Electronic Supplementary Information (ESI)

Activatable fluorescence probes for hydrolase enzymes based on coumarin-hemicyanine hybrid fluorophore with large Stokes shift

Hiroyoshi Fujioka[a,†], Shin-nosuke Uno[a,b,†], Mako Kamiya[c], Ryosuke Kojima[c], Kai Johnsson[*,b,d] and Yasuteru Urano[*,a,c,e]

[a] Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. E-mail: uranokun@m.u-tokyo.ac.jp

[b] Institute of Chemical Sciences and Engineering (ISIC), École Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland.

[c] Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.

[d] Department of Chemical Biology, Max Planck Institute for Medical Research, Jahnstrasse 29, 69120 Heidelberg, Germany.

[e] CREST (Japan) Agency for Medical Research and Development (AMED), 1-7-1 Otemachi, Chiyoda-ku, Tokyo 100-0004, Japan

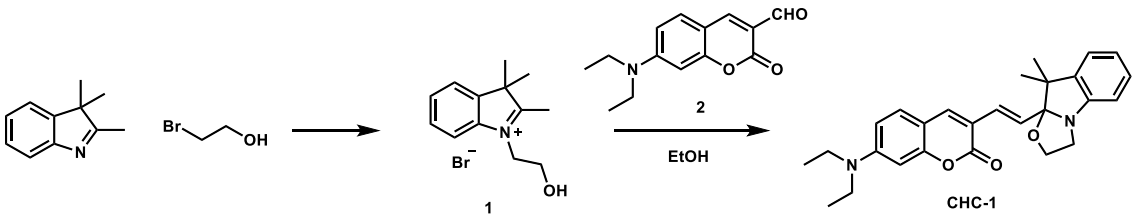
[†] These authors contributed equally to this work.

*Correspondence to: johnsson@mr.mpg.de, uranokun@m.u-tokyo.ac.jp

Materials and general information

General chemicals were purchased from commercial suppliers (Sigma-Aldrich, Fluka, Acros, Tokyo Chemical Industries (TCI)) and were used without further purification. γ-Glutamyltransferase from beef kidney was purchased from Wako (46556003), and esterase from porcine liver was purchased from SIGMA (E3019-3.5KU). The composition of mixed solvents is given as volume ratio (v/v). Flash chromatography was performed with silica gel (230-400 mesh) or using an EPCLC-AI-580S chromatograph (Yamazen, Osaka, Japan). Reverse-phase preparative high-pressure liquid chromatography was performed on a Dionex system equipped with an an UltiMate 3000 pump, an UVD 170U UV-Vis detector, and a Waters SunFire™ Prep C18 OBD™ 5 µm 19 × 150 mm Column, or on an HPLC system equipped with a pump (Jasco PU-2080 or PU-2087), a detector (Jasco MD-2010 or MD-2018), and Inertsil ODS-3 20 mm × 250 mm column (GL Sciences, Tokyo, Japan). Buffer A: 0.1% v/v TFA in H_2O, Buffer B: acetonitrile. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCEIII 400 (400 MHz for ^1H and 101 MHz for ^{13}C) with chemical shifts (δ) reported in ppm relative to the residual solvent signals of $CDCl_3$ (7.26 ppm for ^1H, 77.16 ppm for ^{13}C), CD_3OD (3.31 ppm for ^1H, 49.00 ppm for ^{13}C), or acetone-*d*6 (2.05 ppm for ^1H, 29.84 ppm for ^{13}C). Coupling constants are reported in Hz. High resolution mass spectra (HRMS) were measured on a 6530 Accurate Q-TOF LC/MS spectrometer or a Bruker micrOTOFII with electrospray ionization (ESI).

Synthetic procedures



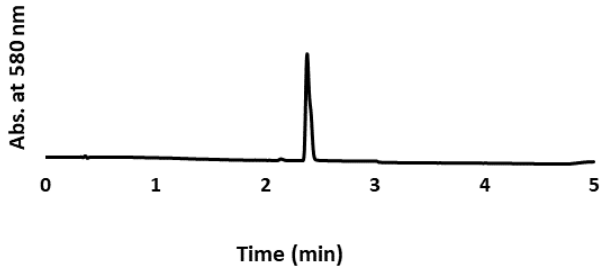
Scheme S1. Synthesis of **CHC-1**.

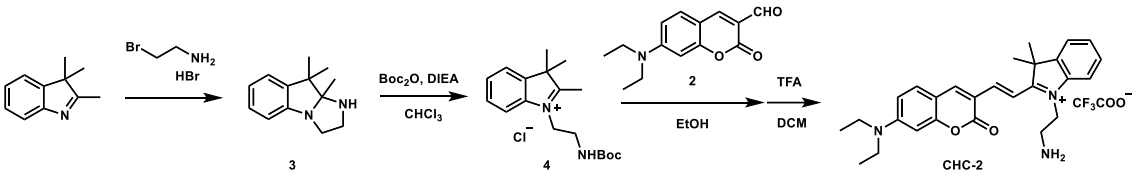
Compound 1:

The mixture of 2,3,3-Trimethylindolenine (3.0 g, 27.7 mmol) and 2-bromoethanol (39.3 g, 31.5 mmol) was stirred at 80 °C under an argon atmosphere for 16 h. The reaction mixture was cooled to room temperature, and poured into Et_2O. Solid was filtered and washed with Et_2O and $CHCl_3$ to give **Compound 1** (3.10 g, 39%).^1H NMR (400 MHz, DMSO-d_6) δ 8.00 – 7.93 (m, 1H), 7.86 (m, 1H), 7.63 (m, 2H), 4.61 (t, *J* = 5.1 Hz, 2H), 3.88 (t, *J* = 5.1 Hz, 2H), 2.83 (s, 3H), 1.56 (s, 6H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 197.7, 141.8, 141.1, 129.3, 128.8, 123.5, 115.6, 57.8, 54.3, 50.3, 22.0, 14.5.; HRMS *(m/z)*: $[M+H]^+$ calcd. for $C_{13}H_{18}NO$, 204.1383; found, 204.1387.

CHC-1:

The solution of **Compound 2**[1)] (31.0 mg, 0.13 mmol) and **Compound 1** (43.1 mg, 0.15 mmol) in EtOH (5 mL) was refluxed with stirring under an argon atmosphere for 17 h. Solvent was evaporated and the residue was purified by flash column chromatography (silica gel, DCM/MeOH = 100/0 to 90/10) to give a green solid (51.2 mg) as a mixture of open and closed forms. The solid was dissolved in $CHCl_3$ and washed with 1 N NaOH aq. and brine, dried over $MgSO_4$. The solvent was evaporated and the residue was purified by flash column chromatography (silica gel, DCM/MeOH = 100/0 to 90/10) to give **CHC-1** (27.5 mg, 43%) as a yellow solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.43 (s, 1H), 8.22 (d, *J* = 15.8 Hz, 1H), 7.97 (d, *J* = 15.8 Hz, 1H), 7.62 – 7.65 (m, 2H), 7.45 - 7.52 (m, 3H), 6.82 (dd, *J* = 9.4, 2.4 Hz, 1H), 6.55 (d, *J* = 2.3 Hz, 1H), 4.55 (t, *J* = 5.0 Hz, 2H), 3.98 (t, *J* = 5.3 Hz, 2H), 3.52 (q, *J* = 7.1 Hz, 4H), 1.75 (s, 6H), 1.18 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR (101 MHz, CD_3CN) δ 161.8, 156.8, 152.0, 151.8, 141.0, 140.7, 130.2, 128.5, 128.2, 127.7, 123.3, 122.4, 117.1, 113.0, 110.9, 110.2, 109.5, 97.5, 64.2, 50.7, 48.5, 45.4, 28.7, 20.6, 12.8; HRMS (*m/z*): $[M]^+$ calcd. for $C_{27}H_{31}N_2O_3$, 431.2335; found, 431.2335.





Scheme S2. Synthesis of **CHC-2**.

Compound 3:

The mixture of 2,3,3-Trimethylindolenine (2.0 g, 18.5 mmol) and 2-bromoethylamine hydrobromide (3.8 g, 18.5 mmol) was stirred at 120 °C under an argon atmosphere for 14 h. The resulting solid was washed with $CHCl_3$, MeOH, and acetone, and dissolved in DCM and washed with sat. $NaHCO_3$ aq. and brine, and dried over $MgSO_4$. The solvent was evaporated and the residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 60/40 to 20/80, and then DCM/MeOH = 100/0 to 90/10) to give **Compound 3** (1.17 g, 31%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.13 (t, *J* = 7.7 Hz,

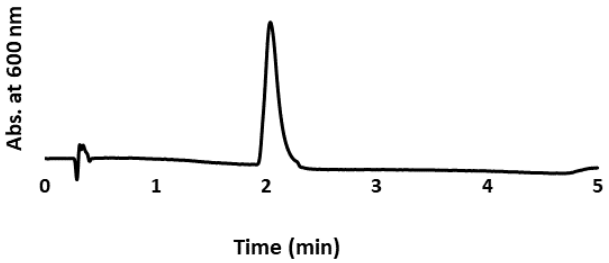
1H), 7.08 (d, *J* = 7.3 Hz, 1H), 6.87 (t, *J* = 7.4 Hz, 1H), 6.66 (d, *J* = 7.8 Hz, 1H), 3.48 - 3.36 (m, 2H), 3.04 (m, 2H), 1.97 (s, 1H), 1.39 (s, 3H), 1.31 (s, 3H), 1.20 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.1, 138.6, 128.0, 122.9, 120.9, 112.5, 94.0, 49.9, 45.6, 44.6, 28.1, 21.0, 17.1.

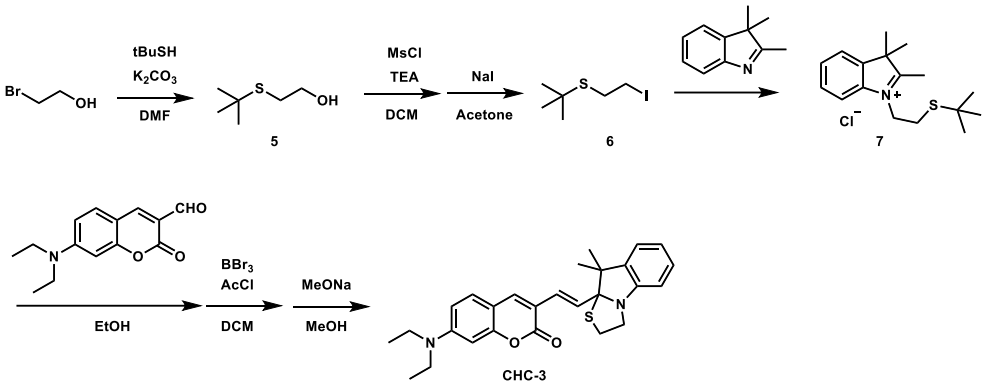
Compound 4:

The mixture of **Compound 3** (276 mg, 0.97 mmol), $(Boc)_2O$ (255 mg, 1.17 mmol), and DIEA (0.25 mL, 1.46 mmol) in $CHCl_3$ (5 mL) was stirred at room temperature under an argon atmosphere for 14 h. The mixture was diluted with DCM, washed with sat. $NaHCO_3$ aq. and brine, and dried over $MgSO_4$. The solvent was evaporated and the residue was purified by flash column chromatography (silica gel, DCM/MeOH = 100/0 to 90/10) to give **Compound 4** (437 mg) as pink oil containing impurities, which was used in next step without further purification.

CHC-2:

The solution of **Compound 2**[1] (50.0 mg, 0.20 mmol) and **Compound 4** (325 mg, 0.96 mmol) in EtOH (5 mL) was refluxed with stirring under an argon atmosphere for 20 h. Solvent was evaporated and the residue was purified by flash column chromatography (silica gel, DCM/MeOH = 100/0 to 90/10) to give the intermediate (19.4 mg). The intermediate was dissolved in DCM (2 mL) and TFA (1 mL) and the solution was stirred at room temperature for 1 h. The solvent was evaporated and the residue was purified by semi-preparative HPLC using eluent A (H_2O with 0.1% TFA) and eluent B (CH_3CN) (A/B = 90/10 to 10/90 for 40 min) to give **CHC-2** (5.7 mg, 14%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.53 (s, 1H), 8.34 (d, *J* = 15.4 Hz, 1H), 7.85 (d, *J* = 15.4 Hz, 1H), 7.62 – 7.66 (m, 2H), 7.46 - 7.55 (m, 3H), 6.86 (dd, *J* = 9.2, 2.4 Hz, 1H), 6.58 (d, *J* = 2.2 Hz, 1H), 4.67 (t, *J* = 7.4 Hz, 2H), 3.55 (q, *J* = 7.1 Hz, 4H), 3.44 (t, *J* = 7.5 Hz, 2H), 1.77 (s, 6H), 1.19 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR (101 MHz, CD_3OD) δ 161.6, 159.9, 156.7, 153.9, 151.9, 144.4, 142.3, 134.3, 130.5, 130.0, 124.2, 114.4, 113.7, 113.1, 112.0, 109.7, 98.0, 53.3, 46.7, 44.2, 40.4, 37.6, 27.4, 12.8; HRMS (*m/z*): $[M]^+$ calcd. for $C_{27}H_{32}N_3O_2$, 430.2495; found, 430.2485.





Scheme S3. Synthesis of **CHC-3**.

Compound 5:

The mixture of 2-bromoethanol (10 g, 80 mmol), 2-methyl-2-propanethiol (10.8 mL, 96 mmol), and K_2CO_3 (16.6 g, 120 mmol) in DMF (40 mL) was stirred at 80 °C under an argon atmosphere for 17 h. The reaction mixture was diluted with DCM, washed with water and brine, and dried over $MgSO_4$. The solvent was evaporated and the residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 100/0 to 40/60) to give **Compound 5** (9.20 g, 86%) as pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.70 (t, *J* = 6.2 Hz, 2H), 2.74 (t, *J* = 6.2 Hz, 2H), 2.33 (s, 1H), 1.31 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 61.5, 42.5, 31.9, 31.2.

Compound 6:

To a solution of **Compound 5** (3.0 g, 22.3 mmol), triethylamine (3.74 mL, 26.8 mmol) in DCM (100 mL), MsCl (2.1 mL, 26.8 mmol) was added dropwise at 0 °C. The reaction mixture was stirred at 0 °C under an argon atmosphere for 1 h. The reaction mixture was diluted with DCM, washed with sat. $NaHCO_3$ aq. and brine, and dried over $MgSO_4$. The solvent was evaporated, and acetone (20 mL) and NaI (27.1 g, 181 mmol) were added to the residue and the mixture was refluxed with stirring for 1 h. The reaction mixture was diluted with DCM, washed with water and brine, and dried over $MgSO_4$. The solvent was evaporated and the residue was purified by flash column chromatography (silica gel, *n*-hexane) to give **Compound 6** (2.98 g, 55%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.35 – 3.16 (m, 2H), 3.07 – 2.89 (m, 2H), 1.32 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 43.6, 32.0, 31.3, 4.1; HRMS (*m/z*): $[M+H]^+$ calcd. for $C_6H_{13}IS$, 244.9861; found, 244.9855.

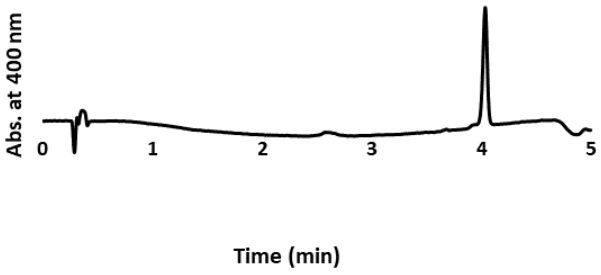
Compound 7:

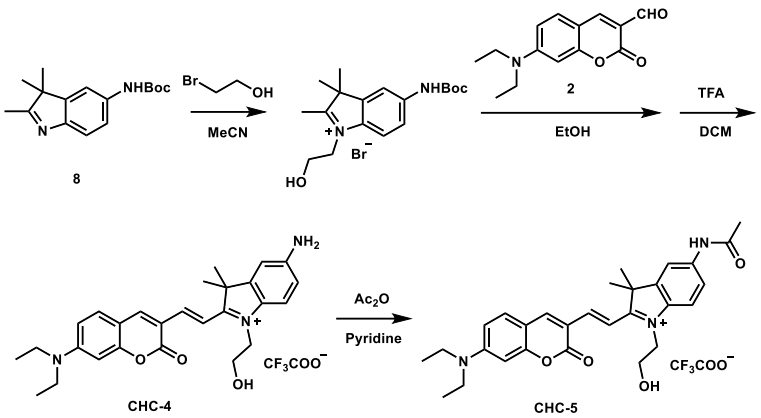
The mixture of 2,3,3-Trimethylindolenine (216 mg, 1.36 mmol) and **Compound 6** (331 mg, 1.36 mmol) was stirred at 120 °C under an argon atmosphere for 16 h. The reaction mixture was cooled to room temperature, and poured into Et_2O. Solid was filtered and washed with Et_2O and $CHCl_3$ to

give **Compound 7** (324 mg) containing impurities, which was used in next step without further purification.

CHC-3:

The solution of **Compound 2**[1] (50.0 mg, 0.20 mmol) and **Compound 7** (324 mg) in EtOH (5 mL) was refluxed with stirring under an argon atmosphere for 3 h. Solvent was evaporated and the residue was purified by flash column chromatography (silica gel, DCM/MeOH = 100/0 to 90/10) to give the crude intermediate (151.3 mg). To the solution of the intermediate in DCM (2 mL), AcCl (3.0 mL) and BBr_3 (1.0 mL) was added at 0 °C, and the solution was stirred at 0 °C for 1 h. The reaction mixture was poured into cold sat. $NaHCO_3$ aq., and extracted with DCM, washed with brine, dried over $MgSO_4$, and the solvent was evaporated. To the solution of the residue in dry MeOH (5 mL), NaOMe (85 mg) was added at 0 °C. The solution was stirred at 0 °C under an argon atmosphere for 20 min. The reaction mixture was diluted with DCM and washed with brine, dried over $MgSO_4$. The solvent was evaporated and the residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 100/0 to 40/60) to give **CHC-3** (24.7 mg, 17%) as an orange solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (s, 1H), 7.25 – 7.27 (m, 2H), 7.11 – 7.17 (m, 2H), 6.96 (t, *J* = 7.4, 1H), 6.66 – 6.80 (m, 3H), 6.58 (dd, *J* = 8.9, 2.4 Hz, 1H), 6.50 (d, *J* = 2.4 Hz, 1H), 4.14 – 4.19 (m, 1H), 3.46 – 3.52 (m, 1H), 3.42 (q, *J* = 7.0 Hz, 4H), 2.90-2.94 (m, 1H), 2.67-2.73 (m, 1H), 1.61 (s, 3H), 1.22 (t, *J* = 7.1 Hz, 6H), 1.15 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 161.4, 155.8, 150.5, 148.9, 139.7, 138.7, 131.1, 128.8, 127.4, 123.2, 122.6, 121.8, 117.3, 110.7, 109.1, 109.0, 99.8, 97.3, 53.0, 48.7, 45.0, 31.8, 29.2, 24.2, 12.6; HRMS (*m/z*): $[M+H]^+$ calcd. for $C_{27}H_{31}N_2O_2S$, 447.2101; found, 447.2097.

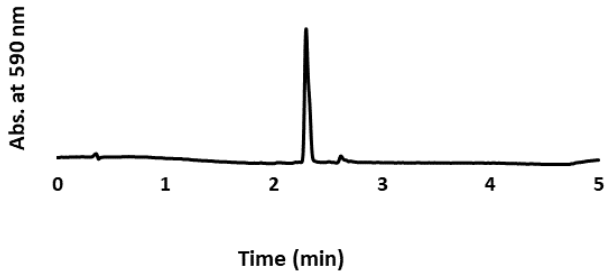




Scheme S4. Synthesis of **CHC-4** and **CHC-5**.

CHC-4:

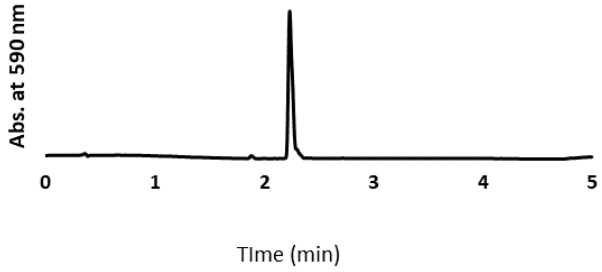
The solution of **Compound 8**[2] (67.1 mg, 0.25 mmol) and 2-bromoethanol (1 mL) in MeCN (5 mL) was refluxed with stirring under an argon atmosphere for 18 h. The solvent was evaporated and the residue was dissolved in EtOH (5 mL) and **Compound 2**[1] (30.0 mg, 0.12 mmol) was added. The reaction mixture was refluxed with stirring under an argon atmosphere for 1 h. The solvent was evaporated and the residue was dissolved in DCM (1 mL) and TFA (1 mL). After stirring for 1 h, the solvent was evaporated and the residue was purified by semi-preparative HPLC using eluent A (H_2O with 0.1% TFA) and eluent B (CH_3CN) (A/B = 90/10 to 10/90 for 40 min) to give **CHC-4** (13.4 mg, 20%) as a blue solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.43 (s, 1H), 8.10 (d, *J* = 15.9 Hz, 1H), 7.96 (d, *J* = 15.9 Hz, 1H), 7.55 (d, *J* = 9.1 Hz, 1H), 7.48 (d, *J* = 8.7 Hz, 1H), 7.00 (d, *J* = 2.2 Hz, 1H), 6.88 (dt, *J* = 9.1, 2.7 Hz, 2H), 6.61 (d, *J* = 2.4 Hz, 1H), 4.57 (t, *J* = 5.1 Hz, 2H), 4.05 (t, *J* = 5.1 Hz, 2H), 3.58 (q, *J* = 7.1 Hz, 4H), 1.79 (s, 6H), 1.26 (t, *J* = 7.1 Hz, 6H). HRMS (*m/z*): $[M]^+$ calcd. for $C_{27}H_{32}N_3O_3$, 446.2444; found, 446.2447.

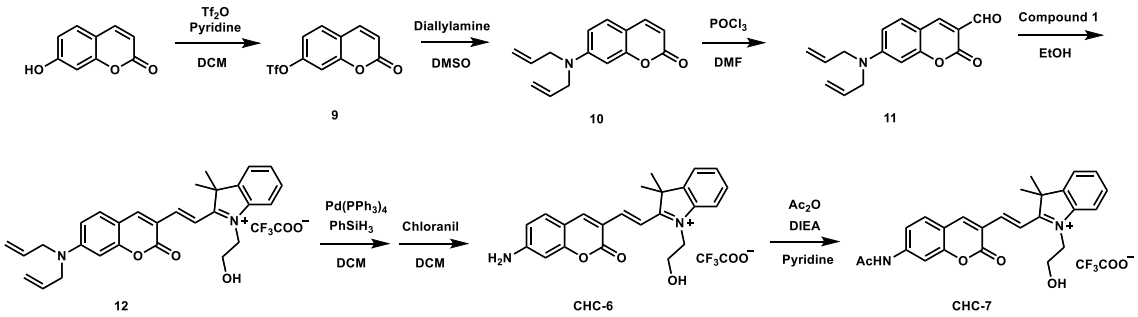


CHC-5:

To a solution of **CHC-4** (3.6 mg, 6 μmol) in pyridine (2 mL), DIEA (1.7 μL, 10 μmol) and Ac_2O (0.7 μL, 10 μmol) were added. The reaction mixture was stirred at room temperature under an argon

atmosphere for 1 h. The solvent was evaporated and the residue was purified by semi-preparative HPLC using eluent A (H_2O with 0.1% TFA) and eluent B (CH_3CN) (A/B = 90/10 to 10/90 for 40 min) to give **CHC-5** (2.8 mg, 72%) as a blue solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.49 (s, 1H), 8.26 (d, *J* = 15.8 Hz, 1H), 8.08 (d, *J* = 1.9 Hz, 1H), 8.04 (d, *J* = 15.8 Hz, 1H), 7.69 (d, *J* = 8.8 Hz, 1H), 7.64 (dd, *J* = 8.8, 1.9 Hz, 1H), 7.57 (d, *J* = 9.1 Hz, 1H), 6.87 – 6.97 (m, 1H), 6.65 (d, *J* = 2.4 Hz, 1H), 4.61 (t, *J* = 5.1 Hz, 2H), 4.07 (t, *J* = 5.0 Hz, 2H), 3.61 (q, *J* = 7.1 Hz, 4H), 2.18 (s, 3H), 1.84 (s, 6H), 1.28 (t, *J* = 7.1 Hz, 6H). HRMS (*m/z*): $[M]^+$ calcd. for $C_{29}H_{34}N_3O_4$, 488.2549; found, 488.2571.





Scheme S5. Synthesis of **CHC-6** and **CHC-7**.

Compound 9:

To the suspension of Umbelliferone (5.0 g, 30.8 mmol) and pyridine (3.48 mL, 43.2 mmol) in DCM (100 mL), Tf_2O (10.4 g, 37.0 mmol) was added dropwise at 0 °C for 5 min. The reactiom mixture was stirred at room temperature for 1 h. The mixture was washed twice with sat. $NaHCO_3$ aq. and brine, dried over $MgSO_4$. The solvent was evaporated and the residue was washed with mixture of DCM and *n*-hexane to give **Compound 9** (5.84 g, 64%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.12 (d, *J* = 9.6 Hz, 1H), 7.93 (d, *J* = 8.6 Hz, 1H), 7.76 (d, *J* = 2.4 Hz, 1H), 7.52 (dd, *J* = 8.6, 2.5 Hz, 1H), 6.60 (d, *J* = 9.6 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.2, 154.1, 150.3, 143.2, 130.5, 119.3, 118.3 (q, $^1J_{C\text{-}F}$ = 322.2 Hz, CF_3), 117.8, 117.4, 110.5.

Compound 10:

The solution of **Compound 9** (1.0 g, 3.40 mmol) and diallylamine (2.1 mL, 17.0 mmol) in DMSO (5 mL) was stirred at 90 °C under an argon atmosphere for 18 h. The reaction mixture was cooled to room temperature and diluted with AcOEt, washed with water and brine, dried over $MgSO_4$. The solvent was evaporated and the residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 100/0 to 40/60) to give **Compound 10** (108.7 mg, 13%) as yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 (d, *J* = 9.4 Hz, 1H), 7.21 (d, *J* = 8.7 Hz, 1H), 6.56 (dd, *J* = 8.8, 2.5 Hz, 1H), 6.50 (d, *J* = 2.5 Hz, 1H), 6.02 (d, *J* = 9.3 Hz, 1H), 5.81 (ddt, *J* = 17.1, 10.4, 4.7 Hz, 2H), 5.22 – 5.07 (m, 4H), 4.00 – 3.91 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.1, 156.4, 151.7, 143.7, 132.3, 128.6, 116.7, 109.9, 109.4, 109.1, 98.5, 53.0; HRMS (*m/z*): $[M+H]^+$ calcd. for $C_{15}H_{16}NO_2$, 242.1176; found, 242.1177.

Compound 11:

Dry DMF (1.0 mL) was added dropwise to $POCl_3$ (1.0 mL) and the mixture was stirred at room temperature under an argon atmosphere for 0.5 h. To the mixture, a solution of **Compound 10** (108 mg, 0.45 mmol) in DMF (2 mL) was added. The reaction mixture was stirred at 60 °C under an argon atmosphere for 1 h. The mixture was poured into ice water, neutralized with 1 N NaOH, extracted with AcOEt three times, washed with brine, dried over $MgSO_4$. The solvent was evaporated and the residue was purified by flash column chromatography (silica gel, *n*-hexane/AcOEt = 100/0 to 40/60) to give **Compound 11** (79.2 mg, 66%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.13 (s, 1H), 8.26 (d, *J* = 0.6 Hz, 1H), 7.41 (d, *J* = 8.9 Hz, 1H), 6.65 (dd, *J* = 8.9, 2.5 Hz, 1H), 6.53 (dd, *J* = 2.5, 0.6 Hz, 1H), 5.84 (m, 2H), 5.26 (m, 2H), 5.17 (m, 2H), 4.04 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 188.1, 161.8, 158.7, 154.6, 145.6, 132.4, 131.4, 117.5, 115.2, 110.9, 109.0, 98.3, 53.3; HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{16}H_{15}NO_3Na$, 292.0944; found, 292.0946.

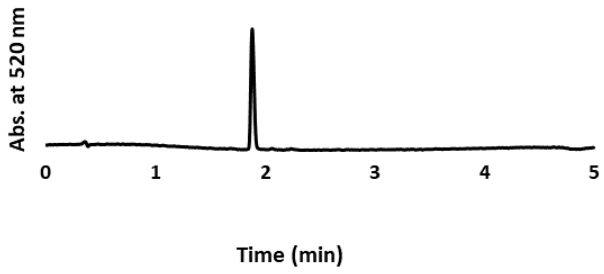
Compound 12:

The solution of **Compound 11** (27.8 mg, 0.10 mmol) and **Compound 1** (147 mg, 0.52 mmol) in EtOH (5 mL) was refluxed with stirring under an argon atmosphere for 16 h. Solvent was evaporated and the residue was diluted with DCM, washed with sat. $NaHCO_3$ aq. and brine, dried over $MgSO_4$. The solvent was evaporated and the residue was purified by flash column chromatography (silica gel, DCM/MeOH = 100/0 to 90/10) and by semi-preparative HPLC using eluent A (H_2O with 0.1% TFA) and eluent B (CH_3CN) (A/B = 90/10 to 10/90 for 40 min) to give **Compound 12** (15.8 mg, 27%) as a yellow solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.55 (s, 1H), 8.32 (d, *J* = 15.8 Hz, 1H), 8.11 (d, *J* = 15.9 Hz, 1H), 7.75 (m, 2H), 7.68 – 7.53 (m, 3H), 6.89 (dd, *J* = 9.1, 2.4 Hz, 1H), 6.64 (d, *J* = 2.3 Hz, 1H), 5.94 (m, 2H), 5.26 (dd, *J* = 10.4, 1.4 Hz, 2H), 5.20 (dd, *J* = 17.2, 1.5 Hz, 2H), 4.67 (t, *J* = 5.1 Hz, 2H), 4.18 (m, 4H), 4.09 (t, *J* = 5.1 Hz, 2H), 1.86 (s, 6H); ^{13}C NMR (101 MHz, CD_3OD) δ 184.5, 161.4, 159.1, 156.9, 151.4, 151.3, 144.8, 142.7, 133.2, 133.1, 130.3, 130.3, 123.9, 117.4, 115.9, 114.8, 113.0, 112.8,

111.7, 98.8, 60.1, 54.3, 53.4, 50.3, 27.1; HRMS (*m/z*): $[M+H]^+$ calcd. for $C_{29}H_{31}N_2O_3$, 455.2329; found, 455.2318.

CHC-6:

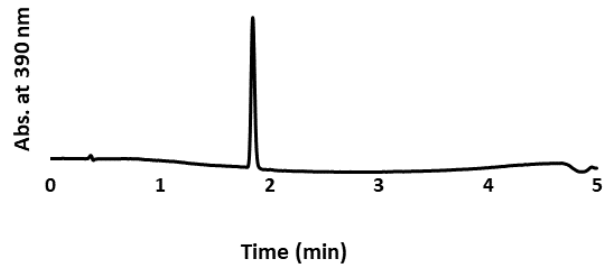
The suspension of **Compound 12** (63.3 mg, 0.14 mmol), $Pd(PPh_3)_4$ (40.1 mg, 0.035 mmol), and $PhSiH_3$ (171 μL, 1.39 mmol) in DCM (10 mL) was stirred at room temperature under argon atmosphere for 1.5 h. Solvent was evaporated and the residue was purified by short column chromatography (silica gel, Hexane/AcOEt = 50/50 to 0/100, then washed with MeOH) to remove excess $PhSiH_3$. The crude intermediate was dissolved in DCM (5 mL) and added chloranil (34.2 mg, 0.14 mmol). The reaction mixture was stirred at room temperature for 20 min. The mixture was poured into sat. $NaHCO_3$ aq., and extracted with DCM, washed with brine, dried over Na_2SO_4, and the solvent was evaporated. The residue was purified with preparative HPLC using eluent A (H_2O with 0.1% TFA) and eluent B (CH_3CN) (A/B = 90/10 to 10/90 for 40 min) to give **CHC-6** (17.3 mg, 33%). ^1H NMR (400 MHz, CD_3OD) δ 8.41 (s, 1H), 8.21 (d, *J* = 15.8 Hz, 1H), 7.98 (d, *J* = 15.8 Hz, 1H), 7.62 – 7.66 (m, 2H), 7.47 – 7.50 (m, 2H), 7.39 (d, *J* = 8.7 Hz, 1H), 6.62 (dd, *J* = 8.7, 2.0 Hz, 1H), 6.41 (d, *J* = 2.1 Hz, 1H), 4.55 (t, *J* = 5.0 Hz, 2H), 3.98 (t, *J* = 5.2 Hz, 2H), 1.75 (s, 6H); ^{13}C NMR (101 MHz, CD_3OD) δ 184.4, 161.6, 159.8, 159.7, 151.8, 151.7, 144.7, 142.8, 134.0, 130.3, 130.1, 123.9, 115.8, 114.9, 113.8, 112.2, 111.8, 99.3, 60.1, 53.3, 50.2, 49.9, 27.2; HRMS (*m/z*): $[M]^+$ calcd. for $C_{23}H_{23}N_2O_3$, 375.17032; found, 375.17011.

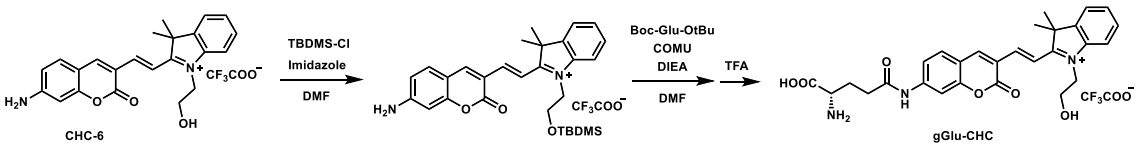


CHC-7:

To a solution of **CHC-6** (4.5 mg, 0.012 mmol) in dry pyridine (5 mL), DIEA (160 μL), and Ac_2O (110 μL) were added. The reaction mixture was stirred at room temperature under an argon atmosphere for 21 h. The solvent was evaporated and the residue was purified by preparative HPLC using eluent A (H_2O with 0.1% TFA) and eluent B (CH_3CN) (A/B = 90/10 to 10/90 for 40 min) to give **CHC-7** (5.4 mg, >99%) as a red solid. ^1H NMR (400 MHz, $CDCl_3$) δ 7.68 – 7.60 (m, 4H), 7.45 (d, *J* = 8.4 Hz, 1H), 7.18 (ddd, *J* = 8.2, 1.2 Hz, 1H), 7.09 (dd, *J* = 7.4, 0.9 Hz, 1H), 6.95 (ddd, *J* = 7.2, 0.9 Hz, 1H), 6.85 –

6.79 (m, 2H), 3.81 – 3.77 (m, 1H), 3.72 – 3.61 (m, 2H), 3.45 – 3.41 (m, 1H), 2.25 (s, 3H), 1.45 (s, 3H), 1.18 (s, 3H); HRMS (*m/z*): $[M]^+$ calcd. for $C_{25}H_{25}N_2O_4$, 417.18088; found, 417.18201.

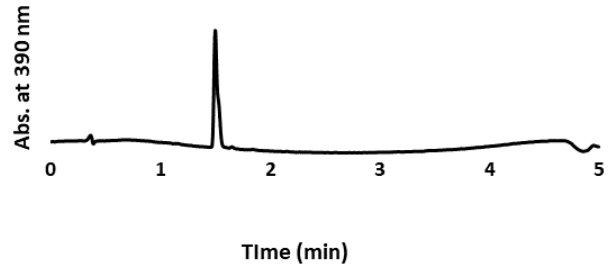


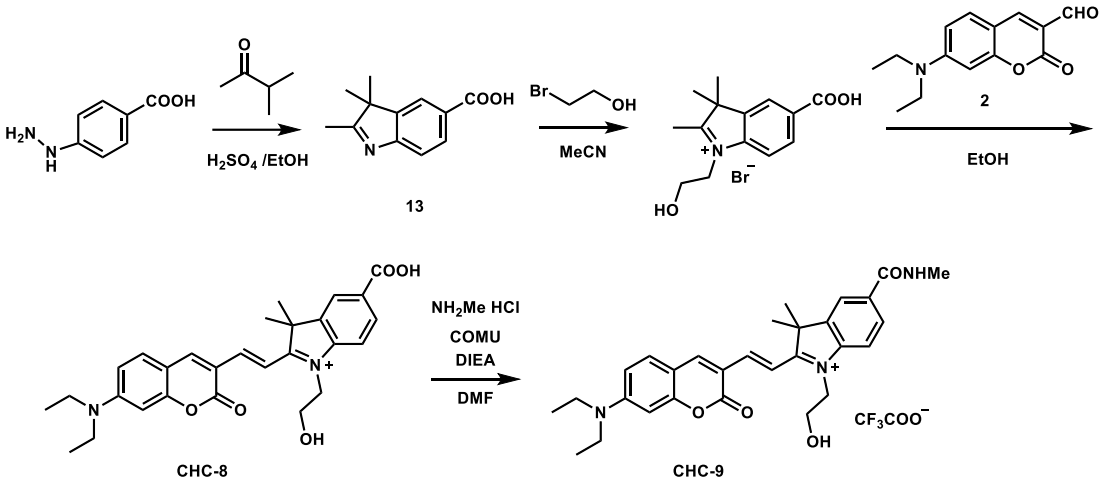


Scheme S6. Synthesis of **gGlu-CHC**.

gGlu-CHC:

The suspension of **CHC-6** (9.7 mg, 0.026 mmol), tert- butyldimethylchlorosilane (39.2 mg, 0.26 mmol) and imidazole (38.7 mg, 0.57 mmol) in dry DMF (3 mL) was stirred at room temperature under argon atmosphere for 16 h. Solvent was evaporated and residue was poured into water, then extracted with DCM, washed with brine, dried over Na_2SO_4, and the solvent was evaporated. The crude intermediate was dissolved in dry DMF (3 mL). To this solution, Boc-Glu-OtBu (111 mg, 0.26 mmol), DIEA (88 µL, 0.52 mmol) and COMU (133 mg, 0.31 mmol) were added. The reaction mixture was stirred at room temperature under argon atmosphere for 22 h. The solvent was evaporated and the residue was poured into sat. $NaHCO_3$ aq., and extracted with DCM, washed with brine, dried over Na_2SO_4, and the solvent was evaporated. The residue was dissolved in TFA (2 mL), and the solution was stirred at room temperature for 17 h. The solvent was evaporated, and the residue was purified by preparative HPLC using eluent A (H_2O with 0.1% TFA) and eluent B (CH_3CN) (A/B = 90/10 to 50/50 for 55 min) to give **gGlu-CHC** (3.4 mg, 26%) as a red solid. ^{1}H NMR (400 MHz, CD_3OD) (close form : open form = 1 : 2.5) δ 8.60 (s, 1H), 8.27 (s, 2.5H), 8.25 (d, *J* = 19.1 Hz, 2H), 7.91 (d, *J* = 1.7 Hz, 1H), 7.84 (d, *J* = 1.9 Hz, 2.5H), 7.77 – 7.70 (m, 7H), 7.66 (d, *J* = 9.2 Hz, 1H), 7.64 (d, *J* = 8.8 Hz, 2.5H), 7.61 – 7.54 (m, 7H), 7.49 (d, *J* = 8.7 Hz, 1H), 7.42 (dd, *J* = 8.6, 1.8 Hz, 1H), 7.38 (dd, *J* = 8.6, 2.0 Hz, 2.5H), 4.66 (t, *J* = 5.3 Hz, 2H), 4.26 (t, *J* = 4.9 Hz, 5H), 4.01 (t, *J* = 4.4 Hz, 2H), 4.02 – 3.94 (m, 3.5H), 3.82 (t, *J* = 5.3 Hz, 5H), 2.70 – 2.60 (m, 7H), 2.23 – 2.12 (m, 7H), 1.78 (s, 6H), 1.58 (s, 15H); HRMS (*m/z*): $[M]^+$ calcd. for $C_{28}H_{30}N_3O_6$, 504.21291; found, 504.21289.





Scheme S7. Synthesis of **CHC-8** and **CHC-9**.

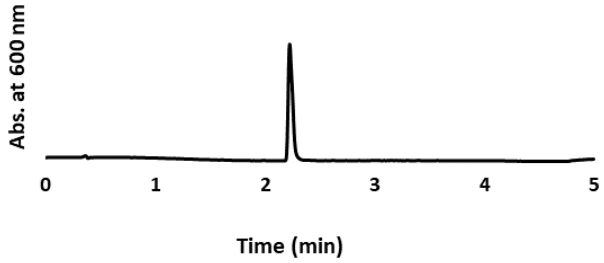
Compound 13:

The solution of 4-Hydrazinobenzoic Acid (5.0 g, 32.9 mmol), 3-Methyl-2-butanone (3.9 mL, 36.1 mmol), and H_2SO_4 (1 mL) in EtOH (120 mL) was refluxed with stirring under an argon atmosphere for 20 h. The reaction mixture was filtered, and the filtrate was evaporated. The residue was dissolved in sat. $NaHCO_3$ and washed with DCM three times, acidified with 1 N HCl, extracted with DCM, washed with brine, and dried over $MgSO_4$ to give **Compound 13** (7.40 g) as a crude brownish solid, which was used in next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (dd, *J* = 8.1, 1.7 Hz, 1H), 8.07 (d, *J* = 1.7 Hz, 1H), 7.69 (d, *J* = 8.1 Hz, 1H), 2.40 (s, 3H), 1.37 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 192.8, 171.2, 156.7, 145.4, 130.9, 127.1, 123.3, 119.6, 53.9, 22.9, 15.5.

CHC-8:

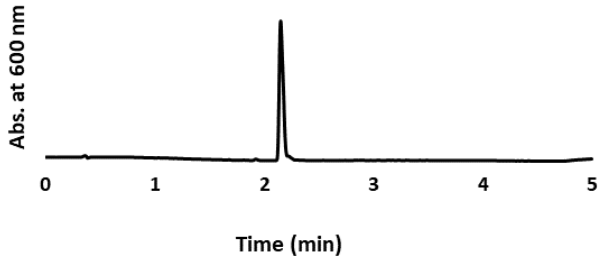
The solution of **Compound 13** (67.1 mg, 0.25 mmol) and 2-bromoethanol (0.5 mL) in MeCN (2 mL) was refluxed with stirring under an argon atmosphere for 18 h. The solvent was evaporated and the residue was dissolved in EtOH (5.0 mL) and **Compound 2**[1] (30.0 mg, 0.12 mmol) was added. The

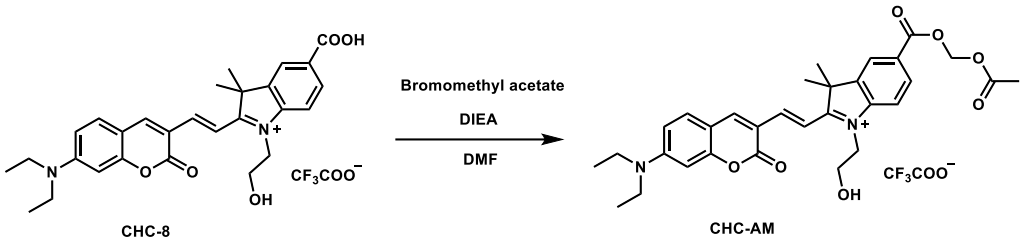
reaction mixture was refluxed with stirring under an argon atmosphere for 1 h. The solvent was evaporated and the residue was purified by semi-preparative HPLC using eluent A (H_2O with 0.1% TFA) and eluent B (CH_3CN) (A/B = 90/10 to 10/90 for 40 min) to give **CHC-8** (24.7 mg, 43%) as a blue solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.49 (s, 1H), 8.26 (d, *J* = 15.8 Hz, 1H), 8.08 (d, *J* = 1.9 Hz, 1H), 8.04 (d, *J* = 15.8 Hz, 1H), 7.69 (d, *J* = 8.8 Hz, 1H), 7.64 (dd, *J* = 8.8, 1.9 Hz, 1H), 7.57 (d, *J* = 9.1 Hz, 1H), 6.94 – 6.88 (m, 1H), 6.65 (d, *J* = 2.4 Hz, 1H), 4.61 (t, *J* = 5.1 Hz, 2H), 4.07 (t, *J* = 5.0 Hz, 2H), 3.61 (q, *J* = 7.1 Hz, 4H), 1.84 (s, 6H), 1.28 (t, *J* = 7.1 Hz, 6H). HRMS (*m/z*): $[M]^+$ calcd. for $C_{28}H_{31}N_2O_5$, 475.2233; found, 475.2261.



CHC-9:

To a solution of **CHC-8** (6.6 mg, 0.014 mmol) in dry DMF (1.0 mL), DIEA (24.2 μL, 0.14 mmol), methylamine hydrochloride (7.5 mg, 0.028 mmol) and COMU (17.9 mg, 0.04 mmol) were added. The reaction mixture was stirred at room temperature under an argon atmosphere for 3 h. The solvent was evaporated and the residue was purified by semi-preparative HPLC using eluent A (H_2O with 0.1% TFA) and eluent B (CH_3CN) (A/B = 90/10 to 10/90 for 40 min) to give **CHC-9** (5.0 mg, 60%) as a blue solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.55 (s, 1H), 8.39 (d, *J* = 15.6 Hz, 1H), 8.14 (d, *J* = 1.6 Hz, 1H), 8.07 (d, *J* = 15.7 Hz, 1H), 8.03 (dd, *J* = 8.5, 1.8 Hz, 1H), 7.80 (d, *J* = 8.5 Hz, 1H), 7.59 (d, *J* = 9.1 Hz, 1H), 6.93 (dd, *J* = 9.2, 2.4 Hz, 1H), 6.66 (d, *J* = 2.3 Hz, 1H), 4.64 (t, *J* = 5.1 Hz, 2H), 4.08 (t, *J* = 5.1f Hz, 2H), 3.63 (q, *J* = 7.1 Hz, 4H), 2.97 (s, 3H), 1.88 (s, 6H), 1.29 (t, *J* = 7.1 Hz, 6H); HRMS (*m/z*): $[M]^+$ calcd. for $C_{29}H_{34}N_3O_4$, 488.2544; found, 488.2541.

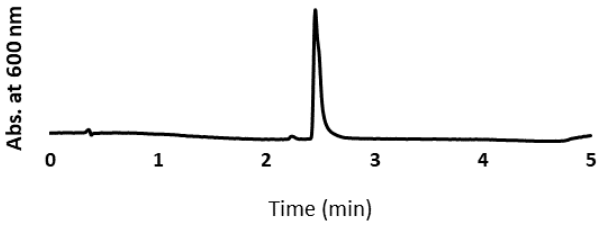




Scheme S7. Synthesis of **CHC-AM**.

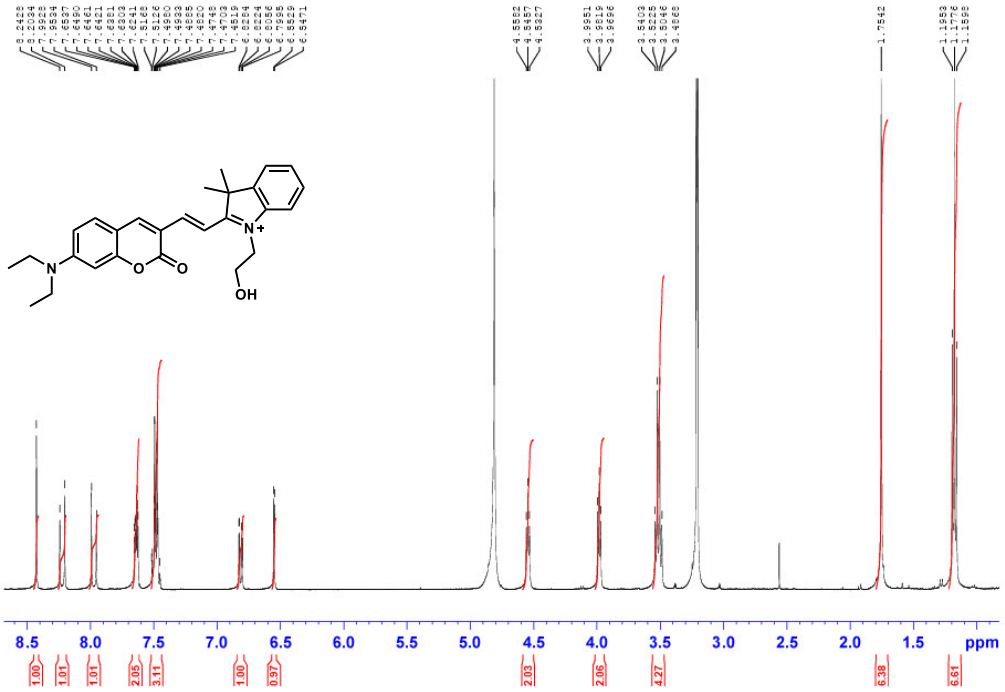
CHC-AM:

To a solution of **CHC-8** (5.2 mg, 0.011 mmol) in dry DMF (2 mL), DIEA (300 µL, 1.7 mmol) was added at 0 °C. Bromometyl acetate (16 µL, 0.16 mmol) was added dropwise, and the solution was stirred at room temperature for 22 h. The solution was poured into 10% (v/v) AcOH, then extracted with DCM, washed with brine, dried over Na_2SO_4, and the solvent was evaporated. The residue was purified by preparative HPLC using eluent A (H_2O with 0.1% TFA) and eluent B (CH_3CN) (A/B = 90/10 to 10/90 for 40 min) to give **CHC-AM** (4.3 mg, 69%) as a blue solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.47 (s, 1H), 8.33 (d, *J* = 15.6 Hz, 1H), 8.25 (d, *J* = 1.4 Hz, 1H), 8.16 (dd, *J* = 8.5, 1.6 Hz, 1H), 7.97 (d, *J* = 15.6 Hz, 1H), 7.73 (d, *J* = 8.5 Hz, 1H), 7.50 (d, *J* = 9.2 Hz, 1H), 6.85 (dd, *J* =9.2, 2.3 Hz, 1H), 6.57 (d, *J* = 2.3 Hz, 1H), 5.92 (s, 2H), 4.54 (t, *J* = 5.1 Hz, 2H), 3.98 (t, *J* = 5.1 Hz, 2H), 3.54 (q, *J* = 7.1 Hz, 4H), 2.03 (s, 3H), 1.79 (s, 6H), 1.19 (t, *J* = 7.1 Hz, 6H); HRMS (*m/z*): $[M]^+$ calcd. for $C_{31}H_{35}N_2O_7$, 547.24388; found, 547.24393.

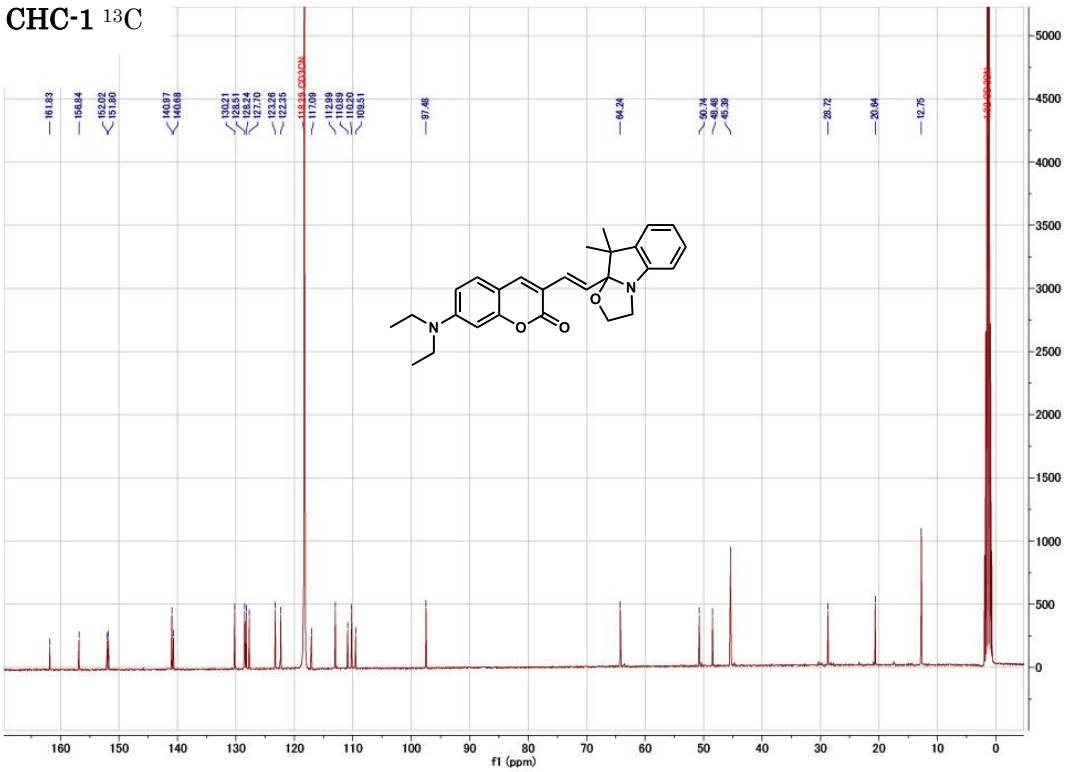


Spectral data

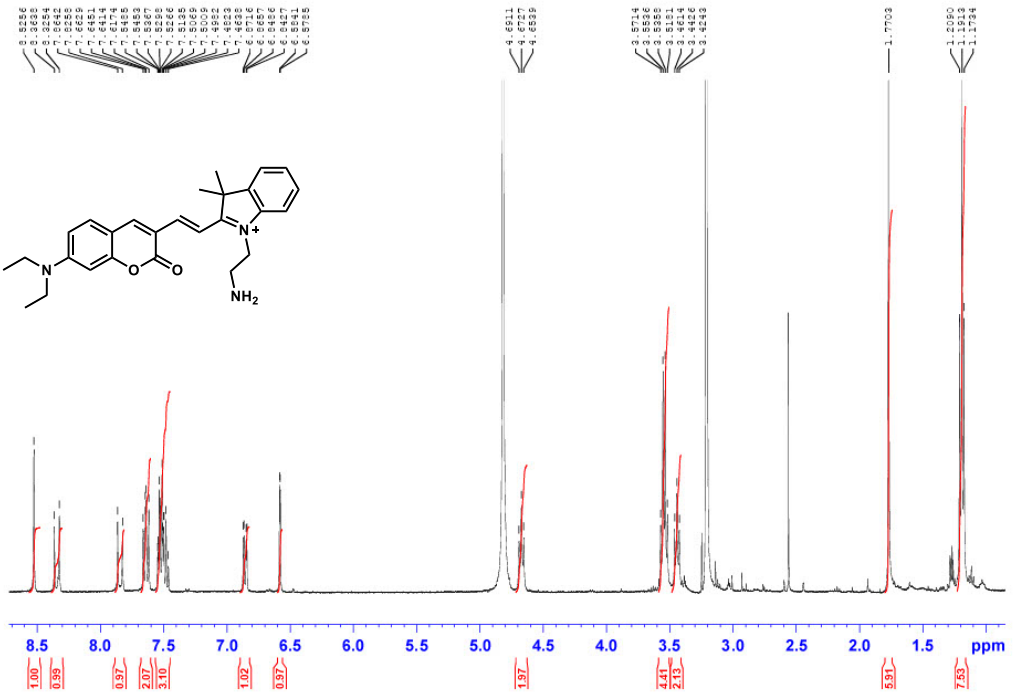
CHC-1 ^{1}H



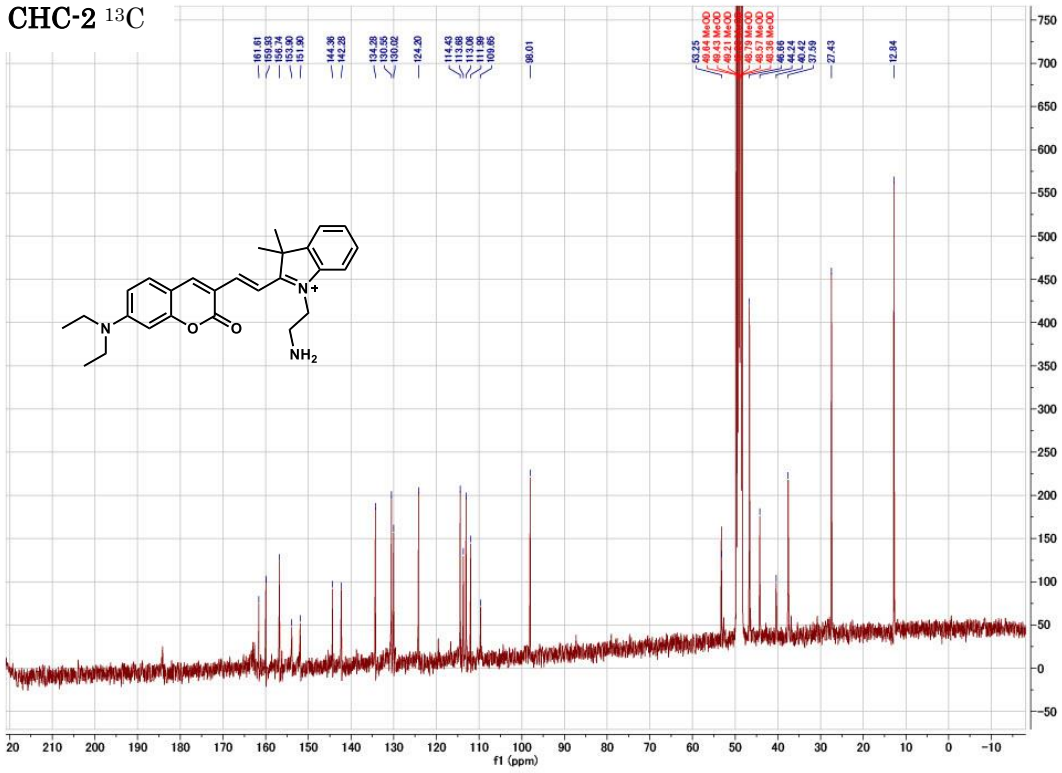
CHC-1 ^{13}C



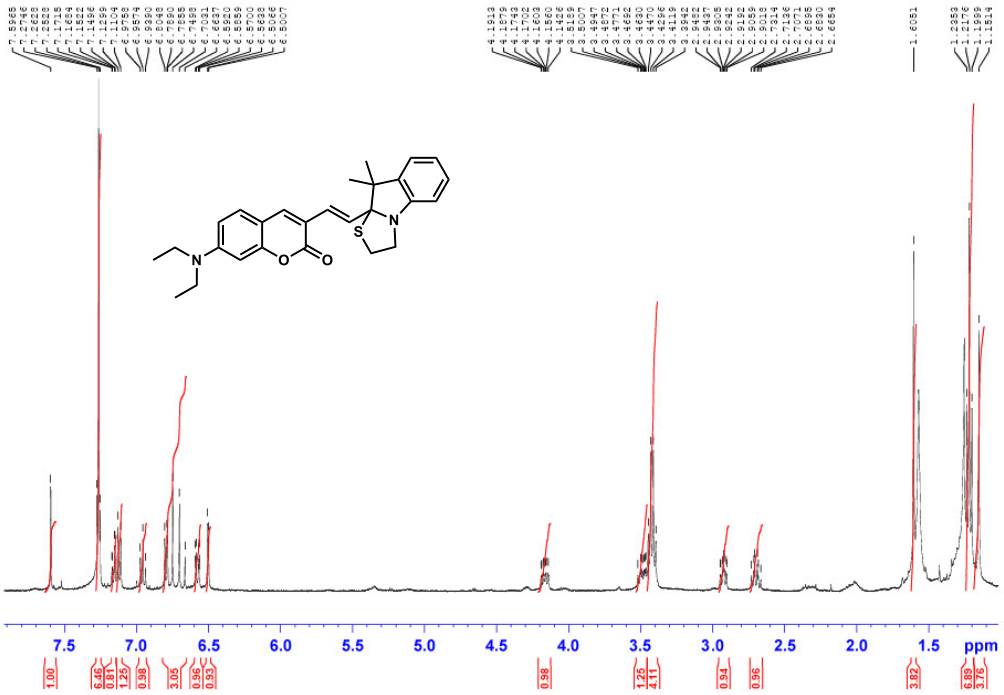
CHC-2 ^{1}H



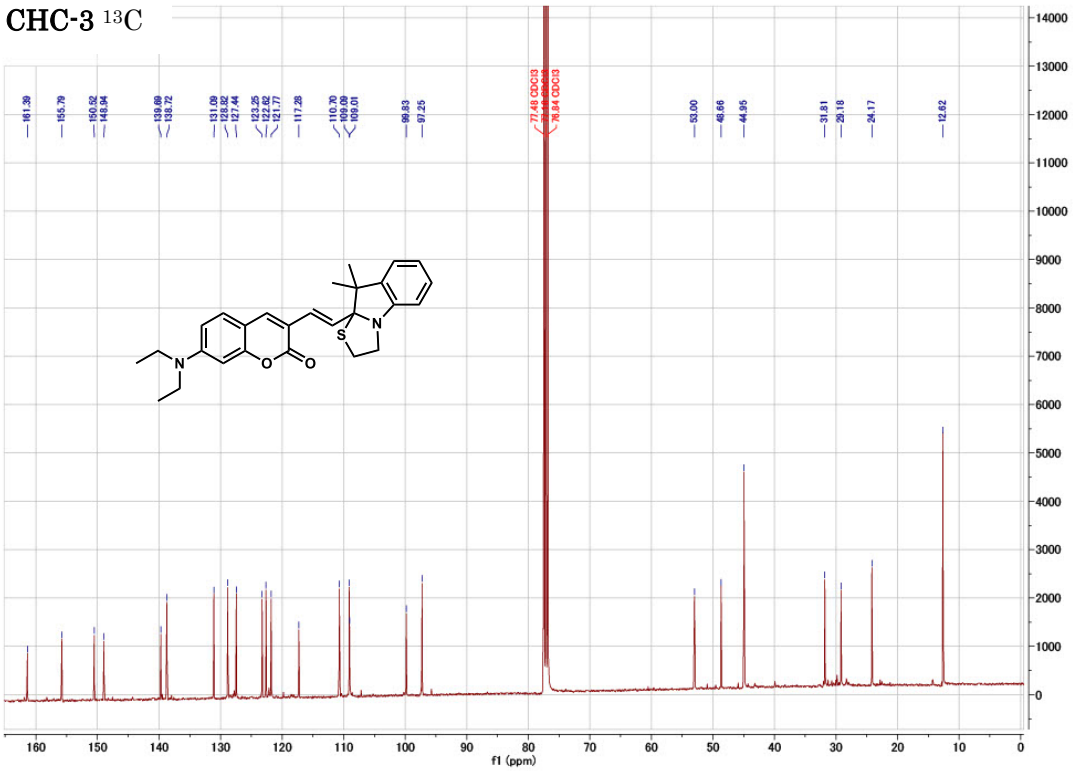
CHC-2 ^{13}C



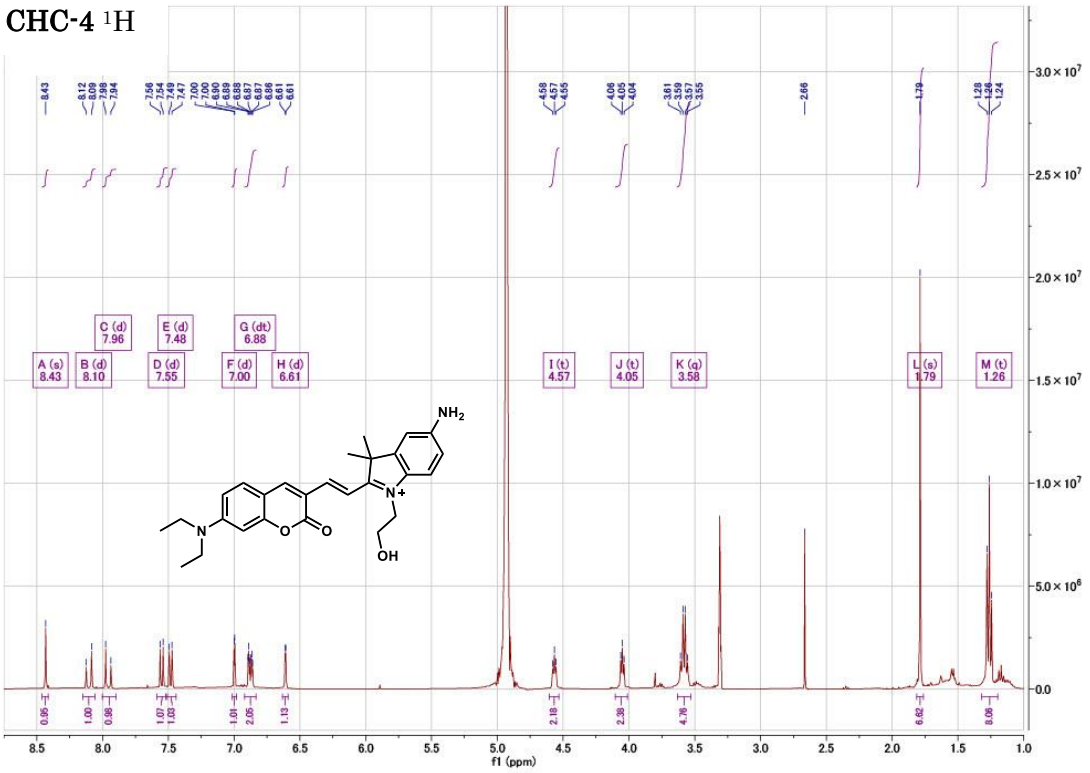
CHC-3 ^{1}H



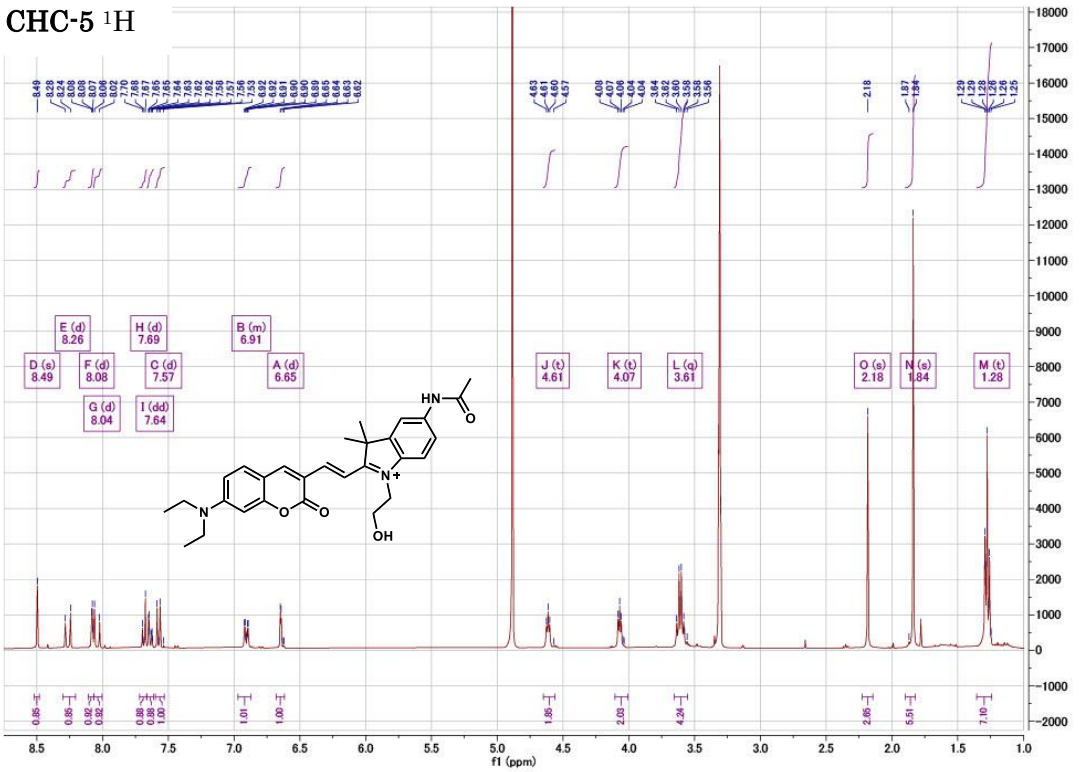
CHC-3 ^{13}C



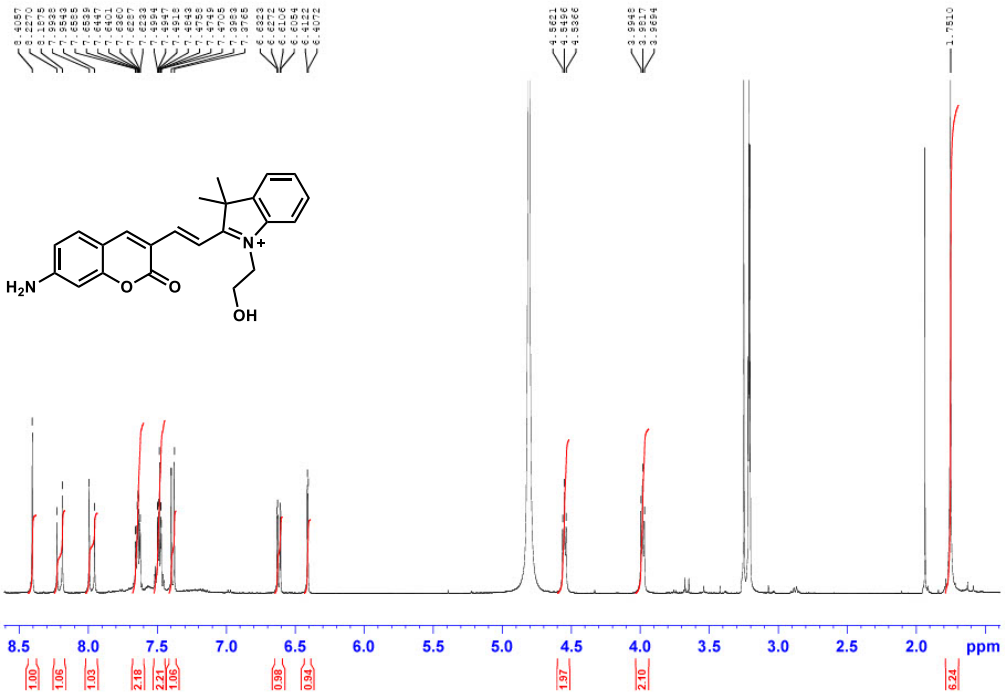
CHC-4 ^{1}H



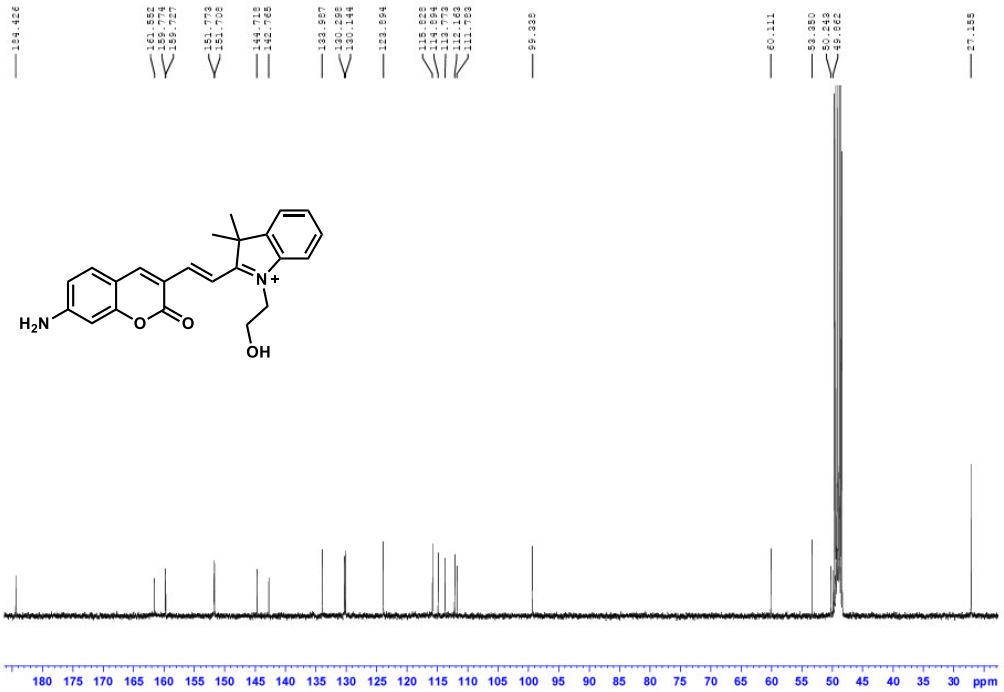
CHC-5 ^{1}H



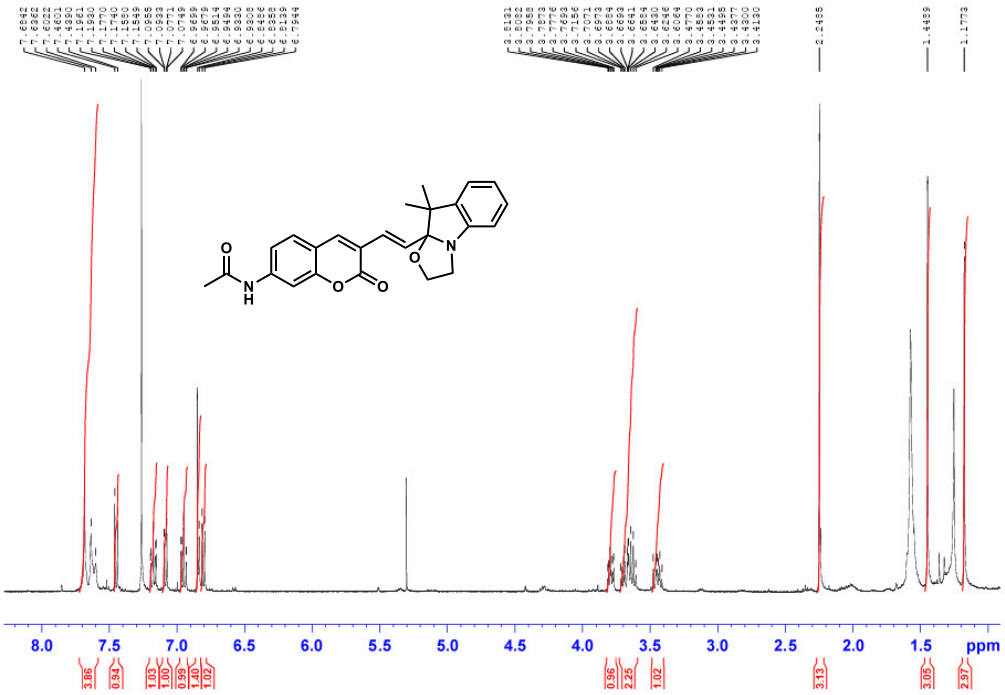
CHC-6 ^{1}H



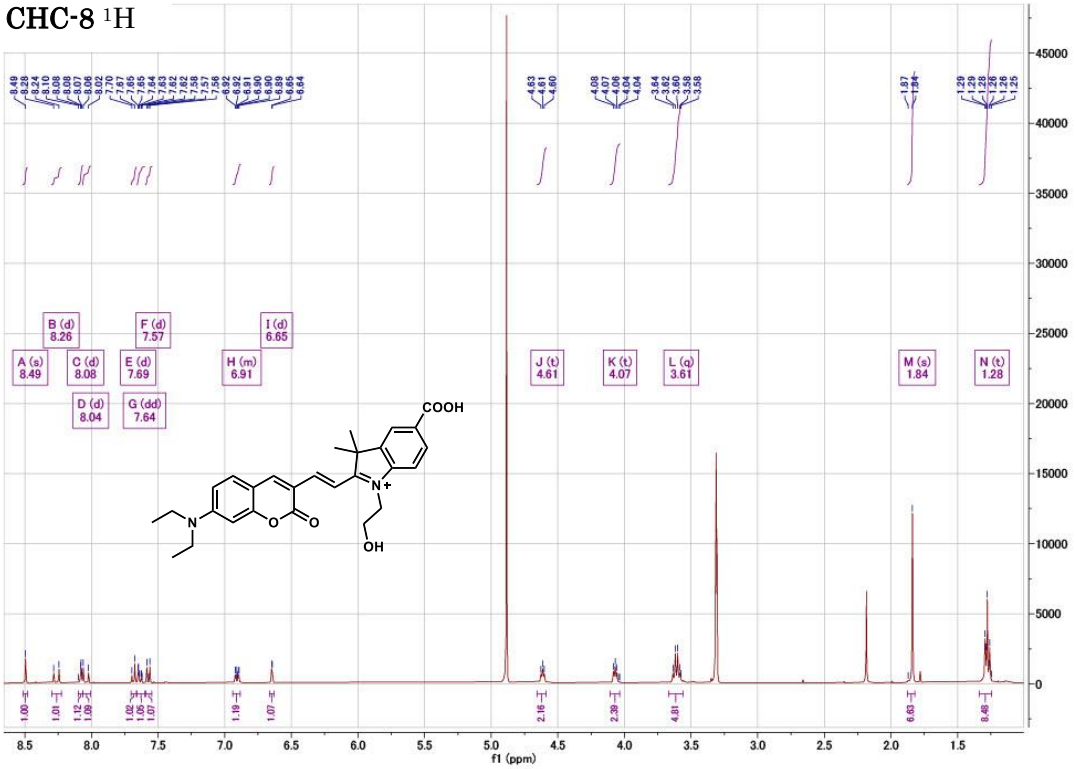
CHC-6 ^{13}C



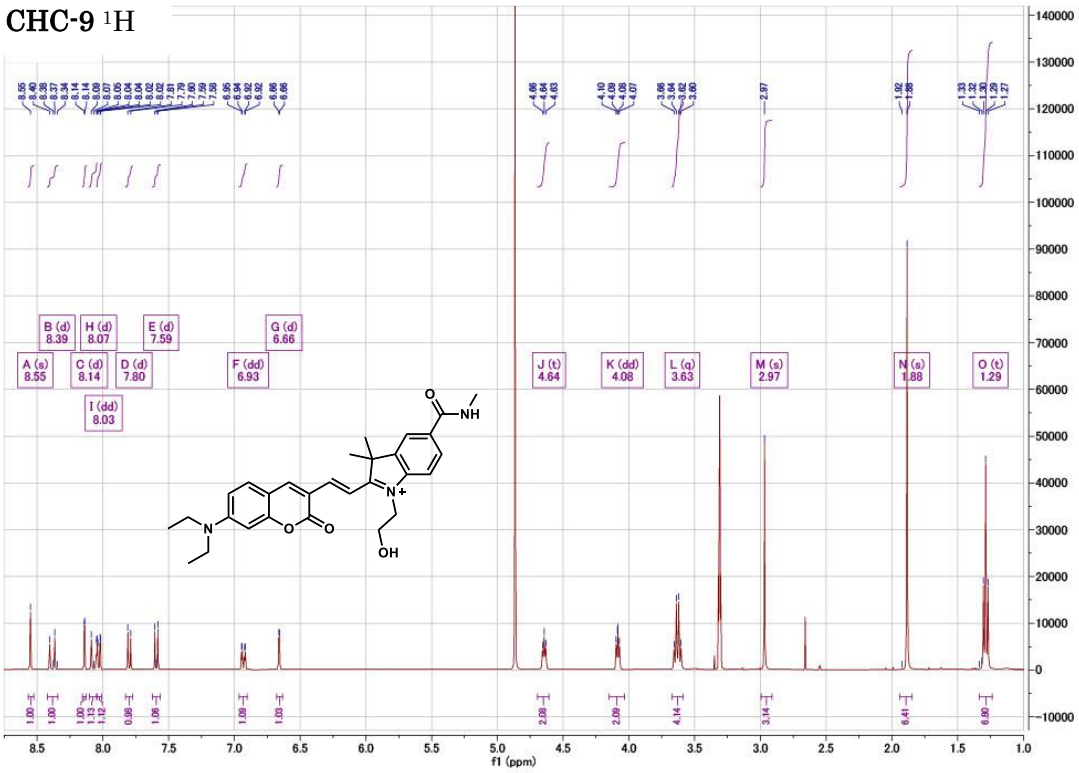
CHC-7 ^{1}H



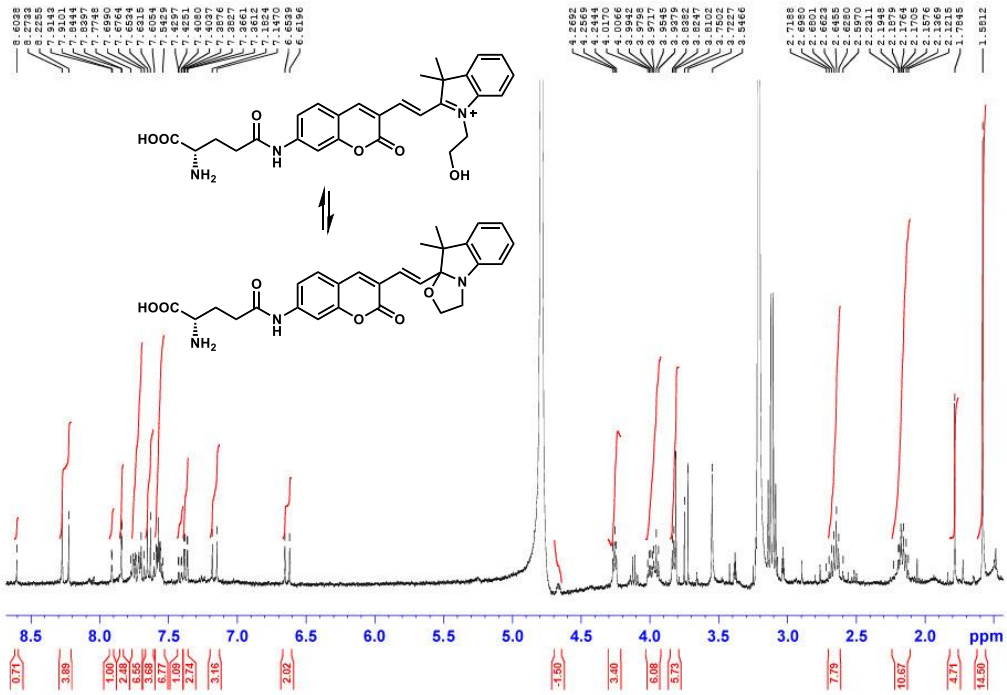
CHC-8 ^{1}H



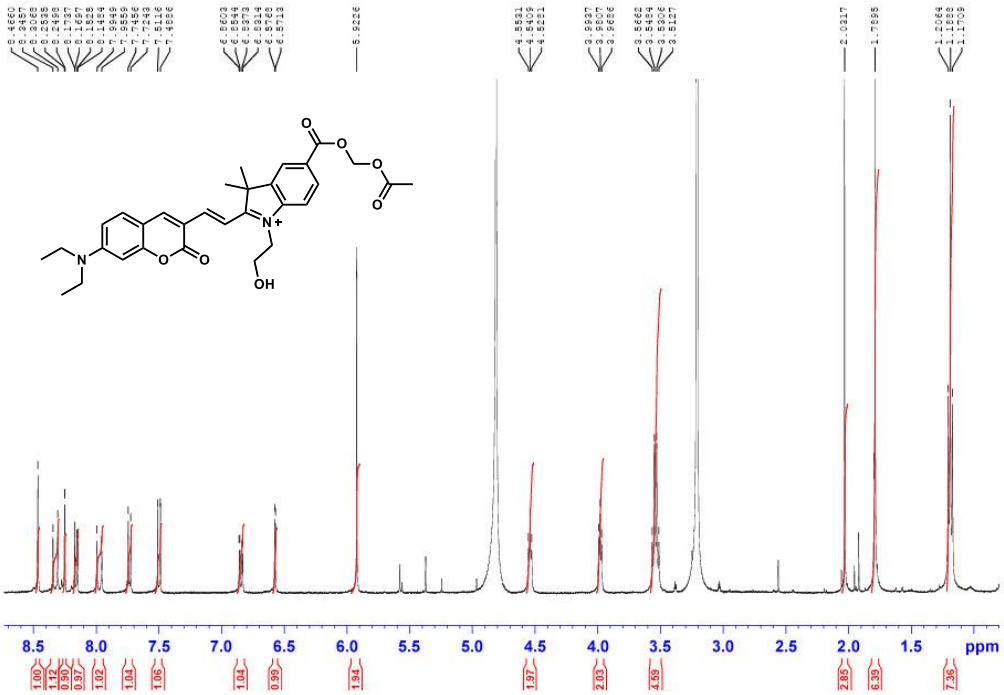
CHC-9 ^{1}H



gGlu-CHC ^{1}H



CHC-AM ^{1}H



Measurements of photophysical properties

Compounds were dissolved in anhydrous DMSO to obtain 10 mM stock solutions. These stock solutions were diluted with 0.2 M sodium phosphate buffer to a final concentration of 5 μM containing 1% DMSO/DMF. Absorption spectra were obtained with a UV-1800 or UV-2450 UV/Vis spectrometer (Shimadzu) and fluorescence spectra were obtained with an F-7000 fluorescence spectrometer (Hitachi) or with an Infinite M1000 spectrofluorometer (TECAN) using 96-well plate. To determine pK_a values for compounds with n acid-base equilibria (n = 1 or 2), pH profiles of absorbance (Abs) or fluorescence intensity (FI) were fitted to the following formula with KaleidaGraph software.

$$\text{Abs or FI} = \frac{c_0 + \sum_{k=1}^{n} c_k \cdot 10^{k\cdot pH - \sum_{l=1}^{k} pK_{al}}}{1 + \sum_{k=1}^{n} 10^{k\cdot pH - \sum_{l=1}^{k} pK_{al}}}$$

(pK_{a1} < pK_{a2} < ••• < pK_{an}; c_n = constant).

LC analysis

In order to confirm the purities of CHC dyes, dyes solution were analyzed with an ACQUITY UPLC H-Class ultra-performance liquid chromatography mass spectroscopy (UPLC-MS) system (Waters), equipped with a column (ACQUITY UPLC BEH C18 1.7 mm, Waters), an autosampler (SMFTN;

186015017, Waters), a pump (QSM; 186015018, Waters), a PDA detector (eλ Detector; 186015033, Waters), and an MS detector (QDa; 186006511, Waters), using 0.1% formate solution (solution A) and acetonitrile (solution B) as eluents. A/B = 95/5 to 5/95 (0 - 3.5 min), 5/95 (3.5 - 4.0 min), 5/95 to 95/5 (4.0 - 4.1 min), 95/5 (4.1-5.0 min). Moreover, in order to confirm that gGlu-CHC and CHC-AM are hydrolyzed to CHC-6 and CHC-8, respectively, upon reaction with GGT and esterase, enzyme reaction solutions were analyzed under the same conditions. For CHC-1, *m/z* = 431.3; for CHC-2, *m/z* = 215.7; for CHC-3, *m/z* = 447.2; for CHC-4, *m/z* = 446.3; for CHC-5, *m/z* = 488.2; for CHC-6, *m/z* = 375.2; for CHC-7, *m/z* = 417.2; for CHC-8, *m/z* = 475.2; for CHC-9, *m/z* = 488.2; for gGlu-CHC, *m/z* = 252.7; for CHC-AM, *m/z* = 547.3.

Cell cultures

A549 cells and H226 cells were purchased from Riken Cell Bank (RCB0098) and American Type Culture Collection, respectively. A549 cells was cultured in DMEM (Wako) and H226 cells were cultured in RPMI (Wako), containing 10% fetal bovine serum (GIBCO), 100 U/mL penicillin, 100 μg/ml streptomycin, at 37 °C in humidified air containing 5% CO_2.

Live-cell imaging

A549 cells and H226 cells were seeded on an 8-chamber plate (Ibidi, μ-slide), and cultured overnight. Then, the medium was replaced with 1 μM gGlu-CHC or CHC-AM solution in HBSS(+) in the presence or absence of 50 μM GGsTop or 1 μM CellTracker™ Green CMFDA for 1 hour at 37 °C. Fluorescence images were captured under a confocal fluorescence microscope (TCS SP8 STED, Leica) equipped with a white light laser and objective lens (HCX PL APO CS 40x/1.25 Oil, Leica). Excitation/emission: 514 nm/580-630 nm for gGlu-CHC, 552 nm/650-700 nm for CHC-AM, 495 nm/505-540 nm for CellTracker™ Green CMFDA.

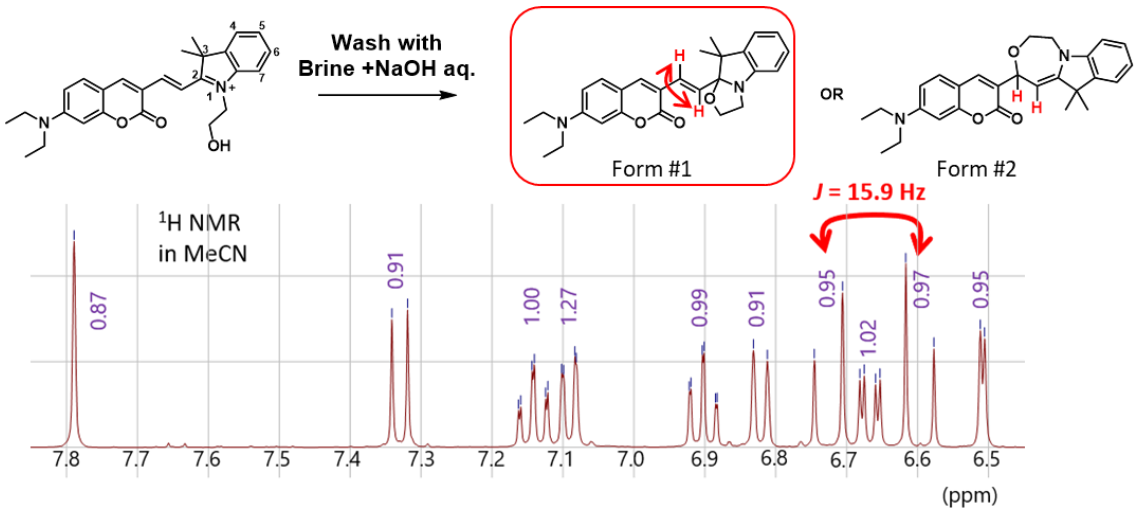


Figure S1. Identification of the spirocyclic form of CHC-1. The ^{1}H NMR spectrum suggested that form #1 is the spirocyclic form, generated by attack of the hydroxyethyl group on the indolenium C2 atom of **CHC-1**.

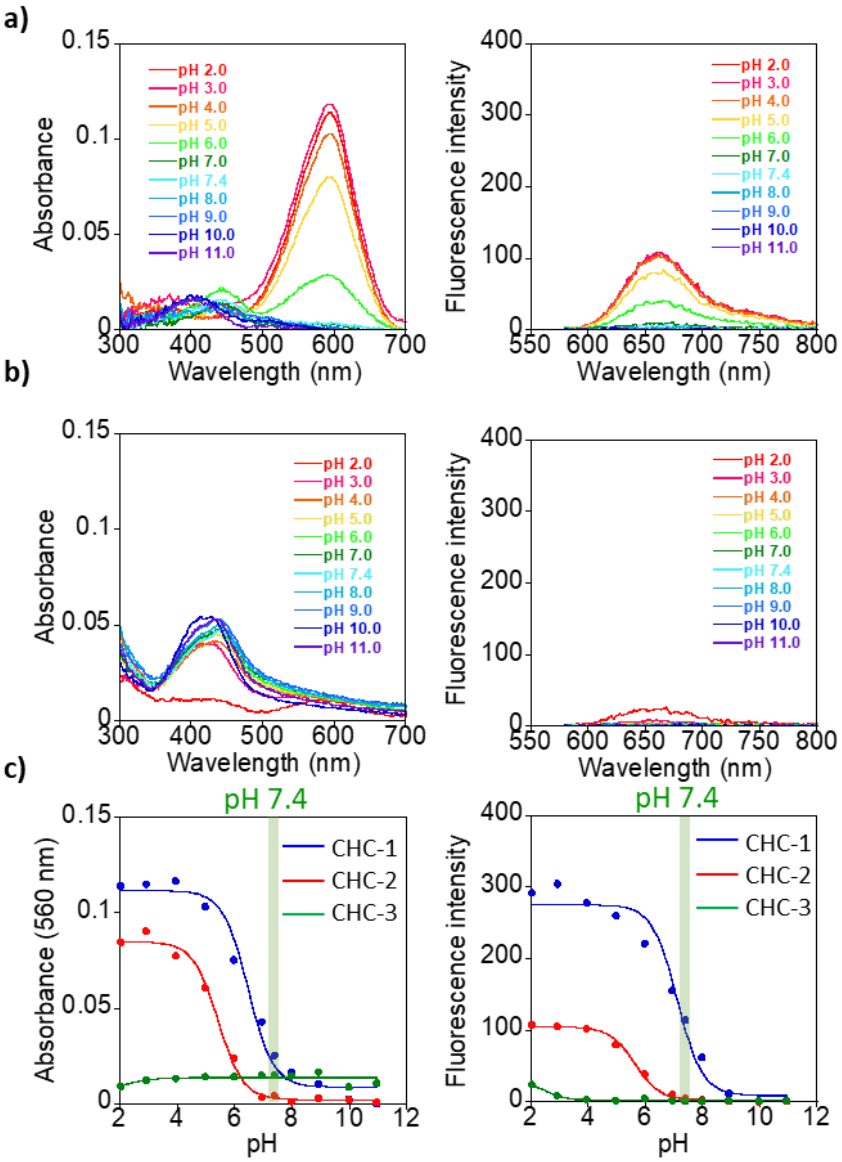


Figure S2. Absorption and fluorescence spectra of 2 µM solution of CHC-2 (a) and CHC-3 (b) at various pH values in 0.2 M sodium phosphate buffer. The excitation wavelength was 560 nm. (c) pH profiles of absorption and fluorescence intensity of CHC-1, CHC-2 and CHC-3. Plots of normalized absorbance at 580 nm, and plots of normalized fluorescence intensity at 660 nm.

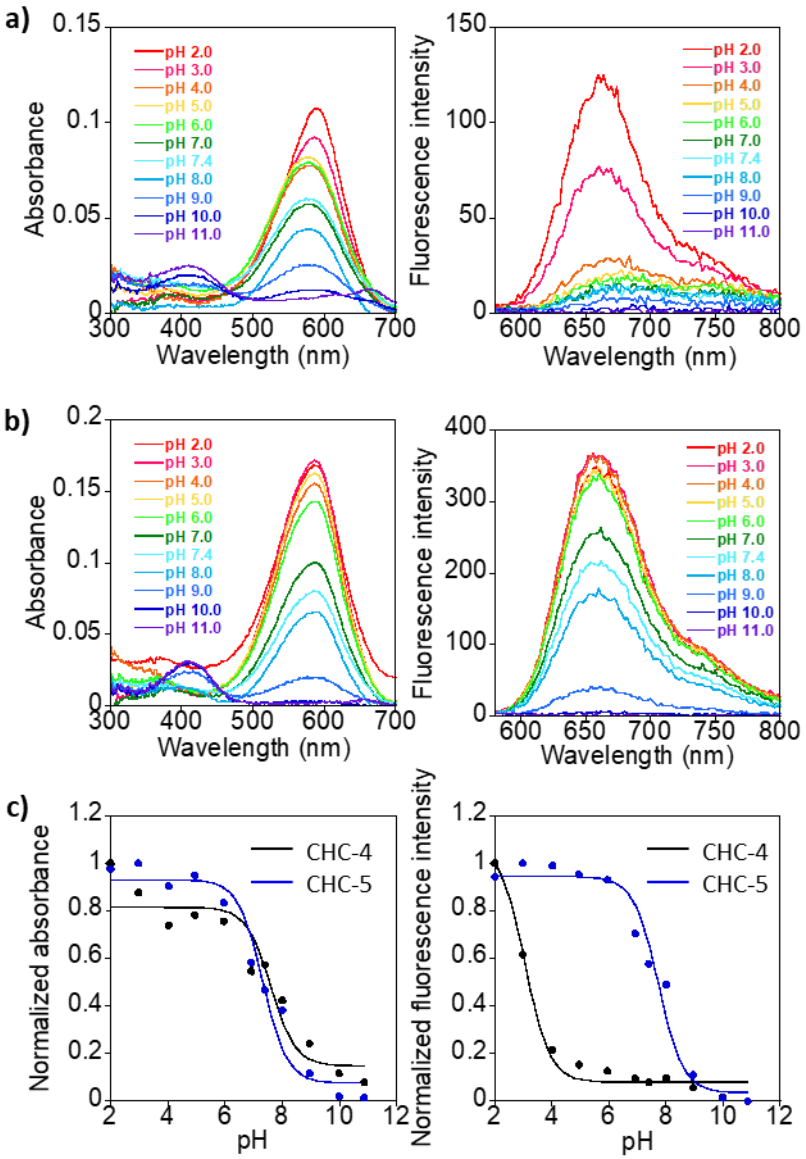


Figure S3. Absorption and fluorescence spectra of 2 μM solution of CHC-4 (a) and CHC-5 (b) at various pH values in 0.2 M sodium phosphate buffer. The excitation wavelength was 560 nm. (c) pH profiles of absorption and fluorescence intensity of CHC-4 and CHC-5. Plots of normalized absorbance at 580 nm, and plots of normalized fluorescence intensity at 660 nm.

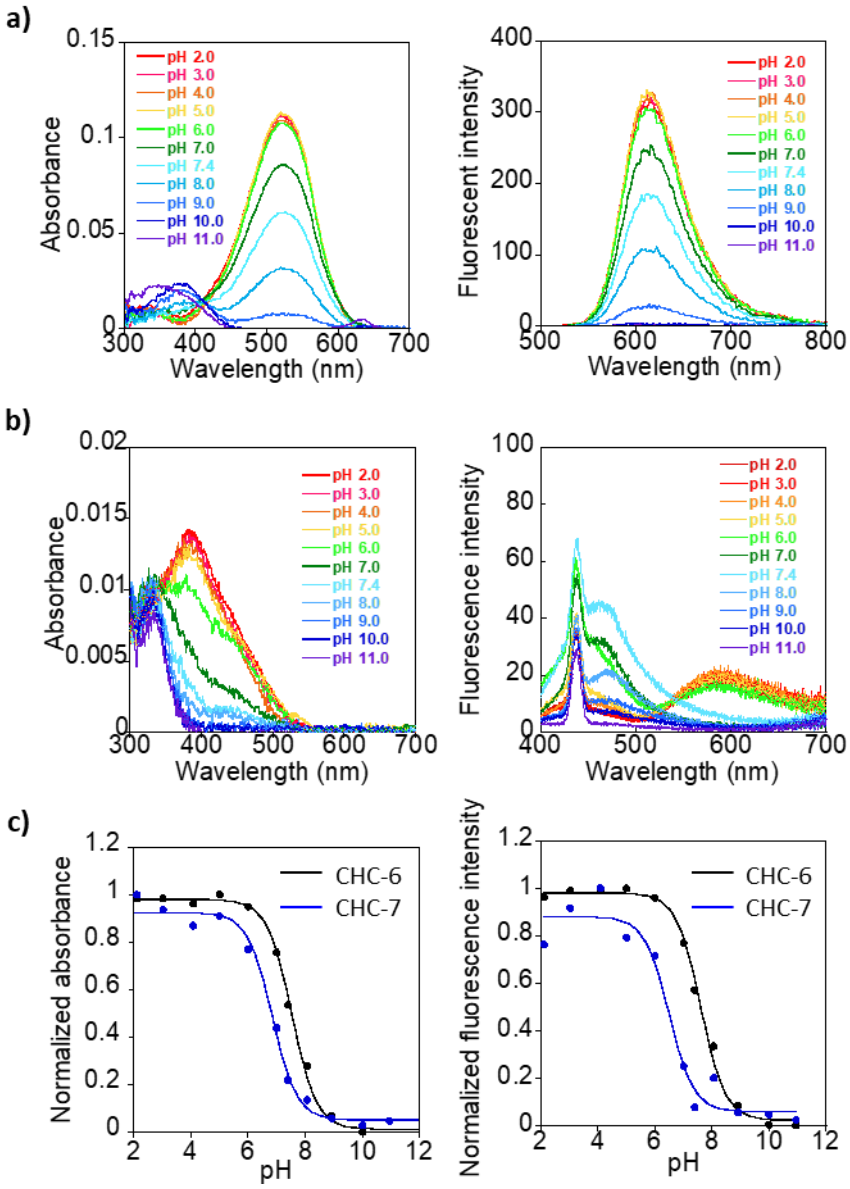


Figure S4. Absorption and fluorescence spectra of 2 μM solution of CHC-6 (a) and 1 μM solution of CHC-7 (b) at various pH values in 0.2 M sodium phosphate buffer. The excitation wavelength was 500 nm (a) and 380 nm (b). (c) pH profiles of absorption and fluorescence intensity of CHC-6 and CHC-7. Plots of normalized absorbance at 520 nm for CHC-6, 380 nm for CHC-7, and plots of normalized fluorescence intensity at 610 nm for CHC-6, 600 nm for CHC-7.

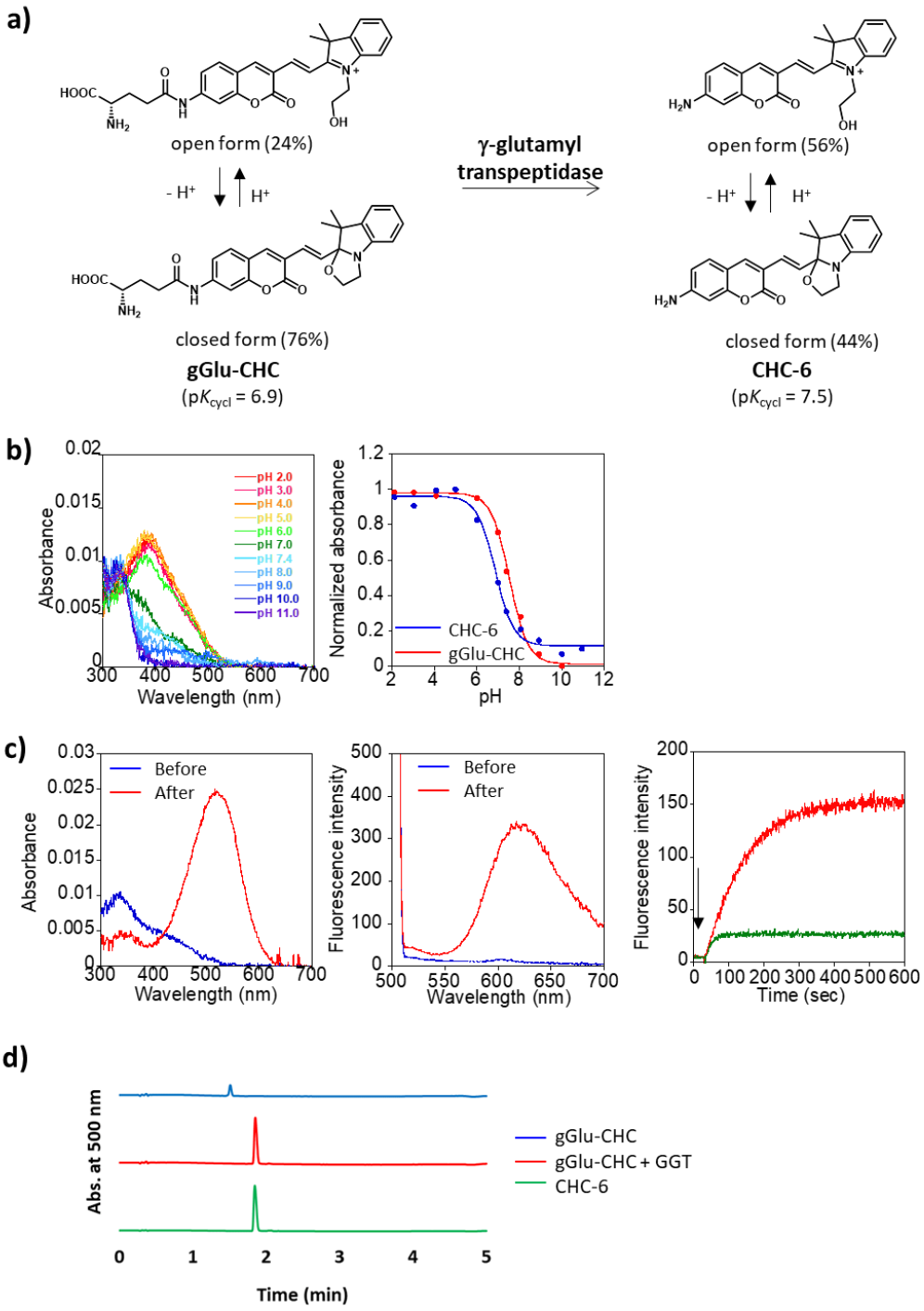


Figure S5. A newly developed fluorescence probe for γ-glutamyltranspeptidase (GGT) based on coumarine-hemicyanine hybrid fluorophore, gGlu-CHC. (a) Acid-base equilibrium of gGlu-CHC and its GGT-catalyzed hydrolysis product (CHC-6). Majority of gGlu-CHC exists in its spirocyclic form, while CHC-6 exist in a 1:1 equilibrium of open form and closed form at pH 7.4. (b) (left) Absorption spectra of 1 μM solution of gGlu-CHC at various pH values in 0.2 M sodium phosphate buffer. (right)

pH profiles of CHC-6 (red) and gGlu-CHC (blue) in 0.2 M sodium phosphate buffer at various pH values. Plots of normalized absorbance at 520 nm for CHC-6 and 380 nm for gGlu-CHC. (c) Absorption (left) and fluorescence (middle) spectra of 1 μM gGlu-CHC before (blue) and after (red) reaction with 1 unit GGT. (right) Time-dependent changes in the fluorescence intensity of gGlu-CHC upon addition of GGT (red, an arrow represents the timing of enzyme addition), and that in the presence of 50 μM GGT specific inhibitor; GGsTop (green). Spectra were measured in PBS (pH 7.4) containing 0.1% DMSO as a cosolvent. The excitation and emission wavelengths were 500 nm and 620 nm, respectively. (d) LC-MS analysis of the reaction solutions of gGlu-CHC with GGT. Absorbance monitored at 500 nm.

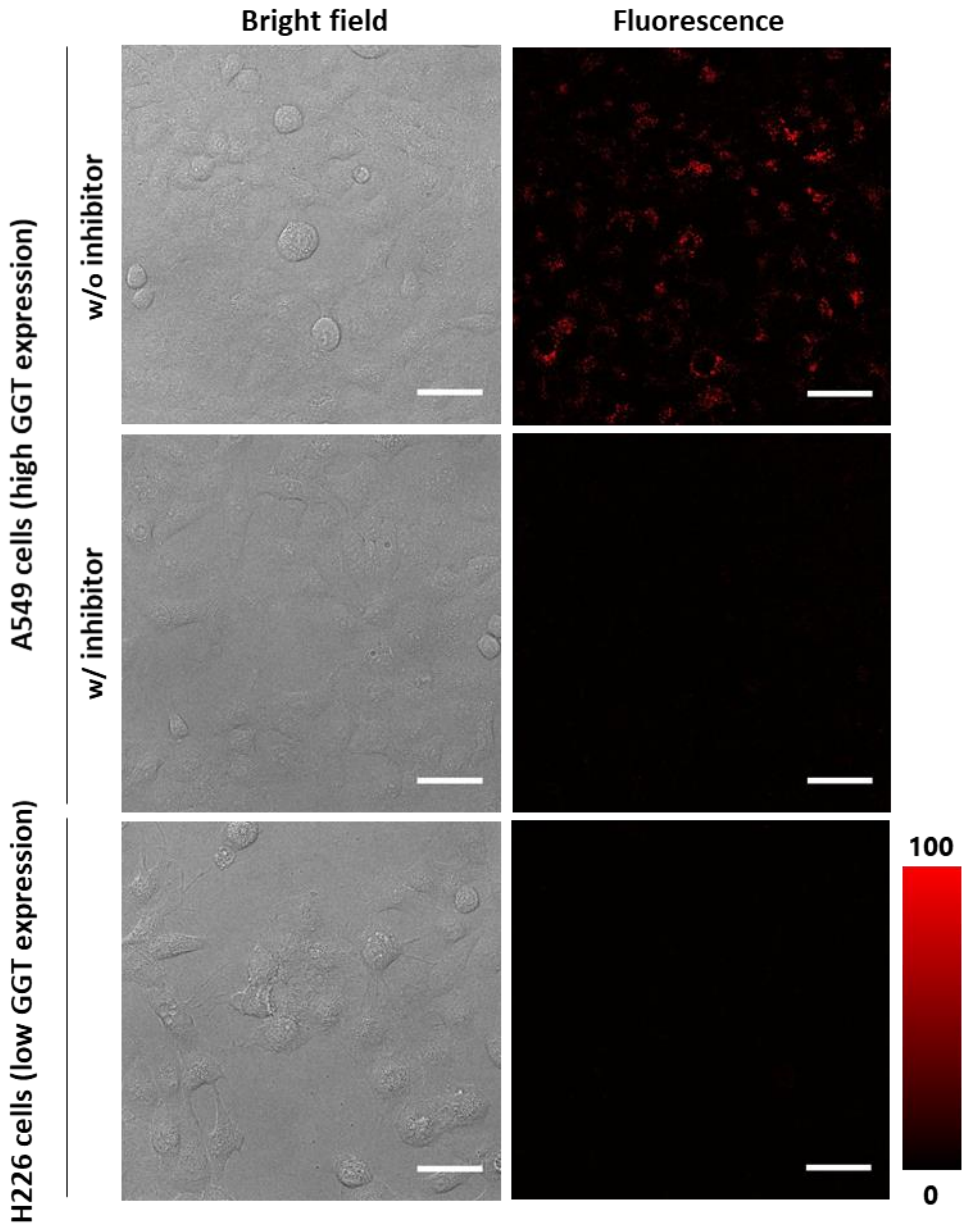


Figure S6. Fluorescence confocal microscopy imaging of GGT activity in live cultured cells. A549 cells with high GGT expression were incubated with 1 µM gGlu-CHC with (middle) or without (upper) 50 µM GGsTop containing <1% DMSO as a cosolvent for 1 h. H226 cells were incubated with 1 µM gGlu-CHC containing 0.1% DMSO as a cosolvent for 1 h (bottom). Excitation and detection wavelengths were 514 nm and 580-630 nm. Scale bar: 50 µm.

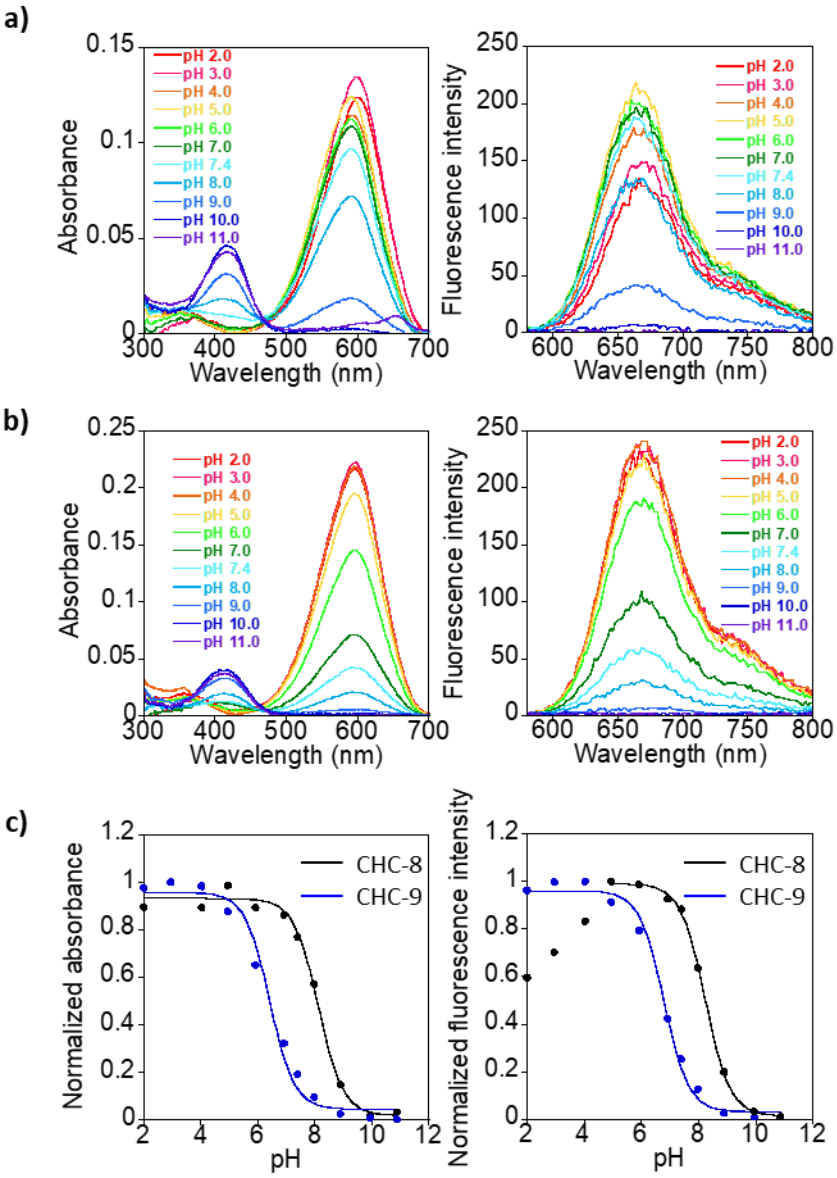


Figure S7. Absorption and fluorescence spectra of 2 μM solution of CHC-8 (a) and CHC-9 (b) at various pH values in 0.2 M sodium phosphate buffer. The excitation wavelength was 560 nm. (c) pH profiles of absorption and fluorescence intensity of CHC-8 and CHC-9. Plots of normalized absorbance at 580 nm for CHC-8 and 600 nm for CHC-9, and plots of normalized fluorescence intensity at 660 nm for CHC-8 and CHC-9.

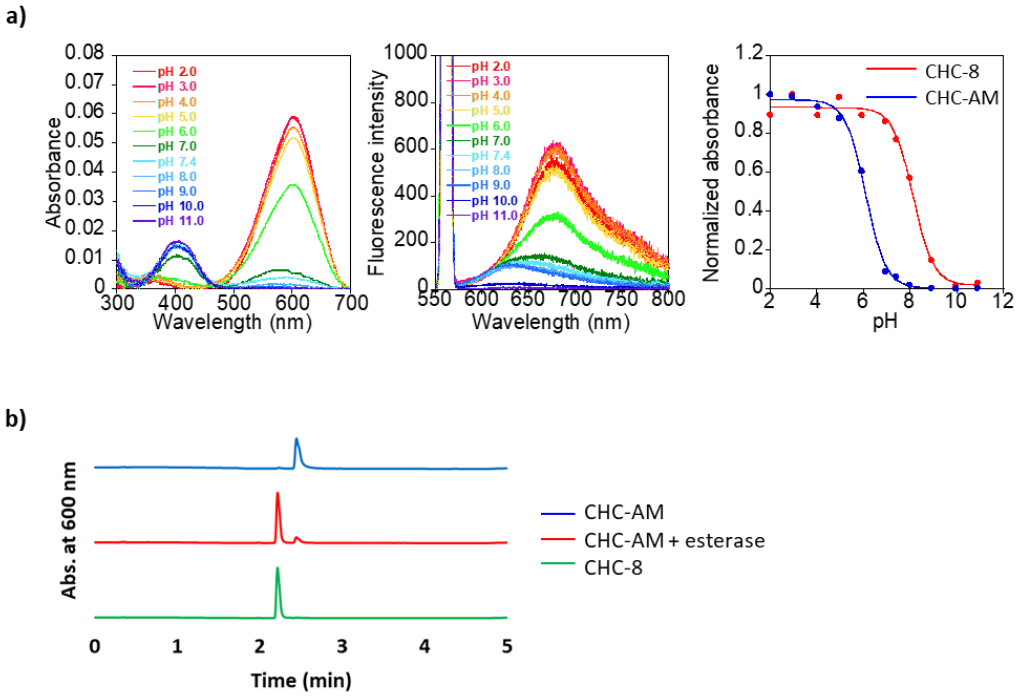


Figure S8. (a) Absorption (left) and fluorescence (middle) spectra of 1 µM solution of CHC-AM at various pH values in 0.2 M sodium phosphate buffer. The excitation wavelength was 560 nm. (right) pH profiles of absorption of CHC-8 (red) and CHC-AM (blue) in 0.2 M sodium phosphate buffer at various pH values. Plots of normalized absorbance at 600 nm. (b) LC-MS analysis of the reaction solutions of CHC-AM with esterase. Absorbance monitored at 600 nm.

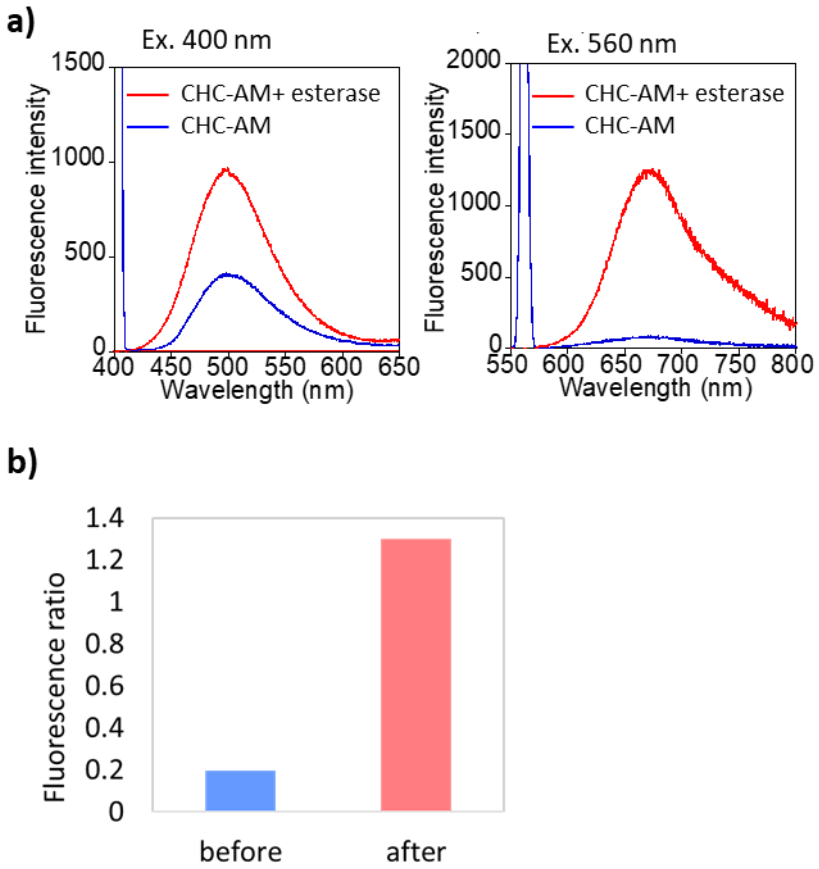


Figure S9. (a) Fluorescence spectra of 1 μM solution of CHC-AM in 0.2 M sodium phosphate buffer at pH 7.4, before (blue) and after (red) addition of esterase (10 units), excited by 400 nm (left) or 560 nm (right). (b) Ratio of fluorescence intensities of 675±5 nm (excited by 560 nm) and 500±5 nm (excited by 400 nm) before and after reaction with esterase.

References

1. L. Yuan, W. Lin and J. Song, *Chem. Comm.*, 2010, **46**, 7930-7932.

2. R. B. Mujumdar, L. A. Ernst, S. R. Mujumdar, and A. S. Waggoner, *Cytom.,* 1989, **10**, 11–19.