Supplementary Information

for

Atom Arrangement Strategy for Designing Turn-On ^{1}H Magnetic Resonance Probe: Dual Activatable Probe for Multimodal Detection of Hypochlorite

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1. Figs S1–S5

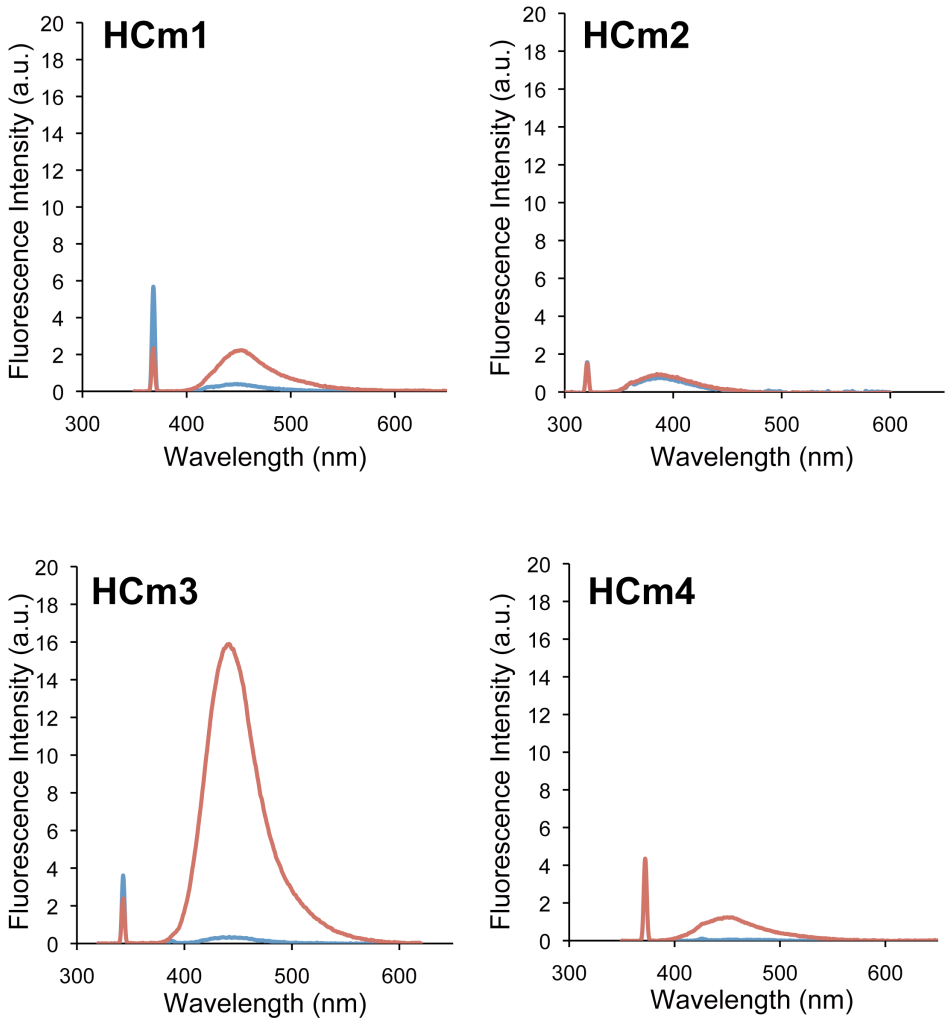


Fig. S1 Fluorescence spectra (excitation at 367 nm for **HCm1**, 320 nm for **HCm2**, 342 nm for **HCm3**, and 371 nm for **HCm4**) of **HCm1–4** (10 μM) without (blue line) or with (red line) incubation with 1 equivalent of $^{-}$OCl in phosphate buffer (pH 7.4, 100 mM) containing 150 mM NaCl and 0.1% DMF at 37 ºC.

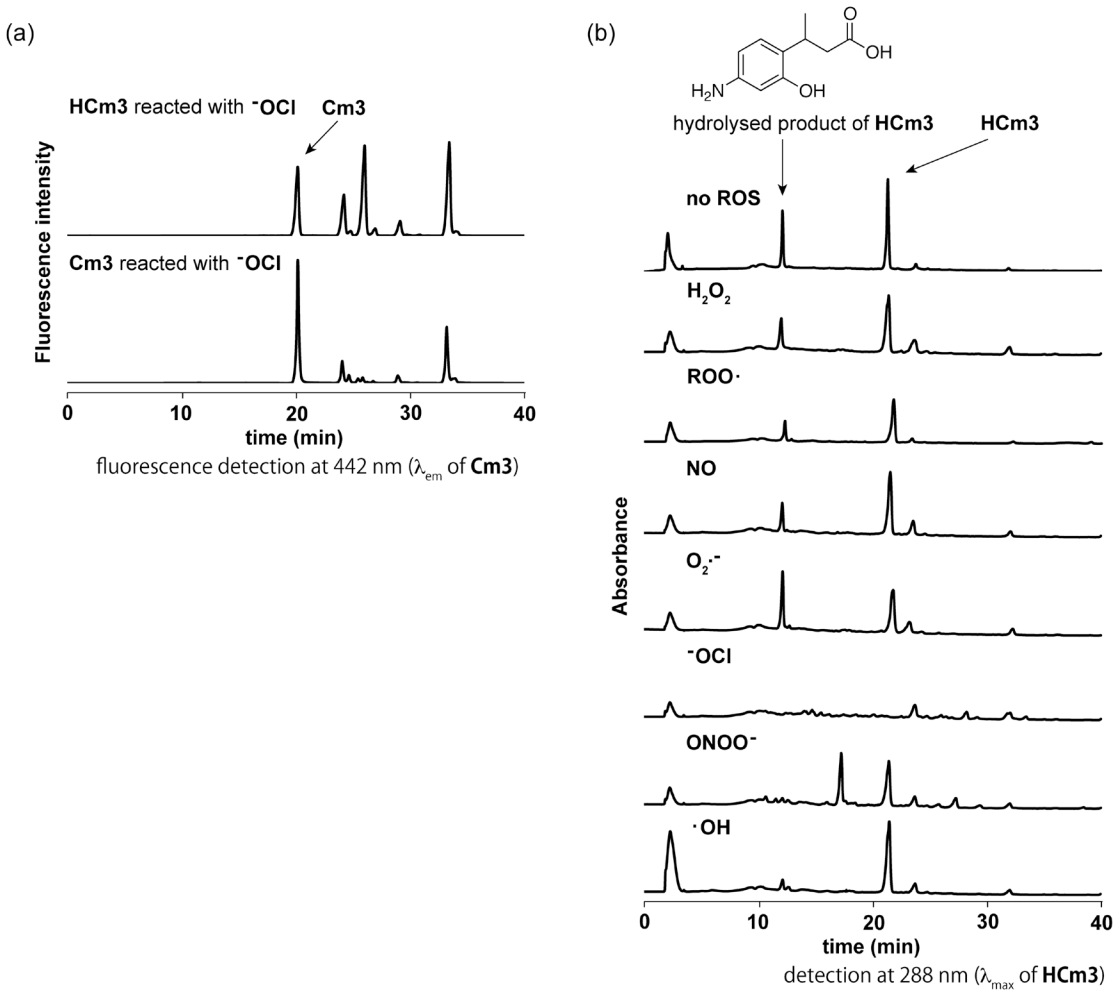


Fig. S2 (a) HPLC-fluorescence profiles (ex. 342 nm, em. 442 nm) on reactions of (upper) **HCm3** (10 μM) with $^{-}$OCl (30 μM) and (lower) **Cm3** (10 μM) with $^{-}$OCl (30 μM). (b) HPLC-UV profiles (detection at 288 nm) on reactions of **HCm3** (10 μM) with various ROS and RNS (30 μM). Reactions were carried out in phosphate buffer (pH 7.4, 100 mM) containing 150 mM NaCl and 0.1% DMF at 37 ºC.

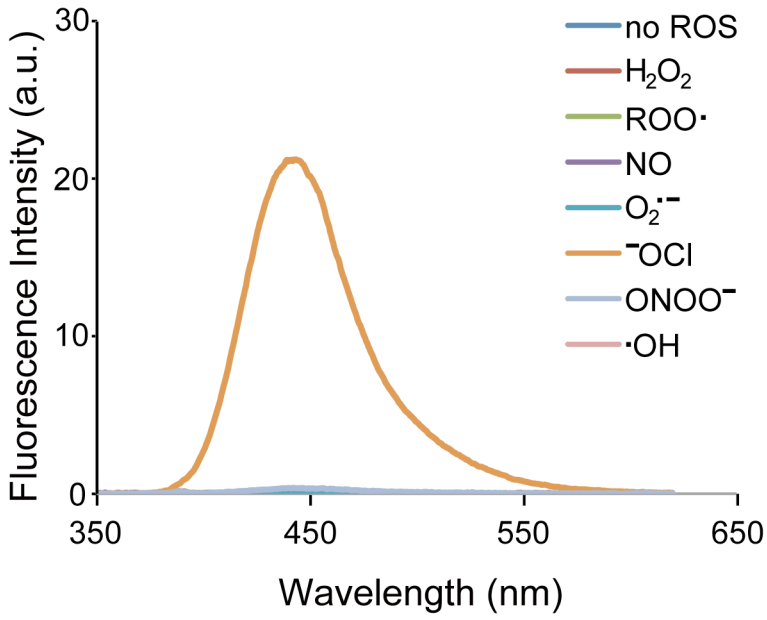


Fig. S3 Typical fluorescence spectra of **HCm3** (10 μM) with various ROS and RNS (30 μM).

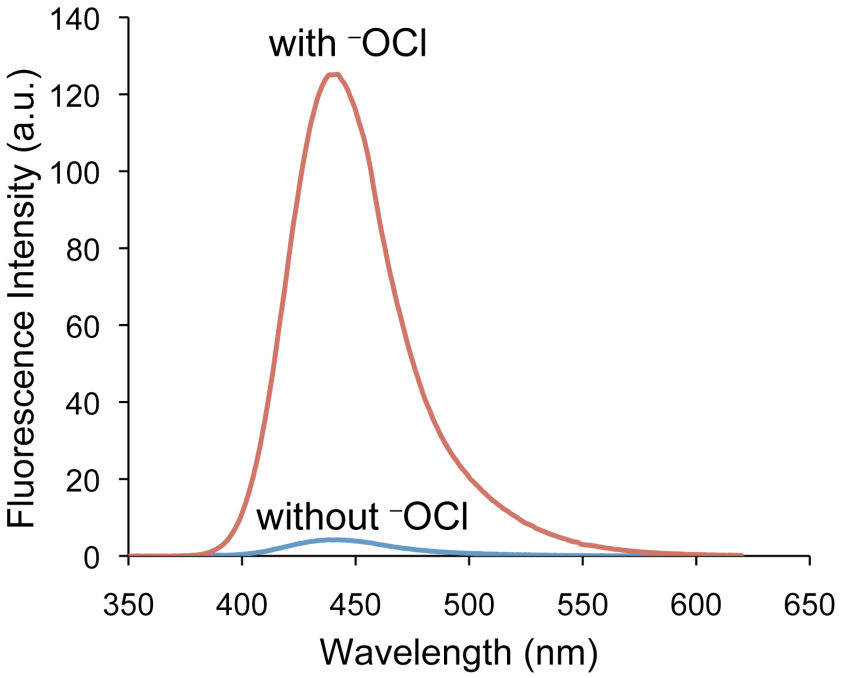


Fig. S4 Fluorescence spectra (excitation at 342 nm) of **HCm3-$^{13}C_2$** (100 μM) solutions with (red line) or without (blue line) ⁻OCl (180 μM) (Fig. 3b).

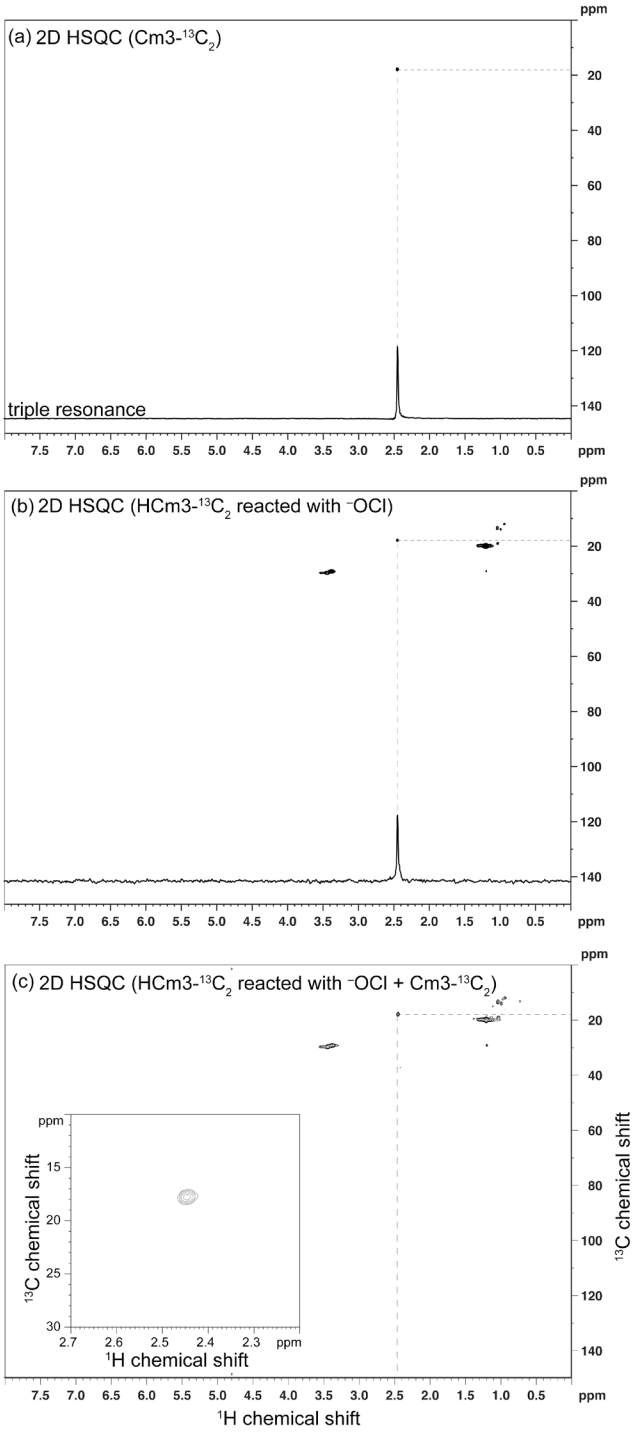


Fig. S5 (a) 2D ^{1}H-^{13}C HSQC spectrum of **Cm3-$^{13}C_2$** in D_2O. The 2D HSQC spectrum is merged with the 1D ^{1}H-{^{13}C-^{13}C$'^{(sp2)}$} triple-resonance spectrum. (b) 2D ^{1}H-^{13}C HSQC spectrum of **HCm3-$^{13}C_2$** reacted with $^{-}$OCl. After reaction of **HCm3-$^{13}C_2$** with $^{-}$OCl (Fig. 3b), the sample was lyophilized, redissolved in D_2O, and subjected to 2D HSQC analysis. The 2D HSQC spectrum is merged with the 1D ^{1}H-{^{13}C-^{13}C$'^{(sp2)}$} triple-resonance spectrum. (c) is ^{1}H-^{13}C HSQC spectrum of $^{-}$OCl-reacted **HCm3-$^{13}C_2$** measured after addition of **Cm3-$^{13}C_2$** as an internal authentic sample, suggesting that the appeared peak (approximately 2.45 ppm for ^{1}H and 18 ppm for ^{13}C) was derived from coumarin scaffold.

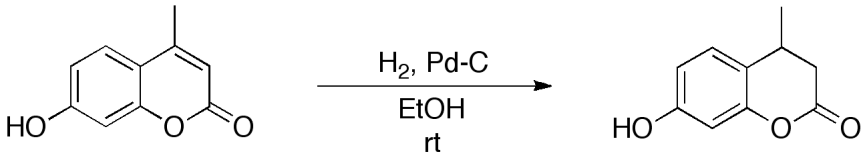
2. Methods

2.1. Synthesis

General

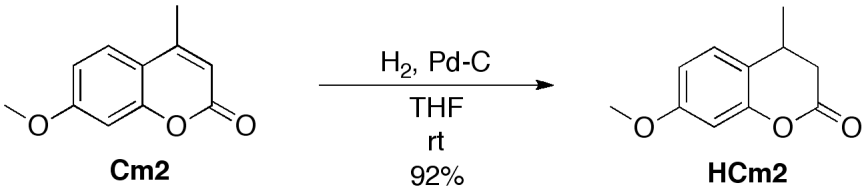
Reagents and solvents were purchased from standard suppliers and were used without further purification. ^{1}H NMR (7.24 ppm for $CDCl_3$ as internal standard) and ^{13}C NMR (39.5 ppm for DMSO-d_6 as internal standard) spectra were acquired using a JEOL LNM-EX270 spectrometer (300 MHz for ^{1}H NMR) at ambient temperature. Mass spectra (MS) were measured using a JEOL JMS-HX110A (FAB).

7-Hydroxy-4-methyl-3,4-dihydrocoumarin (HCm1)



4-Methylumbeliferone (1.00 g, 5.68 mmol) in ethanol (20 mL) was stirred under hydrogen at room temperature for 13 h in the presence of 10% palladium on activated carbon (850 mg). The reaction mixture was filtered through celite, and the filtrate was evaporated to dryness. The resulting residue was purified by silica-gel column chromatography (chloroform) to produce **HCm1** as a white solid (149 mg, 15%). ^{1}H NMR ($CDCl_3$): δ. 7.06 (d, J = 8.1 Hz, 1H), 6.60 (dd, J = 8.1 and 2.6 Hz, 1H), 6.56 (d, J = 2.6 Hz, 1H), 4.96 (s, 1H), 3.15-3.04 (m, 1H), 2.81 (dd, J = 15.8 and 5.3 Hz, 1H), 2.53 (dd, J = 15.8 and 7.5 Hz, 1H), 1.29 (d, J = 7.0 Hz, 3H). ^{13}C NMR (DMSO-d_6): δ. 168.2, 157.1, 151.5, 127.3, 118.3, 111.3, 103.1, 36.5, 27.9, 19.9. HRMS (FAB^{+}): calculated for $C_{10}H_{11}O_3$ (M^{+}) = 179.0708, observed = 179.0714.

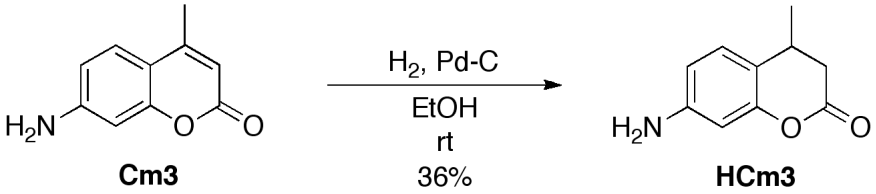
7-Methoxy-4-methyl-3,4-dihydrocoumarin (HCm2)



7-Methoxy-4-methylcoumarin (750 mg, 3.94 mmol) in tetrahydrofuran (40 mL) was stirred under hydrogen at room temperature for 2.5 days in the presence of 10% palladium on activated carbon (310 mg). The solution was filtered through celite, and the filtrate was evaporated to dryness. The resulting residue was purified by silica gel column chromatography (ethyl acetate/*n*-hexane = 1/5) to produce **HCm2** as a colorless oil (698 mg, 92%). ^{1}H NMR ($CDCl_3$): δ. 7.10 (d, J = 8.4 Hz, 1H), 6.66 (dd, J = 8.4 and 2.4 Hz, 1H), 6.60 (d, J = 2.4 Hz, 1H), 3.77 (s, 3H), 3.16-3.05 (m, 1H), 2.81 (dd, J = 15.8 and 5.3 Hz,

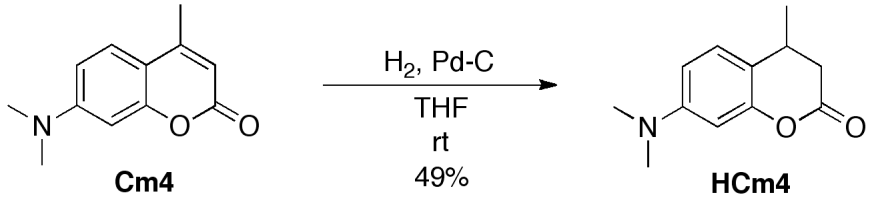
1H), 2.53 (dd, *J* = 15.8 and 7.5 Hz, 1H), 1.29 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (DMSO-d_6): *δ*. 168.1, 159.1, 151.6, 127.4, 120.1, 110.3, 102.1, 55.4, 36.3, 27.9, 19.9. HRMS (FAB$^+$): calculated for $C_{11}H_{13}O_3$ (M^+) = 193.0865, observed = 193.0860.

7-Amino-4-methyl-3,4-dihydrocoumarin (HCm3)



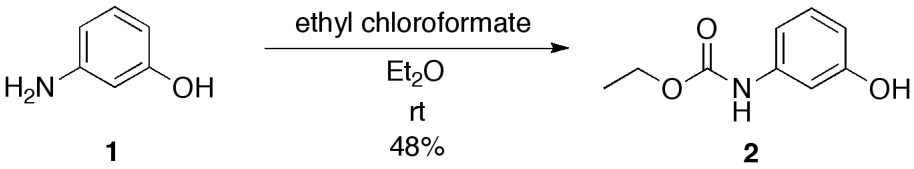
7-Amino-4-methylcoumarin (780 mg, 4.45 mmol) in ethanol (15 mL) was stirred under hydrogen at room temperature for 29 h in the presence of 10% palladium on activated carbon (220 mg). The solution was filtered through celite, and the filtrate was evaporated to dryness. The resulting residue was purified by silica gel column chromatography (chloroform/*n*-hexane = 1/2) to give **HCm3** as a pale yellow solid (286 mg, 36%). ^{1}H NMR ($CDCl_3$): *δ*. 6.98 (d, *J* = 8.1 Hz, 1H), 6.48 (dd, *J* = 8.1 and 2.4 Hz, 1H), 6.42 (d, *J* = 2.4 Hz, 1H), 3.09-3.03 (m, 1H), 2.79 (dd, *J* = 15.8 and 5.5 Hz, 1H), 2.50 (dd, *J* = 15.8 and 7.5 Hz, 1H), 1.27 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (DMSO-d_6): *δ*. 168.5, 151.5, 148.8, 126.8, 114.7, 110.1, 101.1, 36.9, 27.8, 20.1. HRMS (FAB$^+$): calculated for $C_{10}H_{11}O_2N$ (M^+) 177.0790, observed = 177.0786.

7-*N*,*N*-dimethylamino-4-methyl-3,4-dihydrocoumarin (HCm4)



7-*N*,*N*-Dimethylamino-4-methylcoumarin (365 mg, 1.80 mmol) in tetrahydrofuran (15 mL) was stirred under hydrogen at room temperature for 3 days in the presence of 10% palladium on activated carbon (65 mg). The solution was filtered through celite, and the filtrate was evaporated to dryness. The resulting residue was purified by silica gel column chromatography (chloroform/*n*-hexane = 1/5) to give **HCm4** as a colorless oil (180 mg, 49%). ^{1}H NMR ($CDCl_3$): *δ*. 7.04 (d, *J* = 8.4 Hz, 1H), 6.47 (dd, *J* = 8.4 and 2.6 Hz, 1H), 6.39 (d, *J* = 2.6 Hz, 1H), 3.10-3.04 (m, 1H), 2.92 (s, 6H), 2.79 (dd, *J* = 15.6 and 5.3 Hz, 1H), 2.50 (dd, *J* = 15.6 and 7.7 Hz, 1H), 1.27 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (DMSO-d_6): *δ*. 168.5, 151.6, 150.5, 126.9, 115.2, 108.5, 100.0, 40.1, 36.8, 27.7, 20.0. HRMS (FAB$^+$): calculated for $C_{12}H_{16}O_2N$ (M^+) = 206.1181, observed = 206.1179.

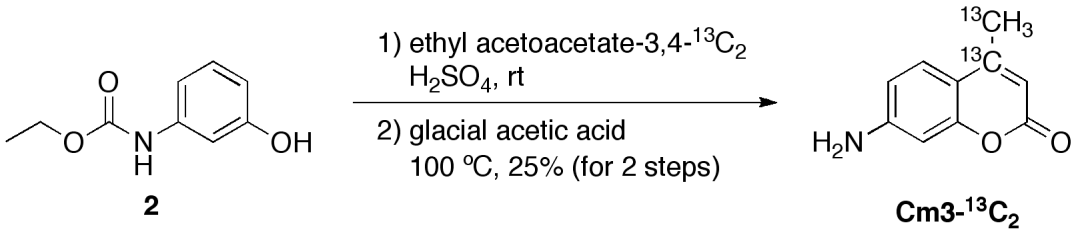
Ethyl (3-hydroxyphenyl)carbamate (2)



1 **2**

Ethyl chloroformate (5.97 g, 55.0 mmol) was added to a solution of 3-aminophenol (5.00 g, 45.8 mmol) in diethyl ether (200 mL) and stirred for 14 h at room temperature. The reaction mixture was filtered, and the filtrate was evaporated to dryness. The resulting residue was washed with *n*-hexane and dried under reduced pressure to produce **2** as a pale yellow crystal (4.00 g, 48%). ^{1}H NMR ($CDCl_3$): δ. 7.22 (brs, 1H), 7.12 (t, J = 8.1 Hz, 1H), 6.67 (dd, J = 8.1 and 2.0 Hz, 1H), 6.58 (brs, 1H), 6.55-6.52 (m, 1H), 5.68 (brs, 1H), 4.21 (q, J = 7.0 Hz, 2H), 1.29 (t, J = 7.0 Hz, 3H). ^{13}C NMR (DMSO-d_6): δ. 157.7, 153.4, 140.3, 129.3, 109.4, 109.0, 105.3, 60.0, 14.5. HRMS (FAB+): calculated for $C_9H_{12}O_3N$ (M^+) = 182.0817, observed = 182.0820.

7-Amino-4-methylcoumarin-$^{13}C_2$ (Cm3-$^{13}C_2$)

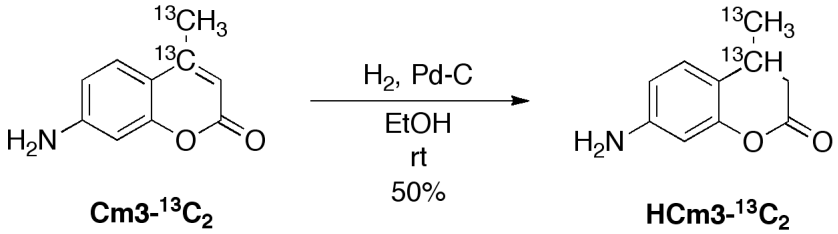


2 **Cm3-$^{13}C_2$**

Ethyl acetoacetate-3,4-$^{13}C_2$ (99% ^{13}C; Isotec Inc.) (425 mg, 3.22 mmol) was added to a solution of **2** (630 mg, 3.48 mmol) in sulfuric acid (2.1 mL) with stirring at room temperature. After stirring for 19 h at room temperature, glacial acetic acid (2.0 mL) was added at room temperature. After stirring for an additional 21 h at 100 °C, the solution was poured into ice-cold water (125 mL). The resulting suspension was made slightly basic (pH 10) with saturated aqueous sodium hydroxide under cooling with ice chips, and the precipitate was separated from the filtrate. The precipitate was dissolved in ethyl alcohol and dried over magnesium sulfate, filtered and evaporated to dryness. The filtrate was extracted with ethyl acetate. The organic phase was dried over magnesium sulfate, filtered, and evaporated to dryness. These residues were combined and purified using silica gel column chromatography (chloroform and then chloroform/methanol = 40/1) to produce **Cm3-$^{13}C_2$** as a pale yellow solid (145 mg, 25%). ^{1}H NMR ($CDCl_3$): δ. 7.35 (dd, J = 8.4 and 3.9 Hz, 1H), 6.55 (dd, J = 8.4 and 2.2 Hz, 1H), 6.53 (d, J = 2.2 Hz, 1H), 6.00 (dd, J = 5.7 and approx. 1Hz, 1H), 4.10 (brs, 2H), 2.33 (ddd, J = 128, 6.2 and approx. 1Hz, 3H). ^{13}C NMR (DMSO-d_6): δ. 160.8, 155.5, 153.8 (d, J = 41.6 Hz), 153.1, 126.2, 111.2,

108.8 (d, J = 53.9 Hz), 107.5 (d, J = 62.0 Hz), 98.5, 18.0 (d, J = 41.6 Hz). HRMS (FAB$^+$): calculated for $^{12}C_8{}^{13}C_2H_9O_2N$ (M^+) = 177.0700, observed = 177.0701.

7-Amino-4-methyl-2,3-dihydrocoumarin-$^{13}C_2$ (HCm3-$^{13}C_2$)



Cm3-$^{13}C_2$ **HCm3-$^{13}C_2$**

Cm3-$^{13}C_2$ (80.7 mg, 0.455 mmol) in ethanol (30 mL) was stirred under hydrogen at room temperature for 28 h in the presence of 10% palladium on activated carbon (150 mg). The solution was filtered through celite, and the filtrate was evaporated to dryness. The resulting residue was purified by silica gel column chromatography and eluted with chloroform to give a pale yellow solid, which was further purified using gel permeation chromatography (methanol) to produce **HCm3-$^{13}C_2$** as a pale yellow solid (40.6 mg, 50%). ^{1}H NMR ($CDCl_3$): δ. 6.97 (dd, J = 8.2 and 3.7 Hz, 1H), 6.42 (dd, J = 8.2 and 2.0 Hz, 1H), 6.36 (d, J = 2.0 Hz, 1H), 3.33-3.25 (m, 0.5H), 2.84-2.73 (m, 0.5H + 1H), 2.55-2.45 (m, 1H), 1.26 (ddd, J = 127, 6.8 and 4.0 Hz, 3H). ^{13}C NMR (DMSO-d_6): δ. 168.7, 151.6, 148.9, 126.9, 114.7 (d, J = 43.8 Hz), 110.1, 101.1, 36.9 (d, J = 31.7 Hz), 27.9 (d, J = 34.7 Hz), 20.1 (d, J = 34.7 Hz). HRMS (FAB$^+$): calculated for $^{12}C_8{}^{13}C_2H_{11}O_2N$ (M^+) = 179.0857, observed = 179.0853.

2.2. Fluorescence measurements

General

Fluorescence spectra were measured at 37 °C using a JASCO FP-6500 fluorescence spectrometer (slit widths were 1 nm for excitation and 3 nm for emission) with a 1 cm cuvette. **HCm1–4** were dissolved in DMF and used as stock solutions.

Reaction with $^{-}$OCl

Aqueous NaOCl solution was prepared by dilution of NaOCl with a 100 mM aqueous NaOH solution, and the concentration of $^{-}$OCl was determined based on the molar extinction coefficient at 292 nm (350 M^{-1} cm^{-1}).

Fig. S1: Aqueous NaOCl solution (final concentration, 10 μM) was added to a solution of **HCm1–4** (10 μM) in phosphate buffer (pH 7.4, 100 mM) containing 150 mM NaCl, 0.1% DMF, and fluorescence spectra were measured (excitation at 367 nm for **HCm1**, excitation at 320 nm for **HCm2**, excitation at 342 nm for **HCm3**, and excitation at 371 nm for **HCm4**) after 5 min of incubation at 37 ºC.

Fig. S4: Aqueous NaOCl solution (final concentration, 180 μM) was added to a solution of **HCm3-$^{13}C_2$** (100 μM) in phosphate buffer (pH 7.4, 1 mM) containing 0.1% DMF, and fluorescence spectra were measured (excitation at 342 nm).

Reactions with various ROS and RNS

Each ROS and RNS was generated according to refs 1 and 2.

(a) no ROS

HCm3 (final concentration, 10 μM) was incubated in 100 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl, 0.1% DMF, and the fluorescence spectrum of the solution was measured after 30 min of incubation at 37 ºC.

(b) H_2O_2

Aqueous H_2O_2 solution (final concentration, 30 μM) was added to a solution of 10 μM **HCm3** in 100 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl, 0.1% DMF, and the fluorescence spectrum of the solution was measured after 30 min of incubation at 37 ºC. The concentration of H_2O_2 was determined based on the molar extinction coefficient at 240 nm (43.6 M^{-1} cm^{-1}).

(c) $O_2\cdot^{-}$

Solid potassium superoxide (final concentration, 30 μM) was added to a solution of 10 μM **HCm3** in 100 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl, 0.1% DMF, and the fluorescence spectrum of the solution was measured after 30 min of incubation at 37 ºC.

(d) ROO·

Aqueous 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) solution (final concentration, 30 μM) was added to a solution of 10 μM **HCm3** in 100 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl, 0.1% DMF, and the fluorescence spectrum of the solution was measured after 30 min of incubation at 37 ºC.

(e) NO

Aqueous NaOCl solution was prepared by dilution of 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1-triazene (NOC7) with a 100 mM aqueous NaOH solution. The aqueous NOC7 solution (final concentration, 30 μM) was added to a solution of 10 μM **HCm3** in 100 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl, 0.1% DMF, and the fluorescence spectrum of the solution was measured after 30 min of incubation at 37 ºC.

(f) $^{-}$OCl

Aqueous NaOCl solution was prepared by dilution of NaOCl with a 100 mM aqueous NaOH solution, and the concentration of $^{-}$OCl was determined based on the molar extinction coefficient at 292 nm (350 M^{-1} cm^{-1}). Aqueous NaOCl solution (final concentration, 30 μM) was added to a solution of 10 μM **HCm3** in 100 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl, 0.1% DMF, and the fluorescence spectrum of the solution was measured after 5 min of incubation at 37 ºC.

(g) $ONOO^{-}$

Aqueous $ONOO^{-}$ solution was prepared by dilution of $ONOO^{-}$ with a 100 mM aqueous NaOH solution, and the concentration of $ONOO^{-}$ was determined based on the molar extinction coefficient at 302 nm (1670 M^{-1} cm^{-1}). Aqueous NaONOO solution (final concentration, 30 μM) was added to a solution of 10 μM **HCm3** in 100 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl, 0.1% DMF, and the fluorescence spectrum of the solution was measured after 5 min of incubation at 37 ºC.

(h) ·OH

Aqueous $Fe(ClO_4)_2$ solution (final concentration, 30 μM) was added to a solution of 10 μM **HCm3** and 0.3 mM H_2O_2 in 100 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl, 0.1% DMF, and the fluorescence spectrum of the solution was measured after 5 min of incubation at 37 ºC.

Reaction with human myeloperoxidase

Human myeloperoxidase (final concentration, 30 nM) was added to **HCm3-$^{13}C_2$** solution (100 μM) in 1 mM sodium phosphate buffer (pH 6.0) containing 5 mM NaCl, 0.1% DMF, and H_2O_2 (final concentration, 3 μM). During incubation for 3 h at 37 °C, H_2O_2 solution was added to the reaction mixture every hour (final concentrations, 6 μM and 9 μM after the first and second additions, respectively), and the fluorescence spectrum of the reaction solution was measured.

2.3. HPLC analyses

General

HPLC analyses were performed on a wakosil 5C18 (4.6 mm × 150 mm) column (Wako) using a HPLC system composed of a pump (LC-20AT, Shimadzu) and a detector (fluorescence: RF-10A XL or UV: SPD-M20A, Shimadzu). Elution was done with eluent A (0.1 M acetic acid/triethylamine buffer (pH 7.0)) and eluent B (CH_3CN).

Reaction with various ROS

Samples were prepared as described in the experimental procedure of fluorescence measurements. They were analyzed using HPLC with a linear gradient elution A/B = from 100/0 to 40/60 in 30 min, and then to 0/100 in 10 min at a flow rate of 1.0 mL/min. Fluorescence was monitored at 442 nm (ex. 342 nm) and UV was monitored at 288 nm (λ_{max} of **HCm3**).

2.4. NMR measurements

General

NMR spectra were acquired at 298 K using a Bruker Avance III 600 spectrometer equipped with a 5 mm TCI CryoProbe. All one-dimensional triple-resonance spectra were obtained using a 3D HCACO pulse sequence,[3] which is commonly used to assign backbone chemical shifts of proteins, with a slight modification for the one-dimensional proton experiments.[4]

Reaction with $^{-}$OCl

Aqueous NaOCl solution was prepared by dilution of NaOCl with a 100 mM aqueous NaOH solution, and the concentration of $^{-}$OCl was determined based on the molar extinction coefficient at 292 nm (350 M^{-1} cm^{-1}). Aqueous NaOCl solution (final concentration, 180 μM) was added to a solution of 100 μM **HCm3-$^{13}C_2$** in sodium phosphate buffer (pH 7.4, 1mM) containing 0.1% DMF. An aliquot of the solution (495 μL) was mixed with D_2O (55 μL) and subjected to NMR analysis (1024 scans).

Reaction with $^{-}$OCl in crude tissue extract

Pork muscle (5 g) was homogenized in an aqueous solution of 200 mM trichloroacetic acid (TCA) using a Qiagen TissueLyser II. The homogenates were centrifuged at 1000 rpm for a period of 3 min at room temperature, and the supernatant was collected after filtration. The aqueous extract was lyophilized to dryness and dissolved in distilled water (30 mL). The pH of the aqueous extract was adjusted with 10 M aqueous sodium hydroxide to pH 5.5. Aqueous NaOCl (final concentration, 800 μM) at room temperature was added to a solution of the aqueous extract containing **HCm3-$^{13}C_2$** (final concentration 100 μM) of and DMF (final 0.1%). An aliquot of the solution (495 μL) was mixed with D_2O (55 μL) and subjected to NMR analysis (2048 scans).

Reaction with human myeloperoxidase

Human myeloperoxidase (final concentration, 30 nM) was added to a solution of **HCm3-$^{13}C_2$** (100 μM) in sodium phosphate buffer (pH 6.0, 1 mM) containing 5 mM NaCl, 0.1% DMF, and H_2O_2 (final concentration, 3 μM). During incubation for 3 h at 37 °C, H_2O_2 was added to the reaction mixture every hour (final concentration, 6 μM and 9 μM after the first and second additions, respectively), and an aliquot of the solution (495 μL) was mixed with D_2O (55 μM) and subjected to NMR analysis (4096 scans).

3. References

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