Supplementary Information

For

Camptothecin Delivery into Hepatoma Cell Line by Galactose-ap pended Fluorescent Drug Delivery System

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Contents

1. Materials and methods

General chemicals and instrumentation

Potassium phthalimide (TCI, Japan), hydrazine monohydrate (TCI, Japan), benzyl chloroformate (TCI, Japan), *β*–D-galactopylanose pentaacetate (Aldrich), boron trifluoridediethyl ether (Aldrich), *p*-toluenesulfonic acid (Aldrich), Pd/C (Aldrich), 2-aminoethanethiol (TCI, Japan), di-tert-butyl dicarbonate (TCI, Japan), 2,2'-dithiodiethanol (Aldrich), 4-nitro-1,8-naphthalic anhydride (Aldrich), phosgene (Aldrich), *N, N*-diisopropylethylamine (TCI, Japan), TFA (TCI, Japan), sodium methoxide (Aldrich) were received and were used without further purification. Galactose moiety and the linker section containing the disulfide bond were synthesized according to previously published procedures. Column chromatography was performed using silica gel 60 (70 ~ 230 mesh) as a stationary phase. Analytical thin layer chromatography was performed using 60 silica gel (precoated sheets with 0.25 mm thickness). The mass spectra were obtained by IonSpec HiResESI mass spectrometer. The NMR spectra were collected by a 300 and 400 MHz spectrometer (AS400, 300, Varian, US).

UV/Vis and fluorescence spectroscopic methods

Stock solution of **1** was prepared in PBS buffer solution (pH = 7.4). Excitation was carried out at 340, 370, 430 nm with all excitation and emission slit widths are 3 nm. The concentrations of each GSH, pH varied, but the total volume was fixed at 3.0 mL.

Cell lines and culture

HepG2 (human hepatoma), A549 (human lung adenocarcinoma), HeLa (human cervical adenocarcinoma), KB (human HeLa contaminant carcinoma) cell lines were used. HepG2 cell was cultured in RPMI containing 10% FBS, 1% penicillin streptomycin and 10^{-9} M Biotin. A549 and KB cell lines were cultured in RPMI medium, HeLa cell was cultured in DMEM medium. All mediums were contained 10% FBS and 1% penicillin streptomycin. Cells were grown at 37 °C under humidified atmosphere 5% CO_2.

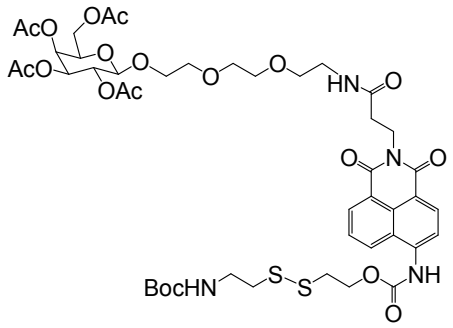
Cell viability measurement

HepG2 cells were plated at 2×10^4/well in a 96 well plate for 24 hrs and the media were replaced wit

h RPMI containing variable concentration of drugs. After incubated for 48 hrs at 37 °C, the media were d ischarged. And, 100 µl of a reaction solution containing 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added to each well followed by incubation for 90 min at 37 °C. Afte r the remaining reagent was removed, the cells were lysed with 100 µl of DMSO for 5min at 37 °C. The absorbance of the dissolved formazan crystals was measured at 570 nm using a microplate reader (VERS Amax, USA).

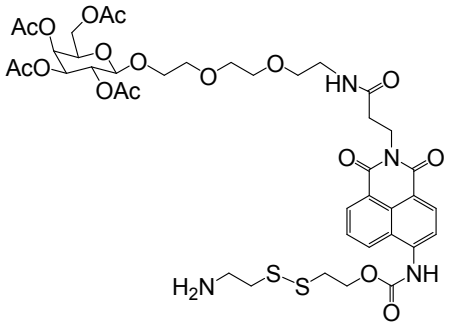
2. Synthesis of compounds

Synthesis of 4



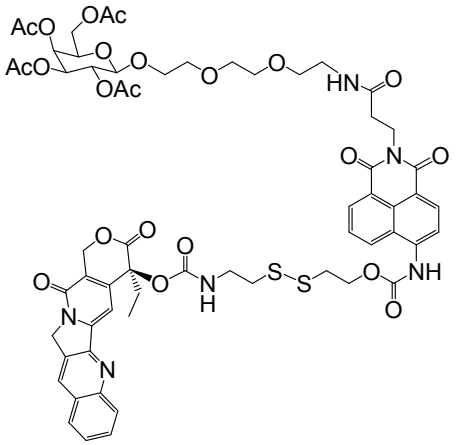
Compound **5a** (0.7 g, 0.93 mmole) was dissolved in distilled DCM (80 mL) in round bottom flask and placed in an ice path under nitrogen gas. Phosgene in toluene (2.90 mL, 20%) was added with a syringe. *N,N*-Diisopropylethyla mine (1.90 mL, 16.6 mmole) was slowly added with a syringe. After 50 minutes of stirring, the mixture was purge d with nitrogen for 30 minutes to eradicate the remaining phosgene gas. Compound **7** (0.240 g, 0.95 mmole) dissol ved in 10 mL of distilled DCM was added to the mixture. The mixture was stirred overnight. The mixture was eva porated and vacuumed. 100 mL of distilled water and 100 mL of ethyl acetate was added, where the organic part w as extracted. After removing the solvent, the product was purified by silica gel chromatography using ethyl acetate only, where the product yielded a lime colored solid of 0.172 g (17.8%). ESI-MS *m/z* (M^+) calcd 1024.33 found 10 47 ($M+Na^+$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.57 (m, 3H), 8.51 (s, 1H), 8.44 (d, 1H, J = 8.7 Hz), 8.28 (d, 1H, J = 7.2 Hz), 7.72 (t, 1H, J = 7.7 Hz), 6.64 (m, 1H), 5.37 (dd, 1H, J = 3.3 Hz), 5.19 (dd, 1H, J = 10.4 Hz J = 8.1 Hz), 5. 02 (d, 1H, J = 3.4 Hz), 4.99 (d, 1H, J = 3.4 Hz), 4.55 (m, 2H), 4.48 (t, 2H, J = 7.8 Hz), δ 4.12 (m, 2H), 3.94 (m, 2H), 3.69 (m, 4H), 3.55 (m, 6H), 3.47 (m, 2H), 3.06 (t, 2H, J = 6.1 Hz), 2.86 (t, 2H, J = 6.8 Hz), 2.68 (t, 2H, J =7.3 H z), 2.14 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.96 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 170.63, 170.54, 170.39, 170.2 8, 169.69, 164.22, 163.71, 156.01, 153.50, 139.85, 132.54, 131.45, 129.02, 127.66, 126.56, 123.72, 122.99, 117.84, 101.44, 70.96, 70.74, 70.68, 70.33, 70.26, 69.91, 69.27, 68.89, 67.13, 62.93, 61.37, 60.51, 39.86, 39.32, 37.79, 37. 71, 36.95, 34.80, 29.80, 28.46, 20.90, 20.80, 20.79, 20.70, 14.30 ppm.

Synthesis of 3



TFA (5 mL) was added to compound **5a** (0.172 g, 0.168 mmole). The mixture was stirred under room temperature for 3 hours. The mixture was evaporated to remove the remaining trifluoroacetic acid gas. The solid was recrystalli zed from MeOH and diethyl ether. Compound **3 (**0.121 g) as a yellow solid was obtained (78% yield). ESI-MS *m/z* (M^+) calcd 924.28 found 925.5 ($M+H^+$), 947.4 ($M+Na^+$), 923.5 ($M - H^+$). ^1H NMR (400 MHz, $CDCl_3$): δ 9.05 (m, 2H), 8.39 (m, 3H), 8.19 (m, 1H), 8.09 (m, 1H), 7.95 (m, 1H), 7.77 (m, 1H), 7.31 (m, 1H), 5.19 (t, 1H, J = 9.35 Hz), 5.05 (t, 1H, J = 9.98 Hz), 4.96 (t, 1H, J = 8.28Hz), 4.57 (d, 1H, J = 8.07 Hz), 4.48 (m, 2H), 4.25 (m, 1H), 4.21 (m, 1H), 4.08 (m, 1H), 3.94 (m, 6H), 3.70 (m, 2H), 3.64-3.40 (m, 12H), 3.10 (m, 4H), 2.62 (m, 2H), 2.05 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.96 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): 171.88, 170.95, 170.43, 169.85, 169.71, 163.98, 1 63.44, 153.97, 140.32, 132.08, 131.24, 128.45, 128.21, 126.26, 123.15, 121.80, 117.34, 116.59, 101.10, 76.30, 76. 00, 72.78, 72.11, 71.91, 71.39, 70.56, 70.27, 69.36, 68.54, 63.38, 62.17, 39.45, 37.32, 36.93, 34.72, 20.92, 20.86, 2 0.80, 20.73, 20.70, 14.30 ppm.

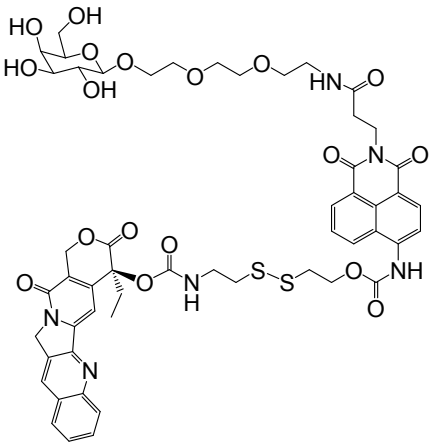
Synthesis of 2



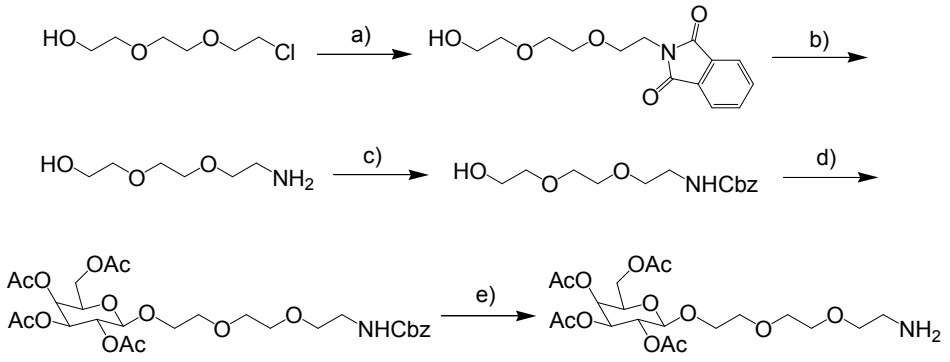
CPT (54 mg, 0.155 mmole) was dissolved in chloroform (60 mL) in the dark under at 40℃ under nitrogen gas. Ph osgene in toluene (1.00 mL, 20%) was added. Next, 4-dimethylaminopyridine (210 mg, 1.72 mmole) was dissolve d in 30 ml of chloroform and was slowly added to the flask by syringe. After the mixture was stirred for 50 minute s, the mixture was purged with nitrogen gas for 30 minutes to remove the remaining phosgene gas. Compound **3** (1 21 mg, 0.118 mmole) was dissolved in chloroform (40 mL) and added with a syringe. The mixture was stirred for 5 hours. The solvent was evaporated *in vacuo* and 100 mL of DCM and 100 mL of distilled water were added whe re only the organic layer was extracted. The product was purified using silica gel chromatography using 10% MeO H in DCM as an eluent. The compound was recrystallized from a mixture of MeOH and diethyl ether to provide 2 4.3 mg of lime colored solid (13.2% yield). ESI-MS *m/z* (M^+) calcd 1298.37 found 1321.6 ($M+Na^+$), 1297.7 (M –

H^+). ^1H NMR (400 MHz, $CDCl_3$): δ 8.51-8.37 (m, 5H), 8.30 (t, 1H, J = 4.48 Hz), 8.23 (d, 1H, J = 8.40Hz), 8.17 (d, 1H, J = 8.40Hz), 8.10 (d, 1H, J = 9.00 Hz), 7.94 (d, 1H, J = 9.00 Hz), 7.8-7.74 (m, 3H), 7.71-7.59 (m, 3H), 7.55 (t, 1H, J = 8.4 Hz), 7.28 (s, 1H), 6.77 (t, 1H, J = 5.60 Hz), 6.70 (t, 1H, J = 6.68 Hz), 6.01 (t, 1H, J = 6.00 Hz), 5.24-5.15 (m, 3H), 5.11 (s, 1H), 5.10-5.05 (m, 2H), 5.05-5.01 (m, 2H), 4.97 (t, 2H, J = 9.44 Hz), 4.60 (d, 1H, J = 8.20 Hz), 4.49-4.43 (m, 6H), 4.09-4.01 (m, 2H), 3.98-3.01 (m, 2H), 4.22 (d, 1H, J = 4.72 Hz), 4.15-4.10 (m, 2H), 3.97-3.92 (m, 2H), 3.75-3.68 (m, 2H), 3.60-3.52 (m, 12H), 3.48-3.44 (m, 2H), 3.10-2.95 (m, 4H), 2.82 (m, 1H), 2.73-2.62 (m, 3H), 2.50-2.36 (m, 2H), 2.07 (s, 3H), 2.05 (s, 3H), 2.01 (s, 3H), 1.06 (t, 2H, J = 7.88 Hz), 0.93 (t, 3H, J = 7.56 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 172.6, 170.9, 170.5, 169.7, 168.5, 164.3, 163.8, 162.2, 157.0, 155.1, 153.9, 153.4, 152.2, 149.0, 147.1, 146.1, 144.7, 139.9, 132.7, 132.5, 131.5, 131.3, 130.0, 129.6, 129.0, 128.9, 128.5, 128.3, 127.7, 123.0, 119.4, 117.8, 117.2, 101.0, 89.6, 76.4, 72.1, 71.9, 71.5, 71.3, 70.8, 70.4, 70.1, 69.3, 68.7, 68.5, 63.0, 62.6, 62.2, 40.6, 39.5, 37.6, 37.1, 34.8, 31.9, 21.0, 20.9, 20.8, 20.7, 7.8 ppm.

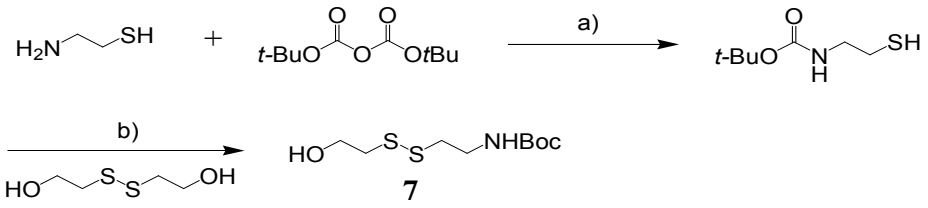
Synthesis of 1



Compound **2** (24 mg, 0.018 mmole) was dissolved in 3 mL of MeOH. 1*N* sodium methoxide (30 μL) in MeOH was added and stirred for 3 hours under nitrogen gas. Cation-exchange resin (H^+) was added and filtered and washed with MeOH. The solvent was evaporated *in vacuo*. The compound **1** was recrystallized from a mixture of MeOH and diethyl ether to give 12 mg of yellow solid in 59% yield. ESI-MS *m/z* (M^+) calcd 1130.32 found 1131.8 ($M+H^+$). ^1H NMR (400 MHz, DMSO): δ 10.3 (m, 1H), 8.64 (m, 1H), 8.58 (m, 1H), 8.47 (m, 1H), 8.40 (m, 1H), 8.15 (d, 1H, J = 9.00 Hz), 8.08 (m, 1H), 8.02 (m, 1H), 7.92-7.77 (m, 2H), 7.76-7.63 (m, 1H), 7.36 (m, 1H), 7.10 (m, 1H), 5.43 (m, 1H), 5.26 (m, 1H), 4.89-4.71 (m, 2H), 4.48 (t, 1H, J = 6.8 Hz) 4.41 (t, 1H, J = 6.84 Hz), 4.22 (m, 2H), 4.14 (d, 1H, J = 7.7 Hz), 3.90-3.82 (m, 2H), 3.68 – 3.44 (m, 22H), 3.37 (m, 2H), 3.21-3.15 (m, 2H), 3.13-3.02 (m, 2H), 2.98-2.91 (m, 2H), 1.97 (m, 2H), 0.92 (m, 3H).



Scheme S1 Synthesis of the galactose unit and naphthalimide fluorescence connected through a disulfide linker. a) Potassium phthalimide, DMF, 15 h, reflux, overnight; b) NH_2NH_2, EtOH, reflux, 2 h; c) $NaHCO_3$, benzyl chlorofo rmate, H_2O, overnight, 0℃; d) *β*-D-galactopylanose pentaacetate, DCM, boron trifluoridediethyl ether, 0°C, overni ght; e) *p*-toluenesulfonic acid, Pd/C, H_2 gas, overnight.



Scheme S2 Synthesis of the disulfide linker (**7**) which connects the naphthalimide and CPT. a) THF, 0℃, 1.5 h; b) i) MeOH/DCM, 12 h, rt; ii) I_2, MeOH.

3. ESI-MS, ^{1}H and ^{13}C NMR spectra

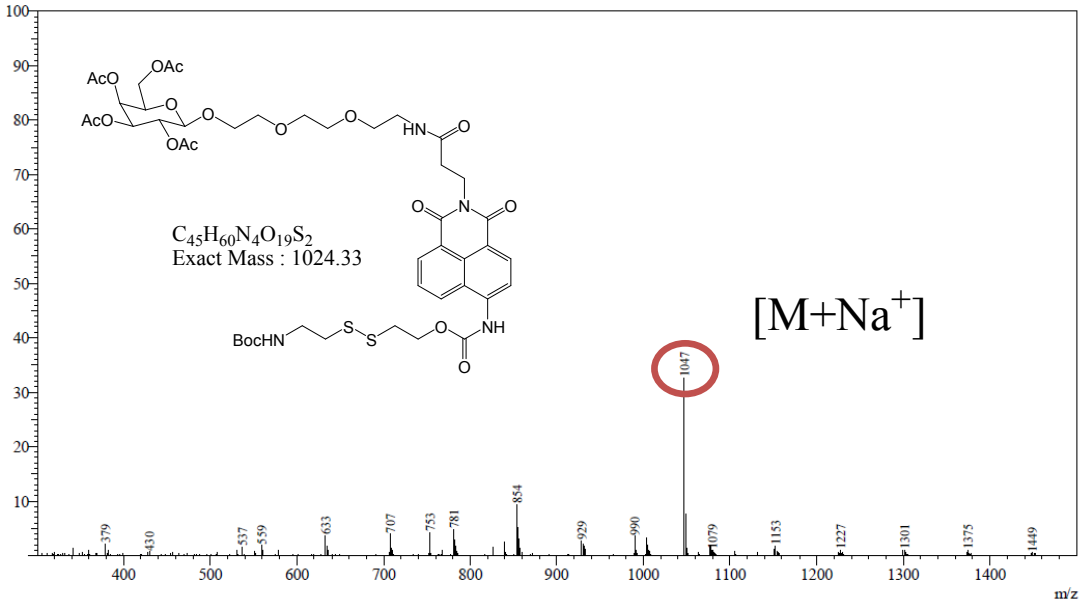


Fig. S1. ESI-MS spectrum of compound **4**.

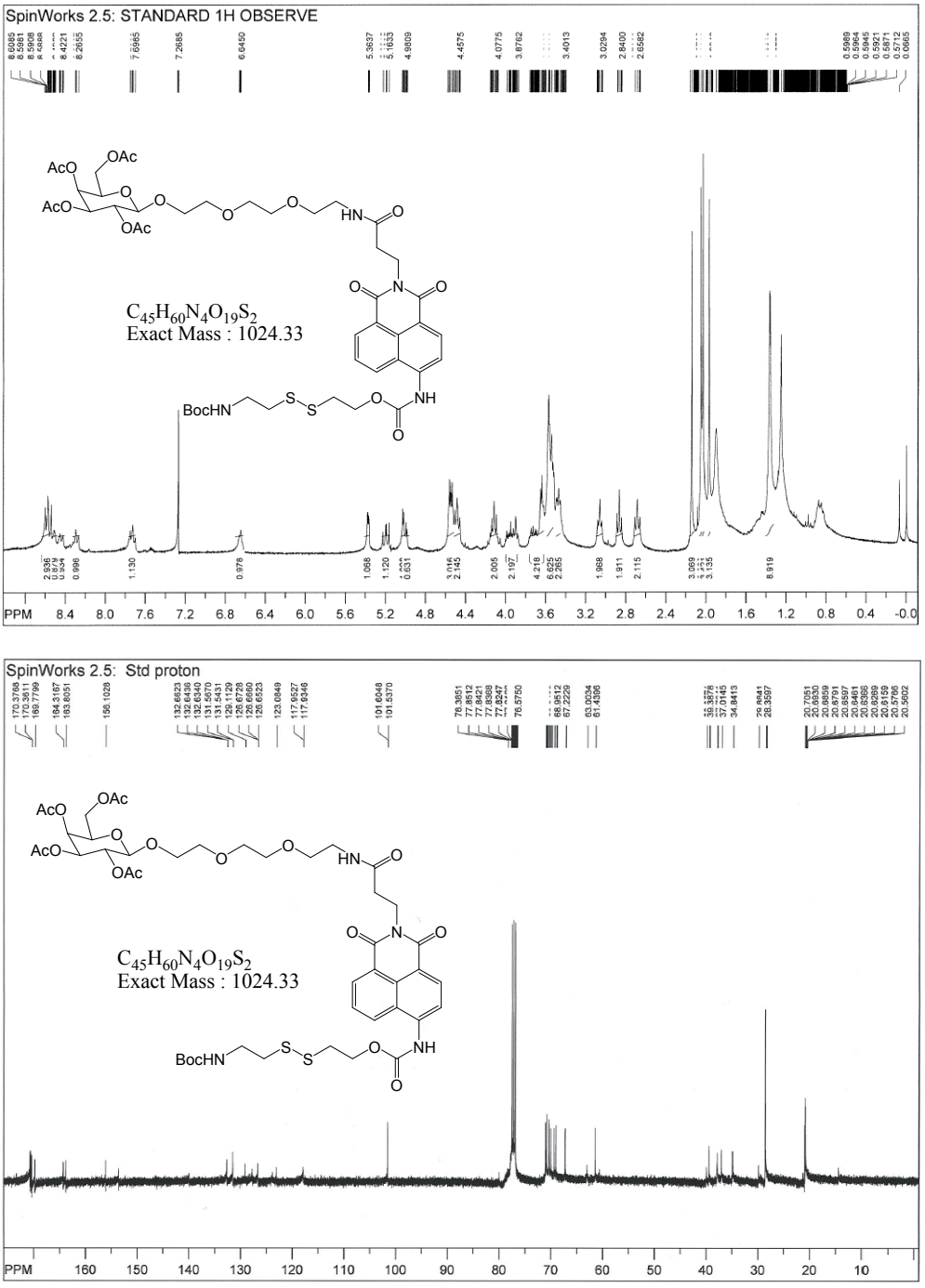


Fig. S2. ^{1}H and ^{13}C NMR spectra of compound **4**.

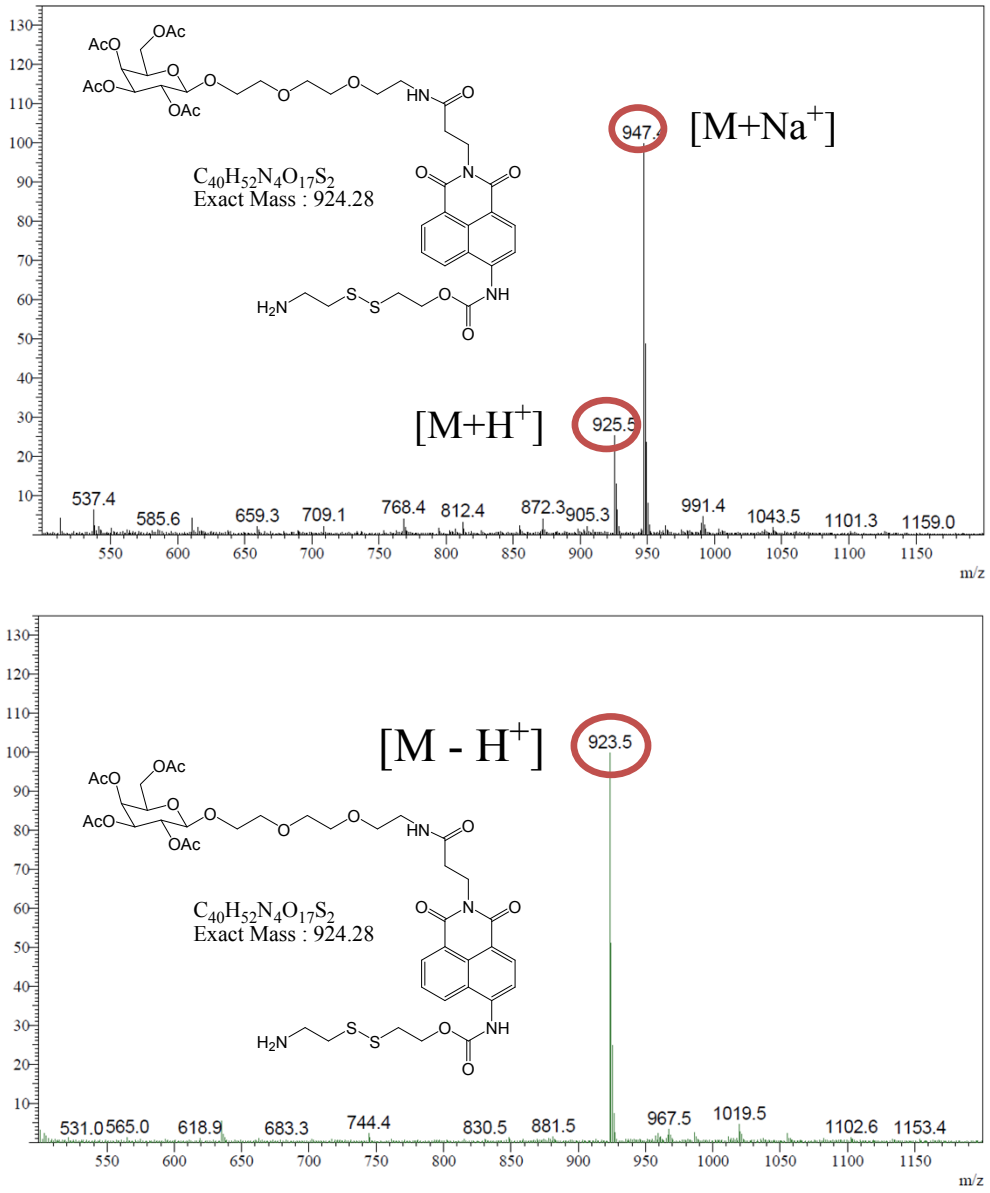


Fig. S3. ESI-MS spectra of compound **3**.

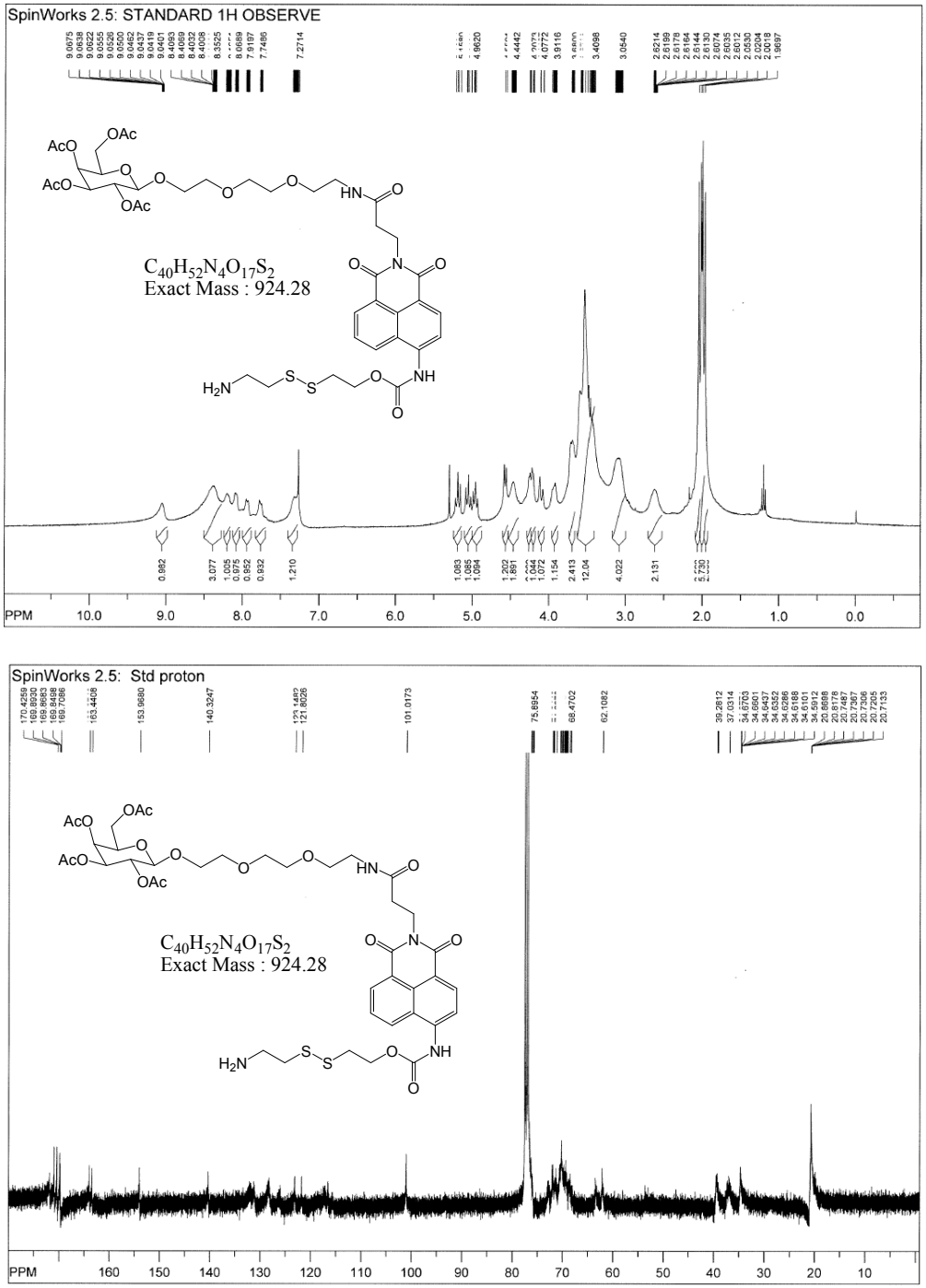


Fig. S4. ^{1}H and ^{13}C NMR spectra of compound **3**.

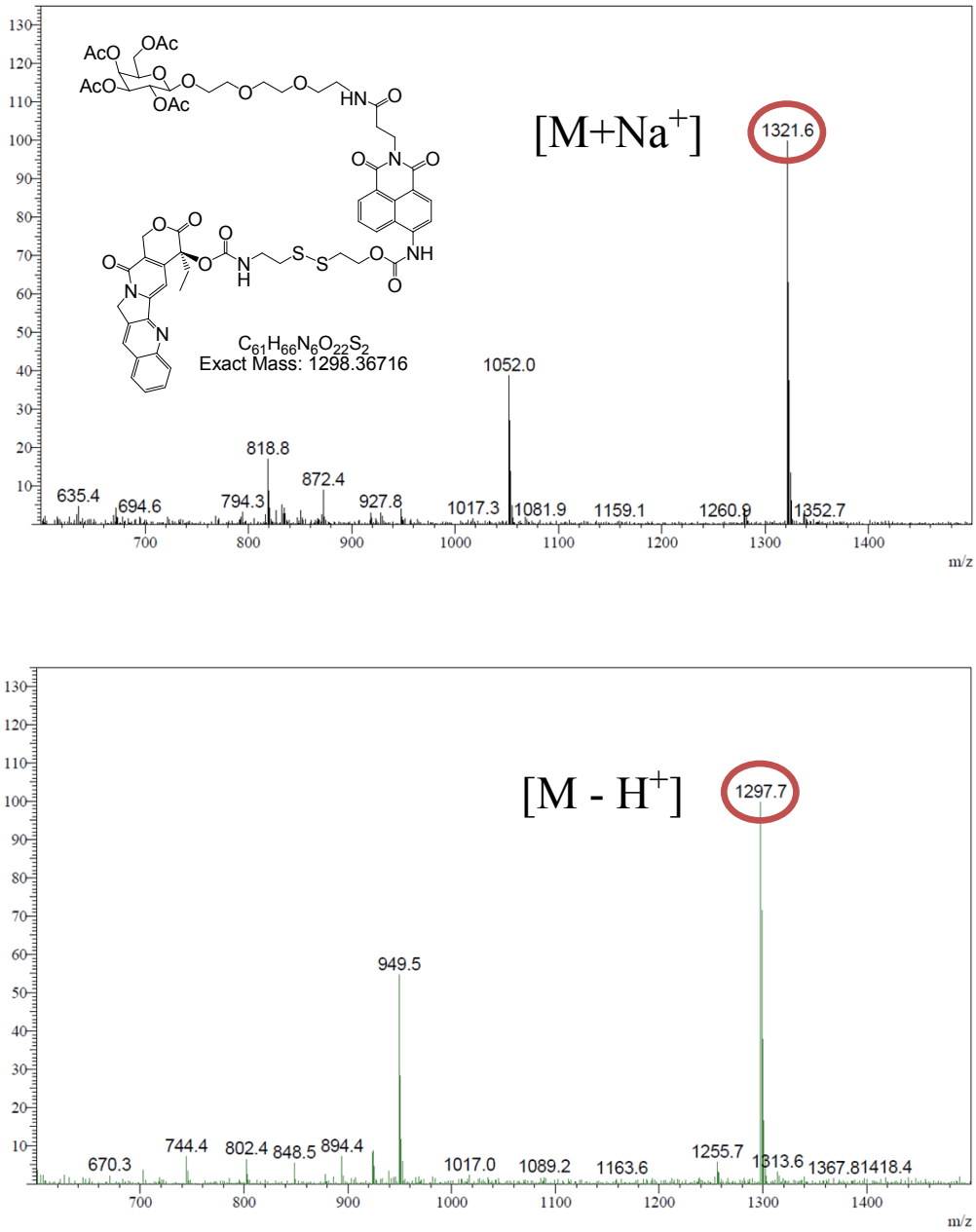


Fig. S5. ESI-MS spectra of compound **2**.

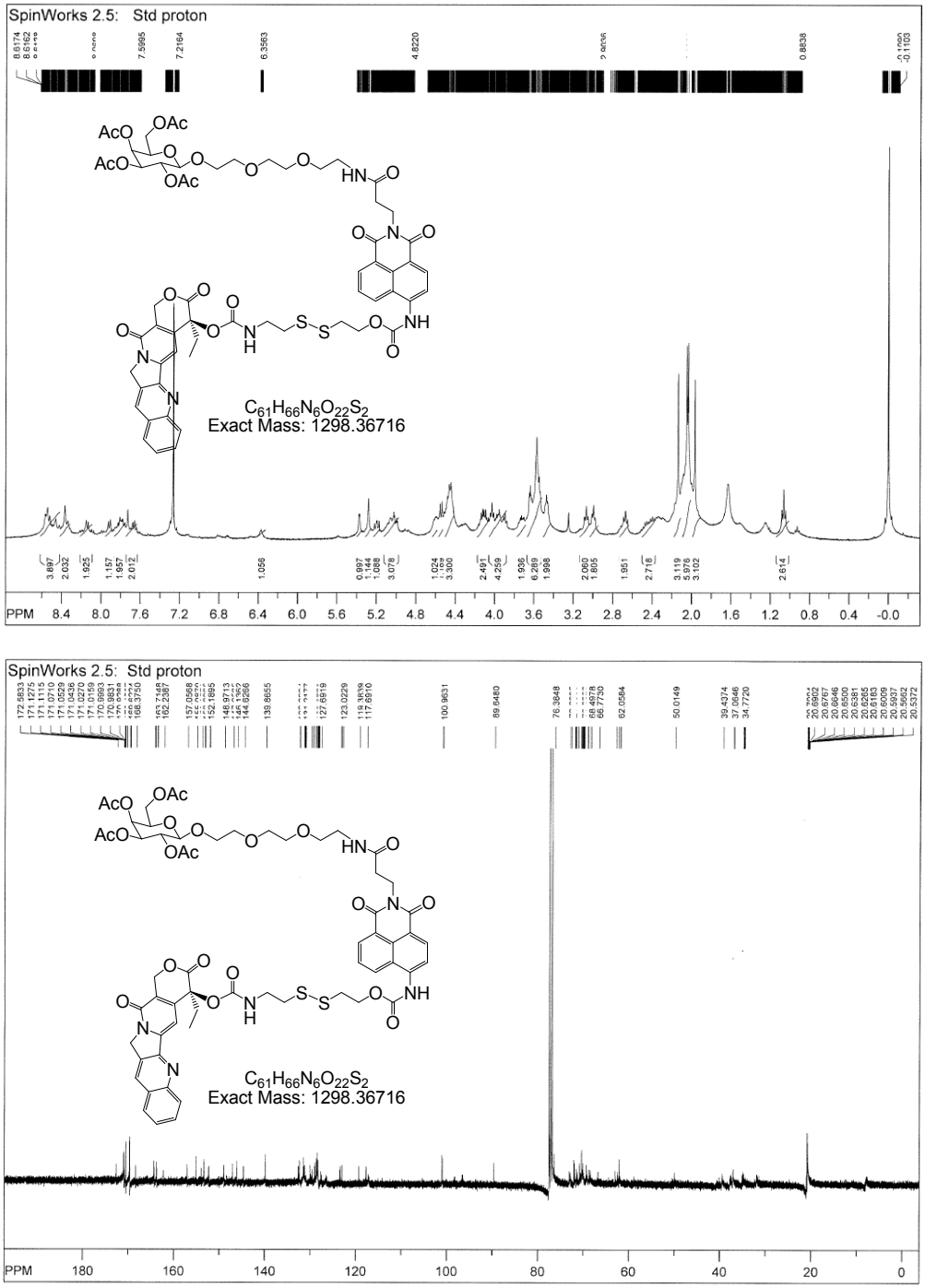


Fig. S6. ^{1}H and ^{13}C NMR spectra of compound **2**.

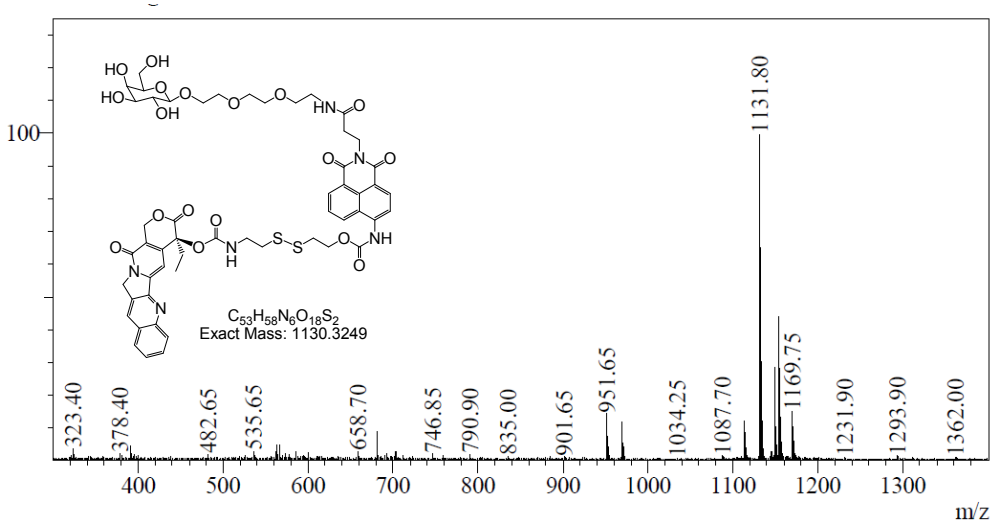


Fig. S7. ESI-MS spectrum of compound **1**.

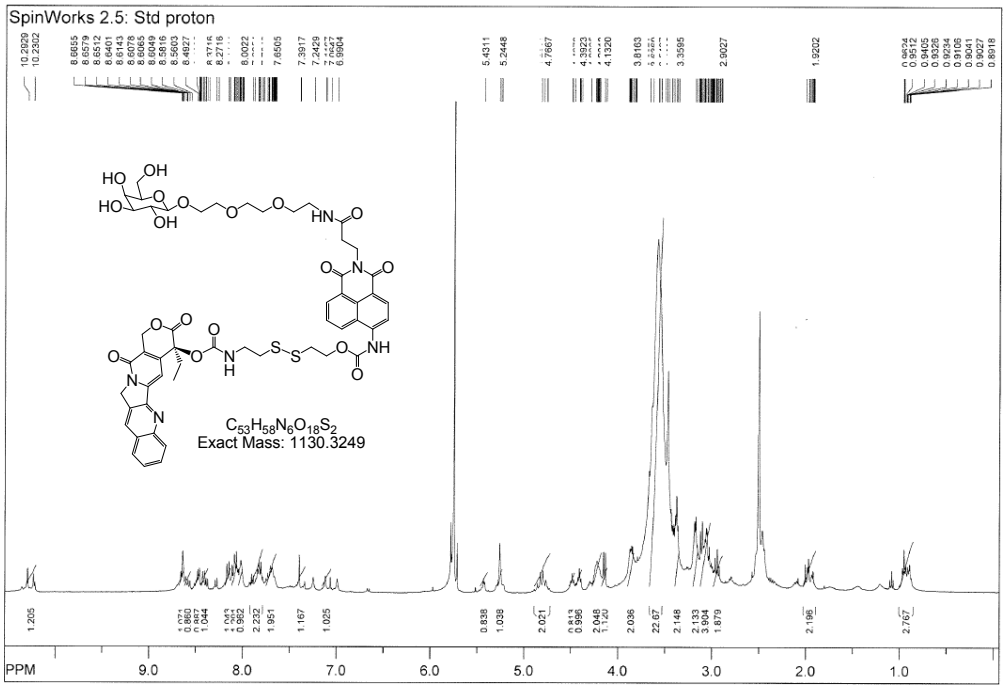


Fig. S8. ^{1}H spectra of compound **1**.

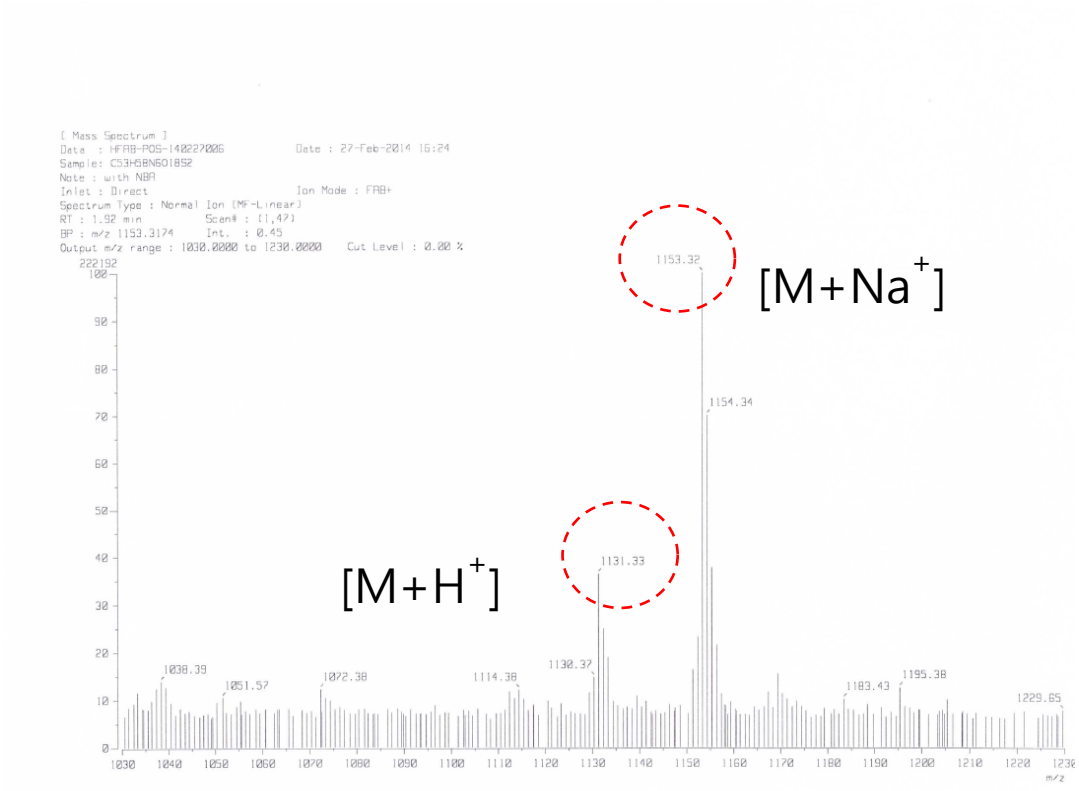


Fig. S9. High resolution mass spectra of compound **1**. Mass peak of compound 1 corresponds to peaks at [M+H$^+$] and [M+Na$^+$].

4. Additional data

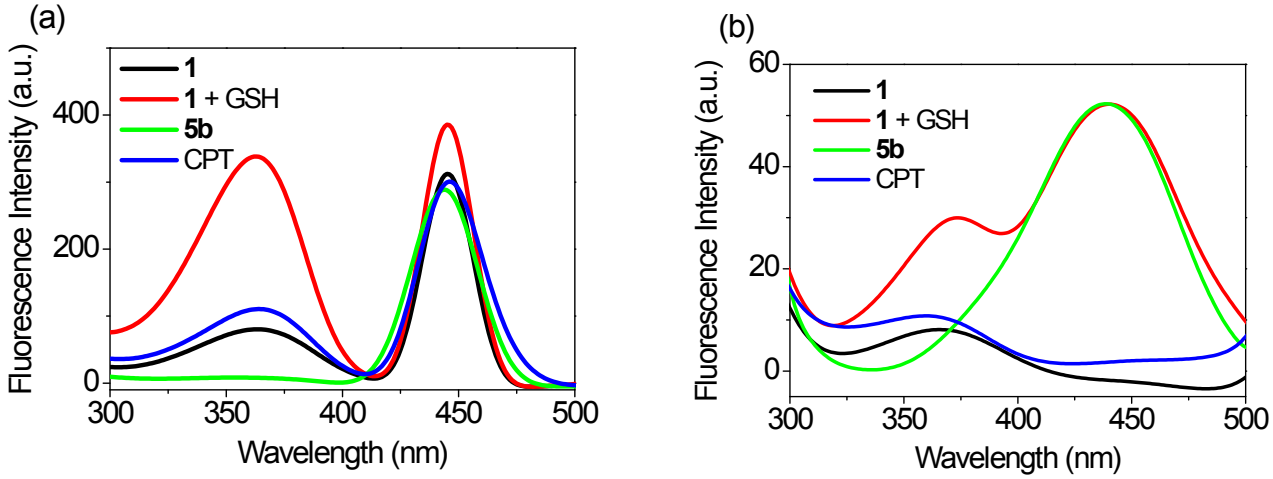


Fig. S10. Excitation spectra of **1** (1.0 μM) measured with and without GSH (5.0 mM), CPT, and **5b**. (a) λ_{em} = 445 nm; (b) λ_{em} = 540 nm. All spectra were acquired 3 h after addition of GSH in PBS buffer (pH 7.4).

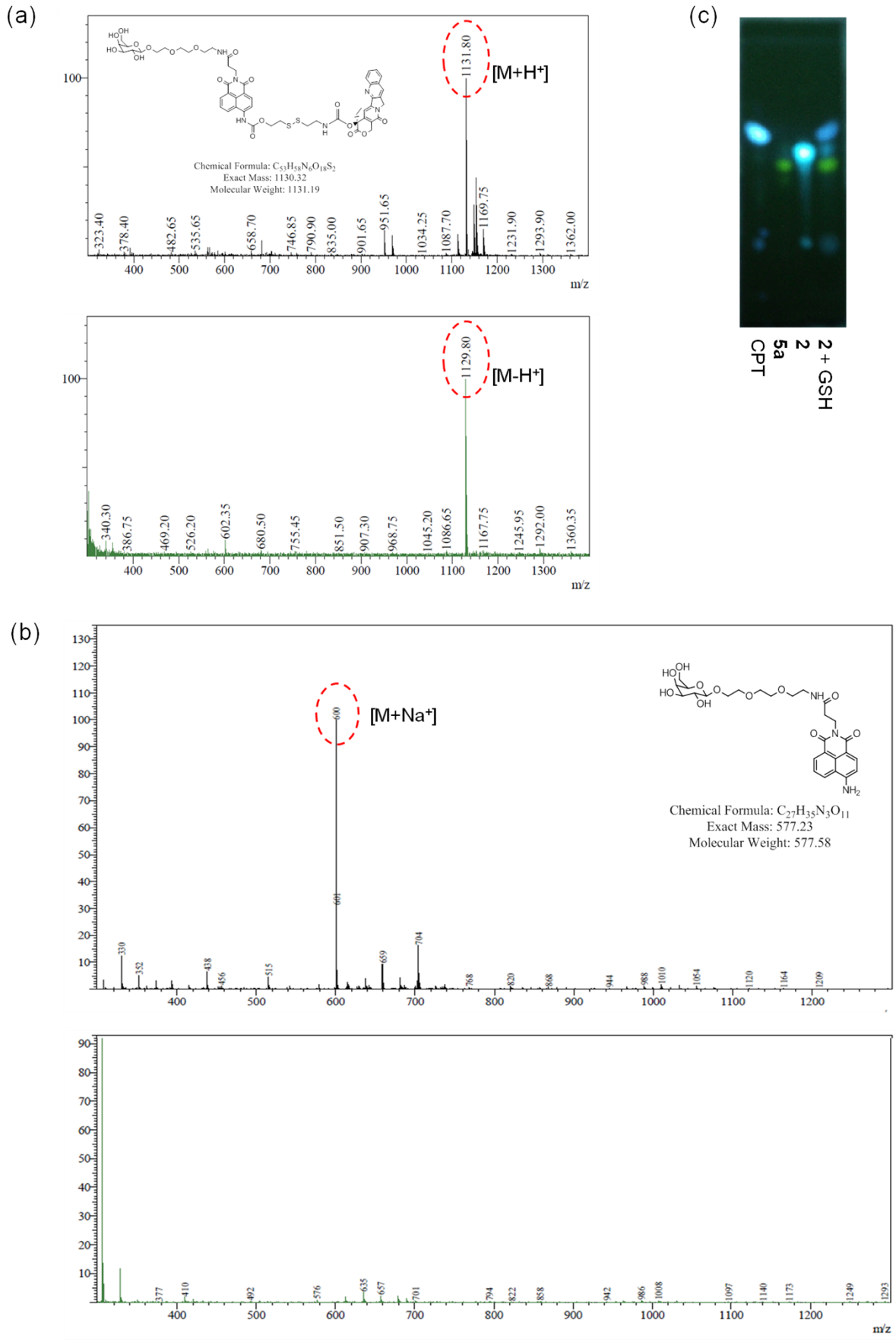


Fig. S11. (a) Mass spectra of compound **1**. Mass peak of compound **1** shown at [M+H$^+$] (upper graph = cationic m easurement) and at [M - H$^+$] (lower graph = anionic measurement) (b) Mass spectra of compound **1** with excess am ount of GSH. Mass peak of compound **5b** is shown at [M+Na$^+$] (c) Thin layer chromatography picture comparing f rom left to right - CPT, compound **5a**, compound **2** and compound **2** with addition of GSH.

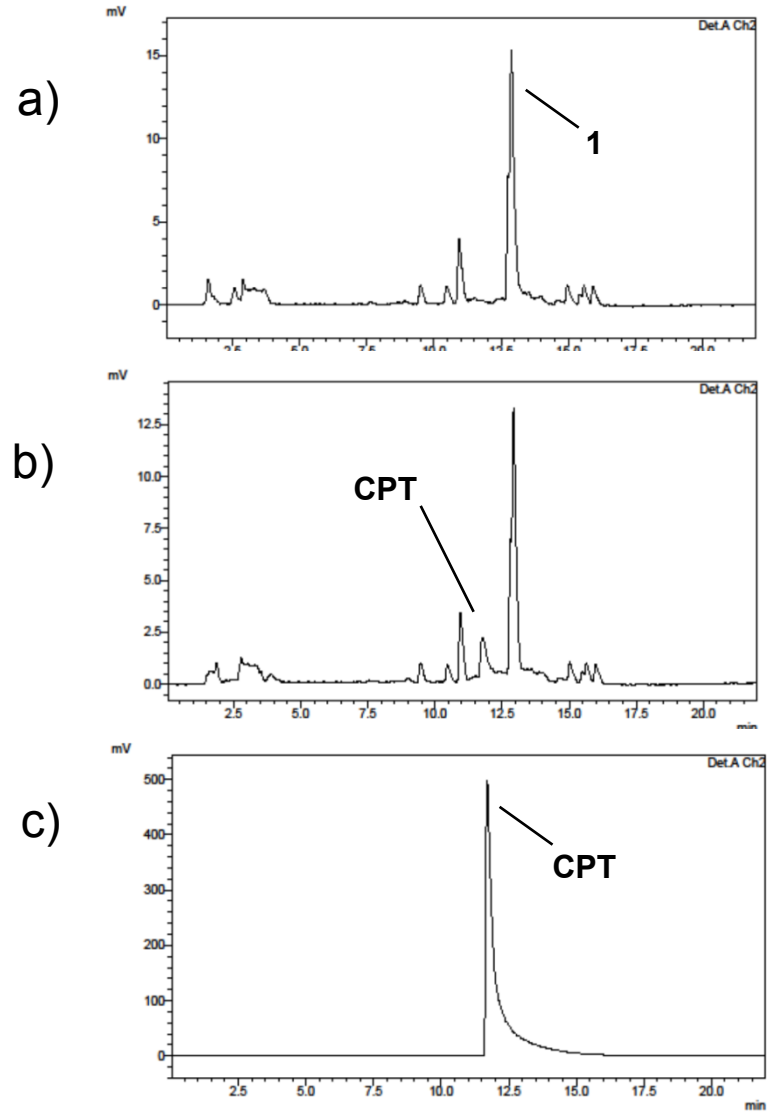


Fig. S12. Reverse-phase HPLC chromatograms. (a) **1,** (b) **1** with GSH, (c) camptothecin. Peaks in the chromatograms were detected at 350 nm.

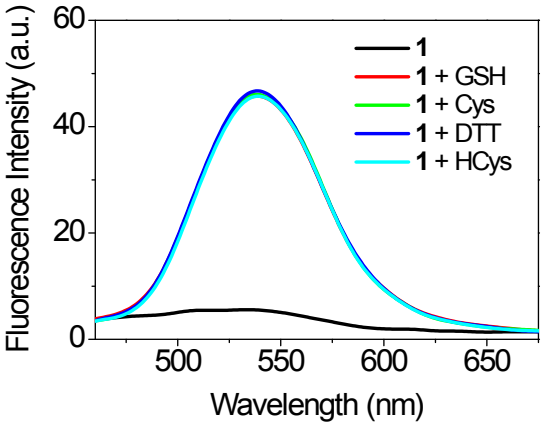


Fig. S13. Fluorescence spectra of compound **1** (1.0 μM) with and without various types of thiols. All spectra were acquired 3 hours after addition of GSH or thiols (5.0 mM) and were recorded in PBS buffer with λ_{ex} = 430 nm.

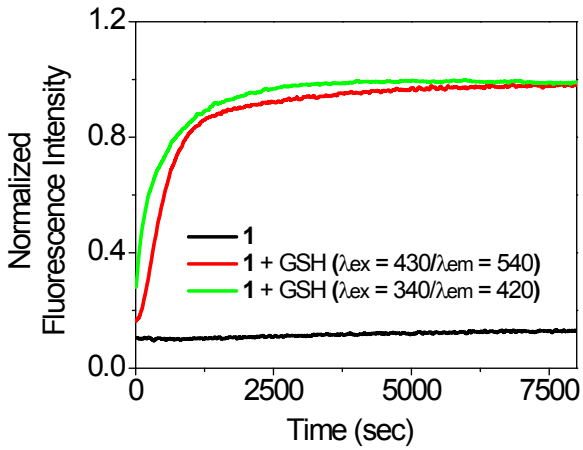


Fig. S14. Time course spectra for reaction of **1** with GSH to give **5b** (λ_{ex} = 430 nm, emission detected at 540 nm) a nd CPT (λ_{ex} = 340 nm, emission detected at 420 nm).

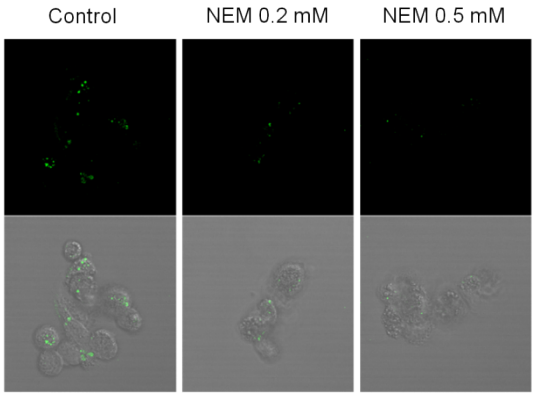


Fig. S15. Dependency of fluorescence from **1** on the concentration of NEM. The HepG2 cells were treated with N EM for 1 h then with **1** (5.0 μM) for 15 min. The cells were washed with PBS and the confocal microscopic image s were taken. The images were obtained by using excitation at 458 nm and emission with LP 505 nm.

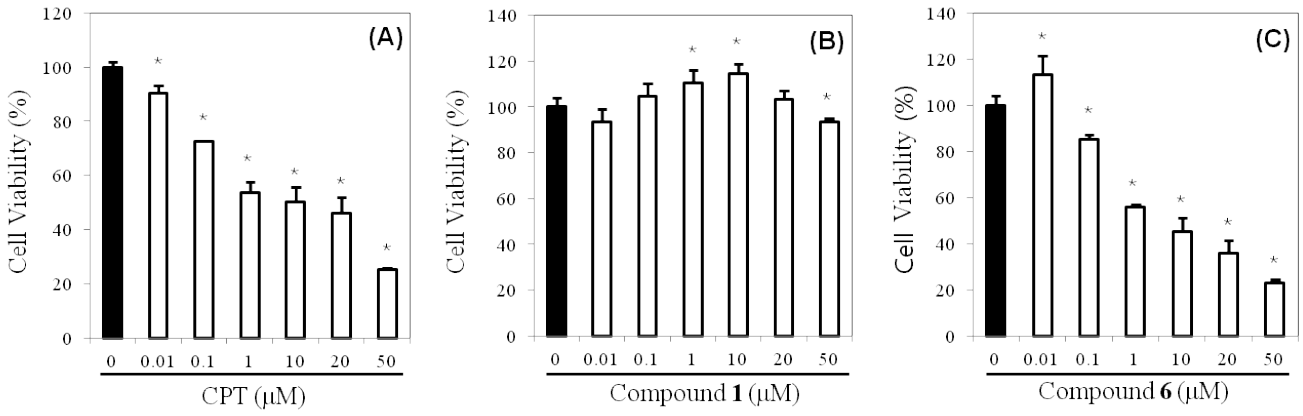


Fig. S16. The anticancer effects of CPT (A), compounds **1** (B) and **6** (C) on the HepG2 cell line. The cell viability was detected via MTT assay. The histograms are based on the average with the standard deviation (n = 4). The stat istical signification was marked as * for $p < 0.05$.