Supplementary information for

Development of an ICT-based ratiometric fluorescent hypochlorite probe suitable for living cell imaging

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Experimental Section

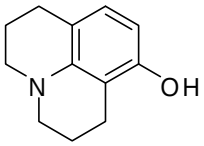
Materials and instruments: Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. Melting points of compounds were measured 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 or Bruker AV-500 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 TE300 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.

Spectroscopic studies of probe 1b: Solutions of different analytes represented by $MgSO_4$, $CuCl_2$, $FeCl_3$, $CoCl_2$, $CaCl_2$, $ZnCl_2$, NaF, Na_2SO_4, $NaHCO_3$, $NaNO_2$, $NaNO_3$, Na_2SO_3, NaCl, Na_3PO_4, KI, NaOCl, hydrogen peroxide, hydroxyl radicals, superoxide, and CH_3COOOH were added to the solution of compound **1b** (final concentration, 3 μM) in PBS buffer/DMF (pH 7.4, 8 : 2). The resulting solution was kept at ambient temperature for 10 min and the fluorescence intensities were recorded with excitation at 464 and 540 nm. Superoxide ($O_2^{\cdot-}$) was added as solid KO_2.[1] Hydroxyl radicals were generated *in situ* by Fenton reaction.[2] To a 5 mL solution of probe **1b** (3 μM) in PBS buffer/DMF (pH 7.4, 8: 2), hydrogen peroxide stock solution (0.01 mL, 18 mM) was added. Aqueous Fe^{2+} (0.01 mL, 100 mM) was then added to the probe **1b**/H_2O_2 solution to generate hydroxyl radicals (60 μM). The time course of the fluorescence spectra of probe **1b** (3 μM) in the presence of NaOCl (40 equiv.) were acquired over various time points at 25 ^oC in PBS/DMF (pH 7.4, 8: 2), and the emission ratio changes (I_{505}/I_{585}) were continuously monitored at time intervals.

Detection limit:[8] The detection limit was calculated based on the fluorescence titration. To determine the S/N ratio, the emission intensity of probe **1b** in the absence of ClO^- was measured by thirty times and the standard deviation of the blank measurement was determined. The detection limit is then calculated by the following equation: detection limit = $3\sigma/m$, where σ is the standard deviation of the blank measurement, and m is the slope for the intensity versus the sample concentration.

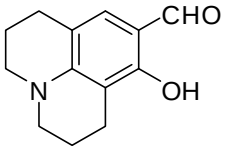
MCF-7 cell imaging using probe 1b: MCF-7 cells were grown in MEM (modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 ^oC. The cells were plated on 12-well plates and allowed to adhere for 24 h. Immediately before the experiments, the cells were washed with PBS buffer. Subsequently, the cells were incubated with probe **1b** (5 μM) for 30 min at 37 ºC, and then washed with phosphate-buffered saline (PBS) three times. After incubating with NaOCl (6 equiv.) for another 30 min at 37 ºC, the Hela cells were rinsed with PBS three times, and the fluorescence images were acquired through a Nikon eclipase TE300 inverted fluorescence microscopy equipped with a cooled CCD camera.

The imaging processing was performed in Nikon EZ-C1 software 3.90 and Image J software.



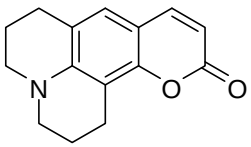
3

Synthesis of compound 3. A mixture of 3-methoxyaniline **2** (1.30 g, 10.0 mmol), 1-bromo-3-chloropropane **(**23.50 g, 150.0 mmol) was stirred at room temperature for 0.5 h under N_2 atmosphere. The mixture was heated at 95 ^oC for 1 h, then at 140 ^oC for 23 h, finally at reflux for 21 h. The progress of the reaction was monitored by TLC. When the raw starting material 3-methoxyaniline disappeared, the reaction was terminated. The reaction mixture was cooled to 80 ^oC and an orange red solid appeared. 7 mL of HI was slowly added to the solid and the mixture was heated to reflux for 6 h. Subsequently, water (20 ml) was added to the solution, and the organic phase was collected. The solvent was removed under reduced pressure to give a residue, which was then purified by column chromatography on silica gel (petroleum ether : CH_2Cl_2 = 2: 1) to afford compound **3** as a white crystal (1.32 g, yield: 70.1%). mp 134-136 ^oC (lit.[5] mp 126-130 ^oC); ^1H NMR ($CDC1_3$, 400 MHz) δ 1.97-2.01 (m, 4 H), 2.64-2.72 (m, 4 H), 3.08-3.12 (m, 4 H), 4.30 (br s, 1 H), 6.08-6.10 (d, 1 H, J = 8.0 Hz), 6.66-6.68 (d, 1 H, J = 8.0 Hz).



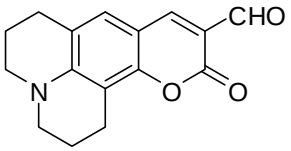
4

Synthesis of 9-Formyl-8-hydroxy-2, 3, 6, 7-tetrahydro-1H, 5H-benzo[*ij*]-quinolizine 4. Fresh distilled DMF (2.0 mL) was added dropwise to $POCl_3$ (2.0 mL) at room temperature under N_2 atmosphere, and the mixture was stirred for 30 min to yield a canary solution. Then compound **3** (500.0 mg, 2.6 mmol, dissolved in 10 mL DMF) was added to this solution as one portion. After being stirred at room temperature for 30 min, the solution was heated at 60 ^oC for an additional 30 min. The reaction mixture was then slowly added to ice water (100 ml), and aged for 2 h. The resulting yellow solid was collected by filtration. mp 70-71 ^oC (lit.[5] mp 71.5-73 ^oC; lit.[6] 70-72 ^oC); ^1H NMR ($CDC1_3$, 400 MHz) δ 1.91-1.96 (m, 4 H), 2.66-2.69 (t, 4 H, J = 6.4 Hz), 3.26-3.30 (m, 4 H), 6.85 (s, 1H), 9.37 (s, 1H), 11.8 (s, 1 H).



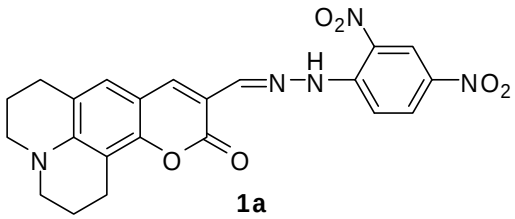
6

Synthesis of compound 6. Compound **4** (500.0 mg, 2.3 mmol) and diethylmalonate (738.2 mg, 4.6 mmol) were dissolved in absolute ethanol (25 mL) with stirring, and then 4 drops of piperidine was added to the mixture. The mixture was heated to reflux for 24 hours. After removal of ethanol under reduced pressure, concentrated HCl (8 mL) and glacial acetic acid (8 mL) were added. The mixture was heated at 80 ^{o}C for 12 hours. The solution was cooled to room temperature and poured into ice water (40 ml). The pH was adjusted to around 7.0 by introduction of a NaOH solution (0.1 M), and a yellow precipitate was formed. After being stirred for 30 min, the mixture was filtered, washed with water, dried, then recrystallized with toluene to give compound **6** (439.8 mg) in 79.1% yield. mp127-129 ^{o}C.; ^{1}H NMR (CDC1$_3$, 400 MHz) δ 1.96-1.99 (m, 4 H), 2.75-2.78 (t, 2 H, J = 6.4 Hz), 2.87-2.90 (t, 2 H, J = 6.4 Hz), 3.24-3.29 (m, 4 H), 5.99 (d, 1 H, J = 9.2 Hz), 6.85 (s, 1H), 7.47 (d, 1 H, J = 9.2 Hz).



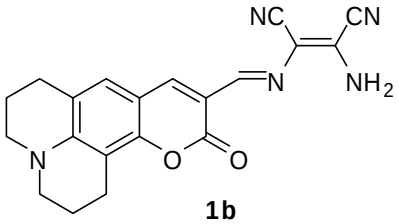
7

Preparation of 10-Oxo-2,3,5,6-tetrahydro-1*H*,4*H*,10*H*-11-oxa- 3a-azabenzo[*de*]anthracene-9-carbaldehyde 7. Fresh distilled DMF (2.0 mL) was added dropwise to $POCl_3$ (2.0 mL) at room temperature under N_2 atmosphere, and the mixture was stirred for 30 min to yield a canary solution. Then compound **6** (600 mg, 0.48 mmol, dissolved in 10 mL DMF) was added to this solution as one portion. After being stirred at room temperature for 30 min, the solution was heated at 60 ^{o}C for an additional 12 hours. The reaction mixture was slowly added to ice water (100 ml), and then a NaOH solution (20%) was added to adjust the pH to around 7.0 to yield a precipitate. The resulting solid was collected by filtration and recrystallized in absolute ethanol to give compound **7** (570 mg) in 85.2% yield. mp = 194-196 ^{o}C (dec) (lit.[7] mp 190-195^{o}C (dec)); ^{1}H NMR (CDC1$_3$, 400 MHz) δ 1.97-2.00 (m, 4H), 2.75-2.78 (t, 2 H, J = 6.4 Hz), 2.87-2.90 (t, 2 H, J = 6.4 Hz), 3.36-3.40 (m, 4H), 6.98 (s, 1H), 8.14 (s, 1H), 10.10 (s, 1H) ppm. MS (EI^{+}): m/z 269.1 (M^{+}).



1a

Synthesis of compound 1a: (2,4-Dinitrophenyl)hydrazine (122.6 mg, 0.62 mmol) and compound **7** (200.0 mg, 0.74 mmol) in 75 mL of mix-solvent (ethanol: acetate = 1: 2) were stirred for 2 hours at room temperature. The progress of the reaction was monitored by TLC (CH_2Cl_2: C_2H_5OH = 5: 1). When the raw material **7** disappeared, the reaction was terminated. The solvent was removed under reduced pressure. The resulting residue was recrystallized from ethanol, and further purified by chromatography on neutral Al_2O_3 using CH_2Cl_2/CH_3OH (v/v 15 : 1) as eluent to give compound **1a** (204.8 mg) in 73.5% yield: mp = 228 – 229 ^oC; ^1H NMR ($CDCl_3$, 400 MHz) δ 2.0 (m, 4H), 2.79 (t, 2 H), 2.91 (t, 2 H, J = 5.6 Hz), 3.36 (m, 4H), 7.01 (s, 1H), 7.40 (s, 1H), 8.22 (s, 1H), 8.36 (s, 1H), 8.68 (s, 1H), 9.15(s, 1H) , 11.38 (s, 1H) ppm; MS (EI^+): m/z 449.1 (M^+). HRMS (EI^+): m/z calcd for $(C_{22}H_{19}N_5O_6)^+$, 449.1335; found, 449.1338; Anal. Calcd for $C_{22}H_{19}N_5O_6$: C 58.80, H 4.26, N 15.58, Found: C 59.01, H 4.15, N 15.49. (Note: Compound **1a** has very poor solubility in a wide variety of solvents (e.g. CH_3Cl, DMF, DMSO, THF, acetone, methanol, pyridine, etc. Thus, a ^{13}C NMR spectrum of compound **1a** with reasonable resolution was not available.).



1b

Synthesis of compound 1b: Diaminomaleonitrile (221.2 mg, 2.0 mmol) and compound **7** (500.1 mg, 1.9 mmol) in 40 mL of absolute ethanol containing two drops of acetate acid as a catalyst were stirred for 3 hours at room temperature. The progress of the reaction was monitored by TLC (CH_2Cl_2: C_2H_5OH = 10: 1). When the raw material **7** disappeared, the reaction was terminated. The solvent was removed under reduced pressure, and the resulting residue was recrystallized from ethanol, and further purified by chromatography on silica using CH_2Cl_2/CH_3OH (v/v 15 : 1) as eluent to give compound **1b** (547.2 mg) in 82.1% yield: mp = 246-248 ^oC; ^1H NMR (d_5-pyridine, 400 MHz) δ 1.50 (m, 4H), 2.32-2.35 (t, 2 H, J = 5.6 Hz), 2.49-2.52 (t, 2 H, J = 5.6 Hz), 2.83- 2.89 (m, 4H), 6.30 (s, 1H), 8.10 (s, 1H), 8.68 (bs, 2H), 8.83(s, 1H) ppm; ^{13}C NMR (d_5-pyridine, 100 MHz) δ 20.73, 21.72, 27.87, 50.13, 50.62, 106.71, 106.88, 109.54, 114.06, 116.29, 120.12, 127.04, 127.75, 141.98, 148.59, 151.69, 162.39; MS (EI^+): m/z 359.1 (M^+). HRMS (EI^+): m/z calcd for $(C_{20}H_{17}N_5O_2)^+$, 359.1377; found, 359.1368.

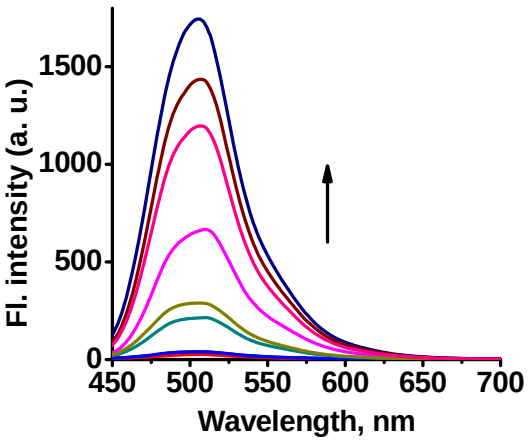


Fig. S1. The fluorescence spectra of probe **1a** (2 μM) with the addition of increasing concentrations of NaOCl (0 to 40 equiv.) in PBS/DMF (pH 7.4, 8: 2). Excitation at 464 nm. *Notably, only a fluorescence turn-on instead of ratiometric response was observed.*

Table S1. Photophysical data of Compounds **1a-b**, and **7**.

Compound	Absorption λ_{max} [nm]	Logε	Emission λ_{max} [nm]	Φ_f
1a	520	4.66	575	-----[a]
1b	514	4.73	585	0.02 [b]
7	464	4.30	505	0.59 [c]

[a] Too small to be measured accurately. [b] Fluorescence quantum yield for **1a** was calculated with Rhodamin B (0.49 in EtOH)[3] as the reference. [c] Fluorescence quantum yield for **7** was calculated with Quinine Sulfate (0.546 in 0.1 M H_2SO_4)[4] as the reference.

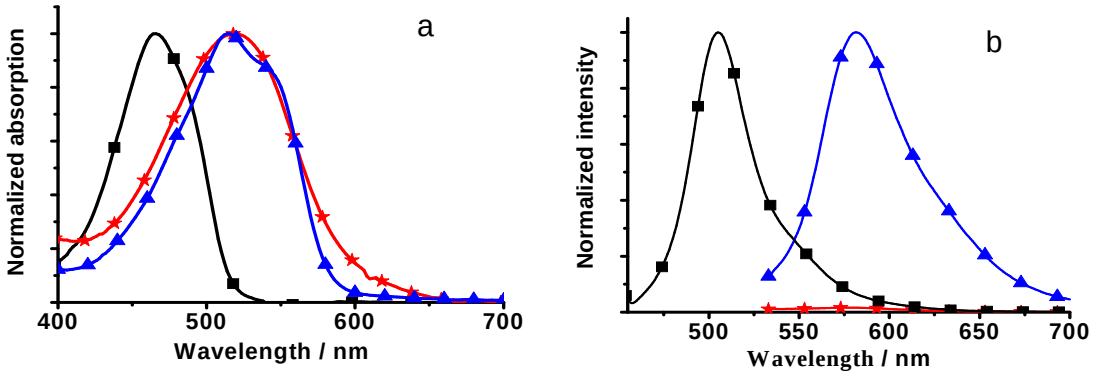


Fig. S2. a) Normalized absorption spectra of compounds **1a** (★), **1b** (▲), and **7** (■) in PBS/DMF (pH 7.4, 8: 2). b) Normalized emission spectra of **1a** (★), **1b** (▲), and **7** (■). The emission spectra were normalized while keeping the peak ratio between **1b** and **1a** unchanged.

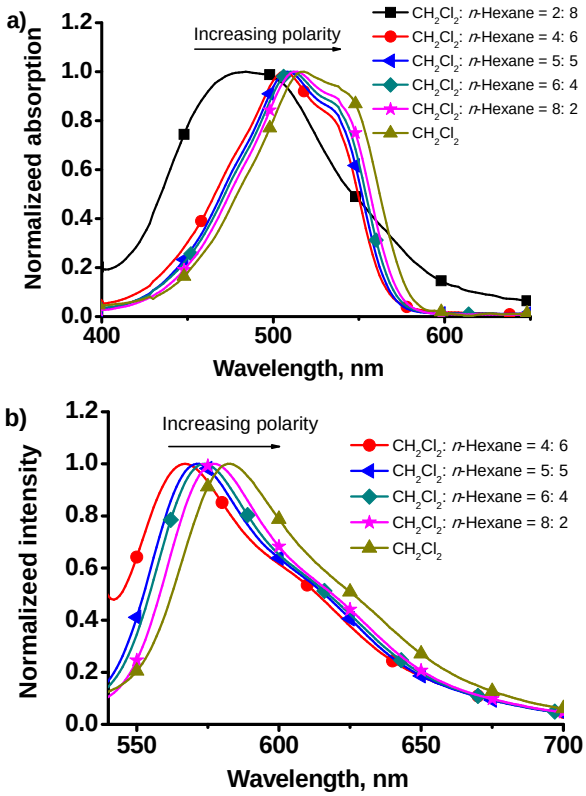


Fig. S3. Absorption (a) and fluorescence (b) spectra of compound **1b** in the mixture of CH_2Cl_2: *n*-Hexane with varying ratios.

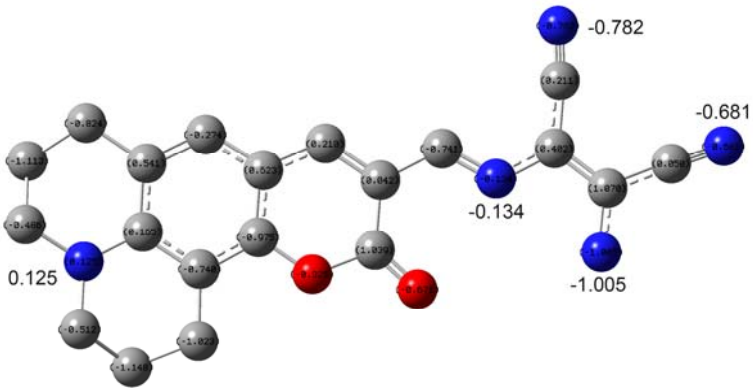


Fig. S4. DFT optimized structure of compound **1b** and the distribution of the Mulliken charges calculated by DFT. In the ball-and-stick representation, carbon, nitrogen, and oxygen atoms are colored in gray, blue, and red, respectively. H atoms were omitted for clarity.

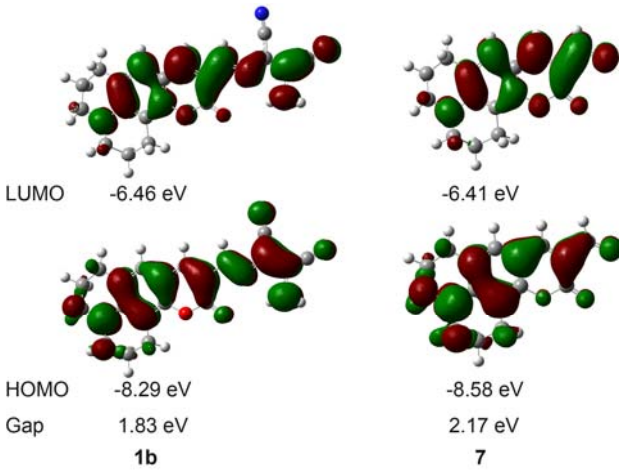


Fig. S5. The HOMO–LUMO energy gaps and the interfacial plots of the orbitals for compounds **1b** and **7**.

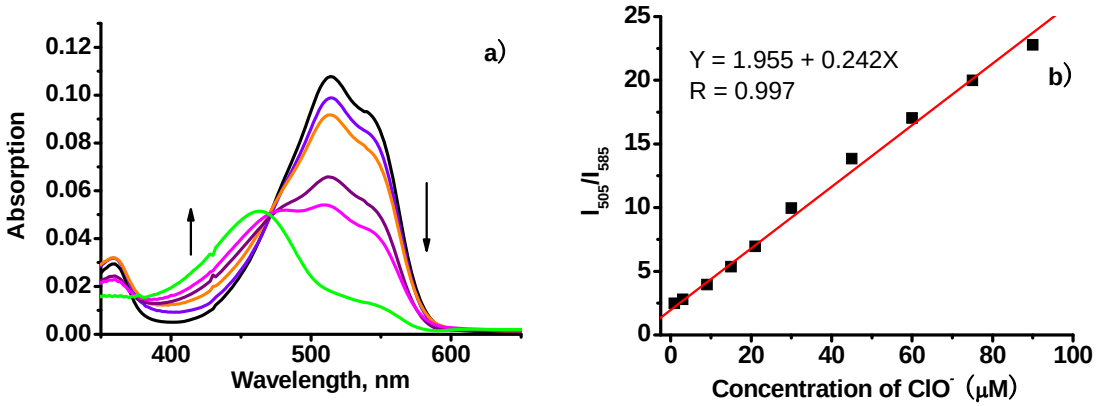


Fig. S6. (a) The absorption spectra of probe **1b** in the presence of different equiv. of ClO^- (0-40 equiv.) in PBS/DMF (pH 7.4, 8: 2). (b) The emission ratio (I_{505}/I_{585}) of probe **1b** (3 μM) as a function of ClO^- concentration in PBS/DMF (pH 7.4, 8: 2).

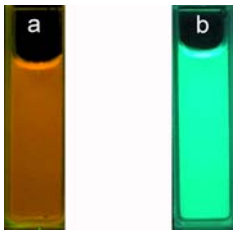


Fig. S7. Visual fluorescence color of compound **1b** (20 μM) in PBS/DMF (pH 7.4, 8/2 v/v) in the absence (a) or presence (b) of NaClO (5 equiv.) on excitation at 365 nm using a handheld UV lamp.

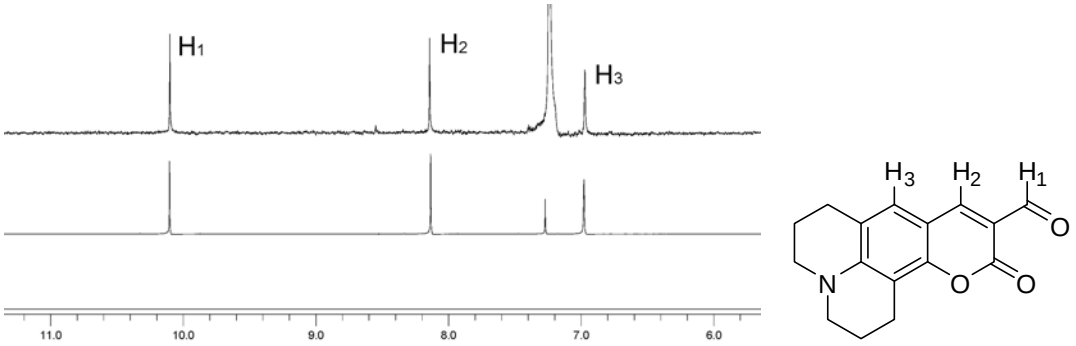


Fig. S8. Partial ^{1}H NMR (400 MHz) spectra of the separated product of probe **1b** + ClO^- (Top) and the standard compound **7** (Bottom) in $CDCl_3$.

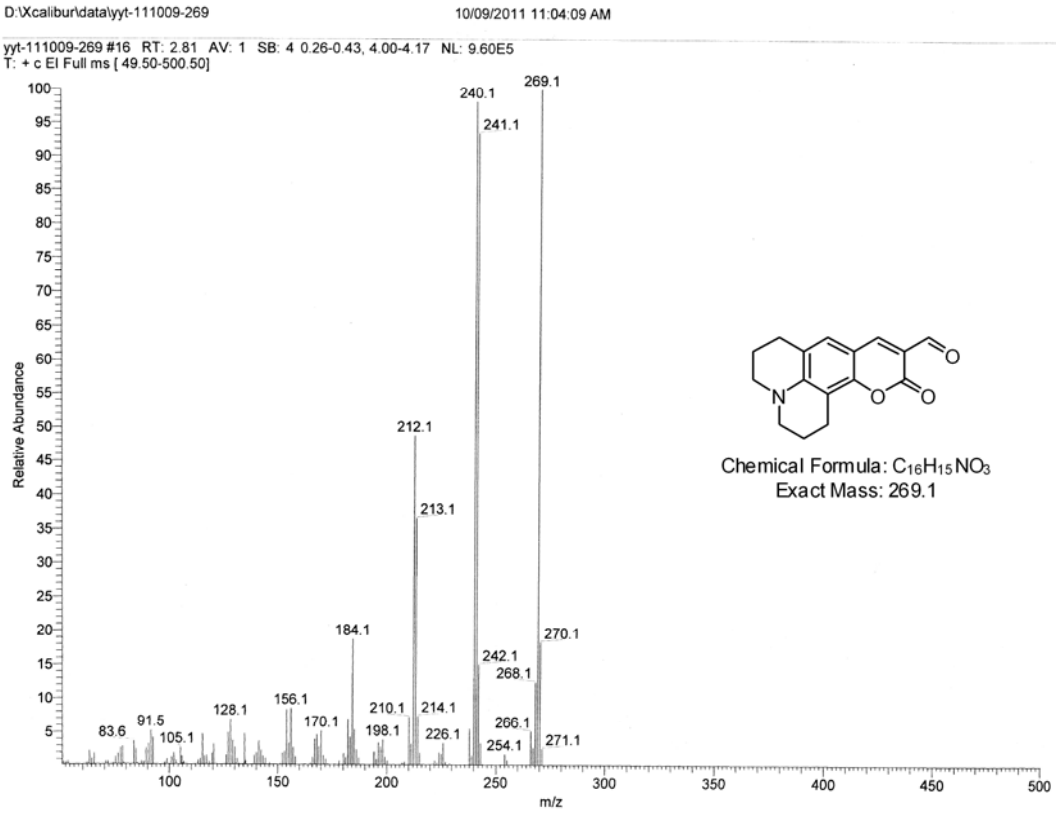


Fig. S9 MS (EI) spectrum of the separated product of probe **1b** + ClO^-

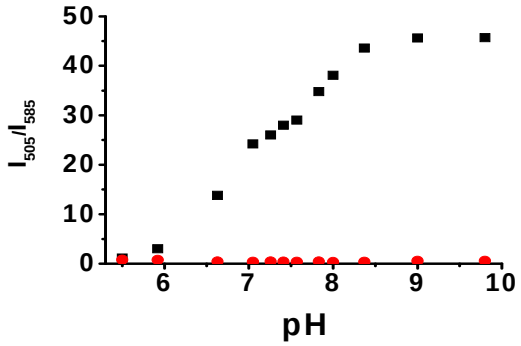


Fig. S10. Emission ratio (I_{505}/I_{585}) of probe **1b** (3 μM) vs. pH values in the absence (●) or presence (■) of OCl^- (40 equiv.) in H_2O/DMF (8: 2).

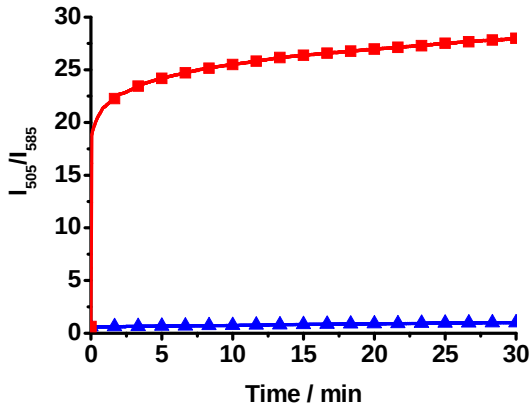


Fig. S11. The time course of probe **1b** (3 μM) in the absence (▲) or presence (■) of 40 equiv. of OCl^- in PBS/DMF (pH 7.4, 8: 2). The time course studies were performed at room temperature. The emission ratio changes (I_{505}/I_{585}) were continuously monitored at time intervals in PBS/DMF (pH 7.4, 8: 2).

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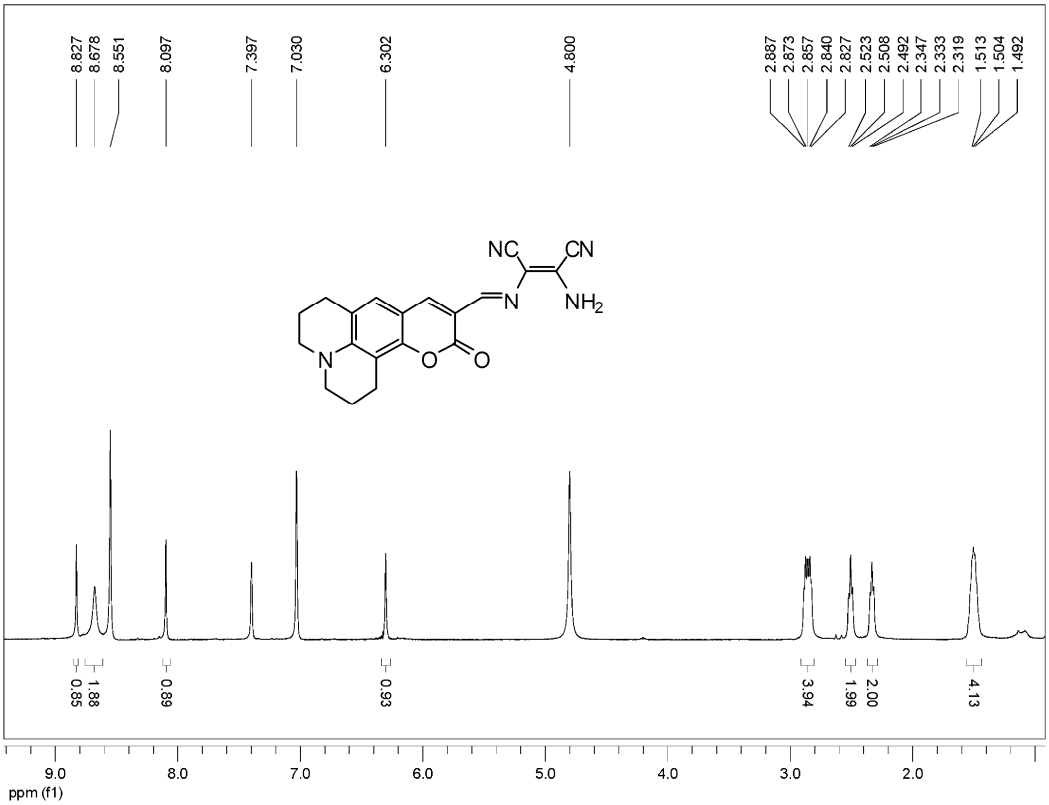
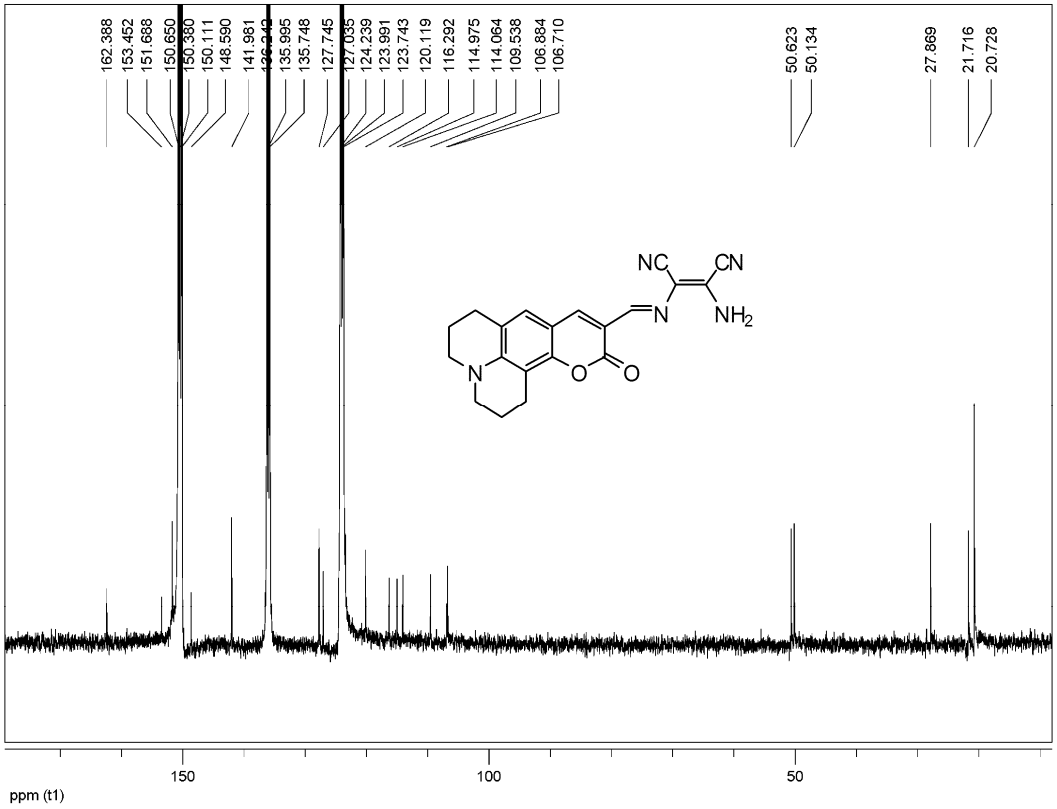


Solvent: d_5-pyridine



Solvent: d_5-pyridine