Supporting Information

For

Biotin-guided fluorescent-peptide drug delivery system for cancer

Taeyoung Kim,[a] Hyun Mi Jeon,[b] Hoa Thi Le,[b] Tae Woo Kim,[b] Chulhun Kang,*[,b] Jong Seung Kim*[,a]

[a] Department of Chemistry, Korea University, Seoul 136-701, Korea

[b] The East-West Medical Science, Kyung Hee University, Yongin 446-701, Korea

*Corresponding authors: kangch@khu.ac.kr (C. Kang); jongskim@korea.ac.kr (J. S. Kim)

Experimental

Materials, Methods, and Instrumentation

All reactions were carried out under nitrogen atmosphere. Column chromatography was performed using silica gel 60 (70–230 mesh) as the stationary phase. Analytical thin layer chromatography was performed using 60 silica gel (precoated sheets, 0.25 mm thick). ^{1}H and ^{13}C NMR spectra were collected in $CDCl_3$, DMSO, and CD_3OD (Cambridge Isotope Laboratories, Cambridge, MA) on Varian 300 and 400 MHz spectrometers. All chemical shifts are reported in ppm by using TMS as an internal reference. ESI mass spectral analyses were carried out using an LC/MS-2020 Series (Shimadzu). Reverse-phase HPLC experiments were conducted using an Agilent HPLC (Agilent 1100 series) with a Zorbax C18 (3.5 μm, 4.6 × 150 mm) column, and a Waters HPLC (Waters 600) with an XBridge ™ C18 (5 μm, 19 × 150 mm) column for preparative separation.

Cell lines and cell culture

In this study, HepG2 (human hepatocellular carcinoma) and WI-38 (human lung diploid cell line) cells were cultured in RPMI (WelGene Inc., Seoul, Korea) supplemented with 10% FBS (WelGene Inc., Seoul, Korea), penicillin (100 units/mL), and streptomycin (100 μg/mL). All cells were maintained in a humidified 5% CO_2 incubator at 37 °C.

Cell viability assay

To determine the cytotoxicity of the peptide or probe from HepG2 cells (biotin receptor-positive cell line) and WI-38 cells (biotin receptor-negative cell line), the cells were plated at 1×10^4 cells/well in a 96-well plate. After overnight incubation, the cells were treated with media containing 0, 25, 50, 75, 100, 150, 200, and 300 μM peptide or probe for 48 h at 37 °C. The media were replaced with 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and incubated for 90 min at 37 °C. The cells were lysed with 100 μL of DMSO for 5 min at 37 °C. The formazan dissolved by DMSO was measured at 570 nm by using a microplate reader (VERSAmax, USA).

Spectroscopic materials and methods

Stock solutions of biologically relevant analytes [thiols, Val, Tyr, Thr, Tau, Ser, Pro, Phe, Met, Lys, Leu, Ile, His, Gly, Gluc, Glu, Gln, Asp, Asn, Arg, Ala, Trp, K(I), Na(I), Zn(II), Mg(II), Fe(II), Fe(III), Cu(II), and Ca(II)] were prepared in triple-distilled water. All spectroscopic measurements were performed under physiological conditions (PBS buffer containing 1% (v/v) DMSO, pH 7.4, 37 °C). Absorption spectra were recorded on an S-3100 (Scinco) spectrophotometer, and fluorescence spectra were recorded using an RF-5301 PC spectrofluorometer (Shimadzu) equipped with a xenon lamp. Samples for absorption and emission measurements were contained in quartz cuvettes (4 mL volume). Excitation was set at 430 nm with excitation and emission slit widths of 3 nm.

Peptide HJ Inhibitor2 was purchased from GenicBio Ltd.

Synthesis

Compounds **7**[17] and **8**[18], and naphthalimide derivatives **12**[19] and **11**[20] were synthesized according to reported procedures.

Synthesis of 6. 4-Nitro-1,8-naphthalic anhydride (0.5 g, 2.06 mmol) and compound **7** were refluxed with stirring for 3 h in 50 mL of ethanol. The solvent was removed under reduced

pressure. The residue was then dissolved in CH_2Cl_2 and washed with H_2O (3 × 50 mL). The organic layer was then dried over $MgSO_4$ and evaporated to yield the crude product. Purification with column chromatography (n-hexanes:EtOAc = 6:1, v/v) yielded 491 mg (yield: 60.05%) as a white powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.85 (d, *J* = 4.58 Hz, 1H), 8.68–8.76 (m, 2H), 8.41 (d, 4.41 Hz, 1H), 8.00 (t, *J* = 7.85 Hz, 1H), 5.14 (s, 1H), 4.28 (t, *J* = 6.98 Hz, 2H), 3.18 (q, *J* = 3.78 Hz, 2H), 1.95 (p, *J* = 6.74 Hz, 2H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 163.7, 162.9, 156.0, 149.8, 132.8, 130.1, 129.2, 126.9, 124.1, 123.8, 122.9, 79.3, 38.3, 37.7, 28.6. MS (ESI) m/z (M^+) calcd 399.4 [M^+], found 400.3 [M + H^+].

Synthesis of 5. Compound **6** (300 mg, 0.75 mmol) was dissolved in ethanol and saturated with hydrogen in the presence of 10% w/w Pd/C. After 24 h, the resulting thick solution was warmed slightly and filtered to remove the catalyst. The reaction liquor was evaporated and the precipitate was collected by filtration. Recrystallization of the precipitate from CH_2Cl_2/n-hexanes gave the title compound as yellow needles (181 mg, 65.53%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.57 (d, *J* = 7.38 Hz, 1H), 8.39 (d, *J* = 8.24 Hz, 1H), 8.15 (d, *J* = 9.08 Hz, 1H), 7.62 (t, *J* = 7.76 Hz, 1H), 6.87 (d, *J* =8.40 Hz, 1H), 5.22 (s, 2H), 4.23 (t, *J* = 5.54 Hz, 2H), 3.15 (br, 2H), 1.92 (t, 5.83 Hz, 2H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 165.1, 164.5, 156.3, 150.0, 134.2, 131.8, 130.0, 127.6, 125.1, 124.9, 122.9, 120.2, 111.5, 109.7, 79.2, 37.8, 37.5, 28.6. MS (ESI) m/z (M^+) calcd 369.4 [M^+], found 368.0 [M - H^+].

Synthesis of 4. To a mixture of **5** (300 mg, 1.23 mmol) and phosgene (1.1 g, 3.69 mmol) in 20 mL of dry toluene, DIPEA (1.06 mL, 6.15 mmol) was added in a dropwise manner. The resulting solution was heated to reflux for 3 h. After cooling to room temperature, the reaction mixture was flushed with nitrogen gas. After removal of unreacted phosgene gas (CAUTION: TOXIC) and neutralization in a NaOH bath, a solution of compound **8** (346.5 mg, 1.85 mmol) in distilled DCM was added to the mixture. The reaction mixture was stirred overnight. The solvent was evaporated, at which point CH_2Cl_2 (100 mL) and water (100 mL) were added, and the organic layer was collected. The CH_2Cl_2 layer was dried over anhydrous $MgSO_4$. After removal of the solvent, the crude product was purified over silica gel by using CH_2Cl_2:EtOAc (6:1, v/v) as the eluent to yield **4** as a white solid (261 mg, 55.21%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.35–8.45 (m. br, 3H), 8.24 (d, *J* = 4.49 Hz, 1H), 8.15 (t, *J* = 8.04 Hz, 2H), 7.58 (br, 2H), 7.00 (t, *J* = 5.78 Hz, 1H), 4.43 (t, *J* = 5.97 Hz, 2H), 4.13 (t, *J* = 5.97 Hz, 2H), 3.05 (t, *J* = 6.0 Hz, 4H), 1.82 (p, *J* = 6.0 Hz, 2H), 1.35 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 164.6, 164.1, 159.7, 156.3, 153.3, 149.9, 139.7, 137.4, 132.7, 131.5, 129.0, 127.2, 126.6, 123.3, 123.0, 121.3, 120.5, 117.6, 117.3, 79.3, 63.9, 37.7, 37.6, 28.6, 19.3. MS (ESI) m/z (M^+) calcd 582.6 [M^+], found 581.0 [M - H^+].

Synthesis of 3. The starting compound **4** (89 mg, 0.153 mmol) was dissolved in CH_2Cl_2. Trifluoroacetic acid was added. The reaction mixture was stirred at room temperature for 3 h. CH_2Cl_2 was evaporated and replaced by diethyl ether, which was then evaporated to azeotrope excess trifluoroacetic acid. This operation was repeated three times to yield oil, which was dried in vacuo. The amine products compound **3** was obtained without further purification as white solid (60 mg. 81.15%). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.31 (d, *J* = 2.34 Hz, 1H), 8.09–8.14 (m, 2H), 7.98 (d, 4.27 Hz, 1H), 7.82 (d, 4.26 Hz, 1H), 7.76 (d, 4.05 Hz, 1H), 7.69 (t, 6.83 Hz, 1H), 7.43 (t, *J* = 7.71 Hz, 1H), 7.11 (t, *J* = 6.08 Hz, 1H), 4.42 (t, *J* = 6.32 Hz, 2H), 4.03 (t, *J* = 6.01 Hz, 2H), 3.12 (t, *J* = 6.12 Hz, 2H), 3.00 (t, *J* = 6.76 Hz, 2H), 2.03 (p, *J* = 6.76 Hz, 2H). ^{13}C NMR (CD_3OD, 100 MHz): δ = 164.3, 163.8, 159.6, 153.9, 149.1, 140.7, 138.2, 131.8, 131.0, 130.9, 128.2, 126.1, 126.0, 123.0, 121.7, 121.4, 121.3, 120.2, 120.1, 116.8, 116.6, 116.2, 63.2, 37.4, 36.8, 26.2. MS (ESI) m/z (M^+) calcd 582.6 [M^+], found 581.0 [M - H^+].

Synthesis of 2. A mixture of **3** (1.83 g, 3.8 mmol), EDCI (0.74 g, 3.8 mmol), and DMAP (0.47 g, 3.8 mmol) in anhydrous DMF was stirred under nitrogen atmosphere for 30 min at room temperature. Biotin (928.37 mg, 3.8 mmol) was then added to the mixture. The reaction mixture was stirred overnight. After removal of solvent, the crude product was purified over silica gel using CH_2Cl_2:MeOH (9:1, v/v) as the eluent to yield **2** as a green solid (1.83 g, 68%). ^{1}H NMR (400 MHz, DMSO): δ = 8.42 (d, *J* = 3.85 Hz, 1H), 8.10 (m, 3H), 8.08 (d, *J* = 4.15 Hz, 1H), 7.77 (m, 3H), 7.18 (t, 5.83 Hz, 1H), 6.34 (d, *J* = 14.40, 2H), 4.39 (t, *J* = 6.23 Hz, 2H), 4.24 (t, *J* = 6.23 Hz, 1H), 4.08 (t, *J* = 5.79 Hz, 1H), 3.98 (t, *J* = 7.24 Hz, 2H), 3.18 (t, *J* = 6.40 Hz, 2H), 3.04 (p, *J* = 6.10 Hz, 3H), 2.74 (q, *J* = 3.57 Hz, 2H), 2.00 (t, *J* = 6.54 Hz), 1.69 (p, *J* = 7.27 Hz, 2H), 1.52–1.61 (br, 2H), 1.38–1.52 (br, 2H), 1.22–1.33 (br, 2H). ^{13}C NMR (100 MHz, DMSO): δ = 172.6, 164.1, 163.6, 163.3, 159.5, 154.4, 150.3, 141.2, 138.5, 132.2, 131.5, 130.0, 129.0, 127.1, 124.7, 124.6, 122.9, 121.9, 120.1, 119.2, 117.9, 63.3, 61.7, 59.8, 56.0, 39.9, 39.7, 39.5, 38.4, 37.7, 37.1, 35.9, 28.7, 25.9. MS (ESI) m/z (M^+) calcd 708.8 [M^+], found 709.2 [M + H^+].

Synthesis of 1. Compound **2** (40 mg, 56.4 mmol) was dissolved in DMF (5 mL) and HJ inhibitor peptide2 (163 mg, 0.169 mmol) was added. The mixture was stirred under nitrogen at room temperature overnight. Solvent was removed and the residue was purified using HPLC (C18, 3.6 μm, 4.6 × 150 mm for the stationary phase; buffer A, H_2O containing 0.1% TFA, buffer B, CH_3CN containing 0.1% TFA for the mobile phase). **1** was eluted at 25-100% of buffer B for 30 min, and lyophilized, affording a green fluffy power (35.6 mg, 40.2%).

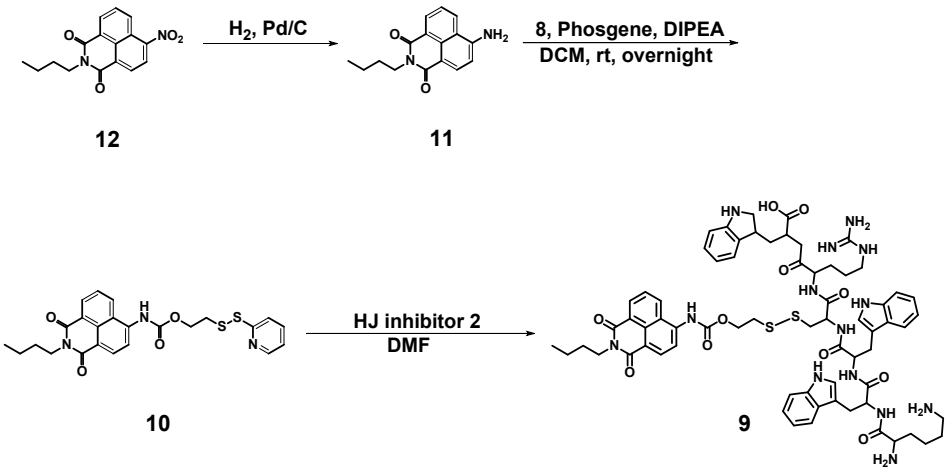
Synthesis of 10. To a stirred solution of compound **9** (210 mg, 0.78 mmol) and DIPEA (300 mg, 2.3 mmol) in dry dichloromethane (DCM) (5.0 mL), a phosgene solution (500 mg, 5.1 mmol) was added. The reaction mixture was stirred at 0 °C under argon atmosphere. After 2 h, the excess phosgene was removed from the reaction mixture by argon purge. Then, compound **8** (300 mg, 1.6 mmol) in dry DCM and DMAP (244 mg, 2.0 mmol) were added to the reaction mixture and was stirred overnight. After completion of the reaction (as monitored by TLC), the reaction mixture was diluted with EtOAc, washed twice with brine, dried over sodium sulfate, and then filtered, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc:DCM 1:1) to give **10** (210 mg, 56% yield) as an ivory solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.65 (d, 1H, *J* = 7.5 Hz), 8.62 (d, 1H, *J* = 8.1 Hz), 8.4 (d, 1H, *J* = 8.4 Hz), 8.3 (d, 1H, *J* = 8.1 Hz), 8.2(d, 1H, *J* = 8.4 Hz), 7.8 (t, 1H, *J* = 7.8 Hz), 7.68 (d, 1H, *J* = 8.1 Hz), 7.62 (d, 1H, *J* = 8.4Hz), 7.5 (s, 1H), 7.1 (t, 1H, *J* = 6.3 Hz), 4.55 (t, 2H, *J* = 5.9 Hz), 4.17 (t, 2H, *J* = 7.5 Hz), 3.16 (t, 2H, *J* = 5.8 Hz), 1.63-1.76 (m, 2H), 1.38-1.48 (m, 2H), 0.9 (t, 3H, *J* = 6.777 Hz). ^{13}C NMR (100 MHz, CDCl3): δ = 164.3, 163.8, 153.0, 149.9, 139.1, 137.4, 132.6, 131.5, 129.1, 126.9, 126.3, 126.1, 123.6, 123.2, 121.3, 120.6, 118.2, 117.1, 64.1, 53.3, 40.4, 37.64, 30.4, 20.6, 14.0. MS (ESI) m/z (M^+) calcd 481.5 [M^+], found 482.3 [M + H^+].

Synthesis of 9. Compound **10** (20 mg, 0.0415 mmol) was dissolved in DMF (3 mL) and HJ inhibitor peptide2 (120 mg, 0.124 mmol) was added. The mixture was stirred under nitrogen at room temperature overnight. Solvent was removed and the residue was purified using HPLC (C18, 3.6 μm, 4.6 × 150 mm for the stationary phase; buffer A, H_2O containing 0.1% TFA, buffer B, CH_3CN containing 0.1% TFA for the mobile phase). **9** was eluted at 25-100% of buffer B for 30 min, and lyophilized, affording a yellowish fluffy power (5 mg, 9.01%).

Synthesis of 14. The starting compound **6** (300 mg, 0.75 mmol) was dissolved in CH_2Cl_2. Trifluoroacetic acid was added. The reaction mixture was stirred at room temperature for 3 h. CH_2Cl_2 was evaporated and replaced by diethyl ether, which was then evaporated to azeotrope excess tifluoroacetic acid. This operation was repeated three times to yield oil, which was dried in vacuo. The crude product was confirmed by MS analysis (Figure S32, Supporting Information) and then used directly for the next reaction. The crude product was dissolved in DMF (2 mL) with EDCI and DMAP. After 30 min at room temperature, biotin was added to the mixture. The reaction mixture was stirred overnight. After removal of solvent, the crude product was purified over silica gel using CH_2Cl_2:MeOH (9:1, v/v) ^{1}H NMR (300 MHz, DMSO): δ = 8.71 (d, *J* = 7.83 Hz, 1H), 8.61 (m, 3H), 8.09 (t, *J* = 8.0Hz, 1H), 7.86 (t, *J* = 5.78 Hz, 1H), 6.39 (d, *J* = 21.70 Hz,

2H), 4.29 (t, *J* = 5.95 Hz, 1H), 4.12(m, 1H), 4.05 (t, *J* = 7.24 Hz, 2H), 3.11 (m, 3H), 2.80 (q, *J* = 8.70 Hz, 2H), 2.05 (t, *J* = 7.46 Hz, 2H), 1.78 (m, 2H),1.49 (m, 4H), 1.31 (m, 2H). ^{13}C NMR (100 MHz, DMSO): δ = 172.74, 163.52, 163.43, 162.73, 149.60, 132.27, 130.70, 130.18, 129.32, 128.85, 127.18, 124.68, 123.26, 61.68, 59.86, 56.08, 38.87, 37.10, 35.93, 31.35, 28.36, 28.69, 28.44, 25.99.

Synthesis of 13. Compound **14** (300mg, 0.57 mmol) was dissolved in ethanol and saturated with hydrogen in the presence of 10% w/w Pd/C. After 24 h, the resulting thick solution was warmed slightly and filtered to remove the catalyst. The reaction liquor was evaporated and the precipitate was collected by filtration. The filtrate was evaporated under reduced pressure and the residue was subjected to flash column chromatography with CH_2Cl_2:MeOH (9:1, v/v) as eluent to give the desired product as a yellow solid (169.49 mg, 60% yield). ^{1}H NMR (300 MHz, DMSO): δ = 8.44 (d, *J* = 7.89 Hz, 2H), 8.34 (d, *J* = 7.89 Hz, 1H), 7.83 (t, *J* = 5.87 Hz, 1H), 7.69 (t, *J* = 7.86 Hz, 1H), 7.20 (d, *J* = 7.89 Hz, 1H), 6.40 (d, *J* = 23.02 Hz, 2H), 4.29 (q, *J* = 6.24 Hz, 1H), 4.13 (m, 1H), 4.02 (t, *J* = 7.02 Hz, 2H), 3.09 (m, 3H), 2.80 (q, *J* = 8.72 Hz, 2H), 2.05 (t, *J* = 7.13 Hz, 2H), 1.73(m, 2H), 1.49 (m, 4H), 1.31(m, 2H). ^{13}C NMR (100 MHz, DMSO): δ = 172.58, 164.37, 163.65, 163.39, 152.97, 134.66, 131.31, 129.42, 128.86, 125.41, 122.48, 118.61, 110.45, 105.29, 61.67, 59.84, 56.06, 56.04, 38.10, 37.17, 35.96, 28.85, 28.69, 25.98.



Scheme S1. Synthetic Route to **9**.

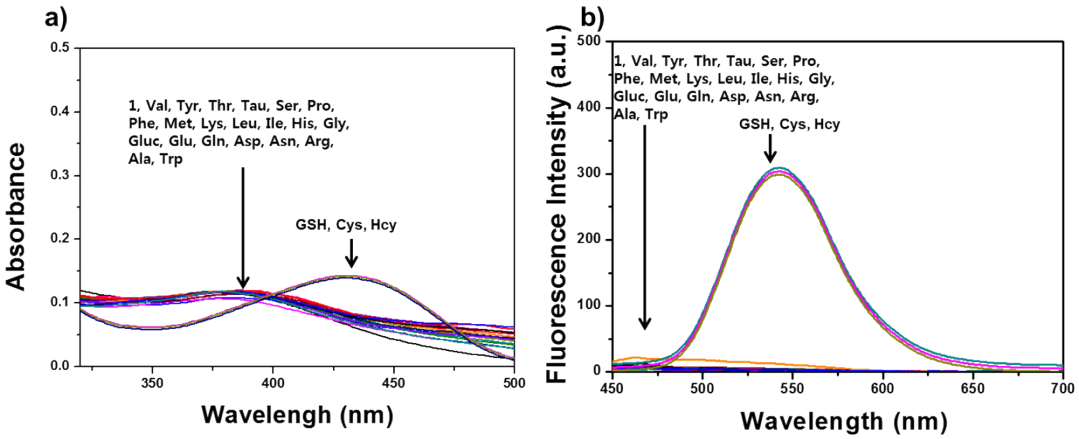


Figure S1. Absorption (a) and fluorescence (b) spectra of **1** (10.0 μM) toward GSH, Cys, Hcy (5.0 mM, respectively) and other amino acids (5.0 mM, respectively) with an excitation at 430 nm. All spectra were acquired 3 h after addition of various analysts in PBS buffer (pH 7.4) containing 1 % (v/v) of DMSO at 37 °C

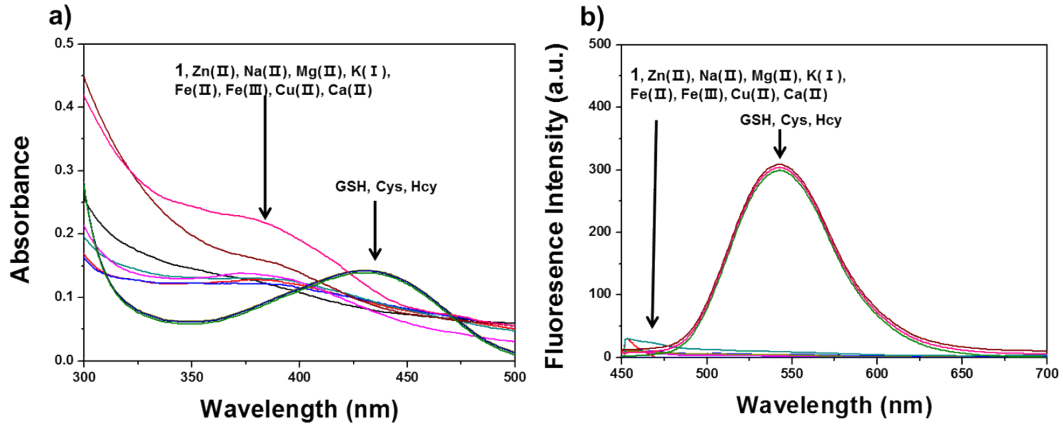
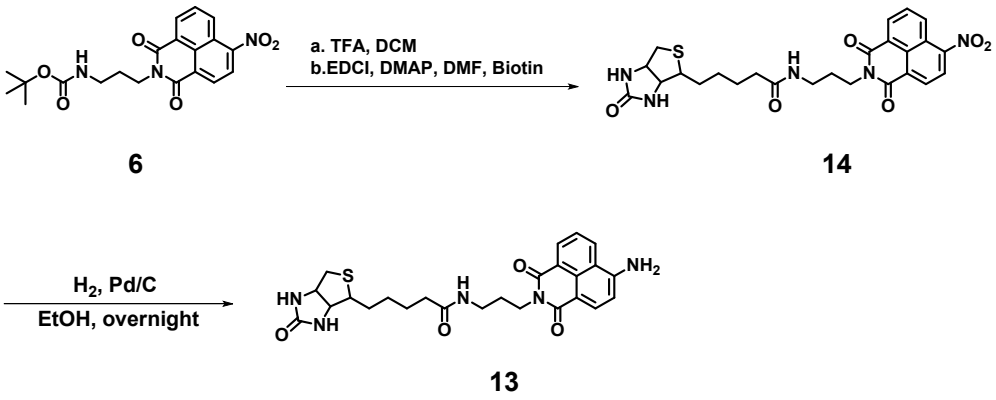


Figure S2. Absorption (a) and fluorescence (b) spectra of **1** (10.0 μM) recorded in the absence and presence of GSH, Cys, Hcy (5.0 mM, respectively) and various metal cations (1.0 mM: monovalent metal ions; 0.1 mM: divalent metal ions, respectively) with excitation effected at 430 nm. All spectra were acquired 3 h after addition of the analytes in question and were recorded in PBS buffer (pH 7.4) containing 1% (v/v) DMSO at 37 °C.



Scheme S2. Synthesis Route to **13**.

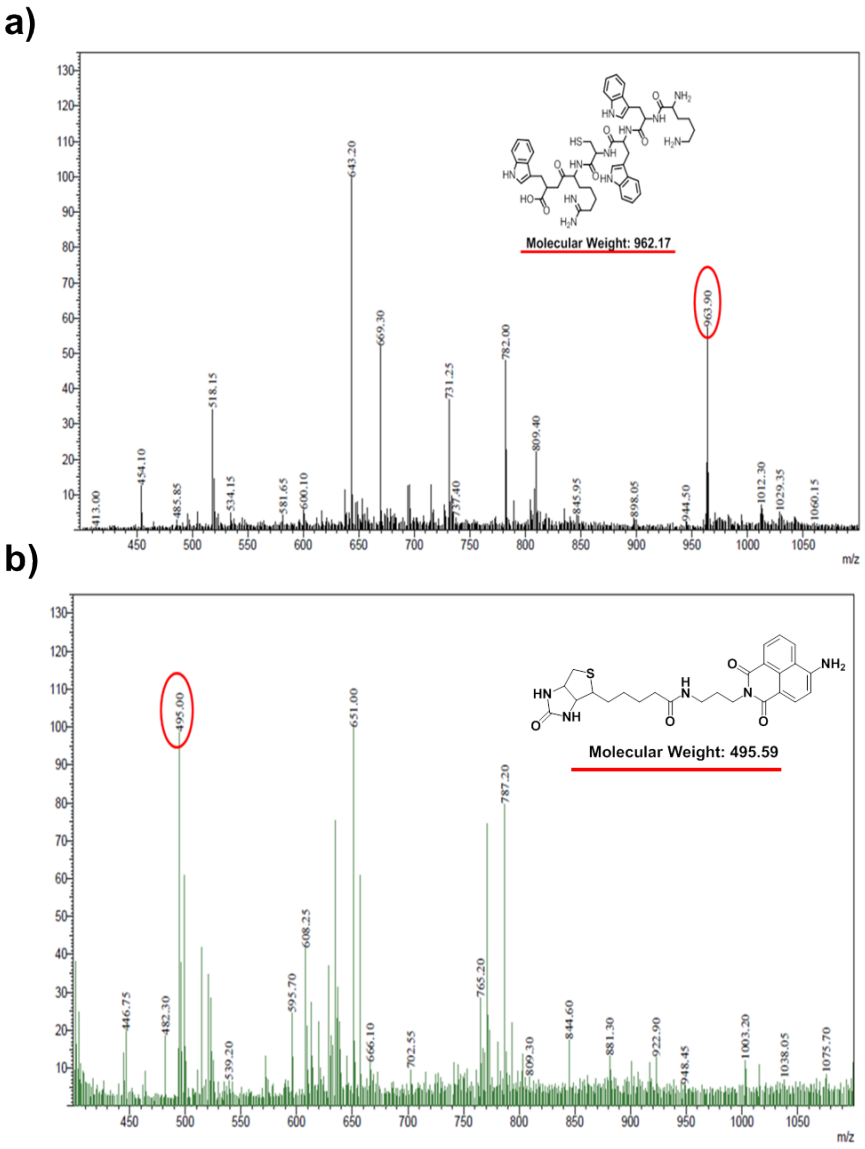


Figure S3. ESI-MS spectrum of the products from the reaction of **1** with GSH. (Upper graph = cationic measurement and lower graph = anionic measurement)

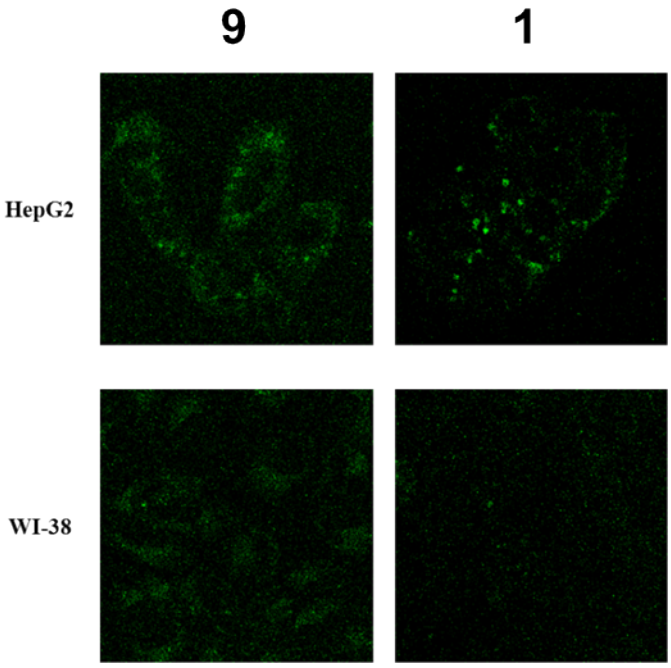


Figure S4. Confocal microscopy analysis of HepG2 and WI-38 cells treated with **9** and **1**. The cells were incubated with media containing **9** (10 μM) and **1** (10 μM). The images were obtained at after incubating for 60 min. Cell images were obtained using excitation at 458 nm and a long-path (>475 nm) emission filter.

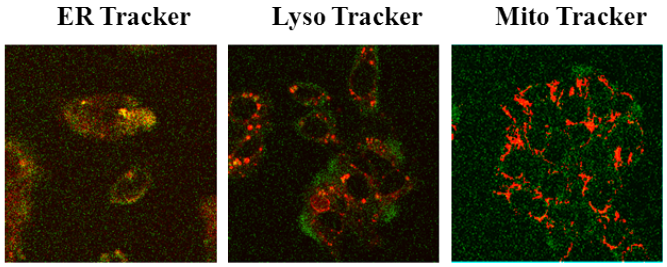


Figure S5. Colocalization of **9** with ER Tracker Red (0.1 μM), LysoTracker Red DND-22 (0.1 μM), or MitoTracker Red FM (0.1 μM) in HepG2 cells. The cells were separately pre-treated with trackers and then **9** (10 μM) was added. Fluorescence from **9** appears as green signals while fluorescence from ER-Tracker, LysoTracker, and MitoTracker appears as red signals. In the merged image, the yellow regions highlight the colocalized areas of the corresponding dyes. Images of the cells were obtained using excitation wavelengths of 458, 543, and 633 nm, and band-path (505−530 nm, green signal) and long-path (>650 nm, red signal) emission filters.

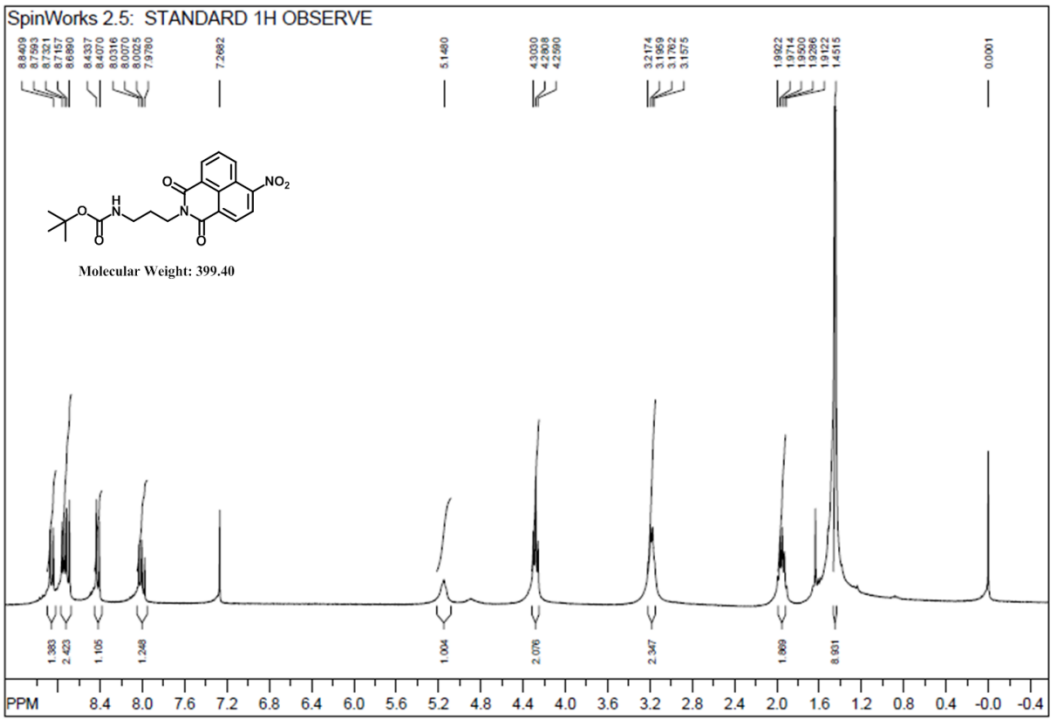


Figure S6. ^{1}H NMR spectrum of compound **6.**

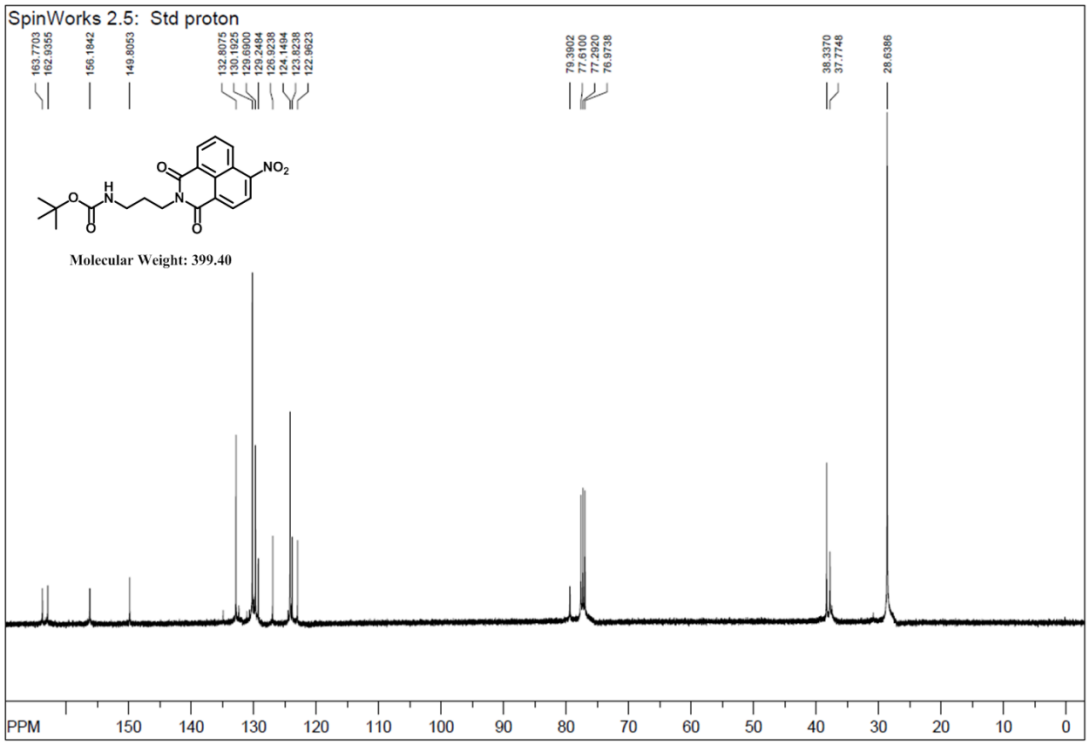


Figure S7. ^{13}C NMR spectrum of compound **6**

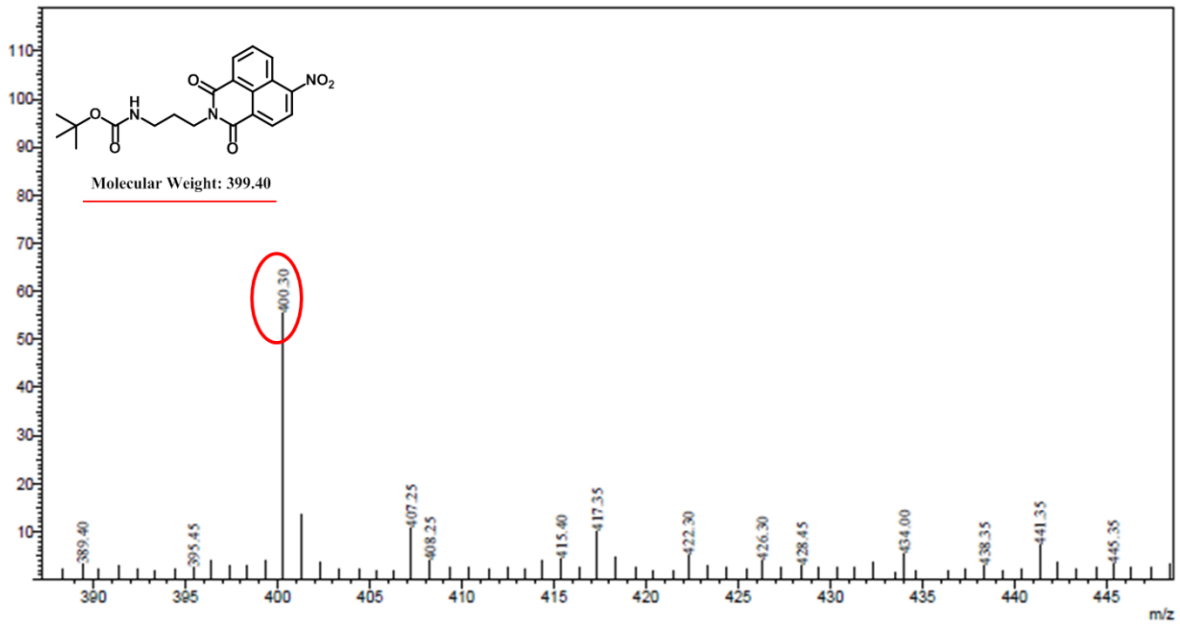


Figure S8. Mass spectrum of compound **6.**

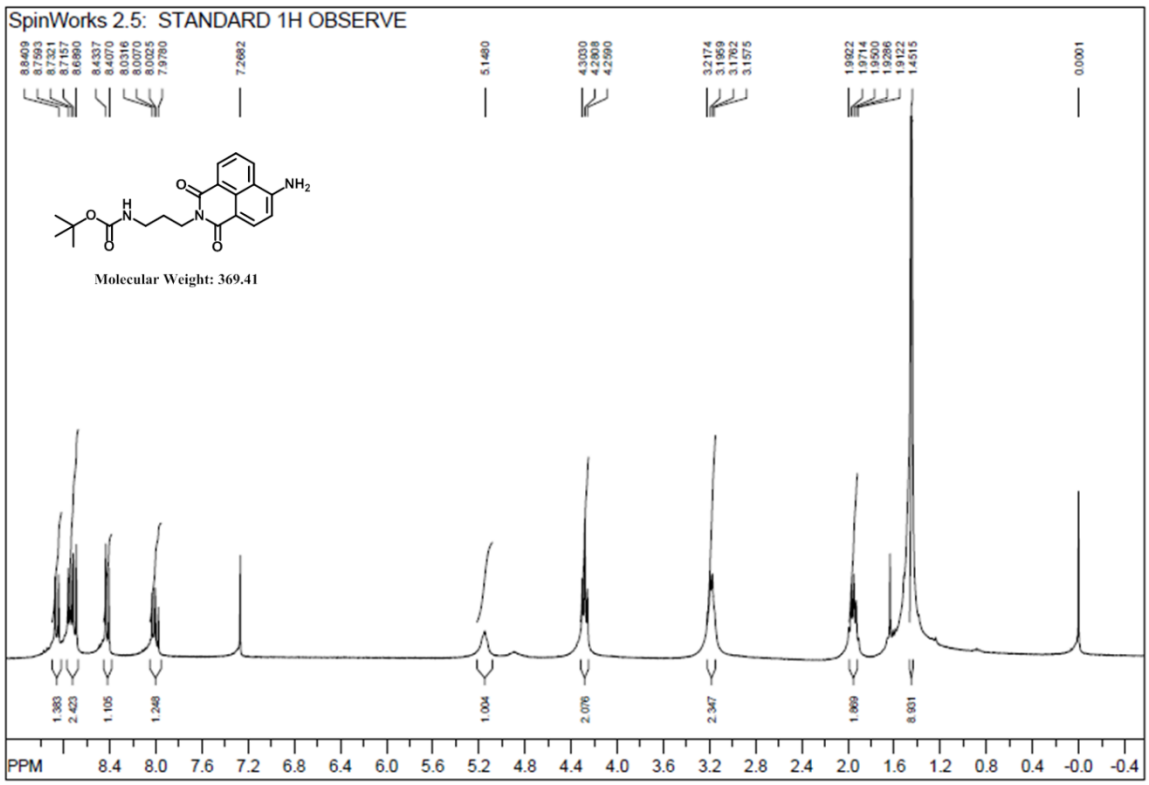


Figure S9. ^{1}H NMR spectrum of compound **5.**

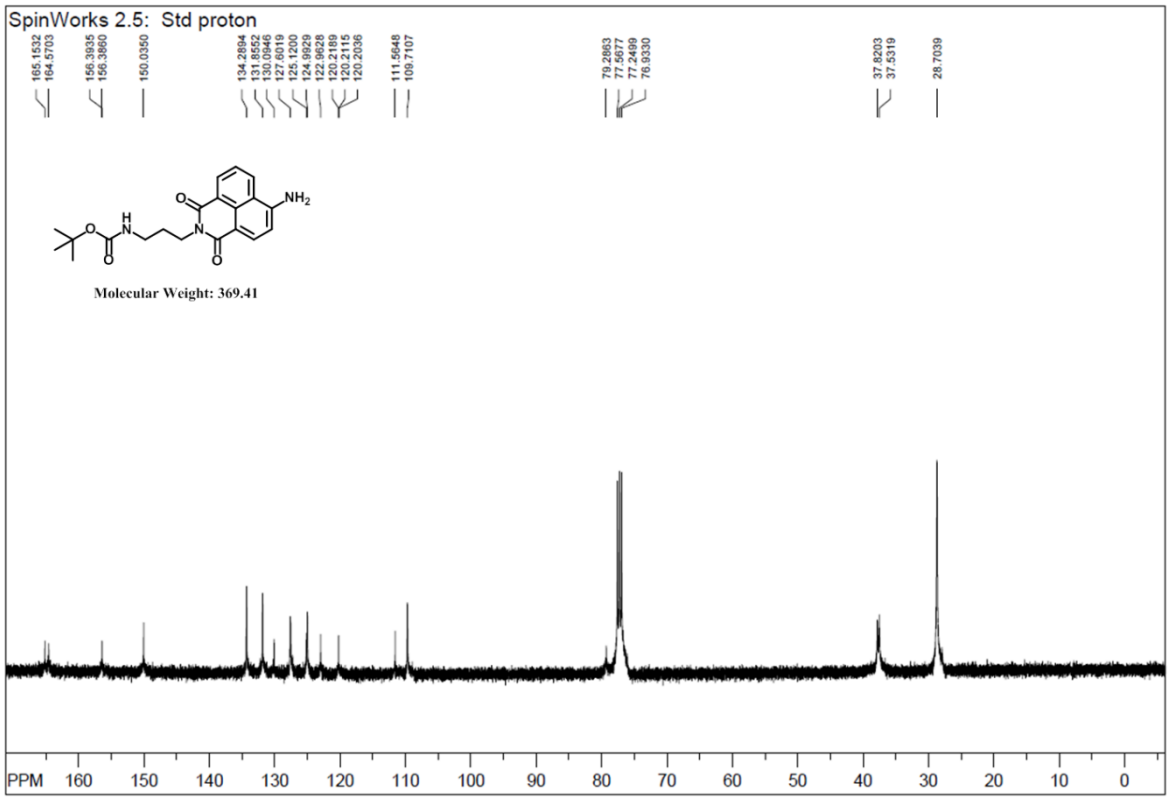


Figure S10. ^{13}C NMR spectrum of compound **5.**

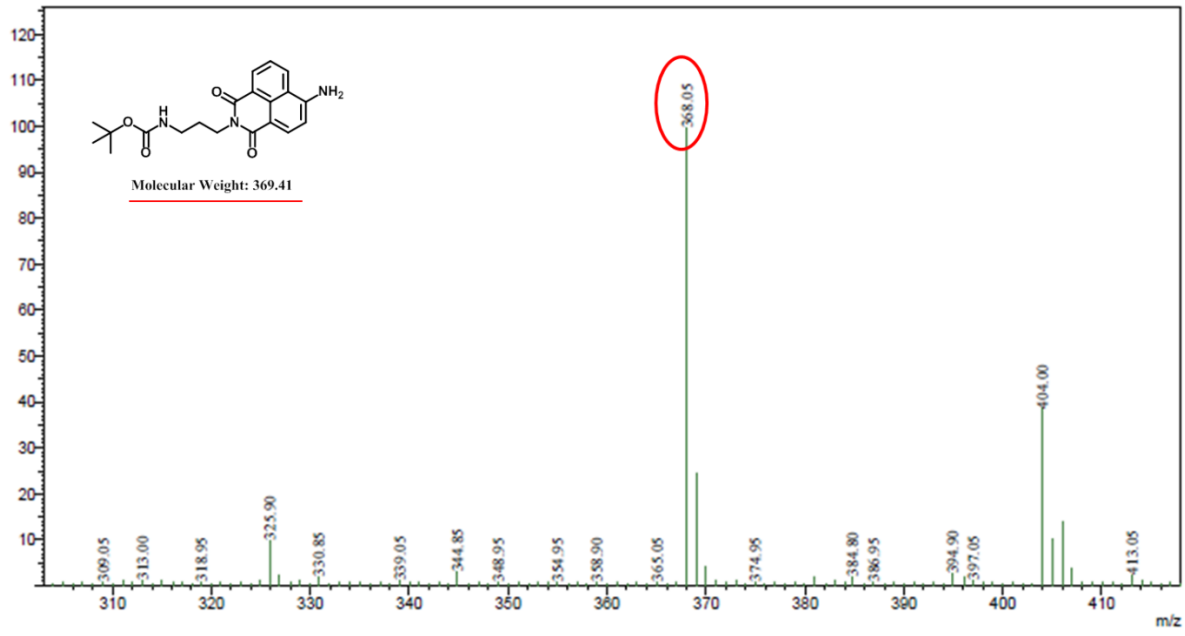


Figure S11. Mass spectrum of compound **5.**

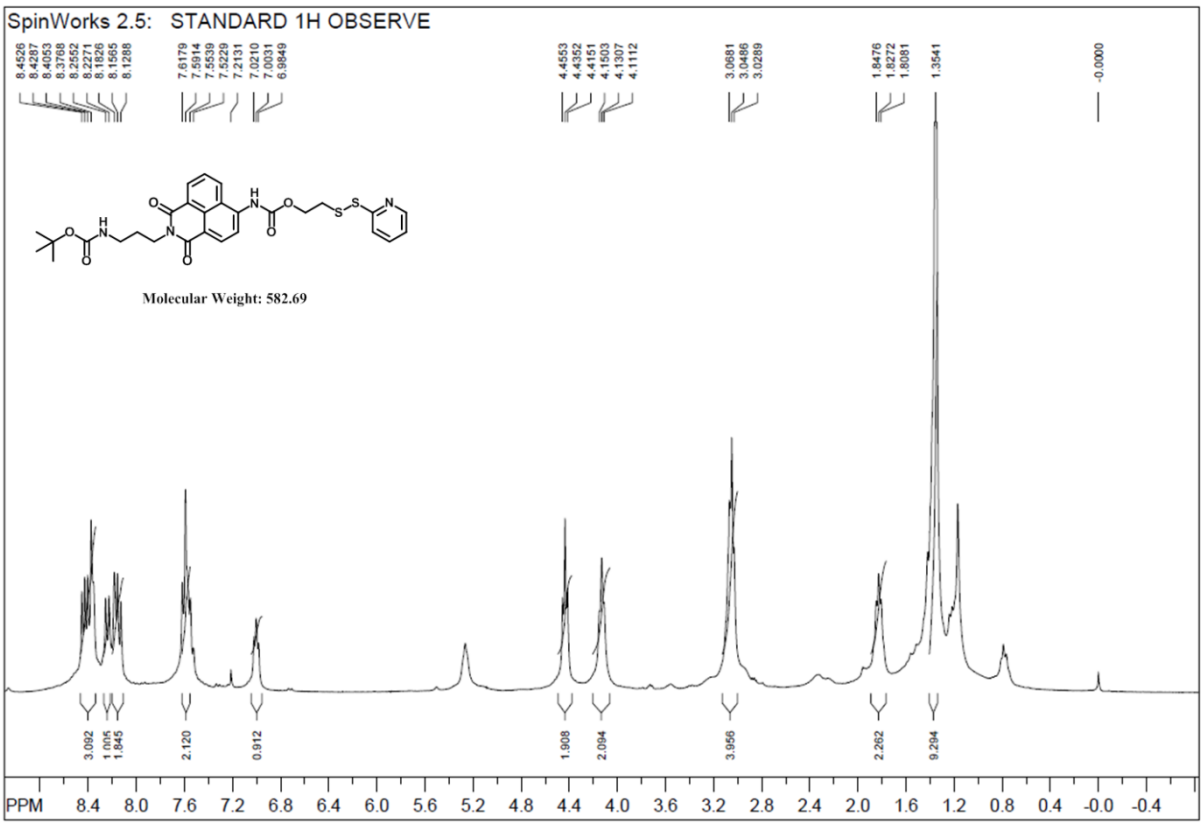


Figure S12. ^{1}H NMR spectrum of compound **4.**

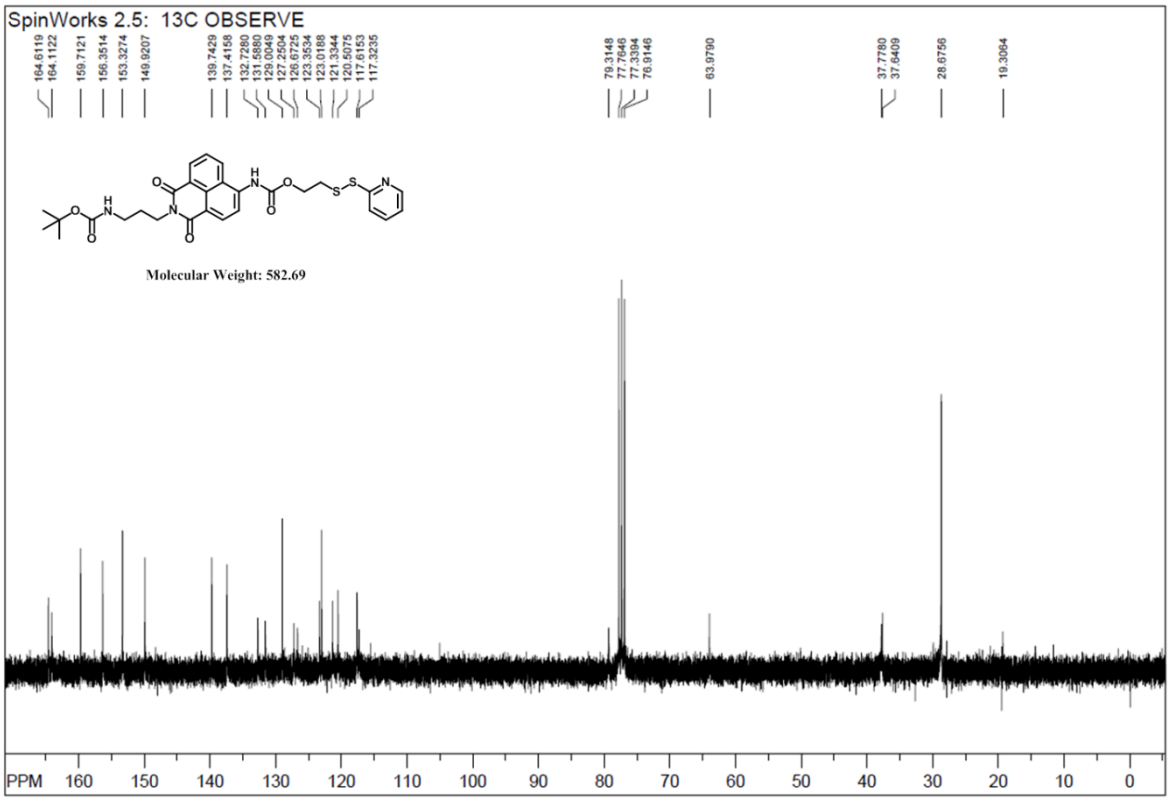


Figure S13. ^{13}C NMR spectrum of compound **4.**

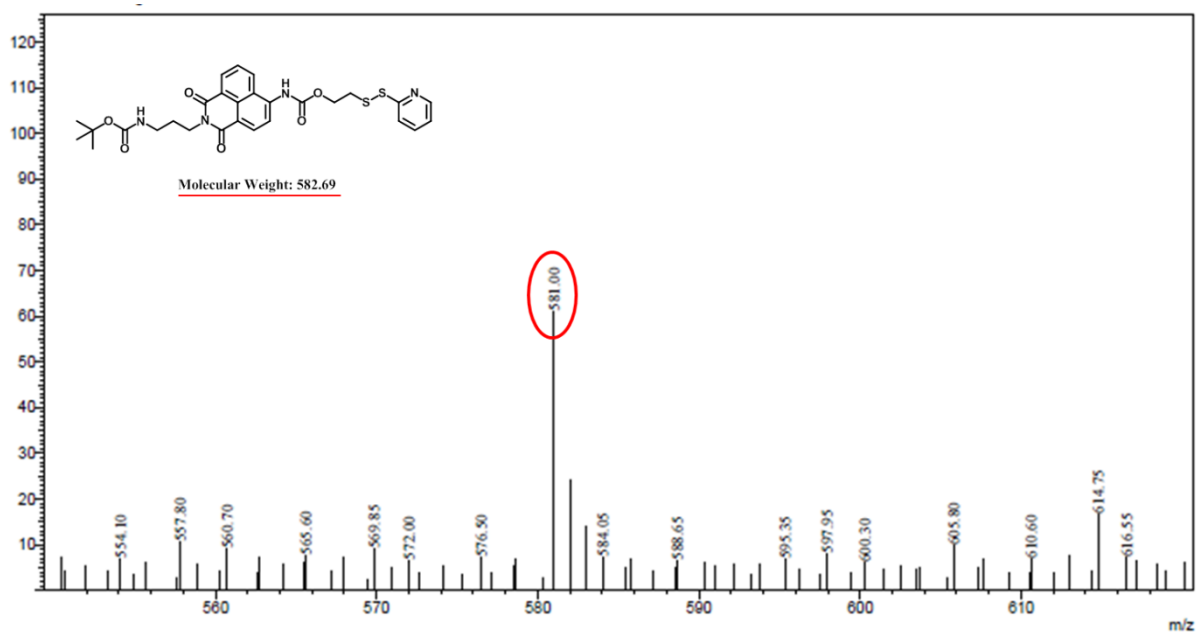


Figure S14. Mass spectrum of compound **4.**

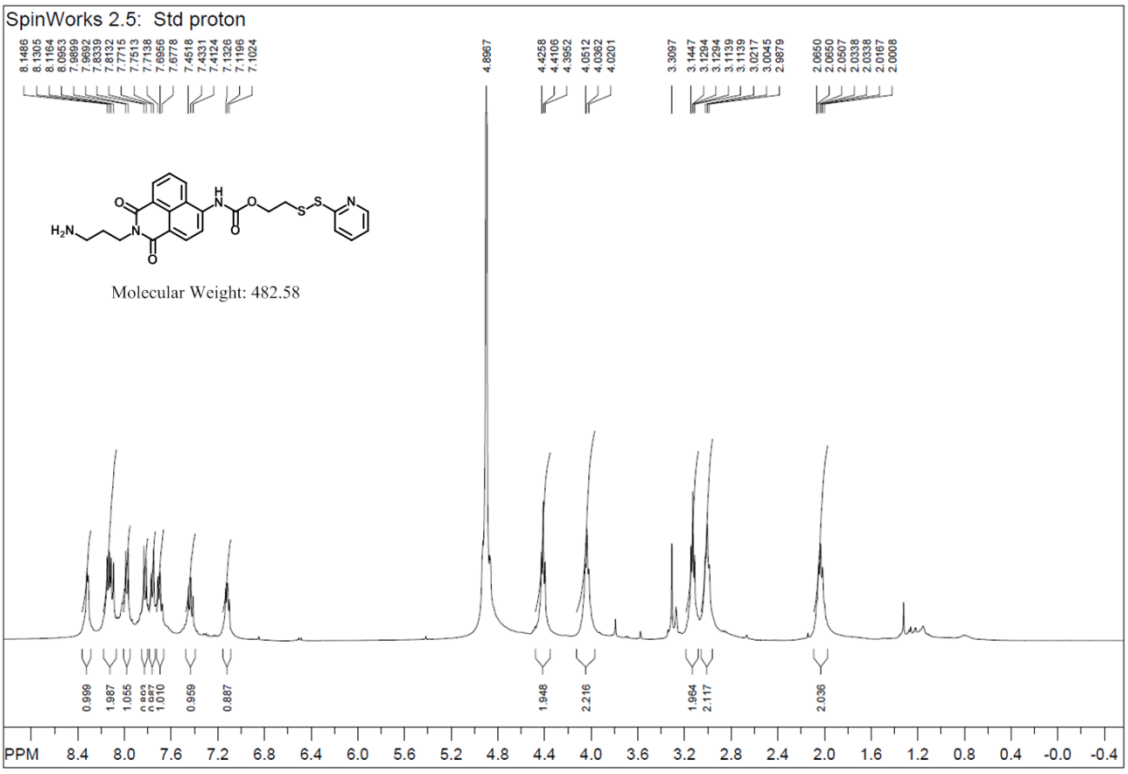


Figure S15. ^{1}H NMR spectrum of compound **3.**

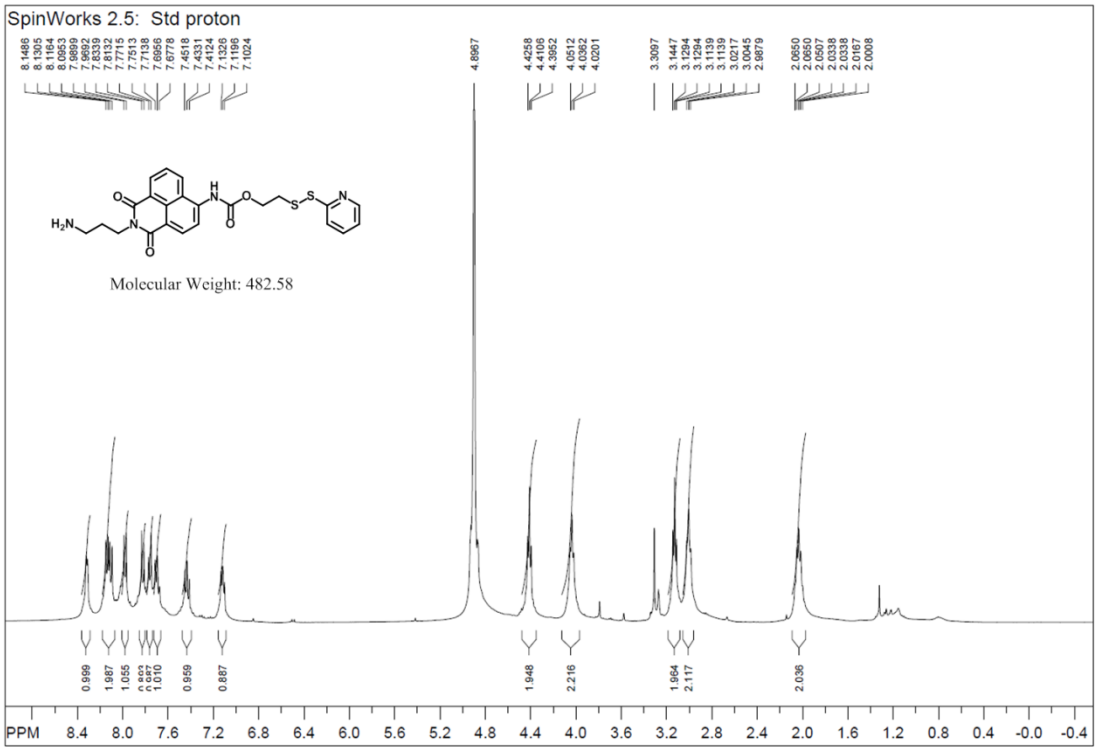


Figure S16. ^{13}C NMR spectrum of compound **3.**

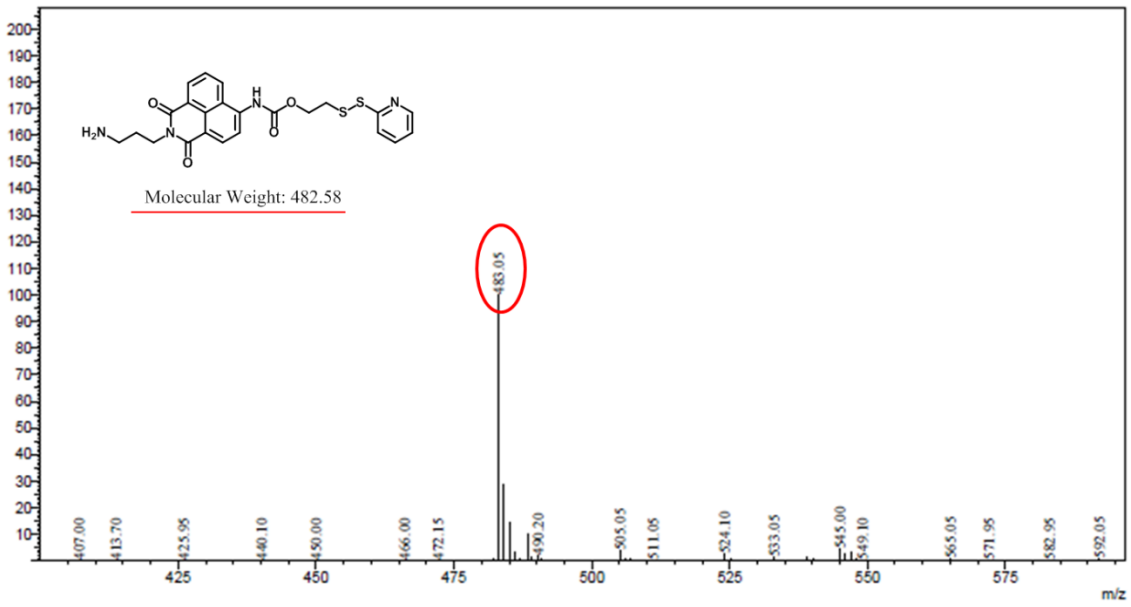


Figure S17. Mass spectrum of compound **3.**

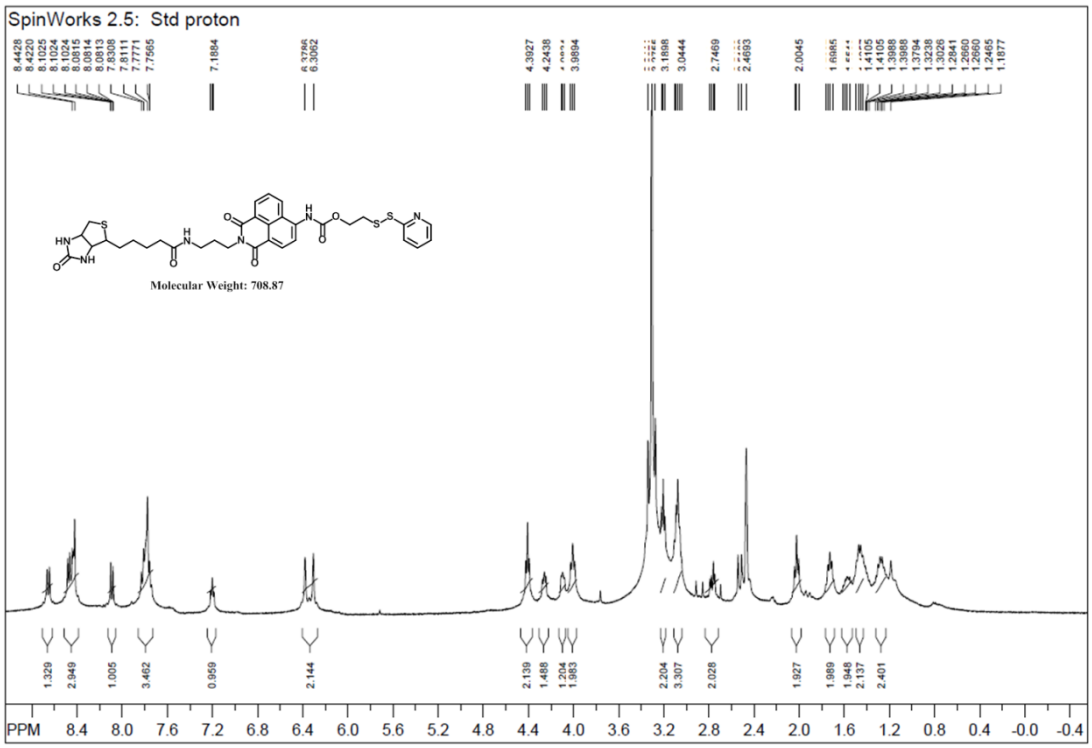


Figure S18. ^{1}H NMR spectrum of compound **2.**

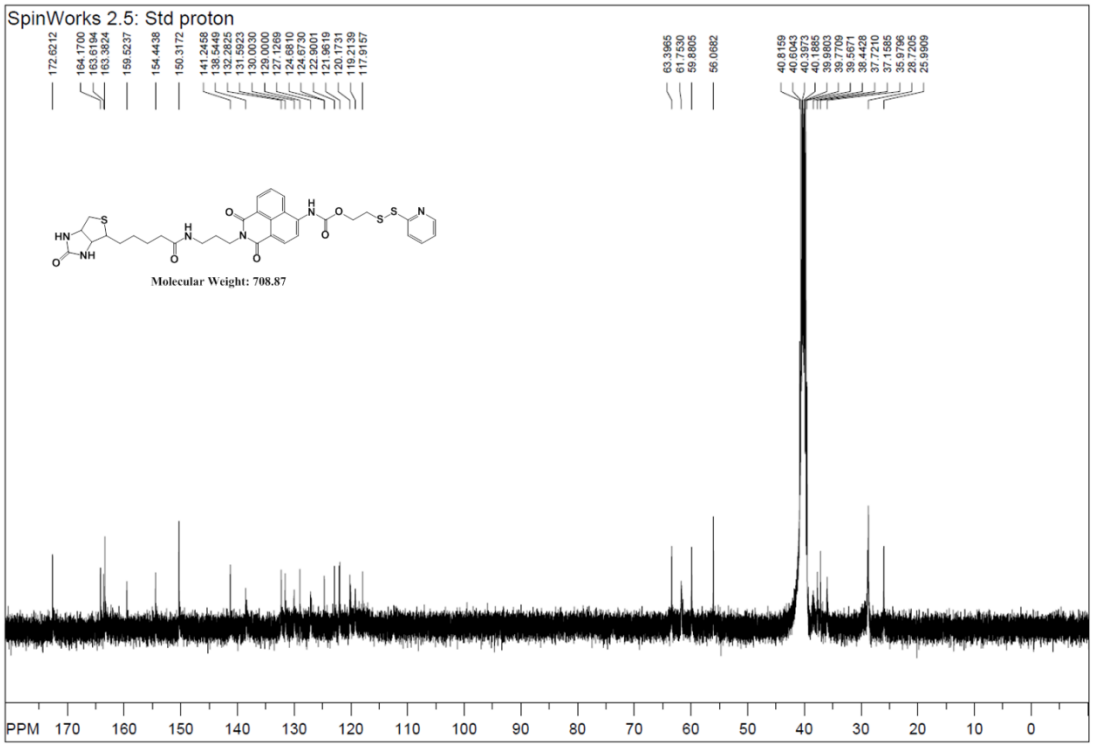


Figure S19. ^{13}C NMR spectrum of compound **2.**

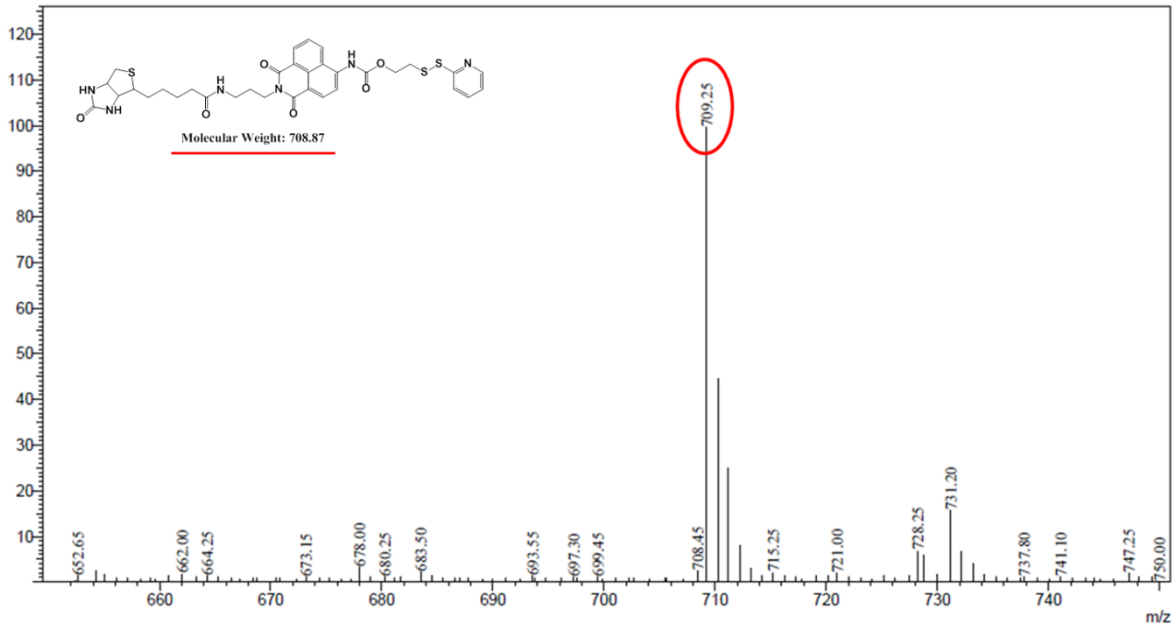


Figure S20. Mass spectrum of compound **2.**

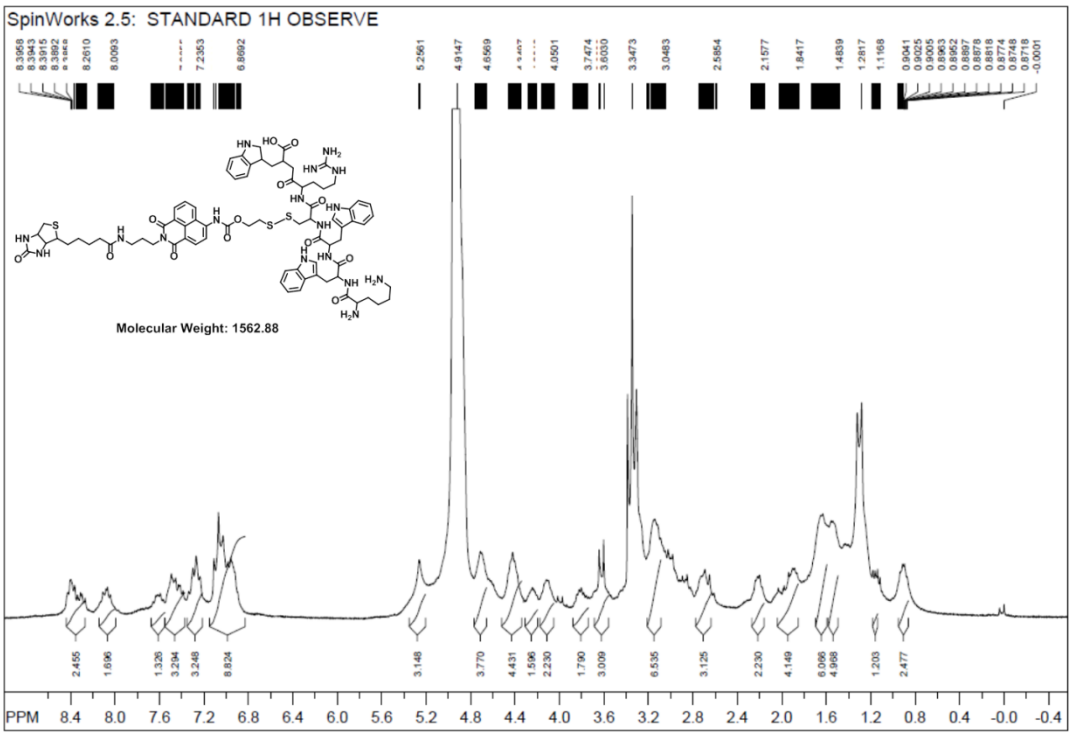


Figure S21. ^{1}H NMR spectrum of **1.**

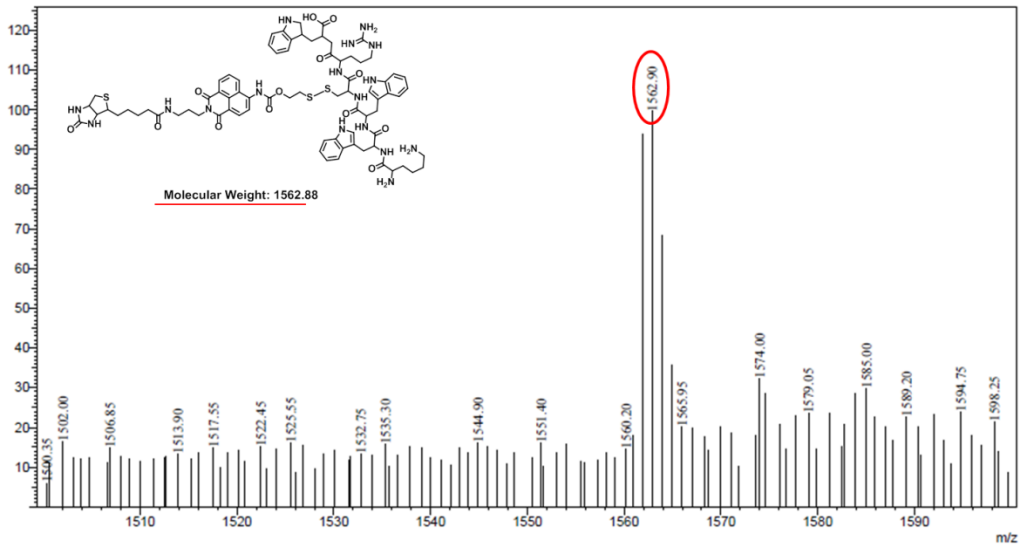


Figure S22. Mass spectrum of **1.**

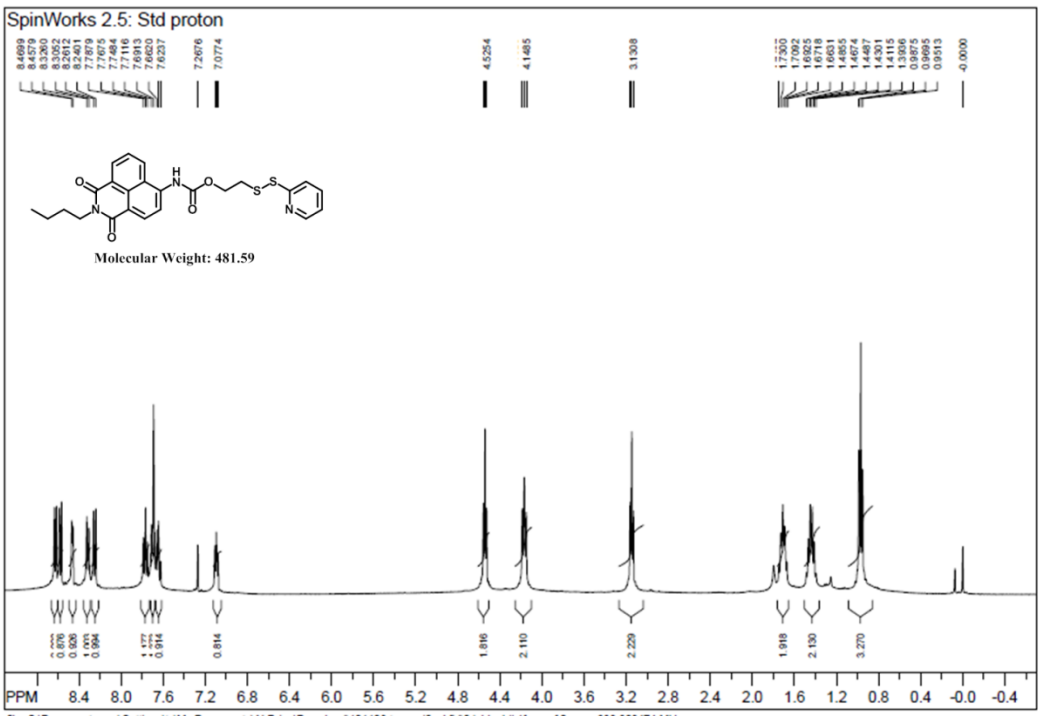


Figure S23. ^{1}H NMR spectrum of compound **10**

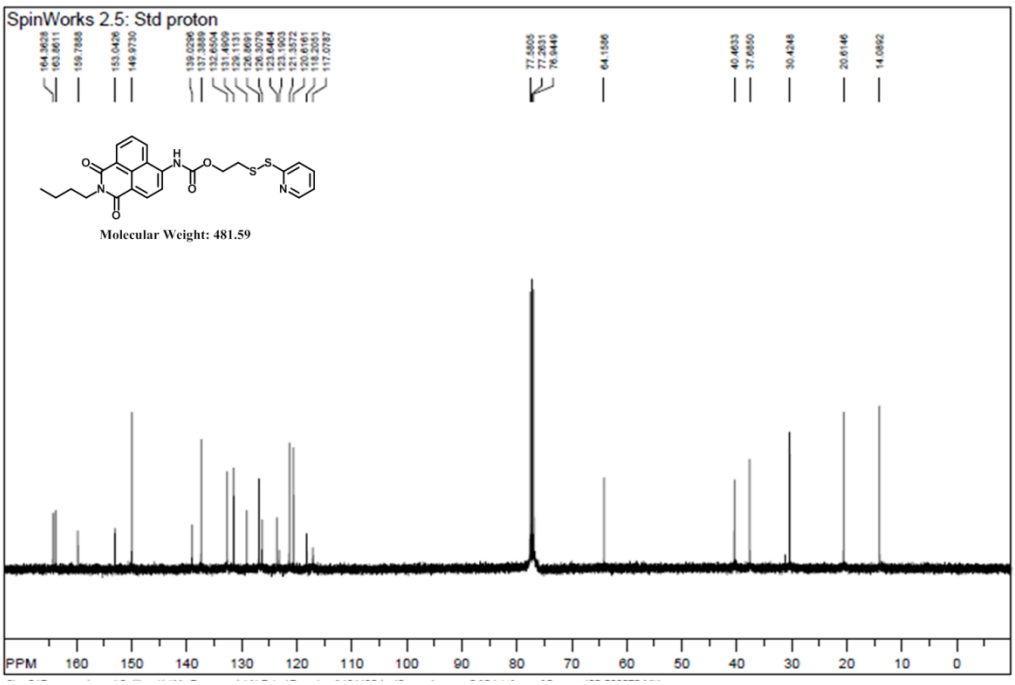


Figure S24. ^{13}C NMR spectrum of compound **10**

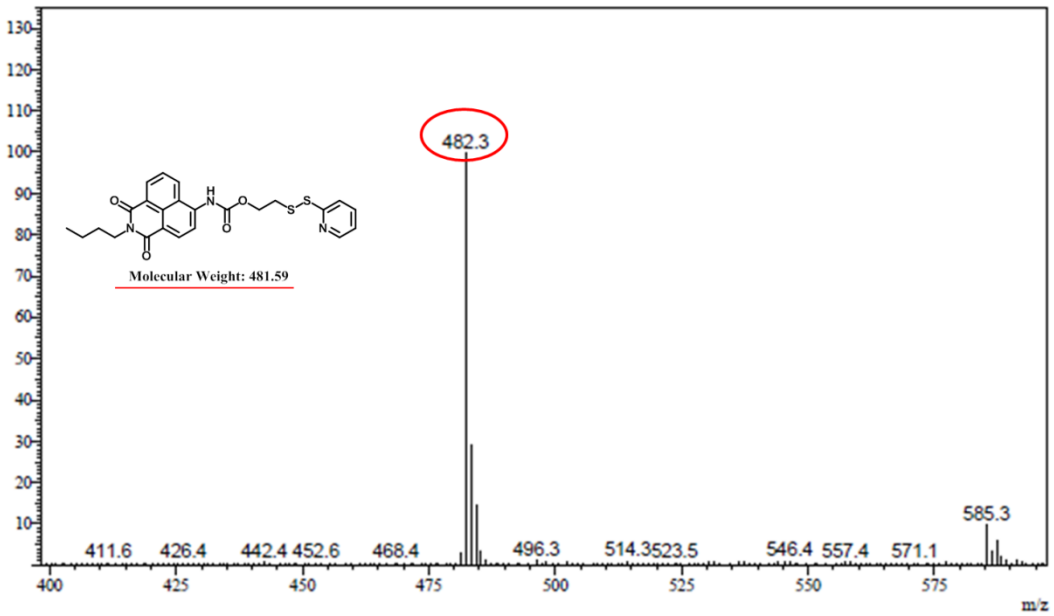


Figure S25. Mass spectrum of compound **10.**

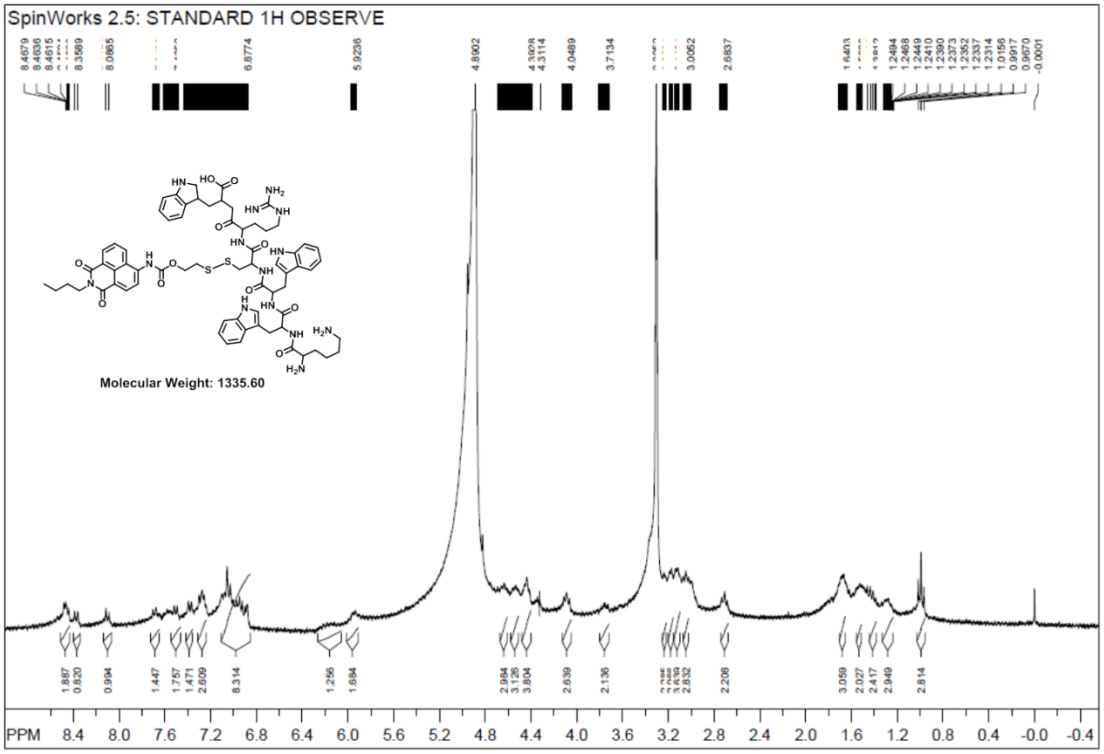


Figure S26. ^{1}H NMR spectrum of **9.**

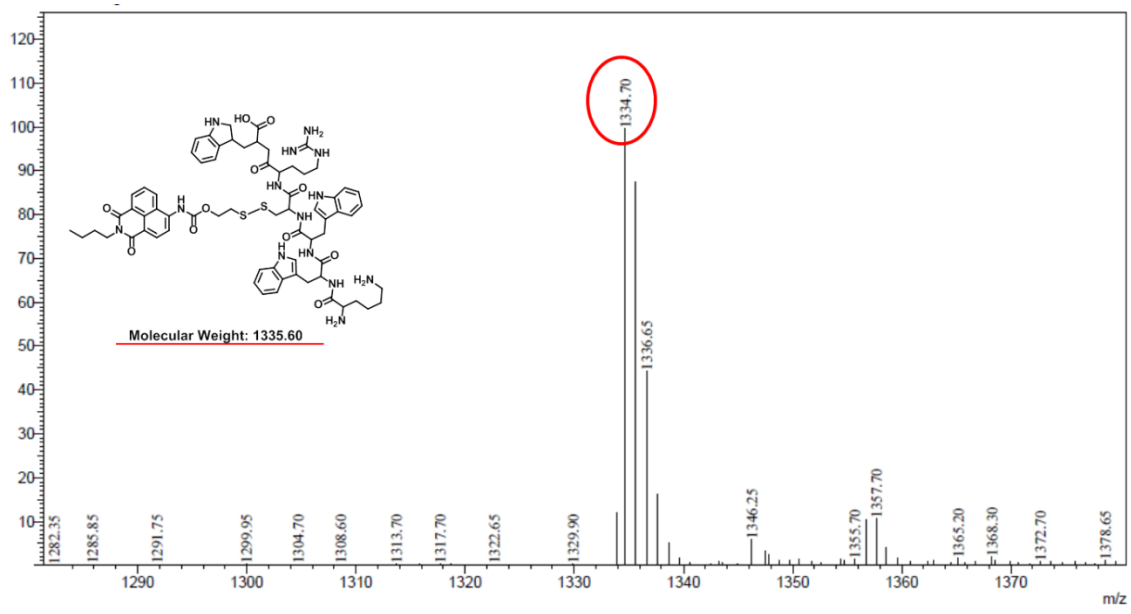


Figure S27. Mass spectrum of **9.**

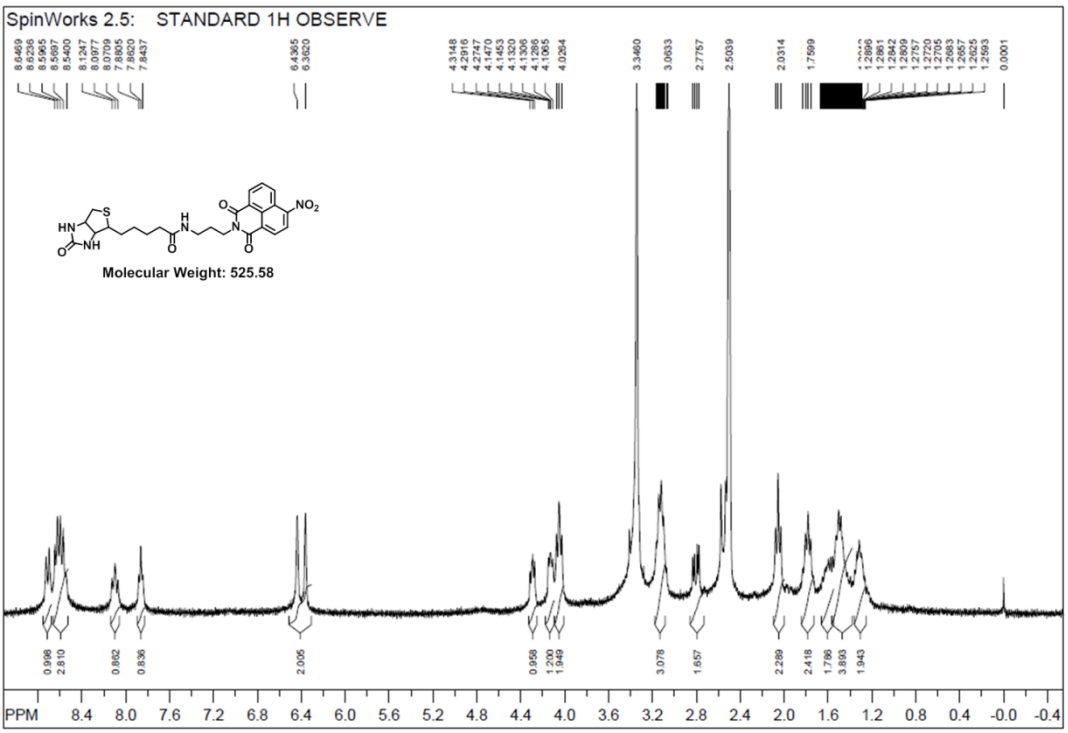


Figure S28. ^{1}H NMR spectrum of compound **14.**

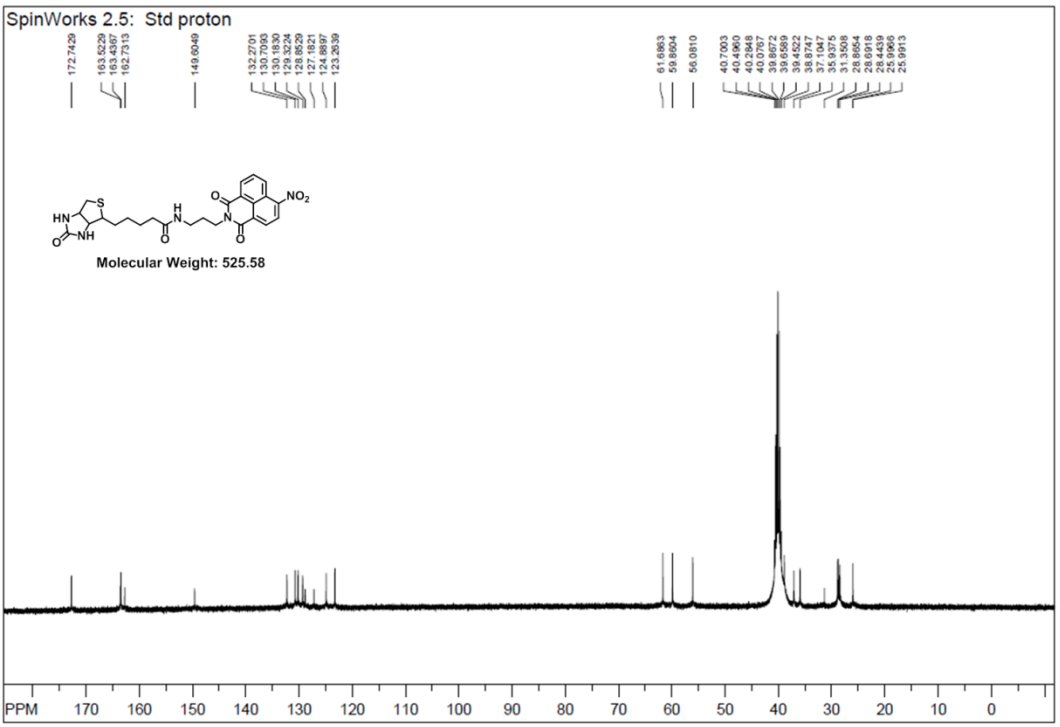


Figure S29. ^{13}C NMR spectrum of compound **14.**

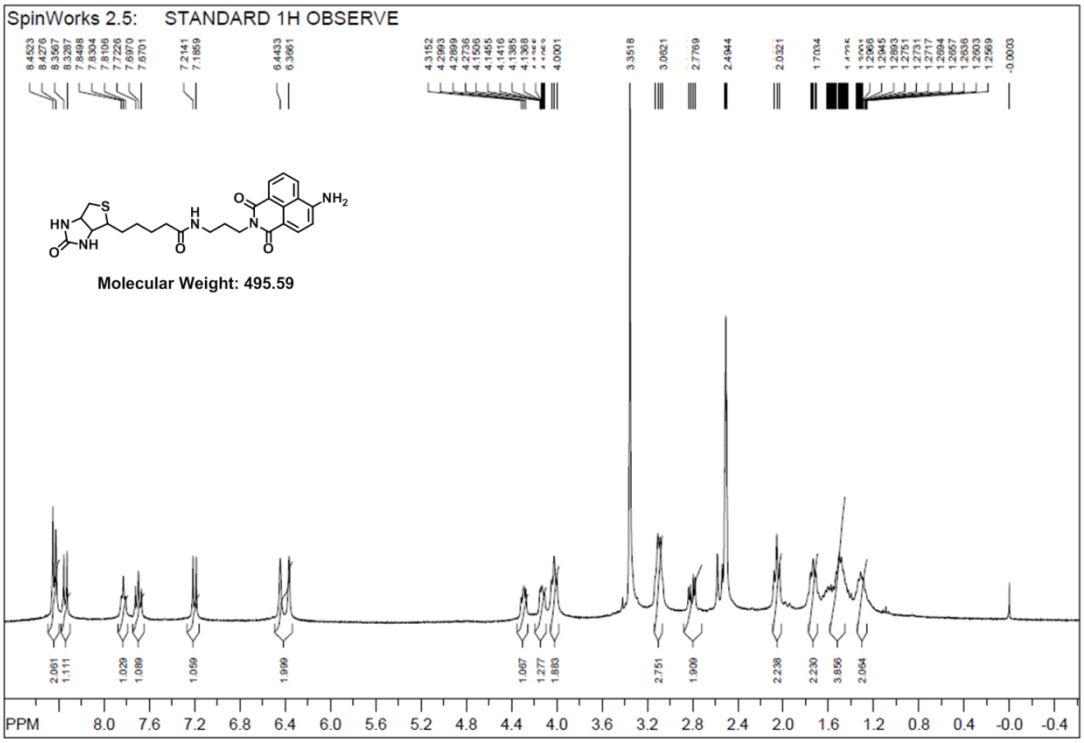


Figure S30. [1]H NMR spectrum of compound **13.**

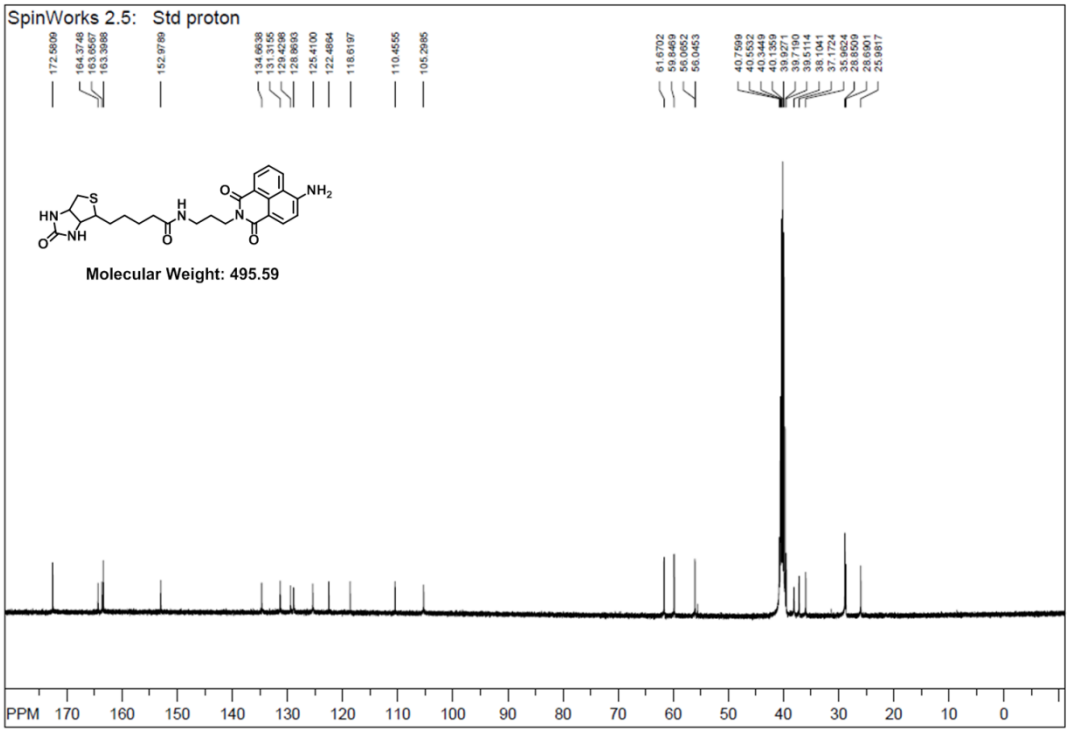


Figure S31. [13]C NMR spectrum of compound **13.**

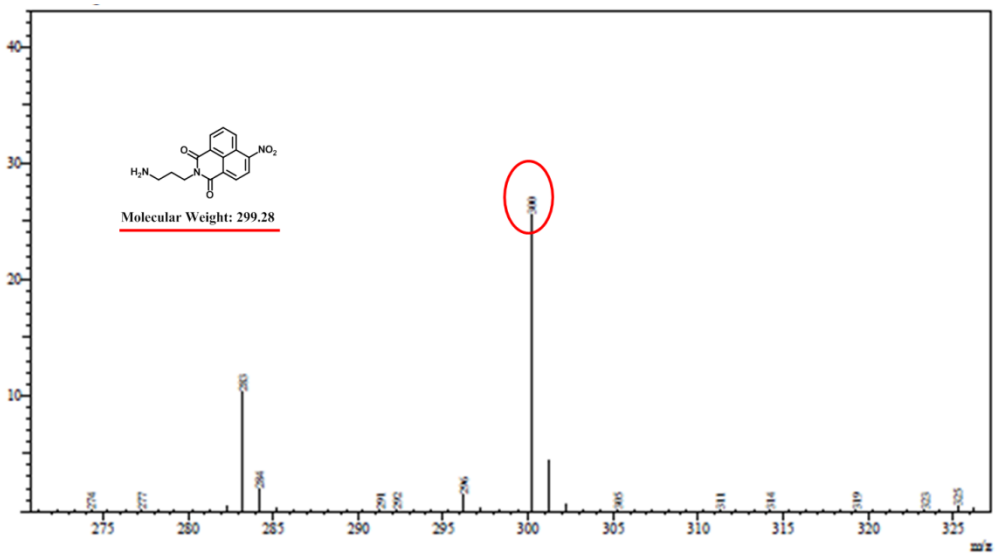


Figure S32. ESI MS spectrum of the depotected **6** with TFA/DCM