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Electronic Supplementary Information

A highly selective ratio-metric fluorescent sensor for visualizing nitroreductase in hypoxic cells

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Contents

1. Experimental section

Reagents and Chemicals

6-nitro-1H,3H-benzo[de]isochromene-1,3-dione, 3-aminopropan-1-ol, triethylamine, tribromophosphane, diethylamino-2-oxo-2H-chromene-3-carboxylic acid, prop-2-yn-1-amine, EDCI, HOBt, Copper sulfate pentahydrate and L-Ascorbic Acid Sodium Salt were purchased from Adamas-Beta Co., Ltd. (Shanghai, China). Petroleum ether (PE), ethyl acetate (EA), dichloromethane (DCM), ethanol (EtOH) and N, N-dimethylformamide (DMF) were bought from General Reagents Co., Ltd. (Shanghai, China). KCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2$, trypsin, bovine serum albumin (BSA), ascorbic Acid (AA) and dopamine (DA) were purchased from Aladdin Chemistry Co. Ltd. (China). Phosphate buffer solution (PBS, pH 7.4) with concentration of 0.1 M was prepared from KH_2PO_4, $K_2HPO_4 \cdot 3H_2O$ and KCl. All aqueous solutions were prepared with Milli-Q water (18.2 M Ω cm, Millipore) and all chemicals were used as purchase without further purification.

Apparatus and Instruments

^1H NMR and ^{13}C NMR spectra were obtained with a 500 MHz NMR spectrometer (Bruker, Germany). HPLC-MS was performed on Agilent 1260 HPLC (U.S.A.) equiped with SolariX 7.0T FT ICR MS (Bruker, Germany).

UV/Vis absorption and fluorescence spectroscopic methods

All UV/Vis absorption and fluorescence spectra were recorded on UV-2600 (Shimadzu Corporation, Kyoto, Kyoto Prefecture, Japan) and RF-6000 (Shimadzu Corporation, Kyoto, Kyoto Prefecture, Japan) spectrophotometer, respectively. Stock solutions of compounds were preparedS3 in DMSO (HPLC grade). All data were recorded in PBS solution (10 mM, pH 7.4) containing 5%(v/v) of DMSO. Excitation wavelength was 405 nm.

Cell culture and confocal microscopic methods

A human cervical cancer cells (HeLa) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Gibco® fetal bovine serum (FBS), and 100 U/mL penicillinstreptomycin. Adenocarcinoma human alveolar basal epithelial cells (A549) and human hepatocellular liver carcinoma cells (HepG2) were cultured Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 10% FBS, and 100 U/mL penicillin-streptomycin. Mouse embryonic fibroblast cells (NIH-3T3) were cultured in DMEM supplemented with 10% Gibco® bovine calf serum (BCS), and 100 U/mL penicillin-streptomycin. At 2 days before the microscopic experiments, the cells were transferred on cover glass-bottom dish. The cells were seeded at 105 per dish and maintained at 37 °C in a humidified atmosphere consisting of 5% (v/v) CO_2 containing air.

MTT assay

Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

assay. Cells at 1.5×104/mL were treated with different concentrations of probe 1 in 96-well plates for 24 h at 37 °C. Then, a solution of MTT in serum free media (5 mg/mL) was added to each well, which was then further incubated for 3 h. The water-insoluble formazan was formed during the incubation, and then DMSO was added to each well. The amount of formazan was then measured by checking the absorbance at 540 nm using a Spectra Max i3x microplate reader (Molecular devices, San Jose, CA). MTT was purchased from Sigma-Aldrich (St. Louis, MO) and used as received without further purification.

Synthesis of NORP

Synthesis of compound 1. A mixture of 6-nitro-1H,3H-benzo[de]isochromene-1,3-dione (486 mg, 2.0 mmol), 3-aminopropan-1-ol (750 mg, 10 mmol), and triethylamine (NEt_3) (505 mg, 5 mmol) in ethanol (EtOH) (20 mL) was stirred for and refluxed for 8 h under N_2. The mixture was cooled, filtered, then removed the solvent by evaporation and dried in vacuo. The residue was purified by column chromatography (DCM: EtOH = 200: 1, v/v) to obtain **compound 1**. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.63 (d, J = 8.7 Hz, 1H), 8.59 – 8.45 (m, 3H), 8.03 (t, J = 8.0 Hz, 1H), 4.09 (t, J = 7.5 Hz, 2H), 3.52 (t, J = 6.4 Hz, 2H), 1.81 (t, J = 7.1 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 163.28, 162.48, 149.37, 132.02, 130.48, 129.93, 129.05, 128.61, 126.98, 124.66, 123.09, 123.06, 59.31, 38.44, 31.20, 31.15.

Synthesis of compound 2. A mixture of **compound 1** (300 mg, 1.0 mmol), tribromophosphane (540 mg, 2.0 mmol) in DCM was stirred for 2h under N_2 at room temperature. Then, the mixture was washed three times with water (20 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was removed by evaporation under reduced pressure, the residue was purified by column chromatography (DCM: EtOH = 500:1 to 100:1, v/v) to obtain **compound 2**. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.69 (d, J = 8.3 Hz, 1H), 8.63 – 8.58 (m, 2H), 8.54 (d, J = 8.8 Hz, 1H), 8.11 – 8.06 (m, 1H), 4.20 – 4.15 (m, 2H), 3.66 – 3.61 (m, 2H), 2.25 – 2.20 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 163.59, 162.80, 149.53, 132.06, 130.52, 129.96, 129.14, 128.86, 127.22, 124.66, 123.33, 123.14, 40.49, 32.74, 31.23.

Synthesis of compound 2-1. A mixture of **compound 2** (363 mg, 1.0 mmol), sodium azide (195 mg, 3.0 mmol) in N, N-dimethylformamide (DMF) (10 mL) was stirring overnight at 90 ^{0}C under N_2. Cool the mixture to room temperature and remove the solvent by reduced pressure distillation. The mixture was diluted with dichloromethane (20 mL), and washed three times with water (20 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was removed by evaporation under reduced pressure to obtain **compound 2-1**.

Synthesis of compound 3. A mixture of 7-(diethylamino)-2-oxo-2H-chromene-3-carboxylic acid (261 mg, 1.0 mmol), prop-2-yn-1-amine (220 mg, 4.0 mmol), 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDCI) (0.37 g, 1.9 mmol), and 1-hydroxybenzotriazole hydrate (HOBt) (0.29 g, 2.4 mmol), triethylamine (NEt_3) (303 mg, 3 mmol) in N,N-dimethylformamide (DMF) (10 mL) was stirring overnight under N_2 at room temperature. The mixture was diluted with dichloromethane (20 mL), and washed three times with water (20 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was removed by evaporation under reduced pressure, the residue was purified by column chromatography (DCM: EtOH = 200:1 to 20:1, v/v) to obtain **compound 3**. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.84 (t, J = 5.6 Hz, 1H), 8.67 (s, 1H), 7.69 (d, J = 9.0 Hz, 1H), 6.81 (dd, J = 9.0, 2.5 Hz, 1H), 6.61 (d, J = 2.4 Hz, 1H), 4.10 (dd, J = 5.6, 2.5 Hz, 2H), 3.48 (q, J = 7.1 Hz, 4H), 3.14 (t, J = 2.5 Hz, 1H), 1.14 (t, J = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 162.53, 162.09, 157.79, 153.05, 148.52, 132.17, 110.66, 109.24, 108.11, 96.34, 81.53, 73.59, 44.82, 28.91, 12.78.

Synthesis of compound NORP. A mixture of **compound 3** (163 mg, 0.5 mmol), **compound 4** (150 mg, 0.5 mmol), Copper sulfate pentahydrate (150 mg, 0.6 mmol) and L-Ascorbic Acid Sodium Salt (240 mg, 1.4 mmol) in anhydrous N, N-dimethylformamide (DMF) (10 mL) was stirring overnight under N_2 at 100 °C. Cool the mixture to room temperature and remove the solvent by reduced pressure distillation. Then, the reaction mixture was concentrated under vacuum, and the crude product was purified by silica column chromatography (DCM: MeOH=100:1, v/v) to obtain Compound **NORP**. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.68 (s, 1H), 8.61 (d, J = 8.3 Hz, 1H), 8.42 (d, J = 7.2 Hz, 1H), 8.19 (d, J = 8.4 Hz, 1H), 8.06 (s, 1H), 7.68 (d, J = 9.0 Hz, 1H), 7.64 (t, J = 7.9 Hz, 1H), 6.84 (d, J = 8.4 Hz, 1H), 6.80 (dd, J = 9.0, 2.5 Hz, 1H), 6.61 (d, J = 2.3 Hz, 1H), 4.54 (s, 2H), 4.42 (t, J = 7.2 Hz, 2H), 4.07 (t, J = 7.0 Hz, 2H), 3.50 (d, J = 7.1 Hz, 2H), 3.47 (d, J = 7.0 Hz, 2H), 2.19 – 2.15 (m, 2H), 1.14 (t, J = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.34, 163.43, 162.69, 162.14, 157.66, 152.89, 148.24, 134.39, 132.00, 131.43, 130.16, 129.73, 124.34, 122.15, 110.53, 109.53, 108.58, 108.05, 107.94, 96.24, 55.33, 48.21, 44.79, 37.27, 14.38, 12.73. HR-EI-MS Calcd for: $C_{32}H_{30}N_7O_7^+$ ($[M+H]^+$): 624.2207; Found: 624.2202.

2. NMR spectra and HR-MS data

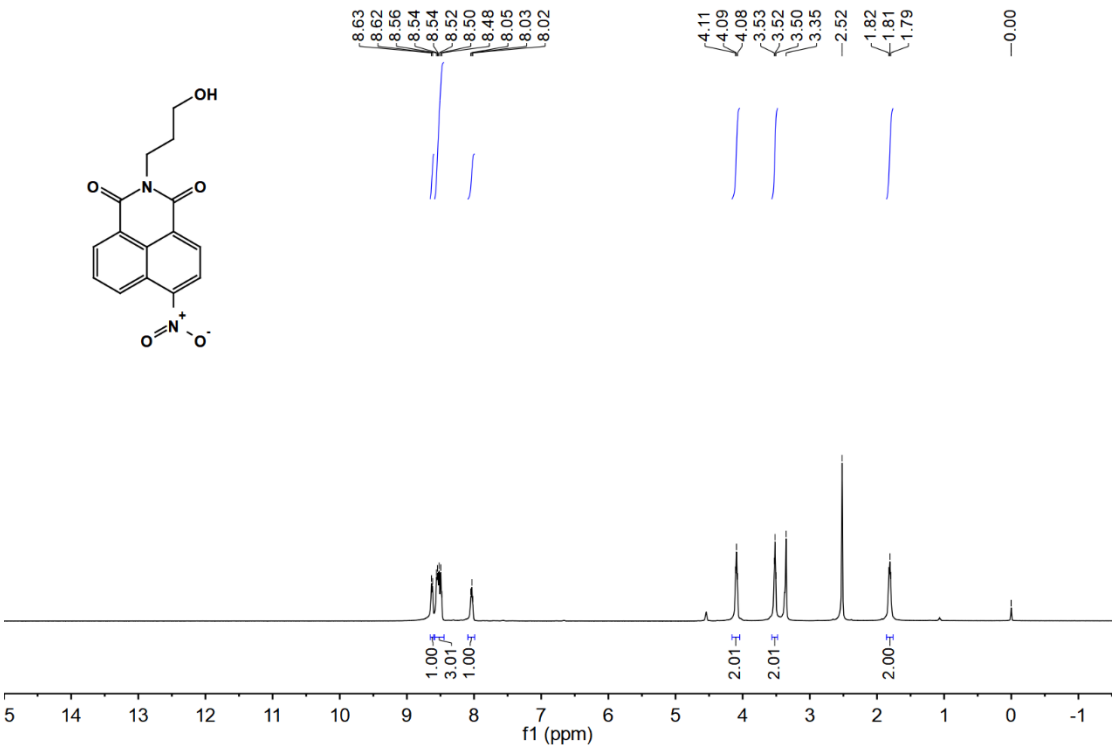

Fig. S1. [1]H NMR spectrum of **Compound 1**.

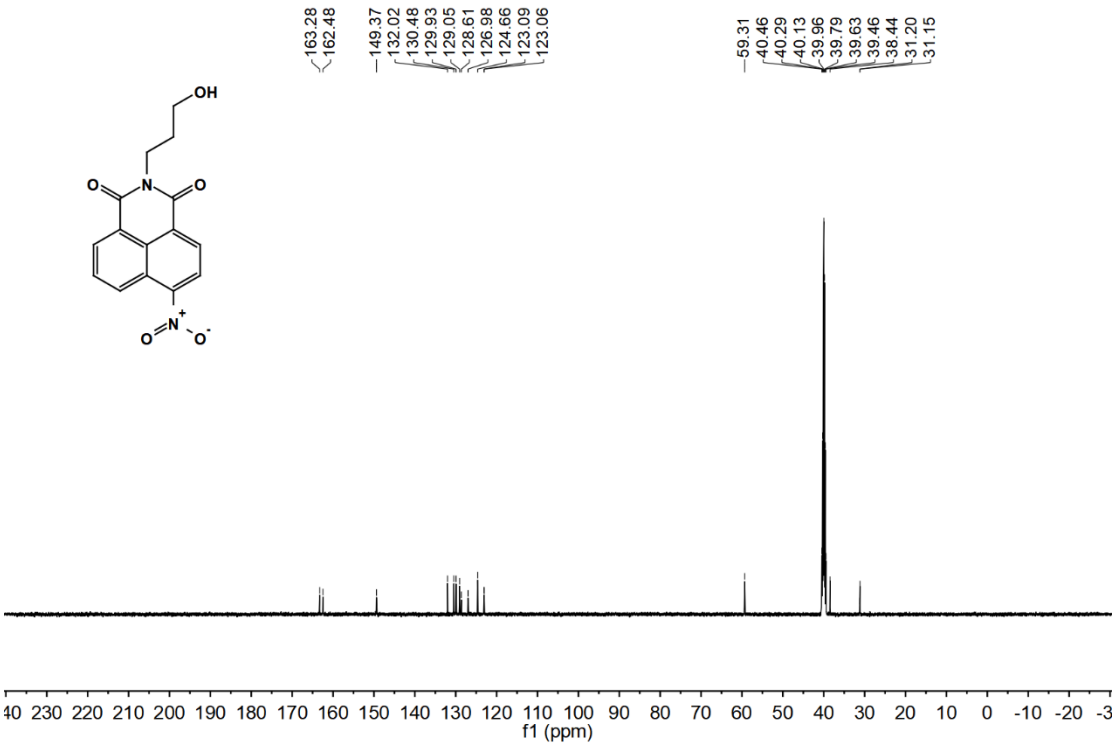

Fig. S2. [13]C NMR spectrum of **Compound 1**.

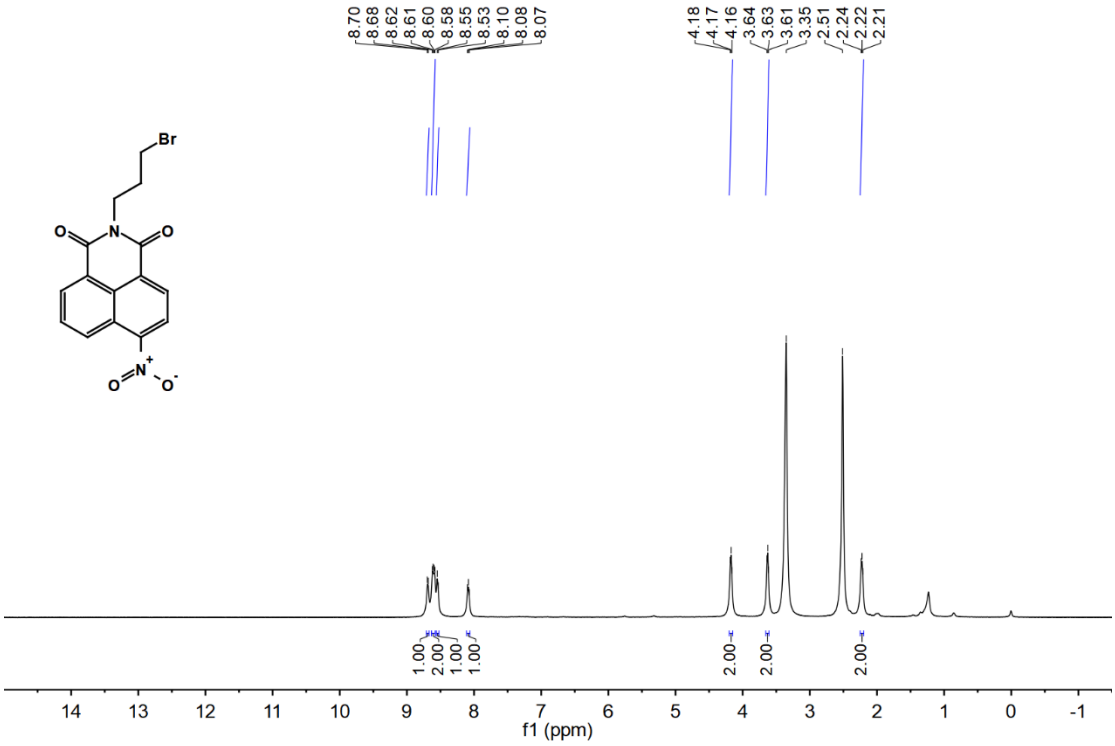

Fig. S3. [1]H NMR spectrum of **Compound 2**.

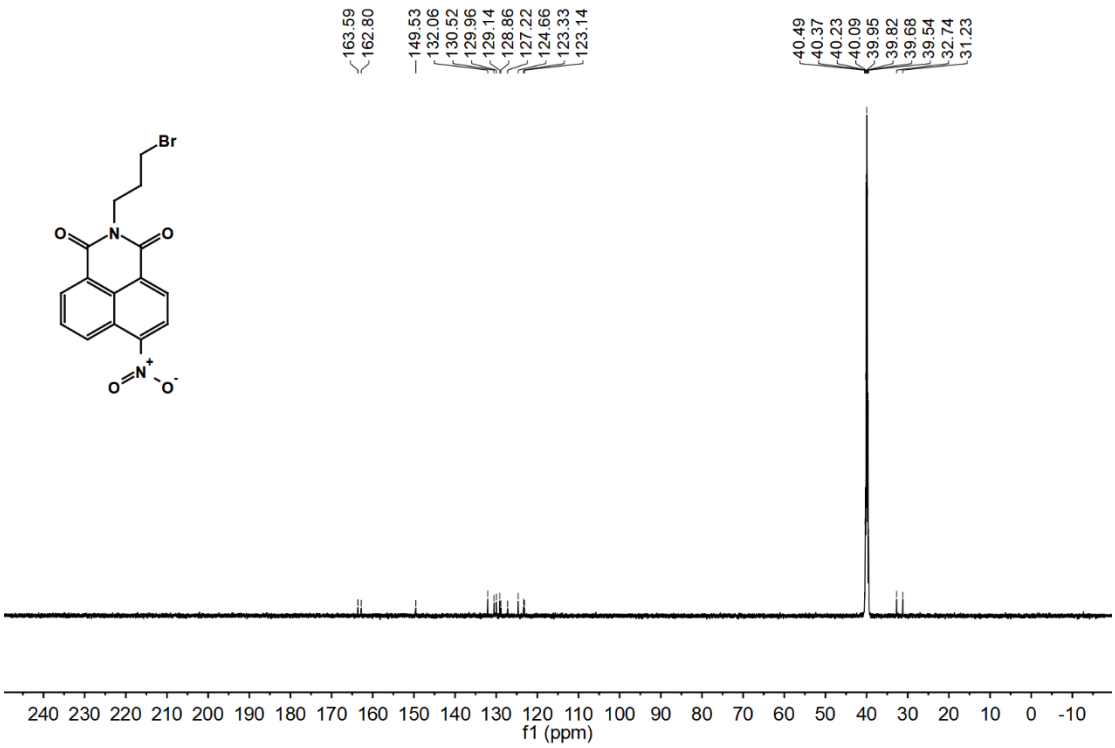

Fig. S4. [13]C NMR spectrum of **Compound 2**.

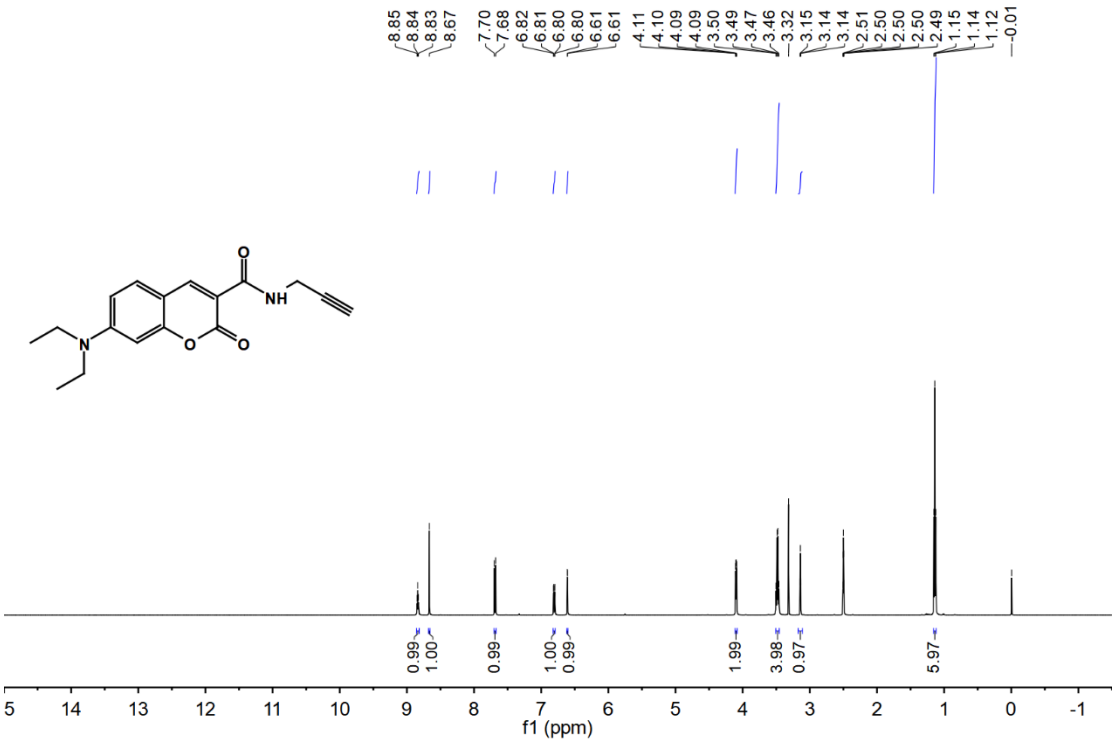

Fig. S5. ^{1}H NMR spectrum of **Compound 3**.

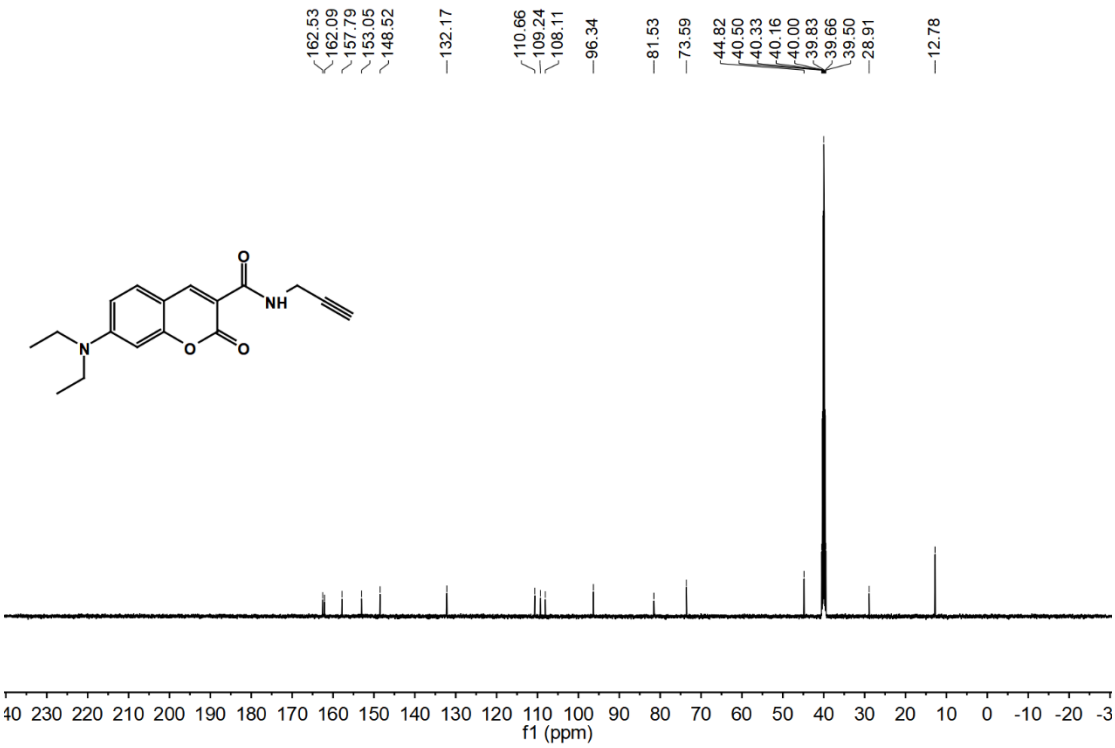

Fig. S6. ^{13}C NMR spectrum of **Compound 3**.

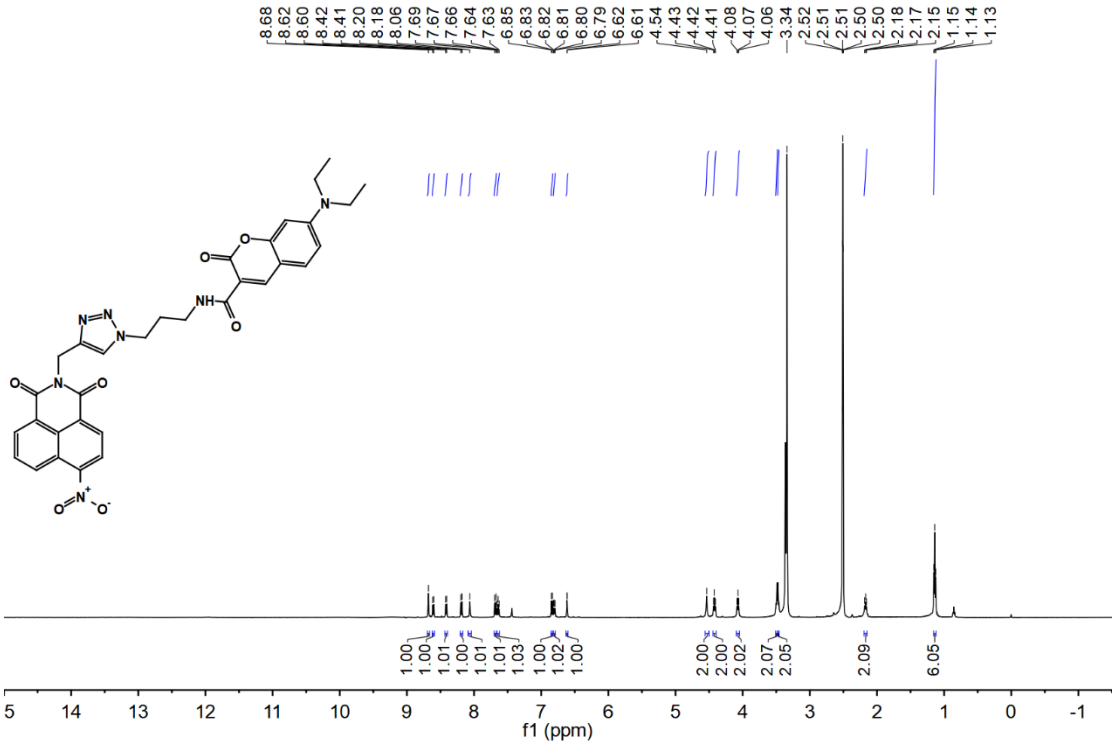

Fig. S7. [1]H NMR spectrum of **Compound NORP**.

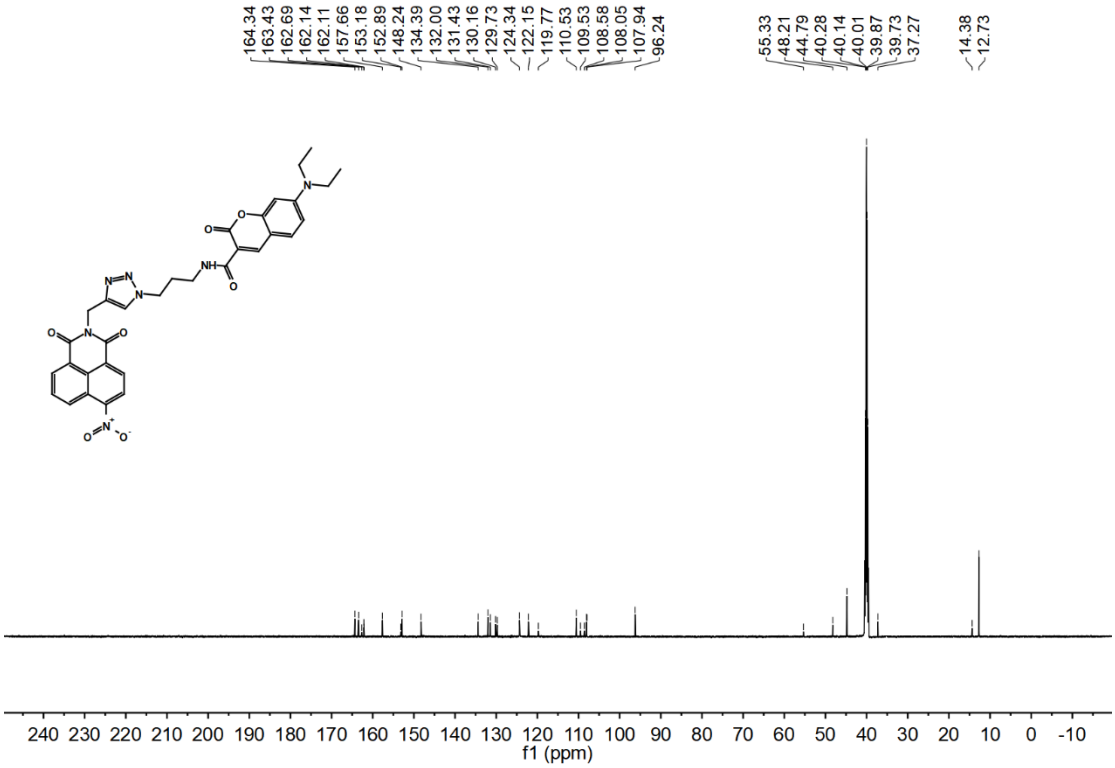

Fig. S8. [13]C NMR spectrum of **Compound NORP**.

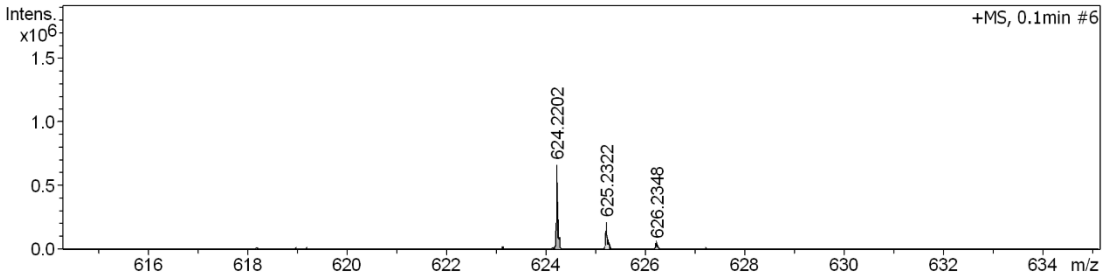

Fig. S9. HR-MS spectrum of **Compound NORP**.

3. Time and pH stabilities of probe NORP

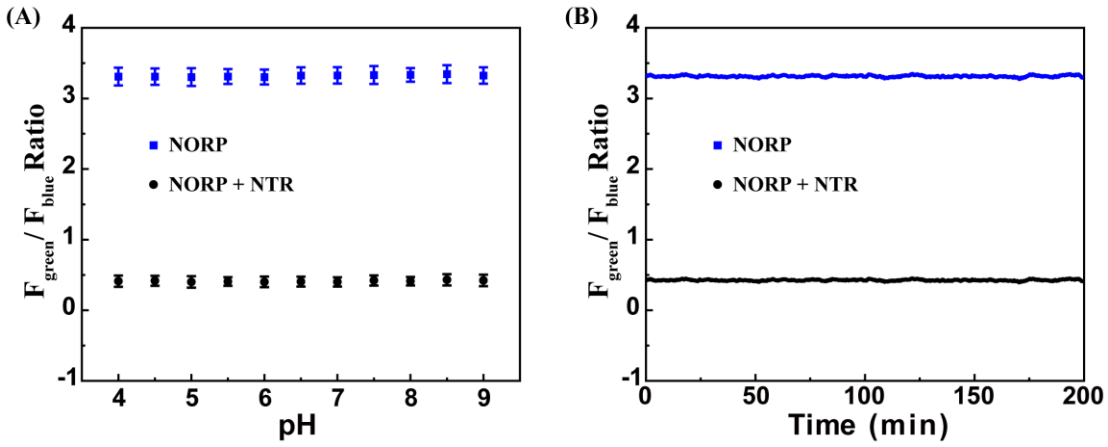

Fig. S10. (c) Time and (d) pH stabilities of probe **NORP** (5 μM) with (red curve) and without (black curve) NTR (10.0 μg mL^{-1}) (error bars, n = 5, S.D.).

4. Competition tests of probe NORP toward determination of NTR

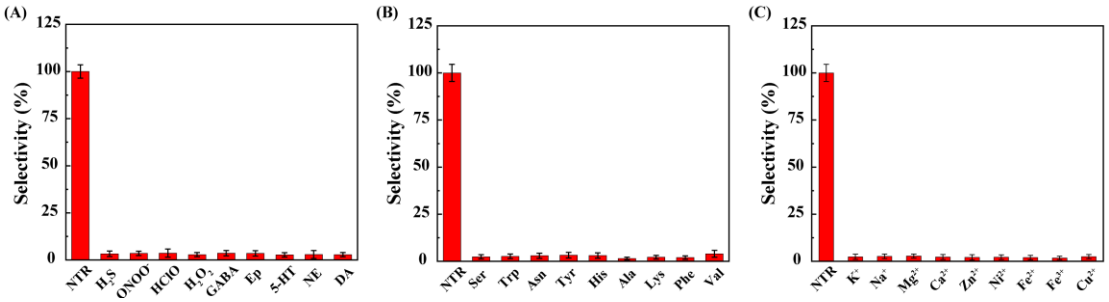

Fig. S11. Competition tests of 5.0 μM probe **NORP** toward NTR followed by addition of (A) ROS and other relative analyts (1.0 mM for each); (B) amino acids (1.0 mM for each); (C) metal ions (1.0 mM for each). (error bars, n = 5, S.D.).

5. MTT measurements of probe NORP in live HeLa cells

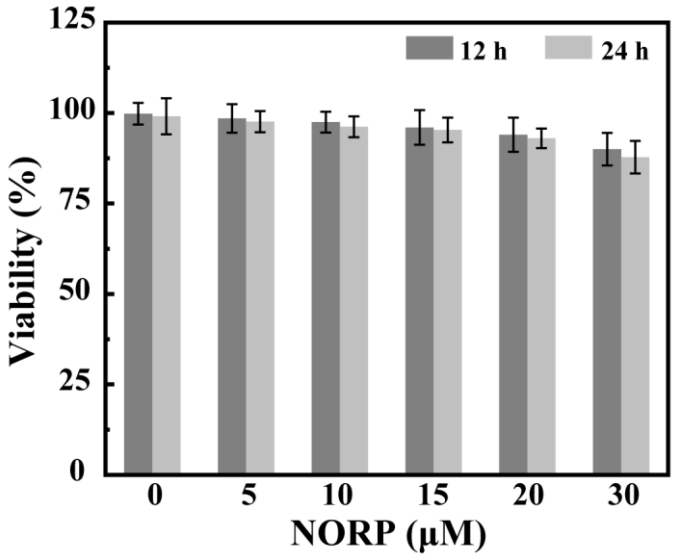

Fig. S12. The MTT assay for HeLa cells upon incubation with **NORP** at different concentrations (0, 5, 10, 15, 20, 25 and 30 μM) after 12 h and 24 h, respectively (error bars, n = 5, S.D.).

6. Confocal microscopy imaging of probe NORP in live HeLa cells

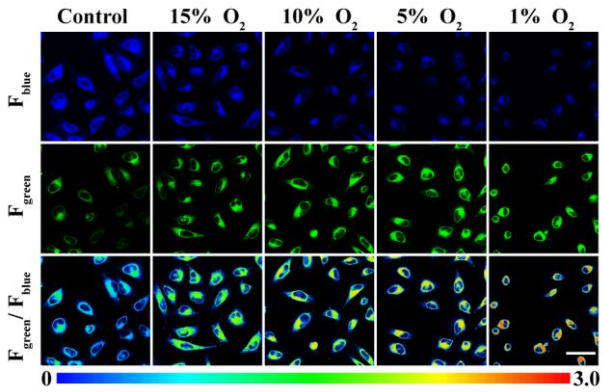

Fig. S13. Confocal microscopy imaging of **NORP** (5 μM) in HeLa cells under different O_2 concentrations (20%, 10%, 5%, 1%). Scale bar: 30 μm.

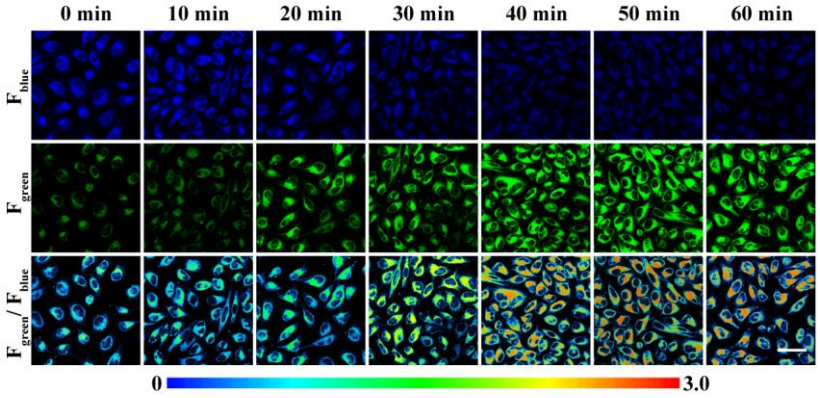

Fig. S14. Confocal microscopy imaging of probe **NORP** (5 μM) in HeLa cells under hypoxia stimulation (1% O_2) for different times (0, 10, 20, 30, 40, 50 and 60 min). Scale bar: 30 μm.