Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Development of a Reversible Fluorescent Probe for Reactive Sulfur Species, Sulfane Sulfur, and its Biological Application

Yoko Takano,[†] Kenjiro Hanaoka,[†,*] Kazuhito Shimamoto,[†] Ryo Miyamoto,[‡] Toru Komatsu,[†,‖] Tasuku Ueno,[†] Takuya Terai,[†] Hideo Kimura,[‡] Tetsuo Nagano,[§] Yasuteru Urano[†,¶,⊥,*]

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

[§] Drug Discovery Initiative, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.

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

[‡] Department of Molecular Pharmacology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8502, Japan

[‖] PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

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

Synthetic materials and instrumentation. General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, Aldrich Chemical Co., Kanto Chemical Co., Alfa Aesar, Watanabe Chemical Industries, Dojindo and Invitrogen Corp., and were used without further purification. All solvents were used after appropriate distillation or purification. NMR spectra were recorded on a JEOL JNM-LA300 instrument at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR or JEOL JNM-AL400 instrument at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. Mass spectra (ESI^{+} or ESI^{-}) were measured with a JEOL JMS-T100LC AccuTOF for ESI. HPLC analyses were performed on an Inertsil ODS-3 (4.6 × 250 mm) column (GL Sciences Inc.) using a HPLC system composed of a pump (PU-2080, JASCO) and a detector (MD-2018 or FP-2025, JASCO). UPLC-MS analyses were performed on an BEH C18 1.7 μm (2.1 × 50 mm) column (ACQUITY UPLC®) using a UPLC system composed of a pump (Acquity UPLC H class, Waters), and a detector (Acquity UPLC and Acquity QDa, Waters). Preparative HPLC was performed on an Inertsil ODS-3 (10 × 250 mm) column (GL Sciences Inc.) using a HPLC system composed of a pump (PU-2080, JASCO) and a detector (MD-2015 or FP-2025, JASCO) or a HPLC system composed of a pump (PU-2086, JASCO) and a detector (MD-2018, JASCO). pH was measured with a F-52 pH meter (HORIBA) electrode.

UV-visible and fluorescence spectra measurements. UV-visible spectra were obtained on a spectrometer (UV-1650, Shimadzu, Japan). Fluorescence spectroscopic studies were performed on a fluorescence spectrometer (F-4500, Hitachi, Japan). The slit width was 5.0 nm for both excitation and emission. The photomultiplier voltage was 700 V. All experiments were carried out at room temperature. Dyes were dissolved in DMSO to obtain stock solutions.
Conditions for Figure 3a and 3b: Fluorescence intensity of SSip-1 was measured in 100 mM sodium phosphate buffer (pH 7.4) containing 0.1% DMSO as a cosolvent and 1 mg/mL BSA.

Determination of relative fluorescence quantum yields. For determination of the relative fluorescence quantum efficiency (Φ_{fl}) of SSip-1, fluorescein in 0.1 N NaOH aq. (Φ_{fl} = 0.85) was used as a standard.[S1] Values were calculated by use of the following equation.

$$\Phi_x/\Phi_{std} = [A_{std}/A_x][n_x^2/n_{std}^2][D_x/D_{std}]$$

std: standard x: sample

A: absorbance at the excitation wavelength

n: refractive index

D: area under the fluorescence spectra on an energy scale

Since the absorbance and fluorescence spectra of two dyes, fluorescein and 2-thio RB, could not be separated, we calculated the apparent fluorescence quantum efficiency by using the following values:

A: absorbance at the excitation wavelength (470 nm)

D: area under the fluorescence spectra on an energy scale (480 nm-670 nm)

Determination of detection limit. The detection limit of SSip-1 was calculated according to the following equation: $3\sigma/S$, in which σ is the standard deviation of blank measurements, $n = 3$, and S is the slope of the regression equation according to Figure 3a.

Cell lines and culture conditions. A549 cells were purchased from RIKEN Bioresource Center cell bank (Tsukuba, Japan). Cells were cultured in DMEM (Dulbecco's modified Eagle's medium) (Gibco 11885), containing 10% fetal bovine albumin (Invitrogen) and 1% penicillin streptomycin (Invitrogen). Cells were maintained at 37 °C under an atmosphere of 5% CO_2 in air.

Fluorescence confocal microscopy. A549 cells seeded on Lab-Tek®II Chambered #1.5 German Coverglass System 8 chambers were washed with 200 μL of Hanks' Balanced Salt Solution (HBSS), then incubated for 1 h at 37 °C in 200 μL DMEM (Gibco 21063) containing 10 μM SSip-1 DA, 0.1% DMSO and 0.03% Pluronic as a cosolvent. After incubation, cells were washed with HBSS, and 200 μL DMEM (Gibco 21063) supplemented with 10% fetal bovine albumin and 1% penicillin streptomycin was added to the cells. Fluorescence images were captured using a Leica Application Suite Advanced Fluorescence (LAS-AF) with a TCS SP5 and a 63× objective lens. The light source was an argon laser. The excitation wavelength was 488 nm and the emission wavelengths were 500-540 nm for fluorescein (PMT; Gain:1000) and 590-650 nm for 2-thio RB (HyD; gain: 100).

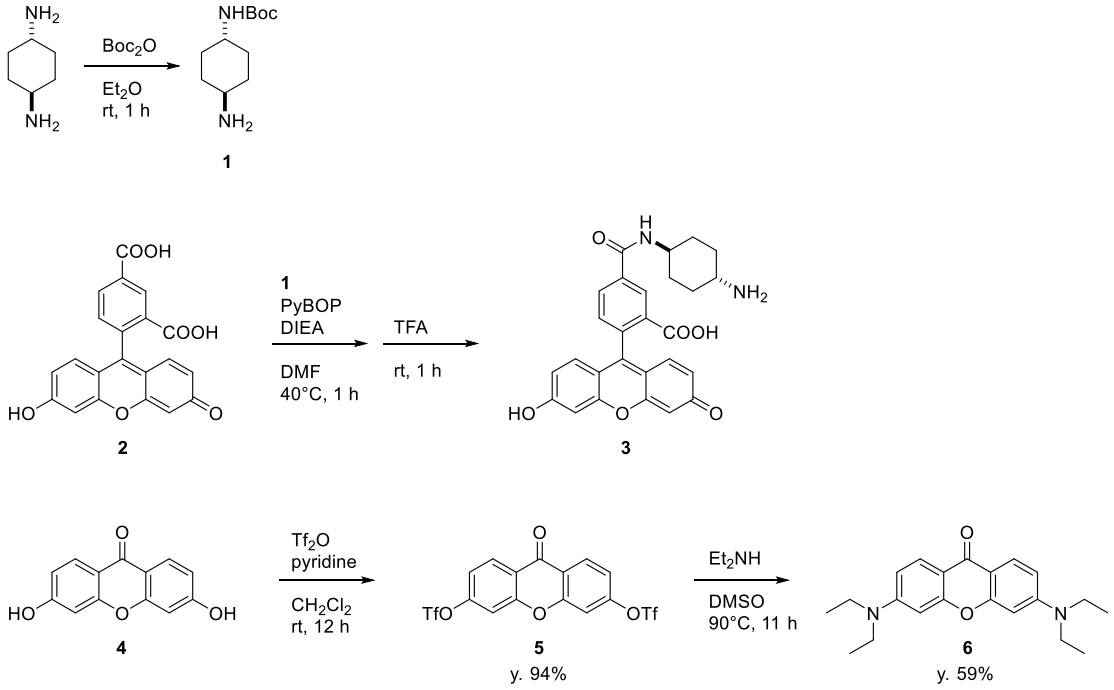
Primary culture of hippocampal astrocytes. All the animal procedures were approved by the Animal Care and Use Committee of The National Institute of Neuroscience (NCNP). Hippocampi were dissected from embryonic day 17 Sprague-Dawley rats (CLEA Japan, Tokyo, Japan). After the meninges were removed, the hippocampal tissues were incubated with 0.25% trypsin (Sigma-Aldrich, St Louis, MO, USA) and 0.1% DNase (Sigma-Aldrich) in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline for 15 min at 37°C. Cells were dispersed by gentle pipetting, suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum and 5% horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin, and seeded onto poly-D-lysine-coated coverslips at a density of 5×10^4 cells/cm^2. The cells were

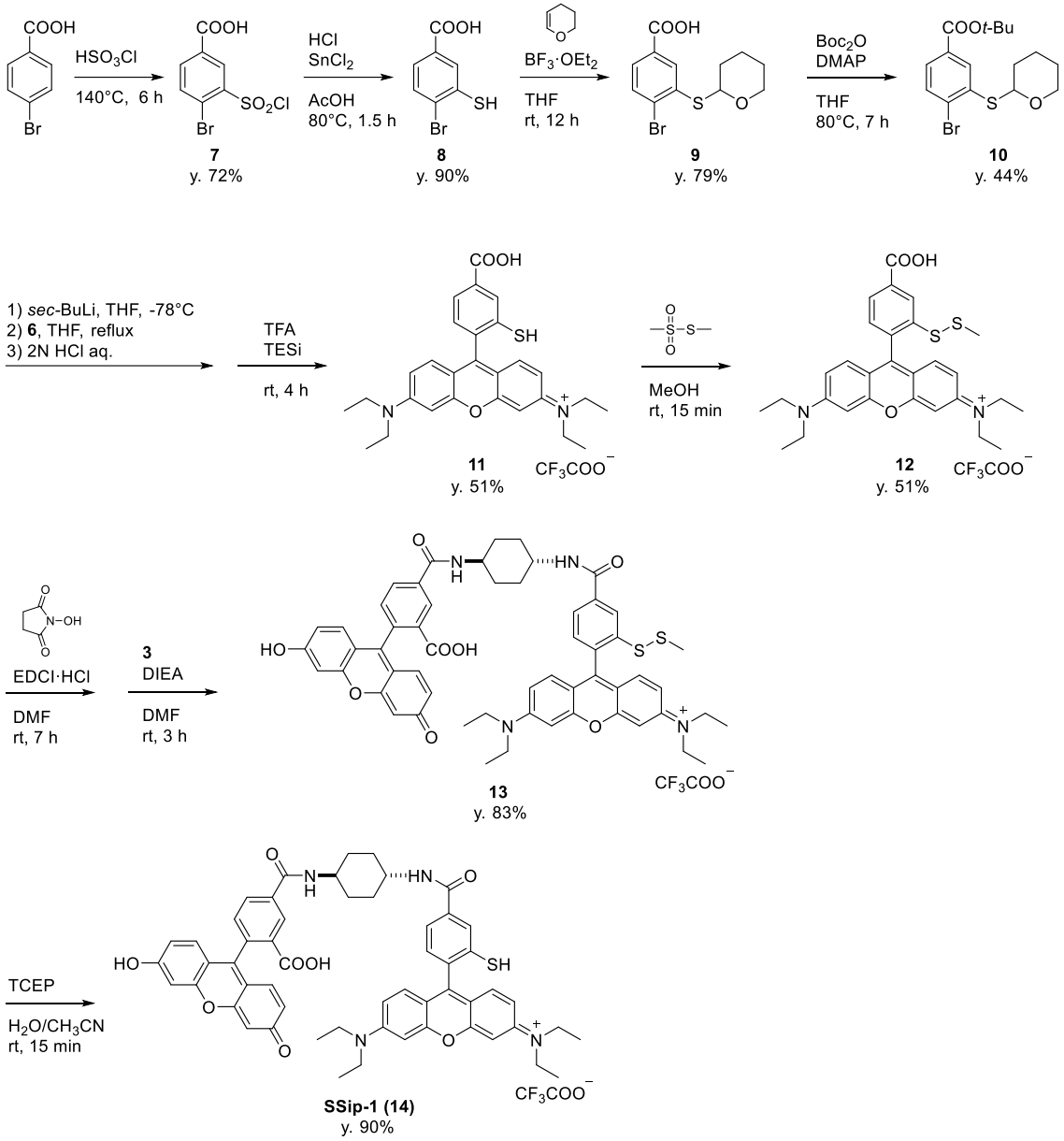
cultured in a humidified atmosphere of 10% CO_2 and 90% air at 37ºC for 14 days. Half of the culture medium was changed twice a week.

Imaging of intracellular calcium and polysulfide. Fluo-4 AM (Thermo Fisher Scientific, Waltham, MA, USA) and SSip-1 DA were diluted in balanced salt solution (BSS; mM): 137 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, 10 HEPES (pH 7.3 with NaOH). Cultured astrocytes were loaded with 2 µM Fluo-4 AM (0.02% Cremophor EL) or 10 µM SSip-1 DA (0.03% Pluronic F-127) for 30 min at room temperature or 60 min at 37ºC, respectively. Incubation was continued in BSS for 15 min at room temperature, then cells placed on a coverslip, which was mounted on an upright microscope (DM LFS, Leica, Heidelberg, Germany) and continuously superfused with BSS at a rate of 1 ml/min. Fluorescence was detected every 5 s through a bandpass filter with excitation at 480/40 nm and emission at 527/30 nm. Fluorescence images were acquired using Aquacosmos 2.6 software (Hamamatsu Photonics, Shizuoka, Japan).

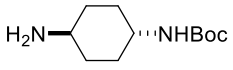
Synthesis and Characterization of Compounds

Scheme S1. Synthetic scheme for SSip-1



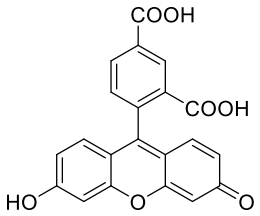


Synthesis of compound 1



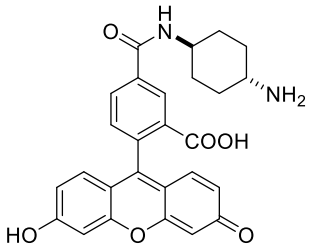
This compound was synthesized according to reference S2.

Synthesis of 5-carboxy fluorescein (2)



This compound was synthesized according to reference S3.

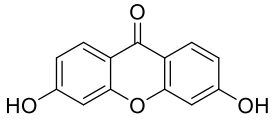
Synthesis of compound 3



To a solution of compound **2** (147 mg, 0.391 mmol) in anhydrous DMF (3.0 mL), compound **1** (167 mg, 0.782 mmol), *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU, 163 mg, 0.430 mmol) and *N*,*N*-diisopropylethylamine (252 mg, 340 μL, 1.95 mmol) were added. The mixture was stirred at 40°C for 1 h. The product was collected by filtration, and then washed with 1 N HCl aq. and H_2O. The resulting residue was dissolved in trifluoroacetic acid (TFA) (3.0 mL) and the solution was stirred at room temperature for 1 h. TFA was evaporated off, and the resulting residue was purified by HPLC (eluent, a 25-min linear gradient, from 25% to 70% eluent B; flow rate, 5.0 ml/min; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA)) to afford compound **3** (63.0 mg, 0.133 mmol, 34% yield) as a yellow solid.

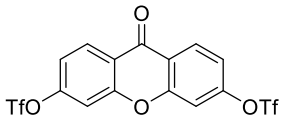
^{1}H NMR (400 MHz, CD_3OD): δ 1.55-1.58 (4H, m), 2.13-2.16 (4H, m), 3.13-3.16 (1H, m), 3.92-3.95 (1H, m), 6.57 (2H, dd, *J* = 8.8, 2.4 Hz), 6.63 (2H, d, *J* = 8.8 Hz), 6.73 (2H, d, *J* = 2.4 Hz), 7.32 (1H, d, *J* = 8.3 Hz), 8.19 (1H, dd, *J* = 8.3, 1.5 Hz), 8.43 (1H, d, *J* = 1.5 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): δ 29.2, 29.7, 47.5, 48.6, 102.2, 109.0, 112.6, 123.2, 124.1, 126.4, 129.0, 134.7, 136.3, 151.8, 154.6, 157.9, 158.2, 159.6, 163.9, 168.1; HRMS (ESI$^+$): Calcd for $[M+H]^+$, 473.1713, Found, 473.1686 (−2.7 mmu).

Synthesis of compound 4



This compound was synthesized according to reference S4

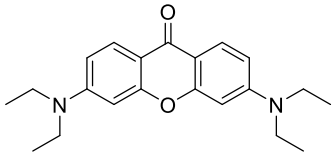
Synthesis of compound 5



To a solution of compound **4** (2.28 g, 10.0 mmol) in CH_2Cl_2 (50 mL), pyridine (7.90 g, 8.1 mL, 100 mmol) was slowly added at 0°C. The mixture was stirred at 0°C for 10 min, then trifluoromethanesulfonic anhydride (8.46 g, 5.0 mL, 30.0 mmol) was slowly added at 0°C. The mixture was warmed to room temperature, then stirred for 12 h, and H_2O was added to it. The organic layer was washed with H_2O, 1 N HCl aq. and brine, dried over Na_2SO_4 and evaporated to dryness. The resulting residue was recrystallized from CH_2Cl_2/hexane to afford compound **5** (4.60 g, 9.35 mmol, 94% yield) as a colorless solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36 (2H, dd, *J* = 8.8, 2.4 Hz), 7.50 (2H, d, *J* = 2.4 Hz), 8.45 (2H, d, *J* = 8.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 111.4, 113.9, 117.1, 118.2, 120.3, 121.4, 123.5, 129.6, 153.4, 156.6, 174.5.

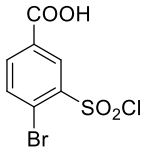
Synthesis of 3,6-bis(diethylamino)xanthone (6)



To a solution of compound **5** (880 mg, 1.80 mmol) in DMSO (5.0 mL), diethylamine (1.31 g, 1.9 mL, 18.0 mmol) was added. The mixture was stirred at 90°C for 11 h, then cooled to room temperature, and H_2O was added to it. 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 resulting residue was purified by column chromatography (silica gel, 1% MeOH/CH_2Cl_2) to afford compound **6** (356 mg, 1.05 mmol, 59% yield) as a yellowish solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.22 (12H, t, *J* = 7.1 Hz), 3.43 (8H, q, *J* = 7.1 Hz), 6.44 (2H, d, *J* = 2.4 Hz), 6.63 (2H, dd, *J* = 9.3, 2.4 Hz), 8.09 (2H, d, *J* = 9.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.5, 44.6, 96.3, 108.5, 111.5, 127.7, 152.0, 158.4, 174.7; HRMS (ESI$^+$): Calcd for $[M+H]^+$, 339.2073, Found, 339.2028 (−4.5 mmu).

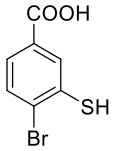
Synthesis of 4-bromo-3-chlorosulfonylbenzoic acid (7)



To 4-bromobenzoic acid (6.01 g, 29.8 mmol), chlorosulfonic acid (15 mL) was slowly added at 0°C. The solution was warmed to 140°C, then stirred for 6 h. The reaction mixture was cooled to room temperature and poured slowly into iced H_2O. The mixture was extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated to dryness. The resulting residue was washed with CH_2Cl_2 to afford compound **7** (6.32 g, 21.3 mmol, 72% yield) as a colorless solid.

^{1}H NMR (400 MHz, acetone-d_6):δ 8.23 (1H, d, J = 8.3 Hz), 8.33 (1H, dd, J = 8.3, 2.0 Hz), 8.74 (1H, d, J = 2.0 Hz); ^{13}C NMR (100 MHz, acetone-d_6): δ 125.8, 132.1, 132.2, 137.8, 138.4, 143.8, 165.0; HRMS (ESI$^-$): Calcd for [M−H]$^-$, 298.8604, Found, 298.8589 (−1.5 mmu).

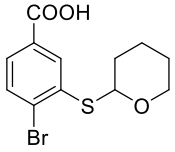
Synthesis of 4-bromo-3-mercaptobenzoic acid (8)



To a solution of compound **7** (5.00 g, 16.7 mmol) in AcOH (50 mL), $SnCl_2{\cdot}2H_2O$ (22.5 g, 100 mmol) in 10 N HCl aq. (25 mL) was added. The resulting mixture was stirred at 80°C for 1.5 h, then cooled to room temperature. The product was collected by filtration, washed with H_2O and dried *in vacuo*, affording compound **8** (3.50 g, 15.1 mmol, 90% yield) as a yellowish solid.

^{1}H NMR (400 MHz, acetone-d_6): δ 5.04 (1H, s), 7.66 (1H, dd, J = 8.3, 2.0 Hz), 7.72 (1H, d, J = 8.3 Hz), 8.14 (1H, d, J = 2.0 Hz); ^{13}C NMR (100 MHz, acetone-d_6): δ 126.7, 128.2, 131.3, 131.5, 134.0, 136.7, 166.5; HRMS (ESI$^-$): Calcd for [M−H]$^-$, 232.9095, Found, 232.9139 (+4.4 mmu).

Synthesis of 4-bromo-3-(tetrahydropyran-2-ylthio)benzoic acid (9)

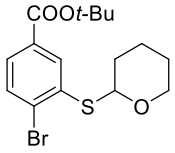


To a solution of compound **8** (3.00 g, 12.9 mmol) and 3,4-dihydro-2*H*-pyran (2.17 g, 2.3 mL, 25.9 mmol) in THF (50 mL), boron trifluoride-ethyl ether complex (1.84 g, 1.6 mL, 12.9

mmol) was slowly added at 0°C. The mixture was warmed to room temperature, then stirred for 12 h, and sat. $NaHCO_3$ aq. (30 mL) was added to it. This mixture was extracted with AcOEt. The aqueous layer was acidified with 2 N HCl aq. and then extracted with AcOEt. The combined organic layer was washed with brine, dried over Na_2SO_4 and evaporated to dryness. The resulting residue was roughly purified by column chromatography (silica gel, 1:1 to 1:4 hexane/AcOEt). The residue was recrystallized from CH_2Cl_2/hexane to afford the pure compound **9** (3.23 g, 10.3 mmol, 79% yield) as a colorless solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.66-1.78 (3H, m), 1.90-1.99 (2H, m), 2.10-2.14 (1H, m), 3.68-3.73 (1H, m), 4.15-4.21 (1H, m), 5.48-5.49 (1H, m), 7.64 (1H, d, *J* = 8.3 Hz), 7.73 (1H, dd, *J* = 8.3, 2.0 Hz), 8.29 (1H, d, *J* = 2.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.2, 25.4, 31.1, 64.1, 83.7, 128.2, 129.1, 129.8, 130.9, 133.0, 138.7, 171.0; HRMS (ESI^-): Calcd for $[M-H]^-$, 314.9691, Found, 314.9724 (+3.3 mmu).

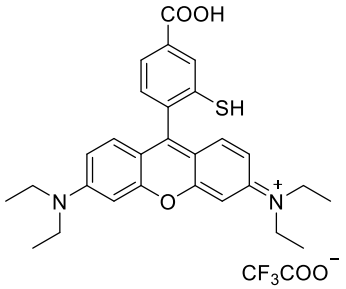
Synthesis of *tert*-butyl 4-bromo-3-(tetrahydropyran-2-ylthio)benzoate (10)



To a solution of compound **9** (2.50 g, 7.94 mmol) in THF (80 mL), 4-dimethylaminopyridine (194 mg, 1.59 mmol) and di-*tert*-butyl dicarbonate (3.46 g, 3.7 mL, 15.9 mmol) were added. The mixture was warmed to 80°C, then stirred for 7 h. The reaction mixture was cooled to room temperature and H_2O was added to it. Stirring was continued for 30 min, and then the mixture was extracted with CH_2Cl_2. The organic layer was washed with sat. $NaHCO_3$ aq., sat. NH_4Cl aq. and brine, dried over Na_2SO_4 and evaporated to dryness. The resulting residue was purified by column chromatography (silica gel, 1:1 to 2:3 hexane/CH_2Cl_2) to afford compound **10** (1.30 g, 3.49 mmol, 44% yield) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.58 (9H, s), 1.66-1.69 (3H, m), 1.90-1.95 (2H, m), 2.06-2.09 (1H, m), 3.66-3.69 (1H, m), 4.16-4.19 (1H, m), 5.36-5.37 (1H, m), 7.57 (1H, d, *J* = 8.3 Hz), 7.62 (1H, dd, *J* = 8.3, 2.0 Hz), 8.19 (1H, d, *J* = 2.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.6, 25.3, 28.1, 31.1, 64.7, 81.4, 83.8, 127.7, 128.3, 130.6, 131.7, 132.5, 137.9, 164.7; HRMS (ESI^+): Calcd for $[M+Na]^+$, 395.0293, Found, 395.0266 (−2.7 mmu).

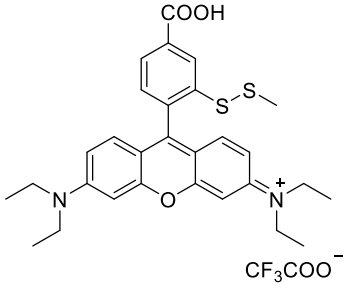
Synthesis of compound 11



To a flame-dried flask flushed with argon, compound **10** (660 mg, 1.78 mmol) and anhydrous THF (20 mL) were added. The solution was cooled to −78°C, and 1 M *sec*-BuLi (1.60 mL, 1.60 mmol) was slowly added. The mixture was stirred at −78°C for 20 min, and a solution of compound **6** (60 mg, 0.178 mmol) in anhydrous THF (10 mL) was slowly added. The mixture was warmed to 70°C, and stirred for 3 h. It was cooled to room temperature and then 2 N HCl aq. (20 mL) was added to it. Stirring was continued for 20 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 resulting residue was partially purified by column chromatography (silica gel, 0% to 10% MeOH/CH_2Cl_2). The partially purified compound and triethylsilane (100 μL) were dissolved in trifluoroacetic acid (TFA) (5.0 mL) and the solution was stirred for 4 h. The mixture was evaporated to dryness. The resulting residue was purified by HPLC (eluent, a 20-min linear gradient, from 50% to 100% eluent B; flow rate, 5.0 ml/min; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA)) to afford compound **11** (52.9 mg, 90.0 μmol, 51% yield) as a red solid.

^{1}H NMR (300 MHz, CD_3OD): δ1.22 (12H, t, *J* = 7.1 Hz), 3.61 (8H, q, *J* = 7.1 Hz), 6.91 (2H, d, *J* = 2.2 Hz), 7.00 (2H, dd, *J* = 9.5, 2.2 Hz), 7.09 (2H, d, *J* = 9.5 Hz), 7.32 (1H, d, *J* = 8.1 Hz), 7.93 (1H, dd, *J* = 8.1, 1.5 Hz), 8.19 (1H, d, *J* = 1.5 Hz); ^{13}C NMR (75 MHz, CD_3OD): δ 12.8, 47.0, 97.6, 114.1, 115.9, 127.7, 131.4, 132.4, 132.6, 134.3, 134.6, 136.4, 155.9, 157.4, 159.6, 168.3; HRMS (ESI$^+$): Calcd for $[M-CF_3COO]^+$, 475.2055, Found, 475.2033 (−2.2 mmu).

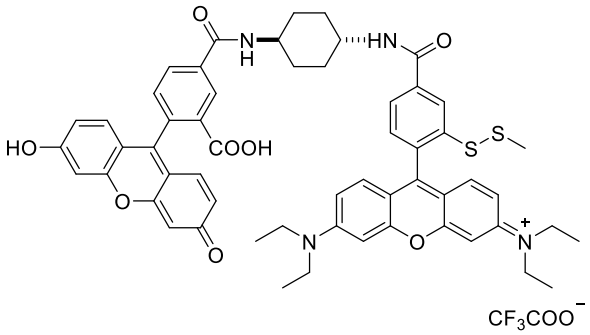
Synthesis of compound 12



To a solution of *S*-methyl methanethiosulfonate (140 mg, 0.10 mL, 1.11 mmol) in methanol (2.0 mL), compound **11** (52.9 mg, 90.0 μmol) in methanol (3 mL) was slowly added. The mixture was stirred at room temperature for 15 min and then evaporated to dryness. The resulting residue was purified by HPLC (eluent, a 20-min linear gradient, from 50% to 100% eluent B; flow rate, 5.0 ml/min; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA)) to afford compound **12** (23.7 mg, 45.5 μmol, 51% yield) as a red solid.

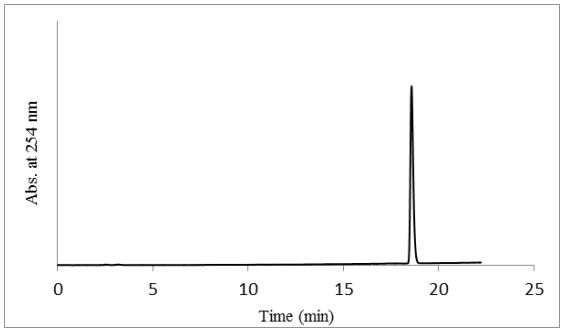
^{1}H NMR (400 MHz, CD_3OD): ^{1}H NMR (400 MHz, CD_3OD): δ1.32 (12H, t, *J* = 7.1 Hz), 2.38 (3H, s), 3.70 (8H, q, *J* = 7.1 Hz), 7.01 (2H, d, *J* = 2.4 Hz), 7.08 (2H, dd, *J* = 9.3, 2.4 Hz), 7.15 (2H, d, *J* = 9.3 Hz), 7.47 (1H, d, *J* = 7.8 Hz), 8.15 (1H, dd, *J* = 7.8, 1.5 Hz), 8.71 (1H, d, *J* = 1.5 Hz); ^{13}C NMR (100 MHz, CD_3OD): δ 12.8, 23.2, 47.0, 97.6, 114.4, 115.9, 129.4, 130.1, 131.6, 132.4, 134.8, 136.9, 138.7, 155.1, 157.5, 159.4, 168.3; HRMS (ESI$^+$): Calcd for [M−CF_3COO]$^+$, 521.1933, Found, 521.1890 (−4.3 mmu).

Synthesis of compound 13

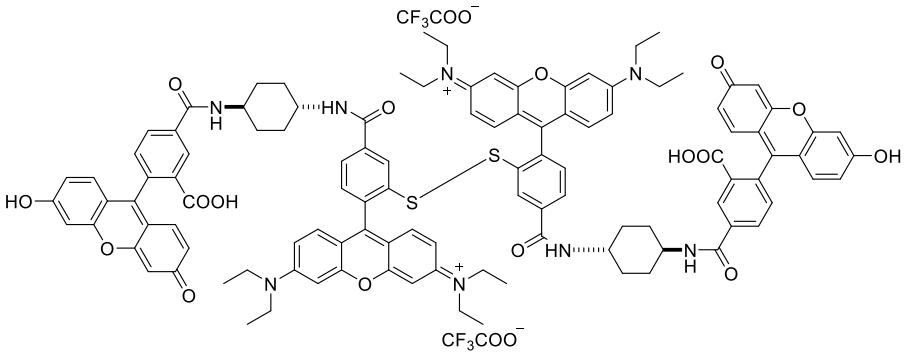


To a solution of compound **12** (11.9 mg, 22.7 μmol) in anhydrous DMF (4.0 mL), *N*-hydroxysuccinimide (26.1 mg, 0.227 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (21.8 mg, 0.114 mmol) were added. The mixture was stirred at room temperature for 7 h, then acidified with 0.1 N HCl aq., and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated to dryness. The resulting residue was partially purified by HPLC (eluent, a 15-min linear gradient from 25% to 100% solvent B; flow rate, 5.0 ml/min; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA)). To a solution of the partially purified compound and compound **3** (16.1 mg, 34.1 μmol) in anhydrous DMF (2.0 mL), *N*,*N*-diisopropylethylamine (29.4 mg, 40 μL, 0.228 mmol) was added. The mixture was stirred at room temperature for 3 h, then 1% TFA aq. was added to it. The resulting mixture was purified by HPLC (eluent, a 20-min linear gradient, from 50% to 100% eluent B; flow rate, 5.0

ml/min; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA)) to afford compound **13** (18.3 mg, 18.8 μmol, 83% yield) as a red solid.
^1H NMR (400 MHz, CD_3OD): δ 1.31 (12H, t, *J* = 7.1 Hz), 1.69-1.72 (4H, m), 2.18-2.20 (4H, m), 2.36 (3H, s), 3.68 (8H, q, *J* = 7.1 Hz), 4.07-4.09 (2H, br m), 6.53 (2H, dd, *J* = 8.8, 2.4 Hz), 6.62 (2H, d, *J* = 8.8 Hz), 6.71 (2H, d, *J* = 2.4 Hz), 6.98 (2H, d, *J* = 2.4 Hz), 7.05 (2H, dd, *J* = 9.8, 2.4 Hz), 7.13 (2H, d, *J* = 9.8 Hz), 7.30 (1H, d, *J* = 7.8 Hz), 7.41 (1H, d, *J* = 7.8 Hz), 7.97 (1H, dd, *J* = 7.8, 1.5 Hz), 8.23 (1H, dd, *J* = 7.8, 1.5 Hz), 8.48 (1H, d, *J* = 1.5 Hz), 8.54 (1H, d, *J* = 1.5 Hz); HRMS (ESI^+): Calcd for $[M-CF_3COO]^+$, 975.3461, Found, 975.3444 (−1.7 mmu). HPLC analysis: retention time, 18.6 min (eluent, a 20-min linear gradient, from 50% to 100% eluent B; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA); flow rate: 1.0 mL/min; detection wavelength, 254 nm).

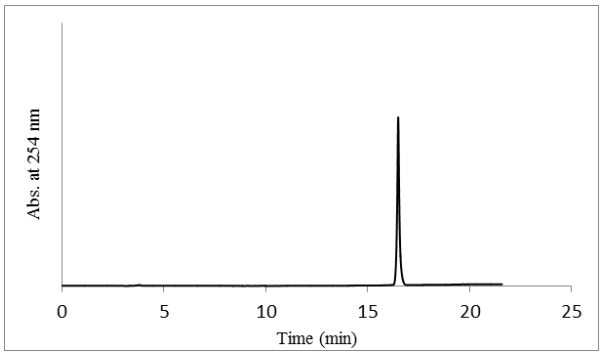


Synthesis of SSip-1 (14)

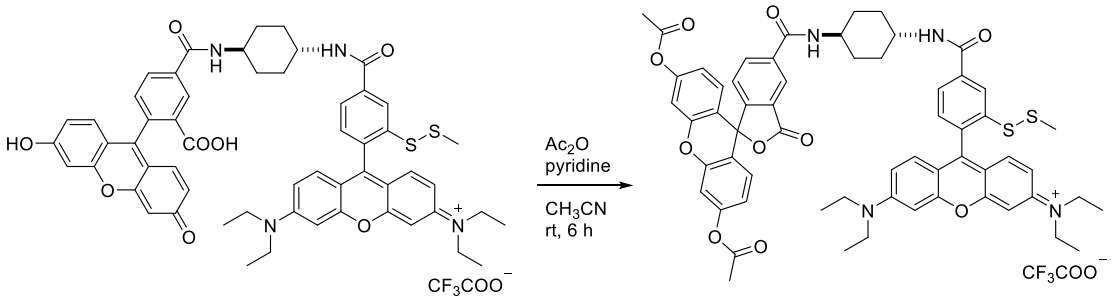


To a solution of compound **13** (3.0 mg, 3.08 μmol) in CH_3CN (1.0 mL) and H_2O (1.0 mL), tris(2-carboxyethyl)phosphine hydrochloride (1.0 mg, 3.86 μmol) was added. The mixture was stirred at room temperature for 15 min, and then purified by HPLC (eluent, a 20-min linear gradient, from 50% to 100% eluent B; flow rate, 5.0 ml/min; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA)) to afford SSip-1 (**14**) (2.6 mg, 1.40 μmol, 91% yield) as a red solid. This compound was readily oxidized, and was isolated as a dimer (see the above chemical structure).

^{1}H NMR (400 MHz, CD_3OD): δ 1.32 (12H, t, *J* = 7.1 Hz), 1.69-1.71 (4H, m), 2.15-2.23 (4H, m), 3.69 (8H, q, *J* = 7.1 Hz), 4.07-4.10 (2H, br m), 6.47 (2H, dd, *J* = 8.8, 2.4 Hz), 6.54 (2H, d, *J* = 8.8 Hz), 6.68 (2H, d, *J* = 2.4 Hz), 6.83 (2H, d, *J* = 9.3 Hz), 6.92 (2H, dd, *J* = 9.3, 2.4 Hz), 6.97 (2H, d, *J* = 2.4 Hz), 7.25 (1H, d, *J* = 7.8 Hz), 7.40 (1H, d, *J* = 8.3 Hz), 8.04 (1H, dd, *J* = 7.8, 1.5 Hz), 8.20 (1H, dd, *J* = 8.3, 1.5 Hz), 8.33 (1H, d, *J* = 1.5 Hz), 8.47 (1H, d, *J* = 1.5 Hz); HRMS (ESI^+): Calcd for $[M-2CF_3COO]^{2+}$, 1857.7045, Found, 1857.7065 (+2.0 mmu). HPLC analysis: retention time, 16.4 min (eluent, a 15-min linear gradient, from 25% to 100% eluent B; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA); flow rate: 1.0 mL/min; detection wavelength, 254 nm).

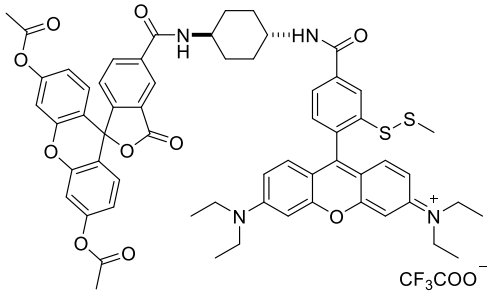


Scheme S2. Synthetic scheme for SSip-1 DA



13 **SSip-1 DA (15)**
y. 85%

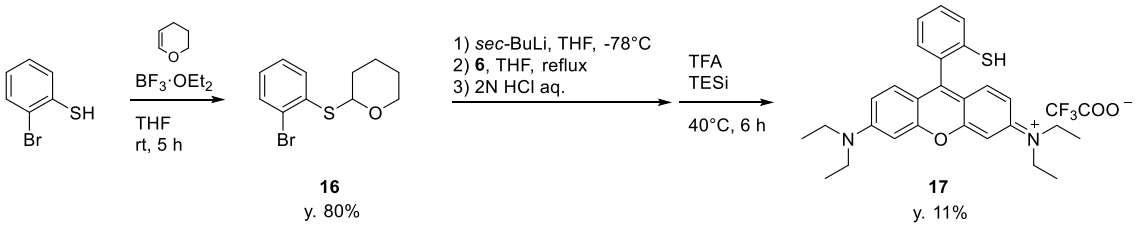
Synthesis of SSip-1 DA (15)



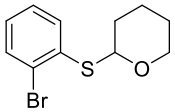
To a solution of compound **13** (15.0 mg, 15.4 μmol) in anhydrous CH_3CN (4.0 mL), acetic anhydride (31.4 mg, 29 μL, 0.308 mmol) and pyridine (24.3 mg, 25 μL, 0.308 mmol) were added. The mixture was stirred at room temperature for 6 h and then evaporated to dryness. The resulting residue was purified by HPLC (eluent, a 15-min linear gradient, from 50% to 100% eluent B; flow rate, 5.0 ml/min; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA)) to afford SSip-1 DA (**15**) (13.8 mg, 13.0 μmol, 85% yield) as a red solid.

^{1}H NMR (400 MHz, CD_3OD): δ 1.31 (12H, t, *J* = 7.1 Hz), 1.65-1.68 (4H, m), 2.16-2.19 (4H, m), 2.29 (6H, s), 2.37 (3H, s), 3.69 (8H, q, *J* = 7.1 Hz), 4.03-4.06 (2H, br m), 6.88-6.90 (4H, m), 7.00 (2H, d, *J* = 2.0 Hz), 7.06 (2H, dd, *J* = 9.3, 2.0 Hz), 7.14 (2H, d, *J* = 9.3 Hz), 7.19 (2H, d, *J* = 2.0 Hz), 7.35 (1H, d, *J* = 8.3 Hz), 7.44 (1H, d, *J* = 7.8 Hz), 7.97 (1H, dd, *J* = 7.8, 1.5 Hz), 8.24 (1H, dd, *J* = 8.3, 1.5 Hz), 8.49 (1H, d, *J* = 1.5 Hz), 8.54 (1H, d, *J* = 1.5 Hz); ^{13}C NMR (100 MHz, CD_3OD): δ12.8, 20.9, 23.3, 32.3, 47.0, 50.2, 83.3, 97.6, 111.7, 114.5, 115.9, 117.2, 119.4, 125.2, 125.5, 127.2, 127.7, 128.7, 130.0, 131.5, 132.4, 135.8, 136.0, 138.6, 138.7, 138.7, 152.8, 154.1, 155.3, 156.5, 157.5, 159.4, 167.6, 168.2, 170.0, 170.5; HRMS (ESI$^+$): Calcd for $[M-CF_3COO]^+$, 1059.3673, Found, 1059.3628 (−4.5 mmu).

Scheme S3. Synthetic scheme for 2-thio Rhodamine B (2-thio RB)



Synthesis of 2-(bromophenyl-2-thioyl)tetrahydro-2*H*-pyran (16)

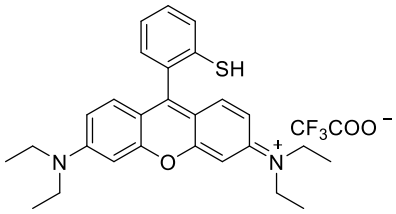


To a solution of 2-bromobenzenethiol (1.00 g, 0.62 mL, 5.29 mmol) and 3,4-dihydro-2*H*-pyran (890 mg, 0.96 mL, 10.6 mmol) in THF (15 mL), boron trifluoride-ethyl ether complex (750 mg, 0.67 mL, 5.29 mmol) was slowly added at 0°C. The mixture was warmed to room temperature, then stirred for 5 h, and sat. $NaHCO_3$ aq. (15 mL) was added to it. The mixture was extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated to dryness. The resulting residue was purified by column

chromatography (silica gel, 1:0 to 1:1 hexane/CH_2Cl_2) to afford compound **16** (1.16 g, 4.25 mmol, 80% yield) as a colorless oil.

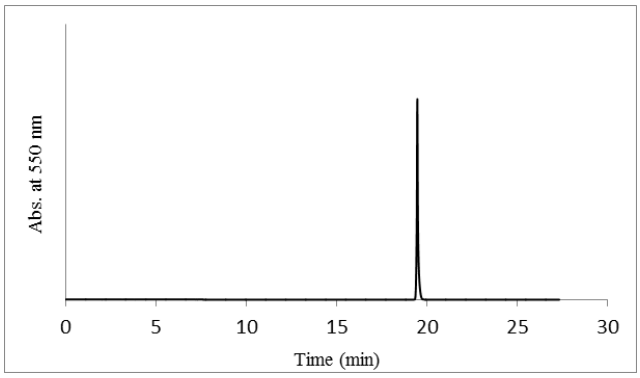
^{1}H NMR (300 MHz, $CDCl_3$): δ 1.62-1.74 (3H, m), 1.86-1.96 (2H, m), 2.07-2.14 (1H, m), 3.59-3.66 (1H, m), 4.13-4.21 (1H, m), 5.37-5.38 (1H, m), 7.04 (1H, td, *J* = 7.7, 1.5 Hz), 7.26 (1H, td, *J* = 7.7, 1.5 Hz), 7.53 (1H, dd, *J* = 7.7. 1.5 Hz), 7.59 (1H, dd, *J* = 7.7. 1.5 Hz); ^{13}C NMR (75 MHz, CD_2Cl_2): δ 21.8, 25.9, 31.7, 64.6, 84.2, 124.0, 127.5, 128.3, 130.4, 133.1, 138.0.

Synthesis of 2-thio RB (17)

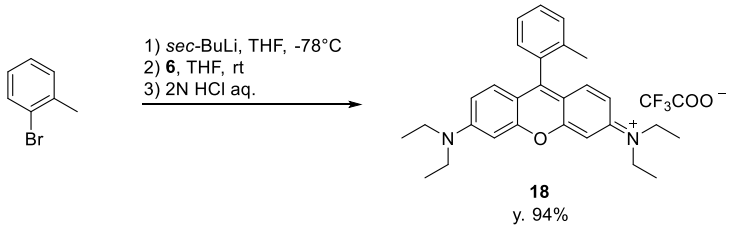


To a flame-dried flask flushed with argon, compound **16** (243 mg, 0.89 mmol) and anhydrous THF (10 mL) were added. The solution was cooled to −78°C, and 1 M *sec*-BuLi (0.80 mL, 0.80 mmol) was slowly added. The mixture was stirred at −78°C for 20 min, and a solution of compound **6** (30 mg, 0.089 mmol) in anhydrous THF (5 mL) was slowly added. The mixture was warmed to 70°C, then stirred for 2 h. It was cooled to room temperature and 2 N HCl aq. (20 mL) was added to it. 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 resulting residue and triethylsilane (100 μL) were dissolved in trifluoroacetic acid (TFA) (3.0 mL). The solution was stirred at 40°C for 4 h and cooled to room temperature. The mixture was evaporated to dryness. The resulting residue was purified by HPLC (eluent, a 15-min linear gradient, from 25% to 100% eluent B; flow rate, 5.0 ml/min; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA)) to afford compound **17** (4.1 mg, 9.5 μmol, 11% yield) as a red solid.

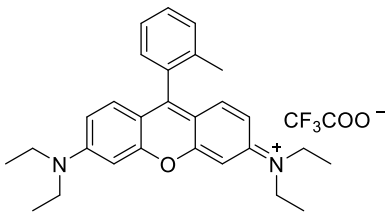
^{1}H NMR (300 MHz, CD_3OD): δ1.32 (12H, t, *J* = 7.0 Hz), 3.70 (8H, q, *J* = 7.0 Hz), 7.00 (2H, d, *J* = 2.2 Hz), 7.09 (2H, dd, *J* = 9.5, 2.2 Hz), 7.21 (2H, d, *J* = 9.5 Hz), 7.28 (1H, dd, *J* = 7.7, 1.5 Hz), 7.43 (1H, td, *J* = 7.7, 1.5 Hz), 7.52 (1H, td, *J* = 7.7, 1.5 Hz), 7.65 (1H, dd, *J* = 7.7, 1.5 Hz); HRMS (ESI$^+$): Calcd for $[M-CF_3COO]^+$, 431.2157, Found, 431.2154 (−0.3 mmu). HPLC analysis: retention time, 19.5 min (eluent, a 15-min linear gradient, from 50% to 100% eluent B; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA); flow rate: 1.0 mL/min; detection wavelength, 550 nm).



Scheme S4. Synthetic scheme for 2-Me Rhodamine B (RB)



Synthesis of 2-Me RB (18)

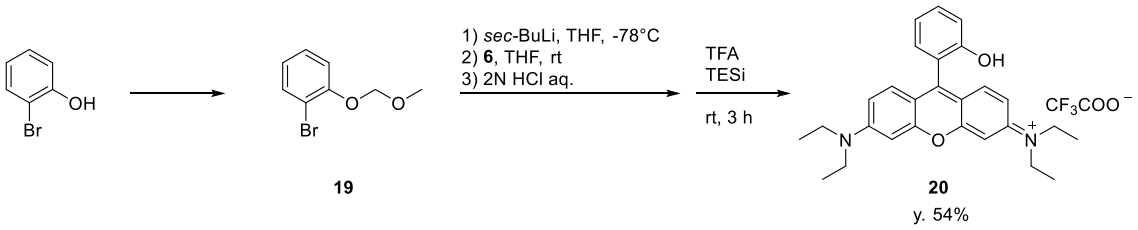


To a flame-dried flask flushed with argon, 2-bromotoluene (152 mg, 0.89 mmol) and anhydrous THF (10 mL) were added. The solution was cooled to −78°C, and 1 M *sec*-BuLi (0.80 mL, 0.80 mmol) was slowly added. The mixture was stirred at −78°C for 20 min, and a solution of compound **6** (30 mg, 0.089 mmol) in anhydrous THF (5 mL) was slowly added. The mixture was stirred at room temperature for 2 h, and 2 N HCl aq. (20 mL) was added to it. 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 resulting residue was purified by HPLC (eluent, a 15-min linear gradient, from 25% to 100% eluent B; flow rate, 5.0 ml/min; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA)) to afford compound **18** (33.5 mg, 81.1 μmol, 94% yield) as a red solid.

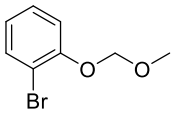
^{1}H NMR (300 MHz, CD_3OD): δ1.32 (12H, t, *J* = 7.0 Hz), 2.07 (1H, s), 3.70 (8H, q, *J* = 7.0 Hz), 7.00 (2H, d, *J* = 2.2 Hz), 7.08 (2H, dd, *J* = 9.5, 2.2 Hz), 7.19 (2H, d, *J* = 9.5 Hz), 7.25 (1H, m), 7.50 (3H, m); ^{13}C NMR (75 MHz, CD_3OD): δ 12.8, 19.6, 46.7, 97.4, 114.7, 115.7, 127.3, 130.1,

131.2, 131.8, 132.7, 133.2, 137.3, 157.3, 159.1, 159.5; HRMS (ESI$^+$): Calcd for [M−CF_3COO]$^+$, 413.2593, Found, 413.2571 (−2.2 mmu).

Scheme S5. Synthetic scheme for 2-OH Rhodamine B (RB)

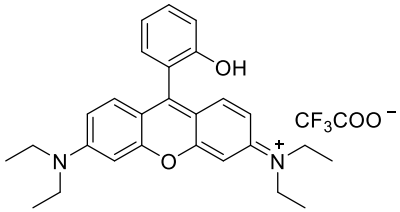


Synthesis of 1-bromo-2-methoxymethoxybenzene (19)



This compound was synthesized according to reference S5.

Synthesis of 2-OH RB (20)

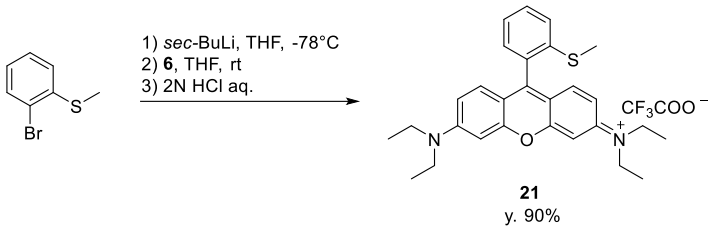


To a flame-dried flask flushed with argon, compound **19** (193 mg, 0.89 mmol) and anhydrous THF (10 mL) were added. The solution was cooled to −78°C, and 1 M *sec*-BuLi (0.80 mL, 0.80 mmol) was slowly added. The mixture was stirred at −78°C for 20 min, and a solution of compound **6** (30 mg, 0.089 mmol) in anhydrous THF (5 mL) was slowly added. Stirring was continued at room temperature for 2 h, and then 2 N HCl aq. (20 mL) 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 resulting residue was purified by HPLC (eluent, a 15-min linear gradient, from 25% to 100% eluent B; flow rate, 5.0 ml/min; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA)) to afford compound **20** (20.0 mg, 48.1 μmol, 54% yield) as a red solid.

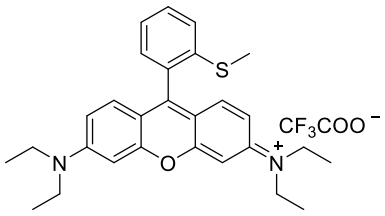
^{1}H NMR (300 MHz, CD_3OD): δ1.28 (12H, t, *J* = 7.1 Hz), 3.63 (8H, q, *J* = 7.1 Hz), 6.91 (2H, d, *J* = 2.2 Hz), 7.02 (2H, dd, *J* = 9.5, 2.2 Hz), 7.09 (2H, m), 7.19 (1H, dd, *J* = 7.7, 1.5 Hz), 7.37

(2H, d, J = 9.5 Hz), 7.48 (1H, td, J = 7.7, 1.5 Hz); ^{13}C NMR (75 MHz, CD_3OD): δ 12.8, 46.7, 97.1, 115.0, 115.2, 117.3, 120.4, 120.7, 131.8, 132.9, 133.3, 156.1, 157.1, 157.8, 159.5; HRMS (ESI^+): Calcd for $[M-CF_3COO]^+$, 415.2386, Found, 415.2366 (−2.0 mmu).

Scheme S6. Synthetic scheme for 2-SMe Rhodamine B (RB)



Synthesis of 2-SMe RB (21)



To a flame-dried flask flushed with argon, 2-bromothioanisole (181 mg, 0.89 mmol) and anhydrous THF (10 mL) were added. The solution was cooled to −78°C, and 1 M *sec*-BuLi (0.80 mL, 0.80 mmol) was slowly added. The mixture was stirred at −78°C for 20 min, and a solution of compound **6** (30 mg, 0.089 mmol) in anhydrous THF (5 mL) was slowly added. The mixture was stirred at room temperature for 2 h, and 2 N HCl aq. (20 mL) was added to it. This mixture was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated to dryness. The resulting residue was purified by HPLC (eluent, a 15-min linear gradient, from 25% to 100% eluent B; flow rate, 5.0 ml/min; eluent A (H_2O containing 0.1% TFA) and eluent B (CH_3CN with 20% H_2O containing 0.1% TFA)) to afford compound **21** (35.6 mg, 80.1 μmol, 90% yield) as a red solid.

^{1}H NMR (300 MHz, CD_3OD): δ1.31 (12H, t, J = 7.2 Hz), 2.41 (3H, s), 3.69 (8H, q, J = 7.2 Hz), 6.98 (2H, d, J = 2.2 Hz), 7.05 (2H, dd, J = 9.5, 2.2 Hz), 7.19 (2H, m), 7.26 (1H, m), 7.44 (1H, td, J = 7.3, 1.5 Hz), 7.60 (1H, dd, J = 7.3, 1.5 Hz), 7.65 (1H, td, J = 7.3, 1.5 Hz); ^{13}C NMR (75 MHz, CD_3OD): δ 12.8, 16.0, 46.8, 97.4, 114.7, 115.5, 126.4, 127.8, 130.6, 131.9, 132.7, 135.6, 139.4, 157.3, 157.6, 159.5; HRMS (ESI^+): Calcd for $[M-CF_3COO]^+$, 445.2314, Found, 445.2281 (−3.3 mmu).

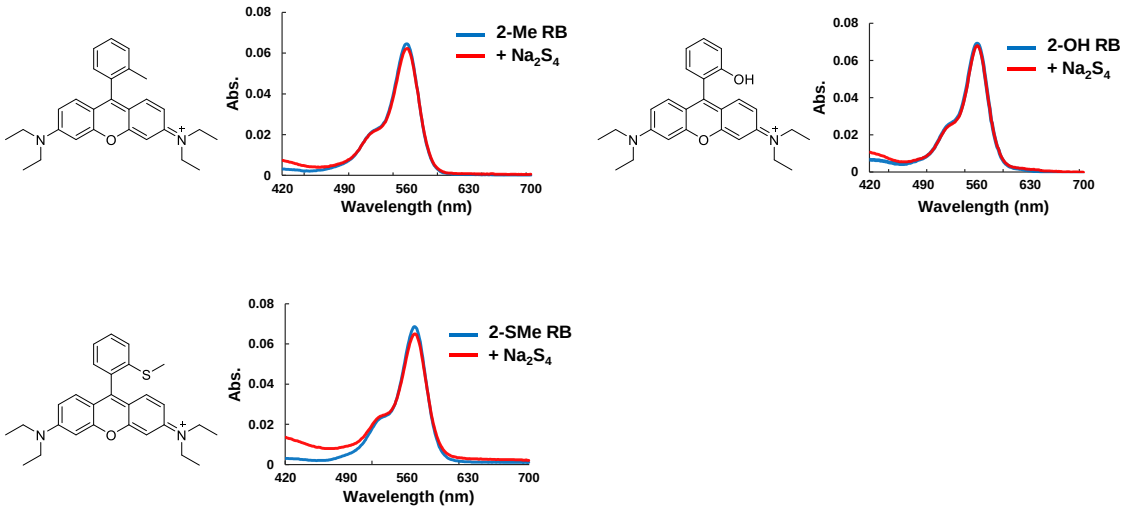


Figure S1. Absorption spectra of 1 μM 2-Me RB, 2-OH RB and 2-SMe RB measured in 100 mM sodium phosphate buffer (pH 7.4) with 100 μM GSH before (blue) and after (red) addition of 50 μM Na_2S_4, containing 0.1% DMSO and 1 mg/mL BSA as a cosolvent. None of the compounds showed a change of absorption spectrum after addition of Na_2S_4.

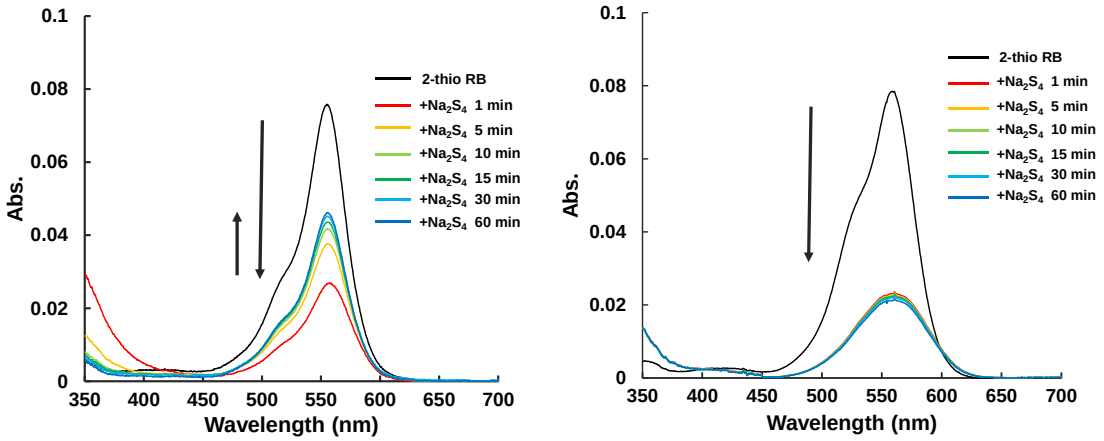


Figure S2. Absorption spectra of 1 μM 2-thio RB measured in 100 mM sodium phosphate buffer (pH 7.4) in the presence (left) and in the absence (right) of 5 mM GSH after addition of 50 μM Na_2S_4, containing 0.1% DMSO and 1 mg/mL BSA as a cosolvent. The absorbance of 2-thio RB in the presence of 5 mM GSH was strongly decreased 1 min after addition of 50 μM Na_2S_4 (black to red), and then subsequently recovered slowly (red (1 min after addition of 50 μM Na_2S_4) to yellow (5 min after addition of 50 μM Na_2S_4) to blue (60 min after addition of 50 μM Na_2S_4)).

The absorption of 2-thiol RB recovered to only half of the original level in the presence of 5 mM GSH, while SSip-1 showed 80 to 90% recovery. We consider that two factors may contribute to this difference of the recovery rate, i.e., hydrophilicity and stability of the spirocyclized form.

i) Since the spirocyclized form of 2-thio RB is thought to be highly hydrophobic, some of the compound may be precipitated. On the other hand, SSip-1 has a fluorescein moiety, which is relatively highly water-soluble.

ii) We also think that the spirocyclized form of SSip-1 is less stable than that of 2-thio RB, because the absorbance decrease of SSip-1 was less than that of 2-thio RB upon addition of 50 μM Na_2S_4.

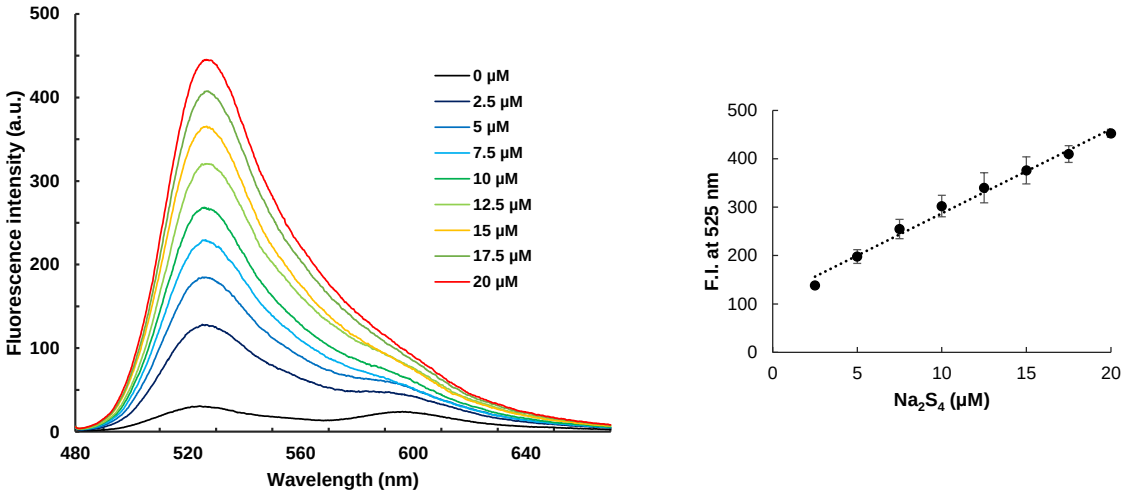


Figure S3. Fluorescence spectra of SSip-1 (1 μM) upon addition of various concentrations of Na_2S_4 (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 μM) in the presence of 5 mM GSH, measured in 100 mM sodium phosphate buffer (pH 7.4) containing 0.1% DMSO as a cosolvent and 1 mg/mL BSA. Spectra were measured 1 min after addition of Na_2S_4.

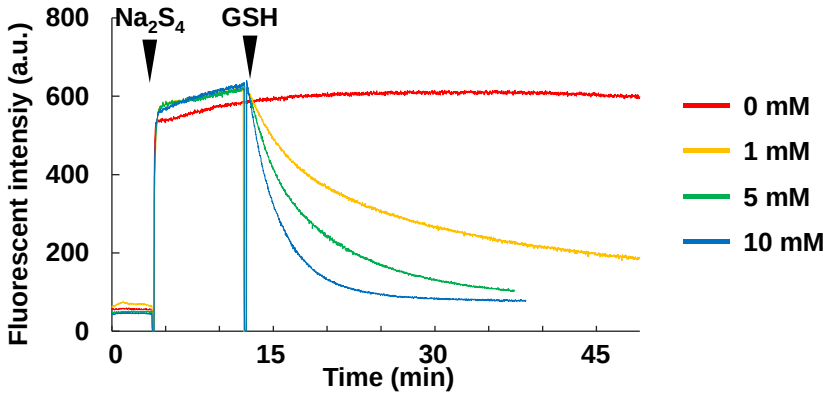


Figure S4. Fluorescence intensity change of SSip-1 (1 μM) after addition of 50 μM Na_2S_4, followed by addition of various concentrations of GSH (0, 1, 5, 10 mM). The fluorescence intensity change at 525 nm of the reaction mixture was monitored in 100 mM sodium phosphate buffer (pH 7.4) containing 0.1% DMSO as a cosolvent and 1 mg/mL BSA. λ_{ex} = 470 nm. The fluorescence decrease was dependent upon the amount of added GSH.

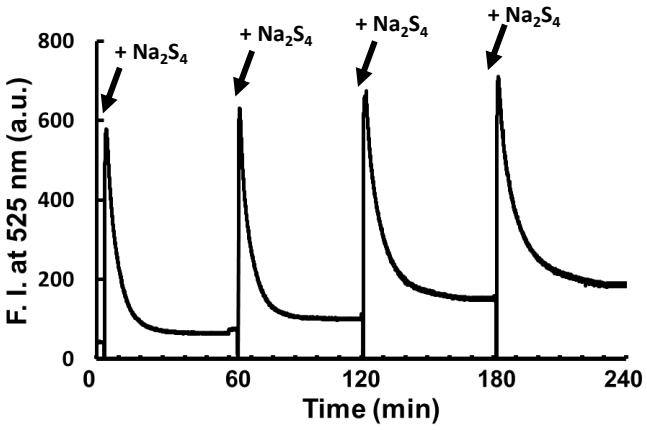


Figure S5. Fluorescence intensity changes of SSip-1 after repeated addition (four times) of 50 μM Na_2S_4 at 1 h intervals in the presence of 5 mM GSH. The fluorescence intensity (525 nm) of the reaction mixture of 1 μM SSip-1, 5 mM GSH and 50–200 μM Na_2S_4 was monitored in 100 mM sodium phosphate buffer (pH 7.4) containing 0.1% DMSO as a cosolvent and 1 mg/mL BSA. λ_{ex} = 470 nm.

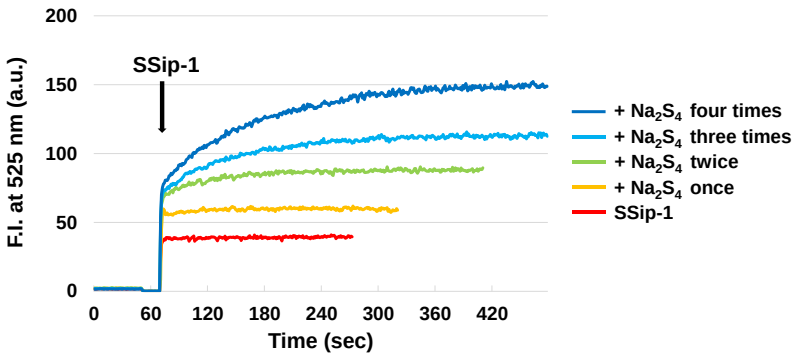


Figure S6. Fluorescence intensity change of SSip-1 after 0 to 4 additions of 50 μM Na_2S_4 to a solution containing 5 mM GSH. The fluorescence intensity change at 525 nm of the reaction mixture of 1 μM SSip-1, 5 mM GSH and 50-200 μM Na_2S_4 was monitored in 100 mM sodium phosphate buffer (pH 7.4) containing 0.1% DMSO as a cosolvent and 1 mg/mL BSA. λ_{ex} = 470 nm. Detailed protocol: We first added 50 μM Na_2S_4 to the reaction mixture (5 mM GSH in 100

mM sodium phosphate buffer) and left the mixture for 60 min. This procedure (Na_2S_4 addition and 60-min waiting time) was repeated from 1 to 4 times (once: +Na_2S_4 once (orange); twice: +Na_2S_4 twice (green); 3 times: +Na_2S_4 three times (blue); 4 times: +Na_2S_4 four times (dark blue)). The red line indicates the fluorescence change of 1 μM SSip-1 in 100 mM sodium phosphate buffer (5 mM GSH) without Na_2S_4 addition. Then, 1 μM SSip-1 was added to the mixture and the fluorescence intensity change (525 nm) was measured. The fluorescence intensity increase was dependent on the number of Na_2S_4 additions (see the above graph).

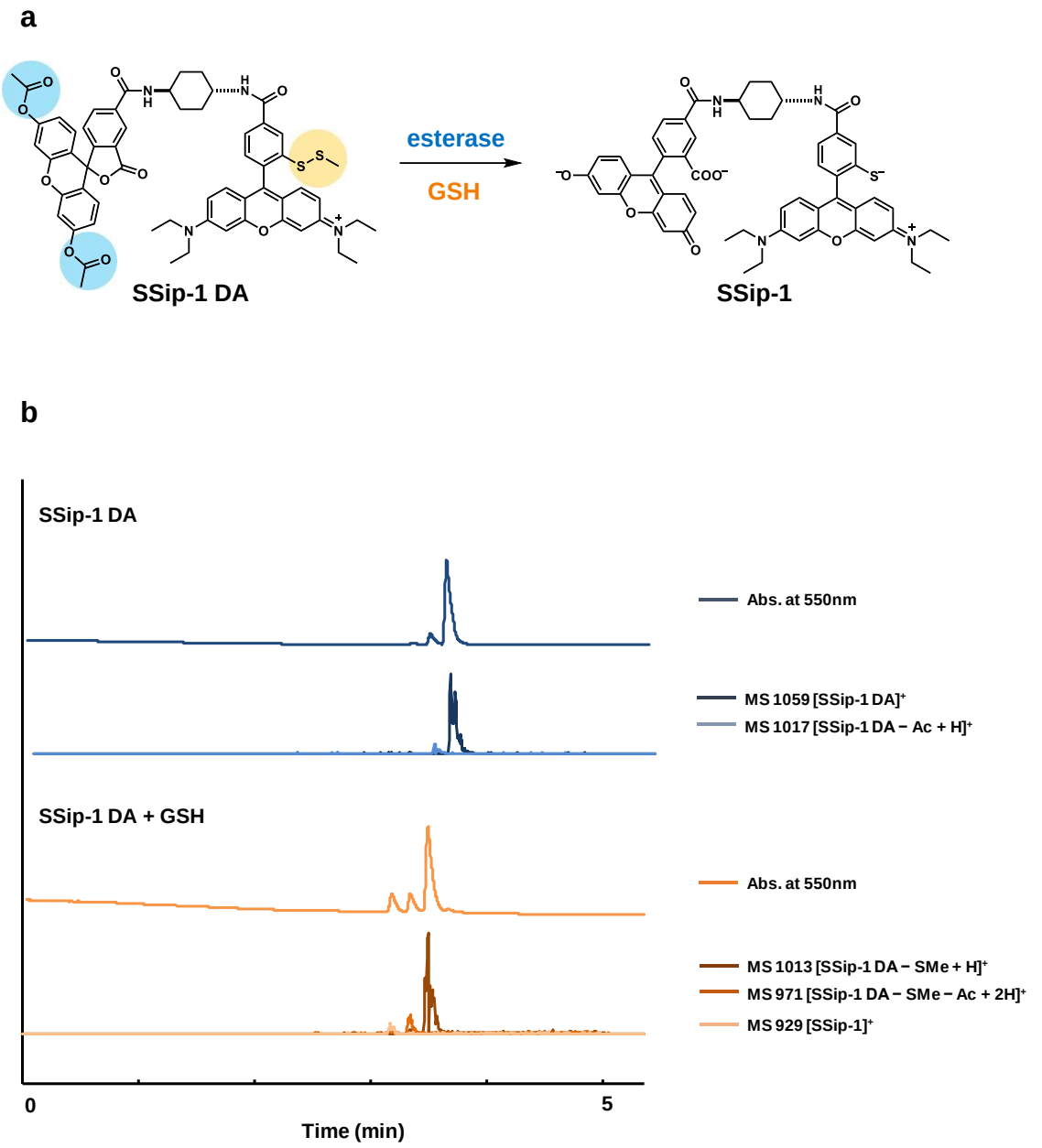


Figure S7. (a) Chemical structure of SSip-1 DA. SSip-1 DA permeates the cell membrane owing to its high lipophilicity, and is converted to SSip-1 by intracellular esterases and reductive conditions (probably mainly related to GSH). SSip-1, produced from SSip-1 DA, stays inside the cell owing to its hydrophilicity. (b) UPLC-MS charts of the reaction mixture of 10 μM SSip-1 DA in 100 mM sodium phosphate buffer containing 0.1% DMSO before (blue) and 60 min after (orange) addition of 5 mM GSH. UPLC condition: (eluent, a 3.5 min linear gradient, from 5% to 95% eluent B; eluent A (H_2O containing 0.1% formic acid) and eluent B (CH_3CN with 20% H_2O containing 0.1% formic acid); detection wavelength, 550 nm).

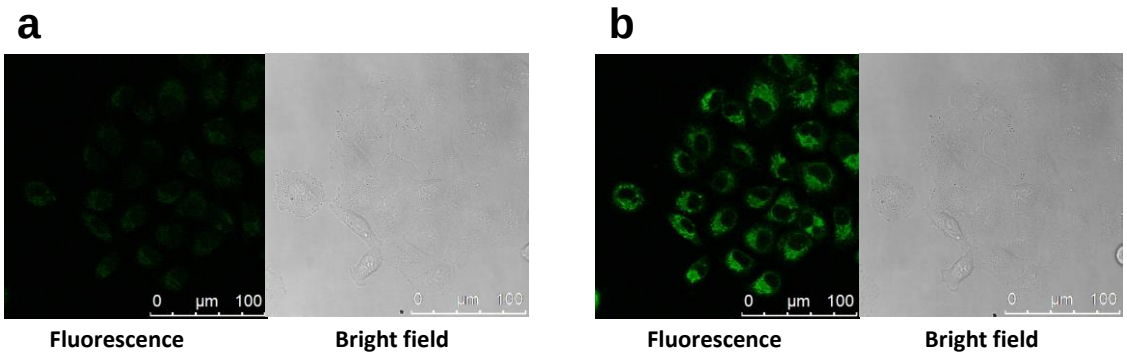


Figure S8. Fluorescence confocal microscopy images of live A549 cells loaded with SSip-1 DA. (a) Cells were incubated with 10 μM SSip-1 DA in DMEM containing 0.1% DMSO for 1 h, then washed with HBSS and placed in fresh DMEM. (b) 10 μM Na_2S_4 was added to the extracellular medium and the cells were incubated for 1 min.

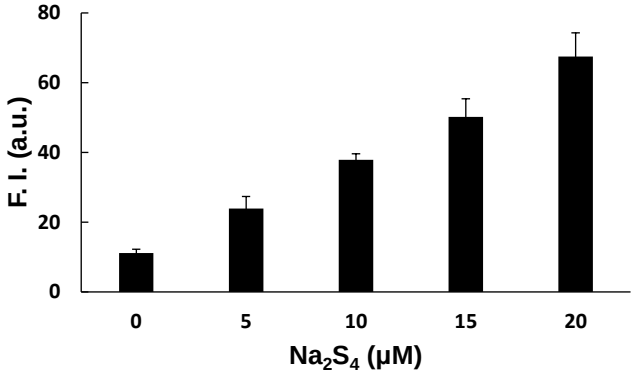


Figure S9. Average fluorescence intensity (F.I.) in fluorescence images 1 min after addition of 0, 5, 10, 15, 20 μM Na_2S_4 in DMEM. Cells were incubated with 10 μM SSip-1 DA containing 0.03% Pluronic F-127 and 0.1% DMSO as a cosolvent for 1 hour. Error bars are ± S.D.

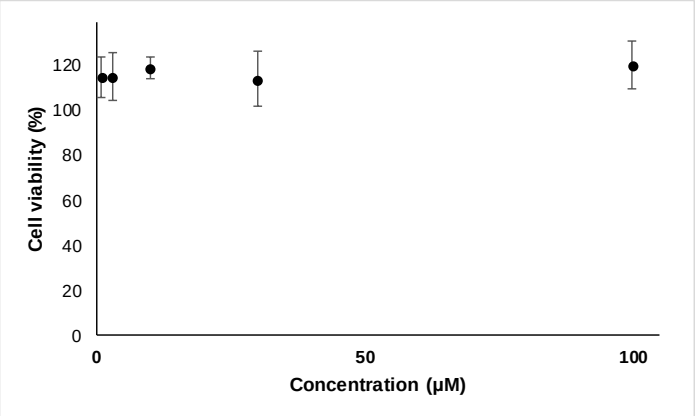


Figure S10. Cytotoxicity of SSip-1 DA to A549 cells. A549 cells were incubated with the indicated concentrations of SSip-1 DA for 3 hours at 37°C under 5% CO_2, and the viability was measured by using CCK-8 assay. Data represent mean ±S.D. from a single experiment (n = 5).

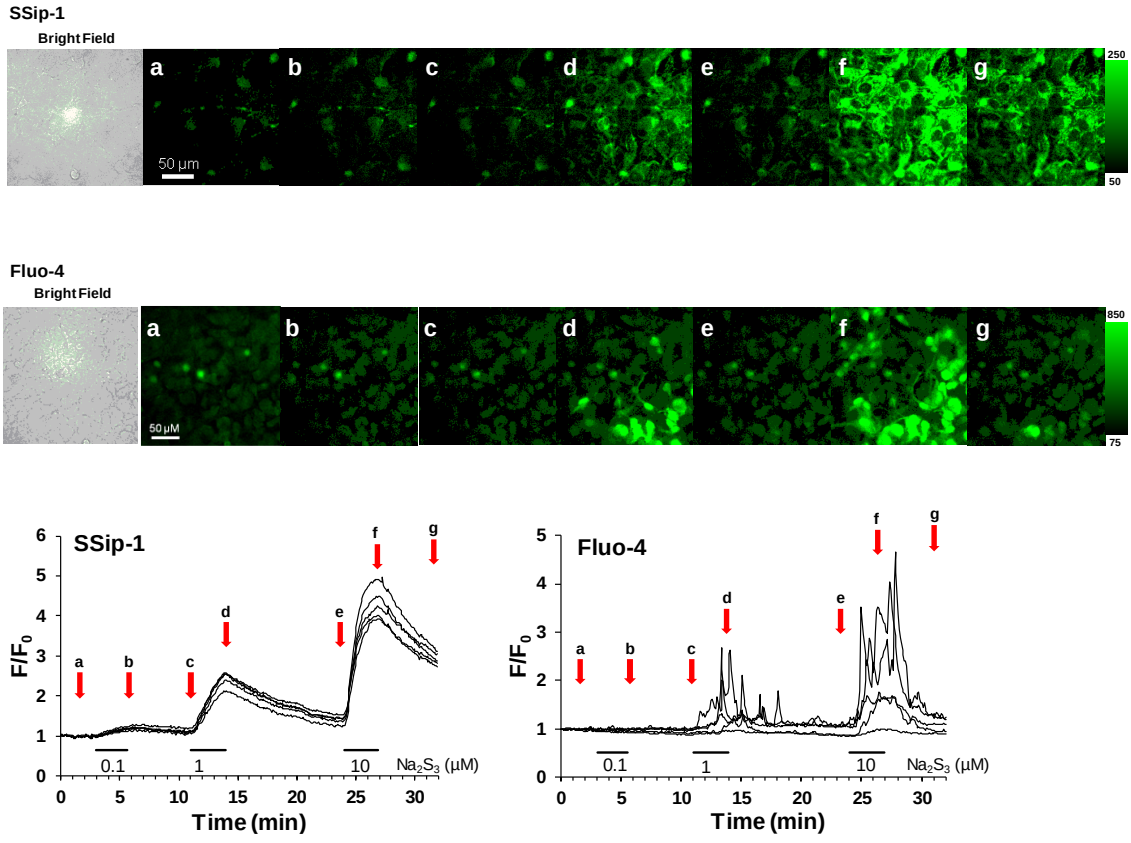
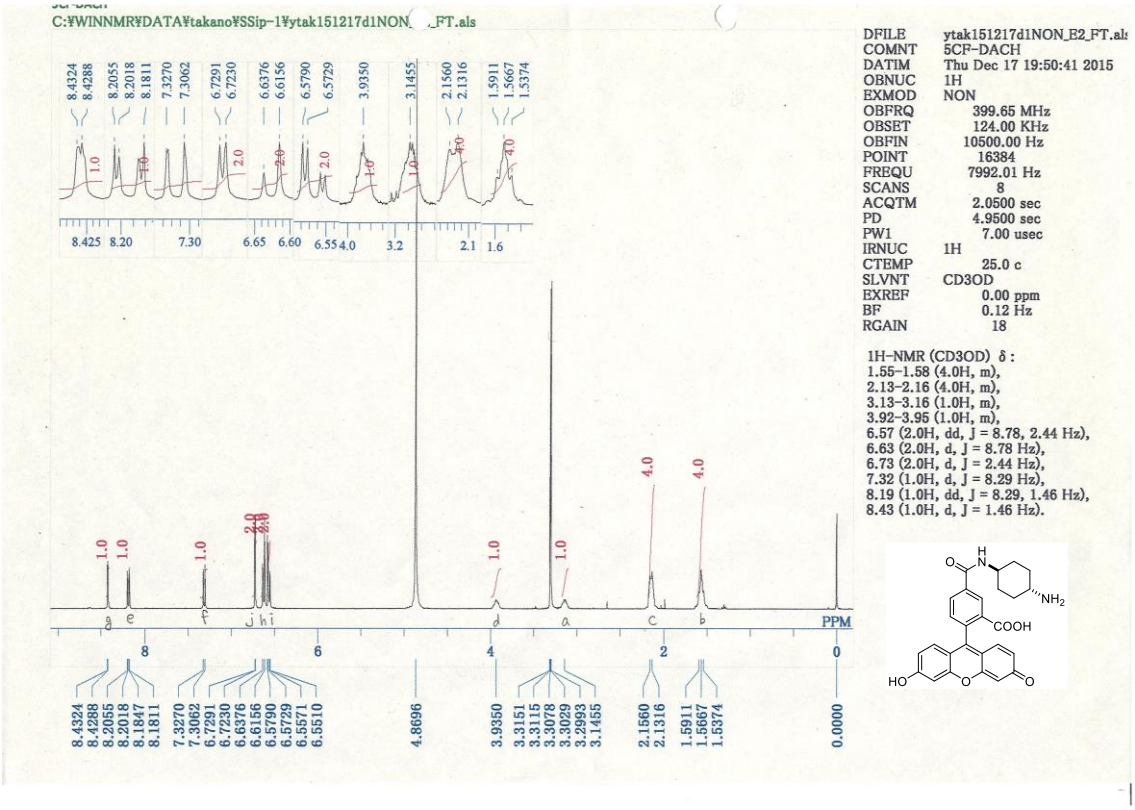
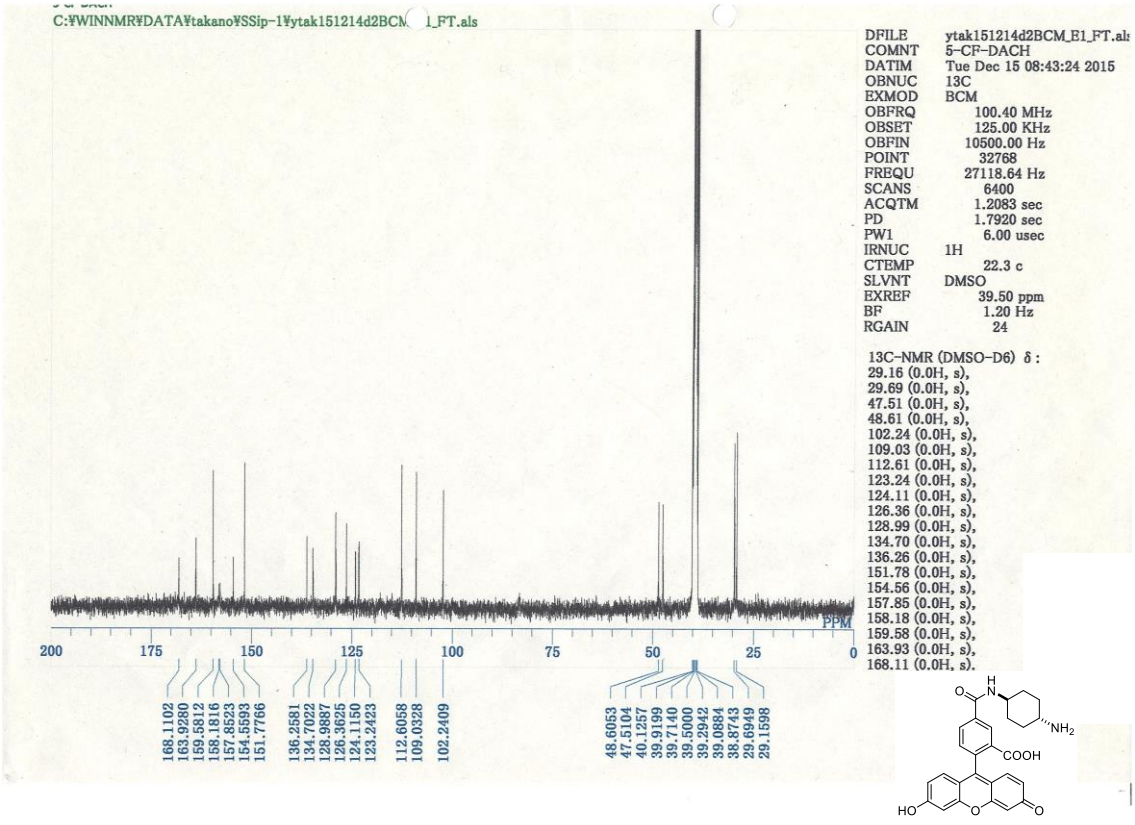
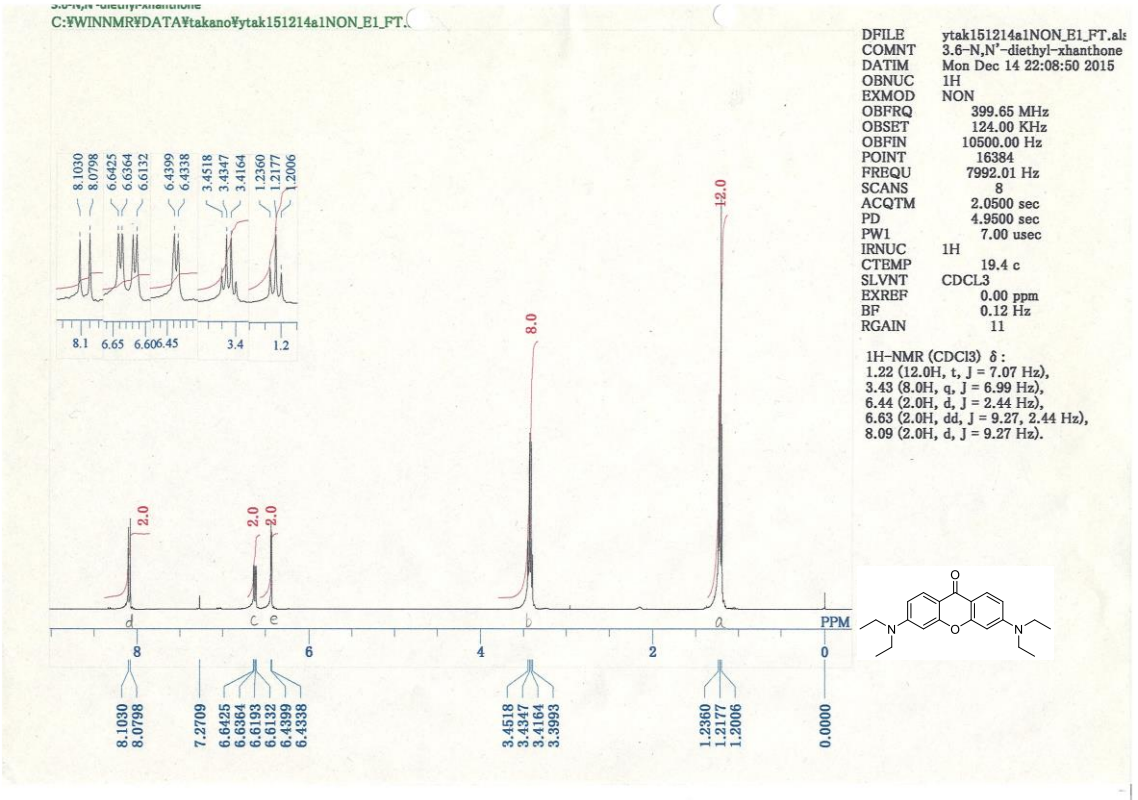


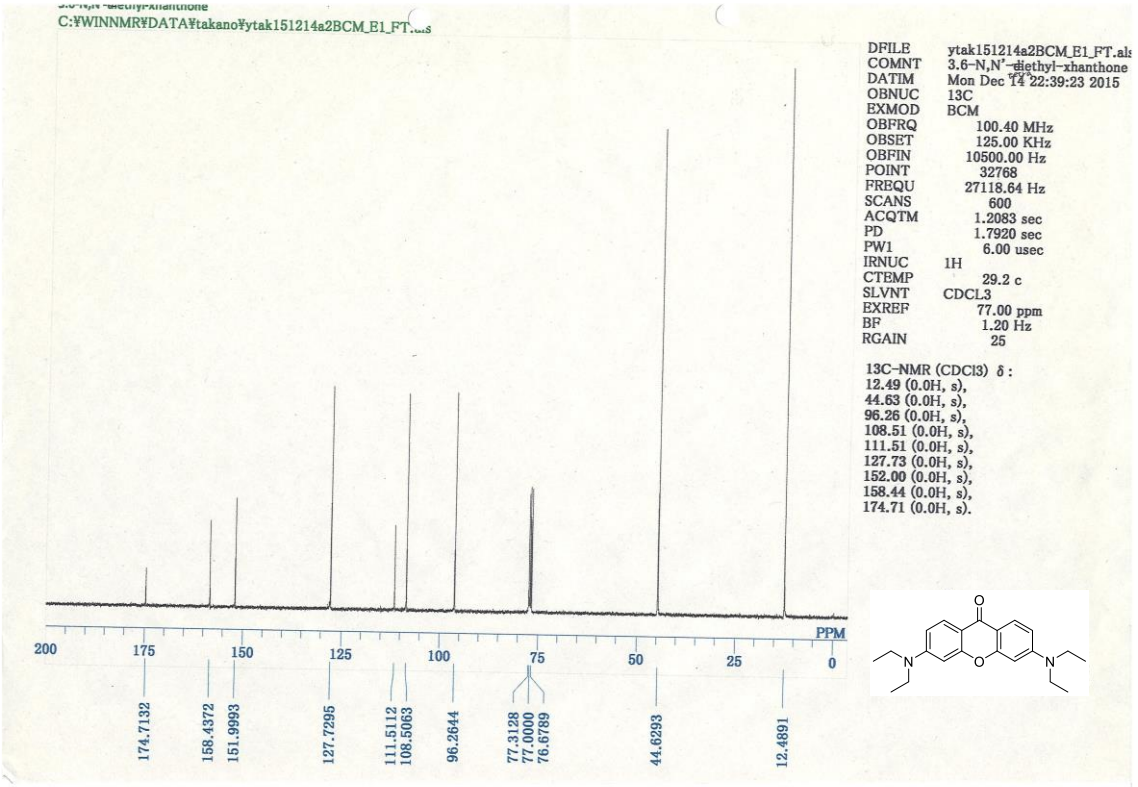
Figure S11. Fluorescence images of cultures of hippocampal astrocytes loaded with SSip-1 DA and Fluo-4.

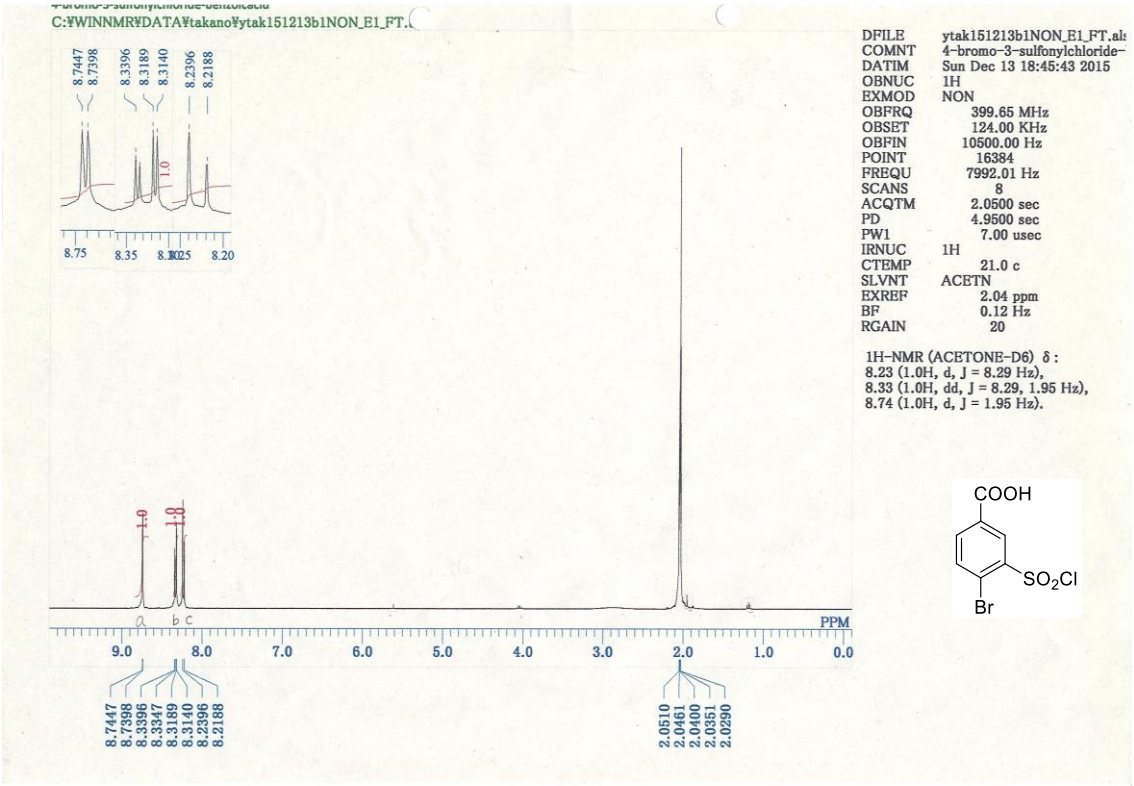
^{1}H and ^{13}C NMR charts of new compounds.

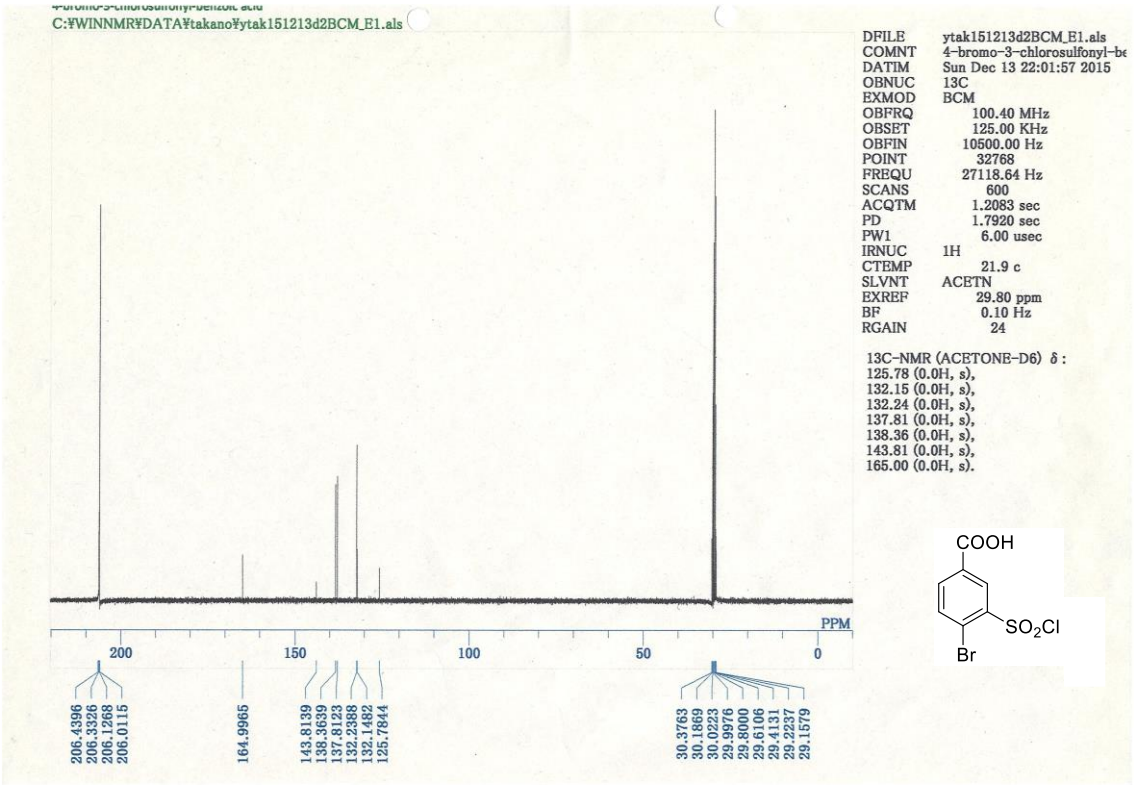


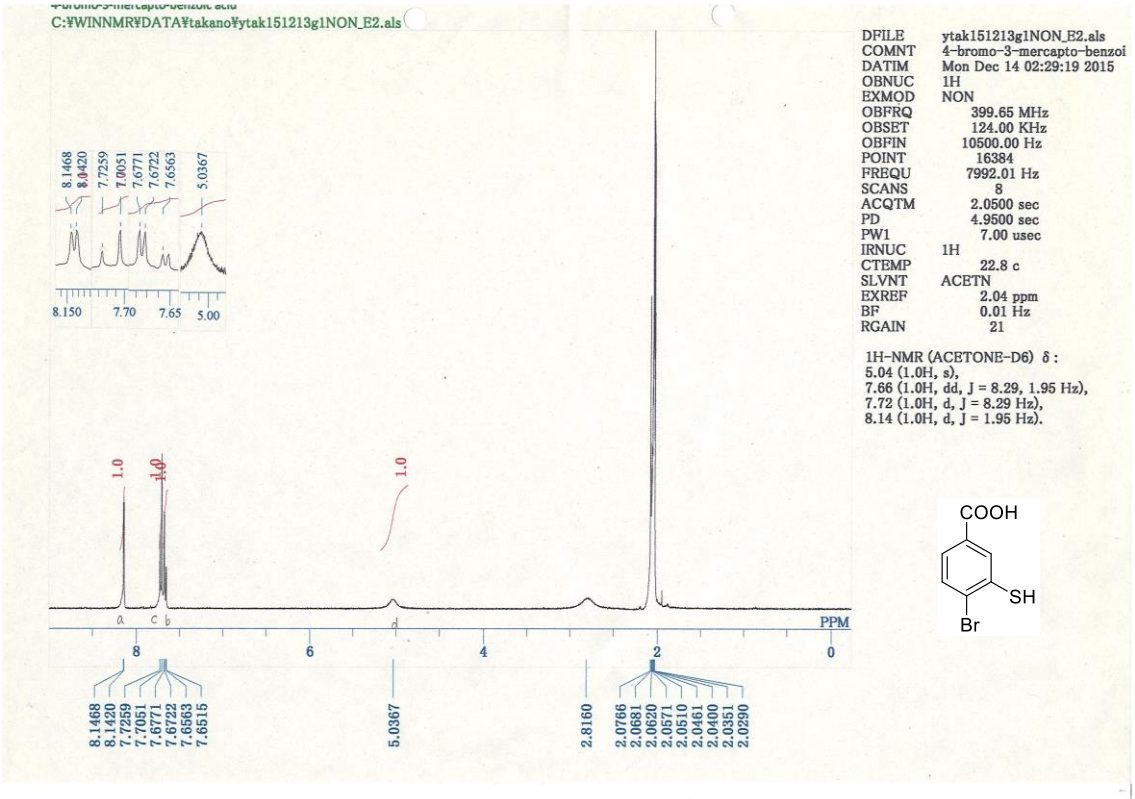


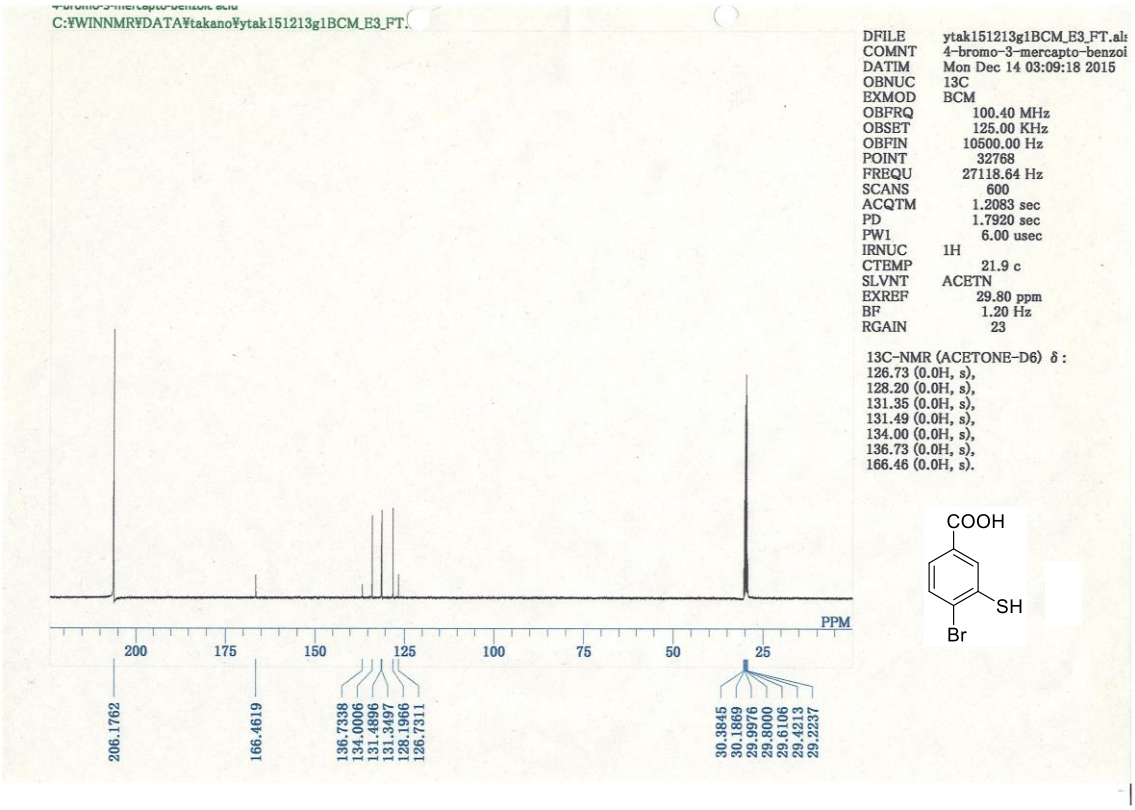

C:¥WINNMR¥DATA¥takano¥ytak151214a1NON_E1_FT.
DFILE ytak151214a1NON_E1_FT.als
COMNT 3.6-N,N'-diethyl-xhanthone
DATIM Mon Dec 14 22:08:50 2015
OBNUC 1H
EXMOD NON
OBFRQ 399.65 MHz
OBSET 124.00 KHz
OBFIN 10500.00 Hz
POINT 16384
FREQU 7992.01 Hz
SCANS 8
ACQTM 2.0500 sec
PD 4.9500 sec
PW1 7.00 usec
IRNUC 1H
CTEMP 19.4 c
SLVNT CDCL3
EXREF 0.00 ppm
BF 0.12 Hz
RGAIN 11
1H-NMR (CDCl3) δ :
1.22 (12.0H, t, J = 7.07 Hz),
3.43 (8.0H, q, J = 6.99 Hz),
6.44 (2.0H, d, J = 2.44 Hz),
6.63 (2.0H, dd, J = 9.27, 2.44 Hz),
8.09 (2.0H, d, J = 9.27 Hz).
8.1030
8.0798
6.6425
6.6364
6.6132
6.4399
6.4338
3.4518
3.4347
3.4164
1.2360
1.2177
1.2006
12.0
8.0
2.0
2.0
2.0
d
c
e
b
a
PPM
8
6
4
2
0
8.1030
8.0798
7.2709
6.6425
6.6364
6.6193
6.6132
6.4399
6.4338
3.4518
3.4347
3.4164
3.3993
1.2360
1.2177
1.2006
0.0000
O
N
O
N

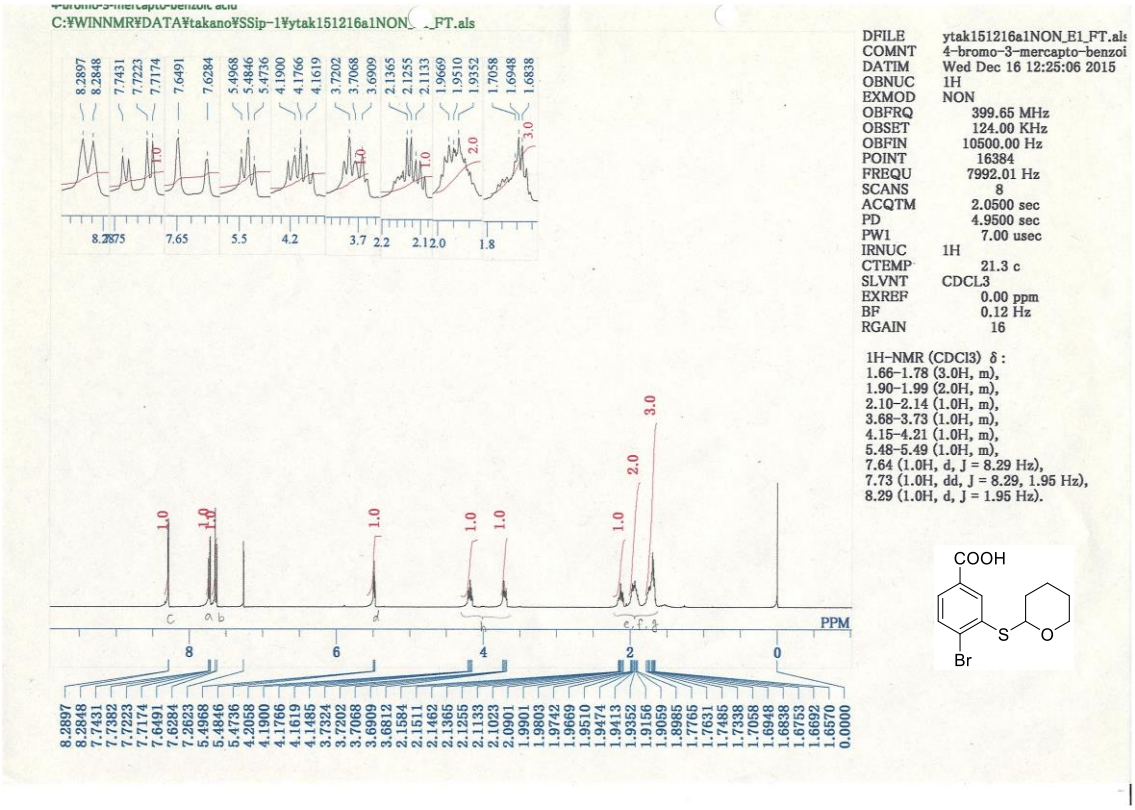

C:¥WINNMR¥DATA¥takano¥ytak151214a2BCM_E1_FT.
DFILE ytak151214a2BCM_E1_FT.als
COMNT 3.6-N,N'-diethyl-xhanthone
DATIM Mon Dec 14 22:39:23 2015
OBNUC 13C
EXMOD BCM
OBFRQ 100.40 MHz
OBSET 125.00 KHz
OBFIN 10500.00 Hz
POINT 32768
FREQU 27118.64 Hz
SCANS 600
ACQTM 1.2083 sec
PD 1.7920 sec
PW1 6.00 usec
IRNUC 1H
CTEMP 29.2 c
SLVNT CDCL3
EXREF 77.00 ppm
BF 1.20 Hz
RGAIN 25
13C-NMR (CDCl3) δ :
12.49 (0.0H, s),
44.63 (0.0H, s),
96.26 (0.0H, s),
108.51 (0.0H, s),
111.51 (0.0H, s),
127.73 (0.0H, s),
152.00 (0.0H, s),
158.44 (0.0H, s),
174.71 (0.0H, s).
PPM
200
175
150
125
100
75
50
25
0
174.7132
158.4372
151.9993
127.7295
111.5112
108.5063
96.2644
77.3128
77.0000
76.6789
44.6293
12.4891
O
N
O
N

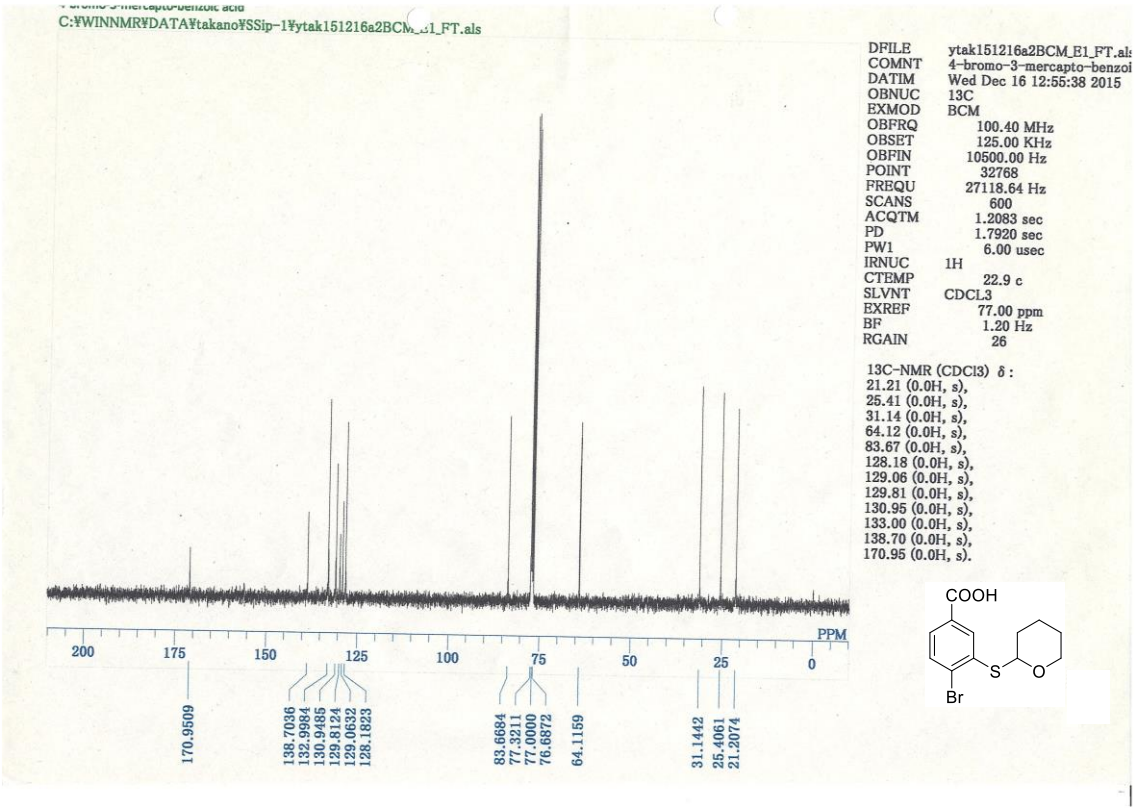

C:¥WINNMR¥DATA¥takano¥ytak151213b1NON_E1_FT.
8.7447
8.7398
8.3396
8.3189
8.3140
8.2396
8.2188
1.0
8.75
8.35
8.20
DFILE ytak151213b1NON_E1_FT.als
COMNT 4-bromo-3-sulfonylchloride-
DATIM Sun Dec 13 18:45:43 2015
OBNUC 1H
EXMOD NON
OBFRQ 399.65 MHz
OBSET 124.00 KHz
OBFIN 10500.00 Hz
POINT 16384
FREQU 7992.01 Hz
SCANS 8
ACQTM 2.0500 sec
PD 4.9500 sec
PW1 7.00 usec
IRNUC 1H
CTEMP 21.0 c
SLVNT ACETN
EXREF 2.04 ppm
BF 0.12 Hz
RGAIN 20
1H-NMR (ACETONE-D6) δ :
8.23 (1.0H, d, J = 8.29 Hz),
8.33 (1.0H, dd, J = 8.29, 1.95 Hz),
8.74 (1.0H, d, J = 1.95 Hz).
COOH
SO2Cl
Br
1.0
1.0
1.0
a
b c
PPM
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
8.7447
8.7398
8.3396
8.3347
8.3189
8.3140
8.2396
8.2188
2.0510
2.0461
2.0400
2.0351
2.0290

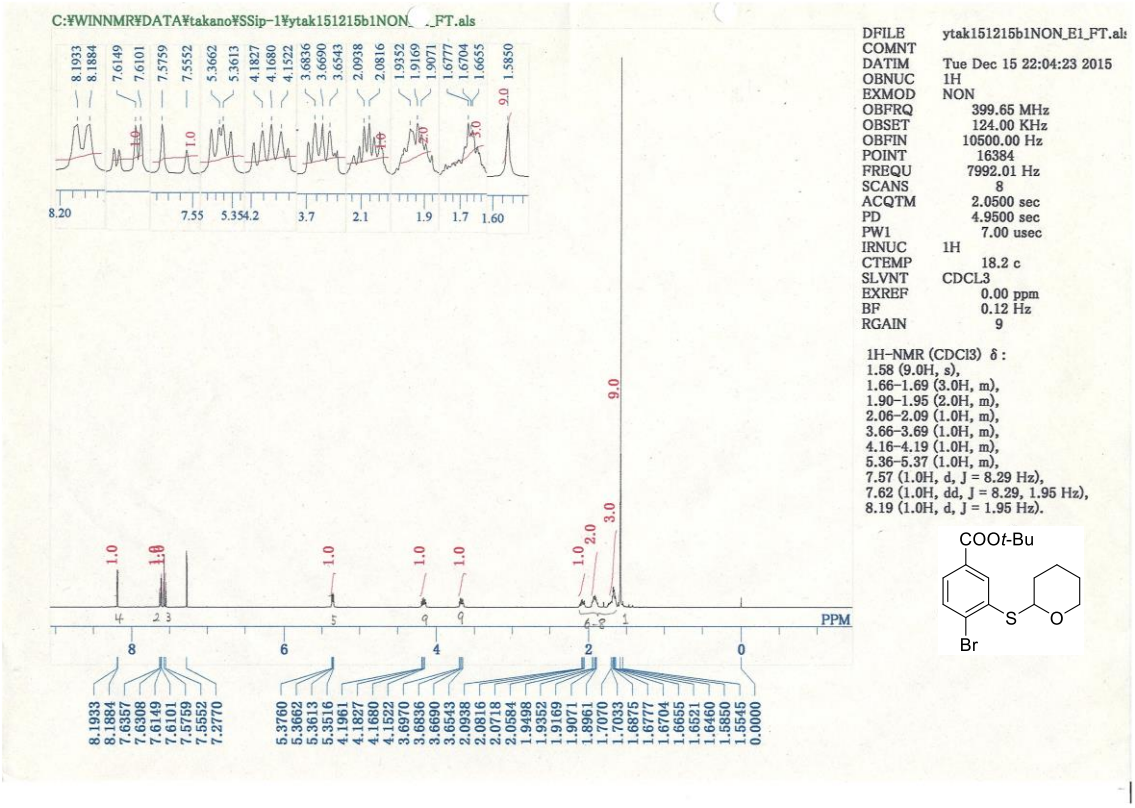

C:¥WINNMR¥DATA¥takano¥ytak151213d2BCM_E1.als
DFILE ytak151213d2BCM_E1.als
COMNT 4-bromo-3-chlorosulfonyl-be
DATIM Sun Dec 13 22:01:57 2015
OBNUC 13C
EXMOD BCM
OBFRQ 100.40 MHz
OBSET 125.00 KHz
OBFIN 10500.00 Hz
POINT 32768
FREQU 27118.64 Hz
SCANS 600
ACQTM 1.2083 sec
PD 1.7920 sec
PW1 6.00 usec
IRNUC 1H
CTEMP 21.9 c
SLVNT ACETN
EXREF 29.80 ppm
BF 0.10 Hz
RGAIN 24
13C-NMR (ACETONE-D6) δ :
125.78 (0.0H, s),
132.15 (0.0H, s),
132.24 (0.0H, s),
137.81 (0.0H, s),
138.36 (0.0H, s),
143.81 (0.0H, s),
165.00 (0.0H, s).
COOH
SO2Cl
Br
PPM
200
150
100
50
0
206.4396
206.3326
206.1268
206.0115
164.9965
143.8139
138.3639
137.8123
132.2388
132.1482
125.7844
30.3763
30.1869
30.0223
29.9976
29.8000
29.6106
29.4131
29.2237
29.1579

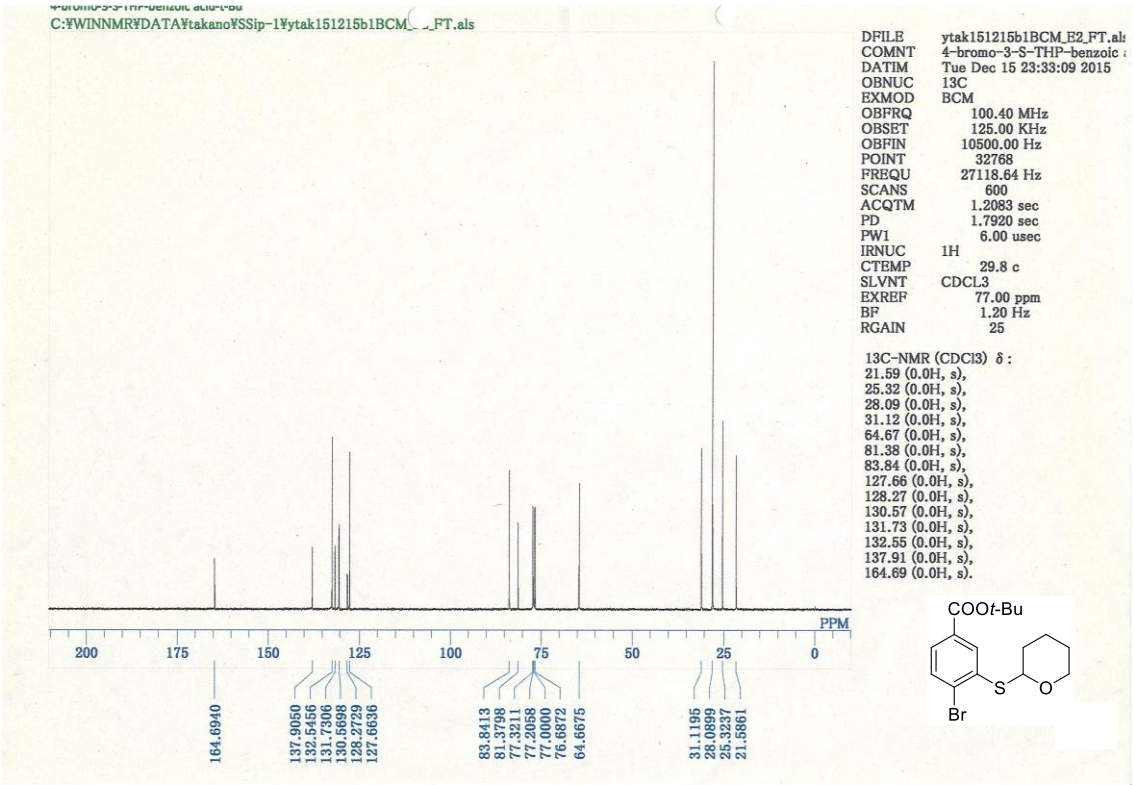

C:¥WINNMR¥DATA¥takano¥ytak151213g1NON_E2.als
DFILE ytak151213g1NON_E2.als
COMNT 4-bromo-3-mercapto-benzoi
DATIM Mon Dec 14 02:29:19 2015
OBNUC 1H
EXMOD NON
OBFRQ 399.65 MHz
OBSET 124.00 KHz
OBFIN 10500.00 Hz
POINT 16384
FREQU 7992.01 Hz
SCANS 8
ACQTM 2.0500 sec
PD 4.9500 sec
PW1 7.00 usec
IRNUC 1H
CTEMP 22.8 c
SLVNT ACETN
EXREF 2.04 ppm
BF 0.01 Hz
RGAIN 21
1H-NMR (ACETONE-D6) δ :
5.04 (1.0H, s),
7.66 (1.0H, dd, J = 8.29, 1.95 Hz),
7.72 (1.0H, d, J = 8.29 Hz),
8.14 (1.0H, d, J = 1.95 Hz).
COOH
SH
Br
PPM
8.1468
8.0420
7.7259
7.7051
7.6771
7.6722
7.6563
5.0367
2.8160
2.0766
2.0681
2.0620
2.0571
2.0510
2.0461
2.0400
2.0351
2.0290

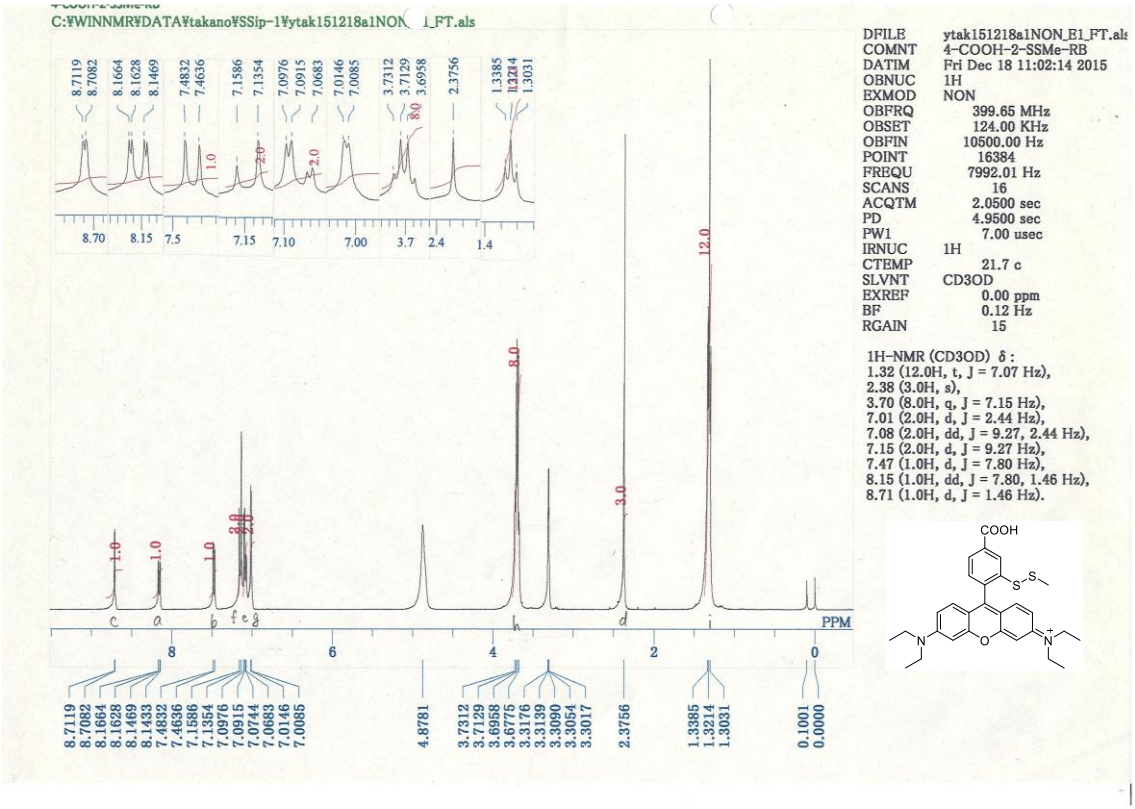

C:¥WINNMR¥DATA¥takano¥ytak151213g1BCM_E3_FT.
DFILE ytak151213g1BCM_E3_FT.als
COMNT 4-bromo-3-mercapto-benzoi
DATIM Mon Dec 14 03:09:18 2015
OBNUC 13C
EXMOD BCM
OBFRQ 100.40 MHz
OBSET 125.00 KHz
OBFIN 10500.00 Hz
POINT 32768
FREQU 27118.64 Hz
SCANS 600
ACQTM 1.2083 sec
PD 1.7920 sec
PW1 6.00 usec
IRNUC 1H
CTEMP 21.9 c
SLVNT ACETN
EXREF 29.80 ppm
BF 1.20 Hz
RGAIN 23
13C-NMR (ACETONE-D6) δ :
126.73 (0.0H, s),
128.20 (0.0H, s),
131.35 (0.0H, s),
131.49 (0.0H, s),
134.00 (0.0H, s),
136.73 (0.0H, s),
166.46 (0.0H, s).
COOH
SH
Br
PPM
206.1762
166.4619
136.7338
134.0006
131.4896
131.3497
128.1966
126.7311
30.3845
30.1869
29.9976
29.8000
29.6106
29.4213
29.2237

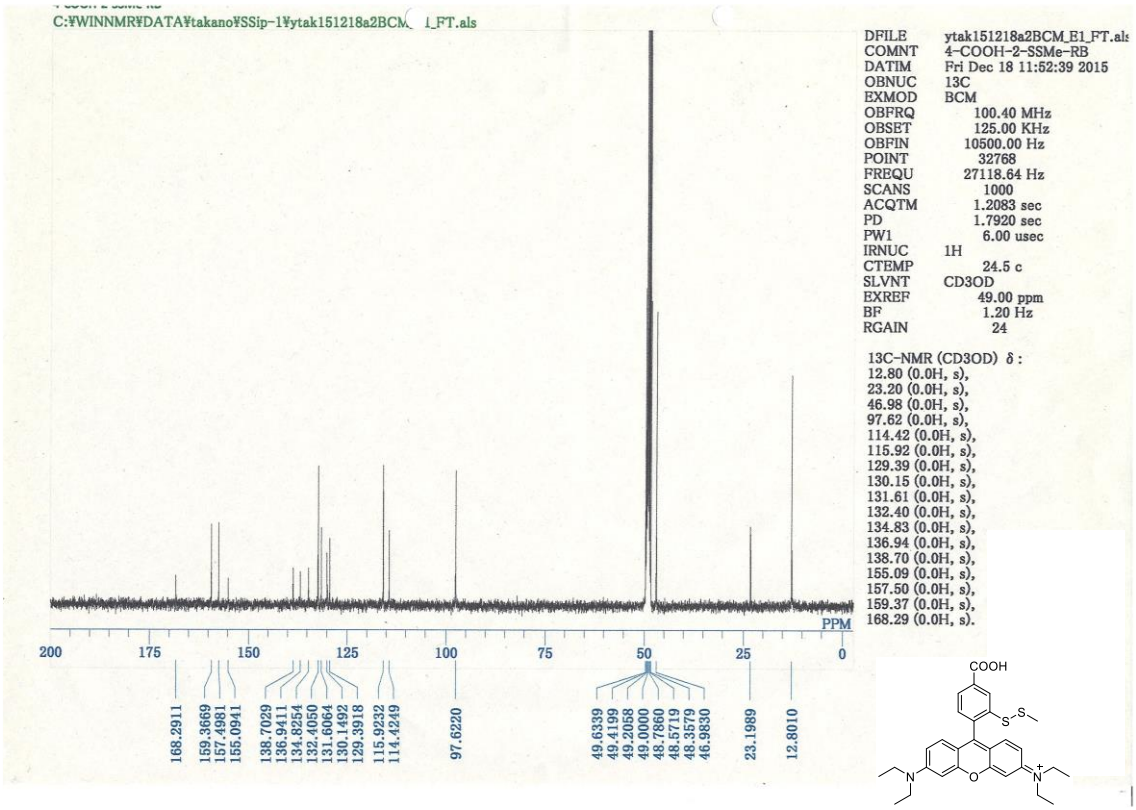

C:¥WINNMR¥DATA¥takano¥SSip-1¥ytak151216a1NON_ _FT.als
DFILE ytak151216a1NON_E1_FT.als
COMNT 4-bromo-3-mercapto-benzoi
DATIM Wed Dec 16 12:25:06 2015
OBNUC 1H
EXMOD NON
OBFRQ 399.65 MHz
OBSET 124.00 KHz
OBFIN 10500.00 Hz
POINT 16384
FREQU 7992.01 Hz
SCANS 8
ACQTM 2.0500 sec
PD 4.9500 sec
PW1 7.00 usec
IRNUC 1H
CTEMP 21.3 c
SLVNT CDCL3
EXREF 0.00 ppm
BF 0.12 Hz
RGAIN 16
1H-NMR (CDCl3) δ :
1.66–1.78 (3.0H, m),
1.90–1.99 (2.0H, m),
2.10–2.14 (1.0H, m),
3.68–3.73 (1.0H, m),
4.15–4.21 (1.0H, m),
5.48–5.49 (1.0H, m),
7.64 (1.0H, d, J = 8.29 Hz),
7.73 (1.0H, dd, J = 8.29, 1.95 Hz),
8.29 (1.0H, d, J = 1.95 Hz).
COOH
S
O
Br
PPM

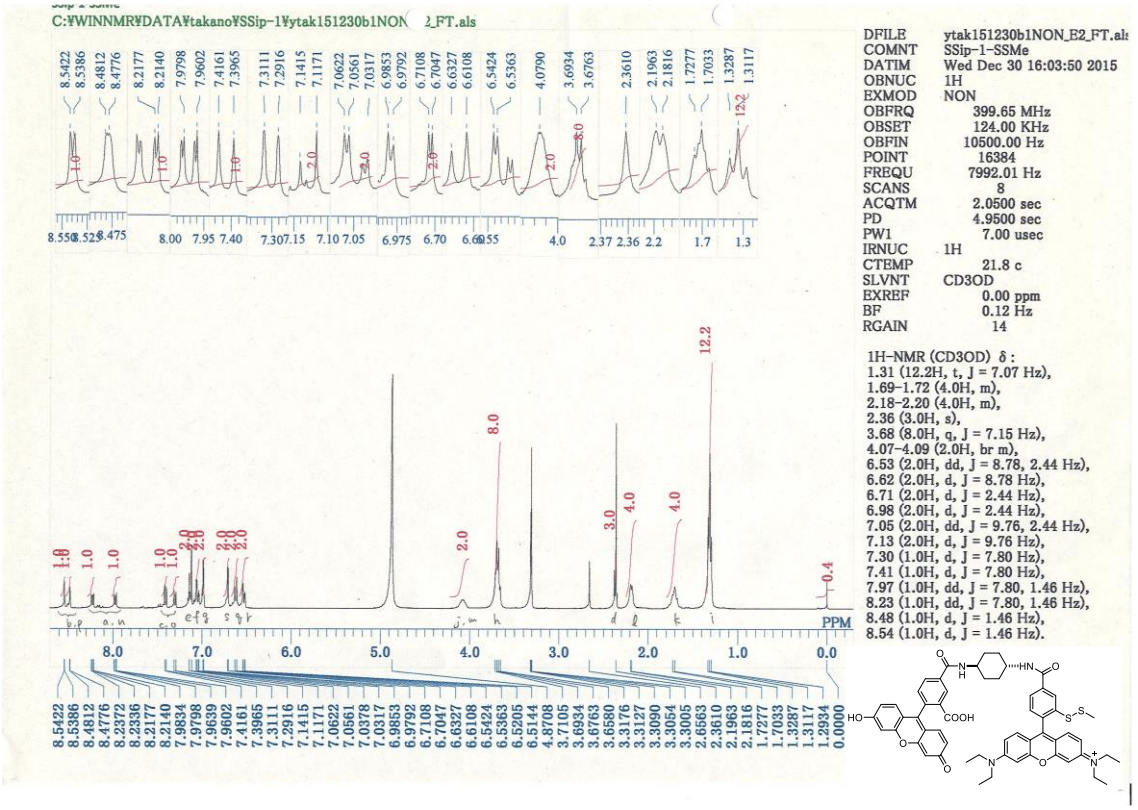

C:¥WINNMR¥DATA¥takano¥SSip-1¥ytak151216a2BCM_E1_FT.als
DFILE ytak151216a2BCM_E1_FT.als
COMNT 4-bromo-3-mercapto-benzoi
DATIM Wed Dec 16 12:55:38 2015
OBNUC 13C
EXMOD BCM
OBFRQ 100.40 MHz
OBSET 125.00 KHz
OBFIN 10500.00 Hz
POINT 32768
FREQU 27118.64 Hz
SCANS 600
ACQTM 1.2083 sec
PD 1.7920 sec
PW1 6.00 usec
IRNUC 1H
CTEMP 22.9 c
SLVNT CDCL3
EXREF 77.00 ppm
BF 1.20 Hz
RGAIN 26
13C-NMR (CDCl3) δ :
21.21 (0.0H, s),
25.41 (0.0H, s),
31.14 (0.0H, s),
64.12 (0.0H, s),
83.67 (0.0H, s),
128.18 (0.0H, s),
129.06 (0.0H, s),
129.81 (0.0H, s),
130.95 (0.0H, s),
133.00 (0.0H, s),
138.70 (0.0H, s),
170.95 (0.0H, s).
COOH
S
O
Br
PPM

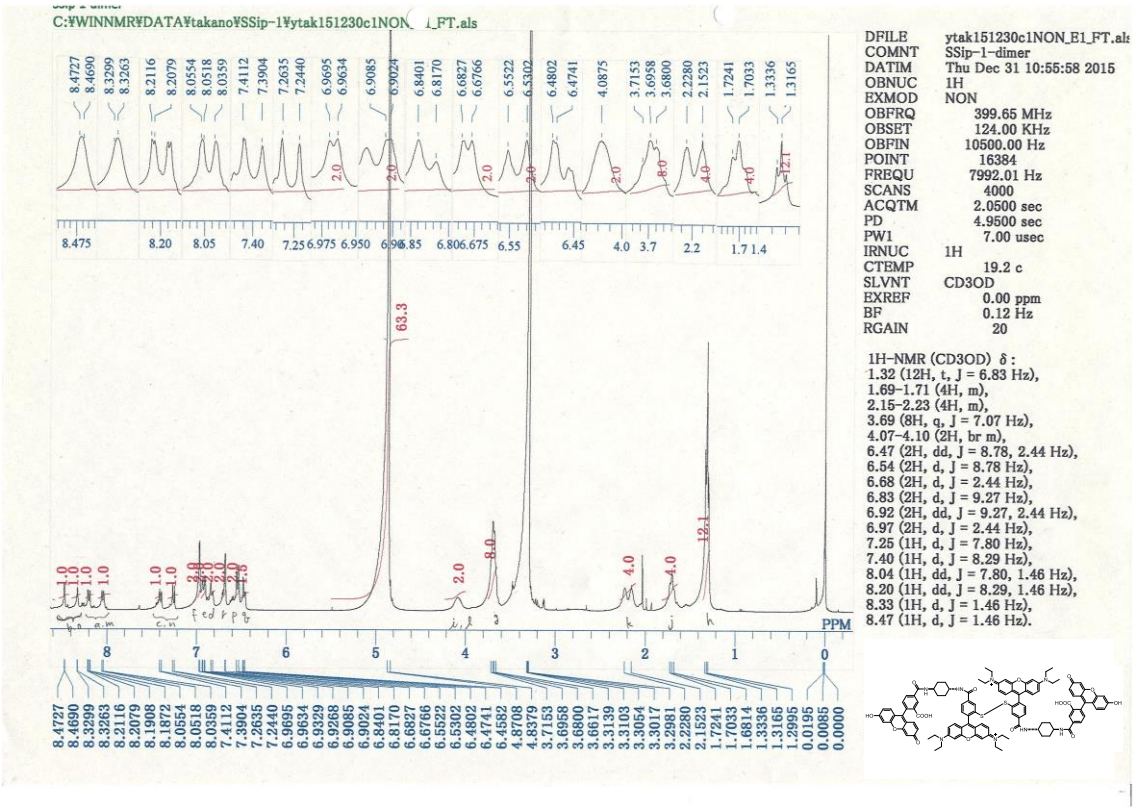

C:¥WINNMR¥DATA¥takano¥SSip-1¥ytak151215b1NON_ _FT.als
DFILE ytak151215b1NON_E1_FT.al
COMNT
DATIM Tue Dec 15 22:04:23 2015
OBNUC 1H
EXMOD NON
OBFRQ 399.65 MHz
OBSET 124.00 KHz
OBFIN 10500.00 Hz
POINT 16384
FREQU 7992.01 Hz
SCANS 8
ACQTM 2.0500 sec
PD 4.9500 sec
PW1 7.00 usec
IRNUC 1H
CTEMP 18.2 c
SLVNT CDCL3
EXREF 0.00 ppm
BF 0.12 Hz
RGAIN 9
1H-NMR (CDCl3) δ :
1.58 (9.0H, s),
1.66-1.69 (3.0H, m),
1.90-1.95 (2.0H, m),
2.06-2.09 (1.0H, m),
3.66-3.69 (1.0H, m),
4.16-4.19 (1.0H, m),
5.36-5.37 (1.0H, m),
7.57 (1.0H, d, J = 8.29 Hz),
7.62 (1.0H, dd, J = 8.29, 1.95 Hz),
8.19 (1.0H, d, J = 1.95 Hz).
COOt-Bu
S
O
Br
PPM

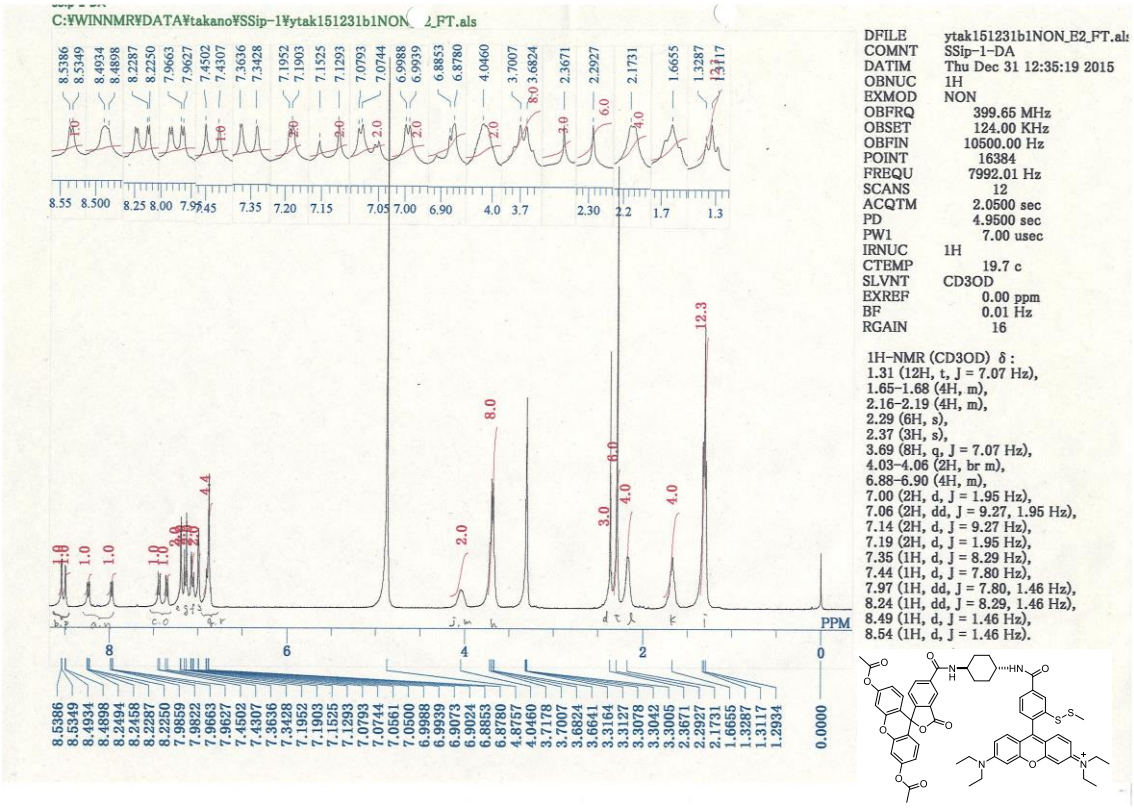

C:¥WINNMR¥DATA¥takano¥SSip-1¥ytak151215b1BCM_ _FT.als
DFILE ytak151215b1BCM_E2_FT.al
COMNT 4-bromo-3-S-THP-benzoic
DATIM Tue Dec 15 23:33:09 2015
OBNUC 13C
EXMOD BCM
OBFRQ 100.40 MHz
OBSET 125.00 KHz
OBFIN 10500.00 Hz
POINT 32768
FREQU 27118.64 Hz
SCANS 600
ACQTM 1.2083 sec
PD 1.7920 sec
PW1 6.00 usec
IRNUC 1H
CTEMP 29.8 c
SLVNT CDCL3
EXREF 77.00 ppm
BF 1.20 Hz
RGAIN 25
13C-NMR (CDCl3) δ :
21.59 (0.0H, s),
25.32 (0.0H, s),
28.09 (0.0H, s),
31.12 (0.0H, s),
64.67 (0.0H, s),
81.38 (0.0H, s),
83.84 (0.0H, s),
127.66 (0.0H, s),
128.27 (0.0H, s),
130.57 (0.0H, s),
131.73 (0.0H, s),
132.55 (0.0H, s),
137.91 (0.0H, s),
164.69 (0.0H, s).
COOt-Bu
S
O
Br
PPM

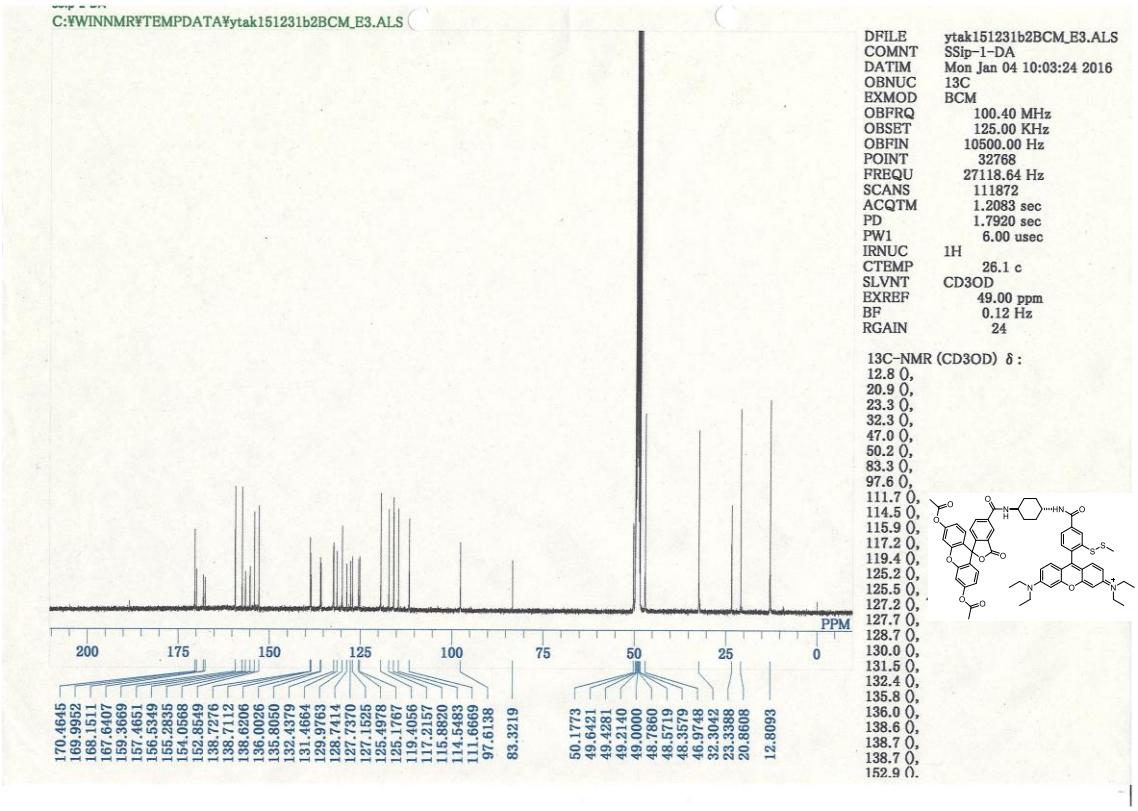

C:¥WINNMR¥DATA¥takano¥SSip-1¥ytak151218a1NON_1_FT.als
DFILE ytak151218a1NON_E1_FT.als
COMNT 4-COOH-2-SSMe-RB
DATIM Fri Dec 18 11:02:14 2015
OBNUC 1H
EXMOD NON
OBFRQ 399.65 MHz
OBSET 124.00 KHz
OBFIN 10500.00 Hz
POINT 16384
FREQU 7992.01 Hz
SCANS 16
ACQTM 2.0500 sec
PD 4.9500 sec
PW1 7.00 usec
IRNUC 1H
CTEMP 21.7 c
SLVNT CD3OD
EXREF 0.00 ppm
BF 0.12 Hz
RGAIN 15
1H-NMR (CD3OD) δ :
1.32 (12.0H, t, J = 7.07 Hz),
2.38 (3.0H, s),
3.70 (8.0H, q, J = 7.15 Hz),
7.01 (2.0H, d, J = 2.44 Hz),
7.08 (2.0H, dd, J = 9.27, 2.44 Hz),
7.15 (2.0H, d, J = 9.27 Hz),
7.47 (1.0H, d, J = 7.80 Hz),
8.15 (1.0H, dd, J = 7.80, 1.46 Hz),
8.71 (1.0H, d, J = 1.46 Hz).
PPM
COOH


C:¥WINNMR¥DATA¥takano¥SSip-1¥ytak151218a2BCM_1_FT.als
DFILE ytak151218a2BCM_E1_FT.als
COMNT 4-COOH-2-SSMe-RB
DATIM Fri Dec 18 11:52:39 2015
OBNUC 13C
EXMOD BCM
OBFRQ 100.40 MHz
OBSET 125.00 KHz
OBFIN 10500.00 Hz
POINT 32768
FREQU 27118.64 Hz
SCANS 1000
ACQTM 1.2083 sec
PD 1.7920 sec
PW1 6.00 usec
IRNUC 1H
CTEMP 24.5 c
SLVNT CD3OD
EXREF 49.00 ppm
BF 1.20 Hz
RGAIN 24
13C-NMR (CD3OD) δ :
12.80 (0.0H, s),
23.20 (0.0H, s),
46.98 (0.0H, s),
97.62 (0.0H, s),
114.42 (0.0H, s),
115.92 (0.0H, s),
129.39 (0.0H, s),
130.15 (0.0H, s),
131.61 (0.0H, s),
132.40 (0.0H, s),
134.83 (0.0H, s),
136.94 (0.0H, s),
138.70 (0.0H, s),
155.09 (0.0H, s),
157.50 (0.0H, s),
159.37 (0.0H, s),
168.29 (0.0H, s).
PPM
COOH


C:¥WINNMR¥DATA¥takano¥SSip-1¥ytak151230b1NON_2_FT.als
DFILE ytak151230b1NON_E2_FT.als
COMNT SSip-1-SSMe
DATIM Wed Dec 30 16:03:50 2015
OBNUC 1H
EXMOD NON
OBFRQ 399.65 MHz
OBSET 124.00 KHz
OBFIN 10500.00 Hz
POINT 16384
FREQU 7992.01 Hz
SCANS 8
ACQTM 2.0500 sec
PD 4.9500 sec
PW1 7.00 usec
IRNUC 1H
CTEMP 21.8 c
SLVNT CD3OD
EXREF 0.00 ppm
BF 0.12 Hz
RGAIN 14
1H-NMR (CD3OD) δ :
1.31 (12.2H, t, J = 7.07 Hz),
1.69-1.72 (4.0H, m),
2.18-2.20 (4.0H, m),
2.36 (3.0H, s),
3.68 (8.0H, q, J = 7.15 Hz),
4.07-4.09 (2.0H, br m),
6.53 (2.0H, dd, J = 8.78, 2.44 Hz),
6.62 (2.0H, d, J = 8.78 Hz),
6.71 (2.0H, d, J = 2.44 Hz),
6.98 (2.0H, d, J = 2.44 Hz),
7.05 (2.0H, dd, J = 9.76, 2.44 Hz),
7.13 (2.0H, d, J = 9.76 Hz),
7.30 (1.0H, d, J = 7.80 Hz),
7.41 (1.0H, d, J = 7.80 Hz),
7.97 (1.0H, dd, J = 7.80, 1.46 Hz),
8.23 (1.0H, dd, J = 7.80, 1.46 Hz),
8.48 (1.0H, d, J = 1.46 Hz),
8.54 (1.0H, d, J = 1.46 Hz).
PPM


C:¥WINNMR¥DATA¥takano¥SSip-1¥ytak151230c1NON_1_FT.als
DFILE ytak151230c1NON_E1_FT.als
COMNT SSip-1-dimer
DATIM Thu Dec 31 10:55:58 2015
OBNUC 1H
EXMOD NON
OBFRQ 399.65 MHz
OBSET 124.00 KHz
OBFIN 10500.00 Hz
POINT 16384
FREQU 7992.01 Hz
SCANS 4000
ACQTM 2.0500 sec
PD 4.9500 sec
PW1 7.00 usec
IRNUC 1H
CTEMP 19.2 c
SLVNT CD3OD
EXREF 0.00 ppm
BF 0.12 Hz
RGAIN 20
1H-NMR (CD3OD) δ :
1.32 (12H, t, J = 6.83 Hz),
1.69-1.71 (4H, m),
2.15-2.23 (4H, m),
3.69 (8H, q, J = 7.07 Hz),
4.07-4.10 (2H, br m),
6.47 (2H, dd, J = 8.78, 2.44 Hz),
6.54 (2H, d, J = 8.78 Hz),
6.68 (2H, d, J = 2.44 Hz),
6.83 (2H, d, J = 9.27 Hz),
6.92 (2H, dd, J = 9.27, 2.44 Hz),
6.97 (2H, d, J = 2.44 Hz),
7.25 (1H, d, J = 7.80 Hz),
7.40 (1H, d, J = 8.29 Hz),
8.04 (1H, dd, J = 7.80, 1.46 Hz),
8.20 (1H, dd, J = 8.29, 1.46 Hz),
8.33 (1H, d, J = 1.46 Hz),
8.47 (1H, d, J = 1.46 Hz).
PPM


C:¥WINNMR¥DATA¥takano¥SSip-1¥ytak151231b1NON_2_FT.als
DFILE ytak151231b1NON_E2_FT.als
COMNT SSip-1-DA
DATIM Thu Dec 31 12:35:19 2015
OBNUC 1H
EXMOD NON
OBFRQ 399.65 MHz
OBSET 124.00 KHz
OBFIN 10500.00 Hz
POINT 16384
FREQU 7992.01 Hz
SCANS 12
ACQTM 2.0500 sec
PD 4.9500 sec
PW1 7.00 usec
IRNUC 1H
CTEMP 19.7 c
SLVNT CD3OD
EXREF 0.00 ppm
BF 0.01 Hz
RGAIN 16
1H-NMR (CD3OD) δ :
1.31 (12H, t, J = 7.07 Hz),
1.65-1.68 (4H, m),
2.16-2.19 (4H, m),
2.29 (6H, s),
2.37 (3H, s),
3.69 (8H, q, J = 7.07 Hz),
4.03-4.06 (2H, br m),
6.88-6.90 (4H, m),
7.00 (2H, d, J = 1.95 Hz),
7.06 (2H, dd, J = 9.27, 1.95 Hz),
7.14 (2H, d, J = 9.27 Hz),
7.19 (2H, d, J = 1.95 Hz),
7.35 (1H, d, J = 8.29 Hz),
7.44 (1H, d, J = 7.80 Hz),
7.97 (1H, dd, J = 7.80, 1.46 Hz),
8.24 (1H, dd, J = 8.29, 1.46 Hz),
8.49 (1H, d, J = 1.46 Hz),
8.54 (1H, d, J = 1.46 Hz).
PPM


C:¥WINNMR¥TEMPDATA¥ytak151231b2BCM_E3.ALS
DFILE ytak151231b2BCM_E3.ALS
COMNT SSip-1-DA
DATIM Mon Jan 04 10:03:24 2016
OBNUC 13C
EXMOD BCM
OBFRQ 100.40 MHz
OBSET 125.00 KHz
OBFIN 10500.00 Hz
POINT 32768
FREQU 27118.64 Hz
SCANS 111872
ACQTM 1.2083 sec
PD 1.7920 sec
PW1 6.00 usec
IRNUC 1H
CTEMP 26.1 c
SLVNT CD3OD
EXREF 49.00 ppm
BF 0.12 Hz
RGAIN 24
13C-NMR (CD3OD) δ :
12.8 (),
20.9 (),
23.3 (),
32.3 (),
47.0 (),
50.2 (),
83.3 (),
97.6 (),
111.7 (),
114.5 (),
115.9 (),
117.2 (),
119.4 (),
125.2 (),
125.5 (),
127.2 (),
127.7 (),
128.7 (),
130.0 (),
131.5 (),
132.4 (),
135.8 (),
136.0 (),
138.6 (),
138.7 (),
138.7 (),
152.9 ().
PPM

References:

(S1) Peaker, C. A.; Rees, W. T. *Analyst*, **1960**, *85*, 587.

(S2) PCT Int. Appl., WO2013141586, 26 Sep 2013.

(S3) Rossi, F. M.; Kao, J. P. Y. *Bioconjugate Chem.* **1997**, *8*, 495.

(S4) Piao, W.; Tsuda, S.; Tanaka, Y.; Maeda, S.; Liu, F.; Takahashi, S.; Kushida, Y.; Komatsu, T.; Ueno, T.; Terai, T.; Nakazawa, T.; Uchiyama, M.; Morokuma, K.; Nagano, T.; Hanaoka, K. *Angew. Chem. Int. Ed.* **2013**, *52*, 13028.

(S5) Deguchi, J.; Sasaki, T.; Hirasawa, Y.; Kaneda, T.; Kusumawati, I.; Shirota, O.; Morita, H. *Tetrahedron Lett.* **2014**, *55*, 1362.