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Rational Design of Fluorescent Probes for Targeted *in vivo* Nitroreductase Visualization

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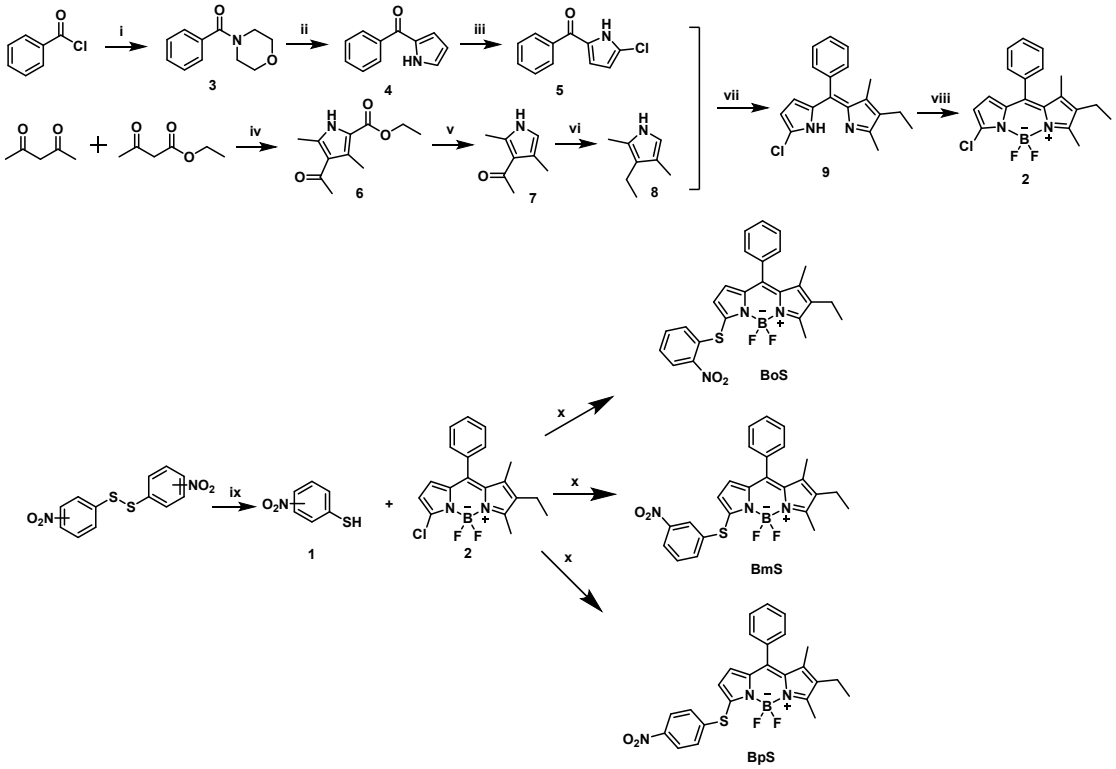
Materials and instruments

All reagents and solvents were purchased from commercial sources and were of the highest grade. NTR from *Escherichia coli* and NADH were purchased from Sigma-Aldrich. All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC). Mass spectra were measured on an HP 1100 LC-MS spectrometer. Cell viability test was obtained on a Thermo Scientific Multiskan. Confocal fluorescence imaging was performed using Carl Zeiss LSM710 with a 60 × oil-immersion objective lens. NIR-I image was collected using the IVIS spectrum imaging system. The following abbreviations were used to explain the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet.

UV−vis and Fluorescence Experiments

All the UV−vis spectra and fluorescence spectra were measured in Tris-HCl containing 25% MeCN (pH = 7.4), with the following procedure. 10 μM **BoS** and 0.5 mM NADH were mixed, and then an appropriate volume of NTR was added at 37 °C.

Synthesis



Scheme S1 Synthetic route. Reagents and conditions: (i) morpholine, Et_3N, CH_2Cl_2, 4 h; (ii) $POCl_3$, pyrrole, CH_2Cl_2, overnight; (iii) $CuCl_2 \cdot 2H_2O$, H_2SO_4, acetonitrile, 24 h; (iv) Acetic acid, zinc dust, 1 h; (v) KOH, ethylene glycol, 4 h; (vi) $LiAlH_4$, THF, 5 h; (vii) $POCl_3$, CH_2Cl_2, 12 h; (viii) Et_3N, $BF_3 \cdot Et_2O$, 4 h; (ix) $NaBH_4$, THF, 3 h; (x) DMAP, MeCN, 5 h.

Synthesis of compound 3 To the solution of morpholine (22.00 mL, 0.25 mol) in CH_2Cl_2 (100 mL) was dropwise added Et_3N (47.00 mL, 0.34 mol) in an ice bath, then dropwise added benzol choride (26.00 mL, 0.23 mol). The reaction was further stirred at r.t. for 4 h. The solids were removed by filtration and the filtrate was washed by water (50 mL × 3), The organic layer was dried over Na_2SO_4 and evaporated in vacuum to afford **3** (40.00 g, 91%) as white crystal. ^{1}H NMR (400 MHz, DMSO-d_6), δ 7.43 (d, 3H), 7.39 (s, 1H), 7.37 (m, 1H), 3.59 (m, 4H), 3.54 (m, 4H).

Synthesis of compound 4[1] Compound **3** (11.50 g, 60.00 mmol) was added to 10.00 mL of $POCl_3$ under nitrogen protection, then the reaction mixture was stirred at r.t. overnight. After dropwise added the solution of pyrrole (6.93 mL, 100.00 mmol) in CH_2Cl_2 (10.00 mL), the reaction was stirred at r.t. for another 24 h. The reaction was quenched by saturated $NaHCO_3$ in an ice bath. The mixture was stirred at r.t. for 30 min, then heated to reflux with stirring for another 2 h. After the reaction was cooled to room temperature, the organic phase was washed by water (50 mL × 3), then dried over Na_2SO_4 and evaporated in vacuum. The residue was purified by silica gel column to afford **4** (3.10 g, 30%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.77 (s, 1H), 7.91 (d, 2H), 7.57 (t, 1H), 7.49 (t, 2H), 7.16 (s, 1H), 6.90 (s, 1H), 6.35 (dd, 1H).

Synthesis of compound 5[1] Compound **4** (0.51 g, 3.10 mmol) and $CuCl_2 \cdot 2H_2O$ (1.53 g, 9.00 mmol) was dissolved in acetonitrile, then the reaction mixture was heated to reflux with stirring for 24 h. After the reaction was cooled to room temperature, H_2SO_4 (10%, 3.05 mL) was added into reaction mixture, and the reaction keep stirring for 70 min. The mixture was extracted by ethyl acetate (50 mL × 3). The organic phase was washed by water (50 mL × 3), then dried over Na_2SO_4 and evaporated in vacuum. The residue was purified by silica gel column to afford **5** (0.30 g, 50%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.90 (s, 1H), 7.79 (d, 2H), 7.63 (t, J = 7.5 Hz, 1H), 7.53 (t, J = 7.5 Hz, 2H), 6.77 (t, 1H), 6.27 (t, 1H).

Synthesis of compound 6[2] To a stirred cooled (0 °C) solution of ethyl acetoacetate (25.27 mL, 0.20 mol) in acetic acid (52.00 mL) was added a cold solution of sodium nitrite (14.90 g, 0.22 mol) in H_2O (10.00 mL). After stirring the mixture for 12 h at 25 °C, acetyl acetone (20.52 mL, 0.20 mmol) and zinc dust (28.12 g, 0.43 mmol) were added in an ice bath, then the mixture was stirred at 60 °C for 1 h. The mixture was cooled to room temperature and extracted with CH_2Cl_2. The organic

phase was washed by water, then dried over Na_2SO_4, concentration of the extract in vacuum followed by crystallization from ethanol gave **6** as a white solid (23.41 g, 56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.03 (s, 1H), 4.31 (q, J = 7.1 Hz, 2H), 2.56 (s, 3H), 2.49 (s, 3H), 2.42 (s, 3H), 1.35 (t, J = 7.1 Hz, 3H).

Synthesis of compound 7 A mixture of **6** (20.00 g, 95.69 mmol) and KOH (7.98 g, 142.58 mmol) in ethylene glycol (40 mL) was heated at 160 °C for 4 h. After cooling, the mixture was extracted with CH_2Cl_2, and organic phase was washed with brine and dried over Na_2SO_4. Concentration of the extract in vacuum gave **7** (11.27 g, 86%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.91 (s, 1H), 6.39 (s, 1H), 2.38 (s, 3H), 2.28 (s, 3H), 2.14 (s, 3H).

Synthesis of compound 8 The solution of **7** (7.00 g, 51.06 mmol) in dry THF was dropwise added suspension of $LiAlH_4$ in dry THF (2.5 M, 24.5 mL, 61.28 mmol) in an ice bath. The mixture was refluxed for 5 h then cooled to 0 °C, and the excess hydride was decomposed with $Na_2SO_4 \cdot 10H_2O$. The mixture was extracted with ethyl acetate, dried over Na_2SO_4, concentrated in vacuum. The crude product after usual work-up was used without further purification.

Synthesis of compound 9 A mixture of compound **5** (1.18 g, 5.75 mmol) and $POCl_3$ (1.60 mL, 17.25 mmol) in dry CH_2Cl_2 was stirred for 1 h at room temperature. To this reaction mixture was added compound **8** (2.12 g, 17.25 mmol) in CH_2Cl_2, and the mixture was further stirred for 12 h. The reaction mixture was slowly poured into saturated aqueous $NaHCO_3$ (50 mL) under ice-cold conditions, washed with water, dried over anhydrous Na_2SO_4, and evaporated in vacuum. The crude product was purified with silica gel flash chromatography to give compound **9** (1.29 g,

72%).^{1}H NMR (400 MHz, DMSO-d_6) δ 12.23 (s, 1H), 7.47 (d, 3H), 7.28 (d, 2H), 6.34 (d, *J* = 3.9 Hz, 1H), 6.30 (d, *J* = 3.9 Hz, 1H), 2.33 (s, 3H), 2.26 (q, 7.4 Hz, 2H), 1.21 (s, 3H), 0.91 (t, *J* = 7.4 Hz, 3H).

Synthesis of compound 2[3] The compound **9** (1.26 g, 4.06 mmol) was dissolved in toluene before Et_3N (1.7 mL, 12.15 mmol) was added and the mixture was stirred for 1 h at room temperature. $BF_3{\cdot}Et_2O$ (2.0 mL, 16.20 mmol) was added at 0 °C, then the reaction was stirred at 105 °C for another 3 h. After cooling to room temperature, the reaction mixture was diluted with CH_2Cl_2 and washed with saturated $NaHCO_3$ solution and brine, dried over Na_2SO_4, and evaporated. The residue was purified by column chromatography to give compound **2** (1.11 g, 76%) as an orange crystalline solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50 (d, *J* = 5.8 Hz, 2H), 7.46 (d, *J* = 5.8 Hz, 1H), 7.32 (d, *J* = 1.4 Hz, 1H), 7.30 (d, *J* = 1.4 Hz, 1H), 6.24 (d, *J* = 3.9 Hz, 1H), 6.20 (d, *J* = 3.9 Hz, 1H), 2.63 (s, 3H), 2.35 (q, *J* = 7.6 Hz, 2H), 1.42 (s, 3H), 1.03 (d, *J* = 7.6 Hz, 3H).

Synthesis of compound 1 Bis (3-nitrophenyl) disulfide, Bis(2-nitrophenyl) disulfide or Bis (4-nitrophenyl) disulfide (0.30 g, 0.97 mmol) was dissolved in anhydrous THF, then $NaBH_4$ (0.14 g, 3.40 mmol) was slowly added under N_2 atmosphere, the mixture was stirred at room temperature for 3 h. Then the reaction was cooled to 0 °C, wherein cooled water was added to quench the reaction, the pH was adjusted to 7 with 1M hydrochloric acid. Then the mixture was extracted with CH_2Cl_2 (3 × 30 mL), washed with brine (3 × 30 mL), dried over anhydrous Na_2SO_4 to give compound **1** (126.30 mg, 84%).

Synthesis of compound BoS, BmS, BpS Under nitrogen protection, compound **2** (0.12 g, 0.34 mmol) and compound **1** (0.16 g, 1.02 mmol) were dissolved in 10 mL anhydrous CH_2Cl_2, followed by addition of DMAP (0.12 g, 1.02 mmol). The mixture was stirred 5 h at room temperature. After

evaporation of the solvent, silica gel column was used to give compound **BoS**, **BmS**, **BpS**, respectively (0.13 g, 82%). **Compound BoS:** ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.18 (d, 1H), 7.55 (m, 4H), 7.44 (d, 2H), 7.35 (t, 1H), 6.90 (d, 1H), 6.57 (d, J = 3.7 Hz, 1H), 6.26 (d, J = 3.7 Hz, 1H), 2.47 (s, 3H), 2.31 (q, J = 7.4 Hz, 2H), 1.38 (s, 3H), 0.90 (t, J = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.44, 144.85, 143.01, 140.33, 139.41, 137.66, 137.20, 136.53, 134.86, 133.63, 133.28, 129.50, 129.39, 128.79, 128.58, 125.46, 125.18, 124.84, 123.94, 17.12, 14.05, 13.63, 12.41. HRMS (ESI, m/z): calculated for $C_{25}H_{23}BF_2N_3O_2S^+$ $[M+H]^+$: 478.1567, found: 478.1573. **Compound BmS:** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (s, 1H), 8.03 (d, 1H), 7.66 (d, 1H), 7.48 (s, 2H), 7.46 (s, 1H), 7.43 (d, 1H), 7.33 (d, 2H), 6.26 (d, 1H), 6.22 (s, 1H), 2.60 (s, 3H), 2.33 (q, J = 7.5 Hz, 2H), 1.43 (s, 3H), 0.99 (t, J = 7.5 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.35, 148.60, 142.33, 140.14, 139.79, 138.76, 138.20, 136.90, 136.65, 135.52, 134.12, 133.73, 129.66, 129.38, 128.85, 128.50, 125.52, 124.41, 121.71, 120.93, 17.13, 14.14, 13.44, 12.33. HRMS (ESI, m/z): calculated for $C_{25}H_{23}BF_2N_3O_2S^+$ $[M+H]^+$: 478.1567, found: 478.1573. **Compound BpS:** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, 2H), 7.52 (d, 3H), 7.36 (dd, 3H), 7.33 (s, 1H), 6.43 (d, J = 3.8 Hz, 1H), 6.32 (d, J = 3.8 Hz, 1H), 2.62 (s, 3H), 2.35 (q, J = 7.6 Hz, 2H), 1.46 (s, 3H), 1.01 (t, J = 7.6 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 165.80, 147.37, 145.72, 142.88, 140.24, 137.47, 136.94, 136.22, 134.64, 133.59, 129.50, 128.79, 128.58, 127.62, 124.99, 123.96, 122.57, 17.13, 14.07, 13.56, 12.39. HRMS (ESI, m/z): calculated for $C_{25}H_{23}BF_2N_3O_2S^+$ $[M+H]^+$: 478.1567, found: 478.1573.

Time-dependent absorption spectra changes of BoS, BmS, BpS

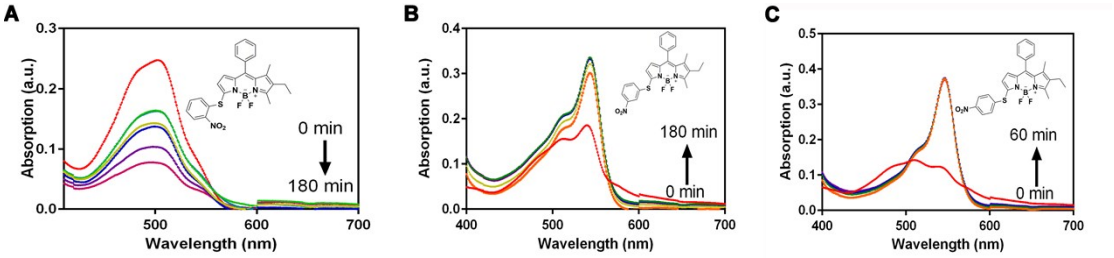


Figure S1 Time-dependent spectra changes of **BoS**, **BmS**, **BpS** (10 μM) in the presence of NTR (10 μg/mL) and NADH (0.5 mM) in buffer (MeCN/Tris-HCl, v/v, 1:3, pH 7.4) at 37 °C. (A) absorption spectral changes of **BoS**, (B) absorption spectral changes of **BmS**, (C) absorption spectral changes of **BpS**.

Detection Limit

The detection limit was calculated based on the fluorescence titration. The linear response (Y = 80.6 X + 140.0 with R^2 = 0.995) of fluorescent intensity (y) with respect to the concentration of NTR (x) was established. The lower detection limit (LDL) was calculated following equation. LDL = 3σ/k (σ = 0.588), The corresponding detection limit was as low as 0.022 μg/mL.

The BoS exhibited high specificity for NTR activity

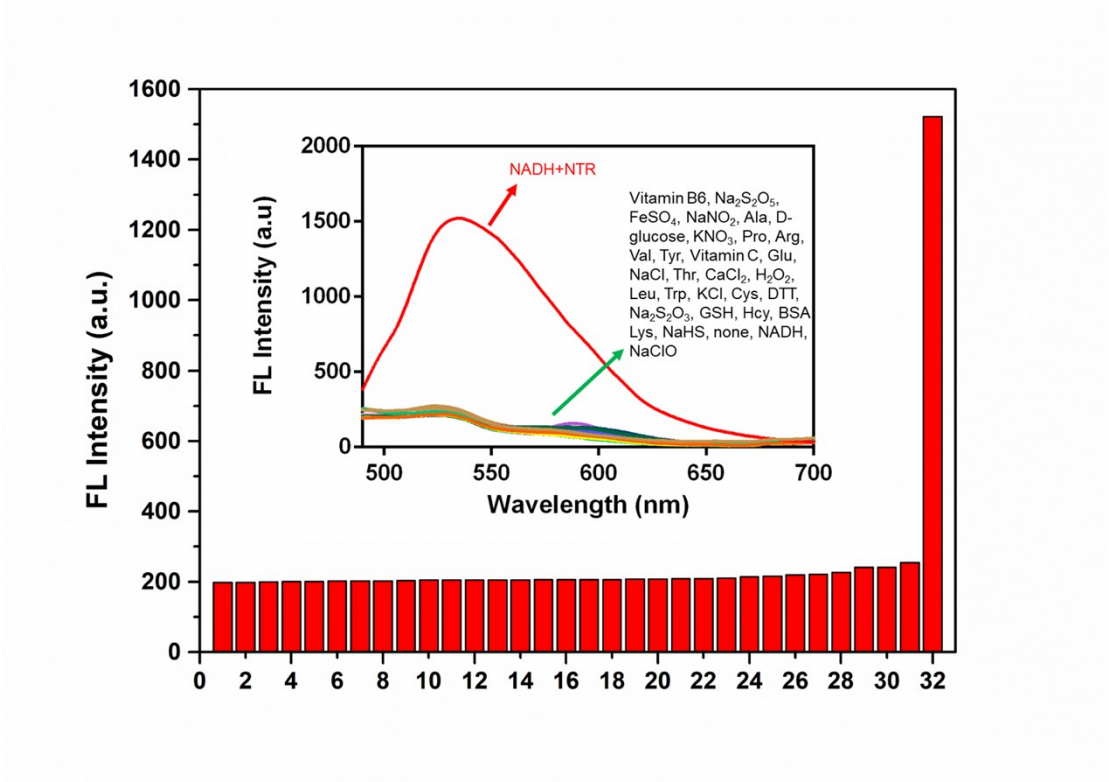


Figure S2 Probe **BoS** (10 μM) exhibited high specificity for NTR activity in buffer (25% MeCN/Tris-HCl, pH 7.4) at 37 °C. The fluorescence intensity at 540 nm was selected for the selectivity discussion. (1)Vitamin B_6 (1 mM), (2) $Na_2S_2O_5$ (1 mM), (3) $FeSO_4$ (1 mM), (4) $NaNO_2$ (1 mM), (5) Ala (1 mM), (6) D-glucose (1 mM), (7) KNO_3 (1 mM), (8) Pro (1 mM), (9) Arg (1 mM), (10) Val (1 mM), (11) Tyr (1 mM), (12) Vitamin C (1 mM), (13) Glu (1 mM), (14) NaCl (1 mM), (15) Thr (1 mM), (16) $CaCl_2$ (1 mM), (17) H_2O_2 (1 mM), (18) Leu (1 mM), (19) Trp (1 mM), (20) KCl (1 mM), (21) Cys (1 mM), (22) DTT (1 mM), (23) $Na_2S_2O_3$ (1 mM), (24) GSH (1 mM), (25) Hcy (1 mM), (26) BSA (1 mM), (27) Lys (1 mM), (28) NaHS (1 mM), (29) none, (30) NADH (0.5 mM), (31) NaClO (1 mM), (32) NADH (0.5 mM)+NTR (10 μg/mL).

NTR-induced the fluorescence enhancement of BoS in the absence or presence of inhibitor

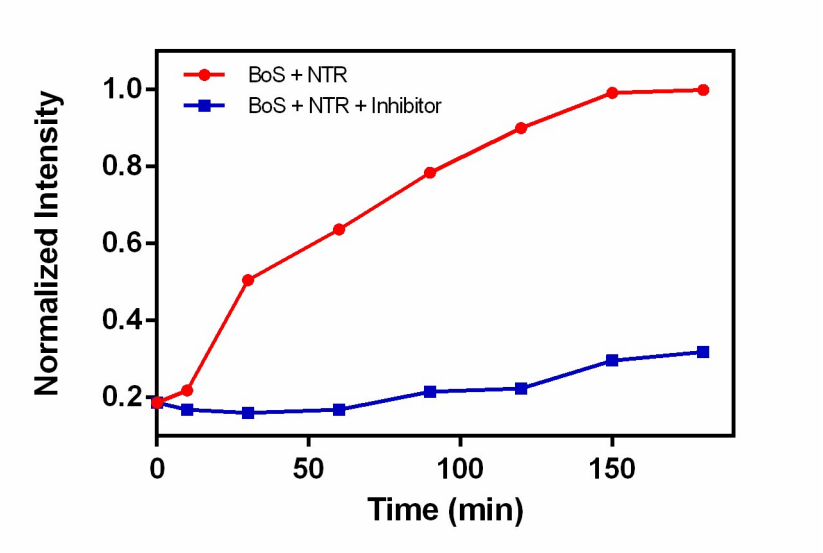


Figure S3 Time dependent fluorescence changes (normalized) at 540 nm of **BoS** in the absence or presence of inhibitors in buffer (MeCN/Tris-HCl, v/v, 1:3, pH 7.4) at 37 °C. Probe: 10 μM; NTR: 10 μg/mL; NADH: 0.5 mM; the inhibitor: NTR + NADH + 0.5 mM dicoumarol.

Fluorescence quantum yields

Table S1 The fluorescence quantum yields of the designed probes.

Compound	**BoS**	**BmS**	**BpS**
Φ_{probe}	0.4%	1.7%	1.5%
$\Phi_{reaction\ with\ NTR}$	1.2%	1.5%	1.3%

Note: The fluorescence quantum yields for **BoS**, **BmS** and **BpS** were obtained in solution of

MeCN/Tris-HCl, v/v, 1:3, pH 7.4.

The chemical stability

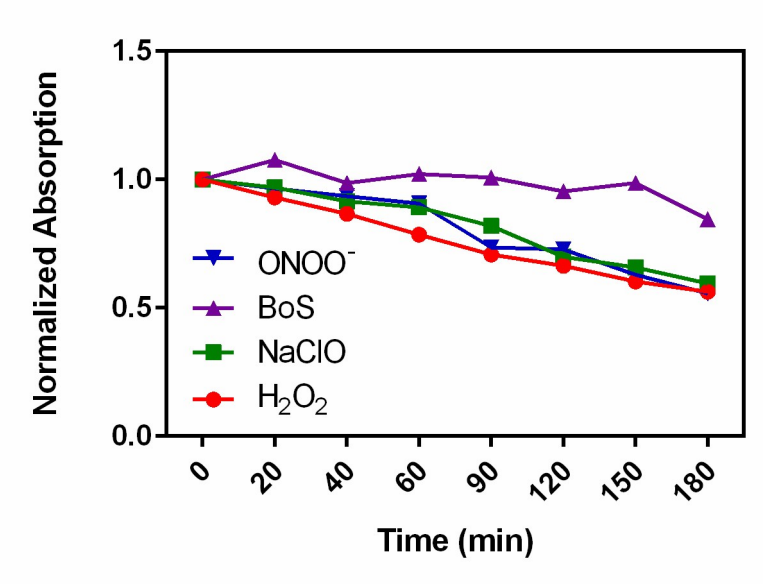


Figure S4 Time course of absorption changes (normalized) of **BoS** after exposure to H_2O_2, ClO^- and $ONOO^-$ in buffer (25% MeCN/Tris-HCl, pH 7.4) at 37 °C: λ_{ab} = 500 nm, **BoS**: 10 μM, H_2O_2, ClO^- and $ONOO^-$: 1mM.

Cell culture and imaging

Hela cells and HepG-2 cells were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% FBS at 37 °C in humidified environment of 5% CO_2. Hela cells and HepG-2 cells were grown on glass-bottom culture dishes in a humidified incubator under normoxic (20% O_2, 5% CO_2) and different hypoxic conditions, respectively. Before use, the adherent cells were washed with PBS (1 mL × 3 times). For fluorescence imaging, the cells were further incubated with **BoS** (10 μM) in DMEM at 37 °C for 30 min under the respective conditions and then washed three times with the PBS buffer (pH 7.4) to remove the free probe.[4] For assay the inhibitory effects of

dicoumarol: Hela cells and HepG-2 cells were pretreated with 1 mM dicoumarol for 1 h, followed by incubation with **BoS** (10 μM) for 30 min. Fluorescence imaging was performed with Carl Zeiss LSM710 with a 60 x oil objective. Green fluorescence was excited at 488 nm and emission was collected at 500-580 nm.

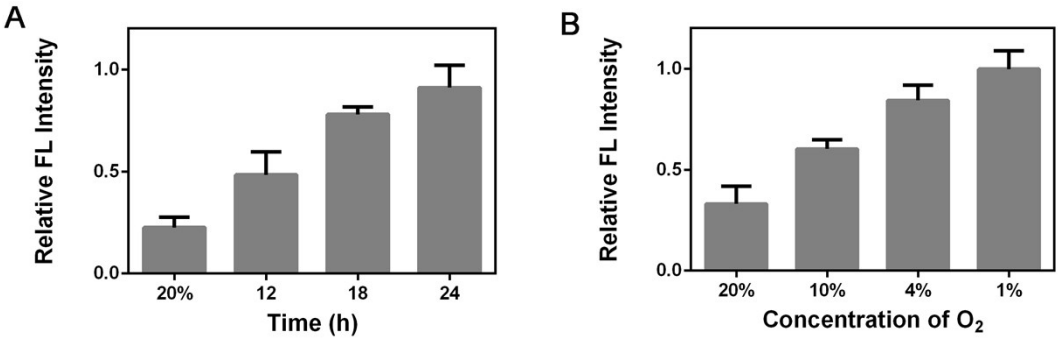


Figure S5 Fluorescence intensities obtained from the images of Hela cells. (A) The Hela cells were grown under normoxic (20% O_2) and hypoxic conditions (1% O_2) for 12 h, 18 h and 24 h, and then incubated with 10 μM probe **BoS** for 30 min under hypoxic conditions (1% O_2), cell images, n=3. (B) The Hela cells were grown under normoxic (20% O_2) and different hypoxic conditions (10%, 4%, and 1% O_2) for 24 h, and then incubated with 10 μM probe **BoS** for 30 min under the respective conditions, The cell images, n=3. The strongest fluorescence intensity from the image of Hela cells under the hypoxic condition of 1% O_2 for 24 h is defined as 1.0.

CCK-8 assays

HeLa cells and HepG-2 cells were seeded in 96-well microplates in DMEM medium supplemented with 10% FBS at 37 °C in humidified environment of 5% CO_2. After 12 h of cell attachment, the plates were washed with PBS, followed by addition of increasing concentrations of probe **BoS** (0-50 μM) in DMEM. The cells were then incubated at 37 °C in humidified environment of 5% CO_2 for 24 h, followed by standard CCK-8 assays.

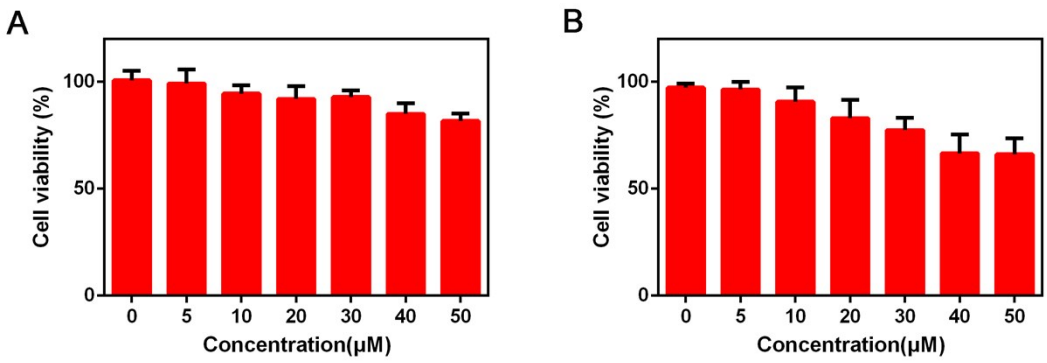


Figure S6 The cytotoxicity of **BoS** toward living cells by CCK-8 assay. (A) Hela cells with various concentrations of **BoS** for 24 h. (B) HepG-2 cells with various concentrations of **BoS** for 24 h.

***In vivo* imaging**

All animal experiments were carried out in compliance with the relevant laws and institutional guidelines for the Care and Use of Research Animals established by Fudan University, and the experimental protocols and procedures were approved by the committee. HepG-2 cells (5×10^7/mL) suspended in 100 μL PBS were implanted subcutaneously into the indicated location in male mice to obtain the HepG-2 subcutaneous xenograft nude mice. When the tumor was about 50 mm^3, *in vivo* fluorescence imaging was performed for targeted cancer visualization. Probe **BoS** (100 nmol) in Tris-HCl were administered to the tumor regions and normal sites via intratumoral injection. Images were acquired at various time points after injection. NIR-I image was collected using the IVIS spectrum imaging system, 460 nm was used as the excitation wavelength and the fluorescence signals were collected between 560 nm-580 nm.

The Costaining imaging

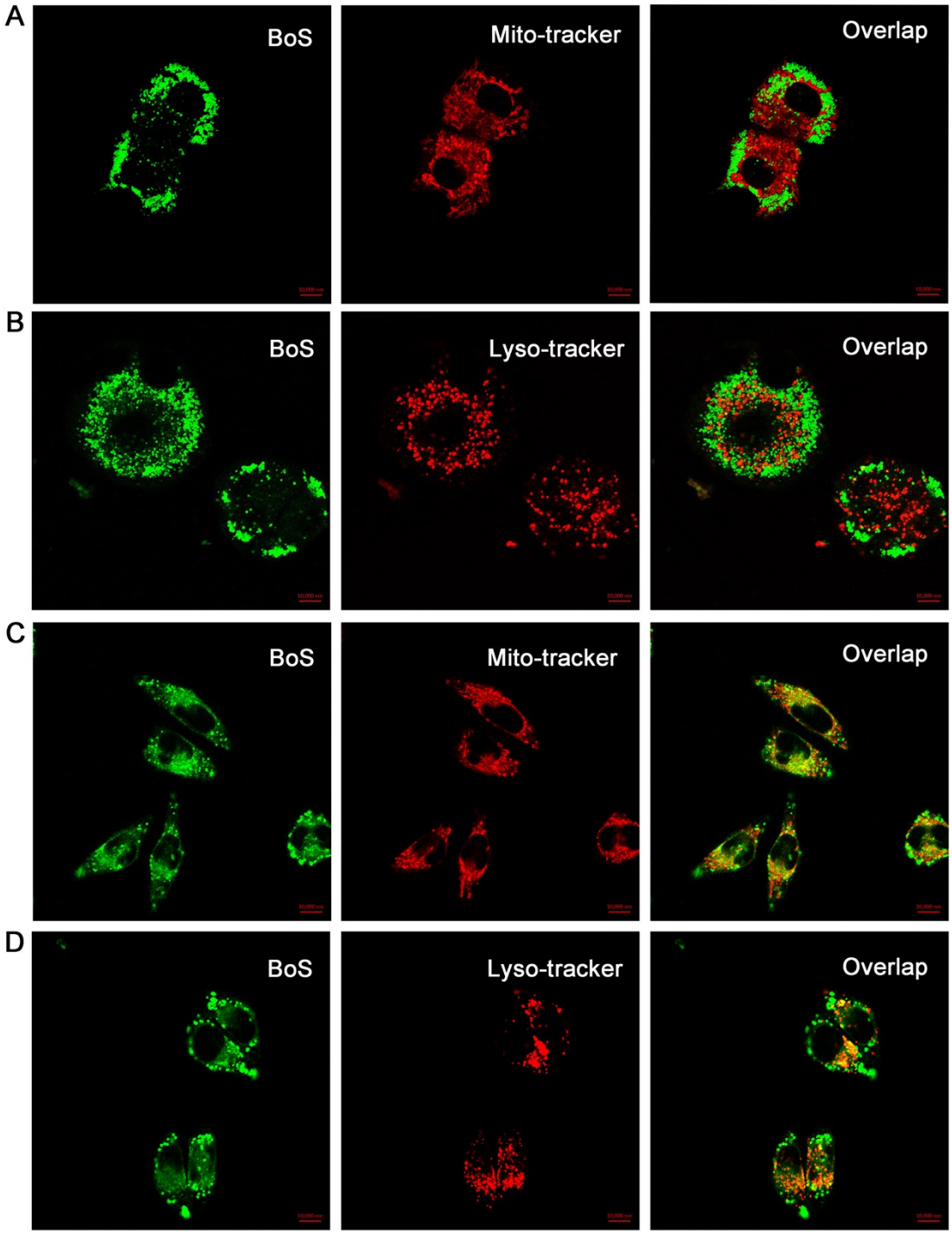


Figure S7 Intracellular localization of **BoS** with confocal fluorescence images in HeLa and HepG-2 cells. The cells were grown under hypoxic conditions (1% O_2) for 24 h, and then incubated with 10 μM probe **BoS** for 30 min under hypoxic condition, then costained with Mito-Tracker Deep Red FM (100 nM) in DMEM medium for 15 min, or LysoTracker Deep Red (50 nM) in DMEM medium

for 30 min. (A-B) HepG-2 cells, (C-D) HeLa cells. Green channel at 500-580 nm with λ_{ex} = 488 nm, red channel at 650-750 nm with λ_{ex} = 633 nm. The scale bar is 10 μm.

^{1}H NMR and ^{13}C NMR characterizations.

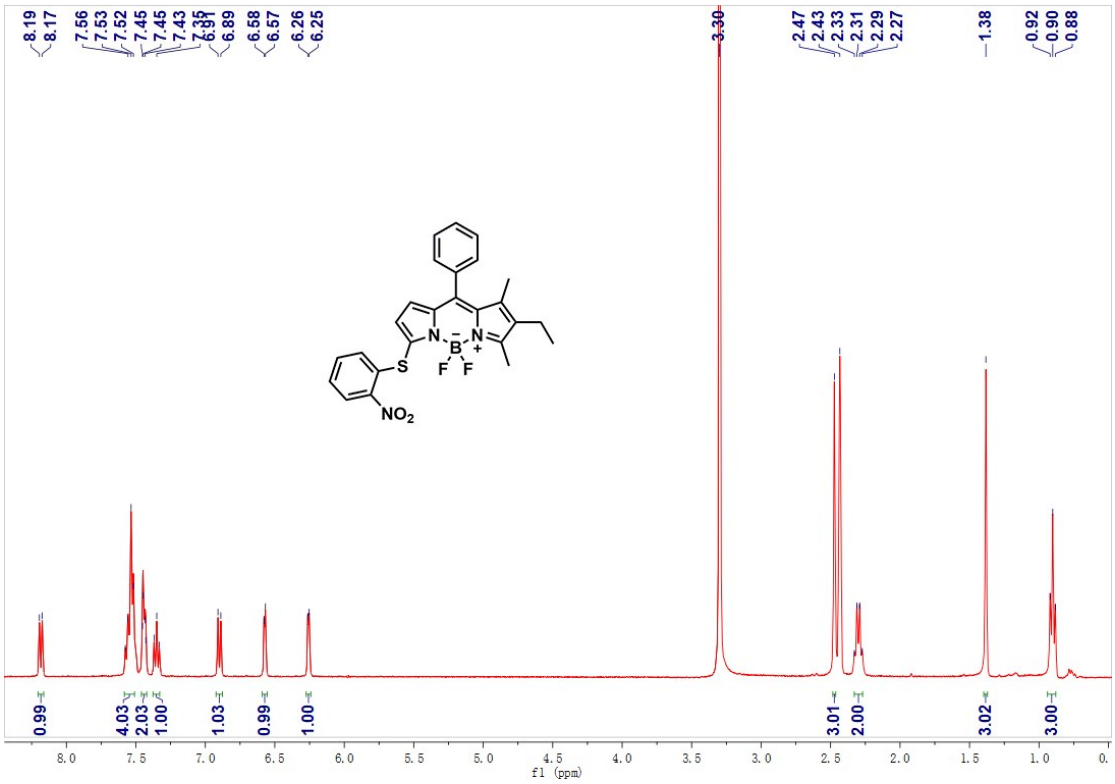


Figure S8 ^{1}H NMR spectrum for compound **BoS.**

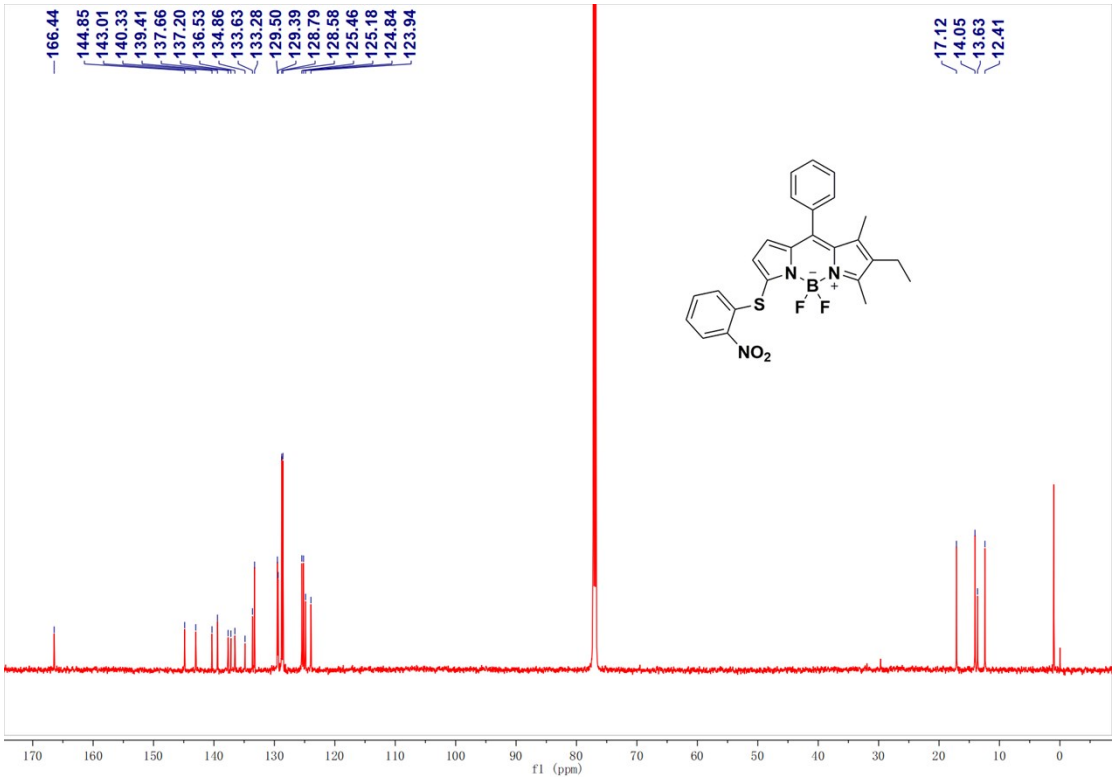


Figure S9 ^{13}C NMR spectrum for compound **BoS**.

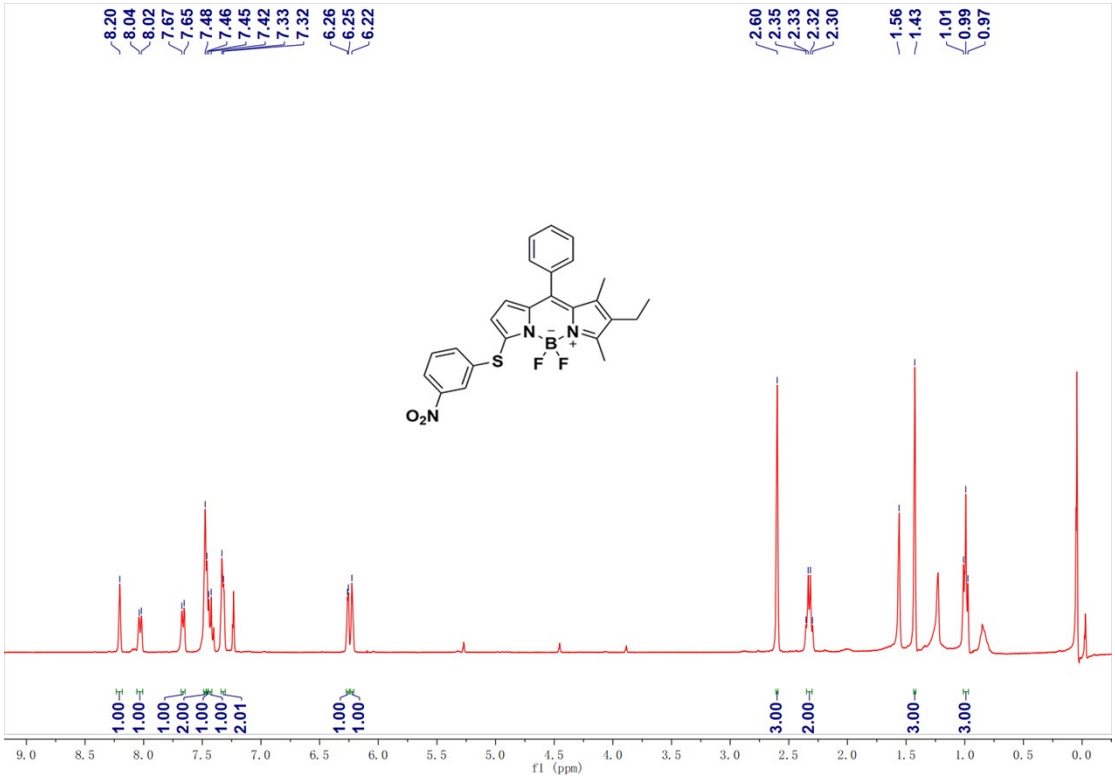


Figure S10 ^{1}H NMR spectrum for compound **BmS.**

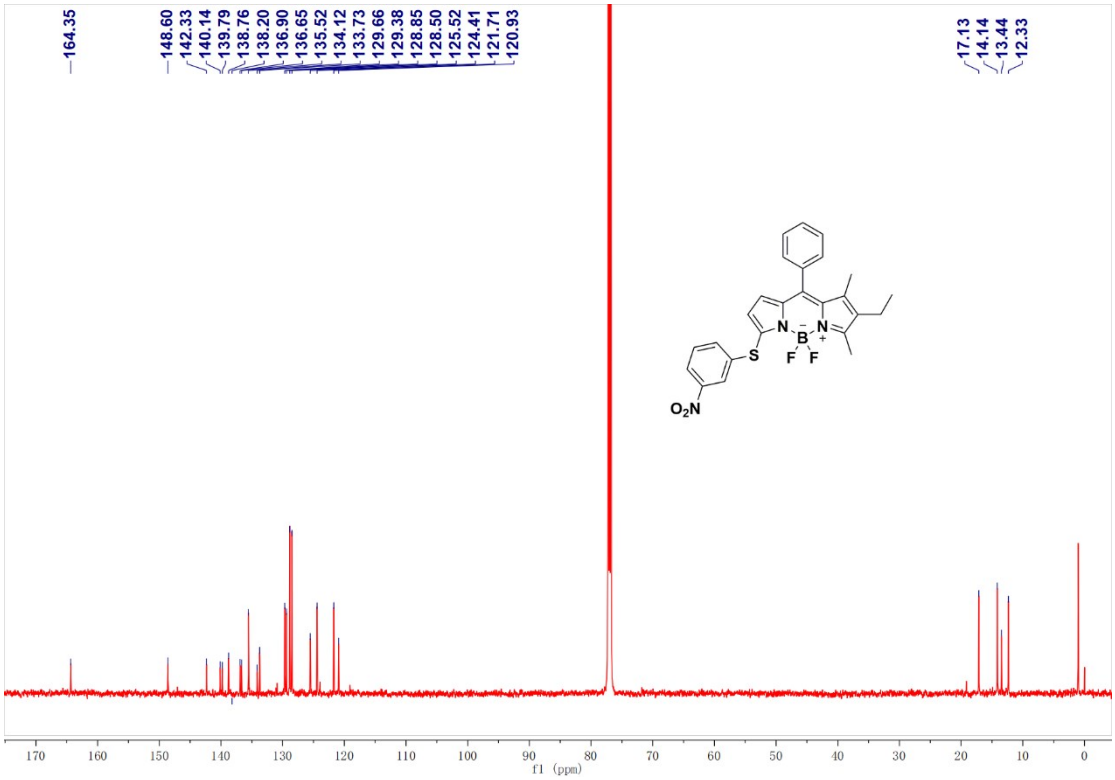


Figure S11 ^{13}C NMR spectrum for compound **BmS.**

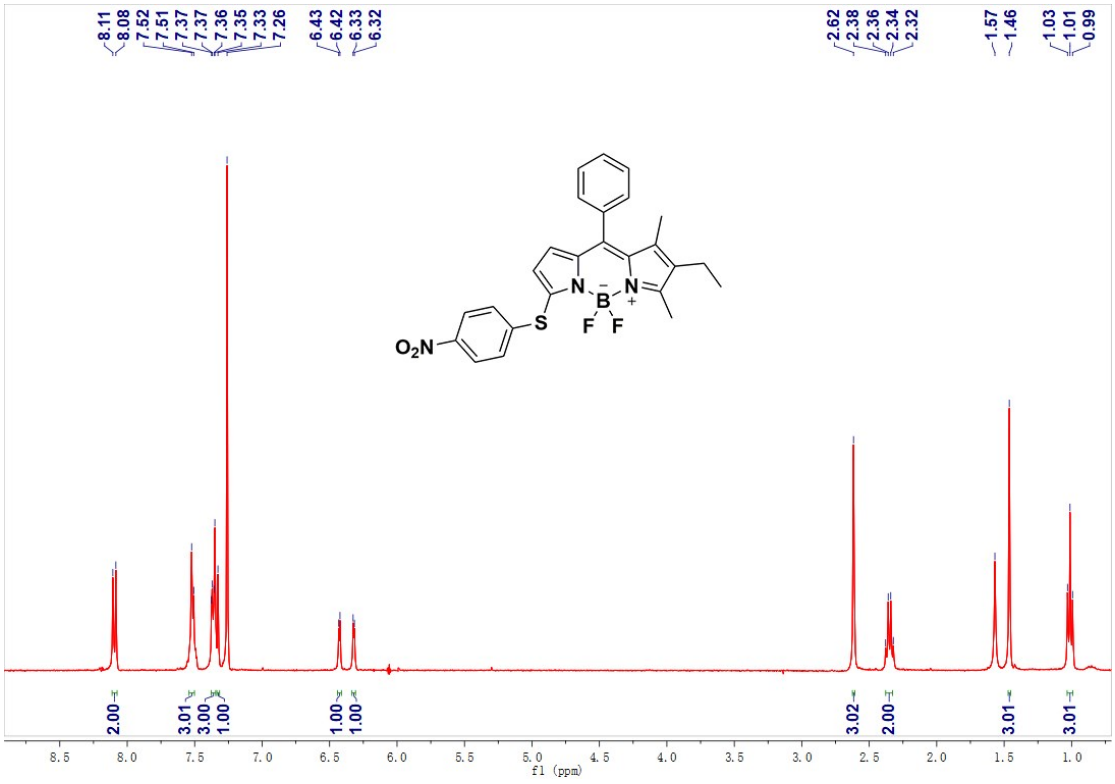


Figure S12 ^{1}H NMR spectrum for compound **BpS**.

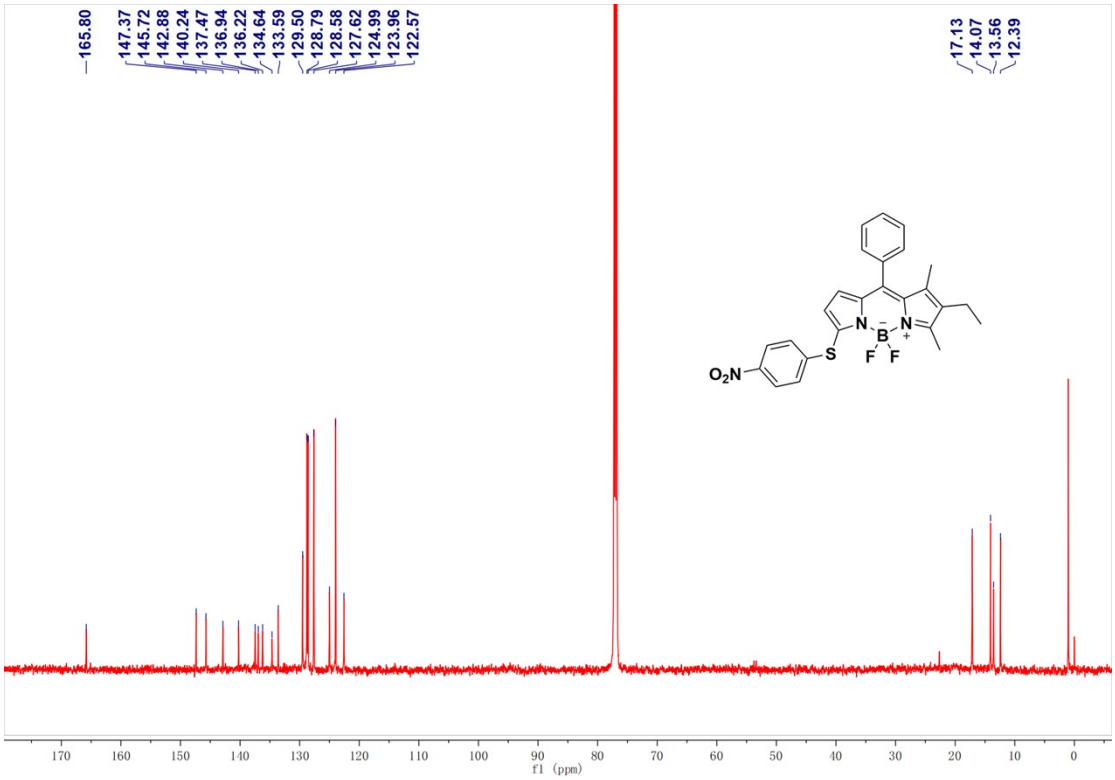


Figure S13 ^{13}C NMR spectrum for compound **BpS**.

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