Supporting Information

A cysteine-activated fluorescent H_2S donor for visualizing H_2S release and alleviating cellular inflammation

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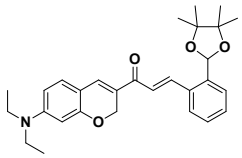
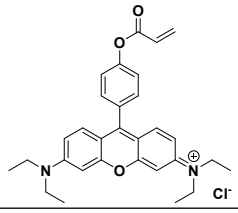
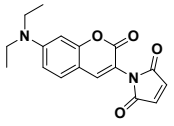
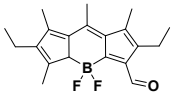
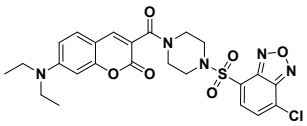
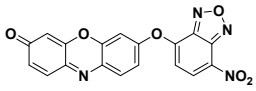
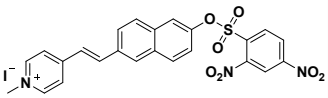
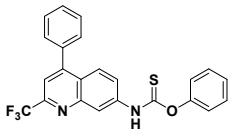
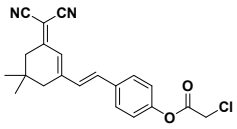
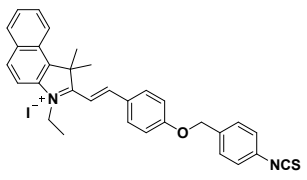
1. Materials and methods

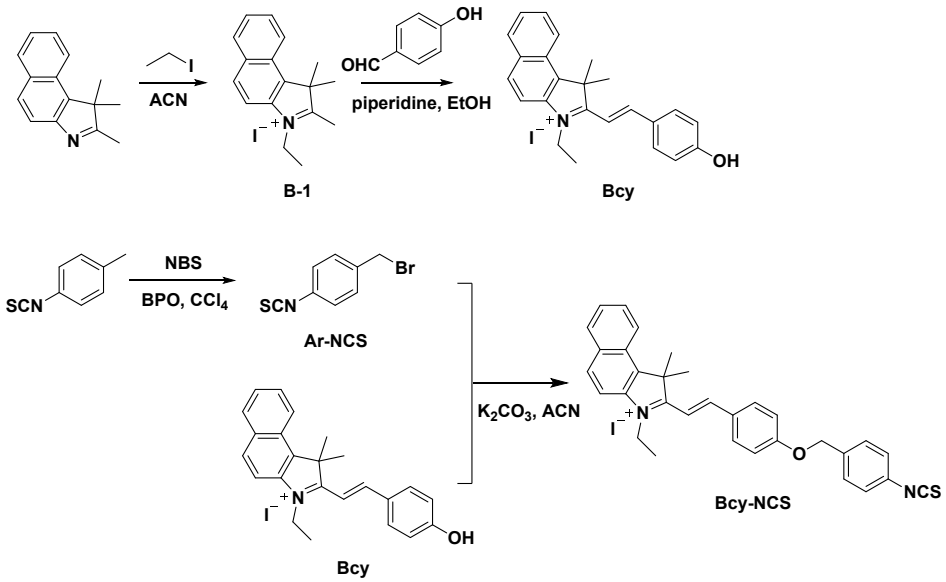
Except for piperidine and diethyl ether, which were provided by the reagent library of Shenyang Pharmaceutical University, anhydrous ACN, which was prepared by distillation, and the H_2S fluorescent probe **AzMC**, which was synthesized according to the literature, all other biological and chemical reagents, raw materials, and solvents were purchased from Adamas or Macklin. The purity of all raw materials was above 98%, and all solvents were of analytical grade or higher, with water content in anhydrous solvents below 50 ppm. Unless otherwise specified, further purification was not necessary prior to use. Lipopolysaccharide (LPS) and the Griess reagent kit were purchased from Beyotime. High-performance thin-layer chromatography plates (model: GF254) were purchased from Qingdao Marine Chemical Factory, and silica gel for column chromatography was 100-200 mesh and 200-300 mesh.

Fluorescence emission spectra were recorded on a fluorescence spectrophotometer (Hitachi F-7100). Absorption spectra were measured on a UV-visible spectrometer (UV-2201 spectrometer). Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker-ARX-600 running at 600 MHz. Chemical shifts (δ) were referenced to residual DMSO-d_6 (^{1}H NMR, 2.50) and $CDCl_3$ (^{1}H NMR, 7.26). High-resolution mass spectra (HRMS) were measured on a mass spectrometer (AB SCIEX Triple TOF5600) and the compound decomposition mechanism was verified. Live cell imaging was performed using an inverted fluorescence microscope (Olympus IX73). The cytotoxicity assay and cellular NO_2^- levels were recorded by measuring absorbance at 450 nm and 540 nm using a microplate reader (Thermo Varioskan Flash).

Table S1 Comparison of **Bcy-NCS** with the reported Cys probes.

Chemical structure	$\lambda_{ex}/\lambda_{em}$ (nm)	Stoke shift	Limit of detection	H_2S release function	Applications	Ref.
	460/498	38 nm	14 μM	–	Cell imaging	1
	549/584	35 nm	0.29 μM	–	Food detection	2
	410/495	85 nm	14 nM	–	Cell imaging	3
	480/547	67 nm	3.3 μM	–	Cell imaging	4
	450/580	130 nm	1.4 μM	–	Cell imaging, Zebrafish imaging	5
	470/540, 585	70 nm, 115 nm	0.13 μM (585 nm)	–	Human plasma detection	6
	370/485	115 nm	0.29 μM	–	Cell imaging	7
	390/518	128 nm	51 nM	Yes	Cell imaging, Zebrafish imaging	8
	430/590	160 nm	0.70 μM	–	Cell imaging	9
	**520/576**	**56 nm**	**0.193 μM**	**Yes**	**Cell imaging, Cellular inflammation alleviation**	**This work**

2. Synthesis



Scheme S1. Synthetic procedure of the **Bcy-NCS**.

Synthesis of B-1[10]

1,1,2-trimethyl-1*H*-benz[*e*]indole (500 mg, 2.39 mmol) was dissolved in 40 mL of ACN. Under a nitrogen atmosphere, iodoethane (250 μL, 3.63 mmol) was added, and the mixture was stirred under reflux at 80 ºC for 24 h. After confirming the completion of the reaction by TLC, the solvent was evaporated under reduced pressure. The crude product was then ground with a small amount of diethyl ether, filtered, and the filter cake was washed 2-3 times with a small amount of cold diethyl ether. The filter cake was dried to yield a silver-gray solid, **B-1** (700 mg, 81%). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.37 (d, J = 8.4 Hz, 1H), 8.30 (d, J = 8.9 Hz, 1H), 8.22 (d, J = 8.2 Hz, 1H), 8.16 (d, J = 8.9 Hz, 1H), 7.79 (t, J = 7.6 Hz, 1H), 7.73 (t, J = 7.5 Hz, 1H), 4.62 (q, J = 7.3 Hz, 2H), 2.94 (s, 3H), 1.76 (s, 6H), 1.51 (t, J = 7.3 Hz, 3H).

Synthesis of Bcy[10]

B-1 (600 mg, 2.5 mmol) and *p*-hydroxybenzaldehyde (370 mg, 3 mmol) were dissolved in 6 mL of anhydrous ethanol. After complete dissolution, 150 μL of piperidine was added. The reaction mixture was then refluxed overnight at 80-85 ºC under a nitrogen atmosphere. Upon confirming the completion of the reaction by TLC, the reaction mixture was filtered under vacuum, and the filter cake was washed with a small amount of cold ethanol. The filter cake was dried to yield a purplish-red solid, **Bcy** (540 mg, 70%).^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.08 (d, J = 9.4 Hz, 1H), 7.91 (t, J = 7.8 Hz, 2H), 7.76 (d, J = 14.0 Hz, 1H), 7.61 – 7.49 (m, 3H), 7.43 (t, J = 7.0 Hz, 1H), 7.28 (s, 1H), 6.56 (d, J = 9.1 Hz, 2H), 6.27 (d, J = 14.0 Hz, 1H), 4.10 (q, J = 7.3 Hz,

2H), 1.99 (s, 6H), 1.46 (t, J = 7.3 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 147.23, 139.29, 132.82, 131.57, 130.56, 130.09, 128.32, 127.72, 124.70, 124.56, 122.12, 121.94, 109.96, 96.32, 77.24, 77.03, 76.82, 50.64, 39.19, 27.68, 12.56.

Synthesis of Ar-NCS[11]

p-tolyl isothiocyanate (1.49 g, 10 mmol) and *N*-bromosuccinimide (1.79 g, 10 mmol) were dissolved in 20 mL of anhydrous CCl_4. A catalytic amount of benzoyl peroxide (24.2 mg, 0.1 mmol) was then added, and the mixture was stirred under reflux at 80 ºC for 12 h. After confirming the completion of the reaction by TLC, the reaction mixture was filtered, and the filtrate was evaporated under reduced pressure. The residue was dissolved in DCM and the solution was washed three times with saturated sodium thiosulfate until the organic layer was free of any apparent reddish-brown color. After removing the solvent under reduced pressure, the product was recrystallized from methanol to obtain white crystals of **Ar-NCS** (1.2 g, 53%).^{1}H NMR (600 MHz, $CDCl_3$) δ: 7.38 (d, J = 8.4 Hz, 2H), 7.19 (d, J = 8.4 Hz, 2H), 4.46 (s, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 136.90, 136.16, 131.20, 130.21, 125.99, 32.17.

Synthesis of Bcy-NCS

Bcy (50 mg, 0.15 mmol) and **Ar-NCS** (66 mg, 0.29 mmol) were dissolved in 5 mL of ACN followed by the addition of K_2CO_3 (40.3 mg, 0.29 mmol) and KI (2.42 mg, 0.015 mmol). The reaction mixture was then reacted for 8 h at 60 ºC under nitrogen protection. The reaction was monitored by TLC until completion, after which the reaction mixture was filtered and the filtrate was evaporated under reduced pressure. The residue was redissolved in DCM and washed 2-3 times with saturated brine, collecting the DCM layer each time. The DCM layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was further purified by silica gel column chromatography (eluent: DCM:MeOH = 40:1) to afford the orange-yellow solid **Bcy-NCS** (36 mg, 36%). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.53 (d, J = 16.2 Hz, 1H), 8.42 (d, J = 8.5 Hz, 1H), 8.28 (t, J = 9.1 Hz, 3H), 8.22 (d, J = 8.2 Hz, 1H), 8.11 (d, J = 8.9 Hz, 1H), 7.81 (t, J = 7.7 Hz, 1H), 7.72 (t, J = 7.6 Hz, 1H), 7.63 – 7.56 (m, 3H), 7.50 (d, J = 8.3 Hz, 2H), 7.26 (d, J = 8.7 Hz, 2H), 5.32 (s, 2H), 4.82 (q, J = 7.3 Hz, 2H), 2.02 (s, 6H), 1.50 (t, J = 7.2 Hz, 3H).^{13}C NMR (150 MHz, DMSO-d_6) δ: 182.31, 163.09, 153.24, 138.75, 138.59, 136.77, 133.62, 133.58, 131.53, 130.52, 130.15, 130.12, 129.74, 128.90, 128.20, 127.59, 127.27, 126.62, 123.55, 116.21, 113.54, 110.00, 69.38, 54.12, 42.60, 26.07, 14.34. HRMS (ESI): *m/z* calcd for $C_{32}H_{29}N_2OS^+$ $[M]^+$ 489.1995. Found 489.1992.

3. Test systems and spectral measurements

Preparation of solutions: An appropriate amount of donor **Bcy-NCS** was weighed and dissolved in DMSO to prepare a 10 mM stock solution. Similarly, an appropriate amount of Cys was weighed and dissolved in deionized water to prepare a 50 mM stock solution. Unless otherwise specified, this concentration of stock solutions was used for subsequent evaluation experiments.

UV absorption and fluorescence testing of donor **Bcy-NCS**: An appropriate amount of the donor stock solution was diluted with PBS buffer (pH = 7.4, 10 mM, 20% DMSO) to a final concentration of 10 μM. Two separate solutions were prepared: one served as a blank, and the other was supplemented with the Cys stock solution to achieve a final concentration of 100 μM. The solutions were then incubated in the dark at 37 ºC for 1 h, after which the UV absorption and fluorescence emission spectra of the two solutions were measured. For the fluorescence testing of donor **Bcy-NCS**, an excitation wavelength of 520 nm was used, with excitation and emission slit widths set to 20 nm and 5 nm, respectively. Fluorescence intensity data were collected in the range of 550-650 nm.

4. Optical response experiment of Bcy-NCS to Cys

Preparation of a PBS buffer solution (pH = 7.4, 10 mM, 20% DMSO) containing **Bcy-NCS** at a final concentration of 10 μM was performed. The Cys stock solution was then added to achieve final concentrations of 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 μM. Each solution was incubated in the dark at 37 ºC for 1 h, and the fluorescence emission spectra were measured. Each concentration was measured in parallel three times.

The detection limit was calculated by plotting the fluorescence intensity of **Bcy-NCS** at 576 nm against the Cys concentration. The linear concentration range of Cys and the corresponding fluorescence intensity was determined, and the linear regression equation was calculated. Limit of detection (LOD) was then calculated using the formula LOD = 3σ/k, where k is the slope of the linear regression equation and σ is the standard deviation obtained from 11 measurements of the fluorescence intensity of the blank solution.

5. Fluorescence response kinetics and photostability experiment of Bcy-NCS

Prepare a PBS buffer solution (pH = 7.4, 10 mM, 20% DMSO) containing **Bcy-NCS** at a final concentration of 10 μM. Add the Cys stock solution to achieve a concentration of 400 μM, and measure the fluorescence emission spectra at 0 min, every 2 min from 1 to 59 min, and at 60 min. Each incubation time was measured in parallel three times.

Photostability Experiment: In the fluorescence kinetics experiments for **Bcy-NCS**, once the fluorescence intensity reached a plateau, the photostability was tested using the time-scan function of the fluorescence spectrophotometer for 1 h.

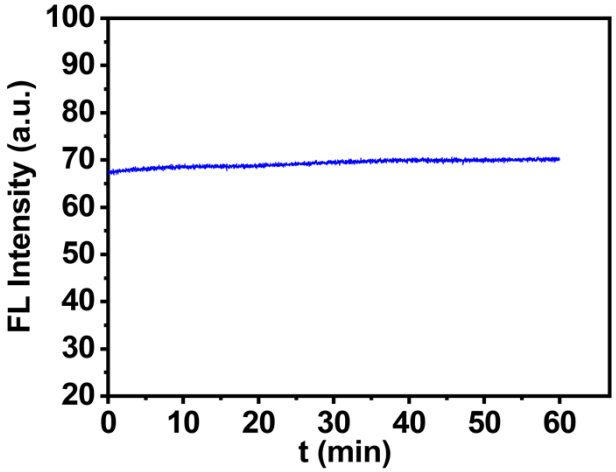
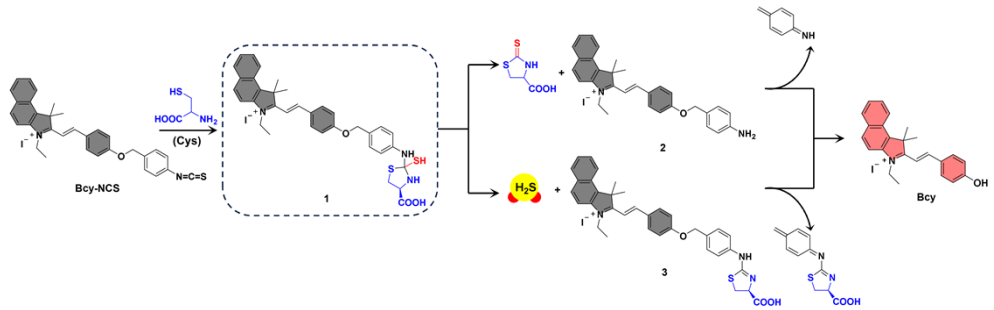


Figure S1. Photostability of **Bcy-NCS** (10 μM) after reacting with Cys (400 μM) for 1 h at the plateau phase. Test conditions: λ_{ex} = 520 nm, slit width: 20/5 nm, PBS buffer solution (pH = 7.4, 10 mM, 20% DMSO) at 37 ºC.

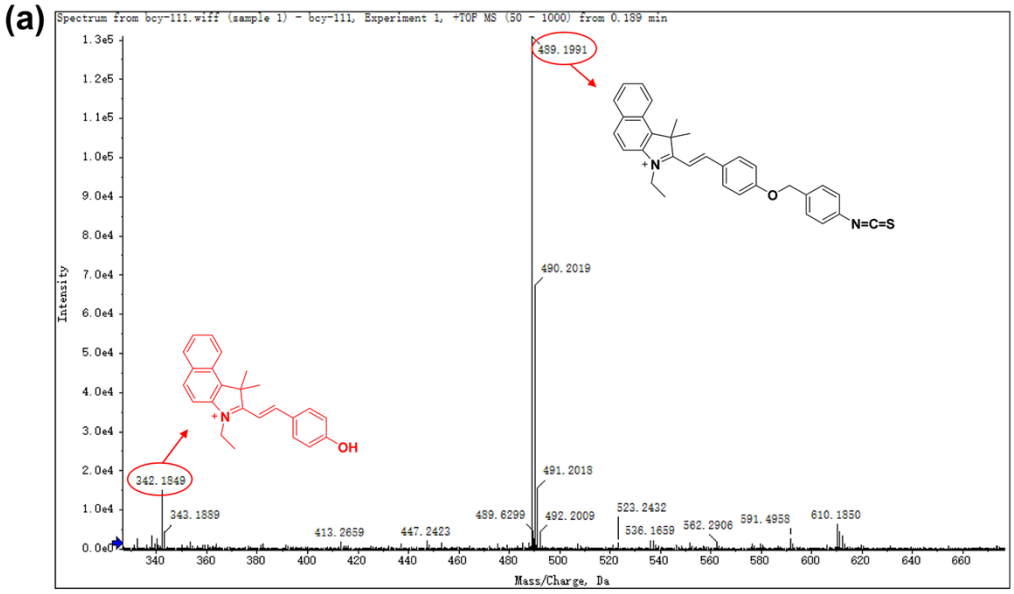
6. Verification experiment for the reaction mechanism between Cys and Bcy-NCS

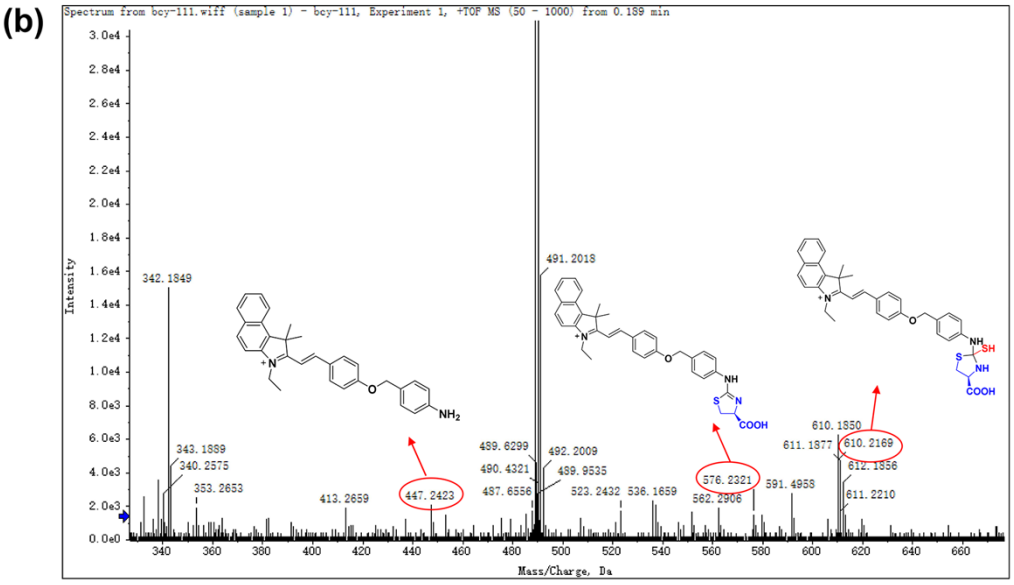
A 10 mM stock solution of the donor Bcy-NCS was prepared in chromatographic-grade ACN. An aliquot of this stock solution was then diluted to 10 μM with an ACN/H_2O (3:7, v/v) solution. Next, a Cys stock solution was added to achieve a final concentration of 100 μM. Two experimental groups were established: one was incubated at 37 °C for 15 min, while the other was incubated at 37 °C for 1 h. After incubation, the mixtures from both groups were extracted and the organic layers were collected. Following solvent evaporation, the residues were re-dissolved in chromatographic-grade ACN. An appropriate amount of each solution was then diluted to 1 μM using ACN. A 1 mL aliquot from each group was filtered through a 0.22 μm

microporous membrane into HPLC vials, and the compositions of the mixtures were then qualitatively analyzed by ESI-MS.



Scheme S2. Reaction mechanism of **Bcy-NCS** with Cys





(c)

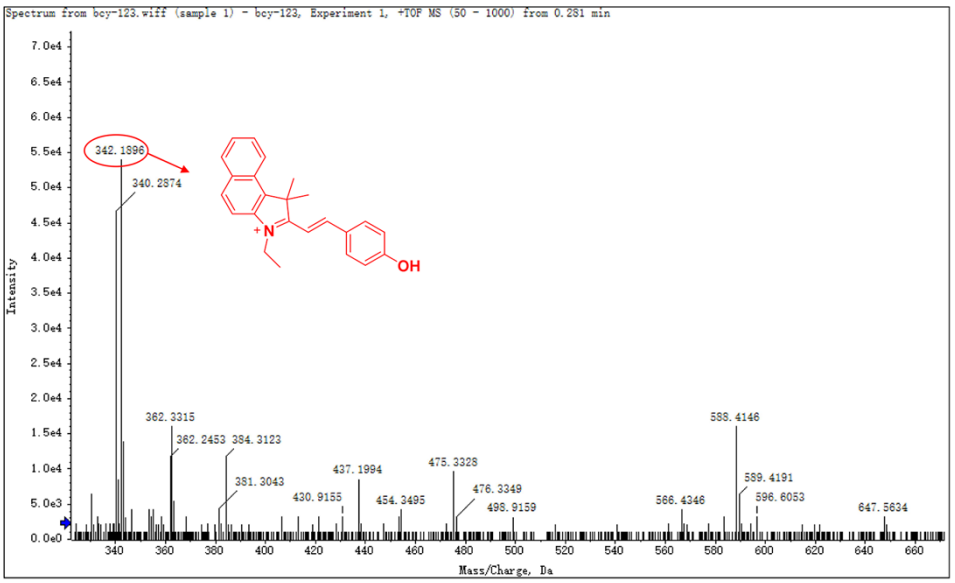


Figure S2. (a) ESI-MS spectrum of **Bcy-NCS** (10 μM) after reacting with Cys (100 μM) for 15 min; (b) magnified view of the selected region from (a); (c) ESI-MS spectrum of **Bcy-NCS** (10 μM) after reacting with Cys (100 μM) for 1 h. Test mode: positive ion mode

7. pH stability and biological media selectivity experiments of Bcy-NCS

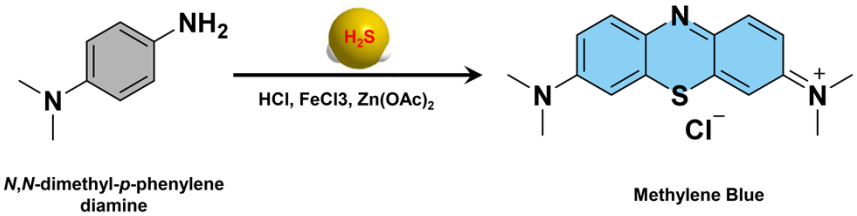
A PBS buffer solution (pH = 7.4, 10 mM, 20% DMSO) containing 10 μM of donor **Bcy-NCS** was prepared, divided into nine portions, and the pH of eight portions was adjusted to 3.5, 4.5, 5.5, 6.5, 8.5, 9.5, 10.5, and 11.5 using a pH meter. The Cys stock solution was added to each portion of the donor **Bcy-NCS** solution to achieve a final concentration of 400 μM. After a 1 h incubation, the fluorescence intensity at 576 nm was measured. The measurements were repeated three times under each pH condition, and the corresponding scatter plots were generated.

Seventeen common biological mediators were selected, including cations (Mg^{2+}, Fe^{2+}, and Fe^{3+}), anions ($S_2O_3^{2-}$, SO_3^{2-}, SO_4^{2-}, and NO_2^-), amino acids (Trp, Tyr, Phe, Gly, Lys, and Ser), biothiols (Hcy and GSH), as well as $Na_2S·9H_2O$ and H_2O_2. All biological mediators were prepared as 50 mM stock solutions for later use. Sixteen separate portions of a PBS buffer solution (pH = 7.4, 10 mM, 20% DMSO) containing 10 μM of donor **Bcy-NCS** were prepared, and each was supplemented with a different biological mediator to a final concentration of 400 μM. After a 1 h incubation, the fluorescence emission spectra were measured. Each measurement was repeated three times for each biological mediator, and the corresponding curves and histograms were generated.

8. H_2S release experiment of Bcy-NCS

8.1 MB method for testing H_2S release

First, the H_2S release standard curve was tested and plotted using the MB method.[12] The detection solution for the MB method consisted of 40% 30 mM ferric chloride, 40% 20 mM *N*,*N*-dimethyl-1,4-phenylenediamine sulfate, and 20% 1% w/v zinc acetate. The detection mechanism is detailed in **Scheme S3**. Next, Na_2S standard solutions were prepared: $Na_2S{\cdot}9H_2O$ was dissolved in deionized water to prepare a 5 mM Na_2S stock solution, which was then diluted with PBS buffer (pH = 7.4, 10 mM, 20% DMSO) to obtain Na_2S solutions at concentrations of 0, 5, 10, 20, 40, 60, and 80 μM. 2 mL of each Na_2S solution was placed in a labeled brown vial, and 1 mL of the MB assay solution was added. The reaction was conducted at room temperature and protected from light for 30 min. After the reaction, 200 μL of the reaction mixture was transferred into a 96-well plate, with three replicate wells, and the absorbance at 670 nm was measured using a microplate reader. The experiment was repeated three times in parallel, and the absorbance curves of Na_2S concentration at 670 nm were plotted. The linear regression equations of the curves were calculated and combined.



Scheme S3. Mechanism of H_2S detection by MB method

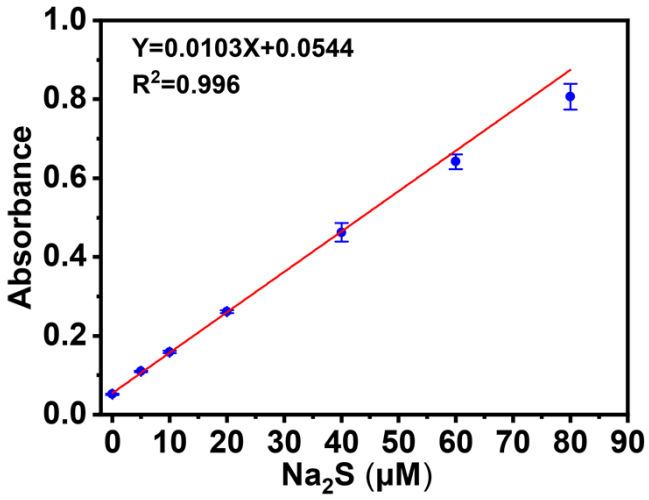


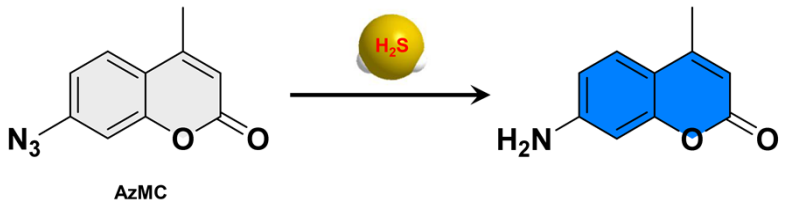
Figure S3. Calibration curve for H_2S determined by the MB method. Test conditions: absorbance measured at 670 nm in PBS buffer (pH 7.4, 10 mM, 20% DMSO) at 37 ºC.

The release of H_2S from donor **Bcy-NCS** was subsequently tested. A PBS buffer solution (pH = 7.4, 10 mM, 20% DMSO) containing 10 μM of donor **Bcy-NCS** was prepared, and the Cys stock solution was added to achieve a final concentration of 100 μM. 2 mL of the donor mixture were placed in labeled brown penicillin vials, and six groups were set up in parallel, each corresponding to a specific incubation time point. The reaction mixtures were incubated at 37 ºC for 0.5, 1, 2, 4, 6, and 8 h. After each incubation period, 1 mL of the MB assay solution was added to the vials, and the reaction was conducted at room temperature for 30 min, protected from light. After the reaction, 200 μL of the reaction solution was transferred into a 96-well plate with three replicate wells, and the absorbance at 670 nm was measured using a microplate reader. The experiments were repeated three times in parallel, and the measured absorbance was then converted to the corresponding H_2S concentration using the H_2S release standard curve. Scatter plots of H_2S concentration versus incubation time were then generated.

8.2 Verification of H_2S release by fluorescent probe method

The classical H_2S fluorescent probe **AzMC** was selected to verify the ability of the donor **Bcy-NCS** to release H_2S. **AzMC** was synthesized following a reported procedure[13], and its structure was confirmed by NMR (Figure S14 and S15). The detection mechanism of **AzMC** is detailed in **Scheme S4**.

Four experimental setups were established: **AzMC**, **AzMC** + Cys, **AzMC** + **Bcy-NCS**, and **AzMC** + **Bcy-NCS** + Cys. The concentrations of the compounds were as follows: **AzMC** (10 μM), **Bcy-NCS** (10 μM), and Cys (100 μM). **Bcy-NCS** was incubated with Cys at 37 ºC for 2 h, after which **AzMC** was added, and the reaction was protected from light. Following incubation with Cys for 2 h at 37 ºC, **AzMC** was added, and the mixture was allowed to react for 30 min under light protection; similarly, **AzMC**, **AzMC** + Cys, and **AzMC** + **Bcy-NCS** were incubated for 30 min as controls. After the reaction, the fluorescence intensity was measured using a fluorescence spectrophotometer, with an excitation wavelength of 350 nm and a detection range of 400-500 nm.



Scheme S4. AzMC mechanism for detecting H_2S

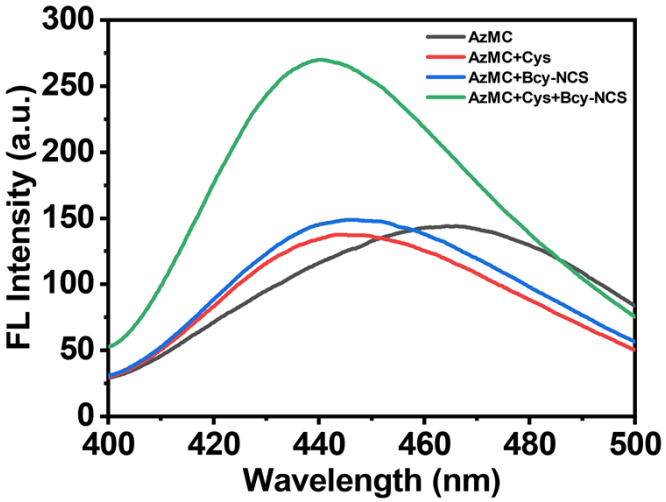


Figure S4. Fluorescence emission spectra of **AzMC** (10 μM) reacting with Cys (100 μM), **Bcy-NCS** (10 μM), and the mixture of Cys (100 μM) and **Bcy-NCS** (10 μM). Test conditions: λ_{ex} = 350 nm, slit width: 5/5 nm, in PBS buffer (pH 7.4, 10 mM, 20% DMSO) at 37 ºC.

9. Cell culture and cytotoxicity assays

HaCat or RAW 264.7 cells were cultured in DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 ºC in a 5% CO_2 environment.

HaCat or RAW 264.7 cells were seeded in 96-well plates and cultured at 37 ºC in a 5% CO_2 environment. After the cells adhered, the original medium was discarded, and the cells were treated with DMEM containing different concentrations of the drug for 12 or 24 h. After incubation, the old DMEM was discarded, and the cells were washed 1-2 times with PBS buffer. The CCK-8 solution was diluted 10-fold with DMEM, and 100 μL of this solution was added to each well of the 96-well plates. The plates were then incubated for an additional 2-4 h at 37 ºC in a 5% CO_2 environment. Finally, the absorbance at 450 nm was measured using a microplate reader. Four replicate wells were set up for each drug concentration, and the experiment was repeated three times in parallel.

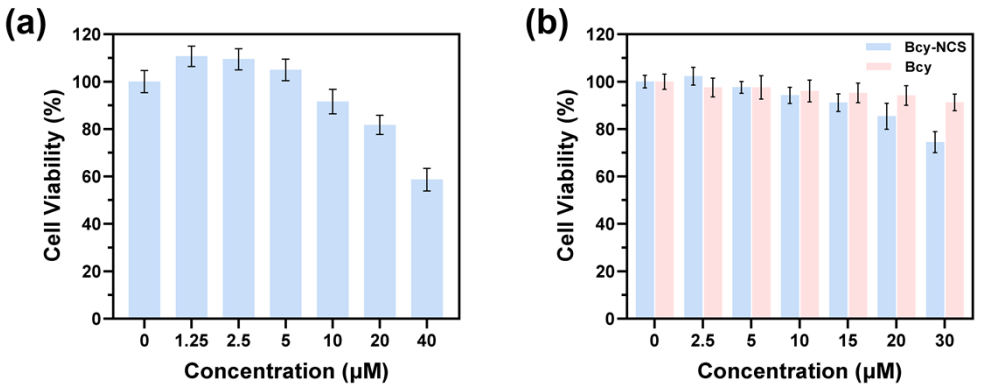


Figure S5. (a) Cell viability of HaCat cells after 12 h treatment with **Bcy-NCS**. (b) Cell viability of RAW 264.7 cells after 24 h treatment with **Bcy-NCS** or **Bcy**. Data are presented as the mean ± SD from three independent experiments.

10. Cellular imaging experiments

The concentrations of compounds used for cell imaging were: **AzMC** (10 μM), **Bcy-NCS** (10 μM), Cys (400 μM), and NEM (500 μM).

HaCat cells were seeded in 96-well plates at a density of 10^4 cells per well and cultured overnight at 37 ºC in a 5% CO_2 environment, with two plates set up in parallel. After the cells adhered to the plate, the original medium was discarded from both plates. The cells in one plate were divided into three groups and treated with DMEM containing only **Bcy-NCS**, DMEM containing **AzMC**, and DMEM containing **Bcy-NCS** + **AzMC**, respectively. The cells in the other plate were pretreated with NEM-containing DMEM for 1 h. After treatment, the old medium was discarded, and the cells were washed 2-3 times with PBS buffer. The cells were then divided into two groups and treated with DMEM containing **Bcy-NCS** + **AzMC** and DMEM containing **Bcy-NCS** + **AzMC** + Cys, respectively. **Bcy-NCS** was incubated for 1 h, Cys for 30 min, and **AzMC** for 30 min. After all incubations were completed, the cells were washed 2-3 times with PBS buffer. Finally, live cell imaging was performed using an inverted fluorescence microscope. Bright field images were recorded, red channel fluorescence images were collected using a green light excitation block, and blue channel fluorescence images were collected using a UV excitation block.

11. Anti-inflammatory studies of Bcy-NCS

The concentrations of compounds used for anti-inflammatory studies were as follows: LPS (500 ng/mL), **Bcy-NCS** (5, 10, 15 μM), DEX (10 μM), and **Bcy** (15 μM).

RAW 264.7 cells were seeded in 96-well plates at a density of 5×10^4 cells/well and cultured at 37 ºC in a 5% CO_2 environment. After the cells adhered, the original medium was discarded, and the cells were divided into seven groups. The control group received no treatment, while the remaining six experimental groups were treated with LPS, LPS + **Bcy-NCS** (5 μM), LPS + **Bcy-NCS** (10 μM), LPS + **Bcy-NCS** (15 μM), LPS + DEX, and LPS + **Bcy**, respectively, for 24 h.

The NO_2^- levels in the cells were measured using a Griess reagent kit. In a new 96-well plate, 50 μL of supernatant from the cell culture of each group, 25 μL of Griess Reagent I, and 25 μL of Griess Reagent II were sequentially added to each well (100 μL total per well). The absorbance was measured at 540 nm, and nitrite levels were

calculated based on a standard curve. Three replicate wells were set up for each drug concentration, and the experiment was repeated three times in parallel.

12. NMR spectra and HRMS

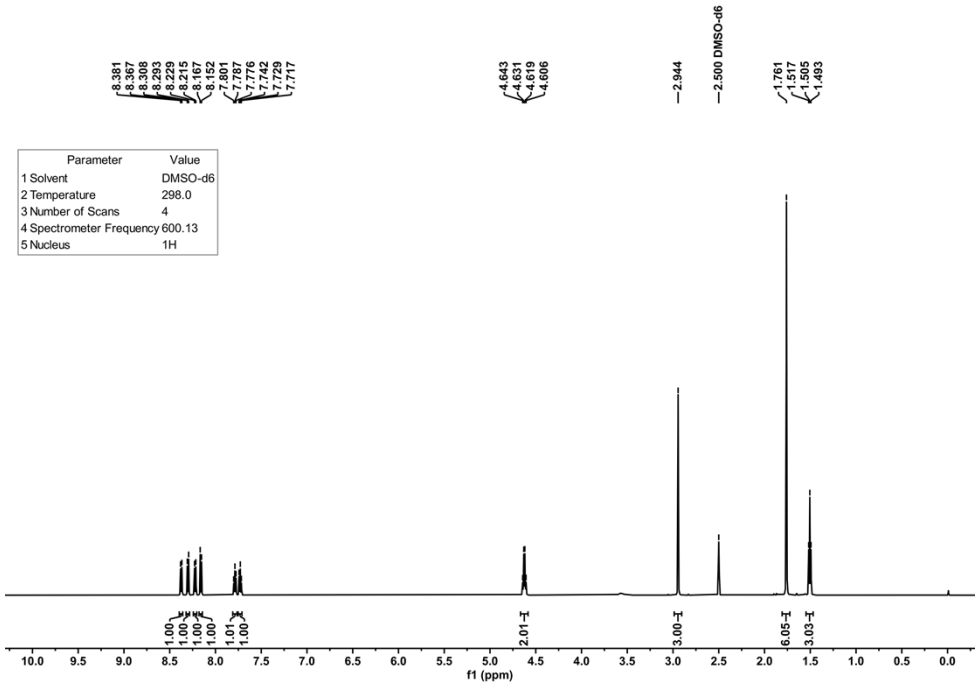


Figure S6. ^{1}H NMR spectrum of **B-1** in DMSO-d_6 (600 MHz)

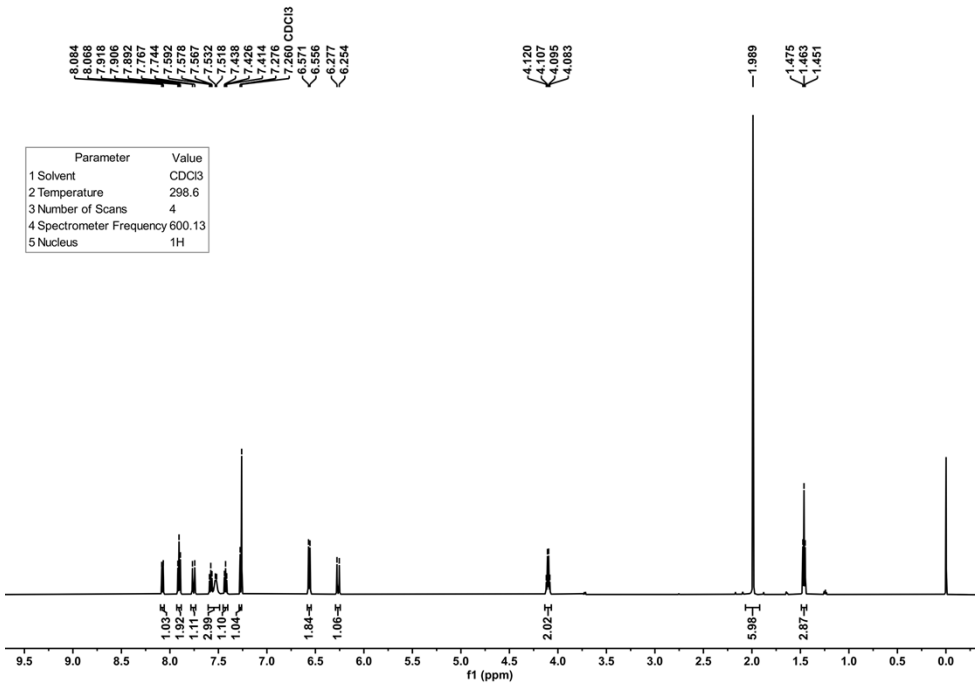


Figure S7. ^{1}H NMR spectrum of **Bcy** in $CDCl_3$ (600 MHz)

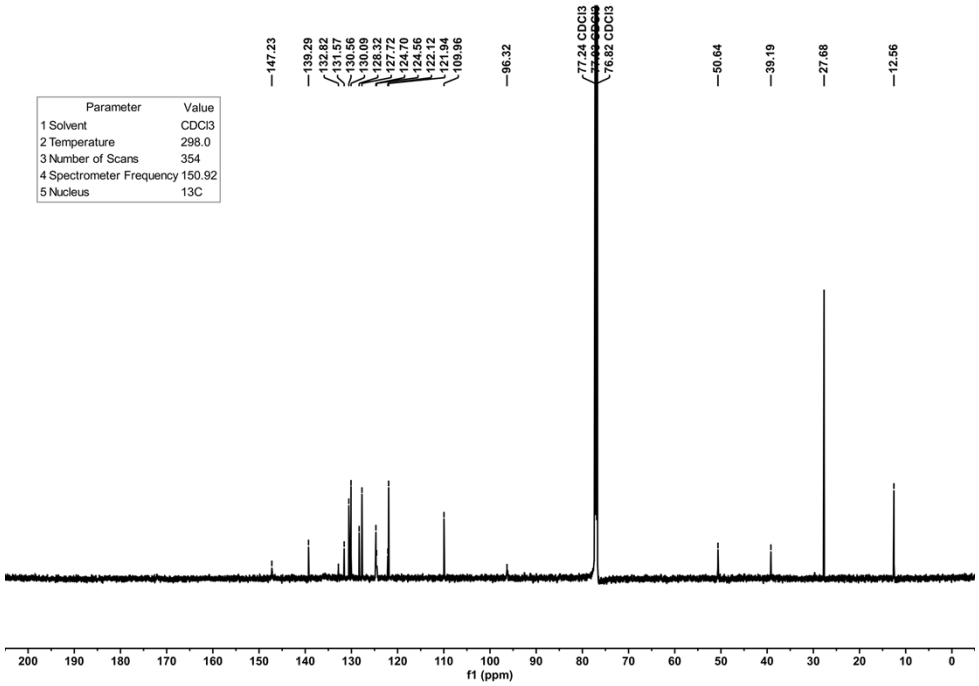


Figure S8. ^{13}C NMR spectrum of **Bcy** in $CDCl_3$ (150 MHz)

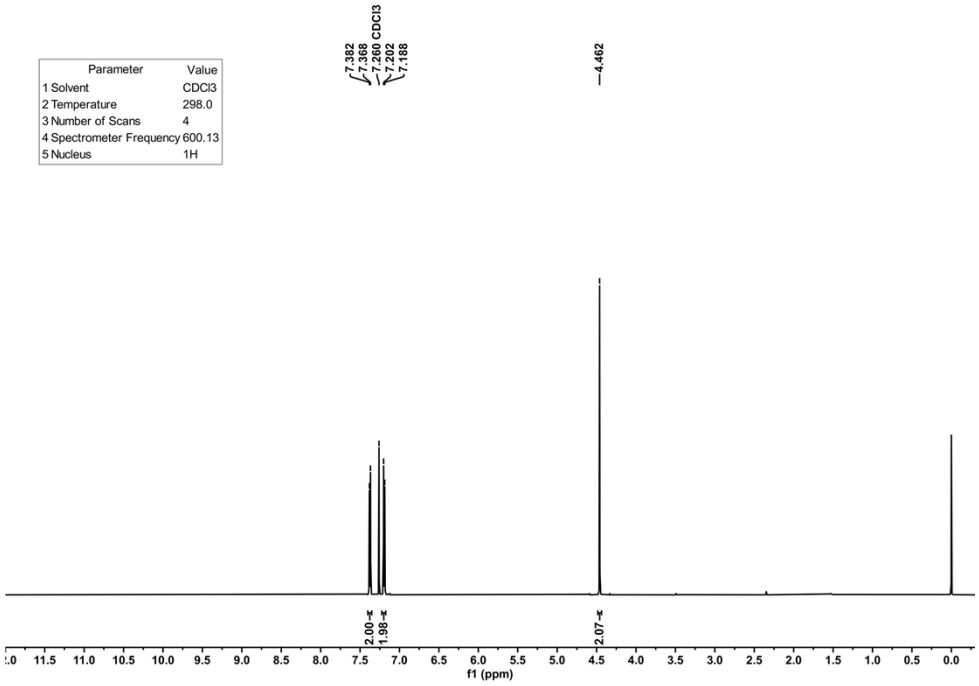


Figure S9. ^{1}H NMR spectrum of **Ar-NCS** in $CDCl_3$ (600 MHz)

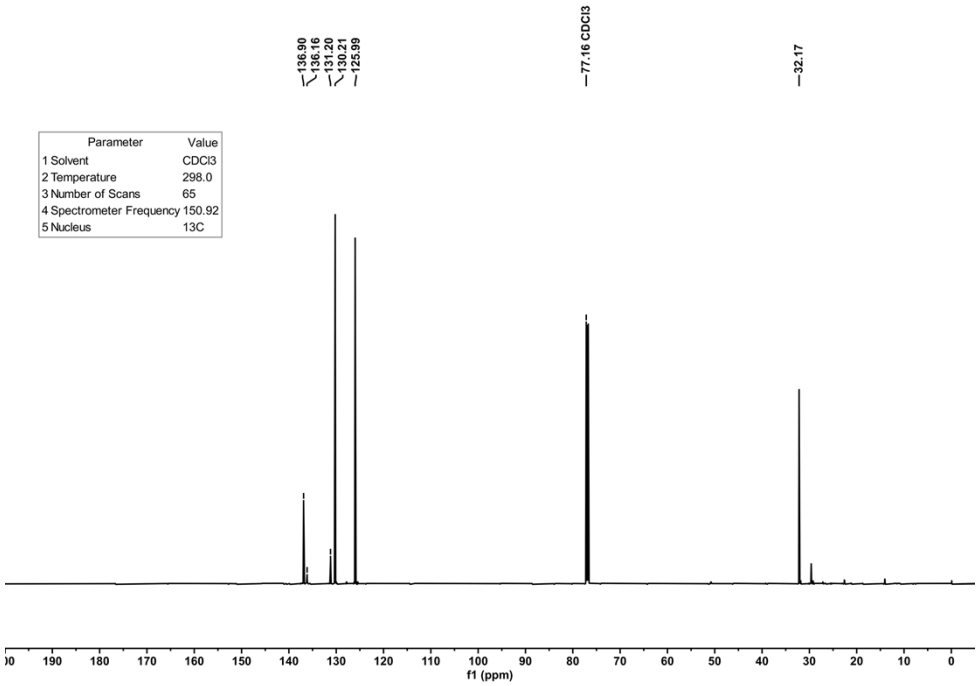


Figure S10. ^{13}C NMR spectrum of **Ar-NCS** in $CDCl_3$ (150 MHz)

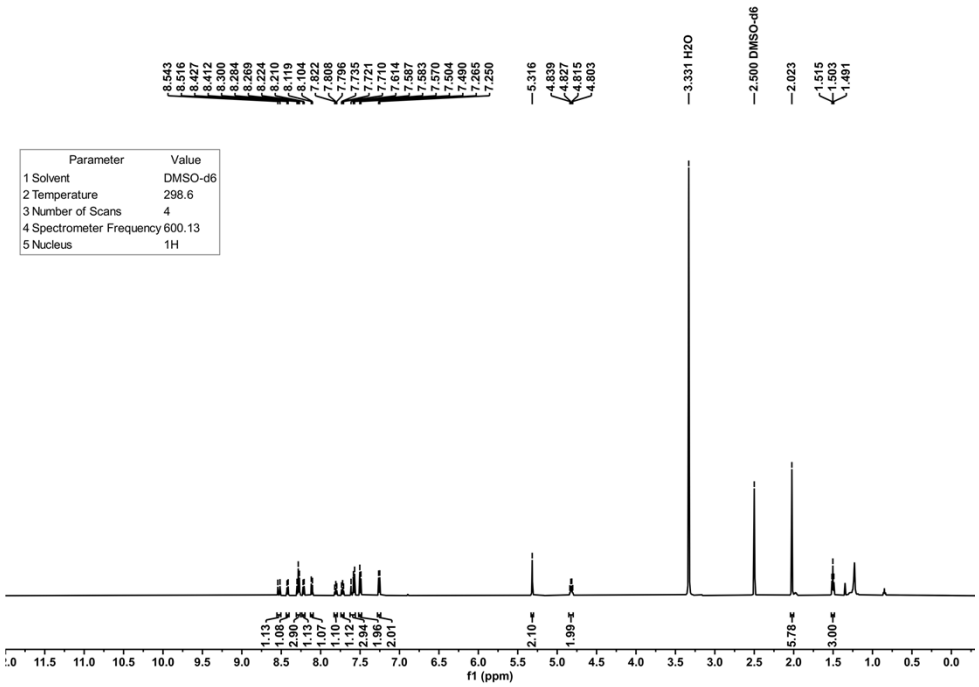


Figure S11. ^{1}H NMR spectrum of **Bcy-NCS** in DMSO-d_6 (600 MHz)

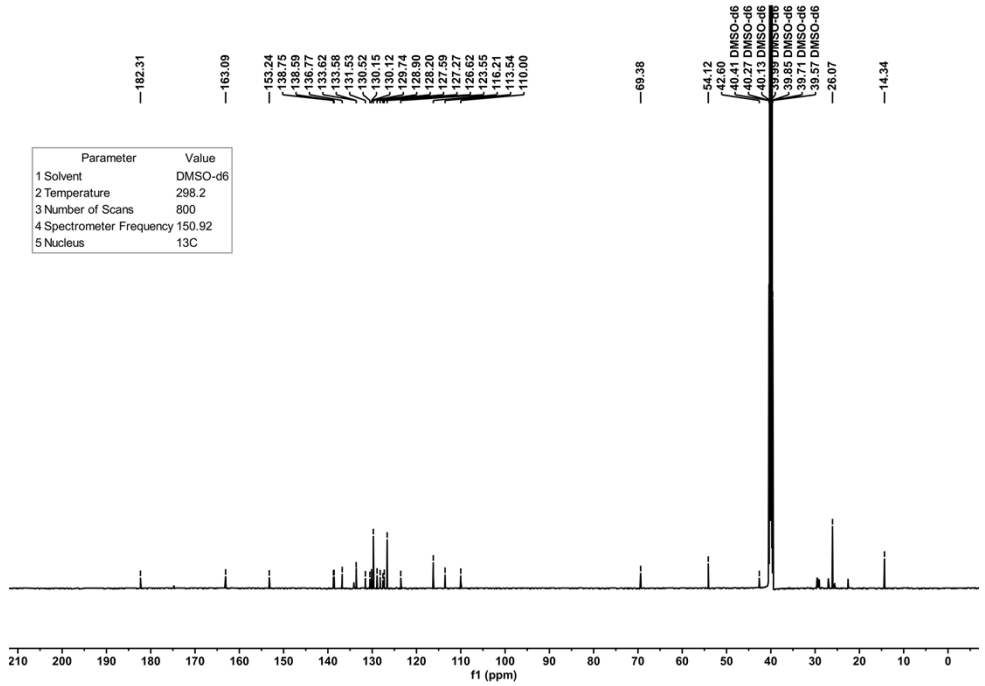


Figure S12. ^{13}C NMR spectrum of **Bcy-NCS** in DMSO-d_6 (150 MHz)

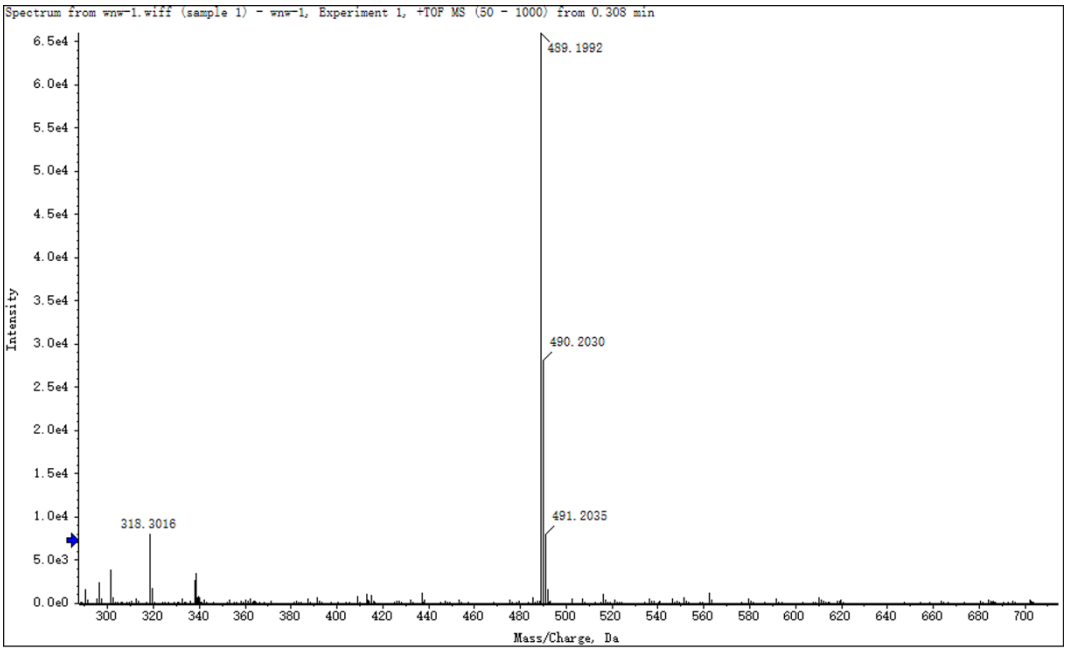


Figure S13. HRMS spectrum of **Bcy-NCS**.

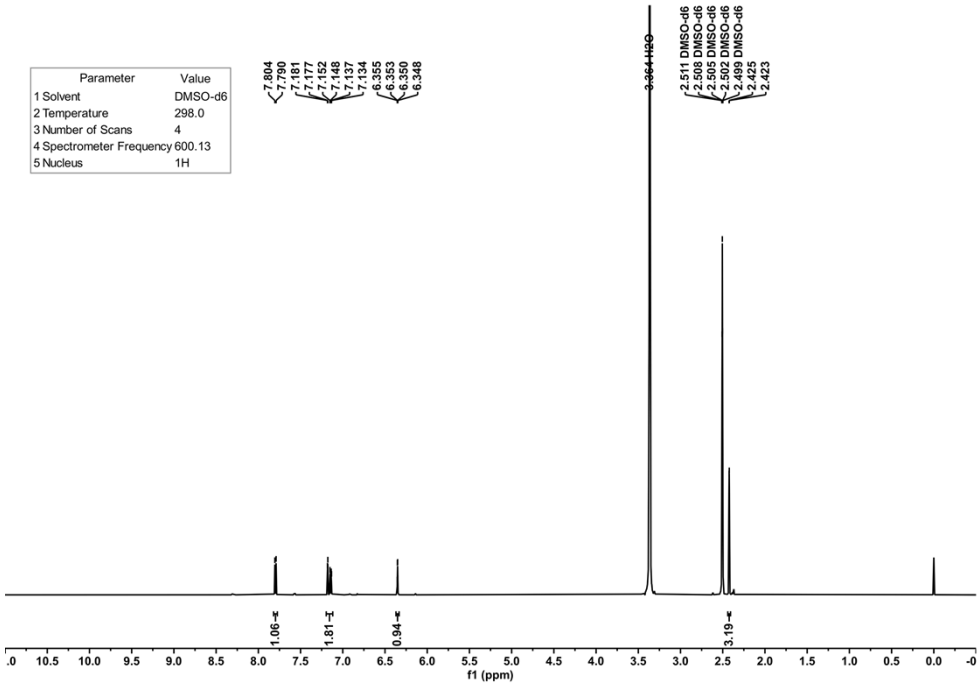


Figure S14. [1]H NMR spectrum of **AzMC** in DMSO-d_6 (600 MHz)

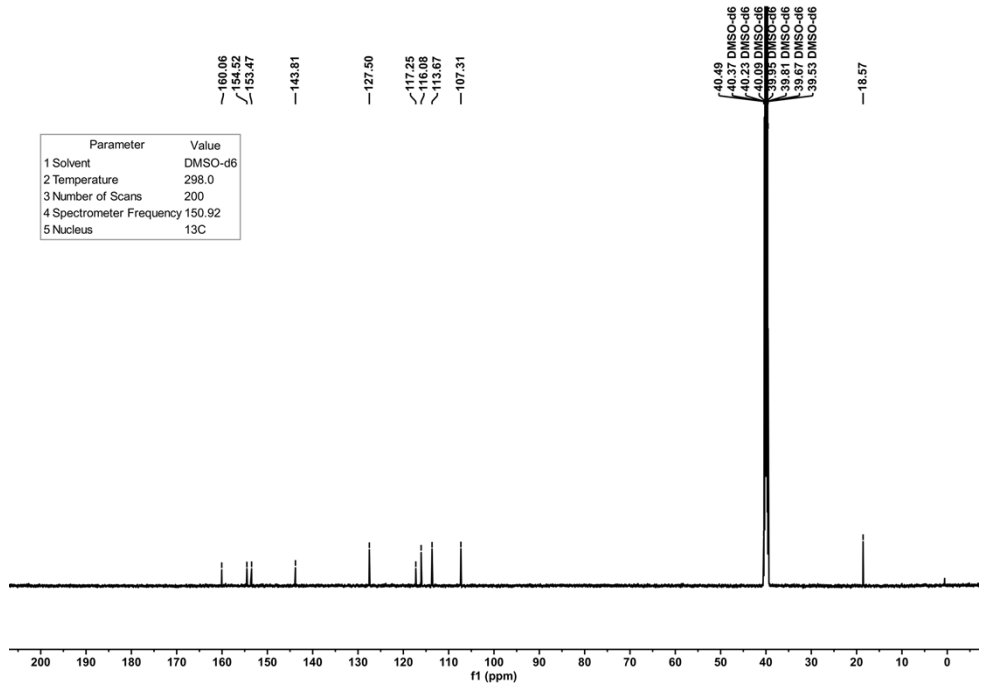


Figure S15. [13]C NMR spectrum of **AzMC** in DMSO-d_6 (150 MHz)

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