

Electronic Supplementary Information

A ratiometric fluorescent probe for the detection of hydroxyl radicals in living cell

Luyan Meng, Yongquan Wu, Tao Yi*

Department of chemistry, Fudan University, 220 Handan Road, Shanghai
200433, China

E-mail: yitao@fudan.edu.cn

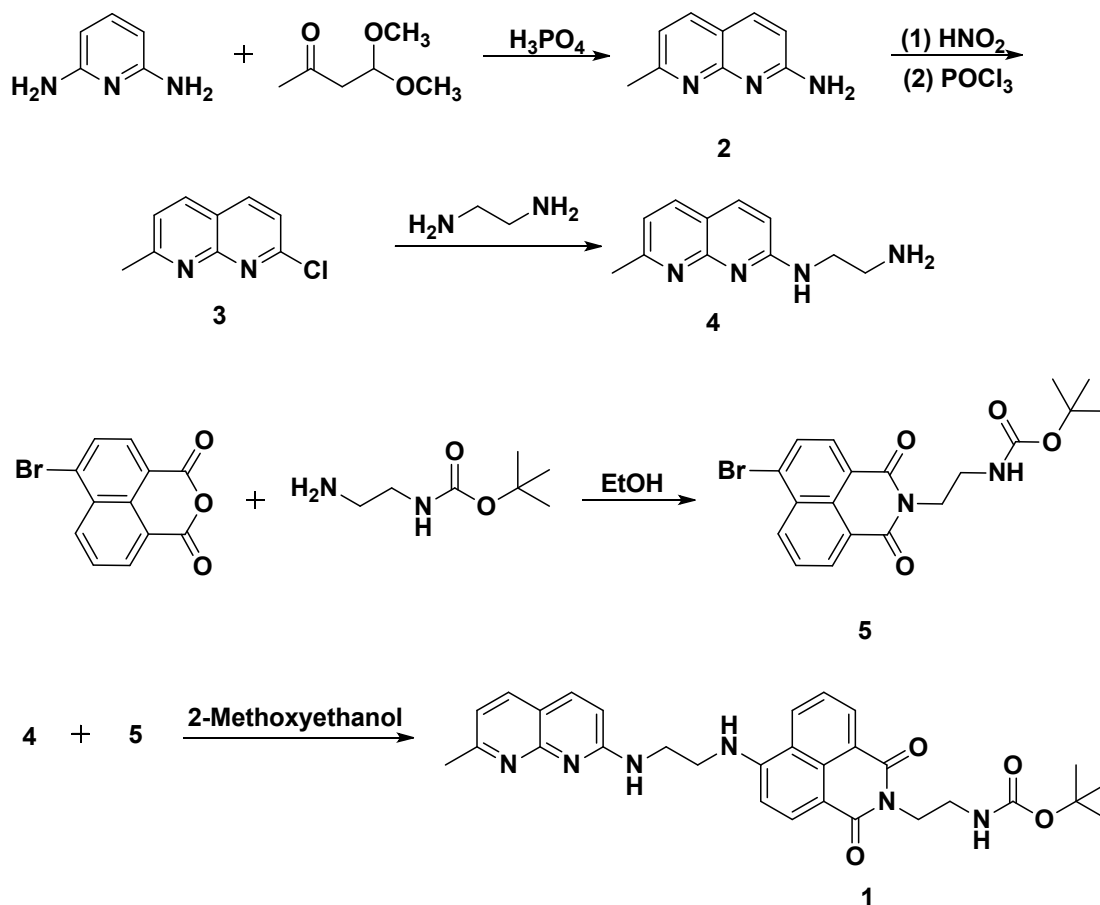
1. General instrument for characterization

All of the starting materials were obtained from commercial suppliers and used as received. Moisture sensitive reactions were performed under an atmosphere of dry argon. 2,6-Diaminopyridine and 4,4-dimethoxybutan-2-one were provided by Acros, 4-bromo-1,8-naphthalic anhydride (95%) and other chemicals were supplied from Sinopharm Chemical Reagent Co., Ltd. (Shanghai). Column chromatography was carried out on silica gel (200–300 mesh). The ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Mercury plus-Varian instrument. Proton chemical shifts are reported in parts per million downfield from tetramethylsilane (TMS). HR-MS was recorded on an LTQ-Orbitrap mass spectrometer (ThermoFisher, San Jose, CA). The UV-visible spectra were recorded on a Shimadzu UV-2550 spectrometer. Steady-state emission experiments at room temperature were measured on an Edinburgh instruments spectrometer (FS-920). The luminescence quantum yields of **1** in DMF were measured with reference to flavin mononucleotide (FMN) as a standard ($\Phi_{\text{QY}} = 0.25$). MALDI-TOF mass spectrum is recorded on a Matrix Assisted Laser Desorption Ionization-Time Of Flight/ Time Of Flight Mass Spectrometer 5800.

2. Synthesis details

The synthesis of **1** is shown in Scheme S1. The synthesis of contrastive compounds **S1** and **S2** is shown in Scheme S2. Compounds **2**, **3**¹ and **5**² were synthesized according to the previous reports and characterized by ^1H NMR. **1**

was synthesized by the reaction of N¹-(7-methyl-1,8-naphthyridin-2-yl) ethane-1,2-diamine (**4**) and tert-butyl (2-(6-bromo-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl) ethyl)carbamate (**5**) and characterized by ¹H NMR, ¹³C NMR and HR-MS analysis. The details of the synthesis were as follows.



Scheme S1 Synthetic routine of **1**

Synthesis of 2-amino-7-methyl-1,8-naphthyridine (2).¹ 2,6-Diaminopyridine (3.0 g, 27.5 mmol) was dissolved in 35 mL of H_3PO_4 at 90 °C under argon atmosphere. 4,4-Dimethoxybutan-2-one (3.7 g, 28.2 mmol) was slowly added from a pressure-equalizing addition funnel. The mixture was heated at 115 °C for 3 h. After cooling, ammonia solution was added until pH = 8. The slurry was extracted with CHCl_3 , and the organic layer was concentrated to yield a dark-red solid which was recrystallized from toluene to afford 2.50 g of **2** (58%). ¹H NMR (400 MHz, CDCl_3 , 298 K), δ (ppm) 7.88 – 7.72 (m, 2H), 7.07 (d, $J = 8.0$ Hz, 1H), 6.72 (d, $J = 8.6$ Hz, 1H), 5.18 (s, 2H), 2.68 (s, 3H).

Synthesis of 2-chloro-7-methyl-1,8-naphthyridine (3).¹ **2** (1.59 g, 10 mmol) was dissolved in 15 mL of 40% H_2SO_4 , then NaNO_2 (1.04g, 15mmol) was added slowly with vigorous stirring at ice-salt bath. The solution was gradually warmed to 80°C for 0.5 h, then cooled to room temperature and neutralized. The resulting solution was extracted with chloroform; the organic fractions were combined and

purified by column chromatography [SiO₂, CH₂Cl₂-MeOH (98: 2, v/v)] to give 2-hydro-7-methyl-1, 8-naphthyridine as a pale pink solid (0.93 g, 58%). ¹H NMR (400 MHz, DMSO-d₆, 298 K) δ 12.01 (s, 1H), 7.99 (d, *J* = 7.9 Hz, 1H), 7.88 (d, *J* = 9.5 Hz, 1H), 7.12 (d, *J* = 7.9 Hz, 1H), 6.49 (d, *J* = 9.4 Hz, 1H), 2.52 (s, 3H).

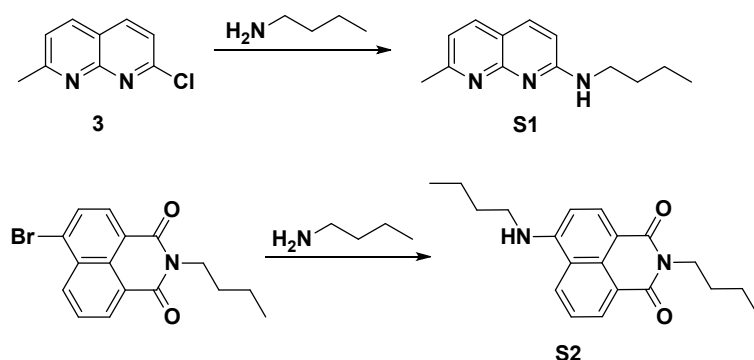
2-hydro-7-methyl-1, 8-naphthyridine (0.48 g, 3 mmol) was refluxed with 10 mL of POC1₃ for 2 h and cooled. The reaction mixture was then added to crushed ice, neutralized with NH₄OH, and extracted with chloroform. The organic fraction was evaporated and purified by column chromatography (SiO₂, CH₂Cl₂) to give **3** as a silvery white solid (0.48 g, 90%). ¹H NMR (400 MHz, DMSO-d₆, 298 K) δ 8.50 (d, *J* = 8.4 Hz, 1H), 8.42 (d, *J* = 8.3 Hz, 1H), 7.66 (d, *J* = 8.4 Hz, 1H), 7.60 (d, *J* = 8.3 Hz, 1H), 2.71 (s, 3H).

Synthesis of N¹-(7-methyl-1,8-naphthyridin-2-yl)ethane-1,2-diamine (4). **3** (0.36 g, 2 mmol) and ethylenediamine (2.0 mL) in ethanol (25 mL) was refluxed for 3 h and cooled to room temperature. The reaction mixture was evaporated and purified by column chromatography [SiO₂, CH₂Cl₂-MeOH (10: 1, v/v)] to give **4** as a buff oil (0.17 g, 42%). ¹H NMR (400 MHz, DMSO-d₆, 298 K) δ 7.93 (s, 1H), 7.86 (d, *J* = 8.7 Hz, 1H), 7.70 (br, 3H), 7.06 (d, *J* = 7.9 Hz, 1H), 6.81 (d, *J* = 8.7 Hz, 1H), 3.66 (s, 2H), 3.09 (t, *J* = 5.7 Hz, 2H), 2.53 (s, 3H). ¹³C NMR (100 MHz, DMSO-d₆, 298 K) δ 160.29, 159.18, 155.92, 136.83, 136.61, 117.85, 114.85, 113.16, 38.68, 38.60, 24.70. HRMS (ESI, *m/z*): calcd for C₁₁H₁₅N₄ [M+H]⁺, 203.1297; found: 203.1271.

Synthesis of compound 5. ² A mixture of 6-bromobenzo[de]isochromene-1,3-dione (2.77 g, 10 mmol) and (2-Aminoethyl) carbamic acid *tert*-butyl ester (2.40 g, 15 mmol) in EtOH (150 mL) was refluxed for 12 h under nitrogen atmosphere. The reaction mixture was cooled to room temperature and condensed to 50 mL. **5** was obtained as white solid by filtration and washed with EtOH several times. Yield, 3.14 g (75%). ¹H NMR (400 MHz, CDCl₃) δ 8.65 (d, *J* = 6.8 Hz, 1H), 8.55 (d, *J* = 8.4 Hz, 1H), 8.40 (d, *J* = 7.8 Hz, 1H), 8.03 (d, *J* = 7.8 Hz, 1H), 7.84 (t, *J* = 7.9 Hz, 1H), 4.96 (s, 1H), 4.35 (t, *J* = 5.3 Hz, 2H), 3.54 (d, *J* = 4.8 Hz, 2H), 1.28 (s, 9H).

Synthesis of compound 1. To the mixture of **4** (0.51 g, 2.5 mmol) and **5** (0.84 g, 2.0 mmol), 2-methoxyethanol (30 mL) was added. The solution was heated at 90°C for 24 h under argon atmosphere. After that, the solvent was removed using a rotary evaporator equipped with high vacuum. The crude product was purified by column chromatography [SiO₂, CH₂Cl₂-MeOH (10: 1, v/v)] to give **1** as a yellow solid (0.51 g, 47%). ¹H NMR (400 MHz, DMSO-d₆, 298 K) δ 8.88 (d, *J* = 8.3 Hz, 1H), 8.51 (s, 1H), 8.39 (d, *J* = 7.1 Hz, 1H), 8.30 (d, *J* = 8.5 Hz, 1H), 8.00 (d, *J* = 7.9 Hz, 1H), 7.92 (d, *J* = 8.8 Hz, 1H), 7.85 (s, 1H), 7.55 (t, *J* = 7.8 Hz, 1H), 7.16 (d, *J* = 7.9 Hz, 1H), 7.03 (d, *J* = 8.6 Hz, 1H), 6.93 – 6.77 (m, 2H), 4.09 (t, *J* = 5.6 Hz, 2H), 3.83 (d, *J* = 5.4 Hz, 2H), 3.60 (d, *J* = 4.3 Hz, 2H), 3.22 (dd, *J* = 11.8, 5.8 Hz, 2H), 2.71 (s, 3H), 1.26 (s, 9H). ¹³C NMR (100

MHz, DMSO-d₆, 298 K) δ 163.97, 163.15, 160.52, 160.01, 155.99, 155.66, 150.50, 137.18, 136.55, 134.24, 130.50, 129.44, 128.78, 124.07, 122.05, 119.93, 117.89, 114.93, 113.07, 108.07, 103.90, 77.39, 44.99, 38.04, 28.13, 25.15. HRMS (ESI, *m/z*): calcd for C₃₀H₃₃N₆O₄ [M+H]⁺, 541.2563; found: 541.2557.



Scheme S2 Synthetic routine of **S1** and **S2**.

Synthesis of N-butyl-7-methyl-1,8-naphthyridin-2-amine (S1). 2-chloro-7-methyl-1,8-naphthyridine (0.36 g, 2 mmol) and n-butylamine (2.0 mL) in ethanol (25 mL) was refluxed for 3 h and cooled to room temperature. The product **S1** was obtained by evaporating the solvent and purified by column chromatography [SiO₂, CH₂Cl₂-MeOH (10: 1, v/v)] to give a yellow oil (0.43 g, 75%). ¹H NMR (400 MHz, DMSO-d₆, 298 K) δ 7.87 (d, *J* = 7.9 Hz, 1H), 7.78 (d, *J* = 8.8 Hz, 1H), 7.29 (t, *J* = 5.2 Hz, 1H), 7.00 (d, *J* = 7.9 Hz, 1H), 6.73 (d, *J* = 8.8 Hz, 1H), 3.49 – 3.37 (m, 2H), 2.52 (s, 3H), 1.62 – 1.51 (m, 2H), 1.38 (dd, *J* = 15.0, 7.4 Hz, 2H), 0.92 (t, *J* = 7.3 Hz, 3H). ¹³C NMR (100 MHz, DMSO-d₆, 298 K) δ 159.97, 159.32, 156.54, 136.32, 136.24, 117.10, 114.50, 113.01, 30.97, 24.76, 19.86, 13.83. HRMS (ESI, *m/z*): calcd for C₁₃H₁₈N₃ [M+H]⁺, 216.1495; found: 216.1513.

Synthesis of 2-butyl-6-(butylamino)-1H-benzo[de]isoquinoline-1,3(2H)-dione (S2). 6-Bromo-2-butyl-1H-benzo[de]isoquinoline-1,3(2H)-dione (0.28 g, 1 mmol) and n-butylamine (2.0 ml) were added in 2-methoxyethanol (15 mL), the solution was heated at 90 °C for 24 h under argon atmosphere. The solvent was then removed using a rotary evaporator equipped with high vacuum. The crude product of **S2** was purified by column chromatography [SiO₂, CH₂Cl₂] to give a yellow solid (0.27 g, 92%). ¹H NMR (400 MHz, DMSO-d₆, 298 K) δ 8.65 (d, *J* = 8.3 Hz, 1H), 8.36 (d, *J* = 7.2 Hz, 1H), 8.19 (d, *J* = 8.5 Hz, 1H), 7.62 (dd, *J* = 19.8, 12.1 Hz, 2H), 6.68 (d, *J* = 8.6 Hz, 1H), 3.97 (t, *J* = 7.2 Hz, 2H), 3.31 (dd, *J* = 12.3, 6.4 Hz, 2H), 1.74 – 1.61 (m, 2H), 1.61 – 1.48 (m, 2H), 1.47 – 1.24 (m, 4H), 0.91 (dt, *J* = 12.0, 7.3 Hz, 6H). ¹³C NMR (100 MHz, DMSO-d₆, 298 K) δ 163.69, 162.85, 150.58, 134.17, 130.52, 129.37, 128.50,

124.06, 121.79, 120.05, 107.46, 103.62, 42.57, 29.96, 29.83, 19.87, 13.75.
HRMS (ESI, m/z): calcd for $C_{20}H_{25}N_2O_2$ $[M+H]^+$, 325.1911; found: 325.1913.

3. Confocal laser microscopy images

Confocal laser microscopy (CLMS) images of RAW264.7 cells incubated with **1** were performed on our modified microscope. The instrument was built on an inverted microscope (Olympus IX81, Japan) and a confocal scanning unit (FV1000, Olympus, Japan). For the luminescence microscopy imaging, the continuous wave laser at 405 nm provided the excitation, and luminescence emission at both blue channel (420–450 nm) and green channel (520–570 nm) were collected as output signals. A photomultiplier tube (R6357 enhanced model, HAMAMATSU, Japan) was used as a detector. A 60 × oil-immersion objective lens was used.

4. Cytotoxicity of **1**

The HeLa cell lines were provided by the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences). The HeLa cells were grown in RPMI 1640 (Roswell Park Memorial Institute's Medium) supplemented with 10% FBS (Fetal Bovine Serum) at 37°C and 5% CO₂. In vitro cytotoxicity was measured by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays on the HeLa cells. Cells were seeded into a 96-well cell culture plate at 5×10^3 /well, and were cultured at 37°C and 5% CO₂ for 12 h; different concentrations of **1** (0, 5, 10, 15 and 20 μM) were then added to the wells. The cells were subsequently incubated for 36 h at 37°C under 5% CO₂. Thereafter, MTT (5 mg/mL) was added to each well and the plate was incubated for an additional 3 h at 37°C under 5% CO₂. The optical density OD₅₇₀ value (Abs.) of each well, with background subtraction at 690 nm, was measured by means of a Tecan Infinite M200 monochromator-based multifunction microplate reader. The following formula was used to calculate the inhibition of cell growth: Cell viability (%) = (mean of Abs. value of treatment group/mean of Abs. value of control) × 100 %.

5. Additional absorption and fluorescent spectra

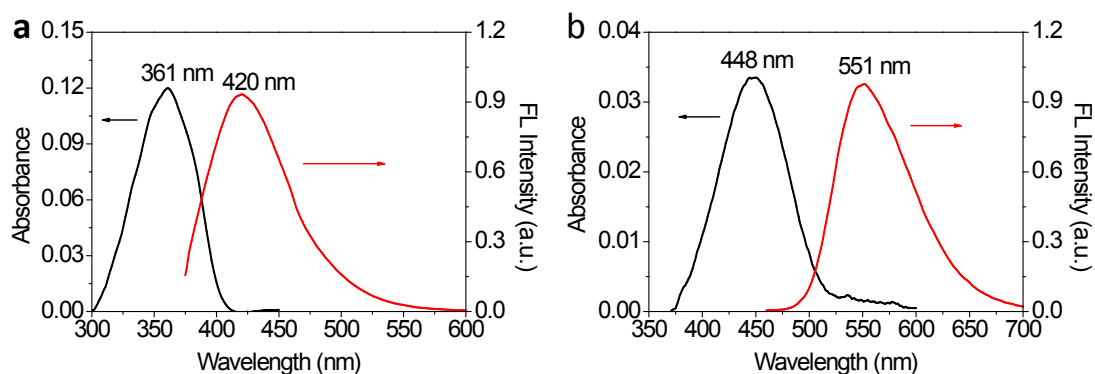


Fig S1. Absorption and fluorescence spectra of (a) S1 (10 μM) and (b) S2 (10 μM) in H₂O-DMF (98:2, v/v).

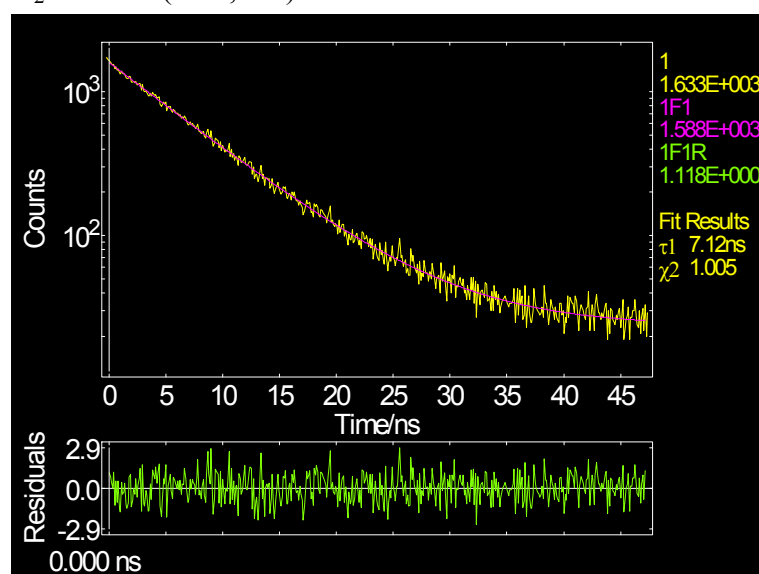


Fig. S2 Fluorescent lifetime of 1

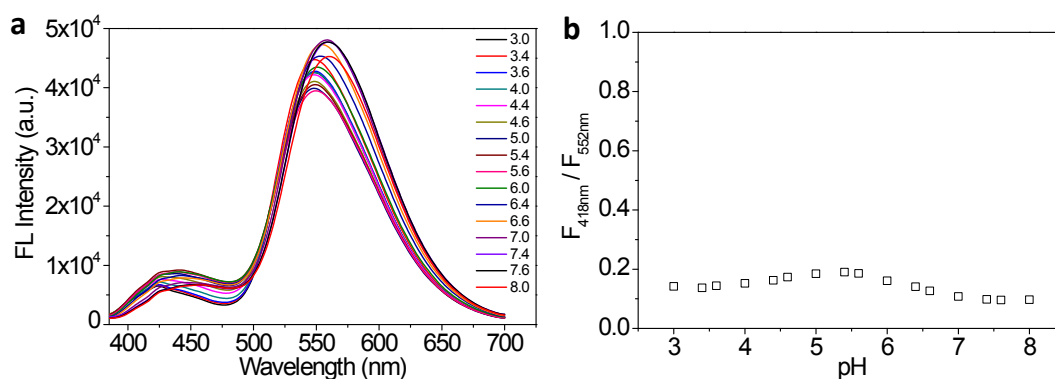


Fig S3. (a) The fluorescence spectra of 1 (10 μM) in Buffer-DMF (98:2, v/v, Na₂HPO₄-Citric acid buffer) under different pH value (from 3 to 8). (b) The ratio of emission intensities at 418 and 552 nm ($F_{418\text{ nm}} / F_{552\text{ nm}}$) under different pH value.

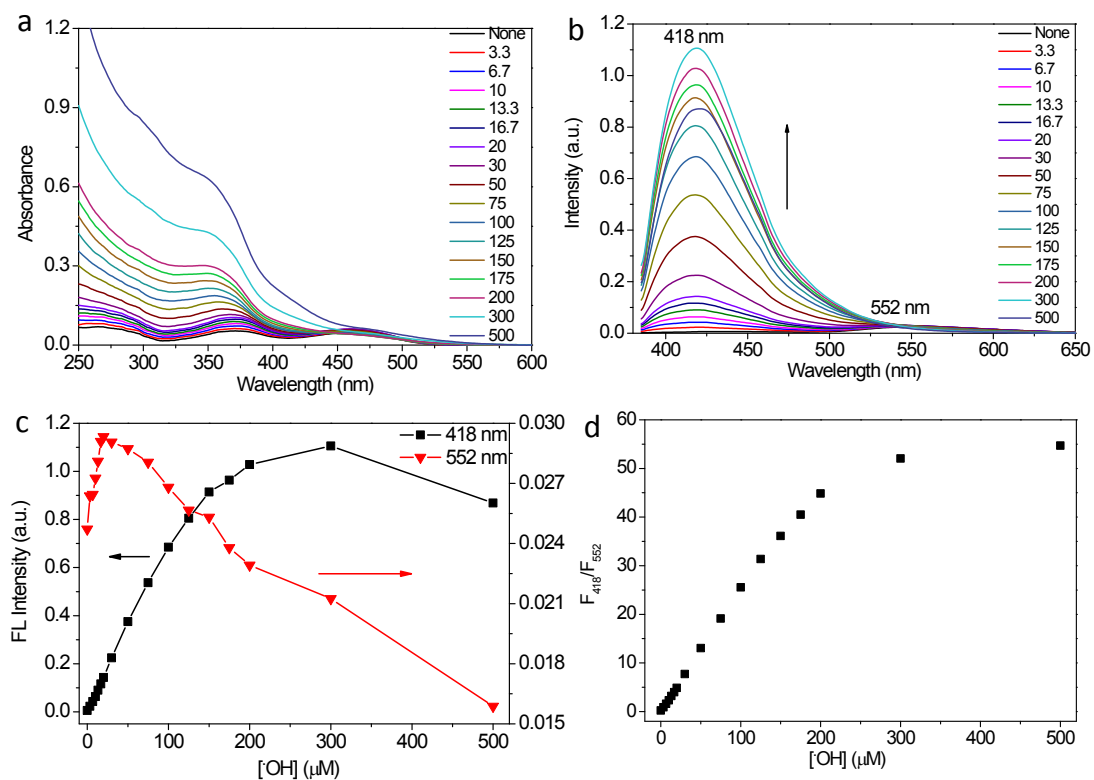


Fig. S4 Changes in the absorption (a) and fluorescence (b) spectra of 10 μM **1** in H_2O -DMF (98:2, v/v) with addition of different concentrations of hydroxyl radicals (from 0 to 500 μM). (c) Change in emission intensities at 418 and 552 nm as a function of $\cdot\text{OH}$ concentration. (d) The ratio of emission intensities (F_{418}/F_{552}) as a function of concentrations of $\cdot\text{OH}$ concentration.

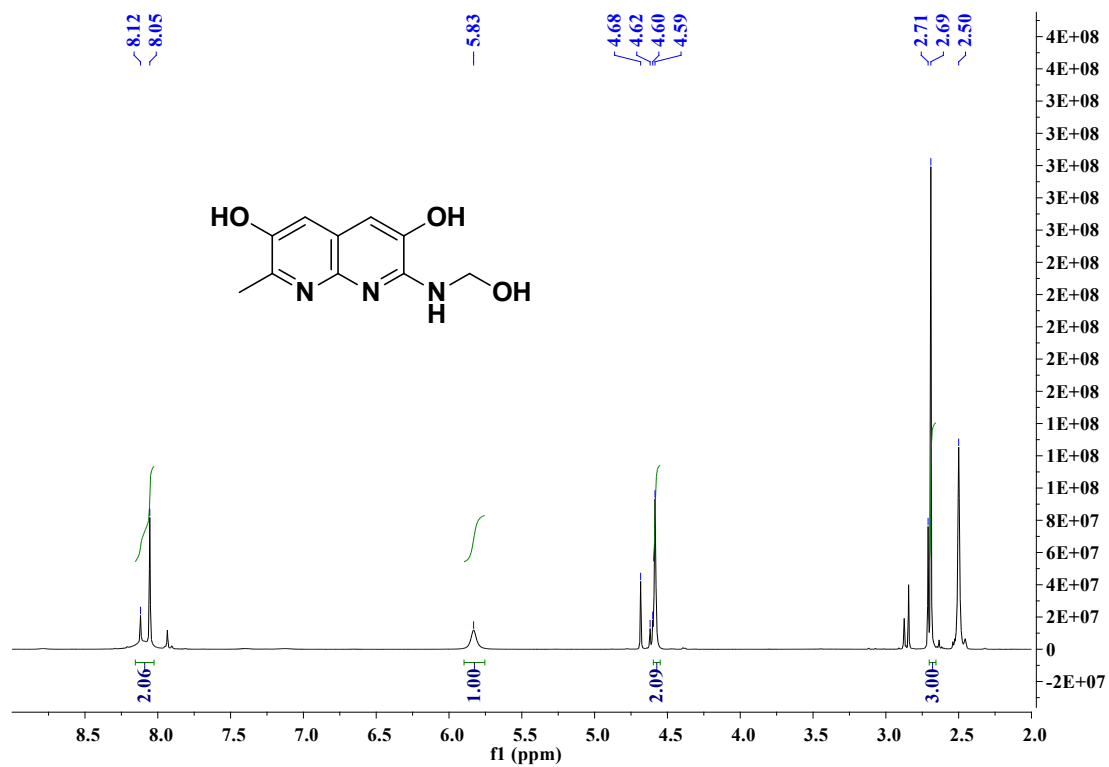


Fig. S5 ¹H NMR and proposed structure of the blue emissive product of probe **1** with hydroxyl radical.

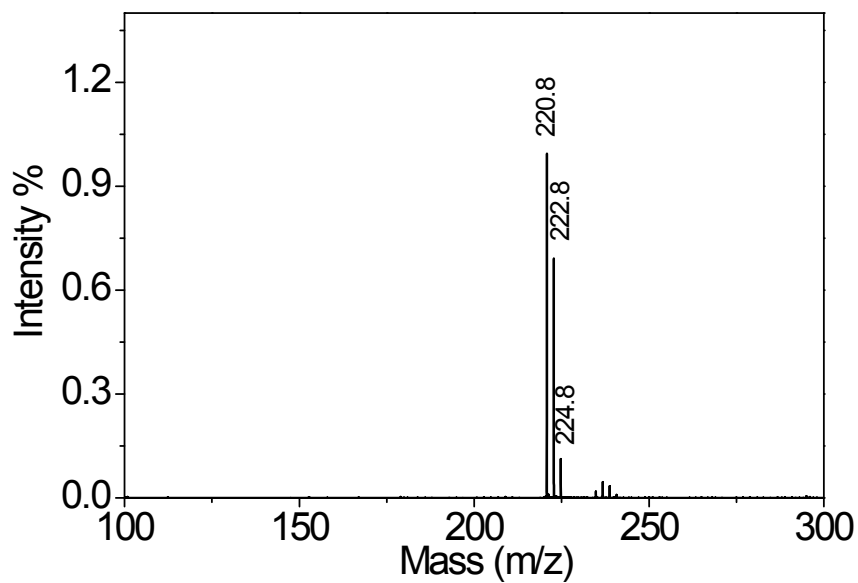


Fig. S6 MALDI-TOF mass spectrum of the reaction product of probe **1** with hydroxyl radical.

6. Distribution of **1** in the cells

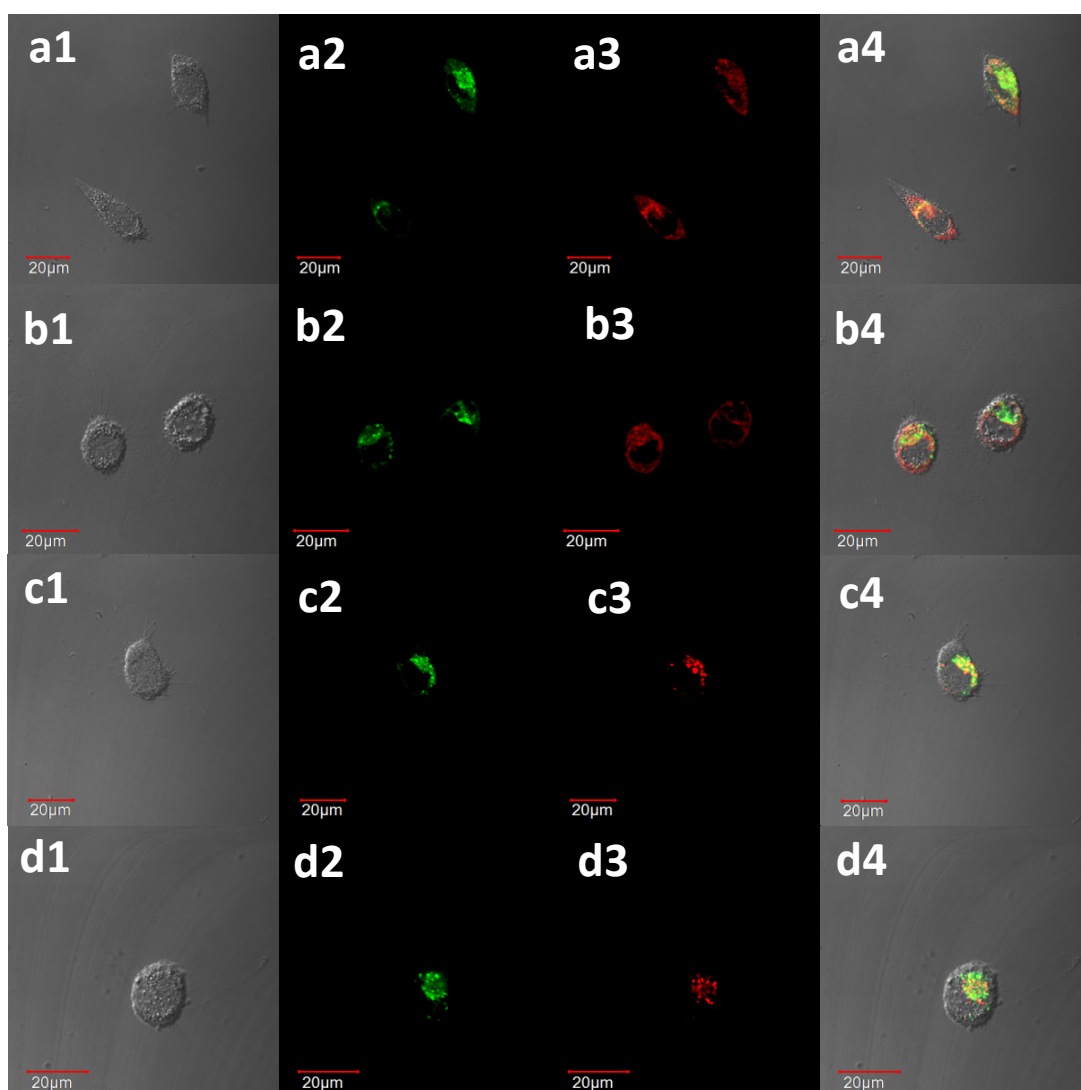


Fig. S7 CLMS images of RAW264.7 cells incubated with (a) **1** ($5 \mu\text{M}$) for 30 min and Mito-Tracker Red for 15 min, (b) **1** ($5 \mu\text{M}$) for 30 min and ER-Tracker Red 15 min, (c) **1** ($5 \mu\text{M}$) for 30 min and Lyso-Tracker Red for 15 min and (d) **1** ($5 \mu\text{M}$) for 30 min and Golgi-Tracker Red for 15 min; 1–4 are bright field images, fluorescence images of **1** in green channel (520–570 nm), location dye in red channel (590–640 nm) and overlay images, respectively.

7. Cellular toxicity of 1

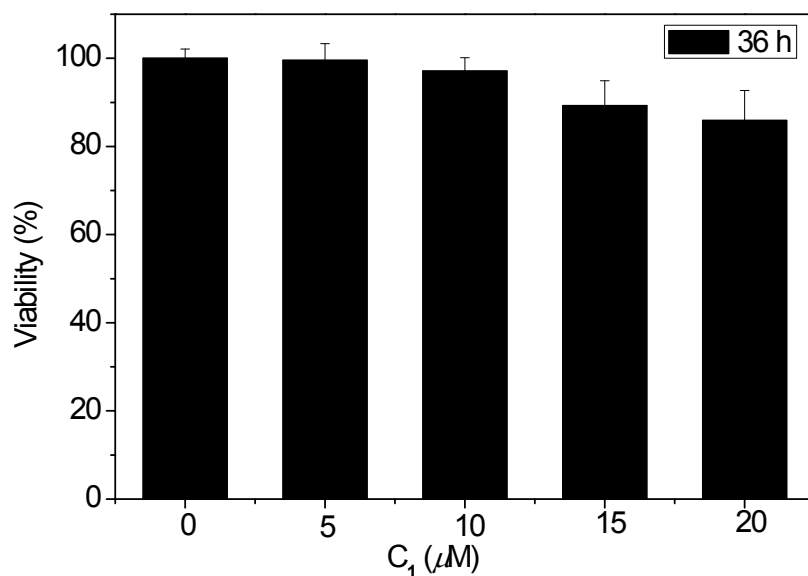


Fig. S8 Cell viability values (%) estimated by MTT assay in HeLa cells, which were cultured in the presence of 0-20 μM **1** for 36 h at 37°C.

8. Characteristics of probe 1

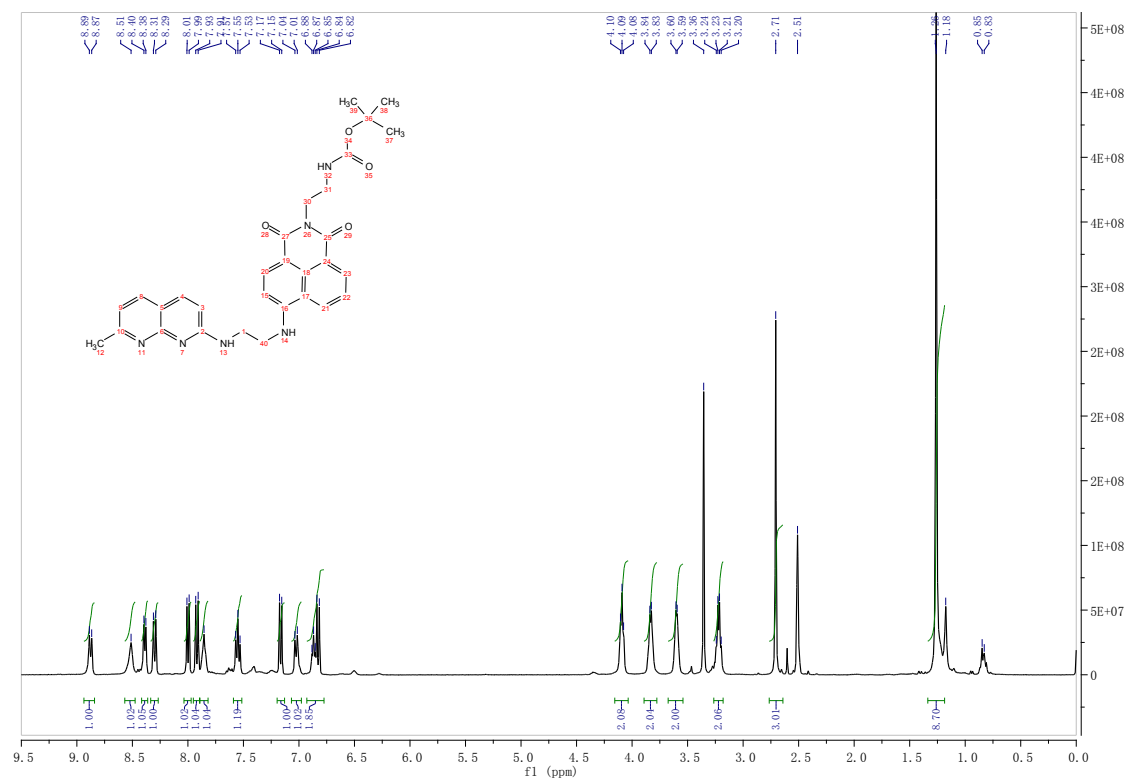


Fig. S9 The ^1H NMR spectrum of **1** in DMSO-d_6

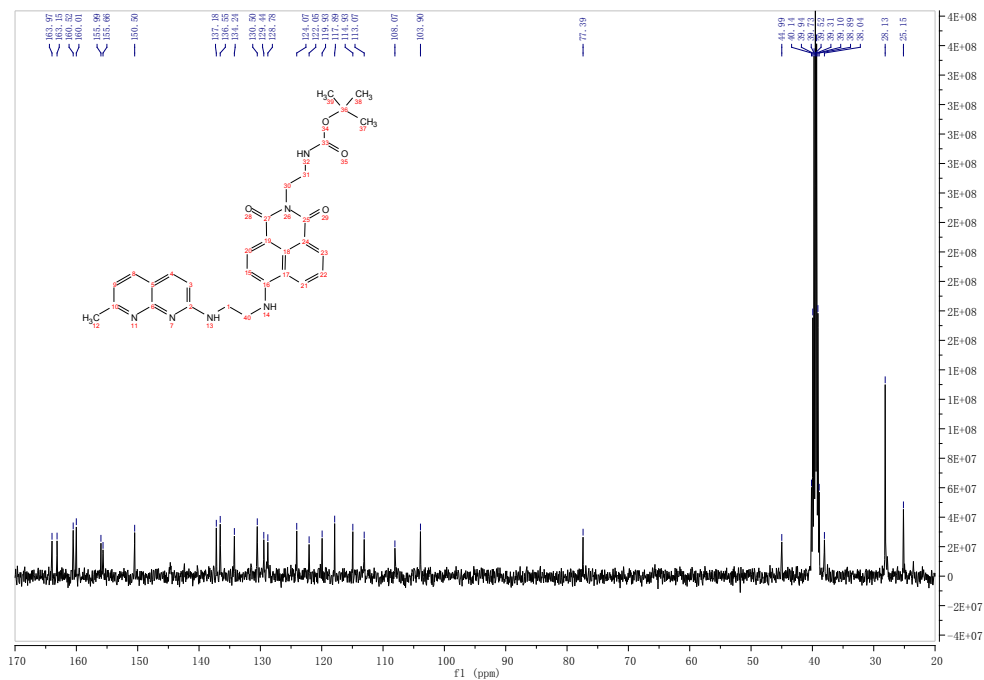


Fig. S10 The ^{13}C NMR spectrum of **1** in DMSO- d_6 .

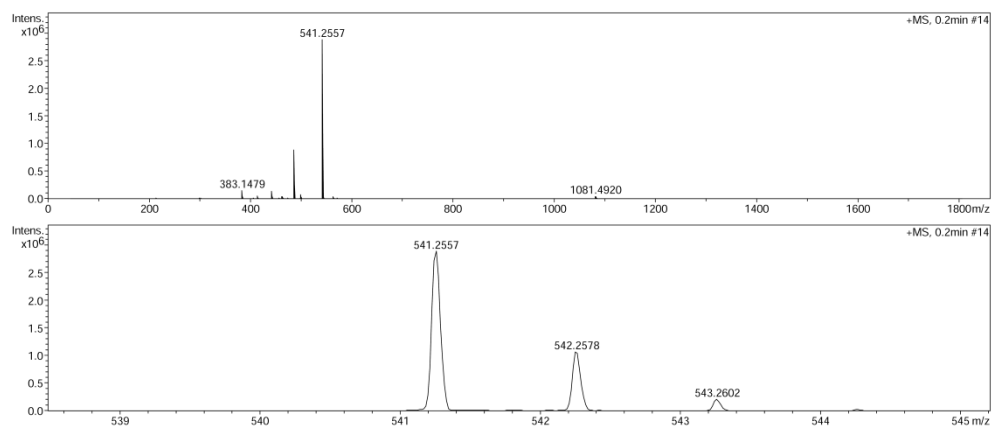


Fig. S11 HR-MS of **1**

References

1. E. V. BROWN, *Journal of Organic Chemistry*, 1965, **30**, 1607-1610.
2. M. Zhang, S. Sun, X. Yu, X. Cao, Y. Zou and T. Yi, *Chemical communications*, 2010, **46**, 3553-3555.