Electronic Supplementary Information for:

In vivo monitoring of hydrogen sulfide using a cresyl violet-based ratiometric fluorescence probe

Qiongqiong Wan, Yanchao Song, Zhao Li, Xinghui Gao and Huimin Ma*

Beijing National Laboratory for Molecular Sciences, Key Laboratory of Analytical Chemistry for Living Biosystems, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China. E-mail: mahm@iccas.ac.cn

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1. Apparatus and reagents

Fluorescence measurements were made on a Hitachi F-2500 fluorimeter (Tokyo, Japan). Fluorescence quantum yield (Φ) was determined by using cresyl violet (Φ = 0.54 in methanol; J. R. Lakowicz, *Principle of Fluorescence Spectroscopy*, 2nd ed., Kluwer Academic/Plenum, New York, 1999) as a standard. A model HI-98128 pH-meter (Hanna Instruments Inc., USA) was used for pH measurements. ^{1}H NMR spectra were measured with a Bruker DMX-400 spectrometer in CD_3OD, and ^{13}C NMR spectra were recorded with a Bruker AVANCE 600 in CF_3COOD. HPLC analyses were carried out with LC-20AT pumps, SPD-20A UV-vis detector (Shimadzu, Japan) and Inertsil

ODS-SP column (5 μm, 4.6 mm × 250 mm, GL Sciences Inc.). Electrospray ionization mass spectra (ESI-MS) were measured with an LC-MS 2010A (Shimadzu) instrument. High resolution electrospray ionization mass spectra (HR-ESI-MS) were recorded on an APEX IV FTMS instrument (Bruker, Daltonics). Absorption spectra were recorded in 1-cm quartz cells with a TU-1900 spectrophotometer (Beijing, China). Fluorescence imaging experiments were performed on a FV 1000-IX81 confocal laser scanning microscope (Olympus, Japan).

Cresyl violet acetate, reduced glutathione (GSH), L-cysteine, reduced nicotinamide adenine dinucleotide (NADH) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Dulbecco's modified eagle media (DMEM), fetal bovine serum, penicillin (100 μg/mL), and streptomycin (100 μg/mL) were obtained from Invitrogen corporation. A phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4) of pH 7.4 was obtained from MatTek Co. Anhydrous NaHS was purchased from Alfa Aesar. All other chemicals used were local products of analytical grade. Ultrapure water (over 18 MΩ·cm) from a Milli-Q reference system (Millipore) was used throughout. The stock solution (1.0 mM) of the probe **1** was prepared by dissolving requisite amount of **1** in deoxygenated DMSO, which should be used freshly. Stock solutions (1-100 mM) of other substances were prepared by dissolving in water.

2. Synthesis of 1

A solution of $NaNO_2$ (69 mg, 1.0 mmol) in water (2 mL) was added dropwise to cresyl violet acetate (321 mg, 1.0 mmol) in 20 mL of 2 M HCl aqueous solution at 0-5 ºC. After stirring for 20 min, an aqueous solution of NaN_3 (75 mg, 1.5 mmol) in 3 mL of water was added, and then the reaction mixture was stirred at room temperature for 24 h. The resulting brown precipitate was filtrated and recrystallized from CH_3CN, affording the probe **1** as a brown solid (255 mg, 80% yield). The ^1H NMR and ^{13}C NMR spectra of **1** are shown below in Fig. S1 and Fig. S2, respectively. ^1H NMR (400 MHz, CD_3OD): δ = 9.06-9.04 (d, J = 8.0 Hz, 1H; Ar-H), 8.47-8.45 (d, J = 8.0 Hz, 1H; Ar-H), 8.13-8.09 (m, 2H; Ar-H), 8.04-8.02 (m, 1H; Ar-H), 7.43-7.35 (m, 2H; Ar-H), 7.00 (s, 1H, Ar-H). ^{13}C NMR (600 MHz, CF_3COOD): δ 165.7, 153.0, 148.7, 145.4, 142.5, 134.9, 132.7, 132.1, 126.6, 123.5, 123.1, 119.2, 105.9, 99.0. HR-ESI-MS, calcd for $[M+H]^+$: *m/z* 288.0880; found: *m/z* 288.0876.

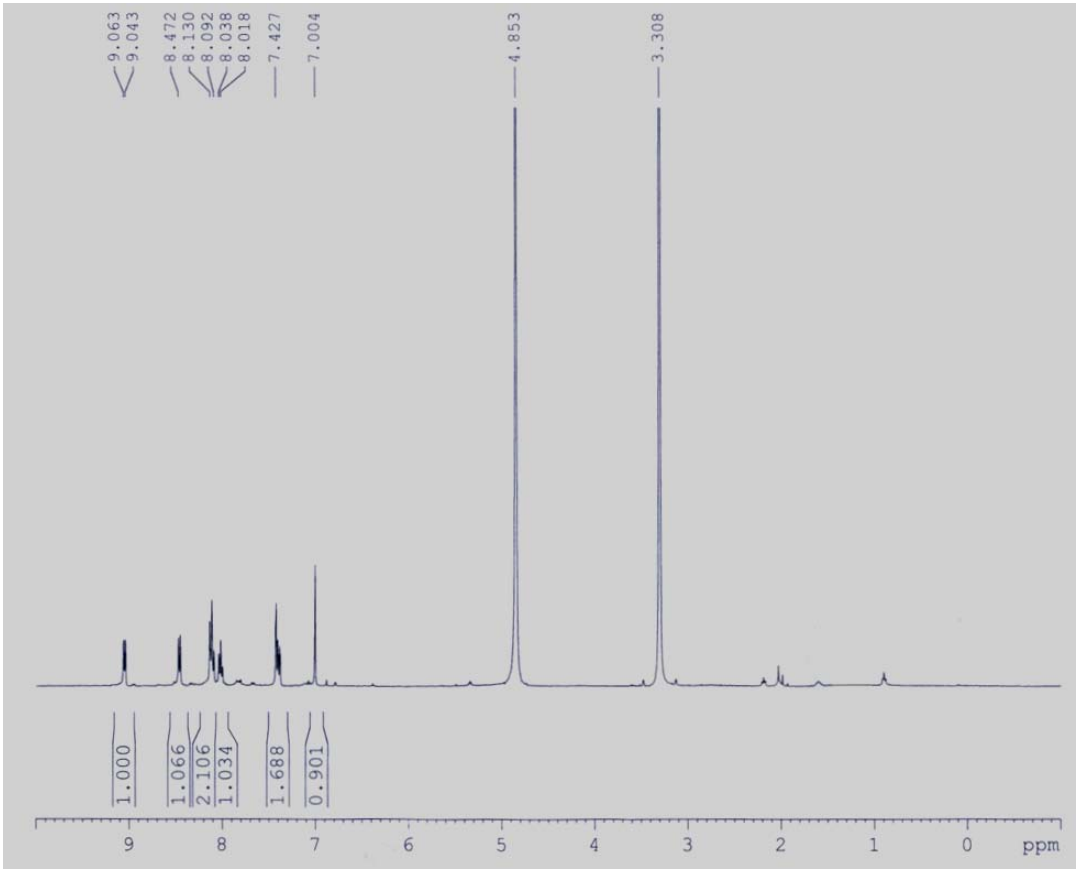


Fig. S1 ^{1}H NMR spectrum of **1** (400 MHz, CD_3OD, 298K).

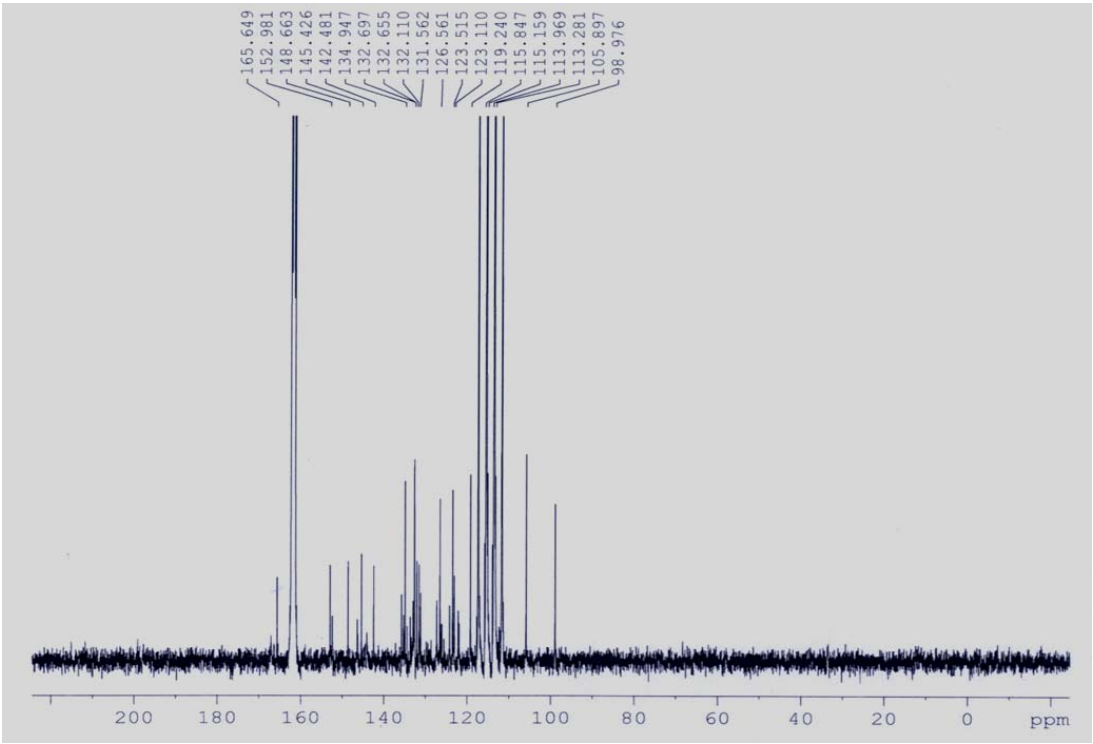


Fig. S2 ^{13}C NMR spectrum of **1** (600 MHz, CF_3COOD, 298K).

3. General procedure for H_2S detection

Unless otherwise noted, all the measurements were made according to the following procedure. In a test tube, 4 mL of 60 mM pH 7.4 Na_2HPO_4-KH_2PO_4 buffer (referred to the phosphate buffer) and 50 μL of the stock solution of **1** were mixed, followed by addition of an appropriate volume of NaHS sample solution. The final volume was adjusted to 5 mL with the phosphate buffer and the reaction solution was mixed well. After standing for 40 min at room temperature, a 3-mL portion of the reaction solution was transferred to a quartz cell of 1-cm optical length to measure absorbance and fluorescence with λ_{ex} = 535 nm (both excitation and emission slit widths were set to 10 nm). In the meantime, a blank solution containing no NaHS was prepared and measured under the same conditions for comparison.

4. Spectral properties of 1

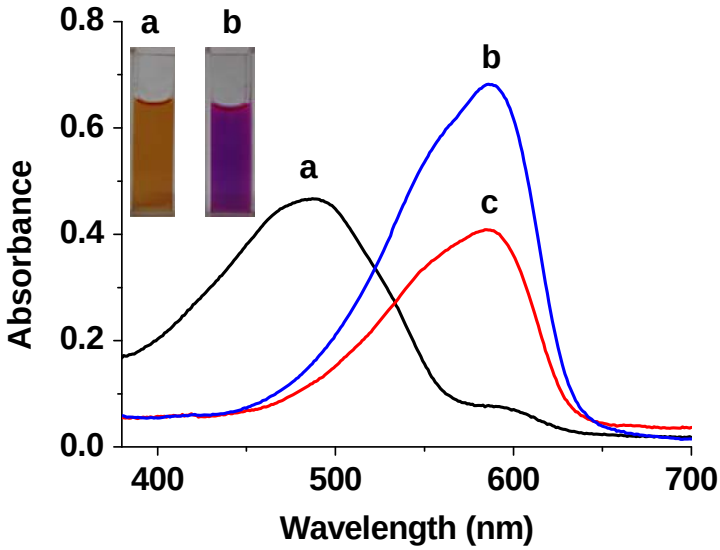


Fig. S3 Absorption spectra and color changes of **1** (25 μM) before (a) and after (b) reaction with NaHS (250 μM) for 40 min at room temperature. The absorption spectrum (c) of cresyl violet acetate (15 μM) is also shown in the figure.

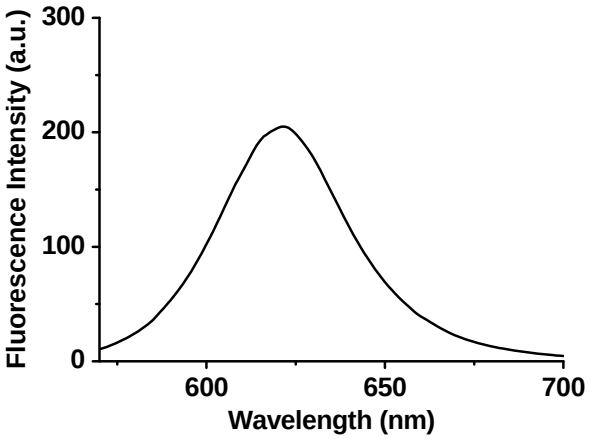
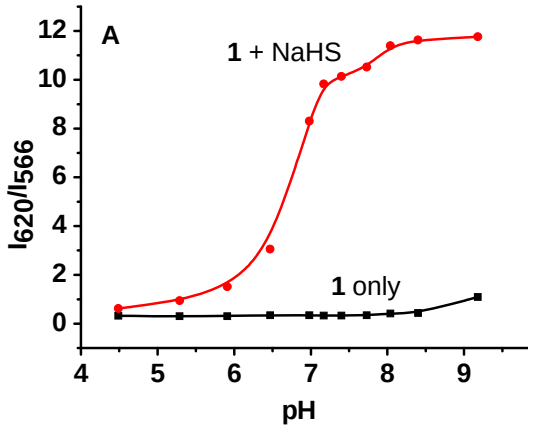
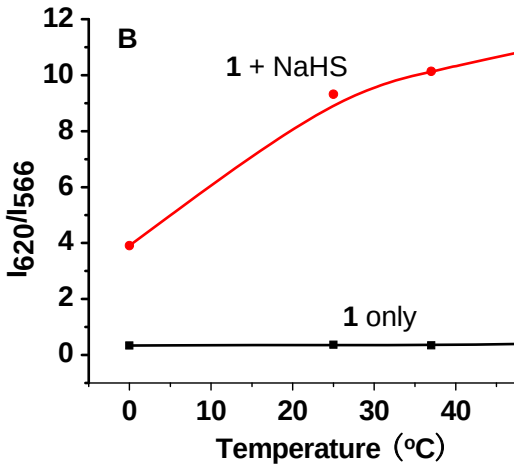


Fig. S4 The fluorescence spectrum (λ_{ex} = 535 nm) of cresyl violet acetate (15 μM) in the phosphate buffer.

5. Optimization of experimental conditions





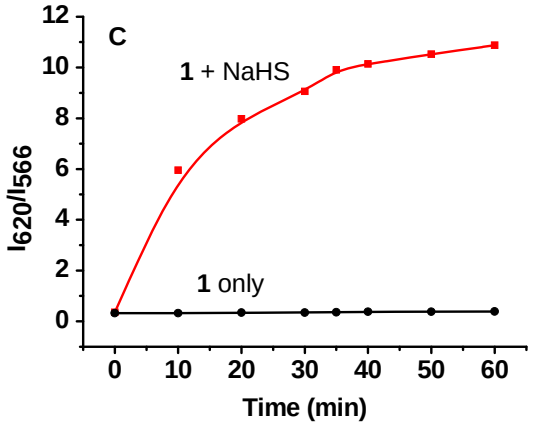


Fig. S5 Effects of pH (A), reaction temperature (B) and time (C) on the fluorescence intensity ratio (I_{620}/I_{566}) of **1** (10 μM) in the absence and prescence of NaHS (100 μM). Conditions: (A) the reaction was performed at room temperature for 40 min in the phosphate buffer with different pH values; (B) the reaction was performed in the phosphate buffer (pH 7.4) for 40 min at different temperatures; (C) the reaction was conducted at room temperature for different lengths of time in the phosphate buffer (pH 7.4). λ_{ex} = 535 nm.

6. Selectivity study

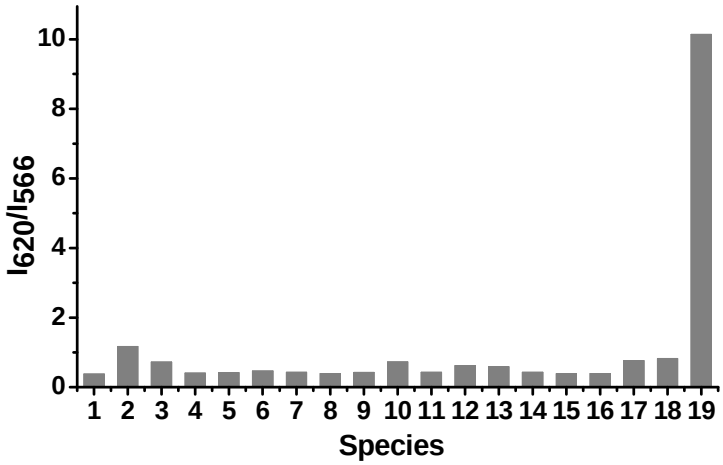


Fig. S6 Fluorescence intensity ratio (I_{620}/I_{566}) of **1** (10 μM) in the presence of various species: (1) **1** only; (2) 5 mM L-cysteine; (3) 5 mM reduced glutathione; (4) 100 μM $Na_2S_2O_3$; (5) 100 μM Na_2SO_3; (6) 100 μM KI; (7) 100 μM $CaCl_2$; (8) 100 μM $CuCl_2$; (9) 100 μM $MgCl_2$; (10) 100 μM H_2O_2; (11) 10 μM HOCl; (12) 100 μM $\cdot O_2^-$; (13) 100 μM ·OH; (14) 100 μM $NaNO_2$; (15) 100 μM BSA; (16) 1 mM $ZnCl_2$; (17) 100 μM $NaHSO_3$; (18) 30 μM NADH; (19) 100 μM H_2S. The reactions were performed in the phosphate buffer (pH 7.4) at room temperature for 40 min; λ_{ex} = 535 nm.

7. Studies on reaction mechanism

In order to investigate the reaction mechanism, the reaction products of **1** with H_2S were subjected to HPLC, mass spectral and absorption spectral analyses. As shown in Fig. S7, the probe **1** gives a chromatographic peak at 16.69 min (peak 3 in curve A), whereas cresyl violet acetate (purchased from Sigma-Aldrich) displays two chromatographic peaks at 7.11 min and 15.40 min (peaks 1 and 2 in curve B), respectively, which is surprising to us. To explore this, the component 1 (for peak 1 in curve B of Fig. S7) and the component 2 (for peak 2 in curve B of Fig. S7) were collected and then subjected to ESI-MS analysis. Interestingly, the two components produced an identical pseudo-molecular ion peak at *m/z* 262 $[M + H]^+$, as depicted in Fig. S8 and Fig. S9, respectively. Furthermore, the absorption spectra of the two components can be changed into each other with acid or base. As shown in Fig. S10, for example, addition of diluted HCl into the solution of the component 1 results in the red-shift of the absorption maximum from 520 nm (curve c) to 595 nm (curve e), which resembles the spectroscopic features of both cresyl violet (curve a) and the component 2 (curve b). Conversely, introduction of diluted NaOH into the solution of the component 2, whose absorption spectrum (curve b) is also similar to that (curve a) of cresyl violet, leads to the blue-shift of the absorption maximum from 595 nm (curve b) to 520 nm (curve d). These studies show that the two chromatographic peaks of cresyl volet may arise from its protonation equilibrium (Scheme S1), which however has not been found in literatures.

After reaction with H_2S for 40 min, the chromatographic peak at 16.69 min of the probe **1** almost vanishes, concomitant with the appearance of the two major characteristic chromatographic peaks of cresyl violet at 7.11 min and 15.40 min (curve C in Fig. S7). This indicates that cresyl violet is formed in the reaction system. ESI-MS analysis further confirmed the generation of cresyl violet, because its corresponding pseudo-molecular ion peak at *m/z* 262 $[M + H]^+$ can be clearly observed in the reaction solution (Fig. S11). Based on the above findings, we propose that the reaction of **1** with H_2S may proceed through the reduction route of the azide group to an amino one, as shown in Scheme S2.

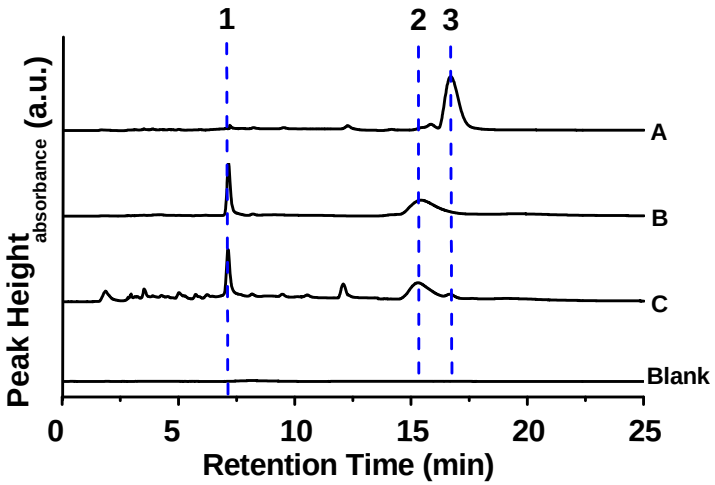


Fig. S7 Chromatograms of different reaction systems. (A) 50 μM probe **1**; (B) 50 μM cresyl violate acetate; (C) the reaction products of 50 μM probe **1** with 500 μM NaHS; (Blank) 100 μM NaHS. HPLC analyses were performed on an Inertsil ODS-SP column (5 μm, 4.6 mm × 250 mm) using a Shimadzu HPLC system that consists of two LC-20AT pumps and a SPD-20A UV-vis detector at 254 nm with methanol (flow rate, 0.8 mL/min) and water (flow rate, 0.2 mL/min) as eluents. The assignments of the peaks: (1) 7.11 min and (2) 15.40 min, cresyl violet; (3) 16.69 min, probe **1**.

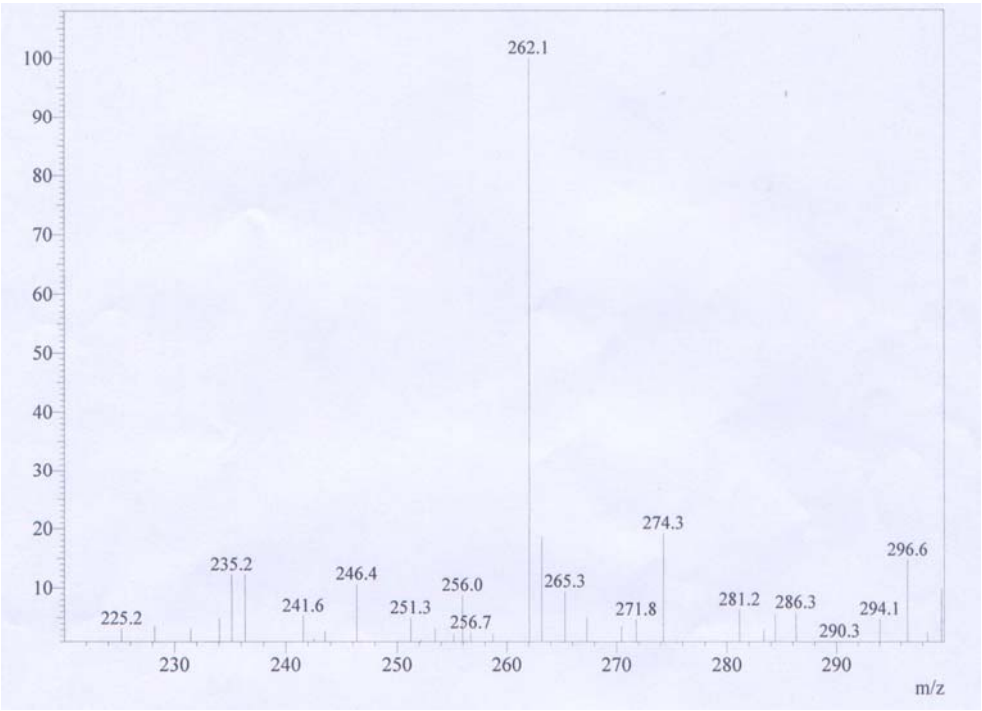


Fig. S8 ESI mass spectrum of the component for peak 1 in curve B of Fig. S7. The major peak at *m/z* 262 $[M+H]^+$ was characterized to be cresyl violet.

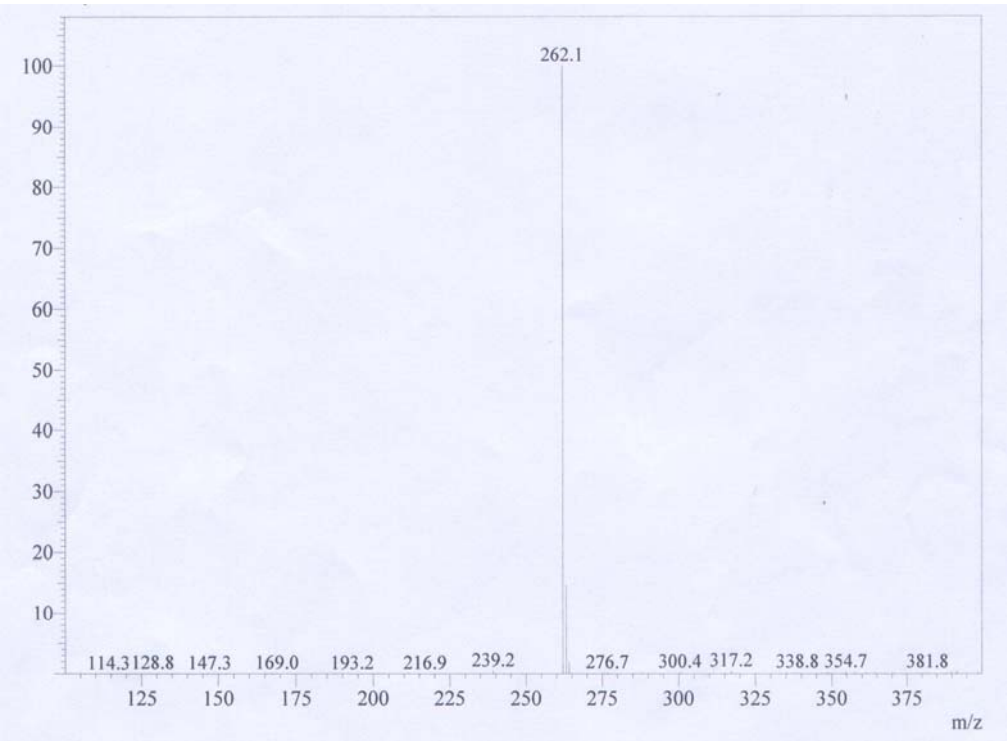


Fig. S9 ESI mass spectrum of the component for peak 2 in curve B of Fig. S7. The major peak at *m/z* 262 $[M+H]^+$ was characterized to be cresyl violet.

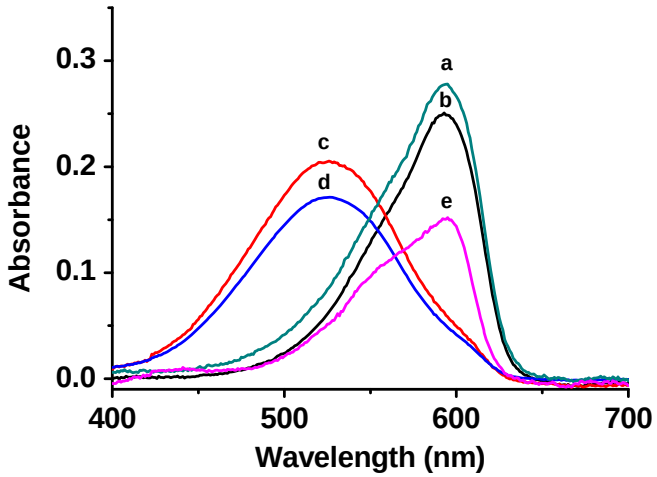
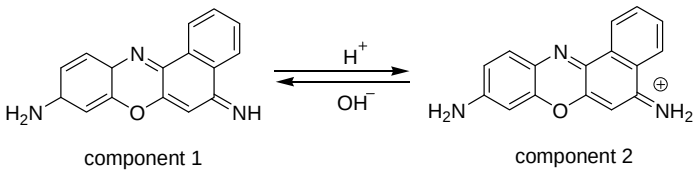


Fig. S10 Absorption spectra. (a) Cresyl violet acetate (10 μM); (b) absorption spectrum of the component 2 (for peak 2 in curve B of Fig. S7); (c) absorption spectrum of the component 1 (for peak 1 in curve B of Fig. S7); (d) absorption spectrum of the component 2 treated with diluted NaOH; (e) absorption spectrum of the component 1 treated with diluted HCl. The concentrations of the two components 1 and 2 were not known because they were collected through HPLC separation (also see Fig. S7), and the absorption spectra were recorded with a TU-1900 spectrophotometer (Beijing, China).



Scheme S1 Protonation equilibrium of cresyl violet.

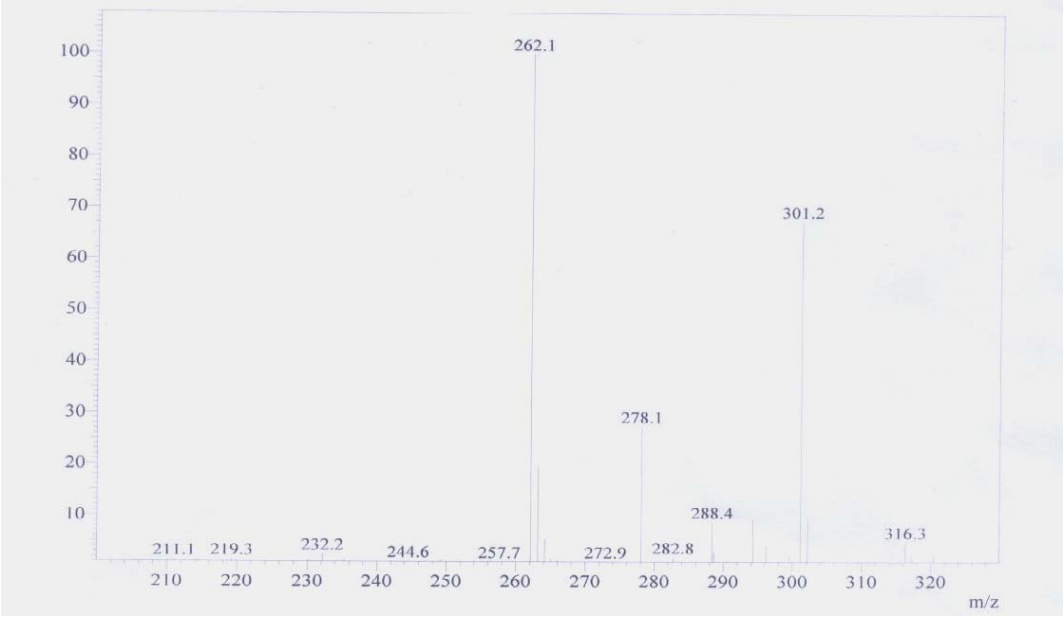
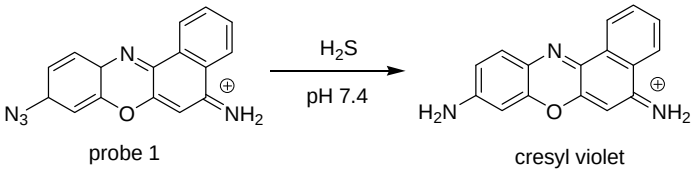


Fig. S11 ESI mass spectrum of the reaction products of **1** with 10 equiv. of NaHS after 30 min. The major peak at *m/z* 262 $[M+H]^+$ was characterized to be cresyl violet, and the peak at *m/z* 288 $[M+H]^+$ was the remained probe **1**.



Scheme S2 Proposed reaction mechanism of **1** with H_2S.

8. Fluorescence imaging of H_2S in MCF-7 cells and zebrafishes

The MCF-7 cells were grown on glass-bottom culture dishes (MatTek Co.) in DMEM supplemented with 10% (v/v) fetal bovine serum, penicillin (100 μg/mL) and streptomycin (100 μg/mL) at 37 °C in a 5% CO_2 incubator. Before use, the adherent cells were washed three times with serum-free DMEM. For H_2S imaging, the cells were first loaded with the probe **1** (10 μM) in serum-free DMEM at 37 °C for 20 min, washed 3 times with PBS solution (pH 7.4) to remove the

excess **1**, and then treated with NaHS (100 μM) in PBS at room temperature for 40 min.

Zebrafishes were obtained from the National Zebrafish Resources of China (Beijing), and maintained in E3 embryo media (15 mM NaCl, 0.5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 0.7 mM $NaHCO_3$, 5-10 % methylene blue; pH 7.5). In fluorescence imaging experiments, 5-day-old zebrafishes were incubated with 10 μM **1** in E3 embryo media for 20 min at 28 ºC. After washing with PBS (pH 7.4) to remove the remaining **1**, the zebrafishes were further treated with different concentrations of H_2S at 28 ºC for 20 min.

Fluorescence imaging experiments were performed on a FV 1000-IX81 confocal laser scanning microscope (Olympus, Japan) with FV5-LAMAR for excitation at 559 nm through a 100× 1.4 NA objective for MCF-7 cells and a 100× 0.4 NA objective for zebrafishes. Optical sections were acquired at 0.8 μm. The fluorescence was collected in the ranges of 570-600 nm (probe **1**) and 640-670 nm (cresyl violet), respectively. Following our previous approach (W. Shi, X. H. Li and H. M. Ma, *Angew. Chem. Int. Ed.*, 2012, 51, 6432-6435), region of interest (ROI) was selected based on peripheries of cells and zebrafishes. Image processing and analysis was performed on Olympus software (FV10-ASW), and the ratio of ROI was calculated pixel-by-pixel. All data were expressed as mean ± standard deviation.

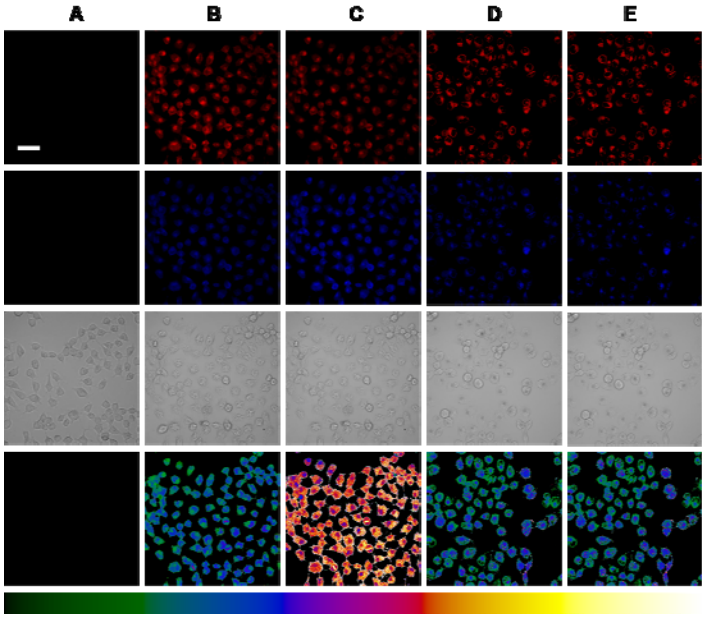


Fig. S12 Fluorescent images of H_2S in MCF-7 cells. (A) MCF-7 cells only (control). (B) MCF-7 cells were incubated with **1** (10 μM) for 20 min and then washed with PBS (pH 7.4) for imaging. (C) The above **1**-loaded

MCF-7 cells (B) were then treated with NaHS (100 μM) for 40 min. (D) MCF-7 cells were pretreated with 1 mM $ZnCl_2$ for 30 min, incubated with **1** (10 μM) for 20 min, and then washed with PBS (pH 7.4) for imaging (another control). (E) The above MCF-7 cells (D) were then treated with NaHS (100 μM) for 40 min. The images of the first row (channel of probe **1**) and second row (channel of cresyl violet) were collected in the ranges of 570-600 nm and 640-670 nm, respectively. The third row shows the corresponding differential interference contrast images. The images of the fourth row (the ratio channel) were generated by Olympus software (FV10-ASW). The bottom color strip represents the pseudocolor change with H_2S. Scale bar, 50 μm.

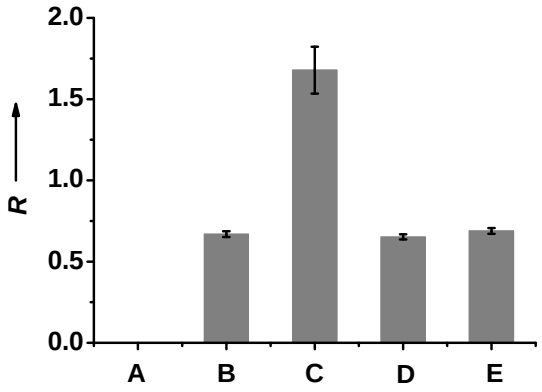


Fig. S13 The relative fluorescence intensity ratio ($R = I_{640–670}/I_{570–600}$) generated by Olympus software (FV10-ASW) according to the corresponding ratiometric images (i.e., the fourth row in Fig. S12) of MCF-7 cells. The R values were obtained from triplicate experiments (n = 3).

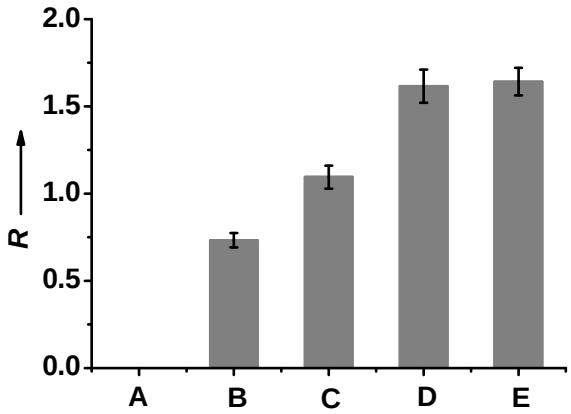


Fig. S14 The relative fluorescence intensity ratio ($R = I_{640–670}/I_{570–600}$) generated by Olympus software (FV10-ASW) according to the corresponding ratiometric images (the fourth row in Fig. 3) of zebrafishes. The R values were obtained from triplicate experiments (n = 3).

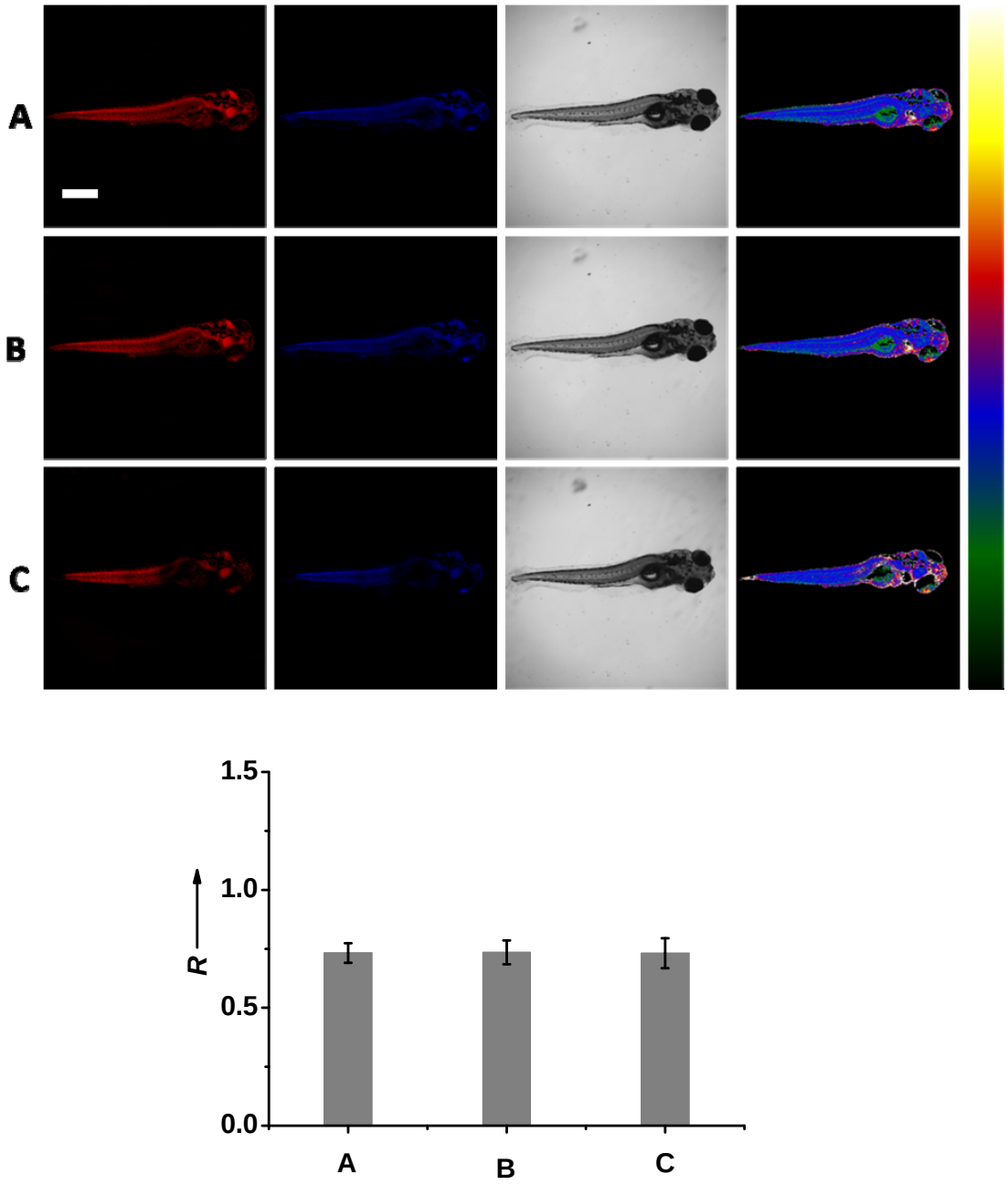


Fig. S15 (Top panel) Fluorescence change of 10 μM **1** itself in 5-day-old zebrafishes with time: (A) 20 min, (B) 40 min, and (C) 60 min. The images of the first column (channel of probe **1**) and second column (channel of cresyl violet) were collected in the ranges of 570-600 nm and 640-670 nm, respectively, upon excitation at 559 nm. The third column shows the corresponding DIC images. The images of the fourth column (the ratio channel) were generated by Olympus software (FV10-ASW). The right color strip represents the pseudocolor change with time. Scale bar, 500 μm. (Bottom panel) The R values (from the ratio channel) were obtained from triplicate experiments (n = 3).