

Supporting Information

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Quantification of Protein Sulfenic Acid Modifications Using Isotope-Coded Dimedone and Iododimedone**

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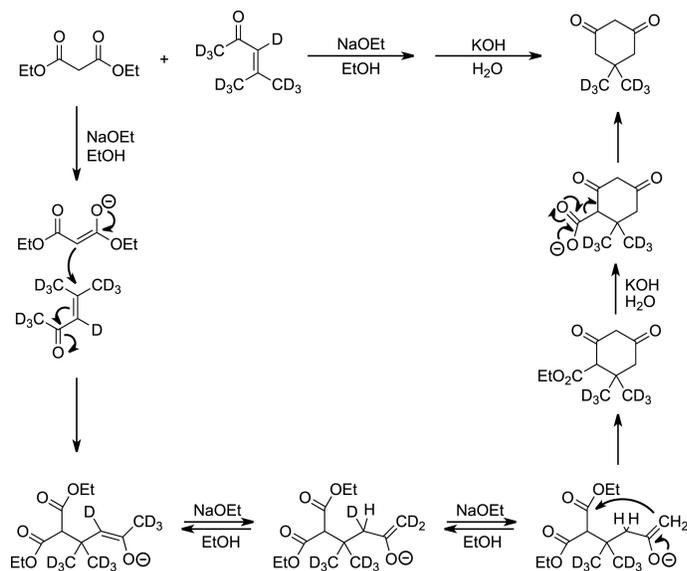
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General Synthetic Procedures

Unless otherwise noted, all reactions were performed under an argon atmosphere in oven-dried glassware. All purchased materials were used without further purification. Thin layer chromatography (TLC) was carried out using Analtech Uniplate silica gel plates. TLC plates were visualized using a combination of UV, *p*-anisaldehyde, ceric ammonium molybdate, ninhydrin, and potassium permanganate staining. Flash chromatography was performed following the method of Still *et al.*,^[1] using Sorbent Technologies Incorporated silica gel (32-63 μM , 60 Å pore size). NMR spectra were obtained on a Varian Inova 400 (400 MHz for ^1H ; 100 MHz for ^{13}C), or a Varian Mercury 300 (300 MHz for ^1H ; 75 MHz for ^{13}C NMR) spectrometer. ^1H and ^{13}C NMR chemical shifts are reported in parts per million (ppm) relative to TMS, with the residual solvent peak used as an internal reference. Low-resolution electrospray ionization (ESI) mass spectra of small-molecules were recorded on a Water-Micromass LCT spectrometer. Reverse-phase HPLC purification was performed using a Beckman Coulter System Gold 126P equipped with System Gold 166P detector ($\lambda= 220$) and a C18 (21.2×150 mm) Beckman Coulter Ultraprep column using a gradient of 0.1% TFA in H_2O and CH_3CN as the mobile phase.

Synthesis of Compounds

*d*₆-Dimedone



The following is a slight modification of the literature protocol. Sodium metal (120 mg, 5.21 mmol) was added to ethanol (3 mL) at rt under argon. After the sodium dissolved completely, diethyl malonate (850 mg, 5.45 mmol) was added to the sodium ethoxide solution followed by a slow addition of mesityl-*d*₁₀ oxide (500mg, 4.62 mmol) at rt. The solution was warmed to reflux (oil bath at 100 °C). After 3 h reflux, the solution was cooled to rt and a solution of potassium hydroxide (625 mg, 11.14 mmol) in H₂O (3 mL) was added. The resulting solution was warmed to reflux (oil bath at 110 °C) for 6 h. The solution was cooled to 0 °C and acidified with 6 N HCl to pH 3. The resulting precipitate was filtered, washed with ice-cold water three times and purified by C18 reverse-phase HPLC (10 μm, 21.2×150 mm, Beckman Coulter) with a gradient of 0% B to 50 % B over 50 min (buffer A: 0.1% TFA in H₂O; buffer B: 0.1% TFA in CH₃CN) at a flow rate of 10 mL/min to provide the title compound (492 mg, 3.37 mmol) in 73 % yield. ¹H

NMR (DMSO- d_6 , 400 MHz): δ 10.95 (s, 1H), 5.19 (s, 1H), 2.11(s, 4H). ^{13}C NMR (DMSO- d_6 , 100 MHz): δ 102.51, 102.50, 46.38, 31.80, 27.19. EI-LRMS calcd. For $\text{C}_8\text{H}_6\text{D}_6\text{O}_2$ $[\text{M}]^+$ 146.1214, found 146.1215.

2-Iododimedone

2-Iodo-5,5-dimethyl-1,3-cyclohexanedione were synthesized according to established literature procedures.^[2]

^1H NMR Time Course Experiment for Iododimedone Stability

100 mM PBS (pH 6, 7, or 8) was prepared in D_2O . Iododimedone (4.0 mg, 15 μmol) was dissolved in PBS at pH 6, 7, or 8 in D_2O with 10% DMSO- d_6 . ^1H -NMR was obtained at 3, 10, 30 min, 1, 2, 4, and 24 h.

ESI-LC/MS Analysis of Lysine Incubated with Iododimedone

To a solution of lysine (4.4 mg, 30 μmol) in 900 μL PBS (pH 7.4) was added a solution of iododimedone (2.7 mg, 10 μmol) in 100 μL DMSO at rt. The resulting solutions were stirred for 2 h at rt and then analyzed using a combination of TLC and ESI-LC/MS using a Shimadzu LCMS-2010 after separation on a Vydac Everest 2.1 \times 250mm, 300 \AA , 5 μm reverse-phase C18 monomeric column with a gradient of 5% to 65% B in 60 min (buffer A: 0.1% formic acid in H_2O ; buffer B: 0.1% formic acid in CH_3CN) at a flow rate of 0.2 mL/min.

Cloning, Expression, and Purification of Recombinant Gpx3

Recombinant Cys82Ser Gpx3 and Cys64Ser Cys82Ser Gpx3 proteins were expressed and purified as described previously.^[3]

Western Blot

His-tagged C64S C82S Gpx3 (105.7 μ M in 50 mM Tris-HCl pH 7.4) was reduced with DTT (10 mM) for 30 min at 0 °C. To remove small-molecules, reactions were passed successively through two Micro Bio-Spin P-30 columns (BioRad). C64S C82S Gpx3 (50 μ M) was then untreated or treated with H₂O₂ (12.5, 25, 50, or 100 μ M) for 10 min followed by dimedone (20 mM) or iododimedone (20 mM). The reactions were incubated for 1 h at rt and excess H₂O₂ and/or iododimedone was quenched by the addition of DTT (100 mM). The resulting samples were subjected to SDS-PAGE and Western blot analyses as reported previously.^[4] Equal protein loading was verified by staining with Ponceau S and α -His antibody (Pierce).

ESI-LC/MS Analysis of Intact Gpx3 Labeled with Iododimedone

C64S C82S Gpx3 and C82S Gpx3 (50 μ M in 50 mM Tris-HCl pH 7.4) were reduced with DTT (10 mM) for 30 min at 0 °C and then treated with 50 mM iododimedone or DMSO for 2 h at rt. Small molecules were removed by passage through a Micro Bio-Spin P-30 column and analyzed using a Shimadzu LCMS-2010 after separation on a PLRP-S polymeric RP-HPLC column (50 \times 2.1mm, 1000Å, 5 μ m) using a gradient of 5% to 35% B in 30 min (buffer A: 0.1% formic acid in H₂O; buffer B: 0.1% formic acid in CH₃CN) at a flow rate of 0.2 mL/min.

Quantitative Analysis of Sulfenic-Acid Modification

C64S C82S Gpx3 or C82S Gpx3 (100 μ M in 50 mM Tris-HCl pH 7.4) were reduced as described above. Gpx3 mutants (50 μ M) were untreated or oxidized with H₂O₂ (12.5, 25, 50, or 100 μ M) in the presence of *d*₆-dimedone (20 mM) for 1 h at rt. Excess H₂O₂ was quenched by the addition of DTT (10 mM) for 10 min at 0 °C. To remove small molecules, reactions were passed successively through two Micro Bio-Spin P-30 columns. The resulting samples were treated with DTT (10 mM) for 30 min at 0 °C followed by iododimedone (50 mM). After 2 h at rt, small molecules were removed and proteins were exchanged into 50 mM NH₄CO₃ (pH 8) using a Micro Bio-Spin P-30 column. The resulting samples were treated with DTT (10 mM) for 1 h at rt and digested with sequencing grade modified trypsin (Promega) for 24 h at 37 °C. Complete digestion was verified by SDS-PAGE. Peptide digests were analyzed on a Shimadzu LCMS-2010 after separation on a Vydac Everest reverse-phase C18 monomeric column (2.1×250mm, 300Å, 5 μ m) with a gradient of 5% to 65% B in 60 min (buffer A: 0.1% formic acid in H₂O; buffer B: 0.1% formic acid in CH₃CN) at a flow rate of 0.2 mL/min. Digests of C82S Gpx3 were prepared as described above except that the protein was digested with sequencing grade chymotrypsin (Promega) for 24 h at 37 °C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from rabbit muscle was obtained as a powder (Sigma). Digests of GAPDH were prepared as described above with the following modifications. After reduction, GAPDH (25 μ M) was untreated or oxidized with H₂O₂ (3.125, 6.25, 12.5, 25, or 50 μ M) in the presence of *d*₆-dimedone (10 mM) for 1 h at rt. Excess H₂O₂ was quenched with DTT (10 mM) for 10 min at 0 °C. Small molecules were removed and protein exchanged into 50 mM NH₄CO₃ (pH 8) using a Micro Bio-Spin P-30 column. Reactions were then treated with DTT (5 mM) for 30 min at 0 °C followed by iododimedone (20 mM). After 2 h at rt, excess iododimedone was

quenched with DTT (30 mM) for 1 h at rt and the protein was digested with Sequencing Grade Modified Trypsin (Promega) for 24 h at 37 °C.

Figure Legends

Figure S1. Possible mechanisms for *S*-alkylation by iododimedone. (A) Nucleophilic attack of the thiolate at the secondary iodine atom in iododimedone furnishes a sulfenyl halide intermediate, which reacts with the dimedone nucleophile to give the *S*-alkylation product. Should the reaction mechanism in Figure S1A be operational, the resulting sulfenyl iodide also has the potential to react with protein thiols (or hydrolyze to the sulfenic acid and then react with protein thiols).^[5] If this mechanism is operational, the absence such side reactions in our reactions is most likely due to the fact that protein labeling was carried out at neutral pH (preventing hydrolysis) and in the presence of excess alkylating reagent. (B) Nucleophilic substitution of the thiolate at the α -carbon in iododimedone to give the *S*-alkylation product.

Figure S2. Iododimedone is stable under experimental conditions. (A) The chemical shifts of iododimedone (15 μ M) in PBS buffered solution (pH 7) with 90% D₂O and 10% DMSO-*d*₆ were monitored by ¹H NMR at 3, 10, 30 min, 1, 2, 4, and 24 h. (B) A plot of chemical shifts (ppm) of iododimedone plotted with respect to time (h).

Figure S3. Iododimedone reacts with the low-molecular weight thiol GSH to afford the alkylation product. Iododimedone (15 μ M) in DMSO (100 μ L) was reacted with GSH (10 μ M) in PBS (pH 7) for 1 h at rt. The reaction was analyzed by LC-MS. (A) Chemical structure of dimedone-labeled GSH (GS-Dimedone). (B) Dimedone-labeled GSH was resolved by reverse-phase C18 column chromatography. (C) MS analysis confirmed the covalent dimedone adduct of GSH (446 Da).

Figure S4. Iododimedone does not cross-react with lysine. (A) Iododimedone (10 μmol) in DMSO (100 μL) was added to a solution of lysine (30 μmol) in 900 μL PBS (pH 7.4) for 2 h at rt. The reaction was resolved by reverse-phase C18 column chromatography to yield two peaks. (B) Peak A had a mass of 174 Da, corresponding to unmodified lysine. (C) Peak B had a mass of 267 Da, consistent with the mass of unreacted iododimedone.

Figure S5. ESI-LC/MS shows quantitative labeling of Gpx3 residues Cys36 and Cys63 by iododimedone. C82S Gpx3 (50 μM) was reduced with DTT (10 mM) for 30 min at 0 $^{\circ}\text{C}$ and then treated with iododimedone (50 mM) or DMSO for 2 hours at rt. The resulting protein was then analyzed by ESI-LC/MS. (A) The observed molecular weight of C82S Gpx3 treated with DMSO only was 22,754.8 Da corresponding to intact, unlabeled C82S Gpx3. (B) The observed molecular weight of C82S Gpx3 treated with iododimedone (50 mM) was 23,034.1, corresponding to C82S Gpx3 with two dimedone adducts (Δm 279.4).

Figure S6. High peroxide concentrations result in sulfinic acid modification of Gpx3. (A) ESI-MS analysis of C64S C82S Gpx3 sulfinic acid-modified peptide 36-43 (closed circle). (B) Mock data illustrating how the fraction sulfenic acid can level off at an endpoint less than one. High peroxide concentrations can decrease the apparent populations of both the RSH and RSOH forms of the protein by proceeding rapidly to the RSO₂H state, which is not detectable by dimedone or iododimedone probes. (C) Graph of mock data presented in (B).

Figure S7. Quantifying peroxide-dependent sulfenic acid modification of C82S Gpx3. The molecular ions corresponding to m/z of 541 and 544 are consistent with the masses of peptide 36-43 tagged with ‘light’ dimedone and sulfenic acid-modified peptide 36-43 tagged by ‘heavy’ dimedone, respectively. (B) To monitor the peptide fragment of Gpx3 containing Cys64, the

protein was digested with sequencing grade chymotrypsin. The molecular ion corresponding to m/z of 639 is consistent with the mass of peptide 58-67 tagged with 'light' dimedone. No 'heavy' dimedone-tagged peptide was observed. (C) Fraction sulfenic acid at Cys36 (circles) and Cys64 (diamonds) plotted as a function of H_2O_2 concentration. Error bars represent \pm s.d. calculated from duplicate experiments.

Figure S8. Quantifying peroxide-dependent sulfenic acid modification of GAPDH. The molecular ion corresponding to m/z of 820 is consistent with the mass of peptide 232-245 tagged with 'light' dimedone; 'heavy' dimedone-tagged peptide was not observed.

References

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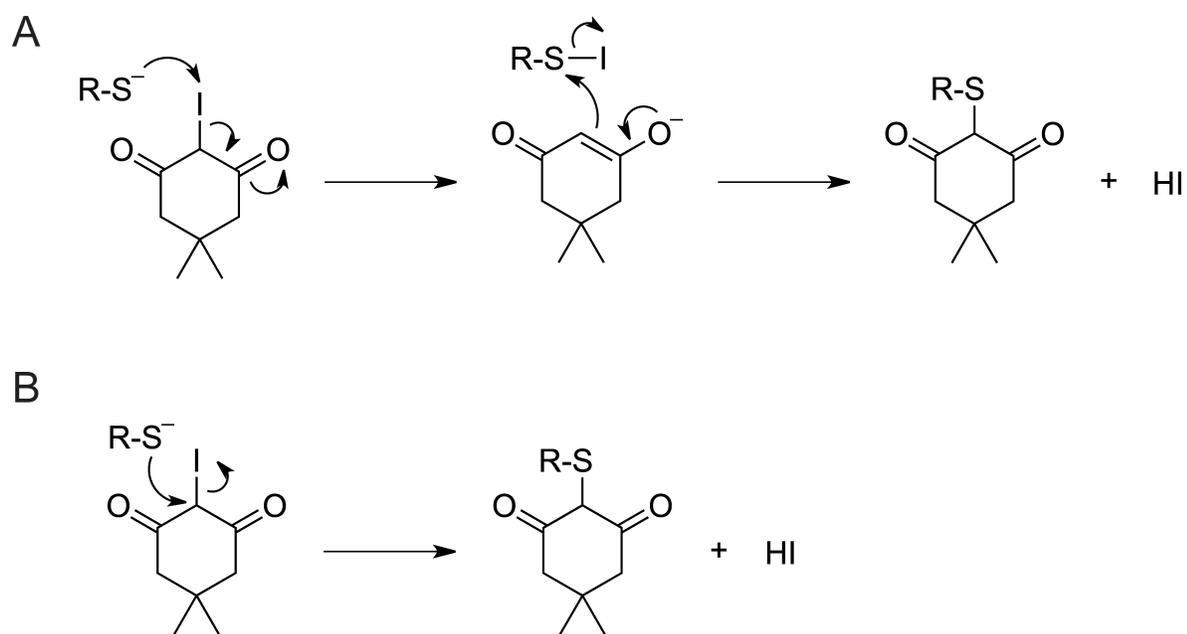


Figure S1

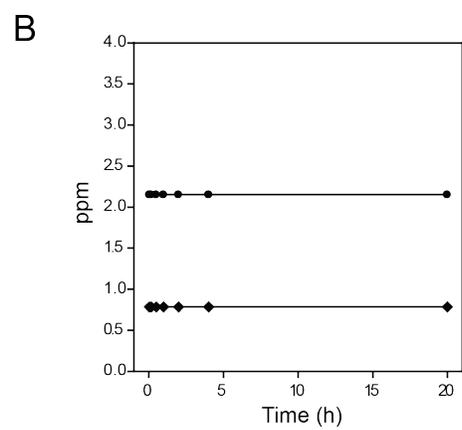
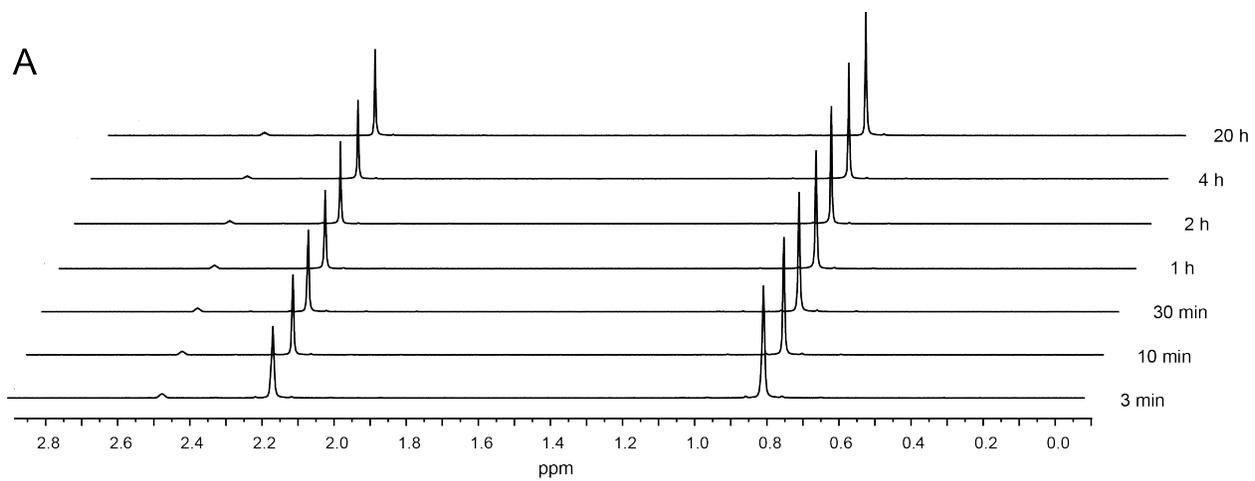


Figure S2

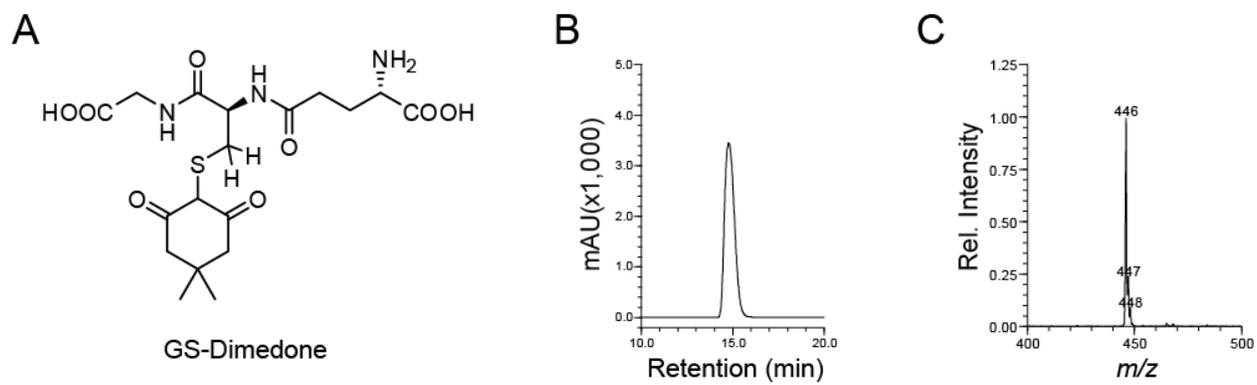


Figure S3

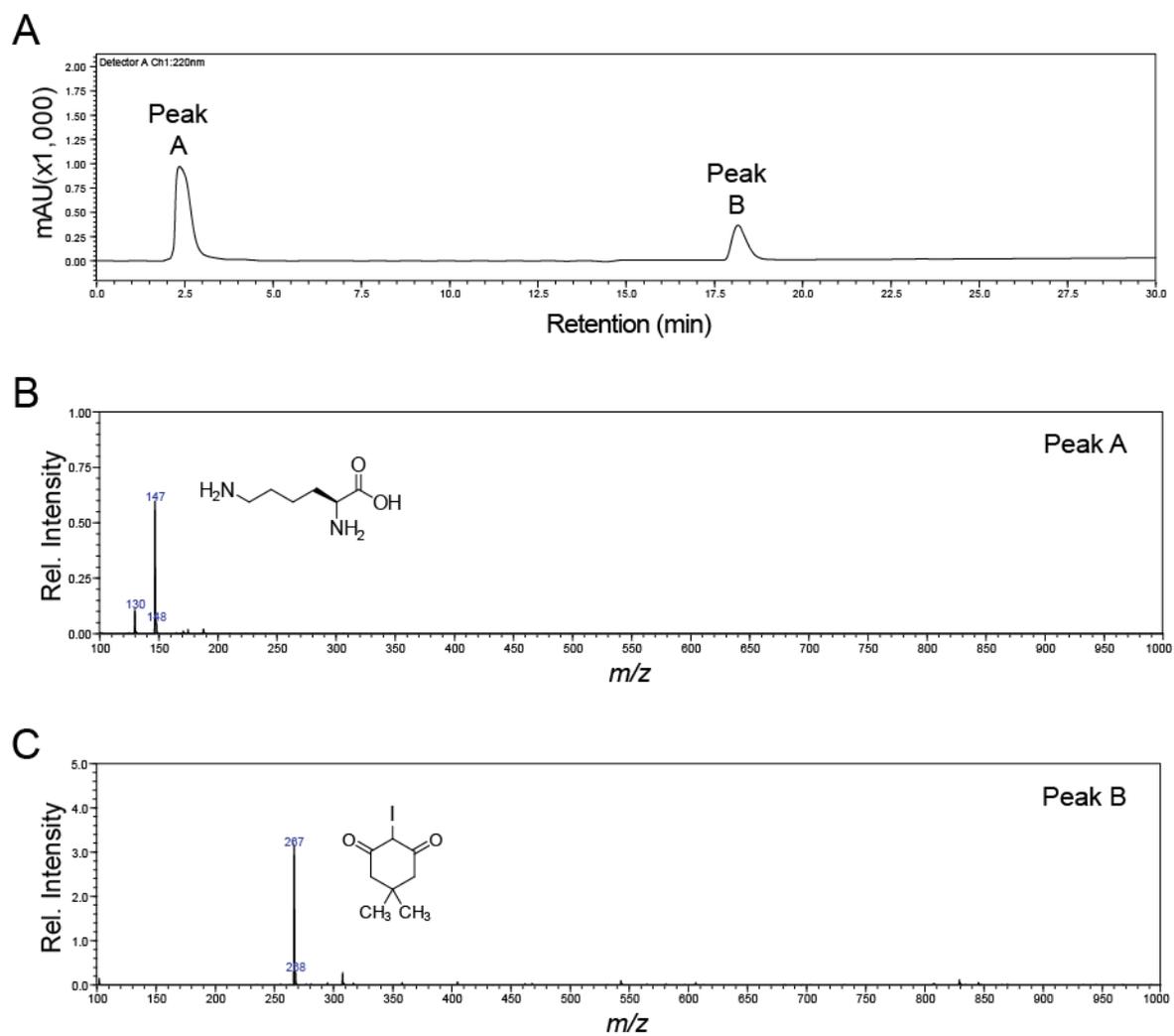


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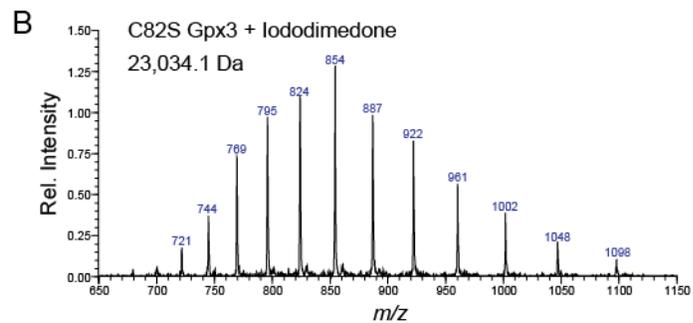
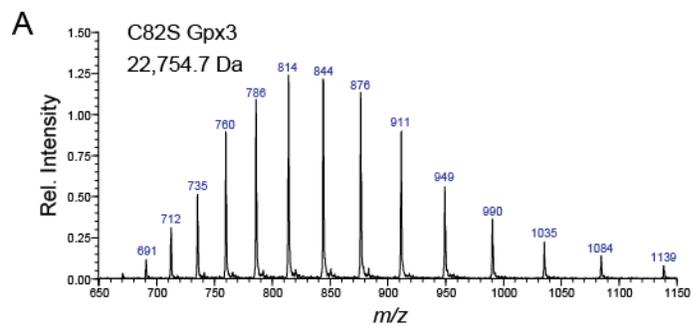
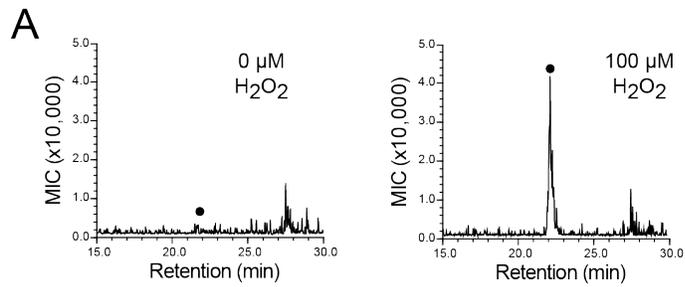


Figure S5



B

H_2O_2 (μM)	RSH (a.u.)	RSOH (a.u.)	RSO ₂ H (a.u.)	RSOH/[RSH+RSOH]
0	100	0	0	0.00
12.5	85	15	0	0.15
25	70	30	0	0.30
50	50	50	0	0.50
100	40	40	20	0.50

C

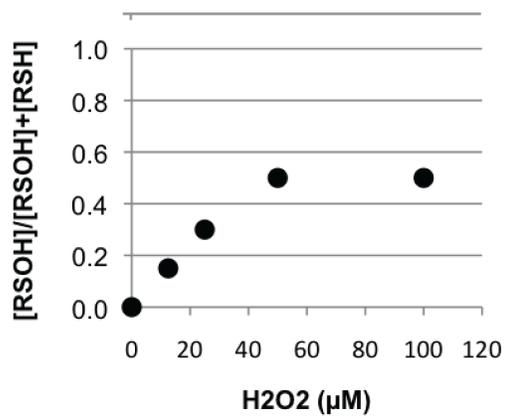


Figure S6

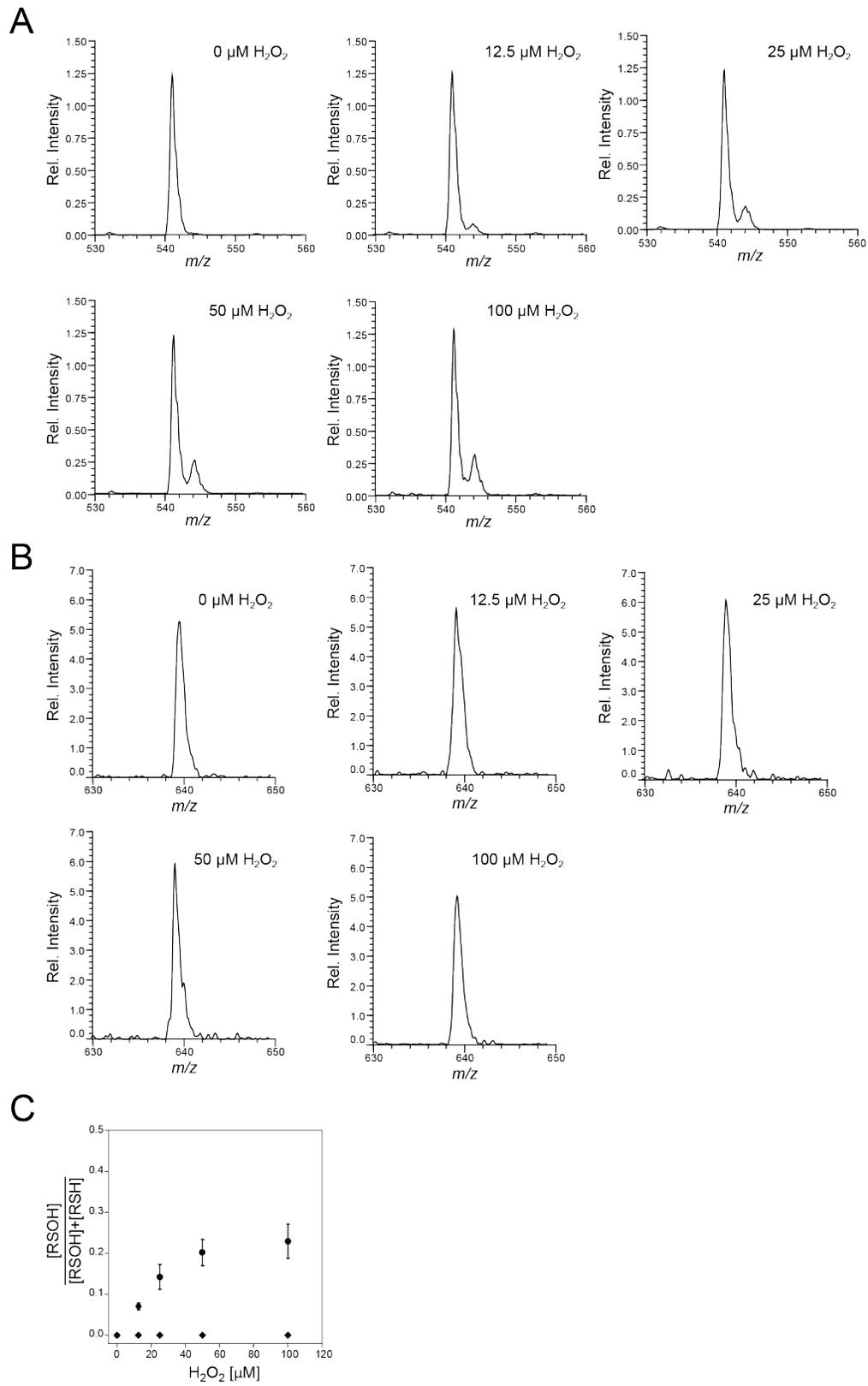


Figure S7

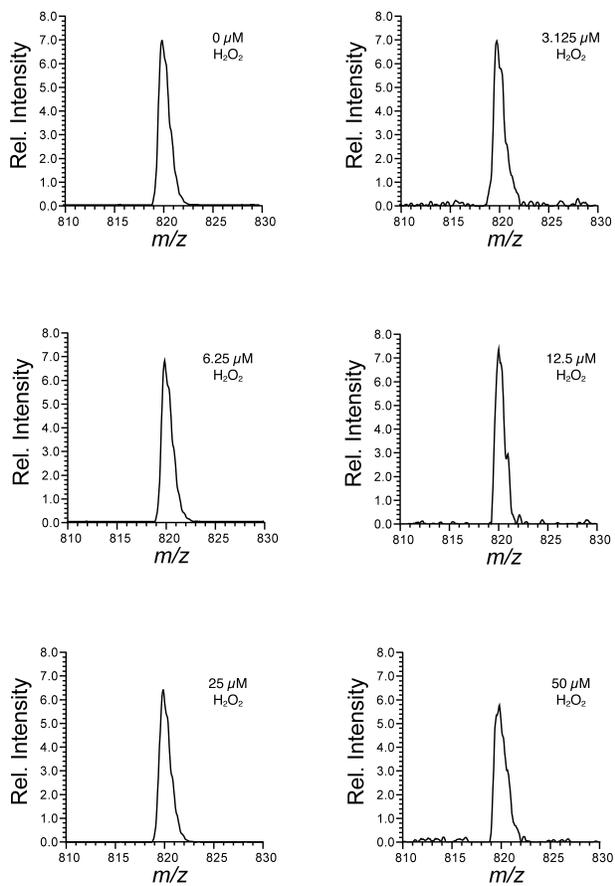


Figure S8