Electronic Supplementary Information For

A Fluorescent Chemodosimeter Specific for Cysteine: Effective Discrimination of Cysteine from Homocysteine

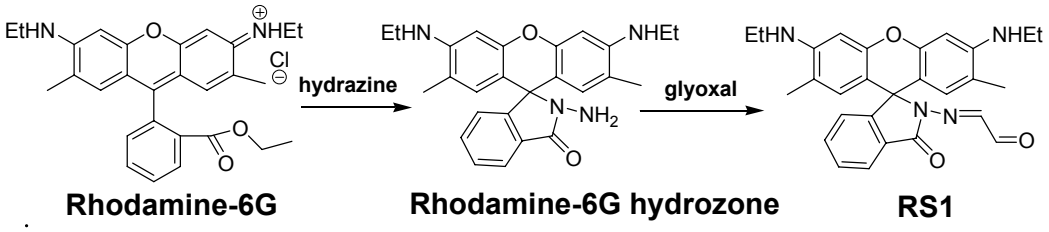
Honglin Li[a], Jiangli Fan*[a], Jingyun Wang*[b], Maozhong Tian[a], Jianjun Du[a], Shiguo Sun[a] Pingping Sun[b] and Xiaojun Peng*[a]

[a] *State Key Laboratory of Fine Chemicals, Dalian University of Technology, Dalian 116012, China. Fax:+86-411-3989-3906; Tel: +86-411-3989-3899; E-mail: pengxj@dlut.edu.cn*
[b] *Dept of Bioscience and Biotechnology, Dalian University of Technology, Dalian 116012, China. E-mail: wangjingyun@dl.cn*

Materials and general methods:

All solvents used were of analytical grade. Analyte solutions were prepared from L-Cysteine (Cys), L-Aspartic acid (Asp), L-Glutamic acid (Glu), L-Asparagine (Asn), L-Glutamine (Gln), L-Tryptophan (Trp), L-isoleucine (Ile), L-Leucine (Leu), L-Valine (Val), L-Methionine (Met), L-Tyrosine (Tyr), L-Histidine (His), L-Lysine (Lys), L-Threonine (Thr), L-Proline(Pro), L-alanine (Ala), L-Arginine (Arg), L-Serine (Ser), L-Phenylalanine (Phe), L-Glycin (Gly), DL-Homocysteine (Hcy), reduced Glutathione (GSH), Glucose (Gluc), Taurine (Tau) by separately dissolved in distilled water. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a VARIAN INOVA-400 spectrometer chemical shifts reported as ppm (in $CDCl_3$ or CD_3OD, TMS as internal standard). Mass spectrometry data were obtained with a HP1100LC/MSD mass spectrometer and a LC/Q-TOF MS spectrometer. Fluorescence measurements were performed on a VAEIAN CARY Eclipse Fluorescence Spectrophotometer (Serial No. FL0812-M018) and the slit width was 5 nm for both excitation and emission. All pH measurements were made with a Model PHS-3C meter.

Synthetic procedures:



Rhodamine-6G **Rhodamine-6G hydrozone** **RS1**

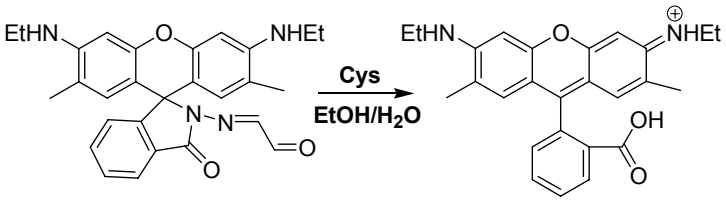
Scheme S1. Synthesis of **RS1**.

Synthesis of Rhodamine-6G hydrozone

Rhodamine 6G hydrozone was prepared as the reference reported.[1]

Synthesis of RS1

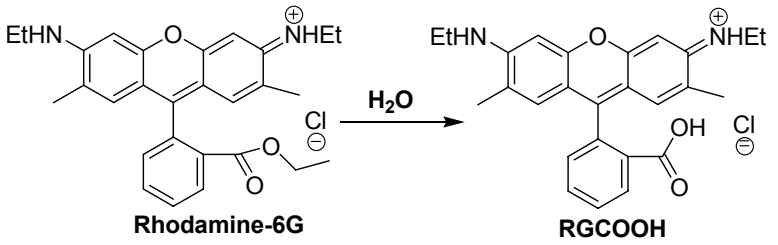
Rhodamine-6G hydrozone (300.0 mg, 0.7 mmol) was dissolved in 20 mL ethanol in a 50-mL flask. 2.0 mL (excess) glyoxal aqueous (40%) was then added dropwises with vigorous stirring at room temperature for overnight. After removal of ethanol under vacuum, the residue was purified by flash chromatography with petroleum/ethyl acetate = 6/1 as eluent to give the yellow powder **RS1** (217 mg, yield: 66.2%), mp. 282-283°C. ^{1}H-NMR (400 MHz, $CDCl_3$), δ_H (ppm): 9.23 (d, 1H, J = 7.6 Hz, CHO), 7.98 (d, 1H, J = 7.2 Hz, C_6H_4), 7.63-7.55 (m, 2H, C_6H_4), 7.44 (d, 1H, J = 7.6 Hz, CHNN), 7.02 (d, 1H, J = 7.6 Hz, C_6H_4), 6.34 (s, 2H, Xanthene-H), 6.28 (s, 2H, Xanthene-H), 4.17 (s, 2H, NH), 3.22-3.16 (m, 4H, CH_2), 1.85 (s, 6H, CH_3), 1.26 (t, 6H, J = 6.8 Hz, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$), δ_C 192.55, 166.17, 153.16, 151.01, 148.19, 140.94, 135.22, 128.78, 127.16, 126.21, 124.227, 118.27, 104.14, 97.03, 76.87, 66.15, 38.40, 16.83, 14.81. IR (KBr, cm^{-1}): 3381, 2968, 2925, 2870, 1669, 1635, 1615, 1548, 1517, 1467, 1357, 1309, 1266, 1221, 1122, 824, 785, 762, 702. Q-TOF MS：$[M+H]^+$ 469.2240; found 469.2229.



Scheme S2. Synthesis of **RGCOOH** by reaction of **RS1** with Cys.

Synthesis of RGCOOH by reaction of RS1 with Cys

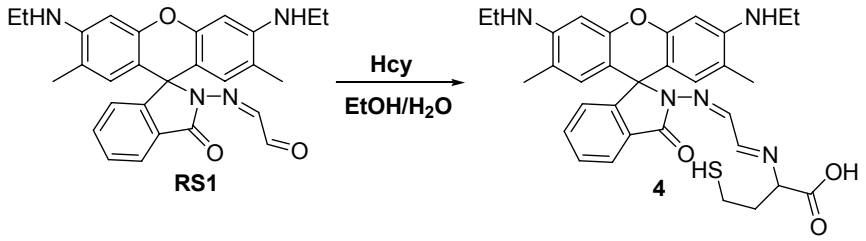
RS1 (100.0 mg, ~0.2 mmol) was dissolved in 300 mL ethanol in a 500 mL flask. Cys aqueous solution (50 mg Cys dissolved in 10 mL H_2O) was then added dropwises with vigorous stirring at 37°C for 1h. After removal of water/ethanol under vacuum, the residue was purified by flash chromatography with CH_2Cl_2/MeOH/acetic acid= 400/25/1 (volume ratio) as eluent to give the red powder **RGCOOH** (3.0 mg, yield: ~3.3%). Q-TOF MS: $[M]^+$ 415.2022; found 415.2029.



Scheme S3. Synthesis of **RGCOOH** by hydrolysis of Rhodamine-6G.

Synthesis of RGCOOH by hydrolysis of Rhodamine-6G

Rhodamine-6G (400.0 mg, 0.84 mmol) and NaOH (34 mg, 0.84 mmol) were mixed in 20mL methanol in a 100 mL flask. The mixture was refluxed overnight with vigorous stirring. After removal of methanol under vacuum, the residue was dissolved in 5 mL H_2O, and then adjusted to pH about 3.0 by addition of hydrochloric acid. The red precipitate was collected, washed with cold water to give the red powder **RGCOOH** (300 mg, yield: 86%). ^{1}H- NMR (400 MHz, CD_3OD), δ_H (ppm): 8.22 (d, 1H, J = 6.4 Hz, C_6H_4), 7.80 (m, 2H, C_6H_4), 7.36 (d, 1H, J = 6.8 Hz, C_6H_4), 6.89 (d, 4H, J = 10.4 Hz, Xanthene-H), 3.50 (q, 4H, J = 7.2 Hz, CH_2), 2.15 (s, 6H, Xanthene-CH_3), 1.35 (t, 6H, J = 6.8 Hz, CH_3).



Scheme S4. The synthetic route of **4**.

Synthesis of 4

To a 500 mL flask, **RS1** (100.0 mg, ~0.2 mmol) was dissolved in 300 mL ethanol, Hcy aqueouse (50 mg Hcy dissolved in 10 mL H_2O) was then added dropwise with vigorous stirring at 37°C for 1h. **4** is unstable when purified by flash chromatography. Q-TOF MS：$[M+H]^+$ 586.2488; found 586.2483.

Experimental procedure

Crystallography

The intensities of the crystal data were collected on a Bruker SMART APEX CCD diffractometer with graphite–monochromated Mo–Kα (λ = 0.71073 Å) using the SMART and SAINT programs.[2] The structure was solved by direct methods and refined on F^2 by full–matrix least–squares methods with SHELXTL version 5.1.[3] All of the non-hydrogen atoms were refined with anisotropic thermal displacement coefficients. Hydrogen atoms were fixed geometrically at calculated distances and allowed to ride on the parent non-hydrogen atoms with the isotropic displacement being fixed at 1.2 and 1.5 times of the aromatic and methyl carbon atoms the attached, respectively.

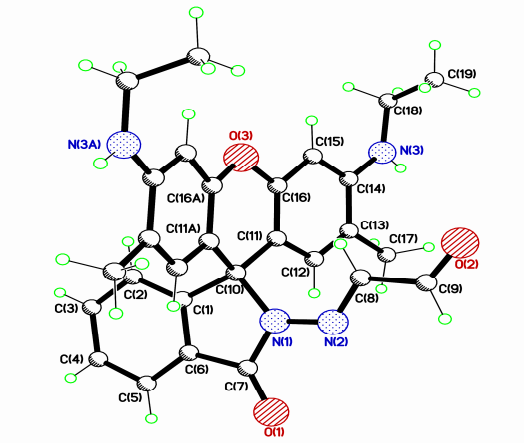


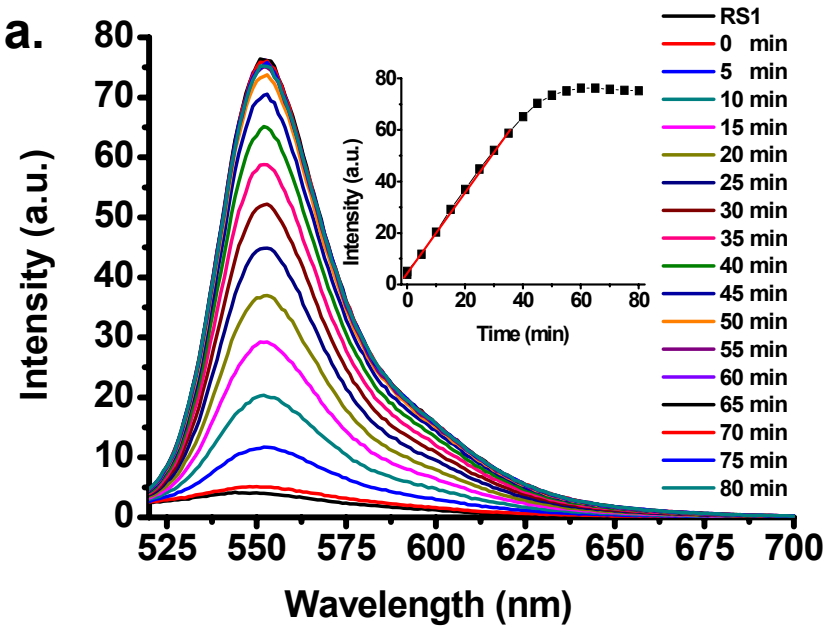
Fig. S1. X-ray crystal structure of **RS1**.

formula	$C_{28}H_{28}N_4O_3$
Mr [g mol^{-1}]	468.54
λ [Å]	0.71073
crystal system	Orthorhombic
space group	Pnma
a [Å]	a=19.352(4)
b [Å]	b=15.755(3)
c [Å]	c=8.3951(17)
V [Å^3]	2559.5 (9)
Z	4
T [K]	298(2)
μ [mm^{-1}]	0.081
F [000]	992
total reflns	7957
reflns observed	2289
parameters	181

goodness-of-fit on F^2	1.082
$R1^a$ [I > 2σ (I)]	0. 0534
ωR^b [I > 2σ (I)]	0. 1493
max peak/hole [e Å^{-3}]	0.414/-0.262
*R*int	0.0339

$R1 = (\sum||F_o| - |F_c||)/(\sum|F_o|)$. $\omega R = [\sum w(F_o^2 - F_c^2)^2/\sum w(F_o^2)^2]^{1/2}$

Table S1. X-ray crystallographic data for **RS1**.



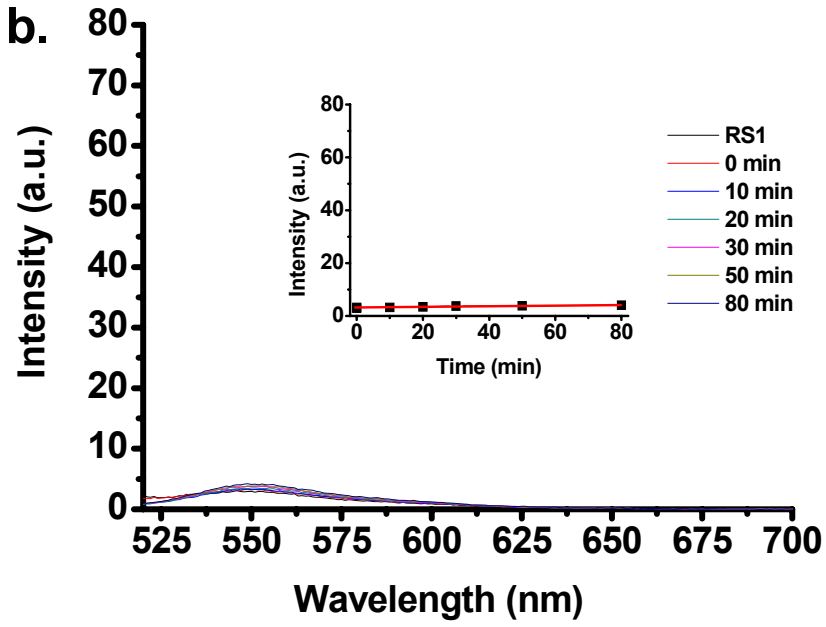


Fig. S2. Time dependent fluorescence intensity changes of **RS1** (10 μM) with 20 equiv of Cys (a) and Hcy (b) in ethanol-PBS (0.1 M, pH = 7.00) solution (3:7, v/v, 37°C), λ_{ex} = 500 nm. Inset: Fluorescence intensity 552 nm.

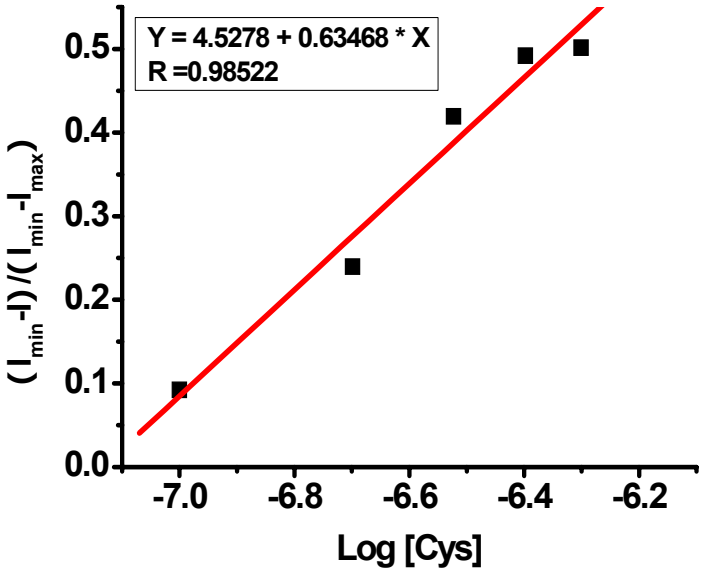


Fig. S3. Normalized response of the fluorescence signal to changing Cys concentrations. A linear regression curve was then fitted to these normalized fluorescence intensity data, and the point at which this line crossed the ordinate axis was considered as the detection limit (7.35×10^{-8} M).[4] Y = 4.5278 + 0.63468 * X, R =0.98522.

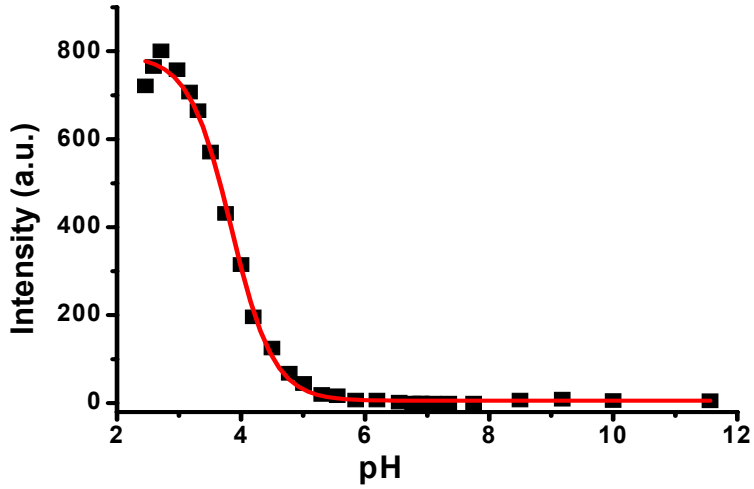


Fig. S4. Influence of pH on fluorescence at 552 nm for **RS1** (10 μM) in ethanol aqueous solution, λ_{ex} = 500 nm. The pH of solution was adjusted by aqueous solution of NaOH (1 M) or HCl (1 M).

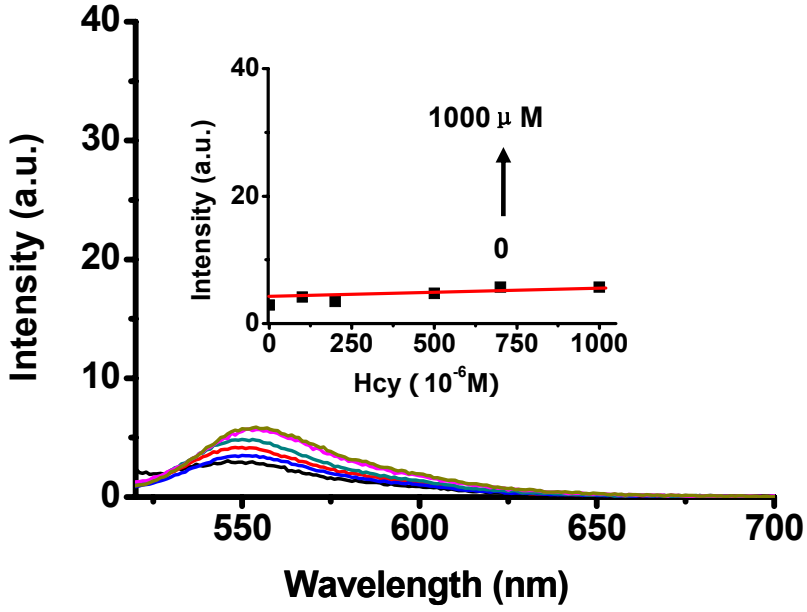


Fig. S5. Fluorescence spectral changes of **RS1** (10 μM) in ethanol-PBS (0.1 M, pH = 7.00) solution (3:7, v/v, 37°C) upon addition of Hcy (0-1000 μM). Each spectrum was recorded after 30 min, λ_{ex} = 500 nm. Inset: Fluorescence intensity (Cys: 0, 100, 200, 500, 700, 1000 μM) at 552 nm.

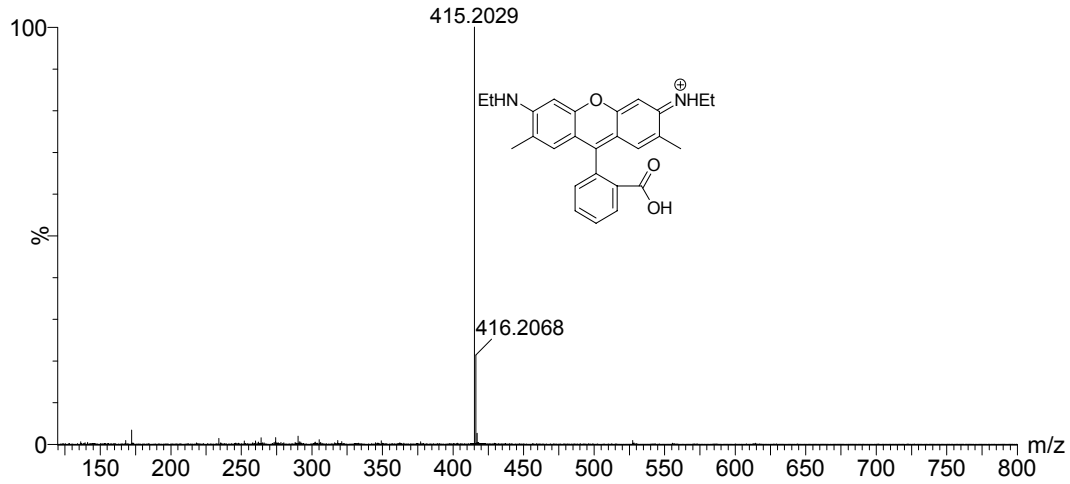


Fig. S6.TOF-MS of **RGCOOH**: Reaction of **RS1** with Cys

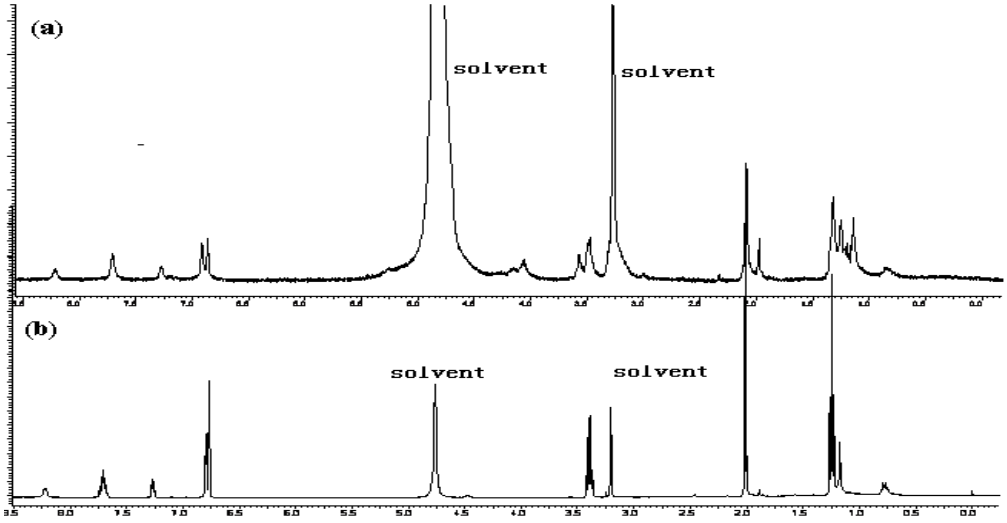


Fig. S7. ^{1}H-NMR of **RGCOOH**: (a) Reaction of **RS1** with Cys (b) Hydrolysis of Rhodamine-6G.

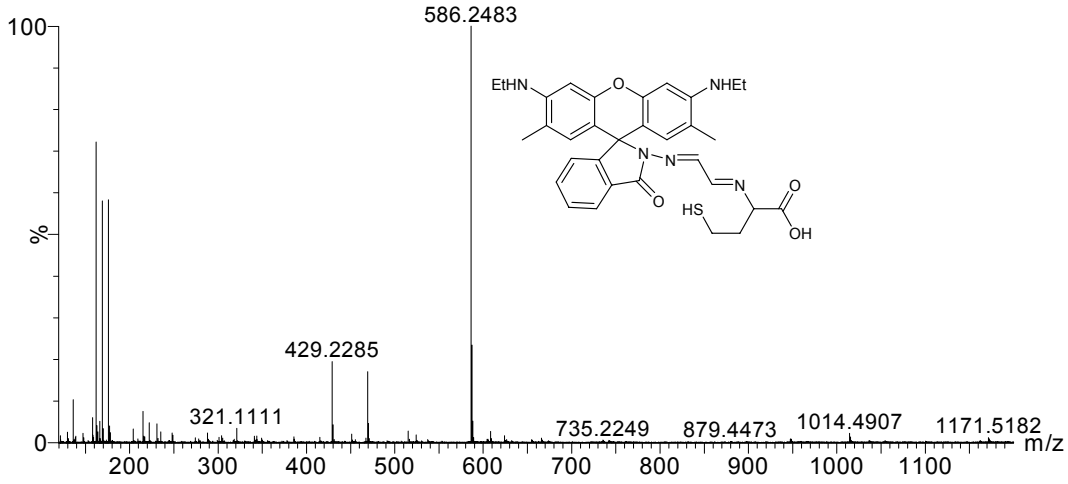
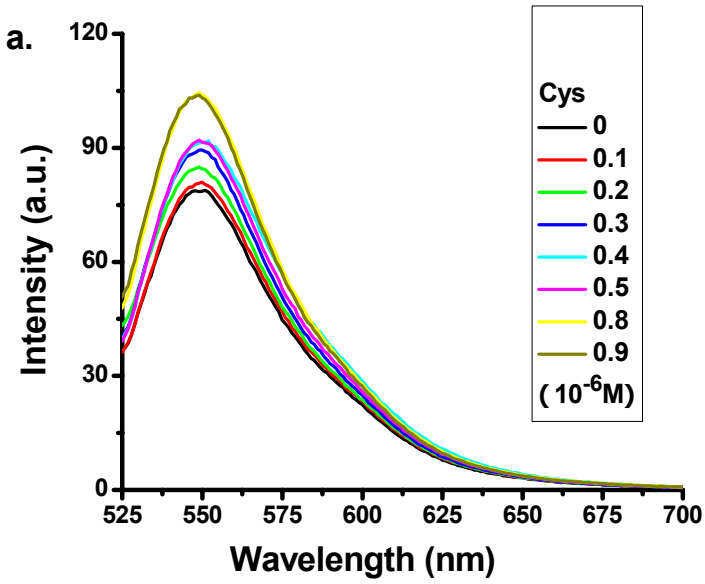


Fig. S8. TOF-MS of **4**



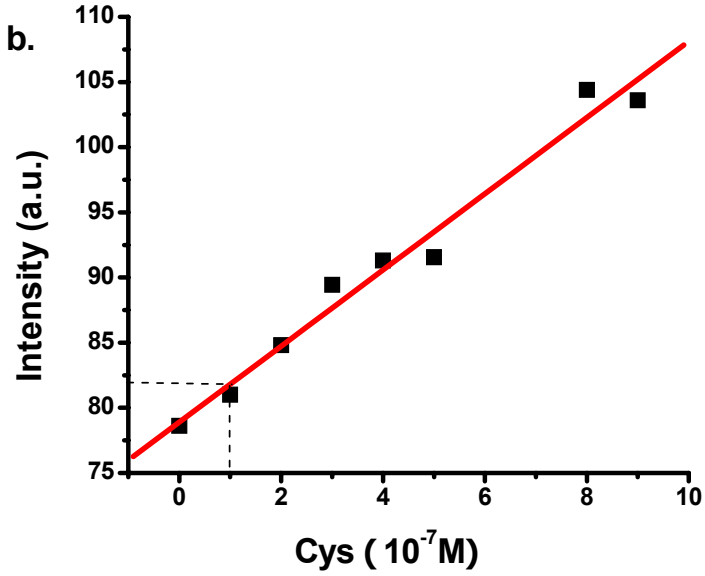


Fig. S9. (a) Fluorescence spectral changes of **RS1** (10 μM) upon addition of Cys (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.8, 0.9 μM). (b) Calibration curves for the determination of Cys concentration in human urine using commercial Cys as an internal standard. Typical standard curve obtained by adding graded amounts of Cys (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.8, 0.9 μM) to **RS1** solution and intensity at 552 nm was recorded. The resulting linear curve gives an intensity-Cys concentration function: $\boldsymbol{I} = 78.89 + 2.93\boldsymbol{C}$ (***I***: intensity (a.u.), ***C***: Cys concentration). Condition: ethanol-PBS (0.1 M, pH=7.00) solution (3:7, v/v, 37°C). Each spectrum and value were recorded after 30 min, λ_{ex} = 500 nm, $Slit_{ex}$= 10 nm, $Slit_{em}$= 20 nm.

Cell incubation and fluorescence imaging

PC12 cells and MCF cells were both cultured in DEME (Invitrogen) supplemented with 10% FCS (Invitrogen). One day before imaging, cells were seeded in 24-well flat-bottomed plates in an atmosphere of 5% CO_2, 95% air at 37°C. Fluorescence imaging of intracelluar cysteine was observed under Nikon eclipase TE2000-5 inverted fluorescence microscope (excited with green light). The microscope settings (brightness, contrast, and exposure time) were held constant before and after pretreatment of cells with N-ethylmaleimide to compare the relative intensity of intracellular cysteine fluorescence.

For fixed cell imaging, cells were incubated in ethanol-PBS (3:7, v/v, pH = 7.20) for 20 min, and then pretreated with a DMSO-PBS (1:49, v/v, pH=7.20) solution containing 60 μM **RS1** for 30 min in an atmosphere of 5% CO_2, 95% air at 37°C. For the control experiment, cells were pretreated with ethanol-PBS (3:7, v/v, pH = 7.20) solution containing 100 μM N-ethylmaleimide for 1.5 hrs, and then incubated with 60 μM **RS1** in DMSO-PBS (1:49, v/v, pH = 7.20) for 30min in an atmosphere of 5% CO_2, 95% air at 37°C. Fluorescence imaging was then carried out after washing the cells with the PBS.

For living cell imaging, cells were incubated with 60 μM **RS1** in culture medium for

30 min in an atmosphere of 5% CO_2, 95% air at 37°C. For the control experiment, cells were pretreated with 30 μM N-ethylmaleimide for 30 min, and then incubated with 60 μM **RS1** in culture medium for 30 min in an atmosphere of 5% CO_2, 95% air at 37°C. Fluorescence imaging was then carried out after washing cells with the PBS.

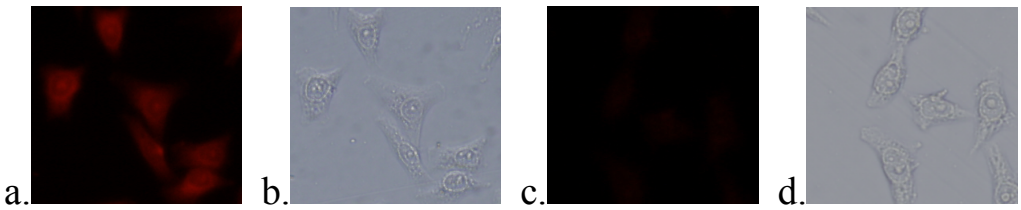


Fig. S10. Fluorescence (a) and brightfield (b) image of fixed MCF cells incubated with 60 μM **RS1** for 30 min; fluorescence (c) and brightfield (d) image of fixed MCF cells pretreated with 100 μM N-ethylmaleimide for 1.5 hrs, and then incubated with 60 μM **RS1** for 30 min. Condition: an atmosphere of 5% CO_2, 95% air at 37°C, exposure time of 1/10 sec (Excited with green light).

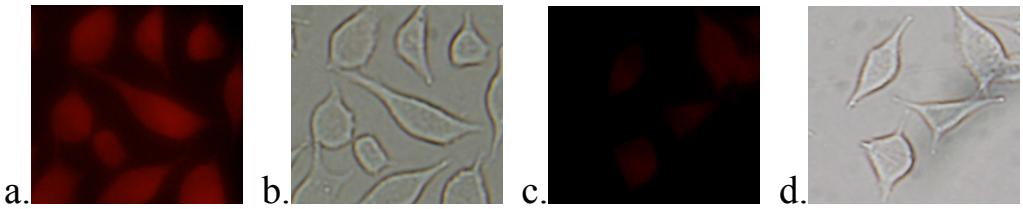


Fig. S11. Fluorescence (a) and brightfield (b) image of fixed PC12 cells incubated with 60 μM **RS1** for 30 min; fluorescence (c) and brightfield (d) image of fixed PC12 cells pretreated with 100 μM N-ethylmaleimide for 1.5 hrs, and then incubated with 60 μM **RS1** for 30 min. Condition: an atmosphere of 5% CO_2, 95% air at 37°C, exposure time of 1/10 sec (Excited with green light).

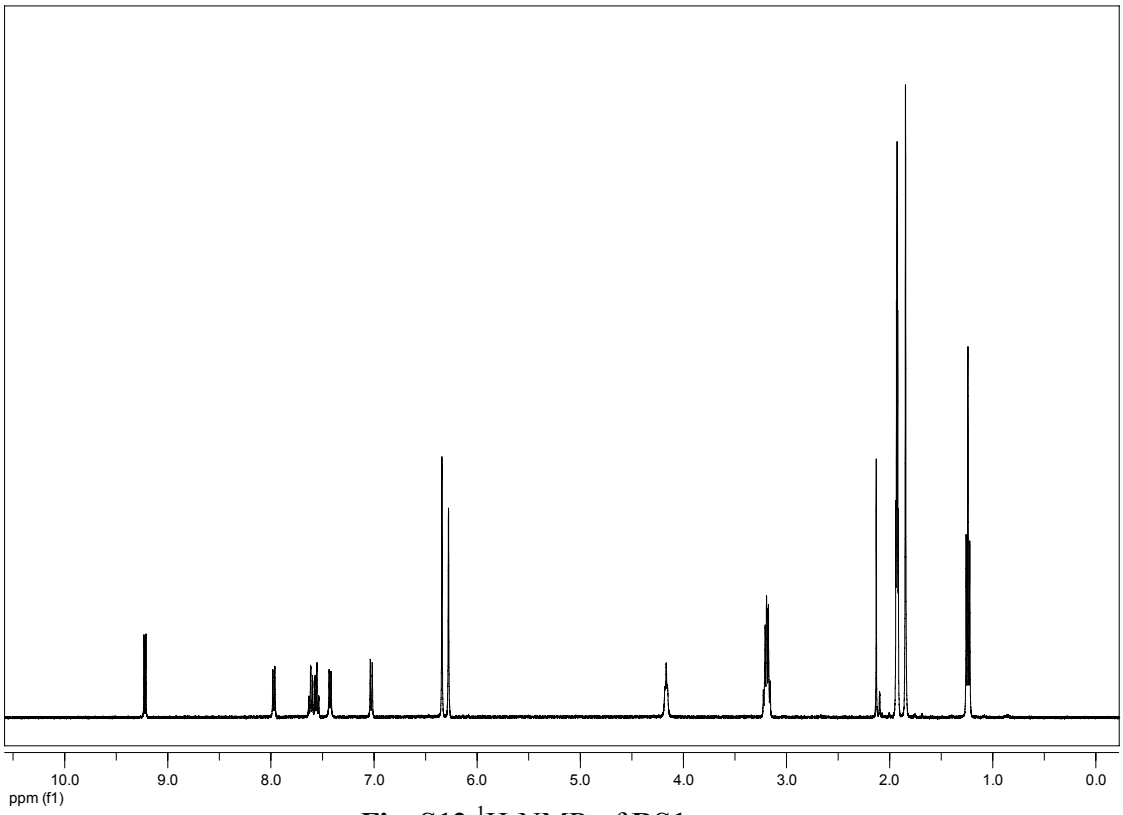


Fig. S12 ^{1}H-NMR of **RS1**

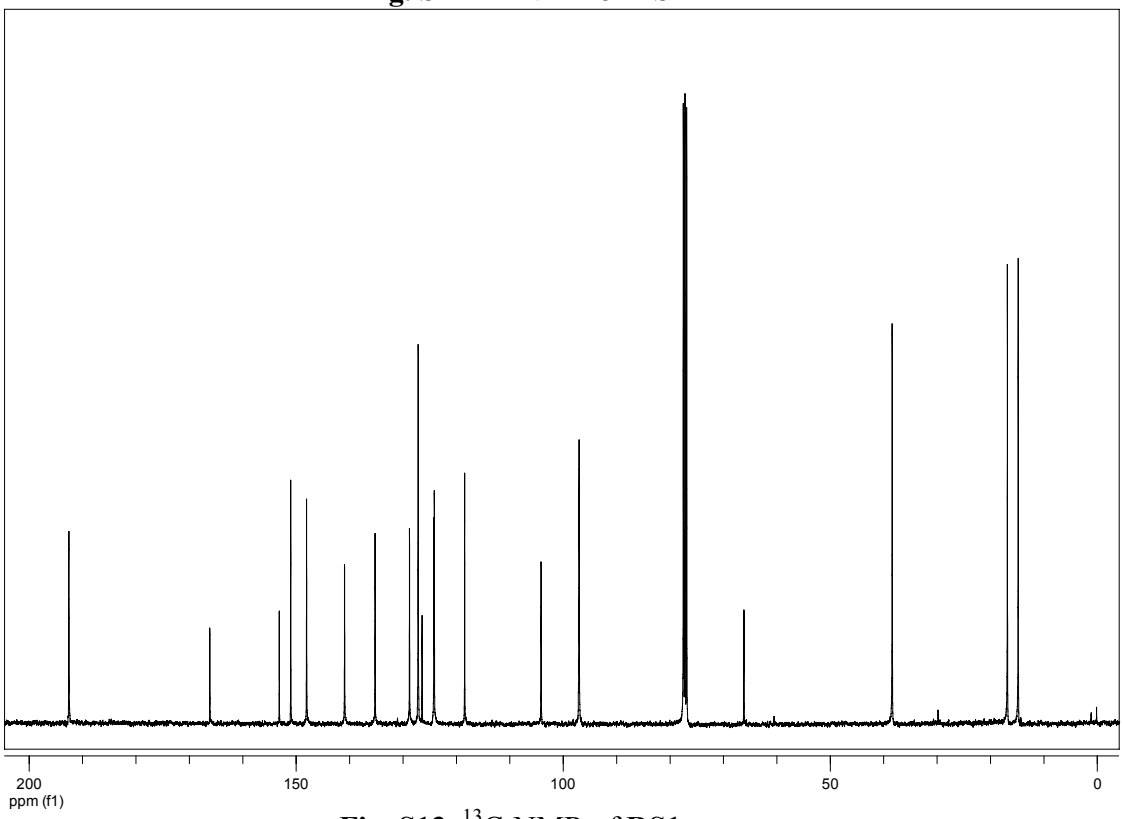


Fig. S13. ^{13}C-NMR of **RS1**

References:

1. Yang Y-K., Yook K-J. and Tae J., *J. Am. Chem. Soc.* 2005, **127***,* 16760.
2. SMART and SAINT, Area Detector Control and Integration Software, Siemens Analytical X-Ray Systems, Inc., Madison, WI, 1996.
3. G. M. Sheldrick, SHELXTL V5.1, Software Reference Manual, Bruker, AXS, Inc.: Madison, WI, 1997
4. M. Shortreed, R. Kopelman, M. Kuhn and B. Hoyland. *Anal. Chem*. 1996, **68**, 1414.