Supporting Information

Visualizing Glutathione Levels in Adipose Tissue: A CBD Aryl Ether Thiolysis-Activated ESIPT Probe for Obesity Research

Liya Jing,[abcd] Lijuan Shan,[a] Zimin Shen,[a] Mengyuan Liu,[a] Junjie Shi,[a] Chunlin Qian,[a] Zihan Jiang,[a] Minxuan Xiong,[a] Xixian Miao,[a] Zhicheng Xie,[a] Jing Xu,[a] Haowen Jiang,[b] Jia Li,[be] Chang Peng,*[e] Jinlong Li*[a] and Wei Liu*[a]

[a]School of Pharmacy, Nantong University, Nantong 226001, China.
[b]State Key Laboratory of Chemical Biology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China.
[c]Lingang Laboratory, Shanghai 201203, China.
[d]School of Life Science and Technology, Shanghai Tech University, Shanghai 201210, China.
[e]Key Laboratory of Glyco-drug Research of Zhejiang Province, School of Pharmaceutical Science and Technology, Hangzhou Institute for Advanced Study, University of Chinese Academy of Sciences, 310024, Hangzhou, China.

1. Material and Methods

1.1 Material and instruments

All chemicals and solvents were obtained from commercial suppliers and used as received without further purification. Reaction progress was monitored by thin-layer chromatography (TLC) on precoated silica gel plates (Merck 60 F_{254}, 250 μm thickness) with visualization under UV light. Flash column chromatography was performed using Merck silica gel 60 (70–200 mesh). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS, δ = 0.00 ppm) as an internal standard or to residual solvent signals ($CDCl_3$: δ = 7.26 ppm; DMSO-d_6: δ = 2.50 ppm). Coupling constants (J) are given in Hertz (Hz), and multiplicities are abbreviated as follows: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet. High-resolution mass spectra (HRMS) were acquired on an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system. UV-Vis absorption spectra were measured on a METASH UV-6000 spectrophotometer. Fluorescence spectra were recorded using an F-280 spectrophotometer (Tianjin Gangdong Sci. & Tech. Development Co., Ltd.).

1.2 Synthesis of probe 1

Synthesis of intermediate 3

A mixture of 2,1,3-benzooxadiazole (1.0 g, 8.33 mmol) and iron powder (0.1 g, 1.60 mmol) was placed in a 25 mL glass reactor. The mixture was heated to 90°C, and bromine (1.2 mL, 25.0 mmol) was added dropwise over 10 minutes. After the addition, heating was continued for another 2 hours. The reaction was then cooled to room temperature and poured into water (100 mL). The aqueous mixture was extracted with dichloromethane (DCM). The organic layer was washed with saturated $NaHCO_3$ solution (50 mL) once, followed by brine (50 mL) twice, and dried over anhydrous Na_2SO_4. After concentration under reduced pressure, the crude product was purified by column chromatography to afford product 3 as a white solid (1.53 g, 66.8% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 149.5, 134.3, 108.8.

Synthesis of intermediate CBD-Br

Compound **3** (532 mg, 1.93 mmol) and CuCN (171.6 mg, 1.93 mmol) were dissolved in anhydrous DMF (5 mL). The mixture was heated at 150°C for 12 hours and then cooled to room temperature. The solvent was removed in vacuo. The residue was taken up in ethyl acetate and washed sequentially with water (50 mL) and brine (50 mL × 2). The organic layer was dried over anhydrous Na_2SO_4, concentrated under reduced pressure, and purified by column chromatography to afford CBD-Br as a white solid (50 mg, 11.7% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.29 (d, J = 7.3 Hz, 1H), 8.14 (d, J = 7.3 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 149.8, 148.2, 142.2, 135.1, 115.7, 114.6, 99.9.

Synthesis of probe 1

A mixture of HBT (34 mg, 0.15 mmol) and CBD-Br (50 mg, 0.23 mmol) was dissolved in anhydrous THF (4 mL), followed by the addition of NaH (0.30 mmol). The reaction was stirred at 60°C for 4 h. After cooling, the mixture was diluted with ethyl acetate and washed sequentially with water (30 mL × 2) and brine (30 mL × 3). The organic layer was dried over anhydrous Na_2SO_4, concentrated in vacuo, and purified by column chromatography to afford product **1** as a white solid (19 mg, 34.5% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (dd, J = 7.8, 1.7 Hz, 1H), 7.97 (d, J = 8.2 Hz, 1H), 7.81 (d, J = 7.9 Hz, 1H), 7.70 (d, J = 7.8 Hz, 1H), 7.64 (td, J = 7.8, 1.7 Hz, 1H), 7.57 (td, J = 7.7, 1.1 Hz, 1H), 7.51 – 7.43 (m, 1H), 7.36 (ddd, J = 5.6, 4.1, 0.9 Hz, 2H), 6.51 (d, J = 7.8 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.0, 152. 5, 151.7, 150.4, 149.4, 144.4, 141.1, 135.6, 132.6, 131.4, 127.9, 126.7, 126.7, 125.9, 123.5, 122.4, 121.7, 114.0, 109.7, 95.1. HRMS (ESI): m/z $[M+H]^+$ calculated for $C_{20}H_{11}N_4O_2S^+$: 371.0597; found: 371.0596.

1.3 General procedure for spectroscopic studies

All measurements were carried out in degassed phosphate-buffered saline (PBS, 50 mM, pH 7.4) containing 30% DMSO. Stock solutions of the compounds (1–10 mM) were prepared in DMSO. Solutions of various analytes (20 mM) were prepared in PBS buffer. An appropriate amount of each bio-relevant species was added to separate aliquots of the probe solution and mixed thoroughly. The mixture was shaken gently

before spectral acquisition. Fluorescence spectra were recorded with an excitation wavelength of 305 nm.

For selectivity experiments, fluorescence spectra of probe **1** (1 μM) were recorded in the presence of different species with or without GSH in PBS buffer. All reactions were incubated for 0.5 h at room temperature. The concentration of GSH was 2 mM, while other species were used at 100 μM. The data were background-subtracted for plotting.

In kinetic experiments, probe **1** (1 μM) was added to PBS buffer, and the fluorescence spectral changes upon reaction with different concentrations of GSH (4, 6, 8, and 10 mM) were monitored.

For the determination of the detection limit, probe **1** was incubated with varying concentrations of GSH (0.1–0.8 mM) for 0.5 h prior to recording the emission spectra.The detection limit was calculated with the following equation:[1]

$$\text{Detection limit} = 3\sigma / k$$

Construction of fitting curves and calculation of parameters including dissociation constant K_d were done with the following equation. Fluorescence intensity at the fluorescence maximum (denoted as F in the following formula) was plotted against GSH concentration. F_{free}, F_{bound} and K_d denote fluorescence intensity of the GSH-free and GSH-bound states and the dissociation constant with GSH, respectively.[2]

$$F = F_{bloud} + \frac{F_{free} - F_{bloud}}{1 + \frac{[GSH]}{K_d}}$$

1.4 HPLC Measurements

For HPLC analysis, a mixture of probe **1** (500 μM) and GSH (10 mM) in PBS (50 mM, pH 7.4) containing 50% acetonitrile was prepared and analyzed at various time points. The analysis was performed on an ANGELA TECHNOLOGIES LC-10F HPLC system equipped with a C18 column (4.6 mm × 250 mm). Detection was carried out at 360 nm. The mobile phase consisted of solvent A (water containing 0.1% v/v trifluoroacetic acid) and solvent B (methanol). The flow rate was 1.0 mL/min. The following gradient program was used: 5–75% B from 0 to 5 min; 75–85% B from 5 to

23 min; 85–5% B from 23 to 30 min.

1.5 Cell culture and MTT assay

The HeLa cell line (human cervical cancer cells) was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 U/mL streptomycin, and 4 mM L-glutamine, and cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

The cytotoxicity of probe **1** was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, HeLa cells were seeded into 96-well plates and cultured for 24 h. The culture medium was then replaced with fresh medium containing different concentrations of probe **1** (0, 5, 10, 20, 30, and 40 μM). After 24 h of incubation, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well, and the plates were incubated for an additional 4 h. The medium was subsequently removed, and the formed formazan crystals were dissolved in 150 μL of DMSO per well. Absorbance at 490 nm was measured using a SpectraMax M2E microplate reader (Molecular Devices)

1.6 Imaging in living cell

The ability of probe **1** to detect intracellular GSH was assessed by fluorescence imaging. HeLa cells were seeded onto glass-bottom dishes placed in a 24-well plate and allowed to adhere for 12 h. Before imaging, the culture medium was replaced with fresh medium. For the experimental group, cells were incubated with probe **1** for 1 h. In the control group, cells were first treated with the thiol-blocking agent N-ethylmaleimide (NEM, 1 mM) for 30 min, followed by incubation with probe **1** for an additional 1 h. After incubation, cells were washed quickly with PBS and fixed with 4% paraformaldehyde for 10 min. Following PBS washes, images were acquired on an Olympus FV1000 confocal microscope using a 40 × objective. Fluorescence was collected in the green channel (450–500 nm) with excitation at 405 nm.

1.7 Stromal vascular fraction (SVF) separation

Male C57BL/6 mice (6–8 weeks old, #202) were obtained from Shanghai Shengchang Biotechnology Co., Ltd. All animal experiments were approved by the

Animal Ethics Committee of the Shanghai Institute of Materia Medica（IACUC, 2025-04-LJ-202）. Mice were kept in a SPF barrier facility under controlled conditions (22 ± 2 °C, 50% humidity, 12/12 h light–dark cycle), with 3–5 mice per ventilated cage. Sterile food and water were provided ad libitum.

Adipose tissue from C57BL/6J mice was digested with a buffer containing 2 mg/mL Collagenase D (Cat#11088882001, Roche), 2.4 mg/mL Dispase II (Cat#4942078001, Roche), and 10 mM $CaCl_2$ at 37℃ for 1 hour. The digestion was stopped by adding DMEM-F12 medium (Cat#12500062, Gibco) supplemented with 10% FBS (Cat#10091-148, Gibco). The cell suspension was filtered through a 70-μm mesh and centrifuged at 500 × g for 10 minutes at 4℃. Following separation, the SVF pellet was treated with red blood cell lysis buffer (Cat#B541001, Sangon Biotech) to remove erythrocytes. Finally, cells were cultured in DMEM-F12 medium supplemented with 10% FBS at 37℃.

1.8 Fluorescence imaging of GSH

Cells were treated with 10 μM GSH probe 1 for 1 hour, fixed with 4% paraformaldehyde for 15 minutes, and then imaged using the Opera Phenix high-content imaging system via the 405 nm channel for detection.

1.9 Cellular senescence model induction and evaluation

To induce senescence, cells were exposed to 5 μM Etoposide or 1 μM Adriamycin for 24 hours. After treatment, the drug-containing medium was replaced with fresh medium, and cells were cultured for an additional 5-7 days.

1.10 Flow cytometry

Cells were washed with PBS, and then stained with probe 1 in the dark for 1 hour. After three washes with cold PBS (500 × g, 3 min each time), cells were resuspended for flow cytometry analysis.

1.11 Western blotting

Total protein was lysed in SDS-PAGE loading buffer, separated by SDS-PAGE, and transferred to NC membranes. After blocking with 5% BSA, membranes were incubated with primary antibody overnight at 4°C, followed by HRP-conjugated secondary antibody for 1 hour. Protein bands were visualized using ECL. The

commercial primary antibodies were used: anti-GAPDH (Cat# 2118, Cell Signaling Technology), anti-P21 (Cat# ab188224, Abcam).

2.Supplementary figures

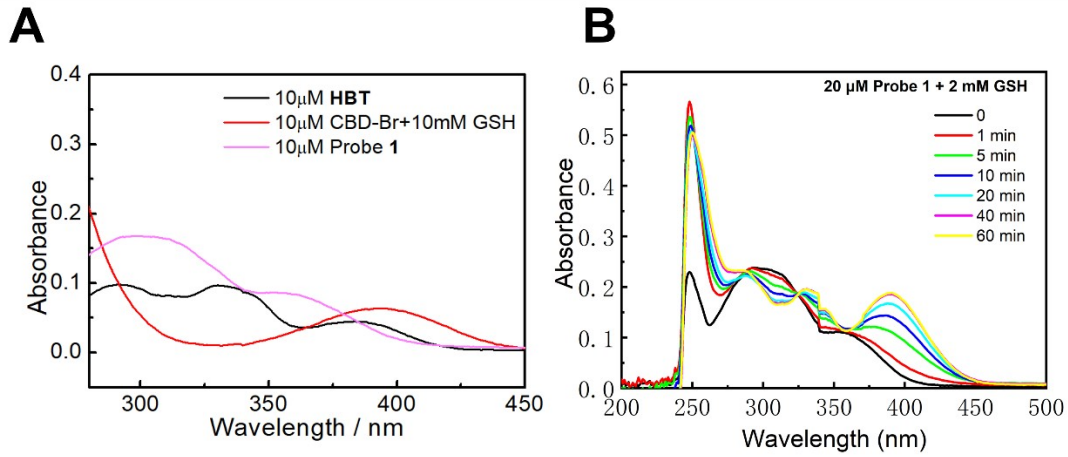


Fig.S1. (A) Comparison of the absorption spectra of HBT (10 μM), CBD-SG (10 μM), and probe **1** (10 μM); (B) Time-dependent UV-vis spectra of probe 1 (20 μM) upon addition of GSH (2 mM) in PBS buffer (pH 7.4, containing 30% DMSO).

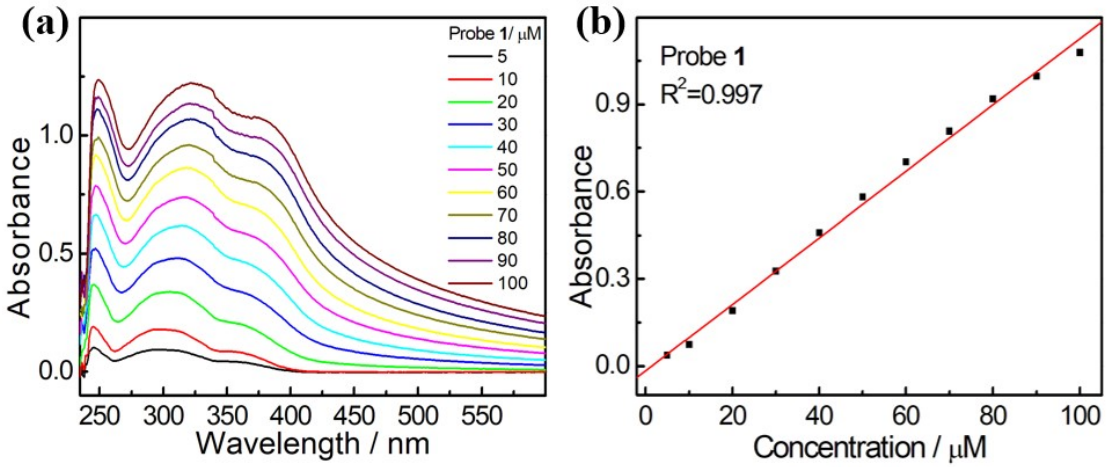


Fig. S2. (a) UV-vis spectra of probe **1** at different concentrations recorded in PBS buffer (50 mM, pH 7.4, containing 30% DMSO). (b) Linear relationship between the absorbance at 367 nm and the concentration of probe **1**.

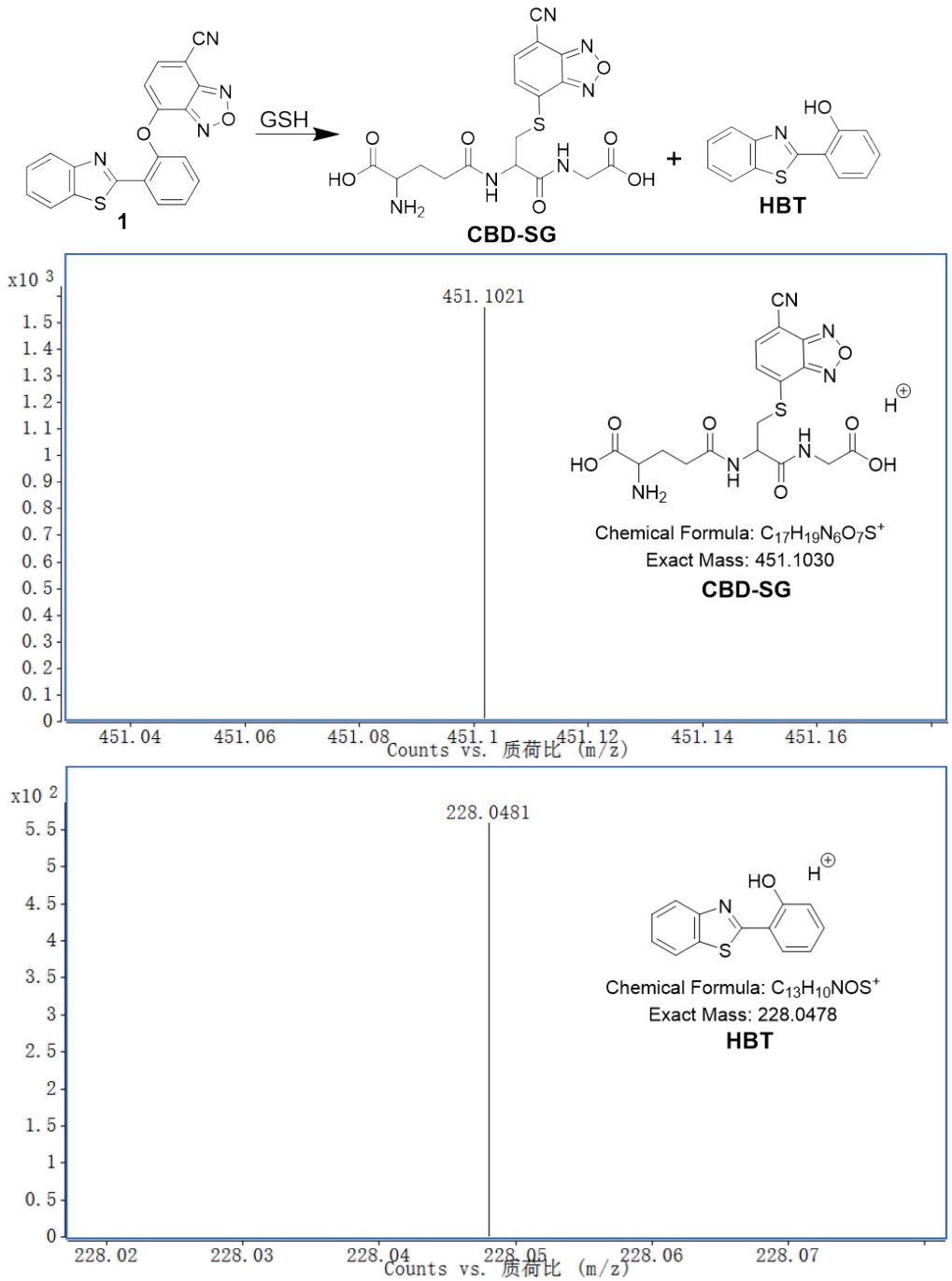


Fig. S3. HRMS analysis of probe **1** (500 μM) after incubation with GSH (10 mM) for 3 h in PBS buffer (25 mM, pH 7.4, containing 50% acetonitrile).

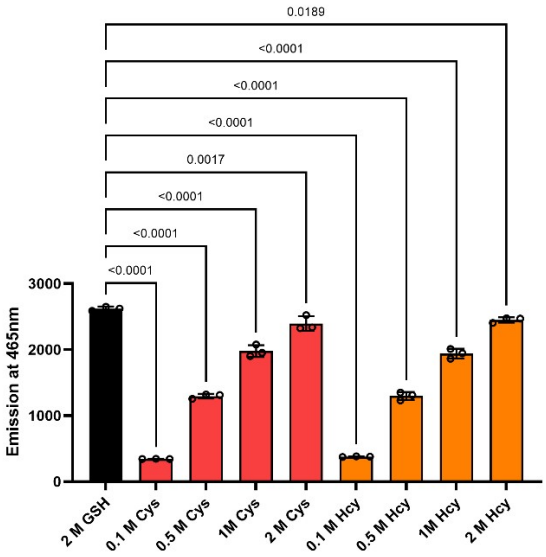


Fig. S4. Fluorescence response of probe **1** (1 μM) to cysteine (Cys) and homocysteine (Hcy) at indicated concentrations in PBS buffer (pH 7.4). λex = 305 nm.

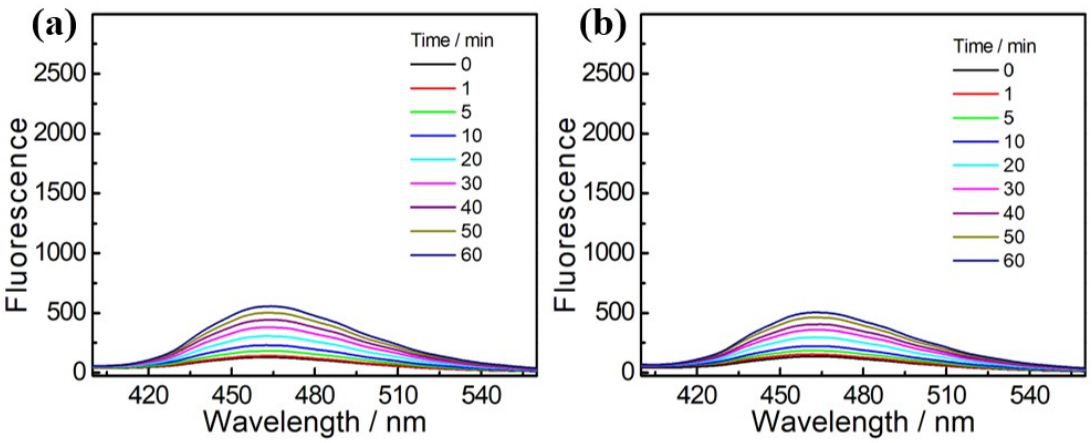


Fig. S5. Fluorescence spectra of probe **1** (1 μM) after incubation with 100 μM Cys (a) or 100 μM Hcy (b) for 1 h.

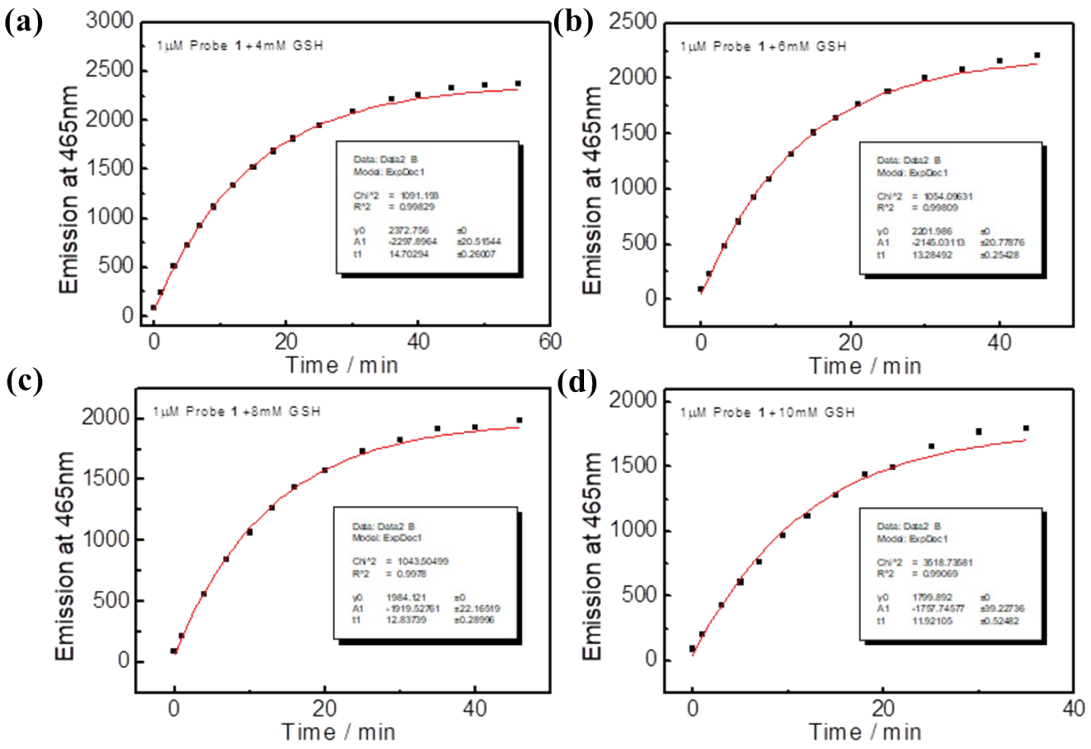


Fig.S6. Time-dependent fluorescence intensities of probe **1** (1 μM) upon treatment with different concentrations of GSH.

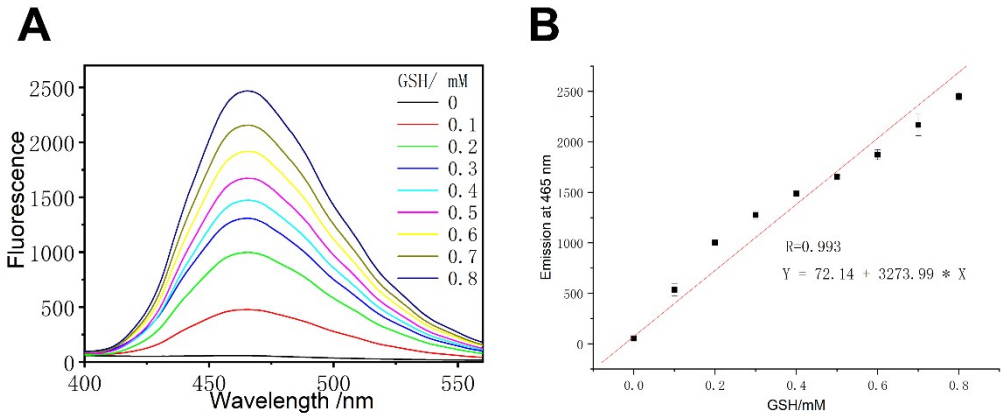


Fig.S7 The fluorescence response of probe **1** (1 μM) upon addition of GSH (0–0.8 mM) in PBS buffer. The detection limit was calculated to be 3.56 μM.

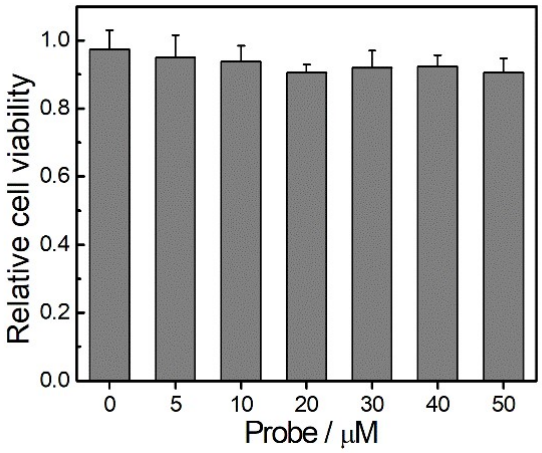
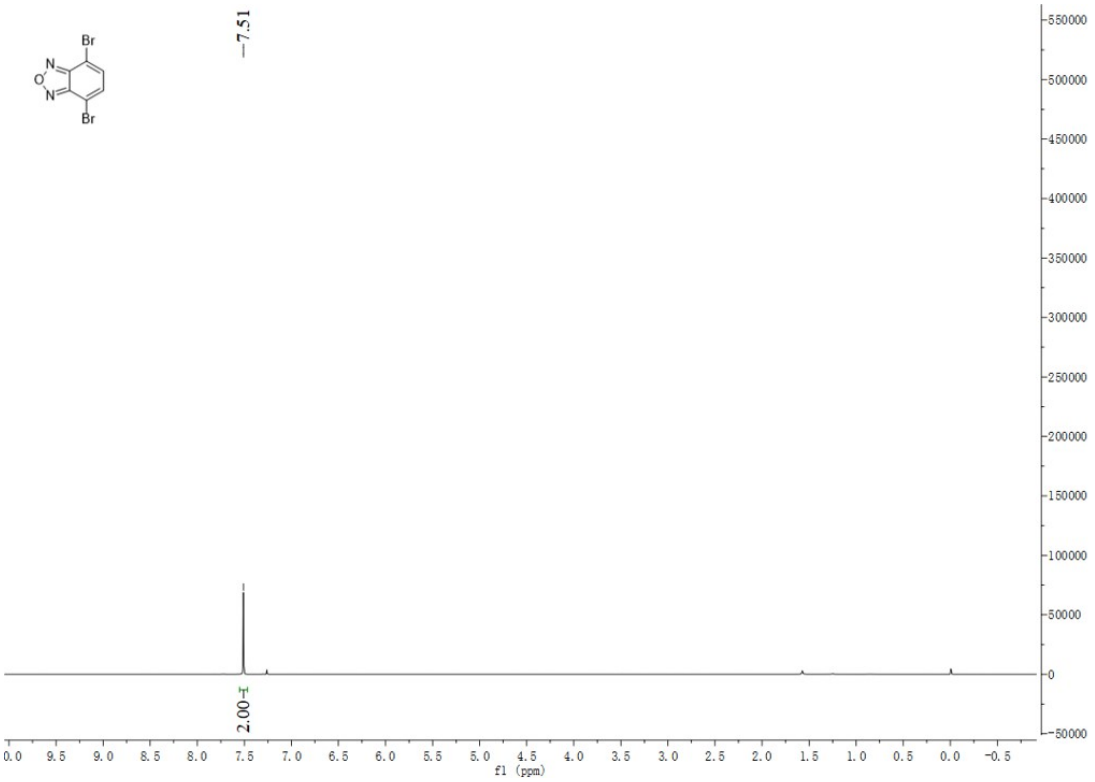
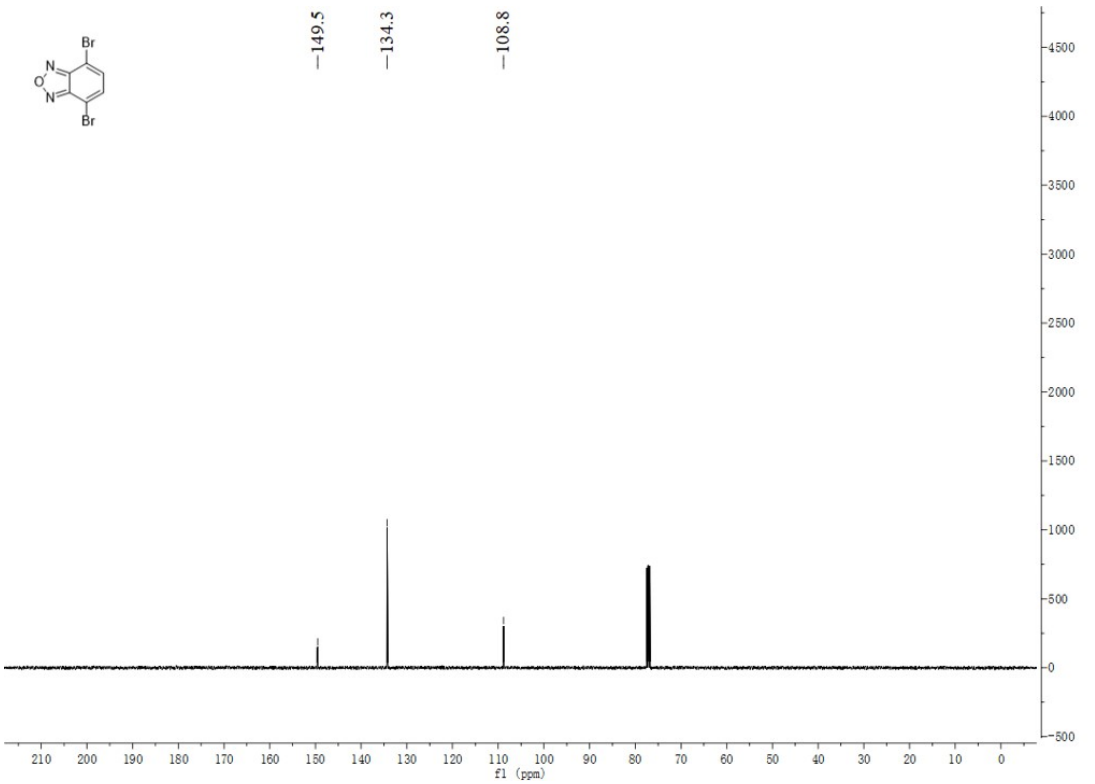
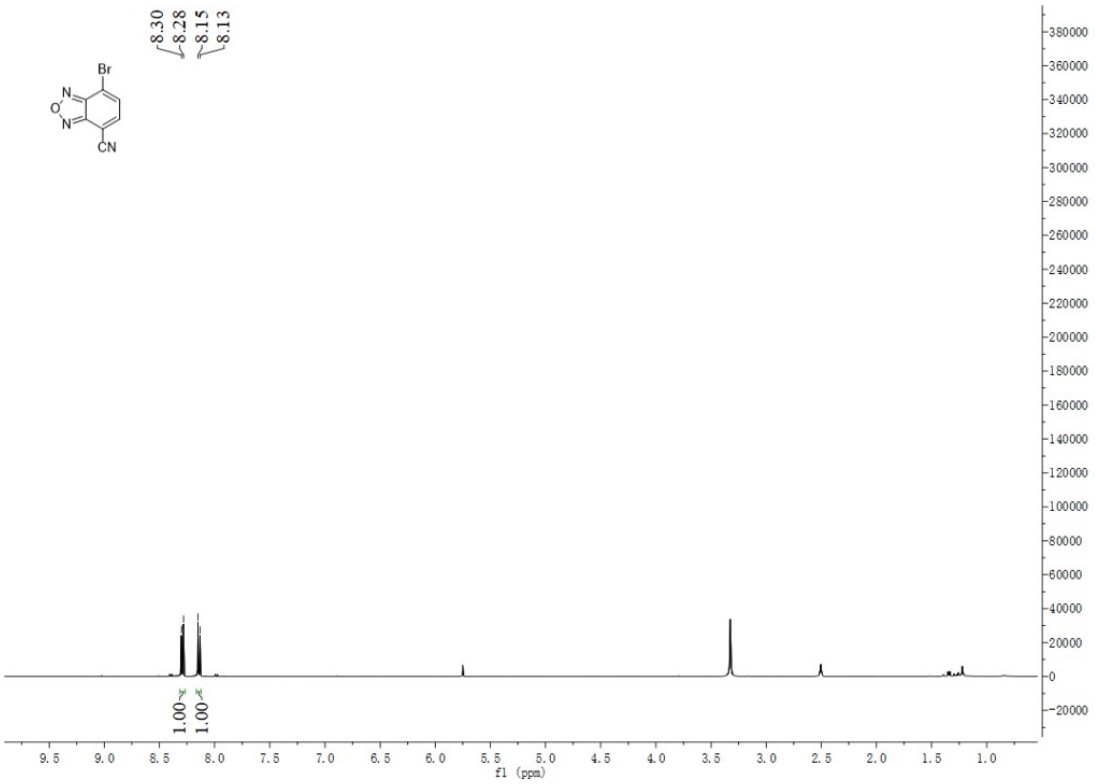


Fig.S8 Relative cell viability of HeLa cells after treatment for 24 h with probe **1** by MTT assay.

3.Supplementary NMR and HR-MS spectra



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8.13
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O
N
CN
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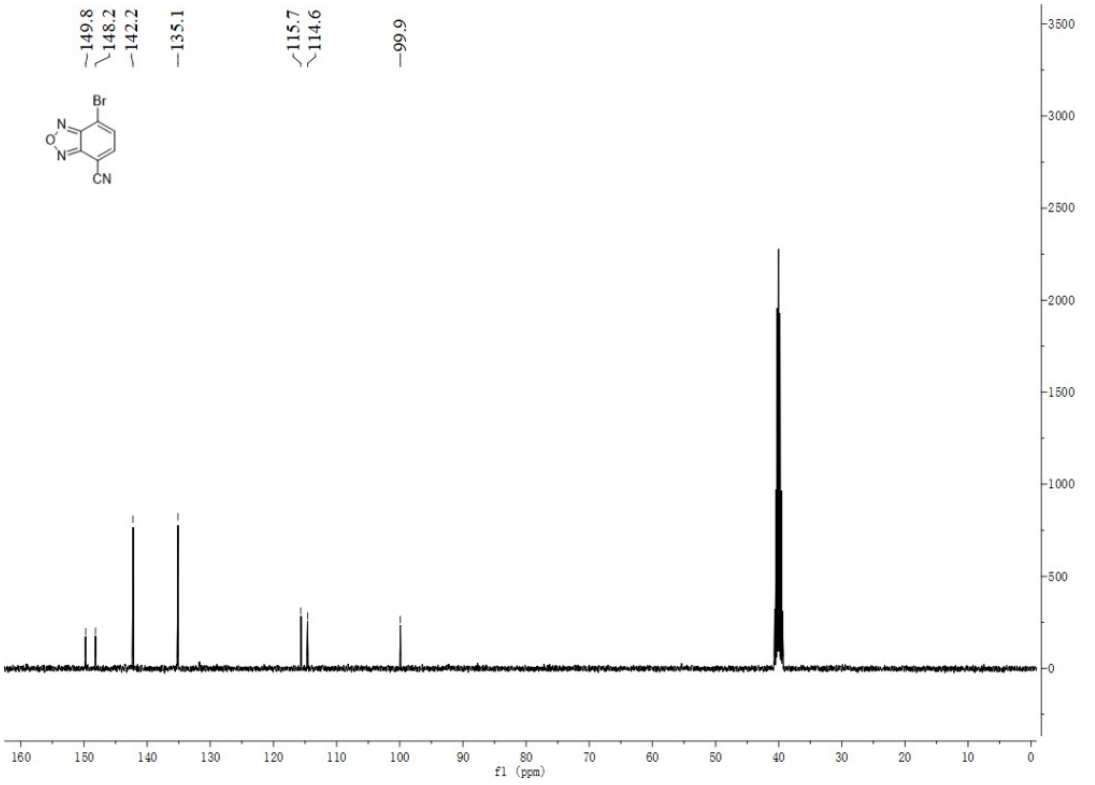
1.00
f1 (ppm)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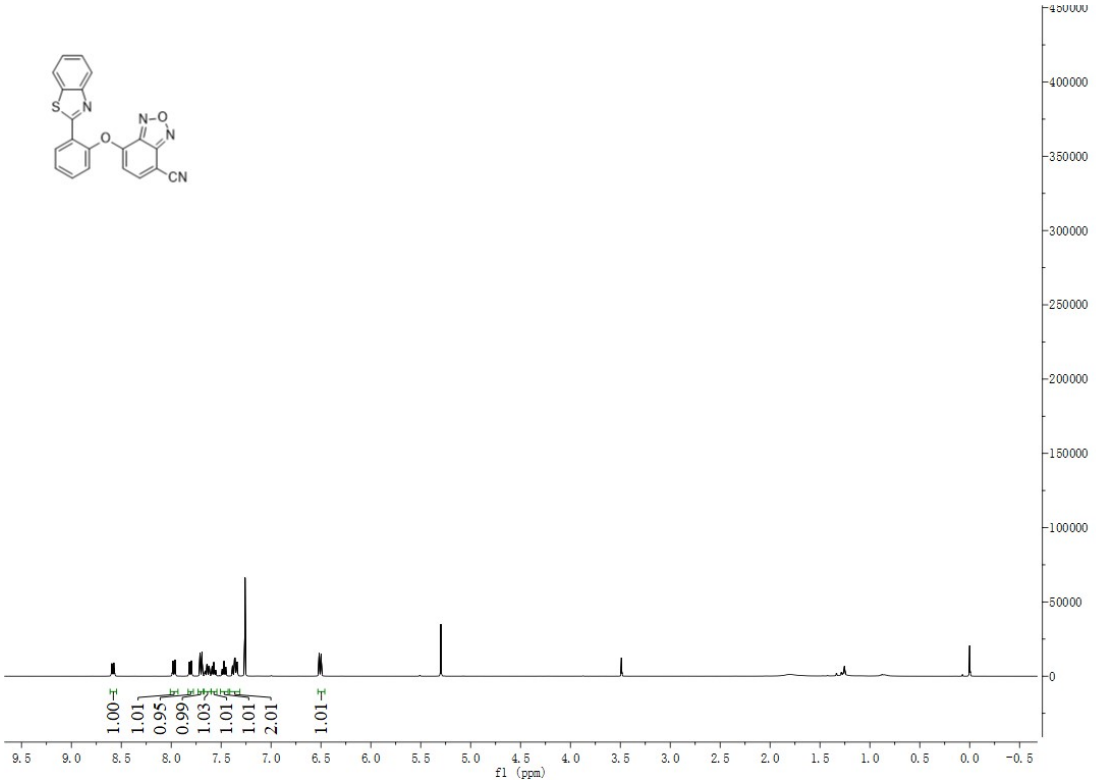
Br
N
O
N
CN
f1 (ppm)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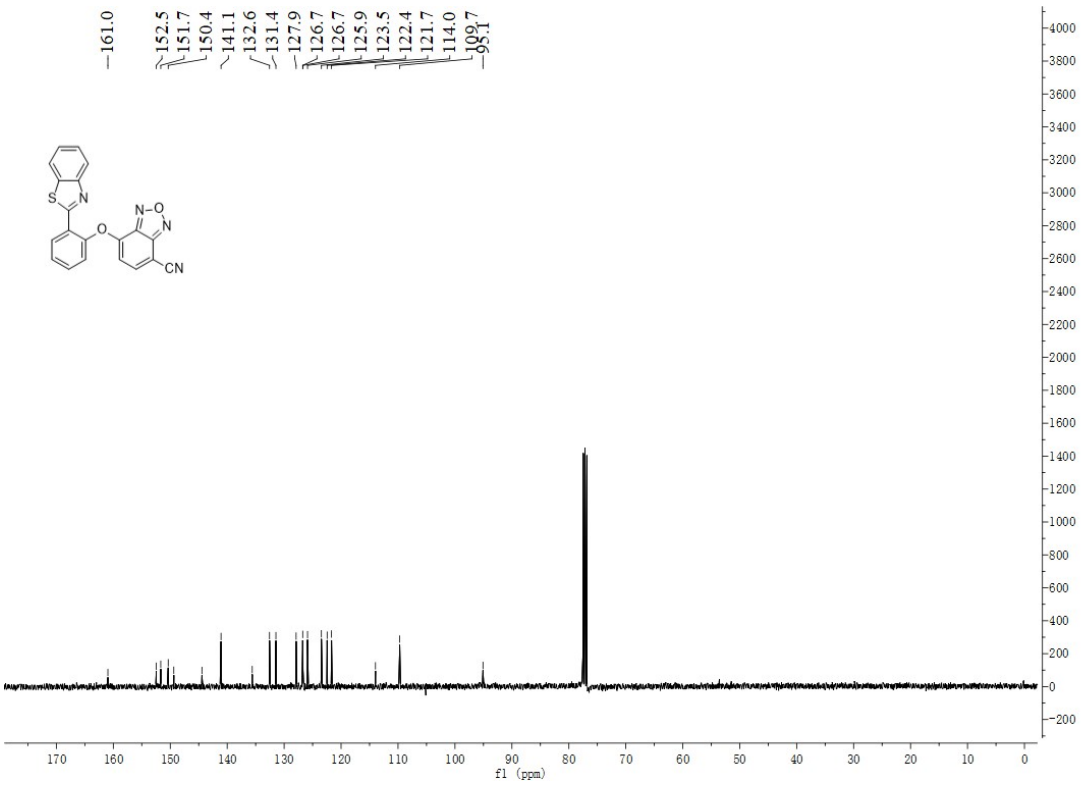
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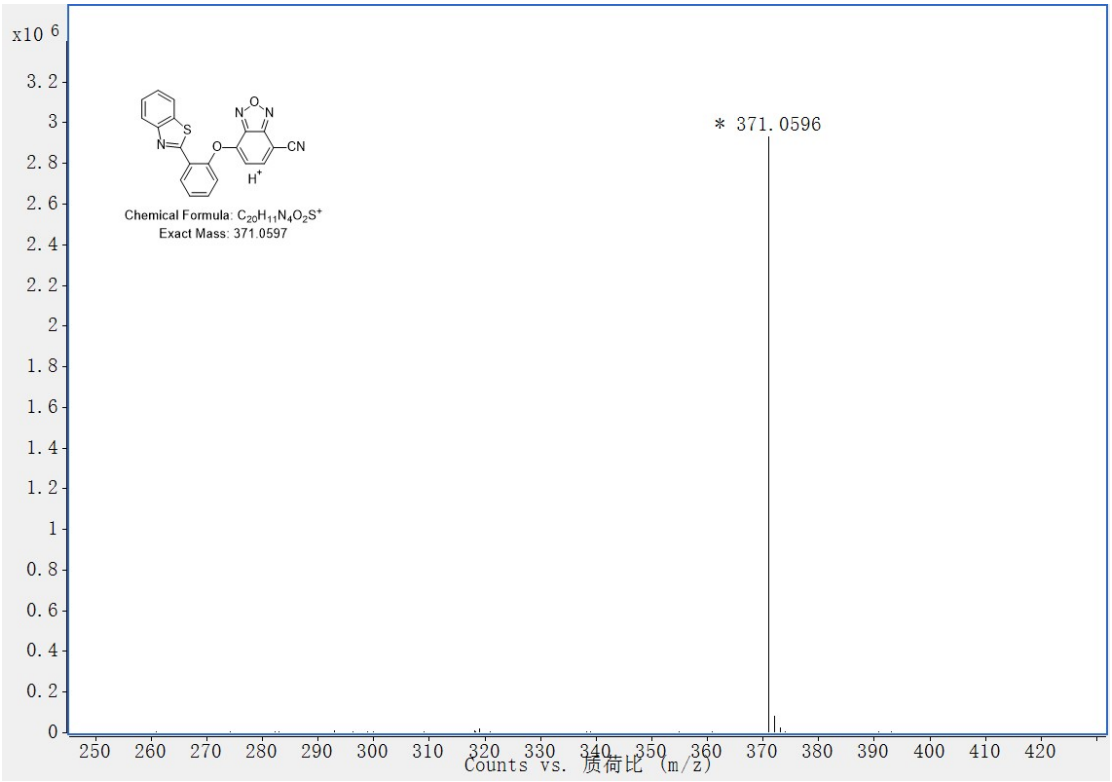
f1 (ppm)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0.6
0.4
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250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420
* 371.0596
Chemical Formula: $C_{20}H_{11}N_4O_2S^+$
Exact Mass: 371.0597
Counts vs. 质荷比 (m/z)