Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Iterative synthetic strategies and gene deletant experiments enable the first identification of polysulfides in Saccharomyces cerevisiae

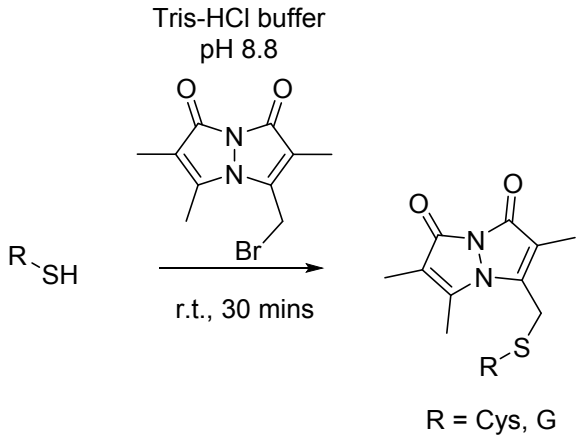
Lisa I. Pilkington,† Rebecca C. Deed, Katie Parish-Virtue, Chien-Wei Huang, Michelle E. Walker, Vladimir Jiranek, David Barker,† Bruno Fedrizzi†

*Corresponding authors: Lisa I. Pilkington, lisa.pilkington@auckland.ac.nz, David Barker, d.barker@auckland.ac.nz, Bruno Fedrizzi, b.fedrizzi@auckland.ac.nz

Table of Contents

Procedure to derivatize hydropolysulfides

To establish a suitable synthetic procedure to derivatise the hydropolysulfides, we first decided to trial the derivatization with both cysteine and glutathione (Scheme S1). Cysteine and glutathione, in turn, were stirred in a Tris-HCl buffer. A solution of monobromobimane in MeCN was then added to the reaction, which was left to stir for 30 mins. Analysis of the reaction mixture confirmed the successful derivatization of cysteine and glutathione, verifying the method planned for the derivatization of any hydropolysulfides formed (see Figure S1).



Scheme S1: Synthesis of CysBim and GBim.

Cysteine-bimane, CysBim: To a solution of cysteine (5 mg, 0.041 mmol) in Tris-HCl buffer (pH 8.8, 100 mmol L^{-1}, 1 mL) at room temperature, a solution of monobromobimane (22 mg, 0.082 mmol) in MeCN (0.3 mL) was added and the reaction stirred for 30 mins. Formation of the derivatized product, CysSBim, was confirmed though LC-MS/MS analysis (see *LC-MS/MS Method* section for details): CysSBim, MS (ESI, positive): 312 (MH^+).

Glutathione-bimane, GBim: To a solution of glutathione (5 mg, 0.016 mmol) in Tris-HCl buffer (pH 8.8, 100 mmol L^{-1}, 1 mL) at room temperature, a solution of monobromobimane (9 mg, 0.032 mmol) in MeCN (0.3 mL) was added and the reaction stirred for 30 mins. Formation of the derivatized product, GSBim, was confirmed though LC-MS/MS analysis (see *LC-MS/MS Method* section for details): GSBim, MS (ESI, positive): 498 (MH^+).

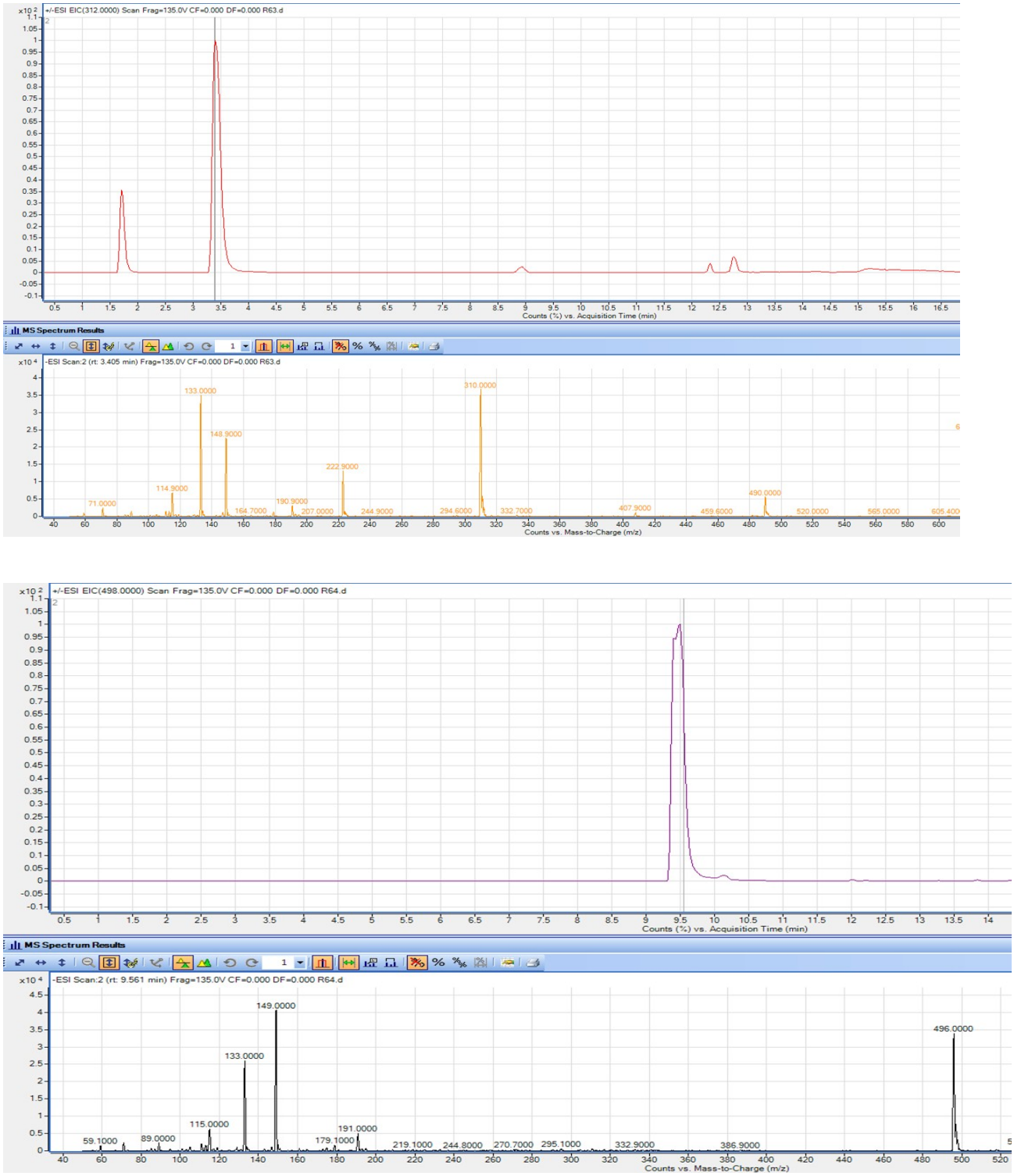
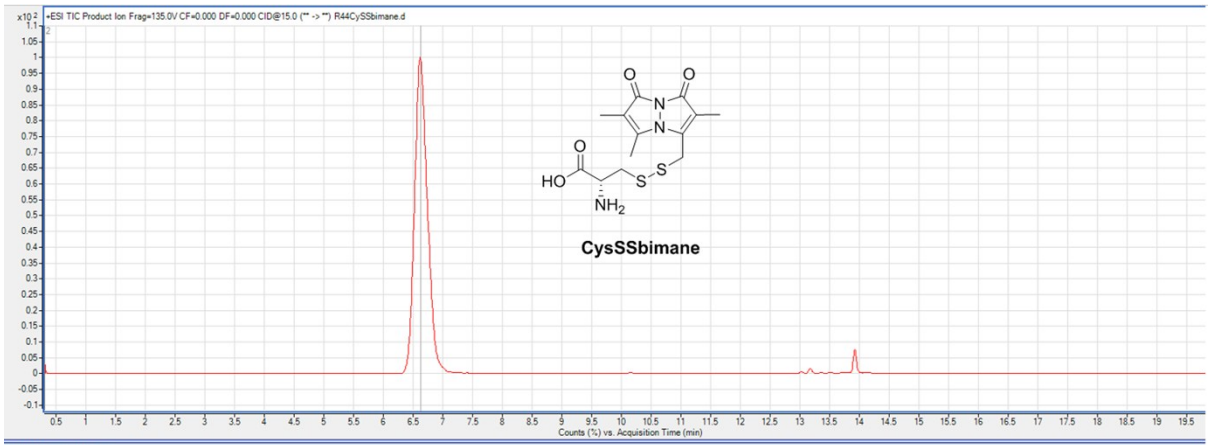
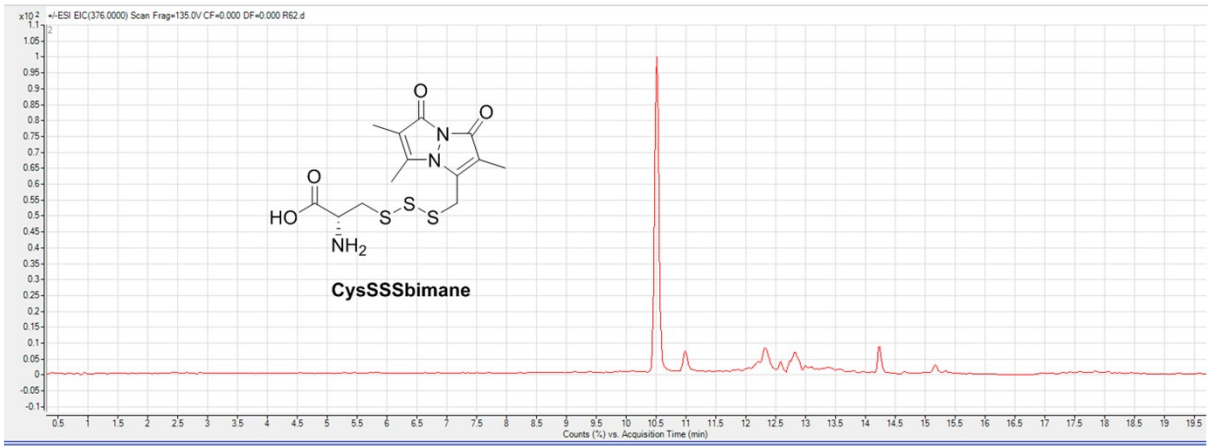


Figure S1: Chromatograms showing the presence and synthesis of derivatized hydropolysulfides; CysSBim (top) and GSBim (bottom).

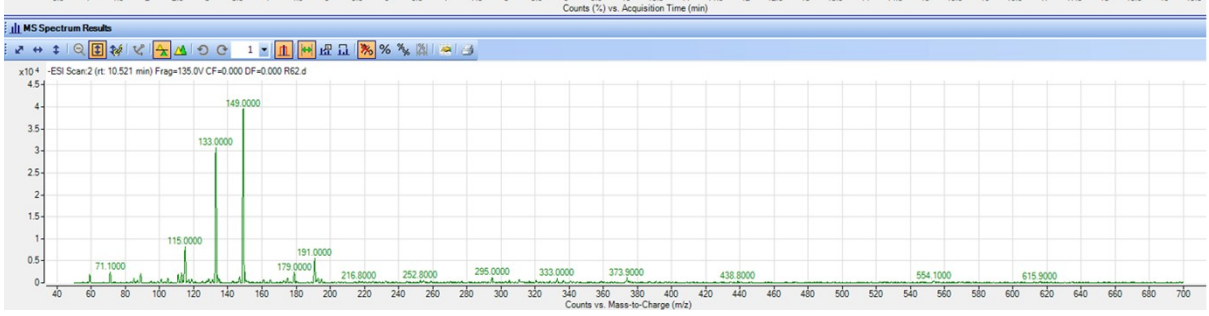

+ESI TIC Product Ion Frag=135.0V CF=0.000 DF=0.000 CID@15.0 (** -> **) R44CySSbimane.d
Counts (%) vs. Acquisition Time (min)

CysSSbimane

MS Spectrum Results
+ESI Product Ion:1 (rt: 6.627 min) Frag=135.0V CF=0.000 DF=0.000 CID@15.0 (344.0000 -> **) R44CySSbimane.d
192.1000
223.1000
53.2000
81.2000
119.3000
149.1000
175.1000
206.3000
256.9000
297.7000
344.0000
Counts vs. Mass-to-Charge (m/z)


+/-ESI EIC(376.0000) Scan Frag=135.0V CF=0.000 DF=0.000 R62.d
Counts (%) vs. Acquisition Time (min)

CysSSSbimane


MS Spectrum Results
-ESI Scan:2 (rt: 10.521 min) Frag=135.0V CF=0.000 DF=0.000 R62.d
149.0000
133.0000
115.0000
71.1000
191.0000
179.0000
216.8000
252.8000
295.0000
333.0000
373.9000
438.8000
554.1000
615.9000
Counts vs. Mass-to-Charge (m/z)

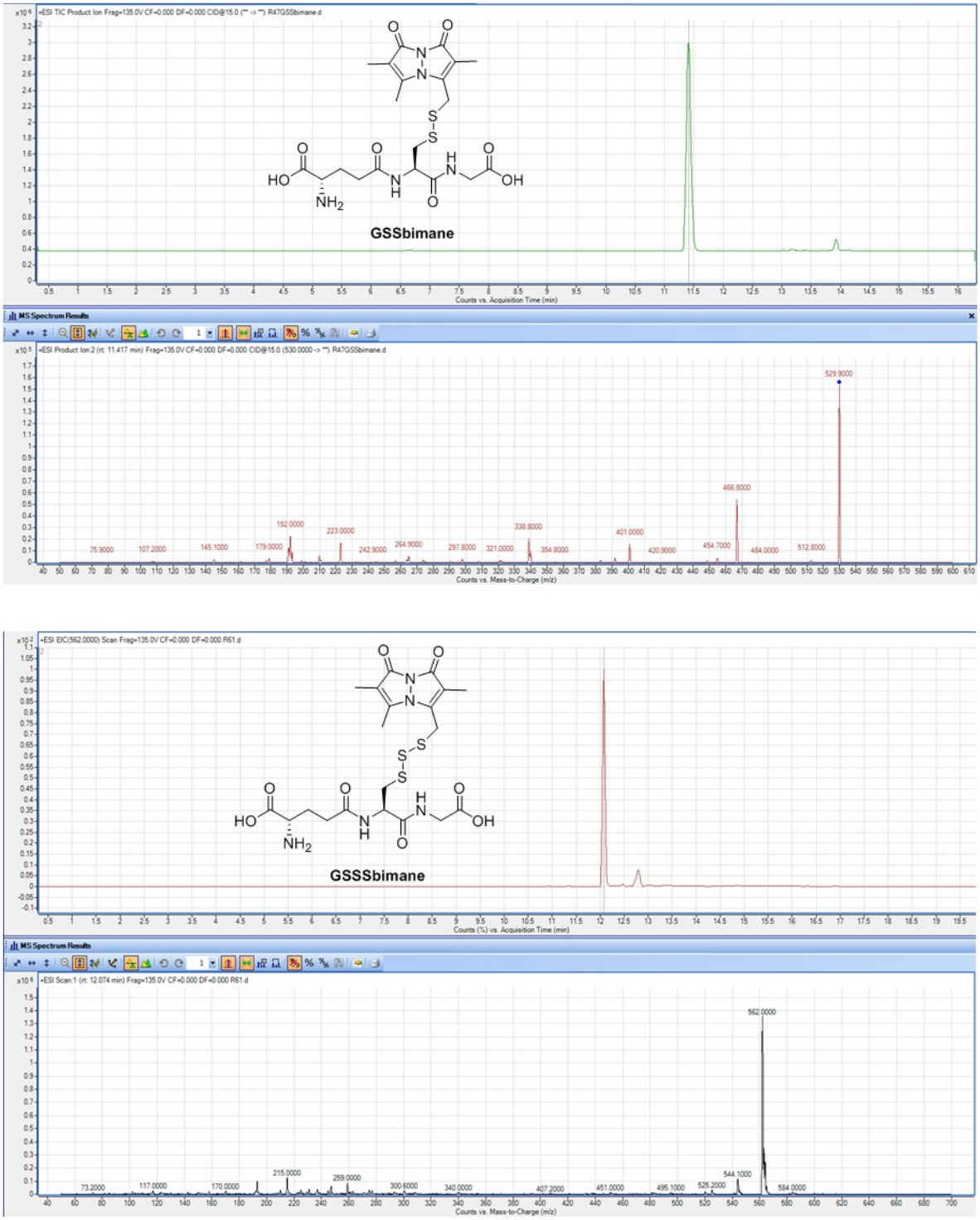
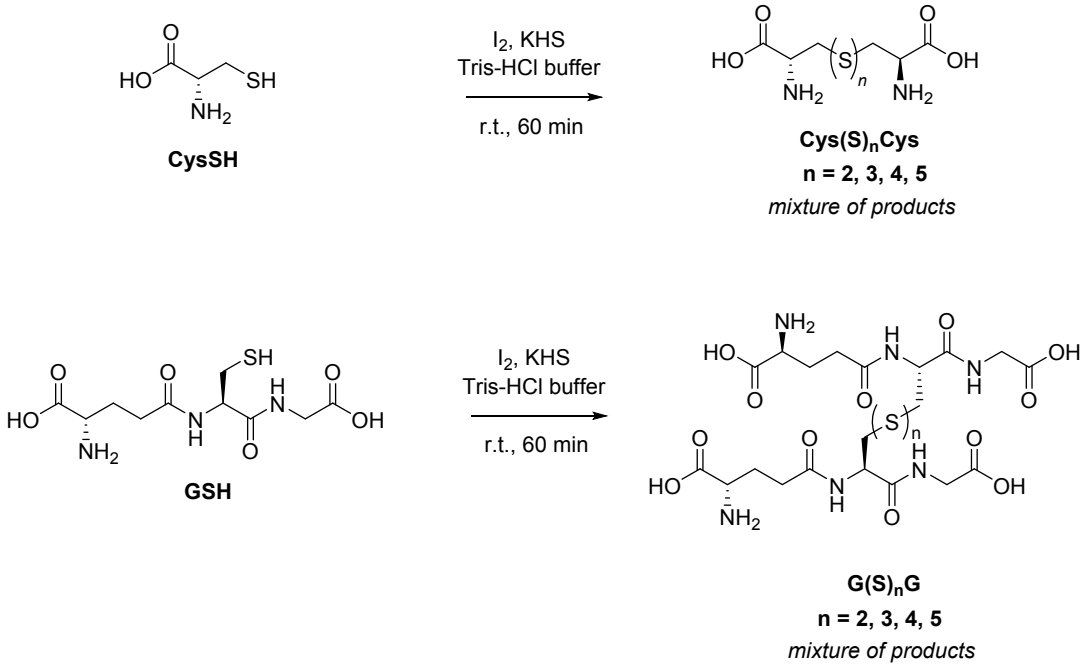


Figure S2: Chromatograms of RSSBim and RSSSBim, R = Cys and G (Glutathione).

Description of synthesis of oxidized polysulfides and hydropolysulfides

In this procedure, cysteine and glutathione, in turn, were reacted with iodine in the presence of KHS as the sulfur-source, in a Tris-HCl buffer (Scheme S2). This reaction successfully yielded oxidized polysulfides $CysS(S)_nCys$ and $GS(S)_nG$, with n = 2-5, verified by the use of LC-MS/MS (see Figure S2 for the chromatograms of the products). To unselectively synthesize the required hydropolysulfides, cysteine and glutathione were first reacted with KHS and NOC-7 to produce the hydropolysulfides, $Cys(S)_nH$ and $G(S)_nH$,[15] which were immediately derivatized *in situ*, using the developed procedure (Scheme S3). As for the oxidized polysulfides, the presence of the desired products was confirmed through LC-MS/MS analysis (Figure S3), verifying the successful synthesis of $Cys(S)_nBim$ and $G(S)_nBim$, where n = 1-4.



Scheme S2: Synthesis of oxidized polysulfides, $CysS(S)_nCys$ and $GS(S)_nG$.

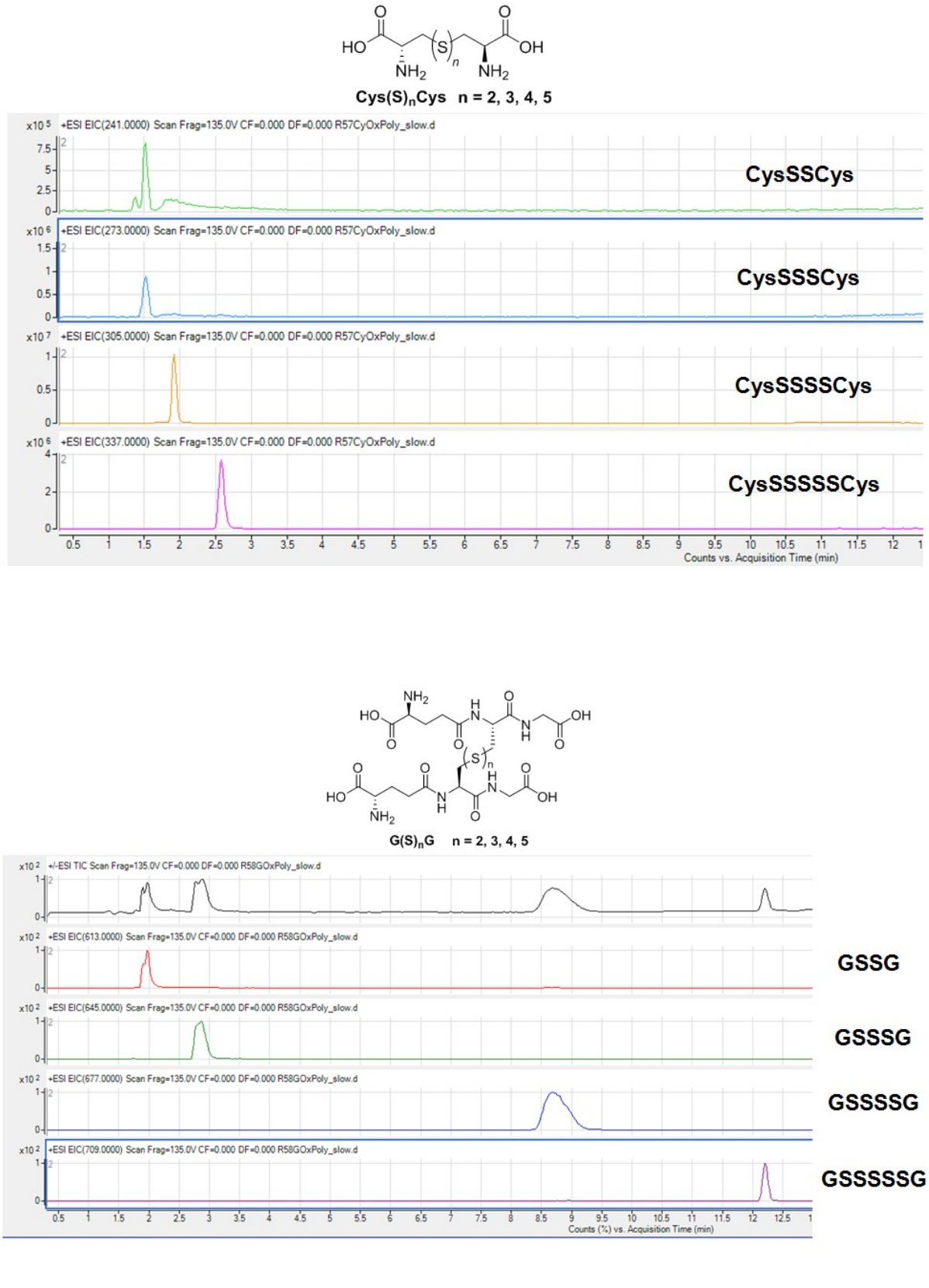
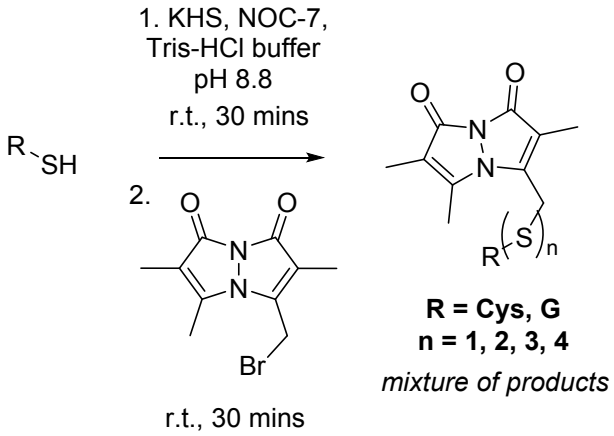


Figure S3: Chromatograms showing the presence and synthesis of oxidized polysulfides; $CysS(S)_nCys$ (top) and $GS(S)_nG$ (bottom).



Scheme S3: Synthesis of $Cys(S)_nBim$ and $G(S)_nBim$.

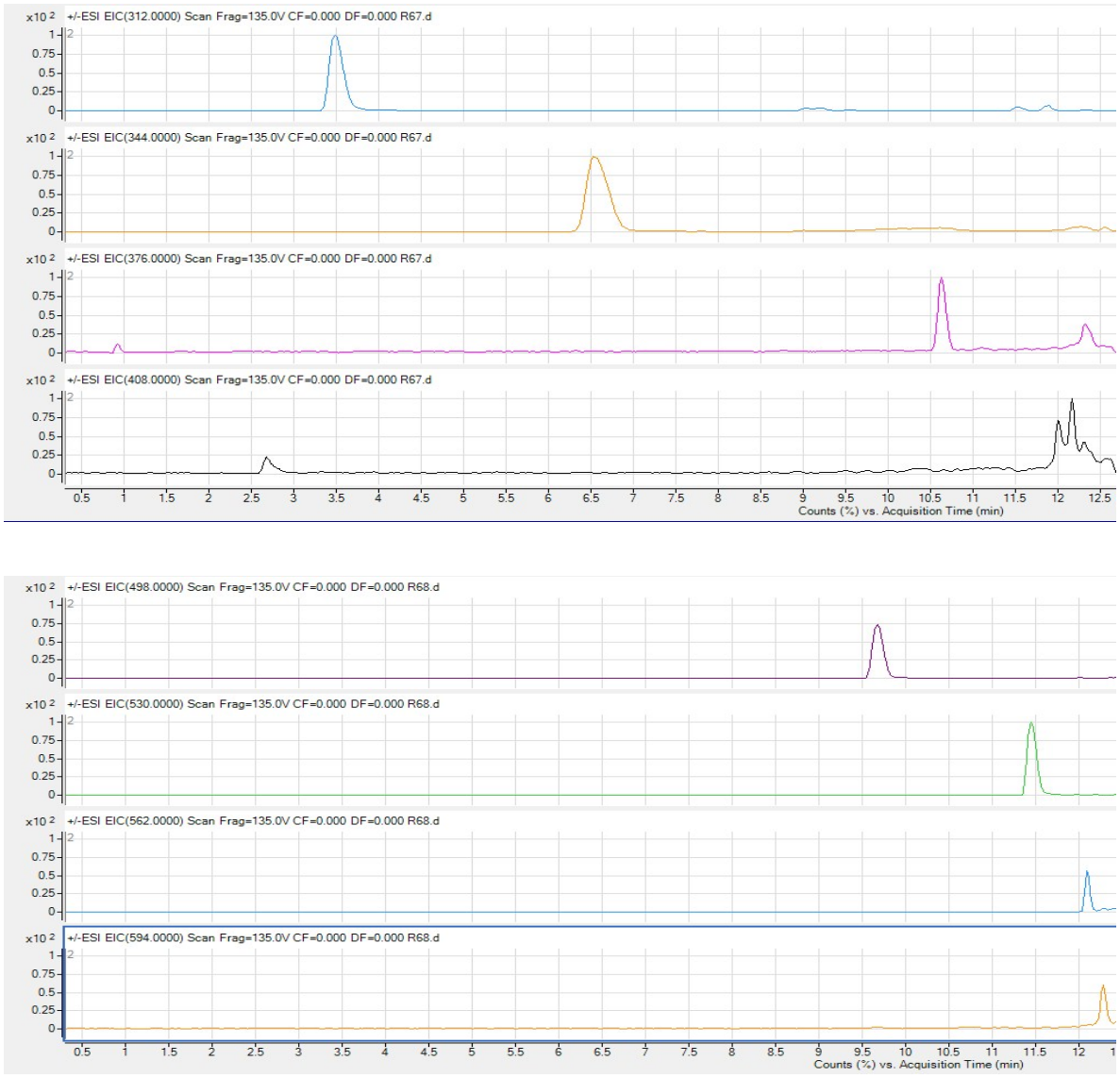
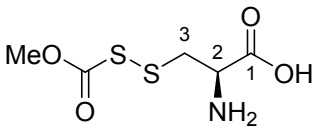


Figure S4: Chromatograms showing the presence and synthesis of the bimane derivatives of hydropolysulfides; $Cys(S)_nBim$ (top) and $G(S)_nBim$ (bottom).

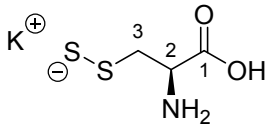
Synthetic experimental procedures

SS-Cysteine O-methyl carbono(dithioperoxoate)



To a cooled (0 °C) suspension of cysteine (0.5 g, 4.13 mmol) in dioxane (10 mL) under an atmosphere of N_2, methoxycarbonylsulfenyl chloride (0.37 mL, 4.13 mmol) was added dropwise. The reaction was then stirred overnight at room temperature. The crude product was then collected by filtration, washed with ether and then dissolved in MeOH to remove any unreacted, insoluble, starting material. The solvent was then removed *in vacuo* to give *the title product* (1.03 g, quant.) as a white solid which was used without further purification. ^{1}H NMR (400 MHz, D_2O) δ = 3.28 (1H, dd, *J* = 8.8 and 15.0 Hz, H-3_a), 3.44 (1H, dd, *J* = 4.0 and 15.0 Hz, H-3_b), 3.96 (3H, s, OCH_3), 4.29 (1H, dd, *J* = 4.0 and 8.8 Hz, H-2); ^{13}C{^{1}H} NMR (100 MHz, D_2O) δ = 38.3 (C-3), 51.9 (C-2), 56.6 (OCH_3), 170.5 (C-1), 171.8 (C=O); *m/z* (ESI^-): 210 (M-H, 60%), 184 (100) and 120 (50); HRMS (ESI^-) Found (M-H): 209.9893, $C_5H_8NO_4S_2$ calcd 209.9900.

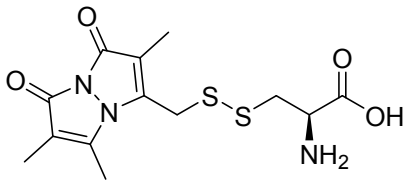
Cysteine persulfide potassium salt, CysSS.K



To a cooled (0 °C) solution of *SS*-cysteine *O*-methyl carbono(dithioperoxoate) (0.4 g, 1.89 mmol) in MeOH (1.6 mL) a solution of KHS (0.27 g, 3.79 mmol) in MeOH (0.8 mL) was added dropwise. The reaction was allowed to warm to room temperature, and stirred for 90 mins. The precipitated product was then collected by filtration, washed with cold MeOH and

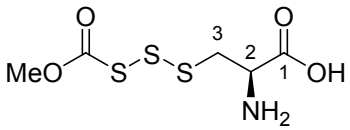
ether, and dried *in vacuo* to give the *title product* (0.36 g, quant.) as a pale green solid which was used without further purification.

Bimane-derivatized cysteine persulfide, CysSSBim



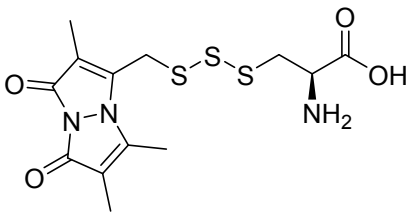
To a solution of CysSS.K (15.4 mg, 0.080 mmol) in Tris-HCl buffer (pH 8.8, 100 mmolL^{-1}, 2.8 mL) at room temperature, a solution of monobromobimane (2.7 mg, 0.01 mmol) in MeCN (1 mL) was added and the mixture stirred at room temperature for 30 mins. Formation of the derivatized persulfides, CysSSBim, was confirmed though LC-MS/MS analysis (see *LC-MS/MS Method* section for details): CysSSBim, MS (ESI, positive): 344 (MH^+).

Methyl 3-cysteinetrisulfane-1-carboxylate:



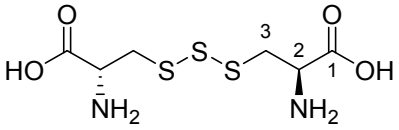
To a cooled (0 °C) suspension of CysSS.K (0.1 g, 0.52 mmol) in dioxane (10 mL) under an atmosphere of N_2, methoxycarbonylsulfenyl chloride (0.047 mL, 0.52 mmol) was added dropwise. The reaction was then stirred for 6 days. The solvent was then removed *in vacuo* to give *the title product* (0.126 g, quant.) as a white solid which was reacted on without further purification. ^{1}H NMR (400 MHz, D_2O) δ = 3.22 (1H, dd, *J* = 8.0 and 15.0 Hz, H-3$_a$), 3.44 (1H, dd, *J* = 4.0 and 15.0 Hz, H-3$_b$), 3.91 (3H, s, OCH_3), 4.29 (1H, dd, *J* = 4.0 and 8.0 Hz, H-2); ^{13}C{^{1}H} NMR (100 MHz, D_2O) δ = 36.7 (C-3), 52.1 (C-2), 56.8 (OCH_3), 169.3 (C-1), 171.2 (C=O); *m/z* (ESI^+): 244 (MH^+, 20%), 212 (100) and 113 (40); HRMS (ESI^+) Found (MH^+): 243.9767, $C_5H_{10}NO_4S_3$ calcd 243.9766.

Bimane-derivatized cysteine hydrotrisulfide, CysSSSBim



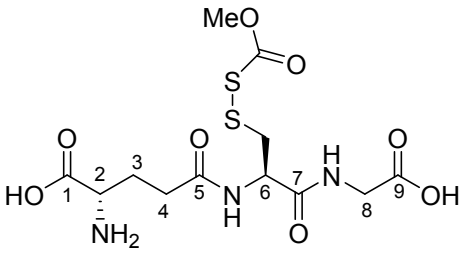
To a solution of methyl 3-cysteinetrisulfane-1-carboxylate (5.0 mg, 0.021 mmol) in MeOH (1 mL) under N_2 at room temperature, KHS (3.0 mg, 0.041 mmol) was added and the mixture stirred for 2 mins. A solution of monobromobimane (28 mg, 0.10 mmol) in MeCN (1 mL) was then added to the reaction which was subsequently left to stir for a further 30 mins. Formation of the derivatized hydrotrisulfide, CysSSSBim, was confirmed though LC-MS/MS analysis (see *LC-MS/MS Method* section for details): CysSSSBim, MS (ESI, positive): 376 (MH^+).

Oxidized cysteine trisulfide, CysSSSCys



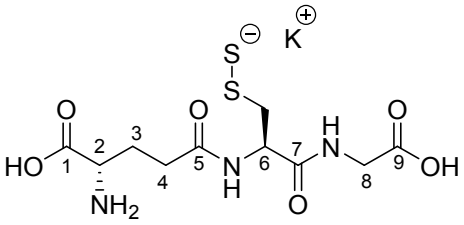
To a suspension of methyl 3-glutathionetrisulfane-1-carboxylate (0.02 g, 0.047 mmol) in MeOH (4 mL) under an atmosphere of N_2 at room temperature glutathione (0.017 g, 0.06 mmol) was added, followed by Et_3N (0.01 mL, 0.06 mmol), and the solution stirred at room temperature for 6 days. The solvent was then removed *in vacuo* to give a light brown solid. The presence of the *title product*, CysSSSCys, was confirmed through LC-MS/MS analysis (see *LC-MS/MS Method* section for details): CysSSSCys, MS (ESI, positive): 273 (MH^+). ^1H NMR (400 MHz, D_2O) δ = 3.30-3.37 (4H, m, H-3), 4.06-4.09 (2H, m, H-2).

SS-Glutathione O-methyl carbono(dithioperoxoate)



To a cooled (0 °C) suspension of glutathione (1.0 g, 3.30 mmol) in dioxane (20 mL) under an atmosphere of N_2, methoxycarbonylsulfenyl chloride (0.29 mL, 3.30 mmol) was added dropwise. The reaction was then stirred overnight at room temperature. The solvent was then removed *in vacuo* to give *the title product* (1.28 g, quant.) as a white solid which was used without further purification. ^{1}H NMR (400 MHz, D_2O) δ = 2.19-2.33 (2H, m, H-3), 2.56-2.69 (2H, m, H-4), 3.15 (1H, dd, *J* = 9.0 and 14.5 Hz, -CH_2SS-), 3.32 (1H, dd, *J* = 4.5 and 14.5 Hz, -CH_2SS-), 3.93 (3H, s, OCH_3), 4.03 (2H, s, H-8), 4.08 (1H, t, *J* = 6.6 Hz, H-2), 4.67 (1H, q, *J* = 4.5 Hz, H-6); ^{13}C{^{1}H} NMR (100 MHz, D_2O) δ = 25.5 (C-3), 31.0 (C-4), 39.5 (-CH_2SS-), 41.2 (C-8), 52.5 (C-6), 56.3 (OCH_3), 171.8 (C-1), 171.9 (C=O), 172.3 (C-7), 172.9 (C-5), 174.4 (C-9); *m/z* (ESI$^+$): 398 (MH^+, 20%), 323 (30), 283 (100), 269 (50) and 166 (60); HRMS (ESI$^+$) Found (MH^+): 398.0692, $C_{12}H_{20}N_3O_8S_2$ calcd 398.0686.

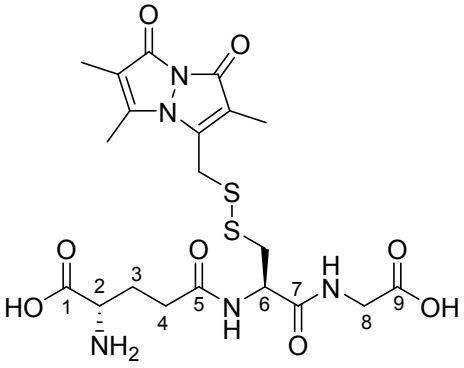
Glutathione persulfide potassium salt, GSS.K.



To a cooled (0 °C) solution of SS-glutathione *O*-methyl carbono(dithioperoxoate) (0.434 g, 1.09 mmol) in MeOH (1.6 mL), a solution of KHS (0.16 g, 2.2.mmol) in MeOH (0.8 mL) was added dropwise. The reaction was allowed to warm to room temperature, and stirred for 90

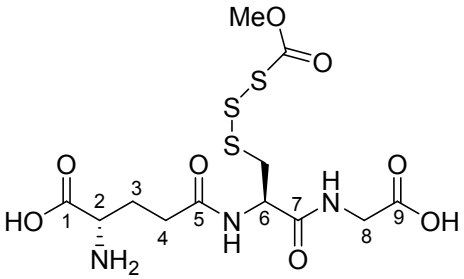
mins. The precipitated product was then collected by filtration, washed with cold MeOH and ether, and dried *in vacuo* to give the *title product* (0.41 g, quant.) as a pale green solid which was used without further purification.

Bimane-derivatized glutathione persulfide, GSSBim



To a solution of GSS.K (30.3 mg, 0.080 mmol) in Tris-HCl buffer (pH 8.8, 100 mmol L^{-1}, 2.8 mL) at room temperature, a solution of monobromobimane (2.7 mg, 0.01 mmol) in MeCN (1 mL) was added and the mixture stirred at room temperature for 30 mins. Formation of the derivatized persulfide GSSBim, was confirmed though LC-MS/MS analysis (see *LC-MS/MS Method* section for details): GSSBim, MS (ESI, positive): 530 (MH^+).

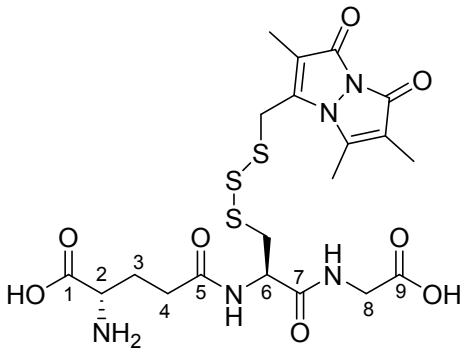
Methyl 3-glutathionetrisulfane-1-carboxylate



To a cooled (0 °C) suspension of GSS.K (0.1 g, 0.26 mmol) in dioxane (5 mL) under an atmosphere of N_2, methoxycarbonylsulfenyl chloride (0.024 mL, 0.26 mmol) was added dropwise. The reaction was then stirred for 6 days. The solvent was then removed *in vacuo* to give *the title product* (0.112 g, quant.) as a white solid which was reacted on without further

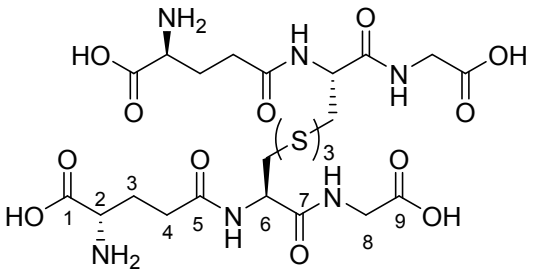
purification. ^{1}H NMR (400 MHz, D_2O) δ = 2.12-2.20 (2H, m, H-3), 2.46-2.59 (2H, m, H-4), 3.06-3.28 (2H, m, -CH_2SS-), 3.90 (3H, s, OCH_3), 3.91-3.97 (3H, m, H-2, H-8), 4.59-4.64 (1H, m, H-6); ^{13}C{^{1}H} NMR (100 MHz, D_2O) δ = 25.8 (C-3), 31.1 (C-4), 38.6 (-CH_2SS-), 41.3 (C-8), 52.6 (C-6), 56.8 (OCH_3), 172.6 (C-1), 172.7 (C=O), 173.0 (C-7), 173.5 (C-5), 174.7 (C-9); *m/z* (ESI$^-$): 428 (M-H, 15%), 396 (50) and 304 (100); HRMS (ESI$^-$) Found (M-H): 428.0248, $C_{12}H_{18}N_3O_8S_3$ calcd 428.0262.

Bimane-derivatized glutathione hydrotrisulfides, GSSSBim



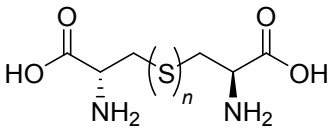
To a solution of methyl 3-glutathionetrisulfane-1-carboxylate (5.0 mg, 0.012 mmol) in MeOH (1 mL) under N_2 at room temperature, KHS (1.7 mg, 0.023 mmol) was added and the mixture stirred for 2 mins. A solution of monobromobimane (16 mg, 0.06 mmol) in MeCN (1 mL) was then added to the reaction which was subsequently left to stir for a further 30 mins. Formation of the derivatized hydrotrisulfide, GSSSBim, was confirmed though LC-MS/MS analysis (see *LC-MS/MS Method* section for details): GSSSBim, MS (ESI, positive): 562 (MH^+).

Oxidized glutathione trisulfide, GSSSG.



To a suspension of methyl 3-cysteinetrisulfane-1-carboxylate (0.01 g, 0.041 mmol) in MeOH (1 mL) under an atmosphere of N_2 at room temperature cysteine (0.006 g, 0.049 mmol) was added, followed by Et_3N (0.01 mL, 0.06 mmol), and the solution stirred at room temperature for 6 days. The solvent was then removed *in vacuo* to give a white solid. The presence of the *title product*, GSSSG, was confirmed through LC-MS/MS analysis (see *LC-MS/MS Method* section for details): GSSSG, MS (ESI, positive): 645 (MH^+). ^1H NMR (400 MHz, D_2O) δ = 2.06-2.15 (4H, m, H-3), 2.44-2.55 (4H, m, H-4), 3.67-3.80 (6H, m, H-2 and H-8), -CH_2SS- and H-6 (obscured); *m/z* (ESI^-): 643 (M-H, 50%), 611 (100) and 418 (50); HRMS (ESI^-) Found (M-H): 643.1167, $C_{20}H_{31}N_6O_{12}S_3$ calcd 643.1168.

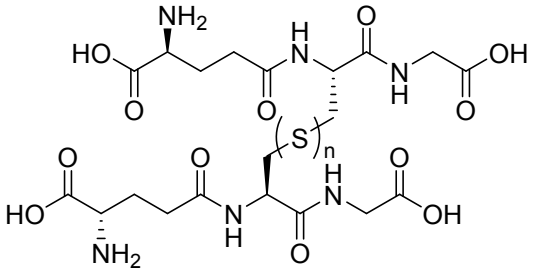
Cysteine oxidized polysulfides, Cys(S)$_n$Cys, n = 2,3,4,5.



To a solution of cysteine (24.2 mg, 0.2 mmol) in a Tris-HCl buffer (pH 7.4, 20 mmol L^{-1}, 10 mL), KHS (14.4 mg, 0.2 mmol) and I_2 (50.8 mg, 0.2 mmol) were added, and the mixture was stirred at room temperature for 20 mins. Formation of products CysSSCys, CysSSSCys, CysSSSSCys and CysSSSSSCys was confirmed though LC-MS/MS analysis (see *LC-MS/MS Method* section for details): CysSSCys, MS (ESI, positive): 241 (MH^+); CysSSSCys, MS (ESI,

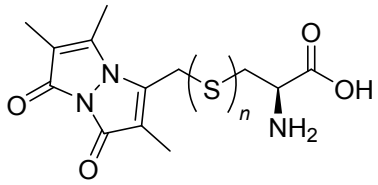
positive): 273 (MH^+); CysSSSSCys, MS (ESI, positive): 305 (MH^+); CysSSSSSCys, MS (ESI, positive): 337 (MH^+).

Glutathione oxidized polysulfides, $G(S)_nG$, n = 2,3,4,5.



A solution of I_2 (50.8 mg, 0.2 mmol) in a Tris-HCl buffer (pH 7.4, 20 mmol L^{-1}, 10 mL) was stirred for 5 mins at room temperature. Following this, glutathione (61.5 mg, 0.2 mmol) and KHS (14.4 mg, 0.2 mmol) was added and the mixture was stirred at room temperature for 20 mins. Formation of products GSSG, GSSSG, GSSSSG and GSSSSSG was confirmed though LC-MS/MS analysis (see *LC-MS/MS Method* section for details): GSSG, MS (ESI, positive): 613 (MH^+); GSSSG, MS (ESI, positive): 645 (MH^+); GSSSSG, MS (ESI, positive): 677 (MH^+); GSSSSSG, MS (ESI, positive): 709 (MH^+).

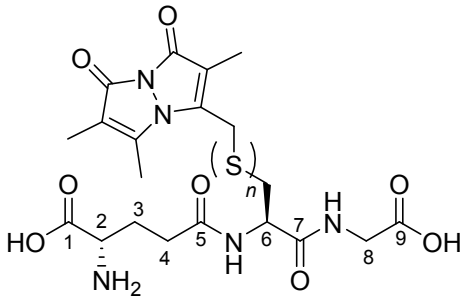
Bimane-derivatized cysteine hydropolysulfides, $Cys(S)_nBim$, n = 2,3,4.



To a solution of cysteine (3.8 mg, 0.031 mmol) in Tris-HCl buffer (pH 8.8, 100 mmol L^{-1}, 1.5 mL) at room temperature, KHS (2.3 mg, 0.031 mmol) and NOC-7 (5 mg, 0.031 mmol) were added, and the mixture stirred for 30 mins. Monobromobimane (85 mg, 0.31 mmol) was then added to the reaction which was subsequently left to stir for a further 30 mins. Formation of the derivatized hydropolysulfides, CysSSBim, CysSSSBim, CysSSSSBim was confirmed

though LC-MS/MS analysis (see *LC-MS/MS Method* section for details): CysSSBim, MS (ESI, positive): 344 (MH^+); CysSSSBim, MS (ESI, positive): 376 (MH^+); CysSSSSBim, MS (ESI, positive): 408 (MH^+); CysSSSSSBim, MS (ESI, positive): 440 (MH^+).

Bimane-derivatized glutathione hydropolysulfides, $G(S)_nBim$, n = 2,3,4.



To a solution of glutathione (9.6 mg, 0.031 mmol) in Tris-HCl buffer (pH 8.8, 100 mmol L^{-1}, 1.5 mL) at room temperature, KHS (2.3 mg, 0.031 mmol) and NOC-7 (3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propanamine; 5 mg, 0.031 mmol) were added, and the mixture stirred for 30 mins. Monobromobimane (85 mg, 0.31 mmol) was then added to the reaction which was subsequently left to stir for a further 30 mins. Formation of the derivatized hydropolysulfides, GSSBim, GSSSBim, GSSSSBim was confirmed though LC-MS/MS analysis (see *LC-MS/MS Method* section for details): GSSBim, MS (ESI, positive): 530 (MH^+); GSSSBim, MS (ESI, positive): 562 (MH^+); GSSSSBim, MS (ESI, positive): 594 (MH^+); GSSSSSBim, MS (ESI, positive): 626 (MH^+).

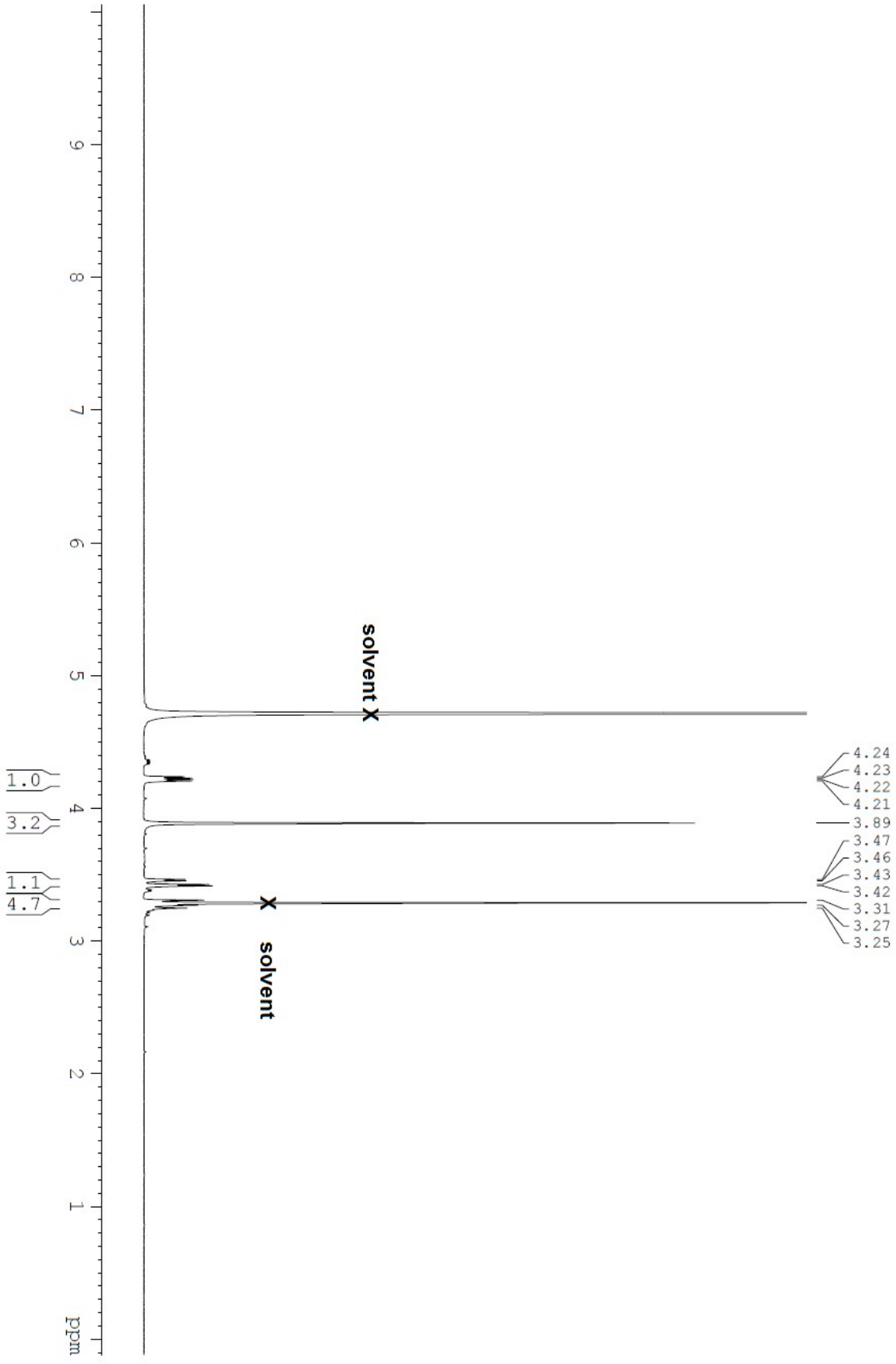


Figure S5: ^{1}H NMR of SS-Cysteine O-methyl carbono(dithioperoxoate) (400 MHz, D_2O)

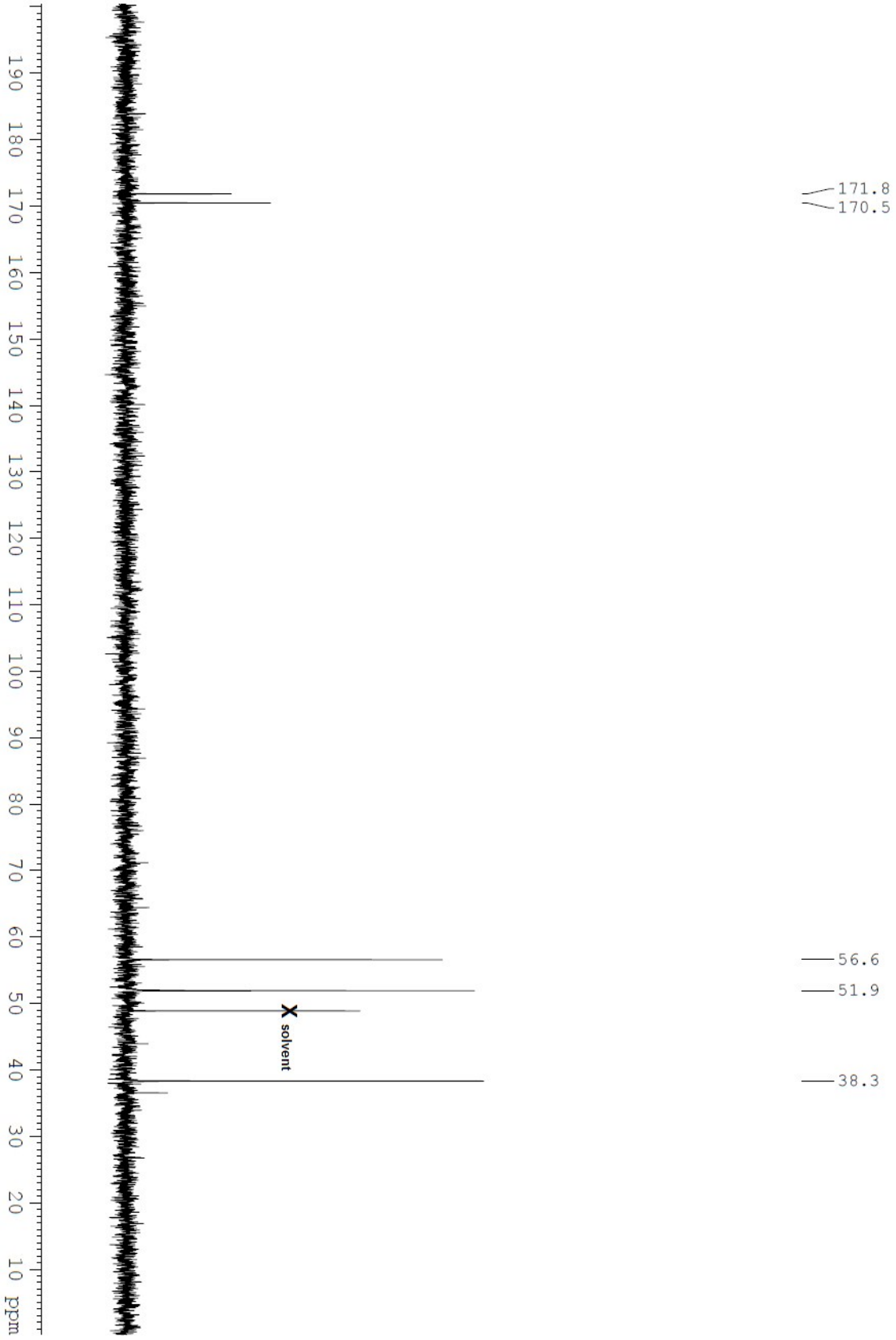


Figure S6: ^{13}C NMR of SS-Cysteine O-methyl carbono(dithioperoxoate) (100 MHz, D_2O)

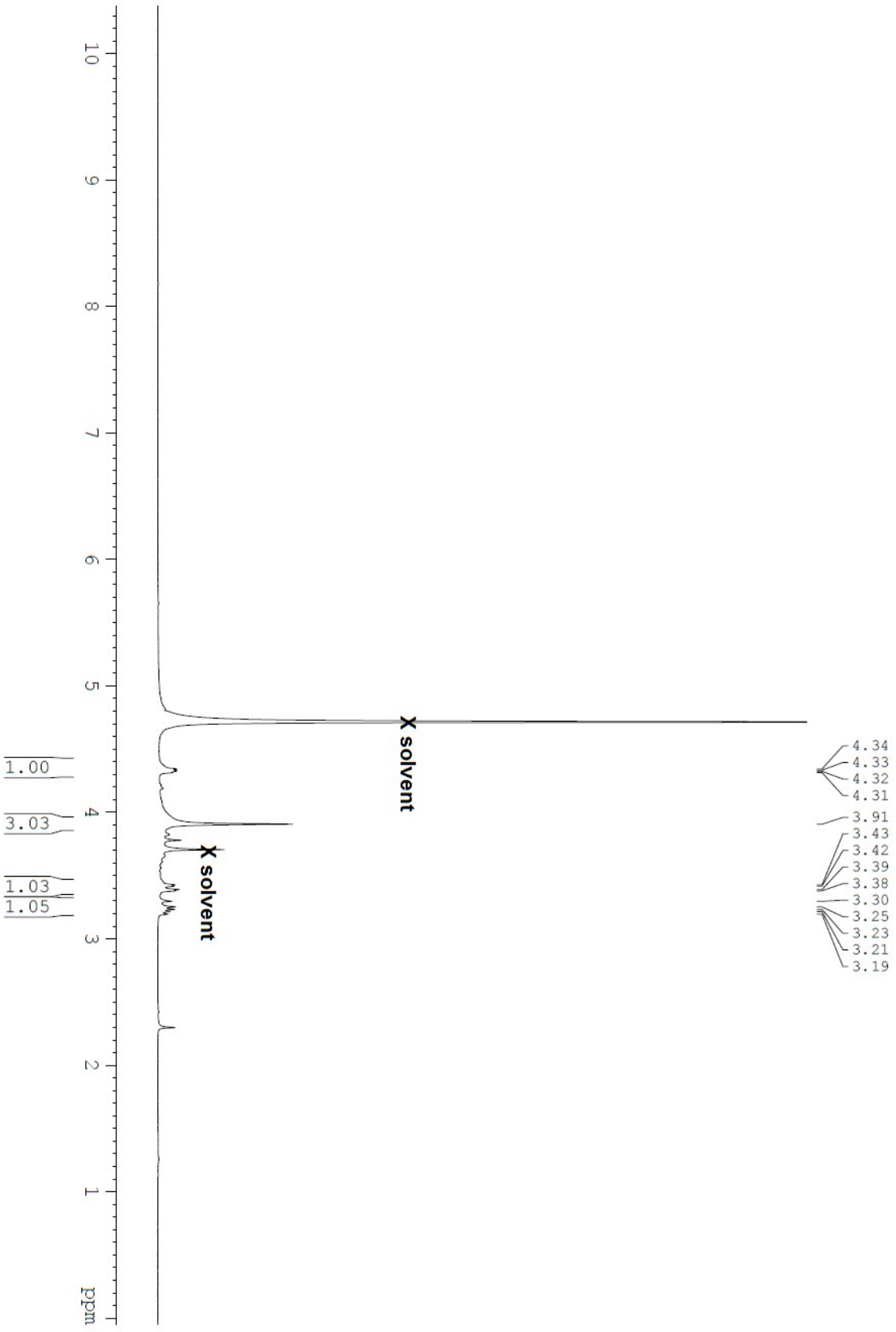


Figure S7: ^{1}H NMR of methyl 3-cysteinetrisulfane-1-carboxylate (400 MHz, D_2O)

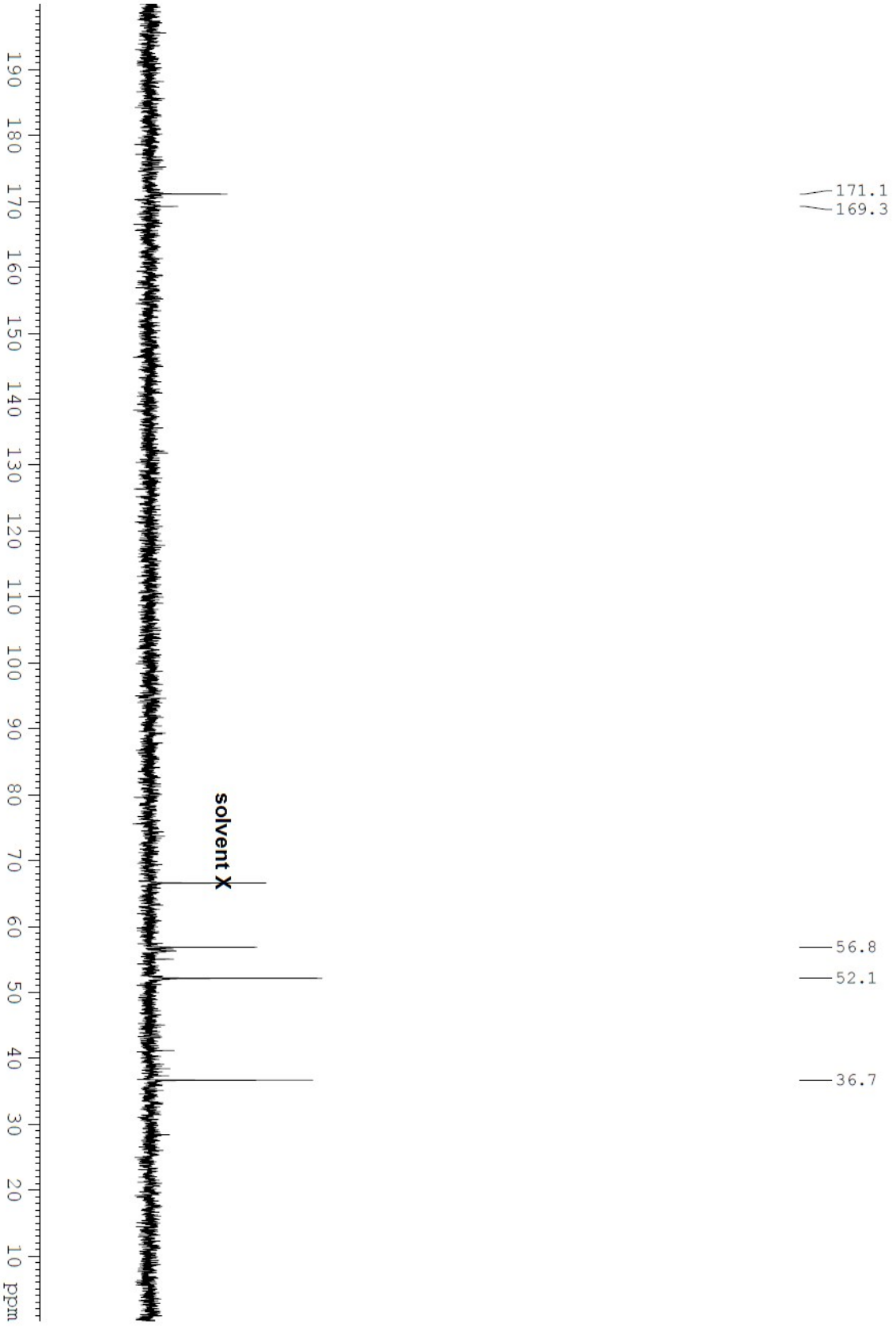


Figure S8: ^{13}C NMR of methyl 3-cysteinetrisulfane-1-carboxylate (100 MHz, D_2O)

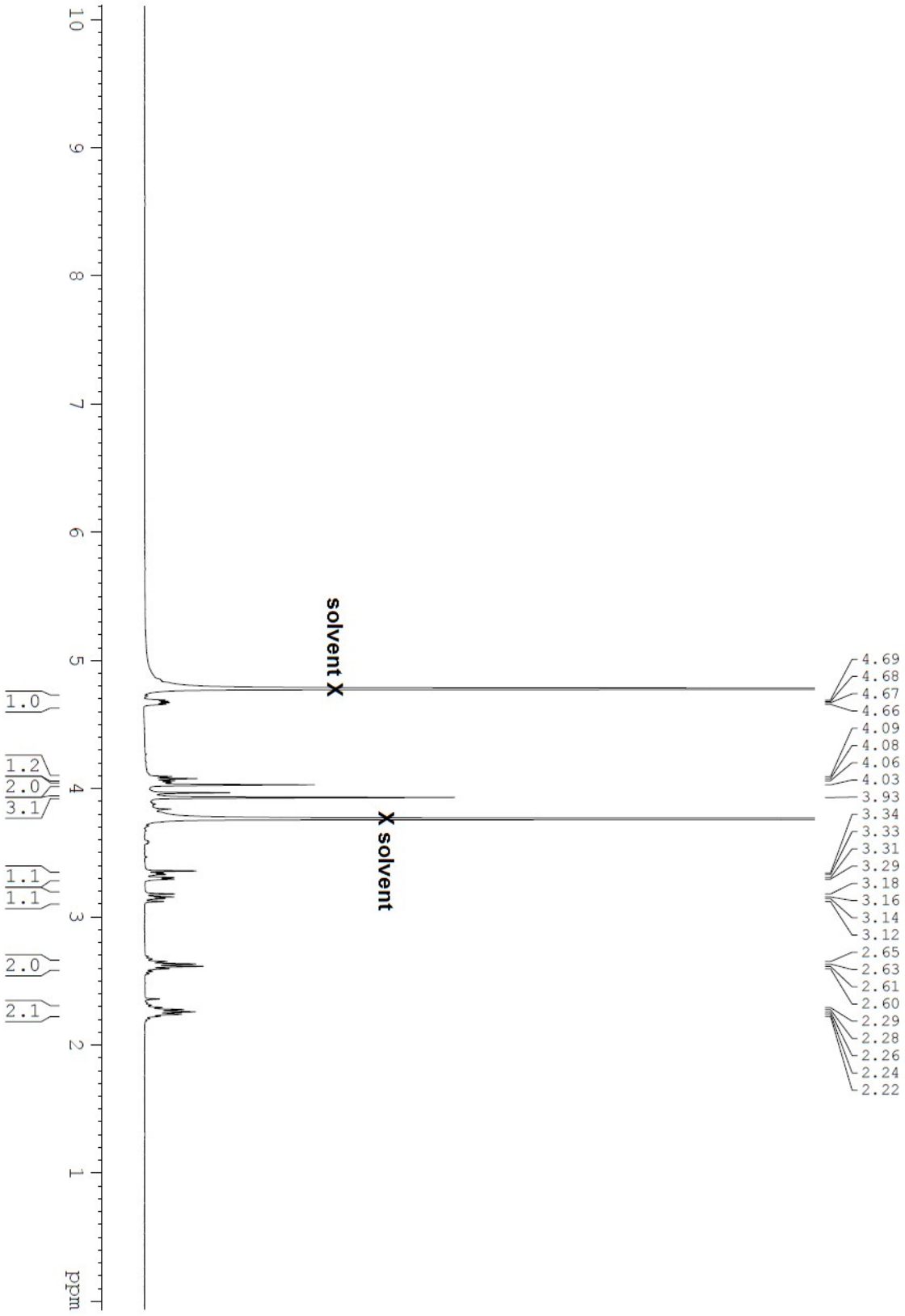


Figure S9: ^{1}H NMR of SS-glutathione O-methyl carbono(dithioperoxoate) (400 MHz, D_2O)

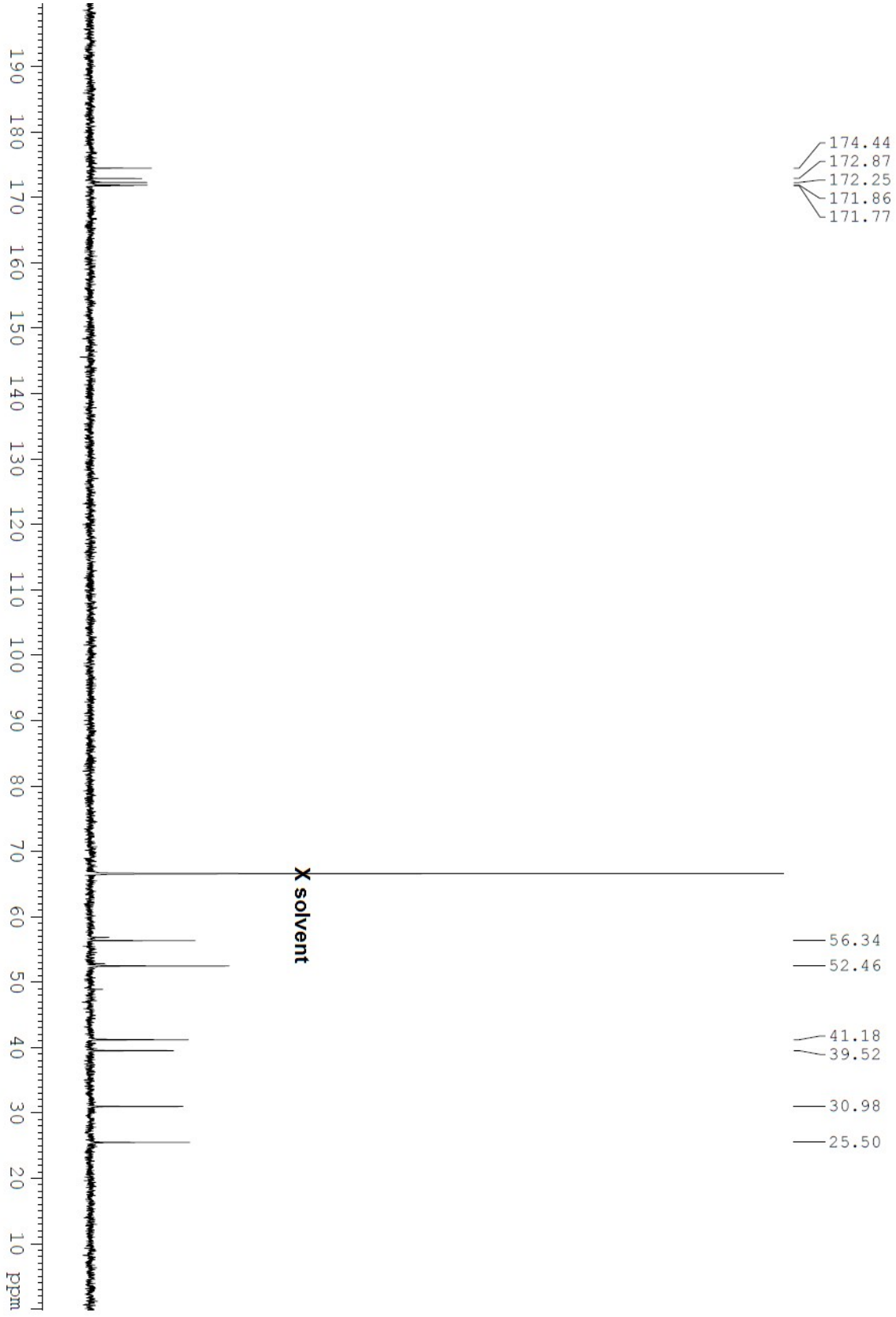


Figure S10: ^{13}C NMR of SS-glutathione O-methyl carbono(dithioperoxoate) (100 MHz, D_2O)

LC-MS/MS Method

Analytical LC-MS/MS data for the polysulfide compounds were acquired using an Agilent 1290 Infinity Liquid Chromatograph (Santa Clara, CA), equipped with a quaternary pump and coupled to an Agilent 6460 Triple Quadrupole mass spectrometer (Santa Clara, CA). Samples (10 μL) were injected onto a Phenomenex (Torrance, CA) Kinetex C18 column (100 x 3 mm ID, 100 Å, 2.6 μm particle size), with the column heater set at 25 °C. The solvents used were 0.1 % aqueous formic acid (solvent A) and 100 % acetonitrile (solvent B). Two methods were used in the analysis: one for the oxidized polysulfides with a flow rate of 0.3 mL min^{-1} and one for the derivatized hydropolysulfides with a flow rate of 0.5 mL min^{-1}. The same gradient was used for both methods with solvent B as follows: 5 % at 0 min, 5% at 6 min, 15 % at 10 min, 80 % at 12 min, 100 % at 15 min, and then back to 5 % at 17 min, for a total run time of 20 mins. All mass spectrometric data were obtained in positive-ion mode using an Agilent (Santa Clara, CA) jet stream electrospray ionisation (ESI) probe. Nitrogen (BOC, Auckland) was used as the desolvation gas at 5 L min^{-1} and 300 °C, the nebulizer was set at 45 psi, with the sheath gas temperature at 250 °C and a flow of 11 L min^{-1}. The ESI capillary, nozzle and fragmentor voltages were set at 3500, 500 and 100 V, respectively. The optimized Multiple Reaction Monitoring (MRM) transitions and collisions energies for each polysulfide are displayed in Table S1.

Table S1: Multiple Reaction Monitoring (MRM) properties of polysulfide compounds synthesized during this project and utilized for the LC-MS analysis of the yeast experiment.

Analyte	**Precursor Ion (*m/z*)**	**Product Ion (*m/z*)***	**Collision Energy (V)****
Oxidized Polysulfides			
CysSSCys	241	152, 122, 120	8, 8, 8
CysSSSCys	273	169, 122, 87	8, 8, 8
CysSSSSCys	305	202, 168, 153	24, 24, 12
CysSSSSSCys	337	201, 185, 120	16, 12, 20
GSSG	613	484, **355**, 276	12, 20, 40
GSSSG	645	516, 387, 231	16, 24, 44
GSSSSG	677	339, 210, 145	28, 36, 44
GSSSSSG	709	**371**, 339, 307	24, 28, 32
Bimane-derivatized Polysulfides			
CysSBim	312	225, 200, 88	16, 12, 20
CysSSBim	344	223, **192**, 175	8, 16, 36
CysSSSBim	376	192, 175, 149	20, 40, 40
CysSSSSBim	408	256, 237, **192**	10, 10, 20
CysSSSSSBim	440	177, 133, 89	10, 20, 30
GSBim	498	435, 369, **225**	20, 20, 32
GSSBim	530	467, 223, **192**	20, 28, 36
GSSSBim	562	317, 280, 192	44, 16, 36
GSSSSBim	594	339, 210, 145	10, 30, 30
GSSSSSBim	626	356, 295, 191	10, 10, 30

*MRM transition used for quantitation of detected polysulfides during the yeast experiment is highlighted in bold
**Respective optimized collision energies for each MRM transition

Bioassay procedures

Yeast strains and culture

Five *S. cerevisiae* strains were used in this study; the laboratory reference strain BY4743 (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met17Δ0/MET17 ura3Δ0/ura3Δ0*) and four BY4743 deletion strains derived from the Saccharomyces Genome Deletion Project (SGDP): BY4743 *Δcys3*, BY4743 *Δcys4* and BY4743 *Δtum1*.[49] Yeast strains were obtained from Euroscarf®. Liquid YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} casein peptone, and 20 g L^{-1} D-glucose) was used to propagate yeast cultures, with orbital shaking at 150 revolutions per min (rpm) and overnight incubation at 28 °C.

Medium to measure growth and polysulfides

To enable experiments to be performed using different sulfur compounds as sole sulfur sources, modified synthetic minimal medium (MSD) was made, based on a medium described by Cherest and Surdin-Kerjan.[50] Table S2 lists the concentrations of each component in the MSD. Na_2SO_4 (1 mM), L-cysteine (0.25 mM), or L-cystine (0.1 mM) were used as the sole sulfur source for each iteration of MSD. Additional L-histidine-HCl (20 mg L^{-1}), L-leucine (30 mg L^{-1}), and uracil (20 mg L^{-1}) were added to the MSD for all treatments in order to supplement the remaining strain auxotrophies.

Table S2: Composition of modified synthetic minimal medium (MSD).

Component	Constituents
Carbon source	2 % D-glucose
Iron	0.05 μg mL^{-1} $FeCl_3.6H_2O$
Mineral salts	0.7 mM $CaCl_2$. H_2O, 6.6 mM KH_2PO_4, 0.5 mM K_2HPO_4, 2 mM $MgCl_2.6H_2O$, 1.7 mM NaCl
Sulfur-free trace salts	0.04 μg mL^{-1} $CuCl_2$. H_2O, 0.5 μg mL^{-1} H_3BO_3, 0.1 μg mL^{-1} KI, 0.19 μg mL^{-1} $ZnCl_2$
Vitamins	0.02 μg mL^{-1} biotin, 2 μg mL^{-1} calcium pantothenate, 0.2 μg mL^{-1} folic acid, 20 μg mL^{-1} myo-inositol, 2 μg mL^{-1} nicotinic acid, 2 μg mL^{-1} pyridoxine-HCl, 0.2 μg mL^{-1} p-aminobenzoic acid, 0.2 μg mL^{-1} riboflavin, 2 μg mL^{-1} thiamine-HCl
Nitrogen source	15 mM NH_4Cl
Sulfur source	1 mM Na_2SO_4, 0.25 mM L-cysteine, or 0.1 mM L-cystine

Yeast growth assay conditions

The BY4743 reference and the BY4743 deletion strains were grown in MSD containing Na_2SO_4, L-cysteine, or L-cystine as the sole sulfur source in the Bioscreen C™ MBR Automated Growth Curve Analysis System operated by BioScreener software (Growth Curves USA). Pre-cultures of each strain were washed twice with sterile water to remove the YPD medium and inoculated into triplicate wells of a 100-well honeycomb plate containing 300 μL of MSD (final cell density of 1×10^6 cells mL^{-1}). Cells were grown at 28 °C for 72 hours. Cell

density was measured at $OD_{480–560\,nm}$ every 15 mins with 75 s shaking on high, prior to reading. Wells containing medium only and no yeast were included as negative controls for growth and polysulfide accumulation.

Detection of yeast intracellular polysulfides via SSP4 staining and fluorescence microscopy

The protocol used for detecting intracellular polysulfides using the fluorescent Sulfane Sulfur Probe 4 or SSP4 (3', 6'-di (O-thiosalicyl) fluorescein; kindly provided by Professor Ming Xian, Washington State University, USA), was adapted from Chen et al. with minor modifications.[8] Yeast cells were grown in 5 mL MSD at 28 °C for 48 h with a single sulfur source. Cells (~2 × 10^7) were harvested, washed twice with phosphate buffered saline (PBS) and incubated with 20 μM SSP4 in dimethyl sulfoxide (DMSO) containing 250 μM cetyltrimethylammonium bromide (CTAB) for 30 min at 28 °C in the dark. Cells not stained with SSP4 were used as negative controls. The cells were then washed twice with PBS. For the positive control, cells were treated with a cysteine polysulfide standard for 30 min at 28 °C and then washed twice with PBS. Fluorescence images were acquired using a Leica DMR microscope (Leica, Germany) equipped with a SPOT Pursuit camera (Diagnostic Instruments, USA) and a GFP light cube (49002, Chroma Technology Corp. USA).

Cell harvesting for measurement of yeast polysulfide biosynthesis via LC-MS/MS

The cell pellet from each yeast strain after 72 hours of growth was harvested in order to semi-quantitate intracellular polysulfides generated by yeast. In order to obtain enough volume for polysulfide quantitation, the triplicates for each treatment were pooled into a 1.5-mL Eppendorf tube and then centrifuged at 3000 *g* for 5 mins. The cell pellets were washed twice with 300 μL sterile water and centrifuged at 3000 *g* for 5 mins to remove any remaining supernatant. Yeast cell pellets were lysed using 5 μL of the enzyme Zymolyase®-20T (0.005 g Zymolyase

(20,000 units/g) in 1 mL of Zymolyase buffer) and 15 µL Zymolyase buffer (1.2 M sorbitol, 0.1 M KH_2PO_4, pH 7.2). Tubes were incubated at 37 °C for 10 mins, followed by the addition of 100 µL monobromobimane (0.2 mL, 10 mmol L^{-1}) and further incubation for 10 mins at 37 °C. A final incubation was then performed for 10 mins at 60 °C to ensure that cells were sufficiently lysed. Lysed cell extracts were frozen at -80 °C before quantitation of polysulfides via LC-MS/MS.

Statistical Analysis

All statistical analysis was performed using R software (version 3.5.1). Two-sample t-tests were used to compare the peak areas relative to cell count of the gene deletant strains versus the reference strain (BY4743) and the z-scores of the tests were used to produce Table 1. The heatmaps shown were produced using the heatmap function available in the base package. The networks shown were produced using the "qgraph" R package. Edges between gene and polysulfide are coloured according to the apparent effect of the gene on the occurrence of polysulfide as determined using the aforementioned comparison tests that are shown in Table 1 and the weighting signifying the strength of the effect (the z-score). Edges between polysulfides are coloured according to the correlation (blue indicates a positive correlation, red a negative correlation) with the line width signifying the strength of the correlation. The correlations being determined using the base R package to compute the pearsons correlation coefficients which were then used by the cor_auto function in the "qgraph" package to create a correlation matrix. The bar graphs in the supporting information were plotted using the ggplot2 R package.

Intracellular polysulfides via SSP4 staining and fluorescence microscopy results

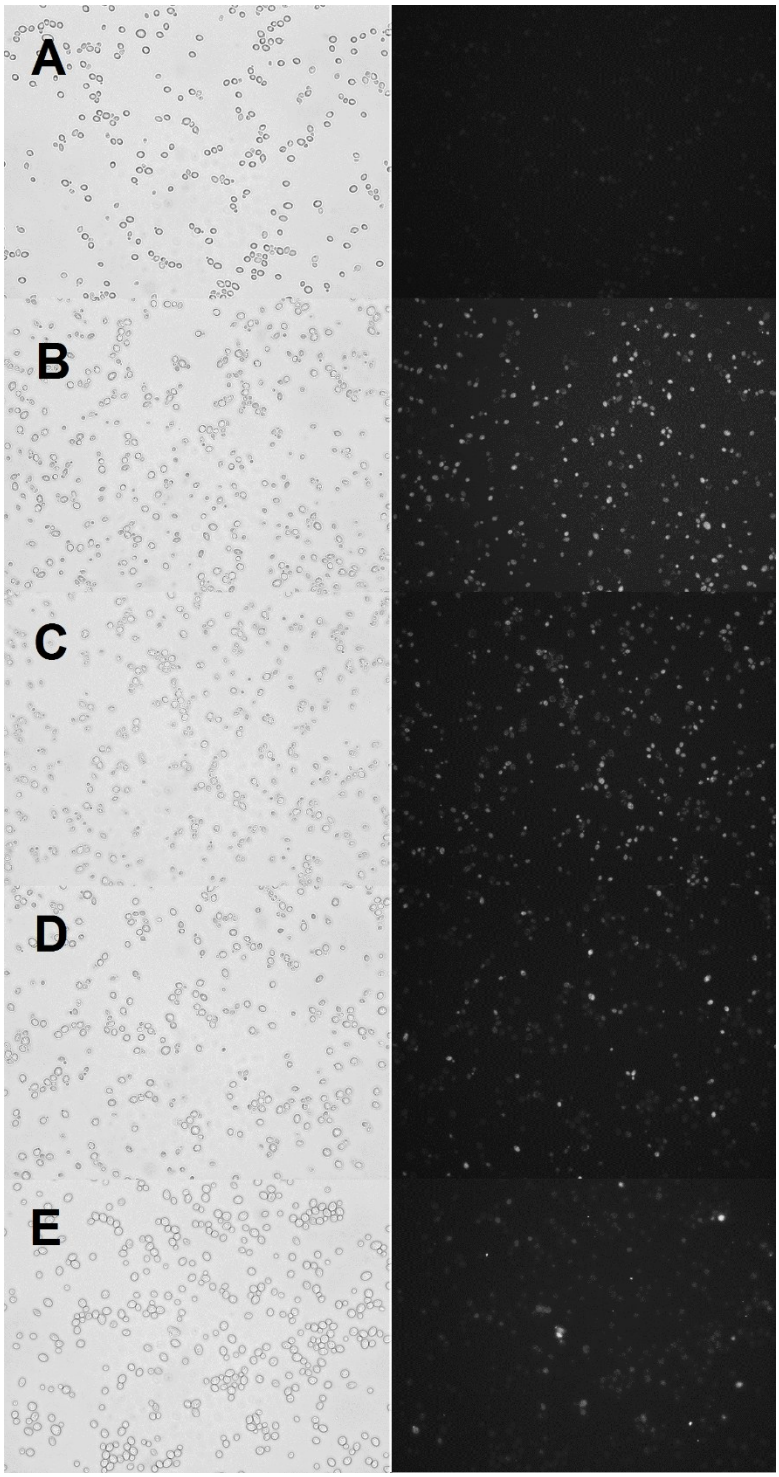
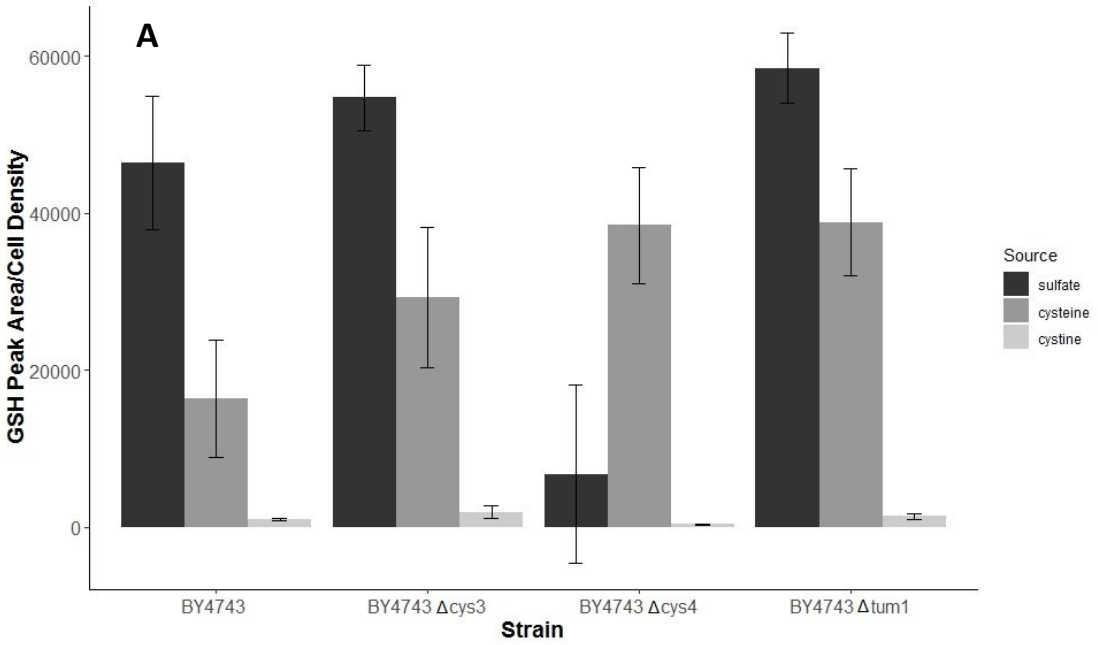
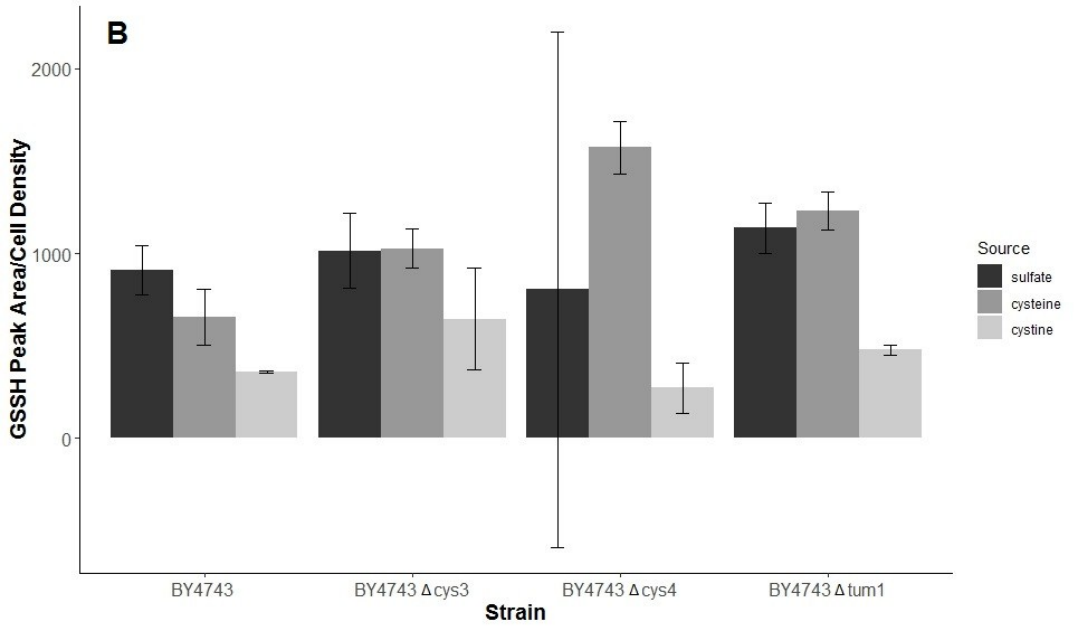
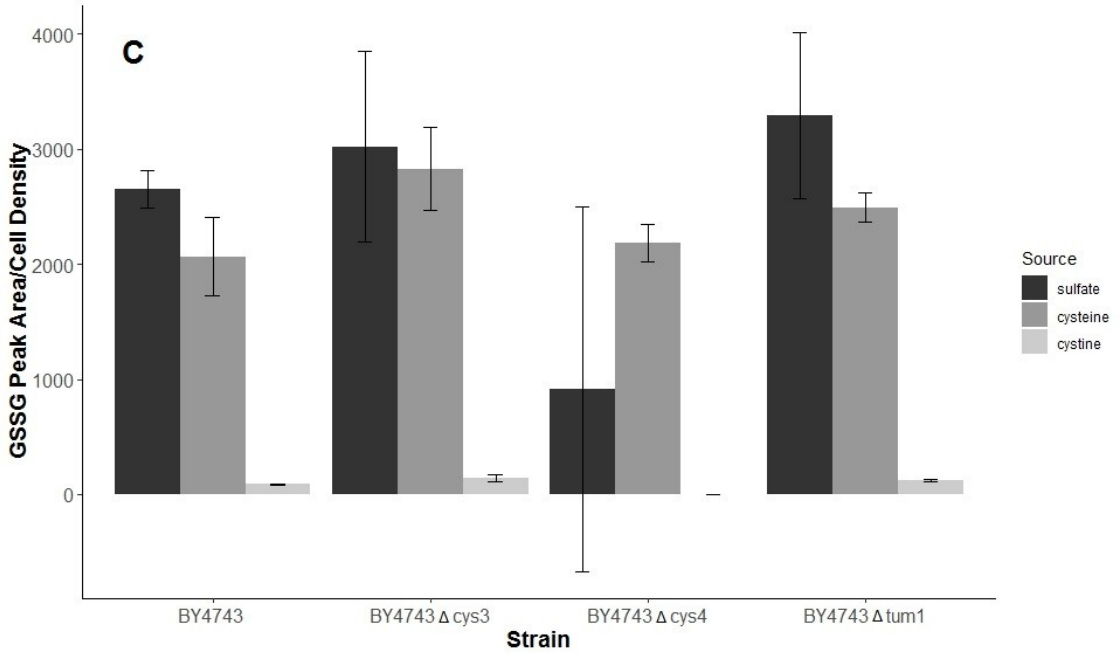


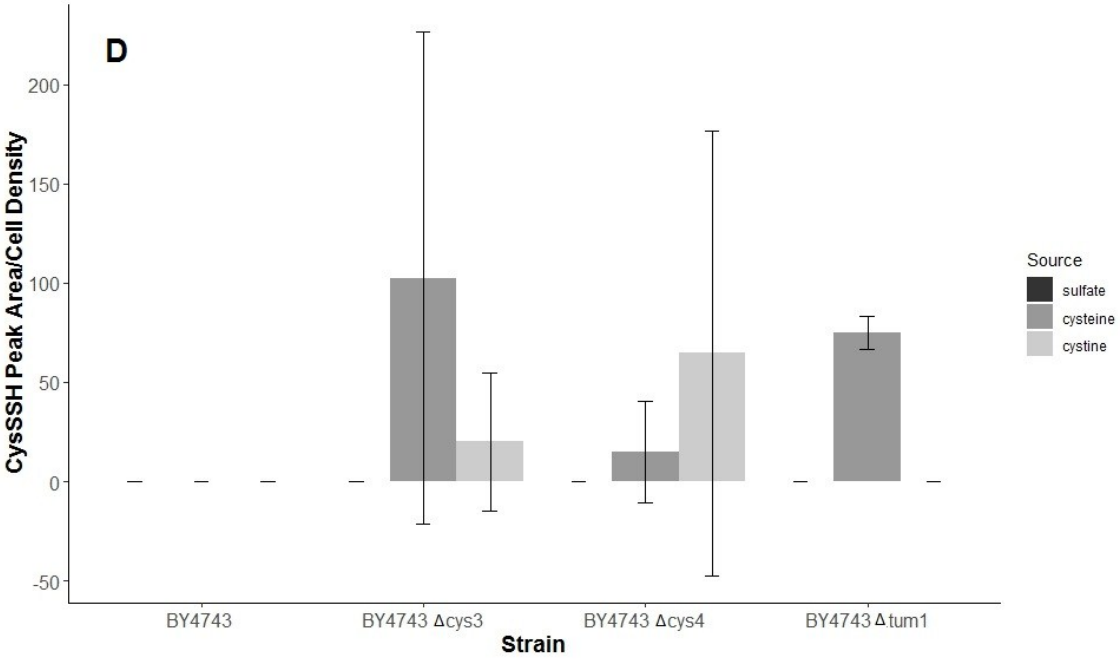
Figure S11: Detection of polysulfides in *S.C.* BY4743 after 72 h growth via staining with SSP4. A) 0.25 mM cysteine, no SSP4. B) 0.25 mM Cys + polysulfides + SSP4. C). 0.25 mM Cys + SSP4. D) 1 mM SO_4^{2-} + SSP4. E) 0.1 mM cystine + SSP4. Magnification 400 ×.

Results of biological assays for the detection of polysulfides






C
GSSG Peak Area/Cell Density
4000
3000
2000
1000
0
BY4743
BY4743 Δ cys3
BY4743 Δ cys4
BY4743 Δ tum1
Strain
Source
sulfate
cysteine
cystine


D
CysSSH Peak Area/Cell Density
200
150
100
50
0
-50
BY4743
BY4743 Δcys3
BY4743 Δcys4
BY4743 Δ tum1
Strain
Source
sulfate
cysteine
cystine

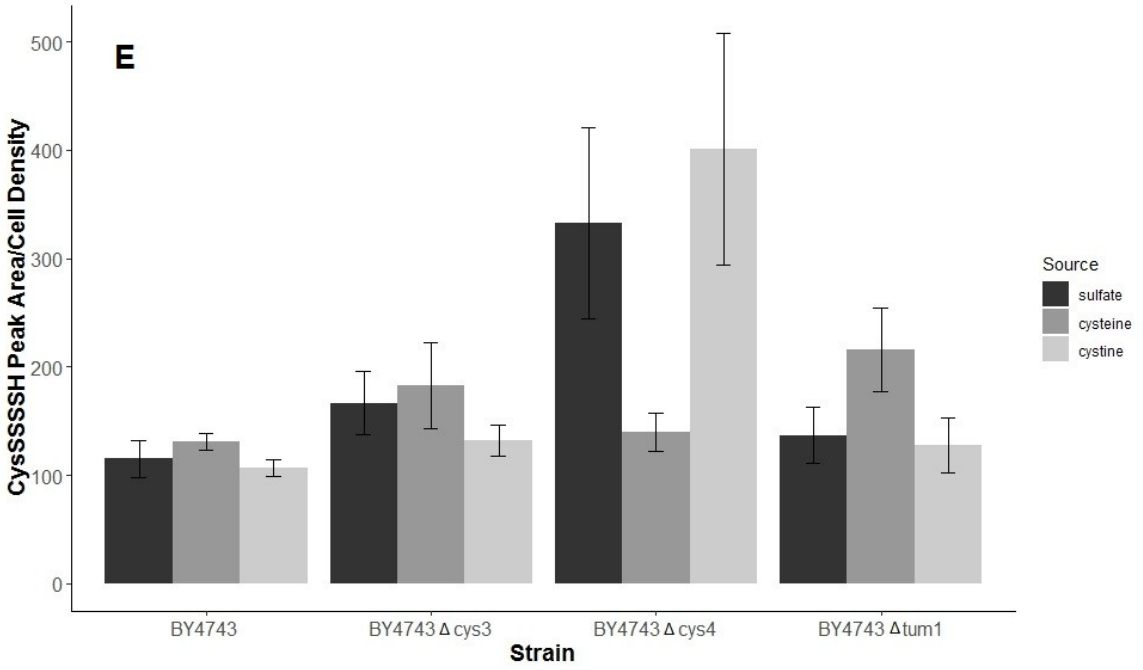
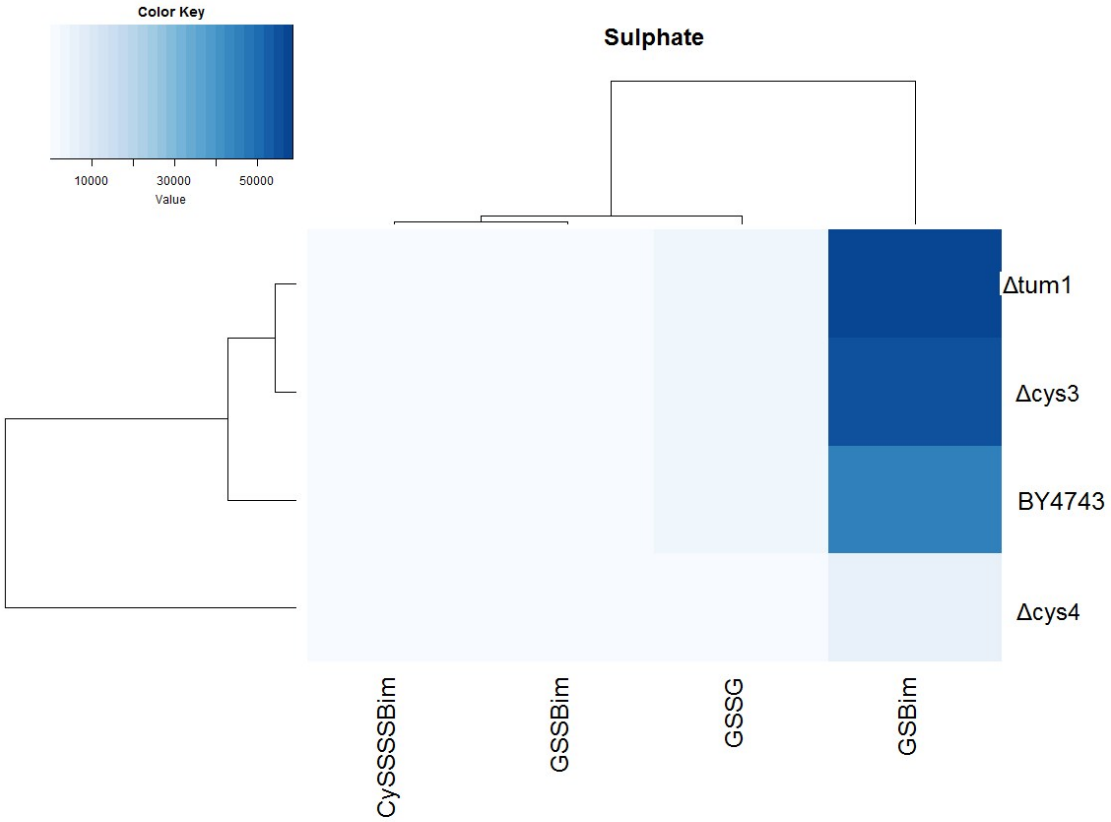
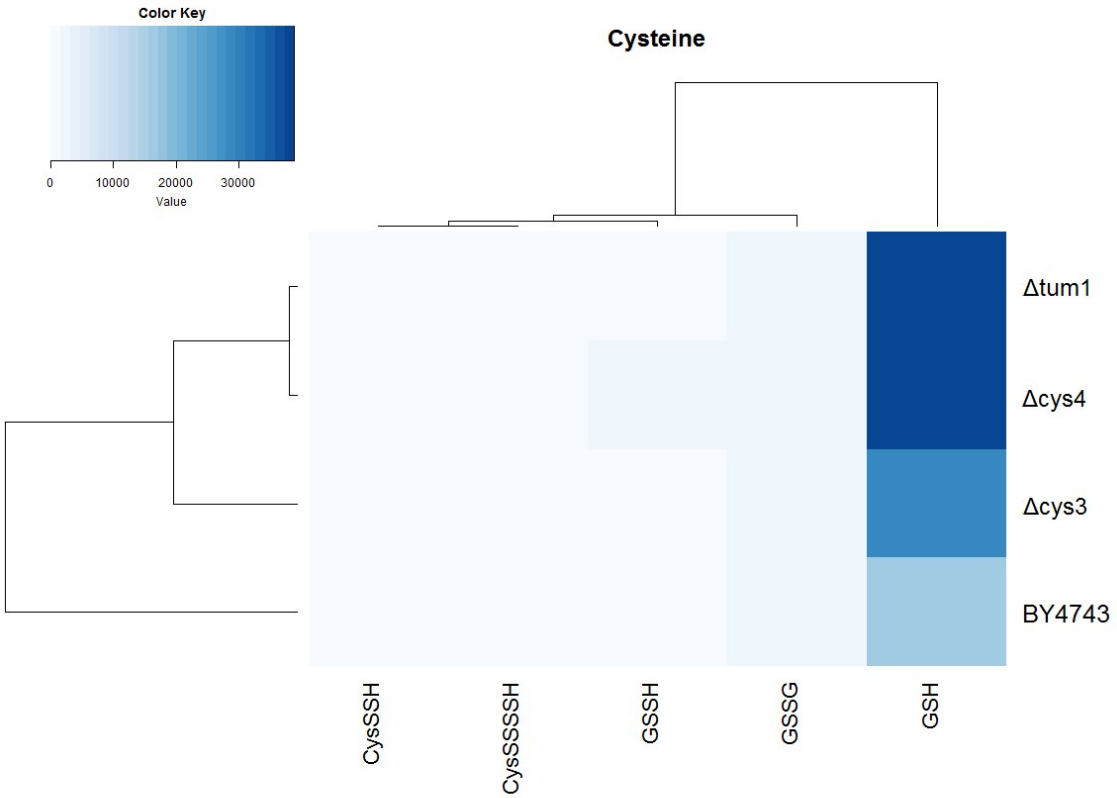


Figure S12: Peak areas of polysulfides A) GSH, B) GSSH, C) GSSG, D) CysSSH and E) CysSSSSH relative to cell density for BY4743, BY4743 *Δcys3*, BY4743 *Δcys4* and BY4743 *Δtum1* grown in MSD medium with sulfate (black), cysteine (dark grey) or cystine (pale grey) as sole sulfur source. Error bars represent the standard deviation of the mean, n =3 (biological replicates).


Color Key
10000
30000
50000
Value
Sulphate
Δtum1
Δcys3
BY4743
Δcys4
CySSSSBim
GSSBim
GSSG
GSBim


Color Key
0
10000
20000
30000
Value
Cysteine
Δtum1
Δcys4
Δcys3
BY4743
CysSSH
CysSSSSH
GSSH
GSSG
GSH

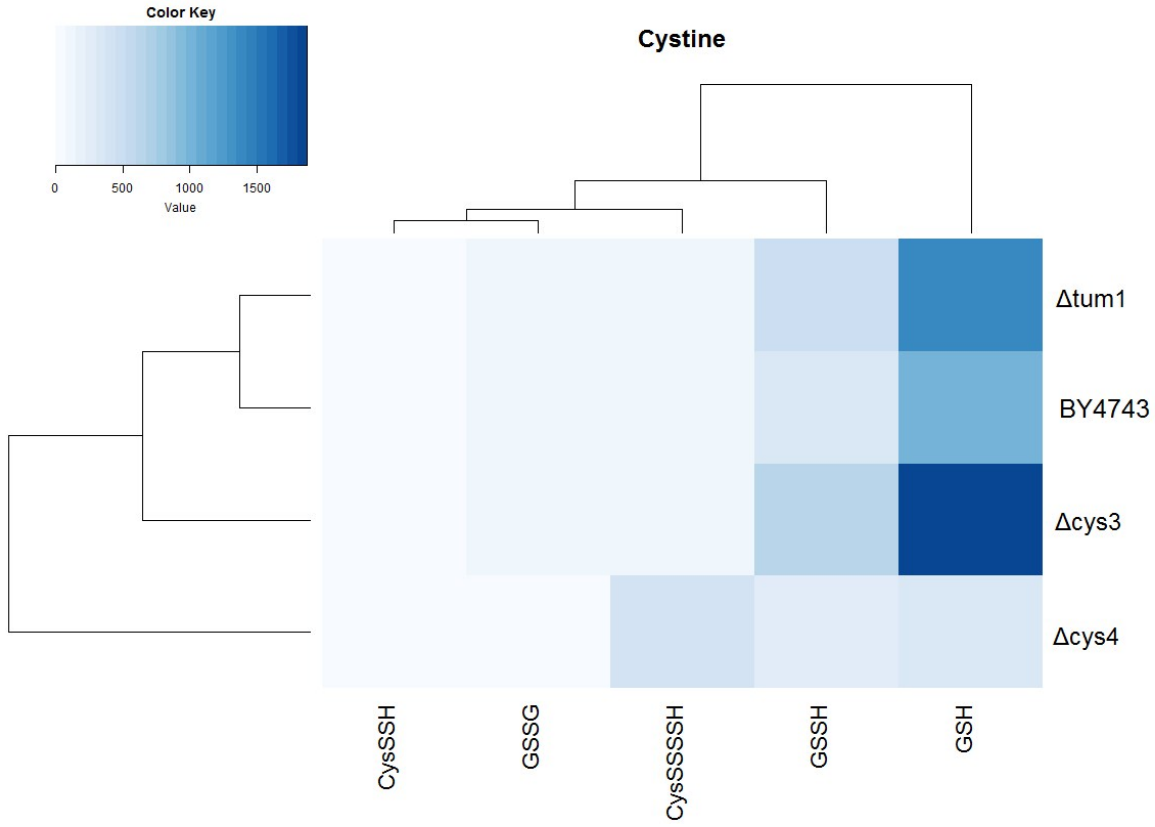


Figure S13: Heatmaps to explore the interrelationships between yeast strain and polysulfides. The row and column order is dictated by the similarities between the yeast strain and polysulfide, respectively.

	Sulfur Source	Average Optical Density	Average Cell Count /mL
BY4743	Sulfate	1.105	3.60×10^7
BY4743 *Δcys3*		1.024	3.21×10^7
BY4743 *Δcys4*		0.464	1.28×10^7
BY4743 *Δtum1*		1.061	3.38×10^7
BY4743	Cysteine	1.275	4.62×10^7
BY4743 *Δcys3*		1.119	3.67×10^7
BY4743 *Δcys4*		1.372	5.36×10^7
BY4743 *Δtum1*		1.250	4.45×10^7
BY4743	Cystine	1.294	4.75×10^7
BY4743 *Δcys3*		1.283	4.67×10^7
BY4743 *Δcys4*		0.521	1.45×10^7
BY4743 *Δtum1*		1.286	4.70×10^7

Table S3: Average final cell count based on optical density, for normalization of polysulfide peak areas.