Supporting Information

Fluorogenic Hydrogen Sulfide (H_2S) Donors Based on Sulfenyl Thiocarbonates Enable H_2S Tracking and Quantification

Yu Zhao, Matthew M. Cerda, and Michael D. Pluth*

Department of Chemistry and Biochemistry
Institute of Molecular Biology
Materials Science Institute
University of Oregon
Eugene, OR 97403, USA

Contact Information:

Michael D. Pluth: pluth@uoregon.edu

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EXPERIMENTAL DETAILS

Methods and Materials

Reagents were purchased from Sigma-Aldrich, Tokyo Chemical Industry (TCI), Fisher Scientific, Combi-Blocks, and VWR and used directly as received. Silica gel (SiliaFlash F60, Silicycle, 230−400 mesh) was used for column chromatography. Deuterated solvents were purchased from Cambridge Isotope Laboratories (Tewksbury, Massachusetts, USA). ^{1}H and ^{13}C NMR spectra were recorded on Bruker 500 MHz NMR instruments at the indicated frequencies. Chemical shifts are reported in ppm relative to residual protic solvent resonances. Mass spectrometric measurements were performed by the University of Illinois, Urbana Champaign MS facility, or on a Xevo Waters ESI LC/MS instrument. Fluorescein intensity was measured using a Quanta Master 40 spectrofluorometer (Photon Technology International) and methylene blue absorbance was monitored by a Cary 60 UV-Vis spectrometer. Compounds 1, 2, and C-Az were synthesized by following the literature reports.[1-2] HeLa cells and RAW 264.7 cells were purchased from ATCC (Manassas, Virginia, USA). Cell imaging experiments were performed on a Leica DMi8 fluorescence microscope, equipped with an Andor Zyla 4.2+ sCMOS detector. NO_2^- levels were obtained by using a Griess Reagent kit (Thermo Fisher Scientific) and the absorbance at 548 nm was measured by using a microplate reader (Tecan Spark 20M).

Synthesis

General procedure for the synthesis of FLD donors. The fluorescein starting material (1.00 equiv.) was added to $CHCl_3$ containing **1** or **2** (3.00 equiv.). After stirring the reaction mixture at 0 ºC for 5 min, DIPEA (3.00 equiv.) was added slowly. The reaction solution was stirred at r.t. until the completion of the reaction as indicated by TLC (usually less than 2 h). The reaction was then quenched by adding brine (25 mL), and the aqueous solution was extracted with ethyl acetate (3 × 15 mL). The organic layers were combined, dried over $MgSO_4$, and evaporated under vacuum. The product was isolated after purification by column chromatography.

FLD-1 was prepared by reacting fluorescein (332 mg, 1.00 mmol) with **1** (654 mg, 3.00 mmol) in the presence of DIPEA (390 mg, 3.00 mmol) using the general synthetic procedure described above. **FLD-1** was isolated as yellow solid by column chromatography using ethyl acetate/hexanes (1/3, v/v, R_f = 0.31) as the eluent (390 mg, 56% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.09 (d, J = 5.0 Hz, 1H), 7.85 (t, J = 5.0 Hz, 1H), 7.79 (t, J = 5.0 Hz, 1H), 7.38 (m, 13H), 7.01 (d, J = 10.0 Hz, 2H), 6.95 (d, J = 10.0 Hz, 2H), 4.18 (s, 4H). $^{13}C\{^{1}H\}$ NMR (125 MHz, DMSO-d_6) δ (ppm): 168.8, 167.8, 152.6, 151.2, 136.5, 131.1, 130.1, 130.0, 129.0, 128.2, 125.7, 125.6, 124.6, 118.4, 117.5, 110.6, 81.1, 42.4. IR (cm^{-1}): 2981, 1744, 1608, 1408, 1420, 1237, 1143, 1107, 1060, 988, 881, 751. HRMS m/z $[M + H]^+$ calcd. For $[C_{36}H_{25}O_7S_4]^+$ 697.0483; found 697.0474.

FLD-2 was prepared by reacting fluorescein (93.0 mg, 0.280 mmol) with **2** (171 mg, 0.840 mmol) in the presence of DIPEA (109 mg, 0.840 mmol) using the general synthetic procedure described above. **FLD-2** was isolated as white solid by column chromatography using ethyl acetate/hexanes (1/2, v/v, R_f = 0.49) as the eluent (105 mg, 56% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.08 (d, J = 5.0 Hz, 1H), 7.83 (t, J = 5.0 Hz, 1H), 7.78 (t, J = 5.0 Hz, 1H), 7.65 (d, J = 10.0 Hz, 4H), 7.55 (s, 2H), 7.43 (m, 7H), 7.14 (d, J = 5.0 Hz, 2H), 6.96 (d, J = 5.0 Hz, 2H). $^{13}C\{^{1}H\}$ NMR (125 MHz, DMSO-d_6) δ (ppm): 168.8, 167.7, 152.6, 151.2, 136.6, 131.1, 130.4, 130.1, 130.0, 129.5, 125.7, 125.6, 124.6, 118.5, 117.7, 110.8, 81.0. IR (cm^{-1}): 3057, 2923, 1747, 1607, 1581,

1489, 1419, 1327, 1285, 1235, 1219, 1142, 1107, 1061, 881, 685. HRMS m/z $[M + H]^+$ calcd. For $[C_{34}H_{21}O_7S_4]^+$ 669.0170; found 669.0173.

FLD-3 was prepared by reacting 3-*O*-methylfluorescein (69.0 mg, 0.207 mmol) with **1** (136 mg, 0.623 mmol) in the presence of DIPEA (81.0 mg, 0.623 mmol) using the general synthetic procedure described above. **FLD-3** was isolated as white solid by column chromatography using ethyl acetate/hexanes (1/1, v/v, $R_f = 0.64$) as the eluent (43.0 mg, 41% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.06 (d, J = 10.0 Hz, 1H), 7.83 (t, J = 5.0 Hz, 1H), 7.77 (t, J = 5.0 Hz, 1H), 7.36 (m, 7H), 6.98 (d, J = 10.0 Hz, 2H), 6.91 (d, J = 10.0 Hz, 2H), 6.77 (d, J = 10.0 Hz, 1H), 6.73 (d, J = 10.0 Hz, 1H), 4.18 (s, 2H), 3.84 (s, 3H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 168.9, 167.8, 161.7, 152.7, 152.4, 151.9, 151.5, 136.5, 13 6.4, 130.9, 130.1, 130.0, 129.5, 129.0, 128.2, 126.1, 125.4, 124.5, 118.0, 117.8, 113.0, 110.9, 110.4, 101.3, 81.9, 56.2, 42.4. IR (cm^{-1}): 2981, 1747, 1607, 1491, 1420, 1241, 1220, 1144, 1103, 1060, 986, 874. HRMS m/z $[M + H]^+$ calcd. For $[C_{29}H_{21}O_6S_2]^+$ 529.0780; found 529.0779.

FLD-4 was prepared by reacting fluorescein (166 mg, 0.500 mmol) with **1** (22.0 mg, 0.100 mmol) in the presence of DIPEA (13.0 mg, 0.100 mmol) using the general synthetic procedure described above. **FLD-4** was isolated as yellow solid by column chromatography using ethyl acetate/hexanes (1/1, v/v, $R_f = 0.52$) as the eluent (8.00 mg, 16% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm): 10.23 (s, 1H), 8.04 (d, J = 10.0 Hz, 1H), 7.83 (t, J = 5.0 Hz, 1H), 7.76 (t, J = 5.0 Hz, 1H), 7.35 (m, 7H), 6.95 (d, J = 10.0 Hz, 1H), 6.87 (d, J = 10.0 Hz, 1H), 6.74 (s, 1H), 6.62 (s, 2H), 4.18 (s, 2H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6) δ (ppm): 169.0, 167.8, 160.2, 152.7, 152.4, 151.9, 151.6, 136.5, 136.3, 130.9, 130.1, 129.9, 129.6, 129.0, 128.2, 126.2, 125.3, 124.5, 118.0, 117.8, 113.7, 110.5, 109.5, 102.7, 82.2, 42.4. IR (cm^{-1}): 3057, 2923, 1747, 1607, 1581, 1489, 1419, 1285, 1219, 1142, 1107, 1061. HRMS m/z $[M + H]^+$ calcd. For $[C_{28}H_{19}O_6S_2]^+$ 515.0623; found 515.0620.

TCN-1. The fluorescein starting material (33.0 mg, 0.100 mmol) was combined with triethyl amine (40.0 mg, 0.400 mmol) in anhydrous THF. After stirring the reaction mixture at 0 ºC for 10 min, benzyl chlorothioformate (75.0 mg, 0.400 mmol) was added slowly. The reaction solution was stirred at r.t. for 2 h. The reaction was then quenched by adding brine (25 mL), and the aqueous solution was extracted with ethyl acetate (3 × 15 mL). The organic layers were combined, dried over $MgSO_4$, and evaporated under vacuum. **TCN-1** was isolated as white solid by column chromatography using ethyl acetate/hexanes (1/2, v/v, $R_f = 0.50$) as the eluent (57.0 mg, 91% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 8.07 (d, J = 5.0 Hz, 1H), 7.68 (m, 2H), 7.38 (m, 8H), 7.31 (m, 2H), 7.19 (m, 3H), 6.88 (m, 4H), 4.22 (s, 4H). ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$) δ (ppm): 169.5, 169.0, 152.9, 152.4, 151.5, 136.4, 135.3, 130.1, 129.1, 129.0, 128.8, 127.8, 126.0, 125.3, 124.0, 117.4, 116.8, 110.2, 81.4, 35.8. IR (cm^{-1}): 2981, 1763, 1716, 1607, 1490, 1419, 1237, 1144, 1062, 989, 752, 687. HRMS m/z $[M + H]^+$ calcd. For $[C_{36}H_{25}O_7S_2]^+$ 633.1042; found 633.1050.

Measurement of Fluorescence Intensity of FLD Donors

A freshly prepared **FLD-1** stock solution (3.00 µL, 10.0 mM in DMSO) was added to 3.00 mL of PBS (Ph 7.40, 10.0 Mm) containing CA (25.0 µg/mL) in a quartz fluorescence cuvette. A Cys stock solution (10.0 mM) was then added to reach the desired working concentration shown in Figure 2. The reaction solution was excited at 490 nm and the fluorescence intensity (500 – 650 nm) was measured and recorded using a Quanta Master 40 spectrofluorometer. The fluorescence intensity of other donors (**FLD-2** – **4**) and the control compound (**TCN-1**) was measured and recorded by following the similar procedure as mentioned above (Figures 3a and 4).

Measurement of H_2S Release from FLD-1 or FLD-3 by MB Assay.

A **FLD-1** stock solution (20.0 μL, 10.0 mM in DMSO) was added to 20.0 mL of PBS (pH 7.40, 10.0 mM) containing CA (25.0 μg/mL) in a 20-mL scintillation vial. A Cys stock solution (20.0 μL, 100 mM) was then added. Next, 300 μL aliquots of the reaction mixture were transferred to UV cuvettes containing 300 μL of MB cocktail (60.0 μL $Zn(OAc)_2$ (1.00% w/v), 120 μL $FeCl_3$ (30.0 mM in 1.20 M HCl), and 120 μL *N,N*-dimethyl-*p*-phenylene diamine (20.0 mM in 7.20 M HCl)) at different time points. The absorbance at 670 nm was then measured after 1 hour and was converted to H_2S concentration by using an H_2S calibration curve (Figure S2). H_2S Release from **FLD-3** was measured by following the same procedure as mentioned above in PBS (pH 7.40, 10.0 mM) in the presence or absence of CA (25.0 μg/mL) (Figure 3a).

Selectivity of FLD-1 to Cellular RSONs.

To 3.00 mL of PBS was added a stock solution of **FLD-1** (3.00 μL, 10.0 mM in DMSO), followed by the addition of RSON stock solution (30.0 μL, 10.0 mM in H_2O) shown in Figure 5. After 2 hours of incubation at r.t., the solution was excited at 490 nm and the fluorescence intensity (500 – 650 nm) was measured and recorded by using a Quanta Master 40 spectrofluorometer. For the NEM-pretreated group, NEM (30.0 μL, 1.00 M) was added to PBS (3.00 mL) containing CA (25.0 μg/mL) and Cys (100 μM). The solution was then incubated for 1 h before adding the **FLD-1** stock solution (3.00 μL, 10.0 mM in DMSO).

Cell Culture and Cellular Imaging of H_2S Delivery from FLD-1

HeLa cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C under 5% CO_2. HeLa cells were then plated in poly-D-lysine coated plates (MatTek) containing 2.00 mL of DMEM and incubated at 37 ºC under 5% CO_2 for 24 h. The confluent cells were washed with PBS and then co-incubated with NucRed nuclear dye (2 drops), C7-Az (50.0 μM), and **FLD-1** (or **TCN-1**) (50.0 μM) for 30 min. Prior to imaging, the cells were washed with PBS and bathed in 2.00 mL of PBS. Cell imaging was performed on a Leica DMi8 fluorescent microscope.

Anti-Inflammatory Activities of FLD-1 and Control Compounds

Macrophage RAW 264.7 cells were seeded in a 24-well plate (5 × 105 cells/well) containing 0.500 mL of DMEM and incubated at 37 ºC under 5% CO_2 for 24 h. The confluent cells were washed with PBS and incubated with either **FLD-1** (0 – 25.0 μM), or GYY4137 (25.0 μM) at 37 ºC for 2 h. Compounds were then removed by washing cells with PBS and these pretreated cells were incubated in FBS-free DMEM containing LPS (0.500 μg/mL) for 24 h. NO_2^- levels were measured by using a Griess Reagent Kit.

Table S1. Spectroscopic Properties of **FLD-1** – **4** and **TCN-1** in PBS (pH 7.4, 10 mM).

Compound	λ_{max} (nm)	ε ($M^{-1}cm^{-1}$)	λ_{em} (nm)	Φ
FLD-1	N/A	N/A	N/A	N/A
FLD-2	N/A	N/A	N/A	N/A
FLD-3	N/A	N/A	N/A	N/A
FLD-4	449	$27{,}300 \pm 2500$	514	0.11 ± 0.01
TCN-1	N/A	N/A	N/A	N/A

Cys-Triggered FLD-1 Activation

A freshly prepared **FLD-1** stock solution (3.00 µL, 10.0 mM in DMSO) was added to 3.00 mL of PBS (pH 7.40, 10.0 mM) containing CA (25.0 µg/mL) in a quartz UV cuvette. A Cys stock solution (30.0 µL, 10.0 mM in H_2O) was then added. The absorbance (350 – 600 nm) was measured for 3 h using a Cary 100 spectrometer.

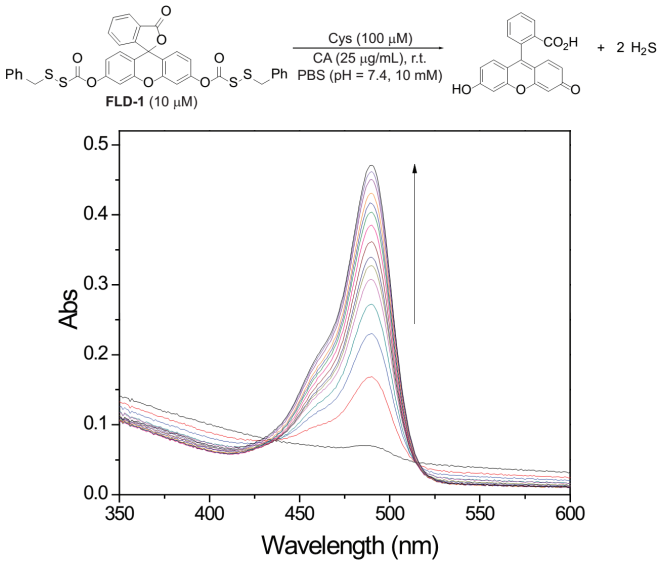


Figure S1. Time-dependent UV-Vis spectra of **FLD-1** (10 µM) in PBS (pH 7.4, 10 mM) containing Cys (100 µM) and CA (25 µg/mL).

Cys-Triggered H_2S Release from FLD-1

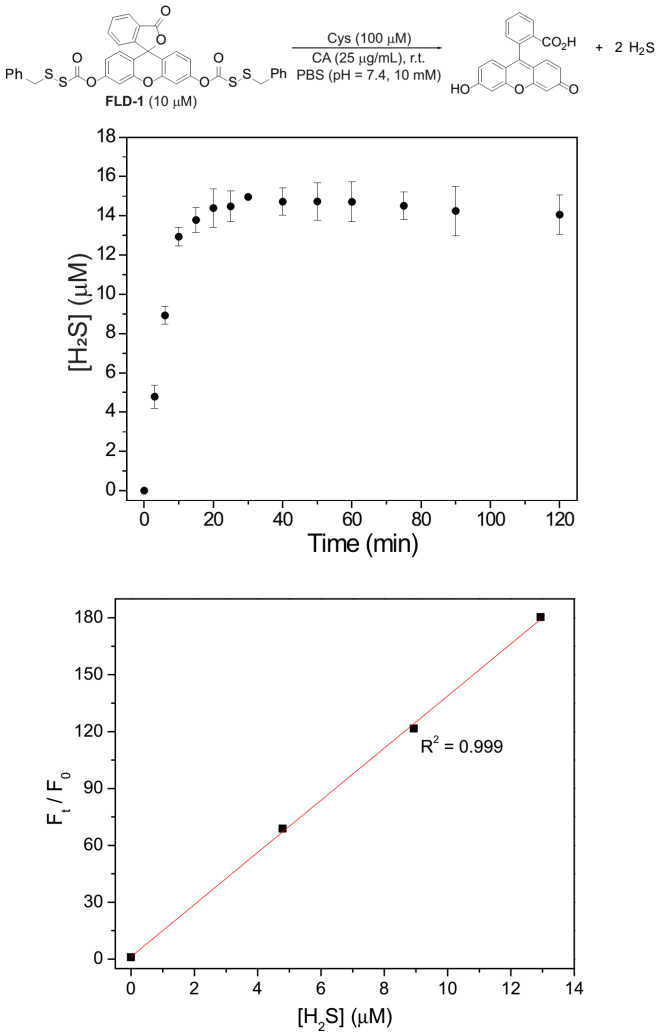
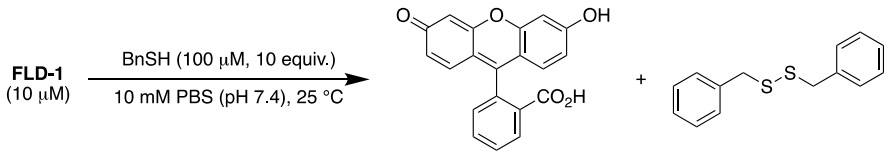


Figure S2. (Top) MB Measurement of H_2S release from **FLD-1** (10 μM) upon Cys (100 μM) activation. (Bottom) Correlation between fluorescence measurement of **FLD-1** in Figure 1B and MB detection in the first 10-min reaction period. The experiments were performed in triplicate. The results are expressed as mean ± SD (n = 3)

Reaction Product Analysis by HPLC



To a 3.00 mL PBS (pH 7.4, 10 mM) containing benzyl mercaptan (100 μM), 3.00 μL of **FLD-1** (10 mM in THF) was added and stirred at room temperature. After 1 h, a 1 mL reaction aliquot was analyzed by HPLC.

HPLC analysis was performed on an Agilent 1260 HPLC instrument with a Poroshell 120 EC-C18 4.6x100 mm column and monitored absorption at 230 nm.

HPLC Method: Solvent A: 95% H_2O, 5% MeOH, Solvent B: 100% MeCN. Gradient: 35% Solvent A/65% Solvent B for 2 min. Change to 100% Solvent B over 4 min and hold for 6.5 min. Change to 35% Solvent A/65% Solvent B over 0.5 min and hold for 4.5 min. Flow Rate: 0.5 mL/min, 2 μL injection, unless stated otherwise.

To confirm the formation of expected reaction products and confirm observed peaks, authentic samples of 20 μM benzyl disulfide, 10 μM **FLD-1**, and 100 μM benzyl mercaptan were prepared in 10 mM PBS (pH 7.4) containing 0.1-1.0% THF and analyzed as described above. Due to the low absorption of **FLD-1** at 230 nm, injection volume was increased to 8.0 μL. Due to poor solubility in THF, an authentic sample of 10 μM fluorescein was prepared in 10 mM PBS (pH 7.4) containing 0.1% DMSO.

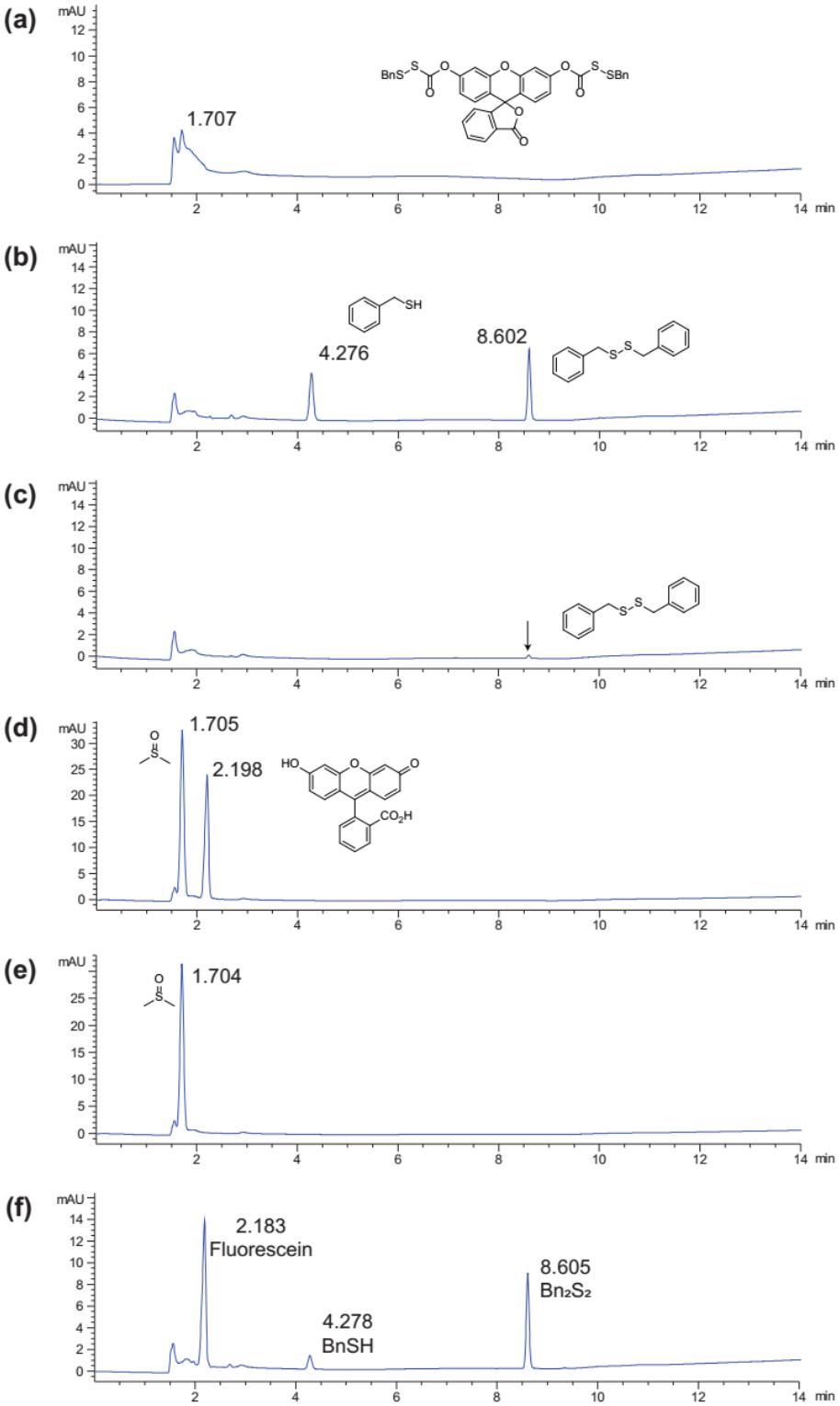
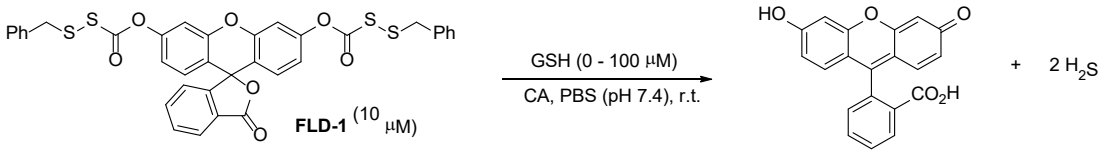


Figure S3. Reaction analysis by HPLC. (a) 10 μM **FLD-1** (b) 100 μM BnSH containing Bn_2S_2 due to aerobic oxidation (c) 20 μM Bn_2S_2 (d) 10 μM fluorescein (e) 0.1% (v/v) DMSO in 10 mM PBS (pH 7.4) (f) Reaction aliquot after 1 h.

GSH-Triggered FLD-1 Activation



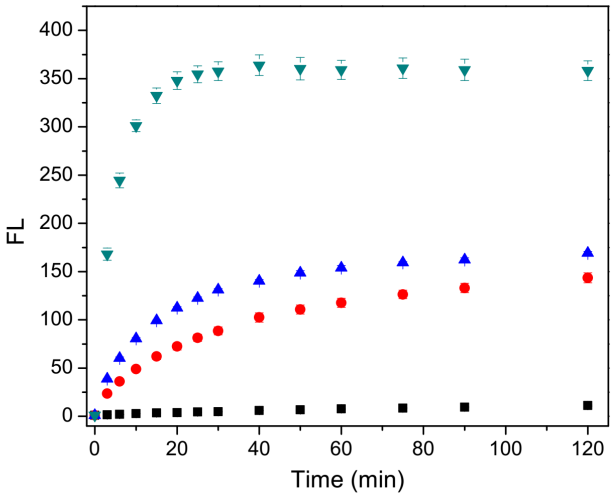


Figure S4. GSH-Dependent (black: 0 μM; red: 20 μM; blue: 50 μM; and green: 100 μM) fluorescence turn on of **FLD-1** (10 μM) in PBS. λ_{ex} = 490 nm, λ_{em} = 500 – 650 nm, and slit width = 0.3 mm. The experiments were performed in triplicate and results are expressed as mean ± SD (n = 3).

Cytotoxicity of FLD-1 in HeLa Cells

Confluent HeLa cells were incubated in FBS-free DMEM containing vehicle (0.5% DMSO), and **FLD-1** (6.25 – 50.0 μM) for 30 min in a 96-well plate. The culture media were then removed and 100 μL of FBS-free DMEM containing 10% CCK-8 solution was added to each well, and cells were incubated for 2 hours at 37 ºC. The absorbance at 450 nm was measured by using a microplate reader. The cell viability was measured and normalized to the vehicle group. The results are expressed as mean ± SEM (n = 6).

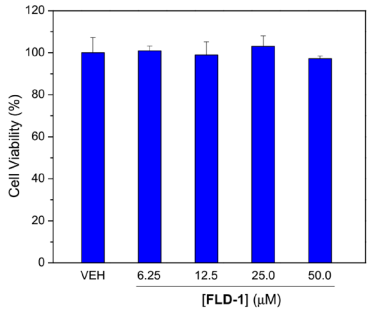


Figure S5. Cytotoxicity of **FLD-1** in HeLa cells. The results are expressed as mean ± SEM (n = 6)

Cytotoxicity of FLD-1, BnSH, and TCN-1 in RAW 264.7 Cells

Confluent RAW 264.7 cells were incubated in FBS-free DMEM containing vehicle (0.5% DMSO), **FLD-1**, **BnSH**, and **TCN-1** (10.0 – 100 µM) for 2 hours in a 96-well plate. The culture media were then removed and 100 µL of FBS-free DMEM containing 10% CCK-8 solution was added to each well, and cells were incubated for 2 hours at 37 ºC. The absorbance at 450 nm was measured by using a microplate reader. The cell viability was measured and normalized to the vehicle group. The results are expressed as mean ± SEM (n = 6).

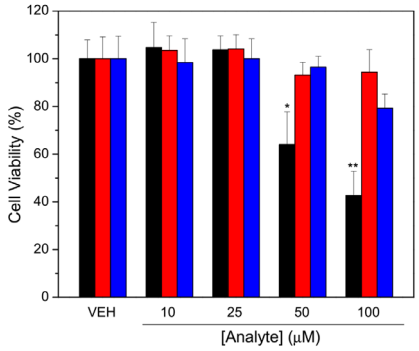


Figure S6. Cytotoxicity of **FLD-1** (black), **TCN-1** (red), and **BnSH** (blue) in RAW 264.7 cells. $^{*}P < 0.05$ vs VEH group and $^{**}P < 0.01$ vs VEH group. The results are expressed as mean ± SEM (n = 6)

Anti-inflammatory Effects of TCN-1, Fluorescein, and Benzyl Mercaptan

Macrophage RAW 264.7 cells were seeded in a 24-well plate (5 × 105 cells/well) containing 0.500 mL of DMEM and incubated at 37 ºC under 5% CO_2 for 24 h. The confluent cells were washed with PBS and incubated with 25 µM of **TCN-1**, fluorescein (FLOH), or benzyl mercaptan (BnSH) at 37 ºC for 2 h. Compounds were then removed by washing cells with PBS and these pretreated cells were incubated in FBS-free DMEM containing LPS (0.500 µg/mL) for 24 h. NO_2^- levels were measured by using a Griess Reagent Kit. As expected, none of these species exhibited anti-inflammatory activities.

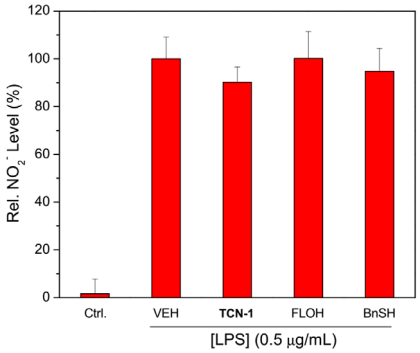
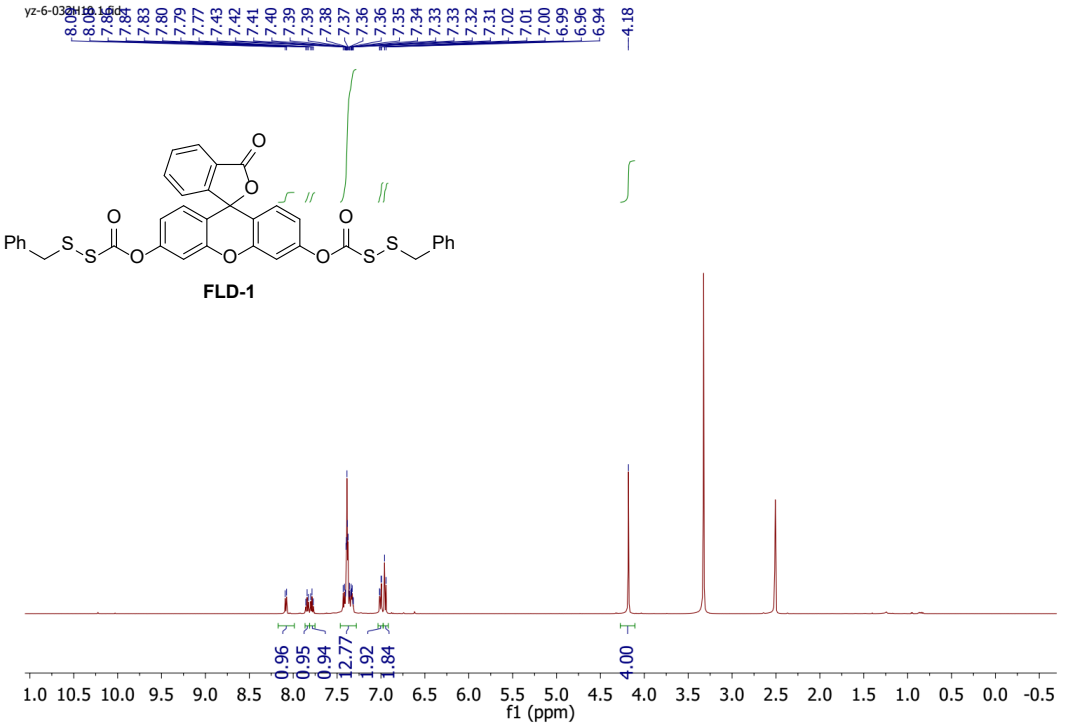
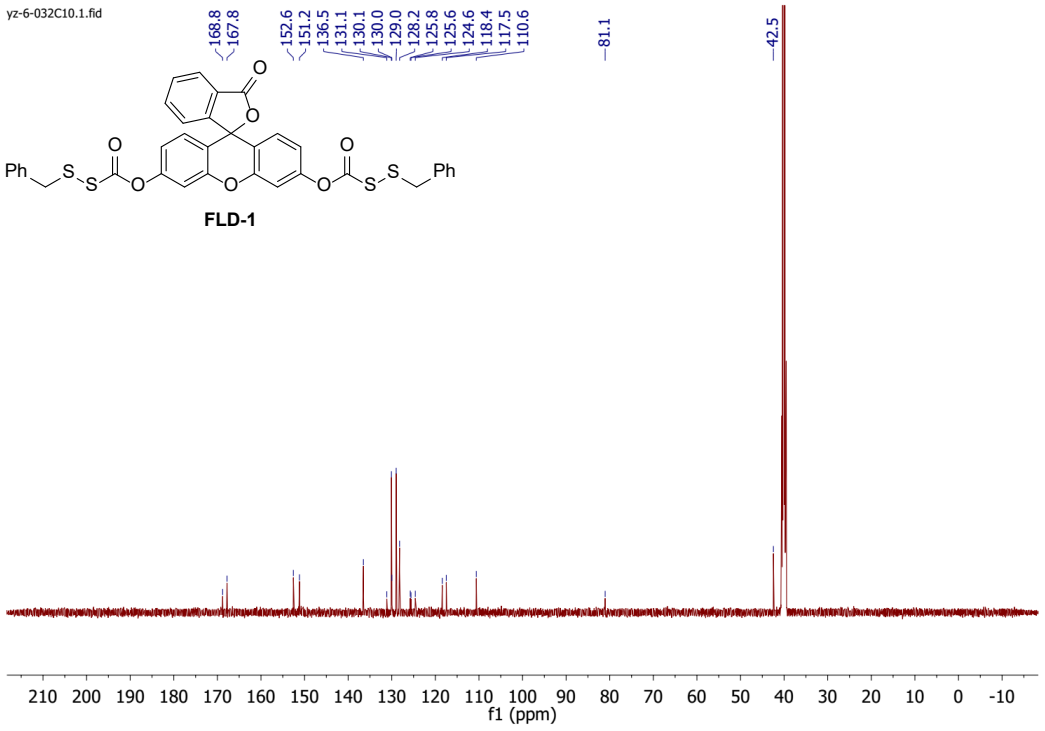


Figure S7. Effects of **TCN-1** and H_2S releasing byproducts on LPS-induced NO_2^- accumulation. RAW 264.7 cells were pretreated with **TCN-1**, fluorescein, and benzyl mercaptan (25 µM) for 2 h, followed by a 24-h treatment of LPS (0.5 µg/mL). Results are expressed as mean ± SD (n = 4).

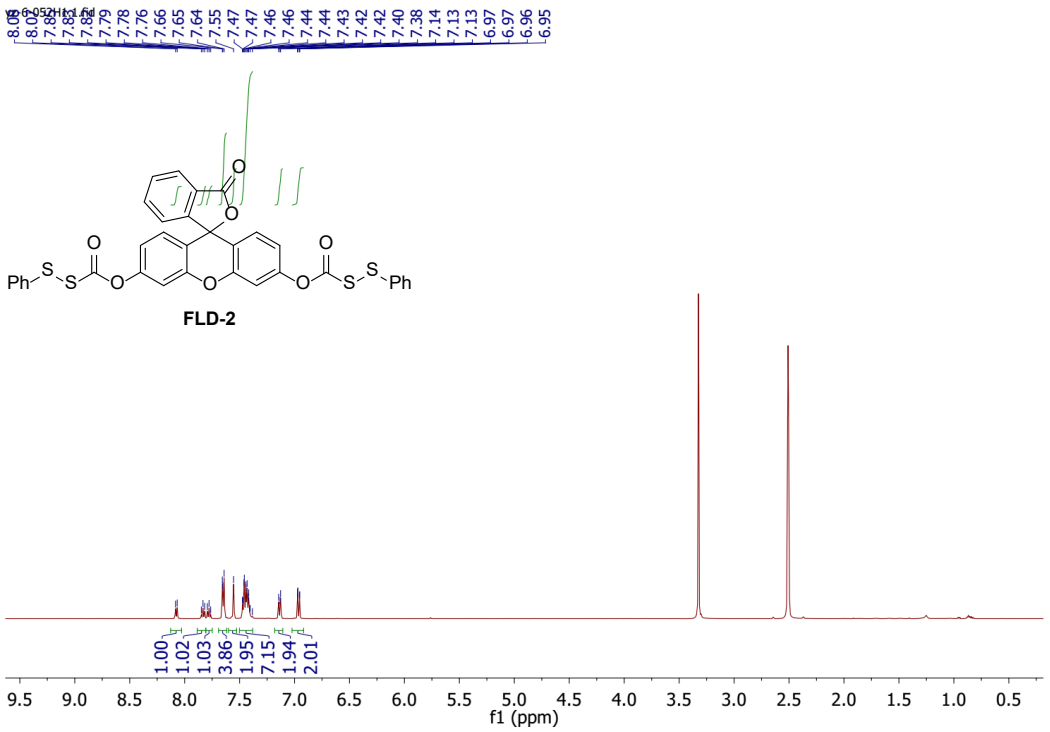
NMR Spectra

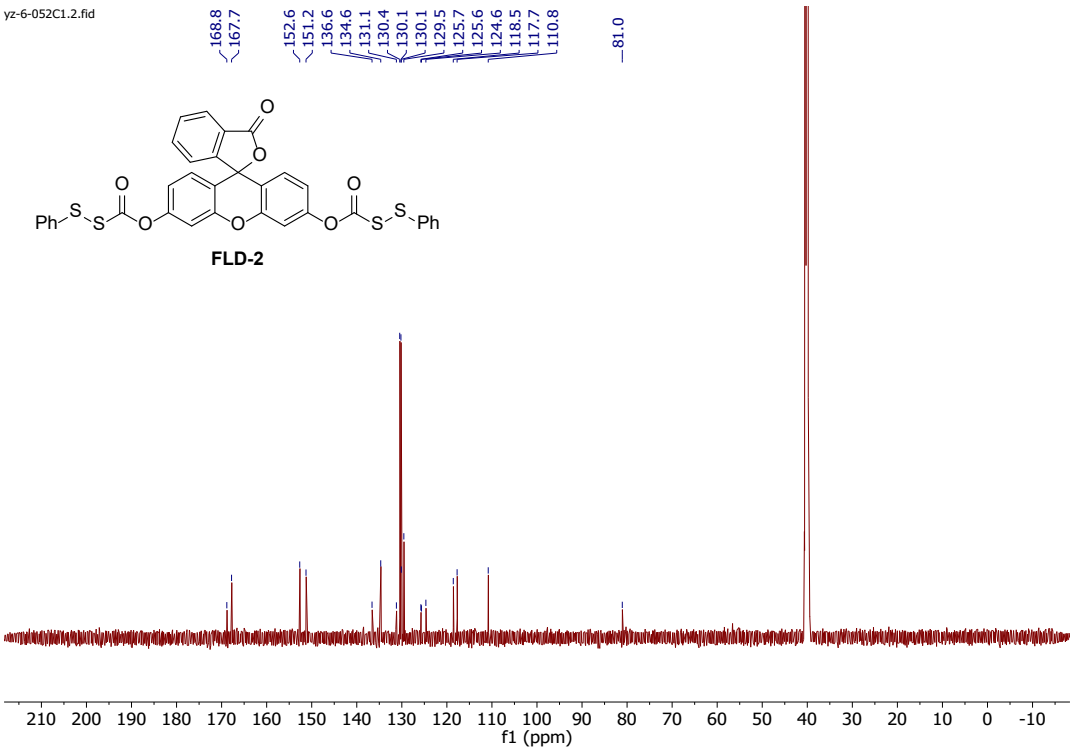
^{1}H (500 MHz, DMSO-d_6) and ^{13}C{1H} (125 MHz, DMSO-d_6) NMR Spectra of **FLD-1**.



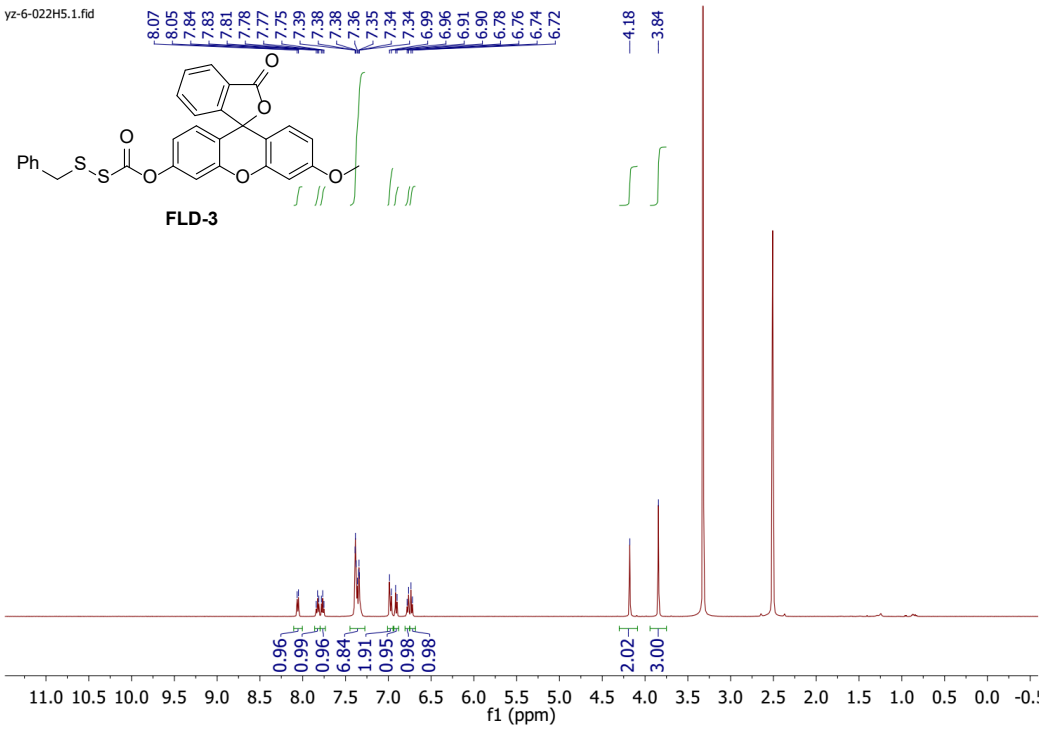


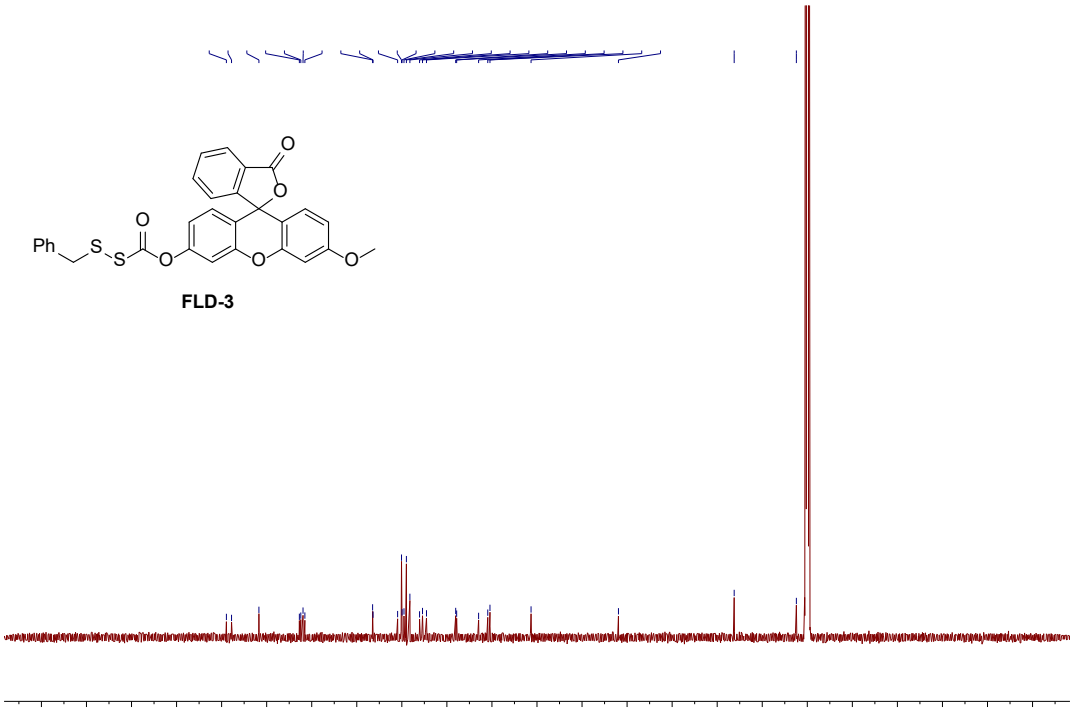
^{1}H (500 MHz, DMSO-d_6) and ^{13}C{1H} (125 MHz, DMSO-d_6) NMR Spectra of **FLD-2**.



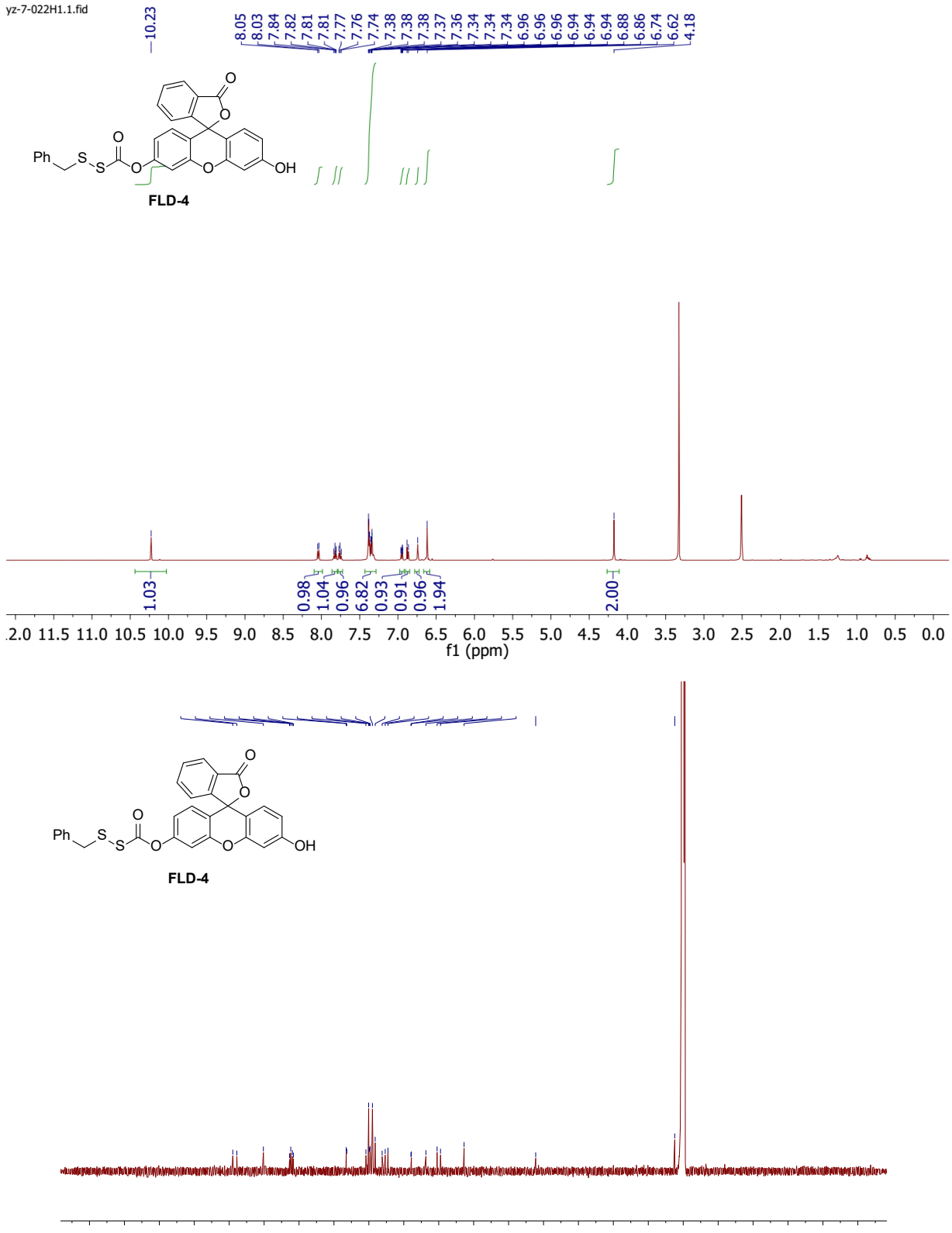


^{1}H (500 MHz, DMSO-d_6) and ^{13}C{1H} (125 MHz, DMSO-d_6) NMR Spectra of **FLD-3**.

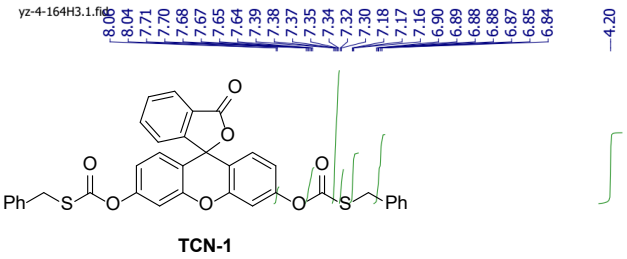




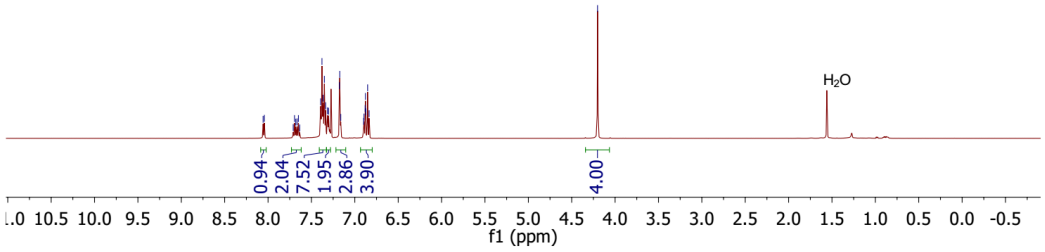
^{1}H (500 MHz, DMSO-d_6) and ^{13}C{1H} (125 MHz, DMSO-d_6) NMR Spectra of **FLD-4**.

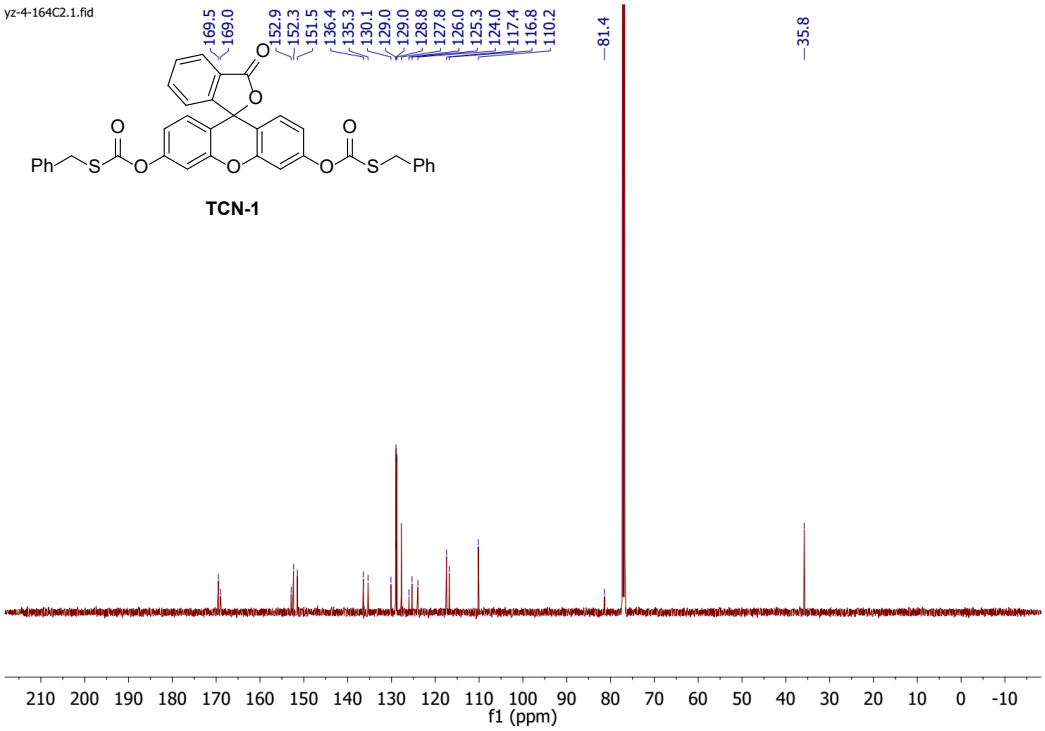


^{1}H (500 MHz, $CDCl_3$) and $^{13}C\{1H\}$ (125 MHz, $CDCl_3$) NMR Spectra of **TCN-1**.



TCN-1





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References

1. Schrader, A. M.; Schroll, A. L.; Barany, G., Synthetic Routes to, Transformations of, and Rather Surprising Stabilities of (N-Methyl-N-phenylcarbamoyl)sulfenyl Chloride, ((N-Methyl-N-phenylcarbamoyl)dithio)carbonyl Chloride, and Related Compounds. *J. Org. Chem.* **2011,** *76* (19), 7882-7892.

2. Chen, B.; Li, W.; Lv, C.; Zhao, M.; Jin, H.; Du, J.; Zhang, L.; Tang, X., Fluorescent probe for highly selective and sensitive detection of hydrogen sulfide in living cells and cardiac tissues. *Analyst* **2013,** *138* (3), 946-951.