A Dual-enzyme Activated Fluorescent Probe for Precise Identification of Tumor Senescence

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Content:

1. General methods

Reagents and Instruments.

4-Bromo-1,8-Naphthalenedioic anhydride, 1-hydroxybenzotriazole, 3-bromopropionic acid, 4-methylquinoline, and 5-nitrosalicylaldehyde are sourced from Anagi Chemicals. Hydroiodic acid was purchased from McLean Biochemical Co., Ltd. 1- (3-dimethylaminopropyl) -3-ethylcarbodiimide hydrochloride (EDCl), N-tert-butoxycarbonyl-1,2-ethylenediamine and other chemical reagents were purchased from Exploration Technology Co., Ltd. Lipopolysaccharide comes from Yuanye Biotechnology Co., Ltd. Cell-Cointing-Kit-8, DMEM high glucose medium, 1640 medium, fetal bovine serum, penicillin, and streptomycin were all obtained from Shengong Bioengineering Co., Ltd. NMR experiments were performed on Bruker Avance II NMR spectrometer (Germany). The fluorescence and absorption spectra were collected on a Hitachi F-7000 spectrophotometer (Japan) and UV-2550 spectrophotometer (Japan), respectively. Cell images were obtained using a Nikon C2 confocal laser scanning microscope (CLSM, Japan).

The stability of the probe HDQ-NA-AFU-Gal

The stability of the engineered probe was also studied by fluorescence analysis. Briefly, the probe HDQ-NA-AFU-Gal was added in 10 mM PBS at pH 6 or 10% FBS-containing DMEM medium and kept at 37 °C for indicated time intervals (1, 4.5, 8, 15, 22, 36, 50 h). Thereafter, the fluorescence spectra (740 nm and 550 nm) were collected by using the fluorescence spectrophotometer (Hitachi, F-7100).

Cell Culture and Confocal Imaging

Human normal hepatocytes (LO_2 cells), human hepatocellular carcinoma cells (HepG2 cells), mouse breast cancer cells (4T1 cells), and human ovarian cancer cells (Ovcar-3 cells) were obtained from the Cell Bank of the Committee for Preservation of Typical Cultures of the Chinese Academy of Sciences (Shanghai, China). Except for Ovcar-3 cells cultured in RPMI-1640 medium, the other mentioned cells were cultured in DMEM high glucose medium containing 10% FBS and 1% antibiotics (penicillin/streptomycin, 100 μg/mL) in an incubator at 37 °C in a 5% CO_2 environment. For the detection of intracellular AFU and β-gal, the probes were

incubated with the cells for 1 h, washed three times with PBS, and then imaged under 488 nm and 640 nm lasers.

Cell Viability Assay

The cytotoxicity of the probe was evaluated using the CCK-8 assay method. The tested cells (5.0×10^3 cells/well) were seeded in a 96-well plate and cultured for 24 h at 37 °C in a 5% CO_2 atmosphere. Subsequently, various concentrations of HDQ-NA-AFU-Gal (0-80 μM) were added to each well. After further incubation for 24 h, 10 μL of CCK-8 solution was added to each well, followed by incubation at 37 °C in a 5% CO_2 environment for 4 h. The optical density (OD) of the solution in the 96-well plate at 450 nm was measured using a microplate reader.

Establishment of Cell Models

To study the fluctuation of AFU and β-gal levels during cell senescence, relevant cell senescence models were established. For LO_2 and HepG2 cells, the cells were co-incubated with H_2O_2 (150 μM) for 3 days to induce senescence. For Ovcar-3 cells, the cells were co-incubated with doxorubicin (DOX, 0.5 μM) for 3 days to induce senescence. For 4T1 cells, cells were cultured with 5 μM palbociclib in DMEM for one week to induce senescence. After establishing the cell models, the probe was co-incubated with the cells for 1 h and imaged using confocal microscopy.

Cell Senescence Assay

The cell senescence staining kit was used according to the manufacturer's instructions to verify the successful establishment of the senescence models. In brief, model cells were washed once with PBS buffer, then 1 mL of β-gal staining fixative was added to each well of a six-well plate. After 15 min, the fixative was removed, and cells were washed every 3 min for a total of 3 times. Subsequently, 1 mL of staining working solution was added to each well, and cells were incubated at 37 °C for 12 h. Cells were then examined under an optical microscope, where blue color indicates β-gal activity.

Establishment of Tumor Senescence Mouse Model

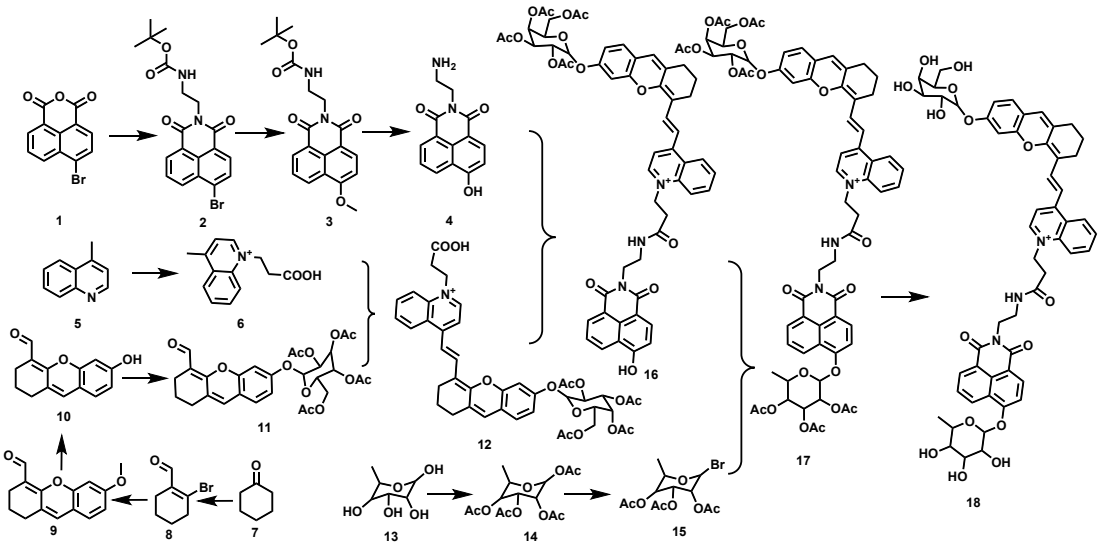
All mouse experiments were conducted according to the protocol approved by the Animal Experiment Ethics Committee of East China Normal University. According to

previous literature,[1,2] an allograft tumor model was established by subcutaneously injecting five hundred thousand 4T1 cells into the right hind limb of each 6-week-old female BALB/c mice. After the tumor grew to 5 mm in diameter, the mice were randomly divided into two groups. Verify the successful establishment of a mouse tumor model through H&E sectioning. One group was treated with Palbociclib (100 mg/kg) by gavage every day for day 7. The other group was treated with saline by gavage every day for day 7. Ki67 staining of tumor tissue was performed after the treatment at day 7. Subsequently, the probe was intravenously injected (i.v.) to the mice, and after 2 h, imaging and analysis were performed using an IVIS spectroscopic imaging system.

Statistical Analysis

Data were expressed as the means ± standard deviation (SD) of three parallel measurements. The significance of the difference was evaluated via one-way analysis of variance. Statistical significance was set at *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

2. Synthesis and characterization of compounds



Scheme S1. The general synthesis routes for HDQ-NA-AFU-Gal probe.

Synthesis of Compound 2

Compound 1 (2.77 g, 10 mmol) and tert-butyl (2-aminoethyl) carbamate (1.6 mL, 20 mmol) were added to a round-bottom flask containing 100 mL of anhydrous ethanol,

then the mixture was heated and refluxed overnight. After completion of the reaction, it was filtered and dried to give compound 2, which was used directly in the next step without any treatment.^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 8.76 - 8.65 (m, 1H), 8.60 (d, J = 8.4 Hz, 1H), 8.45 (d, J = 7.9 Hz, 1H), 8.07 (d , J = 7.9 Hz, 1H), 7.93 - 7.81 (m, 1H), 4.94 (s, 1H), 4.38 (t, J = 5.6 Hz, 2H), 3.56 (d, J = 5.4 Hz, 2H), 1.29 (s, 9H).

Synthesis of Compound 3

Compound 2 (3.03 g, 6.3 mmol) and potassium carbonate (7 g, 50 mmol) were added to a round-bottomed flask containing 100 mL of methanol, after which the mixture was heated and refluxed overnight. Upon completion of the reaction, the mixture was poured into water and a large amount of yellow solid appeared. The crude product was purified by column chromatography (200-300 mesh) using gradient elution with CH_2Cl_2 and MeOH (100:1-30:1, v/v) to afford the yellow solid compound 3. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.76 - 8.65 (m, 1H), 8.60 (d, J = 8.4 Hz, 1H), 8.45 (d, J = 7.9 Hz, 1H), 8.07 (d, J = 7.9 Hz, 1H), 7.93 - 7.81 (m, 1H), 4.94 (s, 1H), 4.38 (t, J = 5.6 Hz, 2H), 3.56 (d, J = 5.4 Hz, 2H), 1.29 (s, 9H).

Synthesis of Compound 4

Compound 3 (1.85 g, 5 mmol) and hydriodic acid (30 mL) were added to a 50 mL round-bottomed flask, and then the mixture was heated to reflux at 140 °C overnight. Upon completion of the reaction, a large yellow solid appeared, which was filtered and washed with plenty of water. The yellow solid obtained was dried under vacuum at 60 °C and used directly in the next step without treatment.^{1}H NMR (500 MHz, DMSO) δ (ppm) 11.97 (s, 1H), 8.57 (dd, J = 8.3, 1.2 Hz, 1H), 8.49 (dd, J = 7.3, 1.2 Hz, 1H), 8.38 (d, J = 8.2 Hz , 1H), 7.80 (dd, J = 8.3, 7.3 Hz, 3H), 7.19 (d, J = 8.2 Hz, 1H), 4.29 (t, J = 5.8 Hz, 2H), 3.14 (dd, J = 11.3, 5.7 Hz, 2H).

Synthesis of Compound 6

4-Methylquinoline (1.43 g, 10 mmol) and 3-bromopropanoic acid (1.51 g, 10 mmol) were added to a round-bottom flask containing 50 mL anhydrous acetonitrile. The mixture was refluxed overnight. Filtered to obtained compound 6, no purification required, directly used for the next step. ^{1}H NMR (500 MHz, DMSO) δ 9.13 – 9.05 (m, 1H), 8.42 (d, J = 8.5 Hz, 1H), 8.22 (d, J = 8.6 Hz, 1H), 8.13 (t, J = 7.7 Hz, 1H), 7.96 –

7.93 (m, 2H), 3.09 – 3.03 (m, 2H), 2.98 (s, 2H), 2.95 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 172.0, 157.9, 144.9, 134.6, 129.9, 128.3, 126.2, 122.9, 122.0, 53.3, 33.6, 19.9.

Synthesis of compound 8

DMF (8 mL) and chloroform (50 mL) were mixed at 0 °C for 30 min, followed by dropwise addition of PBr_3 solution (10 mL) to the mixture. After stirring for 60 min, cyclohexanone (4 mL) was added. The reaction mixture was left at room temperature for 12 h, then poured into water, adjusted to neutral pH, and extracted with CH_2Cl_2. The crude product was used directly for the next step without purification.

Synthesis of compound 9

2-Hydroxy-4-methoxybenzaldehyde (1.52 g, 10 mmol), cesium carbonate (10 g, 31 mmol), and 2-bromo-1-cyclohexene-1-carbaldehyde (1.89 g, 10 mmol) were dissolved in 5 mL anhydrous DMF under nitrogen at 25 °C and stirred overnight. After completion, the solution was poured into water and extracted with CH_2Cl_2. The crude product was purified by column chromatography (200-300 mesh) using a gradient of petroleum ether and methanol (5:1, v/v) to yield a yellow solid.

Synthesis of compound 10

Compound 9 (2.42 g) was dissolved in 20 mL anhydrous CH_2Cl_2 at 0 °C, followed by dropwise addition of diluted BBr_3 (3 mL, dissolved in 5 mL CH_2Cl_2). After stirring at 0 °C for 1 h, the mixture was allowed to react at room temperature overnight. It was then slowly poured into water to collect the solid. ^{1}H NMR (500 MHz, DMSO) δ 10.20 (s, 1H), 7.20 (d, J = 8.3 Hz, 1H), 6.94 (s, 1H), 6.69 – 6.53 (m, 2H), 2.57 – 2.52 (m, 2H), 2.29 (t, J = 6.0 Hz, 2H), 1.66 – 1.56 (m, 2H).

Synthesis of compound 11

Compound 10 (0.228 g, 1 mmol), tetra acetyl-α-D-bromogalactose (1.65 g, 2 mmol), anhydrous sodium sulfate (0.6 g, 4 mmol), and cesium carbonate (1.3 g, 4 mmol) were dissolved in 6 mL anhydrous DMF. After approximately 8 h, the solution was poured into water to precipitate a yellow solid, filtered to obtain compound 11. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.34 (s, 1H), 7.11 (d, J = 8.4 Hz, 1H), 6.79 – 6.72 (m, 2H), 6.67 (s, 1H), 5.50 (dd, J = 8.2, 2.4 Hz, 2H), 5.36 – 5.35 (m, 1H), 5.14 (dd, J = 10.4, 3.4 Hz, 1H),

5.12 – 5.10 (m, 1H), 4.14 – 4.11 (m, 2H), 2.61 – 2.57 (m, 2H), 2.46 (t, J = 6.0 Hz, 2H), 2.21 (s, 3H), 2.18 (d, J = 0.8 Hz, 3H), 2.06 (s, 6H), 1.74 (d, J = 6.2 Hz, 2H).

Synthesis of compound 12

Compound 11 (0.405 g, 1 mmol) and compound 6 (0.232 g, 1 mmol) were dissolved in anhydrous acetonitrile, and then triethylamine (0.14 mL) was added. The mixture was stirred at 85 °C overnight, and the crude product was purified by silica gel column chromatography (CH_2Cl_2: MeOH = 7:1, v/v) to yield compound 12. ^{1}H NMR (500 MHz, DMSO) δ 9.05 (d, J = 6.6 Hz, 1H), 8.84 (d, J = 8.5 Hz, 1H), 8.57 – 8.51 (m, 1H), 8.45 (d, J = 6.7 Hz, 1H), 8.39 (d, J = 8.8 Hz, 1H), 8.13 (d, J = 7.6 Hz, 1H), 8.08 – 8.04 (m, 1H), 7.90 (d, J = 7.7 Hz, 1H), 7.32 (d, J = 8.5 Hz, 1H), 7.18 (s, 1H), 6.89 (s, 1H), 5.69 (d, J = 7.7 Hz, 1H), 5.38 (s, 1H), 5.29 – 5.20 (m, 3H), 5.08 (s, 2H), 4.60 (d, J = 6.2 Hz, 1H), 4.14 (d, J = 5.1 Hz, 2H), 3.09 (d, J = 7.2 Hz, 2H), 2.71 (s, 2H), 2.60 (s, 2H), 2.17 (s, 3H), 2.08 (s, 3H), 2.00 (s, 3H), 1.97 (s, 3H), 1.80 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 172.2, 170.4, 170.3, 170.0, 169.7, 158.0, 154.1, 153.6, 153.1, 147.2, 138.2, 135.1, 130.0, 128.9, 128.7, 126.8, 126.4, 117.4, 115.2, 114.5, 113.3, 112.2, 104.5, 97.6, 70.9, 70.6, 68.7, 67.6, 61.6, 46.1, 33.8, 29.3, 21.1, 21.0, 20.9, 20.8, 9.00.

Synthesis of compound 14

Compound 13 (3 g) was dissolved in acetic anhydride (11 mL) and pyridine (40 mL), then stirred at room temperature for 24 h. After completion, the solution was poured into water, extracted with CH_2Cl_2, and washed with saturated NaCl solution. Evaporation of the solvent yielded Compound 14, which was used directly for the next step without further treatment. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.34 (d, J = 3.1 Hz, 1H), 5.34 (d, J = 3.0 Hz, 2H), 5.31 (s, 1H), 4.28 (d, J = 6.6 Hz, 1H), 2.18 (s, 3H), 2.15 (s, 3H), 2.01 (d, J = 6.3 Hz, 6H), 1.16 (d, J = 6.5 Hz, 3H).

Synthesis of compound 15

Compound 14 was dissolved in a small amount of chloroform, then under nitrogen atmosphere, a solution of hydrogen bromide in acetic acid (33%) was added dropwise. After 3 h, the mixture was diluted with 200 mL CH_2Cl_2, poured into ice water, washed with 1 M sodium bicarbonate solution, and further purified by silica gel column chromatography to yield compound 15. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.70 (d, J = 3.9

Hz, 1H), 5.41 (dd, J = 10.6, 3.3 Hz, 1H), 5.37 (dd, J = 3.3, 1.1 Hz, 1H), 5.03 (dd, J = 10.6, 3.9 Hz, 1H), 4.42 (t, J = 6.5 Hz, 1H), 2.18 (s, 3H), 2.11 (s, 3H), 2.01 (s, 3H), 1.22 (d, J = 6.5 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.3, 170.1, 169.8, 89.3, 70.0, 69.8, 68.4, 67.8, 20.8, 20.6, 20.5, 15.5.

Synthesis of compound 16

Compound 12 (0.38 g, 0.5 mmol), EDCl (0.19 g, 1 mmol), and 1-hydroxybenzotriazole (HOBT, 0.135 g, 1 mmol) were dissolved in a small amount of anhydrous DMF under nitrogen. The mixture was stirred at 0 °C for 30 min, then compound 4 (0.13 g, 0.5 mmol) and 0.13 mL of triethylamine were added and the reaction continued for 24 h. After completion, the solution was poured into water, extracted with CH_2Cl_2, and the crude product was purified by silica gel column chromatography (CH_2Cl_2: MeOH = 20:1, v/v) to yield compound 16. ^{1}H NMR (500 MHz, DMSO) δ 11.87 (s, 1H), 8.53 (d, J = 8.3 Hz, 1H), 8.46 (d, J = 7.0 Hz, 1H), 8.34 (d, J = 8.1 Hz, 2H), 8.20 – 8.09 (m, 2H), 7.96 (s, 2H), 7.83 (d, J = 8.8 Hz, 2H), 7.75 (d, J = 7.8 Hz, 2H), 7.63 (s, 2H), 7.40 (d, J = 8.1 Hz, 2H), 7.17 (d, J = 8.2 Hz, 2H), 6.87 (d, J = 28.5 Hz, 1H), 5.34 (ddd, J = 103.0, 102.4, 46.0 Hz, 4H), 4.61 (d, J = 6.5 Hz, 2H), 4.20 – 3.99 (m, 4H), 3.30 (d, J = 6.1 Hz, 2H), 2.89 (s, 2H), 2.73 (s, 2H), 2.65 (t, J = 6.6 Hz, 2H), 2.06 (dd, J = 68.1, 30.0 Hz, 12H), 1.23 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 169.7, 164.4, 163.7, 162.8, 160.6, 134.4, 133.9, 131.5, 130.4, 129.7, 129.3, 126.0, 124.5, 122.8, 122.4, 115.0, 113.2, 112.5, 110.4, 67.6, 61.7, 44.6, 37.1, 36.3, 35.3, 31.2, 21.0, 20.8.

Synthesis of compound 17

Compound 15 (0.05 g, 0.15 mmol), compound 16 (0.1 g, 0.1 mmol) and anhydrous cesium carbonate (0.06 g, 0.2 mmol) were dissolved in anhydrous acetonitrile under nitrogen, then refluxed overnight. The solvent was evaporated, and the residue in the dichloromethane solution was washed with 1 M NaOH and brine. Cesium carbonate and 4 mL anhydrous DMSO were added to the residue, stirred at room temperature under nitrogen for 48 h. The mixture was diluted with 50 mL CH_2Cl_2, washed with 5% $NaHCO_3$, 5% citric acid, and brine. After solvent removal, the residue was purified by silica gel column chromatography (CH_2Cl_2: MeOH = 10:1, v/v) to yield Compound 17.

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.52 (d, *J* = 6.7 Hz, 1H), 8.56 – 8.48 (m, 3H), 8.47 – 8.38 (m, 3H), 8.29 (d, *J* = 6.7 Hz, 1H), 8.17 (s, 1H), 8.07 (t, *J* = 7.7 Hz, 1H), 7.85 – 7.79 (m, 1H), 7.72 – 7.67 (m, 1H), 7.23 (d, *J* = 8.3 Hz, 1H), 7.17 – 7.13 (m, 1H), 7.12 (d, *J* = 8.6 Hz, 1H), 6.79 (dd, *J* = 8.4, 2.3 Hz, 1H), 6.70 (s, 1H), 5.72 (dd, *J* = 10.5, 7.9 Hz, 1H), 5.52 (t, *J* = 6.8 Hz, 2H), 5.40 (t, *J* = 5.4 Hz, 2H), 5.28 – 5.17 (m, 4H), 4.49 (s, 1H), 4.37 (t, *J* = 5.5 Hz, 2H), 4.28 (dd, *J* = 11.2, 6.7 Hz, 1H), 4.18 (dd, *J* = 12.3, 6.1 Hz, 2H), 3.65 (s, 2H), 3.16 (d, *J* = 7.3 Hz, 4H), 2.74 – 2.61 (m, 4H), 2.25 (s, 3H), 2.20 (s, 3H), 2.06 (dd, *J* = 25.4, 7.7 Hz, 15H), 1.93 (d, *J* = 6.6 Hz, 2H), 1.46 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 178.2, 170.2, 170.1, 169.9, 169.5, 164.7, 164.1, 159.3, 158.2, 157.6, 153.9, 153.4, 150.7, 146.3, 138.2, 134.9, 132.9, 131.8, 129.4, 128.5, 128.2, 126.6, 126.3, 125.5, 123.5, 122.3, 118.5, 116.9, 112.6, 108.9, 105.0, 103.6, 98.83, 98.2, 70.1, 69.9, 68.7, 68.4, 67.0, 62.7, 61.2, 46.1, 38.6, 29.7, 29.6, 29.6, 20.9, 20.8, 20.8, 20.7, 20.6, 20.6, 16.2, 8.6. HRMS: m/z [$C_{67}H_{68}N_3O_{22}^+$], calcd, 1266.4289; found [M]$^+$: 1266.4309.

Synthesis of compound 18 (HDQ-NA-AFU-Gal)

Compound 17 (0.13 g) was dissolved in 2 mL of a mixed solution of CH_2Cl_2/MeOH and then sodium methanol (5.4 mol/L, 0.2 mL,) was added. After stirring for 3 h, the solvent was evaporated and the residue was purified by silica gel column (CH_2Cl_2: MeOH = 15: 1, v/v) to give compound 18 (HDQ-NA-AFU-Gal). ^{1}H NMR (500 MHz, DMSO) δ 8.82 (d, *J* = 4.6 Hz, 1H), 8.38 (dd, *J* = 15.9, 8.2 Hz, 2H), 8.27 – 8.13 (m, 2H), 8.00 (d, *J* = 8.4 Hz, 1H), 7.97 – 7.91 (m, 2H), 7.81 (d, *J* = 4.8 Hz, 1H), 7.76 (s, 1H), 7.62 (t, *J* = 7.6 Hz, 1H), 7.25 (t, *J* = 7.6 Hz, 2H), 7.11 (d, *J* = 8.5 Hz, 1H), 6.92 (s, 1H), 6.70 (d, *J* = 8.2 Hz, 1H), 6.53 (s, 1H), 6.14 (dd, *J* = 9.5, 6.6 Hz, 2H), 6.06 (d, *J* = 2.1 Hz, 1H), 5.55 (dd, *J* = 10.1, 2.1 Hz, 2H), 5.32 (s, 1H), 4.87 (d, *J* = 7.6 Hz, 2H), 4.23 – 4.02 (m, 6H), 3.96 (d, *J* = 7.0 Hz, 1H), 3.74 (s, 2H), 3.62 – 3.56 (m, 4H), 3.30 – 3.21 (m, 5H), 3.16 – 3.01 (m, 2H), 2.65 (s, 2H), 2.08 – 1.94 (m, 2H), 1.83 – 1.75 (m, 2H), 1.59 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 135.9 (s), 132.4 (s), 130.3 (s), 129.6 (s), 125.3 (s), 124.2 (s), 120.6 (s), 104.8 (s), 74.1 (s), 71.5 (s), 70.9 (s), 70.6

(s), 70.4 (s), 56.2 (s), 38.8 (s), 37.6 (s), 32.0 (s), 26.2 (s), 17.1 (s). HRMS: m/z [$C_{53}H_{54}N_3O_{15}^+$], calcd, 972.3549; found [M+Na]$^+$: 995.3380.

3. Absorption spectroscopy study of HDQ-NA-AFU-Gal

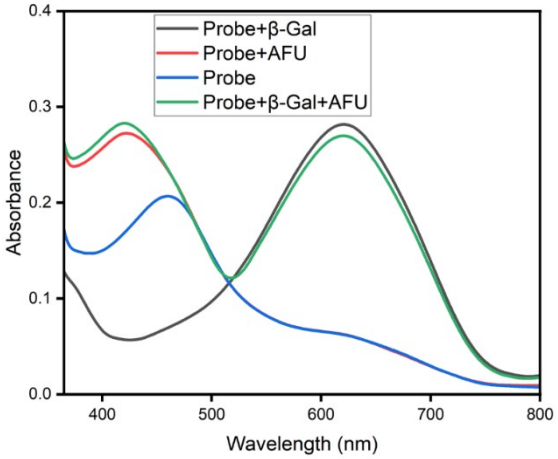


Figure S1. UV-Vis spectra for probe HDQ-NA-AFU-Gal, HDQ-NA-AFU-Gal+β-gal, HDQ-NA-AFU-Gal+AFU, and HDQ-NA-AFU-Gal+β-gal+AFU in DMSO: PBS buffer (1: 9, v/v).

4. The stability of HDQ-NA-AFU-Gal

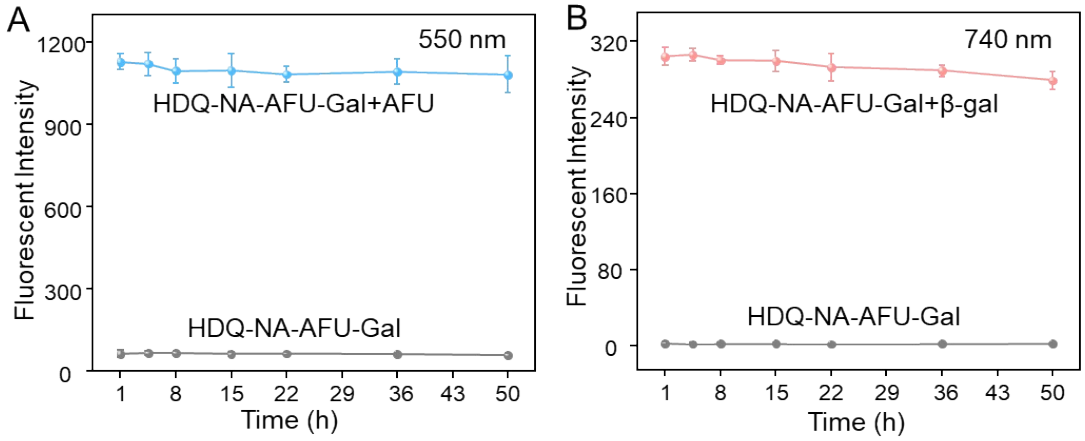


Figure S2. The stability of the probe in 10 mM PBS at pH 6. The fluorescence intensity of the probe was measured at 550 nm and 740 nm before and after the addition of AFU and β-gal, respectively. the data were displayed as mean ± standard deviation (n=3).

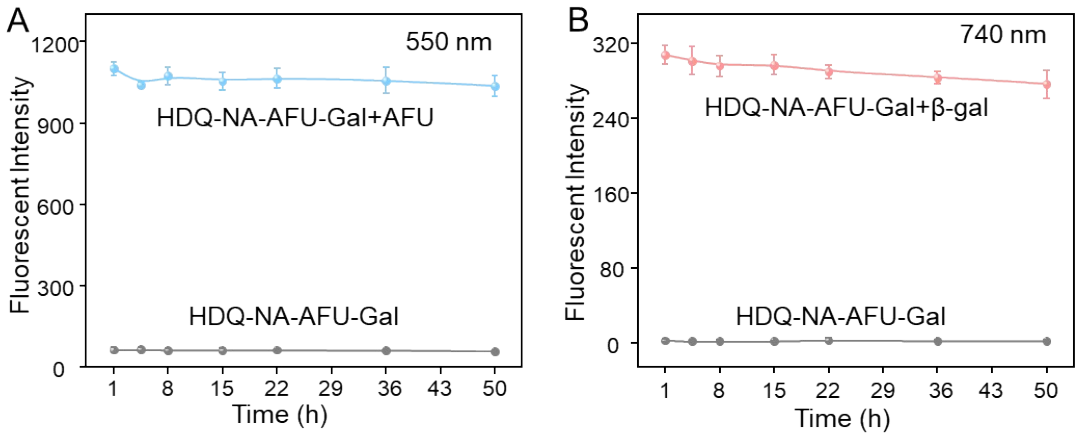


Figure S3. The stability of the probe in DMEM medium containing 10% FBS. The fluorescence intensity of the probe was measured at 550 nm and 740 nm before and after the addition of AFU and β-gal, respectively. The data were displayed as mean ± standard deviation (n=3).

5. Fluorescence spectra of probe in the presence of both AFU and β-gal.

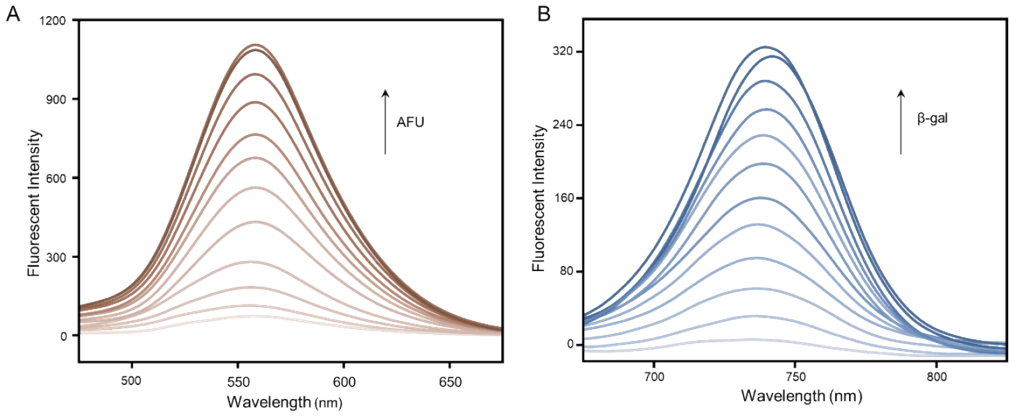


Figure S4. Fluorescence spectra of HDQ-NA-AFU-Gal against AFU (A) (1-100 U/L) in the presence of β-gal (500 U/L), and (B) β-gal (5-500 U/L) in the presence of AFU (100 U/L).

6. Selective research of HDQ-NA-AFU-Gal

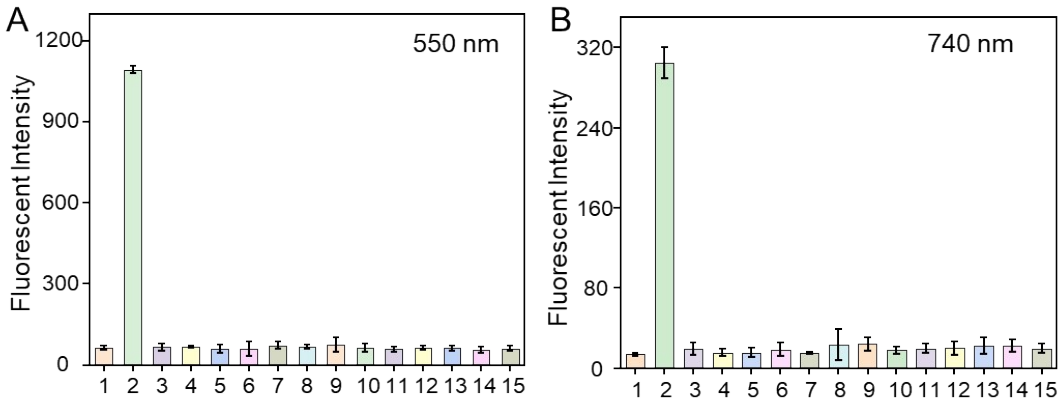


Figure S5. Selectivity of the probe for (A) AFU and (B) β-gal 1. Blank, 2. AFU (100 U/L) or β-gal (500 U/L), 3. Try (100 μM), 4. Met (100 μM), 5. Leu (100 μM), 6. Thr (100 μM), 7. Gly (100 μM), 8. Ala (100 μM), 9. Fe^{3+} (100 μM), 10. Ca^{2+} (100 μM), 11. Mg^{2+} (100 μM), 12. Zn^{2+} (100 μM), 13. SO_4^{2-} (100 μM), 14. CO_3^{2-} (100 μM), 15. NO_3^- (100 μM). The fluorescent intensity was measured at 550 nm and 740 nm, respectively. The data were displayed as mean ± standard deviation (n=3).

7. Kinetics of HDQ-NA-AFU-Gal to β-gal and AFU

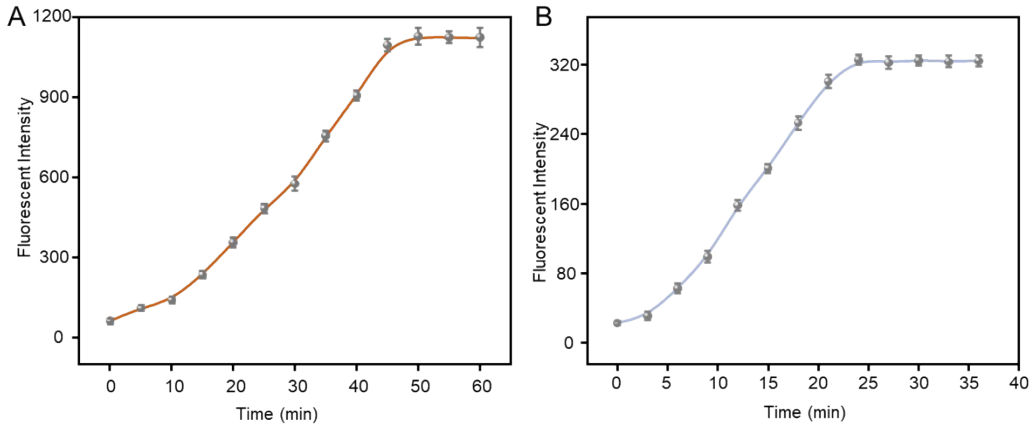


Figure S6. Time-dependent changes of emission intensity of HDQ-NA-AFU-Gal towards (A) AFU (100 U/L, λ_{em} = 550 nm) and (B) β-gal (500 U/L, λ_{em} = 740 nm),

respectively. Error bars represent mean ± S.D. from four independent replicates (n=4).

8. Effect of pH on the performance of HDQ-NA-AFU-Gal

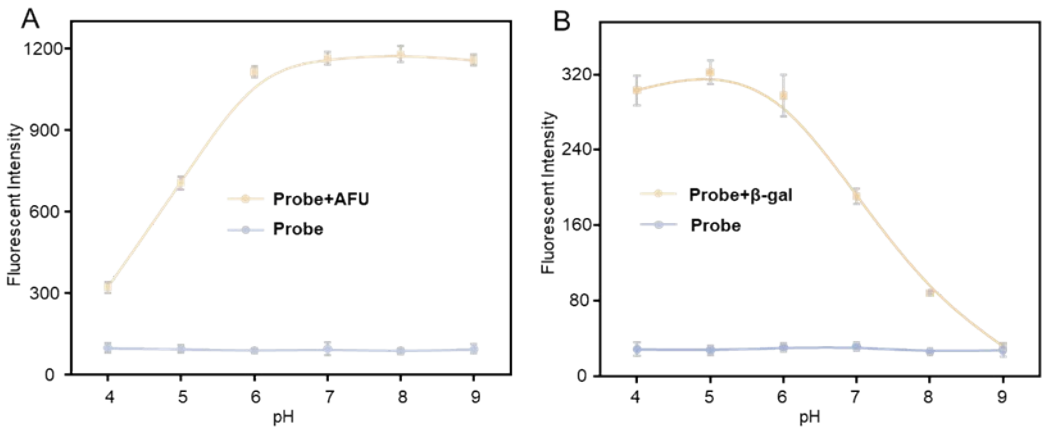


Figure S7. The changes of fluorescent intensity of HDQ-NA-AFU-Gal towards (A) AFU (100 U/L, λ_{em} = 550 nm) and (B) β-gal (500 U/L, λ_{em} = 740 nm) under different pH. Error bars represent mean ± S.D. from four independent replicates (n=4).

9. Cytotoxicity assays and imaging study

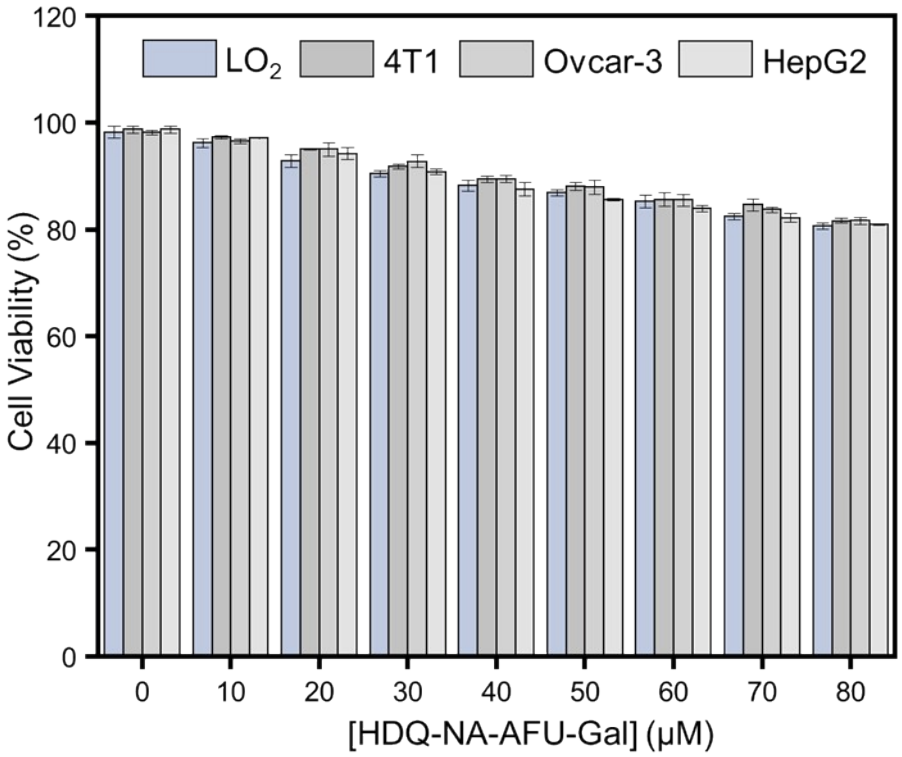


Figure S8. Cell viability of LO_2, 4T1, Ovcar-3 and HepG2 incubated with different concentrations of HDQ-NA-AFU-Gal probe. Error bars represent mean ± S.D. from

four independent replicates (n=4).

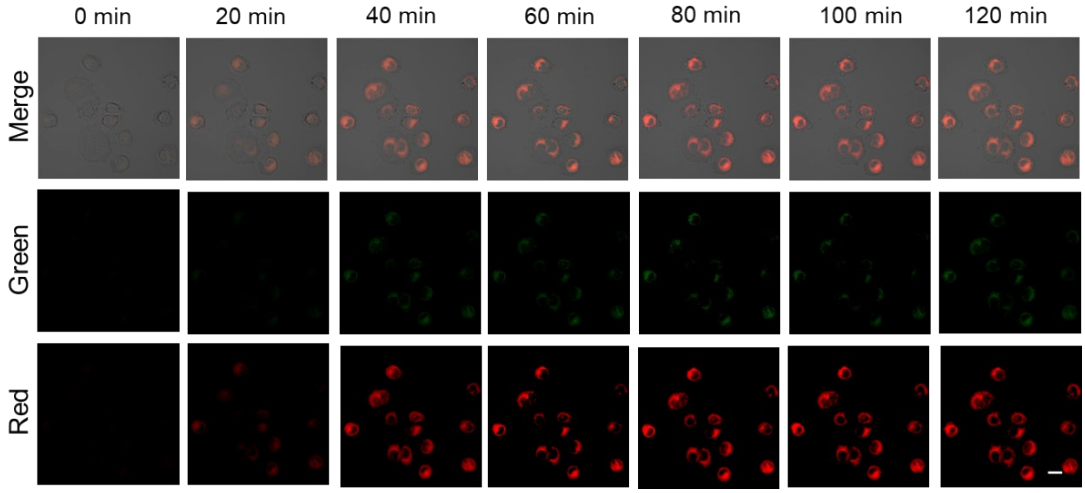


Figure S9. HDQ-NA-AFU-Gal probe used for visualization Ovacar-3 cells at different time points. Green channel, 500-560 nm (λ_{ex}= 488 nm), and red channel, 700-780 nm (λ_{ex}= 640 nm). Scale bar: 50 μm.

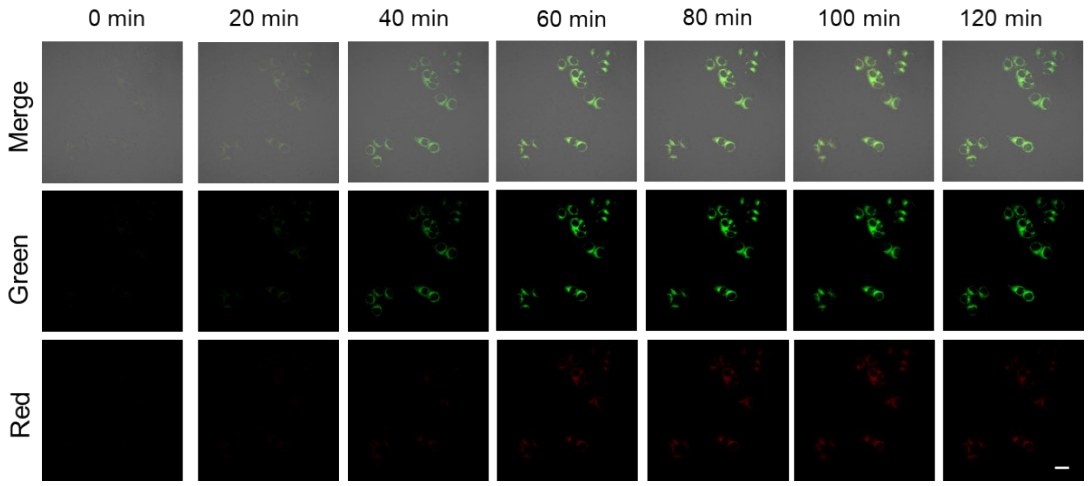


Figure S10. HDQ-NA-AFU-Gal probe used for visualization HepG2 cells at different time points. Green channel, 500-560 nm (λ_{ex}= 488 nm), and red channel, 700-780 nm (λ_{ex}= 640 nm). Scale bar: 50 μm.

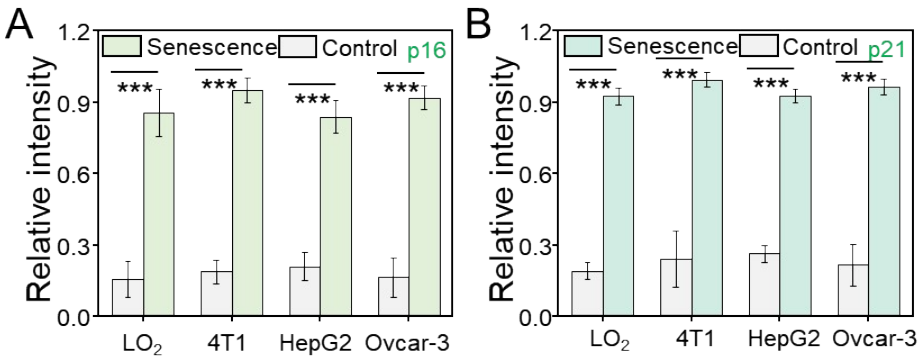


Figure S11. The levels of (A) p16 and (B) p21 in normal or senescent LO_2 cells, 4T1 cells, HepG2 cells and Ovcar-3 cells. Error bars represent mean ± S.D. from four independent replicates. Statistical significance was calculated using one way ANOVA by Tukey's multiple comparisons test. *p < 0.05, **p < 0.01, ***p < 0.001, and ns: no significant difference.

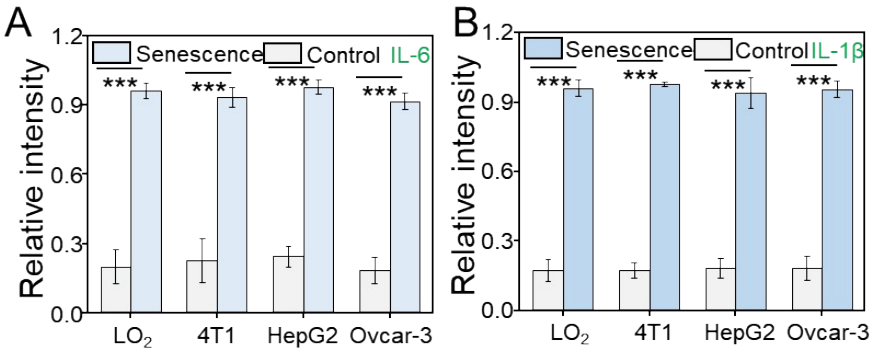


Figure S12. The levels of (A) IL-6 and (B) IL-1β in normal or senescent LO_2 cells, 4T1 cells, HepG2 cells and Ovcar-3 cells using ELISA detection kit. Error bars represent mean ± S.D. from four independent replicates. Statistical significance was calculated using one way ANOVA by Tukey's multiple comparisons test. *p < 0.05, **p < 0.01, ***p < 0.001.

10. ^{1}H NMR, ^{13}C NMR and HRMS

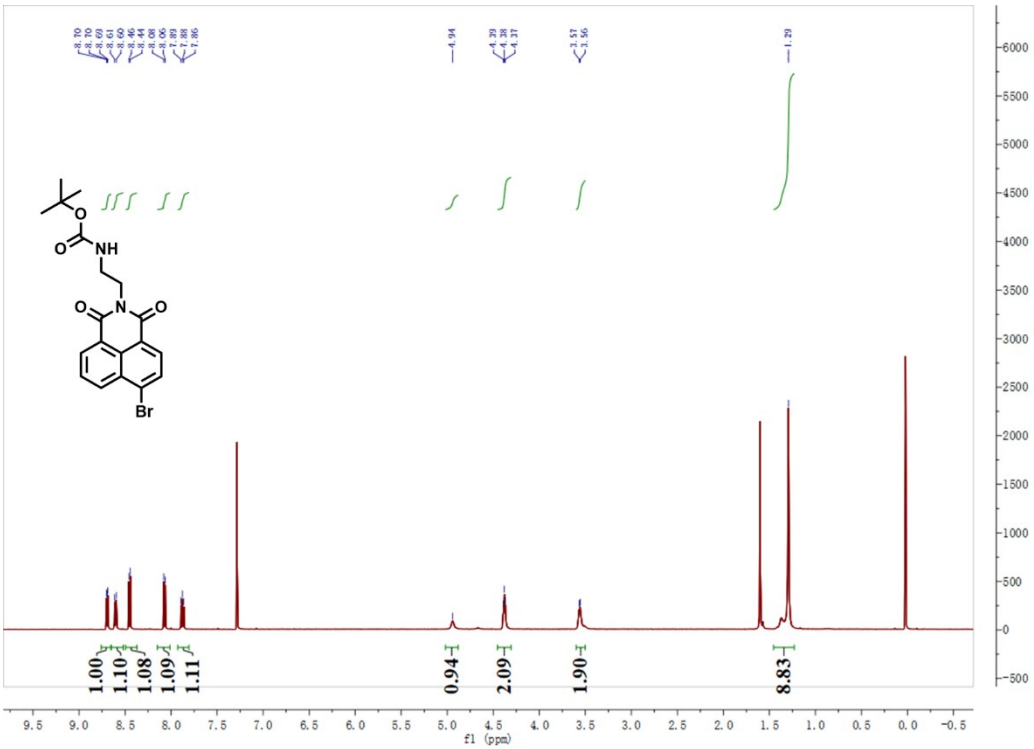


Figure S13. ^{1}H NMR of compound 2.

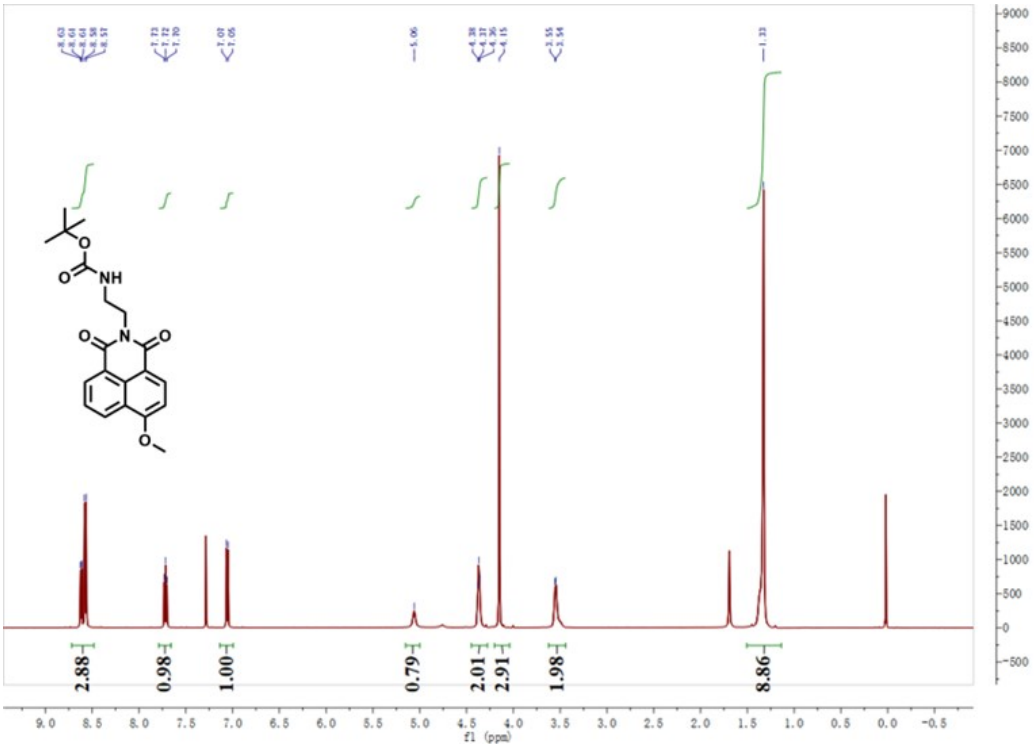


Figure S14. ^{1}H NMR of compound 3.

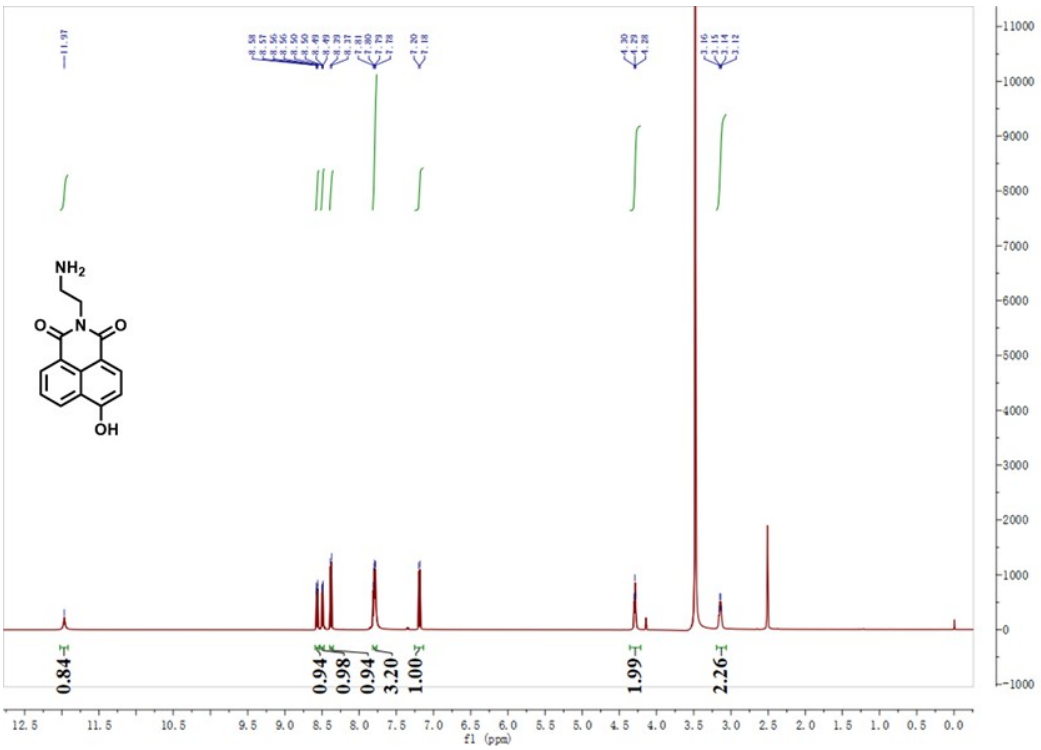


Figure S15. [1]H NMR of compound 4.

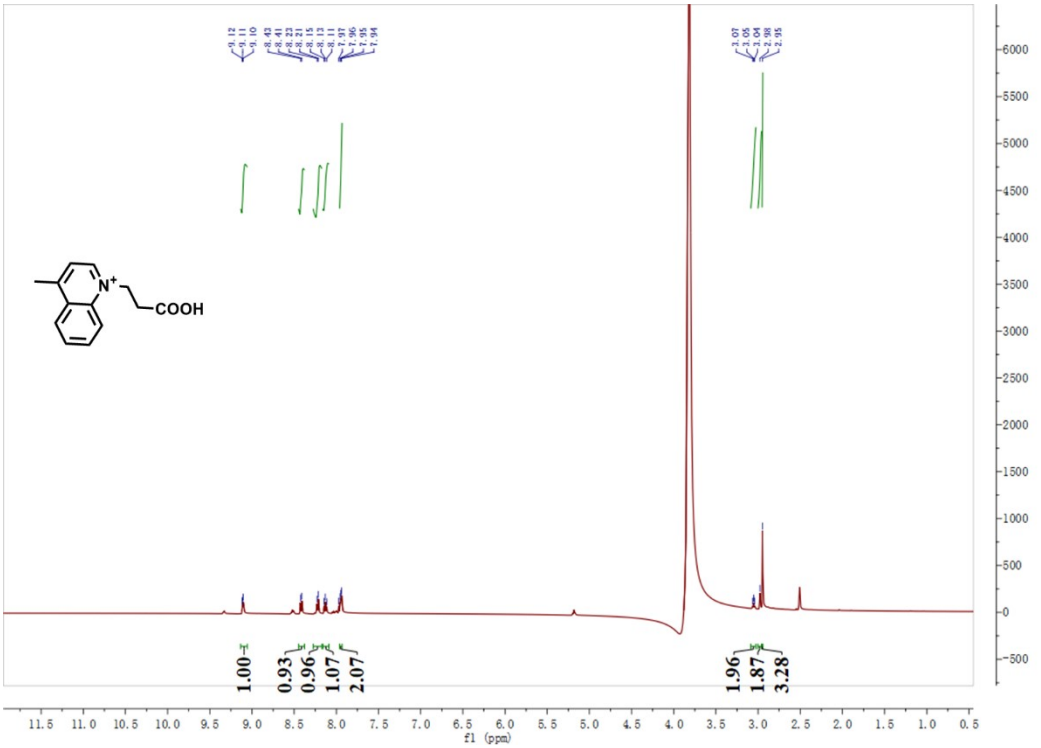


Figure S16. [1]H NMR of compound 6.

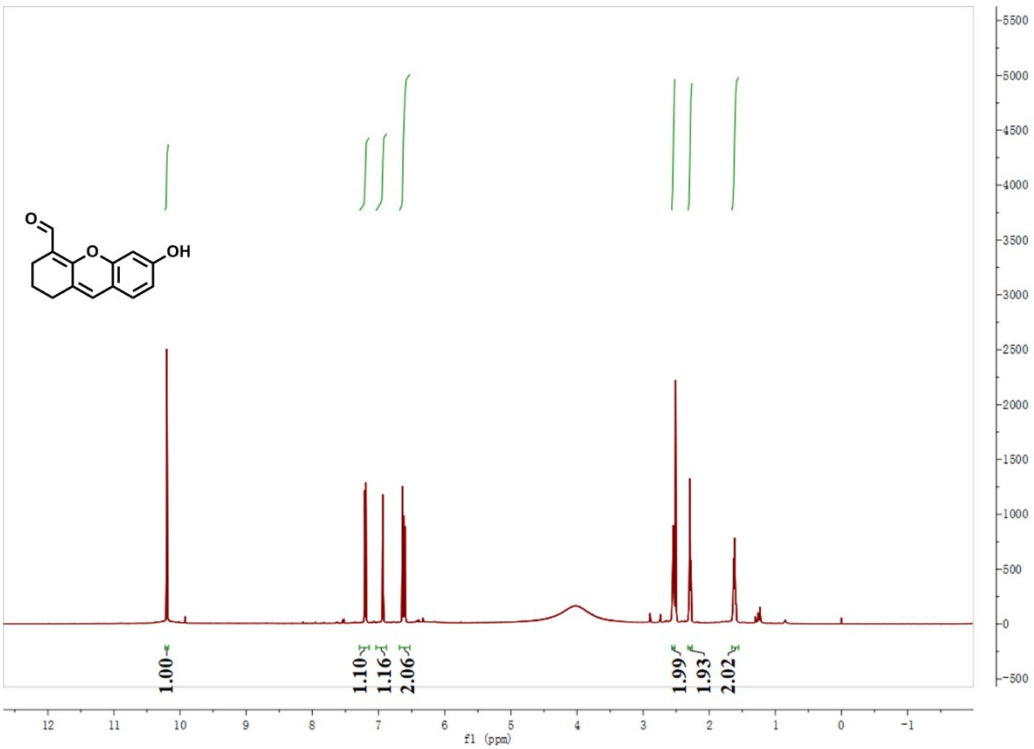


Figure S17. [1]H NMR of compound 10.

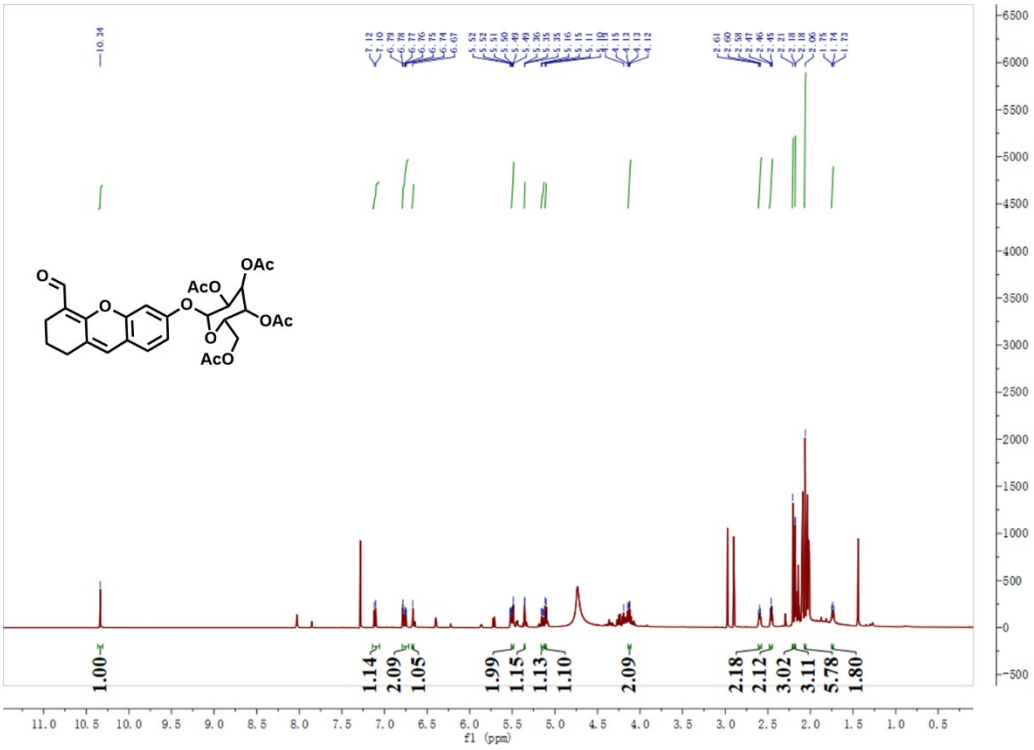


Figure S18. [1]H NMR of compound 11.

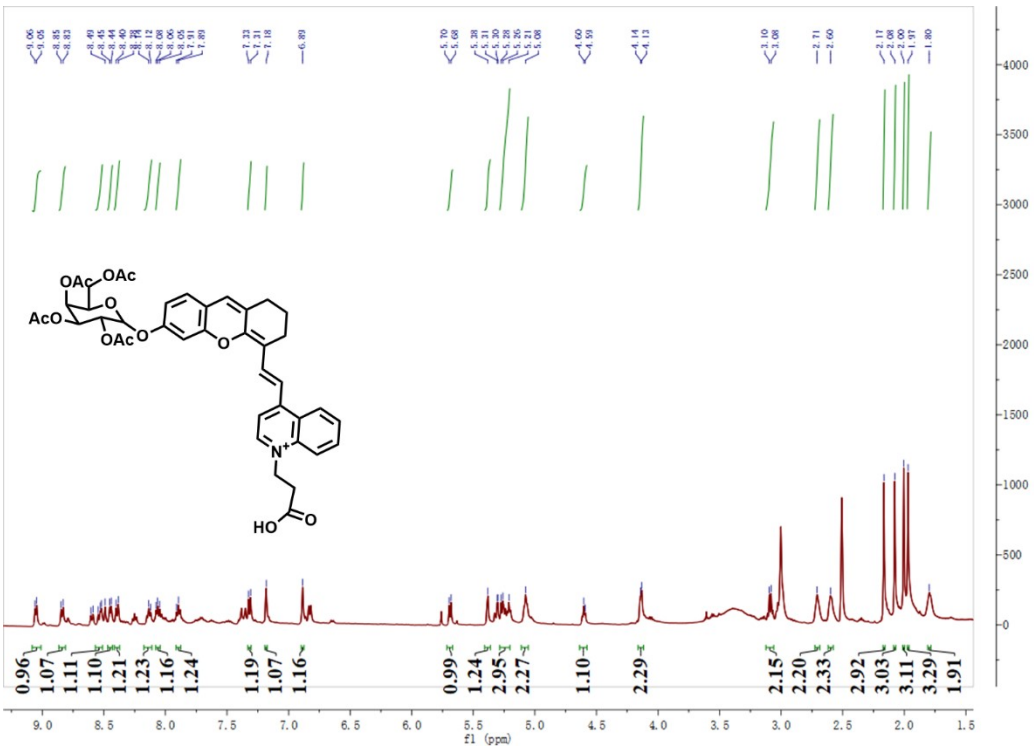


Figure S19. ^{1}H NMR of compound 12.

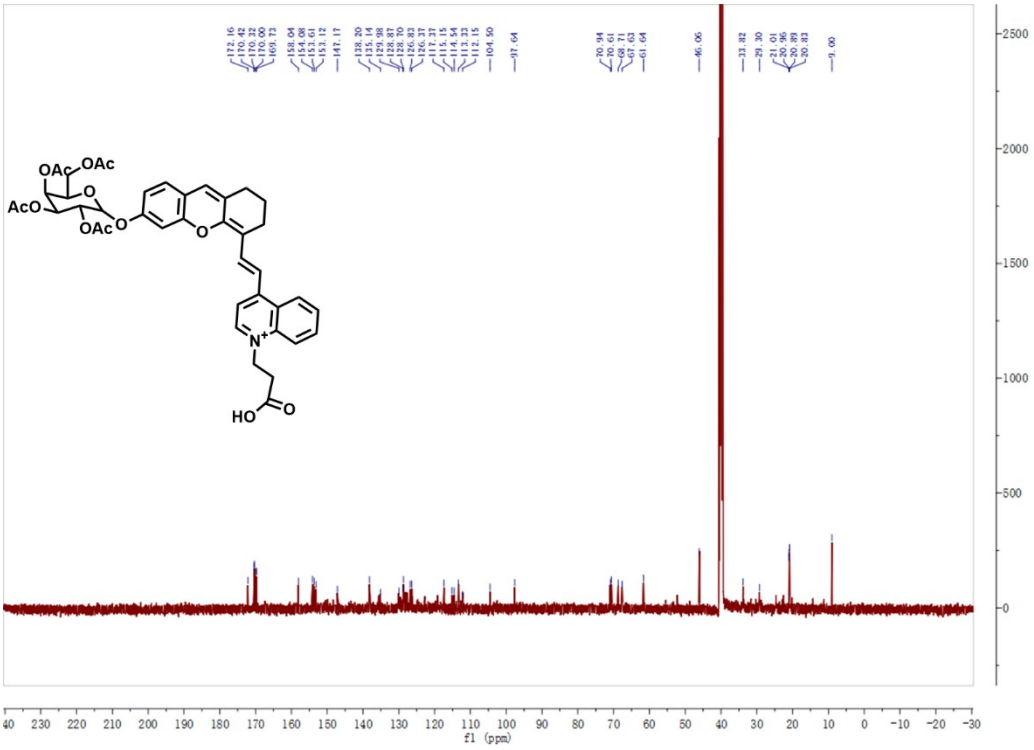


Figure S20. ^{13}C NMR of compound 12.

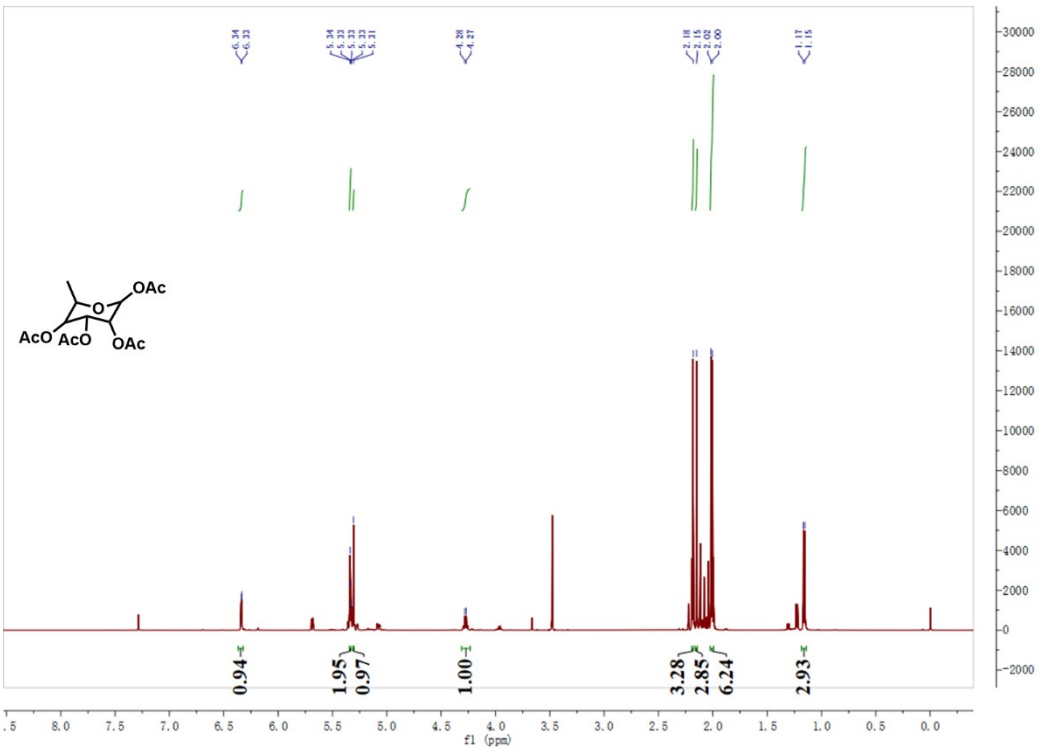


Figure S21. ^{1}H NMR of compound 14.

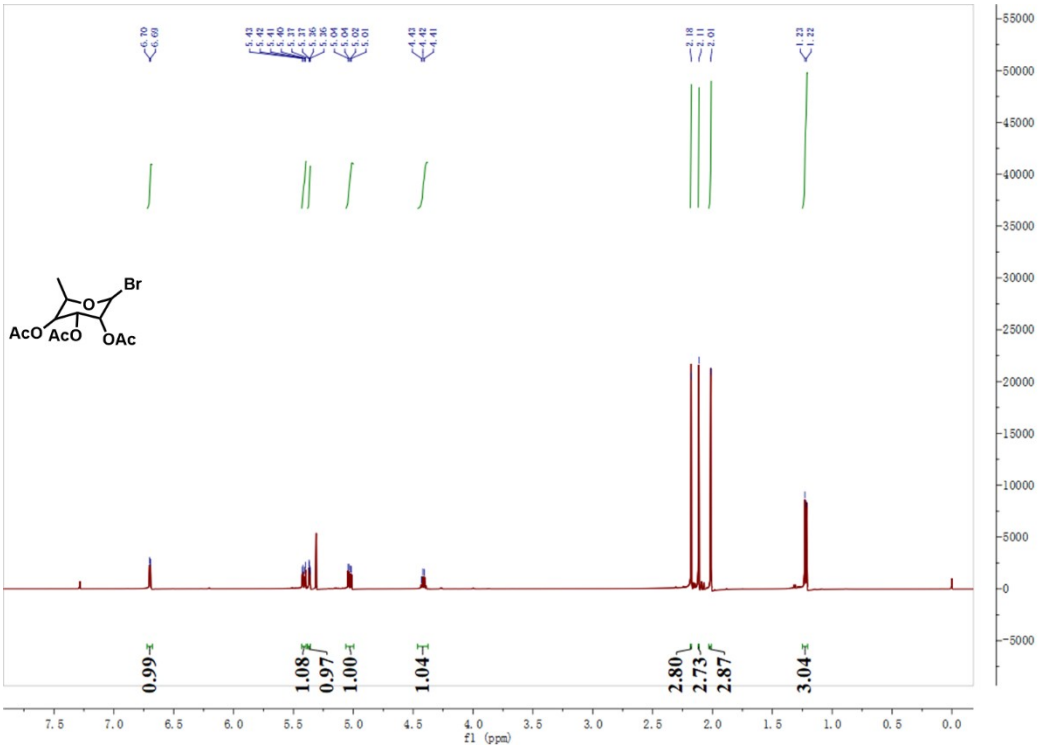


Figure S22. ^{1}H NMR of compound 15.

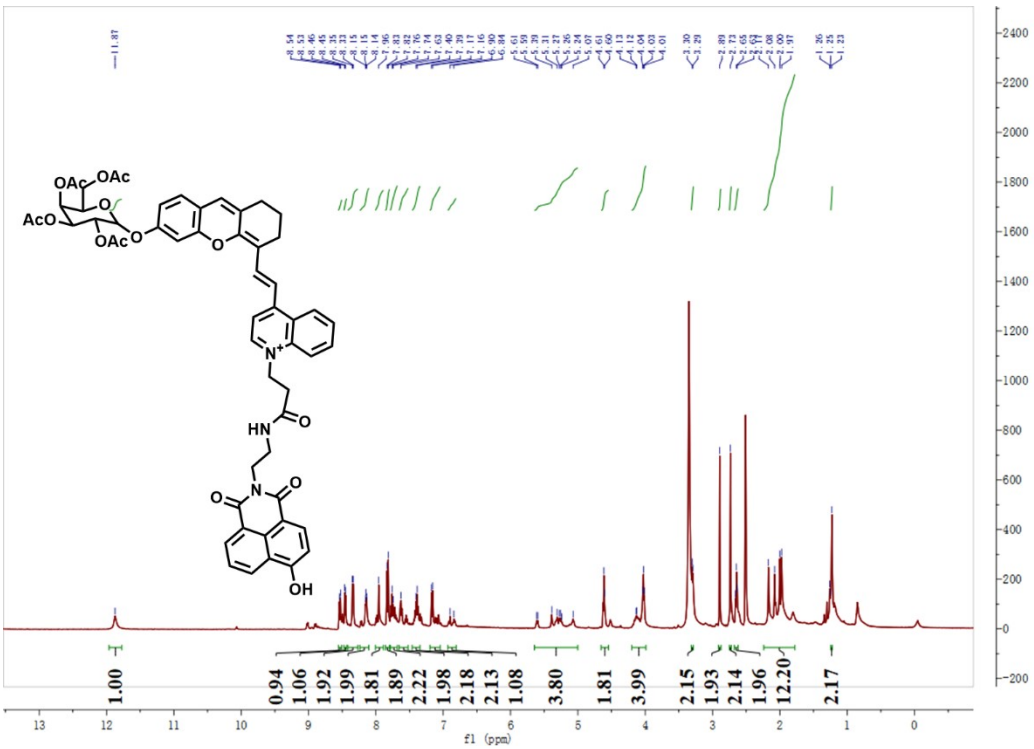


Figure S23. ^{1}H NMR of compound 16.

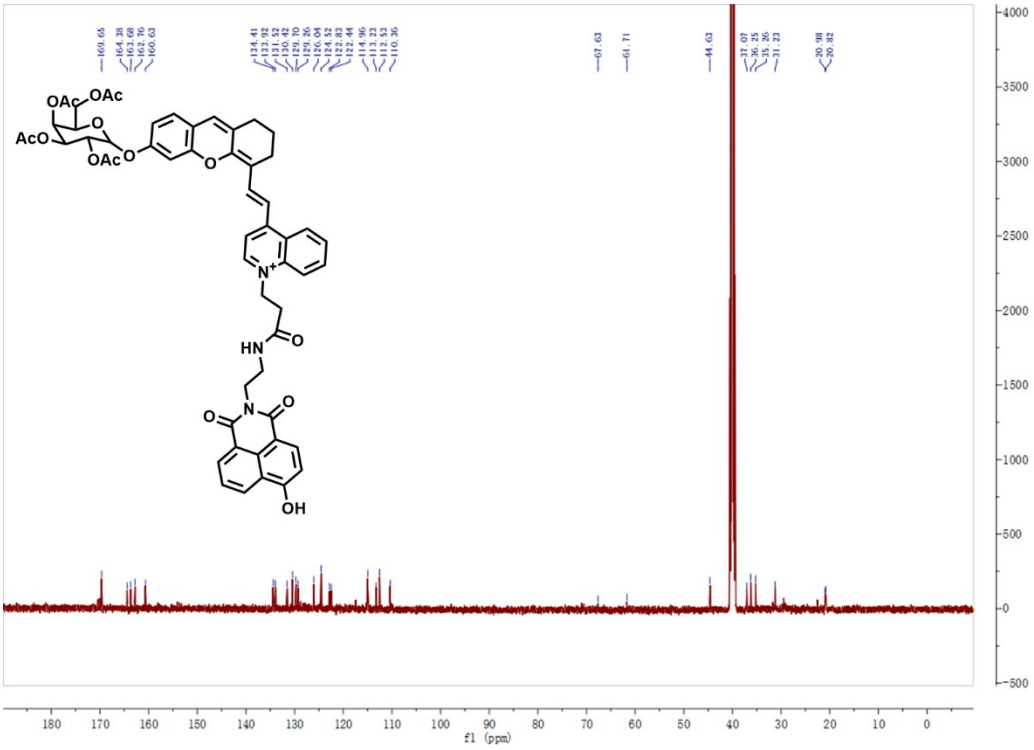


Figure S24. ^{13}C NMR of compound 16.

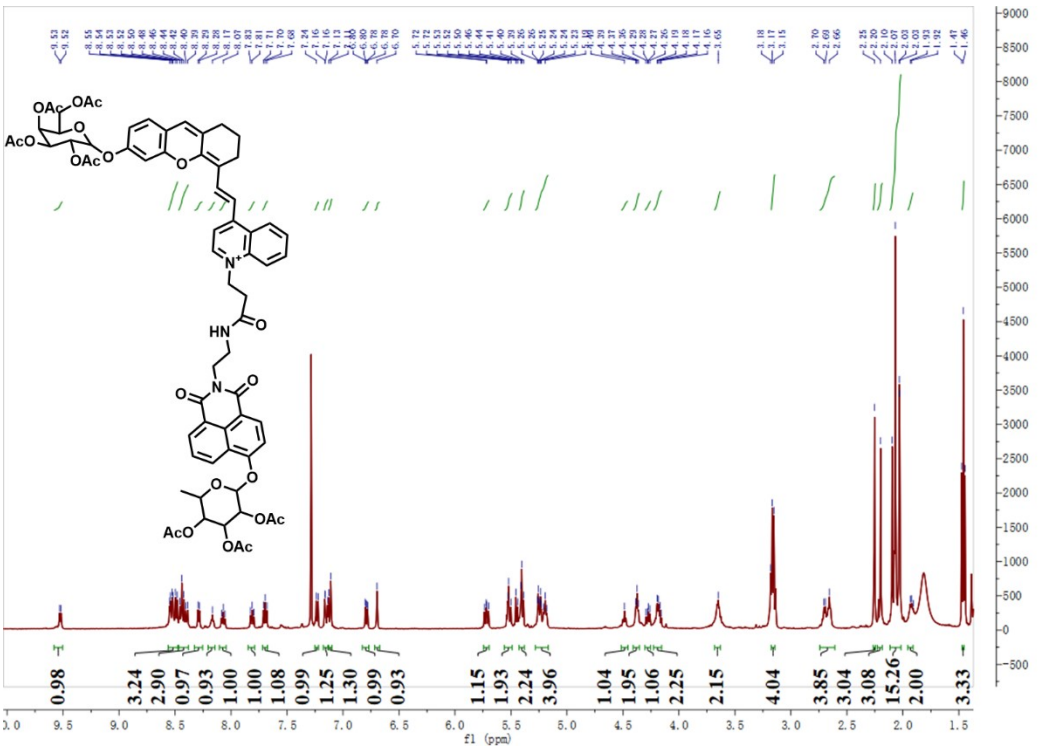


Figure S25. ^{1}H NMR of compound 17.

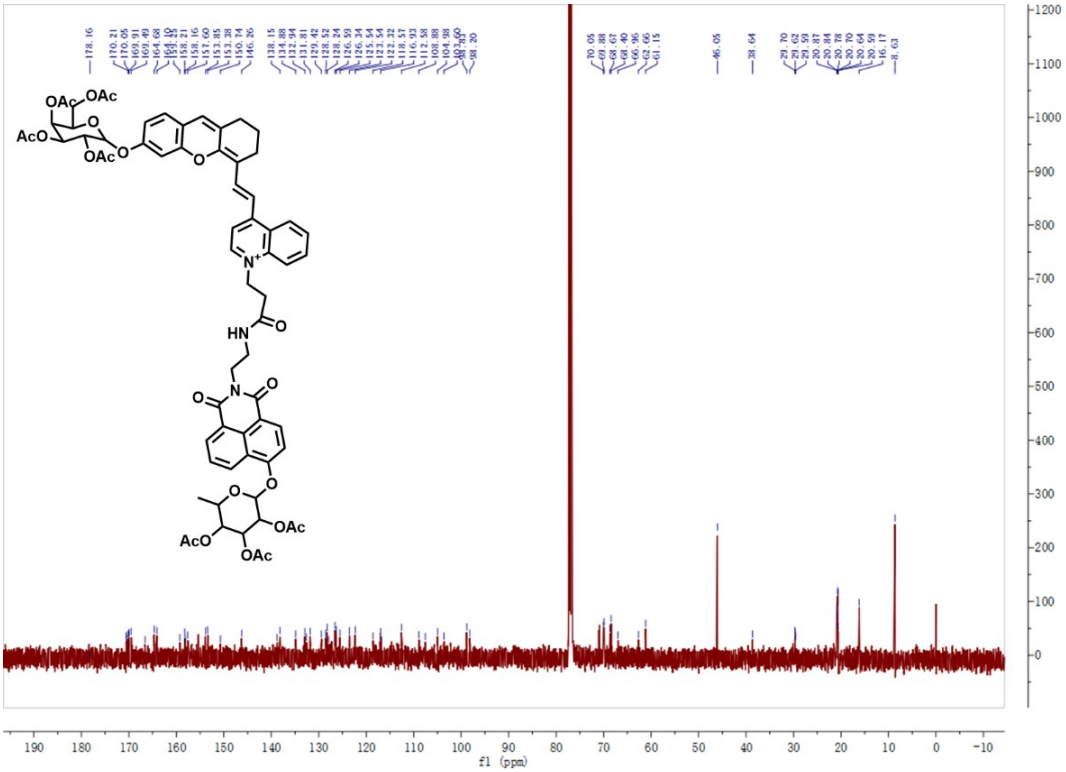


Figure S26. ^{13}C NMR of compound 17.

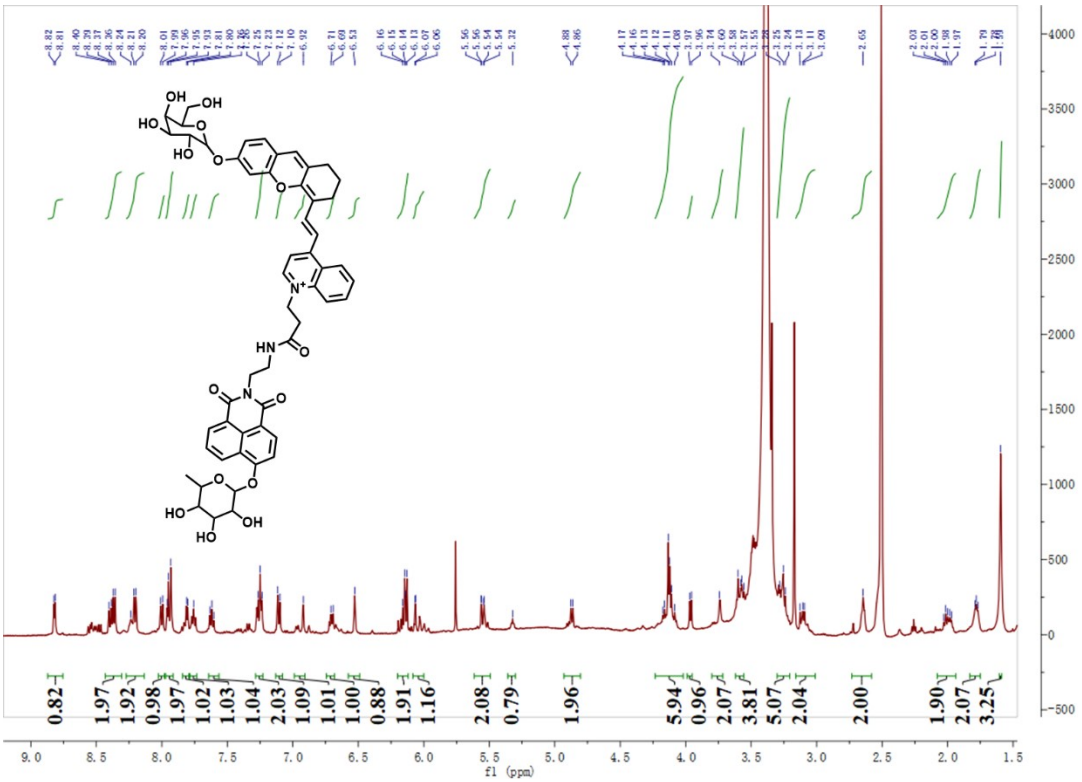


Figure S27. ^{1}H NMR spectrum of HDQ-NA-AFU-Gal.

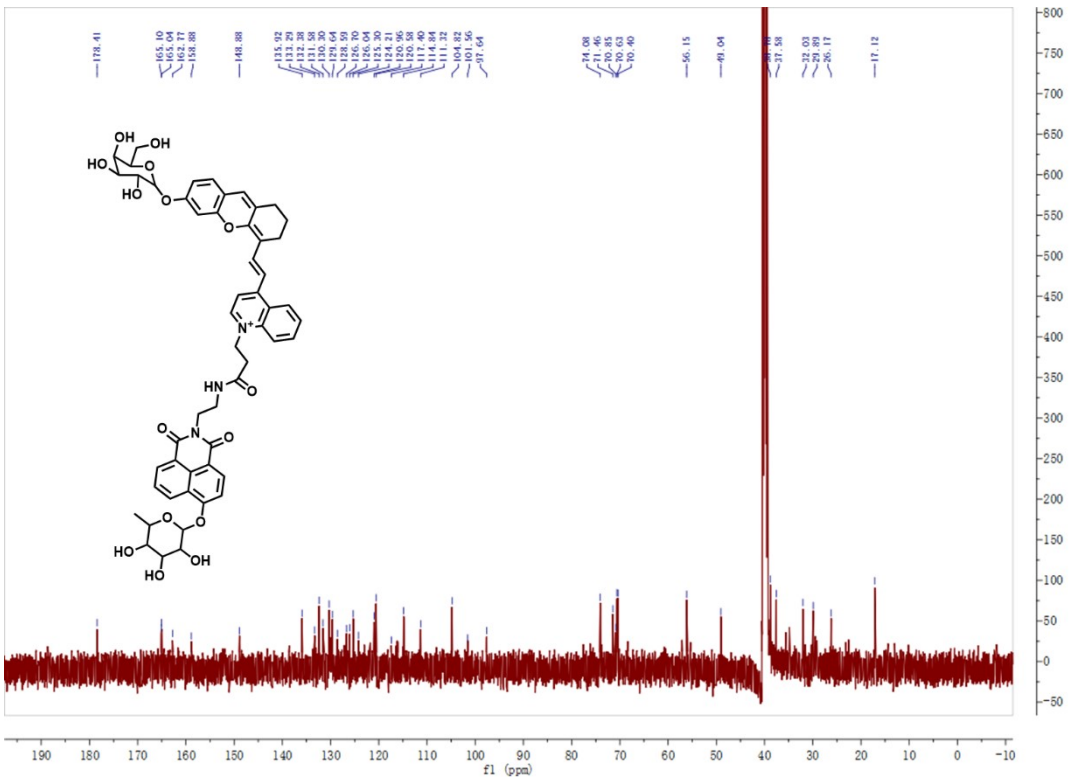


Figure S28. ^{13}C NMR spectrum of HDQ-NA-AFU-Gal.

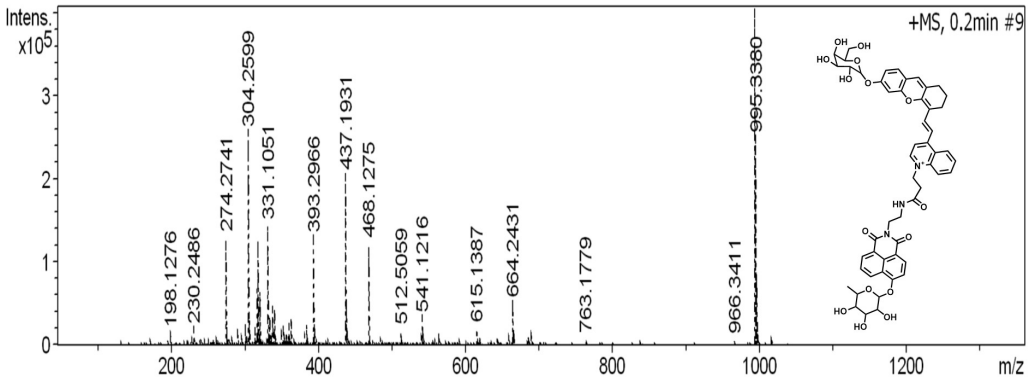


Figure S29. HR-MS spectrum of HDQ-NA-AFU-Gal.

11. References

1. B. Lozano-Torres, J. F. Blandez, I. Galiana, A. García-Fernµndez, M. Alfonso, M. D. Marcos, M. Orzµez, F. Sancenón, and R. Martínez-Manez, *Angew. Chem. Int. Ed.* 2020, **59**, 15152–15156.

2. F. Wu, J. Liu, M. Tao, M. Wang, X. Ren, Z. Hai, *Anal. Chem.* 2023, **95**, 10481−10485.