Supporting Information

GSH Activated Biotin-tagged Near-Infrared Probe for Efficient Cancer Imaging

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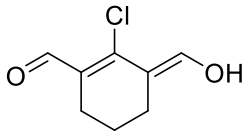
Chemical information

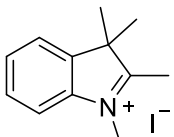
*Synthesis of (E)-2-chloro-3-(hydroxymethylene)cyclohex-1-ene-1-carbaldehyde (**1a**):*



1a

A mixed solution of N,N-dimethylformamide (DMF, 20 mL, 0.198 mol) and dichloromethane (40 mL) in a round bottom single-neck flask was cooled over ice, and phosphorus oxychloride (19 mL, 0.2 mol) was added in the above solution dropwise with stirring, followed by the addition of cyclohexanone (5.0 g, 0.050 mol). The mixture was refluxed for 2 h. After cooled to room temperature, the solution was poured into 200 g ice and allowed to stand overnight. A yellow solid was recrystallized and collected (6.03 g, 36 %). The product was used directly for the next reaction without purification. ESI–MS: calcd for $C_8H_9ClO_2$ 172.0291; found: 173.20.

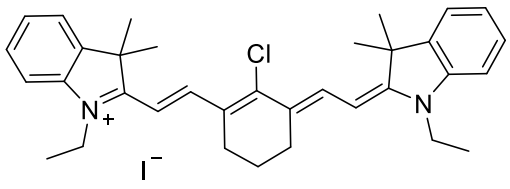
*Synthesis of 1-ethyl-2,3,3-trimethyl-3H-indol-1-ium iodide **(1b)**:*



1b

2,3,3-Trimethyllindolenine and ethyl iodide were dissolved in 30 mL toluene, the mixture was stirred at 100 ℃ for 20 h. Then the reaction was cooled to room temperature and filtered. The solid was washed with ester acetic to afford 1b (8.54 g, 71 %) as pink solid without further purification for next step. ESI–MS: calcd for $C_{13}H_{18}N^+$ 188.1434; found: 188.20.

*Synthesis of 2-((E)-2-((E)-2-chloro-3-(2-((E)-1-ethyl-3,3-dimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1-ethyl-3,3-dimethyl-3H-indol-1-ium iodide **(2)**:*

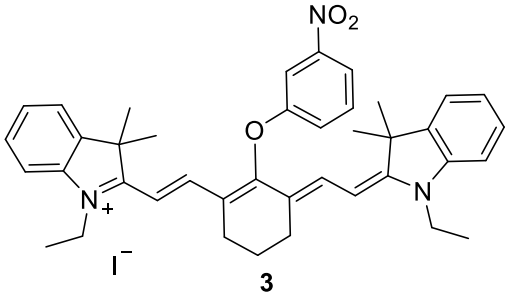


2

Under nitrogen atmosphere, to a round bottom double-neck flask containing compound **1a**(2.58 g, 14 mmol) in 80 ml acetic anhydride, 1-ethyl-2,3,3-trimethyl-3H-indol-1-ium iodide (10 g, 31.7 mmol) and sodium acetate (1.28 g, 15 mmol) were added. The solution was heated to 130 ºC and refluxed for 8 h. The generated mixture was filtered and the precipitate was washed thoroughly with diethyl ether and potassium iodide aqueous solution. A dark green solid **2** with metallic luster was obtained (7.56 g, 85 %). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.34 (d, *J* = 14.1 Hz, 2H), 7.41 – 7.33 (m, 4H), 7.23 (dd, *J* = 7.5, 1.0 Hz, 2H), 7.16 (d, *J* = 8.0 Hz, 2H), 6.23 (d, *J* = 14.0 Hz, 2H), 4.25 (q, *J* = 7.5 Hz, 4H), 2.74 (t, *J* =

6.0 Hz, 4H), 1.97 (p, *J* = 6.0 Hz, 2H), 1.70 (s, 12H), 1.44 (t, *J* = 7.0 Hz, 6H). ESI–MS: calcd for $C_{34}H_{40}C_lN_2$ 511.2875; found: 511.25 ESI–MS: calcd for $C_{34}H_{40}C_lN_2$ 511.2875; found: 511.25.

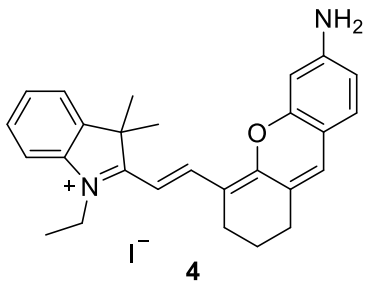
*Synthesis of 1-ethyl-2-((E)-2-((E)-3-(2-((E)-1-ethyl-3,3-dimethylindolin-2-ylidene)ethylidene)-2-(3-nitrophenoxy)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-3H-indol-1-ium iodide **(3)**:*



3

Triethylamine (576 μL, 4.16 mmol) was added to a stirred solution of **3-Nitrophenol** (580 mg, 4.16 mmol) in DCM (30 mL) at room temperature under nitrogen atmosphere, and the resulting mixture was stirred for 10 min in room temperature. Then a solution of Cy7Cl (1.33 g, 2.08 mmol) in DCM (30 mL) was added to the above mixture, and the reaction mixture was heated at 50 °C for 4 h. Eventually the solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography (CH_2Cl_2/CH_3OH = 20:1 to 10:1) on silica gel, affording the desired compound **3** as a purple solid (1.34 g, 87%).^{1}H NMR (500 MHz, $CDCl_3$) δ 7.94 – 7.88 (m, 2H), 7.78 (d, *J* = 14.0 Hz, 2H), 7.65 (t, *J* = 8.0 Hz, 1H), 7.51 – 7.47 (m, 1H), 7.35 (m, 2H), 7.25 (d, *J* = 7.0 Hz, 2H), 7.18 (m, 2H), 7.12 (d, *J* = 8.0 Hz, 2H), 6.10 (d, *J* = 14.0 Hz, 2H), 4.17 (q, *J* = 7.0 Hz, 4H), 2.78 (t, *J* = 6.0 Hz, 4H), 2.11 – 2.03 (m, 2H), 1.41 (t, *J* = 7.0 Hz, 6H), 1.32 (s, 12H). ESI–MS: calcd for $C_{40}H_{44}N_3O_3$+ 614.3377; found: 614.30.

*Synthesis of (E)-2-(2-(6-amino-2,3-dihydro-1H-xanthen-4-yl)vinyl)-1-ethyl-3,3-dimethyl-3H-indol-1-ium iodide **(4)**:*

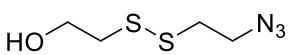


4

$SnCl_2 \cdot H_2O$ (11.25 g, 50 mmol) and hydrochloric acid (8.3 mL, 100 mmol) were added to a solution of **compound 3** (7.41 g, 10 mmol) in methanol (100 mL). The mixture was stirred at 75 °C under nitrogen for 24 h. Methanol was evaporated, and the residue was separated in CH_2Cl_2 and water. The aqueous layer was alkalified by 1 M NaOH aqueous solution to pH 9, then the mixture was extracted with CH_2Cl_2. The combined organic layers were dried over anhydrous sodium sulfate and evaporated. The crude product was washed by ethyl

acetate and further purified *via* chromatography (silica gel) eluting with CH_2Cl_2:MeOH (20:1 to 10: 1) to afford 3.0 g (58 %) of compound **4**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (d, *J* = 14.0 Hz, 1H), 7.46 (d, *J* = 7.5 Hz, 1H), 7.43 – 7.38 (m, 2H), 7.26 (dd, *J* = 8.0, 4.0 Hz, 2H), 7.16 – 7.09 (m, 2H), 6.93 (d, *J* = 8.0 Hz, 1H), 5.95 (d, *J* = 13.5 Hz, 1H), 4.13 (s, 1H), 3.51 (s, 2H), 2.79 – 2.75 (m, 2H), 2.67 (t, *J* = 6.0 Hz, 2H), 1.97 – 1.92 (m, 2H), 1.79 (s, 6H), 1.50 (s, 3H). ESI–MS: calcd for $C_{27}H_{29}N_2O^+$ 397.2274; found: 397.35.

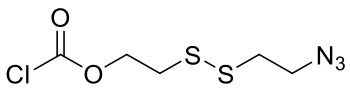
*Synthesis of 2-((2-azidoethyl)disulfanyl)ethan-1-ol **(5)**:*



5

Triethylamine (5.4 mL, 38.8 mmol) was added to a solution of 2-Hydroxyethyl disulfide (5.0 g, 32.4 mmol) in tetrahydrofuran (20 mL) firstly. The mixture was stirred at 0 °C under nitrogen protection for 10 min. Then a solution of methanesulfonyl chloride (2.5 mL, 32.3 mmol) in tetrahydrofuran (10 mL) was added dropwise to the above mixture, and the reaction mixture was closely monitored by TLC until the crude material was fully consumed. Then the reaction was quenched by water and extract by ethyl acetate (50 mL ×2). The combined organic layers were dried over anhydrous sodium sulfate and evaporated. The intermediate was directly dissolved in DMF (15 mL), sodium azide was carefully added to the solution and the mixture was heated at 60 °C overnight. After fully reacted, the mixture was dilute in water (150 mL) and extract with ethyl acetate (50 mL ×3). The combined organic layers were dried over anhydrous sodium sulfate and evaporated. The crude product was further purified *via* chromatography (silica gel) eluting with PE: EA (5: 1) to afford 2.0 g (35 %) of compound **5**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.92 – 3.88 (m, 2H), 3.61 (t, *J* = 7.0 Hz, 2H), 2.88 (m, 4H), 1.89 (s, 1H). ESI–MS: calcd for $C_4H_9N_3OS_2$ 179.0187 found: 180.15

*Synthesis of 2-((2-azidoethyl)disulfanyl)ethyl carbonochloridate **(6)**:*

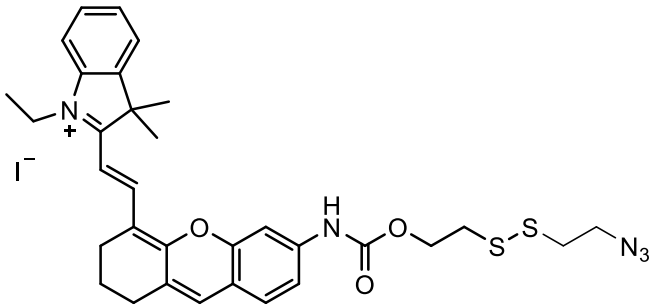


6

Triethylamine (320 μL, 2.23 mmol) was added to a solution of **compound 5** (400 mg, 2.23 mmol) in anhydrous toluene (3 mL). The mixture was stirred at 0 °C for 10 min. Then a solution of triphosgene (220 mg, 0.74 mmol) in anhydrous toluene (2 mL) was added dropwise to the above mixture, and the

reaction mixture was closely monitored by TLC until the crude material was fully consumed (approximately 10 min). Then the mixture was directly purified via fast chromatography (silica gel) eluting with PE to PE: EA (10: 1) to get compound **6** as colorless oil (0.2 g, 37%) and directly used in the next step.

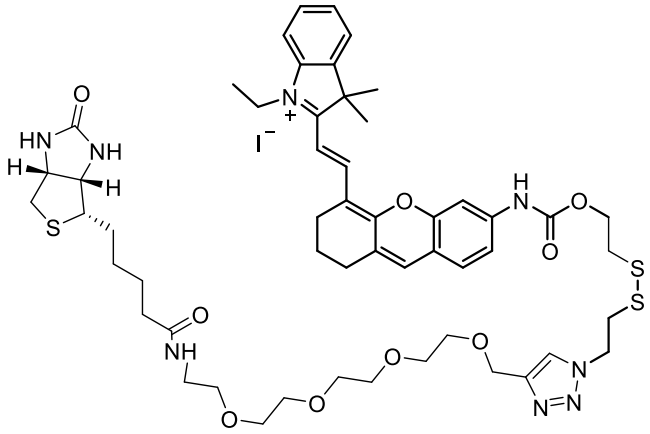
*Synthesis of (E)-2-(2-(6-((((2-((2-azidoethyl)disulfanyl)ethoxy)carbonyl)amino)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-1-ethyl-3,3-dimethyl-3H-indol-1-ium iodide **(7)**:*



7

Compound 4 (216 mg, 0.5 mmol), anhydrous triethylamine (138 μL, 1 mmol) and DMAP (6.1mg, 0.05 mmol) were added to a solution of **compound 6** (242 mg, 1 mmol) in anhydrous DCM (15 mL). The mixture was stirred in room temperature overnight. Then the mixture was purified *via* chromatography (silica gel) eluting with CH_2Cl_2:MeOH (20:1 to 10: 1) to afford 2.0 g (35 %) of compound **7**. ^{1}H NMR (500 MHz, MeOD) δ 8.78 (d, *J* = 15 Hz, 1H), 7.91 (s, 1H), 7.68 (d, *J* = 7.5 Hz, 1H), 7.59 – 7.52 (m, 2H), 7.45 (d, *J* = 8.5 Hz, 2H), 7.37 (s, 1H), 7.22 (dd, *J* = 8.5, 2.0 Hz, 1H), 6.54 (d, *J* = 15.0 Hz, 1H), 4.47 (d, *J* = 2.0 Hz, 2H), 4.42 (d, *J* = 7.5 Hz, 2H), 3.65 – 3.58 (m, 2H), 3.11 – 3.05 (m, 2H), 2.95 (t, *J* = 6.5 Hz, 1H), 2.79 (s, 2H), 2.73 (s, 2H), 1.99 – 1.91 (m, 2H), 1.84 (s, 6H), 1.50 (t, *J* = 7.0 Hz, 3H), 1.17 (d, *J* = 7.0 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.70, 162.40, 153.60, 153.45, 146.02, 143.63, 141.99, 140.95, 134.76, 129.05, 127.98, 127.40, 127.11, 122.92, 116.97, 116.93, 111.83, 105.55, 77.31, 77.27, 77.06, 76.81, 63.17, 50.87, 50.04, 46.24, 41.08, 37.81, 37.09, 29.14, 28.52, 20.36, 12.82, 8.78. HRMS (ESI) *m*/*z*: $[M + H]^+$ calcd for $C_{32}H_{36}N_5O_3S_2^+$, 602.2254; found, 602.2260.

Synthesis of 1-ethyl-3,3-dimethyl-2-((E)-2-(6-(((2-((2-(4-(15-oxo-19-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-2,5,8,11-tetraoxa-14-azanonadecyl)-1H-1,2,3-triazol-1-yl)ethyl)disulfanyl)ethoxy)carbonyl)amino)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3H-indol-1-ium iodide ***(P6)****:*



P6

Biotin-PEG4-Alkyne (25.1 mg, 55 μm) and sodium ascorbate (4mg, 40 mol) were added to a solution of **compound 7** (36.5mg, 50umol) in EtOH : MeOH (2 : 1, 3.0 mL), were added. The reaction mixture as degassed for 15 min by purging argon gas. Then 19 mg (75 umol) of $CuSO4 \cdot 5H_2O$ in 2 mL water was added to the reaction mixture. The reaction was continued overnight at room temperature. Then the crude reaction mixture was directly purified through prepare C18 column chromatography using Acetonitrile/Water with 0.1% TFA as eluent to afford 14 mg (24%) of **P6** ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.91 (s, 1H), 8.69 (d, *J* = 14.0 Hz, 1H), 7.85 (d, *J* = 10.0 Hz, 2H), 7.67 (d, *J* = 8.0 Hz, 1H), 7.50 (d, *J* = 7.5 Hz, 1H), 7.46 (t, *J* = 7.5 Hz, 1H), 7.39 (d, *J* = 7.5 Hz, 1H), 7.37 – 7.31 (m, 2H), 7.28 (s, 1H), 6.65 (s, 1H), 6.23 (d, *J* = 12.5 Hz, 1H), 5.51 (s, 6H), 4.74 (t, *J* = 6.5 Hz, 2H), 4.67 (s, 2H), 4.53 (s, 1H), 4.42 (t, *J* = 6.0 Hz, 2H), 4.34 (s, 1H), 4.25 (dd, *J* = 14.0, 7.0 Hz, 2H), 3.67 (s, 2H), 3.62 (m, 10H), 3.54 (t, *J* = 4.5 Hz, 2H), 3.40 (d, *J* = 4.0 Hz, 2H), 3.21 (t, *J* = 6.5 Hz, 2H), 3.12 (d, *J* = 4.5 Hz, 1H), 3.03 (t, *J* = 6.0 Hz, 2H), 2.88 (dd, *J* = 12.7, 4.5 Hz, 1H), 2.73 (t, *J* = 9.0 Hz, 3H), 2.64 (d, *J* = 6.0 Hz, 2H), 2.20 (t, *J* = 7.0 Hz, 2H), 1.97 – 1.87 (m, 2H), 1.76 (s, 6H), 1.65 (m, 3H), 1.50 (t, *J* = 7.0 Hz, 3H), 1.44 – 1.35 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.54, 162.80, 153.96, 153.53, 146.01, 143.89, 141.80, 140.90, 135.22, 130.30, 130.30, 129.11, 128.29, 127.39, 127.21, 125.57, 122.89, 117.11, 116.99, 114.56, 111.57, 105.11, 101.74, 77.23, 70.15, 70.13, 69.86, 69.39, 64.30, 62.66, 62.20, 60.72, 55.40, 50.65, 48.94, 40.32, 39.14, 37.50, 37.46, 35.30, 29.71, 29.10, 28.32, 28.32, 28.05, 27.84, 27.72, 27.43, 25.37, 20.30, 12.48. HRMS (ESI) *m*/*z*: $[M + H]^+$ calcd for $C_{53}H_{71}N_8O_9S_3^+$, 1059.4501; found, 1059.4499.

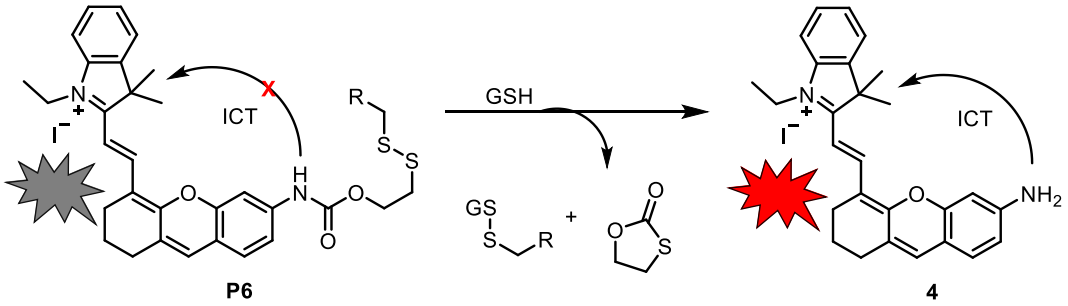


Figure S1. Working mechanism of P6. **P6** was activated in the presence of intracellular GSH to release the fluorophore.

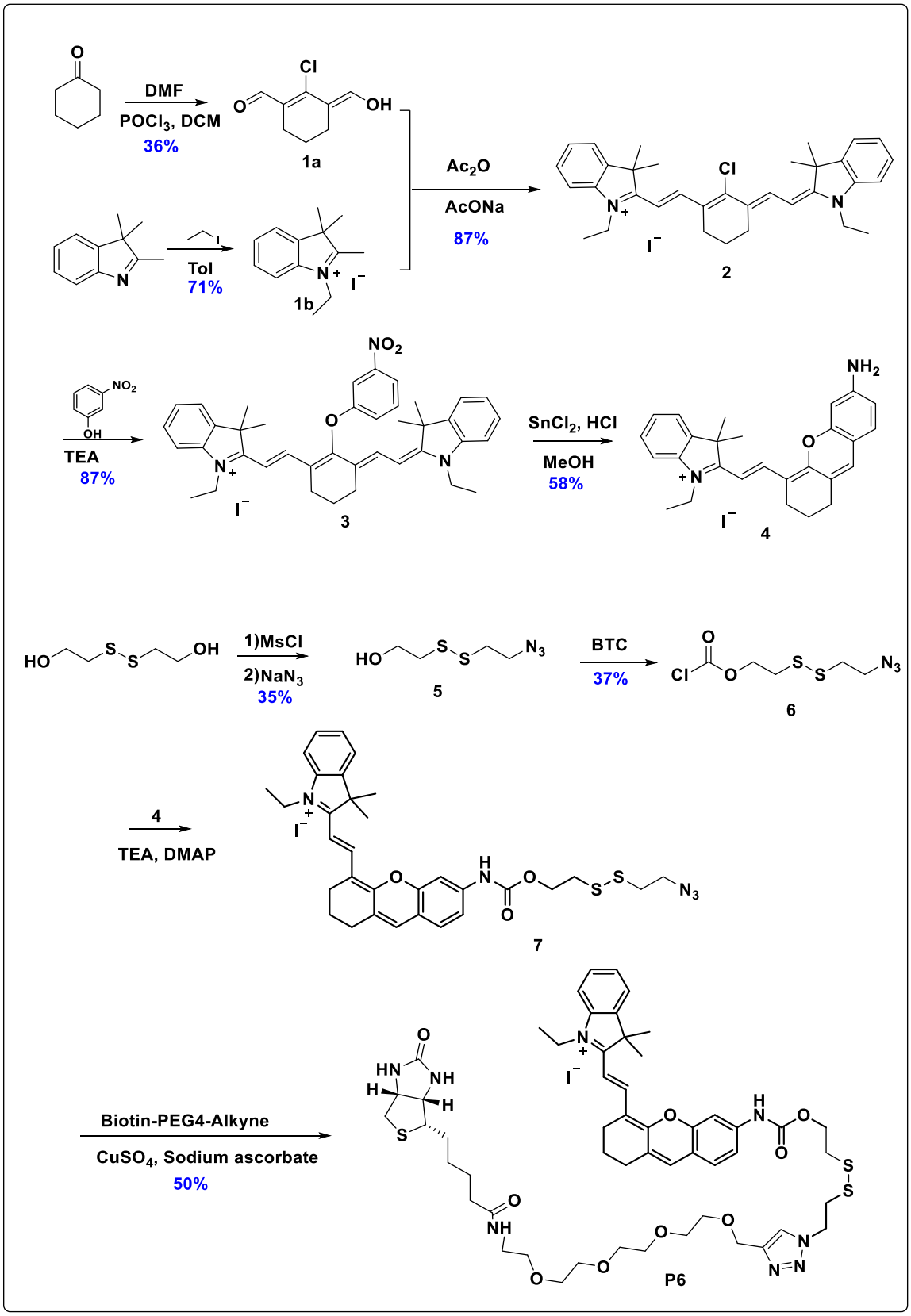


Figure S2. Synthesis of compound P6

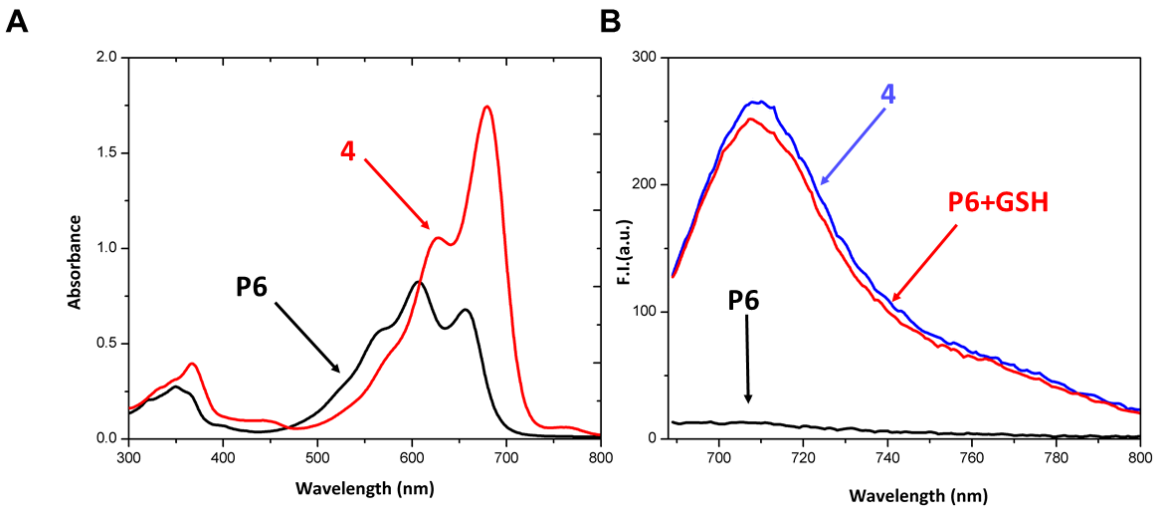


Figure S3. (A) Absorption spectra of P6 (20 μM) and compound 4 (20 μM) in PBS (0.1 M, pH 7.4 with 20% Acetonitrile). (B) Fluorescence spectra of P6 (20 μM) before and after GSH (200 μM) incubation compared to compound 4 (20 μM) in PBS (0.1M, pH 7.4 with 20% Acetonitrile).

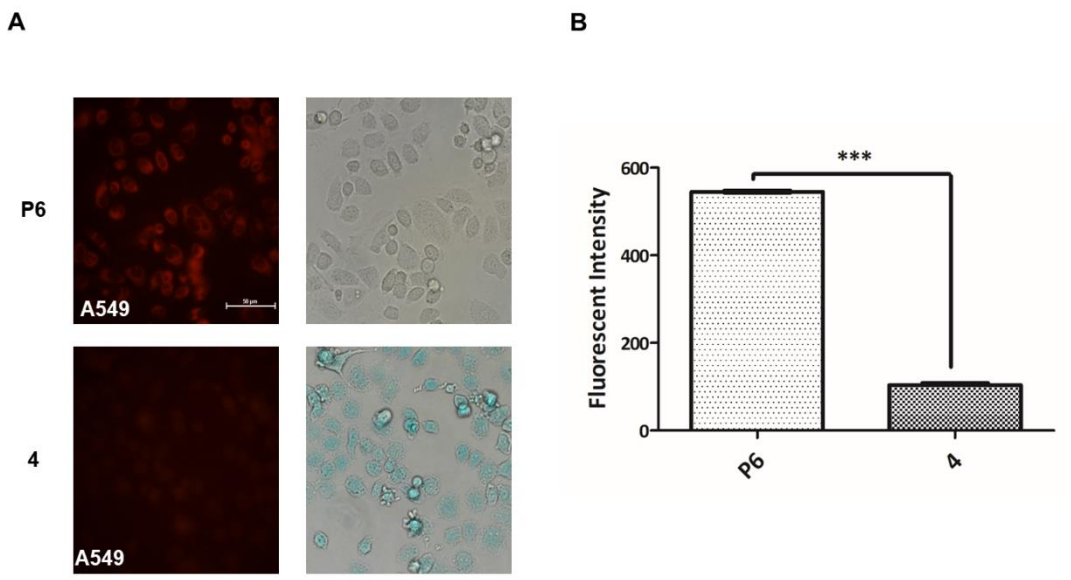


Figure S4. P6 uptake in biotin receptor-positive cancer cells. (A) Representative fluorescence microscopy images of A549 cells incubated with P6 and 4 (5μM) at 37°C for 4h. (B) Fluorescence intensity of A549 cells after incubation with P6 and 4 at 37°C for 4 h.

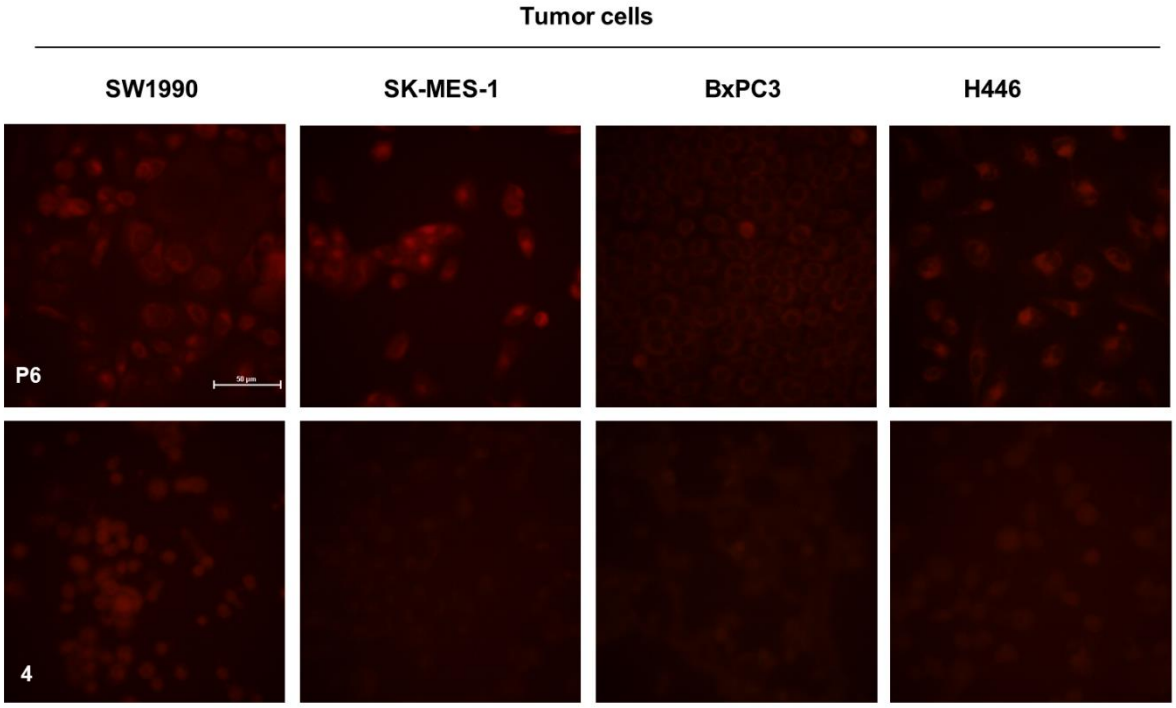


Figure S5. Microscopy images in different cancer cells incubated with P6 and 4 at 37 °C for 4 h, signals were collected in red channel. scar bar (50 μM)

Table S1. GSH concentration in different cell lines

		GSH(UM/l)	protien(g/l)	G/P(UM/G)
HGC-27	Control	89.78474	0.235384	381.4399
	P6	56.38617	0.172754	326.3952
SW480	Control	329.576	0.514456	640.6305
	P6	456.3862	0.60307	756.772
H460	Control	215.5512	0.507412	424.8049
	P6	198.8519	0.480095	414.1927
BxPC3	Control	111.2851	0.30953	359.5291
	P6	56.2818	0.179322	313.8592
BEAS-2B	Control	47.61905	0.156954	303.3946
	P6	42.66145	0.198263	215.1761
HFL1	Control	27.84083	0.120976	230.1359
	P6	27.2668	0.131636	207.138
THP1	Control	341.8395	0.439738	777.3705
	P6	363.0267	0.509316	712.7733

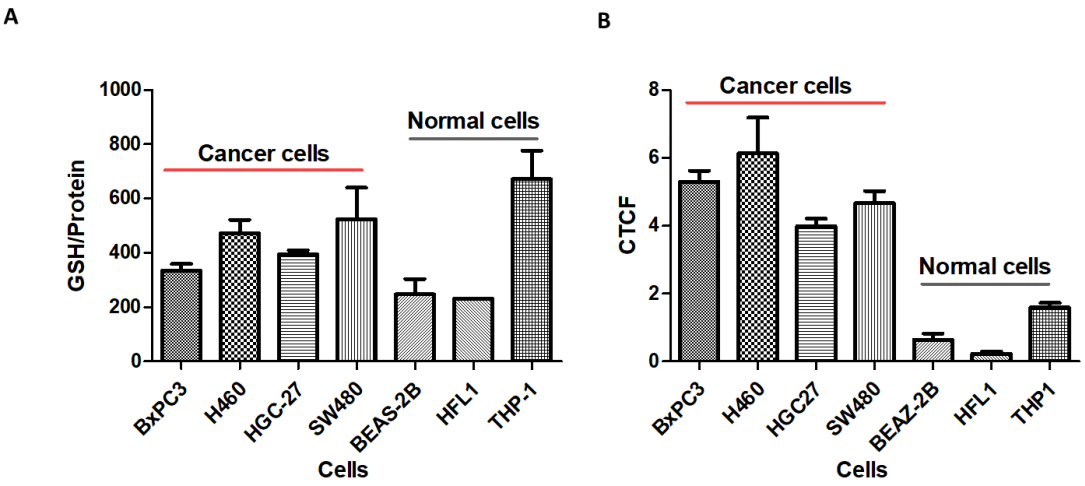
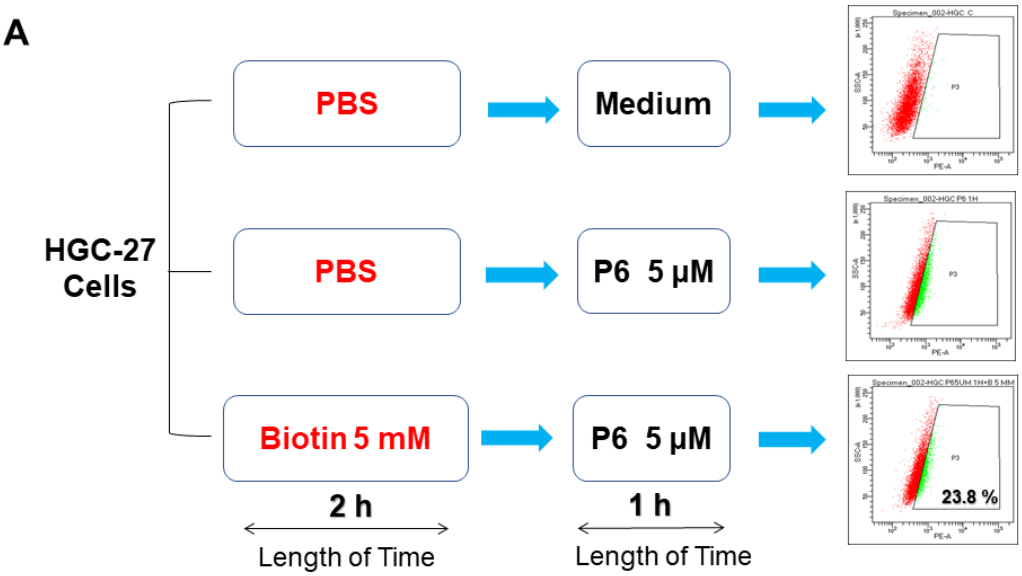


Figure S6. Calibration of cellular GSH levels and the corrected total cell fluorescence (CTCF). A) GSH concentration. B) CTCF

A



B

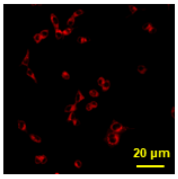
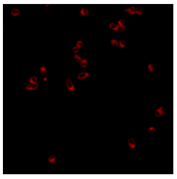
Items	HGC-27	
	P6	Biotin+P6
Total fluorescence (A.U.)	3,341,520	1,769,445
Number of cells	30	23
Mean fluoresence per cell (A.U.)	111,384	76,932
Representative images		

Figure S7. Biotin competitive experiment in HGC-27 cells. A) Illustration of experiment process and results of the flow cytometry. B) Fluorescence of HGC-27 cells with or without pre-treatment of free biotin.

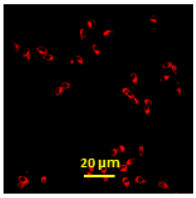
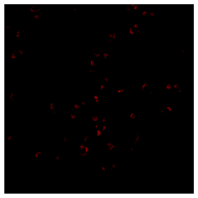
Items	HGC-27	
	P6	Fillipin III+P6
Total fluorescence (A.U.)	6,092,740	2,708,745
Number of cells	34	45
Mean fluoresence per cell (A.U.)	179,181	60,914
Representative images		

Figure S8. Addition of Fillipin III diminished intracellular P6 in HGC-27 cells.

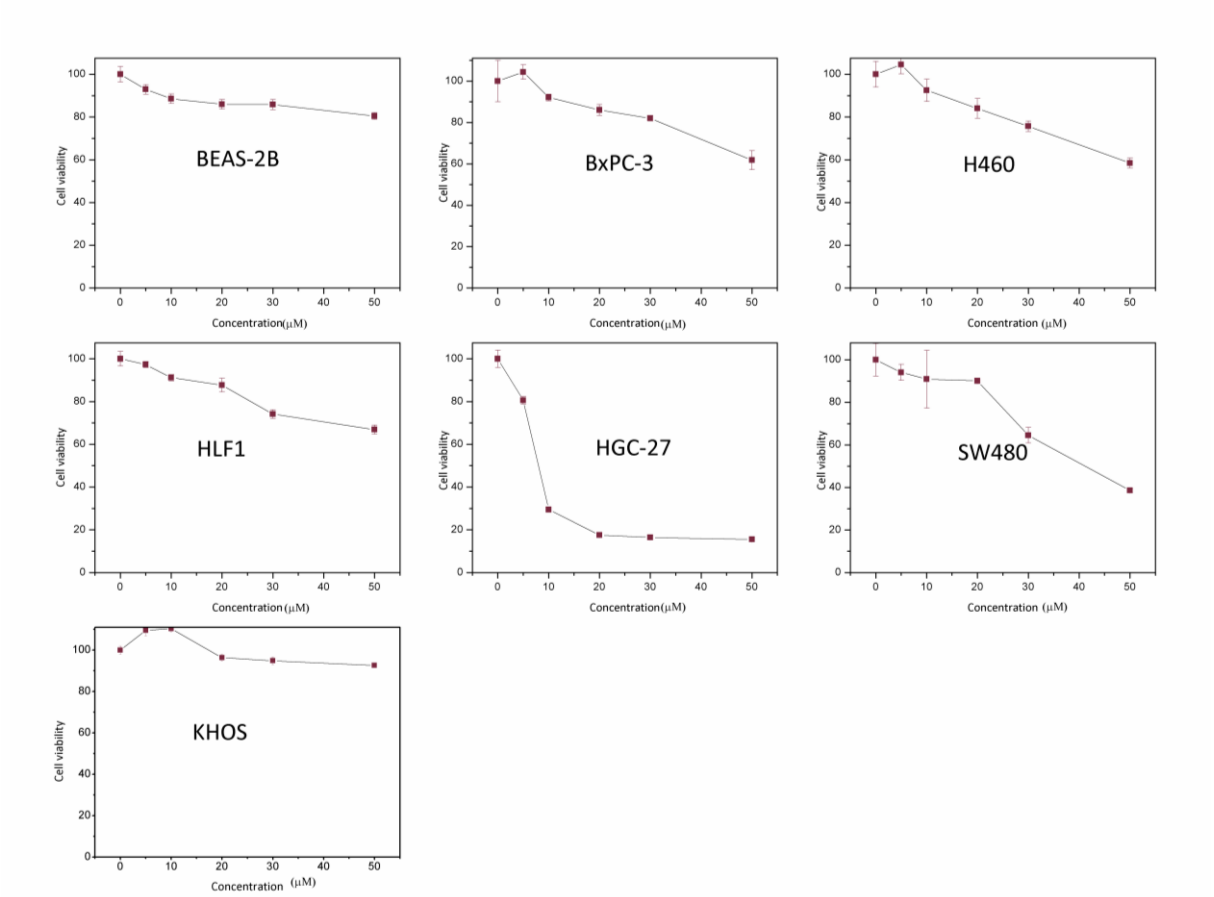


Figure S9. Cell viability results using CCK8 assay.（P6 concentration 0 to 50 μM）

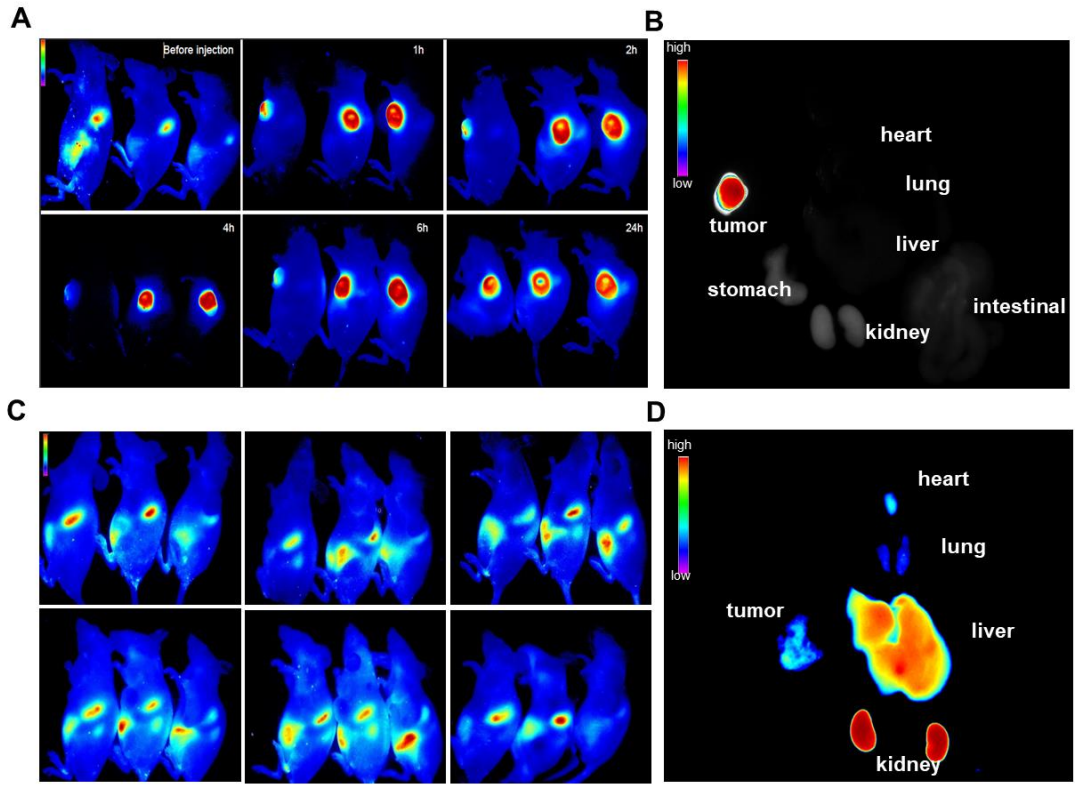


Figure S10. *In vivo* NIR fluorescence imaging in HGC-27 tumor-bearing mice with P6. (A) Representative fluorescence images of mice before and 1h, 2h, 4h, 6h and 24h after intra-tumoral injection of **P6** (0.1 mg/kg). (B) Representative fluorescence images of kidney, lung, spleen, liver, heart, intestinal, stomach brain, and tumor 24h post injection. (C) Representative fluorescence images of mice before and 1h, 2h, 4h, 6h and 24h after intravenous injection of **P6** (0.1 mg/kg). (D) Representative fluorescence images of individual organs and tumors 24h post I/V injection with **P6**.

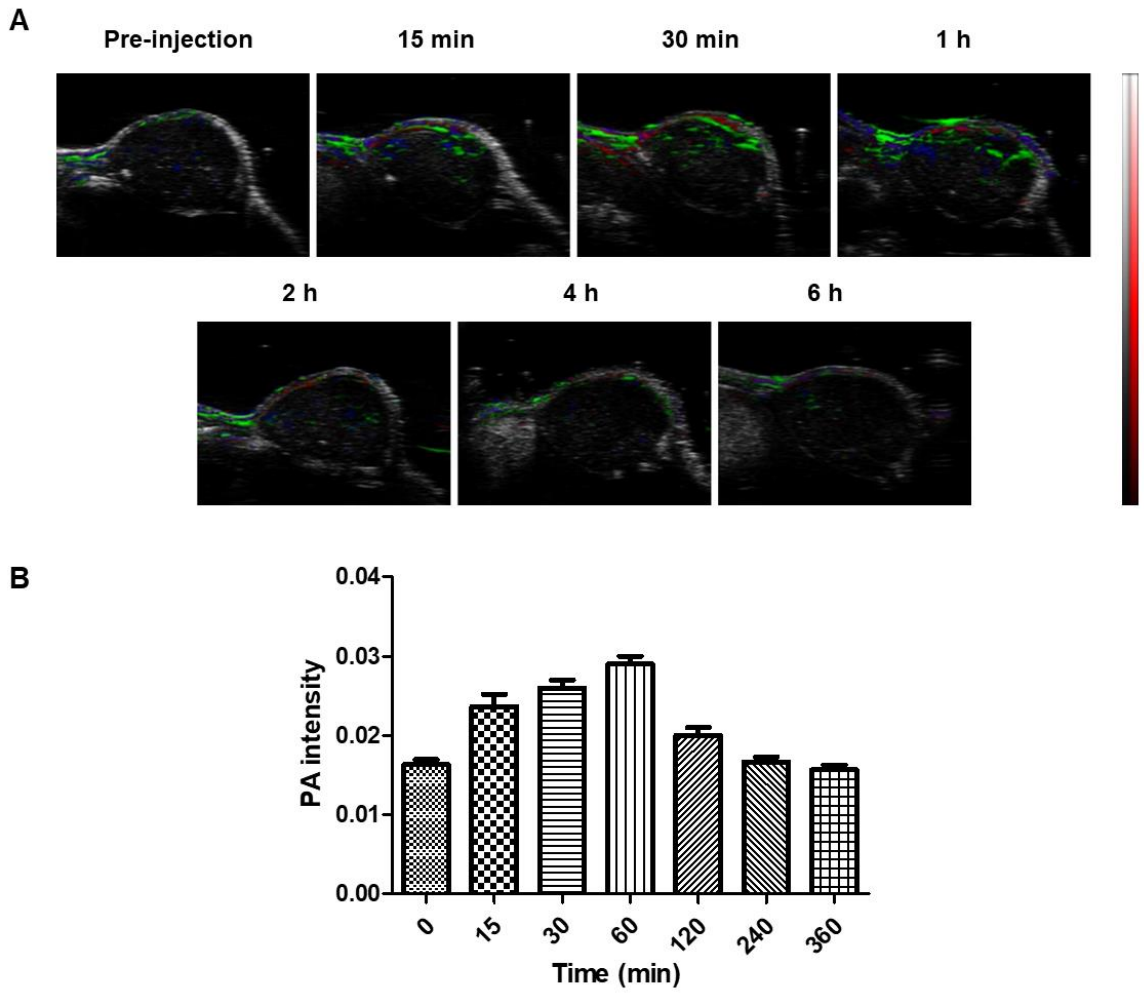
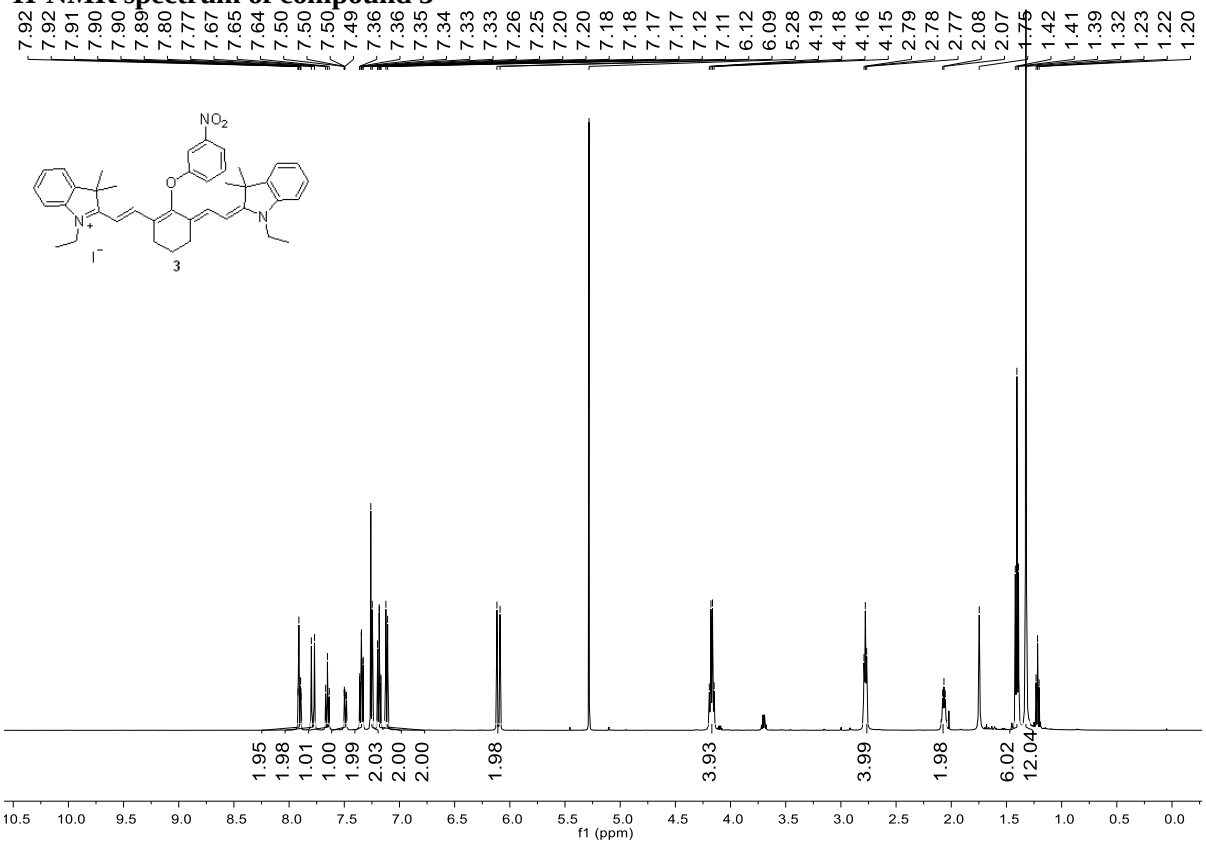


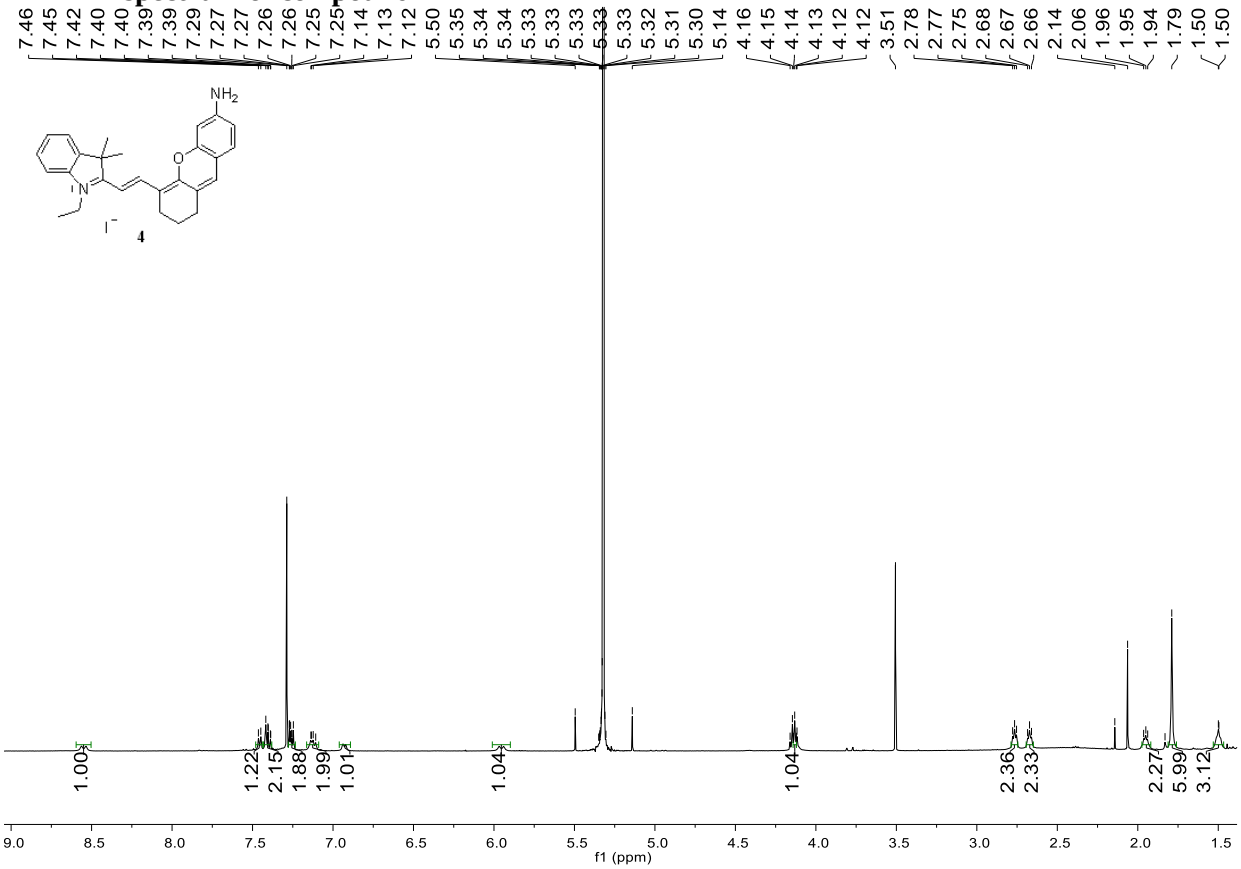
Figure S11. ***In vivo*** **photoacoustic imaging of P6 in tumor-bearing mice.** (A) Representative images of live mice before and after intratumoral injection of **P6** (0.1mg/kg in 5% DMOS saline) for 15 min, 30 min, 1h, 2h, 4h and 6h. P6 signal (800nm) in green, vein signal (carboxy-hemoglobin) in blue, artery (oxyhemoglobin) in red. (B) Time-dependent dynamics of PA intensity at the tumor site before and after intratumoral injection. All the signals were quantified three times for Statistic/error analysis.

Spectra:

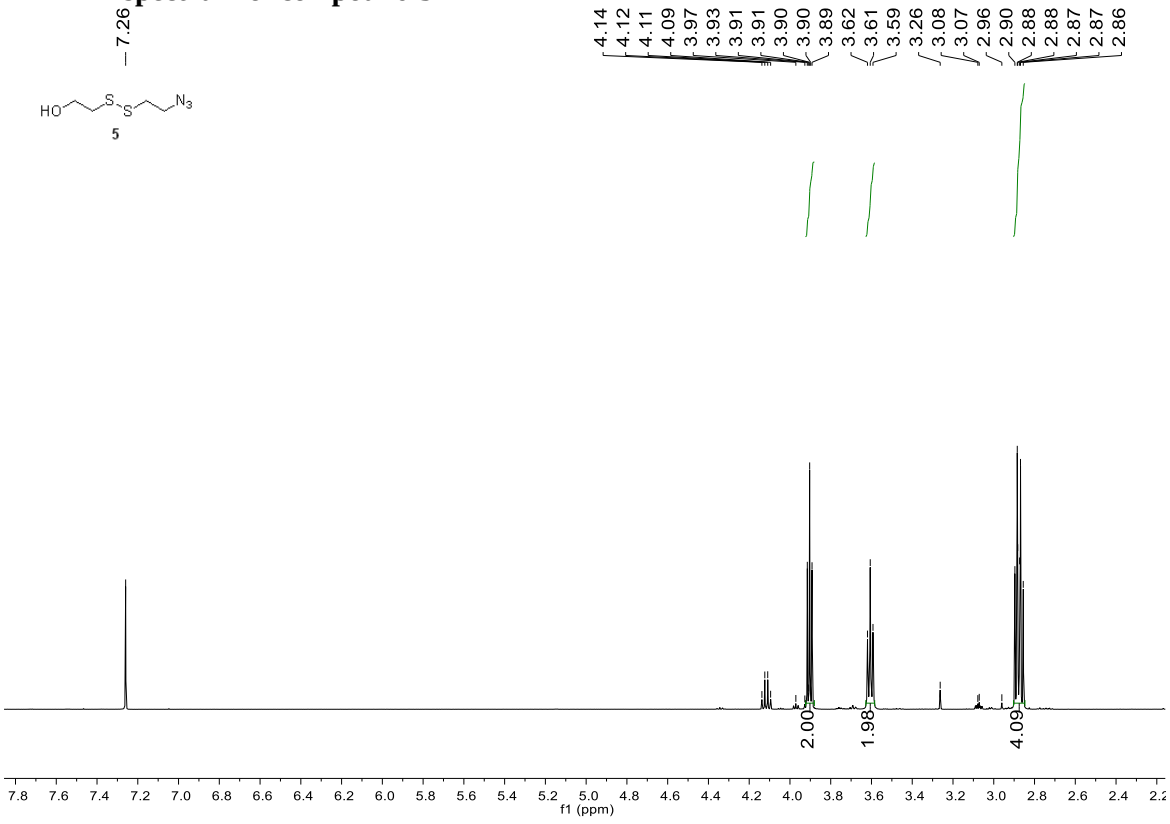
^{1}H-NMR spectrum of compound 3



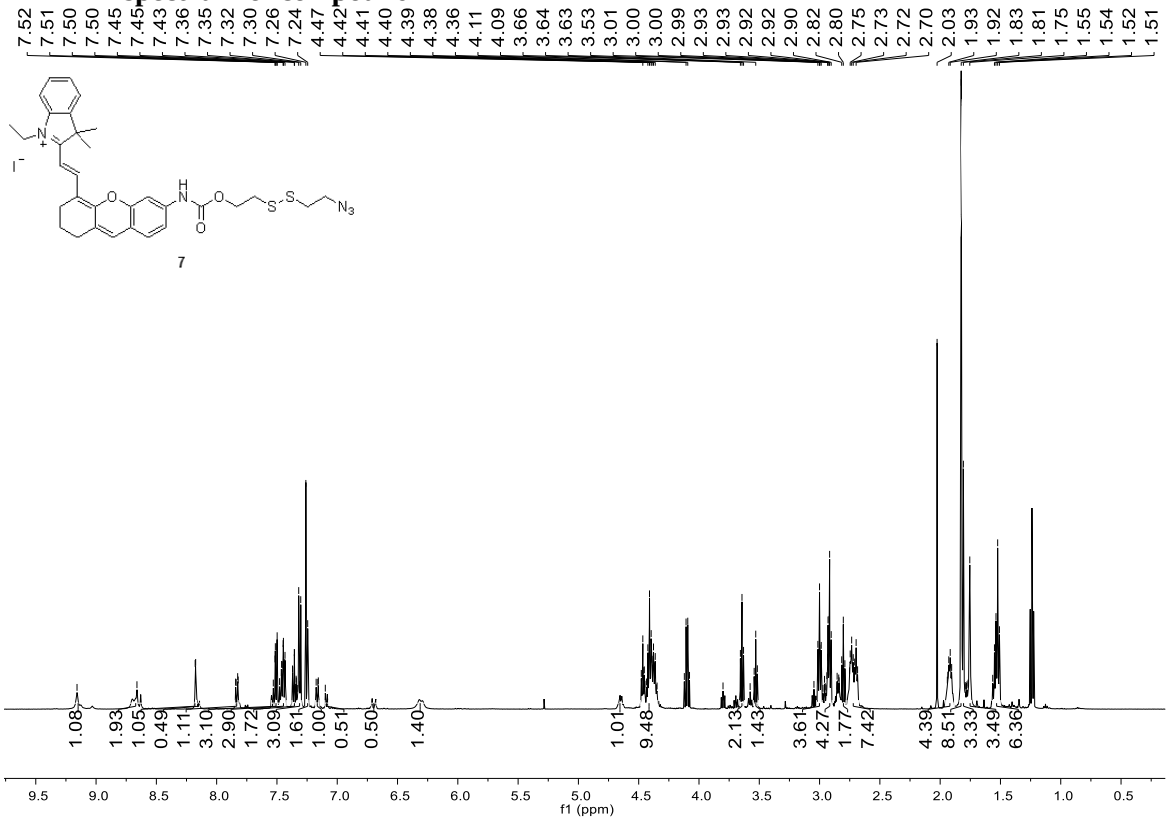
^{1}H-NMR spectrum of compound 4



^{1}H-NMR spectrum of compound 5

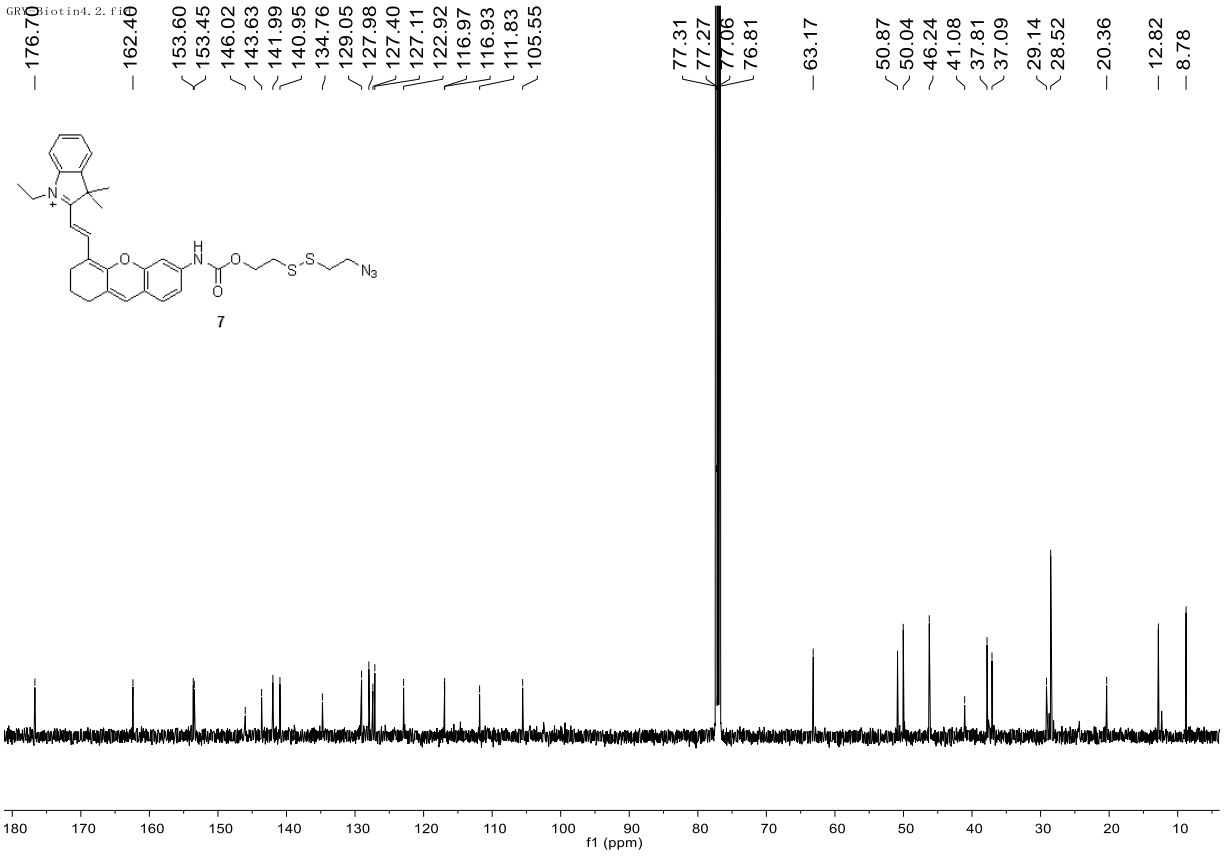


^{1}H-NMR spectrum of compound 7



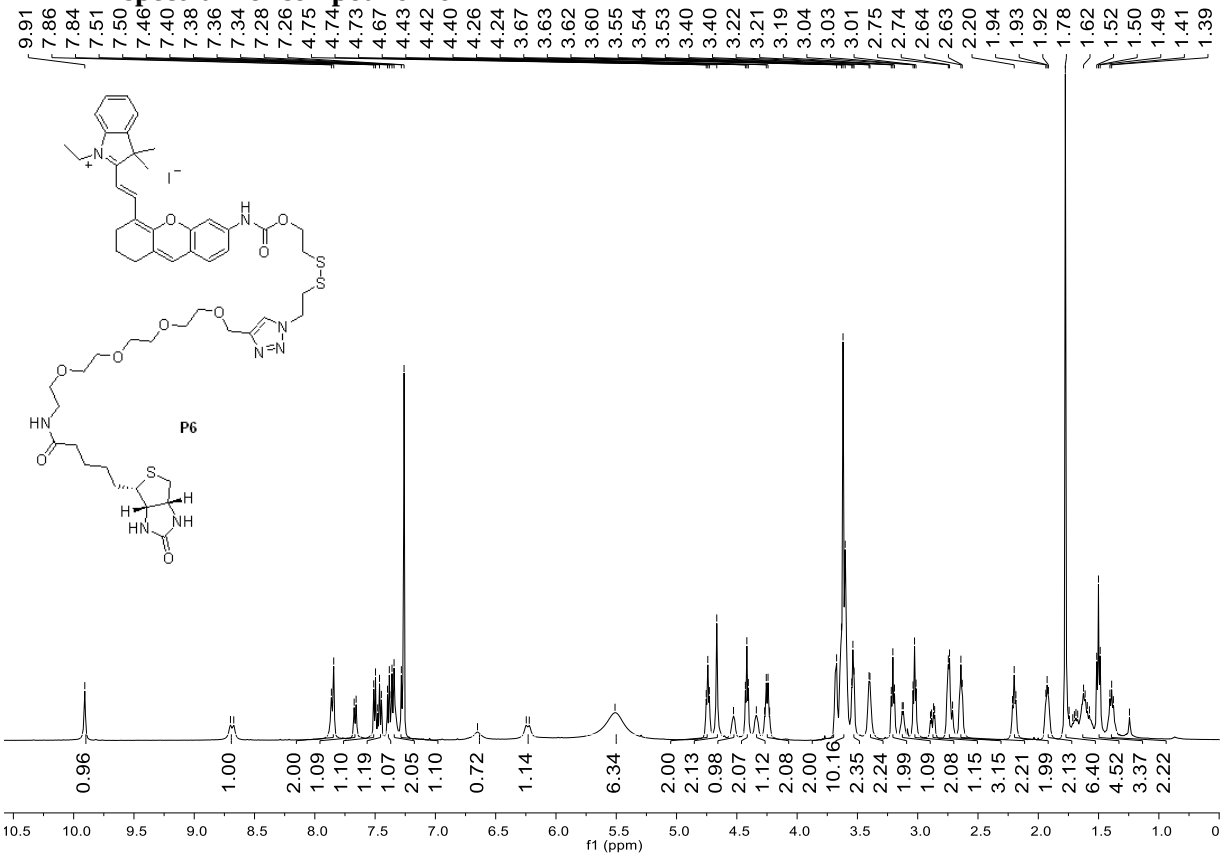
7

^{13}C-NMR spectrum of compound 7

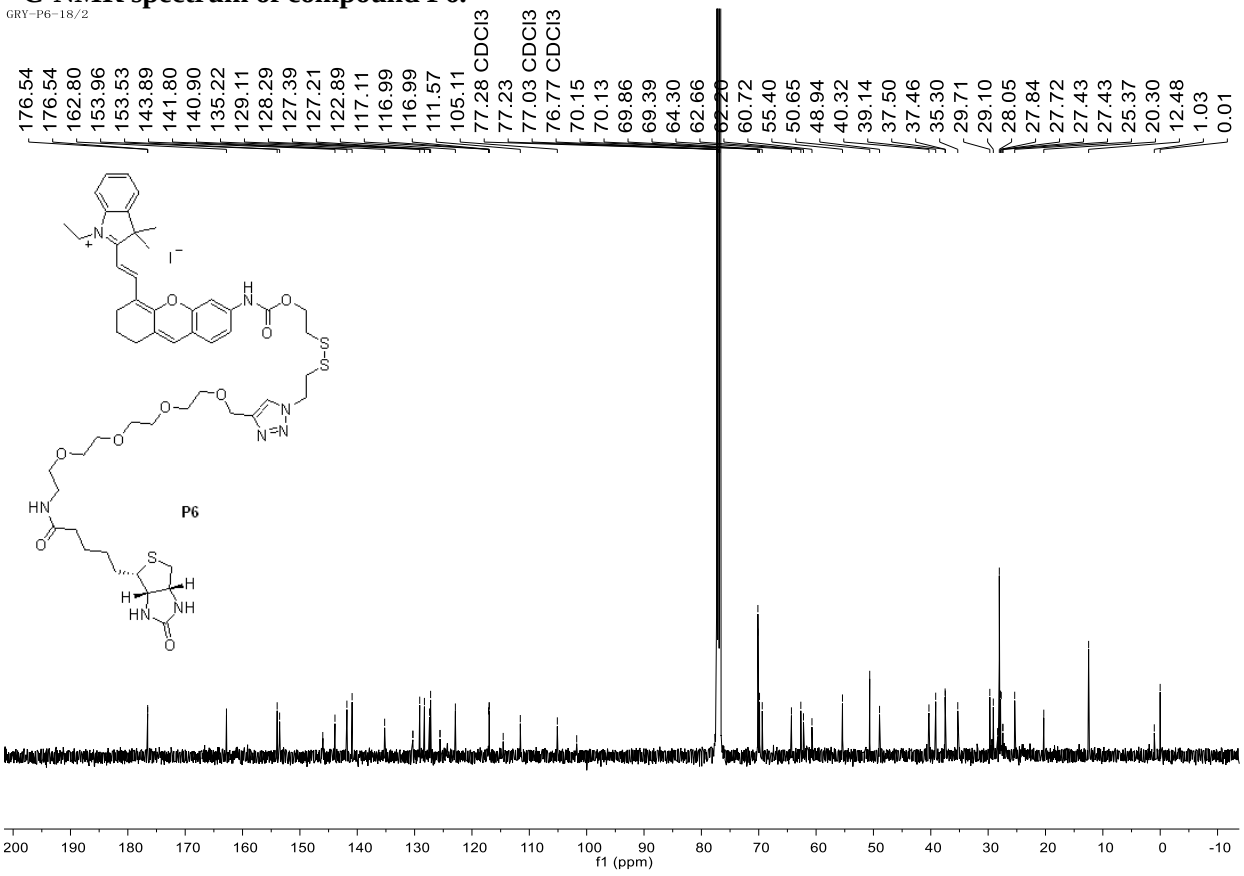


7

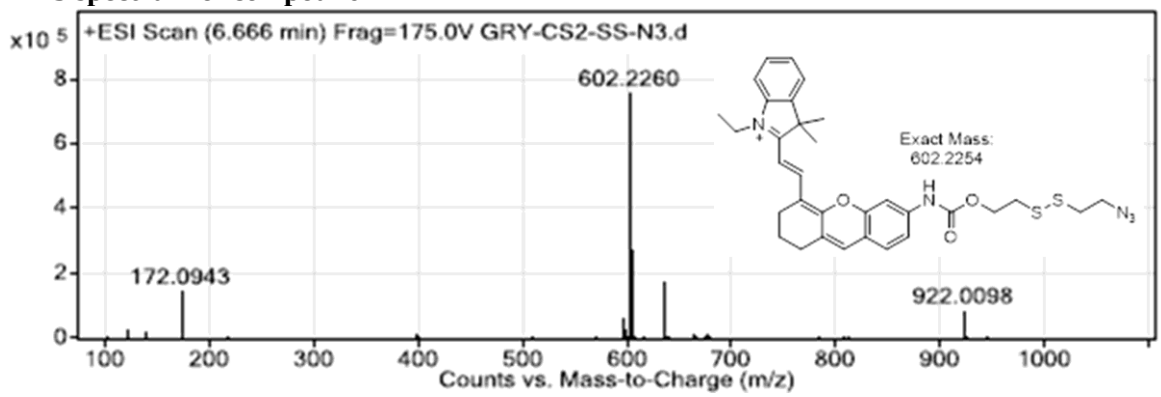
^{1}H-NMR spectrum of compound P6



^{13}C-NMR spectrum of compound P6.



HRMS spectrum of compound 7



HRMS spectrum of compound P6