Supporting Information

Shining a Light on SSP4: A Comprehensive Analysis and Biological Applications for the Detection of Sulfane Sulfurs

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Materials and Instrumentation:

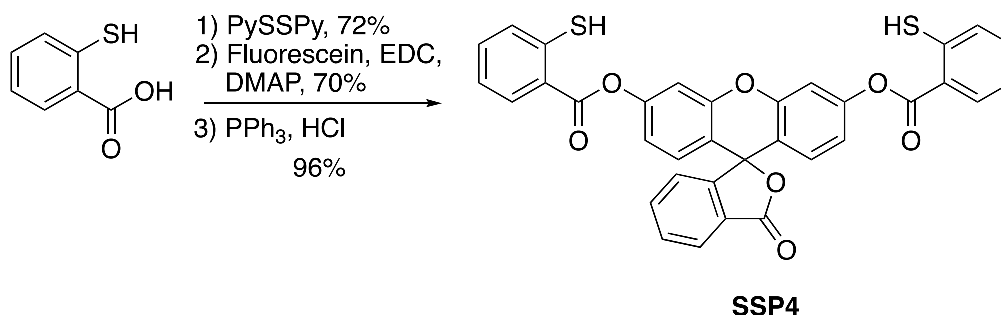
Reagents and solvents were of the highest grade available. Sodium disulfide (#SB02) was from Dojindo. Elemental sulfur was from Acros Organics (#201250250). Fluorescein was from Tokyo Chemical Industry (#F0095). Anhydrous sodium sulfate (Fisher Chemical, #S421-500), sodium thiosulfate (Acros Organics, #450622500), sodium sulfite (Sigma-Alrdich, #S0505), L-cysteine (Alfa Aesar, #A10435), reduced glutathione (GSH) (Acros Organics #120000050), DL-homocysteine (Tokyo Chemical Industry, #H0159), L-methionine (Acros Organics, #166161000), oxidized glutathione (GSSG) (Tokyo Chemical Industry, G#G0073), magnesium chloride (Argos Organics, #223211000), calcium chloride dihydrate (Fisher Chemical, #C79-500), anhydrous zinc chloride (Alfa Aesar, #A16281), anhydrous copper (II) sulfate (Alfa aesar, #A13986), iron (III) chloride hexahydrate (Fisher Chemical, #I88-500), and iron (II) chloride (Sigma-Aldrich, #372870) were purchased. Glycine, tyrosine, tryptophan and arginine were obtained from Acros Organics. Cell culture media (DMEM/F12 (1:1)) and phenol-red free media (Fluorobrite DMEM) were from Gibco (Invitrogen; Darmstadt, Germany). Papain (from *Carica papaya*, #P76220), bovine serum albumin (BSA, # A2153), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, from rabbit muscle, #G2267) were obtained from Sigma-Aldrich. Recombinant human protein disulfide isomerase was provided by the Flaumenhaft Lab.

Absorption spectra were recorded on a Thermo Scientific Evolution 350 UV-Vis Spectrophotometer using a 1 cm quartz cuvette. Fluorescence emission spectra were measured on a Cary Eclipse Fluorescence Spectrophotometer using a 1 cm quartz cuvette and Molecular Devices SpectraMax iD3 Multi-Mode Microplate Reader using a 96-well black/clear flat-bottomed

plate (ThermoFisher Scientific, #165305). Cell images were obtained using the Keyence All-in-One Fluorescence Microscope (BZ-X810) or a confocal laser scanning microscope (Nikon C2 plus, NIS elements Version 4.01 software). Fluorescence intensities (a.u.) of images were calculated using ImageJ software (National Institutes of Health).

Chemical reactions were magnetically stirred and monitored by thin layer chromatography (TLC) with 0.25 mm pre-coated silica gel plates. Flash chromatography was performed with silica gel 60 (particle size 0.040-0.062 mm). ^{1}H NMR and ^{13}C NMR spectra were recorded at 600 MHz and 150 MHz, respectively and are reported in parts per million (ppm) on the δ scale relative to $CDCl_3$. Purity was verified by TLC and NMR.

Chemical Synthesis and Characterization



The first two steps followed our reported protocols.[1] For the last step: PPh_3 (162.0 mg, 0.68 mmol) was added to a solution of the *S*-pyridium disulfide precursor (110.0 mg, 0.12 mmol) in THF/H_2O slowly at 0°C. The mixture was allowed to warm to room temperature (rt) and stirred for 45 min. THF was removed under reduced pressure and 10 mL HCl (1 N) was added to acidify the solution. The mixture was extracted with ethyl acetate (40 mL). The organic layer was separated and washed with brine. After drying with anhydrous Na_2SO_4, the solvent was removed under reduced pressure, and the resulting residue was purified by flash column chromatography. SSP4 was obtained as a white solid (70 mg, 96% yield). mp 211-214 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.28 (d, *J* = 8.0 Hz, 2H), 8.08 (d, *J* = 7.7 Hz, 1H), 7.88 – 7.58 (m, 2H), 7.42 (dt, *J* = 13.7, 7.6 Hz, 4H), 7.30 – 7.23 (m, 5H), 7.09 – 6.86 (m, 4H), 4.68 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.2, 164.6, 153.1, 152.1, 151.6, 139.8, 135.3, 133.4, 132.3, 131.2, 130.1, 129.0, 126.0, 125.2, 124.9, 124.5, 124.1, 118.0, 116.7, 110.7, 81.6; HRMS calculated for $C_{34}H_{21}O_7S_2$ $[M+H]^+$ 605.0729, found 605.0702.

SSP2 and cysteine polysulfide were prepared according to literature procedures.[2]

Fluorescence measurements

General:

Probes were dissolved in DMSO to obtain a 1 mM stock solution. CTAB was dissolved in ethanol to yield a 5 mM stock solution. Analytes were dissolved in MilliQ water, 50 mM PBS (pH 7.4), or THF to make 10 mM stock solutions. Buffer was added to a 4.5 mL vial, followed by surfactant (25 µM), SSP4 (5 µM), and other species to a final volume of 4 mL. Samples were vortexed briefly and incubated in the dark at room temperature (rt) for 20 min before 3 mL was transferred to a 1 cm quartz cuvette and measured on a Cary Eclipse Fluorescence spectrophotometer (emission 514 nm; excitation 482 nm). Samples measured on a Molecular Devices SpectraMax iD3 Multi-Mode Microplate Reader (emission 525 nm; excitation 485 nm; integration time 400s, low PMT sensitivity, attenuation 1, read height 1.00 mm) followed the same general procedure as above, though with a final volume of 200 µL (unless otherwise stated) added to a black polystyrene, flat-bottomed clear 96-well plate.

Time-dependent fluorescence assays:

Solutions were prepared by the method described above, though they were added directly to the cuvette, SSP4 was added last, and the final volume was 4 mL. CTAB (5 mM) was prepared in ethanol, Triton X-100 in 50 mM PBS (pH 7.4), and SDS in MilliQ H_2O. The cuvette was inverted to mix and placed into the fluorescence spectrophotometer. The fluorescent signals of solutions were measured every 0.25 min for 45 min.

Various biologically relevant buffers:

Solutions were prepared by the method described above, though volumes were added directly to a microplate, shaken at 120 rpm for 1 min, and then incubated for 20 min before loading onto the microplate reader.

Selectivity assay:

Cys-polysulfide and S_8 were prepared in THF. Amino acids, GSSG, and GSH were prepared in MilliQ H_2O. Na_2S_2, Na_2SO_3, Na_2S, Na_2SO_4, and $Na_2S_2O_3$ were prepared in MilliQ H_2O.

Interference assays:

Solutions were prepared by the methods described above with metal ion solutions in MilliQ H_2O. S_8 was prepared in THF to obtain a 10 mM stock solution. The order of preparation was as follows: 50 mM PBS (pH 7.4), CTAB (25 µM), amino acid (or metal ion solution), S_8 (25 µM), vortex briefly,

SSP4 (5 μM), vortex briefly, and incubate in the dark at rt for 20 min. 3 mL of samples were then transferred to a 1 cm quartz cuvette and measured on a Cary Eclipse Fluorescence spectrophotometer.

Protein-SSH measurements:

Papain

Papain was persulfidated according to literature procedures[3] with the following modifications. A solution of papain (~25 mg) in 1 mL of Tris-HCl (pH 7.4, 100 mM) was incubated with cysteine (2.3 mg) at rt for 10 min. Protein-containing fractions were pooled from a PD-10 column (500 μL, 400 μL, 600 μL, 500 μL) and quantified by UV-Vis with a 100-fold dilution based on the absorbance at 280 nm (ε_{mM} = 57.6). The highest concentration fraction (300 μM, 580 μL) was incubated with DTNB (58 μL, 4 mM) at rt for 20 min and then purified with a PD-10 column. Protein-containing fractions were pooled from a PD-10 column (500 μL), quantified, then the highest concentration fraction (480 μL, >150 μM) was incubated in Na_2S solution (30 mM, 11.2 μL) at rt for 10 min and then purified. The resulting papain persulfide was then used immediately. Experiments were performed in triplicate.

50 mM PBS (pH 7.4) was added to a ½ dram vial. The probe was then added, followed by the persulfidated papain at the appropriate concentrations. The final volume was 250 μL. Solutions were mixed by gentle pipetting, followed by incubation in the dark at rt for 30 min. 200 μL of solution was then transferred to a 96-well microplate, and the relative fluorescent units (RFU) at 525 nm was measured on the microplate reader with excitation at 485 nm.

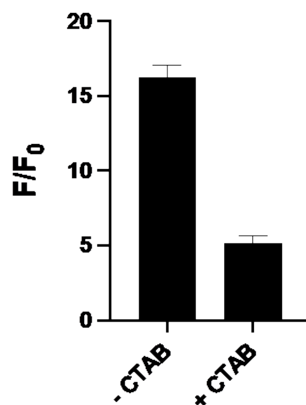


Fig. S1. Mean fluorescence enhancement of SSP4 (5 μM) in the presence of persulfidated papain (5 μM) ± CTAB (25 μM). Reactions were carried out for 30 min at rt in PBS buffer (50 mM, pH 7.4). Results are expressed as mean ± SEM (*n* = 3).

BSA

The mixture of BSA (69 mg) in 1 mL sodium phosphate buffer (pH 8.0) with 2-mercaptoethanol (8.2 mg) was parafilmed and incubated in the dark at rt for 1 hr in an Eppendorf tube. Excess reductant was removed with a PD-10 column equilibrated with the sodium phosphate buffer. Then, reduced BSA (~630 μM, 300 μL) was persulfidated by incubating with H_2O_2 (4 mM, 12.8 μL) at 37°C for 4 min with shaking at 100 rpm. Catalase (~ 1 mg/mL, 100 μL) was immediately added and solution was incubated at rt for 10 min. Na_2S was then added (final concentration = 2 mM). The tube was incubated at rt for 15 min in the dark. The obtained persulfidated BSA and reduced BSA were then diluted to 30 μM and 5 μM concentrations with PBS (50 mM, pH 7.4) to a final volume of 250 μL and incubated with 5 μM SSP4 for 30 min. The measurement method was the same as that for papain.

GAPDH

A solution of GAPDH (5 mg) in 278 μL PBS (50 mM, pH 7.4) was prepared to a concentration of 500 μM. DTT was added (10 mM, 14 μL), and the solution was incubated in the dark at rt for 30 min. Zeba 7K MWCO spin columns were prepared and used according to manufacturer's instructions with 3 washes of PBS. Excess reductant was removed by column purification with the Zeba 7K MWCO columns. The resulting reduced protein concentration was determined by the absorbance at 280 nm (ε_{mM} = 30.4 mM^{-1} cm^{-1}). The solution (260 μL, 600 μM) was diluted to 233 μM with PBS (50 mM, pH 7.4) then incubated with Na_2S_2 (26 μL, 10 mM) in the dark at rt for 30 min. The solution was then purified by Zeba 7K MWCO columns and quantified by absorbance. The GAPDH persulfide was then incubated with SSP4 (final concentration = 5 μM) and measured on the microplate reader according to the same procedures above.

PDI

Human recombinant PDI (PDIA1) was generated and reduced by the Flaumenhaft Lab.[4] Proteins were quantified by absorbance at 280 nm with Nanodrop (ThermoFisher Scientific) (MW = 56 kDa, 1% ε_{280} = 8.638, calculated ε_{mM} = 48.4 $mM^{-1}cm^{-1}$) and diluted to equal concentrations (12 μM) with PBS (10 mM, pH 7.4). Na_2S_2 (10 mM, final concentration = 97 μM) was then added to the PDIs, incubated in the dark at rt for 30 min, and then purified by Zeba 7K MWCO spin columns. The

resulting solutions were quantified by Nanodrop. Reduced and treated proteins were diluted to 5 μM with PBS, incubated with 5 μM SSP4, and measured on the microplate reader according to the procedures above.

Cell viability and cytotoxicity assay

HeLa cells were cultured in DMEM/F12 (1:1) (Gibco, Invitrogen, #11330-032) medium supplemented with 10% fetal bovine serum (FBS) at 37°C, 5% CO_2 for 24 hrs. Cells were then inoculated in a 96-well black/clear flat-bottomed plate (ThermoFisher Scientific, #165305) at 37°C, 5% CO_2 overnight. Media was then aspirated from wells, and cells were washed twice with 1X PBS. SSP4 in FBS-free Fluorobrite DMEM was then administered, and cells were cultured for 2 hrs or 22 hrs. WST-8 from the CCK-8 assay was then administered to the cells and incubated for 2 hrs at 37°C, 5% CO_2 for a total incubation time of 4 or 24 hrs. The absorbance at 450 nm was measured with a microplate reader. The optical density (OD) of the wells (3 wells per condition) were used to calculate the relative cell viability (%) according to the following formula:

Cell Viability (%) = $(OD_{treatment\ group} / OD_{control\ group}) * 100$

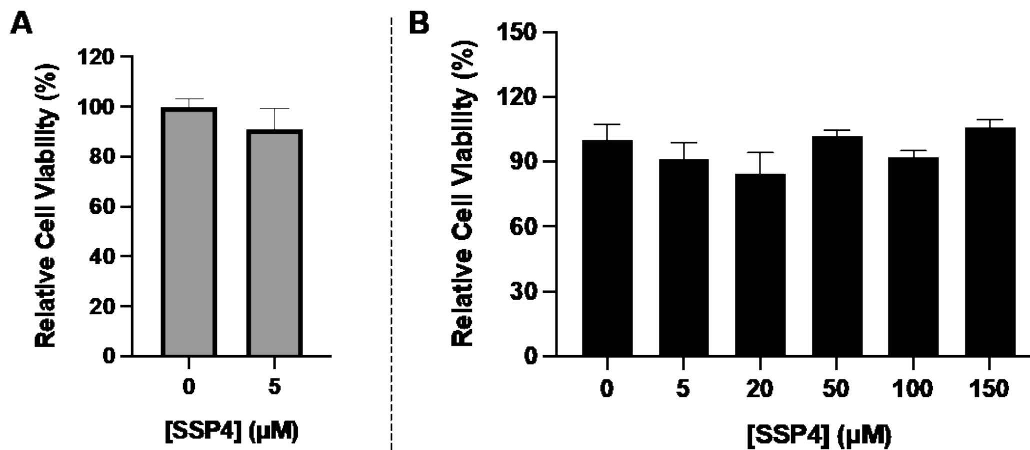


Fig. S2. Cell viability and cytotoxicity of SSP4 in HeLa cells with the CCK-8 assay. Cells were treated with **A)** 0 or 5 μM SSP4 and incubated for 24 hrs at 37°C, 5% CO_2 or **B)** different concentrations of SSP4 (0-150 μM) and incubated for 4 hrs at 37°C, 5% CO_2. After the initial incubation period, WST-8 was added, and cells were incubated for another 2 hrs at 37°C, 5% CO_2. Absorbance was measured at 450 nm. Results are expressed as mean ± SEM (*n* = 3).

Cell culturing and bioimaging

HeLa cells

HeLa cells were seeded in a 48-well clear flat-bottomed plate at a density of 4 x 10^5 cells per well and cultured in DMEM supplemented with 10% FBS at 37°C under 5% CO_2 for 24 hrs. Culturing media was then aspirated, and cells were washed twice with 1X PBS. SSP4 (5 μM, 0.5% DMSO in serum-free Fluorobrite DMEM) was then added to the wells, and cells were incubated for 20 min at 37°C under 5% CO_2. The solution was removed post-incubation, and cells were washed once with PBS. Cetyltrimethylammonium bromide (CTAB) (100 μM) and Na_2S_2 (50 μM) in serum-free Fluorobrite DMEM was then added to the cells. Cells not treated with Na_2S_2 were used as negative controls. After 20 min incubation at 37°C under 5% CO_2, cells were washed once with PBS and then taken for fluorescence imaging using the Keyence All-in-One Fluorescence Microscope (BZ-X810) (excitation: 470/40 nm; emission: 525/50 nm).

COS-7 and HEK293T cells

COS-7 and HEK293T cells were seeded in eight-well glass chamber slides at a density of 4 x 10^5 cells per well. In some experiments, cells were pre-treated with CSE-expression plasmid. The CSE overexpression and *CARS2* KO cells were produced according to the methods reported previously.[5,6] The cells were washed once with serum-free DMEM, followed by incubation with 20 μM SSP4 in serum-free DMEM containing 100 μM CTAB at 37ºC for 30 min. Cells not treated with probe were used as negative controls. After removing the excess probes from the cells and washing them with PBS, they were incubated in PBS for 30 min at 37ºC. Cells were then washed twice with PBS, followed by measurement of fluorescence in images, at an excitation wavelength of 488 nm, with a confocal laser scanning microscope (Nikon C2 plus, NIS elements Version 5.01 software). Fluorescence intensities (a.u.) of images were calculated by using Image J software (National Institutes of Health).

HeLa cell lysate assay:

HeLa cells were cultured in DMEM/F12 (1:1) (Gibco, Invitrogen, #11330-032) medium supplemented with 10% fetal bovine serum (FBS) at 37°C, 5% CO_2 in a 75 cm^2 flask for 48 hrs. Cells were lysed with RIPA Lysis and Extraction Buffer (ThermoFisher Scientific) following manufacturer's instructions with sonication of the pellet for 30 seconds with a 50% pulse. Protein yield (~375 μg/mL) was determined by BCA assay. Lysate was diluted to 20% by adding 600 μL to 2400 μL PBS (50 mM, pH 7.4) and pipetting up and down to mix. Diluted lysate was then added

to a 96-well microplate followed by Na_2S_2 (final concentrations = 1, 3, 5, 10, 20, 30, 50, 75, 100 μM), and SSP4 (final concentration = 5 μM). The final volume was 100 μL. The microplate was then shaken at 120 rpm for 1 min at rt. After a 30 min incubation in the dark at rt, the plate was measured by the microplate reader according to the general procedures above. The results are shown in **Fig. S3** below.

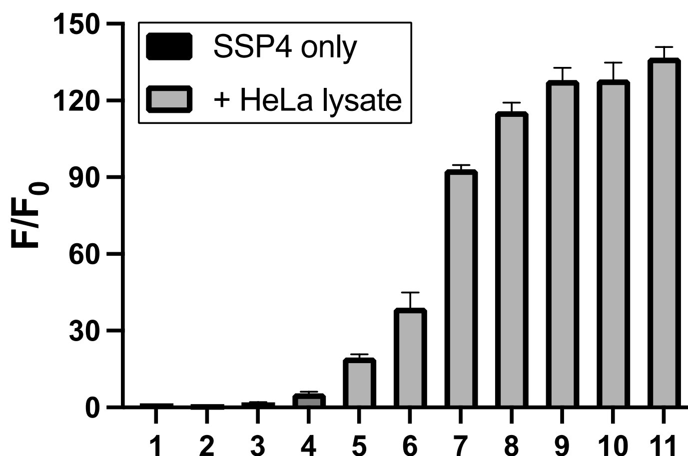


Fig. S3. Fluorescence enhancement (F/F_0) of 5 μM SSP4 in the presence of: (1) probe only; (2) HeLa lysate; and HeLa lysate with increasing concentrations of Na_2S_2: (3) 1 μM; (4) 3 μM; (5) 5 μM; (6) 10 μM; (7) 20 μM; (8) 30 μM; (9) 50 μM; (10) 75 μM; (11) 100 μM. Reactions were carried out for 30 min at rt. Results are expressed as mean ± SEM (*n* = 3).

Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. Statistical evaluations were performed on GraphPad Prism with either one-way ANOVA or Student's *t*-test.

References

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