Supporting Information

In Vivo Multicolor Imaging with Fluorescent Probes Revealed the Dynamics and Function of Osteoclast Proton Pumps

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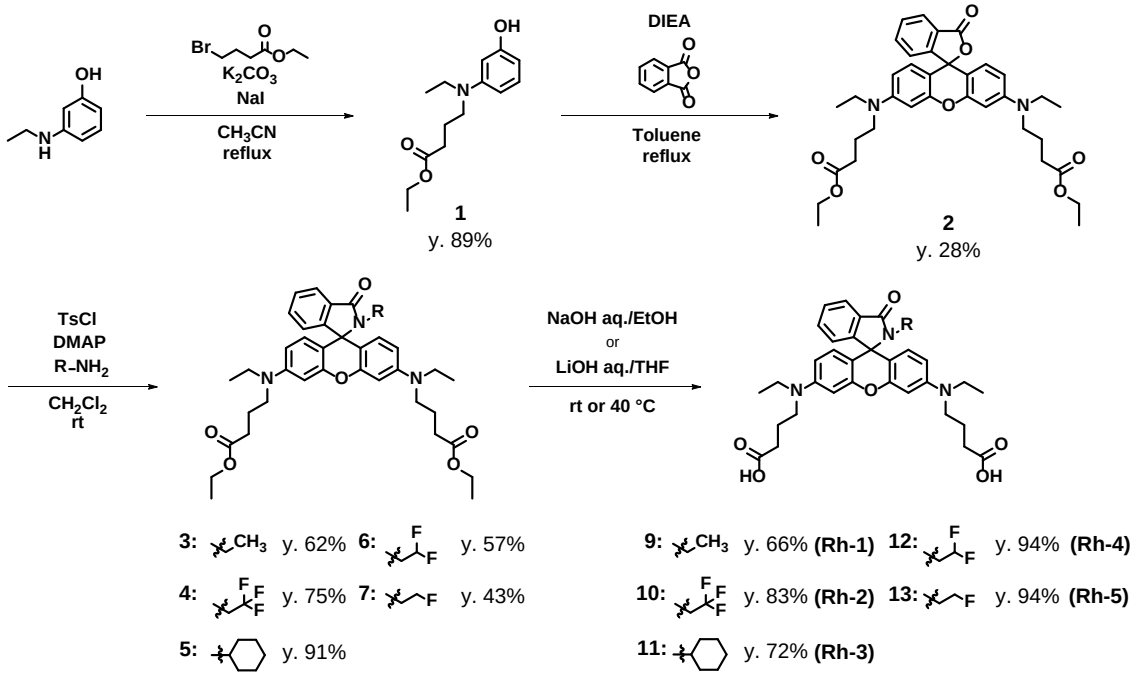
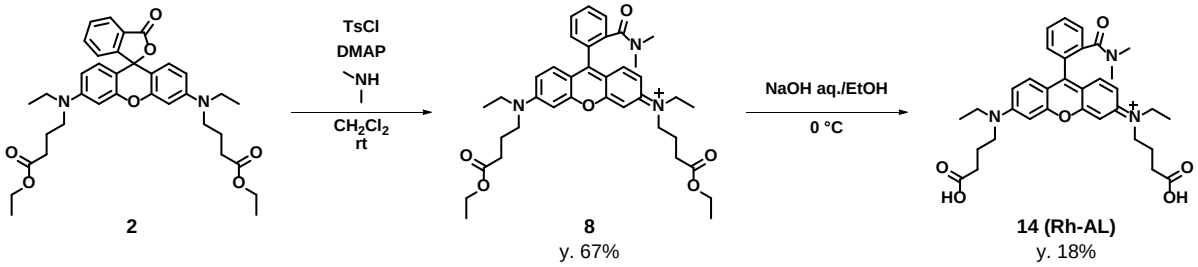
Materials and Methods

Reagents. Unless otherwise specified, all reagents were purchased from the chemical suppliers Tokyo Chemical Industries (Tokyo, Japan), Wako Pure Chemical (Osaka, Japan), or Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and used without further purification.

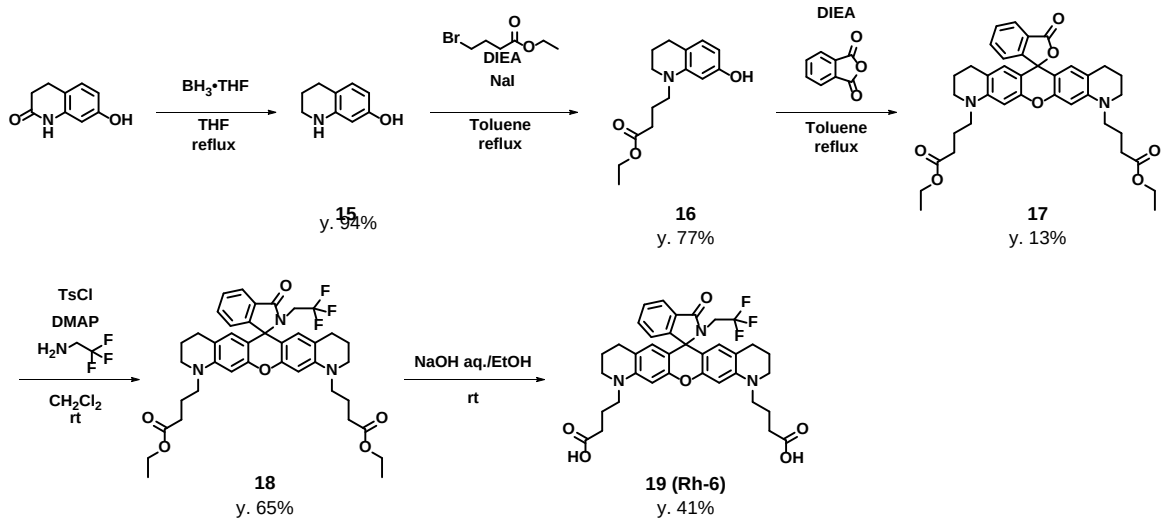
Synthesis and Material Characterization. Analytical thin-layer chromatography was performed on 60F254 silica plates (Merck & Co., Inc., Kenilworth, NJ, USA) and visualized under UV light. Flash auto purification was carried out using an Isolera Spectra (Biotage) with ZIP sphere cartridges, SNAP Ultra cartridges and SNAP C18 cartridge. Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis or purification were performed with Inertsil ODS-3 columns (4.6 × 250 mm and 10.0 × 250 mm; GL Sciences) using an HPLC system composed of a pump (PU-2080 and PU-2087; JASCO) and a detector (MD-2010 plus, UV-2075 and FP-2020; JASCO). Buffer A was composed of 0.1% formic acid in H_2O or 50 mM triethylammonium acetate in H_2O; buffer B was composed of 0.1% formic acid or 50 mM triethylammonium acetate in acetonitrile. Detailed synthetic procedures and characterizations of all compounds are described in the Supporting Information. Nuclear magnetic resonance (NMR) spectra were recorded on an AVANCE500HD instrument (Bruker, Billerica, MA, USA) at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR using tetramethylsilane as an internal standard. Mass spectra were measured on a JMS-700 mass spectrometer (JEOL) for FAB and on a LCT-Premier XE mass spectrometer (Waters, Milford, MA, USA) for electrospray ionization (ESI).



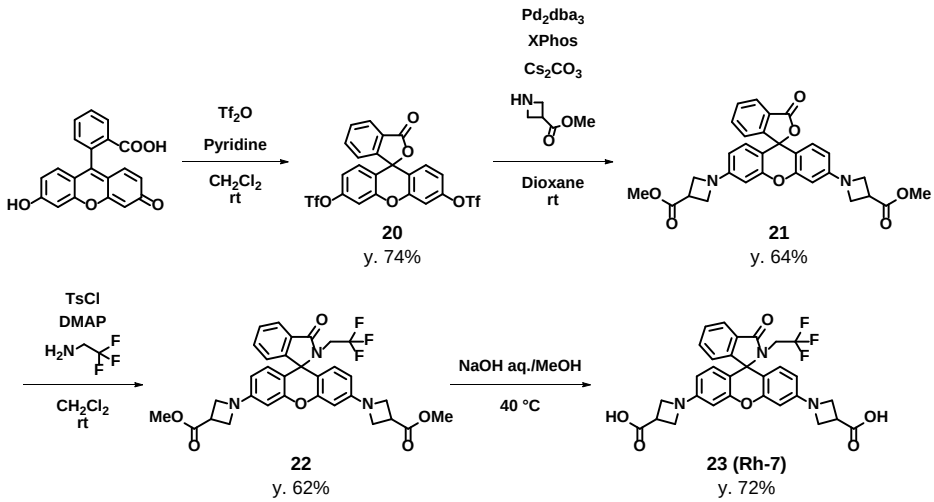
Scheme S1. Synthesis of **Rh-1-5**



Scheme S2. Synthesis of **Rh-AL**

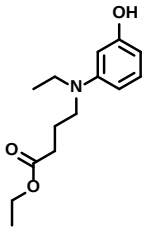


Scheme S3. Synthesis of **Rh-6**



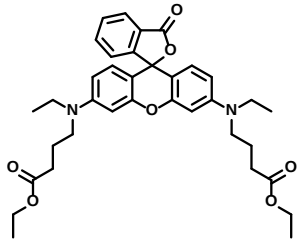
Scheme S4. Synthesis of **Rh-7**

Compound **1**



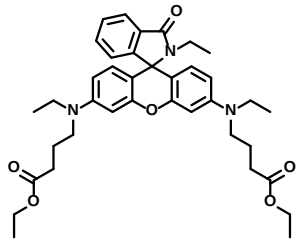
A mixture of 3-(ethylamino)phenol hemisulfate (1.00 g, 5.37 mmol), ethyl 4-bromobutanoate (2.31 mL, 16.1 mmol), *N*,*N*-diisopropylethylamine (9.35 mL, 53.7 mmol), and NaI (885 mg, 5.91 mmol) in toluene (40 mL) was refluxed for 12 h. The solvent was evaporated off and the residue was added water (50 mL). The aqueous layer was extracted with EtOAc (3 × 50 mL). The organic layer was combined, washed with brine (50 mL), dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (silica-packed 25 g snap column) to afford **1** (1.20 g, 4.78 mmol, 89%) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.04 (t, *J* = 8.0 Hz, 1H), 6.27 (dd, *J* = 8.0, 2.0 Hz, 1H), 6.20 (t, *J* = 2.0 Hz, 1H), 6.14 (dd, *J* = 8.0, 2.0 Hz, 1H), 4.15 (q, *J* = 7.0 Hz, 2H), 3.33 (q, *J* = 7.0 Hz, 2H), 3.27 (t, *J* = 7.0 Hz, 2H), 2.35 (t, *J* = 7.0 Hz, 2H), 1.91 (quin, *J* = 7.0 Hz, 2H), 1.26 (t, *J* = 7.0 Hz, 3H), 1.13 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.7, 157.0, 149.4, 130.1, 104.7, 102.8, 99.0, 60.6, 49.5, 45.1, 31.6, 22.7, 14.2, 12.3. HRMS (FAB+): calcd for $[M]^+$ 251.1521, found 251.1526.

Compound **2**



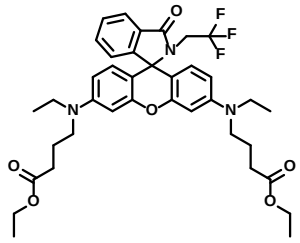
A mixture of **1** (752 mg, 2.99 mmol) and phthalic anhydride (886 mg, 5.98 mmol) in dry toluene (12 mL) was refluxed under an N_2 atmosphere. After stirring for 24 h, dry *N*,*N*-diisopropylethylamine (521 µL, 5.98 mmol) was added and the mixture was refluxed for 24 h. The solvent was evaporated off, after which 2 M HCl aq. (100 mL) was added, and the aqueous layer was extracted with CH_2Cl_2 (6 × 50 mL). The organic layer was combined, washed with brine (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (silica-packed 25 g snap column) to afford **2** (257 mg, 419 µmol, 28%) as a green solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.99 (d, *J* = 7.5 Hz, 1H), 7.63 (t, *J* = 7.5 Hz, 1H), 7.57 (t, *J* = 7.5 Hz, 1H), 7.20 (d, *J* = 7.5 Hz, 1H), 6.56 (d, *J* = 9.0 Hz, 2H), 6.45 (d, *J* = 2.5 Hz, 2H), 6.36 (dd, *J* = 9.0 Hz, 2.5 Hz, 2H), 4.14 (q, *J* = 7.5 Hz, 4H), 3.37 (q, *J* = 7.0 Hz, 4H), 3.31 (t, *J* = 7.0 Hz, 4H), 2.35 (t, *J* = 7.0 Hz, 4H), 1.92 (quin, *J* = 7.0 Hz, 4H), 1.26 (t, *J* = 7.5 Hz, 6H), 1.16 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.2, 169.8, 153.2, 153.1, 149.5, 134.5, 129.2, 129.0, 127.8, 124.7, 124.1, 108.2, 106.3, 97.9, 85.5, 60.5, 49.5, 45.1, 31.4, 22.7, 14.2, 12.2. HRMS (FAB+): calcd for $[M]^+$ 615.3065, found 615.3068.

Compound **3**



To a stirred solution of **2** (50.0 mg, 81.2 µmol) in dry CH_2Cl_2 (10 mL) was added toluenesulfonylchloride (31.0 mg, 162 µmol) under an N_2 atmosphere. To this stirred mixture after 15 minutes was added 4-dimethylaminopyridine (24.8 mg, 203 µmol) at room temperature. After stirring the mixture after 15 minutes, ethylamine (*ca*. 10% in THF, 61 µL, 122 µmol) was added and the reaction mixture was stirred for 19 h. The mixture was quenched with saturated $NaHCO_3$ aq. and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was combined, washed with brine (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (silica-packed 10 g snap column) to afford **3** (32.4 mg, 50.3 µmol, 62%) as a colorless solid. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.86-7.84 (m, 1H), 7.52-7.50 (m, 2H), 7.05-7.03 (m, 1H), 6.47 (d, J = 2.5 Hz, 2H), 6.31 (dd, J = 9.0 Hz, 2.5 Hz, 2H), 6.35 (d, J = 9.0 Hz, 2H), 4.13 (q, J = 7.0 Hz, 4H), 3.36 (q, J = 7.0 Hz, 4H), 3.31 (t, J = 7.0 Hz, 4H), 3.13 (d, J = 7.5 Hz, 2H), 2.37 (t, J = 7.0 Hz, 4H), 1.89 (quin, J = 7.0 Hz, 4H), 1.24 (t, J = 7.0 Hz, 6H), 1.14 (t, J = 7.0 Hz, 6H), 0.765 (t, J = 7.5 Hz, 3H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 173.7, 168.3, 153.7, 153.4, 149.1, 132.5, 131.0, 128.3, 128.1, 123.6, 122.0, 108.2, 105.2, 97.9, 65.5, 60.2, 49.0, 44.5, 34.7, 30.8, 22.3, 13.2, 12.6, 11.0. HRMS (FAB+): calcd for $[M]^+$ 642.3538, found 642.3532.

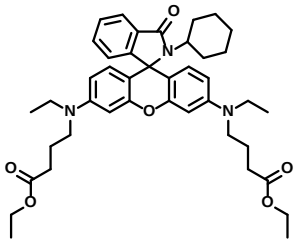
Compound **4**



To a stirred solution of **2** (50.0 mg, 81.2 µmol) in dry CH_2Cl_2 (10 mL) was added toluenesulfonylchloride (31.0 mg, 162 µmol) under an N_2 atmosphere. To this stirred mixture after 15 minutes was added 4-dimethylaminopyridine (24.8 mg, 203 µmol) at room temperature. After stirring the mixture after 15 minutes, a solution of 2,2,2-trifluoroethylamine (9.6 µL, 122 µmol) in CH_2Cl_2 (1 mL) was added and the reaction mixture was stirred for 19 h. The mixture was quenched with saturated $NaHCO_3$ aq. and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was combined, washed with brine (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (silica-packed 10 g snap column) to afford **4** (42.4 mg, 60.9 µmol, 75%) as a colorless solid. ^{1}H NMR (500 MHz,

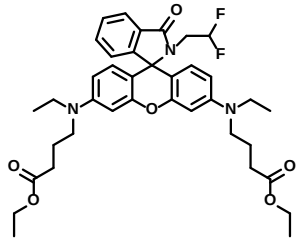
Methanol-d_4): δ 7.91-7.89 (m, 1H), 7.60-7.52 (m, 2H), 7.07-7.05 (m, 1H), 6.47 (d, *J* = 2.5 Hz, 2H), 6.37 (dd, *J* = 9.0, 2.5 Hz, 2H), 6.33 (d, *J* = 9.0 Hz, 2H), 4.13 (q, *J* = 7.0 Hz, 4H), 3.70 (q, *J* = 9.5 Hz, 2H), 3.39 (q, *J* = 7.0 Hz, 4H), 3.33 (t, *J* = 7.0 Hz, 4H), 2.37 (t, *J* = 7.0 Hz, 4H), 1.89 (quin, *J* = 7.0 Hz, 4H), 1.24 (t, *J* = 7.0 Hz, 6H), 1.14 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 173.7, 169.8, 154.1, 153.4, 149.2, 133.5, 129.1, 128.4, 128.3, 124.1, 122.5, 108.5, 104.3, 97.8, 66.1, 60.2, 49.0, 44.5, 41.3, 41.1, 30.7, 22.3, 13.2, 11.1. HRMS (FAB+): calcd for $[M]^+$ 696.3255, found 696.3260.

Compound **5**



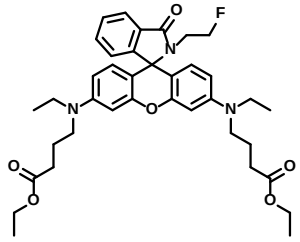
To a stirred solution of **2** (50.0 mg, 81.2 µmol) in dry CH_2Cl_2 (10 mL) was added toluenesulfonylchloride (31.0 mg, 162 µmol) under an N_2 atmosphere. To this stirred mixture after 15 minutes was added 4-dimethylaminopyridine (24.8 mg, 203 µmol) at room temperature. After stirring the mixture after 15 minutes, a solution of cyclohexylamine (14 µL, 122 µmol) in CH_2Cl_2 (1 mL) was added and the reaction mixture was stirred for 24 h. The mixture was quenched with saturated $NaHCO_3$ aq. and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was combined, washed with brine (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (silica-packed 10 g snap column) to afford **5** (51.5 mg, 73.9 µmol, 91%) as a pale pink solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.85 (dd, *J* = 7.0 Hz, 1.5 Hz, 1H), 7.40-7.37 (m, 2H), 6.97 (dd, *J* = 7.0 Hz, 1.5 Hz, 1H), 6.50 (d, *J* = 9.0 Hz, 2H), 6.38 (d, *J* = 2.5 Hz, 2H), 6.27 (dd, *J* = 9.0 Hz, 2.5 Hz, 2H), 4.15 (q, *J* = 7.5 Hz, 4H), 3.35 (t, *J* = 7.0 Hz, 4H), 3.30 (q, *J* = 7.0 Hz, 4H), 2.94-2.88 (m, 1H), 2.35 (t, *J* = 7.0 Hz, 4H), 2.19-2.11 (m, 2H), 1.93 (quin, *J* = 7.0 Hz, 4H), 1.59-1.56 (m, 1H), 1.46-1.43 (m, 1H), 1.26 (t, *J* = 7.5 Hz, 6H), 1.16 (t, *J* = 7.0 Hz, 6H), 1.46-1.08 (m, 4H), 0.98-0.90 (m, 2H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 173.7, 167.9, 153.4, 153.3, 149.1, 132.3, 132.1, 128.8, 128.0, 123.6, 121.7, 108.0, 105.1, 97.8, 66.3, 60.2, 54.2, 48.9, 44.5, 30.8, 29.2, 25.9, 25.0, 22.3, 13.2, 11.0. HRMS (FAB+): calcd for $[M]^+$ 696.4007, found 696.3992.

Compound **6**



To a stirred solution of **2** (50.0 mg, 81.2 µmol) in dry CH_2Cl_2 (10 mL) was added toluenesulfonylchloride (31.0 mg, 162 µmol) under an N_2 atmosphere. To this stirred mixture after 15 minutes was added 4-dimethylaminopyridine (24.8 mg, 203 µmol) at room temperature. After stirring the mixture after 15 minutes, a solution of 2,2-difluoroethylamine (8.5 µL, 122 µmol) in CH_2Cl_2 (1 mL) was added and the reaction mixture was stirred for 17 h. The mixture was quenched with saturated $NaHCO_3$ aq. and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was combined, washed with brine (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (silica-packed 10 g snap column) to afford **6** (31.4 mg, 46.3 µmol, 57%) as a colorless solid. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.90-7.88 (m, 1H), 7.59-7.52 (m, 2H), 7.08-7.07 (m, 1H), 6.49 (d, *J* = 2.5 Hz, 2H), 6.42 (dd, *J* = 9.0, 2.5 Hz, 2H), 6.36 (d, *J* = 9.0 Hz, 2H), 5.18 (tt, *J* = 61.3 Hz, 4.8 Hz, 1H), 4.13 (q, *J* = 7.0 Hz, 4H), 3.44-3.37 (m, 6H), 3.34 (t, *J* = 7.0 Hz, 4H), 2.37 (t, *J* = 7.0 Hz, 4H), 1.89 (quin, *J* = 7.0 Hz, 4H), 1.24 (t, *J* = 7.0 Hz, 6H), 1.14 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 173.7, 169.0, 153.4, 153.3, 149.3, 133.2, 129.9, 128.3, 128.2, 123.9, 122.4, 113.1, 108.5, 104.4, 97.8, 65.6, 60.2, 49.0, 44.5, 42.1, 30.7, 22.3, 13.2, 11.0. HRMS (FAB+): calcd for $[M]^+$ 678.3349, found 678.3358.

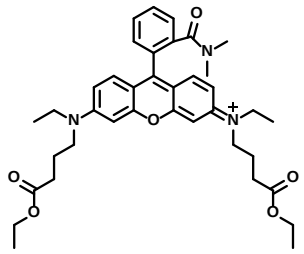
Compound **7**



To a stirred solution of **2** (50.0 mg, 81.2 µmol) in dry CH_2Cl_2 (10 mL) was added toluenesulfonylchloride (31.0 mg, 162 µmol) under an N_2 atmosphere. To this stirred mixture after 15 minutes was added 4-dimethylaminopyridine (24.8 mg, 203 µmol) at room temperature. After stirring the mixture after 15 minutes, 2-fluoroethylamine hydrochloride (12.1 mg, 122 µmol) was added and the reaction mixture was stirred for 19 h. The mixture was quenched with saturated $NaHCO_3$ aq. and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was combined, washed with brine (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (silica-packed 10 g snap column) to afford **7** (23.1 mg, 34.9 µmol, 43%) as a colorless solid. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.87-7.86 (m, 1H), 7.55-7.50 (m, 2H), 7.05-7.04 (m, 1H), 6.48 (d, *J* = 2.5 Hz, 2H), 6.40 (dd, *J* = 9.0 Hz, 2.5 Hz, 2H), 6.36 (d,

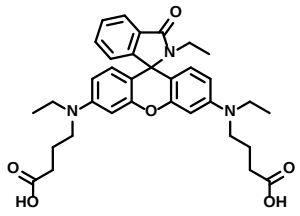
J = 9.0 Hz, 2H), 4.12 (q, *J* = 7.0 Hz, 4H), 3.96 (td, *J* = 6.3 Hz, 46.8 Hz, 2H), 3.42-3.35 (m, 6H), 3.33 (t, *J* = 7.0 Hz, 4H), 2.37 (t, *J* = 7.0 Hz, 4H), 1.89 (quin, *J* = 7.0 Hz, 4H), 1.24 (t, *J* = 7.0 Hz, 6H), 1.14 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 173.7, 169.0, 153.7, 153.3, 149.2, 132.9, 130.3, 128.2, 128.1, 123.7, 122.2, 108.4, 104.8, 97.9, 79.5, 65.5, 60.2, 49.0, 44.5, 39.6, 30.7, 22.3, 13.2, 11.0. HRMS (FAB+): calcd for $[M]^+$ 660.3443, found 660.3455.

Compound **8**



To a stirred solution of **2** (50.0 mg, 81.2 µmol) in dry CH_2Cl_2 (10 mL) was added toluenesulfonylchloride (31.0 mg, 162 µmol) under an N_2 atmosphere. To this stirred mixture after 15 minutes was added 4-dimethylaminopyridine (24.8 mg, 203 µmol) at room temperature. After stirring the mixture after 15 minutes, dimethylamine (*ca*. 10% in THF, 61 µL, 122 µmol) was added and the reaction mixture was stirred for 21 h. The mixture was quenched with saturated $NaHCO_3$ aq. and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was combined, washed with brine (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (silica-packed 10 g snap column) to afford **8** (31.9 mg, 54.4 µmol, 67%) as a green solid. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.77-7.74 (m, 2H), 7.69-7.67 (m, 1H), 7.51-7.50 (m, 1H), 7.29 (d, *J* = 9.5 Hz, 2H), 7.14 (dd, *J* = 9.5 Hz, 2.0 Hz, 2H), 7.06 (d, *J* = 2.0 Hz, 2H), 4.17 (q, *J* = 7.0 Hz, 4H), 3.71 (q, *J* = 7.0 Hz, 4H), 3.65 (t, *J* = 7.0 Hz, 4H), 2.97 (s, 3H), 2.74 (s, 3H), 2.48 (t, *J* = 7.0 Hz, 4H), 2.00 (quin, *J* = 7.0 Hz, 4H), 1.31 (t, *J* = 7.0 Hz, 6H), 1.27 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 173.2, 169.3, 158.0, 156.2, 135.9, 131.8, 130.8, 130.3, 129.8, 129.6, 128.4, 127.5, 125.6, 114.0, 113.6, 96.2, 60.4, 49.9, 45.9, 38.6, 33.8, 30.1, 13.2, 11.3. HRMS (FAB+): calcd for $[M]^+$ 642.3538, found 642.3532.

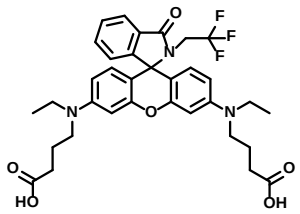
Compound **9** (**Rh-1**)



To a stirred solution of **3** (25.7 mg, 40.0 µmol) in EtOH (4 mL) was added 2 M NaOH aq. (8 mL) at room temperature. After stirring for 3 h, the mixture was quenched with 2 M HCl aq. and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (C18 12 g snap column) to afford **9**

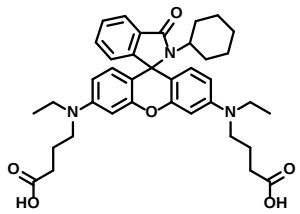
(15.5 mg, 26.4 µmol, 66%) as a purple solid. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.86-7.84 (m, 1H), 7.54-7.51 (m, 2H), 7.07-7.05 (m, 1H), 6.49 (d, *J* = 2.5 Hz, 2H), 6.41 (dd, *J* = 9.0 Hz, 2.5 Hz, 2H), 6.35 (d, *J* = 9.0 Hz, 2H), 3.40 (q, *J* = 7.0 Hz, 4H), 3.34 (t, *J* = 7.5 Hz, 4H), 3.13 (q, *J* = 7.0 Hz, 2H), 2.35 (t, *J* = 7.0 Hz, 4H), 1.88 (quin, *J* = 7.0 Hz, 4H), 1.15 (t, *J* = 7.0 Hz, 6H), 0.767 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 175.7, 168.2, 153.7, 153.4, 149.1, 132.5, 131.0, 128.3, 128.1, 123.6, 122.0, 108.2, 105.2, 97.9, 65.5, 49.1, 44.5, 34.7, 30.5, 22.3, 12.6, 11.1. HRMS (FAB+): calcd for $[M]^+$ 586.2912, found 586.2928.

Compound **10** (**Rh-2**)



To a stirred solution of **4** (27.9 mg, 40.0 µmol) in EtOH (4 mL) was added 2 M NaOH aq. (8 mL) at 40 °C. After stirring for 22 h, the mixture was quenched with 2 M HCl aq. and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (C18 12 g snap column) to afford **10** (21.3 mg, 33.2 µmol, 83%) as a purple solid. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.89 (d, *J* = 7.0 Hz, 1H), 7.60-7.55 (m, 2H), 7.09 (d, *J* = 7.5 Hz, 1H), 6.48 (d, *J* = 2.5 Hz, 2H), 6.38 (dd, *J* = 9.0 Hz, 2.5 Hz, 2H), 6.34 (d, *J* = 9.0 Hz, 2H), 3.70 (d, *J* = 9.5 Hz, 2H), 3.40 (q, *J* = 7.0 Hz, 4H), 3.35 (t, *J* = 7.0 Hz, 4H), 2.35 (t, *J* = 7.0 Hz, 4H), 1.88 (quin, *J* = 7.0 Hz, 4H), 1.15 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 169.8, 154.1, 153.4, 149.2, 133.5, 129.1, 128.4, 128.3, 124.1, 122.4, 108.4, 104.3, 97.8, 66.2, 49.1, 48.2, 44.5, 41.2, 41.1, 30.5, 22.3, 11.1. HRMS (FAB+): calcd for $[M]^+$ 640.2629, found 640.2627.

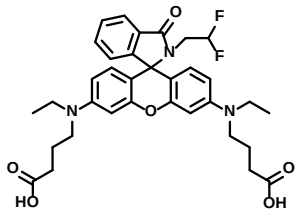
Compound **11** (**Rh-3**)



To a stirred solution of **7** (27.8 mg, 40.0 µmol) in EtOH (4 mL) was added 2 M NaOH aq. (8 mL) at 40 °C. After stirring for 4 h, the mixture was quenched with 2 M HCl aq. and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (C18 12 g snap column) to afford **11** (18.4 mg, 28.8 µmol, 72%) as a purple solid. ^{1}H NMR (500 MHz, Methanol-d_4 + TFA): δ 8.07 (d, *J* = 8.0 Hz, 1H), 7.83-7.80 (m, 1H), 7.76-7.74 (m, 2H), 7.47-7.45 (m, 1H), 7.29 (d, *J* = 9.5 Hz, 2H), 7.12 (d, *J* = 9.5 Hz, 2H), 7.05 (s, 2H), 3.70 (t, *J* = 7.0 Hz, 4H), 3.65 (q, *J* = 7.5 Hz, 4H), 3.48-3.40 (m, 1H), 2.45 (t, *J* = 7.0 Hz, 4H), 1.99 (quin, *J* = 7.0 Hz, 4H), 1.65-1.56 (m, 5H), 1.30 (t, *J* = 7.5 Hz, 6H), 1.22-1.09 (m, 5H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 175.1, 158.0,

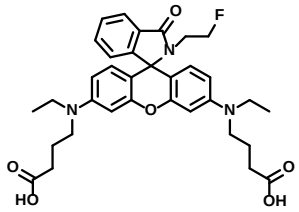
156.0, 153.4, 137.2, 131.6, 131.3, 130.3, 130.1, 127.6, 123.6, 121.7, 114.0, 113.6, 96.1, 50.0, 45.9, 32.0, 29.9, 29.2, 25.9, 24.7, 22.2, 11.2. HRMS (FAB+): calcd for $[M]^+$ 640.3381, found 640.3391.

Compound **12** (**Rh-4**)



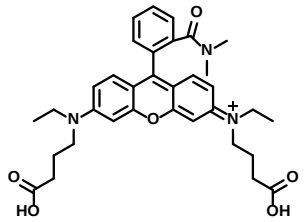
To a stirred solution of **5** (27.1 mg, 40.0 µmol) in THF (5 mL) was added a solution of lithium hydroxide monohydrate (16.8 mg, 400 µmol) in H_2O (4 mL) at room temperature. After stirring for 6 h, the mixture was quenched with 2 M HCl aq. and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (C18 12 g snap column) to afford **12** (23.4 mg, 37.6 µmol, 94%) as a purple solid. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.89-7.87 (m, 1H), 7.59-7.52 (m, 2H), 7.09-7.07 (m, 1H), 6.51 (d, J = 2.5 Hz, 2H), 6.43 (dd, J = 9.0 Hz, 2.5 Hz, 2H), 6.35 (d, J = 9.0 Hz, 2H), 5.17 (tt, J = 56.5 Hz, 4.8 Hz, 1H), 3.44-3.38 (m, 6H), 3.34 (t, J = 7.0 Hz, 4H), 2.35 (t, J = 7.0 Hz, 4H), 1.88 (quin, J = 7.0 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 175.7, 169.0, 153.5, 153.4, 149.4, 133.2, 129.9, 128.3, 128.2, 123.9, 122.4, 113.1, 108.5, 104.3, 97.8, 65.6, 49.1, 44.5, 42.1, 30.5, 22.3, 11.1. HRMS (FAB+): calcd for $[M]^+$ 622.2723, found 622.2724.

Compound **13** (**Rh-5**)



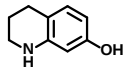
To a stirred solution of **6** (26.4 mg, 40.0 µmol) in THF (5 mL) was added a solution of lithium hydroxide monohydrate (16.8 mg, 400 µmol) in H_2O (4 mL) at room temperature. After stirring for 6 h, the mixture was quenched with 2 M HCl aq. and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (C18 12 g snap column) to afford **13** (22.7 mg, 37.6 µmol, 94%) as a purple solid. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.87-7.85 (m, 1H), 7.56-7.50 (m, 2H), 7.06-7.05 (m, 1H), 6.49 (d, J = 2.5 Hz, 2H), 6.41 (dd, J = 9.0 Hz, 2.5 Hz, 2H), 6.36 (d, J = 9.0 Hz, 2H), 3.96 (td, J = 6.3 Hz, 46.8 Hz, 2H), 3.42-3.33 (m, 10H), 2.35 (t, J = 7.0 Hz, 4H), 1.88 (quin, J = 7.0 Hz, 4H), 1.15 (t, J = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 175.7, 169.0, 153.7, 153.3, 149.2, 132.9, 130.3, 128.2, 123.7, 122.2, 108.4, 104.8, 97.9, 80.3, 78.9, 65.5, 49.1, 44.5, 39.6, 30.5, 22.3, 11.1. HRMS (FAB+): calcd for $[M]^+$ 604.2817, found 604.2817.

Compound **14** (**Rh-AL**)



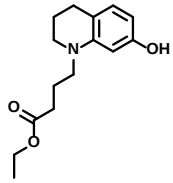
To a stirred solution of **8** (25.7 mg, 40.0 µmol) in EtOH (4 mL) was added 2 M NaOH aq. (8 mL) at 0 °C. After stirring for 1 h, the mixture was quenched with 2 M HCl aq. and concentrated under reduced pressure. The residue was purified by reversed phase HPLC under the following conditions: A/B = 25/75 (0 min), 45/55 (30 min) (solvent A: 50 mM TEAA aq.; solvent B: CH_3CN). After lyophilization, a purple powder of **14** (422 µg, 7.20 µmol, 18%) was obtained. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.77-7.74 (m, 2H), 7.69-7.67 (m, 1H), 7.51 (m, 1H), 7.29 (d, J = 9.5 Hz, 2H), 7.14 (dd, J = 9.5 Hz, 2.0 Hz, 2H), 7.06 (d, J = 2.0 Hz, 2H), 4.17 (q, J = 7.0 Hz, 4H), 3.71 (q, J = 7.0 Hz, 4H), 3.65 (t, J = 7.0 Hz, 4H), 2.97 (s, 3H), 2.74 (s, 3H), 2.48 (t, J = 7.0 Hz, 4H), 2.00 (quin, J = 7.0 Hz, 4H), 1.31 (t, J = 7.0 Hz, 6H), 1.27 (t, J = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 168.7, 158.4, 157.0, 156.6, 145.6, 139.5, 137.1, 132.5, 131.3, 130.8, 130.3, 129.9, 128.9, 128.1, 126.3, 114.5, 114.2, 51.0, 46.6, 39.3, 34.5, 20.9. HRMS (FAB+): calcd for $[M]^+$ 586.2912, found 586.2915.

Compound **15**



Compound **15** was prepared by the literature method[S1].

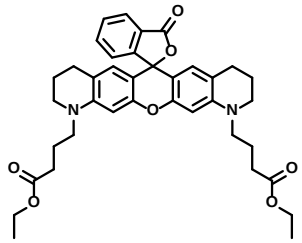
Compound **16**



A mixture of **15** (1.00 g, 6.70 mmol), ethyl 4-bromobutanoate (2.88 mL, 20.1 mmol), *N*,*N*-diisopropylethylamine (11.7 mL, 67.0 mmol), and NaI (1.10 g, 7.37 mmol) in toluene (40 mL) was refluxed for 18 h. The solvent was evaporated off and the residue was added water (50 mL). The aqueous layer was extracted with EtOAc (3 × 50 mL). The organic layer was combined, washed with brine (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (silica-packed 25 g snap column) to afford **16** (1.36 g, 5.16 mmol, 77%) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.77 (d, J = 8.0 Hz, 1H), 6.12 (d, J = 2.5 Hz, 1H), 6.04 (dd, J = 8.0 Hz, 2.5 Hz, 1H), 4.83 (br, 1H), 4.15 (q, J = 7.0 Hz, 2H), 3.25-3.22 (m, 4H), 2.66 (t, J = 6.5 Hz, 2H), 2.35 (t, J = 7.0 Hz, 2H), 1.94-1.88 (m, 4H), 1.26 (t, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz,

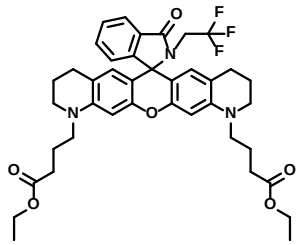
$CDCl_3$): δ 173.5, 155.1, 146.1, 130.0, 114.8, 102.2, 97.7, 60.5, 50.7, 49.4, 31.6, 27.4, 22.4, 21.6, 14.2. HRMS (FAB+): calcd for $[M]^+$ 263.1521, found 263.1523.

Compound **17**



A mixture of **16** (360 mg, 1.37 mmol) and phthalic anhydride (405 mg, 2.73 mmol) in dry toluene (10 mL) was refluxed under an N_2 atmosphere. After stirring for 24 h, dry *N*,*N*-diisopropylethylamine (476 µL, 2.73 mmol) was added and the mixture was refluxed for 24 h. The solvent was evaporated off, after which 2 M HCl aq. (100 mL) was added, and the aqueous layer was extracted with CH_2Cl_2 (6 × 50 mL). The organic layer was combined, washed with brine (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (silica-packed 25 g snap column) to afford **17** (57.0 mg, 89.1 µmol, 13%) as a green solid. ^{1}H NMR (500 MHz, Methanol-d_4): δ 8.26 (dd, *J* = 9.0Hz, 1.5Hz, 1H), 7.79-7.73 (m, 2H), 7.33 (dd, *J* = 8.5 Hz, 1.5 Hz, 1H), 7.02 (s, 2H), 6.80 (s, 2H), 4.20 (q, *J* = 7.0 Hz, 4H), 3.63 (t, *J* = 7.0 Hz, 4H), 3.58 (t, *J* = 6.0 Hz, 4H), 2.70 (t, *J* = 6.0 Hz, 4H), 2.50 (t, *J* = 7.0 Hz, 4H) 2.03 (quin, *J* = 7.0 Hz, 4H), 1.94 (quin, *J* = 6.0 Hz, 4H), 1.28 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 173.3, 167.1, 157.8, 157.1, 153.9, 134.1, 132.3, 131.4, 131.0, 130.1, 129.8, 127.5, 125.4, 113.5, 94.9, 60.4, 51.1, 49.8, 30.3, 27.1, 20.7, 20.6, 13.2. HRMS (FAB+): calcd for $[M]^+$ 639.3065, found 639.3074.

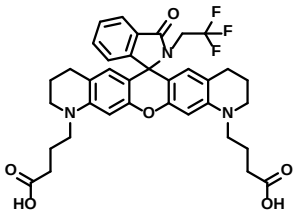
Compound **18**



To a stirred solution of **17** (50.0 mg, 78.2 µmol) in dry CH_2Cl_2 (10 mL) was added toluenesulfonylchloride (29.8 mg, 156 µmol) under an N_2 atmosphere. To this stirred mixture after 15 minutes was added 4-dimethylaminopyridine (23.9 mg, 195 µmol) at room temperature. After stirring the mixture after 15 minutes, a solution of 2,2,2-trifluoroethylamine (9.2 µL, 117 µmol) in CH_2Cl_2 (1 mL) was added and the reaction mixture was stirred for 16 h. The mixture was quenched with saturated $NaHCO_3$ aq. and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was combined, washed with brine (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (silica-packed 10 g snap column) to afford **18** (37.1 mg, 50.8 µmol, 65%) as a pale pink solid. ^{1}H NMR (500 MHz,

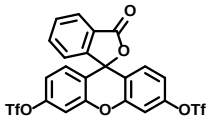
Methanol-d_4): δ 7.90-7.88 (m, 1H), 7.60-7.52 (m, 2H), 7.07-7.05 (m, 1H), 6.37 (s, 2H), 6.01 (s, 2H), 4.15 (q, J = 7.0 Hz, 4H), 3.69 (q, J = 9.5 Hz, 2H), 3.34 (t, J = 7.0 Hz, 4H), 3.26 (t, J = 6.0 Hz, 4H), 2.51-2.44 (m, 4H), 2.41 (t, J = 7.0 Hz, 4H), 1.94 (quin, J = 7.0 Hz, 4H), 1.84-1.78 (m, 4H), 1.27 (t, J = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 173.7, 169.7, 154.1, 151.9, 146.3, 133.5, 129.1, 128.3, 126.9, 124.0, 122.5, 118.9, 103.7, 96.6, 66.3, 60.2, 50.1, 48.7, 41.4, 41.1, 30.9, 27.1, 21.8, 20.9, 13.2. HRMS (FAB+): calcd for $[M]^+$ 720.3255, found 720.3256.

Compound **19** (**Rh-6**)



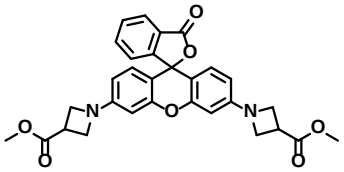
To a stirred solution of **18** (28.8 mg, 40.0 μmol) in EtOH (4 mL) was added 2 M NaOH aq. (8 mL) at room temparature. After stirring for 7 h, the mixture was quenched with 2 M HCl aq. and the aqueous layer was extracted with CH_2Cl_2 (5 × 50 mL). The organic layer was combined, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by reversed phase HPLC under the following conditions: A/B = 30/70 (0 min), 50/50 (30 min) (solvent A: 0.1% HCOOH in H_2O; solvent B: 0.1% HCOOH in CH_3CN). After lyophilization, a purple solid of **19** (12.7 mg, 19.1 μmol, 41%) was obtained. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.88-7.86 (m, 1H), 7.79-7.77 (m, 2H), 7.45-7.44 (m, 1H), 7.02 (s, 2H), 6.86 (s, 2H), 3.81 (q, J = 9.5 Hz, 2H), 3.65 (t, J = 7.0 Hz, 4H), 3.59 (t, J = 6.0 Hz, 4H), 2.71 (t, J = 6.0 Hz, 4H), 2.48 (t, J = 7.0 Hz, 4H), 2.03 (quin, J = 7.0 Hz, 4H), 1.98-1.90 (m, 4H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 177.5, 169.8, 154.2, 151.9, 146.4, 133.5, 129.1, 128.2, 126.8, 124.1, 122.4, 118.8, 103.5, 96.6, 66.4, 50.4, 48.8, 16.4, 32.2, 27.1, 21.8, 21.5, 7.82. HRMS (FAB+): calcd for $[M]^+$ 664.2629, found 664.2636.

Compound **20**



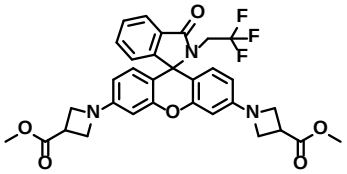
Compound **20** was prepared by the literature method[S2].

Compound **21**



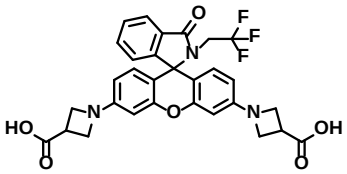
Compound **21** was prepared by the literature method[S2].

Compound **22**

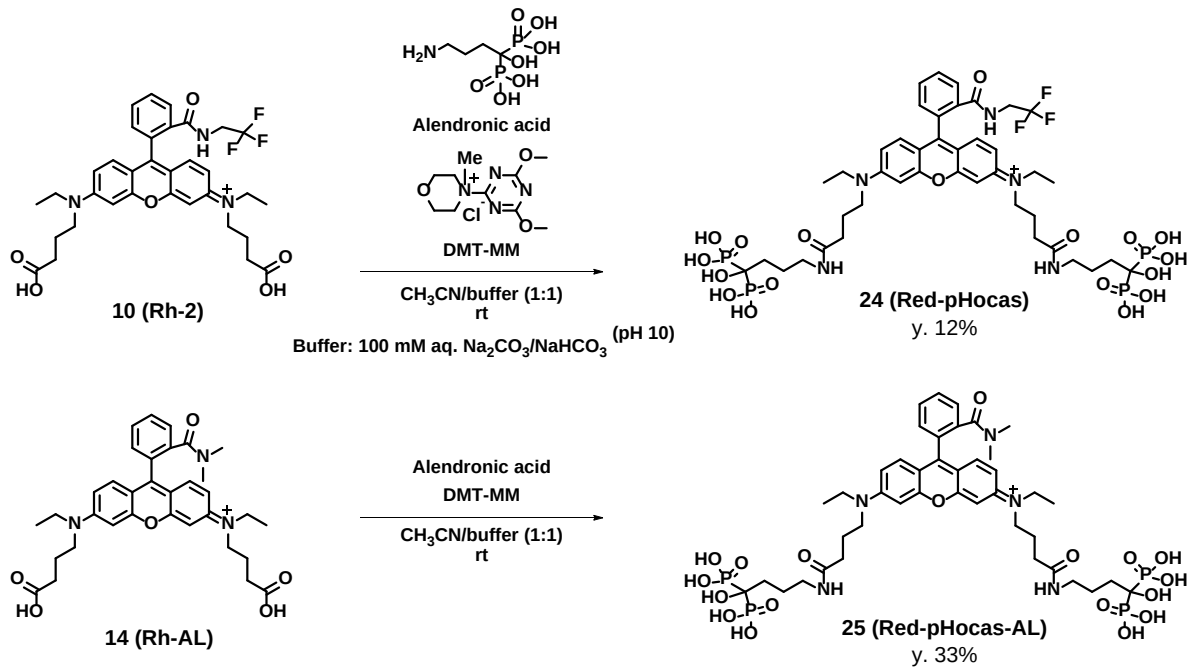


To a stirred solution of **21** (50.0 mg, 94.8 µmol) in dry CH_2Cl_2 (10 mL) was added toluenesulfonylchloride (36.1 mg, 190 µmol) under an N_2 atmosphere. To this stirred mixture after 15 minutes was added 4-dimethylaminopyridine (29.0 mg, 237 µmol) at room temperature. After stirring the mixture after 15 minutes, a solution of 2,2,2-trifluoroethylamine (11 µL, 142 µmol) in CH_2Cl_2 (1 mL) was added and the reaction mixture was stirred for 22 h. The mixture was quenched with saturated $NaHCO_3$ aq. and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was combined, washed with brine (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (silica-packed 10 g snap column) to afford **22** (35.8 mg, 58.8 µmol, 62%) as a colorless solid. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.92-7.90 (m, 1H), 7.58-7.53 (m, 2H), 7.02-7.00 (m, 1H), 6.37 (d, J = 8.5 Hz, 2H), 6.23 (d, J = 2.3 Hz, 2H), 6.15 (dd, J = 8.5 Hz, 2.3 Hz, 2H), 4.09-4.06 (m, 4H), 3.99-3.95 (m, 4H), 3.73 (s, 6H), 3.68 (q, J = 9.0 Hz, 2H), 3.62-3.56 (m, 2H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 173.5, 169.7, 154.0, 152.9, 152.8, 133.7, 128.9, 128.5, 128.4, 124.9, 124.0, 122.7, 122.6, 108.0, 106.7, 97.8, 65.9, 54.1, 51.3, 33.0. HRMS (FAB+): calcd for $[M]^+$ 608.2003, found 608.2002.

Compound **23** (**Rh-7**)

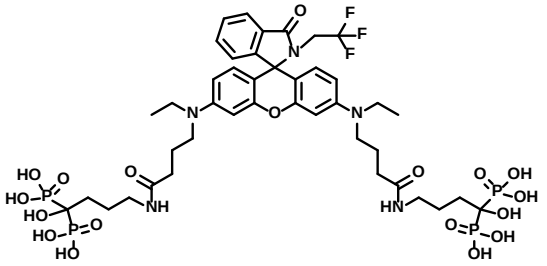


To a stirred solution of **22** (40.0 µmol) in MeOH (4 mL) was added 2 M NaOH aq. (8 mL) at 40 °C. After stirring for 4 h, the mixture was quenched with 2 M HCl aq. and concentrated under reduced pressure. The residue was purified by Biotage isolera Flash Purification System (C18 12 g snap column) to afford **23** (18.4 mg, 28.8 µmol, 72%) as a purple solid. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.92-7.90 (m, 1H), 7.60-7.53 (m, 2H), 7.04-7.02 (m, 1H), 6.38 (d, J = 8.6 Hz, 2H), 6.24 (d, J = 2.3 Hz, 2H), 6.16 (dd, J = 8.6 Hz, 2.3 Hz, 2H), 4.09-4.06 (m, 4H), 4.00-3.97 (m, 4H), 3.69 (q, J = 9.5 Hz, 2H), 3.58-3.53 (m, 2H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 175.1, 169.7, 154.0, 153.0, 152.9, 133.6, 128.9, 128.5, 128.4, 125.0, 124.0, 122.6, 108.0, 106.6, 97.8, 65.9, 54.2, 33.2, 29.3. HRMS (FAB+): Calcd for $[M]^+$, 580.1690, found 580.1694.



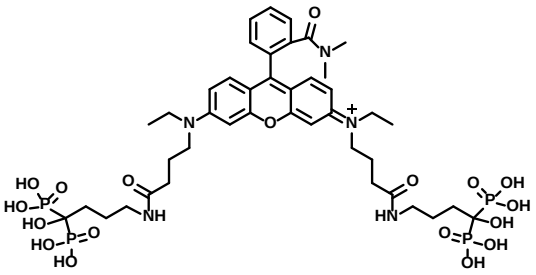
Scheme S5. Synthesis of **Red-pHocas** and **Red-pHocas-AL**

Compound **24 (Red-pHocas)**



To the solution of **10** (10.0 mg, 15.6 µmol) in CH_3CN (5 mL) was added 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (17.3 mg, 62.4 µmol). After stirring for 1 h, alendronic acid (15.5 mg, 62.4 µmol) in aq. carbonate/bicarbonate buffer (5 mL, pH 10.0) was added at room temperature. These manipulations ware repeated three times every 24 h. After stirring for 72 h, the mixture was quenched with acetic acid and concentrated under reduced pressure. The residue was purified by reversed phase HPLC under the following conditions: A/B = 30/70 (0 min), 50/50 (30 min) (solvent A: 50 mM TEAA aq.; solvent B: CH_3CN). After lyophilization, a purple powder of **24** (2.63 mg, 1.87 µmol, 12%) was obtained. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.90 (dd, J = 7.5 Hz, 1.0 Hz, 1H), 7.61 (td, J = 7.5 Hz, 1.0 Hz, 1H), 7.55 (td, J = 7.5 Hz, 1.0 Hz, 1H), 7.08 (dd, J = 7.5 Hz, 1.0 Hz, 1H), 6.45 (d, J = 2.5 Hz, 2H), 6.40 (dd, J = 9.0 Hz, 2.5 Hz, 2H), 6.34 (d, J = 9.0 Hz, 2H), 3.70 (q, J = 9.5 Hz, 2H), 3.41 (q, J = 7.0 Hz, 4H), 3.36-3.32 (m, 4H), 3.21-3.14 (m, 28H), 2.23 (t, J = 7.5 Hz, 4H), 2.01-1.99 (m, 4H), 1.96-1.91 (m, 4H), 1.87 (quin, J = 7.5 Hz, 4H), 1.29 (t, J = 7.0 Hz, 36H), 1.15 (t, J = 7.0 Hz, 6H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 173.9, 170.0, 154.1, 153.4, 149.2, 133.5, 129.1, 128.4, 128.3, 124.1, 122.4, 108.4, 104.2, 97.7, 73.3, 66.2, 49.3, 48.2, 44.6, 40.1, 32.8, 31.5, 23.5, 23.3, 21.8, 11.1. HRMS: (ESI−) Calcd for $[M-H]^-$, 1100.2607, found 1100.2596.

Compound **25 (Red-pHocas-AL)**



To the solution of **14** (9.15 mg, 15.6 μmol) in CH_3CN (5 mL) was added 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (17.3 mg, 62.4 μmol). After stirring for 1 h, alendronic acid (15.5 mg, 62.4 μmol) in aq. carbonate/bicarbonate buffer (5 mL, pH 10.0) was added at room temperature. These manipulations ware repeated three times every 24 h. After stirring for 72 h, the mixture was quenched with acetic acid and concentrated under reduced pressure. The residue was purified by reversed phase HPLC under the following conditions: A/B = 20/70 (0 min), 50/50 (30 min) (solvent A: 50 mM TEAA aq.; solvent B: CH_3CN). After lyophilization, a purple powder of **25** (6.97 mg, 5.15 μmol, 33%) was obtained. ^{1}H NMR (500 MHz, Methanol-d_4): δ 7.79-7.76 (m, 2H), 7.70-7.67 (m, 1H), 7.55-7.51 (m, 1H), 7.31 (d, J = 9.5 Hz, 2H), 7.16 (d, J = 9.5 Hz, 2H), 7.06 (s, 2H), 3.73 (q, J = 7.0 Hz, 4H), 3.65 (t, J = 7.0 Hz, 4H), 3.25 (t, J = 6.5 Hz, 4H), 3.18 (q, J = 7.0 Hz, 18H), 2.98 (s, 3H), 2.76 (s, 3H), 2.35 (t, J = 7.0 Hz, 4H), 2.09-1.95 (m, 12H), 1.35-1.30 (m, 33H). ^{13}C NMR (125 MHz, Methanol-d_4): δ 173.2, 169.2, 157.9, 156.1, 156.0, 136.0, 131.8, 130.7, 130.4, 129.8, 129.6, 127.5, 114.0, 113.6, 96.2, 73.4, 50.3, 46.0, 40.1, 38.7, 33.8, 31.9, 31.5, 23.4, 22.9, 11.3. HRMS (ESI−): calcd for $[M-H]^-$, 1046.2889, found 1046.2842.

UV-Vis Absorption and Fluorescence Spectroscopy. UV-vis absorption spectra (sample concentration: 10 μM in citrate-phosphate buffer) were recorded on a V-650 UV-VIS spectrometer (JASCO, Japan) at 37 °C. Fluorescence spectra (sample concentration: 0.2 μM in citrate-phosphate buffer, excited at 535 nm) were measured by using a fluorescence spectrophotometer F-7000 (Hitachi, Japan) at 37 °C. The slit width was 5.0 nm for both excitation and emission, and the photomultiplier voltage was 700 V. pH profile was plotted within the pH range from 8.0 to 4.0 (regarding Rh-5, from 8.0 to 3.0). The citrate-phosphate buffer was prepared by mixing a 0.1 M solution of citric acid with a 0.1 M solution of Na_2HPO_4. pK_a values at the absorption maximum or emission maximum were calculated with a curve fitting based on Henderson–Hasselbalch equation.

Measurement of Turn-On Kinetic Constant. The kinetic values were measured by using a V-650 UV-VIS spectrometer or a rapid scan stopped-flow system (Unisoku, Japan) constructed with a Xe or halogen light source. Solutions containing Rh-series or Red-pHocas (20 μM) in 10 mM citrate-phosphate buffer (pH 8.0) were mixed with the same volume of 100 mM citrate-phosphate buffer (pH 3.7). The final probe concentration was 10 μM and

the pH was 4.0. Transient absorption changes were recorded at 37 °C and the kinetic constants were calculated with curve fitting.

Measurement of Extinction Coefficient and Quantum Yield. Solutions of Rh-series, Red-pHocas or Red-pHocas-AL containing 0.5% SDS or not in citrate-phosphate buffer (pH 4.0) were prepared (regarding Rh-7, the pH value was 3.0). UV-vis absorption spectra were recorded at 37 °C and extinction coefficients were calculated from absorption maxima. Fluorescence quantum yields of the dyes were determined in aqueous solution using Rhodamine 6G in EtOH as a standard ($\Phi = 0.95$, excited at 530 nm)[S3].

Measurement of pH Reversibility. To a solution of 0.2 μM Red-pHocas in 2 mL citrate-phosphate buffer (pH 7.1), 17 μL of 6 M HCl aq. and 6 M NaOH aq. were repeatedly added at 37 °C. At each cycle, the pH value was confirmed using a pH meter (SevenEasy, Mettler-Toledo International, Inc.). Fluorescence intensity was measured repeatedly 5 minutes after the addition of these solutions.

Photostability under Xe Laser. Solutions of Red-pHocas or pHocas (0.2 μM, 2.0 mL) in citrate-phosphate buffer (pH 6.0) were continuously irradiated by a xenon light source (5 mW/cm^2, MAX-303, Asahi Spectra, Japan) equipped with a bandpass filter (Red-pHocas: 550/5 nm, pHocas: 500/5 nm). Fluorescence intensity was measured every 5 minutes for 30 minutes.

***In Vitro* Hydroxyapatite Binding Test and Photostability under Confocal Laser.** Hydroxyapatite (5 mg/mL) was vortexed in 5 μM aqueous solutions of Red-pHocas or pHocas (1 mL) for 30 min at room temperature. The mixture was centrifuged and washed twice with water. A portion of the residual powder was soaked in citrate-phosphate buffer (400 μL) at various pH values on a glass-bottom dish. Fluorescence images were then observed using a confocal laser scanning microscope (Olympus, FLUOVIEW FV10i) equipped with a 60× lens. To confirm the binding reversibility, Red-pHocas-AL (20 pmol)-adsorbed hydroxyapatite was incubated at 37 °C in the absence and presence of 50 μM alendronate (20 nmol) in citrate-phosphate buffer (pH 7.0). Fluorescence images were obtained immediately after incubation (0 h), after 1 h, and 24 h. The excitation wavelength was 559 nm (Red-pHocas-AL), and the emission was filtered with a 570-670 nm bandpass filter. To measure the photostability, Red-pHocas or pHocas adsorbed on hydroxyapatite was continuously irradiated by confocal laser (excited at 559 nm and 473 nm, respectively, 11.9 mW). Fluorescence images were collected every 5 min for 30 min and the emissions were filtered with a 570–670 nm bandpass filter (Red-pHocas) and 490–590 nm bandpass filter (pHocas). Fluorescence intensity was analyzed using Image J software (NIH, Bethesda, MD, USA).

Cell Culture. Bone marrow cells were obtained from femurs of *a*3-GFP mice, and were cultured with M-CSF (10 ng/mL, R&D) in minimal essential medium containing 10% FCS. After 3 days, cells were cultured with M-CSF (10 ng/mL) and sRANKL (50 ng/mL, Peprotech) for additional 3 days.

Two-Photon Excitation Spectra. To obtain excitation spectra, two-photon fluorescence images of Red-pHocas adsorbed on hydroxyapatite and *in vitro* differentiated osteoclasts expressing GFP-fused V-ATPase *a*3 subunit were collected. Next, the wavelength of the laser source was changed from 800 nm to 960 nm with the same laser intensity. The signal intensity of each fluorophore was extracted by Image J software.

Histological Analysis. Histological analysis in bone tissue was performed via Kawamoto's film method, according to the manufacture's protocol[S4]. The dissected bone tissue from mice administered Red-pHocas was fixed with 4% paraformaldehyde (PFA) at 4 °C overnight. Samples were embedded in super cryoembedding medium (Section-LAB Co. Ltd) and were then frozen. Frozen samples were cut into 10 μm sections with a cryostat (Lesica, CM3050), following which they were incubated with DAPI for staining nucleus (Roche). The treated sections were soaked into citrate-phosphate buffer at various pH values (from pH 8.0 to pH 4.0) and fluorescence images were acquired with an A1 confocal microscope (Nikon) equipped with an objective (Plan Apo VC 20× DIC N2, Nikon). The excitation wavelengths were 405 nm for DAPI, 488 nm for EGFP, and 561 nm for Red-pHocas. Bandpass filters were 450/50 nm for DAPI, 525/50 nm for EGFP, and 595/50 nm for Red-pHocas.

Bone Morphometric Analysis. Trabecular bone morphometry within the metaphyseal region of the distal femur was quantified by micro-computed tomography (μCT) system (ScanXmate-RX; Comscantechno Co., Ltd., Japan). Three-dimensional microstructural image data were reconstructed, and bone morphometric analysis was performed using TRI/3D-BON software (RATOC System Engineering Co., Ltd., Japan).

Intravital Imaging. V-type H^+ ATPase *a*3 subunit-green fluorescent protein (GFP) fusion knock-in mice (*a*3-GFP, C57BL/6 background) and TRAP-tdTomato mice (C57BL/6 background) were previously described[S5, S6]. All animal experiments were performed in compliance with the guidelines for the Animal Experimental Committee of Osaka University. All experiments involved 10–14-week-old *a*3-GFP mice[S6]. Red-pHocas or Red-pHocas-AL (10 mg/kg) dissolved in PBS was injected subcutaneously into the mice daily for 3 days before acquiring the images. Intravital microscopy of mouse calvaria bone tissue was performed using a modification of a protocol described in a previous study[S6]. Mice were anesthetized with isoflurane (Escain; 2% (vol/vol) vaporized in 100% (vol/vol) oxygen); the frontoparietal regions of the skull bones were exposed and then the internal surfaces of bones (adjacent to the bone marrow cavity) were observed using two-photon excitation microscopy. The imaging system comprised a multiphoton microscope (LSM 780 NLO; Carl Zeiss AG) driven by a laser (Chameleon Vision II Ti: Sapphire; Coherent, Inc.) and an upright microscope equipped with a 20× water immersion objective (W Plan-Apochromat, NA 1.0; Carl Zeiss). The microscope was enclosed in an environmental chamber in which the anesthetized mice were warmed by hot air. When the osteoclast activity was investigated under inhibition of V-ATPase, *a*3-GFP knock-in mice were treated with bafilomycin A1 (Aldrich). Bafilomycin A1 (0.5 mg/kg) dissolved in PBS (containing 1% DMSO) was injected intravenously into mice during the imaging experiments.

Data Analysis. After acquiring true-color images, fluorescence spectra of the probe, fluorescent protein, second harmonic generation, and autofluorescence were obtained using the ZEN software (Carl Zeiss) by manually selecting the appropriate pixels on true-color images. These spectral libraries were initially saved on a computer and utilized for spectral unmixing algorithms to create unmixed images that excluded autofluorescence. Raw imaging data were processed using Imaris (Bitplane, Belfast, UK) to create fluorescence images on a maximum intensity projection. Kymograph reconstruction was performed using EBImage package of R/Bioconductor with visual inspection, and smoothed mean intensities of Red-pHocas and *a*3-GFP were calculated for plotting and analysis. 3D surface plots and Otsu's binarized images were produced from the manually segmented cell areas.

Statistics. The data were analyzed using one-way analysis of variance or the Mann–Whitney rank-sum test. Data represent means ± standard deviation (SD) unless otherwise specified. A P-value < 0.05 was considered to reflect statistical significance.

Supporting Figures and Tables

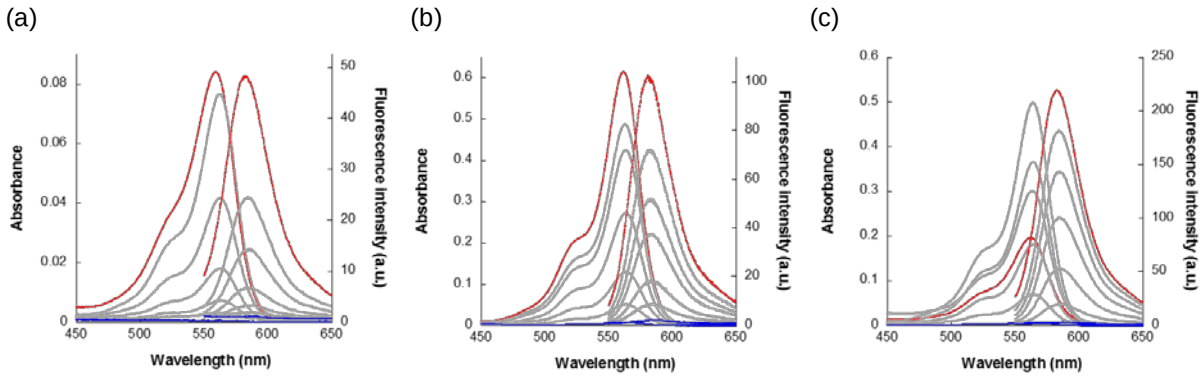


Figure S1. Absorption and fluorescence spectra of (**a**) **Rh-1**, (**b**) **Rh-2**, and (**c**) **Rh-3** (excited at 535 nm) at different pH values. Probe: (absorption) 10 µM and (fluorescence) 0.2 µM solution in 0.1 M citrate-phosphate buffer containing 1% and 0.02% DMSO, respectively. Temperature: 37 °C.

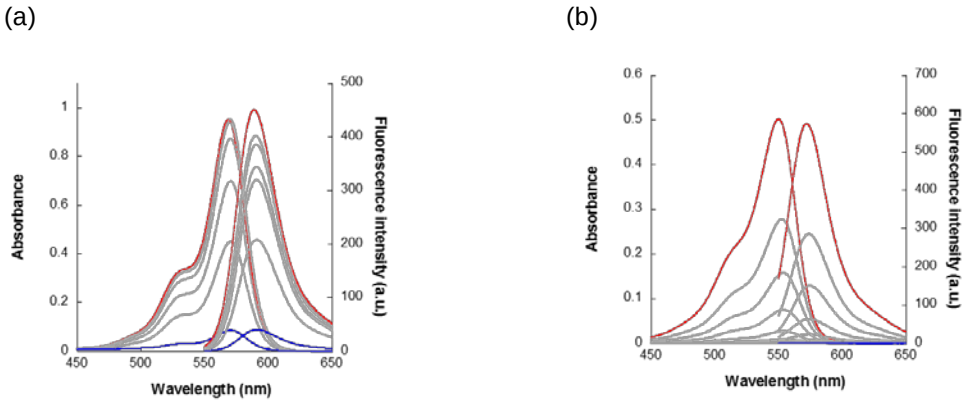


Figure S2. Absorption and fluorescence spectra of (**a**) **Rh-6** and (**b**) **Rh-7** (excited at 535 nm) at different pH values. Probe: (absorption) 10 µM and (fluorescence) 0.2 µM solution in 0.1 M citrate-phosphate buffer containing 1% and 0.02% DMSO, respectively. Temperature: 37 °C.

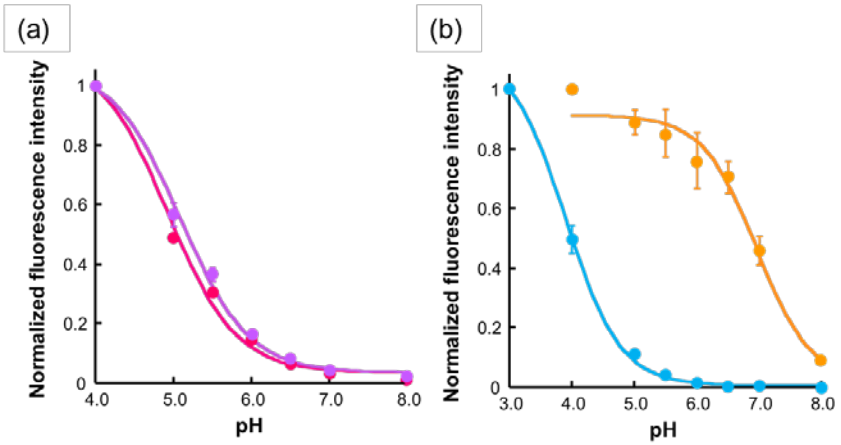


Figure S3. pH profile of fluorescence intensity of (**a**) **Rh-4** (magenta), **Rh-5** (purple) and (**b**) **Rh-6** (orange), **Rh-7** (light blue). The dyes (0.2 μM) were excited at 535 nm in 0.1 M citrate-phosphate buffer at 37 °C. pK_a values were calculated by Henderson–Hasselbalch equation (N = 3).

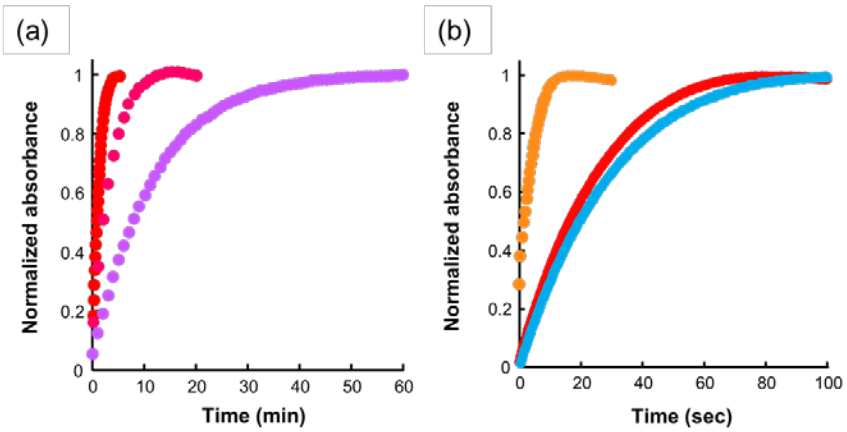


Figure S4. Time-course absorbance of (**a**) **Rh-2** (red), **Rh-4** (magenta), **Rh-5** (purple), and (**b**) **Rh-2** (red), **Rh-6** (orange), and **Rh-7** (light blue) at the maximum absorption wavelengths upon pH-jump from 8.0 to 4.0. The absorbance of each dye (10 μM) was monitored at 37 °C. The changes in absorbance were normalized to the maximum absorbance (N = 3).

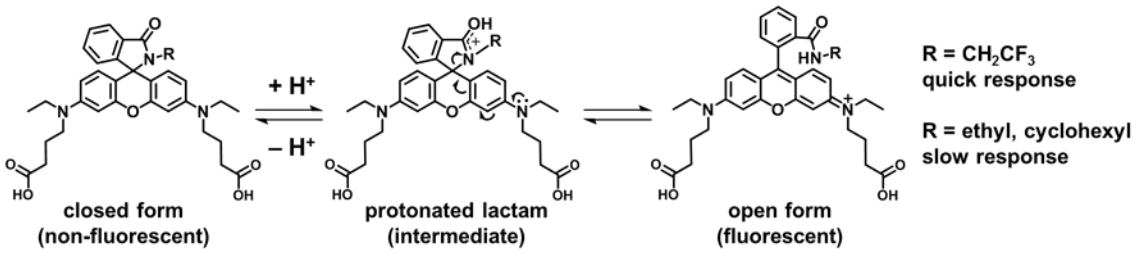


Figure S5. Plausible pH-activation mechanism of Rh-dyes.

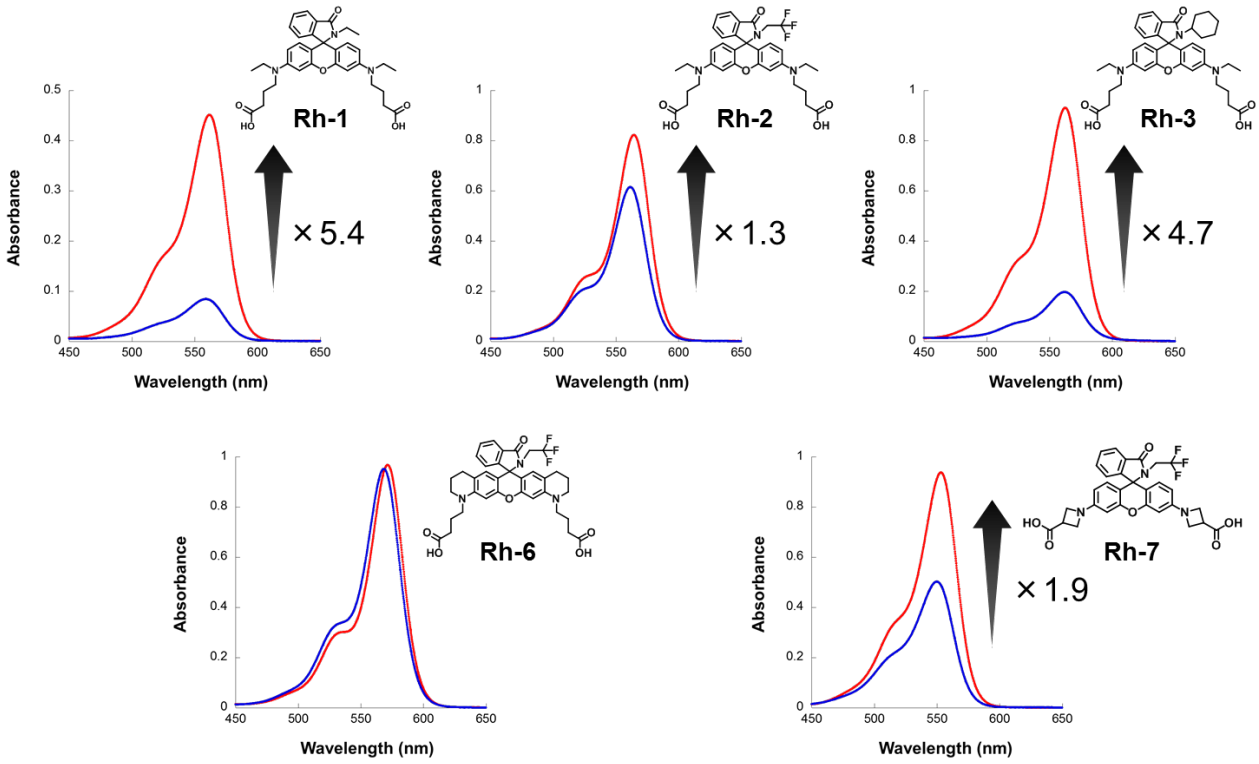


Figure S6. Absorption spectra of **Rh-1**, **Rh-2**, **Rh-3**, **Rh-6**, and **Rh-7** (10 μM) in 0.1 M citrate-phosphate buffer (pH 4.0) containing 1% DMSO with (red line) or without (blue line) 0.5% sodium dodecyl sulfate (SDS). Regarding **Rh-7**, pH value was 3.0. Temperature: 37 °C.

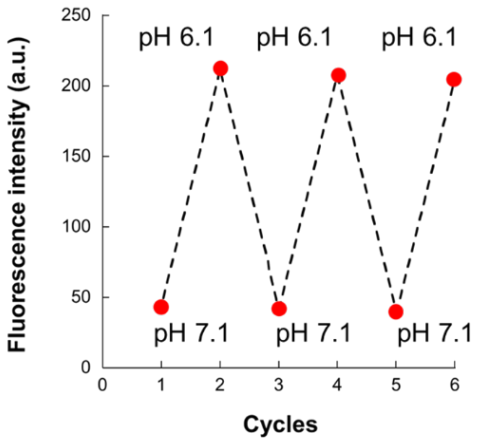


Figure S7. Fluorescence reversibility of **Red-pHocas** during pH cycling between pH 7.1 and 6.1. Probe: 0.2 μM in 0.1 M citrate-phosphate buffer. Temperature: 37 °C. (N = 3)

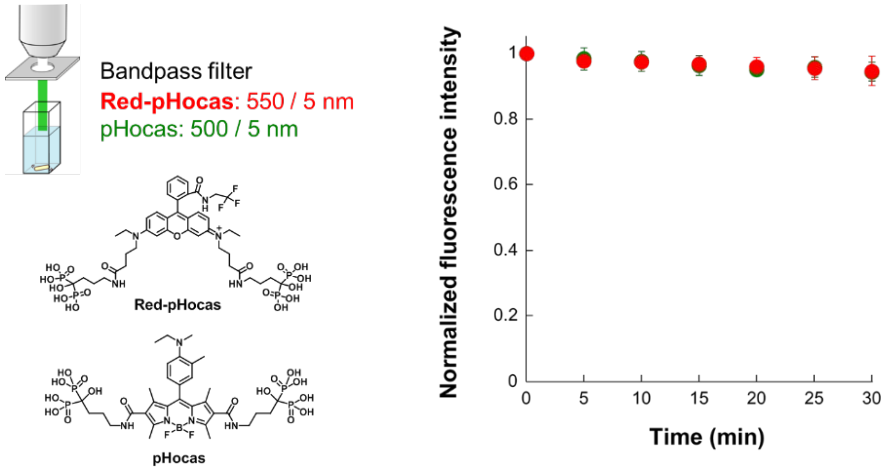


Figure S8. Photostability of **Red-pHocas** and **pHocas** (at pH 6.0) during continuous irradiation (5 mW/cm^2) for 30 min. The changes in fluorescence intensity were measured and normalized to the initial intensity. Probe: 0.2 μM in 0.1 M citrate-phosphate buffer. Temperature: 37 °C. (N = 3)

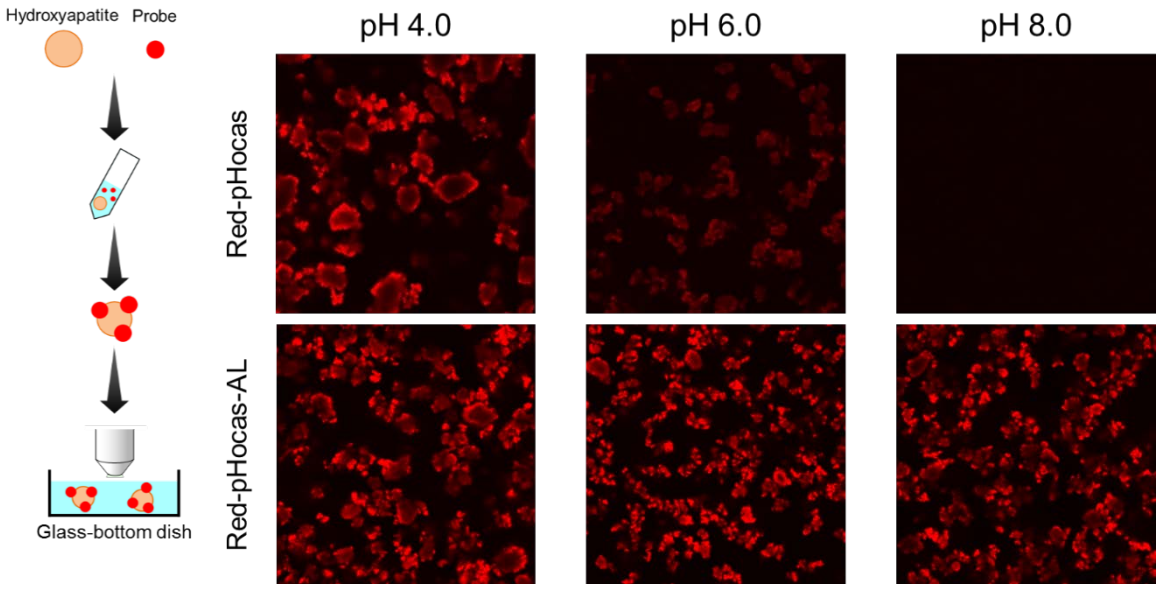


Figure S9. Fluorescence images of **Red-pHocas** and **Red-pHocas-AL** adsorbed on hydroxyapatite with confocal laser scanning microscopy. Temperature: 37 ºC. Excited at 559 nm. Bandpass filter: 570–670 nm.

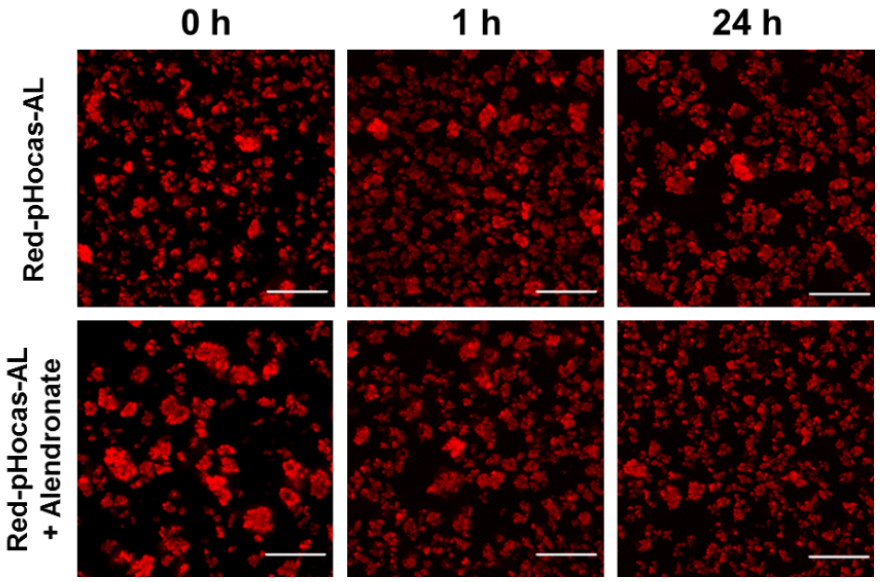


Figure S10. Confocal fluorescence images of Red-pHocas-AL adsorbed on hydroxyapatite in the absence and presence of 50 μM alendronate. Temperature: 37 ºC. Excitated at 559 nm. Bandpass filter: 570–670 nm. Scale bars: 50 μm.

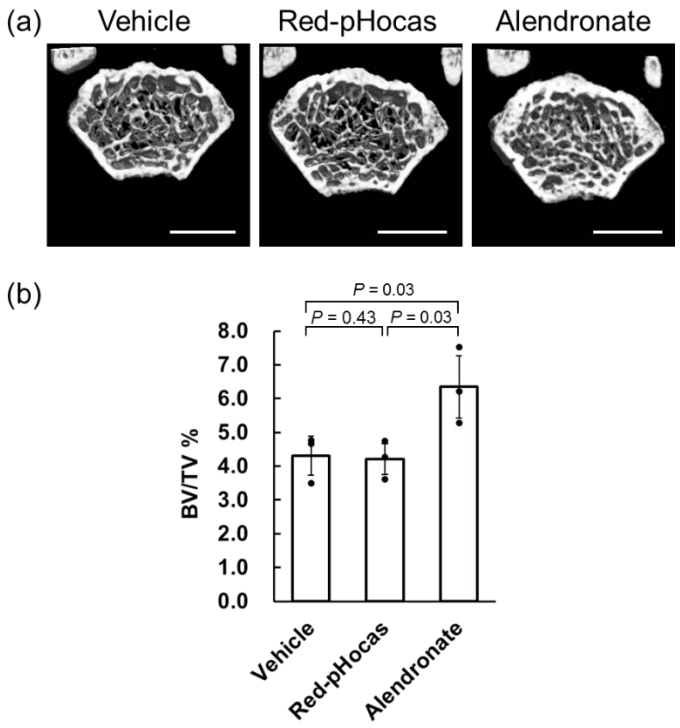


Figure S11. (**a**) Representative μCT images of the metaphyseal region of the distal femur. Wild-type mice in which vehicle, Red-pHocas (10 mg/kg) or alendronate (5.9 mg/kg) had been subcutaneously injected for three days. Scale bars: 1 mm. (**b**) Analysis of the bone volume (BV/TV, n = 3 for each). Error bars represent means ± SD.

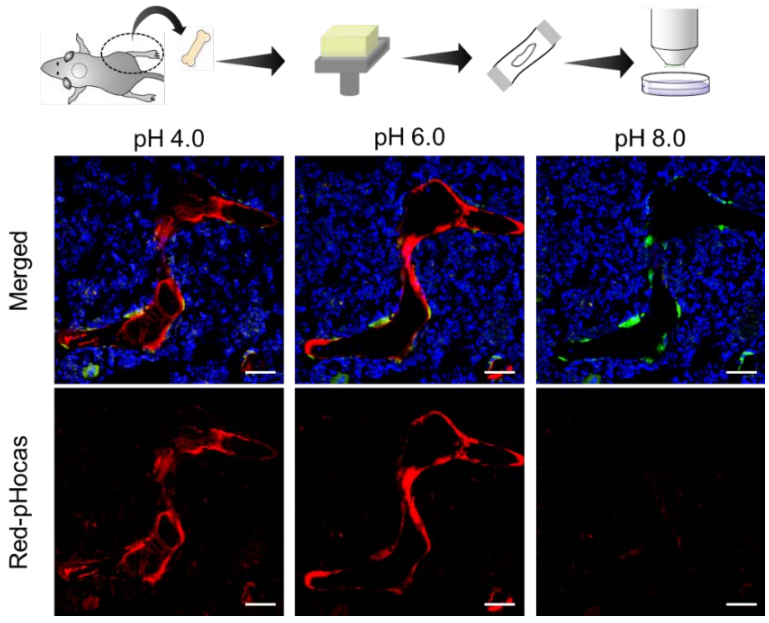


Figure S12. Confocal images of bone tissue. The sliced tissue soaked into citrate-phosphate buffer (pH 4.0, 6.0, and 8.0) was imaged after staining cell nuclei with DAPI. Scale bars: 50 μm. Blue, DAPI (excited at 405 nm); green, *a*3-GFP (excited at 488 nm); red, **Red-pHocas** (excited at 561 nm).

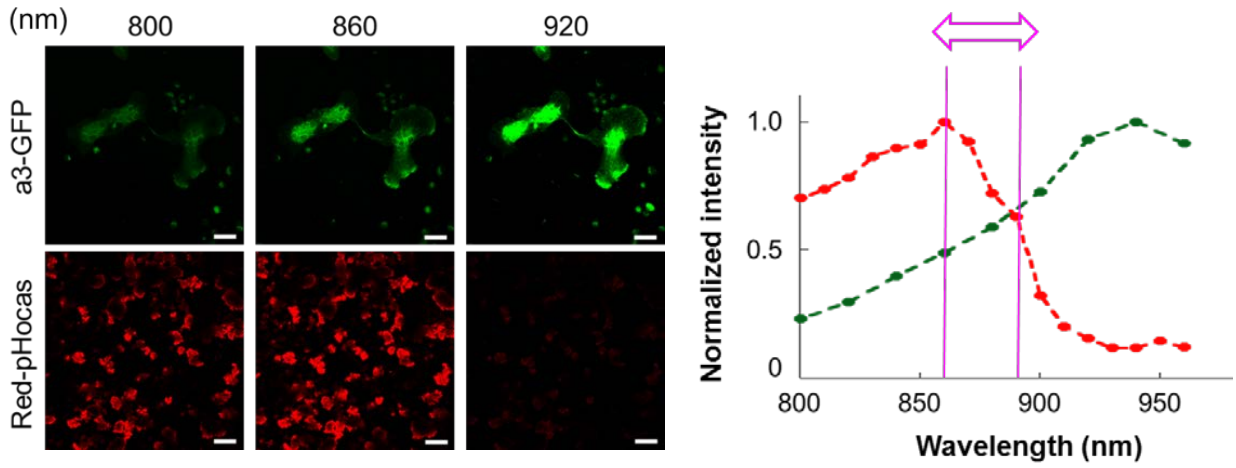


Figure S13. Two-photon excitation spectrum of **Red-pHocas** (pH 6.0) and *a*3-GFP. The excitation wavelength was changed from 800 to 960 nm, keeping its intensity constant. Scale bars: 30 μm.

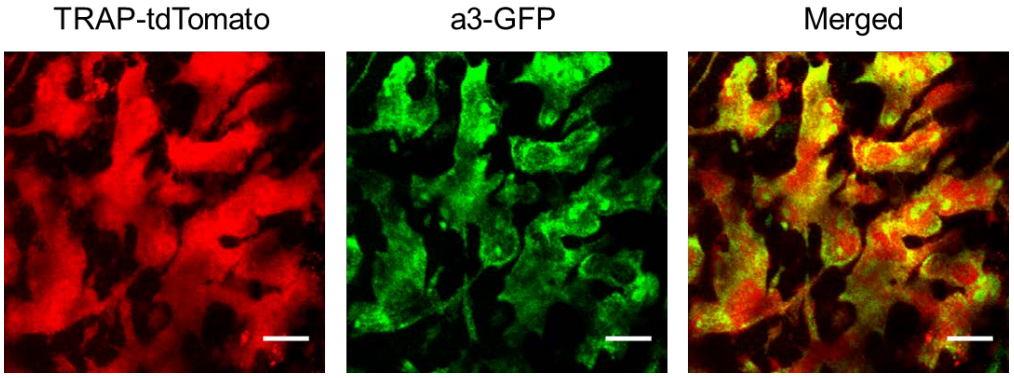


Figure S14. Two-photon fluorescence images of osteoclasts expressing TRAP-tdTomato and *a*3-GFP. Scale bars: 20 μm.

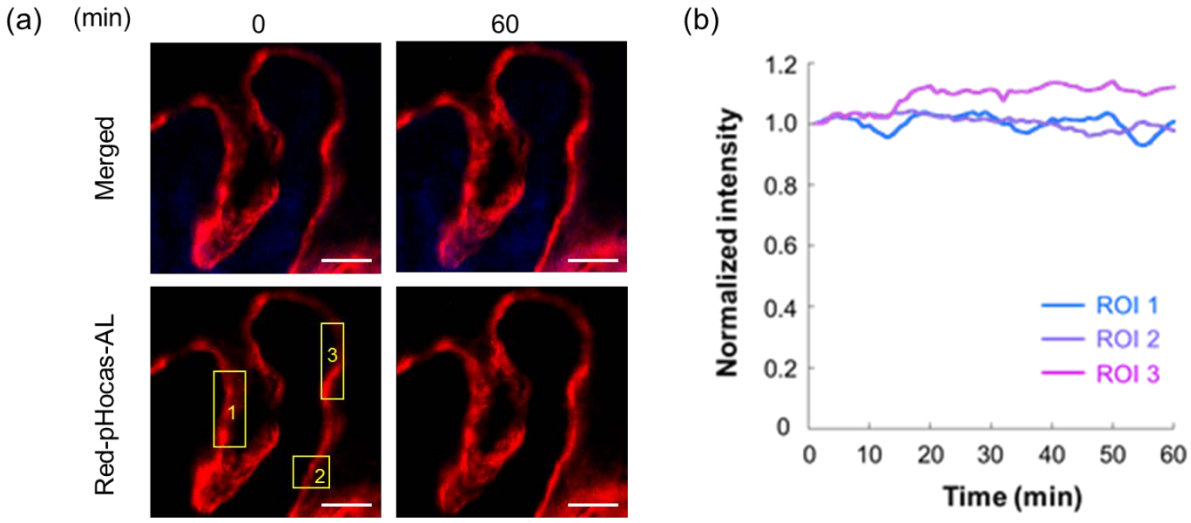


Figure S15. (**a**) Two-photon fluorescence images after injection of **Red-pHocas-AL**. Excited at 860 nm. Scale bars: 30 μm. Dose: 10 mg/kg (3 days). Red, **Red-pHocas**; blue, bone tissue. (**b**) Changes in fluorescence intensity normalized to the initial intensity in three regions of interest selected randomly.

Table S1. Spectroscopic data of rhodamine spirolactams **Rh-1–7**

	λ_{abs} (nm)	λ_{em} (nm)	ε ($M^{-1}cm^{-1}$)[a]	ε_{Max} ($M^{-1}cm^{-1}$)[b]	Φ[c]	pK_a	k_{obs} (10^{-2} s^{-1})[d]
Rh-1	561	582	8300	45000	0.36	4.9	0.027
Rh-2	562	581	61000	81000	0.25	5.5	4.1
Rh-3	561	582	20000	93000	0.55	5.9	0.014
Rh-4	558	579	29000	96000	0.33	4.9	0.46
Rh-5	561	582	27000	63000	0.27	5.1	0.14
Rh-6	569	589	95000	95000	0.53	7.0	25
Rh-7	553	572	49000	94000	0.62	< 3.9	3.5

Table S2. Spectroscopic data of **Red-pHocas** and **Red-pHocas-AL**

	λ_{abs} (nm)	λ_{em} (nm)	ε ($M^{-1}cm^{-1}$)[a]	ε_{Max} ($M^{-1}cm^{-1}$)[b]	Φ[c]	pK_a	k_{obs} (10^{-2} s^{-1})[d]
Red-pHocas	562	583	77000	82000	0.27	5.6	3.1
Red-pHocas-AL	564	586	100000	110000	0.26	–	–

k_{obs}, kinetic constant of ring-opening reaction. [a] measured in citrate-phosphate buffer (pH 4.0) containing 1% DMSO. [b] measured in citrate-phosphate buffer (pH 4.0) containing 1% DMSO and 0.5% SDS. [c] Rhodamine 6G in EtOH (Φ 0.95) was used as the fluorescence standard (excited at 530 nm)[S3]. [d] measured by pH-jump from pH 8.0 to pH 4.0 (dyes except for **Rh-7**), from pH 8.0 to pH 3.0 (**Rh-7**).

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Movie S1-S3 Captions

Movie S1. Two-photon time-lapse imaging of bone tissue after injection of **Red-pHocas-AL** at an interval of 1 min for 60 min. Excited at 860 nm. Red fluorescence signals from Red-pHocas-AL. Scale bar: 30 μm. Playback speed, 300×.

Movie S2. Two-photon time-lapse imaging of bone tissue after injection of **Red-pHocas** with spectral unmixing at an interval of 1 min for 270 min. Excited at 860 nm. Green, mature osteoclasts expressing *a*3-subunit-fused GFP; red, fluorescent signals from Red-pHocas. Scale bars: 20 μm. Playback speed, 300×.

Movie S3. Two-photon time-lapse imaging of bone tissue after injection of **Red-pHocas** with spectral unmixing at an interval of 5 min for 3 h. Excited at 860 nm. Green, mature osteoclasts expressing *a*3-subunit-fused GFP; red, fluorescent signals from Red-pHocas. Scale bars: 20 μm. Playback speed, 300×.