Electronic Supplementary Information (ESI) to:

Targeted combinational therapy inducing mitochondrial dysfunction

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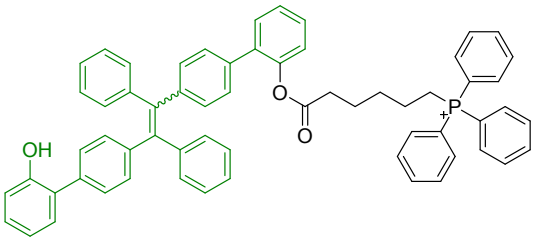
Synthesis

Materials and methods for the synthesis. The reagents used in this study were purchased from Alfa-Aesar, Aldrich, TCI, Carbsynth, Duksan, and Acros and used without further purification. Silica gel 60 (Merck, 0.040-0.063 mm) was used for column chromatography and Merck 60 F254 silica gel plates were used for analytical thin-layer chromatography. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Varian (400 MHz) and Bruker (500 MHz) instrument. The 500 MHz NMR data were collected in the NMR laboratory of Center for Molecular Spectroscopy and Dynamics, Institute of Basic Science, in Korea University. Reverse-phase HPLC experiments were performed on a SunFire C18 column (5μm, 250 × 4.6 mm) with a Young Lin HPLC system (YL9100) using a mobile phase consisting of a binary gradient of solvent A (water with 0.1% *v*/*v* TFA) and solvent B (acetonitrile with 0.1% *v*/*v* TFA). ESI mass spectrometric analyses were carried out using an LC/MS-2020 Series (Shimadzu) instrument.



11

Synthesis of 11. This compound was prepared as described before.[S1]

Synthesis of 9. This compound was synthesized, according to a previously reported procedure,[S2] with a 99 % yield.

Synthesis of 8. This compound was synthesized, according to a previously reported procedure,[S3] with a 99 % yield.

Synthesis of 7. EDC·HCl (427 mg, 2.75 mmol) was added to a solution of **9** (120 mg, 1.37 mmol), **8** (571 mg, 1.51 mmol) and DMAP (336 mg, 2.75 mmol) in CH_2Cl_2 (40 mL) at room temperature. After 12 h the mixture was washed with an aqueous 1*N* HCl solution, the organic layer was dried with $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography yielded 414 mg (67 % yield) of the title product. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.85 – 7.73 (m, 15H), 4.21 (t, J = 5 Hz, 2H), 3.72 – 3.66 (m, 2H), 3.49 (t, J =

5.15 Hz, 2H), 2.33 (t, J = 7.3 Hz, 2H), 1.75 – 1.63 (m, 6H) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ 22.11, 22.15, 22.56, 23.97, 29.41, 29.54, 33.36, 49.59, 62.75, 117.54, 118.22, 130.40, 130.50, 133.38, 133.46, 135.04, 135.06, 172.83 ppm.

Synthesis of 6. This compound was synthesized, according to a previously reported procedure,[S4] with a 77 % yield.

Synthesis of 5. This compound was synthesized, according to a previously reported procedure,[S1] with a 81 % yield.

Synthesis of 4. Propargyl bromide (46.1 mg, 0.38 mmol) was added to a suspension of compound **5** (200.0 mg, 0.38 mmol) and potassium carbonate (80.35 mg, 0.58 mmol) in MeCN (50 mL). After being stirred for 8 h under reflux conditions, the reaction mixture was concentrated under reduced pressure and diluted with 100 ml of CH_2Cl_2. The organic layer was washed with 1*N* HCl solution (3 × 20 mL), dried over $MgSO_4$ and concentrated *in vacuo*. Purification by column chromatography yielded 132 mg (62 % yield) of the title product. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.32 – 6.93 (m, 26H), 5.21 (d, J = 20.92 Hz, 1H), 4.60 (d, J = 6.84 Hz, 2H), 2.45 (d, J = 32.76 Hz, 1H) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 56.40, 56.45, 75.43, 75.44, 78.74, 114.21, 115.85, 115.86, 120.45, 122.09, 122.14, 126.46, 126.52, 127.63, 127.73, 128.28, 128.34, 128.67, 128.79, 130.97, 130.98, 131.41, 131.44, 131.47, 131.82, 143.72, 154.48 ppm.

Synthesis of 3. A solution of compound **4** (100mg, 0.18 mmol) with TEA (18mg, 0.18 mmol) in DCM (30 mL) was added dropwise into a phosgene solution (30 mL, 20% in toluene) in an ice bath. After 1h the solution was evaporated. A solution of 2-Hydroxyethyl disulfide (111.19mg, 0.72 mmol) with TEA (72 mg, 0.72 mmol) in DCM (10 mL) was added to the residue at room temperature. After stirring for 3h, the reaction mixture was extract with 1*N* HCl solution (3 × 20 mL) concentrated *in vacuo*. Purification by column chromatography yielded 33 mg (25 % yield) of the title product. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.40 – 7.05 (m, 26 H), 4.60 (d, J = 2.36 Hz, 1H), 4.58 (d, J = 2.4 Hz, 1H), 4.32 (t, J = 6.84 Hz, 1H), 4.25 (t, J = 6.68 Hz, 1H), 3.83 (t, J = 5.72 Hz, 1H), 3.78 (t, J = 5.64 Hz, 1H), 2.86 – 2.69 (m, 4H), 2.46 (t, J = 2.36 Hz, 0.5H), 2.40(t, J = 2.4 Hz, 0.5H) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 36.56, 36.57, 41.76, 41.84, 56.60, 56.68, 60.38, 60.41, 66.44, 66.50, 75.70, 75.77, 79.06, 79.07, 114.20, 114.39, 122.36, 122.38, 122.45, 126.73, 126.79, 126.98, 127.00, 127.91, 127.96, 127.98, 128.04, 128.31, 128.40, 128.74, 128.95, 129.02, 131.24, 131.27, 131.66, 131.69, 131.72, 131.76, 134.48, 134.55, 135.09, 135.21, 136.42, 136.47, 140.70, 141.44, 141.45, 142.45, 143.35, 143.42, 143.89, 143.95, 143.96, 144.02, 148.15, 148.16, 153.23, 153.28, 154.72, 154.73 ppm. ESI-MS: the calculated value (calcd) for $C_{46}H_{38}O_5S_2Na$ ($M + Na^+$): 757.21, $C_{46}H_{38}O_5S_2K$ ($M + K^+$): 773.18, found 757.20, 773.20.

Synthesis of 2. EDC·HCl (42 mg, 0.27 mmol) was added to a solution of **3** (100 mg, 0.13 mmol), Chlorambucil (62 mg, 0.20 mmol) and DMAP (33 mg, 0.27 mmol) in CH_2Cl_2 (15 mL) at room temperature. After 12 h the mixture was washed with an aqueous 1*N* HCl solution, the organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography yielded 114 mg (83 % yield) of the title product. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.40 – 7.03 (m, 28H), 6.60 (d, J = 8.44 Hz, 2H), 4.59 (d, J = 2.4 Hz, 1H), 4.58 (d, J = 2.36 Hz, 1H), 4.32 – 4.20 (m, 4H), 3.68 – 3.57 (m, 8H), 2.89 (t, J = 6.52 Hz, 1H), 2.84 (q, J = 6.32 Hz, 2H), 2.70 (t, J = 6.72 Hz, 1H), 2.53 (t, J = 7.44 Hz, 2H), 2.45 (t, J = 2.28 Hz, 0.5H), 2.39 (t, J = 2.32 Hz, 0.5H), 2.33 – 2.28 (m, 2H), 1.90 – 1.85 (m, 2H) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 14.34, 14.35, 22.89, 26.85, 26.89, 29.91, 29.94, 31.82, 33.65, 33.68, 34.13, 34.16, 36.74, 37.49, 37.51, 40.64, 40.68, 53.83, 53.86, 56.67, 62.21, 62.24, 66.36, 66.43, 75.62, 75.65, 75.72, 76.92, 76.94, 77.23, 77.26, 77.55, 112.46, 114.17, 114.38, 122.32, 122.44, 126.75, 126.93, 127.91, 128.28, 128.37, 128.55, 128.66, 128.88, 128.95, 129.92, 129.95, 131.22, 131.63 ppm. ESI-MS: the calculated value (calcd) for $C_{60}H_{55}Cl_2NO_6S_2Na$ (M + Na^+): 1042.27, $C_{60}H_{55}Cl_2NO_6S_2K$ (M + K^+): 1058.25, found 1042.20, 1056.20.

Synthesis of 1. Compound **2** (20 mg, 0.02 mmol), **7** (10 mg, 0.023 mmol), $CuSO_4 \cdot 5H_2O$ (2.44 mg, 0.001 mmol), and sodium ascorbate (2 mg, 0.001 mmol) were mixed in DMF (5 ml) and then stirred at room temperature under argon for 4 h. At this point, the volatiles were removed under reduced pressure and the reaction mixture was purified by HPLC, to yield 26 mg (91 % yield) of the title product. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.72 – 7.61 (m, 15H), 7.28 – 6.96 (m, 26H), 6.56 (d, J = 7.96 Hz, 2H), 5.05 (br.s, 2H), 4.55 – 4.16 (m, 8H), 3.64 – 3.53 (m, 8H), 2.90 – 2.77 (m, 4H), 2.65 (t, J = 6.36 Hz, 1H), 2.49 (t, J = 7.52 Hz, 2H), 2.29 – 2.24 (m, 2H), 2.20 – 2.17 (m, 2H), 1.86 – 1.82 (m, 2H), 1.62 – 1.46 (m, 8H) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 14.35, 22.22, 22.50, 22.85, 24.27, 26.87, 29.62, 29.79, 29.89, 31.78, 33.65, 33.72, 34.11, 36.69, 36.76, 36.81, 37.32, 37.45, 40.79, 53.75, 62.21, 66.35, 112.33, 114.21, 117.92, 118.77, 122.02, 122.44, 124.82, 126.72, 127.87, 127.93, 128.27, 128.92, 128.98, 129.90, 130.59, 130.69, 130.81, 131.13, 131.59, 133.74, 133.84, 135.32, 135.35, 140.74, 141.22, 142.41, 143.26, 144.53, 148.10, 153.10, 155.32 ppm. ESI-MS: the calculated value (calcd) for $C_{86}H_{84}Cl_2N_4O_8S_2P^+$ (M + H^+): 1465.48, found 1466.95.

Cell studies

Human normal and cancer cells. Human cancer cell lines (HCT-116, HeLa, LNCaP, and PC-3) and the normal human dermal fibroblast NHDF cells were purchased from American Type Culture Collection (Rockville, MD, USA) or Korea Cell Line Bank (Seoul, South Korea). The cells were maintained in Roswell Park Memorial Institute 1640 or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc., Gaithersburg, MD) at 37 ˚C in a humidified atmosphere with 5% CO_2. N-acetyl-L-cysteine (NAC) was purchased from Sigma-Aldrich (St. Louis, MO).

Cell growth assay. To measure cellular growth, cells were seeded in 6-well plate at the density of 0.8 x 10^5 cells per well in triplicate and maintained in the presence of 10% FBS for 48 h. Cells were washed twice with PBS, and medium containing 10% FBS with added chemical compounds. Cell numbers were counted using a hemocytometer after 24 h incubation.

Cell viability assay. Cell viability was determined using MTT assay. The cells were seeded at a density of 5 x 10^3 per well in a 96-well microplate, stabilized for 48 h, and then exposed to synthesized compounds, chlorambucil, or NAC. After 24 h of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added (20 μl/well of a 5 mg/ml solution in PBS) for 3 h. Solubilisation of the converted purple formazan dye was accomplished by adding 50 μl/well of 0.04 N HCl/isopropyl alcohol. The reaction product was quantified by measuring the absorbance at 570 nm using a Bio-Rad 680 microplate reader (Bio-Rad)

Flow cytometric analysis of apoptosis. Cells were seeded at the density of 0.8 x 10^5 cells in 6-well plate and maintained in medium with 10% serum for 48 h. Cells were washed twice with PBS, and medium containing 10% FBS and synthesized compounds were added and incubated for 24 h. Cells were fixed with 70% ethanol and resuspended in 1 ml of PBS containing 50 μg/ml RNase and 50 μg/ml propidium iodide (Sigma). The assay was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and sub-G1 fraction was analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA). For the Annexin V assay, equally treated cells were trypsinized and stained with Annexin V-FITC (0.5 μg/ml) and propidium iodide and analyzed using a FACScan flow cytometer.

siRNA and transfection. Short-interfering RNA (siRNA) duplexes against glutathione synthetase (GSS) (si-GSS: 5'-CAUCAAACAGGAUGACUUU-3') and control siRNA duplex that served as a negative control were synthesized by Dharmacon Research (Lafayette, CO, USA). Transfection of siRNAs or expression plasmids was performed using

Microporator™ (Neon transfection system, Invitrogen). Knockdown of GSS expression was verified using quantitative RT-PCR assay. Briefly, 1 μg of total cellular RNA was converted to cDNA by reverse transcription using random hexamer primers and MoMuLV reverse transcriptase (Invitrogen). PCR was performed in 1.5 mM $MgCl_2$-containing reaction buffer for 35 cycles using 1:4 diluted cDNA (12.5 ng per 50 μl of PCR) and primers for *GSS* (sense; 5′-TCCAGCACCATCAAACAGGAT-3′ and antisense; 5′-ATCACATGGATGTTCCTGGC-3′) and an endogenous expression standard gene *GAPDH*. 10 μl of PCR products were resolved on 2% (*wt*/*vol*) agarose gels.

Immunoblotting assay. Cells were washed twice in ice-cold PBS and lysed in a radioimmunoprecipitation assay buffer containing 50 mM tris·HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, and protease inhibitor mixture. After sonification, the lysate was centrifuged, and supernatant was recovered and loaded on an 8% SDS–polyacrylamide gel for electrophoresis. Immunoblot analysis was performed using antibodies specific for cleaved PARP and β-tubulin (Santa Cruz Biotechnology).

Co-localisation experiment. HeLa cells were incubated with 20 μM of compound **1** (0.4% DMSO in PBS, pH 7.4) for 20 min, and were then treated with 0.5 μM of Mito-tracker deep Red for an additional 10 min. After cells were washed three times with saline (PBS, pH 7.4), cell images were obtained with a confocal microscope (Leica TCS SP2 model) fitted with a 100× oil lens (numerical aperture = 1.30). Fluorescence images for compound **1** were collected at 400–600 nm range following two photon excitation at 740 nm, and fluorescence images for Mito-tracker deep Red were collected at 650-700 nm range following excitation at 630 nm.

Cellular fluorescence intensity determination

NHDF and PC3 cells were seeded in 6-well plate at the density of 0.8 x 10^5 cells per well and maintained in the presence of 10% FBS for 24 h. Cells were washed twice with PBS, and medium containing 10% FBS with added compound **1**. After 24h incubation, the fluorescence intensity of trypsinized cells were measured by SpectraMAX I3x (Molecular devices) at an excitation wavelength of 350 nm and an emission wavelength of 460 nm in 96-well black plate with flat, black bottom.

Supplementary references

S1 W. S. Shin, M.-G. Lee, P. Verwilst, J. H. Lee, S.-G. Chi, J. S. Kim, *Chem. Sci.*, 2016, ***7***, 6050–6059.

S2 O. Norberg, L. Deng, T. Aastrup, M. Yan, O. Ramström, *Anal. Chem.*, 2011, ***83***, 1000–1007.

S3 C. Trapella, R. Voltan, E. Melloni, V. Tisato, C. Celeghini, S. Bianco, A. Fantinati, S. Salvadori, R. Guerrini, P. Secchiero, G. Zauli, *J. Med. Chem.*, 2016, ***59***, 147–156.

S4 Y. Liu, C. Deng, L. Tang, A. Qin, R. Hu, J. Z. Sun and B. Z. Tang, *J. Am. Chem. Soc.*, 2011, ***133***, 660–663.

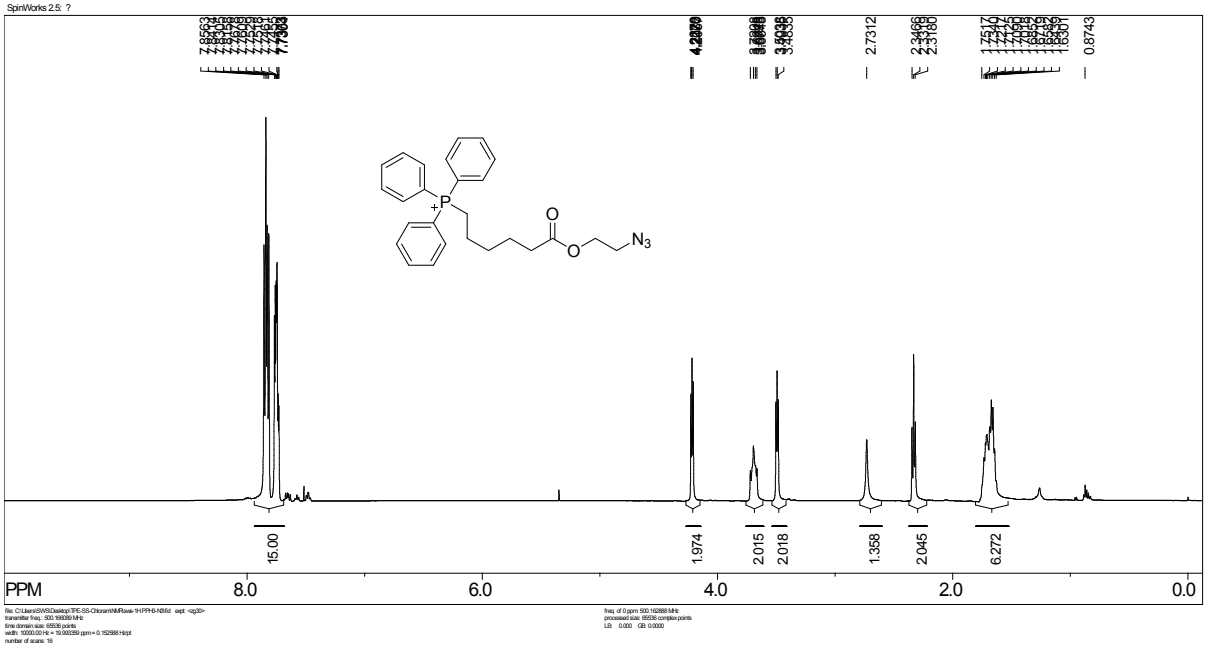


Figure S1. ^{1}H NMR spectrum of compound **7**

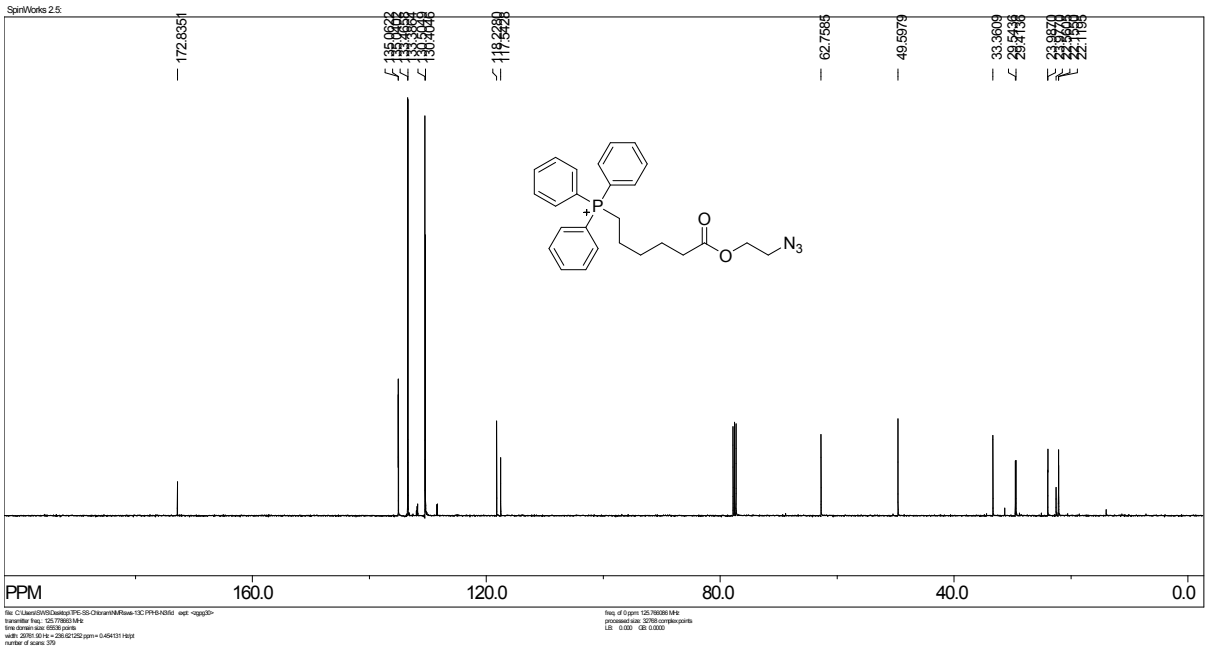


Figure S2. ^{13}C NMR spectrum of compound **7**

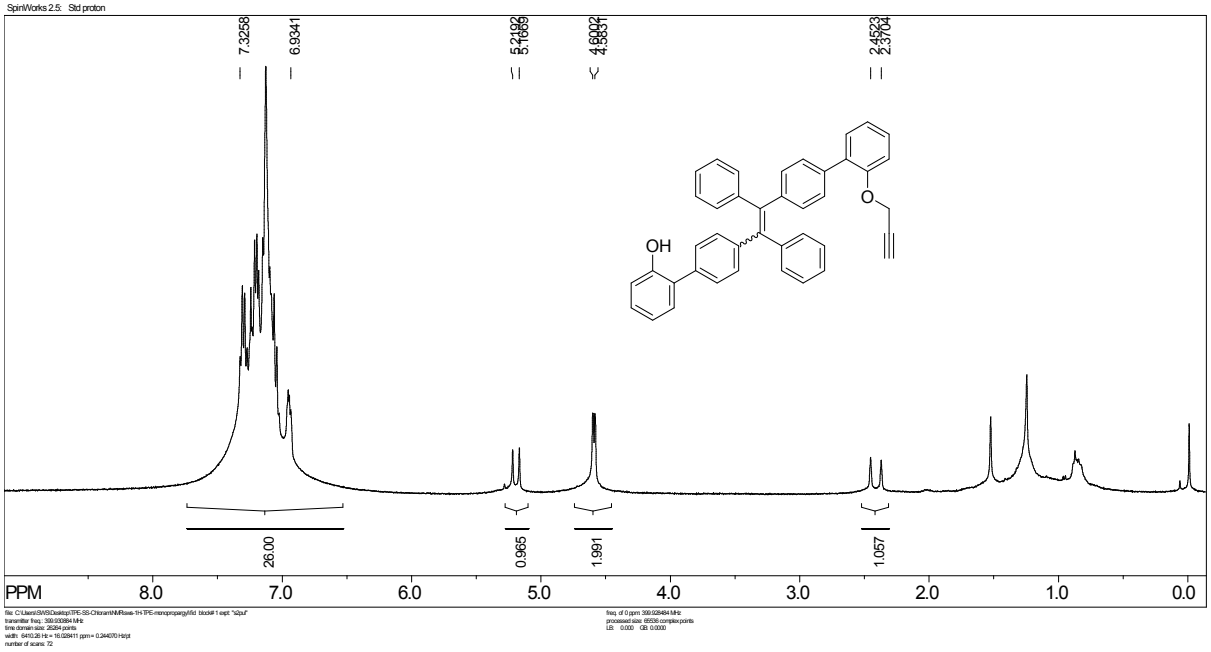


Figure S3. ^{1}H NMR spectrum of compound **4**

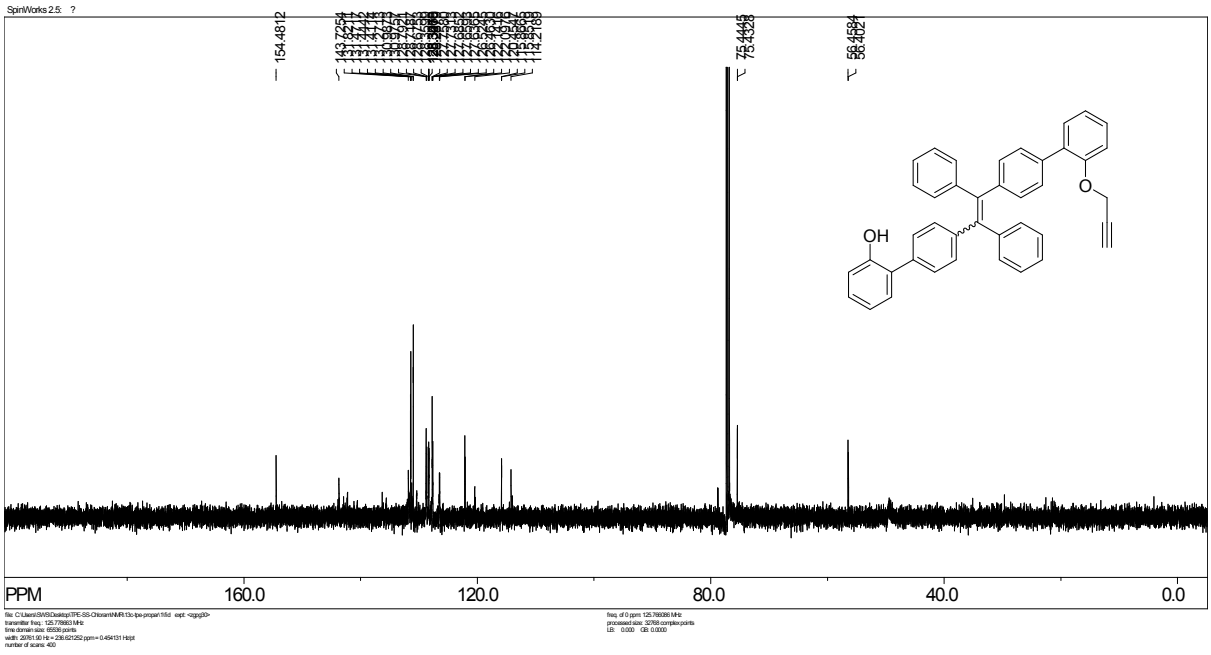


Figure S4. ^{13}C NMR spectrum of compound **4**

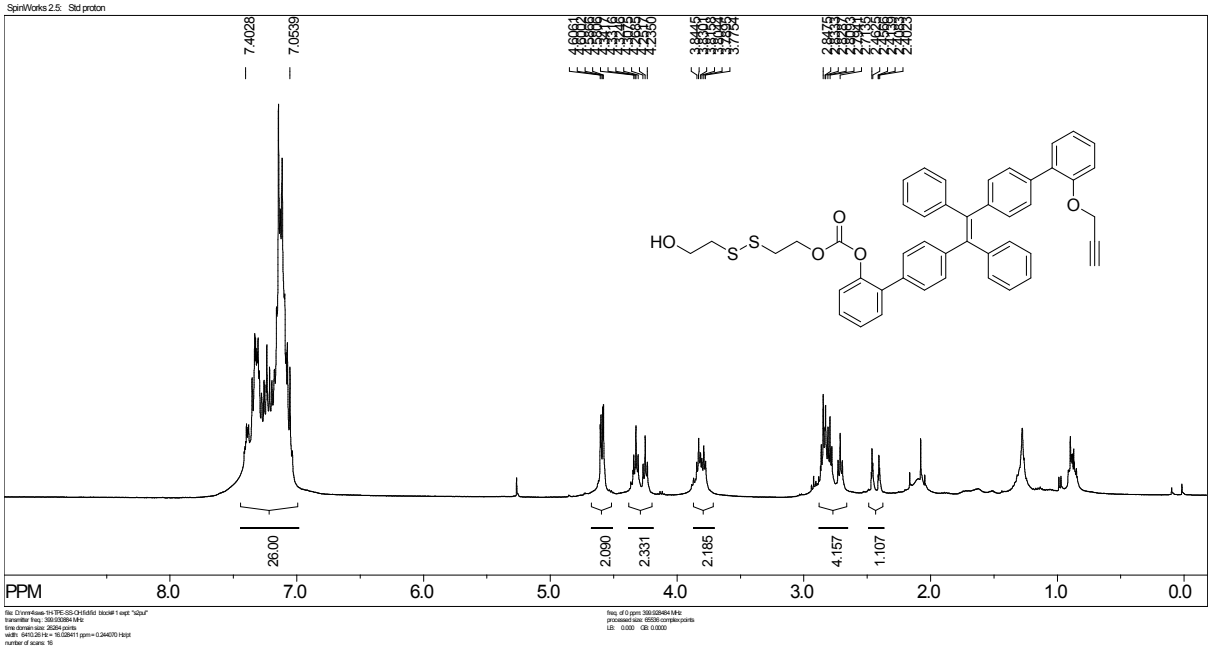


Figure S5. ^{1}H NMR spectrum of compound **3**

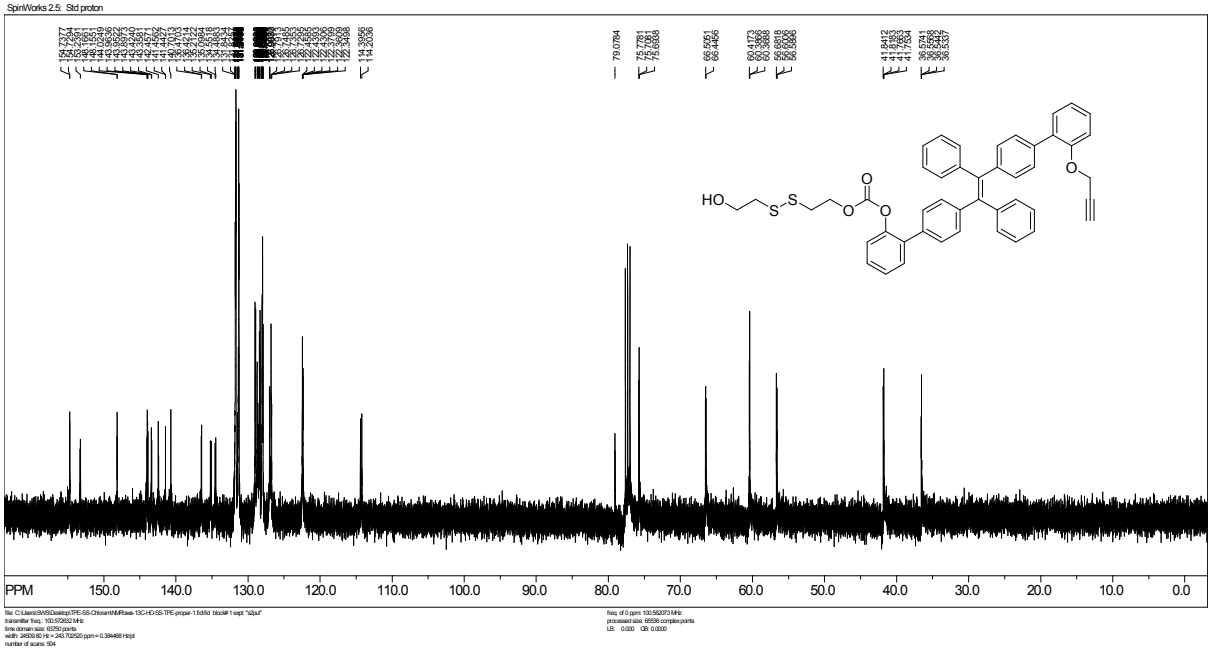


Figure S6. ^{13}C NMR spectrum of compound **3**

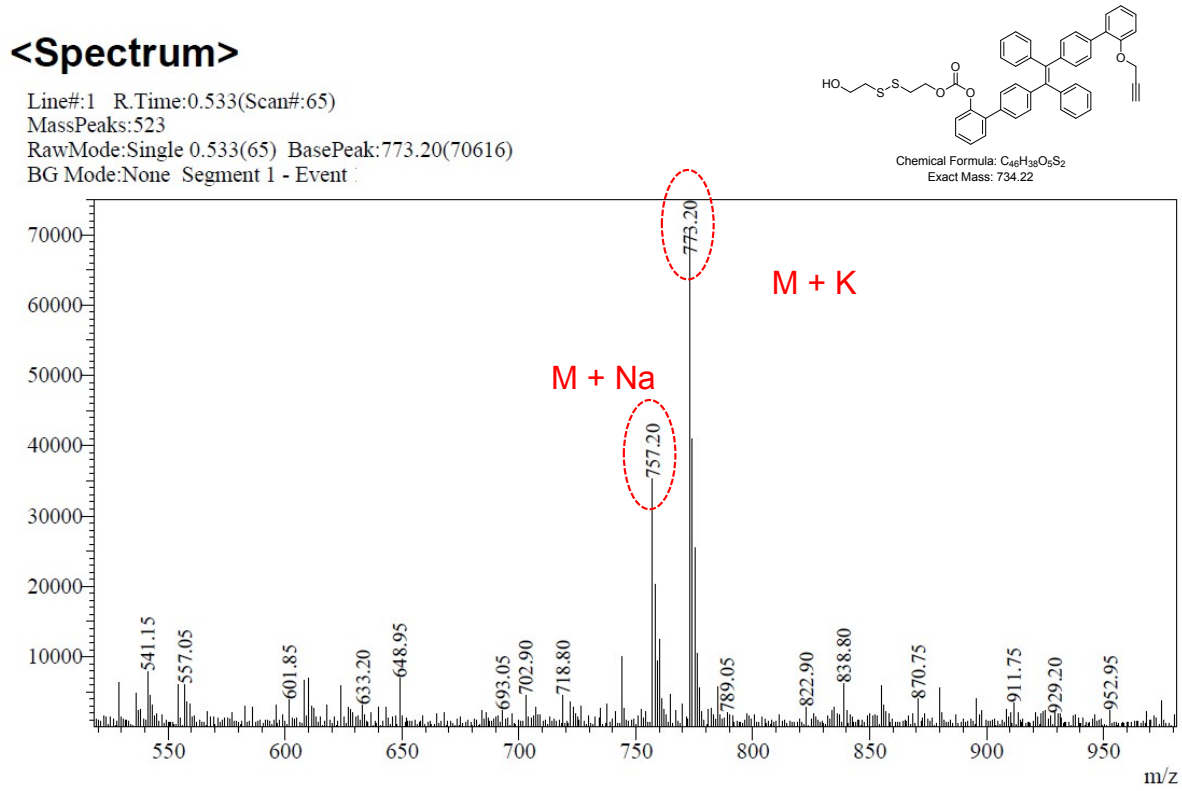


Figure S7. Mass spectrum of compound **3**

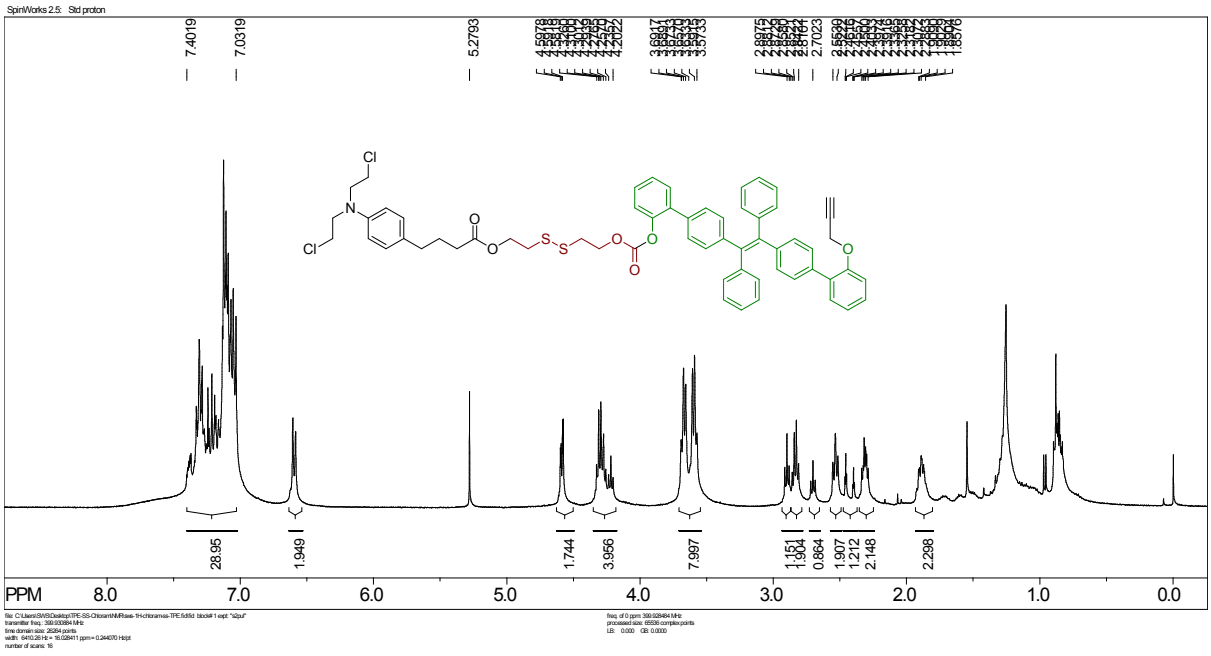


Figure S8. ^{1}H NMR spectrum of compound **2**

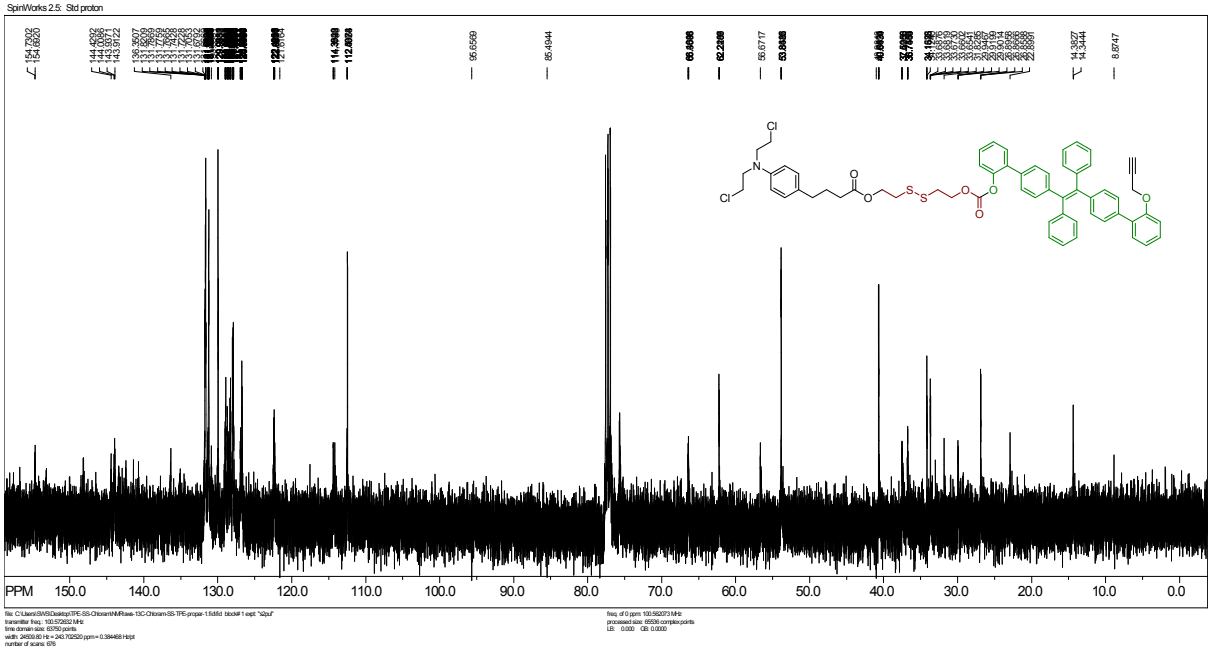


Figure S9. ^{13}C NMR spectrum of compound **2**

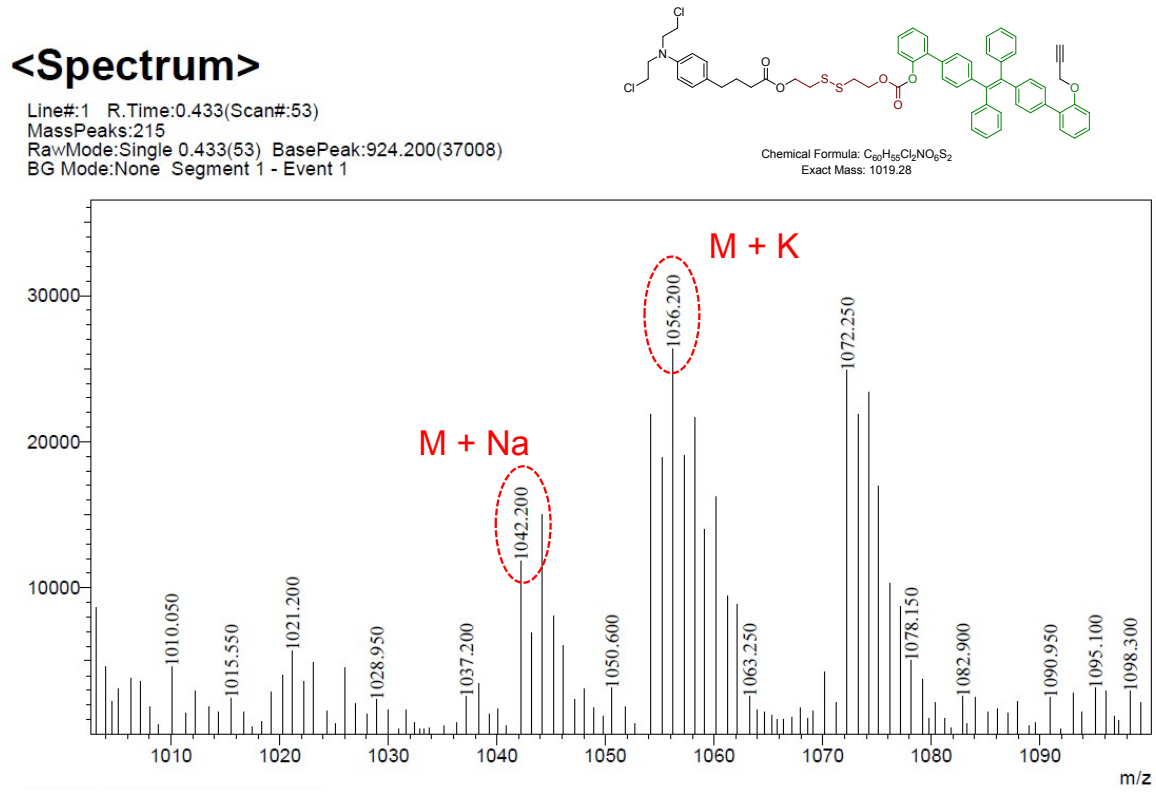


Figure S10. Mass spectrum of compound **2**

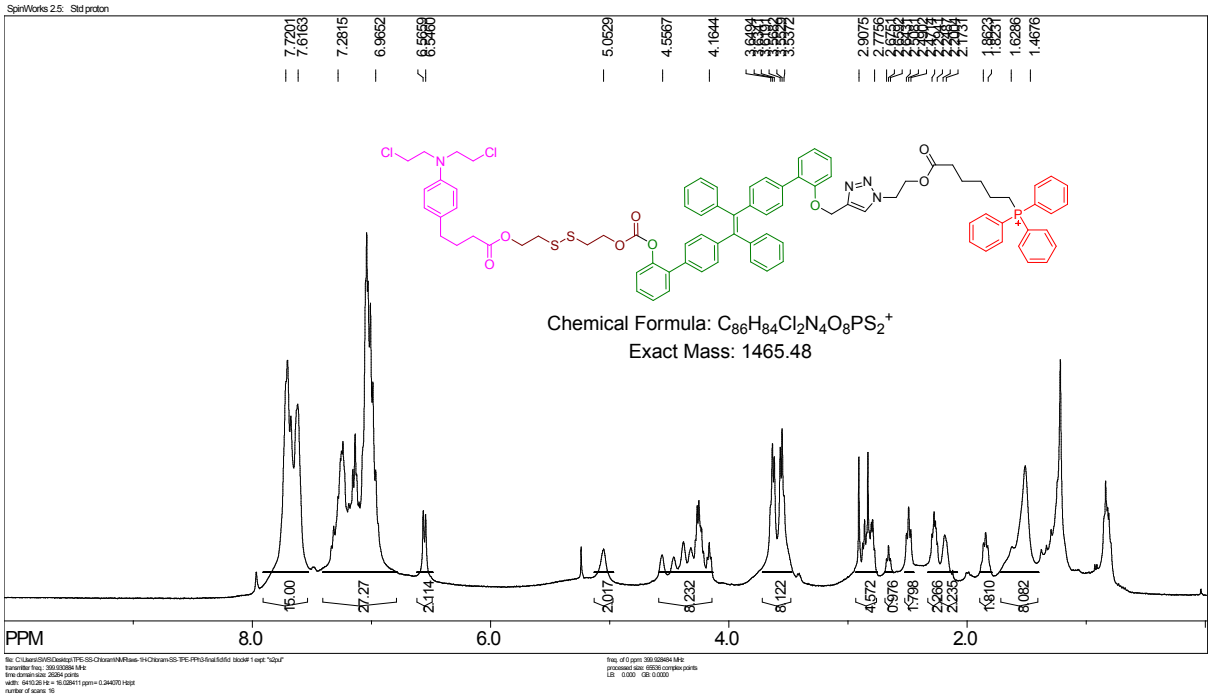


Figure S11. ^{1}H NMR spectrum of compound **1**

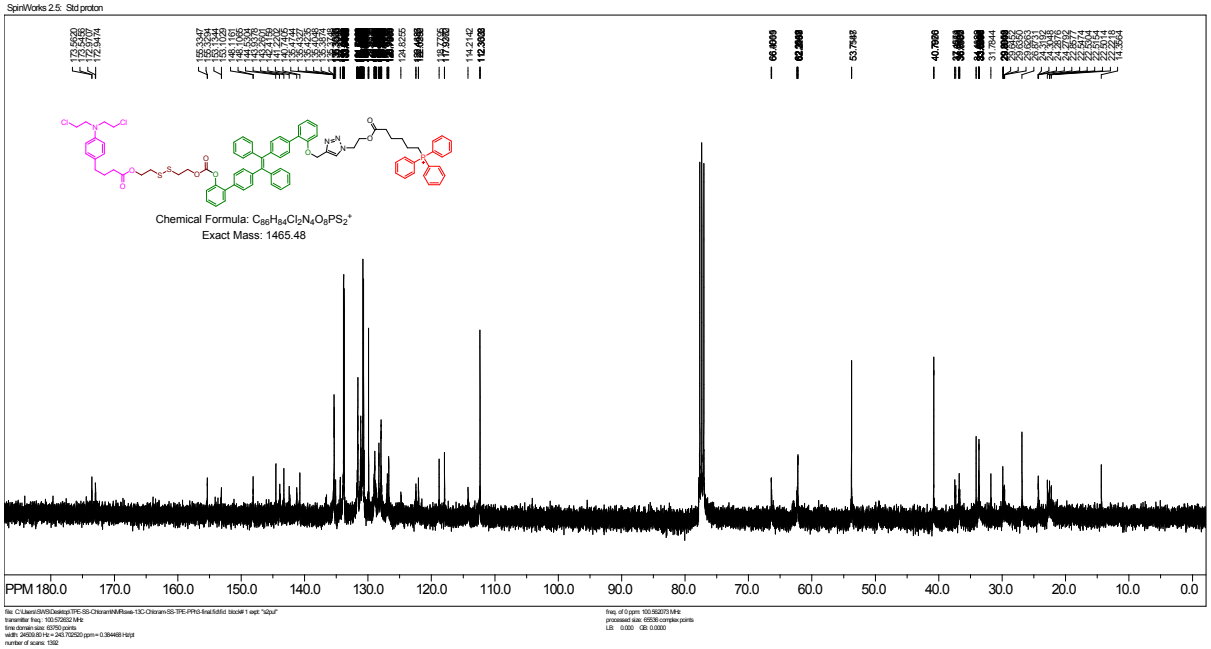


Figure S12. ^{13}C NMR spectrum of compound **1**

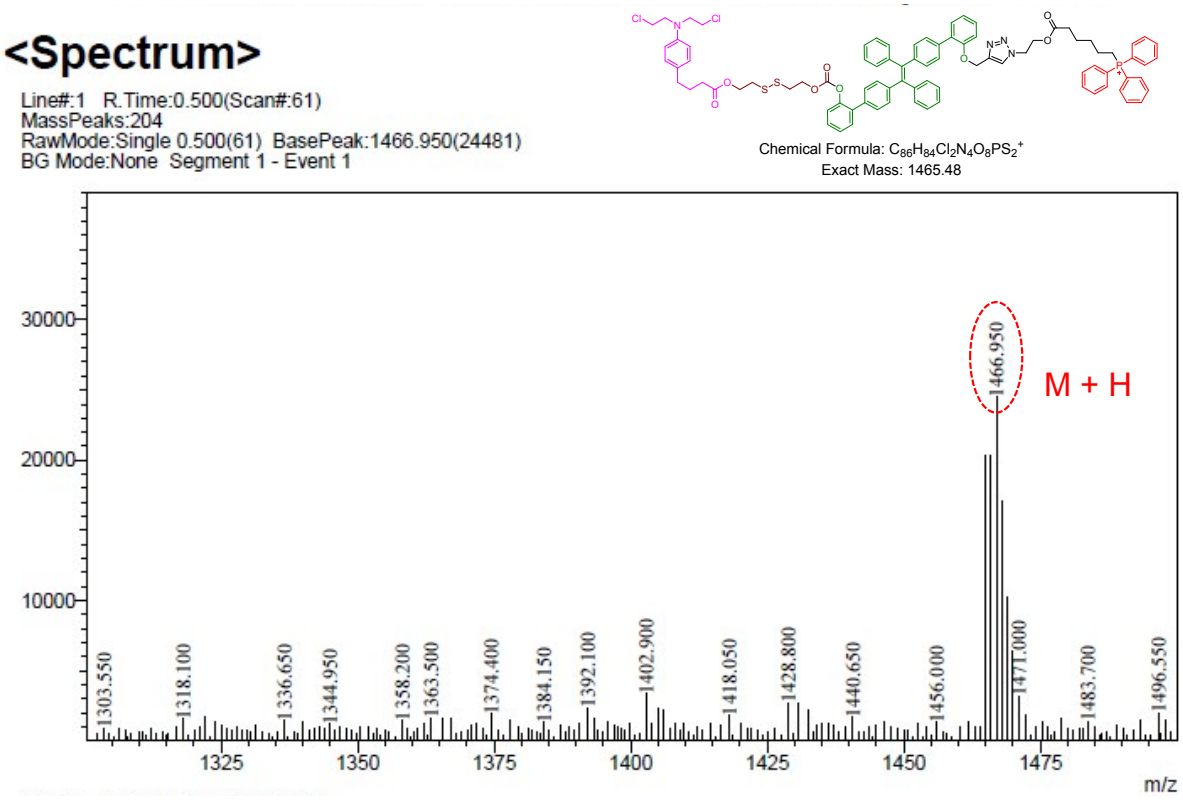


Figure S13. Mass spectrum of compound **1**

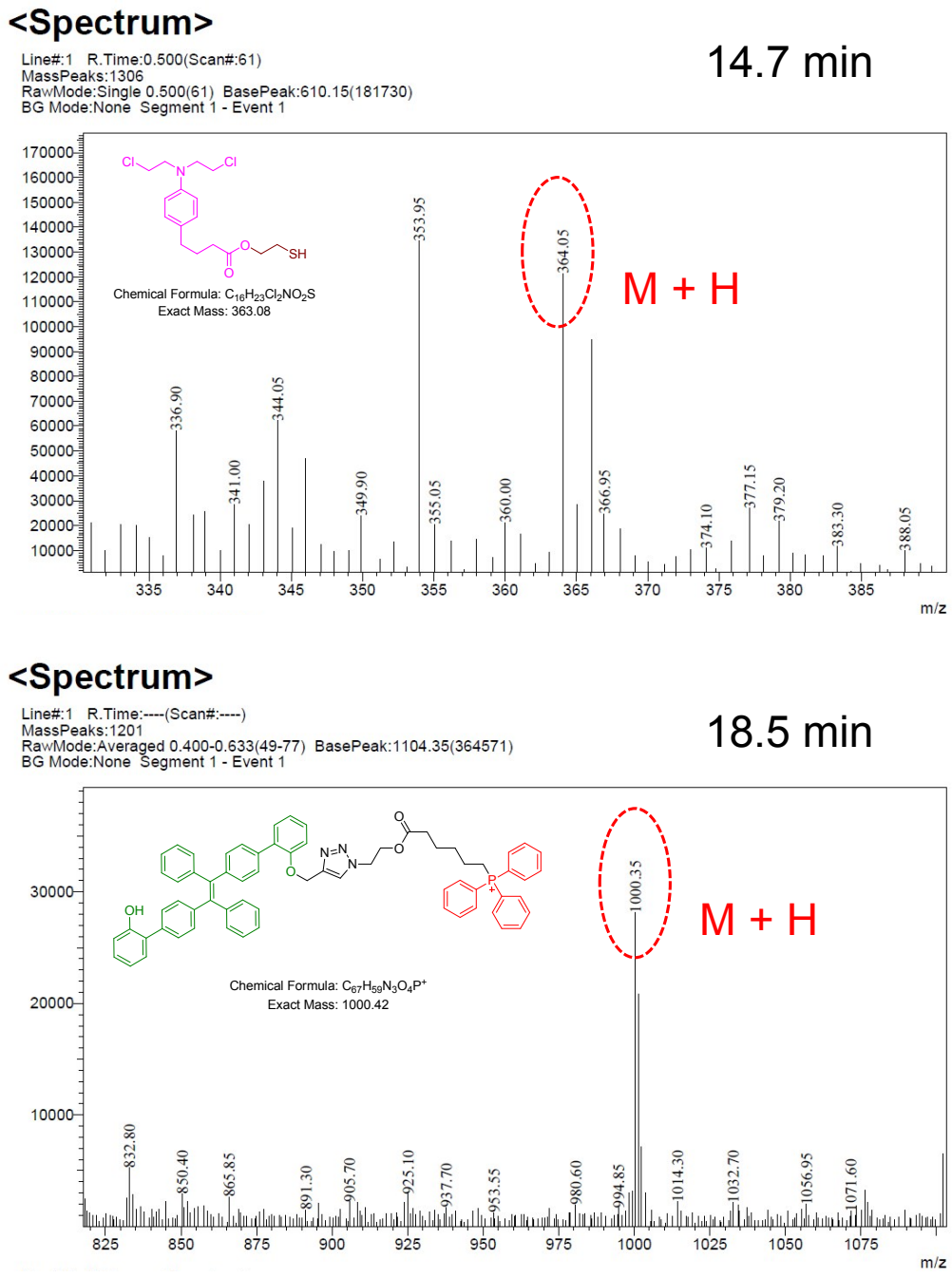


Figure S14. Mass spectra after GSH reduction.

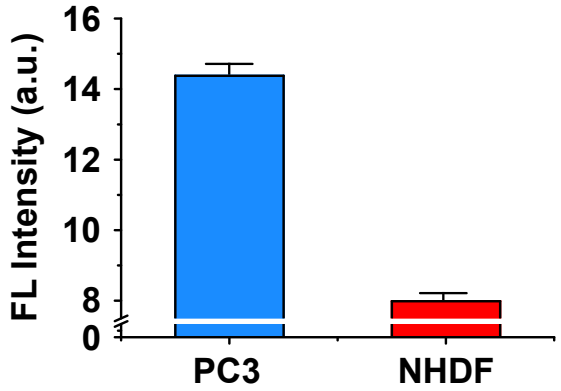


Figure S15. The fluorescence intensity of 1 in PC3 (cancer cell) and NHDF (normal cell). Fluorescence intensity of suspended cells were detected at an excitation wavelength of 350 nm and an emission wavelength of 460 nm.

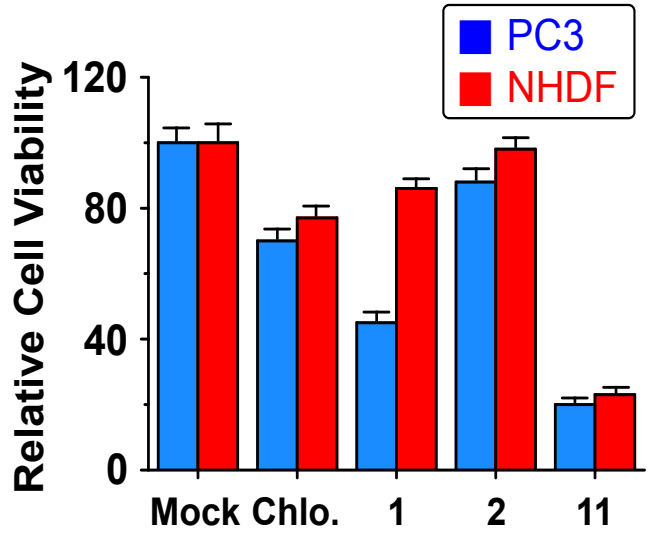


Figure S16. Comparison of compounds **1, 2, 11** and chlorambucil on cell viability. MTT assays were performed after 24 h of treatment (20 μM).

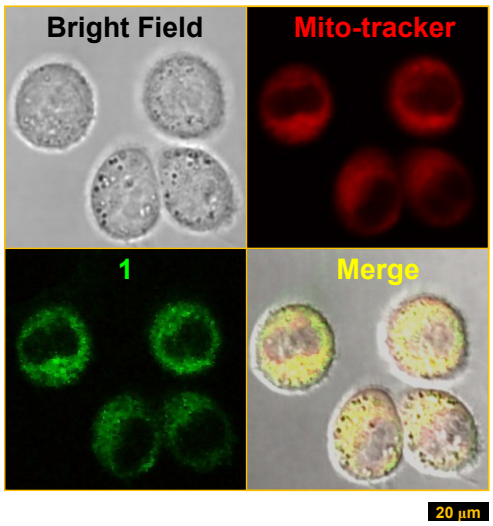


Figure S17. Intracellular co-localisation image of Compound 1 in PC3 cells using confocal microscopy. Cell images were taken after incubation with 20 µM of compound **1** for 2 hours and co-stained with 0.5 µM of Mito-tracker deep red for 30 min. The red fluorescence originates from Mito-tracker deep red (λ_{ex} = 633 nm, λ_{em} = 650–700 nm) and the green fluorescence originates from compound **1** (λ_{ex} = 740 nm, λ_{em} = 400–600 nm) using two photon excitation.