Supporting Information to Accompany "**A Small Molecule Two-Photon Probe for Hydrogen Sulfide in Live Tissues**"

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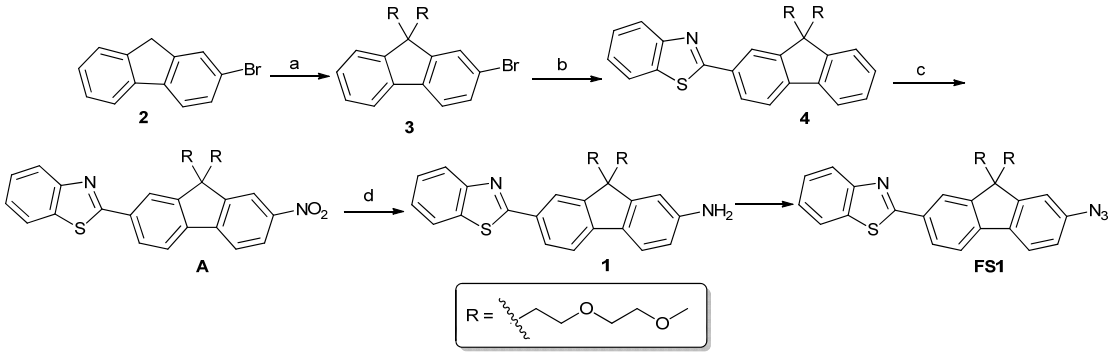
Synthesis:

General. Melting points were determined with a hot stage apparatus. All dry reactions were carried out under argon or nitrogen in oven-dried glassware using standard gas-light syringes, cannulas, and septa. All solvents were dried prior to use according to the standard methods. Commercial reagents were used

without further purification unless otherwise stated. Progress of reactions was monitored by TLC on precoated Merck silica gel plates (60F-254). Visualization of reactants and products was accomplished with UV light. High resolution mass spectra were recorded using FAB (fast atom bombardment) and ^{1}H NMR and ^{13}C NMR spectra were recorded either at 300MHz and 100 MHz, respectively.

Scheme S1. Synthesis of **1** and **SF1**[a]



[a]*Reagents and conditions*: (a) $CH_3OCH_2CH_2OCH_2CH_2OTs$, KOH, anhyd. DMSO, rt, overnight, 90 %; (b) benzothiazole, Cs_2CO_3, $Pd(OAc)_2$, CuI, t-Bu_3P, anhyd. DMF, 145 ^{o}C, overnight, 86 %; (c) Fuming HNO_3, Ac_2O/AcOH (1:1), rt, 45 min, 84 %; (d) Fe, NH_4Cl, EtOH/H_2O, 85 ^{o}C, 2 h, 90 %; (d) (i) 4(N) H_2SO_4, $NaNO_2$, H_2O, 0 ^{o}C, 15 min, (ii) NaN_3, H_2O, 0 ^{o}C-rt, 45 min, 91 %.

Compound 3. A stirred solution of 2-bromofluorene **2** (10.0 g, 40.8 mmol) and diethyleneglycol monomethyl ether tosylate (25.7 g, 93.8 mmol) in anhydrous DMSO (150 mL) was purged with dry nitrogen by repeated filling and deflating of a nitrogen balloon. To this was added freshly prepared anhydrous powdered KOH (5.72 g, 102 mmol). The resulting mixture was then degassed, and again purged with dry nitrogen. The reaction mixture was stirred vigorously for overnight under nitrogen atmosphere at RT. It was then poured into a beaker containing 300 mL 1N HCl. Diethyl ether (200 mL) was added to it and stirred vigorously for 5 min. The organic layer was separated, washed by saturated aq. Na_2CO_3 solution (100 mL), brine (100 mL), dried ($MgSO_4$), and filtered. Evaporation of the solvent under reduced pressure gave the crude product which was subjected to silica gel column chromatography (30-50% ethyl acetate in *n*-hexane) to get compound **3** (16.5 g, 90.0 %) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.65-7.63 (m, 1H), 7.57-7.52 (m, 2H), 7.46-7.39 (m, 2H), 7.33-7.31 (m, 2H), 3.29-3.15 (m, 14H), 2.78-2.74 (m, 4H), 2.41-2.37 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 150.8, 148.2, 139.1, 139.0, 130.1, 127.5, 127.2, 126.3, 122.9, 120.9, 120.8, 119.6, 71.4, 69.6, 66.6, 58.7, 51.1, 39.2.

Compound 4: A stirred solution of compound **3** (15.0 g, 33.4 mmol) and benzothiazole (5.50 mL, 50.4 mmol) in anhydrous DMF (150 mL) was purged with dry nitrogen by repeated filling and deflating of a nitrogen balloon. To this were added successively Cs_2CO_3 (16.5 g, 50.6 mmol, $Pd(OAc)_2$ (1.12 g, 5.00 mmol), CuI (952 mg, 5.00 mmol), and t-Bu_3P (15.0 mL, 10 wt. % in hexanes, 5.00 mmol). The resulting mixture was then degassed, and again purged with dry nitrogen. The reaction mixture was stirred vigorously for overnight under nitrogen atmosphere at 145 ^{o}C. It was then filtered, and the

residue was washed by ethyl acetate (300 mL). The combined filtrate was concentrated under reduced pressure to get a black residue which was re-dissolved in ethyl acetate (200 mL), washed by 1N HCl (100 mL), brine (100 mL), dried ($MgSO_4$), and filtered. Solvent was removed under reduced pressure and silica gel column chromatography (20-60% ethyl acetate in *n*-hexane) of the crude product gave a solid which was recrystallized from ethyl acetate-hexane to get compound **4** (14.5 g, 86.0 %) as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.19 (s, 1H), 8.10 (d, *J* = 8.0 Hz, 2H), 7.93 (d, *J* = 8.0 Hz, 1H), 7.79 (d, *J* = 8.0 Hz, 1H), 7.75-7.73 (m, 1H), 7.53-7.37 (m, 5H), 3.29-3.17 (m, 14H), 2.85-2.75 (m, 4H), 2.59-2.44 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.1, 154.1, 149.7, 149.5, 143.3, 139.4, 134.9, 132.5, 128.4, 127.4, 127.2, 126.3, 125.1, 123.3, 123.0, 122.0, 121.6, 120.3, 120.2, 71.6, 69.8, 66.9, 58.9, 51.4, 39.5. HRMS (FAB) Calcd. for $C_{30}H_{34}NO_4S$ $[M+H]^+$: 504.2209. Found: 504.2209.

Compound A. To a stirred solution of compound **4** (10.0 g, 19.9 mmol) in AcOH (50 mL) and Ac_2O (50 mL) was added 93% HNO_3 (1.7 mL, 39.7 mmol) dropwise for 10 min at rt. The reaction was monitored (TLC) very closely until the starting material had been completely consumed (~80-100 min). It was then poured into a beaker containing 200 mL water. Ethyl acetate (200 mL) was added to it and stirred vigorously for 5 min. The organic layer was separated, washed by water (100 mL), brine (100 mL), dried ($MgSO_4$), and filtered. Solvent was removed under reduced pressure and silica gel column chromatography (20-50% ethyl acetate in *n*-hexane) of the crude product gave a semi-solid which was recrystallized from ethyl acetate-hexane to get compound **5** (9.15 g, 84.0 %) as a yellow solid.[1] M.p.-80−81 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.38 (d, *J* = 1.6 Hz, 1H), 8.30 (dd, *J* = 1.6, 8.4 Hz, 1H), 8.27 (d, *J* = 0.8 Hz, 1H), 8.15 (dd, *J* = 0.8, 7.6 Hz, 1H), 8.11 (d, *J* = 7.6, 1H), 7.94 (d, *J* = 7.6, 1H), 7.89 (d, *J* = 7.6, 1H), 7.85 (d, *J* = 8.4, 1H), 7.52 (t, *J* = 7.2 Hz, 1H), 7.41 (t, *J* = 7.2 Hz, 1H), 3.27-3.12 (m, 14H), 2.98-2.81 (m, 4H), 2.59-2.56 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.5, 153.4, 151.1, 150.7, 146.8, 145.0, 140.2, 134.4, 133.6, 125.9, 124.8, 122.6, 121.6, 121.1, 121.0, 119.8, 118.5, 70.9, 69.2, 66.2, 58.1, 51.7, 38.5.

Compound 1. Fe (4.60 g, 82.4 mmol) powder was added to a solution of compound **5** (9.00 g, 16.4 mmol) and NH_4Cl (2.63 g, 49.20 mmol) in EtOH (150 mL) and H_2O (50 mL) and the resulting mixture was then refluxed for 2 h under nitrogen atmosphere. After cooling to rt, the reaction mixture was filtered through celite and the residue was washed by ethyl acetate (150 mL). The combined filtrate was concentrated under reduced pressure to get a yellow residue which was redissolved in ethyl acetate (200 mL) and H_2O (100 mL). The organic layer was separated, washed by brine (100 mL), dried ($MgSO_4$), and filtered. Solvent was removed under reduced pressure and silica gel column chromatography (60-90 % ethyl acetate in *n*-hexane) of the crude product gave a solid which was recrystallized from ethyl acetate-hexane to get compound **1** (7.65 g, 90.0 %) as a yellow solid.[1] M.p.-134−135 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.11 (d, *J* = 1.2 Hz, 1H), 8.08-8.05 (m, 1H), 8.02 (dd, *J* = 1.6, 7.8 Hz, 1H), 7.93-7.86 (m, 1H), 7.60 (d, *J* = 7.8 Hz, 1H), 7.52-7.46 (m, 2H), 7.40-7.35 (m, 1H), 6.76 (d, *J* = 2.1 Hz, 1H), 6.68 (dd, *J* = 2.1, 8.4 Hz, 1H), 3.90 (s, br, 2H), 3.32-3.20 (m, 14H), 2.83-2.78 (m, 4H), 2.54-2.32 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.2, 153.8, 151.3, 148.3, 147.3, 143.8,

134.5, 130.4, 129.6, 127.1, 125.9, 124.6, 122.5, 121.3, 121.25, 121.18, 118.4, 114.1, 109.1, 71.3, 69.5, 66.6, 58.6, 50.6, 39.5. HRMS (FAB) Calcd. for $C_{30}H_{34}N_2O_4S$ $[M+H]^+$: 519.2318. Found 519.2319.

Compound FS1. To an ice-cooled stirred solution of compound **5** (1.00 g, 1.92 mmol) in 4N H_2SO_4 (20 mL) was added dropwise an ice-cooled solution of $NaNO_2$ (160 mg, 2.32 mmol) in water (5 mL) for 5 min. The reaction was stirred for 10 min at 0 ^oC, and then a solution of NaN_3 (626 mg, 9.64 mmol) in water (15 mL) was added dropwise. After the addition was complete, the cooling bath was removed and the reaction mixture was vigorously stirred for additional 45 min at rt. Ethyl acetate (50 mL) was added to it and stirred vigorously for 5 min. The organic layer was separated, washed by water (30 mL), brine (30 mL), dried ($MgSO_4$), and filtered. Solvent was removed under reduced pressure and column chromatography (20-40% ethyl acetate in *n*-hexane) of the crude product through a short pad of silica gel was done rapidly to obtain compound **FS1** (955 mg, 91.0 %) as a light yellow semi-solid. ^1H NMR (300 MHz, $CDCl_3$): δ 8.16 (d, J = 1.1 Hz, 1H), 8.08 (dd, J = 1.6, 8.0 Hz, 2H), 7.92 (d, J = 8.0 Hz, 1H), 7.71 (t, J = 8.6 Hz, 2H), 7.54-7.37 (m, 2H), 7.12 (d, J = 1.9 Hz, 1H), 7.04 (dd, J = 1.9, 8.2 Hz, 1H), 3.30-3.17 (m, 14H), 2.87-2.75 (m, 4H), 2.59-2.39 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.2, 153.5, 151.4, 149.0, 141.9, 139.4, 135.9, 134.3, 131.8, 126.9, 125.7, 124.5, 122.4, 121.4, 121.0, 119.4, 117.9, 113.6, 71.0, 69.3, 66.3, 58.2, 51.1, 38.9. HRMS (FAB) Calcd. for $C_{30}H_{33}N_4O_4S$ $[M+H]^+$: 545.2223. Found 545.2225.

Water solubility. Small amount of dye was dissolved in DMSO to prepare the stock solutions (1.0 × 10-3 M). The solution was diluted to ($6.0 \times 10^{-3} \sim 6.0 \times 10^{-5}$) M and added to a cuvette containing 3.0 mL of H_2O by using a micro syringe. In all cases, the concentration of DMSO in H_2O was maintained to be 0.2 %.[2] The plots of fluorescence intensity against the total amount of the dye injected to the cuvette were linear at low dye content and showed downward curvature as more dye was added (Figure S1). The maximum point in the linear region was taken as the solubility. The solubility of **FS1** and **1** in water is 5.0 μM.

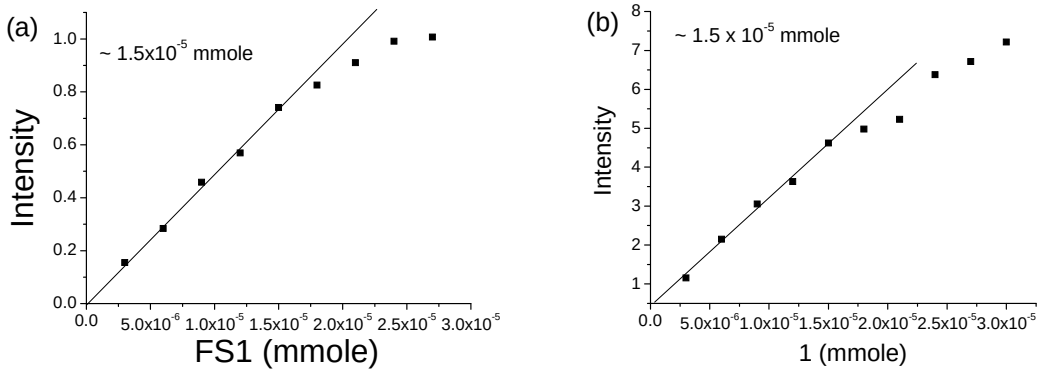


Figure S1. Plot of fluorescence intensity against the total amount of the dye for **FS1** (a) and **1** (b) in 3.0 mL HEPES buffer. The excitation wavelength was 363 nm.

Spectroscopic measurements. Absorption spectra were recorded on a Hewlett-Packard 8453 diode array spectrophotometer, and fluorescence spectra were obtained with Amico-Bowman series 2 luminescence spectrometer with a 1-cm standard quartz cell. The fluorescence quantum yield was determined by using Coumarin 307 and Rhodamine B as the reference by the literature method.[3] The spectral data obtained under various conditions are summarized in Figure S2 and Table S1.

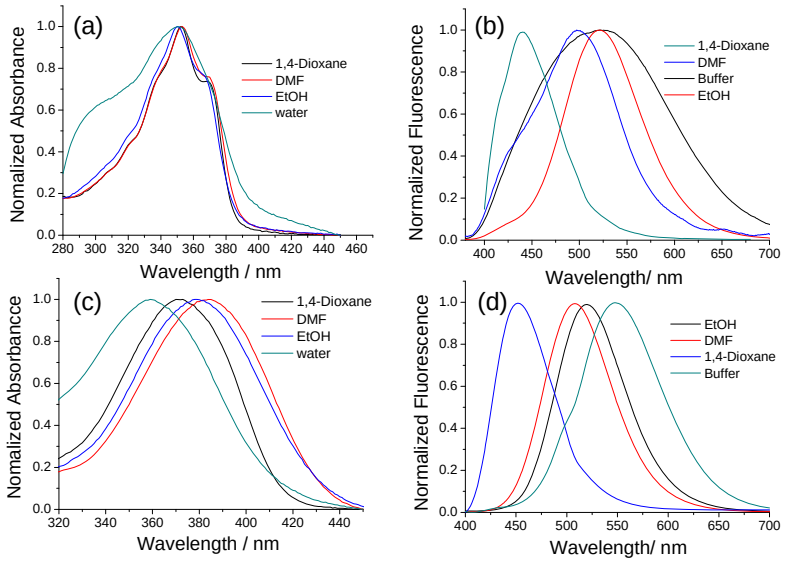


Figure S2. (a, c) Normalized absorption and (b, d) emission spectra of **FS1** (a, b) and **1** (c, d) in 1,4-dioxane, DMF, EtOH, and H_2O.

Table S1. Photophysical properties of **FS1** and **1** in various solvents

Solvent (E_T^N)[a]	$\lambda_{max}^{(1)}$ [b]		λ_{max}^{fl} [b]		Φ[c]	
	FS1	**1**	**FS1**	**1**	**FS1**	**1**
Dioxane (0.164)	353	373	440	453	5.5×10^{-2}	1.00
DMF (0.386)	353	384	498	507	3.2×10^{-2}	1.00
EtOH (0.654)	350	381	521	520	1.7×10^{-1}	0.87
H_2O (1.00)	352	363	529	548	2.5×10^{-2}	0.46

[a] The numbers in the parenthesis are normalized empirical parameter of solvent polarity.[4] [b] λ_{max} of the one-photon absorption and emission spectra in nm. [c] Fluorescence quantum yield. The uncertainty is ± 15 %.

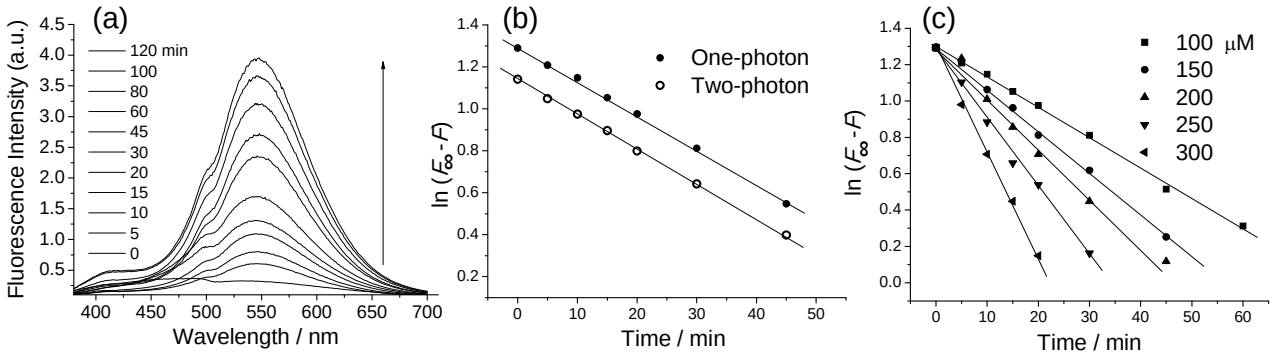


Figure S3. (a) The change of one-photon fluorescence intensity with time for the reaction of **FS1** (5 μM) with Na_2S (100 μM) in HEPES buffer. (b) Plots of $\ln(F_\infty - F)$ vs time for the reaction of **FS1** (5 μM) with Na_2S (100 μM) in HEPES buffer for the one- and two-photon processes, where F_∞ and F are the fluorescence intensity measured at infinity (> 10 half lives) and at a given time, respectively. (c) Plots of $\ln(F_\infty - F)$ vs time for the reaction of **FS1** (5 μM) with various Na_2S (100–300 μM) in HEPES buffer.

Product analysis. The reaction of **FS1** (5 μM) with Na_2S (300 μM) was carried out for 2 hr at 37°C in HEPES buffer (3 mL, pH = 7.2). The LC-MS traces of **FS1**, **1**, and the reaction are shown below (**Figure S4**). The result shows that **1** is the only product.

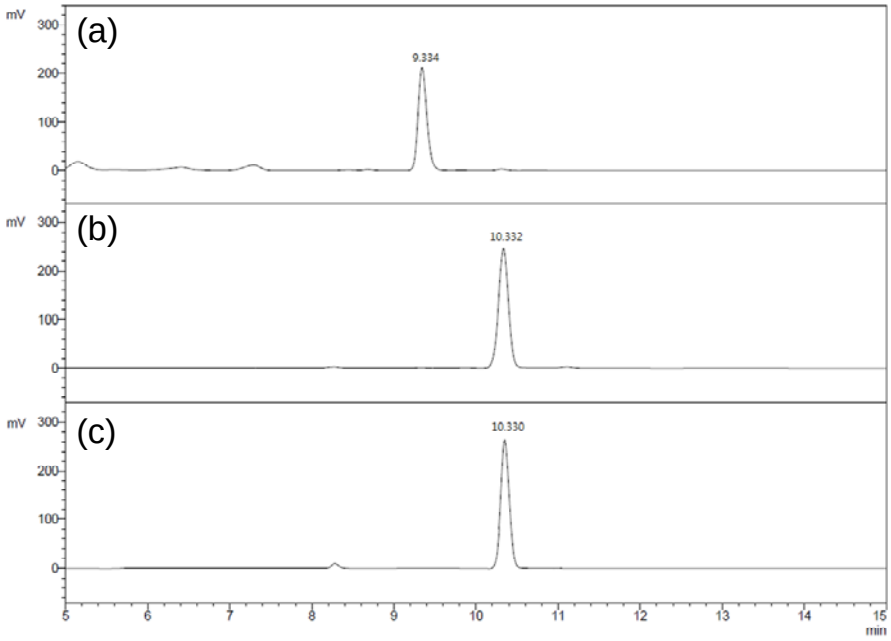


Figure S4. LC-MS traces of (a) **FS1**, (b) **1,** (c) the reaction product between **FS1** and Na_2S. Assigned peaks were confirmed with ESI^+. LC-MS Conditions: 5.0 mL/min flow rate, 10 % A to 100% A over 20 min, detected at 350 nm. Solvent A is water with 0.1% TFA and solvent B is acetonitrile.

Detection limit: The detection limit of **FS1** was determined as the concentration of Na_2S that resulted in a statistically significant increase in fluorescence intensity after 120 min with a p-value < 0.01 when compared with a blank control.

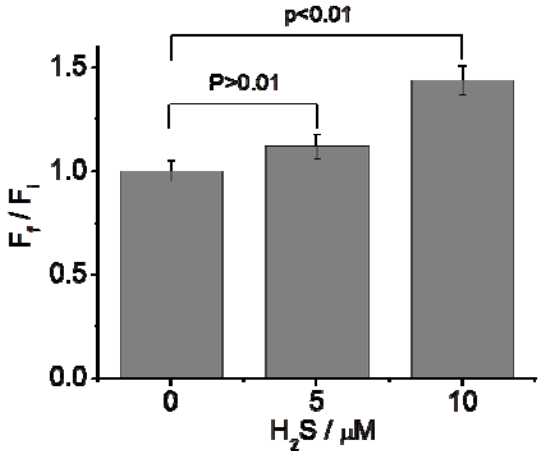


Figure S5. Fluorescence responses of 5 μM **FS1** to 0, 5, and 10 μM Na_2S after 120 min. Data were acquired at 37 ºC in HEPES buffer (pH 7.4) with excitation at λ_{ex} 363 nm. Statistical analyses were performed with a two-tailed Student's *t*-test (n = 3). Error bars are ± standard deviation.

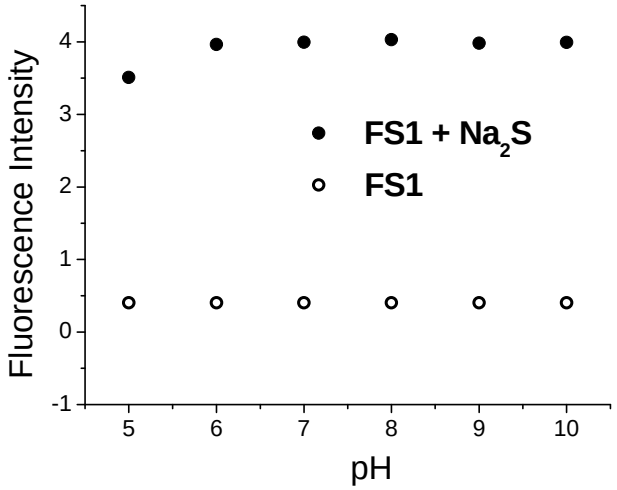


Figure S6. Effect of the pH on the one-photon fluorescence intensity of **FS1** (○) and the reaction product between **FS1** and Na_2S (●) in HEPES buffer. The reaction of **FS1** (5 μM) with Na_2S (100 μM) was carried out for 2 hr at 37°C in HEPES buffer (3 mL). The excitation wavelength was 356 nm.

Measurement of two-photon cross section. The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique as described.[5] **FS1** and **1** were dissolved in 20 mM HEPES buffer (pH 7.2) and ethanol at concentrations of 5.0×10^{-6} M and then the two-photon induced fluorescence intensity was measured at 740–940 nm by using Rhodamine 6G as the reference, whose two-photon property has been well characterized in the literature.[6] The intensities of

the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using $\delta = \delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s)$: where the subscripts *s* and *r* stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as *S*. Φ is the fluorescence quantum yield. ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as *c*. δ_r is the TPA cross section of the reference molecule.

Cell culture and imaging. HeLa human cervical carcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM (WelGene Inc, Seoul, Korea) supplemented with heat-inactivated 10% FBS (WelGene), penicillin (100 units/mL), and streptomycin (100 μg/mL). All the cell lines were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Two days before imaging, the cells were detached and were replated on glass-bottomed dishes (MatTek). For labeling, the growth medium was removed and replaced with DMEN without FBS. The cells were incubated with **FS1** (5 μM), or pretreated with Cysteine (100 μM), GSH (100 μM), or 2 μL (1 μg/mL) PMA for 30 min. Following this incubation, the cells were washed three times with DMEM without FBS and imaged.

Two-photon fluorescence microscopy. Two-photon fluorescence microscopy images of probe-labeled cells and tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP2) with a ×100 (NA = 1.30 OIL) and ×10 (NA = 0.30 DRY) objective lens, respectively. The two-photon fluorescence microscopy images were obtained with a DM IRE2 Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 750 nm and output power 1380 mW, which corresponded to approximately 10 mW average power in the focal plane. To obtain images at 400-680 nm range, internal PMTs were used to collect the signals in an 8 bit unsigned 1024 × 1024 pixels at 400 Hz scan speed.

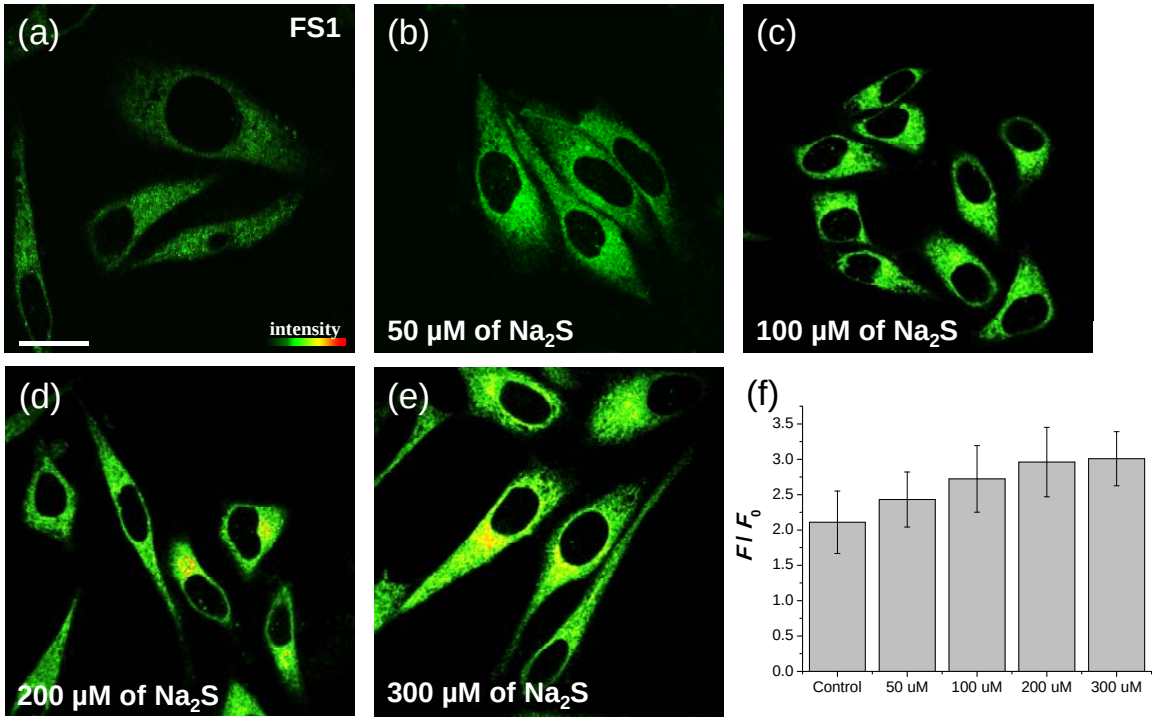


Figure S7. TPM images (a-e) of HeLa cells labeled with 5 μM **FS1** (a) for 30 min at 37°C. (b-e) TPM images of HeLa cells pretreated with Na_2S 50-300 μM. (f) The average and standard deviation of the two-photon excited fluorescence intensity from the cells (a-e). Two-photon excitation was provided at 750 nm with femto-second pulses. Cells shown are representative images from replicate experiments (n = 5). The TPEF were collected at 400-680 nm. Scale bar, 30 μm.

Cell viability. To confirm that the tracker couldn't affect the viability of HeLa cells in our incubation condition, we used CCK-8 kit (Cell Counting Kit-8, Dojindo, Japan) according to the manufacture's protocol. The results are shown in **Figure S7.**

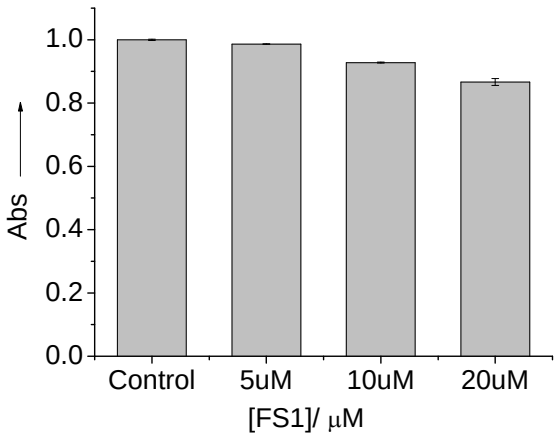


Figure S8. Viability of HeLa cells in the presence of **FS1** as measured by using CCK-8 kit. The cells were incubated with 5–20 μM **FS1** for 1 h.

Preparation and staining of fresh rat Hippocampal slices. Slices were prepared from the hippocampi of 2-weeks-old rat (SD). Coronal slices were cut into 400 μm-thick using a vibrating-blade microtome in artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10 mM D-glucose, 2.4 mM $CaCl_2$, and 1.3 mM $MgSO_4$). Slices were incubated with 20 μM **FS1** in ACSF bubbled with 95% O_2 and 5% CO_2 for 2 hr at 37 °C. Slices were then washed three times with ACSF and transferred to glass-bottomed dishes (MatTek) and observed in a spectral confocal multiphoton microscope. To assess the effect of Na_2S, glutathione (GSH), cysteine (Cys) and phorbol myristate acetate (PMA), the slices were treated with 1 mM of Na_2S, GSH, Cys and 10 μL of PMA (1 μg/mL) for 50 min before **FS1** was added.

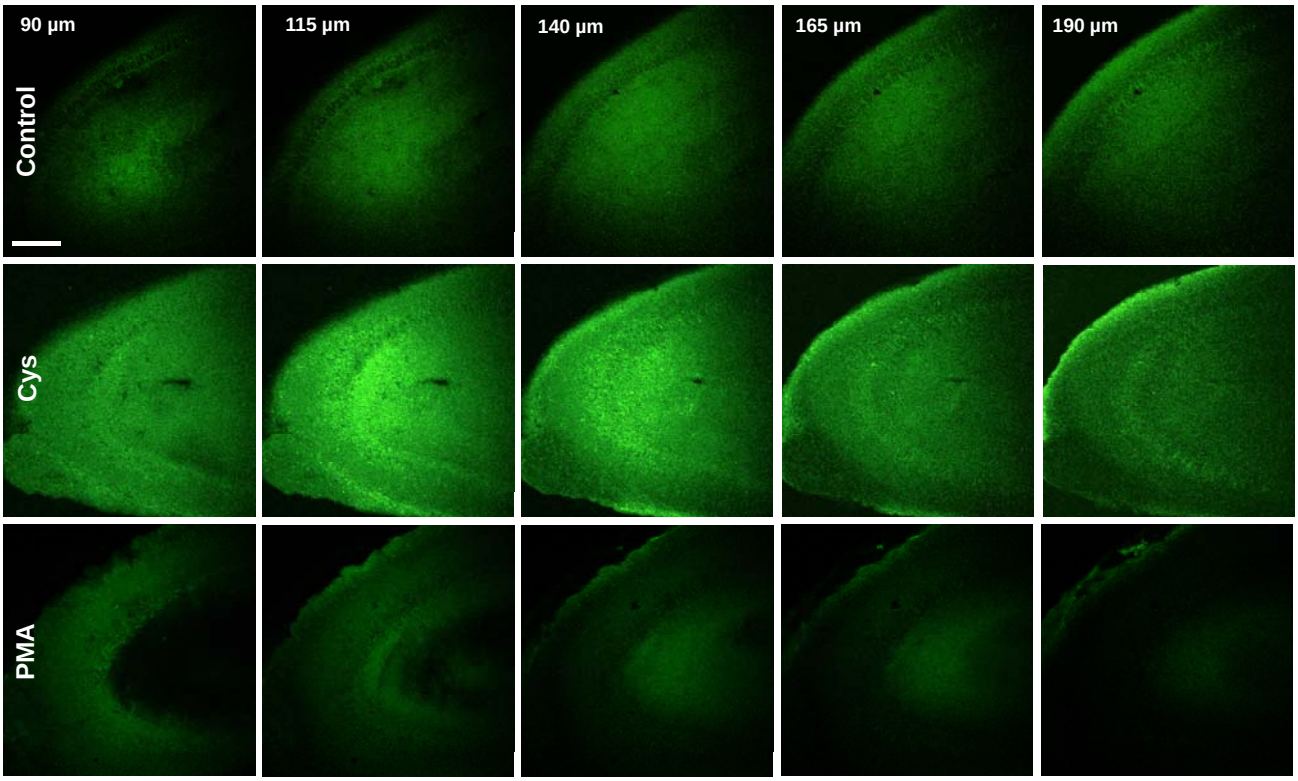


Figure S9. TPM images a fresh rat hippocampal slice stained with 20 μM **FS1**. 10 TPM images were taken at depths of ~90-190 μm with magnification at 10×. The images shown are representative images. The TPEF were collected at 400-680 nm upon excitation at 750 nm with fs pulses. Scale bar, 300 μm.

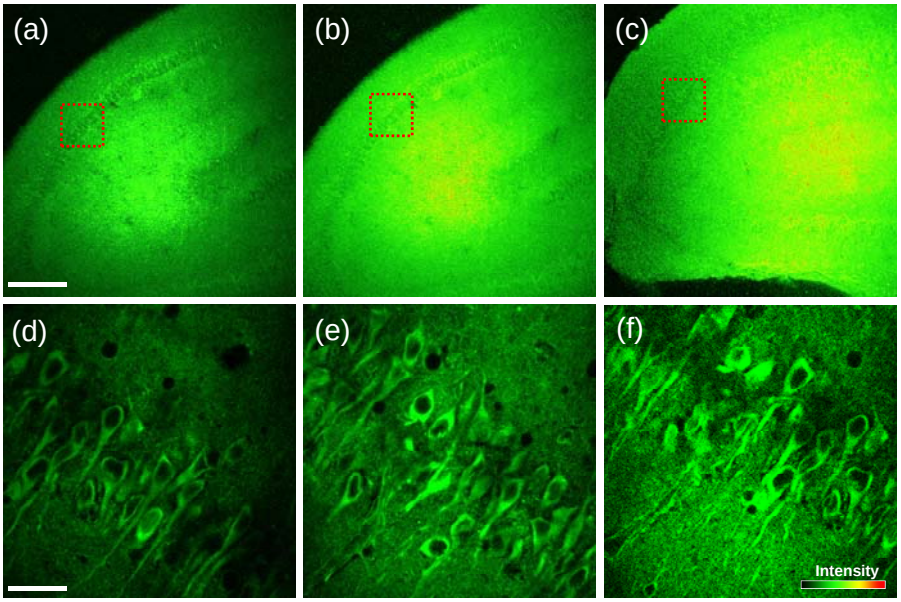


Figure S10. TPM images of a rat hippocampal slice stained with 20 μM **FS1** for 2 h which were (a) not treated and pretreated with (b) Na_2S (1 mM) and (c) GSH (1 mM) for 50 min. Ten TPM images were accumulated along the z direction at depths of 90–190 μm with 10x magnification. (d-f) Enlarged images of the red boxes in (a-c) at a depth of 120 μm with 100x magnification. The TP fluorescence emission was collected at 400-680 nm upon excitation at 750 nm with a femtosecond pulse. Scale bars: 300 μm.

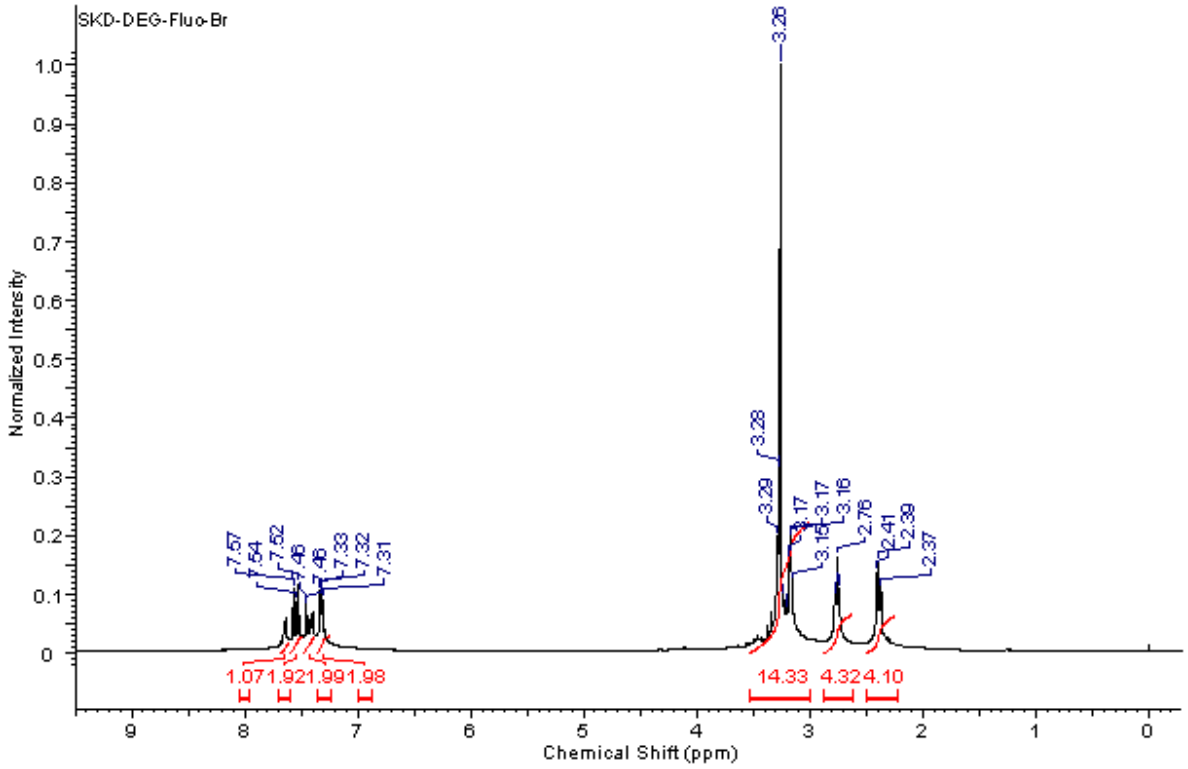


Figure S11. ^1H NMR (400 MHz, $CDCl_3$) of compound **3**

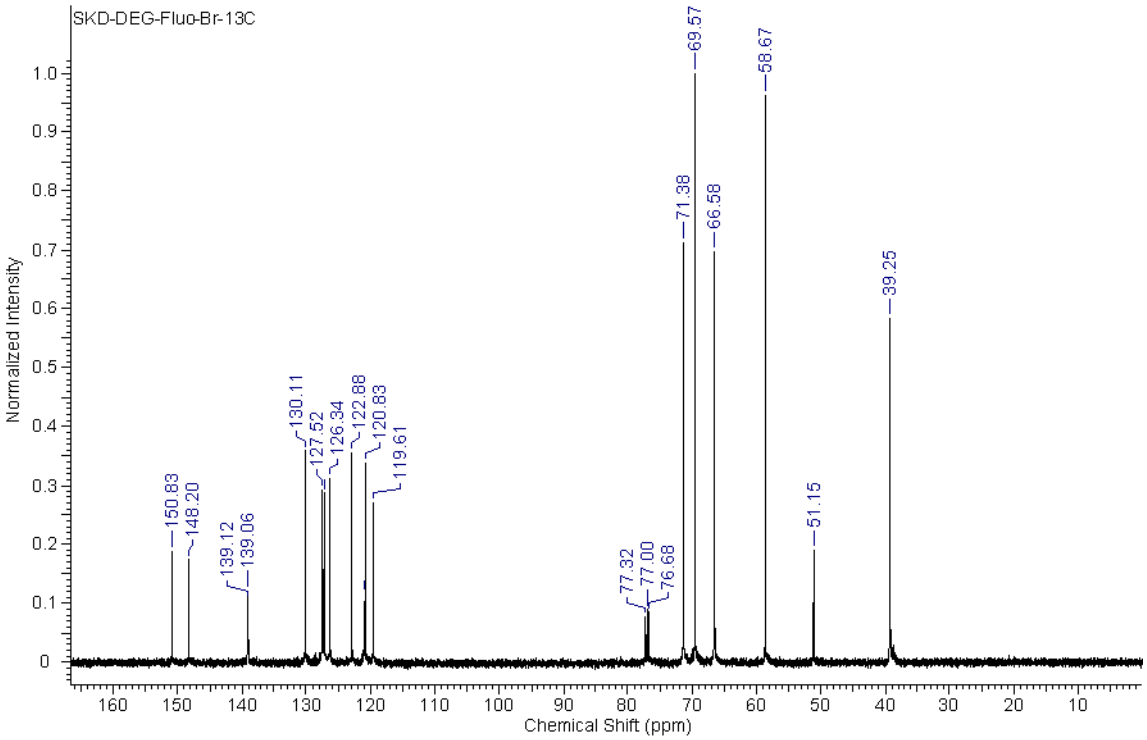


Figure S12. ^{13}C NMR (100 MHz, $CDCl_3$) of compound **3**

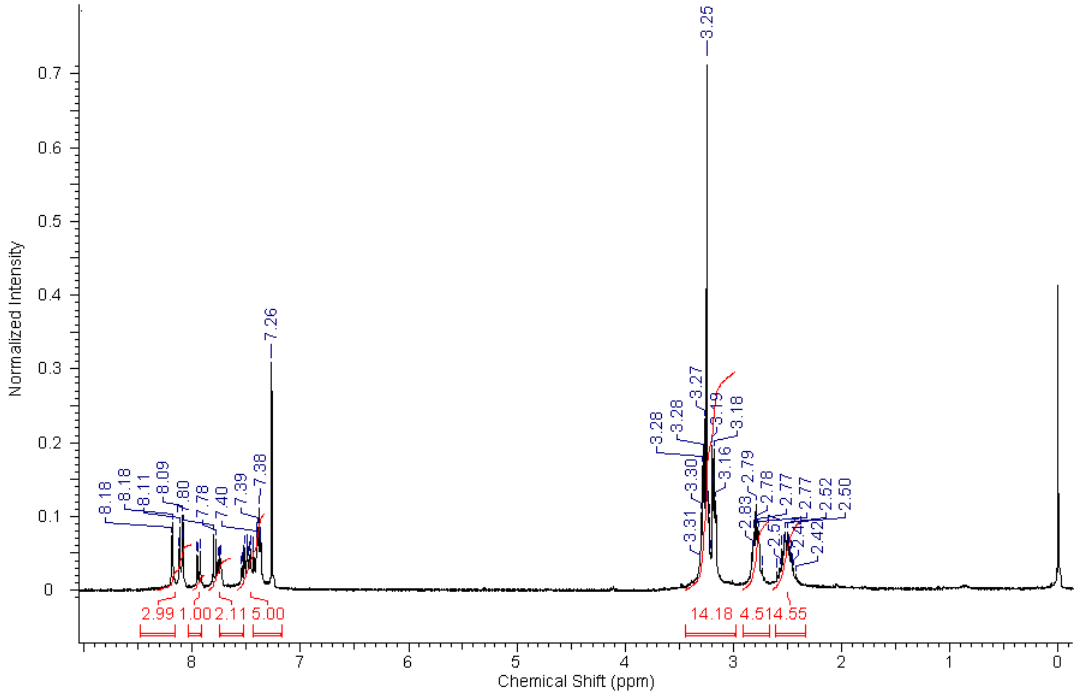


Figure S13. ^{1}H NMR (300 MHz, $CDCl_3$) of compound **4**

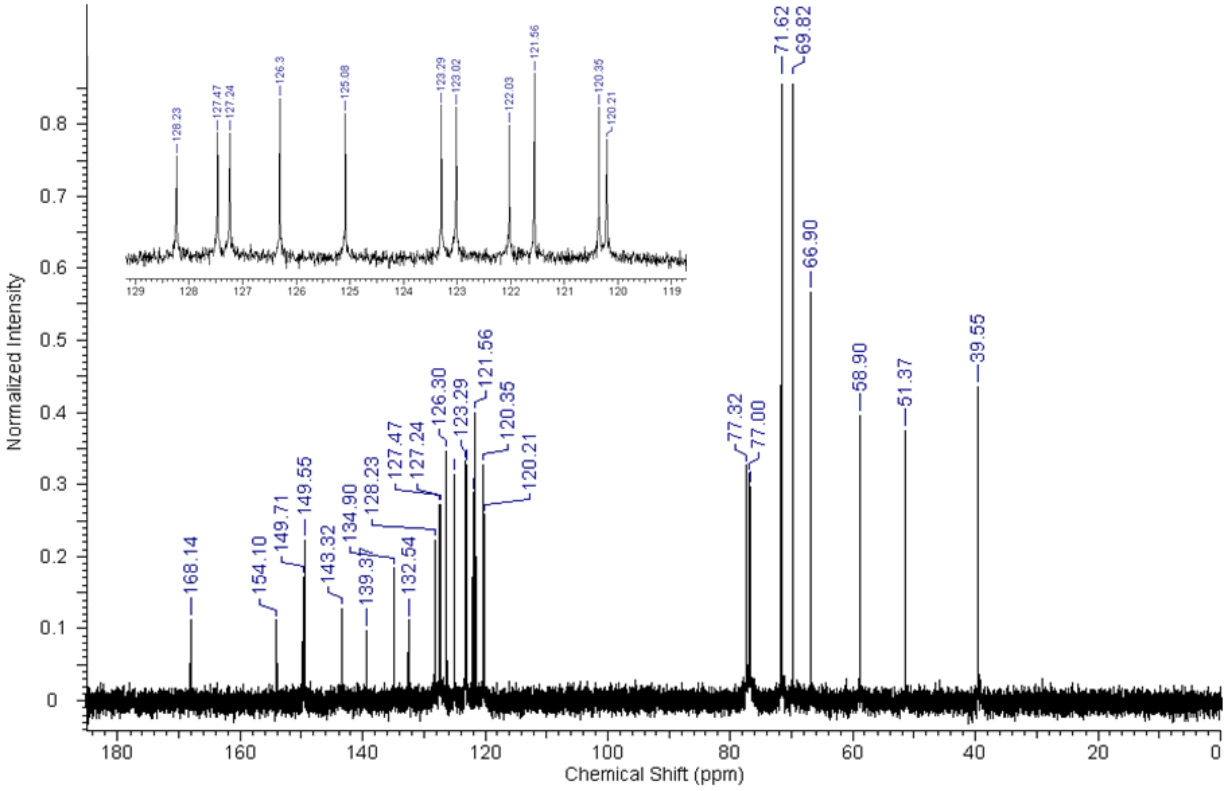


Figure S14. ^{13}C NMR (100 MHz, $CDCl_3$) of compound **4**

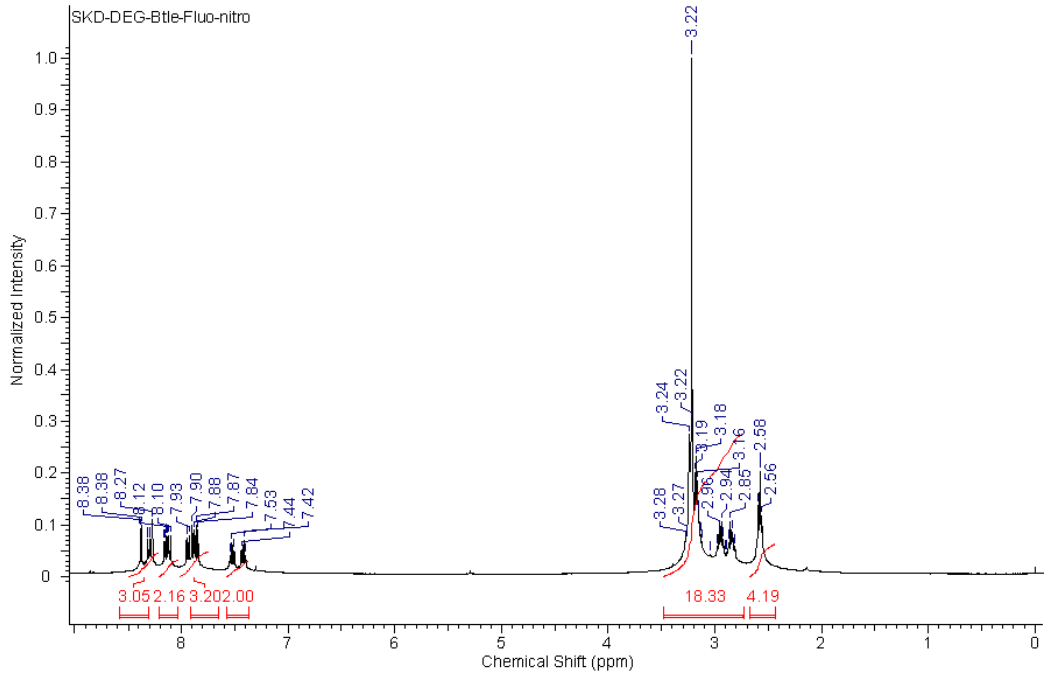


Figure S15. ^{1}H NMR (400 MHz, $CDCl_3$) of compound **A**

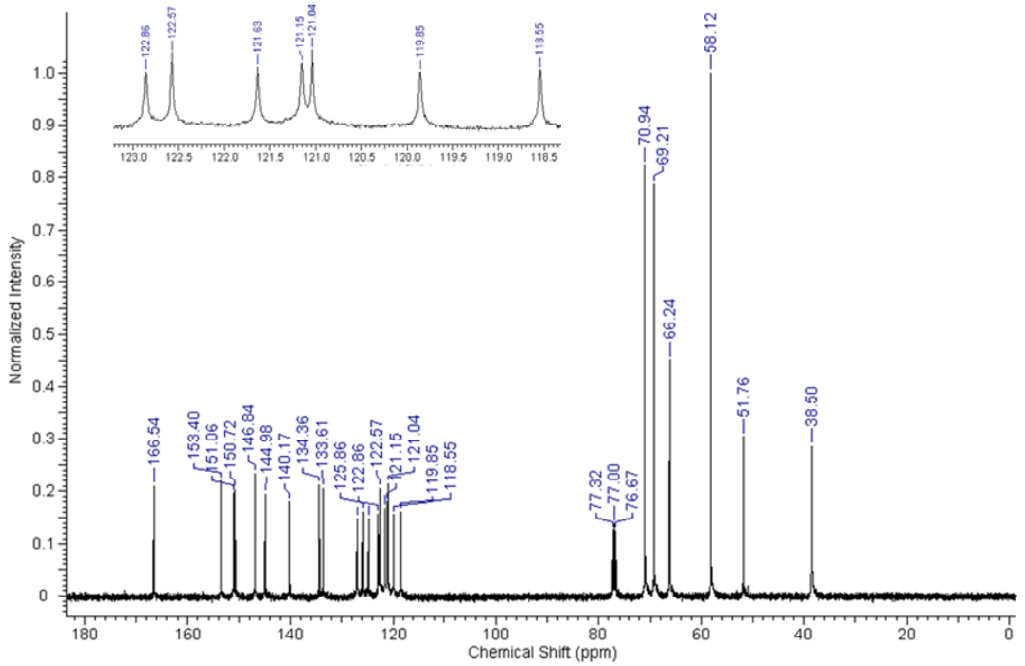


Figure S16. ^{13}C NMR (100 MHz, $CDCl_3$) of compound **A**

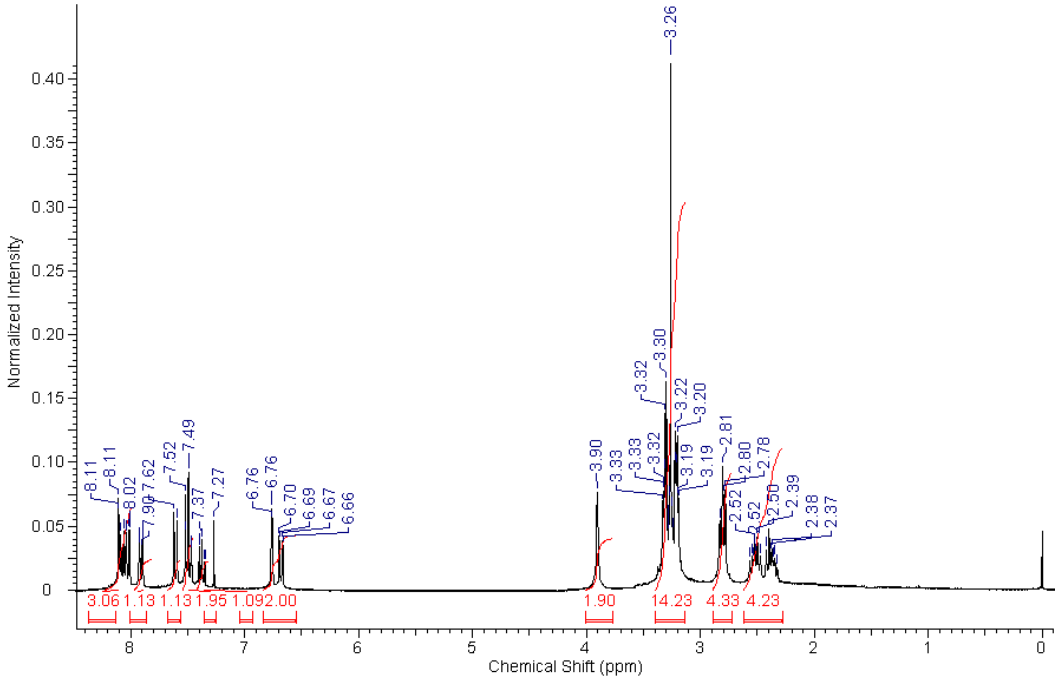


Figure S17. ^{1}H NMR (300 MHz, $CDCl_3$) of compound **1**

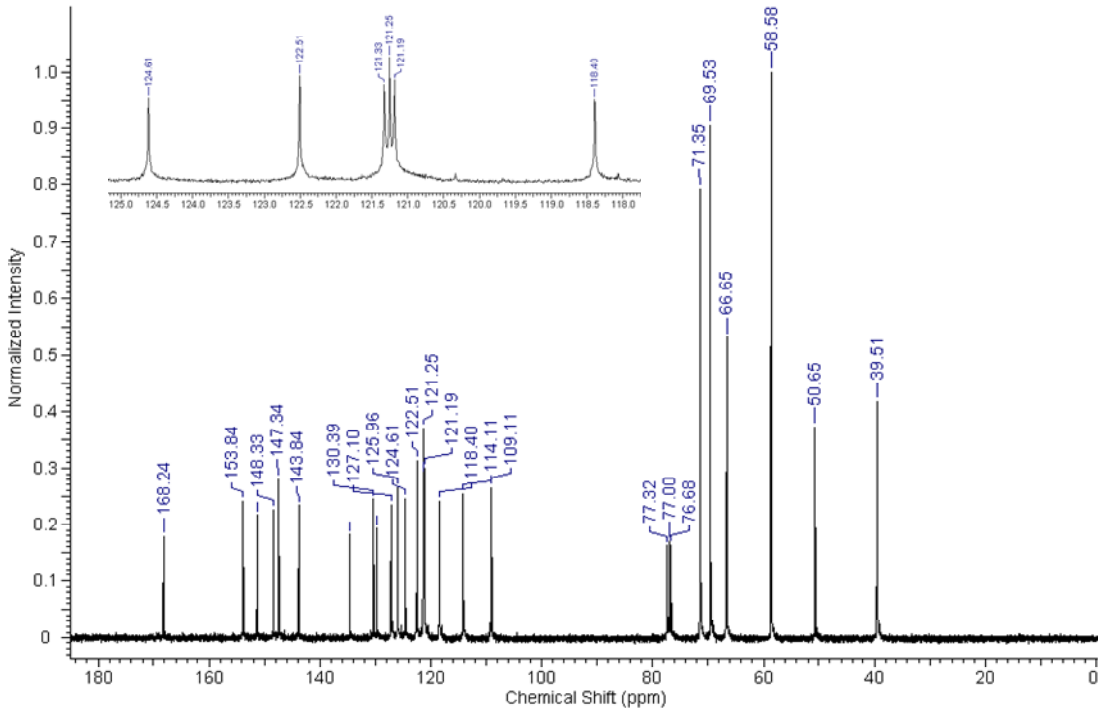


Figure S18. ^{13}C NMR (100 MHz, $CDCl_3$) of compound **1**

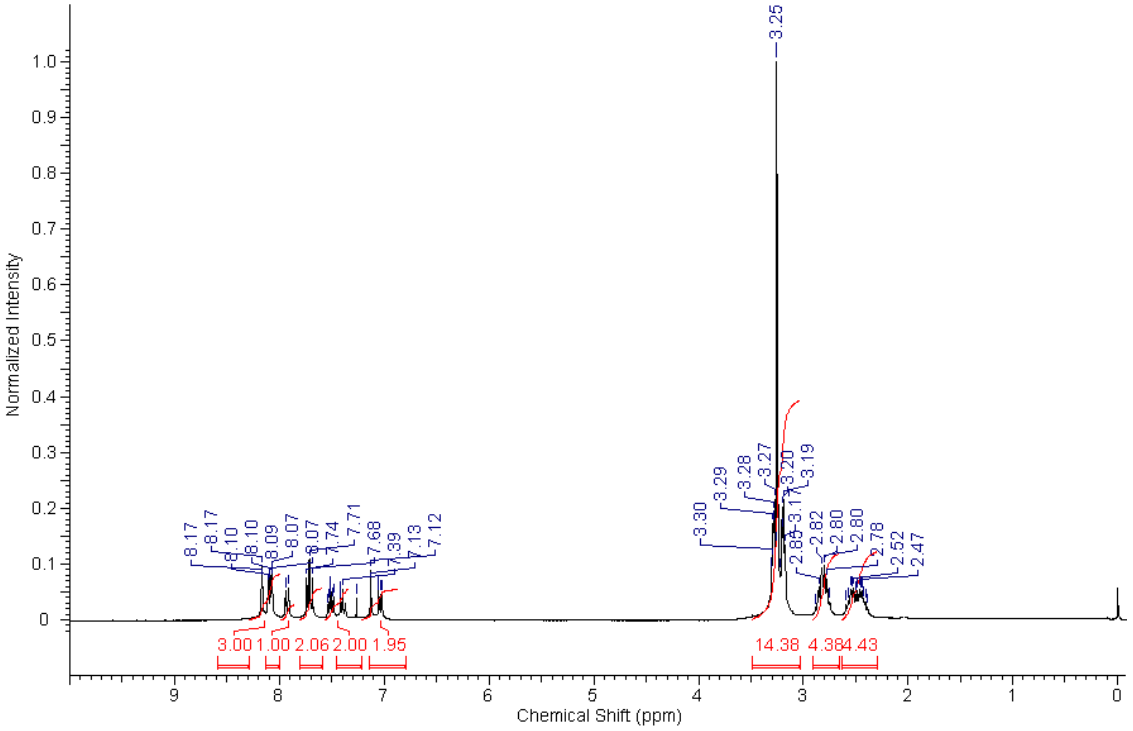


Figure S19. ^{1}H NMR (300 MHz, $CDCl_3$) of compound **FS1**

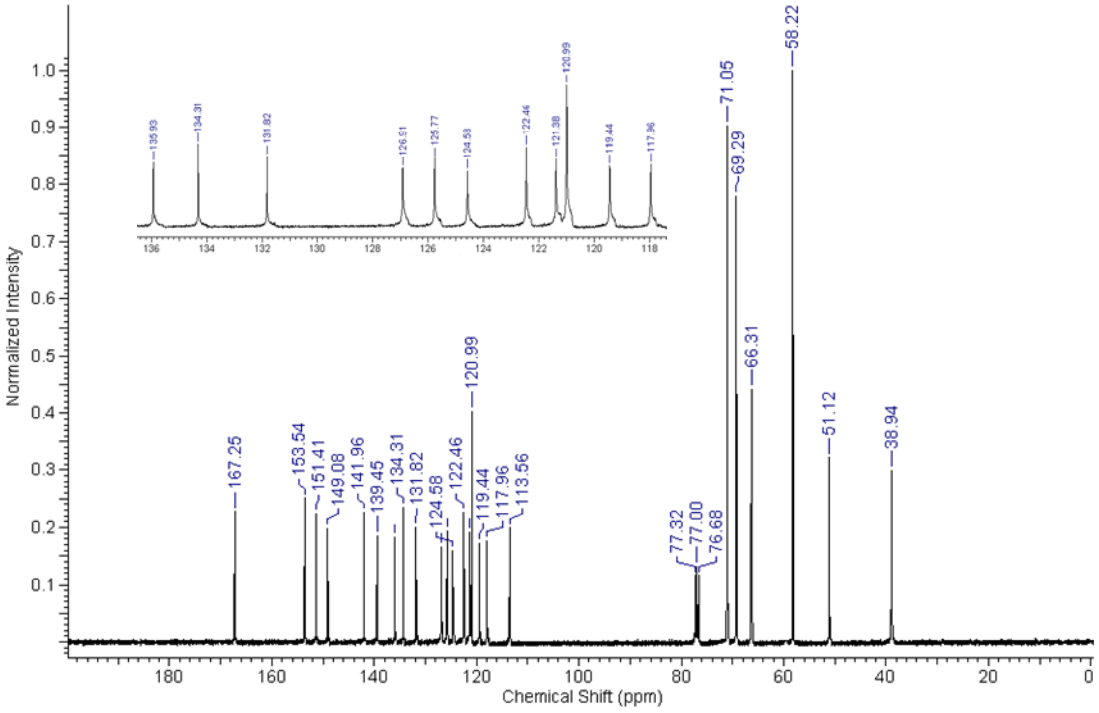


Figure S20. ^{13}C NMR (100 MHz, $CDCl_3$) of compound **FS1**

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