Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

Commonly Used Alkylating Agents Limit Persulfide Detection by Converting Protein Persulfides into Thioethers

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SUPPORTING INFORMATION

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Chemicals

Diamide (D3648), Iodoacetamide (I1149), Iodoacetic acid (I4386), *N*-(*tert*-butyl)-2-iodoacetamide (PH000979), *N*-ethylmaleimide (04259), sodium sulfide (407410), potassium cyanide (60178), sodium mercaptopyruvate dihydrate (90374), pentafluorobenzyl bromide (101052), tetrabutylammonium bisulfate (86868), biotin (B4501), benzonase (9025-65-4), AEBSF HCl (30827-99-7) and leupeptin (103476-89-7) were obtained from Sigma-Aldrich. 2-Iodo-*N*-phenethylacetamide (EN300-249647) and *N*-ethyl-2-iodoacetamide (EN300-7003350) were obtained from Enamine. L-Glutathione disulfide (3542) was obtained from Merck. Monobromobimane (sc-214629) was obtained from Santa Cruz Biotechnology. Sodium disulfide (SB02-10) was obtained from Dojindo Molecular Technologies and hydrogen peroxide 30% (9683-1) from Roth. B-Per extraction reagent (78243), Alexa Fluor 594 C5 maleimide (A10256) and peracetic acid (Acros 257751000) were obtained from Thermo Scientific.

Cell culture

HEK293 MSR (GripTite™, Thermo Fisher Scientific) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies), supplemented with 10% (v/v) bovine calf serum (Life technologies), and 50 units/ml penicillin and streptomycin (Life Technologies), 50 units/ml Geneticin (Life Technologies). All cell lines were confirmed to be free of mycoplasma, viral infections and contaminations with other cell lines, based on multiplex PCR and SNP profiling.

Bacterial expression plasmids

The pQE-60 bacterial expression plasmids encoding hTrx1(**C**SAAA)-SBP-His and hTrx1(**S**SAAA)-SBP-His have been described previously[1]. The amino acid sequence of hTrx1(**C**SAAA)-SBP-His is:
MVKQIESKTAFQEALDAAGDKLVVVDFSATW**C**GPSKMIKPFFHSLSEKYSNVIFLEVDVDDAQDVASEAEVKAMPTFQFFKKGQKVGE
FSGANKEKLEATINELVRSMDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREPGS*HHHHHH*
The single cysteine is indicated in bold, the SBP tag is underlined, and the hexahistidine tag is shown in italic.

Protein expression and purification

Plasmids were expressed under the control of an IPTG-inducible T7 promoter in *Escherichia coli* BL21(DE3) (Thermo Scientific, Cat#EC0114). Chemically competent bacteria (50 µL) were thawed on ice and gently mixed with 1 ng of plasmid. The mixture was kept on ice for 20 min, then incubated at 42 °C for 1 min, then placed back on ice for 2 min. After adding 250 µL of LB-medium, the bacterial suspension was plated on LB agar plates supplemented with 100 µg/mL ampicillin. The plates were incubated overnight at 37 °C. A single colony was used for inoculating 100 mL of LB medium supplemented with 0.1 mg/mL ampicillin. The pre-culture was incubated overnight at 37 °C with constant shaking at 300 rpm. A volume of 50 mL of pre-culture was transferred to 1 L LB medium supplemented with 0.1 mg/mL ampicillin. Bacteria were grown at 37 °C with constant shaking until they reached an OD of 0.6 – 0.7 and then incubated with 1 mM IPTG at 37 °C for another 4 h. Afterwards, the bacterial culture was centrifuged for 10 min at 4000 rcf and 4 °C. The dried bacterial pellet was solubilized in 20 mL of B-PER solution, supplemented with protease inhibitors (1 µg/mL leupeptin, 1 µg/mL AEBSF HCl, 1 µL benzonase), 10 mM imidazole, and 0.5 mM DTT, and transferred to a 50 mL centrifuge tube. The resulting lysate was carefully mixed for 10 min at 4°C and then centrifuged at 16000 rcf for 30 min at 4 °C. The supernatant was collected. Ni-NTA agarose beads (QIAGEN, Cat#30210) were washed three times with NH_4HCO_3 buffer (50 mM NH_4HCO_3, pH 7.4). Then 1 mL Ni-NTA agarose beads were added to the bacterial lysis supernatant and incubated for 1 h at 4°C under gentle rotation. The Ni-NTA bead suspension was transferred into 5 mL disposable chromatography columns (Thermo Scientific, Cat#29922). Beads were washed three times with 5 mL of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.5 mM DTT, 1 µg/mL leupeptin, 1 µg/mL AEBSF HCl, 1 µL benzonase, pH 8.0). The protein was eluted in two steps, first with 5 mL of elution buffer containing 100 mM imidazole (50 mM NaH_2PO_4, 300 mM NaCl, 0.5 mM DTT, 100 mM imidazole, pH 8.0), and then with 5 mL of elution buffer containing 500 mM imidazole (50 mM NaH_2PO_4, 300 mM NaCl, 0.5 mM DTT, 500 mM imidazole, pH 8.0). Both eluates contained pure protein as demonstrated by Coomassie-stained SDS-PAGE and were therefore combined. The protein sample was placed in a 10 kDa cut-off dialysis cassette (Thermo Scientific, Cat#66382) pre-equilibrated with NH_4HCO_3 buffer for 5 min. The cassette was then incubated in 2 L of NH_4HCO_3 buffer under constant stirring. Dialysis was performed overnight at 4 °C. Finally, 1 mL aliquots of dialyzed protein were stored at -80 °C.

Immobilization of recombinant Trx1CS/SS on streptavidin beads

For each protein sample, streptavidin (SA) sepharose beads (Cytvia, Cat#17-5113-01) from 300 µL of a 50% slurry in storage buffer (50 mM Tris-HCl, pH 8) were washed three times with 10 mL of NH_4HCO_3 buffer (50 mM NH_4HCO_3, pH 7.4). All centrifugation steps were performed at 20 rcf and 4 °C for 3 min. After the final washing step, SA beads were resuspended in 300 µL NH_4HCO_3 buffer to obtain a 50% slurry. SA beads were then incubated with 150 µL of protein solution (180 µM Trx1CS or 160 µM Trx1SS) for 1 h at 4 °C with gentle rotation. Beads were then incubated with DTT (10 mM final concentration) for 30 min at 4 °C. Then, the beads were washed five times with 10 mL NH_4HCO_3 buffer to remove DTT. After the final washing step, protein-saturated beads were resuspended in 2 mL NH_4HCO_3 buffer to obtain a suspension containing a final concentration of 13.5 µM Trx1CS and 12 µM Trx1SS, respectively. Unless otherwise stated, all experiments were performed in NH_4HCO_3 buffer adjusted to pH 7.4, prepared just before use. We used a freshly prepared buffer for each reaction and washing step. Alkylating agents were directly dissolved in the buffer and the pH was checked after dissolving the compound.

Treatment of Trx1CS with GSSG, $GS^{34}SSG$, and GSSSSG

2 mL of bead suspension (corresponding to 13.5 µM immobilized Trx1CS) were incubated under rotation with 300 µM of either GSSG, $GS^{34}SSG$, or GSSSSG, for 30 min at room temperature. The reaction was quenched by the addition of 5 mL NH_4HCO_3 buffer (50 mM NH_4HCO_3, pH 7.4) containing either 1 mM IAM or 1 mM MBB. The samples were then washed once with NH_4HCO_3 buffer containing 1 mM of alkylating agent and then incubated with 1 mM alkylating agent in NH_4HCO_3 buffer for 30 min at room temperature. Finally, all samples were washed three times with 10 mL of ice-cold NH_4HCO_3 buffer and kept on ice.

Persulfidation of Trx1CS using H_2O_2 and Na_2S

2 mL of bead suspension (corresponding to 13.5 µM immobilized Trx1CS) were incubated under rotation with 50 µM H_2O_2 and 500 µM $Na_2{}^{34}S$ for 5 min at room temperature. Stable isotope labeled $Na_2{}^{34}S$ was used to distinguish the mass of a single sulfur atom from the combined mass of two ^{16}O atoms. The reaction was quenched by the addition of 5 mL of either 1 mM IAM or 1 mM MBB in NH_4HCO_3 buffer. The samples were then washed once with NH_4HCO_3 buffer containing 1 mM of alkylating agent and then incubated with 1 mM alkylating agent in NH_4HCO_3 buffer for 30 min at room temperature. Finally, all samples were washed three times with 10 mL of ice-cold NH_4HCO_3 buffer and kept on ice. For the specific experiment shown in Supplementary Figure S1F, two samples were treated with 50 µM H_2O_2 and 500 µM $Na_2{}^{34}S$, and one sample with only 50 µM H_2O_2. Two of the samples were subsequently incubated with 10 mM DTT for 30 min at room temperature.

Persulfidation of Trx1CS using the MPST/3MP system

Recombinantly expressed and purified TUM1 (thiouridine modifying protein 1, the yeast homologue of MPST) and inactive mutant TUM1(C259S) were kindly provided by Dr. Brandán Pedre Pérez (DKFZ, Heidelberg). Purified TUM1 and TUM1(C259S) were pre-treated with 10 mM DTT for 30 min at 4 °C. Meanwhile, Zeba™ spin desalting columns (Thermo Scientific, Cat#89882) were equilibrated by the repeated addition (3x 300 µL) of phosphate buffer (100 mM NaH_2PO_4, 0.1 mM diethylenetriaminepentaacetic acid (DTPA), pH 7.4) and subsequent centrifugation at 1500 rcf for 2 min. The desalting column was then loaded with pre-reduced TUM1 or TUM1(C259S) and centrifuged at 1500 rcf for 2 min. The flowthrough was applied to a second desalting column which was centrifuged in the same way. Following the second desalting step, the protein concentration was determined by measuring absorbance at 280 nm using a NanoDrop spectrophotometer (Thermo Scientific). The molar extinction coefficient of TUM1/TUM1(C259S) was taken as 50880 $M^{-1}\,cm^{-1}$, based on the ProtParam tool (https://web.expasy.org/protparam/). TUM1 or TUM1(C259S) (10 µM final concentration) was added to 2 mL of bead suspension (corresponding to 13.5 µM immobilized Trx1CS) in phosphate buffer. The mixture was then incubated with 40 µM 3MP for 5 min at room temperature. The reaction was quenched by the addition of 5 mL of either 1 mM IAM, 1mM HPE-IAM or 1 mM MBB in NH_4HCO_3 buffer. The samples were washed once with NH_4HCO_3 buffer (50 mM NH_4HCO_3, pH 7.4) containing 1 mM of the respective alkylating agent and then incubated with 1 mM alkylating agent in NH_4HCO_3 buffer for 30 min at room temperature. Finally, all samples were washed three times with 10 mL of ice-cold NH_4HCO_3 buffer and kept on ice.

Persulfidation of Trx1CS using Na_2S_2

Na_2S_2 was freshly dissolved in purified water (Milli-Q, Millipore) at a final concentration of 100 mM. It was then added to 2 mL of bead suspension (corresponding to 13.5 µM immobilized Trx1CS), to a final concentration of 300 µM. The sample was incubated under rotation for 5 min at room temperature. The reaction was quenched by the addition of 5 mL of NH_4HCO_3 buffer (50 mM NH_4HCO_3, pH 7.4) containing 1 mM of alkylating agent. The samples were then washed once with NH_4HCO_3 buffer containing 1 mM of alkylating agent and then incubated with 1 mM alkylating agent in NH_4HCO_3 buffer for 30 min at room temperature. Finally, all samples were washed three times with 10 mL of ice-cold NH_4HCO_3 buffer and kept on ice.

Elution of Trx1CS/SS from SA beads

Following centrifugation at 20 rcf for 3 min, SA beads were resuspended in 300 µL of NH_4HCO_3 buffer (50 mM NH_4HCO_3, pH 7.4) containing 4 mM biotin. The biotin solution was adjusted to the same pH under which the last step of an experiment was performed (typically 7.4). Proteins were eluted by incubation with the biotin solution for 45 min at 4 °C under rotation. Beads were then removed by applying the suspension to an empty microcentrifuge spin column (Thermo Scientific, Cat#89868) and centrifuging for 2 min at 100 rcf. The collected flow-through was stored at -80 °C until analyzed by mass spectrometry.

Whole protein mass spectrometry

Whole protein mass spectrometry analysis was conducted using a liquid chromatography (LC) system equipped with a POROS 10R1 column (Applied Biosystems) connected to a Bruker maXis time-of-flight (TOF) mass spectrometer with an ESI source operating in positive ion mode. Samples were injected into the LC system *via* a loading pump using 0.3% formic acid (FA) as the mobile phase. After 3 min the mobile phase was switched to 50% of 0.3% FA and 50% of a 80% isopropanol/10% acetonitrile/0.3% FA mixture and maintained under these conditions for 15 min. The target protein eluted between 10 and 12 min. The data were deconvoluted and processed with Data Analysis 4.2 and ESI Compass 1.3 software. Processed data were normalized to the peak with the highest detected intensity and plotted using Origin (Pro) Version 2021b.

Treatment of alkylated persulfides with KCN

Following Na_2S_2-mediated protein persulfidation, 10 mL of bead suspension (corresponding to 13.5 µM immobilized Trx1CS) was washed five times with 10 mL of NH_4HCO_3 buffer (50 mM NH_4HCO_3, pH 7.4). The bead suspension was then divided into 5 samples. Two samples were alkylated with 1 mM MBB in 5 mL of NH_4HCO_3 buffer at pH 7.4. Three samples were alkylated with 1 mM IAM in 5 mL of NH_4HCO_3 buffer adjusted to pH 9.5. All samples were incubated for 30 min at room temperature under rotation. One of the two MBB-alkylated samples was then incubated with NH_4HCO_3 buffer (pH 7.4), the other with 0.5 mM KCN in NH_4HCO_3 buffer (pH 7.4). One of the three IAM-alkylated samples was incubated with NH_4HCO_3 buffer (pH 7.4), one with 0.5 mM KCN in NH_4HCO_3 buffer (pH 7.4), and one with 0.5 mM KCN in NH_4HCO_3 buffer adjusted to pH 9.5. All samples were incubated for 30 min at room temperature under rotation, then washed three times with ice-cold NH_4HCO_3 buffer (10 mL). Finally, proteins were eluted and analyzed by whole protein MS, as described above.

Sample preparation for thiocyanate quantitation

To quantify the amount of thiocyanate released from alkylated protein persulfides during cyanide treatment, Trx1CS was persulfidated with the MPST/3MP system and alkylated with 1 mM of alkylating agent (as described above). After incubation for 3 min at room temperature, samples were washed three times with 3 mL ice-cold phosphate buffer (100 mM NaH_2PO_4, 0.1 mM DTPA, pH 7.4). Thereafter, the buffer was aspirated, and the beads were resuspended in 400 µL of phosphate buffer. 350 µL bead suspension was supplemented with 1 mM KCN, incubated for 30 min at room temperature and then used for gas chromatography-based thiocyanate measurements (see below). The remaining 50 µL of bead suspension was used for the quantification of labile sulfane sulfur with SSP4 (see below).

Quantitation of thiocyanate by gas chromatography

Thiocyanate was derivatized with pentafluorobenzyl bromide (PFBBr) as described previously[2]. Briefly, 200 µL of sample was mixed with 500 µL of phase transfer reagent tetrabutylammonium bisulfate (TBAS) in saturated sodium borate (pH 9.2) and with 500 µL of PFBBr in ethyl acetate. Samples were incubated at 70 °C with constant shaking, spun down at 10.000 g and the organic layer transferred to GC-MS vials. A calibration curve was prepared with KSCN and KCN dissolved in 0.1 M NaOH. Samples were analyzed on a gas chromatograph (Agilent 7890A) coupled to a mass spectrometer (Agilent 5975C). The conditions were as follows: 5 µL of sample was injected, with 1 to 5 split ratio. Heater temperature was 250 °C. Starting column temperature was 50 °C, which was increased to 200 °C over 10 min and then to 300 °C in 2 min and maintained for 1 min. Separation was performed with a HP-5MS column (Agilent 19091S-433) with 1 mL/min He flow. MS settings were as follows: gain factor - 25, mode - SIM (181 m/z - 100 ms dwell time; 239 m/z - 300 ms dwell time). Data was analyzed with ChemStation (G1701EA) and MestReNova (v. 14.2.1) software. The 239 m/z ion corresponding to ion PFB-SCN was used for identification and 181 m/z corresponding to fragment ion was used for quantification.

Quantitation of labile sulfane sulfur with SSP4

Following protein persulfidation and alkylation, as described above, remaining 50 µL of sample was mixed with 50 µL of phosphate buffer (100 mM NaH_2PO_4, 0.1 mM DTPA, pH 7.4). SSP4 was added to a final concentration of 100 µM. Fluorescein released from SSP4 was measured with a fluorescence plate reader (Pherastar, BMG), using opaque black 96-well plates.

Colorimetric quantitation of thiocyanate

CAM-SS-CAM was generated by air oxidation of 2-mercaptoacetamide. CAM-SS-CAM and cystamine were dissolved in DMSO at a final concentration of 50 mM. To 50 µL of the obtained solution, 50 µL of aqueous 0.5 M KCN was added. The disulfides of 3MP and 3MPA were generated from the corresponding thiols by the addition of the thiol oxidant diamide (tetramethylazodicarboxamide). 5 mM of thiol was mixed with 3 mM of diamide and with 50 mM of KCN in PBS. The reactions were performed in a transparent 96-well plate. The mixtures were incubated for 30 min followed by the addition of 100 µL of Goldstein reagent, prepared as described previously[3]. The product $[Fe(SCN)(H_2O)_5]^{2+}$ was detected by measuring absorbance at 460 nm with a microplate reader (FLUOstar, BMG Labtech). Thiocyanate concentrations were determined by use of a KSCN standard curve.

LC-MS analysis of small molecules

A solution of CAM-SS-CAM in methanolic ammonia was obtained by air oxidation of 2-mercaptoacetamide. CAM-SS-CAM and cystamine (50 mM each) were incubated with 100 mM KCN for 30 min. The solution was diluted 1:100 in water and 5 µL injected into an HPLC/MS Agilent 1260 Infinity system equipped with a 6120 Quadrupole mass detector (equipped with ESI) and a evaporative light scattering detector (ELSD). Separation was performed on a Kinetex 2.6 µm C18 100 Å LC column (50 x 2.1 mm) at 40 °C using a flow rate of 0.6 mL/min. Solvent A: H_2O, 0.01% HCOOH; Solvent B: MeCN, 0.01% HCOOH. Gradient: 100% A for 2 min, change to 10% A within 10 min, 1% A for 2 min. The data was processed with MestReNova (v. 14.2.1) software.

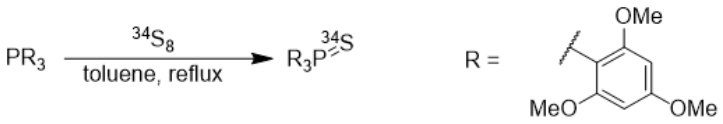
Generation and alkylation of L-cysteine persulfide

L-cysteine (1 mM) was persulfidated with Na_2S_2 (2 mM) in NH_4HCO_3 buffer (50 mM, pH 7.4) at RT. After 5 min, MBB or NEM was added to a final concentration of 10 mM. Samples were incubated at RT and in the dark for 30 min and subsequently analyzed by LC-MS.

Detection of protein persulfides in whole cell lysates

6 x 10^6 HEK293 MSR cells were seeded into a 10 cm petri dish and grown in serum-free DMEM for 24 hrs, reaching 80%-90% confluence. Cells were then gently washed twice with PBS. 1 mL of cold hypotonic lysis buffer (20 mM HEPES, 2 mM EDTA, 2 mM $MgCl_2$, 1% protease inhibitor) supplemented with or without alkylating agents (10 mM NEM or 10 mM MBB) was added and cells were incubated for 20 min with shaking at 4 °C. Cells were then lysed by gentle scraping. The lysates were collected and centrifuged (16000 g, 10 min, 4 °C) to remove cell debris. Proteins were precipitated with methanol/chloroform using a lysate:MeOH:$CHCl_3$ ratio of 4:4:1 (v/v/v). Following centrifugation (14000 g, 10 min, 4 °C) proteins collect between the aqueous and organic layers. Both layers were aspirated. A H_2O/MeOH/$CHCl_3$ mixture (4:4:1; v/v/v) was added to the precipitate and again centrifuged (14000 g, 10 min, 4 °C). Following aspiration of the supernatant, the protein pellet was washed 2 times with methanol (14000g, 5 min, 4 °C). The pellet was then resuspended in 50 mM HEPES, 2% SDS with or without 10 mM alkylating agent and incubated for 20 min. Proteins were again precipitated using methanol/chloroform, as described above. The protein pellet was resuspended in 50 mM HEPES, 2% SDS with or without 50 mM DTT and incubated for 20 min. The solution was precipitated using methanol/chloroform and resuspended in 50 mM HEPES, 2% SDS with or without 5 µM Alexa Fluor™ 594 C5 maleimide (Thermo Fisher A10256) for 20 min. To remove excess Alexa Fluor™ 594 C5 maleimide, the solution was again precipitated using methanol/chloroform and redissolved in 50 mM HEPES, 2% SDS. The protein concentration was measured and 20 µg of protein was loaded on a 12% SDS polyacrylamide gel. Labeled thiols were visualized in the gel by fluorescence (600 nm channel, LI-COR Odyssey Fc). Band intensity was quantified with ImageJ (version 1.53k).

Synthesis of tris(2,4,6-trimethoxyphenyl)phosphine sulfide-^{34}S ($R_3P^{34}S$)

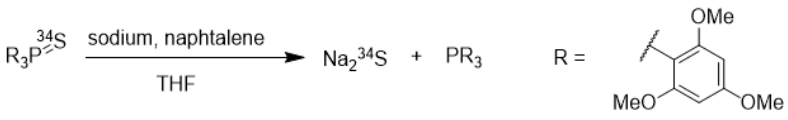


$R_3P^{34}S$ was prepared according to a procedure by[4,5]. Briefly**,** a mixture of $^{34}S_8$ (50.0 mg, 1.47 mmol, 1.0 equiv S_1) and tris(2,4,6-trimethoxyphenyl)phosphane (780 mg, 1.47 mmol, 1.0 equiv) in toluene (15 mL) was refluxed under nitrogen atmosphere at rt for 5 h, then concentrated to dryness to obtain $R_3P^{34}S$ as a white solid (570 mg, 1.006 mmol, 69% yield), which showed >95% purity and was used without further purification.

^{1}H NMR (600 MHz, $CDCl_3$) δ 6.04 (d, *J* = 4.2 Hz, 6H), 3.79 (s, 9H), 3.54 (s, 18H) ppm.
^{31}P NMR (243 MHz, $CDCl_3$) δ 11.33 ppm.
LC/MS (*m/z*): $[M+H]^+$ 567.2

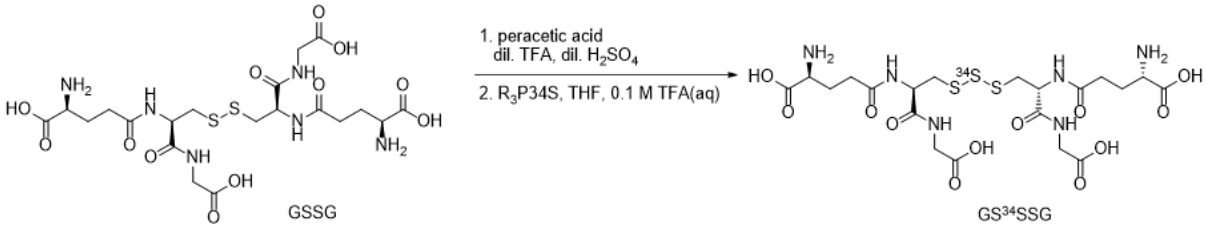
Analytical data is in accordance with reported literature[4].

Synthesis of sodium sulfide-^{34}S ($Na_2{}^{34}S$)



$Na_2{}^{34}S$ was prepared based on the procedure described in[6]. To a stirring solution of naphthalene (180 mg, 1.40 mmol, 2.7 equiv) in dry THF (15 mL) was added freshly cut sodium metal (33 mg, 1.43 mmol, 2.7 equiv) under nitrogen atmosphere. After the appearance of a dark green color, a solution of $R_3P^{34}S$ (300 mg, 0.53 mmol, 1.0 equiv) in dry THF (5 mL) was added dropwise, then the reaction mixture was stirred at room temperature for 30 min until the dark green color persisted. The reaction was quenched by slow addition of water (5 mL), then washed with diethyl ether (20 mL). The aqueous phase was collected and the concentration of $Na_2{}^{34}S$ was assayed with the methylene blue method[7] indicating a total amount of approx. 0.5 mmol (approx. 94% yield).

Synthesis of glutathione trisulfide (GS^{34}SSG)

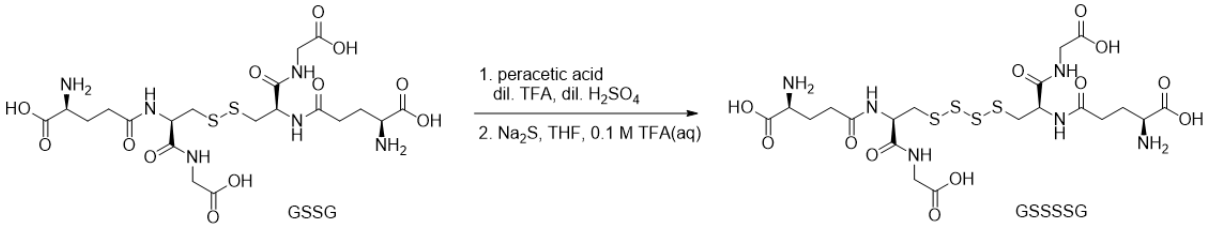


To an ice-cooled solution of glutathione disulfide (GSSG, 300 mg, 0.49 mmol, 2.77 equiv) in a mixture of 0.1 M $TFA_{(aq)}$ (3.5 mL) and 1 M $H_2SO_{4(aq)}$ (0.5 mL) was added peracetic acid (110 µL, 30wt% in dilute acetic acid, 0.43 mmol, 2.46 equiv). After 2 h, HPLC/MS reaction control indicated complete conversion to the respective S-oxide and ice-cold acetone (15 mL) was added to the reaction mixture. The sticky precipitate was washed with acetone and redissolved in degassed 0.1 M $TFA_{(aq)}$ (3 mL). A solution of $R_3P^{34}S$ (100 mg, 0.177 mmol, 1.0 equiv) in THF (5 mL) was added under a nitrogen atmosphere and the mixture was stirred at 40 °C in a sealed flask under nitrogen for 96 h. GS^{34}SSG was purified by preparative HPLC (gradient: 1 → 30% B in 11 min), to provide the 2 x TFA salt of a GS^{34}SSG as a fluffy white powder (30 mg, 34 µmol, 19% yield) after lyophilization. HPLC/MS analysis indicates >95% purity.

HR-MS-MS (*m/z*): $[M+H]^+$ calcd for $C_{20}H_{33}N_6O_{12}S_2{}^{34}S^+$: 647.1271; found: 647.1283. MS2 fragmentation pattern is matching literature data[8].

GS^{34}SSG was further used as a solution in PBS and the concentration was assayed with the cold cyanolysis[3] method directly before use.

Synthesis of glutathione tetrasulfide (GSSSSG)



GSSSSG synthesis was based on the procedure of CysSSSCys synthesis[9]. GSSG S-oxide was prepared as described in the synthesis of GS^{34}SSG from glutathione disulfide (GSSG, 97 mg, 0.158 mmol, 1.0 equiv) by oxidation with peracetic acid (36 µL, 30wt% in dilute acetic acid, 0.14 mmol, 0.9 equiv) in a mixture of 0.1 M $TFA_{(aq)}$ (1.14 mL) and 1 M $H_2SO_{4(aq)}$ (0.163 mL). After precipitation and washing with acetone, the GSSG S-oxide was dissolved in 0.1 M TFA (1 mL), then anhydrous Na_2S (18 mg, 0.230 mmol, 1.45 equiv) was added and the solution was stirred at room temperature for 3 h. The resulting polysulfide mixture was separated by preparative HPLC (gradient: 1 → 30% B in 11 min) to provide the 2 x TFA salt of a GSSSSG as a fluffy white powder (4.0 mg, 4.4 µmol, 3% yield) after lyophilization. HPLC/MS analysis indicates >95% purity.

HR-MS-MS (*m/z*): $[M+H]^+$ calcd for $C_{20}H_{33}N_6O_{12}S_4^+$: 677.1034; found: 677.1001. MS2 fragmentation pattern is matching literature data[8].

For use, GSSSSG was dissolved in PBS and the concentration was determined with the cold cyanolysis method[3] directly before use.

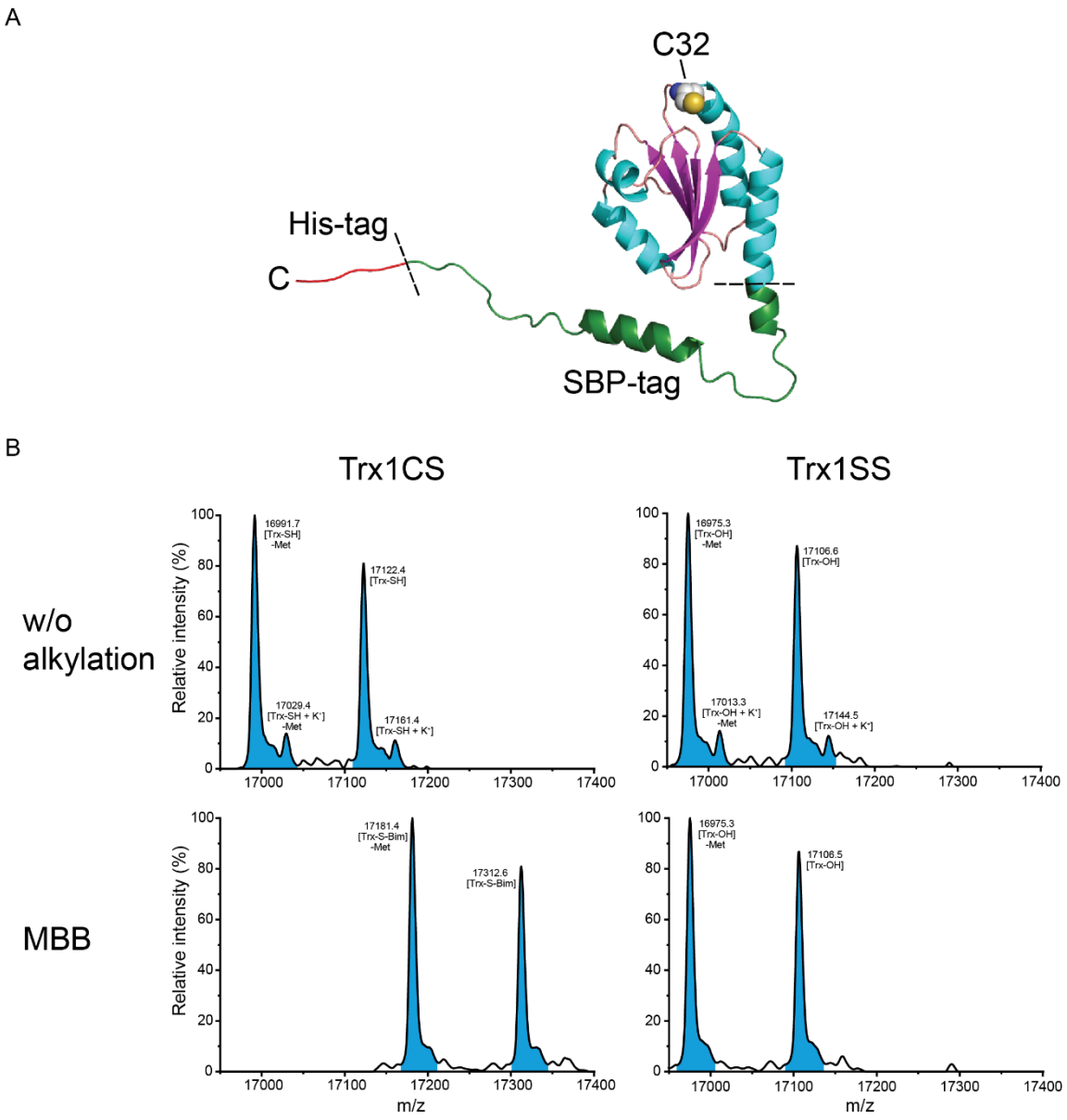
NMR spectroscopy

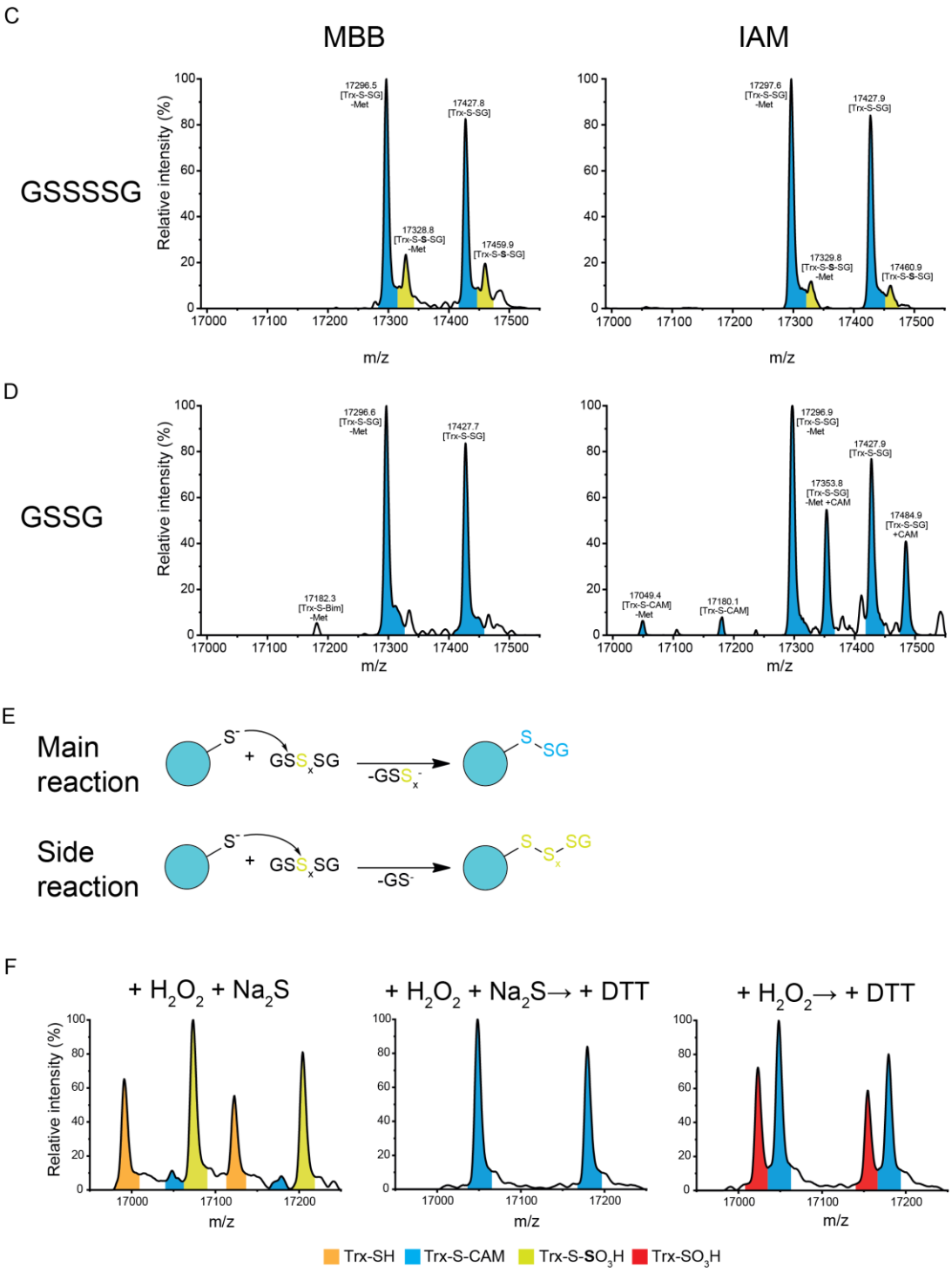
CAM-SS-CAM was dissolved in deuterated DMSO and NMR spectra were recorded on a Bruker Avance III 14.1 T NMR spectrometer operating at 600 for 1H nuclei. Spectra were recorded at 298.1 K.

Preparative HPLC

Preparative HPLC was performed on an Agilent 1260 Infinity system equipped with a Kinetex 5 µm C18 100 Å, AXIA packed 250 x 21.2 mm column, operated at 20 °C with a flow rate of 15.0 mL/min, using water with 0.05% TFA as solvent A and MeCN with 0.05% TFA as solvent B. UV-signals were detected at 254 and 230 nm. Solvent of combined product fractions was removed by lyophilization with a Christ alpha 2-4 LD plus freeze-dryer.

Supplementary Figures




C
MBB
IAM
GSSSSG
Relative intensity (%)
17296.5 [Trx-S-SG] -Met
17427.8 [Trx-S-SG]
17328.8 [Trx-S-S-SG] -Met
17459.9 [Trx-S-S-SG]
17297.6 [Trx-S-SG] -Met
17427.9 [Trx-S-SG]
17329.8 [Trx-S-S-SG] -Met
17460.9 [Trx-S-S-SG]
m/z
D
GSSG
17296.6 [Trx-S-SG] -Met
17427.7 [Trx-S-SG]
17182.3 [Trx-S-Bim] -Met
17296.9 [Trx-S-SG] -Met
17427.9 [Trx-S-SG]
17353.8 [Trx-S-SG] -Met +CAM
17484.9 [Trx-S-SG] +CAM
17049.4 [Trx-S-CAM] -Met
17180.1 [Trx-S-CAM]
E
Main reaction
Side reaction
-GSSx-
-GS-
F
+ H2O2 + Na2S
+ H2O2 + Na2S→ + DTT
+ H2O2→ + DTT
Trx-SH
Trx-S-CAM
Trx-S-SO3H
Trx-SO3H

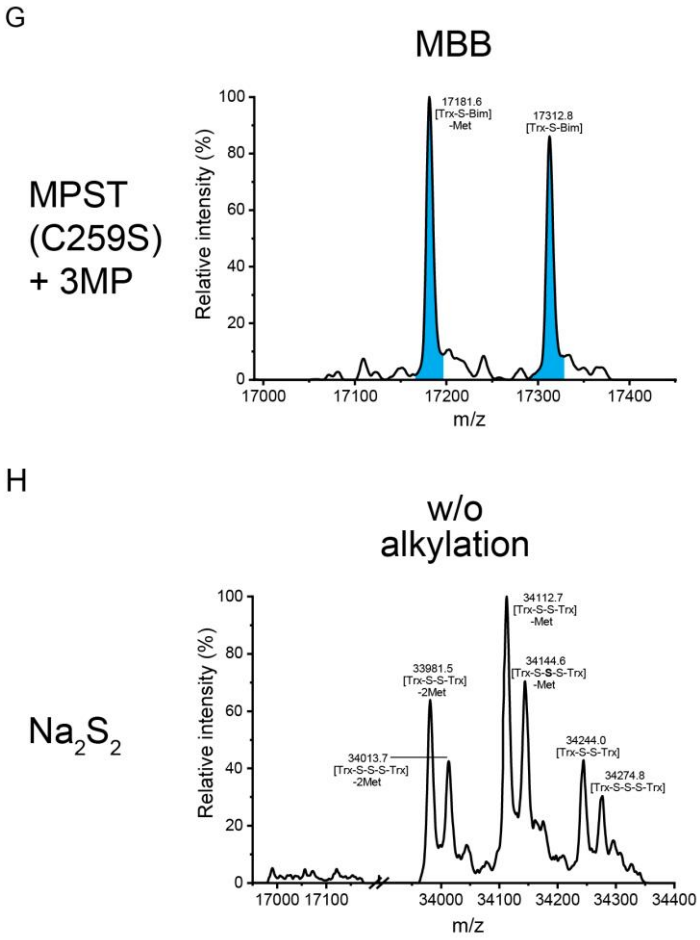


Figure S1. Preparation, persulfidation and alkylation of Trx1CS.

(A) Three-dimensional structure of Trx1(CSAAA)-SBP-His as predicted by the AlphaFold algorithm (https://alphafold.ebi.ac.uk/). The C-terminal hexahistidine tag is shown in red, the SBP tag in green, and the surface-exposed single cysteine residue is shown in space-filling mode. (B) Whole protein mass spectra of Trx1CS (left panels) and Trx1SS (right panels) (13.5 µM), before (upper panels) and after alkylation with MBB (1 mM) (lower panels). (C-D) Whole protein mass spectra of Trx1CS treated with GSSSSG (C) or GSSG (D) (300 µM each) and alkylated with either MBB (left panels) or IAM (right panels) (1 mM each). In (D) additional IAM overalkylation peaks were observed. Peaks representing CAM modified glutathionylated protein (D, right panel) result from the alkylation of residues other than cysteine. (E) Scheme for the reaction between protein thiols and glutathione polysulfides, explaining the generation of the observed major (P-S-SG) and minor product (P-S-S-SG). (F) Whole protein mass spectra of Trx1CS (13.5 µM) treated with H_2O_2 (50 µM) plus $Na_2{}^{34}S$ (500 µM) (left panel), H_2O_2 plus $Na_2{}^{34}S$, followed by DTT (10 mM) (middle panel), and H_2O_2 followed by DTT (right panel), and subsequently alkylated by IAM (1 mM). Peaks corresponding to thiol-alkylated proteins are highlighted in blue and peaks corresponding to per-/polysulfide-alkylated proteins are highlighted in yellow. In addition, peaks corresponding to hyperoxidized thiols are highlighted in red. Peaks highlighted in orange correspond to unmodified thiols, presumably resulting from the decomposition of hyperoxidized persulfides. (G) Whole protein mass spectrum of Trx1CS (13.5 µM) incubated with MPST(C259S) (10 µM) plus 3MP (40 µM) and alkylated with MBB (1 mM). (H) Whole protein mass spectrum of Trx1CS (13.5 µM) reacted with Na_2S_2 (300 µM), without subsequent alkylation.

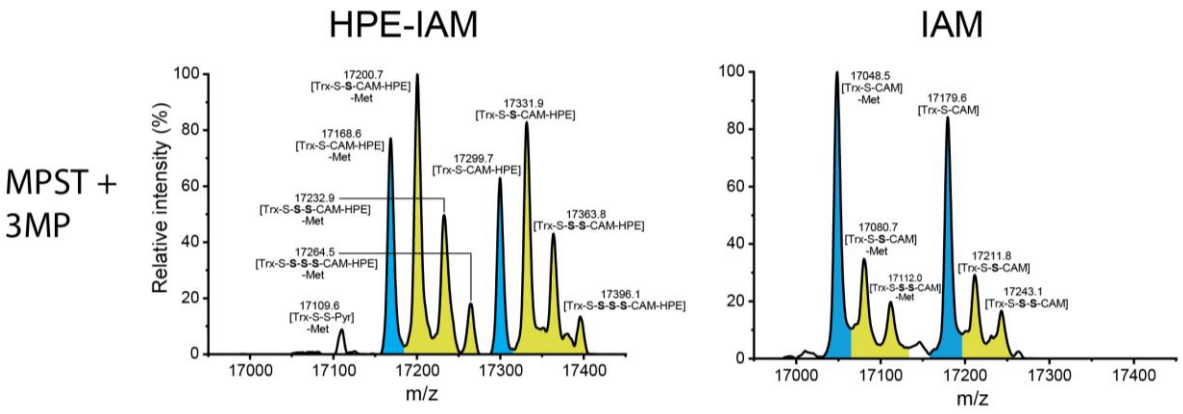


Figure S2. Comparison of different alkylating agents.

Whole protein mass spectra of Trx1CS (13.5 µM) persulfidated with MPST (10 µM) plus 3MP (40 µM) and alkylated with either HPE-IAM or IAM (1 mM each). Peaks corresponding to unmodified or thiol-alkylated proteins are highlighted in blue and peaks corresponding to per-/polysulfide-alkylated proteins are highlighted in yellow.

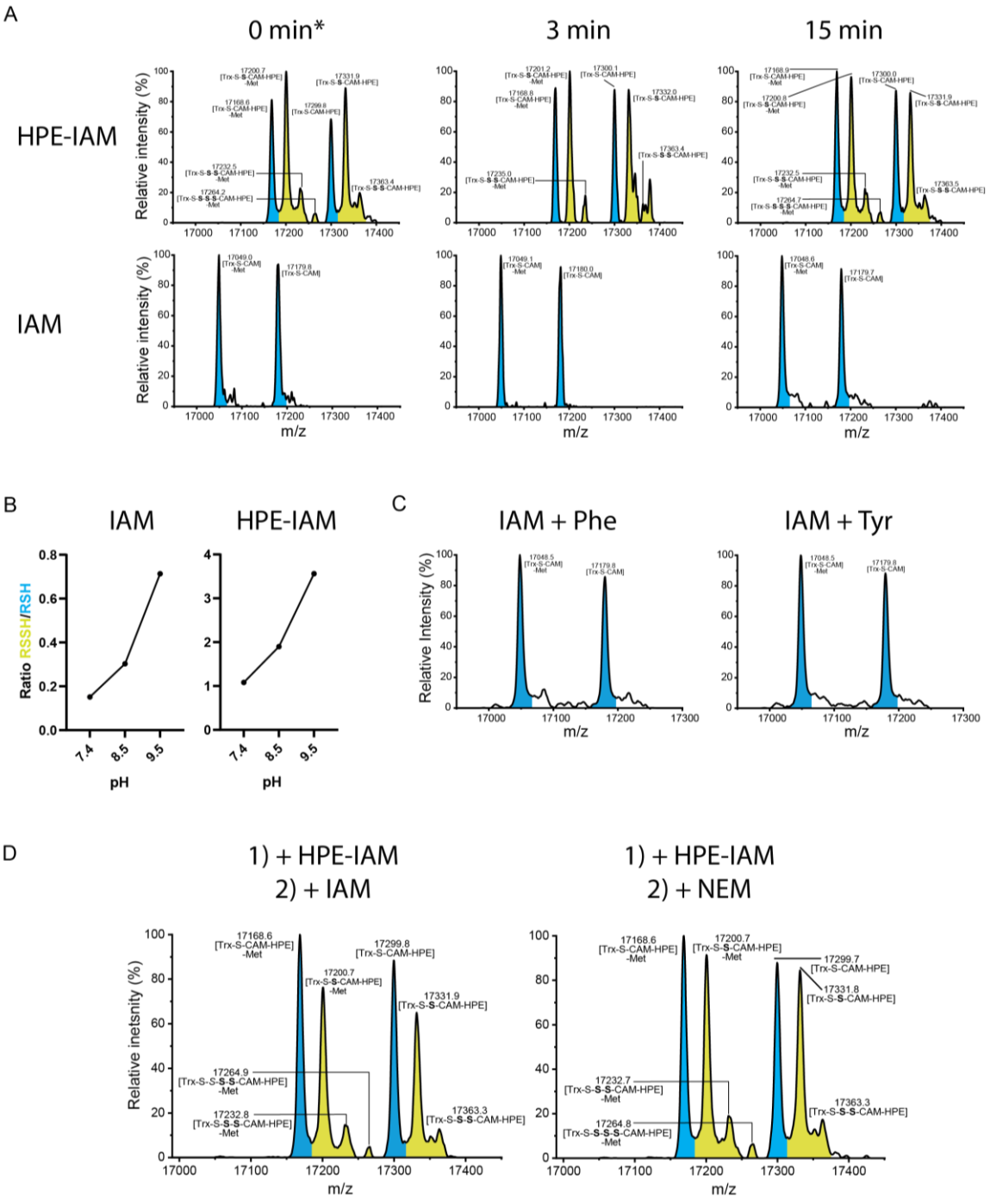


Figure S3. Comparison of IAM and HPE-IAM.

(A) Whole protein mass spectra of Trx1CS (13.5 µM) persulfidated with Na_2S_2 (300 µM) and alkylated with either HPE-IAM (upper panels) or IAM (lower panels) (1 mM each) for different time periods (0, 3, and 15 min). *The 0 min time point represents the shortest possible incubation period. (B) Quantification of alkylated persulfides for main Figure 3A. (C) Whole protein mass spectra of Trx1CS persulfidated with Na_2S_2 and alkylated with IAM in the presence of either phenylalanine (left panel) or tyrosine (right panel) (1 mM each). (D) Whole protein mass spectra of Trx1CS persulfidated with Na_2S_2, alkylated with HPE-IAM and then incubated with either IAM (left panel) or NEM (right panel) (1 mM each). Peaks corresponding to unmodified or thiol-alkylated proteins are highlighted in blue and peaks corresponding to per-/polysulfide-alkylated proteins are highlighted in yellow.

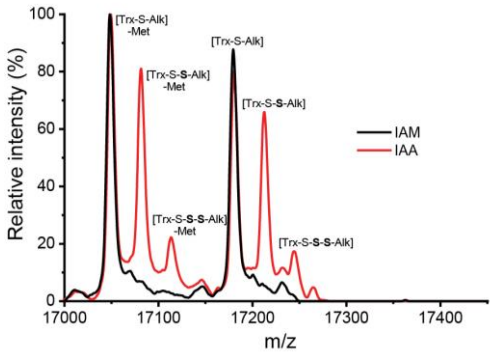


Figure S4. Comparison of IAM and IAA.

Overlay of whole protein mass spectra of Trx1CS (13.5 µM) persulfidated with Na_2S_2 (300 µM) and alkylated with either IAM or IAA (1 mM each).

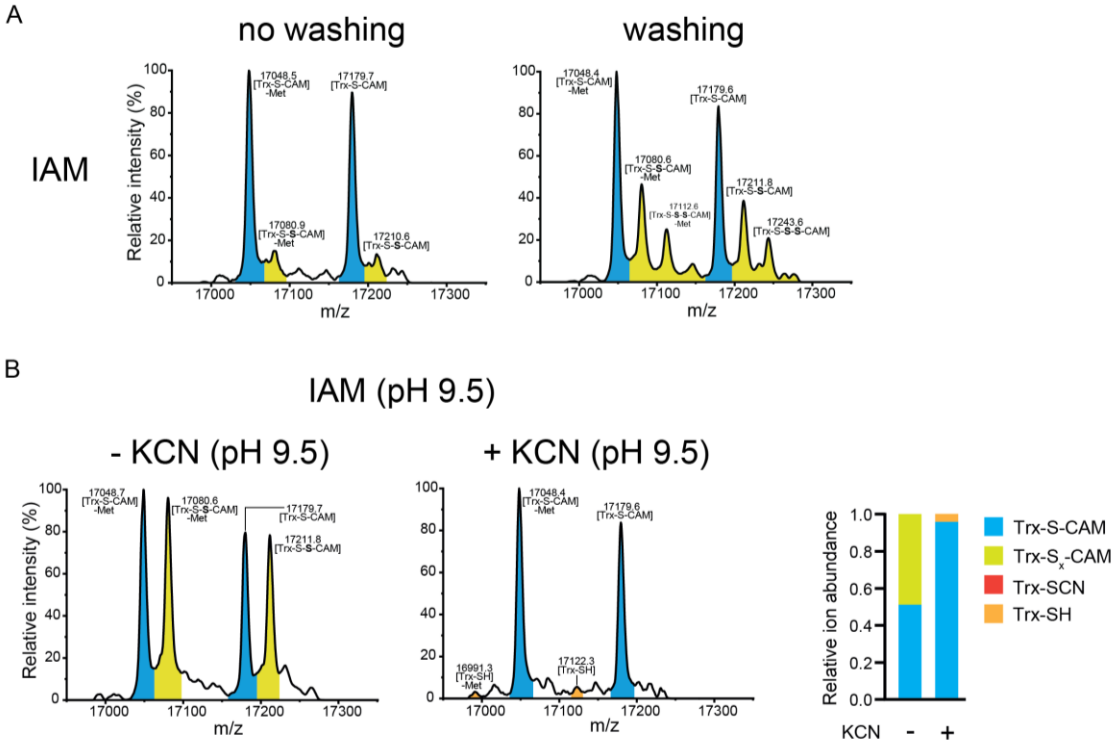


Figure S5. Trapping of persulfides with IAM.

(A) Whole protein mass spectra of Trx1CS (13.5 µM) persulfidated with Na_2S_2 (300 µM), either directly alkylated with IAM (1 mM) (left panel, same as Fig. 3A) or washed 3 times with buffer prior to alkylation with IAM (1 mM) (right panel). (B) Whole protein mass spectra of Trx1CS persulfidated with Na_2S_2, alkylated with IAM at pH 9.5, and then treated with buffer (left panel) or KCN (0.5 mM) (right panel) at pH 9.5. Quantification of product species formed with and without cyanide (right panel). Peaks corresponding to unmodified or thiol-alkylated proteins are highlighted in blue and peaks corresponding to per-/polysulfide-alkylated proteins are highlighted in yellow.

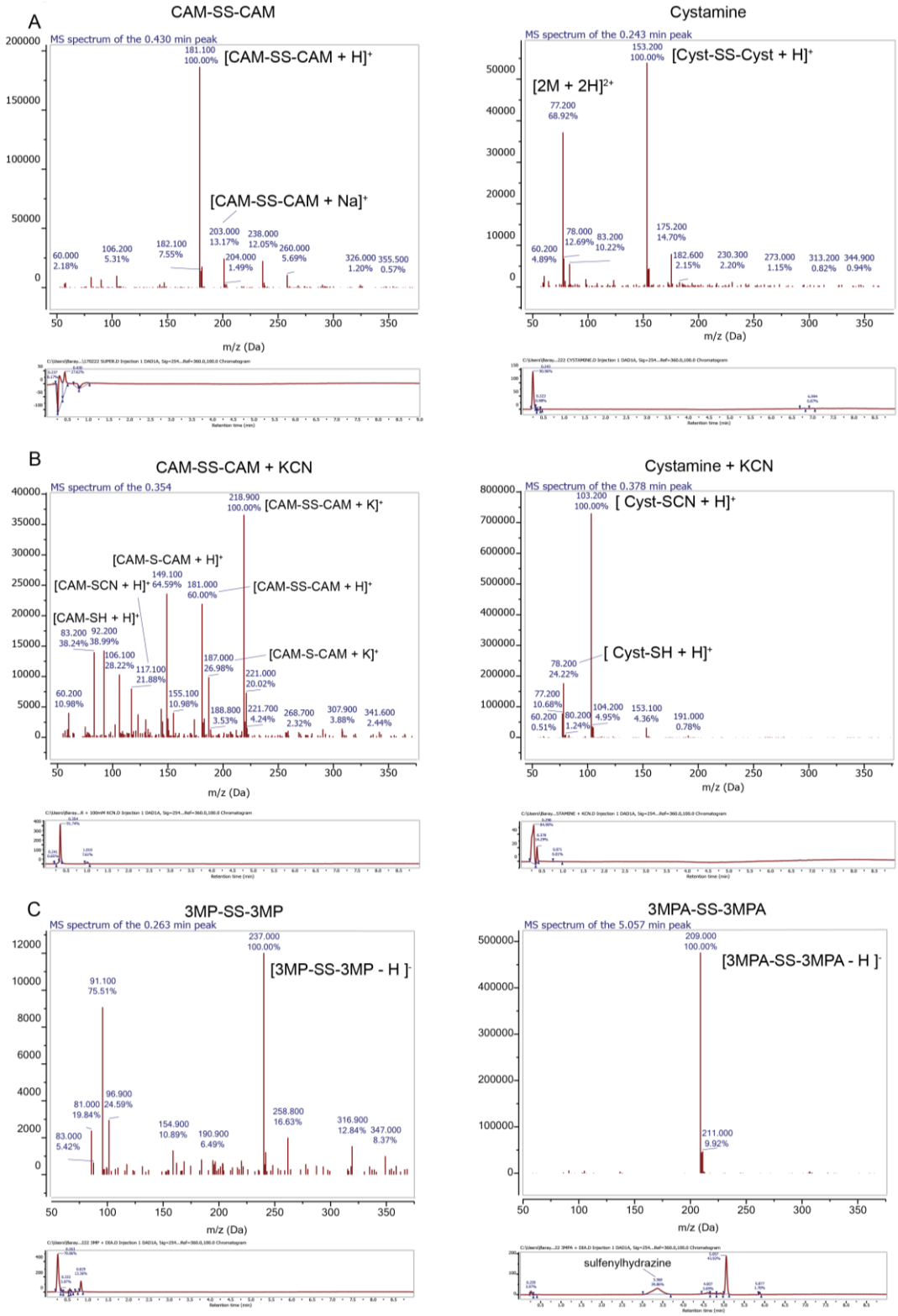


Figure S6. 2-mercaptoacetamide disulfide reacts with cyanide to form the corresponding thioether.

(A) Mass spectra of 2-mercaptoacetamide disulfide (CAM-SS-CAM, left panel) and cystamine (right panel) (25 mM each) in the absence of KCN. (B) Mass spectra of 2-mercaptoacetamide disulfide (CAM-SS-CAM, left panel) and cystamine (right panel) (25 mM each) in the presence of KCN. (C) Mass spectra of 3-mercaptopyruvate disulfide (3MP-SS-3MP) (left panel), and 3-mercaptopropionic acid disulfide (3MPA-SS-3MPA) (right panel), (2.5 mM each).

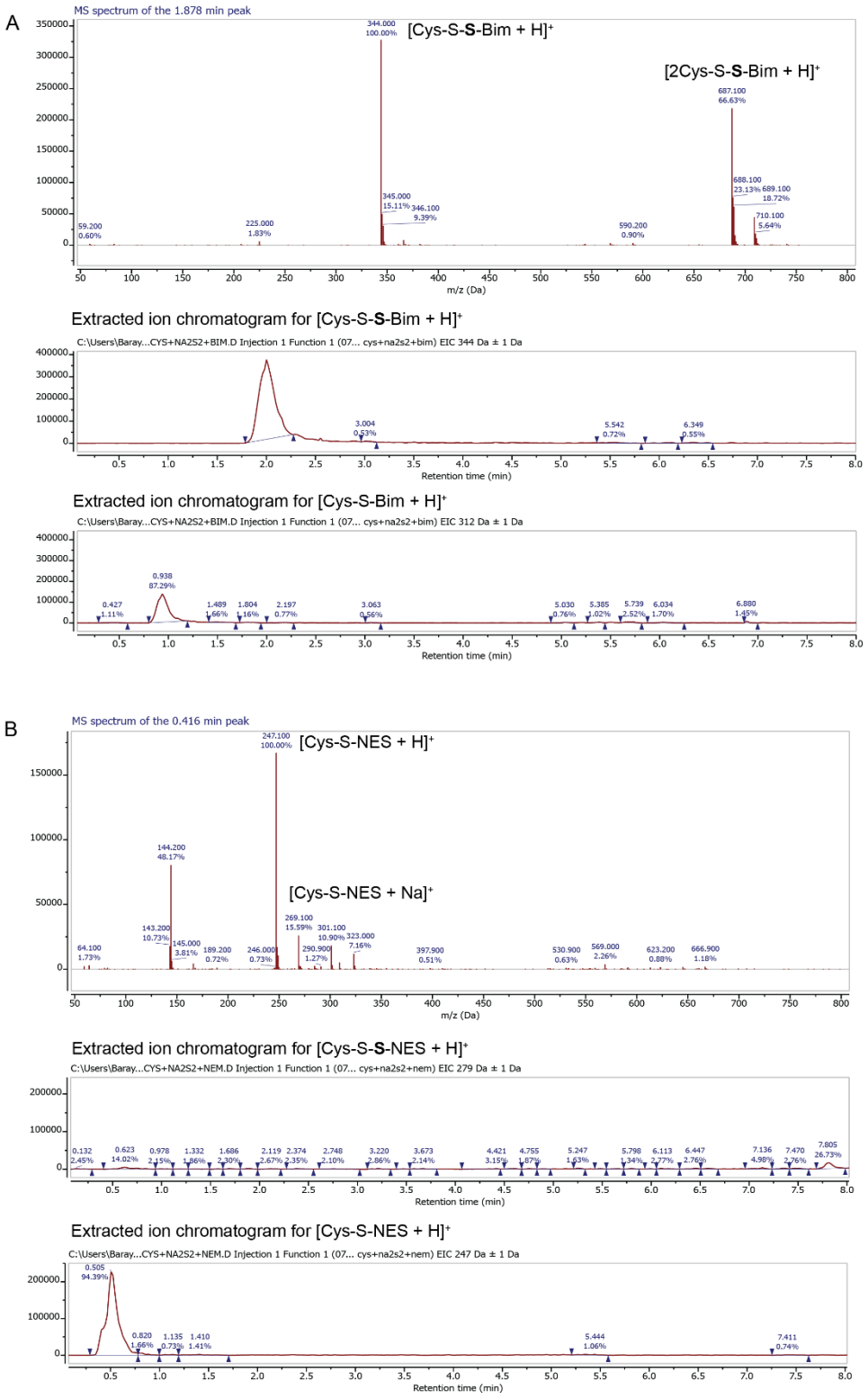


Figure S7. Cysteine persulfide can be trapped with MBB but not with NEM.

(A) Mass spectra of cysteine persulfide (1 mM) alkylated with MBB (upper panel) (10 mM). Extracted ion chromatograms for Cys-S-S-Bim and Cys-S-Bim (lower panels). (B) Mass spectra of cysteine persulfide (1 mM) alkylated with NEM (upper panel) (10 mM). Extracted ion chromatograms for Cys-S-S-NEM and Cys-S-NEM (lower panels)

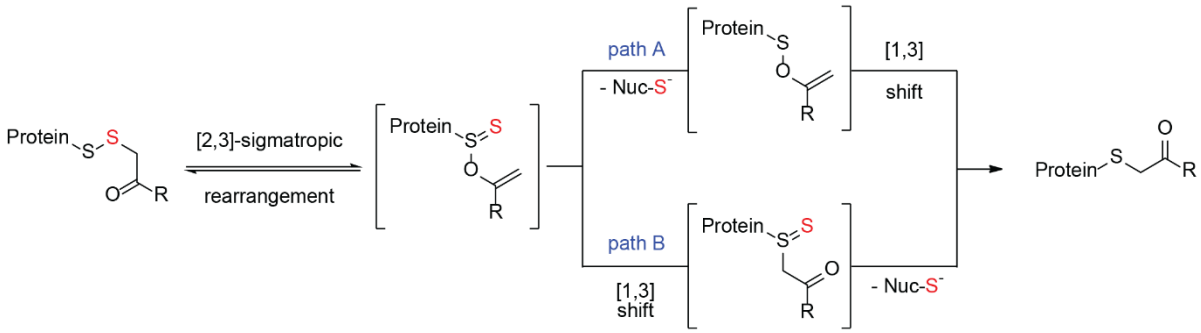


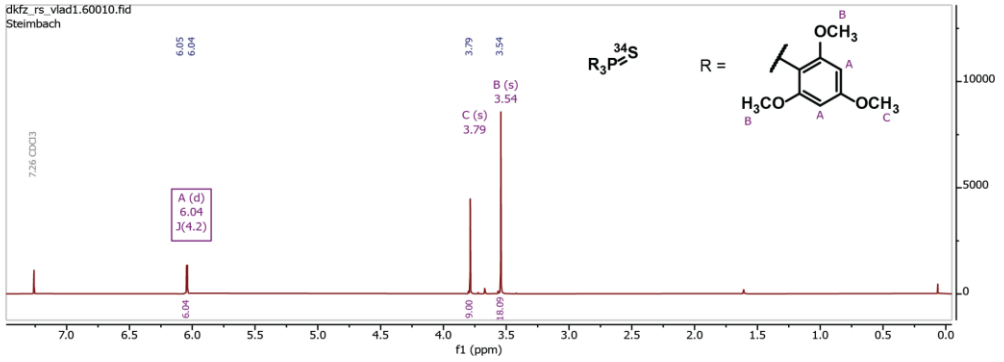
Figure S8. Hypothetical mechanism of disulfide-thiosulfoxide tautomerization and desulfurization.

A [2,3]-sigmatropic rearrangement leads to an unstable thiosulfoxide, which may first desulfurize and then undergo a [1,3]-shift to the thioether (path A), or first undergo a [1,3]-shift and then desulfurize to the thioether (path B).

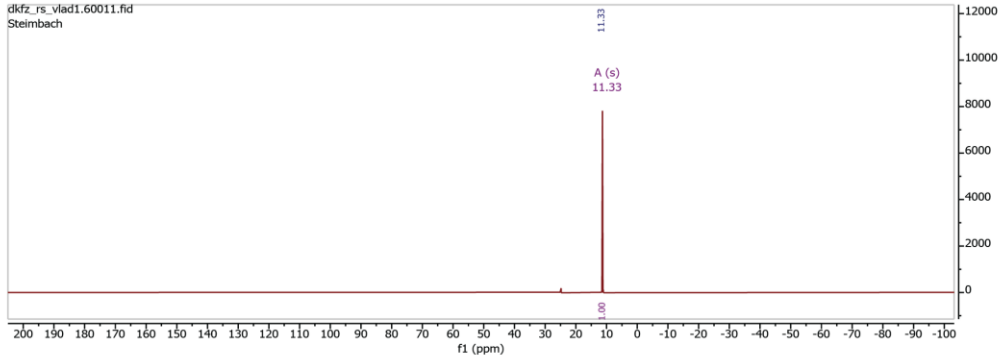
SUPPORTING INFORMATION

NMR and MS spectra

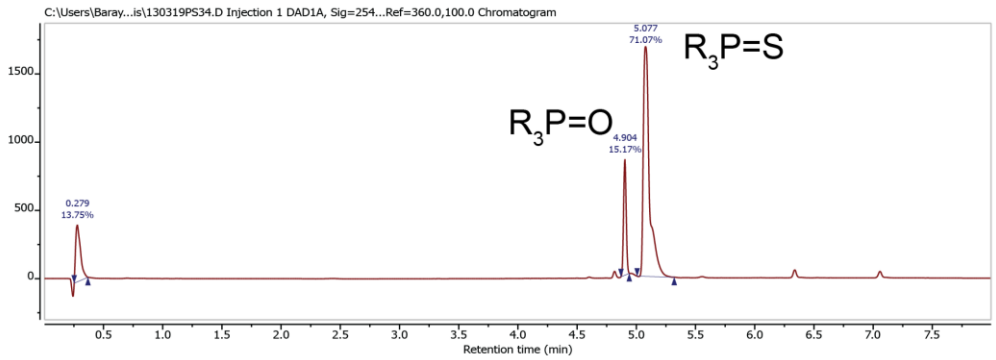
^{1}H NMR



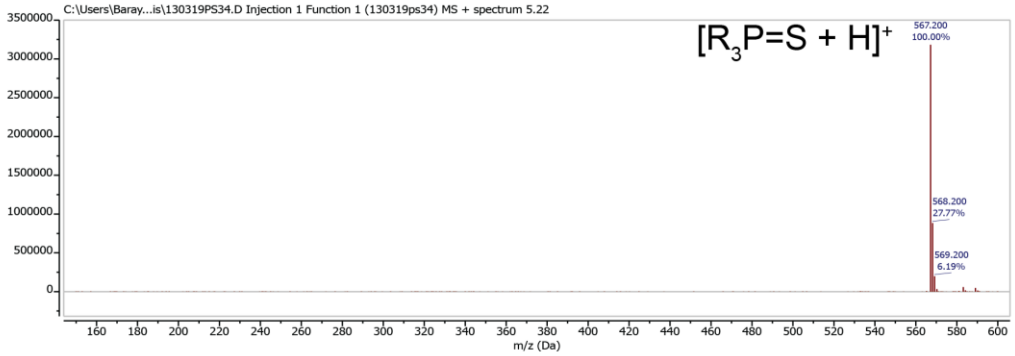
^{31}P NMR



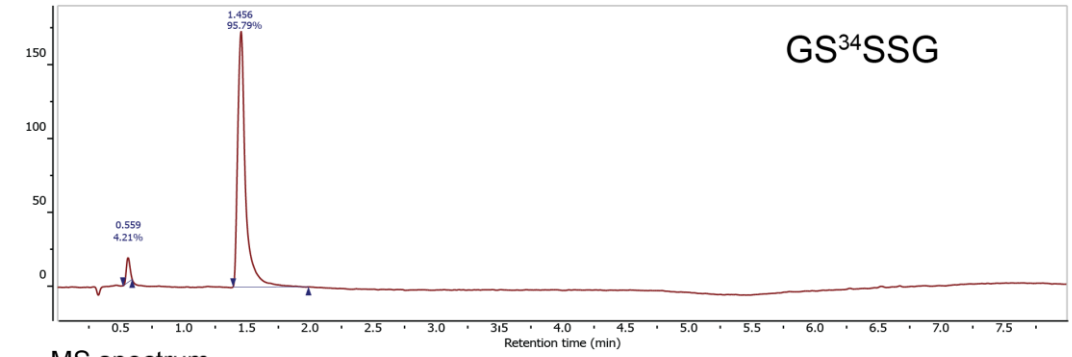
HPLC chromatogram (254 nm)



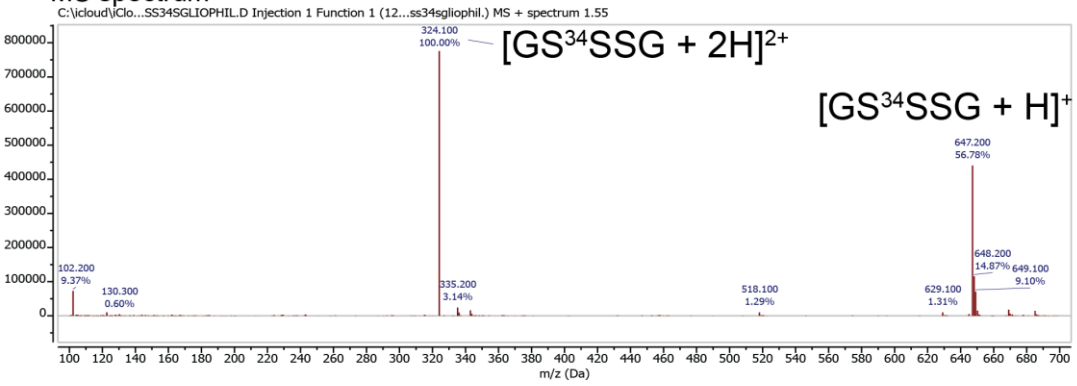
MS spectrum



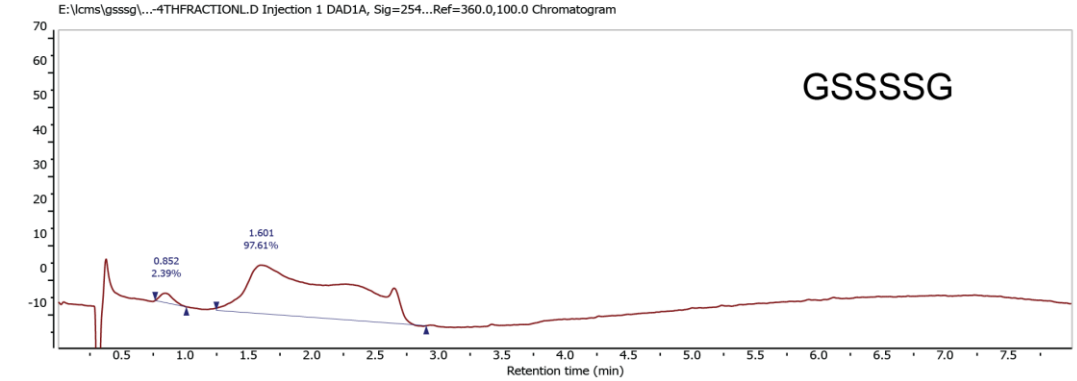
HPLC chromatogram (254 nm)



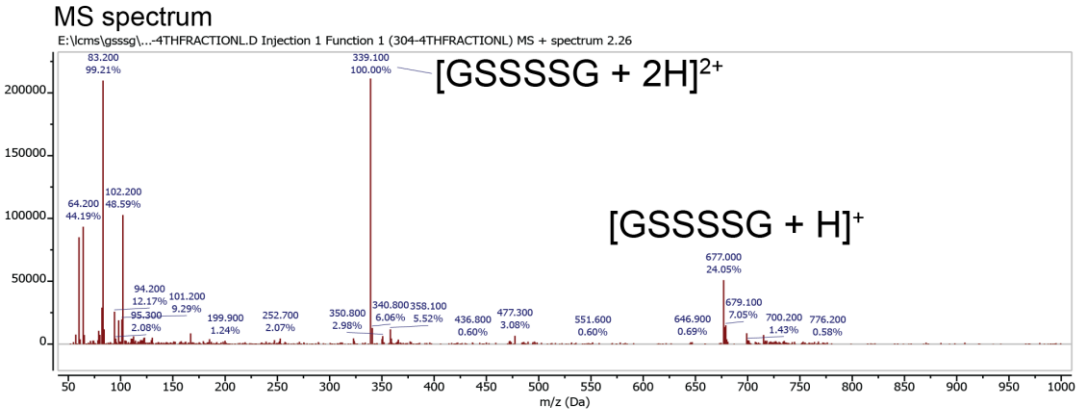
MS spectrum



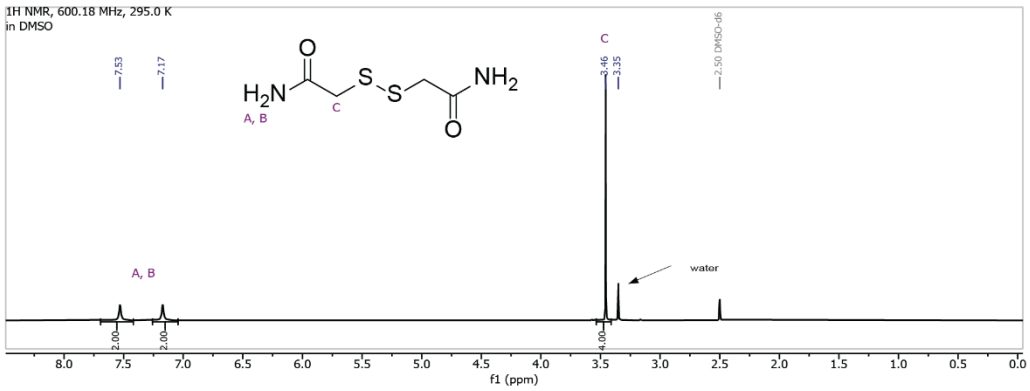
HPLC chromatogram (254 nm)



MS spectrum



^{1}H NMR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in DMSO
7.53
7.17
3.46
3.35
2.50 DMSO-d6
A, B
C
water
2.00
2.00
4.00
f1 (ppm)

References

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Author Contributions

Funding acquisition: TPD; Conceptualization: DS, UB, TPD; Methodology: DS, UB, RRS, DT, AKM, TPD; Supervision: AKM, TPD; Investigation: DS, UB, RRS, DT; Formal Analysis: DS, UB, RRS, AKM, TPD; Visualization: DS, UB, RRS; Writing: DS, UB, RRS, DT, AKM, TPD.