*Supplementary Information for*

# Ratiometric fluorescent detection of intracellular hydroxyl radicals based on a hybrid coumarin-cyanine platform

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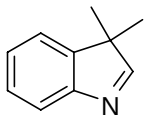
**Materials and instruments:** Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. Melting points of compounds were measured on a Beijing Taike XT-4 microscopy melting point apparatus, and all melting points were uncorrected; Mass spectra were performed using an LCQ Advantage ion trap mass spectrometer from Thermo Finnigan or Agilent 1100 HPLC/MSD spectrometer; NMR spectra were recorded on an INOVA-400 spectrometer, using TMS as an internal standard; Electronic absorption spectra were obtained on a LabTech UV Power spectrometer; Photoluminescent spectra were recorded with a HITACHI F4600 fluorescence spectrophotometer; Cells imaging was performed with a Nikon Eclipse TE2000U inverted microscope; TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals.

**Cell Culture and Reactive Oxygen Species (ROS) Fluorescence Imaging using Probe 1:** Hela cells were grown in MEM (modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% $CO_2$ and 95% air at 37 $^oC$. The cells were plated on 6-well plates and allowed to adhere for 24 h. Immediately before the experiments, the cells were washed with PBS buffer, and then the cells were incubated with PMA (2 ng $mL^{-1}$) for 2 h, with or without TEMPOL (5 mM) for 1 h, and then with probe **1** (20 μM) for 30 min at 37ºC. The cells were washed with PBS buffer, and the fluorescence images were acquired through a Nikon Eclipse TE2000U inverted fluorescence microscope equipped with a cooled CCD camera and a 20 × objective lens with blue or green excitation.

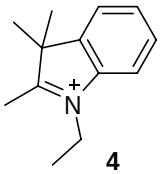
**Spectroscopic studies of probe 1:** Various analytes were added to the solution of compound **1** (20 μM) in $H_2O$/methanol (1: 1 v/v). The resulting solution was kept at

ambient temperature for 1-2 hours and the fluorescence intensities were recorded with excitation at 460 nm. Superoxide ($O_2^{\cdot-}$) was added as solid $KO_2$.[1] Singlet oxygen $^1O_2$ was prepared by addition NaClO to $H_2O_2$ according to a literature procedure.[2] Solutions of different analytes (final concentration: 500 μM) represented by hydrogen peroxide, hypochlorite, $CH_3COOOH$, $FeCl_2$, $CaCl_2$, $CoCl_2$, $ZnCl_2$, $NaNO_2$, $NaNO_3$, and GSH were added to the solution of compound **1** (final concentration, 20 μM). Hydroxyl radicals were generated *in situ* by Fenton reaction.[3] To a 2 mL solution of probe **1** (20 μM) in $H_2O$/methanol (1: 1 v/v), hydrogen peroxide stock solution (0.02 mL, 100 mM) was added. Aqueous $Fe^{2+}$ (0.01 mL, 100 mM) was then added to the probe **1**/$H_2O_2$ solution to generate hydroxyl radical (500μM, 25 equiv.). The time course of the fluorescence and absorption spectra of probe **1** (20 μM) in the presence of hydroxyl radicals (25 equiv.) were acquired over various time points at 25 °C in $H_2O$-$CH_3OH$ (1:1, v/v), with excitation λ = 460 nm.



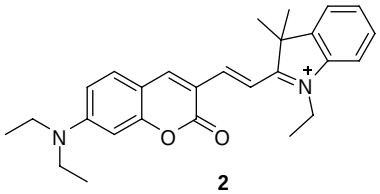

**5**

**Synthesis of 2, 3, 3-trimethyl-3H-indole 5.** Isopropylmethyl ketone (31.8 g, 369.2 mmol) and phenylhydrazine (10.0 g, 92.6 mmol) were dissolved in acetic acid (15 ml), and then the mixture was heated at 125 °C for 12 h. After being cooled to room temperature, the mixture was washed three times with water. The organic layer was collected and dried under vacuum to afford compound **5**: $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 1.29 (s, 6H), 2.27 (s, 3H), 7.17-7.21 (t, 1H), 7.26-7.31(m, 2H), 7.53 (d, $J$ = 7.6 Hz, 1H); MS (ESI): ($M + H^+$) 160.1.



**4**

**Synthesis of N-ethyl-2, 3, 3-trimethyl-3H-indolium iodide 4.** 2, 3, 3-trimethyl-3H-indole (5.3 g, 32.8 mmol) and ethyl iodide (12.9 g, 82.5 mmol) were placed in a 50 mL round-bottomed flask. The mixture was heated under an $N_2$ atmosphere at 75 °C for 24 h. After being cooled to room temperature, the solid was filtered off and dried under vacuum to afford compound **4** (9.9 g, yield: 94.9%), which was then used for next step reaction without further purification. Mp 165-166 $^{o}$C. $^{1}$HNMR (400 MHz, $CDCl_3$) $\delta$ 1.62-1.66 (t, $J$ = 7.6 Hz, 3H), 1.67 (s, 6H), 3.17 (s, 3H), 4.76-4.81(q, 2H), 7.58-7.64 (m, 3H), 7.71-7.75 (m, 1H); MS (ESI): ($M^+$) 188.1.



**2**

**Synthesis of compound 2:** 1-ethyl-2,3,3-trimethyl-3H-indolium iodide **4** (63.0 mg, 0.2 mmol) was treated with the corresponding coumarin aldehydes **3** (49.1 mg, 0.2 mmol) in anhydrous ethanol (10 mL). The reaction mixture was then refluxed for 10 h, and the solvent was removed under reduced pressure. The resulting residue was purified by column chromatography on silica gel ($CH_2Cl_2$ to $CH_2Cl_2$ /acetone = 50: 1) to afford the compound **2** as a reddish brown powder (78.2 mg, yield: 72.1%). Mp 236-237 $^{o}$C. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 1.29 (t, $J$ = 7.2 Hz, 6H), 1.63 (t, $J$ = 7.4 Hz, 3H), 1.85 (s, 6H), 3.49-3.55 (q, 4H), 4.83-4.88 (q, 2H), 6.46 (d, $J$ = 2.4 Hz, 1H), 6.68-6.71 (dd, $J$ = 7.2 Hz, 2.4 Hz, 1H), 7.45-7.48 (t, $J$ = 7.6 Hz, 1H), 7.50-7.57 (m, 3H), 7.99 (d, $J$ = 15.6 Hz, 1H), 8.19 (d, $J$ = 10.0 Hz, 1H), 8.58-8.62 (d, $J$ = 16.0 Hz, 1H), 10.16 (s, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 180.30, 161.19, 158.90, 154.63, 150.98, 143.28, 140.51, 134.58, 129.35, 128.65, 122.77, 113.31, 112.50, 111.02, 108.51, 96.81, 51.69, 45.66, 42.91, 27.69, 14.10, 12.64. MS (ESI): ($M^+$) 415.1. HRMS (EI) m/z calcd for $C_{27}H_{31}N_2O_2$ ($M^+$): 415.2380. Found 415.2401.

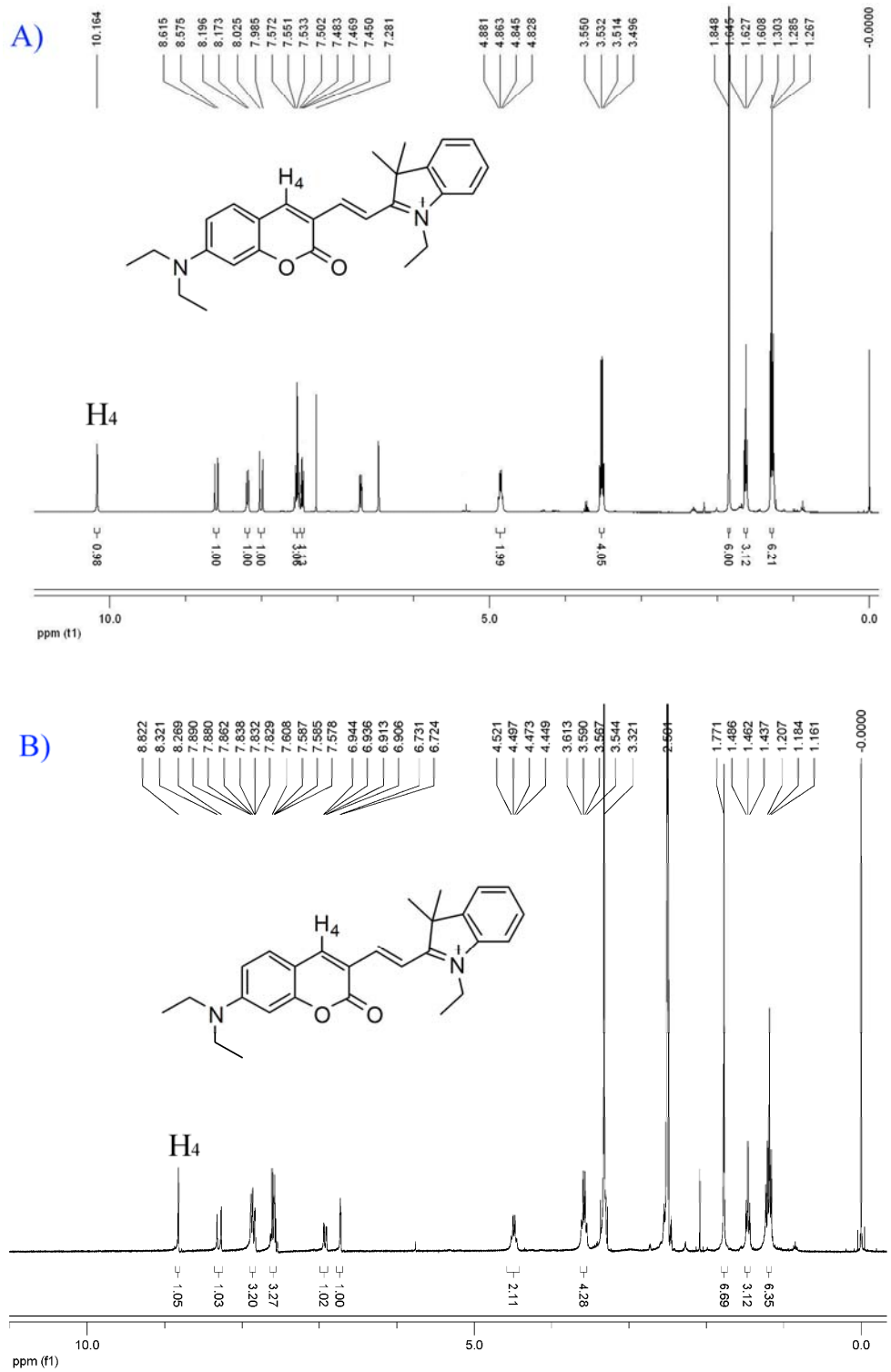



**Figure S1**. The $^1$H NMR spectra of compound **2** in $CDCl_3$ (A) and $d_6$-DMSO (B). Notably, the $^1$H NMR spectrum (B) of compound **2** in $d_6$-DMSO is in good agreement with those of other hybrid coumarin-cyanine derivatives reported (*Org. Lett.* 2008, **10**, 4175). The relatively downshift of some resonance signals may be ascribed to the deshielding effect in light of the resonance delocalization of the positive charge on the nitrogen atom of compound **2** (Scheme S1).

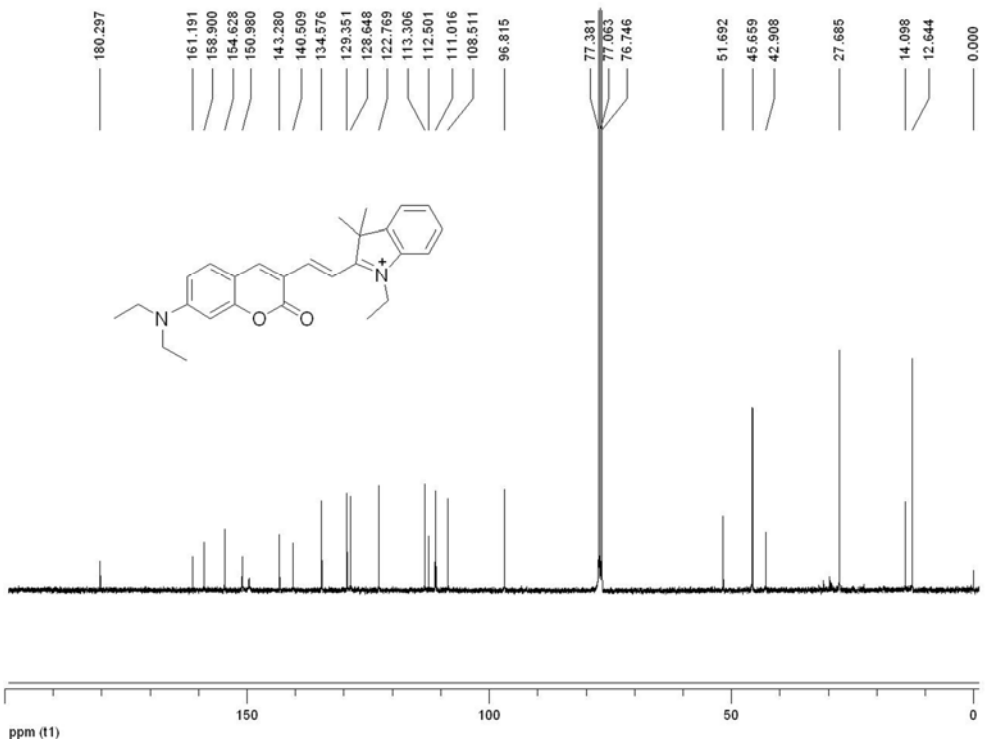
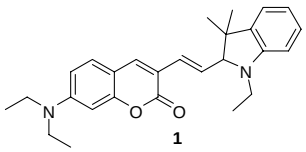



**Figure S2**. The $^{13}C$ NMR spectrum of compound **2**. The relatively downshift of some resonance signals may be ascribed to the deshielding effect in light of the resonance delocalization of the positive charge on the nitrogen atom of compound **2** (Scheme S1).



**1**

**Synthesis of compound 1.** Compound **2** (50.0 mg, 0.092 mmol) was dissolved in ethanol (1 mL), and then $NaBH_4$ (1.4 mg, 0.037 mmol in 0.5 mL ethanol) was added drop-wise over 10 min. Subsequently, the reaction mixture was stirred at room temperature for 20 min, and the solvent was removed under reduced pressure. The resulting residue was purified on a silica gel column ($CH_2Cl_2$ /petroleum ether = 3: 7) to afford compound **1** as a yellow powder (36.5 mg, yield: 95%). Mp 145-146 $^oC$. $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 1.05-1.09 (t, $J$ = 7.6 Hz, 3H), 1.11 (s, 3H), 1.20-1.24 (t, $J$ = 7.6 Hz, 6H), 1.32 (s, 3H), 3.11-3.17 (m, 1H), 3.29-3.37 (m, 1H), 3.39-3.45 (q, 4H), 3.72-3.74 (d, $J$ = 9.2 Hz, 1H), 6.46-6.53 (m, 3H), 6.57-6.60 (dd, $J$ = 8.4, 2.4 Hz, 1H),

6.67 (s, 1H), 6.71 (s, 1H), 7.03 (d, $J$ = 7.2 Hz, 1H), 7.08-7.12 (t, $J$ = 7.6 Hz, 1H), 7.27-7.29 (d, $J$ = 8.8 Hz, 1H), 7.66 (s, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 10.35, 12.47, 24.33, 25.89, 39.85, 44.49, 44.84, 97.11, 108.77, 109.03, 109.75, 117.11, 117.57, 121.82, 127.46, 128.82, 137.90, 150.42, 155.77, 161.59; MS (ESI): ($M + H^+$) 417.3; HRMS (EI) m/z calcd for $C_{27}H_{32}N_2O_2$ (M): 416.2458. Found 416.2424.

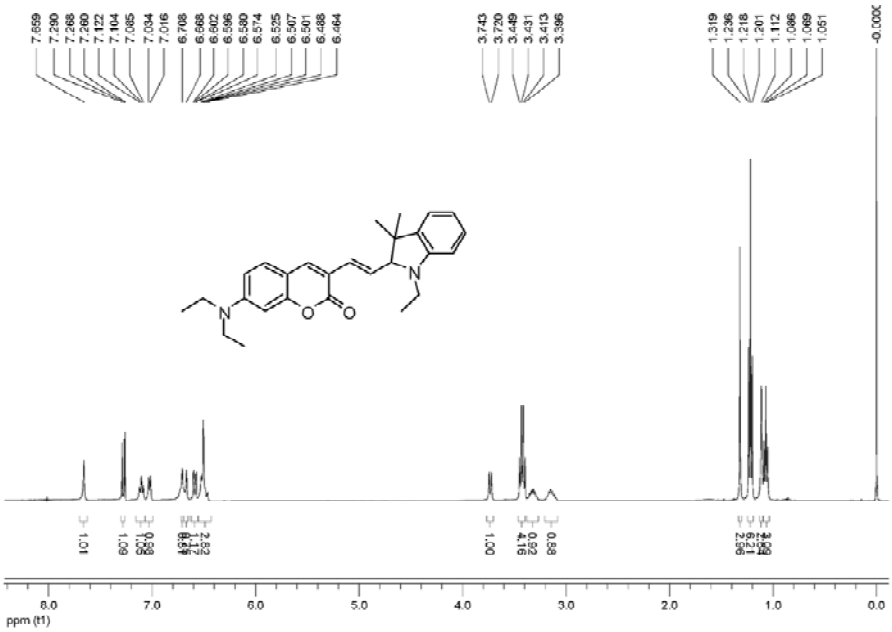



**Figure S3**. The $^1$H NMR spectrum of compound **1**.

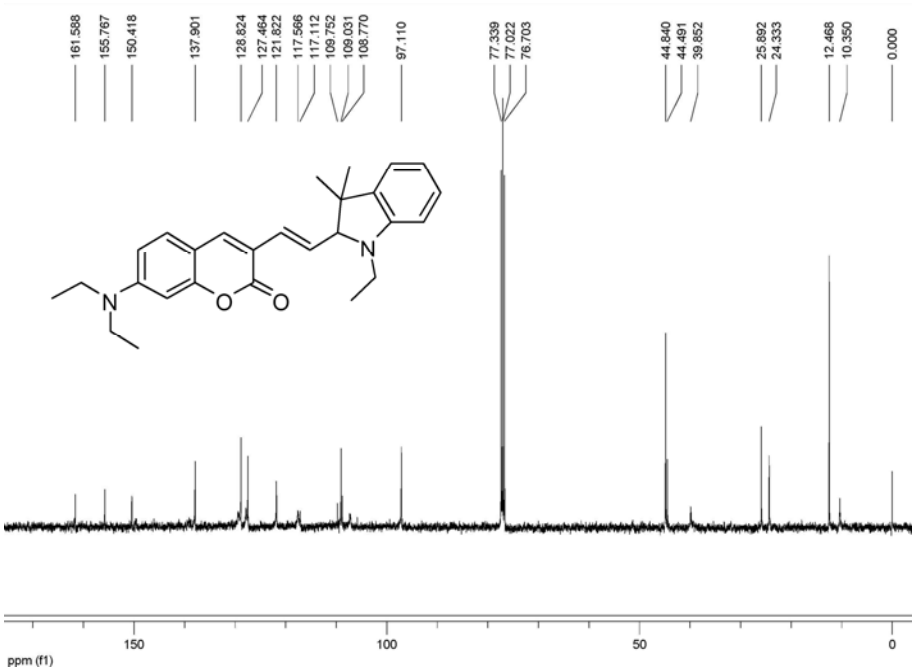
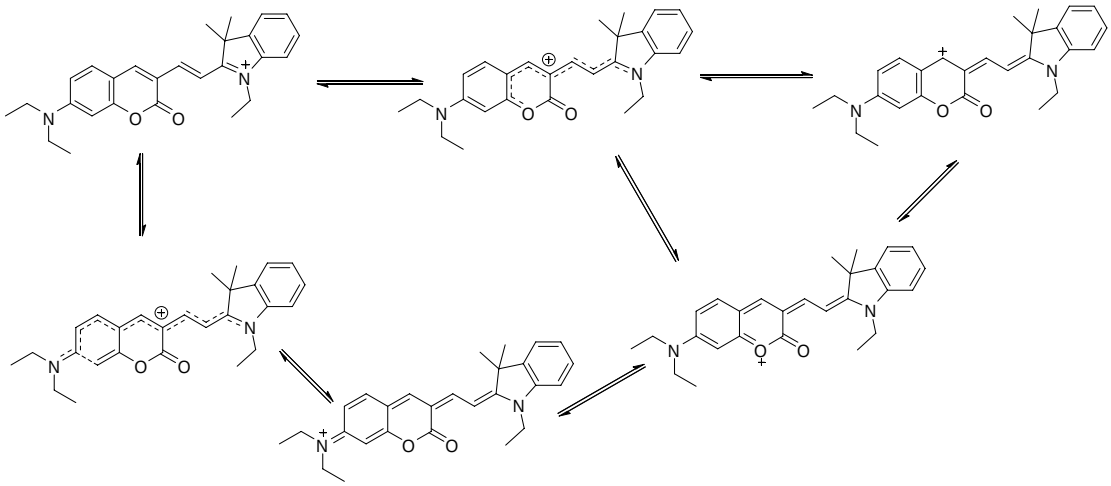



**Figure S4**. The $^{13}C$ NMR spectrum of compound **1**.



***Scheme S1.*** Resonance delocalization of the positive charge on the nitrogen atom of compound **2**.

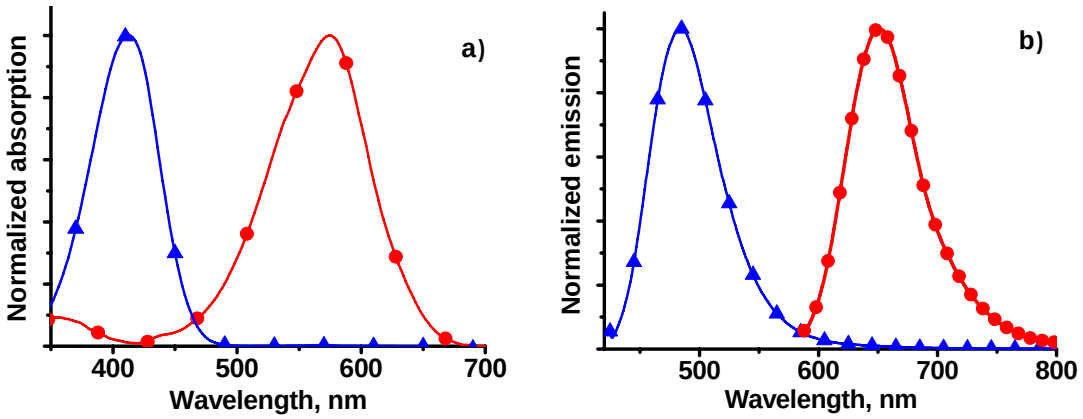



**Figure S5**. The normalized absorption (a) and emission (b) spectra of compounds **1** (▲) and **2** (●) in PBS/$CH_3CN$ (v/v 1:1, pH 7.4). The emission spectra were recorded by excitation at the corresponding maximal absorption of each compound.

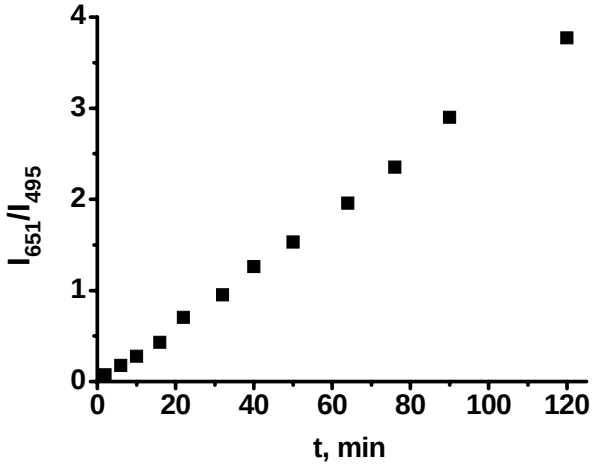



**Figure S6.** The fluorescent ratio ($I_{651}/I_{495}$) of probe **1** (20 μM) to hydroxyl radical (25 equiv.) at various incubation time (0 -120 min). The data were acquired after incubation of probe **1** with hydroxyl radical for 0 to 120 min at 25 $^oC$ in $H_2O$-$CH_3OH$ (1:1, v/v). Excitation was provided at 460 nm, and the ratio of emission intensities at 651 nm and 495 nm was measured over various incubation time points.

**Kinetic Studies** [4]: The reaction of probe **1** (10 μM) with hydroxyl radical (1 mM) in $H_2O$/MeOH (1: 1) was monitored using the fluorescence intensity at 651 nm ($\lambda_{ex}$ = 575 nm). The reaction was carried out at 25 $^oC$. The apparent rate constant for the reaction was determined by fitting the fluorescence intensities to the pseudo first-order equation (S1):

$$\text{Ln}\,[(F_{max} - F_t) / F_{max}) \quad = - k't \qquad \text{(S1)}$$

Where $F_t$ and $F_{max}$ are the fluorescence intensities at 651 nm at times *t* and the maximum value obtained after the reaction was complete. k' is the observed rate constant. Figure S7 is the pseudo first-order plot for the reaction of **1** with hydroxyl radical (1 mM). The negative slope of the line provides the observed rate constant: k' = $5.14 \times 10^{-4}$ $s^{-1}$.

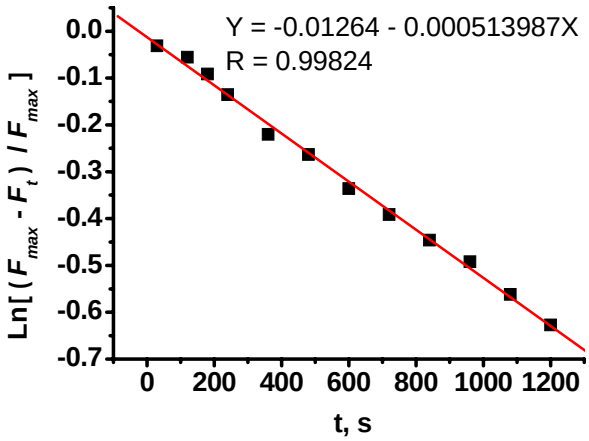



**Figure S7.** Pseudo first-order kinetic plot of reaction of compound **1** (10 μM) with hydroxyl radicals (1 mM). Slope = - $5.14 \times 10^{-4}$ $s^{-1}$.

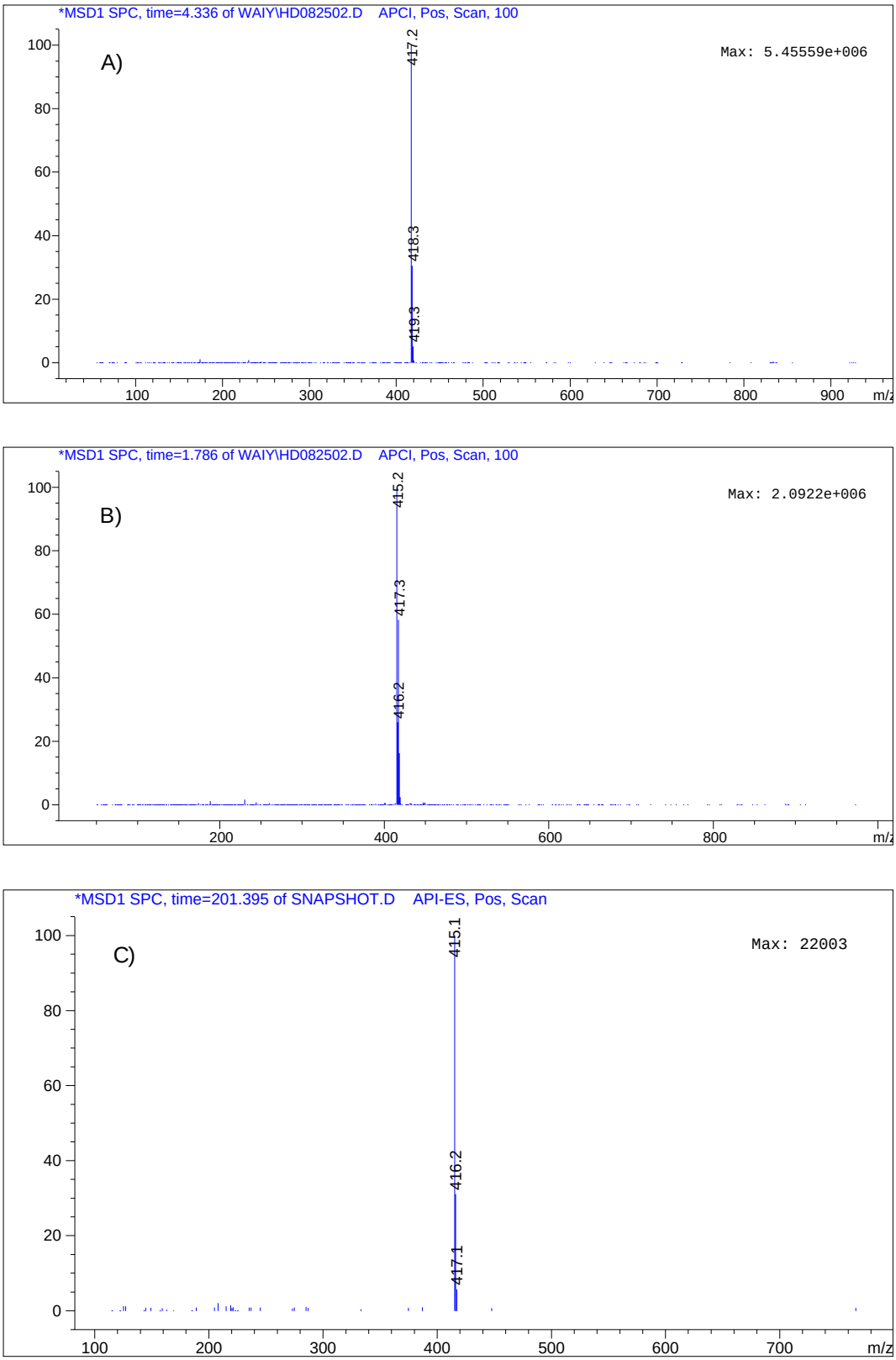



**Figure S8.** A) Mass spectrum of compound **1** ($[\mathbf{1} + H]^+ = 417.2$); B) Mass spectrum of compound **1** + hydroxyl radicals (Notably, two main peaks are present in the spectrum. $[\mathbf{1} + H]^+ = 417.3$, $[\mathbf{2}]^+ = 415.2$); C) Mass spectrum of the isolated product of compound **1** + hydroxyl radicals ($[\mathbf{2}]^+ = 415.1$).

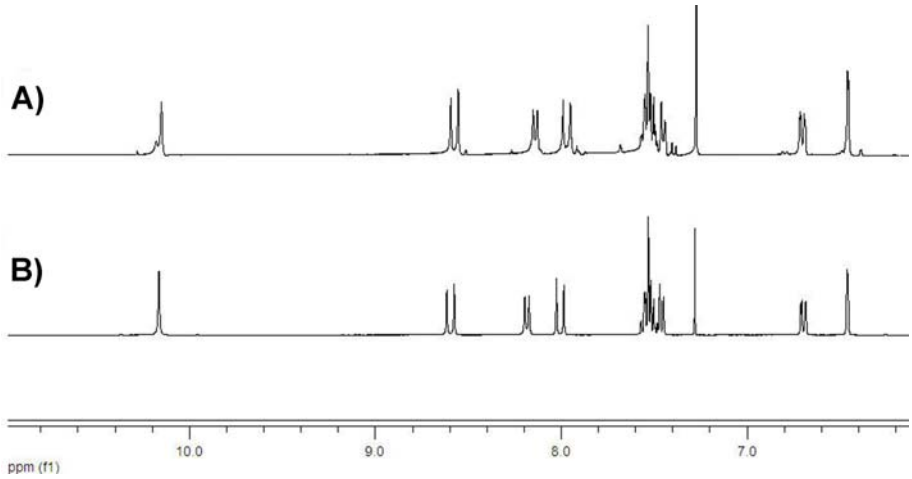



**Figure S9.** (A) The partial $^{1}H$ NMR spectrum of the isolated product of compound **1** + hydroxyl radical; (B) The partial $^{1}H$ NMR spectrum of the standard compound **2**. The $^{1}H$ NMR spectra were recorded in $CDCl_3$.

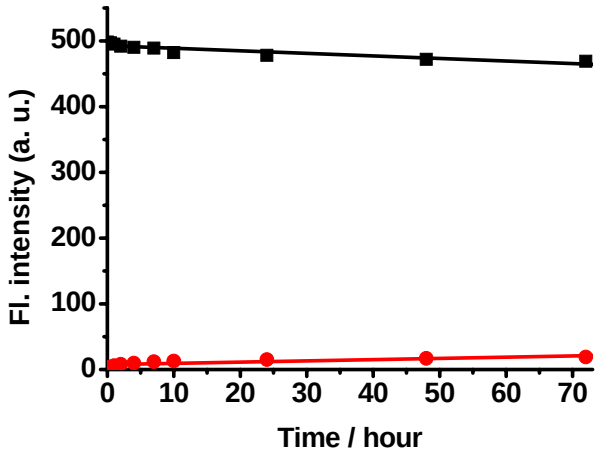



**Figure S10.** Time-profile of the emission intensities of compound **1** in PBS buffer (pH 7.4, containing 50% $CH_3OH$ as co-solvent) at room temperature. The fluorescent data were collected at 495 nm (■) (excited at 408 nm) and 651 nm (●) (excited at 575 nm), respectively.

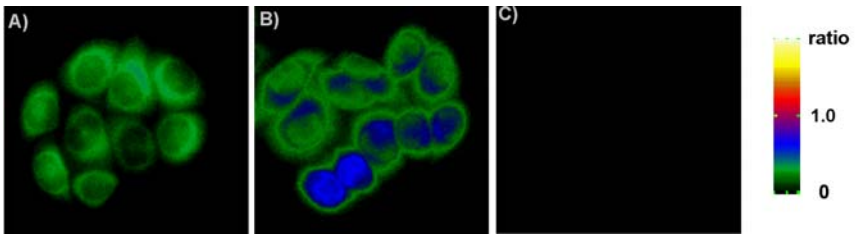



**Figure S11.** Ratio fluorescence ($I_{651}$ / $I_{495}$) images of Hela cells incubated with probe **1** (20 μM): (A) Hela cells incubated with only probe **1 (**20 μM) for 30 min; (B) Hela cells pre-treated with PMA (2 ng/ mL) for 2 h and then incubated with probe **1 (**20 μM) for 30 min; (C) Hela cells incubated sequentially with PMA (2 ng/ mL) for 2 h, then with TEMPOL (5 mM) for 1 h, and finally with probe **1 (**20 μM) for 30 min.

## References

(1) (a) Kundu, K.; Knight, S.F.; Willett, N.; Lee, S.; Taylor, W. R.; Murthy, N. *Angew. Chem. Int. Ed.* **2009**, *48*, 299-303. (b) Albers, A. E.; Okreglak, V. S.; Chang, C. J. *J. Am. Chem. Soc.* **2006**, *128*, 9640-9641.

(2) Xu, K.; Liu, X.; Tang, B.; Yang, G.; Yang, Y.; An, L. *Chem. Eur. J.* **2007**, *13*, 1411-1416.

(3) Fenton, H. H. *Chem. News*, **1876**, *33*, 190.

(4) Dale, T. J.; Rebek, J. *J. Am. Chem. Soc*. **2006**, *128*, 4500-4501.