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Supporting Information

Thiobenzophenones: tunable hydrolysis-based donors for intracellular H_2S delivery

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1. Reagents and instruments

All chemicals and solvents used for synthesis were purchased from commercial suppliers and applied directly in the experiments without further purification. The progress of the synthetic reactions was monitored by TLC on pre-coated silica plates (Merck 60F-254, 250 μm in thickness), and spots were visualized by UV light. Merck silica gel 60 (70-200 mesh) was used for general column chromatography purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm) or residual solvent peaks ($CDCl_3$ = 7.26 ppm, DMSO-d_6 = 2.50 ppm). ^{1}H NMR coupling constants (J) are reported in Hertz (Hz), and multiplicity is indicated as the following: s (singlet), d (doublet). High-resolution mass spectrum (HRMS) was obtained on an Agilent 6540 UHD Accurate-Mass Q-TOFLC/MS. The UV-visible spectra were recorded on a UV-6000 UV-VIS-NIR-spectrophotometer (METASH, China). Fluorescence studies were carried out using F-280 spectrophotometer (Tianjin Gangdong Sci & Tech., Development. Co., Ltd). ANGELA TECHNOLOGIES HPLC LC-10F was employed for HPLC analysis. The cellular bioimaging was carried out on confocal microscopes (Olympus FV 1000).

2. Synthetic procedures of donors 1 and 2

Synthesis of 4,4'-diaminothiobenzophenone (1). To a solution of 4,4'-diaminobenzophenone (106 mg, 0.5 mmol) in tetrahydrofuran (20 mL), Lawesson's Reagent (LR, 202 mg, 0.5 mmol) was added at room temperature.[1] The mixture was stirred at 60 ℃ for 4 h under nitrogen gas protection. After cooling and filtration, the filtrate was evaporated under reduced pressure to remove the solvent. The residue was purified by flash column chromatography, eluting with 10-50% ethyl acetate/petroleum ether to give a dark red solid product **1** (89 mg, 78%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.45 (d, J = 8.7 Hz, 4H), 6.52 (d, J = 8.7 Hz, 4H), 6.16 (s, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 226.2, 153.4, 135.3, 132.9, 112.1. HRMS (ESI): m/z $[M+H]^+$ calculated for $C_{13}H_{12}N_2S^+$: 229.0794; found: 229.0801.

Synthesis of 4,4'-dimethoxythiobenzophenone (2). To a solution of 4,4'-dimethoxybenzophenone (121 mg, 0.5 mmol) in toluene (20 mL), LR (202 mg, 0.5 mmol) was added at room temperature. The mixture was stirred at 90 ℃ for 4 h under nitrogen gas protection. After cooling and filtration, the filtrate was evaporated under reduced pressure to remove the solvent. The residue was purified by flash column chromatography, eluting with 10-30% ethyl acetate/petroleum ether to give a blue solid product **2** (94 mg, 73%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.73 (d, J = 8.8 Hz, 4H), 6.88 (d, J = 8.8 Hz, 4H), 3.88 (s, 6H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 233.4, 163.2, 140.9, 132.2, 113.3, 55.6. HRMS (ESI): m/z $[M+H]^+$ calculated for $C_{15}H_{15}O_2S^+$: 259.0787; found: 259.0790.

3. General procedure for spectroscopic and HPLC studies

All measurements were performed in degassed phosphate buffer (PBS, 50 mM, pH 7.4, containing 50% CH_3CN). Compounds were dissolved into CH_3CN to prepare the stock solutions with concentrations of 5 mM. The reaction mixture was shaken uniformly before spectra were measured.

For HPLC studies, thiocamphor (0.3 mM) in PBS (50 mM, pH = 7.4; 50% CH_3CN) was analyzed by HPLC at different reaction time. Conditions: ANGELA TECHNOLOGIES HPLC LC-10F; C18 column with 4.6 mm x 250 mm; flow 1.0 mL min^{-1}; buffer a: 0.1% trifluoroacetic acid in water; buffer b: MeOH. Elution condition: 0-3 min, buffer b: 5-80%; 3-20 min, buffer b: 80-95%; 20-25 min, buffer b: 95-5%. Detection wavelength: 250 nm.

4. Methylene blue assay

H_2S concentration was determined via the widely used methylene blue assay (MBA).[2] The stock solutions of 1% $Zn(OAc)_2$ in water, 30 mM $FeCl_3$ in 1.2 M HCl, 20 mM *N*,*N*-dimethyl-*p*-phenylene diamine in 7.2 M HCl were prepared. The methylene blue cocktail solution was freshly prepared by mixing $Zn(OAc)_2$, $FeCl_3$,

and *N*,*N*-dimethyl-p-phenylene diamine solutions with a 1:2:2 ratio (v:v:v). A solution (1.8 mL) containing 0.9 mL of the methylene blue cocktail and 0.9 mL degassed PBS in cuvettes was used as MBA background solution. A 250 mM stock solution of Na_2S in degassed PBS was prepared on ice and diluted to 10 mM. Immediately after dilution, Na_2S solutions was added to the 1.8 mL MBA to give final concentrations of 10, 20, 30, 40, and 50 μM, respectively. Solutions were mixed thoroughly, and incubated at 25 ℃ for 1 h. Absorbance at 670 nm were measured for the H_2S calibration curve.

5. Cell culture, cell cytotoxicity assay and bioimaging

HeLa (human cervical cancer) cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM medium with 10% fetal bovine serum, and 1% penicillin/streptomycin, at 37 ℃ in a 5% CO_2 atmosphere.

The cell-trappable H_2S probe **AM-BODIPY-NBD** developed by our group was used to visualized the H_2S releasing by donor **1** via cell imaging.[3] Briefly, the cover-slips were added to a 24-well plate before cells were seeded. Then, HeLa cells were seeded into 24-well plate with a density of 8×10^4 cells/well and cultured for overnight. After that, cells were pretreated with 10 μM **AM-BODIPY-NBD** for 0.5 h, and incubated with donor **1** for 4h. Then the cells were washed with PBS three times and then fixed with 4% paraformaldehyde solution for 10 min. Following the 4% paraformaldehyde fixing, the cells was stained with DAPI for 7 min. Finally, the cells were washed with PBS and imaged on a confocal microscope (Olympus FV1000) with a 20×objective lens. Emission was collected at the green channel (500-550 nm) with 488 nm excitation.

The cytotoxicity of donor **1** was assessed by Cell Counting Kit-8 (CCK-8) cell viability assay. Briefly, the HeLa cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and grown to confluence. Then, varying concentrations of donor **1** were added to the cells and incubation for 24 h. After that, the culture medium was

refreshed with medium containing CCK-8 solution (10 μL/well) and incubated with cells for 2 h at 37 ℃. Then, absorbance of each well was measured by a microplate reader at 450 nm.

Evaluation of antioxidant activity of donor 1. The HeLa cells were seeded into a 96-well plate at a density of 1×10^4 cells/well and cultured for 24 h. Then cells were incubated 200 μM H_2O_2 mixed with different concentrations (0, 50, 75, 100 μM) of donor **1** dissolved in fresh culture medium for 2 h. Subsequently, the culture medium was replaced with fresh medium containing CCK-8 solution (10 μL/well) and incubated with cells for 2 h at 37 ℃. After that, the absorbance of each well was measured by a microplate reader at 450 nm.

6. References

1. R. J. Hewitt, M. J. H. Ong, Y. W. Lim and B. A. Burkett, *Eur. J. Org. Chem.*, 2015, **2015**, 6687-6700.

2. M. M. Cerda, Y. Zhao and M. D. Pluth, *J. Am. Chem. Soc.*, 2018, **140**, 12574-12579.

3. H. Ye, L. Sun, Z. Pang, X. Ji, Y. Jiao, X. Tu, H. Huang, X. Tang, Z. Xi and L. Yi, *Anal. Chem.*, 2022, **94**, 1733-1741.

7. Supplementary figures

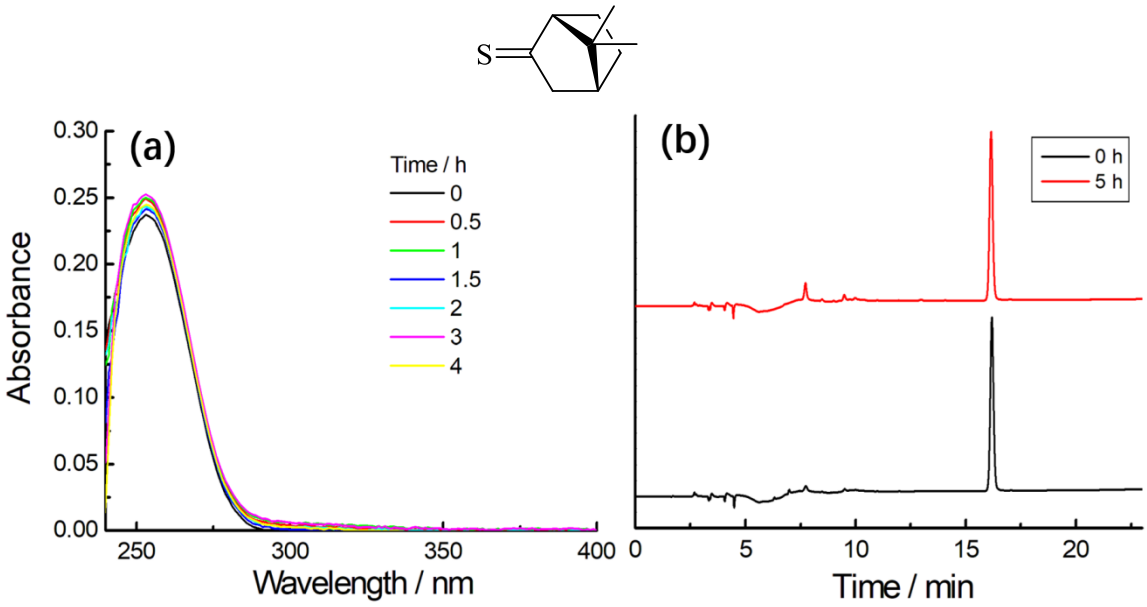

Fig. S1 Time-dependent absorbance spectra of thiocamphor (30 μM) (a) and HPLC traces of thiocamphor (300 μM) (b) in PBS buffer (pH 7.4, 50 mM; containing 30% DMSO).

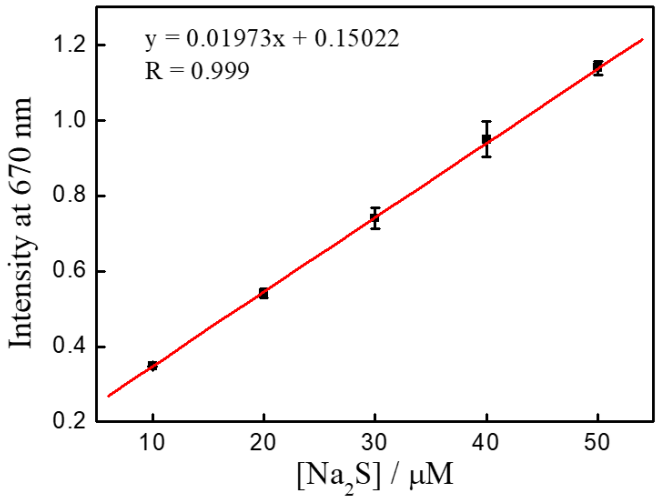

Fig. S2 H_2S calibration curve for the MB assay. An Na_2S stock solution (10 mM) was added to fresh MB solution to make the final H_2S concentrations of 10, 20, 30, 40 and 50 μM. The MB solution was allowed to react with H_2S for 1 h before measuring the absorbance at 670 nm.

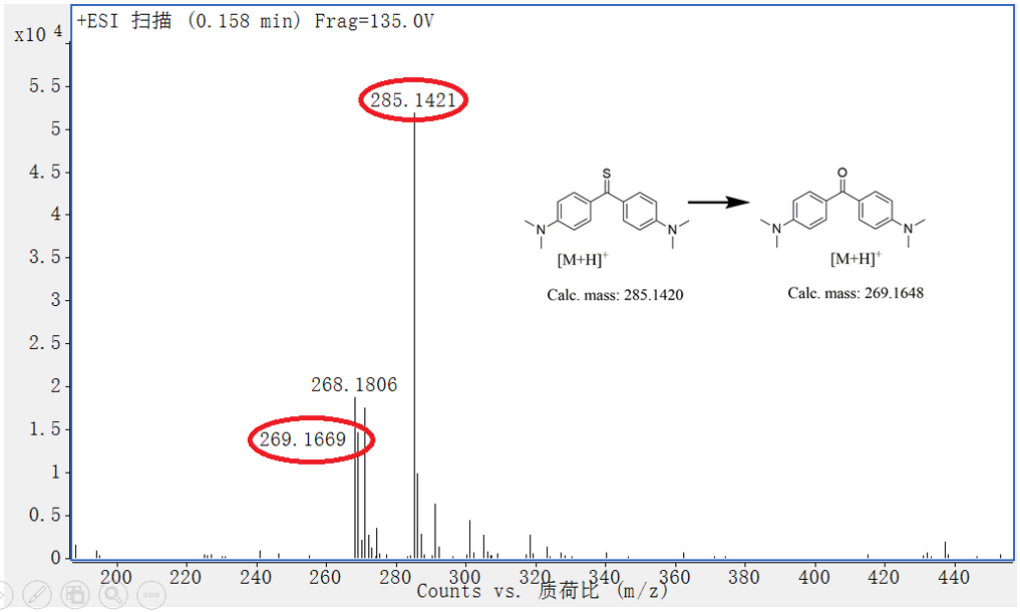

Fig. S3 HRMS spectra of thiomichler's ketone (5 mM) for 4 h in PBS buffer (pH = 7.4).

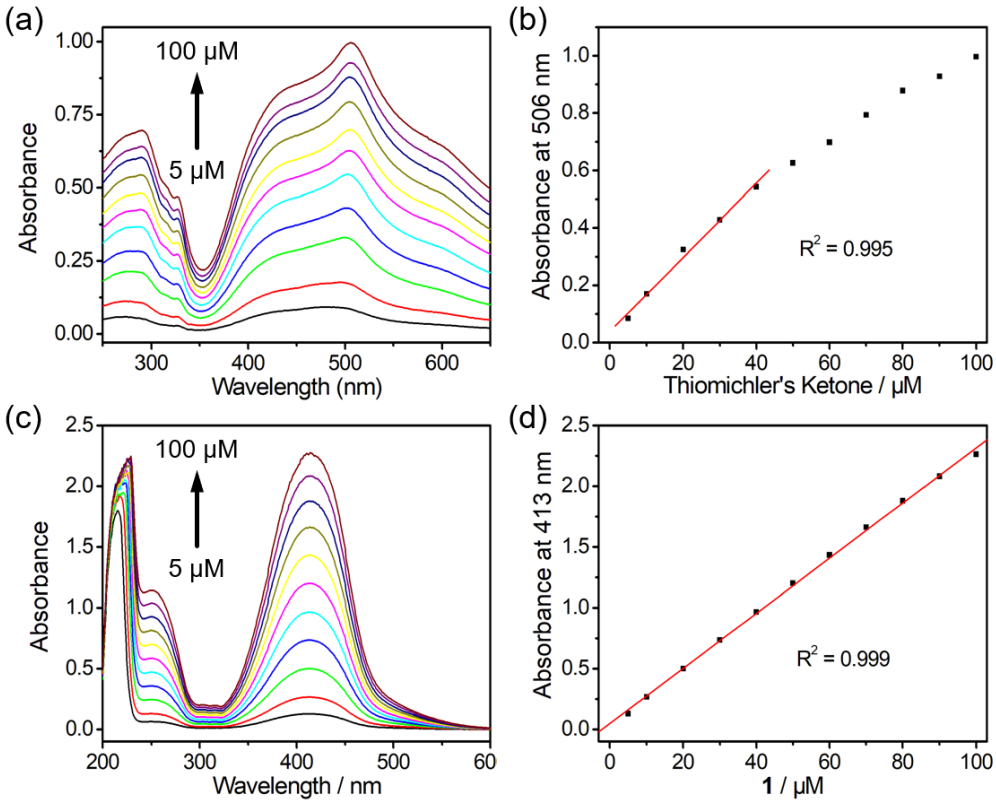

Fig. S4 (a, c) Concentration-dependent absorption spectra of thiomichler's ketone and donor **1** in PBS buffer (50 mM, pH = 7.4). (b, d) The linear relationship between absorbance of thiomichler's ketone at 506 nm and its concentration (b), of donor **1** at 413 nm and its concentration (d). For thiomichler's ketone, the linear range was 0-40 μM; for donor **1**, the linear range was at least 0-100 μM.

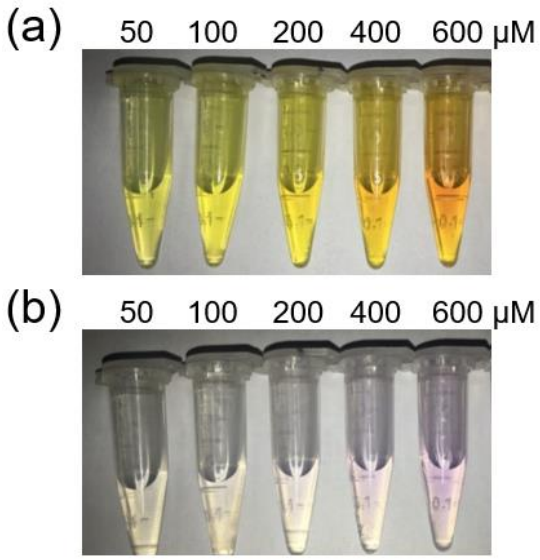

Fig. S5 Clarity of different concentrations of donor **1** (a) and donor **2** (b) solutions in PBS buffer (50 mM, pH = 7.4) presented by photos. Both donors are soluble up to 400 μM, and a small number of insoluble substances appeared at 600 μM for both donors.

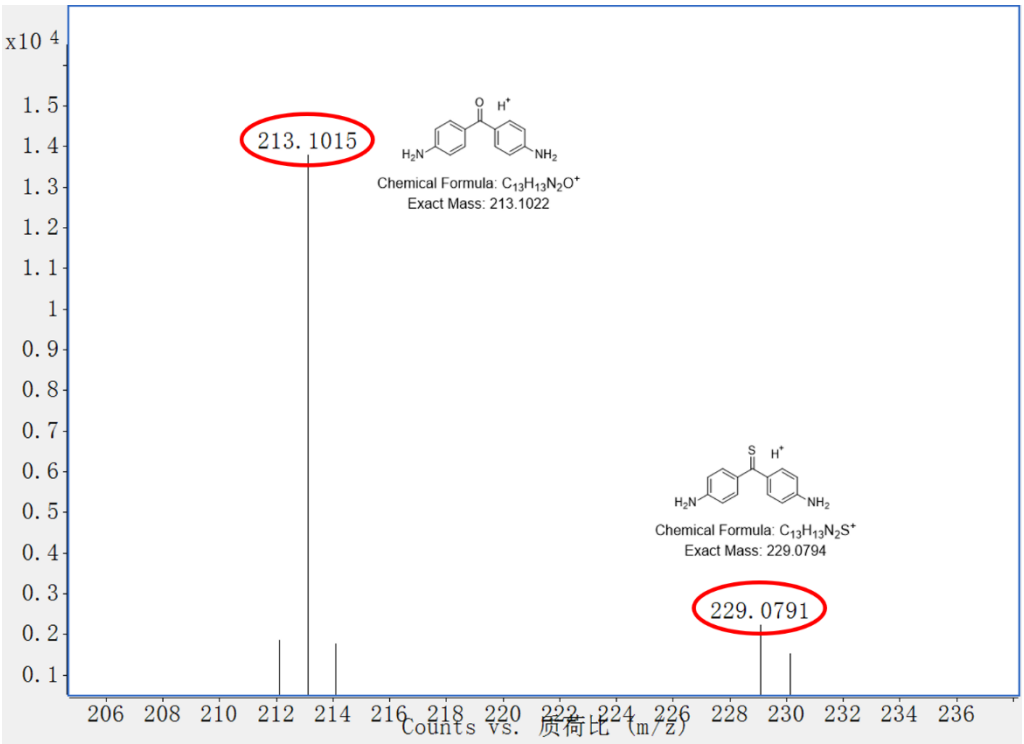

Fig. S6 HRMS spectra of donor **1** (5 mM) in PBS buffer (pH = 7.4) after overnight incubation.

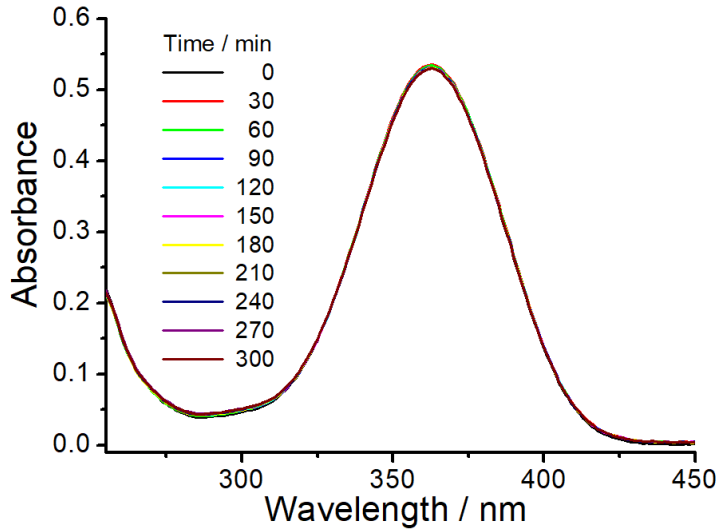

Fig. S7 Time-dependent absorbance spectra of donor **2** (30 μM) in PBS buffer (pH 7.4, 50 mM; 30% DMSO).

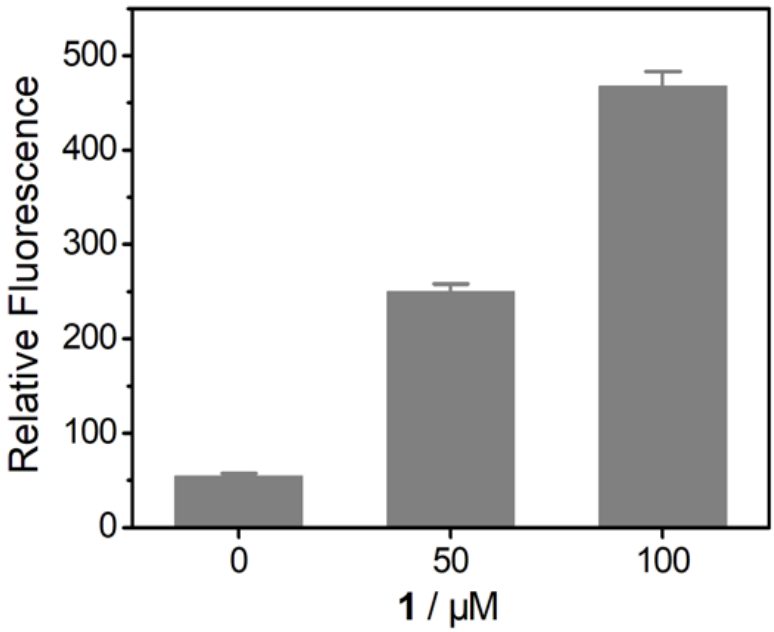

Fig. S8 Relative fluorescence intensity of images from Figure 4b. *N* = 3 fields of cells, error bars are means ± S.D.

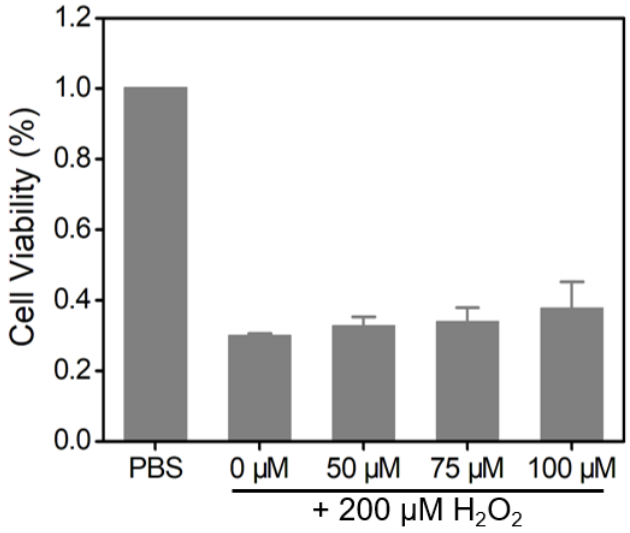

Fig. S9 Relative cell viability of HeLa cells treated with 200 μM H_2O_2 for 2 h assessed by CCK-8 assay after pre-incubated with varying concentration of donor **1** (0, 50, 75, 100 μM) for 12 h. Error bars are means ± S.D. (n = 3).

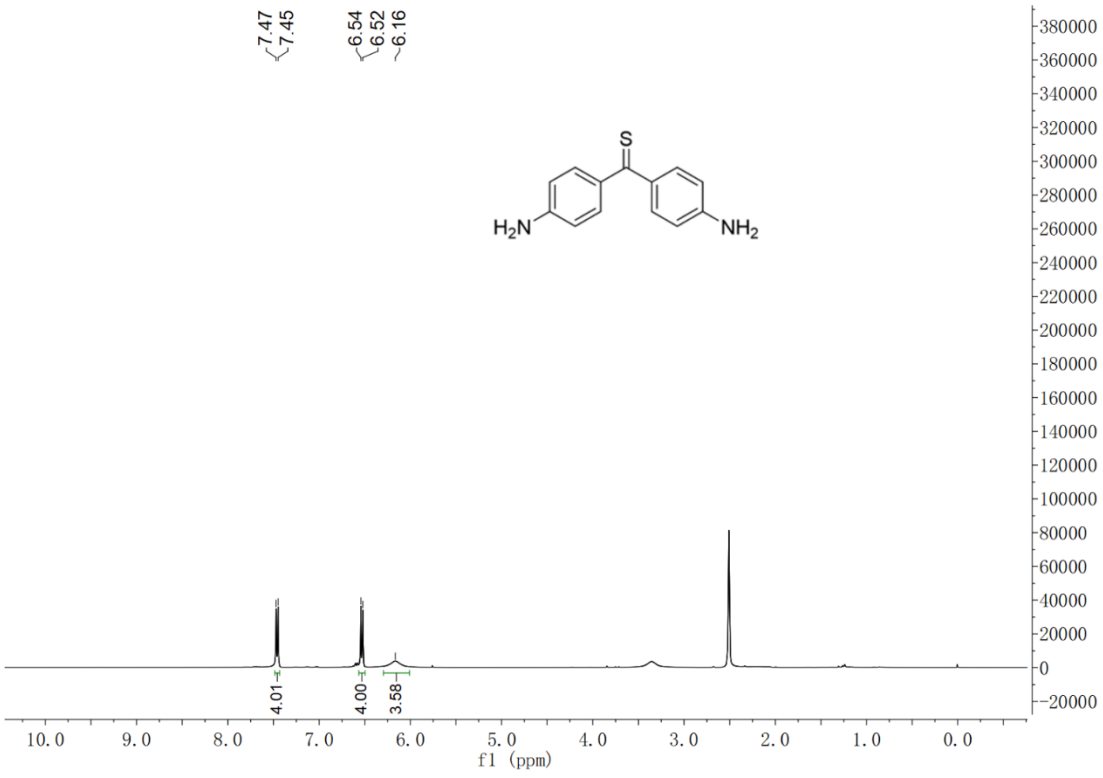

Fig. S10 ^{1}H NMR spectrum of donor **1**.

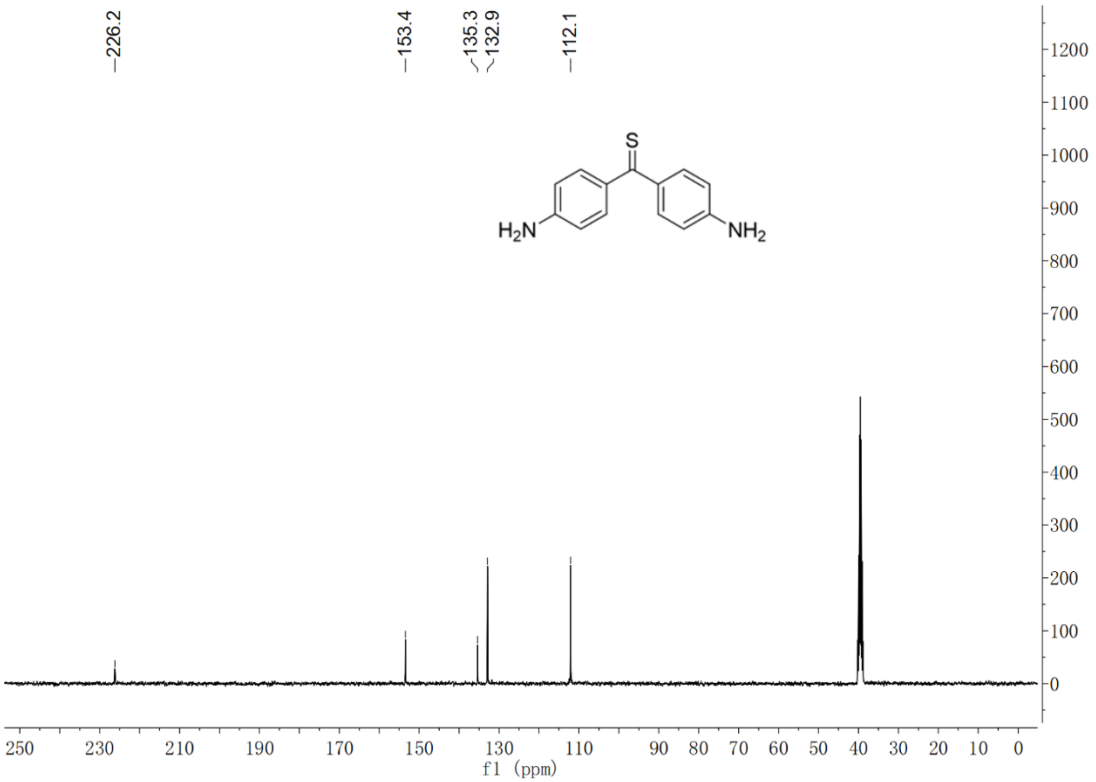

Fig. S11 ^{13}C NMR spectrum of donor **1**.

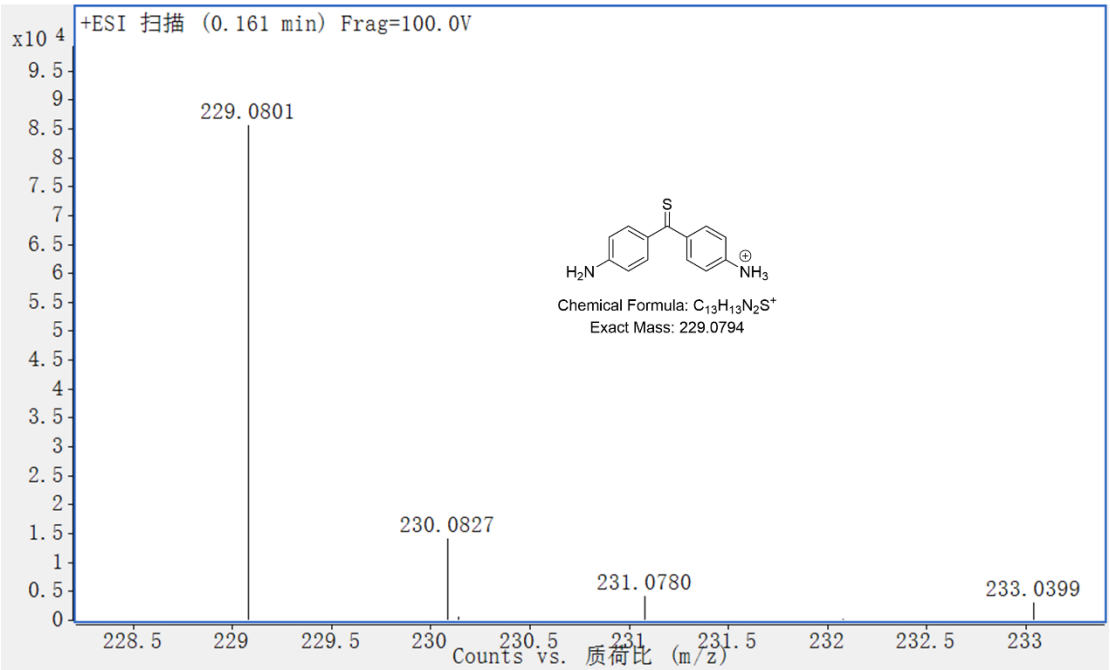

Fig. S12 HRMS spectrum of donor **1**.

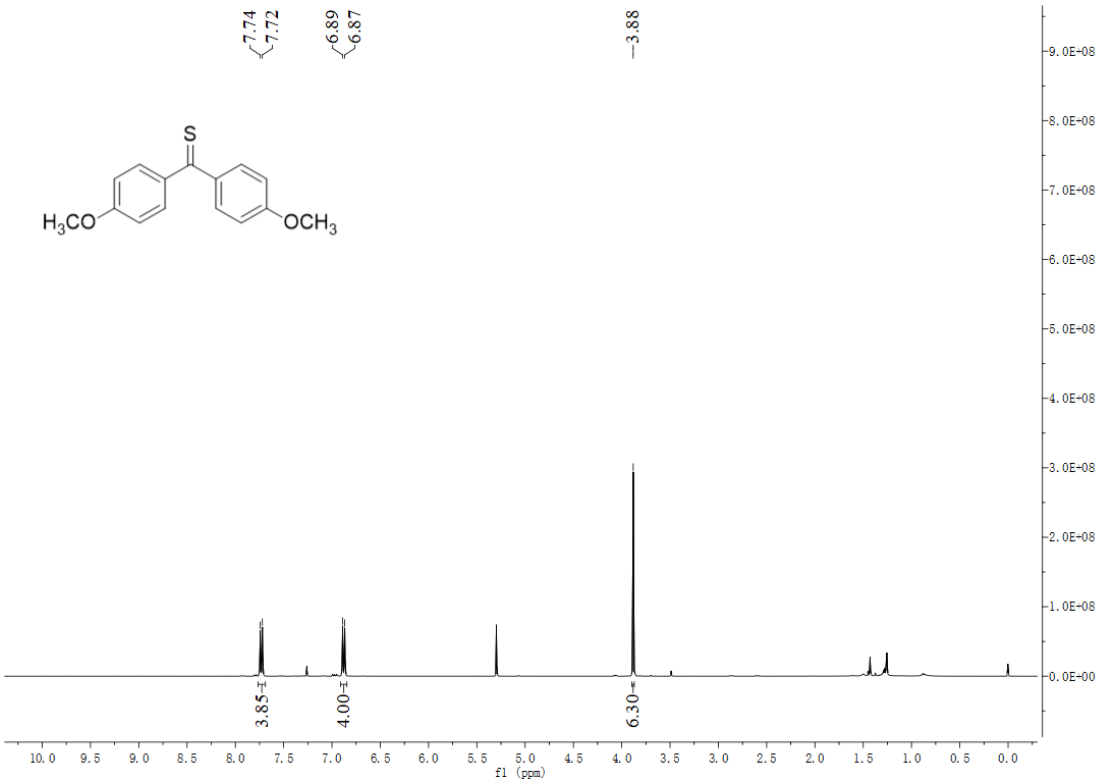

Fig. S13 ^{1}H NMR spectrum of donor **2**.

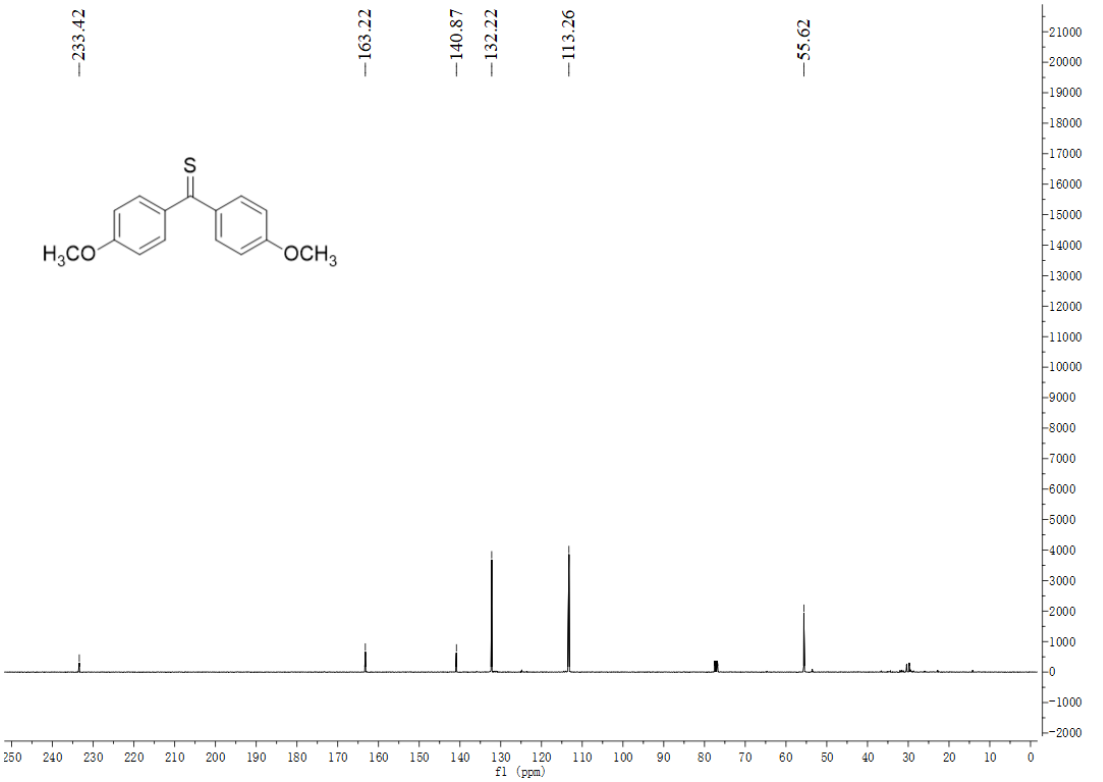

Fig. S14 ^{13}C NMR spectrum of donor **2**.

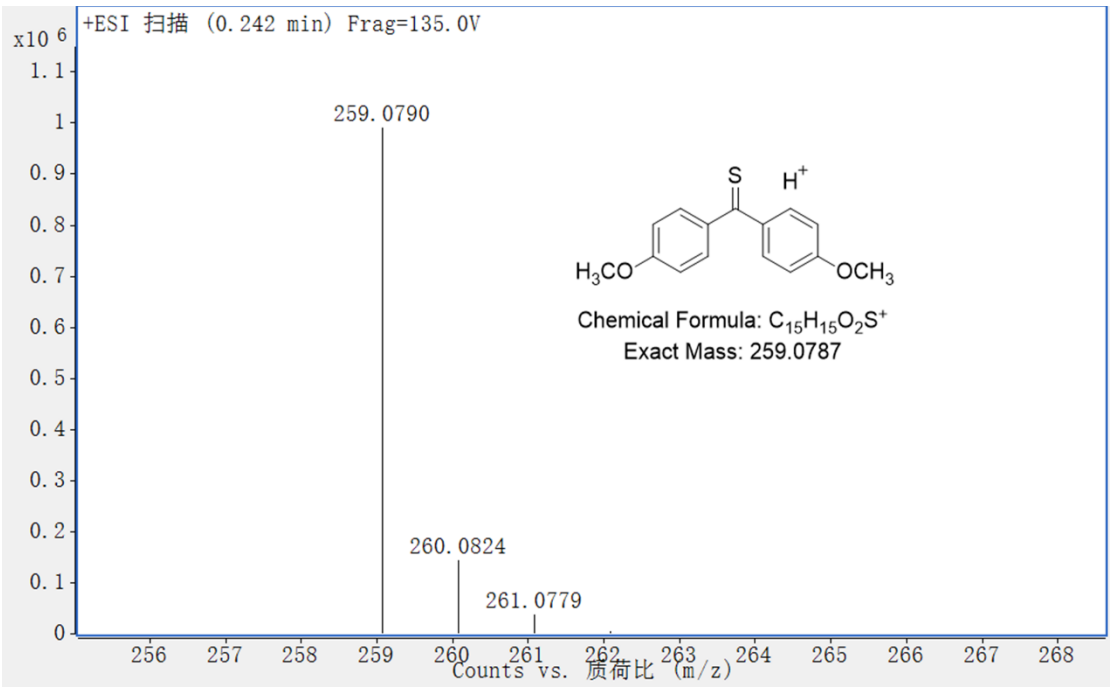

Fig. S15 HRMS spectrum of donor **2**.