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Supporting Information

A new ratiometric fluorescent probe for rapid, sensitive and selective detection of endogenous hydrogen sulfide in mitochondria

Xiao Feng[a,#], Tao Zhang[b,#], Jin-Ting Liu [a], Jun-Ying Miao[b,*], Bao-Xiang Zhao[a ,*]

[a] *Institute of Organic Chemistry, School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, P.R. China. E-mail: bxzhao@sdu.edu.cn (B.X. Zhao); jintliu@sdu.edu.cn (J.T. Liu); Tel.: +86 531 88366425; fax: +86 531 88564464;*

[b] *Institute of Developmental Biology, School of Life Science, Shandong University, Jinan 250100, P.R. China. E-mail: miaojy@sdu.edu.cn (J.Y. Miao)*

[#] *Equal contribution*

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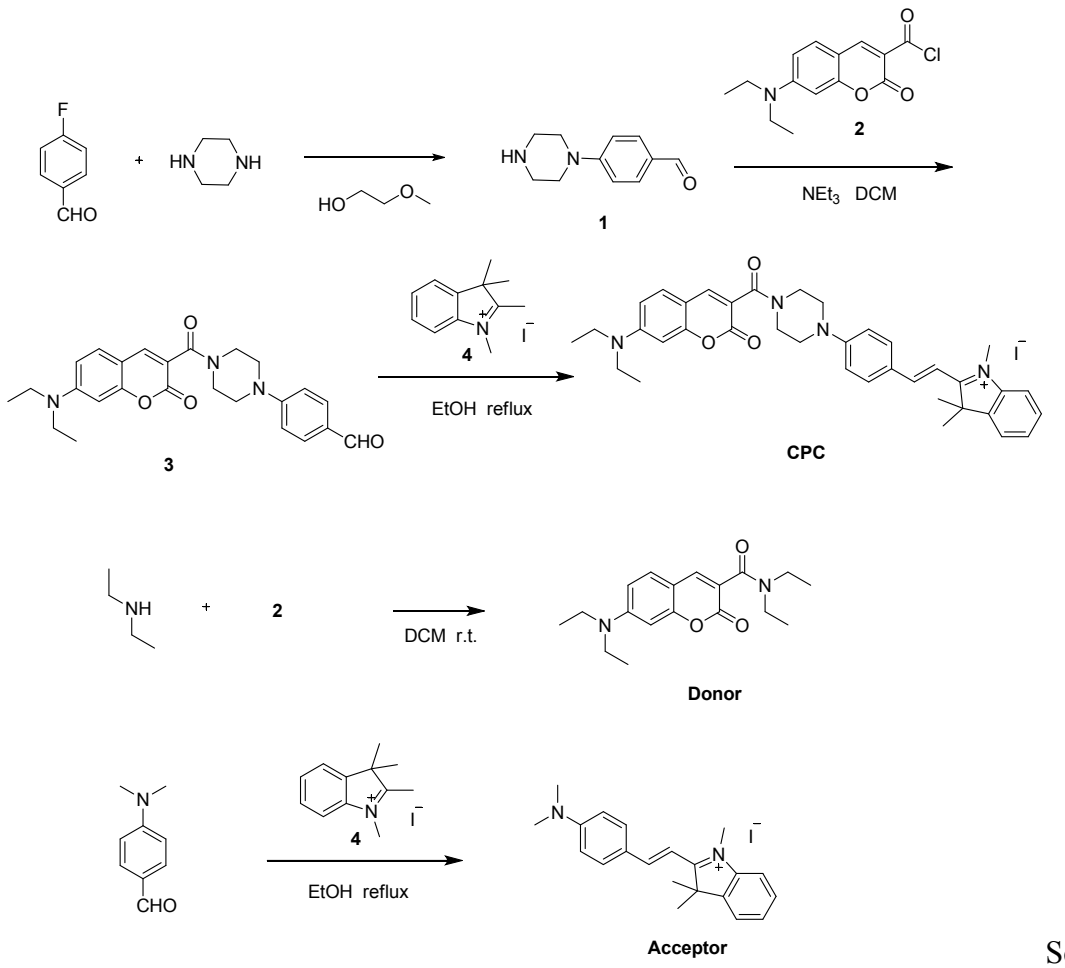
References

Apparatus and chemicals

^{1}H NMR (300 MHz) and ^{13}C NMR (100 MHz) spectra were acquired on a Bruker Avance 300 or 400 spectrometer, with DMSO-*d6* used as a solvent and tetramethylsilane (TMS) as an internal standard. Mass spectrometry (MS) involved High-resolution mass spectrometry (HRMS) Q-TOF6510 spectrograph (Agilent) and Thermo LCQ Fleet mass spectrometer (Thermo). Melting points of compounds were measured on an XD-4 digital micro-melting-point apparatus. IR spectra were recorded with an IR spectrophotometer VERTEX 70 FT-IR (Bruker Optics). UV–vis spectra were measured by a Hitachi U-4100 spectrophotometer. Fluorescent measurements were performed on a Perkin-Elmer LS-55 luminescence spectrophotometer. Quartz cuvettes with a 1-cm path length and 3-mL volume were used for all measurements. The pH was determined with a model PHS-3C pH meter. Unless otherwise stated, all reagents were purchased from Aladdin, J&K or Sinopharm Chemical Reagent Co. and used without further purification. Twice-distilled water was used throughout all experiments. The salts used in stock aqueous solutions of metal ions were KNO_3, $Ca(NO_3)_2{\bullet}4H_2O$, $NaNO_3$, KCl, KBr, KI, NaClO, $NaHCO_3$, Na_2SO_4, CH_3COONa, Na_2SO_3.

Synthesis



Scheme S1 Synthesis of probe CPC, **Donor** and **Acceptor**

Synthesis of compound 1

Piperazine (1.5 g, 17.41 mmol) was dissolved in H_2O (18 mL) and 2-methoxyethanol (20 mL). The mixture was heated to reflux, then the solution of 4-fluorobenzaldehyde (0.5 mL, 4.64 mmol) dissolved in 2-methoxyethanol (5 mL) was added dropwise over a period of 0.5 h. Then the mixture continued to reflux for 3 h. After cooling down to room temperature, the mixture was poured into H_2O (50 mL) to afford a yellow precipitate. The precipitate was filtered and dried to give a yellow solid **1** (0.59 g, 66.7%)，m.p. 180–182 °C.

Compound 2 was synthesized according to the reported method.[1]

Synthesis of compound 3

Compound **1** (0.118 g, 0.618 mmol) was dissolved in dry DCM (20 mL) and NEt_3 (0.3 mL) under nitrogen atmosphere in ice-salt bath. Then compound **2** (0.157 g, 0.562 mmol) was added to the mixture in batches. Then the mixture reacted in ice-salt bath for 0.5 h further in room temperature for 3.5 h. The reaction completed as shown TLC analysis. The solvent was then evaporated under reduced pressure, and the resulting residue was purified by flash column chromatography (dichloromethane : ethyl acetate : methanol = 50 : 10 : 1, V/V/V) to afford target product **3** (0.140 g, 57.6%), m.p. 195-197 °C. ^{1}H NMR (300 MHz, DMSO-*d6*): δ 9.73 (s, 1H), 8.02 (s, 1H), 7.74 (d, *J* = 9 Hz, 2H), 7.51 (d, *J* = 9 Hz, 1H), 7.06 (d, *J* = 9 Hz, 2H), 6.76 (dd, *J* = 9, 2.4 Hz, 1H), 6.57 (d, *J* = 2.4 Hz, 1H), 3.72 - 3.46 (m, 12H), 1.14 (t, *J* = 7.2 Hz, 6 H).

Synthesis of compound 4

2,3,3-Trimethyl-3H-indole (3.20 g, 20 mmol), iodomethane (5.68 g, 40 mmol) were mixed and dissolved in acetonitrile (10 mL). Then the mixture was refluxed for 11 h under nitrogen atmosphere. The mixture was cooled down to room temperature to afford a light pink precipitate. After filtered, the filter cake was washed with ethyl acetate (10 mL) for 3 times and dried in vacuum to give a light pink solid **4** (5.8 g, 96.0%).

Synthesis of probe CPC

Compound **3** (0.135 g, 0.311 mmol) and compound **4** (0.085 g, 0.282 mmol) were mixed and dissolved in ethanol (20 mL). Then the mixture was refluxed for 3 h. The solvent was then evaporated under reduced pressure, and the resulting residue was

purified by flash column chromatography (dichloromethane : ethyl acetate : methanol = 10 : 1 : 1, V/V/V) to afford a pure **CPC** (0.137 g, 68.0%). IR (KBr, cm^{-1}): 3449.5, 2973.2, 2935.4, 1713.6, 1577.9, 1522.5; ^{1}H NMR (300 MHz, DMSO-*d6*): δ 8.32 (d, *J* = 15.9 Hz, 1H), 8.11 (d, *J* = 9 Hz, 2H), 8.04 (s, 1H), 7.78 (dd, *J* = 14.1, 7.2 Hz, 2H), 7.60 - 7.49 (m, 3H), 7.36 (d, *J* = 15.9 Hz, 1H), 7.11 (d, *J* = 9 Hz, 2H), 6.77 (dd, *J* = 9, 2.2 Hz, 1H), 6.58 (d, *J* = 2.2 Hz, 1H), 4.02 (s, 3H), 3.75-3.58 (m, 8H), 3.47 (q, *J* = 6.9 Hz, 4H), 1.76 (s, 6H), 1.15 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ 206.88, 180.80, 164.82, 158.95, 157.19, 154.46, 154.20, 151.90, 144.75, 143.30, 142.48, 134.19, 130.67, 129.23, 128.48, 124.32, 123.15, 116.09, 114.46, 114.13, 109.96, 107.64, 107.37, 96.80, 51.67, 44.66, 33.87, 31.14, 26.50, 12.78; HRMS: m/z $[M]^+$ calcd for $C_{37}H_{41}N_4O_3^+$: 589.3179, found: 589.3159.

Synthesis of Donor

Compound **2** (0.110 g, 0.393 mmol) was added to a mixture of $HNEt_2$ (1 mL) and DCM (6 mL) with stirring. Then the mixture was stirred for 13 h. The solvent was then evaporated under reduced pressure, and the resulting residue was purified by flash column chromatography (petroleum ether : ethyl acetate = 2 : 1, V/V) to afford a pure **Donor** (0.055 g, 44.4%). ^{1}H NMR (300 MHz, DMSO-*d6*): δ 7.90 (s, 1H), 7.48 (d, *J* = 9.0 Hz, 1H), 6.74 (dd, *J* = 9.0 and 2.4 Hz, 1H), 6.56 (d, *J* = 2.4 Hz, 1H), 3.48 - 3.34 (m, 6H), 3.24 (q, *J* = 6.9 Hz, 2H), 1.15 - 1.03 (m, 12H); HRMS: m/z $[M+H]^+$ calcd for $[C_{18}H_{25}N_2O_3]^+$: 317.1865, found: 317.1864.

Synthesis of Accepter

4-Dimethylaminobenzaldehyde (0.112 g, 0.75 mmol), compound **4** (0.150 g, 0.50

mmol) were mixed and dissolved in ethanol (10 mL). Then the mixture was refluxed for 4.5 h under nitrogen atmosphere. The solvent was then evaporated under reduced pressure, and the resulting residue was purified by flash column chromatography (DCM : MeOH = 20:1, V/V) to afford a pure **Accepter** (0.194 g, 89.8%). ^{1}H NMR (300 MHz, DMSO-*d6*) δ 8.31 (d, *J* = 15.9 Hz, 1H), 8.07 (d, *J* = 9 Hz, 2H), 7.77 (d, *J* = 7.8 Hz, 1H), 7.70 (d, *J* = 7.8 Hz, 1H), 7.58-7.45 (m, 2H), 7.26 (d, *J* = 15.9 Hz, 1H), 6.89 (d, *J* = 9 Hz, 2H), 3.97 (s, 3H), 3.16 (s, 6H), 1.75 (s, 6H); ^{13}C NMR (100 MHz, DMSO-*d6*): 206.93, 180.06, 154.90, 154.52, 143.02, 142.54, 129.15, 128.00, 123.08, 122.74, 114.04, 112.65, 105.66, 51.33, 33.57, 31.17, 26.71; HRMS: m/z $[M]^+$ calcd for $C_{21}H_{25}N_2^+$: 305.2018, found: 305.2059.

Preparation of the test solution

Sodium sulfide ($Na_2S•9H_2O$) was diluted from the commercially available solution to 0.01 M in water. A stock solution of probe **CPC** (1×10^{-3} M) was prepared in DMF. Test solutions were prepared by displacing 50 μL of the stock solution into a 10-mL volumetric flask. The solution was diluted to 10 mL in a mixture of phosphate buffer (pH 7.4, 0.01 M) and DMF (6 : 4, V/V). The solutions of various testing species were prepared from Cys, GSH, Hcy, H_2O_2, KNO_3, $Ca(NO_3)_2•4H_2O$, $NaNO_3$, KCl, KBr, KI, NaClO, $NaHCO_3$, Na_2SO_4, CH_3COONa, Na_2SO_3, $N_2H_4•H_2O$, $NH_3•H_2O$, $CO(NH_2)_2$, $CS(NH_2)_2$, EDA, NO, O_2^-, respectively. Nitric oxide (NO) was generated from potassium nitroprusside dehydrate.[2] Superoxide (O_2^-) was prepared from KO_2. Small aliquots of each testing species solution were added. The resulting solution was

shaken well and incubated for 1 h at room temperature before recording the spectra.

The UV-vis absorption and fluorescence property of probe CPC was measured in a mixture of PBS buffer (pH 7.4, 0.01 M) : DMF (6: 4, v/v) except the selectivity test for hydrogen sulfide and sulfite in PBS buffer (pH 7.4, 0.01 M) : DMF (1: 9, v/v).

Determination of fluorescence quantum yield

Quantum yield (Φ_F) was determined by the relative comparison procedure, with quinine sulfate dehydrate (Φ_{Fs} = 0.56, 0.1 N H_2SO_4)[3] or rhodamine B (Φ_{Fs} = 0.89, in ethyl alcohol)[4] as the main standard. The general equation used in the determination of relative quantum yields from earlier research is Eq. (1).[5]

$$\Phi_F = (\Phi_{Fs})(F_{Au})(A_s)(\eta^2_u) / (F_{As})(A_u)(\eta^2_s), \quad (1)$$

Where Φ_F and F_A are fluorescence quantum yield and integrated area under the corrected emission spectrum, respectively; A is absorbance at the excitation wavelength; η is the refractive index of the solution; and subscripts u and s refer to the unknown and the standard, respectively.

Calculation of energy transfer efficiency

Energy transfer efficiency (*E*) was calculated using the following equation as reported.[6,7]

$$E = 1 - F_{DA} / F_D$$

Where, F_{DA} and F_D denote the donor fluorescence intensity with and without an acceptor, respectively.

Cell culture and imaging of cells

Hela cells were seeded in a 6-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C.

Imaging of exogenous H_2S in living cells

Hela cells were incubated with **CPC (**3.0 μM) for 1 h and then treated with Na_2S (0.5, 1, 5 mM) for 1 h. Subsequently, the cells were imaged using a confocal microscope (LSM 700) at 405 nm excitation and emission channels of 405-560 nm (blue channel) and 560-700 nm (red channel).

Imaging of endogenous H_2S in living cells

HeLa cells incubated with CPC (1 μM) for 1 h or cells incubated with Cys (200 μM) for 1 h followed by CPC (1 μM) for 1 h. Inhibition test was carried out by following procedure: cells incubated with PAG (1 mM), AOAA (1 mM) and both PAG and AOAA for 1 h, respectively, followed by further incubation with Cys (200 μM) for 1 h, then incubated with CPC (1 μM) for 1h. Subsequently, the cells were imaged using a confocal microscope (LSM 700) at 405 nm excitation and emission channels of 405-560 nm (blue channel) and 560-700 nm (red channel).

Co-localization imaging of cells

Hela cells were incubated with **CPC (**1 μM) for 1 h at 37 ℃. Then, Mito Tracker Deep Red (0.3 μM) was added and incubated for another 0.5 h and the confocal fluorescent images were captured.

Table S1. The spectroscopic data of compounds **CPC, Donor** and **Acceptor**

Compound	λ_{abs} (nm)	Logε ($M^{-1}cm^{-1}$)	λem (nm)	Φ_F
CPC	418	4.58	474	0.005[a]
	518	4.81	587	0.01[b]
Donor	406	4.41	475	0.139[a]
Acceptor	546	4.90	588	0.004[b]
CPC + H_2S 10 eq	--	--	474	0.015[a]

[a] λ_{ex} = 370 nm [b] λ_{ex} = 495 nm

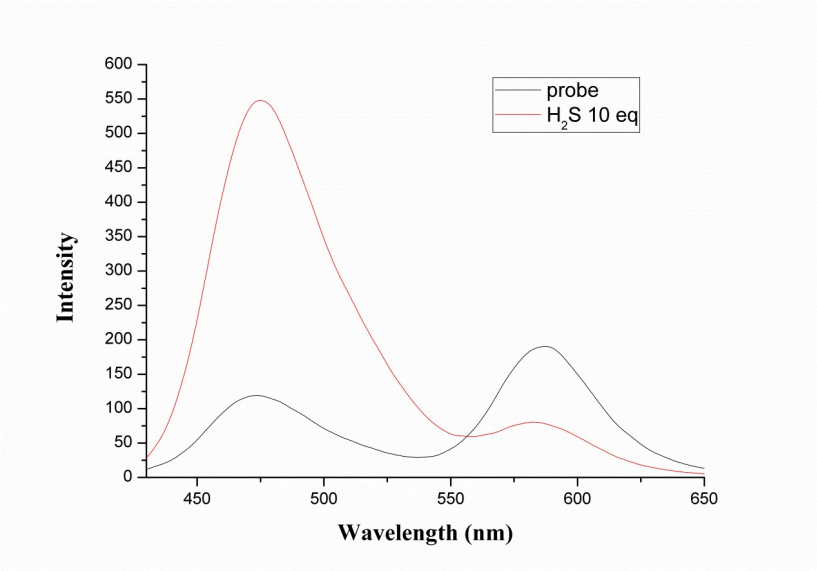


Fig. S1 Fluorescence spectra of the probe (5 μM) in the absence and presence of hydrogen sulfide (50 μM); λ_{ex} = 410 nm, slit: 12 nm/10 nm.

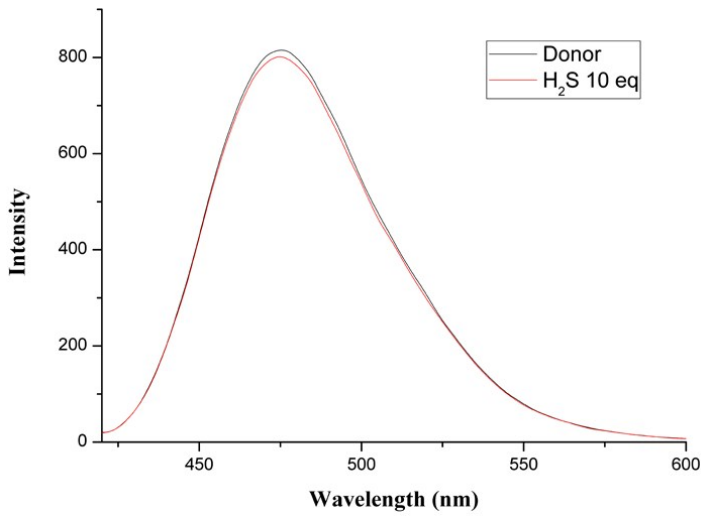


Fig. S2 Fluorescence spectra of the **donor** (5 μM) in the absence and presence of hydrogen sulfide (50 μM); λ_{ex} = 410 nm, slit: 12 nm/4.5 nm.

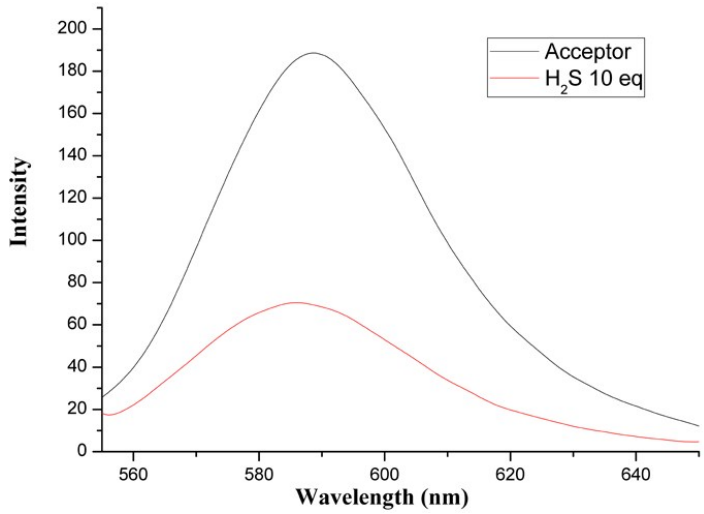


Fig. S3 Fluorescence spectra of the **acceptor** (5 μM) in the absence and presence of hydrogen sulfide (50 μM); λ_{ex} = 540 nm, slit: 12 nm/10 nm.

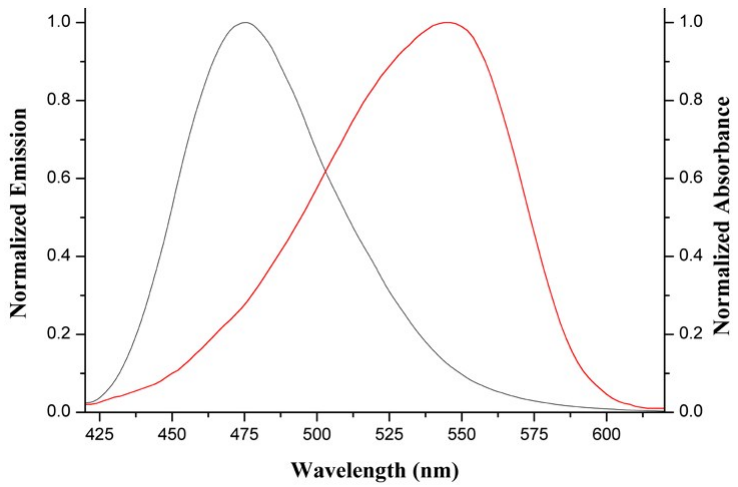


Fig. S4 The normalized fluorescence spectrum of the **donor** (5 μM) (black) and the normalized absorption spectra of the **acceptor** (5 μM) (red). λ_{ex} = 410 nm, slit: 12 nm/4.5 nm.

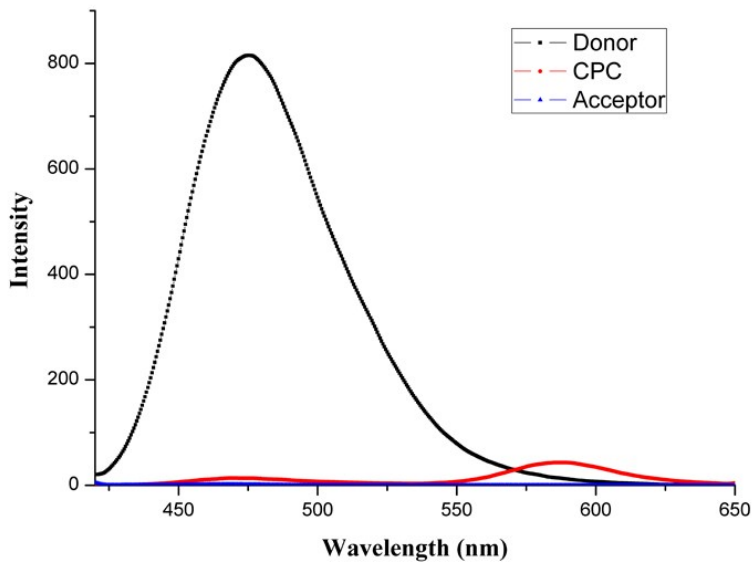


Fig. S5 The fluorescence emission spectra of the probe (5 μM) (red), the reference donor (5 μM) (black), and the reference acceptor (5 μM) (blue); λ_{ex} = 410 nm, slit: 12 nm/4.5nm. E = 98.35%.

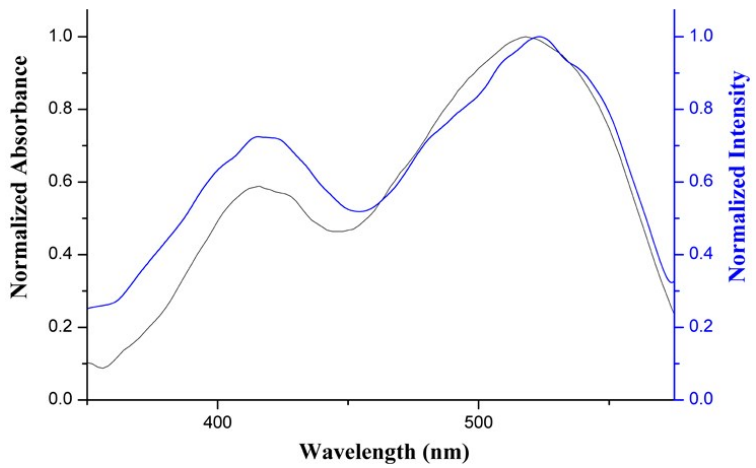


Fig. S6 The absorption (black) and corrected excitation (blue) spectra of probe **CPC** (5 μM); λ_{em} = 587 nm, slit: 12 nm/10 nm.

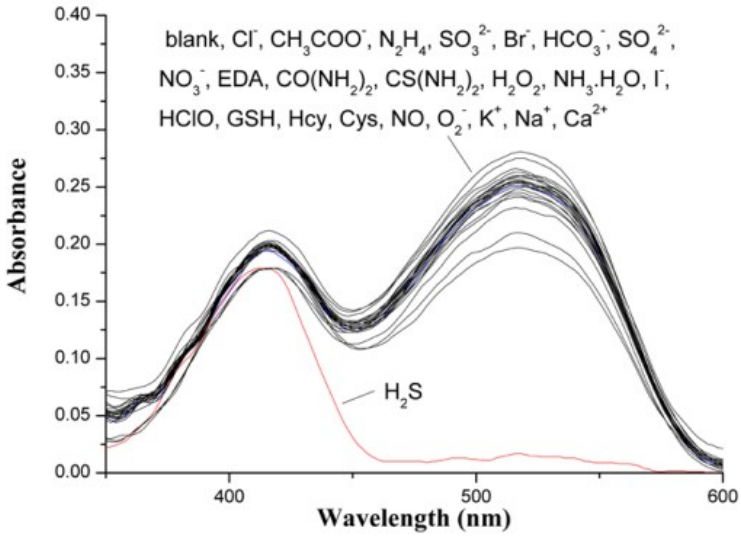


Fig. S7 Absorption spectra of the probe (5 μM) toward Na_2S (10 equiv.) and various biologically relevant species (20 equiv.).

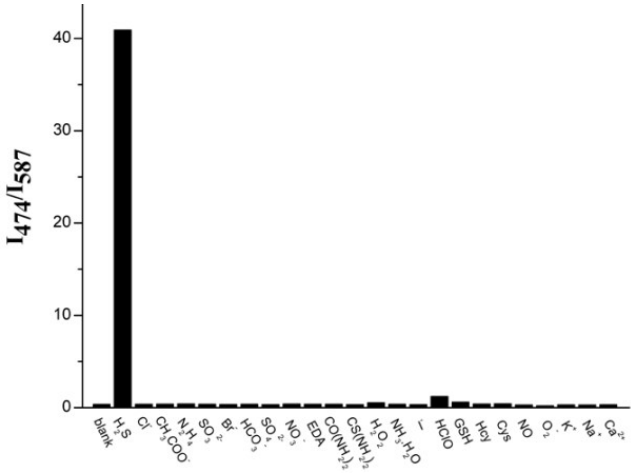


Fig. S8 Fluorescence intensity ratio (I_{474}/I_{587}) changes of the probe (5 μM) to Na_2S (50 μM), GSH (5 mM), Hcy (1 mM), Cys (1 mM) and other biologically relevant species (100 μM). λex = 410 nm , slit: 12 nm/10 nm.

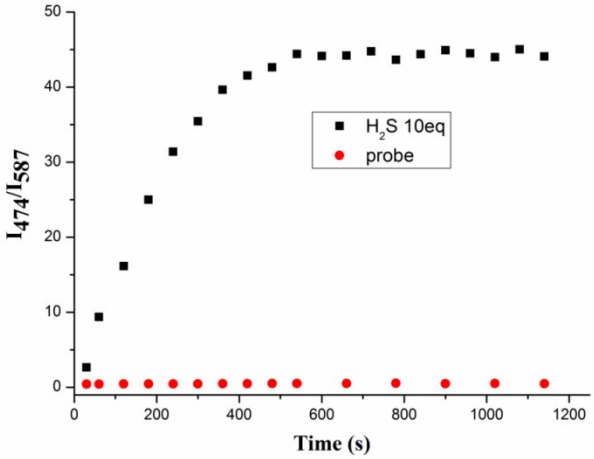


Fig. S9 Time-dependent fluorescence intensity ratio (I_{474}/I_{587}) of the probe (5 μM) with Na_2S 10 equiv.; λ_{ex} = 410 nm, slit: 12 nm/10 nm.

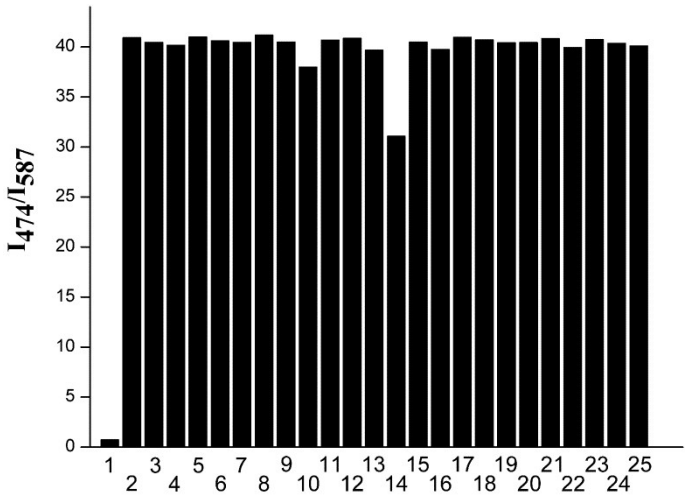


Fig. S10 Fluorescence intensity ratio (I_{474}/I_{587}) of the probe (5 μM) upon addition of Na_2S (50 μM) in the presence of various species, 1 free probe, 2 Na_2S at 50 μM. 3 Cl^-, 4 $CH_3CO_2^-$, 5 NO_3^-, 6 SO_3^{2-}, 7 Br^-, 8 HCO_3^-, 9 SO_4^{2-}, 10 EDA, 11 N_2H_4, 12 $CO(NH_2)_2$, 13 $CS(NH_2)_2$, 14 H_2O_2, 15 $NH_3.H_2O$, 16 I^- and 17 HClO at 100 μM. 18 GSH at 5 mM, 19 Hcy at 1 mM, 20 Cys at 1 mM. 21 NO, 22 O_2^-, 23 K^+, 24 Na^+ and 25 Ca^{2+} at 100 μM; λ_{ex} = 410 nm, slit: 12 nm/10 nm.

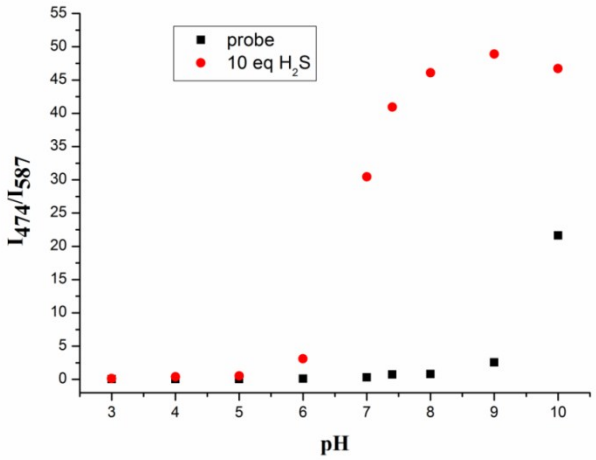


Fig. S11 pH-dependent fluorescence intensity ratio changes of the probe (5 μM);λ_{ex} = 410 nm , slit: 12 nm/10 nm.

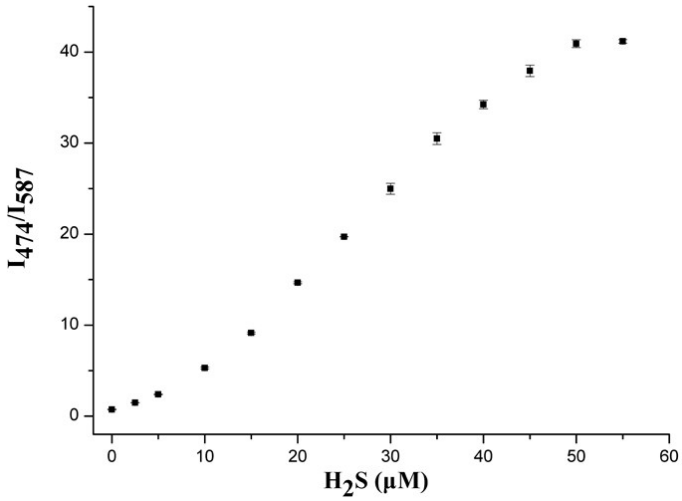


Fig. S12 Fluorescence intensity ratio (I_{474}/I_{587}) changes of the probe (5 μM) with the addition of Na_2S (0–55 μM); λ_{ex} = 410 nm, slit: 12 nm/10 nm.

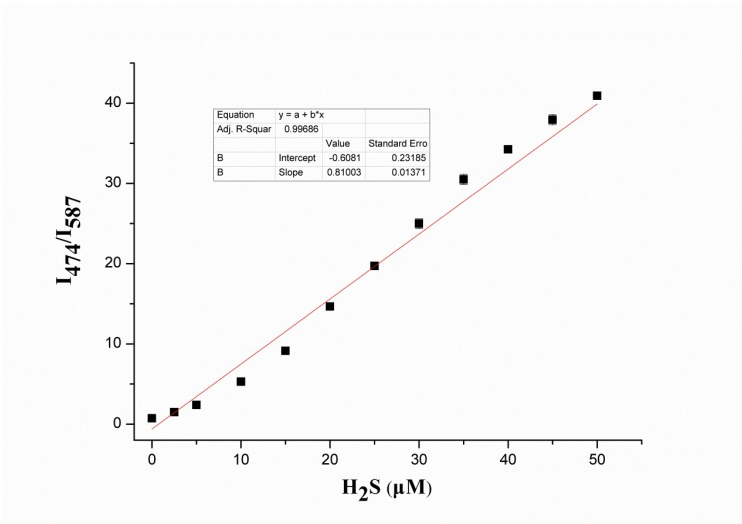


Fig. S13 Linearity of the ratio (I_{474}/I_{587}) of the probe with Na_2S from 0 to 50 μM. λ_{ex} = 410 nm, slit: 12 nm/10 nm.

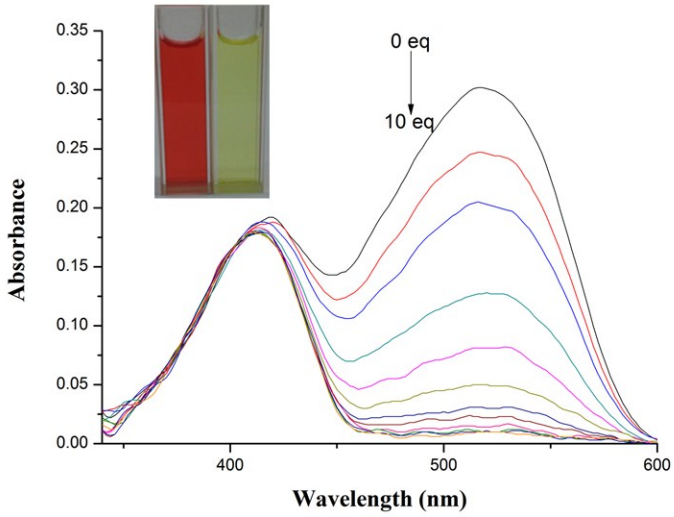


Fig. S14 Absorption spectra of the probe (5 μM) upon addition of Na_2S (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 equiv.). Inset: the color change observed by the naked eye of the probe with the addition of H_2S.

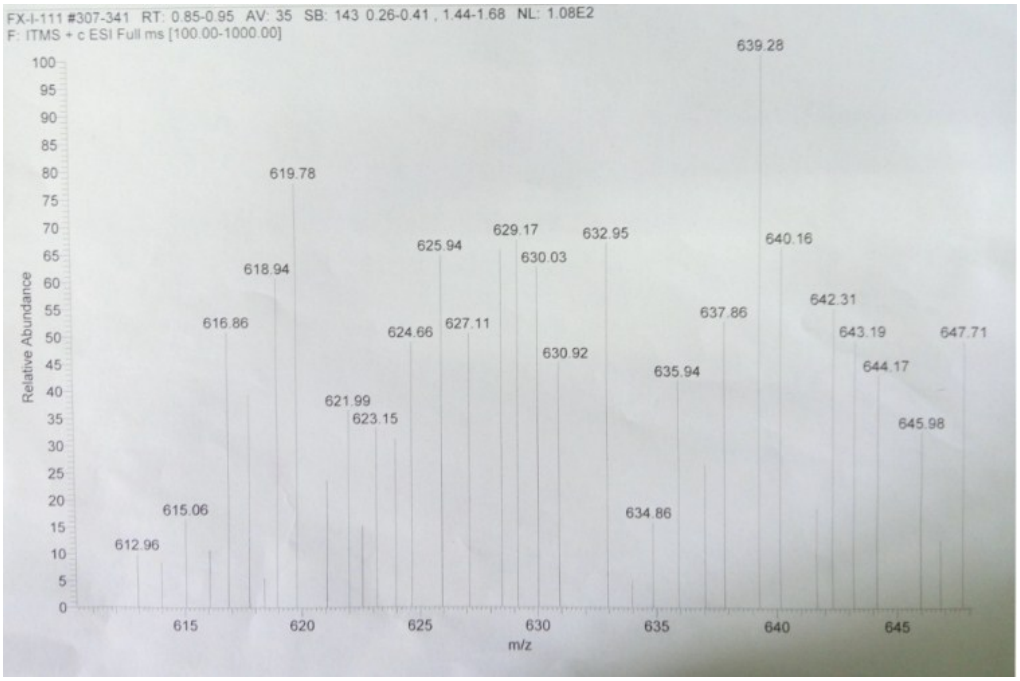


Fig. S15 Thermo LCQ Fleet mass spectrum of the **CPC** (50 μM) solution in the presence of excessive Na_2S (500 μM).

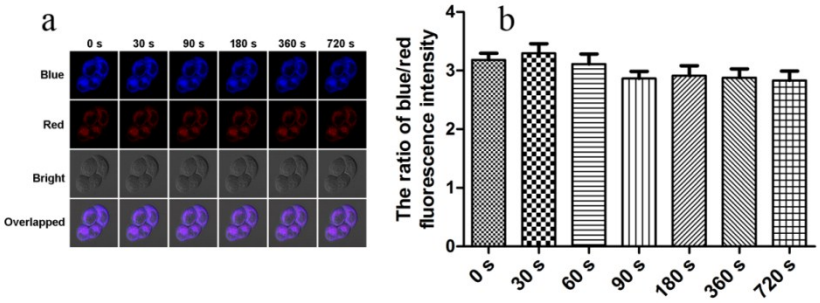


Fig. S16 (a) Photostability of probe **CPC** for fluorescence images of HeLa cells. The

cells were incubated with the probe (3 μM) for 1 h. First line: fluorescence images at the blue channel (405–560 nm), second line: fluorescence images at the red channel (560–700 nm), third line: bright field images, fourth line: overlay images of the first, second and third lines. λ_{ex} = 405 nm. (b) The ratio of blue/red fluorescence intensity of probe **CPC** for fluorescence images of HeLa cells.

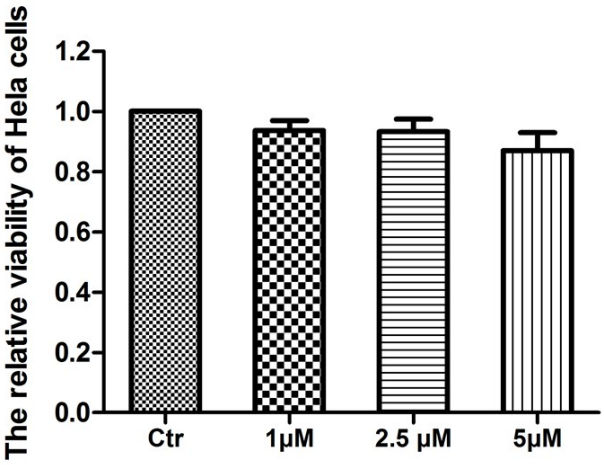


Fig. S17 Cytotoxicity of probe **CPC** evaluated on living HeLa cells by the standard SRB assay. The cells were incubated with the **CPC** (1, 2.5 and 5 μM) for 6 h.

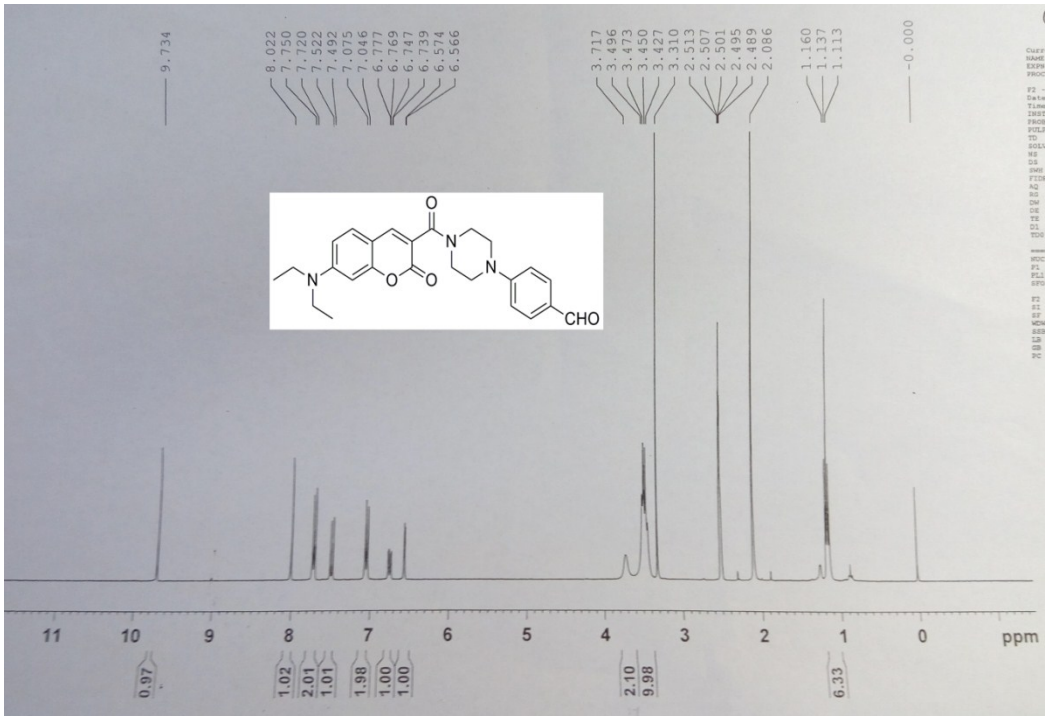


Fig. S18 ^{1}H NMR spectrum of compound **3** in DMSO-*d6*.

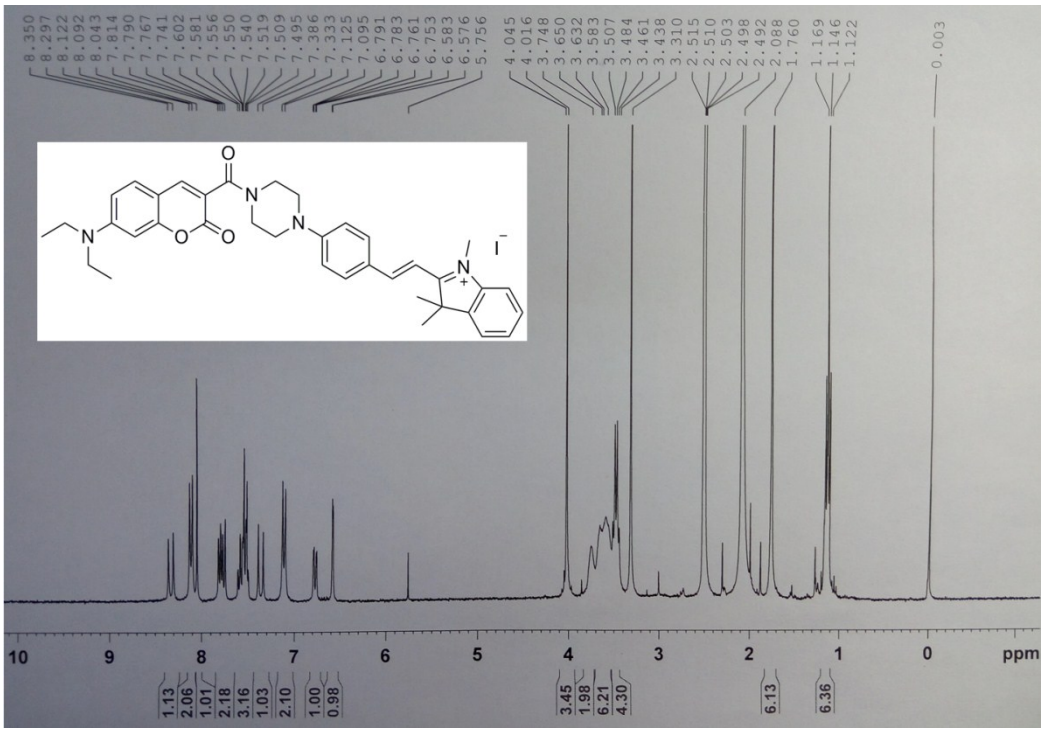


Fig. S19 ^{1}H NMR spectrum of probe **CPC** in DMSO-*d6*.

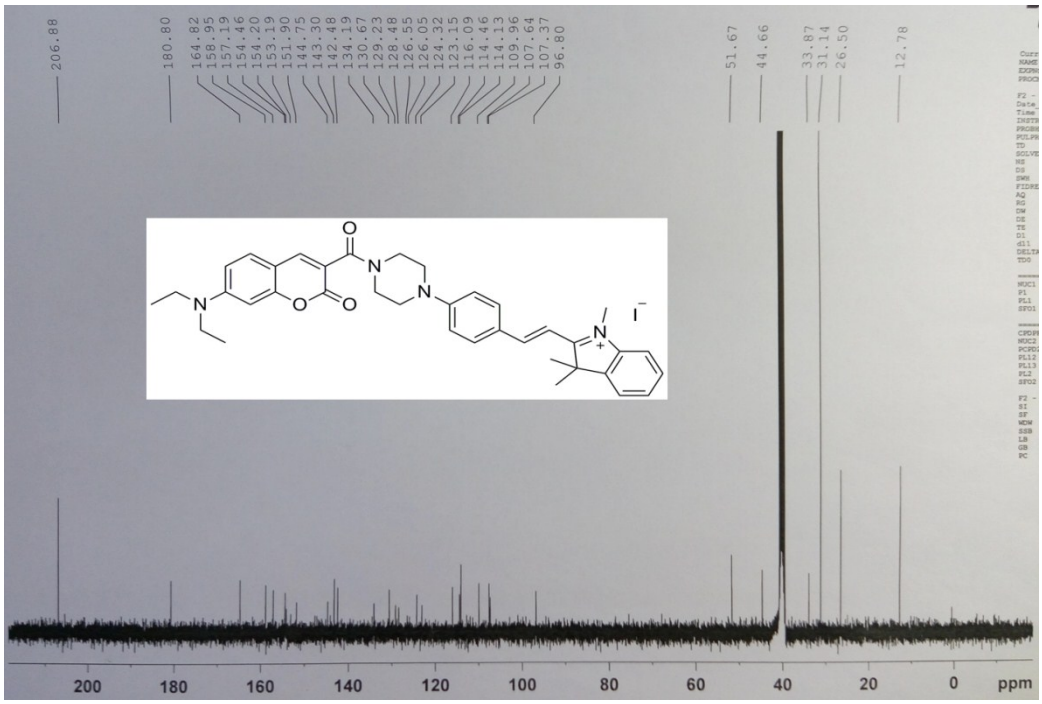


Fig. S20 ^{13}C NMR spectrum of probe **CPC** in DMSO-*d6*.

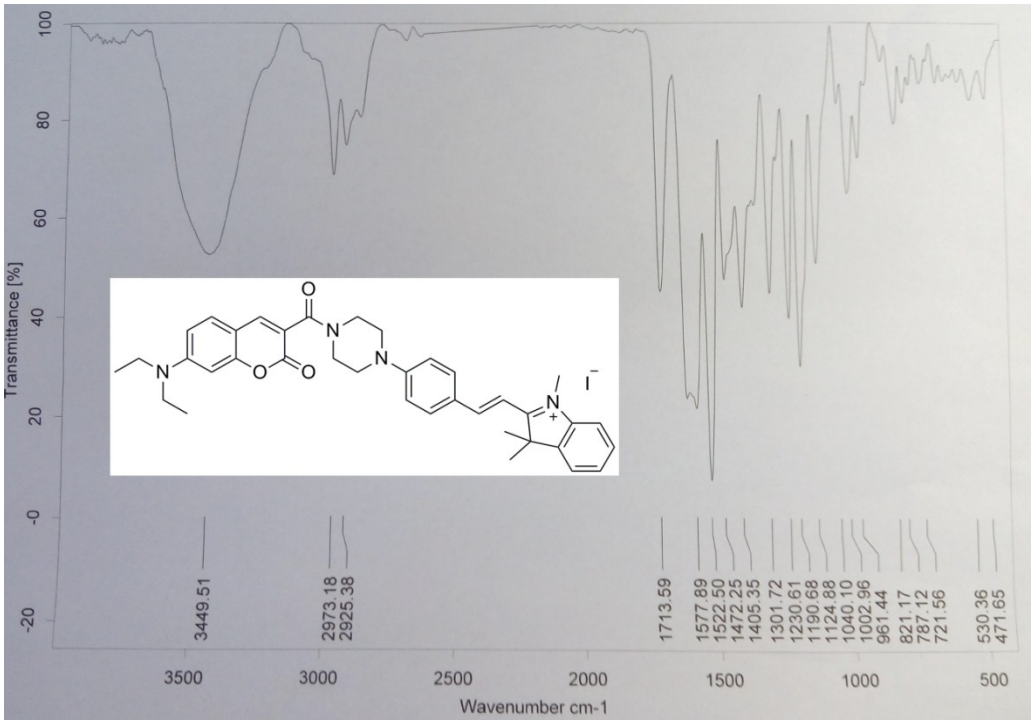


Fig. S21 IR spectrum of probe **CPC**.

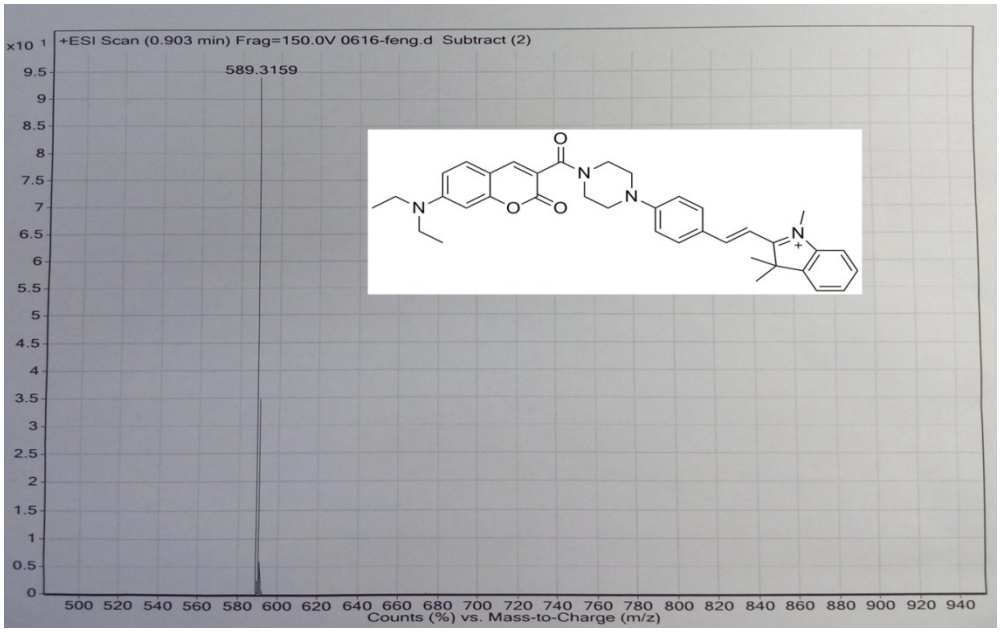


Fig. S22 HRMS spectrum of probe **CPC**.

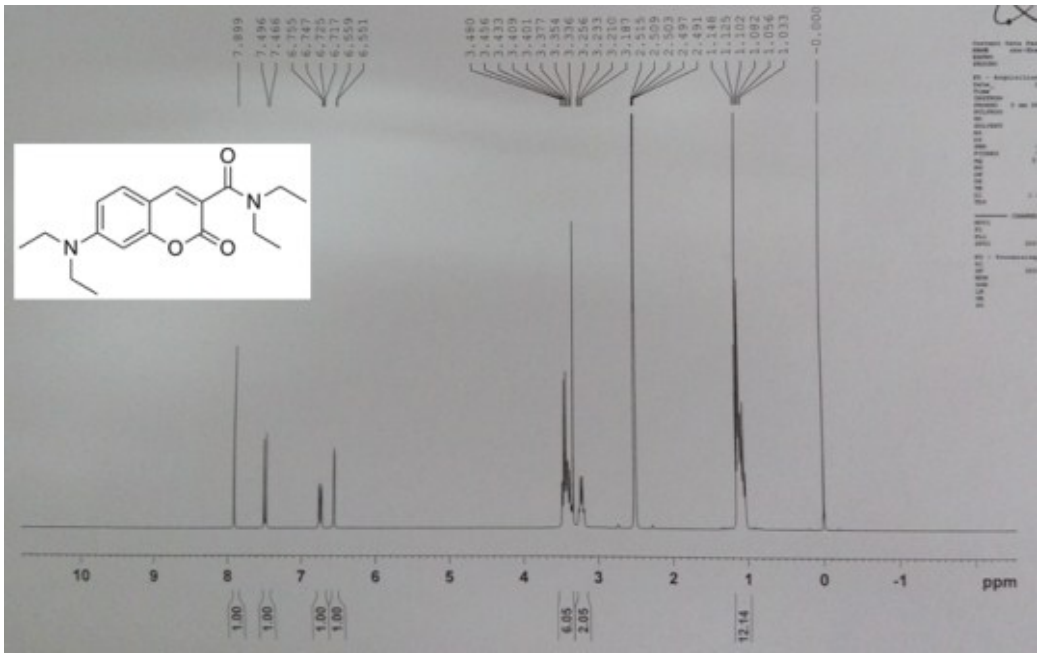


Fig. S23 ^{1}H NMR spectrum of **Donor** in DMSO-*d6*.

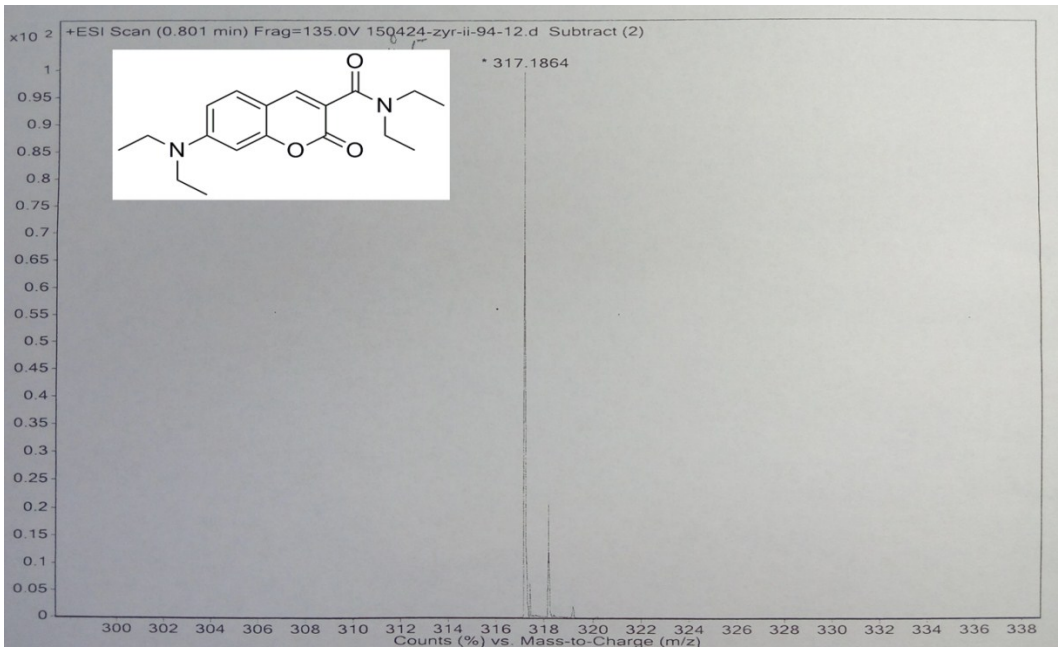


Fig. S24 HRMS spectrum of **Donor**.

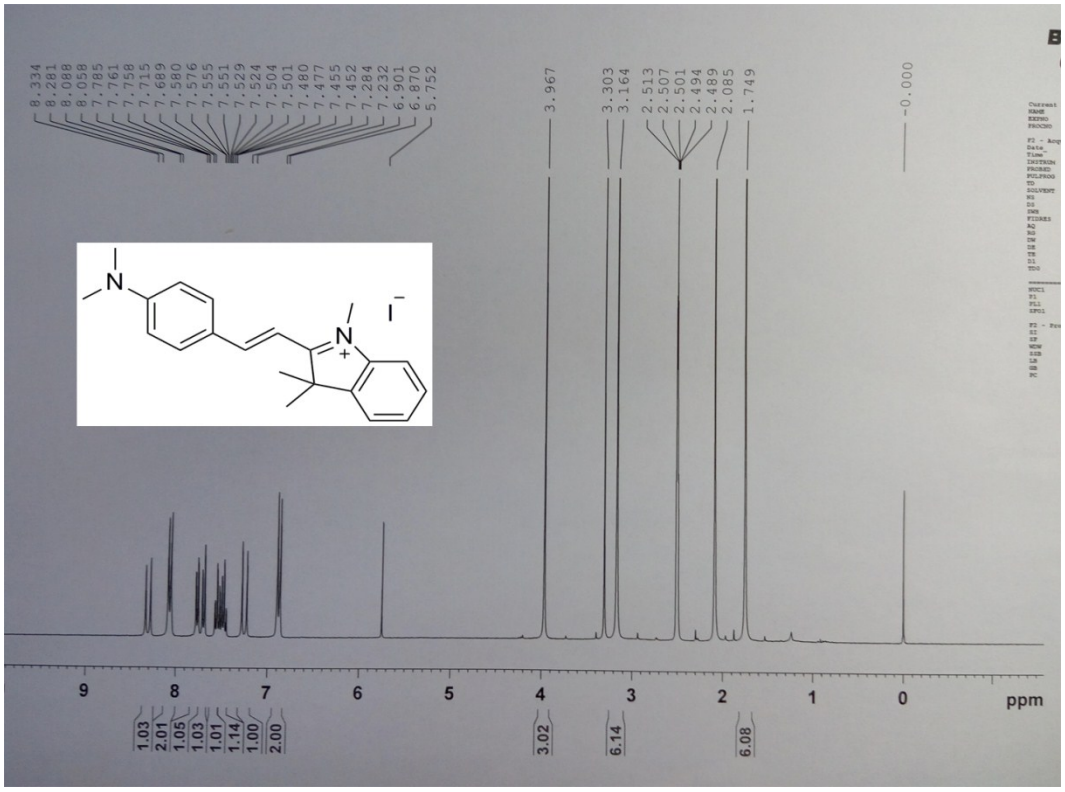


Fig. S25 ^{1}H NMR spectrum of **Acceptor** in DMSO-*d6*.

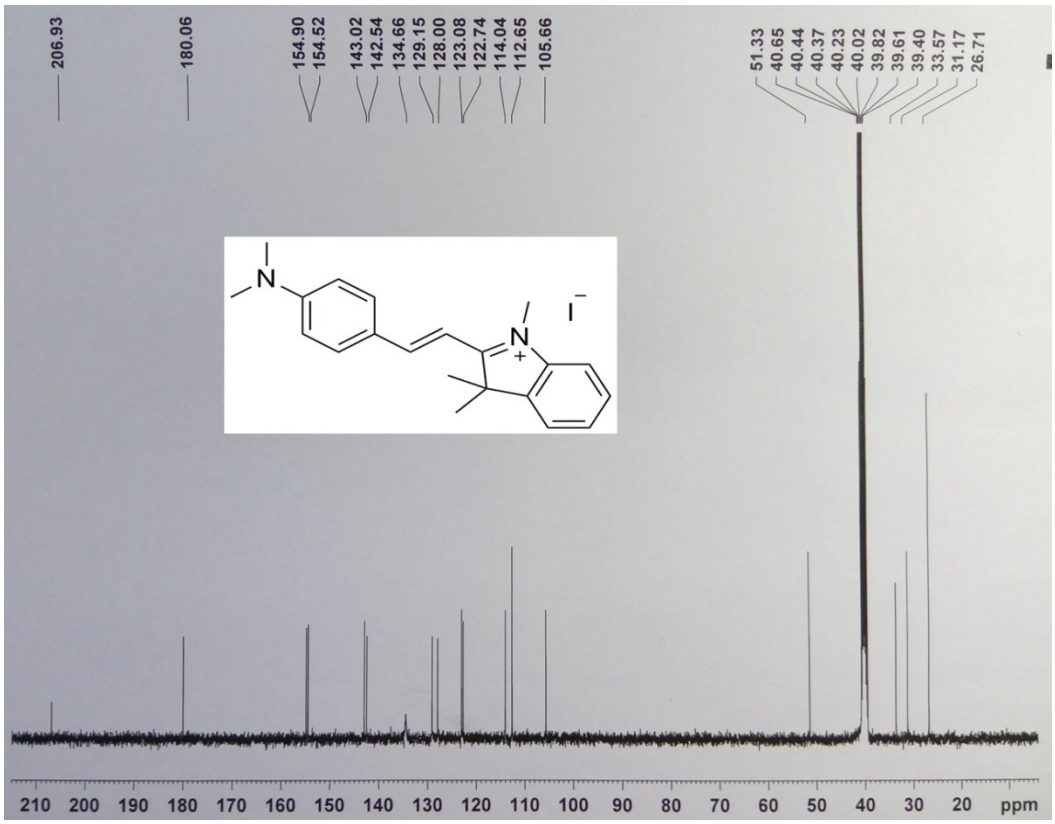


Fig. S26 ^{13}C NMR spectrum of **Acceptor** in DMSO-*d6*.

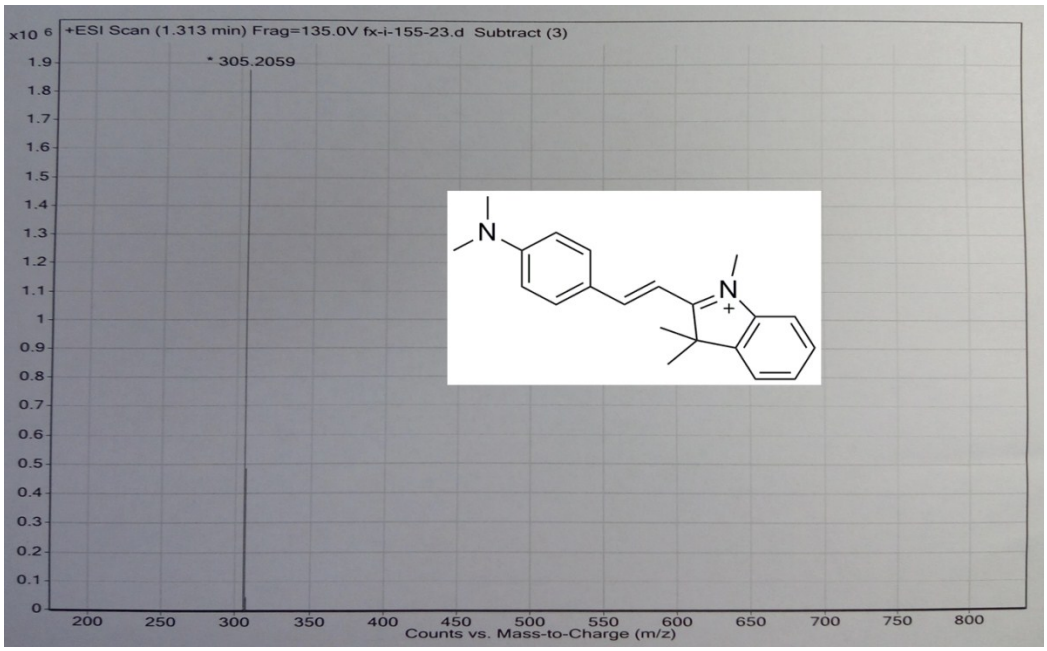


Fig. S27 HRMS spectrum of **Acceptor**.

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