Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Acidic pH/reduction Dual-stimuli Responsive Nanoprobe for Enhanced CT Imaging of Tumours *in vivo*

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1. General methods

All the starting chemicals were purchased from commercial suppliers and used without further purification, unless indicated otherwise. All NMR spectra (^{1}H-NMR, ^{13}C-NMR) were obtained on a Bruker ADVANCE III NMR spectrometer. HPLC analyses were performed on carried out on Agilent 1260 semi-prep high performance liquid chromatography. Dynamic light scattering (DLS) measurements were performed on a particle size analyzer (Nano ZS90, Malvern) at room temperature. TEM images were taken on an electron microscope (Tecnai G2 Spirit, FEI). Photoluminescence (PL) spectra were recorded on Edinburgh FLS980 spectrofluorometer. UV-Vis absorption spectra were taken on UV spectrometer (UV-3600, Shimadzu). MTT cell proliferation cytotoxicity assay kit was purchased from Sigma.

Cytotoxicity Assay

Mouse fibroblast cell line 3T3 and murine breast cancer cells 4T1 were cultured in standard cell media containing 10% heat-inactivated fetal bovine serum and maintained in a humidified 37 ^{o}C incubator with 5% CO_2. 1×10^4 Cells were seeded into 96 well plates and incubated with different concentrations (0, 5, 10, 20, 40, and 60 μM **1** or **1-Scr** for 24 h. The relative cell viabilities were finally determined by the standard MTT assay.

CLSM Images of 4T1 Cells

4T1 cells were cultured on the 8-well confocal dish (culture area: 0.8 cm^2/well), at the density of 5×10^3 cells per well. After the growth in cell incubator for 24 h, 4T1 cells were incubated with **1** or **1-Scr** at 20 μM for different times (1, 2, 4 h). Then the culture medium was removed and the cells were washed with phosphate buffered saline (PBS) for three times. Finally, adding the fresh culture medium into the wells for CLSM.

Live Cell Imaging Co-stained with Lyso-Tracker

Cells were firstly incubated with **1** (20 μM) for 2 h in the presence and absence of inhibitor DEM (50 μM) and then washed with PBS for three times before incubation with 60 nM of Lyso-Tracker Red for 30 min at 37 °C. After three times of PBS rinsing, the cells were treated with fresh medium for CLSM.

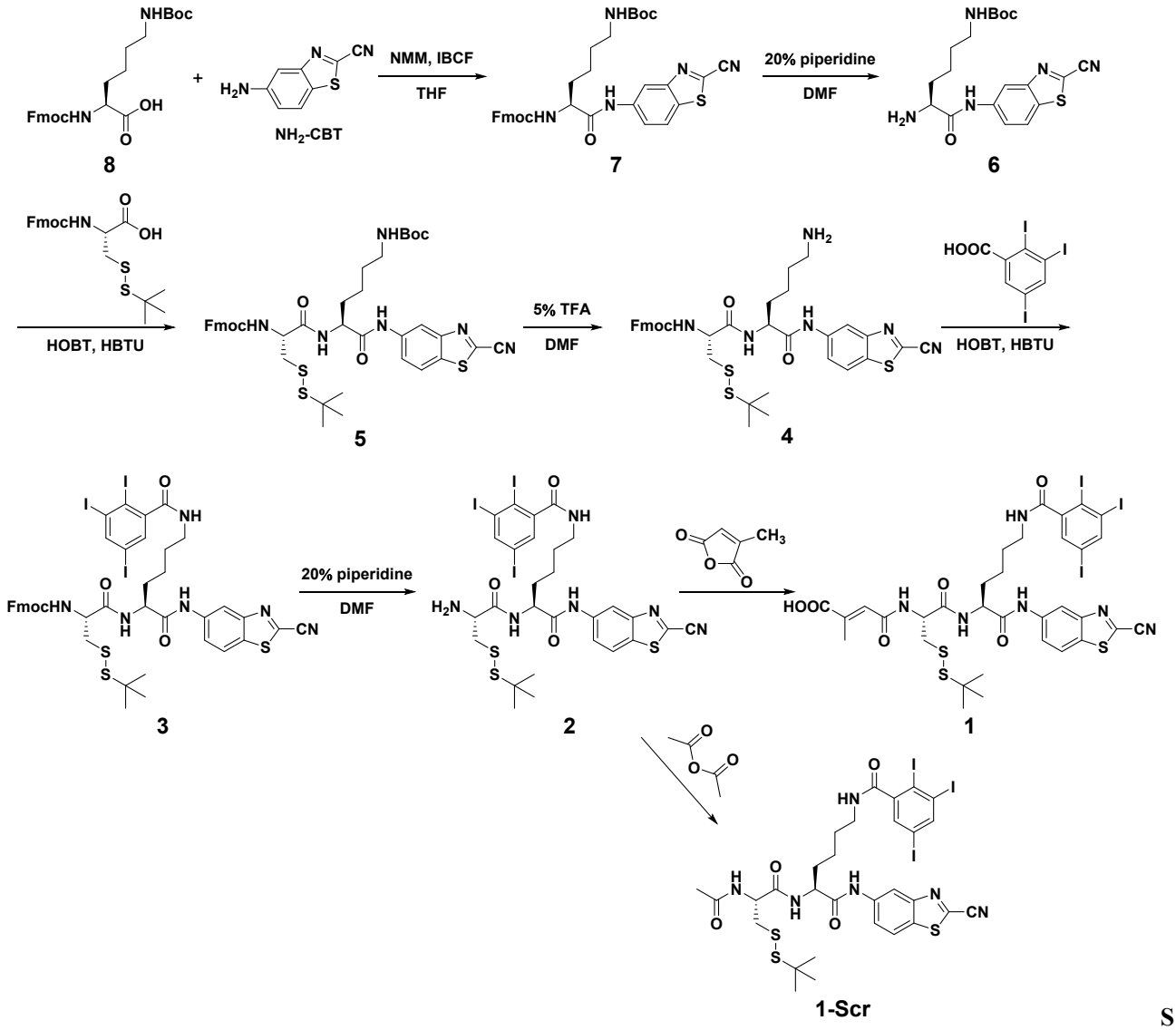
In Vitro CT Signal Measurement

The potential of **1** and **1-Scr** in CT imaging was illustrated by comparison with commercial iopromide agent. The aqueous solutions of **1**, **1-Scr** and iopromide with different concentrations (0.125, 0.25, 0.5, 1, 2 and 4 mg I/mL) were loaded into capsules for measuring Hounsfield units to evaluate the CT imaging effect. The images were scanned in the accurate mode using full angle, 3 frames averaging, 615 mA tube current, and 55 kV tube voltage.

CT Imaging of 4T1 Tumour Xenografts *In Vivo*

All animal experiments were approved by the Animal Care and Use Committee of Soochow University (P. R. China), and all protocols of animal studies conformed to the Guide for the Care and Use of Laboratory Animals. All the mice were scanned on the same machine. The images were obtained at an X-ray voltage of 55 kVp, and anode current of 615 A in accurate mode using full angle, 3 frames averaging. For *in vivo* CT imaging, 4T1 tumour-bearing mice were prepared and placed in an animal bed under anesthetic for CT imaging. The injection volume of contrast agents for each mouse was 200 μL with a dose of 21.69 mg I equiv./kg. The CT imaging was taken at 0, 12, 30, 60, 72, 120, 180 and 240 min post-injection.

2. Synthesis of The Probe



cheme S1. Synthetic route for compound **1** and **1-Scr**.

Synthesis of the compound 7: The isobutyl chloroformate (175 mg, 1.28 mmol) was added to a mixture of **8** (400 mg, 0.85 mmol) and NMM (4-methylmorpholine, 130 mg, 1.28 mmol) in THF (10 mL) at 0 °C and the reaction mixture was then stirred for 30 min. The solution of 2-cyano-6-aminobeno-thiazole (179 mg, 1.00 mmol) was added to the reaction mixture and further stirred for 1 h at 0 °C. After further reaction at room temperature overnight, the solvent was removed under vacuum. The residue was dissolved in ethyl acetate (50 mL) and washed with water and aqueous $NaHCO_3$ (50 mL × 3). The organic phase was dried with Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by silica gel chromatography with the eluent of petroleum ether (PE) : ethyl acetate (EA) = 2 : 1 to afford the compound **7** (531.88 mg, yield: 85%). ^{1}H NMR (600 MHz, Methanol-d4) δ 8.59 (s, 1H), 8.04 (d, J = 9.0 Hz, 1H), 7.74 (d, J = 7.6 Hz, 2H), 7.63 (dt, J

= 14.8, 5.0 Hz, 3H), 7.33 (t, *J* = 7.5 Hz, 2H), 7.26 (t, *J* = 7.7 Hz, 2H), 4.36 (d, *J* = 6.9 Hz, 2H), 4.24 (dd, *J* = 9.0, 5.4 Hz, 1H), 4.17 (t, *J* = 7.0 Hz, 1H), 3.02 (dt, *J* = 9.8, 4.5 Hz, 2H), 1.83 (dp, *J* = 15.1, 5.4 Hz, 1H), 1.73 (tq, *J* = 11.7, 7.9, 6.3 Hz, 1H), 1.48 (p, *J* = 9.4, 8.3 Hz, 2H), 1.37 (s, 9H), 1.30–1.18 (m, 2H). ^{13}C NMR (151 MHz, Methanol-d4) δ 172.33, 157.18, 148.38, 143.84, 143.70, 141.80, 141.14, 139.09, 136.58, 135.25, 127.33, 126.72, 124.76, 124.49, 120.82, 119.49, 112.63, 111.40, 78.44, 66.51, 55.84, 47.00, 39.57, 31.51, 29.20, 27.36, 22.80.

*Synthesis of the compound **6**:* To remove the protecting group Fmoc, piperidine (2 mL) was added to a solution of compound **7** (500 mg, 0.80 mmol) in DMF (8 mL) for 5 min at 0 °C. Then the solvent was evaporated under reduced pressure. The crude product was purified by silica gel chromatography with the eluent of DCM : MeOH = 80 : 1 to get the compound **6** (290.18 mg, yield: 90%). ^{1}H NMR (600 MHz, Methanol-d4) δ 8.59–8.45 (m, 1H), 8.07–7.93 (m, 1H), 7.73–7.54 (m, 1H), 3.47 (q, *J* = 6.7 Hz, 1H), 3.06–2.97 (m, 2H), 1.85–1.58 (m, 2H), 1.49 (h, *J* = 7.1 Hz, 3H), 1.37 (d, *J* = 5.9 Hz, 9H), 1.26 (d, *J* = 3.5 Hz, 1H). ^{13}C NMR (151 MHz, Methanol-d4) δ 174.81, 161.86, 157.07, 149.04, 141.79, 137.45, 137.07, 123.84, 119.90, 112.02, 78.37, 55.30, 39.62, 34.65, 29.43, 27.32, 22.52.

*Synthesis of the compound **5**:* To a solution of compound **6** (250 mg, 0.62 mmol) in dry DMF (10 mL), Fmoc–Cys(StBu)-OH (321.04 mg, 0.74 mmol), HBTU (282.15 mg, 0.74 mmol), HOBT (100.44 mg, 0.74 mmol) and DIPEA (213.68 μL) were added. The resulting solution was stirred for 2 h at room temperature and then evaporated under reduced pressure. The residue was dissolved in ethyl acetate (25 mL) and washed with water and aqueous $NaHCO_3$ (50 mL × 3). The organic phase was dried with Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by silica gel chromatography with the eluent of PE : EA = 2 : 1 to get the compound **5** (405.26 mg, yield: 80%). ^{1}H NMR (600 MHz, Methanol-d4) δ 8.37 (d, *J* = 2.1 Hz, 1H), 7.84 (d, *J* = 9.0 Hz, 1H), 7.71 (dd, *J* = 9.0, 2.1 Hz, 1H), 7.58 (d, *J* = 7.5 Hz, 1H), 7.52 (dd, *J* = 11.0, 7.6 Hz, 2H), 7.45 (d, *J* = 7.5 Hz, 1H), 7.38–7.27 (m, 1H), 7.22 (q, *J* = 7.1 Hz, 2H), 7.16 (t, *J* = 7.4 Hz, 1H), 4.52 (dd, *J* = 9.9, 4.5 Hz, 1H), 4.42 (t, *J* = 7.3 Hz, 1H), 4.28 (qd, *J* = 10.6, 7.5 Hz, 2H), 4.08 (q, *J* = 7.1 Hz, 1H), 3.18–3.02 (m, 2H), 3.02 (s, 2H), 1.99 (s, 1H), 1.77–1.68 (m, 1H), 1.53–1.42 (m, 2H), 1.38 (s, 9H), 1.34 (s, 11H). ^{13}C NMR (151 MHz, Methanol-d4) δ 172.13, 171.21, 157.15, 157.05, 148.27, 143.49, 143.32, 141.02, 140.78, 138.75, 136.27, 135.19, 127.25, 126.78, 124.74, 124.18, 120.92, 119.34, 112.64,

111.50, 78.43, 66.92, 55.08, 54.09, 46.76, 40.65, 39.75, 37.46, 30.87, 29.03, 28.86, 27.39, 22.96. MS (ESI) Calcd for: $C_{41}H_{48}N_6O_6S_3$ ($[M+H]^+$) : 817.2800, found: 817.2865.

*Synthesis of the compound **3**:* The Boc protecting group of **5** (400 mg, 0.49 mmol) was cleaved with 5% TFA in DMF. After 1 h incubation, the solvent and TFA were removed by evaporation under reduced pressure. Without purified, 2, 3, 5-triiodobenzoic acid (294.89 mg, 0.59 mmol), HBTU (223.61 mg, 0. 59 mmol), HOBT (79.65 mg, 0.59 mmol) and DIPEA (168.87 μL) were then added to the above crude product in DMF. The resulting solution was stirred for 2 h at room temperature and then evaporated under reduced pressure. The residue was dissolved in ethyl acetate (50 mL) and washed with water and aqueous $NaHCO_3$ (50 mL × 3). The organic phase was dried with Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by silica gel chromatography with the eluent of DCM : MeOH = 90 : 1 to get the compound **3** (381.21 mg, yield: 65%). ^{1}H NMR (600 MHz, DMSO-d6) δ 10.55–10.25 (m, 1H), 8.73–8.65 (m, 1H), 8.55–8.25 (m, 2H), 8.20 (dd, *J* = 5.6, 2.0 Hz, 1H), 8.18–8.09 (m, 1H), 7.86 (d, *J* = 7.6 Hz, 1H), 7.82 (t, *J* = 7.9 Hz, 1H), 7.78–7.74 (m, 1H), 7.71 (ddd, *J* = 12.2, 8.8, 6.0 Hz, 1H), 7.68–7.64 (m, 1H), 7.44 (dd, *J* = 3.9, 1.9 Hz, 1H), 7.41–7.32 (m, 2H), 7.28 (dq, *J* = 13.7, 7.2 Hz, 2H), 4.48–4.39 (m, 1H), 4.34 (ddt, *J* = 13.6, 8.6, 4.5 Hz, 1H), 4.32–4.21 (m, 2H), 4.18 (t, *J* = 7.2 Hz, 1H), 3.17–3.09 (m, 2H), 3.09–2.91 (m, 2H), 1.86–1.62 (m, 2H), 1.50 (tt, *J* = 10.3, 6.8 Hz, 2H), 1.26 (d, *J* = 12.7 Hz, 9H), 1.19 (d, *J* = 4.4 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d6) δ 171.50, 170.60, 168.19, 156.32, 148.12, 146.31, 144.14, 141.13, 139.62, 137.11, 135.49, 134.83, 128.07, 127.47, 125.72, 125.18, 121.26, 120.49, 113.99, 113.78, 111.93, 107.97, 95.73, 66.29, 54.66, 54.05, 48.24, 47.01, 39.24, 38.69, 31.77, 30.01, 28.84, 23.28. MS (MALDI-TOF) Calcd for: $C_{43}H_{41}I_3N_6O_5S_3$ ($[M+Na]^+$): 1220.944, found: 1220.955

*Synthesis of the compound **2**:* To remove the protecting group Fmoc, piperidine (2 mL) was added to a solution of compound **3** (350 mg, 0.29 mmol) in DMF (8 mL) for 5 min at 0 °C. Then the solvent was evaporated under reduced pressure. The crude product was purified by silica gel chromatography with the eluent of DCM: MeOH = 50:1 to get the compound **2** (220.1 mg, yield: 80%). ^{1}H NMR (600 MHz, DMSO-d6) δ 10.56 (s, 1H), 8.70 (d, *J* = 2.1 Hz, 1H), 8.38 (t, *J* = 5.5 Hz, 1H), 8.27 (d, *J* = 8.0 Hz, 1H), 8.20 (d, *J* = 2.0 Hz, 1H), 8.17 (d, *J* = 9.0 Hz, 1H), 7.74 (dd, *J* = 9.0, 2.2 Hz, 1H), 7.45 (dd, *J* = 7.7, 2.0 Hz, 1H), 4.48 (d, *J* = 7.2 Hz, 1H), 3.48 (dd, *J* = 8.0, 4.8 Hz, 1H), 3.14 (dq, *J* = 9.5, 3.2, 2.6 Hz, 2H), 3.03 (dd, *J* = 12.7, 4.7 Hz, 1H), 2.83 (dd, *J* = 12.8, 7.8 Hz, 1H), 1.81–

1.63 (m, 2H), 1.51 (p, *J* = 7.3 Hz, 2H), 1.39 (s, 2H), 1.24 (s, 9H). ^{13}C NMR (151 MHz, DMSO-d6) δ 173.41, 171.54, 168.21, 148.16, 148.05, 146.31, 139.69, 137.10, 135.54, 134.83, 125.25, 121.33, 114.00, 113.78, 111.99, 108.02, 95.73, 60.18, 54.79, 48.08, 46.76, 39.21, 32.44, 30.01, 28.76, 23.16. MS (MALDI-TOF) Calcd for: $C_{28}H_{31}I_3N_6O_3S_3$ ([M+H]$^+$): 976.875, found: 976.961.

*Synthesis of the compound **1**:* Citraconic anhydride (0.14 mL, 0.3 mmol) was added dropwise into 10 mL anhydrous DMF solution of **2** (100 mg, 0.2 mmol). After reacting at room temperature overnight, the solvent was removed under vacuum. The crude product was purified by silica gel chromatography with the eluent of DCM : MeOH = 100 : 1 to get the compound **1** (72.53 mg, yield: 65%). ^{1}H NMR (400 MHz, DMSO-d6) δ 13.44 (s, 1H), 10.48 (s, 1H), 8.77 (d, *J* = 2.1 Hz, 1H), 8.72 (d, *J* = 8.0 Hz, 1H), 8.45 (s, 1H), 8.27 (d, *J* = 2.0 Hz, 1H), 8.23 (d, *J* = 9.0 Hz, 1H), 7.81 (dd, *J* = 9.0, 2.1 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 1H), 6.14 (t, *J* = 1.8 Hz, 1H), 4.65 (td, *J* = 8.4, 4.8 Hz, 1H), 4.46 (td, *J* = 8.0, 5.6 Hz, 1H), 3.20 (d, *J* = 3.6 Hz, 2H), 3.17 (dd, *J* = 8.4, 4.5 Hz, 1H), 3.00 (dd, *J* = 13.0, 8.8 Hz, 1H), 1.99 (d, *J* = 1.6 Hz, 3H), 1.94–1.60 (m, 2H), 1.59–1.52 (m, 2H), 1.38 (s, 2H), 1.30 (s, 9H). MS (ESI) Calcd for: $C_{33}H_{35}I_3N_6O_6S_3$ ([M-H]$^-$): 1086.891, found: 1086.810.

*Synthesis of compound **1-Scr**:* Acetic anhydride (0.15 mL, 0.3 mmol) and triethylamine (0.29 mL, 0.3 mmol) were added dropwise into 10 mL anhydrous DMF solution of **2** (100 mg 0.2 mmol). After reacting at room temperature overnight, the solvent was removed under vacuum. The crude product was purified by silica gel chromatography with the eluent of DCM : MeOH = 100 : 1 to get the compound **1-Scr** (73.08 mg, yield: 70%). ^{1}H NMR (400 MHz, DMSO-d6) δ 10.50 (s, 1H), 8.78 (d, *J* = 2.1 Hz, 1H), 8.45 (t, *J* = 5.6 Hz, 1H), 8.34 (d, *J* = 7.6 Hz, 1H), 8.31–8.25 (m, 2H), 8.23 (d, *J* = 9.0 Hz, 1H), 7.80 (dd, *J* = 9.0, 2.1 Hz, 1H), 7.51 (d, *J* = 2.0 Hz, 1H), 4.59 (td, *J* = 8.4, 4.9 Hz, 1H), 4.46 (q, *J* = 7.8 Hz, 1H), 3.20 (q, *J* = 6.6 Hz, 2H), 3.13 (dd, *J* = 12.9, 4.9 Hz, 1H), 2.97 (dd, *J* = 12.9, 8.9 Hz, 1H), 1.92 (s, 3H), 1.89–1.66 (m, 2H), 1.56 (q, *J* = 7.0 Hz, 2H), 1.52–1.31 (m, 2H), 1.30 (s, 9H). ^{13}C NMR (101 MHz, DMSO) δ 170.99, 170.00, 169.65, 167.77, 147.66, 147.58, 145.85, 139.27, 136.66, 135.05, 134.37, 124.79, 120.81, 113.55, 113.33, 111.41, 107.53, 95.29, 53.71, 52.31, 47.68, 42.63, 38.78, 31.40, 29.50, 28.34, 22.82, 22.57. ^{13}C NMR (101 MHz, DMSO-d6) δ 170.99, 170.00, 169.65, 167.77, 147.66, 147.58, 145.85, 139.27, 136.66, 135.05, 134.37, 124.79, 120.81, 113.55, 113.33, 111.41, 107.53, 95.29, 53.71, 52.31, 47.68, 42.63, 38.78, 31.40, 29.50, 28.34, 22.82, 22.57. MS (MALDI-TOF) Calcd for: $C_{30}H_{33}I_3N_6O_4S_3$ ([M+Na]$^+$): 1040.886, found: 1040.789.

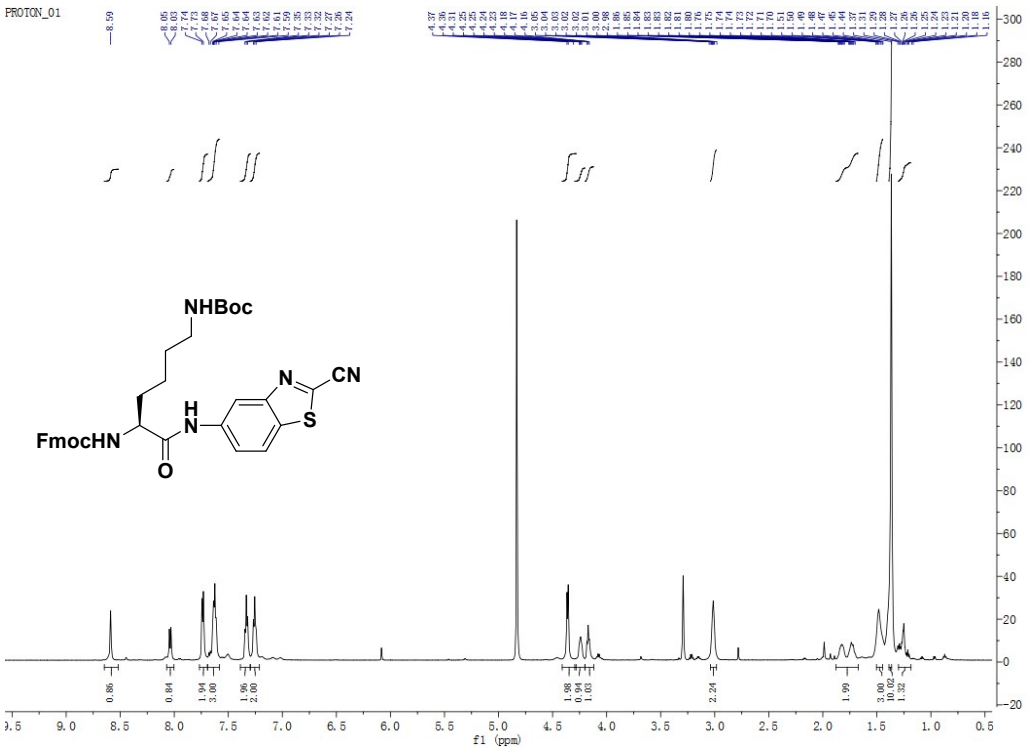


Fig. S1 ^{1}H NMR spectrum of **7**.

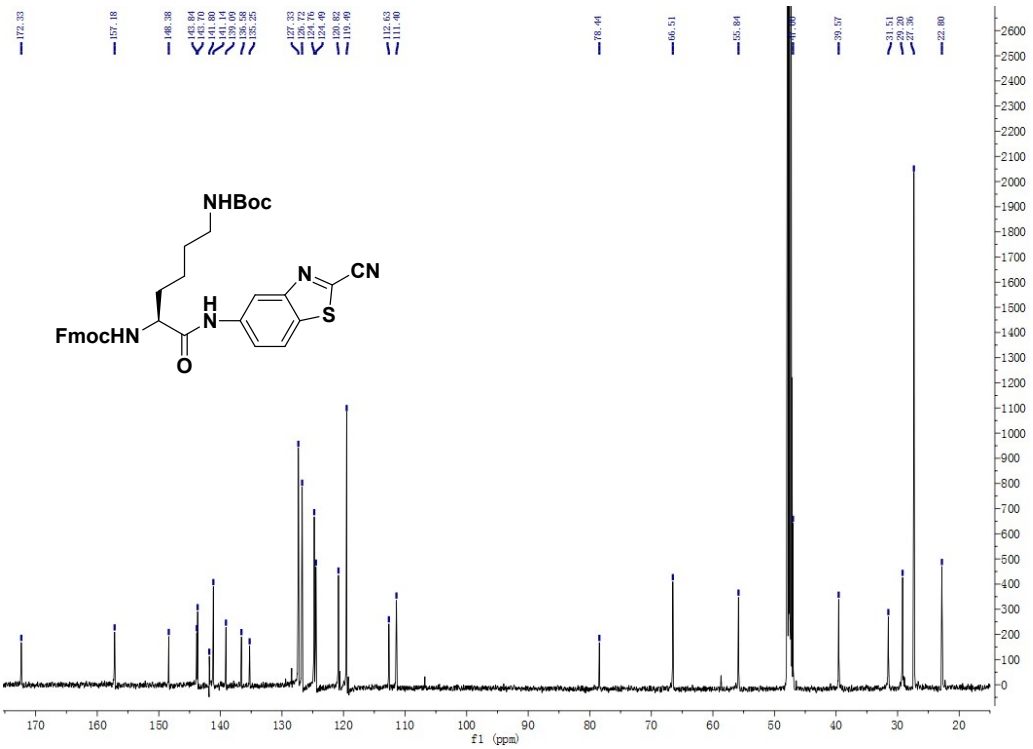


Fig. S2 ^{13}C NMR spectrum of **7**.

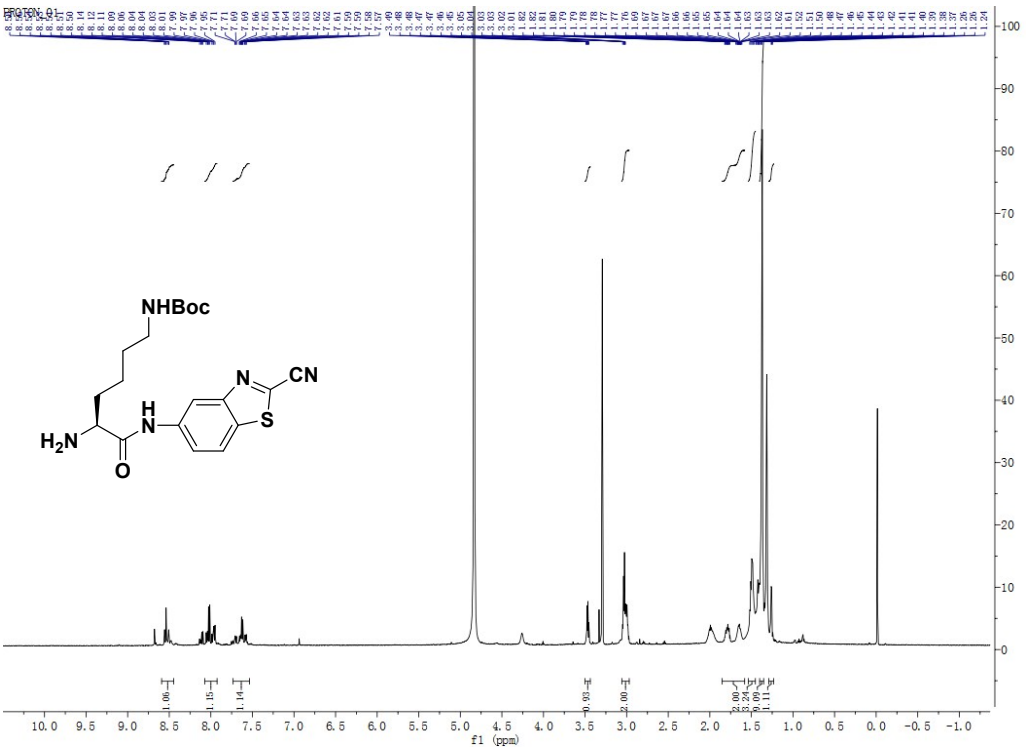


Fig. S3 ^{1}H NMR spectrum of **6**.

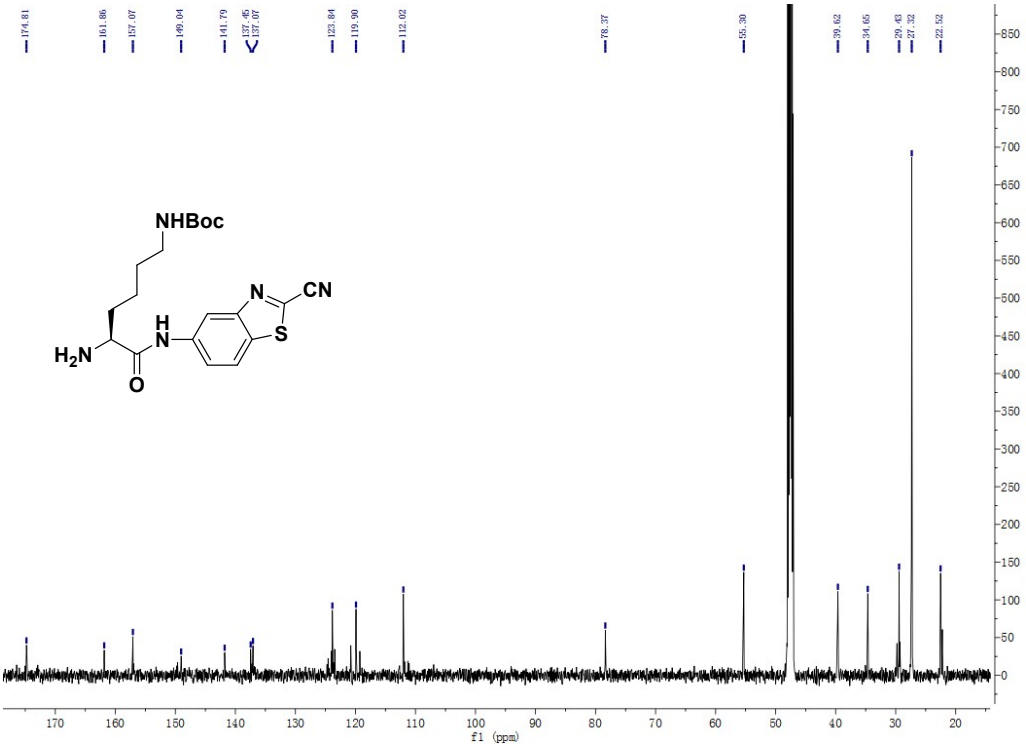


Fig. S4 ^{13}C NMR spectrum of **6**.

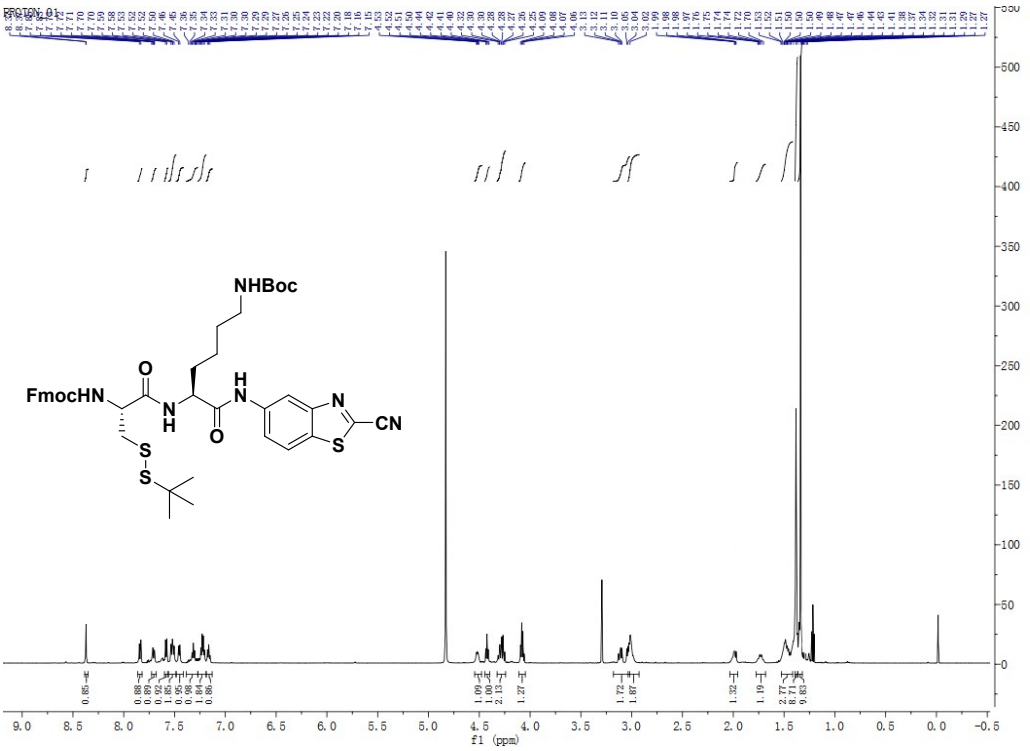


Fig. S5 ^{1}H NMR spectrum of **5**.

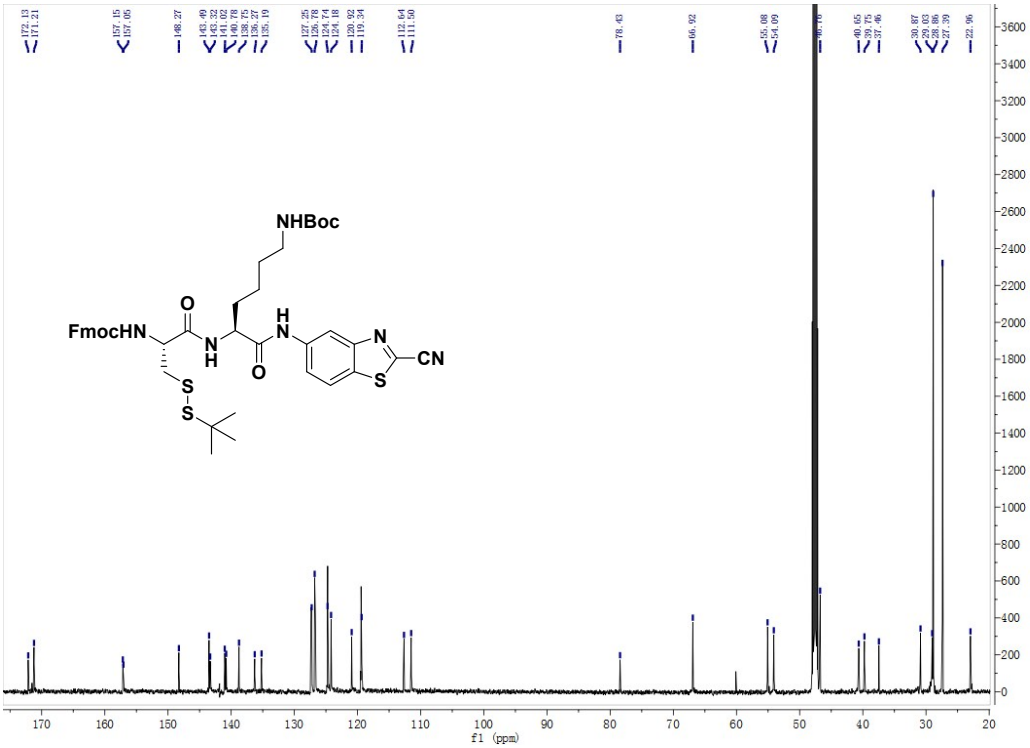


Fig. S6 ^{13}C NMR spectrum of **5**.

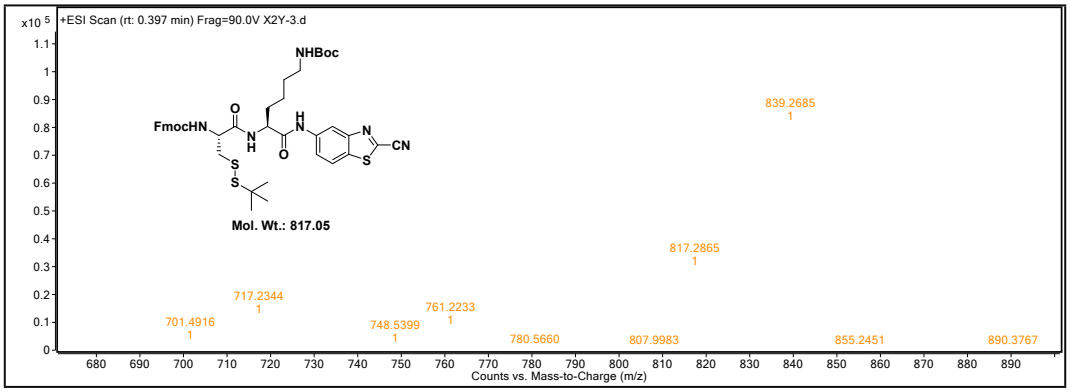


Fig. S7 ESI-MS spectrum of compound **5**.

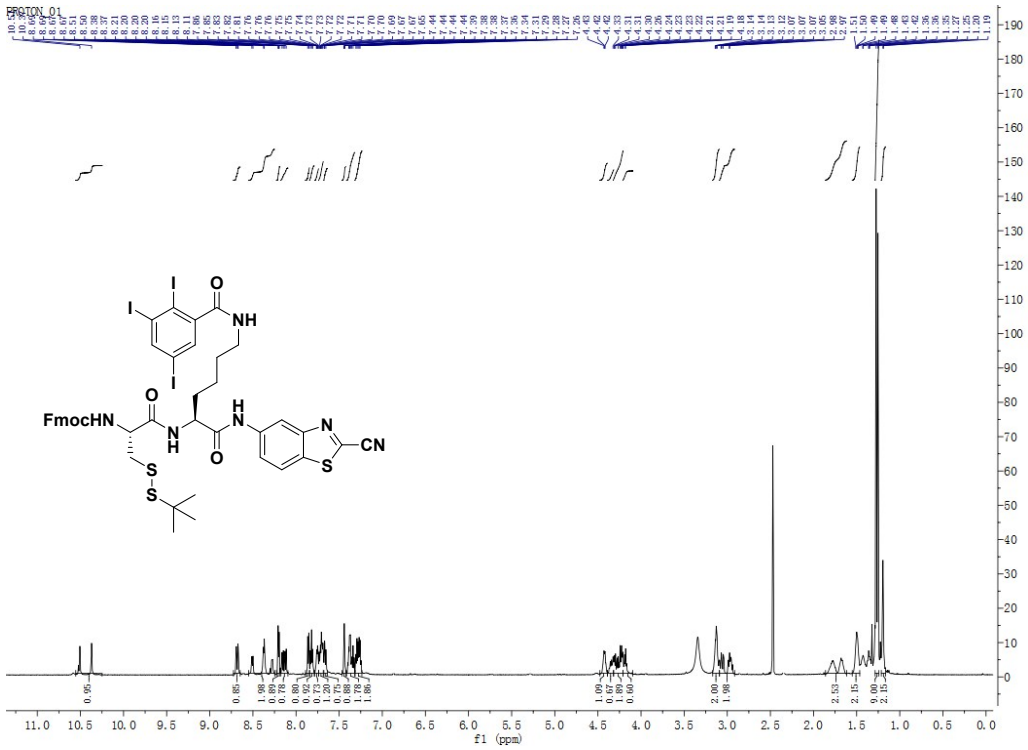


Fig. S8 ^{1}H NMR spectrum of **3**.

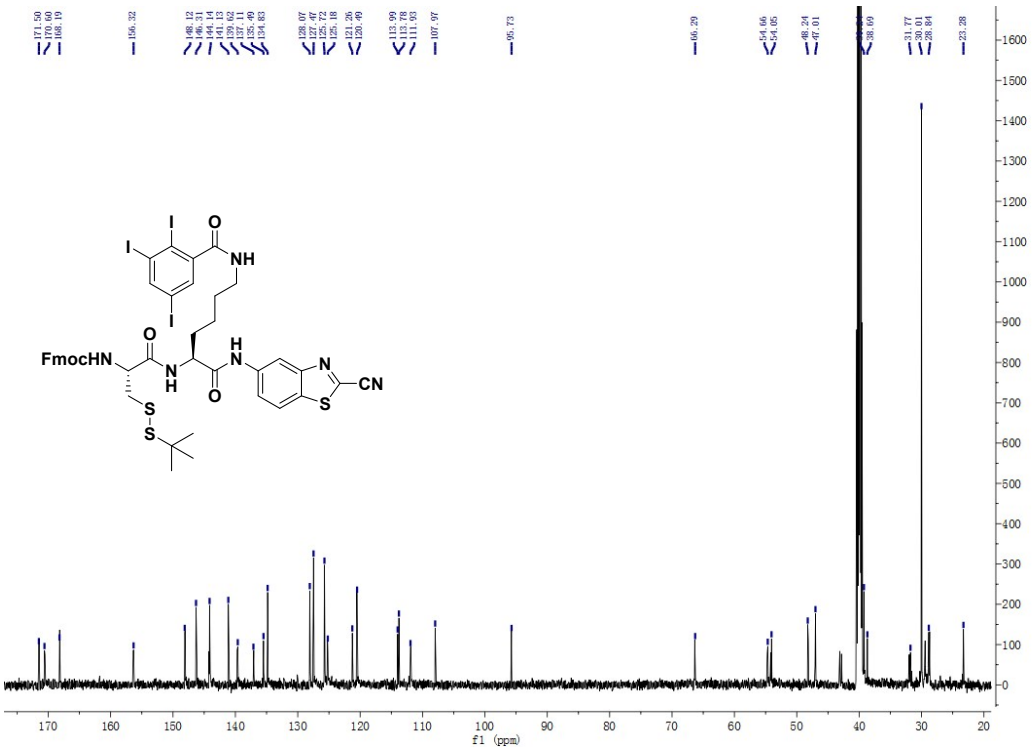


Fig. S9 ^{13}C NMR spectrum of **3**.

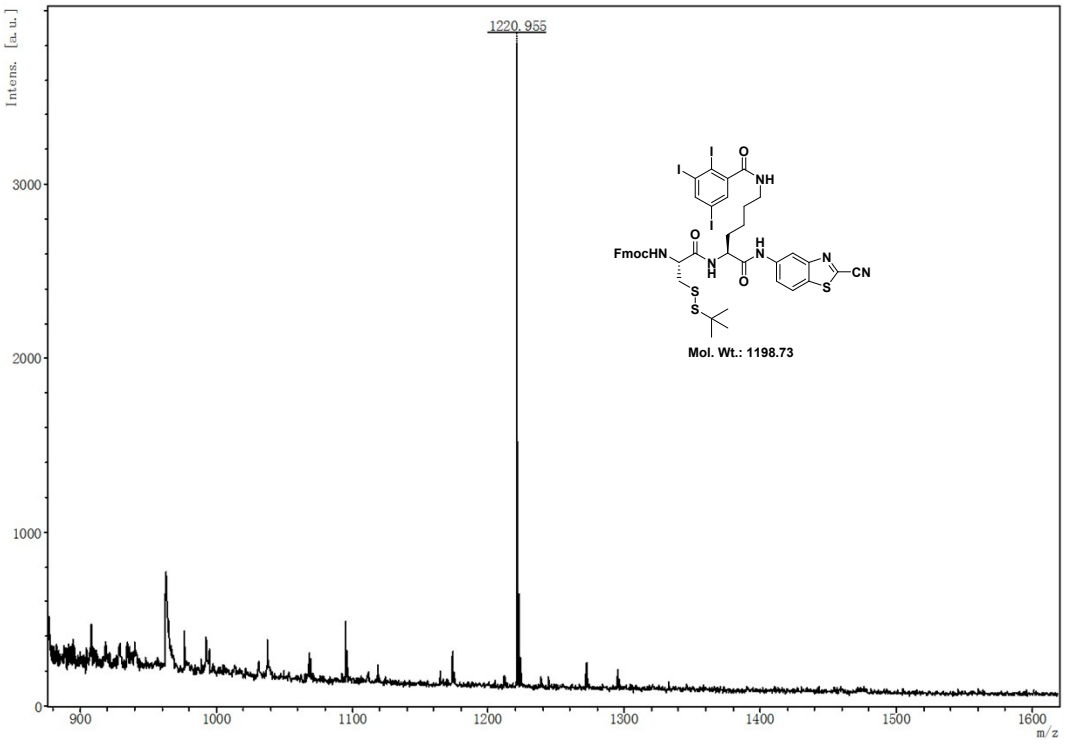


Fig. S10 MALDI-TOF/MS spectrum of compound **3**.

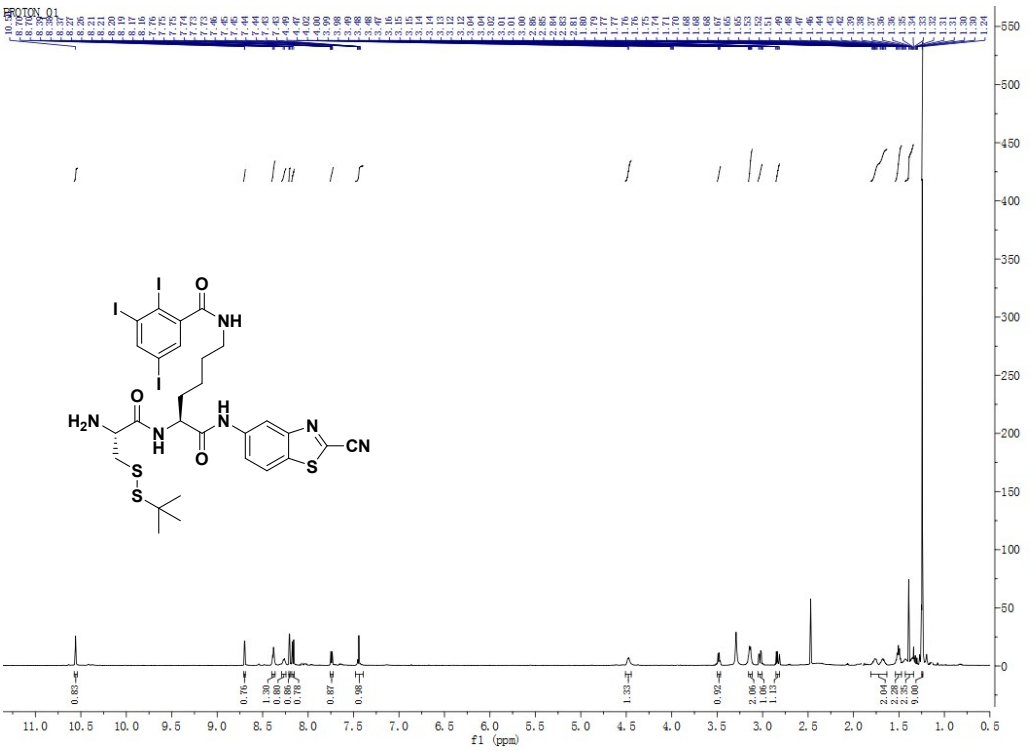


Fig. S11 ^{1}H NMR spectrum of **2**.

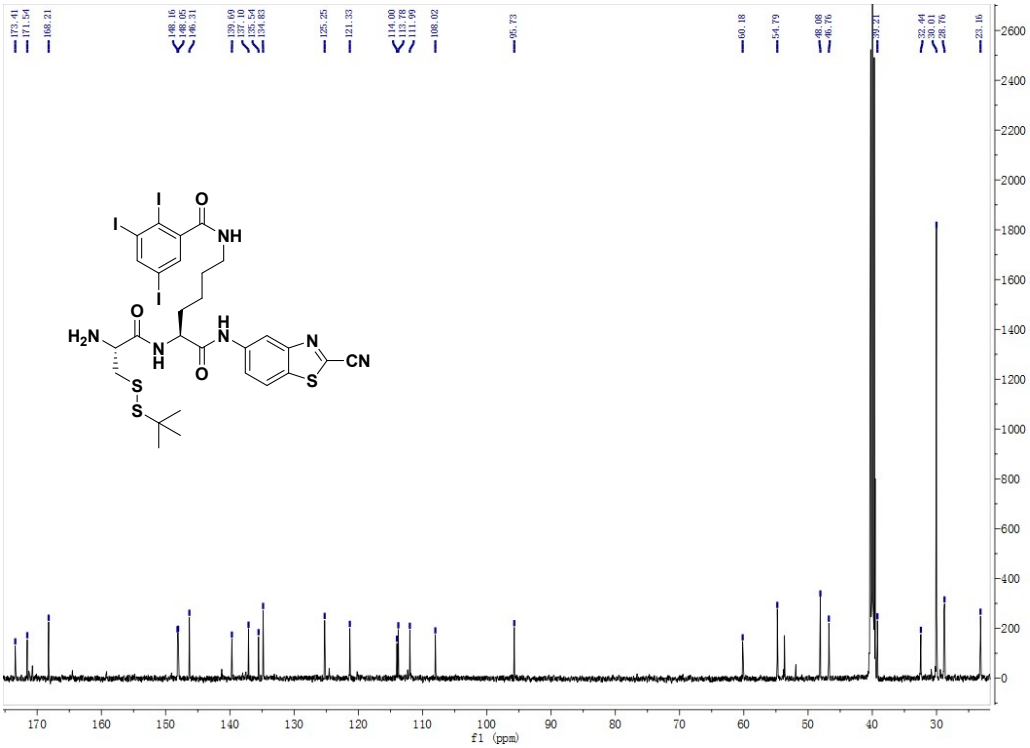


Fig. S12 ^{13}C NMR spectrum of **2**.

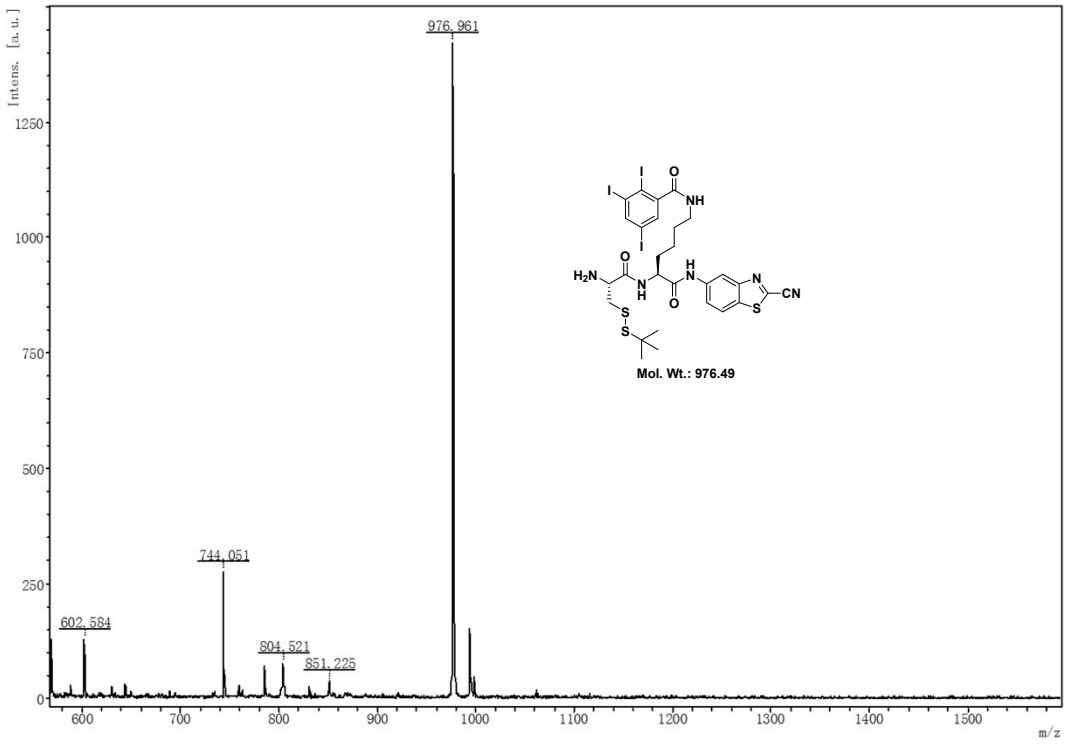


Fig. S13 MALDI-TOF/MS spectrum of compound **2**.

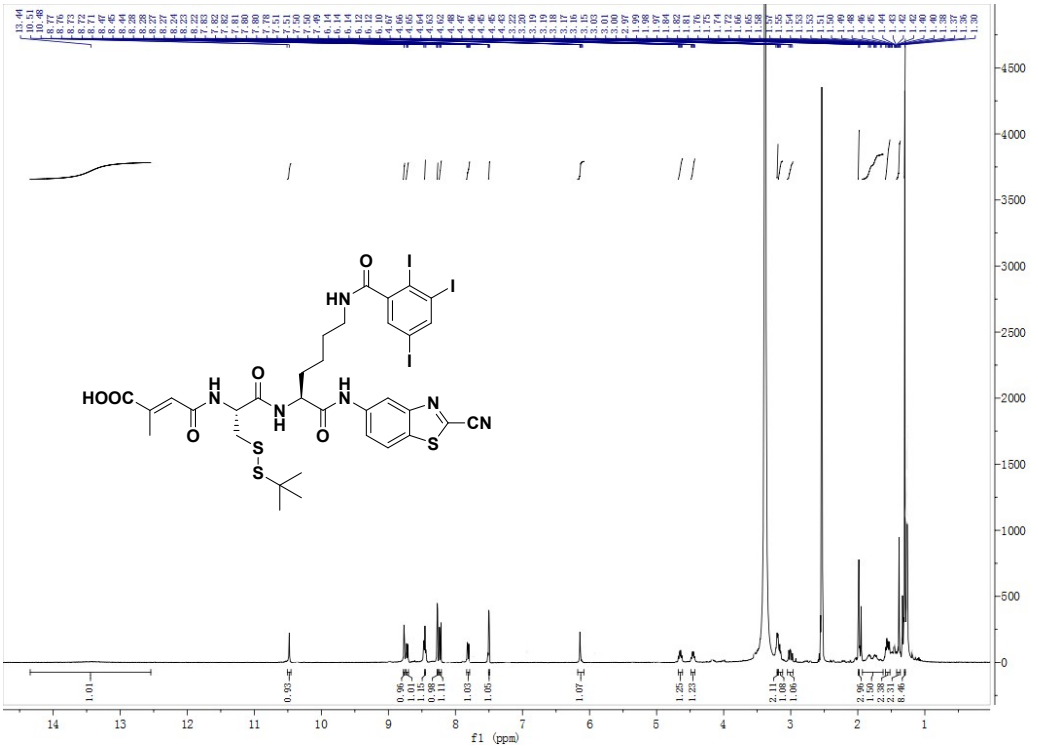


Fig. S14 ^{1}H NMR spectrum of **1**.

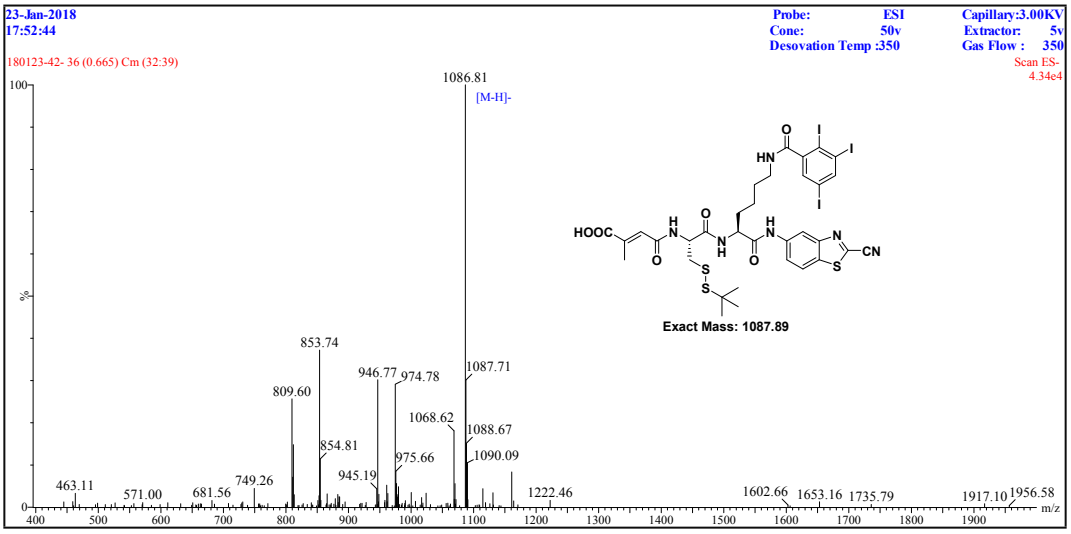


Fig. S15 ESI-MS spectrum of compound **1**.

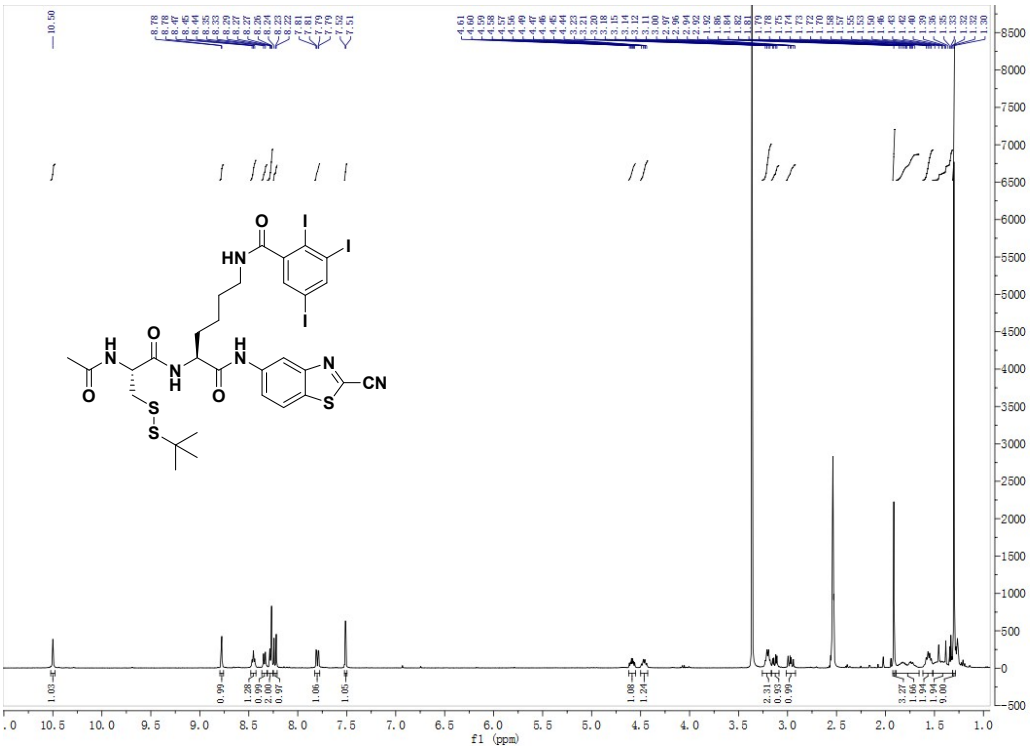


Fig. S16 ^{1}H NMR spectrum of **1-Scr**.

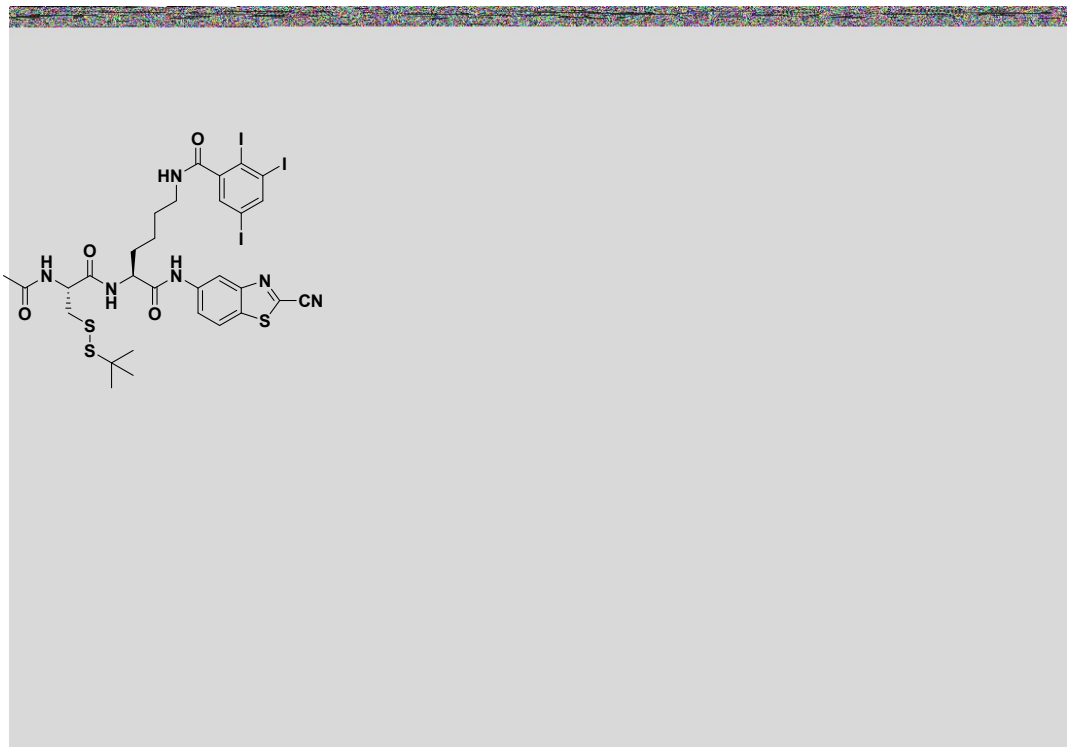


Fig. S17 ^{13}C NMR spectrum of **1-Scr**.

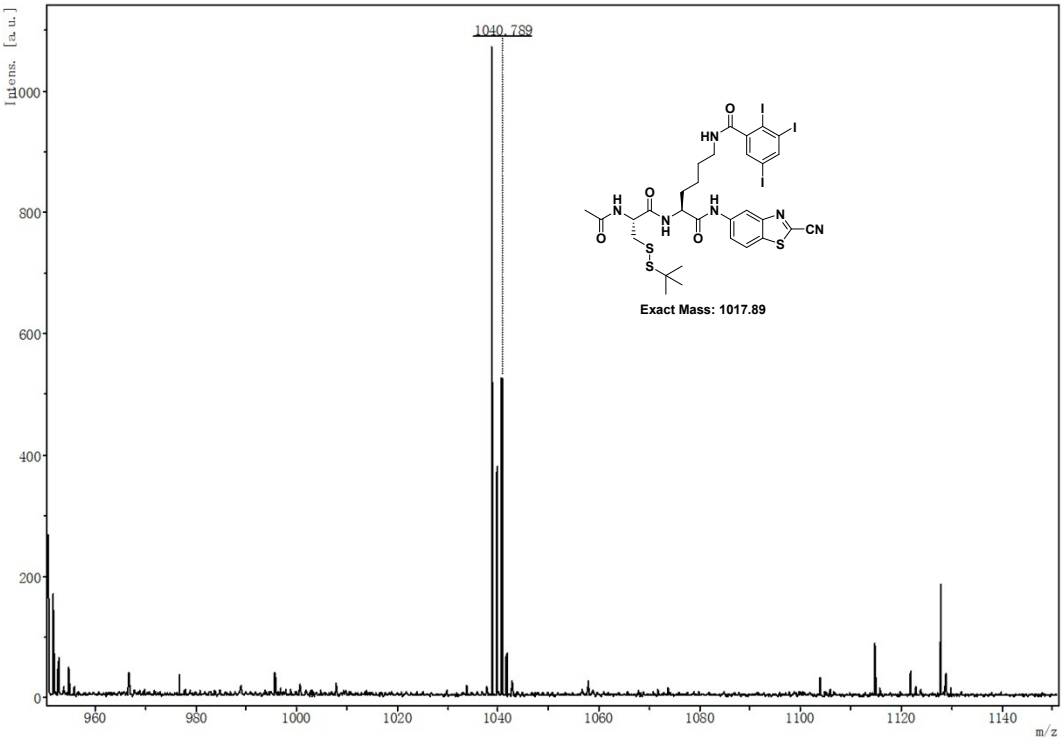


Fig. S18 MALDI-TOF/MS spectrum of compound **1-Scr**.

3. Supporting Figures and Tables

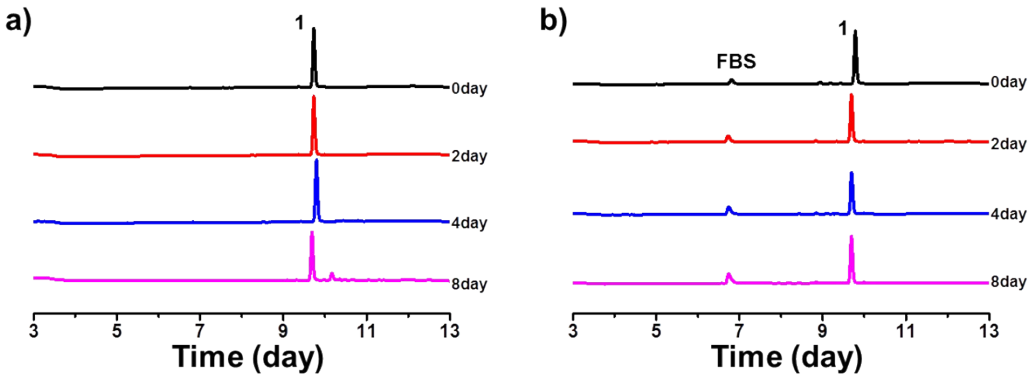


Fig. S19 HPLC spectra of probe **1** in PBS (pH 7.0) (a) and FBS (b) for 8 days.

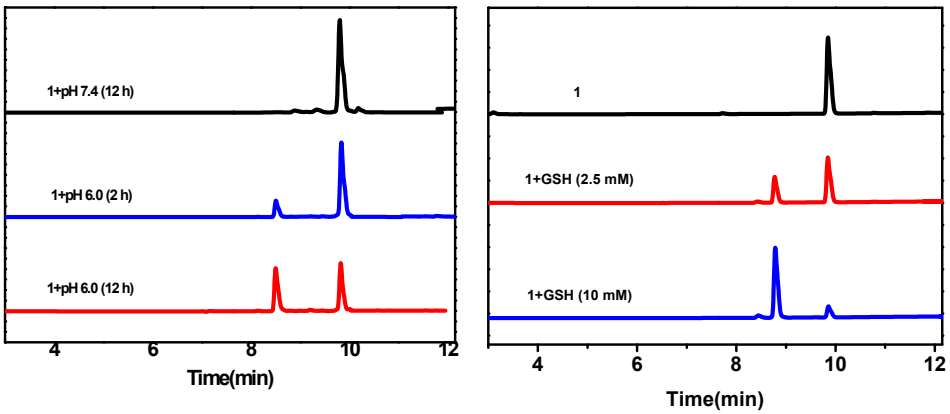
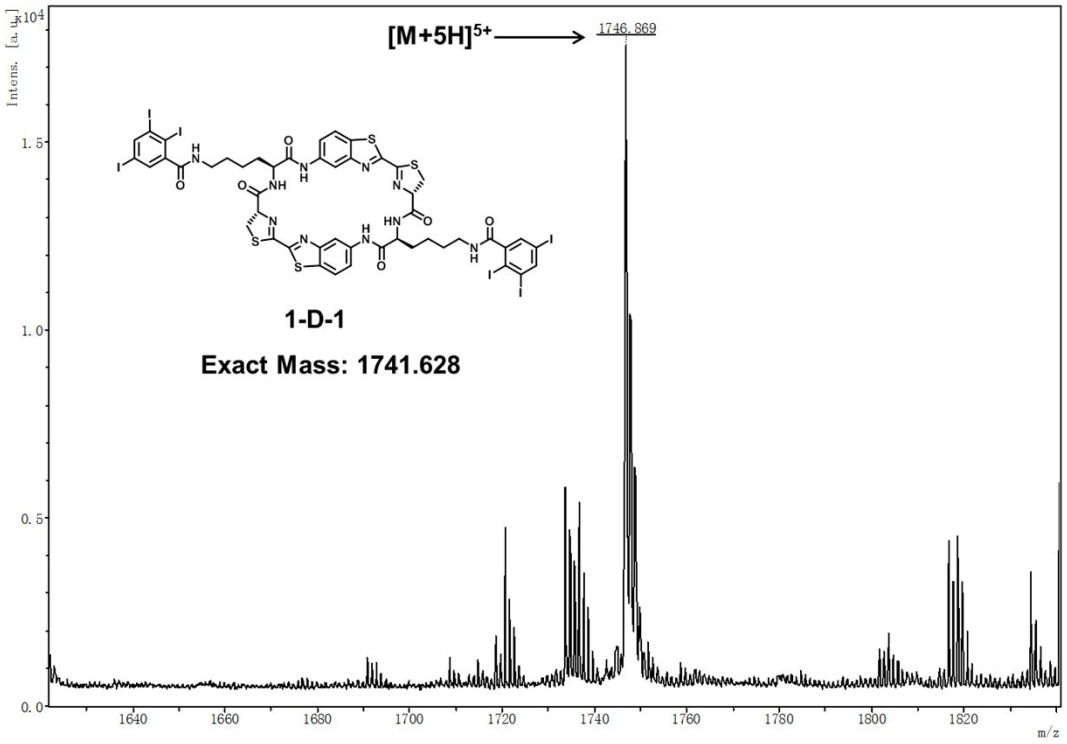
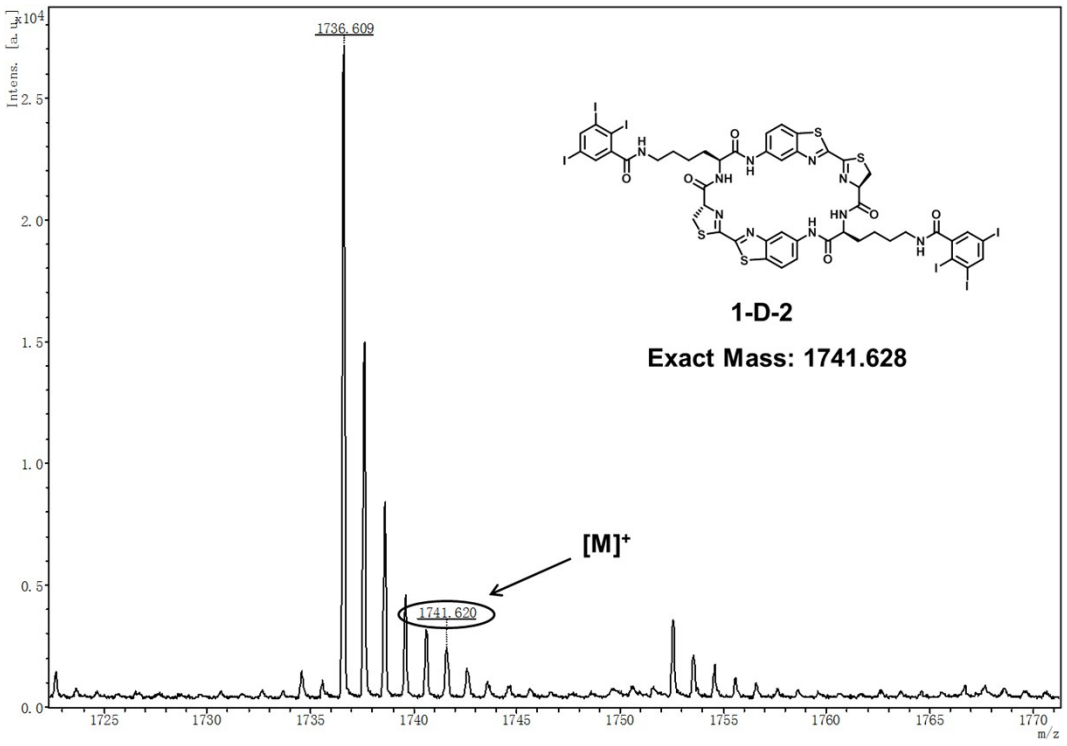


Fig. S20 (a) HPLC trace of the reaction of probe **1** in PBS buffer (pH 7.4) at 37 °C for 12 h (black), probe **1** in PBS buffer (pH 6.0) for 2 h (blue) and 12 h (red), respectively. (b) HPLC trace of the solutions of probe **1** (black), probe **1** + 2.5 mM GSH (red), probe **1** + 10 mM GSH (blue) in PBS buffer (pH 7.4) after 1 h incubation. Absorbance wavelength: 350 nm.


[M+5H]5+
1746.869
1-D-1
Exact Mass: 1741.628
Intens. [a.u.]
m/z


1736.609
1-D-2
Exact Mass: 1741.628
[M]+
1741.620
Intens. [a.u.]
m/z

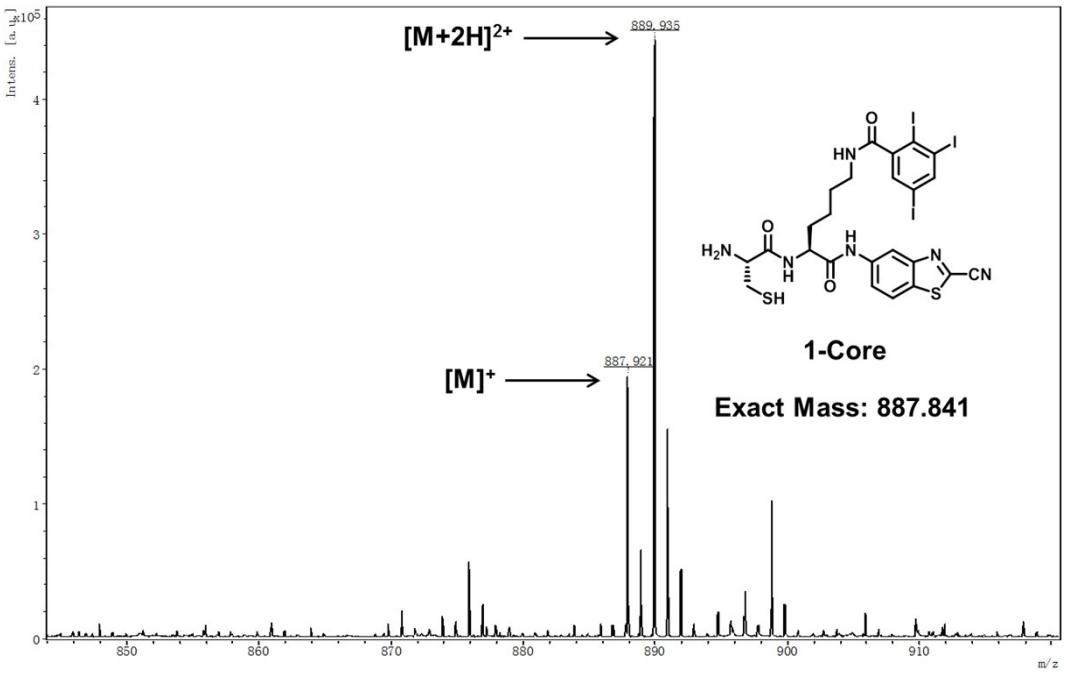


Fig. S21 MALDI mass spectra of HPLC peaks at 6.19 min (**1-D-1**), 7.64 min (**1-D-2**) and 5.89 min (**1-Core**) in Fig. 1b respectively.

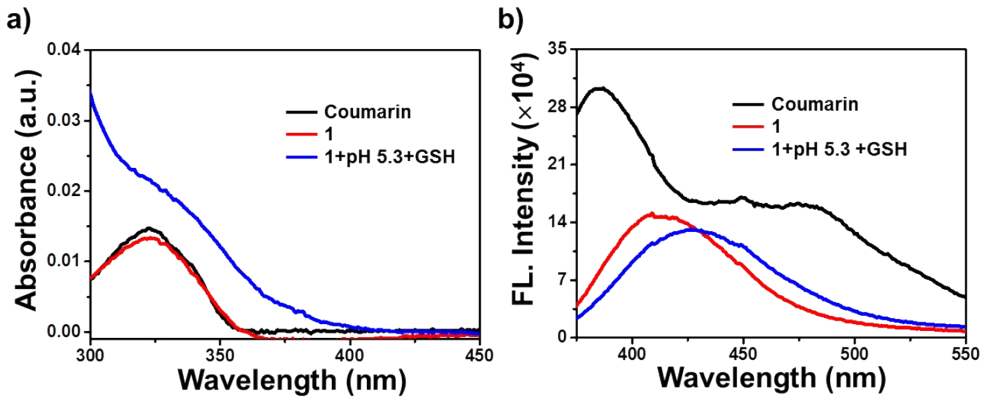


Fig. S22 (a) Absorption spectra of coumarin (black), **1** before (red) and after CBT condensation (blue) in methanol. (b) Fluorescence spectra of coumarin (black), **1** before (red) and after CBT condensation (blue) in methanol. λ_{ex}: 325 nm.

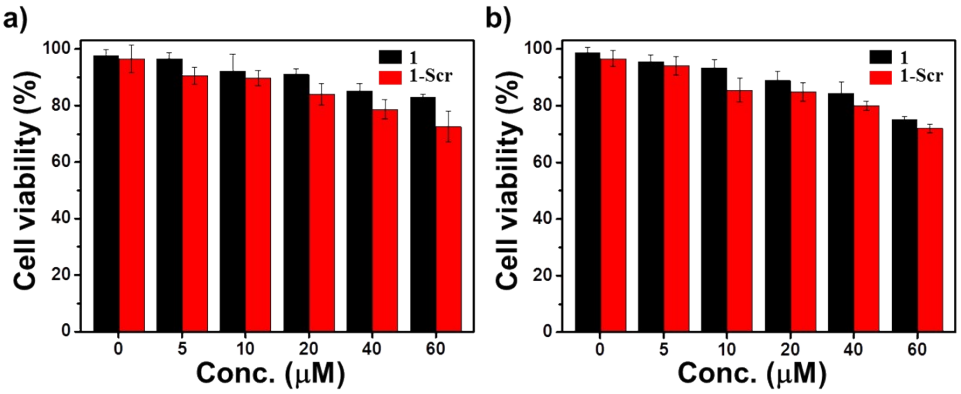


Fig. S23 Cell viability of 3T3 (a) and 4T1 (b) cells incubated with different concentrations of **1** and **1-Scr** for 24 h.

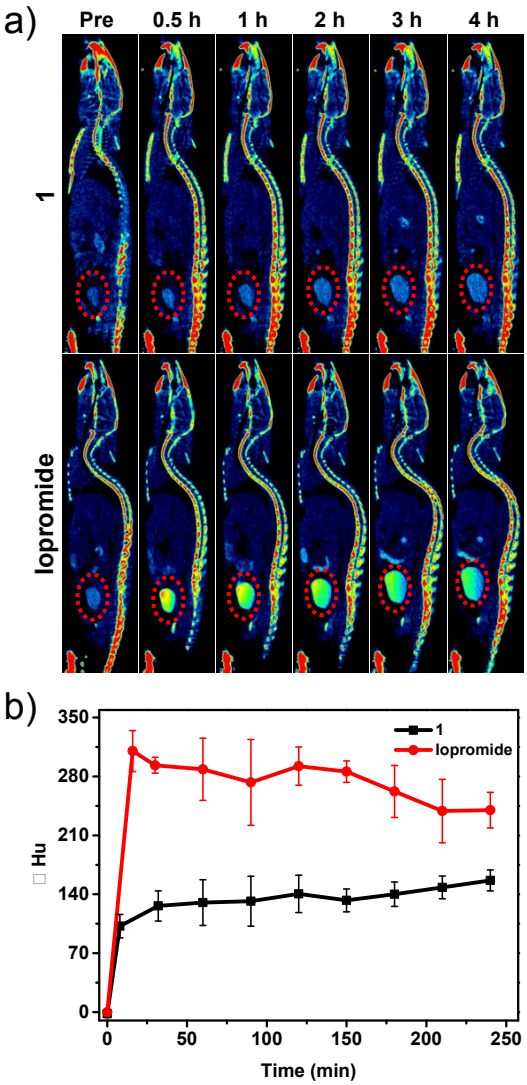


Fig. S24 Representative CT images (a) and dynamic contrast enhanced density (ΔHu) (b) of of bladders at different time intervals after intravenous injection of probe **1** and Iopromide via tail veins. The injected dose was 43.58 mg I/kg for both agents.

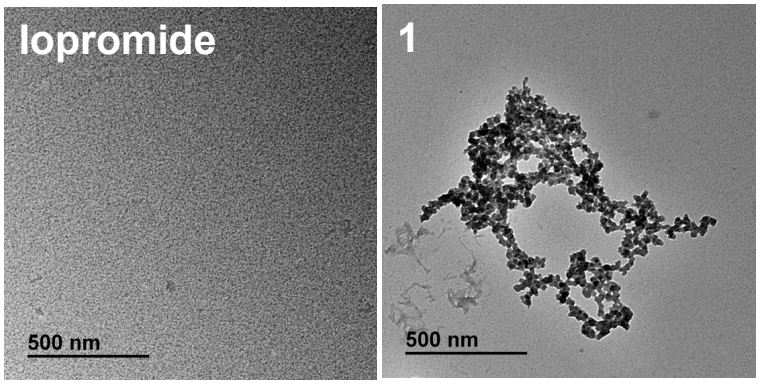


Fig. S25 TEM images of Iopromide (left) and probe **1** (right).

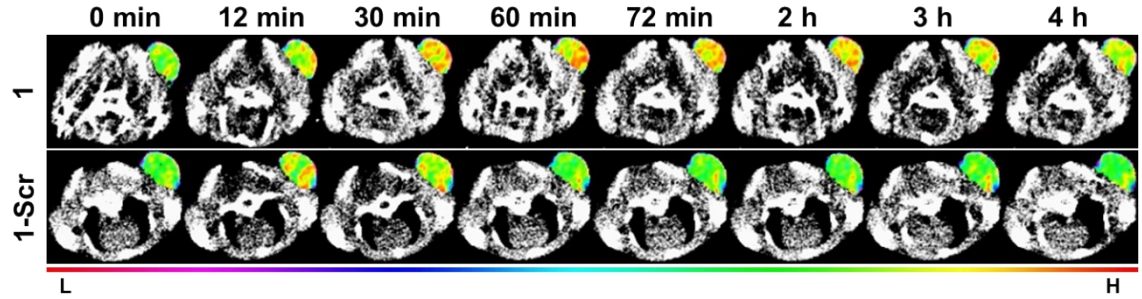


Fig. S26 Representative CT images of mice with subcutaneously xenografted 4T1 tumours at 0 min, 12 min, 30 min, 60 min, 72 min, 90 min, 2 h, 3 h and 4 h after intravenous injections of **1** (upper) or **1-Scr** (lower) via tail veins.