Supporting Information for

Development a Two-Photon Fluorescent Probe for Imaging Hydrogen Sulfide (H_2S) in the Living Cells and Zebrafish

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Materials and instruments

All chemicals such as 4-Bromo-1,8-naphthalic Anhydride (Shanghai Macklin Biotechnology Co., Ltd.), N-Hydroxyphthalimide (NOP) (Shanghai Macklin Biotechnology Co, Ltd.) were purchased directly from the supplier and used without further purification. The solvents used in the experiments were purified by standard methods. The solvents used for chromatographic column separation, such as dichloromethane, methanol, ethyl acetate, petroleum ether, etc., were purchased from the supplier (Fuyu Chemical) without further purification. The cells used in the experiment were HepG2 cells of the fifth generation, and the culture medium used was DMEM/HIGH GLUCOSE (HyClone). The water used in the spectroscopy test was twice-distilled water. The experimental apparatus used was the same as the one used in our previous reports in the literature. TLC analysis was performed on silica gel plates and column chromatography was performed on silica gel (grid 200-300), both obtained from Qingdao Ocean Chemicals. Photoluminescence spectra were recorded with a Hitachi F4600 fluorescence spectrophotometer with a 1 cm standard quartz cell; fluorescence imaging of the cells was formed with a Leica SP8 confocal microscope. All experiments were performed in compliance with the relevant laws and institutional guidelines of the Animal Ethical Experimentation Committee of Qilu University of Technology (Shandong Academy of Sciences), and in accordance with the requirements of the National Law on the Use of Laboratory Animals (China).

Synthesis procedures

*1. Synthesis and characterization of compound **2***

Mix 4-bromo-1,8-naphthalic anhydride (1.1 g, 4.0 mmol, 1.0 eq.) with ethylamine (70 % aqueous solution, 0.28 mL, 4.8 mmol, 1.2 eq.) and dichloromethane (50 mL) in a 250 mL round-bottom flask and react at room temperature for 7 h. At the end of the reaction, pour the reaction solution into a certain amount of distilled water, stir well, and then filter under reduced pressure, and the filtrate is washed with

distilled water three times. The residue was washed 3 times with distilled water, and the residue was collected and placed in a condensation drier to remove water. Finally, compound **2** was obtained. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.68 (d, J = 7.3 Hz, 1H), 8.59 (d, J = 8.5 Hz, 1H), 8.44 (d, J = 7.8 Hz, 1H), 8.06 (d, J = 7.8 Hz, 1H), 7.86 (dd, J = 8.5, 7.3 Hz, 1H), 4.26 (q, J = 7.1 Hz, 2H), 1.35 (t, J = 7.1 Hz, 3H).

*2. Synthesis and characterization of compound **3***

Compound **2** (500mg, 1.65 mmol, 1.0 eq.) and N-hydroxyphthalimide (324 mg, 1.98 mmol, 1.2 eq.) were dissolved in 20 mL of dimethyl sulfoxide (DMSO) solution using a 50 mL round-bottom flask, then potassium carbonate (685 mg, 4.95 mmol, 3.0 eq.) was added, a condensing reflux device was built, and the reaction was heated and maintained at 100°C in an oil bath for 6 h. At the end of the reaction, the reaction solution was cooled to room temperature, then 50 mL of distilled water was added to the reaction solution and the pH of the mixture was adjusted with hydrochloric acid solution to equal to 3. Finally, the liquid was filtered out by reduced pressure filtration, and the residue was washed more than 3 times with distilled water. The filter residue was transferred to a condensation drier to remove water to obtain compound **3**.^{1}H NMR (400 MHz, DMSO-d_6) δ 11.87 (s, 1H), 8.51 (dd, J = 22.0, 7.8 Hz, 2H), 8.36 (d, J = 8.2 Hz, 1H), 7.76 (t, J = 7.8 Hz, 1H), 7.16 (d, J = 8.2 Hz, 1H), 4.06 (q, J = 7.0 Hz, 2H), 1.20 (q, J = 6.9 Hz, 3H).

*3. Synthesis and characterization of end product **T-HS***

2-(Azidomethyl)benzoic acid (100 mg, 0.57 mmol, 1.0 eq.) was dissolved in 5 mL of dichloromethane (DCM) in a 25 mL double-necked flask and mixed thoroughly, then oxalyl chloride (143 mg, 1.13 mmol, 1.9 eq.) was added under nitrogen protection and the reaction was carried out for 4 h at room temperature. After the reaction was complete, dichloromethane (DCM) was withdrawn under the protection of nitrogen using a reduced pressure filtration device to obtain crude product. The crude product was then diluted with

3 mL of dichloromethane (DCM) and a few drops of triethylamine (TEA) were added, and the reaction solution was cooled to 0°C in an ice water bath, and compound 3 (136.2 mg, 0.22 mmol, 0.39 eq.) was added and reacted at room temperature for 12 h. At the end of the reaction, the reaction solution was diluted with a certain amount of distilled water and the organic layer was purified with dichloromethane (DCM). Finally, the desired end product **T-HS** was separated by column chromatography (petroleum ether: ethyl acetate = 1:1).^{1}H NMR (400 MHz, DMSO-d_6) δ 8.59 (dd, J = 12.3, 7.6 Hz, 2H), 8.46 (d, J = 8.1 Hz, 1H), 7.97 – 7.87 (m, 2H), 7.84 (t, J = 7.5 Hz, 1H), 7.77 – 7.62 (m, 2H), 4.91 (s, 2H), 4.11 (q, J = 7.0 Hz, 2H), 1.23 (t, J = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.74 – 164.66 (m, 1H), 163.61 – 163.48 (m, 1H), 163.07 – 162.98 (m, 1H), 151.72 – 151.66 (m, 1H), 138.47 – 138.34 (m, 2H), 134.71 – 134.54 (m, 2H), 132.20 (s, 2H), 131.78 (d, J = 8.1 Hz, 5H), 131.12 (s, 2H), 129.33 (s, 2H), 129.06 (s, 1H), 128.41 (d, J = 7.2 Hz, 5H), 127.65 (s, 1H), 125.40 (s, 1H), 123.04 (s, 1H), 120.83 (d, J = 4.7 Hz, 4H), 52.57 (s, 2H), 35.33 (s, 2H), 13.57 (s, 2H); HRMS (ESI) m/z calcd for $C_{22}H_{16}N_4O_4$ [M + H^+]: 401.1251; found 401.1239.

Cytotoxicity assays

The in vitro cytotoxicity of HepG2 cells was determined using the colorimetric methyl thiazolyl tetrazolium (MTT) assay. Cells were inoculated into 96-well tissue culture plates with 100μL of DMEM high sugar medium (supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin), incubated overnight in a sterile cell culture incubator at 37 ℃ and 5% CO_2, and then incubated in the presence of different concentrations (0, 1, 2, 3, 5, 10, 20 μM) of **T-HS** for 24 h. Cells were then buffered with PBS cells were washed and 100μL of supplemented DMEM medium was added. Subsequently, 10μL of MTT (5 mg/mL) was added to each well and incubated for 4 h. Violet formazan was dissolved in 100μL of biotech-grade DMSO. The absorbance of the solution was measured at 492 nm using an enzyme

marker. Cell viability was determined by assuming that the cell viability of the cells without **T-HS** was 100%.

Tab. S1. Specific data on the toxicity assay of T-HS on HepG2 cells.

Conc./μM	Average Abs.	Abs. SD.	Normalization	Survival rate/%
0	0.34174	0.0462	1	100.000
1	0.3335	0.0282	0.975888102	97.589
2	0.3211	0.0150	0.939603207	93.960
3	0.3174	0.0657	0.928776263	92.878
5	0.2878	0.0547	0.842160707	84.216
10	0.2718	0.0496	0.795341488	79.534
20	0.2502	0.0178	0.732135542	73.214

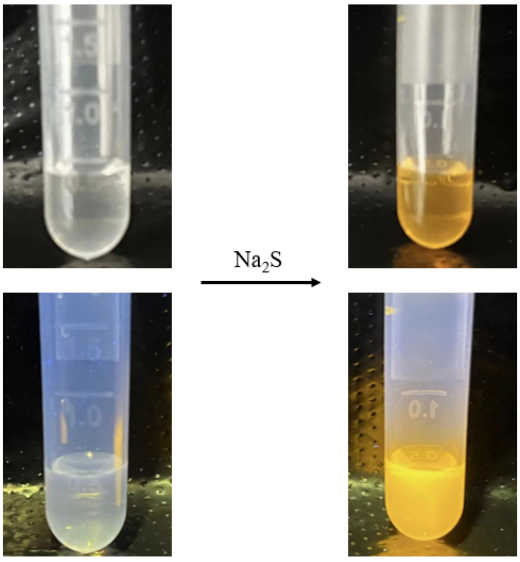

Fig. S1. The color and fluorescence changes of the probe **T-HS** solution (1.0 mM) with the addition of Na_2S (10 eq) in DMSO with 365 nm ultraviolet light.

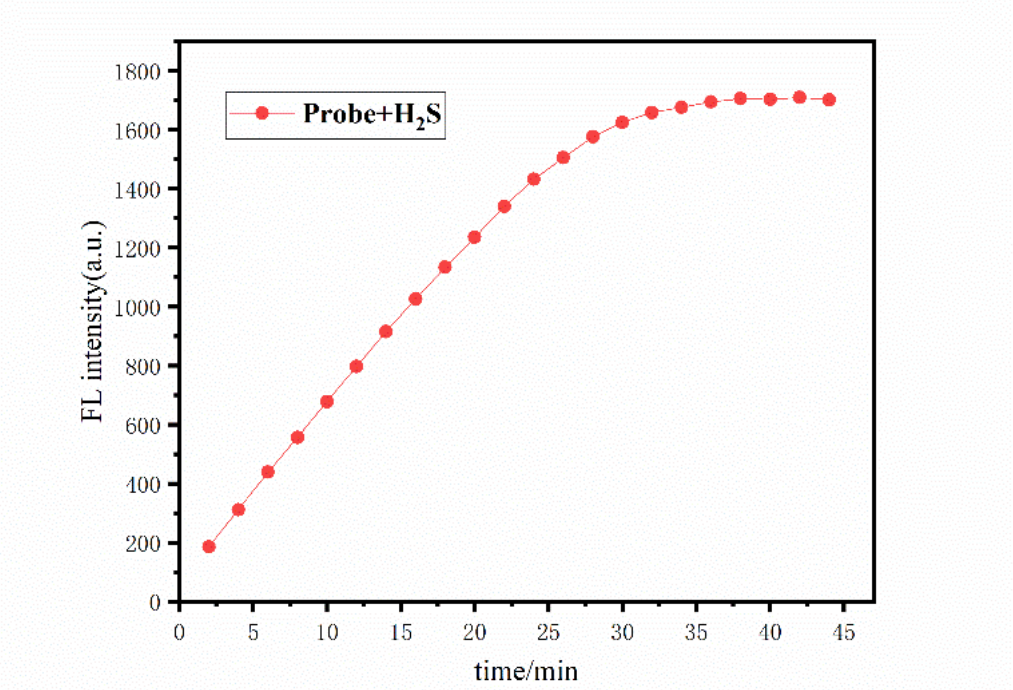

Fig. S2. Fluorescence intensity of **T-HS** (10 μM) at 570 nm in the presence of Na_2S (100 μM) in PBS buffer (pH=7.4, containing 50% DMSO as a cosolvent) over 45 min.

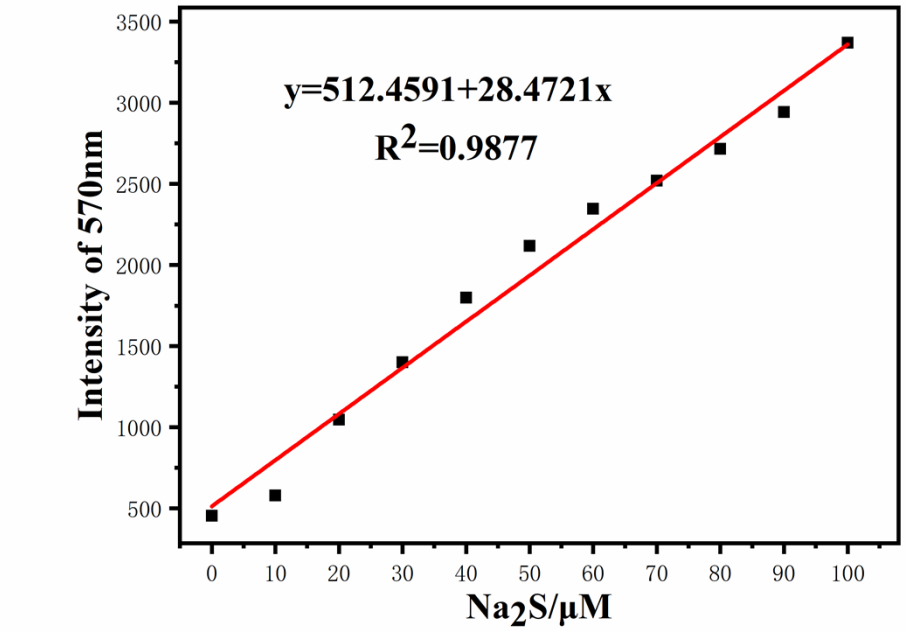

Fig. S3. Relationship between fluorescence intensity at 570 nm of **T-HS** (10 μM) and the amount of Na_2S (0-100 μM).

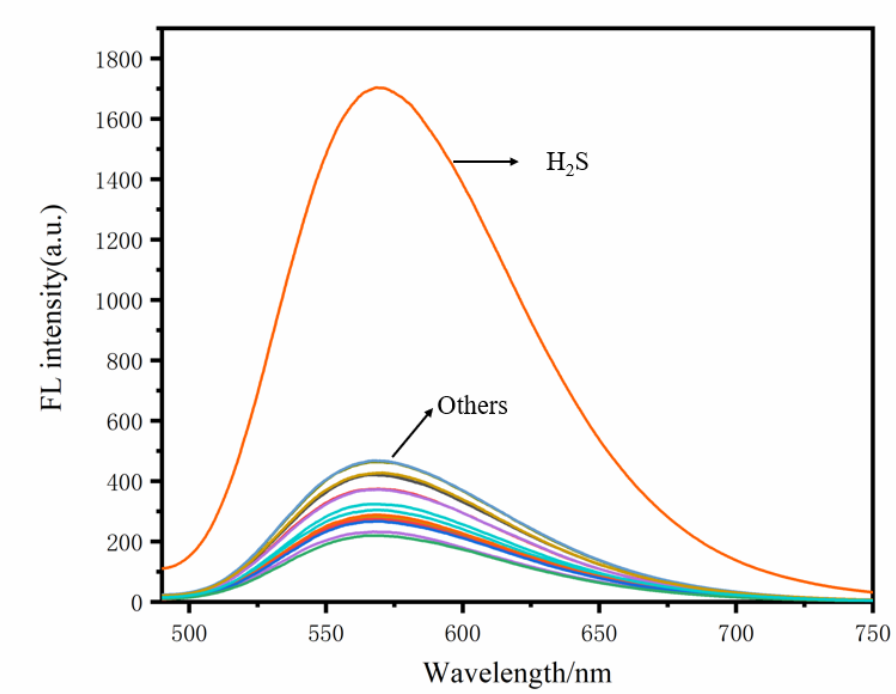

Fig. S4. Probe **T-HS** (10μM) in PBS buffer (pH=7.4, containing 50% DMSO as a cosolvent) in the presence of various analytes (100 μM, Blank, $AgNO_3$, $CaCl_2$, $CuCl_2$, $FeCl_3$, $FeSO_4$, KI, Na_2SO_4, $NaHSO_3$, NH_4Cl, $Pb(NO_3)_2$, $Zn(NO_3)_2$, H_2O_2, L-Cys, L-Trp, Leu, Na_2S) fluorescence intensity.

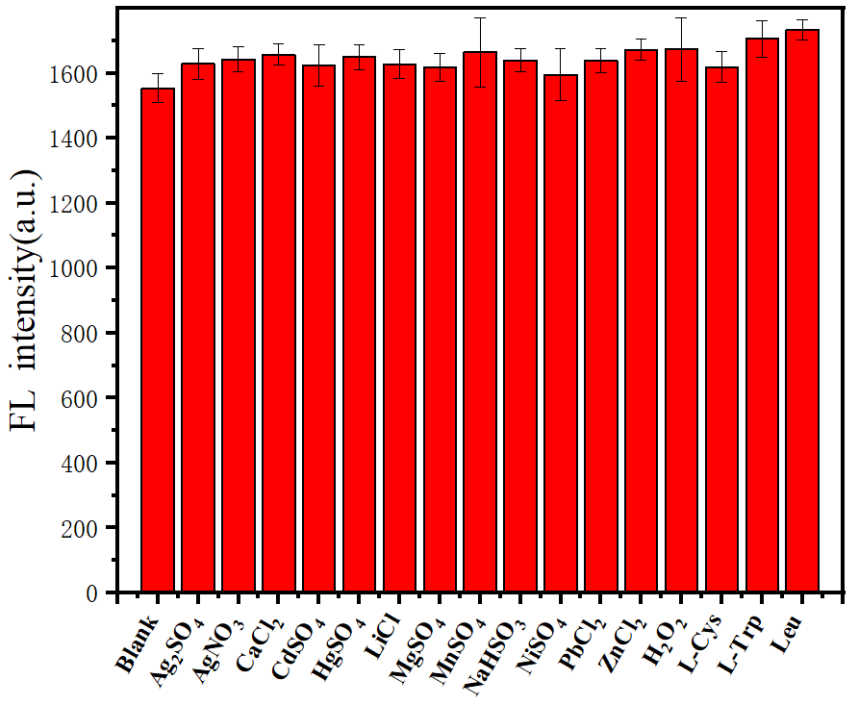

Fig. S5. The fluorescence intensity of Na_2S (100 μM) and **T-HS** (10 μM) was added when various interferors were present.

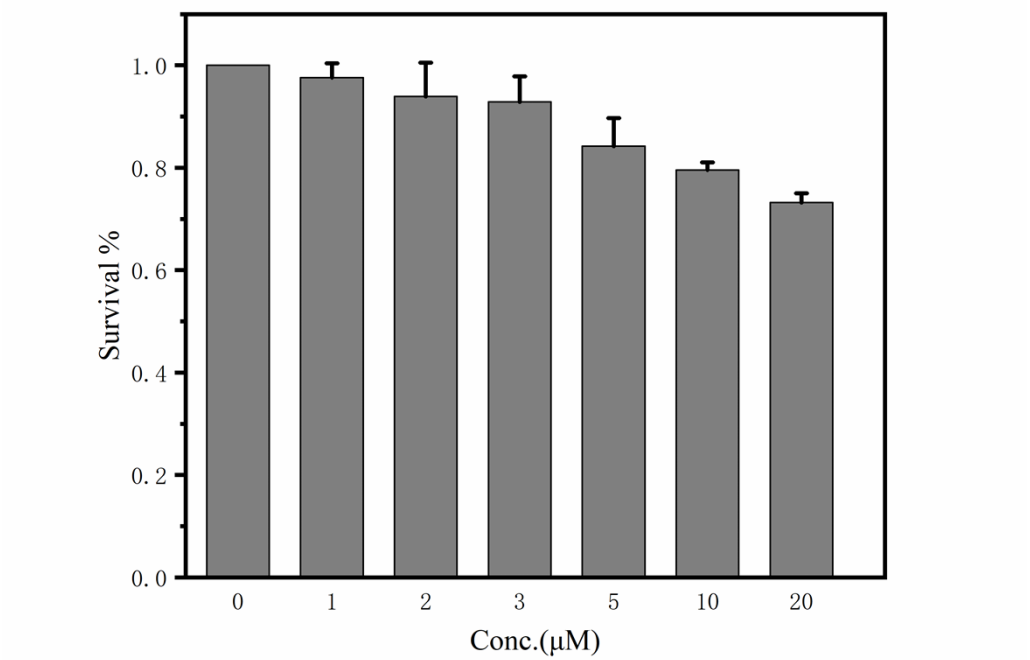

Fig. S6. Cytotoxicity assays of **T-HS** at different concentrations for HepG2 cells.

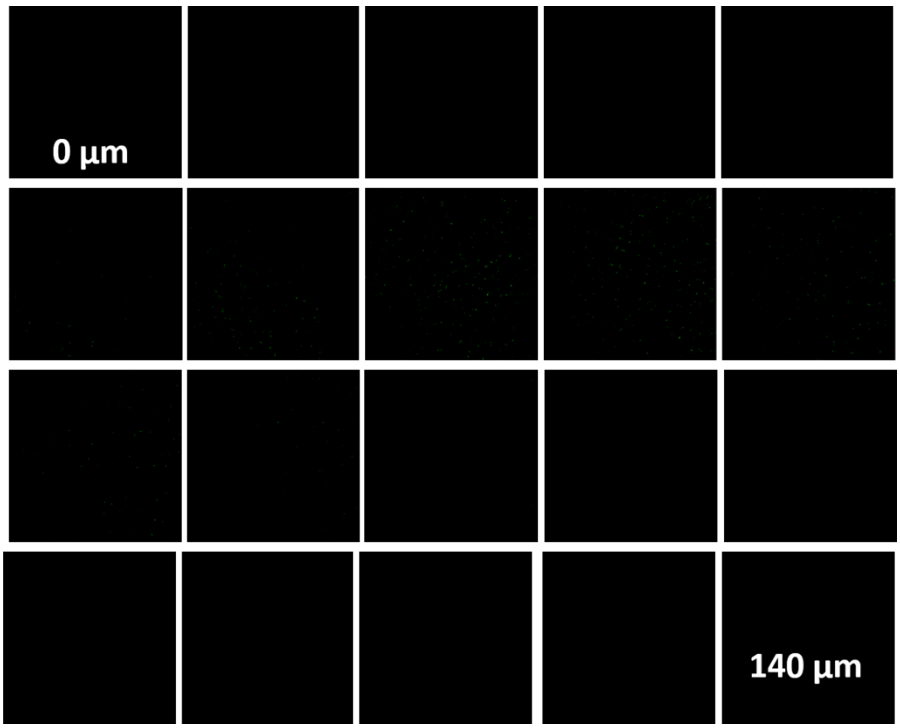

Fig. S7. Two-photon fluorescence images of a fresh rat liver slice incubated with 30 μM **T-HS** in the absence of H_2S at the depths of approximately 0~140 μm. Excitation at 780 nm.

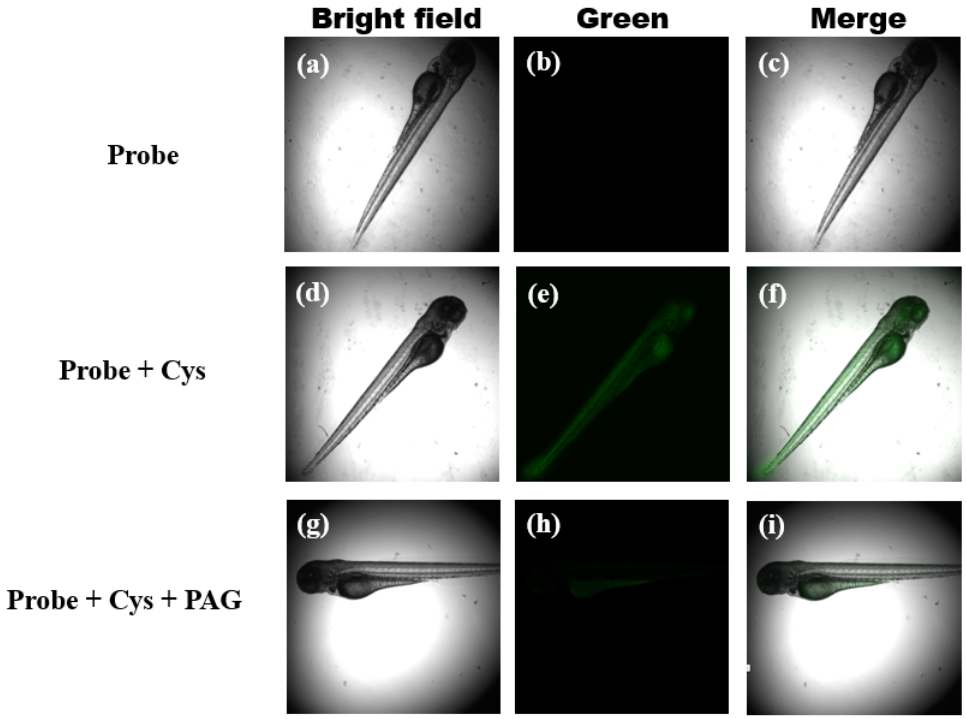

Fig. S8. Imaging of endogenous H_2S in zebrafish stained with probe **T-HS** (10 μM).

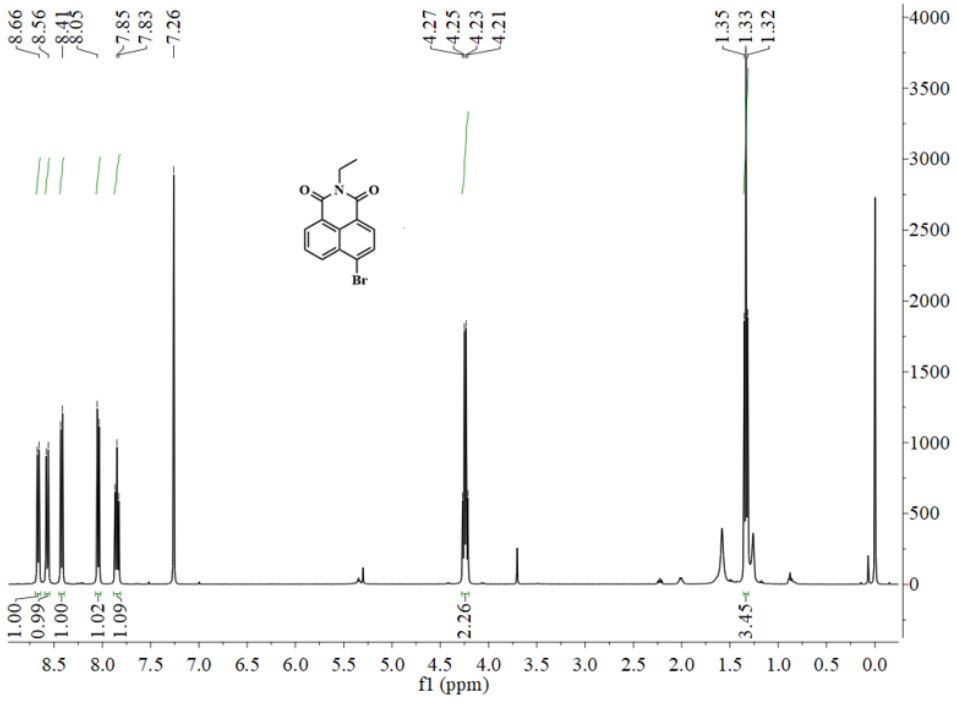

Fig. S9. ^{1}H-NMR (Chloroform-d) spectrum of compound 2.

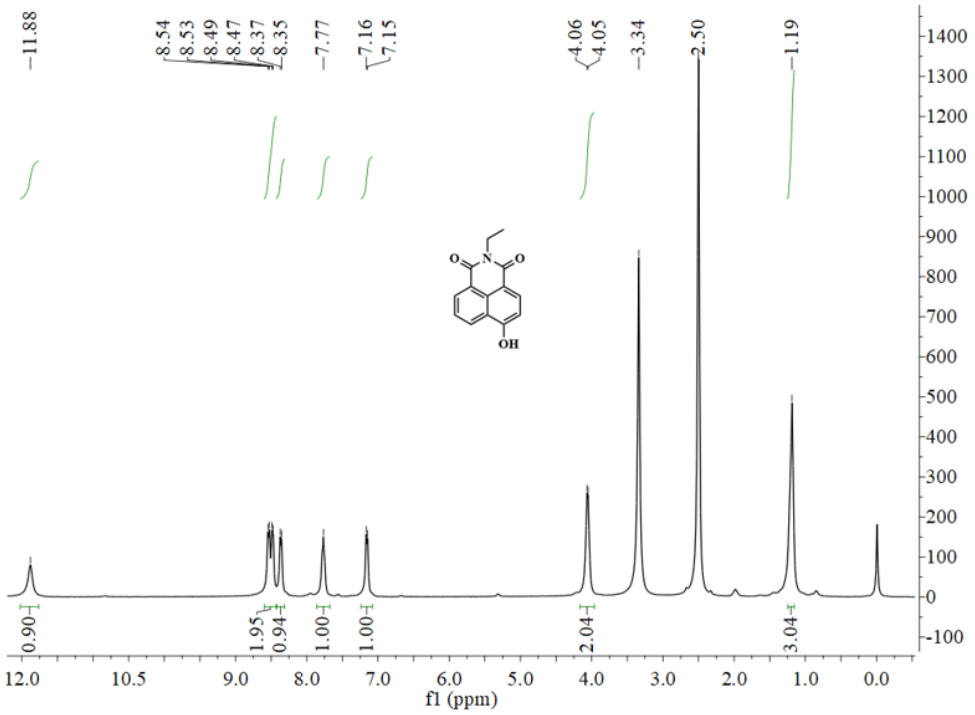

Fig. S10. [1]H-NMR (DMSO-d_6) spectrum of compound 3.

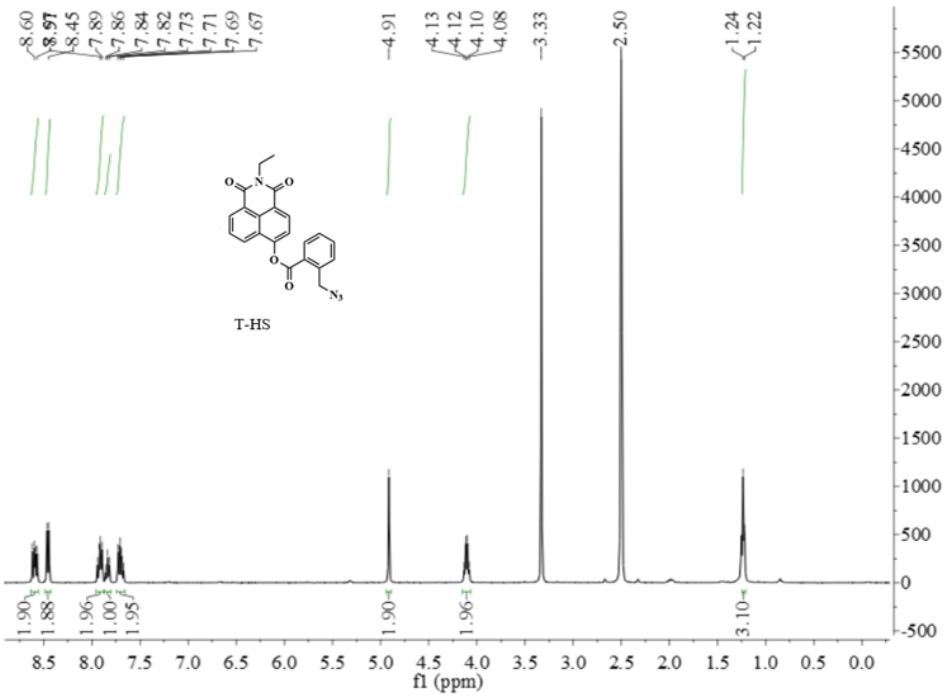

Fig. S11. [1]H-NMR (DMSO-d_6) spectrum of **T-HS.**

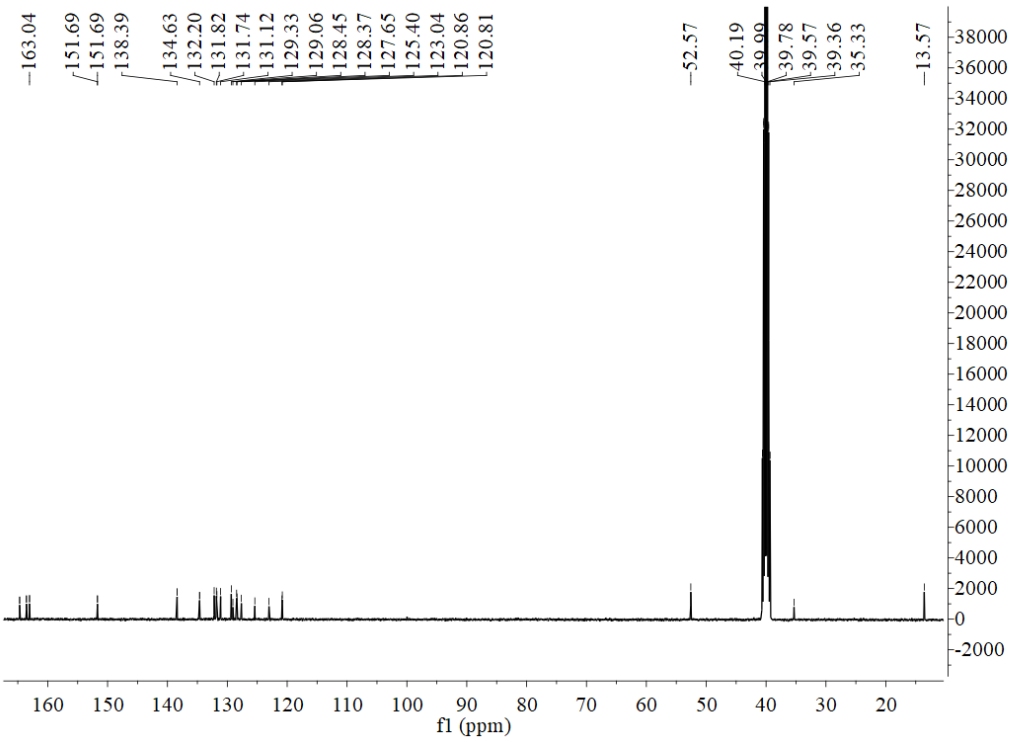

Fig. S12. ^{13}C-NMR (DMSO-d_6) spectrum of **T-HS.**

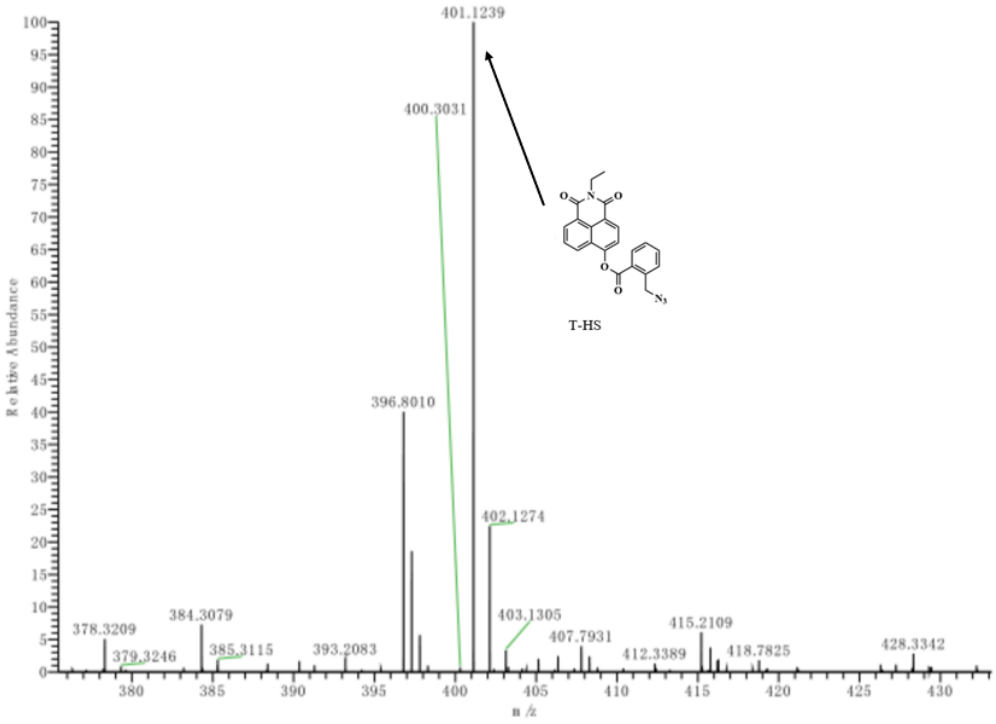

Fig. S13. HRMS (ESI) spectrum of **T-HS.**

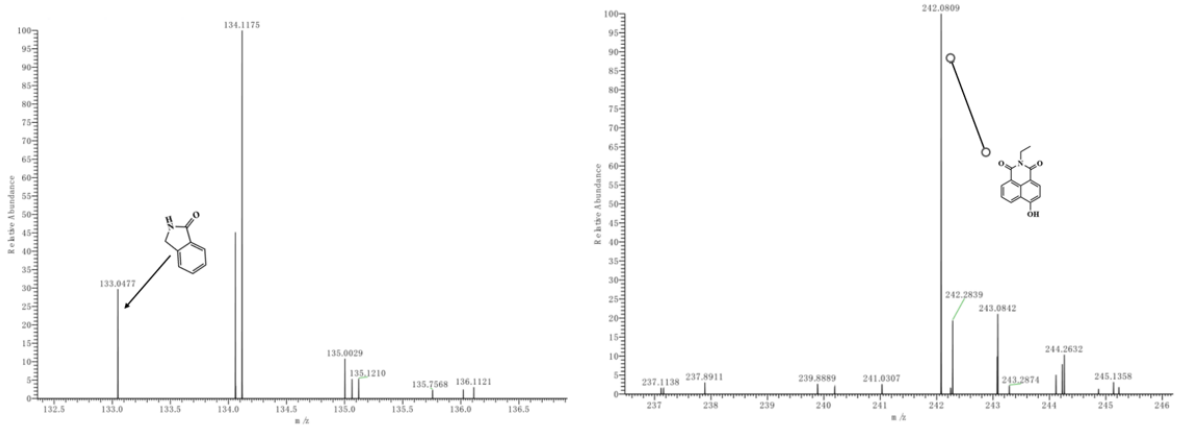

Fig. S14. HRMS (ESI) spectrum of **T-HS**+Na_2S**.**