Electronic Supplementary Information

A simple two-photon turn-on fluorescent probe for selective detection of cysteine based on dual PeT/ICT mechanism

Xiani Chen,[#,a] Hang Xu,[#,a] Shengnan Ma,[a] Hongjuan Tong,[a,b] Kaiyan Lou,*[a] and Wei Wang*[a,c]

[a] Shanghai Key Laboratory of New Drug Design, Shanghai Key Laboratory of Chemical Biology, School of Pharmacy, and State Key Laboratory of Bioengineering Reactor, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China

[b] School of Pharmacy, Shaanxi Institute of International Trade & Commerce, Xi'an 712046, China

[c] Department of Chemistry and Chemical Biology, University of New Mexico, Albuquerque, NM 87131-0001, USA

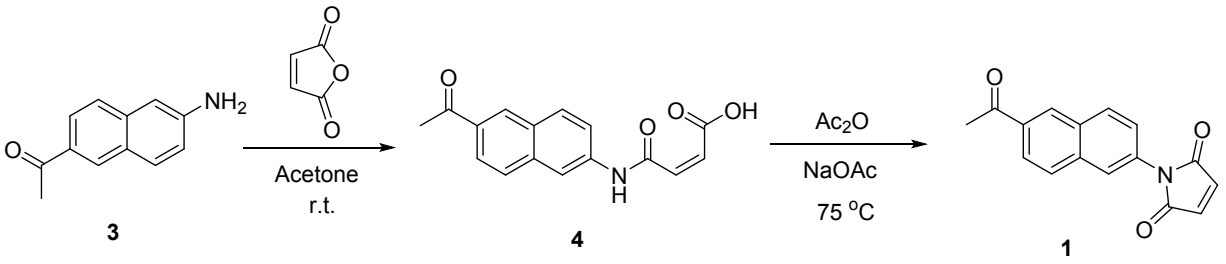
Table of Contents

General Information

Commercial reagents were purchased from commercial suppliers and used as received, unless otherwise stated.

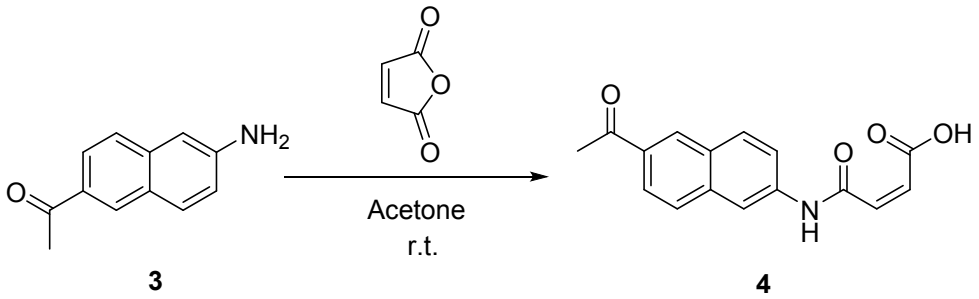
^{1}H and ^{13}C NMR spectra were recorded on Bruke DRX 400 (400 MHz). Data for ^{1}H are reported as follows: chemical shift (ppm), and multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet). Data for ^{13}C NMR are reported as ppm. Mass Spectra were obtained from East China University of Science and Technology LC-Mass spectral facility. HPLC-MS was performed using Waters e2695 using gradient elution of MeCN and H_2O with a flow rate 0.8 mL/min. UV-Vis spectra were collected on a Shimadzu UV-1800 spectrophotometer. Fluorescence spectra were collected on a FluoroMax-4 (Horiba Scientific) fluorescence spectrophotometer with slit widths were set at 2 nm both for excitation and emission. The pH measurements were carried out with a FE20 plus (Mettler Toledo) pH meter.

Part II Synthetic Procedures and Structural Determinations:



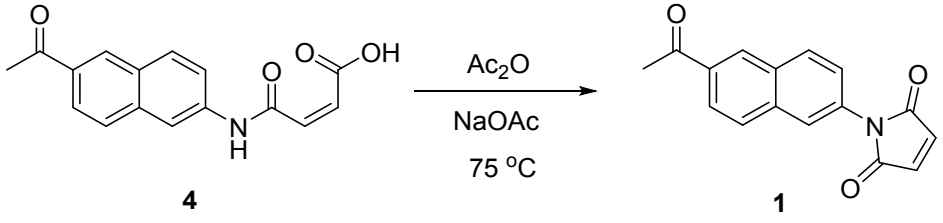
Scheme S1 Synthesis of the probe **1**

(*Z*)-4-((6-acetylnaphthalen-2-yl)amino)-4-oxobut-2-enoic acid (4)



To a solution of the amine **3** (200 mg, 1.08 mmol) in 5 mL acetone, was added maleic anhydride (127.1 mg, 1.30 mmol). The reaction mixture was stirred at room temperature for 7 h. The precipitate was isolated by filtration and washed with toluene to give the title compound **4** (171 mg, 56% yield) as pale green solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.98 (bs, 1H), 10.71 (s, 1H), 8.59 (s, 1H), 8.44 (s, 1H), 8.10 (d, J = 8.9 Hz, 1H), 8.00~7.88 (m, 2H), 7.69 (dd, J = 8.9, 1.9 Hz, 1H), 6.53 (d, J = 12.0 Hz, 1H), 6.37 (d, J = 12.0 Hz, 1H), 2.68 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 197.6, 167.1, 163.7, 138.7, 135.8, 133.1, 131.5, 130.5, 130.5, 130.1, 129.0, 127.8, 124.2, 120.7, 115.2, 16.7; EI-HRMS: m/z $M^{\cdot+}$ calcd for $C_{16}H_{13}NO_4$: 283.0845, found: 283.0844.

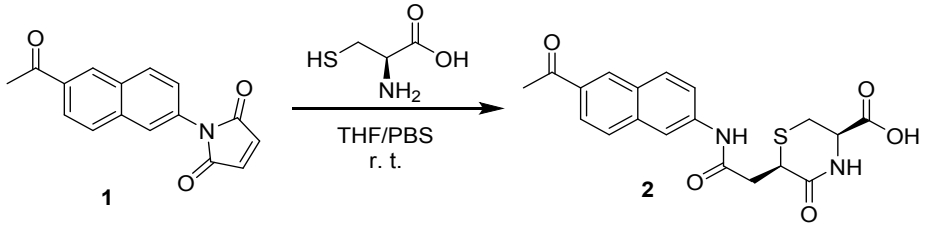
***N*-(6-acetylnaphthalen-2-yl)-maleimide (1)**



To a suspension of compound **4** (170 mg, 0.60 mmol) in 5 mL acetic anhydride 5 mL was added sodium acetate (74 mg, 0.90 mmol). The reaction mixture was stirred at 75 °C for 4 h, then concentrated under vacuum to remove acetic anhydride. The residue was washed with saturated sodium bicarbonate solution, extracted with methylene chloride. The organic layer was then washed with brine, dried over anhydrous Na_2SO_4, concentrated. The crude product was purified with flash column chromatography (silica gel) eluted with EtOAc/hexanes (1:2, V/V) to give the title probe **1** (106 mg, 67% yield) as

yellow-green solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.48 (s, 1H), 8.07 (dd, *J* = 8.6, 1.7 Hz, 1H), 8.06 (d, *J* = 8.8 Hz, 1H), 7.92 (d, *J* = 8.6 Hz, 1H), 7.91 (s, 1H), 7.58 (dd, *J* = 8.8, 2.1 Hz), 6.93 (s, 2H), 2.74 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.0, 169.5, 135.6, 135.2, 134.5, 131.5, 131.2, 130.8, 129.9, 128.8, 124.8, 124.5, 124.4, 26.9; ESI-HRMS: m/z $[M-H]^-$ calcd for $C_{16}H_{10}NO_3$: 264.0661, found: 264.0656.

(3*R*,6*R*)-6-(2-((6-acetylnaphthalen-2-yl)amino)-2-oxoethyl)-5-oxothiomorpholine-3-carboxylic acid (2)



Scheme S2 Synthesis of the product **2**

To a stirred solution of probe **1** (200 mg, 0.75 mmol) in 5 mL THF at room temperature, a solution of Cys (92 mg, 0.76 mmol) in 50 mL PBS was added dropwise. The reaction mixture was stirred at room temperature for 30 min. The white precipitate formed was filtered and dried to obtain the title product **2** (186 mg, 64% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.83 (s, 1H), 8.57 (s, 1H), 8.43 (s, 1H), 8.06 (d, *J* = 8.9 Hz, 1H), 7.96-7.83 (m, 3H), 7.75 (d, *J* = 8.0 Hz, 1H), 4.25 (dd, *J* = 5.4, 4.4 Hz, 1H), 3.77 (dd, *J* = 9.0, 4.9 Hz, 1H), 3.22 (dd, *J* = 13.2, 4.4 Hz, 1H), 3.02 (dd, *J* = 15.5, 4.9 Hz, 1H), 2.97 (dd, *J* = 13.2, 5.4 Hz, 1H), 2.90 (dd, *J* = 15.5, 9.0 Hz, 1H), 2.66 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 197.7, 171.8, 169.2, 168.1, 139.3, 135.9, 132.8, 130.4, 130.3, 128.7, 127.7, 124.1, 120.7, 114.6, 56.3, 39.8, 37.4, 26.7, 26.6; ESI-HRMS: m/z $[M+H]^+$ calcd. for $C_{19}H_{19}N_2O_5S$: 387.1015, found: 387.1016.

Part III: Fluorescence Response Studies

3.1 Spectroscopic materials

All aqueous solutions were prepared using double distilled water. Enantiomerically pure natural amino acids were used in all experiments, except for Hcy. All solutions of the biological analytes were freshly prepared (100 μM in distilled water, unless otherwise stated) and used within 12 hours. Probe **1** stock solution (1 mM in dry DMSO) was prepared and stored at -20 °C. All fluorescence and absorption spectroscopic measurements were performed in 10 mM phosphate buffer pH = 7.4 at 25 °C unless otherwise stated. Samples for absorption and fluorescence measurements were contained in 1 cm×1 cm quartz cuvettes (3.5 mL volume).

3.2 Fluorescence spectra of probe 1 upon addition of 1 equiv. Cys

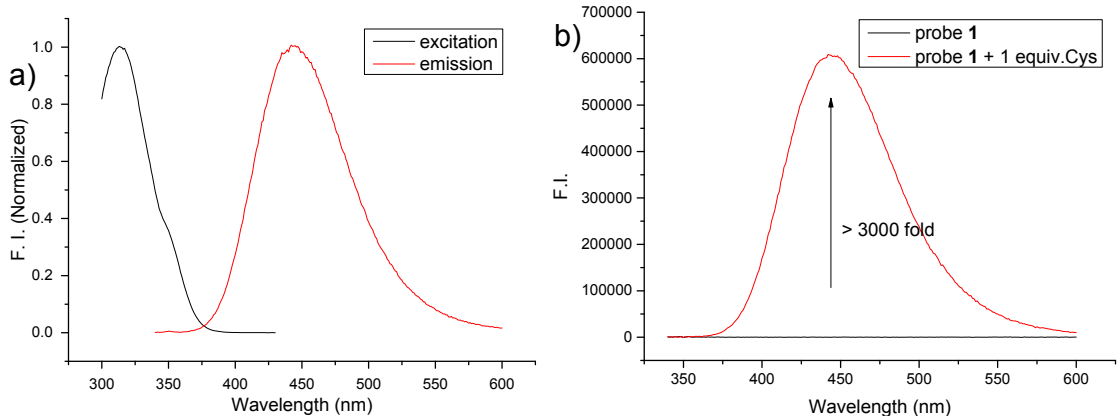


Figure S1 a) Normalized fluorescence excitation (λ_{em} = 446 nm) and emission (λ_{ex} = 314 nm) spectra of probe **1** (10 μM) after reaction with 1 equiv. of Cys; b) Fluorescence turn-on response (λ_{ex} = 314) of probe **1** (2 μM) before and after incubation with 1 equiv. of Cys. (All measurements were taken in 10 mM PBS buffer, pH=7.4 at 25 °C and incubation time = 30 min).

3.2 Time-dependent fluorescence intensity change (446 nm) of probe 1 upon addition of 1 equiv. Cys and reaction kinetic analysis.

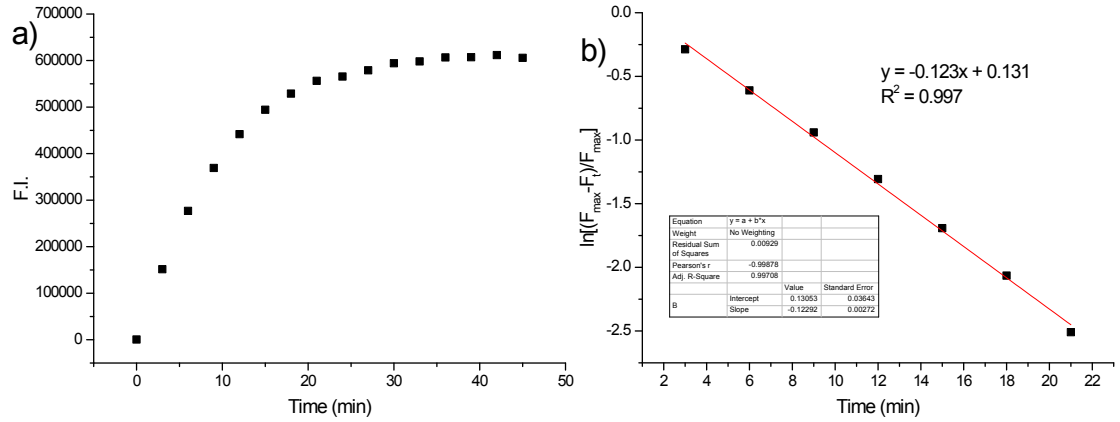


Figure S2 a) Time-dependent fluorescence intensity (λ_{ex}/ λ_{em} = 314/446 nm) of probe **1** (2 μM) upon

addition of 1 equiv. Cys in PBS buffer (10 mM, pH 7.4 and at 25 °C); b) The corresponding linear regression analysis of $Ln[(F_{max}-F_t)/F_{max}]$ versus t (0-21 min) of the time-dependent fluorescence intensity at 446 nm of probe **1** (2 μM) in PBS buffer at pH 7.4 with 1 equiv. Cys. The *pseudo*-first-order reaction constant was calculated as 0.213 min^{-1} (half-time = 3.25 min).

3.3 Concentration-dependent fluorescence emission spectra of probe 1 towards Cys.

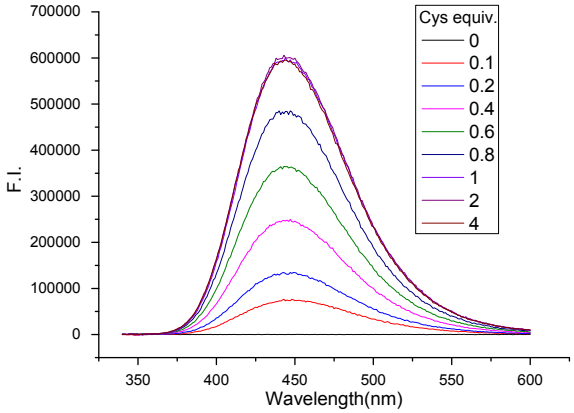


Figure S3 Concentration-dependent fluorescence spectra of probe **1** (2 μM) upon addition of various amount (0 to 4 equiv.) of Cys. (All measurements were taken in 10 mM PBS buffer, pH=7.4 at 25 °C with λ_{ex}=314 nm and incubation time = 30 min).

3.4 Job's Plot

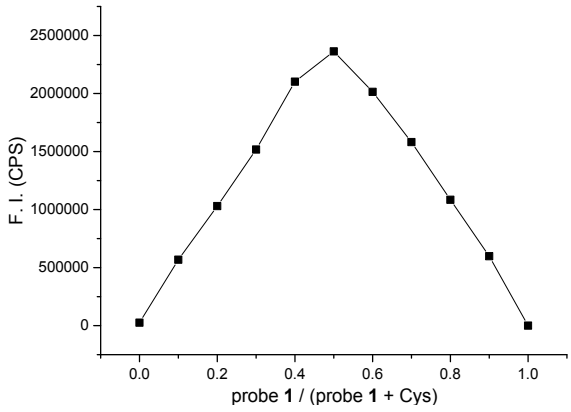


Figure S4 Fluorescence intensity (F. I.) at 446 nm versus the mole ratio of (probe **1**)/(probe **1** + Cys) when a total concentration of probe **1** and Cys was 10 μM. (All measurements were taken in 10 mM PBS buffer, pH=7.4 at 25 °C with λ_{ex}=314 nm and incubation time = 30 min).

3.4 Determination of detection limit of probe 1 towards Cys

The detection limit of probe **1** (2 μM) towards Cys was derived from the fluorescence titration experiment (446 nm, excited at 314 nm) with increasing amount of Cys (0.2-2.0 μM) as show in **Figure 3d**. An excellent linear relationship (R^2 = 0.999) was obtained. And then the fluorescence intensity data of free probe **1** (2 μM) at 446 nm were collected ten times as the blank measurements for calculation of the standard deviation. The detection limit was calculated using the following the

equation: detection limit = 3σbi/m, where σbi is the standard deviation of the blank measurements (σbi = 132.89); m is the slope obtained from linear regression of fluorescence intensity at 476 nm versus Cys concentration. The detection limit was calculated to be 1.4 nM at S/N = 3.

3.5 Selectivity studies of probe 1 for detection of Cys over other species

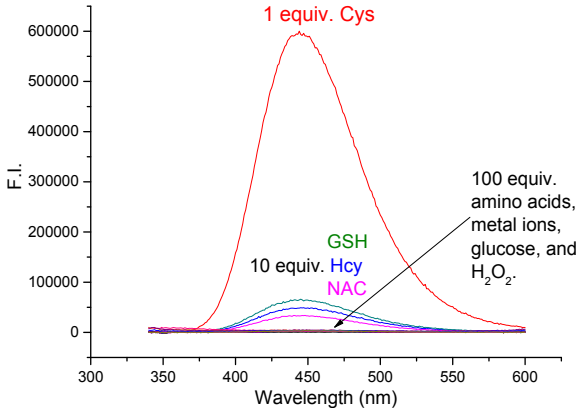
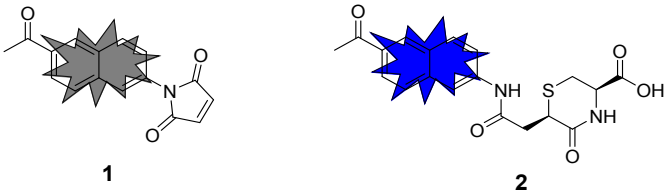


Figure S5 Fluorescence spectra of probe **1** (2 μM) upon addition of various species: 1 equiv. of Cys, 10 equiv. of other thiols (Hcy, GSH, NAC), 100 equiv. of different amino acids (Val, Gly, Ile, Lys, Leu, His, Asn, Met, Pro, Ser, Ala, Thr, Arg, Gln, Asp, Glu, Tyr, Trp, Phe), 100 equiv. of glucose, 100 equiv. of H_2O_2, and 100 equiv. of common metal ions in biological systems (Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+}, Cu^{2+}, Zn^{2+}). (All measurements were taken in 10 mM PBS buffer, pH=7.4 at 25 °C with λ_{ex}=314 nm).

3.6 Determination of fluorescence quantum yields of probe 1 and the product 2

The quantum yield of **1** and **2** were determined according to the literature.[1] Quinine sulfate with 0.1 M H_2SO_4 was chosen as standard (ϕ = 0.557, λ_{ex}= 320 nm). The quantum yields of **1** and **2** were measured in distilled water with 0.5% DMSO, λ_{ex}= 320 nm. Fluorescence quantum yields were obtained with the following equation: $\phi_s = \phi_b I_s A_b \eta_s / I_b A_s \eta_b$. Where ϕ is quantum yield; I is integrated area under the corrected emission spectra; A is absorbance at the excitation wavelength; η is the refractive index of the solution; the subscripts s and b refer to the sample and the standard, respectively. Generally, in diluted solution, $\eta_s \approx \eta_b$ then fluorescence quantum yields were obtained with the following equation $\phi_s = \phi_b I_s A_b / I_b A_s$.



1 ϕ = 0.002

2 ϕ = 0.782

Figure S6 Fluorescence quantum yields of probe 1 and the product 2.

3.7 pH dependence of fluorescence response of probe 1 for Cys

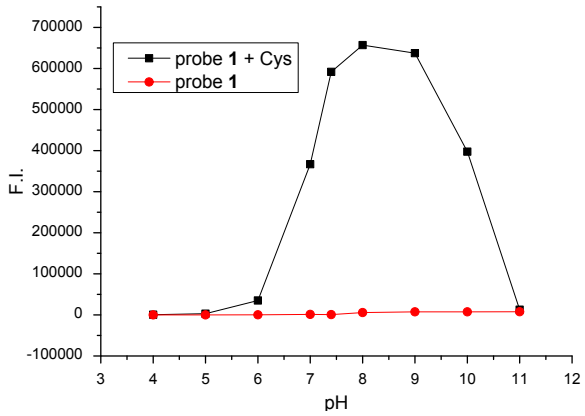
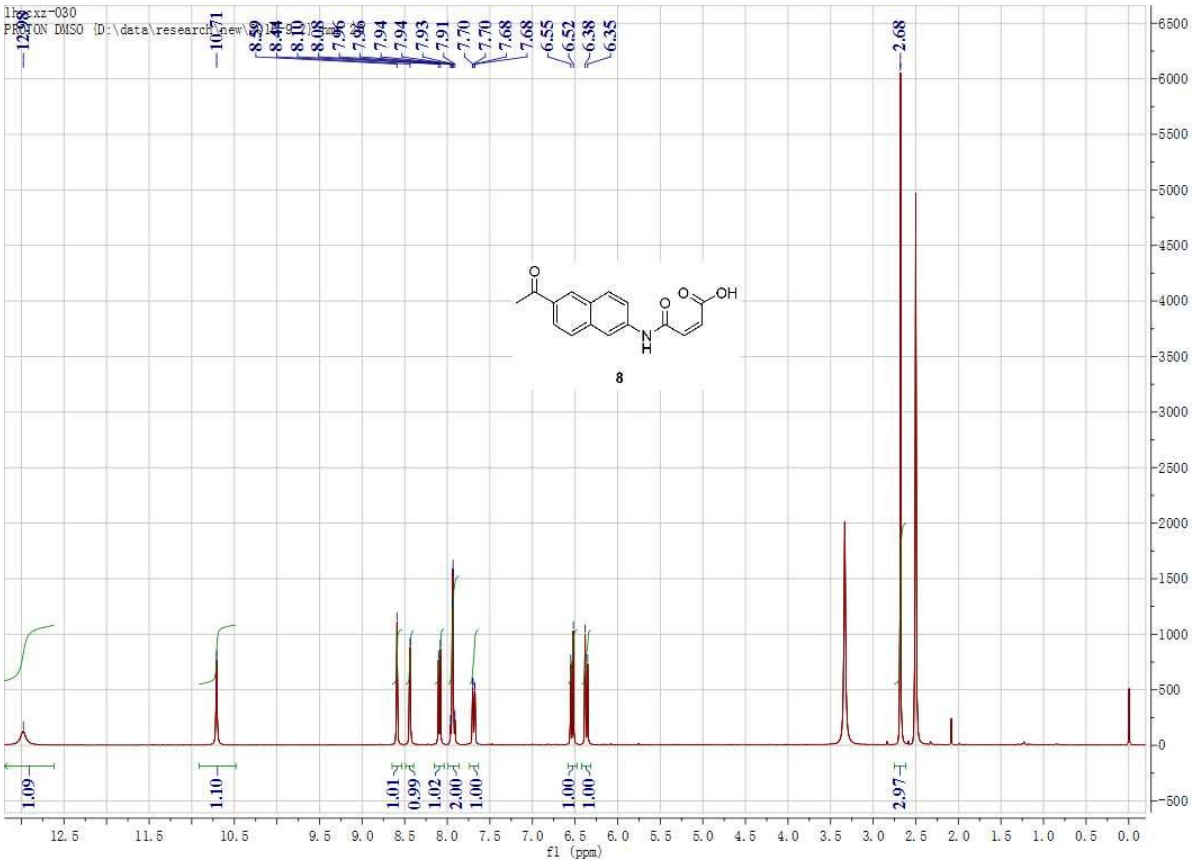


Figure S7 pH-dependent fluorescence intensity at 446 nm (λ_{ex} = 314 nm) for probe **1** (2 μM), and probe **1**(2 μM) after incubation with 500 μM of Cys for 30 min at various pHs from 4-11.

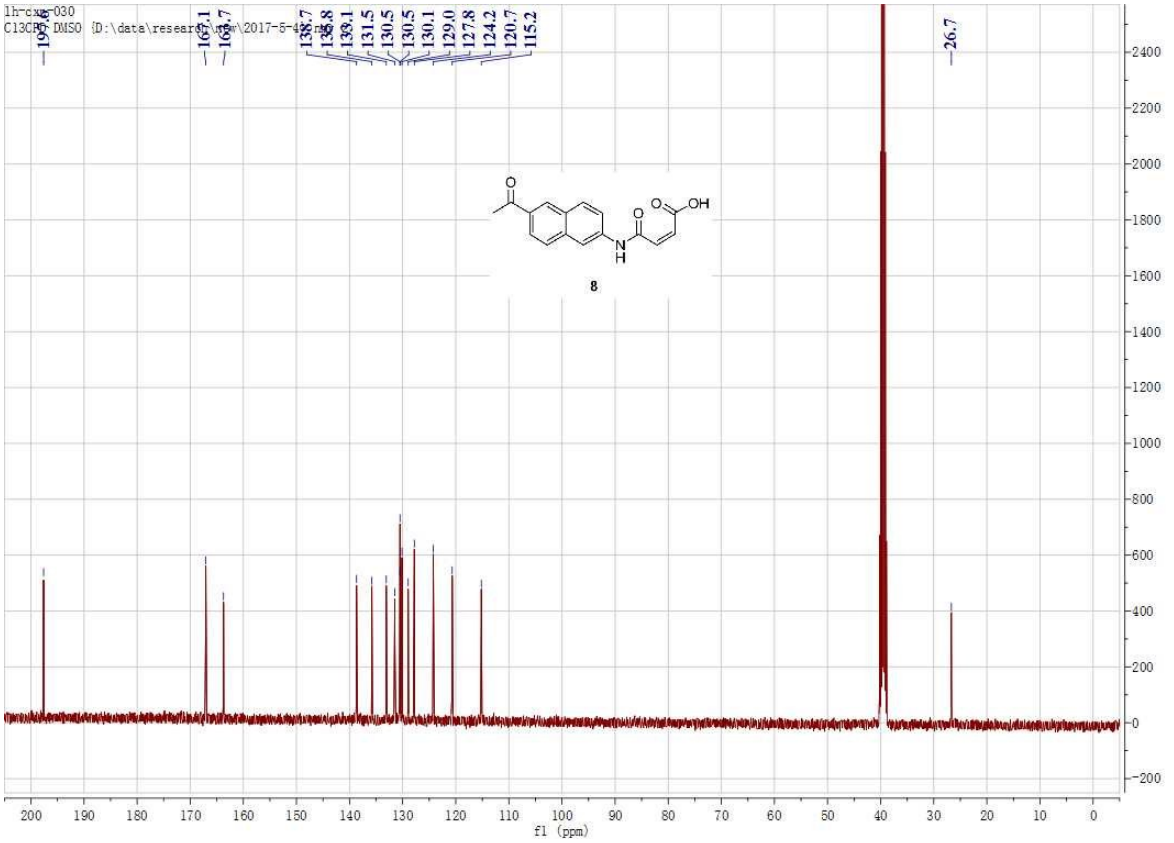
Part IV: Two-photon Imaging Studies

HeLa cells were seeded in a 6-well glass tissue culture dish at the cell culture facility of East China University of Science and Technology (ECUST) and cultured in DMEM medium (with phenol red, Gibco/Invitrogen) with 10% Fetal Bovine Serum and 1% Glutamine at 37 ˚C for 24 hours. Before incubation with probe **1** and other reagents, the upper medium in the culture dish was removed, and the cultured cells were washed with PBS buffer three times. The first group cells were preincubated with a solution of *N*-ethylmaleimide (0.5 mM in distilled water) for 30 min to reduce the concentration of all thiols including cysteine, then washed with PBS buffer three times before incubated with probe **1** in DMEM medium (10 μM, containing 1‰ DMSO, prepared from 10 mM stock solution in DMSO) at 37 ºC for 30 min. The second to fourth group cells were first pretreated with 0.5 mM *N*-ethylmaleimide for 30 min, then washed with PBS buffer three times and then treated with 1 mM Cys, Hcy and GSH for 30 min respectively, finally washed with PBS buffer three times again and incubated with probe **1** solution (10 μM) following the same procedure described above for 30 min. Two-photon fluorescence images was obtained using a Leica SP8 confocal microscope.

Part V: NMR and HRMS Data



8



8

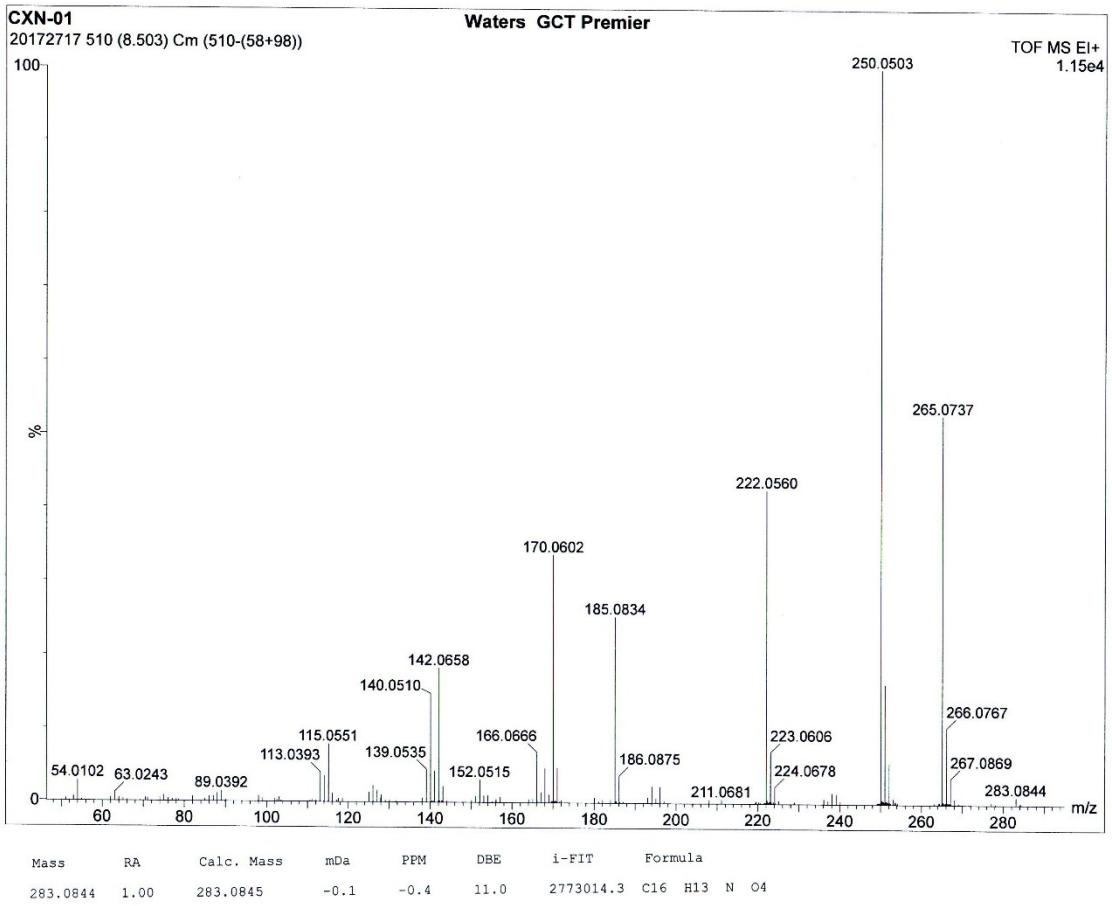
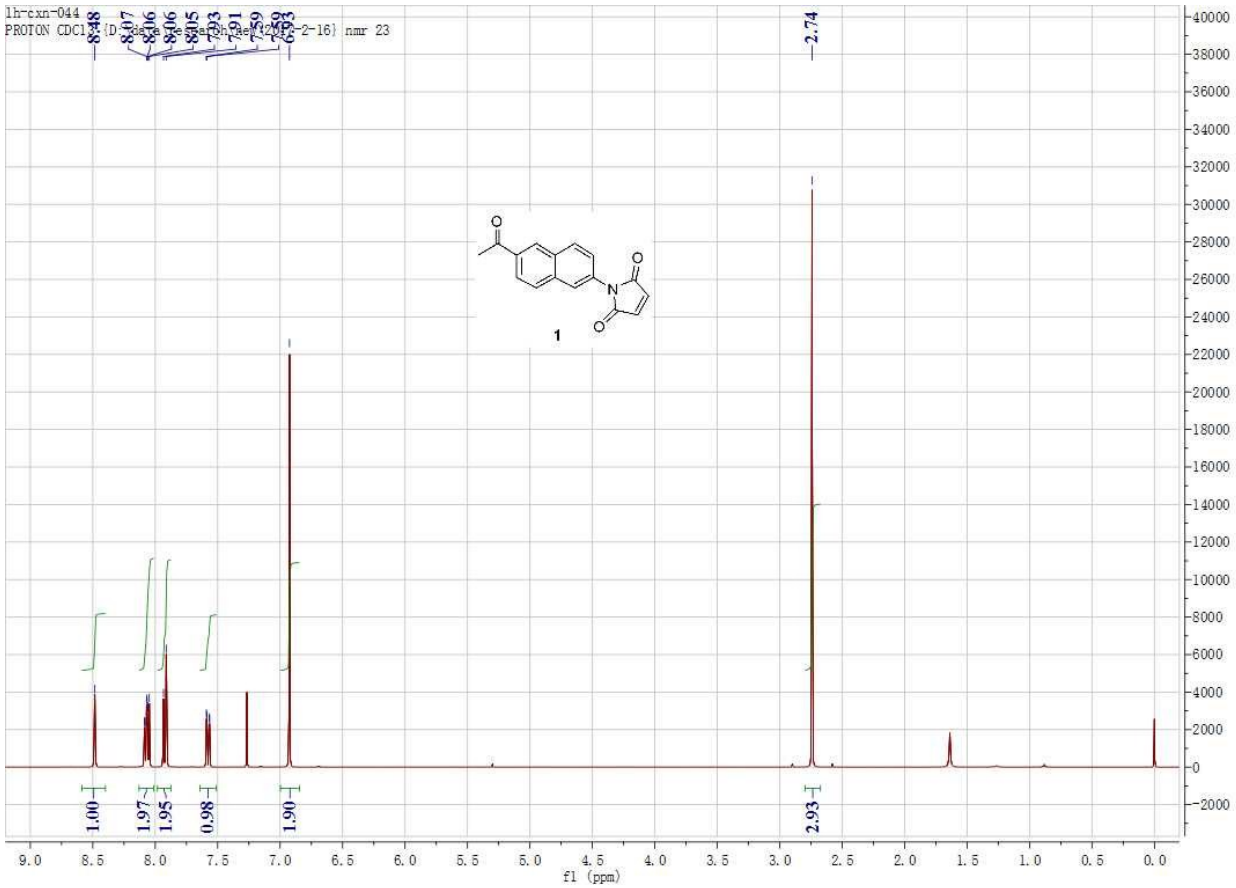
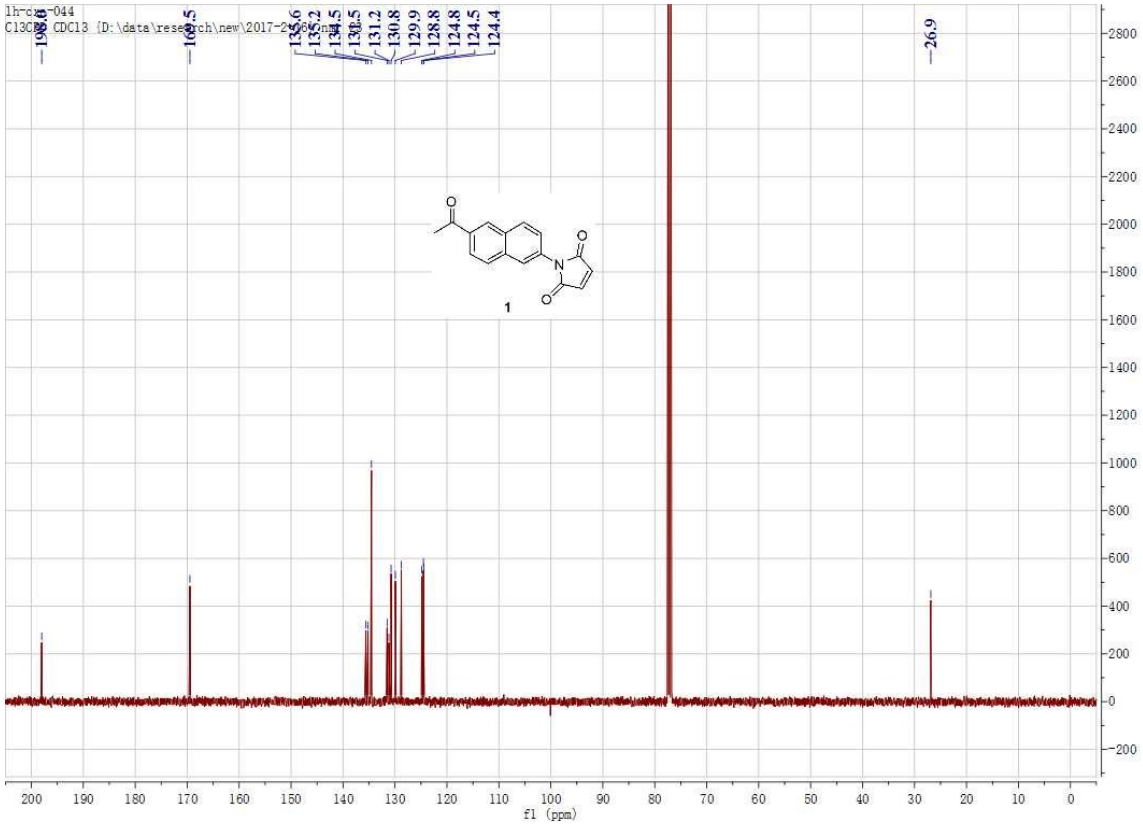


Figure S8 ^{1}H NMR, ^{13}C NMR and HRMS of the intermediate **4**.

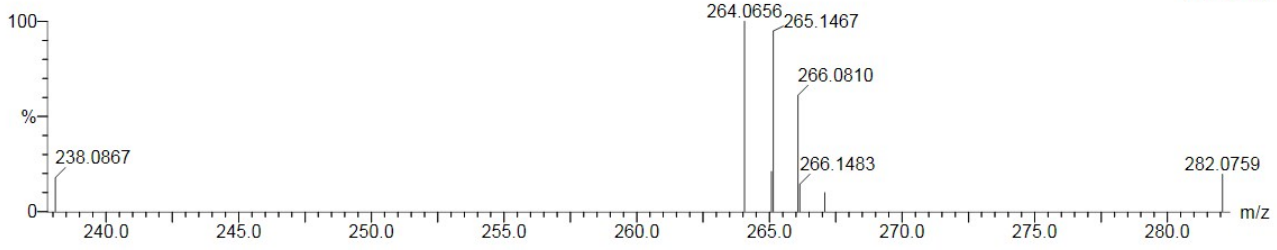




Single Mass Analysis
Tolerance = 50.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

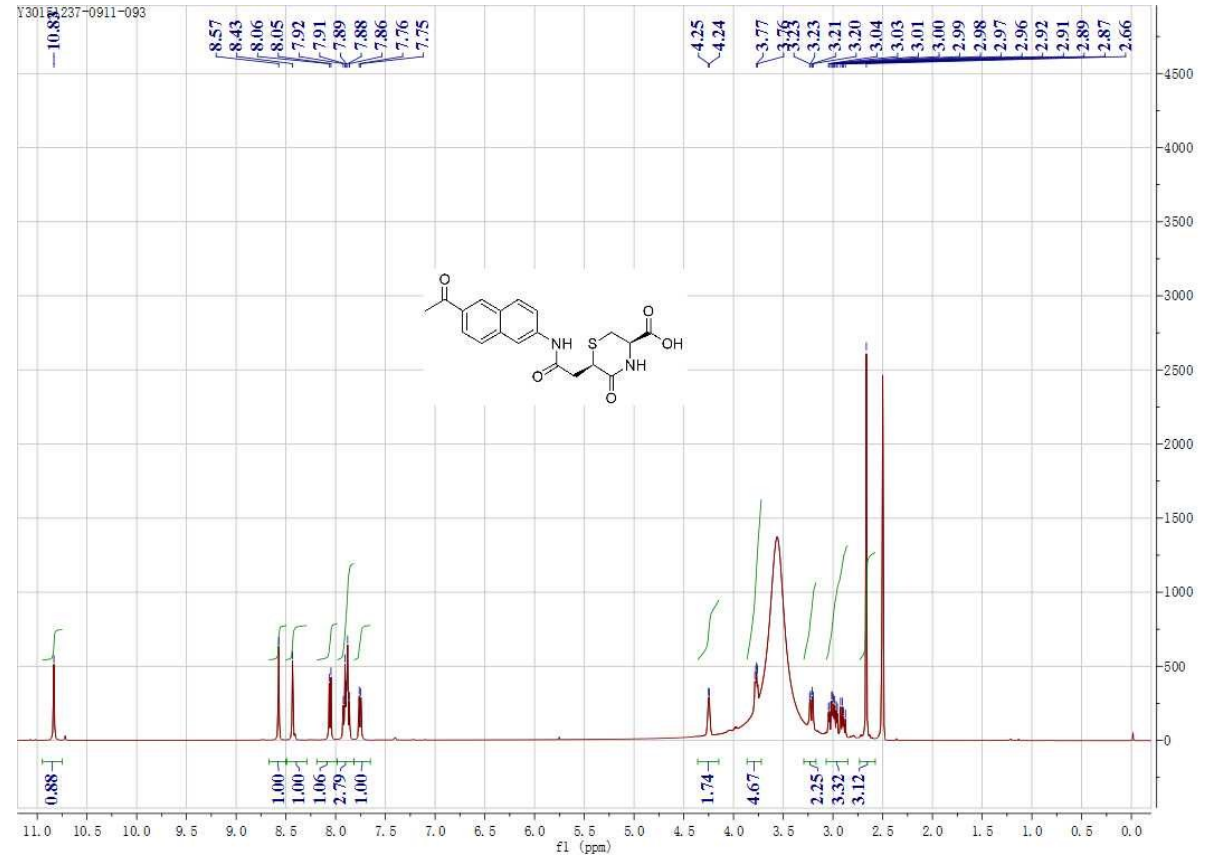
Monoisotopic Mass, Even Electron Ions
4 formula(e) evaluated with 1 results within limits (up to 1 best isotopic matches for each mass)
Elements Used:
C: 0-16 H: 0-10 N: 0-1 O: 0-3

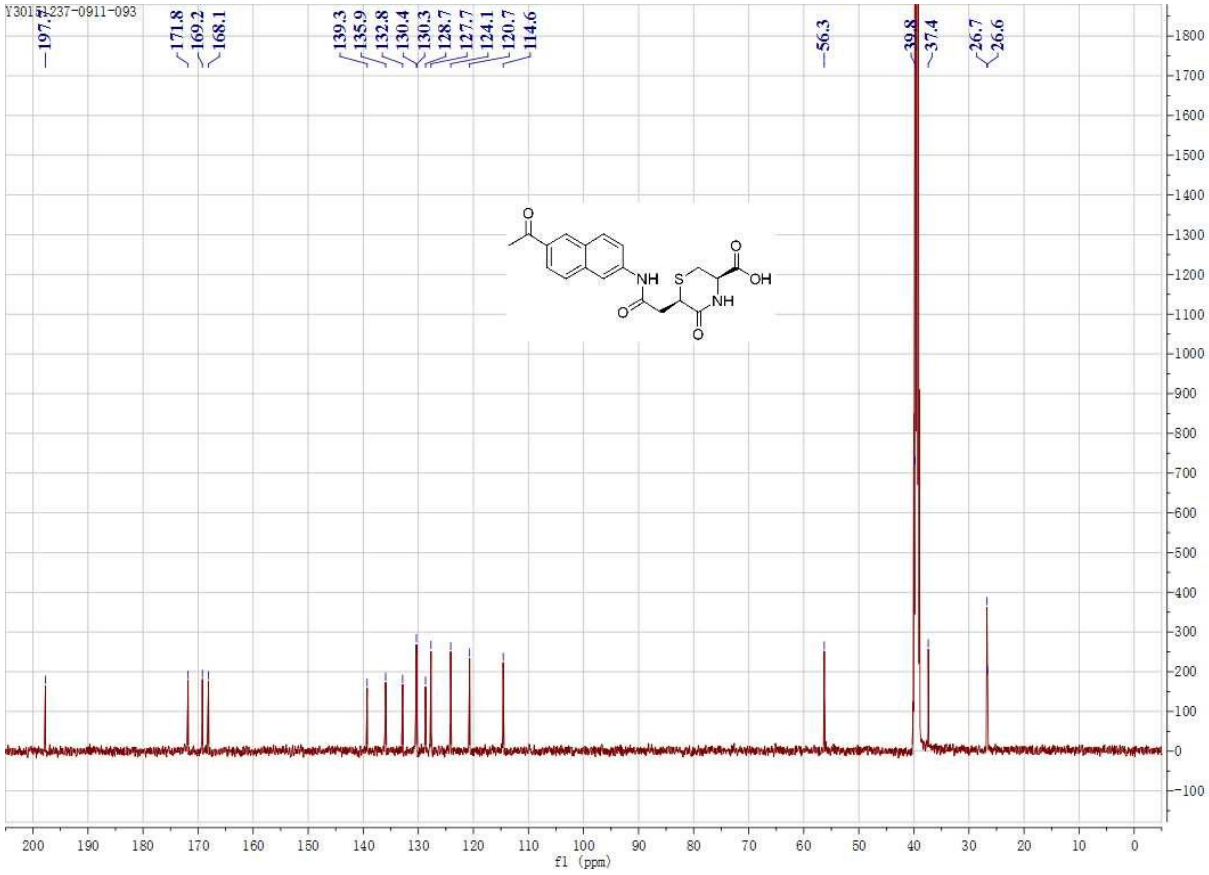
H-LI ECUST institute of Fine Chem 22-Mar-2017 21:18:57
LH-CXN-044 13 (0.346) Cm (11:13) 1: TOF MS ES- 2.89e+003

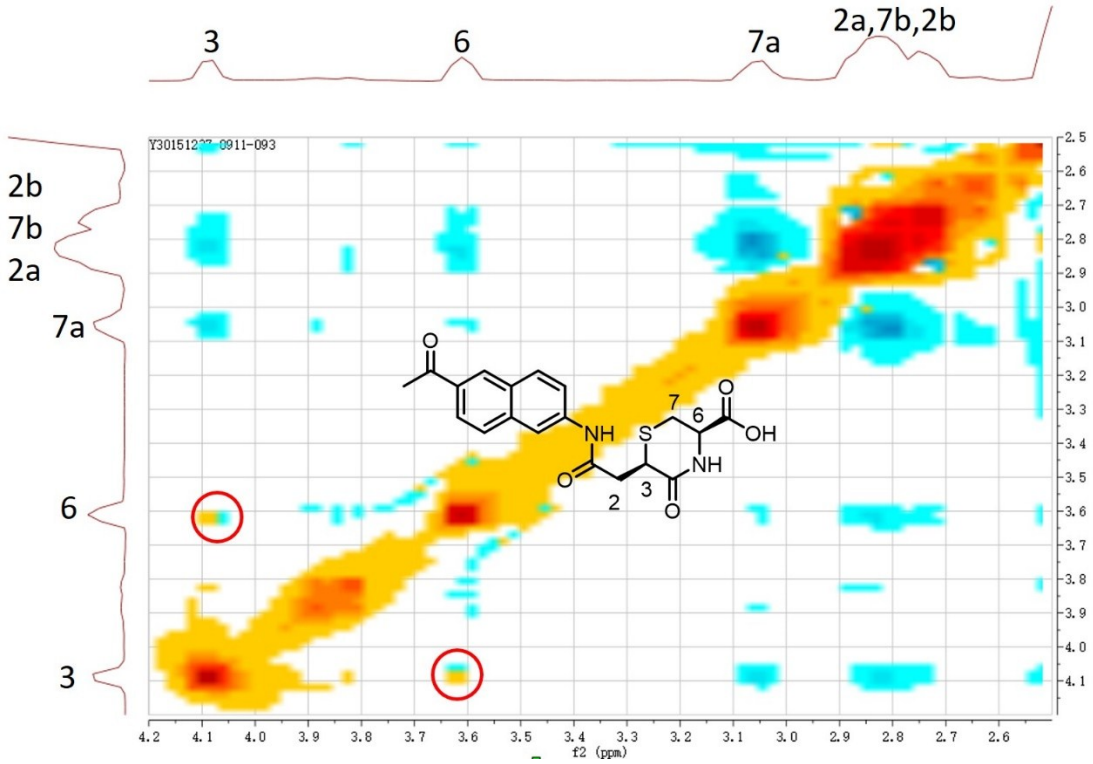


Minimum:				-1.5			
Maximum:		30.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
264.0656	264.0661	-0.5	-1.9	12.5	55.2	0.0	C16 H10 N O3

Figure S9 ^{1}H NMR, ^{13}C NMR, and HRMS of probe **1**.


Y3011237-0911-093
10.83
8.57
8.43
8.06
8.05
7.92
7.91
7.89
7.88
7.86
7.76
7.75
4.25
4.24
3.77
3.76
3.23
3.21
3.20
3.04
3.03
3.01
3.00
2.99
2.98
2.97
2.96
2.92
2.91
2.89
2.87
2.66
0.88
1.00
1.00
1.06
2.79
1.00
1.74
4.67
2.25
3.32
3.12
f1 (ppm)

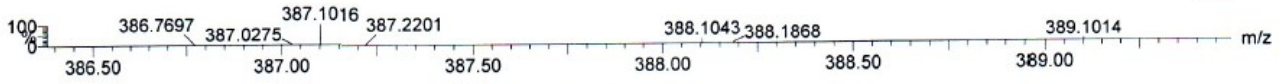

Y3011237-0911-093
197.7
171.8
169.2
168.1
139.3
135.9
132.8
130.4
130.3
128.7
127.7
124.1
120.7
114.6
56.3
39.8
37.4
26.7
26.6
f1 (ppm)



Elemental Composition Report Page 1

Multiple Mass Analysis: 2 mass(es) processed
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
12696 formula(e) evaluated with 14 results within limits (all results (up to 1000) for each mass)
Elements Used:
12C: 0-50 13C: 0-1 H: 0-50 N: 0-6 O: 0-10 S: 0-1
Y30151237-02
20180047 78 (0.784) Cm (78-(518+527))
XEVO-G2TOF#NotSet
08-Jan-201810:40:53
1: TOF MS ES+
1.49e+005



Minimum:	20.00				-1.5				
Maximum:	100.00		5.0	5.0	50.0				
Mass	RA	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
387.1016	100.00	387.1015	0.1	0.3	3.5	66.1	1.075	34.13	12C9 13C H20 N5 O9 S

Figure S10 ^{1}H NMR, ^{13}C NMR, 2D-ROESY, and HRMS of probe **1**. (The cross-peaks between protons 3-H and 6-H circled in red in the 2D-ROESY spectrum indicated that the two protons are on the same side of the six-membered thiomorpholinone ring[2])

References

1. L. Cui, Y. Zhong, W. Zhu, Y. Xu and X. Qian, Chem. Commun., 2010, 46, 7121.
2. X. Li, Y. Zheng, H. Tong, R. Qian, L. Zhou, G. Liu, Y. Tang, H. Li, K. Lou and W. Wang, Chem. Eur. J., 2016, 22, 9247.