Supporting Information for

A ratiometric fluorescent probe for specific detection of cysteine over homocysteine and glutathione based on the drastic distinction in the kinetic profiles

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Materials and instruments: Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. Melting points of compounds were measured on a Beijing Taike XT-4 microscopy melting point apparatus, all melting points were uncorrected; Low resolution mass spectra were performed using an LCQ Advantage ion trap mass spectrometer from Thermo Finnigan or Agilent 1100 HPLC/MSD spectrometer; High resolution mass spectrometric (HRMS) analyses were measured on a Finnigan MAT 95 XP spectrometer. NMR spectra were recorded on an INOVA-400 spectrometer, using TMS as an internal standard; Electronic absorption spectra were obtained on a LabTech UV Power spectrometer; Photoluminescent spectra were recorded with a HITACHI F4600 fluorescence spectrophotometer; Cells imaging were performed with a Nikon eclipase TE300 inverted fluorescence microscopy; The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter; TLC analyses were performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals.

Preparation of the test solution: A stock solution of probe **Ratio-Cys** (5×10^{-4} M) was prepared in CH_3CN. The test solution of the probe (5 μM) in pH 7.4 PBS (containing 10% CH_3CN as a co-solvent) was prepared by placing 0.03 mL of the probe stock solution in CH_3CN, 0.27 mL CH_3CN, and 2.70 mL of pH 7.4 PBS. The solutions of various testing species were prepared from Cys, Arg, Hcy, Phe, Pro, Tyr, Val, Ala, Gly, Lys, Leu, Glu, Ser, glucose, NaCl, KCl, $CaCl_2$, $MgCl_2$, H_2O_2, NADH, Vitamin C, and GSH, respectively. The resulting solution was shaken well and incubated for 30 min at room temperature before recording the spectra.

Cell culture and fluorescence imaging: Live MCF-7 cells were seeded in a 12-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum for 24 h. Before imaging, the cells were washed with PBS three times and then incubated with 5 μM **Ratio-Cys** in PBS (containing 1% CH_3CN as a co-solvent) for 30 min in an atmosphere of 5% CO_2, 95% air at 37°C. For the control experiment 1, the cells were pretreated with 1 mM N-ethylmaleimide (MEM) for 30 min, and then incubated with 5 μM **Ratio-Cys** in PBS for 30

min in an atmosphere of 5% CO_2, 95% air at 37°C. For the control experiment 2, the cells were pretreated with 600 μM Cys for 30 min and then incubated with 5 μM **Ratio-Cys** in PBS for 30 min in an atmosphere of 5% CO_2, 95% air at 37°C. After washing with PBS three times to remove the remaining probe, the fluorescence images were acquired under Nikon eclipase TE2000 inverted fluorescence microscope.

Calculation of fluorescence quantum yield [1-2]

Fluorescence quantum yield was determined using Rhodamine 6G in water (Φ_r = 0.95) as standard and it was calculated by equation 1 as reported. [1-2]

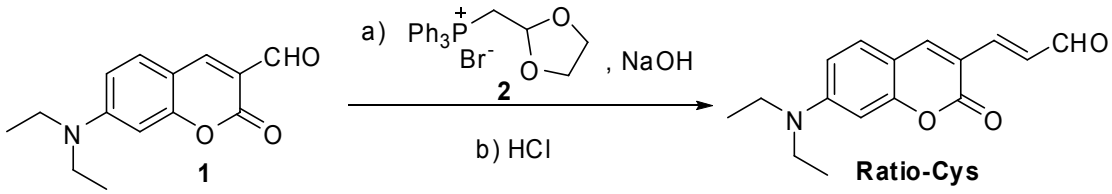
$$\Phi_s = \Phi_r (A_r F_s / A_s F_r) (\eta_s^2 / \eta_r^2) \quad \text{..... (S1)}$$

where, s and r denote sample and reference, respectively, A is the absorbance, F is the relative integrated fluorescence intensity, and η is the refractive index of the solvent.

References

[1] I. B. Berlman, Handbook of fluorescence spectra of aromatic molecules, Academic Press, New York, 1971.

[2] D. Magde, G. E. Rojas and P. Seybold, *Photochem. Photobiol*. 1999, **70**, 737.



Synthesis of compound 3-(7-(diethylamino)-2-oxo-2H-chromen-3-yl)acrylaldehyde (**Ratio-Cys**). 7-(diethylamino)-2-oxo-2H-chromene-3-carbaldehyde **1** (100.1 mg, 0.41 mmol) and (1,3-dioxolan-2-ylmethyl)-triphenylphosphonium bromide **2** (210 mg, 0.49 mmol, 1.2 eq) were dissolved in dry CH_2Cl_2 (8 mL). The mixture was stirred at room temperature for 30 min, and then NaOH (150.2 mg, 1.1 eq) dissolved in water (0.5 mL) was slowly added. After the resulting reaction mixture was stirred at room temperature for 3 h, concentrated HCl (1 mL) was added, and the mixture was further stirred at room temperature for 12 min. Deionized water and CH_2Cl_2 were added. The organic layer was separated and dried over Na_2SO_4. After concentration under reduced pressure, the crude product was purified by chromatography on a silica gel column with a mixture of CH_2Cl_2/ petroleum ether (1/1, v /v) as the mobile phase, affording compound **Ratio-Cys** as a salmon pink solid (95.0 mg, 85.5%). m.p. 205-207 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 1.23-1.26 (t, *J* = 7.2 Hz, 6H, $(CH_3CH_2)_2N$), 3.44-3.49 (q, 4H, $(CH_3CH_2)_2N$), 6.51 (d, *J* = 2.4 Hz, 1H, Ar*H*), 6.65-6.67 (dd, *J* = 8.8, 2.4 Hz, 1H, Ar*H*), 6.98-7.04 (dd, *J* = 15.6, 7.6 Hz, 1H, CH=C*H*CHO), 7.34-7.36 (d, *J* = 8.8 Hz, 1H, Ar*H*), 7.43-7.47 (d, *J* = 15.6 Hz, 1H, C*H*=CHCHO), 7.83 (s, 1H), 9.63-9.65 (d, *J* = 7.6 Hz, 1H, C*H*O); ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.42, 45.32, 97.33, 108.87, 109.98, 114.18, 128.66, 130.38, 132.50, 143.68, 145.36, 147.14, 151.97, 157.01, 160.18, 193.99; MS (EI) m/z 271.1 (M^+); HRMS (EI) *m/z* calcd for $C_{16}H_{17}O_3N_1$ (M^+): 271.1203. Found 271.1203.

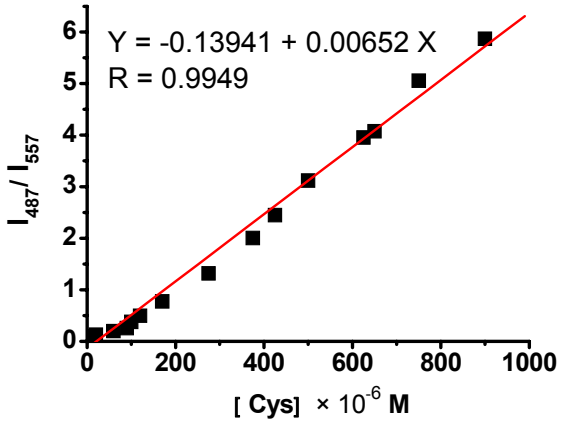


Fig. S1. The emission ratio (I_{487}/I_{557}) of probe **Ratio-Cys** (5 μM) to various concentrations (2.0 × 10^{-6} – 9.0 × 10^{-4} M) of Cys. The data were acquired after incubation of probe **Ratio-Cys** with Cys for 30 min in pH 7.4 PBS (containing 10% CH_3CN as a cosolvent). Excitation was provided at 435 nm, and the ratio of emission intensities at 487 nm and 557 nm was measured.

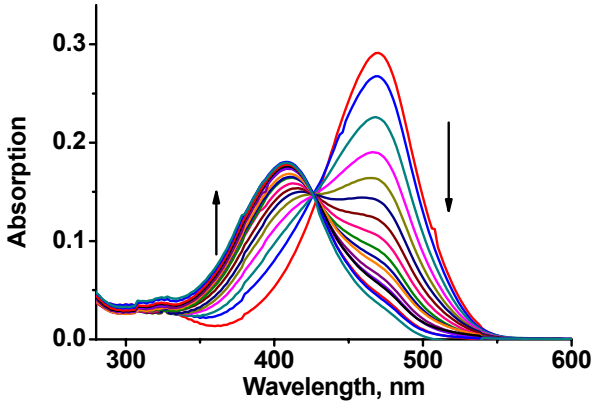


Fig. S2 (a) Absorption spectral changes of **Ratio-Cys** (5 μM) upon addition of Cys (0-250 equiv.) in pH 7.4 PBS (containing 10% CH_3CN as a cosolvent). The spectra were recorded after incubation of the probe with Cys for 30 min.

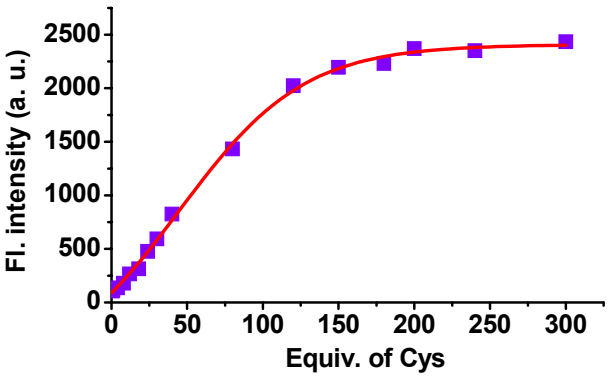


Fig. S3 Fluorescence intensity of **Ratio-Cys** (5 μM) at 487 nm upon addition of Cys (0-300 equiv.). The spectra were recorded after incubation of the probe with Cys for 30 min. Excitation at 435 nm.

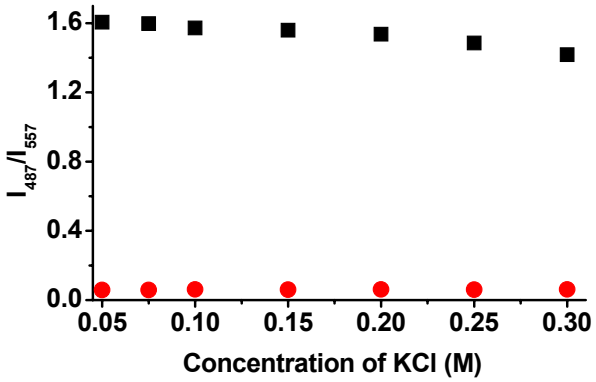


Fig. S4 Effect of ionic strength (different concentrations of KCl: 0.05, 0.07 0.10, 0.15, 0.20, 0.25, 0.30 M) on the emission ratio (I_{487}/I_{557}) of probe **Ratio-Cys** (5 μM) in the absence (●) or presence of 300 μM Cys (■). The spectra were recorded after incubation of the probe with Cys for 30 min. Excitation at 435 nm.

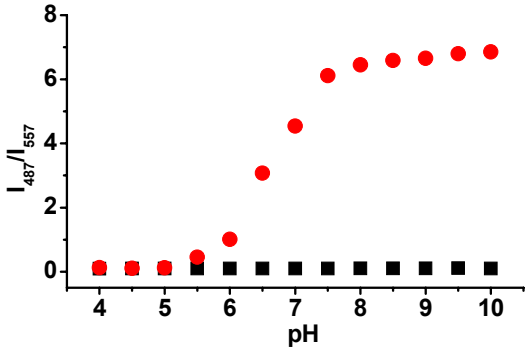


Fig. S5 The fluorescent ratio (I_{487}/I_{557}) of probe **Ratio-Cys** (5 μM) at various pH values in the absence (■) or presence (●) of Cys (200 equiv.) in pH 7.4 PBS/CH_3CN (9: 1). The spectra were recorded after incubation of the probe with Cys for 30 min. Excitation at 435 nm.

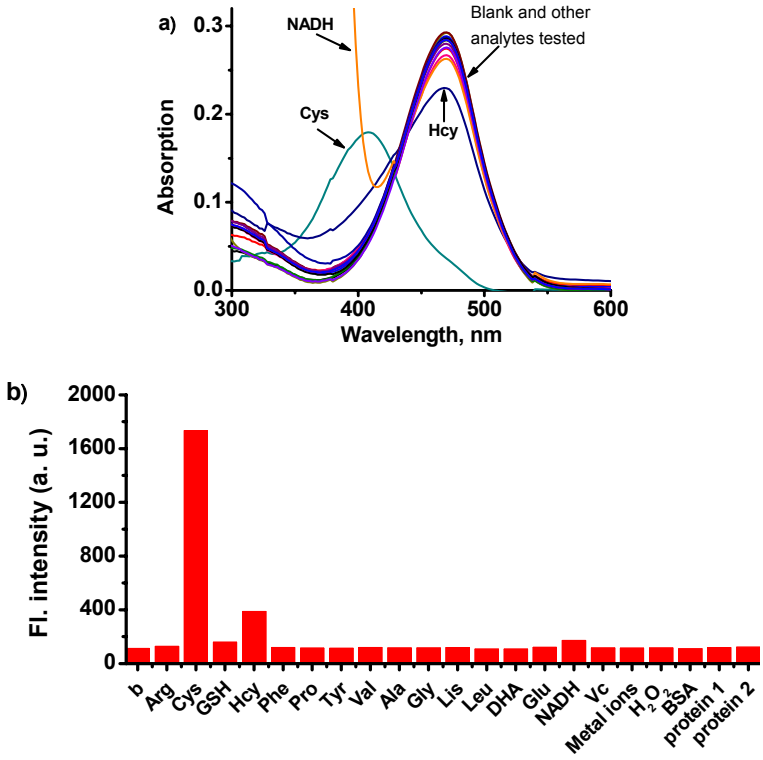


Fig. S6 **(a)** Absorption spectra of **Ratio-Cys** (5 μM) to various species (1 mM) including amino acids (Arg, Cys, GSH, Hcy, Phe, Pro, Tyr, Val, Ala, Gly, Lys, Leu, Glu, Ser), glucose, metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}), a reactive oxygen species (H_2O_2), and reducing agents (NADH, Vitamin C) in pH 7.4 PBS (containing 10% CH_3CN as a cosolvent). Notably, NADH itself has significant UV absorption at < 400 nm. (b) Fluorescence responses of **Ratio-Cys** (5 μM) at 487 nm to various species (1 mM) in pH 7.4 PBS (containing 10% CH_3CN as a cosolvent). Metal ions include Na^+, K^+, Ca^{2+}, and Mg^{2+}. Protein 1 is Hela cell total protein, and protein 2 is $HepG_2$ Cell total protein.

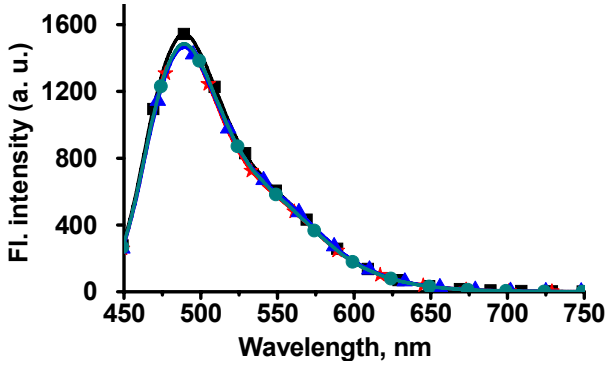


Fig. S7 The fluorescence spectra of **Ratio-Cys** (5 μM) in the presence of Cys (■), Cys + BSA (▲), Cys + Hela cell total protein (●), Cys + $HepG_2$ cell total protein (□). The concentration of proteins are 1 g/L, and the concentration of Cys is 750 μM.

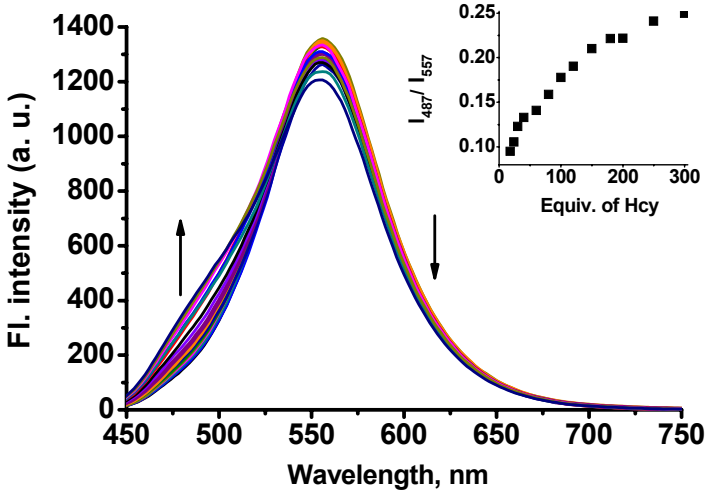


Fig. S8. Fluorescence spectral changes of **Ratio-Cys** (5 μM) upon addition of Hcy (0 - 300 equiv.) in pH 7.4 PBS (containing 10% CH_3CN as a cosolvent). Inset: Emission ratio (I_{487}/I_{557}) of **Ratio-Cys** (5 μM) upon addition of Hcy (0-300 equiv.). The spectra were recorded after incubation of the probe with Hcy for 30 min. Excitation at 435 nm.

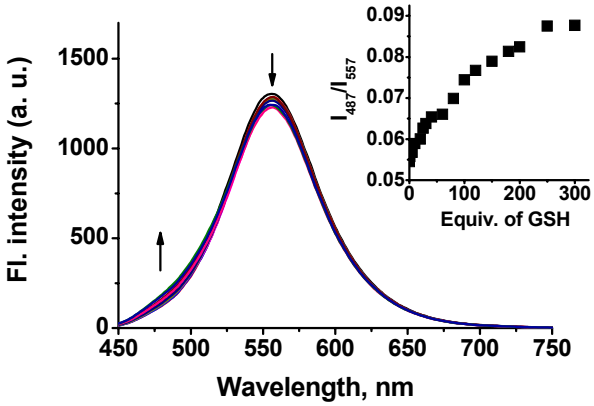


Fig. S9. Fluorescence spectral changes of **Ratio-Cys** (5 μM) upon addition of GSH (0-300 equiv.) in pH 7.4 PBS (containing 10% CH_3CN as a cosolvent). Inset: Emission ratio (I_{487}/I_{557}) of **Ratio-Cys** (5 μM) upon addition of GSH (0-300 equiv.). The spectra were recorded after incubation of the probe with GSH for 30 min. Excitation at 435 nm.

Kinetic Studies:

The reaction of probe **Ratio-Cys** (5 μM) with Cys (100-250 equiv.) in pH7.4 PBS (containing 10% CH_3CN as cosolvent) was monitored using the fluorescence intensity at 487 nm. The reaction was carried out at room temperature. The *pseudo*-first-order rate constant for the reaction was determined by fitting the fluorescence intensities of the samples to the *pseudo* first-order equation (S2):

$$\text{Ln}\,[(F_{max} - F_t) / F_{max}] = -k't \qquad \text{(S2)}$$

Where F_t and F_{max} are the fluorescence intensities at 487 nm at time t and the maximum value obtained after the reaction was complete. k' is the *pseudo*-first-order rate constant. The *pseudo* first-order plots for the reaction of **Ratio-Cys** with 150 equiv. of Cys, Hcy, and GSH, are shown in Figures S10-12, respectively. The negative slope of the line provides the *pseudo*-first-order rate constant for Cys, Hcy, and GSH: $k' = 5.8\times10^{-2}$ min^{-1}, 3.5×10^{-3} min^{-1}, and 1.3×10^{-3} min^{-1}, (Figures S10-12), respectively.

The *pseudo*-first-order (apparent) rate constant, k', is related to the second-order rate constant, k ($M^{-1}min^{-1}$), by equation S3:

$$k' = k\,[M] \qquad \text{(S3)}$$

Where M is the concentration of Cys (or Hcy, GSH). The second-order rate constant for this reaction is thus the slope of the linear plot of k' versus the concentration of Cys (or Hcy, GSH) (Figs. S13-15): $k = 2.0\times10^{-2}$ M^{-1} min^{-1}, 1.8×10^{-5} M^{-1} min^{-1}, and 1.1×10^{-6} M^{-1} min^{-1} for Cys, Hcy, and GSH), respectively.

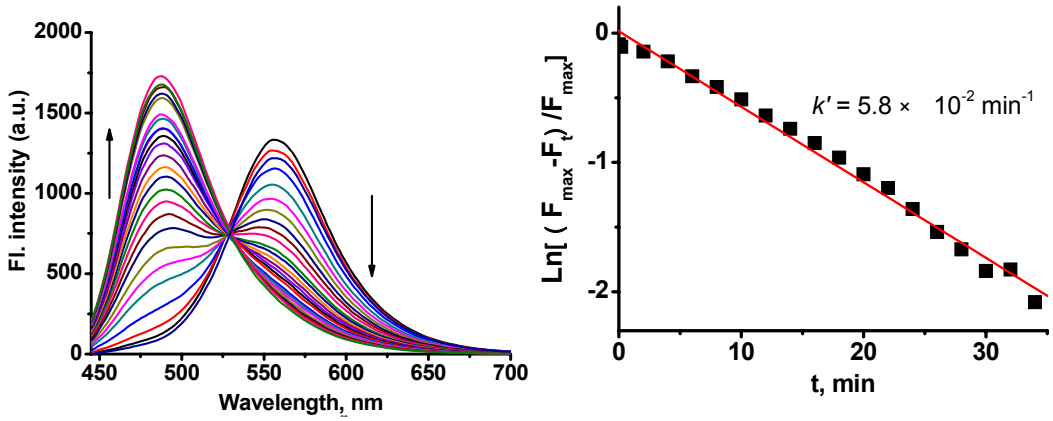


Fig. S10 (a) The fluorescence intensity of compound **Ratio-Cys** (5 μM) incubated with Cys (150 equiv.) for 0-50 min. (b) *Pseudo* first-order kinetic plot of the reaction of **Ratio-Cys** (5 μM) with Cys (150 equiv.) in pH 7.4 PBS (containing 10% CH_3CN as cosolvent). Slope = 5.8×10^{-2} min^{-1}.

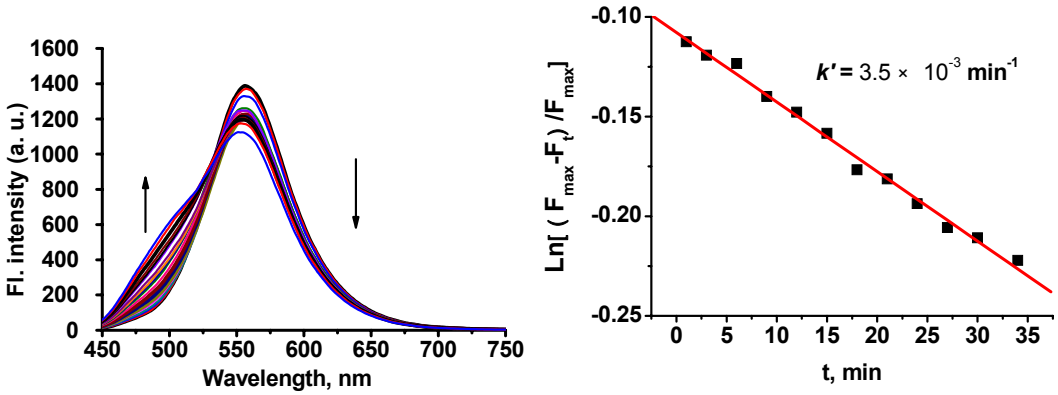


Fig. S11 (a) The fluorescence intensity of compound **Ratio-Cys** (5 μM) incubated with Hcy (150 equiv.) for 0-60 min. (b) *Pseudo* first-order kinetic plot of the reaction of **Ratio-Cys** (5 μM) with Hcy (150 equiv.) in pH7.4 PBS (containing 10% CH_3CN as cosolvent). Slope = 3.5×10^{-3} min^{-1}.

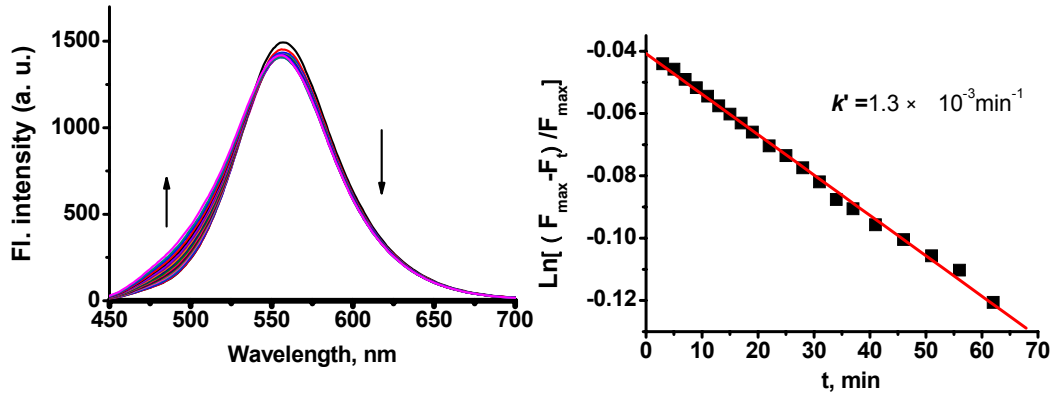


Fig. S12 (a) The fluorescence intensity of compound **Ratio-Cys** (5 μM) incubated with GSH (150 equiv.) for 0-70 min. (b) *Pseudo* first-order kinetic plot of the reaction of **Ratio-Cys** (5 μM) with GSH (150 equiv.) in pH 7.4 PBS (containing 10% CH_3CN as cosolvent). Slope = 1.3×10^{-3} min^{-1}.

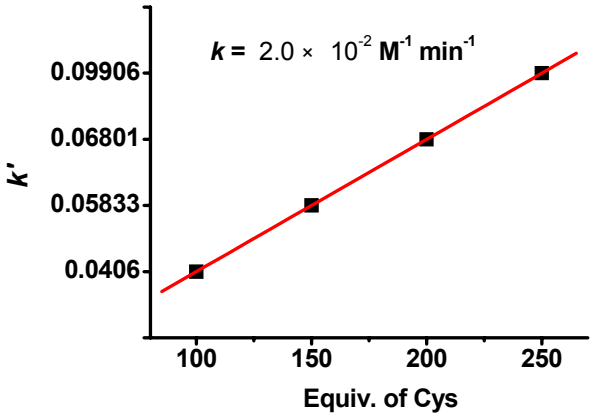


Fig. S13 Plot of the apparent rate constant *k'* versus the concentrations of Cys. Slope = 2.0 × 10^{-2} M^{-1} min^{-1}. Thus, second-order rate constant, k = 0.02 M^{-1} min^{-1}.

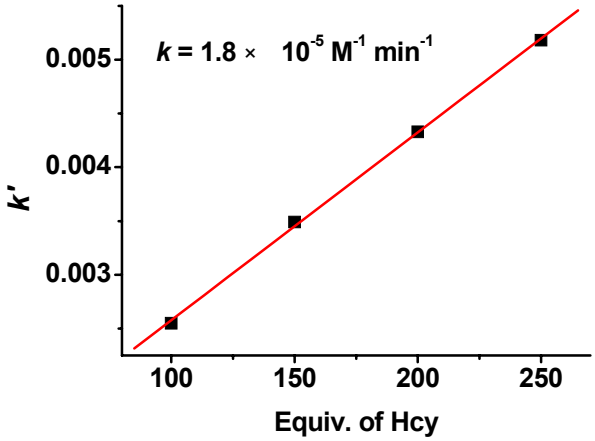


Fig. S14 Plot of the apparent rate constant *k'* versus the concentrations of Hcy. Slope = 1.75 × 10^{-5} M^{-1} min^{-1}. Thus, second-order rate constant, k =1.8 × 10^{-5} M^{-1} min^{-1}.

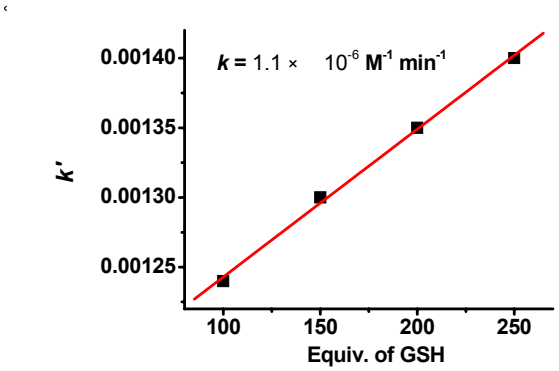
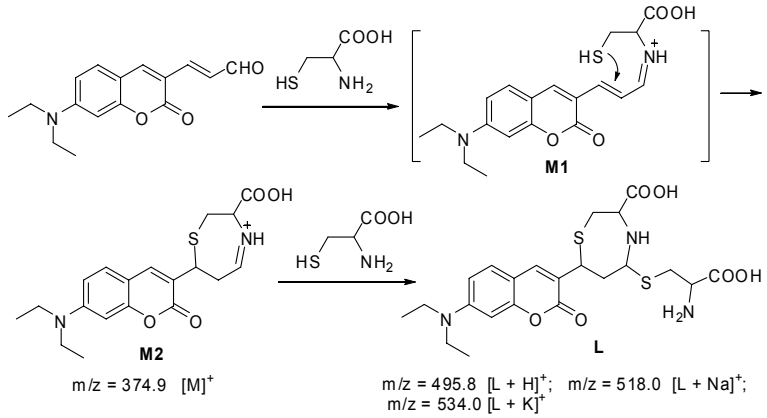


Fig. S15 Plot of the apparent rate constant *k'* versus the concentrations of GSH. Slope = 1.06 × 10^{-6} M^{-1} min^{-1}. Thus, second-order rate constant, k = 1.1 × 10^{-6} M^{-1} min^{-1}.



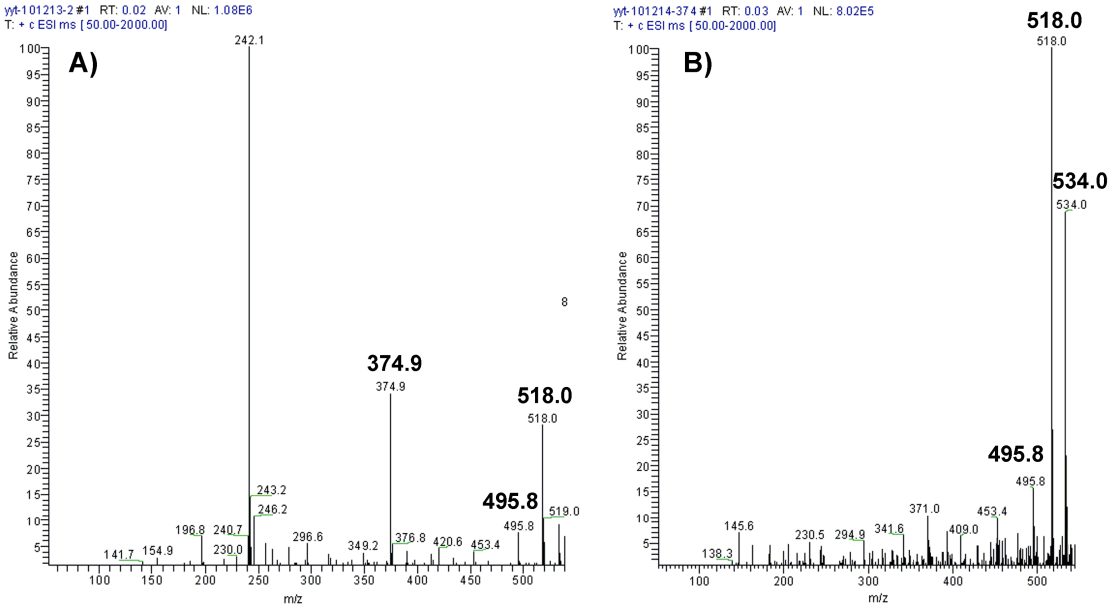


Fig. S16 ESI-MS (positive ion mode) spectra of **Ratio-Cys** (5 μM) after treatment with Cys (150 μM) in pH 7.4 PBS/CH_3CN (9: 1) for 30 min (A) and 120 min (B). Peak at m/z 374.9 corresponds to the intermediate (**M2**); Peaks at m/z 495.8, 518.0, and 534.0, correspond to (**L**+ H)$^+$, the sodium salt (**L** + Na)$^+$, and potassium salt (**L** + K)$^+$, respectively; Peak at m/z 242.1 corresponds to Cys-Cys.

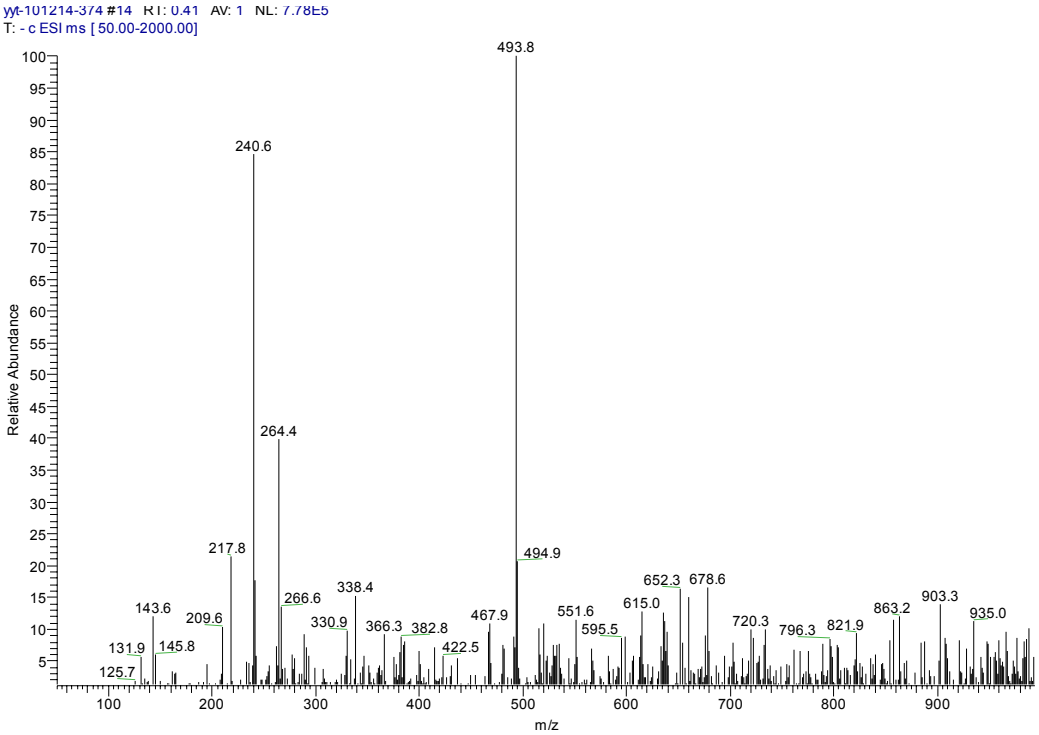


Fig. S17 ESI-MS (negative ion mode) spectrum of **Ratio-Cys** (5 μM) after treatment with Cys (150 μM) in pH 7.4 PBS/CH_3CN (9: 1) for 120 min. Peak at m/z 493.8 corresponds to $(\mathbf{L} - H)^+$; Peak at m/z 240.6 corresponds to Cys-Cys.

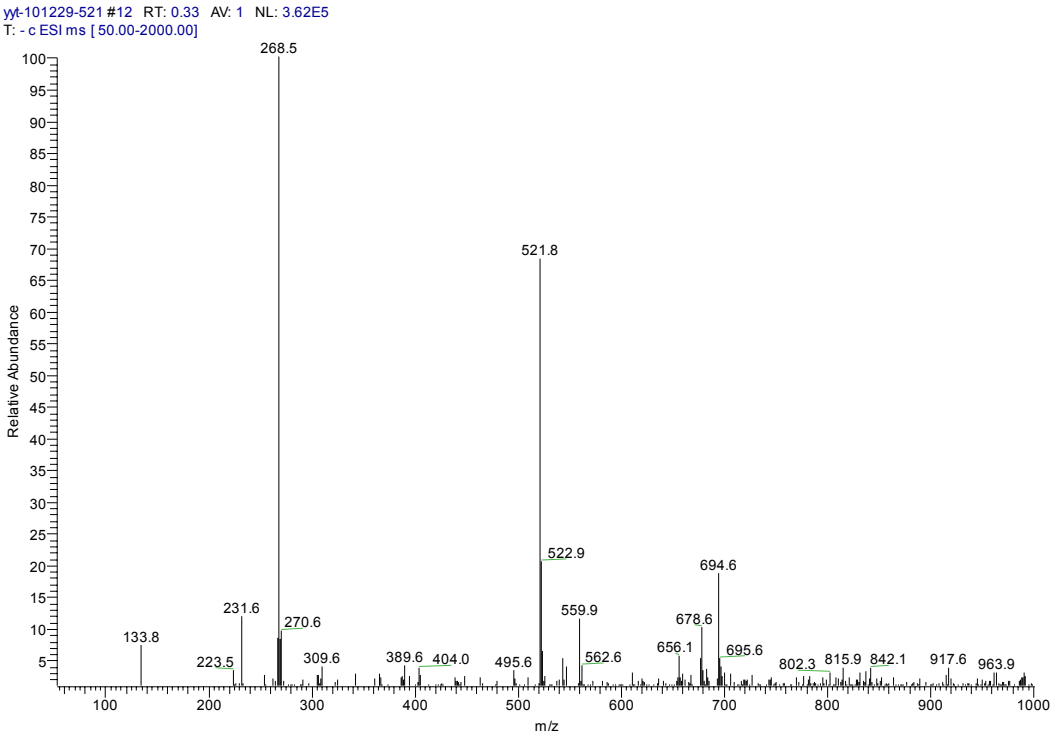
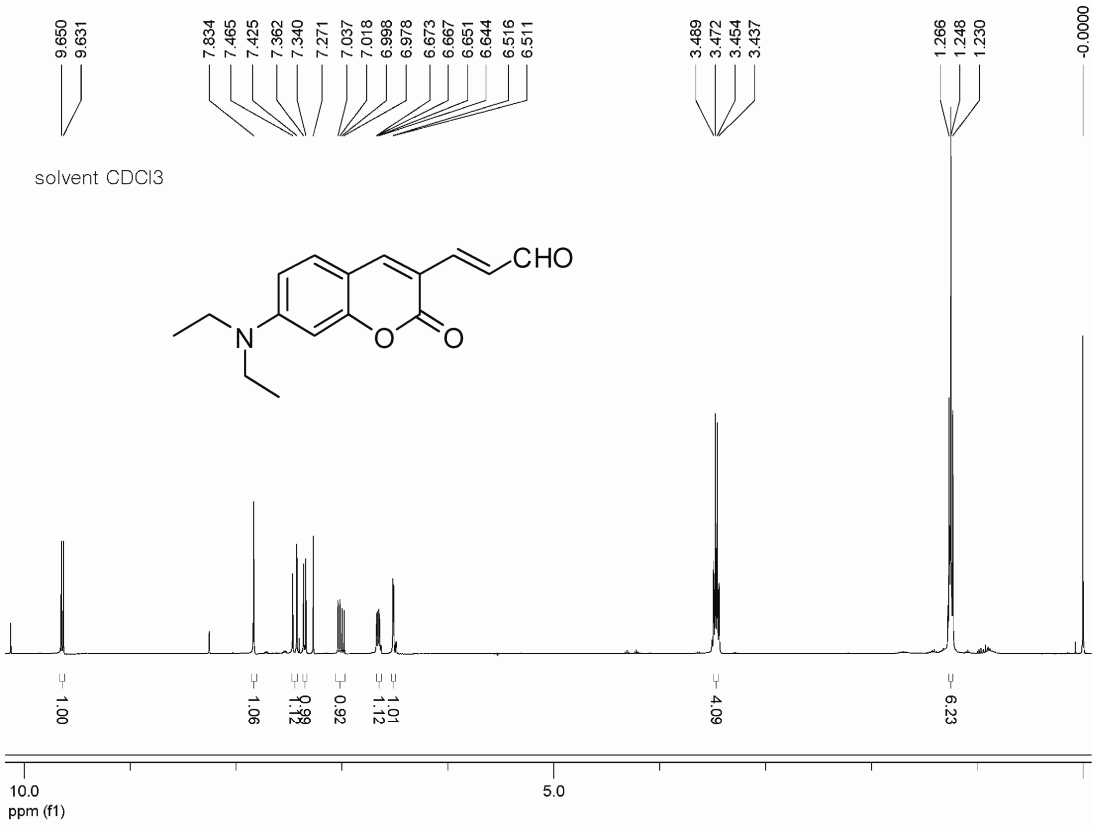


Fig. S18. ESI-MS (negative ion mode) spectrum of **Ratio-Cys** (5 μM) after treatment with Hcy (150 μM) in pH 7.4 PBS/CH_3CN (9: 1) for 24 hours. Peak at m/z 521.8 corresponds to 1: 2 adduct between **Ratio-Cys** and Hcy; Peak at m/z 268.5 corresponds to Hcy-Hcy.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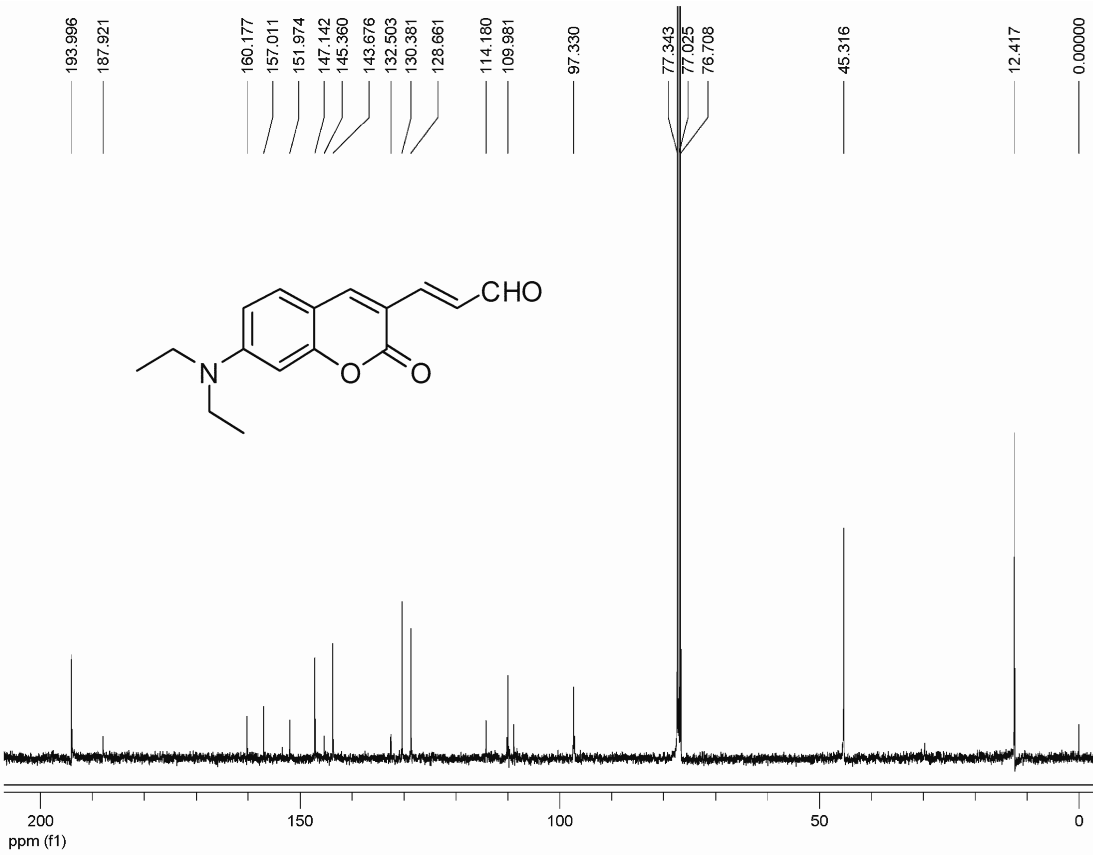
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