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An HBT-based fluorescent probe for nitroreductase determination and its application in *Escherichia coli* cell imaging

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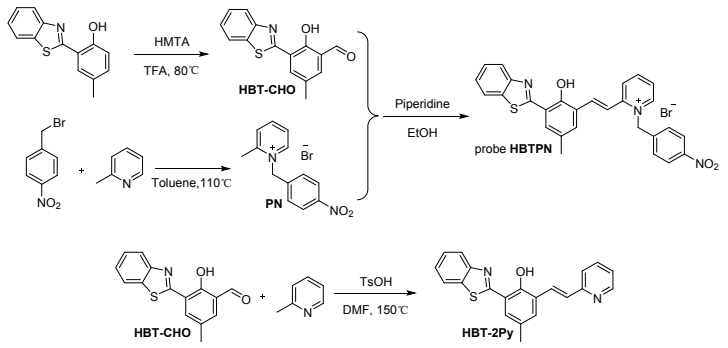
1. General procedure

All reagents were obtained commercially without further purification. Both NTR and NADH were obtained from Sigma-Aldrich Co. Ltd.. Nitroreductase solution was prepared by dissolving the lyophilized powder into ultrapure water and then divided into several parts and stored at -20 °C to keep the enzyme activity. NMR spectra were measured on a Bruker 400M NMR spectrometer and ESI mass spectra were measured on a Bruker micrOTOF-Q II mass spectrometry. The stock solution of probes was prepared in dimethyl sulfoxide (DMSO) with a concentration of 0.5 mM. UV-absorption spectra were carried out on a Perkin Elmer Lambda-35 UV-visible double beam scanning spectrophotometer. Solution fluorescence spectral studies were measured on a FluoroMax-3 spectrofluorometer (HORIBA Jobin Yvon, Inc., NJ, USA). *Escherichia coli* (DH5a) was received from the laboratory of Prof. B. Qing at Lanzhou Institute of Chemical Physics. All fluorescence images were recorded on Olympus Fluoview 1200 confocal fluorescence microscope using a 60× oil immersed optics.

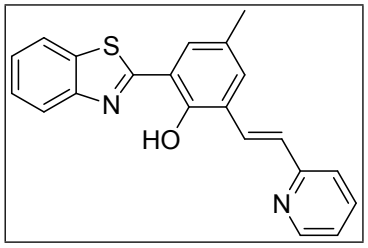
2. Synthesis



Scheme S1 Synthesis routes of probe **HBTPN** and dye **HBT-2Py**.

Compound HBT-CHO were obtained following the previously reported procedures in good yield. In brief, 2-Aminothiophenol (1.00 g, 8 mmol), 2-Hydroxy-5-methylbenzaldehyde (1.088 g, 8 mmol) and aq HCl (37%, 2 mL) were dissolved in

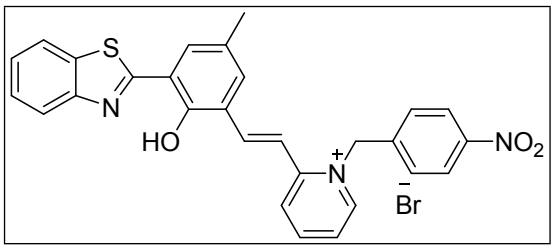
ethanol (30 mL). After stirring for 5 min, aq H_2O_2 (30%, 5 mL) was added and continue to stir at room temperature for 1 h. The precipitate was then filtered, washed with cold ethanol, dried in vacuo to give compound HBT as white powder (1.308 g, yield: 67.8%). Hexamethylenetriamine (HMTA, 1.68 g, 12 mmol) and compound HBT (964 mg, 4 mmol) dissolved in 15 ml trifluoroacetic acid (TFA) and the solution was refluxed for 6 h. After being cooled to room temperature, the mixture was evaporated under reduced pressure. The residues were dissolved in CH_2Cl_2, washed with water, dried over anhydrous $MgSO_4$ and then dried in vacuo to give compound HBT-CHO as light-yellow powder (962 mg, yield: 89.4%).



2-(benzo[d]thiazol-2-yl)-4-methyl-6-(2-(pyridin-2-yl)vinyl)phenol (HBT-2Py)

2-Methylpyridine (1.4 mmol, 137 μL) and compound HBT-CHO (1.0 mmol, 269 mg) were dissolved in DMF (10 mL), after which *p*-toluenesulfonic acid monohydrate (323mg 1.7mmol) was added. The mixture was refluxed at 150 °C for 6 h with stirring under an argon atmosphere. After being cooled to room temperature, the mixture was diluted with CH_2Cl_2, washed with water, dried with $MgSO_4$ and then evaporated in vacuo. The residues were purified by flash chromatography (CH_2Cl_2/ethyl acetate, 3/1, v/v) to afford fluorophores **HBT-2Py** as a yellow powder (132 mg, yield: 38.3%). M.P. 216.4-216.9 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 13.03 (s, 1H, Ar-O*H*), 8.62 (d, J=4 Hz, 1H, Ar-*H*), 7.13-8.02 (m, 11H, Ar-*H,* vinyl-*H*), 2.37 (s, 3H, Ar-CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ:169.49, 156.27, 154.29, 151.77, 149.43, 136.56, 132.66, 131.62, 129.28, 128.51, 128.43, 127.70, 126.71, 125.55, 125.51, 122.14, 121.87, 121.71, 121.50, 116.73, 20.61. HRMS: m/z $[M+1]^+$

calcd for: $C_{21}H_{17}N_2OS^+$ 345.1056, found 345.1053.



(E)-2-(3-(benzo[d]thiazol-2-yl)-2-hydroxy-5-methylstyryl)-1-(4-nitrobenzyl)pyridin-1-ium bromide (probe HBTPN)

2-Methylpyridine (3.03 mmol, 300 μL) and 4-nitrobenzyl bromide (3.0 mmol, 648 mg) were dissolved in toluene (20 mL). The mixture was refluxed at 110 °C for 12 h. After being cooled to room temperature, the precipitate was filtered, washed with toluene, and dried in vacuo to afford pure compound **NP** as a white powder. (790 mg, yield: 85.1%). This compound was used without further purification. Subsequently, compound **NP** (1.0 mmol, 309 mg) and compound HBT-CHO (1.0 mmol, 269 mg) were dissolved in ethanol (20 mL), after which 1.0 mmol piperidine was added. The mixture was refluxed for 1-2 h and then evaporated in vacuo. The residues were purified by flash chromatography (CH_2Cl_2/MeOH, 40/1, v/v) to afford probe **HBTPN** as a yellow powder (130 mg, yield: 23.2 %). M.P. 194.6-195.5 °C. ^{1}H-NMR (400 MHz, DMSO) δ: 13.04 (s, 1H, Ar-O*H*), 9.22 (d, J=4 Hz, 1H, Ar-*H*), 7.52-8.67 (m, 15H, Ar-*H,* vinyl-*H*), 6.32 (s, 2H, N-CH_2), 2.39 (s, 3H, Ar-CH_3). ^{13}C-NMR (100 MHz, DMSO) δ:168.52, 154.52, 153.08, 151.25, 147.97, 146.90, 146.18, 141.90, 138.38, 133.40, 133.06, 131.74, 129.66, 129.24, 127.67, 126.60, 126.45, 124.60, 123.59, 122.96, 122.46, 118.96, 117.76, 60.11, 20.41. HRMS: m/z [M-Br]$^+$ calcd for: $C_{28}H_{22}N_3O_3S^+$ 480.1376, found 480.1371.

3. NTR detection assay

Unless specially stated, spectroscopic evaluation of **HBTPN** was conducted in Tris-HCl buffer solution (50 mM, pH 7.4). In a 10 mL tube, 50 μL probe stock solution (0.5

mM in DMSO), 50 μL NADH solution (2.5 mM in water), and 50 μL of NTR sample solution (appropriate concentration) were added, and dilute the solution to 5 mL with Tris-HCl buffer. After being incubated at 37 °C for 10 min, part of the mixture was filled into the cuvette to test absorbance or emission signal.

4. *E. coli* cell imaging experiments

Escherichia coli (DH5α) were cultured in Luria–Bertani (LB) culture media (pH 7.4) media at 37 °C for 12 h. After that the cells were harvested and washed twice with Tris-HCl buffer (pH 7.4). Bacteria were then resuspended in Tris-HCl buffer without lysis to test the absorbance and subsequently incubated with **HBTPN** (5 μM) at 37 ℃ for 10 min to test the emission spectra.

E. coli cells were transformed to special coverslips and incubated in LB culture media overnight. Then the cells were washed with Tris-HCl buffer three times, incubated with **HBTPN** (10 μM) for 10 min. Fluorescence images were acquired using an Olympus FV1200 confocal fluorescence microscope with a 60 × oil–immersion objective lens.

5. DFT and docking calculations

The density functional theory (DFT) calculations were performed for molecules in water phase by using the Gaussian 09 package. PBEPBE/6-31+G*functional was chosen to study the properties of the ground state and singlet excited state in the time–dependent DFT (TDDFT) method.

The molecular docking study was conducted on AutoDock suite program (version 4.2.6) and the crystal structure of NTR could be download from Protein Data Bank with a code of 4DN2. All the binding affinity calculations and the graphics were generated using AutoDock and LigPlot^{+} software.

6. Table and spectral profile

Table S1 Comparison of various fluorescent probes for nitroreductase detection.

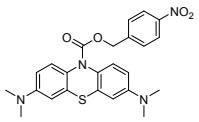
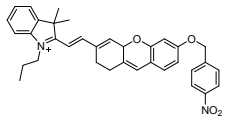
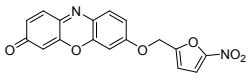
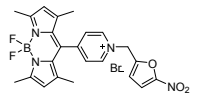
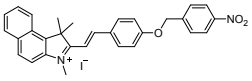
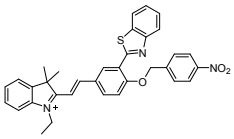
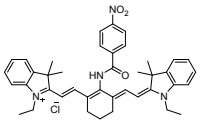
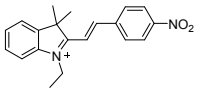
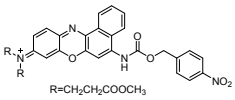
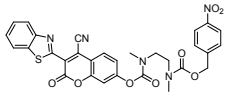
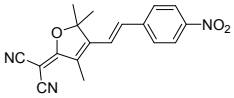
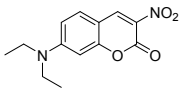
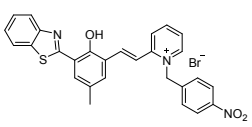
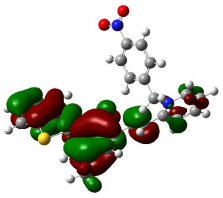
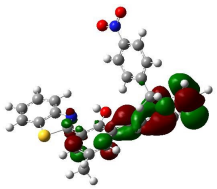
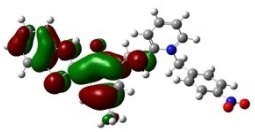
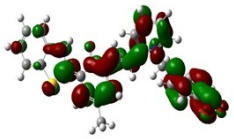
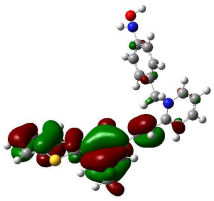
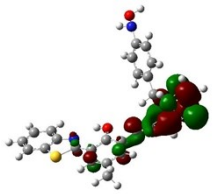
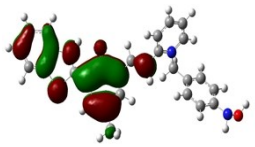
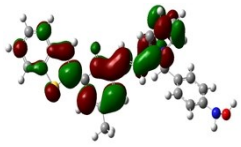
NTR probe	Stokes Shift	Emission Wavelength	Response Time	Reference
	20 nm	685 nm	>4 hour	Chem. Commun., 2015, **51**, 12787
	35 nm	705 nm	15 min	Biosens. Bioelectron., 2015, **63**, 112
	35 nm	585 nm	30 min	Anal. Chem., 2013, **85**, 3926
	40 nm	520 nm	5 min	Analyst., 2015, **140**, 574
	47 nm	570 nm	30 min	Org. Biomol. Chem., 2017, **15**, 4383
	50 nm	580 nm	2 min	Dyes. Pigments., 2017, **136**, 627
	52 nm	782 nm	10 min	J. Am. Chem. Soc., 2015, **137**, 6407
	66 nm	556 nm	30 min	RSC Adv., 2014, **4**, 56207
	78 nm	658 nm	60 min	Chem. Commun., 2013, **49**, 10820
	95 nm	595 nm	60 min	Biosens. Bioelectron., 2011, **26**, 3511
	109 nm	609 nm	Not mentioned	Chem. Sci., 2013, **4**, 220
	131 nm	511 nm	30 min	Sensors. Actuators B., 2017, **252**, 927
	236 nm	633 nm	10 min	This work

Table S2 Optical properties of **HBT-2Py** and probe **HBTPN** in different solvents.

Solvent	HBT-2Py				HBTPN			
	λ_{ex} (nm)	λ_{em} (nm)	Stokes shift	quantum yield (φ)	λ_{ex} (nm)	λ_{em} (nm)	Stokes shift	quantum yield (φ)
H_2O	397	595	198	13.5%	397	638[a]	241	0.26%
CH_3CN	377	596	218	4.2%	397	665	268	13.4%
CH_2Cl_2	379	598	219	6.4%	411	643	232	26.1%
DMSO	365	570	205	6.0%	397	673	276	1.9%
CH_3OH	376	594	218	3.8%	397	667	270	5.4%

[a] Weak fluorescence emission in water.

Table S3 Natural bond orbitals (NBOs) representing transitions contributing to the lowest energy absorption and singlet emission of probe **HBTPN** and hydroxylamine compound in water phase obtained at the TD DFT/PBEPBE/6-31+G* level of theory.

	Optical properties simulation	Holes	Electrons	Energy gap
Probe **HBTPN**	Absorption			3.2123 eV
	Emission			1.9242 eV
Hydroxylamine Compound	Absorption			3.3282 eV
	Emission			1.9305 eV

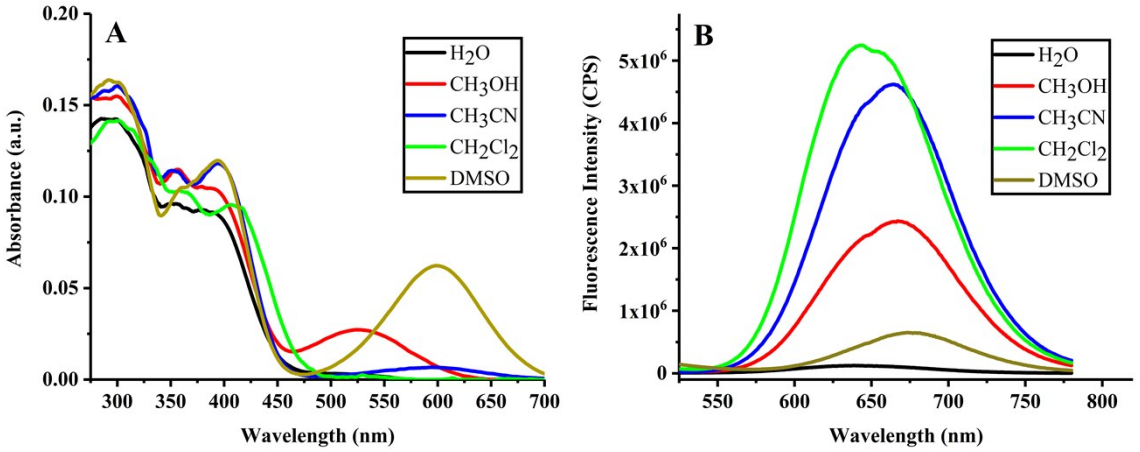


Fig. S1 (A) Absorption spectra of **HBTPN** (10 μM) in various solvents. (B) Emission spectra of **HBTPN** (5 μM) with excitation at their maximum excitation wavelength.

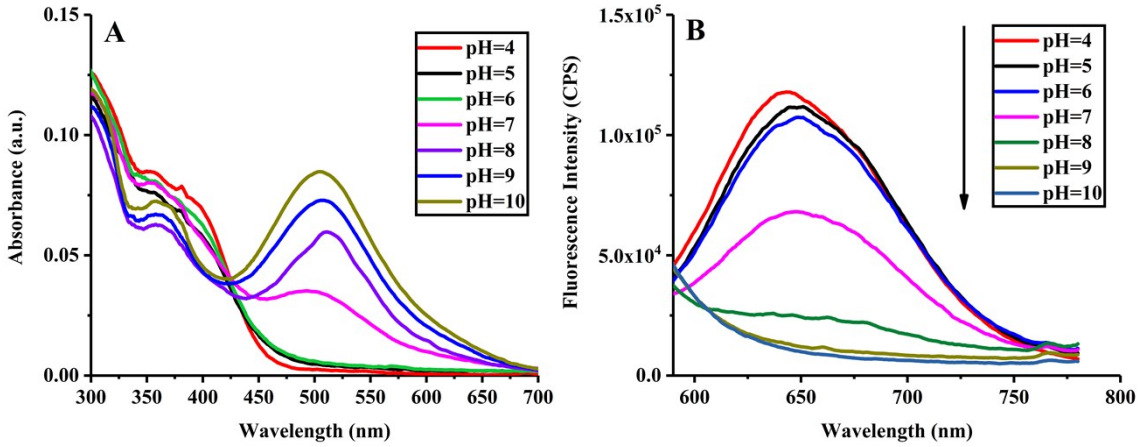


Fig. S2 (A) Absorption spectra of **HBTPN** (10 μM) at different pH in water. (B) Emission spectra of **HBTPN** (5 μM) at different pH in water, λ_{ex}=397 nm.

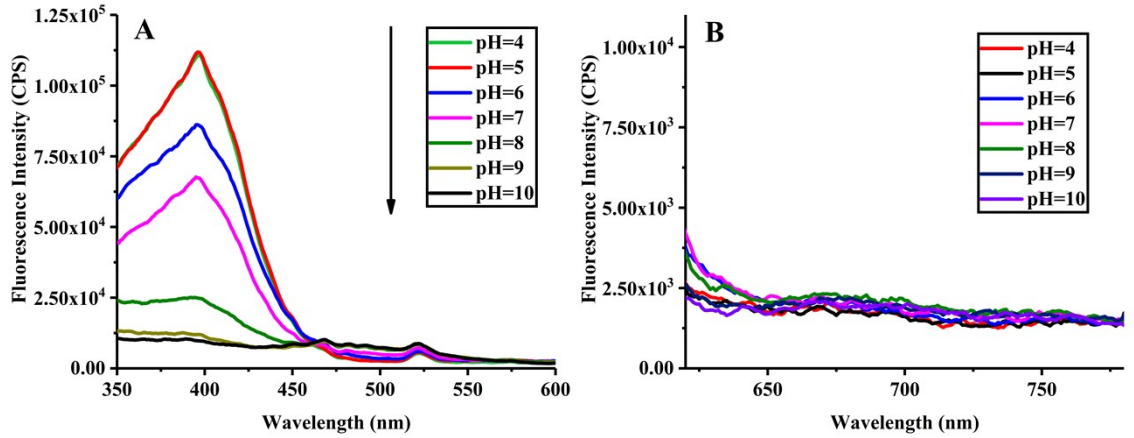


Fig. S3 (A) Excitation spectra of **HBTPN** (5 μM) at different pH in water, λ_{em}=638 nm. (B) Emission spectra of **HBTPN** (5 μM) at different pH in water, λ_{ex}=600 nm.

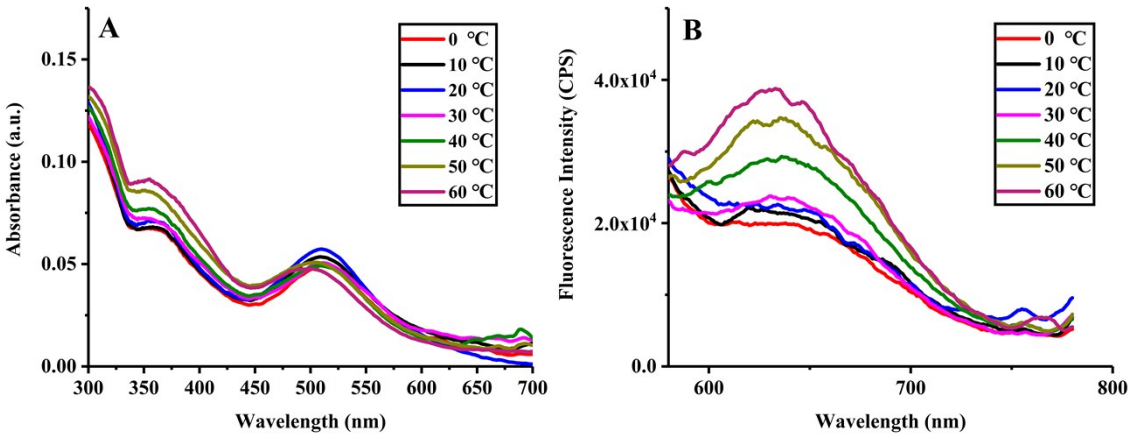


Fig. S4 (A) Absorption spectra of **HBTPN** (10 μM) at different temperature in Tris-HCl, pH=7.4; (B) Emission spectra of **HBTPN** (5 μM) at different temperature in Tris-HCl, pH=7.4, λ_{ex}=397 nm.

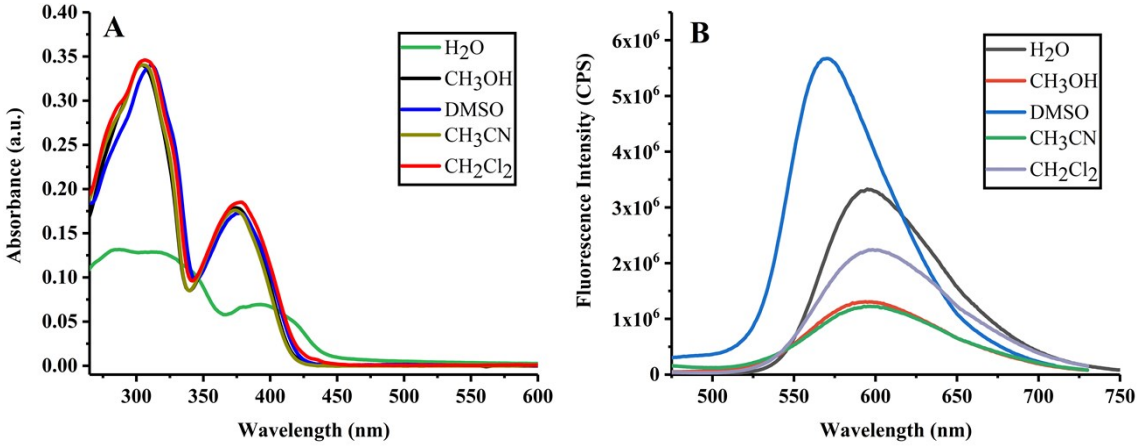
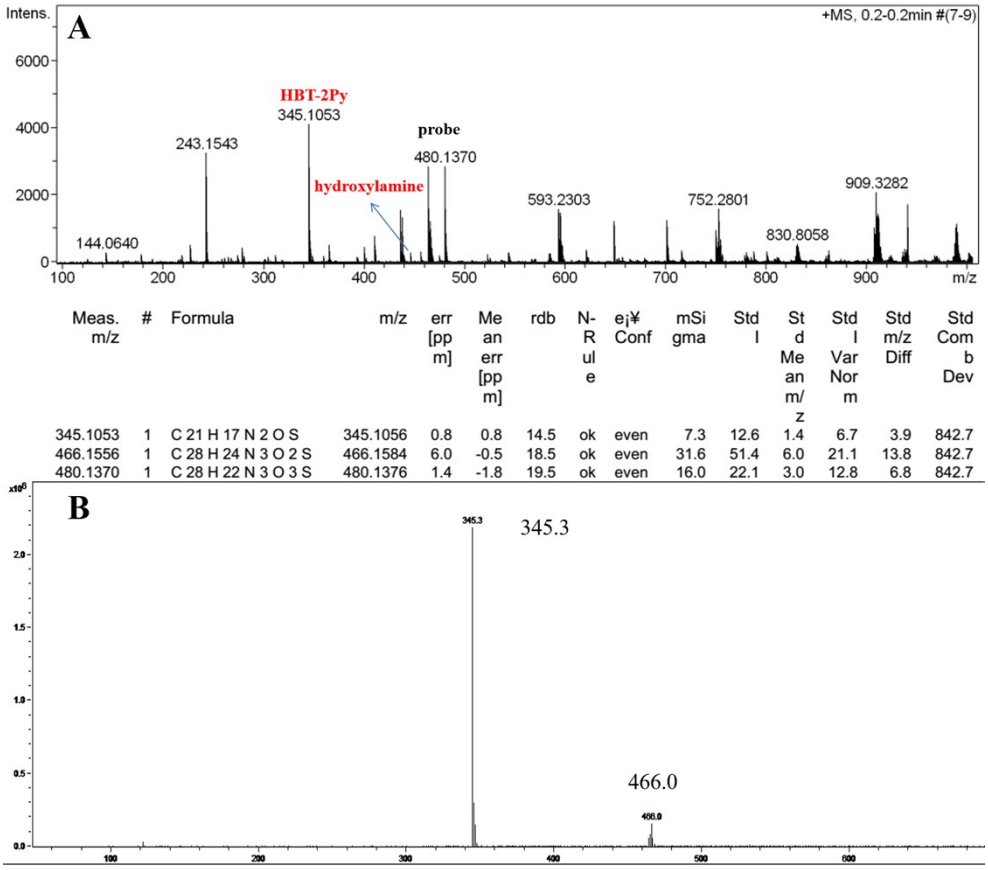


Fig. S5 (A) Absorption spectra of **HBT-2Py** (10 μM) in various solvents. (B) Emission spectra of **HBT-2Py** (5 μM) with excitation at their maximum excitation wavelength.



Meas. m/z	#	Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	ei¥ Conf	mSigma	Stdl	Std Mean m/z	Stdl Varnorm	Std m/z Diff	Std Combb Dev
345.1053	1	C 21 H 17 N 2 O S	345.1056	0.8	0.8	14.5	ok	even	7.3	12.6	1.4	6.7	3.9	842.7
466.1556	1	C 28 H 24 N 3 O 2 S	466.1584	6.0	-0.5	18.5	ok	even	31.6	51.4	6.0	21.1	13.8	842.7
480.1370	1	C 28 H 22 N 3 O 3 S	480.1376	1.4	-1.8	19.5	ok	even	16.0	22.1	3.0	12.8	6.8	842.7

Fig. S6 (A) HRMS spectra of the reaction solution of probe **HBTPN** (50 μM) with NTR (5 μg/ mL). (B) The LC-MS spectra of the peak at 3.56 min.

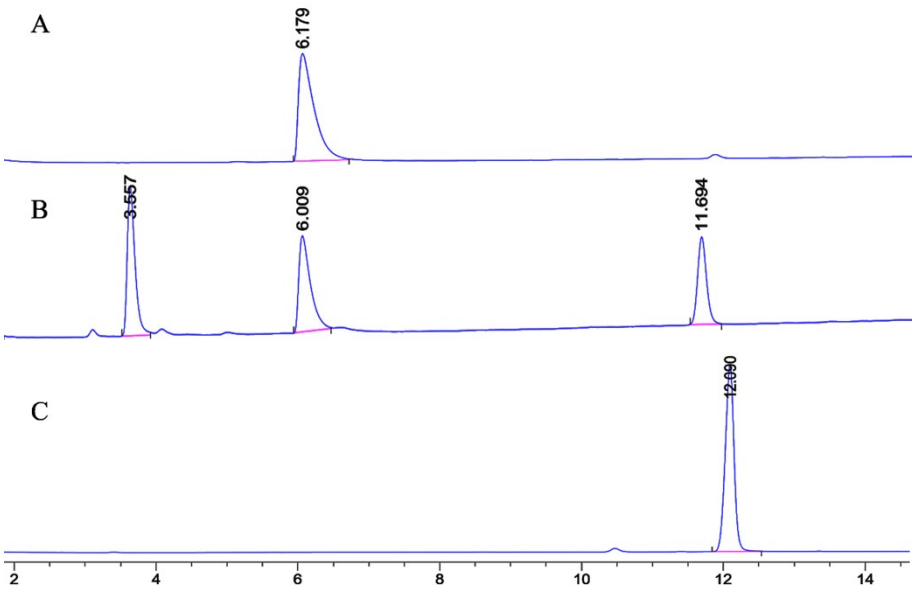


Fig. S7 Chromatograms of different reaction systems. (A) probe **HBTPN** (B) the reaction system of 50 μM probe **HBTPN** with 5 μg/ mL nitroreductase in the presence of 500 μM NADH for 10 min; (C) **HBT-2Py**. The assignments of the peaks: (1) 6.18 min, probe **HBTPN**; (2) 12.09 min, **HBT-2Py**; (3) 3.56 min, hydroxylamine compound. Mobile phase: 0 min from CH_3OH–H_2O (1‰ HCOOH), 70:30 (v/v) to 14 min CH_3OH–H_2O (1‰ HCOOH), 100: 0 (v/v). Detection: UV-vis (300 nm) detector. Flow rate: 1 mL/min. 20 °C. Injection volume: 10 μL. Chromatographic column: Agilent Eclipse XDB-C18 4.6X150 mm 5μm.

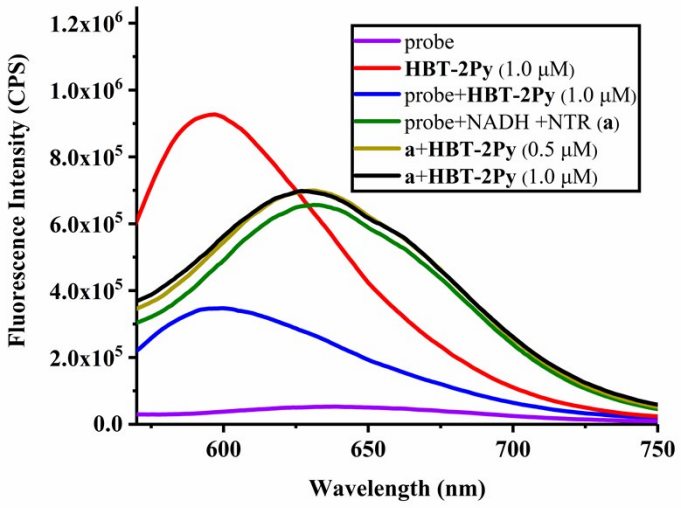


Fig. S8 Emission spectra of probe **HBTPN**, **HBT-2Py**, mixture solution of **HBT-2Py** and probe **HBTPN** (5 μM), and different reaction system in Tris-HCl buffer solution; NTR (0.6 μg/ mL), λ_{ex}=397 nm.

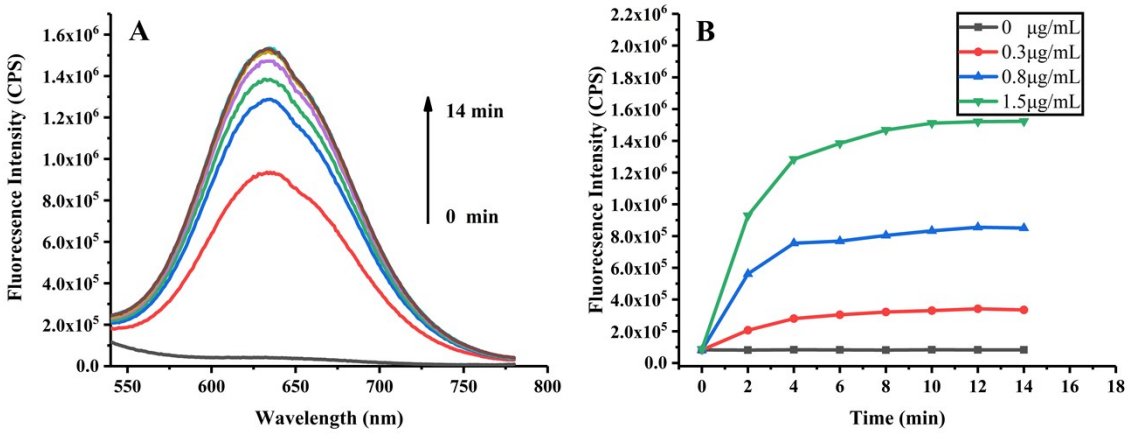


Fig. S9 (A) Emission spectroscopic changes of probe **HBTP**N (5 μM) after addition of NTR (1.5 μg/mL); (B) Emission spectroscopic changes at 633 nm along with time for probe (5 μM) incubated with different concentration NTR, λ_{ex}=397 nm.

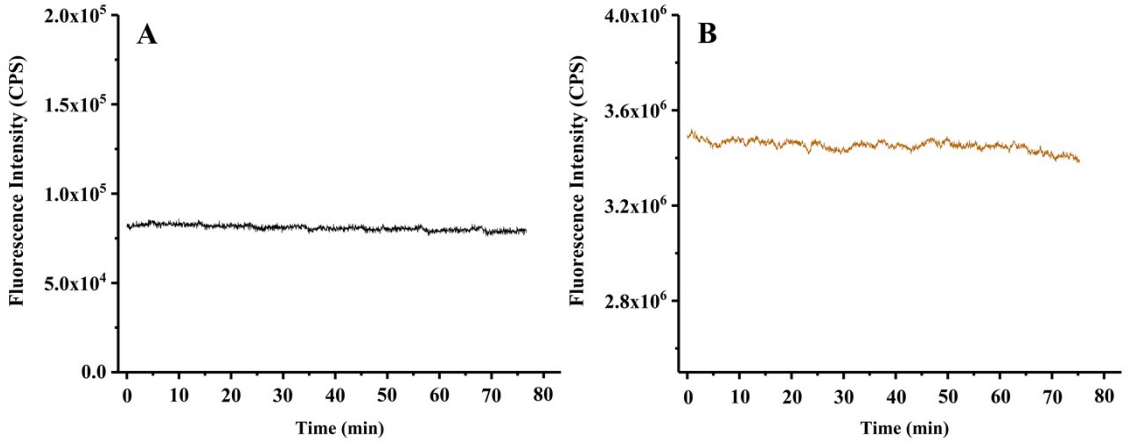


Fig. S10 (A) Fluorescence intensity of **HBTPN** (5 μM in H_2O) at 638 nm as a function of time, λ_{ex}= 397 nm. (B) Fluorescence intensity of **HBT-2Py** (5 μM in H_2O) at 595 nm as a function of time, λ_{ex}= 397 nm.

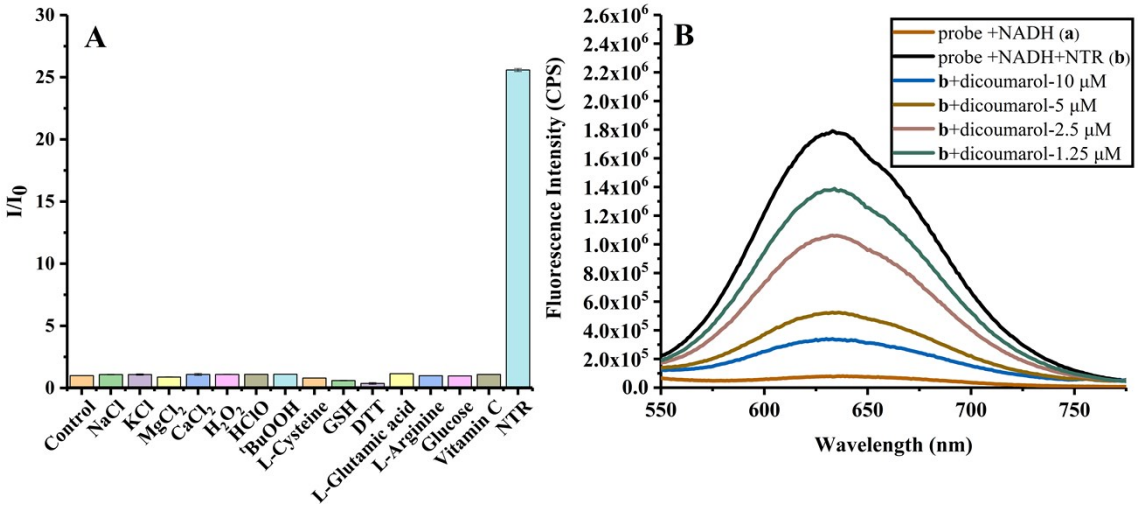


Fig. S11 (A) Fluorescence responses of **HBTPN** (5 μM) to various species: control (**HBTPN** +NADH); NaCl, 10 mM; KCl, 10 mM; $MgCl_2$, 2.5 mM; $CaCl_2$, 2.5 mM; H_2O_2, 1 mM; HClO, 10 mM; t-BuOOH, 1 mM; L-Cysteine, 1 mM; GSH, 1 mM; DTT, 1 mM; L-Glutamic acid, 1 mM; L-Arginine, 1 mM; Glucose, 1 mM; Vitamin C, 1 mM; NTR, 2.5 μg/mL. The results are the mean standard deviation of three separate measurements. (B) Emission spectra of detective solution with or without dicoumarol.

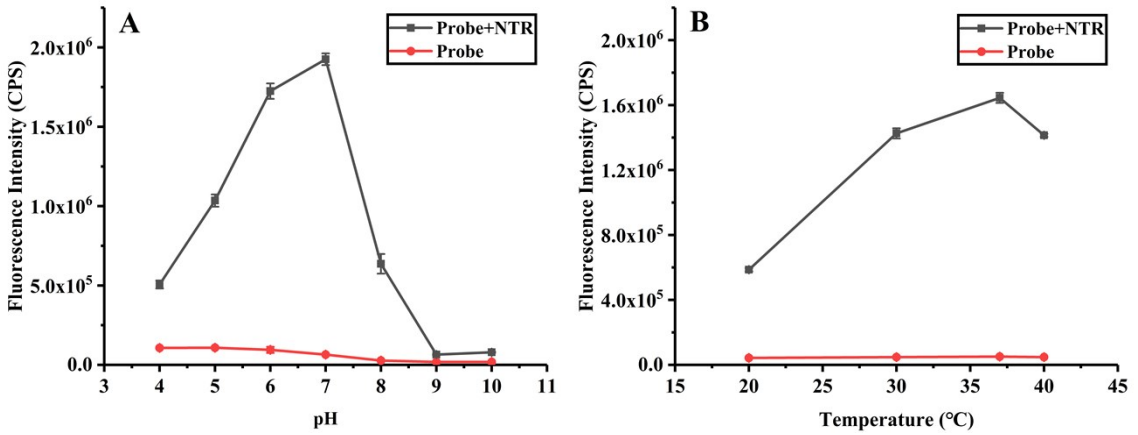


Fig. S12 Effects of pH (A) and temperature (B) on the reaction system of probe **HBTPN** (5 μM). All measurements were acquired in Tris-HCl buffer solution with NTR (1.8 μg/ mL), $\lambda_{ex/em}$=397/633 nm. Every data point was the mean of three measurements. Error bars stand for the mean standard deviation of three results.

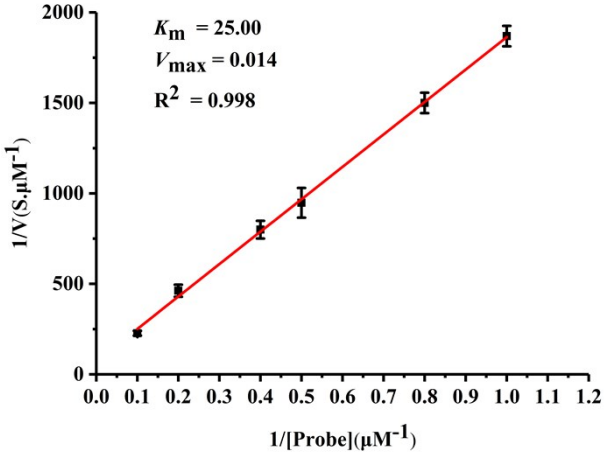


Fig. S13 Lineweaver-Burk double reciprocal plot for the enzyme-catalyzed reaction. The Michaelis-Menten equation was described as: $V=V_{max}$ [probe]/(K_m+[probe]), where V is the reaction rate, [probe] is **HBTPN** concentration (substrate), and K_m is the Michaelis constant. Conditions: 1 μg/mL NTR, 1-10 μM **HBTPN** probe. All measurements were performed in Tris-HCl at 37 °C, $\lambda_{ex}/_{em}$ = 397/633 nm.

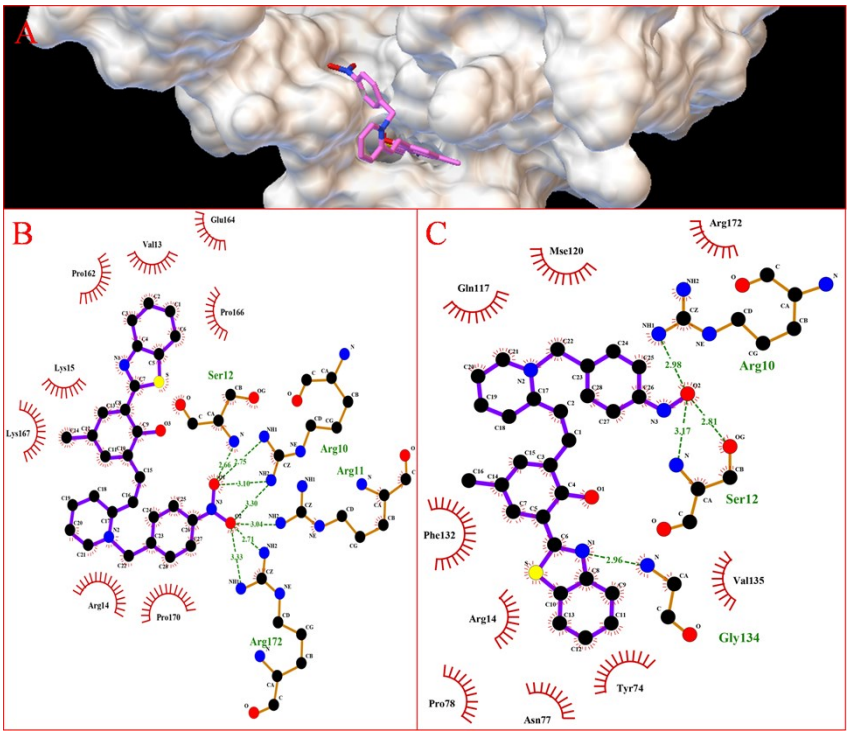


Fig. S14 (A) Stereoview of probe binding with NTR. Calculated binding model of probe (B) and hydroxylamine compound (C) with NTR. The hydrogen bonds are shown as green dashed.

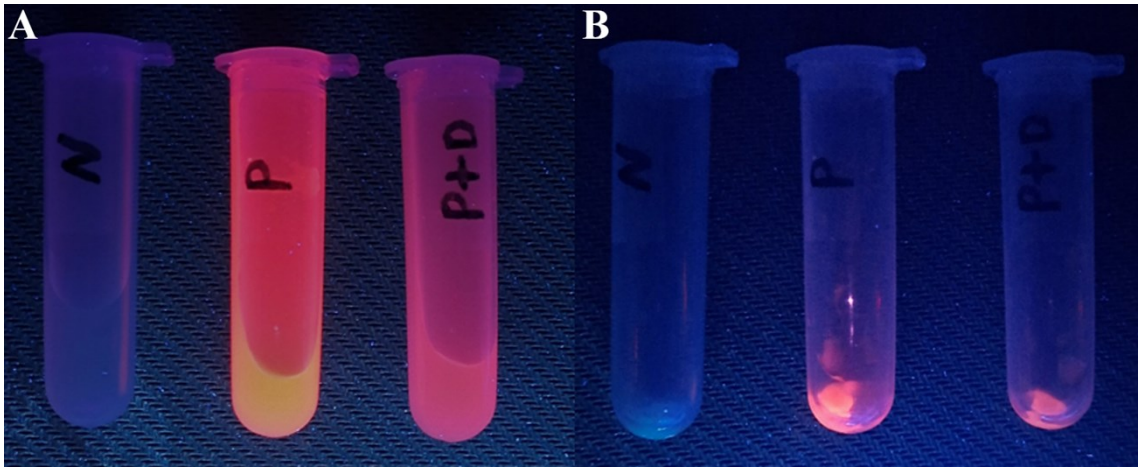
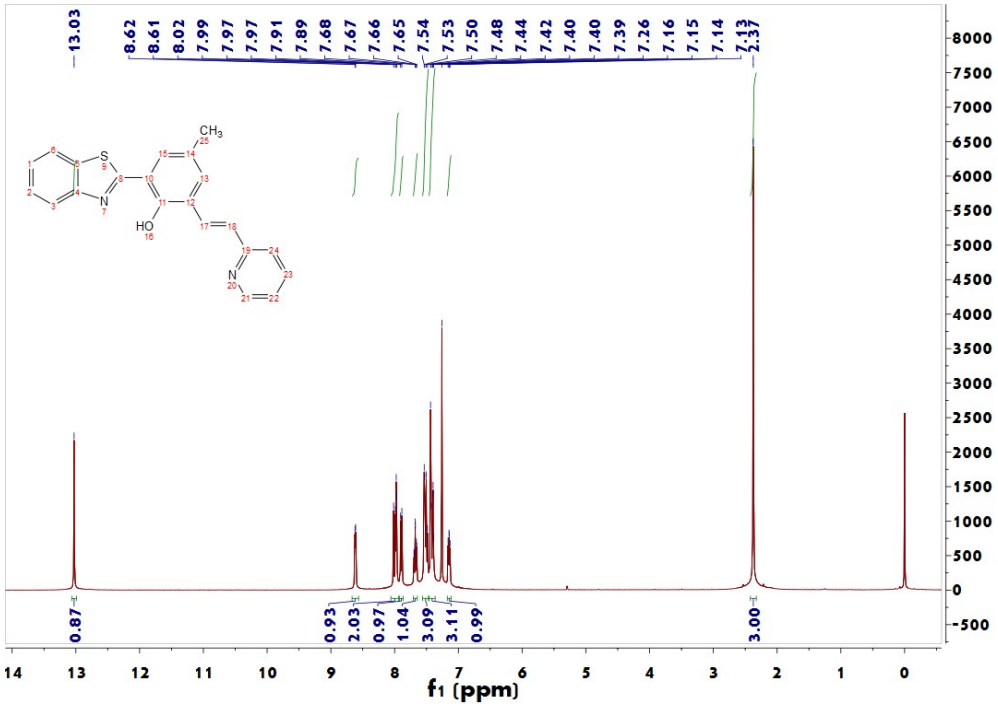
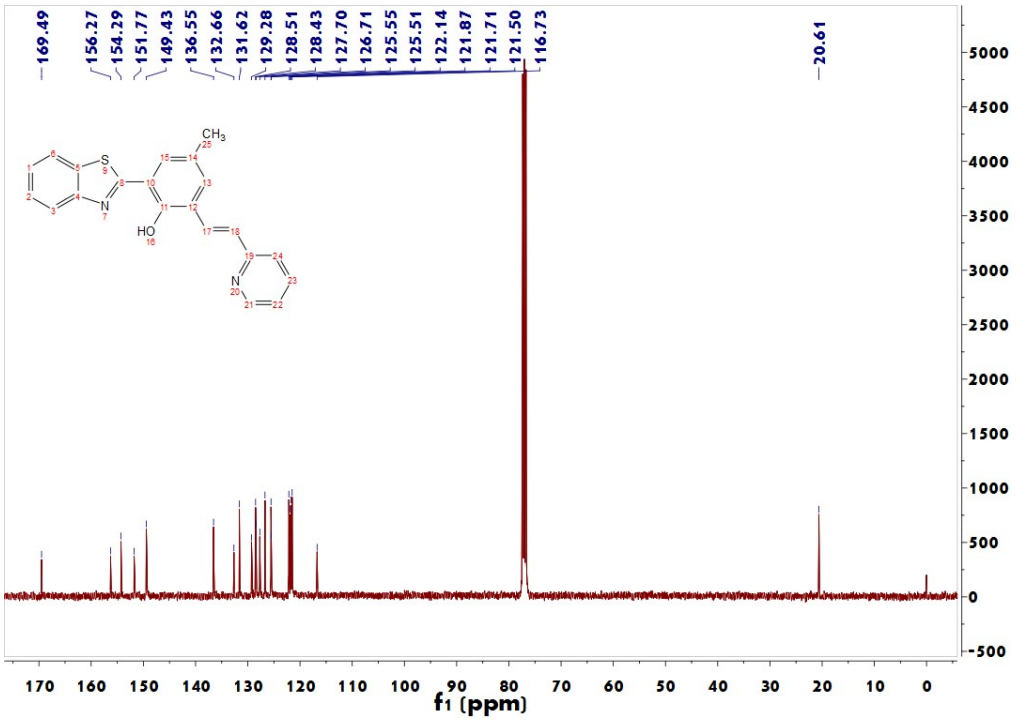


Fig. S15 (A) Fluorescence photograph of the *E. coli* (OD_{600}=1.2) in different reaction system; **N**: negative, only *E. coli* in tris-HCl buffer; **P**: probe **HBTPN** incubated with *E. coli* for 10 min; **P+D**: probe **HBTPN** incubated with *E. coli* in the present of dicoumarol. (B) Fluorescence photograph of the *E. coli* cells after centrifugation. λ_{ex} =365 nm.

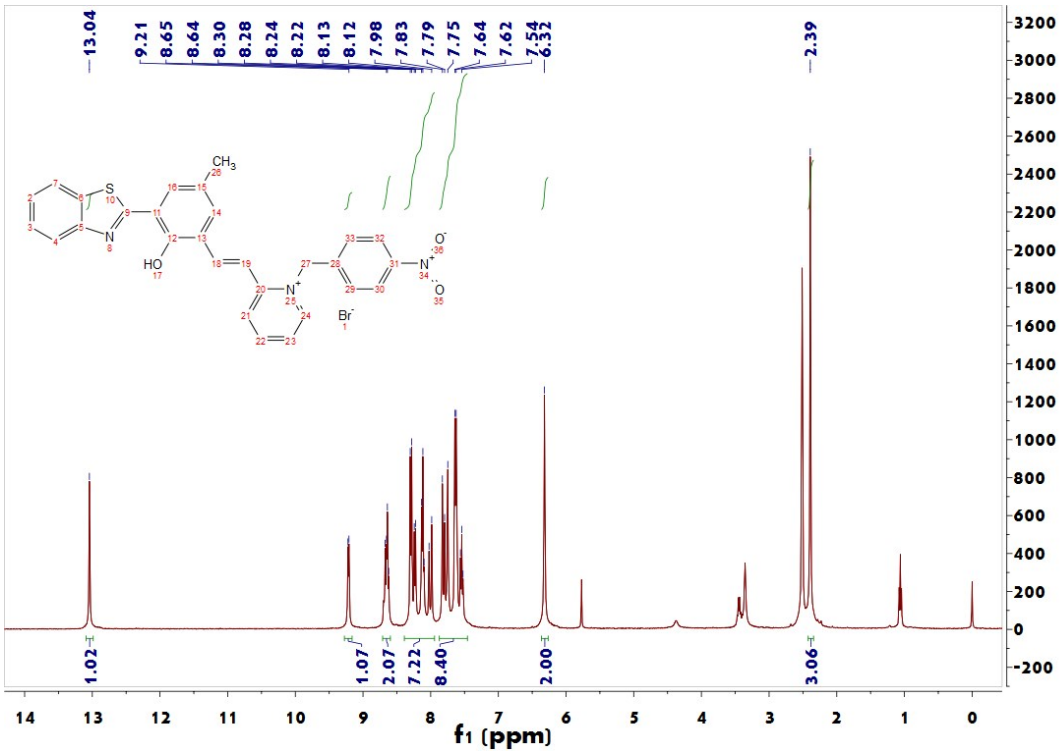
7. [1]HNMR and [13] CNMR spectra



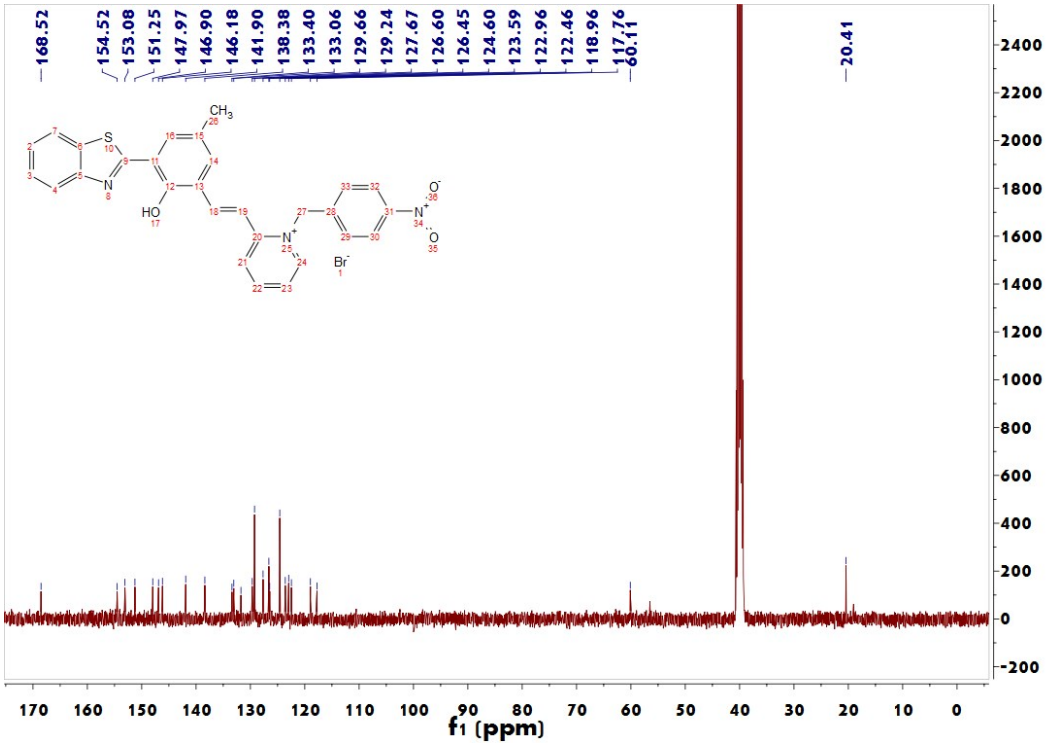
[1]H NMR spectra of compound **HBT-2Py**



[13]C NMR spectra of compound **HBT-2Py**



^{1}H NMR spectra of compound probe **HBTPN**



^{13}C NMR spectra of compound probe **HBTPN**