Supporting Information

Multi-functional probe to discriminate Lys, Arg, His, Cys, Hcy and GSH from common amino acids

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Contents

General Information

All chemicals were purchased from Adamas, Alfa Aesar, Acros and used without further purification. Deuterated solvents were purchased from Cambridge Isotope Laboratories. A549 cell line and Hela cell line were received from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences.

All non-aqueous reactions were carried out using oven-dried (110 °C) or heat gun dried glassware under a positive pressure of dry argon unless otherwise noted. Toluene, THF, DMF, and dichloromethane were purified by distillation and dried over activated molecular sieves (4 Å) under an argon atmosphere.

High purified water was taken from Millipore elix / RIOs water purified system.

UV-Visible spectra were collected on a SHIMADZU UV-2550 UV-visible spectrophotometer. Fluorescence spectra were collected on SHIMADZU RF-5301PC fluorescence spectrophotometer.

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AMX-400 NMR spectrometer, and BrukerAscend-600 NMR spectrometer, using TMS as an internal standard. LC-MS was obtained on Aglient 6120 Quadrupole LC-MS spectrometer. ESI-HRMS (high resolution mass spectrometry) spectra were obtained on AB SCIEX TRIPLE TOF 5600^{+} mass spectrometer.

Microscope images were taken by Opera (Perkin Elmer) 20X water object.

In all experiments enantiomerically pure natural amino acids were used except for Hcy which was used as the racemate.

Preparation of amino acids solutions for fluorescence study

Stock solutions (10 mM) of amino acids and other anylates TSC, Gly, Glu, Leu, Met, Ala, His, Tyr, Asp, Lys, Gln,Phe, Trp, Ser, Thr, Pro, Arg, $FeCl_3$, $ZnCl_2$, NaHS, Cys, Hcy and GSH in water were prepared. Stock solutions of probe **1** (1 mM) and probe **2** (1 mM) were also prepared in water and acetonitrile respectively. In a typical experiment, solutions for the measurement were prepared by placing 30 μL of the analytes stock solution into a test tube, diluting the solution to 3 mL with saline, phosphate buffer (pH 7.26), CH_3CN-phosphate buffer (pH 7.26, 2:8 v/v) or Acetone, and adding 30 μL of the probe stock. Normally, excitation was at 478 nm. The excitation and emission slit widths were 2.5 nm/2.5 nm. Fluorescence spectra and UV-Visible spectra were measured after addition of analyte for 5 min, 1h or 5h. For low concentration titration of Lys, Arg, Cys, Hcy and GSH fluorescence spectra were measured after addition of thiols for 10 min, and the excitation and emission slit widths were 2.5 nm/5nm.

Cell culture and fluorescence imaging

A 549 cells were cultured in culture media (GIBCO F-12 supplemented with 10% FBS) and Hela cells were cultured in culture media (GIBCO DMEM supplemented with 10% FBS) at 37 °C in a humidified incubator, and culture media were replaced with fresh media every day. The cells were seeded at a density of 2×10^6 cells per mL in culture media. After 24 h, the cells were treated without or with 500 μM NEM in culture media for 30 min at 37 °C. After washing with phosphate buffered saline (PBS) to remove the remaining NEM, the cells were further incubated with 10 μM of 1in culture media for 30 min at 37 °C. The cells were imaged by Opera (Perkin Elmer) 20X water object.

Preparation of NMR samples in D_2O for mechanistic studies

a) Preparation of D_2O solutions of Lys and Arg and probe **1**

Probe **1** (2 mg) and Lys (5 mg) or Arg (5 mg) were mixed in D_2O (0.6 ml). The spectra were measured 30 min later.

b) Preparation of D_2O-PBS buffer

Na_2HPO_4 (39.47mg) and KH_2PO_4 (4.49 mg) were added into D_2O (20 ml) under shaken until all the solids dissolved.

c) Preparation of D_2O-PBS buffer solutions of Cys, Hcy, GSH and probe **1**

Probe **1** (2 mg) and bio-thiol (5 mg) were mixed in D_2O-PBS buffer (0.6 ml). The spectra were measured 30 min later.

All ^{1}H-NMR spectra in D_2O were measured with BrukerAscend-600 NMR spectrometer.

Synthetic procedures of probes 1 and 2

(*E*)-pyridine-2-acrylic acid (3)

Pyridine-2-aldehyde (1.9 g, 17 mmol, 1.0 equiv) and malonic acid (5.3 g, 51 mmol, 3.0 equiv) were dissolved in pyridine (20 mL) in argon. To the resulting suspension was added piperidine (0.5 mL, 5.3 mmol, 0.3 equiv), warmed to reflux and stirred for 6 h. Then the mixture was cooled to room temperature, and pyridine was evaporated under reduced pressure. Then $CHCl_3$ (10 mL) was added to the brown oil and stirred for 0.5 h, during which white solid precipitate formed. The resulted white solid was filtered to afford **2** as a white solid (1.2 g, 8.0 mmol, 47% yield) which was used directly in the next step without further purification.^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.61 (s, 1H), 8.64 (dd, J = 4.7, 0.8 Hz, 1H), 7.86 (td, J = 7.7, 1.8 Hz, 1H), 7.73 (d, J = 7.8 Hz, 1H), 7.60 (d, J = 15.7 Hz, 1H), 7.41 (ddd, J = 7.5, 4.8, 1.1 Hz, 1H), 6.83 (d, J = 15.7 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 167.72, 152.84, 150.44, 143.57, 137.65, 125.06, 124.93, 123.11.

Fluorescein bis[(*E*)-(3-pyridin-2-yl)acrylate] (2)

(*E*)-pyridine-2-acrylic acid (**2**) (950mg, 6.3 mmol, 2.5 equiv) was added into a mixture of THF (50 mL) and DMF (25 mL) under argon flow. Then DMAP (125 mg, 1.0 mmol, 0.4 equiv) and EDC/HCl (1.9 g, 10.0 mmol, 4.0 equiv) were added and stirred for 1 h. At last fluorescein (716 mg, 2.5 mmol, 1.0 equiv) was added and stirred for 16 h at room temperature. The color of the mixture changed into black from red brown. Then THF and DMF were evaporated under reduced pressure. The crude product was purified by column chromatography directly (eluted with PE : EA = 2:1~1:1) to afford **3** as a white foam (840 mg, 1.5 mmol, 61% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.71 (d, *J* = 4.6 Hz, 2H), 8.09 (d, *J* = 7.5 Hz, 1H), 7.96–7.89 (m, 4H), 7.89–7.83 (m, 3H), 7.80 (td, *J* = 7.5, 0.8 Hz, 1H), 7.45–7.49 (m, 3H), 7.44 (d, *J* = 2.3 Hz, 2H), 7.14 (d, *J* = 15.7 Hz, 2H), 7.09 (dd, *J* = 8.7, 2.3 Hz, 2H), 6.95 (d, *J* = 8.7 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm): 168.88, 164.64, 152.66, 152.22, 151.34, 150.68, 146.43, 137.85, 136.52, 131.07, 129.67, 125.93, 125.73, 125.53, 124.64, 120.57, 119.08, 116.81, 111.00, 81.49. HRMS (ESI$^+$): $[M+H]^+$ calcd. for $C_{36}H_{23}N_2O_7$: 595.1500; found: 595.1515.

Fluorescein-bis-[(*E*)-(3-(*N*-methylpyridinium-2-yl)acrylate) iodide] (1)

Fluorescein bis[(*E*)-(3-pyridin-2-yl)acrylate] (**3**) (120 mg, 0.22 mmol) was added into methyl iodide (5 mL). The mixture was stirred at reflux for 24 h. Then excess of methyl iodide was evaporated *in vacuo*, and the brick red solid residue was washed by Et_2O, filtered and 140 mg crude product was obtained. Recrystallization from water obtained **1** as a yellow solid (60 mg, 0.072 mmol, 33% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.10 (d, *J* = 5.9 Hz, 2H), 8.65 (t, *J* = 7.9 Hz, 2H), 8.57 (d, *J* = 7.6 Hz, 2H), 8.15 (t, *J* = 6.4 Hz, 2H), 8.11–8.00 (m, 3H), 7.92–7.71 (m, 3H), 7.47 (t, *J* = 5.0 Hz, 2H), 7.39 (d, *J* = 15.8 Hz, 2H), 7.10 (dd, *J* = 8.7, 2.2 Hz, 2H), 6.99 (d, *J* = 8.7 Hz, 2H), 6.60–6.90 (m, 1H), 4.41 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm):168.4, 162.80, 152.10, 151.50, 150.80, 148.93, 147.21, 145.49, 136.09, 134.80, 130.66, 130.35, 129.47, 128.10, 127.07, 125.72, 125.36, 125.09, 124.81, 124.09, 118.41, 117.84, 117.06, 116.65, 110.28, 80.81, 46.36. HRMS (ESI$^+$): M^{2+} calcd. for $(C_{38}H_{28}N_2O_7)/2$: 312.0943; found: 312.0963.

Spectroscopic studies and images

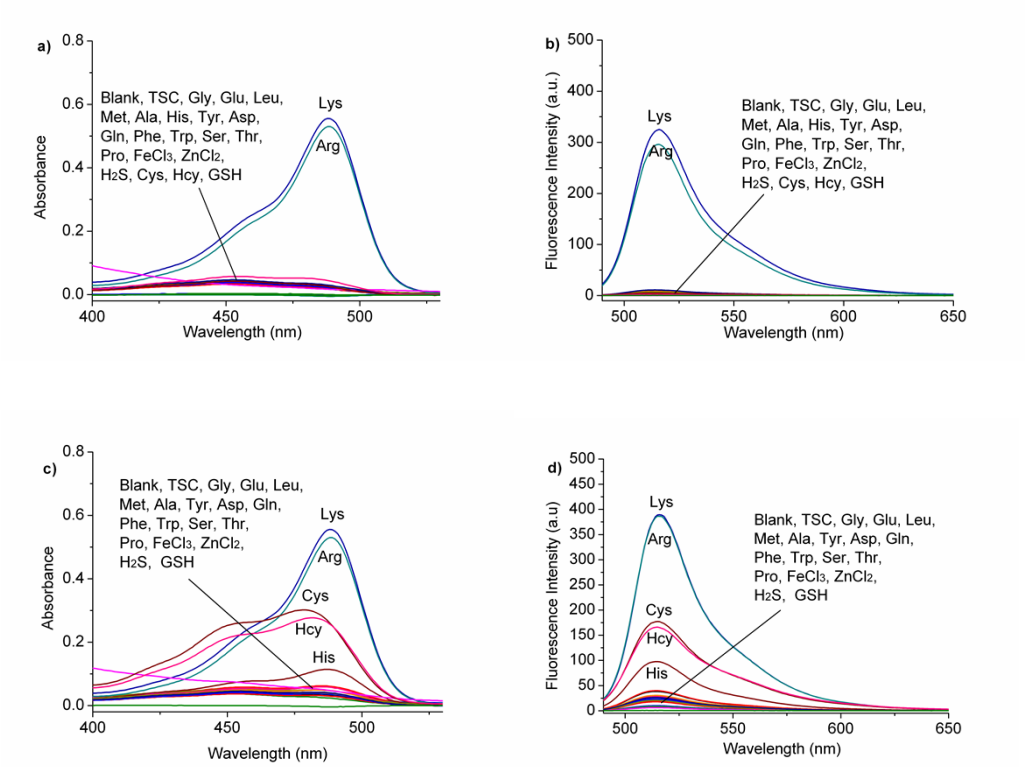


Figure S1. UV-vis and fluorescence spectra (λ_{ex} = 478 nm, slit: 2.5 nm/2.5 nm) of **1** (10 μM) upon addition of different biological analytes (100 μM) in physiological saline at room temperature. a) UV-vis spectra and b) Fluorescence spectra recorded after 5 min; c) UV-vis spectra and d) Fluorescence spectra recorded after 5 h.

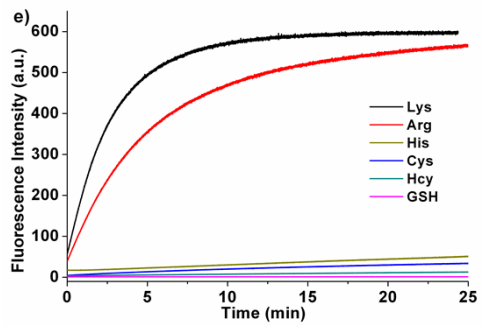


Figure S2. Time course fluorescence response of **1** (10 μM) upon addition of Lys, Arg, His, Cys, Hcy, and GSH (100μM) in physiological saline at room temperature, monitored at 513 nm (λ_{ex} = 478 nm, slit: 2.5 nm/2.5 nm).

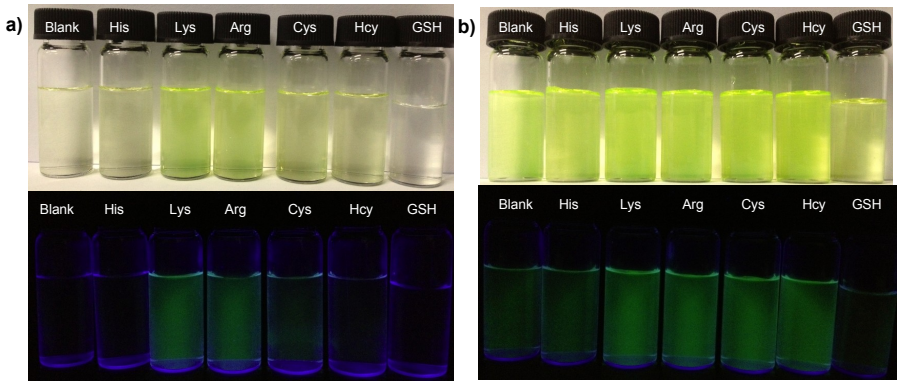


Figure S3. Visualized images of probe **1** (10 μM) was added to various biological analytes (Lys, Arg, His, Cys, Hcy, GSH) (100 μM) at room temperature in physiological saline under ambient light (upper) and under UV irradiation (365nm). a) Photographs taken after 5 min; b) Photographs taken after 5 h.

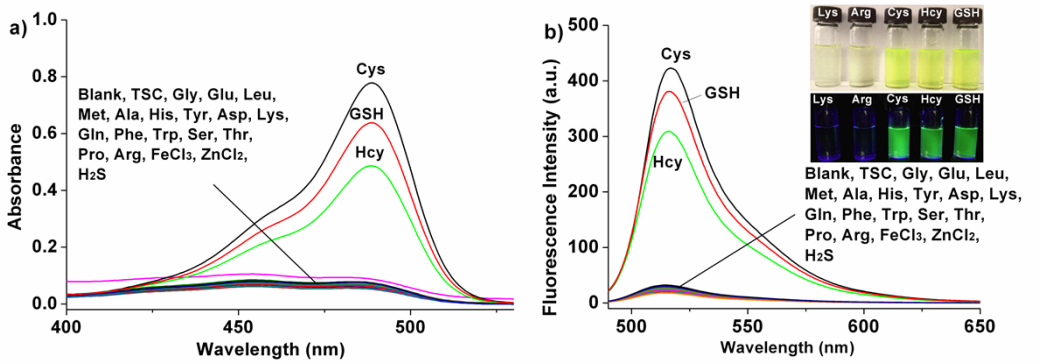


Figure S4. Responses of probe **1** (10 μM) to various analytes (100 μM each) in PBS buffer (pH 7.26) at room temperature. a) UV-vis spectra obtained 5 min after addition. b) Fluorescence spectra (λ_{ex} = 478 nm, slit: 2.5 nm/2.5 nm) obtained 5 min after addition. *Inset:* Photographs of **1** after addition of Lys, Arg, Cys, Hcy, GSH respectively visualized under ambient light (upper) and UV light of 365 nm (lower).

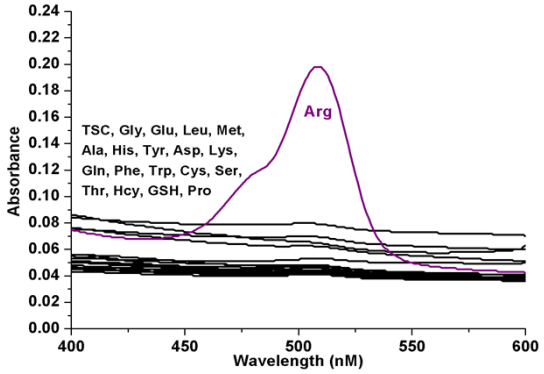


Figure S5. UV-vis spectra of **1** (10 μM) responded to various analytes (100 μM each) in aqueous acetone at room temperature. Data were recorded 1 h after addition.

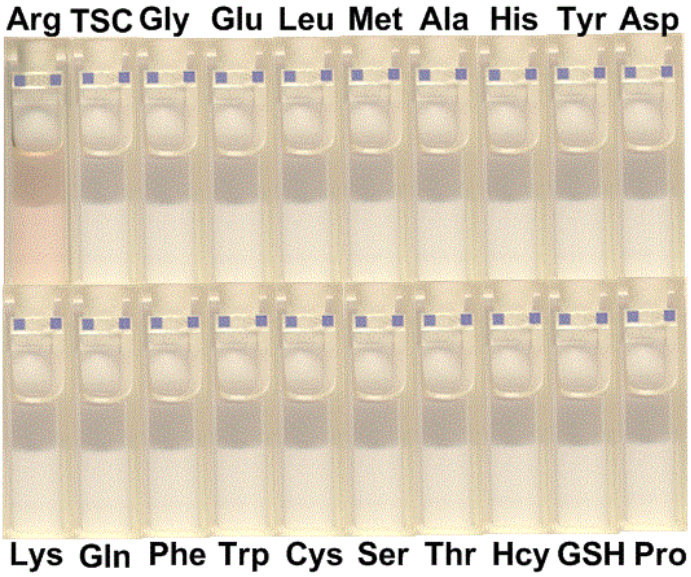


Figure S6. Images of probe **1** (10 μM) responded to various biological analytes (100 μM) in acetone at room temperature under ambient light. Photographs taken after 1 h.(**1** was dissolved in purified water (1 mM) and analytes were dissolved in water (10 mM) and then placed 30 μL of the analytes stock solution into a test tube, diluting the solution to 3 mL with acetone, and at last 30 μL of the probe **1** stock solution was added.)

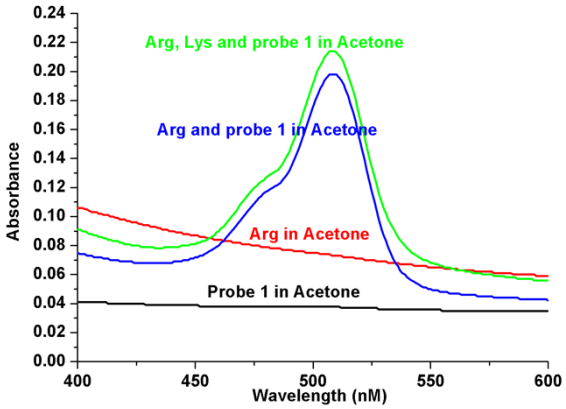


Figure S7. UV-vis spectra of **1** (10 μM) upon addition of various analytes (100 μM each) in acetone at room temperature. Data recorded 1 h after addition (**1** was diluted to 1 M in water and analytes were diluted to 10 M in water and then diluted 100 folds in acetone prior to the test.)

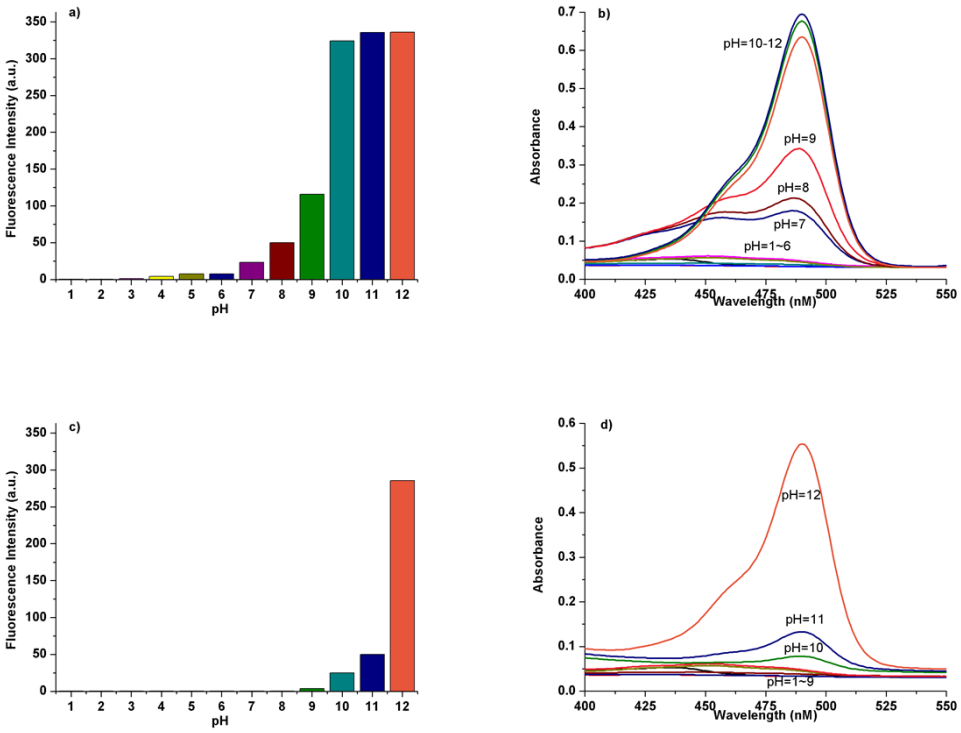


Figure S8. The fluorescence intensities and absorption behaviors of fluorescent probes **1** and **2** in various pH values of water. Data was recorded at 20 minutes after addition. a) and c): Emission response (λ_{ex} = 478nm) at 513 nm of **1** (10 μM) and **2** (10 μM) respectively; b) and d) UV-vis spectra of **1** (10 μM) and **2** (10 μM) responded in different pH value in water at room temperature respectively.

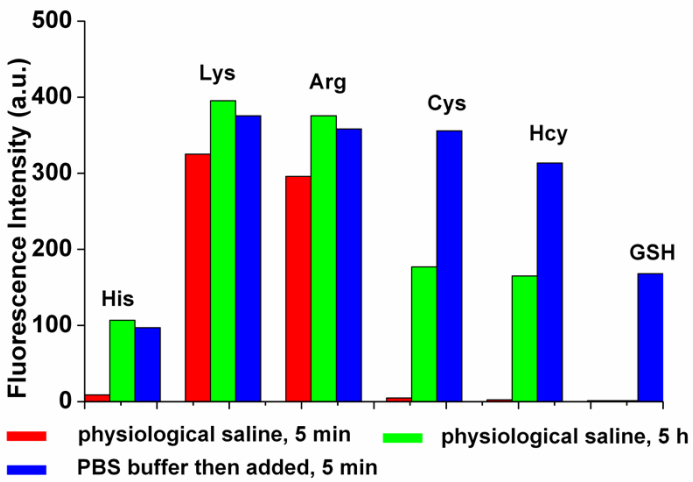


Figure S9. Fluorescence responses of probe **1** (10 μM) and various biological analytes (Lys, Arg, Cys, Hcy, GSH) (100 μM) at room temperature in physiological saline (3 mL) 5 min, 5 h, and then 5 min after PBS buffer solution (pH 7.26) (1 mL) was added (λ_{ex} = 478 nm, λ_{em} = 513 nm, slit: 2.5 nm/2.5 nm).

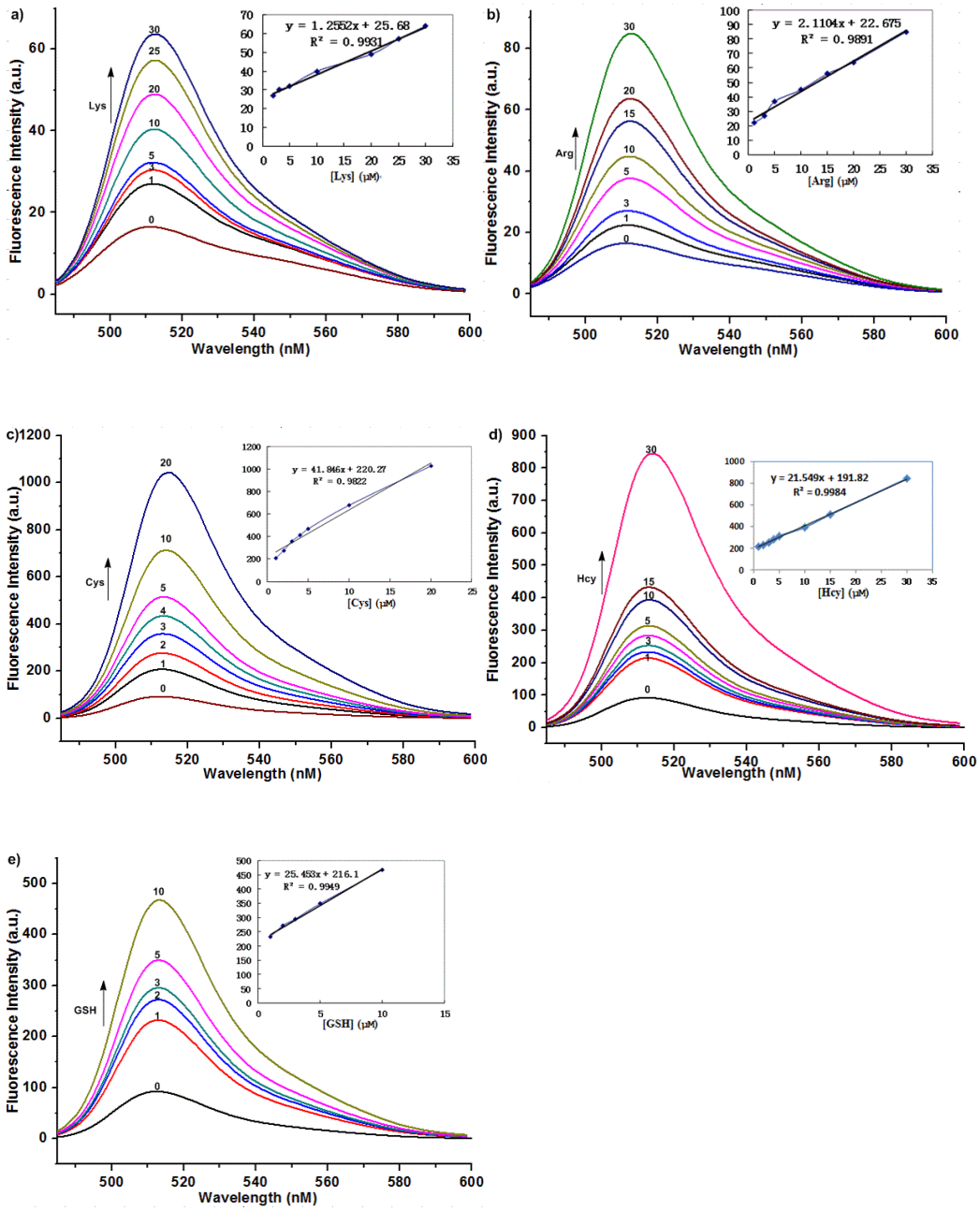


Figure S10. Fluorescence spectra of probe **1** (10 μM) toward various concentrations of Lys, Arg, Cys, Hcy and GSH (0-30μM for each, λ_{ex} = 478 nm, slit:2.5 nm/5 nm). *Inset:* the linear relationship between fluorescence intensity and Lys, Arg, Cys, Hcy and GSH concentrations in the ranges from 1-30μM for each. (λ_{ex} = 478 nm, λ_{em} = 513 nm slit: 2.5 nm/5 nm). Lys and Arg were tested in saline, Cys, Hcy and GSH were tested in PBS buffer solution (pH 7.26). Data were recorded at 10 min after addition. a) Fluorescence spectra of Lys; b) Fluorescence spectra of Arg; c) Fluorescence spectra of Cys; d) Fluorescence spectra of Hcy; e) Fluorescence spectra of GSH.

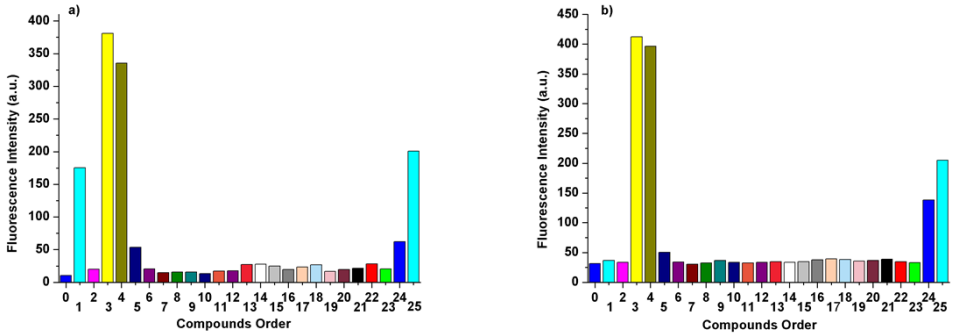
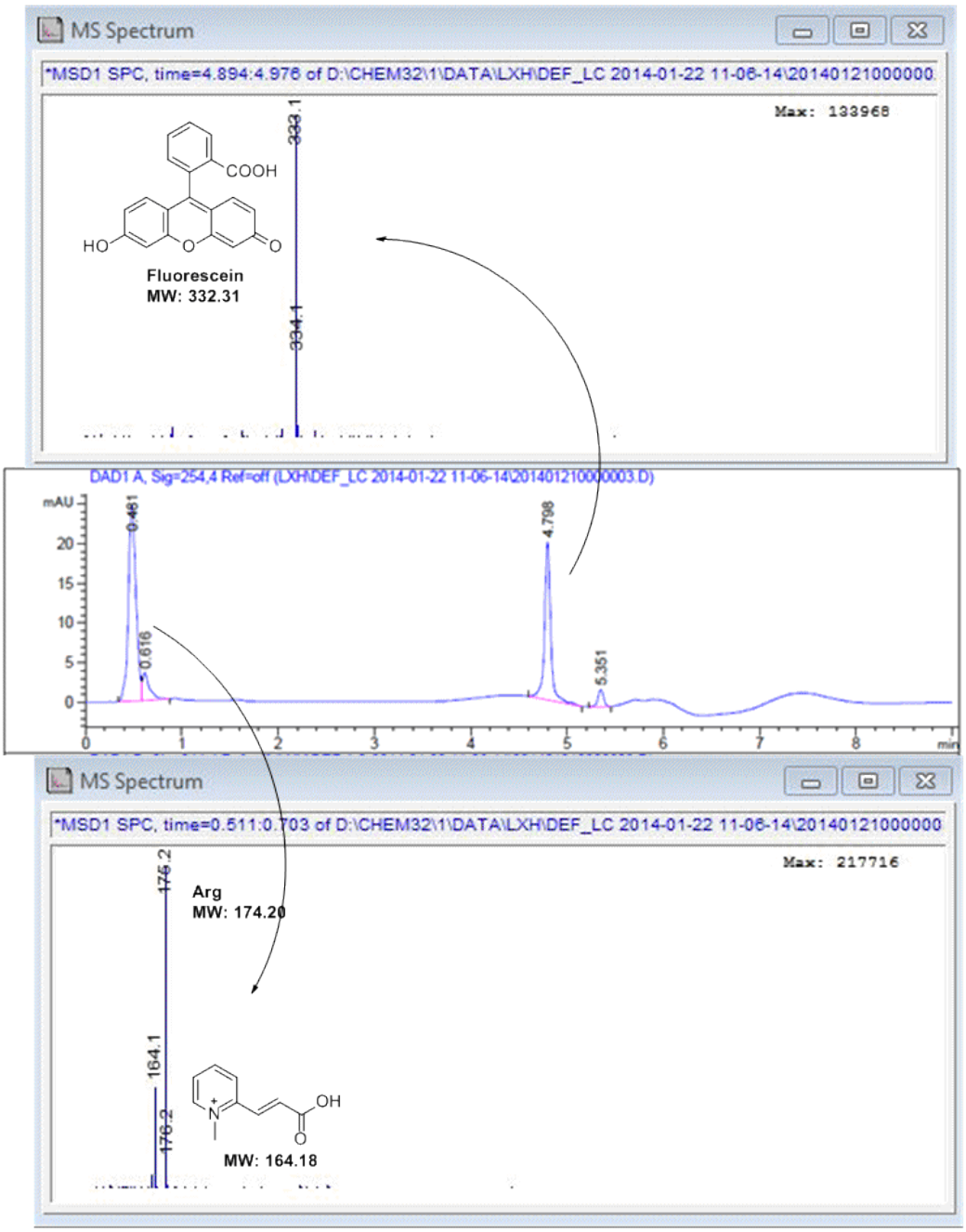


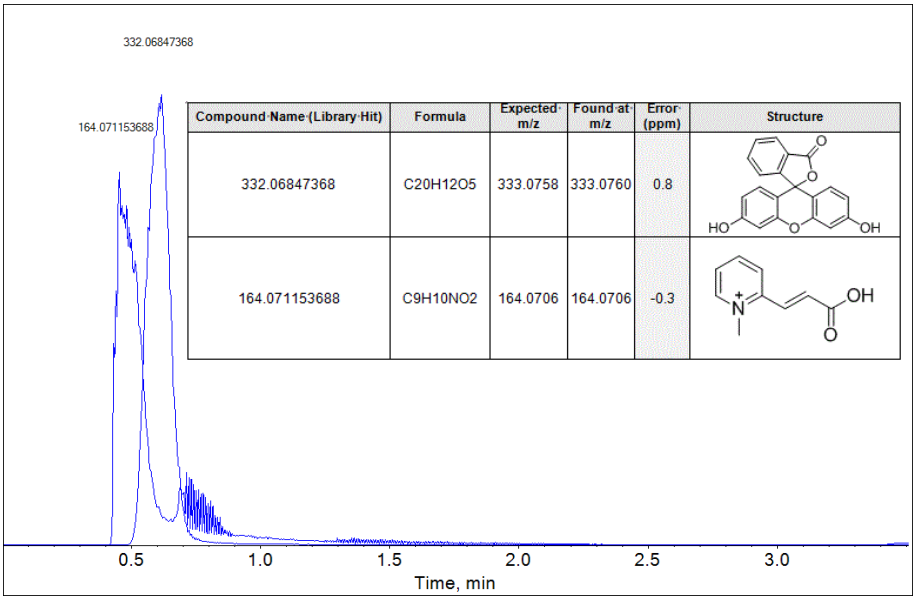
Figure S11. Emission responses (λ_{ex} = 478nm) at 513 nm of **1** (10 μM) upon addition of various analytes (100 μM, except cases 24 and 25) in physiological saline (a) and PBS buffer (b, pH = 7.26) at room temperature. 0: blank, 1: Benzylamine, 2: Aniline, 3: Benzylmercaptan, 4: Thiophenol, 5: Et_3N, 6: *tert*-Butyl mercaptan, 7: D-Ribose, 8: D (+)-Glucose, 9: Lactose, 10: D (+)-Galactose, 11: Thymine, 12: Cytosine, 13: Uracil, 14: Guanine, 15: Adenine, 16: Guanosine, 17: Cytidine, 18: Thymidine, 19: Uridine, 20: Adenosine, 21: Deoxy-guanosine, 22: Deoxy-adenosine, 23: Deoxycytidine, 24: Albumin from bovine serum (BSA, 100 μg/ml), 25: Fetal Bovine Serum (FBS) filtered by 0.22 μm membrane and diluted with equal volume of solvents. Data were recorded 20 min after addition of analyate.

LC-MS,HRMS and ^{1}H-NMRresults for mechanistic studies

a)



b)



Compound Name (Library Hit)	Formula	Expected m/z	Found at m/z	Error (ppm)	Structure
332.06847368	C20H12O5	333.0758	333.0760	0.8	
164.071153688	C9H10NO2	164.0706	164.0706	-0.3	

c)

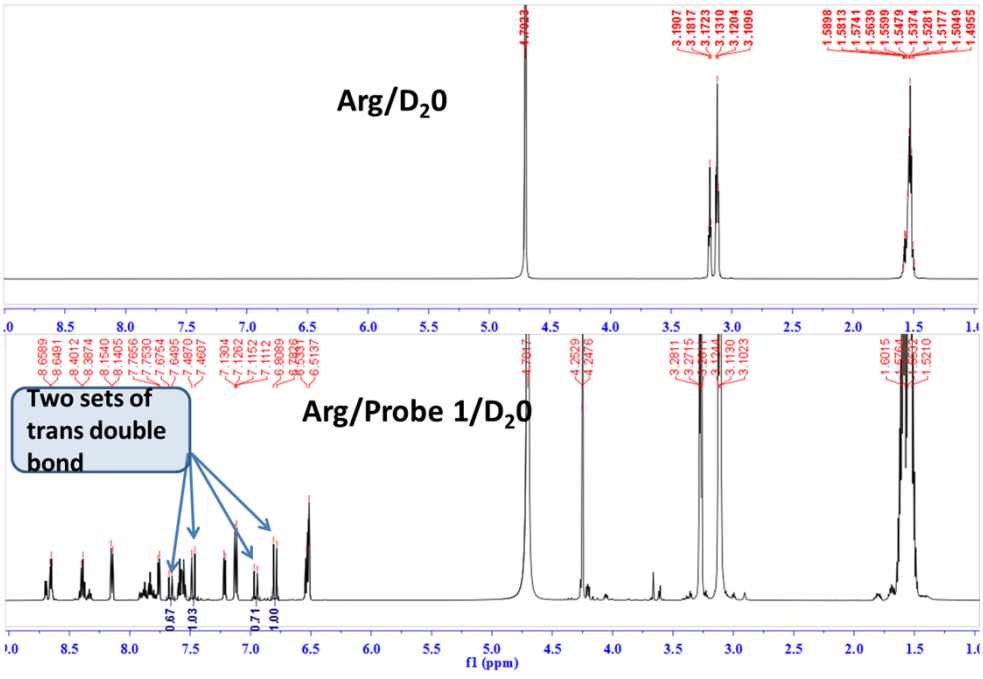


Figure S12. a) HPLC-MS spectra of Arg and probe **1** in physiological saline; b) HRMS spectra of Arg and probe **1** in physiological saline; c) ^{1}H-NMR spectra of probe **1** and Arg in D_2O.

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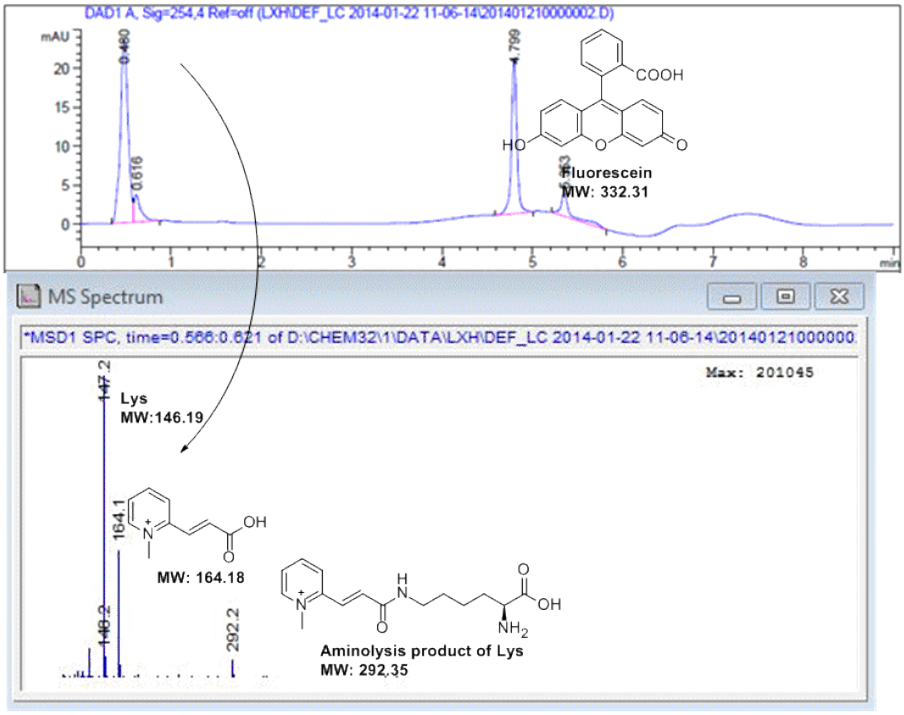
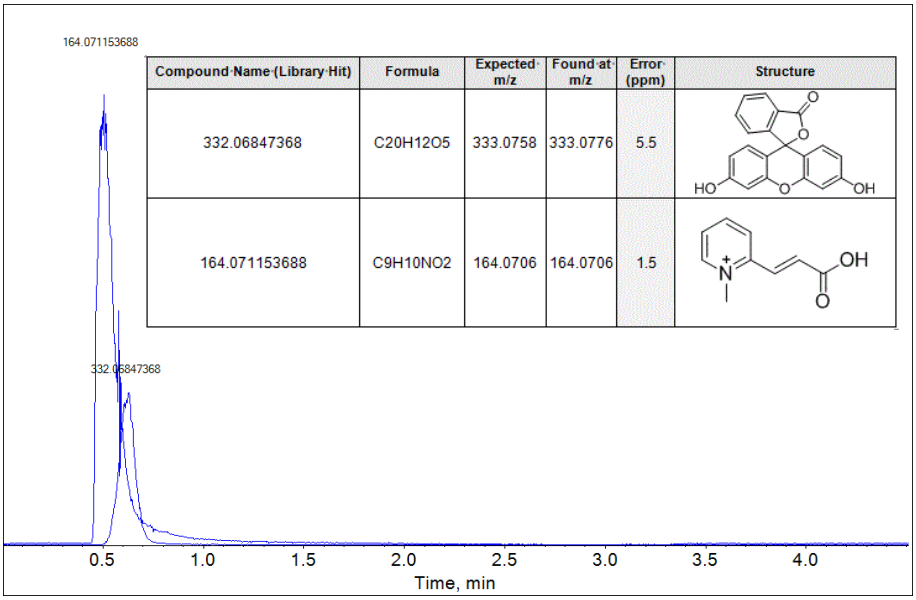
a)



b)



Compound Name (Library Hit)	Formula	Expected m/z	Found at m/z	Error (ppm)	Structure
332.06847368	C20H12O5	333.0758	333.0776	5.5	
164.071153688	C9H10NO2	164.0706	164.0706	1.5	

c)

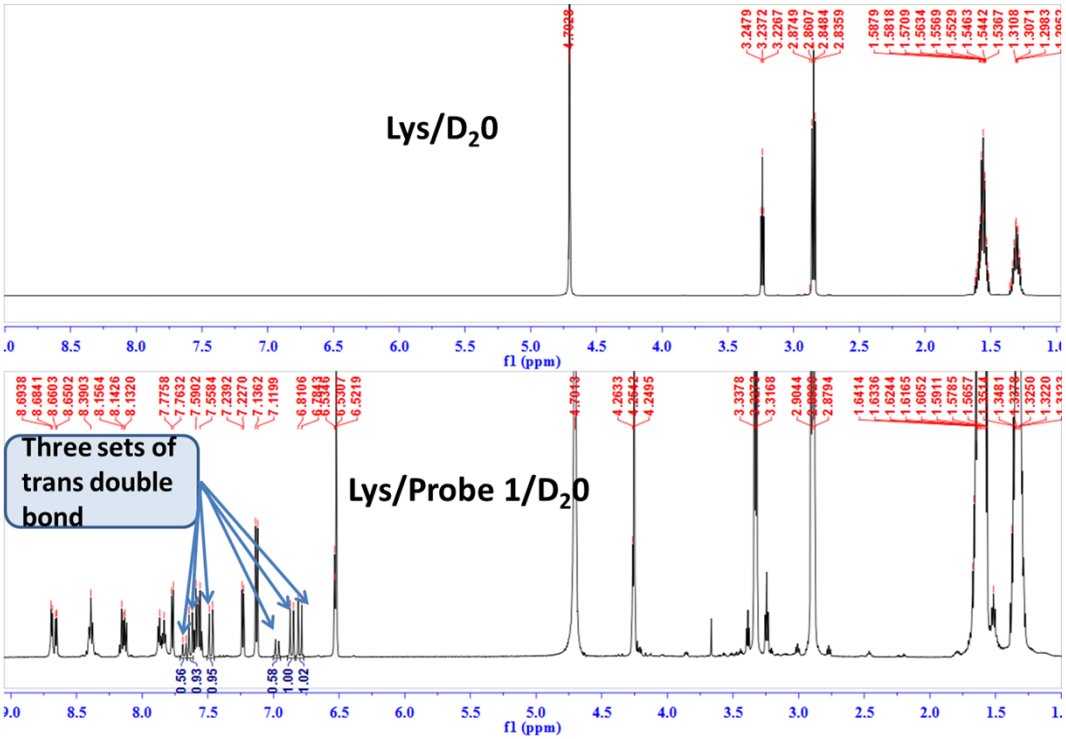
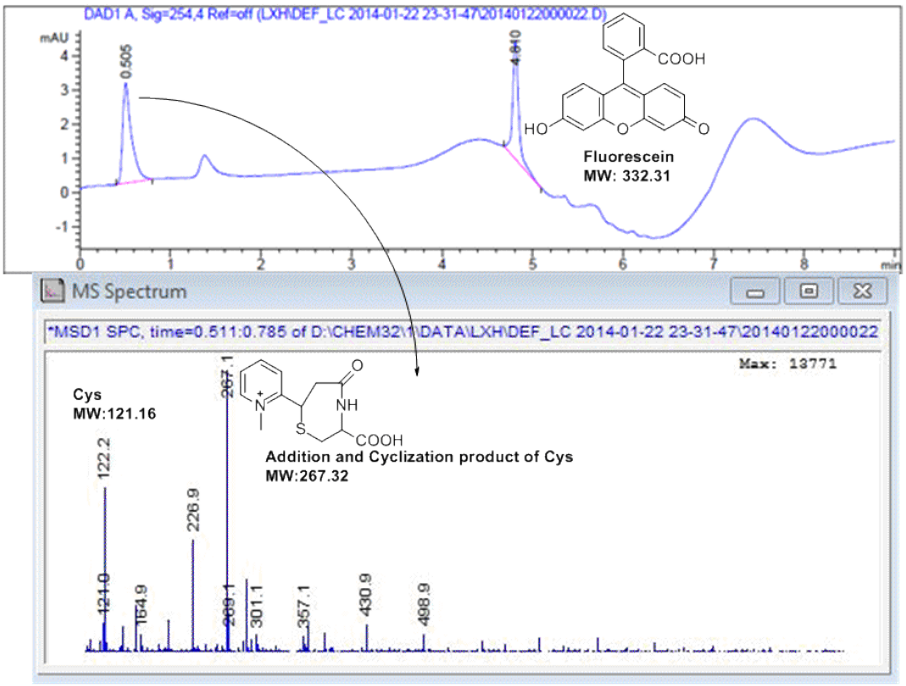
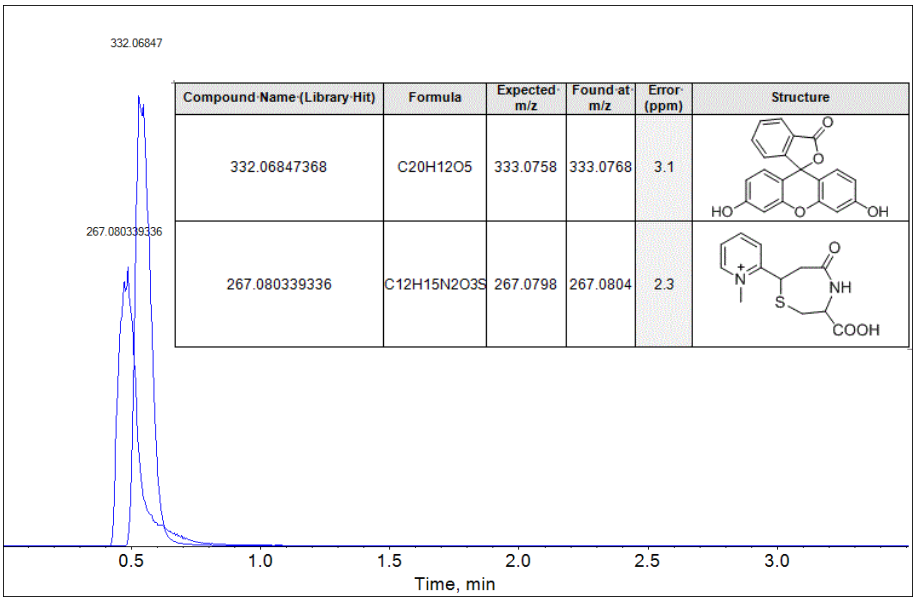


Figure S13 a) HPLC-MS spectra of Lys and probe **1** in physiological saline; b) HRMS spectra of Lys and probe **1** in physiological saline; c) ^{1}H-NMR spectra of probe **1** and Lys in D_2O.

a)



b)



Compound Name (Library Hit)	Formula	Expected m/z	Found at m/z	Error (ppm)	Structure
332.06847368	C20H12O5	333.0758	333.0768	3.1	
267.080339336	C12H15N2O3S	267.0798	267.0804	2.3	

c)

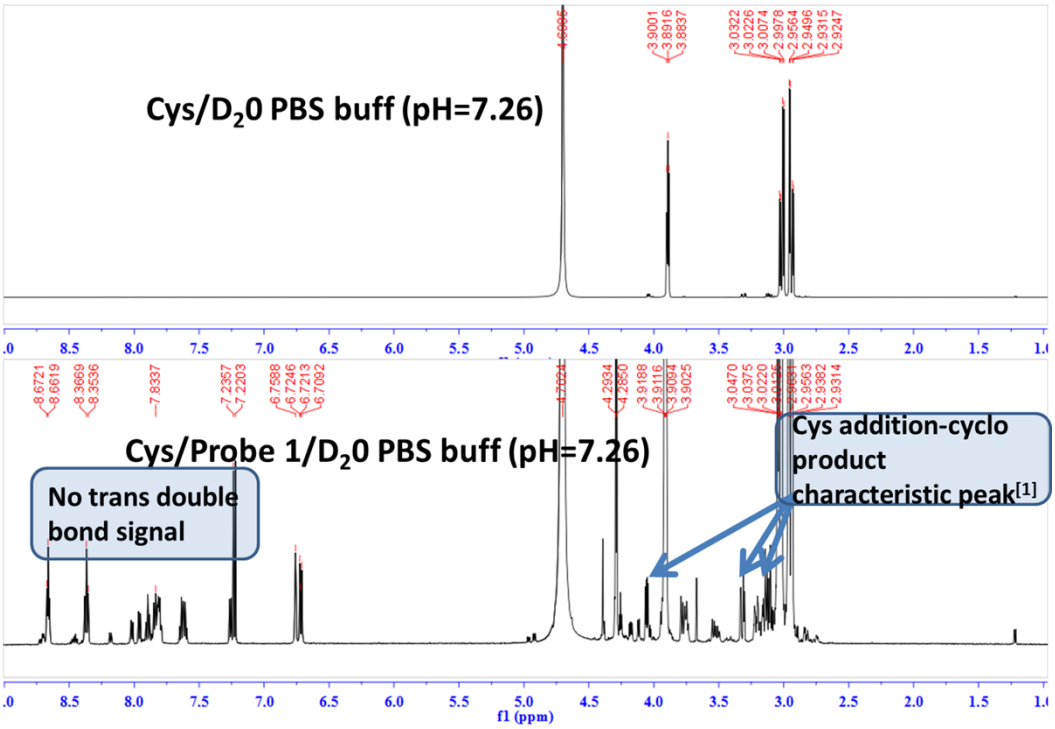
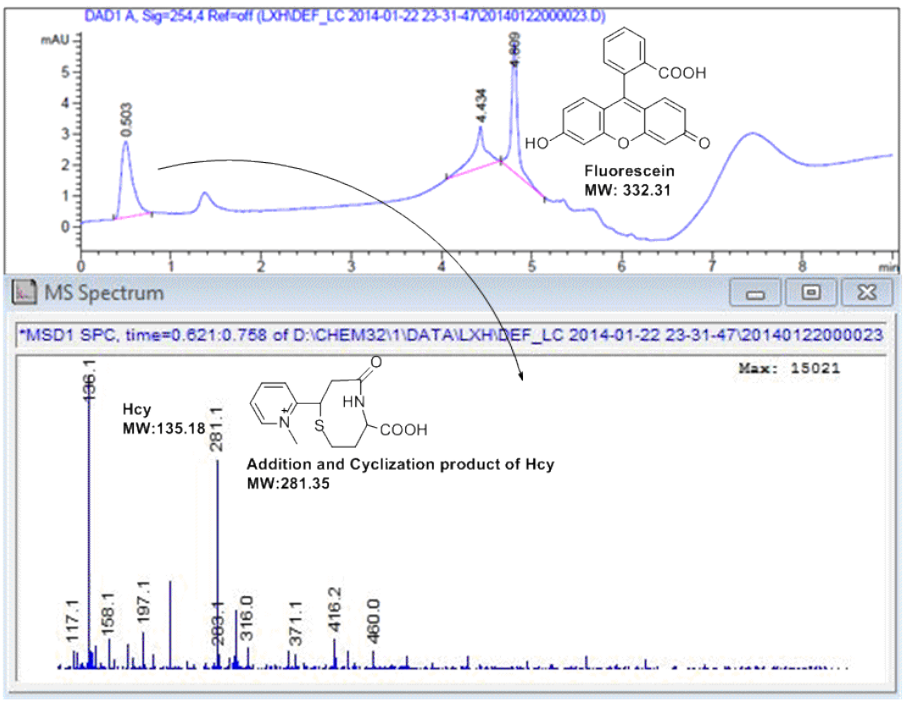
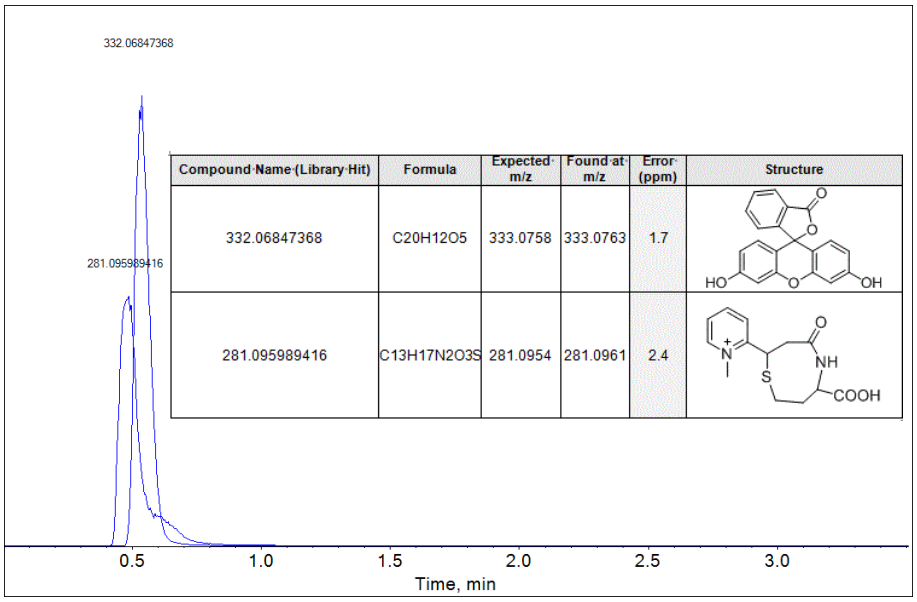


Figure S14. a) HPLC-MS spectra of Cys and probe **1** in PBS buffer (pH 7.26); b) HRMS spectra of Cys and probe **1** in PBS buffer; c) ^{1}H-NMR of probe **1** and Cys in D_2O-PBS buffer.

a)



b)



Compound Name (Library Hit)	Formula	Expected m/z	Found at m/z	Error (ppm)	Structure
332.06847368	C20H12O5	333.0758	333.0763	1.7	
281.095989416	C13H17N2O3S	281.0954	281.0961	2.4	

c)

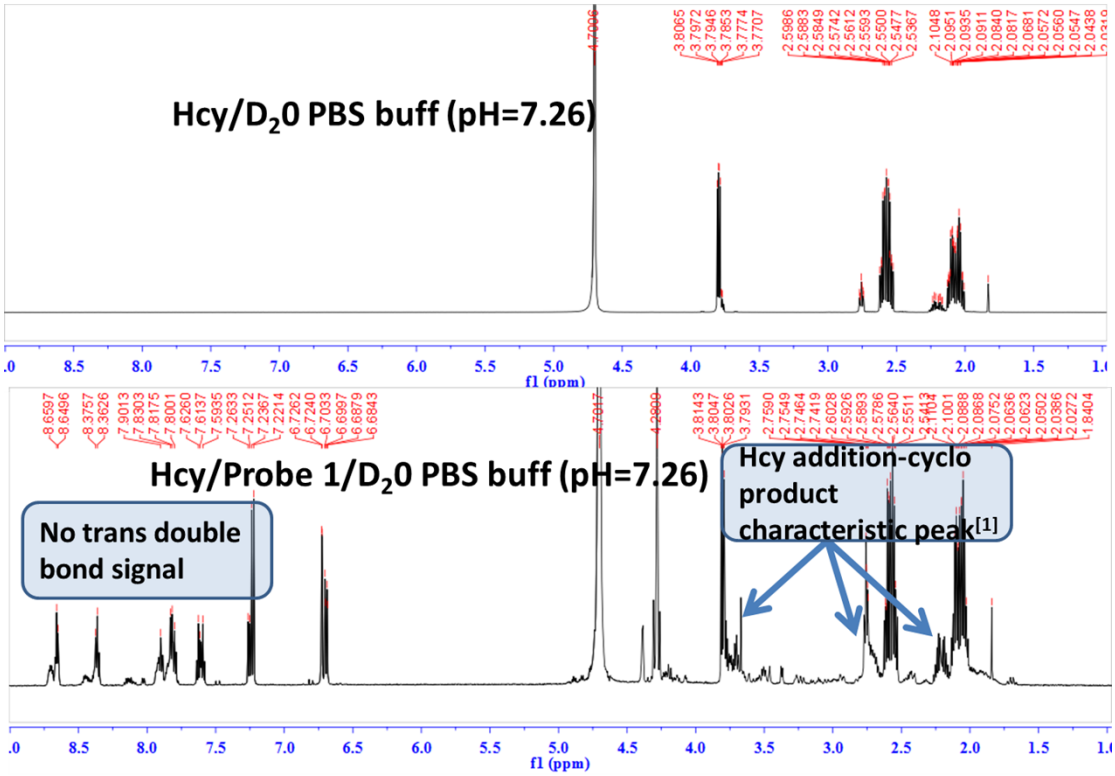
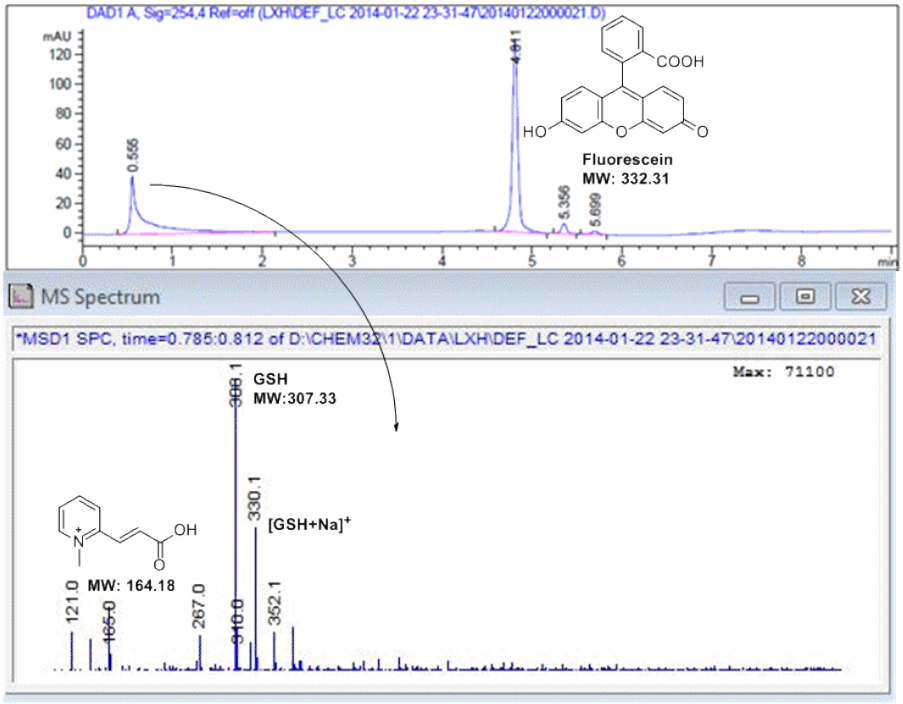
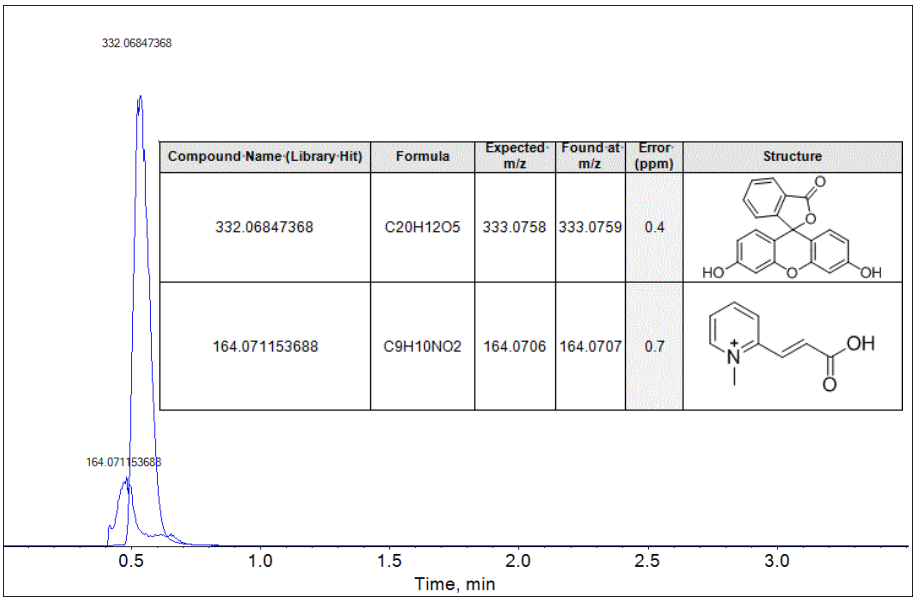


Figure S15. a) HPLC-MS spectra of Hcy and probe **1** in PBS buffer (pH 7.26); b) HRMS spectra of Hcy and probe **1** in PBS buffer; c) ^{1}H-NMR of probe **1** and Hcy in D_2O-PBS buffer.

a)



b)



Compound Name (Library Hit)	Formula	Expected m/z	Found at m/z	Error (ppm)	Structure
332.06847368	C20H12O5	333.0758	333.0759	0.4	
164.071153688	C9H10NO2	164.0706	164.0707	0.7	

c)

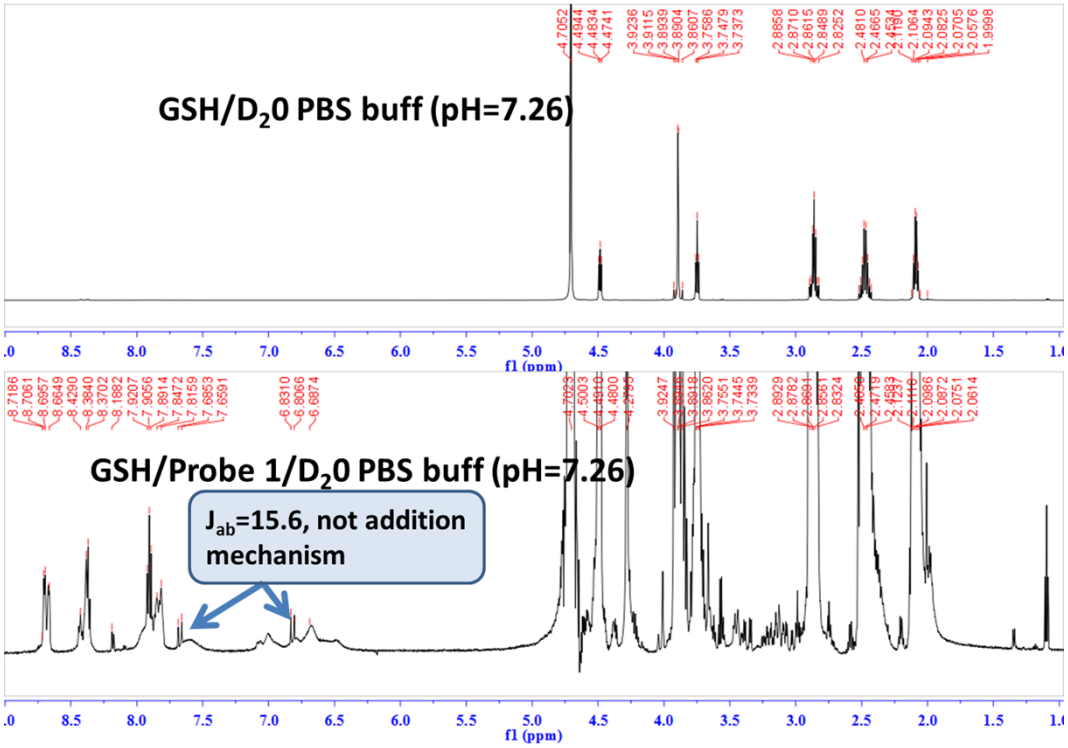
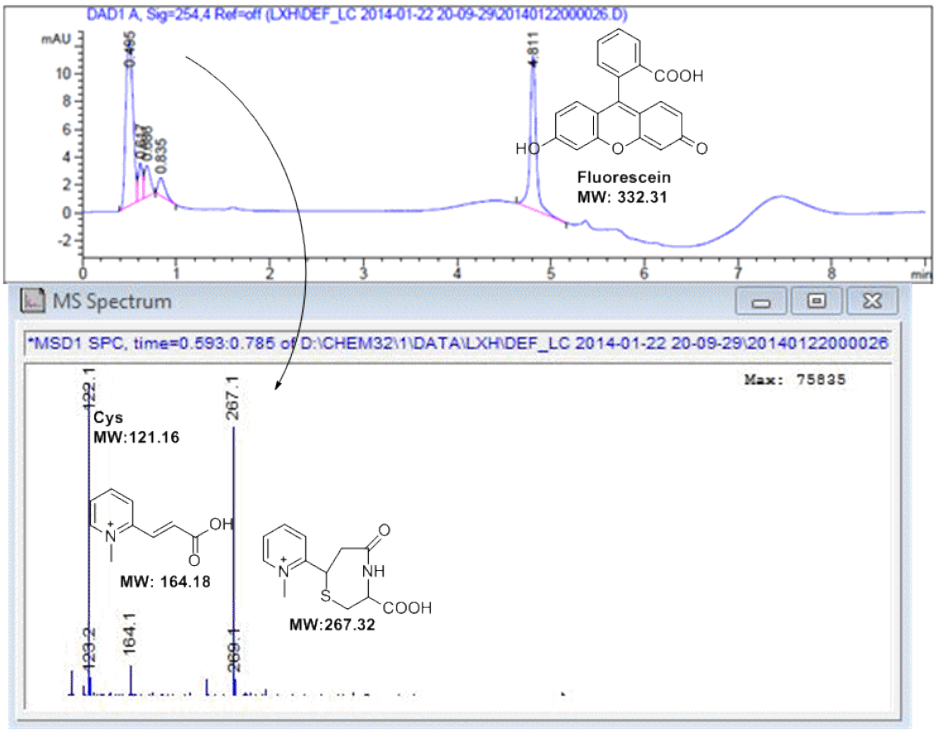
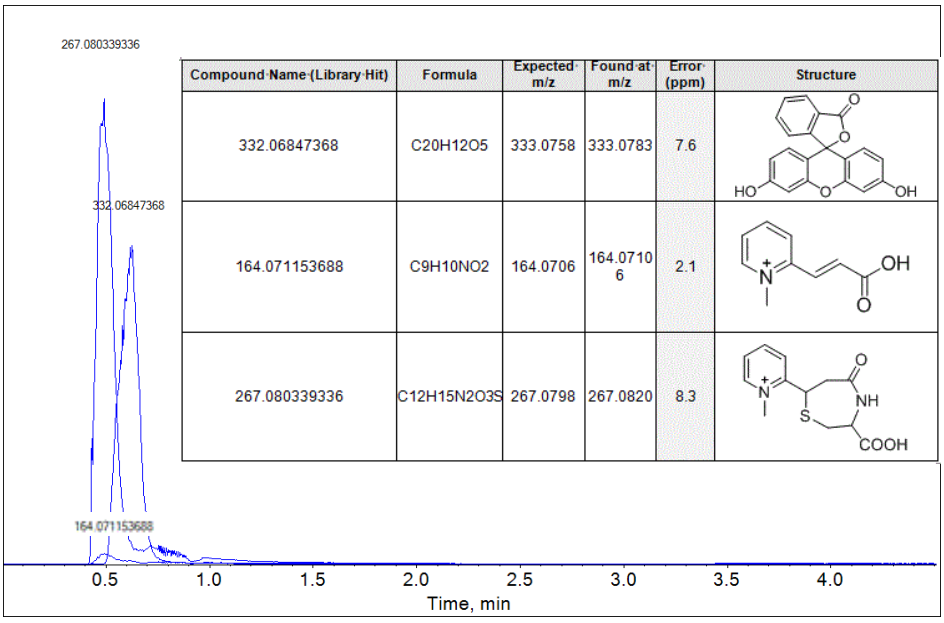


Figure S16. a) HPLC-MS spectra of GSH and probe **1** in PBS buffer (pH 7.26); b) HRMS spectra of GSH and probe **1** in PBS buffer; c) ^{1}H-NMR of probe **1** and GSH in D_2O-PBS buffer.

a)



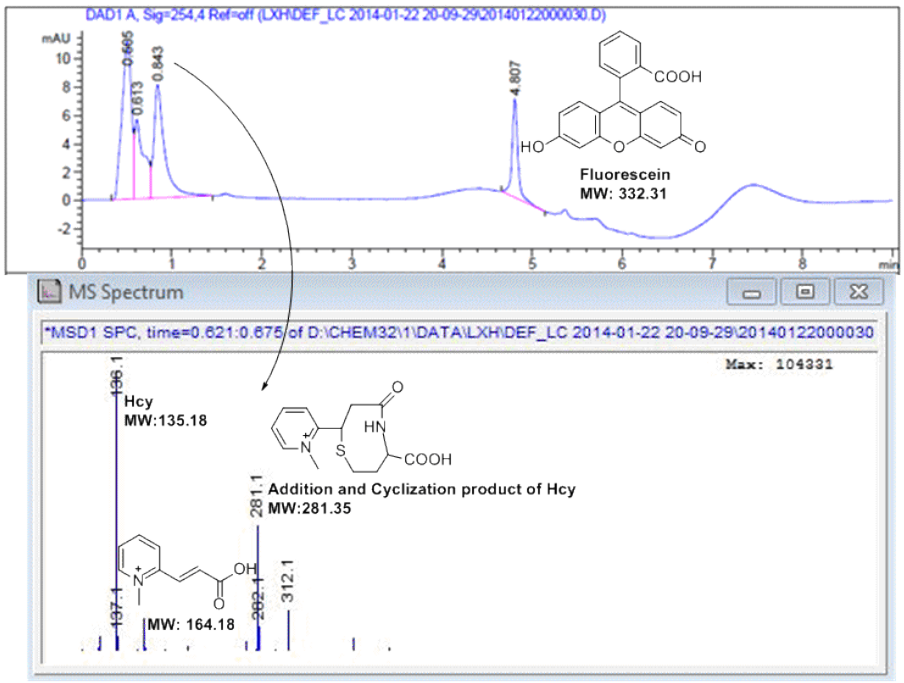
b)



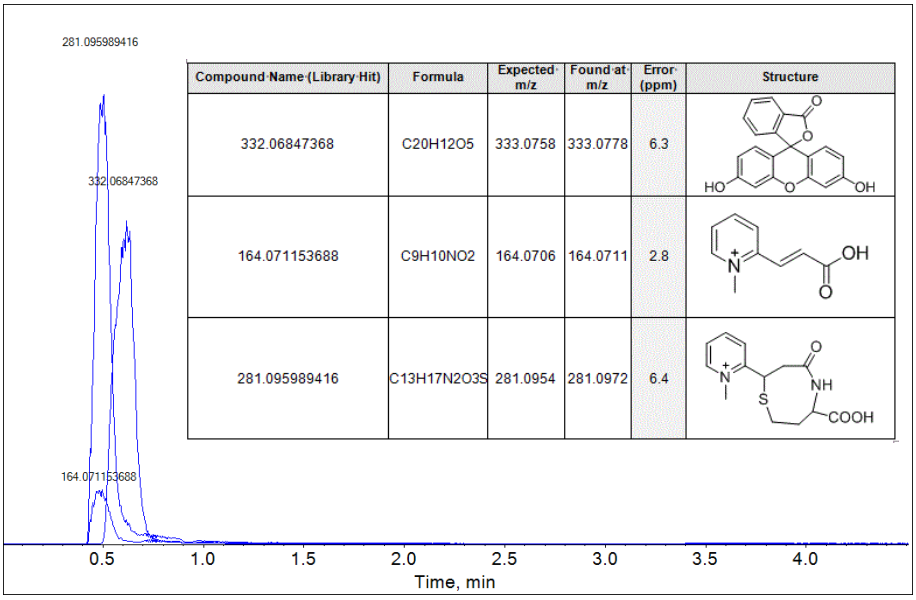
Compound Name (Library Hit)	Formula	Expected m/z	Found at m/z	Error (ppm)	Structure
332.06847368	C20H12O5	333.0758	333.0783	7.6	
164.071153688	C9H10NO2	164.0706	164.07106	2.1	
267.080339336	C12H15N2O3S	267.0798	267.0820	8.3	

Figure S17. HPLC-MS (a) and HRMS spectra (b) of Cys and probe **1** in physiological saline.

a)



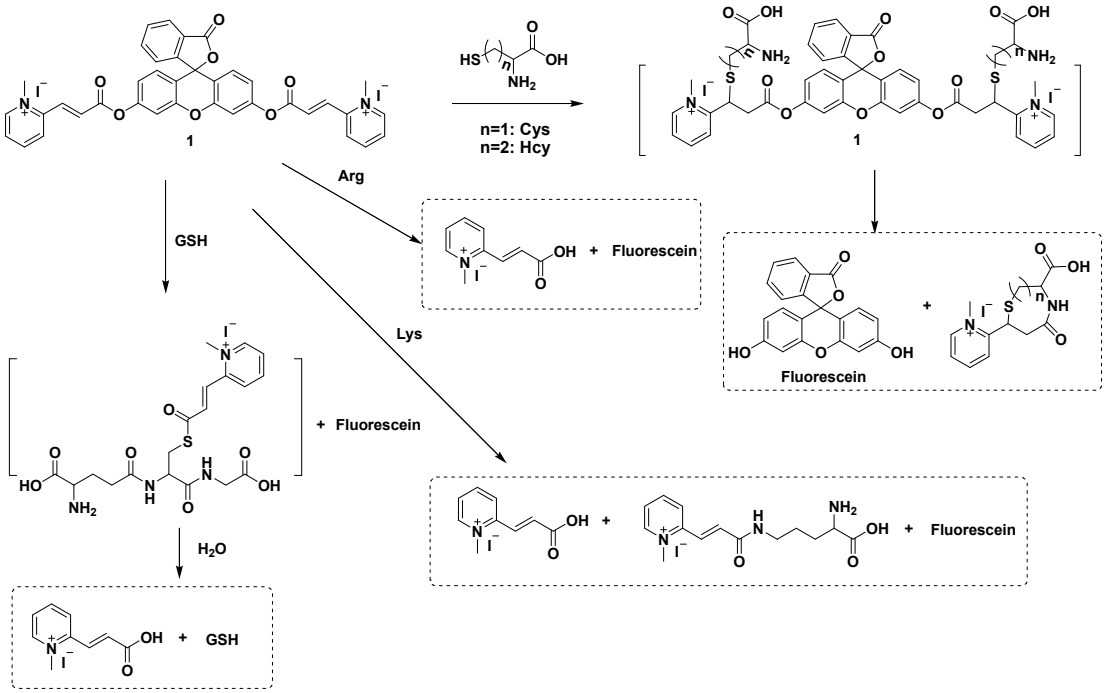
b)



Compound Name (Library Hit)	Formula	Expected m/z	Found at m/z	Error (ppm)	Structure
332.06847368	C20H12O5	333.0758	333.0778	6.3	
164.071153688	C9H10NO2	164.0706	164.0711	2.8	
281.095989416	C13H17N2O3S	281.0954	281.0972	6.4	

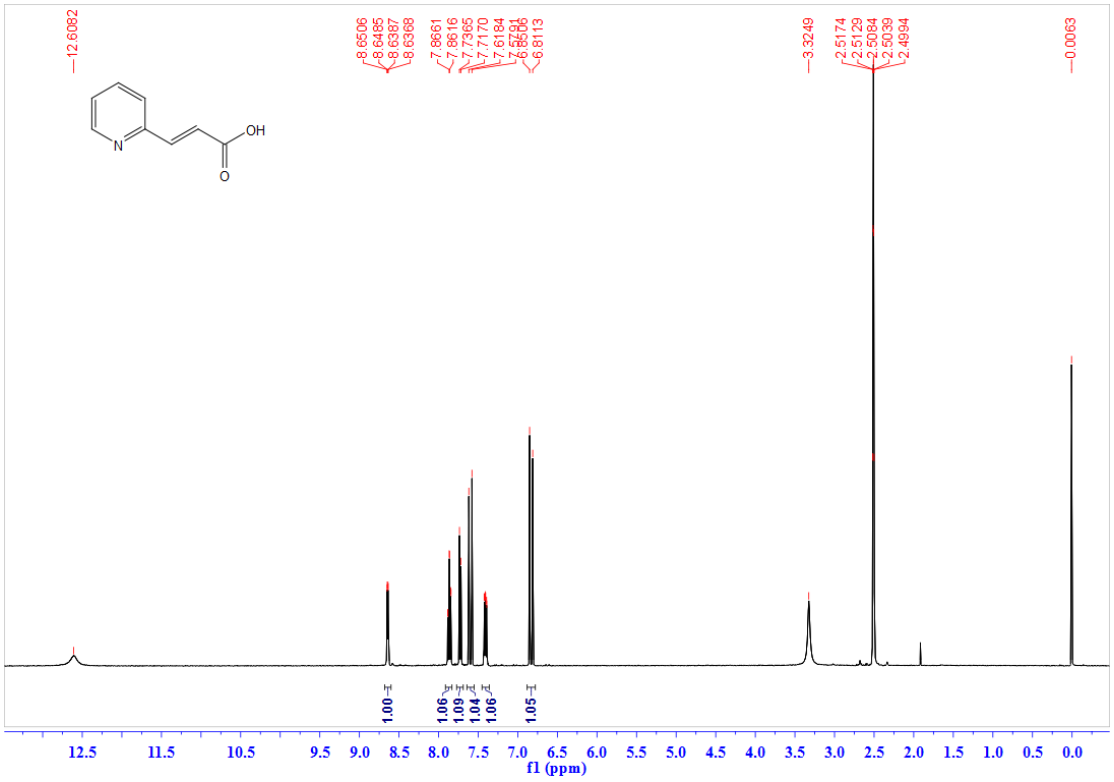
Figure S18. HPLC-MS (a) and HRMS (b) spectra of Hcy and probe **1** in physiological saline.

The proposed sensing mechanism of probe 1



Scheme S1. The proposed sensing mechanism of probe **1**.

Copies of NMR spectra



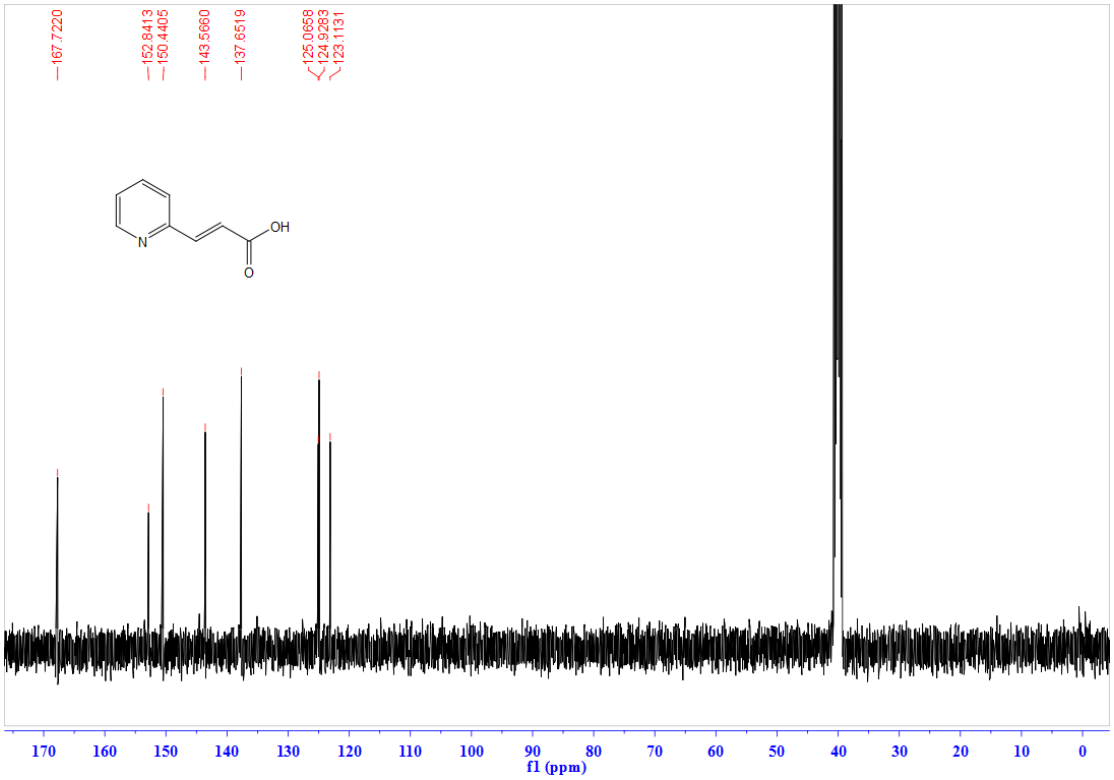


Figure S19. ^{1}H NMR and ^{13}C NMR spectra of compound **3**.

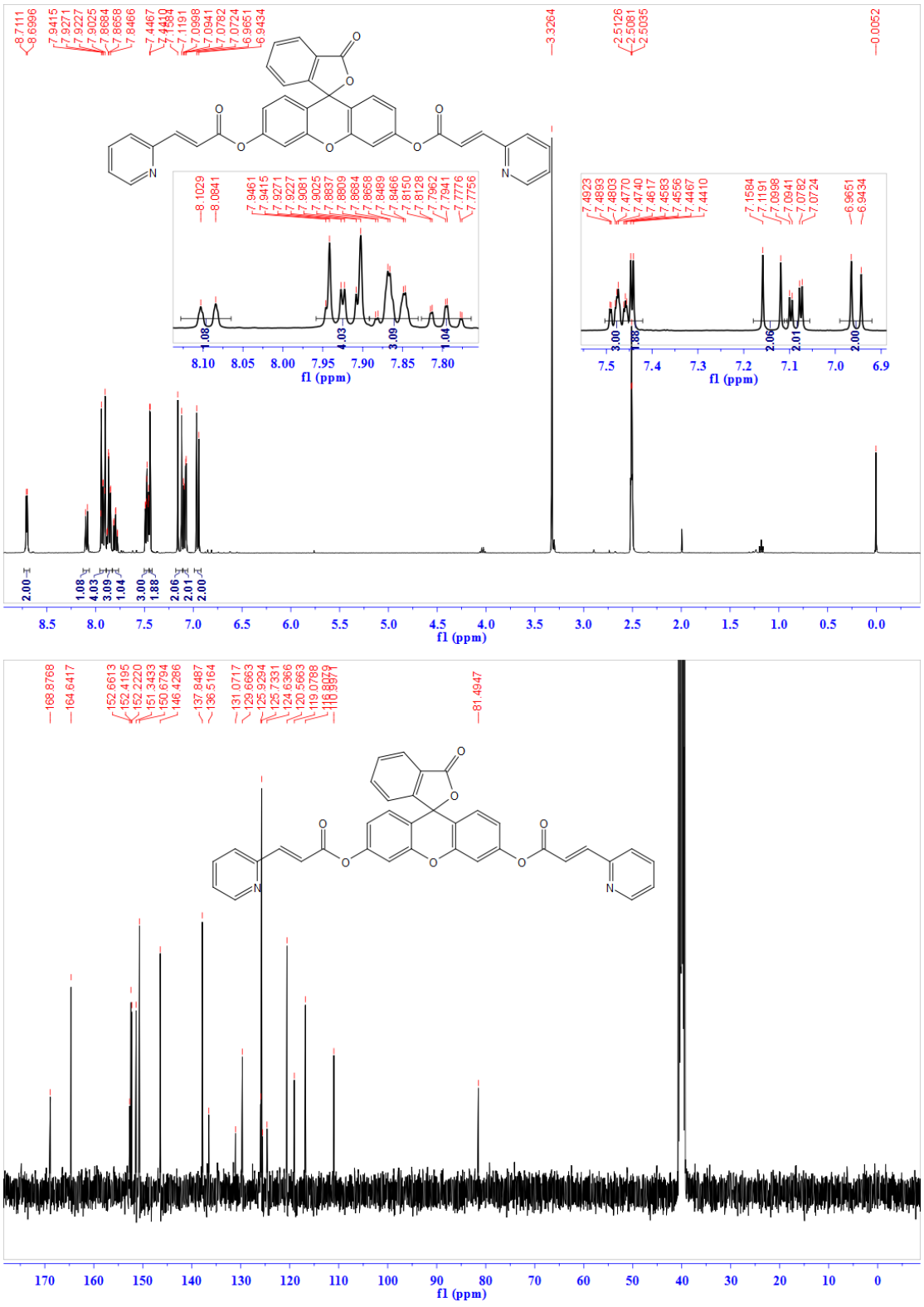
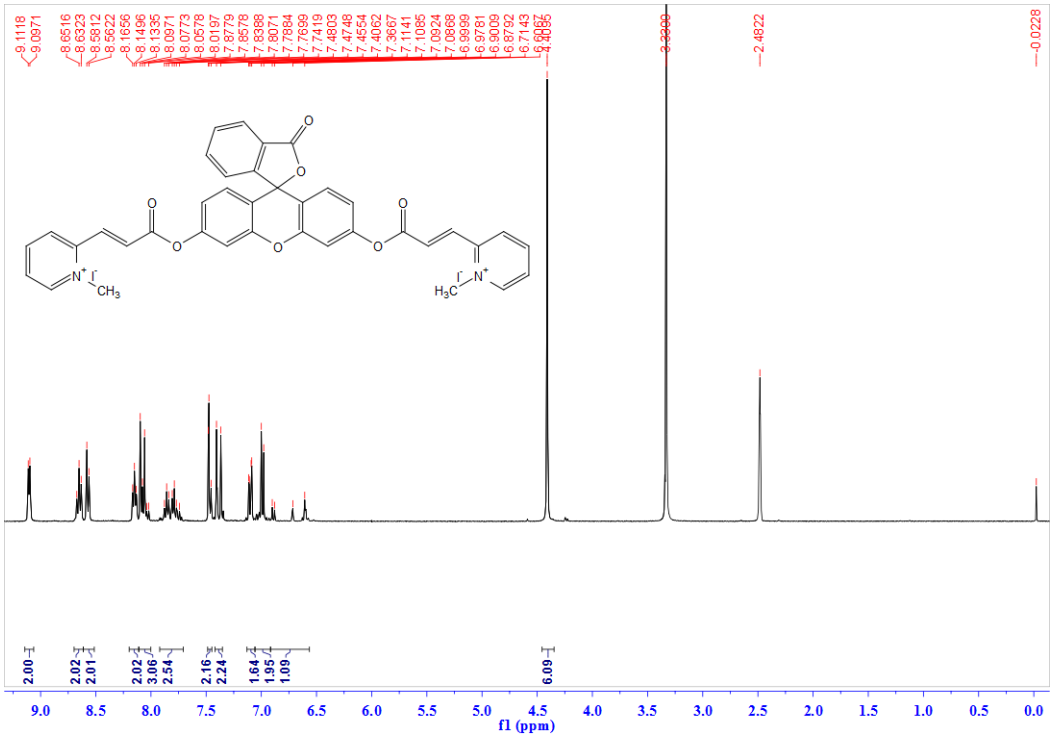


Figure S20. [1]HNMR and [13]C NMR spectra of compound **2**.



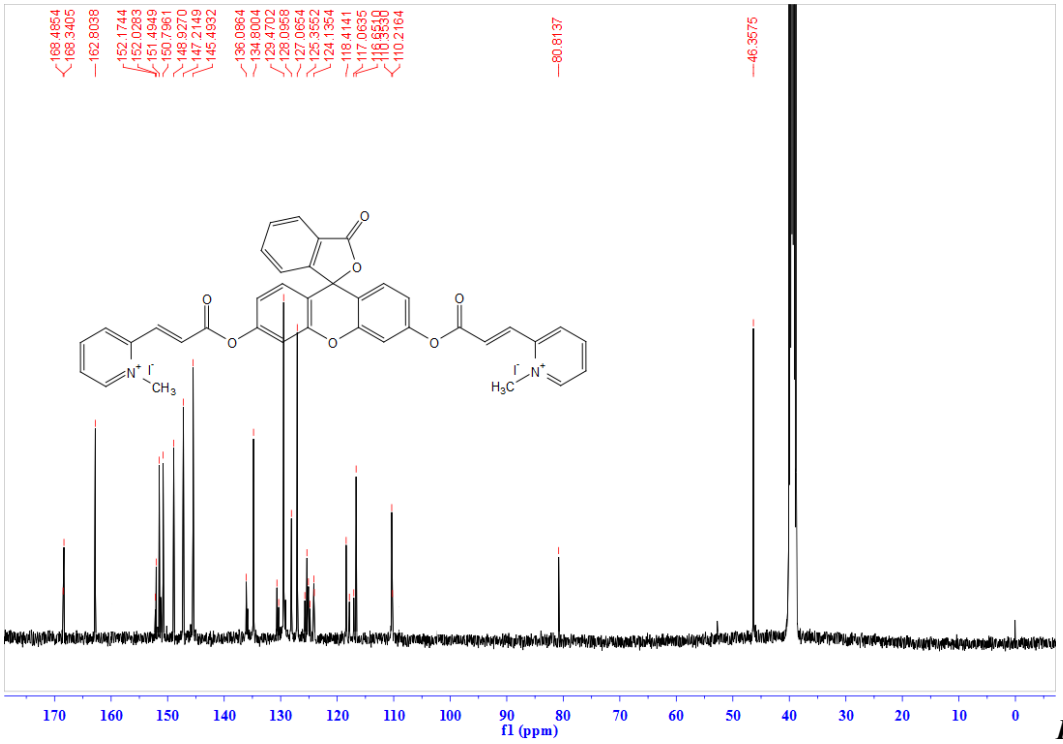


Figure S21. ^{1}H NMR and ^{13}C NMR spectra of compound **1**.

Reference

[1]. The characteristic peaks on cyclized lactam species can be found in: X. Yang, Y. Guo and R. M. Strongin, *Angew. Chem., Int. Ed.*, 2011, **50**, 10690.