Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Highly Sensitive Long-wavelength Fluorescence Probe for Nitroreductase and Hypoxia: Selective Detection and Quantification

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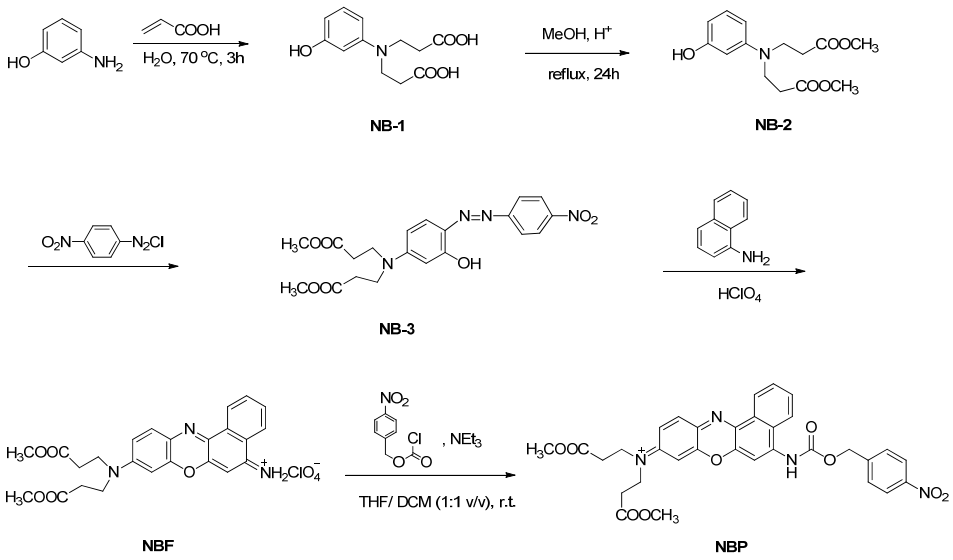
1. Materials and Instruments

Silica gel P60 (Qingdao) was used for column chromatography. All chemicals were purchased from TCI and Aladdin reagent Co without further purification except otherwise stated. All the organic solvents were of analytical grade. Acetonitrile were distilled by CaH_2 to remove the water before used. Water was purified by a Milli-Q system.

^{1}H-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 Analysis and Test Center of East China University of Science and Technology (ECUST).

2. Synthesis



Scheme S1 Synthesis of fluorophore **NBF** and probe **NBP**

3-[(2-carboxyethyl)(3-hydroxyphenyl) amino]propionic acid (NB-1)

A solution of 3-aminophenol (5.0 g, 0.05mol) in acrylic acid (85 mL, 1.24 mol) and water (90 mL) was heated to 70 ^{o}C for 3 h. The reaction mixture was cooled, and then ethanol (180 mL) was added. The result solution was kept at 5 ^{o}C for 12 hrs. The white precipitate that formed was filtered, washed with ethanol (50 mL) and dried to obtain dicarboxylic acid (9.3 g, 80%). The compound **NB-1** was used without any further purification.

methyl 3-(3-hydroxy-2-(3-methoxy-3-oxopropyl) phenylamino)propanoate (NB-2)

A solution of the compound **NB-1** (5.0 g, 19.7 mmol) in methanol (250 mL) along with HCl (10.0 M, 0.5 mL) was refluxed for 12 hrs. The reaction mixture was cooled and methanol was

evaporated under reduced pressure. The residue was dissolved in EtOAc (50 mL) and the organic layer was washed with water (3×20 mL). The organic layer was evaporated to dryness under reduced pressure to yield compound **NB-2** as a yellow semisolid (3.4 g, 61%). [S1]

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.58 (t, 4H, *J* = 7.2 Hz), 3.61 (t, *J* = 7.2, 4H), 3.67 (s, 6H), 6.25-6.23 (m, 2H), 6.58 (s, 1H), 7.07-7.03 (m, 1H).

Dimethyl 3,3'-(3-hydroxy-4-((4-nitrophenyl) diazenyl) phenylazanediyl) dipropanoate (NB-3)

A solution of 4-nitroaniline (0.1 g, 0.8 mmol) was dissolved in a mixture of 0.5 mL of concentrated hydrochloric acid and 0.5 mL of water. The reaction flask was cooled in an ice-bath and sodium nitrite (55 mg, 0.8 mmol) dissolved in 100 mL of water was added slowly. The reaction mixture was stirred at 0 ^{o}C for an additional 20 min. At the end of the reaction, the solution turned orange. Then a solution of compound **NB-2** (247 mg, 0.7 mmol) in 0.2 mL of methanol was added to the reaction flask. The reaction mixture was stirred for 30 min at room temperature. The red precipitate was filtered and washed with cold ethanol. The crude product was recrystallized in ethanol. After drying, compound **NB-3** (264 mg, 75%) was obtained as an orange red solid. [S2]

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.70 (t, *J* = 7.0 Hz, 4H), 3.72 (s, 6H), 3.81 (t, *J* = 7.0 Hz, 3H), 6.06 (s, 1H), 6.48 (d, *J* = 9.2 Hz, 1H), 7.51 (d, *J* = 8.8 Hz, 2H), 7.77 (d, *J* = 8.4 Hz, 2H), 8.29 (d, *J* = 4.0 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 32.4, 47.0, 52.0, 98.8, 108.2, 120.1, 125.1, 136.1, 146.1, 152.8, 153.3, 171.6.

9-(bis (3-methoxy-3-oxopropyl) amino)-5H-benzo[a]phenoxazin-5-iminium perchlorate (NBF)

A mixture of compound **NB-3** (86 mg, 0.17 mmol), 1-aminonaphthalene (27 mg, 0.19 mmol) and 3 mL of DMF containing perchloric acid (0.1 mL, 70%) was heated to 155-160 ^{o}C for 15 min with stirring. The color of the reaction mixture changed from brown to deep blue. The reaction was monitored by silica gel TLC. After cooling, the DMF was evaporated to dryness under reduced pressure. The crude perchlorate salt was purified by flash column chromatography (silica gel, 1:40 methanol/dichloromethane) to afford **NBF** as a blue solid (88 mg, 85%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 2.74 (t, *J* = 7.2 Hz, 4H), 3.64 (s, 6H), 3.88 (t, *J* = 7.2 Hz, 4H), 6.88 (s, 1H), 7.11 (s, 1H), 7.28 (d, *J* = 9.4 Hz, 1H), 7.92 (dd, J_1 = 15.0, J_2 = 7.6 Hz, 2H), 8.03 (t, *J* = 7.6 Hz, 1H), 8.50 (d, *J* = 8.0 Hz, 1H), 8.84 (d, *J* = 7.2 Hz, 1H), 10.05-10.12 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6,) δ 31.8, 47.2, 52.1, 97.2, 97.5, 115.0, 123.4, 124.8, 129.2, 130.7, 131.9, 132.7, 133.4, 135.9, 147.8, 152.1, 152.2, 153.7, 162.3, 171.9. HRMS (ESI): Calcd for $C_{24}H_{24}N_3O_5$

(M^+) 434.1716; Found, 434.1719.

3-methoxy-N-(3-methoxy-3-oxopropyl)-N-(5-((4-nitrobenzyloxy)carbonylamino)-9H-benzo[a]phenoxazin-9-ylidene)-3-oxopropan-1-aminium (NBP)

Compound **NBF** (100.0 mg, 0.18 mmol) was dissolved in 10 mL of dry THF/DCM (1:1 v/v), then cooled to 0 ^oC, and NEt_3 (0.10 mL, 1.4 mmol) was added. The reaction mixture was stirred for 10 min, and then a solution of 4-nitrophenylchloroformate (146.0 mg, 0.68 mmol) in dry THF/DCM (1:1 v/v) (5 mL) was added in a period of 10 min. The reaction mixture was stirred for 1 h at the same temperature and 1 h at room temperature. Finally it was filtered off and purified by flash column chromatography (silica gel, 50:1 CH_2Cl_2/ CH_3OH) to afford **NBP** (49.4 mg, 43%) as a purple solid.

^1H NMR (400 MHz, $CDCl_3$) δ 2.68 (t, *J* = 7.2 Hz, 4H), 3.72 (s, 6H), 3.79 (t, *J* = 7.2 Hz, 4H), 5.42 (s, 2H), 6.45 (d, *J* = 2.4 Hz, 1H), 6.58 (s, 1H), 6.67-6.70 (m, 1H), 7.60-7.72 (m, 5H), 8.25 (d, *J* = 8.4 Hz, 2H), 8.48 (d, *J* = 7.6 Hz, 1H), 8.64 (d, *J* = 8.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$,) δ 32.1, 47.0, 52.0, 66.4, 97.3, 100.4, 110.1, 123.8, 124.0, 125.6, 126.1, 128.7, 130.3, 131.1, 131.2, 131.6, 141.5, 143.8, 146.4, 147.7, 149.5, 149.9, 162.1, 163.4, 171.8. HRMS (ESI): Calcd for $C_{32}H_{29}N_4O_9$ (M^+) 613.1929; Found, 613.1927.

3. Methods

3.1 Spectroscopic materials and methods

Double distilled water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 0.01 M PBS buffer (pH 7.4, 1% DMSO) at 37 ^oC. All pH measurements were made with a Sartorius basic pH-Meter PB-10. Absorption spectra were recorded using a Varian Cary100 Bio UV-Visible spectrophotometer. Fluorescence spectra were recorded using a Varian Cary Eclipse scanning spectrofluorometer equipped with a Xenon flash lamp. Samples for absorption and fluorescence measurements were contained in 1 cm×1 cm quartz cuvettes (3.5 mL volume) or 400 µL Fluor Micro Cell (Varian Cary FLR).

3.2 Determination of quantum yield

$$\Phi_1 = \frac{\Phi_B I_1 A_B \lambda_{exB} \eta_1}{I_B A_1 \lambda_{ex1} \eta_B}$$

Where Φ is quantum yield; I is integrated area under the corrected emission spectra; A is absorbance at the excitation wavelength; λex is the excitation wavelength; η is the refractive index of the solution; the subscripts 1 and B refer to the unknown and the standard, respectively.

We chose Rhodamine B in ethanol as standard, which has the quantum yield of 0.97. [S3]

Table S1. Spectroscopic data for **NBF** and **NBP**

Compds	λ_{abs} (nm)	ε [$M^{-1}cm^{-1}$]	λ_{em} (nm)	Φ_f
NBF	613	8.2×10^4	668	0.02
NBP	529	2.0×10^4	-	-

3.3 Nitroreductase assay

Nitroreductase activity experiments were performed in 400 μL Fluor Micro Cell and employed recombinant forms of Nitroreductase (expressed in Escherichia coli. purchased from Sigma-Aldrich). Stock solutions of probe **NBP** and fluorophore **NBF** were prepared in pure DMSO (1 mM) and diluted in phosphate buffered saline (pH 7.4) with 50 eq NADH. **NBP** was added to a final protein concentration of 12.5 μg/mL enzyme per 400 μL well at 37 ºC, and emission intensity was collected from 600 nm to 800 nm with excitation at 580 nm.

3.4 HPLC for probe NBP with nitroreductase

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% buffer (acetic acid and ammonium acetate pH=6.0). 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 (265 nm and 613 nm)

3.5 Detection limit

The detection limit was calculated based on the fluorescence titration. The fluorescence enhancement of **NBP** was dose-dependent with respect to nitroreductase. The linear response (y = 5.173 x + 3.718 with R = 0.995) of fluorescent intensity (y) with respect to the concentration (x) of nitroreductase was established. The lower detection limit (LDL) was calculated following equation. LDL = 3S/m (S is the ratio signal and noise, which is the standard deviation of blank measurements, n = 11; m is the slope of linear equation). The detection limit was determined to be 180 ng/mL.

3.6 Cell Viability Assays

Cell proliferation was evaluated by MTT assay. A549 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated overnight, and then treated with **NBP** and **NBF** at different concentration for 48 h. After that, 20 μL MTT solution (5 mg/mL) was added to each well and incubated for another 4 h. The crystals were dissolved in 100 μL of DMSO to each well. Absorbance values were measured at 490 nm with BioTek Synergy 2 multifunction microplate reader (USA). The cell viability was calculated according to the following equation: Cell viability (%) = $A_{490\ (sample)}/A_{490\ (control)} \times 100$.

3.7 Cell Culture and Imaging

Lung carcinoma cell line A549 cells were obtained from American Type Culture collection and were grown in F12 supplemented with 10% FBS and 1% L-Glutamine. Cells were incubated in a 5% CO_2 humidified incubator at 37 ^oC and typically passaged with sub-cultivation ratio of 1:4 every two days. A549 cells were seeded in in 24-well plates in culture medium. For fluorescence microscopy, the cells were incubated under normoxic (20 % pO_2) and hypoxic (5 %, 1% pO_2) condition for 8 h at 37 ^oC. Then the cells were washed with PBS buffer (pH 7.4) and were treated with 1μM **NBP** in FBS-free F12 for 2 h, 1h and 0.5 h. Before taken images, A549 cells were washed three times with PBS buffer. Fluorescence imaging was performed with Nikon. The fluorescent field was collected with 2 s exposure time using a Texas Red filter and with 300 ms exposure time for bright field.

4. Data

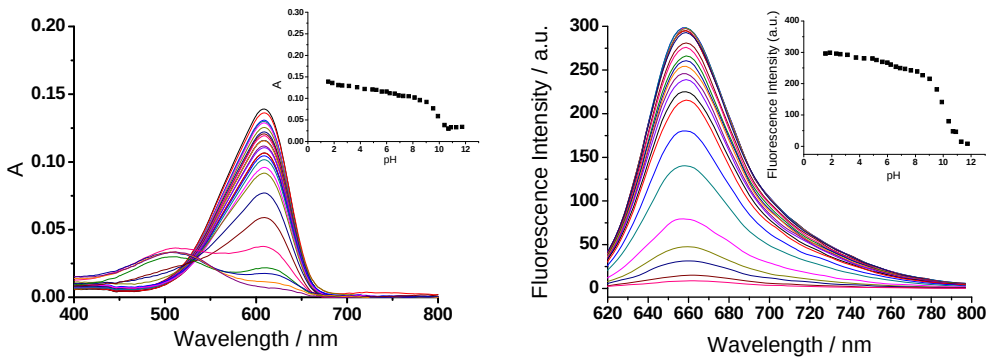


Fig. S1 The pH titration of fluorophore **NBF** (1.2 μM) in H_2O (1% DMSO as co-solution).

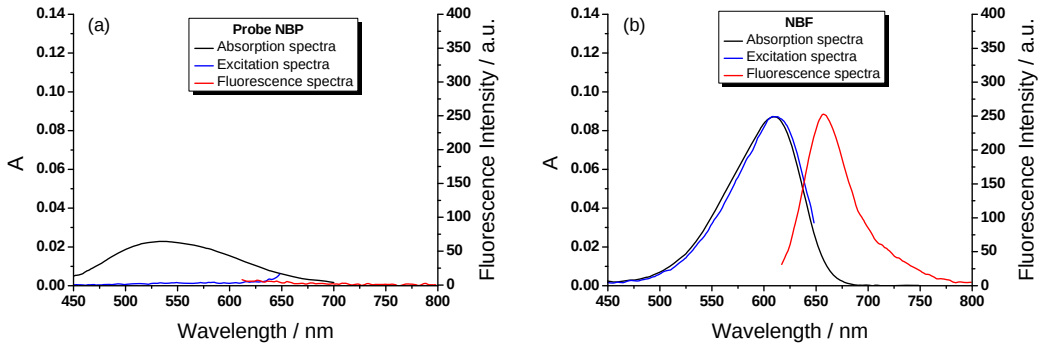


Fig. S2 The UV absorption/ excitation spectra and fluorescent emission spectra of (a) 1.2 μM probe **NBP** and (b) 1.2 μM **NBF** in 0.01 M PBS buffer (pH 7.4, 1% DMSO). Excitation wavelength was 580 nm; slit (5/5).

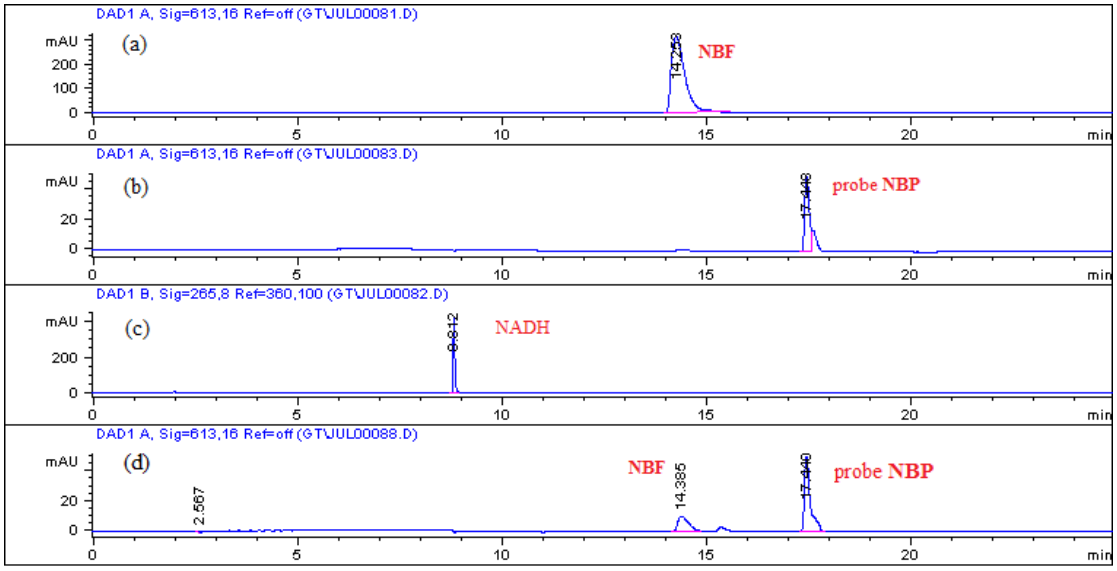


Fig. S3 HPLC chromatogram of probe **NBP** (10 μM) and NADH (0.5 mM) upon treatment with Nitroreductase for 60 min. Chromatogram of dye **NBF** (a), unreacted probe **NBP** (b) and 0.5 mM NADH (c) are also showed. HPLC profiles were detected by UV/Vis at 265 nm and 613 nm.

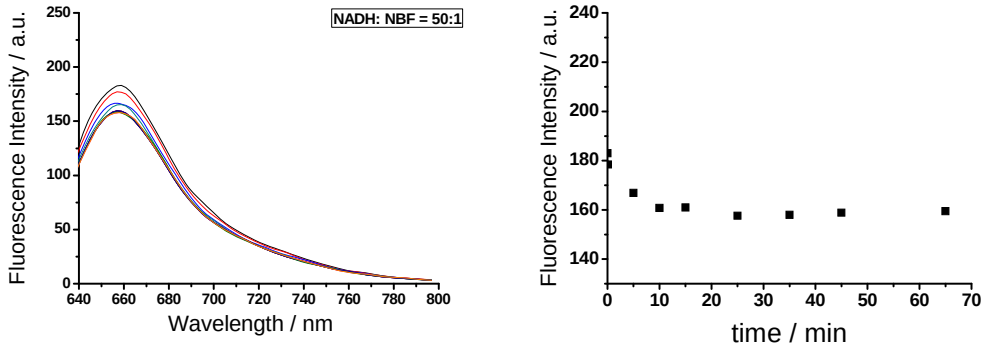


Fig. S4 Reaction–time profile of dye **NBF** (1 μM) in the presence of 50 equiv of NADH in 0.01 M PBS buffer (pH 7.4, 1% DMSO). Excitation wavelength was 580 nm.

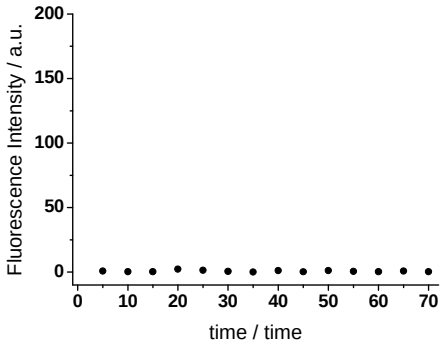


Fig. S5 Reaction–time profile of the probe **NBP** (1 μM) in the presence of 50 equiv of NADH in 0.01 M PBS buffer (pH 7.4, 1% DMSO). Excitation wavelength was 580 nm.

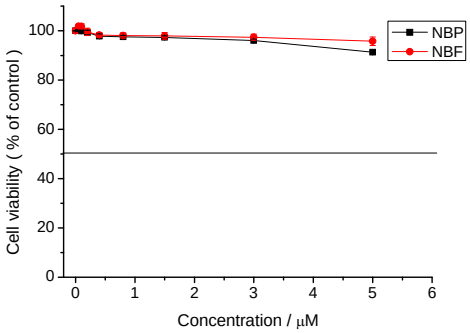


Fig. S6 Cell Viability Assays of **NBP** and **NBF**

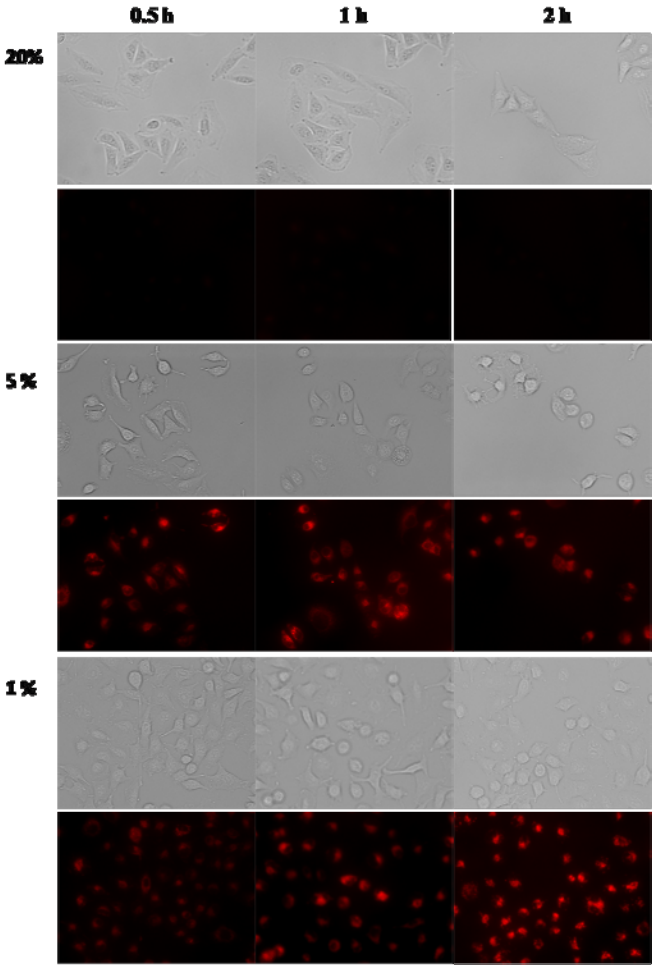


Fig. S7 Fluorescence and bright field images of A549 cells to different oxygen concentration. The cells were incubated under normoxic (20% pO_2) and different hypoxia (5%, 1% pO_2) condition for 8 h, and then treated with **NBP** (1 μM) for 2 h, 1 h and 0.5 h.

5. NMR spectra

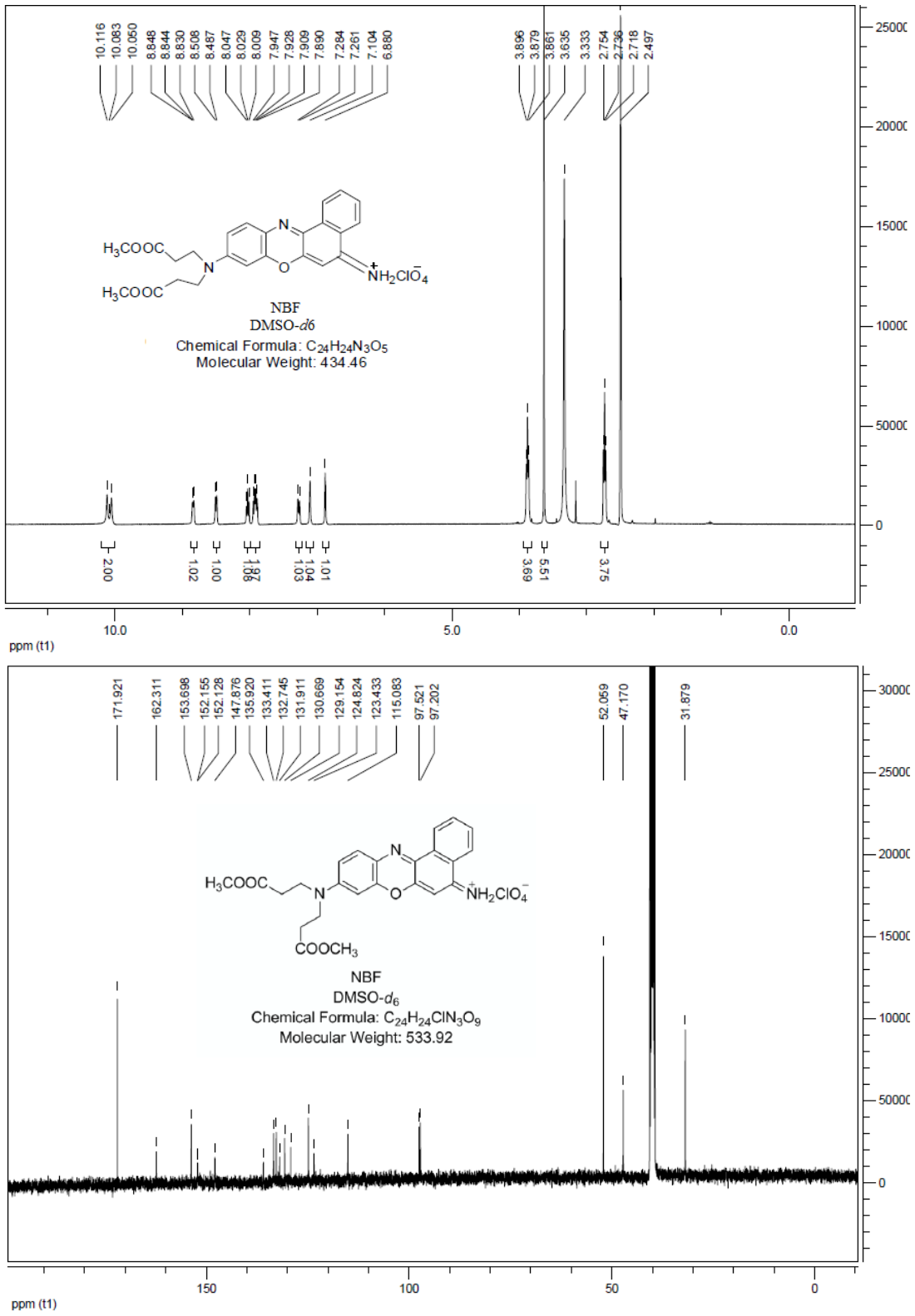


Fig. S8 The ^{1}H-NMR and ^{13}C-NMR spectrum of **NBF**

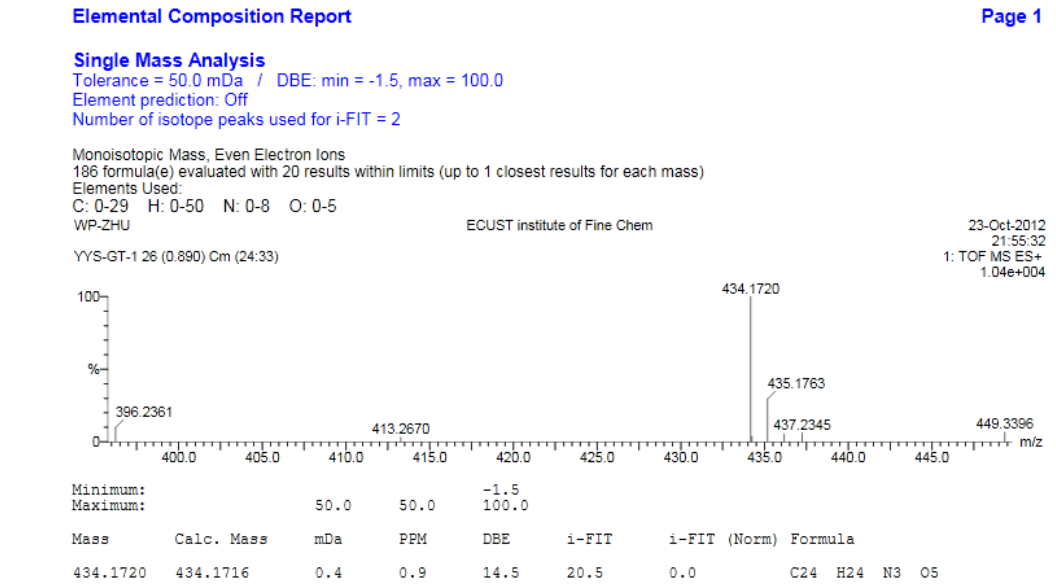
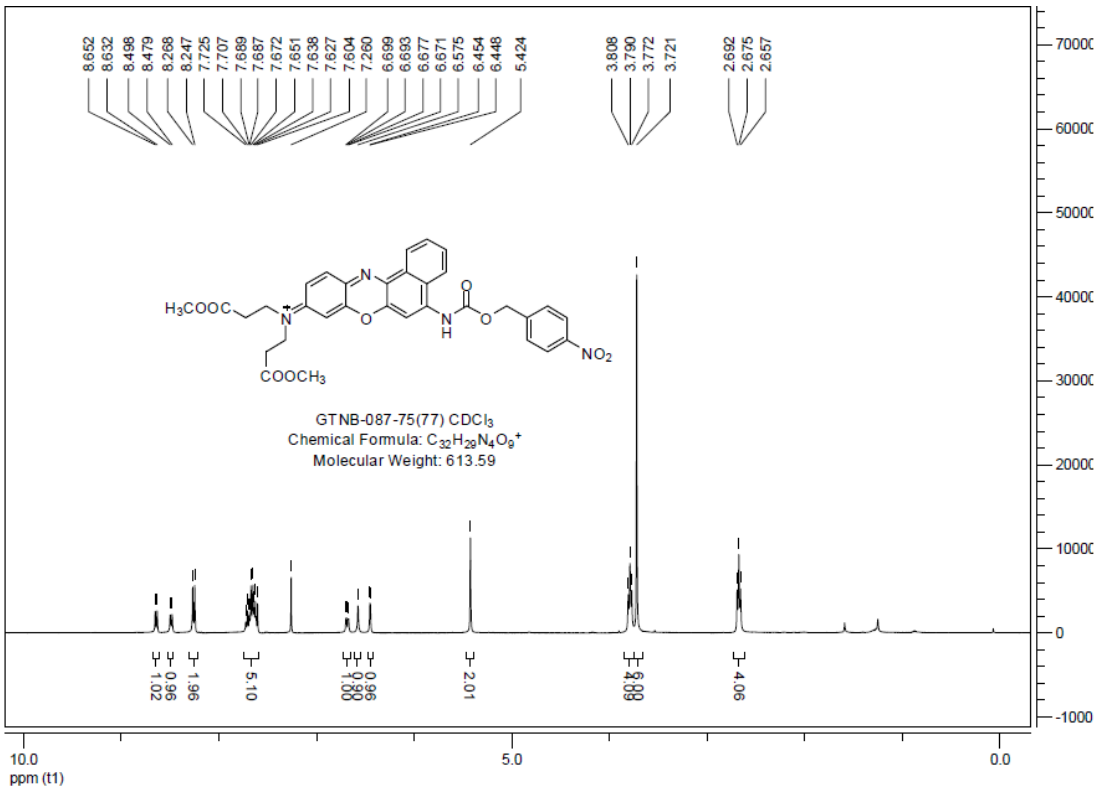


Fig. S9 The HR-MS spectrum of **NBF**



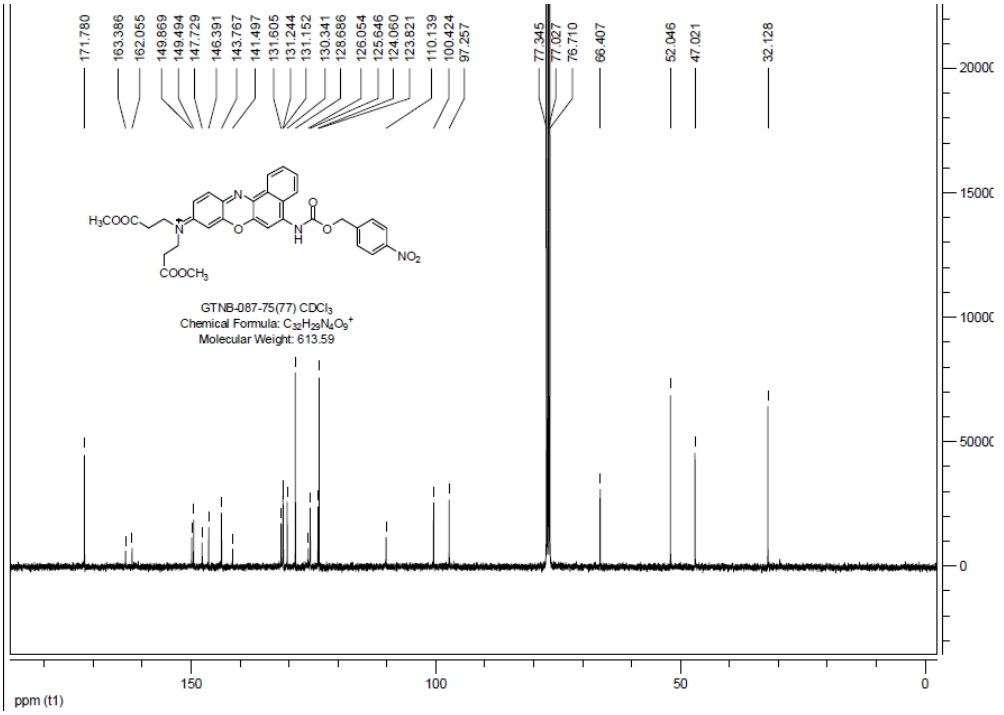


Fig. S10 The ^{1}H-NMR and ^{13}C-NMR spectrum of **NBP**

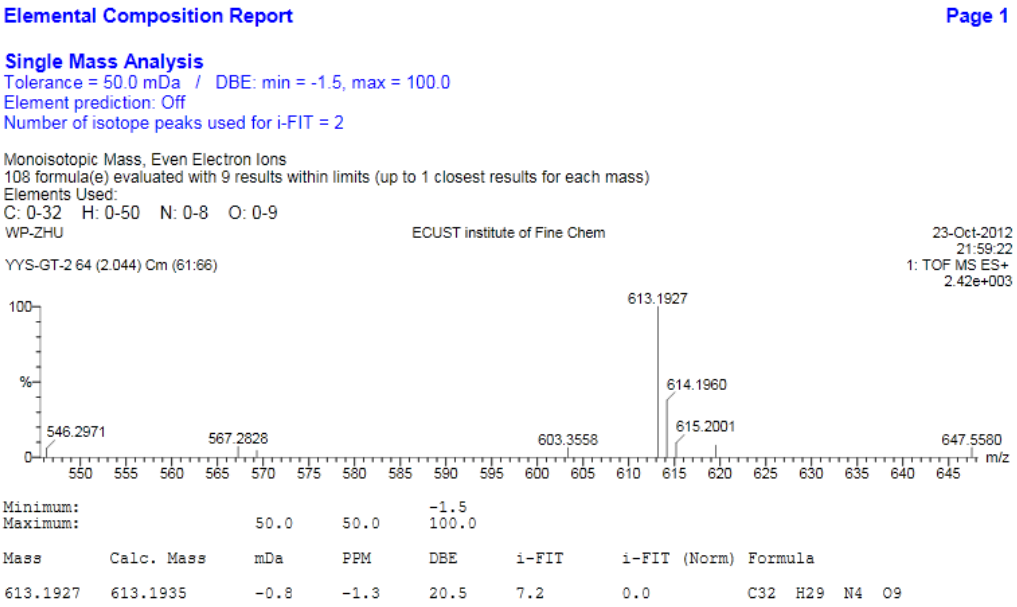


Fig. S11 The HR-MS spectrum of **NBP**

6. References

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(S2) N.-h. Ho, R. Weissleder and C.-H. Tung, Tetrahedron, **2006**, 62, 578.

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