Electronic Supplementary Information

A novel dual-emission fluorescent probe for simultaneous detection of H_2S and GSH

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1. Materials and methods

1.1. Materials

All chemicals were purchased from Sigma-Aldrich and Aladdin reagent Co. (Shanghai, China) and used without further purification. Column chromatography was conducted over silica gel (200-300 mesh) obtained from the Qingdao Ocean Chemicals. Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents were purified and dried by standard methods prior to use. Double-distilled water was used throughout the experiments. In all experiments enantiomerically pure natural amino acids were used except for Hcy, which was used as the racemate.

1.2. Instruments

The fluorescence spectra and relative fluorescence intensity were measured with a Shimadzu RF-5301 spectrofluorimeter with a 10 mm quartz cuvette. The absolute fluorescence quantum yield was determined by an interacting sphere (Edinburgh Instruments FLS-980 fluorimeter) under appropriate excitation from a 150 W xenon lamp. Absorption spectra were recorded using a Shimadzu UV-2550 spectrophometer. High-resolution mass spectra were recorded in negative mode with a HRMS apex ultra 7.0T US+ in electrospray ionization (ESI) mode. ^1H and ^{13}C NMR spectra were recorded on a Bruker AVIII 600 NMR spectrometer, using tetramethylsilane (TMS) as the internal standard. The pH measurements were carried out on a REX-PHS-3C PH Meter. The absorbance for MTT analysis was recorded on a microplate spectrophotometer (Molecular Devices, VersaMax, USA). Fluorescence imaging experiments were performed on confocal microscope (Olympus, IX81, JPN).

2. Synthesis of probe 1

The probe **1** was synthesized from malonic acid, 2,4,6-trichlorophenol, DMF, $POCl_3$, and malononitrile by substitution, perkin, Vilsmeie-Haack, and Knoevenagel condensation reaction (as shown in **Scheme S1**).

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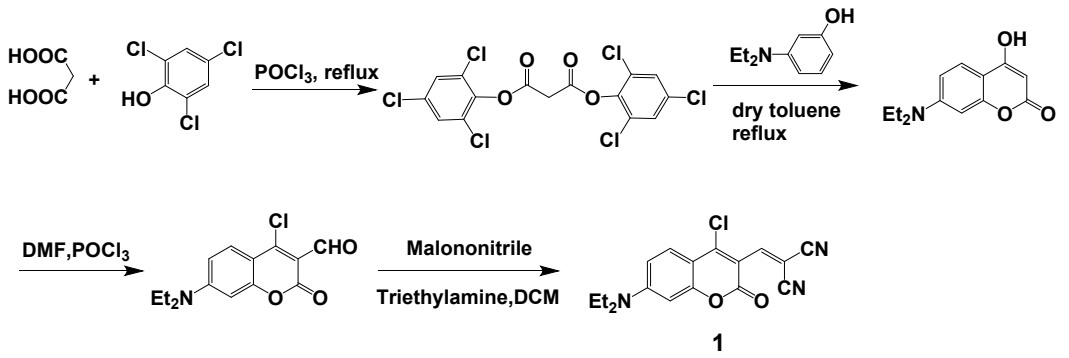


Scheme S1 Synthetic route of probe **1**

2.1 Bis(2,4,6-trichlorophenyl) malonate

Malonic acid (0.52 g, 5.0 mmol), 2,4,6-trichlorophenol (1.58 g, 8.0 mmol) and $POCl_3$ (1 mL, 1.645 g, 10.5 mmol) were added into the reactor (topped with a $CaSO_4$ drying tube) in a constant temperature oil bath at 100 ^oC. After stirring for 4 h with constant stirring, the reaction system was cooled to room temperature. Then the mixture was slowly poured into 75 mL CH_2Cl_2 and washed with iced, saturated $NaHCO_3$ solution (3×100 mL). The organic layer was then dried over Na_2SO_4 and concentrated to yield bis(2,4,6-trichlorophenyl) malonate as a white solid (1.75 g, 92.7% yield, and M.p 183.9-185.0 ^oC).[1]

2.2 7-(Diethylamino)-4-hydroxy-coumarin

3-Diethylaminophenol (1.65 g, 10 mmol), bis(2,4,6-trichlorophenyl) malonate (4.63 g, 10 mmol) and dry toluene (10 mL) were added into the reactor. The mixture was heated brought to reflux with constant stirring for 2 h. Then the reaction mixture was cooled to room temperature and filtered. The resultant grey solid was washed with toluene and dried under high vacuum to generate 7-(diethylamino)-4-hydroxy-coumarin (1.63 g, 70.1% yield, M.p 155.2-156.8 ^oC).[1]

2.3 7-(Diethylamino)-4-chloro-3-formyl-coumarin

Under nitrogen, fresh distilled DMF (2.8 mL) was added dropwise to $POCl_3$ (2.8 mL) at room temperature and stirred for 30 min to obtain a red solution. Then a portion of 7-(diethylamino)-4-hydroxy-coumarin (2.33 g, 10 mmol, dissolved in 13.2 mL DMF) was added dropwise to the above solution and yield a scarlet suspension. After the mixture was stirred at 60 ºC for 12 h, it was poured into 100 mL of ice water. NaOH solution (20 %) was used to adjust the pH to obtain a large amount of precipitate. The crude product was subjected to column chromatography (silica: hexanes/EtOAc=1/1) to give 7-(diethylamino)-4-chloro-3-formyl-coumarin as an orange solid (0.83 g, 61.4% yield, M.p 138.2-139.3 ^oC [1]). ^1H NMR ($CDCl_3$) δ ppm: 10.28 (s, 1H), 7.82 (d, J = 6.2 Hz, 2H), 6.69 (d, J = 6.2 Hz, 1H), 6.42 (d, J = 2.4 Hz, 1H), 3.50 (q, J = 7.2 Hz, 4H) 1.27 (t, J = 9.2 Hz, 6H).

2.4 2-((4-Chloro-7-(diethylamino)-2-oxo-2H-chromen-3-yl)methylene)malononitrile (1)

To a stirred solution of 7-(diethylamino)-4-chloro-3-formyl-coumarin (560 mg, 2 mmol) and malononitrile (135 mg, 2 mmol) in CH_2Cl_2 (30 mL), triethylamine (1.0 mL) was added at room temperature under argon. The reaction mixture was stirred at room temperature for 3 h.[2] The solvent was evaporated under reduced pressure, and the residue was purified by silica gel column chromatography (eluent: CH_2Cl_2/PE=1/1) to afford **1** as a black brown solid (336 mg, 51.2% yield, M.p 174.6-175.8 ^oC). ^1H NMR (600 MHz, $CDCl_3$) δ ppm: 7.81 (s, 1H), 7.72 (d, J = 9.0 Hz, 1H), 6.72 (dd, J = 9.0 Hz, 2.4 Hz, 1H), 6.46 (d, J = 2.4 Hz, 1H), 3.51 (q, J = 7.2 Hz, 4H), 1.28 (t, J = 7.2 Hz, 6H).

^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 156.46, 156.13, 154.13, 153.01, 150.74, 129.13, 114.71, 111.94, 111.01, 109.48, 107.37, 96.71, 86.40, 45.50, 12.48. HRMS (ESI) *m/z* calcd for $C_{17}H_{14}ClN_3O_2$ $[M+Na]^+$: 350.06668, found: 350.06698.

3. Spectrometer Measurement

In a set of 10 mL volumetric tubes containing 1.0 mL phosphate buffer (10 mM, pH 7.4), 3.0 mL of DMF and 50 μL of probe **1** (1.6 mM), different concentrations of analytes were added and the reaction mixture was diluted to 10 mL with H_2O. The resulting solution was well-mixed and kept at 25 °C for 10 min, and then the absorption or fluorescence spectra were recorded. The fluorescence emission intensity was measured at the excitation wavelength of 390 or 515 nm.

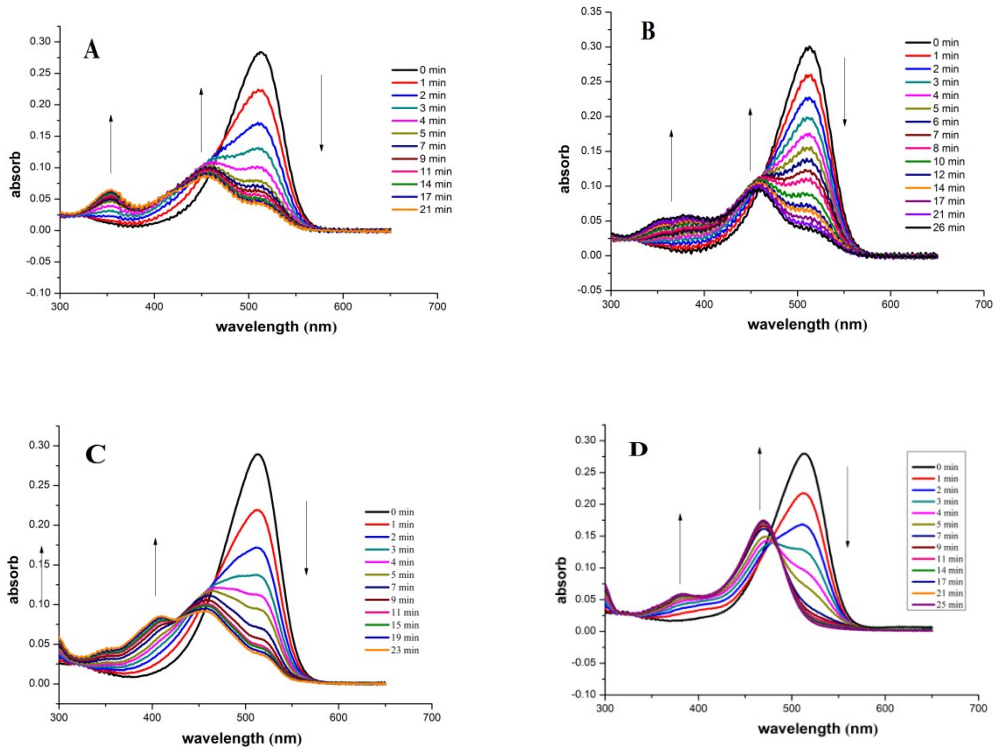
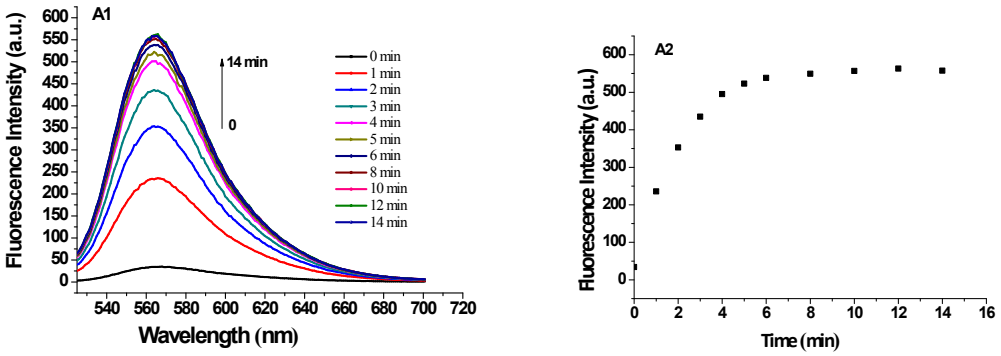


Fig. S1 (**A**-**D**)Time-dependent absorption spectra of **1** (5 μM) in the presence of 10 equiv of H_2S (**A**), Cys (**B**), GSH (**C**), and Hcy (**D**) in DMF/phosphate buffer (3:7 *V/V*, 10 mM, pH 7.4) at r.t.



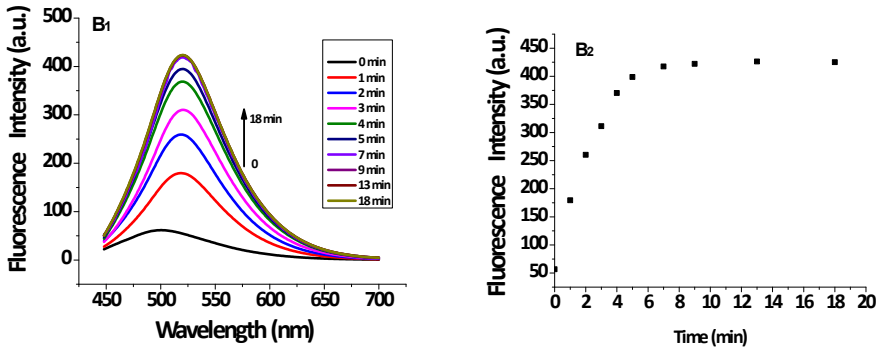


Fig. S2 Time-dependent fluorescence response of probe **1** (5 μM) toward 10 equiv of H_2S (**A**: excited at 515 nm, Slits: 5/5 nm) and GSH (**B**: excited at 430 nm, Slits: 10/10 nm) in PBS buffer (10 mM, pH 7.4) at r.t.

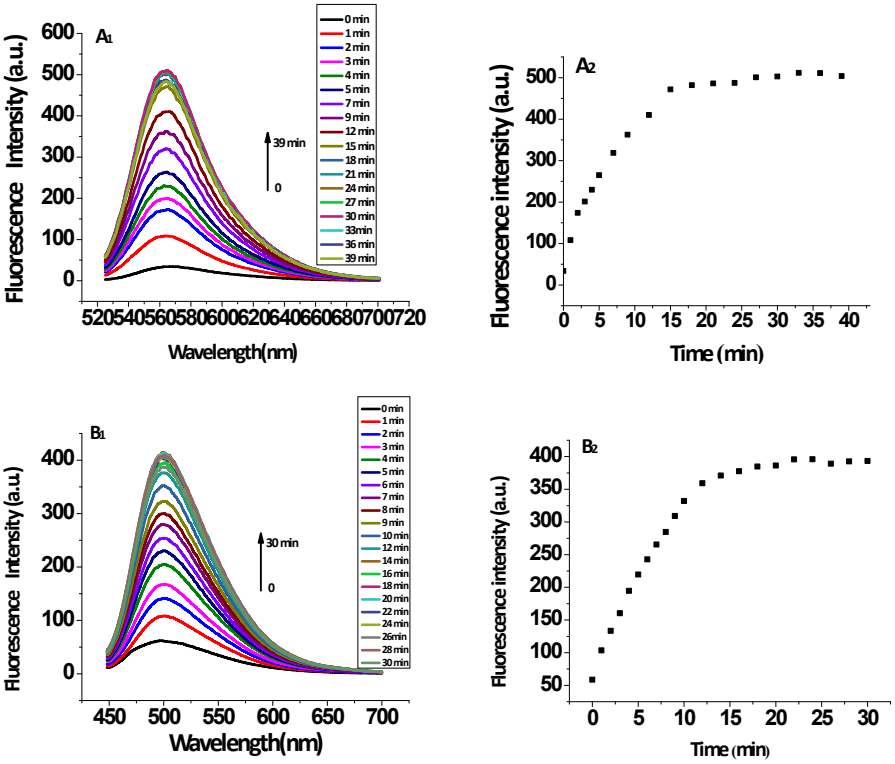


Fig. S3 Time-dependent fluorescence response of probe **1** (5 μM) toward H_2S upon addition 10 equiv of Cys, Hcy and GSH (**A**: excited at 515 nm, Slits: 5/5 nm) and GSH upon addition of10 equiv of H_2S, Cys, and Hcy (**B**: excited at 430 nm, Slits: 10/10 nm) in PBS buffer (10 mM, pH 7.4) at r.t.

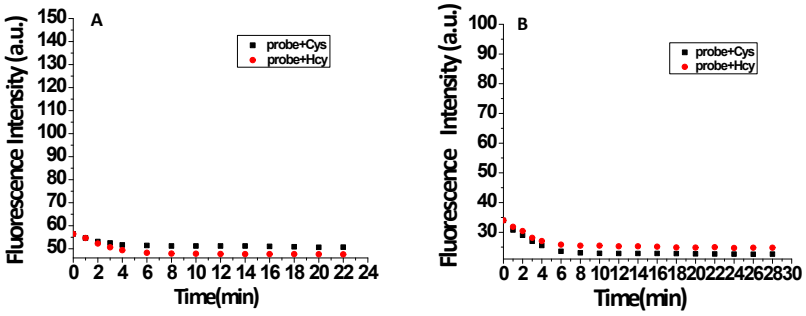


Fig. S4 Time-dependent fluorescence response of probe **1** (5 μM) toward 10 equiv of Cys and Hcy (**A**: excited at 430 nm, Slits: 10/10 nm) and 10 equiv of Cys and Hcy (**B**: excited at 515 nm, Slits: 5/5 nm) in PBS buffer (10 mM, pH 7.4) at r.t.

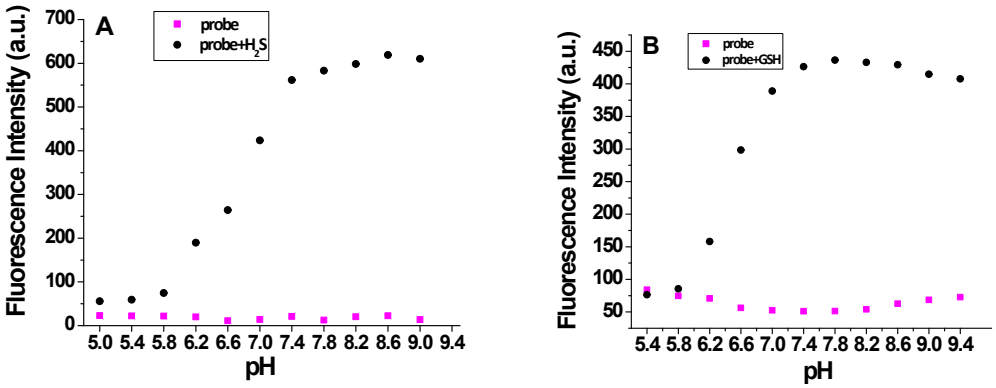


Fig. S5 The fluorescence increment of probe (5 μM) in the presence of H_2S (5 equiv) and GSH (10 equiv) at various pH values (5-9)

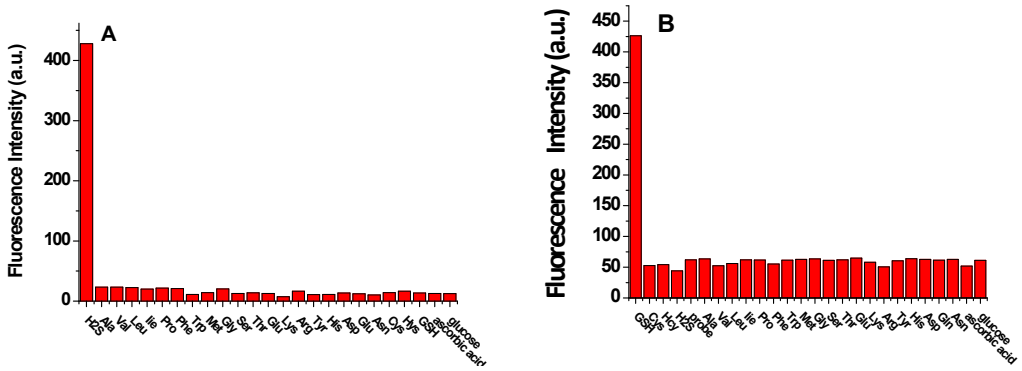


Fig. S6 Fluorescence intensity changes of probe (5 μM) upon addition of H_2S or GSH with various species in PBS buffer (10 mM, pH 7.4) at r.t.
A: 10 equiv. of H_2S and 20 equiv. of various amino acids biologically related Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Gly, Ser, Thr, Glu, Lys, Arg, Tyr, His, Asp, Glu, Asn, Cys, Hcy, and GSH
B: 10 equiv. of GSH and 20 equiv. of various amino acids biologically related Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Gly, Ser, Thr, Glu, Lys, Arg, Tyr, His, Asp, Glu, Asn, H_2S, Cys, and Hcy

4. MTT assay

The effect of probe **1** on cells viability was determined using the MTT assay.[3] In brief, MCF-7 cells were seeded in 96 well culture plates at density of 2×10^4 cells per well and incubated overnight. Then probe **1** was added to the wells to achieve final concentrations. Control wells were prepared by addition of culture medium. Wells containing culture medium without cells were used as blanks. At the end of incubation, 10 μL of MTT (5.0 mg/mL) was added into each well and incubation for another 4 h. Then, the supernatant was removed and 100 μL DMSO was added to each well for dissolve the MTT formazan. The optical density (*OD*) of formazan solutions produced was recorded on a microplate spectrophotometer (Molecular Devices, Versa Max, USA) at 570 nm. The cell viability was presented as the fold over the control group and was calculated according to the following formula: cell viability (%) = $(OD_{sample}\text{-}OD_{blank})/(OD_{control}\text{-}OD_{blank}\times100)$.

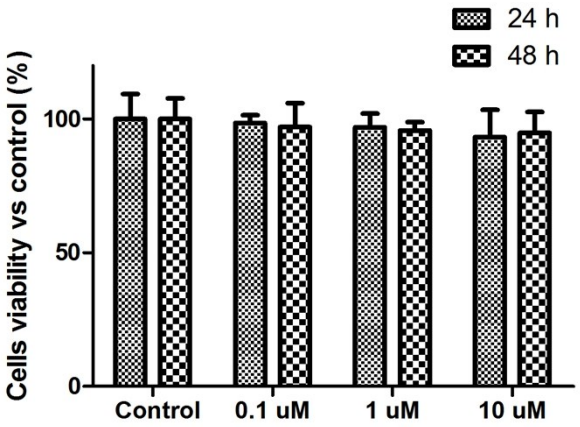


Fig. S7 Cell viability of MCF-7 cells treated by probe **1** at different concentrations for different time

5. Confocal microscopy experiments

MCF-7 cells were seeded in 6-well culture plates containing sterile coverslips and were cultured in RPMI 1640 medium supplemented with 10% (*V/V*) fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in a 95% humidity atmosphere under 5% CO_2 for 24 h. Before imaging, the cells were washed with PBS (pH 7.4) three times and then incubated with probe **1** (10 μM) in PBS (pH 7.4) at 25 °C for 30 min. After that, the samples were rinsed three times with PBS (pH 7.4) to remove the remaining probe. For the control experiment, the cells were pretreated with NEM (1 mM) at 37 °C for 30 min. After washing with PBS (pH 7.4) three times, the cells were further incubated with probe **1** (10 μM) in a DMSO/PBS (5/95, *V/V*) solution at 25°C for 30 min. Fluorescence imaging was performed by an Olympus FV1000 confocal laser scanning microscope. Emission was collected at 450-510 nm for the green channel (λ_{ex}=405 nm) and at 550-630 nm for the red channel (λ_{ex} = 515 nm).

6. Proposed mechanism for the reaction of probe 1 with Cys and Hcy

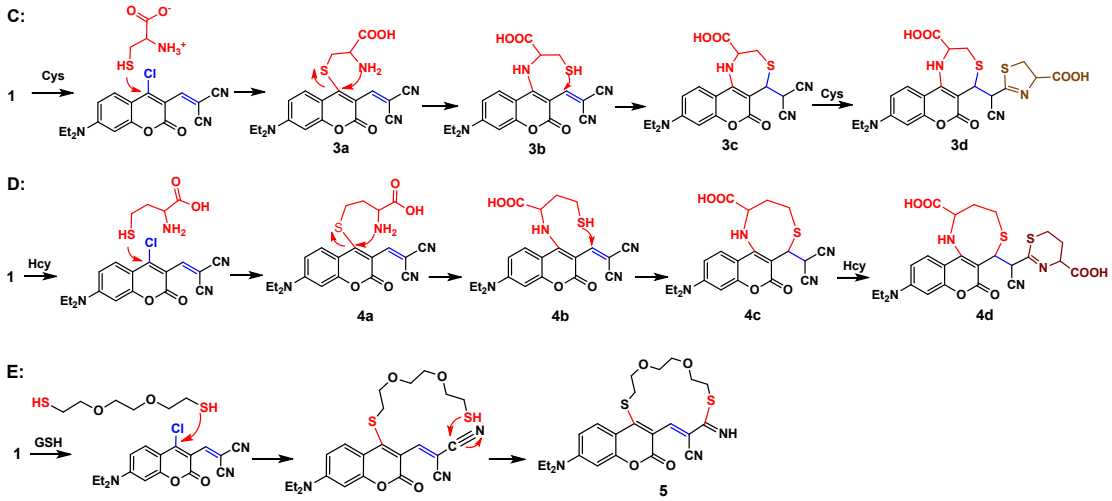


Fig. S8 Proposed mechanism for the reaction of probe **1** with Cys (**C**), Hcy (**D**) and DODT (**E**)

7. $^{1}H/^{13}C$ NMR and MS spectra

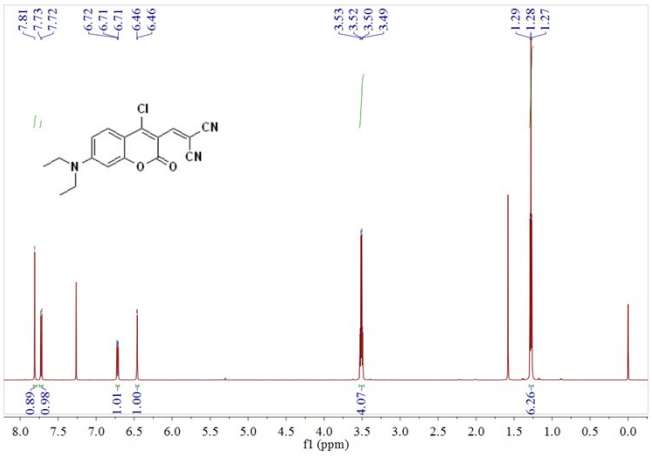


Fig. S9 The ^{1}H NMR spectrum of probe **1** in $CDCl_3$

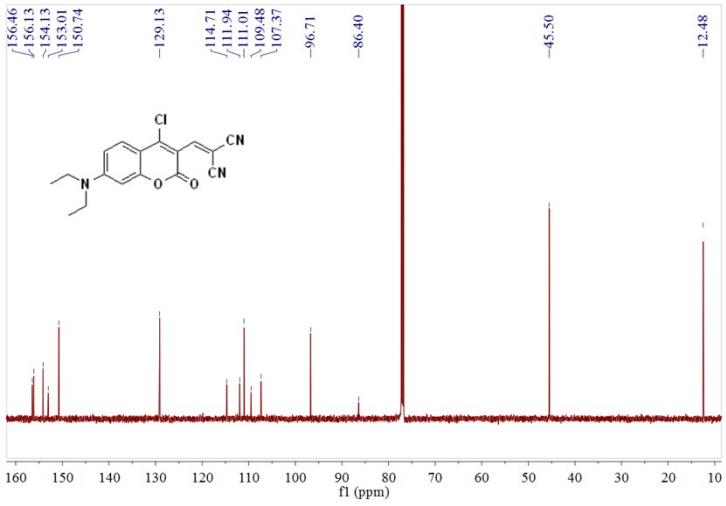


Fig. S10 The ^{13}C NMR spectrum of probe **1** in $CDCl_3$

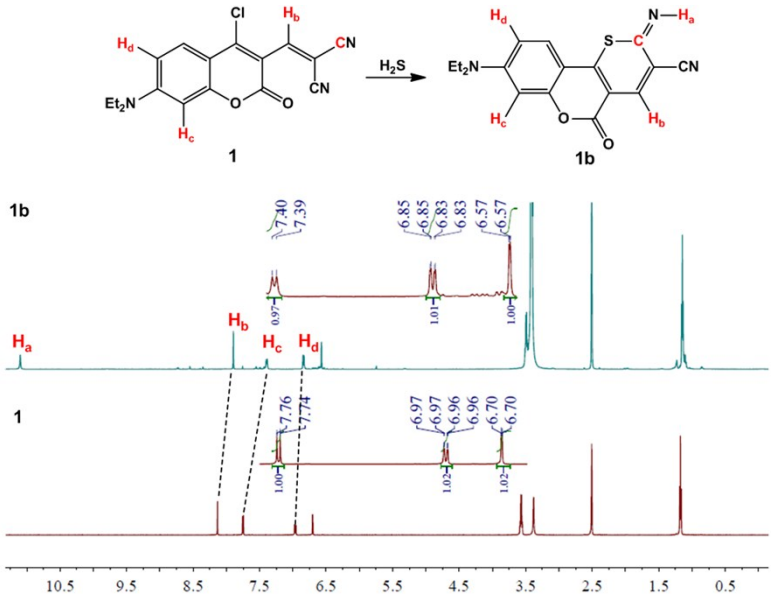


Fig. S11 ^{1}H NMR spectra of probe **1** upon addition of NaHS (1.2 equiv.) in DMSO-d_6/H_2O (*V/V*, 100/1)

(1) Only probe **1**; (2) probe **1** + NaHS

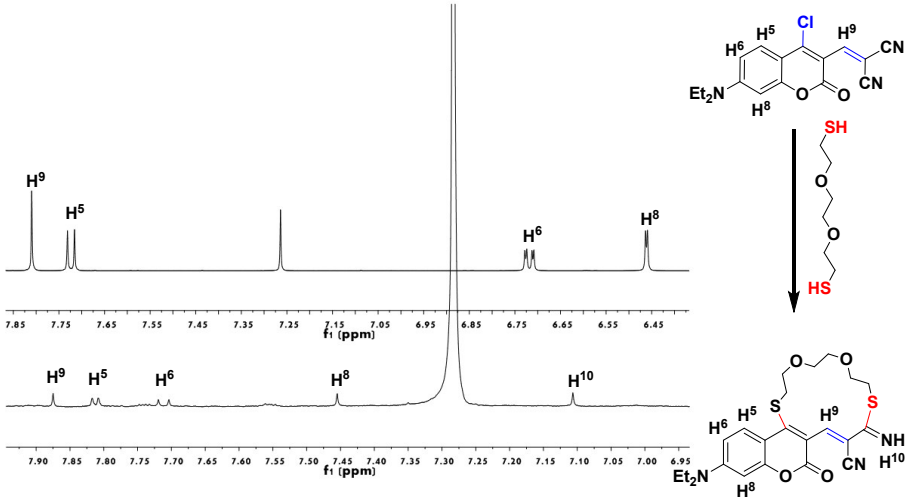
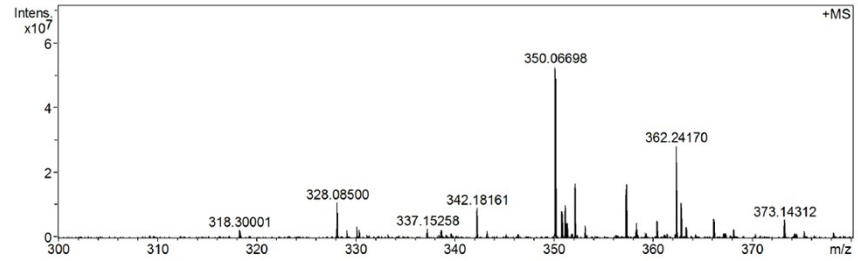


Fig. S12 ^{1}H NMR spectra of probe **1** upon addition of DODT

Acquisition Parameter

Polarity	Positive	Source	ESI	No. of Laser Shots	20
Averaged Scans	2	No. of Cell Fills	1	Laser Power	51.0 %
Broadband Low Mass	100.3 m/z	End Plate	3500.0 V	MALDI Plate	300.0 V
Broadband High Mass	3000.0 m/z	Capillary Entrance	4000.0 V	Imaging Spot Diameter	2000.0 µm
Acquisition Mode	Single MS	Skimmer 1	20.0 V		
Pulse Program	basic	Drying Gas Temperature	180.0 °C	Calibration Date	Mon Dec 15 09:21:48
Source Accumulation	0.0 sec	Drying Gas Flow Rate	4.0 L/min	Data Acquisition Size	201072
Ion Accumulation Time	0.1 sec	Nebulizer Gas Flow Rate	1.0 L/min	Apodization	Sine-Bell Multiplication
Flight Time to Acq. Cell	0.0 sec				

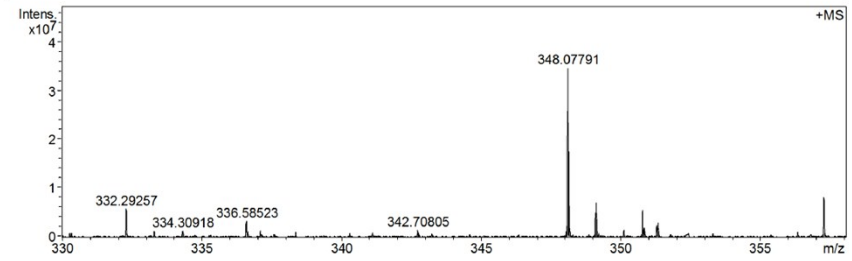


Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e¡¥ Conf	N-Rule
350.06698	1	C 17 H 14 Cl N 3 Na O 2	100.00	350.06668	-0.3	-0.9	10.0	11.5	even	ok

Fig. S13 HRMS of probe **1**

Acquisition Parameter

Polarity	Positive	Source	ESI	No. of Laser Shots	20
Averaged Scans	2	No. of Cell Fills	1	Laser Power	51.0 %
Broadband Low Mass	100.3 m/z	End Plate	3500.0 V	MALDI Plate	300.0 V
Broadband High Mass	3000.0 m/z	Capillary Entrance	4000.0 V	Imaging Spot Diameter	2000.0 µm
Acquisition Mode	Single MS	Skimmer 1	20.0 V		
Pulse Program	basic	Drying Gas Temperature	180.0 °C	Calibration Date	Mon Dec 15 09:21:48
Source Accumulation	0.0 sec	Drying Gas Flow Rate	4.0 L/min	Data Acquisition Size	201072
Ion Accumulation Time	0.2 sec	Nebulizer Gas Flow Rate	1.0 L/min	Apodization	Sine-Bell Multiplication
Flight Time to Acq. Cell	0.0 sec				



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e¡¥ Conf	N-Rule
348.07791	1	C 17 H 15 N 3 Na O 2 S	100.00	348.07772	-0.2	-0.5	15.1	11.5	even	ok

Fig. S14 HRMS of **1b**

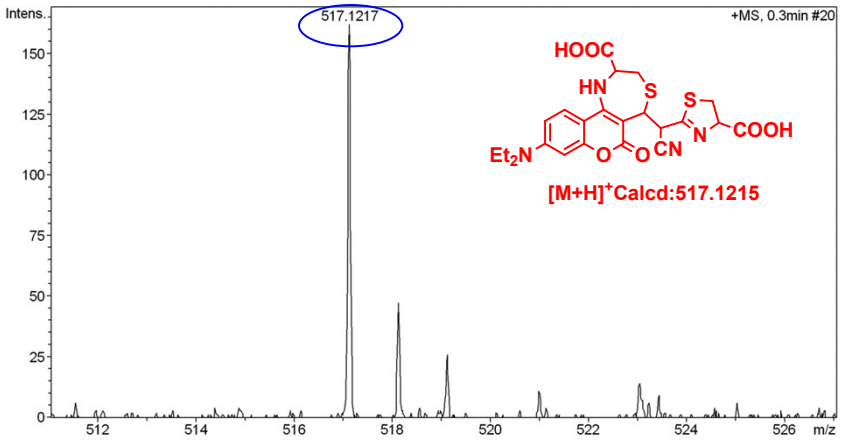


Fig. S15 HRMS of **3d**

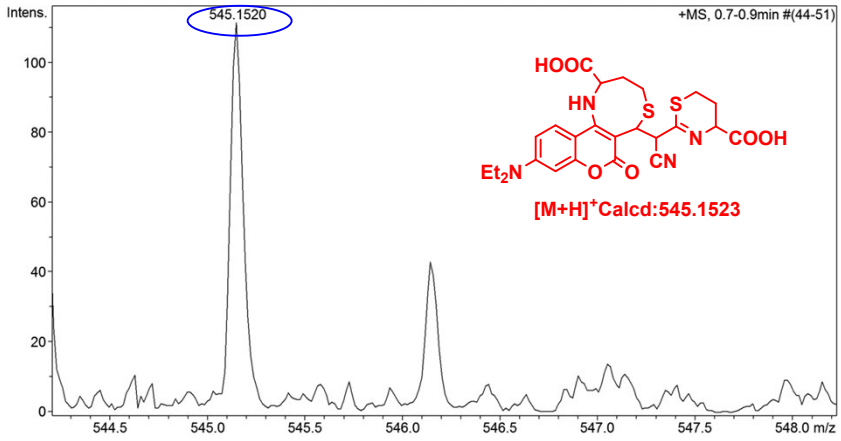


Fig. S16 HRMS of **4d**

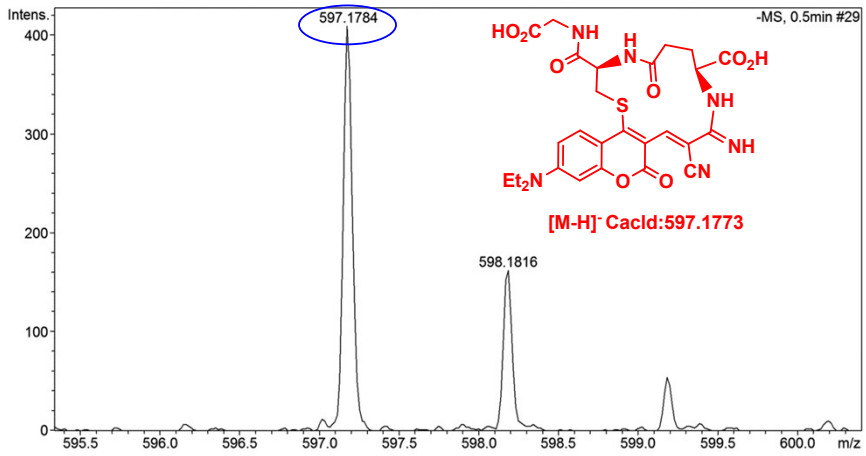


Fig. S17 HRMS of **2b**

References

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2 D. Kim, S. Singha, T. Wang, E. Seo, J. Lee, S. J. Lee, K. H. Kim, K. H. Ahn, *Chem. Commun.*, 2012, **48**, 10243.

3. Mosmann. T. *J. Immunol. Methods*, 1983, **65**, 55.