Supporting Information

NBD-based fluorescent probes for separate detection of cysteine and biothiols via different reactivities

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[b] *Tianjin Key Laboratory on Technologies Enabling Development of Clinical Therapeutics and Diagnostics (Theranostics), School of Pharmacy, Tianjin Medical University, Tianjin 300070, China. E-mail: sunlu@tmu.edu.cn*
[‡] *These authors contributed equally to this work.*

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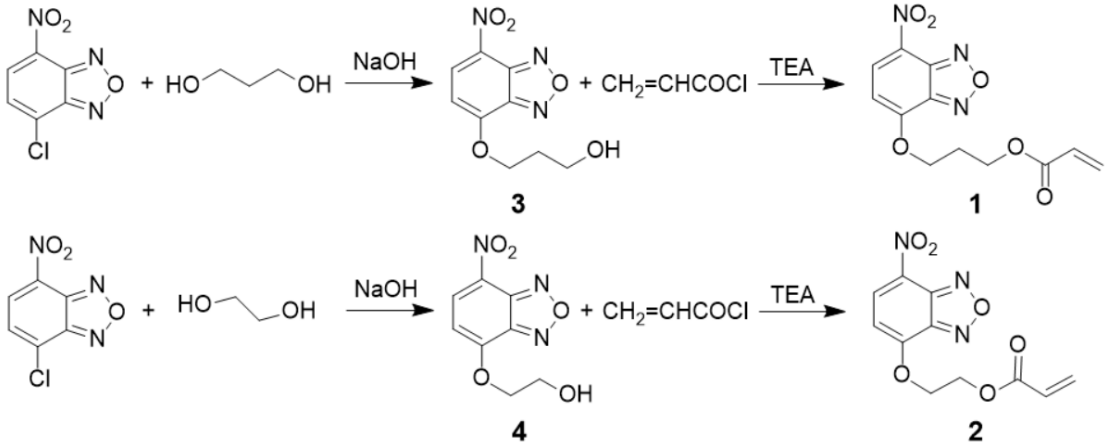
1. Reagents and instruments

All chemicals and solvents used for synthesis were purchased from commercial suppliers and applied directly in the experiment without further purification. The progress of the reaction was monitored by TLC on pre-coated silica plates (Merck 60F-254, 250 μm in thickness), and spots were visualized by UV light. Merck silica gel 60 (70-200 mesh) was used for general column chromatography purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm) or residual solvent peaks ($CDCl_3$ = 7.26 ppm, CD_3OD = 3.31 ppm). ^{1}H NMR coupling constants (*J*) are reported in Hertz (Hz), and multiplicity is indicated as the following: s (singlet), d (doublet), dd (doublet doublet), t (triplet), m (multiplet). High-resolution mass spectrum (HRMS) was obtained on an Agilent 6540 UHD Accurate-Mass Q-TOFLC/MS. The UV-visible spectra were recorded on a UV-6000 UV-VIS-NIR-spectrophotometer (METASH, China). Fluorescence studies were carried out using F-280 spectrophotometer (Tianjin Gangdong Sci & Tech., Development. Co., Ltd). ANGELA TECHNOLOGIES HPLC LC-10F was employed for HPLC study.

2. Synthesis of probes



Synthesis of 3.

Compound **3** was synthesized according to a known procedure.[1] NBD-Cl (200 mg, 1 mmol) was suspended in propylene glycol (2 mL) and treated with a solution of NaOH

(120 mg, 3 mmol) in propylene glycol (6 mL) at room temperature. The reaction mixture was stirred for 3 h and then acidified with 3 M HCl to adjust the pH to around 3 at 0 °C. The resultant aqueous layer was dissolved with EtOAc, which was washed sequentially with water and brine. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The resulting residue was purified by column chromatography on silica gel with CH_2Cl_2/MeOH = 100/1 to give a brownish yellow oil **3** (89 mg, 37%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (d, J = 8.4 Hz, 1H), 6.74 (d, J = 8.4 Hz, 1H), 4.55 (t, J = 6.2 Hz, 2H), 3.94 (t, J = 5.8 Hz, 2H), 2.27 - 2.20 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.8, 145.2, 143.9, 134.3, 129.5, 104.6, 68.1, 58.6, 31.2.

Synthesis of 1.

Compound **1** was synthesized according to a previous reported method with minor modifications.[2] Acryloyl chloride (23 μL, 0.28 mmol) was added to a solution of **3** (44 mg, 0.18 mmol) in anhydrous CH_2Cl_2. After that, TEA (52 μL, 0.37 mmol) was added drop-by-drop at 0 °C. After that, the mixture was stirred at room temperature overnight. The organic solution was evaporated under reduced pressure. The resulting residue was purified by column chromatography on silica gel with CH_2Cl_2 to give a yellow oil **1** (50 mg, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (d, J = 8.3 Hz, 1H), 6.72 (d, J = 8.4 Hz, 1H), 6.39 (dd, J = 17.3, 1.3 Hz, 1H), 6.10 (dd, J = 17.3, 10.4 Hz, 1H), 5.84 (dd, J = 10.4, 1.3 Hz, 1H), 4.51 (t, J = 6.2 Hz, 2H), 4.42 (t, J = 6.0 Hz, 2H), 2.42 - 2.34 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.0, 154.6, 145.2, 144.0, 134.1, 131.4, 129.8, 128.0, 104.7, 67.9, 60.6, 28.2. HRMS (ESI): m/z $[M-H]^-$ calculated for $C_{12}H_{10}N_3O_6^-$: 292.0575; found: 292.0605.

Synthesis of 4.

NBD-Cl (200 mg, 1 mmol) was suspended in ethylene glycol (2 mL) and treated with a solution of NaOH (120 mg, 3 mmol) in ethylene glycol (6 mL) at room temperature. The reaction mixture was stirred for 3 h and then acidified with 3 M HCl to adjust the pH to around 3 at 0 °C. The resultant aqueous layer was dissolved with EtOAc, which was washed sequentially with water and brine. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The resulting residue was purified by column chromatography on silica gel with CH_2Cl_2/MeOH = 100/5 to give a brownish

yellow oil **4** (130 mg, 58%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.65 (d, J = 8.4 Hz, 1H), 7.00 (d, J = 8.4 Hz, 1H), 4.53 (t, J = 4.4 Hz, 2H), 4.07 (t, J = 4.4 Hz, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 156.1, 146.8, 145.4, 136.1, 130.8, 106.5, 73.8, 60.9.

Synthesis of 2.

To a solution of **4** (54 mg, 0.24 mmol) in anhydrous CH_2Cl_2, acryloyl chloride (30 μL, 0.36 mmol) was added. Then, TEA (67 μL, 0.48 mmol) was added drop-by-drop at 0 °C. The mixture was stirred at room temperature overnight. The organic solution was evaporated under reduced pressure. The resulting residue was purified by column chromatography on silica gel with CH_2Cl_2 to give a yellow oil **2** (33 mg, 49%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (d, J = 8.3 Hz, 1H), 6.77 (d, J = 8.3 Hz, 1H), 6.44 (dd, J = 17.3, 1.3 Hz, 1H), 6.14 (dd, J = 17.3, 10.5 Hz, 1H), 5.88 (dd, J = 10.5, 1.3 Hz, 1H), 4.68 (bs, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.9, 154.3, 145.2, 144.0, 133.9, 132.2, 130.2, 127.6, 105.2, 68.9, 61.7. HRMS (ESI): m/z $[M-H]^-$ calculated for $C_{11}H_8N_3O_6^-$: 278.0419; found: 278.0423.

3. General procedure for spectroscopic studies

All measurements were performed in degassed phosphate buffer (PBS, 50 mM, pH 7.4, containing 2% DMSO). Compounds were dissolved into DMSO to prepare the stock solutions with a concentration of 1-10 mM. Various stock solutions (20 mM) of different analytes were prepared in PBS buffer. Appropriate amount of bio-relevant species was added to separate portions of the probe solution and mixed thoroughly. The reaction mixture was shaken uniformly before spectra were measured. All measurements were performed in a 3 mL corvette with 2 mL solution at room temperature and all the fluorescence spectra were obtained by excitation at 470 nm.

For time-dependent optical spectra, the probes were treated with 20-50 eq. of Cys, Hcy GSH or β-ME, respectively, and the profiles were recorded at different points. For the selectivity experiment, fluorescence spectra of **2** (10 μM) toward different species with or

without Cys in PBS buffer were monitored. All reactions were performed for 30 min at room temperature. Cys and all other species were 200 μM.

For the determination of the detection limit, probe **2** (10 μM) was incubated with various concentrations of Cys (0-10 μM) for 30 min before recording the emission profiles. The detection limit was calculated with the $3\sigma/k$ method.[3]

4. HPLC Measurements

HPLC analysis was performed by ANGELA TECHNOLOGIES HPLC LC-10F using C18 column with 4.6 mm X 250 mm. Buffer A: 0.1% (v/v) trifluoroacetic acid in water; buffer B: methanol; flow: 1 mL/min. The elution conditions were: 0-5 min, buffer B: 5-40%; 5-25 min, buffer B: 40-95%; 25-30 min, buffer B: 95-5%. The detection wavelength for HPLC is 254 nm.

5. Cell cultures and MTT assay of probe 2

HUVEC (human umbilical vein endothelial cells) cell line and HT-1080 (human fibrosarcoma) cell line were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin under standard cell culture conditions at 37 °C in a humidified CO_2 incubator.

The cytotoxicity of probe **2** was determined via a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay by using the HUVEC cells. Briefly, the HUVEC cells were transferred to the 96-well plate and cultured for one night before experiments. After that, the culture medium was replaced with a fresh one and the HUVEC cells were incubated with probe **2** (10-25 μM) for 24 h. Then, 5 mg/mL MTT in PBS (20 μL) was added to each well and incubated for another 4 h. Finally, the medium was replaced with 150 μL of DMSO to dissolve the purple formazan crystals. The absorbance intensity in each well was detected at 490 nm by a microplate spectrophotometer (SpectraMax M2E (Molecular Device, Inc.)).

6. Cell imaging

The feasibility of **2** for detecting Cys in cells was evaluated via fluorescence imaging. Briefly, glass bottom dishes were added into a 24-well plate before cells were seeded. Then, the HT-1080 cells were transferred to the 24-well plate and cultured for one night before the experiments. After that, the culture medium was replaced with the fresh one and the cells were treated with the desired reagents. After incubation, the HT-1080 cells were quickly washed with PBS three times, and then fixed with 4% paraformaldehyde solution for 10 min. Finally, the HT-1080 cells were washed with PBS and imaged using a confocal microscope (Olympus FV1000) with a 40 × objective lens. Emission was collected at the green channel (500-550 nm) with 488 nm excitation.

7. References

1. I. Cummins, D. J. Wortley, F. Sabbadin, Z. He, C. R. Coxon, H. E. Straker, J. D. Sellars, K. Knight, L. Edwards, D. Hughes, S. S. Kaundun, S.-J. Hutchings, P. G. Steel, and R. Edwards, *P. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 5812.
2. X. F. Yang, Y. X. Guo and R. M. Strongin, *Angew. Chem. Int. Ed.*, 2011, **50**, 10690.
3. J. Radford-Knoery and G. A. Cutter, *Anal. Chem.*, 1993, **65**, 976.

8. Supplementary figures

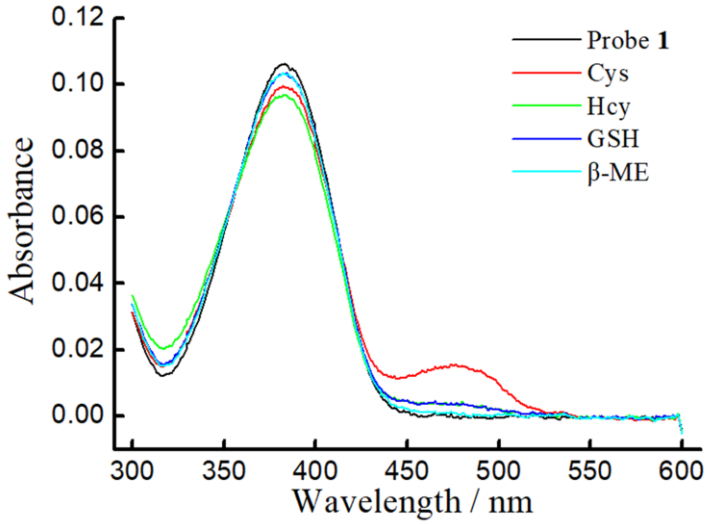


Fig. S1 Absorption spectra of **1** (10 μM) and its reaction with thiols (200 μM, inset) for 30 min.

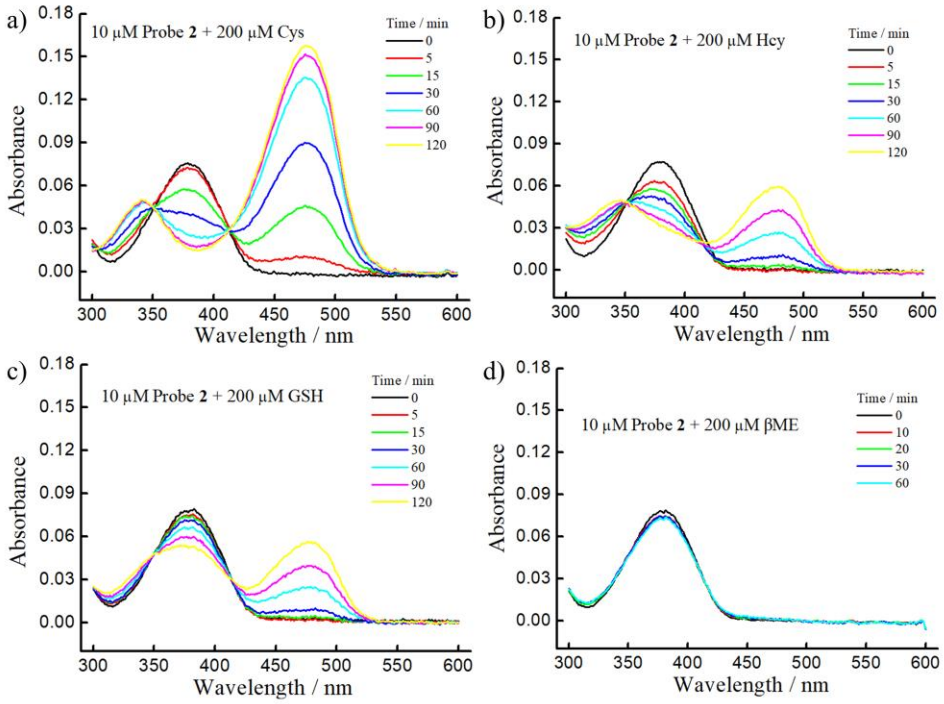


Fig. S2 Time-dependent Absorption spectra of 10 μM **2** upon addition of different thiols (200 μM).

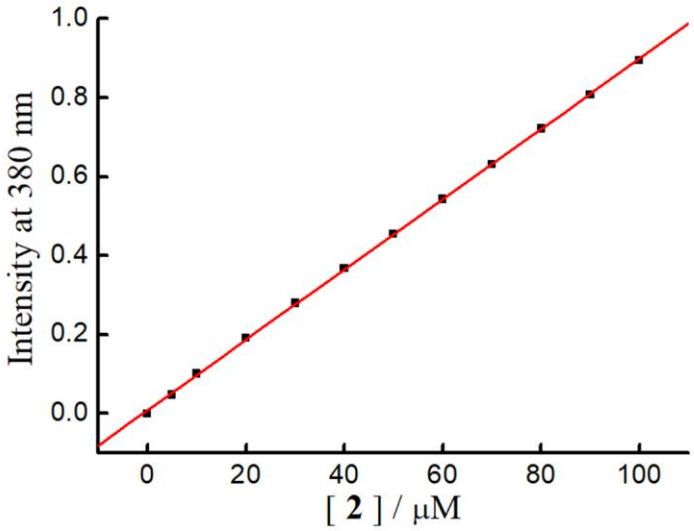


Fig. S3 Concentration-dependent absorption at 380 nm of **2** in PBS buffer. The wide linear range implies the good solubility (up to 100 μM) of **2** in aqueous buffer.

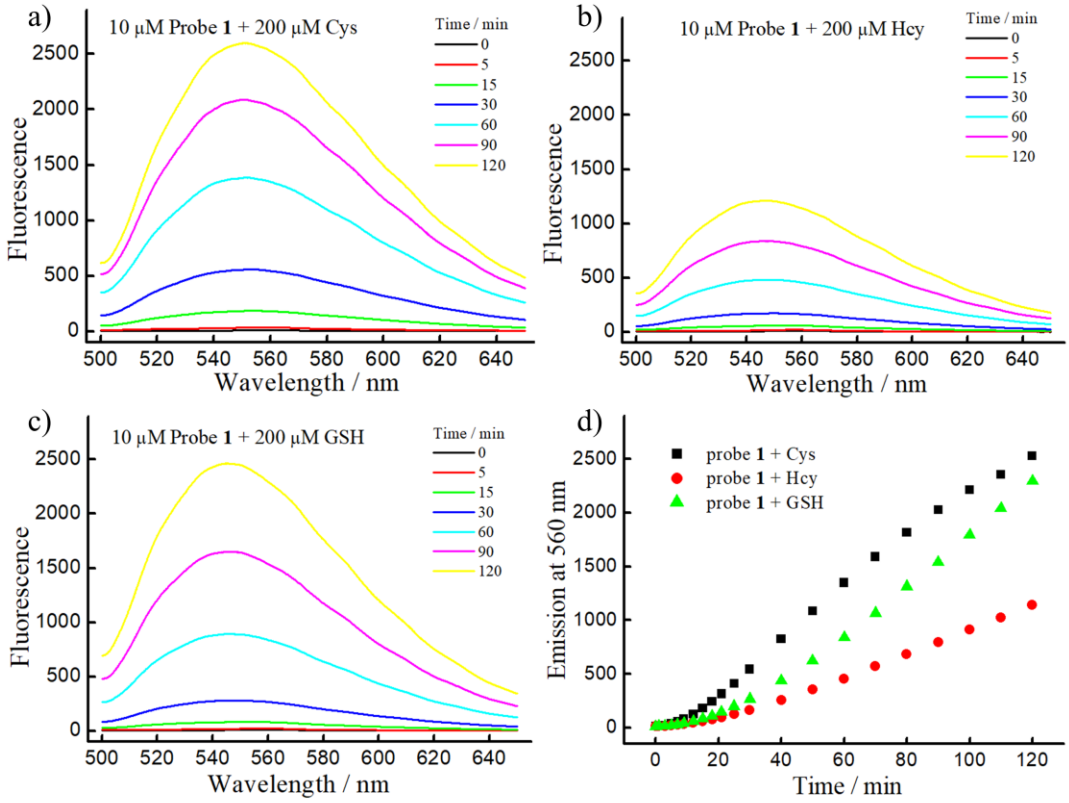


Fig. S4 Time-dependent fluorescence spectra of **1** (10 μM) toward biothiols (200 μM). (a) Cys; (b) Hcy; (c) GSH. (d) Spot chart of fluorescent intensity changes at 560 nm (λ_{ex} = 470 nm).

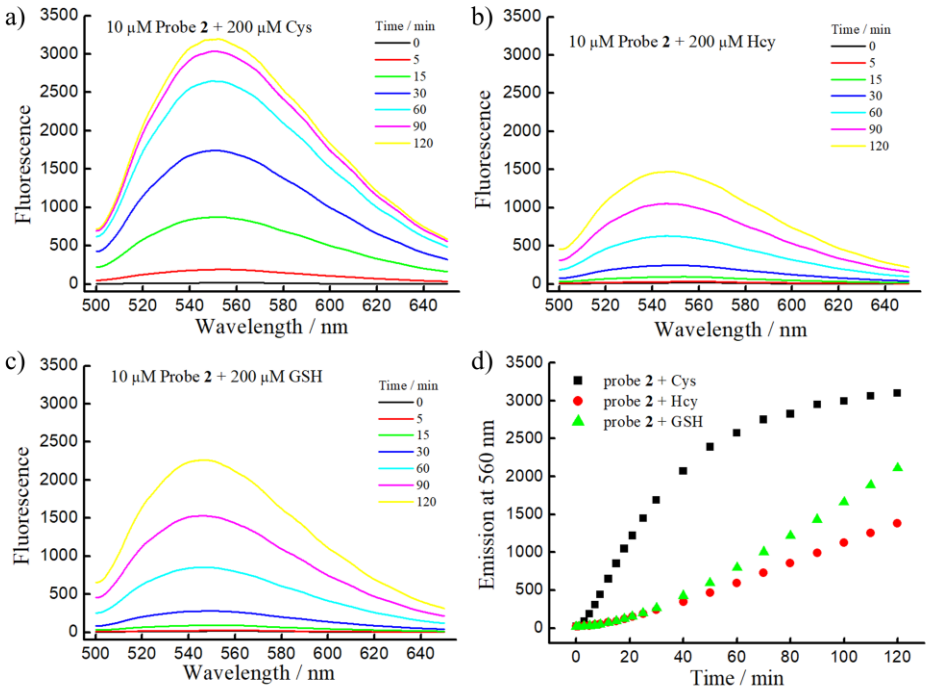


Fig. S5 Time-dependent fluorescence spectra of **2** (10 μM) toward biothiols (200 μM). (a) Cys; (b) Hcy; (c) GSH (d) Spot chart of fluorescent intensity changes at 560 nm (λ_{ex} = 470 nm).

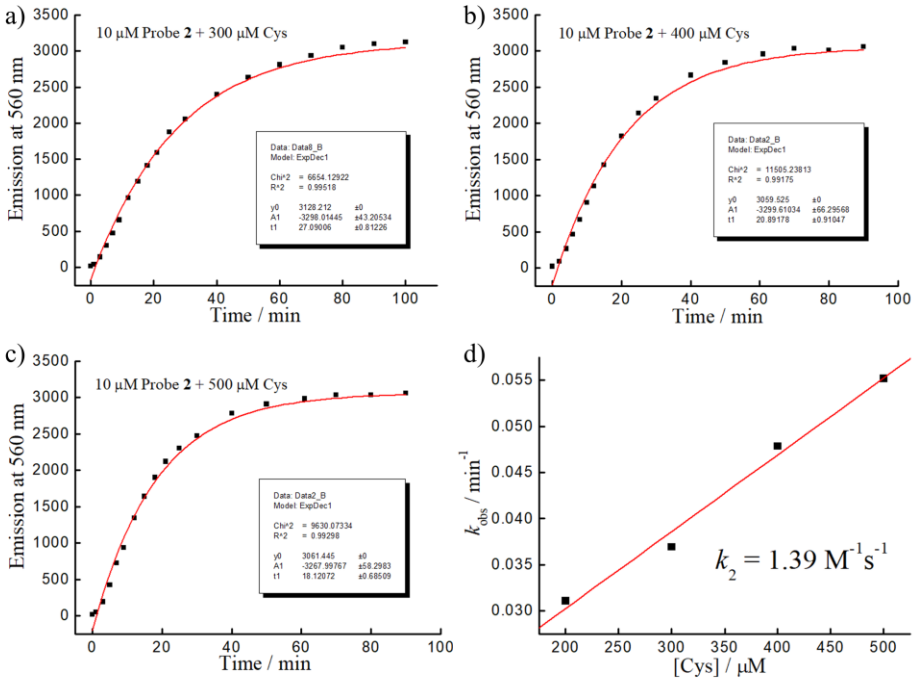


Fig. S6 (a-c) Time-dependent fluorescence intensity at 560 nm of **2** (10 μM) in the presence of different concentrations of Cys. (d) The linear relationship between concentrations of Cys and k_{obs} values. The red line represents the best single-exponential fitting.

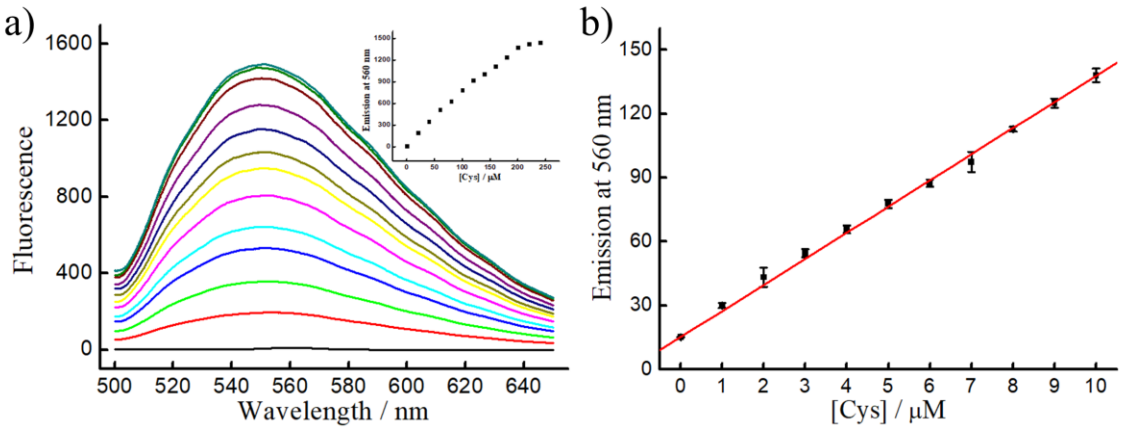


Fig. S7 (a) The fluorescence changes of **2** (10 μM) responses to various concentrations of Cys. Inset: Spot chart of fluorescence intensity changes at 560 nm. λ_{ex} = 470 nm. (b) The linear relationship between concentrations of Cys and emission at 560 nm. The detection limit was calculated to be 0.17 μM.

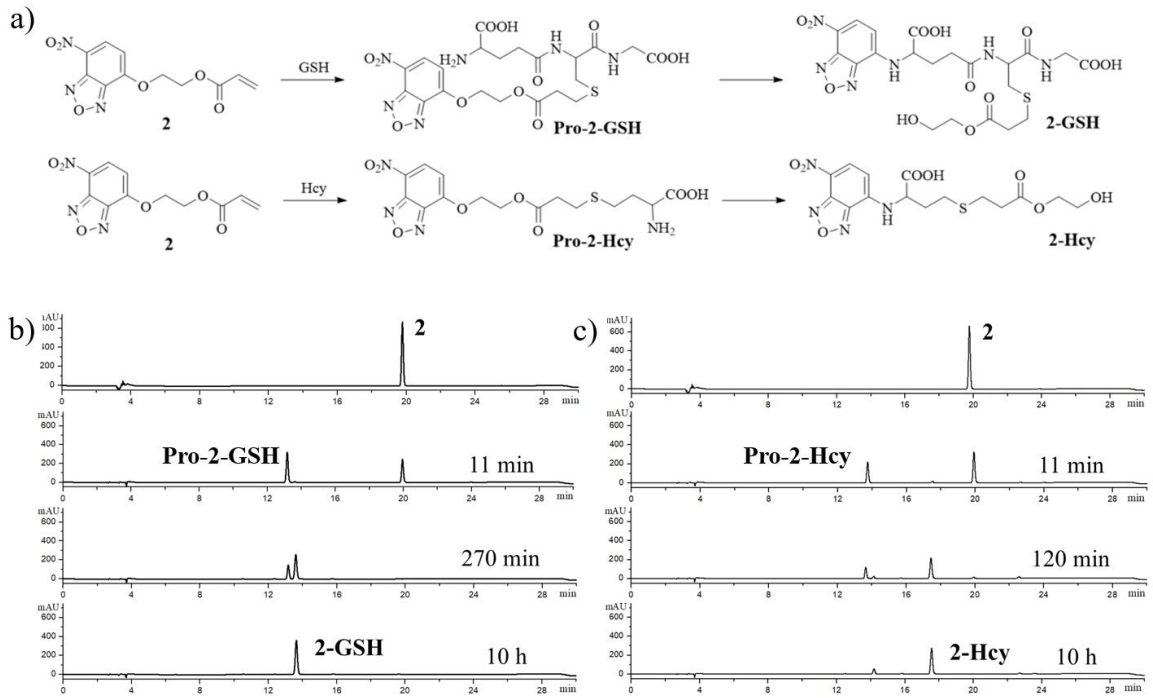


Fig. S8 (a) Structures and reactions of probe **2** with GSH and Hcy. Time-dependent HPLC traces of **2** (1 mM) treated with (b) GSH (2 mM) or (c) Hcy (2 mM).

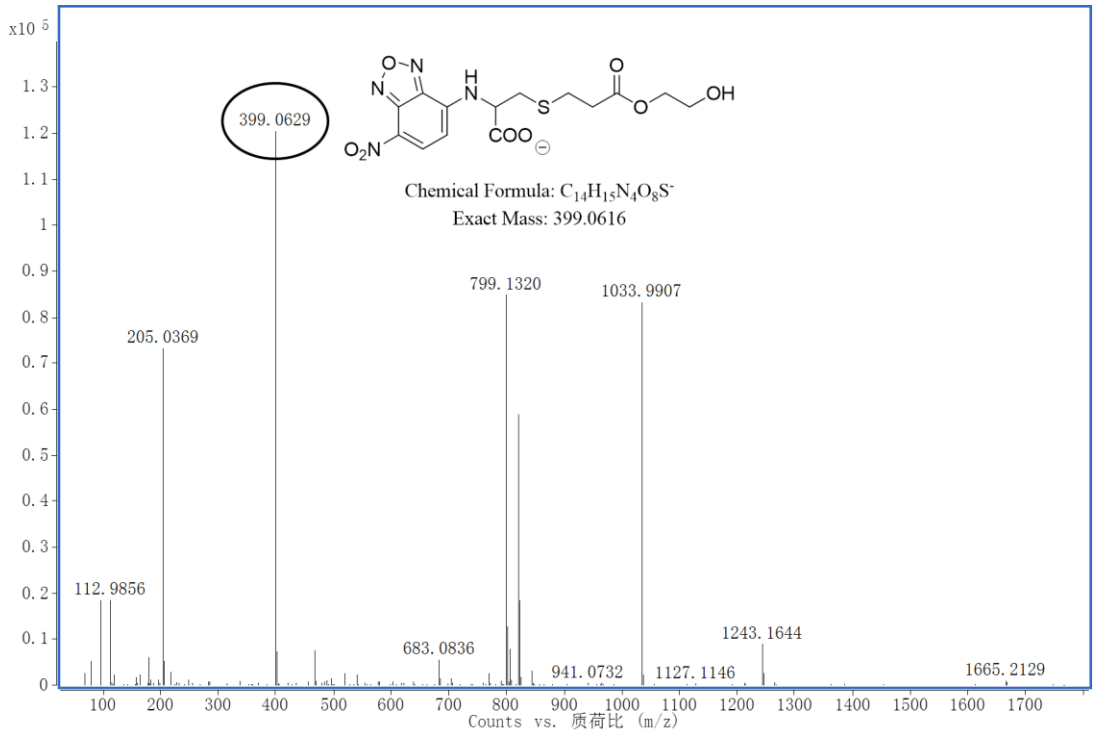


Fig. S9 HRMS spectra of probe **2** (2 mM) incubated with Cys (4 mM) in PBS buffer (containing 20% DMSO) over night.

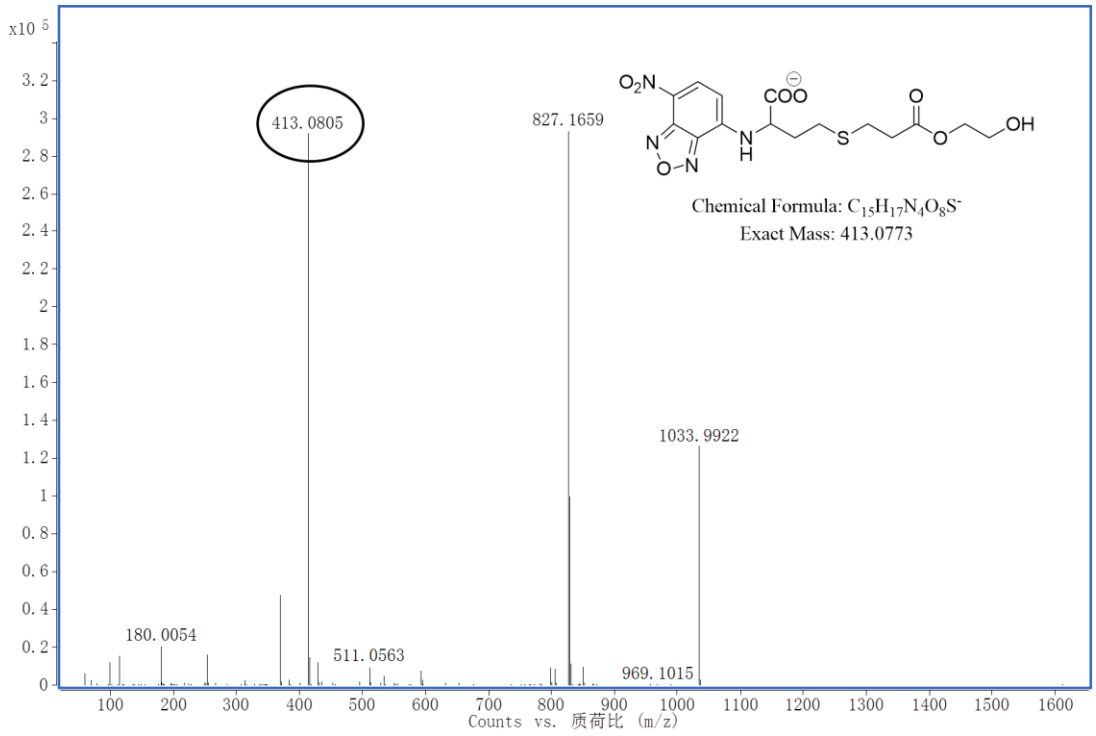


Fig. S10 HRMS spectra of probe **2** (2 mM) incubated with Hcy (4 mM) in PBS buffer (containing 20% DMSO) over night.

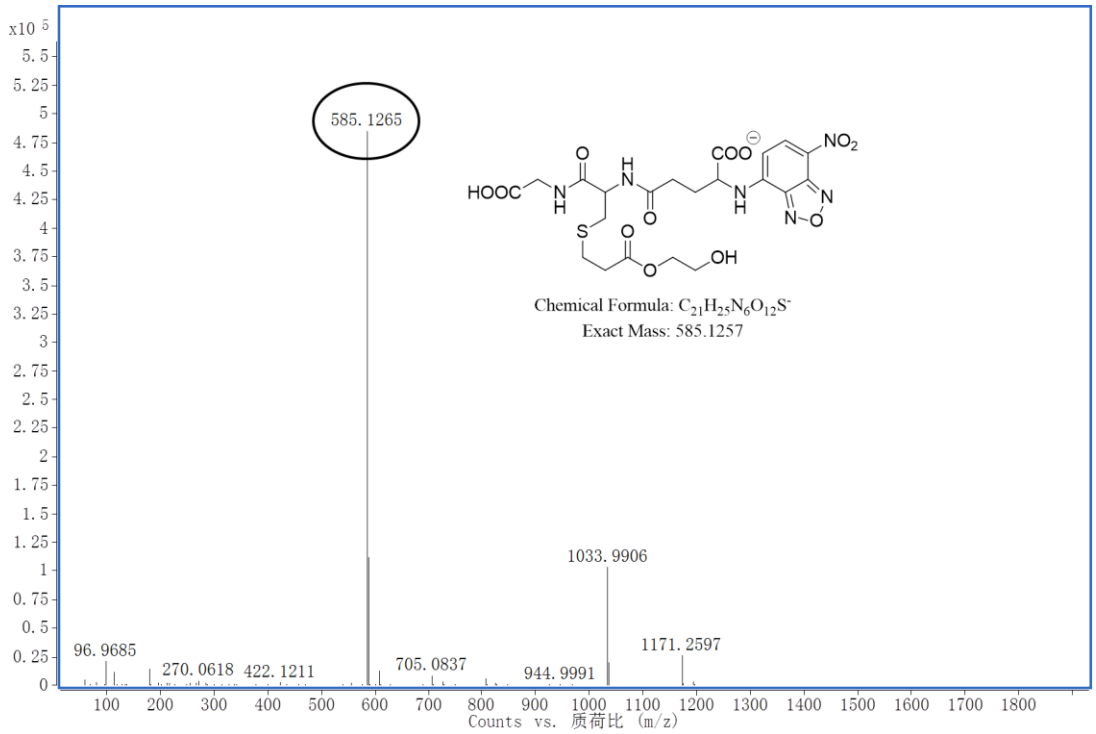


Fig. S11 HRMS spectra of probe **2** (2 mM) incubated with GSH (4 mM) in PBS buffer (containing 20% DMSO) over night.

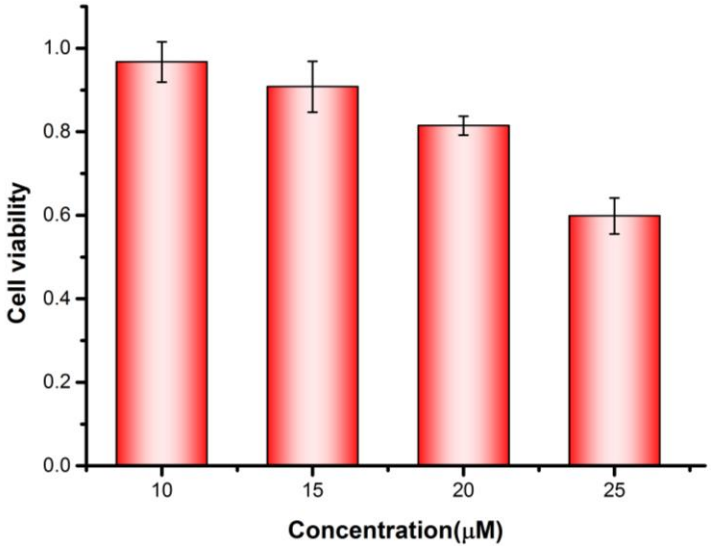


Fig. S12 Cell viability study of probe 2 with HUVEC cells.

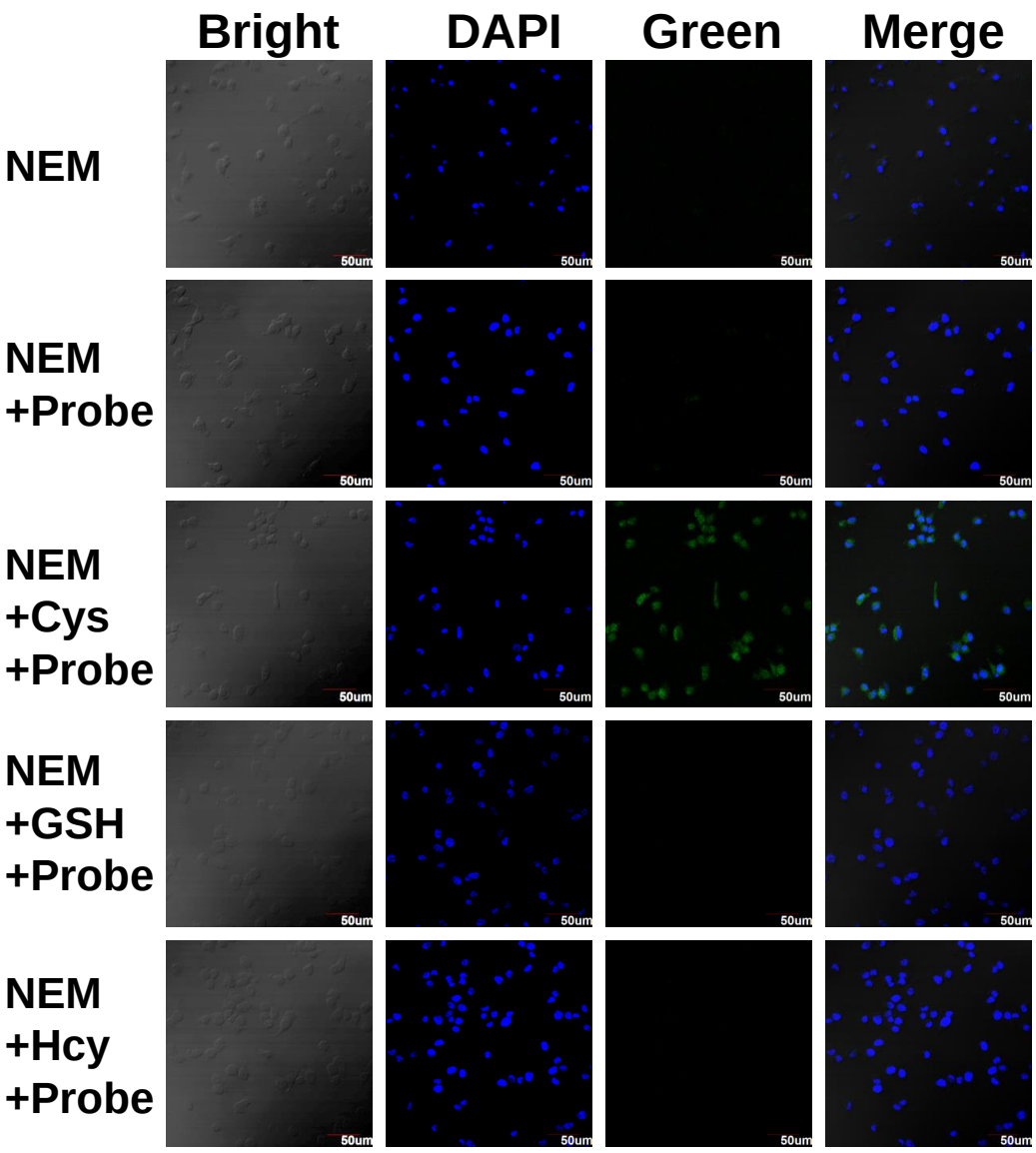


Fig. S13 Confocal microscopy images of HT-1080 pretreated with probe **2** (10 μM) and Cys/Hcy/GSH (200 μM) for 30 min in living cells. All cells were treated with NEM (1 mM) first for 30 min to eliminate endogenous biothiols. (λ_{ex} = 488 nm, λ_{em} = 500-550 nm for the green channel. Scale bar, 50 μm).

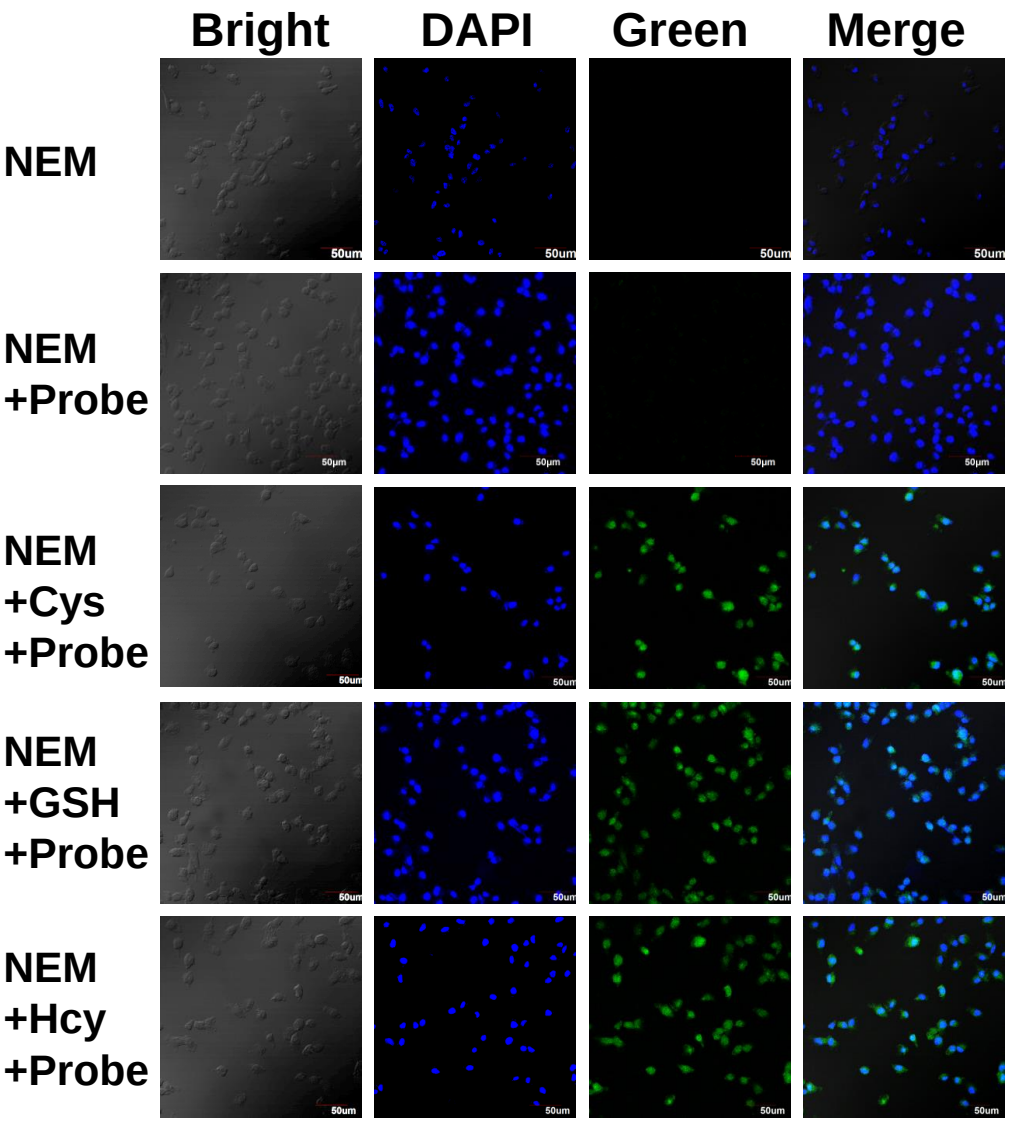


Fig. S14 Confocal microscopy images of HT-1080 pretreated with probe **2** (10 μM) and Cys/Hcy/GSH (200 μM) for 3 h in living cells. All cells were treated with NEM (1 mM) first for 30 min to eliminate endogenous biothiols. (λ_{ex} = 488 nm, λ_{em} = 500-550 nm for the green channel. Scale bar, 50 μm).

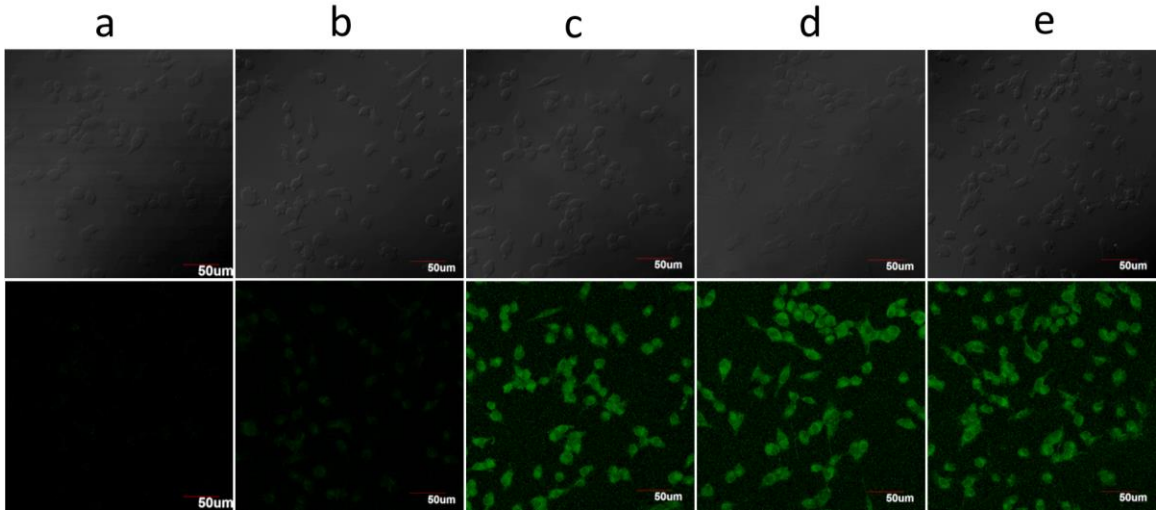
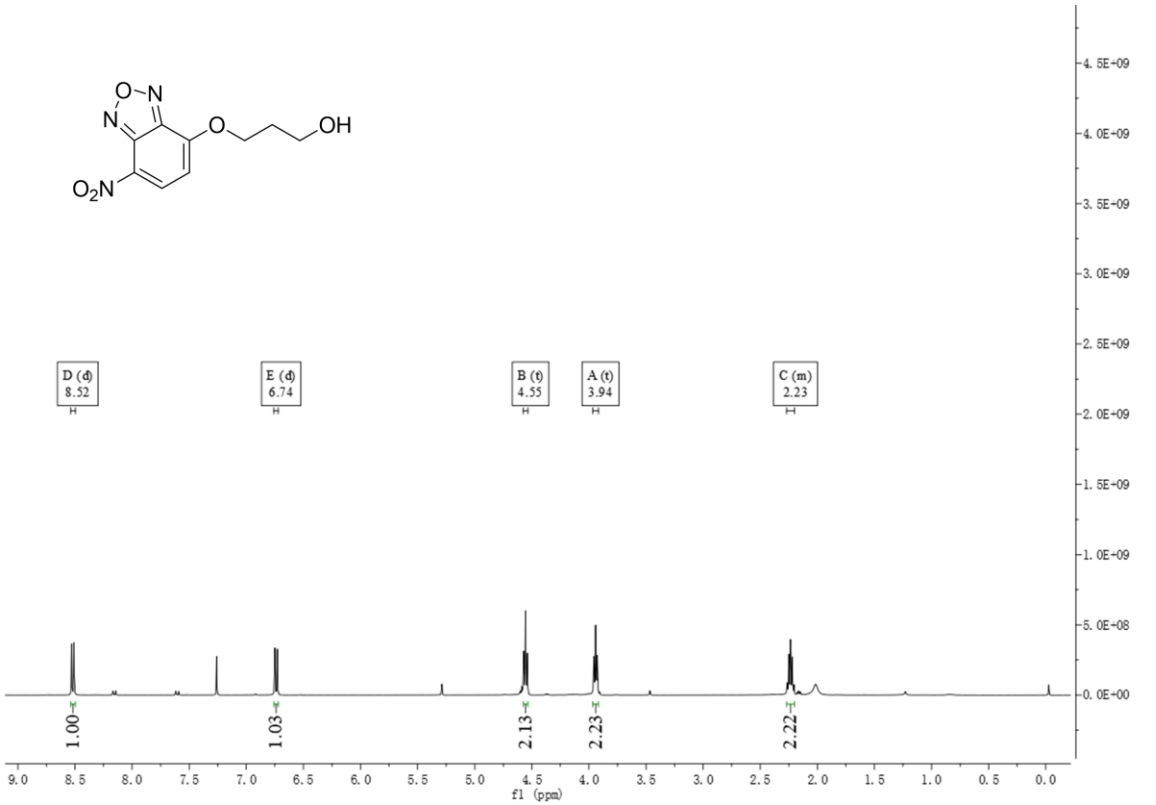
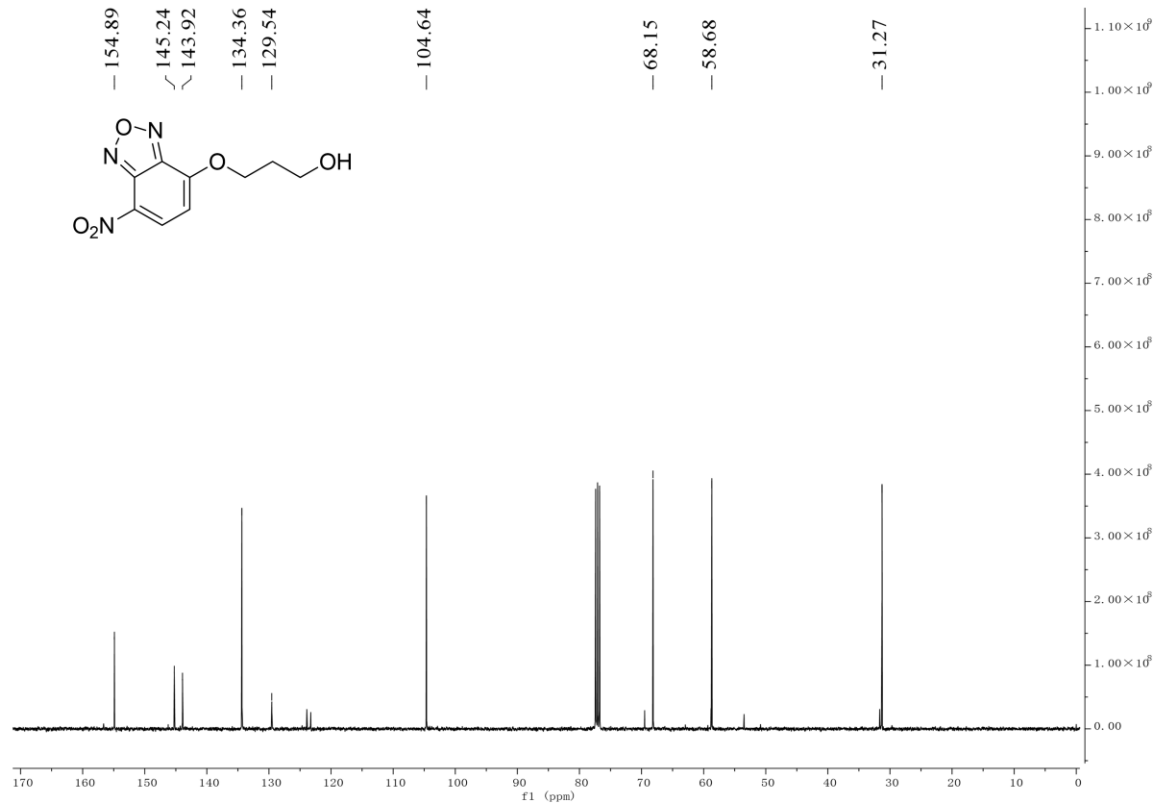
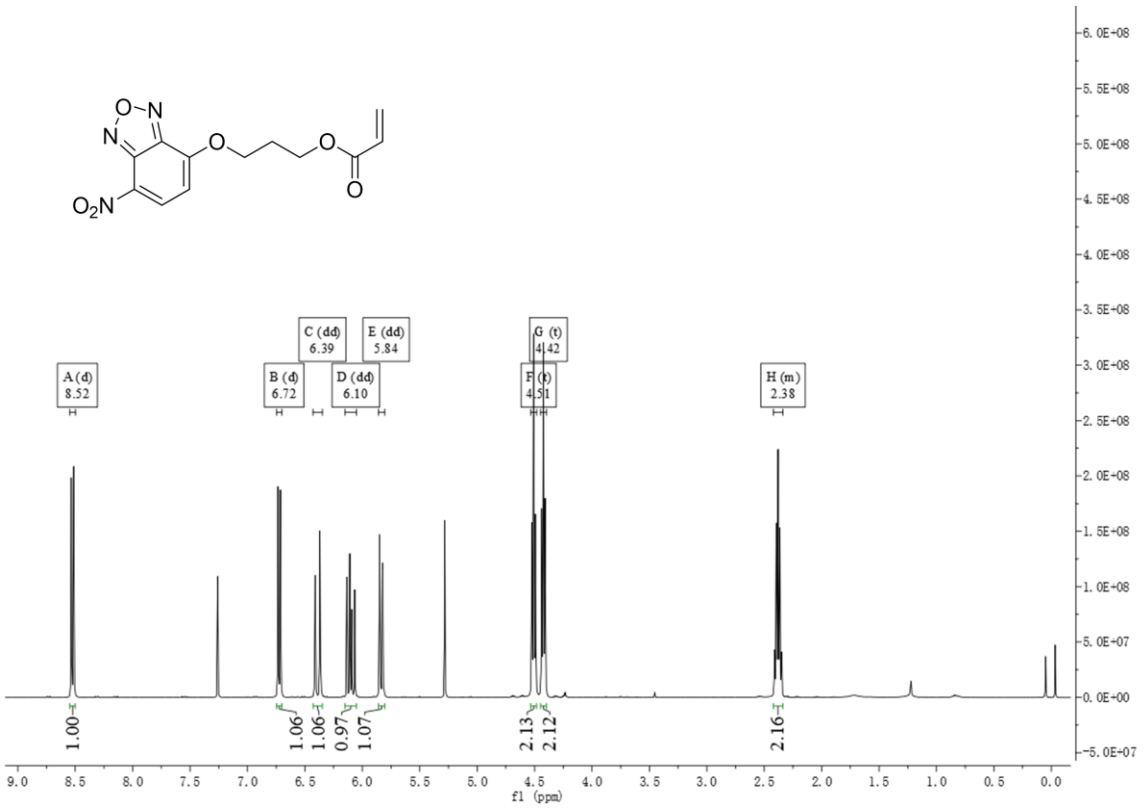


Fig. S15 Confocal microscopy images of HT-1080 cells. Cells pretreated with NEM for 30 min, then (a) probe **2** and Hcy; (b) probe **2** and GSH; (c) probe **2** and Cys; (d) probe **2**, Cys and Hcy; (e) probe **2**, Cys and GSH for 30 min. Probe **2** was 10 μM; Cys and Hcy was 200 μM; NEM and GSH was 1 mM. (λ_{ex} = 488 nm, λ_{em} = 500-550 nm for the green channel. Scale bar, 50 μm.)

9. Supplementary NMR and MS spectra



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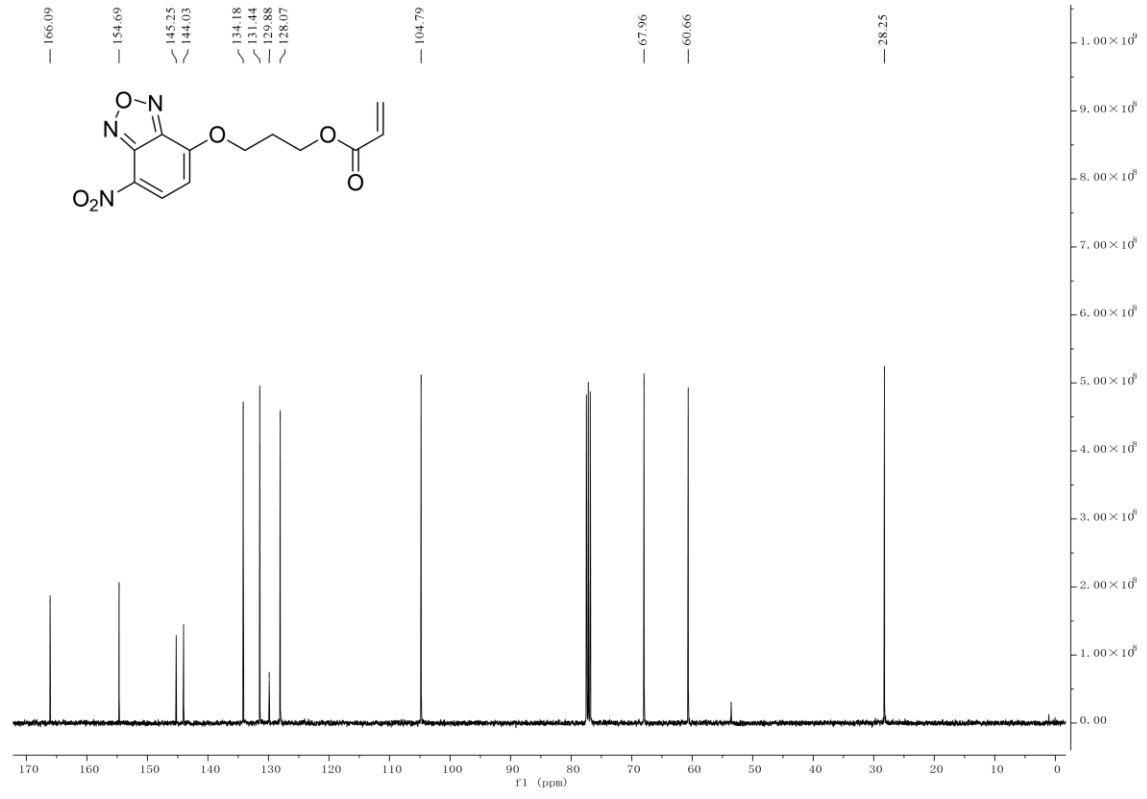
f1 (ppm)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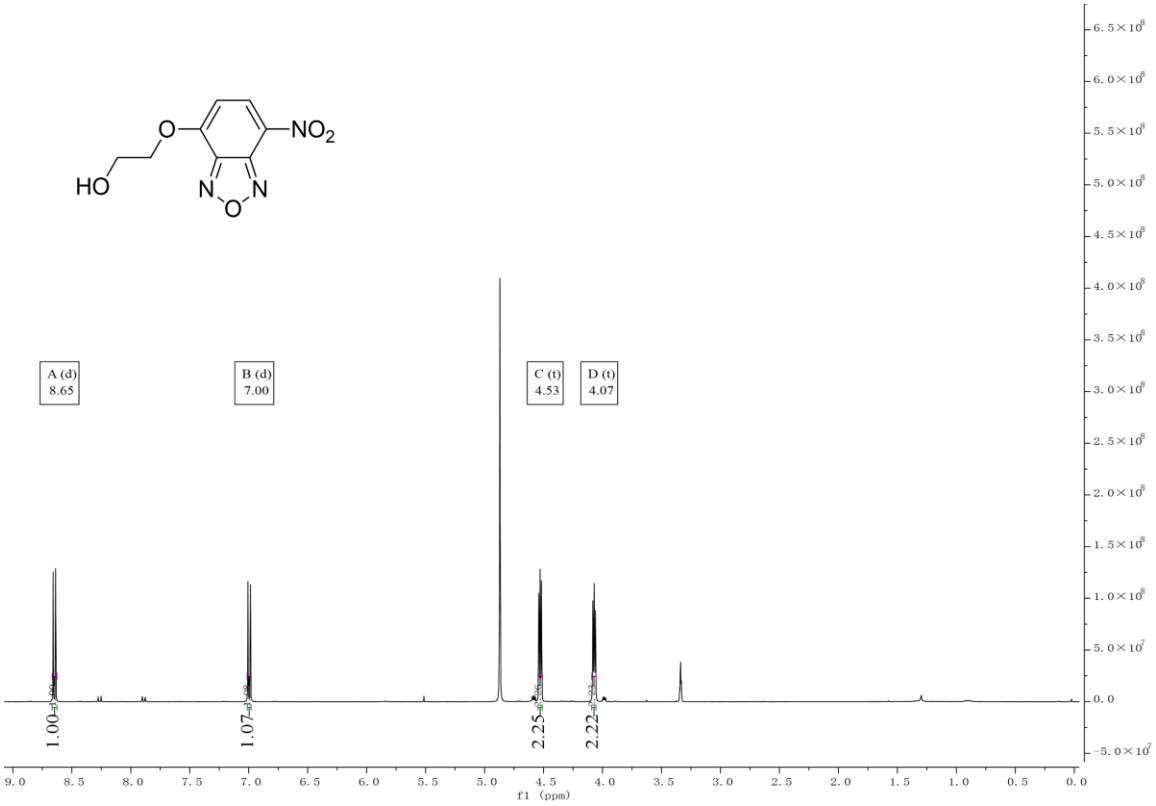
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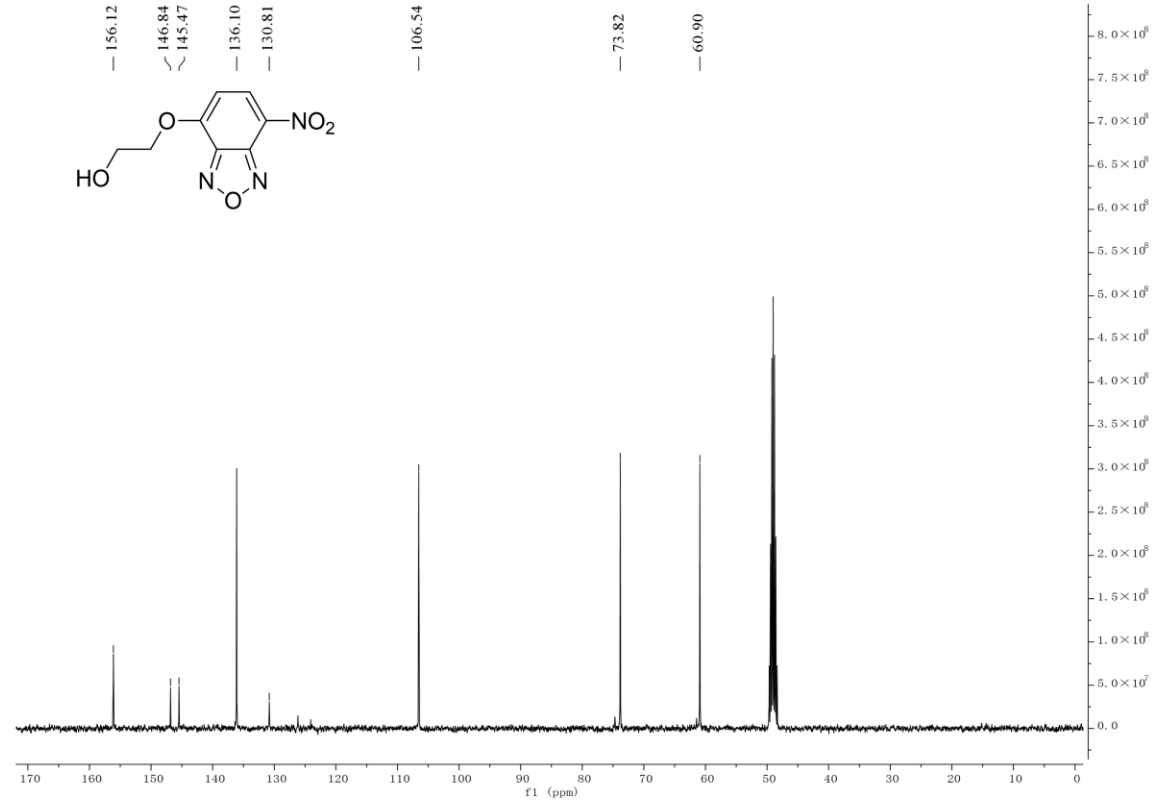
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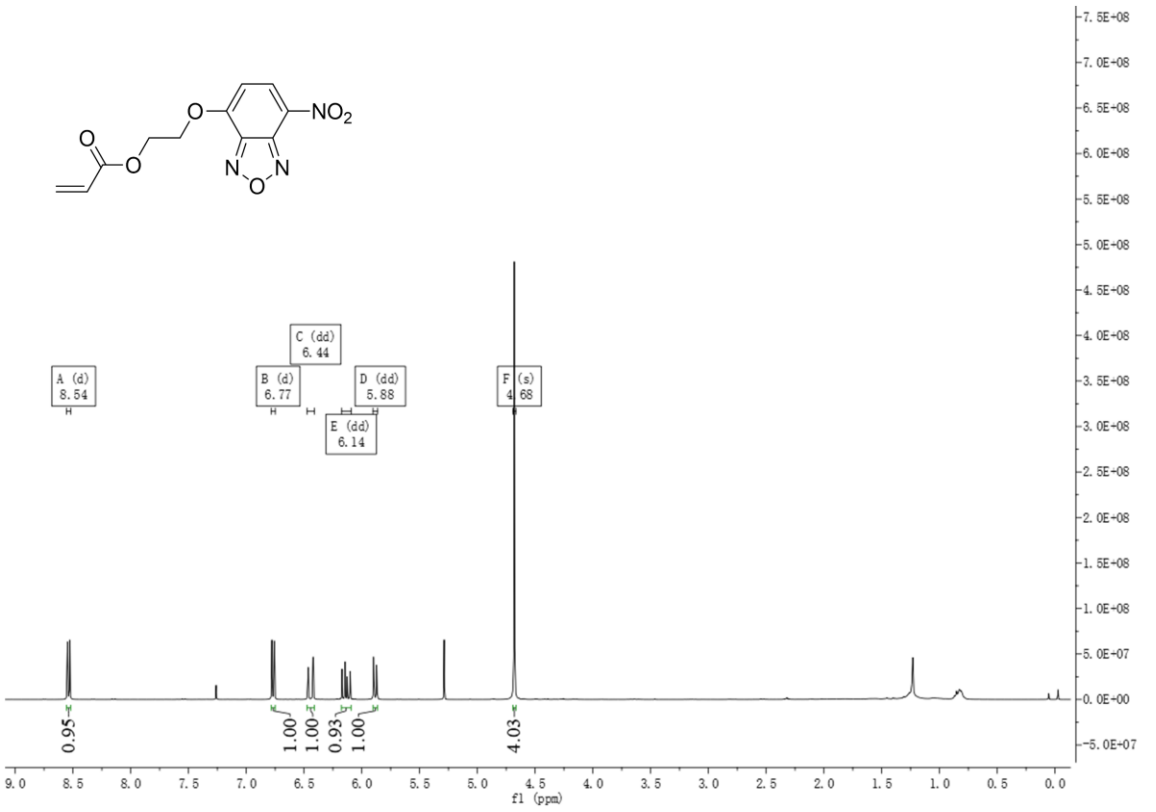
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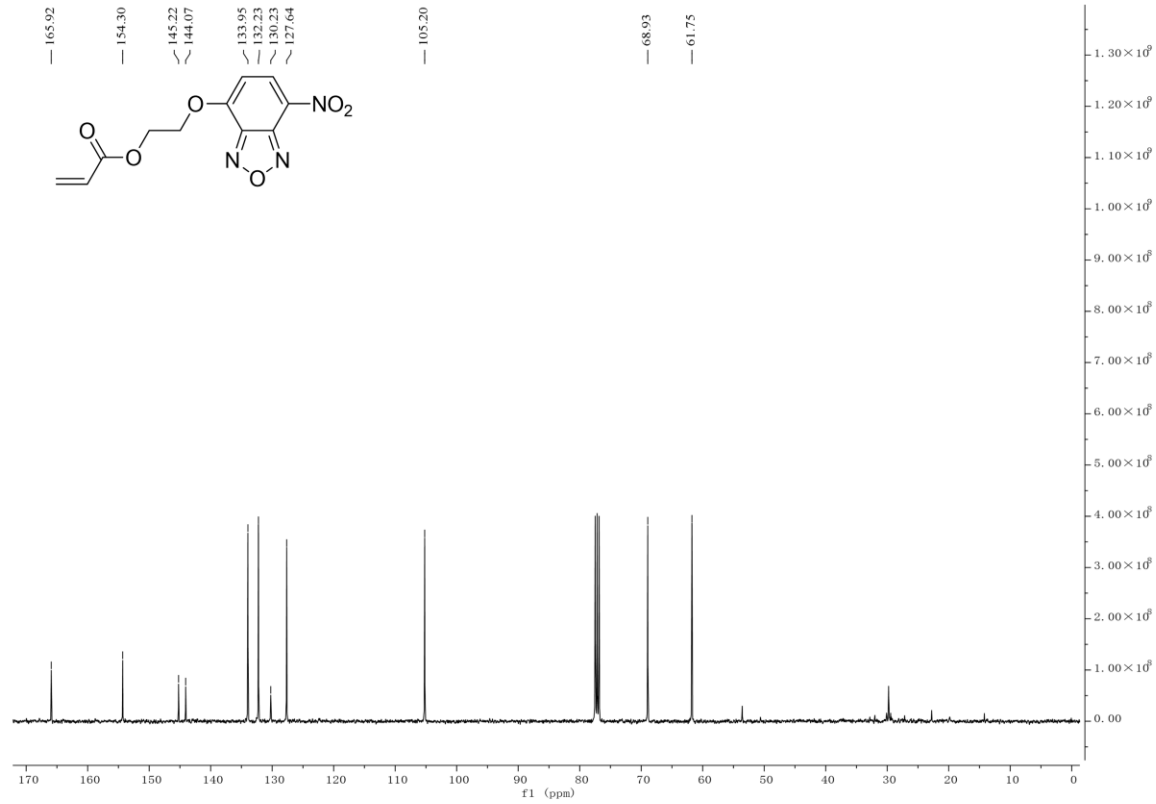
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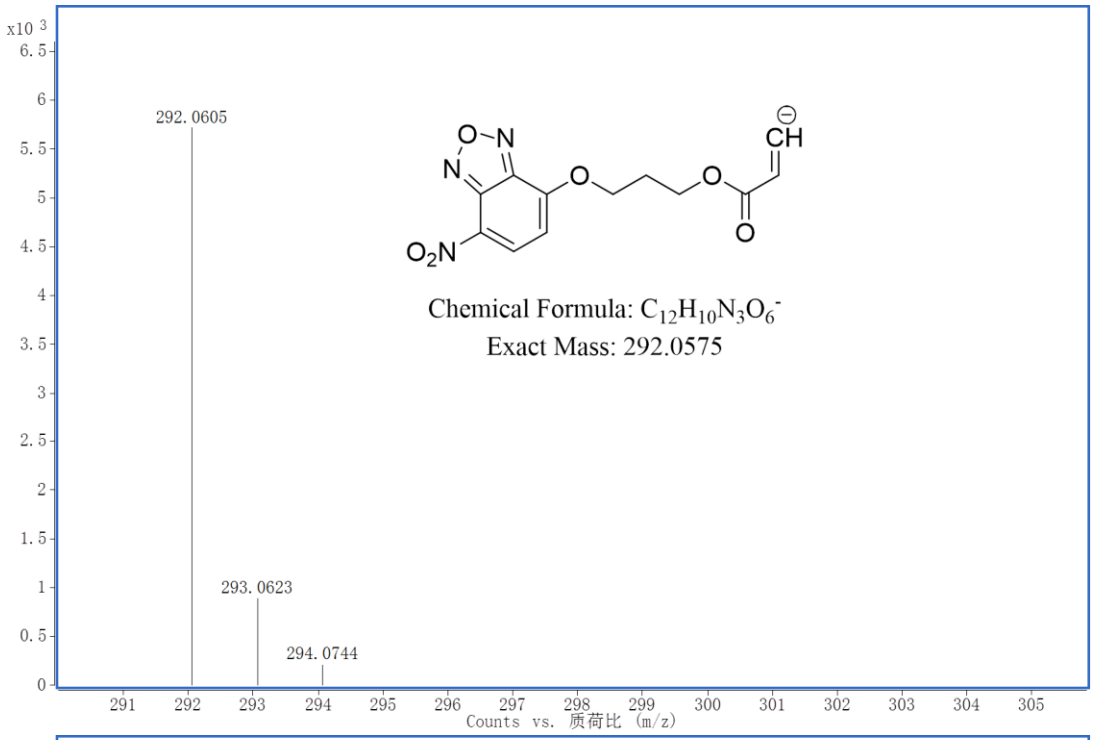
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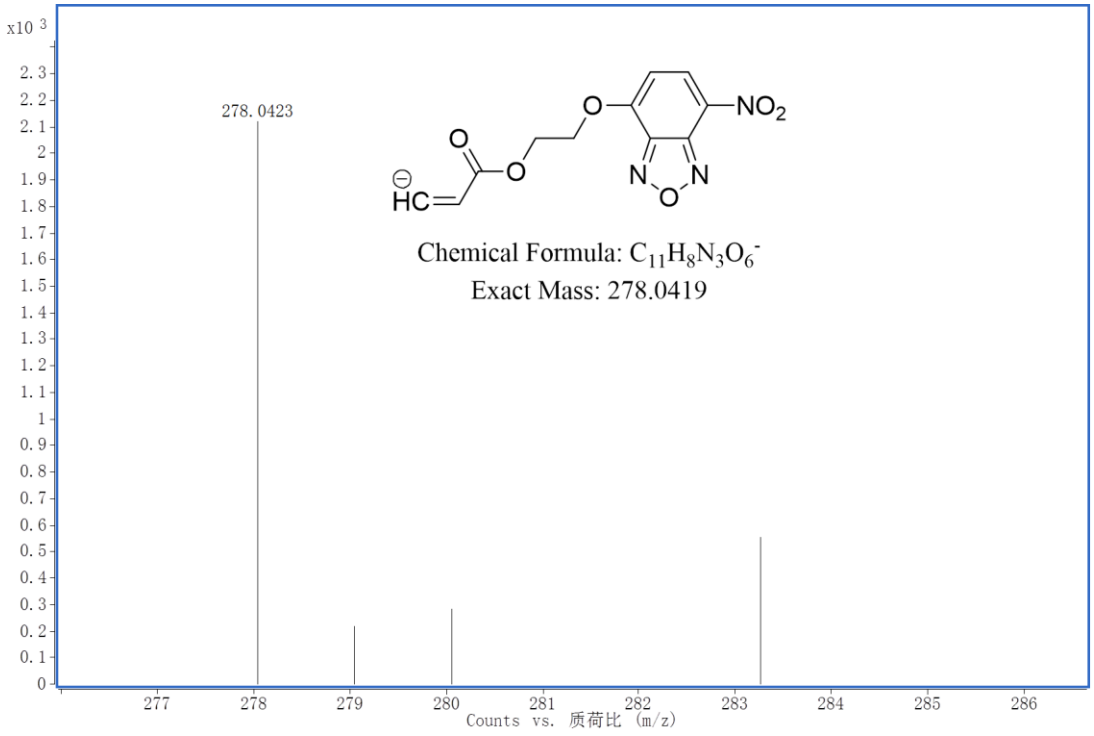
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