Supplementary Information

A cytosolically localized far-red to near-infrared rhodamine-based fluorescent probe for calcium ions

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General Information. Reagents and solvents were of the best grade available, supplied by Tokyo Chemical Industries, Co., Ltd., Wako Pure Chemical Industries, Ltd., Sigma-Aldrich Co., LLC., Kanto Chemical Co., Inc. and Toronto Research Chemicals Inc., and were used without further purification. Silica gel column chromatography was performed by using Silica gel 60 (Spherical) (Kanto Chemical Co., Inc.). ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL INM-LA300 instrument (300 MHz), JNM-LA400 or ECZ-400S instrument (400 MHz); δ values are in ppm relative to tetramethysilane (TMS). Mass spectra (MS) were measured with a JEOL JMS-T100LC AccuTOF (ESI). UV-vis spectra were obtained on a UV-2550 or UV-1850 (Shimadzu). Fluorescence spectroscopic studies were performed with a Hitachi F-7000 or F-7100. The slit widths were 2.5 nm for both excitation and emission. The photomultiplier voltage was 700 V. Absolute fluorescence quantum yields were measured with Quantaurus-QY C11347 (Hamamatsu K.K.). HPLC analysis performed on an Inertsil ODS-3 column (GL Science Inc.; 4.6 mm × 250 mm) using an HPLC system composed of a pump (PU-980, JASCO) and a detector (MD-2015, JASCO). HPLC purifications were performed on an Inertsil ODS-3 column (GL Science Inc., 10.0 mm× 250 nm) equipped with a pump (PU-2080, JASCO) and a detector (MD-2015, JASCO). MPLC purifications were performed on a Yamazen Smart Flash EPCLC AI-5805.

Methods

Fluorescence Imaging of Cultured Cells. A confocal imaging system (TCSSP5 or TCSSP8; Leica) equipped with a white-light laser (TCSSP5) and a He-Ne laser (TCSSP8) was used. Fluorescence images were captured with excitation and emission wavelengths of 488/500-535 nm for LysoTracker and MitoTracker, and 650/670-750 nm (TCSSP5) or 633/670-750 nm (TCSSP8) for CaSiR-1 AM, CaSiR-2 AM and other Si-rhodamine dyes. Human epithelial carcinoma cell line HeLa cells (RIKEN Bioresource Center cell bank, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Thermo Fischer Scientific, Inc.), supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen, Thermo Fischer Scientific, Inc.) and penicillin (100 units/mL)-streptomycin (100 μg/mL) liquid (Invitrogen, Thermo Fischer Scientific, Inc.) at 37°C in a humidified incubator containing 5% CO_2 in air. Cells were plated on a 35-mm poly-L-lysine coated glass-bottomed dish (Matsunami Glass Ind., Ltd.) in DMEM supplemented with 10% (v/v) FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL). Before loading dyes, DMEM was removed, and the dish was washed with Hanks' balanced salt solution (HBSS) three times. Then the

Si-rhodamine dye 1 or 2 (1 μM) in HBSS containing 0.3% DMSO as a cosolvent was added. After incubation at 37°C for 30 min, the medium was removed and the dish was washed with HBSS three times. The cells were observed in HBSS. For costaining with LysoTracker Green or MitoTracker Green, the cells were incubated with Si-rhodamine dye 2 (1 μM) and 75 nM LysoTracker Green DND-26 (Invitrogen, Thermo Fischer Scientific, Inc.) or MitoTracker Green FM (Invitrogen, Thermo Fischer Scientific, Inc.) in HBSS containing 0.3% DMSO as a cosolvent. The medium was warmed to 37°C before all procedures.

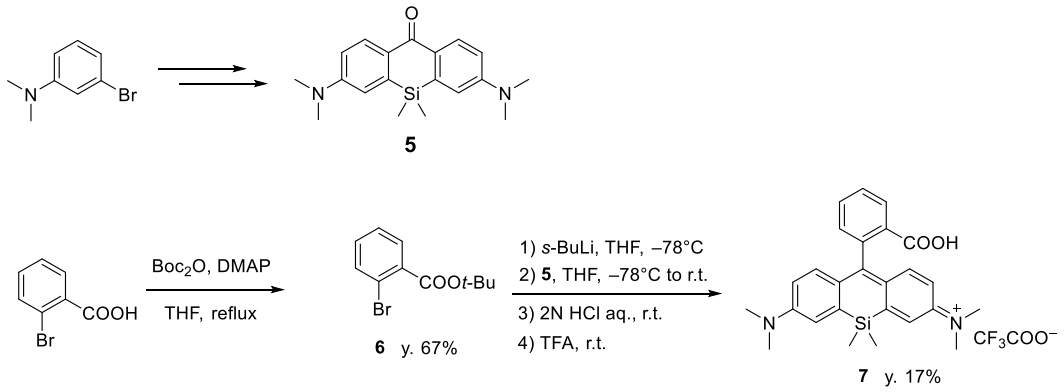
Fluorescence Imaging of Intracellular Ca^{2+} Oscillations in Cultured Cells. Cells were plated onto a 35-mm poly-L-lysine-coated glass-bottomed dish in DMEM supplemented with 10% (v/v) FBS, 1% penicillin and 1% streptomycin. Before loading dyes, DMEM was removed, and the dish was washed with HBSS three times. Then CaSiR-1 AM (GORYO Chemical Inc., Sapporo, Japan) or CaSiR-2 AM (3 μM) in HBSS containing 0.3% DMSO as a cosolvent and 0.03% Pluronic F-127 (Invitrogen, Thermo Fischer Scientific, Inc.) was added. After incubation at 37°C for 30 min, the medium was removed and the dish was washed with HBSS three times, then the cells were observed in HBSS. For the stimulation of cells with histamine, 11 μM histamine dihydrochloride (FUJIFILM Wako Pure Chemical Industries, Ltd.) in HBSS was added to the medium (final concentration: 1 μM). For the stimulation of cells with ATP (adenosine 5'-triphosphate), 1.1 mM ATP disodium salt trihydrate (FUJIFILM Wako Pure Chemical Industries, Ltd.) in HBSS was added to the medium (final concentration: 100 μM). Then, 60 μM ionomycin from *Streptomyces conglobatus* (Sigma-Aldrich Co. LLC) in HBSS was added to the medium (final concentration: 5 μM). The medium was warmed at 37°C before all procedures.

Single-Crystal X-ray Analysis. Single-crystal X-ray analysis of 2-COOH SiR650 was performed with a CCD-based X-ray diffractometer (Bruker AXS, SMART APEX2 Ultra-Cu) with Cu *K*α radiation (λ = 1.54178 Å). The crystal was cooled using a low temperature controller (Japan Thermal Engineering, TC-190CP-CS-K). The diffraction frames were integrated with the Bruker SAINT program. The cell constants were determined by global refinement. The molecular structure was solved by the direct method and refined by the full-matrix least-squares method using the SHELXL-2014 program. The positions of all hydrogen atoms were calculated geometrically and refined by means of the riding model. Crystal data of 2-COOH SiR650. $C_{26}H_{28}N_2O_2Si$, T = 93(2) K, orthorhombic $Pna2_1$, a = 7.5919(2) Å, b = 32.0914(6) Å, c = 21.4030(5) Å, V = 5214.5(2) $Å^3$, Z = 8, R_1 ($I > 2\sigma(I)$) = 0.0591, wR_2 ($I > 2\sigma(I)$) = 0.1614. The crystallographic data can be obtained free of charge from the Cambridge Crystallographic Data Centre (reference number CCDC 1579600).

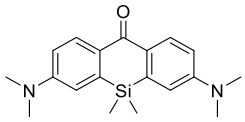
Fluorescence Imaging of Ca^{2+} in Rat Hippocampal Slices. Hippocampal organotypic slices were prepared from Wistar/ST rats (SLC) as described elsewhere.[SR1] Rat pups were anaesthetized with hypothermia and isoflurane, and decapitated. The brains were removed and placed in ice-cold oxygenated Gey's balanced salt solution supplemented with 25 mM glucose. Each brain was horizontally sliced at a thickness of 300 μm using a vibratome (DTK-1500, Dosaka). The entorhinal-hippocampal regions were trimmed using a surgical microknife. The slices were placed on Omnipore membrane filters (JHWP02500, Merck Millipore) and incubated in 5% CO_2 at 35°C. The culture medium, which was composed of 50% minimal essential medium (Invitrogen, Thermo Fischer Scientific, Inc.), 25% HBSS, 25% horse serum (Gibco), and antibiotics, was changed every 3.5 days. Experiments were performed on slices after 8−12 days *in vitro*. The slices were transferred to a 35-mm dish filled with 2 mL of dye solution and incubated for 40 min in a humidified incubator at 37°C in 5% CO_2 with 0.0005% **CaSiR1-AM** or **CaSiR2-AM**, 0.01% Pluronic F-127 (Invitrogen, Thermo Fischer Scientific, Inc.), and 0.005% Cremophor EL (Sigma-Aldrich). The slices were stabilized for 30 min in oxygenated artificial cerebrospinal fluid (aCSF) consisting of (in mM) 127 NaCl, 26 $NaHCO_3$, 3.3 KCl, 1.24 KH_2PO_4, 1.2 $MgSO_4$, 1.2 $CaCl_2$, and 10 glucose, bubbled with 95% O_2 and 5% CO_2. In some experiments, 200 nM MitoTracker Green FM or 75 nM LysoTracker Green DND-26 was added to aCSF during stabilization. A slice was mounted in a recording chamber and perfused with aCSF at a flow rate of 1.5–2.0 ml/min. The hippocampal CA3 pyramidal cell layer was imaged at 10 Hz using a Nipkow-disk confocal microscope (CSU-X1, Yokogawa Electric) equipped with a cooled complementary metal-oxide-semiconductor camera (C11440-22CU, Hamamatsu Photonics) and an upright microscope with a water-immersion objective lens (40×, 0.8 numerical aperture, Nikon). Fluorophores were excited at 488 nm and 633 nm with laser diodes and visualized with 507-nm long-pass and 665-705 nm band-pass emission filters, respectively.[SR2] The fluorescence change was measured as $(F_t–F_0)/F_0$, where F_t is the fluorescence intensity at time t, and F_0 is the fluorescence intensity averaged from –10 to 10 s relative to t.

Synthesis and characterization

Scheme S1. Synthetic scheme for 2-COOH SiR650.

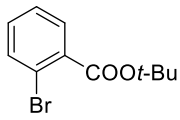


Synthesis of compound 5



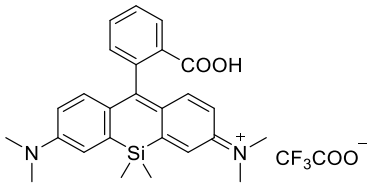
The compound was synthesized according to reference SR3.

Synthesis of compound 6

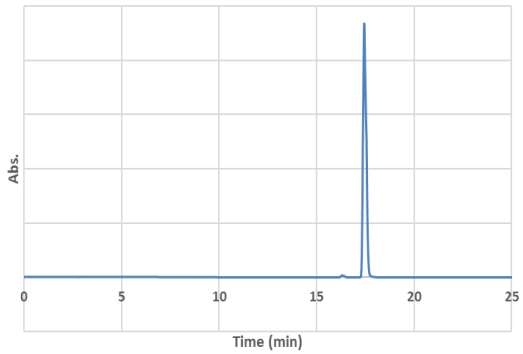


2-Bromobenzoic acid (5.65 g, 28.3 mmol), Boc_2O (8.65 g, 39.5 mmol) and DMAP (765 mg, 6.27 mmol) were dissolved in anhydrous THF (40 mL). The solution was refluxed overnight, cooled to room temperature, and evaporated to dryness. The residue was dissolved in ethyl acetate, and the solution was washed with sat. $NaHCO_3$ aq. and brine, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by silica gel column chromatography (EtOAc/*n*-hexane = 1/10), affording **6** (4.89 g, 19.0 mmol, y. 67%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.61 (s, 9H), 7.25-7.29 (m, 1H), 7.31-7.35 (m, 1H), 7.61 (dd, *J* = 8.0 Hz, 1.2 Hz, 1H), 7.68 (dd, *J* = 7.8 Hz, 2.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.1, 82.5, 120.9, 127.0, 130.8, 131.8, 134.0, 134.3, 165.7; HRMS (ESI$^+$): Calcd for $[M + Na]^+$ 278.9997, Found, 278.9979 (-1.8 mmu).

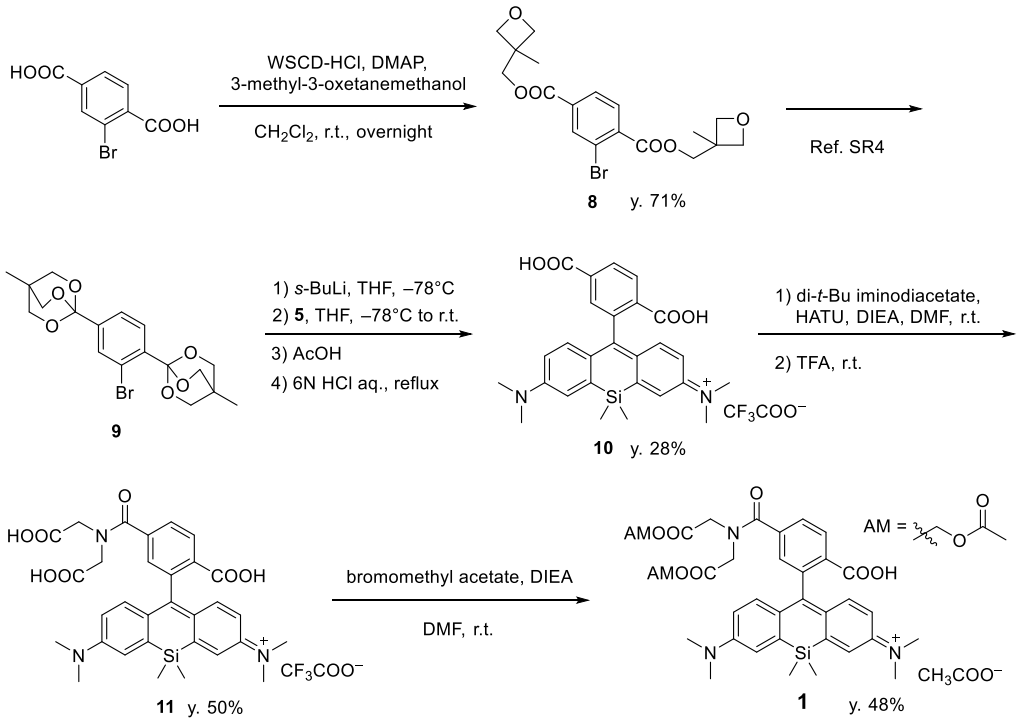
2-COOH SiR650 (7)



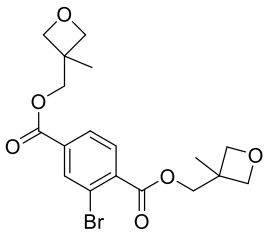
Compound **6** (186 mg, 0.723 mmol) dissolved in anhydrous THF (5.0 mL) was added to a flame-dried flask that had been flushed with argon. The solution was cooled to –78°C, and 1 M *sec*-BuLi in hexane (0.3 mmol) was added. The mixture was stirred for 10 min, and then **5** (33.5 mg, 0.103 mmol) dissolved in anhydrous THF (5.0 mL) was slowly added to the solution at the same temperature. The mixture was warmed to room temperature, then stirred for 2.5 h, and 2 N HCl aq. (10 mL) was added to it. Stirring was continued for 20 min, then the mixture was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved in trifluoroacetic acid (TFA) (1.5 mL) and the solution was stirred for 2 h. TFA was removed by evaporation, and the resulting residue was purified by HPLC (ODS silica gel, H_2O/CH_3CN, 0.1% TFA) to give 2-COOH SiR650 (**7**) (6.3 mg, 0.012 mmol, 12% yield). ^{1}H NMR (300 MHz, acetone-d_6): δ = 0.11 (s, 3H), 0.21 (s, 3H), 2.61 (s, 12H), 6.31 (dd, *J* = 9.2 Hz, 2.6 Hz, 2H), 6.40 (d, *J* = 9.5 Hz, 2H), 6.83 (d, *J* = 2.9 Hz, 2H), 6.87 (d, *J* = 7.2 Hz, 1H), 7.19-7.24 (m, 1H), 7.32 (m, 1H), 7.54 (d, *J* = 7.3 Hz, 1H); HRMS (ESI$^+$): Calcd for $[M]^+$ 429.1998, Found, 429.2006 (+0.8 mmu); HPLC analysis: ODS silica gel; eluent: A/B = 80/20 to 0/100, 20 min, linear gradient; solvent A: H_2O, 0.1% TFA; solvent B: CH_3CN/H_2O = 80/20, 0.1% TFA; flow rate, 1.0 mL/min; detection wavelength 650 nm.



Scheme S2. Synthetic scheme for compound **1**.

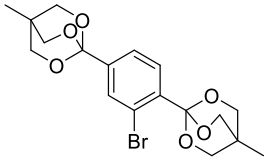


Synthesis of compound 8



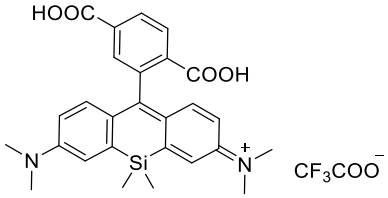
2-Bromoterephthalic acid (5.02 g, 20.5 mmol), WSCD·HCl (8.43 g, 44.1 mmol), DMAP (715 mg, 5.86 mmol), and 3-methyl-3-oxetanemethanol (5.0 mL, 51.0 mmol) were dissolved in dry CH_2Cl_2. The solution was stirred at room temperature overnight, then washed with sat. $NaHCO_3$ aq. and brine, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by silica gel column chromatography (EtOAc/*n*-hexane = 1/1) to obtain **8** (6.02 g, 14.6 mmol, 71% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.43 (s, 3H), 1.44 (s, 3H), 4.44 (s, 2H), 4.47 (s, 4H), 4.49 (s, 2H), 4.61 (d, *J* = 2.1 Hz, 2H), 4.63 (d, *J* = 1.2 Hz, 2H) 7.85 (d, *J* = 8.1 Hz, 1H), 8.05 (dd, *J* = 8.1 Hz, 1.5 Hz, 1H), 8.31 (d, *J* = 1.5 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 21.0, 21.2, 39.1, 39.2, 69.9, 70.1, 79.3, 79.4, 121.3, 128.1, 131.1, 133.5, 135.1, 136.2, 164.3, 165.6; HRMS (ESI$^+$): Calcd for $[M + H]^+$ 413.0600, Found, 413.0580 (-2.0 mmu).

Synthesis of compound 9

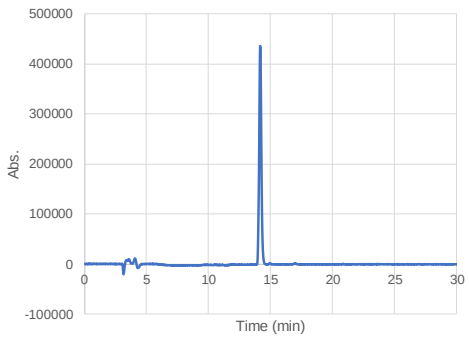


The compound was synthesized according to reference SR4.

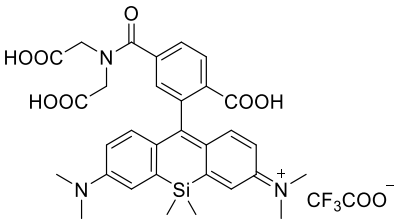
Synthesis of compound 10



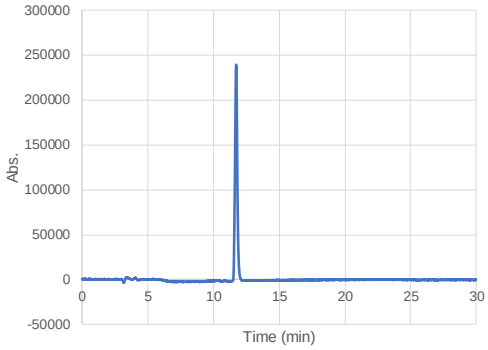
Compound **9** (406 mg, 0.983 mmol) and anhydrous THF (10 mL) were added to a flame-dried flask that had been flushed with argon. The solution was cooled to –78°C, and 1 M *sec*-BuLi in hexane (0.98 mmol) was added. The mixture was stirred for 1 h, then **5** (106 mg, 0.327 mmol) dissolved in anhydrous THF (10 mL) was slowly added to it at the same temperature. The resulting mixture was warmed to room temperature, then stirred for 3.5 h, and AcOH (5.0 mL) was added to it. The whole was evaporated to dryness. The residue was dissolved in 6 N HCl aq. and the solution was stirred under reflux overnight, cooled to room temperature, and evaporated to dryness. The resulting residue was purified by HPLC (ODS silica gel, H_2O/CH_3CN, 0.1% TFA) to obtain **10** (54.3 mg, 0.093 mmol, 28% yield). ^{1}H NMR (300 MHz, CD_3OD): δ = 0.58 (s, 3H), 0.66 (s, 3H), 3.30 (s, 12H), 6.77 (dd, *J* = 9.5 Hz, 2.9 Hz, 2H), 6.97 (d, *J* = 9.5 Hz, 2H), 7.34 (d, *J* = 2.9 Hz, 2H), 7.83 (s, 1H), 8.30 (s, 2H); HRMS (ESI^+): Calcd for $[M]^+$ 473.1897, Found, 473.1937 (+4.0 mmu); HPLC analysis: ODS silica gel; eluent: A/B = 80/20 to 0/100, 20 min, linear gradient; solvent A: H_2O, 0.1% TFA; solvent B: CH_3CN/H_2O = 80/20, 0.1% TFA; flow rate, 1.0 mL/min; detection wavelength 650 nm.



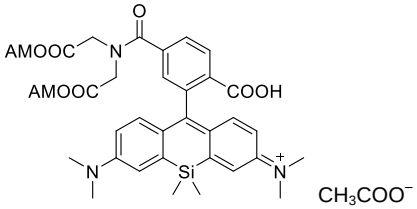
Synthesis of compound 11



Compound **10** (15.0 mg, 0.026 mmol), di-*tert*-butyl iminodiacetate (9.4 mg, 0.038 mmol), HATU (30.1 mg, 0.077 mmol) and DIEA (17 μL, 0.098 mmol) were dissolved in DMF (3.0 mL). The solution was stirred at room temperature for 4.5 h, and evaporated to dryness. The residue was dissolved in TFA (2.0 mL) and the resulting solution was stirred at room temperature for 3 h. TFA was evaporated off, and the residue was purified by HPLC (ODS silica gel, H_2O/CH_3CN, 0.1% TFA) to obtain **11** (9.2 mg, 0.0131 mmol, 50% yield). ^{1}H NMR (300 MHz, acetone-d_6): δ = 0.44 (s, 3H), 0.55 (s, 3H), 2.96 (s, 12H), 4.09 (s, 2H), 4.20 (s, 2H), 6.63 (dd, *J* = 9.2 Hz, 2.6 Hz, 2H), 6.72 (d, *J* = 8.8 Hz, 2H), 7.17 (d, *J* = 2.2 Hz, 2H), 7.25 (s, 1H), 7.58-7.61 (m, 1H), 7.99 (d, *J* = 8.1 Hz, 1H); HRMS (ESI^+): Calcd for $[M]^+$ 588.2166, Found, 588.2182 (+1.6 mmu); HPLC analysis: ODS silica gel; eluent: A/B = 80/20 to 0/100, 20 min, linear gradient; solvent A: H_2O, 0.1% TFA; solvent B: CH_3CN/H_2O = 80/20, 0.1% TFA; flow rate, 1.0 mL/min; detection wavelength 650 nm.

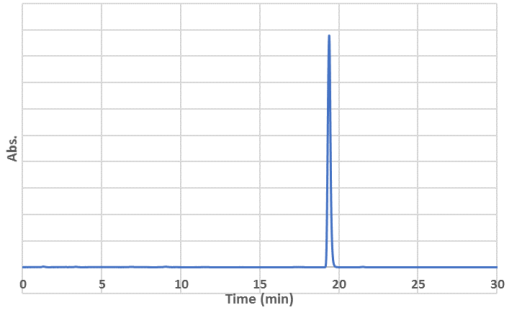


Synthesis of compound 1

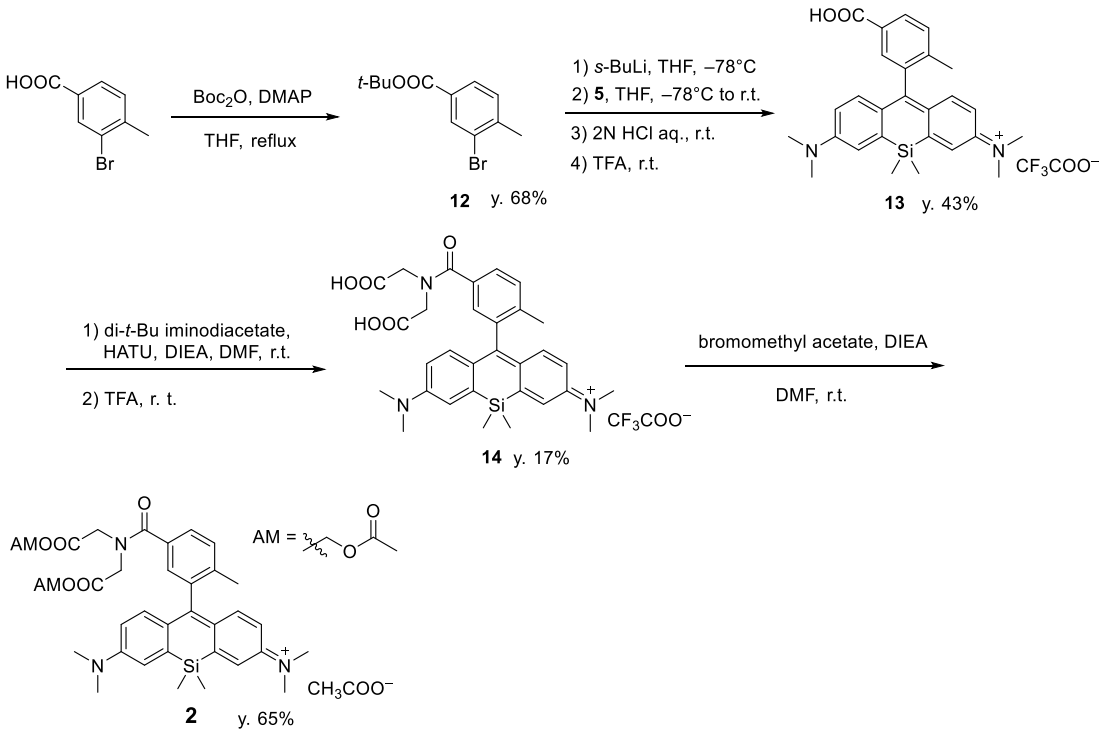


To a solution of **11** (9.2 mg, 0.0131 mmol) in DMF (3.0 mL), bromomethyl acetate (8.8 μL, 0.0897 mmol) and DIEA (28 μL, 0.160 mmol) were added. The mixture was stirred at room

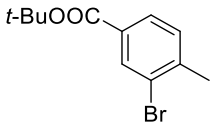
temperature for 6 h, then evaporated to dryness, and the residue was purified by HPLC (ODS silica gel, H_2O/CH_3CN, 0.1% AcOH) to obtain **1** (5.3 mg, 0.00627 mmol, 48% yield). ^{1}H NMR (300 MHz, CD_3OD): δ = 0.55 (s, 3H), 0.63 (s, 3H), 1.94 (s, 3H), 2.04 (s, 3H), 3.04 (s, 12H), 4.20 (s, 2H), 4.34 (s, 2H), 5.45 (s, 2H), 5.77 (s, 2H), 6.67 (dd, *J* = 8.8 Hz, 2.9 Hz, 2H), 6.75 (d, *J* = 8.8 Hz, 2H), 7.09 (d, *J* = 2.2 Hz, 2H), 7.23 (s, 1H), 7.66 (dd, *J* = 8.1 Hz, 1.5 Hz, 2H), 8.10 (d, *J* = 7.3 Hz, 1H); HRMS (ESI$^+$): Calcd for $[M]^+$ 732.2583, Found, 732.2605 (+2.2 mmu); HPLC analysis: ODS silica gel; eluent: A/B = 50/50 to 0/100, 25 min, linear gradient; solvent A: H_2O, 0.1% AcOH; solvent B: CH_3CN/H_2O = 80/20, 0.1% AcOH; flow rate, 1.0 mL/min; detection wavelength 650 nm.



Scheme S3. Synthetic scheme for compound **2**.

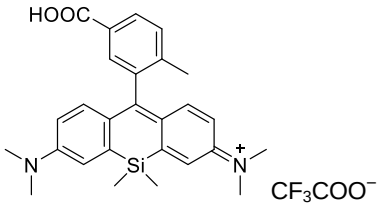


Synthesis of compound 12

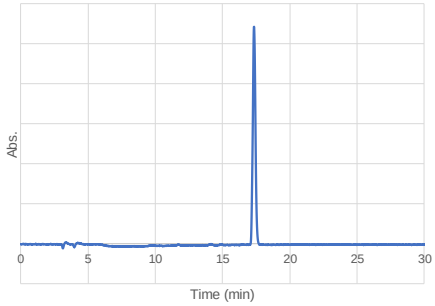


Compound **12** was synthesized from 4.07 g (18.9 mmol) of 3-bromo-4-methylbenzoic acid by using the same method as compound **6** (3.47 g, 12.8 mmol, 68% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.58 (s, 9H), 2.44 (s, 3H), 7.27 (d, *J* = 8.1 Hz, 1H), 7.81 (dd, *J* = 8.1 Hz, 1.5 Hz, 1H), 8.12 (d, *J* = 1.5 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 23.1, 28.1, 81.4, 124.6, 128.2, 130.5, 131.3, 133.2, 142.6, 164.5.

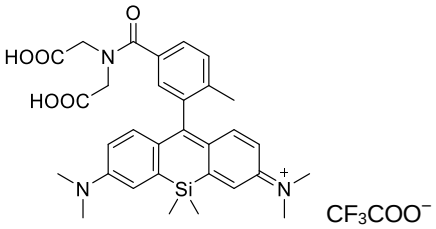
Synthesis of compound 13



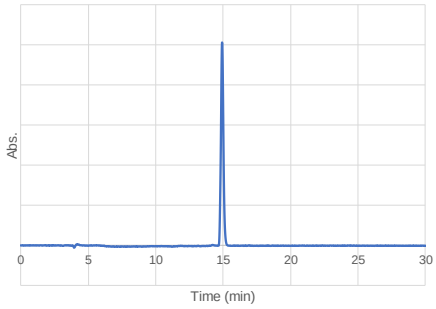
Compound **13** was synthesized from 627 mg (2.31 mmol) of **12** and 72.3 mg (0.223 mmol) of **5** by using the same method as described for 2-COOH SiR650 (**7**) (53.5 mg, 0.0961 mmol, 43% yield). ^{1}H NMR (300 MHz, CD_3OD): δ = 0.61 (s, 3H), 0.63 (s, 3H), 2.11 (s, 3H), 3.35 (s, 12H), 6.78 (dd, *J* = 9.9 Hz, 2.6 Hz, 2H), 7.03 (d, *J* = 9.5 Hz, 2H), 7.37 (d, *J* = 2.9 Hz, 2H), 7.54 (d, *J* = 8.1 Hz, 1H), 7.73 (s, 1H), 8.11 (d, *J* = 8.1 Hz, 1H); HRMS (ESI$^+$): Calcd for $[M]^+$ 443.2154, Found, 443.2158 (+0.4 mmu); HPLC analysis: eluent: A/B = 80/20 to 0/100, 20 min, linear gradient; solvent A: H_2O, 0.1% TFA; solvent B: CH_3CN/H_2O = 80/20, 0.1% TFA; flow rate, 1.0 mL/min; detection wavelength 650 nm.



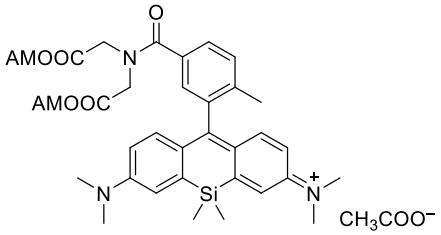
Synthesis of compound 14



Compound **14** was synthesized from 40.0 mg (0.0718 mmol) of **13** by using the same method as described for **11** (8.0 mg, 0.0119 mmol, 17% yield). ^{1}H NMR (300 MHz, CD_3OD): δ = 0.59 (s, 3H), 0.61 (s, 3H), 2.08 (s, 3H), 3.35 (s, 12H), 4.19 (s, 2H), 4.28 (s, 2H), 6.78 (dd, *J* = 9.5 Hz, 2.9 Hz, 2H), 7.06 (d, *J* = 10.3 Hz, 2H), 7.18 (s, 1H) 7.37 (d, *J* = 2.9 Hz, 2H), 7.54-7.55 (m, 2H); HRMS (ESI$^+$): Calcd for $[M]^+$ 558.2424, Found, 558.2431 (+0.7 mmu); HPLC analysis: eluent: A/B = 80/20 to 0/100, 20 min, linear gradient; solvent A: H_2O, 0.1% TFA; solvent B: CH_3CN/H_2O = 80/20, 0.1% TFA; flow rate, 1.0 mL/min; detection wavelength 650 nm.

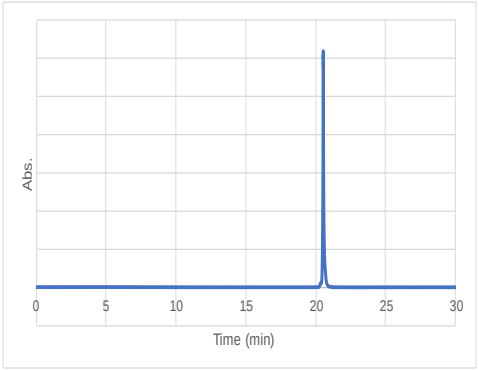


Synthesis of compound 2

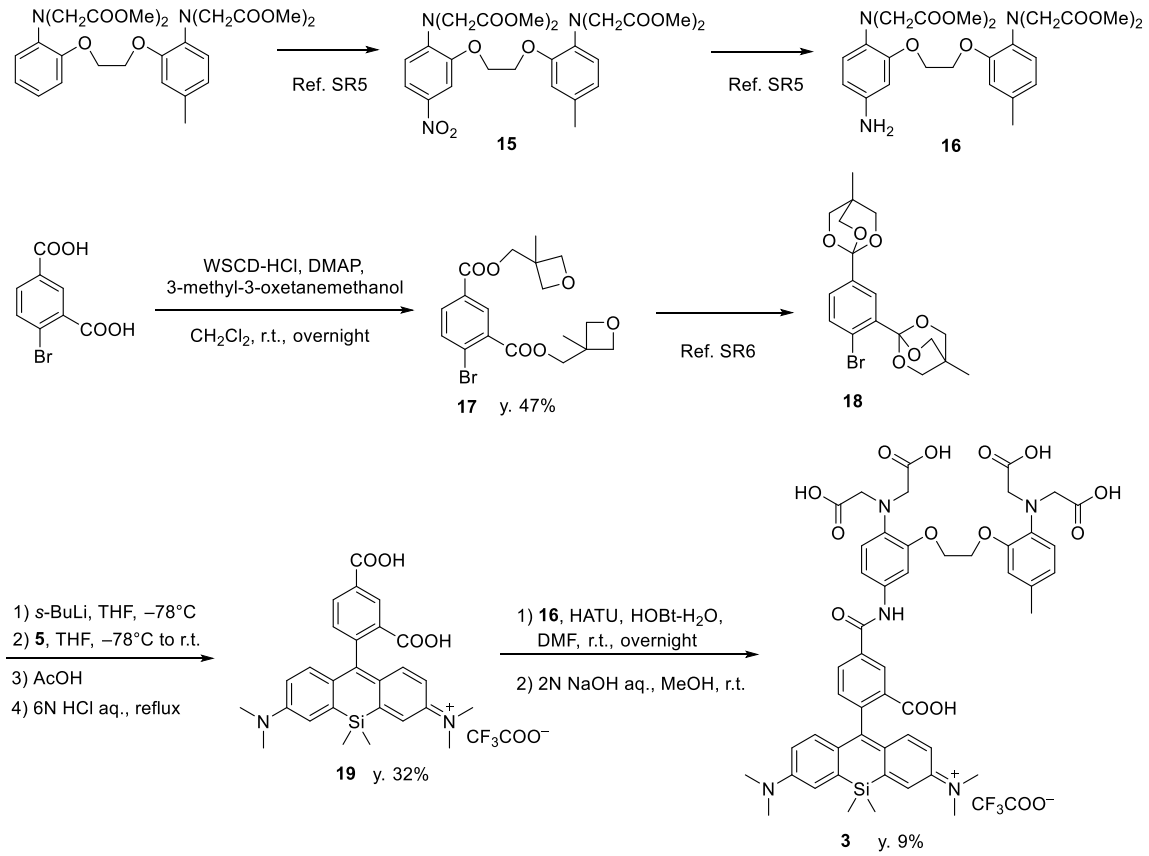


Compound **2** was synthesized from 11.2 mg (0.0167 mmol) of **14** by using the same method as described for **1** (8.9 mg, 0.0109 mmol, 65% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.60 (s, 3H), 0.61 (s, 3H), 2.08-2.09 (m, 6H), 2.12 (s, 3H), 3.38 (s, 12H), 4.24 (s, 2H), 4.37 (s, 2H), 5.64 (s, 2H), 5.80 (s, 2H), 6.65 (dd, *J* = 9.6 Hz, 1.6Hz, 2H), 6.98-7.01 (m, 2H), 7.19-7.23 (m, 3H), 7.42 (d, *J* = 8.4 Hz, 1H), 7.50-7.52 (m, 1H); HRMS (ESI$^+$): Calcd for $[M]^+$ 702.2847, Found, 702.2816 (–3.1 mmu); HPLC analysis: eluent: A/B = 80/20 to 0/100, 20 min, linear gradient;

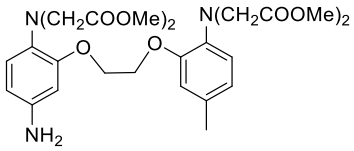
solvent A: H_2O, 0.1% AcOH; solvent B: CH_3CN/H_2O = 80/20, 0.1% AcOH; flow rate, 1.0 mL/min; detection wavelength 650 nm.



Scheme S4. Synthetic scheme for compound **3**.

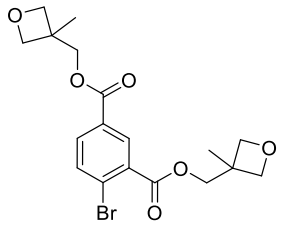


Synthesis of compound 16



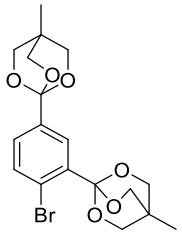
The compound was synthesized according to reference SR5.

Synthesis of compound 17



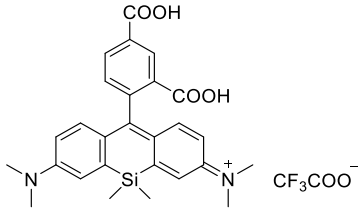
Compound **17** was synthesized from 5.04 g of 4-bromoisophthalic acid by using the same method as described for **8** (4.03 g, 9.75 mmol, 47% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.43 (s, 3H), 1.45 (s, 3H), 4.43-4.48 (m, 8H), 4.61-4.64 (m, 4H), 7.79 (d, *J* = 8.4 Hz, 1H), 8.00 (dd, *J* = 8.4 Hz, 2.4 Hz, 1H), 8.45 (d, *J* = 2.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.1, 21.2, 39.1, 39.2, 69.5, 70.1, 79.3, 79.4, 127.1, 129.2, 132.3, 132.4, 133.1, 134.8, 164.9, 165.3; HRMS (ESI^+): Calcd for $[M + H]^+$ 413.0600, Found, 413.0605 (+0.5 mmu).

Synthesis of compound 18



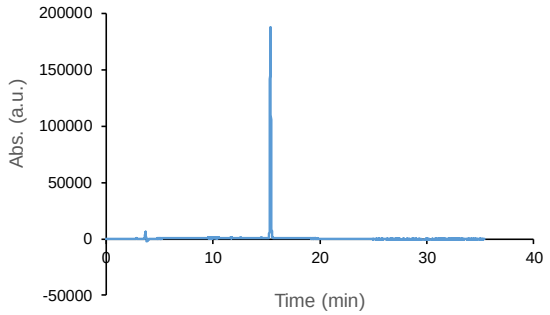
The compound was synthesized according to reference SR6.

Synthesis of compound 19

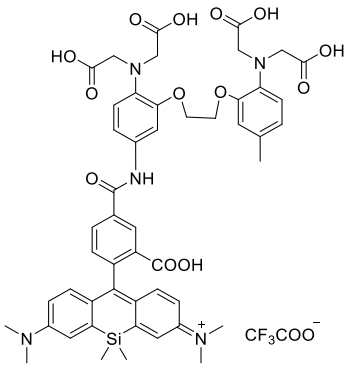


Compound **19** was synthesized from 476 mg (1.15 mmol) of **18** and 49.0 mg (0.151 mmol)

of **5** by using the same method as described for **10** (25.5 mg, 0.0435 mmol, 29% yield). ^{1}H NMR (400 MHz, CD_3OD): δ = 0.15 (s, 3H), 0.22 (s, 3H), 2.85 (s, 12H), 6.31 (dd, *J* = 7.5 Hz, 2.1 Hz, 2H), 6.49 (d, *J* = 9.8 Hz, 2H), 6.74 (d, *J* = 2.4 Hz, 1H), 6.86 (d, *J* = 2.1 Hz, 1H), 6.98 (d, *J* = 7.8 Hz, 1H), 7.93 (dd, *J* = 8.0 Hz, 1.7 Hz, 3H), 8.36-8.38 (m, 1H); HRMS (ESI$^+$): Calcd for $[M]^+$ 483.1896, Found, 473.1877 (-1.9 mmu); HPLC analysis: eluent: A/B = 80/20 to 0/100, 20 min, linear gradient; solvent A: H_2O, 0.1% TFA; solvent B: CH_3CN/H_2O = 80/20, 0.1% TFA; flow rate, 1.0 mL/min; detection wavelength 650 nm.

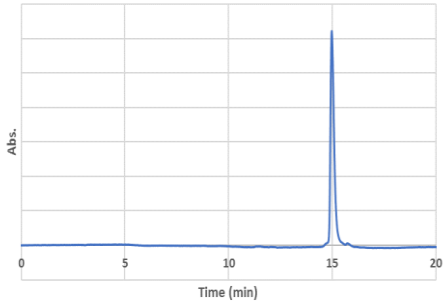


Synthesis of compound 3

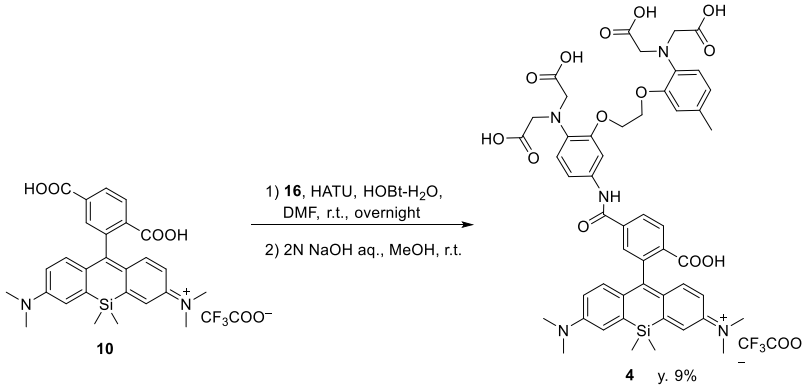


Compound **19** (25.5 mg, 0.044 mmol), **16** (20.0 mg, 0.035 mmol), HATU (86.0 mg, 0.226 mmol) and HOBt·H_2O (48.0 mg, 0.30 mmol) in DMF (3.0 mL) was stirred at room temperature overnight. The solution was evaporated to dryness, then 2 N HCl aq. was added to the residue and the mixture was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in 2 N NaOH aq./MeOH (1.5 mL/1.5 mL), and the solution was stirred at room temperature for 5 h. The whole was purified by HPLC (ODS silica gel, H_2O/CH_3CN, 0.1% TFA) to obtain **3** (3.1 mg, 3.2 μmol, 9% yield). ^{1}H NMR (400 MHz, CD_3OD): δ = 0.57 (s, 3H), 0.65 (s, 3H), 2.27 (s, 3H), 3.30 (s, 12H), 3.82 (s, 4H), 3.90 (s, 4H), 4.38-4.41 (m, 4H), 6.66 (dd, *J* = 9.0 Hz, 2.7 Hz, 3H), 6.74 (d, *J* = 9.3 Hz, 2H), 6.88 (dd, *J* = 8.3 Hz, 4.9 Hz, 2H), 6.98 (d, *J* = 9.3 Hz, 1H), 7.05 (d, *J* = 2.9 Hz, 2H),

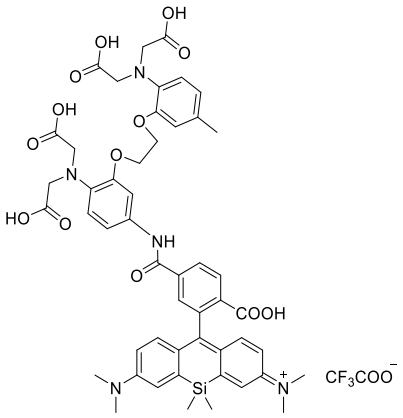
7.37-7.42 (m, 2H), 8.31 (dd, J = 8.3 Hz, 1.5 Hz, 1H) 8.51 (d, J = 1.0 Hz, 1H); HRMS (ESI^+): Calcd for $[M]^+$ 960.3487, Found, 960.3461 (-2.6 mmu); HPLC analysis: eluent: A/B = 80/20 to 0/100, 20 min, linear gradient; solvent A: H_2O, 0.1% TFA; solvent B: CH_3CN/H_2O = 80/20, 0.1% TFA; flow rate, 1.0 mL/min; detection wavelength 650 nm.



Scheme S5. Synthetic scheme for compound **4**.

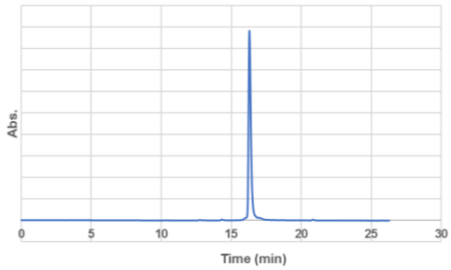


Synthesis of compound 4

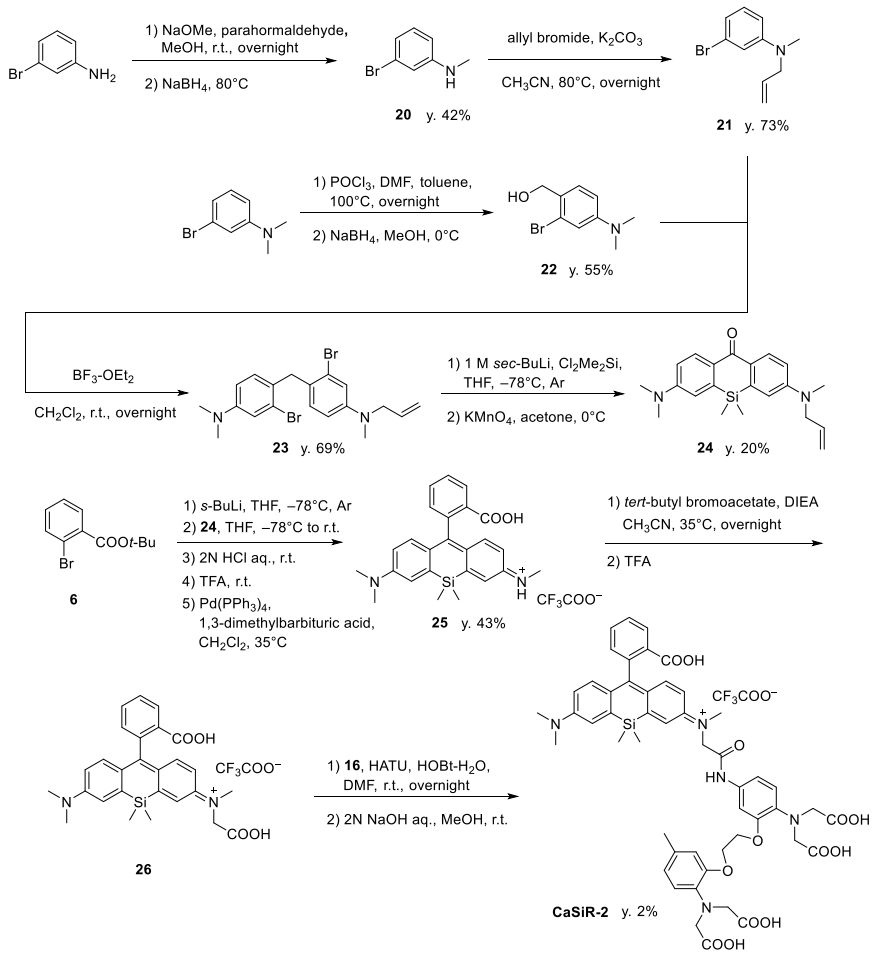


Compound **4** was synthesized from 8.3 mg of **10** and 23.3 mg of **16** by using the same method as described for compound **3** (1.2 mg, 1.12 μmol, 8% yield). ^1H NMR (400 MHz,

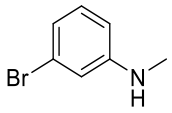
CD_3OD): δ = 0.53 (s, 3H), 0.64 (s, 3H), 2.22 (s, 3H), 2.94 (s, 12H), 3.79 (s, 4H), 3.92 (s, 4H), 4.29-4.30 (m, 4H), 6.61-6.64 (m, 3H), 6.72 (d, *J* = 8.7 Hz, 2H), 6.79-6.83 (m, 2H), 6.86 (d, *J* = 8.7 Hz, 1H), 7.02 (d, *J* = 2.7 Hz, 2H), 7.27 (d, *J* = 2.3 Hz, 1H), 7.33 (dd, *J* = 8.7 Hz, 2.3 Hz, 1H), 7.79 (s, 1H), 8.03 (d, *J* = 8.2 Hz, 1H) 8.16 (dd, *J* = 8.2 Hz, 1.4 Hz, 1H); HRMS (ESI^+): Calcd for $[M]^+$ 960.3487, Found, 960.3507 (+2.0 mmu); HPLC analysis: eluent: A/B = 80/20 to 0/100, 20 min, liner gradient; solvent A: H_2O, 0.1% TFA; solvent B: CH_3CN/H_2O = 80/20, 0.1% TFA; flow rate, 1.0 mL/min; detection wavelength 650 nm.



Scheme S6. Synthetic scheme for **CaSiR-2**.

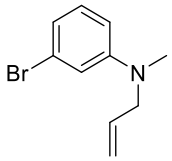


Synthesis of compound 20



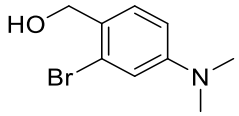
To a solution of 3-bromoaniline (5.00 g, 29.1 mmol) in MeOH (90 mL), NaOMe (11.25 g, 208 mmol) and paraformaldehyde (13.1 g, 437 mmol) were added, and the mixture was stirred at room temperature overnight. The solution was cooled to 0°C, then $NaBH_4$ was slowly added at the same temperature. After that, the mixture stirred at 80°C for 2 h was cooled to room temperature, and 2 N NaOH aqueous solution was added. The mixture was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue was purified by silica gel column chromatography (EtOAc/*n*-hexane = 1/4) to obtain **20** (2.27 g, 12.2 mmol, 42% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.81 (s, 3H), 3.78 (br, 1H), 6.51 (dd, *J* = 8.1, 2.2 Hz, 1H), 6.73-6.74 (m, 1H), 6.80 (d, *J* = 8.1 Hz, 1H), 6.99-7.04 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 30.5, 111.2, 114.7, 119.9, 123.3, 130.4, 150.5; HRMS (ESI^+): Calcd for $[M + H]^+$ 185.9918, Found, 185.9895 (-2.3 mmu).

Synthesis of compound 21



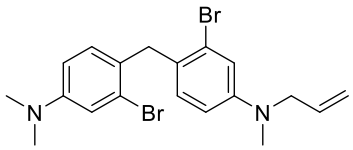
Compound **20** (2.27, 12.2 mmol), K_2CO_3 (4.20 g, 30.4 mmol) and allyl bromide (4.00 g, 33.1 mmol) were suspended in CH_3CN (50 mL), and the mixture was stirred at 80°C overnight. After cooling to room temperature, the solution was filtered, and the filtrate was evaporated to dryness. The resulting oil was purified by silica gel column chromatography (CH_2Cl_2/*n*-hexane = 1/2) to obtain **21** (2.02 g, 8.94 mmol, 73% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.93 (s, 3H), 3.89-3.91 (m, 2H), 5.10-5.18 (m, 2H), 5.74-5.87 (m, 1H), 6.60 (dd, *J* = 8.4 Hz, 2.6 Hz, 1H), 6.78-6.81 (m, 2H), 7.05 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 38.1, 55.1,110.9, 115.0, 116.5, 119.1, 123.5, 130.4, 133.1, 150.7; HRMS (ESI^+): Calcd for $[M + H]^+$ 226.0231, Found, 226.0210 (+2.1 mmu).

Synthesis of compound 22



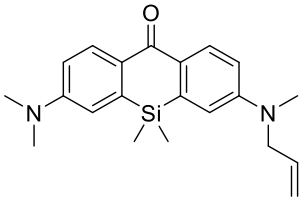
A mixture of DMF (2.0 mL, 25.8 mmol) and $POCl_3$ (2.6 mL, 28.0 mmol) was stirred at 100°C, then *N*,*N* dimethyl-3-bromoaniline (5.02 g, 25.1 mmol) in toluene (130 mL) was added to it. The resulting solution was stirred at the same temperature overnight, then allowed to cool to room temperature, and 2 N NaOH aqueous solution was added to it. The mixture was stirred for 2 h, and then extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue was dissolved in MeOH (100 mL), and $NaBH_4$ was added to the solution at 0°C. The mixture was stirred at the same temperature for 5.5 h, then quenched with H_2O, and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue was purified by silica gel column chromatography (AcOEt/*n*-hexane = 1/4) to obtain **22** (3.20 g, 13.9 mmol, 55% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.87 (t, *J* = 6.6 Hz, 1H), 2.94 (s, 6H), 4.64 (d, *J* = 5.9 Hz, 2H), 6.63 (dd, *J* = 8.4, 2.6 Hz, 1H), 6.88 (d, *J* = 2.9 Hz, 1H), 7.25 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 40.3, 65.1, 111.4, 116.0, 124.4, 127.0 130.4, 151.1; HRMS (ESI$^+$): Calcd for $[M + H]^+$ 230.0181, Found, 230.0164 (-1.7 mmu).

Synthesis of compound 23



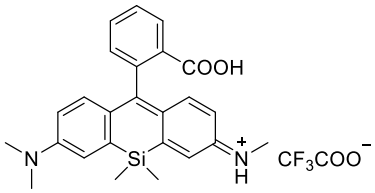
Compound **21** (958 mg, 4.24 mmol), **22** (650 mg, 2.83 mmol) and $BF_3{\cdot}OEt_2$ (452 μmol, 423 mmol) were dissolved in CH_2Cl_2 (10 mL), and the mixture was stirred at room temperature overnight. The reaction was quenched with H_2O, and the solution was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue was purified by silica gel column chromatography (CH_2Cl_2/*n*-hexane = 1/3) to obtain **23** (876 mg, 2.00 mmol, 69% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.88 (s, 9H), 3.83-3.85 (m, 2H), 3.98 (s, 2H), 5.10-5.15 (m, 2H), 5.74-5.83 (m, 1H), 6.52-6.58 (m, 2H), 6.80-6.85 (m, 2H), 6.90-6.93 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 38.1, 39.9, 40.6, 55.2, 111.8, 111.9, 116.1, 116.3, 116.5, 125.7, 127.0, 127.2, 130.9, 133.4, 149.0, 150.1; HRMS (ESI$^+$): Calcd for $[M + H]^+$ 437.0228, Found, 437.0200 (-2.8 mmu).

Synthesis of compound 24



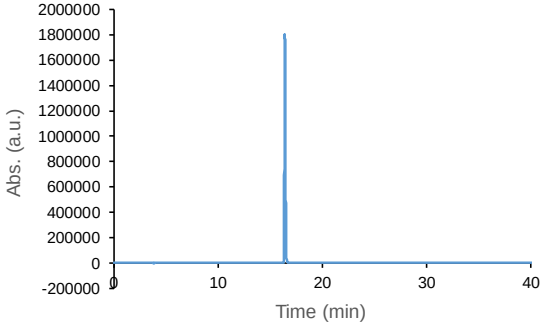
To a flame-dried flask, a solution of **23** (1.17 g, 5.68 mmol) in THF (10 mL) was added under an Ar atmosphere. The solution was cooled to –78°C, and 1 M *sec*-BuLi (6.5 mL, 6.50 mmol) was added to it. Dichlorodimethylsilane (696 μL, 8.04 mmol) in THF (3.0 mL) was added, and the mixture was warmed to room temperature. Then, 2 N HCl aq. was added to the mixture, followed by sat. $NaHCO_3$ aq.. The mixture was extracted with CH_2Cl_2, and the organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue was dissolved in acetone (30 mL), and the solution was cooled to 0°C. $KMnO_4$ (2.54 g, 16.1 mmol) was added to it in small portions. The mixture was filtered through a Celite pad, and the filtrate was evaporated to dryness. The residue was purified by silica gel column chromatography (*n*-hexane/AcOEt = 4/1) to obtain **24** (187 mg, 0.533 mmol, 20% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.45 (s, 6H), 3.08 (s, 3H), 3.09 (s, 6H), 4.03-4.05 (m, 2H), 5.15-5.21 (m, 2H), 5.80-5.92 (m, 1H), 6.78-6.85 (m, 4H), 8.37 (d, *J* = 4.5 Hz, 1H), 8.40 (d, *J* =4.5 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = –1.1, 38.0, 40.0, 54.6, 113.1, 113.2, 114.2, 114.4, 116.5, 129.6, 131.5, 131.6, 132.7, 140.4, 140.5, 150.6, 151.4, 185.2; HRMS (ESI$^+$): Calcd for $[M + H]^+$ 351.1893, Found, 351.1877 (-1.6 mmu).

Synthesis of compound 25

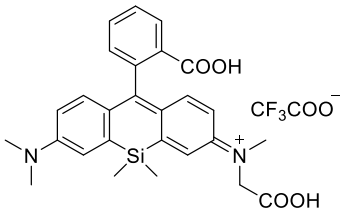


Compound **6** (395 mg, 1.54 mmol) and anhydrous THF (3.0 mL) were added to a flame-dried flask that had been flushed with argon. The solution was cooled to –78°C, and 1 M *sec*-BuLi (1.3 mmol) was added to it. Then, the mixture was stirred for 4 min. At the same temperature, **24** (94.0 mg, 0.267 mmol) dissolved in anhydrous THF (4.0 mL) was slowly added to the solution. The mixture was warmed to room temperature, then stirred for 2 h, and 2 N HCl aq. (5.0 mL) was added. Stirring was continued for 30 min, and then the mixture was extracted

with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue was dissolved in TFA (5.0 mL). This solution was stirred for 3 h, then evaporated to dryness, and the residue was roughly purified by silica gel column chromatography. Fractions containing the intermediate compound (MS (ESI^+): 455) were combined and evaporated to dryness. To the residue in CH_2Cl_2 (30 mL), 1,3-dimethylbarbituric acid (144 mg, 0.923 mmol) and $Pd(PPh_3)_4$ (99 mg, 0.086 mmol) were added, and the mixture was stirred at 35°C overnight. The whole was evaporated to dryness, and the residue was purified by HPLC (ODS silica gel, H_2O/CH_3CN, 0.1% TFA) to obtain **25** (60.6 mg, 0.115 mmol, 43% yield). ^1H NMR (300 MHz, CD_3OD): δ = 0.56 (s, 3H), 0.61 (s, 3H), 3.03(s, 3H), 3.28 (s, 6H), 6.61 (d, *J* = 9.5 Hz, 2H), 6.74 (d, *J* = 9.5 Hz, 1H), 6.97-7.00 (m, 2H), 7.20-7.32 (m, 3H), 7.67 (m, 2H), 8.23 (d, *J* = 7.3 Hz, 1H); HRMS (ESI^+): Calcd for $[M]^+$ 415.1842., Found, 415.1843 (+0.1 mmu); HPLC analysis: eluent: A/B = 80/20 to 0/100, 20 min, linear gradient; solvent A: H_2O, 0.1% TFA; solvent B: CH_3CN/H_2O = 80/20, 0.1% TFA; flow rate, 1.0 mL/min; detection wavelength 650 nm.



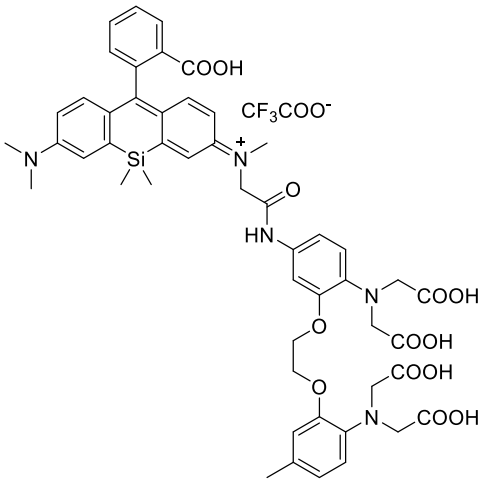
Synthesis of compound 26



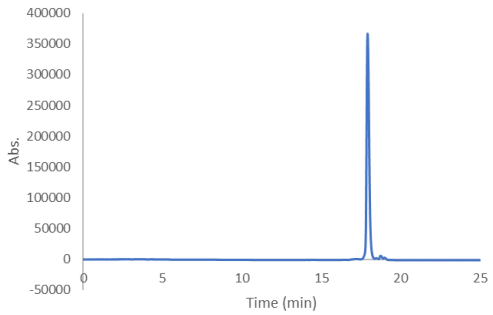
To a solution of **25** (22.0 mg, 41.6 μmol) in CH_3CN (5.0 mL), *tert*-butyl bromoacetate (13.8 μL, 102 μmol) and *N*,*N*-diisopropylethylamine (14.2 μL, 81.5 μmol) were added. The mixture was stirred at 35°C overnight, and then evaporated to dryness. The residue was dissolved in TFA (5.0 mL), and the mixture was stirred at room temperature for 1.5 h. The solution was evaporated to dryness, and the residue was roughly purified by HPLC (ODS silica gel, H_2O/CH_3CN, 0.1% TFA) to obtain crude **26** (10.3 mg). The compound was used to the next step

without further purification.

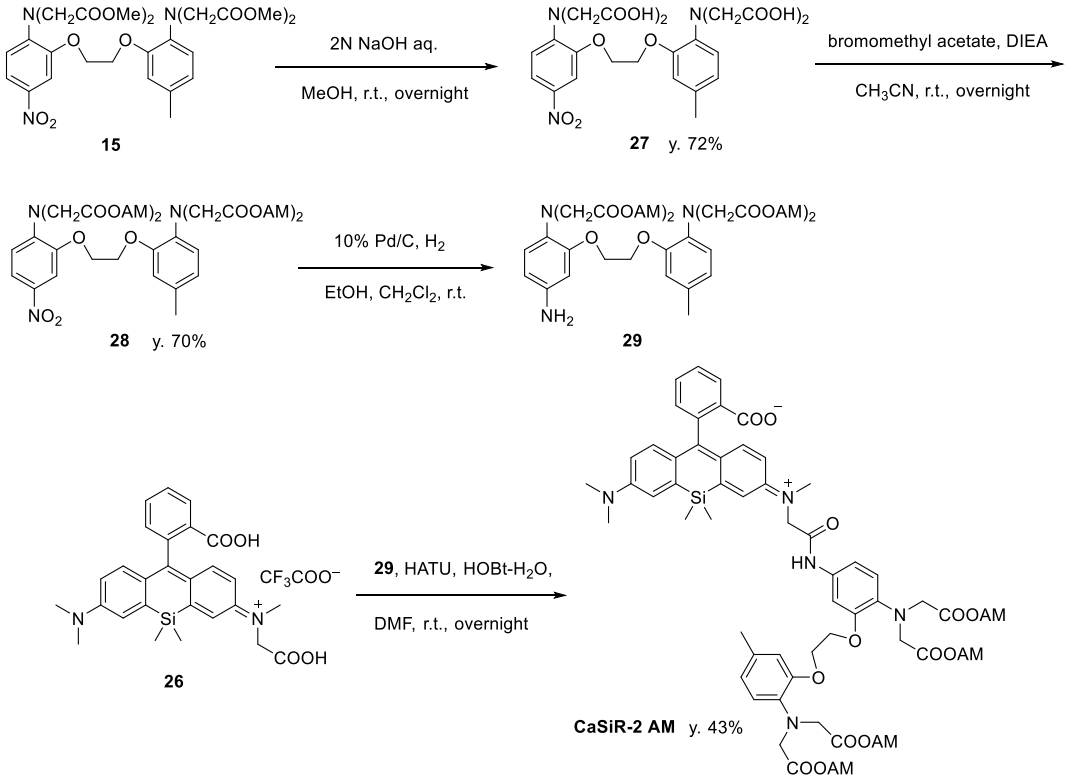
Synthesis of CaSiR-2



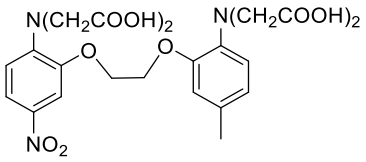
CaSiR-2 was synthesized from 3.7 mg (0.0063 mmol) of **26** and 6.0 mg (0.0107 mmol) of **16** by using the same method as described for compound **3** (0.1 mg, 0.000093 mmol, 1.5% yield). ^{1}H NMR (400 MHz, CD_3OD): δ = 0.50 (s, 3H), 0.57 (s, 3H), 2.22 (s, 3H), 2.90 (s, 6H), 3.08 (s, 3H), 3.46 (s, 4H), 3.50 (s, 4H), 4.09 (s, 2H), 4.22-4.26 (m, 4H), 6.57 (dd, *J* = 9.1 Hz, 3.2 Hz, 1H), 6.61-6.62 (m, 2H), 6.65 (d, *J* = 8.7 Hz, 1H), 6.68 (d, *J* = 9.1 Hz, 1H), 6.73 (d, *J* = 1.4 Hz, 1H), 6.89 (d, *J* = 8.2 Hz, 1H), 6.94 (d, *J* = 8.7 Hz, 1H), 6.98 (dd, *J* = 1.4 Hz, 1.4 Hz, 2H), 7.02 (dd, *J* = 9.4 Hz, 2.5 Hz, 1H), 7.21 (d, *J* = 2.3 Hz, 1H), 7.25 (q, *J* = 7.8 Hz, 1H), 7.59 (ddd, *J* = 7.5 Hz, 7.5 Hz, 1.2 Hz 1H), 7.71 (ddd, *J* = 7.5 Hz, 7.5 Hz, 1.2 Hz, 1H), 7.89 (d, *J* = 7.8 Hz, 1H), 8.49 (br, 1H); HRMS (ESI^+): Calcd for $[M]^+$ 960.3487, Found, 960.3453 (-3.4 mmu); HPLC analysis: eluent: A/B = 80/20 to 0/100, 20 min, linear gradient; solvent A: H_2O, 0.1% TFA; solvent B: CH_3CN/H_2O = 80/20, 0.1% TFA; flow rate, 1.0 mL/min; detection wavelength 650 nm.



Scheme S7. Synthetic scheme for **CaSiR-2 AM**.

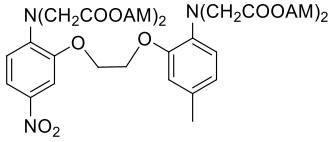


Synthesis of compound 27



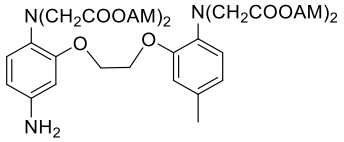
To a solution of **15** (259 mg, 0.438 mmol) in MeOH (5.0 mL), 2 N NaOH aq. (8.0 mL) was added. The mixture was stirred at room temperature overnight, neutralized with 2N HCl aq., and evaporated to dryness. The resulting residue was purified by HPLC (ODS silica gel, H_2O/CH_3CN, 0.1% TFA) to give **27** (170 mg, 0.318 mmol, 72% yield). ^{1}H NMR (400 MHz, CD_3OD): δ =2.27 (s, 3H), 4.12 (s, 4H), 4.24-4.34 (m, 8H), 6.67-6.71 (m, 3H), 6.77 (d, *J* = 7.3 Hz, 1H), 7.73 (d, *J* = 2.9 Hz, 1H), 7.83 (dd, *J* = 8.8, 2.9 Hz, 1H); ^{13}C NMR (75 MHz, CD_3OD): δ = 21.0, 68.1, 69.3, 109.5, 115.7, 116.5, 119.2, 120.8, 122.8, 134.5, 137.1, 141.7, 147.0, 149.8, 151,9, 174.4, 175.3; HRMS (ESI^+): Calcd for $[M + Na]^+$, 558.1336, Found, 558.1340 (+0.4 mmu).

Synthesis of compound 28



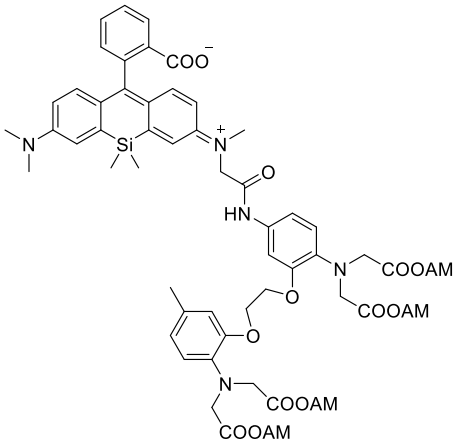
To a solution of **27** (147.8 mg, 0.276 mmol) in CH_3CN (5.0 mL), *N*,*N*-diisopropylethylamine (420 μL, 2.41 mmol) and bromomethyl acetate (120 μL, 1.2 mmol) were added. The mixture was stirred at room temperature overnight. The reaction mixture was acidified with AcOH aq. and the whole was purified by HPLC (ODS silica gel, H_2O/CH_3CN, 0.1% AcOH) to give **28** (158 mg, 0.192 mmol, 70% yield). ^{1}H NMR (300 MHz, CD_2Cl_2): δ =2.03 (s, 6H), 2.05 (s, 6H), 2.27 (s, 3H), 4.13 (s, 4H), 4.28-4.31 (m, 6H), 4.36-4.39 (m, 2H), 5.58 (s, 4H), 5.61 (s, 4H), 6.70-6.75(m, 3H), 6.80 (d, *J* = 8.1 Hz), 7.76 (d, *J* = 2.9 Hz, 1H), 7.81 (dd, *J* = 9.2, 2.6 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 20.5, 20.8, 53.3, 53.4, 66.7, 67.7, 79.0, 79.4, 108.2, 114.9, 116.4, 118.0, 120.4, 122.3, 133.0, 136.2, 141.3, 144.6, 148.7, 150.3, 169.1, 169.3, 169.3, 169.9; HRMS (ESI$^+$): Calcd for $[M + Na]^+$, 846.2181, Found, 846.2173 (-0.8 mmu).

Synthesis of compound 29

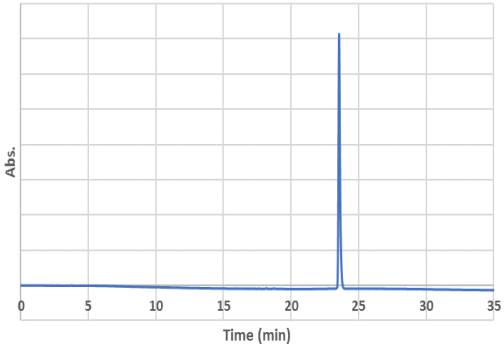


To a solution of **28** (147.9 mg, 0.179 mmol) in EtOH (5.0 mL) and CH_2Cl_2 (5.0 mL), Pd/C (10%) was added, and the mixture was stirred under H_2 at room temperature for 3 h. Pd/C was removed by filtration, and the filtrate was evaporated to dryness. The resulting residue was roughly purified by HPLC to give crude **29** (77.0 mg).

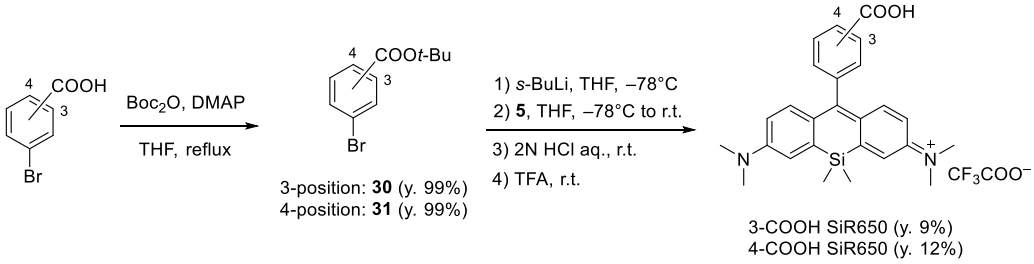
Synthesis of CaSiR-2 AM



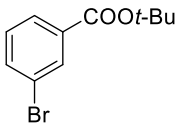
A solution of **26** (4.1 mg, 7.0 μmol), **29** (35.0 mg, 41 μmol), HATU (15.7 mg, 41.3 μmol) and HOBt·H_2O (3.6 mg, 23.5 μmol) in DMF (2.0 mL) was stirred at room temperature overnight, and then neutralized with AcOH aq. The solution was purified by HPLC (ODS silica gel, H_2O/CH_3CN, 0.1% AcOH) to give **CaSiR-2 AM** (4.0 mg, 3.06 μmol, 43% yield). ^{1}H NMR (400 MHz, CD_3OD): δ = 0.55 (s, 3H), 0.62 (s, 3H), 2.00 (s, 6H), 2.03 (s, 6H), 2.28 (s, 3H), 2.98 (s, 6H), 3.17 (s, 3H), 4.13-4.17 (m, 10H), 4.26 (s, 4H), 5.58 (s, 4H), 5.60 (s, 4H), 6.63-6.84 (m, 8H), 7.01-7.08 (m, 3H), 7.29-7.33 (m, 2H), 7.64-7.68 (m, 1H), 7.75-7.78 (m, 1H), 7.96 (d, *J* = 7.8 Hz, 1H); HRMS (ESI$^+$): Calcd for $[M]^+$ 1248.4332., Found, 1248.4289 (-4.3 mmu); HPLC analysis: eluent: A/B = 80/20 to 0/100, 20 min, liner gradient; solvent A: H_2O, 0.1% AcOH; solvent B: CH_3CN/H_2O = 80/20, 0.1% AcOH; flow rate, 1.0 mL/min; detection wavelength 650 nm.



Scheme S8. Synthetic scheme for 3-COOH SiR650 and 4-COOH SiR650.



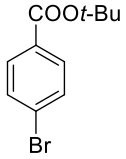
Synthesis of compound 30



Compound **30** was synthesized from 1.02 g of 3-bromobenzoic acid by using the same method as described for compound **6** (1.30 g, 5.06 mmol, 99% yield). ^{1}H NMR (400 MHz,

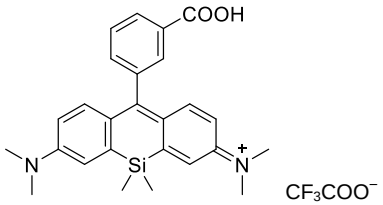
$CDCl_3$): δ = 1.59 (s, 9H), 7.29 (d, *J* = 7.7 Hz, 1H), 7.64 (dd, *J* = 8.1, 2.2 Hz, 1H), 7.92 (d, *J* = 7.8 Hz, 1H), 8.11 (d, *J* = 1.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.0, 81.1, 122.3, 128.1, 129.9, 132.5, 134.0, 135.4, 164.4.

Synthesis of compound 31

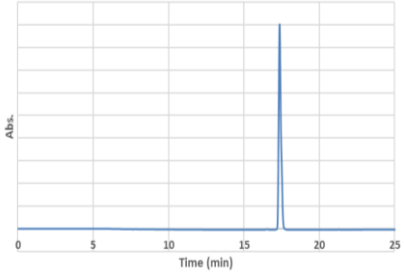


Compound **31** was synthesized from 998 mg of 4-bromobenzoic acid by using the same method as described for compound **6** (1.30 g, 5.06 mmol, 99 % yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.59 (s, 9H), 7.55 (d, *J* = 8.8 Hz, 2H), 7.84 (d, *J* = 8.1 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.2, 81.6, 127.5, 131.0, 131.1, 131.6, 165.1.

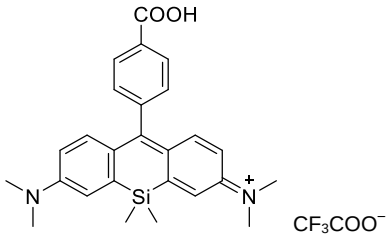
Synthesis of 3-COOH SiR650



3-COOH SiR650 was synthesized from 153 mg (0.595 mmol) of compound **30** and 23.0 mg of **5** (0.071 mmol) by using the same method as described for 2-COOH SiR650 (**7**) (3.5 mg, 0.0065 mmol, 9% yield). ^{1}H NMR (400 MHz, acetone-d_6): δ = 0.15 (s, 6H), 2.95 (s, 12H), 6.40 (dd, *J* = 9.8, 2.9 Hz, 2H), 6.63 (d, *J* = 9.8 Hz, 2H), 7.08-7.12 (m, 3H), 7.27-7.30 (m, 1H), 7.45 (s, 1H), 7.76 (d, *J* = 7.8 Hz, 1H); HRMS (ESI$^+$): Calcd for $[M]^+$, 429.1998, Found, 429.2014 (+1.6 mmu); HPLC analysis: ODS silica gel; eluent: A/B = 80/20 to 0/100, 20 min, linear gradient; solvent A: H_2O, 0.1% TFA; solvent B: CH_3CN/H_2O = 80/20, 0.1% TFA; flow rate, 1.0 mL/min; detection wavelength 650 nm.



Synthesis of 4-COOH SiR650



4-COOH SiR650 was synthesized from 153 mg (0.595 mmol) of compound **31** and 21.4 mg (0.0659 mmol) of **5** by using the same method as described for 2-COOH SiR650 (**7**) (4.2 mg, 0.0077 mmol, 12% yield). ^{1}H NMR (400 MHz, acetone-d_6): δ = 0.15 (s, 6H), 2.96 (s, 12H), 6.40 (dd, *J* = 9.3, 2.9 Hz, 2H), 6.64 (d, *J* = 9.3 Hz, 2H), 6.99 (d, *J* = 7.8 Hz, 2H), 7.09 (d, *J* = 2.9 Hz, 2H), 7.74 (d, *J* = 8.3 Hz, 2H); HRMS (ESI$^+$): Calcd for [M]$^+$, 429.1998, Found, 429.2014 (+1.6 mmu); HPLC analysis: ODS silica gel; eluent: A/B = 80/20 to 0/100, 20 min, linear gradient; solvent A: H_2O, 0.1% TFA; solvent B: CH_3CN/H_2O = 80/20, 0.1% TFA; flow rate, 1.0 mL/min; detection wavelength 650 nm.

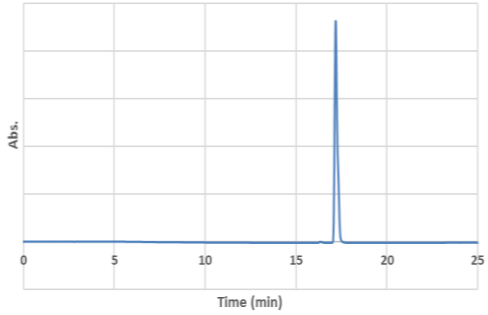


Table S1. Photophysical properties of **3**, **4** and **CaSiR-2** [a]

	$[Ca^{2+}] = 0$ μM			$[Ca^{2+}] = 39$ μM			
	λ_{abs} (nm)	λ_{em} (nm)	Φ_{fl} [b]	λ_{abs} (nm)	λ_{em} (nm)	Φ_{fl} [b]	K_d [μM]
3	646	673	0.10	646	674	0.25	0.30
4	648	671	0.04	647	672	0.29	0.37
CaSiR-2	637	664	0.01	636	661	0.26	0.31

[a] Measurements were made in a buffer under the conditions described in Fig. S4 or Fig. 3 in the absence and presence of free Ca^{2+} ions. [b] The absolute fluorescence quantum yields were determined with Hamamatsu Photonics Quantaurus-QY C11347.

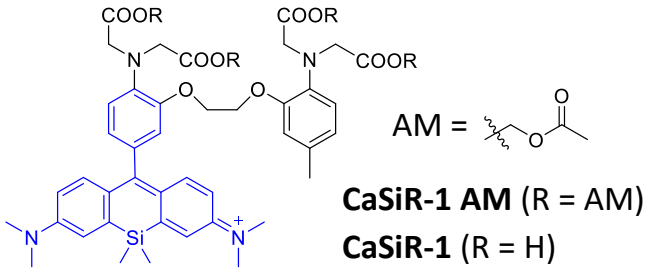


Fig. S1. Structures of **CaSiR-1 AM** and **CaSiR-1**.

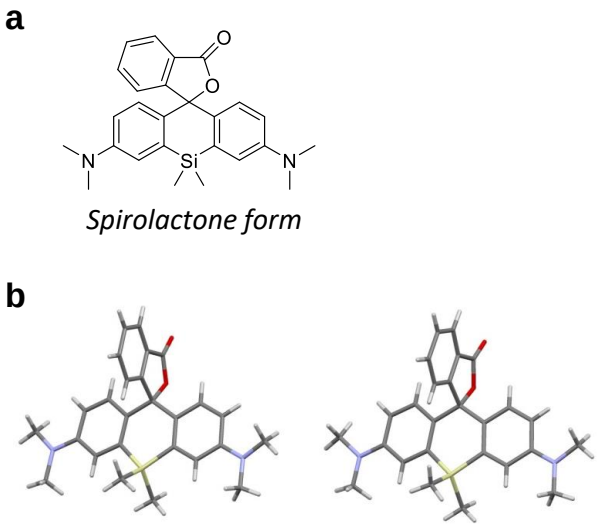


Fig. S2. (a) Structure of the spirolactone form of 2-COOH SiR650. (b) Result of single-crystal X-ray analysis of 2-COOH SiR650. The asymmetric unit contains two crystallographically independent molecules. 2-COOH SiR650 takes an intramolecularly spirocyclized structure as shown in (a).

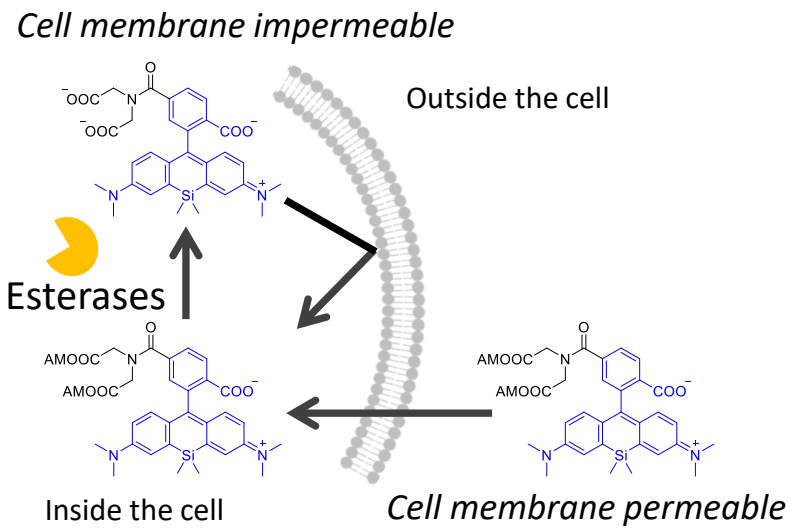


Fig. S3. Proposed mechanism of cytosolical retention of **1**.

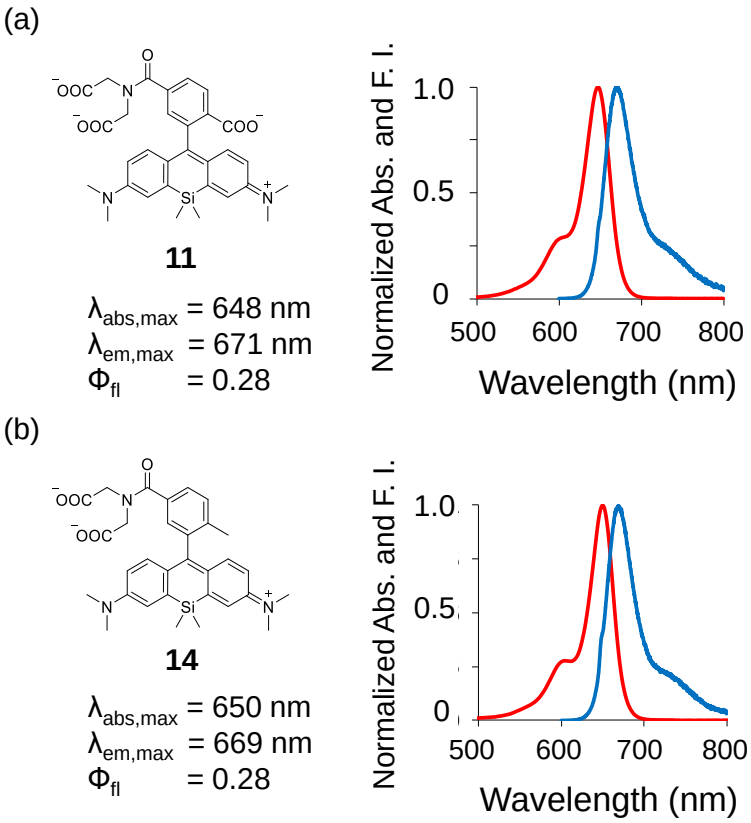


Fig. S4. Structures, absorption maximum ($\lambda_{abs.}$), emission maximum ($\lambda_{em.}$) and fluorescence quantum yield (Φ_{fl}) of **11** (a) and **14** (b) in sodium phosphate buffer (pH 7.4) (**11**: ex. 648 nm, **14**: ex. 650 nm). For determination of Φ_{fl}, SiR650 in PBS (pH 7.4) (Φ_{fl} = 0.31) was used as a fluorescence standard.[SR7] The absorption (red) and emission (blue) spectra of 1 μM **11** and **14** in sodium phosphate buffer (pH 7.4) containing 0.1% DMSO as a cosolvent are also shown.

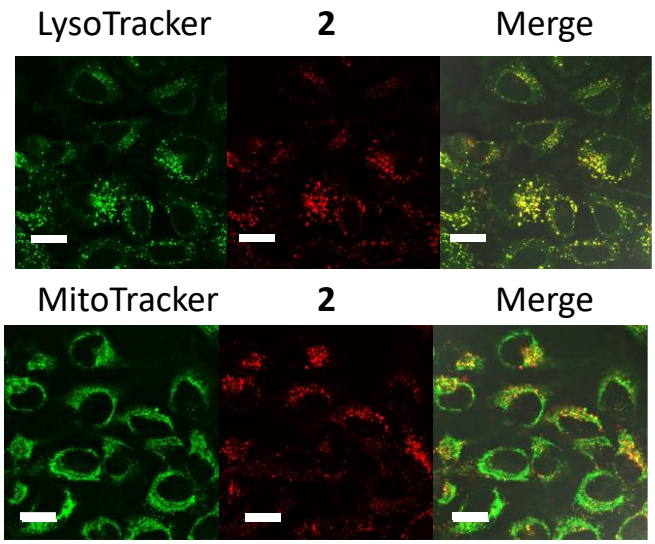


Fig. S5. Fluorescence images of HeLa cells incubated with 1 μM **2** and 75 nM LysoTracker or 75 nM MitoTracker for 1 hr. Ex. 650 nm; Em. 670-750 nm. Scale bar: 20 μm.

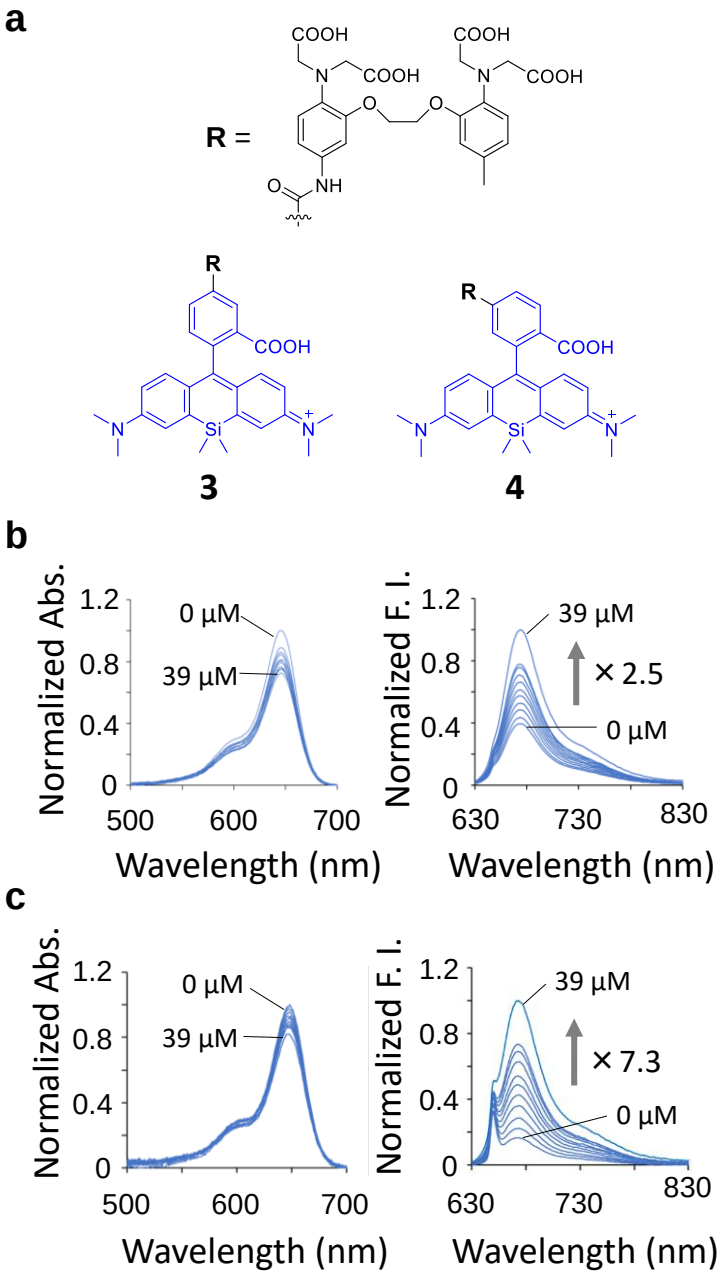


Fig. S6. (a) Structures of candidate 2-COOH SiR650-based fluorescence probes for Ca^{2+}, **3** and **4**. (b,c) Normalized absorption (left) and fluorescence (right) spectra of 1 μM **3** (b) and **4** (c) in the presence of various concentrations of free Ca^{2+} (0, 0.017, 0.038, 0.065, 0.100, 0.150, 0.225, 0.351, 0.602, 1.35, 39 μM) in 30 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer containing 100 mM KCl and 10 mM ethylene glycol tetraacetic acid (EGTA), pH 7.2. The excitation wavelength was 646 nm (**3**) or 648 nm (**4**).

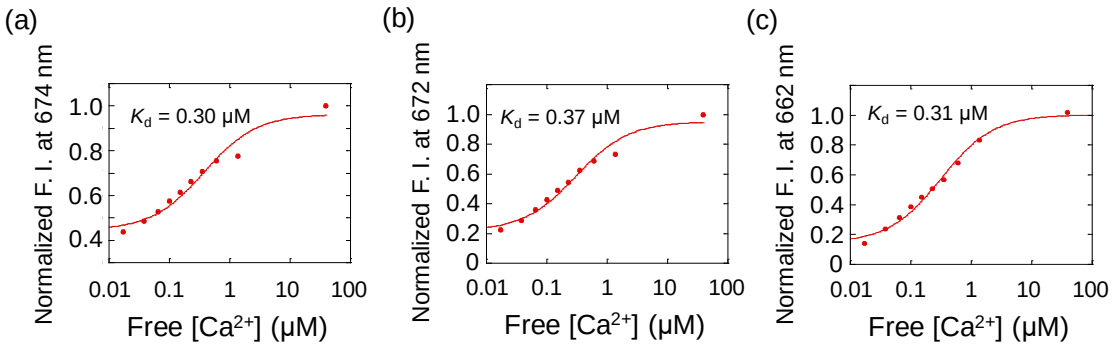


Fig. S7. Plots of fluorescence intensity of **3**, **4** and **CaSiR-2** in the presence of various concentrations of free Ca^{2+} (0, 0.017, 0.038, 0.065, 0.100, 0.150, 0.225, 0.351, 0.602, 1.35, 39 mM) in 30 mM 3-(*N*- morpholino)propanesulfonic acid (MOPS) buffer containing 100 mM KCl and 10 mM ethylene glycol tetraacetic acid (EGTA), pH 7.2. The excitation wavelength was 646 nm (**3**), 648 nm (**4**) and 635 nm (**CaSiR-2**), respectively. The dissociation constant K_d of each compound with Ca^{2+} was determined by curve fitting with KaleidaGraph 4.1.

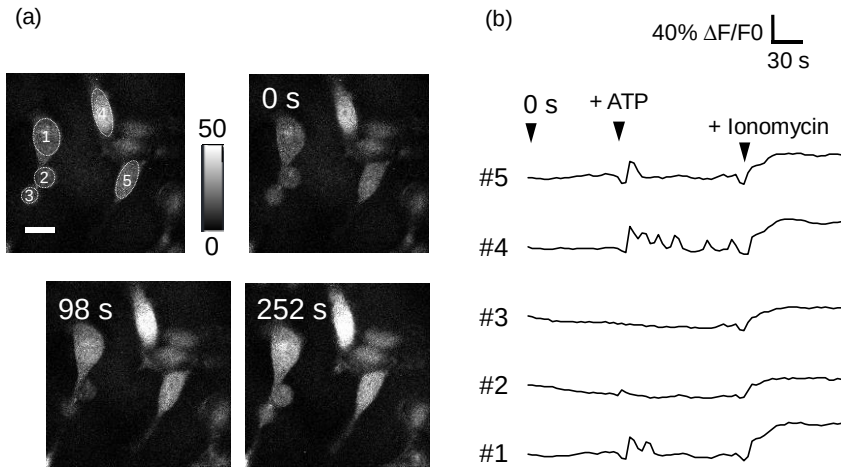


Fig. S8. (a) Fluorescence imaging of ATP-induced calcium oscillations in HeLa cells utilizing **CaSiR-2 AM**. HeLa cells were incubated with 3 μM **CaSiR-2 AM** in HBSS (Hank's Balanced Salt Solution) containing 0.03% Pluronic and 0.45% DMSO as a cosolvent at 37˚C for 30 min. Then, the dyes were washed out three times, and the fluorescence imaging was started. Cells were stimulated with 100 μM ATP at 90 s and 5 μM ionomycin at 210 s. Fluorescence images were taken at the time points indicated in each fluorescence image. (b) Fluorescence intensity changes in ROIs of individual cells numbered 1-5 in (a) are shown. Scale bars: 20 μm.

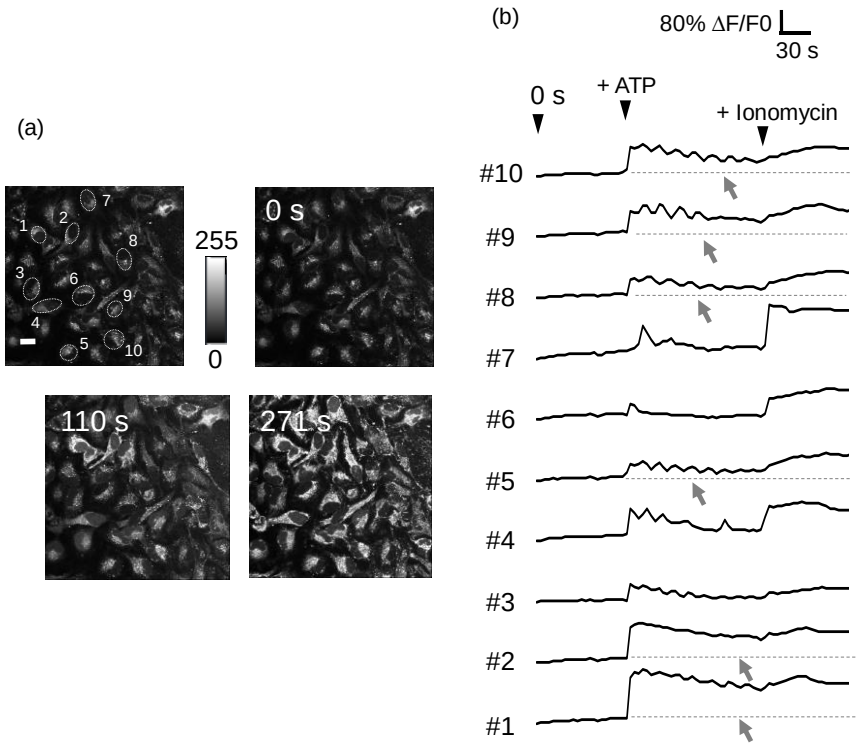


Fig. S9. Fluorescence imaging of ATP-induced calcium oscillations in HeLa cells with **CaSiR-1 AM**. HeLa cells were incubated with 3 μM **CaSiR-1 AM** in HBSS containing 0.03% Pluronic and 0.45% DMSO as a cosolvent at 37˚C for 30 min. Then, the cells were washed three times and the fluorescence imaging was started. Cells were stimulated with 100 μM ATP at 90 s and 5 μM ionomycin at 210 s. Fluorescence images were taken at the time points indicated in each fluorescence image (a). Fluorescence intensity changes in ROIs of individual cells numbered 1-10 in (a) are shown in (b). Gray arrows indicate the background fluorescence increase after the stimulation with ATP. The cells numbered 1, 2, 9 and 10 especially showed the large background fluorescence increase. Scale bars: 20 μm.

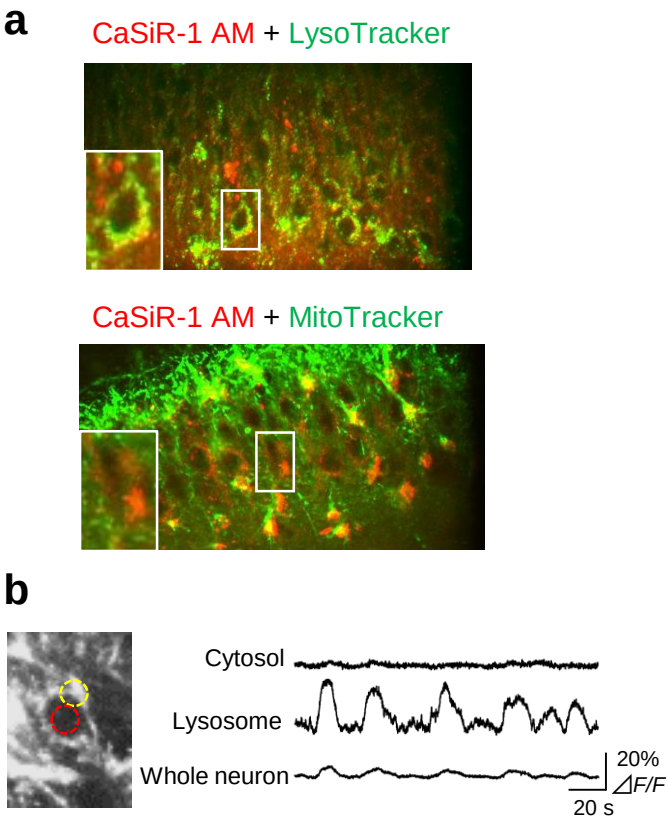


Fig. S10. (a) Costaining with **CaSiR-1 AM**, and LysoTracker or MitoTracker. Rat brain slices were incubated with 5 μM **CaSiR-1 AM** and 75 nM LysoTracker Green DND-26 or 200 nM MitoTracker Green FM in artificial cerebrospinal fluid (aCSF) at 37˚C for 30 min. Merged fluorescence images of **CaSiR-1 AM** (red) and LysoTracker (green) or MitoTracker (green) are shown. (b) Fluorescence traces of cytosol, lysosome and whole neuron of rat brain slice stained with **CaSiR-1 AM** are shown. Red and yellow ROIs indicate the cytosolic and lysosomal regions, respectively. The ROI of the whole neuron includes the ROIs of both cytosolic and lysosomal regions.

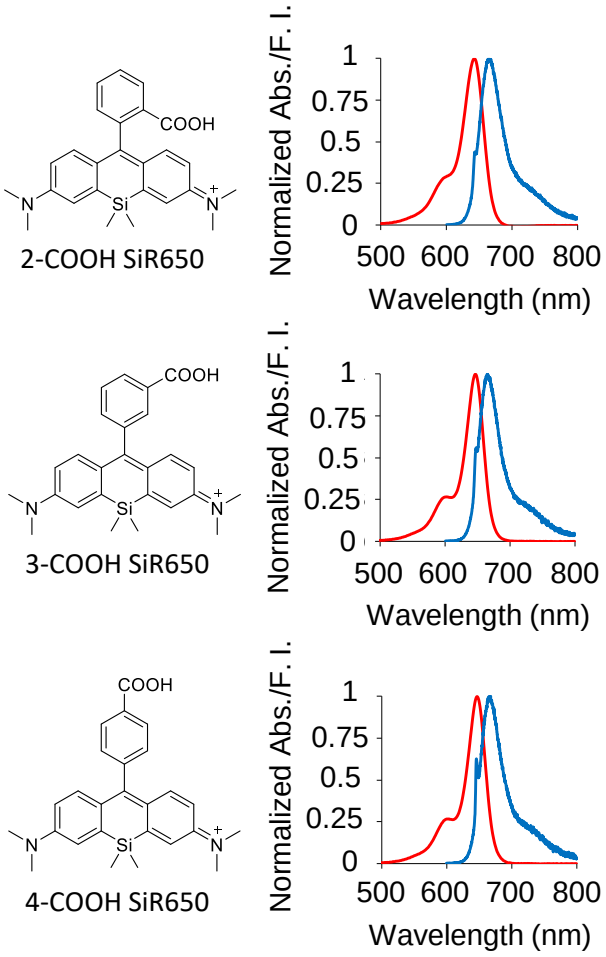


Fig. S11. Structures of 2-COOH SiR650, 3-COOH SiR650 and 4-COOH SiR650, and the absorption (red) and fluorescence (blue) spectra of 0.5 μM 2-COOH SiR650, 3-COOH SiR650 and 4-COOH SiR650 in 100 mM sodium phosphate buffer (pH 7.4) containing 0.05% DMSO as a cosolvent. The three SiR650 derivatives had similar absorbance and emission spectra ($\lambda_{abs} \approx$ 650 nm and $\lambda_{em} \approx$ 670 nm) in 100 mM sodium phosphate buffer (pH 7.4).

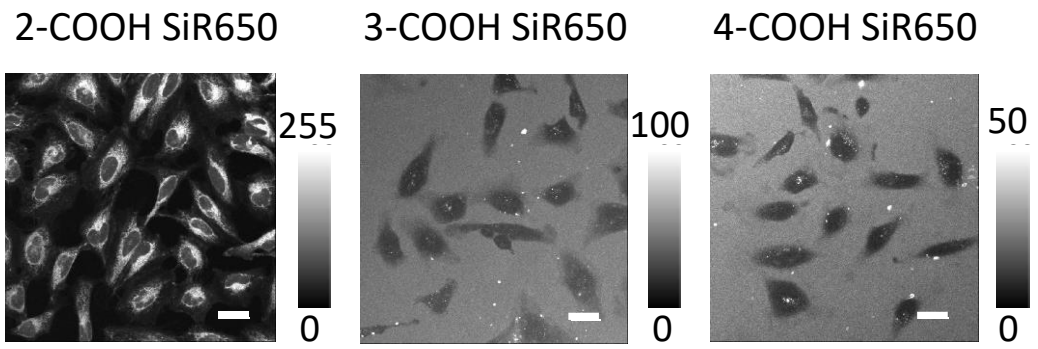


Fig. S12. Fluorescence images of HeLa cells incubated with 1 μM 2-COOH SiR650, 3-COOH SiR650 or 4-COOH SiR650 for 1 hr. Ex. 633 nm; Em. 670-750 nm. Scale bar: 30 μm. The strong fluorescence was observed throughout the cells, except for the nucleus, after staining with 2-COOH SiR650, whereas the fluorescence signals of 3-COOH SiR650 and 4-COOH SiR650 were mainly observed in the extracellular medium, but not inside of the cells. These results indicate that 3-COOH SiR650 and 4-COOH SiR650 cannot permeate through the cell-membrane, whereas 2-COOH SiR650 does enter the cells and is distributed throughout the intracellular regions. Only 2-COOH SiR650 can take a spirolactone form.

Table S2. Photophysical properties of 2-, 3- and 4-COOH SiR650 [a]

Compound	λ_{abs} (nm)	λ_{em} (nm)	Φ_{fl} [b]
2-COOH SiR650	643	664	0.31
3-COOH SiR650	647	665	0.21
4-COOH SiR650	647	668	0.11

[a] Measurements were made in 100 mM sodium phosphate buffer (pH 7.4) containing 0.05% DMSO as a cosolvent. [b] For determination of Φ_{fl}, 2-Me SiR650 in PBS (pH 7.4) (Φ_{fl} = 0.31) was used as a fluorescence standard.[SR7]

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