

Supporting Information

A nitroreductase responsive and photoactivated fluorescent probe for dual-controlled tumor hypoxia imaging

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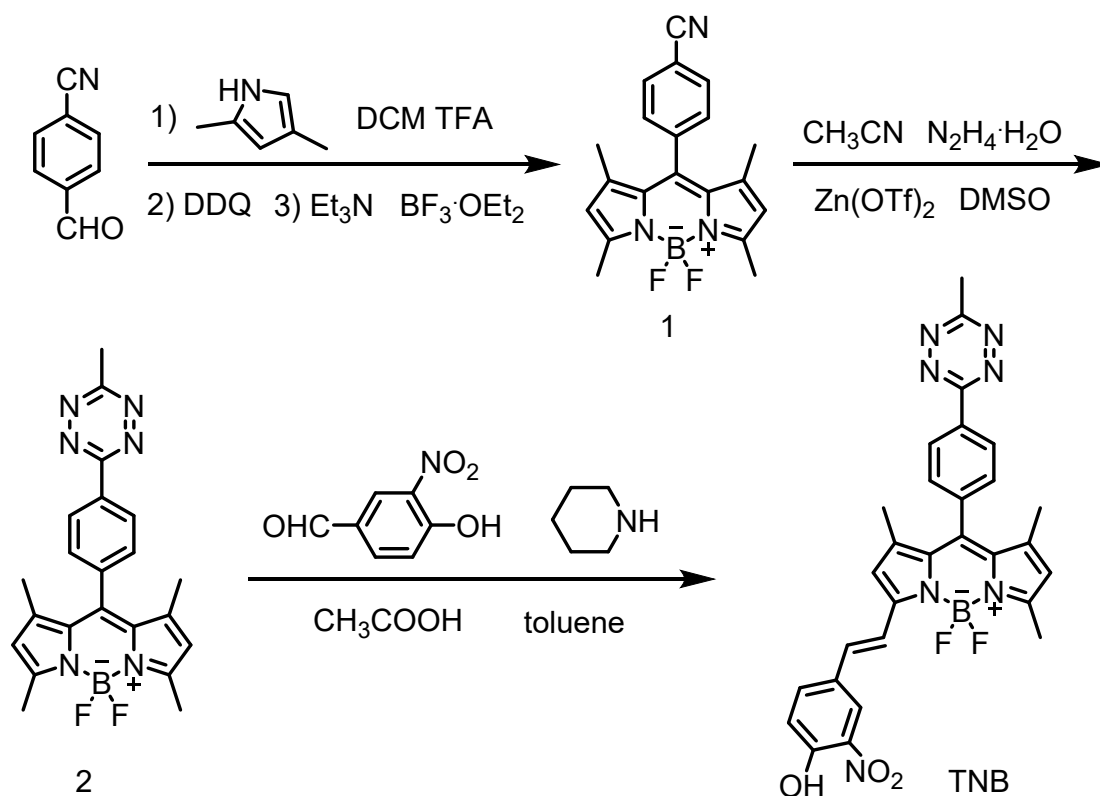
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1 Materials and Apparatus

Nitroreductase purified from *Escherichia coli* was purchased from Sigma-Aldrich. ^1H NMR and ^{13}C NMR spectra were measured on Bruker AVANCE III 500 NMR spectrometer and Bruker AVANCE III 400 NMR spectrometer, internally referenced to the residual proton signals of deuterated solvents. ESI-MS spectra were recorded on a Mariner System 5304 Mass spectrometer and AB SCIEX API 3000. Absorption and Fluorescence spectra were measured on SpectraMax M3. BODIPY dyes were photoactivated using OmniCure S2000 at 400-500 nm with 30% intensity and fluorescence imaging was performed on OLYMPUS FV3000 confocal microscope 405 nm light at 100% laser intensity. All reagents were purchased from commercial suppliers and used without further purification unless otherwise indicated.

2 Synthesis and Characterization



Scheme S1. Synthesis route of probe TNB.

Synthesis of compound 1. 4-Cyanobenzaldehyde (2.0 g, 15.25 mmol) and 2,4-dimethylpyrrole (2.9 g, 30.50 mmol) were dissolved in dichloromethane (300 mL) under nitrogen in a 1000 mL flask, and 0.1 mL of TFA were added and stirred at room temperature for 12 h. 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (4.2 g, 18.30 mmol) dissolved in 300 mL of dichloromethane was added to the reaction stirred for 30 min at room temperature, followed by Et₃N (21.2 mL, 150.52 mmol) and stirred for another 15 min. The mixture was cooled in an ice bath, and BF₃·Et₂O (19.2 mL, 152.52 mmol) was added dropwise while keeping the solution temperature below 10°C during the dropping process. The reaction mixture was stirred in an ice-water bath for 12 h. The mixture was diluted with dichloromethane than washed with a half-saturated aqueous sodium chloride solution. Through collecting the organic

phase and drying it with anhydrous Na_2SO_4 , evaporating the solvent dichloromethane under reduced pressure, and further separating the crude product by 200-300 mesh silica gel column chromatography (dichloromethane: Ethyl acetate = 100:20), an orange solid, compound 1 (1.98 g, yield: 37.4%) was finally obtained. ^1H NMR (500 MHz, Chloroform- d) δ 7.81 (d, J = 8.2 Hz, 2H), 7.45 (d, J = 8.2 Hz, 2H), 6.00 (s, 2H), 2.55 (s, 6H), 1.35 (s, 6H). ^{13}C NMR (101 MHz, Chloroform- d) δ 156.66, 142.69, 140.15, 138.78, 133.01, 130.82, 129.45, 121.93, 121.90, 118.23, 113.36, 14.73. MS: calc. M^+ = 349.1562, obsvd. HR-MS: $(M+H)^+$ = 350.1551.

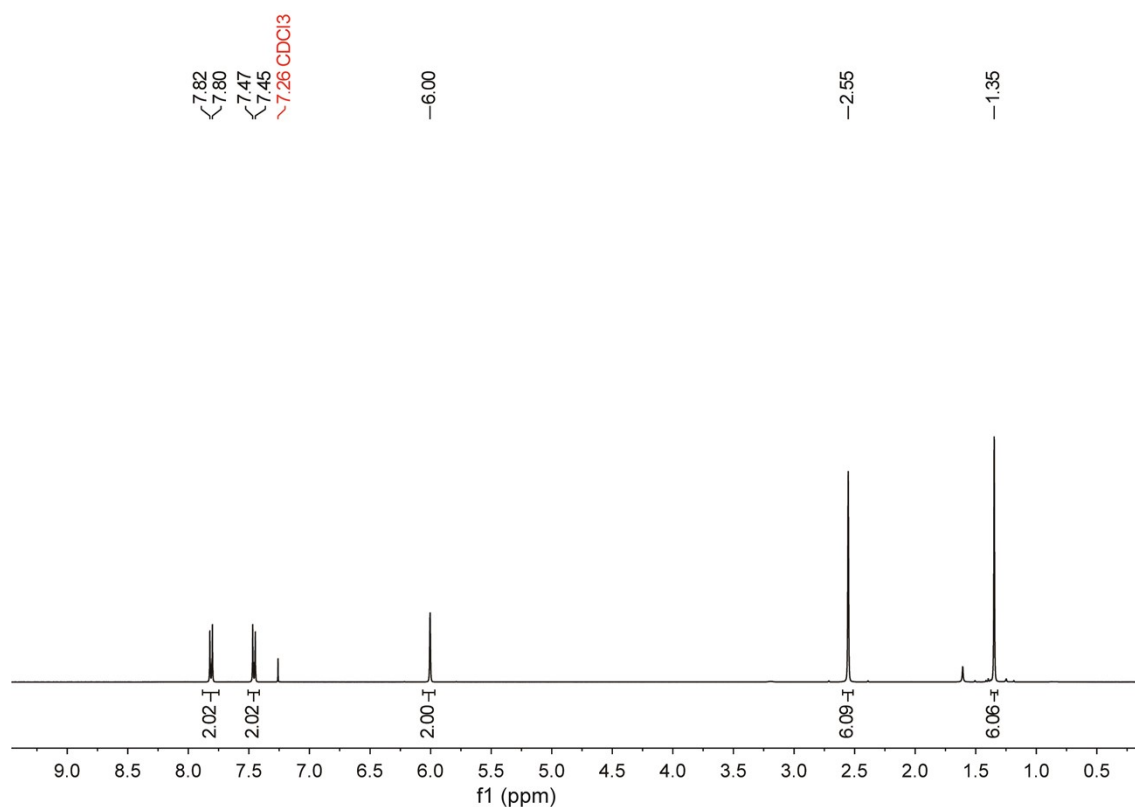


Figure S1 ^1H NMR spectra of compound 1.

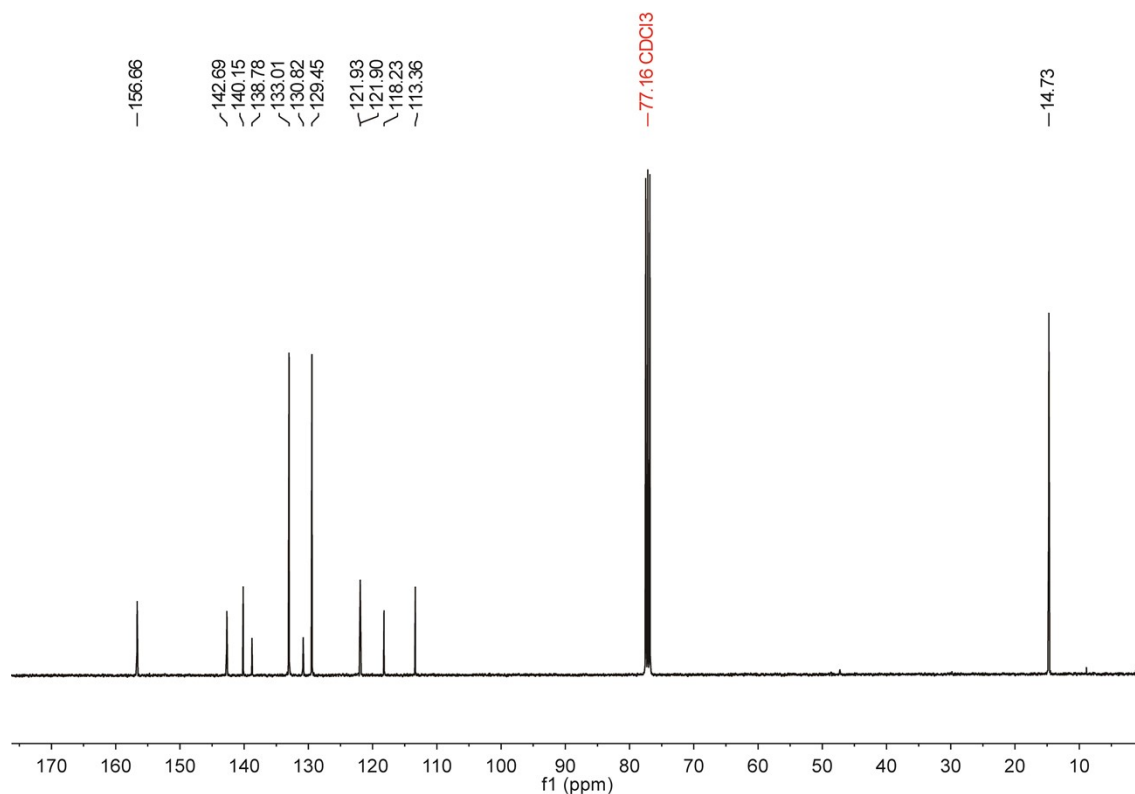


Figure S2 ¹³C NMR spectra of compound 1.

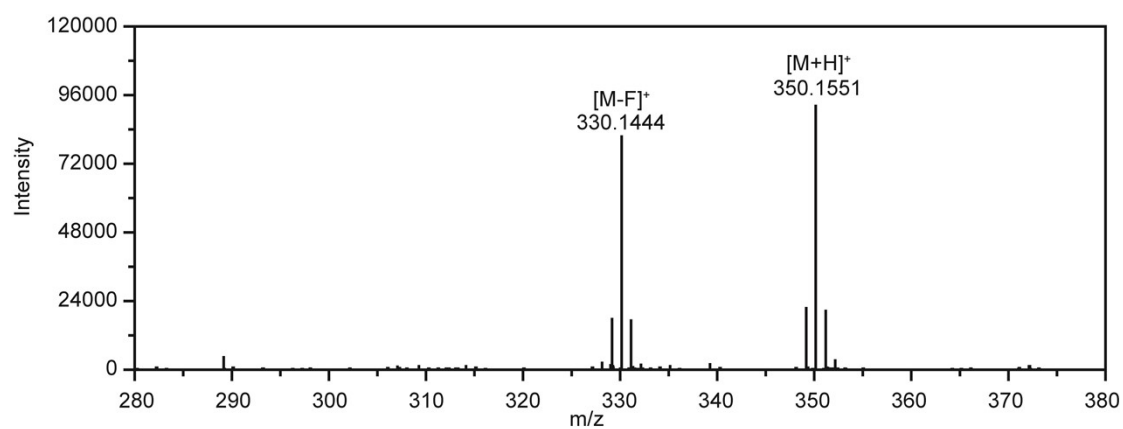


Figure S3 ESI mass spectrum of compound 1.

Synthesis of compound 2. Compound 1 (0.8 g, 2.29 mmol), DMSO (22 mL), Zn(OTf)₂ (0.42 g, 1.15 mmol) were loaded into a sealed tube, added with acetonitrile (1.2 mL, 22.91 mmol) and 1.8 mL of 80% hydrazine monohydrate. The reaction was stirred for 36 h at 60°C, then stopped and cooled to 0-10°C in the ice bath, and 0.6 g/mL sodium nitrite aqueous solution was added and the pH was adjusted to 3 with

1M HCl (during this period there were lots of nitrogen oxides generated, pay attention to protect and carry it out in a fume hood). The mixture was extracted with dichloromethane, dried over anhydrous Na_2SO_4 , further evaporating the solvent under reduced pressure, and then purified by column chromatography (dichloromethane: petroleum ether = 100:80). Compound 2 (0.28 g, yield: 28.7%) was obtained as a red solid. ^1H NMR (500 MHz, Chloroform- d) δ 8.75 (d, J = 8.3 Hz, 2H), 7.56 (d, J = 8.3 Hz, 2H), 6.01 (s, 2H), 3.13 (s, 3H), 2.57 (s, 6H), 1.45 (s, 6H). ^{13}C NMR (101 MHz, Chloroform- d) δ 167.72, 156.18, 143.01, 140.30, 139.62, 132.69, 131.13, 129.37, 128.71, 121.67, 121.63, 21.35, 14.78. MS: calc. M^+ = 418.1889, obsvd. HR-MS: $(M+H)^+ = 419.1848$.

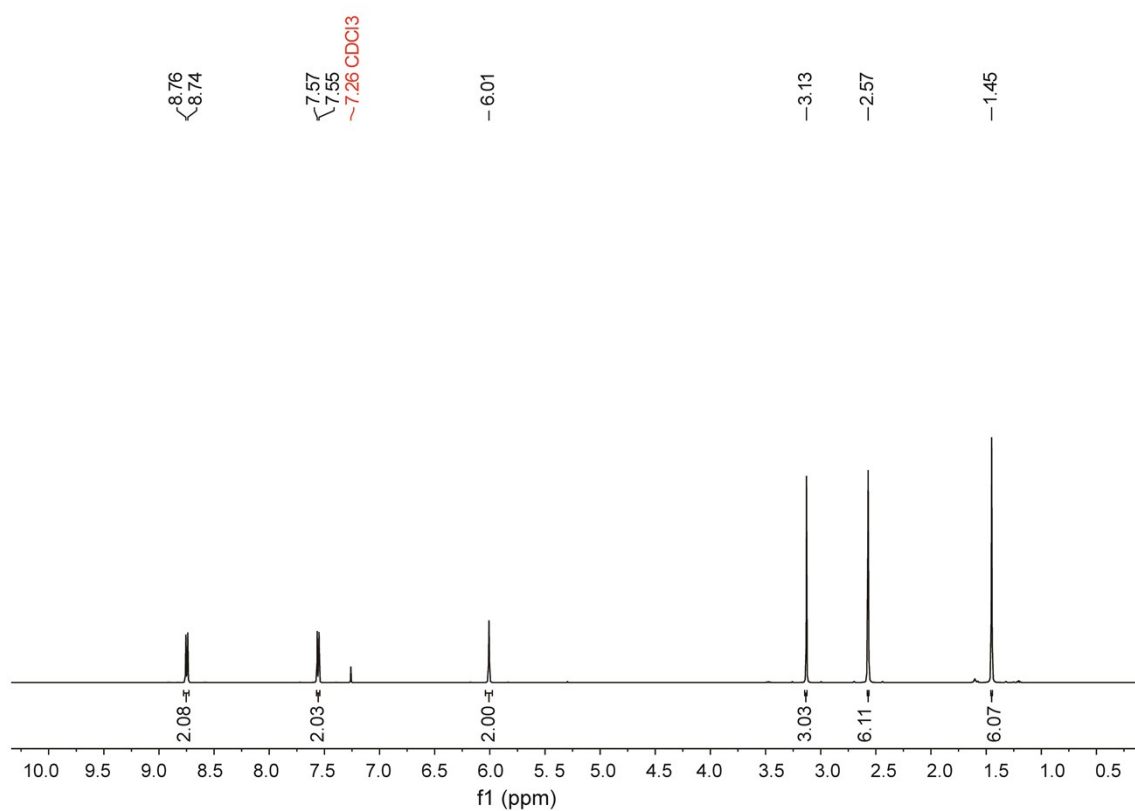


Figure S4 ^1H NMR spectra of compound 2.

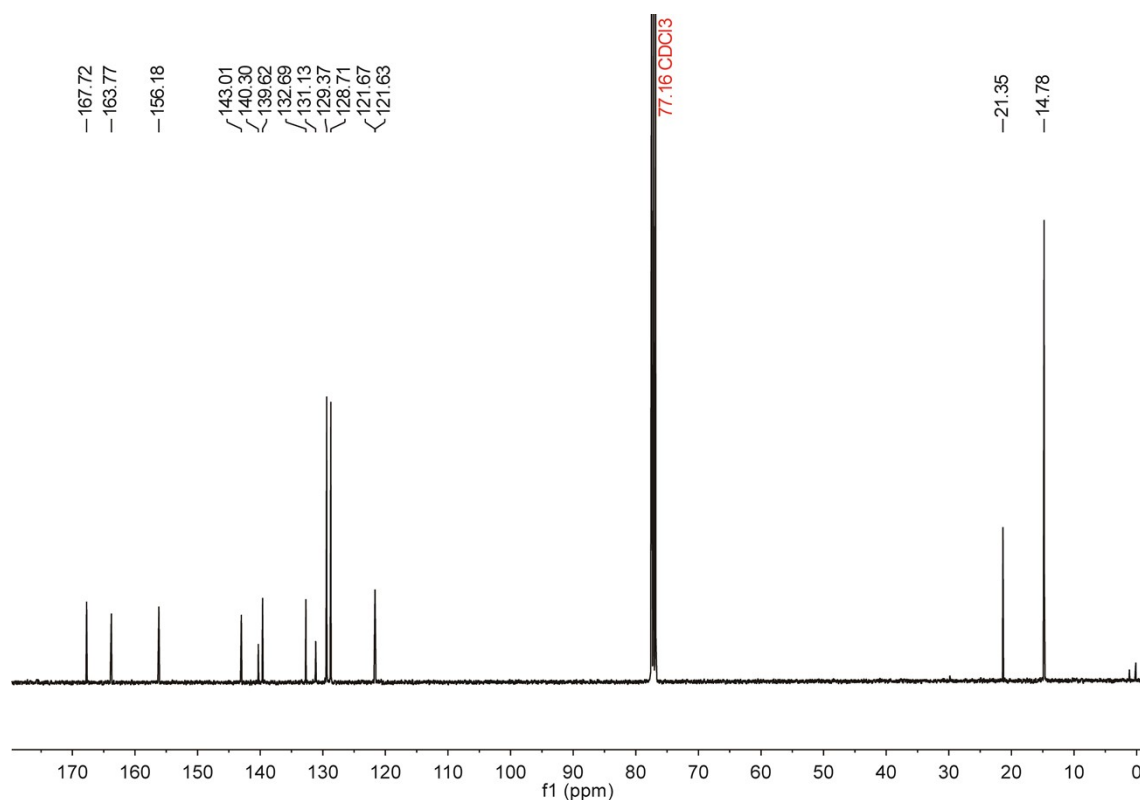


Figure S5 ^{13}C NMR spectra of compound 2.

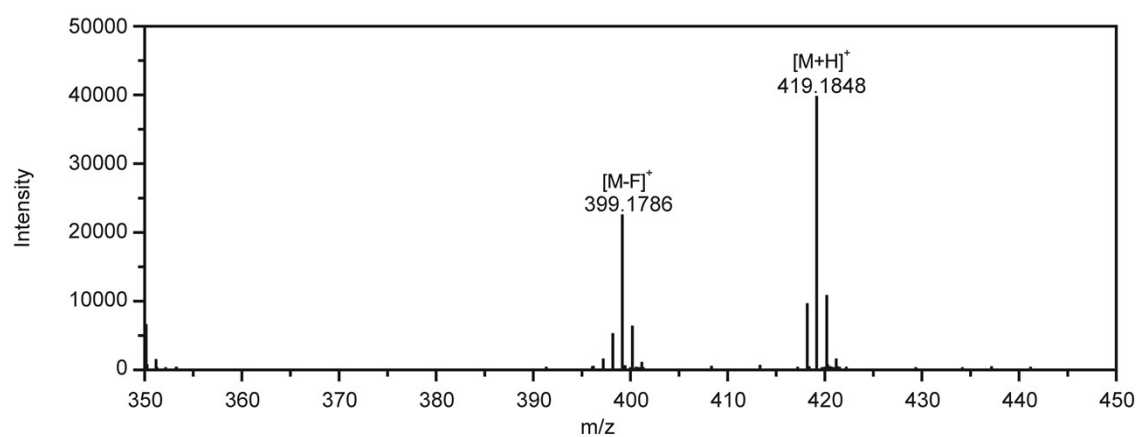


Figure S6 ESI mass spectrum of compound 2.

Synthesis of probe TNB. A solution of compound **2** (0.45 g, 1.08 mmol) in toluene (20 mL) was loaded into a Schlenk tube, adding 3-nitro-4-phenol hydroxybenzaldehyde (0.22 g, 1.29 mmol), 0.6 mL of pyridine and 0.6 mL of acetic acid. The reaction was stirred for 5 h at 110°C, then stopped, and the solvent was removed under reduced pressure. The residue was purified by column

chromatography (dichloromethane:petroleum ether = 100:50) to afford compound TNB (91 mg, yield: 14.9%). ^1H NMR (500 MHz, Chloroform-*d*) δ 10.70 (s, 1H), 8.77 (d, J = 7.6 Hz, 2H), 8.17 (s, 1H), 7.94 (d, J = 8.6 Hz, 1H), 7.62 (s, 1H), 7.59 (d, J = 7.0 Hz, 2H), 7.17 (dd, J = 22.6, 12.4 Hz, 2H), 6.60 (s, 1H), 6.07 (s, 1H), 3.14 (s, 3H), 2.62 (s, 3H), 1.51 (s, 3H), 1.48 (s, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.77, 157.45, 155.41, 151.67, 143.75, 142.01, 139.51, 139.47, 135.31, 133.73, 132.85, 132.72, 132.43, 132.04, 129.72, 129.51, 128.77, 124.17, 122.34, 120.76, 120.00, 117.78, 21.39, 15.00, 14.93. MS: calc. M^+ = 567.2002, obsvd. HR-MS: $(M+H)^+ = 568.1880$.

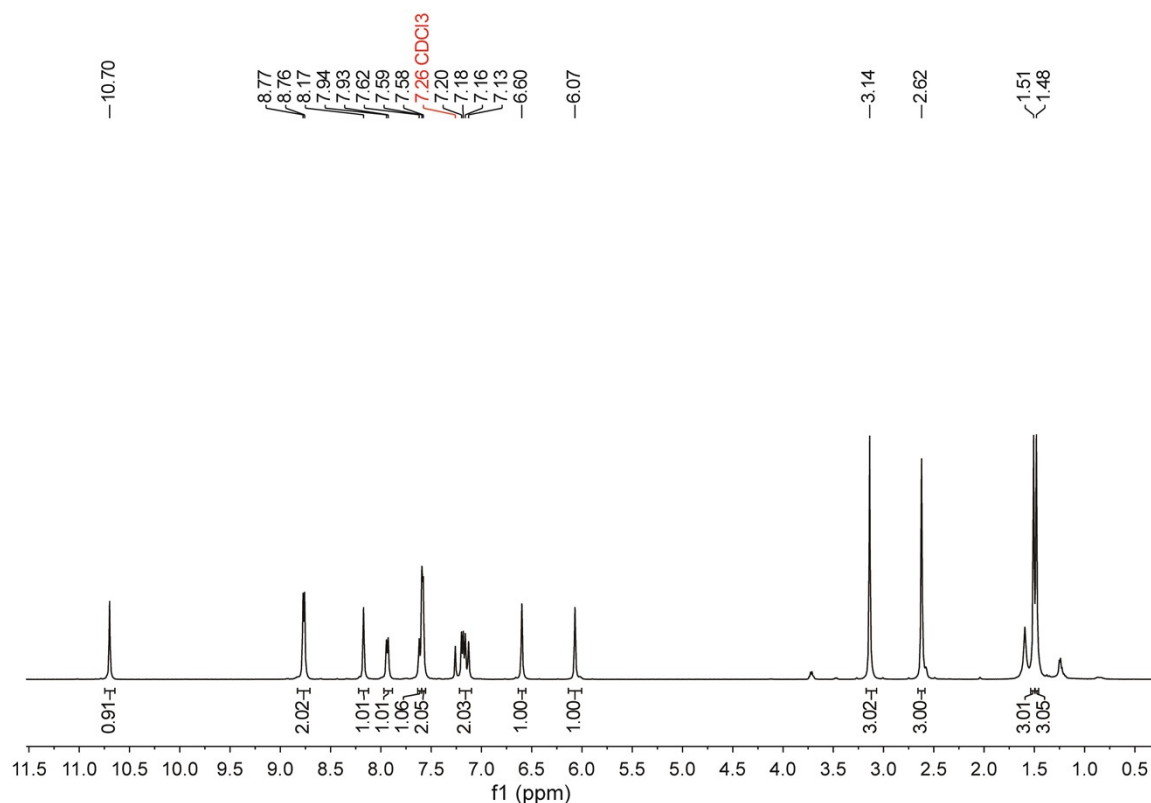


Figure S7 ^1H NMR spectra of compound TNB.

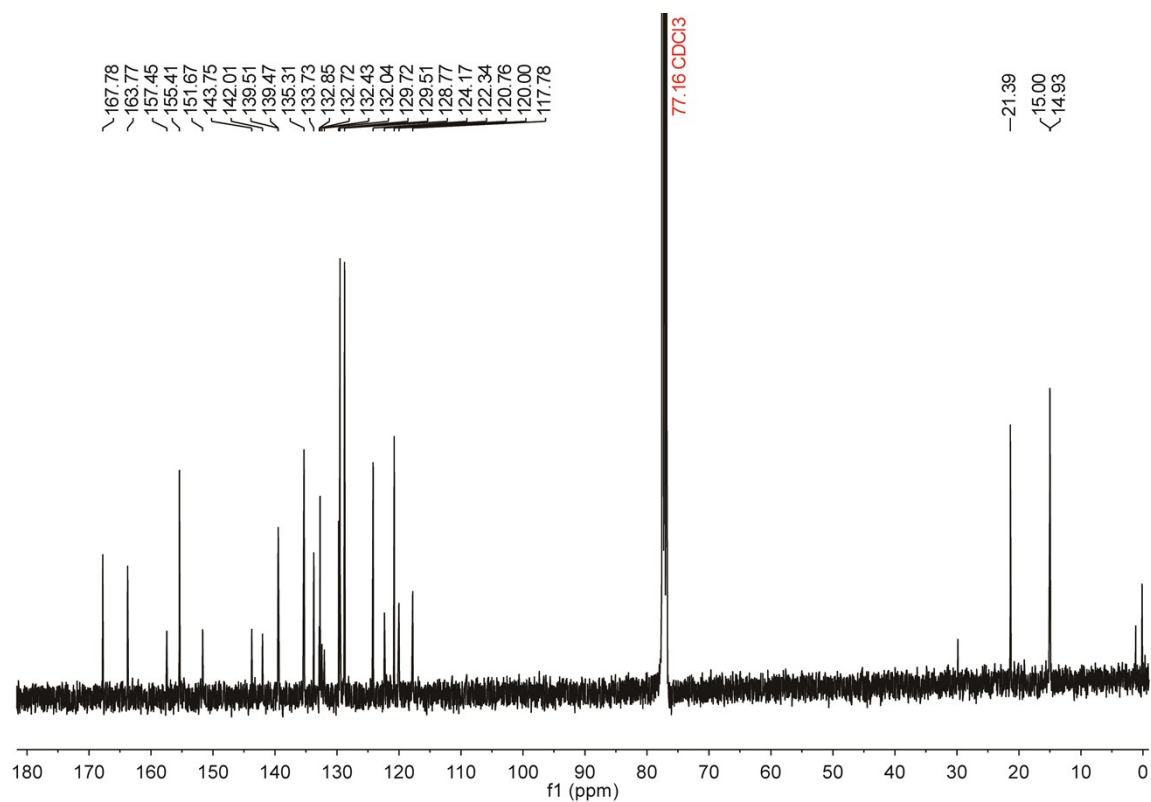


Figure S8 ¹³C NMR spectra of compound TNB.

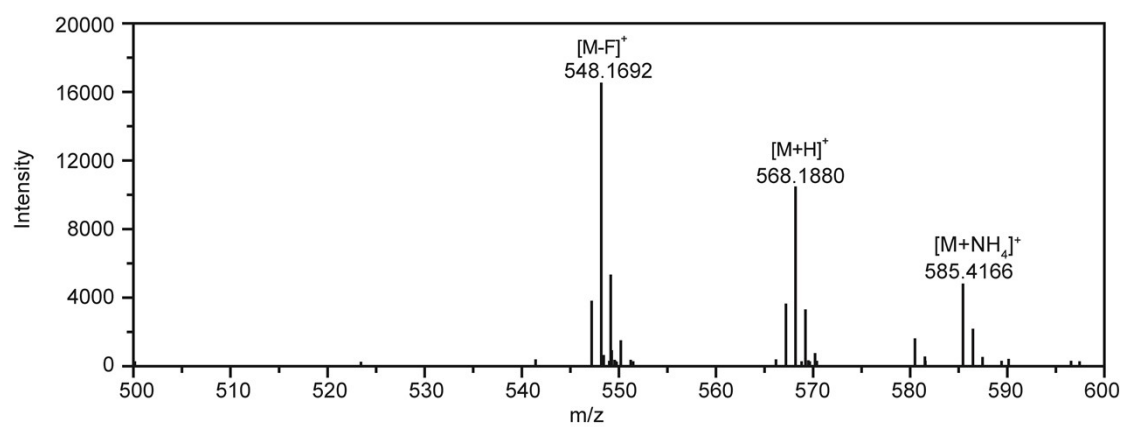
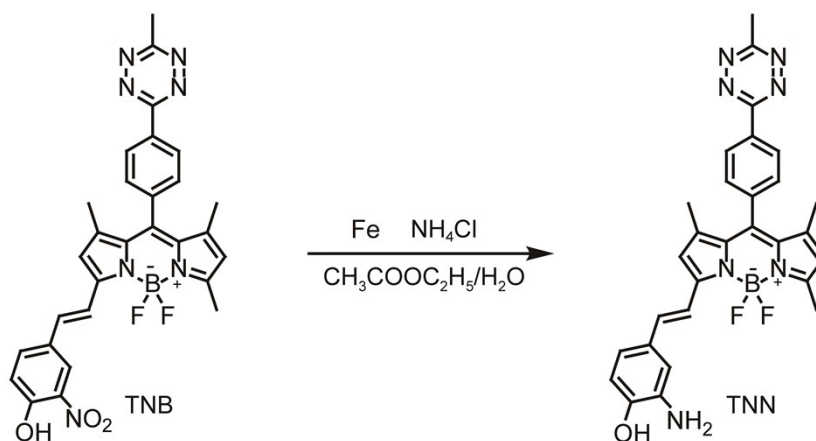


Figure S9 ESI mass spectrum of compound TNB.



Scheme S2 Synthesis route of compound TNN.

Synthesis of compound TNN. A solution of ammonium chloride (98 mg, 1.76 mmol) and iron powder (98 mg, 1.76 mmol) in H₂O (10 mL) was slowly added to a solution of TNB (50 mg, 88.13 μ mol) in ethyl acetate (10 mL). The mixture was heated to 80°C for 2 h, and then was cooled to room temperature, extracted with dichloromethane, washed twice with saturated NaHCO₃, and dried over anhydrous Na₂SO₄. The solvent was removed under reduced pressure to obtain a blue solid without further purification (10 mg, 21%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.21 (s, 1H), 8.65 (d, *J* = 8.3 Hz, 2H), 7.73 (d, *J* = 8.3 Hz, 2H), 7.41 (d, *J* = 16.2 Hz, 1H), 7.25 (d, *J* = 16.1 Hz, 1H), 6.96 (s, *J* = 2.4 Hz, 2H), 6.72 - 6.67 (m, 2H), 6.18 (s, 1H), 4.84 (s, 2H), 3.02 (s, 3H), 1.48 (s, 3H), 1.42 (s, 3H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 167.27, 162.86, 154.57, 152.75, 146.83, 142.52, 140.40, 139.69, 138.01, 137.47, 132.65, 131.99, 129.65, 128.08, 127.67, 120.92, 119.06, 118.55, 114.34, 113.94, 110.78, 26.35, 20.89. MS: calc. *M*⁺ = 537.2260, obsvd. HR-MS: (*M*+H)⁺ = 538.2379.

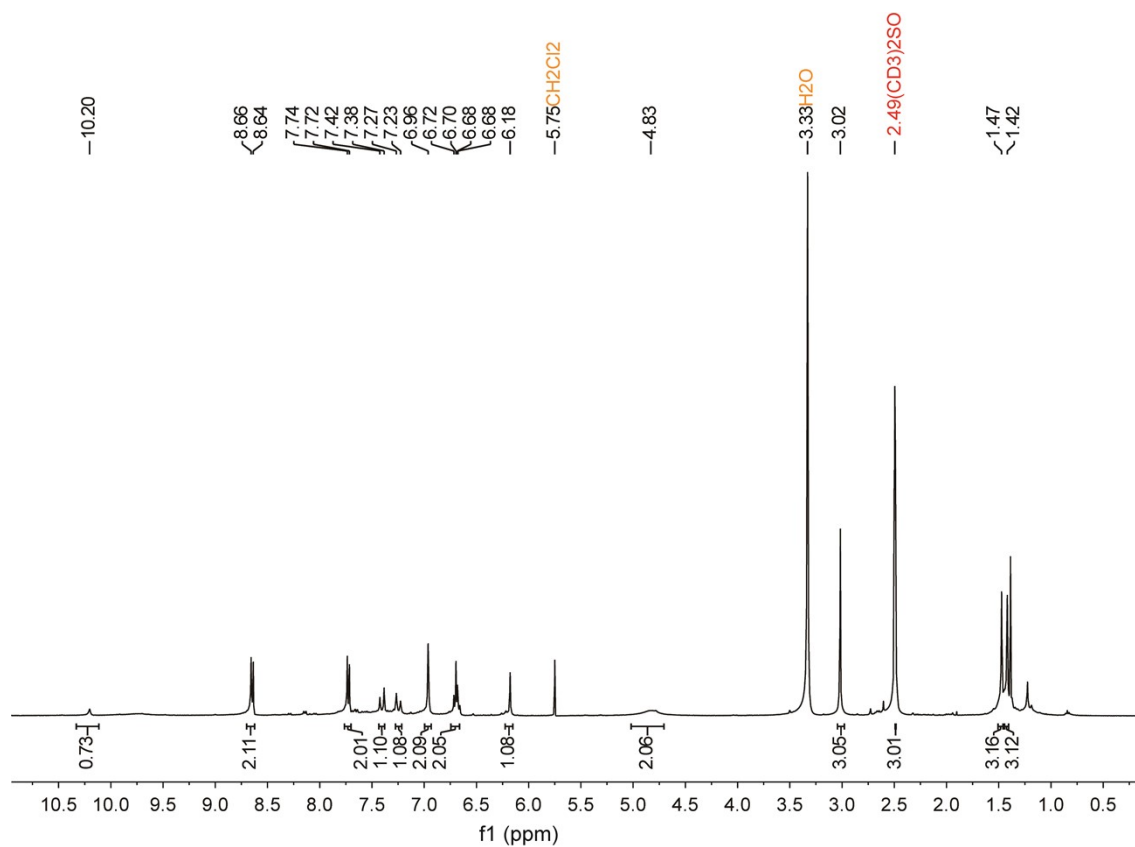


Figure S10 ¹H NMR spectra of compound TNN.

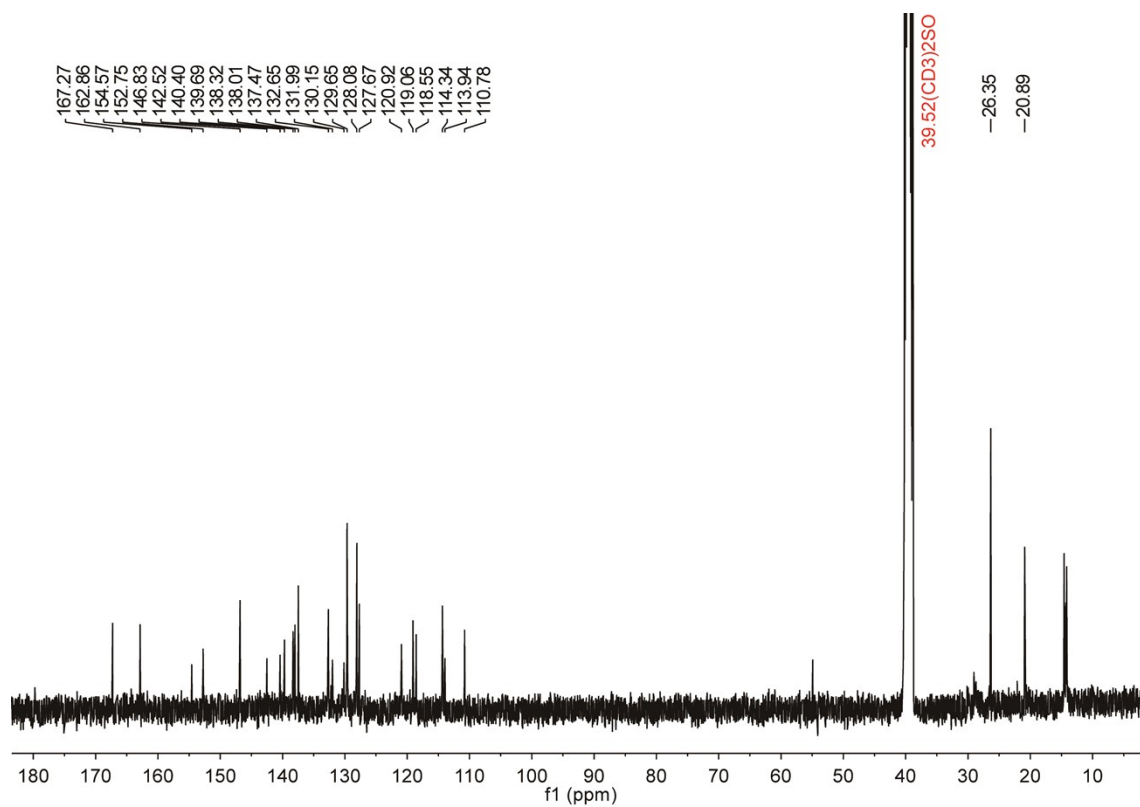


Figure S11 ¹³C NMR spectra of compound TNN.

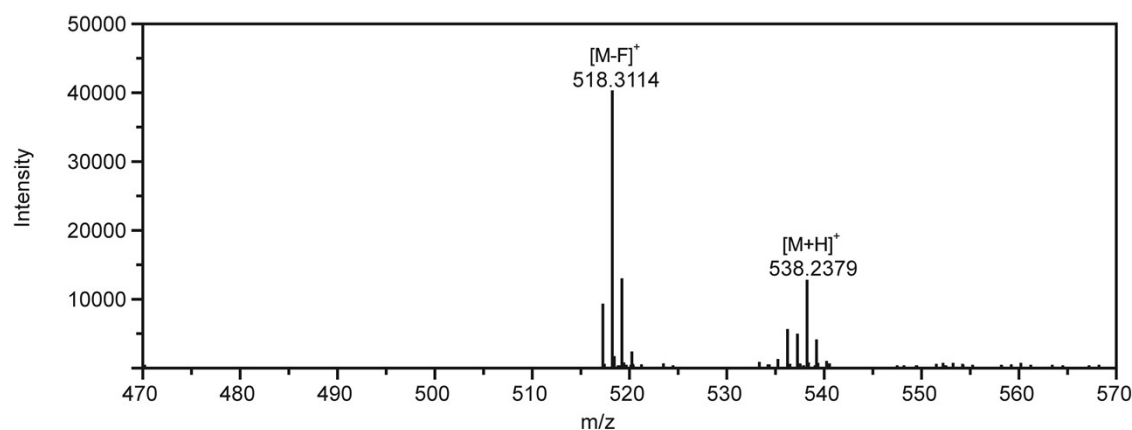
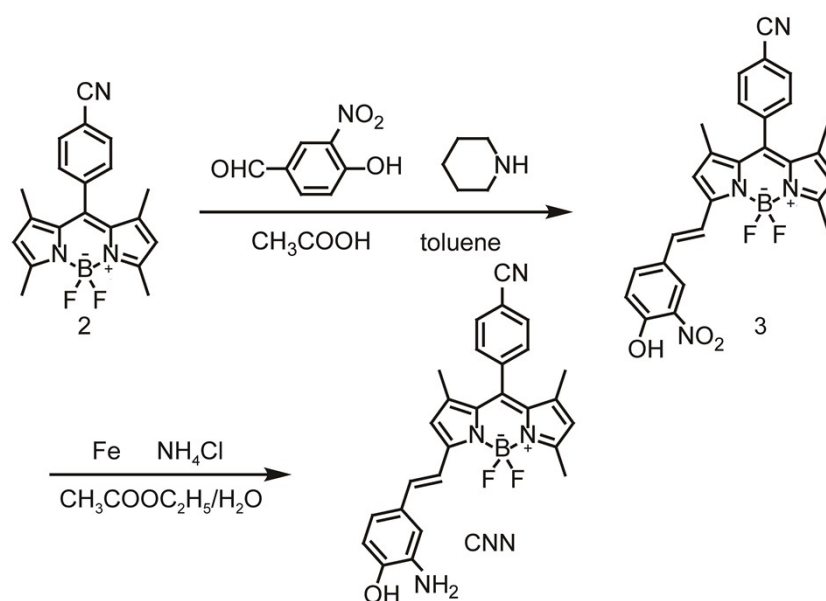


Figure S12 ESI mass spectrum of compound TNN.



Scheme S3 Synthesis route of compound CNN.

Synthesis of compound 3. Compound 2 (0.45 mg, 1.08 mmol) was added to a solution of 3-nitro-4-phenol hydroxybenzaldehyde (0.22g, 1.29 mmol) in 20 mL of toluene, then added pyridine (0.6 mL) and acetic acid (0.6 mL). The mixture was reflux at 110°C for 4 h. The reaction solvent was removed under reduced pressure and the residue was further purified by silica gel column chromatography (dichloromethane : petroleum ether = 100:40, v:v) to yield dark red solid (91 mg, 14.9%). ¹H NMR (500 MHz, Chloroform-*d*) δ 10.70 (s, 1H), 8.18 (d, *J* = 2.1 Hz, 1H),

7.92 (dd, $J = 8.8, 2.2$ Hz, 1H), 7.84 (d, $J = 8.1$ Hz, 2H), 7.58 (d, $J = 16.3$ Hz, 1H), 7.50 (d, $J = 8.1$ Hz, 2H), 7.23 - 7.11 (m, 2H), 6.59 (s, 1H), 6.07 (s, 1H), 2.61 (s, 3H), 1.41 (s, 3H), 1.38 (s, 3H). ^{13}C NMR (101 MHz, Chloroform- d) δ 157.51, 155.18, 151.78, 143.00, 141.41, 139.74, 137.61, 135.00, 133.42, 132.87, 132.76, 129.30, 129.26, 123.94, 122.25, 120.48, 119.47, 117.72, 113.22, 14.66, 14.57. MS: calc. $\text{M}^+ = 498.1675$, obsvd. HR-MS: $(\text{M}+\text{H})^+ = 499.1588$.

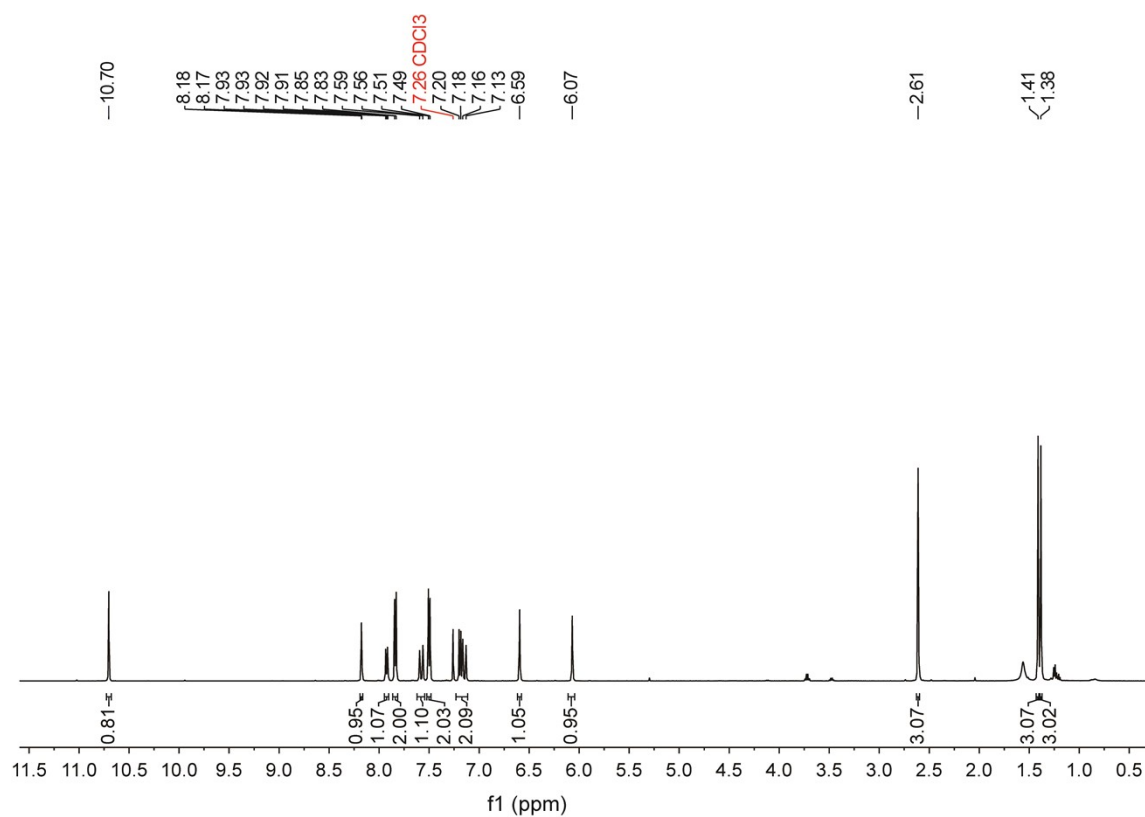


Figure S13 ^1H NMR spectra of compound 3.

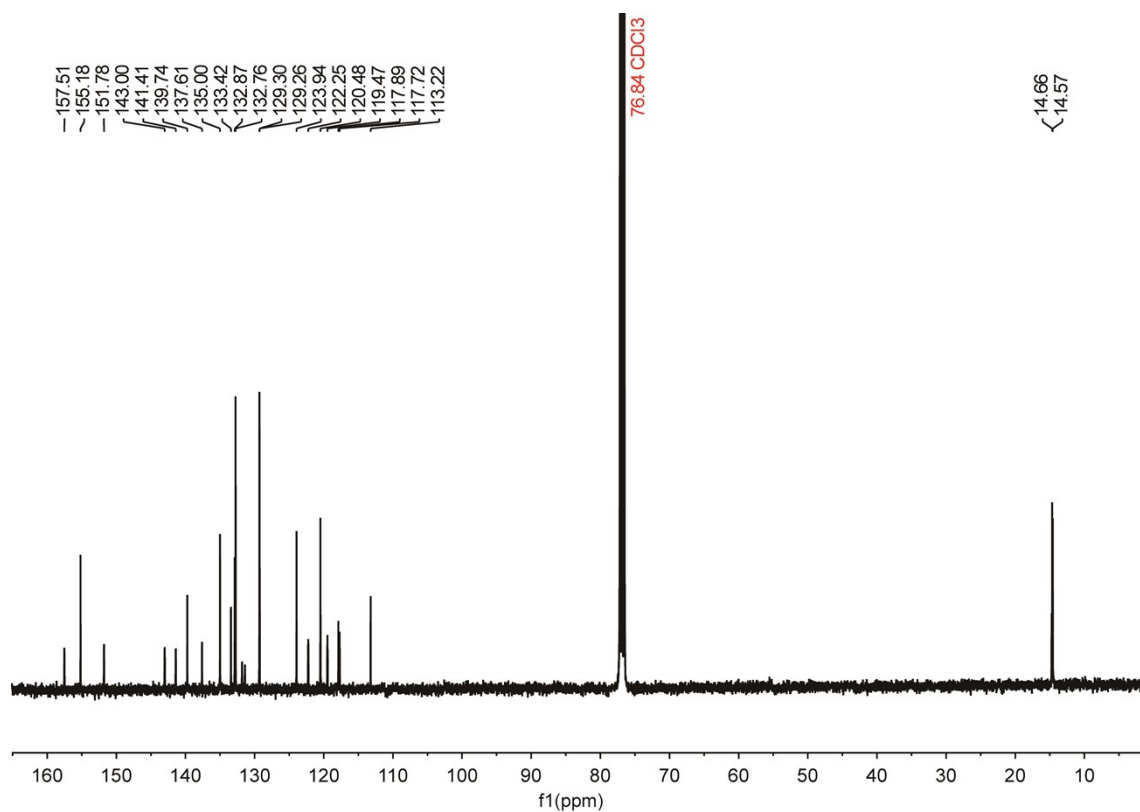


Figure S14 ¹³C NMR spectra of compound 3.

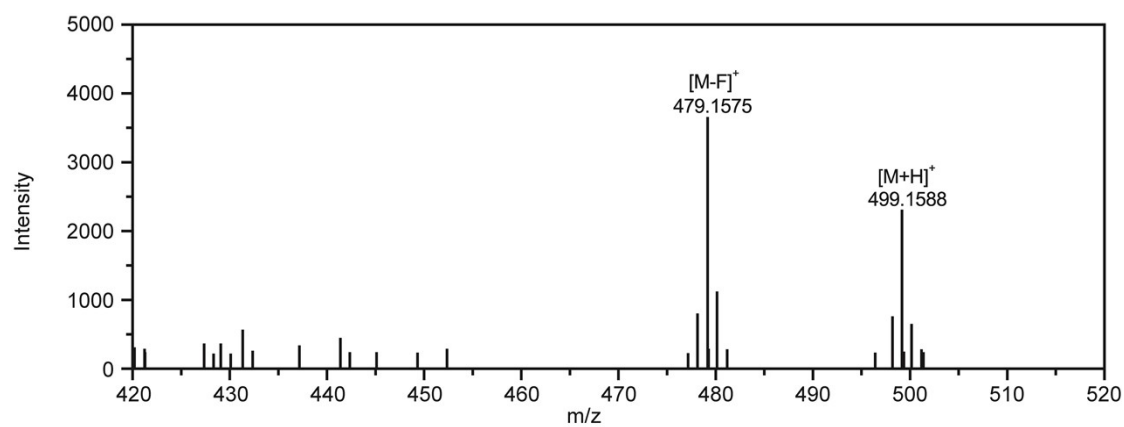


Figure S15 ESI mass spectrum of compound 3.

Synthesis of compound CNN. A solution of ammonium chloride (35 mg, 662.26 μ mol) and iron powder (37 mg, 662.26 μ mol) in H₂O (6 mL) was slowly added to a solution of compound **3** (33 mg, 66.23 μ mol) in ethyl acetate (6 mL). The mixture was heated to 80°C for reacting 2 h, and then was cooled to room temperature, extracted with dichloromethane, washed twice with saturated NaHCO₃, and dried over

anhydrous Na₂SO₄. The solvent was removed under reduced pressure to obtain a blue solid without further purification (23 mg, 76%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.73 (s, 1H), 8.04 (d, *J* = 8.1 Hz, 2H), 7.72 - 7.65 (d, 2H), 7.40 (d, *J* = 16.1 Hz, 1H), 7.22 (d, *J* = 16.2 Hz, 1H), 6.95 (s, 2H), 6.72 - 6.64 (m, 2H), 6.16 (s, 1H), 4.83 (s, 2H), 2.46 (s, 3H), 1.36 (s, 3H), 1.31 (s, 3H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 154.77, 152.86, 146.89, 142.42, 140.22, 139.92, 139.31, 137.47, 136.92, 133.12, 131.79, 129.87, 129.79, 127.64, 120.99, 119.15, 118.70, 118.45, 114.34, 113.85, 112.09, 110.78, 14.52, 14.09. MS: calc. M⁺ = 468.1933, obsvd. HR-MS: (M+H)⁺ = 469.1890.

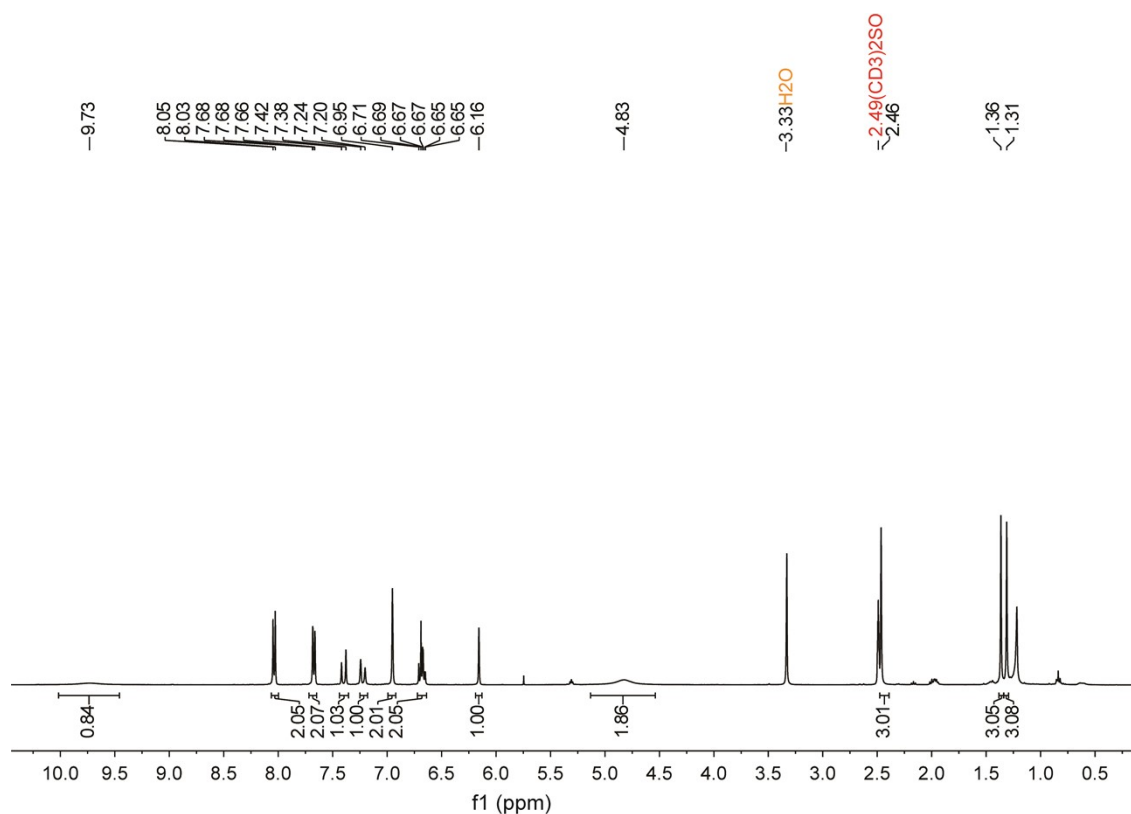


Figure S16 ¹H NMR spectra of compound CNN.

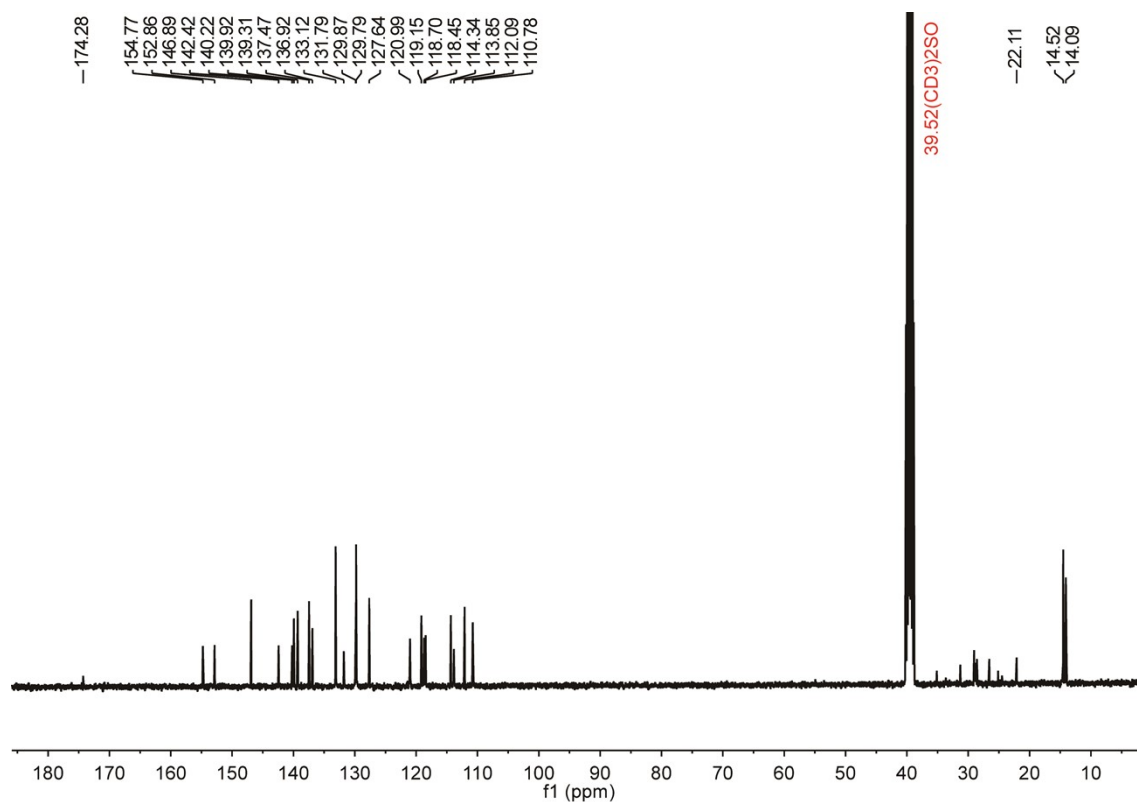


Figure S17 ¹³C NMR spectra of compound CNN.

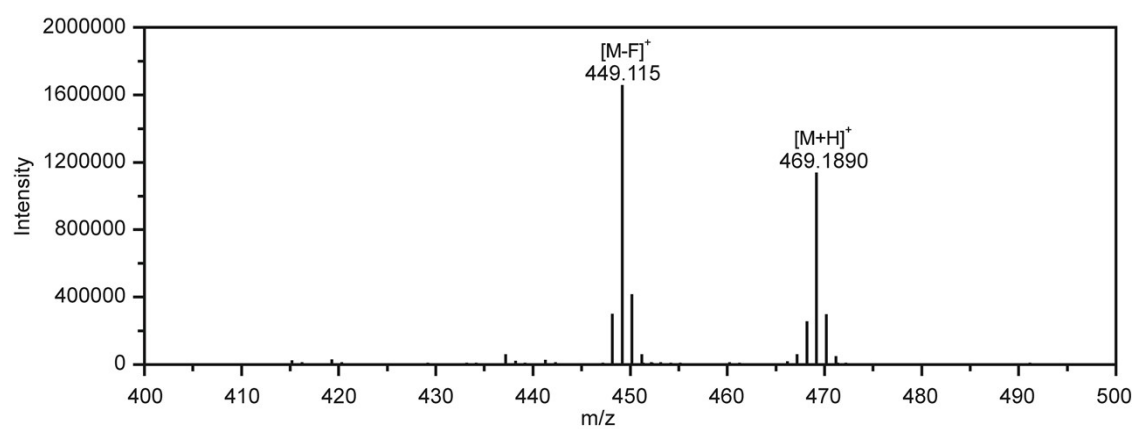


Figure S18 ESI mass spectrum of compound CNN.

3 Absorption and Fluorescence assays

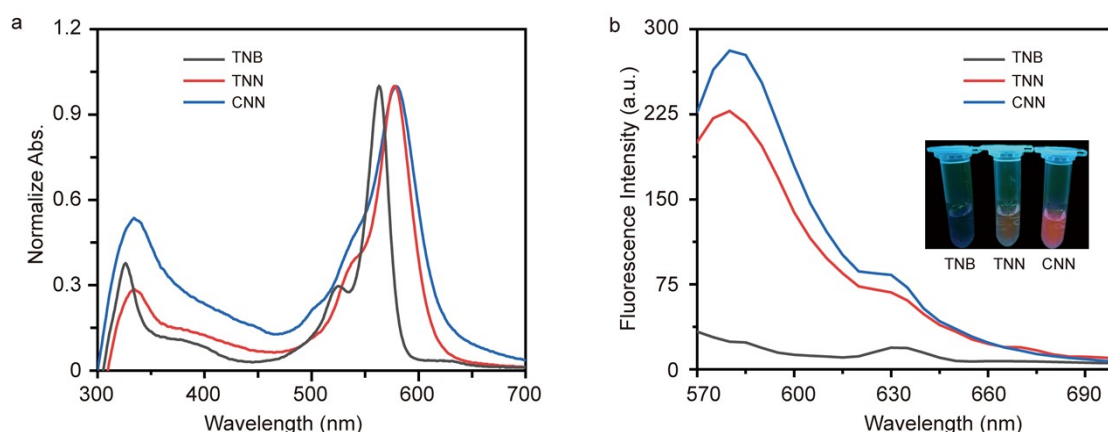


Figure S19 (a) Normalized UV-vis absorbance spectra of TNB (grey), TNN (red), CNN (blue) 25 μM in ethanol; (b) fluorescence emission spectra of CNN, TNN, TBN, 5 μM in ethanol (λ_{ex} = 560 nm). Inset: the solution color of compounds TNB, TNN, CNN in ethanol under 254 nm UV light.

The UV-vis absorption spectra of ethanol solution (25 μM) of TNB, TNN, CNN was measured between 450 and 650 nm. Fluorescence spectral characteristics of TNB, TNN, CNN in ethanol solution (5 μM) were investigated from 550 to 700 nm with excitation wavelength of 400 nm. Compound TNB TNN and CNN have two absorption peaks at about 350 nm and 570 nm. In order to avoid the influence of compound TNB and TNN on CNN at 572 nm, the short wavelength regional peak is selected as the excitation wavelength. Considering that prolonged UV exposure can cause damage to cells, so we chose 400 nm as the excitation wavelength because we also found that the fluorescence of CNN at 520 nm was stronger than that of TNN and TNB.

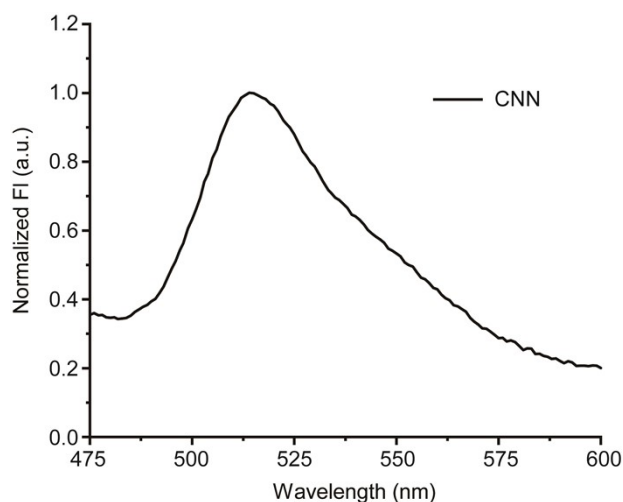


Figure S20 Normalized fluorescence emission spectra of CNN in 30% EtOH and 70% PBS ($\lambda_{\text{ex}} = 400$ nm).

4 Quantum yield determination

Relative fluorescence quantum yields were determined by using Rhodamine B ($\lambda_{\text{ex}} = 495$ nm, $\Phi_{\text{F}} = 0.89$ in ethanol) as a fluorescence standard. The quantum yield, Φ , was calculated using equation as following:

$$\frac{\Phi_{\text{sample}}}{\Phi_{\text{Rho}}} = \frac{I_{\text{sample}} OD_{\text{Rho}} d_{\text{sample}}^2}{I_{\text{Rho}} OD_{\text{sample}} d_{\text{Rho}}^2}$$

Φ : quantum yield

I : integrated emission intensity

OD : optical density at the excitation wavelength

d : refractive index of solvents, $d_{\text{ethanol}} = 1.36$

Table S1 Photophysical data of compounds TNB, TNN, CNN.

Compound number	Compound	λ_{abs} (nm)	λ_{em} (nm)	$\epsilon \times 10^4$ ($\text{M}^{-1}\text{cm}^{-1}$)	Φ_{EtOH}
1	TNB	560	628	1.73	0.0004
2	TNN	576	631	1.74	0.004
3	CNN	578	630	2.97	0.035

Compounds TNB, TNN, CNN fluorescein in EtOH (1 mM) , Rhodamine B in EtOH (1 mM) was used as a reference for measuring the quantum yields.

5 HPLC for Nitroreductase metabolism assay

Metabolic conditions of nitroreductase. The reactions were carried out in 1×PBS (pH=7.4) buffer solution containing 30% EtOH. In a 2 mL tube, EtOH (300 µL) and 1 mM TNB (50 µL) were mixed, and then newly prepared 5 mM NADH (100 µL) was added to reach a final concentration of 500 µM. Nitroreductase (5 µg/mL) was added to the sample solution. The solution volume was adjusted to 1 mL with 1×PBS. After rapid mixing, stirring the reaction solution at 37°C for 60 min. After the reaction finished, 100 µL of the reaction solution was heated in a water bath at 100°C for 5 min to inactivate the nitroreductase, and then the sample was detected by HPLC detection.

HPLC analysis conditions. The reaction solution was analyzed by high-performance liquid chromatography (HPLC) after filtering through a filter (13 mm PTFE Hydrophilic Syringe Filter 0.45 µm, ALWSCL Corporation). HPLC was performed on a ZORBAX SB-Aq (Analytic 4.6 × 250 mm, 5µm, Agilent) with Shimadzu LC-20AT HPLC system. The conditions were as follows: volume ratio of methanol/10 mM NaH₂PO₄ = 5:95 (0 min) to 95:5 (52 min), as shown in Table S2; flow rate 0.8 mL/min; detection under UV light at 254 nm.

Table S2 Table of gradient elution of the reaction solutions.

Time (min)	MeOH	10 mM NaH ₂ PO ₄
0	5	95
10	30	70
15	70	30
35	95	5
40	95	5
42	5	95
52	5	95

HPLC profiled of TNB incubated with NTR and blue light. The reactions were carried out in 1×PBS (pH=7.4) buffer solution containing 30% EtOH. In a 2 mL tube, EtOH (300 µL) and 1 mM TNB (50 µL) were mixed, and then newly prepared 5 mM NADH (100 µL) was added to reach a final concentration of 500 µM. Nitroreductase was dissolved in H₂O (200 µg/mL), and 25 µL nitroreductase was added to the sample solution. The final solution volume was adjusted to 1 mL with 1×PBS. After rapid mixing, the reaction solution was agitated at 37°C, and 100 µL of the reaction solution was sampled at 0, 1, 3, 5, 10, 15 min respectively, heated in a water bath at 100°C for 5 min to inactivate the nitroreductase, and then detected by HPLC.

Under the same conditions, incubate at 37°C for 30 min, inactivate nitroreductase with water bath, take 100 µL, using OmniCure S2000 at 400-500 nm with 30% intensity to light 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 min. Then the fluorescence spectrum was detected.

6 Cell Culture

HeLa cells, A549 cells and HSF cells were incubated in complete medium Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Solarbio). HUVEC cells were cultured by RPMI 1640 medium containing 10% FBS and 1% antibiotics of penicillin-streptomycin at 37 °C in a humidified incubator containing 5% CO₂.

7 Cytotoxicity Assay

The in vitro cytotoxicity of probe TNB was measured by using a standard CCK-8 assay in A549 and HeLa cell lines. When cell growth entered the log phase, 8×10^3 cells per well were seeded in a 96-well cell culture plate within a humidified 5% CO₂ atmosphere at 37°C for 12 h. The probe TNB (100 µL/well) at concentrations of 2-20 µM was added to the wells of each group, six replicates made for each measurement, while 100 µL/well DMSO diluted in DMEM at final concentration of 0.5% served as the negative control group. The cells were incubated for 12 h at 37°C under normoxia (20% O₂) or hypoxia (1% O₂). One hypoxia group was irradiated with a 405 nm laser with the power intensity of 50 mW/cm² for 5 min (Lasers used in this study). The other groups were cultured without laser irradiation. Remove the medium and add 100 µL of CCK-8 diluted ten times by DMEM to each well, and measure the OD value at 450 nm using a multifunctional microplate reader (SpectraMax M3, Molecular Devices) after incubation for 1 h at 37°C. The following formula was used to calculate the cell viability:

$$\text{Cells viability (\%)} = (\text{OD dye} - \text{OD blank}) / (\text{OD control} - \text{OD blank}) \times 100\%$$

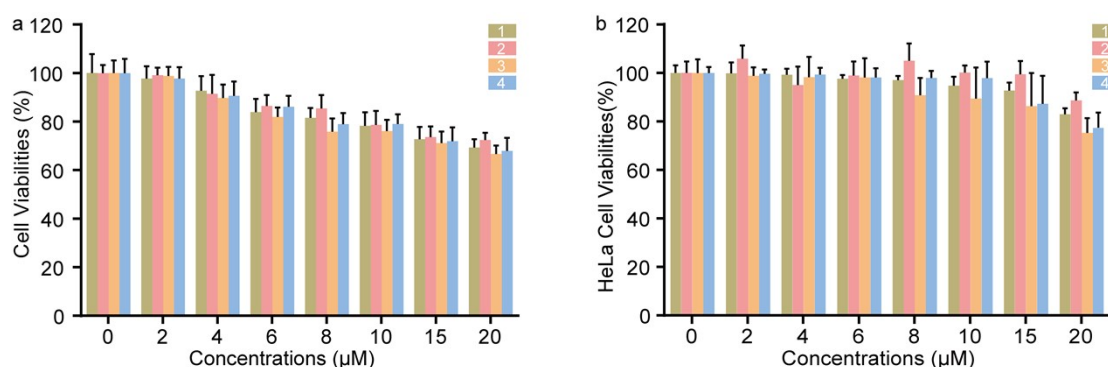


Figure S21 Cell viabilities of A549 and HeLa induced by probe TNB after 12 h in CCK-8 assay. A549 cells (a) and HeLa cells (b) were incubated with 0~20 μM TNB at 37°C. (1) normoxia without laser, (2) normoxia with laser (3) hypoxia without laser, (4) hypoxia with laser. The error bars represent standard errors (n = 6).

8 Confocal Fluorescence Imaging for Living Cells

TNB was prepared to a concentration of 1 mM with anhydrous DMSO and filtered through a filter (13 mm PTFE Hydrophilic Syringe Filter 0.45 μm, ALWISCL Corporation). The solution was diluted to the working concentration of 5 μM by complete growth medium. HeLa (8×10^3 /mL, 2.5 mL) and A549 cells (10^4 /mL, 2.5 mL) were seeded on Glass Bottom Cell Culture Dish (Glass Diameter 15 mm, NEST) at 37°C under 5% CO₂ for 12 h, and then incubated with 5 μM TNB for 6 h under normoxia (20% O₂) or hypoxia (1% O₂) conditions at 37°C. Cells were washed twice with 1×PBS (pH 7.4) and turned to confocal laser scanning microscope (CLSM, FV3000, Olympus, Japan) with a 20×objective lens. TNB probes were photoactivated using CLSM 405 nm laser at 100% laser intensity for 60 s. The integrated optical density (IOD) intensity per cell was calculated by Image J.

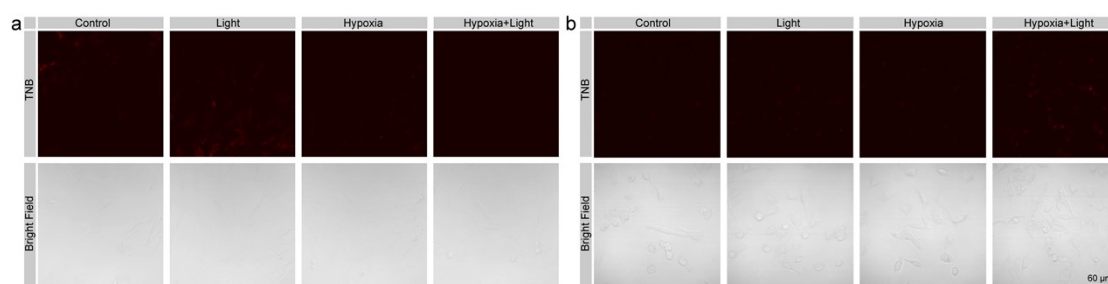


Figure S22 Confocal fluorescence microscopy imaging of HSF (a) and HUVEC (b) cells incubated with TNB under different conditions. Scale bar: 60 μm . Control: normoxia without laser, Light: normoxia with laser, Hypoxia: hypoxia without laser, Hypoxia+Light: hypoxia with laser.

9 4T1 tumor section imaging

Animal Experiments. BALB/c mice aged 6-8 weeks, weighing about 20-22 g, were purchased from Yangzhou University Medical Center (Yangzhou, China). The animals were cared and used in accordance with the regulations of the Institutional Animal Care and Use Committee (IACUC) of Nanjing University. During the study period, the animals had free access to food and water. The right abdominal tumor model of 4T1 mice was established by injecting 5×10^5 cells in 50 μL high glucose medium. When the tumor size reached to $\sim 100 \text{ mm}^3$, TNB (20 μM , 100 μL) was dissolved in serum-free DMEM medium and injected intratumoral. Tumor tissue was collected one hour later, and 4T1 tumors were embedded with an cold cutting temperature compound (OCT) for the frozen section. The sections were then fixed with cold acetone, washed with PBS for 3 times to remove OCT, and the nuclei were stained with DAPI. Frozen section turned to confocal laser scanning microscope (CLSM, FV3000, Olympus, Japan) with a 20 \times objective lens. The mean optical density was calculated by Image J.

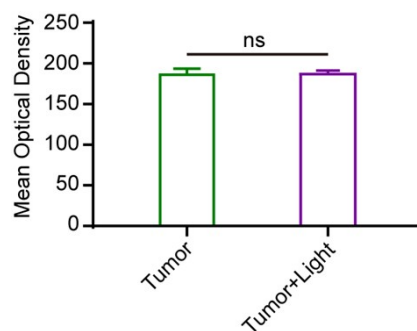


Figure S23 Mean optical density quantification of DAPI before and after light irradiation as shown in figure 4a. Data were given as mean \pm S.D. (n=4).

10 Statistics Analysis

The one-way analysis of variance (ANOVA) test was used for IOD per cell analyses.

Mean Optical Density between experimental and control groups were analyzed by the unpaired Student's *t*-test. $P < 0.05$, 0.01, 0.001, 0.0001 were considered a statistically difference and remarked with *, **, ***, ****, respectively.