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Supporting Information

A new dual functional H_2S donor for fluorescence imaging and anti-inflammatory application

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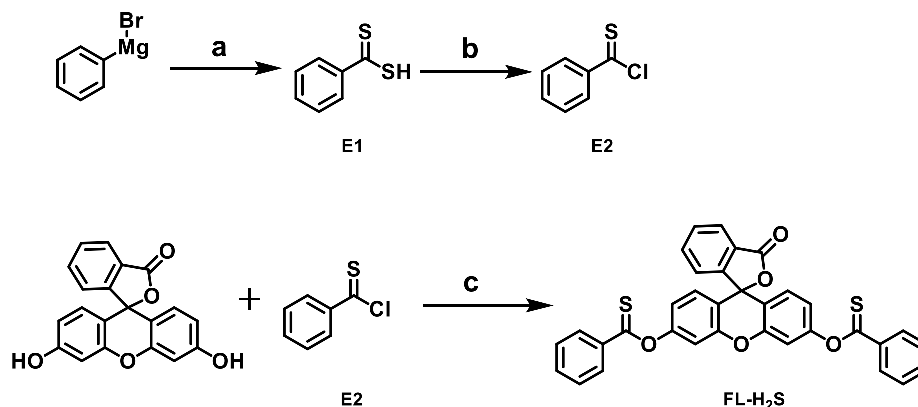
1. Experimental Section

1.1 Materials and Methods

Phenylmagnesium bromide were purchased from Energy Chemical&3A (Shanghai, China). Carbon disulfide and thionyl chloride were purchased from Aladdin. Cysteine (Cys) were purchased from Sangon Biotech. Co., Ltd. (Shanghai, China). NEM was obtained from bidepharm (Shanghai, China). Lipopolysaccharide (LPS) was purchased from Sigma Aldrich. Carrageenan was purchased from Adamas. Unless for special needs, all other reagents were obtained from qualified commercial suppliers and were used without further purification. Commercial ELISA kit of IL-6 and TNF-α were obtained from Cell Signaling Technology (CST). BCA protein assay kit, Nuclear and cytoplasmic protein extraction kit, β-Actin Rabbit Monoclonal Antibody, Lamin B Rabbit Monoclonal Antibody and HRP-labeled Goat Anti-Rabbit IgG(H+L) were obtained from Beyotime (Haimen, China). NF-kB p65(Ser536, Ab-AF5006) was purchased from Affinity Biosciences.

Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker 400 MHz spectrometer. Mass spectrometry was performed with PC Sciex API 150EX ESI-MS system and Thermo TSQ Endura Triple Quadrupole Mass Spectrometer. UV-vis absorption spectra were recorded on Shimadzu UV-3600 Plus UV-VIS-NIR Spectrophotometer. Fluorescence spectra were acquired with a FluoroMax-4 fluorescence photometer. Cell imaging was performed by a ZEISS LSM 800 With Airscan Confocal Laser Scanning Microscope. The levels of proinflammatory cytokine (TNF-α and IL-6) were measured using an ELISA kit from Cell Signaling Technology (Shanghai, China), and absorbance at 450 nm was tested using a microplate reader (Biorad). Milli-Q water was applied in all experiments.

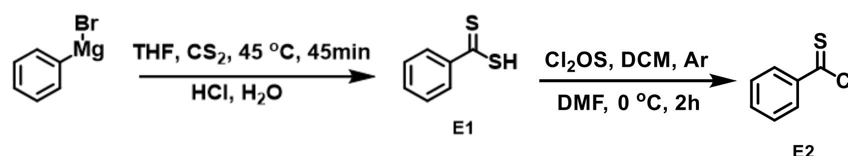
1.2 Synthesis and Characterization of FL-H_2S



Scheme S1 The synthetic route of **FL-H_2S**. (a) CS_2, 45 ºC, HCl, THF, H_2O, Ar; (b) Cl_2OS, DCM, DMF, 0 ºC, Ar.; (c) DMAP, DCM, Ar.

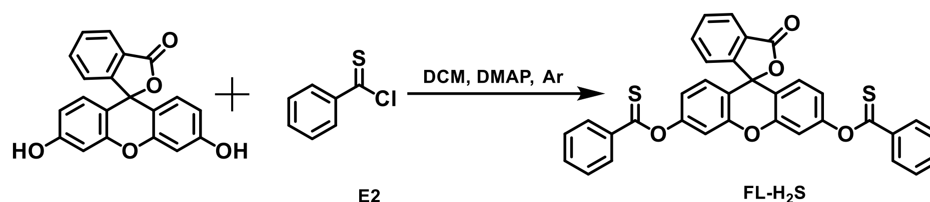
1.2.1 Synthesis of intermediates

The intermediates E1 and E2 were prepared following the procedure shown in Scheme S1 using the method described in previous report.



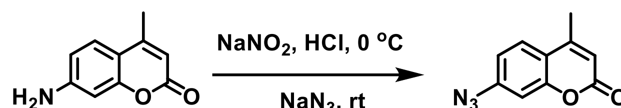
Synthesis of compound E1and E2: 9.3 mL of dry THF and 7.5 mL of a 2 M phenylmagnesium bromide solution in THF (15 mmol) were heated to 40 °C in a nitrogen atmosphere. 2.06 g (27 mmol) of carbon disulfide was then slowly injected through a septum. After stirring for 45 min at 45 °C, it was poured onto a mixture of 110 g of ice and 50 mL of concentrated hydrochloric acid. The violet mixture was then extracted several times with diethyl ether; the organic phases were combined and dried over magnesium sulfate. After evaporation of the solvent, E1, a violet liquid of characteristic odor, was obtained of without purification (2.10 g, 91%). Then, E1 (924 mg, 6.0 mmol) were dissolved in dry DCM (10 mL). Thionyl chloride (1.5 mL) and DMF (1 crystal) were added to the solution at 0 ºC. The resulting solution was stirred for 2 h under Ar atmosphere. The reaction mixture was removed under vacuum to gain the product E2 and used directly for the next step without the purification.

1.2.2 Synthesis of FL-H_2S



Fluorescein (332 mg, 1.0 mmol) and E2 (624 mg, 4.0 mmol) were dissolved in dry DCM. DMAP (488 mg, 4 mmol) was then placed into the reaction flask under Ar atmosphere. The resulting solution was stirred overnight under Ar atmosphere. The reaction mixture was diluted DCM (60 mL). The organic layer was washed with saturated saline and dried with anhydrous Na_2SO_4. After removing the organic solvent under vacuum, the crude product was purified by silica gel column chromatography (ethyl acetate/hexane=1/4, v/v) to obtain yellow compound **FL-H_2S**. ^{1}HNMR (400 MHz, DMSO-d^6) δ: 8.30 (d, J = 8 Hz, 4H), 8.11 (d, J = 8 Hz, 1H), 7.89 (t, J = 8 Hz, J = 8 Hz, 1H), 7.82 (d, J = 8 Hz, 1H), 7.77 (t, J = 8 Hz, J = 8 Hz, 2H), 7.59 (t, J = 8 Hz, J = 8 Hz, 4H), 7.48 (m, 3H), 7.10 (dd, J = 8 Hz, J = 4 Hz, 2H), 7.02 (d, J = 8 Hz, 2H). ^{13}CNMR (400 MHz, DMSO-$d6$) δ 209.47, 168.05, 156.23, 155.46, 151.73, 151.09, 148.42, 143.65, 136.84, 135.34, 133.36, 130.50, 129.99, 128.74, 127.98, 125.52, 124.68, 123.63, 118.39, 116.57, 110.77, 109.82, 107.88, 80.88. ESI-MS: m/z calculated for $C_{34}H_{20}N_5O_2$ =572.0752; found =573.0843 $[M+H]^+$.

1.2.3 Synthesis of Cou-N_3



7-Amino-4-methylcoumarin (175 mg, 1.0 mmol) was dissolved in HCl under ice bath. Then $NaNO_2$ (83 mg, 1.2 mmol) dissolved in water was slowly dropped in above solution and reacted 30 min. After that, NaN_3 (78 mg, 1.2 mmol) was also placed into the reaction and stirred overnight. The mixture was extracted several times with $NaHCO_3$ and ethyl acetate; the organic phases were combined and dried over magnesium sulfate. After evaporation of the solvent and silica gel column chromatography purification (ethyl acetate/hexane=1/4, v/v), Cou-N_3 was obtained as

light-yellow solid. ^{1}HNMR (400 MHz, $CDCl_3$) δ: 7.57 (d, J = 8 Hz, 1H), 6.97 (m, 2H), 6.23 (s, 1H), 2.42 (s, 3H). ^{13}CNMR (101 MHz, $CDCl_3$) δ 154.66, 153.43, 132.04, 129.99 120.95, 114.48. ESI-MS: m/z calculated for $C_{10}H_7N_3O_2$ =201.0538; found =202.0586 $[M+H]^+$.

1.3 General procedure for absorption and fluorescence measurement

10 mM stock solution of **FL-H$_2$S** and Cou-N_3 were firstly prepared in appropriate DMSO, respectively. Serine, S-methyl-L-cysteine, acetylcysteine, 2-aminothiophenol, 1-propanethiol, allyl mercaptan, 1,2-ethanedithiol, 2-mercaptoethanol, Cys, and Glutathione (GSH) were prepared as 500 mM stock solution in water, respectively. For UV and fluorescent titration measurement, **FL-H$_2$S** and Cou-N_3 was dissolved into the mixture solution of ACN/PBS (pH = 7.4, 20 mM, v/v, 3/7) and the UV/fluorescence spectra was determined. After that, Cys was added in and the UV/ fluorescence spectrum was determined after 1 hour reaction at 37 °C. UV-vis absorption studies were performed on a UV-2600 spectrophotometer using 1 cm path length quartz cuvette and the UV-Vis spectra in the range of 300–650 nm was recorded. For fluorescence measurement, the fluorescence spectra of **FL-H$_2$S** and Cou-N_3 were recorded in the range of 450-650 nm with an excitation wavelength of 480 nm, and 370-570 nm with an excitation wavelength of 340 nm. All the slit widths are 5 nm.

1.4 General procedure of cell culture and MTT assay

Hela cells were cultured in DMEM containing 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin (10000 U/mL) in the constant-temperature incubation under a humidified 5% CO_2 atmosphere at 37 °C. The MTT assay was used to measure the cytotoxicity of **FL-H$_2$S**. About 2.0×10^4 cells/well was seeded into a 96-well cell-culture plate. After 24 h for attachment, the old medium was removed and fresh medium containing various concentrations of **FL-H$_2$S** (1, 2, 5, 10, 15 and 20 μM) were added to the wells. After another 24 h, MTT solution (5 mg/mL, DMEM) was added to each well and incubated at 37 °C for 4 h. Remove the MTT solution and purple precipitates (formazan) observed in plates were dissolved in 150 μL DMSO. Microplate reader was used to measure the absorbance at 490 nm for each well. The assays were performed in six sets for each concentration.

1.5 Living cell imaging experiments

To investigate the H_2S release capacity of **FL-H_2S**, fluorescent probe Cou-N_3 was used to image the release of H_2S in cells. 10 mM stock solution of **FL-H_2S** and Cou-N_3 were diluted into working solution by cell culture medium at a concentration of 10 μM. 4T1 cells were first pretreated with or without N-ethylmaleimide (NEM, 100 μM) for 30 min at 37 °C; then, the cells were treated with **FL-H_2S** (10 μM) and Cou-N_3**.** For comparison, the cells were treated exogenous thiols for another 30 min in DMEM followed by washing with PBS twice before imaging. Cell imaging was carried out with the excitation/emission wavelength of 488/550 ± 50 nm for Green Channel and 405/455 ± 25 nm for Blue Channel.

1.6 ELISA

In the ELISA experiments, macrophage RAW 264.7 cells were seeded in two 12-well plate (5×10^5 cells/well) containing 3 mL of DMEM and incubated at 37 °C under 5% CO_2 for 24 h. The LPS (0.5 μg/mL)-pretreated cells were incubated with **FL-H_2S** (20 μM), and Na_2S (20 μM) for 2 h. Thereafter, the cell culture supernatant (100 μL) was collected, and the levels of IL-6 and TNF-α in each well were measured using corresponding commercial ELISA kit according to the manufacturer's protocols, respectively. The OD values were measured using the Microplate reader at 450 nm.

1.7 Western bolt

The LPS (1 μg/mL)-pretreated Raw264.7cells were incubated with different concentration of **FL-H_2S** (0, 10, 20, 30 μM), and NaHS (30 μM) for 2 h. The treated cells were collected, and protein was extracted in the presence of protease inhibitor cocktail with a Nuclear and Cytoplasmic Protein Extraction Kit (Cat#: P0027; Beyotime). Protein concentration was measured using a BCA protein assay kit (Beyotime, Haimen, China) according to the manufacturer's instructions. Equal amounts (30 μg) of protein were then boiled in Loading buffer and resolved on 10%-15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and were subsequently transferred onto a polyvinylidene fluoride. After blocking with Blocking Buffer (50 ml TBST, 2.5 g skimmed milk) at room temperature for 2 hours, membranes were incubated with the primary antibodies (NF-kB p65, β-Actin Rabbit Monoclonal Antibody, Lamin B Rabbit Monoclonal Antibody) at a 1:1000 dilution overnight at 4°C.

After washing with TBST, the membranes were incubated with secondary antibodies (HRP-labeled Goat Anti-Rabbit IgG) at room temperature for 2 hours. Visualization was achieved with an ECL chemiluminescence kit (Millipore). The protein bands were exposed using a Bio-Rad imaging system (Bio-Rad), and band intensities were quantified using Image J.

1.8 Rat foot-swelling experiment

Nine SPF qualified Sprague-Dawley (SD) adult rats (180±20 g) were randomly divided into the PBS control group, **FL-H_2S** group and Dex group. The inflammatory model was established by subcutaneous injection of 100 μL carrageenan (1%) into the pad of the posterior foot with a 1 mL syringe. After 0.5 h of the last administration, 100 μL of PBS, **FL-H_2S (**50 μg/kg**)** or Dex (50 μg/kg) was injected subcutaneously. The foot circumference of the posterior foot was measured with a string. at 0.5 h and 3 h after modelling. Photographs were taken to record the data, a ruler was used as a scale, and the swelling inhibition rate was calculated. The foot swelling was defined as the difference in the value of the right posterior foot circumference before and after administration. The rate of the foot swelling degree $R_{swelling}$ (%) and the inhibition rate of the foot swelling degree $IR_{swelling}$ (%) was calculated using the following equation:

$$R_{swelling}\ (\%) = (C_{foot(after)} - C_{foot\ (before)})\ /\ C_{foot(before)} \times 100\%$$

$$IR_{swelling}\ (\%) = (R_{swelling(Drugs)} - R_{swelling(\ PBS)})\ /\ R_{swelling\ (\ PBS)} \times 100\%$$

2. Supplementary Figures

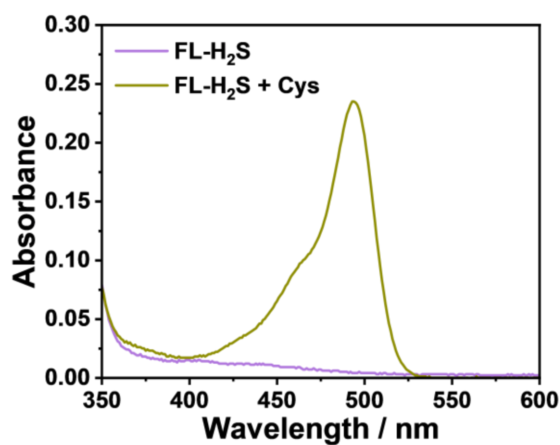


Fig.S1 Absorption spectra of **FL-H_2S** (5 μM) with or without Cys (100 μM). Solvent: ACN/PBS (pH = 7.4, 20 mM, v/v, 3/7).

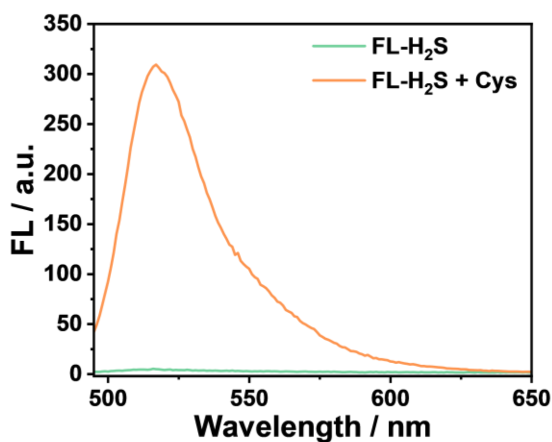


Fig.S2 Fluorescence spectra of **FL-H_2S** (5 μM) with or without Cys (100 μM). Ex=480 nm. Solvent: ACN/PBS (pH = 7.4, 20 mM, v/v, 3/7).

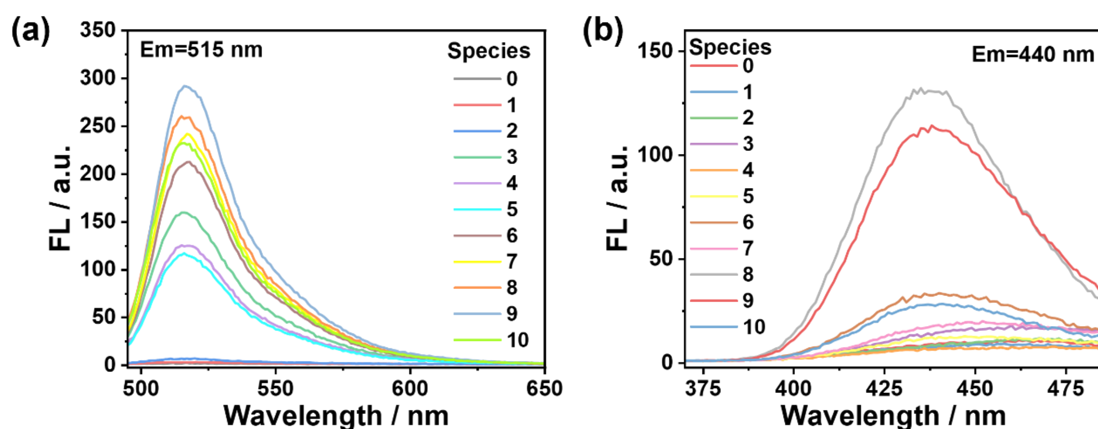


Fig.S3 Fluorescence spectra of **FL-H_2S** (a) and Cou-N_3 (b) toward different species (500 μM). **FL-H_2S** (5 μM), Ex/Em: 480/515 nm; Cou- N_3 (10 μM), Ex/Em: 340/440. Solvent: PBS /ACN =7/ 3 (pH=7.4, 20 mM). 0, control; 1, serine; 2, S-methyl-L-cysteine; 3, acetylcysteine; 4, 2-aminothiophenol; 5, 1-propanethiol; 6, allyl mercaptan; 7, 2-mercaptoethanol; 8, 1,2-ethanedithiol; 9, cysteine; 10, glutathione.

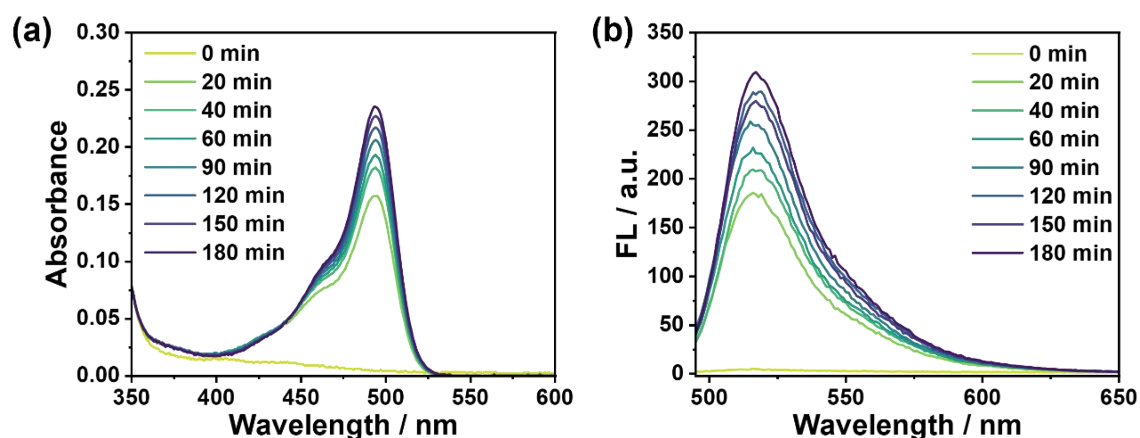


Fig.S4 Absorption spectra and fluorescence spectra of **FL-H_2S** with 500 μM Cys within 180 min. Solvent: PBS /ACN (pH = 7.4, 20 mM, v/v, 3/7).

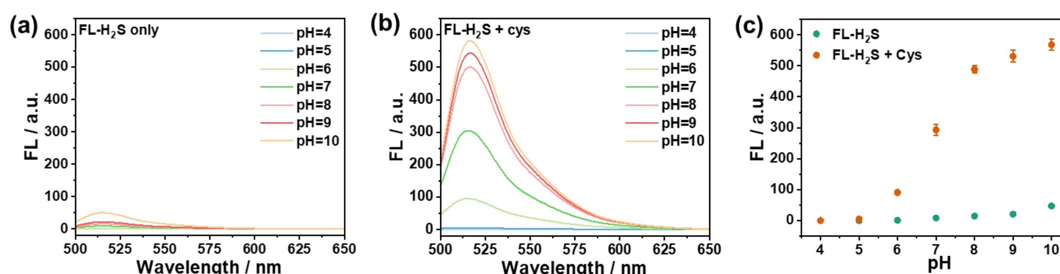


Fig. S5 Fluorescence spectra of (a) **FL-H2S** (5 μM), (b) **FL-H2S** (5 μM) with Cys (500 μM) in different pH. (c) The plot of fluorescence intensity changes at 515 nm in Fig. R2a and R2b. Ex=480 nm. Solvent: ACN/PBS (pH = 7.4, 20 mM, v/v, 3/7). **FL-H2S** exhibited significant reactivity with Cys in neutral and basic solutions, representing the pH values commonly encountered in biological systems. Furthermore, **FL-H2S** alone exhibited exceptional stability in solutions within the physiological pH range.

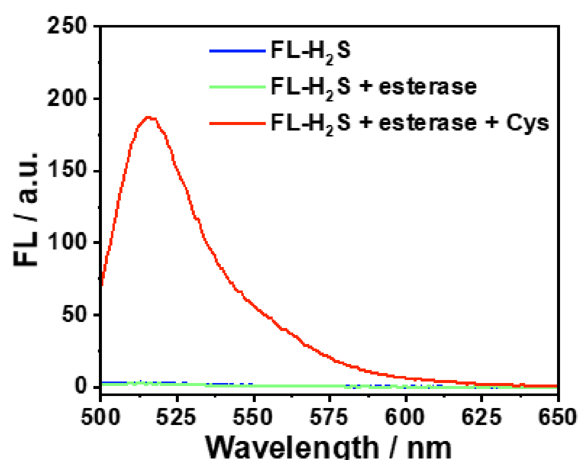


Fig. S6 Fluorescence spectra of **FL-H2S** (5 μM) with or without esterase (200 U/ml) and **FL-H2S** with esterase (200 U/ml) and Cys (500 μM). Ex=480 nm. Solvent: ACN/PBS (pH = 7.4, 20 mM, v/v, 3/7). When **FL-H2S** was mixed with esterase, no fluorescence was observed.

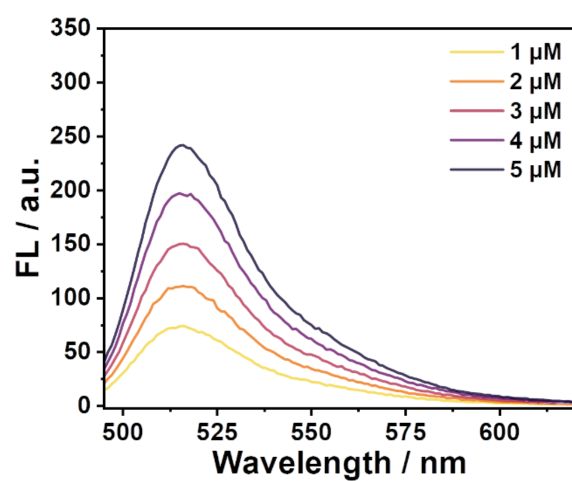


Fig. S7 Fluorescence spectra of fluorescein (1-5 μM) in PBS /ACN =7/ 3 (pH=7.4). Ex= 480 nm.

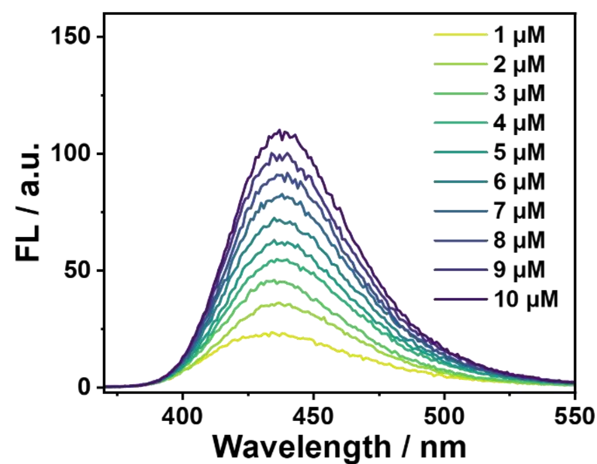


Fig. S8 Fluorescence spectra of **Cou-N_3** with (1-5 μM) NaHS. Solvent: PBS /ACN =7/ 3 (pH=7.4). Ex=340 nm.

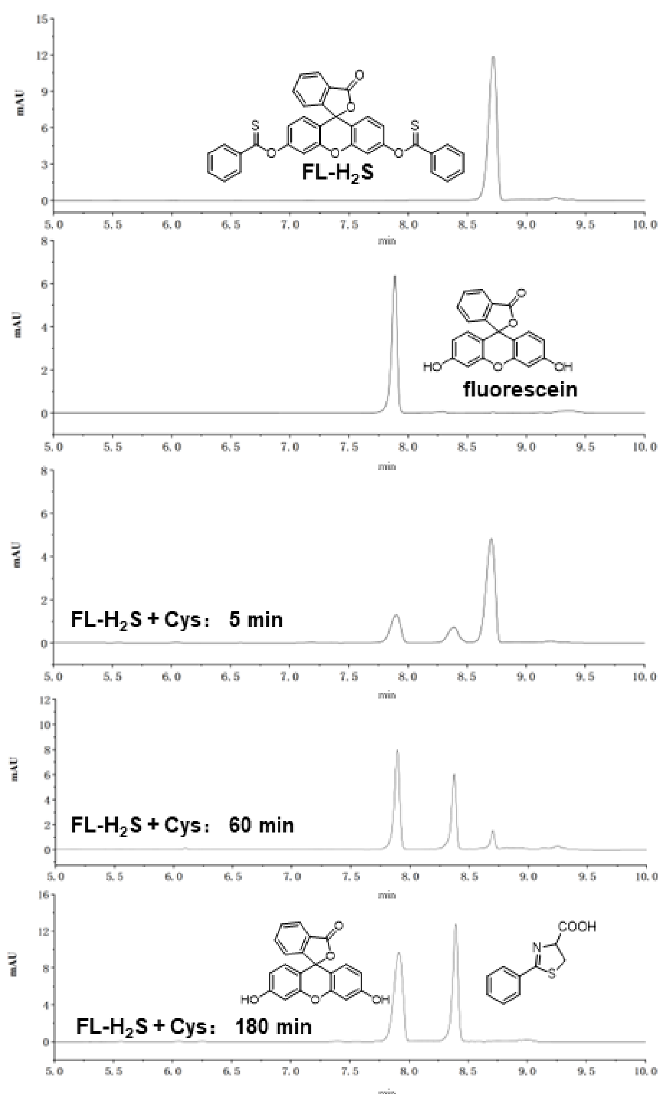


Fig. S9 Reversed-phase UPLC chromatograms of the solution of various substrates in ACN/PBS (pH = 7.4, 20 mM, v/v, 3/7). (a) **FL-H$_2$S** (50 μM) only; (b) fluorescein only (50 μM); (c) a mixture of **FL-H$_2$S** (50 μM) and Cys (2 mM) and incubate for 5 min; (d) a mixture of **FL-H$_2$S** (50 μM) and Cys (2 mM) and incubate for 60 min; (e) a mixture of **FL-H$_2$S** (50 μM) and Cys (2 mM) and incubate for 180 min. The absorption wavelength was recorded at 254 nm. H_2O-ACN, from 50:50 to 0:100 in 10 min; flow rate: 0.2 mL/min; column: 2.1×50 mm.

Table S1 Release efficiency calculated by substituting a linear fitted curve method.

	1 μM **FL-H$_2$S**	2 μM **FL-H$_2$S**	3 μM **FL-H$_2$S**	4 μM **FL-H$_2$S**	5 μM **FL-H$_2$S**	Average

Release efficiency[a]	94 %	90 %	94 %	94 %	93 %	93 %
Release efficiency[b]	93 %	91 %	97 %	97 %	92 %	94 %
Average	93 %	90 %	95 %	95 %	93 %	93 %

a: calculated by linear fitted curve of fluorescein;

b: calculated by linear fitted curve of Cou-N_3 and H_2S.

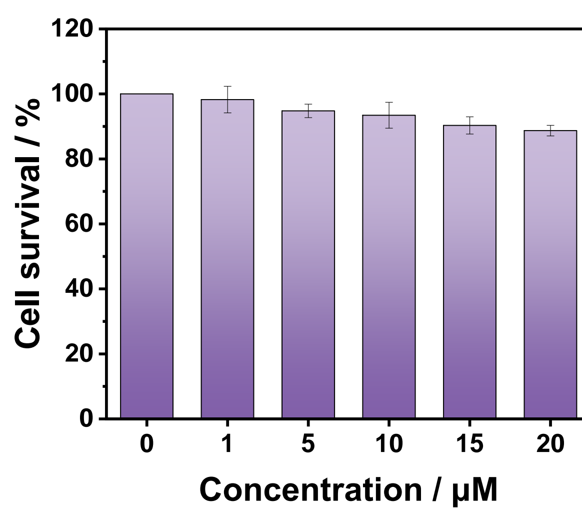


Fig. S10 Cytotoxicity data of **FL-H_2S** in HeLa cells.

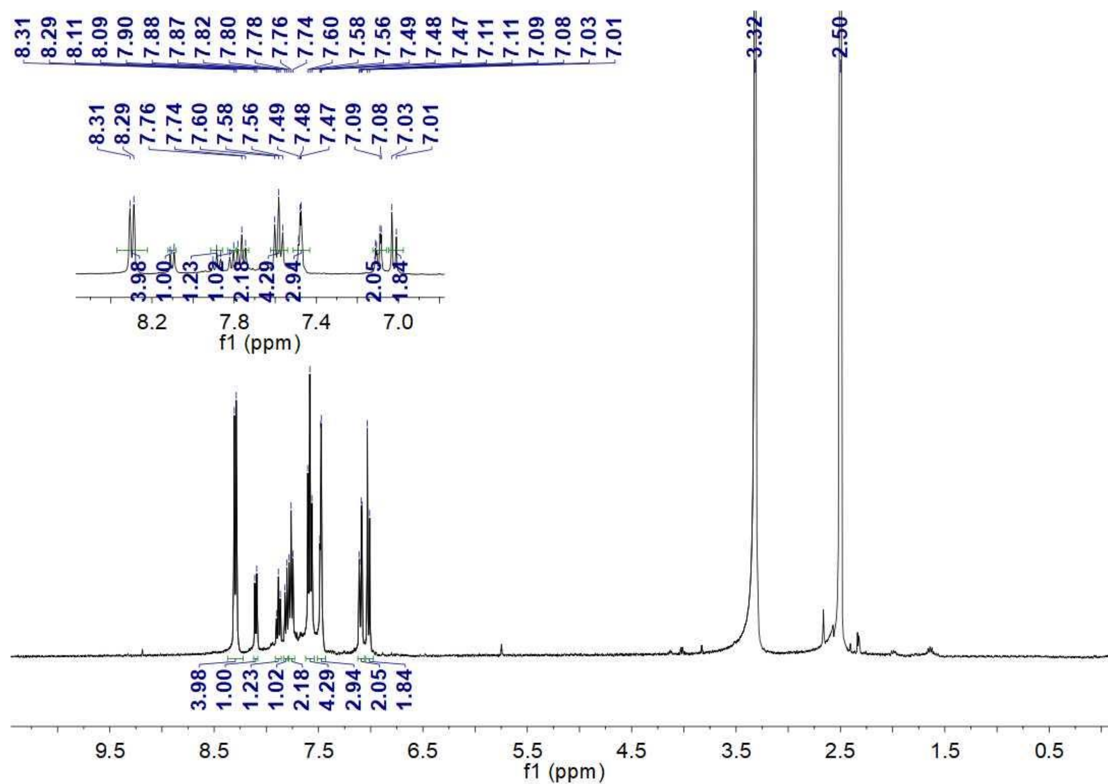


Fig. S11 ^{1}H NMR spectrum of **FL-H$_2$S** (DMSO-d^6).

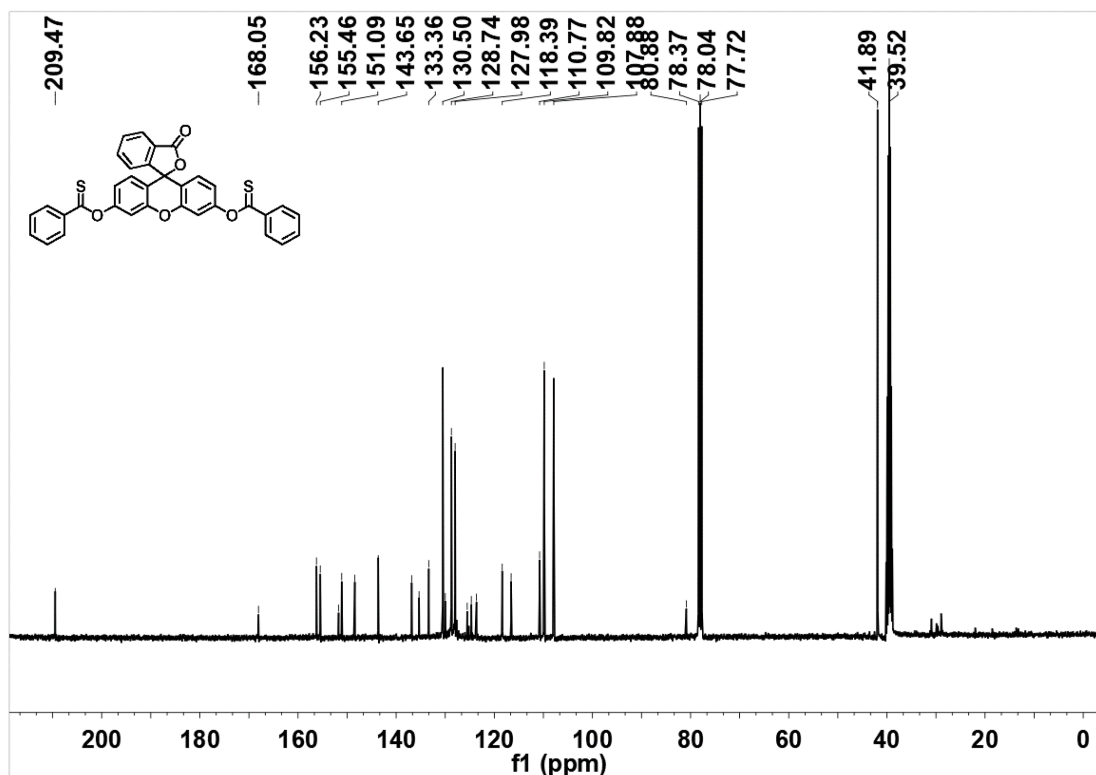


Fig. S12 ^{13}C NMR spectrum of **FL-H$_2$S** (DMSO-d^6+$CDCl_3$).

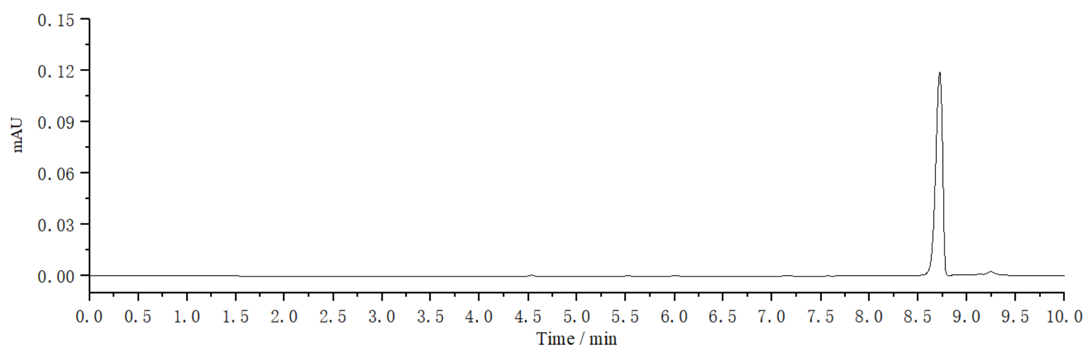


Fig. S13 Reversed-phase UPLC chromatograms of FL-H_2S.

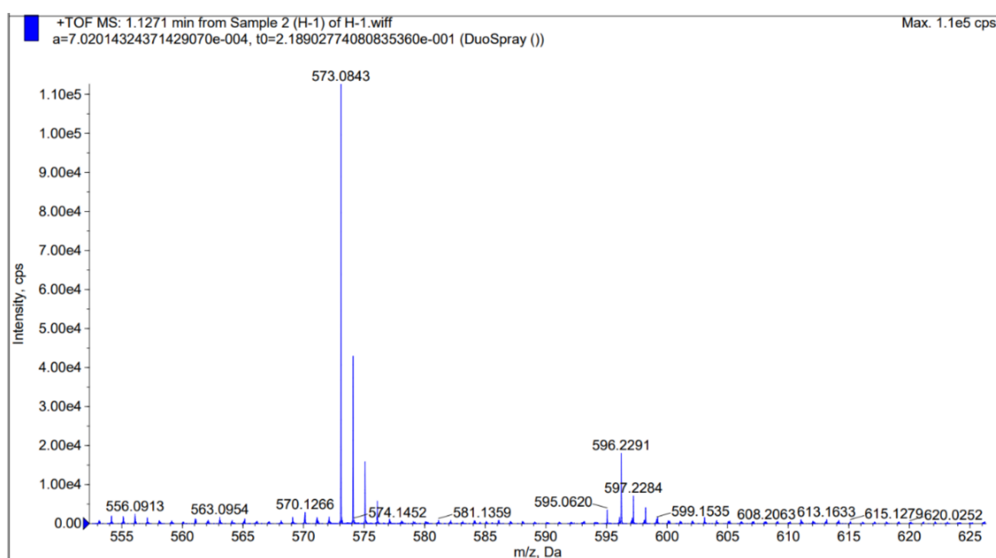


Fig. S14 ESI-MS spectrum of **FL-H_2S**.

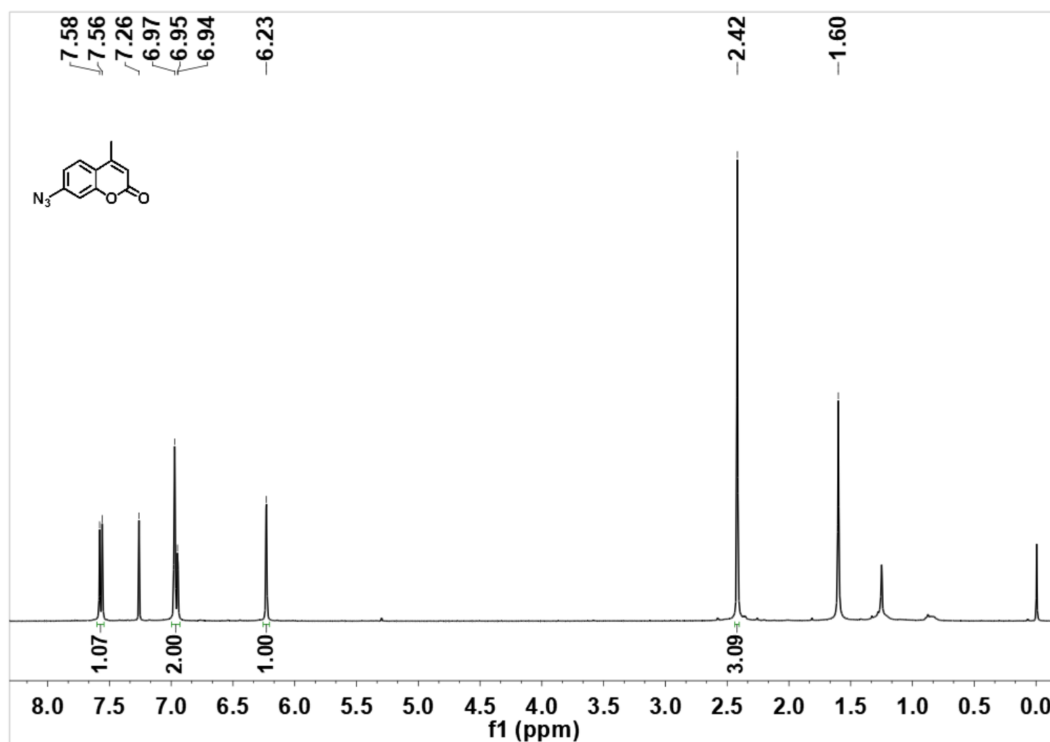


Fig. S15 ^{1}H NMR spectrum of Cou-N_3 ($CDCl_3$).

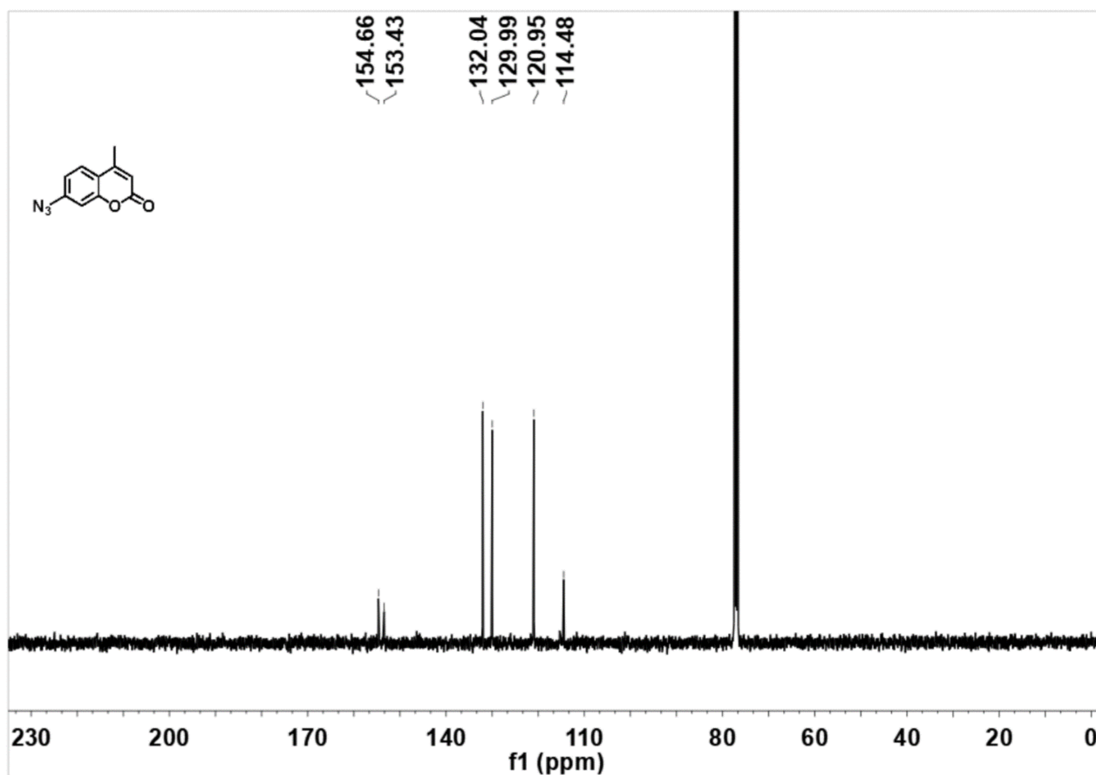


Fig. S16 ^{13}C NMR spectrum of Cou-N_3 ($CDCl_3$).

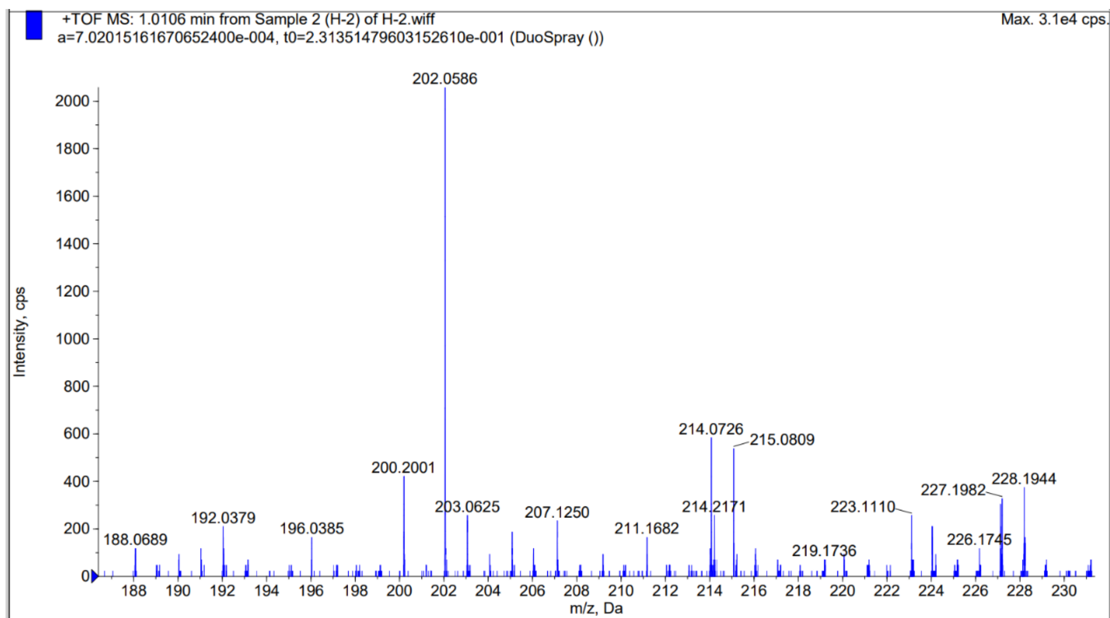


Fig. S17 ESI-MS spectrum of Cou-N_3.