Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Clickable glutathione using tetrazine-alkene bioorthogonal chemistry for detecting protein glutathionylation

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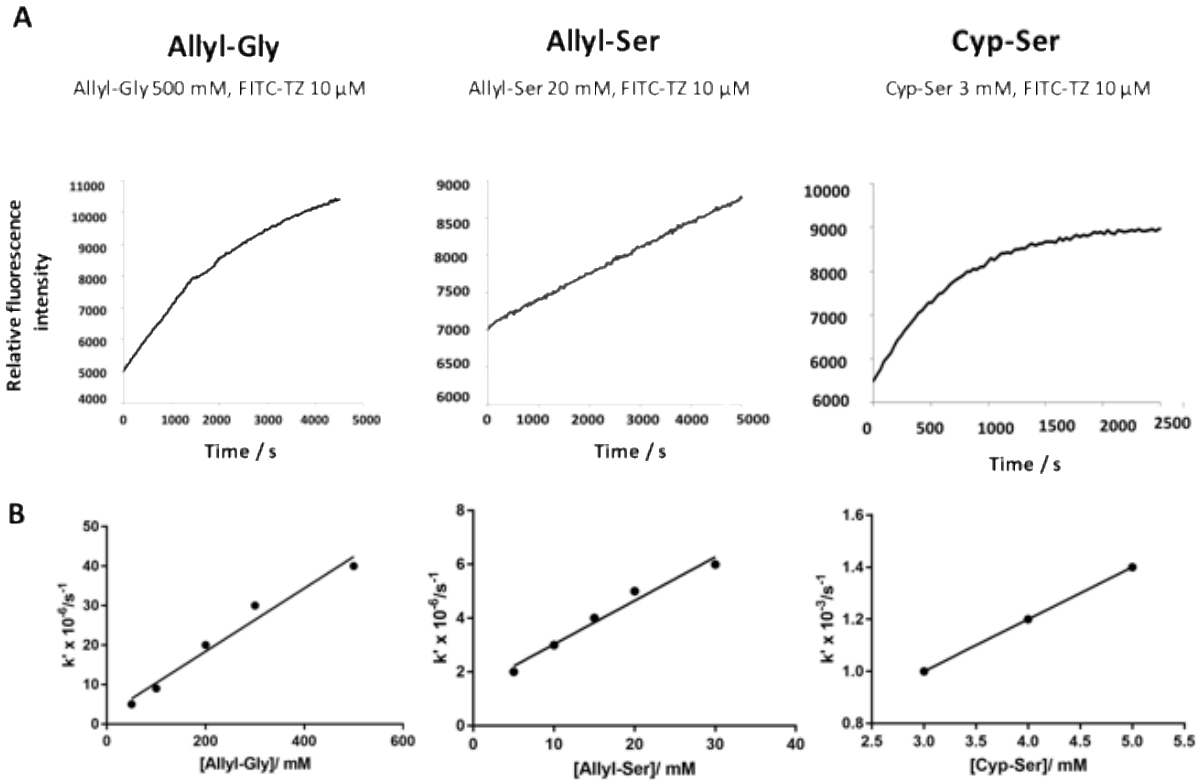


Figure S1. The second-order rate constants for reactions of alkene-containing amino acids with tetrazine-FITC. (A) The relative fluorescence increase as a function of time at a given concentration was fitted to a single exponential equation, $F = (F_1\text{-}F_2)\, e^{(-k't)}$, where F value is the detected fluorescence, $F_1\text{-}F_2$ value is a background fluorescence intensity and k′ value is a pseudo first order rate constant. (B) The pseudo-first order rate constants of the reaction with different concentrations of dienophiles was fitted to an equation, $k' = k\,[\text{Gly surrogate}] + C$, where k is the second order rate constant.

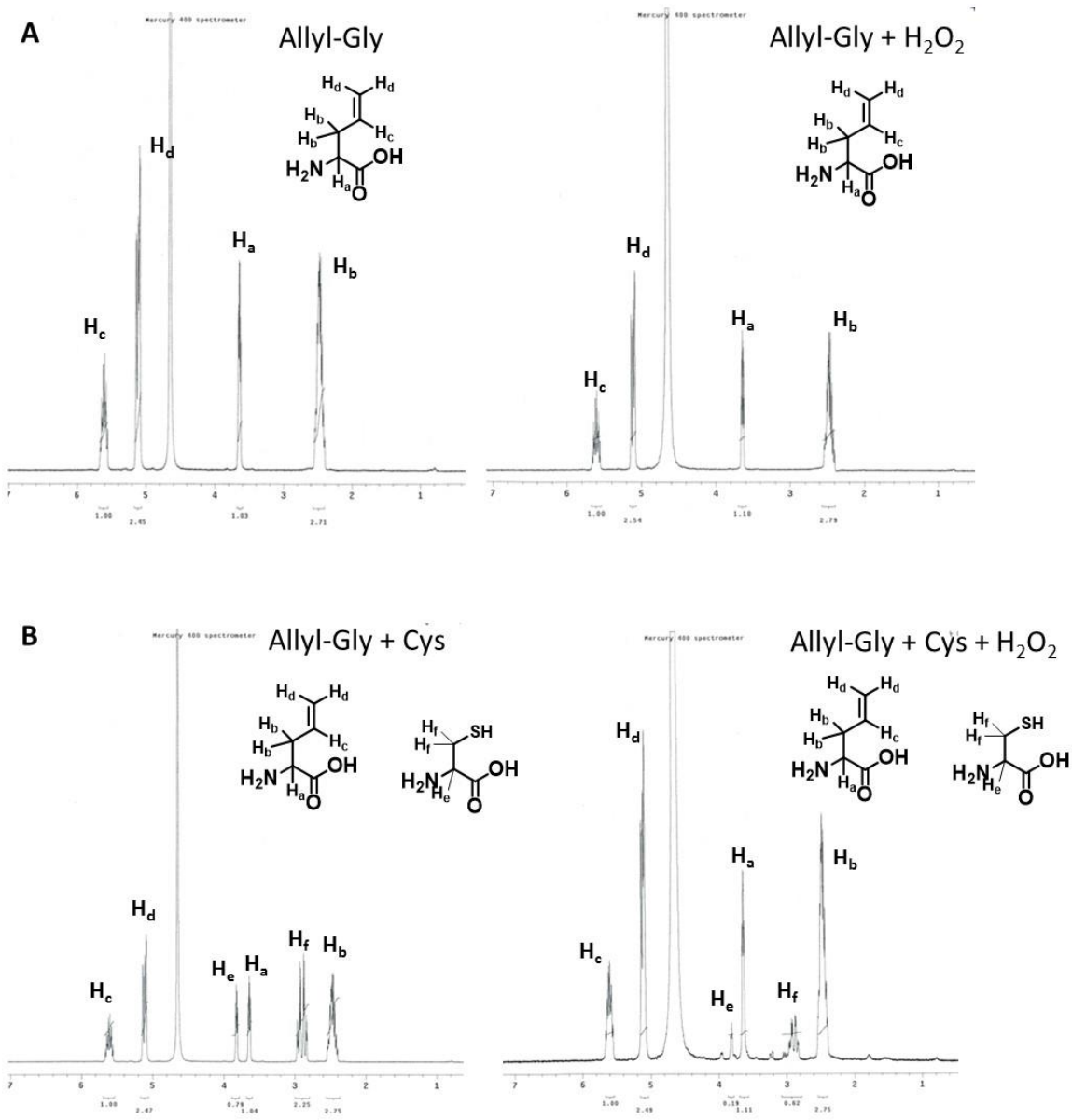


Figure S2. NMR experiments for determining the stability of allyl-Gly in the presence of Cys and/or hydrogen peroxide. (A) Allyl-Gly in D_2O without (left) and with (right) incubation of H_2O_2 (50 mM) for 15 min. (B) A mixture of allyl-Gly and cysteine (1:1 molar equivalents) in D_2O without (left) and with (right) incubation of H_2O_2 (50 mM) for 15 min. There is no chemical shift for allyl-Gly while Cys is oxidized.

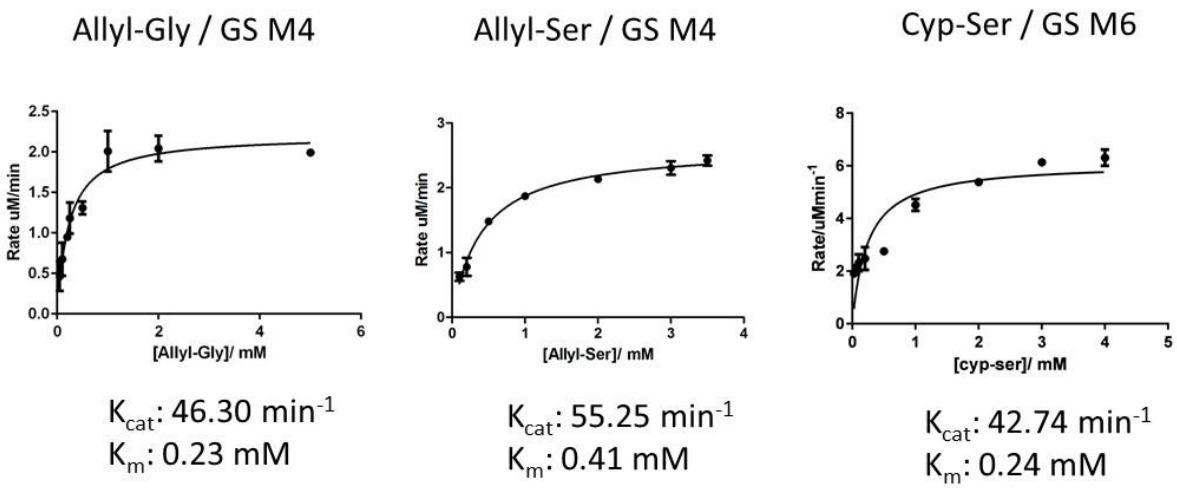
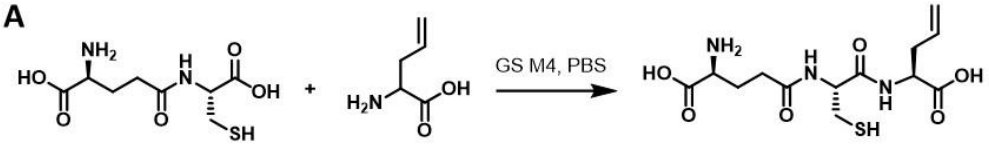
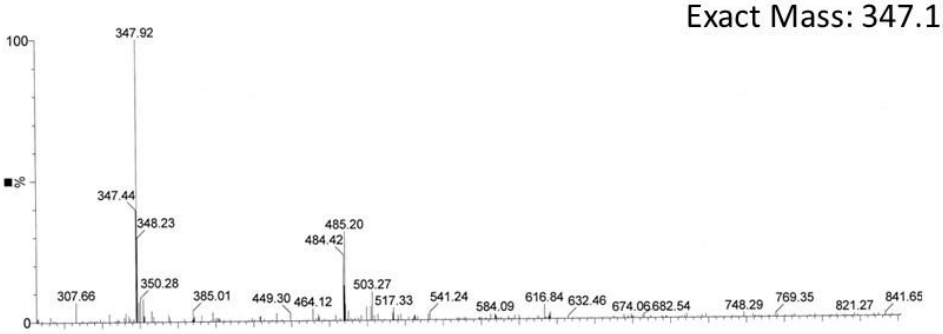


Figure S3. Kinetic values of alkene-containing amino acids for GS M4 or M6. To calculate k_{cat} and K_m values, the initial reaction rates were plotted with a range of amino acid concentrations by using Graphpad Prism.

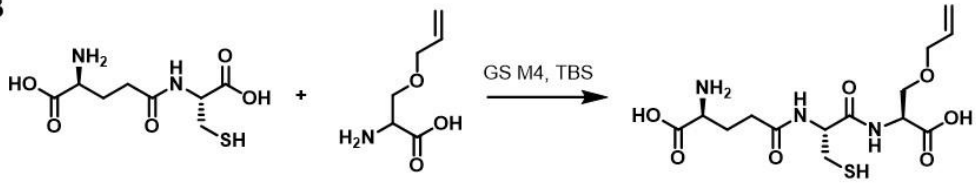
A



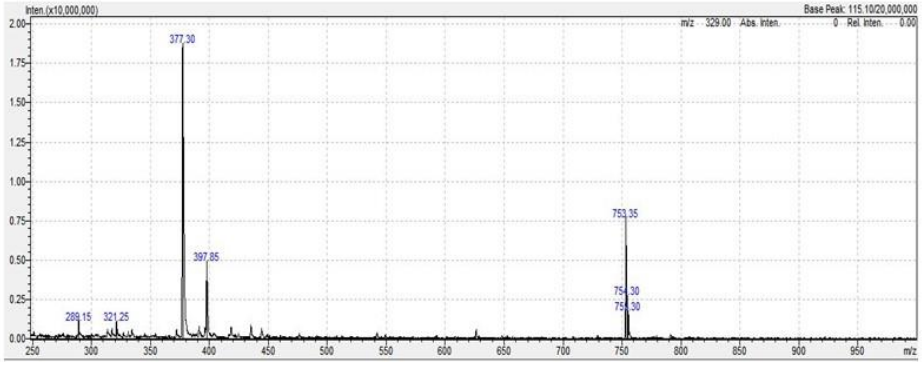
Allyl-glutathione
Exact Mass: 347.12



B



Allyl-O-glutathione
Exact Mass: 377.13



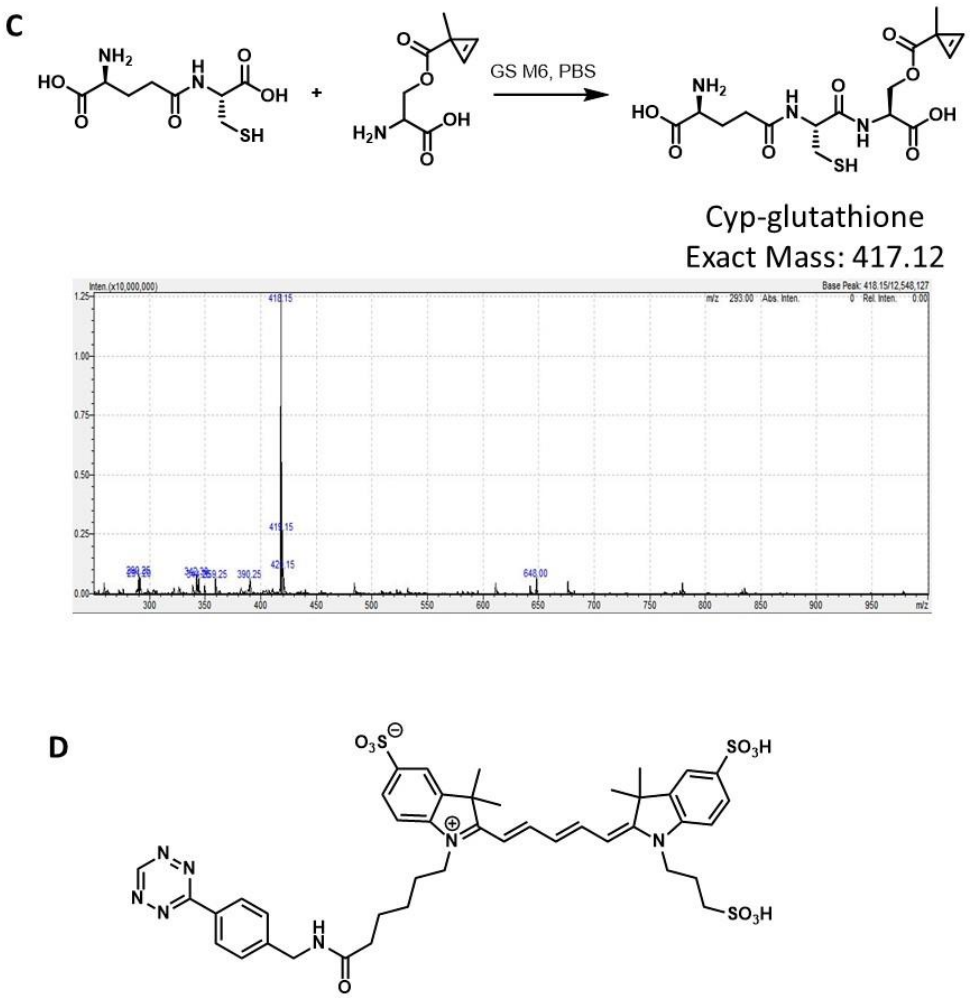


Figure S4. Synthesis of alkene-containing glutathione derivatives. (A) allyl-glutathione using GS M4. (B) allyl-O-glutathione using GS M4. (C) cyp-glutathione using GS M6. (D) A structure of tetrazine-Cy5.

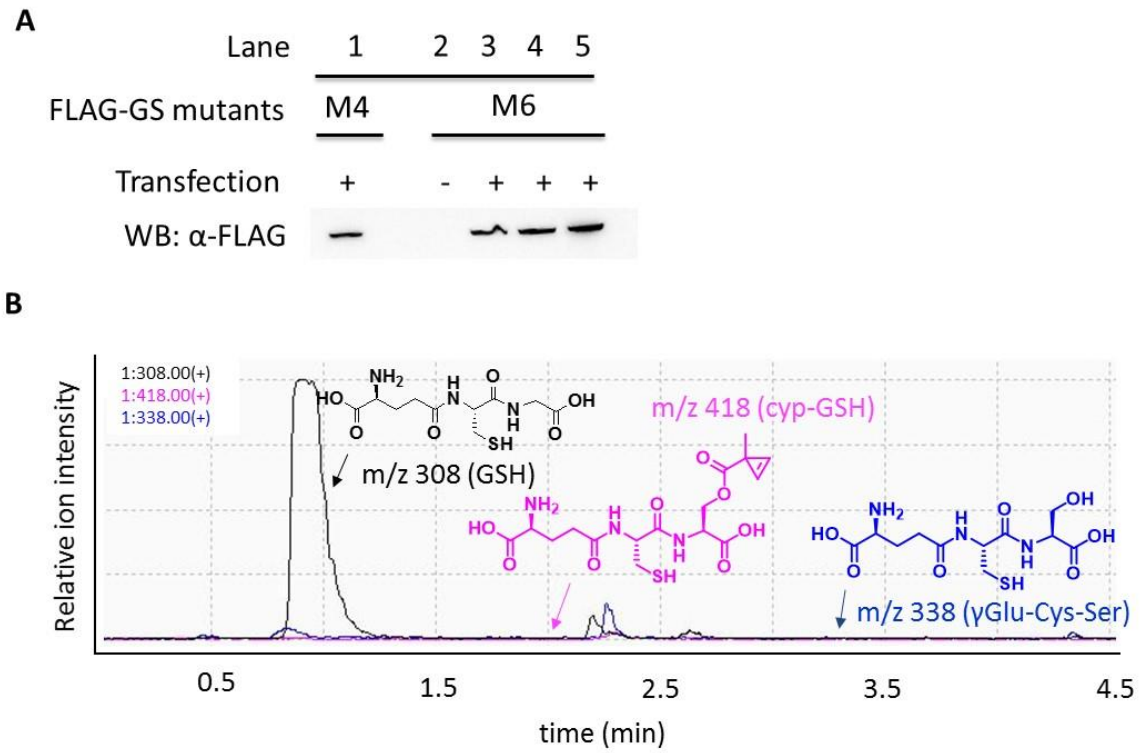


Figure S5. LC-MS analysis for biosynthesis of glutathione derivatives containing cyp-Ser. After incubation of cyp-Ser (1 mM) into HEK293 transfected with GS M6, cells were collected for LC-MS analysis. (A) Western blotting for an expression level of GS M6 in HEK293 in comparison to the level of GS M4 in HEK293 cells stably expressing GS M4. (B) LC-MS traces for extracted ion masses for indicated glutathione derivatives

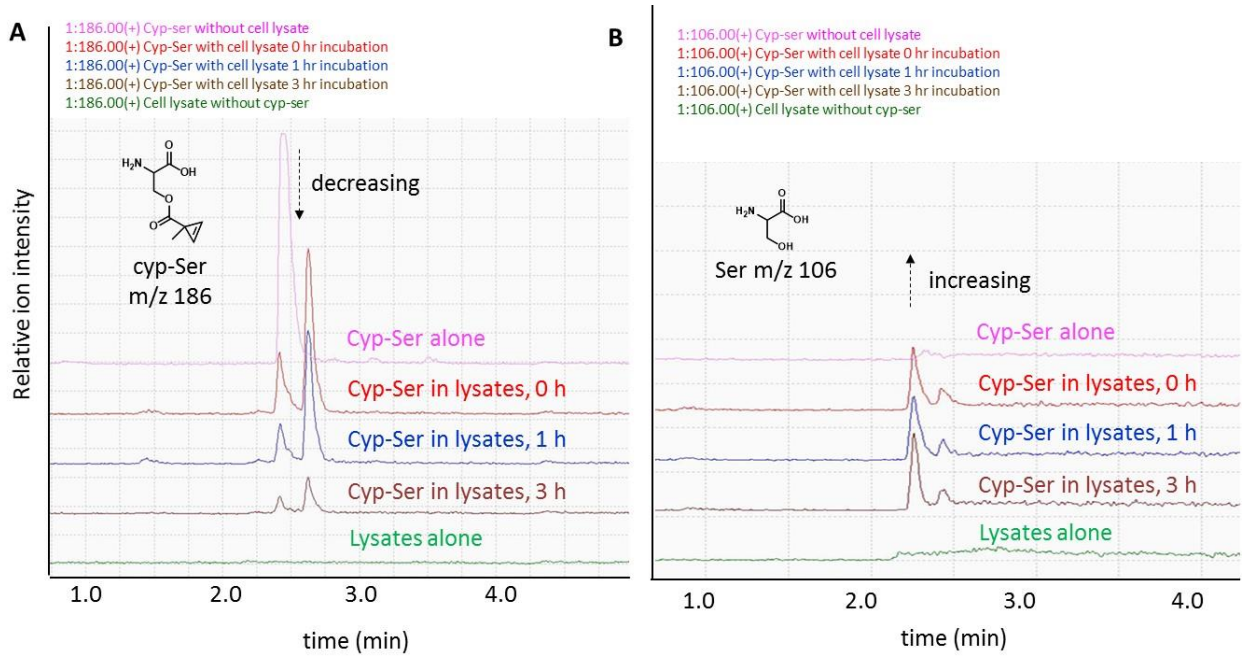


Figure S6. LC-MS analysis for stability of cyp-Ser in cell lysates. (A) LC-MS traces for extracted ion mass of cyp-Ser. (B) LC-MS traces for extracted ion mass corresponding to Ser, a hydrolyzed product of cyp-Ser. Cyp-Ser was added to freshly-prepared cell lysates. Lysates were collected at different time points (0-3 h). After precipitation of proteins, the supernatant was analyzed by LC-MS.

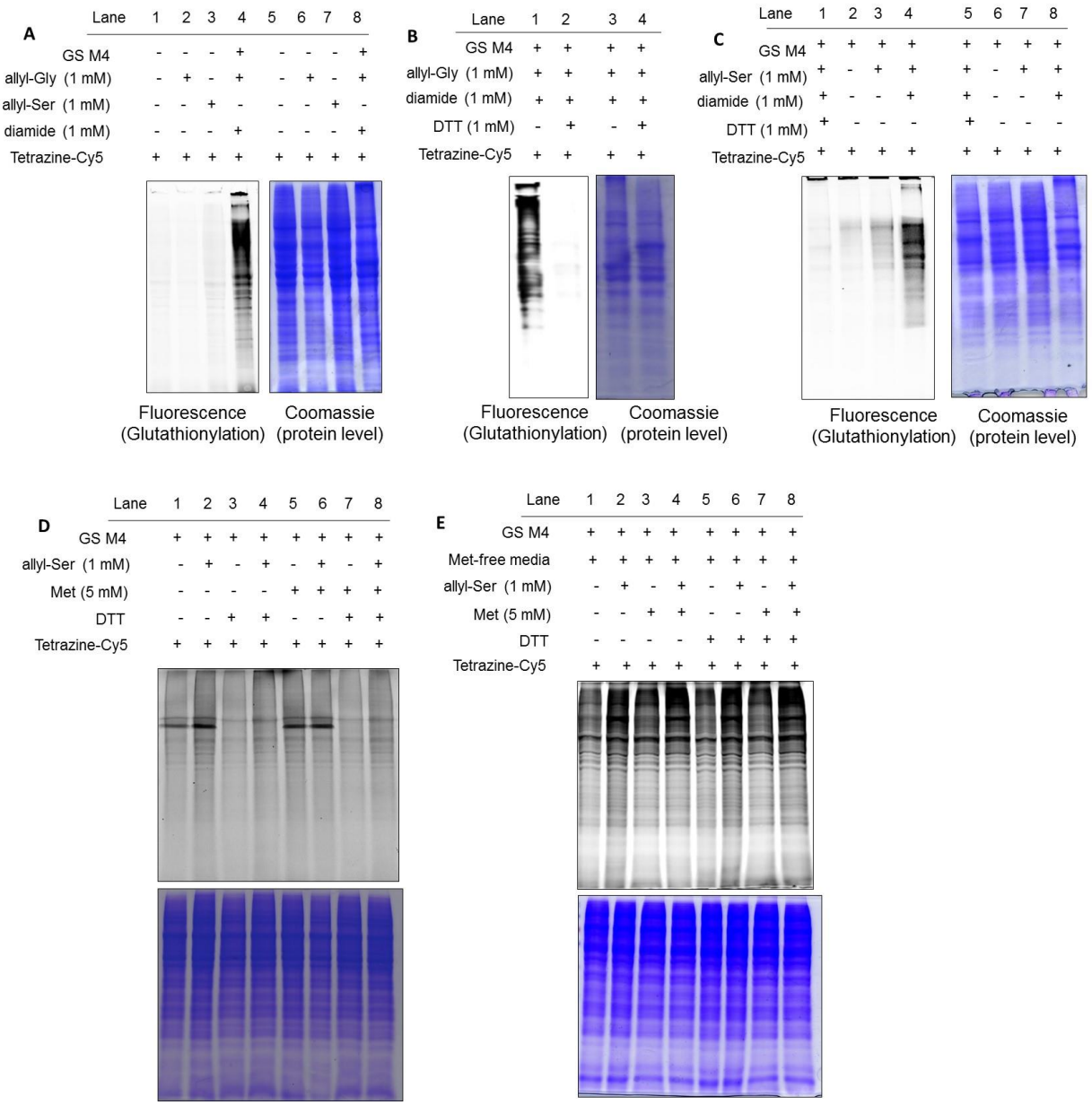


Figure S7. In-gel fluorescence detection of glutathionylation. (A) An incubation of allyl-Gly or allyl-Ser alone without GS M4 does not show any apparent signal that results from labeling intracellular proteins. (B) Glutathionylation signals detected upon incubation of allyl-Gly and H_2O_2 disappear upon treatment of DTT. (C) Glutathionylation signals detected upon incubation of allyl-Ser and H_2O_2 disappear upon treatment of DTT (D) High exposure in-gel fluorescence upon incubation of allyl-Ser. The number of protein bands are apparently same between lane 1 vs 2, lane 5 vs 6, and lane 2 vs 6, indicating that allyl-Ser does not label proteins. (E) High exposure in-gel fluorescence upon incubation of allyl-Ser in Met-free DMEM. The number of protein bands are apparently same in the absence or presence of Met (lane 2 vs 4; lane 6 vs 8), indicating that allyl-Ser does not label proteins.

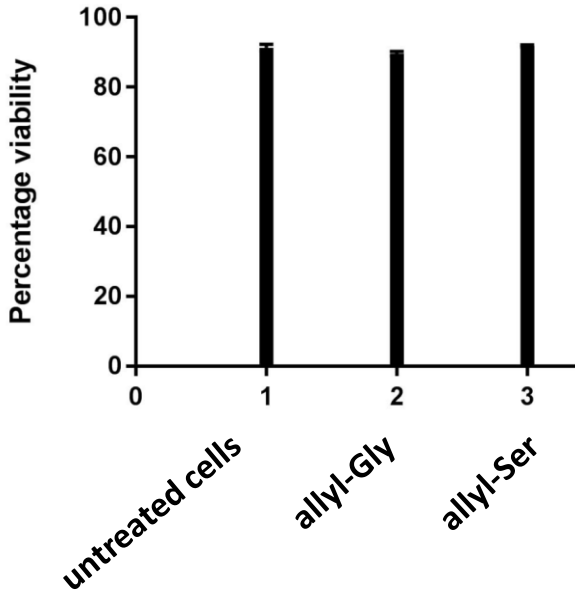


Figure S8. Cell viability upon incubation of allyl-Gly or allyl-Ser. HEK293/GS M4 cells were incubated with allyl-Gly (1 mM) or allyl-Ser (1 mM) for 24 h. Cells were collected for measuring the viability by Trypan blue assay.

Experimental Methods

General. All reagents and solvents were purchased from Sigma-Aldrich. All cell culture reagents were purchased from Life technologies. The mass spectroscopic data were obtained from Water micromass ZQ LC-MS and Shimadzu LC-8040 Triple Quad. NMR spectra were recorded on a 400 MHz Bruker units.

Synthesis of allyl-Ser (2). A solution of N-Boc-Serine (0.5 g, 2.4 mmol) in DMF (8 mL) was slowly added to a solution of sodium hydride (144 mg, 6 mmol) in DMF (2 mL) at 0 °C. Allyl-bromide (260 μL, 3 mmol) was then added and stirred for 3 h at room temperature. Excess sodium hydride was quenched by an addition of water (0.5 ml), and DMF was removed under reduced pressure. The mixture was dissolved in water and acidified with 4 M HCl (2 mL), and the aqueous layer was extracted five times with ethyl acetate (10 mL each). The combined organic layer was dried over anhydrous sodium sulfate and concentrated to give Boc-allyl-Serine. The crude Boc-allyl-Serine was dissolved in dichloromethane (4 mL). After adding 4 N HCl in dioxane (1 mL), the mixture was stirred for 1 h. The solution was concentrated, and cold diethyl ether was added to precipitate the product. The solid product was washed with diethyl ether and dried under vacuum to obtain allyl-Ser (2) (150 mg, 48%). ^{1}H-NMR (400 MHz, D_2O): δ 5.7-5.8 (m, 1 H), δ 5.55 (d, *J* = 16 Hz, 1H), 5.45 (d, *J* = 18 Hz, 1H), 4.20 (t, *J* = 6.5 Hz, 2H), 3.93 (t, *J* = 5.0 Hz,1H), 3.86 (d, *J* = 3.5 Hz, 1H), 3.83 (d, *J* = 3.5 Hz, 1H). ESI-MS (m/z): [M+H] for $C_6H_{11}NO_3$ 146. 07 found 146. 10.

Synthesis of cyp-Ser (3). 1-methylcyclopro-2-ene carboxylic acid was synthesized according to the reported procedure.[S1] To a solution of N-Boc Serine (34 mg, 0.133 mmol), N,N'-diisopropylcarbodiimide (18 mg, 0.146 mmol) and 4-dimethylaminopyridine (5 mg, 0.0013 mmol) in DMF (2 mL) was added dropwise a solution of 1-methylcyclopro-2-ene carboxylic acid (13 mg, 0.133 mmol) in DMF (2 mL) at 0°C. The reaction mixture was stirred overnight at room temperature. The reaction mixture was diluted with ethyl acetate (50 mL), and the organic layer was washed with water (20 mL), 1 M HCl (20 mL), brine (20 mL), and dried over anhydrous sodium sulfate. The mixture was concentrated and purified with silica gel column chromatography (10-50% ethyl acetate in hexane). The esterified Boc-cyp-Ser was dissolved in dichloromethane (4 mL). After adding 4 N HCl in dioxane (1 mL), the mixture was stirred for 1 h. The mixture was concentrated, and the product was precipitated by addition of cold diethyl ether. After washing with cold-diethyl ether, the solid product was dried under vacuum to obtain cyp-Ser (3) (13 mg, 54%). ^{1}H-NMR (400 MHz, D_2O): δ 6.98 (d, *J* = 4.8 Hz, 2H), 4.32 (d, *J* = 12 Hz, 1H), 4.25 (d, *J* = 4 Hz, 1H), 3.97- 3.81 (m, 1H), 1.18 (s, 3H); ESI-MS (m/z): [M+H] for $C_8H_{11}NO_4$ 186. 07, found 186.15.

Plasmid. pET28a bacterial expression vector containing the GS M4 was used for quick change mutagenesis to construct the GS mutants M5 and M6. Primers for site-directed mutagenesis are shown below. Grx3 gene was amplified by PCR using the forward primer (shown below) with NdeI restriction site and the reverse primer with XhoI restriction site. Amplified PCR product was sub-cloned into the pET28a bacterial expression vector. All cloned plasmids were confirmed by DNA sequencing of entire open reading frames. PTP1B plasmid was kindly provided by Dr. Anthony Bishop (Amherst College).

GS mutant	Primer sequence (5′-3′)
GSM5 F152A/S151G/V461A	F: GAGCATGCAGATGGTGGTGCGGCAGCGGGAGTGGCA GTC R: GACTGCCACTCCCGCTGCCGCACCACCATCTGCATGCTC
GSM6	F: CAAGCACGTGGGGCATGCACTTCGAACCAAAGCC

F152G/S151G/L448A	R: GGCTTTGGTTCGAAGTGCATGCCCCACGTGCTTG
Grx3	F: GGTGGTCATATGGCGGCGGGGGCGGCTGA R: GGTGGTCTCGAGCTAATTTTCTCCTCTCAGTATAGGCAGCAA TTCACC

Protein expression and purification. The bacterial expression vector pET28a/GS mutant, pET28a/Grx3, and pET21/PTP1B were transformed into *E. coli* BL21(DE3) cells and transformed cells were inoculated in 5 mL LB media containing 50 μg/ml kanamycin at 37°C overnight. Inoculated culture was diluted into 1 L LB media containing kanamycin and cells were allowed to grow at 37°C in an incubating shaker until OD (600 nm) reached at 0.8. The growing culture was then induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG) (0.4 mM) at 16°C and continued incubation for 20 h at 16°c. Cells were then collected by centrifugation at 4,000 rpm for 20 min at 4°C, re-suspended the pellet in lysis buffer (50 mM Tris–HCl pH 8.3, 300 mM NaCl, 10 mM imidazole), and lysed by passing twice through a chilled French press at 1,000 psi. The lysates were centrifuged to remove debris at 14,000 rpm for 30 min at 4°C. The supernatant with soluble proteins was incubated with Ni-NTA beads for 1 h at 4°C. After draining solutions, the beads were washed with the washing buffer (50 mM Tris-HCl pH 8.3, 300 mM NaCl, 25 mM imidazole). Bound proteins were eluted by addition of an elution buffer (50 mM Tris-HCl pH 8.3, 300 mM NaCl, 300 mM imidazole). Fractions that contain GS mutant were combined and dialyzed against a buffer (50 mM Tris-HCl pH 7.4, 10% glycerol) with 1 mM DTT. The protein was concentrated using a centrifugal filter device and the concentration was determined with Bradford assay.

Trypan blue cell viability assay. HEK293 cells expressing GS M4 at 70-80% confluency in 3 cm dishes were incubated with 1 mM allyl-Gly or allyl-Ser (dissolved in water). After 20 h, cells were washed with PBS and detached with 0.05% trypsin for 5 min. Collected cells were centrifuged at 2,000 rpm for 2 min, and the cell pellet was re-dissolved in DMEM (1 mL). Suspended cells (100 μL) were mixed with 0.4% solution of trypan blue (100 μL). The mixture (20 μL) was loaded on to the slide and percentage viability of the cells were measured using TC20 automated cell counter (Biorad).

References

S1. L. Huang and W. D. Wulff, *J. Am. Soc*., 2011, **133**, 8892-8895.