Supporting Information

A Turn-on Fluorescent Probe for Tumor Hypoxia Imaging in Living Cells

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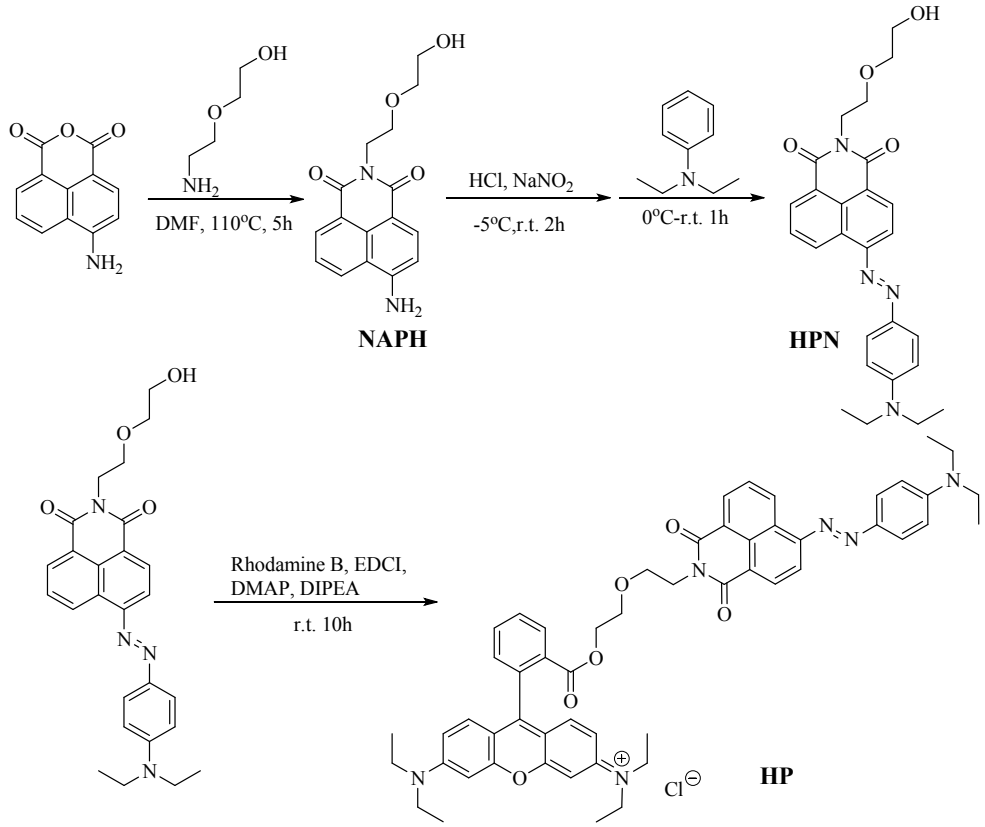
1. Materials and Instruments

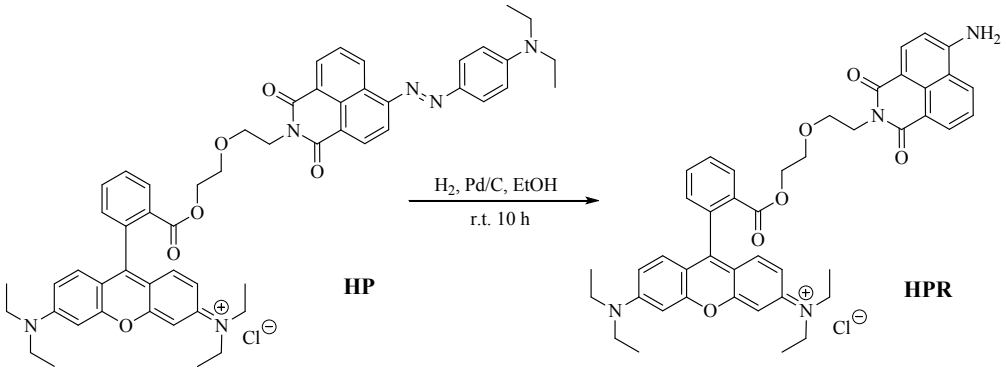
Silica gel G254 (Qingdao) was used for column chromatography. All chemicals were purchased from TCI and J&K reagent Co. Ltd. without further purification except otherwise stated. Cytochrome P450 reductase and NADPH were purchased from Sigma-Aldrich. All organic solvents were of analytical grade. Water was purified by a Milli-Q system.

^{1}H-NMR and ^{13}C-NMR spectra were collected in $CDCl_3$ and DMSO-d_6 at 25 ºC on a Bruker AV-400 spectrometer at NMR Facility of East China University of Science and Technology (ECUST), from which chemical shifts reported in ppm (TMS as internal standard). Mass spectral analyses were carried out at The Institute of Fine Chemicals of East China University of Science and Technology (ECUST) with mass spectrometer HP 5989A.Fluorescence imaging was performed with inverted fluorescence microscope (Nikon Eclipse Ti-E 2000).

2. Synthesis



Scheme S1 Synthesis of quencher **HPN** and probe **HP**



Scheme S2 Synthesis of probe reduction product **HPR**

N-(2-(2-hydroxyethoxy) ethyl)-4-amino-1,8-naphthalimide (NAPH)

4-nitroaniline (430 mg, 2.0 mmol) was dissolved in N, N-dimethyl-formamide (DMF, 4.0 mL), then 2-(2-aminoethoxy) ethanol (210mg, 2.0mmol) was added dropwise into the above solution. The action mixture was stirred at reflux for 5 h. After the reaction completed, the reaction solution was poured into potassium bisulfate solution (5%, m/m). After standing overnight the yellow precipitate was filtered and washed with cold water. The crude product was recrystallized in ethanol/water to give **NAPH** as yellow solid (415 mg, 69% yield, m.p. 226.1-227.4 ^{o}C). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.62 (d, 1H, J = 8.4 Hz), 8.43 (dd, 1H, J_1 = 6.4 Hz, J_2 = 8.0 Hz), 8.20 (d, 1H, J = 8.4 Hz), 7.66 (t, 1H, J = 8.0 Hz), 7.46 (s, 2H), 6.85(d, 1H, J = 8.4 Hz), 4.55-1.58 (m, 1H), 4.21 (t, 2H, J = 6.8 Hz), 3.62 (t, 2H, J = 6.8 Hz), 3.44-1.47 (m, 4H); HRMS (ESI) calcd. for $C_{16}H_{16}N_2O_4$ $[M+H]^+$ 301.1188, found 301.1190.

(E)-6-((4-(diethylamino)phenyl)diazenyl)-2-(2-(2-hydroxyethoxy)ethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (HPN)

NAPH (90mg, 0.3mmol) was dissolved in a mixture of concentrated hydrochloric acid (2.0 mL) and water (1.0 mL). After cooled in an ice-bath for 0.5 h, then sodium nitrite (30 mg, 0.4mmol) dissolved in water (0.5 mL) was added slowly. The action mixture was stirred at 0 ^{o}C for an additional 1h. Then a solution of N, N-diethylaniline (60 mg, 0.4 mmol) in Acetic Acid (0.5 mL) was added to the above reaction solution. The reaction mixture was stirred for 30 min at room temperature and then treated with saturated solution of sodium acetate. After the reaction completed, the black precipitate was filtered and washed with hot water. [S2] The crude product was purified by chromatography on a silica gel (DCM : MeOH, v/v, 100:1) to give **HPN** as a black solid (81 mg, 58% yield, m.p. 144.3-144.9^{o}C) . ^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.22 (dd, 1H, J_1 = 1.2 Hz, J_2 = 8.8 Hz), 8.62-8.66 (m, 2H), 8.02 (dd, 2H, J_1 = 2.0 Hz, J_2 = 7.2 Hz), 7.94 (d, 1H, J = 8.0 Hz), 7.81(dd, 1H, J_1 = 7.2 Hz, J_2 = 8.4 Hz), 6.78(dd, 2H, J_1 = 2.0 Hz, J_2 = 7.2 Hz), 4.47 (t, 2H, J = 5.6 Hz), 3.89 (t, 2H, J = 6.0 Hz), 3.68-3.71 (m, 4H), 3.52 (q, 4H, J = 7.2 Hz), 1.29 (t, 6H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 164.82, 164.36, 152.26, 151.39, 144.36, 134.10, 132.16, 131.54, 130.97, 129.32, 129.15, 126.96, 126.82, 126.64, 122.23, 121.48, 112.21, 111.23, 72.29, 68.52, 61.88, 44.98, 39.53, 12.71; HRMS (ESI) calcd. for $C_{26}H_{28}N_4O_4$ $[M+H]^+$ 461.2189, found 461.2177.

(E)-N-(6-(diethylamino)-9-(2-((2-(2-(6-((4-(diethylamino)phenyl)diazenyl)-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)ethoxy)ethoxy)carbonyl)phenyl)-3H-xanthen-3-ylidene)-N-ethylethanaminium chloride (HP)

HPN (80 mg, 0.17mmol) was dissolved in CH_2Cl_2 (10 mL) , then rhodamine B (100 mg, 0.2 mmol), 1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride(EDCI, 50 mg, 0.25 mmol), dimethylaminopyridine(DMAP,13 mg, 0.1mmol) and N,N-diisopropylethylamine (DIPEA, 150 μL) were added into the mixture solution respectively. The reaction mixture was stirred for 10 h at room temperature. After the reaction completed, the solvent was removed under vacuum. The dark purple oil was dissolved in AcOEt (50 mL) then washed with washed with water (2 × 50mL), and dried with anhydrous sodium sulfate. The organic solvent was removed under reduced pressure. The crude product was purified by chromatography on a silica gel (DCM : MeOH , v/v, 80:1) to give **HP** as dark purple solid (70 mg, 45% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.22 (t, 1H, J = 8.4 Hz), 8.50-8.57 (m, 2H), 8.18 (d, 1H, J = 8.0 Hz), 8.01 (dd, 2H, J_1 = 8.8 Hz, J_2 = 4.0 Hz), 7.90 (d, 1H, J = 8.0 Hz), 7.80 (t, 1H, J = 8.0 Hz), 7.75 (t, 1H, J = 7.6 Hz), 7.59 (t, 1H, J = 8.0 Hz), 7.26 (t, 1H, J = 8.4 Hz), 6.98 (dd, 2H, J_1 = 8.4 Hz, J_2 = 1.6 Hz), 6.78-6.81 (m, 6H), 4.36 (t, 2H, J = 6.4 Hz), 4.15 (br, 2H), 3.70 (t, 2H, J = 6.0 Hz), 3.50-3.60 (m, 14H), 1.26-1.31 (m, 18H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 164.96, 164.42, 164.01, 158.55, 157.76, 155.48, 152.28, 151.62, 144.27, 133.49. 133.00, 131.89, 131.37, 131.22, 131.05, 130.30, 130.12, 129.71, 129.21, 129.14, 127.02, 126.95, 126.70, 122.09, 121.18, 114.02, 113.48, 112.05, 111.29, 96.28, 96.25, 68.15, 67.78, 64.61, 46.00, 45.02, 38.79, 12.71, 12.54; HRMS (ESI) calcd. for $C_{54}H_{57}N_6O_6^+$ $[M\text{-}Cl]^+$ 885.4334, found 885.4304.

N-(9-(2-((2-(2-(6-amino-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)ethoxy)ethoxy)carbonyl)phenyl)-6-(diethylamino)-3H-xanthen-3-ylidene)-N-ethylethanaminium chloride (HPR)

HP (46 mg, 50μmol) and Pd/C (palladium on charcoal, 10 wt %, water 55%, 10 mg) were dissolved in ethanol (10 mL). The mixture reaction was stirred under hydrogen over night at room temperature. After the reaction completed, the solvent was removed under reduced pressure. The crude product was purified by chromatography on a silica gel (DCM : MeOH , v/v, 60:1) to give **HPR** as red solid (32 mg, 83% yield).^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.39 (d, 1H, J = 7.2 Hz), 8.18 (d, 2H, J = 8.4 Hz), 8.12 (d, 1H, J = 8.0 Hz), 7.76 (t, 1H, J = 7.6 Hz), 7.49-7.56 (m, 2H), 7.21 (d, 1H, J = 7.6 Hz), 6.91 (d, 1H, J = 6.4 Hz), 6.71-6.80 (m, 5H), 4.29 (t, 2H, J = 5.6 Hz), 4.12 (t, 2H, J = 4.4 Hz), 3.66 (t, 2H, J = 6.0 Hz), 3.52-3.57 (m, 10H), 1.26 (t, 12H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 165.05, 164.70, 163.92, 158.84, 157.71, 155.44, 150.80, 133.98, 133.32. 132.97, 131.30, 131.20, 130.32, 130.05, 129.78, 129.72, 128.15, 124.54, 122.18, 119.79, 113.99, 113.46, 109.99, 109.22, 96.17, 68.05, 67.89, 64.62, 45.96, 38.44, 12.53; HRMS (ESI) calcd.for $C_{44}H_{45}N_4O_6^+$ $[M\text{-}Cl]^+$ 725.3334, found 725.3340.

3. Method

3.1 Spectroscopic Materials and Methods

All pH measurements were made with a Sartorius basic pH-Meter PB-20. Fluorescence spectra were determined using a Varian Cary Eclipse fluorescence spectrometer. Absorption spectra were determined by a Varian Cary 100 UV-vis spectrophotometer. Milli-Q Water was used to prepare all aqueous solutions. Cytochrome P450 Reductase was purchased from Sigma. Other chemical reagents were of analytical grade and commercially available.

3.2 Cyclic Voltammetry Potential

Cyclic voltammetry potential was examined in an electrochemical analyzer, which contained three separate-electrodes. We selected the Ag/Ag+ electrode as a reference electrode, the Pt wire as an auxiliary electrode, the glassy carbon electrode as a working electrode and 5 mM potassium hexacyanoferrate(III) as the reference solution. After bubbling pure nitrogen for 30 min, 100 mM tetrabutylammonium perchlorate in 5 mL acetonitrile was chosen as the bare solution, then 5 mmol **HP** was added.

3.3 Determination of Quantum Yield

The fluorescent quantum yield was calculated using rhodamine B as a standard (Φ_F = 0.65 in EtOH)[S3] by the following equation:

$$\Phi_{(sample)} = \Phi_{(standard)} \times m_{(sample)} / m_{(standard)} \times n^2_{(sample)} / n^2_{(standard)}$$

In which Φ is quantum yield; m is the slope of integrated fluorescent intensity and corresponding UV absorbance; n is refractive index of test solvent[S4].Ex: 530 nm; Slit: 2.5 nm, 5 nm; Intergration scope: 550 nm-600 nm.

3.4 Chemical reduction assay

Chemical reduction was created by adding hydrochloric acid (1 N) and stannous chloride (100 μM) in ethanol solution of **HP**. The concentration of **HP** was 10 μM with reaction time 10 minutes. The reaction solution was extracted with water and dichloromethane, the organic layer was collected and dried over anhydrous sodium sulfate and evaporated. Then the crude product was dissolved in the same volume of ethanol to test.

3.5 Cytochrome P450 reductase assay

Cytochrome P450 reductase (Rabbit liver, purchased from Sigma-Aldrich) experiments [S5] were performed in 1 cm×1 cm quartz cuvettes. Stock solutions of probe **HP** was prepared in pure DMSO (1 mM). Experimental system is PBS buffer (0.1 mM, pH=7.0) in 37°C water bath. The concentration of **HP** in solution is 10 μM. The dosage of Cytochrome P450 reductase is 1 U/mL and NADPH is 100 μM. Argon gas was bubbled into the **HP** solution for one hour to create the hypoxic environment. The emission intensity was collected from 540 nm to 700 nm with excitation at 530 nm (The fluorescence intensity in trend figure (Fig. 2 b) was collected at 581 nm) [S6].

3.6 HPLC for Probe HP with Cytochrome P450 Reductase

HPLC was performed on a ZoRBAX RX-C18 column (Analytical 4.6×250 mm 5-Micron, Agilent) with a **HP** 1100 system. The HPLC solvents employed were 15% acetonitrile and 85% methanol. HPLC conditions were as follows: solvent A: solvent B = 0:100 (0 min)-100:0 (20 min), flow rate 1 mL/min, detection by UV/Vis (254 nm and 560 nm). The reaction solution was extracted with water and dichloromethane, the organic layer was collected and dried over anhydrous sodium sulfate and evaporated. The crude product was analyzed by HPLC.

3.7 Cell Viability Assays

Cell proliferation was evaluated by MTT assay. HeLa cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated overnight, and then treated with **HP** and **HPR** at different concentration for 48 h. After that, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated for another 4 h. Then abandon the supernatant and redissolved in 100 μL of DMSO for each well. Absorbance values were measured at 490 nm with Bio Tek Synergy 2 multifunction microplatereader (USA). The cell viability was calculate according to the following equation: Cell viability (%) = $A_{490\ (sample)}/A_{490\ (control)} \times 100\%$.

3.8 Cell Culture and Imaging

HeLa cells were obtained from American Type Culture collection and were grown in RPMI-

1640 (Hyclone) supplemented with 10% FBS (Gibco), 2 mM L-Glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were incubated in a 5% CO_2 humidified incubator at 37 °C and typically passaged with sub-cultivation ratio of 1:4 every two days. Hela cells were seeded in 24-well plates in culture medium. For fluorescence microscopy, the cells were incubated under normoxic (20% pO_2) and hypoxic (10%, 5%, 3%, and 1% pO_2) condition for 6 h at 37 °C. Then the cells were washed with PBS buffer (pH 7.4) and treated with 2 μM **HP** in FBS-free RPMI-1640 for 0.5 h. Before taken images, Hela cells were washed three times with PBS buffer before imaging by inverted fluorescence microscope (Nikon Eclipse Ti-E 2000). The fluorescent field was collected with 100 ms exposure time using a Texas Red filter and with 300 ms exposure time for bright field.

4. Figures

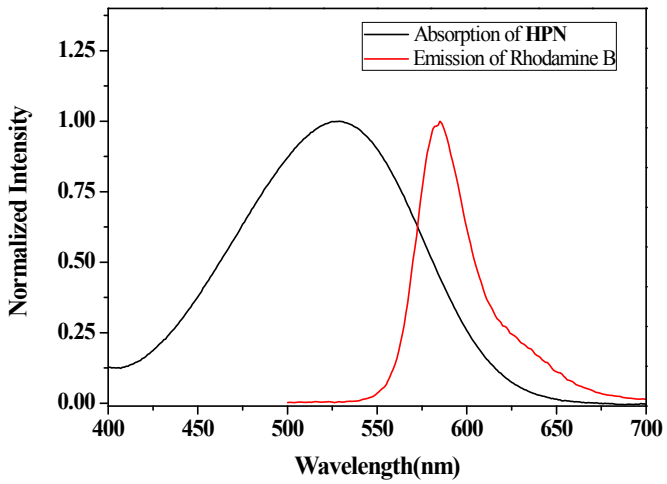


Fig. S1 Normalized absorption of **HPN** and normalized emission of rhodamine B

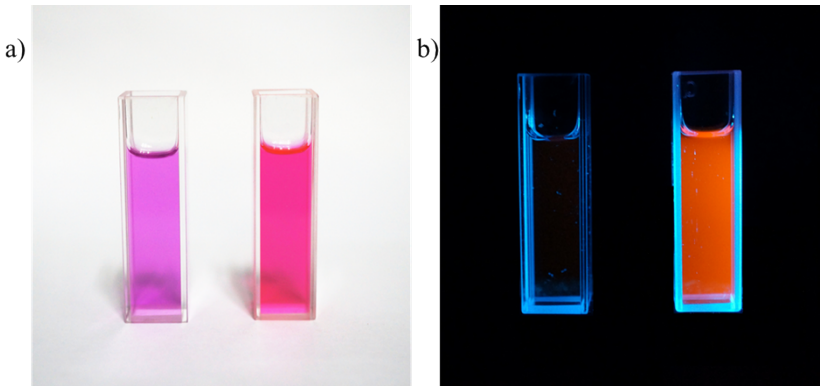


Fig. S2 The bright field and fluorescence changes of probe **HP** before/after the chemical reduction reaction. (a) Bright field, left: before reduction, right: after reduction; b) fluorescence, left: before reduction, right: after reduction; Concentration: 10 μM; Ex: 365 nm; Solvent: Ethanol.

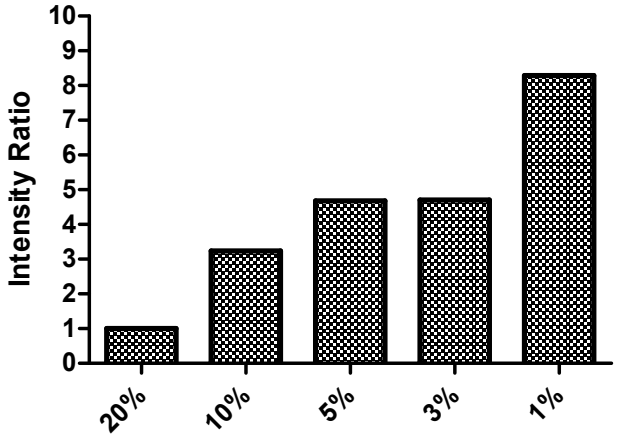


Fig. S3 Fluorescence difference of probe **HP** under different oxygen concentration. HeLa cells were incubated with **HP** (5 μM) for 30 min under different conditions. Cells were cultured under hypoxic condition or normoxic condition for 6 h firstly, then treated with **HP**. The concentration of DMSO in the culture medium is 1%. Filter type is Tex-Red (Ex: 540-560 nm, Em: 570-600 nm). Processing software: Imagine J.

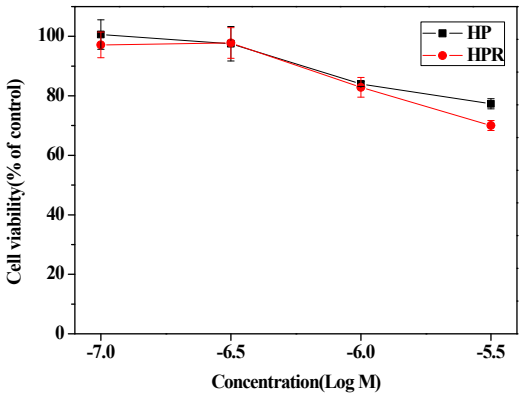
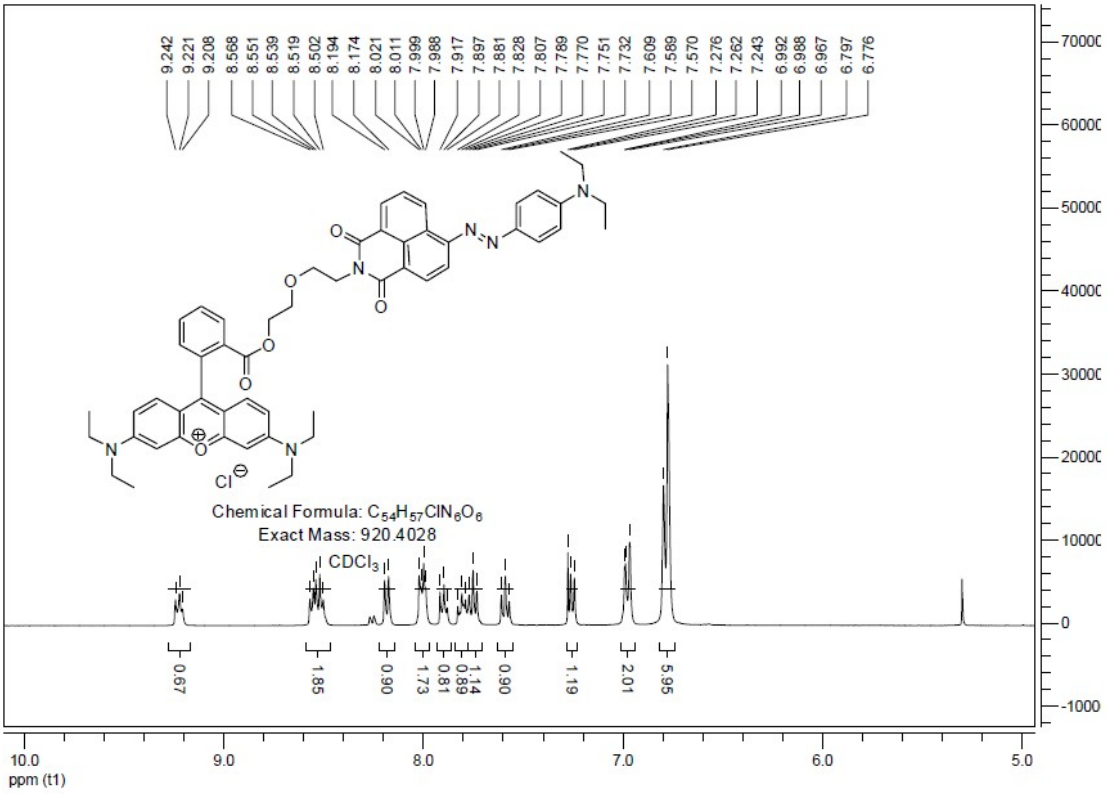
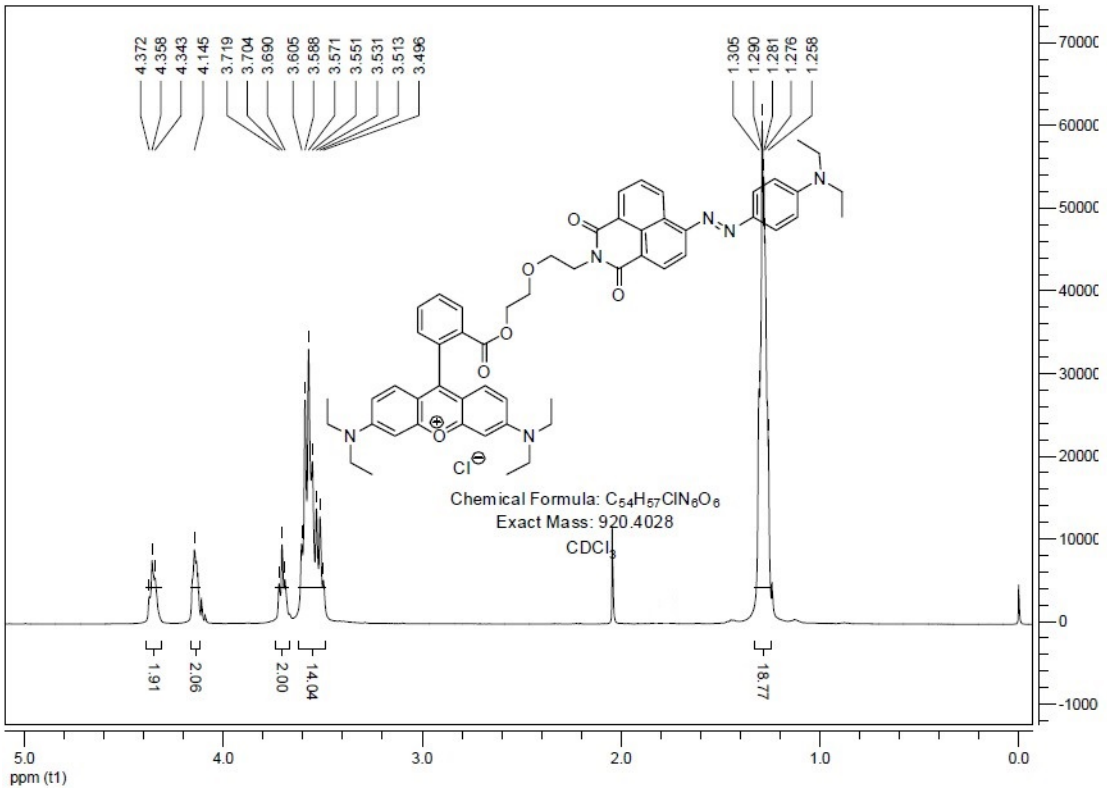


Fig. S4 The MTT test of hypoxia probe **HP** and reduction product **HPR**.

5. NMR spectra





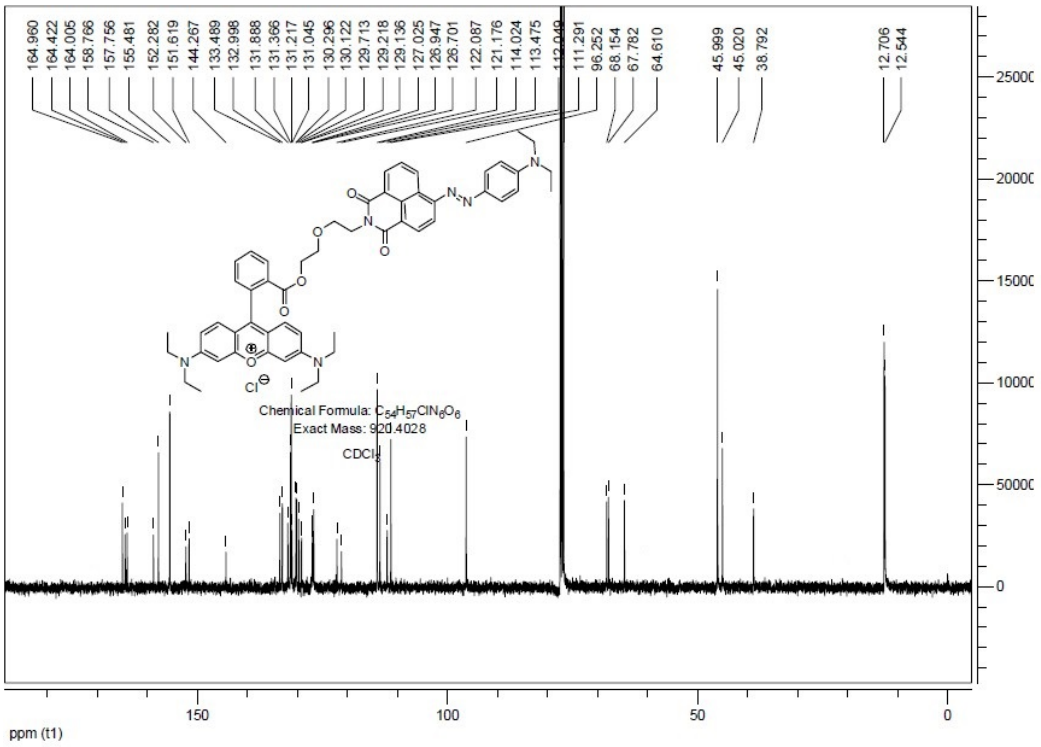


Fig. S5 The ^{1}H-NMR and ^{13}C-NMR spectrum of **HP**

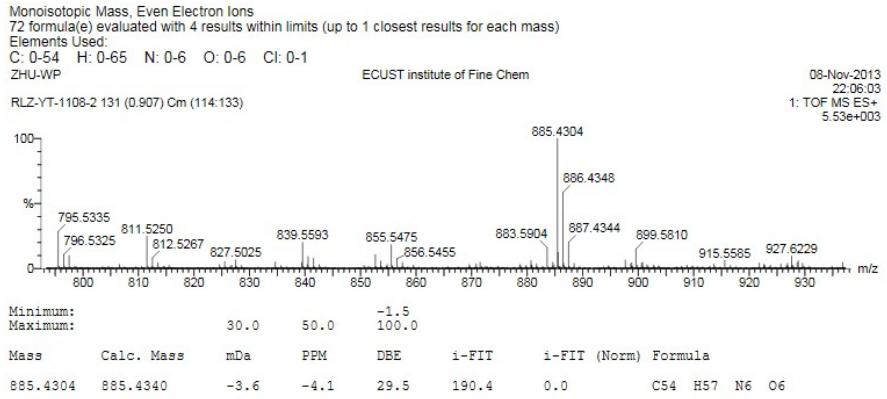


Fig. S6 The HR-MS spectrum of **HP**

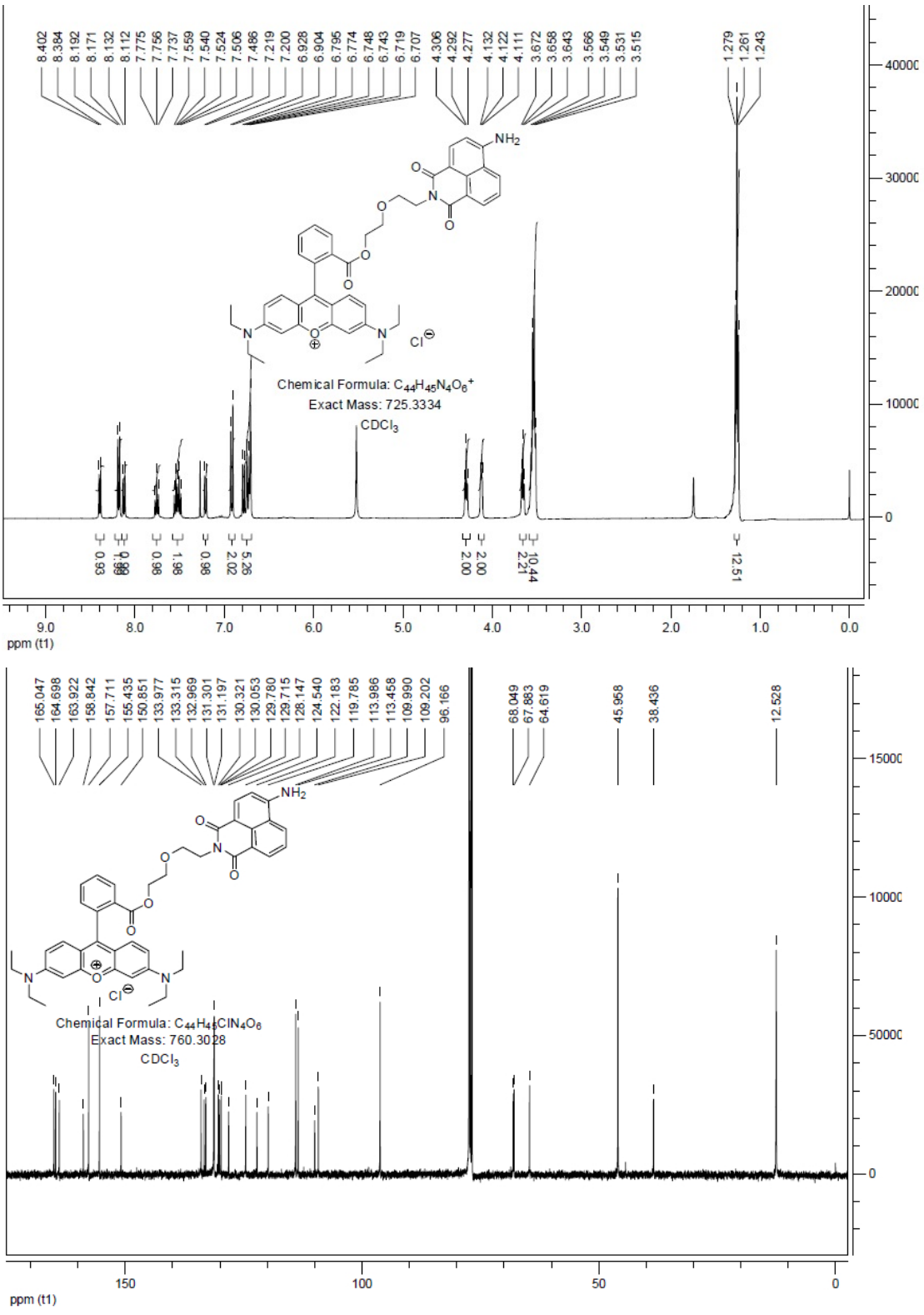


Fig. S7 The ^{1}H-NMR and ^{13}C-NMR spectrum of **HPR**

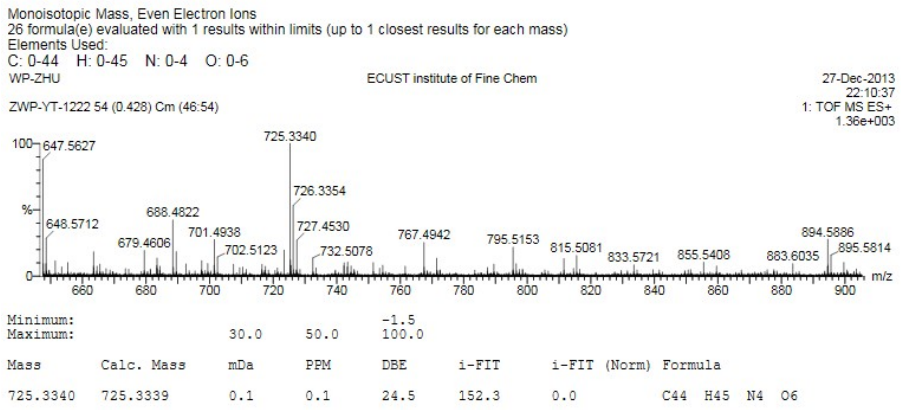


Fig. S8 The HR-MS spectrum of **HPR**

6. References

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