Supporting Information

Construction of Near-Infrared Fluorescent Probe for Cysteine and Its Application in Oxidative Stress Research

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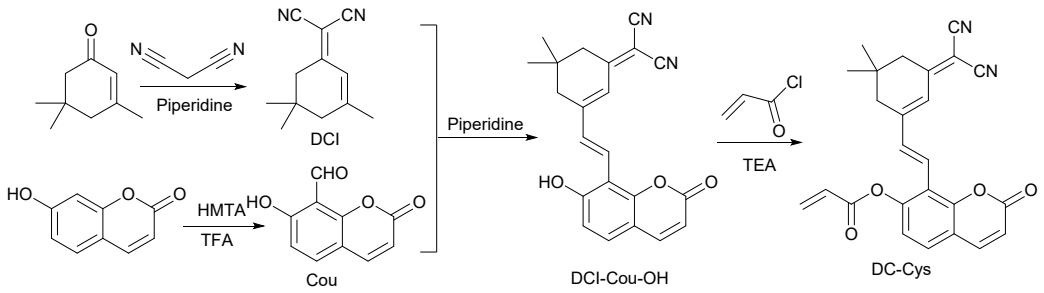
1 Synthesis and characterization



Synthesis Route of DC-Cys

Synthesis of compound DCI:

In a 50 mL flask, isophorone (4.5 mL, 30 mmol) and malononitrile (5.7 mL, 90 mmol) were added, followed by 20 mL of anhydrous ethanol for dissolution. Then, piperidine (300 μL) was added to the system, and the reaction was stirred under reflux at 80°C for 6 hours. After the reaction was completed, the system was cooled to room temperature and the solvent was removed under vacuum.The residue was purified by column chromatography using petroleum ether and ethyl acetate (v/v, 10:1) as the eluent, and 4.4 g of white solid was finally obtained with a yield of 77%. 1H NMR (400 MHz, Chloroform-d) δ 6.61 (q, J = 1.5 Hz, 1H), 2.51 (s, 2H), 2.17 (s, 2H), 2.04 – 2.00 (m, 3H), 1.00 (s, 6H). 13C NMR (101 MHz, Chloroform-d) δ 170.55, 159.94, 120.69, 113.33, 112.54, 45.78, 42.74, 32.50, 27.94, 25.46.

The synthesis of compound Cou:

Using an analytical balance, 7-hydroxycoumarin (3.3 g, 20 mmol) and hexamethylenetetramine (HMTA) (5.6 g, 40 mmol) were accurately weighed and placed in a 100 mL round-bottom flask. Then, 20 mL of trifluoroacetic acid (TFA) was added to dissolve them completely. The reaction mixture was heated to reflux at 80°C for 8 hours. After cooling to room temperature, 20 mL of dilute hydrochloric acid (2 mol/L) and 60 mL of water were added to quench the reaction. The mixture was extracted with ethyl acetate three times. The organic phase was washed with saturated sodium bicarbonate solution to adjust the pH to neutral and then extracted with ethyl acetate three times. The organic phase was dried, and the solvent was removed under vacuum. The residue was purified by column chromatography using

petroleum ether and ethyl acetate (v/v, 1:1) as the eluent, and finally 2.5 g of white solid was obtained with a yield of 77%.1H NMR (400 MHz, Chloroform-d) δ 12.21 (s, 1H), 10.59 (s, 1H), 7.66 (d, J = 9.6 Hz, 1H), 7.60 (d, J = 8.8 Hz, 1H), 6.89 (s, 1H), 6.34 (s, 1H). 13C NMR (101 MHz, Chloroform-d) δ 193.10, 165.63, 159.30, 156.85, 143.56, 136.18, 114.85, 113.54, 110.99, 108.78.

The synthesis of compound DCI-Cou-OH:

In a 50 mL round-bottom flask, compound DCI (1.14 g, 6 mmol) was added and dissolved in 20 mL of anhydrous ethanol. Then, Cou (1.08 g, 5.7 mmol) was accurately weighed and added to the reaction flask. Subsequently, 1.5 mL of piperidine was added to the stirring reaction system, and the reaction temperature was raised to 80°C. Then, 1.5 mL of acetic acid was added, and reflux stirring was carried out for 6 hours. After the reaction was completed, the solvent was removed by rotary evaporation, and the product was separated by flash column chromatography to obtain an orange-yellow solid (0.96 g), with a yield of 47%. 1H NMR (400 MHz, DMSO-d6) δ 11.48 (s, 1H), 7.97 (d, J = 9.5 Hz, 1H), 7.68 (d, J = 16.4 Hz, 1H), 7.54 (d, J = 8.6 Hz, 1H), 7.45 (d, J = 16.4 Hz, 1H), 6.96 (d, J = 8.5 Hz, 1H), 6.71 (s, 1H), 6.30 (d, J = 9.4 Hz, 1H), 2.62 (s, 2H), 2.56 (s, 2H), 1.06 (s, 6H). 13C NMR (101 MHz, DMSO-d6) δ 160.90, 160.38, 156.61, 153.91, 145.53, 133.97, 130.70, 127.01, 113.52, 110.61, 77.15, 42.85, 38.30, 32.22, 27.92.

Synthesis of probe DC-Cys:

Accurately weigh compound DCI-Cou-OH (0.18 g, 0.5 mmol) using an analytical balance and dissolve it in DCM (10 mL). Add acryloyl chloride (135 μL, 1.5 mmol) at 0°C, followed by triethylamine (75 μL, 0.5 mmol). React at room temperature for 12 h. After the reaction, add an appropriate amount of water to remove excess acryloyl chloride, and then pour it into a separatory funnel to take the lower organic phase. Add an appropriate amount of water to remove excess acryloyl chloride, and then pour it into a separatory funnel to take the lower organic phase. Wash the crude product repeatedly with saturated brine, and then dry the sample with anhydrous sodium sulfate. Place it in a vacuum drying oven overnight. After drying

overnight, separate and purify it by column chromatography (pure DCM), and finally obtain 0.1 g of yellow solid with a yield of 56%. 1H NMR (400 MHz, Chloroform-d) δ 7.76 (d, J = 9.6 Hz, 1H), 7.51 (d, J = 8.5 Hz, 1H), 7.38 – 7.23 (m, 2H), 7.16 (d, J = 7.5 Hz, 1H), 6.87 – 6.67 (m, 2H), 6.55 – 6.34 (m, 2H), 6.22 (d, J = 12.0 Hz, 1H), 2.64 (d, J = 5.3 Hz, 4H), 1.11 (s, 6H). 13C NMR (101 MHz, Chloroform-d) δ 169.14, 163.56, 159.60, 153.58, 152.76, 151.11, 143.35, 136.26, 134.58, 128.33, 127.00, 124.98, 124.37, 119.80, 118.11, 117.19, 116.28, 113.22, 112.40, 43.13, 38.79, 32.08, 28.04.

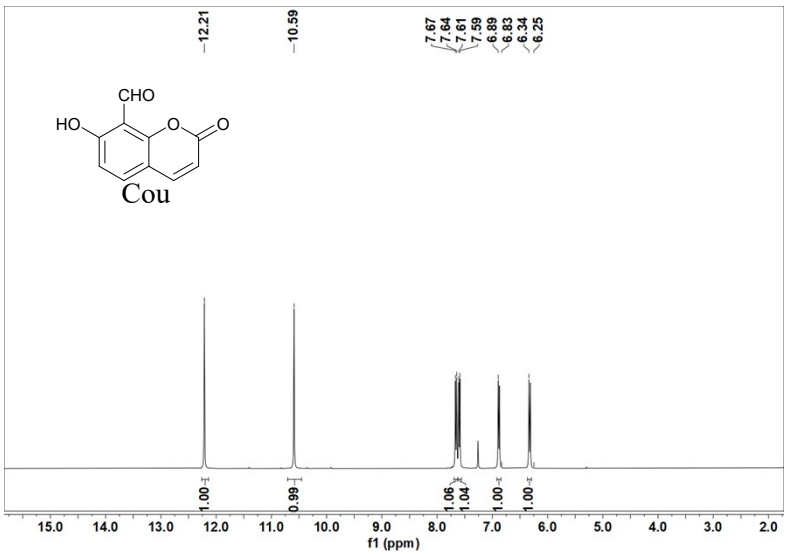


Fig S1 ^{1}H NMR spectrum of compound Cou in $CDCl_3$

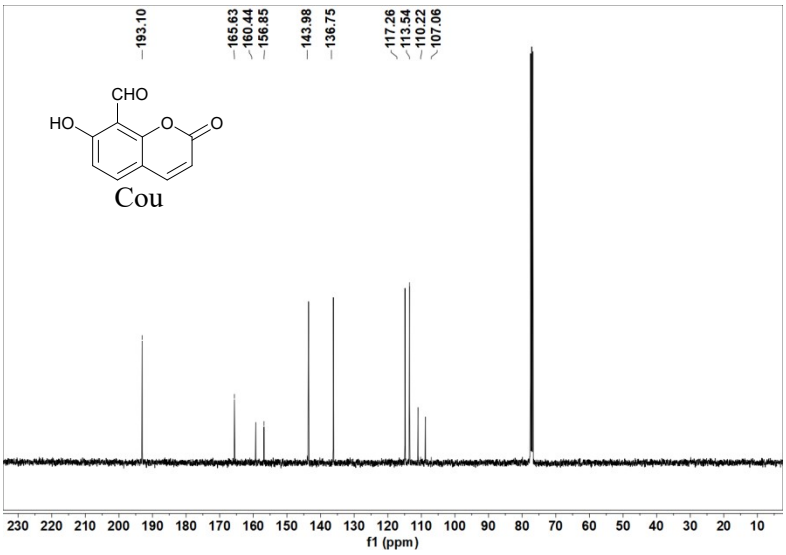


Fig S2 ^{13}C NMR spectrum of compound Cou in $CDCl_3$

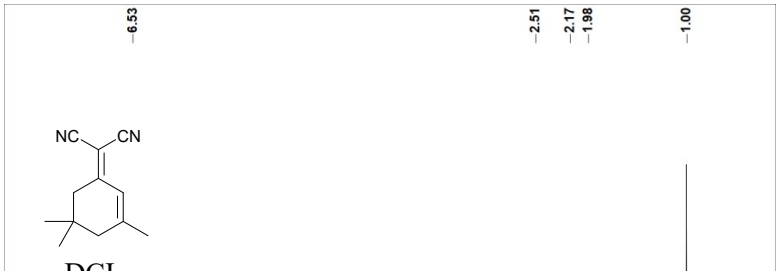


Fig S3 ^{1}H NMR spectrum of compound DCI in $CDCl_3$

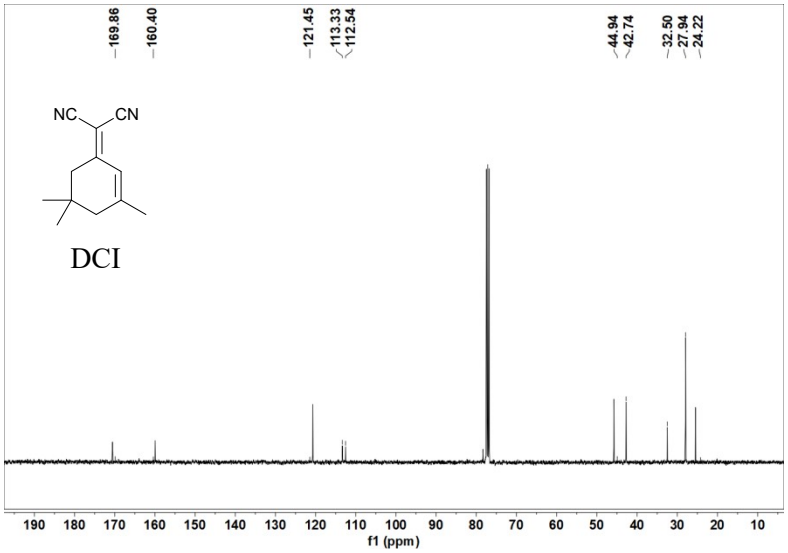


Fig S4 ^{13}C NMR spectrum of compound DCI in $CDCl_3$

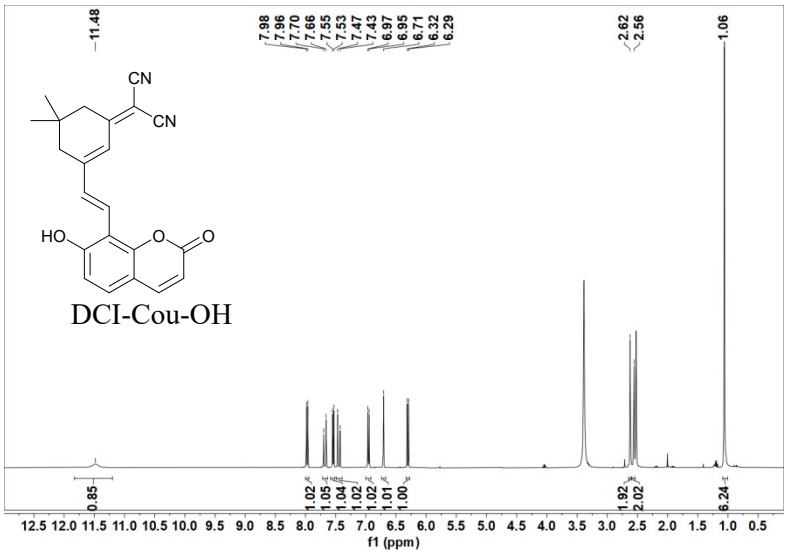


Fig S5 ^{1}H NMR spectrum of compound DCI-Cou-OH in DMSO-*d6*

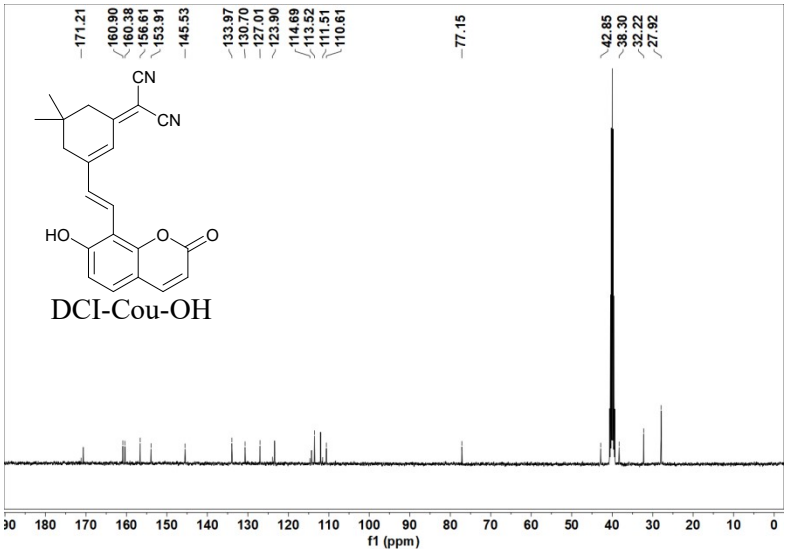


Fig S6 ^{13}C NMR spectrum of compound DCI-Cou-OH in DMSO-*d6*

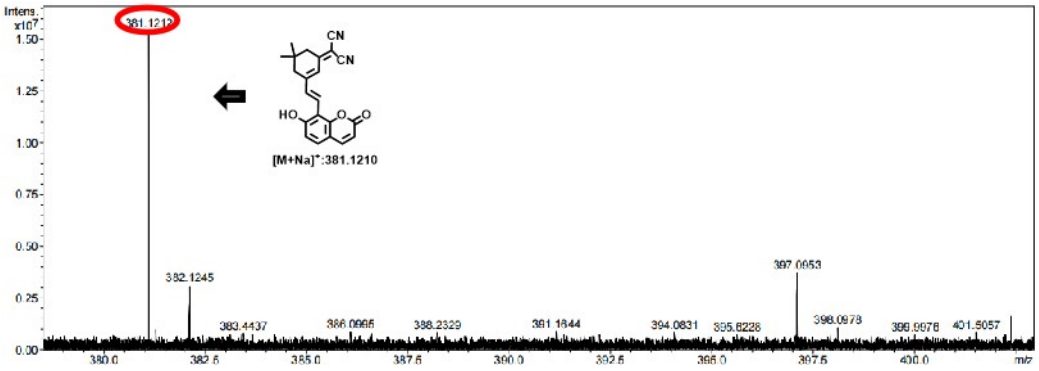


Fig S7 HRMS spectrum of compound DCI-Cou-OH

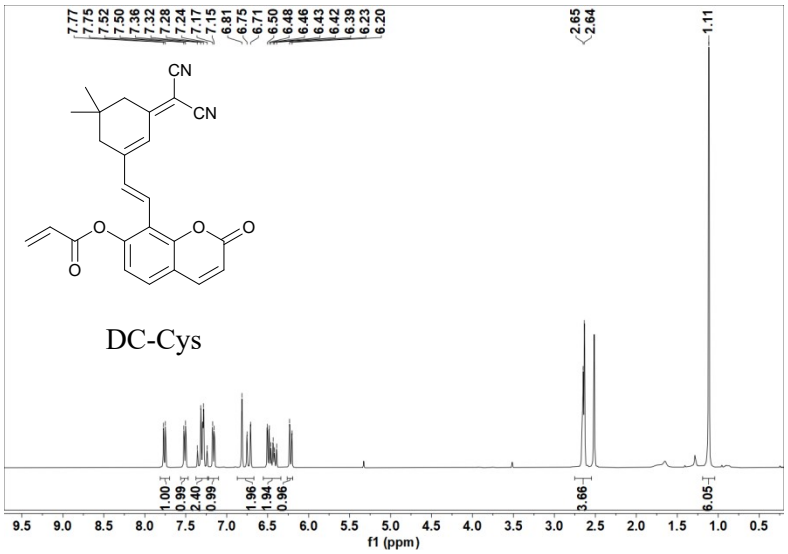


Fig S8 ^{1}H NMR spectrum of compound DC-Cys in DMSO-*d6*

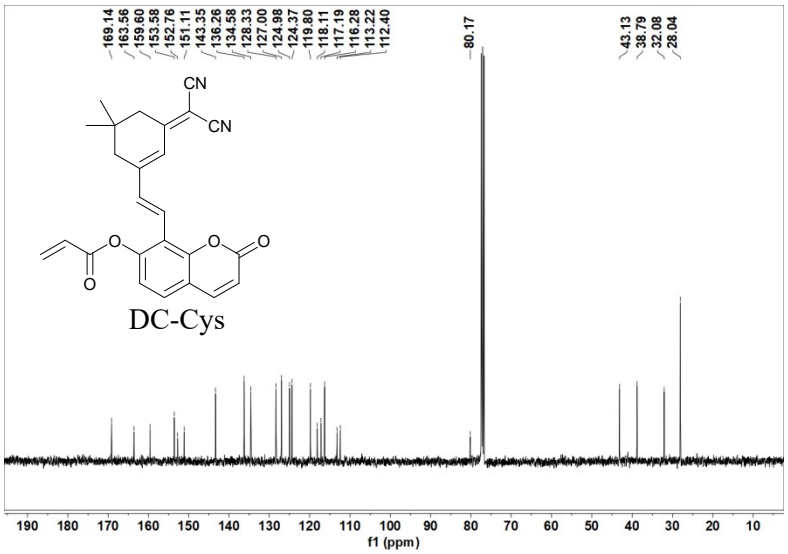


Fig S9 ^{13}C NMR spectrum of compound DC-Cys in DMSO-*d6*

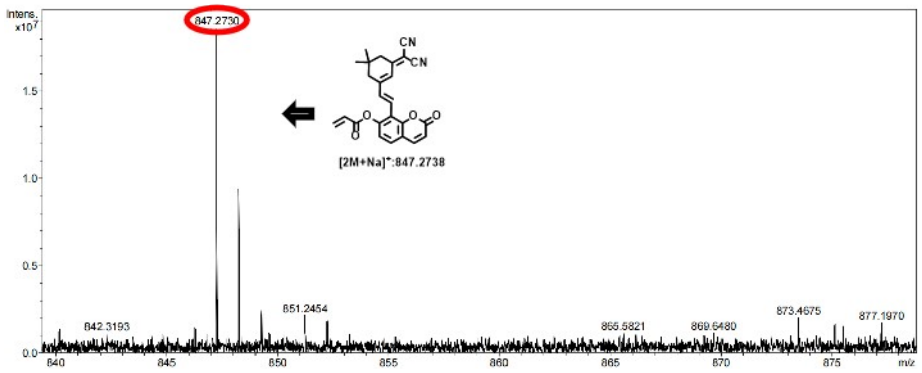
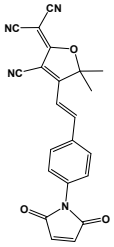


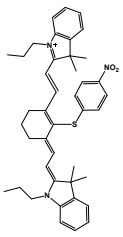
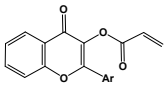
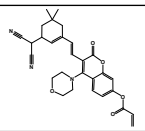
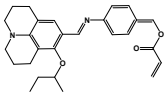
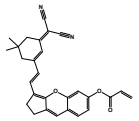
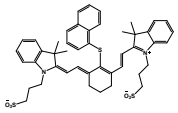
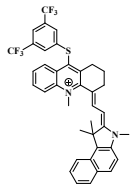
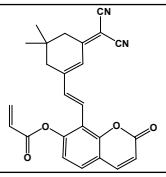
Fig S10 HRMS spectrum of compound DC-Cys

Table 1 Identification and comparison of key NMR signals of compound DCI-Cou-OH and DC-Cys

Compound	Functional group	^{1}HNMR δ（ppm）	Structural attribution meaning
DCI-Cou-OH	Hydroxyl group	11.48 (s,1H)	The appearance of a new hydroxyl signal confirms the formation of hydroxyl groups.
DC-Cys	Allyl ester	6.55 – 6.34 (m, 2H)	The emergence of characteristic terminal alkene signals confirmed the introduction of the allyl ester fragment.

Table 2 Summary of near-infrared fluorescent probes for specific detection of Cys.

Serial Number	Probe structure	λem/LOD	Stokes shift	Selectivity	Applications	References
1		580 nm 0.26 μM	140 nm	Specifically recognize Cys	Test strips, staining reagents, food testing	[1]

2		750 nm 1.26 μM	100 nm	Specifically recognize Cys	Cell	[2]
3		527 nm 5.25 × 10^{-7} M，	187 nm	Specifically recognize Cys	Cell	[3]
4		680 nm 53 nM	170 nm	Specifically recognize Cys	Cell	[4]
5		520 nm 1.5 nM	100 nm	Specifically recognize Cys	Food, cell,	[5]
6		851 nm 10.6 nM	191 nm	Specifically recognize Cys	Cell, Living tumor	[6]
7		820 nm 14 nM	110 nm	Specifically recognize Cys	Cell, Animal imaging	[7]
8		675 nm 74 nM	135 nm	Specifically recognize Cys	Cell, food inspection	[8]
This work		730 nm 23 nM	210 nm	Specifically recognize Cys	Cells, zebrafish	

2、Materials and Methods

All reagents used in the synthesis of compounds, such as petroleum ether (PE), ethyl acetate (EA), dichloromethane (DCM), methanol (MeOH), anhydrous ethanol, anhydrous tetrahydrofuran, acetonitrile, N,N-dimethylformamide (DMF), trifluoroacetic acid (TFA), and dimethyl sulfoxide (DMSO), were of analytical grade and purchased from Comiow Reagent Company. 7-Hydroxycoumarin, acryloyl chloride, and diethyl malonate were purchased from Jiangsu Aikang Biomedical Co., Ltd. Piperidine and hexamethylenetetramine (HMTA) were purchased from Anaiji and Alfa Aesar Reagent Company. All experimental water used in the experiments was self-made deionized water in the laboratory, and all reagents were used directly without further purification.

Human liver cancer cells (HepG2), human cervical cancer cells (HeLa), and human lung cancer cells (A549) were purchased from Wuhan Ponsure Life Science & Technology Co., Ltd. Zebrafish embryos and E3 medium (containing phenylthiourea PTU) were purchased from Nanjing Yishu Lihua Biotechnology Co., Ltd.

UV-2600 ultraviolet-visible spectrophotometer (Shimadzu, Japan) was used to measure the ultraviolet-visible absorption spectra; Edinburgh FS5 fluorescence spectrometer (Tianmei Scientific Instruments Co., Ltd.) was used to measure the fluorescence spectra; BUXI-I NMR nuclear magnetic resonance spectrometer/400 MHz (Zhongke Oxford Pop) was used to measure the nuclear magnetic resonance spectra; Apex Ultra Fourier transform ion cyclotron resonance ultra-high resolution mass spectrometer (Bruker Daltonik, America) was used to measure the high-resolution mass spectra of each compound; Countess II cell counter (Thermo Scientific) was used for cell counting; Multiskan FC microplate reader (Thermo Scientific) was used to test cell toxicity; Steri-Cycle i160 carbon dioxide incubator (Thermo Scientific) was used to culture cells; ZEISS LSM 880 NLO with Airyscan high-resolution & two-photon laser confocal microscope (Zeiss, Germany) was used for cell and zebrafish imaging.

Optical Properties Testing of Probe DC-Cys

All spectral tests were conducted in 10 mmol/L phosphate buffered saline (PBS, pH = 7.4, containing 50% DMSO). Cys was prepared in triple-distilled water to form stock

solutions of 2 mmol/L and 20 mmol/L, respectively. The probe was dissolved in dimethyl sulfoxide (DMSO) to form a 2 mmol/L stock solution for spectral testing. All analytes were prepared as 20 mmol/L analyte stock solutions.

Verification of the Mechanism of DC-Cys Probe for Cys Recognition

To explore the mechanism of the probe's recognition of Cys, DC-Cys was dissolved in acetonitrile/H_2O (v/v = 1/1), and 0.5 equivalents of Cys were added to allow for complete reaction.

Cell culture

When conducting cell experiments, HeLa, HepG2 and A549 cells were cultured in DMEM medium containing fetal bovine serum. The culture dishes were placed in a carbon dioxide incubator with a temperature set at 37°C and a carbon dioxide concentration of 5%.

Toxicity determination of probe DC-Cys

During the cytotoxicity detection experiment, three cell lines, HeLa, HepG2 and A549, were seeded in 96-well plates at a density of approximately 1×105 cells/mL per well. After thorough shaking, the plates were placed in an incubator for overnight growth. When the cells adhered completely, the original culture medium was removed and culture medium containing probe DC-Cys at concentrations of 0, 1, 5, 10, 15 and 20 μmol/L was added to each well. The plates were then incubated in the incubator for 24 hours. Subsequently, 10 μL of CCK-8 reagent was added to each well and incubated for another 2 hours. The absorbance values were measured using a microplate reader. Cell viability at each concentration was calculated based on the absorbance values. The test was repeated three times to ensure the accuracy of the data results.

Imaging of Endogenous and Exogenous Cys in Living Cells

HepG2 cells were randomly divided into groups. The Control group was first cultured in a medium containing 20 μmol/L of the probe for 30 min. The other groups were cultured in a medium containing 50 μmol/L NEM (N-ethylmaleimide, a thiol scavenger) for 30 min to eliminate endogenous biological thiols. After elimination, one group was incubated with a

medium containing 50 μmol/L Cys for 30 min, and another group was stimulated with 50 μmol/L dithiothreitol (DTT) to generate exogenous Cys. The last three groups were then incubated with a medium containing 20 μmol/L DC-Cys probe for 30 min. After three washes with PBS to remove residual cell debris or other impurities, fresh medium was added for imaging.

Construction of Oxidative Stress Cell Model

Prepare 1 mL of culture medium containing a final concentration of 20 μmol/L of the probe DC-Cys and incubate HepG2 cells for 30 minutes as the Control group. The remaining culture dishes were treated with LPS at concentrations of 1.0, 1.5, and 2.0 μg/mL for 12 hours, then replaced with culture medium containing 20 μmol/L of the probe DC-Cys and incubated for another 30 minutes before imaging.

Application of Probe DC-Cys in Oxidative Stress Cell Model

According to the above modeling method, HepG2 cells were cultured in a CO_2 incubator and randomly divided into 6 groups. The cells in the first group were only incubated with 20 μmol/L probe DC-Cys, while the cells in the other groups were co-incubated with 2 μg/mL LPS for 12 hours. Subsequently, the cells in the control group were co-incubated with the medium containing 20 μmol/L probe DC-Cys for 30 minutes. The cells in the alpha-lipoic acid (ALA) group were co-incubated with the medium containing 1 mmol/L ALA for 4 hours, then washed three times with PBS buffer, and co-incubated with the medium containing 20 μmol/L probe for 30 minutes before imaging. The N-acetylcysteine (NAC) group, GSH group, and vitamin C (Vc) group were all treated in the same way.

In vivo imaging of endogenous and exogenous Cys in zebrafish with probe DC-Cys

First, zebrafish were randomly divided into four groups. The Control group was incubated with probe DC-Cys (20 μmol/L) for 30 min; the other three groups were pretreated with NEM (1 mmol/L) for 30 min to eliminate endogenous thiols. After elimination, two of the three groups were further incubated with two concentrations of cysteine, 20 μmol/L and 50 μmol/L, for 30 min. Then, all groups were incubated with 20 μmol/L of the probe for 30

min before imaging.

Construction of Zebrafish Oxidative Stress Model

Zebrafish were randomly and evenly divided into five experimental groups. The blank control group (Control) was treated with culture medium containing 20 μmol/L DC-Cys probe for 30 minutes, washed three times with E3 medium, and then imaged. The other four experimental groups were incubated with 2 μg/mL LPS solution for 1 h, 3 h, 6 h, and 12 h, respectively. After the predetermined time points, all experimental groups were treated with culture medium containing 20 μmol/L probe for 30 minutes and then imaged.

Application of DC-Cys Probe in Zebrafish Oxidative Stress Model

According to the above-mentioned zebrafish modeling method, 15 three-day-old zebrafish were randomly selected and incubated with 2 μg/mL LPS for 12 hours. Then, they were randomly divided into 5 groups. The Control group was incubated with a culture medium containing 20 μmol/L DC-Cys probe for 30 minutes, washed three times with E3 culture medium, and then imaged. The NAC group was incubated with a culture medium containing 1 mmol/L NAC for 4 hours, washed three times with E3 culture medium, and then incubated with 20 μmol/L probe for 30 minutes before imaging. Similarly, the ALA group, GSH group, and Vc group were treated in the same way.

3、Spectral experiment

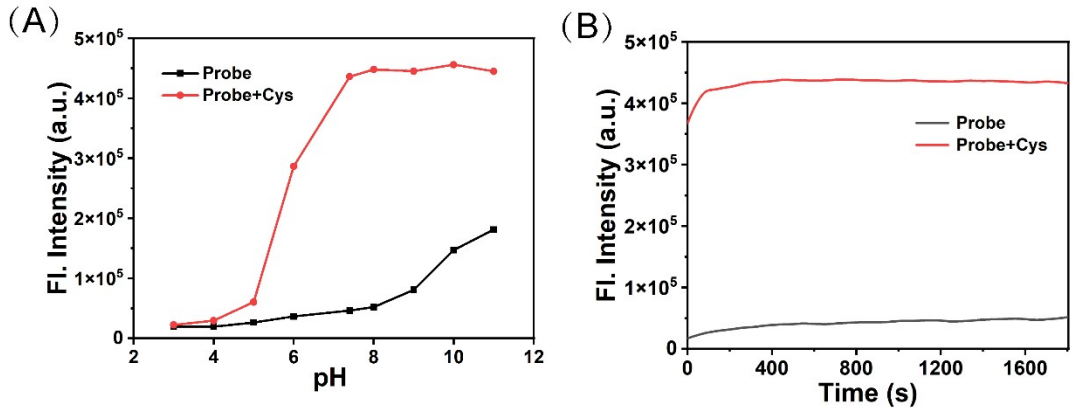


Fig S11 (A) Fluorescence changes of the probe DC-Cys before and after reaction with Cys under different pH test conditions (B) Kinetic spectra of the reaction between the probe DC-Cys and Cys

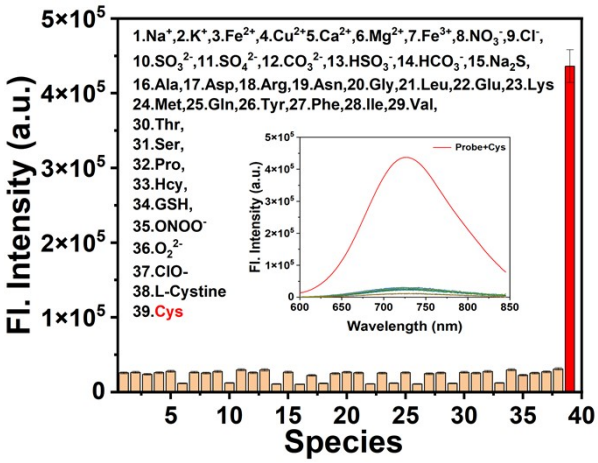


Fig S12 The fluorescence spectral responses of DC-Cys (15 μmol/L) to different analytes (300 μmol/L)

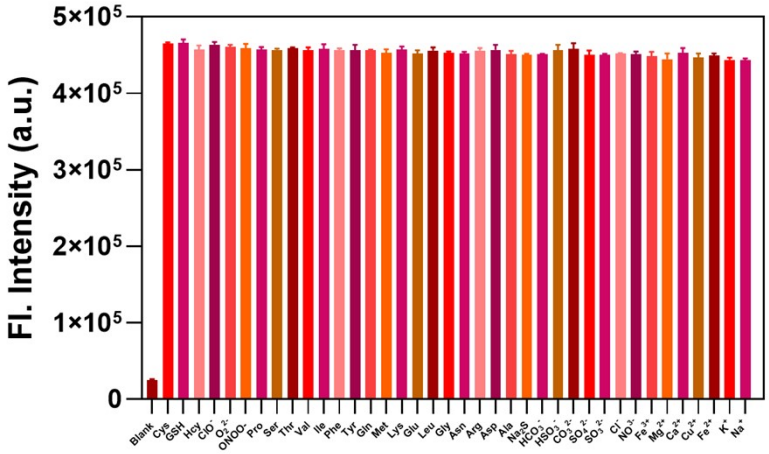


Fig S13 The fluorescence response of probe DC-Cys (15 μmol/L) at 735 nm after the addition of 300 μmol/L Cys and 300 μmol/L other analytes

4、Mechanism verification

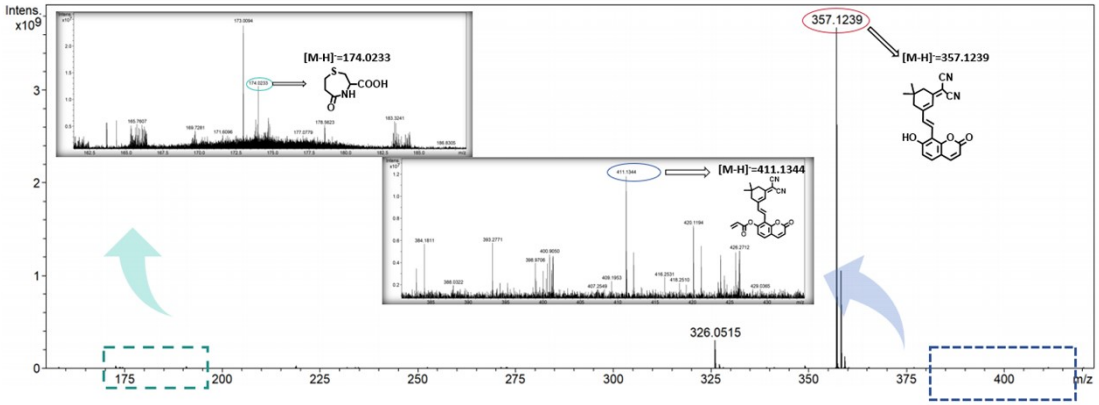


Fig 14 HRMS spectrum of probe DC-Cys after recognition of Cys.

5、Cell experiments

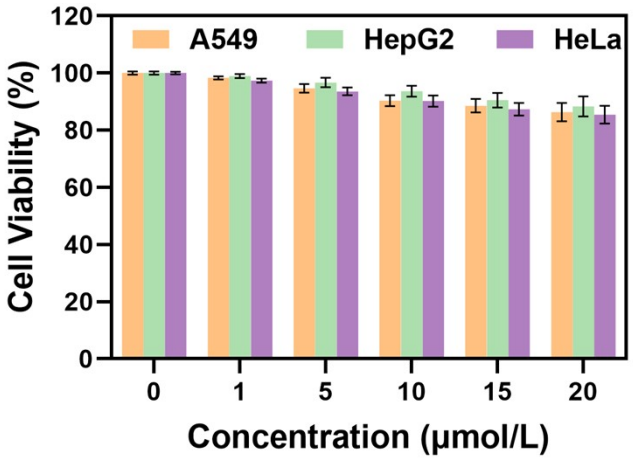


Fig S15 Toxicity test of cells incubated with different concentrations of probe DC-Cys for 24 h

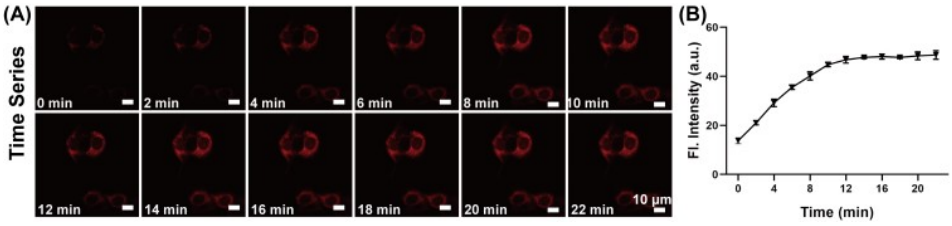


Fig S16 Real-time imaging of probe DC-Cys in HepG2 cells for 22 consecutive minutes; (B) Fluorescence intensities corresponding to the imaging of each group of cells in Figure A; λ_{ex} = 561 nm, λ_{em} = 640 - 750 nm, scale bar: 10 μm.

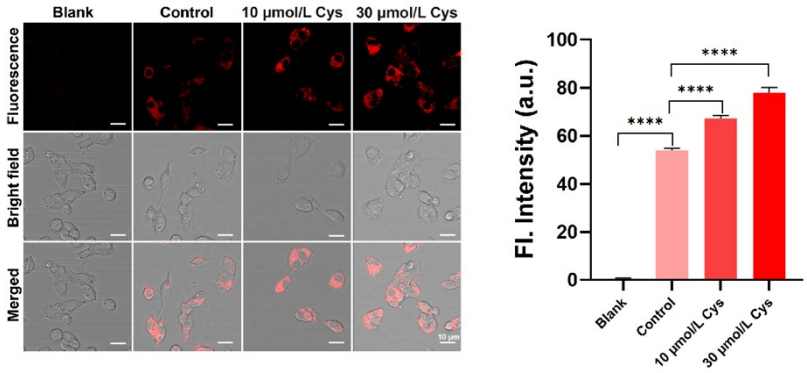


Figure S17 Cell imaging with different concentrations of Cys added.λ_{ex} = 561 nm, λ_{em} = 640 - 750 nm, scale bar: 10 μm.

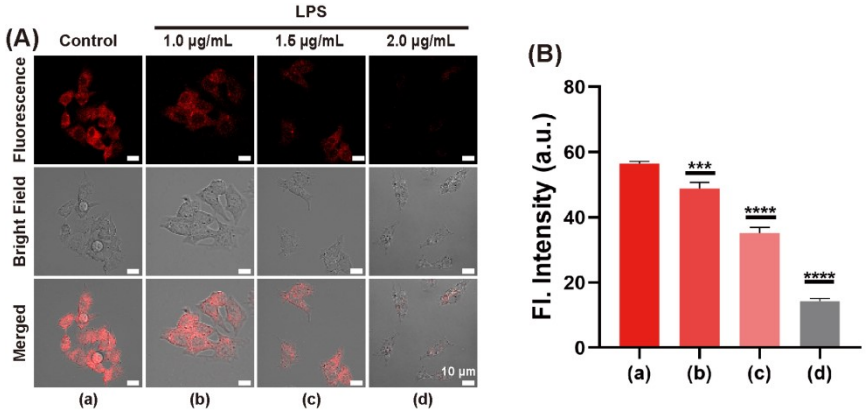


Fig S18 Imaging of cells treated with LPS at different times.λ_{ex} = 561 nm, λ_{em} = 640 - 750 nm, scale bar: 10 μm.

6、Zebrafish experiment

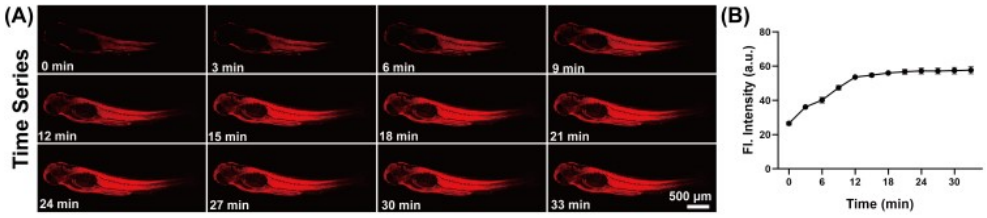


Fig S19 (A) Real-time imaging of probe DC-Cys in zebrafish for 33 consecutive minutes; (B) Fluorescence intensity corresponding to each group of zebrafish in Figure A; λ_{ex} = 561 nm, λ_{em} = 640 - 750 nm, scale bar: 500 μm.

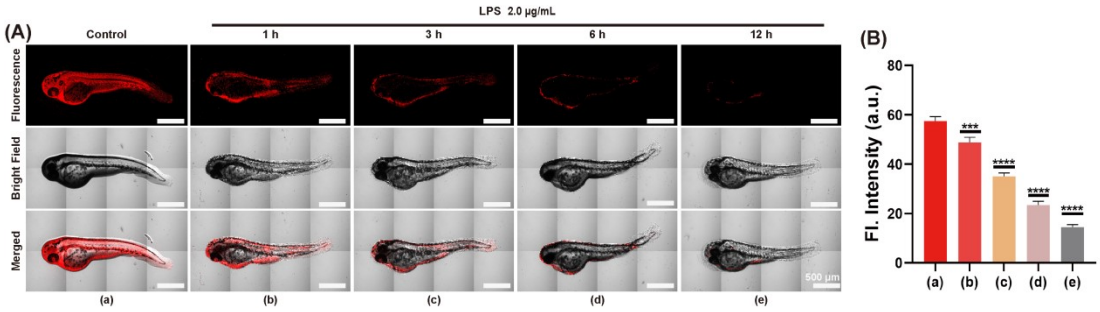


Fig S20 Imaging of the probe in zebrafish at different times after LPS treatment. λ_{ex} = 561 nm, λ_{em} = 640 - 750 nm, scale bar: 500 μm.

7□Reference

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