Supporting Information for

Original article

Pretheranostic agents with extraordinary NIRF/photoacoustic imaging performance and photothermal oncotherapy efficacy

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Received 6 April 2024; received in revised form 19 June 2024; accepted 16 July 2024

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1. Materials and apparatus

The raw materials used in organic synthesis are purchased from Macklin, Bidepharmatech and Aladdin, and all raw materials do not require further purification. amino acid and metal-ion reagents were purchased from Macklin. The pH of the solution was determined with the pH detector (ST 3100, OHAUS). Glass bottom cell culture dish (Φ20 mm, TC) was purchased from Nest. N-Ethylmaleimide was purchased from Shanghai Taoshu Biotechnology Co., Ltd. SuperKind ™ Hypersensitive cell proliferation detection reagent (CCK-8) was purchased from Guangzhou Sishijia Biotechnology Co., Ltd. nude mice (6-week-old) were purchased from Guangzhou Southern Medical University Laboratory Animal Technology Development Co., Ltd. (Guangzhou, China), and used according to the regulation of the Local Institutional Animal Care and Use Committee (IACUC). ^{1}H NMR and ^{13}C NMR spectra were obtained on a 400 MHz nuclear magnetic resonance instrument (AVANCE III 400, Bruker), and the mass spectra and liquid phase spectra were obtained on triple quadrupole liquid mass spectrometry (Prelude SPLC+TSQ Quantiva LC-MS/MS, Thermo Fisher Scientific). The absorption spectrum was measured with the UV-visible spectrophotometer (Hitachi U-3010, Japan), while the emission spectrum was obtained from the fluorescence spectrophotometer (FLS 980, Edinburgh), and the photoacoustic signal and intensity were measure from MSOT in Vision 128, iThera Medical Germany. The cytotoxicity was measured in a microplate reader (Multiskan FC, Thermo Fisher Scientific). Cell imaging was obtained on a 63X lens of the inverted confocal laser microscope (LSM 880 with AiryScan, Carl Zeiss). Animal imaging was obtained on the multi-mode small animal live imager (FX PRO, Bruker). Photothermal imaging were performed on 660 nm laser （RAL660T1-2.0W, Beijing Leiyuan Technology Co., Ltd）and photothermal images were recorded by an IR thermal camera (FLUKE-VT08/CN, Fluke Testing Instruments (Shanghai) Co., Ltd).

2. Synthesis of S-NBD

Synthesis of compound 2. Put 11.2 mL of DMF and 50 mL of $CHCl_3$ into a flask, slowly drip 12.4 mL of PBr_3 (130.5 mmol) at 0 ℃, stir for 45 min, and then add 5 mL of Cyclohexanone (48.4 mmol). After stirring at room temperature for 8 hours, the reaction solution was poured into 200 mL of ice water, and $NaHCO_3$ solid was added. The pH was adjusted to around 7. The mixture was extracted with CH_2Cl_2, and the organic phase was dried with anhydrous sodium sulfate. A silica gel chromatographic column was used to obtain 5.6 g of a yellow oil, with a yield of 61%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.91 (s, 1H), 2.66 (t, *J* = 6.2 Hz, 2H), 2.17 (t, *J* = 6.1 Hz, 2H), 1.68 (dd, *J* = 11.4, 5.9 Hz, 2H), 1.60 (q, *J* = 5.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 193.32, 143.46, 135.06, 38.66, 24.86, 24.13, 20.95. MS: m/z calculated for $C_7H_9BrO_3$ ($[M-H]^+$):188.9; found:188.9.

Synthesis of compound 3. 204 mg of compound 2 (1.08 mmol) was dissolved in 6 mL of DMF, 180 mg of 4-bromo-2-hydroxybenzaldehyde (0.90 mmol) and 880 mg of Cs_2CO_3 (2.70 mmol) were weighed into the solution, stirred at room temperature for 16 h, the reaction solution was filtered, extracted and passed through a silica gel chromatographic column, and 132 mg of the compound was purified, with a yield of 42%.^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.30 (s, 1H), 7.27 (s, 1H), 7.20 (d, *J* = 8.1 Hz, 1H), 7.01 (d, *J* = 8.1 Hz, 1H), 6.62 (s, 1H), 2.60 – 2.54 (m, 2H), 2.44 (t, *J* = 6.0 Hz, 2H), 1.72 (p, *J* = 6.1 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 187.75, 159.48, 152.23, 130.20, 127.46, 126.90, 125.62, 122.89, 120.17, 118.69, 113.73, 30.07, 21.37, 20.11. MS: m/z calculated for $C_{14}H_{11}BrO_2$ ($[M-H]^+$):290.9; found:290.9.

Synthesis of compound 4. Dissolve 100 mg of compound 3 (0.34 mmol) in 20 mL of 1,4-dioxane, weigh 4-methoxy-N-phenylaniline (100 mg, 0.5 mmol), $Pd_2(dba)_3$ (46 mg, 0.05 mmol), x-phos (74 mg) and Cs_2CO_3 (277 mg, 0.85 mmol) into the solution, heat and reflux at 100 ℃ for 16 h under nitrogen protection. After filtering the reaction solution, a silica gel chromatographic column was used to purify 82 mg of the compound, with a yield of 58%.^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.17 (s, 1H),

7.30 (d, *J* = 7.4 Hz, 1H), 7.09 (q, *J* = 9.7, 8.8 Hz, 6H), 6.95 (d, *J* = 8.1 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, 2H), 6.65 (t, *J* = 10.3 Hz, 3H), 3.82 (s, 3H), 2.58 – 2.53 (m, 2H), 2.42 (t, *J* = 5.6 Hz, 2H), 1.69 (p, *J* = 6.4 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 187.46, 160.85, 156.97, 153.10, 150.14, 146.75, 139.40, 129.39, 127.93, 126.94, 126.88, 126.14, 124.70, 123.73, 116.07, 114.98, 114.62, 112.26, 106.44, 55.44, 29.94, 21.49, 20.47. MS: m/z calculated for $C_{27}H_{23}NO_3$ ($[M-H]^+$):410.1;found:410.1.

Synthesis of compound 5. 500 mg of compound 4 (1.22 mmol) was dissolved in 20 mL of dichloromethane solution, PBr_3 2.27 mL (24.4 mmol) was slowly added dropwise in an ice bath, stirred for 4 h, and finally 454 mg of compound was obtained by silica gel chromatography column, with a yield of 94%.^{1}H NMR (400 MHz, DMSO-d_6) δ 10.09 (s, 1H), 9.55 (s, 1H), 7.34 (t, *J* = 7.8 Hz, 2H), 7.17 (d, *J* = 9.1 Hz, 1H), 7.11 (d, *J* = 5.2 Hz, 2H), 7.08 (s, 1H), 7.00 (d, *J* = 8.3 Hz, 2H), 6.91 (s, 1H), 6.81 (d, *J* = 8.4 Hz, 2H), 6.55 (s, 2H), 2.54 (s, 2H), 2.27 (t, *J* = 5.5 Hz, 2H), 1.61 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 186.52, 160.53, 155.72, 152.84, 150.37, 146.75, 137.45, 130.02, 128.91, 128.07, 127.64, 125.62, 124.96, 124.26, 117.00, 115.39, 114.20, 111.76, 105.07, 29.44, 21.67, 20.44. MS: m/z calculated for $C_{26}H_{21}NO_3$ ($[M-H]^+$): 396.1;found:396.1.

Synthesis of compound 7. Dissolve 8 mL (50 mmol) of isophorone, 3.96 g (60 mmol) of Malononitrile and 0.54 mL (5 mmol) of piperidine in 50 mL of anhydrous acetonitrile. Heat and reflux at 81℃ for 12 hours under nitrogen protection conditions. After passing through a silica gel chromatographic column, the compound was recrystallized with n-hexane to obtain 4.9 g of compound with a yield of 74%.^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.61 (s, 1H), 2.51 (s, 2H), 2.17 (s, 2H), 2.03 (s, 3H), 1.01 (s, 6H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 170.34, 159.74, 120.51, 113.13, 112.35, 45.60, 42.56, 32.31, 27.75, 25.27. MS: m/z calculated for $C_{12}H_{14}N_2$ ($[M-H]^+$): 187.1;found:187.1.

Synthesis of Compound 8（S-OH）. Compoud 5 (200 mg, 0.5 mmol), compund 7（113 mg, 0.6 mmol）and 100 μL piperidine were dissolved in 15 ml acetonitrile, heated at 81°C in the nitrogen atmosphere and refluxed for 10 hours, the organic phase

was concentrated to obtain 103 mg of purple product with a yield of 36%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.56 (s, 1H), 7.49 (d, J = 15.5 Hz, 1H), 7.29 (t, J = 7.6 Hz, 2H), 7.07 (d, J = 8.4 Hz, 1H), 7.05 (s, 1H), 7.01 (d, J = 8.0 Hz, 2H), 6.96 (d, J = 8.5 Hz, 2H), 6.79 (d, J = 8.5 Hz, 2H), 6.65 (d, J = 11.6 Hz, 3H), 6.59 – 6.47 (m, 2H), 2.51 (s, 4H), 2.43 (s, 4H), 1.79 – 1.60 (m, 2H), 0.94 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.49, 157.41, 155.49, 153.58, 151.75, 149.66, 147.21, 137.76, 132.80, 129.86, 128.52, 127.71, 127.50, 126.07, 123.82, 123.40, 120.93, 116.91, 115.87, 115.15, 112.10, 106.71, 72.44, 55.32, 42.70, 38.51, 32.00, 29.39, 27.74, 24.69, 20.82. MS: m/z calculated for $C_{38}H_{33}N_3O_2$ ([M-H]$^-$):562.2; found:562.2.

Synthesis of probe 9 (S-NBD). 200 mg (0.36 mmol) of S-OH was dissolved in 10 mL of dichloromethane solution. 85 mg (0.43 mmol) of 4-chloro-7-nitro-2,1,3-benzoxadiazole was added under 0 ℃ ice bath conditions, followed by 3-5 drops of triethylamine. Stirring for 2 hours, 121 mg of product was obtained through a silica gel column with a yield of 47%.^{1}H NMR (400 MHz, DMSO-d_6) δ 8.62 (d, J = 8.4 Hz, 1H), 7.53 – 7.47 (m, 1H), 7.40 – 7.31 (m, 4H), 7.16 (dq, J = 21.1, 8.1, 6.5 Hz, 6H), 6.85 (d, J = 8.6 Hz, 2H), 6.67 (d, J = 11.9 Hz, 3H), 6.58 (s, 1H), 2.51(s,4H), 2.42 (s, 4H), 1.69 (s, 2H), 0.94 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.61, 157.26, 153.93, 153.61, 151.28, 148.59, 148.05, 146.71, 145.75, 145.50, 144.72, 135.85, 132.95, 130.55, 130.26, 129.25, 127.79, 126.14, 125.18, 124.67, 122.62, 122.52, 121.30, 118.90, 117.60, 115.05, 114.19, 112.48, 109.81, 109.31, 42.72, 42.70, 38.49, 31.99, 29.49, 27.74, 20.76. MS: m/z calculated for $C_{44}H_{34}N_6O_5$ ([M-H]$^-$):725.3; found: 725.3.

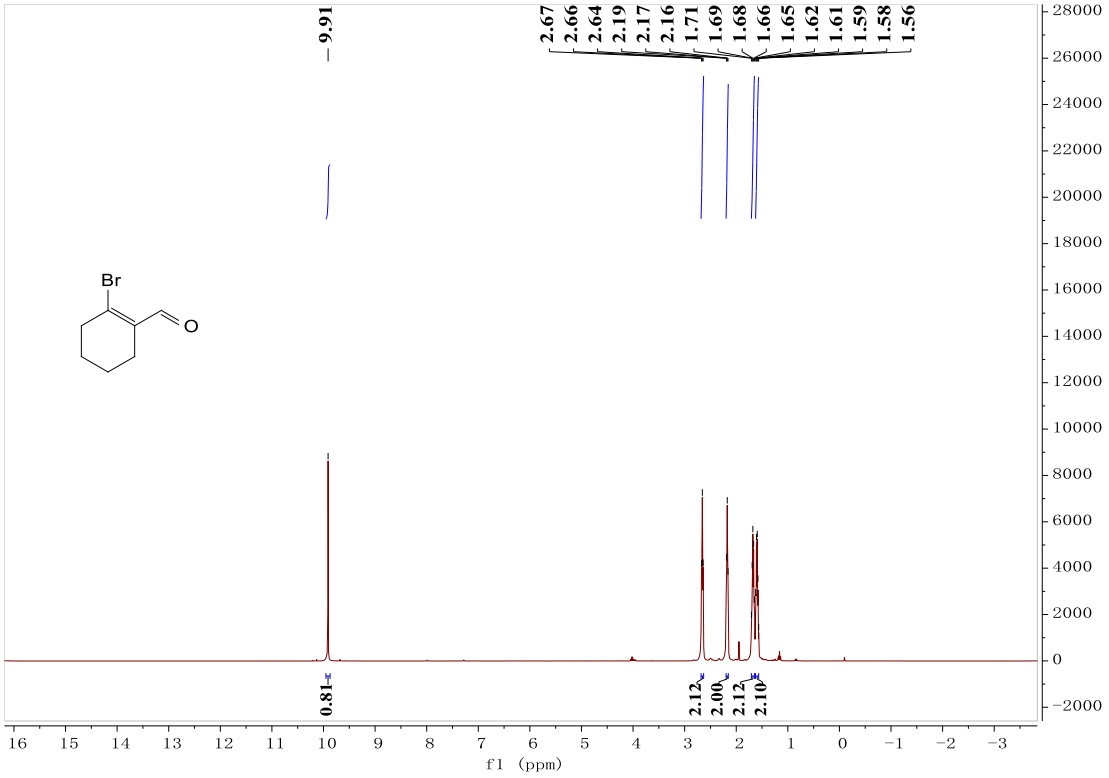

Figure S1 ^{1}H NMR spectra of Compound 2 in $CDCl_3$.

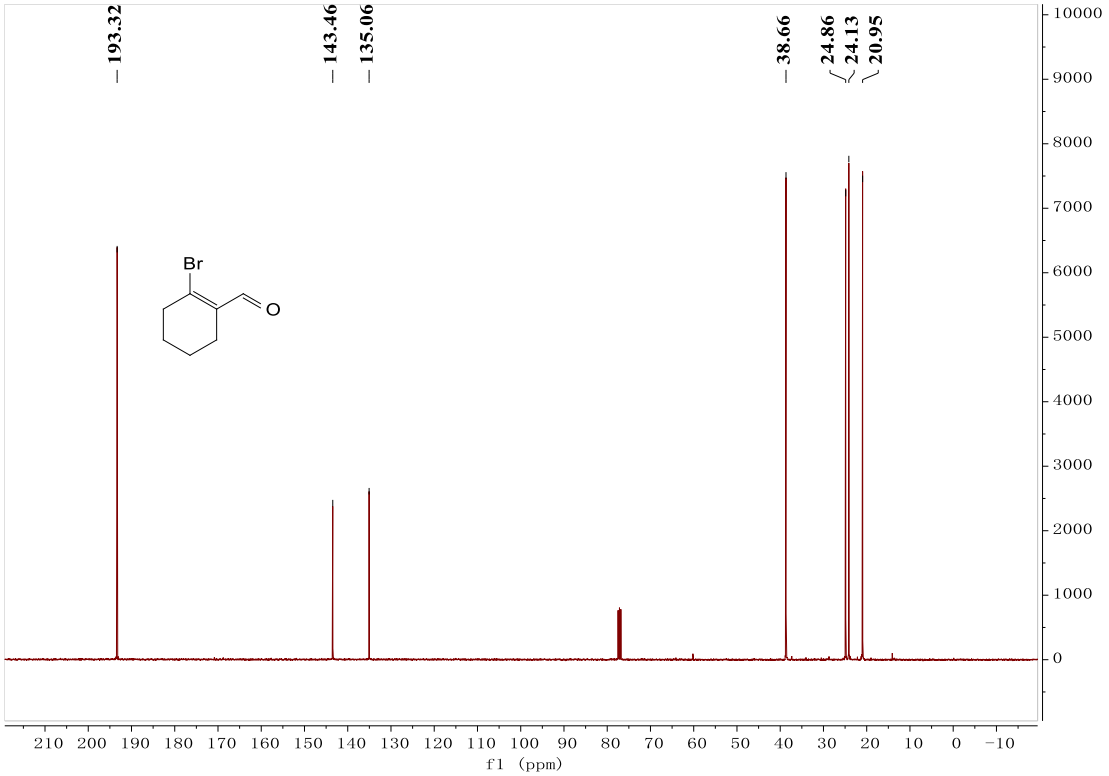

Figure S2 ^{13}C NMR spectra of Compound 2 in $CDCl_3$.

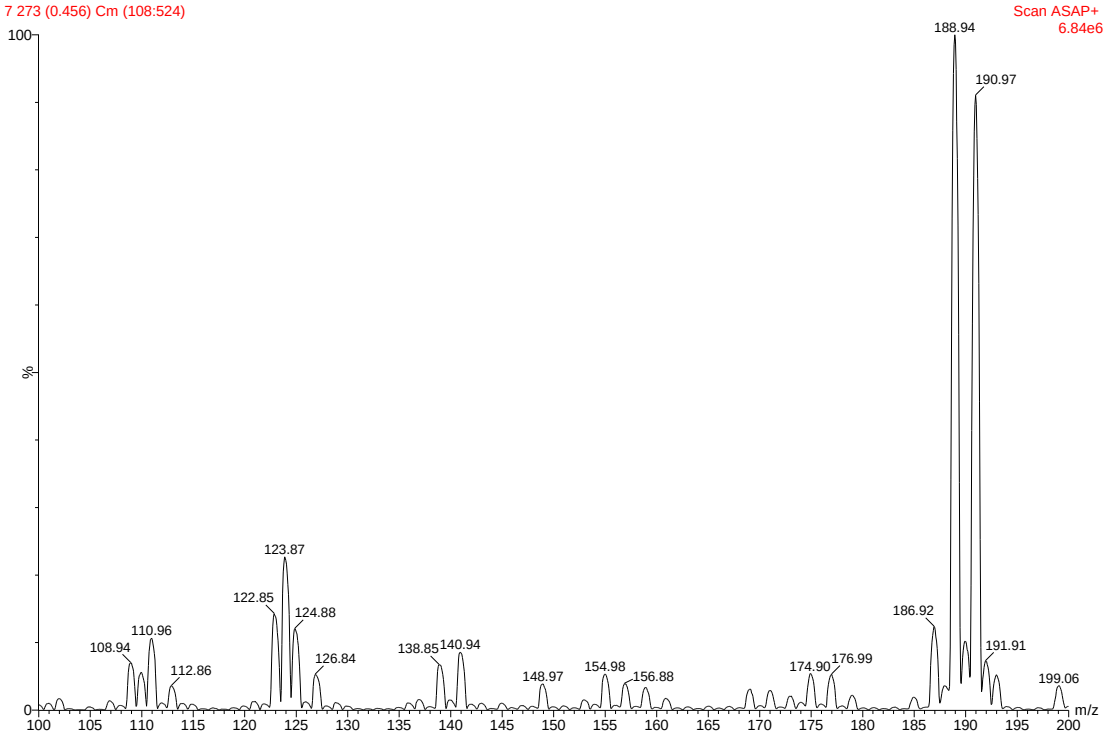

Figure S3 Mass spectra of Compound 2.

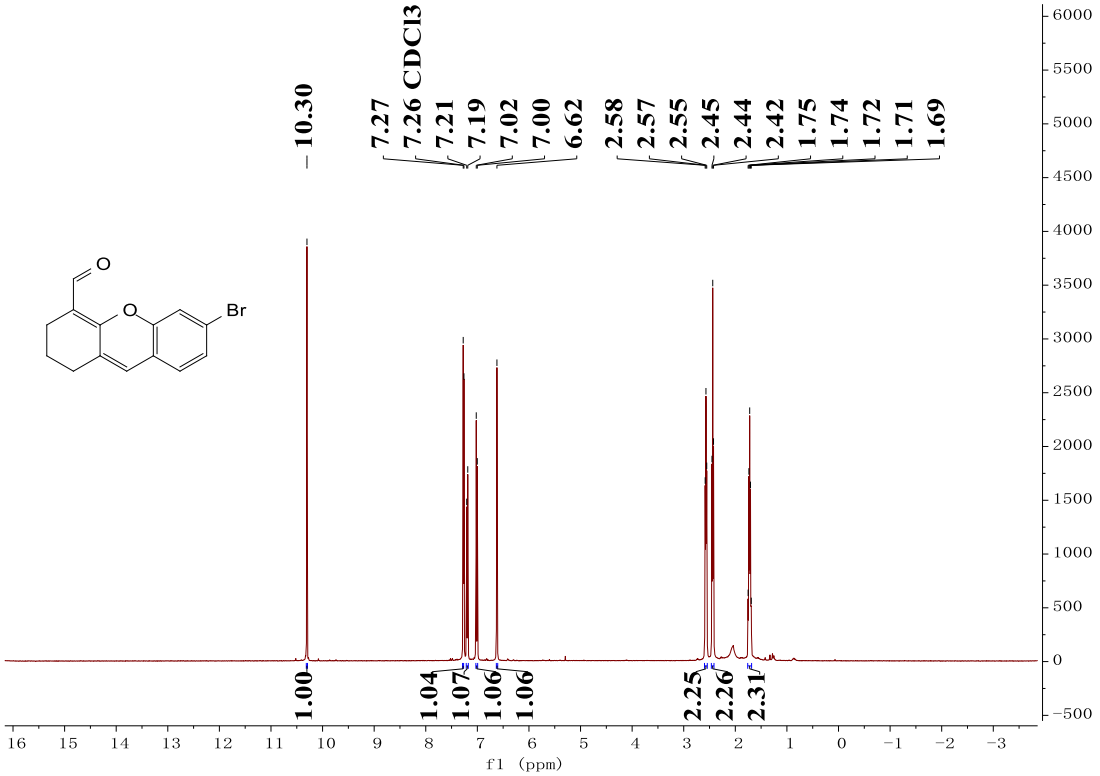

Figure S4 ^{1}H NMR spectra of Compound 3 in $CDCl_3$.

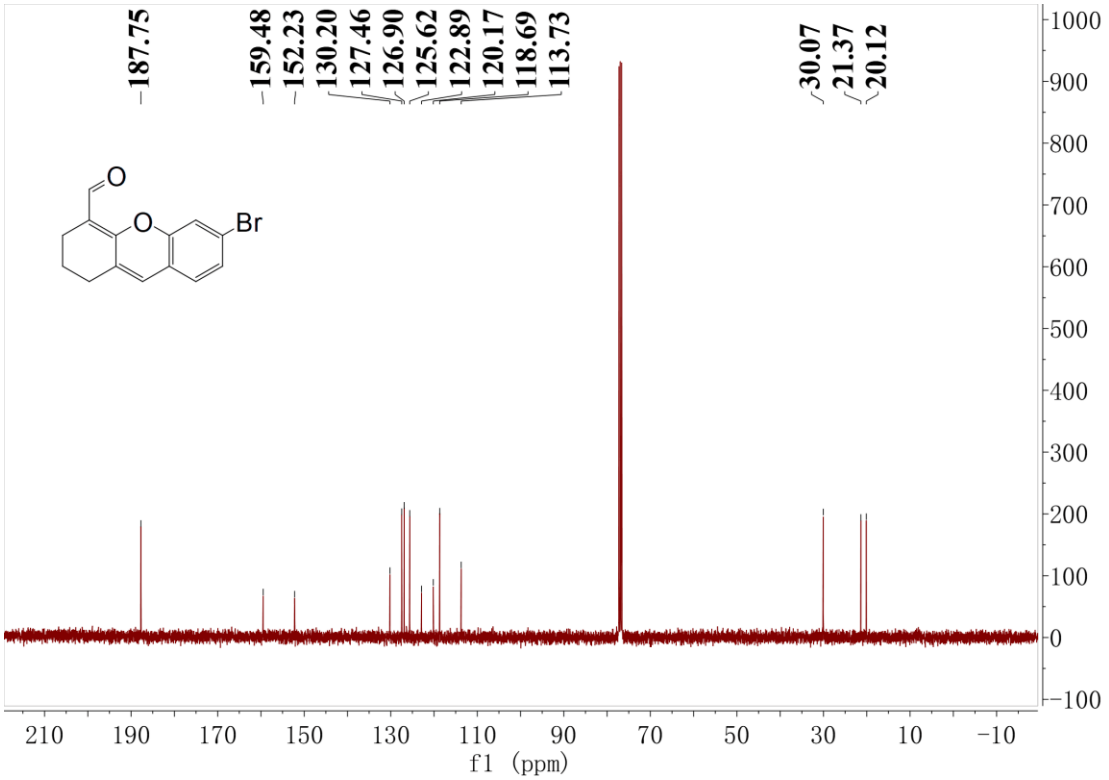

Figure S5 ^{13}C NMR spectra of Compound 3 in $CDCl_3$.

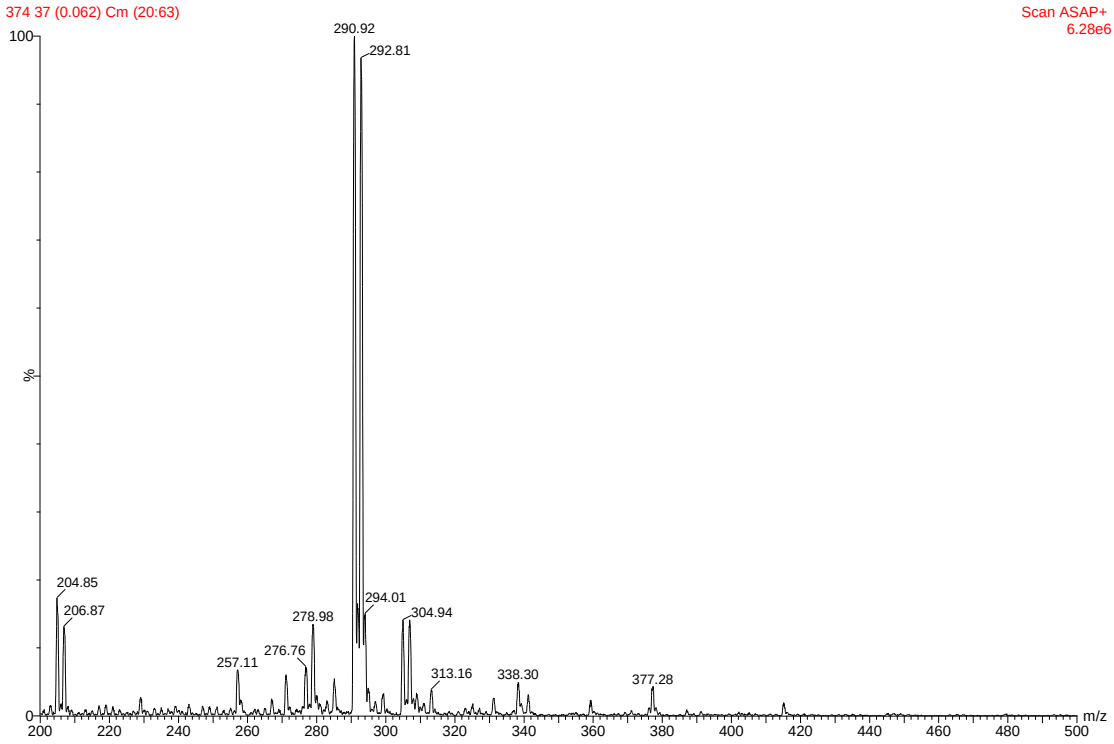

Figure S6 Mass spectra of Compound 3.

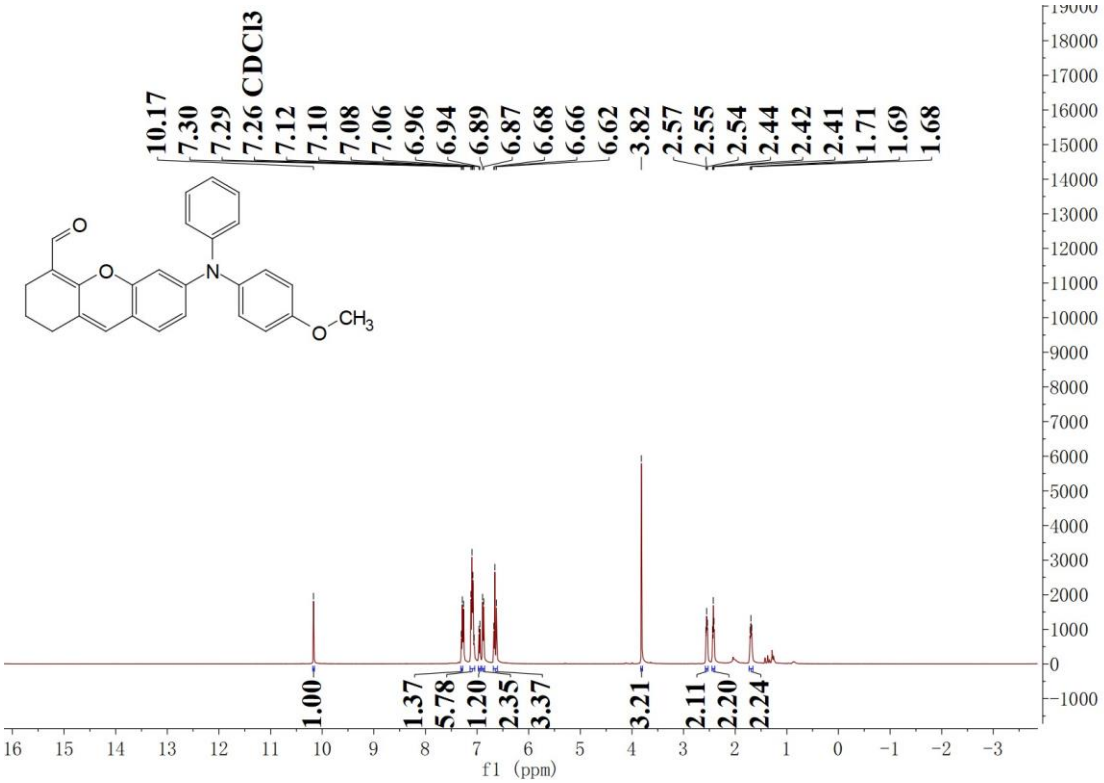

Figure S7 ^{1}H NMR spectra of Compound 4 in $CDCl_3$.

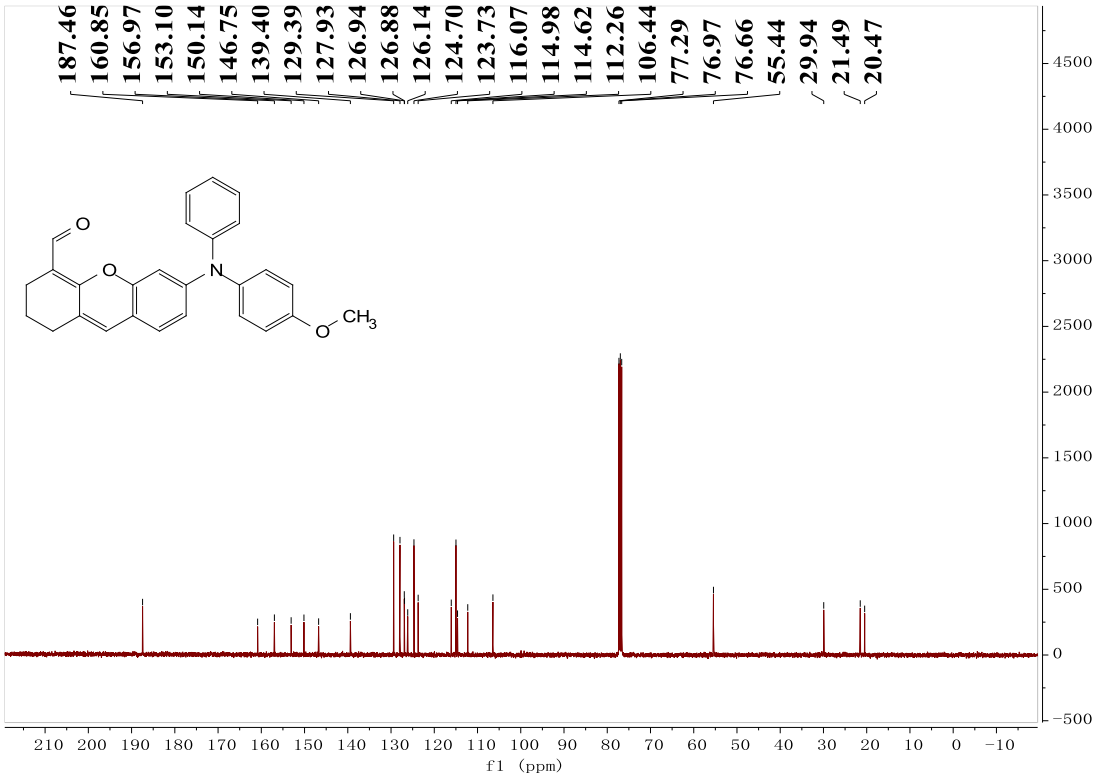

Figure S8 ^{13}C NMR spectra of Compound 4 in $CDCl_3$.

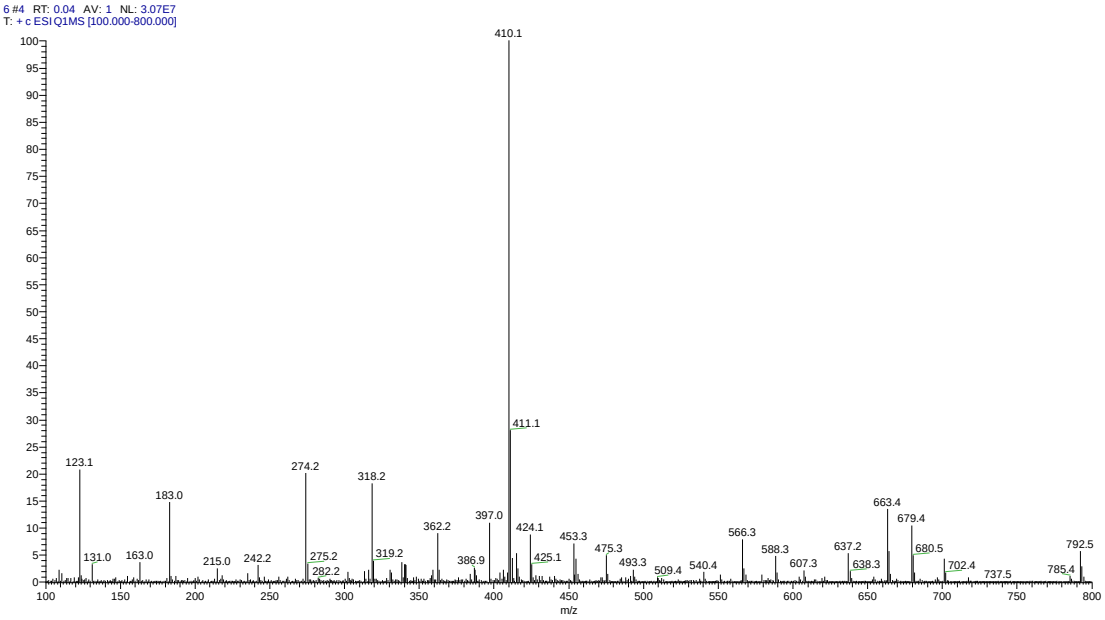

Figure S9 Mass spectra of Compound 4.

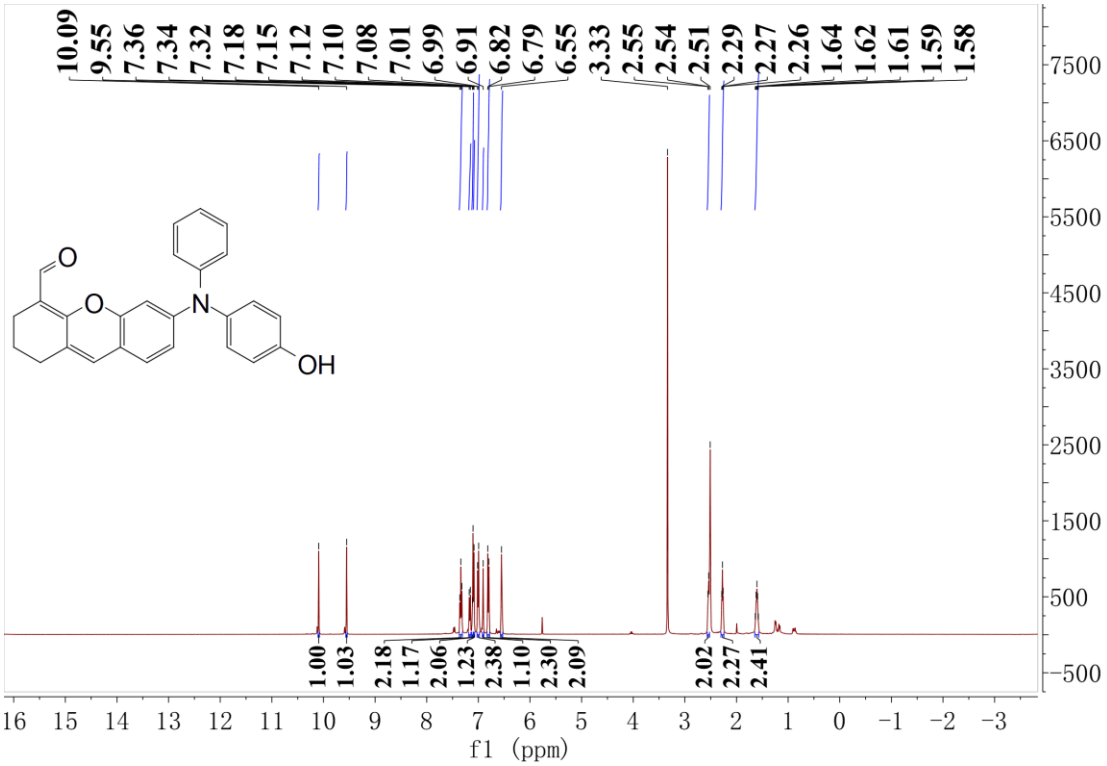

Figure S10 ^{1}H NMR spectra of Compound 5 in DMSO-d_6.

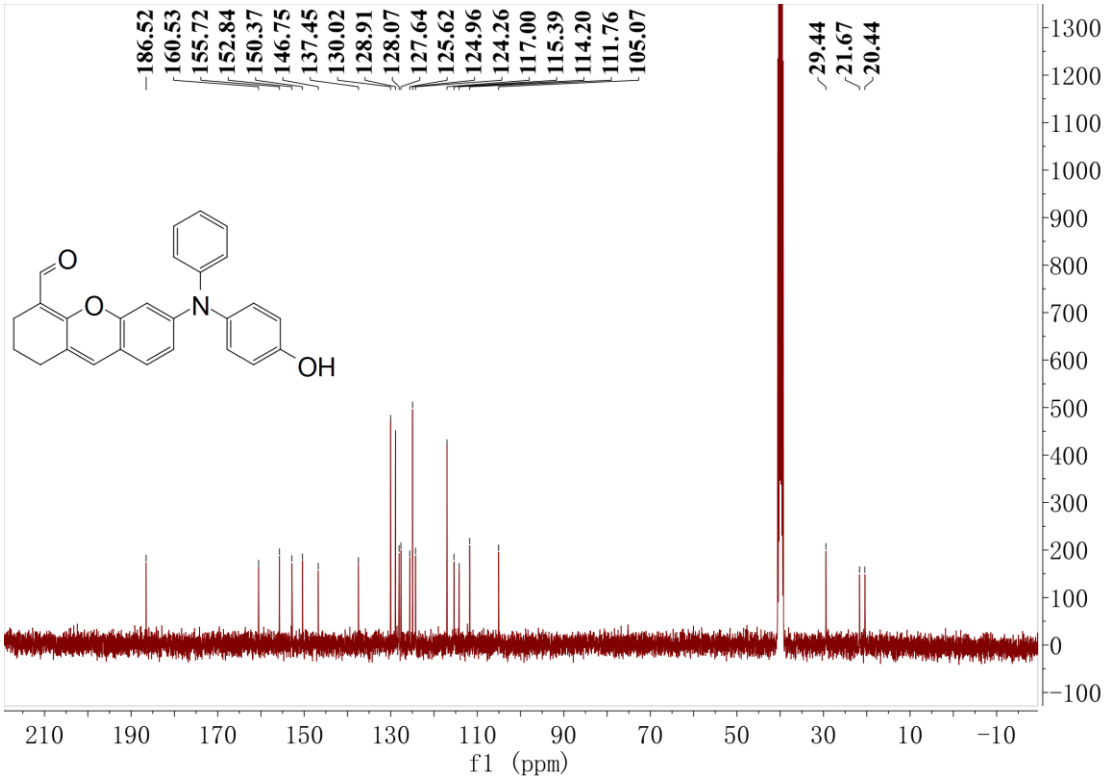

Figure S11 ^{13}C NMR spectra of Compound 5 in DMSO-d_6.

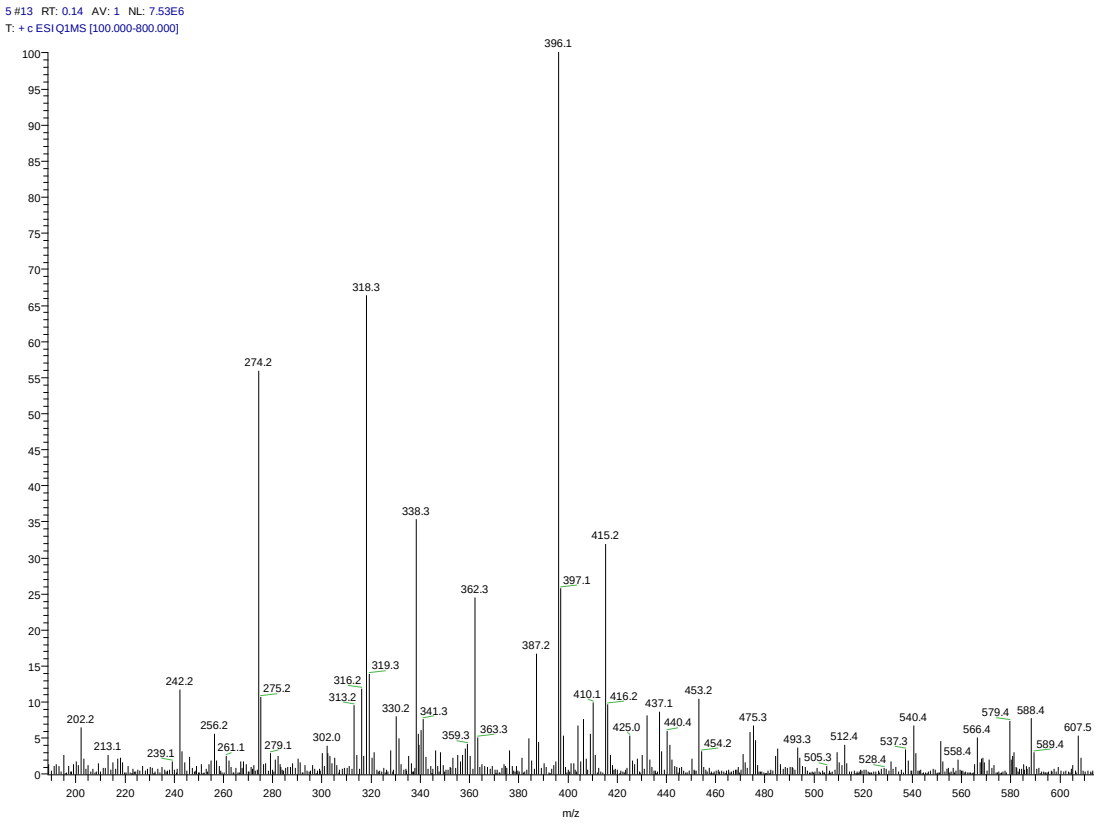

Figure S12 Mass spectra of Compound 5.

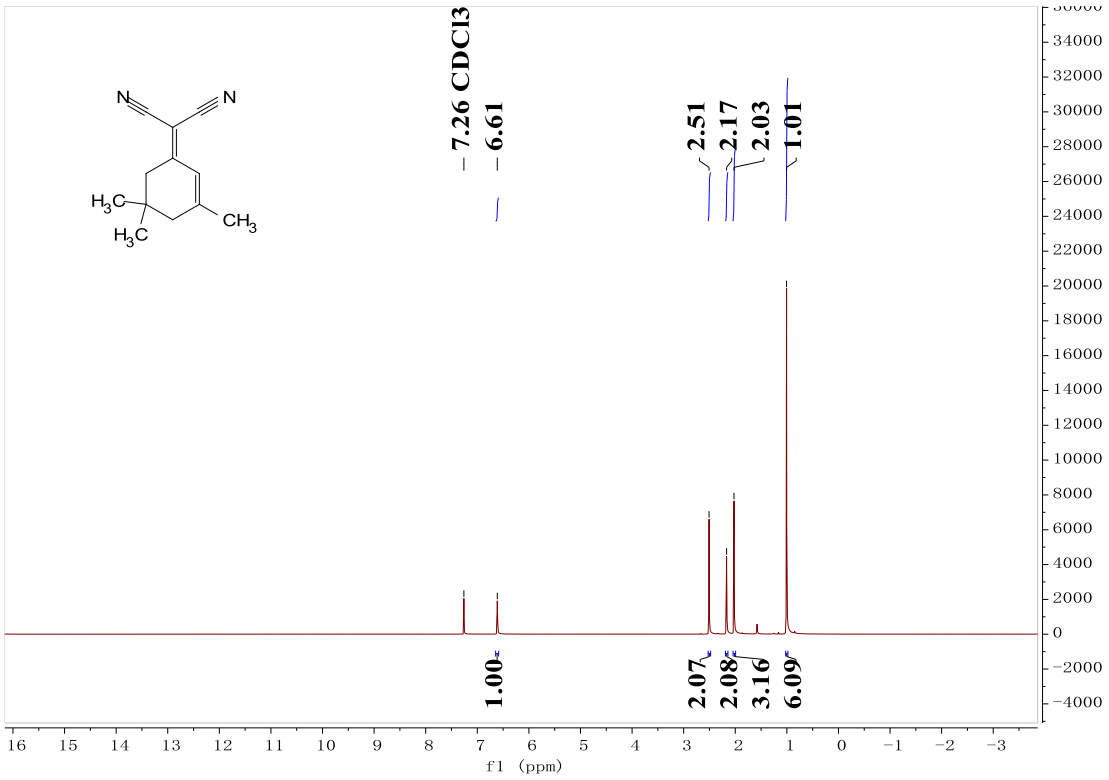

Figure S13 ^{1}H NMR spectra of Compound 7 in $CDCl_3$.

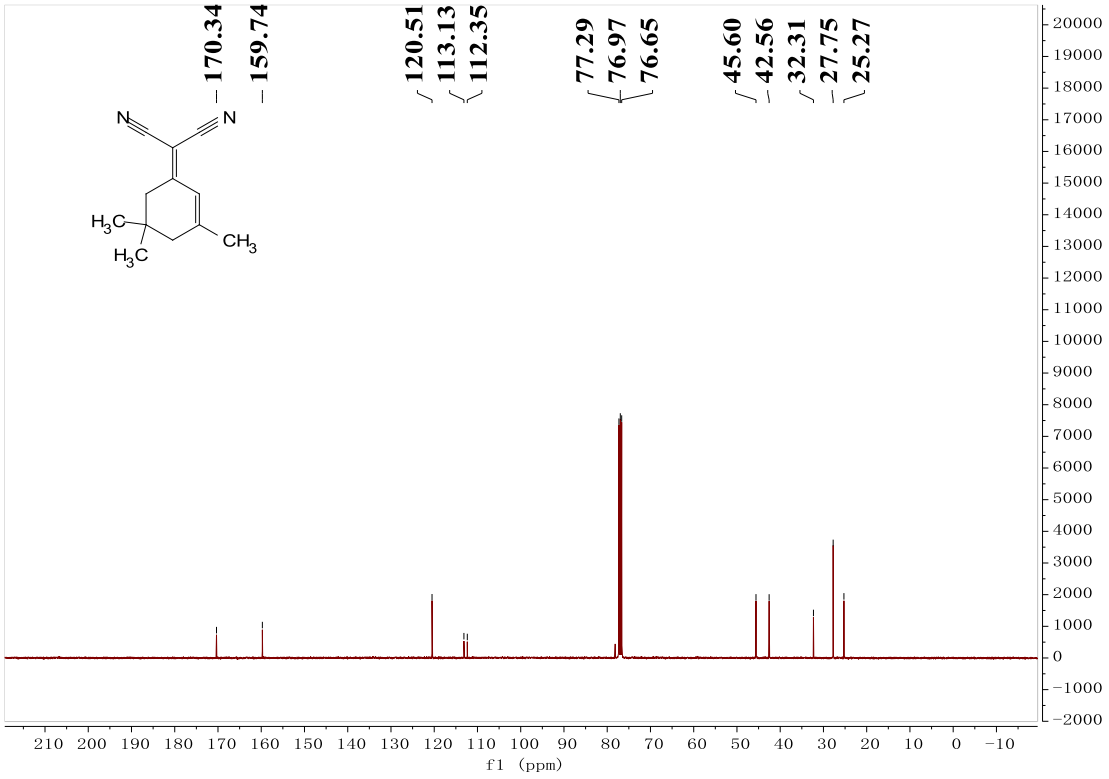

Figure S14 ^{13}C NMR spectra of Compound 7 in $CDCl_3$.

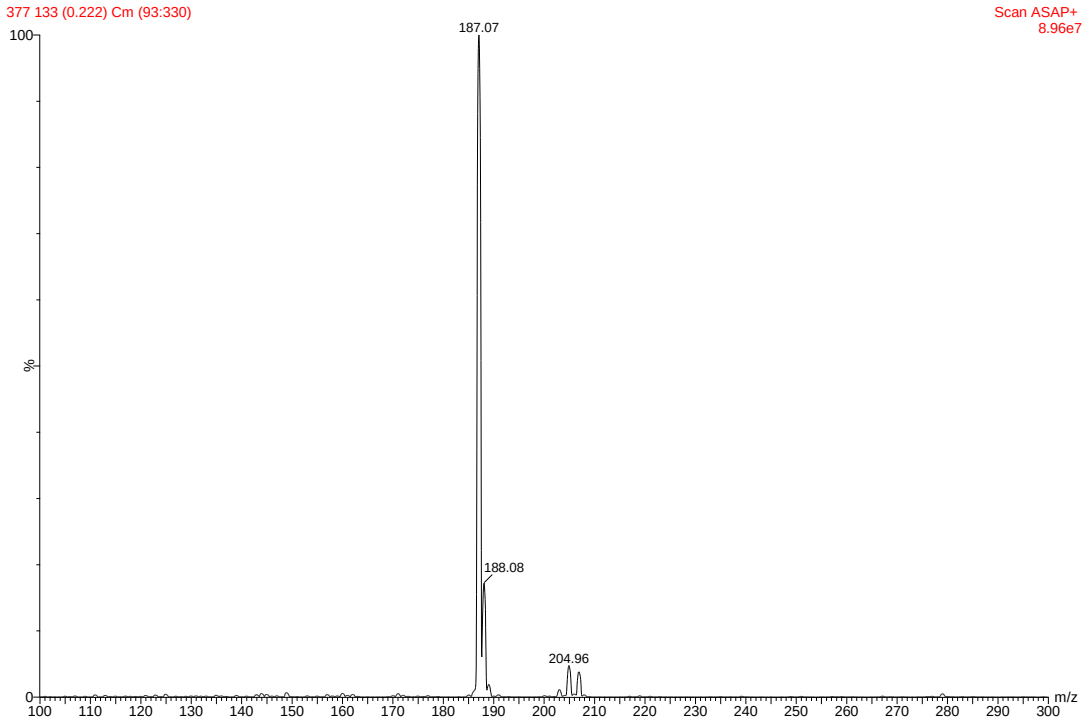

Figure S15 Mass spectra of Compound 7.

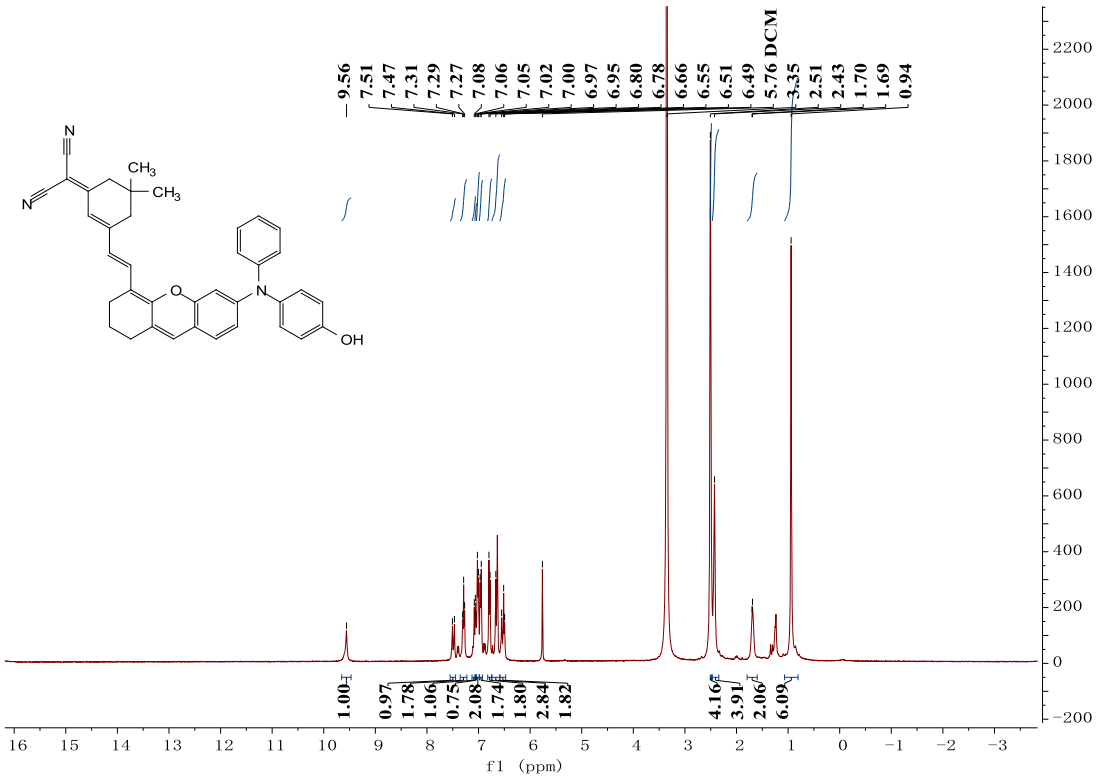

Figure S16 ^{1}H NMR spectra of S-OH in DMSO-d_6.

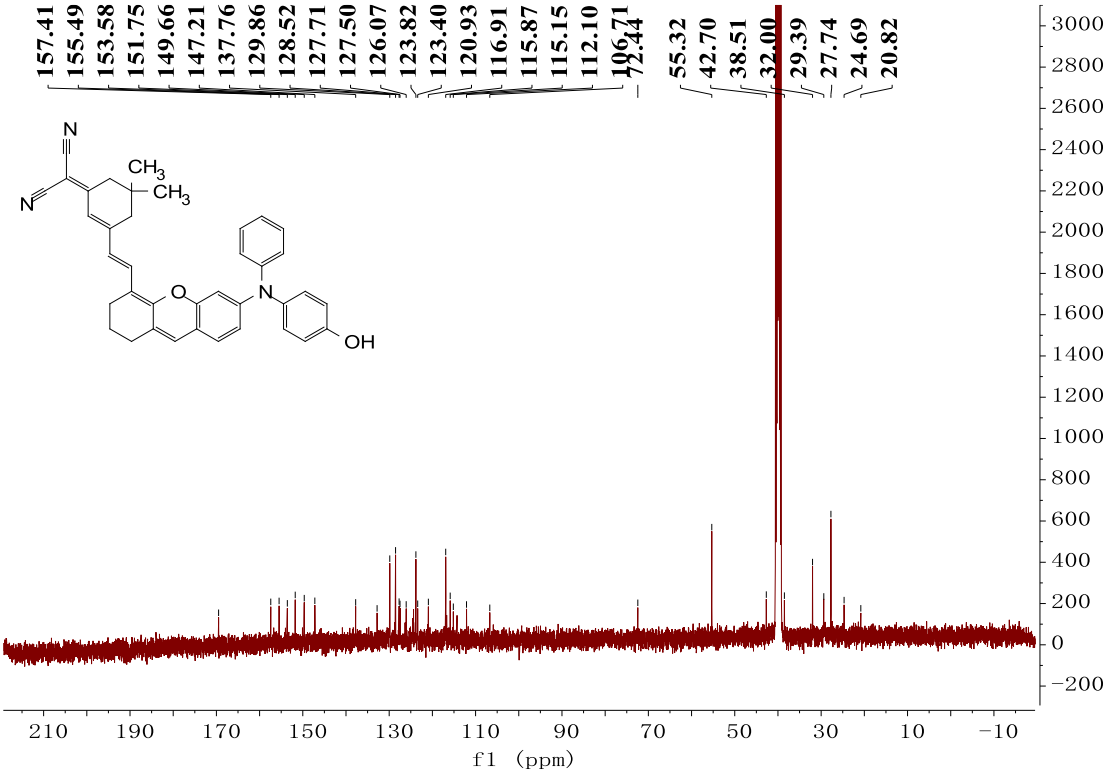

Figure S17 ^{13}C NMR spectra of S-OH in DMSO-d_6.

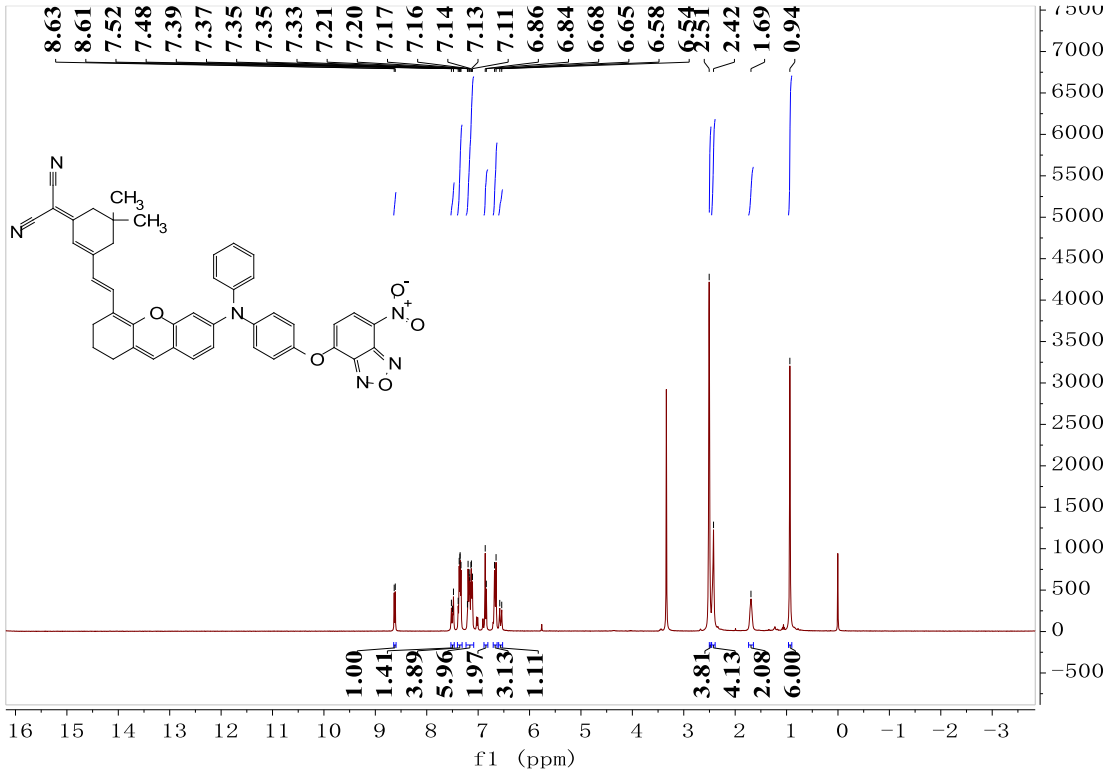

Figure S18 ^{1}H NMR spectra of probe S-NBD in DMSO-d_6.

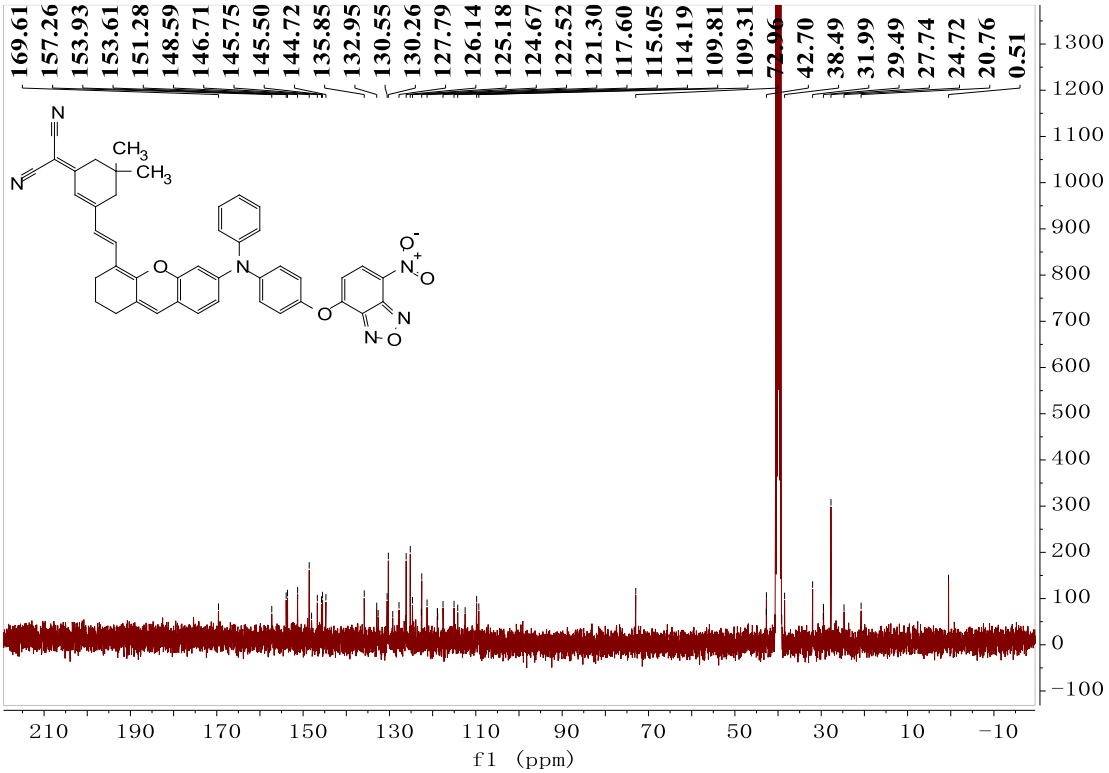

Figure S19 ^{13}C NMR spectra of probe S-NBD in DMSO-d_6.

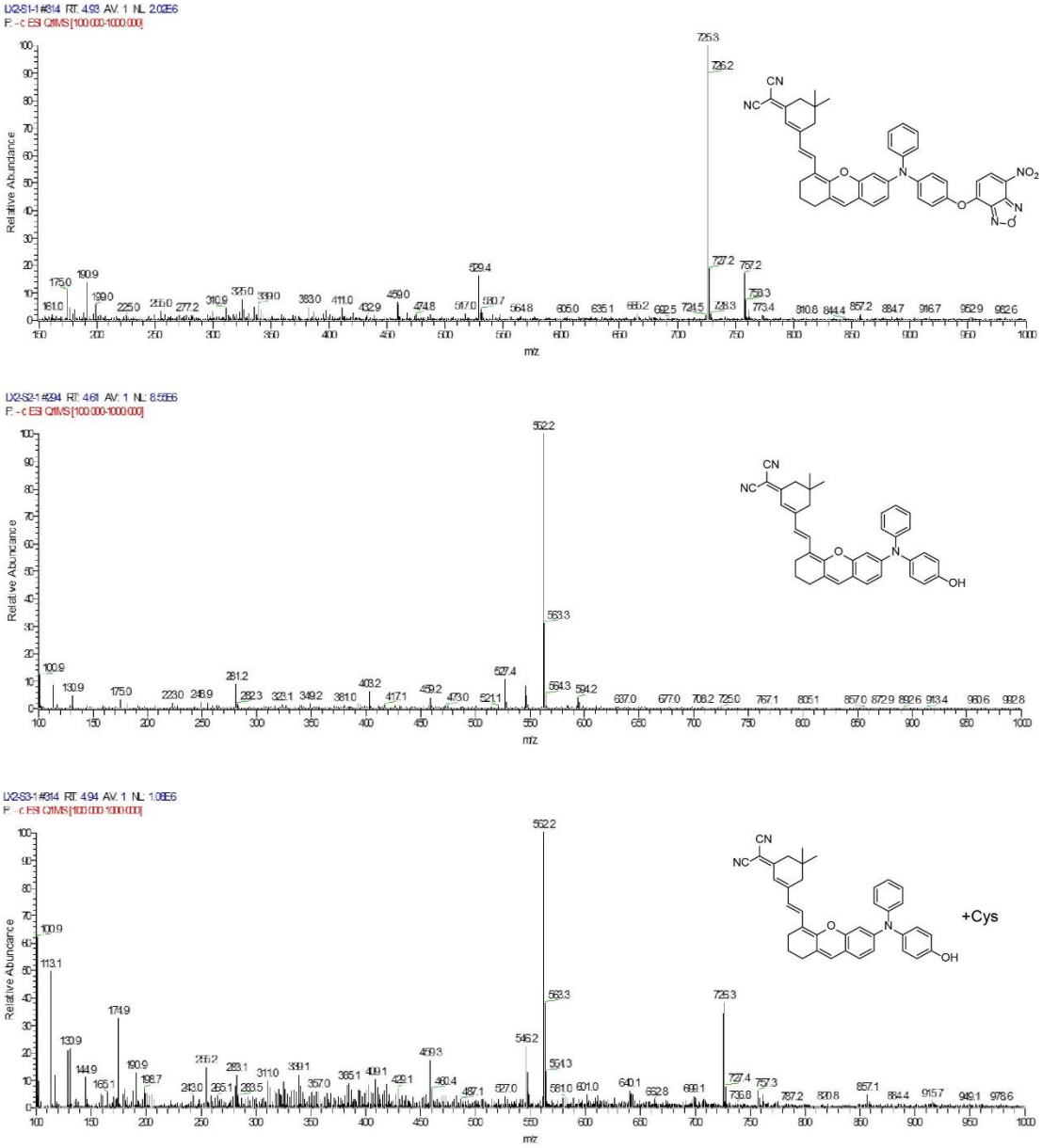

Figure S20 Mass spectra of Probe S-NBD, S-OH and Probe S-NBD + Cys.

3. Spectrometric Studies of S-NBD

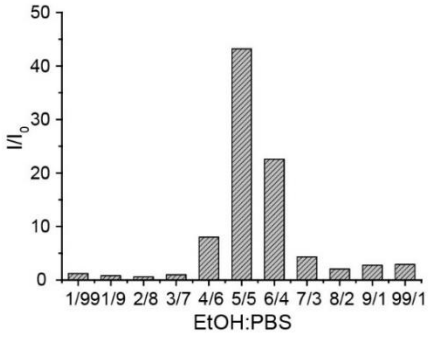

Figure S21 Fluorescence intensity at 838 nm of S-NBD in the absence or presence of Hcy in different ratios of EtOH/PBS solutions (v/v, 1/99, 1/9, 2/8, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2, 9/1, 99/1).

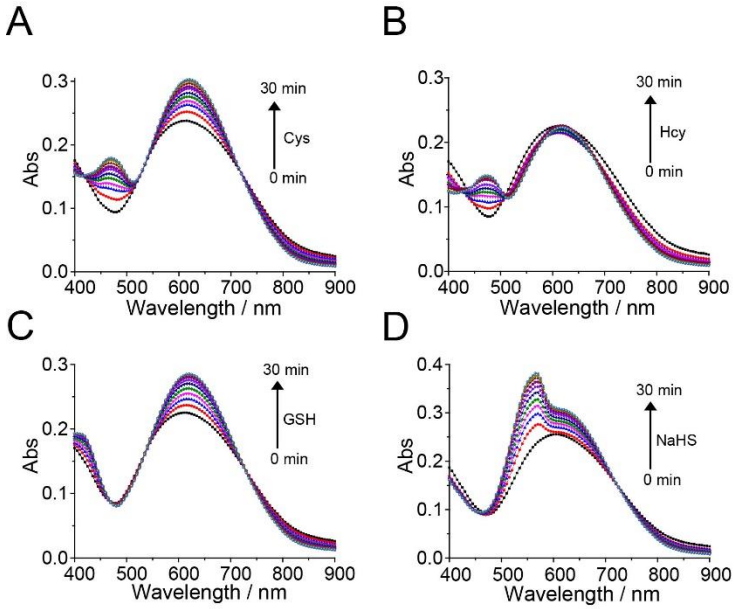

Figure S22 Time-dependent absorption spectra of S-NBD (10 μmol/L) in the presence of (A)10 equiv. of Cys, (B) 10 equiv. of Hcy, (C) 10 equiv. of GSH and (D)10 equiv. of NaHS in EtOH-PBS (pH 7.4, v/v, 5/5).

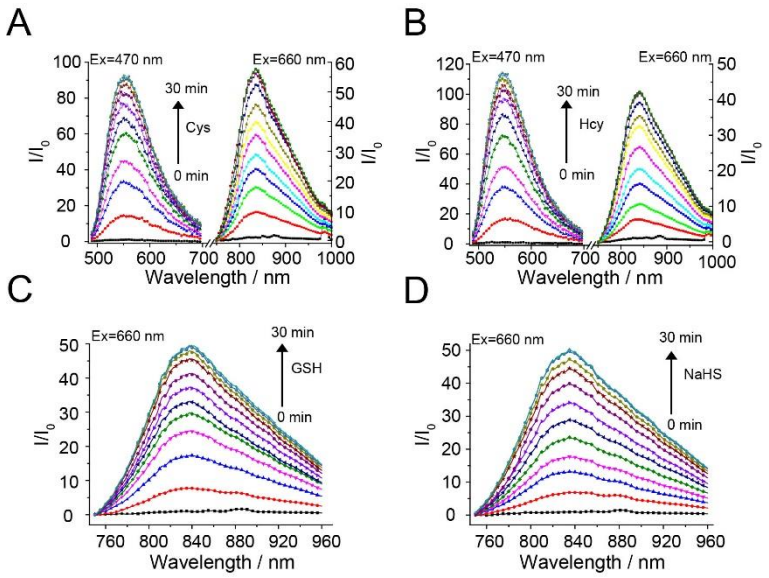

Figure S23 Time-dependent fluorescence spectra of S-NBD (10 μmol/L) in the presence of (A)10 equiv. of Cys, (B) 10 equiv. of Hcy, (C) 10 equiv. of GSH and (D)10 equiv. of NaHS in EtOH-PBS (pH 7.4, v/v, 5/5).

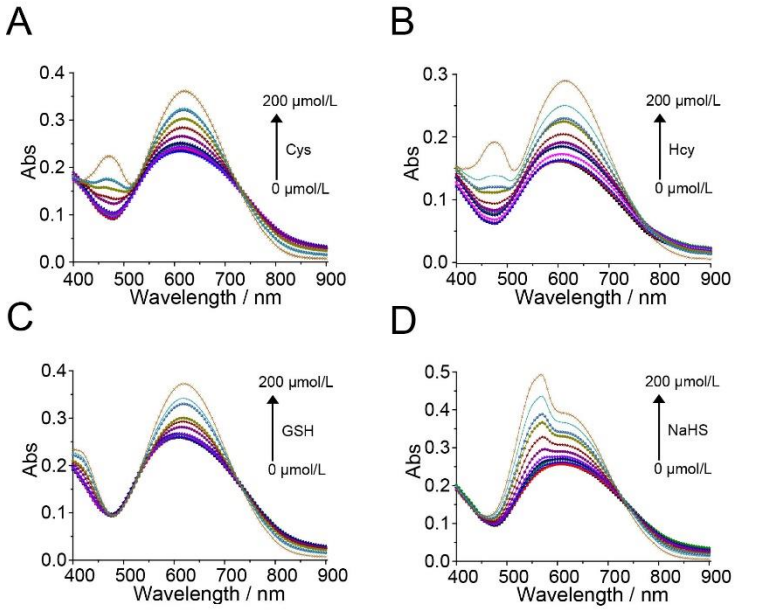

Figure S24 Absorption spectra of S-NBD (10 μmol/L) reacted with Cys /Hcy/GSH and NaHS (0, 1, 2, 4, 6, 8, 10, 20, 40, 60, 80,100 and 200 μmol/L).

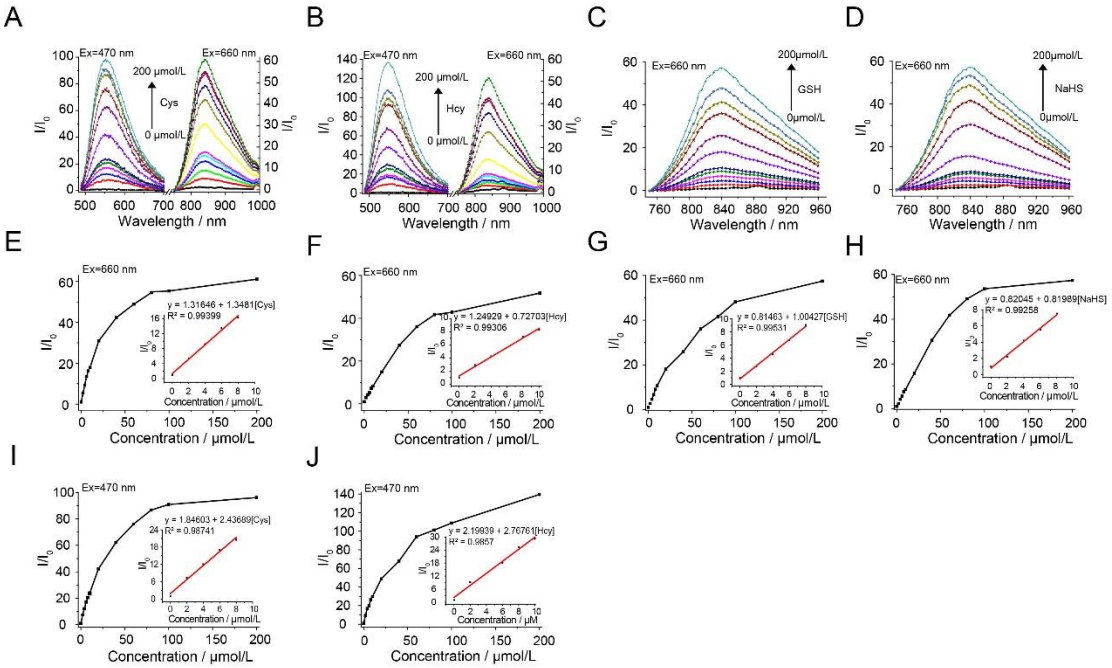

Figure S25 Fluorescence spectra of S-NBD. (A) Fluorescence spectra of S-NBD (10 μmol/L) upon the addition of Cys, (B) Hcy, (C) GSH and (D) NaHS (0–200 μmol/L). Relationship between fluorescence intensity and concentrations of (E) Cys, (F) Hcy, (G) GSH and (H) NaHS at 838 nm (λ_{ex} = 660 nm); The linear relationship between fluorescence intensity and low concentration of biothiols. The plot of fluorescence intensity against (I) Cys's and (J) Hcy's at 550 nm (λ_{ex} = 470 nm) concentration; linearity plot of the fluorescence intensity versus low concentrations of (I) Cys and (J) Hcy.

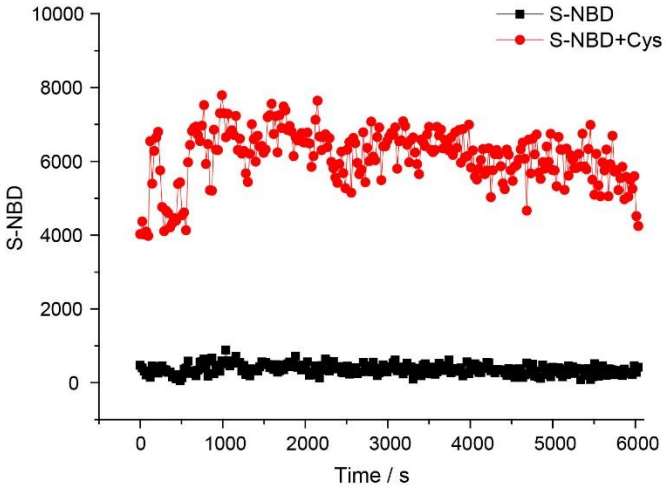

Figure S26 Photo-stability of Probe S-NBD.

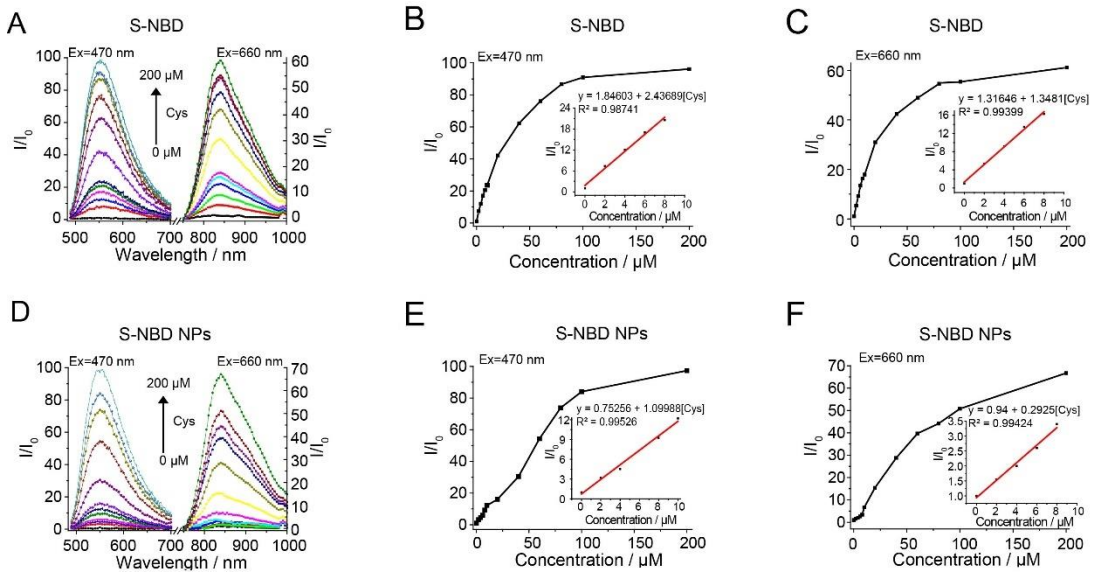

Figure S27 Fluorescence spectra of S-NBD and S-NBD NPs. Fluorescence spectra of (A) S-NBD (10 μmol/L) after Cys (0–200 μmol/L) was added. Relationship between Cys concentrations and fluorescence intensity (B) at 550 nm (λ_{ex} = 470 nm) and (C) at 838 nm (λ_{ex} = 660 nm). Fluorescence spectra of (D) S-NBD NPs (10 μmol/L) upon the addition of Cys (0–200 μmol/L). Relationship between fluorescence intensity and concentrations of Cys (E) at 550 nm (λ_{ex} = 470 nm) and (F) at 838 nm (λ_{ex} = 660 nm). The linear relationship between fluorescence intensity and low concentration of Cys.

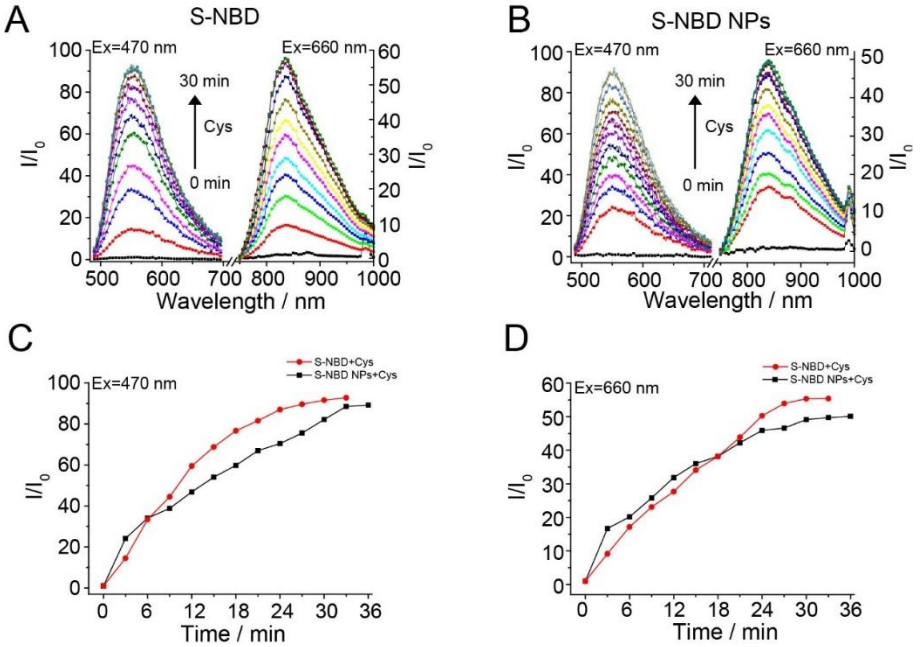

Figure S28 Time-dependent fluorescence spectra of (A) S-NBD (10 μmol/L) and (B) S-NBD NPs (10 μmol/L) in the presence of 10 equiv. of Cys in EtOH-PBS (pH 7.4, v/v, 5/5). Time-dependent fluorescence spectra of S-NBD and S-NBD NPs (10 μmol/L) in the presence of 10 equiv. of of Cys (C) at 550 nm (λ_{ex} = 470 nm) and (D) at 838 nm (λ_{ex} = 660 nm).

4. Photoacoustic Imaging for Biothiols *in Vitro*

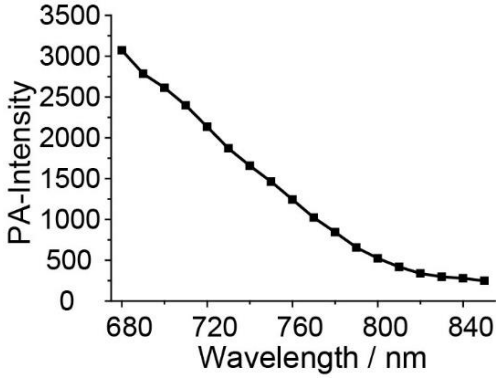

Figure S29 PA intensity of S-NBD (10 μmol/L) reacted with Cys (100 μmol/L) in EtOH/PBS (pH 7.4, v/v, 5/5) at different wavelength (680–850 nm).

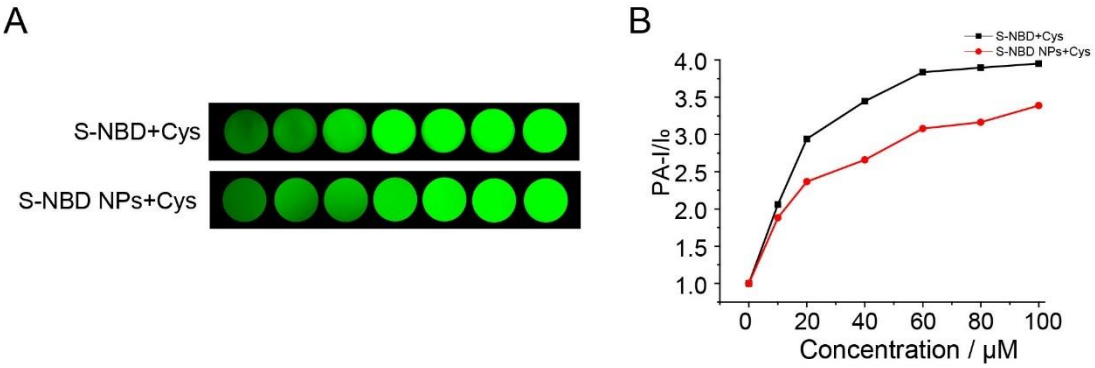

Figure S30 (A) PA images of S-NBD and S-NBD NPs reacted with different concentrations of Cys (0, 10, 20, 40, 60, 80 and 100 μmol/L) in EtOH/PBS (pH 7.4, v/v, 5/5). (B) PA signal intensity of images in (A).

5. Photothermal Imaging for Biothiols *in Vitro*

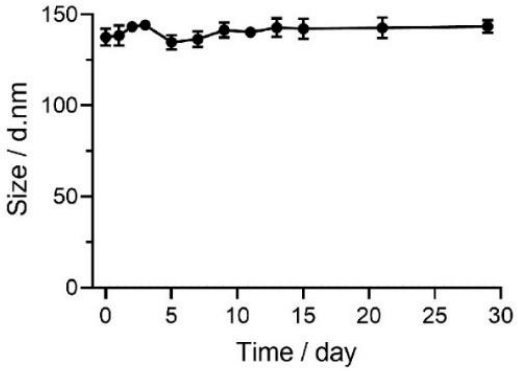

Figure S31 Stability of Probe S-NBD NPs.

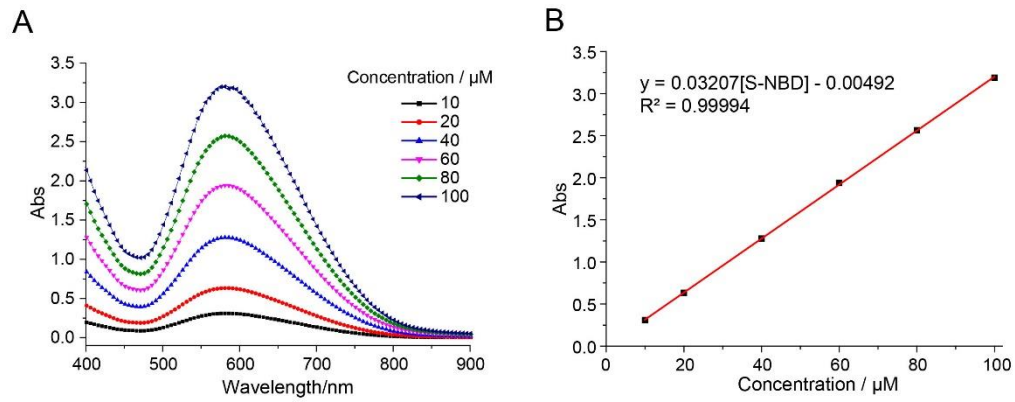

Figure S32 The UV absorption curve as the concentration of S-NBD changes in THF. (B) Standard curve of S-NBD.

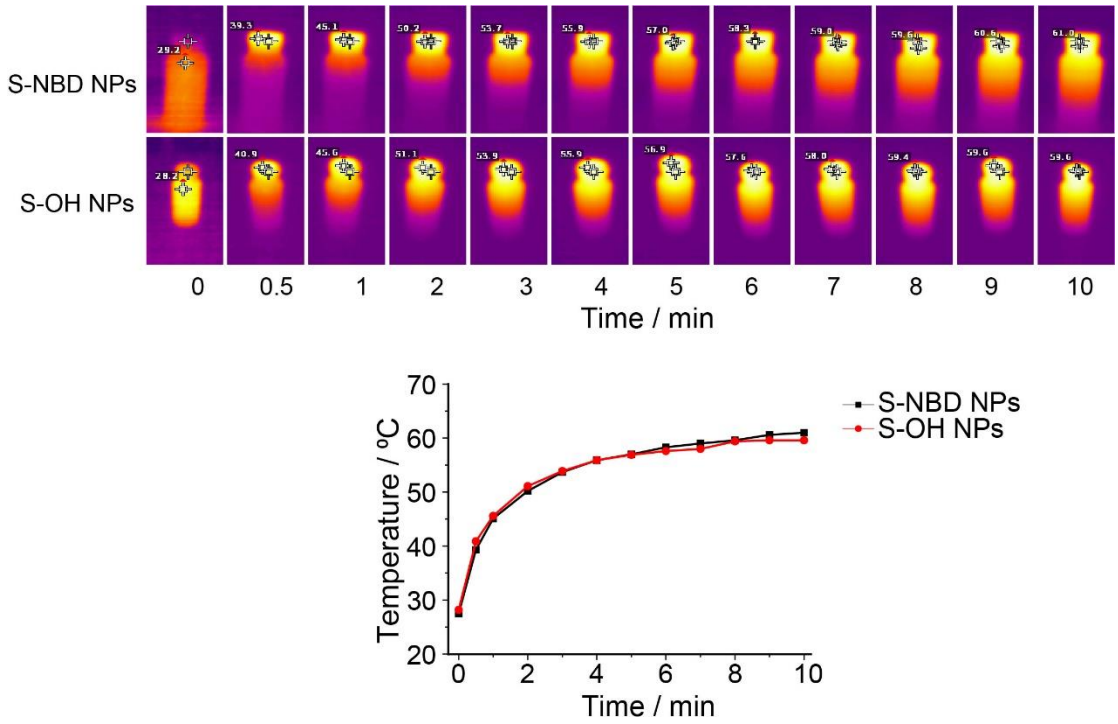

Figure S33 The temperature changes of S-NBD NPs and S-OH NPs (100 μmol/L, 1 W/cm^2, 10 min) irradiated by 660 nm laser.

6. Cell Cytotoxicity by CCK-8 Assay

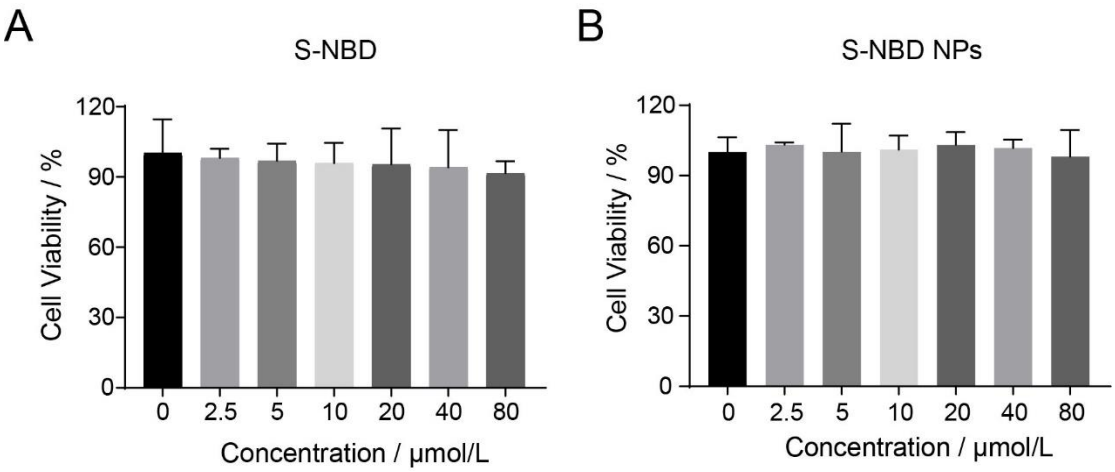

Figure S34 CCK-8 assay for estimating cell viability (%) of HeLa cells treated with various concentrations of S-NBD and S-NBD NPs (0–80 μmol/L) after 24 h incubation.

7. Fluorescence Microscopic Imaging

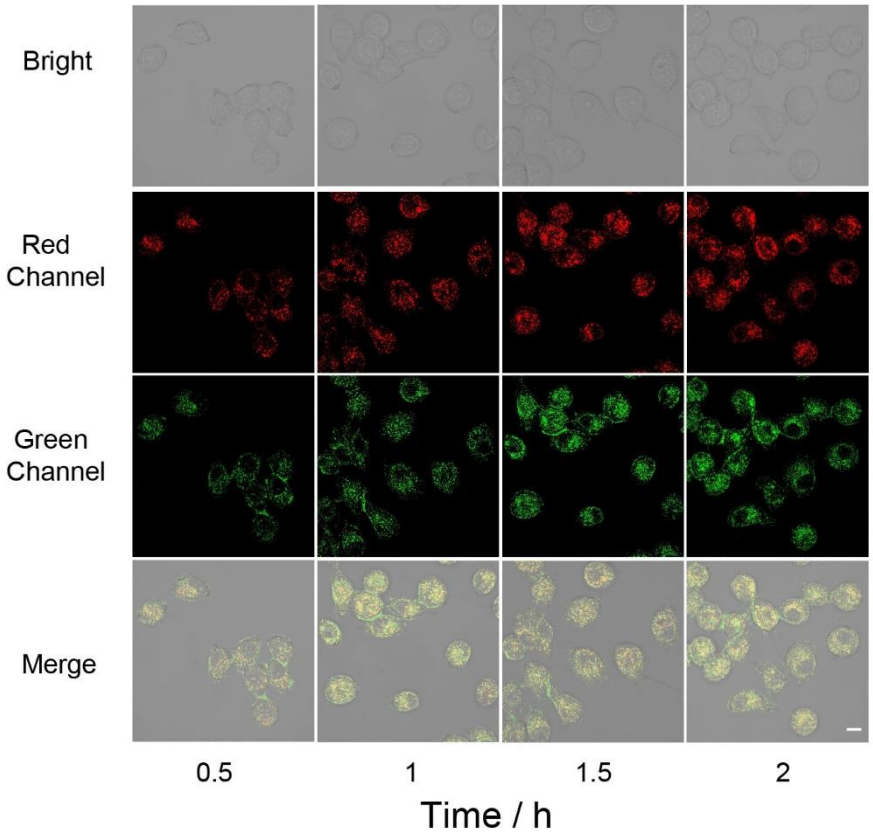

Figure S35 Fluorescence images of HeLa cells incubated with S-NBD (10 μmol/L) for different times (0.5, 1, 1.5, 2 hours). Scale bar = 10 μm.

8. Photothermal Therapy in Cells

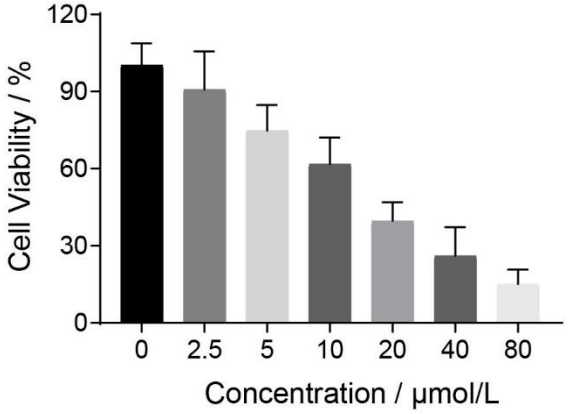

Figure S36 CCK-8 assay for estimating cell viability (%) of HeLa cells treated with various concentrations of S-NBD NPs (0–80 μmol/L) after 24 h incubation and then treated with S-NBD NPs with 660 nm laser irradiation (0.2 W/cm^2, 20 min).