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Supporting Information (SI)

A near-IR ratiometric fluorescent probe for the precise tracking of senescence: A multidimensional sensing assay of biomarkers in cell senescence pathways

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Contents

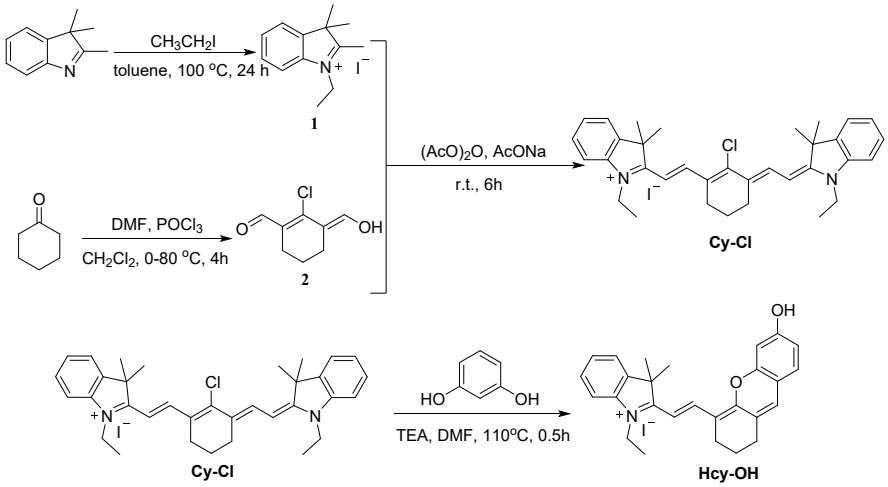
1. Experimental section

1.1 Material information

All of the reagents used for organic synthesis, such as 2,3,3-trimethylindolenine, iodoethane, $POCl_3$, 4-hydroxybenzaldehyde, cesium carbonate, sodium methylate, sodium borohydride, phosphorus tribromide, methanol, acetonitrile, dichloromethane and ethyl acetate were purchased from Energy Chemical (Shanghai, China). They were used as received without further purification. Etra-O-acetyl-α-D-galactopyranosyl-1-bromide and β-galactosidase were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). Isoflavones are purchased from Shanghai Eternal Biological Co., Ltd. and sesame oil is purchased from Spectrum Co. Ltd. Senescence-associated β-galactosidase staining kit were purchased from Beyotime (C0602, Beyotime, Jiangsu, China), ROS including t-$BuOO^-$, ClO^-, $^1O_2$, H_2O_2, $ONOO^-$, $O_2^{\cdot-}$, ·OH were prepared according to previous work.[1-3]

The water used for optical spectra was deionized water produced from Milli-Q Advantage A10 with a conductivity of 0.055 μS/cm. ^1H NMR and ^{13}C NMR spectra were taken on a 500 MHz NMR spectrometer (Bruker, Karlsruhe, Germany) using DMSO-d_6 as solvent, chemical shift relative to tetramethylsilane (TMS, δ scale, with solvent resonance as internal standard) are reported as ppm downward. An FT-ICR-MS mass spectrometer was used for high-resolution mass spectra (HR-MS) analysis. Optical analysis of absorption and fluorescence spectra was performed on a UH5300 UV-Vis and a Hitachi F-4600 spectrophotometer, respectively, in a 1 cm quartz cell. Intracellular fluorescence imaging was obtained on a BioTek CYTATION 5 imager. Cell Counting Kit-8 (CCK-8) assays were performed on a Bio-Tek ELX800 microplate reader to assess the cytotoxic effects of the probes. The in *vivo* imaging experiments were carried out at Jiangsu Keygen Biotech Co., Ltd. using an IVIS Lumina LT Series III PerkinElmer.

1.2 Synthesis of the Probes SA-HCy-1



Compounds **1**, **2** and **Cy-Cl** were synthesized according to previous work.[4, 5]

Synthesis of compound 1

2,3,3-Trimethylindolenine (10.0 g, 62.8 mmol) and iodoethane (14.6 g, 93.6 mmol) were dissolved in toluene (20 mL). The mixture was stirred at 100 °C for 24 hours leading to product. The reaction was cooled to room temperature. The product was filtered, and washed with ether and dried to give 18.6 g (yield: 94%) of compound **1** as a pink solid.

Synthesis of compound 2

A solution of $POCl_3$ (18.0 mL, 215.6 mmol) in dissolved CH_2Cl_2 (15 mL) was slowly added to a solution of DMF (20.0 mL, 258.6 mmol) dissolved in CH_2Cl_2 (20 mL) at 0 °C. The reaction continue for two hours after the addition was finished. Then cyclohexanone (5.0 g, 52.6 mmol) was added in via syringe. The resulted reaction mixture was refluxed at 80 ℃ for three hours. The mixture was resting overnight in ice water (200 mL), and the precipitate was filtered. Then washed with water and dried to give 3.9 g (yield: 43%) of compound **2** as yellow crystalline solid to reserve in the cool temperature.

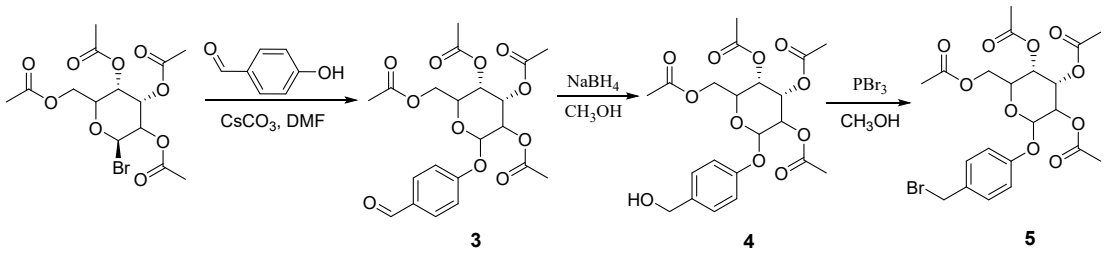
Synthesis of compound Cy-Cl

Compound **1** (0.72 g, 2.28 mmol) and compound **2** (0.20 g, 1.14 mmol) were dissolved in acetic anhydride (15 mL). Sodium acetate (1.2 g, 14.6 mmol) was then added to the mixture as a catalyst. The mixture was reacted under the protection of nitrogen at room temperature for six hours. Product was filtered, and washed with water

and dried to give 1.07 g (yield: 73%) of **Cy-Cl** as green solid.

Synthesis of HCy-OH

Into a flask attached with Dean-Stark trap and a condenser were added compound **3** (0.64 g, 1.0 mmol), resorcinol (0.82 g, 7.4 mmol), triethylamine (1.386 mL, 10 mmol) and anhydrous DMF (15 mL). The mixture was heated for 0.5 h at 110 ℃, resulting in a blue solution. Then the solvent was removed by a rotavapor. The crude product was purified by silica gel chromatography (CH_2Cl_2/MeOH: 60:1) to afford 0.36 g (yield: 68%) of **HCy-OH** as a dark blue solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) 10.79 (s, 1H), 8.61 (d, J = 15.0 Hz, 1H), 7.78 (d, J = 7.4 Hz, 1H), 7.68 (d, J = 8.1 Hz, 1H), 7.55 (d, J = 11.2 Hz, 2H), 7.50 (d, J = 8.5 Hz, 1H), 7.46 (t, J = 7.4 Hz, 1H), 6.94 (s, 1H), 6.87 (d, J = 8.5 Hz, 1H), 6.53 (d, J = 14.9 Hz, 1H), 4.43 (d, J = 7.3 Hz, 2H), 2.74 (s, 2H), 2.70 (s, 2H), 1.85 (s, 2H), 1.76 (s, 6H), 1.39 (t, J = 7.1 Hz, 3H). ESI-MS m/z: M^+ Calcd for $C_{27}H_{28}NO_2^+$ 398.2115, Found 398.2113.



Synthesis of compound 3

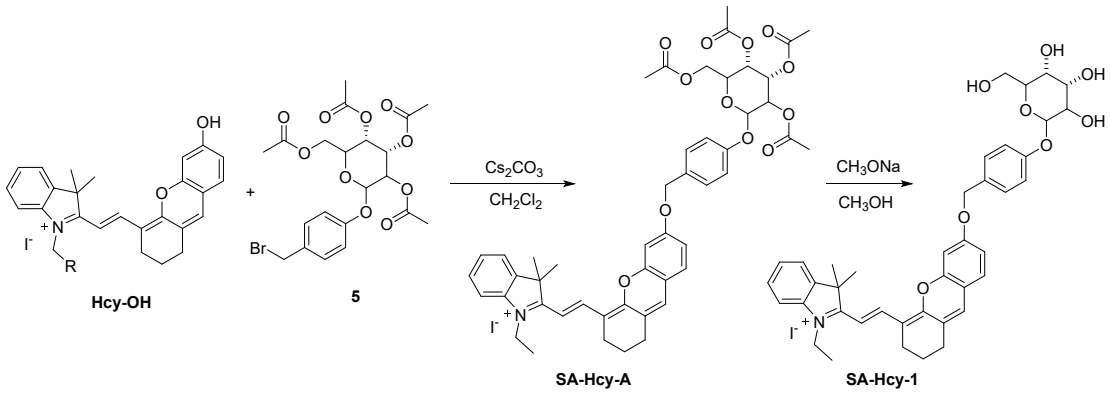
4-hydroxybenzaldehyde (162.3 mg, 1.33 mmol), tetra-O-acetyl-α-D-galactopyranosyl-1-bromide (822.4 mg, 2.00 mmol) and Cs_2CO_3 (1.96 g, 6.00 mmol) were dissolved in anhydrous acetonitrile (20 mL). The system was stirred at room temperature for 16 hours under the protection of nitrogen. After reaction was completed, the content was filtered and collected the filtrate. The solvent was removed under reduced pressure, and the residue was taken up in salt. 20 mL deionized water was added and then extracted with CH_2Cl_2. The organic phase was collected. After dried over anhydrous Na_2SO_4, the solvent was removed by evaporation, and the crude product was purified by silica gel chromatography (CH_2Cl_2/MeOH: 100:1) to obtain the desired product compound **3** (1.12 mmol, yield: 84%).

Synthesis of compound 4

Sodium borohydride (41.6 mg, 1.10 mmol) was added into a stirred solution of compound **3** (248.8 mg, 0.55 mmol) in 20 mL MeOH at 0℃. The reaction was monitored by TLC. After stirred for 5 min, the solution was diluted with acetic ether and washed with saturated ammonium chloride solution and saturated NaCl solution. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The resulting crude residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH: 90:1) to afford compound **4** (232.4 mg, yield: 93%). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) 7.30 (d, J = 7.8 Hz, 2H), 6.99 (d, J = 7.8 Hz, 2H), 5.47 (dd, J = 19.4, 5.5 Hz, 2H), 5.11 (dd, J = 10.3, 2.5 Hz, 1H), 5.04 (d, J = 7.5 Hz, 1H), 4.64 (s, 2H), 4.23 (dd, J = 11.0, 7.0 Hz, 1H), 4.16 (dd, J = 11.1, 6.2 Hz, 1H), 4.07 (t, J = 6.4 Hz, 1H), 2.18 (d, J = 4.4 Hz, 3H), 2.08 – 2.05 (m, 6H), 2.01 (d, J = 4.6 Hz, 3H), 1.90 (s, 1H).

Synthesis of compound 5

PBr_3 (58 μL, 0.61 mmol) was added into a stirred solution of compound **4** (231.6 mg, 0.51 mmol) dissolved in 20.0 mL CH_2Cl_2 at 0 ℃. The reaction solution was stirred for 30 minutes at 0 ℃. After the reaction was completed, the solution was diluted with CH_2Cl_2 and washed by cold water and saturated NaCl solution. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. Then the product was purified by column chromatography on silica gel (CH_2Cl_2/MeOH: 100:1) to afford compound **5**. (192.6 mg, yield: 73%)



Synthesis of compound SA-HCy-A

Compound **HCy-OH** (53 mg, 0.10 mmol) and Cs_2CO_3 (163 mg, 0.50 mmol) were

dissolved in dry CH_2Cl_2 in a round-bottom flask and stirred at room temperature under a nitrogen atmosphere for 15 minutes. Compound **5** (155 mg, 0.31 mmol) was then added, and the reaction mixture was stirred at room temperature for another 12 hours. After that, the solvent was removed under reduced pressure, and the resulting residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH: 20:1) to obtain compound **SA-HCy-A** as a blue solid (60.3 mg, yield: 63%). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) 8.59 (d, J = 14.8 Hz, 1H), 7.76 (d, J = 7.3 Hz, 1H), 7.69 (d, J = 8.0 Hz, 1H), 7.57 - 7.53 (m, 2H), 7.48 (dd, J = 19.5, 7.2 Hz, 4H), 7.18 (s, 1H), 7.06 (t, J = 9.3 Hz, 3H), 6.57 (d, J = 15.0 Hz, 1H), 5.49 (d, J = 7.8 Hz, 1H), 5.34 (s, 2H), 5.28 (d, J = 3.4 Hz, 1H), 5.26 - 5.18 (m, 4H), 4.44 (d, J = 7.4 Hz, 2H), 4.12 – 4.08 (m, 2H), 2.73 (s, 2H), 2.69 (s, 2H), 2.14 (s, 3H), 2.03 (s, 3H), 1.99 (s, 3H), 1.95 (s, 3H), 1.83 (s, 2H), 1.76 (s, 6H), 1.38 (t, J = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm) 177.53, 170.43, 170.29, 170.03, 169.70, 161.88, 160.85, 156.85, 154.22, 145.46, 142.72, 141.50, 133.54, 131.09, 130.34, 129.39, 127.49, 123.27, 116.93, 116.01, 114.42, 113.54, 104.72, 102.35, 98.11, 70.82, 70.59, 70.16, 68.82, 67.67, 61.72, 55.39, 50.90, 28.91, 27.88, 20.96, 20.92, 20.87, 20.83, 13.15. ESI-MS m/z: M^+ Calcd for $C_{48}H_{52}NO_{12}^+$ 834.3484, Found 834.3492.

Synthesis of SA-HCy-1

Compound **SA-HCy-A** (60.0 mg, 0.0624 mmol) was dissolved in dry methanol (7 mL). Sodium methylate (33.7 mg, 0.624 mmol) in dry methanol (3 mL) was added dropwise into the solution, and the solution was stirred for 3 hours at room temperature. When reaction was completed, the residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH: 10:1) to obtain probe **SA-HCy-1** as a blue solid (28.8 mg, yield: 58%). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) 8.60 (d, J = 15.3 Hz, 1H), 7.76 (d, J = 7.5 Hz, 1H), 7.69 (d, J = 8.1 Hz, 1H), 7.54 (t, J = 8.3 Hz, 2H), 7.50 (s, 1H), 7.50 - 7.40 (m, 3H), 7.19 (s, 1H), 7.07 (d, J = 8.4 Hz, 3H), 6.56 (d, J = 15.0 Hz, 1H), 5.22 (s, 2H), 5.16 (d, J = 5.0 Hz, 1H), 4.88 (d, J = 5.6 Hz, 1H), 4.84 (d, J = 7.6 Hz, 1H), 4.66 (d, J = 5.4 Hz, 1H), 4.53 (d, J = 4.6 Hz, 1H), 4.44 (d, J = 7.3 Hz, 2H), 3.70 (s, 2H), 3.56 (s, 2H), 3.48 (s, 1H), 3.40 (s, 1H), 2.72 (s, 2H), 2.69 (s, 2H), 1.83 (s, 2H), 1.76 (s, 6H), 1.37 (t, J = 7.2 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm) 177.47, 161.96,

160.88, 157.94, 154.21, 145.42, 142.70, 141.47, 133.60, 130.12, 129.70, 129.38, 127.59, 127.42, 123.29, 116.71, 115.96, 114.41, 114.08, 113.51, 104.63, 102.31, 101.33, 75.94, 73.84, 70.85, 70.38, 68.39, 60.67, 50.89, 49.04, 31.75, 29.49, 29.16, 27.88, 27.01, 22.56, 13.14. ESI-MS m/*z*: M^+ Calcd for $C_{40}H_{44}NO_8^+$ 666.3061, Found 666.3074.

1.3 General Procedures for Spectra Measurements

The stock solutions of **SA-HCy-1** (5.0 mM) were prepared in spectroscopic pure DMSO and stored at -18 °C in a refrigerator. The stock solution was diluted into 10 μM with PBS (10 mM) for absorption and fluorescence spectral exploration.

1.4 Measurement of detection limit

The limit of detection (LOD) was calculated using the following equation:

$$LOD = 3.3\ \sigma/k$$

where σ and k are the standard deviation of eleven blank measurements and the standard curve slope, respectively.

1.5 Cell culture

RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% streptomycin and 1% penicillin at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air. B16 cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) and 1% streptomycin and 1% penicillin at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air. Before the imaging experiments, the cells were seed in 35-mm glass-bottomed dishes at a density of 2×10^5 cells per dish in 2 mL of culture medium and incubate them inside an incubator containing 5% CO_2 and 95% air at 37 °C, and incubated the cells for another 24 hours.

1.6 Cytotoxicity assays

RAW 264.7 cells were seeded into 96-well plates, and 0, 5, 8, 10, 15, 20, 30 μM (final concentration) of the probe **SA-HCy-1** were added respectively. Subsequently, the cells were cultured at 37 °C in an atmosphere of CO_2 (5%) and air (95%) for 24 hours. Then RAW 264.7 cells were washed with PBS for three times. Next, Cell Counting Kit-8 (CCK-8) solution (10% in serum free DMEM) was added per well and incubated for 12 hours. After the incubation, absorption at 450 nm was recorded on a

microplate reader. The cell viability was determined by assuming 100% cell viability for cells without incubated with **SA-HCy-1**.

1.7 X-gal staining of cells

According to the senescence-associated β-galactosidase staining kit, the X-gal staining assay was investigated. The cells were rinsed once with PBS and fixed with 1 mL of β-galactosidase stationary liquid at room temperature for 10 minutes. Then, the samples were rinsed with PBS for three times (3 minutes each time). After the PBS was removed, 1 mL of X-gal dye solution was added into each well. Subsequently, the cells were incubated at 37 ºC overnight (without CO_2). After that, the development of blue color was observed under a light microscope.

1.8 Cells treatment by DOX

RAW 264.7 and B16 cells were treated with DOX (0.1 μM) for different days (1-3 days) to induce their senescence. After that, the cells were incubated with **SA-HCy-1** (10 μM) for 30 minutes and then washed with PBS before fluorescent imaging. Living cell images were acquired by using a BioTek CYTATION 5 Imaging reader with a 20 × objective.

1.9 RAW 264.7 cells treatment by LPS+PMA, or NAC

Normal RAW 264.7 cells and senescent RAW 264.7 cells were pretreated with LPS (1.0 μg•mL^{-1}) and PMA (0.5 μg mL^{-1}) for 60 minutes or NAC (10 mM) for 60 min, then they were incubated with **SA-HCy-1** (10 μM) for 30 minutes and washed with PBS before fluorescence imaging.

1.10 Senescent RAW 264.7 cells treatment by azithromycin and vitamin E (VE)

Senescent cells (after 3 days of treatment with 0.1 μM DOX) were pretreated with azithromycin (100 μM) or vitamin E (100 μM) for 60 minutes, then incubated with **SA-HCy-1** (10 μM) for 30 minutes and washed with PBS before fluorescence imaging.

1.11 Living cell fluorescent imaging

For intracellular imaging, fluorescent living cell images were acquired using a BioTek CYTATION 5 Imaging reader with a 20 × objective. For NIR channel: λ_{ex} = 628 nm, λ_{em} = 670-730 nm; for cyan channel: λ_{ex} = 377 nm, λ_{em}= 450-500 nm. Scale bar = 100 μm.

1.12 In *vivo* imaging of ultraviolet light-induced skin photoaging

The animal studies were approved by the Medical Ethics Committee of Qingdao University of Science and Technology. The animal procedures were carried out in accordance with the guidelines for the care and use of Laboratory Animals of Qingdao University of Science and Technology. Blab/c nude mice were obtained from Shanghai Slac Laboratory Animal Co., Ltd. at 6 months of age. The in *vivo* imaging experiments were carried out using an IVIS Lumina LT Series III PerkinElmer.

After a week of stable raising, the mice were first pretreated with pure substrate (sesame oil) (100 μL) or 3 % isoflavone preparation (100 μL) made by diluting sesame oil as a substrate on each side (left and right side in a 1.5×1.5 cm area) of the back for 15 minutes. Then the mice were placed in a 5×10 cm rectangular experimental chamber and irradiated with a handheld UV lamp with a wavelength of 365 nm. The mice were irradiated with UV light for 5 times a week (from Monday to Friday), 20, 40 and 60 minutes per irradiation from week 1 to week 3, and 80 minutes per irradiation from week 4 and week 5, for a total of 5 weeks.

For the whole body in *vivo* imaging, **SA-HCy-1** (10 μL, 1.0 mM dissolved in DMSO) was applied to the pretreatment area for 30 minutes before imaging. Then the fluorescent images were collected every Sunday afternoon during the irradiation period. The fluorescence images were recorded both in the NIR and cyan channels. For NIR channel: λ_{ex} =600 nm; λ_{em} = 710 nm; for cyan channel: λ_{ex} =420 nm; λ_{em} = 480 nm.

2. ^{1}H, ^{13}C NMR and HRMS spectra

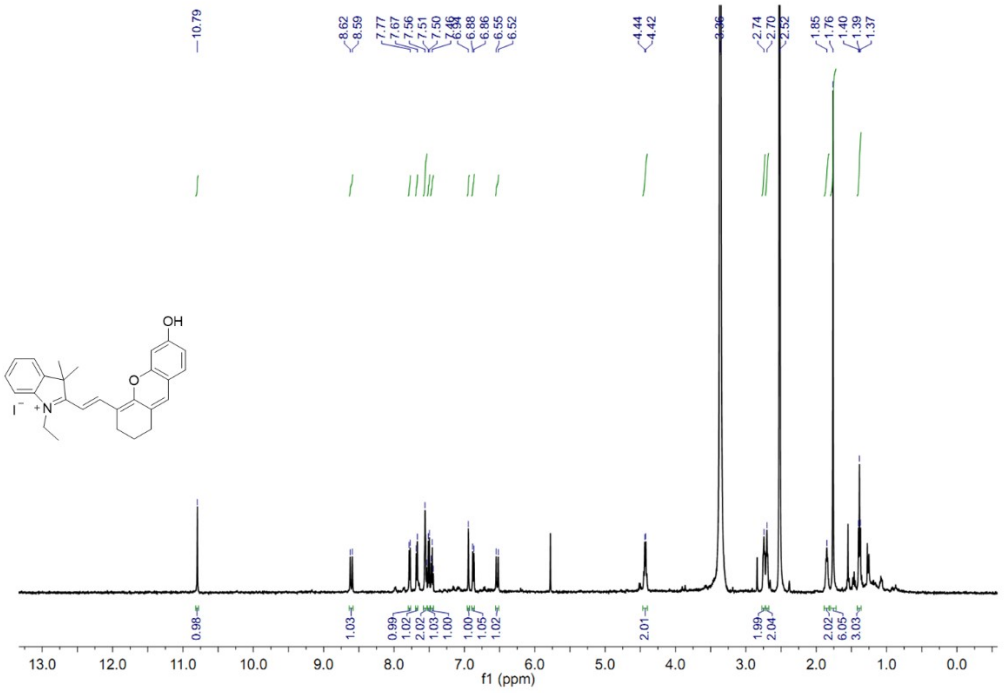


Figure S1. ^{1}H NMR spectrum of **HCy-OH** in DMSO-d_6.

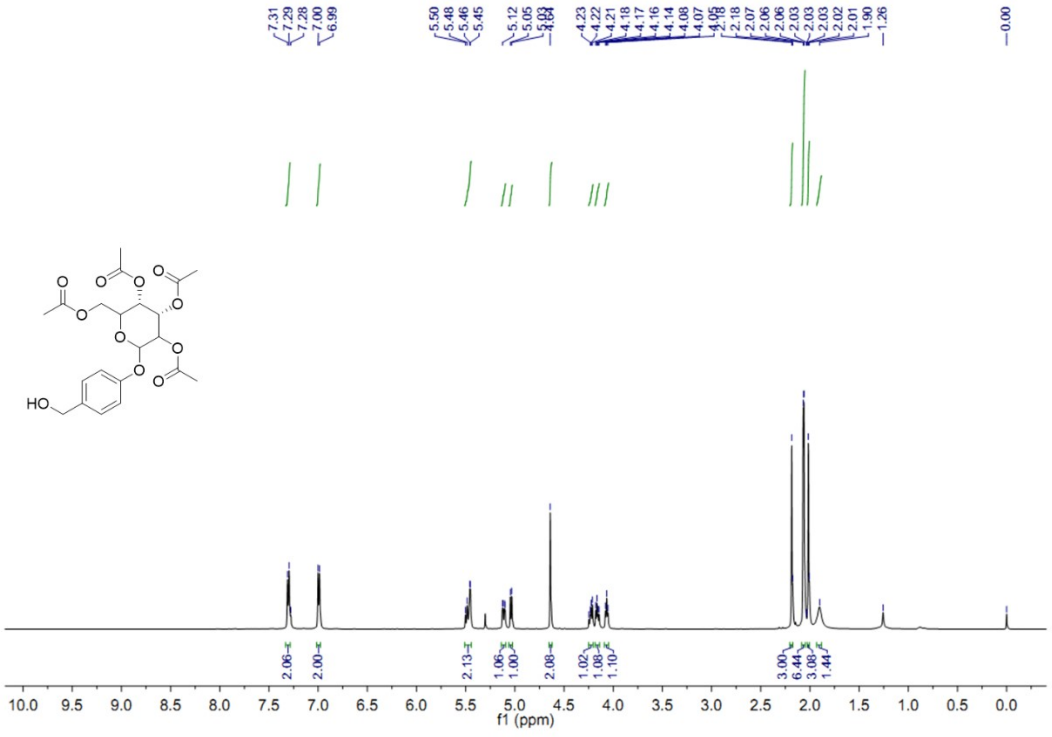


Figure S2. ^{1}H NMR spectrum of compound **4** in DMSO-d_6.

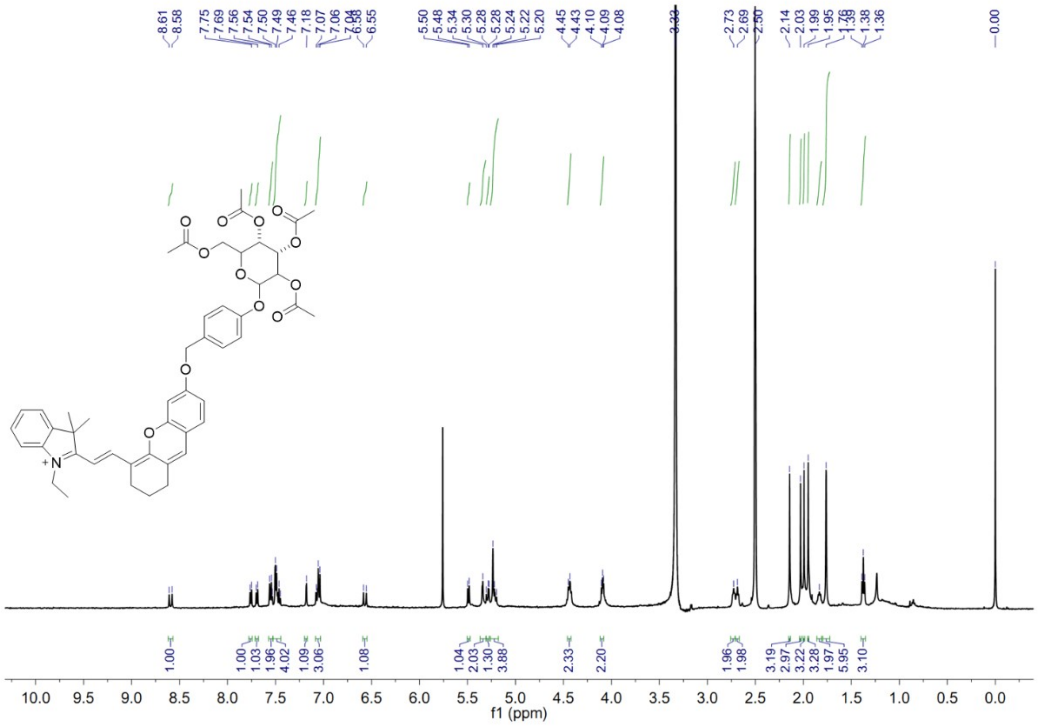


Figure S3. ^{1}H NMR spectrum of **SA-HCy-A** in DMSO-d_6.

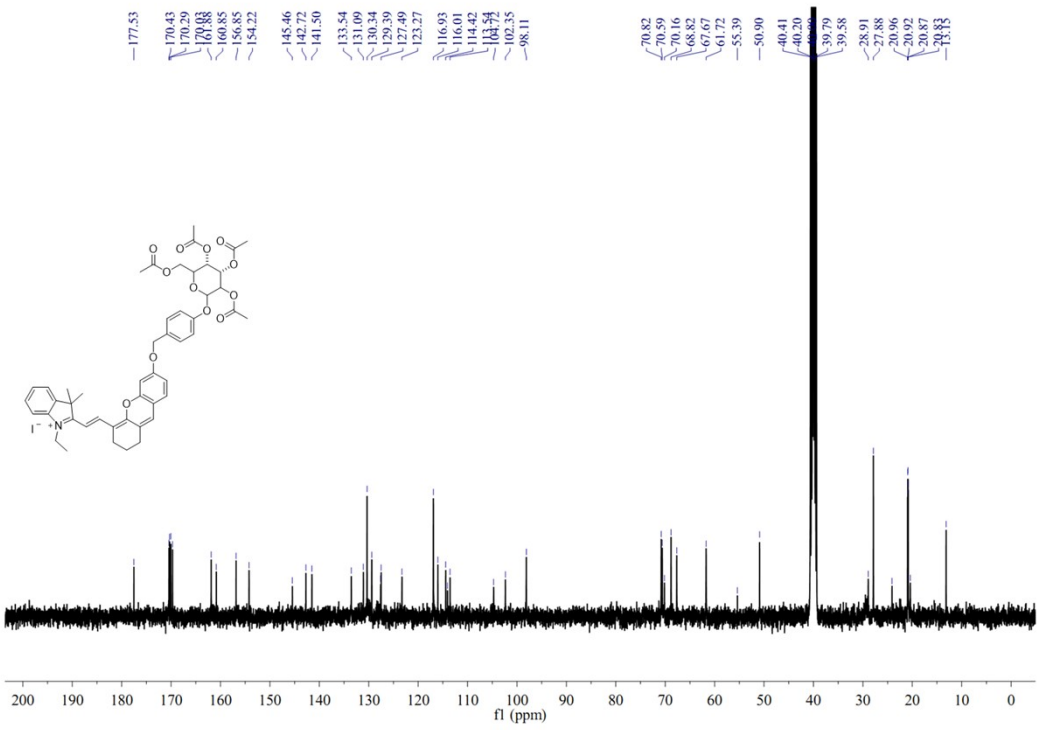


Figure S4. ^{13}C NMR spectrum of **SA-HCy-A** in DMSO-d_6.

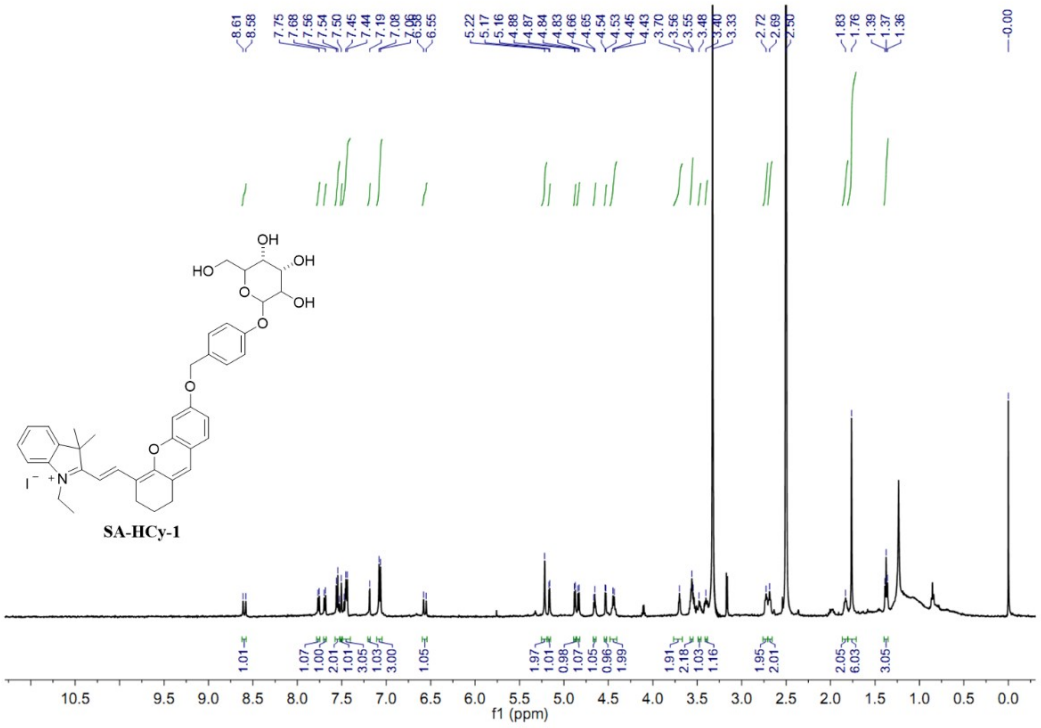


Figure S5. ^{1}H NMR spectrum of **SA-HCy-1** in DMSO-d_6.

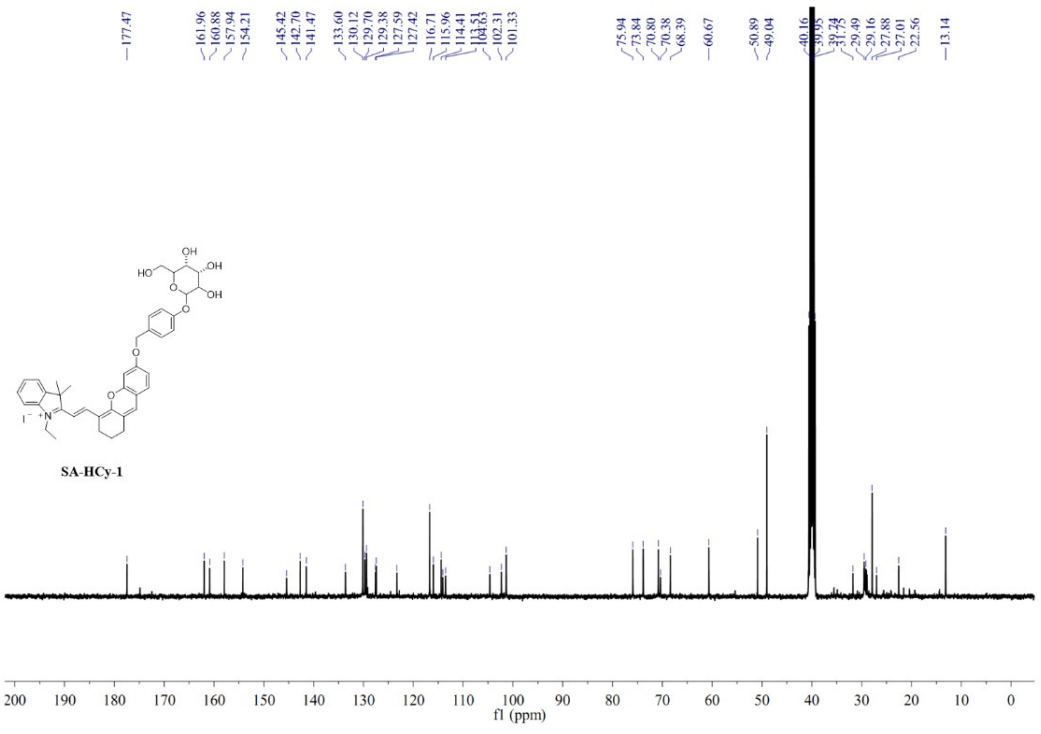


Figure S6. ^{13}C NMR spectrum of **SA-HCy-1** in DMSO-d_6.

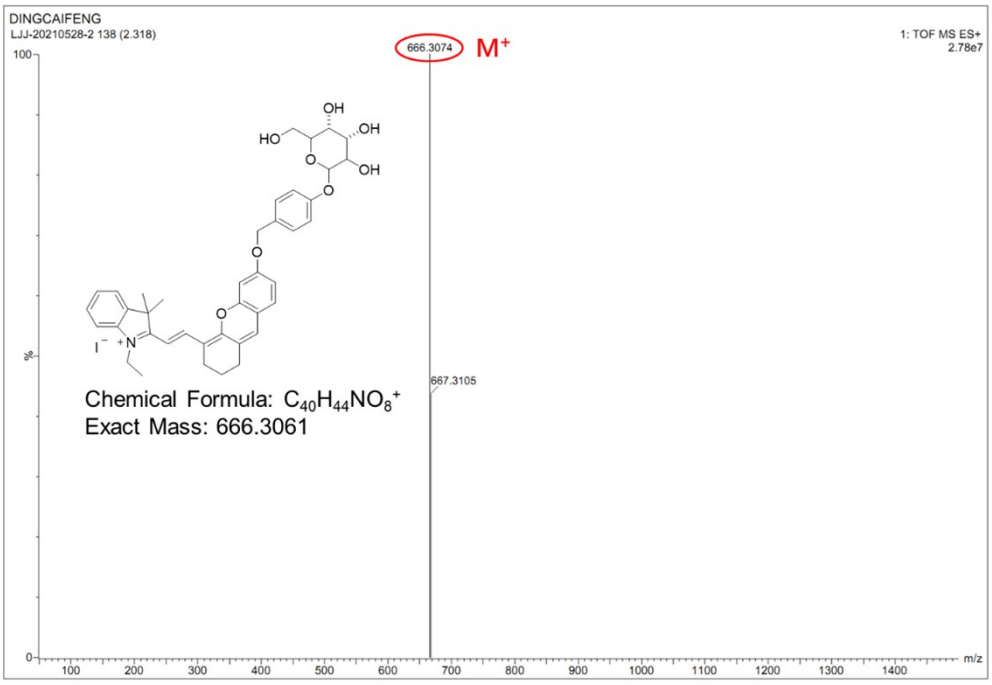


Figure S7. HR-MS spectrum of **SA-HCy-1** in MeOH.

3. Spectral profiles

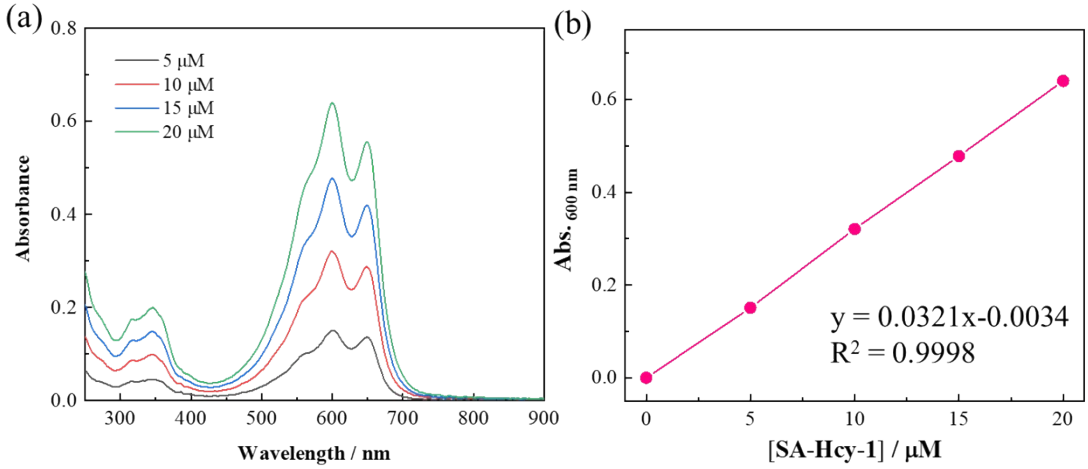


Figure S8. (a) Absorbance spectra of **SA-HCy-1** of increasing concentrations from 0 to 20 μM in 10 mM PBS of pH 8.0. (b) Linear relationship of absorption at 600 nm and **SA-HCy-1** concentrations from 0 to 20 μM.

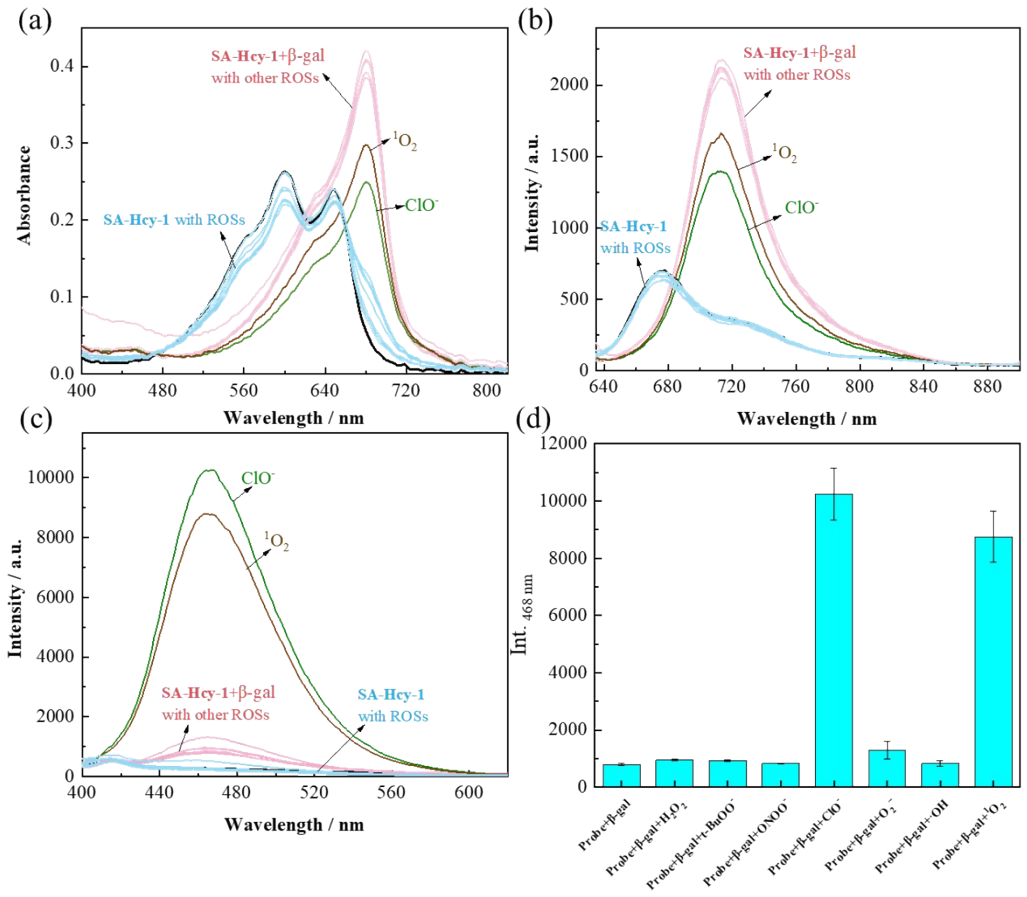


Figure S9. (a) Absorbance and (b & c) fluorescence spectra of **SA-HCy-1** treated with 100 μM different ROSs (blue lines) or 100 mU mL^{-1} β-gal and 100 μM ROSs (green line for ClO$^-$; brown line for $^1O_2$ and pink lines for other ROSs) in 10 mM PBS of pH 8.0. [**SA-HCy-1**] = 10 μM, λ_{ex}= 600 nm for (b) and 360 nm for (c). (d) Fluorescence intensity of **SA-HCy-1** at 468 nm upon incubated with 100 mU mL^{-1} β-gal and 100 μM ROSs (blank, H_2O_2, t-BuOO$^-$, OONO$^-$, ClO$^-$, O_2^-, ·OH and $^1O_2$) in 10 mM PBS of pH 8.0 for 60 minutes.

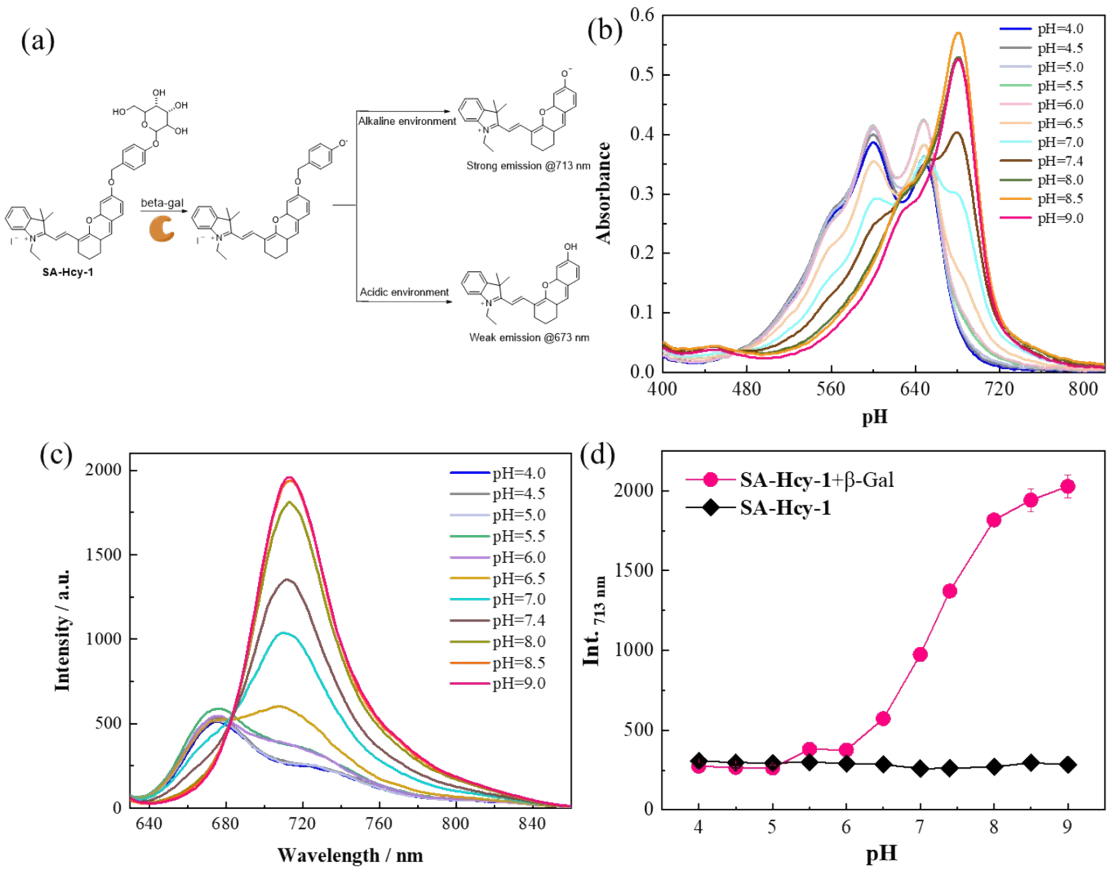


Figure S10. (a) Reaction scheme of **SA-HCy-1** with β-gal in acidic and alkaline environments. UV-vis absorption (b) and fluorescence (c) spectra of **SA-HCy-1** upon the incubation with 100 mU mL^{-1} β-gal for 30 min in 10 mM PBS of different pH values from 4.0 to 9.0. (d) Fluorescence intensity of **SA-HCy-1** at 713 nm before (black squares) and after (red dots) the incubation with 100 mU mL^{-1} β-gal for 30 min in 10 mM PBS of different pH values from 4.0 to 9.0. [**SA-HCy-1**] = 10 μM, λ_{ex}= 600 nm.

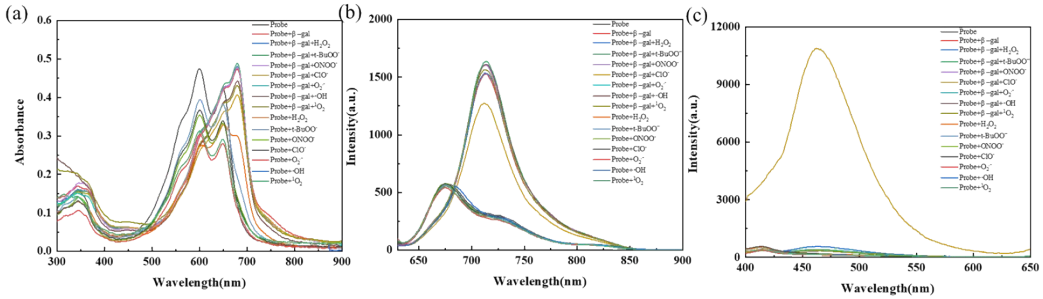


Figure S11. (a) Absorbance and (b & c) fluorescence spectra of **SA-HCy-1** treated with 100 μM different ROSs (blank, H_2O_2, t-$BuOO^-$, $OONO^-$, ClO^-, O_2^-, ·OH and $^1O_2$) or 100 mU mL^{-1} β-gal and 100 μM ROSs (yellow line for ClO^-) in 10 mM PBS of pH 7.4. [**SA-HCy-1**] = 10 μM, λ_{ex}= 600 nm for (b) and 360 nm for (c).

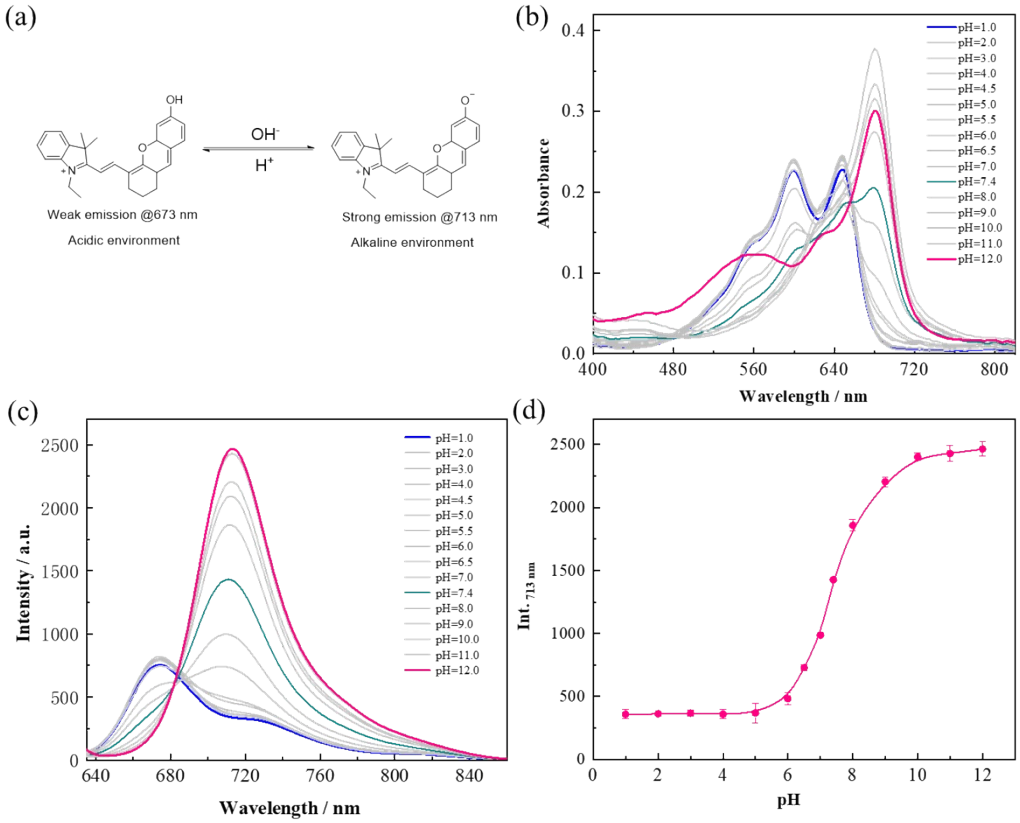


Figure S12. (a) Chemical structure of **HCy-OH** in acidic and alkaline environments. pH dependence of UV-vis absorption (b) and fluorescence (c) spectra of **HCy-OH** in 10 mM PBS of different pH values from 1.0 to 12.0. (d) Fluorescence intensity of **HCy-OH** at 713 nm in 10 mM PBS of different pH values from 1.0 to 12.0. [**HCy-OH**] = 10 μM, λ_{ex}= 600 nm.

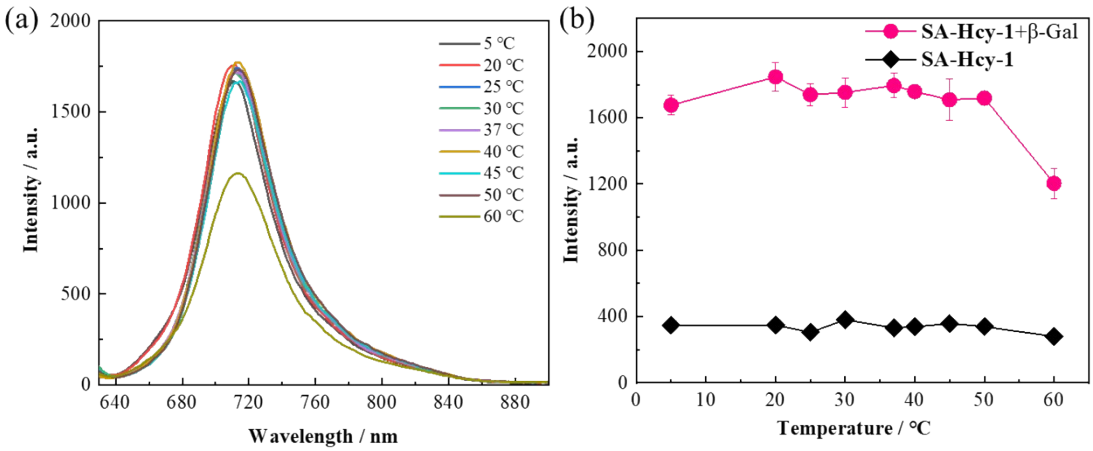


Figure S13. (a) Fluorescence spectra of **SA-HCy-1** upon the incubation with 100 mU

mL^{-1} β-gal at different temperature from 5 to 60 °C for 30 minutes in 10 mM PBS of pH 8.0. (b) Fluorescence intensity of **SA-HCy-1** at 713 nm before (black squares) and after (red dots) the incubation with 100 mU mL^{-1} β-gal at different temperature from 5 to 60 °C for 30 minutes in 10 mM PBS of pH 8.0. [**SA-HCy-1**] = 10 μM, λ_{ex}= 600 nm.

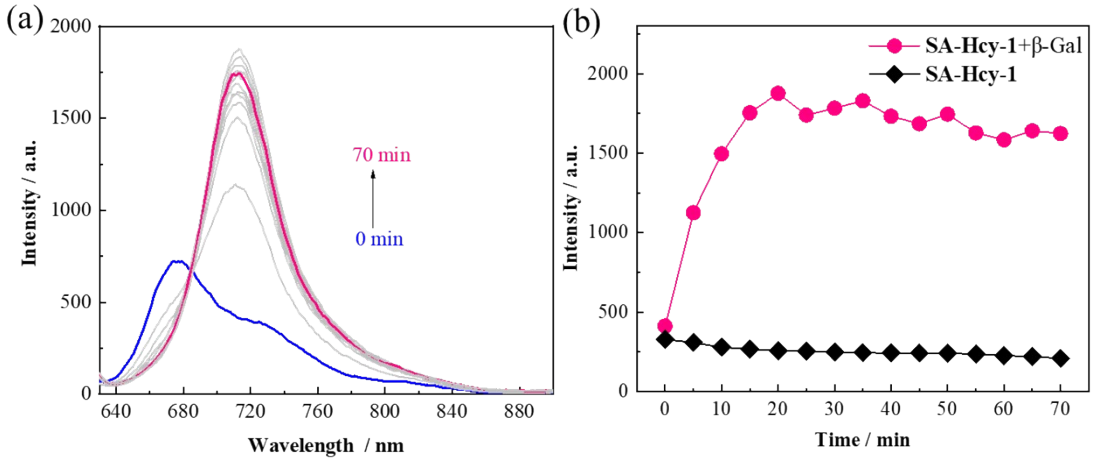


Figure S14. (a) Fluorescence spectra of **SA-HCy-1** upon the incubation with 100 mU mL^{-1} β-gal at 37 °C for 0 to 70 minutes in 10 mM PBS of pH 8.0. (b) Fluorescence intensity of **SA-HCy-1** at 713 nm before (black squares) and after (red dots) the incubation with 100 mU mL^{-1} β-gal at 37 °C from 0 to 70 minutes in 10 mM PBS of pH 8.0. [**SA-HCy-1**] = 10 μM, λ_{ex}= 600 nm.

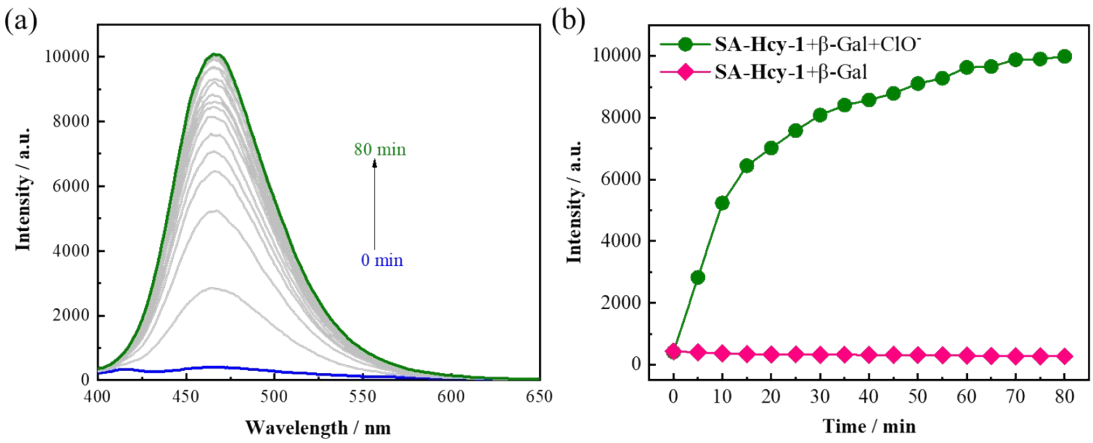


Figure S15. (a) Fluorescence spectra of **SA-HCy-1** upon the incubation with 100 mU mL^{-1} β-gal and 100 μM ClO^- at 37 °C for 0 to 80 minutes in 10 mM PBS of pH 8.0. (b) Time-dependent fluorescence intensity of **SA-HCy-1** at 468 nm upon the incubation

with 100 mU mL^{-1} β-gal with (green dots) or without (red squares) 100 μM ClO^- at 37 ^oC for 0 to 80 minutes in 10 mM PBS of pH 8.0. [**SA-HCy-1**] = 10 μM, λ_{ex}= 360 nm.

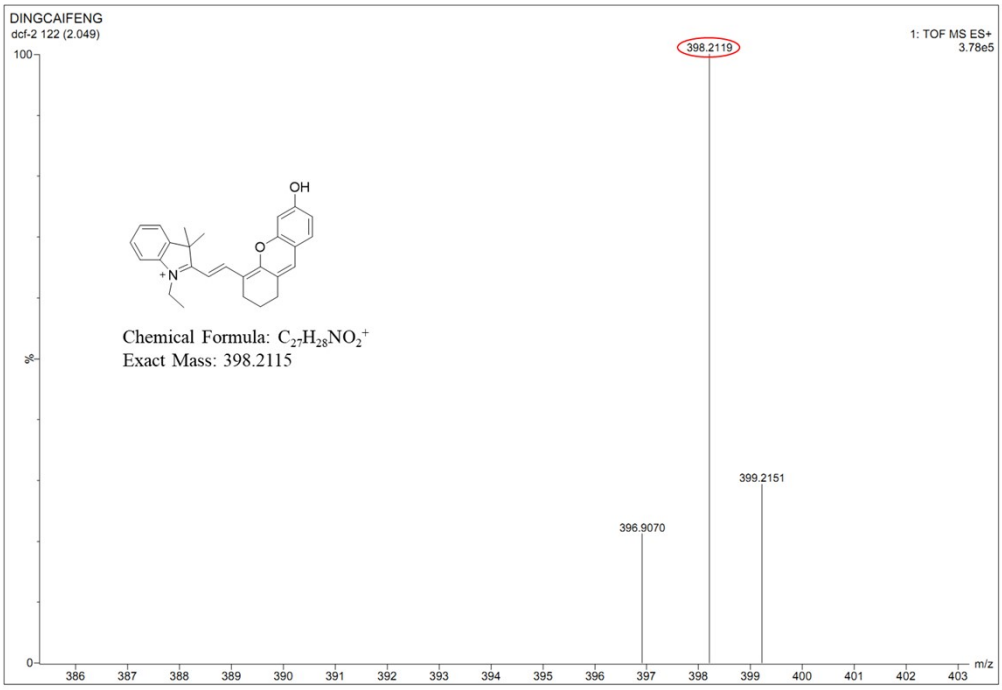


Figure S16. HR-MS spectrum of probe **SA-HCy-1** upon incubated with β-gal in PBS.

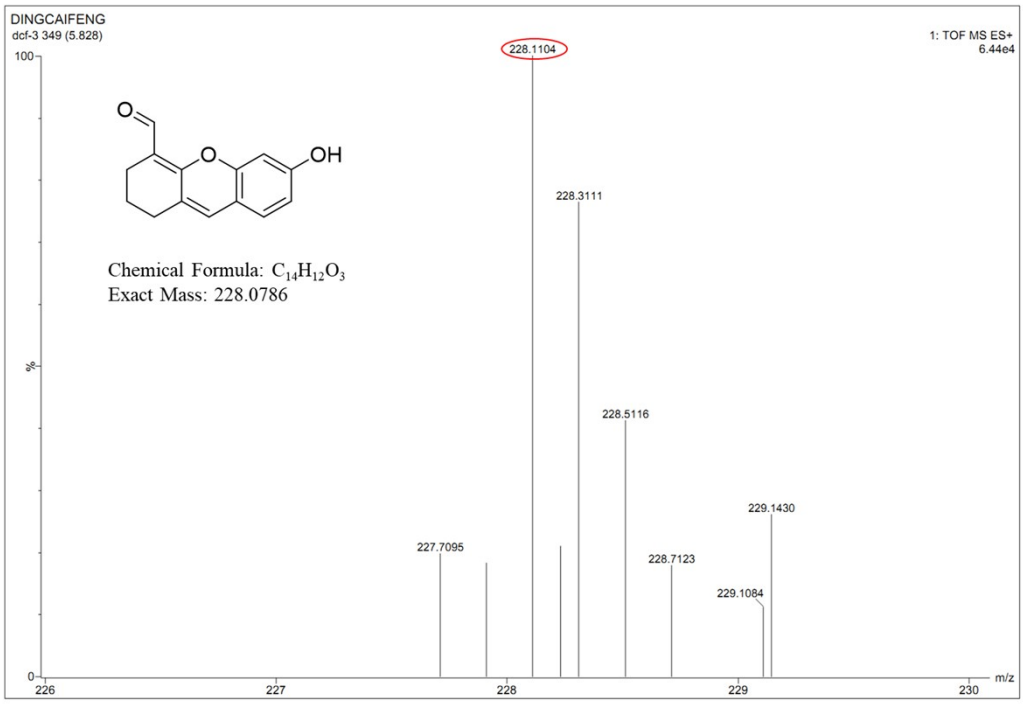


Figure S17. HR-MS spectrum of probe **SA-HCy-1** upon co-incubated with β-gal and ClO^- in PBS.

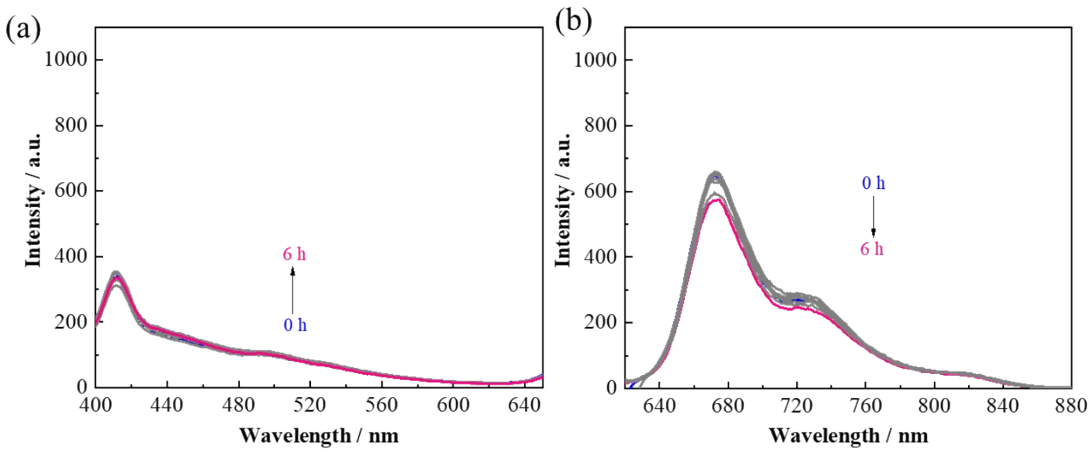


Figure S18. Fluorescence spectra of **SA-HCy-1** incubated in 10 mM PBS of pH 6.5 at 37 °C for 0 to 6 hours. [**SA-HCy-1**] = 10 μM, λ_{ex}= 360 nm for (a) and 600 nm for (b).

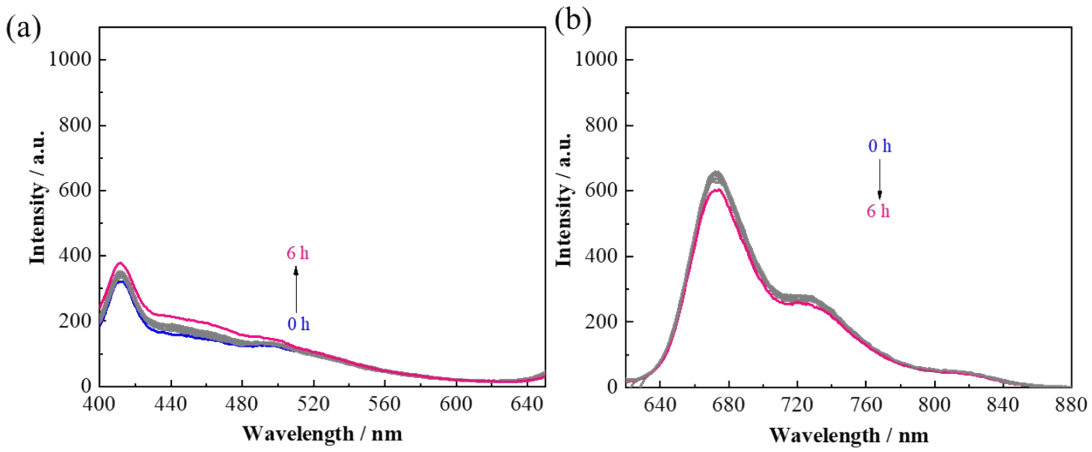


Figure S19. Fluorescence spectra of **SA-HCy-1** incubated in 10 mM PBS of pH 7.4 at 37 °C for 0 to 6 hours. [**SA-HCy-1**] = 10 μM, λ_{ex}= 360 nm for (a) and 600 nm for (b).

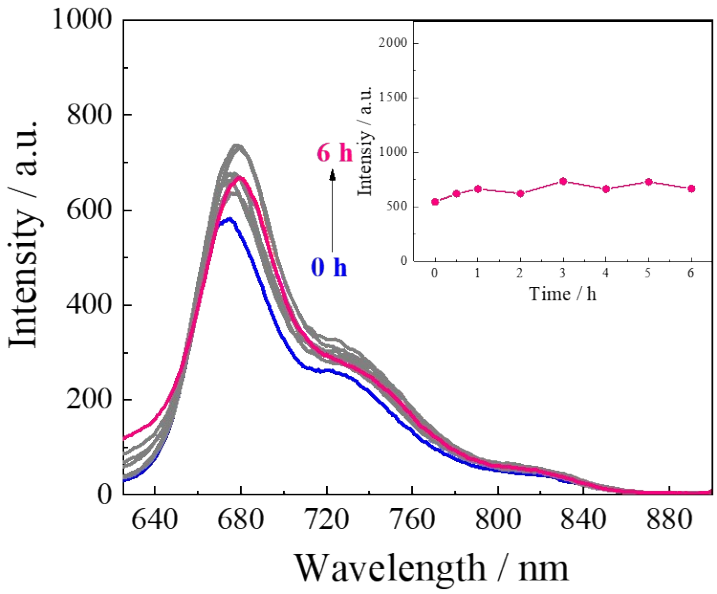


Figure S20. Fluorescence spectra of **SA-HCy-1** incubated in DMEM at 37 °C for 0 to 6 hours. Inset: Fluorescence intensity of **SA-HCy-1** at 713 nm from 0 to 6 hours in DMEM. [**SA-HCy-1**] = 10 μM, λ_{ex}= 600 nm.

4. Cytotoxicity of probe SA-HCy-1

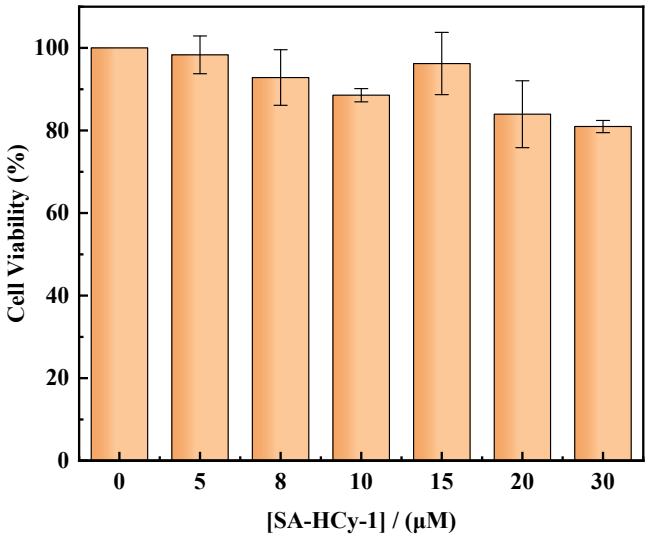


Figure S21. Cytotoxicity of probe **SA-HCy-1** against RAW 264.7 cells determined by Cell Counting kit-8 (CCK-8) assay. The cells were treated with **SA-HCy-1** (0-30 μM) for 12 hours. The data were given as mean ± SD (n = 5).

5. Living cells imaging

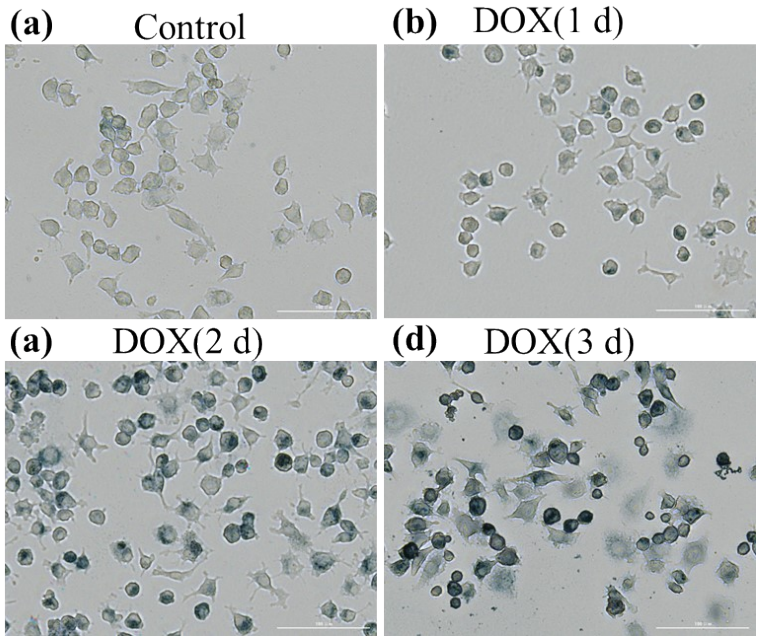


Figure S22. Images of Sudan black B staining of RAW 264.7 cells before (a) and after (b-d) treated with 0.1 μM DOX for 1-3 days.

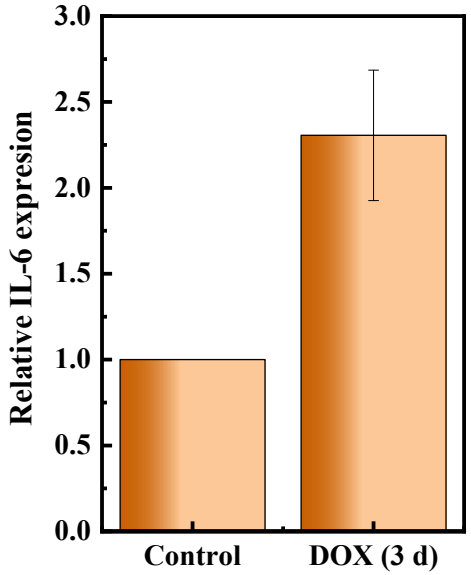


Figure S23. The qPCR data of IL-6 (a kind of typical senescence-associated secretory phenotype) from RAW 264.7 cells before (the control bar) and after (the DOX bar) treated with 0.1 μM DOX for 3 days.

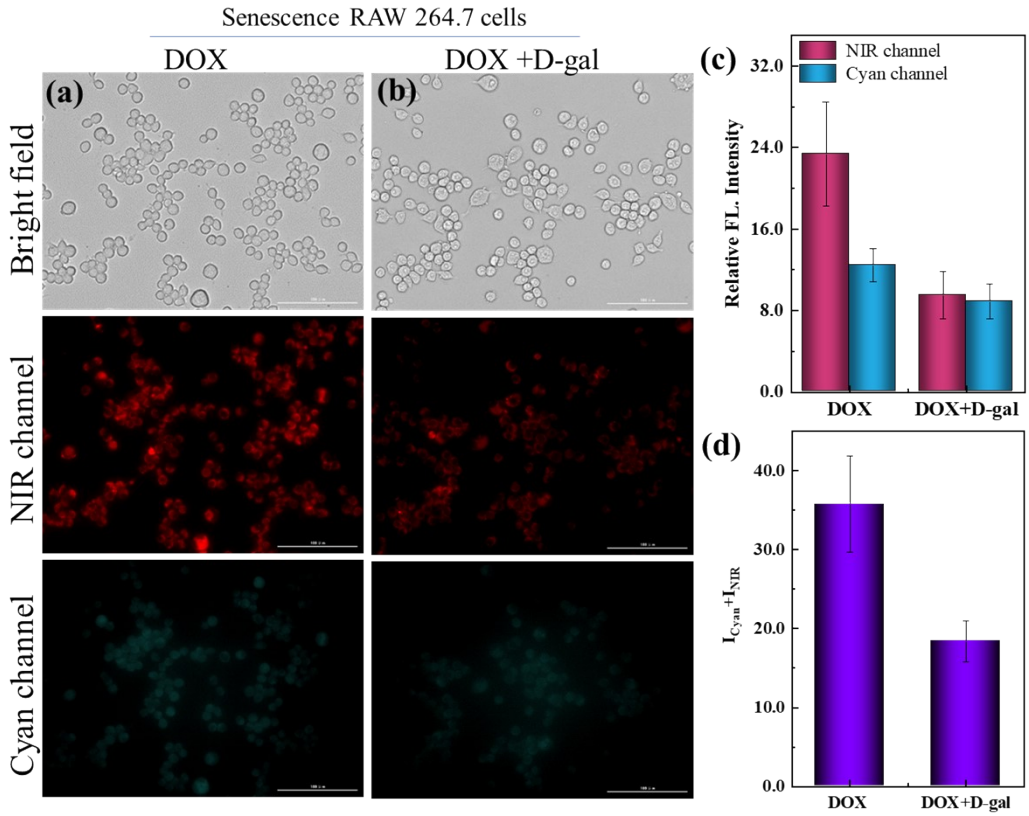


Figure S24. (a-b) Fluorescent images of senescence RAW 264.7 cells (the cells were pretreated with 0.1 μM DOX for 3 days) after the incubation with 10 μM **SA-HCy-1**. (a) Senescence RAW 264.7 cells incubated with **SA-HCy-1**; (b) The senescence RAW 264.7 cells were pertreated with 50 μM D-galactose for 60 minutes and then incubated with **SA-HCy-1**. (c) Relative fluorescence intensities of (a)-(b) from NIR (red bars) and cyan (blue bars) channels. (d) Quantification of fluorescence intensity sums (I_{Cyan} + I_{NIR}) of the images from (a)-(b). For NIR channel: λ_{ex} = 628 nm, λ_{em} = 670-730 nm; for cyan channel: λ_{ex} = 377 nm, λ_{em} = 450-500 nm, scale bar = 100 μm. Error bars represent the standard deviation (± S.D.), n = 3.

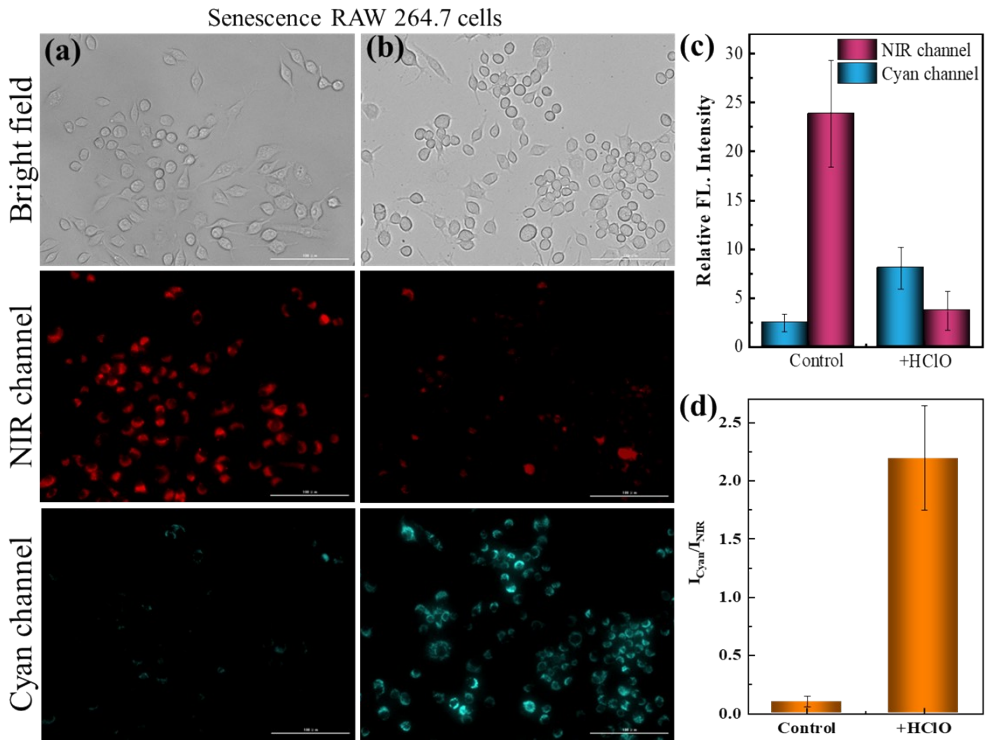


Figure S25. (a-b) Fluorescent images of senescence RAW 264.7 cells (the cells were pretreated with 0.1 μM DOX for 3 days) after the incubation with 10 μM **SA-HCy-1**. (a) The control group (senescence RAW 264.7 cells incubated with **SA-HCy-1**); (b) The senescence RAW 264.7 cells were pertreated with 100 nM HClO for 30 minutes and then incubated with **SA-HCy-1**. (c) Relative fluorescence intensities of (a)-(b) from NIR (red bars) and cyan (blue bars) channels. (d) Quantification of fluorescence intensity ratios (I_{Cyan} / I_{NIR}) of the images from (a)-(b). For NIR channel: λ_{ex} = 628 nm, λ_{em} = 670-730 nm; for cyan channel: λ_{ex} = 377 nm, λ_{em} = 450-500 nm, scale bar = 100 μm. Error bars represent the standard deviation (± S.D.), n = 3.

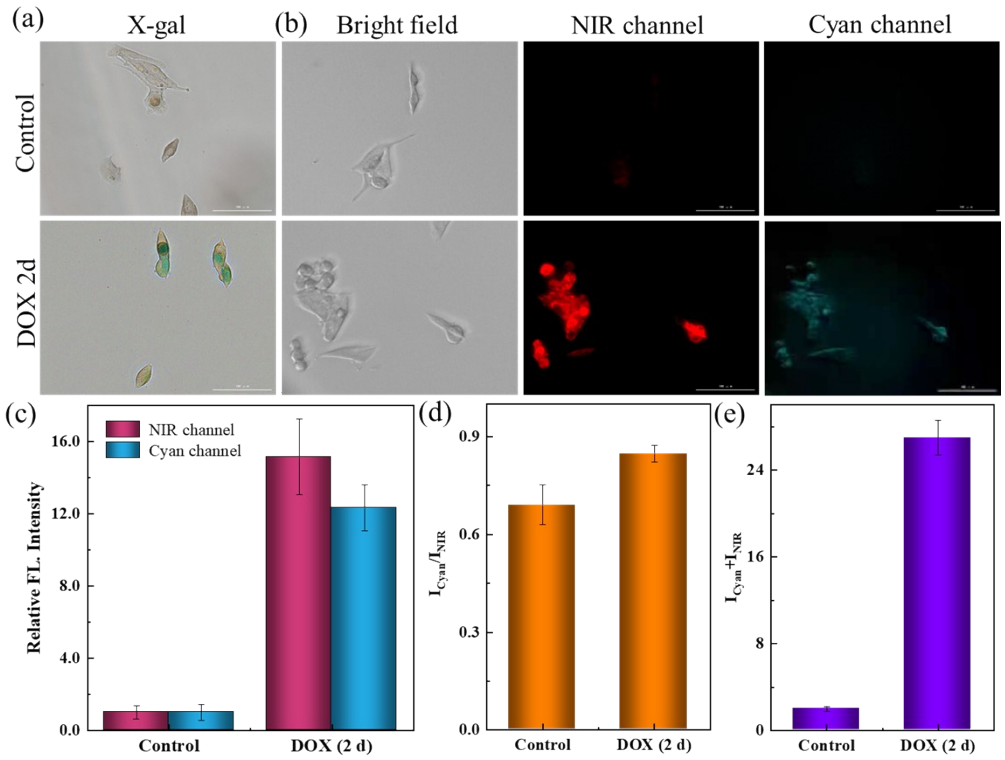


Figure S26. (a) X-gal staining of B16 cells before (above) and after (below) treated with 0.1 μM DOX for 2 days. (b) Fluorescent images of B16 cells with (above) or without (below) pretreated with 0.1 μM DOX for 2 days and then incubated with 10 μM **SA-HCy-1**. For NIR channel: λ_{ex} = 628 nm, λ_{em} = 670-730 nm; for cyan channel: λ_{ex} = 377 nm, λ_{em} = 450-500 nm, scale bar = 100 μm. (c) Quantification of fluorescence intensities of NIR (red bars) and cyan (blue bars) channels from the images in (b). (d) Quantification of fluorescence intensity ratios (I_{Cyan} / I_{NIR}) from (b). (e) Quantification of fluorescence intensity sums (I_{Cyan} + I_{NIR}) from (b). Error bars represent the standard deviation (± S.D.), n = 3.

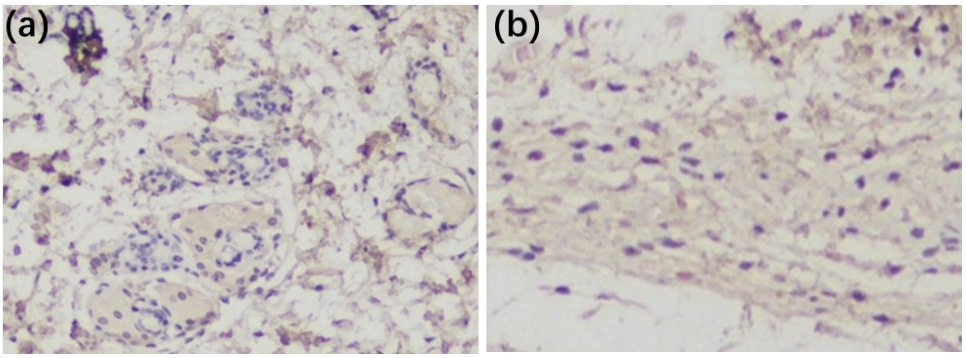


Figure S27. X-gal staining of the skin in the left (a) and right (b) side of the photoaged mice.

6. Comparison of representative probes for senescence with SA-HCy-1

Table S1. Comparison of the representative senescence probes with **SA-HCy-1**.

Probe name	Detection marker	Cell sources and stress types	Mouse aging model	Reference
HCyXA-βGal	β-Gal	WI-38 (Unclear)	Not study	Chin. Chem. Lett. 2023, 34 (12).
KSA01 and KSA02	β-Gal and pH	MRC5 (continuous culture), Hep G2 (XL413), HL-7702(DOX), Hep3B (LY3177833)	Natural aging mice	Angew. Chem. Int. Ed. 2021, 60, 10756–10765
HeckGal	β-Gal	4T1 (palbociclib),A549 (cisplatin), BJ (DOX),SK-Mel-103 (palbociclib)	Drug-induced senescence tumor model, renal fibrosis mouse mode	Anal. Chem. 2021, 93, 3052−3060
AHGa	β-gal	SK-Mel-103 (palbociclib)	Drug-induced senescence tumor model	J. Am. Chem. Soc. 2017, 139, 8808-8811
NBGal	β-gal	4T1 (palbociclib) ,SK-Mel-103 (palbociclib)	Drug-induced senescence tumor model	Anal. Chem. 2023, 95, 1643–1651
Sia-RQ	Sialidase	Huh7 (palbociclib)	Not study	Chem. Commun. 2018, 54, 11566-11569
QM-NHafuc	α-L -fucosidase	HDFs (continuous culture), HCT116 (AZD, t-BHP, UVA)	Drug-induced senescence tumor model	Chem. Sci. 2021, 12, 10054-10062
SBB-N_3 +BODIPY	Lipofuscin	SK-Mel-103 (palbociclib), MDA-MB-231 (palbociclib), WI-38 (palbociclib)	Not study	FEBS. J. 2022. 290 (5), 1314-1325.
XZ1208	β-gal	HEL (continuous culture), HDF (continuous culture)	Natural aging mice, total body irradiated (TBI) mice	Aging Cell. 2023; 22: e13896
SRP	β-gal	HUVECs (H_2O_2)	Not study	Sens. Actuat. B: Chem. 2018, 274, 194-200
SA-HCy-1	β-gal, pH and ROS	RAW 246.7 (continuous culture and DOX), B16 (DOX)	Photoaging mice model (ratiometric fluorescence imaging and slice X-gal staining)	This work

7. References

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