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Supporting Information

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Traceless Ligation of Cysteine Peptides using Selective Deselenization**

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Supporting Information

Experimental Section:

Supporting Materials and Methods.

Buffers for kinetic measurements were prepared using de-ionized water. KH_2PO_4 and K_2HPO_4 were purchased from Fisher Biotech. Deuterated solvents (DMSO-d_6, $CDCl_3$) were purchased from Aldrich Chem. Co. ^{1}H-NMR spectra were recorded on a Varian Mercury-300, Bruker DRX-500 spectrometers, using $CDCl_3$ as a solvent.

All Boc-amino acids were obtained from CS Bio Co. (Menlo Park, CA), with the following side chain protecting groups: Arg(Tos), Asp(OcHxl), Glu(OcHxl), Ser(Bzl), Thr(Bzl), Cys(MeBzl), Lys(2Cl-Z), Tyr(2Br-Z), Asn(Xan) (Tos = 4-toluenesulfonyl, OcHxl = cyclohexyl; Bzl = benzyl; MeBzl = 4-methylbenzyl; 2Cl-Z = 2-chlorobenzyloxycarbonyl; 2Br-Z = 2-bromobenzyloxycarbonyl; Xan = N-Xanthyl). 1*H*-Benzotriazolium-1-[bis(dimethylamino)methylene]-5-chloro,hexafluorophosphate(1-),3-oxide (HCTU), *S*-Trityl-β-mercaptopropionic acid-Leu-OCH_2-Pam (TMPAL-Pam) resin, were obtained from Peptides International (Louisville, KY). All solvents; HPLC-grade, *N,N*-dimethylformamide (DMF), dichloromethane, and acetonitrile (ACN), were purchased from Fisher. Trifluoroacetic acid (TFA) was obtained from Halocabon Products (River Edge, NJ). Anhydrous HF was purchased from Matheson Gas (Cucamonga, CA). Anisole and *N,N*-diisopropylethyl amine (DIEA) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were obtained from Fisher or Sigma-Aldrich, Inc.

High Performance Liquid Chromatography (HPLC). Analytical reversed-phase HPLC was performed on a Hitachi-Merck La Chrom HPLC with 214 nm UV detection using a Phenomenex C_4 column (Jupiter 5 μm, 300 Å, 0.46 × 15 cm) or C_9 (Jupiter 4 μm, 90 Å, 0.46 × 15 cm). Semi-preparative reversed-phase HPLC was performed on a Waters HPLC system using a Phenomenex C_4 column (5 μm, 1.0 × 25 cm). Preparative reversed-phase HPLC was performed on Waters HPLC system using Phenomenex C_9 column (10 μm, 2.12 × 25 cm). Linear gradients of acetonitrile in water with 0.1% TFA were used for all systems to elute bound peptides. The flow rates were 1 mL/min (analytical), 5 mL/min (Semi-preparative), and 20 mL/min (preparative). Buffer A is MilliQ water containing 0.1% TFA; buffer B is acetonitrile with 10% water and 0.09% TFA.

Mass Spectrometry. Electrospray ionization MS was performed on an API-III triple quadruple mass spectrometer (Sciex, Thornhill, ON, Canada). Peptide masses were calculated from the experimental mass to charge (*m/z*) ratios from all of the observed multiply charged species of a peptide.

Synthesis of protected Sec, Boc-Sec(4-MeBzl)-OH. The synthesis was performed as described elsewhere.[1]

Peptides synthesis. Peptides were prepared by manual solid-phase peptide synthesis (SPPS), typically on a 0.3 mmol scale using the in situ neutralization/HCTU activation procedure for Boc-SPPS.[2] The peptide coupling was carried out with 5-fold excess of activated amino acid for 20 min. The C-Terminal β-mercaptopropionic acid–leucine (MPAL) thioester peptides were synthesized after removal of trityl protecting group of the TAMPAL-Pam resin (Peptides International Inc., Louisville, KY) by a mixture of TFA:triisopropylsilane:H_2O, 95:2.5:2.5. Carboxy-amidated peptides were synthesized on a MBHA resin (*p*-methyl benzhydrylamine, Peninsula Laboratories Inc., San Carlos, CA) for Boc-chemistry. The coupling of the Boc-Sec(4-

MeBzl)-OH was carried out manually using a DIC/HOBt activation method, using Boc-Sec(4-MeBzl)-OH (0.5 mmol in 2 mL of 50% DCM/DMF) and activated with DIC (0.5 mmol) in the presence of HOBt (0.52 mmol) at 0 °C for 5 min. The Boc group on resin-bound peptide was deprotected, neutralized with DIEA (2 × 1 min), and washed with DMF. The activated Sec was then added to the resin, and the mixture was kept at room temperature for 1 h. After chain assembly, peptide side-chain deprotection and cleavage from the resin (200 mg) was carried out by standard HF protocol, (with the addition of Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB - 50 mg only for peptides containing Sec) and 1 mL anisole. The crude peptide products were precipitated and washed with cold anhydrous ether, dissolved in aqueous acetonitrile and immediately purified by preparative, reversed-phase HPLC.

Ligation reactions. Typical ligation: 1.1 mg Ac-LYRAG-SR thioester **2** (1.34 µmol, 2.68 mM) and 1.35 mg diselenide-peptide **1**, UGLEFRSI-amide (0.696 µmol, 1.39 mM) and 19 mg 4-mercaptophenylacetic acid (MPAA, 113 µmol, ~220 mM) dissolved in 0.5 mL argon-degassed PB (200 mM, 6 M GdmCl, pH 7.8) in 3 mL round-flask kept under argon, pH was adjusted to 7.5 by NaOH (1 M) and the reaction mixed by micromagnetic stirrer. The progress of the reaction was followed by analytical HPLC, affording the desired ligated product as selenylsulfide (**3a**, with MPAA) in good yields (1.33 mg, 57% recovered after 24 h).

Deselenization of ligated product, 3a. 1 mg MPAA-ligated product **3a**, (0.574 µmol) dissolved in 844 µL buffer (100 mM PB, pH 5.1) and 112 µL buffer (200 mM, 6 M GdmCl, pH 8.5) and 112 µL DTT (25.2 mM stock solution in PB, pH 5.1) and kept for 1 h. Then 120 µL TCEP solution (200 mM stock solution in PB, pH 5.1) were added. The deselenized product **4** was isolated after 17 h in good yield (0.45 mg, 56% recovery).

Deselenization of peptide 5b. 1.6 mg peptide **5b** (cyclic monomer with an intramolecular selenylsulfide, 1.453 µmol, 1.453 mM) and 2.1 mg DTT (13.6 µmol, 9.4 equiv, 13.6 mM) dissolved in 1 mL PB (200 mM, pH 5.1). After 1 h, 1 mg TCEP (3.19 µmol, 3.2 mM) were added and the deselenized product, **6**, was isolated after 2 h in high yield (1.2 mg, 80% recovery).

Dimer peptide 1 reaction with TCEP or H_2O_2. 1 mg dimer peptide **1** (0.5 µmol, 1 mM) dissolved in 0.5 mL PB (200 mM, pH 5.1) and 0.75 mg DTT (4.85 µmol, 9.7 mM) added. After 1 h, 0.7 mg TCEP (2.5 µmol, 5 mM) were added giving deselenized product **10** after 2 h, which is confirmed by ESI-MS. Separately, 1 mg dimer peptide **1** (0.5 µmol, 1 mM) dissolved in 0.5 mL PB (200 mM, pH 8.5) and H_2O_2 was added (final concentration 50 mM), giving the dehydroalanyl peptide, **8**, which was rapidly hydrolyzed under the reaction conditions to the corresponding pyruvoyl peptide **9a** (in equilibrium with its hydrate form **9b**) as confirmed by and ESI-MS.

Deselenization of peptide 5b in D_2O. 1.7 mg peptide **5b** (1.544 µmol, 1.544 mM) and 2 mg DTT (12.9 µmol, 8.4 equiv, 13 mM) dissolved in 1 mL PB (200 mM K_2HPO_4 in D_2O and pD switched to 5.1 by DCl). After 1 h, 3 mg TCEP (3.19 µmol, 3.2 mM) were added and after 12 h the major product was deselenized deuterated-peptide, **6-*d*** (~65%) with minor doubly-deuterated deselenized-desulfurized side-product, **7-*d_2*** (~20%).

Deselenization of dimer FKUSD-amide. 0.47 mg dimer FKUSD (0.365 µmol, 1.55 mM) dissolved in 52 µL PB (200 mM, pH 5.1) and 182 µL TCEP (20 mM in PB, 10 equiv) were added, and the reaction was followed by HPLC and MS. The reaction was complete within 2h, giving only FK(*l*)ASD-amide, as judged by HPLC retention time.

Grx3(1-38)(C11U-C14U-A38C). 9.7 mg Grx3(1-37)(C11U-C14U)-SR thioester (2.23 µmol, 2.23 mM) and 1 mg Cys·HCl (6.34 µmol, 6.34 mM) dissolved in 1 mL PB (200 mM, 6 M GdmCl, pH 8.3) and 1 µL thiophenol added. The progress of the NCL reaction was followed by analytical HPLC, and the desired ligated product Grx3(1-38)(C11U-C14U-A38C) was purified after 24 h by preparative HPLC (7.3 mg, 77% recovery). Similarly, Grx3(1-38)(A38C) was prepared using Grx3(1-37)-SR thioester and Cys·HCl. The synthesis of Grx3(1-37)(C11U-C14U)-SR and Grx3(1-37)-SR thioesters was published earlier.[1]

Deselenization of the diselenide peptide Grx3(1-38)(C11U-C14U-A38C). 0.4 mg peptide (0.094 µmol, 4250 Da, 0.784 mM) dissolved in 94 µL DTT solution (100 mM, ~120 equiv, in 200 mM PB, pH 5.1,) and 48 µL PB (200 mM, pH 5.1) and after 2 h, 1.5 mg TCEP (5.2 µmol, 50 equiv) added, the progress of the reaction was followed by analytical HPLC, and after 21 h the doubly deselenized product Grx3(1-38)(C11A-C14A-A38C) (4092 Da) was the major product with minor fully reduced peptide (4061 Da).

Figures and Schemes:

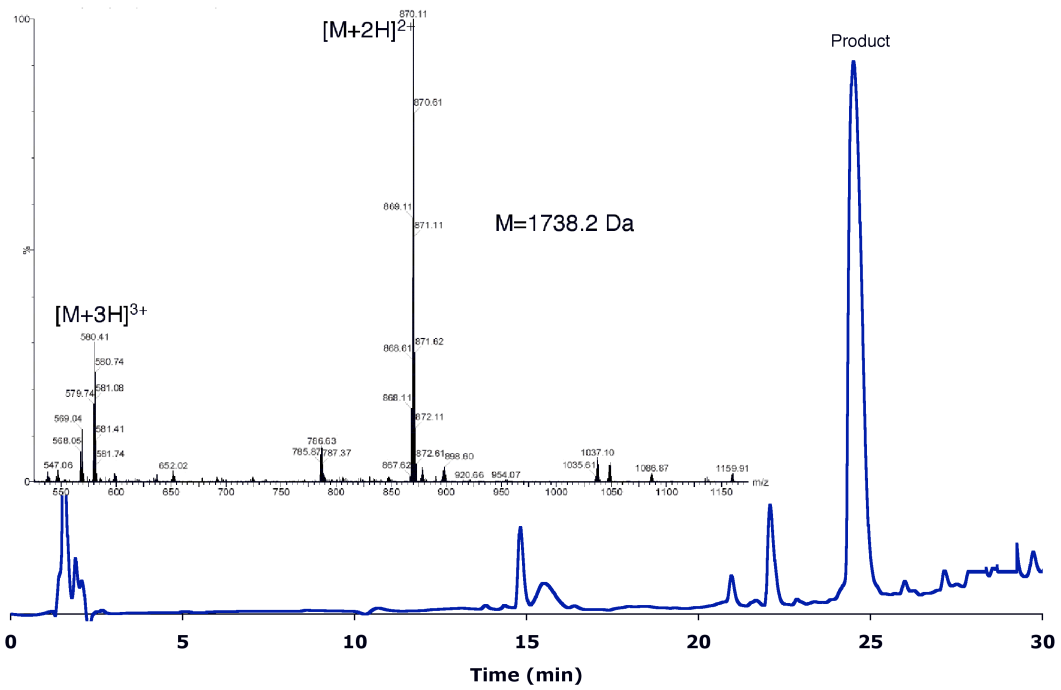


Figure S1. HPLC chromatogram for the crude ligation of C-terminal thioester peptide **2** (Ac-LYRAG-SR) and diselenide dimer peptide **1** (UGLEFRSI.amide) after 24 h. The ligated product contain intermolecular selenylsulfide bond with MPAA, **3**.

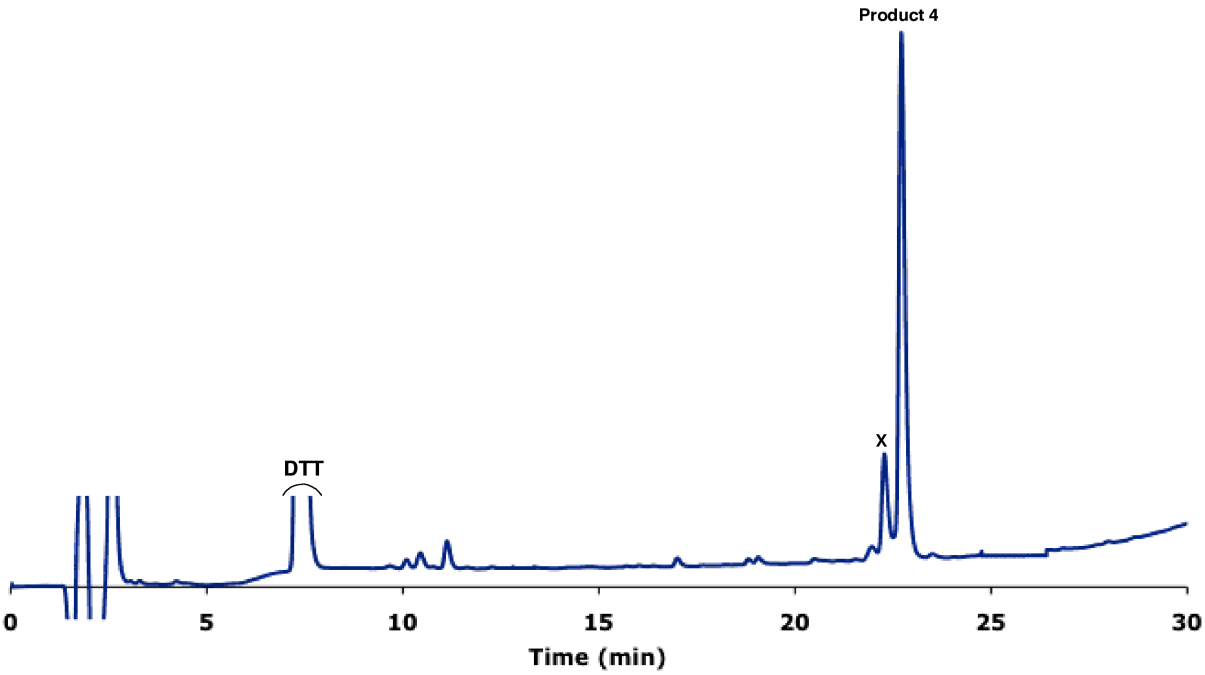


Figure S2. HPLC chromatogram for the deselenization of purified dimer peptide **3b**, with excess TCEP after 17 h. The deselenized product (Sec→Ala), **4**, was the major product with minor side product (peak x, Sec → Ser).

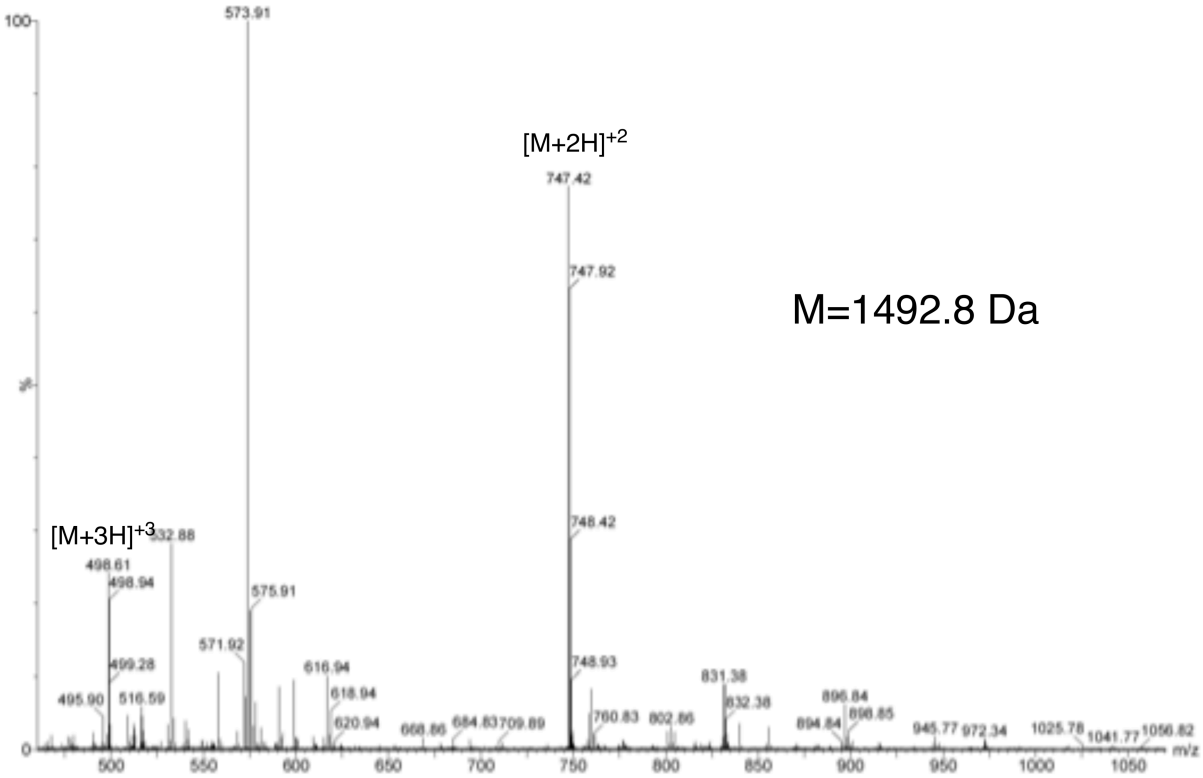


Figure S3. MS of the deselenized product, **4**.

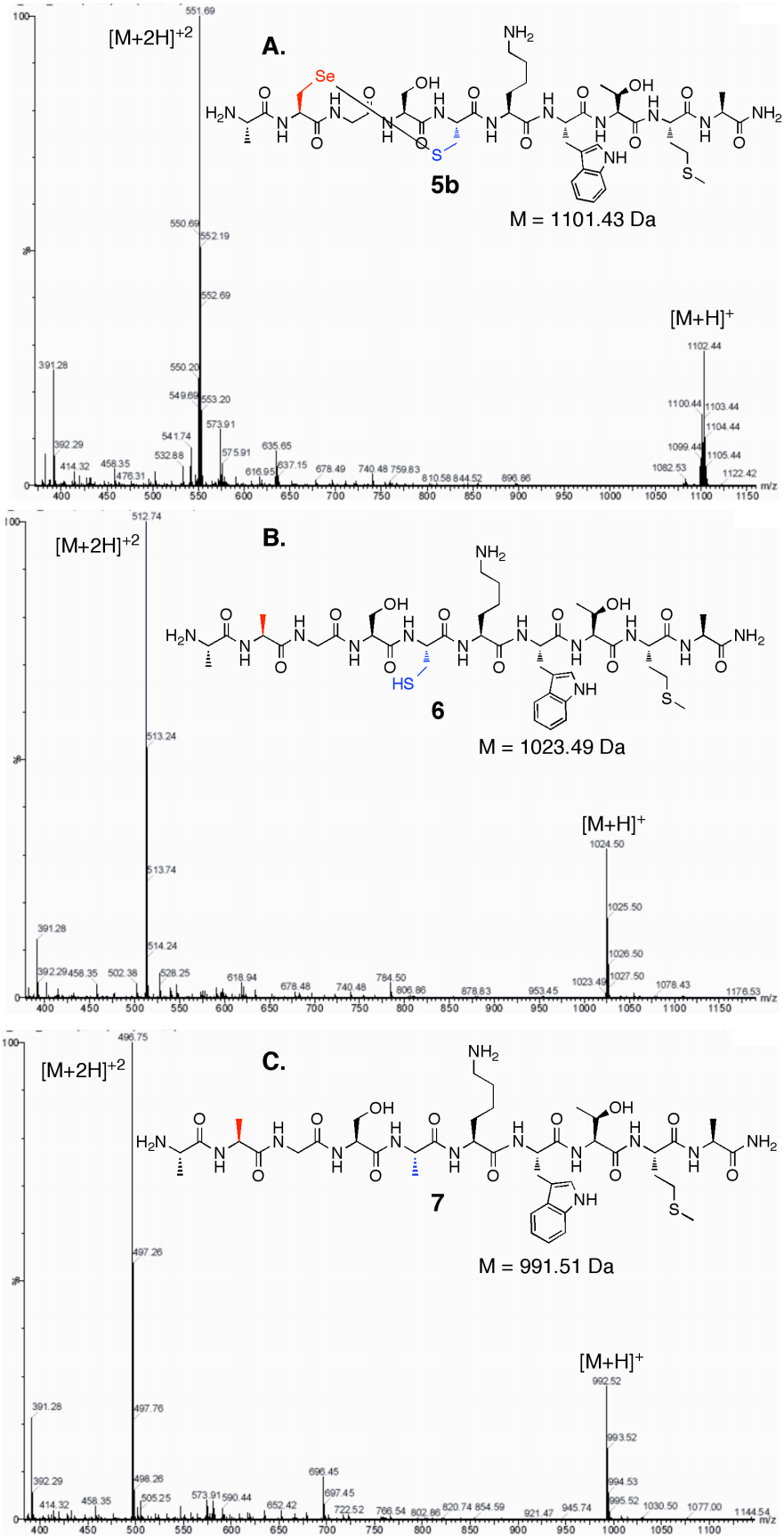


Figure S4. MS data of A. peptide **5b**; B. deselenized product, **6**; C. deselenized-desulfurized side-product, **7**.

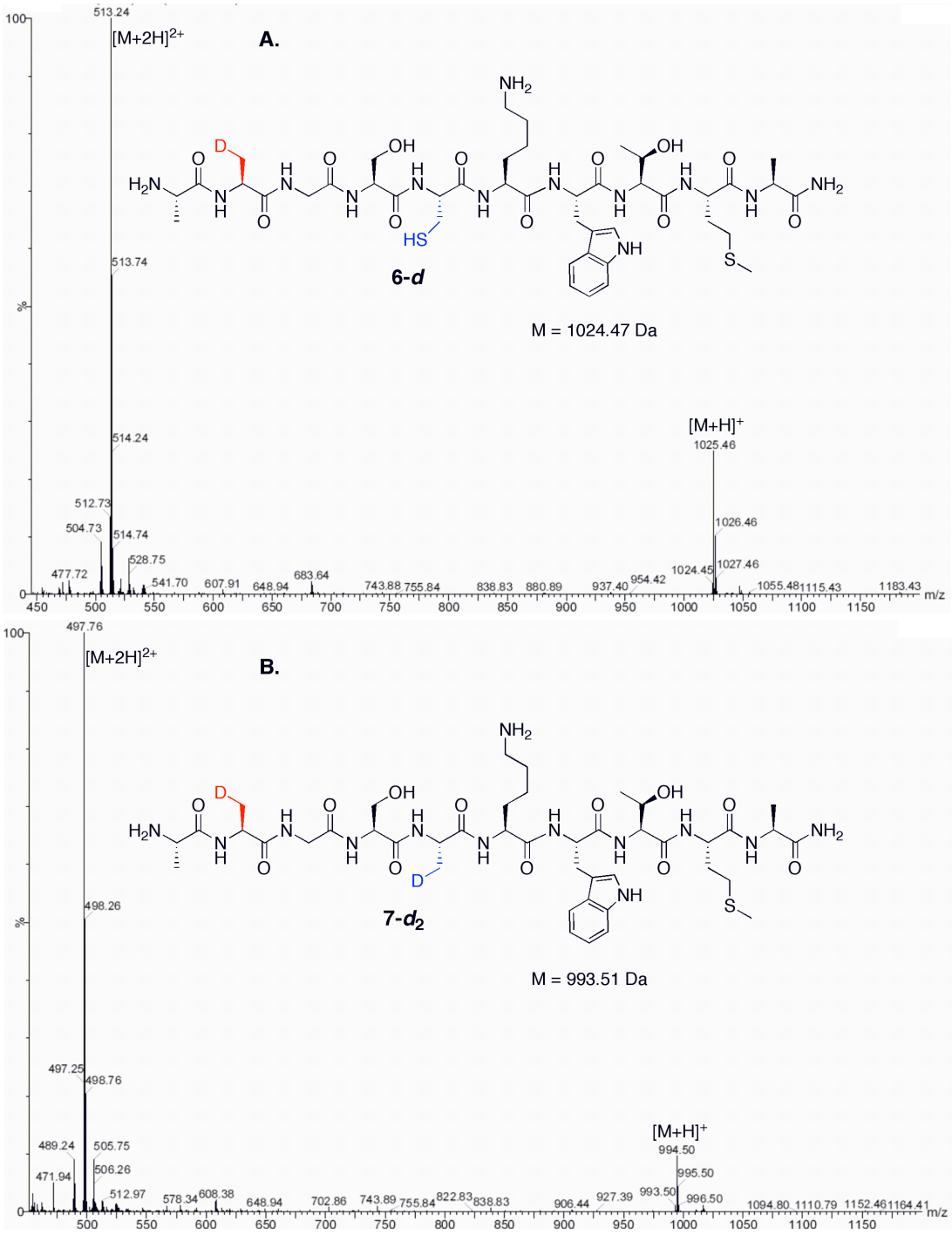


Figure S5. Deselenization of peptide **5b** in PB in D_2O by TCEP forms two products; (A) the deselenized mono-deuterated product, **6-d** as the major product and (B) the doubly-deuterated deselenized-desulfurized minor product, **7-d_2**.

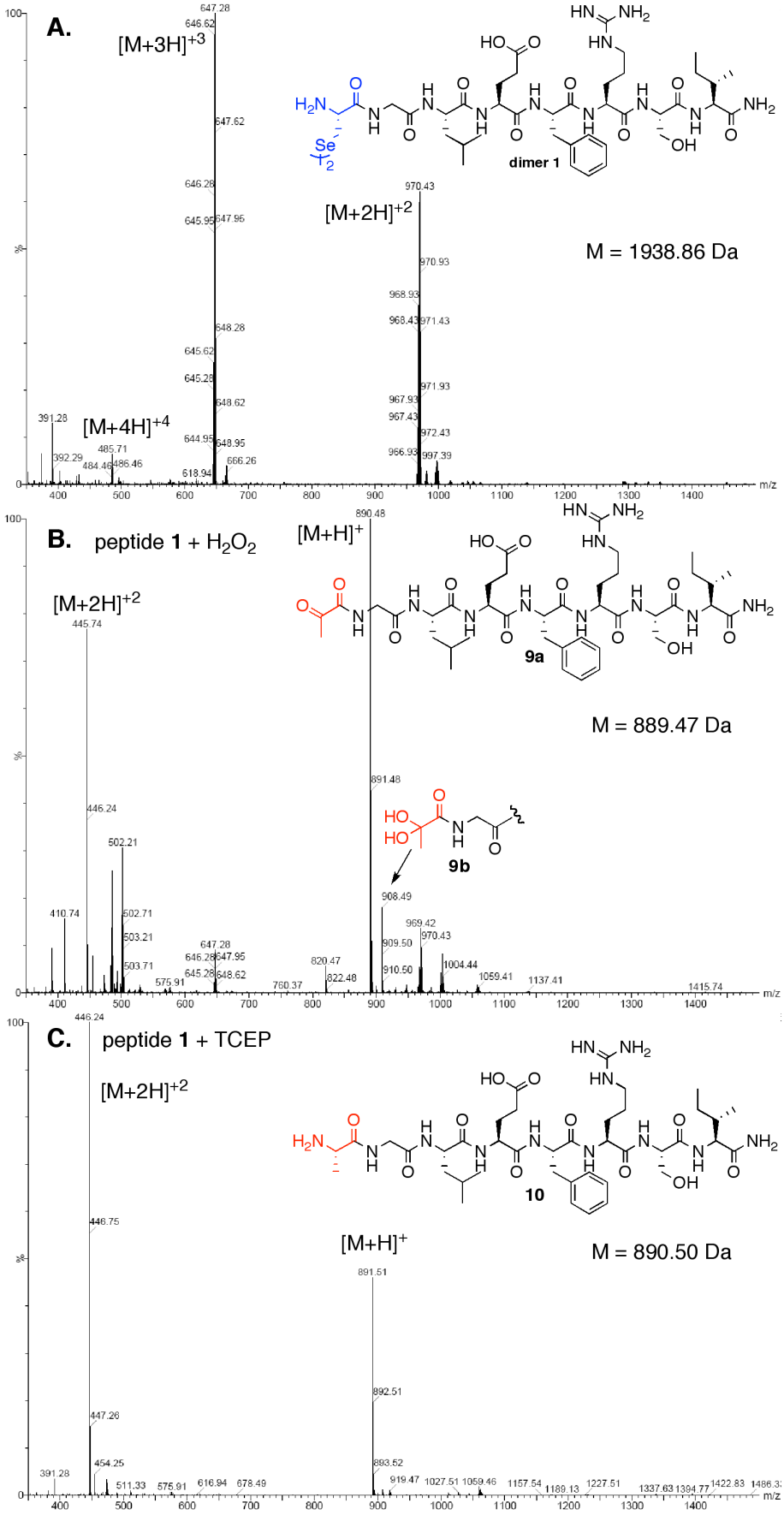


Figure S6. A. MS dimer peptide **1**. B. MS of the product of reaction dimer **1** with H_2O_2 to give the pyruvoyl product, **9a** (with the hydrate **9b**). C. MS of the product of reaction dimer **1** with TCEP to produce product **10**.

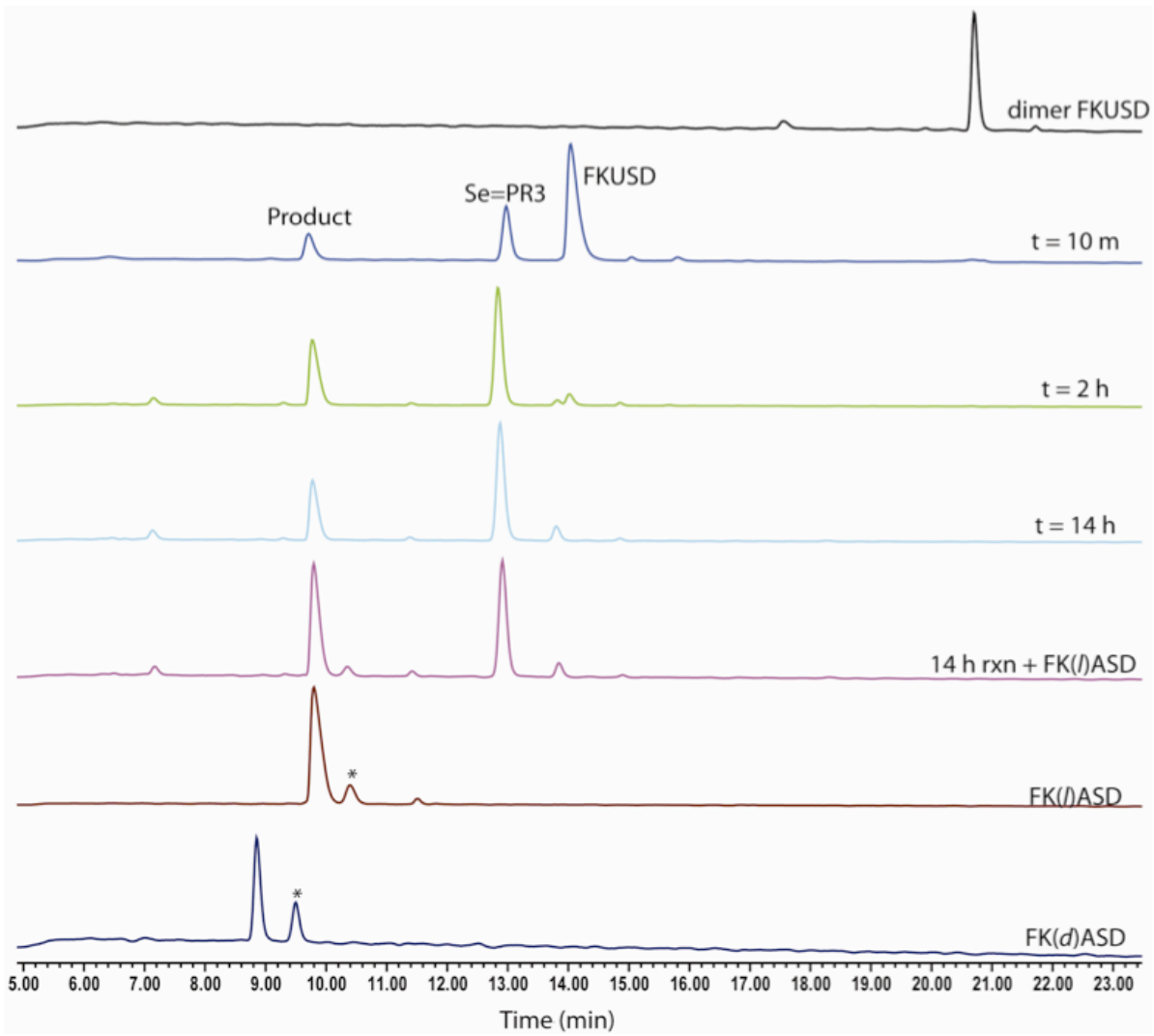


Figure S7. HPLC chromatogram of the deselenization of dimer FKUSD-amide. The reaction is complete within 2 h, and the deselenized product co-elute with authentic FK(*l*)ASD. Antistrike indicates the aspartmide isomer, a synthetic by-product.

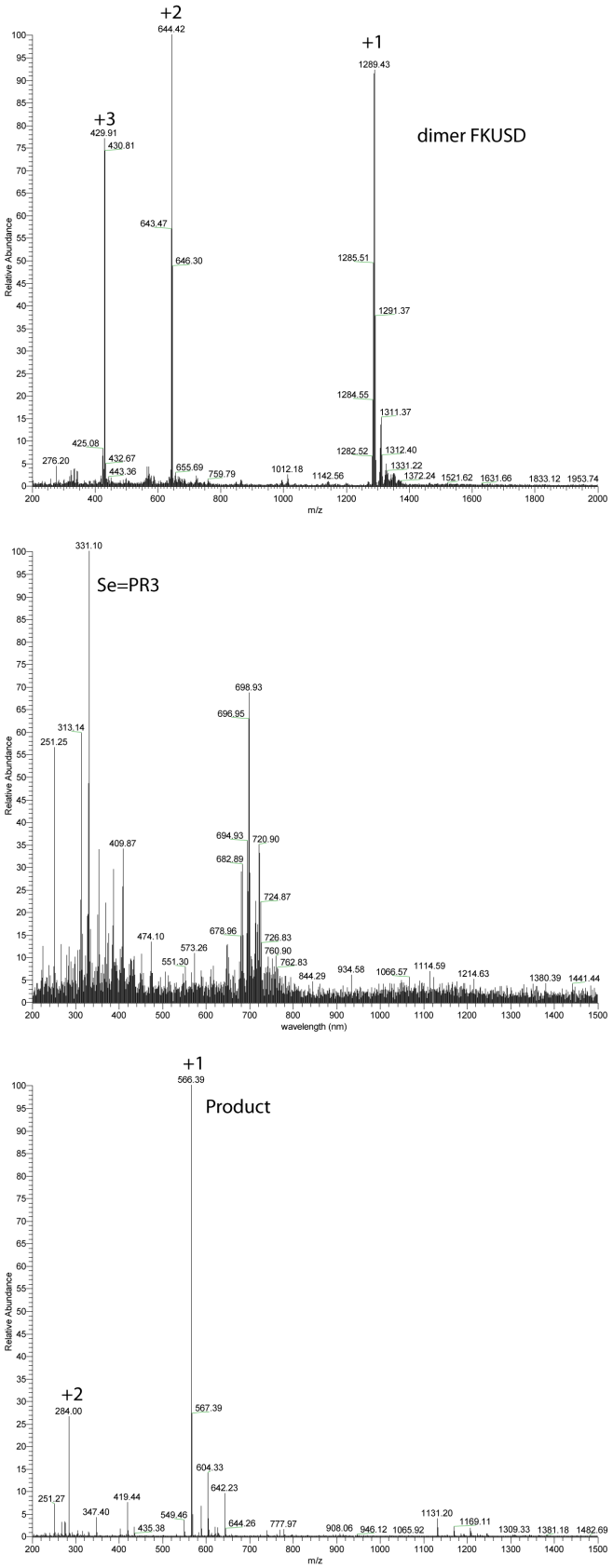
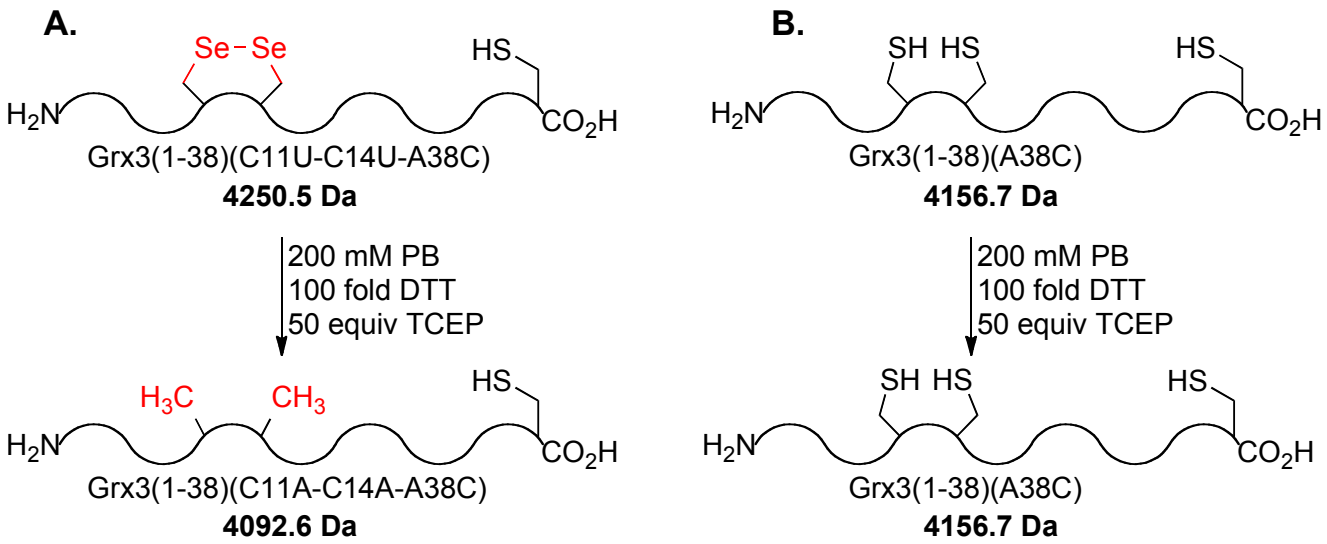
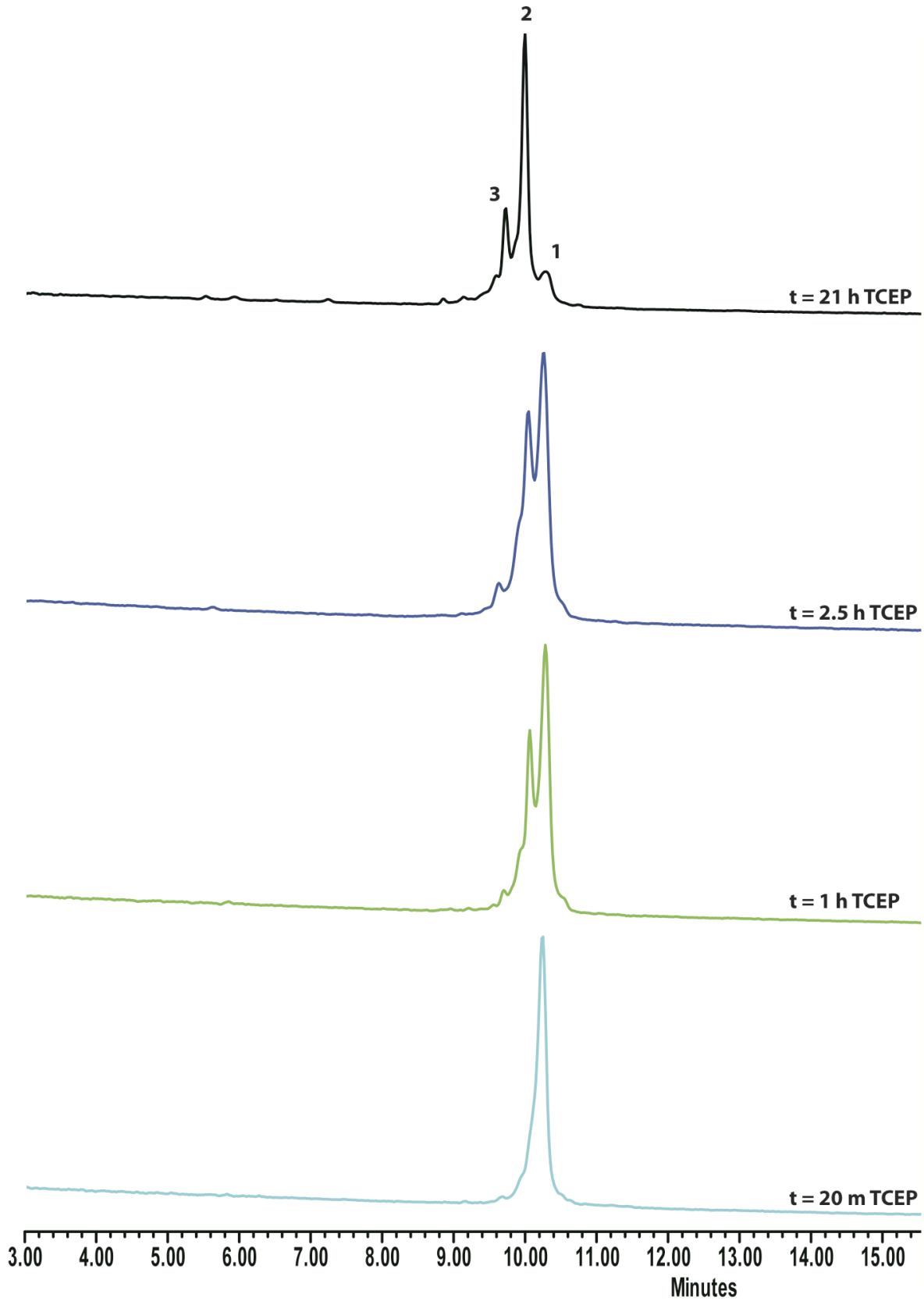


Figure S8. MS data of the dimer FKUSD, Se=PR_3 (R = $C_2H_4CO_2H$, from TCEP) and the deselenized product, FK(*l*)ASD.



Scheme S1. A. The deselenization of Grx3(1-38)(C11U-C14U-A38C) with TCEP gave doubly-deselenized product Grx3(1-38)(C11A-C14A-A38C) as the major product. B. The Cys-analog Grx3(1-38)(A38C) was not affected by reaction with TCEP.



Fisgure S9. HPLC chromatogram of the deselenization reaction of Grx3(1-38)(C11U-C14U-A38C) (peak 1) with TCEP at different time-points gave Grx3(1-38)(C11A-C14A-A38C) (peak 2) peptide as a major product with minor side-product (peak 3) Grx3(1-38).

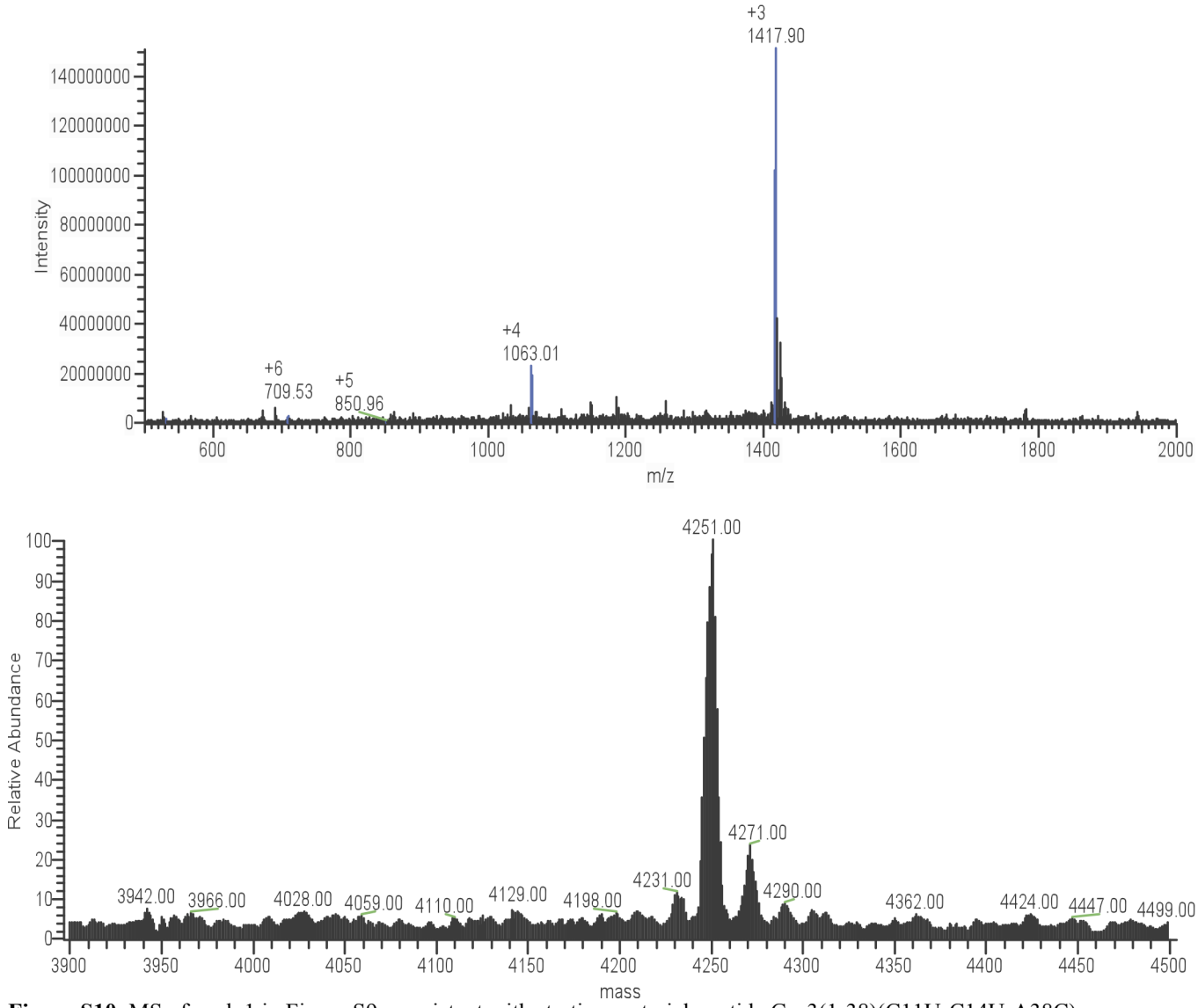


Figure S10. MS of peak 1 in Figure S9, consistent with starting material peptide Grx3(1-38)(C11U-C14U-A38C).

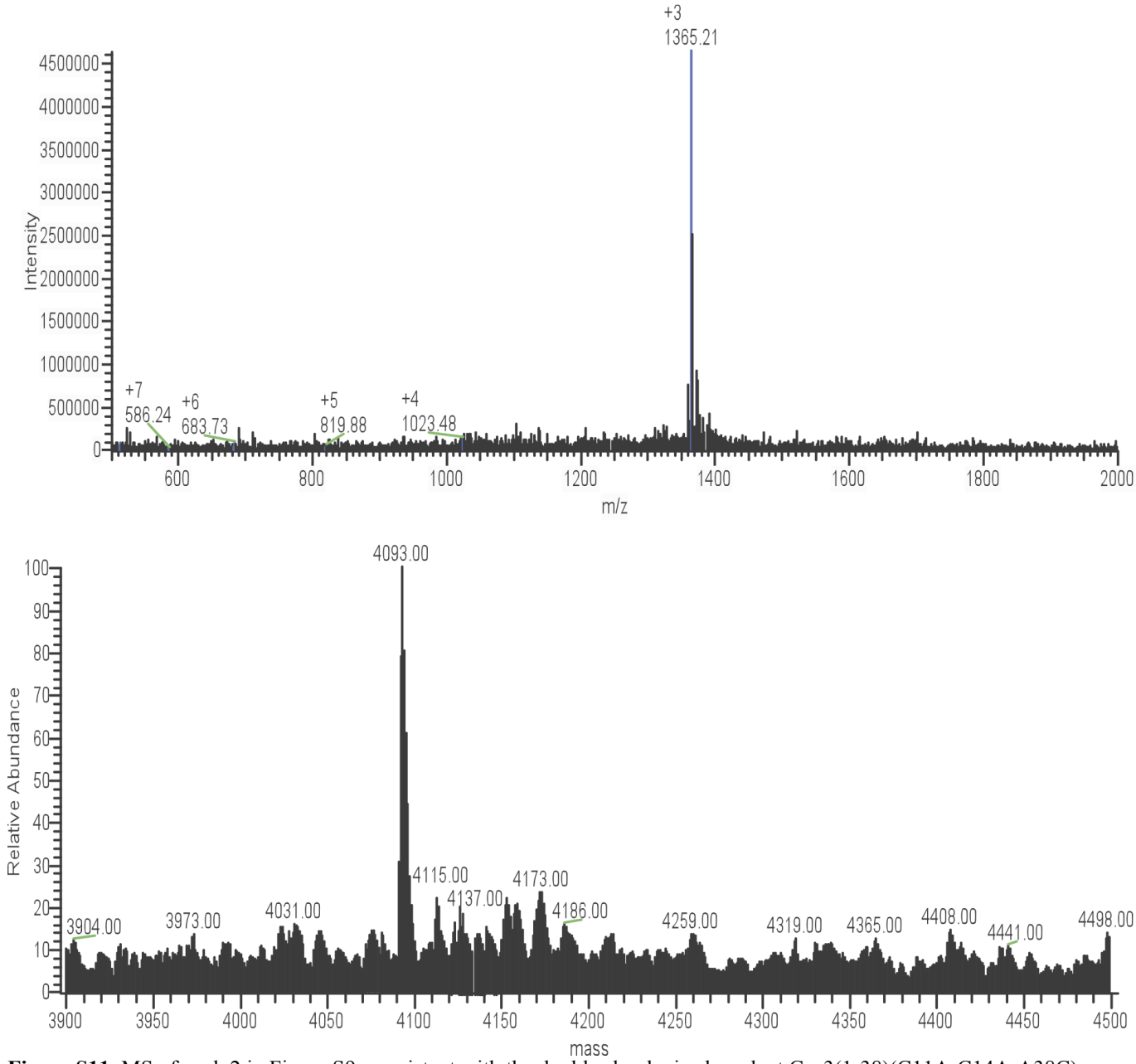


Figure S11. MS of peak 2 in Figure S9, consistent with the doubly-deselenized product Grx3(1-38)(C11A-C14A-A38C).

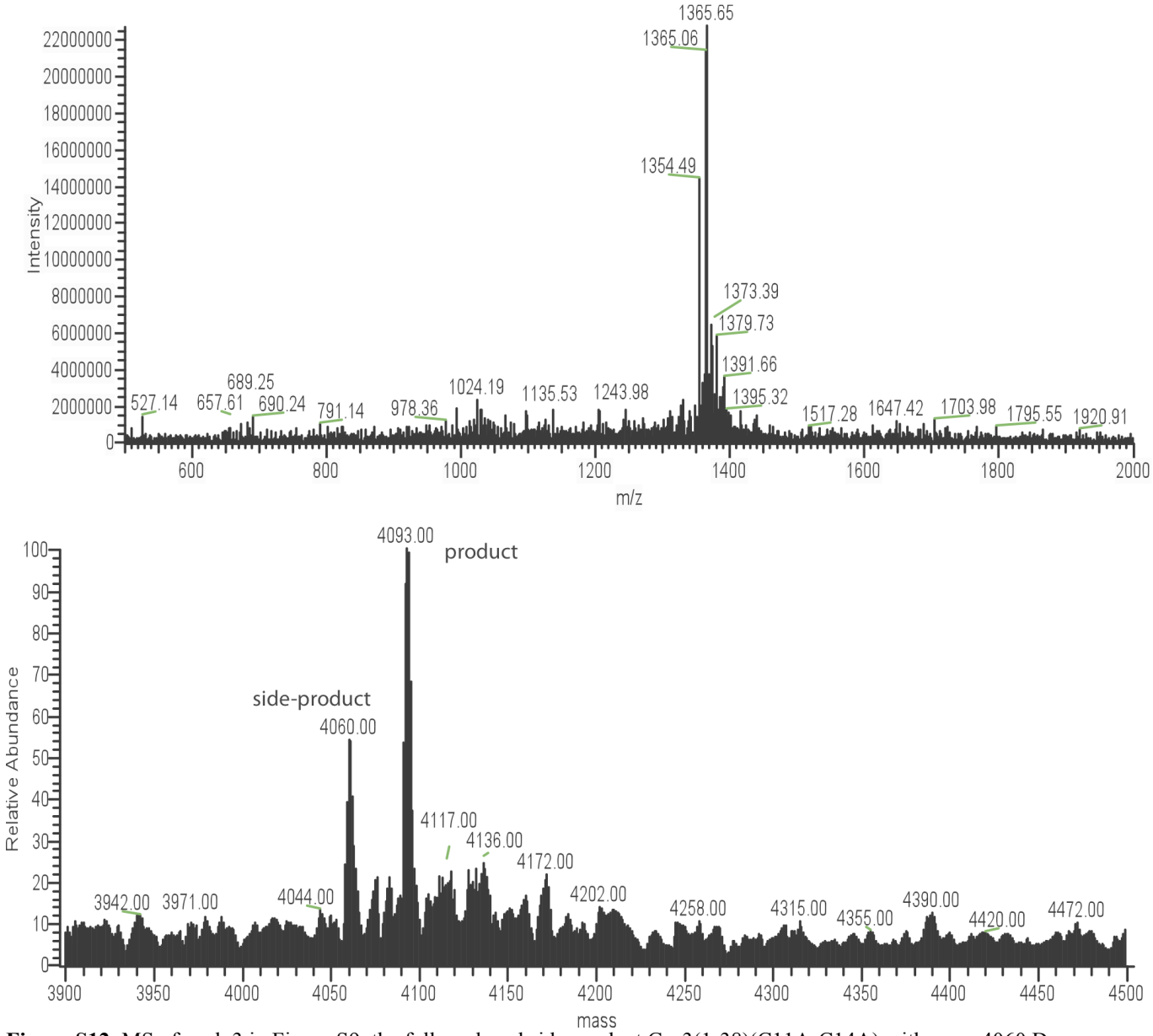


Figure S12. MS of peak 3 in Figure S9, the fully reduced side-product Grx3(1-38)(C11A-C14A) with mass 4060 Da.

1) N. Metanis, E. Keinan, P. E. Dawson, *J. Am. Chem. Soc.* **2006**, *128*, 16684-16691.

2) a) M. Schnolzer, P. F. Alewood, A. Jones, D. Alewood, S. B. H. Kent, *Int. J. Pept. Protein Res.* **1992**, *40*, 180-193; b) M. Schnolzer, P. F. Alewood, A. Jones, D. Alewood, S. B. H. Kent, *Int. J. Pept. Protein Res. Ther.* **2007**, *13*, 31-44.