Supplementary data

Real Time Detection of ESKAPE Pathogens by a Nitroreductase-triggered Fluorescence Turn-on Probe

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Contents

Abbreviations

ATCC = American Type Culture Collection

BHI = Brain Heart Infusion Broth

CICC = China Center of Industrial Culture Collection

Cy = Cyanine Dyes

Cy 5.5-NHS = Cyanine 5.5-*N*-hydroxysuccinimide ester

DCM = Dichloromethane

DIPEA = Diisopropyl-ethyl amine

DMF = Dimethylformamide

DMSO = Dimethyl sulfoxide

ESI-MS = Electrospray Ionisation Mass Spectrometry

HPLC = High Performance Liquid Chromatography

HRMS = High Resolution Mass Spectrometry

LB = Luria-Bertani

LC-MS = Liquid Chromatography Mass Spectrometry

m.p. = Melting Point

MRS = M.R.S. Broth

NB = Nutrient Broth

NMR = Nuclear Magnetic Resonance

NHS = *N*-hydroxysuccinimide

OD = Optical Density

rpm = Revolutions Per Minute

r.t. = Room Temperature

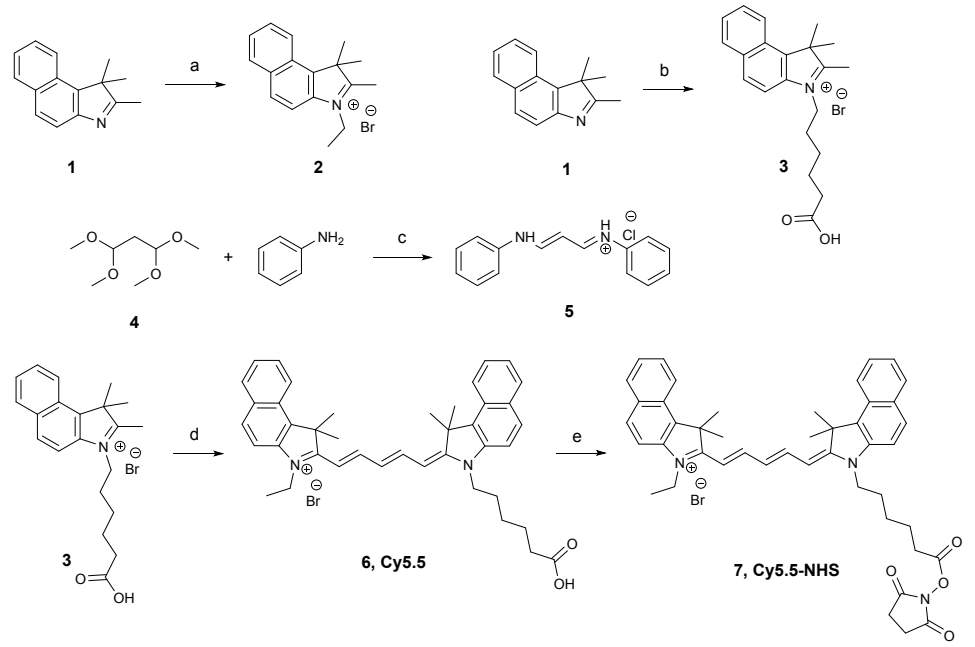
Tris = Tris(hydroxymethyl)aminomethane

TSB = Tryptone Soya Broth

General methods

All the chemicals were purchased from J&K. Commercially available reagents were used without further purification. Nitroreductase (≥100 units/mg) from *Escherichia coli* and reduced nicotinamide adenine dinucleotide (NADH) were purchased from Sigma-Aldrich. Six bacterial strains (*Escherichia coli* (*E. coli*) (ATCC 25922), *Staphylococcus aureus* (*S. aureus*) (ATCC 29213), *Klebsiella pneumoniae* (*K. pneumoniae*) (ATCC 700603), *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC 27853), *Enterobacter cloacae* (*E. cloacae*) (ATCC 13047), Methicillin resistant *Staphylococcus aureus* (MRSA) (ATCC 33592)) were purchased from American Type Culture Collection (ATCC), USA. *Enterococcus faecium* (*E. faecium*) (CICC 10840) and *Acinetobacter baumannii* (*A. baumannii*) (CICC 22933) were purchased from China Center of Industrial Culture Collection, CICC®. Fluorescence emission spectra and full wavelength absorption spectra were performed on Tecan Spark™ 10M Multimode Microplate Reader. OD values were recorded in a 10 mm path quartz cell on a Metash UV-5100B spectrometer. Confocal laser scanning microscope imaging were conducted with ZEISS LSM 710 Confocal Microscope. All ^{1}H NMR spectra were recorded at 300, 400, 500 or 600 MHz, ^{13}C NMR spectra were recorded at 75, 100 or 150 MHz, respectively. Mass spectra (MS) were measured with Thermo LCQ Deca XP Max mass spectrometer for electrospray ionization mass spectra (ESI).

Synthetic procedures and characterized data



Scheme S1. Synthesis of Cy 5.5 and Cy 5.5-NHS. Reagents and conditions: (a) bromoethane, *o*-dichlorobenzene, 110 °C, 24 h, for **2**; (b) 6-bromohexanoic acid, *o*-dichlorobenzene, 110 °C, 24 h, for **3**; (c) hydrochloric acid, H_2O, 50 °C, 2 h, for **5**; (d) **3** and **5**; AcOH/Ac_2O, reflux, 4 h, evaporate, then with **2**, AcOH/pyridine, reflux, 2 h, for **6**; (e) *N*, *N*′-disuccinimidyl carbonate, DIPEA, DMF, r.t., overnight, for **7**.

Compound **2**

Compound **1** (2.0 g) was dissolved in *o*-dichlorobenzene (20 mL). Bromoethane (2.08 g) was added to the solution, and the mixture was heated at 110 °C under an argon atmosphere overnight. The precipitate was collected by filtration, and washed with diethylether to afford the desired product 1.72 g, yield 56.5 %. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.37 (d, J = 8.4 Hz, 1H, -Ar), 8.30 (d, J = 9.1 Hz, 1H, -Ar), 8.22 (d, J = 8.2 Hz, 1H, -Ar), 8.16 (d, J = 9.0 Hz, 1H, -Ar), 7.81–7.71 (m, 2H, -Ar), 4.63 (q, J = 7.6 Hz, 2H, -CH_2-), 2.94 (s, 3H, -CH_3), 1.76 (s, 6H, -CH_3), 1.50 (t, J = 7.4 Hz, 3H, -CH_3). HRMS (*m*/*z*) (M^+): calcd. for $C_{17}H_{20}N^+$ 238.1590, found 238.1584.

Compound **3**

Compound **1** (2.0 g) was dissolved in *o*-dichlorobenzene (20 mL). 6-Bromohexanoic acid (2.8 g) was added to the solution, and the mixture was heated at 110 °C under an argon atmosphere for 24 h. The precipitate was collected by filtration, and washed with diethylether to afford the desired product 2.2 g, yield 57.0 %. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.37 (d, J = 8.4 Hz, 1H, -Ar), 8.29 (d, J = 8.8 Hz, 1H, -Ar), 8.22 (d, J = 8.0 Hz, 1H, -Ar), 8.16 (d, J = 8.8 Hz, 1H, -Ar), 7.81–7.71 (m, 2H, -Ar), 4.58 (t, J = 7.6 Hz, 2H, -CH_2-), 2.95 (s, 3H, -CH_3), 2.23 (t, J = 7.2 Hz, 2H, -CH_2-), 1.94

–1.87 (m, 2H, -CH_2-), 1.76 (s, 6H, -CH_3), 1.61–1.54 (m, 2H, -CH_2-), 1.50–1.42 (m, 2H, -CH_2-). HRMS (*m*/*z*) (M^+): calcd. for $C_{21}H_{26}O_2N^+$ 324.1958, found 324.1954.

Compound **5**

Compound **4** (4.9 g) and hydrochloric acid (4.25 mL) were added to distilled water (90 mL), and stirred at 50 °C. Then the solution of aniline (3.7 mL), hydrochloric acid (5 mL) and distilled water (70 mL) was added dropwise to the reaction and continued to stir at 50 °C for 2 h. After cooling, and filtration, a pure solid was dried and isolated and determined to be the desired product 4.58 g, yield 59.3 %. ^{1}H NMR (500 MHz, Methanol-*d*4) δ 8.69 (d, J = 14.5 Hz, 2H, -Ar), 7.50 (t, J = 10.0, 4H, -Ar), 7.39 (d, J = 9.5, 4H, -Ar), 7.32 (t, J = 9.5, 2H, -C*H*-), 6.26 (t, J = 14.5 Hz, 1H, -C*H*-). HRMS (*m*/*z*) (M^+): calcd. for $C_{15}H_{15}N_2^+$ 223.1230, found 223.1234.

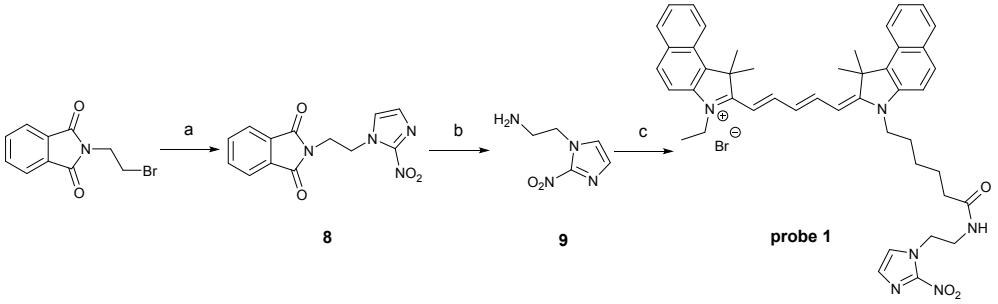
Compound **6** (Cy 5.5)

Compound **3** (508 mg) was dissolved in AcOH/Ac_2O (1.5 mL/1.5 mL). To this solution, Compound **5** (340 mg) was added, and the mixture was stirred under reflux for 4 h. The solvent was removed under reduced pressure, and the residue was dissolved in pyridine/AcOH (1.5 mL/1.5 mL). Compound **2** (318 mg) was added to the solution, and the mixture was refluxed for 2 h. The solvent was removed under reduced pressure, and the crude product was purified by silica gel column chromatography (DCM : MeOH = 30 : 1) to afford the desired product 375 mg, yield 55.3 %. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.31–8.23 (m, 2H, -Ar), 8.16–8.13 (m, 2H, -Ar), 7.90 (t, J = 7.4 Hz, 4H, -Ar), 7.61–7.55 (m, 2H, -Ar), 7.43 (q, J = 7.2 Hz, 2H, -Ar), 7.35 (t, J = 9.2 Hz, 2H, -C*H*), 6.89 (t, J = 12.4 Hz, 1H, -C*H*), 6.47 (d, J = 13.6 Hz, 1H, -C*H*), 6.33 (d, J = 13.6 Hz, 1H, -C*H*), 4.27 (q, J = 7.2 Hz, 2H, -CH_2-), 4.13 (t, J = 7.6 Hz, 2H, -CH_2-), 2.50 (t, J = 7.2 Hz, 2H, -CH_2-), 2.04 (s, 12H, -CH_3), 1.86 (t, J = 7.6 Hz, 2H, -CH_2-), 1.80–1.73 (m, 2H, -CH_2-), 1.62–1.54 (m, 2H, -CH_2-), 1.46 (t, J = 7.2 Hz, 3H, -CH_3). HRMS (*m*/*z*) (M^+): calcd. for $C_{41}H_{45}O_2N_2^+$ 597.3476, found 597.3462.

Compound **7** (Cy 5.5-NHS)

The compound **6** (339 mg), *N*, *N*′-disuccinimidyl carbonate (192 mg) and DIPEA (194 mg) were added to DMF (10 mL) and the mixture was stirred at r.t. overnight. After the reaction was complete, the product was purified by silica gel column chromatography (DCM : MeOH = 30 : 1) to give deep blue solid 350 mg, yield 75.0 %. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.36 (t, J = 12.7 Hz, 2H, -Ar), 8.19 (d, J = 8.8 Hz, 2H, -Ar), 7.91 (t, J = 7.2 Hz, 3H, -Ar), 7.60 (t, J = 7.6 Hz, 2H, -Ar), 7.48–7.43 (m, 2H, -Ar), 7.36 (dd, J = 11.2, 8.8 Hz, 2H, -C*H*), 6.86 (t, J = 12.4 Hz, 1H, -C*H*), 6.44–6.39 (m, 2H, -C*H*), 4.30 (q, J = 7.2 Hz, 2H, -CH_2-), 4.21 (t, J = 7.6 Hz, 2H, -CH_2-), 2.96 (s, 2H, -CH_2-), 2.88 (s, 2H, -CH_2-), 2.84 (s, 2H, -CH_2-), 2.71 (s, 6H, -CH_3-), 2.66 (t, J = 7.2

Hz, 2H, -CH_2-), 2.07 (s, 6H, -CH_3), 1.93–1.83 (m, 2H, -CH_2-), 1.70–1.64 (m, 2H, -CH_2-), 1.48 (t, J = 7.2 Hz, 3H, -CH_3). HRMS (*m*/*z*) (M^+): calcd. for $C_{45}H_{48}O_4N_3^+$ 694.3639, found 694.3631.[1,2]



Scheme S2. Synthesis of probe **1**. Reagents and conditions: (a) 2-nitroimidazole, dry DMF, K_2CO_3, 110 °C, 2 h, for **8**; (b) EtOH, $N_2H_4 \cdot H_2O$, reflux, 2h, for **9**; (c) **7**, dry DMF, DIPEA, r.t., 3 h, for probe **1**.

Compound **9**

According to a reported literature,[3] we synthesized compound **9**, as shown in Scheme S2. A stirred solution of 2-nitroimidazole (200 mg), 2-bromoethylphthalimide (470 mg), and K_2CO_3 (257 mg) in DMF (5 mL) was heated at 110 °C for 2 h. The solvent was removed under reduced pressure, and the residue was poured into water (200 mL). The precipitate was collected, washed with water (100 mL), and dried to give compound **8**. The mixture was used in next step directly.

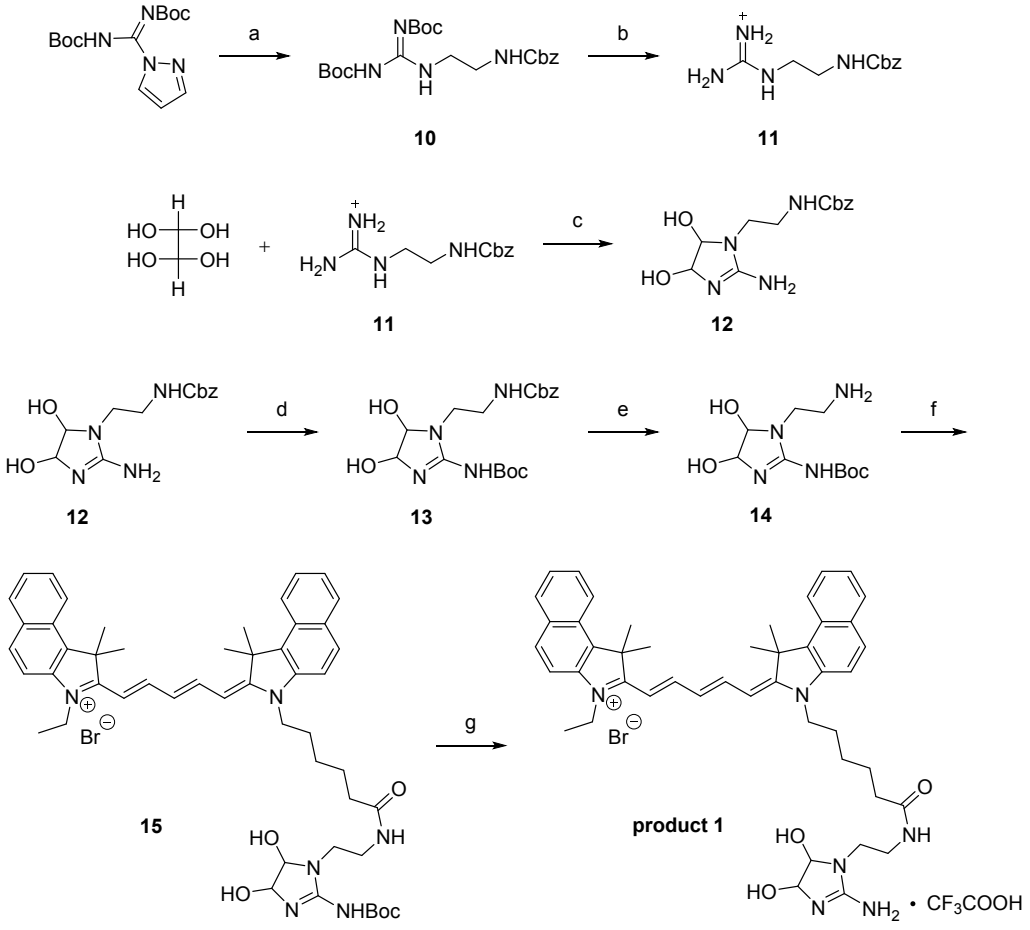
A stirred solution of compound **8** (670 mg) and hydrazine monohydrate (200 mg) in EtOH (10 mL) was heated under reflux for 2 h. The resulting suspension was cooled to 0 °C and filtered, and the fiitrate was evaporated to dryness under reduced pressure. The residue was dissolved in 1 N HCl (15 mL) and filtered, the solvent was removed under reduced pressure, and the residue was crystallized from MeOH / EtOAc to give compound **9** 200 mg, yield 58.7 %. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.29 (s, 2H, -NH_2), 7.76 (s, 1H, -Ar), 7.22 (s, 1H, -Ar), 4.68 (t, J = 4.8 Hz, 2H, -CH_2-), 3.30 (q, J = 4.4 Hz, 2H, -CH_2-). HRMS (*m*/*z*) (M+H): calcd. for $C_5H_9N_4O_2$ 157.0720, found 157.0715.

probe **1**

To a solution of compound **9** (78 mg) in dry DMF, Cy 5.5-NHS (380 mg) and DIPEA (647 mg) were added and stirred at r.t. for overnight. The reaction mixture was then partitioned between water and ethyl acetate. The aqueous layer (30 mL) was separated and extracted with ethyl acetate (3 × 30 mL). The ethyl acetate layer was dried over Na_2SO_4, concentrated in vacuum and the residue was purified by silica gel column chromatography (DCM : MeOH = 30 : 1) to afford probe **1** 72 mg, yield 17.6 %, purity > 95 %. m.p.: 101-103 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.10 (t, J = 8.4 Hz, 2H, -Ar), 7.95–7.93 (m, 6H, -Ar, -C*H*-), 7.64–7.59 (m, 2H, -Ar), 7.50–7.45

(m, 2H, -Ar), 7.36 (t, J = 8.4 Hz, 2H, -Ar), 7.11 (d, J = 7.6 Hz, 1H, -C*H*-), 6.92 (t, J = 10.8 Hz, 1H, -C*H*-), 6.80 (d, J = 8.0 Hz, 1H, -C*H*-), 6.42 (d, J = 8.0 Hz, 2H, -C*H*-), 4.76 (t, J = 4.4 Hz, 2H, -CH_2-), 4.23 (d, J = 7.2 Hz, 2H, -CH_2-), 4.12 (t, J = 7.6 Hz, 2H, -CH_2-), 3.79 (s, 2H, -CH_2-), 2.37 (t, J = 7.6 Hz, 2H, -CH_2-), 2.00 (d, J = 7.6 Hz, 12H, -CH_3), 1.83 (t, J = 7.6 Hz, 2H, -CH_2-), 1.72 (t, J = 6.4 Hz, 2H, -CH_2-), 1.50–1.44 (m, 5H, -CH_2-, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 174.15 (-C=O-), 174.01 (-C=N$^+$-), 173.05 (-CN-), 151.75 (-CH-), 151.45 (-CH-), 139.14 (-Ar), 138.94 (-Ar), 133.72 (-Ar), 133.60 (-Ar), 131.86 (-Ar), 131.72 (-Ar), 130.80 (-Ar), 130.67 (-Ar), 130.10 (-Ar), 130.03 (-Ar), 130.01 (-C=N-), 129.00 (-C-OH), 128.13 (-Ar), 127.98 (-Ar), 127.75 (-Ar), 127.71 (-Ar), 127.44 (-C-OH), 126.00 (-CH-), 125.09 (-Ar), 124.98 (-Ar), 121.91 (-Ar), 121.87 (-Ar), 110.55 (-Ar), 110.09 (-Ar), 103.59 (-CH-), 102.75 (-CH-), 51.02 (-C-), 50.89 (-C-), 49.13 (-CH_2-), 44.46 (-CH_2-), 39.35 (-CH_2-), 38.72 (-CH_2-), 35.63 (-CH_2-), 29.59 (-CH_3), 29.25 (-CH_3), 27.65 (-CH_3), 27.63 (-CH_3), 26.96 (-CH_2-), 26.05 (-CH_2-), 24.70 (-CH_2-), 12.61 (-CH_3). HRMS (*m/z*) (M^+): calcd. for $C_{46}H_{51}N_6O_3^+$ 735.4017, found 735.4028.

Synthesis of product 1 by chemical methods



Scheme S3. Synthesis of product **1**. Reagents and conditions: (a) *N*-Cbz-Ethylenediamine hydrochloride, DIPEA, THF, r.t., overnight, for **10**; (b) TFA, DCM, 0 °C, 30 min, then r.t., 4 h, for

11; (c) H_2O, Et3N, pH 8, r.t., 2 days, for **12**; (d) $(Boc)_2O$, Et3N, THF/H_2O, 0 °C, 30 min, then 60 °C, 5 h, for **13**; (e) 10 % Pd/C, MeOH, H_2, r.t., 4 h, for **14**; (f) Cy 5.5-NHS, DIPEA, DCM/DMF, r.t., 2 h, for **15**; (g) TFA, 0 °C, 45 min, for product **1**.

Compound **10**

To a solution of *N*-Cbz-Ethylenediamine hydrochloride (580 mg) in 15 mL of THF was added DIPEA (975 mg) and solid *N*, *N*′-di-Boc-1H-pyrazole-1-carboxamidine (780 mg). The reaction mixture was stirred for overnight and then partitioned between water and ethyl acetate. The aqueous layer (30 mL) was separated and extracted with ethyl acetate (3 × 30 mL). The ethyl acetate layer was dried over Na_2SO_4, concentrated in vacuum and the residue was purified by silica gel column chromatography (PE : EA = 3:1) to afford compound **10** 649 mg, yield 59.1 %. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 11.42 (s, 1H, -N*H*), 8.51 (t, *J* = 6.0 Hz, 1H, -N*H*), 7.29–7.23 (m, 5H, -Ar), 6.09 (t, *J* = 6.0 Hz, 1H, -N*H*), 5.03 (s, 2H, -CH_2-), 3.51–3.47 (m, 2H, -CH_2-), 3.34–3.30 (m, 2H, -CH_2-), 1.44 (s, 9H, -CH_3), 1.41 (s, 9H, -CH_3). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 162.96 (-C=O-), 156.78 (-C=N-), 156.67 (-C=O-), 152.91 (-C=O-), 136.54 (-Ar), 128.38 (-Ar), 127.94 (-Ar), 127.92 (-Ar), 83.28 (-C-), 79.40 (-C-), 66.52 (-C-Ar), 41.28 (-CH_2-), 40.38 (-CH_2-), 28.10 (-CH_3), 27.93 (-CH_3). HRMS (*m*/*z*) (M+H): calcd. for $C_{21}H_{33}N_4O_6$ 437.2395, found 437.2393.

Compound **11**

To an ice-cold solution of compound **10** (500 mg) in 8 mL of CH_2Cl_2 was added via syringe over a 5 min period 8 mL of TFA. The reaction mixture was stirred for 4 h, then concentrated under reduced pressure to a colorless oil. The isolated material was dissolved in 10 mL of CH_2Cl_2 and concentrated to give the desired product as a colorless oil compound **11** 269 mg, yield 99.0 %. This product was deemed pure by ^{1}H NMR and used in the subsequent reaction without further purification. ^{1}H NMR (300 MHz, D_2O) δ 7.41 (s, 5H, -Ar), 5.11 (s, 2H, -CH_2-), 3.29 (s, 4H, -CH_2-). ^{13}C NMR (75 MHz, D_2O) δ 158.65 (-C=NH_2^+), 157.08 (-C=O-), 136.57 (-Ar), 128.89 (-Ar), 128.49 (-Ar), 127.75 (-Ar), 67.10 (-C-Ar), 41.06 (-CH_2-), 39.37 (-CH_2-). HRMS (*m*/*z*) (M^+): calcd. for $C_{11}H_{17}N_4O_2^+$ 237.1346, found 237.1345.[4]

Compound **12**

Compound **11** (475 mg) and aqueous glyoxal (122 mg) were mixed in a total volume of 10 mL of water, and the pH was adjusted to 8. After 2 days the water was removed under reduced pressure. The residual oil was collected, washed with water (10 mL), and concentrated to give the desired product compound **12**. The mixture was used in next step directly. HRMS (*m*/*z*) (M+H): calcd. for $C_{13}H_{19}N_4O_4$ 295.1401, found 295.1399.[5]

Compound **13**

The mixture of compound **12** (500 mg) in THF/H_2O (5 mL/5 mL) was stirred and Et3N (344 mg) was added. This mixture was treated with $(Boc)_2O$ (556 mg) at 0 °C for 30 min, then the reaction solution was heated to 60 °C for 5 h. After cooling to r.t., the volatiles were removed in vacuo. The residue was purified by Pre-TLC (DCM : MeOH = 10 : 1) to afford compound **13** 120 mg, yield 15.2 %. ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.60 (s, 1H, -N*H*), 8.43 (s, 1H, -N*H*), 7.33–7.28 (m, 5H, -Ar), 6.07 (s, 1H, -O*H*), 5.15–5.00 (m, 4H, -C*H*-, -CH_2-), 3.72 (s, 1H, -O*H*), 3.51–3.19 (m, 4H, -CH_2-), 1.44 (s, 9H, -CH_3). ^{13}C NMR (75 MHz, Chloroform-*d*) δ 161.26 (-C=O-), 158.54 (-C=O-), 158.22 (-C=N-), 136.33 (-Ar), 128.80 (-Ar), 128.52 (-Ar), 128.32 (-Ar), 127.96 (-Ar), 127.84 (-Ar), 87.38 (-C-OH), 84.30 (-C-OH), 79.66 (-C-), 67.42 (-C-Ar), 41.66 (-CH_2-), 39.88 (-CH_2-), 29.94 (-CH_3), 28.49 (-CH_3), 28.03 (-CH_3). HRMS (*m*/*z*) (M+H): calcd. for $C_{18}H_{27}N_4O_6$ 395.1925, found 395.1921.

Compound **14**

To a solution of compound **13** (100 mg) in methanol (8 mL), 10% Pd/C was added. The reaction was hydrogenated at r.t. and atmospheric pressure for 3 h. Then the catalyst was filtered off through Celite, and the clear solution, taken to dryness, afforded the desired product compound **14**. The reaction was monitored by TLC and, for stability problems, the amine was directly used in the subsequent reaction. HRMS (*m*/*z*) (M+H): calcd. for $C_{10}H_{21}N_4O_4$ 261.1518, found 261.1521.

Compound **15**

To a solution of compound **14** (60 mg) in dry DCM/DMF (3 mL/3 mL), Cy 5.5-NHS (87 mg) and DIPEA (89 mg) were added and stirred at r.t. for 2 h. The reaction mixture was then partitioned between water and ethyl acetate. The aqueous layer (30 mL) was separated and extracted with ethyl acetate (3 × 30 mL). The ethyl acetate layer was dried over Na_2SO_4, concentrated in vacuum and the residue was purified by Pre-TLC (DCM : MeOH = 10 : 1) to afford compound **15** 61 mg, yield 26.3 %. ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.65 (s, 1H, -N*H*), 8.45 (s, 1H, -N*H*), 8.31 (s, 1H, -Ar), 8.11 (d, 2H, *J* = 8.4 Hz, -Ar), 7.98–7.91 (m, 5H, -Ar, -C*H*-), 7.61 (t, *J* = 8.1 Hz, 2H, -Ar), 7.46 (t, *J* = 7.8 Hz, 2H, -Ar), 7.35 (t, *J* = 8.4 Hz, 2H, -Ar), 6.90–6.78 (m, 1H, -C*H*-), 6.46–6.32 (m, 2H, -C*H*-), 5.33–5.20 (m, 2H, -C*H*-), 4.19 (t, *J* = 8.1 Hz, 4H, -CH_2-), 3.72–3.48 (m, 4H, -CH_2-), 2.31 (t, *J* = 8.1 Hz, 2H, -CH_2-), 2.00 (s, 12H, -CH_3), 1.83 (t, *J* = 7.5 Hz, 2H, -CH_2-), 1.71 (t, *J* = 6.6 Hz, 2H, -CH_2-), 1.46–1.41 (m, 14H, -CH_2-, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 173.97 (-C=O-), 173.54 (-C=N$^+$-), 173.20 (-CN-), 152.06 (-C=O-), 151.98 (-C=N-), 151.69 (-CH-), 139.36 (-Ar), 139.13 (-Ar), 131.89 (-Ar), 131.79 (-Ar), 130.77 (-CH-), 130.67 (-Ar), 130.14 (-CH-), 130.09 (-Ar), 128.27 (-Ar), 128.24 (-Ar), 128.12 (-Ar), 127.78 (-Ar), 127.76 (-Ar), 125.12 (-Ar), 125.06 (-Ar), 124.98 (-Ar), 122.05 (-Ar), 122.03 (-Ar), 110.73 (-Ar), 110.20 (-Ar), 103.84 (-CH-), 102.96 (-CH-), 87.80 (-C-OH), 84.63 (-C-OH), 81.87 (-C-), 51.12 (-C-), 51.08 (-C-), 44.41 (-CH_2-),

42.02 (-CH_2-), 40.94 (-CH_2-), 39.34 (-CH_2-), 36.19 (-CH_2-), 29.70 (-CH_2-), 28.39(-CH_3), 28.36 (-CH_3), 27.81 (-CH_3), 27.67 (-CH_3), 27.66 (-CH_3), 27.59 (-CH_3), 27.31 (-CH_3), 26.08 (-CH_2-), 25.03 (-CH_2-), 12.66 (-CH_3). HRMS (*m/z*) (M^+): calcd. for $C_{51}H_{63}N_6O_5^+$ 839.4854, found 839.4848.

product **1**

The TFA (3 mL) was added to compound **15** (30 mg) at 0 °C and the reaction mixture was stirred for 45 min. Then the solvent was evaporated under reduced pressure and and the residue was purified by Pre-TLC (DCM : MeOH = 10 : 1) to afford product **1** 19 mg, yield 62.4 %), purity >95 %. ^{1}H NMR (600 MHz, Methanol-*d*4) δ 8.37 (t, *J* = 10.5 Hz, 2H, -Ar), 8.25 (d, 2H, *J* = 7.8 Hz, -Ar), 8.04–7.99 (m, 4H, -Ar, -C*H*-), 7.65 (t, *J* = 7.8 Hz, 2H, -Ar), 7.60 (t, *J* = 8.1 Hz, 2H, -Ar), 7.50–7.48 (m, 2H, -Ar), 6.69 (t, *J* = 12.6 Hz, 1H, -C*H*-), 6.36 (t, *J* = 14.4 Hz, 2H, -C*H*-), 5.34 (t, *J* = 4.8 Hz, 1H, -C*H*-), 5.25 (q, *J* = 6.0 Hz, 1H, -C*H*-), 4.31–4.28 (m, 2H, -CH_2-), 4.24 (t, *J* = 7.2 Hz, 2H, -CH_2-), 3.49–3.36 (m, 4H, -CH_2-), 2.26 (t, *J* = 7.2 Hz, 2H, -CH_2-), 2.03 (s, 12H, -CH_3), 1.92–1.87 (m, 2H, -CH_2-), 1.75–1.70 (m, 2H, -CH_2-), 1.53–1.51 (m, 2H, -CH_2-), 1.46 (t, *J* = 7.2 Hz, 3H, -CH_3). ^{13}C NMR (150 MHz, Methanol-*d*4) δ 175.35 (-C=O-), 174.37 (-C=N^+-), 157.07 (-C=N-), 153.14 (-CN-), 152.89 (-CH-), 139.61 (-Ar), 139.13 (-Ar), 132.08 (-CH-), 131.99 (-Ar), 130.52 (-CH-), 130.40 (-Ar), 130.31 (-Ar), 129.73 (-Ar), 129.70 (-Ar), 128.11 (-Ar), 128.06 (-Ar), 127.35 (-Ar), 127.33 (-Ar), 124.70 (-Ar), 124.68 (-Ar), 121.94 (-Ar), 110.70 (-Ar), 110.47 (-Ar), 99.99 (-CH-), 91.26 (-CH-), 89.89 (-C-OH), 84.13 (-C-OH), 53.40 (-C-), 51.10 (-C-), 43.47 (-CH_2-), 40.71 (-CH_2-), 38.80 (-CH_2-), 36.88 (-CH_2-), 35.24 (-CH_2-), 29.33 (-CH_3), 29.19 (-CH_3), 27.10 (-CH_2-), 26.27 (-CH_3), 26.14 (-CH_3), 26.02 (-CH_2-), 24.99 (-CH_2-), 11.50 (-CH_3). HRMS (*m*/*z*) (M^+): calcd. for $C_{46}H_{55}N_6O_3^+$ 739.4330, found 739.4324.

Multiple sequence alignment and phylogenetic tree of the nitroreductases from *E.coli* and ESKAPE pathogens

The sequence identities were obtained via alignment of the amino acid sequences performed with the program "BLAST 2 SEQENCES" (www.ncbi.nlm.nih.gov). The NTR proteins of *E. coli* and ESKAPE pathogens were aligned using Clustal W.[6] From the alignment, a consensus phylogenetic tree was generated by the maximum likelihood method using MEGA6.[7]

(a)

(b)

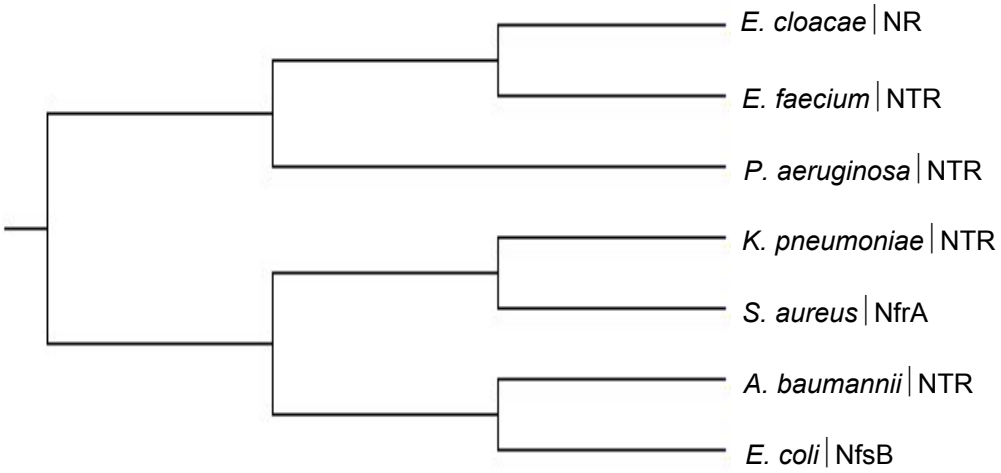


Fig. S1 (a) Multiple sequence alignment of the nitroreductases from *E. coli* and ESKAPE pathogens. Identical and similar residues are highlighted in green and red, respectively. (b) Phylogenetic tree of the bacterial nitroreductases. Shown are: NR (*E. cloacae*; KP715468.1); NTR (*K. pneumoniae*; AOGO01000001.1); NfrA (*S. aureus*; AM980656.1); NTR (*A. baumannii*; NZ_CP018664.1); NTR (*P. aeruginosa*; NZ_CP015117.1); NfsB (*E. coli*; NZ_CP009072.1); NTR (*E. faecium*; CP014449.1).

Bacteria cell culture

Eight wild-type bacteria strains: *E. faecium*, methicillin sensitive and resistant *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *E. cloacae* and *E. coli* were used in this study. Tryptone Soya Broth (TSB) medium was used for culture of methicillin sensitive *S. aureus* and *P. aeruginosa*. Nutrient broth (NB) was used for culture of methicillin resistant *S. aureus*, *K. pneumoniae* and *E. cloacae*. Brain Heart Infusion Broth (BHI) was used for culture of *A. baumannii*. M.R.S. Broth (MRS) was used for culture of *E. faecium*. Luria-Bertani (LB) medium was used for culture of *E. coli*. Single colony from the stock agar plate was added to 10 mL of liquid medium, then was grown at 37 °C on a shaker incubator (180 rpm) overnight followed by a subculture until an OD_{600} of approximately 0.5 - 0.7 was reached.

Confocal imaging of bacteria treated with Cy 5.5[8]

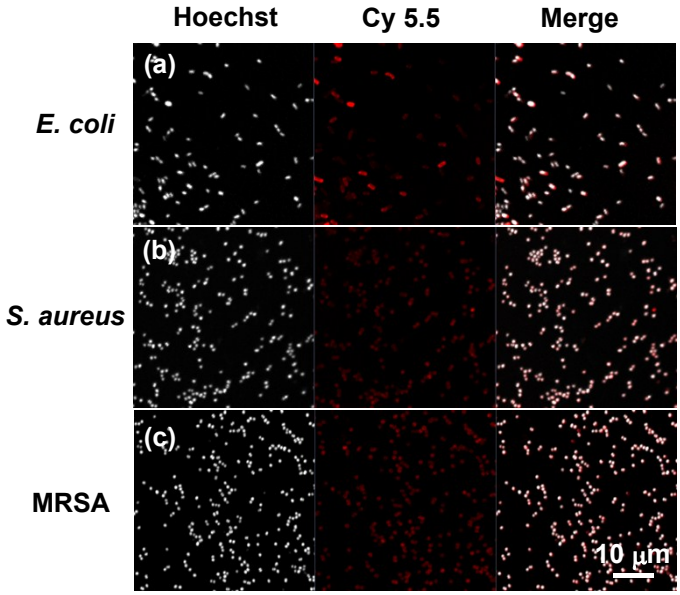


Fig. S2 Fluorescence images of *E. coli* (a), *S. aureus* (b) and MRSA (c) cells staining with Cy 5.5. All the bacterial cells were incubated with 5 μM Cy 5.5 at 37 °C in 0.05 M Tris buffer (pH 7.4) for 1 h prior to fluorescence imaging. λ_{ex} = 405 ± 20 nm and λ_{em} = 460 ± 25 nm; λ_{ex} = 633 ± 20 nm and λ_{em} = 690 ± 25 nm. Scale bar = 10 μm.

E. coli, *S. aureus* and MRSA cells were cultured for 12 h in respective media at 37 °C. Bacterial strains cultured overnight in respective solution were harvested and washed twice with Tris buffer (pH 7.4). The washed cells were resuspended in Tris buffer (pH 7.4) with an OD_{600} of 0.5 - 0.7. Then 500 μL aliquots were treated with 5 μM of Cy 5.5. After incubation at 37 °C for 1 h, the cells were washed with Tris buffer (pH 7.4) by centrifugation to remove the unbound reagents and

treated with 20 μg/mL of Hoechst 33258 at 37 °C for 30 min. Then a drop of the suspension was added into an 8-well chamber followed by covering with agarose pads.[9] Fluorescence images were acquired with ZEISS LSM 710 Confocal Microscope (Nikon Eclipse TE2000-E, CFI Plan-Apochromat VC 63 × oil immersedoptics), using a high pressure He-Ne lamp and diode laser for excitation (λ_{ex} = 633 ± 20 nm and λ_{em} = 690 ± 25 nm; λ_{ex} = 405 ± 20 nm and λ_{em} = 460 ± 25 nm).

UV-vis absorption and fluorescence spectra

The concentration of DMSO stock solution of probe **1** was diluted to 15 μM in Tris buffer. The UV-Visible spectra were recorded using a Tecan Spark™ 10M Multimode Microplate Reader. Wavelength interval: 5.0 nm. Fluorescence spectroscopic studies were also performed at the excitation wavelength of 658 nm. Wavelength interval: 2.0 nm.

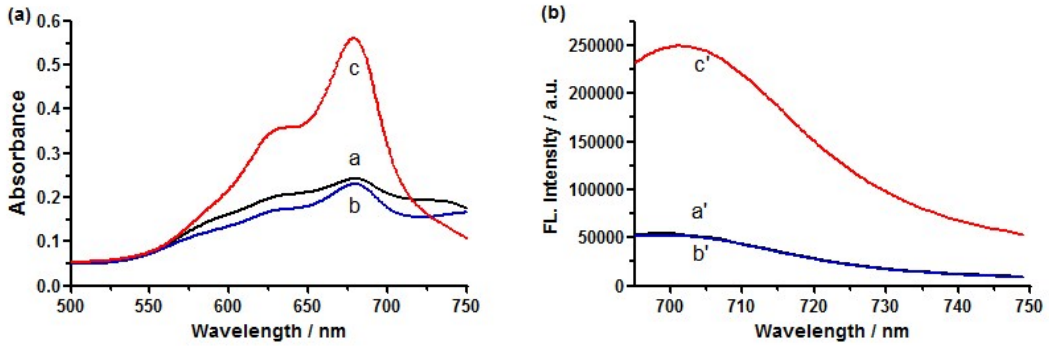


Fig. S3 (a) Absorption and (b) fluorescence emission spectra of 15 μM probe **1** and NTR (5 μg/mL) with or without 500 μM NADH in 0.05 M Tris buffer (pH 7.4) with 1.5% DMSO as co-solvent at 37 °C after an incubation time of 30 min. a and a': probe **1** in pH 7.4 Tris buffer (control); b and b': the system (a) + 500 μM NADH (another control); c and c': the system (b) + NTR (5 μg/mL). λ_{ex} = 658 nm.

HRMS proof for the sensing mechanism

Probe **1** and the reaction product (product **1**) of probe **1** with nitroreductase were characterized by ESI-MS in Fig. S4. A proposed detecting mechanism was shown in Scheme S4 according to one reported literature.[10]

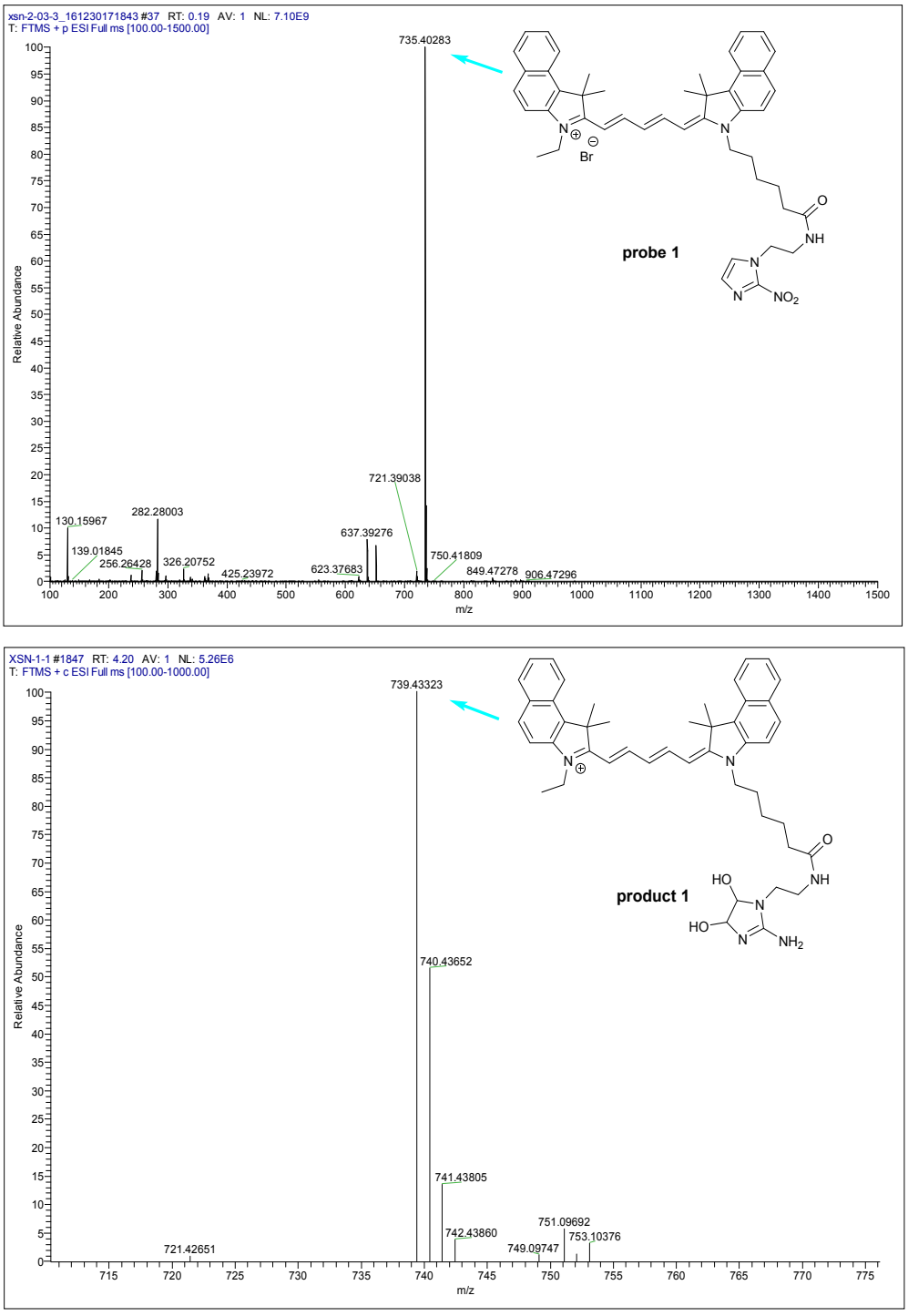
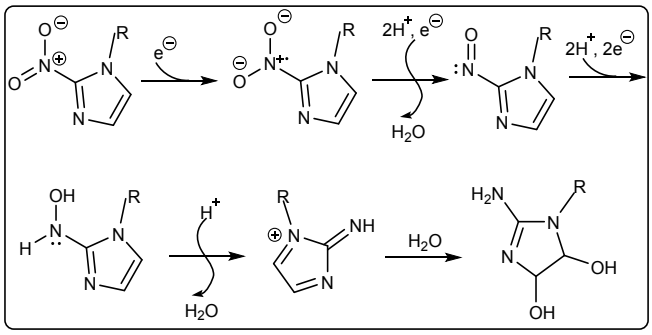


Fig. S4 ESI-MS spectrum of probe **1** and the reaction solution of probe **1** (50 μM) with NTR (10 μg/mL) after 60 min, in the presence of 500 μM NADH at 37 °C.



Scheme S4 The deduced mechanism of probe **1** activated by nitroreductase.

Effects of pH and temperature

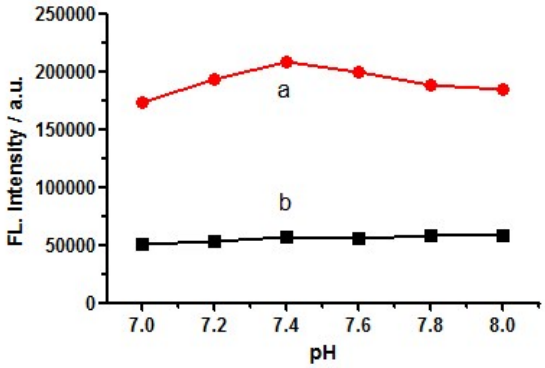


Fig. S5 Effect of pH on the fluorescence intensity of probe **1** (15 μM) reacted with nitroreductase. a: probe **1** (15 μM) + nitroreductase (1 μg/mL) + NADH (500 μM) in 0.05 M Tris buffer for 20 min at 37 °C. b: probe **1** (15 μM) + NADH (500 μM) in 0.05 M Tris buffer for 20 min at 37 °C. $\lambda_{ex}/\lambda_{em}$ = 658/699 nm.

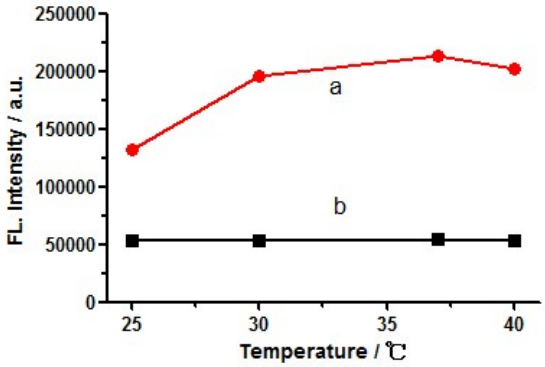


Fig. S6 Effect of temperature on the fluorescence intensity of probe **1** (15 μM) reacted with nitroreductase. a: probe **1** (15 μM) + nitroreductase (1 μg/mL) + NADH (500 μM) in 0.05 M Tris buffer for 20 min at 37 °C. b: probe **1** (15 μM) + NADH (500 μM) in 0.05 M Tris buffer for 20 min at 37 °C. $\lambda_{ex}/\lambda_{em}$ = 658/699 nm.

Apparent kinetic parameters for the enzymatic reaction of probe 1

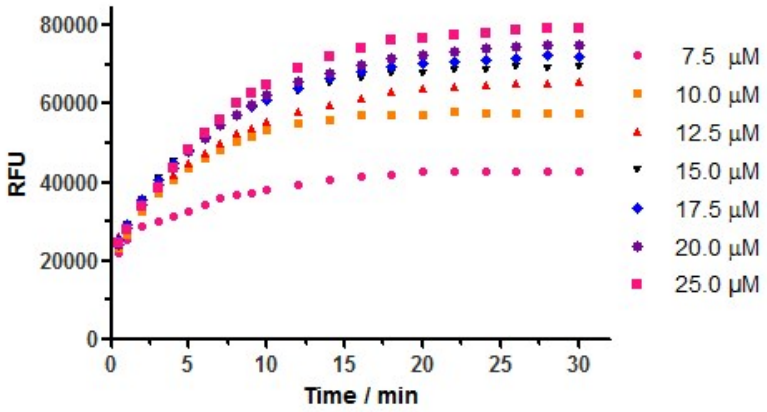


Fig. S7 A plot of fluorescence intensity at 699 nm over an incubation time of 30 min in the presence of different amounts of probe **1**: 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 25.0 μM. The measurements were performed at 37 °C in 0.05 M Tris buffer (pH 7.4) with 1.5% DMSO as co-solvent with 5 μg/mL NTR and 500 μM of NADH. λ_{ex} = 658 nm.

Lineweaver-Burk plot for the enzyme-catalyzed reaction

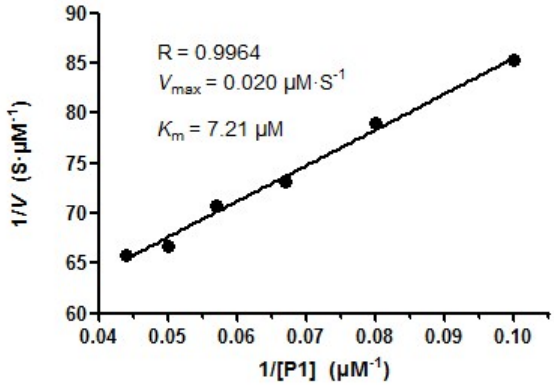


Fig. S8 Lineweaver-Burk plot for the enzyme-catalyzed reaction. The Michaelis-Menten equation was described as: $V = V_{max}$ [probe]/(K_m + [probe]), where V is the reaction rate, [probe] is the probe concentration (substrate), and K_m is the Michaelis constant. Conditions: 5 μg/mL NTR, 500 μM NADH, 7.5-25 μM of the probe, $\lambda_{ex}/\lambda_{em}$ = 658/699 nm. Points are fitted using a linear regression model (correlation coefficient: r = 0.9964).

Inhibition studies

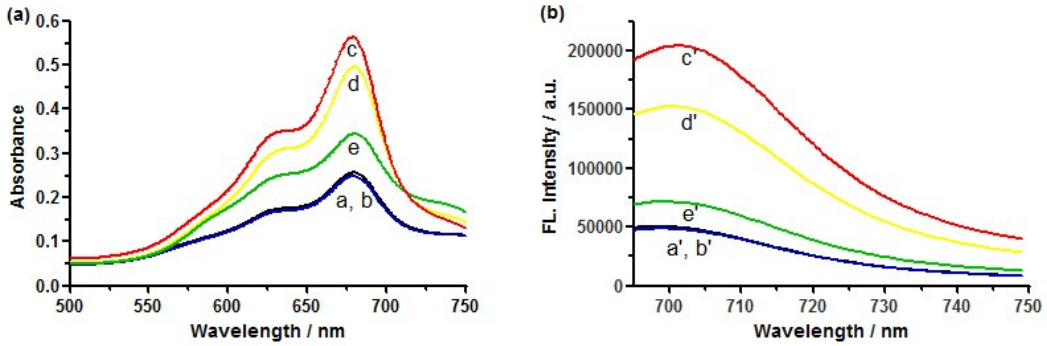


Fig. S9 (a) Absorption and (b) fluorescence emission spectra of probe **1** (15 μM) in the different reaction systems. a and a': probe **1** in pH 7.4 Tris buffer (control); b and b': the system (a) + 500 μM NADH (another control); c and c': the system (b) + NTR (1 μg/mL); d and d': the system (c) + dicoumarin (2.5 μM); e and e': the system (c) + dicoumarin (5.0 μM). All measurements were carried out after mixing 20 min. λ_{ex} = 658 nm.

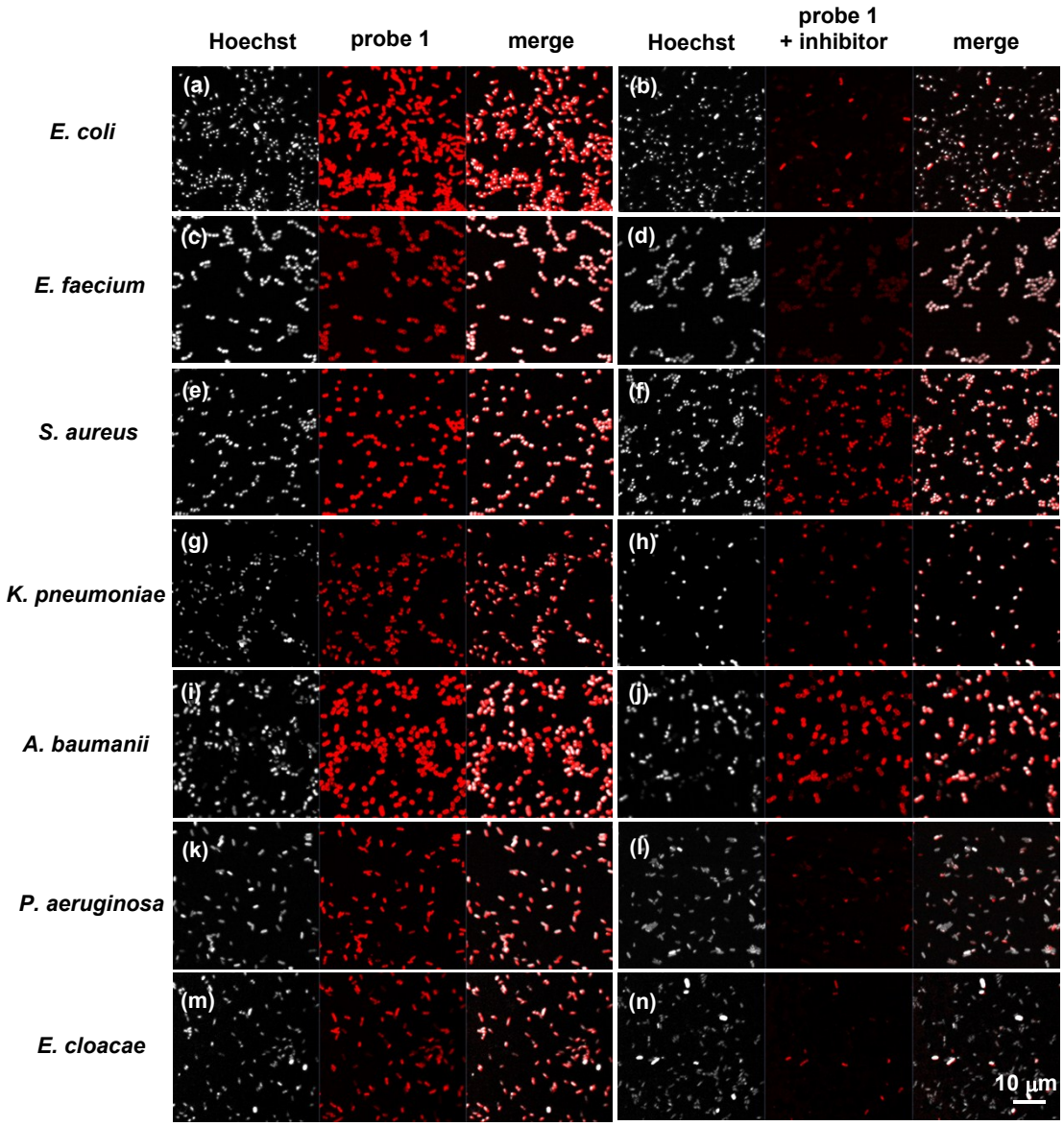
Measurements of nitroreductase activity in bacterial lysate

To measure nitroreductase activity in cell lysates, *E. coli* (ATCC 25922) cells were cultured for 12 h in Luria-Bertani (LB) medium at 37 °C, and then harvested and washed twice with Tris buffer (pH 7.4). The washed cells were resuspended in Tris buffer with an OD_{600} of 2.0, 1.0, 0.5, respectively. The cell suspension was then aliquoted and treated with 5 μM of probe **1** for 1h with or without dicoumarin (0.1 mM). Samples were then lysed by sonication, then fluorescence of the reaction solutions were measured. OD_{600} measurement was done by using the Metash UV-5100B spectrometer.

Confocal imaging of bacteria treated with probe 1

E. faecium, methicillin sensitive and resistant *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *E. cloacae* and *E. coli* cells were cultured for 12 h in respective media at 37 °C. Bacterial strains cultured overnight in respective solution were harvested and washed twice with Tris buffer (pH 7.4). The washed cells were resuspended in Tris buffer (pH 7.4) with an OD_{600} of 0.5 - 0.7. Then 500 μL aliquots were treated with 5 μM of probe **1** in the presence or absence of the enzyme inhibitor, dicoumarin (0.1 mM). After incubation at 37 °C for 1 h, the cells were washed with Tris buffer (pH 7.4) by centrifugation to remove the unbound reagents and treated with 20 μg/mL of Hoechst 33258 at 37 °C for 30 min. Then a drop of the suspension was added into an 8-well chamber followed by covering with agarose pads. Fluorescence images were

acquired with ZEISS LSM 710 Confocal Microscope (Nikon Eclipse TE2000-E, CFI Plan-Apochromat VC 63 × oil immersedoptics), using a high pressure He-Ne lamp and diode laser for excitation (λ_{ex} = 633 ± 20 nm and λ_{em} = 690 ± 25 nm; λ_{ex} = 405 ± 20 nm and λ_{em} = 460 ± 25 nm).



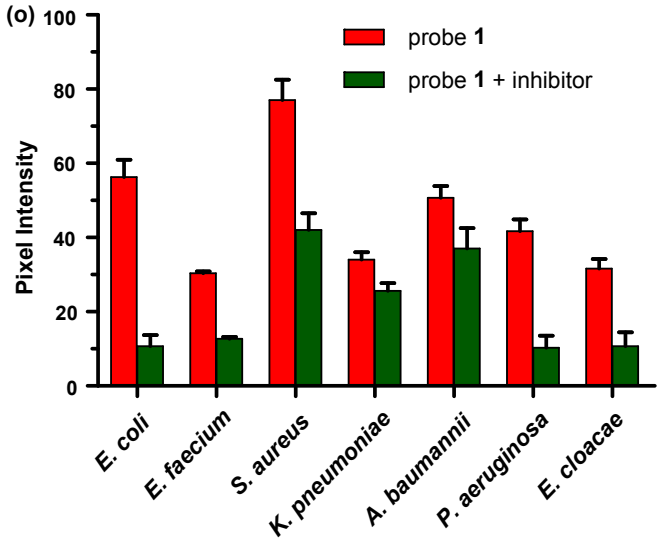


Fig. S10 (a - n) Fluorescence images of *E. coli* and ESKAPE cells staining with probe **1** in the absence or presence of the enzyme inhibitor, dicoumarin. All the bacterial cells were incubated with 5 μM probe **1** with or without dicoumarin (0.1 mM) at 37 °C in 0.05 M Tris buffer (pH 7.4) for 1 h prior to fluorescence imaging. λ_{ex} = 405 ± 20 nm and λ_{em} = 460 ± 25 nm; λ_{ex} = 633 ± 20 nm and λ_{em} = 690 ± 25 nm. Scale bar = 10 μm. (o) Relative pixel intensity obtained from the corresponding fluorescence images a - n by using the software ImageJ. The data are expressed as the mean ± SD (n = 3).

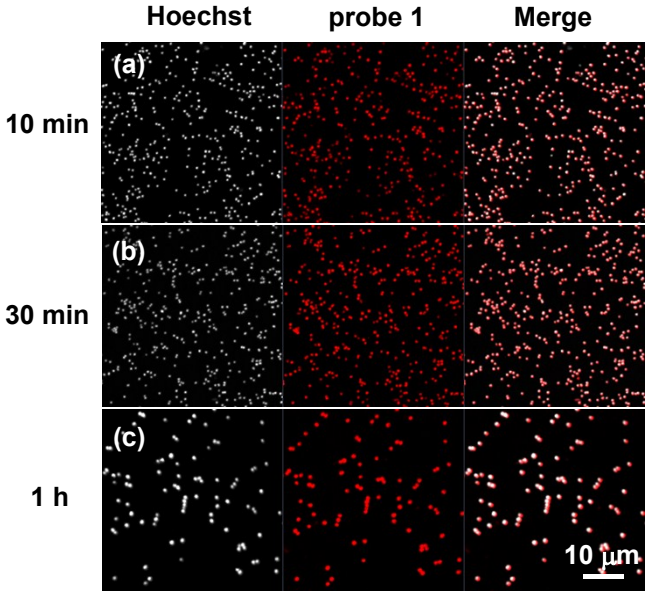


Fig. S11 Fluorescence images of MRSA cells staining with probe **1** for different incubation time. The bacterial cells were treated with 5 μM probe **1** at 37 °C in 0.05 M Tris buffer (pH 7.4) for 10 min, 30 min and 1 h, respectively. λ_{ex} = 405 ± 20 nm and λ_{em} = 460 ± 25 nm; λ_{ex} = 633 ± 20 nm and λ_{em} = 690 ± 25 nm. Scale bar = 10 μm.

Loading abilities of ESKAPE pathogens of product 1

In order to explore the loading abilities of ESKAPE pathogens of product **1**, following experiments have now been carried out .

1. Create a calibration curve with a plot of absorbance *vs.* concentration of product **1**.

   According to Beer's Law, A = εbc, under ideal conditions, a substance's concentration and its absorbance are directly proportional. A calibration curve with a plot of absorbance *vs.* concentration was made. As is shown in Fig. S12a, a gradual increase in absorbance was observed with the increase in the product **1** concentrations, and a good linearity was obtained in the concentration range of 0.5 to 10 μM, with a linear equation of A = 0.046 C (μM) + 0.006 (R = 0.9949).

2. Calibration the loading abilities of ESKAPE pathogens of product **1**.

   Bacteria incubation: ESKAPE pathogens and *E. coli* cells were cultured for 12 h in respective media at 37 °C. Bacterial strains cultured overnight in respective solution were harvested and washed twice with Tris buffer (pH 7.4). The washed cells were resuspended in Tris buffer (pH 7.4) with an OD_{600} of 0.5. Then 500 μL aliquots were treated with or without (blank) 5 μM of product **1** at 37 °C for 1 h, and the supernatant was collected (removed from the buffer) by micro-centrifugation at 12000g for 30s. OD_{600} measurement was done by using the Metash UV-5100B spectrometer.

   Calculation: The concentrations of product **1** in the buffer before and after incubated with ESKAPE pathogens and *E. coli* were determined by the concentration calibration curve. The concentration difference of product **1** between before and after bacteria incubation presented the amount of product **1** that was absorbed by bacterial cells. As is shown in Fig. S12c,d, with an OD_{600} of 0.5, ESKAPE pathogens and *E. coli* showed comparable binding capacity to product **1** with (*E. coli* - 2.27 μmol; *E. faecium* - 2.405 μmol; *S. aureus* - 2.41 μmol; MRSA - 2.33 μmol; *K. pneumoniae* - 1.95 μmol; *A. baumannii* - 2.375 μmol; *P. aeruginosa* - 2.055 μmol; *E. cloacae* - 2.155 μmol). Therefore, we deduced different fluorescence turn-on response of probe **1** to ESKAPE pathogens most likely be due to the different amount and/or types of NTRs in respective strains.

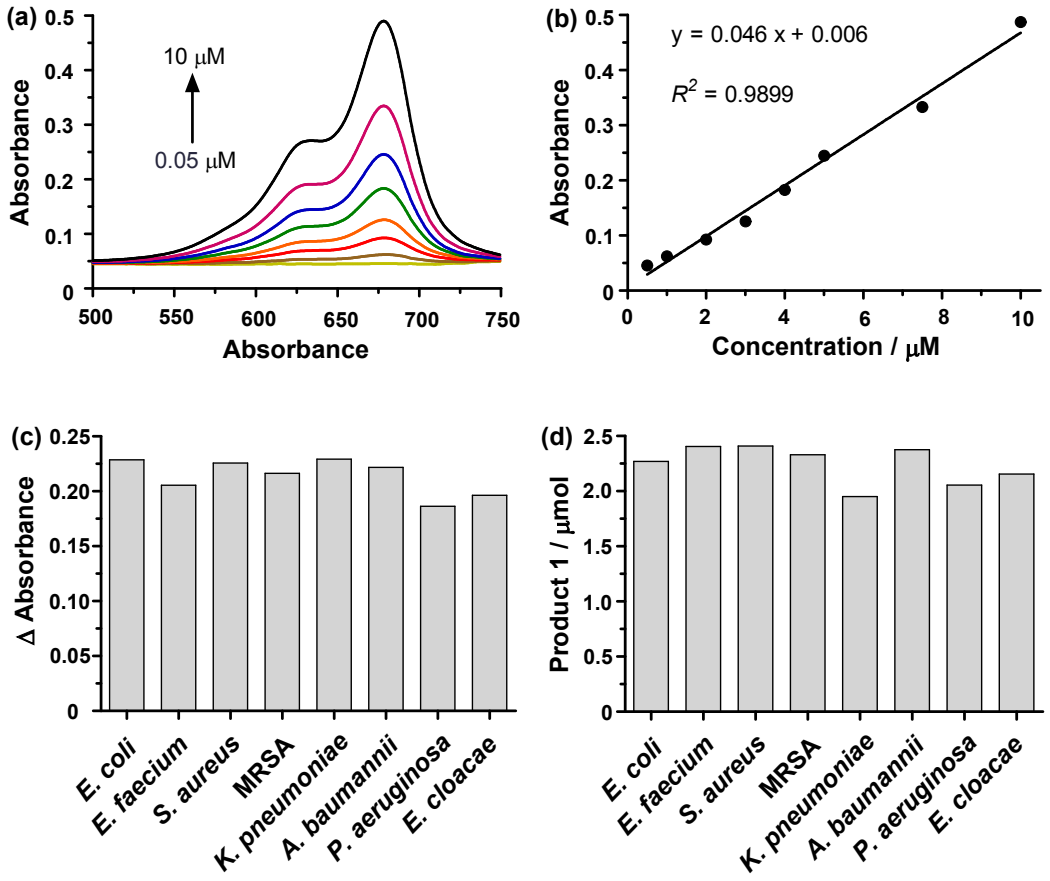
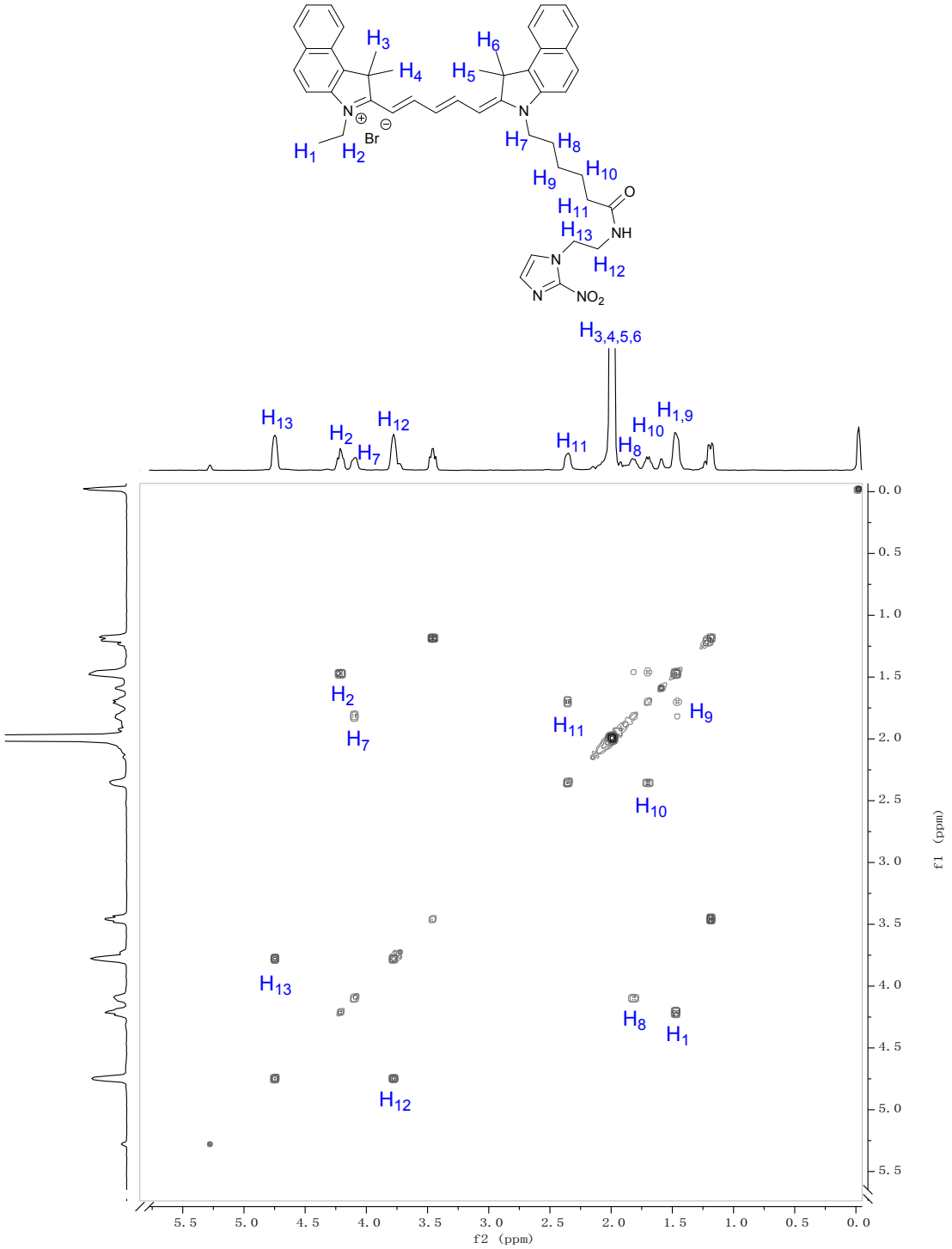
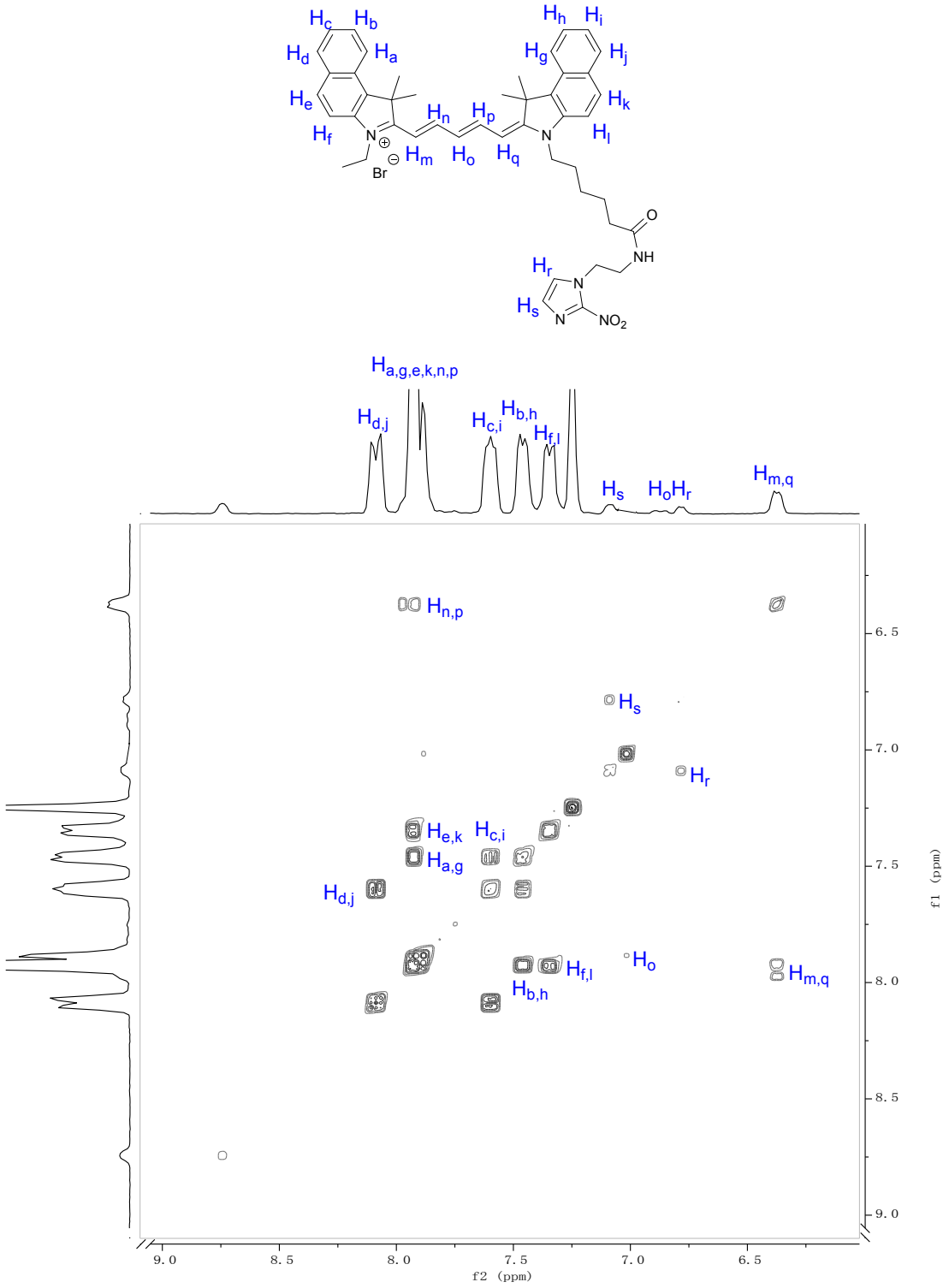


Fig. S12 (a) Absorption spectra of product **1** at varied concentrations (0.5, 1, 2, 3, 4, 5, 7.5 and 10 μM). (b) Linear correlation between the concentration and the absorbance of product **1**. (c) ΔAbsorption at λ_{max} of product **1** (5 μM) incubated with different bacteria at the same initial concentration at 37 °C for 1 h. (d) The number of moles of product **1** that was absorbed by different bacterial strains.

COSY, TOCSY and HSQC NMR analysis of probe 1




Hc Hb
Hd Ha
He
Hf
N
Br
Hn Hp
Hm Ho Hq
Hh Hi
Hg Hj
Hk
Hl
N
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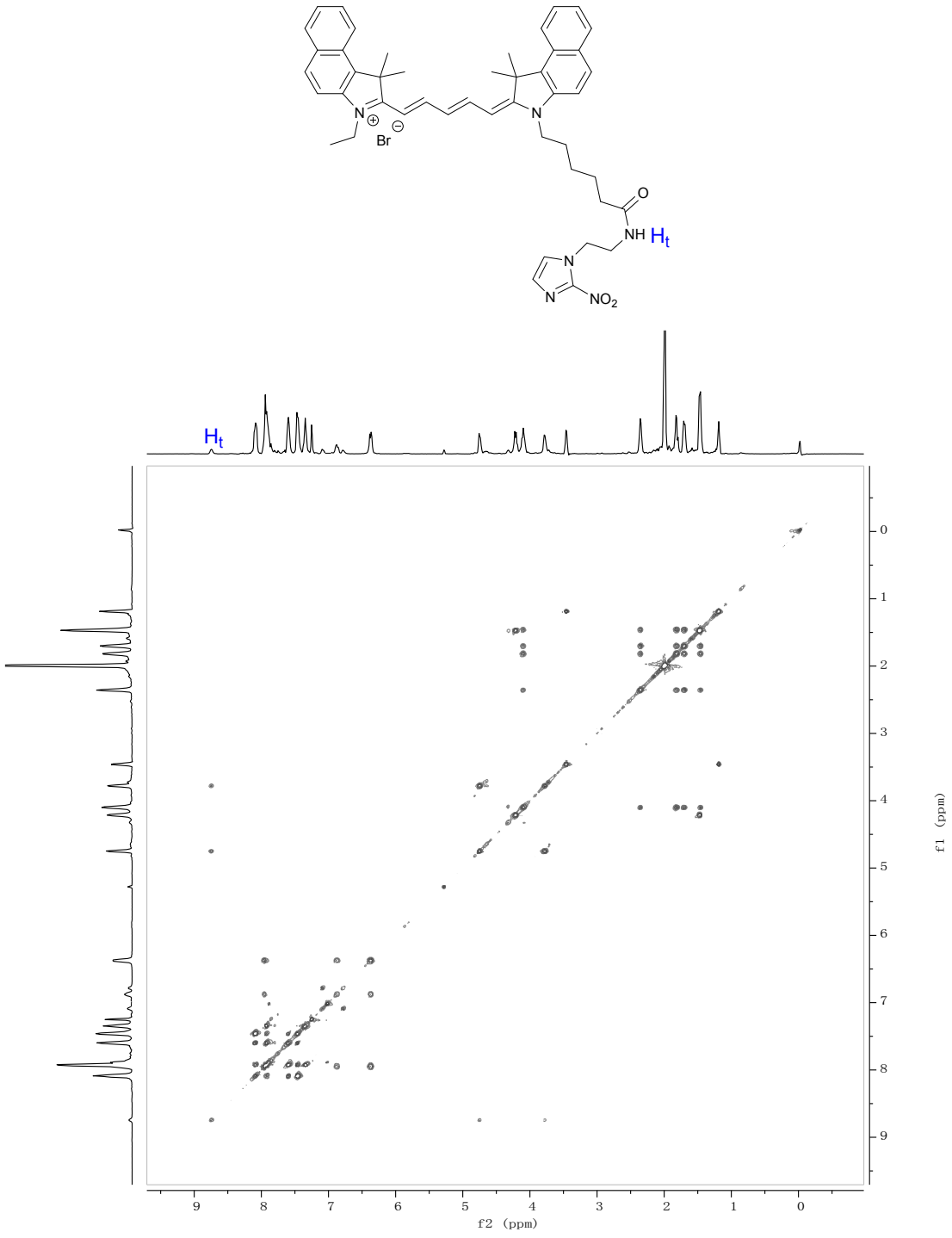
NH
Hr
N
Hs
N
NO2
Ha,g,e,k,n,p
Hd,j
Hc,i
Hb,h
Hf,l
Hs
HoHr
Hm,q
Hn,p
Hs
Hr
He,k
Hc,i
Ha,g
Hd,j
Hf,l
Ho
Hm,q
Hb,h
6.5
7.0
7.5
8.0
8.5
9.0
f1 (ppm)
9.0
8.5
8.0
7.5
7.0
6.5
f2 (ppm)


Br
O
NH
Ht
N
N
NO2
Ht
f2 (ppm)
f1 (ppm)

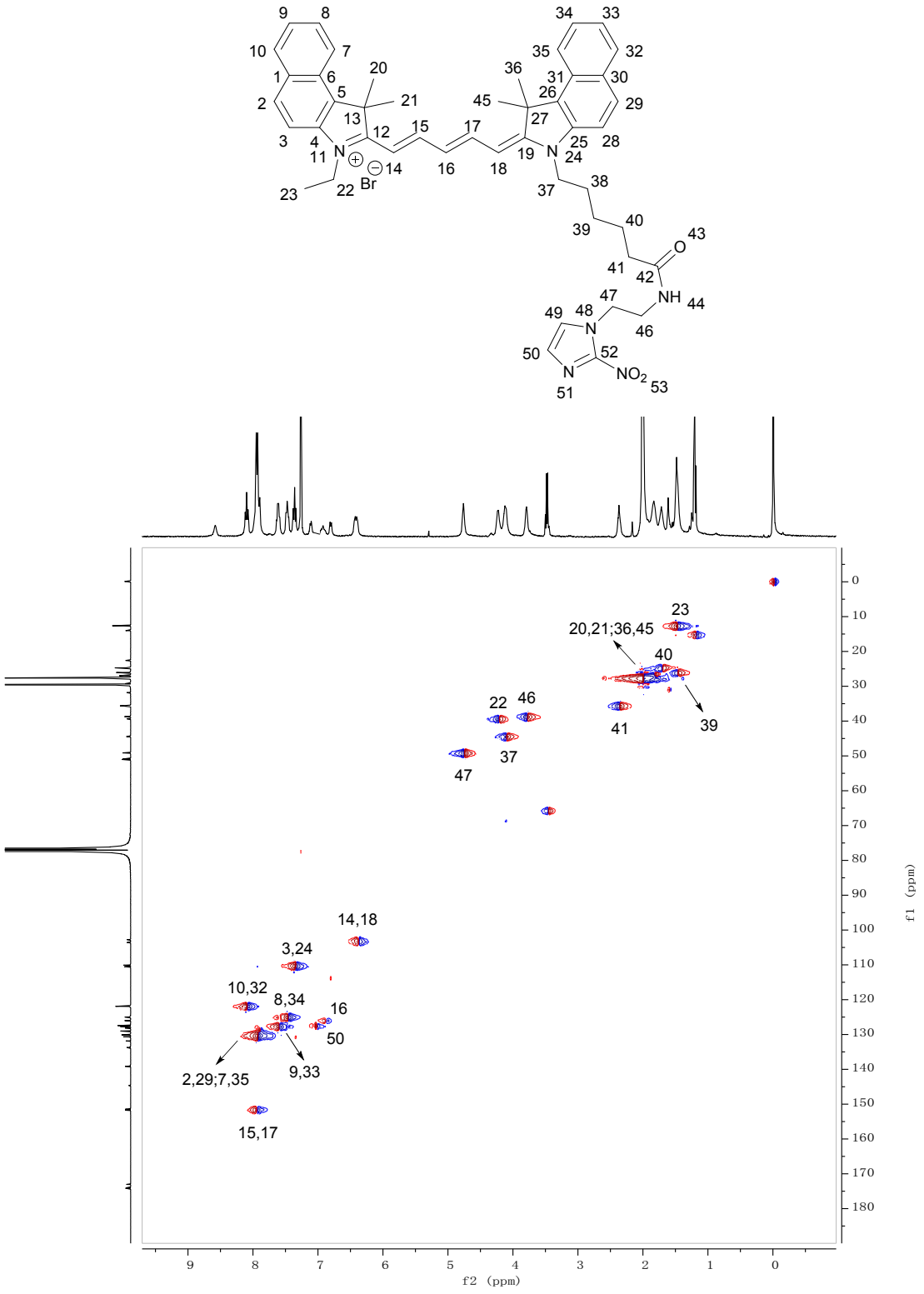


Fig. S13 COSY, TOCSY and HSQC spectra of probe **1**

Comparison analysis of spectroscopic properties between probe 1 and product 1

In order to clarify the mechanism of the quenching of probe **1**, UV-vis and fluorescence emission spectra of probe **1** and product **1** were measured in MeOH and Tris buffer, respectively. As shown in Fig. S14, probe **1** displayed larger absorption and fluorescence intensities in MeOH than in Tris buffer, which reveals that probe **1** forms a fluorescent aggregate in Tris buffer. Probe **1** and product **1** exhibited similar absorbance spectra in MeOH; however, the fluorescence intensity of probe **1** showed 20% lower than product **1**. Therefore, we inferred fluorescence quenching of probe **1** is caused by PET and aggregation, synergistically.

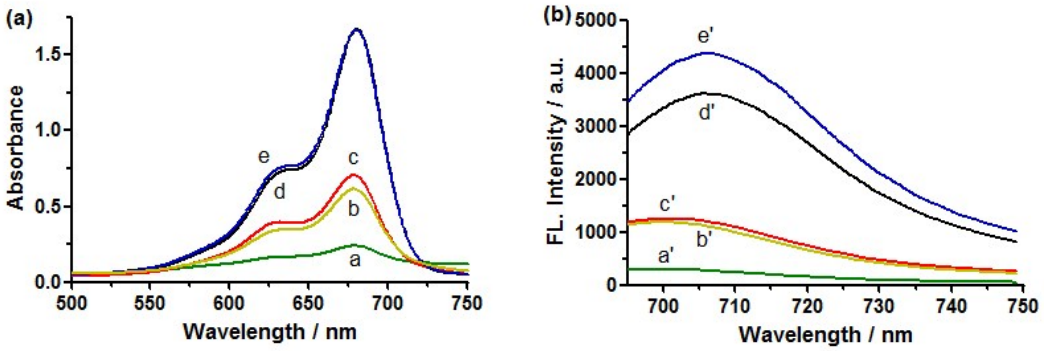


Fig. S14 (a) Absorption and (b) fluorescence emission spectra. a and a': 15 μM probe **1** in Tris buffer (pH 7.4, 1.5% DMSO); b and b': 15 μM probe **1** mixed with 5 μg/mL NTR in the presence of 500 μM NADH at 37 °C for 1 h in Tris buffer; c and c': 15 μM product **1** in Tris buffer; d and d': 15 μM probe **1** in MeOH; e and e': 15 μM product **1** in MeOH. λ_{ex} = 658 nm.

HPLC analysis for the reaction system

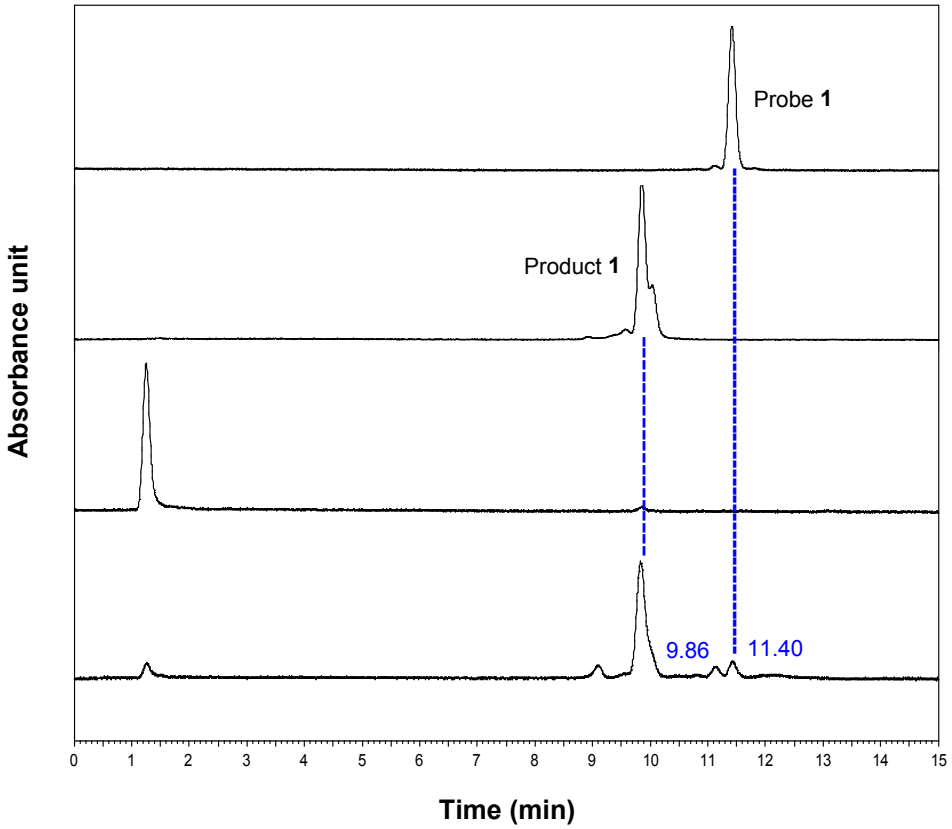


Fig. S15 HPLC chromatogram profiles of (a) 50 μM probe **1**, (b) 50 μM product **1**, (c) control: 500 μM NADH and 5 μg/mL NTR, and (d) the reaction products of 50 μM probe **1** mixed with 5 μg/mL NTR in the presence of 500 μM NADH for 1 h. The assignments of the peaks: 11.40 min, probe **1** and 9.86 min, product **1**. As can be seen, product **1** and probe **1** have a retention time of 9.86 min and 11.40 min respectively; after reaction with nitroreductase, the peak of probe **1** at 11.40 min decreases markedly, accompanied by the appearance of the peak at 9.86 min indicative of product **1**.

References:

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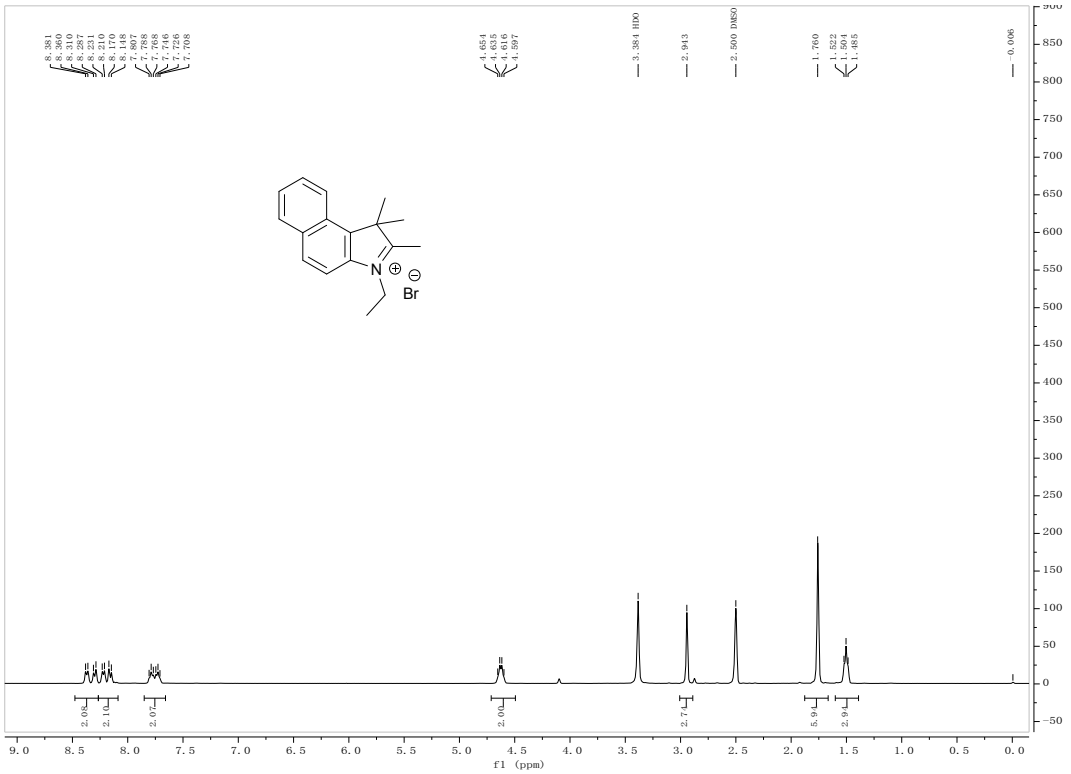
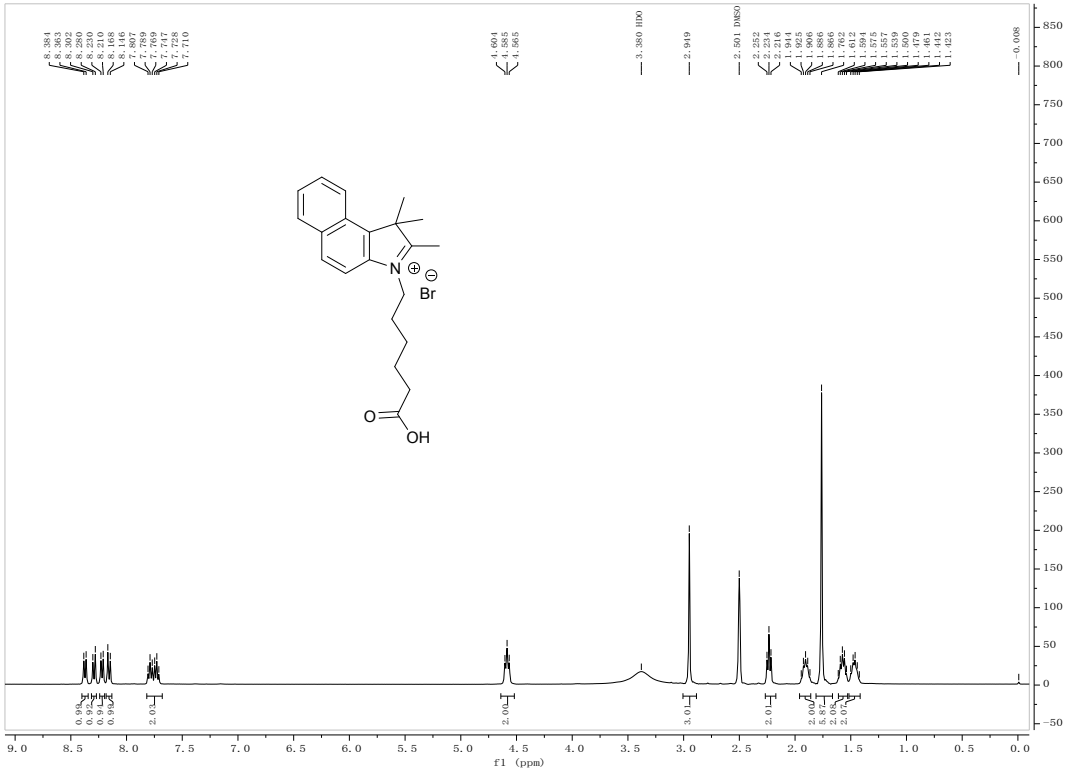
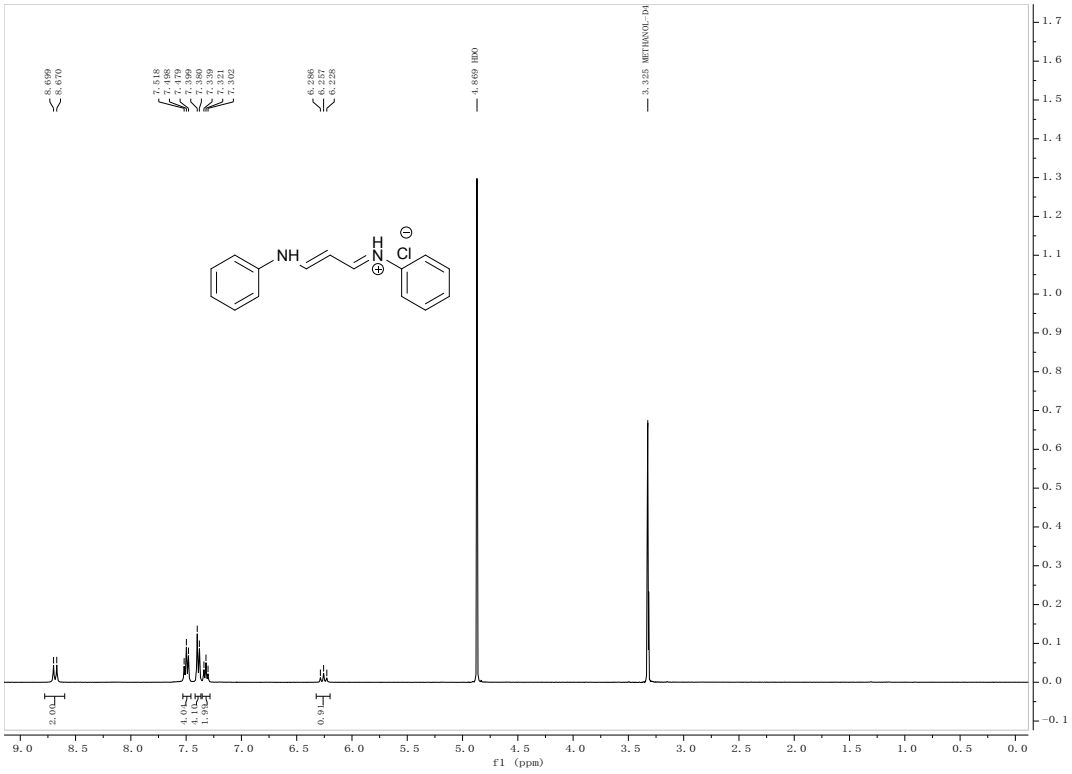
Copies of ^{1}H NMR and ^{13}C NMR spectrum of compounds



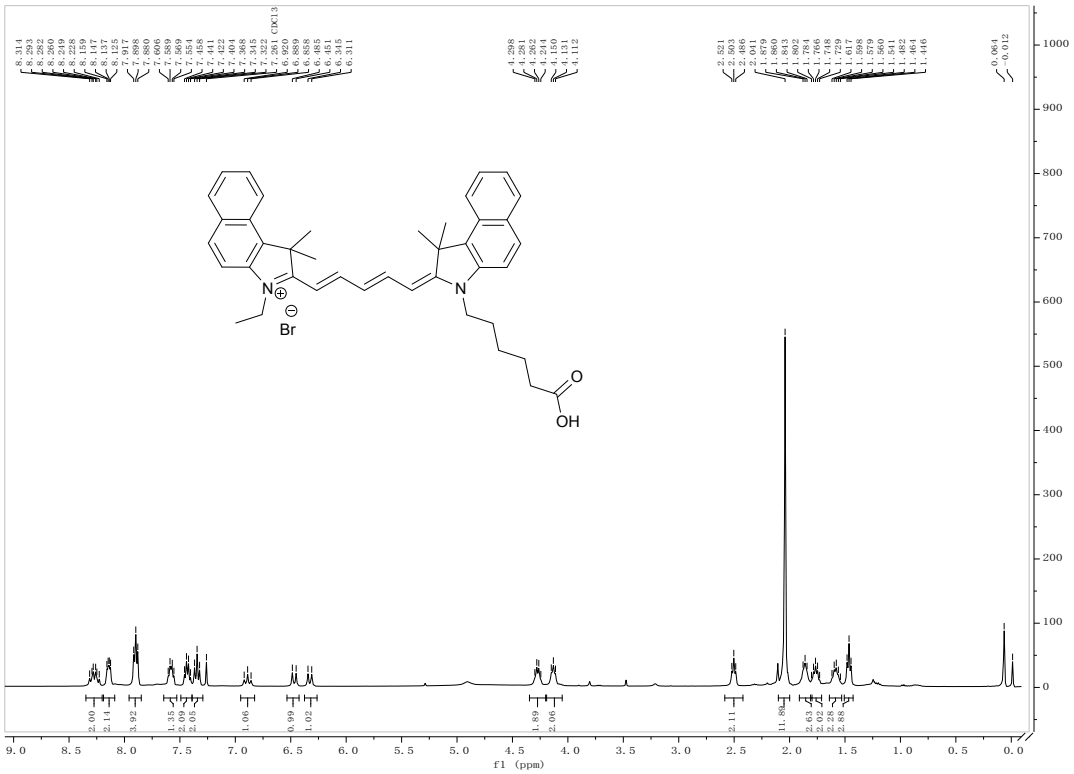
^{1}H NMR (400 MHz, DMSO-*d*6) of compound **2**



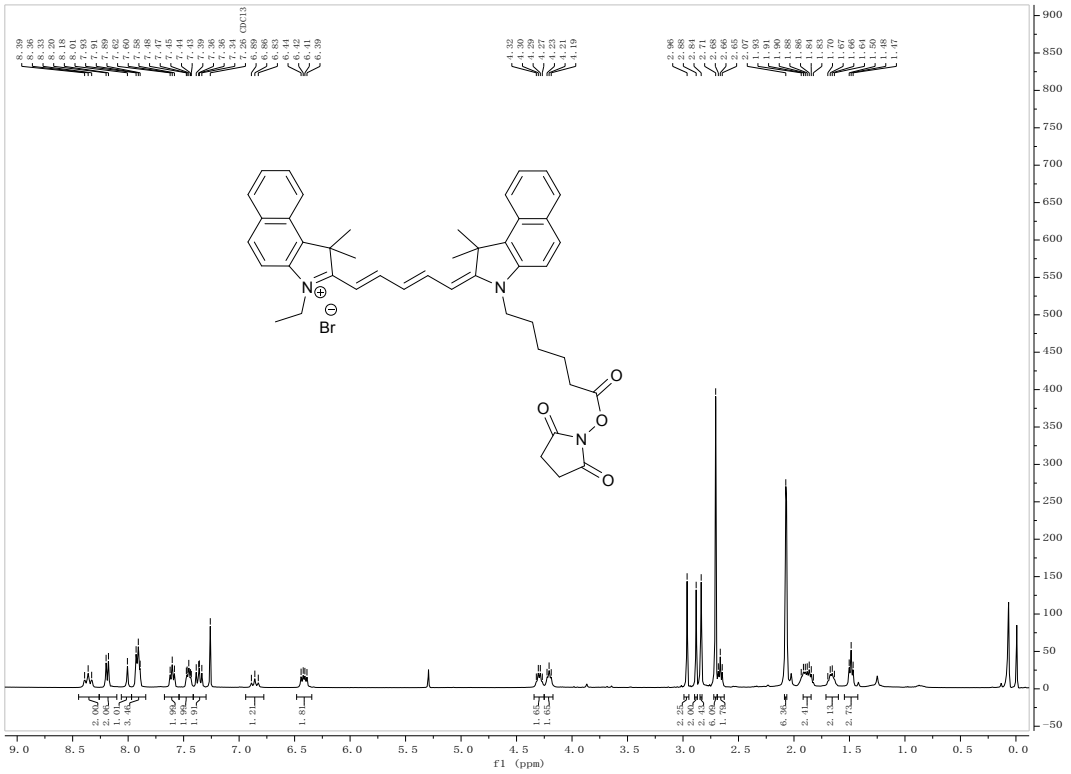
^{1}H NMR (400 MHz, DMSO-*d*6) of compound **3**



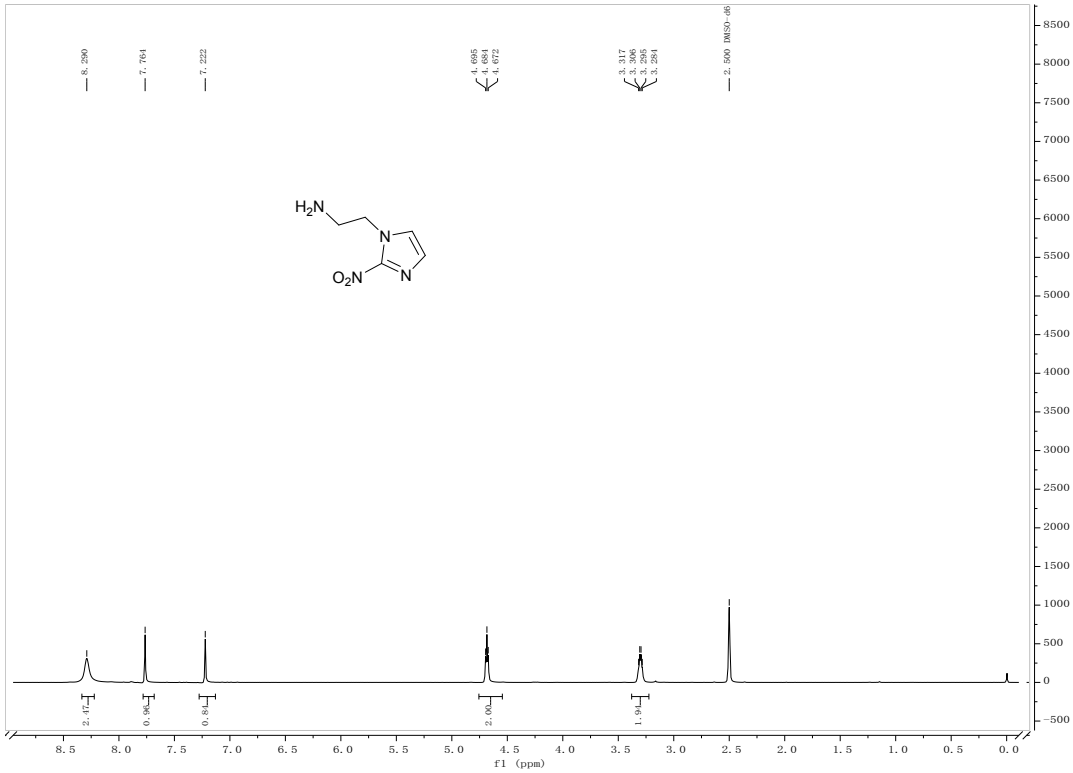
^{1}H NMR (500 MHz, Methanol-*d*4) of compound **5**



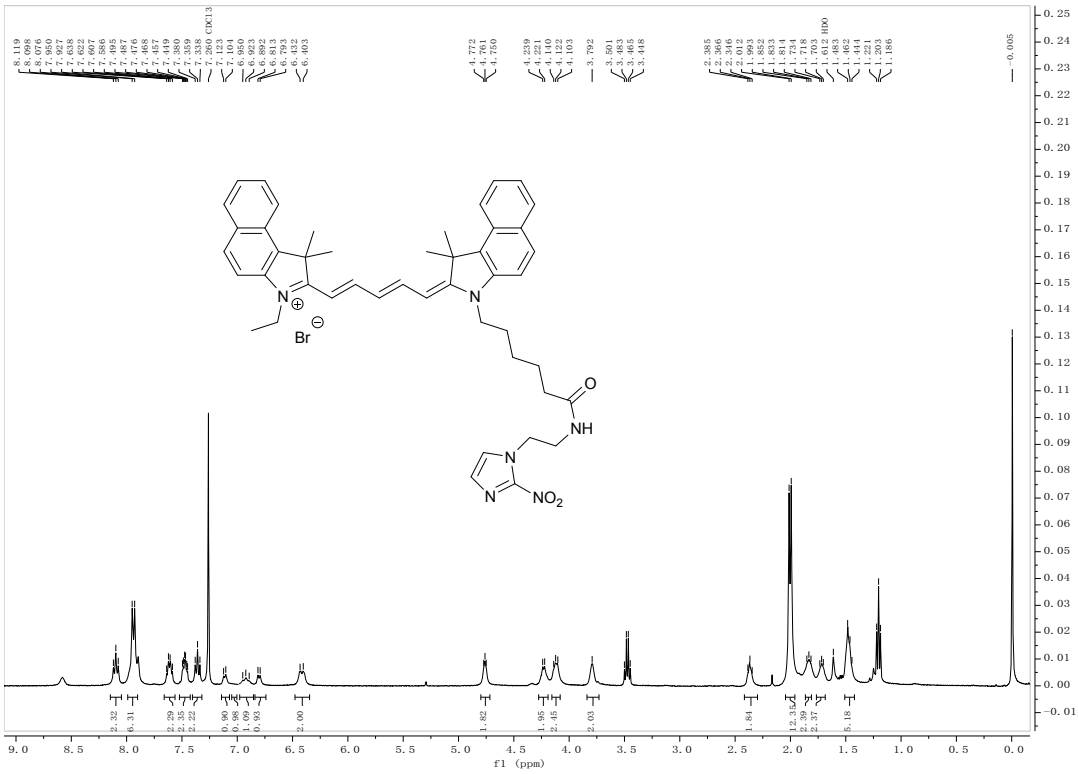
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **6**



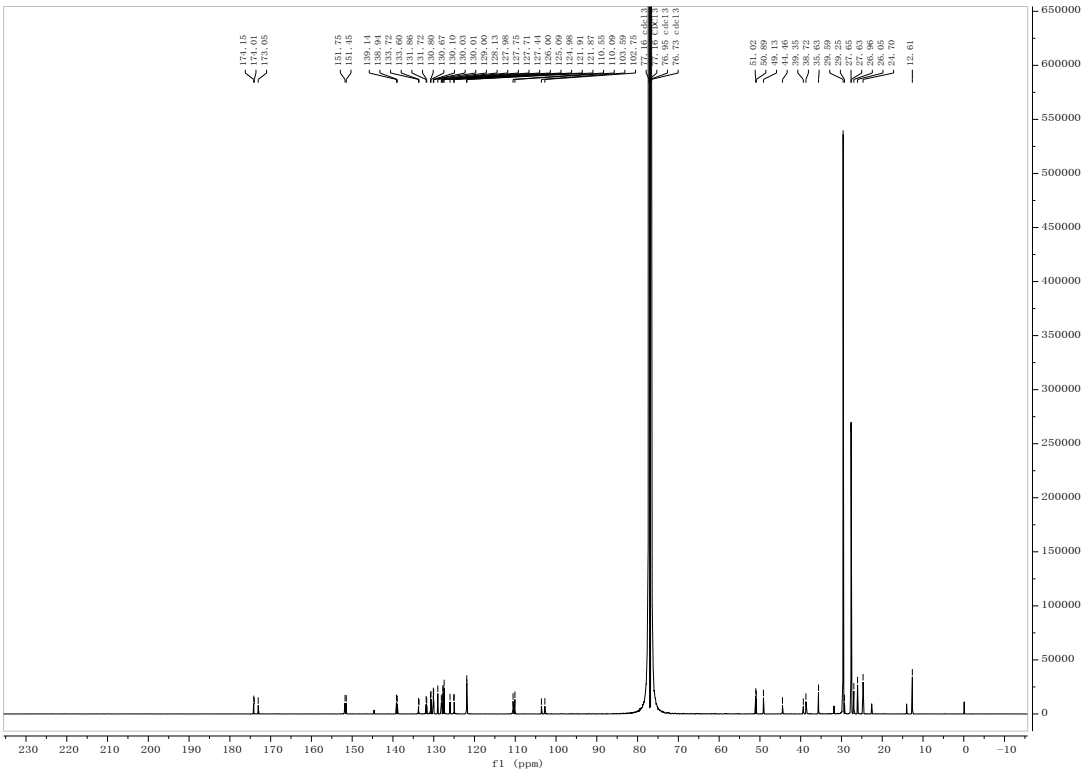
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **7**



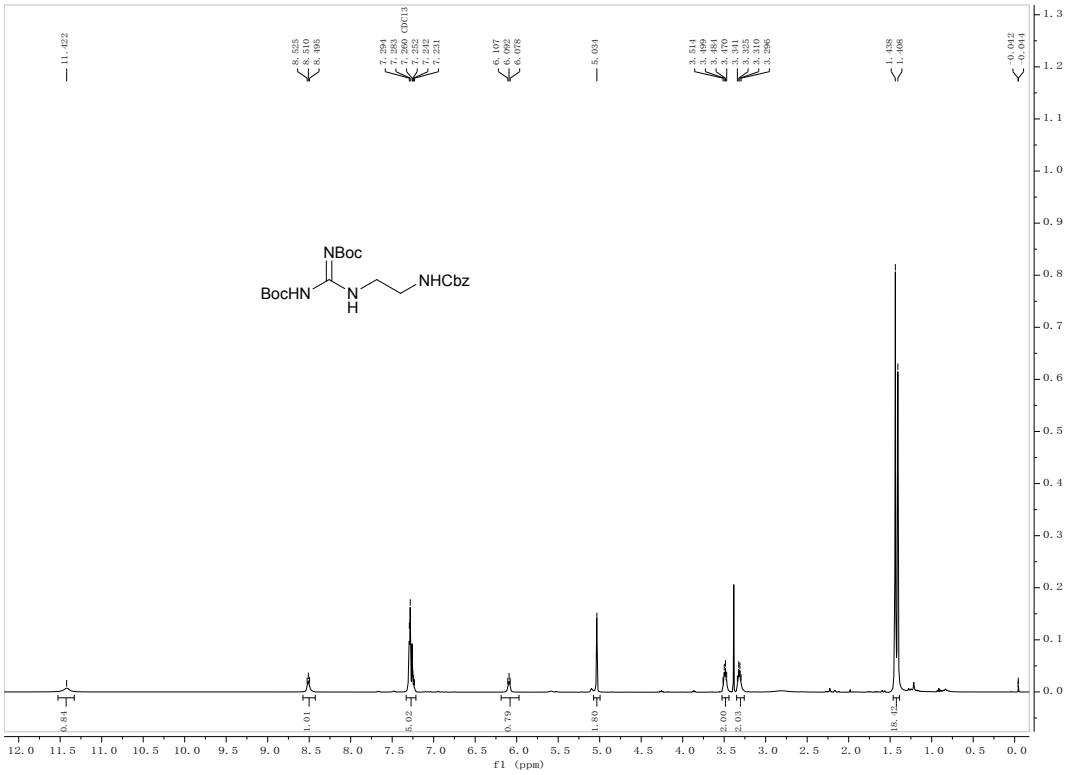
^{1}H NMR (400 MHz, DMSO-*d6*) of compound **9**



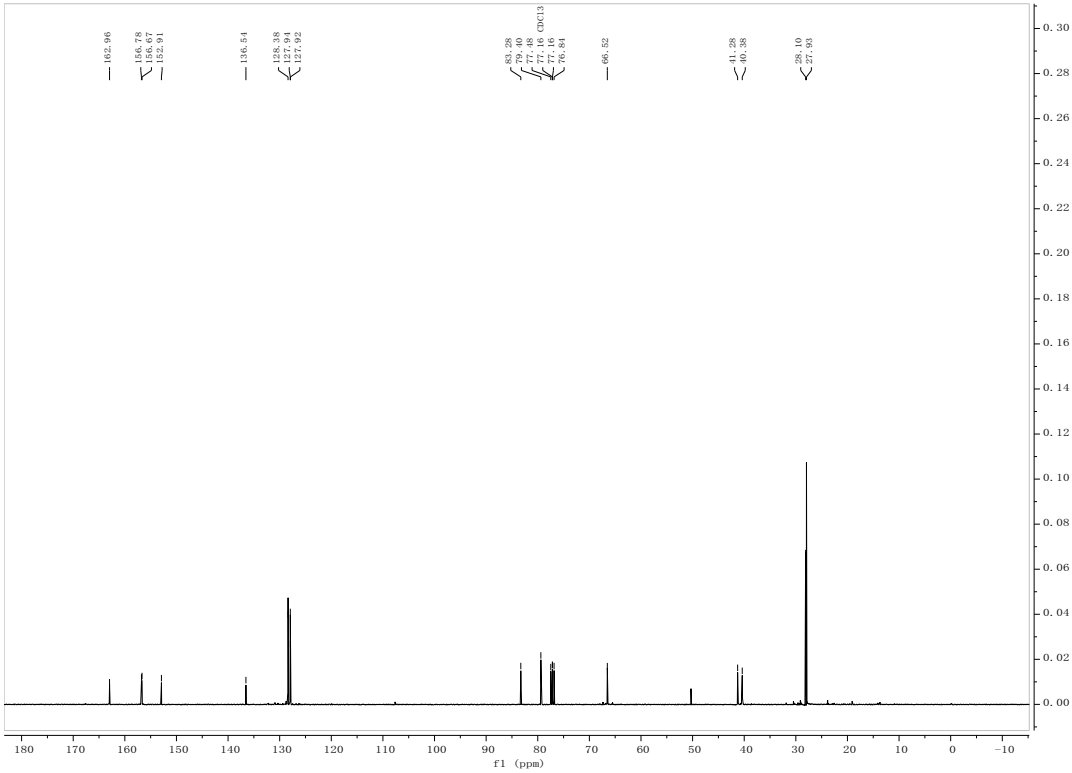
^{1}H NMR (400 MHz, Chloroform-*d*) of probe **1**



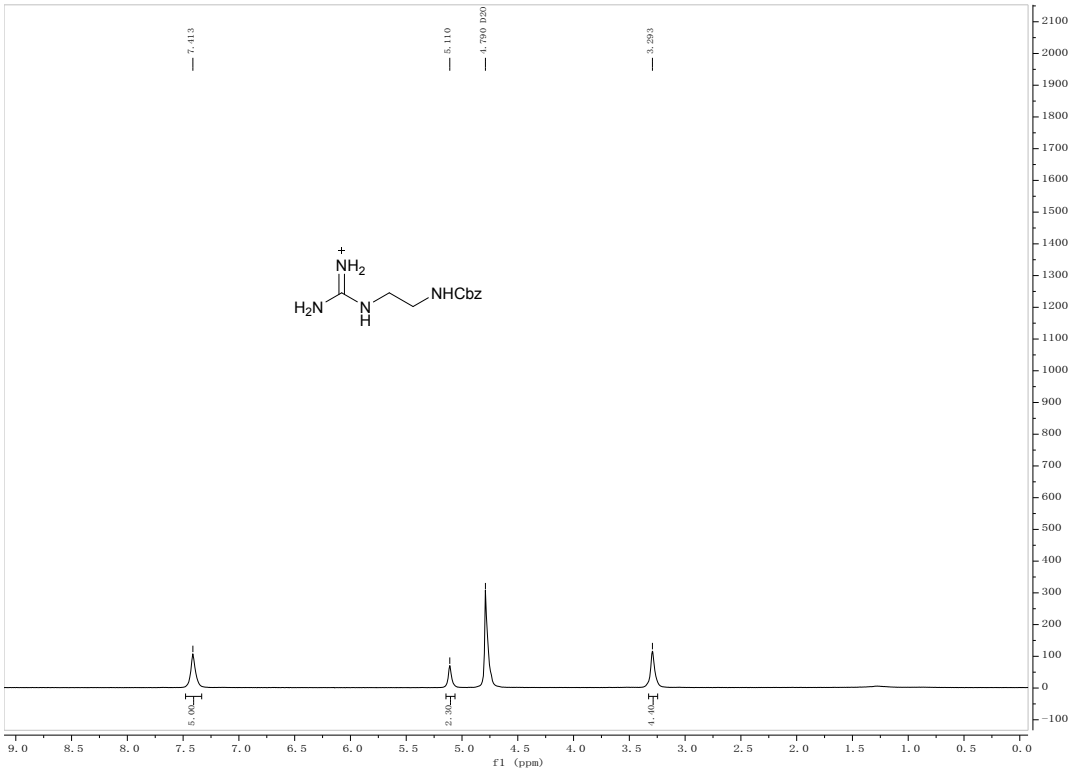
^{13}C NMR (150 MHz, Chloroform-*d*) of probe **1**



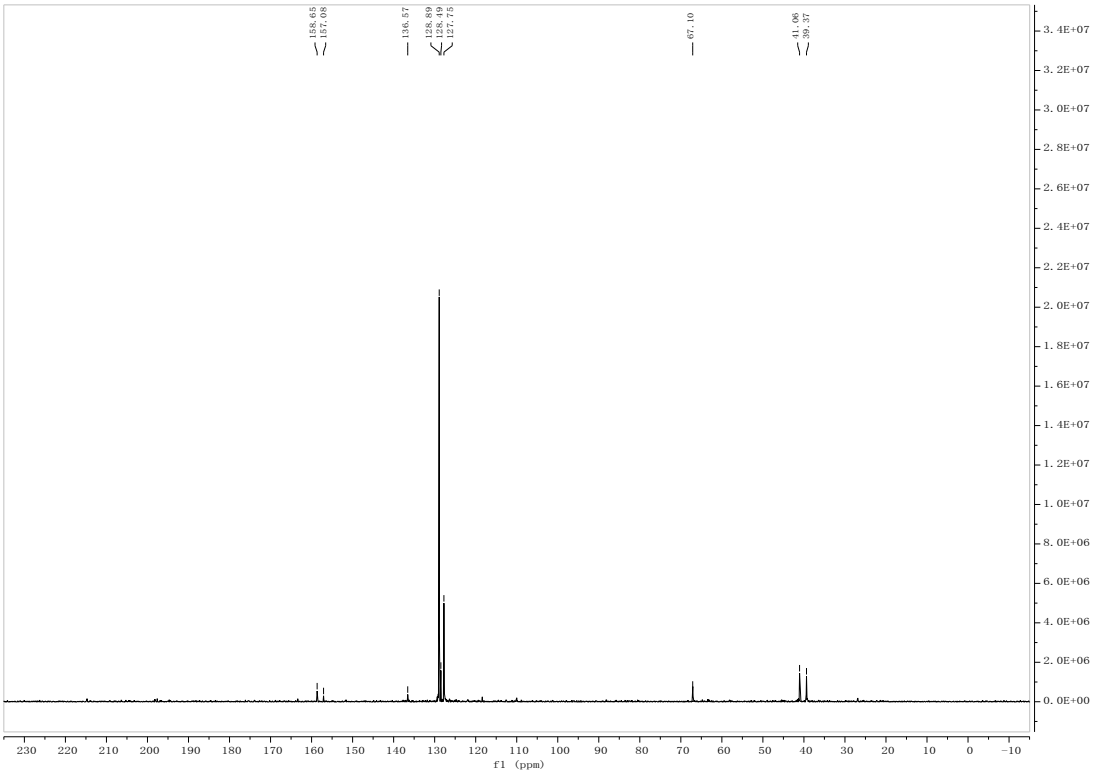
^{1}H NMR (400 MHz, Chloroform-*d*) of compound **10**



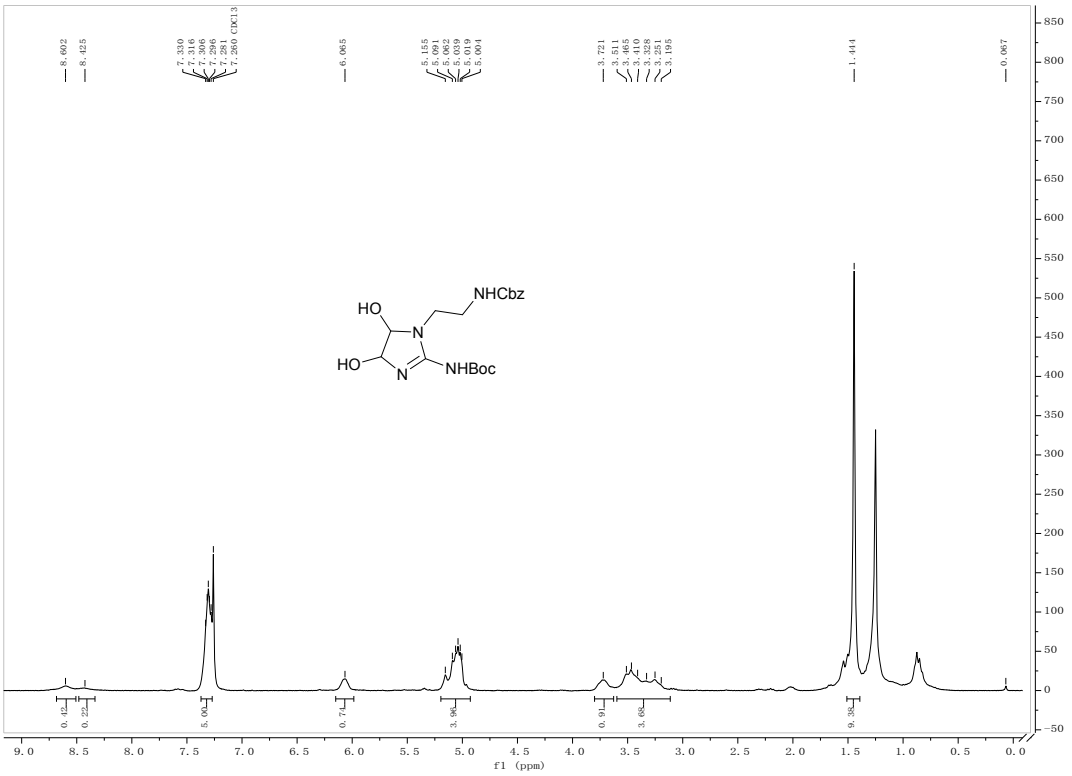
^{13}C NMR (100 MHz, Chloroform-*d*) of compound **10**



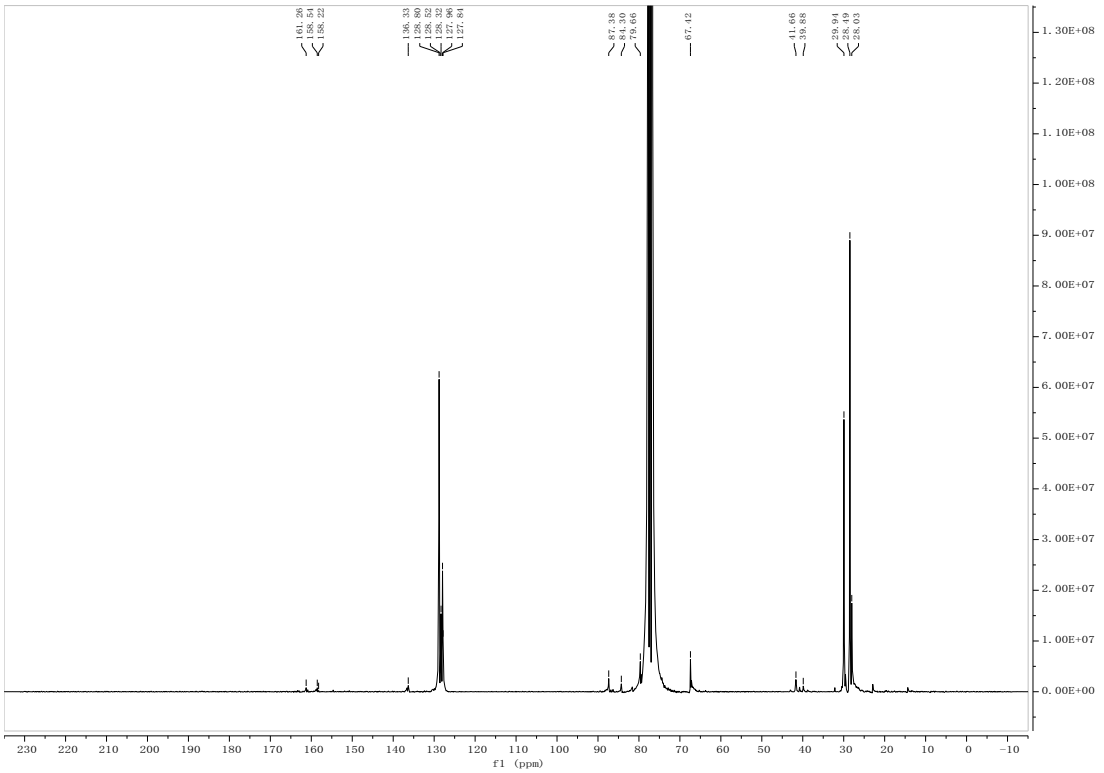
^{1}H NMR (300 MHz, D_2O) of compound **11**



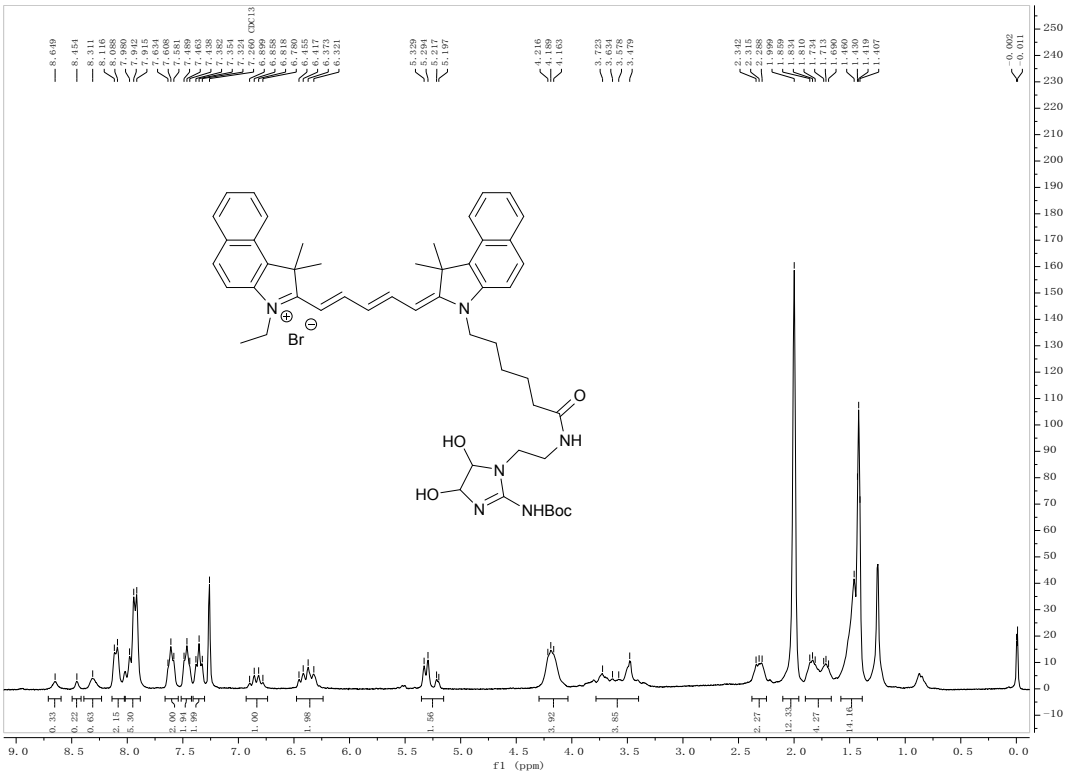
^{13}C NMR (75 MHz, D_2O) of compound **11**



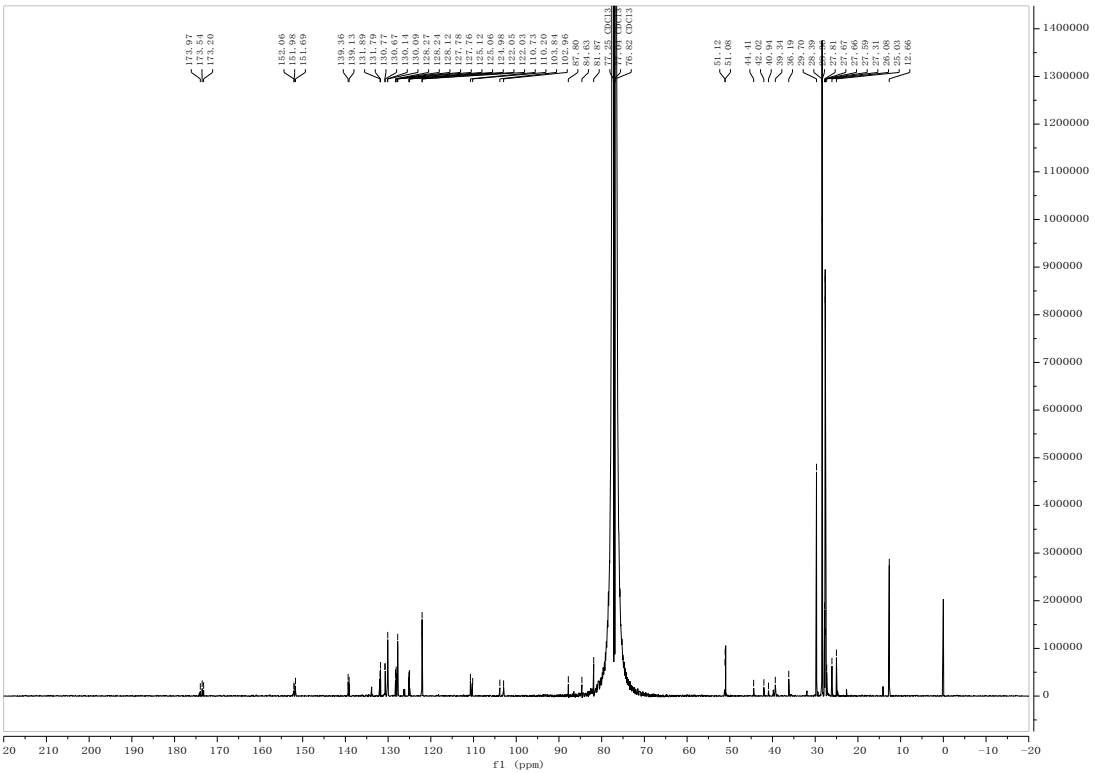
^{1}H NMR (300 MHz, Chloroform-*d*) of compound **13**



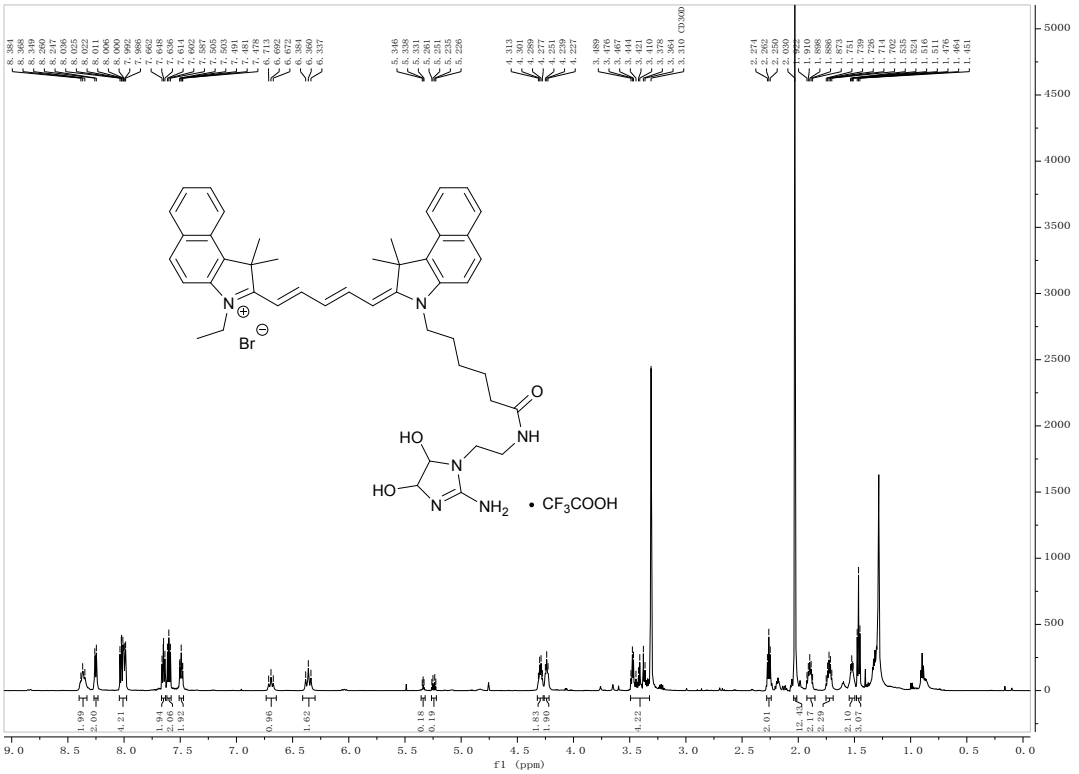
^{13}C NMR (75 MHz, Chloroform-*d*) of compound **13**



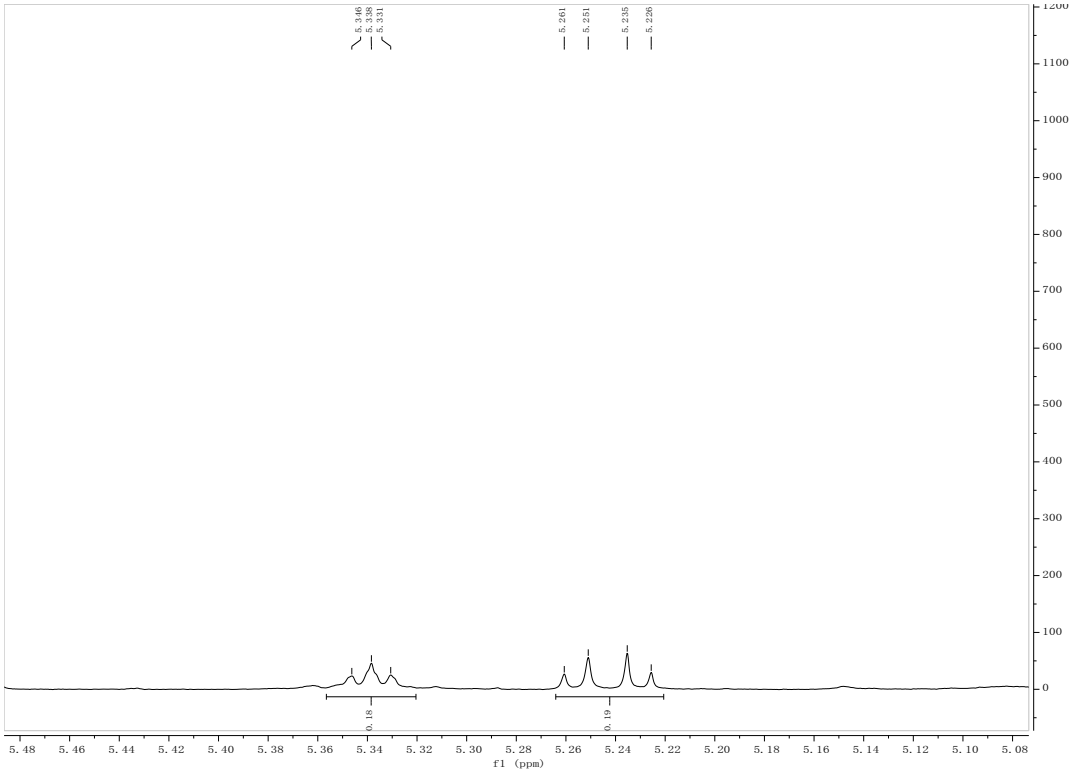
^{1}H NMR (300 MHz, Chloroform-*d*) of compound **15**



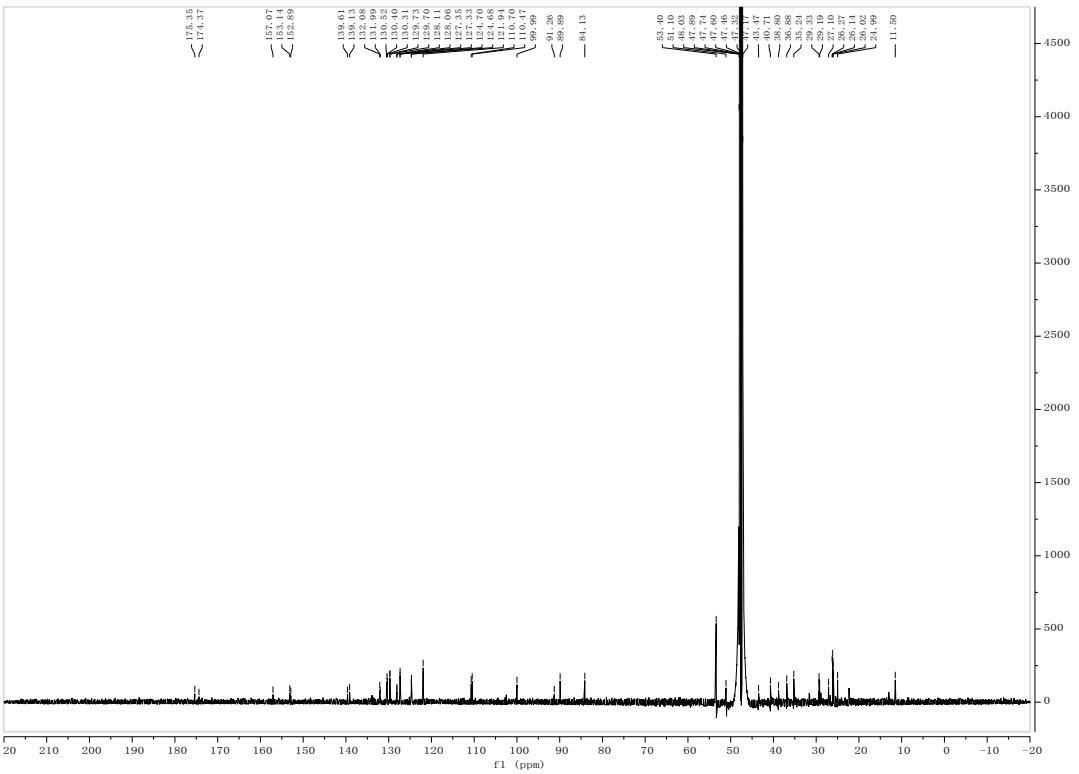
^{13}C NMR (150 MHz, Chloroform-*d*) of compound **15**



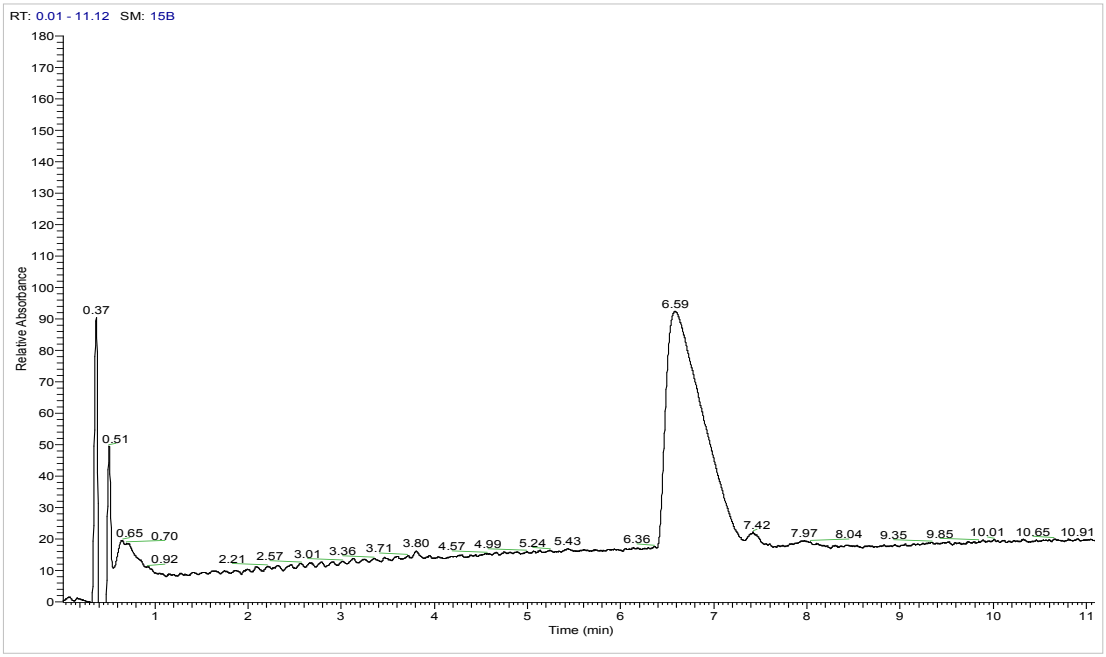
^{1}H NMR (600 MHz, Methanol-*d*4) of product **1**



^{1}H NMR (600 MHz, Methanol-*d*4) of product **1** (δ 5.48–5.08)



^{13}C NMR (150 MHz, Methanol-*d*4) of product **1**



The purity of probe **1** by HPLC