Supporting Information

for

Dual-target-activated dual-color molecular probe for imaging analysis of cellular senescence

Linlin Wang[a], Donghui Hong[a], Jili Li*[a], Lu Wu[a], Yinghao Xia[a,b], Xinjia Di[a], Jian Wang[a], Yuqi Xie[a], Jun Da[a], and Yanlan Liu*[a]

[a]Molecular Science and Biomedicine Laboratory (MBL), State Key Laboratory of Chemo and Biosensing, College of Chemistry and Chemical Engineering, Aptamer Engineering Center of Hunan Province, Hunan University, Changsha, Hunan 410082, China
[b]Hebi Polytechnic, Hebi, Henan 458000, China

[*] Corresponding authors.
E-mail addresses: lijili@hnu.edu.cn (J LI), ylliu@hnu.edu.cn (Y Liu)

Table of Contents

1. Experimental section

Materials and characterizations

All reagents and solvents for organic synthesis were obtained from commercially available sources and used without further purification unless otherwise stated. Doxorubicin hydrochloride (DOX) and β-galactosidase (β-gal) were purchased from Sigma-Aldrich. Oxidized low density lipoprotein (ox-LDL) was purchased from Yiyuan Biotechnologies. SPiDER-β-Gal was purchased from Dojindo Molecular Technologies. The Senescence β-Galactosidase Staining Kit was purchased from Beyotime. The molecular weights of compounds were determined by mass spectrometry using a Bruker Daltonics UltrafleXtreme matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS). Super Total RNA Extraction Kit was purchased from Promega. The RevertAid First Strand cDNA Synthesis Kit was purchased from Thermo Fisher Scientific. SYBR Green qPCR Master Mix (Low ROX) was purchased from Bimake.

Analytical thin-layer chromatography (TLC) was performed on silica gel GF254 plates (Shanghai Titan Technology Co, Ltd., Shanghai, China). Flash column chromatography was performed with silica gel (Shanghai Titan Technology Co, Ltd., Shanghai, China, 200–300 mesh). The composition of mixed solvents was given by the volume ratio (v/v). NMR spectra were recorded by a Bruker 400 MHz NMR spectrometer. Optical measurements and imaging studies were carried out by using a fluorescence spectrophotometer (Hitachi, F-7100), ultraviolet spectrophotometer (Shimadzu, UV-2450), flow cytometer (Cytek Dxp Athena), and confocal microscope (Zeiss, LSM880).

Synthesis of the senoprobe

The synthesis for compounds was shown in Figure 1 These compounds for the proposed senoprobe were synthesized according to previous reports.[1-3]

Synthesis of compound 3: Dichloromethane (20 mL) and ddH_2O (10 mL) were introduced into a flask containing methylene blue (1 g, 3.52 mmol) and Na_2CO_3 (285 mg, 2.71 mmol) under nitrogen protection. Following this, sodium dithionite (934 mg, 5.36 mmol), dissolved in 10 mL of water, was directly injected into the reaction mixture using a syringe. The mixture was stirred at 40 °C under the nitrogen atmosphere for 40 min until the solution became yellow. Subsequently, the mixture was

cooled down to 0 °C, after which a dichloromethane solution (10 mL) containing triphosgene (550 mg) was added. This new mixture was stirred continuously at 0 °C for 3 h. Upon completion, the solvents were carefully removed through reduced pressure distillation, leaving behind the crude product. Purification was achieved via column chromatography on silica gel, employing a gradient elution system composed of petroleum ether and ethyl acetate in a 5:1 ratio to produce compound 3 in 53% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.41 (d, J = 9.0 Hz, 2H), 6.72 (d, J = 2.7 Hz, 2H), 6.64 (dd, J = 8.9, 2.8 Hz, 2H), 2.98 (s, 12H). ^{13}C NMR (101 MHz, Chloroform-d) δ149.33, 127.89, 110.68, 110.19, 40.50.

Synthesis of compound 5: 4-Bromo-1,8-naphthalic anhydride (compound 4, 3 g, 10.9 mmol) and tert-butyl N-(2-aminoethyl) carbamate (3.2 g, 20.0 mmol) were dissolved in ethanol (50 mL). The resultant mixture was then stirred and heated to approximately 90 °C for 6 h. Following this, the solvent was evaporated under reduced pressure, and the crude product was subsequently purified using column chromatography on silica gel (petroleum ether/ethyl acetate 10/1), yielding compound 5 in 78% yield. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.66 (d, J = 7.2 Hz, 1H), 8.57 (d, J = 8.6 Hz, 1H), 8.42 (dd, J = 8.0, 1.8 Hz, 1H), 8.04 (dd, J = 7.9, 1.8 Hz, 1H), 7.96 – 7.69 (m, 1H), 4.37 (t, J = 5.7 Hz, 2H), 3.55 (q, J = 6.6, 6.0 Hz, 2H), 1.30 (s, 9H), ^{13}C NMR (101 MHz, Chloroform-d) δ 163.93, 156.04, 133.36, 132.20, 131.37, 131.09, 130.58, 130.38, 129.05, 128.06, 122.92, 122.05, 79.15, 77.22, 40.04, 39.52, 28.21.

Synthesis of compound 6: Compound 5 (2660 mg, 6.36 mmol), N-hydroxysuccinimide (805 mg, 7.0 mmol, 1.1 eqv.), and potassium carbonate (K_2CO_3) (2905 mg, 21.01 mmol, 3.3 eqv.) were dissolved in DMSO (60 mL) under an argon atmosphere. The reaction mixture was then heated to 80 °C and stirred continuously for 6 h. After the completion of the heating period, the mixture was allowed to cool down to room temperature. The pH of the solution was subsequently adjusted to 3 by the careful addition of 1 M hydrochloric acid (HCl). The crude production was purified using column chromatography with petroleum ether/ ethyl acetate (3:1) as the eluent to obtain compound 6 (1699 mg, 75%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.82 (s, 1H), 8.47 (dd, J = 26.0, 7.7 Hz, 2H), 8.32 (d, J = 8.1 Hz, 1H), 7.74 (t, J = 7.8 Hz, 1H), 7.14 (d, J = 8.2 Hz, 1H), 6.86 (t, J = 6.4 Hz, 1H), 4.10 (t, J = 6.1 Hz, 2H), 3.23 (d, J = 6.7 Hz, 2H), 1.23 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d6) δ 164.35, 163.66, 160.47, 133.78, 131.38, 129.79, 129.10, 125.94, 122.78, 122.55, 113.38, 110.26, 77.85, 38.34, 28.54.

Synthesis of compound 7: Compound 6 (890 mg, 2.5 mmol), 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl-bromide (2100 mg, 5 mmol), K_2CO_3 (1340 mg, 9.7 mmol), and acetonitrile (15 mL) were added to a round-bottom flask. The reaction mixture was then stirred at 70 °C for 4 h. After the mixture was cooled down to room temperature, it was poured into water and subsequently filtered. The filter residue was washed three times with water to remove any impurities. Following this, the residue was dissolved in ethyl acetate. The solvent was then evaporated under reduced pressure, and the residue was purified by column chromatography on silica gel with petroleum ether-ethyl acetate (8:1) as eluent, yielding compound 7 as a white solid (960 mg, 56%). ^{1}H NMR (400 MHz, Chloroform-d) δ 8.65 – 8.61 (m, 1H), 8.56 (d, J = 8.3 Hz, 1H), 8.46 (d, J = 8.4 Hz, 1H), 7.76 (t, J = 7.9 Hz, 1H), 7.25 (d, J = 8.3 Hz, 1H), 5.75 (dd, J = 10.5, 7.8 Hz, 1H), 5.56 (d, J = 3.4 Hz, 1H), 5.40 (d, J = 7.9 Hz, 1H), 5.31 (s, 1H), 5.25 (dd, J = 10.5, 3.4 Hz, 1H), 5.01 (s, 1H), 4.36 (t, J = 5.7 Hz, 2H), 4.30 – 4.24 (m, 2H), 3.53 (q, J = 5.9 Hz, 2H), 2.24 (s, 3H), 2.11 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 1.30 (s, 9H). ^{13}C NMR (101 MHz, Chloroform-d) δ170.33, 170.13, 170.02, 169.65, 163.98, 157.49, 132.77, 132.08, 126.84, 123.53, 108.86, 98.98, 71.66, 70.41, 68.32, 66.75, 61.40, 28.22, 20.77, 20.70, 20.66, 20.59.

Synthesis of compound 8: To a solution of compound 7 (823 mg, 1.2 mmol) in dichloromethane (10 mL), trifluoroacetic acid (5 mL) was added. The mixture was stirred at room temperature for 6 h. Subsequently, the solvent was removed by evaporation, and the residual trifluoroacetic acid was eliminated through azeotropic distillation with toluene. The crude product was then purified using silica gel column chromatography, employing gradient mixtures of petroleum ether and ethyl acetate as the eluent. Compound 8 was thus obtained (435 mg, 62%). ^{1}H NMR (400 MHz, DMSO-d6) δ 8.49 (dd, J = 8.1, 2.5 Hz, 2H), 8.30 (d, J = 8.4 Hz, 1H), 7.88 (t, J = 7.9 Hz, 1H), 7.43 (d, J = 8.3 Hz, 1H), 5.90 (d, J = 7.2 Hz, 1H), 5.76 (s, 2H), 5.50 – 5.40 (m, 3H), 4.63 (t, J = 6.4 Hz, 1H), 4.17 (dt, J = 9.3, 5.2 Hz, 2H), 4.04 (t, J = 7.0 Hz, 2H), 2.78 (t, J = 6.9 Hz, 2H), 2.18 (s, 3H), 2.05 (d, J = 1.9 Hz, 6H), 2.01 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d6) δ 170.45, 170.36, 170.22, 170.02, 164.08, 163.47, 157.10, 132.85, 131.62, 129.11, 123.13, 122.78, 117.04, 109.89, 97.93, 71.39, 70.19, 68.70, 61.74, 55.37, 20.99, 20.95, 20.87, 20.84.

Synthesis of compound 9: Under an atmosphere of nitrogen, triethylamine (101 mg, 1 mmol) was introduced into a round-bottom flask containing compound 3 (173 mg, 0.5 mmol), compound 8 (293 mg, 0.5 mmol), and 30 mL of dichloromethane. The mixture was stirred at room temperature until

the reaction was complete as indicated by TLC analysis. The reaction mixture was then poured into 50 mL of ice-water with stirring, and the resulting mixture was extracted with three 30 mL portions of ethyl acetate. The combined extracts were washed with brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure on a rotary evaporator to afford an oily residue. This residue was purified by column chromatography (ethyl acetate/petroleum ether = 1/8) to yield compound 9 (345 mg, 77%). ^{1}H NMR (400 MHz, Chloroform-d) δ 8.62 – 8.43 (m, 4H), 7.75 (dd, J = 8.4, 7.3 Hz, 1H), 7.24 (dd, J = 8.6, 4.3 Hz, 3H), 6.64 (d, J = 2.8 Hz, 2H), 6.55 (dd, J = 8.8, 2.8 Hz, 2H), 5.76 (dd, J = 10.5, 7.9 Hz, 1H), 5.56 (d, J = 3.4 Hz, 1H), 5.42 (dd, J = 14.4, 6.7 Hz, 2H), 5.28 (dd, J = 10.5, 3.4 Hz, 1H), 4.34 (t, J = 5.8 Hz, 2H), 4.28 – 4.22 (m, 2H), 3.67 – 3.59 (m, 2H), 2.91 (s, 12H), 2.24 (s, 3H), 2.12 (s, 3H), 2.10 (s, 3H), 2.08 (s, 3H). ^{13}C NMR (101 MHz, Chloroform-d) δ 170.00, 169.75, 164.43, 163.69, 157.48, 148.86, 134.10, 132.73, 131.93, 128.45, 128.42, 127.31, 126.72, 123.53, 122.30, 117.18, 111.30, 110.87, 108.91, 98.98, 71.59, 70.44, 68.38, 66.84, 61.48, 53.46, 40.72, 39.74, 39.61, 20.83, 20.73, 20.68, 20.61.

Synthesis of compound 10: Compound 9 (270 mg, 0.3 mmol) was dissolved in 20 mL of CH_3OH, followed by the addition of CH_3ONa (108 mg, 2 mmol) at 0 °C. The mixture was stirred for 20 min, after which the solution was neutralized with Amberlite IRC-50 resin for 10 min and then filtered through a pad of cotton to remove the resin. The solvent was subsequently evaporated under reduced pressure, and the resulting crude material was purified by column chromatography on silica gel to afford compound 10 in 81% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.74 (dd, J = 8.5, 1.2 Hz, 1H), 8.55 (dd, J = 7.3, 1.2 Hz, 1H), 8.48 (d, J = 8.3 Hz, 1H), 7.88 (t, J = 7.9 Hz, 1H), 7.51 (d, J = 8.4 Hz, 1H), 7.11 (d, J = 8.8 Hz, 2H), 6.61 (d, J = 2.7 Hz, 2H), 6.44 (dd, J = 8.9, 2.8 Hz, 2H), 6.23 (t, J = 5.8 Hz, 1H), 5.51 (d, J = 5.2 Hz, 1H), 5.23 (d, J = 7.7 Hz, 1H), 5.02 (d, J = 5.6 Hz, 1H), 4.72 (t, J = 5.5 Hz, 1H), 4.66 (d, J = 4.5 Hz, 1H), 4.20 (s, 2H), 3.88 – 3.83 (m, 1H), 3.79 (t, J = 4.2 Hz, 1H), 3.76 (d, J = 6.3 Hz, 1H), 3.60 (td, J = 10.2, 9.3, 4.6 Hz, 1H), 3.56 – 3.51 (m, 2H), 3.42 – 3.37 (m, 2H), 2.82 (s, 12H). ^{13}C NMR (101 MHz, DMSO-d6) δ 164.40, 163.79, 158.67, 155.50, 148.75, 133.44, 133.13, 131.46, 129.29, 129.18, 128.81, 127.69, 126.76, 123.50, 122.78, 116.15, 111.48, 110.60, 101.62, 76.33, 73.42, 70.71, 68.48, 60.70, 40.67, 40.66, 38.80. MALDI-TOF-MS(m/z): calcd. for $C_{37}H_{39}N_5O_9S$ $[M+Na]^+$: 752.25, found: 752.01.

Optical measurements

For optical measurements, the senoprobe was dissolved in DMSO to obtain the stock solution (1 mM). For sensitivity and selectivity studies, the probe was diluted to 10.0 μM with PBS buffer (10 mM, pH=7.4) and then incubated with NaClO, β-gal, and other analytes (i.e., carbonic anhydrase (CA), cysteine (Cys), glutathione (GSH), H_2O_2, lysozyme (LZ), ferrous chloride (Fe^{2+}), and cobaltous chloride (Co^{2+}) Superoxide($\cdot O_2^-$) , peroxynitrite ($ONOO^-$)), at 37 ℃ for 2 h, respectively. The absorption and fluorescence spectra were then recorded. For fluorescence analysis, the limit of detection (LoD) of the probe was calculated using the following equation:

$LoD = 3\sigma/k$

where σ is the standard deviation of eleven blank measurements, and k is the slope of the linear equation.

The preparation methods for $\cdot O_2^-$ and $ONOO^-$ are as follows: $\cdot O_2^-$ was generated by dissolving KO_2 and 18-crown-6 ether (2.5 eq) in DMSO, yielding a 0.25 M solution. $ONOO^-$, on the other hand, was prepared through the rapid mixing of a solution containing KNO_2 (0.6 M), HCl (0.6 M), and H_2O_2 (0.7 M) with a pre-cooled NaOH solution (3 M) at 0 °C. The concentration of $ONOO^-$ was determined spectrophotometrically using its characteristic extinction coefficient of 1670 cm^{-1} M^{-1} at 302 nm in sodium hydroxide (0.1 M).

Cell culture

Raw264.7 cells and Hela cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and maintained at 37 °C with 5% CO_2. HCT116 cells, OVCAR cells and A549 cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin, and maintained under the same conditions of 37 °C with 5% CO_2.

Cell viability

The $P_{\beta\text{-gal-HClO}}$ cytotoxicity on Raw264.7 cells using the CCK-8 assay. Cells were seeded in 96-well plates and allowed to adhere for 12 h. Then, cells were treated with $P_{\beta\text{-gal-HClO}}$ at concentrations of 0, 1.25, 2.5, 5, 10, 20, 40, and 80 μM for 24 h. After adding CCK-8, absorbance was measured at 450 nm.

Detection of endogenous and exogenous HClO in living cells

Raw264.7 cells were used as the cell model for this study. Briefly, Raw264.7 cells were seeded at a density of 2 × 10^5 cells/well in 12-well plates. After the cells were adhered to the wall, the plates were pretreated with 100 μM NaOCl for 1 h. For imaging studies, CTAB (Cetyl trimethyl ammonium bromide) was premixed with the probe as a co-solvent and subsequently diluted in serum-free medium to achieve final concentrations of 50 mM CTAB and 20 μM probe. Subsequently, the cells were washed and treated with the probe for 2 h. Images were collected using confocal microscopy. For imaging exogenous HClO, Raw264.7 cells were seeded at the same density of 2 × 10^5 cells/well and stimulated with 20 μg/mL LPS for 3 h. The medium was replaced with fresh medium containing the probe, and the cells were cultured for additional 2 h. After washing, the cells were imaged using confocal microscopy. Green channel (λ_{ex} = 488 nm; λ_{em} = 510-590 nm), red channel (λ_{ex} = 633 nm; λ_{em} =650-730 nm).

Detection of endogenous β-gal in living cells

OVCAR-3 and HeLa cells with different β-gal expression levels were seeded in 12-well plates at the density of 2×10^5 cells per well. Once the cells adhered, they were incubated with $P_{\beta\text{-gal-HClO}}$ for 2 h, followed by confocal microscopy imaging. For better imaging, CTAB was premixed with the probe as a co-solvent and subsequently diluted in serum-free medium to achieve final concentrations of 50 mM CTAB and 20 μM probe. A commercial β-gal probe, SPiDER-β-gal, was used as the control for imaging according to the manufacture's protocols. Imaging was conducted using the green fluorescence channel (λ_{ex} = 488 nm; λ_{em} = 510-590 nm).

Development and characterization of senescent cell models

Foam cells with senescent phenotype were established using Raw264.7 cell, while senescent cancer cell models were developed using A549 and HCT116 cells. Briefly, Raw264.7 cells were exposed to 80 μg/mL of ox-LDL for 72 h. Similarly, A549 and HCT116 cells were treated with 200 nM DOX for 3-4 days, followed by continued cultivation in fresh medium to facilitate subsequent characterization of cellular senescence. To validate the foam cell model, both ox-LDL-treated Raw264.7 cells and control Raw264.7 cells were seeded in 12-well plates at a density of 2×10^5 cells/well. The cells were fixed with 4% paraformaldehyde and then incubated in 60% isopropanol for 2 min. Subsequently, Oil red O staining was performed. To do this, a stock solution of oil red O

was first prepared by dissolving 0.5 g oil red O powder in 100 mL of isopropanol, followed by filtration. For cell staining, the oil red O stock solution was diluted in a ratio of 3:2 with distilled water to create the working solution. The cells were then incubated with this working solution at 37°C for 5 min. After washing twice with 60% isopropanol and rinsing with ultrapure water, imaging was conducted using inverted microscope. In paralleled experiments, senescent cells (Raw264.7, A549, HCT116) and their corresponding proliferating cells were seeded in 12-well plates at a density of 2×10^5/well and stained using a senescent β-galactosidase kit. The levels of SA-β-gal were examined under an inverted microscope. Additionally, EdU staining was carried out according to the the instructions provided with the kit, and fluorescence images were captured using a cell microplate monitoring system.

Reverse transcription quantitative PCR analysis

Total RNA in senescent cells and controls was obtained using the Eastep Super Total RNA Extraction Kit. Subsequently, cDNA was generated from the obtained RNA using the Revert Aid First Strand cDNA Synthesis Kit. RT-qPCR was conducted on a 7500 RT-qPCR System using SYBR Green qPCR Master Mix. The primers for this experiment were summarized in Table S1.

Table S1. The primer DNA sequences used for RT-qPCR.

Gene (Human)	**Forward (5'-3')**	**Reverse (5'-3')**
P16	GCTGCCC AACGCACCGAATA	ACCACCA GCGTGTCCA
P21	GACAGCAGAGGAAGACCATGTGGAC	GAGTGGTAGAAATCTGTCATGCTG
P53	CAGCACATGACGGAGGTTGT	TCATCCAAATACTCCACACGC
IL-6	CCAGGAGC CCAGCTATGAAC	CCCAGGGAGAAGGCAACTG
IL-8	AGACAGCAGAGCACACAAGC	ATGGTTCCTTCCGGTGGT
GADPH	GAAGGTGAAGGTCGGAGTC	TTGAGG TCA ATGAAGGGG
MMP-3	AGGGAACTTGAGCGTGAATC	TCACTTGTCTGTTGCACACG
IL-1α	TGGTAGTAGCAACCAACGGGA	ACTTTGATTGAGGGCGTCATTC
Gene (Mouse)	**Forward (5'-3')**	**Reverse (5'-3')**
P21	CCTGGTGATGTCCGACCTG	CCATGAGCGCATCGCAATC

IL-6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
IL-1β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

Flow cytometry analysis

CTAB was premixed with the probe as a co-solvent and subsequently diluted in serum-free medium to achieve final concentrations of 50 mM CTAB and 20 μM probe. Raw264.7 cells were inoculated into 6-well plates at a density of 2×10^5 cells/well. After the cells were adhered to the plats, they were treated with 80 μg/mL ox-LDL for 48 h. Subsequently, $P_{\beta\text{-gal-HClO}}$ was added at the specified concentration and incubated for a total duration of 2 h. After incubation, the cells were collected and analyzed for fluorescence intensity using flow cytometry.

Simultaneous imaging of HClO and SA-β-gal in different types of senescent cells

CTAB was premixed with the probe as a co-solvent and subsequently diluted in serum-free medium to achieve final concentrations of 50 mM CTAB and 20 μM probe. Raw264.7-derived foam cells and senescent HCT116 and A549 cells, along with their proliferating counterparts, were seeded in confocal dishes at a density of 2×10^5 cells per dish. After 12 h of incubation, $P_{\beta\text{-gal-HClO}}$ was added, and cells were co-incubated for additional 2 h. After washing, confocal imaging was then conducted in both green and red fluorescence channels. Green channel: λ_{ex} = 488 nm; λ_{em} = 510-590 nm. Red channel: λ_{ex} = 633 nm; λ_{em} = 650-730 nm.

2. Supplementary Figures

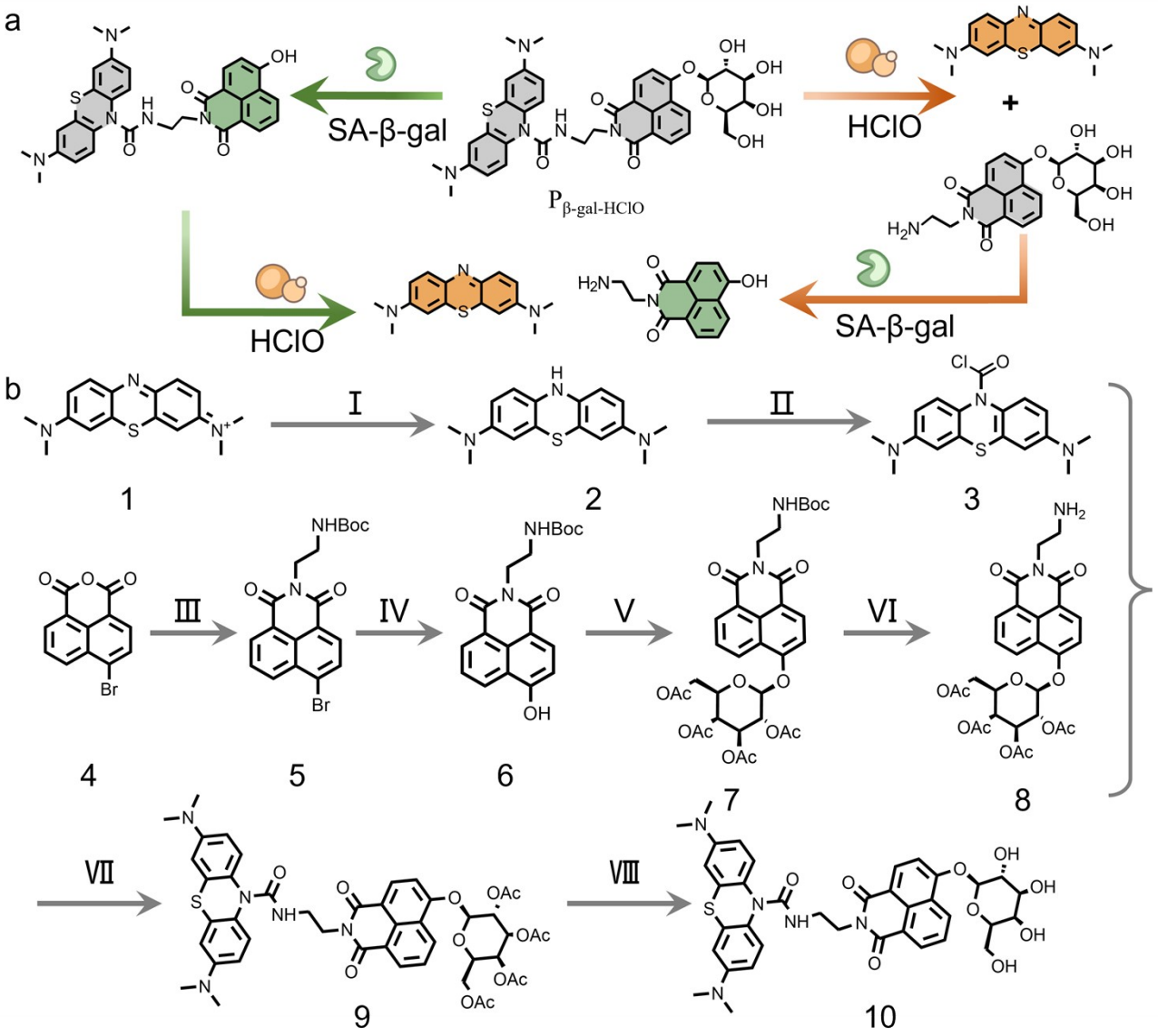

Fig. S1. (a) The proposed imaging mechanism and (b) synthesis routes of the probe $P_{β\text{-gal-HClO}}$. (I) Na_2CO_3, sodium dithionite, H_2O, and dichloromethane. (II) Triphosgene, H_2O, and dichloromethane. (III) Tert-butyl N-(2-aminoethyl) carbamate and ethanol. (IV) N-hydroxysuccinimide, K_2CO_3, and DMSO. (V) 2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl-bromide, K_2CO_3, and acetonitrile. (VI) Trifluoroacetic acid and dichloromethane. (VII) Compound 3, triethylamine, and dichloromethane. (VIII) CH_3OH and CH_3ONa.

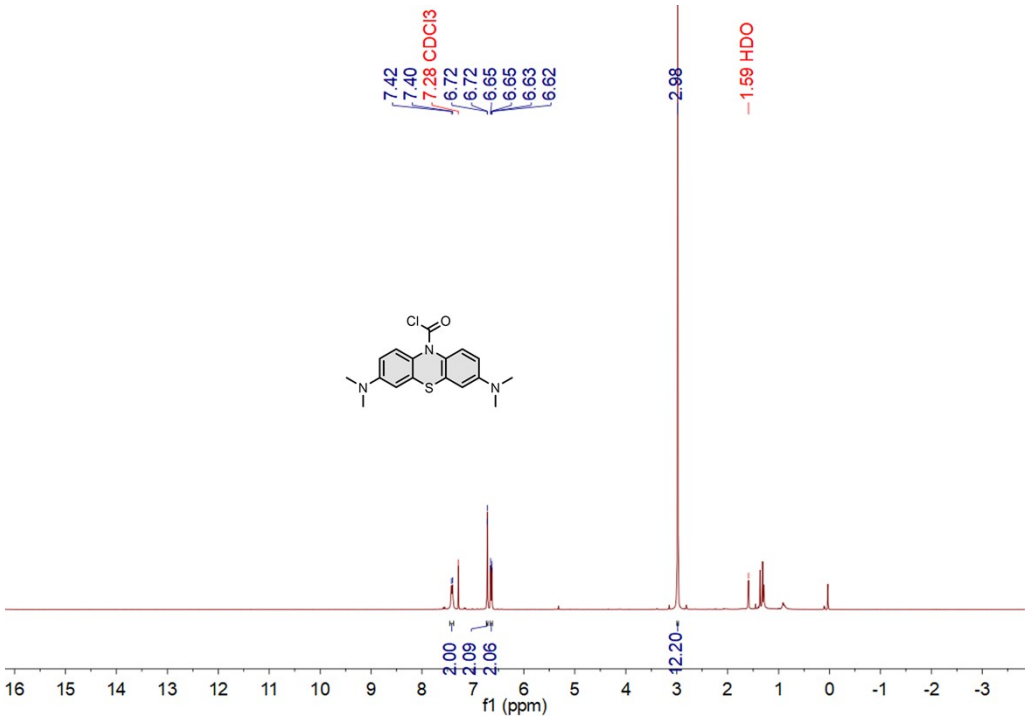

Fig. S2. [1]H NMR spectrum of compound 3.

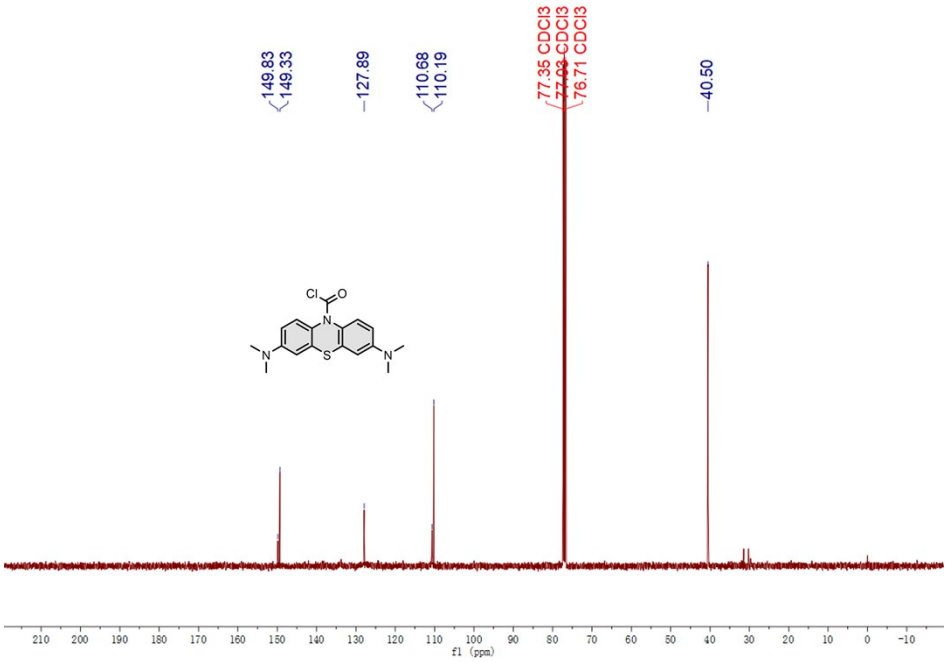

Fig. S3. [13]C NMR spectrum of compound 3

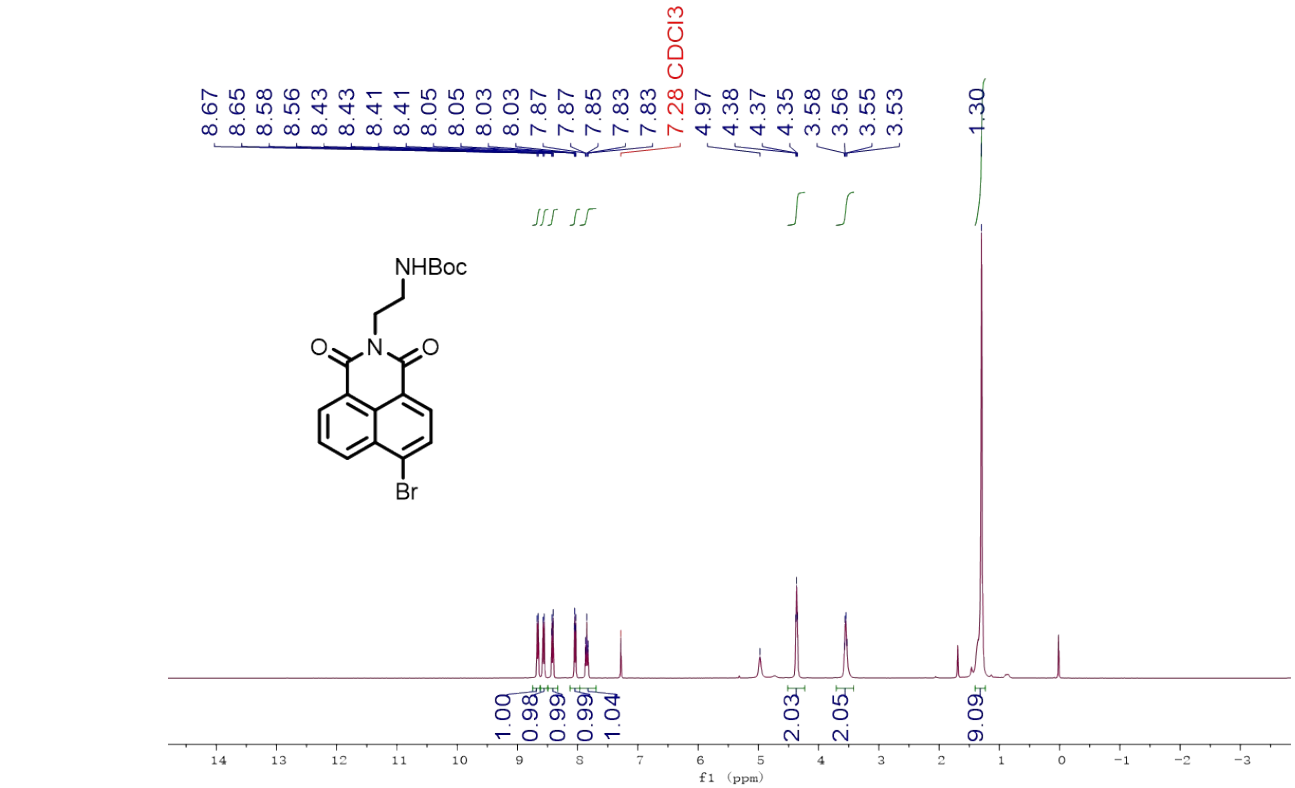

Fig. S4. ^{1}H NMR spectrum of compound 5.

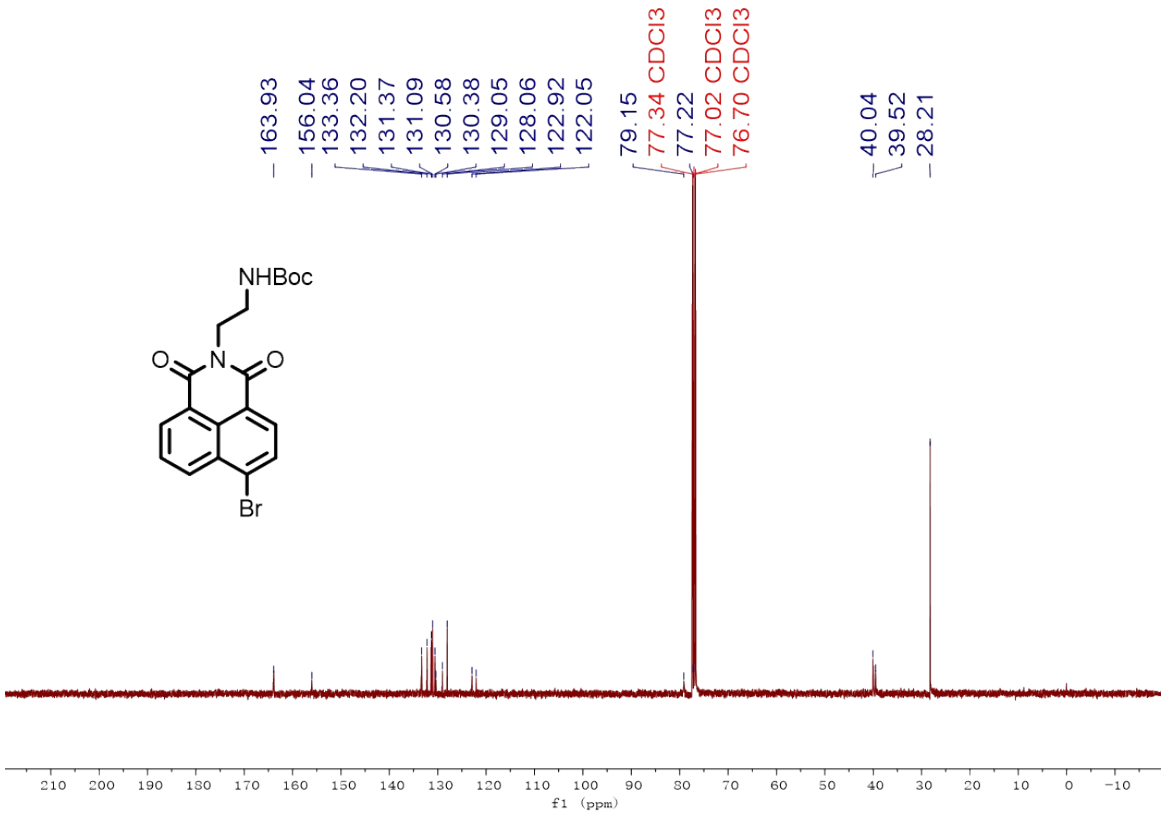

Fig. S5. ^{13}C NMR spectrum of compound 5.

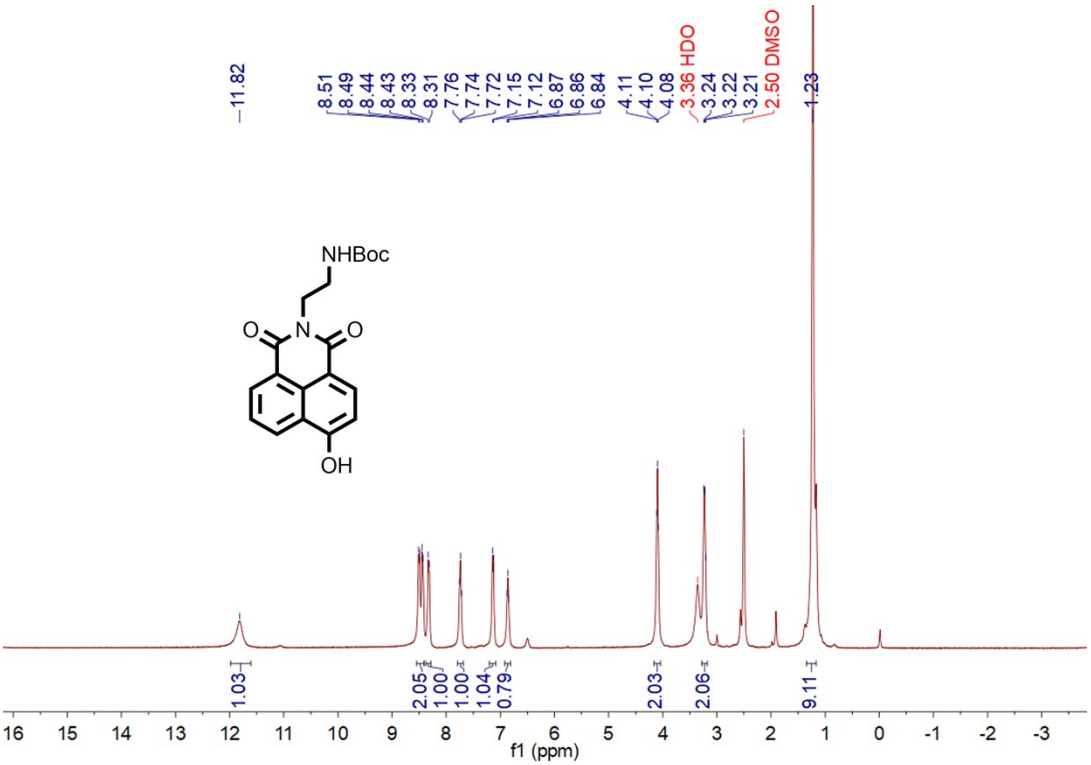

Fig. S6. [1]H NMR spectrum of compound 6.

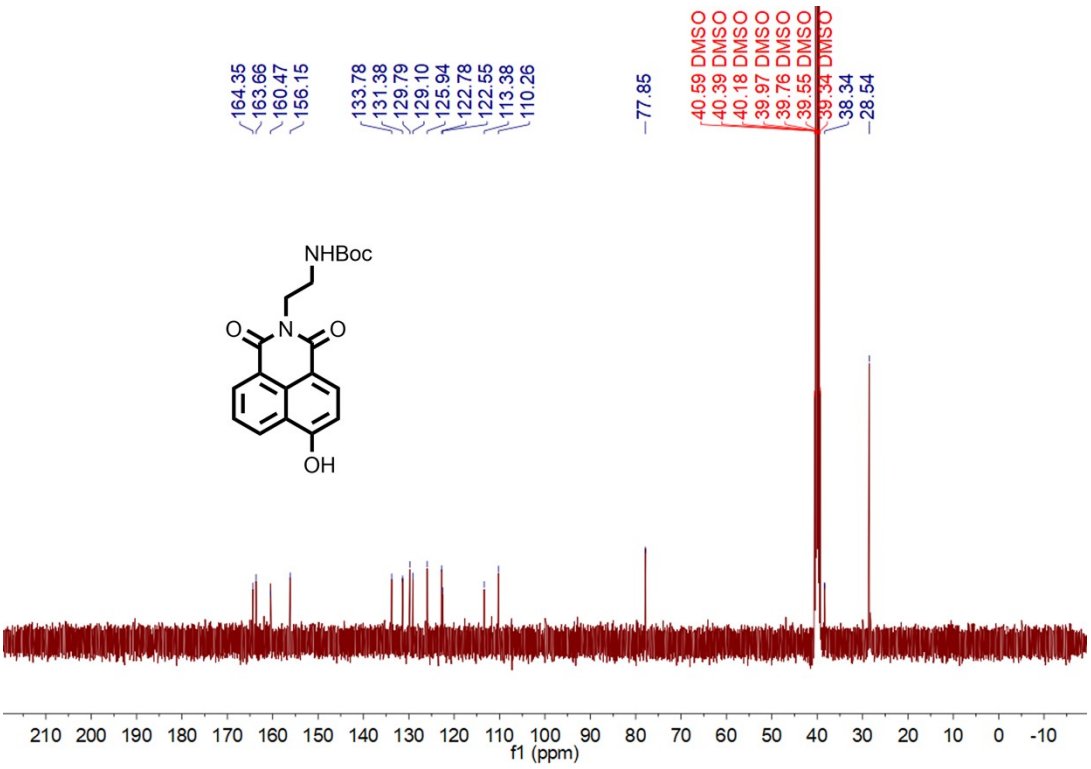

Fig. S7. [13]C NMR spectrum of compound 6.

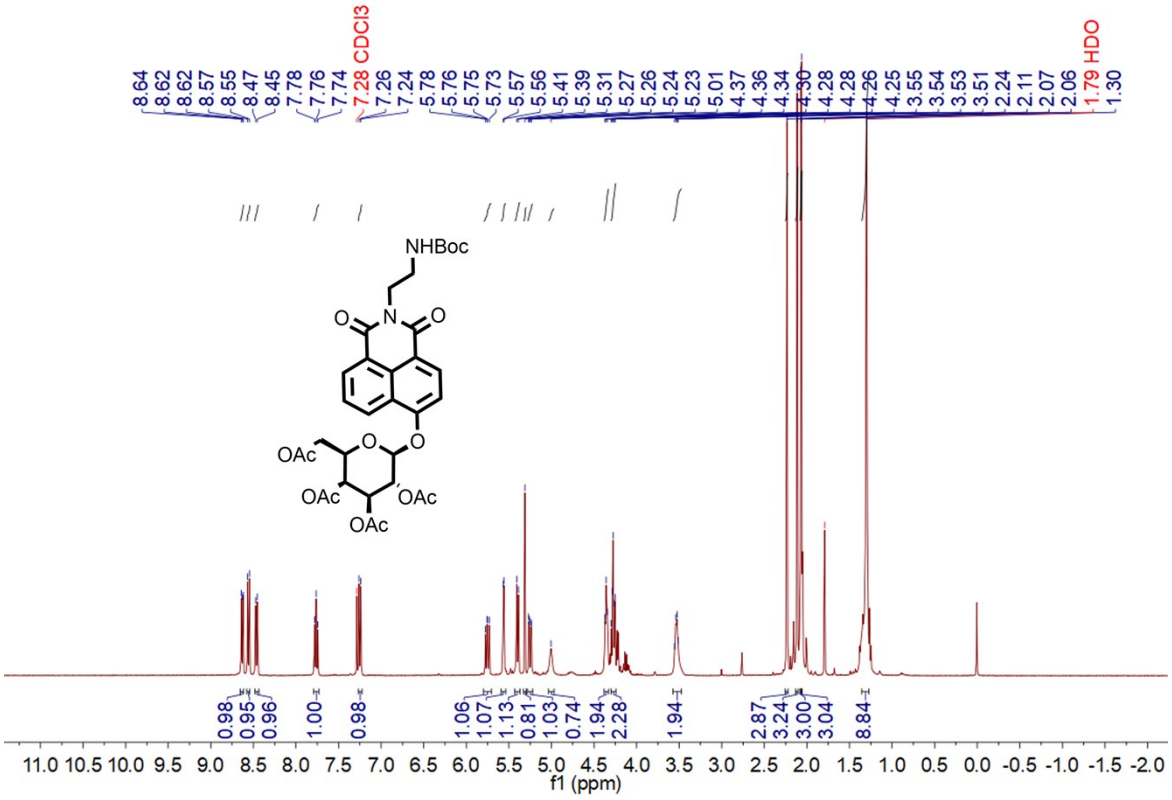

Fig. S8. ^{1}H NMR spectrum of compound 7.

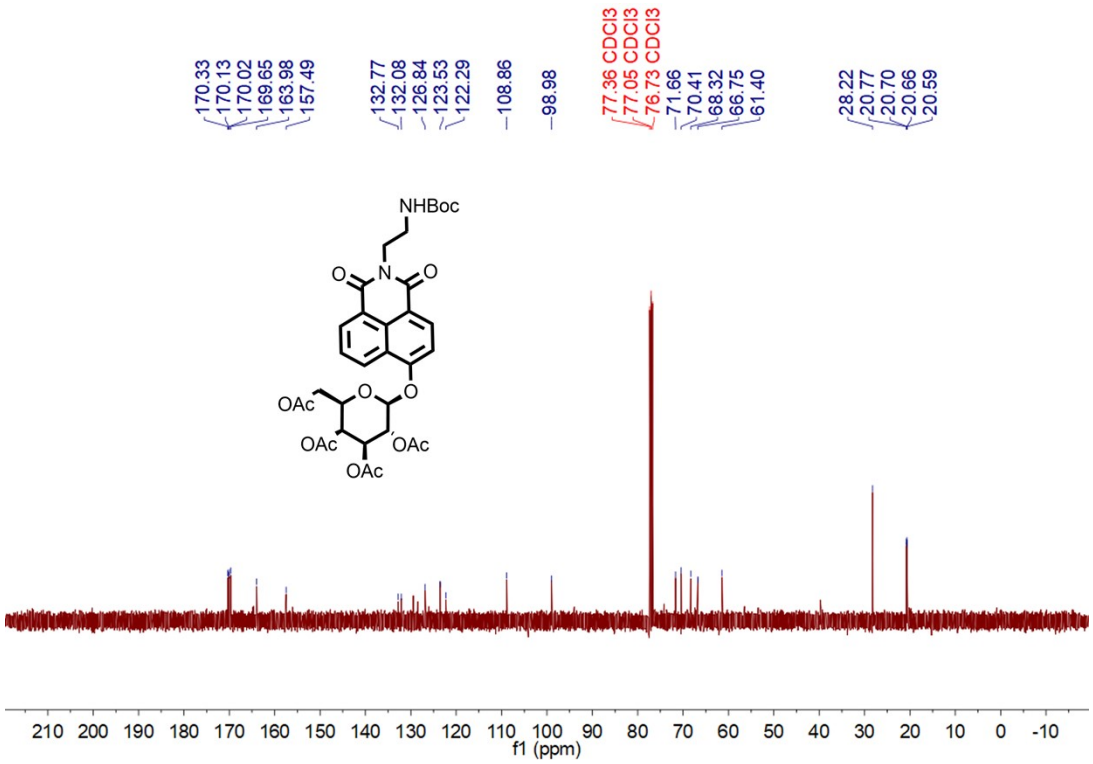

Fig. S9. ^{13}C NMR spectrum of compound 7.

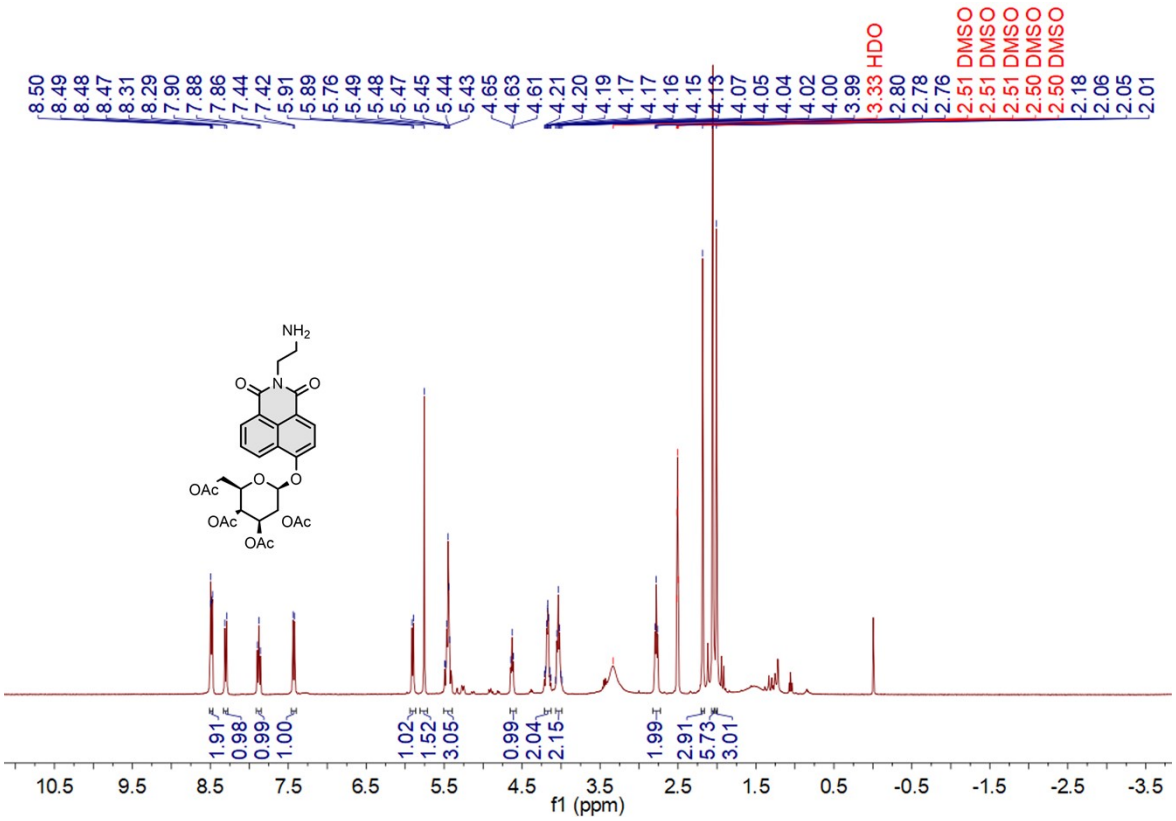

Fig. S10. ^{1}H NMR spectrum of compound 8.

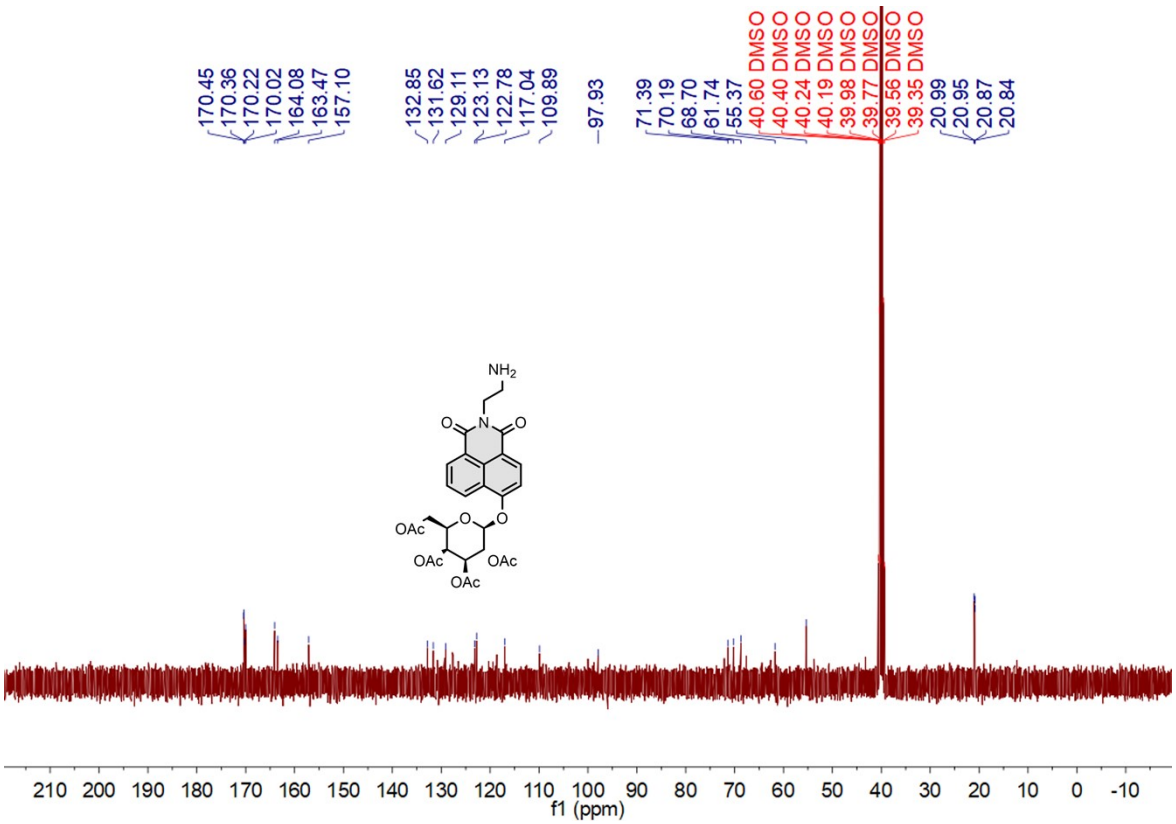

Fig. S11. ^{13}C NMR spectrum of compound 8.

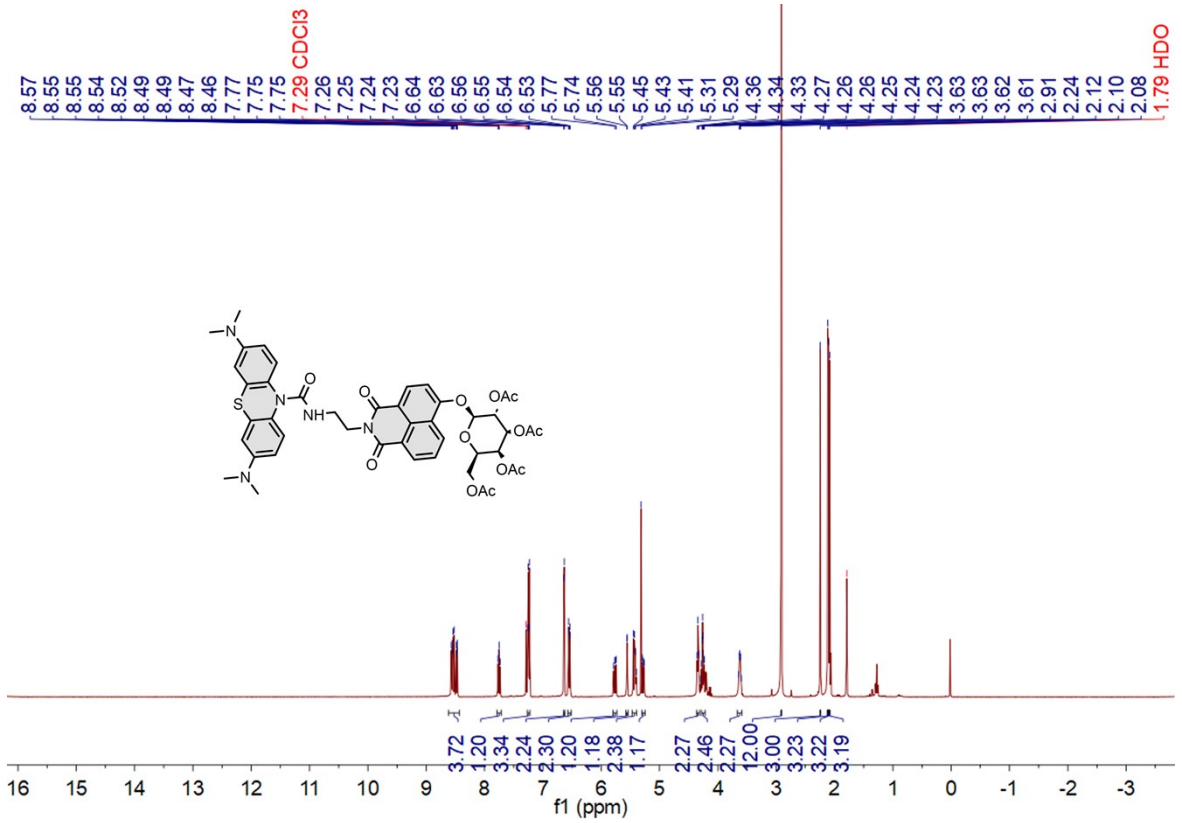

Fig. S12. ^{1}H NMR spectrum of compound 9.

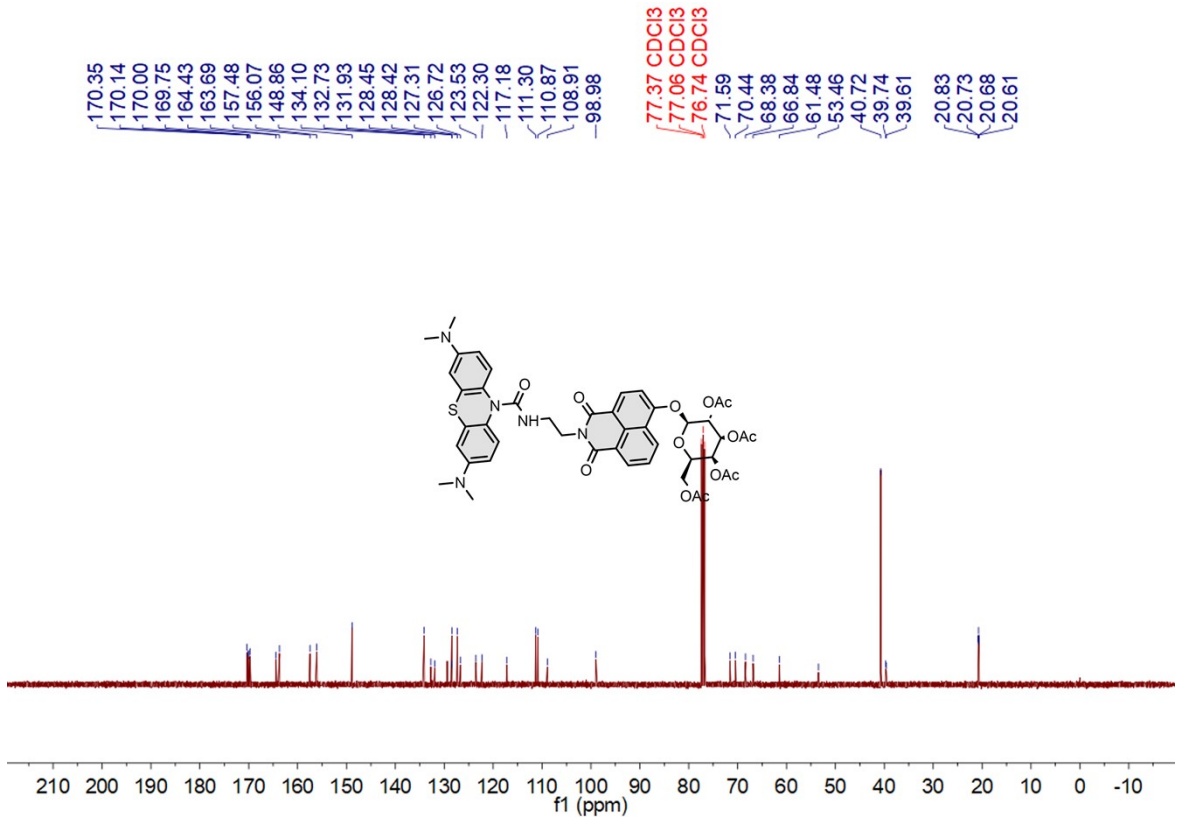

Fig. S13. ^{13}C NMR spectrum of compound 9.

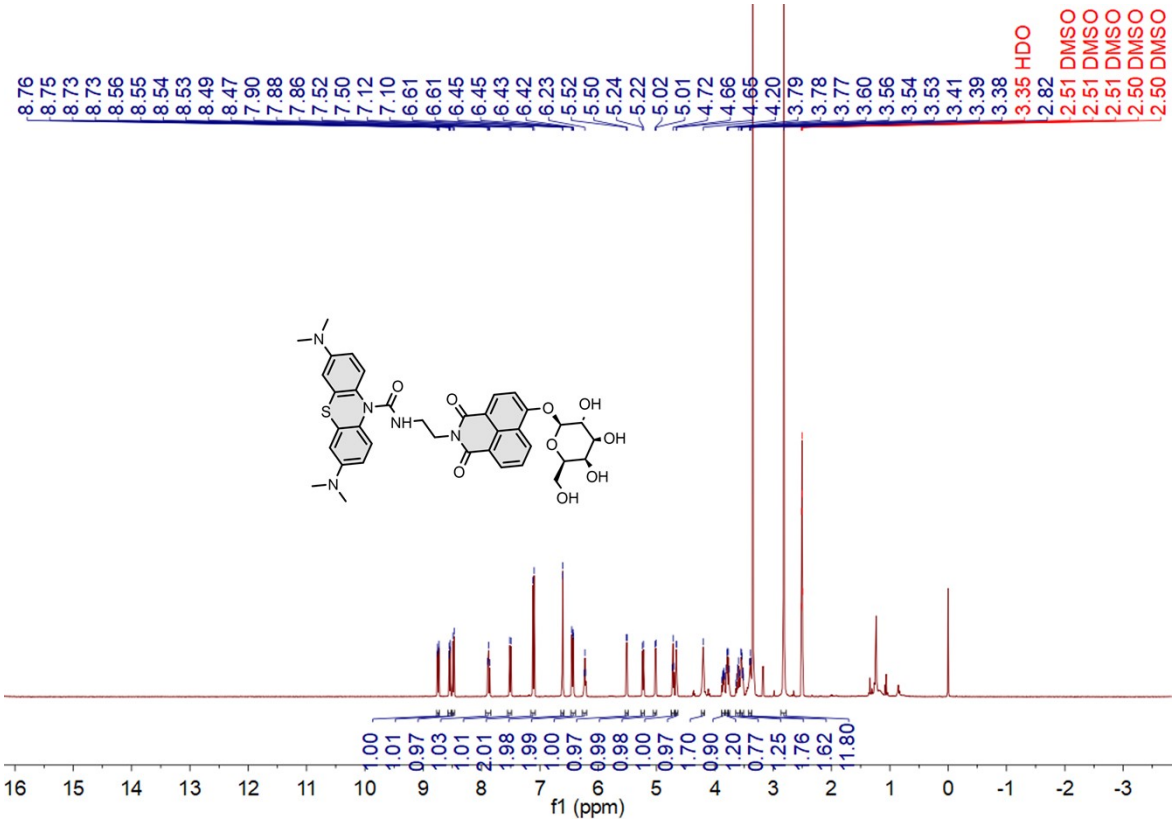

Fig. S14. ^{1}H NMR spectrum of compound 10.

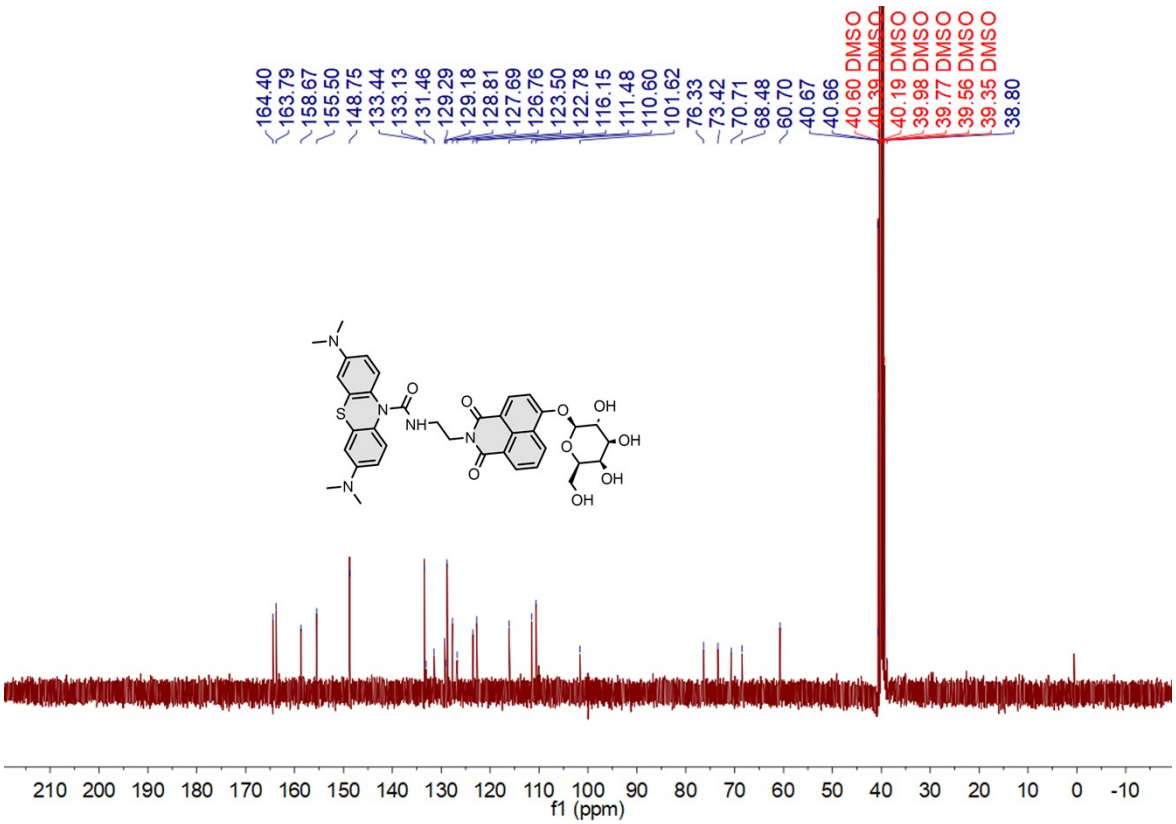

Fig. S15. ^{13}C NMR spectrum of compound 10.

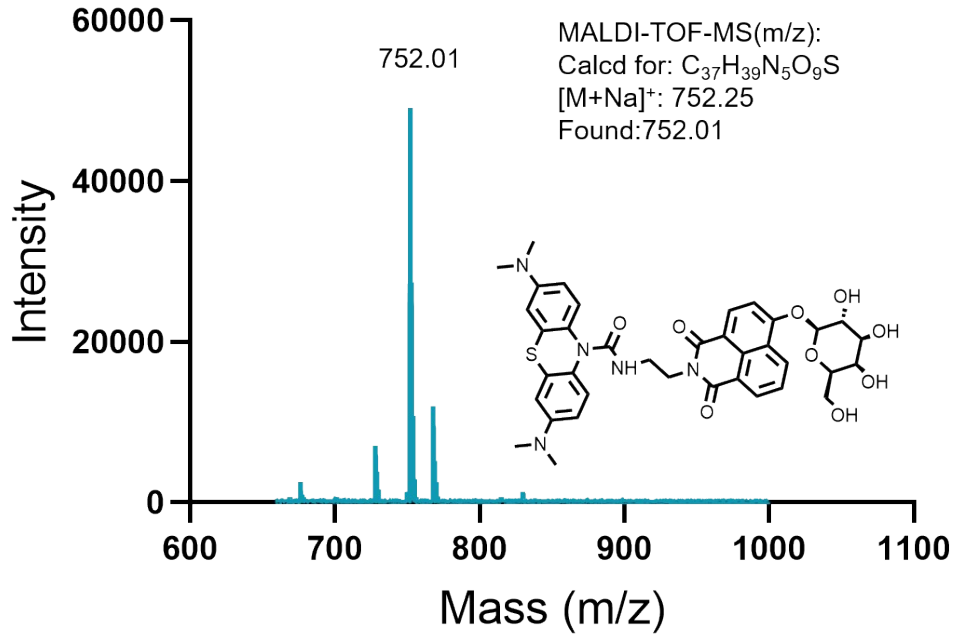

Fig. S16. MALDI-TOF-MS spectrum of $P_{\beta\text{-gal-HClO}}$.

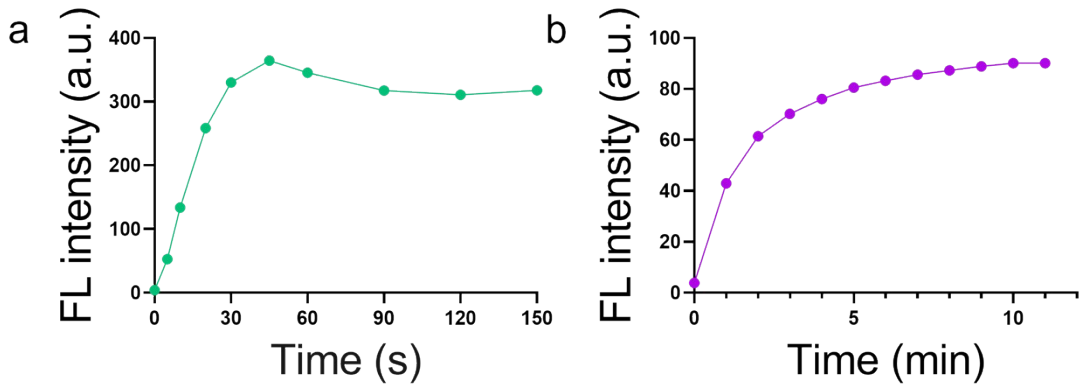

Fig. S17. (a) Time-dependent fluorescence responses of $P_{\beta\text{-gal-HClO}}$ (10 μM) at 700 nm after incubation with NaClO (50 μM). λ_{ex} = 450 nm. (b) Time-dependent fluorescence responses of $P_{\beta\text{-gal-HClO}}$ (10 μM) at 560 nm after treatment with 0.5 U/mL β-gal. λ_{ex} = 630 nm.

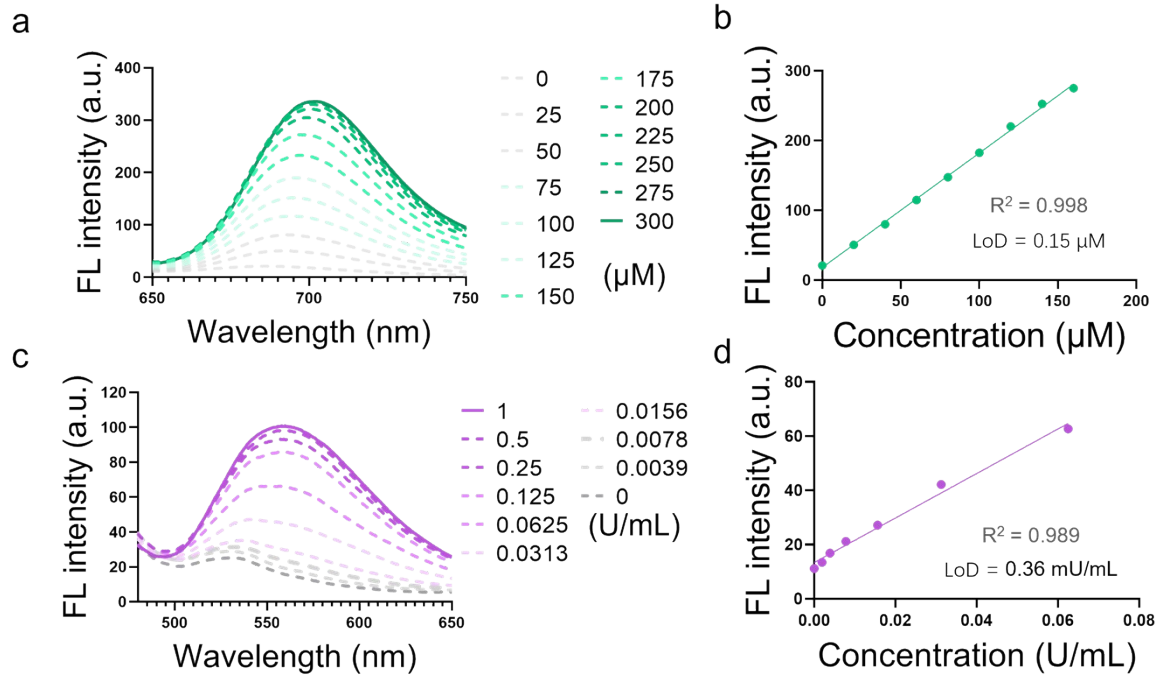

Fig. S18. (a) Fluorescent spectra of $P_{\beta\text{-gal-HClO}}$ (10 μM) in the presence of varying concentrations of NaClO. (b) The corresponding linear relationship between the maximum fluorescent intensity at 700 nm and the concentration of NaClO. (c) Fluorescent spectra of $P_{\beta\text{-gal-HClO}}$ (10 μM) in the presence of varying concentrations of β-gal. (d) The corresponding linear relationship between the minimum fluorescent intensity at 560 nm and the concentration of β-gal.

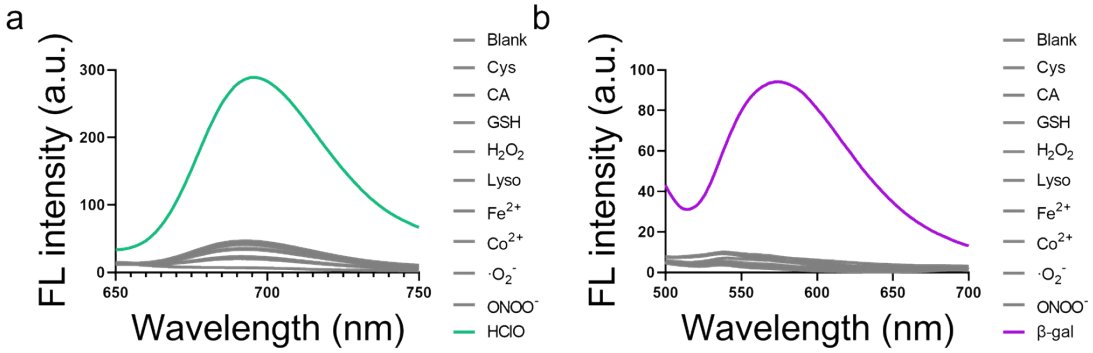

Fig. S19. Fluorescent spectra of $P_{\beta\text{-gal-HClO}}$ (10 μM) under the excitation wavelength (λ_{ex}) of (a) 450 nm and (b) 630 nm, following incubation with Cys (1 mM), CA (0.5 U/mL), GSH (1 mM), H_2O_2 (1 mM), LZ (0.5 U/mL), Fe^{2+}(1 mM), Co^{2+}(1 mM), O_2^- (100 μM), $ONOO^-$ (100 μM), and β-gal (0.5 U/mL), respectively.

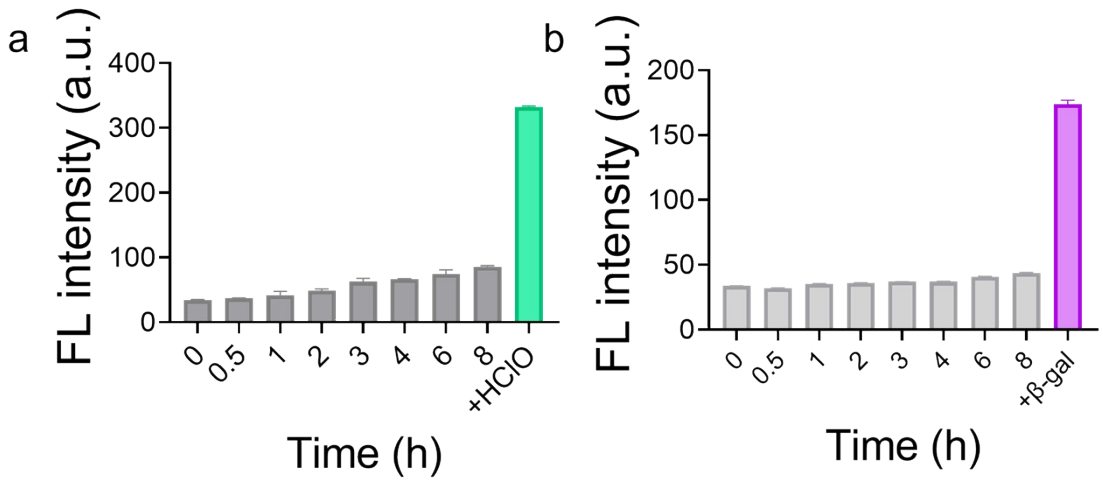

Fig. S20. (a) Fluorescence intensity of $P_{\beta\text{-gal-HClO}}$ at 700 nm after incubation in the RPMI 1640 medium supplemented with 10% FBS for indicated time intervals. λ_{ex} = 630 nm. (b) Fluorescence intensity of $P_{\beta\text{-gal-HClO}}$ at 560 nm after incubation under similar conditions. λ_{ex} = 450 nm. Fluorescence variations in the presence of individual targets were examined as controls. Data are shown as mean ± SD (n = 3).

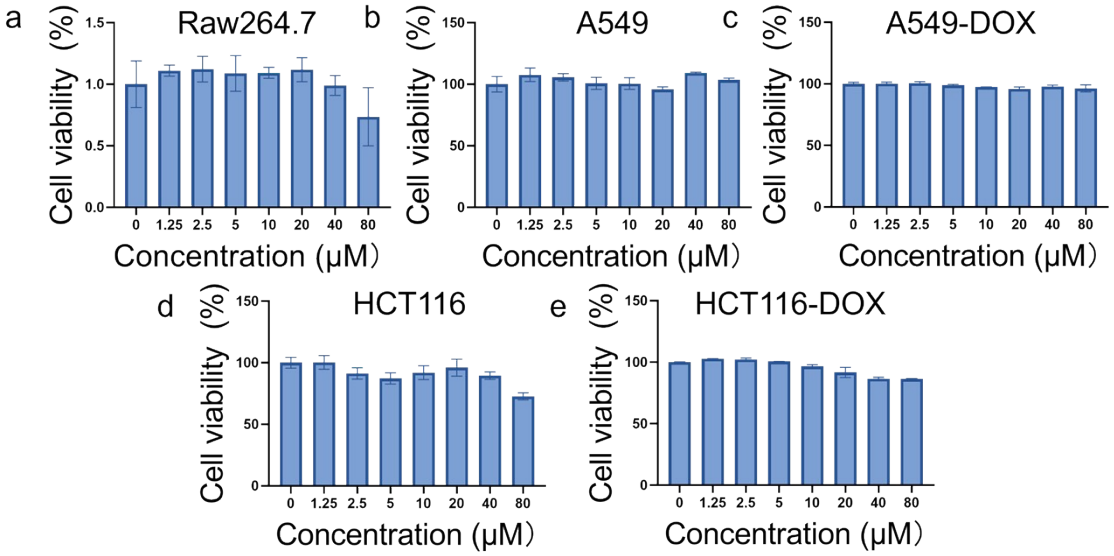

Fig. S21. Cell viability of (a) Raw264.7 cells, (b) A549 cells, (c) DOX-induce senescent A549 cells, (d) HCT116 cells, and (e) DOX-induce senescent HCT116 cells, after treatment with $P_{\beta\text{-gal-HClO}}$ at varied concentrations for 24 h. Data are shown as mean ± SD (n = 6).

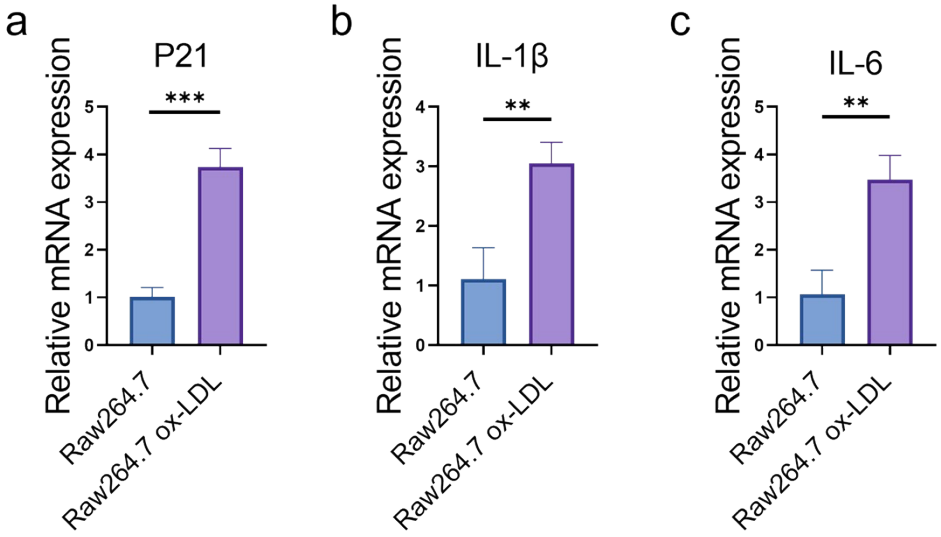

Fig. S22. Relative mRNA expression of (a) P21, (b) IL-1β, and (c) IL-6 in nontreated Raw264.7 cells and ox-LDL-induced senescent Raw264.7 cells. Data are shown as mean ± SD (n = 3). $^{**}P < 0.01$ and $^{***}P < 0.001$.

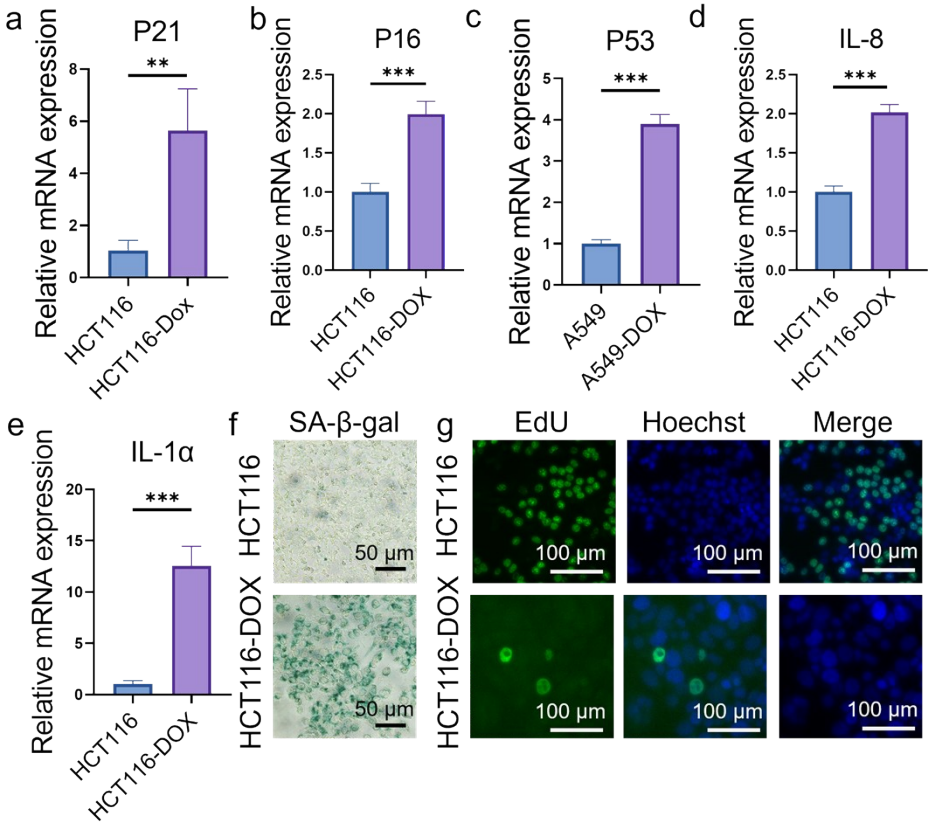

Fig. S23. (a-e) Relative mRNA expression of P21, P16, P53, IL-8, and IL-1α in nontreated and DOX-treated HCT116 cells. Data are shown as mean ± SD (n = 3). (f) Representative SA-β-gal staining images and (g) EdU staining images of HCT116 cells before and after DOX treatment. $^{**}P < 0.01$ and $^{***}P < 0.001$.

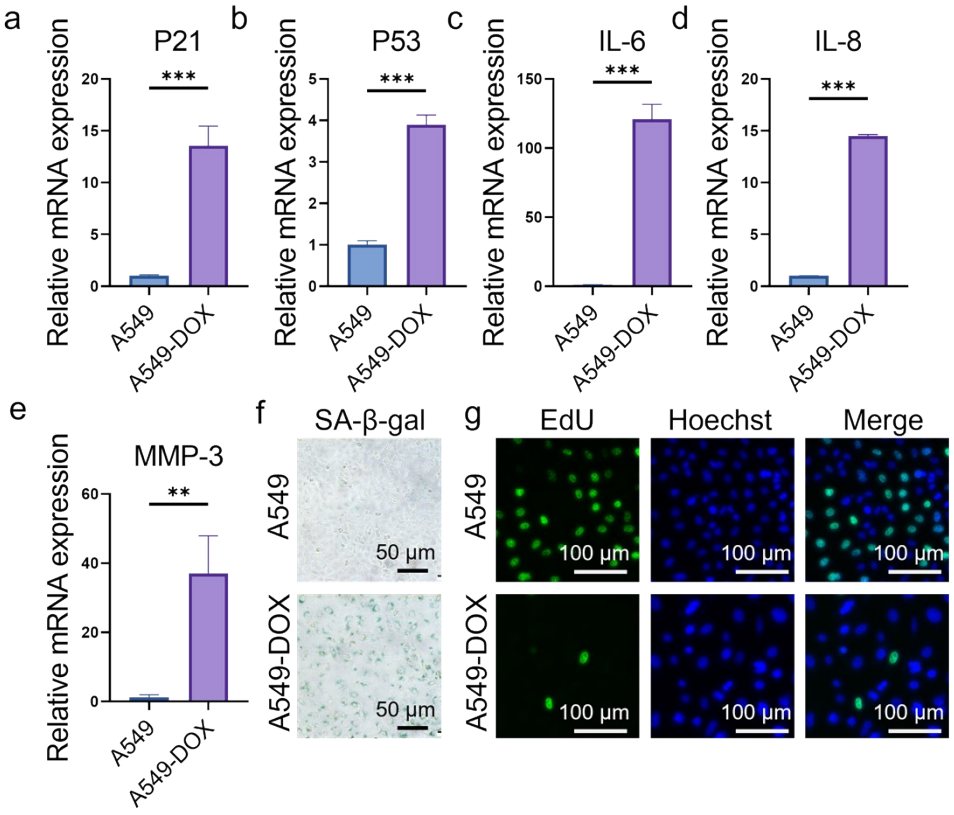

Fig. S24. (a-e) Relative mRNA expression of P21, P53, IL-6, IL-8, and MMP-3 in nontreated and DOX-treated A549 cells. Data are shown as mean ± SD (n = 3). (f) Representative SA-β-gal staining images and (g) EdU staining images of A549 cells before and after DOX treatment. $^{***}P < 0.001$.

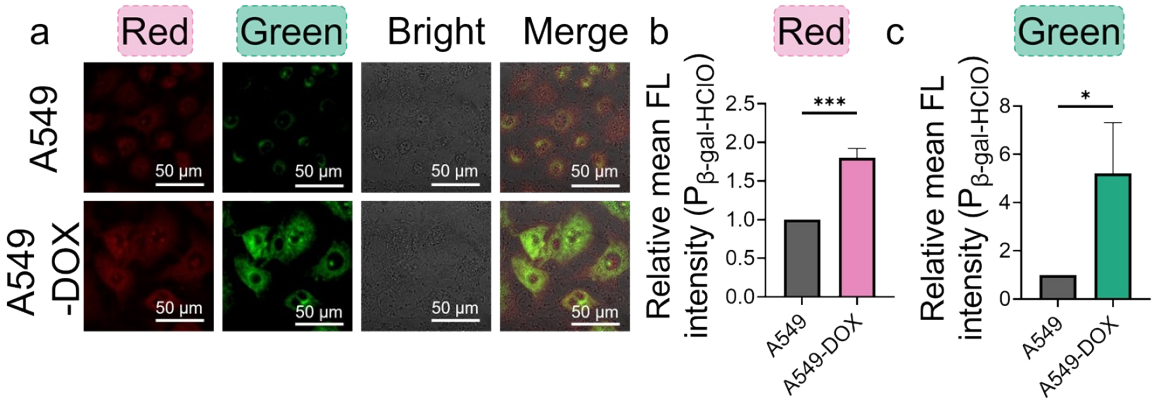

Fig. S25. (a) Confocal imaging of DOX-induced senescent A549 cells and proliferating A549 cells after incubation with $P_{β\text{-gal-HClO}}$ in both red and green fluorescence channels. (b,c) Relative mean fluorescence intensity of the (b) red channel (λ_{ex} = 633 nm; λ_{em} =650-730 nm) and (c) green channel (λ_{ex} = 488 nm; λ_{em} = 510-590 nm) for panel a. Data are shown as mean ± SD (n = 3). $^{*}P < 0.05$ and $^{***}P < 0.001$.

Table S2. Overview of representative dual-targeted imaging probes toward cellular senescence.

Probe name	Detection marker	Response mechanisms	Signal output pathways	The reference
$P_{\beta gal\text{-}MAO\text{-}A}$	MAO-A and β-gal	Sequential activation principle	Single signal output	Anal. Chem. 2023, 95, 3996-4004
PF-Torin1-Ferro	β-gal and Fe^{2+}	Sequential activation principle	Single signal output	Anal. Chem. 2024, 96, 17154-17164
$P_{Gal\text{-}FA}$	β-gal and FA	Sequential activation principle	Single signal output	Chem. Sci., 2021, 12, 13483-13491
Gal-HCy-Biotin	β-gal and biotin receptor	Sequential activation principle	Single signal output	Anal. Chem. 2023, 95, 10481-10485
Ap-βgal-Fret	β-gal and L1CAM	Sequential activation principle	Ratiometric signal output	Anal. Chem. 2023, 95, 3996-4004
ROKS.	PhSH and HClO	Sequential activation principle	Ratiometric signal output	Chem. Sci., 2021, 12, 13483-13491
KSA02	β-gal and pH	Sequential activation principle	Ratiometric signal output	Angew. Chem., Int. Ed. 2021, 60, 10756-10765
$P_{\beta\text{-}gal\text{-}HClO}$	β-gal and HClO	Simultaneous activation principle	Dual signal output	This work

3. References

[1] P. Wei, W. Yuan, F. F. Xue, W. Zhou, R. H. Li, D. T. Zhang and T. Yi, *Chem. Sci.*, 2018, **9**, 495-501.

[2] P. Zhang, C. X. Fu, H. H. Liu, X. J. Guo, Q. Zhang, J. Gao, W. J. Chen, W. Yuan and C. F. Ding, *Anal. Chem.*, 2021, **93**, 11337-11345.

[3] X. Q. Kong, M. Li, B. L. Dong, Y. G. Yin, W. H. Song and W. Y. Lin, *Anal. Chem*., 2019, **91**, 15591-15598.