

Electronic Supplementary Information for:

7-((5-Nitrothiophen-2-yl)methoxy)-3*H*-phenoxazin-3-one as a spectroscopic off-on probe for highly sensitive and selective detection of nitroreductase

Zhao Li, Xinghui Gao, Wen Shi, Xiaohua Li and Huimin Ma*

Beijing National Laboratory for Molecular Sciences, Key Laboratory of Analytical Chemistry for Living Biosystems, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China. E-mail: mahm@iccas.ac.cn

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1. Apparatus and reagents

^1H NMR and ^{13}C NMR spectra were measured on a Bruker DMX-600 spectrometer in CF_3COOD . Electrospray ionization mass spectrum was recorded in negative mode with a Shimadzu LC-MS 2010A instrument (Kyoto, Japan). Elemental analyses were performed on a Flash EA 1112 instrument. A model HI-98128 pH meter (Hanna Instruments Inc.) was employed for pH measurements. Absorption spectra were recorded in 1 cm quartz cells with a TU-1900 spectrophotometer (Beijing, China). Fluorescence measurements were performed on a Hitachi F-2500 spectrofluorimeter in 10×10 mm quartz cells (Tokyo, Japan), with a 400 V PMT voltage. Fluorescence quantum yield (Φ) was determined by using resorufin ($\Phi = 0.75$ in aqueous solutions) as a standard.

Resorufin sodium salt, 5-nitrothiophene-2-carbaldehyde, nitroreductase (≥ 100 units/mg) from *Escherichia coli*, and reduced nicotinamide adenine dinucleotide (NADH) were purchased from Sigma-Aldrich. The lyophilized powder of nitroreductase was dissolved in pure water, and the solution was divided into 20 parts as suitable amounts for daily experiments. All these enzyme solutions were frozen immediately at $-20\text{ }^\circ\text{C}$ for storage and allowed to thaw before use according to the known procedure (E. Mylon and S. Roston, *Am. J. Physiol.*, 1953, **172**, 612-616). A phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , pH 7.4) solution was obtained from Invitrogen Company. All other chemicals used were local products of analytical grade. A stock solution (1 mM) of probe **1** was prepared by dissolving an appropriate amount of **1** in DMSO. Ultrapure water (over $18\text{ M}\Omega\cdot\text{cm}$) from a Milli-Q reference system (Millipore) was used throughout.

2. Synthesis of **1**

Firstly, the raw material **2** was prepared as follows. A solution of 5-nitrothiophene-2-carbaldehyde (157 mg, 1 mmol) in methanol (20 mL) was stirred at $0\text{ }^\circ\text{C}$, and then powdered sodium borohydride (60 mg, 1.5 mmol) was added. The mixture was stirred for 5 h at $30\text{ }^\circ\text{C}$, and the solvent was removed under reduced pressure. Then, water (20 mL) was added and the resulting mixture was extracted with dichloromethane ($30\text{ mL}\times 3$). The extracts were washed with water ($30\text{ mL}\times 3$) and brine ($30\text{ mL}\times 3$), and then dried over Na_2SO_4 . The

solvent was removed by evaporation. The obtained crude product [(5-nitrothiophen-2-yl)methanol (160 mg, 1 mmol)] and tribromophosphine (0.14 mL, 1.5 mmol) were dissolved in dichloromethane (20 mL) at 0 °C, and the resulting reaction solution was stirred for 5 h at 30 °C. Then, the mixture was washed with saturated NaHCO₃ (10 mL) and brine (30 mL × 3). The organic layer was dried over Na₂SO₄ and filtered. After removal of the solvent by evaporation, the obtained crude product, 2-(bromomethyl)-5-nitrothiophene (**2**), was directly used in the following reaction.

To a suspension of resorufin sodium salt (0.24 g, 1.0 mmol) in anhydrous DMF (10 mL), K₂CO₃ (0.21 g, 1.5 mmol) was added, followed by stirring at 40 °C for 10 min under an Ar atmosphere. Then, a solution of **2** (0.23 g, 1.0 mmol) in DMF (2 mL) was added dropwise. The resulting mixture was stirred at 40 °C for 2 h and then diluted with dichloromethane (20 mL). The organic layer was separated, washed with water (50 mL × 3) and brine (50 mL × 3), and then dried over Na₂SO₄. The solvent was removed by evaporation, and the residue was subjected to silica gel chromatography, eluted with petroleum ether (b.p. 60-90 °C)/ethyl acetate (v/v, 1:1), affording 7-((5-nitrothiophen-2-yl)methoxy)-3*H*-phenoxazin-3-one (**1**) as an orange solid (0.14 g, 40%). The ¹H NMR and ¹³C NMR spectra of **1** are given below in Figs. S1 and S2, respectively. ¹H NMR (600 MHz, 298 K, CF₃COOD): δ 8.40 (d, *J* = 9.4 Hz, 1H), 8.36 (d, *J* = 9.4 Hz, 1H), 7.92 (d, *J* = 4.2 Hz, 1H), 7.69 (t, *J* = 2.8 Hz, 1H), 7.67 (t, *J* = 2.8 Hz, 1H), 7.56 (d, *J* = 2.5 Hz, 1H), 7.48 (d, *J* = 2.3 Hz, 1H), 7.20 (d, *J* = 4.2 Hz, 1H), 5.62 (s, 2H). ¹³C NMR (150 MHz, 298 K, CF₃COOD): δ 175.2, 170.5, 151.3, 150.6, 149.3, 144.4, 138.6, 136.2, 136.1, 134.7, 129.0, 126.4, 125.2, 123.2, 101.7, 99.2, 66.5. Elemental analysis, calcd. for **1** (C₁₇H₁₀N₂O₅S): C 57.62, H 2.84, N 7.91, S 9.05%; found: C 57.18, H 2.93, N 8.00, S 8.98%.

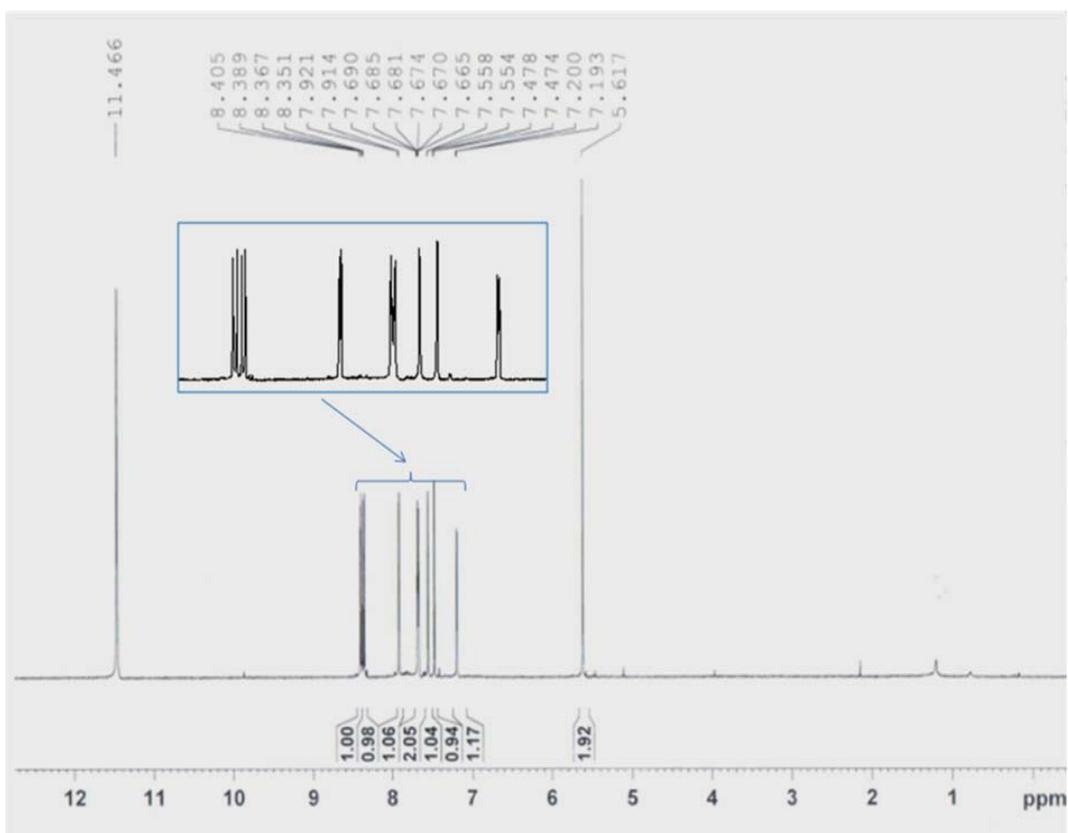


Fig. S1 ^1H NMR spectrum of **1** (600 MHz, CF_3COOD , 298K).

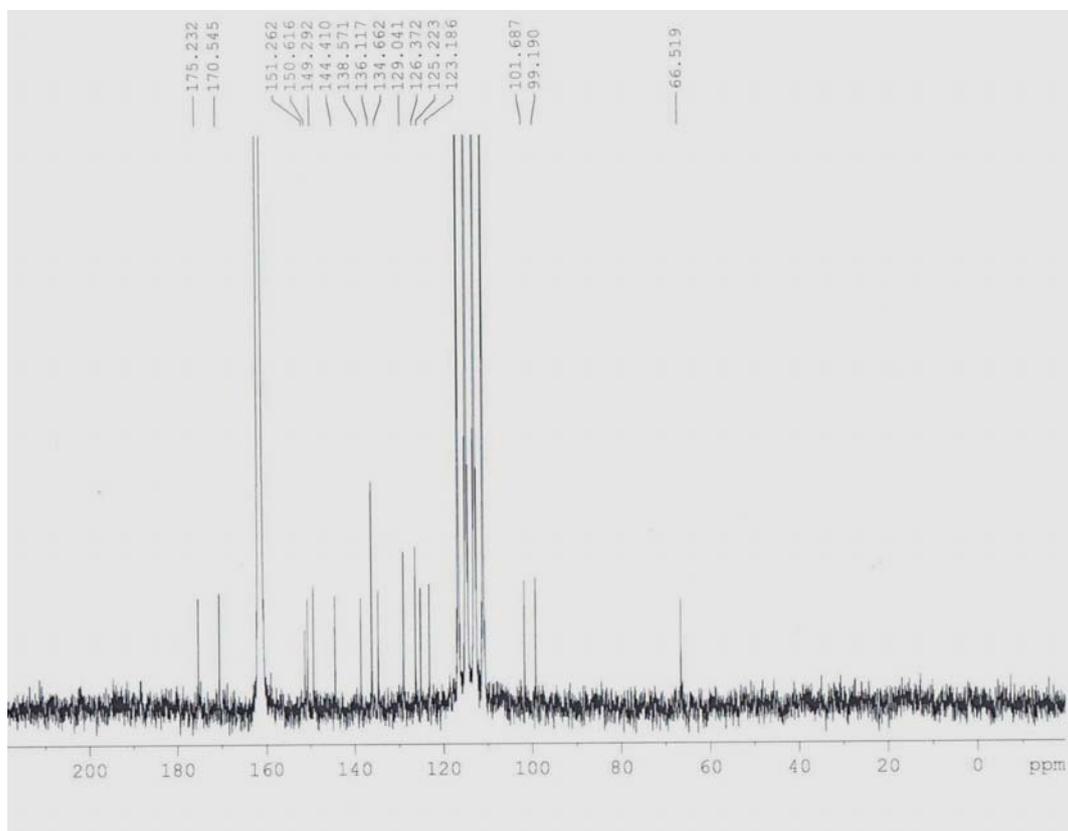


Fig. S2 ^{13}C NMR spectrum of **1** (150 MHz, CF_3COOD , 298K).

3. General procedure for nitroreductase detection

Unless otherwise stated, all the fluorescence measurements were made in 10 mM PBS (pH 7.4) according to the following procedure. In a 10 mL tube, 5 mL of PBS and 50 μ L of 1 mM **1** were mixed, followed by addition of NADH (final concentration, 50 μ M) and an appropriate volume of nitroreductase sample solution. The final volume was adjusted to 10 mL with PBS and the reaction solution was mixed rapidly. After incubation at 37 $^{\circ}$ C for 20 min in thermostat, a 3 mL portion of the reaction solution was transferred to a quartz cell of 1 cm optical length to measure absorbance or fluorescence with $\lambda_{\text{ex/em}} = 550/585$ nm and both excitation and emission slit widths of 10 nm (note that the resorufin's maximum excitation wavelength of 570 nm was not used to decrease the effect of scattered light). In the meantime, a blank solution containing no nitroreductase (control) was prepared and measured under the same conditions for comparison.

4. Effects of pH and temperature

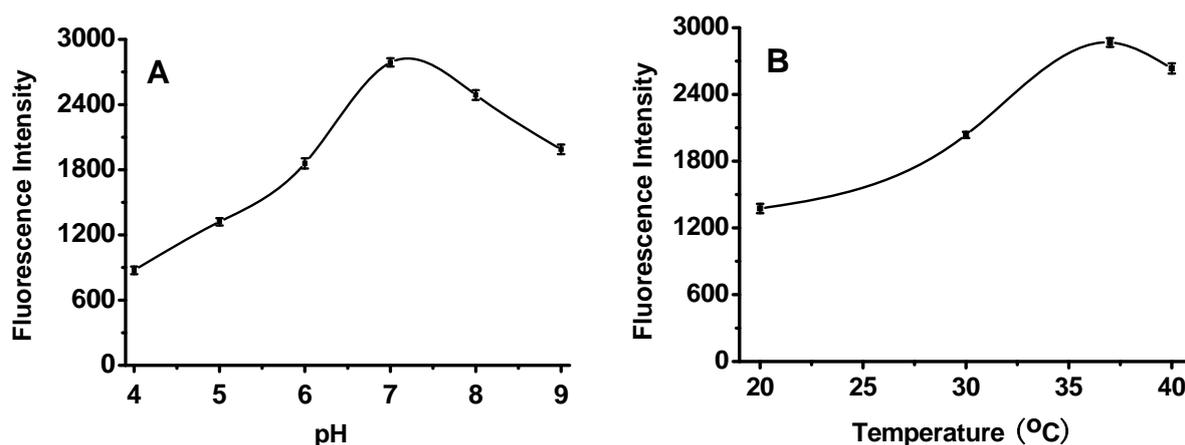


Fig. S3 Effects of (A) pH and (B) temperature on the fluorescence ($\lambda_{\text{ex/em}} = 550/585$ nm) of **1** (5 μ M) reacting with nitroreductase (1 μ g/mL) in the presence of 50 μ M NADH. The results are the mean \pm standard deviation of three separate measurements.

5. Fluorescence kinetic curves of **1** reacting with nitroreductase

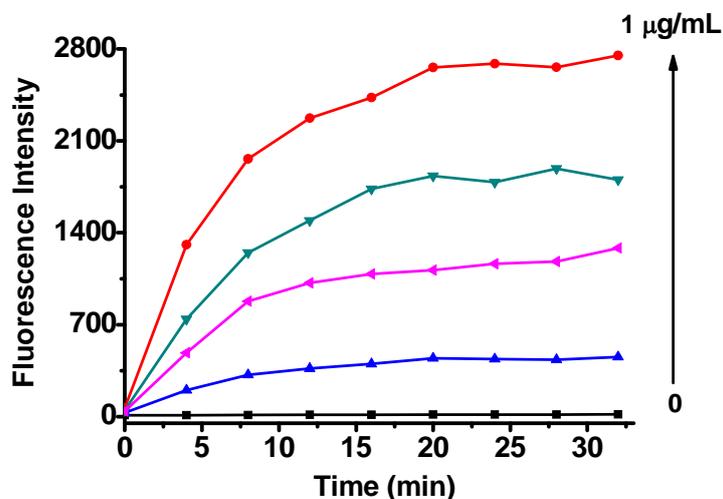


Fig. S4 Plots of fluorescence intensity of **1** (5 μM) vs. the reaction time in the presence of varied concentrations of nitroreductase (from bottom to top): 0 (control), 0.05, 0.25, 0.5 and 1 μg/mL. The measurements were performed at 37 °C in 10 mM PBS (pH 7.4) with $\lambda_{\text{ex/em}} = 550/585$ nm.

6. Electrospray ionization mass spectrum of the reaction solution of **1**

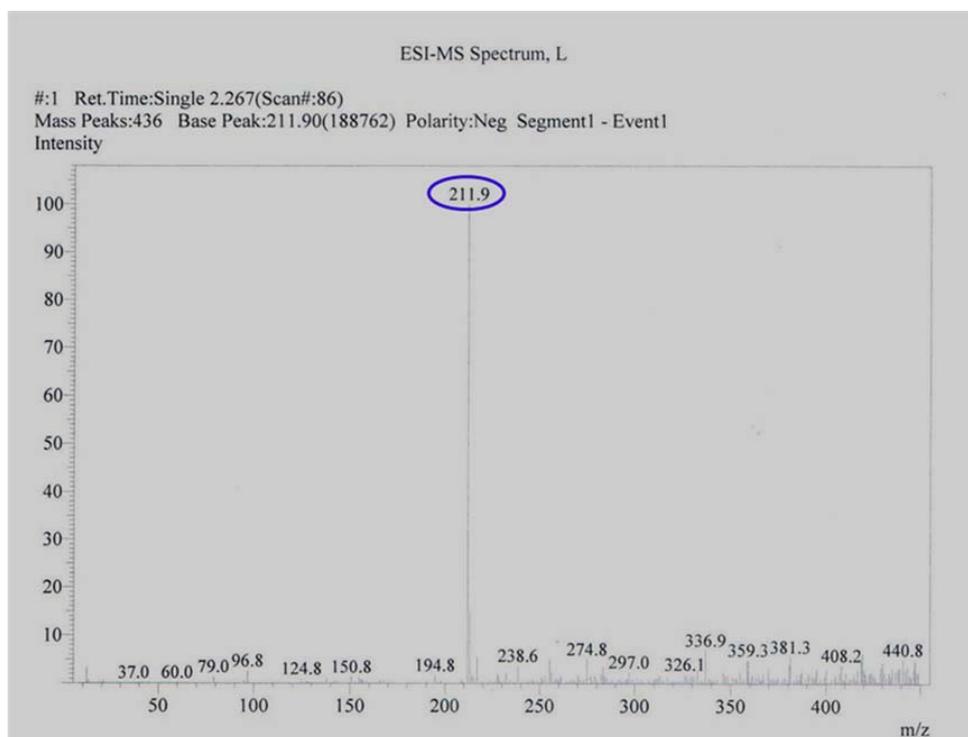


Fig. S5 Electrospray ionization mass spectrum of the reaction solution of **1** (50 μM) with nitroreductase (1 μg/mL).

7. Kinetic parameters for the enzymatic cleavage reaction of **1**

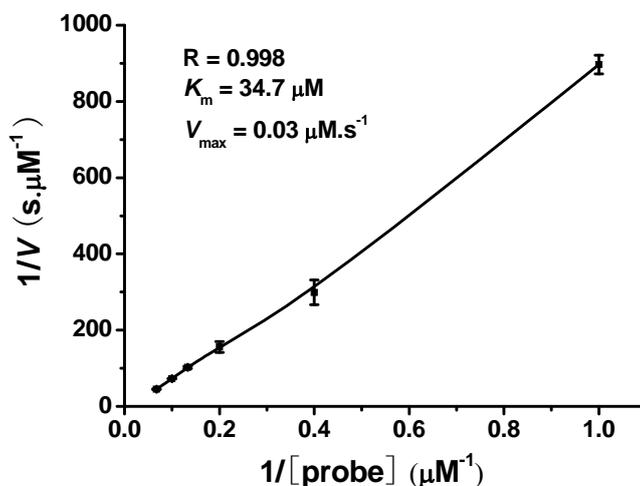


Fig. S6 Lineweaver-Burk plot for the enzyme-catalyzed reaction. The Michaelis-Menten equation was described as: $V = V_{\max}[\text{probe}]/(K_m + [\text{probe}])$, where V is the reaction rate, $[\text{probe}]$ is the probe concentration (substrate), and K_m is the Michaelis constant. Conditions: 0.20 $\mu\text{g}/\text{mL}$ nitroreductase, 50 μM NADH, 1 - 20 μM of probe **1**, $\lambda_{\text{ex/em}} = 550/585$ nm. Reaction at each probe concentration was repeated three times, and the error bars represent standard deviations. Points were fitted using a linear regression model (correlation coefficient $R = 0.998$).

8. Docking study on the complex of nitroreductase with probe **1**

Surflex-dock module, which is available on SYBYL version 1.1 (Tripos Inc.), was used to evaluate the binding affinity between **1** and nitroreductase. The crystal structure of nitroreductase complex was collected from PDB under code 4DN2.

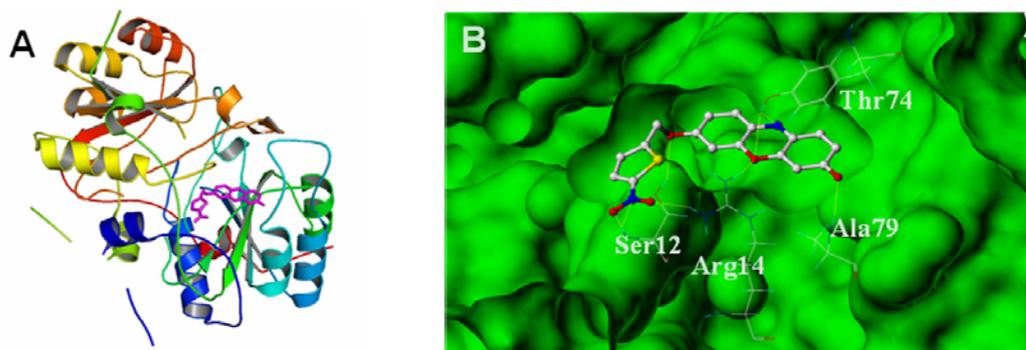


Fig. S7 (A) The docked conformer of **1** at the binding cleft of nitroreductase (generated *via* Surflex docking-scoring combinations). (B) The possible binding of the four amino acids residues (Ser12, Arg14, Thr74 and Ala79) of nitroreductase with **1**.

9. Selectivity study

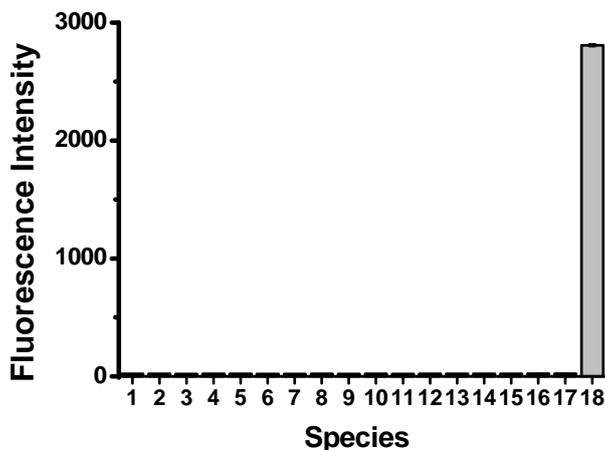


Fig. S8 Fluorescence responses of **1** (5 μM) in the presence of NADH (50 μM) to various species: (1) control (probe **1** + NADH), (2) 150 mM KCl, (3) 2.5 mM CaCl₂, (4) 2.5 mM MgCl₂, (5) 10 mM glucose, (6) 1 mM vitamin C, (7) 1 mM vitamin B₆, (8) 100 μM HSA, (9) 10 μM H₂O₂, (10) 10 μM $\cdot\text{OH}$, (11) 1 mM glutamic acid, (12) 1 mM arginine, (13) 1 mM serine, (14) 5 mM glutathione, (15) 1 mM cysteine, (16) 1 mM homocysteine, (17) 1 mM dithiothreitol, (18) 0.5 $\mu\text{g/mL}$ nitroreductase. The results are the mean \pm standard deviation of three separate measurements. $\lambda_{\text{ex/em}} = 550/585$ nm.

10. Effect of dicoumarin on the activity of nitroreductase

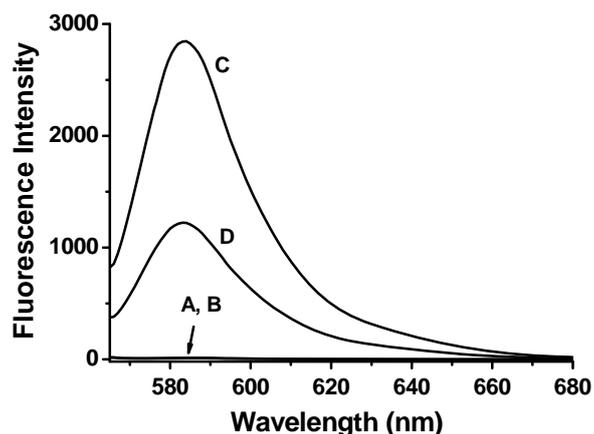


Fig. S9 Fluorescence emission spectra ($\lambda_{\text{ex}} = 550$ nm) of different reaction systems. (A): probe **1** (5 μM) in PBS buffer of pH 7.4 (control); (B): the system (A) + 50 μM NADH (another control); (C): the system (B) + nitroreductase (0.5 $\mu\text{g/mL}$); (D): the system (C) + dicoumarin (0.1 mM). All the reactions were performed at 37 $^{\circ}\text{C}$ for 20 min.

11. Real-time detection of nitroreductase produced by *Escherichia coli*

Escherichia coli (DH5 α) was purchased from Beijing Biofuture Institute of Bioscience & Biotechnology Development. The Luria–Bertani (LB) culture medium was prepared by dissolving 10 g bactotryptone, 5 g bacto yeast extract, and 10 g NaCl in 1 L water, followed by adjusting the pH to 7.4 with 1 M of NaOH. The culture medium was autoclaved prior to use.

For determining nitroreductase generated by *Escherichia coli*, the bacteria was first grown at 37 °C in LB culture media (pH 7.4) for 12 h. Then, the bacterial colonies were harvested using a sterile swab and inoculated into 50 mL of fresh LB culture media with an OD₆₀₀ of 0.2. An appropriate volume (typically 3 mL) of the 50 mL LB culture media was taken and then pre-incubated at 37 °C in a rotary shaker for different periods of time (0 – 4 h). After addition of 15 μ L of probe **1** (final concentration: 5 μ M) and then reaction for 20 min, both absorbance and fluorescence of the reaction solutions were measured. It should be noted that no NADH was added to the reaction solution containing *Escherichia coli*, because *Escherichia coli* itself also produces NADH (e.g., see Sanchez, et al., *J. Biotechnol.*, 2005, **117**, 395-405). On the other hand, a corresponding calibration curve of **1** reacting with nitroreductase in the cell-free LB culture media containing 50 μ M NADH was constructed to eliminate any possible interference, and with the calibration curve the concentration of nitroreductase generated by *Escherichia coli* was quantitatively determined in real time (see Table 1).

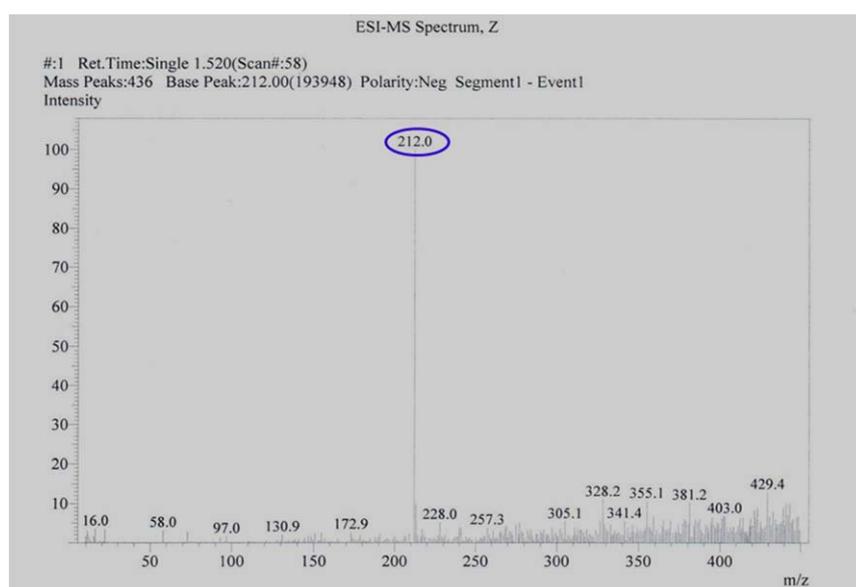


Fig. S10 Electrospray ionization mass spectrum of the reaction solution of **1** (50 μ M) with *Escherichia coli* in the LB culture media.

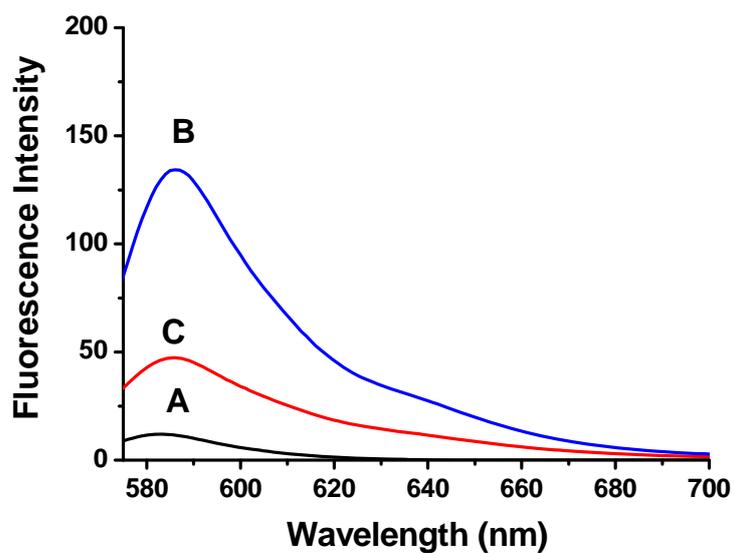


Fig. S11 Fluorescence emission spectra ($\lambda_{\text{ex}} = 550 \text{ nm}$) of different reaction systems. (A): probe **1** ($5 \mu\text{M}$) in LB culture media of pH 7.4 (control); (B): the system (A) + *Escherichia coli* ($\text{OD}_{600} = 0.2$); (C): the system (B) + dicoumarin (0.1 mM). All the reactions were performed at $37 \text{ }^\circ\text{C}$ for 20 min.