Supporting Information

Theranostic Prodrug Vesicles for Imaging Guided Codelivery of Camptothecin and siRNA in Synergetic Cancer Therapy

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Experimental section

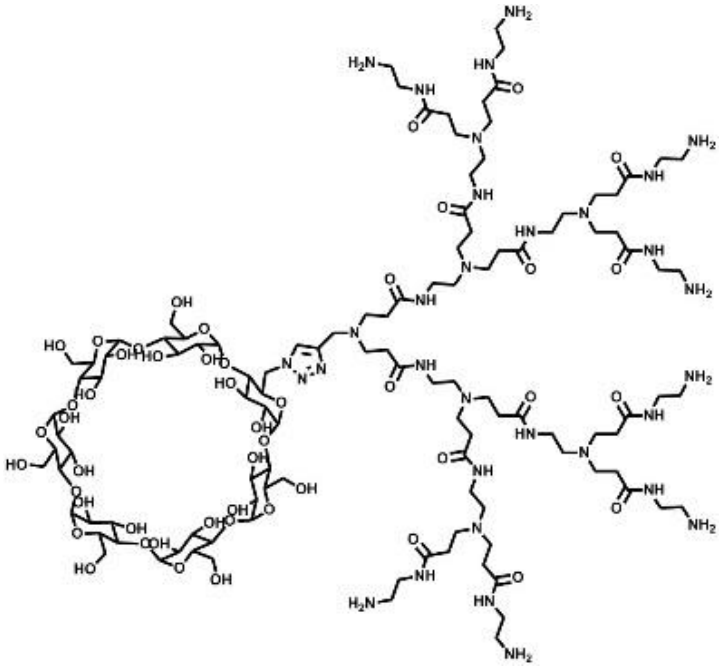
Materials

THF and toluene were refluxed with sodium overnight before uses. DCM was refluxed with CaH_2 overnight before uses. DMF was refluxed with CaH_2 overnight and distilled under the reduced pressure before uses. Ethanol was refluxed with magnesium and iodine overnight and distilled before uses. siPlK1 with the sequence of (sense strand, 5-UGAAGAAGAU-CACCCUCCUUAdTdT-3; antisense strand, 5-UAAGGAGGGUGAUCUUCUUCAdTdT-3) and scrambled siRNA (siNonsense) with the sequence of (sense strand, 5-UUCUCCGAACGUGUCACGUdTdT-3; antisense strand, 5-ACGUGACACGUUCGGAGAAdTdT-3) were supplied by Su Zhou Ribo Life Science Co. Ltd. China. Cy3 labeled siRNA with the sequence of (sense strand, 5'-UUCUCCGAACGUGUCACGUdTdT-3'; antisense strand, 5'-ACGUGACACGUUCGGAGAAdTdT-3', labeled with Cy3 at 5 position) was supplied from Shanghai Genepharma China. Adamantanecarboxylic acid chloride, *L*-ascorbic acid sodium salt, N,N-diisopropylethylamine (DIPEA), RNase A, 4-dimethylaminopyridine (DMAP), di-tert-butyl pyrocarbonate, 2,2'-dithiodiethanol, ethylenediamine, hydrazine hydrate (70%-80 %), methyl acrylate, 4-nitro-1,8-naphthalic anhydride, palladium on carbon, propargylamine, sodium azide, and triphosgene were obtained from Sigma Aldrich and used without further purification. Dendrimer H2 and 4-nitro-N-(2-aminoethyl)-1,8-naphthalimide were synthesized according to literature reported procedures.[S1,S2]

Characterizations

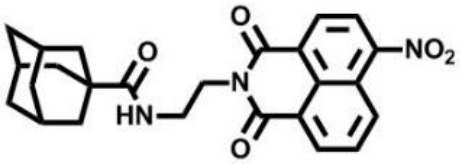
^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectrum was measured on a Bruker BBFO 400 spectrometer using deuterated chloroform ($CDCl_3$) or deuterated dimethyl sulphoxide ($DMSO\text{-}d_6$) as the solvents. The electronic spray ionization (ESI) mass spectra were recorded on a ThermoFinnigan LCQ quadrupole ion trap mass spectrometer. High-resolution mass spectrometry (HR-MS) was performed on a Waters Q-tof Premier MS spectrometer. Transmission electron microscopy (TEM) images were collected on JEM-1400 (JEOL) at 100 kV. The fluorescence emission spectra were recorded on a Shimadzu RF-5301pc fluorescence spectrophotometer. UV–vis spectra were recorded from Shimadzu UV-3600 spectrophotometer. Zeta potential values were determined by Malvern Instruments Zetasizer Nano-S at 25 ^{o}C. DLS size distributions were measured on a Malvern Instruments Zetasizer Nano-S at 25 ^{o}C. MTT assay was recorded on a Tecan Infinite M200. The drug release behavior was determined by HPLC (Shimadzu 20A). CLSM was recorded on a Carl Zeiss LSM 800. Gel retardation assay was recorded on Horizontal Electrophoresis system (Bio-Rad, Singapore).

Synthesis of compound H1



Dentrimer H2 (1.6 g, 1 mmol) and β-cyclodextrin-N_3 (1.6 g, 1.38 mmol) were dissolved in H_2O (40 mL). $CuSO_4 \bullet 5H_2O$ (750 mg, 3 mmol) was added into the solution. The solution was then stirred at room temperature before *L*-ascorbic acid sodium salt (2 g, 10 mmol) was added in portions over a period of 1 h under the protection of inert N_2 gas. The solution was stirred under N_2 atmosphere at 50 °C for 48 h. After cooling down to room temperature, the mixture was filtered to remove copper(I) salt, and the filtrate was dialysis against water for five days using dialysis membrane of MWCO 2000. The water was removed by lyophilization to give the compound H1 as beige solid (920 mg, 32.8 %). ^{1}H NMR (400 MHz, D_2O, 298 K): δ 8.02 (s, 1H), 4.9~5.3 (m, 14H), 2.32~4.05 (m, 155H). HRMS (TOF) m/z $[M+H]^+$, calcd for $C_{115}H_{214}N_{32}O_{48}$, 2812.5367; found, 2812.5264.

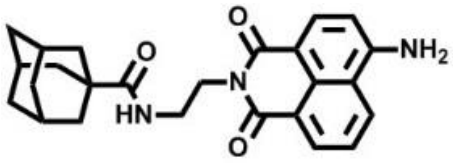
Synthesis of compound 4



To a solution of compound **5** (1.85 g, 6.5mmol) in dry DCM (20 mL), TEA (1.3 g, 13mmol) was added, and the mixture was allowed stirred under the ice bath. Adamantanecarboxylic acid chloride (2 g, 10mmol) dissolved in dry DCM (10 mL) was added dropwise into the solution over 30 min. The solution was then allowed to stir at room temperature overnight. Following which, the solution was washed with water for three times. The organic layer was collected and dried over anhydrous sodium sulfate. After the removal of the solvent, the crude product was purified with silica gel chromatography using hexane/ethyl acetate (v/v, 1:1) as the eluent. The solvent was removed to afford the product **4** as beige solid (2.4 g, 82.8%). ^{1}H

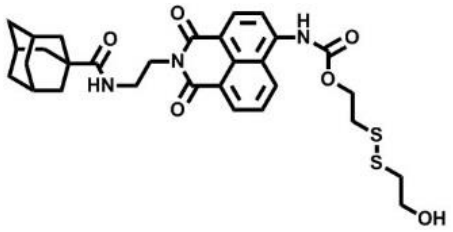
NMR (400 MHz, $CDCl_3$, 298 K): δ 8.85 (d, J 8.8, 1H), 8.73 (d, J 7.2, 1H), 8.69 (d, J 8, 1H), 8.41 (d, J 8, 1H), 8.00 (t, J 16, 1H), 6.17 (s, 1H), 4.42 (m, J 10, 2H), 3.69 (s. 2H), 1.96 (s, 3H), 1.78~1.68 (m, J 28.8, 12H). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ 178.6, 164.0, 163.0, 149.7, 132.6, 130.0, 129.5, 129.2, 126.7, 123.9, 123.7, 122.9, 40.5, 40.1, 39.0, 36.4, 28.0. HRMS (TOF) m/z $[M+H]^+$, calcd for $C_{25}H_{25}N_3O_5$, 448.1872; found, 448.1877.

Synthesis of compound 3



To a stirred cloudy solution of compound **4** (1 g, 2.23mmol) in ethanol, catalytic amount palladium on carbon was added. The hydrazine hydrate (70-80 %, 2mL) was then added, and the solution was stirred under the reflux overnight. After cooling down to room temperature, the solution was filtered to remove Pd@C, and the filtrate was collected and evaporated under the reduced pressure to afford the crude product. The crude product was purified by silica gel chromatography using hexane/ethyl acetate (v/v, 1:1) as the eluent. The solvent was removed to afford the product **3** as light yellow solid (700 mg, 75%). ^1H NMR (400 MHz, $CDCl_3$, 298 K): δ 8.60 (d, J 7.2, 1H), 8.41 (d, J 8, 1H), 8.12 (d, J 8.4, 1H), 7.64 (t, J 15.6, 1H), 6.87 (d, J 8, 1H), 6.53 (s, 1H), 5.05 (s, 2H), 4.41 (m, J 10.8, 2H), 3.63 (dd. J 15.2, 2H), 1.98 (s, 3H), 1.77 (d, J 2.4, 6H), 1.66 (t, J 32.8, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ 178.5, 165.2, 164.6, 134.1, 131.7, 130.0, 127.3, 124.9, 122.8, 120.0, 111.5, 109.5, 40.5, 39.9, 39.1, 36.6, 28.2. HRMS (TOF) m/z $[M+H]^+$, calcd for $C_{25}H_{27}N_3O_3$, 418.2131; found, 418.2148.

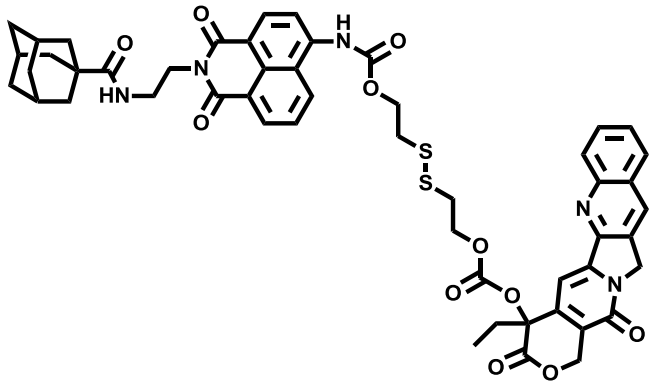
Synthesis of compound 2



The mixture of compound **3** (208 mg, 0.5mmol), and DIPEA (194 mg, 1.5mmol) in anhydrous toluene (10 mL) was added dropwise to a solution of triphosgene (445 mg, 1.5 mmol) in toluene (8 mL). The mixture was refluxed for 3 h and then allowed to cool down to room temperature. The solvent was removed under the reduced pressure. A solution of 2,2'-dithiodiethanol (1.23g, 4 mmol) in anhydrous THF (30 mL) was added to the flask. The reaction mixture was stirred under room temperature overnight. The solvent was first evaporated, followed by the addition of DCM (100 mL) and water (100 mL). Then, the organic layer was collected, and dried using anhydrous Na_2SO_4. After the removal of the solvent, the crude product was purified over silica gel using hexane/ethyl acetate (v/v, 3:1) as

the eluent to remove the impurity and then hexane/ethyl acetate (v/v, 1:1) as the eluent to yield compound **3** as a light yellow solid (300 mg, 65.5%). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ 8.59 (m, J 13.6, 2H), 8.36 (d, J 8, 1H), 8.26 (d, J 8.4, 1H), 7.88 (s, 1H), 7.73 (t, J 15.6, 1H), 6.37 (s, 1H), 4.57 (t, J 12.8, 2H), 4.41 (m, J 10.8, 2H), 3.95 (d. J 4, 2H), 3.65 (dd, J 15.6, 2H), 3.08 (t, J 12.8, 2H), 2.96 (t, J 11.6, 2H), 2.35 (s, 1H), 1.97 (s, 3H), 1.73 (t, J 2.8, 6H), 1.66 (t, J 36.4, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ 178.5, 164.6, 164.3, 153.1, 139.4, 132.7, 131.5, 129.0, 16.6, 123.4, 117.3, 117.0, 63.9, 60.0, 41.6, 40.5, 39.5, 39.4, 39.1, 37.6, 36.5, 28.1. HRMS (TOF) m/z $[M+H]^+$, calcd for $C_{30}H_{35}N_3O_6S_2$, 598.2046; found, 598.2047.

Synthesis of compound G1



Camptothecin (100 mg, 0.287 mmol) and DMAP (110 mg, 0.9 mmol) were dispersed in dry DCM (15 mL). Then, triphosgene (30 mg, 0.1 mmol) was added into the mixture, and the mixture was allowed to stir for 30 min. During this period, the mixture turned from turbid to transparent. Compound **2** (158 mg, 0.264 mmol) dissolved in dry DCM (5 mL) was dropwise added into the solution. The solution was stirred under the room temperature for overnight. The solution was washed with water for 3 times and dried over anhydrous sodium sulfate. The solvent was removed under the reduced pressure to give crude product. The crude produce was purified by silica gel chromatography using ethyl acetate as eluent. The solvent was removed under the vacuum to afford the pure product as light yellow powder (245 mg, 95.35%). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ 8.55 (t, J 14.4, 2H), 8.41 (s, 1H), 8.29 (d, J 9.2, 1H), 8.20 (dd, J 22.8, 2H), 8.04 (s, 1H), 7.96 (d, J 8.4, 1H), 7.85 (t, J 16.4, 1H), 7.68 (dd, J 20.4, 2H), 7.30, (s, 1H), 6.51 (s, 1H), 5.2~5.05 (dd, J 26, 4H), 4.6~4.3 (m, 6H), 3.69 (d. J 26.4, 2H), 3.08 (m, J 58, 4H), 2.185 (t, J 36.8, 1H), 2.13 (ddd, J 68.8, 1H), 1.98 (s, 3H), 1.79 (s, 6H), 1.66 (m, J 38.8, 6H), 0.98 (t, J 14.8, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ = 178.45, 167.22, 164.78, 164.35, 156.91, 153.88, 152.89, 152.02, 148.88, 146.47, 145.70, 139.56, 132.54, 131.33, 130.89, 129.55, 128.96, 128.33, 127.06, 126.42, 123.00, 119.70, 117.49, 116.71, 95.88, 77.93, 66.61, 62.76, 49.96, 40.49, 39.42, 39.04, 37.67, 36.56, 32.29, 31.71, 28.13, 7.65. HRMS (TOF) m/z $[M+H]^+$, calcd for $C_{51}H_{49}N_5O_{11}S_2$, 972.2948; found, 972.2905.

Preparation of vesicles and siRNA-loaded vesicles

Guest prodrug G1 was dissolved in DMSO to prepare a stock solution with the concentration of 4 mM. The host dendritic cyclodextrin H1 was dissolved in deionized water to obtain a stock solution with the concentration of 2 mM. The stock solution of the host molecule was diluted 20 folds to 100 μM. Upon the sonication, guest stock solution (25 μL) was slowly added into the diluted host solution (975 μL) over 5 min to obtain a cloudy solution. The solution was allowed to stand at room temperature overnight for sufficient assembly, followed by dialysis against water for 2 h to remove trace DMSO. The obtained solution was stored at 4 °C. To prepare the siRNA-loaded vesicles, siPlK1 was added into the freshly prepared vesicle solution, followed by shaking to mix well, and then allowed to stay at 37 °C over 30 min for sufficient binding.

Gel retardation assay and siRNA-loaded vesicle protection assay

For the gel retardation assay, siRNA and vesicles with proper concentration were prepared. For each tube, siRNA (200 ng) was mixed with respective amounts of vesicles based on N/P ratios of 1/2.5, 1/1, 2.5/1, 5/1, 10/1, 15/1, 20/1 and 25/1. The final volume of each tube was made up to 10 μL with nuclease free H_2O. The samples were incubated at 37 °C for 30 min before running on 2% agarose gel. Bio-Rad Gel-Doc was used for visualization of the gel.

For the siRNA-loaded vesicle protection assay, siRNA and vesicles were incubated at 37 °C for 30 min. RNase A with final concentration of 0.01 μg/μL was added with continuing incubation at 37 °C. Aliquots containing 4 μL of the mixture (with 200 ng of siRNA) were withdrawn to be mixed with 1.5 μL of RNase free 1% sodium dodecyl sulfate (SDS) at time points 0, 5, 10, 20, 30, 45, 60, 75, 90, 105 and 120 min. The aliquots were kept on ice prior electrophoresis analysis. SDS was used to disrupt the complex formed between the siRNA and vesicle. For naked siRNA, the same procedure was carried out except the incubation with vesicle. Aliquots prepared at different time points were analyzed using electrophoresis with 2% agarose gel.

***In vitro* drug release**

The drug release behavior was investigated by using HPLC. The vesicle solution was prepared in PBS buffer (pH 7.4) with the concentration of 100 μM, followed by adding GSH with the concentration of 5 mM. The mixture was incubated under 37 °C over 200 min. The 100 μL solution was drawn out and diluted with methanol to the concentration of 20 μM. Acetonitrile and water were employed as eluents in HPLC, and the ratio of the two kinds of the solvents was changed along the time. The ratio of acetonitrile to water was 95 to 5 at 0~25 min, 5 to 95 at 25~32 min, and finally 95 to 5 till 40 min. The chromatogram was recorded at a wavelength of 270 nm.

Cellular uptake study

HeLa cells were cultured with Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin (PS) under 5% CO_2 atmosphere at 37 °C. In confocal studies, HeLa cells were seeded in a 6-well tissue culture plate (2 mL medium) with a density of 2.0×10^5 cells per well on the slides. After culturing for 24 h, the vesicles were added into the culture medium with the final concentration of 2 μM. After incubated with vesicles at different time periods, the culture medium was removed, washed with PBS for 3 times, and then fixed with 4% formaldehyde at room temperature for 15 min. The samples were finally visualized.

***In vitro* cytotoxicity study**

MTT assay was used to investigate the cytotoxicity of prodrug G1, H1, supramolecular amphiphile vesicles, and free drug CPT. HeLa cells were seeded in 96-well plates (200 μL medium) and incubated for 24 h. After the cell density reached 60%-70%, the cells were incubated with these samples having concentrations of 0, 0.08, 0.16, 0.32, 0.63, 1.25, 2.5, 5.0 10, and 40 μM, and then incubated for 72 h, respectively. After which, the medium was removed and fresh medium with 10% MTT dye was added, and the mixture was incubated for another 4 h. The medium was removed carefully, followed by the addition of 100 μL DMSO to dissolve the formazan crystals. Finally, optical densities of the samples were measured using a microplate reader at the two wavelengths of 570 nm and 490 nm.

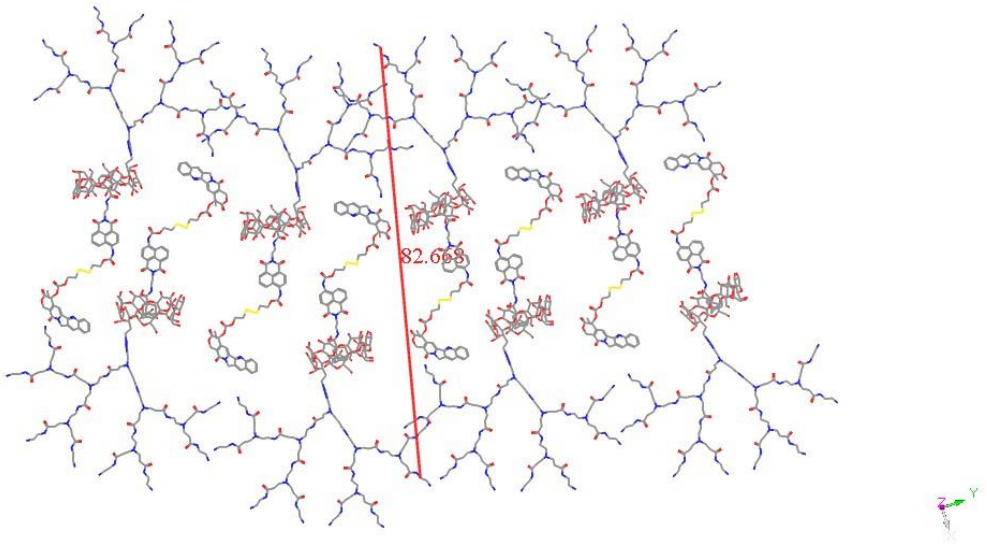


Figure S1: Simulation about alternating stacking of the supramolecular amphiphiles formed from H1 and G1. The thickness of the wall is about 8.4 nm. The width of the vesicular wall via the molecular mechanics results (Geometry Optimazation) with a Dreiding Force field by using materials studio 6.0 software. Total energy: 3247.78 kcal/mol.

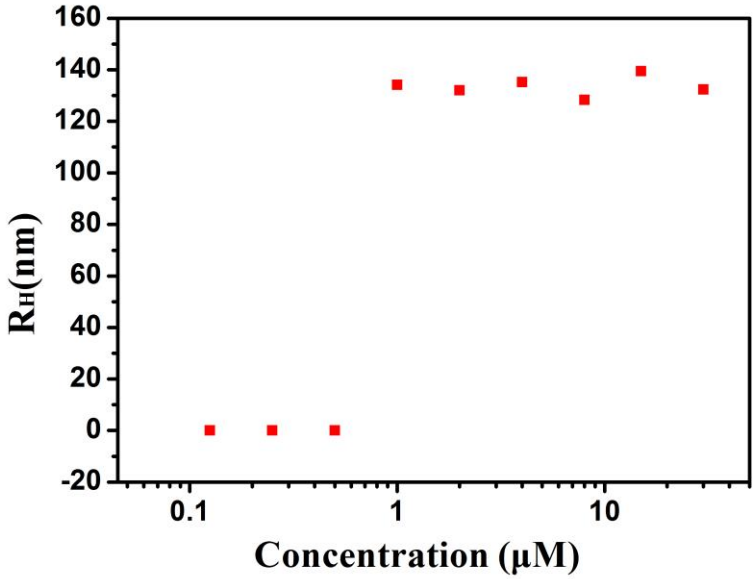


Figure S2: Hydrodynamic diameter (D_H) changes upon increasing the concentration of the supramolecular amphiphilic vesicles from 0.1 μM to 40 μM.

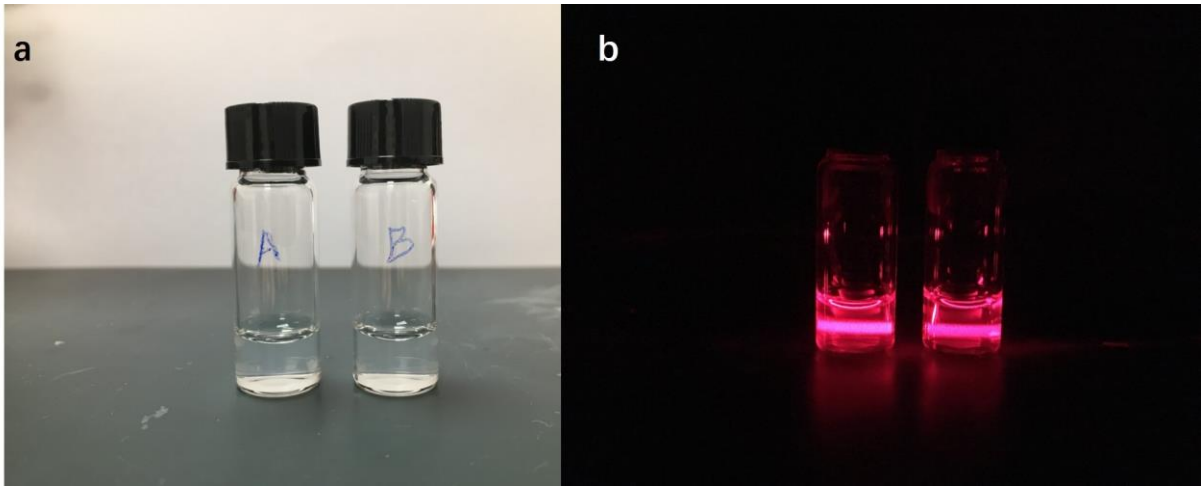


Figure S3: (a) Photography of the supramolecular amphiphilic vesicle solution. A: newly prepared, B: stocked over 4 months. (b) Photography showing the Tyndall effect of the supramolecular amphiphilic vesicle solution. Left: A, right: B.

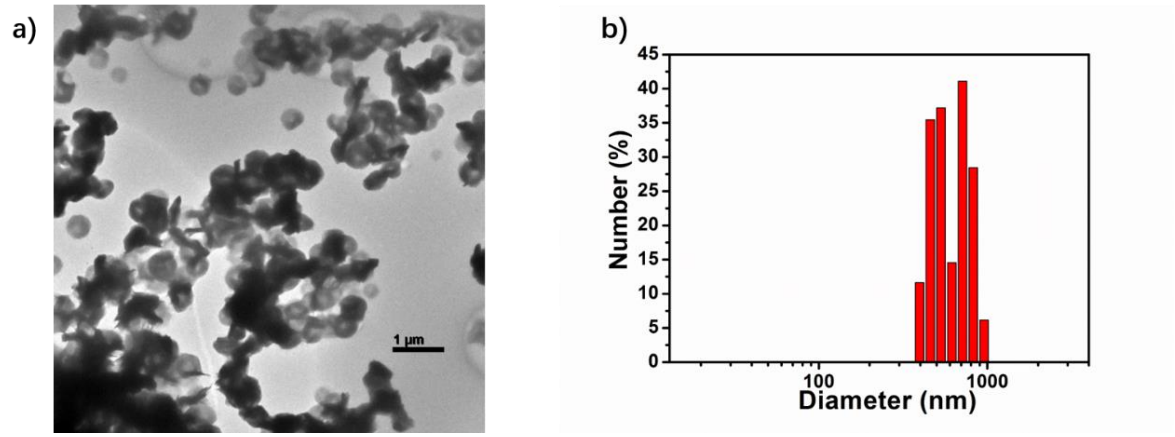


Figure S4: (a) TEM image of the supramolecular amphiphilic vesicles after the treatment with GSH, scale bar = 1 μm. (b) DLS data of the supramolecular amphiphilic vesicles after the treatment with GSH.

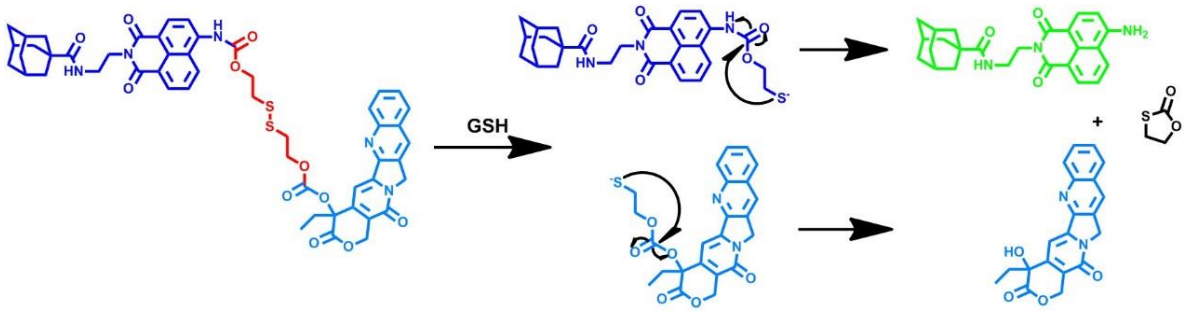


Figure S5: Proposed reaction mechanism of G1 treated with GSH under physiological conditions.

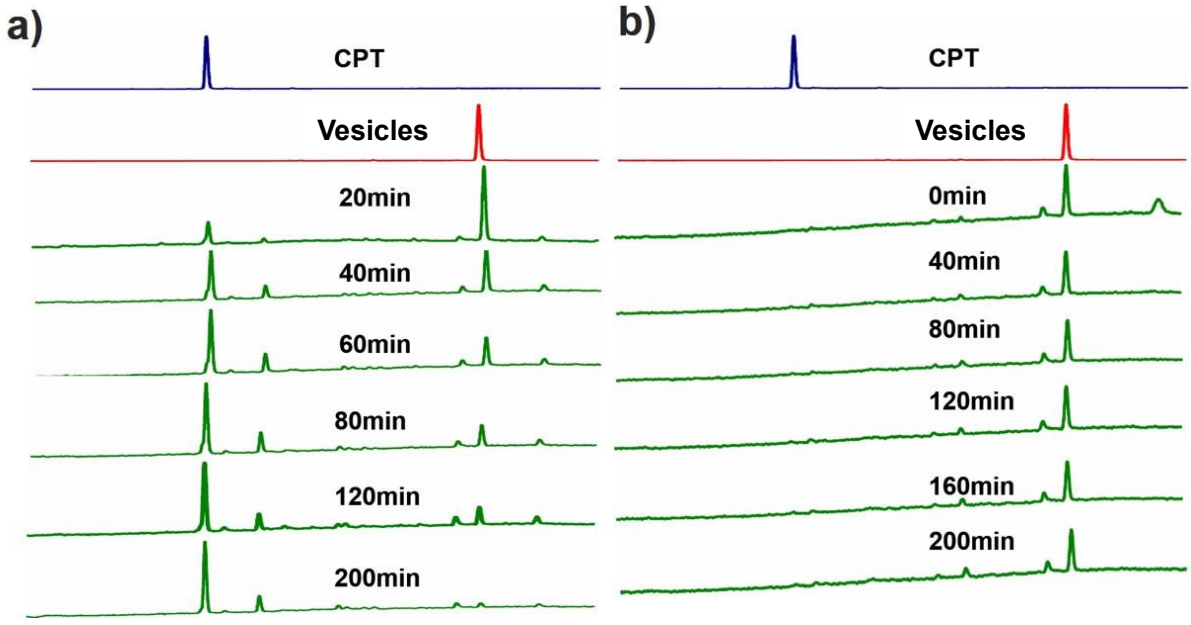


Figure S6: HPLC study of supramolecular vesicles incubated (a) with GSH and (b) without

GSH for 0, 20, 40, 60, 80, 120 and 200 min.

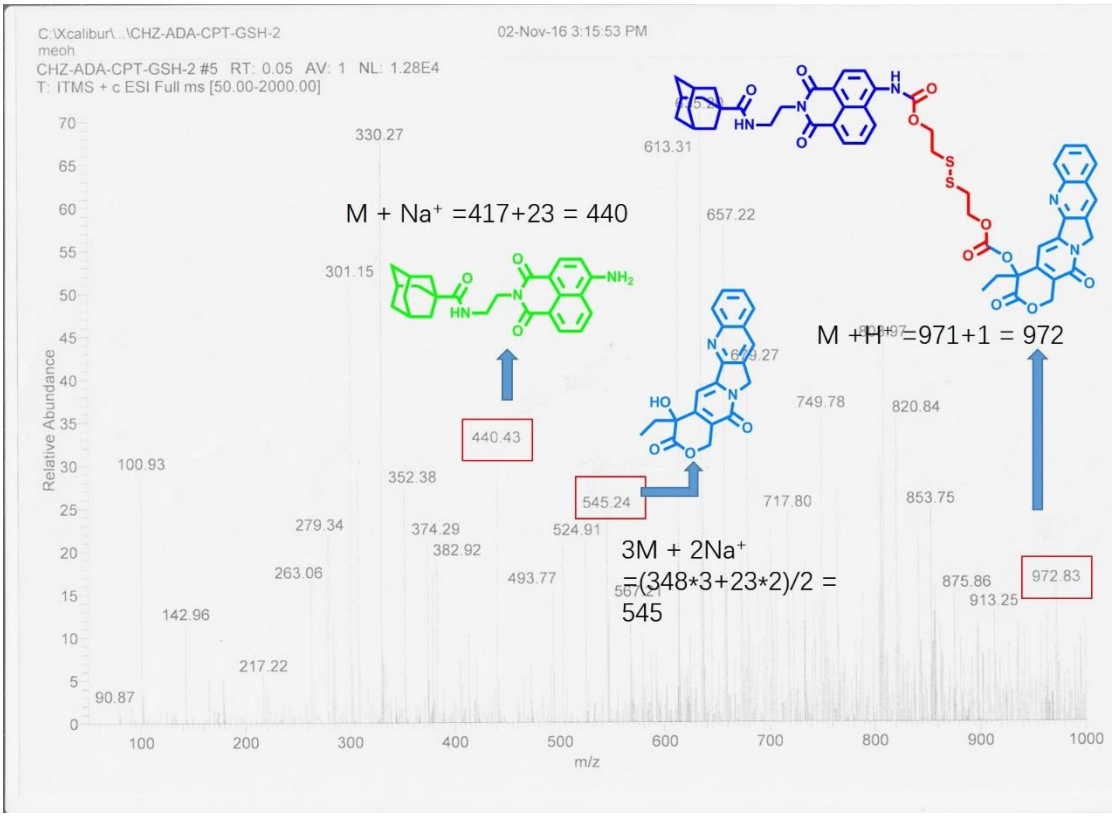


Figure S7: ESI-MS of the supramolecular amphiphilic vesicles after the incubation with GSH (5 mM) in PBS buffer (pH = 7.4) at 37 °C for 1 h.

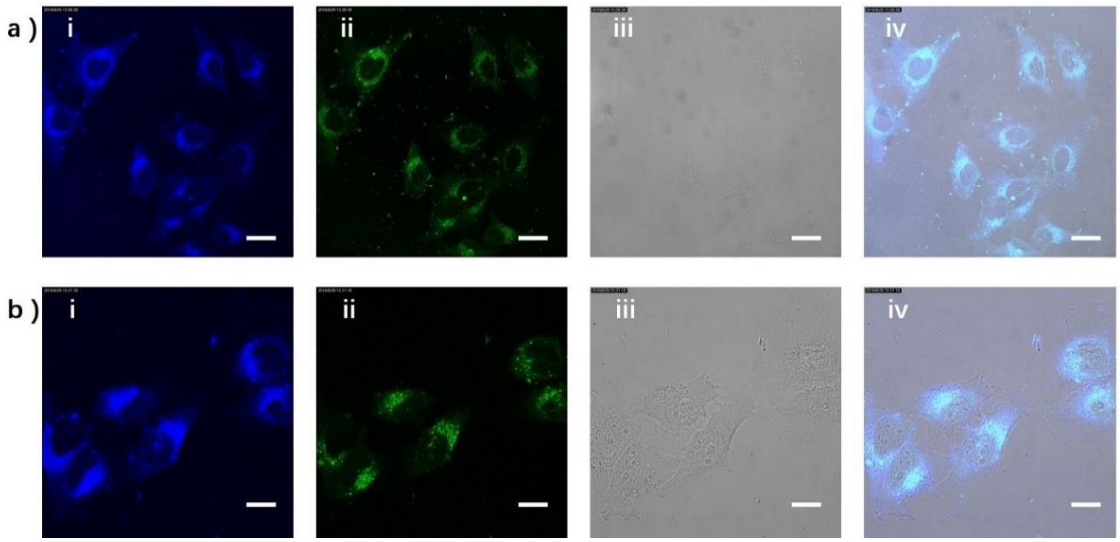


Figure S8: Confocal fluorescence images of HeLa cells incubated with the vesicles (2 μM) for 12 h: (i) blue channel at 450 ± 35 nm, (ii) green channel at 515 ±30 nm, (iii) bright-field transmission image, (iv) overlap image generated from (i) and (ii). Scale bar = 20 μm. (b) Enlarged confocal fluorescence images of corresponding HeLa cells. Scale bar = 10 μm.

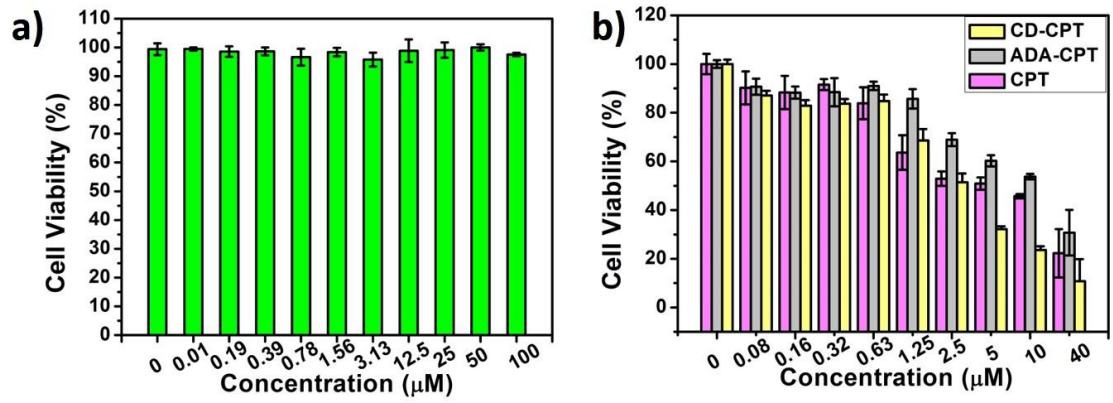


Figure S9: (a) Cell viability of H1 at different concentrations. (b) Cell viability of free CPT, prodrug G1 (ADA-CPT), and supramolecular amphiphilic vesicles (CD-CPT) at different concentrations. HeLa cells were incubated at 37 °C for 72 h.

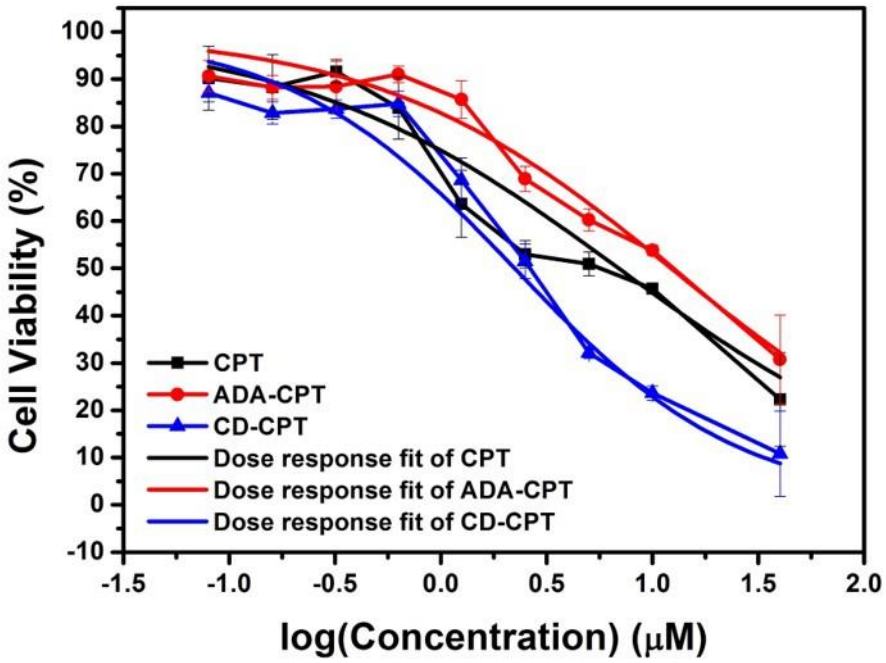


Figure S10: IC50 values of the CPT (6.903 μM), ADA-CPT (12.269 μM) and CD-CPT vesicles (2.229 μM).

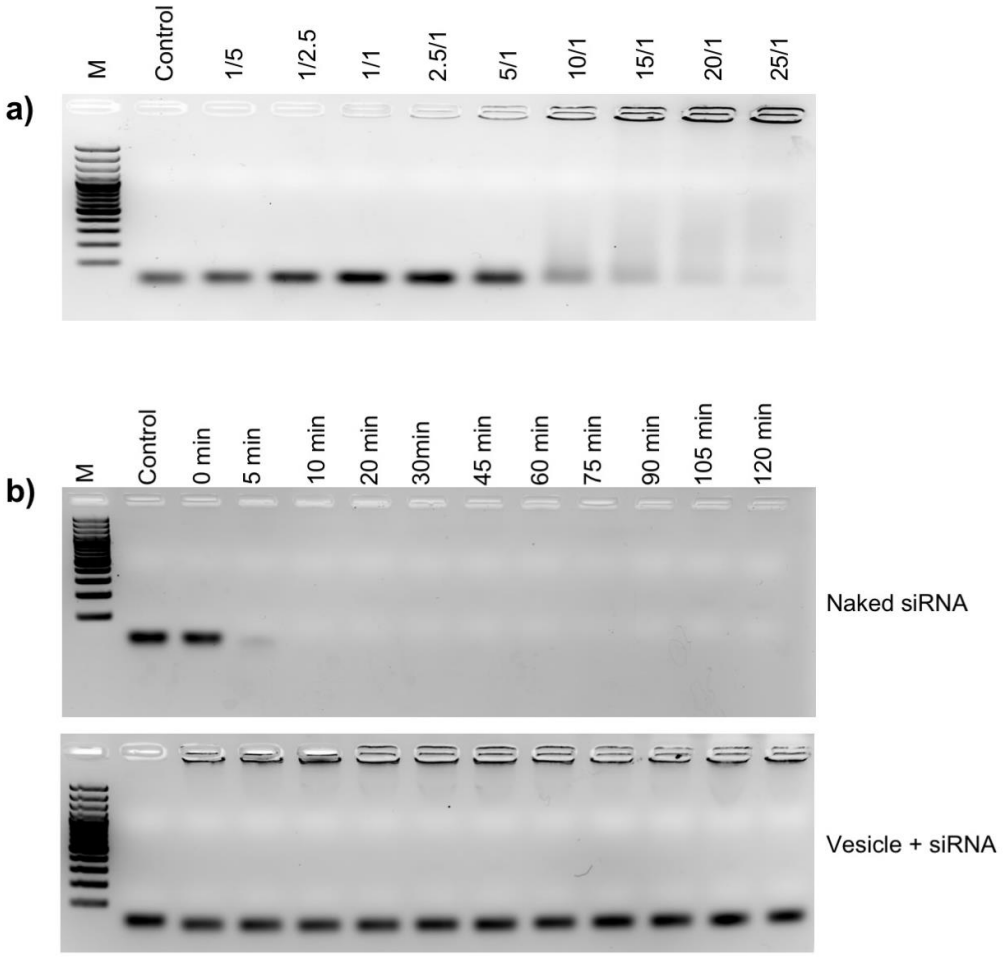


Figure S11: (a) Gel retardation of 200 ng siRNA in agarose gel with vesicles at N/P ratios ranging from 1:5 to 25:1. (b) Resulted siRNA-loaded vesicles (with N/P ratio of 25/1) protect siRNA from RNase degradation. 200 ng siRNA was used as the control.

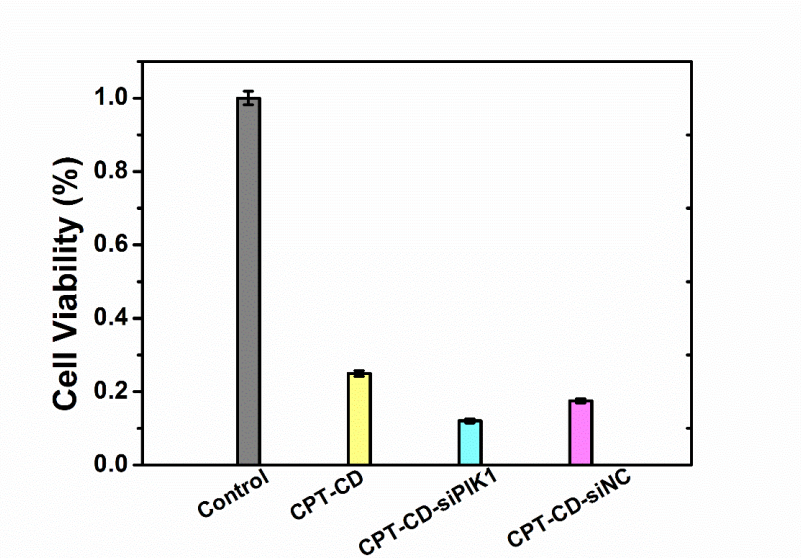


Figure S12: Cell viability of CPT-CD (supramolecular amphiphilic vesicles), CPT-CD-siPlK1 (siPlK1-loaded vesicles), and CPT-CD-siNC (siNC-loaded vesicles) with the vesicle

concentration of 10 μM and siRNA concentration of 100 nM. HeLa cells were incubated at 37 °C for 72 h.

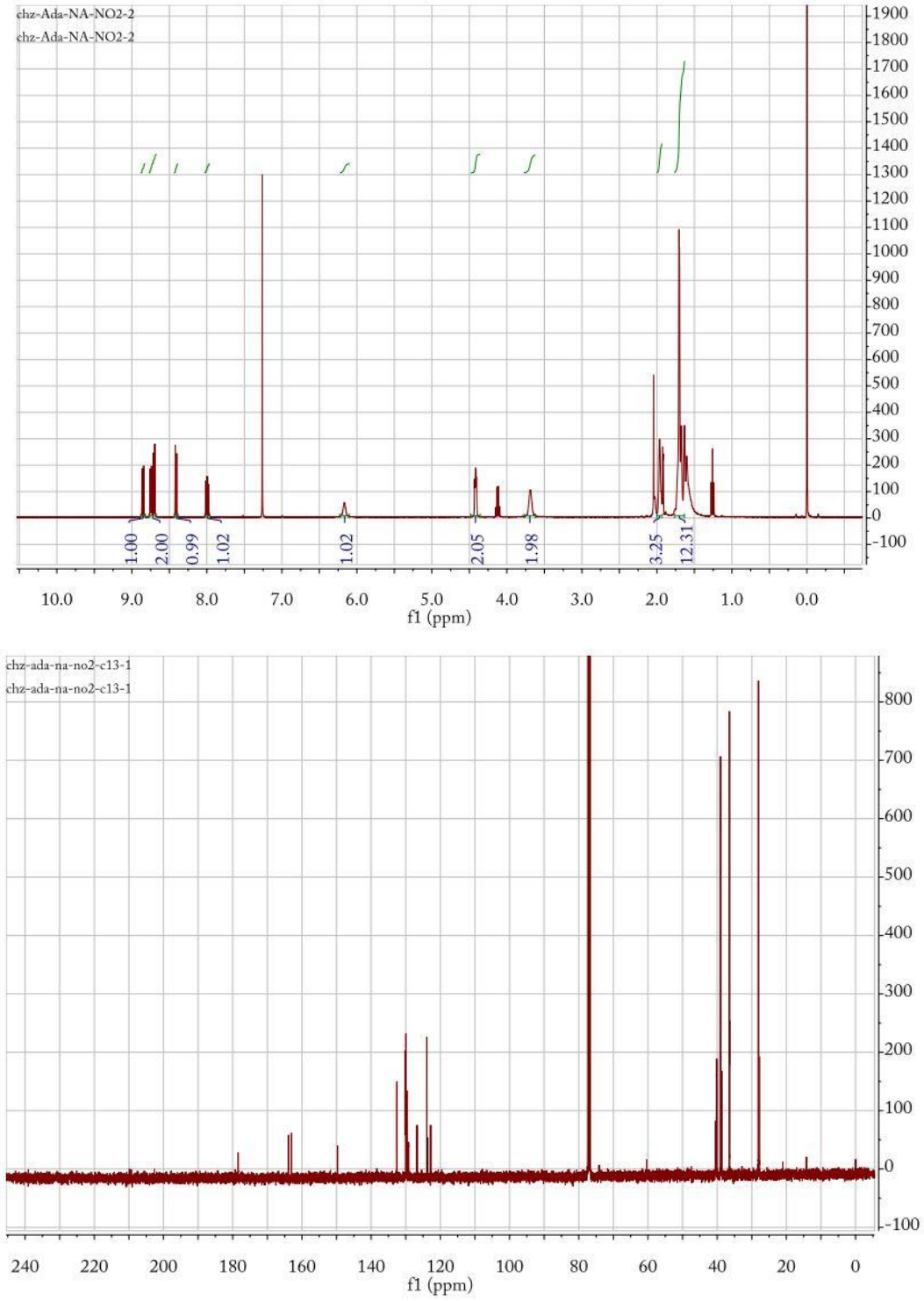


Figure S13: ^{1}H (top) and ^{13}C NMR (down) spectra of the compound **4** in $CDCl_3$.

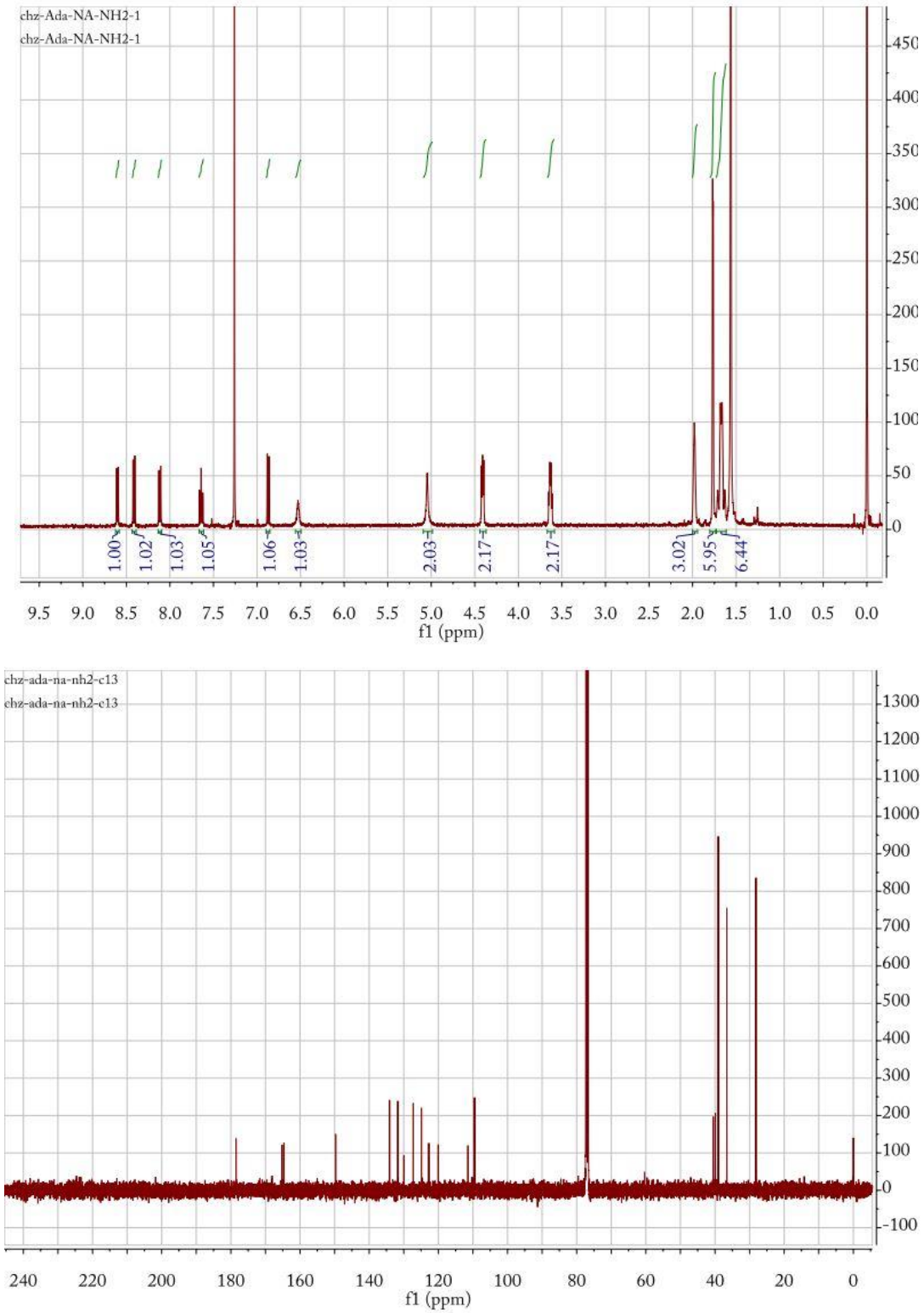


Figure S14: ^{1}H (top) and ^{13}C NMR (down) spectra of the compound **3** in $CDCl_3$.

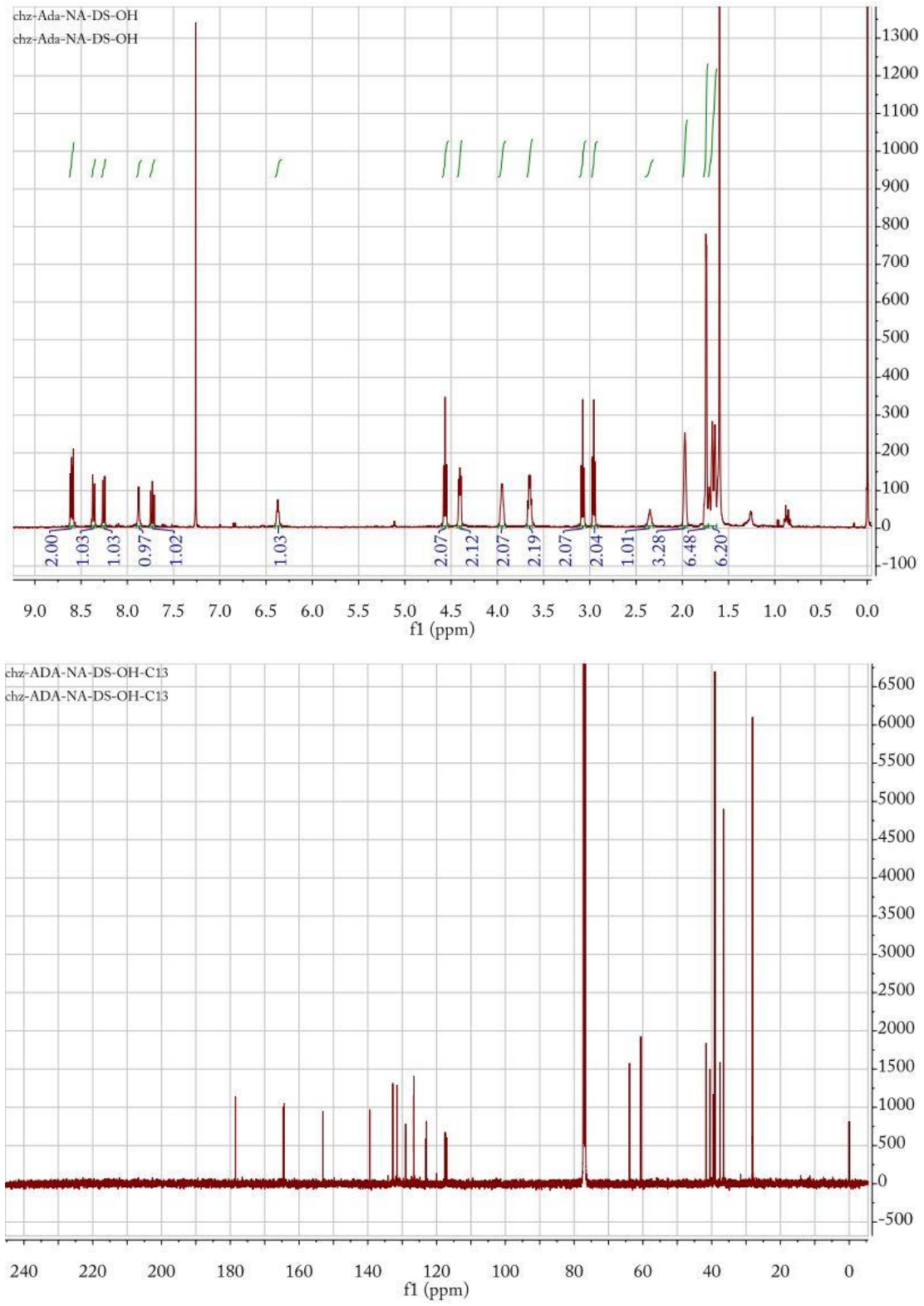


Figure S15: ^{1}H (top) and ^{13}C NMR (down) spectra of the compound **2** in $CDCl_3$.

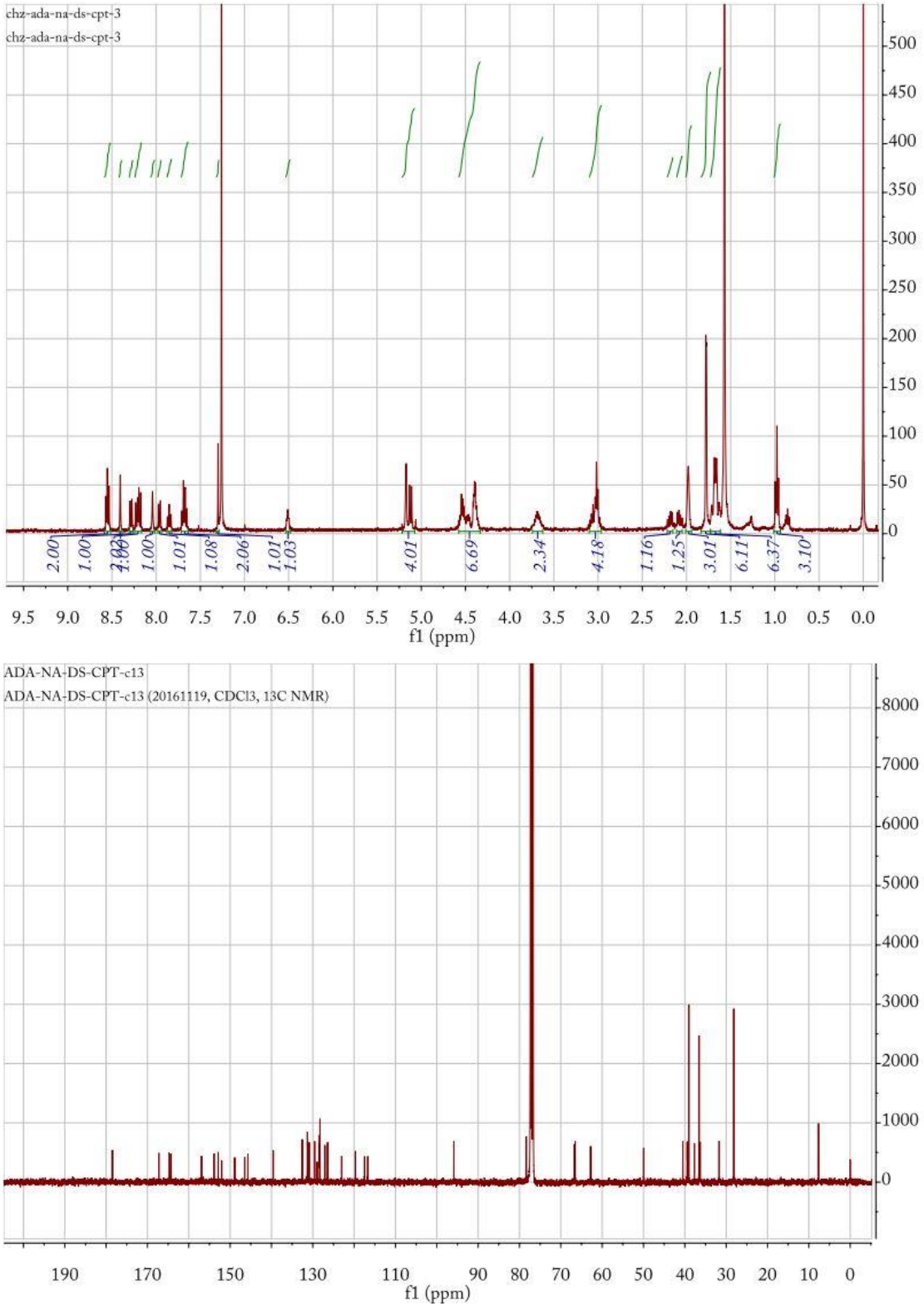


Figure S16: ^{1}H (top) and ^{13}C NMR (down) spectra of the compound G1 in $CDCl_3$.

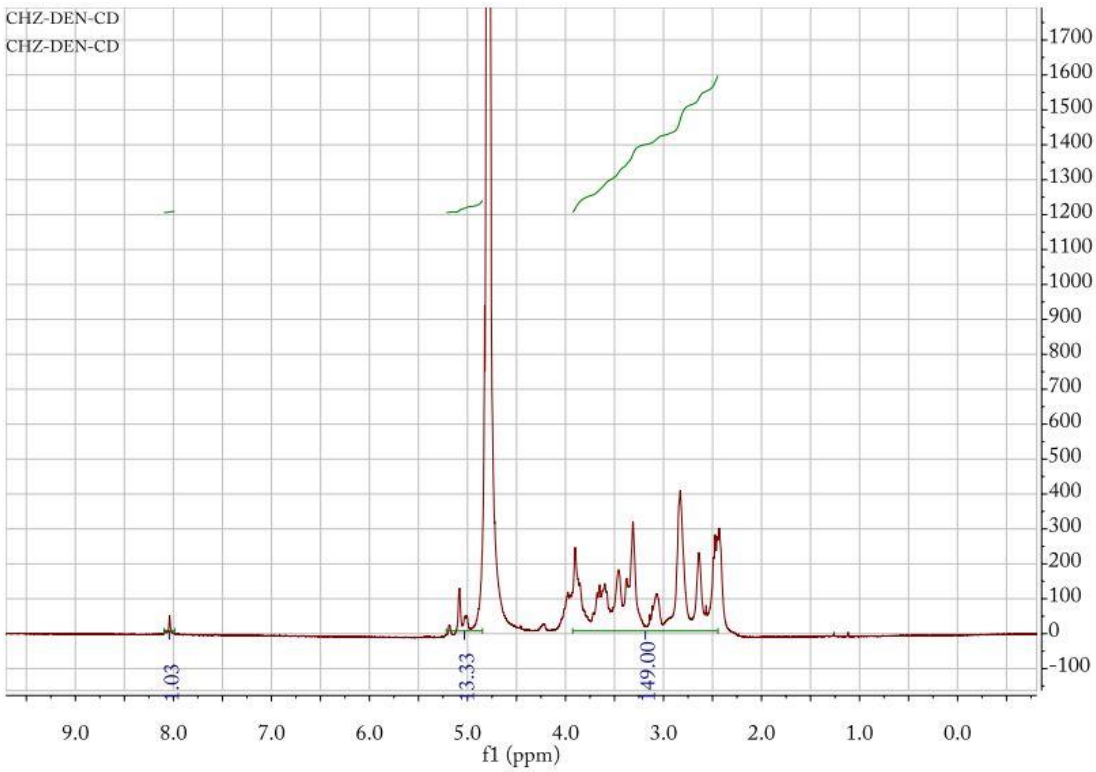


Figure S17: ^{1}H NMR spectrum of H1 in D_2O.

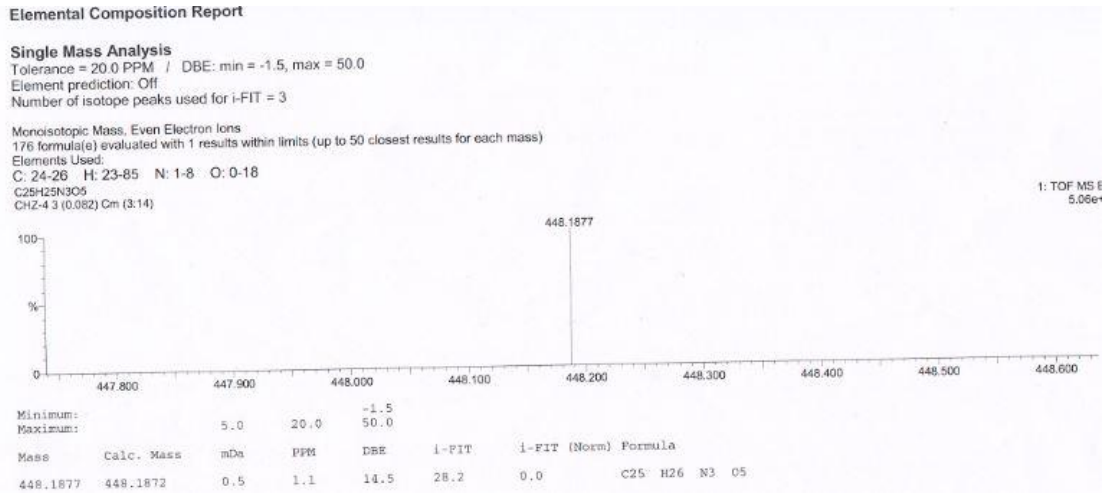


Figure S18: HRMS of the compound **4.**

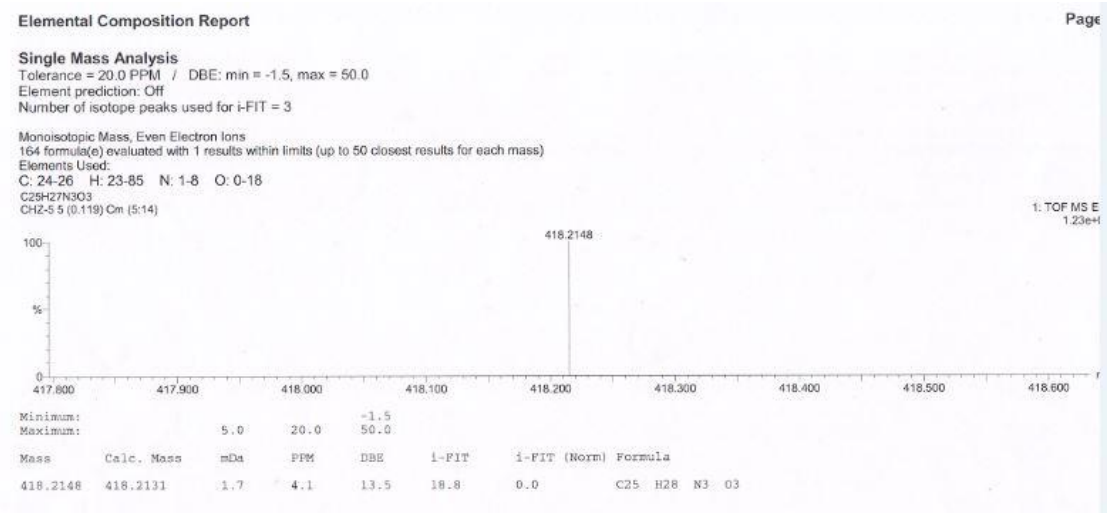


Figure S19: HRMS of the compound **3.**

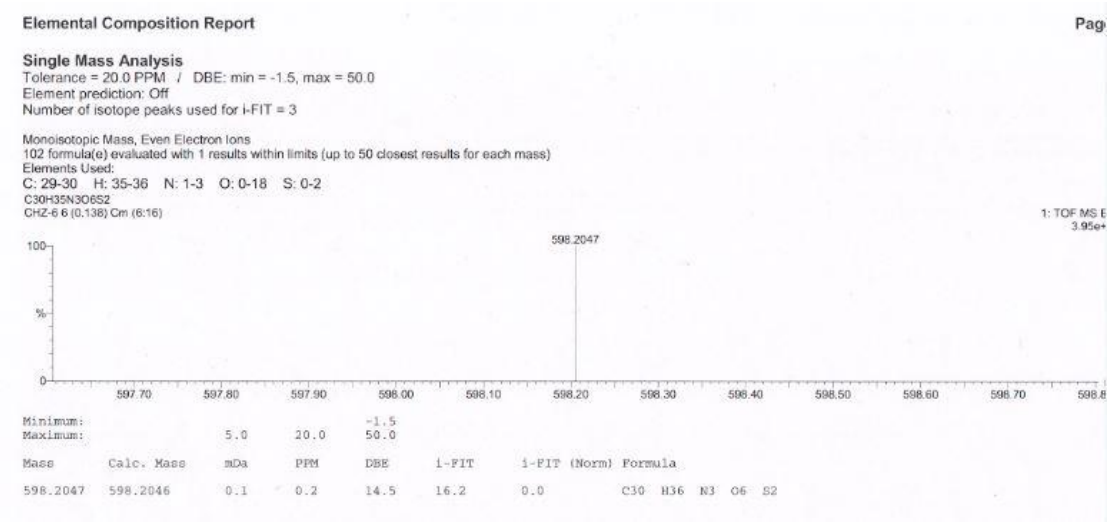


Figure S20: HRMS of the compound **2**.

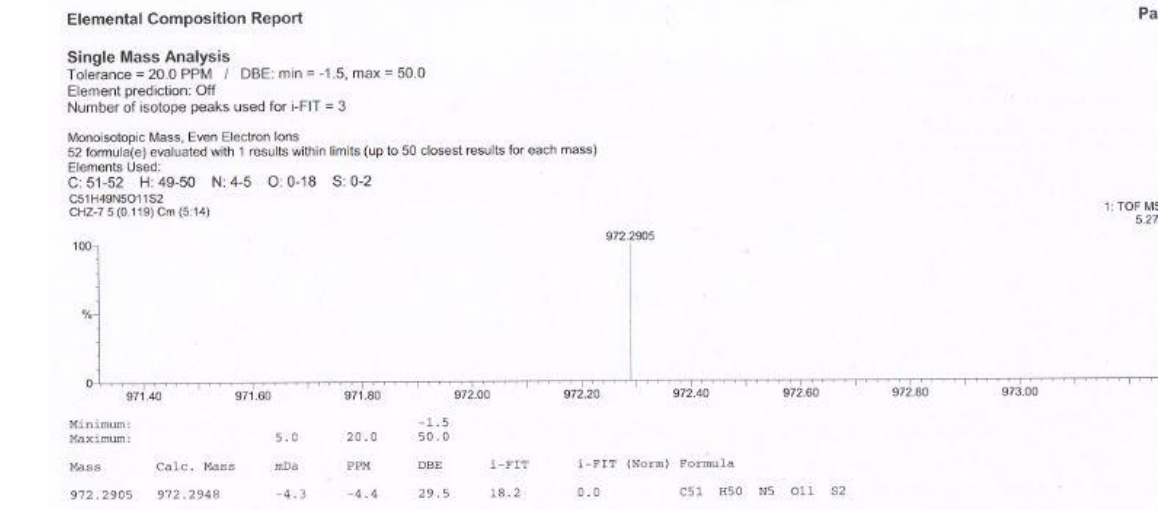


Figure S21: HRMS of the compound G1.

Figure S22: HRMS of dendritic cyclodextrin H1.

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