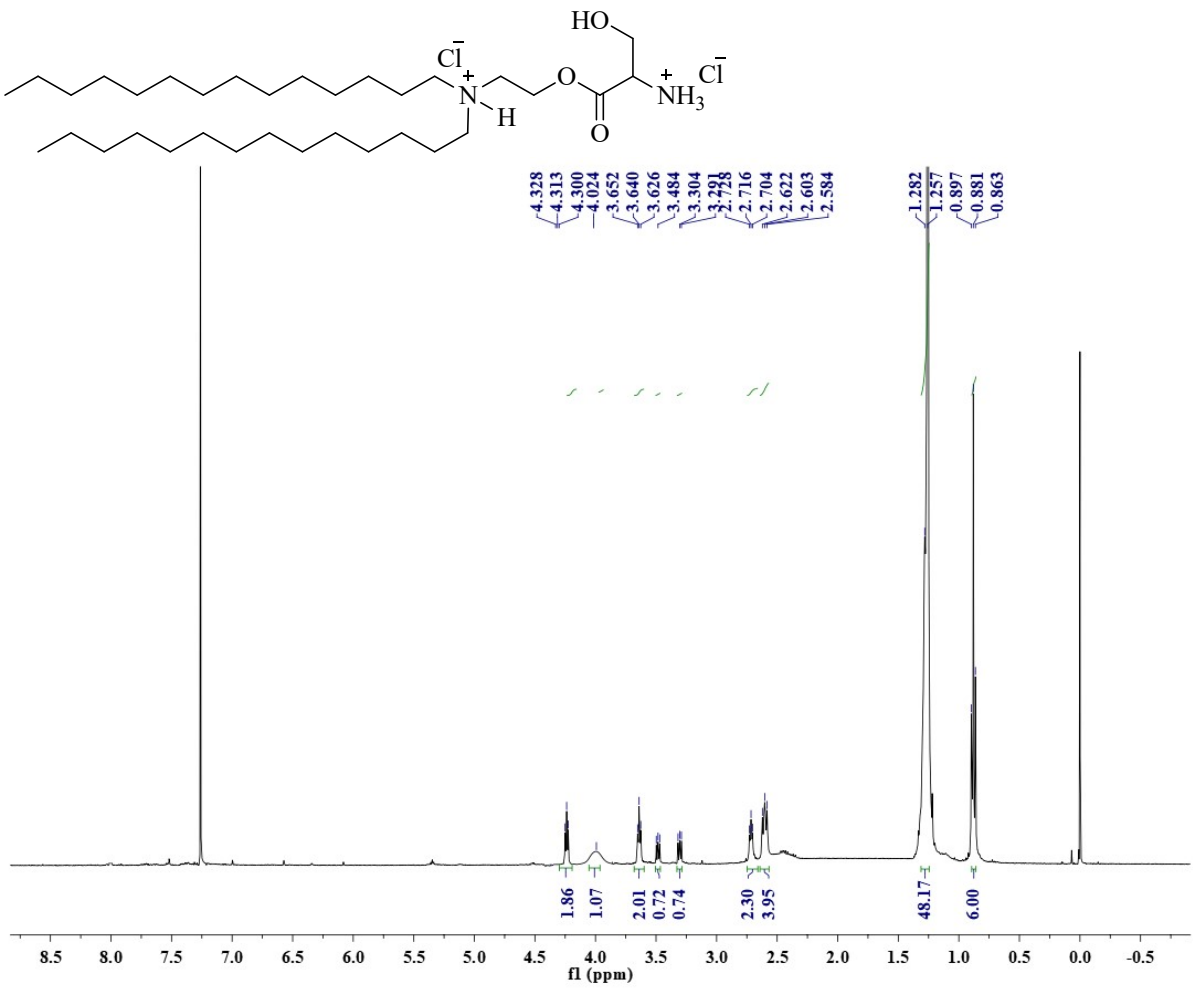


Figure S19: ^{1}H NMR Spectrum of **C14-S**

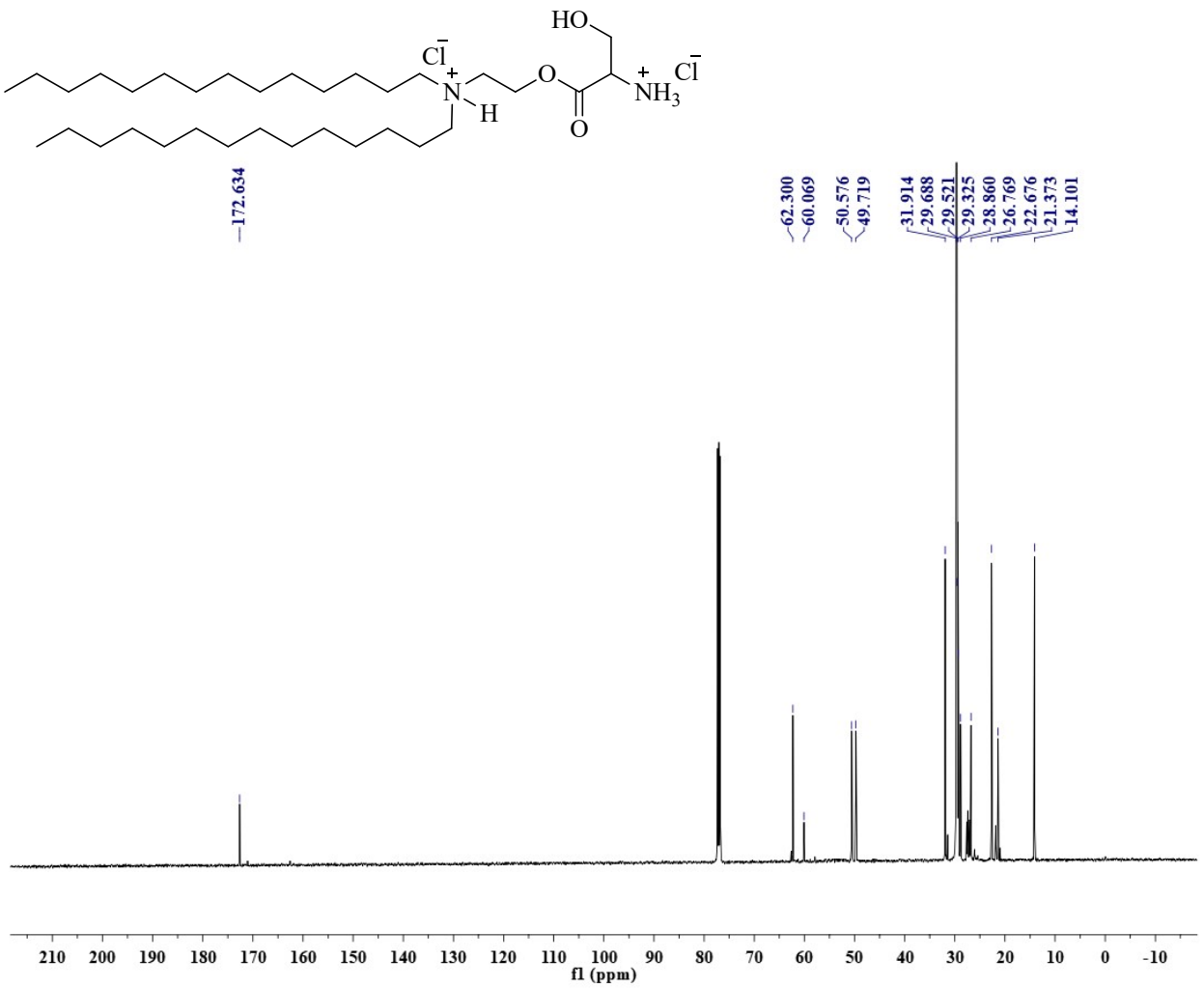
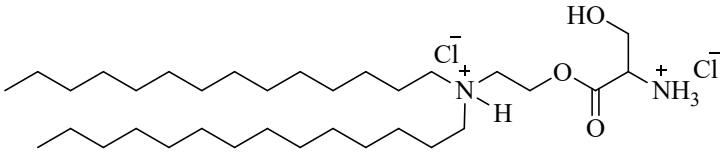


Figure S20: ^{13}C NMR Spectrum of **C14-S**



Chemical Formula: $C_{33}H_{70}N_2O_3^{2+}$

Exact Mass: 542.5375

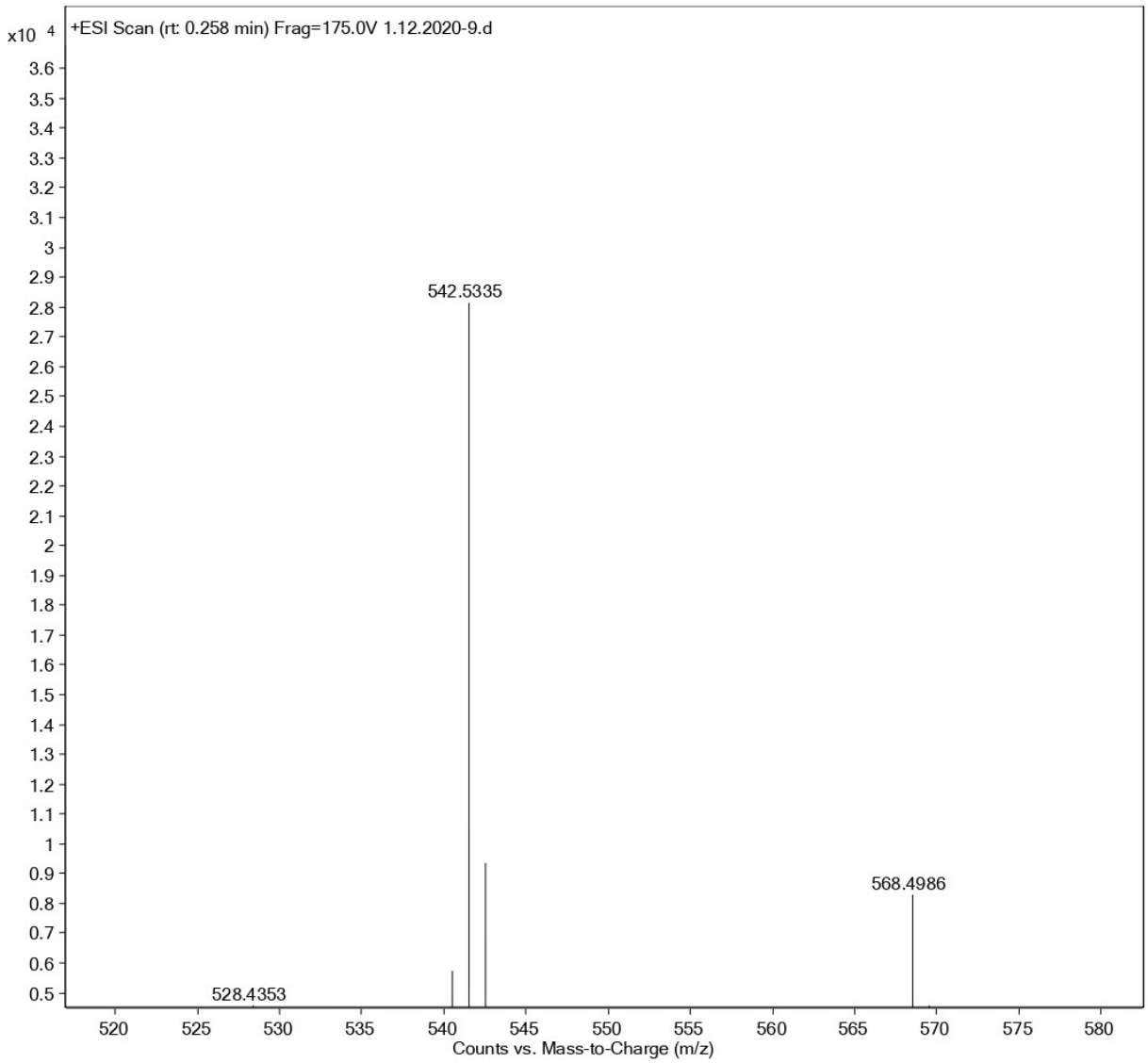


Figure S21: HRMS Spectrum of **C14-S**

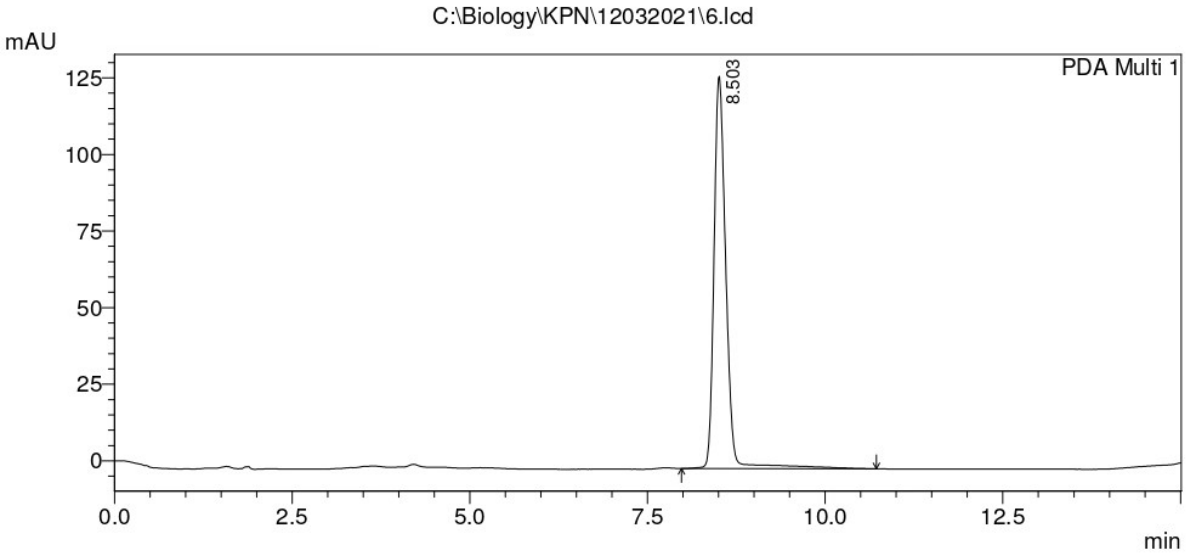


Figure S22: HPLC chromatogram of C14-P

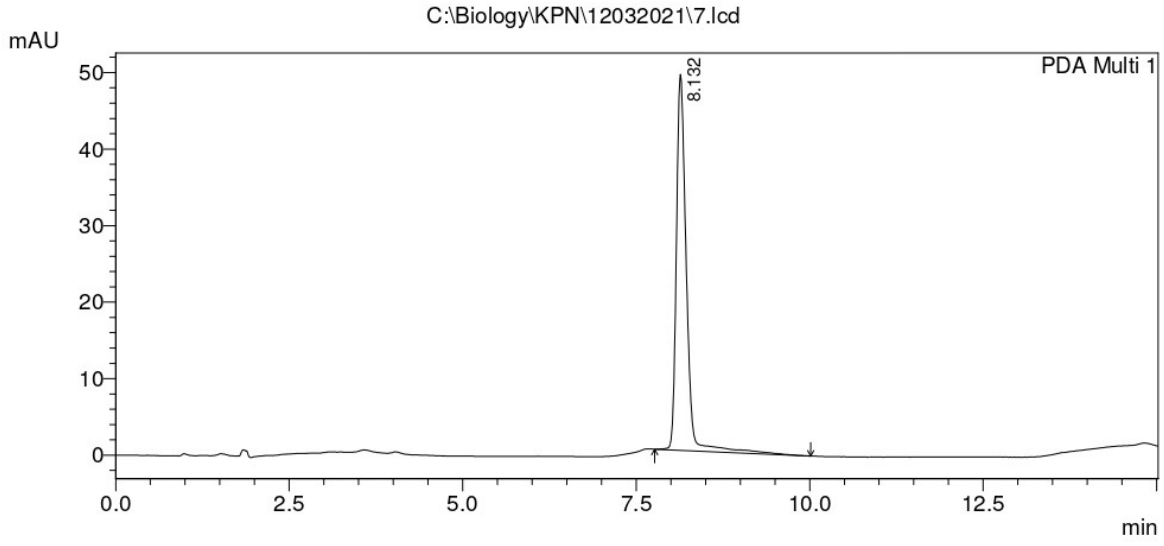


Figure S23: HPLC chromatogram of C14-M

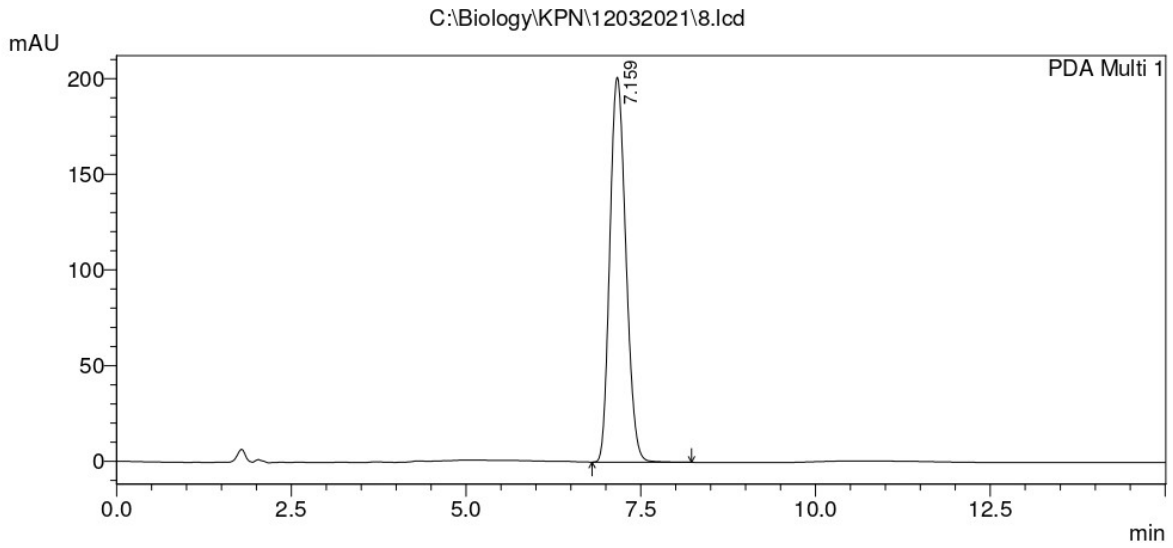


Figure S24: HPLC chromatogram of C14-S

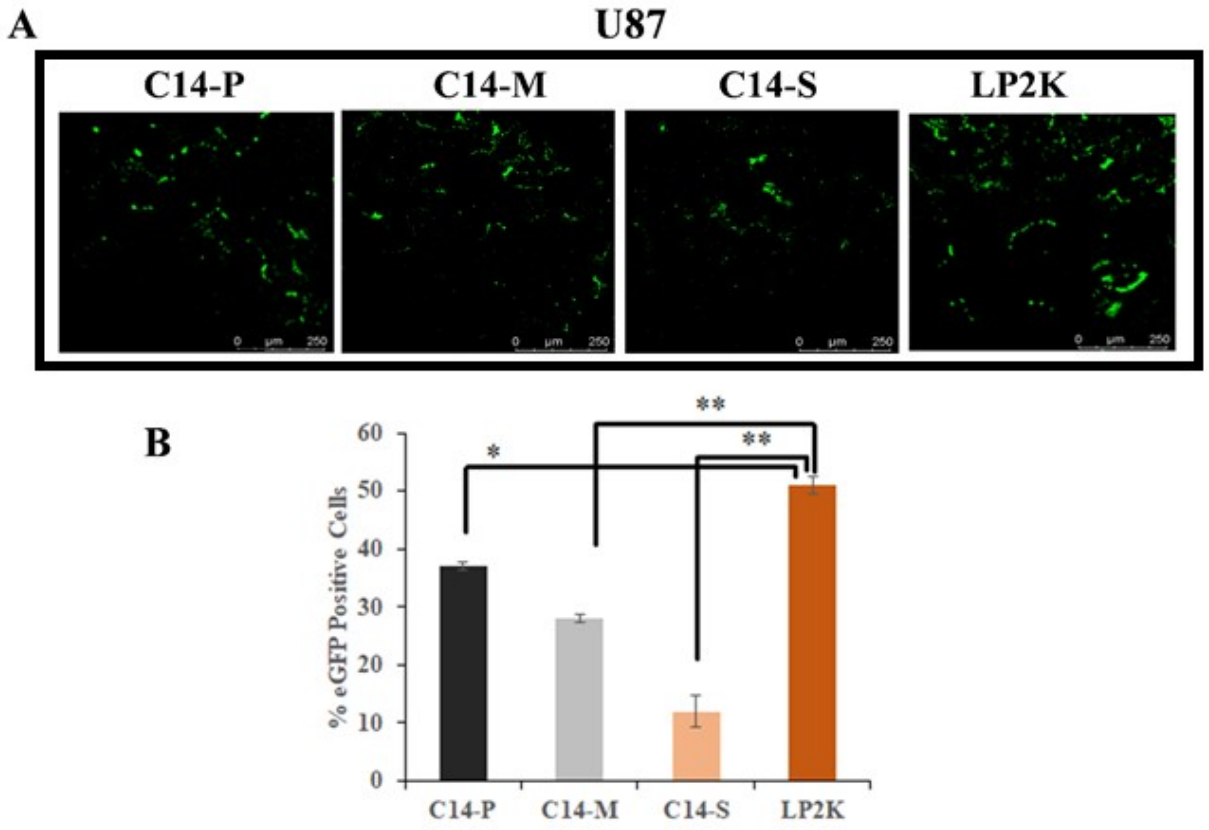


Figure S25. (A) Green fluorescence images of C14-P, C14-M, and C14-S lipoplexes (lipid/pEGFP) at 2:1 N/P ratio in CAL-27; (B) quantitative analysis transfection efficiencies in terms of percentage eGFP positive cells. Statistical analysis was performed by Two-way ANOVA (n=3, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

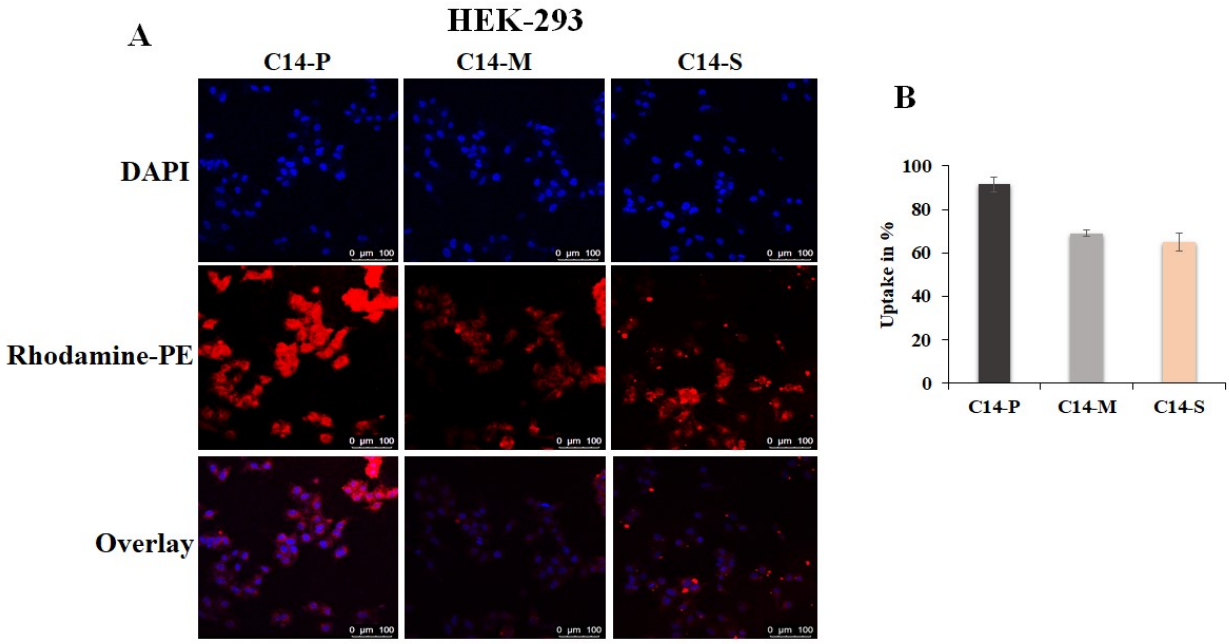


Figure S26: (A) Confocal microscopic images (B) quantitative uptake percentage of HEK-293 cell lines transfected with rhodamine-labelled lipoplexes of lipids C14-P, C14-M, and C14-S prepared at higher *in vitro* transfection lipid/pDNA N/P ratio 2:1; nucleus (DAPI), and cytoplasm (Rh-PE).