Supporting Information for

A Fluorescent Probe for Hypochlorite Based on Modulating the Unique Rotation of N-N Single Bond in Acetohydrazide

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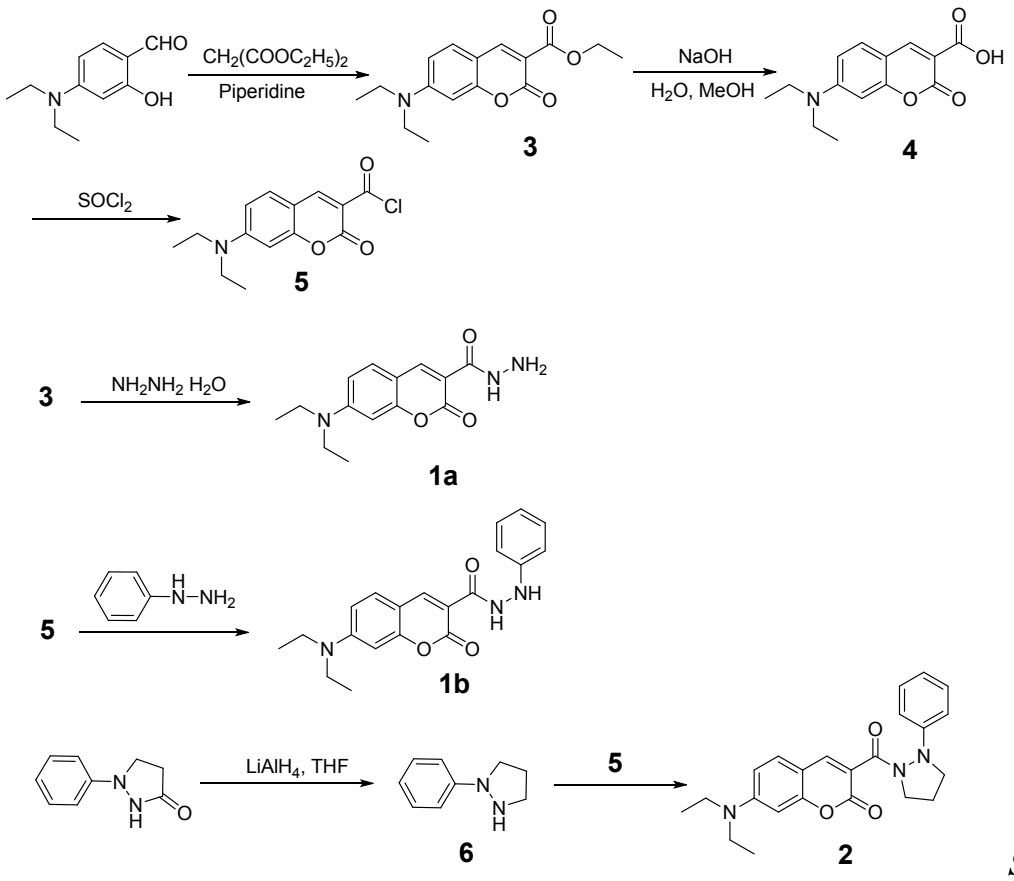
Table of contents

Materials and Instruments: Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents were purified and dried by standard methods prior to use. Twice-distilled water was used throughout all experiments. The solutions of OCl^-, H_2O_2 and *t*-BuOOH were prepared from 5.2% NaClO，30% H_2O_2, and 70% *t*-BuOOH respectively. $^1O_2$ was chemically generated from the H_2O_2/MoO_4^{2-} system in alkaline media.[1, 2] Hydroxyl radical was generated by Fenton reaction.[3, 4] Nitric oxide was generated from SNP (sodium nitroferricyanide (III) dihydrate).[5, 6] TLC analyses were 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. 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 recorded on a LXQ Spectrometer (Thermo Scientific) operating on ESI. ^1H and ^{13}C-NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz and 100 MHz respectively. The Crystallographic data were collected on a Saturn 724^+ CCD X-ray diffractometer by using graphite monochromated Mo $K\alpha$ (λ = 0.71070 Å). Elemental (C, H, N) analysis were carried out using Flash EA 1112 analyzer. Electronic absorption spectra were obtained on a SHIMADZU UV-2450 spectrometer. Fluorescence spectra were measured on a Photon Technology International (PTI) Quantamaster fluorometer with 3 nm excitation and emission slit widths. Confocal fluorescence microcopy imaging experiments were performed on a Leica TCS SP5 II laser confocal scanning microscope. The pH measurements were performed with a pH-3c digital pH-meter (Shanghai ShengCi Device Works, Shanghai, China) with a combined glass-calomel electrode.



Scheme S1. The synthetic procedure of compound **1a**, **1b**, and **2**.

Synthesis of ethyl 7-(diethylamino)-2-oxo-2H-chromene-3-carboxylate (3)

A solution of 4-(diethylamino)-2-hydroxybenzaldehyde (3.86 g, 20 mmol), diethyl malonate (6.4 g, 40 mmol) and piperidine (2 mL) in 60 mL of ethanol was refluxed for 24 h. After cooling to room temperature, the solvent was evaporated under vacuum. The resulting residue was purified by column chromatography (ethyl acetate: petroleum ether = 1:4, v/v), yielding a yellow solid (4.02g, 69.5%). Mp: 75-76 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.45 (s, 1 H), 7.30 (d, J = 9.2 Hz, 1 H), 6.56 (dd, J = 2.4 Hz, 9.2 Hz, 1 H), 6.40 (d, J = 2.4 Hz, 1 H), 4.34 (q, J = 7.2 Hz, 2 H), 3.42 (q, J =7.2 Hz, 4 H), 1.37 (t, J = 7.2 Hz, 3 H), 1.22 (t, J = 7.2 Hz, 6 H); MS (ESI) m/z 290.01 $[M+H]^+$.

Synthesis of 7-(diethylamino)-2-oxo-2H-chromene-3-carboxylic acid (4)

Compound **3** (2.89 g, 10 mmol) and NaOH (0.4g, 10 mmol) were suspended in 50% aqueous MeOH (40 mL) and heated to reflux for 15 min. The reaction mixture was then cooled to room temperature and neutralized with 3 M HCl carefully to pH = 2. The precipitate was then filtered and washed with H_2O and cold MeOH. After dried

over vacuum, the compound **4** was obtained as orange solid (1.88g, 71.9%). Mp: 229-233°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.38 (s, 1H), 8.67 (s, 1H), 7.47 (d, J = 9.2 Hz, 1 H), 6.73 (dd, J = 2.4 Hz, 8.8 Hz, 1H), 6.54 (d, J = 2.4 Hz, 1H), 3.51 (q, J = 7.2 Hz, 4H), 1.29 (t, J = 7.2 Hz, 6H); MS (ESI) m/z 260.02 $[M-H]^-$.

Synthesis of 7-(diethylamino)-2-oxo-2H-chromene-3-carbonyl chloride (5)

Under nitrogen atmosphere, compound **4** (1.15 g, 4.40 mmol) was added to dry $SOCl_2$ solution (9 ml) and the suspension was stirred at room temperature for 3 hours. Then the mixture was filtered and washed with ethyl ether to afford the yellow solid **5** (1.0 g, 81.3 %), which was not purified and used for the next step reaction immediately.

Synthesis of 7-(diethylamino)-2-oxo-2H-chromene-3-carbohydrazide (1a)

Hydrazine monohydrate (1.5 mL, 85%) was added to a solution of compound **3** (3.0 g, 10.3 mmol) in EtOH (20 mL). The reaction mixture was stirred at room temperature for 1 hour under nitrogen atmosphere. After cooling in an ice bath for 20 min, the resulting precipitate was filtered and washed 3 times with cold ethanol (3×50 mL). Then the precipitate was further dried under vacuum to give **1a** as orange needles (1.55 g, 54.7%). Mp: 160-165 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 9.74 (s, 1H), 8.69 (s, 1H), 7.45 (d, J = 8.8 Hz, 1H), 6.67 (dd, J = 2.4 Hz, 8.8 Hz, 1H), 6.51 (d, J = 2.4 Hz, 1H), 4.15 (d, J = 4.8 Hz, 2H), 3.47 (q, J = 7.2 Hz, 4H), 1.26 (t, J = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 163.9, 162.1, 157.6, 152.7, 148.0, 131.1, 110.0, 109.1, 108.2, 96.6, 45.1, 12.4; MS (ESI) m/z 276.07 $[M+H]^+$; Elemental analysis calcd (%) for $C_{14}H_{17}N_3O_3$: C 61.08, H 6.22, N 15.26; found C 60.85, H 6.25, N 15.19.

Synthesis of 7-(diethylamino)-2-oxo-N'-phenyl-2H-chromene-3-carbohydrazide (1b)

Under nitrogen atmosphere, triethylamine (1 mL) was added dropwise to a solution of compound **5** (0.839g, 3.0 mmol) and phenylhydrazine (0.324g, 3.0 mmol) in dry CH_2Cl_2 (30 mL). The mixture was stirring at room temperature for 16 hours. After removed the solvent under reduced pressure, the residue was purified by column chromatography (dichloromethane: petroleum ether: methanol = 90:30:1, v/v), affording a yellow solid (0.554 g, 52.6%). Mp: 201-203 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.97 (s, 1H), 8.65 (s, 1H), 7.98 (s, 1H), 7.70 (d, J = 9.2 Hz, 1H), 7.16 (t, J = 8.4 Hz, 1H), 6.80 (m, 4H), 6.65 (d, J = 2.4 Hz, 1H), 3.49 (q, J = 7.2 Hz,

4H), 1.15 (t, J = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 163.3, 161.8, 157.8, 153.1, 149.5, 148.4, 132.1, 129.2, 119.2, 112.8, 110.7, 109.5, 108.1, 96.4, 44.8, 12.8; MS (ESI) m/z 352.0 $[M+H]^+$; Elemental analysis calcd (%) for $C_{20}H_{21}N_3O_3$: C 68.36, H 6.02, N 11.96; found C 68.15, H 6.05, N 11.91.

Synthesis of 1-phenylpyrazolidine (6)

The compound **6** was synthesized according to a reported procedure.[7] Under nitrogen atmosphere, a solution of 1-phenylpyrazolidin-3-one (0.5 g, 3.1 mmol) and Lithium Aluminium Hydride (0.46 g, 12.1 mmol) in dry THF (12 mL) was refluxed for 18 hours. After cooling to room temperature, 30 mL ether and 30 mL water were added. The white solid was removed by filtration. The organic phase was collected by separation funnel and concentrated under reduced pressure to afford the compound **6** (0.376 g), which was used for the next step without further purification.

Synthesis of 7-(diethylamino)-3-(2-phenylpyrazolidine-1-carbonyl)-2H-chromen -2-one (2)

Under nitrogen atmosphere, triethylamine (1 mL) was added dropwise to a solution of compound **5** (0.4025 g, 1.44 mmol) and compound **6** (0.133 g, 0.897 mmol) in dry CH_2Cl_2 (30 mL). The mixture was stirring at room temperature for 3 hours. After removed the solvent under reduced pressure, the resulting residue was purified by column chromatography (dichloromethane: petroleum ether: methanol = 100:25:1, v/v), affording a yellow solid (0.259 g, 73.7 %). Mp: 204-208 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.61 (s, 1H), 7.23(t, J = 7.2 Hz, 2H), 7.14 (d, J = 7.6 Hz, 1H), 6.93 (m, 3H), 6.50 (d, J = 8.0 Hz, 1H), 6.42 (s, 1H), 3.66 (t, J = 6.4 Hz, 2H), 3.39 (q, J = 7.2 Hz, 4H), 2.10 (s, 2H), 1.63 (m, 2H), 1.19 (t, J = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 167.8, 159.2, 156.8, 151.1, 150.5, 141.3, 129.5, 129.1, 122.1, 115.6, 108.8, 107.5, 97.1, 54.6, 44.8, 43.1, 24.7, 12.4; MS (ESI) m/z 392.10 $[M+H]^+$; Elemental analysis calcd (%) for $C_{23}H_{25}N_3O_3$: C 70.57, H 6.44, N 10.73; found C 70.26, H 6.47, N 10.69.

Preparation of the test solution: The stock solution of probe **1b** (5.0×10^{-4} M) was prepared in CH_3CN, and the stock solution of various testing species (1×10^{-3} M) was prepared by dissolving an appropriate amount of testing species in water. The test solution of the probe **1b** (10 μM) in 20 mM potassium phosphate buffer (pH 7.4, containing 20% CH_3CN as co-solvent) was prepared by placing 0.1 mL of the probe

1b stock solution, 0.9 mL CH_3CN, and an appropriate aliquot of each testing species stock into a 5 mL volumetric flask, and then diluting the solution to 5 mL with 20 mM potassium phosphate buffer (pH 7.4). The resulting solution was shaken well and incubated at room temperature for 10 min before recording the spectra.

Determination of fluorescence quantum yield: Fluorescence quantum yield was determined using the solutions of quinine sulfate (Φ_F = 0.546 in 1N H_2SO_4)[8] as a standard. The quantum yield was calculated using the following equation:[9-11]

$$\Phi_{F(X)} = \Phi_{F(S)} (A_S F_X / A_X F_S) (n_X / n_S)^2$$

Where Φ_F is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, F is the area under the corrected emission curve, and n is the refractive index of the solvents used. Subscripts S and X refer to the standard and to the unknown, respectively.

Determination of the detection limit: The detection limit was determined from fluorescence titration data based on a reported method.[12-15] According to the result of titration experiment, the graph of $(I_{min}-I) / (I_{min}-I_{max})$ versus log $[OCl^-]$ was plotted, where the I is the fluorescence intensity at 468 nm, I_{min} and I_{max} are the minimum and maximum fluorescence intensity at 468 nm respectively. A linear regression curve was then fitted (Fig. S6), and the intercept of the line at x-axis was taken as detection limit.

Preparation of the Polymer Films: Poly(-methylmethacrylate) polymer (1.0 g, 99.8% (w/w)) and compound **1b** (2.0 mg, 0.2% (w/w)) were dissolved in CH_2Cl_2. The polymer film for testing fluorescence spectra was prepared by spin-coating the mixture solution at 1000 rpm on a quartz glass. After evaporation of the solvent at room temperature, the fluorescence spectra were conducted. Then the polymer film for taking visual fluorescence color photo was prepared by pouring the mxiture solution into a glass dish. After evaporation of the solvent at room temperature, the photo was taken under a handheld 365 nm UV lamp.

Cell culture and fluorescence imaging: HepG2 cells were seeded in a 24-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal

bovine serum for 24 h. Then the cells were incubated with probe **1b** (2 μM) in the culture medium for 30 min at 37°C. After washing with PBS three times to remove the remaining probe **1b** in culture medium, the cells were further incubated with NaClO (0 μM, 10 μM, 50 μM) for 20 min at 37°C. Subsequently, fluorescent images were acquired on a Leica TCS SP5 II laser confocal scanning microscope with an objective lens (×40). The excitation wavelength was set to 405 nm and 633 nm for probe **1b** and nuclear staining (DRAQ5), respectively.

Computational details： The calculations were performed with Gaussian 09 program. The parameter referred to the previous work.[16] The ground state structures of the **1a** and **1b** were optimized using density functional theory (DFT) with B3LYP functional and 6-31+G** basis set. The frontier molecular orbitals of **1a** and **1b** and the energy profiles were shown in Fig. S1. It is clear that the energy level of hydrazine or phenylhydrazine (donor) orbital is lower than that of the 7-diethylamino-coumarin fluorochrome (acceptor) orbital in **1a** and **1b**, respectively. This result demonstrates that there is no PET process in **1a** or **1b**.

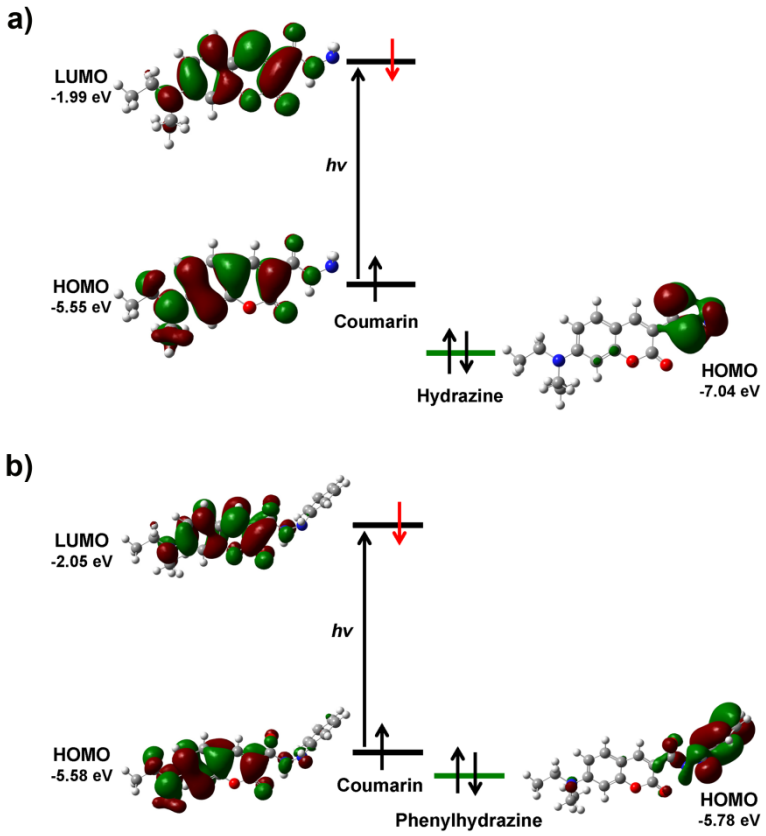


Figure S1. Frontier molecular orbital energy illustrations show the relative energetic dispositions of the orbitals of **1a** (a) and **1b** (b). Calculations were performed with the

DFT method (B3LYP/6-31G**) using Gaussian 09 program.

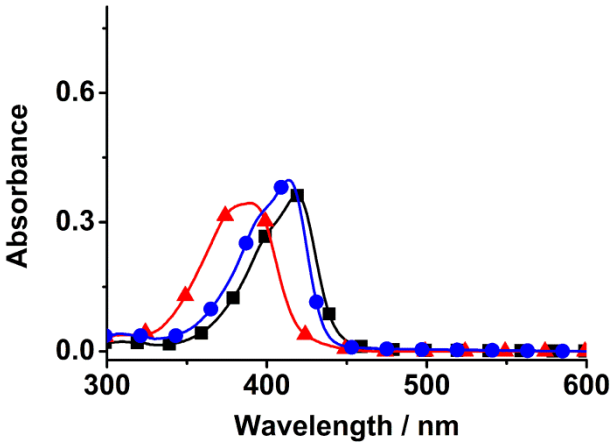


Figure S2. The absorption spectra of 10 μM **1a (●)**, **1b (■)**, and **2** (▲) in toluene.

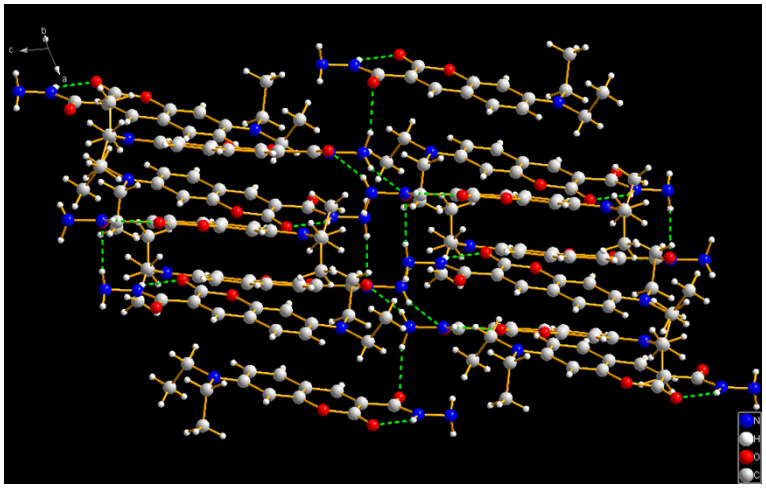


Figure S3. Packing diagram of **1a** (ball-and-stick representation); the green dash lines represent hydrogen bonds.

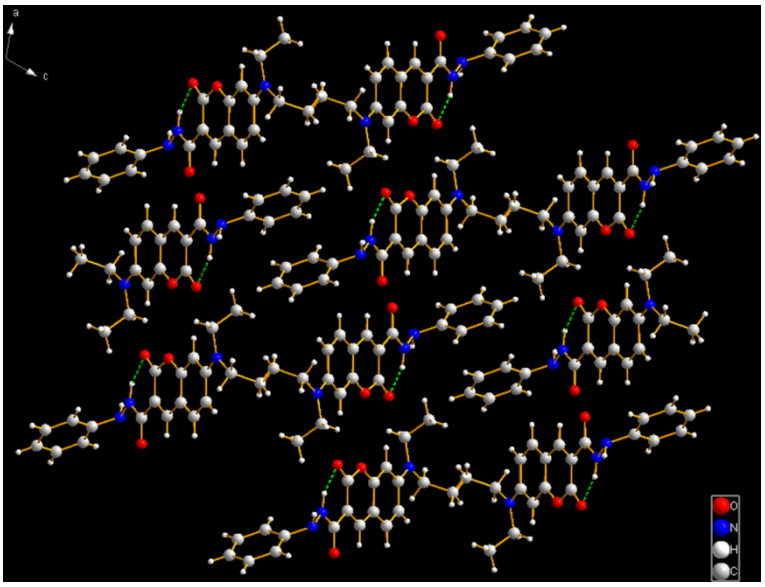


Figure S4. Packing diagram of **1b** (ball-and-stick representation); the green dash lines represent hydrogen bonds.

Table S1 Hydrogen-bond geometry of **1a**.

D-H	d(D-H) (Å)	d(H..A) (Å)	<DHA (º)	d(D..A) (Å)	A
N2-H2	0.860	1.999	138.36	2.703	O2
N3-H3A	0.919	2.109	156.53	2.975	O3[a]
N3-H3B	0.932	2.122	152.84	2.981	O6[b]
N5-H5	0.860	2.043	136.73	2.734	O5
N6-H6B	0.914	2.158	164.14	3.047	O3[c]

Symmetry codes: [a] [-x+1, -y, -z+1]; [b] [x-1, y-1, z-1]; [c] [-x+1, -y+1, -z+2].

Table S2 Hydrogen-bond geometry of **1b**.

D-H	d(D-H) (Å)	d(H..A) (Å)	<DHA (º)	d(D..A) (Å)	A
N2-H2A	0.923	1.992	135.65	2.730	O2

Table S3. Crystallographic parameters for compound **1a**

Compound	**1a**
CCDC	1036454
Chemical formula	$C_{14}H_{17}N_3O_3$
formula weight	275.31
Wavelength (Å)	0.71073
Crystal system	Triclinic
Space group	P-1
T (K)	293(2)
a (Å)	9.2120(18)
b (Å)	12.624(3)
c (Å)	12.927(3)
α(°)	94.78(3)
β(°)	109.99(3)
γ(°)	105.42(3)
V(Å^3)	1336.5(5)
Z	4
D (mg/m^{-3})	1.368
F(000)	584
μ(Mo *K*a)(mm^{-1})	0.098
θ range (º)	3.38 ~ 25.00
Goodness of fit on F^2	1.040
R_1, wR_2 [$I > 2\sigma(I)$]	0.0350, 0.0912
Reflections collected / unique	11095 / 4609 [R(int) = 0.0191]
R indices (all data)	0.0424, 0.0962

$R = \Sigma||F_0| - |F_c||/\Sigma|F_0|$, $wR_2 = \{\Sigma[w(F_0^2 - F_c^2)^2]/\Sigma[w(F_0^2)^2]\}^{1/2}$

Table S4. Crystallographic parameters for compound **1b**

Compound	**1b**
CCDC	1036457
Chemical formula	$C_{20}H_{21}N_3O_3$
formula weight	351.40
Wavelength (Å)	0.71073
Crystal system	Monoclinic
Space group	P21/c
T (K)	293(2)
a (Å)	13.1553(7)
b (Å)	6.9401(4)
c (Å)	21.0792(14)
α(°)	90.00
β(°)	109.629(5)
γ(°)	90.00
V(Å^3)	1812.67(19)
Z	4
D (mg/m^{-3})	1.288
F(000)	744
μ(Mo *K*a)(mm^{-1})	0.088
θ range (º)	3.03 ~ 26.00
Goodness of fit on F^2	1.052
R_1, wR_2 [$I > 2\sigma(I)$]	0.0559, 0.1421
Reflections collected / unique	9709 / 3485 [R(int) = 0.0329]
R indices (all data)	0.0960, 0.1604

$R = \Sigma||F_0| - |F_c||/\Sigma|F_0|$, $wR_2 = \{\Sigma[w(F_0^2 - F_c^2)^2]/\Sigma[w(F_0^2)^2]\}^{1/2}$

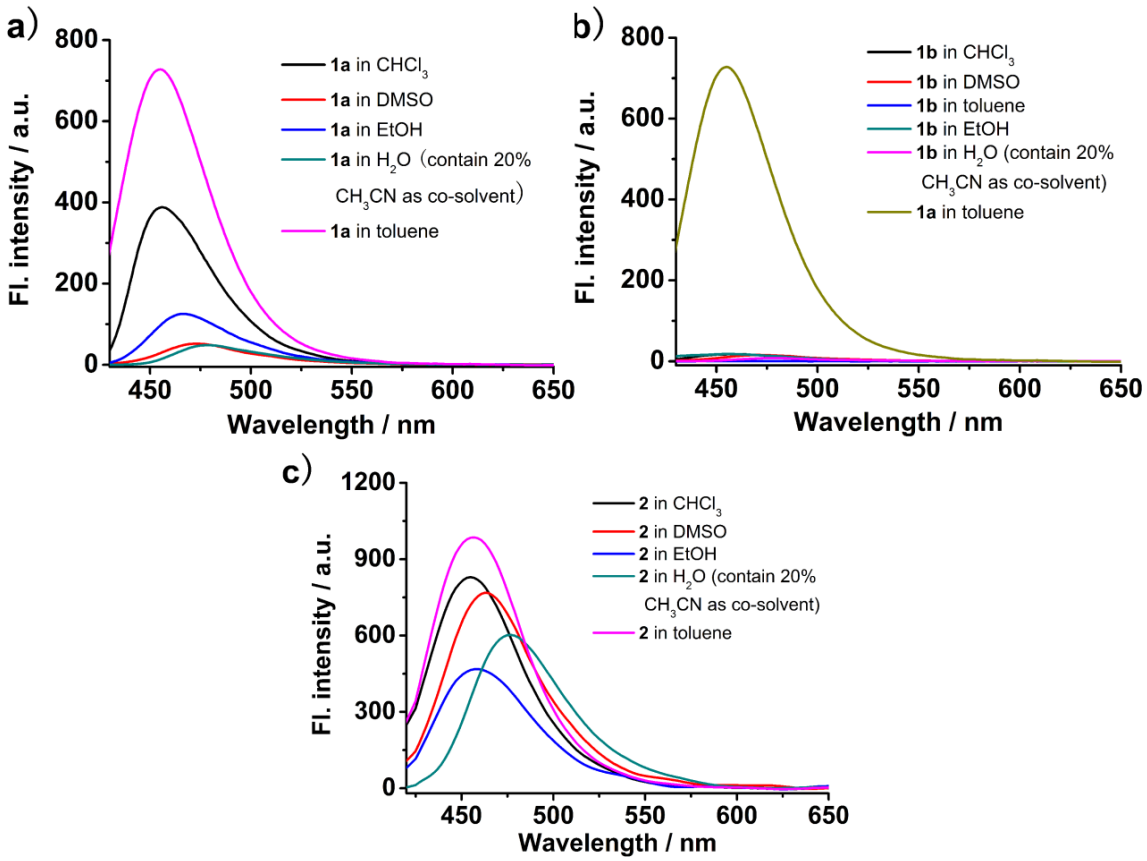


Figure S5. The fluorescence emission spectra of 10 μM **1a** (a), **1b** (b), and **2** (c) in various solvents. The emissions of **1b** in different solvents were significantly quenched, thus the emission of **1a** in toluene was shown in (b) for comparison.

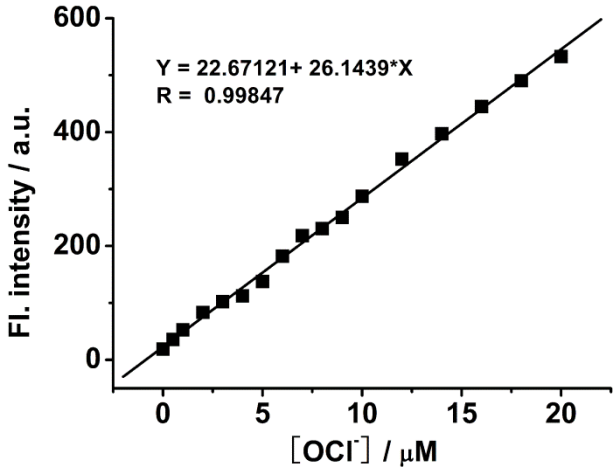


Figure S6. The fluorescence intensity of probe **1b** (10 μM) to various amount of OCl^- (0 to 20 μM). The data were acquired after incubation of probe **1b** with OCl^- for 10 min in 20 mM potassium phosphate buffer (pH 7.4, containing 20% CH_3CN as co-solvent) at room temperature. Excitation was provided at 398 nm.

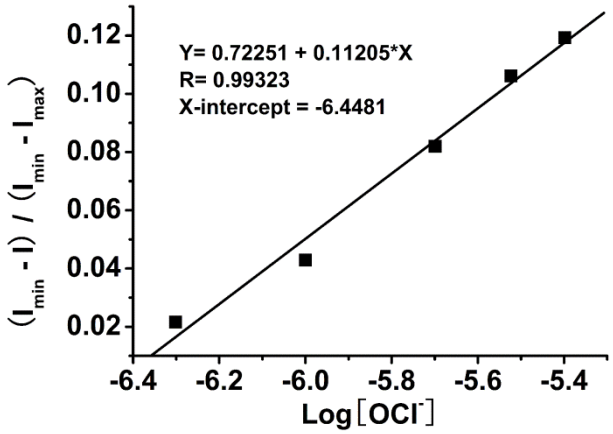


Figure S7. Plot of $(I_{min}- I) / (I_{min}-I_{max})$ versus log [OCl^-] for probe **1b**. Calculated detection limit = 3.56×10^{-7} M.

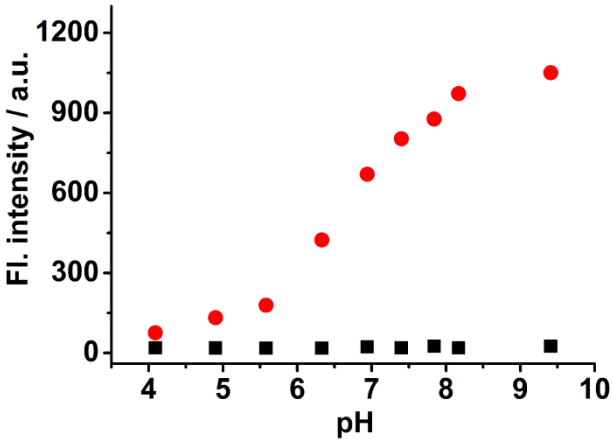


Figure S8. The variations of fluorescence intensity of probe **1b** (10 μM) in the absence (■) or presence (●) of OCl^- (60 μM) as a function of pH (λ_{ex}=398 nm, λ_{em}= 468 nm).

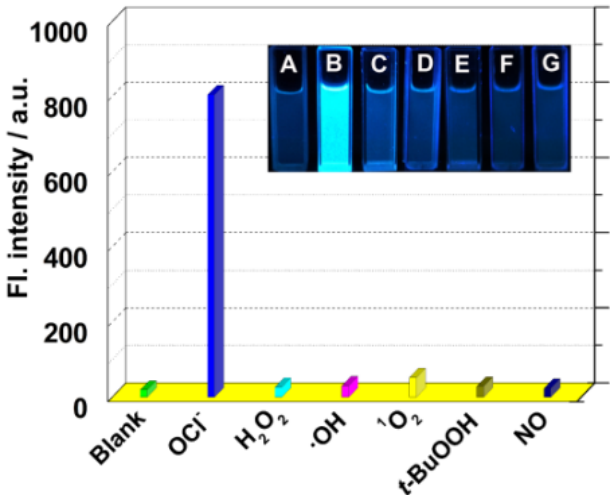


Figure S9. Fluorescence responses of probe **1b** (10 μM) in the presence of 60 μM various species in 20 mM potassium phosphate buffer (pH 7.4, containing 20% CH_3CN as co-solvent) (λ_{ex}=398 nm, λ_{em}= 468 nm). Inset: visual fluorescence color changes of probe **1b** (10 μM) in the presence of 60 μM various species (A-G: Blank, OCl^-, H_2O_2, •OH, $^1O_2$, *t*-BuOOH, NO•.).

Figure S10. ^{1}H NMR spectra of the isolated product of probe **1a** + NaOCl.

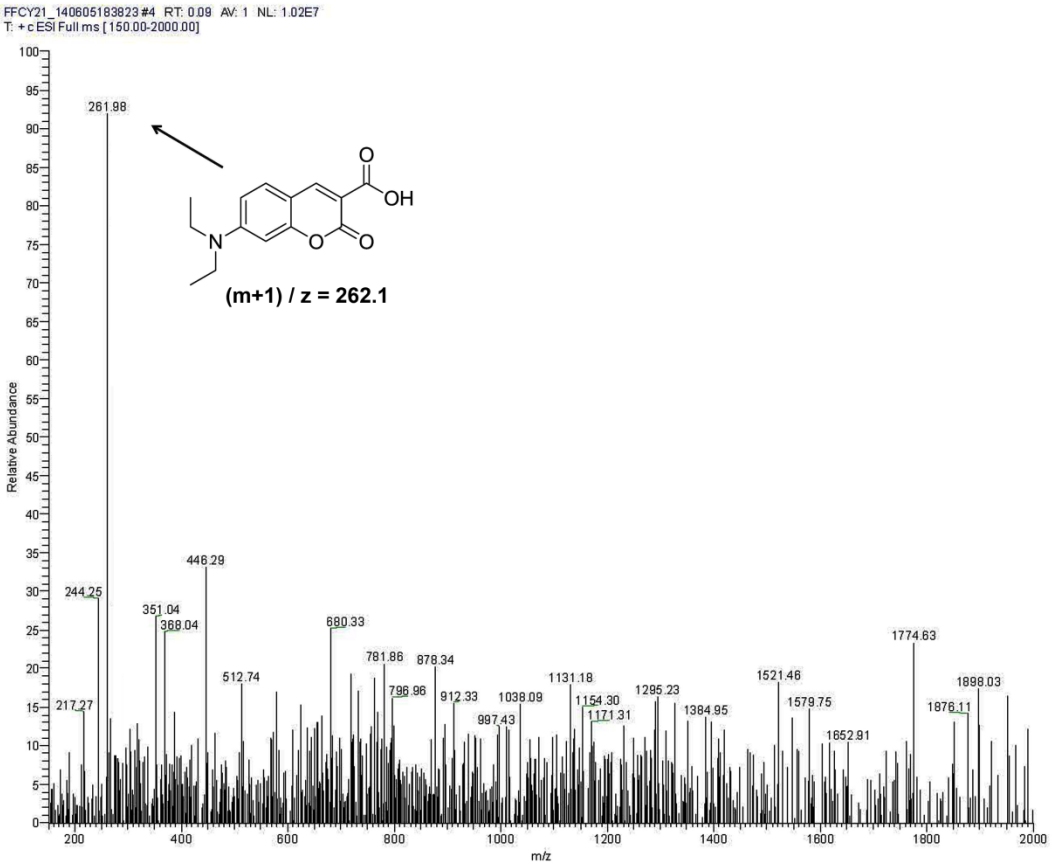


Figure S11. The ESI-Ms spectra of the isolated product of probe **1a** + NaOCl.

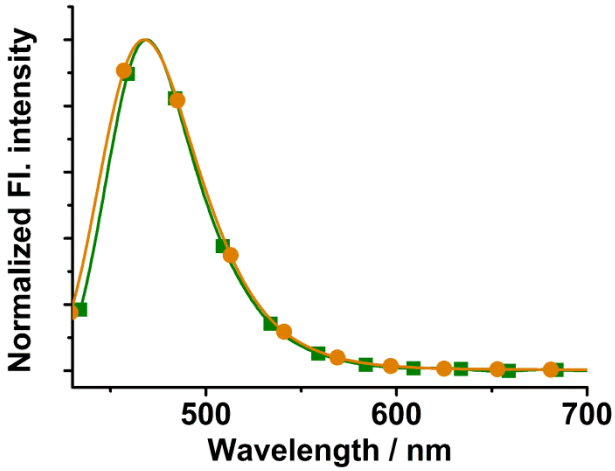


Figure S12. Normalized fluorescence emission spectra of 7-(Diethylamino)coumarin -3-carboxylic acid (●) and the isolated product of probe **1b** + NaOCl (■) (λ_{ex}=398 nm).

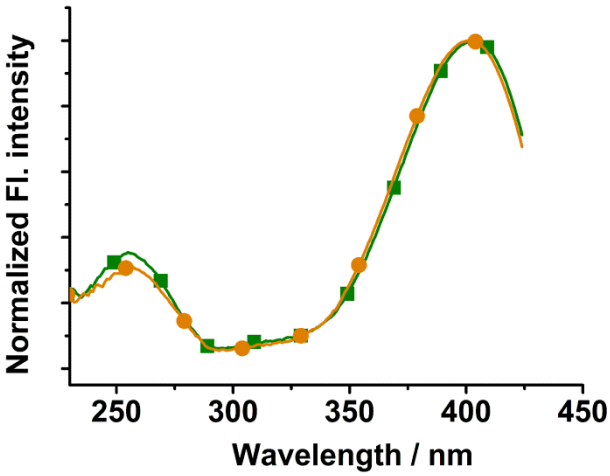


Figure S13. Normalized fluorescence excitation spectra of 7-(Diethylamino)coumarin -3-carboxylic acid (●) and the isolated product of probe **1b** + NaOCl (■) (λ_{em}= 468 nm).

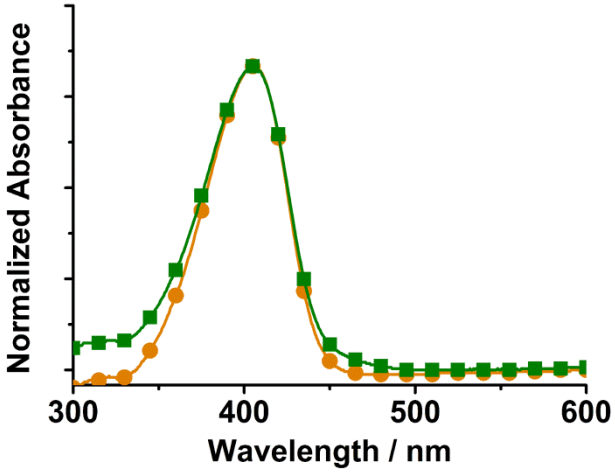


Figure S14. Normalized absorption spectra of 7-(Diethylamino)coumarin-3-carboxylic acid (●) and the isolated product of probe **1b** + NaOCl (■).

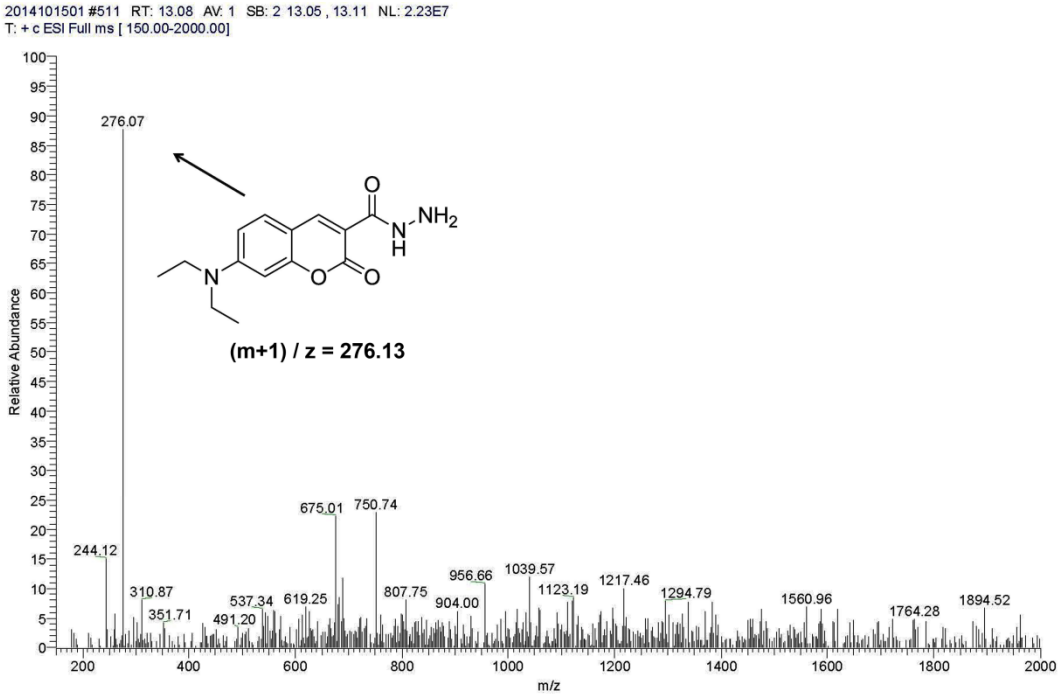


Figure S15. ESI-Ms spectra of **1a**.

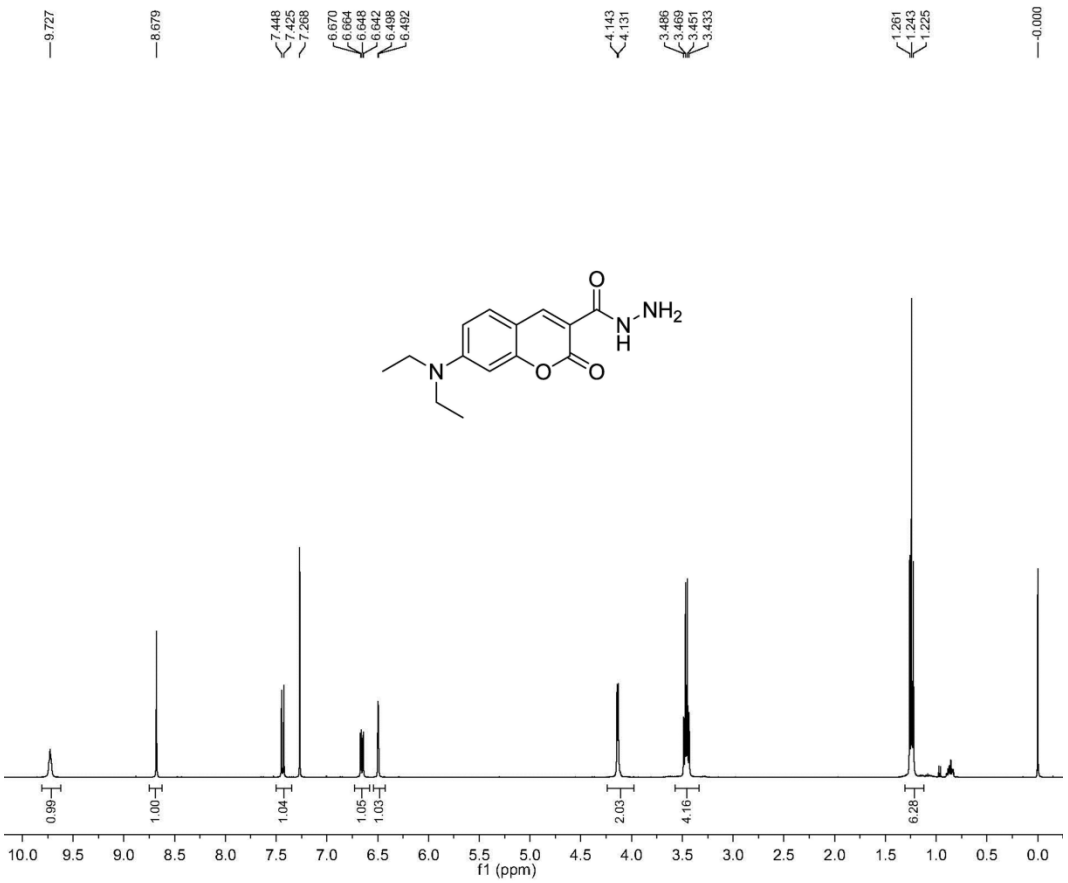


Figure S16. [1] H NMR spectra of **1a**.

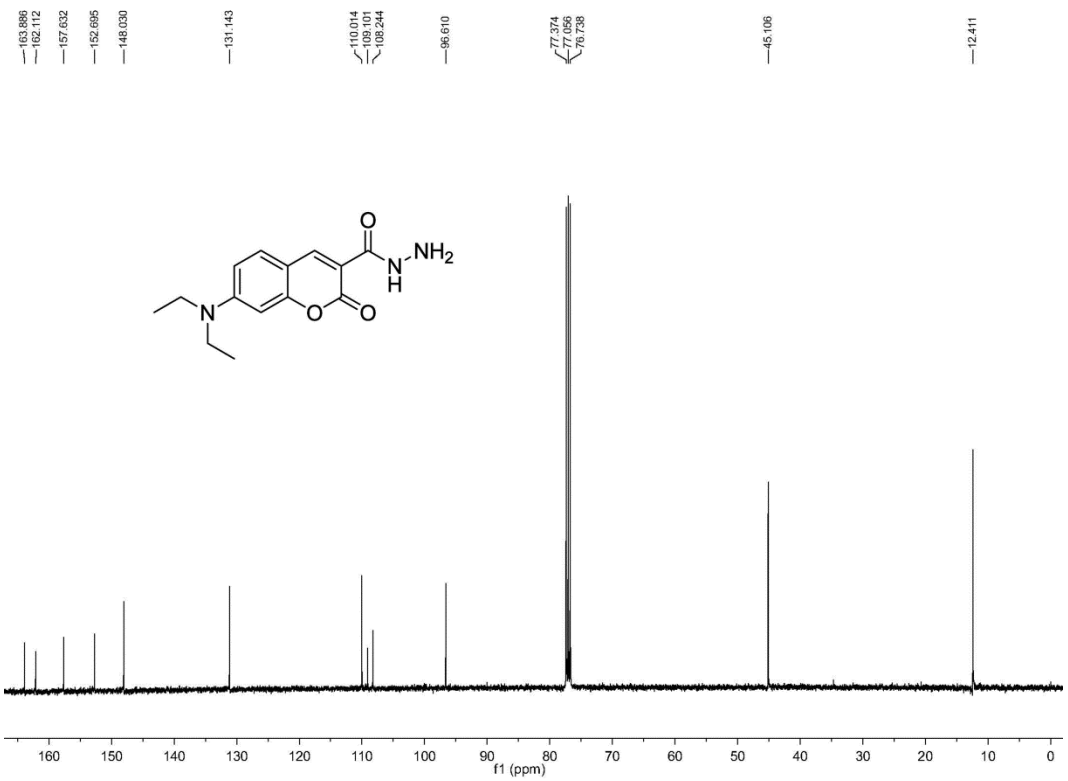


Figure S17. ^{13}C NMR spectra of **1a**.

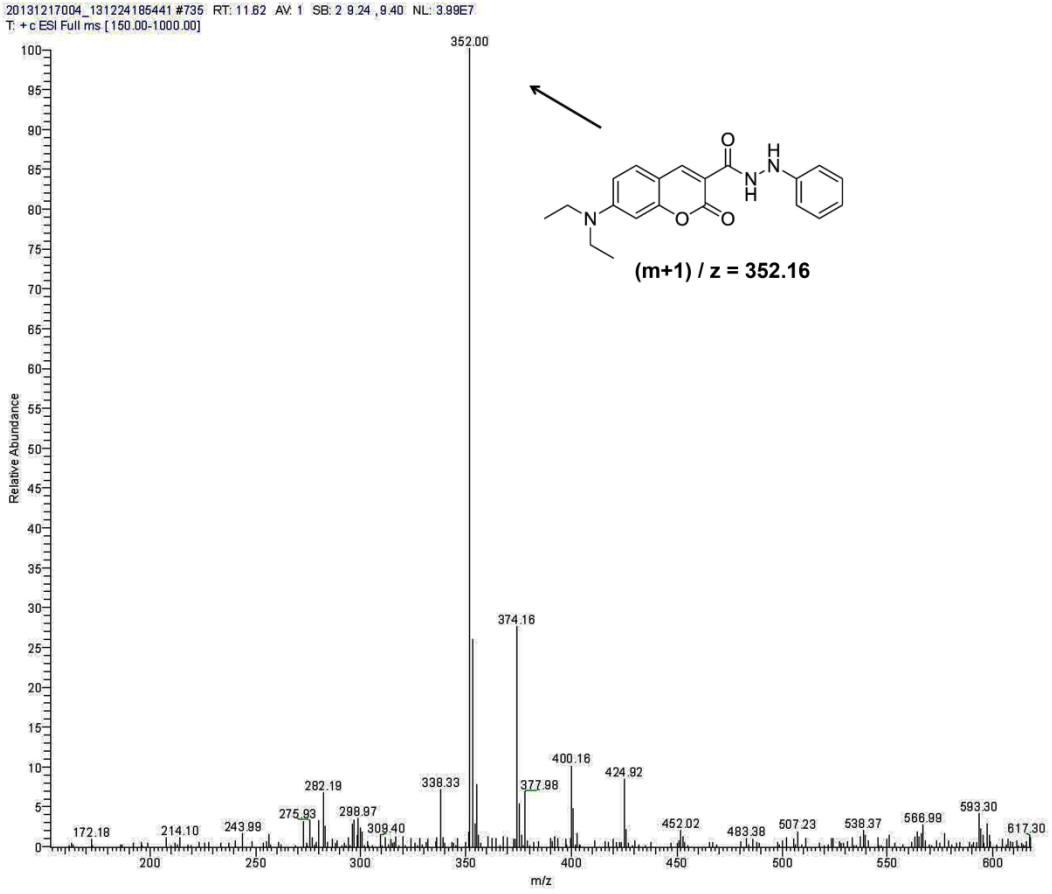


Figure S18. ESI-Ms spectra of **1b**.

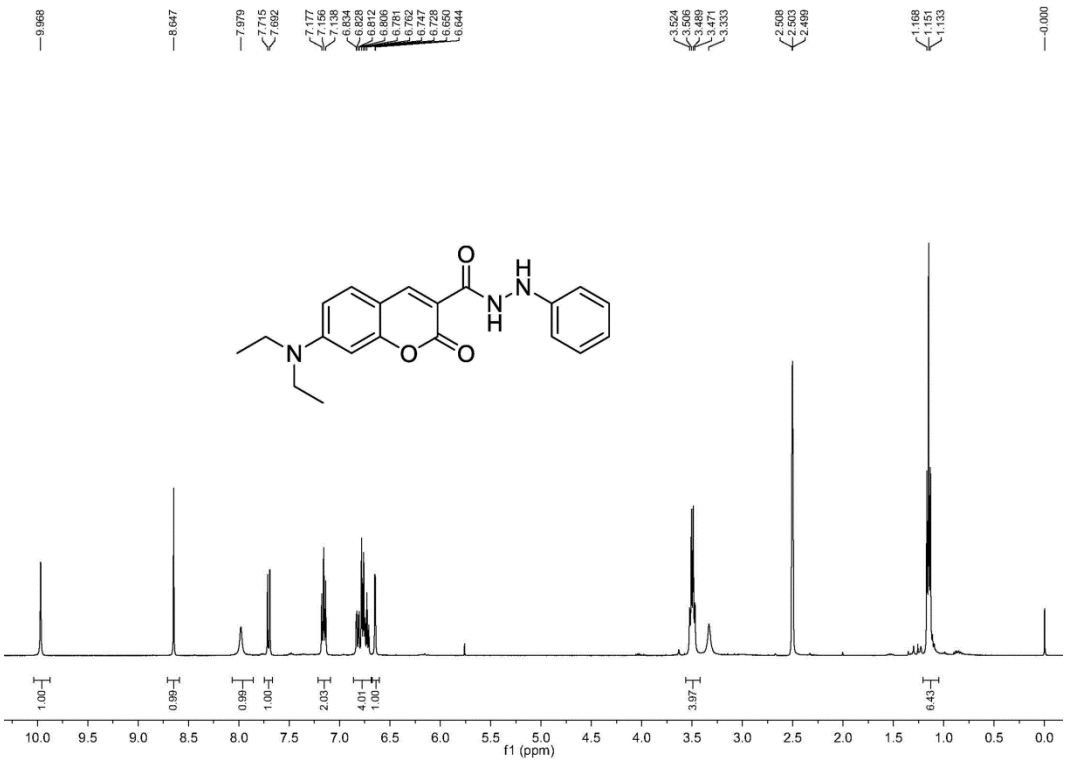


Figure S19. [1] H NMR spectra of **1b**.

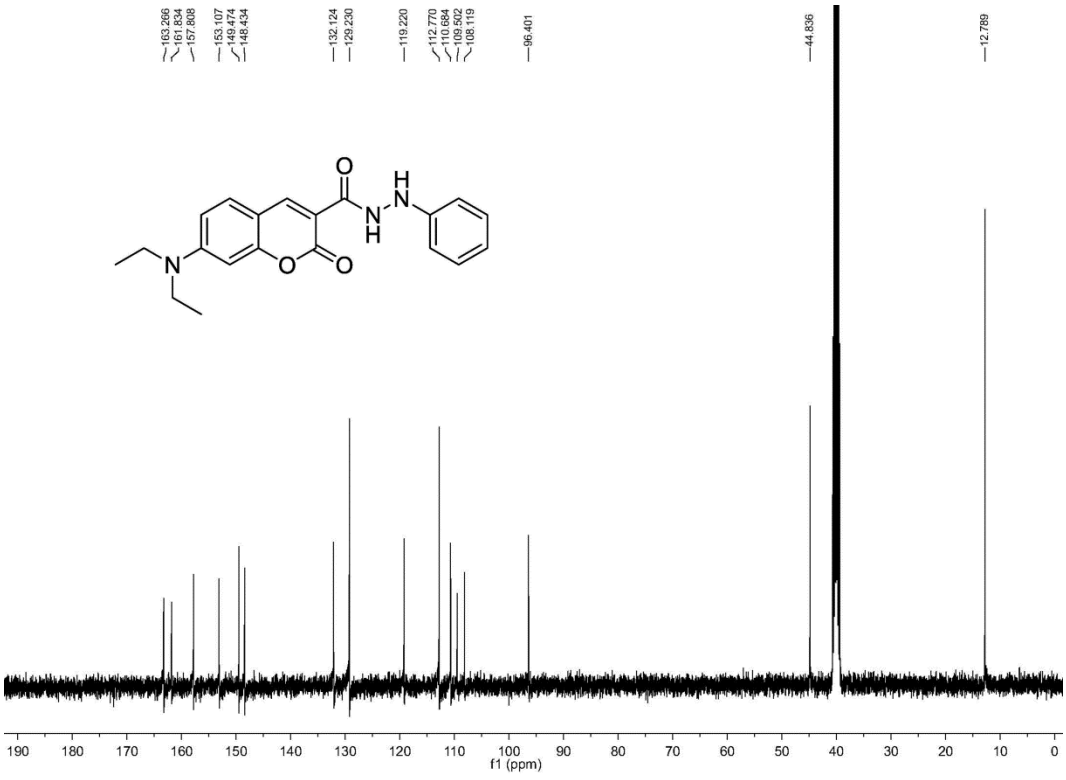


Figure S20. [13]C NMR spectra of **1b**.

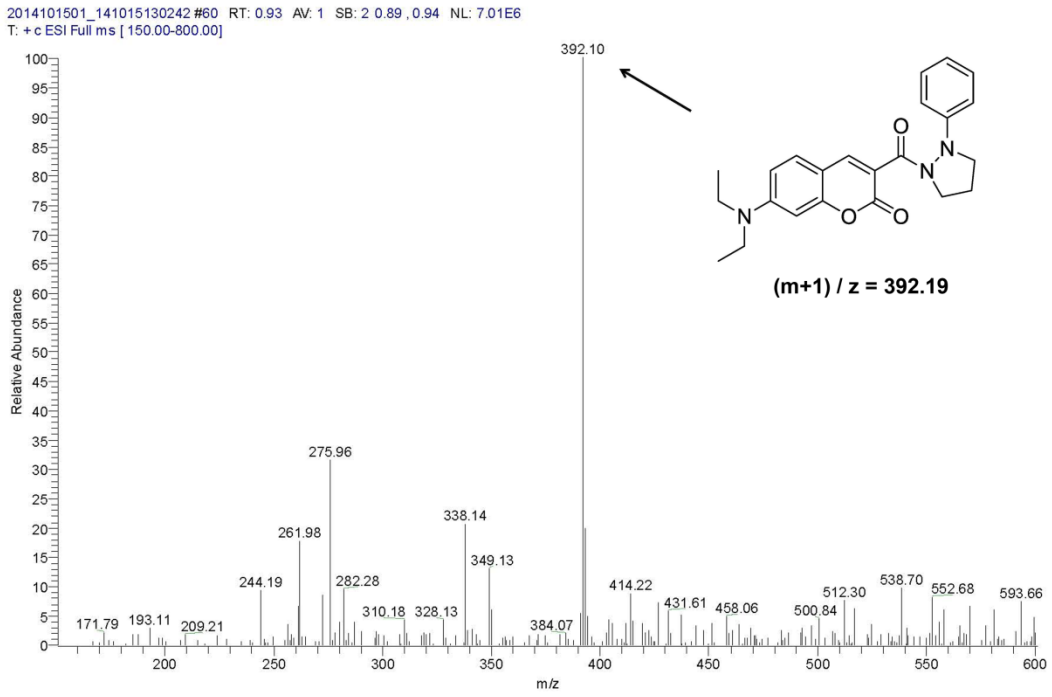


Figure S21. ESI-Ms spectra of **2**.

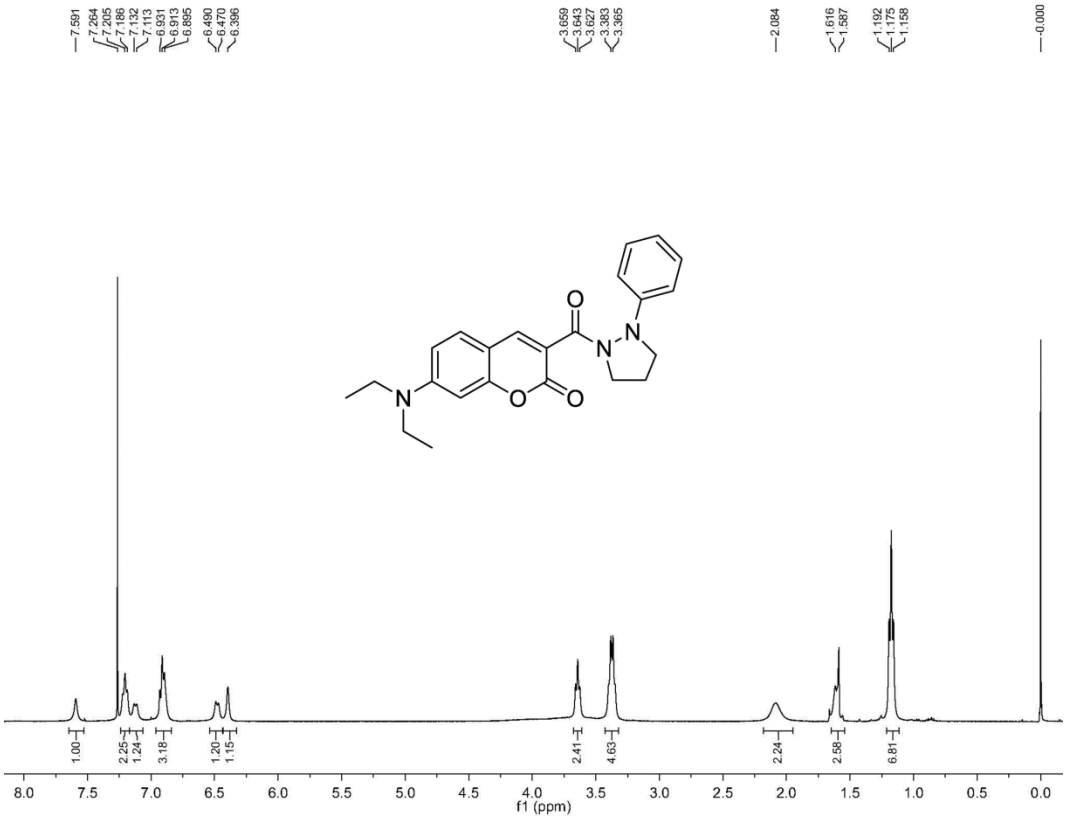


Figure S22. [1] H NMR spectra of **2**.

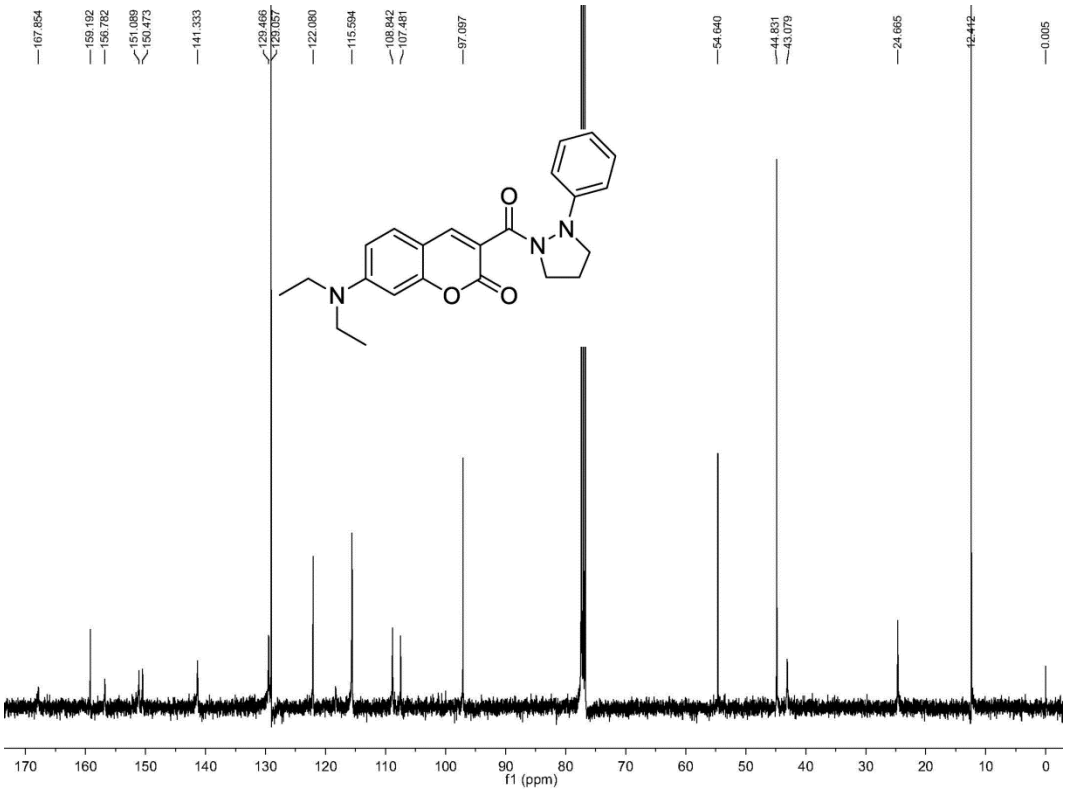


Figure S23. ^{13}C NMR spectra of **2**.

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