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Supplementary Information for

Rapid Assembly and Profiling of an Anticoagulant Sulfoprotein Library

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Materials

Peptide grade dimethylformamide (DMF) was obtained from Labscan. Amino acids, coupling reagents and resins for Fmoc-solid-phase peptide synthesis (SPPS) were obtained from either Novabiochem or GL Biochem. SPPS was performed in polypropylene syringes equipped with Teflon filters, purchased from Torviq. Analytical reversed-phase high-performance liquid chromatography (HPLC) was performed on either a Waters Acquity UPLC system equipped with PDA eλ detector (λ = 210 – 400 nm), Sample Manager FAN and Quaternary Solvent Manager (H-class) modules or a Waters System 2695 separations module with a 2996 photodiode array detector. Peptides were analyzed using an XBridge BEH 5 µm, 2.1 x 150 mm wide-pore column (C-18) at a flow rate of 0.2 mL min^{-1} on the HPLC system or Waters Acquity UPLC BEH 1.7 µm 2.1 x 50 mm column (C-18) at a flow rate of 0.6 mL min^{-1} on the UPLC system. Both instruments were run using a mobile phase composed of 0.1% trifluoroacetic acid in H_2O (Solvent A) and 0.1% trifluoroacetic acid in acetonitrile (Solvent B) in a linear gradient as indicated. The analysis of the chromatograms was conducted using Empower 3 Pro software (2010) and retention times (R_t min) of pure peptides and proteins are reported with the gradients specified.

Preparative and semi-preparative reversed-phase HPLC was performed using a Waters 600E Multisolvent Delivery System with a Rheodyne 7725i Injection valve (4 mL loading loop) and Waters 500 pump with a Waters 490E programmable wavelength detector operating at 214, 230, 254 or 280 nm. Preparative reversed-phase HPLC was performed using a Waters Sunfire C18 column (5 µm, 10 × 250 mm) at a flow rate of 7 mL min^{-1}. Semi-preparative reversed-phase HPLC was performed using a Waters XBridge-BEH300 wide-pore C18 column (5 µm, 10 x 250 mm) at a flow rate of 4 mL min^{-1}. Peptides and proteins bearing free sTyr residues were purified using a mobile phase of 0.1% Formic Acid in water (Solvent A) and 0.1% Formic Acid in acetonitrile (Solvent B) using the indicated linear gradient. A mobile phase of 0.1% trifluoroacetic acid in water (Solvent A) and 0.1% trifluoroacetic acid in acetonitrile (Solvent B) was used in all other cases, using the linear gradients specified. After lyophilization, peptides were isolated as TFA or formate salts depending on the chromatographic eluent.

LC-MS was performed either on a Shimadzu LC-MS 2020 instrument consisting of a LC-M20A pump and a SPD-20A UV/Vis detector coupled to a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode unless otherwise stated, or a Shimadzu UPLC-MS equipped with the same modules as the LC-MS system except for a SPD-M30A diode array detector. Separations were performed on the LC-MS system either on a Waters Sunfire 5 µm, 2.1 x 150 mm column (C-18), or wide-pore equivalent operating at a flow rate of 0.2 mL min^{-1}. Separations on the UPLC-MS system were performed using a Waters Acquity UPLC BEH 1.7 µm 2.1 x 50 mm column (C-8) at a flow rate of 0.6 mL min^{-1}. Separations were performed using a mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) and a linear gradient of 0-50% B over 30 min on the LC-MS System and 0-50% B over 8 min on the UPLC-MS system.

Low-resolution mass spectra were recorded on a Shimadzu 2020 mass spectrometer (ESI) operating in positive or negative mode as indicated. Low Resolution MALDI-TOF mass spectra were recorded on a Bruker Autoflex™ Speed MALDI-TOF mass spectrometer operating in linear mode using a matrix of 10 mg/mL sinapinic acid in water/acetonitrile (1:1 v/v) with no TFA.

General Peptide Synthesis Procedures

Fmoc-Strategy SPPS General Procedures (100-200 µmol scale)

2-Chlorotrityl Chloride Resin Loading

2-Chlorotrityl chloride resin (1.22 mmol/g loading) was swollen in dry CH_2Cl_2 for 30 min, followed by washing with CH_2Cl_2 (10x 3 mL). A solution of Fmoc-Xaa-OH (0.7 mmol/g resin) and *i*Pr_2NEt (4 eq. relative to resin functionalization) in CH_2Cl_2 (final concentration 0.125 M of amino acid) was added to the resin, which was shaken at room temperature for 16 h. The resin was then washed with CH_2Cl_2 (5x 3 mL), DMF (5x 3 mL) and CH_2Cl_2 (5x 3 mL). The resin was then capped *via* treatment with 17:2:1 v/v/v CH_2Cl_2:MeOH:*i*Pr_2NEt (5 mL) for 40 mins at room temperature. The resin was then washed again with CH_2Cl_2 (5x 3 mL), DMF (5x 3 mL) and CH_2Cl_2 (5x 3 mL) prior to determination of the estimated loading of the first amino acid.

Rink Amide Resin Loading

Rink Amide resin (0.7 mmol/g loading) was swollen in dry CH_2Cl_2 for 30 min, followed by washing with CH_2Cl_2 (10x 3 mL). The resin was treated with piperidine/DMF (1:4 v/v, 3 mL, 2x 5 min) and then washed with DMF (5x 3 mL), CH_2Cl_2 (5x 3 mL) and DMF (5x 3 mL). A solution of Fmoc-Xaa-OH (0.7 mmol/g resin), PyBOP (4 eq. relative to resin functionalization) and N-methyl-morpholine (8 eq. relative to resin functionalization) in DMF or NMP (final concentration 0.125 M of amino acid) was added to the resin, which was shaken at room temperature for 2 h. The resin was then washed with DMF (5x 3 mL) and CH_2Cl_2 (5x 3 mL) prior to capping *via* treatment with 9:1 v/v pyridine:Ac_2O (5 mL) for 5 mins at room temperature. The resin was then washed again with CH_2Cl_2 (5x 3 mL), DMF (5x 3 mL) and CH_2Cl_2 (5x 3 mL) prior to determination of the estimated loading of the first amino acid.

Loading Estimation of the First Amino Acid

The resin was treated with piperidine/DMF (1:4 v/v, 3 mL, 2x 5 min) and then washed with DMF (5x 3 mL), CH_2Cl_2 (5x 3 mL) and DMF (5x 3 mL). The combined deprotection solutions were then made up to 10 mL with fresh piperidine/DMF (1:4 v/v). The solution was diluted 50-100 fold with fresh piperidine/DMF (1:4 v/v) and the UV absorbance of the piperidine-fulvene adduct measured (λ = 301 nm, ε = 7800 $M^{-1}cm^{-1}$) to estimate the amount of amino acid loaded onto the resin.

Peptide Assembly via Iterative SPPS

Peptides were assembled by stepwise Fmoc-SPPS on a 0.05-0.2 mmol scale. Coupling of each amino acid (4 eq.) was achieved using PyBOP (4 eq.) and NMM (8 eq.) in DMF (0.1 M) over 1 h at room temperature. Capping steps were introduced after all steps unless otherwise specified using 0.3 M acetic anhydride and 0.3 M *i*Pr_2NEt in DMF for 3 min at room temperature. Fmoc-deprotection steps were performed by treatment with 20% v/v piperidine in DMF at room temperature (2x 5 min). Following each coupling, capping or deprotection step, the resin was washed with DMF (5x 3 mL), CH_2Cl_2 (5x 3 mL) and DMF (5x 3 mL).

Coupling of Fmoc-Tyr(SO_3-nP)-OH

A solution of amino acid (2.0 eq.), DIC (2.0 eq.) and HOAt (2.0 eq.) in DMF (0.1 M) was added to the resin and shaken at room temperature for 16 h. The resin was then washed with DMF (5x 3 mL), CH_2Cl_2 (5x 3 mL) and DMF (5x 3 mL) and a capping step was performed as described above. Synthesis of the desired fragment was then completed using iterative Fmoc-SPPS.

Coupling of Boc-Asp(SePMB)-OH or Boc-Sec(PMB)-OH

A solution of amino acid (1.5 eq.), DIC (1.5 eq.) and HOAt (1.5 eq.) in DMF (0.05 M) was added to the resin and shaken at room temperature for 16 h. The resin was then washed with DMF (5x 3 mL), CH_2Cl_2 (5x 3 mL) and DMF (5x 3 mL).

Peptide Assembly via Iterative Fully Automated Microwave-Assisted SPPS

Peptides were assembled by stepwise microwave assisted Fmoc-SPPS on a Protein Technologies Symphony peptide synthesiser, operating on a 0.1-0.2 mmol scale. Activation of entering Fmoc-protected amino acids (0.3 M solution in DMF) was performed using 0.3 M Oxyma in DMF / 0.3 M DIC in DMF (1:1:1 molar ratio), with a 4 equivalent excess over the initial resin loading. Coupling steps were performed for 45 mins at 25 °C. Capping steps were introduced after each coupling step unless otherwise specified and performed by treatment with a 0.3 M acetic anhydride / 0.3 M *i*Pr_2NEt solution in DMF (1x 3 min). Fmoc-deprotection steps were performed by treatment of the resin with a 20% v/v piperidine solution in DMF at room temperature (2x 3 min). Following each coupling, capping or deprotection step, the resin was washed with DMF (4x 30 sec). Upon complete assembly of the peptide chain, the resin was washed with CH_2Cl_2 (5x 30 sec) and gently dried under nitrogen flow.

Cleavage from the Resin

Fully Deprotected Peptide

The resin-bound peptide was treated with an ice-cold TFA:*i*Pr_3SiH:water mixture (90:5:5 v/v/v, 5 mL) and allowed to shake at room temperature for 2 h. At this point, the resin was filtered and washed with fresh cleavage cocktail. The combined cleavage solutions were worked-up as described below.

Side Chain Protected Peptide

The resin was washed with CH_2Cl_2 (5x 3 mL) before treating with a solution of HFIP/CH_2Cl_2 (3:7 v/v, 4 mL) and shaken for 40 mins at room temperature. The resin was filtered and washed with CH_2Cl_2 (3x 3 mL). The combined cleavage solutions and washes were concentrated under nitrogen flow, to afford the crude sidechain protected product.

Solution-Phase Selenoesterification

A solution of fully sidechain protected peptide in dry DMF (20 mM) was treated with diphenyl diselenide (10 eq.) and tributyl phosphine (10 eq.) at -30 °C for 4-5 h under an argon atmosphere. The solution was then concentrated under nitrogen flow and subject to the acidic deprotection conditions described for the fully deprotected peptide.

Work-up and Purification

Ice-cold diethyl ether (30 mL) was added dropwise to the concentrated cleavage solution to precipitate the crude peptide. The precipitate was then collected via centrifugation and washed with further diethyl ether to remove any remaining scavengers. Residual diethyl ether was removed under gentle nitrogen flow and dissolved in 0.1% v/v TFA aqueous buffer (with minimal addition of MeCN to aid dissolution, if necessary). The crude peptide was analysed by LC-MS (ESI) and purified by RP-HPLC.

On resin Boc Protection, Allyl Deprotection and Selenoesterification

After the synthesis of the sequence was completed, Fmoc deprotection was carried out as described above. The resin (10-90 μmol) was then treated with Boc Anhydride (2 eq., 4-40 mg) and pyridine (2 eq., 2-14.5 μL) in DMF (0.01-0.03 M) for 4 h at room temperature. The resin was then washed, as above, and treated with $Pd(PPh_3)_4$ (0.9 eq., 10-100 mg) and phenylsilane (40 eq., 50-450 μL) in DCM (0.01-0.03 M) for 1 h at room temperature. This procedure was then repeated once and the resin washed, as above. Finally, the resin was treated with DPDS (30 eq., 90-850 mg) and Bu_3P (30 eq., 75-675 μL) in DMF (0.01-0.03 M) for 3 h at 0 °C. Once again, the resin was washed before being submitted to global cleavage conditions, as described above.

PMB Deprotection of Ct Fragments and Purification

The crude C-terminal fragments were dissolved (7 mg/mL) in a 12:5:3 v/v/v mixture of TFA:buffer (6 M Gn·HCl, 100 mM HEPES, pH 4-5):DMSO and allowed to stir at room temperature for 1-4 h before dilution in water prior to purification by RP-HPLC.

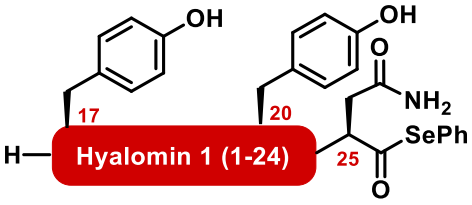
General Protocol for One Pot Ligation-Deselenization

A 5 mM solution of peptide diselenide dimer (1.0 eq.) in ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, adjusted to pH 6.3) was added to the appropriate peptide selenoester (2.0-3.0 eq.). The resulting solution was carefully readjusted to pH 6.2-7.5 with 1 M KOH and the reaction incubated at 25 °C for 20 min. Upon complete conversion to the corresponding ligation product (judged through UPLC-MS monitoring), a solution of TCEP (500 mM) and dithiothreitol (50 mM) in buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, pH 4-5) was added to give a final 250 mM concentration of TCEP, a 25 mM concentration of dithiothreitol, and a 2.5 mM final concentration with respect to the peptide diselenide dimer starting material. The reaction was then incubated at 25 °C for 10 min. After UPLC-MS analysis showed completed conversion to the deselenized product the reaction mixture was incubated at 37 °C for 6 h to remove the neopentyl groups. The ligation mixture was then diluted and purified by RP-HPLC.

Synthesis and Characterization of Tick-Derived Fragments

Hyalomin 1 Fragment Synthesis

Hya1 Un (1-25) Selenoester



H-KPNLQSRSDDGVDESDYDTYPDDNN-SePh

Hya1 Un (1-25) selenoester was prepared by sidechain loading of Fmoc-Asp-OAll to rink amide resin (24 µmol) and extended via Fmoc-strategy SPPS, through a combination of automated and manual synthesis, followed by Fmoc-deprotection, on-resin Boc Protection and on-resin allyl ester deprotection and solid-phase selenoesterification with DPDS (850 mg, 30 eq.) as outlined in the general procedures. Cleavage from resin and removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/ iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) afforded **Hya1 Un (1-25)** selenoester as a fluffy white solid after lyophilization (16.7 mg, 23% yield).

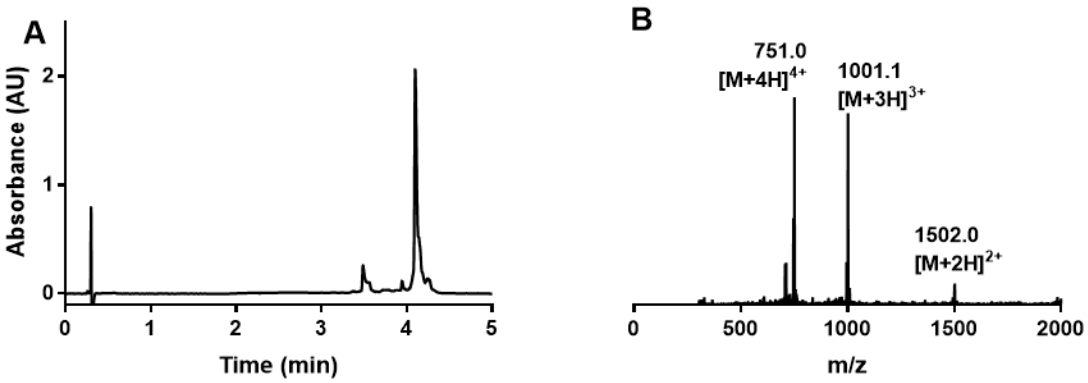
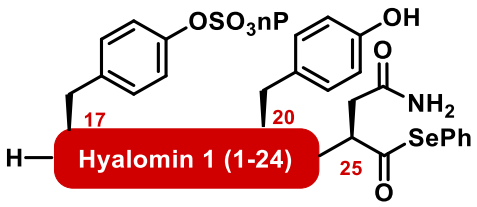


Figure S1: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **Hya1 Un (1-25)** selenoester. Analytical HPLC Rt 4.10 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1500.4, $[M+3H]^{3+}$: 1000.6, Mass Found (ESI) 1502.0 $[M+2H]^{2+}$, 1001.1 $[M+3H]^{3+}$.

Hya1 Y17 (1-25) Selenoester



H-KPNLQSRSDDGVDESDsYDTYPDDNN-SePh

Hya1 Y17 (1-25) selenoester was prepared by sidechain loading of Fmoc-Asp-OAll to rink amide resin (24 µmol) and extended via Fmoc-strategy SPPS, through a combination of automated and manual synthesis, followed by Fmoc-deprotection, on-resin Boc Protection and on-resin allyl ester deprotection and solid-phase selenoesterification with DPDS (850 mg, 30 eq.) as outlined in the general procedures. Cleavage from resin and removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) afforded **Hya1 Y17 (1-25)** selenoester as a fluffy white solid after lyophilization (19.0 mg, 25% yield).

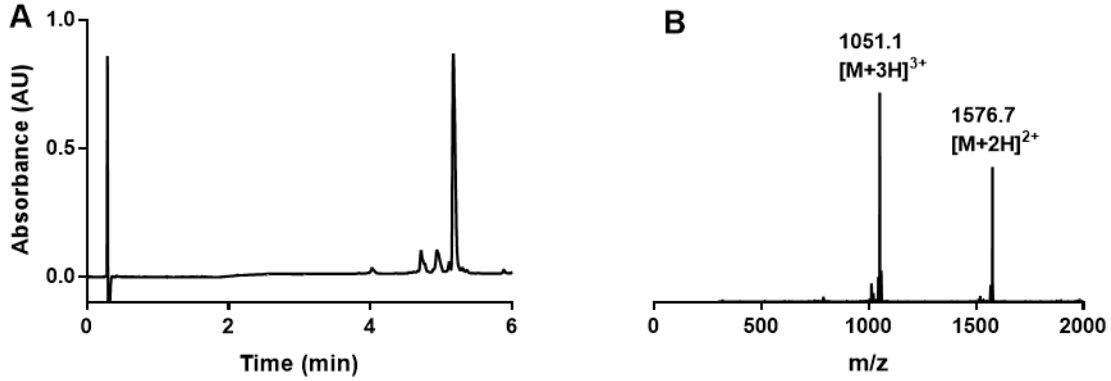
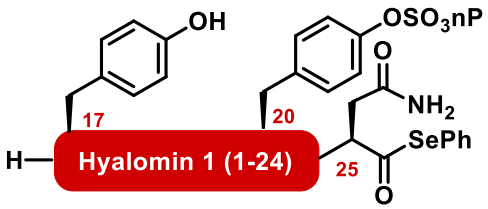


Figure S2: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **Hya1 Y17 (1-25)** selenoester. Analytical HPLC Rt 5.16 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1575.9, $[M+3H]^{3+}$: 1051.0, Mass Found (ESI) 1576.7 $[M+2H]^{2+}$, 1051.1 $[M+3H]^{3+}$.

Hya1 Y20 (1-25) Selenoester



H-KPNLQSRSDDGVDESDYDTsYPDDNN-SePh

Hya1 Y20 (1-25) selenoester was prepared by sidechain loading of Fmoc-Asp-OAll to rink amide resin (24 µmol) and extended via Fmoc-strategy SPPS, through a combination of automated and manual synthesis, followed by Fmoc-deprotection, on-resin Boc Protection and on-resin allyl ester deprotection and solid-phase selenoesterification with DPDS (480 mg, 30 eq.) as outlined in the general procedures. Cleavage from resin and removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) afforded **Hya1 Y20 (1-25)** selenoester as a fluffy white solid after lyophilization (5.8 mg, 8% yield).

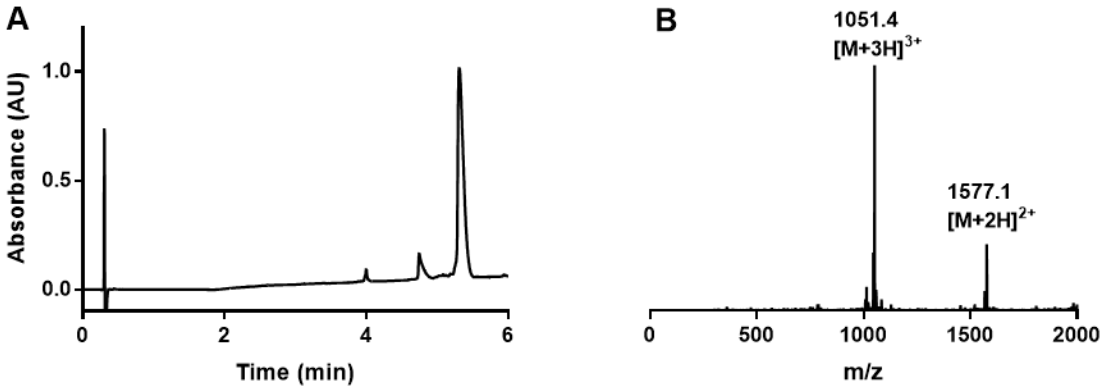
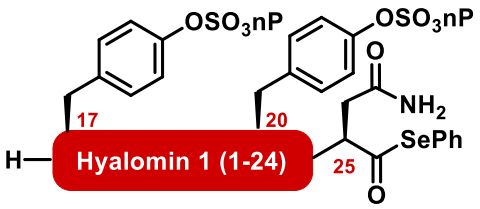


Figure S3: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **Hya1 Y20 (1-25)** selenoester. Analytical HPLC Rt 5.28 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1575.9, $[M+3H]^{3+}$: 1051.0, Mass Found (ESI) 1577.1 $[M+2H]^{2+}$, 1051.4 $[M+3H]^{3+}$.

Hya1 DS (1-25) Selenoester



H-KPNLQSRSDDGVDESDsYDTsYPDDNN-SePh

Hya1 DS (1-25) selenoester was prepared by sidechain loading of Fmoc-Asp-OAll to rink amide resin (24 µmol) and extended via Fmoc-strategy SPPS, through a combination of automated and manual synthesis, followed by Fmoc-deprotection, on-resin Boc Protection and on-resin allyl ester deprotection and solid-phase selenoesterification with DPDS (480 mg, 30 eq.) as outlined in the general procedures. Cleavage from resin and removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) afforded **Hya1 DS (1-25)** selenoester as a fluffy white solid after lyophilization (6.8 mg, 9% yield).

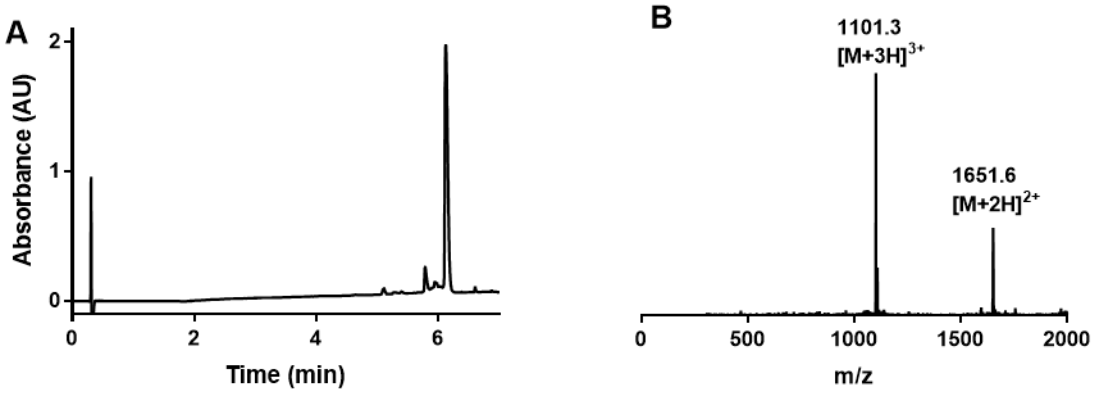


Figure S4: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **Hya1 DS (1-25)** selenoester. Analytical HPLC Rt 6.09 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1651.4, $[M+3H]^{3+}$: 1101.3, Mass Found (ESI) 1651.6 $[M+2H]^{2+}$, 1101.3 $[M+3H]^{3+}$.

Hya1 (26-59) Diselenide

H-DDSGERNGGSEPAKPRLPVPGSGRDSERIPVPVD-OH

Hya1 (26-59) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (45 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) afforded **Hya1 (26-59)** diselenide as a fluffy white solid after lyophilization (15.7 mg, 10% yield).

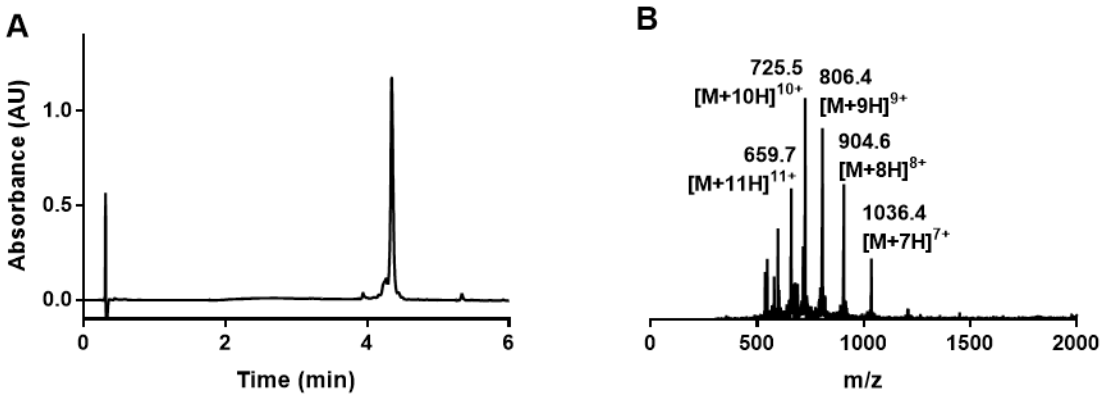
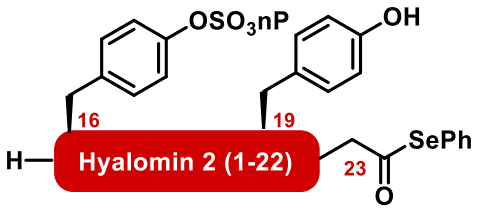


Figure S5: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **Hya1 (1-25)** diselenide. Analytical HPLC Rt 4.34 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass (as the diselenide) $[M+6H]^{6+}$: 1208.6 $[M+7H]^{7+}$: 1036.1 $[M+8H]^{8+}$: 906.7 $[M+9H]^{9+}$: 806.1 $[M+10H]^{10+}$: 725.6 $[M+11H]^{11+}$: 659.7; Mass Found (ESI) 1209.0 $[M+6H]^{6+}$, 1036.4 $[M+7H]^{7+}$, 904.6 $[M+8H]^{8+}$, 806.4 $[M+9H]^{9+}$, 725.5 $[M+10H]^{10+}$, 659.7 $[M+11H]^{11+}$.

Hyalomin 2 Fragment Synthesis

Hya2 Y16 (1-23) Selenoester



H-KPNLQSRTGSDDDDEsYDMYESDG-SePh

Hya2 Y16 (1-23) selenoester was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (25 µmol), followed by solution-phase selenoesterification with DPDS (80 mg, 10 eq.) as outlined in the general procedures. Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 80% B over 30 min, 0.1% v/v TFA) afforded **Hya2 Y16 (1-23)** selenoester as a fluffy white solid after lyophilization (1.6 mg, 2% yield).

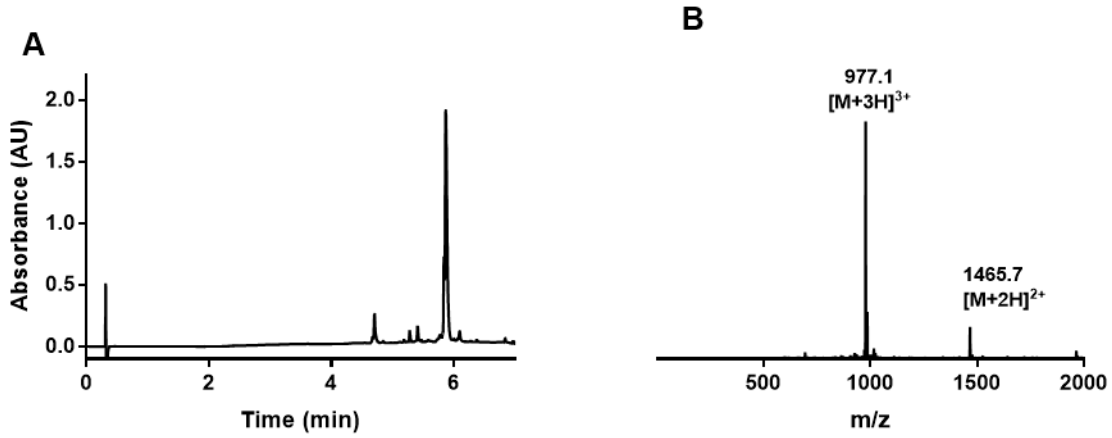
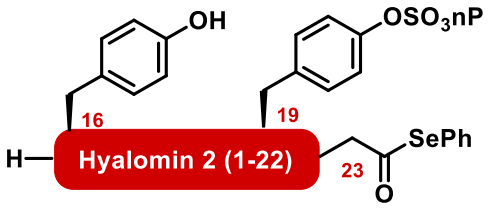


Figure S6: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **Hya2 Y16 (1-23)** selenoester. Analytical HPLC Rt 5.37 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1464.5, $[M+3H]^{3+}$: 976.7, Mass Found (ESI) 1465.7 $[M+2H]^{2+}$, 977.1 $[M+3H]^{3+}$.

Hya2 Y19 (1-23) Selenoester



H-KPNLQSRTGSDDDDEYDMsYESDG-SePh

Hya2 Y19 (1-23) selenoester was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (25 µmol), followed by solution-phase selenoesterification with DPDS (80 mg, 10 eq.) as outlined in the general procedures. Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 80% B over 30 min, 0.1% v/v TFA) afforded **Hya2 Y19 (1-23)** selenoester as a fluffy white solid after lyophilization (3.1 mg, 4% yield).

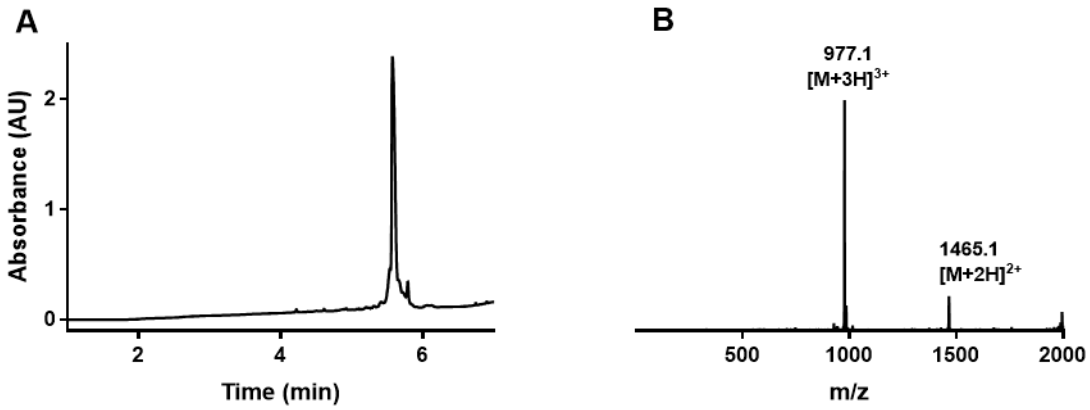
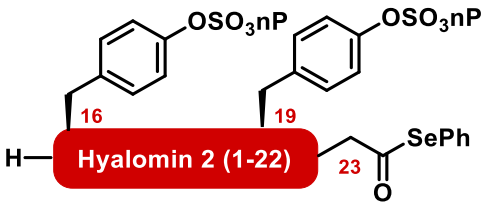


Figure S7: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **Hya2 Y19 (1-23)** selenoester. Analytical HPLC Rt 5.56 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1464.5, $[M+3H]^{3+}$: 976.7, Mass Found (ESI) 1465.1 $[M+2H]^{2+}$, 977.1 $[M+3H]^{3+}$.

Hya2 DS (1-23) Selenoester



H-KPNLQSRTGSDDDDEsYDMsYESDG-SePh

Hya2 DS (1-23) selenoester was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (25 µmol), followed by solution-phase selenoesterification with DPDS (80 mg, 10 eq.) as outlined in the general procedures. Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (20 to 100% B over 40 min, 0.1% v/v TFA) afforded **Hya2 DS (1-23)** selenoester as a fluffy white solid after lyophilization (4.6 mg, 6% yield).

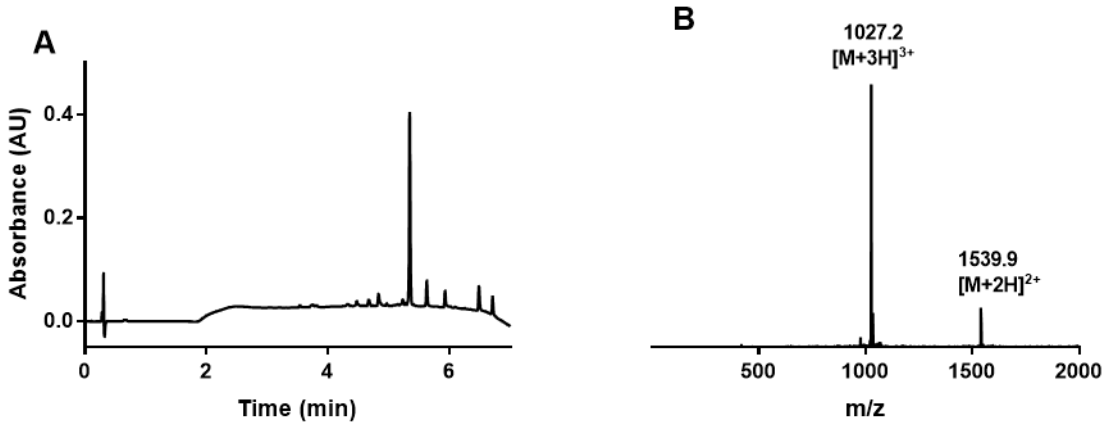
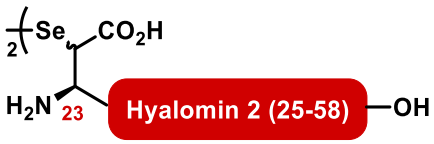


Figure S8: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **Hya2 DS (1-23)** selenoester. Analytical HPLC Rt 5.35 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1540.0, $[M+3H]^{3+}$: 1027.0, Mass Found (ESI) 1539.9 $[M+2H]^{2+}$, 1027.2 $[M+3H]^{3+}$.

Hya2 (24-58) Diselenide



H-DSNEGNDNDEFETAVPRLPNPNSGRDSEHIPMPVN-OH

Hya2 (24-58) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (25 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide with a solution of TFA/iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 30 min, 0.1% v/v TFA) afforded **Hya2 (24-58)** diselenide as a fluffy white solid after lyophilization (11.2 mg, 16% yield).

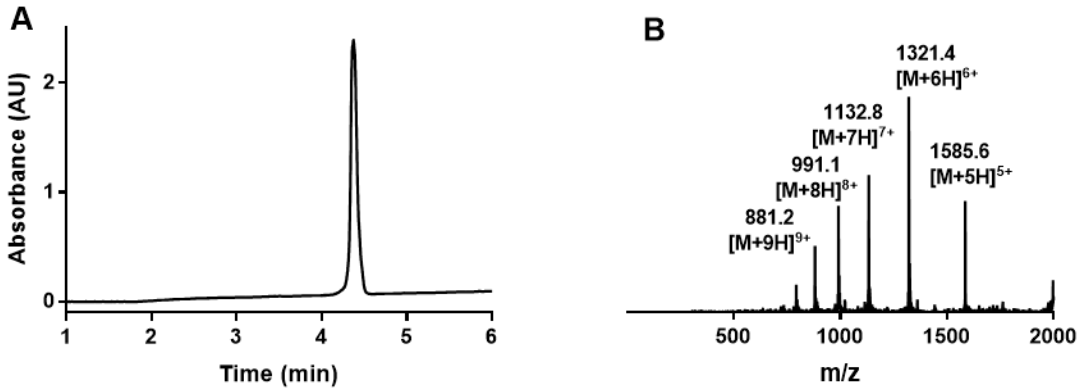
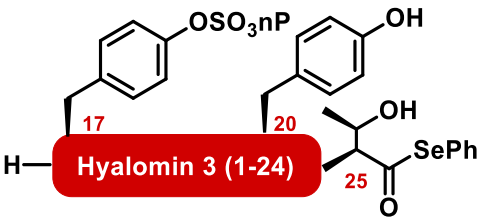


Figure S9: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **Hya2 (24-58)** diselenide. Analytical HPLC Rt 4.35 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass (as the diselenide) $[M+5H]^{5+}$: 1585.0, $[M+6H]^{6+}$: 1321.0, $[M+7H]^{7+}$: 1132.4, $[M+8H]^{8+}$: 991.0, $[M+9H]^{9+}$: 881.0, Mass Found (ESI) 1585.6 $[M+5H]^{5+}$, 1321.4 $[M+6H]^{6+}$, 1132.8 $[M+7H]^{7+}$, 991.1 $[M+8H]^{8+}$, 881.2 $[M+9H]^{9+}$.

Hyalomin 3 Fragment Synthesis

Hya3 Y17 (1-25) Selenoester



H-KPNLQSRNGDGVAETS sY EEYPDDST-SePh

Hya3 Y17 (1-25) selenoester was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (25 µmol), followed by solution-phase selenoesterification with DPDS (80 mg, 10 eq.) as outlined in the general procedures. Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/*i*Pr_3SiH/H_2O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 30 min, 0.1% v/v TFA) afforded **Hya3 Y17 (1-25)** selenoester as a fluffy white solid after lyophilization (2.6 mg, 4% yield).

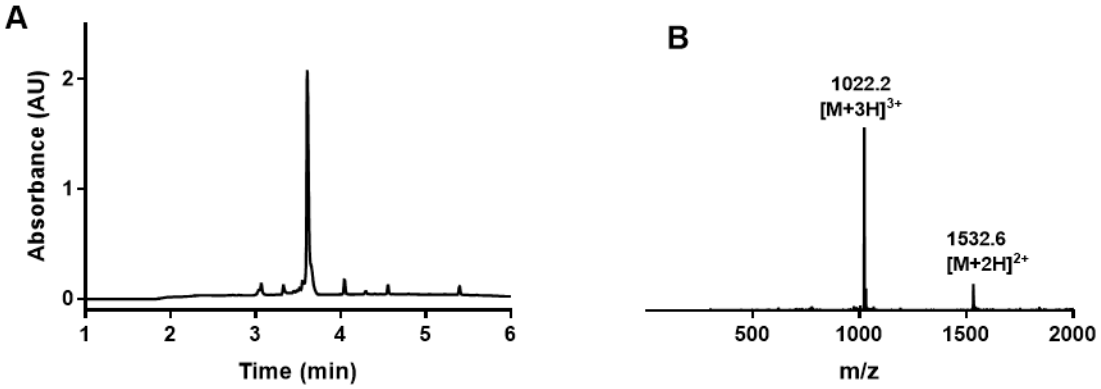
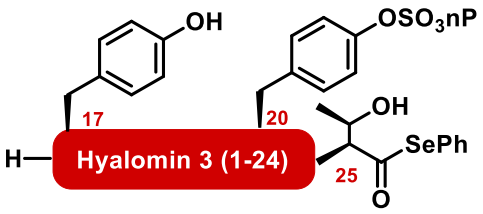


Figure S10: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **Hya3 Y17 (1-25)** selenoester. Analytical HPLC Rt 3.58 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1531.4 $[M+3H]^{3+}$: 1021.3, Mass Found (ESI) 1532.6 $[M+2H]^{2+}$, 1022.2 $[M+3H]^{3+}$.

Hya3 Y20 (1-25) Selenoester



H-KPNLQSRNGDGVAETSYEE sYPDDST-SePh

Hya3 Y20 (1-25) selenoester was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (25 µmol), followed by solution-phase selenoesterification with DPDS (80 mg, 10 eq.) as outlined in the general procedures. Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 30 min, 0.1% v/v TFA) afforded **Hya3 Y20 (1-25)** selenoester as a fluffy white solid after lyophilization (1.5 mg, 2% yield).

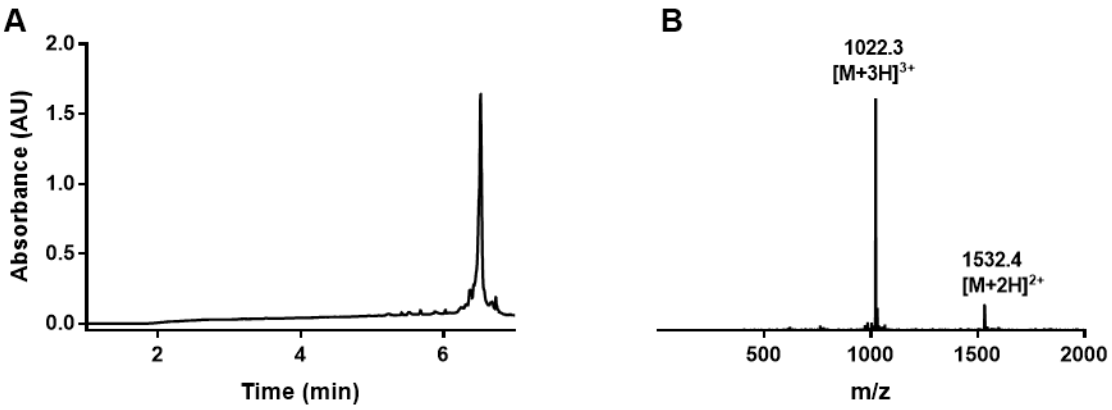
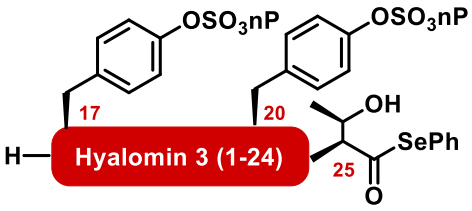


Figure S11: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **Hya3 Y20 (1-25)** selenoester. Analytical HPLC Rt 6.50 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1531.4 $[M+3H]^{3+}$: 1021.3, Mass Found (ESI) 1532.4 $[M+2H]^{2+}$, 1022.3 $[M+3H]^{3+}$.

Hya3 DS (1-25) Selenoester



H-KPNLQSRNGDGVAETS sY EE sY PDDST-SePh

Hya3 DS (1-25) selenoester was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (25 µmol), followed by solution-phase selenoesterification with DPDS (80 mg, 10 eq.) as outlined in the general procedures. Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (20 to 100% B over 40 min, 0.1% v/v TFA) afforded **Hya3 DS (1-25)** selenoester as a fluffy white solid after lyophilization (3.5 mg, 4% yield).

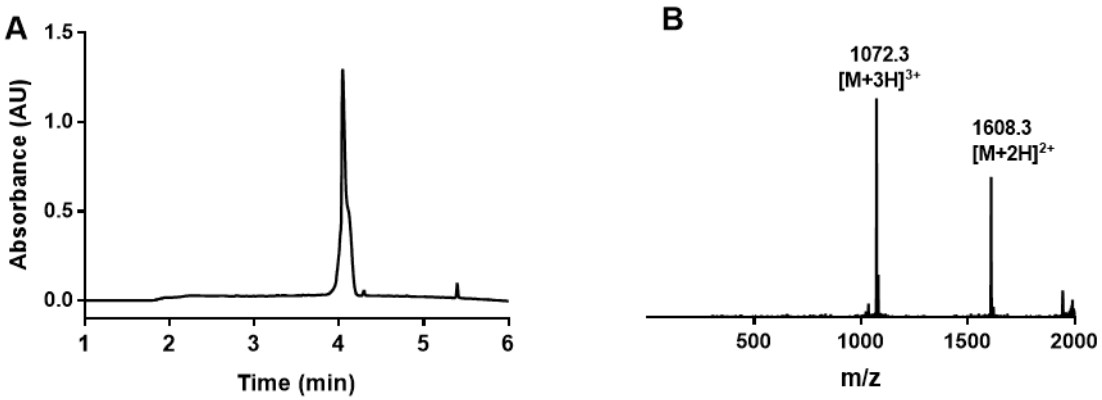
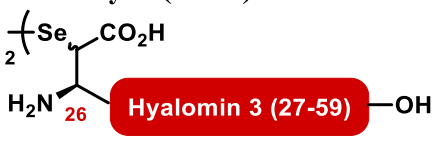


Figure S12: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **Hya3 DS (1-25)** selenoester. Analytical HPLC Rt 4.03 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1606.9 $[M+3H]^{3+}$: 1071.6, Mass Found (ESI) 1608.3 $[M+2H]^{2+}$, 1072.3 $[M+3H]^{3+}$.

Hya3 (26-59) Diselenide



H-DNSGGSSEGSDKAVPRLPSSGSGHDSDPIPVPVN-OH

Hya3 (26-59) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (25 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 30 min, 0.1% v/v TFA) afforded **Hya3 (26-59)** diselenide as a fluffy white solid after lyophilization (12.9 mg, 15% yield).

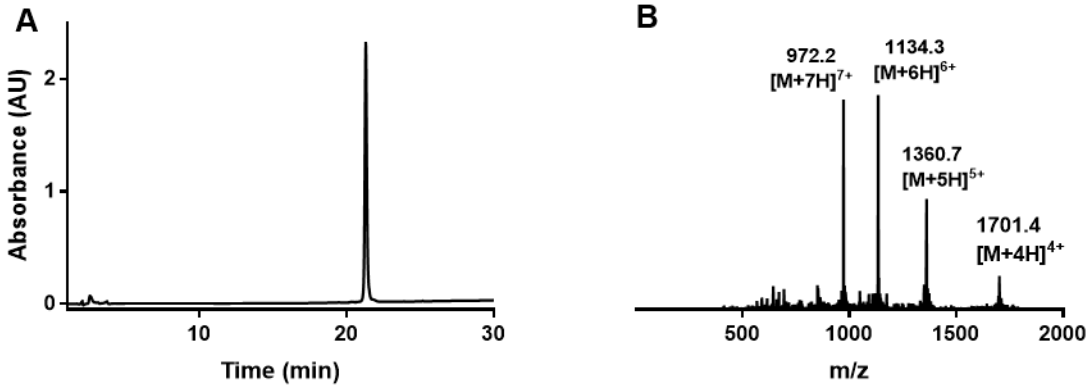
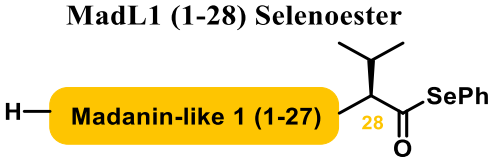


Figure S13: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **Hya3 (26-59)** diselenide. Analytical HPLC Rt 21.20 min (0 to 50% over 30 min, 0.1% v/v TFA, λ = 230 nm); Calculated Mass (as the diselenide) $[M+4H]^{4+}$: 1700.3, $[M+5H]^{5+}$: 1360.4, $[M+6H]^{6+}$: 1133.8, $[M+7H]^{7+}$: 972.0, Mass Found (ESI) 1701.4 $[M+4H]^{4+}$, 1360.7 $[M+5H]^{5+}$, 1134.3 $[M+6H]^{6+}$, 972.2 $[M+7H]^{7+}$.

Madanin-like 1 Fragment Synthesis



H-YPERDSAKDGNEEQERALPVNVQERGEV-SePh

MadL1 (1-28) selenoester was prepared by loading of Fmoc-Val-OH to 2-chlorotritylchloride resin (62.5 µmol) and extended via Fmoc-strategy SPPS, through a combination of automated and manual synthesis, with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment, HFIP-mediated cleavage and solution-phase selenoesterification with DPDS (590 mg, 30 eq.) were completed as outlined in the general procedures. Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/*i*Pr_3SiH/H_2O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0% B for 5 min, 0% to 15% B over 5 min followed 15% to 35% B over 40 min, 0.1% v/v TFA) afforded **MadL1 (1-28)** selenoester as a fluffy white solid after lyophilization (56 mg, 27% yield).

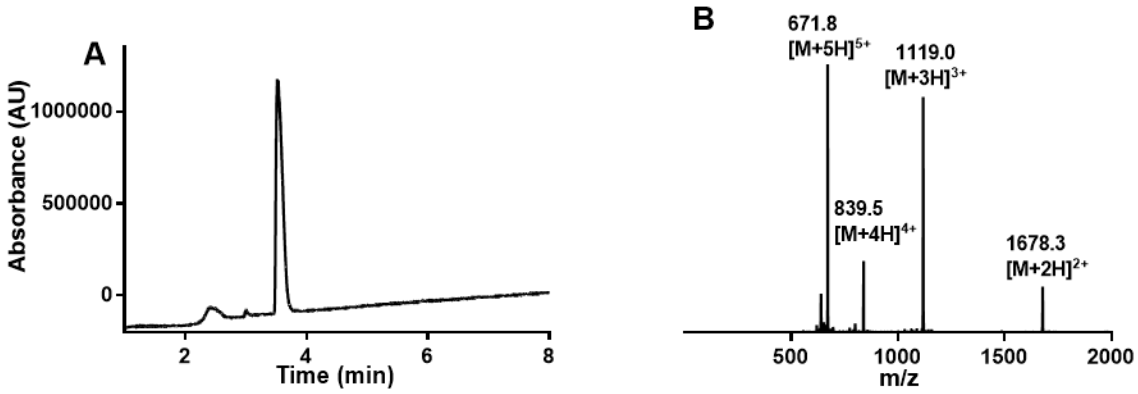
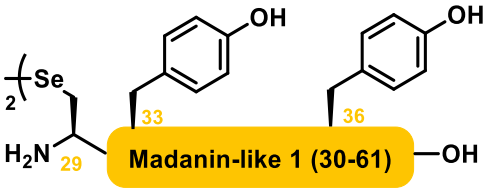


Figure S14: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **MadL1 (1-28)** selenoester. Analytical HPLC Rt 3.5 min (0 to 60% over 5 min, 0.1% v/v TFA, λ = 254 nm); Calculated Mass $[M+2H]^{2+}$: 1678.2, $[M+3H]^{3+}$: 1119.1, $[M+4H]^{4+}$: 839.6, $[M+5H]^{5+}$: 671.9; Mass Found (ESI) 1678.3 $[M+2H]^{2+}$, 1119.0 $[M+3H]^{3+}$, 839.5 $[M+4H]^{4+}$, 671.8 $[M+5H]^{5+}$.

MadL1 Un (29-61) Diselenide



H-UDADYDDYDEEGTTPTVDPTAQTARPRLQGNQS-OH

MadL1 Un (29-61) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (50 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 15% B over 5 min, 15% B for 5 min, followed by 15% to 25% B over 40 min, 0.1% v/v TFA) afforded **MadL1 Un (29-61)** diselenide as a fluffy white solid after lyophilization (5.3 mg, 3% yield).

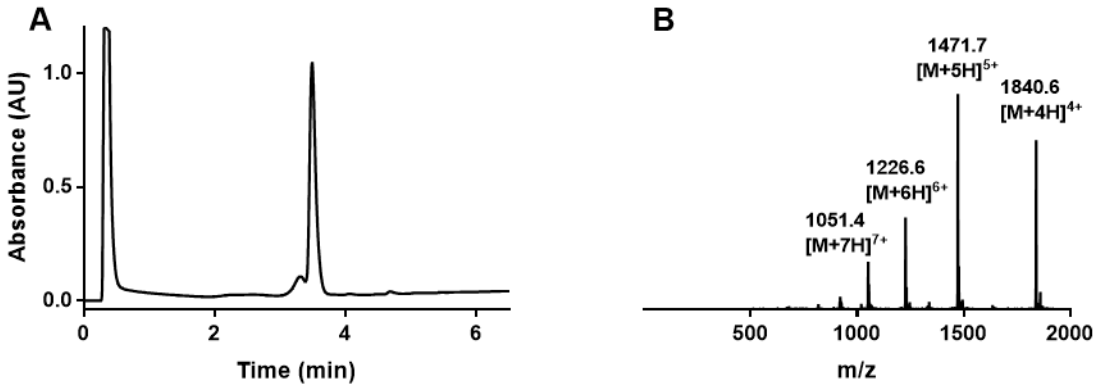
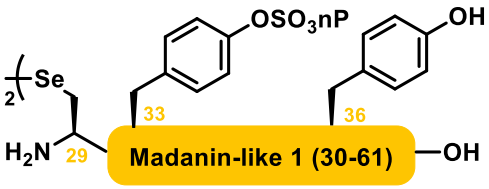


Figure S15: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **MadL1 Un (29-61)** diselenide. Analytical HPLC Rt 3.49 min (0 to 60% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass (as the diselenide) $[M+4H]^{4+}$: 1839.3 $[M+5H]^{5+}$: 1471.6 $[M+6H]^{6+}$: 1226.5 $[M+7H]^{7+}$: 1051.5 $[M+8H]^{8+}$: 920.2 Mass Found (ESI) 1840.6 $[M+4H]^{4+}$, 1471.7 $[M+5H]^{5+}$, 1226.6 $[M+6H]^{6+}$, 1051.4 $[M+7H]^{7+}$, 920.2 $[M+8H]^{8+}$.

MadL1 Y33 (29-61) Diselenide



H-UDADsYDDYDEEGTTPTVDPTAQTARPRLQGNQS-OH

MadL1 Y33 (29-61) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (50 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0% B for 5 min, 0% to 20% B over 5 min followed by 20% to 40% B over 40 min, 0.1% v/v TFA) afforded **MadL1 Y33 (29-61)** diselenide as a fluffy white solid after lyophilization (3.2 mg, 2% yield).

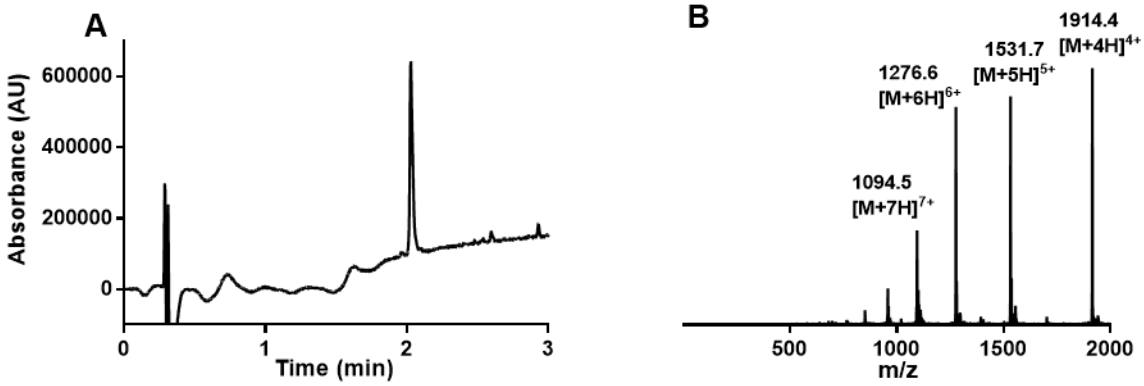
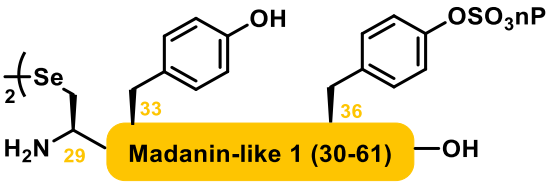


Figure S16: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **MadL1 Y33 (29-61)** diselenide. Analytical HPLC Rt 2.06 min (0 to 50% over 3 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass (as the diselenide) $[M+4H]^{4+}$: 1914.4 $[M+5H]^{5+}$: 1531.7 $[M+6H]^{6+}$: 1276.6 Mass Found (ESI) 1914.7 $[M+4H]^{4+}$, 1531.8 $[M+5H]^{5+}$, 1276.8 $[M+6H]^{6+}$.

MadL1 Y36 (29-61) Diselenide



H-UDADYDDsYDEEGTTPTVDPTAQTARPRLQGNQS-OH

MadL1 Y36 (29-61) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (50 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0% B for 5 min, 0% to 20% B over 5 min followed by 20% to 40% B over 40 min, 0.1% v/v TFA) afforded **MadL1 Y36 (29-61)** diselenide as a fluffy white solid after lyophilization (9.5 mg, 5% yield).

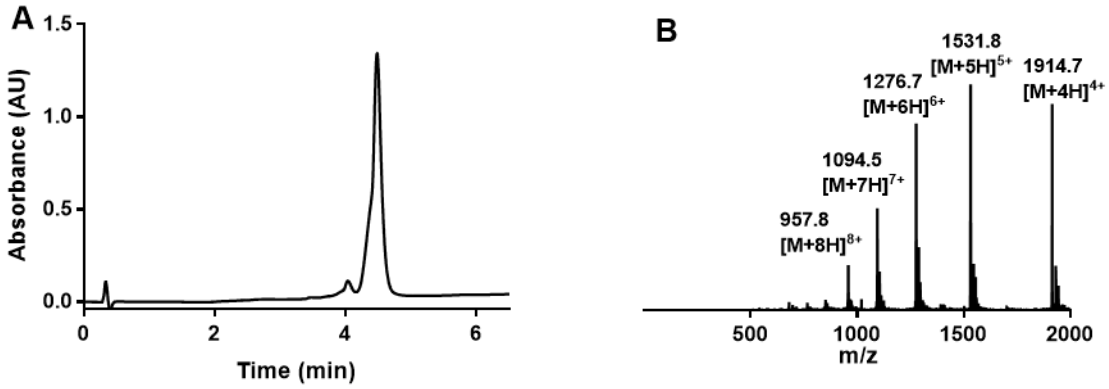
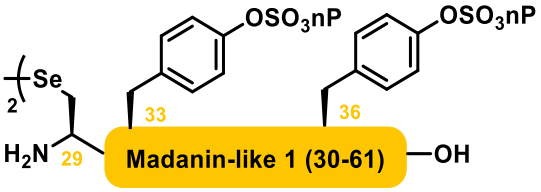


Figure S17: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **MadL1 Y36 (29-61)** diselenide. Analytical HPLC Rt 4.48min (0 to 60% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass (as the diselenide) $[M+4H]^{4+}$: 1914.4 $[M+5H]^{5+}$: 1531.7 $[M+6H]^{6+}$: 1276.6 Mass Found (ESI) 1914.7 $[M+4H]^{4+}$, 1531.8 $[M+5H]^{5+}$, 1276.7 $[M+6H]^{6+}$.

MadL1 DS (29-61) Diselenide



H-UDADsYDDsYDEEGTTPTVDPTAQTARPRLQGNQS-OH

MadL1 DS (29-61) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (50 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 35% B over 5 min, 35% B for 5 min followed by 35% to 55% B over 40 min, 0.1% v/v TFA) afforded **MadL1 DS (29-61)** diselenide as a fluffy white solid after lyophilization (6.2 mg, 3% yield).

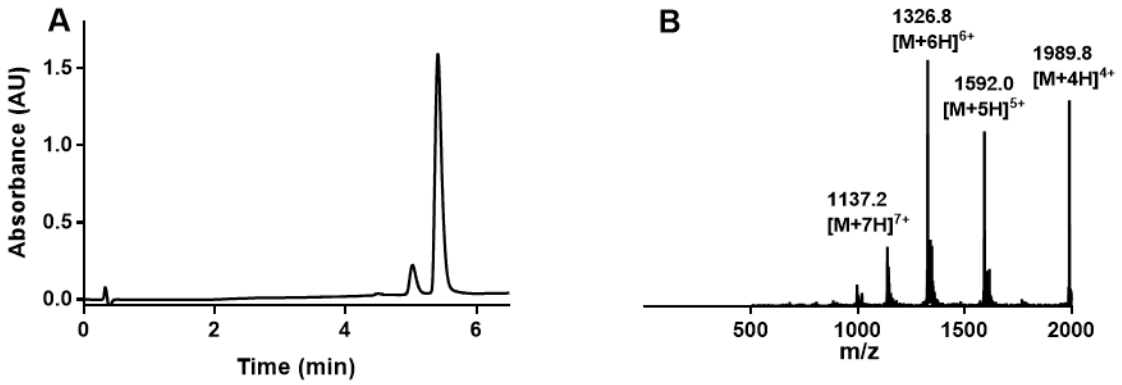
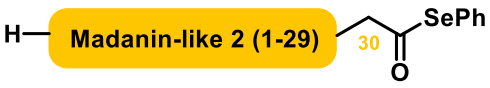


Figure S18: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **MadL1 DS (29-61)** diselenide. Analytical HPLC Rt 5.41 min (0 to 60% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass (as the diselenide) $[M+4H]^{4+}$: 1989.5 $[M+5H]^{5+}$: 1591.8 $[M+6H]^{6+}$: 1326.7 Mass Found (ESI) 1989.8 $[M+4H]^{4+}$, 1592.0 $[M+5H]^{5+}$, 1326.8 $[M+6H]^{6+}$.

Madanin-like 2 Fragment Synthesis

MadL2 (1-30) Selenoester



H-YPESDSAKDDGNQEKEKALLVKVQERSDDG -SePh

MadL2 (1-30) selenoester was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis on 2-chlorotrityl chloride resin (100 µmol), with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment, HFIP-mediated cleavage and solution-phase selenoesterification with DPDS (927 mg, 30 eq.) were completed as outlined in the general procedures. Removal of all acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/*i*Pr_3SiH/H_2O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 30% B over 40 min, 0.1% v/v TFA) afforded **MadL2 (1-30)** selenoester as a fluffy white solid after lyophilization (27.88 mg, 7.98% yield).

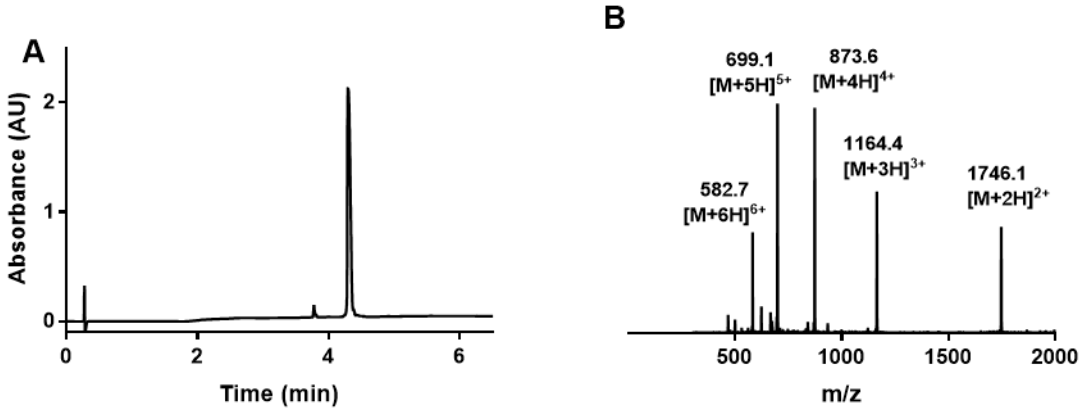
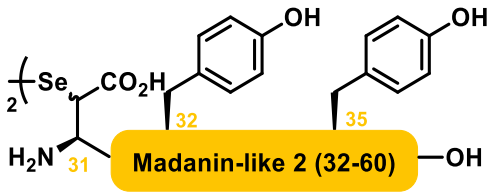


Figure S19: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **MadL2 (1-30)** selenoester. Analytical HPLC Rt 4.29 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1746.3, $[M+3H]^{3+}$: 1164.5, $[M+4H]^{4+}$: 873.6, $[M+5H]^{5+}$: 699.1, $[M+6H]^{6+}$: 582.8; Mass Found (ESI) 1746.1 $[M+2H]^{2+}$, 1164.4 $[M+3H]^{3+}$, 873.6 $[M+4H]^{4+}$, 699.1 $[M+5H]^{5+}$, 582.7 $[M+6H]^{6+}$.

MadL2 Un (31-60) Diselenide



H-DYDEYDNDETTHTPDPSAPTARPRIREHQA-OH

MadL2 Un (31-60) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (50 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 40% B over 40 min, 0.1% v/v TFA) afforded **MadL2 Un (31-60)** diselenide as a fluffy white solid after lyophilization (34.59 mg, 19.34% yield).

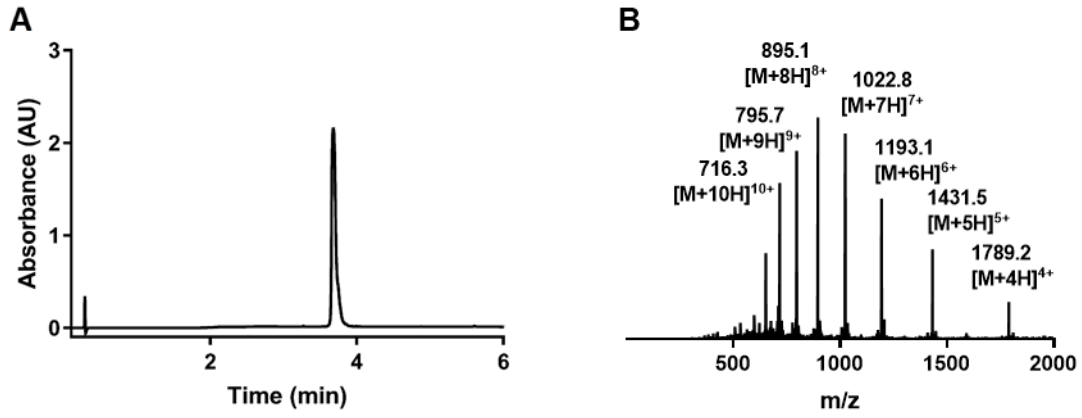
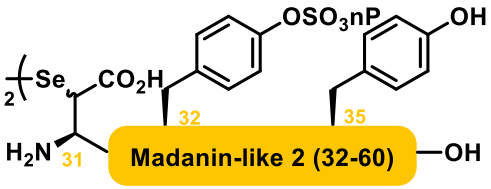


Figure S20: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **MadL2 Un (31-60)** diselenide. Analytical HPLC Rt 3.71 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass (as the diselenide) $[M+4H]^{4+}$: 1789.3 $[M+5H]^{5+}$: 1431.6 $[M+6H]^{6+}$: 1193.2 $[M+7H]^{7+}$: 1022.9 $[M+8H]^{8+}$: 895.2 $[M+9H]^{9+}$: 795.8 $[M+10H]^{10+}$: 716.3; Mass Found (ESI) 1789.2 $[M+4H]^{4+}$, 1431.5 $[M+5H]^{5+}$, 1193.1 $[M+6H]^{6+}$, 1022.8 $[M+7H]^{7+}$, 895.1 $[M+8H]^{8+}$, 795.7 $[M+9H]^{9+}$, 716.3 $[M+10H]^{10+}$.

MadL2 Y32 (31-60) Diselenide



H-DsYDEYDNDETTHTPDPSAPTARPRIREHQA-OH

MadL2 Y32 (31-60) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (50 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) afforded **MadL2 Y32 (31-60)** diselenide as a fluffy white solid after lyophilization (6.05 mg, 3.25% yield).

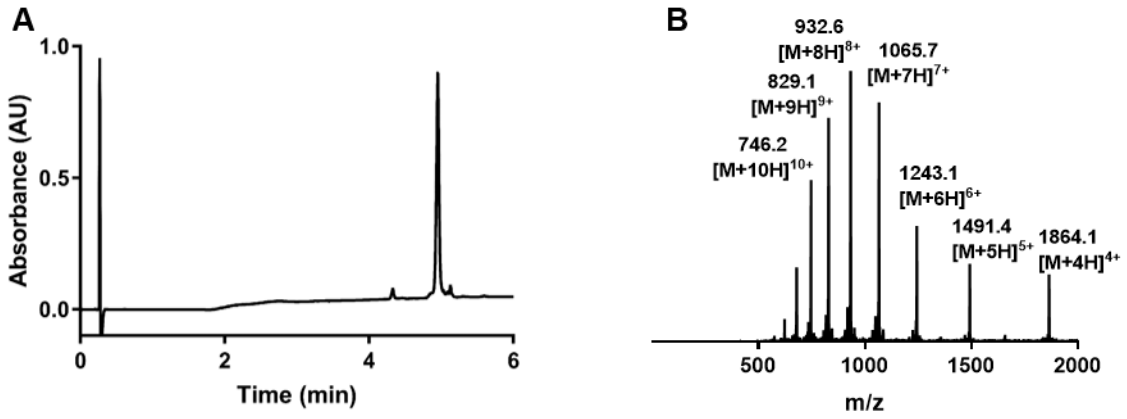
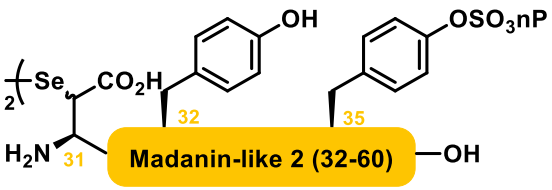


Figure S21: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **MadL2 Y32 (31-60)** diselenide. Analytical HPLC Rt 4.98 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass (as the diselenide) $[M+4H]^{4+}$: 1864.5 $[M+5H]^{5+}$: 1491.8 $[M+6H]^{6+}$: 1243.3 $[M+7H]^{7+}$: 1065.8 $[M+8H]^{8+}$: 932.7 $[M+9H]^{9+}$: 829.2 $[M+10H]^{10+}$: 746.4; Mass Found (ESI) 1864.1 $[M+4H]^{4+}$, 1491.4 $[M+5H]^{5+}$, 1243.1 $[M+6H]^{6+}$, 1065.7 $[M+7H]^{7+}$, 932.6 $[M+8H]^{8+}$, 829.1 $[M+9H]^{9+}$, 746.2 $[M+10H]^{10+}$.

MadL2 Y35 (31-60) Diselenide



H-DYDEsYDNDETTHTPDPSAPTARPRIREHQA-OH

MadL2 Y35 (31-60) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (50 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) afforded **MadL2 Y35 (31-60)** diselenide as a fluffy white solid after lyophilization (3.82 mg, 2.05% yield); Analytical HPLC Rt 5.02 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm).

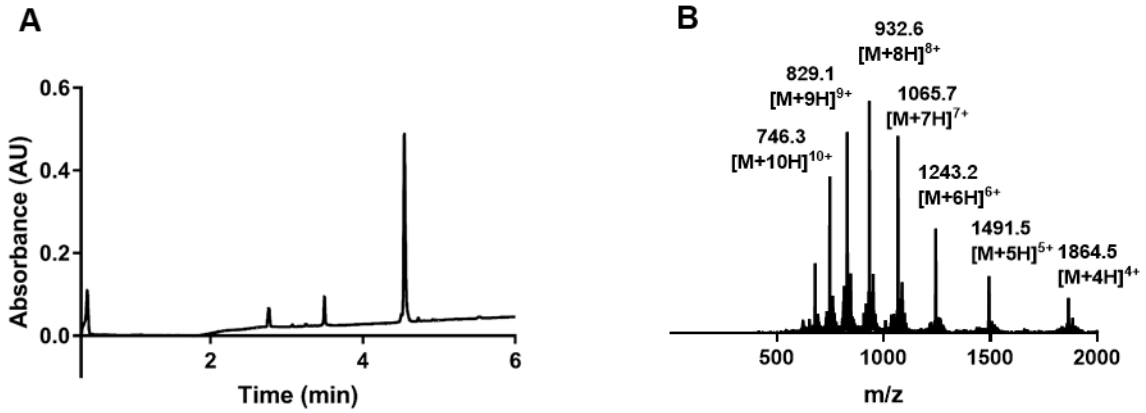
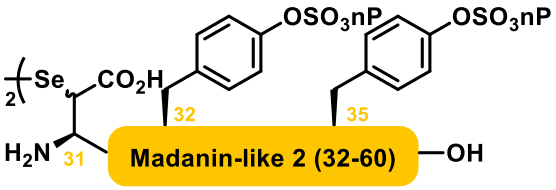


Figure S22: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **MadL2 Y35 (31-60)** diselenide. Analytical HPLC treated with 10% TCEP for 30 min Rt 4.54 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm) ; Calculated Mass (as the diselenide) $[M+4H]^{4+}$: 1864.5 $[M+5H]^{5+}$: 1491.8 $[M+6H]^{6+}$: 1243.3 $[M+7H]^{7+}$: 1065.8 $[M+8H]^{8+}$: 932.69 $[M+9H]^{9+}$: 829.2 $[M+10H]^{10+}$: 746.4; Mass Found (ESI) 1864.5 $[M+4H]^{4+}$, 1491.5 $[M+5H]^{5+}$, 1243.2 $[M+6H]^{6+}$, 1065.7 $[M+7H]^{7+}$, 932.6 $[M+8H]^{8+}$, 829.1 $[M+9H]^{9+}$, 746.3 $[M+10H]^{10+}$.

MadL2 DS (31-60) Diselenide



H-DsYDEsYDNDETTHTPDPSAPTARPRIREHQA-OH

MadL2 DS (31-60) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (50 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide with a solution of TFA/iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) afforded **MadL2 DS (31-60)** diselenide as a fluffy white solid after lyophilization (4.36 mg, 2.24% yield).

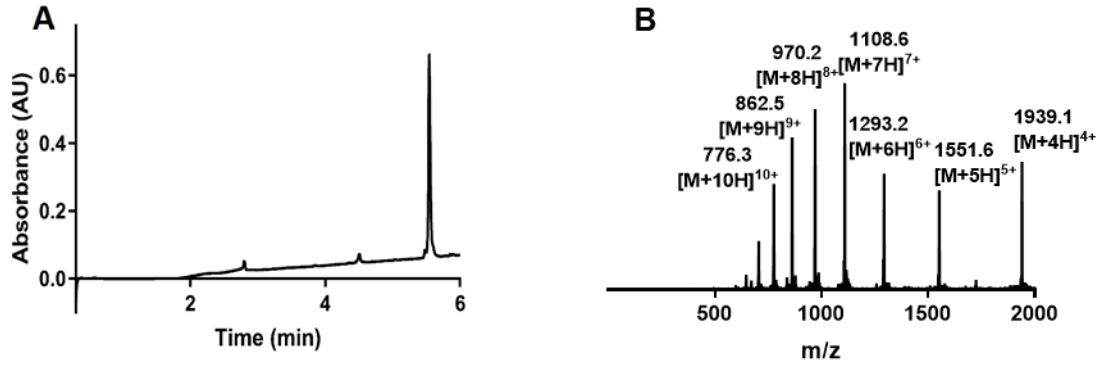
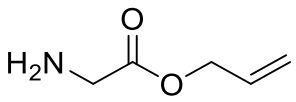


Figure S23: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **MadL2 DS (31-60)** diselenide. Analytical HPLC Rt 5.57 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm). Calculated Mass (as the diselenide) $[M+4H]^{4+}$: 1939.5 $[M+5H]^{5+}$: 1551.8 $[M+6H]^{6+}$: 1293.3 $[M+7H]^{7+}$: 1108.7 $[M+8H]^{8+}$: 970.2 $[M+9H]^{9+}$: 862.5 $[M+10H]^{10+}$: 776.4; Mass Found (ESI) 1939.1 $[M+4H]^{4+}$, 1551.6 $[M+5H]^{5+}$, 1298.7 $[M+6H]^{6+}$, 1112.6 $[M+7H]^{7+}$, 973.6 $[M+8H]^{8+}$, 862.5 $[M+9H]^{9+}$, 776.3 $[M+10H]^{10+}$.

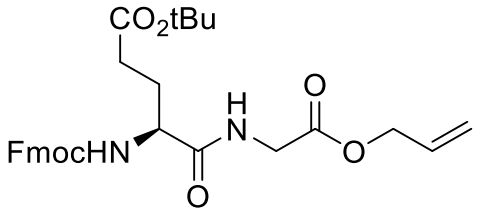
Synthesis of Fmoc-Glu-Gly-OAll Dipeptide

H_2N-Gly-OAll



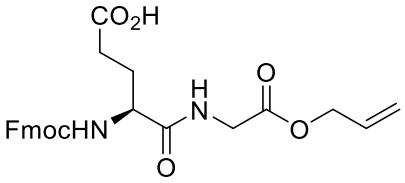
Boc-Gly-OH (2.0 g, 11.4 mmol) was dissolved in DMF (40 mL) and cooled to 0 °C. *i*Pr_2NEt (6.0 mL, 34.2 mmol) was added slowly, followed by allyl bromide (1.1 mL, 12.6 mmol). The solution was allowed to warm to room temperature, and stirred 16 h. The reaction mixture was diluted with EtOAc (120 mL) and then washed with water (2x 60 mL) and brine (60 mL). The organic phase was dried with anhydrous $MgSO_4$, concentrated *in vacuo*, and treated with a 1:1 v/v mixture of TFA in CH_2Cl_2 (10 mL). The reaction mixture was stirred at room temperature for 1 h, after which the TFA/CH_2Cl_2 solvent mixture was concentrated *in vacuo*, azeotroping with toluene, to afford the desired compound as a yellow oil. Yield (1.30 g, quant). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 5.90 (m, 1H), 5.25, (m, 2H), 4.69 (d, *J* = 5.7 Hz, 2H), 3.82 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 167.5, 130.5, 119.8, 67.2, 40.4. LRMS (ESI-) 112.95 $[M-H]^-$; IR (ATR): ν^{max} = 3006, 1752, 1674, 1200, 1135 cm^{-1}.

Fmoc-Glu(OtBu)-Gly-OAll



A solution of Fmoc-Glu(OtBu)-OH (4.38 g, 10.3 mmol), HATU (3.92 g, 10.3 mmol) and NMM (4.6 mL, 41.2 mmol) in DMF (final concentration 0.1 M) was added to a reaction vessel containing the glycine allylester (1.30 g, 8.6 mmol). The reaction was stirred for 2 h, after which the solvent was blown off under a stream of nitrogen. The residue was dissolved into CH_2Cl_2 (400 mL), washed with water (2x 30 mL), 2 M HCl (200 mL), saturated aqueous $NaHCO_3$ solution (200 mL), and brine (300 mL). The organic phase was dried with anhydrous $MgSO_4$, concentrated *in vacuo*, and purified by flash column chromatography (eluent 3:1 v/v to 1:1 v/v hex:EtOAc) to afford the desired dipeptide. Yield (2.93 g, 65%). $[\alpha]^{25}_D$ -7.7 (c 0.30 CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.76 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.5 Hz, 2H), 7.4 (dd, *J* = 1.0, 7.2 Hz, 2H), 7.31 (dd, *J* = 1.0, 7.5 Hz, 2H), 5.87 (m, 1H), 5.77 (d, *J* = 7.7 Hz, 1H), 5.33 (d, *J* = 17.7 Hz, 1H), 5.26 (d, *J* = 10.2 Hz, 1H), 4.65 (d, *J* = 5.4 Hz, 2H), 4.39 (d, *J* = 7.0 Hz, 2H), 4.27 (m, 1H), 4.24 (d, *J* = 7.5Hz, 1H), 4.07 (m, 1H), 2.40 (m, 2H), 2.05-1.94 (m, 2H), 1.46 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 175.5, 170.9, 168.2, 163.0, 142.8, 140.3, 132.0, 126.7, 126.1, 124.1, 119.0, 109.2, 82.3, 66.7, 65.1, 54.4, 46.2, 41.5, 31.8, 28.2, 27.1; LRMS (ESI+) 545.3$[M+Na]^+$; HRMS: (+ESI) Calc. for 545.22582 $[M+Na]^+$, Found: 545.22582 $[M+Na]^+$ diff. = < 0.1 ppm; IR (ATR): ν^{max} = 3314, 1726, 1532, 1450, 1154, 741 cm^{-1}.

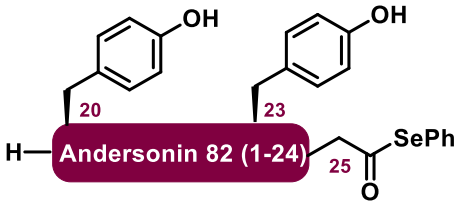
Fmoc-Glu-Gly-OAll



The protected dipeptide (2.93 mg, 5.6 mmol) was treated with a 1:1 mixture of TFA in CH_2Cl_2 (16 mL). The reaction mixture was stirred at room temperature for 1 h, after which the TFA/CH_2Cl_2 solvent mixture was concentrated *in vacuo*, azeotroping with toluene to afford the desired product as a yellow oil. Yield (2.05 g, 79%). $[\alpha]^{25}_D$ - 4.3 (c 0.30, CH_2Cl_2); ^{1}H NMR (MeOD, 300 MHz) δ (ppm): 7.80 (d, *J* = 7.4 Hz, 2H), 7.67 (m, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.32 (t, *J* = 7.2 Hz, 2H), 5.91 (m, 1H), 5.33 (d, *J* = 17.1 Hz, 1H), 5.21 (d, *J* = 10.0 Hz, 1H), 4.62 (d, *J* = 4.6 Hz, 2H), 4.39 (t, *J* = 8.3 Hz, 2H), 4.22 (m, 2H), 3.95 (s, 2H), 2.42 (t, *J* = 7.5 Hz, 2H), 2.11 (m, 2H), 1.92 (m, 2H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ (ppm): 176.8, 174.8, 170.9, 158.8, 145.8, 142.6, 133.3, 128.7, 128.1, 126.1, 120.8, 118.7, 68.0, 66.8, 55.7, 47.3, 42.0, 31.0, 28.5; LRMS (ESI+) 489.2 $[M+Na]^+$; HRMS: (+ESI) Calc. for 489.16322 $[M+Na]^+$, Found: 489.16329 $[M+Na]^+$, diff. = 0.1 ppm; IR (ATR): ν^{max} = 3307, 3069, 2935, 1711, 1670, 1534, 1202, 741 cm^{-1}.

Andersonin 82 Fragment Synthesis

And82 Un (1-25) Selenoester



H-KQRRDLVEARSSENANEHDYDDYQG-SePh

And82 Un (1-25) selenoester was prepared by sidechain loading of Fmoc-GluGly-OAll to Rink amide resin (60 µmol) and extended via Fmoc-strategy SPPS, through a combination of automated and manual synthesis, with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment, on-resin allyl ester deprotection and solid-phase selenoesterification with DPDS (561.8 mg, 30 eq.) were completed as outlined in the general procedures. Cleavage from resin and removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/*i*Pr_3SiH/H_2O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 40% B over 50 min, 0.1% v/v TFA) afforded **And82 Un (1-25)** selenoester as a fluffy white solid after lyophilization (9.46 mg, 5% yield).

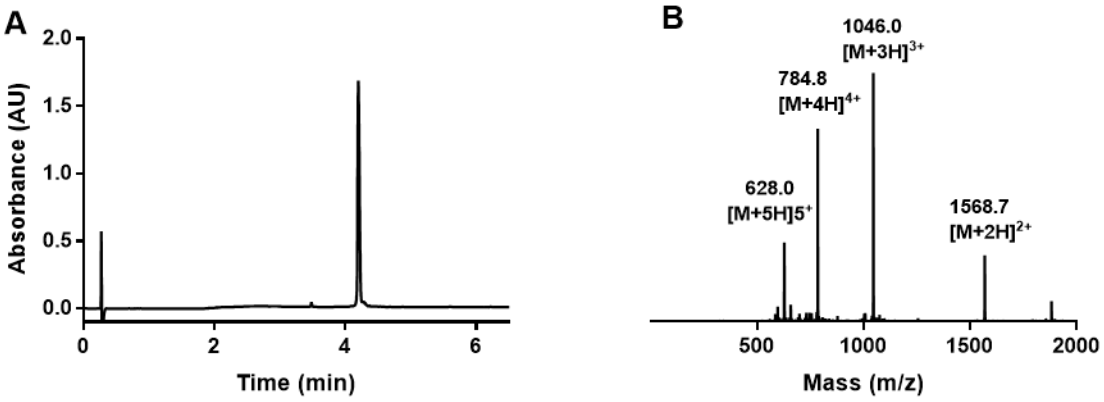
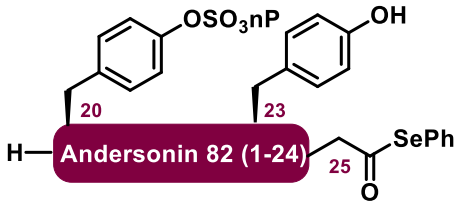


Figure S24: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **And82 Un (1-25)** selenoester. Analytical HPLC Rt 4.15 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1568.2, $[M+3H]^{3+}$: 1045.8, $[M+4H]^{4+}$: 784.6, $[M+5H]^{5+}$: 627.9, Mass Found (ESI) 1568.7 $[M+2H]^{2+}$, 1046.0 $[M+3H]^{3+}$, 784.8 $[M+4H]^{4+}$, 628.0 $[M+5H]^{5+}$.

And82 Y20 (1-25) Selenoester



H-KQRRDLVEARSSENANEHDsYDDYQG-SePh

And82 Y20 (1-25) selenoester was prepared by sidechain loading of Fmoc-GluGly-OAll to Rink amide resin (45 µmol) and extended via Fmoc-strategy SPPS, through a combination of automated and manual synthesis, with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment, on-resin allyl ester deprotection and solid-phase selenoesterification with DPDS (421.4 mg, 30 eq.) were completed as outlined in the general procedures. Cleavage from resin and removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 35% B over 50 min, 0.1% v/v TFA) afforded **And82 Y20 (1-25)** selenoester as a fluffy white solid after lyophilization (12 mg, 8% yield).

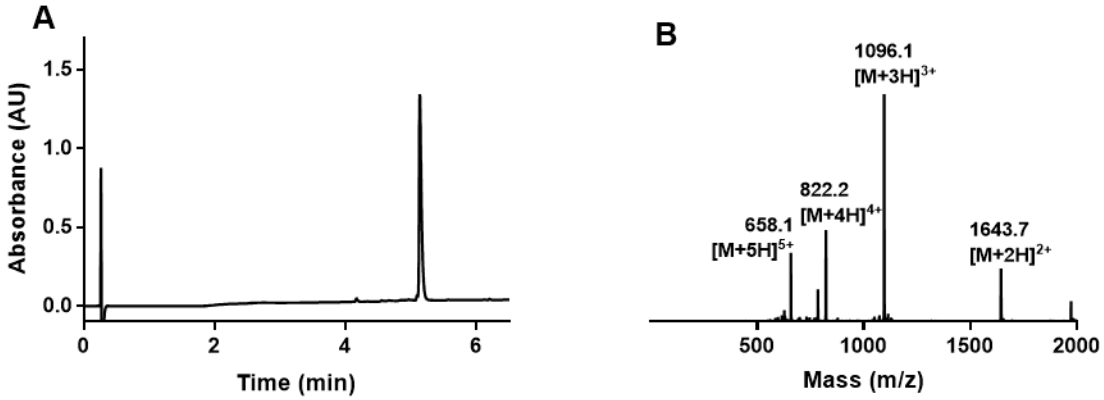
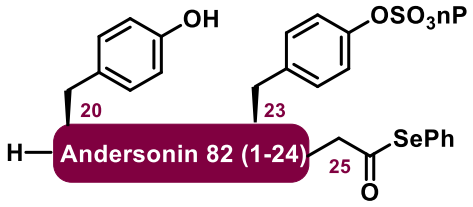


Figure S25: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **And82 Y20 (1-25)** selenoester. Analytical HPLC Rt 5.06 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1643.2, $[M+3H]^{3+}$: 1095.8, $[M+4H]^{4+}$: 822.1, $[M+5H]^{5+}$: 657.9, Mass Found (ESI) 1643.7 $[M+2H]^{2+}$, 1096.1 $[M+3H]^{3+}$, 822.2 $[M+4H]^{4+}$, 658.1 $[M+5H]^{5+}$.

And82 Y23 (1-25) Selenoester



H-KQRRDLVEARSSENANEHDYDD sY QG-SePh

And82 Y23 (1-25) selenoester. was prepared by sidechain loading of Fmoc-GluGly-OAll to Rink amide resin (45 µmol) and extended via Fmoc-strategy SPPS, through a combination of automated and manual synthesis, with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment, on-resin allyl ester deprotection and solid-phase selenoesterification with DPDS (421.4 mg, 30 eq.) were completed as outlined in the general procedures. Cleavage from resin and removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 35% B over 50 min, 0.1% v/v TFA) afforded **And82 Y23 (1-25)** selenoester. as a fluffy white solid after lyophilization (32.1 mg, 22% yield).

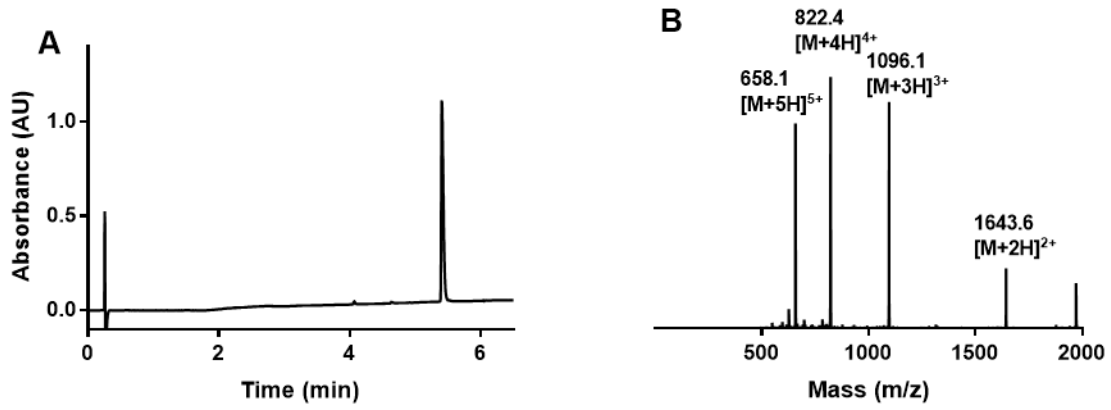
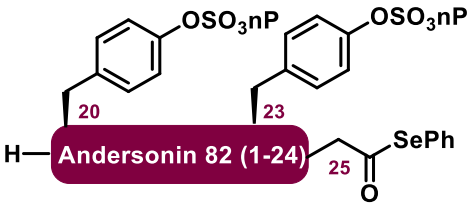


Figure S26: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **And82 Y23 (1-25)** selenoester. Analytical HPLC Rt 5.37 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1643.2, $[M+3H]^{3+}$: 1095.8, $[M+4H]^{4+}$: 822.1, $[M+5H]^{5+}$: 657.9, Mass Found (ESI) 1643.6 $[M+2H]^{2+}$, 1096.1 $[M+3H]^{3+}$, 822.4 $[M+4H]^{4+}$, 658.1 $[M+5H]^{5+}$.

And82 DS (1-25) Selenoester



H-KQRRDLVEARSSENANEHD sY DD sY QG-SePh

And82 DS (1-25) selenoester was prepared by sidechain loading of Fmoc-GluGly-OAll to Rink amide resin (45 µmol) and extended via Fmoc-strategy SPPS, through a combination of automated and manual synthesis, with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment, on-resin allyl ester deprotection and solid-phase selenoesterification with DPDS (421.4 mg, 30 eq.) were completed as outlined in the general procedures. Cleavage from resin and removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 45% B over 50 min, 0.1% v/v TFA) afforded **And82 DS (1-25)** selenoester as a fluffy white solid after lyophilization (12 mg, 8% yield).

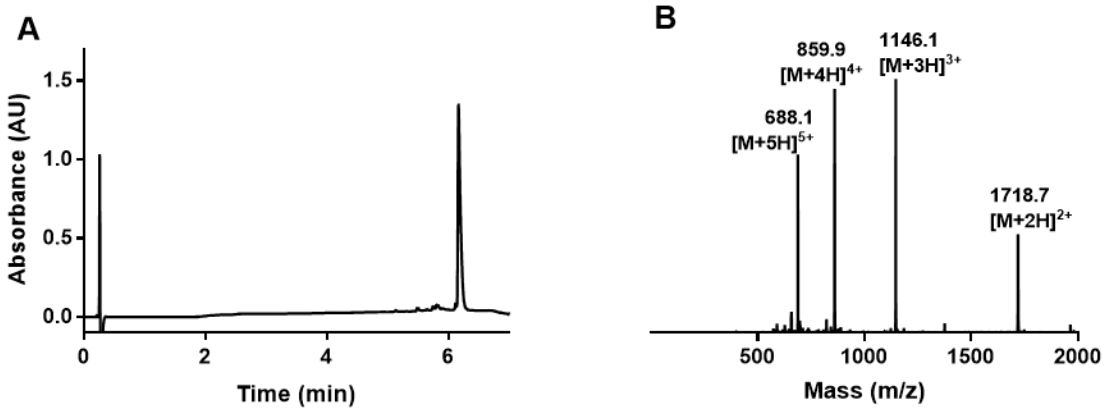


Figure S27: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **And82 DS (1-25)** selenoester. Analytical HPLC Rt 6.10 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1718.2, $[M+3H]^{3+}$: 1145.8, $[M+4H]^{4+}$: 859.6, $[M+5H]^{5+}$: 687.9, Mass Found (ESI) 1718.7 $[M+2H]^{2+}$, 1146.1 $[M+3H]^{3+}$, 859.9 $[M+4H]^{4+}$, 688.1 $[M+5H]^{5+}$.

And82 (26-59) Diselenide

H-DGEPTEGSGTPAIPRLHRTGSGGNDGFEQIDGVA-OH

And82 (26-59) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on Wang resin (67 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide with a solution of TFA/*i*Pr_3SiH/H_2O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 35% B over 50 min, 0.1% v/v TFA) afforded **And82 (26-59)** diselenide as a fluffy white solid after lyophilization (18.6 mg, 8% yield).

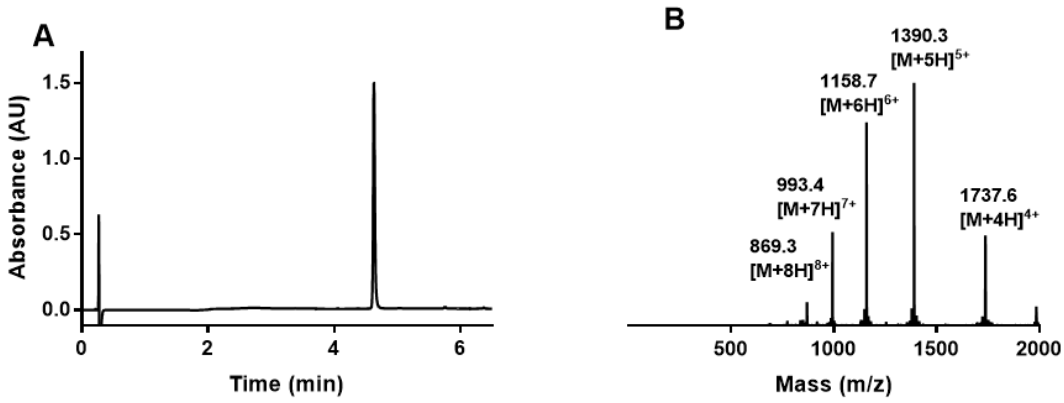
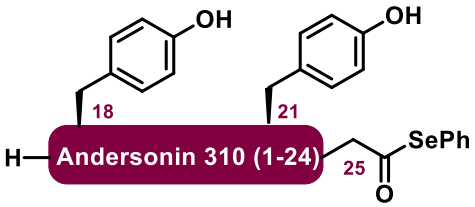


Figure S28: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **And82 (26-59)** diselenide. Analytical HPLC Rt 4.4 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass (as the diselenide) $[M+4H]^{4+}$: 1737.2, $[M+5H]^{5+}$: 1390.0, $[M+6H]^{6+}$: 1158.5, $[M+7H]^{7+}$: 993.1, $[M+8H]^{8+}$: 869.1, Mass Found (ESI) 1737.6 $[M+4H]^{4+}$, 1390.3 $[M+5H]^{5+}$, 1158.7 $[M+6H]^{6+}$, 993.4 $[M+7H]^{7+}$, 869.3 $[M+8H]^{8+}$.

Andersonin 310 Fragment Synthesis

And310 Un (1-25) Selenoester



H-KQRKPLEKIVHEREGTDYDDYEGEG-SePh

And310 Un (1-25) selenoester was prepared by sidechain loading of Fmoc-GluGly-OAll to 2-CTC resin (50 µmol) and extended via Fmoc-strategy SPPS, through a combination of automated and manual synthesis, with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment, on-resin allyl ester deprotection and solid-phase selenoesterification with DPDS (850 mg, 30 eq.) were completed as outlined in the general procedures. Cleavage from resin and removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) afforded **And310 Un (1-25)** selenoester as a fluffy white solid after lyophilization (25.2 mg, 15% yield).

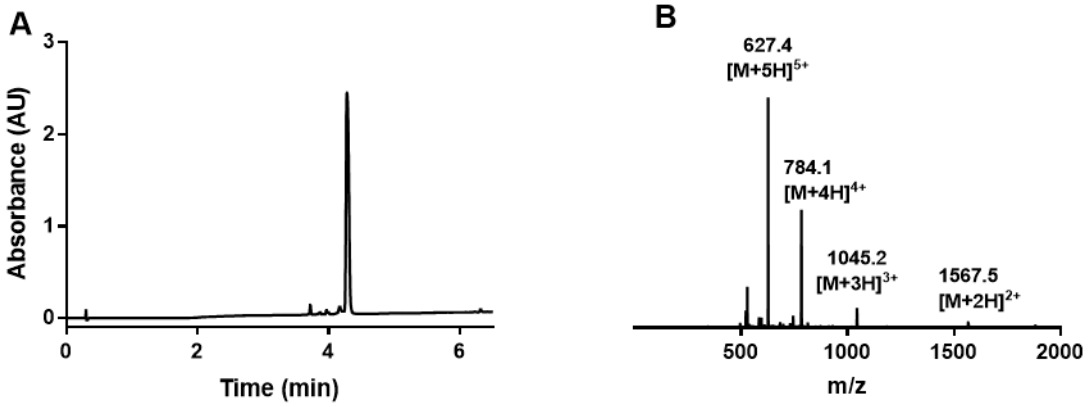
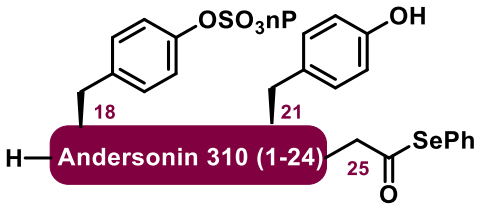


Figure S29: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **And310 Un (1-25)** selenoester. Analytical HPLC Rt 4.17 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1566.7, $[M+3H]^{3+}$: 1044.8, $[M+4H]^{4+}$: 783.9, $[M+5H]^{5+}$: 627.3; Mass Found (ESI) 1567.5 $[M+2H]^{2+}$, 1045.2 $[M+3H]^{3+}$, 784.1 $[M+4H]^{4+}$, 627.4 $[M+5H]^{5+}$.

And310 Y18 (1-25) Selenoester



H-KQRKPLEKIVHEREGTDsYDDYEGEG-SePh

And310 Y18 (1-25) selenoester was prepared by sidechain loading of Fmoc-GluGly-OAll to 2-CTC resin (25 µmol) and extended via Fmoc-strategy SPPS, through a combination of automated and manual synthesis, with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment, on-resin allyl ester deprotection and solid-phase selenoesterification with DPDS (850 mg, 30 eq.) were completed as outlined in the general procedures. Cleavage from resin and removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/*i*Pr_3SiH/H_2O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) afforded **And310 Y18 (1-25)** selenoester as a fluffy white solid after lyophilization (2.4 mg, 3% yield).

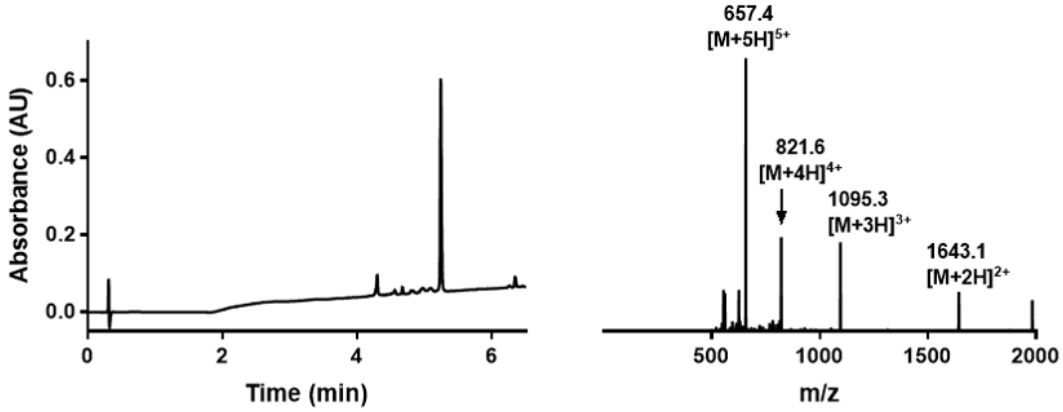
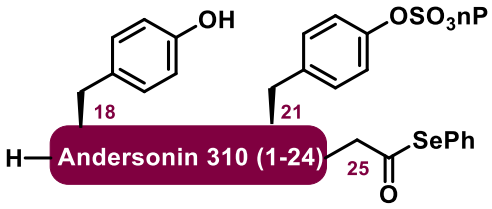


Figure S30: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **And310 Y18 (1-25)** selenoester. Analytical HPLC Rt 5.23 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1642.1, $[M+3H]^{3+}$: 1095.2, $[M+4H]^{4+}$: 821.7, $[M+5H]^{5+}$: 657.6; Mass Found (ESI) 1643.1 $[M+2H]^{2+}$, 1095.3 $[M+3H]^{3+}$, 821.6 $[M+4H]^{4+}$, 657.4 $[M+5H]^{5+}$.

And310 Y21 (1-25) Selenoester



H-KQRKPLEKIVHEREGTDYDDsYEGEG-SePh

And310 Y21 (1-25) selenoester was prepared by sidechain loading of Fmoc-GluGly-OAll to 2-CTC resin (40 µmol) and extended via Fmoc-strategy SPPS, through a combination of automated and manual synthesis, with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment, on-resin allyl ester deprotection and solid-phase selenoesterification with DPDS (850 mg, 30 eq.) were completed as outlined in the general procedures. Cleavage from resin and removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/*i*Pr_3SiH/H_2O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) afforded **And310 Y21 (1-25)** selenoester as a fluffy white solid after lyophilization (9.1 mg, 7% yield).

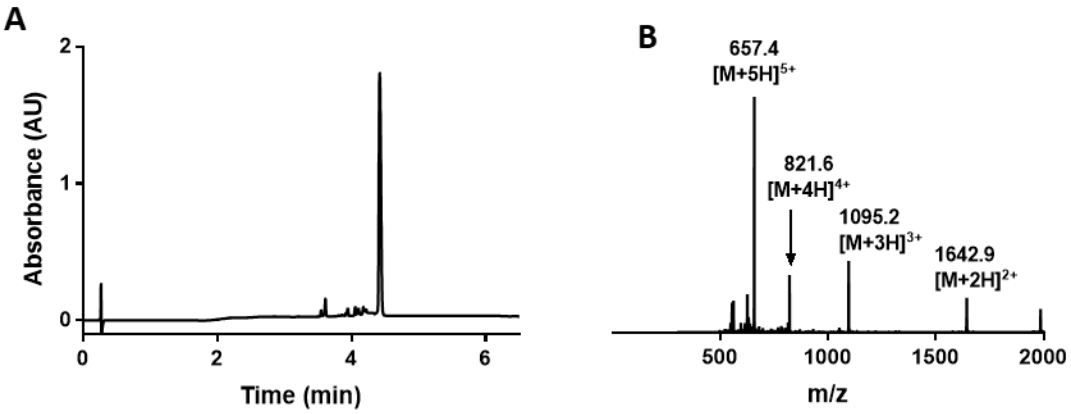
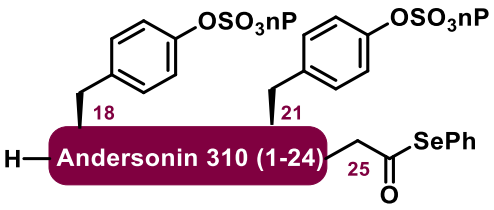


Figure S31: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **And310 Y21 (1-25)** selenoester. Analytical HPLC Rt 4.42 min (0 to 70% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1642.1, $[M+3H]^{3+}$: 1095.2, $[M+4H]^{4+}$: 821.7, $[M+5H]^{5+}$: 657.6; Mass Found (ESI) 1642.9 $[M+2H]^{2+}$, 1095.2 $[M+3H]^{3+}$, 821.6 $[M+4H]^{4+}$, 657.4 $[M+5H]^{5+}$.

And310 DS (1-25) Selenoester



H-KQRKPLEKIVHEREGTDsYDDsYEGEG-SePh

And310 DS (1-25) selenoester was prepared by sidechain loading of Fmoc-GluGly-OAll to 2-CTC resin (40 µmol) and extended via Fmoc-strategy SPPS, through a combination of automated and manual synthesis, with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment, on-resin allyl ester deprotection and solid-phase selenoesterification with DPDS (850 mg, 30 eq.) were completed as outlined in the general procedures. Cleavage from resin and removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/*i*Pr_3SiH/H_2O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude selenoester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) afforded **And310 DS (1-25)** selenoester as a fluffy white solid after lyophilization (13.7 mg, 10% yield).

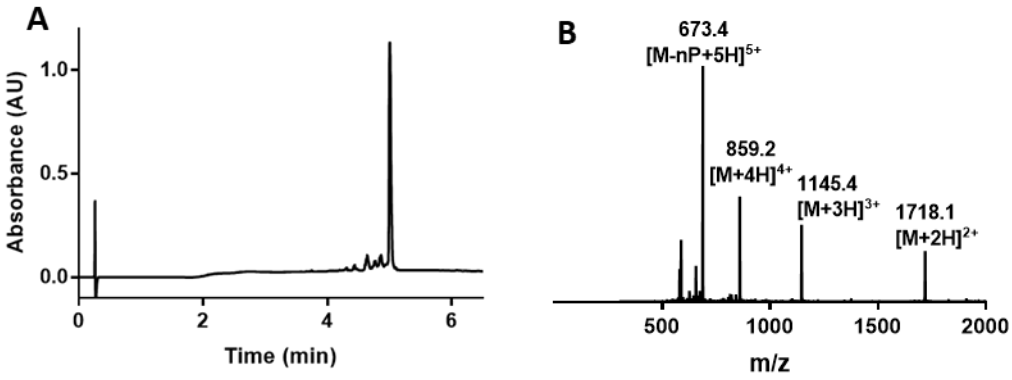


Figure S32: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **And310 DS (1-25)** selenoester. Analytical HPLC Rt 5.10 min (0 to 70% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass $[M+2H]^{2+}$: 1717.5, $[M+3H]^{3+}$: 1145.3, $[M+4H]^{4+}$: 859.2; Mass Found (ESI) 1718.1 $[M+2H]^{2+}$, 1145.4 $[M+3H]^{3+}$, 859.2 $[M+4H]^{4+}$.

And310 (26-62) Diselenide

H-DGGTTERSAHPAQPKLARHPSGSGGGFDEIPHDAIDE-OH

And310 (26-62) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (25 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 h at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) afforded **And310 (26-62)** diselenide as a fluffy white solid after lyophilization (24.7 mg, 25% yield).

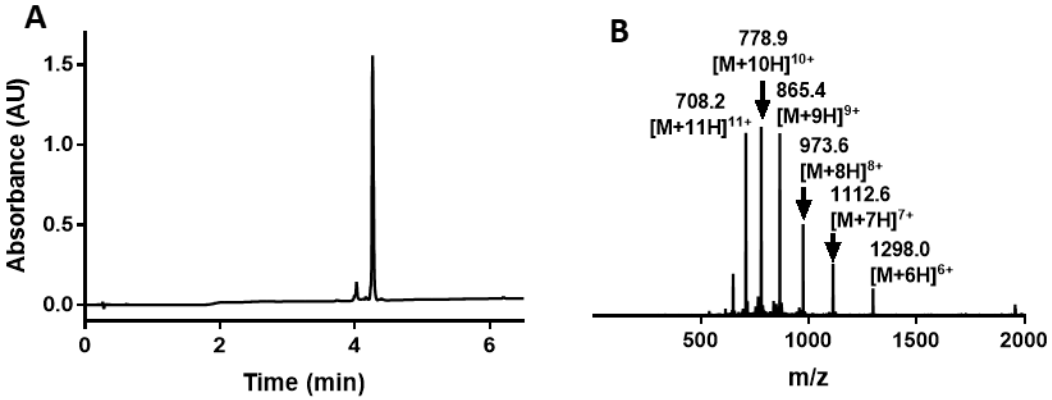


Figure S33: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **And310 (26-62)** diselenide. Analytical HPLC Rt 4.26 min (0 to 50% over 5 min, 0.1% v/v TFA, λ = 214 nm); Calculated Mass (as the diselenide) $[M+6H]^{6+}$: 1297.7 $[M+7H]^{7+}$: 1112.4 $[M+8H]^{8+}$: 973.5 $[M+9H]^{9+}$: 865.4 $[M+10H]^{10+}$: 779.0 $[M+11H]^{11+}$: 708.3; Mass Found (ESI) 1298.7 $[M+6H]^{6+}$, 1112.6 $[M+7H]^{7+}$, 973.6 $[M+8H]^{8+}$, 865.4 $[M+9H]^{9+}$, 778.9 $[M+10H]^{10+}$, 708.2 $[M+11H]^{11+}$.

Synthesis and Characterization of Tick-Derived Anticoagulants *via* DSL

Hya1 Un

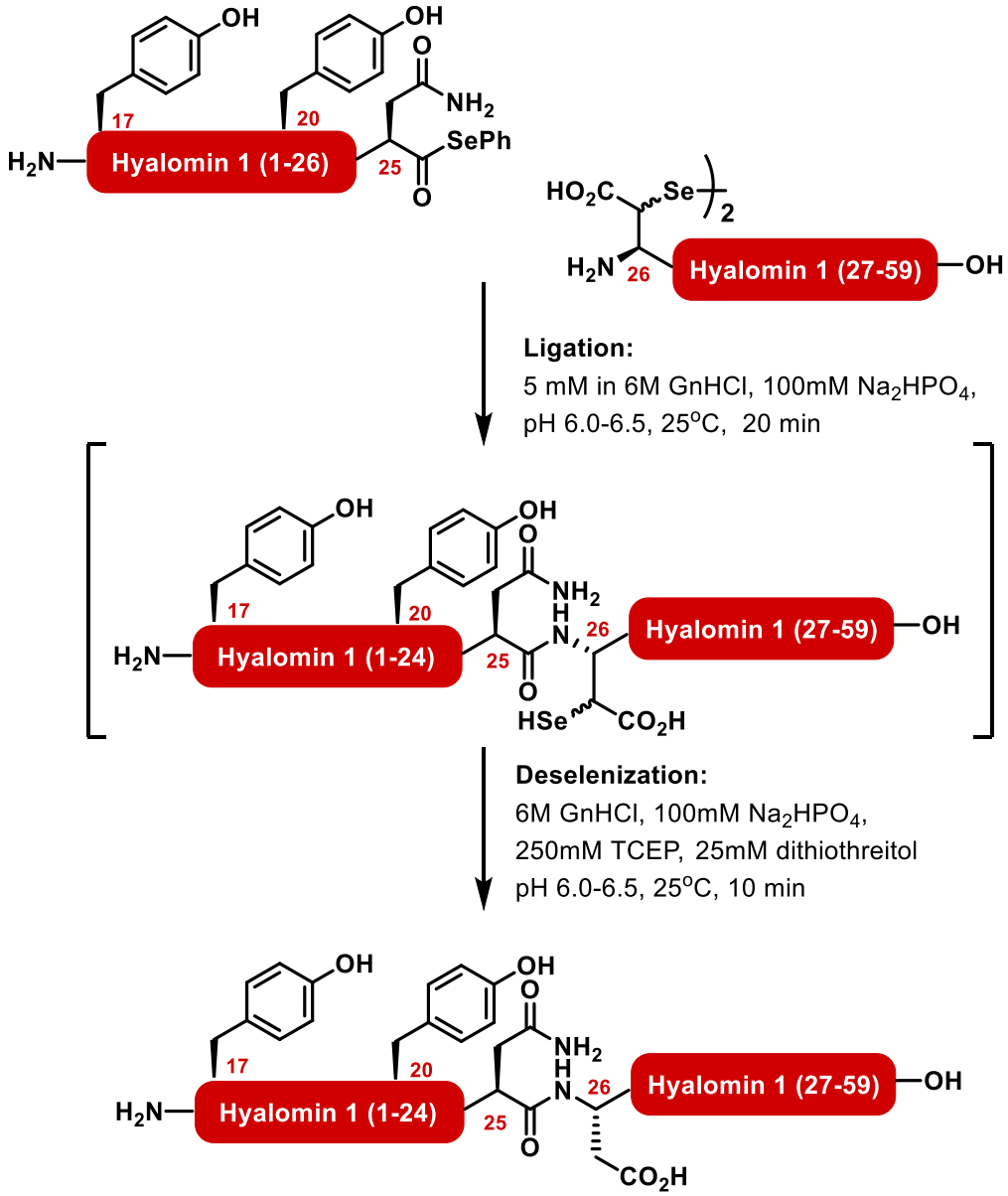


Figure S34: Synthesis of **Hya1 Un** via DSL-deselenization.

The one-pot peptide ligation of **Hya1 Un (1-25)** selenoester (2.4 mg, 0.84 µmol) and **Hya1 (26-59)** diselenide (2.0 mg, 0.56 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0 to 50% B over 40 min, 0.1% v/v TFA) followed by lyophilization afforded final protein **Hya1 Un** as a white solid (2.0 mg, 56%). Analytical HPLC (purified final product): R_t 4.10 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+5H]^{5+}$: 1278.1 $[M+6H]^{6+}$: 1065.3, $[M+7H]^{7+}$: 913.2 $[M+8H]^{8+}$: 799.2 $[M+9H]^{9+}$: 710.5 Mass Found (ESI) 1278.9 $[M+5H]^{5+}$, 1065.8 $[M+6H]^{6+}$, 913.6 $[M+7H]^{7+}$, 799.5 $[M+8H]^{8+}$, 710.6 $[M+9H]^{9+}$. High Res (ESI+): calcd for $C_{263}H_{416}N_{81}O_{105}$: $[M+7H]^{7+}$, 913.28283. Found: 918.28356 $[M+7H]^{7+}$. Difference: 0.9 ppm.

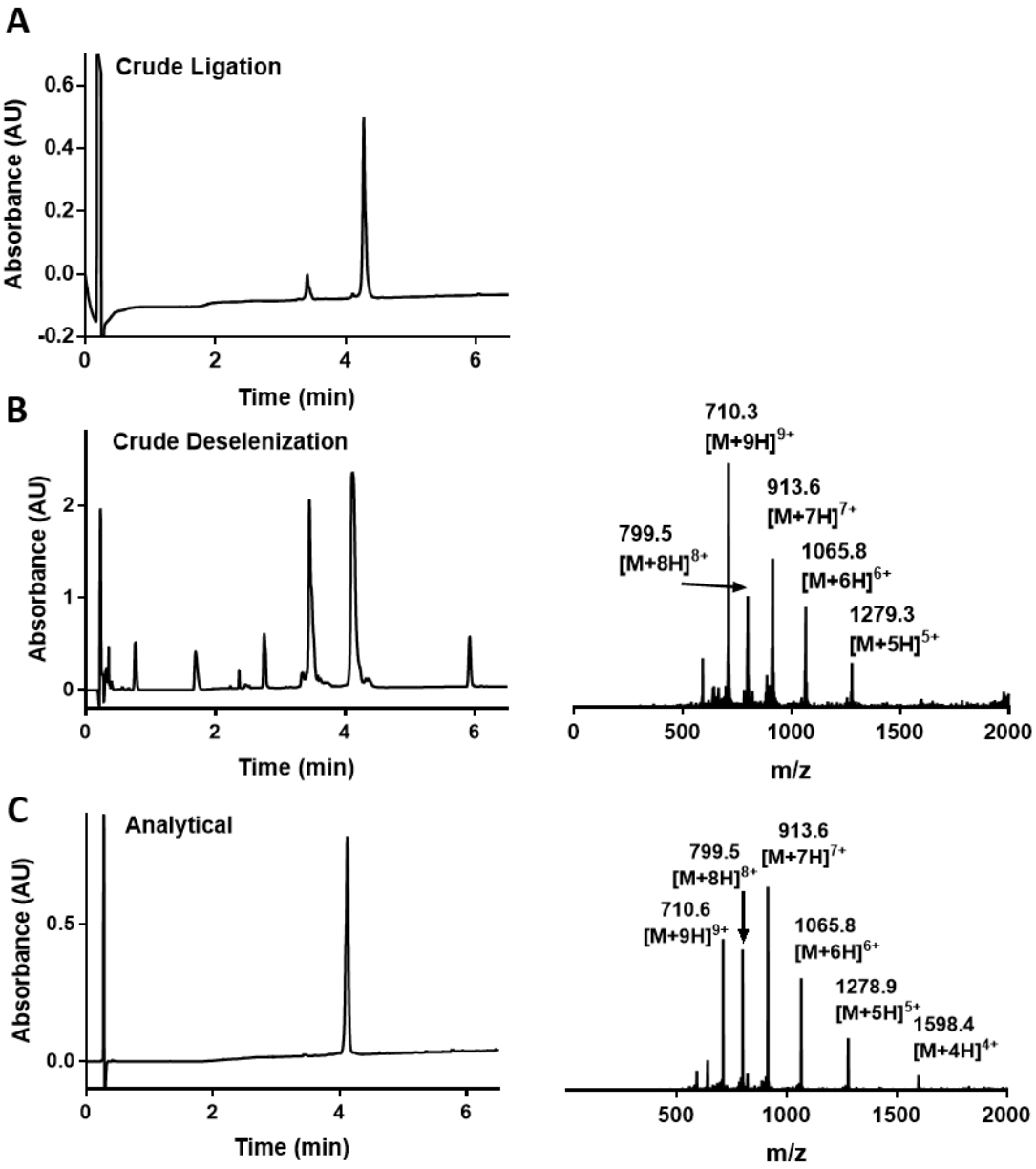


Figure S35: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization and (*C*) purified **Hya1 Un**.

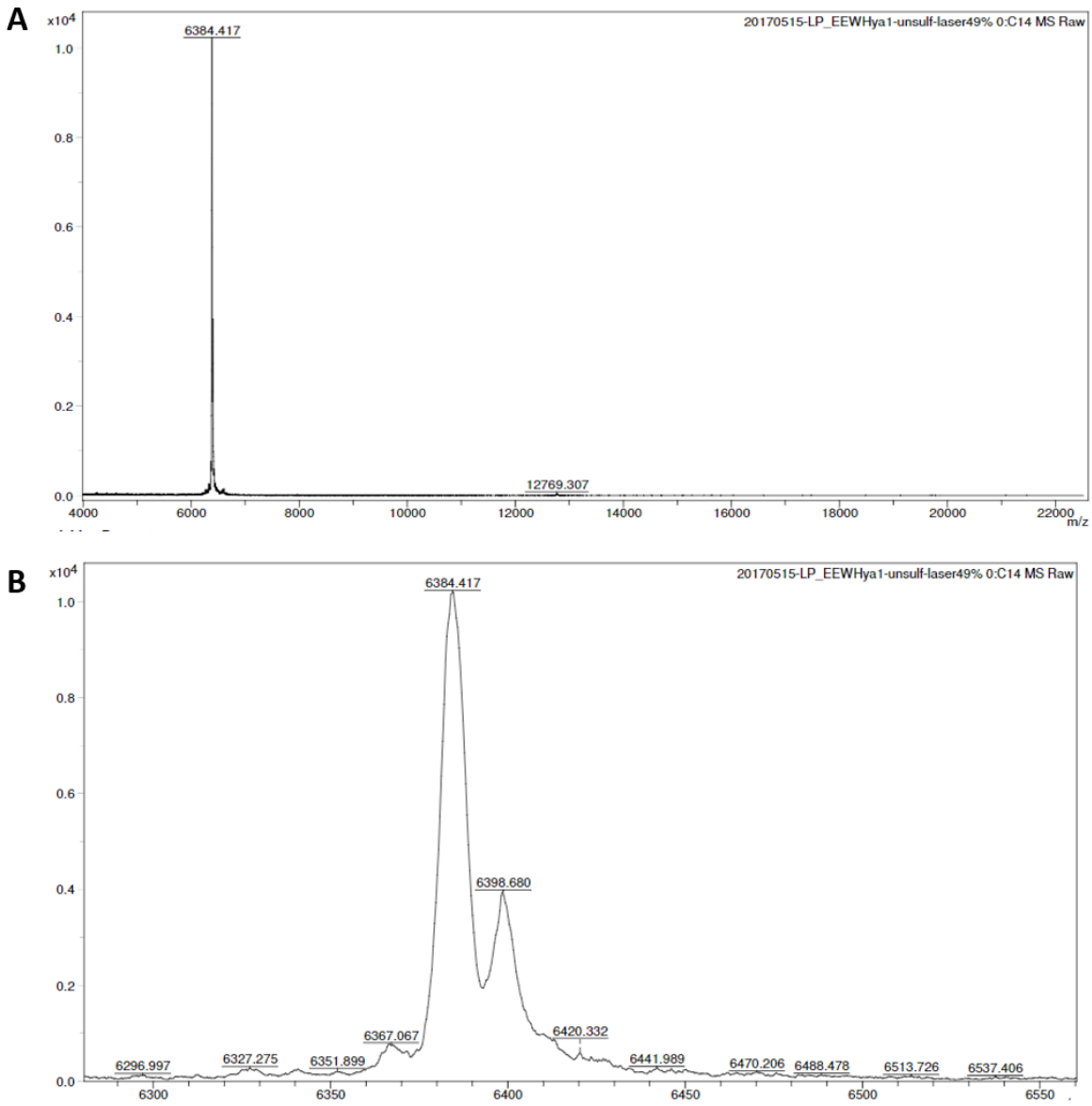


Figure S36: MALDI-TOF mass spectrum of **Hya1 Un** (*A*) 4000-22000 m/z and (*B*) 6300-6500 m/z.

Hya1 Y17

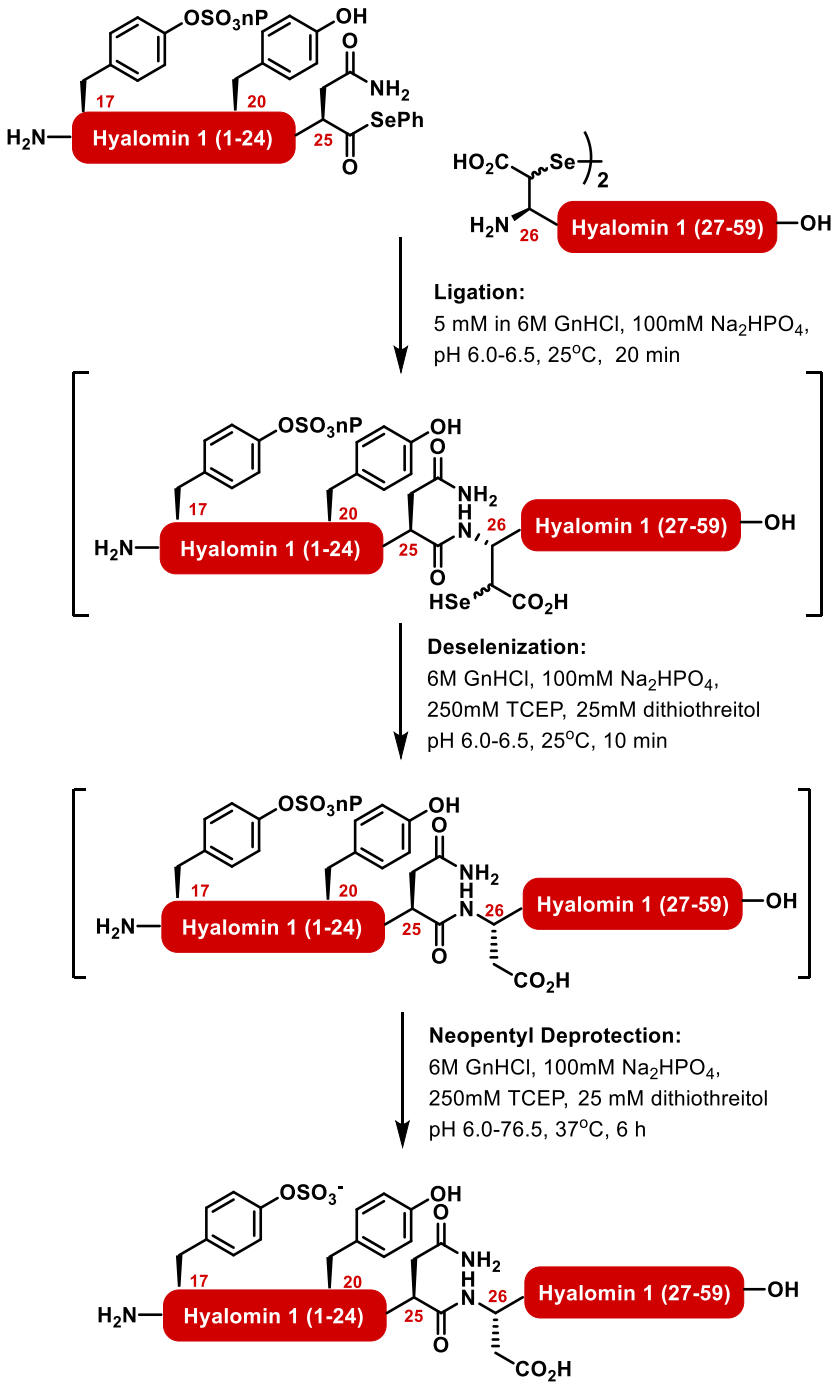


Figure S37: Synthesis of **Hya1 Y17** via DSL-deselenization.

The one-pot peptide ligation of **Hya1 Y17 (1-25)** selenoester (2.5 mg, 0.84 µmol) and **Hya1 (26-59)** diselenide (2.0 mg, 0.56 µmol) followed by *in situ* deselenization was performed according to the general procedure. Neopentyl deprotection was the performed as described in the general procedure, followed by purification via preparative HPLC (0 to 40% B over 40 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **Hya1 Y17** as a white solid (2.3 mg, 64%). Analytical HPLC (purified final product): R_t 4.08 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+5H]^{5+}$: 1294.1 $[M+6H]^{6+}$: 1078.6 $[M+7H]^{7+}$: 924.7 Mass Found (ESI) 1294.8$[M+5H]^{5+}$, 1079.2 $[M+6H]^{6+}$, 925.0 $[M+7H]^{7+}$. High Res (ESI+): calcd for $C_{263}H_{415}N_{81}O_{108}S$: $[M+6H]^{6+}$, 1078.48610. Found: 1078.48807 $[M+6H]^{6+}$. Difference: 1.8 ppm.

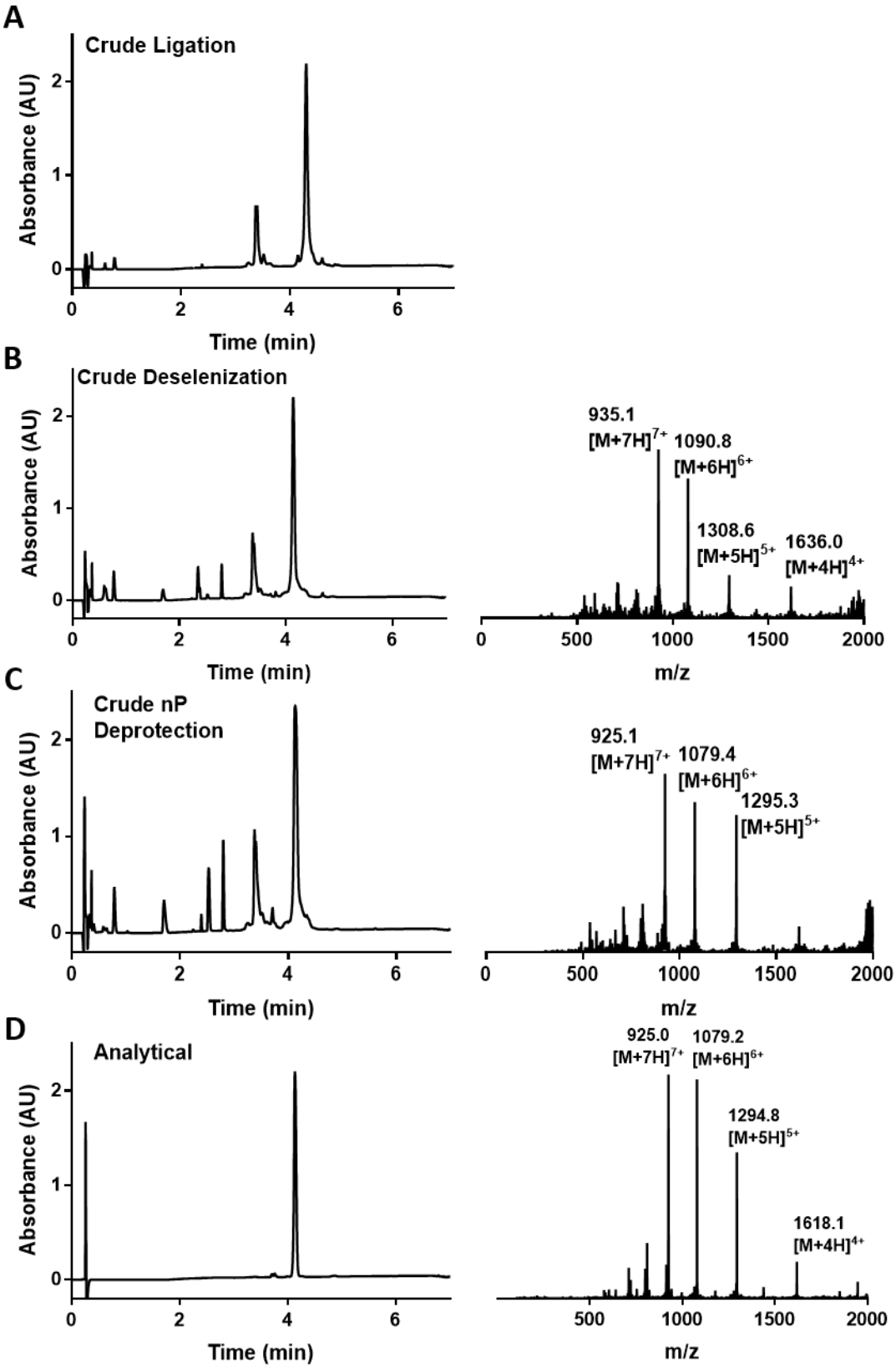


Figure S38: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **Hya1 Y17**.

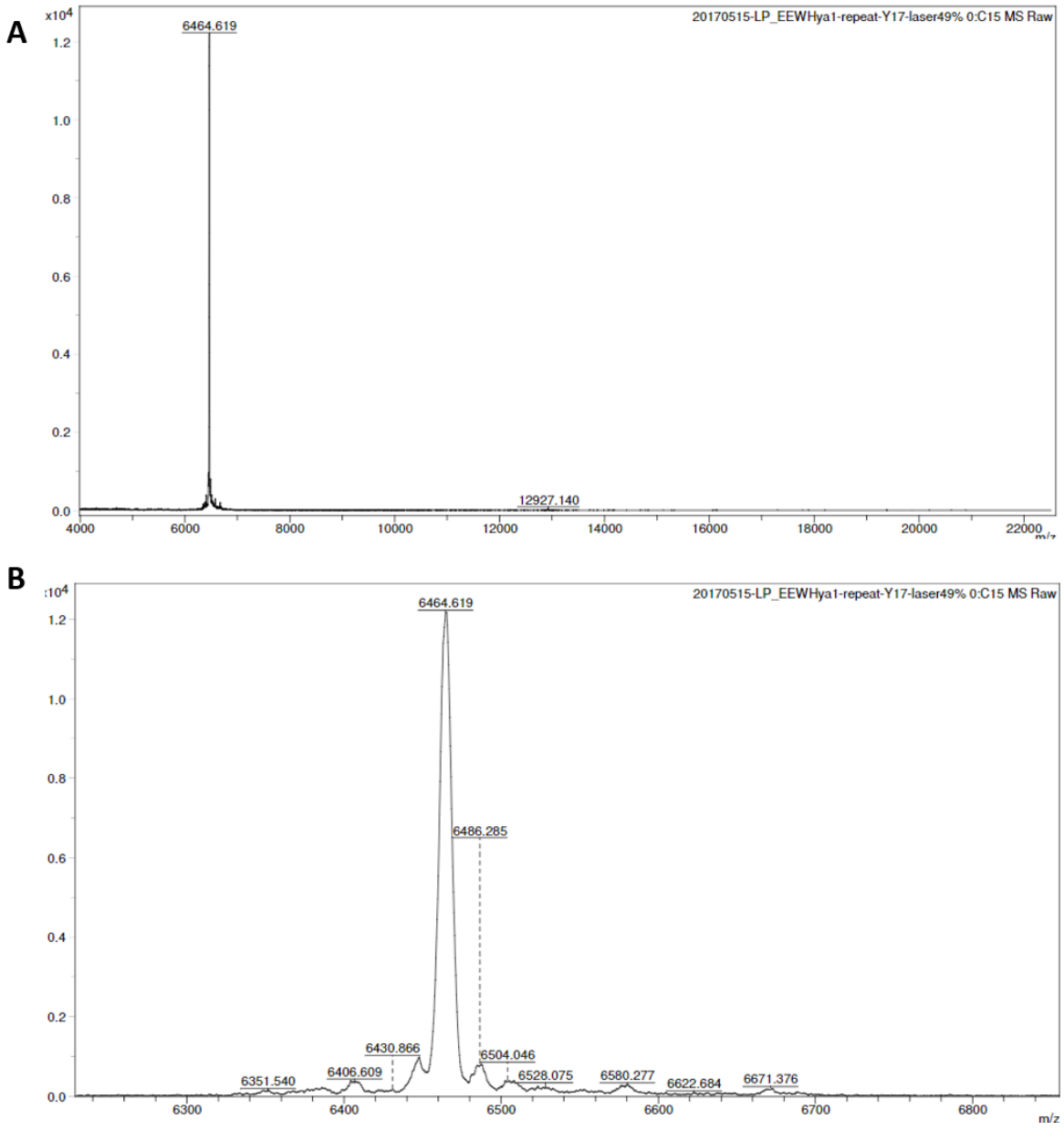


Figure S39: MALDI-TOF mass spectrum of **Hya1 Y17** (*A*) 4000-22000 m/z and (*B*) 6300-6800 m/z.

Hya1 Y20

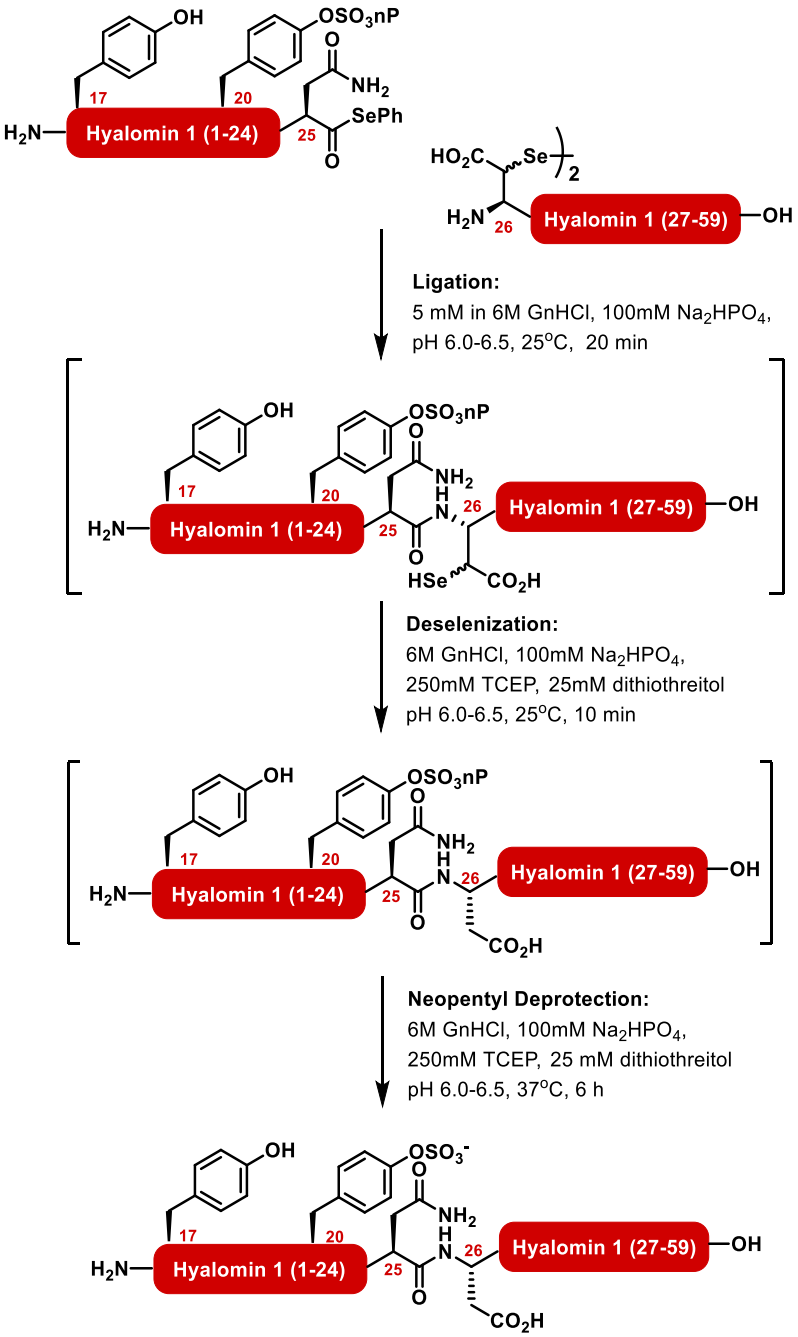


Figure S40: Synthesis of **Hya1 Y20** via DSL-deselenization.

The one-pot peptide ligation of **Hya1 Y20 (1-25)** selenoester (2.5 mg, 0.84 µmol) and **Hya1 (26-59)** diselenide (2.0 mg, 0.56 µmol) followed by *in situ* deselenization was performed according to the general procedure. Neopentyl deprotection was the performed as described in the general procedure, followed by purification via preparative HPLC (0 to 40% B over 40 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **Hya1 Y20** as a white solid (1.9 mg, 52%). Analytical HPLC (purified final product): R_t 4.10 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm Calculated Mass $[M+5H]^{5+}$: 1294.1 $[M+6H]^{6+}$: 1078.6 $[M+7H]^{7+}$: 924.7 Mass Found (ESI) 1294.6 $[M+5H]^{5+}$, 1078.6 $[M+6H]^{6+}$, 924.6 $[M+7H]^{7+}$. High Res (ESI+): calcd for $C_{263}H_{415}N_{81}O_{108}S$: $[M+6H]^{6+}$, 1078.48743 Found: 1078.48886 $[M+6H]^{6+}$. Difference: 1.3 ppm.

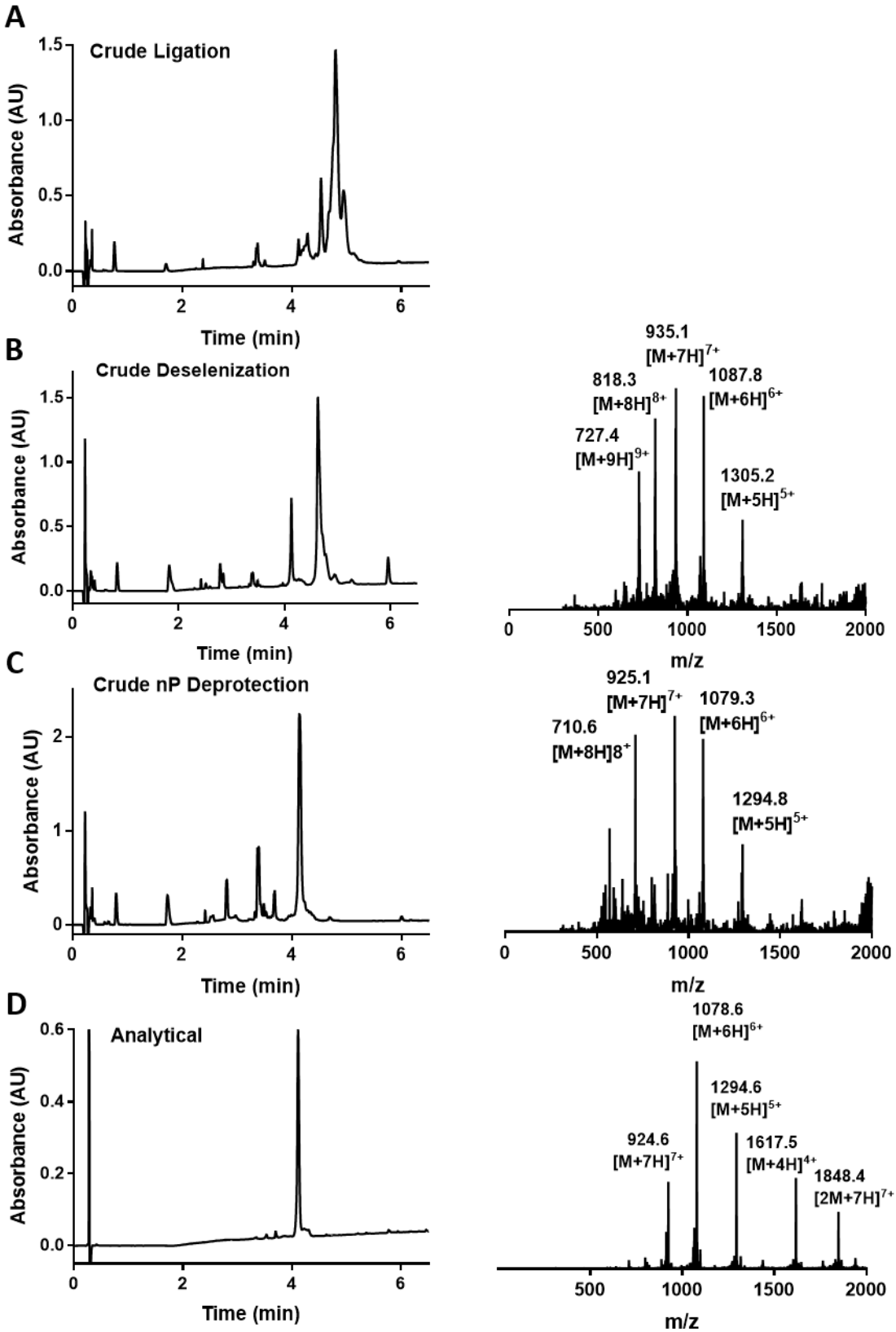


Figure S41: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **Hya1 Y20**.

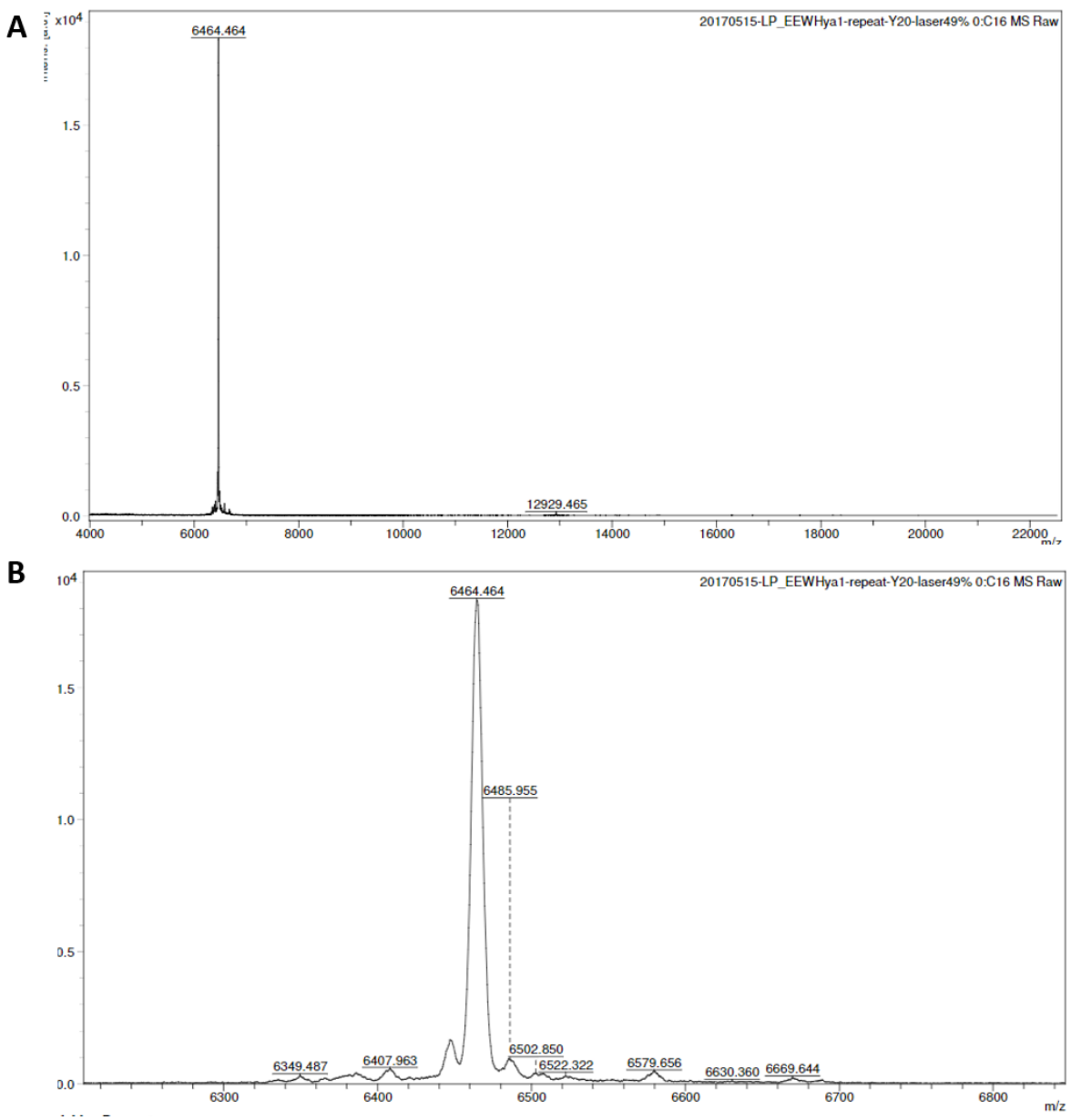


Figure S42: MALDI-TOF mass spectrum of **Hya1 Y20** (*A*) 4000-22000 m/z and (*B*) 6300-6800 m/z.

Hya1 DS

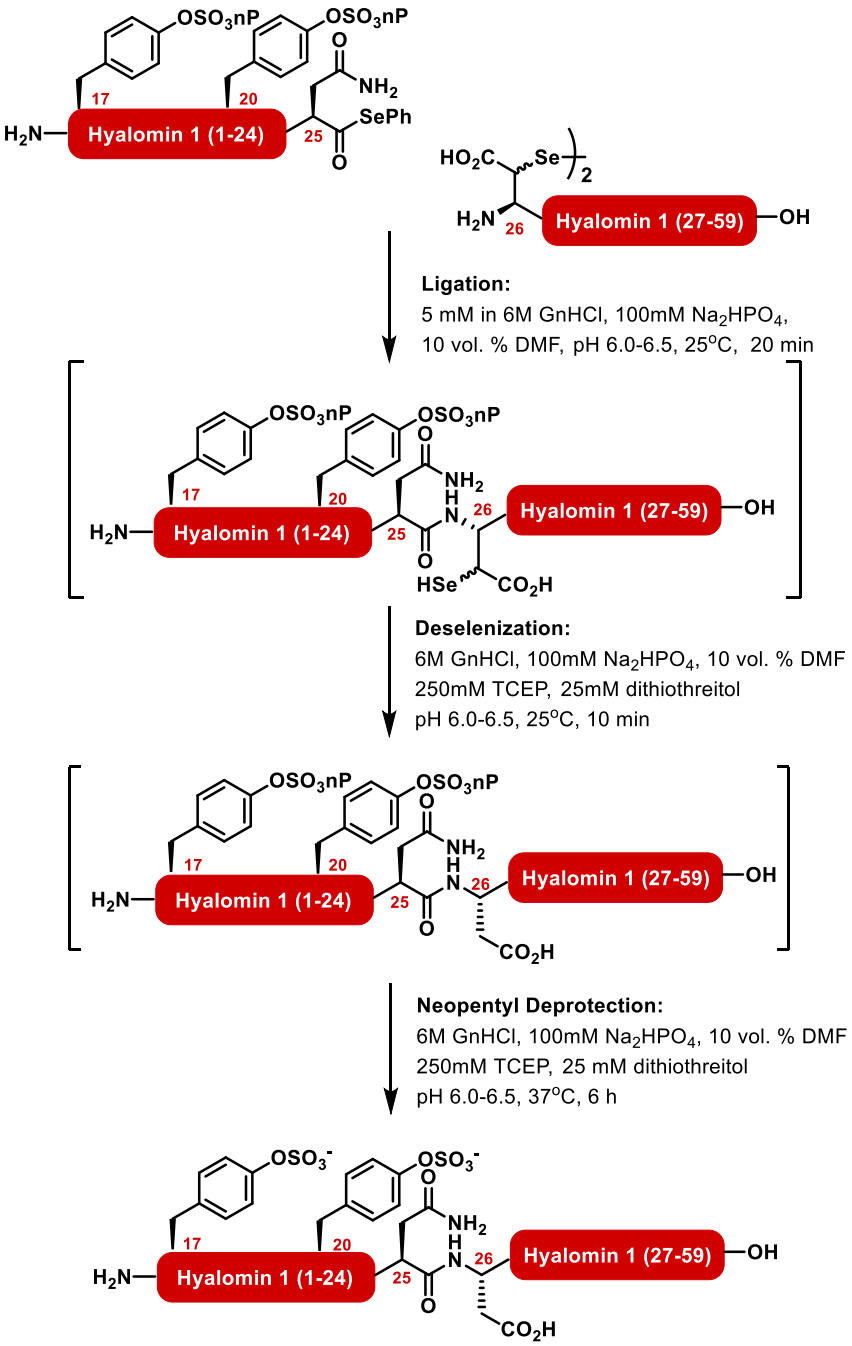


Figure S43: Synthesis of **Hya1 DS** via DSL-deselenization.

The one-pot peptide ligation of **Hya1 DS (1-25)** selenoester (2.7 mg, 0.84 µmol) and **Hya1 (26-59)** diselenide (2.0 mg, 0.56 µmol) followed by *in situ* deselenization was performed according to the general procedure, replacing 10% of the buffer volume with DMF. Neopentyl deprotection was the performed as described in the general procedure, followed by purification via preparative HPLC (0 to 40% B over 40 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **Hya1 DS** as a white solid (1.8 mg, 50%). Analytical HPLC (purified final product): R_t 4.12 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1637.4 $[M+5H]^{5+}$: 1310.1 $[M+6H]^{6+}$: 1091.9 Mass Found (ESI) 1638.5 $[M+4H]^{4+}$, 1310.8 $[M+5H]^{5+}$, 1092.5 $[M+6H]^{6+}$. High Res (ESI+): calcd for $C_{263}H_{415}N_{81}O_{111}S_2$: $[M+6H]^{6+}$, 1091.81356. Found: 1091.81582 $[M+6H]^{6+}$. Difference: 2.0 ppm.

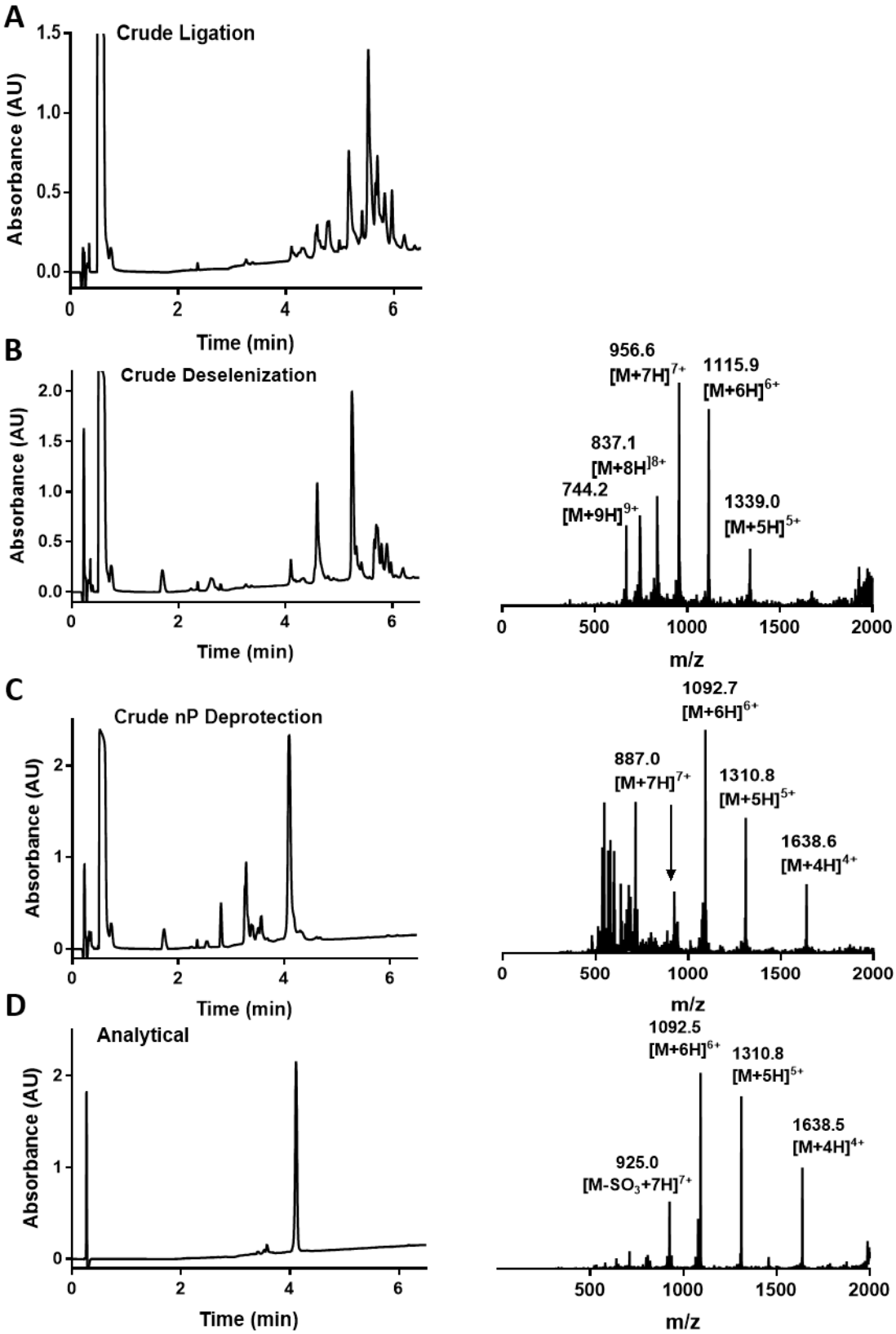


Figure S44: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **Hya 1 DS**.

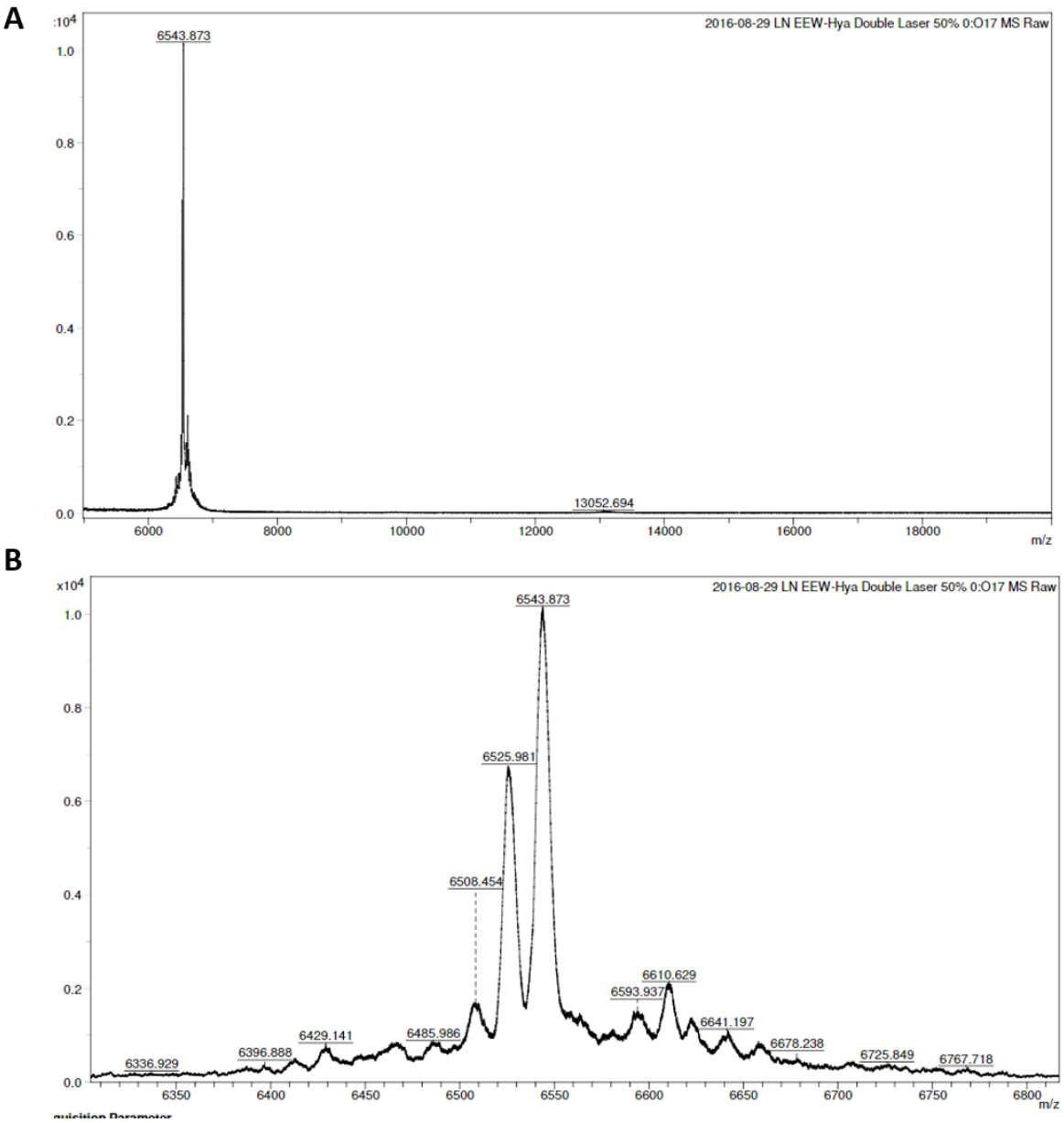


Figure S45: MALDI-TOF mass spectrum of **Hya1 DS** (*A*) 6000-18000 m/z and (*B*) 6350-6800 m/z.

Hya2 Y16

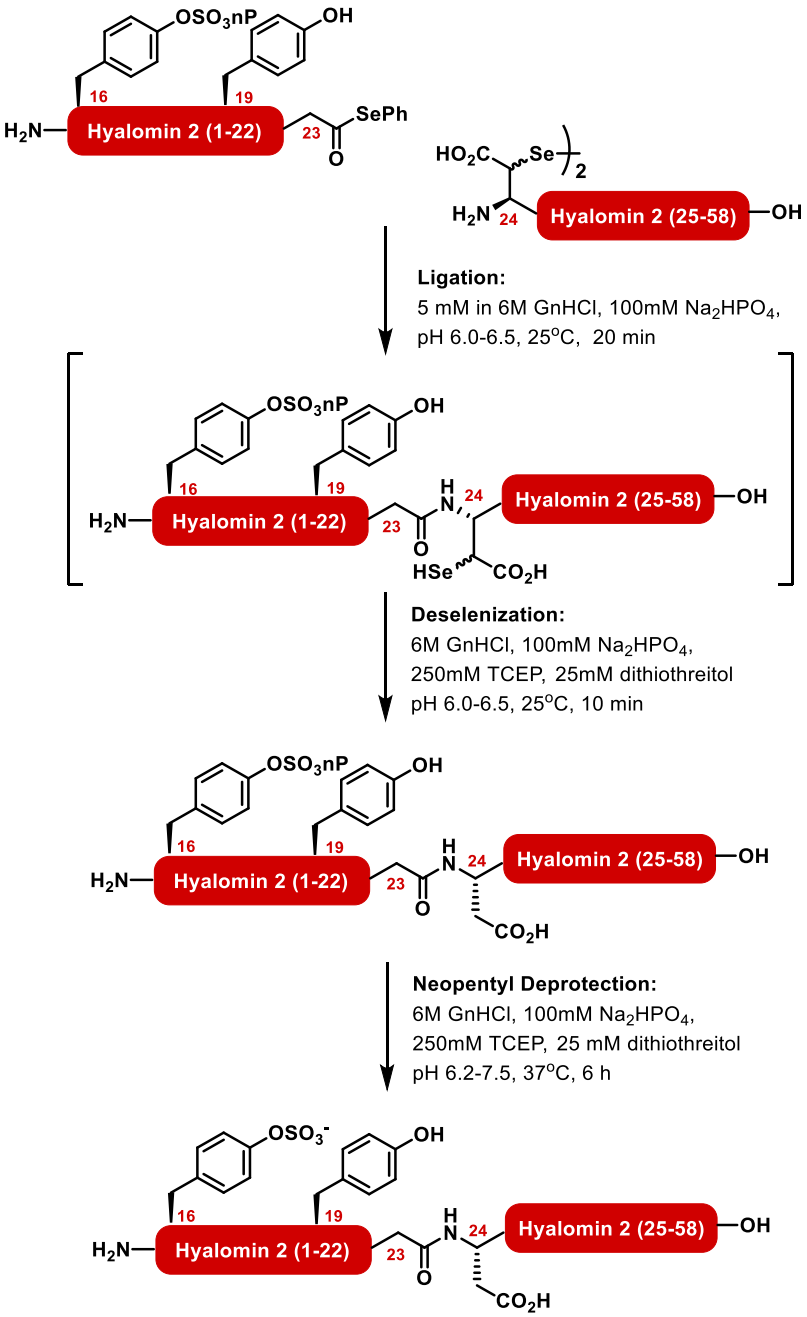


Figure S46: Synthesis of **Hya2 Y16** via DSL-deselenization.

The one-pot peptide ligation of **Hya2 Y16 (1-23)** selenoester (1.9 mg, 0.63 µmol) and **Hya2 (24-58)** diselenide (1.0 mg, 0.25 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0 to 50% B over 30 min, 0.1% v/v TFA followed by lyophilization afforded the neopentyl protected **Hya2 Y16** as a white solid (0.8 mg, 50 %). Neopentyl deprotection was then performed as described in the general procedure, followed by purification via preparative HPLC (0 to 50% B over 30 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **Hya2 Y16** as a white solid (0.4 mg, 88%). Analytical HPLC (purified final product): R_t 4.18 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1642.2, $[M+5H]^{5+}$: 1313.9, $[M+6H]^{6+}$: 1095.1, Mass Found (ESI) 1643.2 $[M+4H]^{4+}$, 1314.8 $[M+5H]^{5+}$, 1095.8 $[M+6H]^{6+}$. High Res (ESI+): calcd for $C_{265}H_{401}N_{78}O_{112}S_3$: $[M+5H]^{5+}$, 1313.34592. Found: 1313.34740 $[M+5H]^{5+}$. Difference: 1.0 ppm.

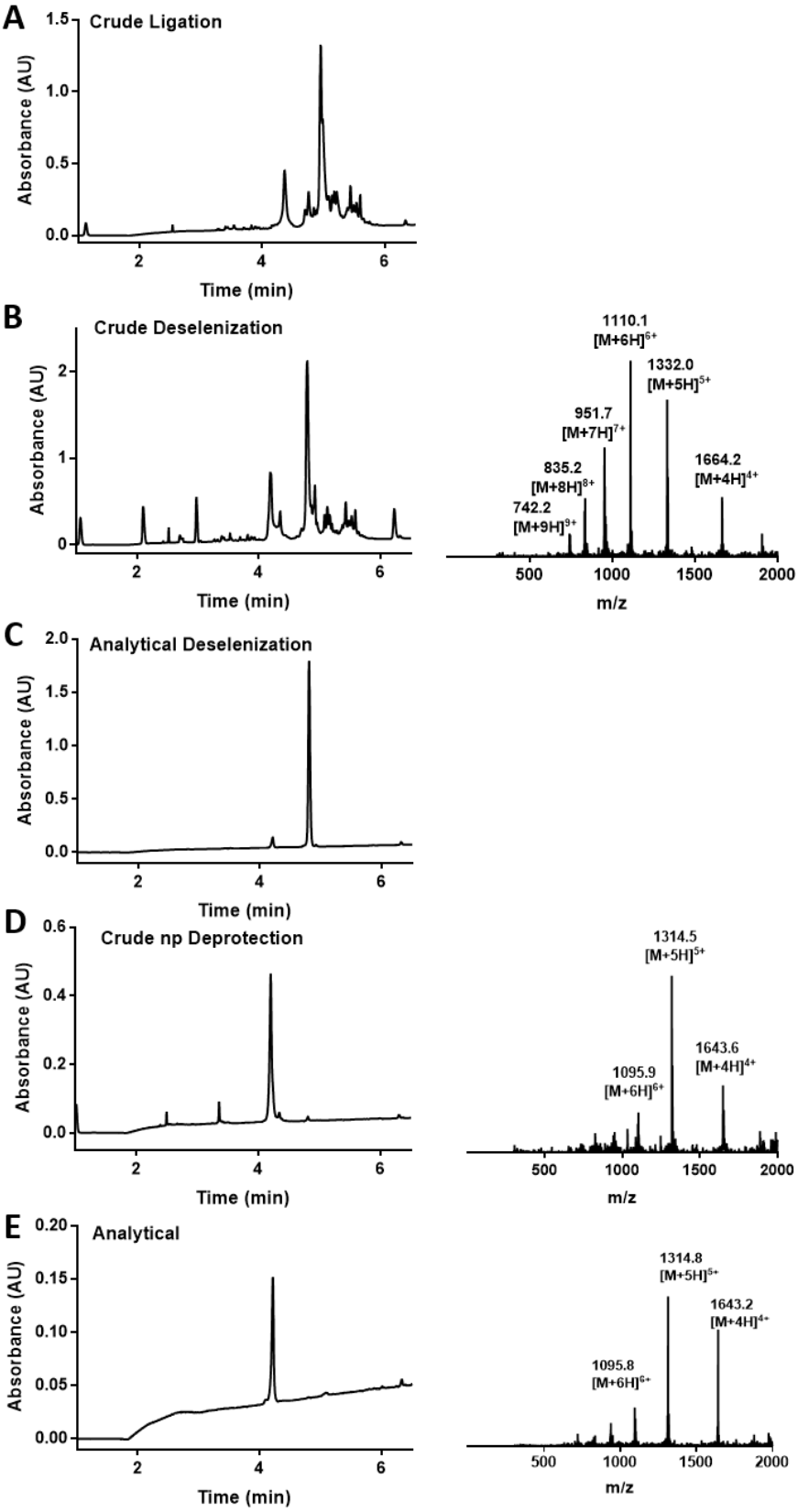


Figure S47: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) analytical deselenization, (*D*) crude nP deprotection and (*E*) purified **Hya2 Y16**.

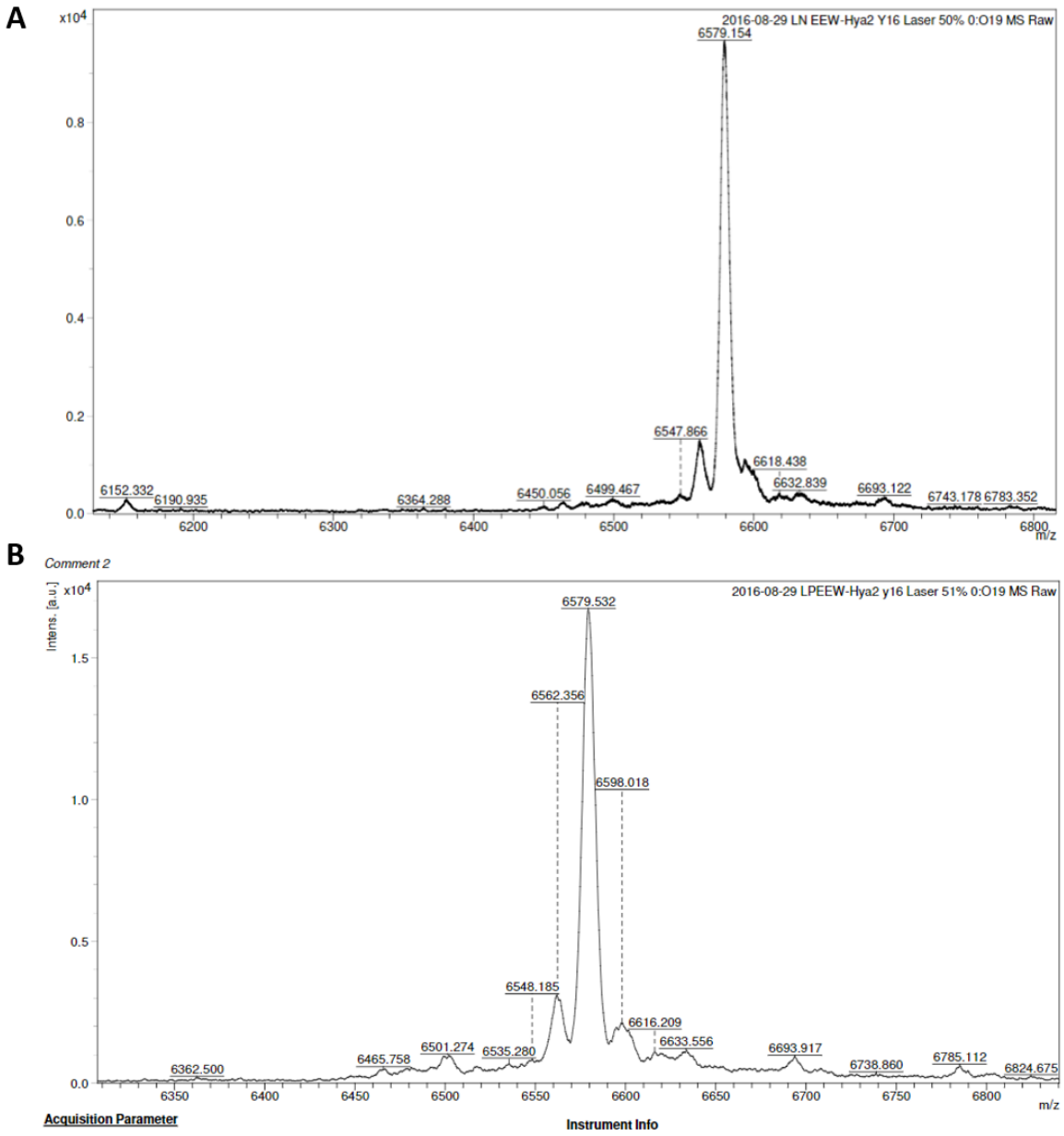


Figure S48: MALDI-TOF mass spectrum of **Hya2 Y16** (*A*) 6200-6800 m/z and (*B*) 6350-6800 m/z.

*Due to the oxidative cleavage conditions required for PMB removal in C-terminal fragment, M55 was oxidized, thus the C-terminal fragment was isolated as the M(S=O) form and this was submitted to the subsequent ligations. Due to the acid lability of the tyrosine sulfates, previously reported reductive conditions (1) were not compatible with these substrates. However, partial reduction of the M(S=O) back to M(S-H) by TCEP was observed during the neopentyl deprotection step. At best a 1:1 mixture of oxidized:reduced material could be obtained and reduction could not be prevented. Yields are therefore quoted for the combined pure masses of the oxidized and reduced material, each of which were isolated separately. Reduced material was submitted for all mass spectrometry studies, analytical traces and biological evaluation.

Hya2 Y19

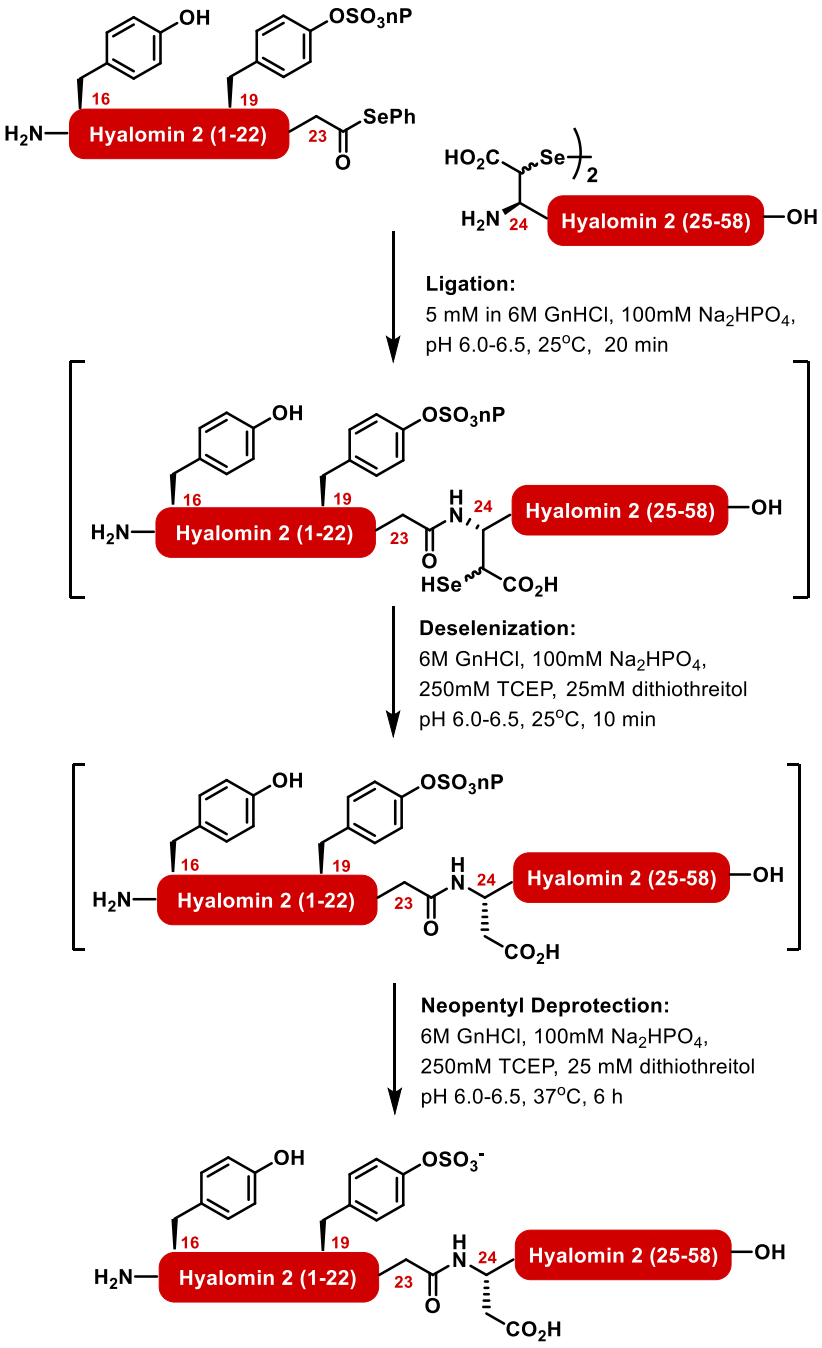


Figure S49: Synthesis of **Hya2 Y19** via DSL-deselenization.

The one-pot peptide ligation of **Hya2 Y19 (1-23)** selenoester (3.5 mg, 1.21 µmol) and **Hya2 (24-58)** diselenide (1.9 mg, 0.48 µmol) followed by *in situ* deselenization was performed according to the general procedure. Neopentyl deprotection was then performed as described in the general procedure, followed by purification via preparative HPLC (0 to 50% B over 30 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **Hya2 Y19** as a white solid (2.0 mg, 65%). Analytical HPLC (purified final product): R_t 4.18 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1642.2, $[M+5H]^{5+}$: 1313.9, $[M+6H]^{6+}$: 1095.1, $[M+7H]^{7+}$: 938.8, Mass Found (ESI) 1643.3 $[M+4H]^{4+}$, 1314.8 $[M+5H]^{5+}$, 1095.7 $[M+6H]^{6+}$, 939.1 $[M+7H]^{7+}$. High Res (ESI+): calcd for $C_{265}H_{401}N_{78}O_{112}S_3Na$: $[M+5H+Na]^{6+}$, 1098.28658. Found: 1098.28678 $[M+5H]^{5+}$. Difference: 0.2 ppm.

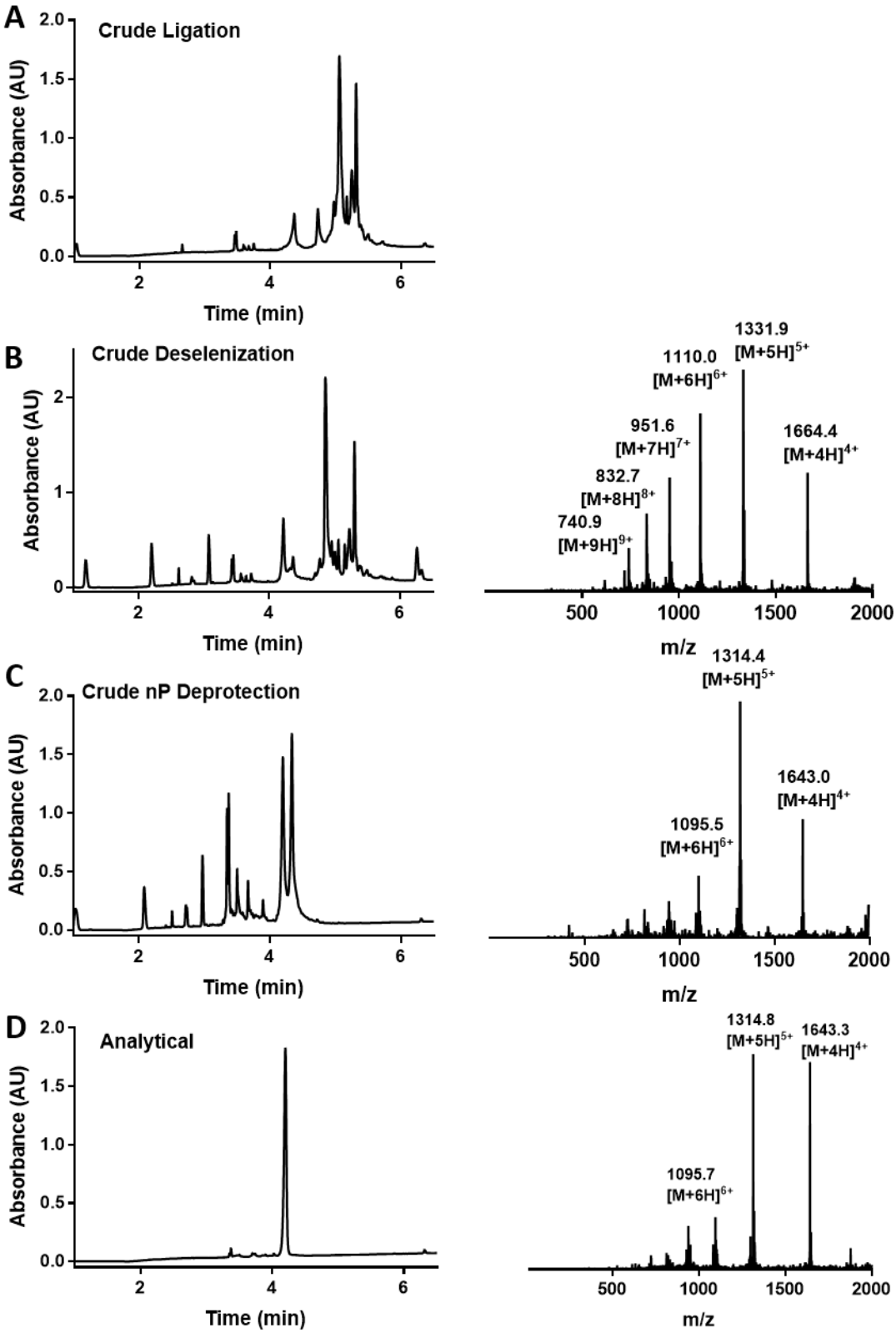


Figure S50: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **Hya2 Y19**.

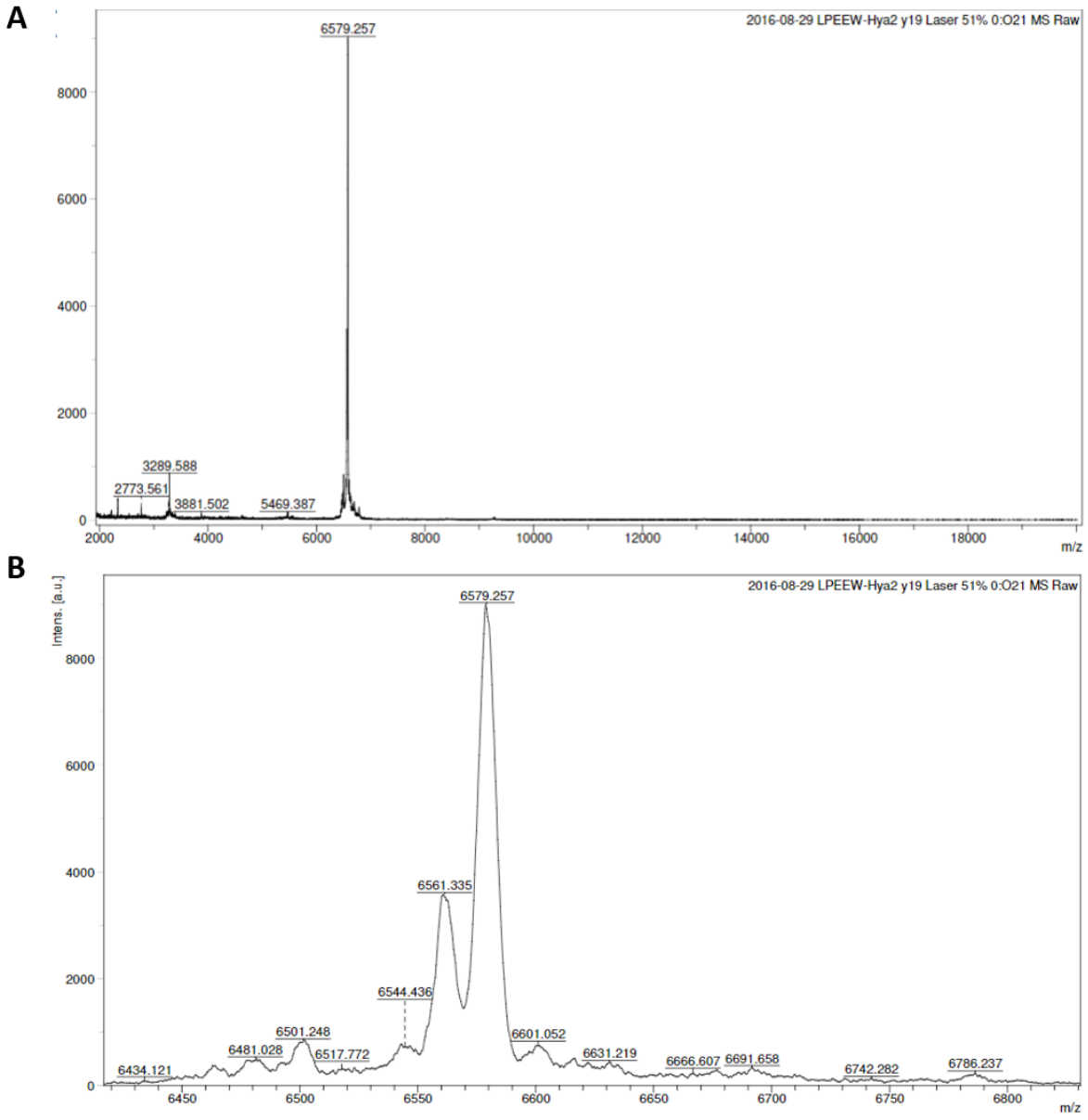


Figure S51: MALDI-TOF mass spectrum of **Hya2 Y19** (*A*) 2000-20000 m/z and (*B*) 6450-6800 m/z.

Hya2 DS

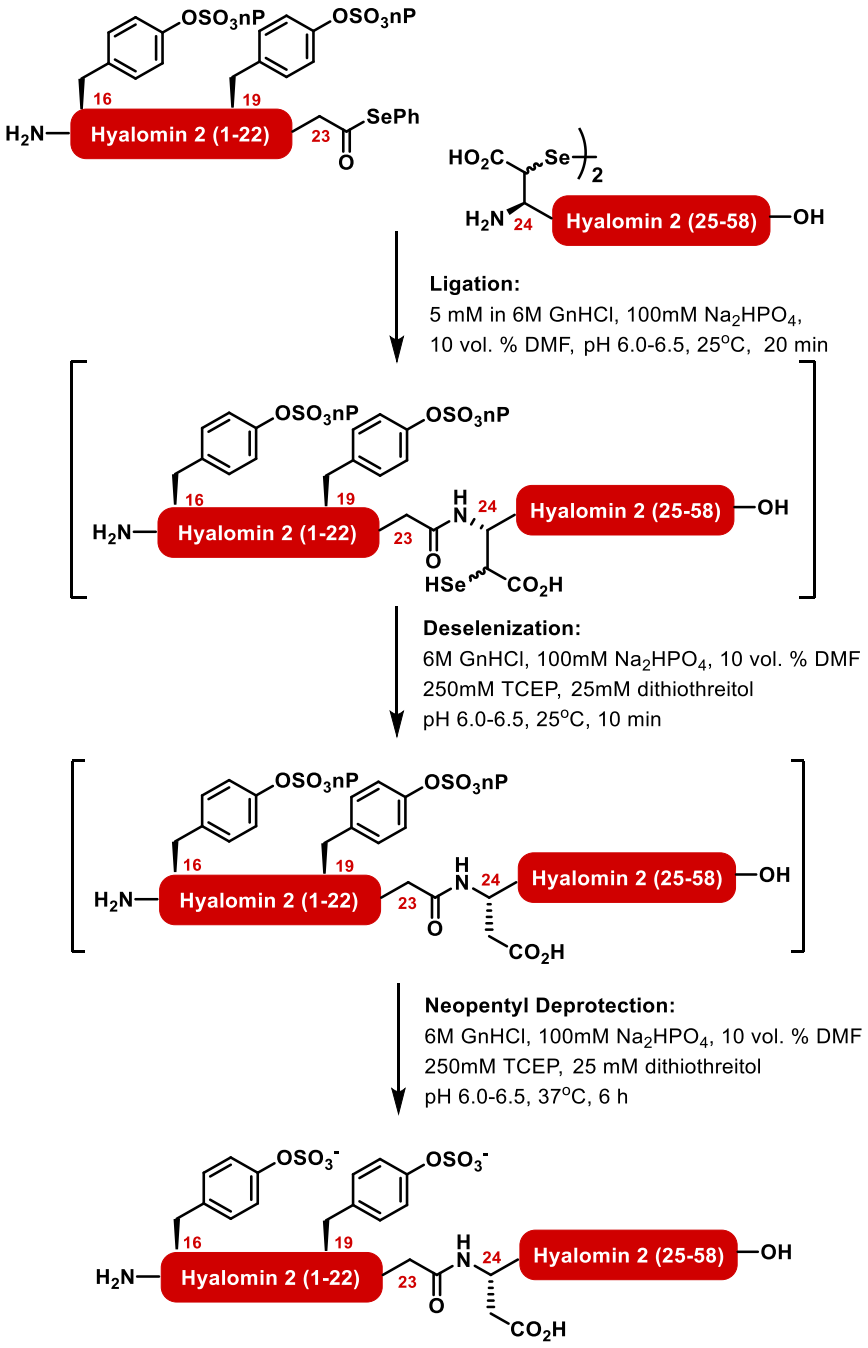


Figure S52: Synthesis of **Hya2 DS** via DSL-deselenization.

The one-pot peptide ligation of **Hya2 DS (1-23)** selenoester (3.9 mg, 1.26 µmol) and **Hya2 (24-58)** diselenide (2.0 mg, 0.51 µmol) followed by *in situ* deselenization was performed according to the general procedure, replacing 10% of the buffer volume with DMF. Neopentyl deprotection was the performed as described in the general procedure, followed by purification via preparative HPLC (0 to 50% B over 30 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **Hya2 DS** as a white solid (1.7 mg, 51%). Analytical HPLC (purified final product): R_t 4.17 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1662.2, $[M+5H]^{5+}$: 1330.0, $[M+6H]^{6+}$: 1095.3 (one sulfate off), Mass Found (ESI) 1663.2 $[M+4H]^{4+}$, 1330.5 $[M+5H]^{5+}$, 1095.5 $[M+6H]^{6+}$. High Res (ESI+): calcd for $C_{265}H_{402}N_{78}O_{115}S_4$: $[M+4H]^{4+}$, 1661.92354. Found: 1661.92469 $[M+4H]^{4+}$. Difference: 0.7 ppm.

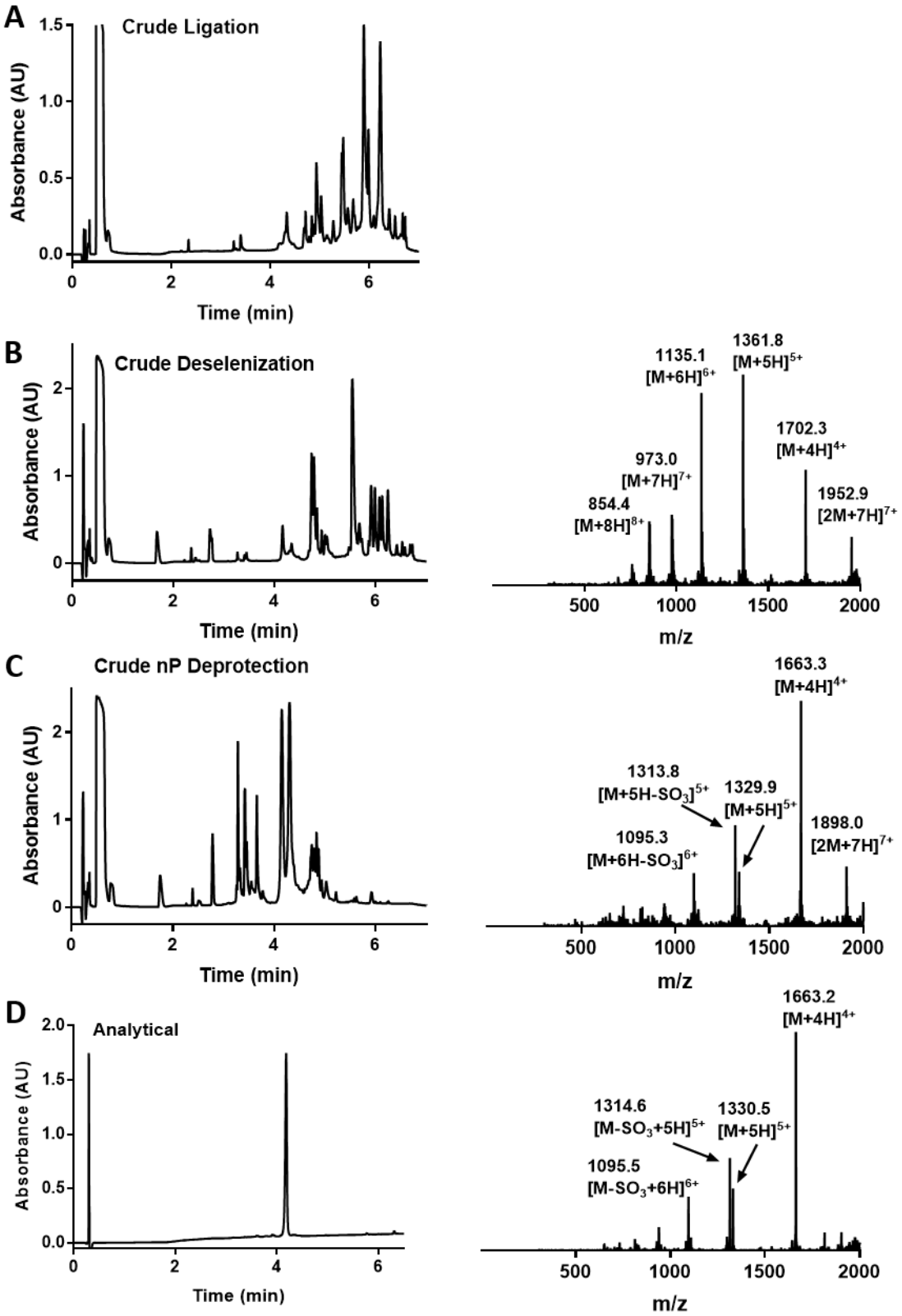


Figure S53: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **Hya2 DS**.

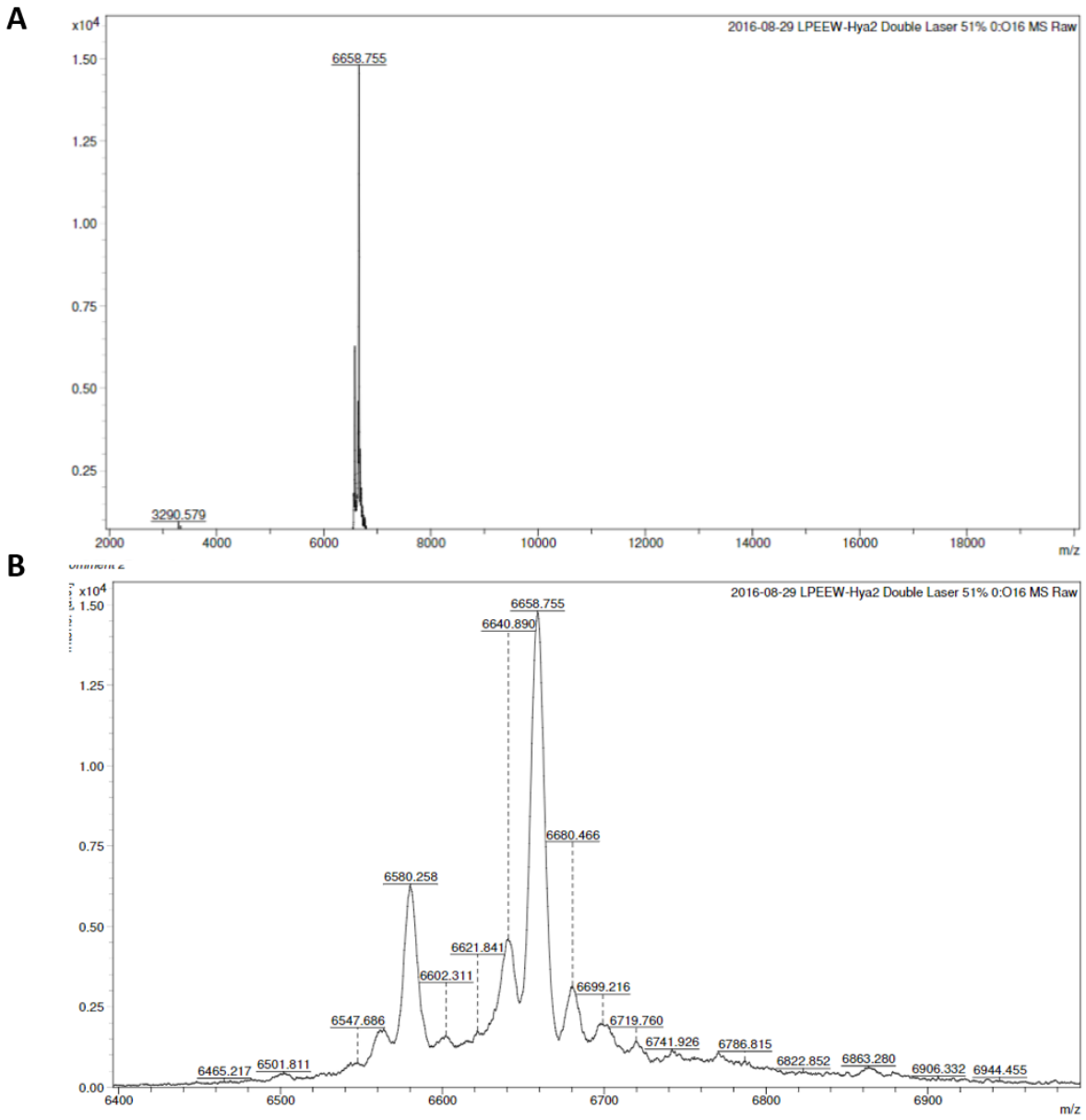


Figure S54: MALDI-TOF mass spectrum of **Hya2 DS** (*A*) 2000-20000 m/z and (*B*) 6400-6900 m/z.

Hya3 Y17

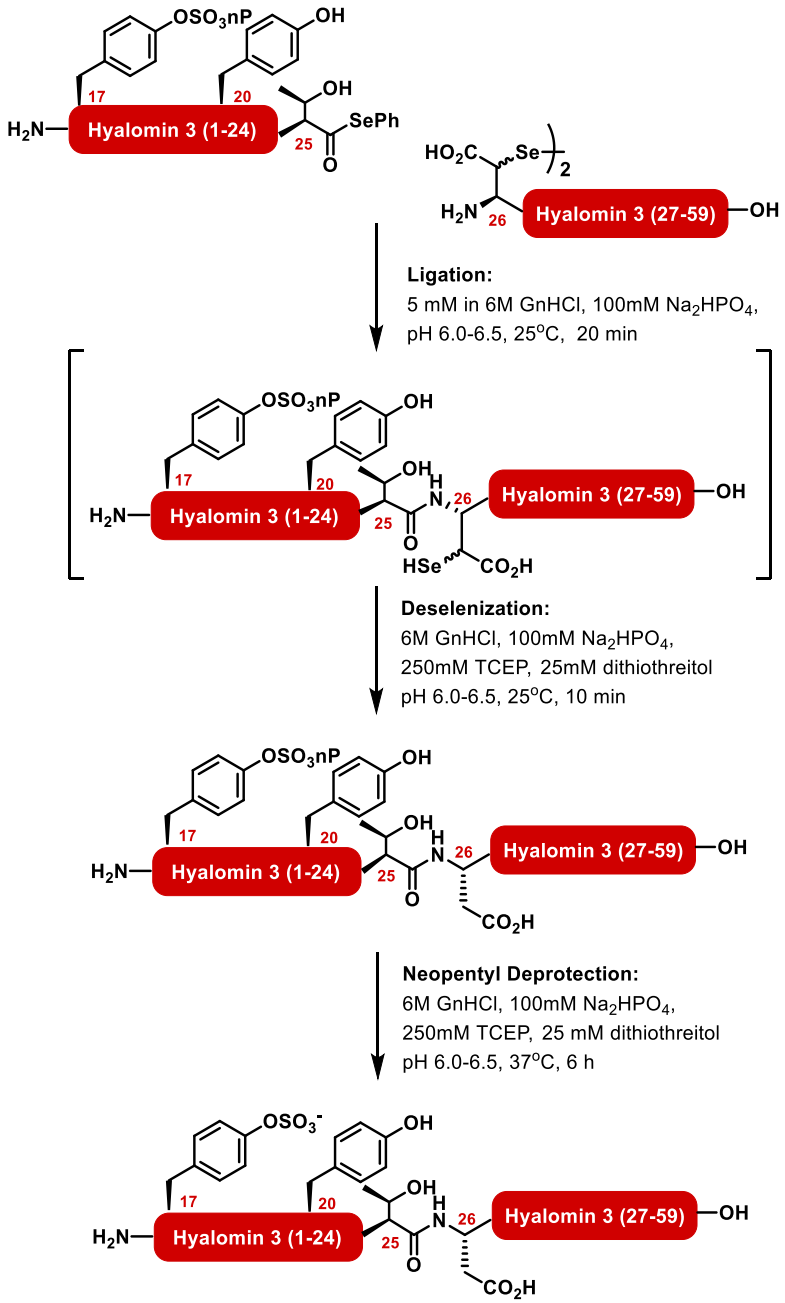


Figure S55: Synthesis of **Hya3 Y17** via DSL-deselenization.

The one-pot peptide ligation of **Hya3 Y17 (1-25)** selenoester (3.4 mg, 1.12 µmol) and **Hya3 (26-59)** diselenide (1.2 mg, 0.35 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0 to 50% B over 30 min, 0.1% v/v TFA) followed by lyophilization afforded the neopentyl protected **Hya3 Y17** as a white solid (1.1 mg, 50 %). Neopentyl deprotection was the performed as described in the general procedure, followed by purification via preparative HPLC (0 to 5% B over 30 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **Hya3 Y17** as a white solid (0.8 mg, 69%). Analytical HPLC (purified final product): R_t 4.08 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1539.5 $[M+5H]^{5+}$: 1231.8 $[M+6H]^{6+}$: 1026.7 Mass Found (ESI) 1540.3 $[M+4H]^{4+}$, 1232.5 $[M+5H]^{5+}$, 1027.2 $[M+6H]^{6+}$. High Res (ESI+): calcd for $C_{251}H_{388}N_{74}O_{105}S$: $[M+6H]^{6+}$, 1026.62585 Found: 1026.62678 $[M+6H]^{6+}$. Difference: 0.9 ppm.

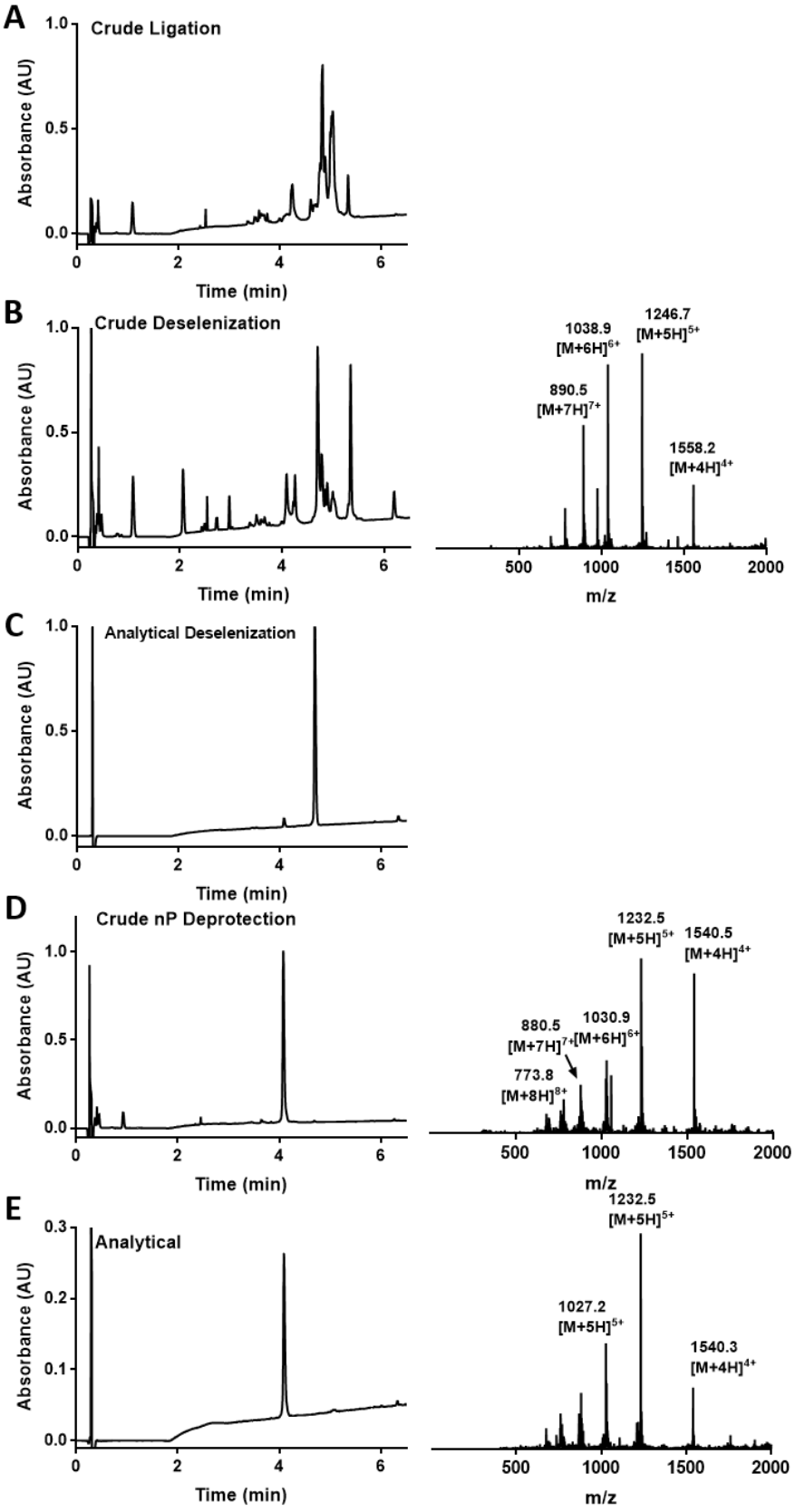


Figure S56: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) Analytical deselenization, (*D*) crude nP deprotection and (*E*) purified **Hya3 Y17**.

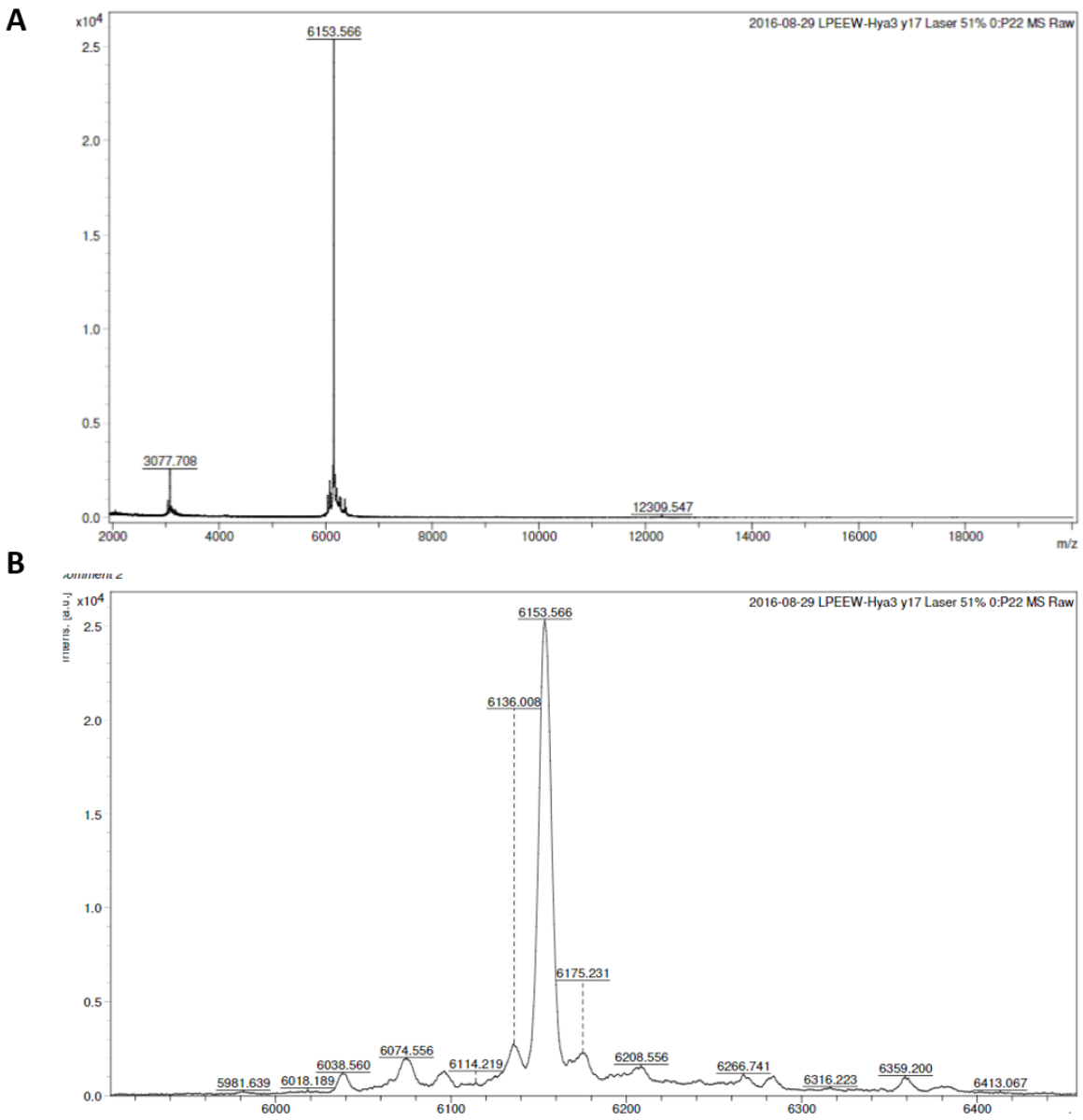


Figure S57: MALDI-TOF mass spectrum of **Hya3 Y17** (*A*) 2000-20000 m/z and (*B*) 6000-6400 m/z.

Hya3 Y20

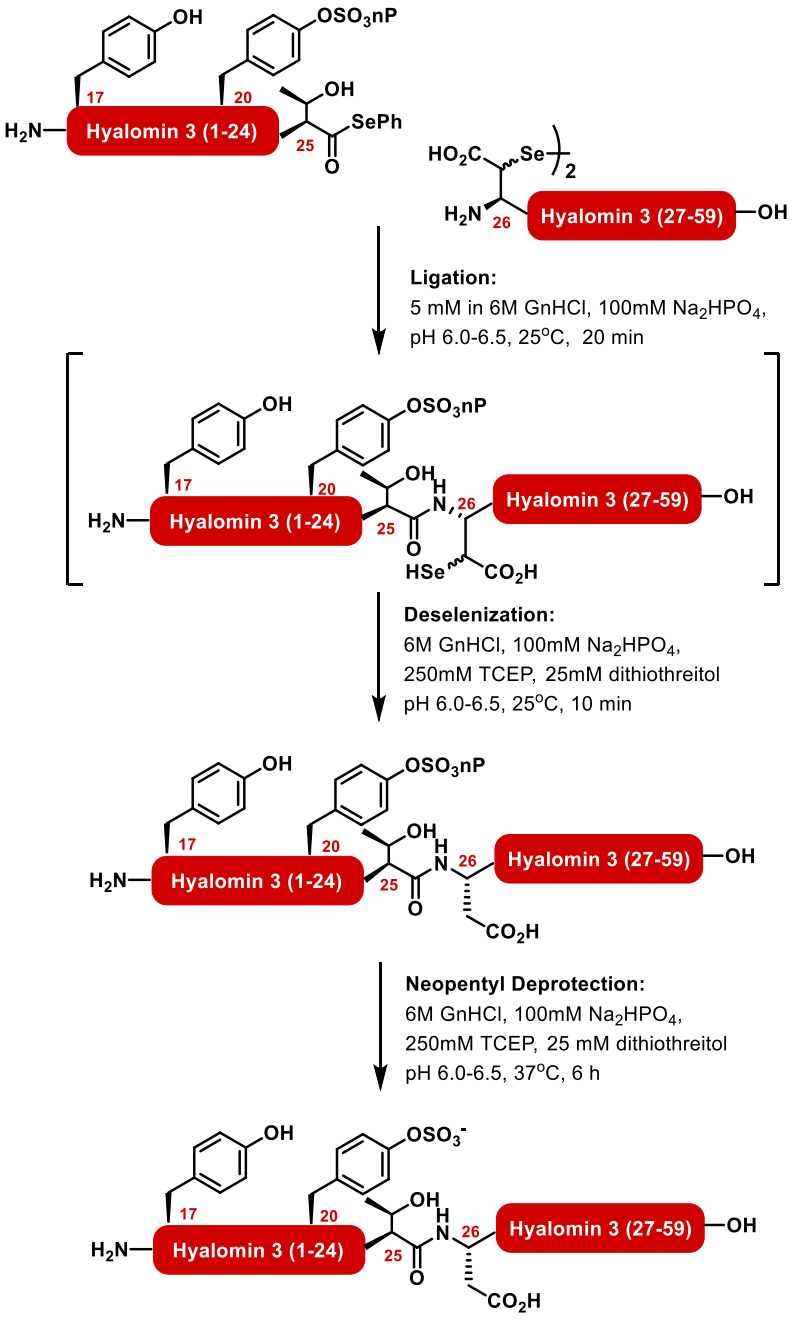


Figure S58: Synthesis of **Hya3 Y20** via DSL-deselenization.

The one-pot peptide ligation of **Hya3 Y20 (1-25)** selenoester (3.0 mg, 1.0 µmol) and **Hya3 (26-59)** diselenide (1.7 mg, 0.50 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0 to 50% B over 30 min, 0.1% v/v TFA) followed by lyophilization afforded the neopentyl protected protein **Hya3 Y20** as a white solid (1.0 mg, 50 %). Neopentyl deprotection was then performed as described in the general procedure, followed by purification via preparative HPLC (0 to 50% B over 30 min, 0.1% v/v Formic acid) followed by lyophilization afforded **Hya3 Y20** as a white solid (0.64 mg, 65 %). Analytical HPLC (purified final product): R_t 4.08 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1539.5 $[M+5H]^{5+}$: 1231.8 $[M+6H]^{6+}$: 1026.7 Mass Found (ESI) 1540.5 $[M+4H]^{4+}$, 1232.5 $[M+5H]^{5+}$, 1027.2 $[M+6H]^{6+}$. High Res (ESI+): calcd for $C_{251}H_{388}N_{74}O_{105}S$: $[M+5H]^{5+}$, 1231.74957. Found: 1231.75075 $[M+5H]^{5+}$. Difference: 1.0 ppm.

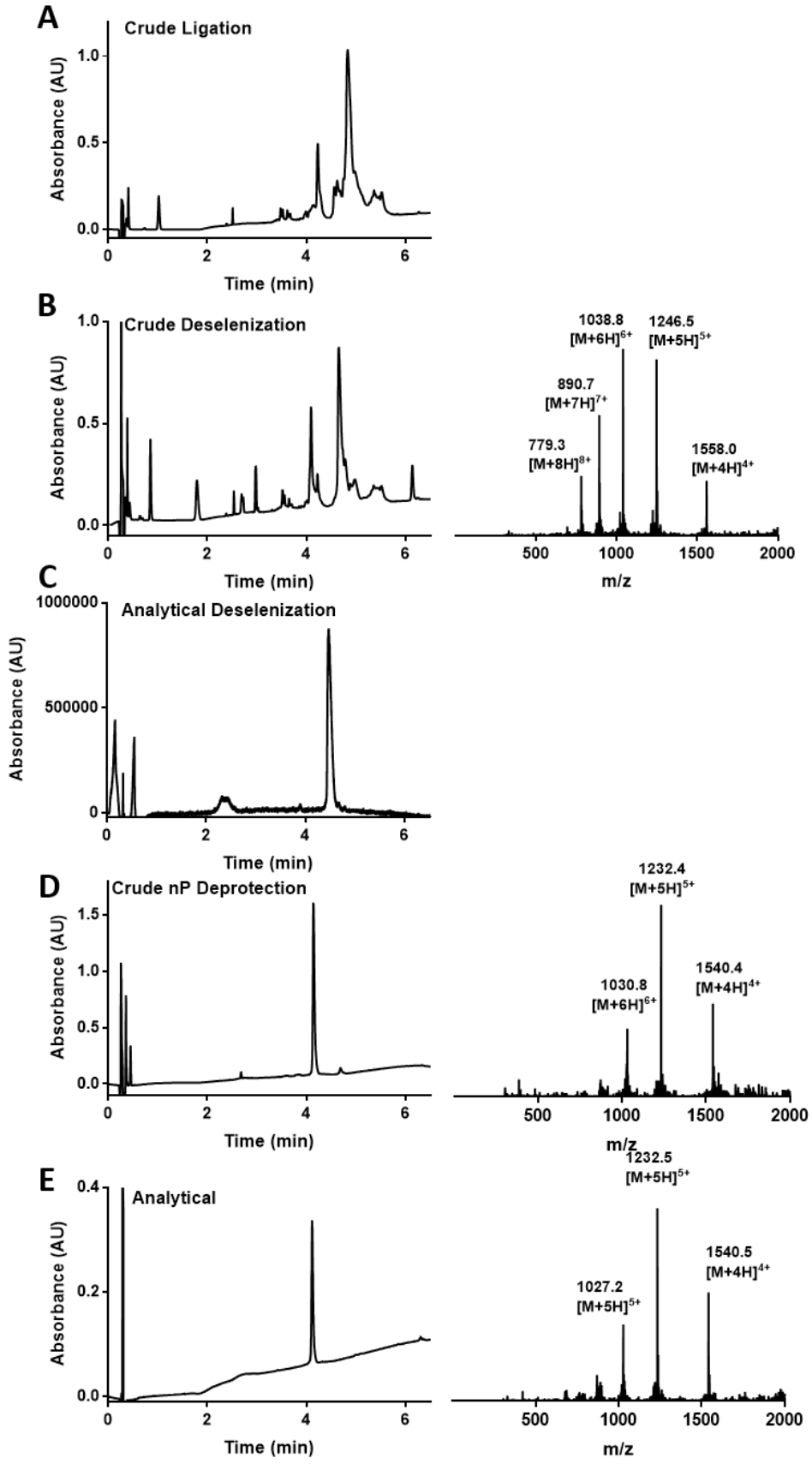


Figure S59: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) Analytical deselenization, (*D*) crude nP deprotection and (*E*) purified **Hya3 Y20**.

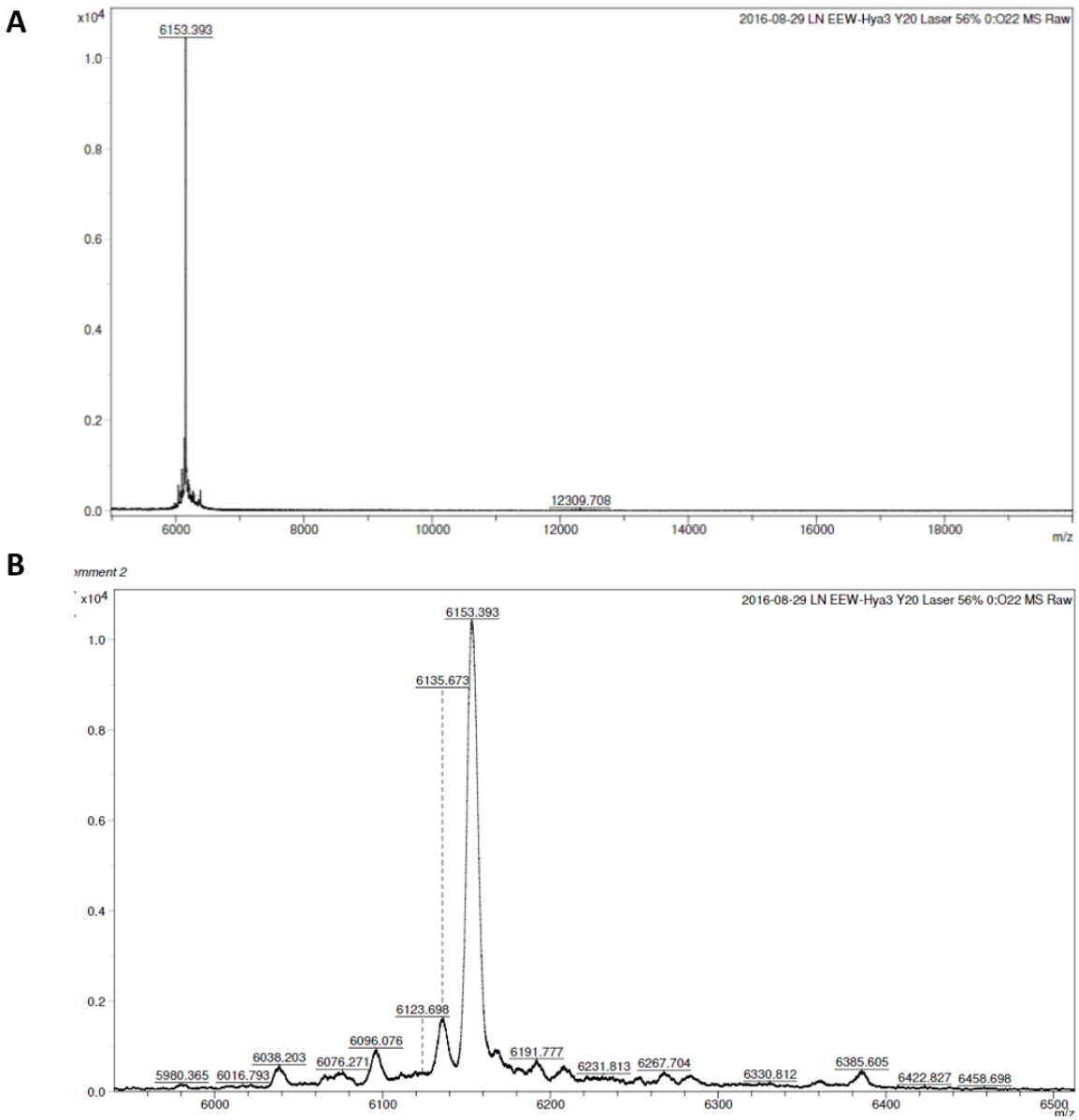


Figure S60: MALDI-TOF mass spectrum of **Hya3 Y20** (*A*) 6000-20000 m/z and (*B*) 6000-6500 m/z.

Hya3 DS

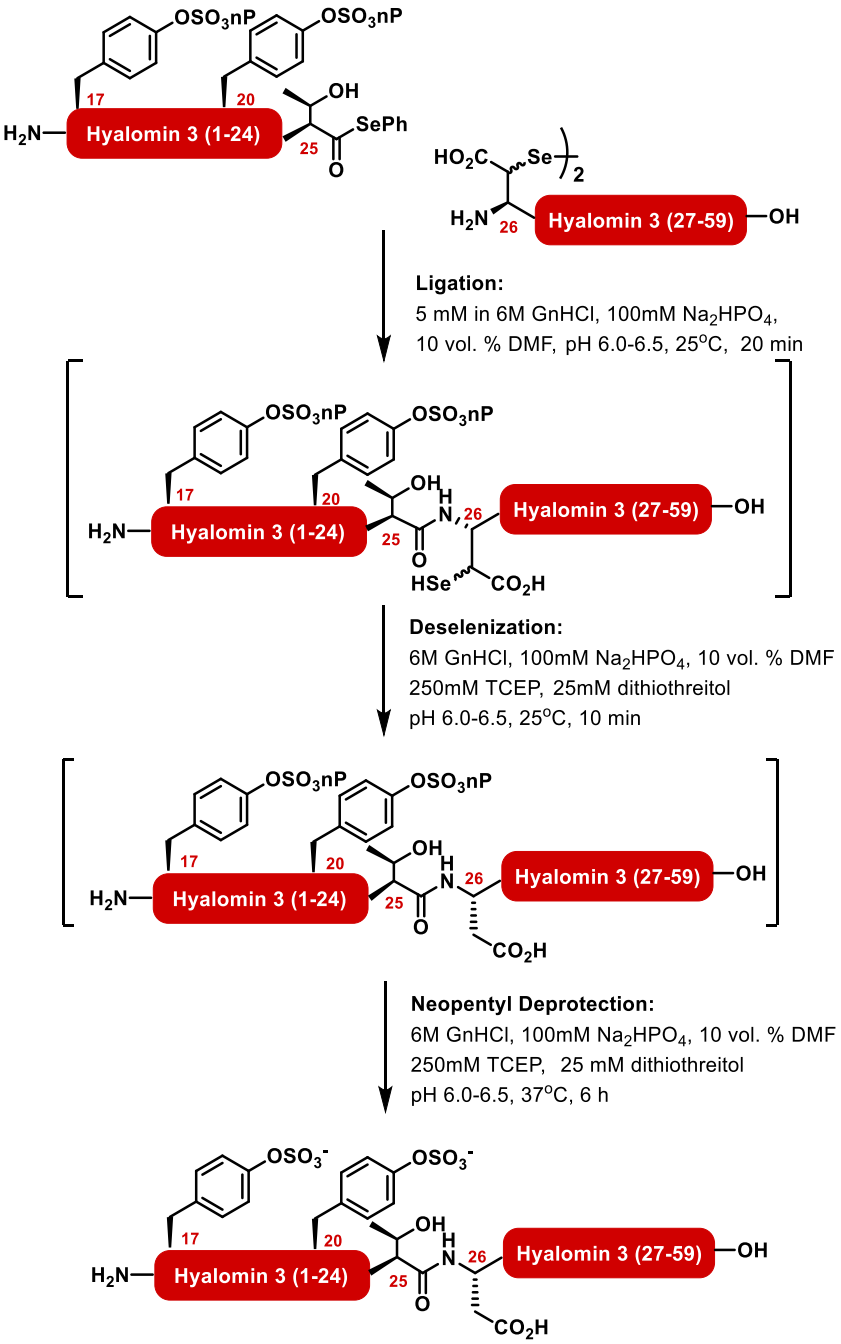


Figure S61: Synthesis of **Hya3 DS** via DSL-deselenization.

The one-pot peptide ligation of **Hya3 DS (1-25)** selenoester (3.8 mg, 1.18 µmol) and **Hya3 (26-59)** diselenide (2.0 mg, 0.59 µmol) followed by *in situ* deselenization was performed according to the general procedure, replacing 10% of the buffer volume with DMF. Neopentyl deprotection was then performed as described in the general procedure, followed by purification via preparative HPLC (0 to 50% B over 30 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **Hya3 DS** as a white solid (1.9 mg, 52%). Analytical HPLC (purified final product): R_t 4.07 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M-6H]^{6-}$: 1038.1 $[M-7H]^{7-}$: 889.6 $[M-8H]^{8-}$: 778.3 $[M-9H]^{9-}$: 691.7 Mass Found (ESI) 1038.5 $[M-6H]^{6-}$, 890.0 $[M-7H]^{7-}$, 778.6 $[M-8H]^{8-}$, 691.7 $[M-9H]^{9-}$. High Res (ESI+): calcd for $C_{251}H_{388}N_{74}O_{108}S_2$: $[M+5H]^{5+}$, 1247.74057. Found: 1247.74151 $[M+5H]^{5+}$. Difference: 0.7 ppm.

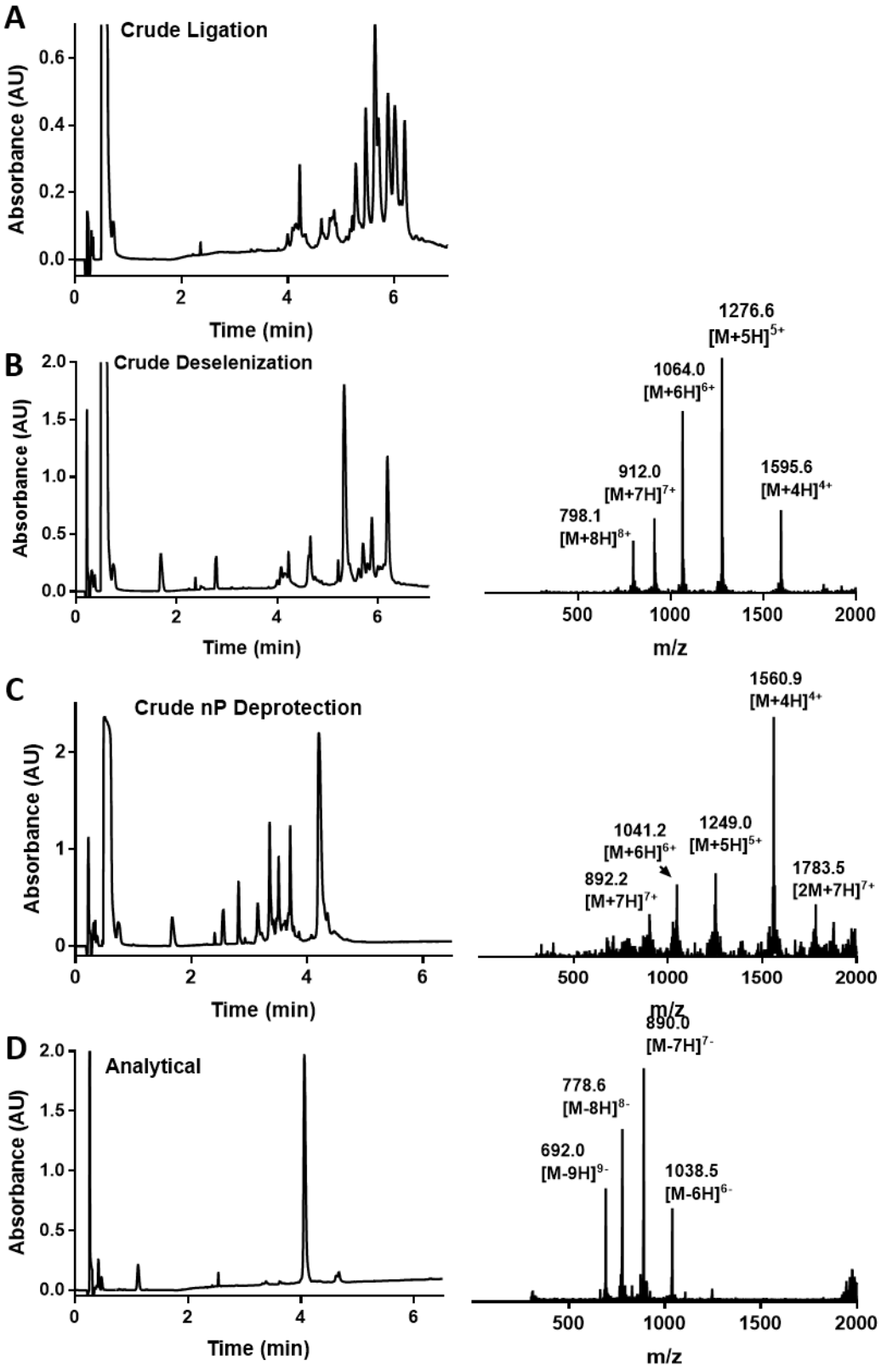


Figure S62: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **Hya3 DS**.

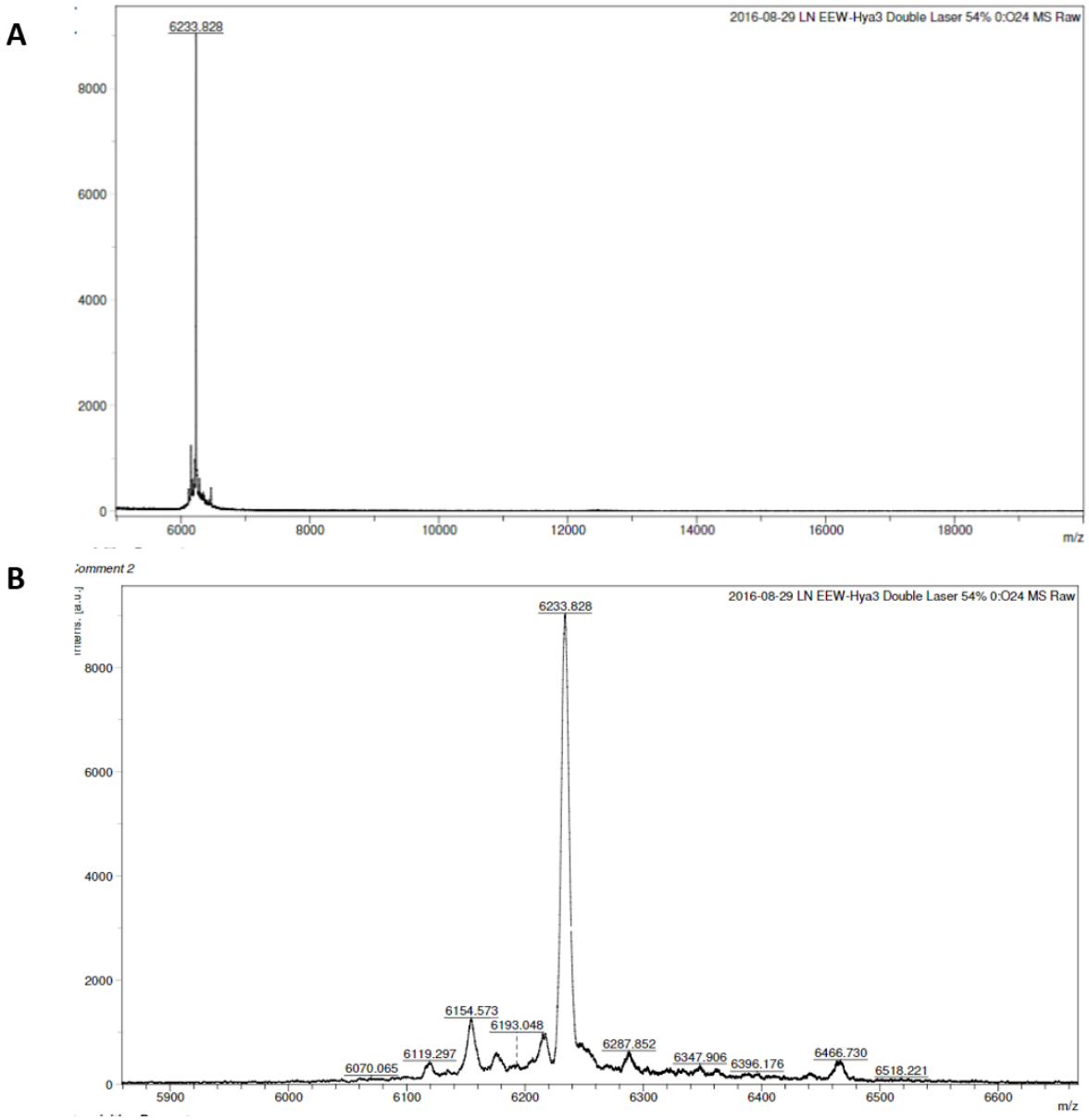


Figure S63: MALDI-TOF mass spectrum of **Hya3 DS** (*A*) 6000-20000 m/z and (*B*) 5900-6600 m/z.

MadL1 Un

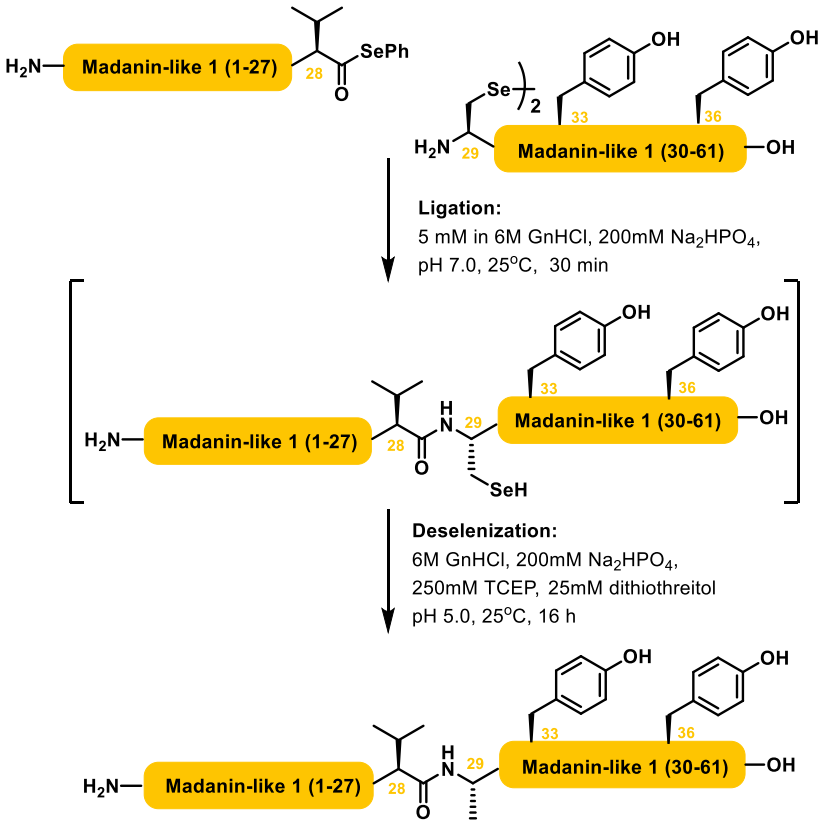


Figure S64: Synthesis of **MadL1 Un** via DSL-deselenization.

The one-pot peptide ligation of **MadL1 (1-28)** selenoester (3.4 mg, 1.01 µmol) and **MadL1 Un (29-61)** diselenide (2.5 mg, 0.68 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0% for 5 min, 0% to 40% B over 40 min, 0.1% v/v TFA) followed by lyophilization afforded final protein **MadL1 Un** as a white solid (6.8 mg, 74%). Analytical HPLC (purified final product): R_t 26.2 min (0 to 50% B over 30 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+5H]^{5+}$ 1360.2 $[M+6H]^{6+}$: 1133.7 $[M+7H]^{7+}$: 971.9 $[M+8H]^{8+}$: 850.5 $[M+9H]^{9+}$: 756.1; Mass Found (ESI) 1360.3 $[M+5H]^{5+}$, 1133.7 $[M+6H]^{6+}$, 971.9 $[M+7H]^{7+}$, 850.4 $[M+8H]^{8+}$, 756.0 $[M+9H]^{9+}$.

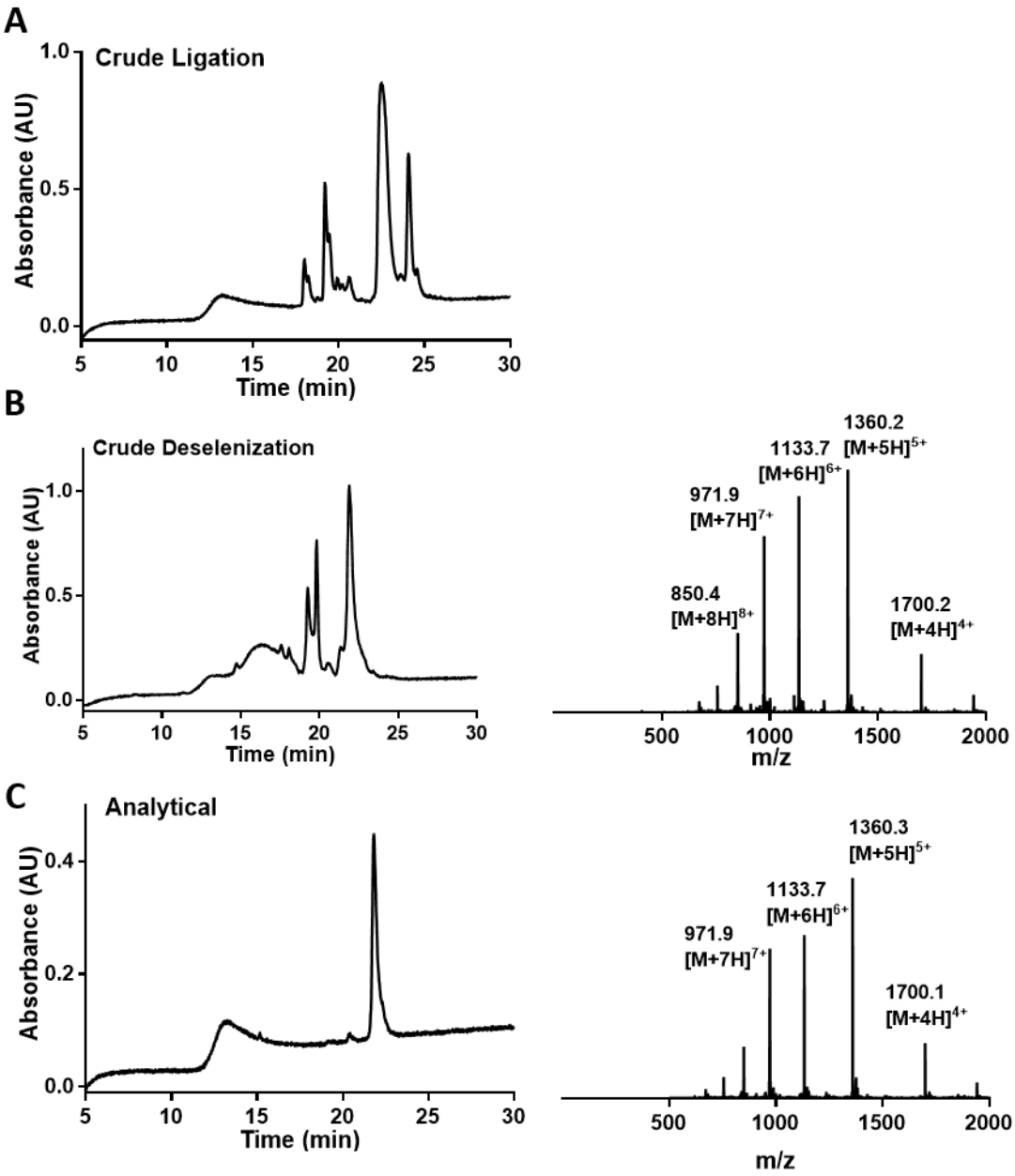


Figure S65: Analytical HPLC trace (0 to 50% B over 30 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization and (*C*) purified **MadL1 Un**.

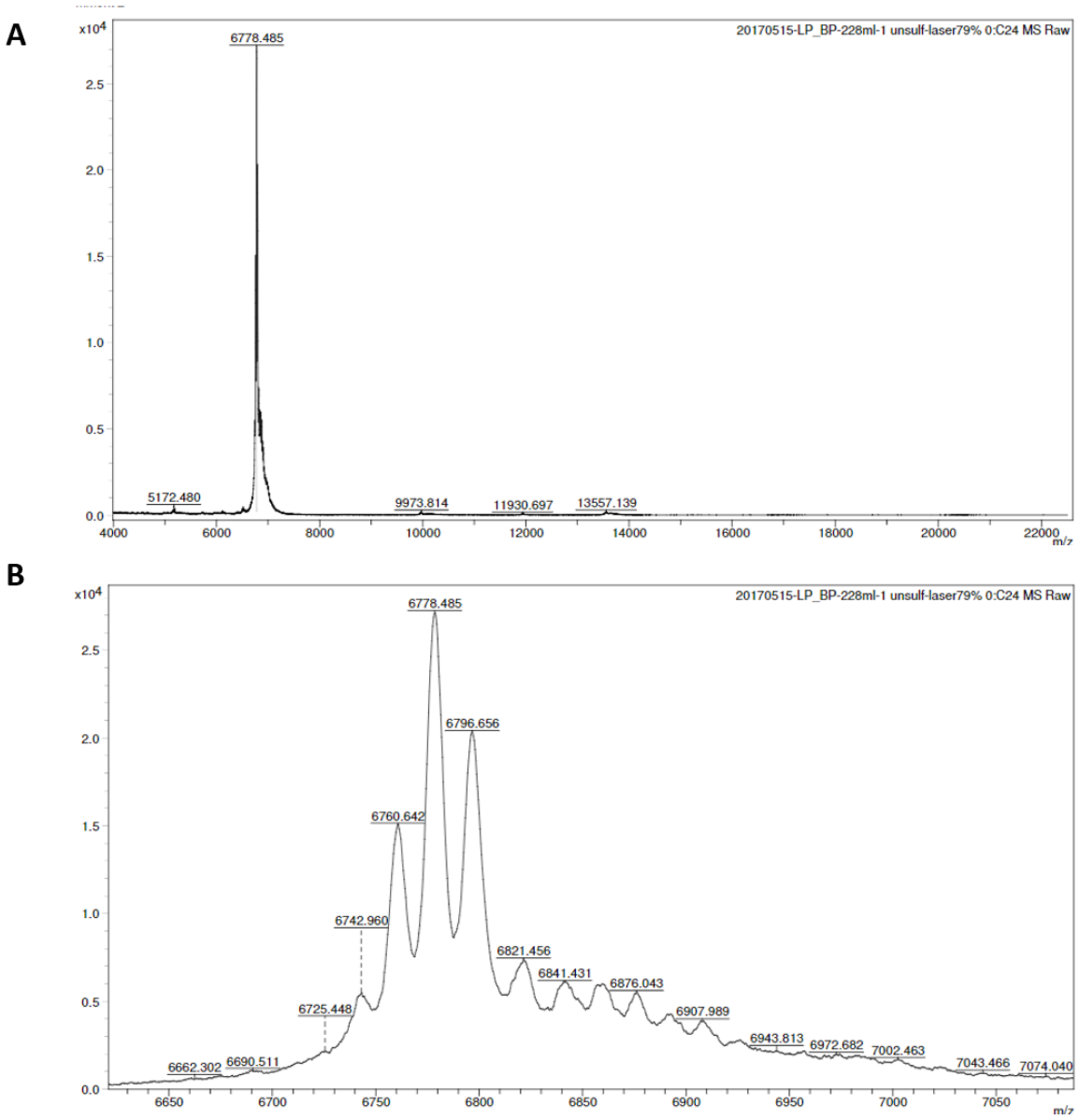


Figure S66: MALDI-TOF mass spectrum of **MadL1 Un** (*A*) 4000-22000 m/z and (*B*) 6650-7050 m/z.

MadL1 Y33

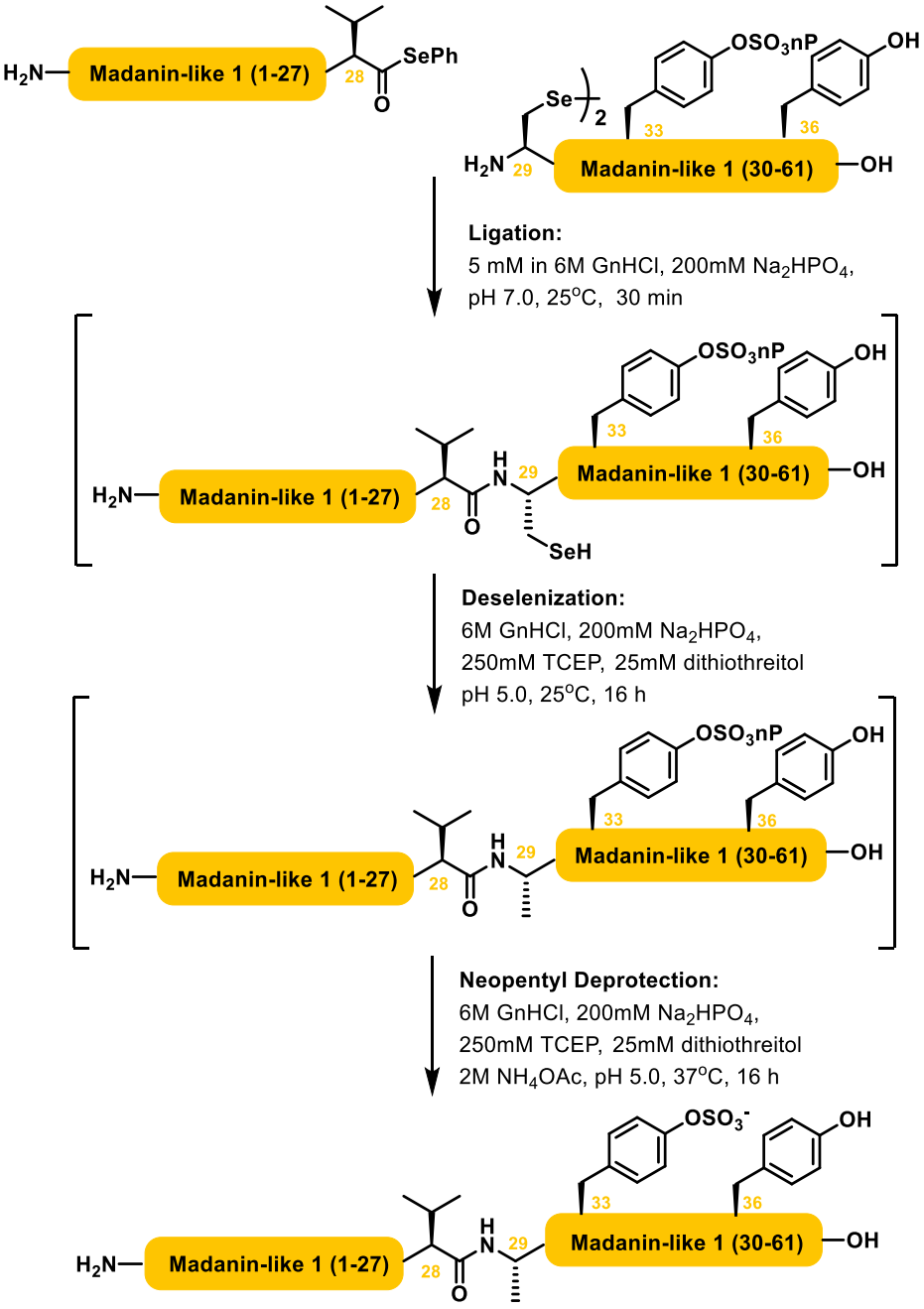


Figure S67: Synthesis of **MadL1 Y33** via DSL-deselenization.

The one-pot peptide ligation of **MadL1 (1-28)** selenoester (4.2 mg, 1.25 µmol) and **MadL1 Y33 (29-61)** diselenide (3.2 mg, 0.84 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0% for 5 min, 0% to 40% B over 40 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **MadL1 Y33** as a white solid (2.3 mg, 53%). Analytical HPLC (purified final product): R_t 26.0 min (0 to 50% B over 30 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1720.0 $[M+5H]^{5+}$: 1376.2 $[M+6H]^{6+}$: 1147.0 Mass Found (ESI) 1720.2 $[M+4H]^{4+}$, 1376.2 $[M+5H]^{5+}$, 1147.1 $[M+6H]^{6+}$.

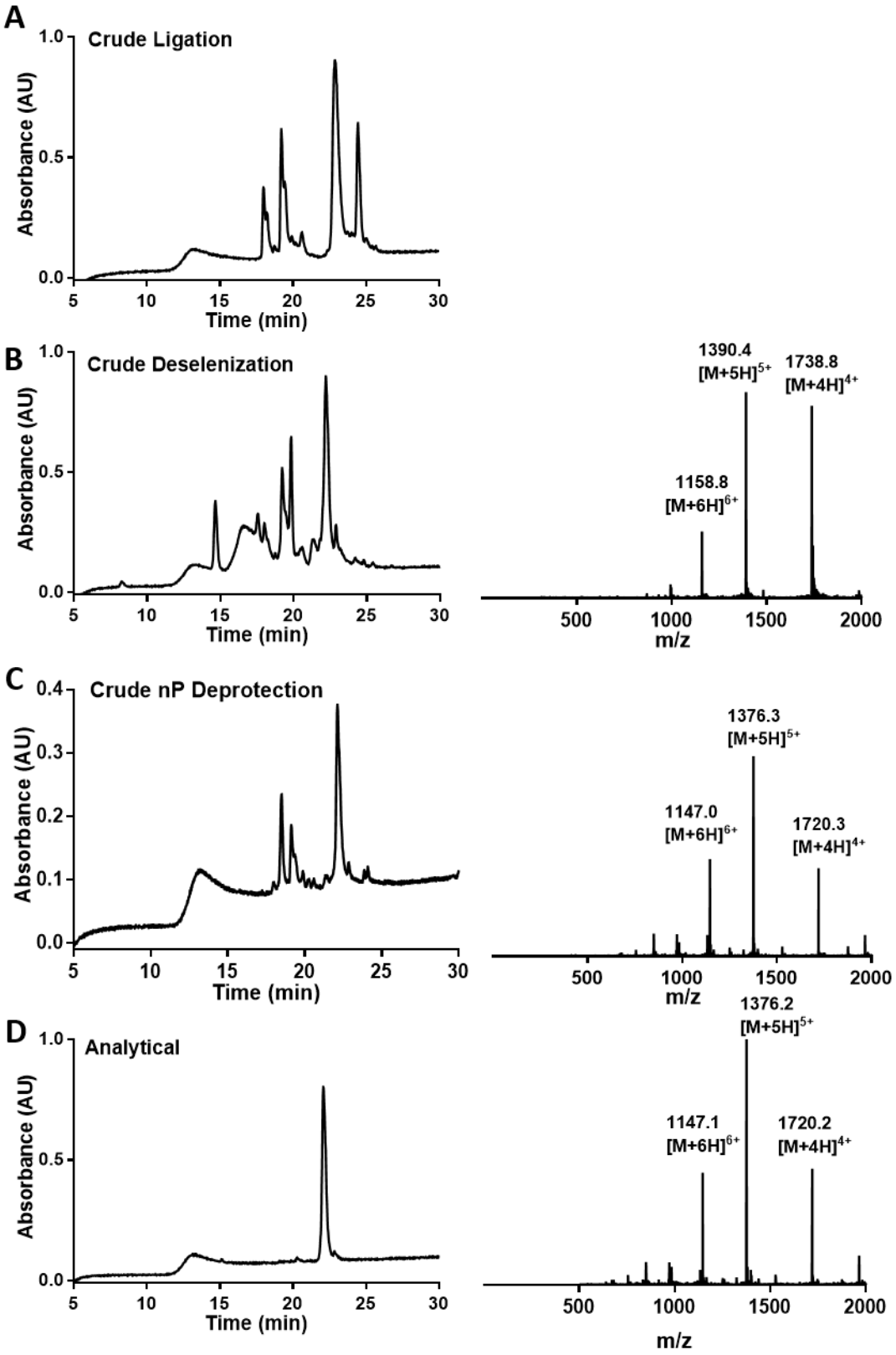


Figure S68: Analytical HPLC trace (0 to 50% B over 30 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **MadL1 Y33**.

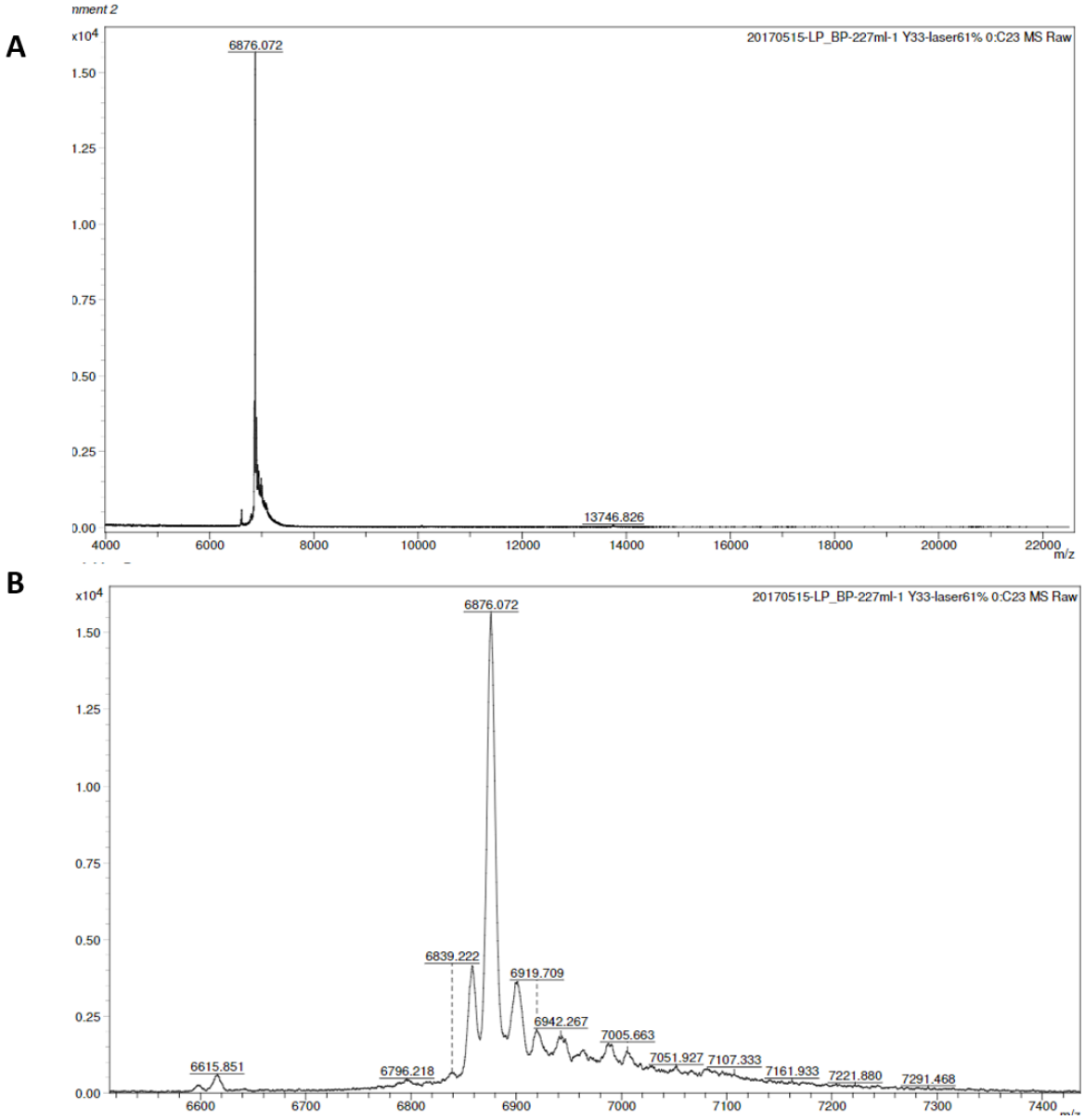


Figure S69: MALDI-TOF mass spectrum of **MadL1 Y33** (*A*) 4000-22000 m/z and (*B*) 6600-7400 m/z.

MadL1 Y36

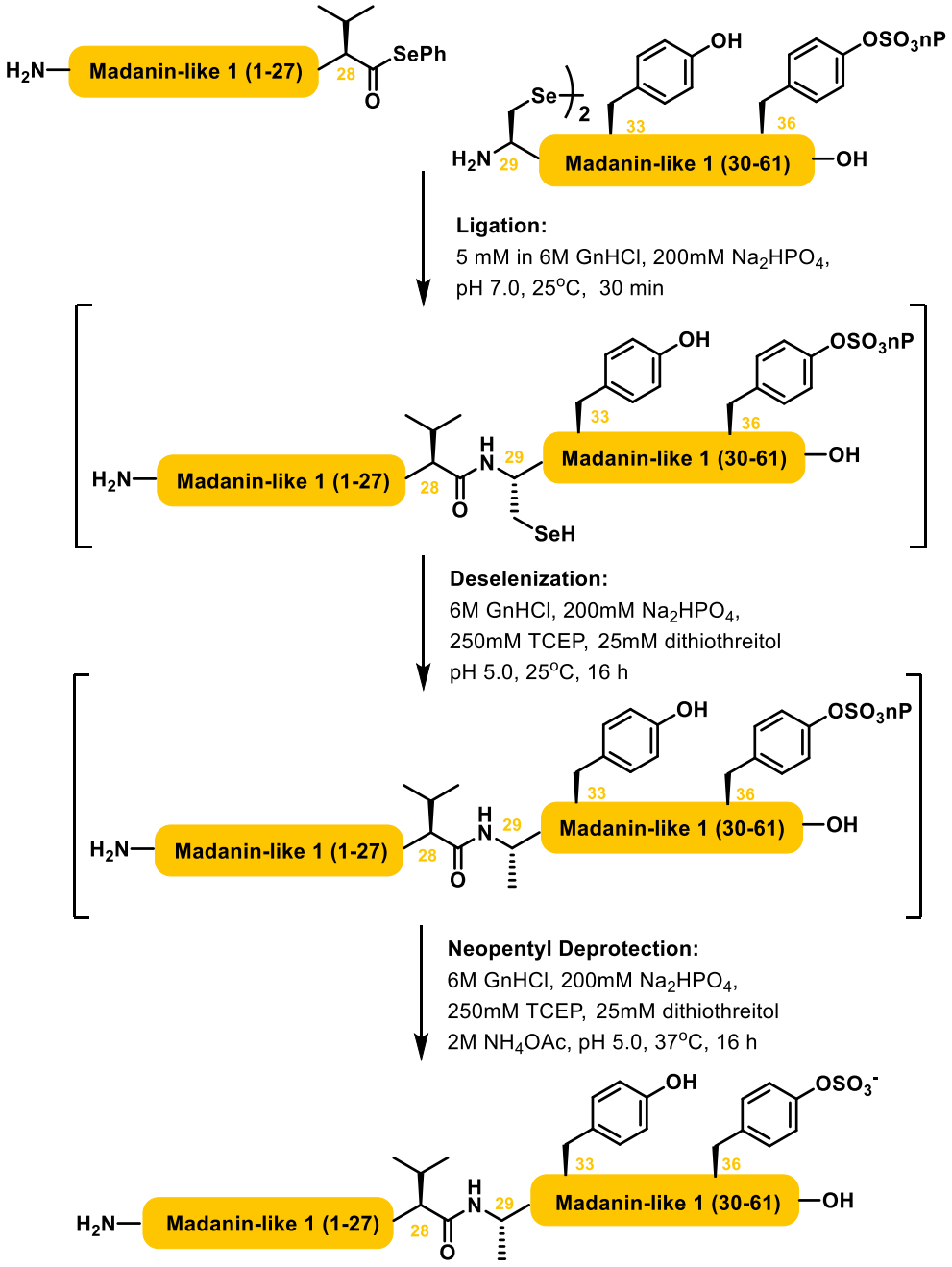


Figure S70: Synthesis of **MadL1 Y36** via DSL-deselenization.

The one-pot peptide ligation of **MadL1 (1-28)** selenoester (4.1 mg, 1.22 µmol) and **MadL1 Y36 (29-61)** diselenide (3.1 mg, 0.81 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0% for 5 min, 0% to 40% B over 40 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **MadL1 Y36** as a white solid (2.1 mg, 61%). Analytical HPLC (purified final product): R_t 26.00 min (0 to 50% B over 30 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1720.0 $[M+5H]^{5+}$: 1376.2 $[M+6H]^{6+}$: 1147.0 Mass Found (ESI) 1720.2 $[M+4H]^{4+}$, 1376.3 $[M+5H]^{5+}$, 1147.2 $[M+6H]^{6+}$.

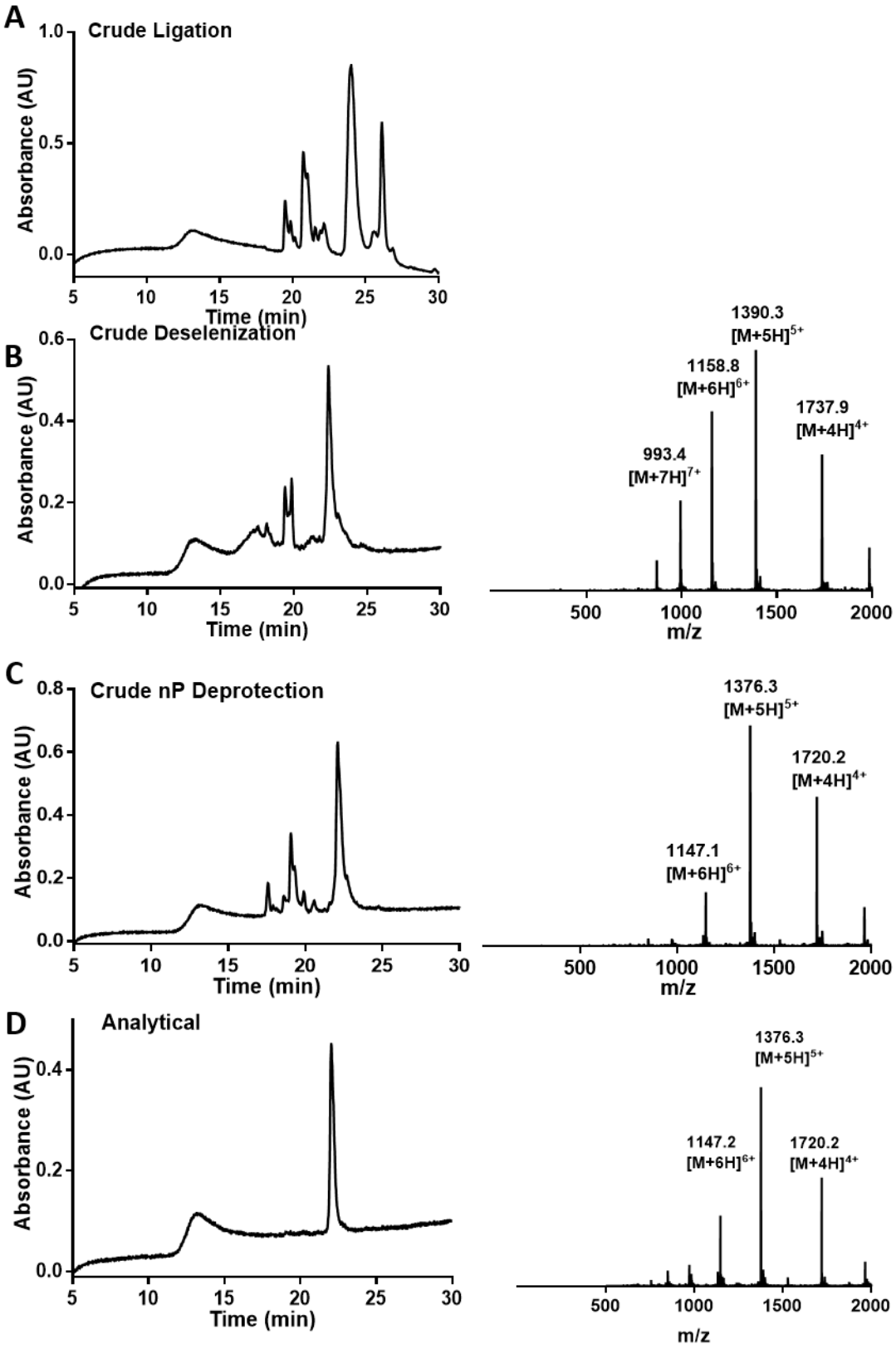


Figure S71: Analytical HPLC trace (0 to 50% B over 30 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **MadL1 Y36**.

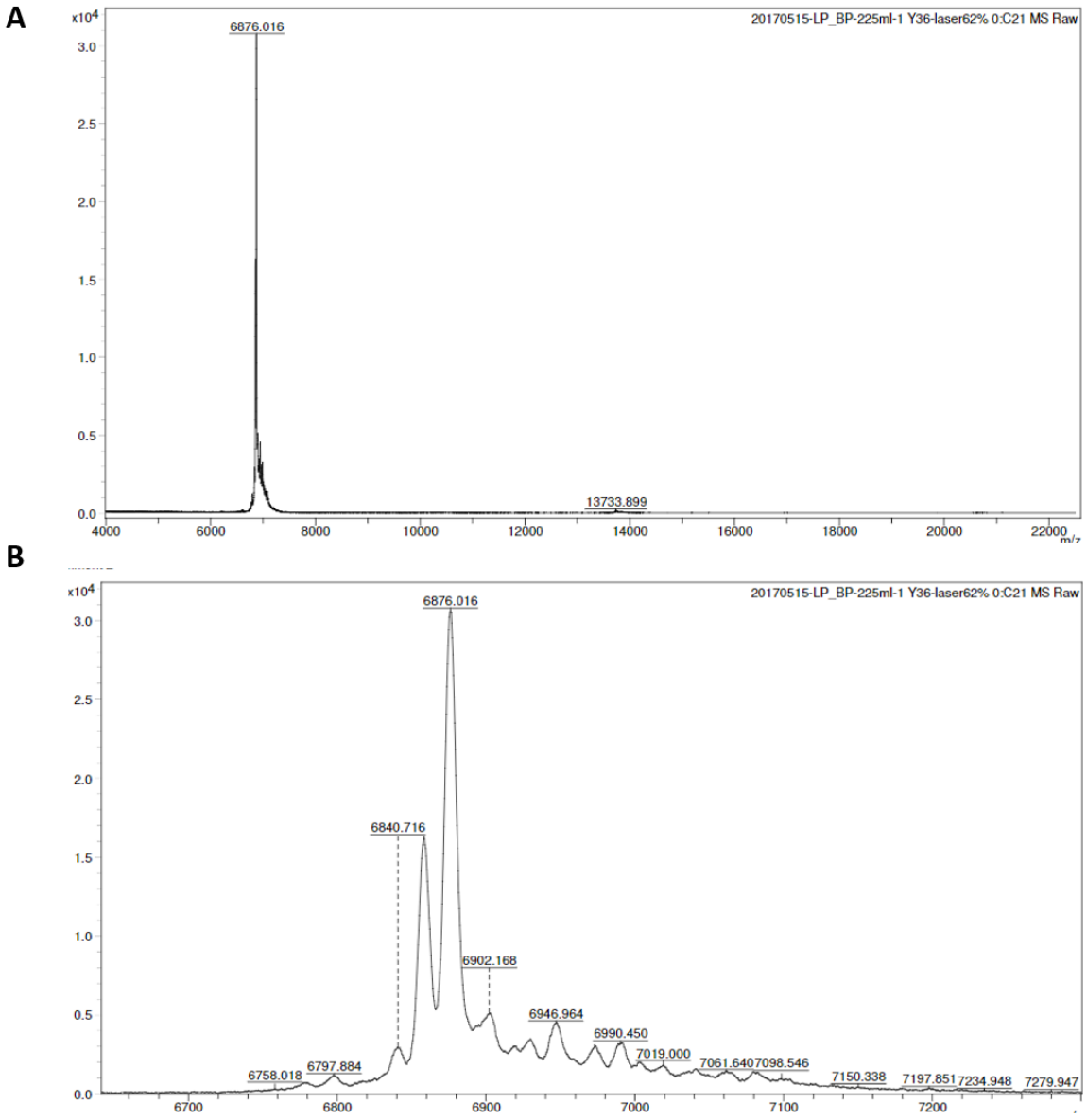


Figure S72: MALDI-TOF mass spectrum of **MadL1 Y36** *(A)* 4000-22000 m/z and *(B)* 6700-7200 m/z.

MadL1 DS

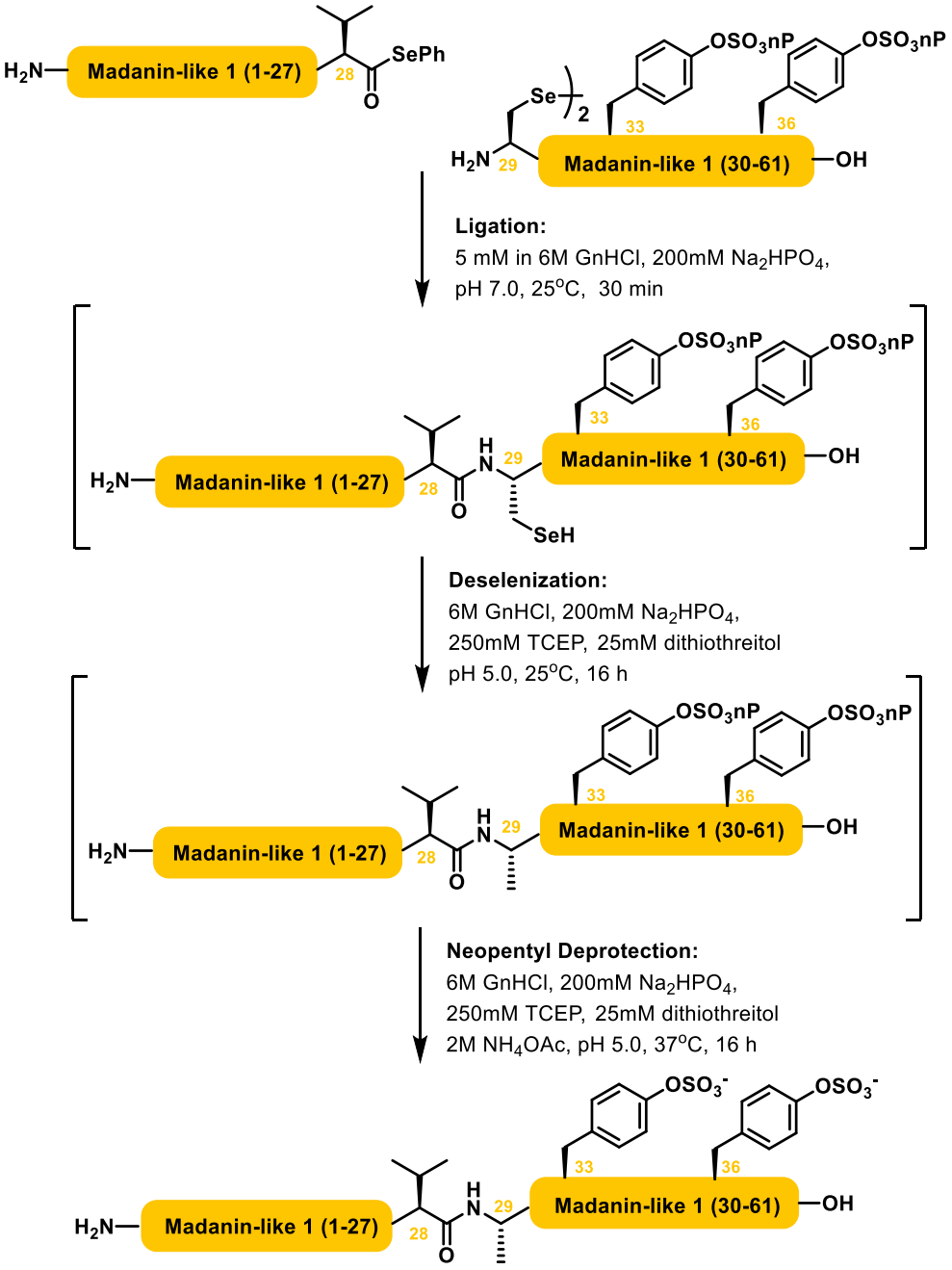


Figure S73: Synthesis of **MadL1 DS** via DSL-deselenization.

The one-pot peptide ligation of **MadL1 (1-28)** selenoester (2.5 mg, 0.75 µmol) and **MadL1 DS (29-61)** diselenide (2.0 mg, 0.50 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0% for 5 min, 0% to 40% B over 40 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **MadL1 DS** as a white solid (2.1 mg, 67%). Analytical HPLC (purified final product): R_t 26.0 min (0 to 50% B over 30 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1740.0 $[M+5H]^{5+}$: 1392.2 $[M+6H]^{6+}$: 1160.4 Mass Found (ESI) 1740.3 $[M+4H]^{4+}$, 1392.4 $[M+5H]^{5+}$, 1160.5 $[M+6H]^{6+}$.

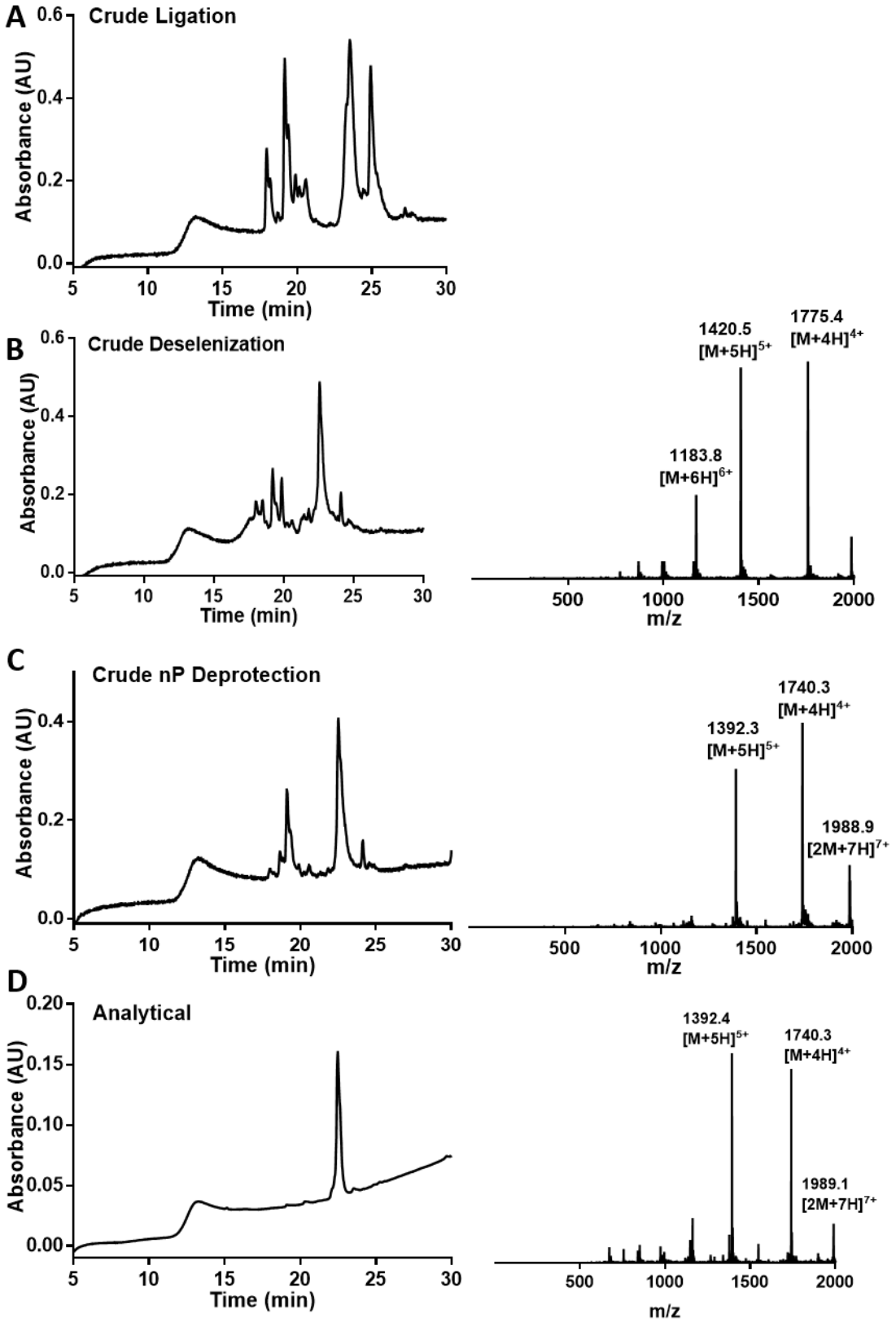


Figure S74: Analytical HPLC trace (0 to 50% B over 30 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **MadL1 DS**.

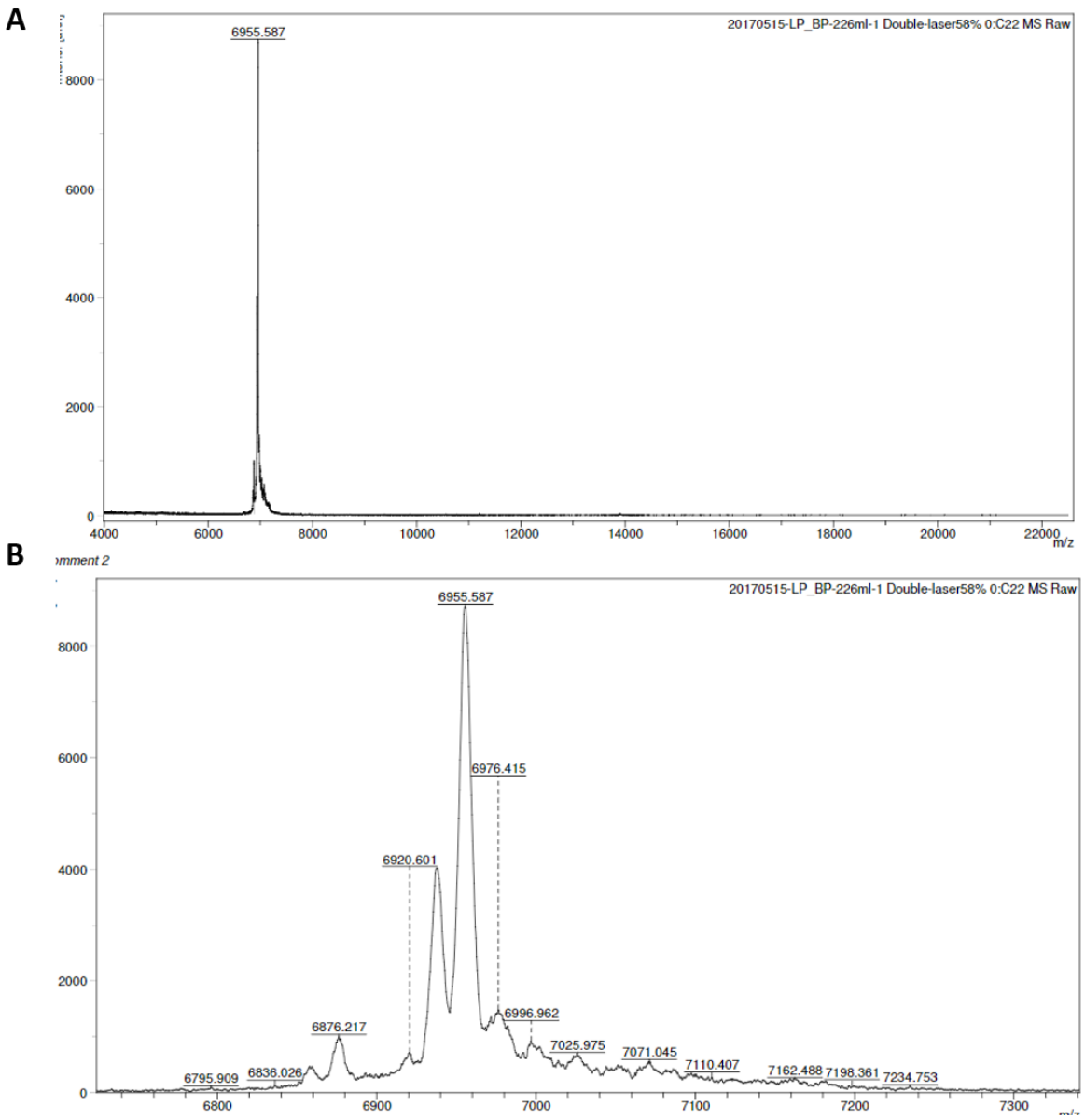


Figure S75: MALDI-TOF mass spectrum of **MadL1 DS** (*A*) 4000-22000 m/z and (*B*) 6800-7300 m/z.

MadL2 Un

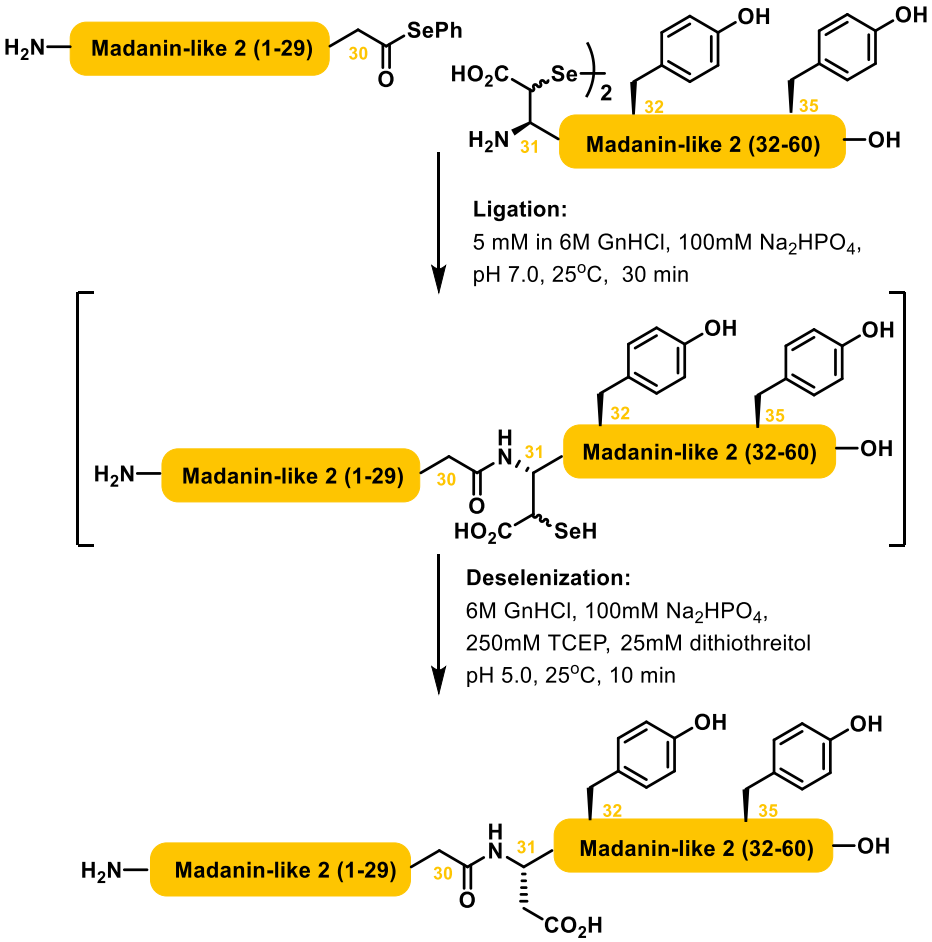


Figure S76: Synthesis of **MadL2 Un** via DSL-deselenization.

The one-pot peptide ligation of **MadL2 (1-30)** selenoester (2.92 mg, 0.83 µmol) and **MadL2 Un (31-60)** diselenide (2.0 mg, 0.56 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0 to 40% B over 40 min, 0.1% v/v TFA) followed by lyophilization afforded final protein **MadL2 Un** as a white solid (1.99 mg, 52%). Analytical HPLC (purified final product): R_t 3.91 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1709.1 $[M+5H]^{5+}$: 1367.4 $[M+6H]^{6+}$: 1139.70 $[M+7H]^{7+}$: 977.03 $[M+8H]^{8+}$: 855.02 $[M+9H]^{9+}$: 760.13 $[M+10H]^{10+}$: 684.22 Mass Found (ESI) 1708.8 $[M+4H]^{4+}$, 1367.3 $[M+5H]^{5+}$, 1139.6 $[M+6H]^{6+}$, 976.9 $[M+7H]^{7+}$, 855.0 $[M+8H]^{8+}$, 760.1 $[M+9H]^{9+}$, 684.1 $[M+10H]^{10+}$. High Res (ESI+): calcd for $C_{285}H_{439}N_{85}O_{111}$: $[M+8H]^{8+}$, 855.02516. Found: 855.02591 $[M+8H]^{8+}$. Difference: 1.0 ppm.

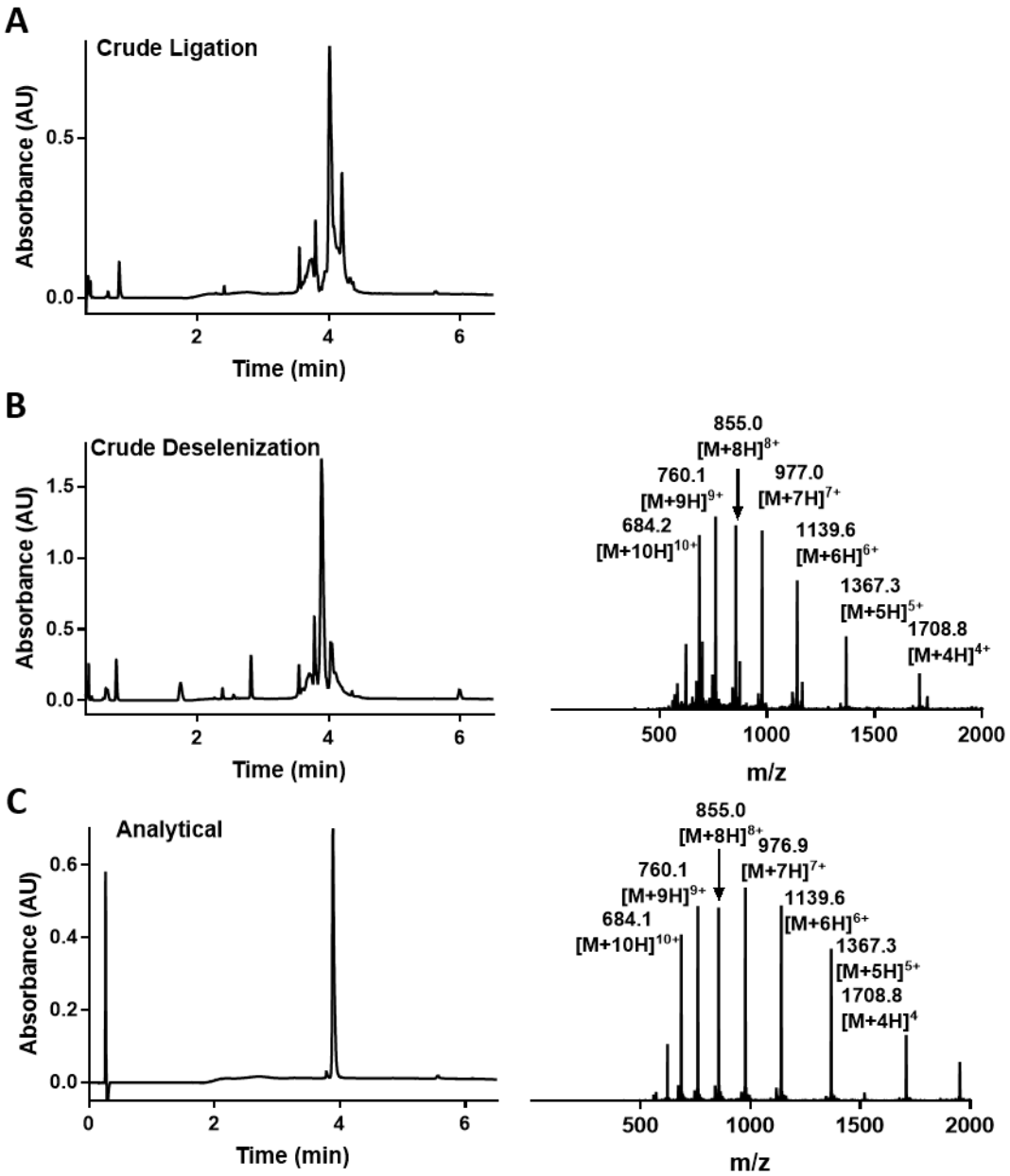


Figure S77: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, and (*C*) purified **MadL2 Un**.

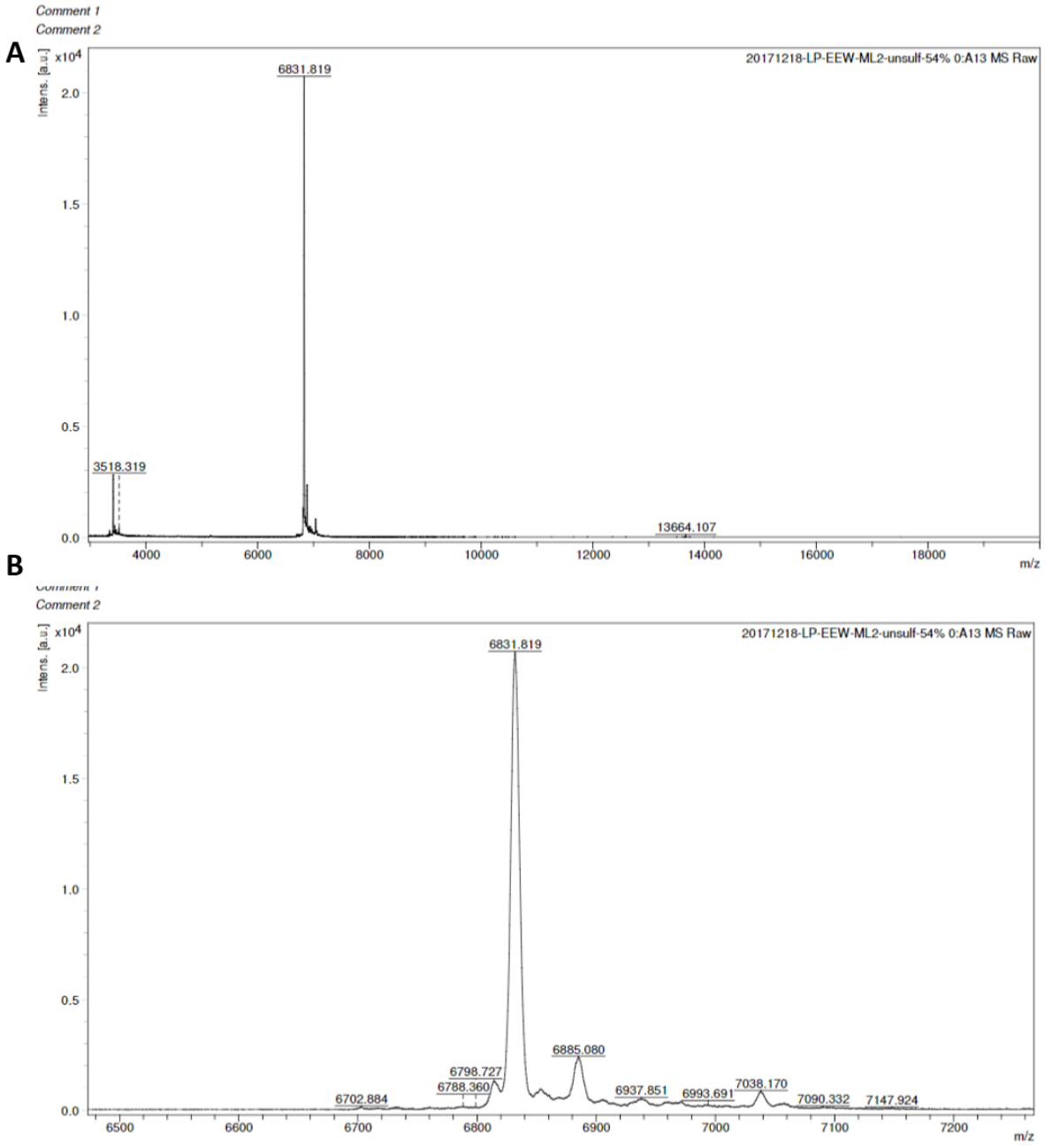


Figure S78: MALDI-TOF mass spectrum of **MadL2 Un** (*A*) 4000-20000 m/z and (*B*) 6500-7200 m/z.

MadL2 Y32

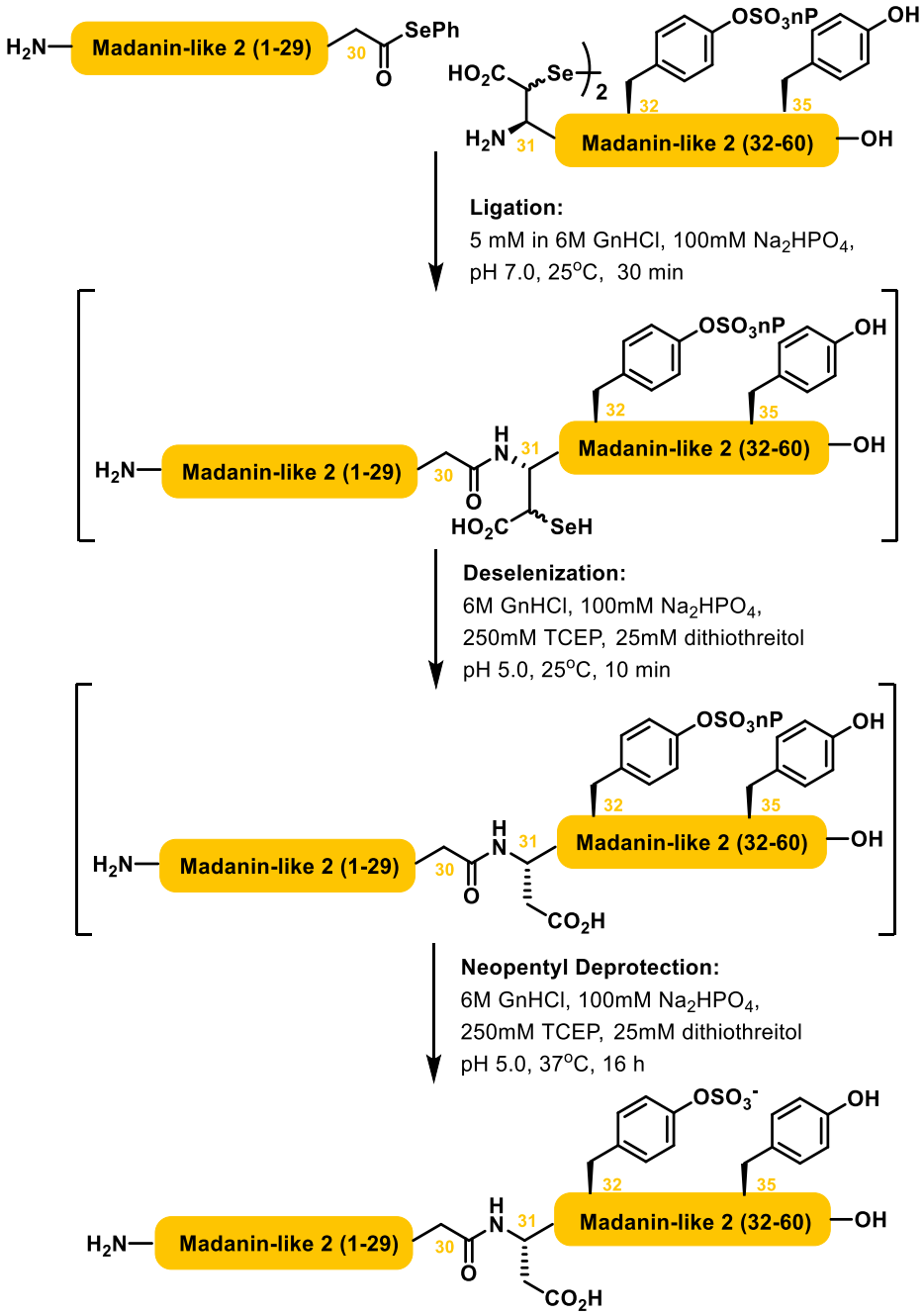


Figure S79: Synthesis of **MadL2 Y32** via DSL-deselenization.

The one-pot peptide ligation of **MadL2 (1-30)** selenoester (2.83 mg, 0.81 µmol) and **MadL2 (31-60)** diselenide (2.0 mg, 0.54 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0 to 40% B over 40 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **MadL2 Y32** as a white solid (1.9 mg, 51%). Analytical HPLC (purified final product): R_t 3.84 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1727.8 $[M+5H]^{5+}$: 1382.4 $[M+6H]^{6+}$: 1152.2 $[M+7H]^{7+}$: 987.7 $[M+8H]^{8+}$: 864.4 $[M+9H]^{9+}$: 768.5 Mass Found (ESI) 1728.5 $[M+4H]^{4+}$, 1383.1 $[M+5H]^{5+}$, 1152.9 $[M+6H]^{6+}$, 988.4 $[M+7H]^{7+}$, 865.0 $[M+8H]^{8+}$, 768.9 $[M+9H]^{9+}$. High Res (ESI+): calcd for $C_{285}H_{438}N_{85}O_{114}S$: $[M+8H]^{8+}$, 865.09172 Found: 865.01997 $[M+8H]^{8+}$. Difference: 0.3 ppm.

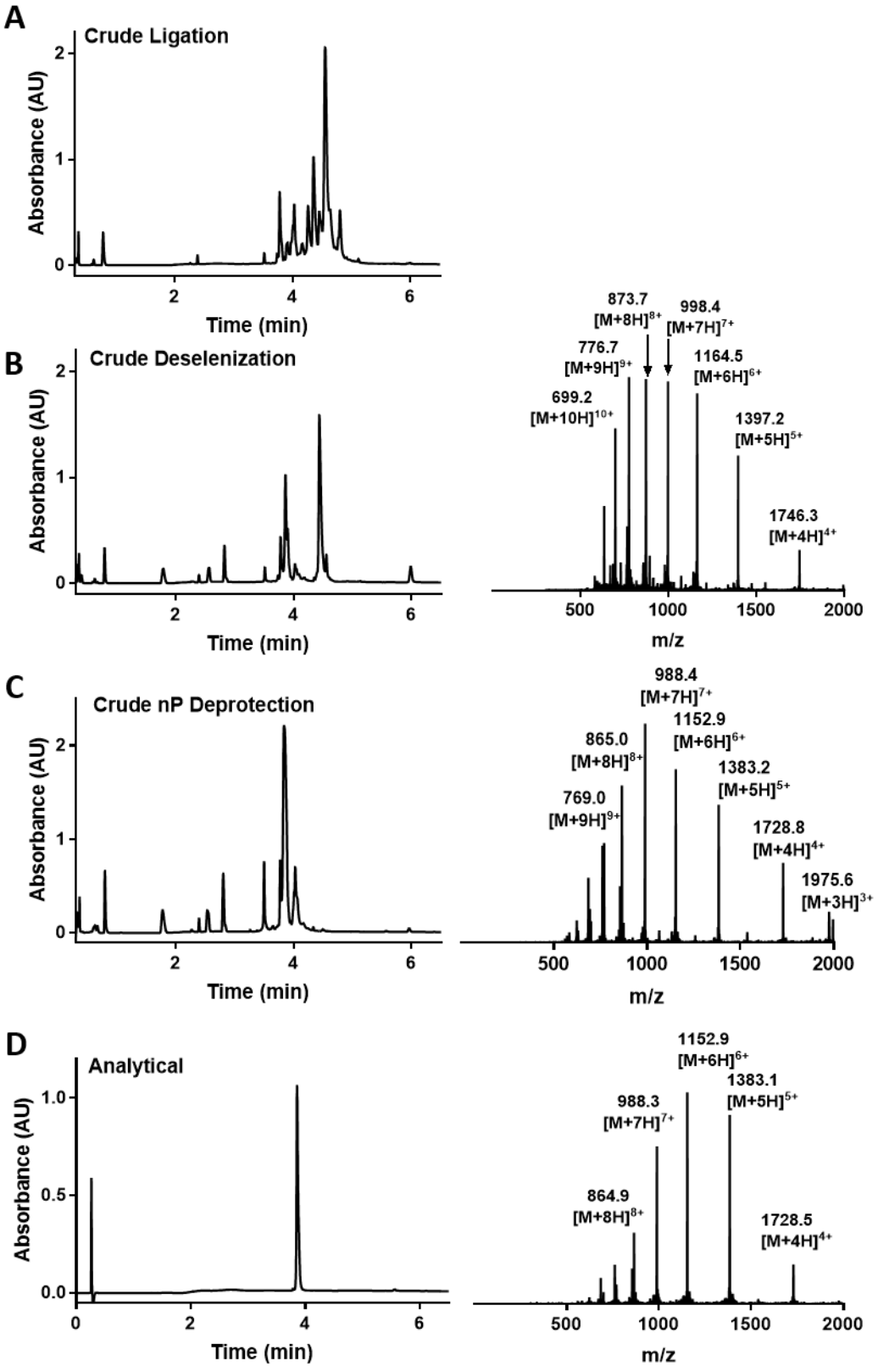


Figure S80: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **MadL2 Y32**.

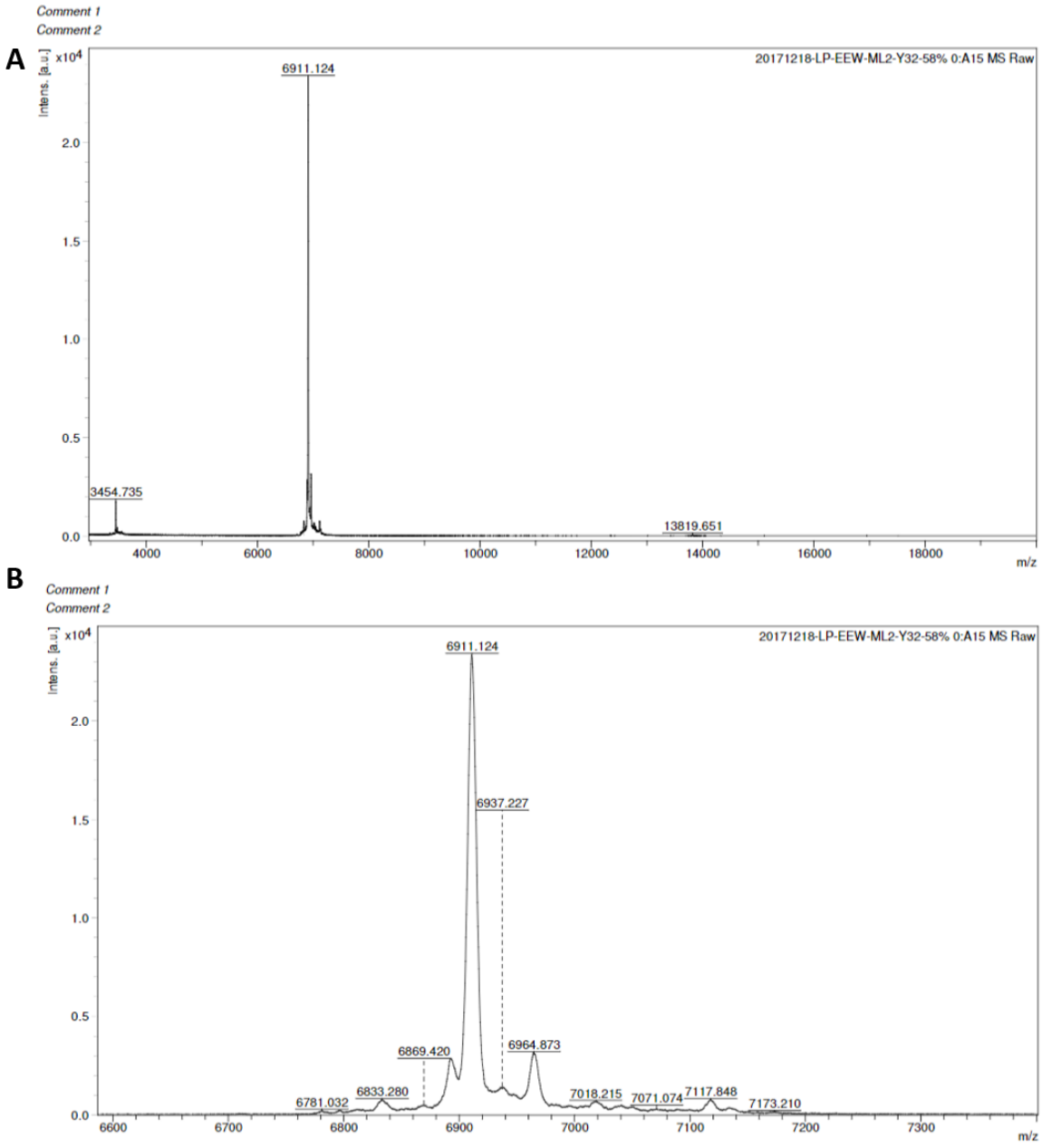


Figure S81: MALDI-TOF mass spectrum of **MadL2 Y32** (*A*) 4000-20000 m/z and (*B*) 6600-7300 m/z.

MadL2 Y35

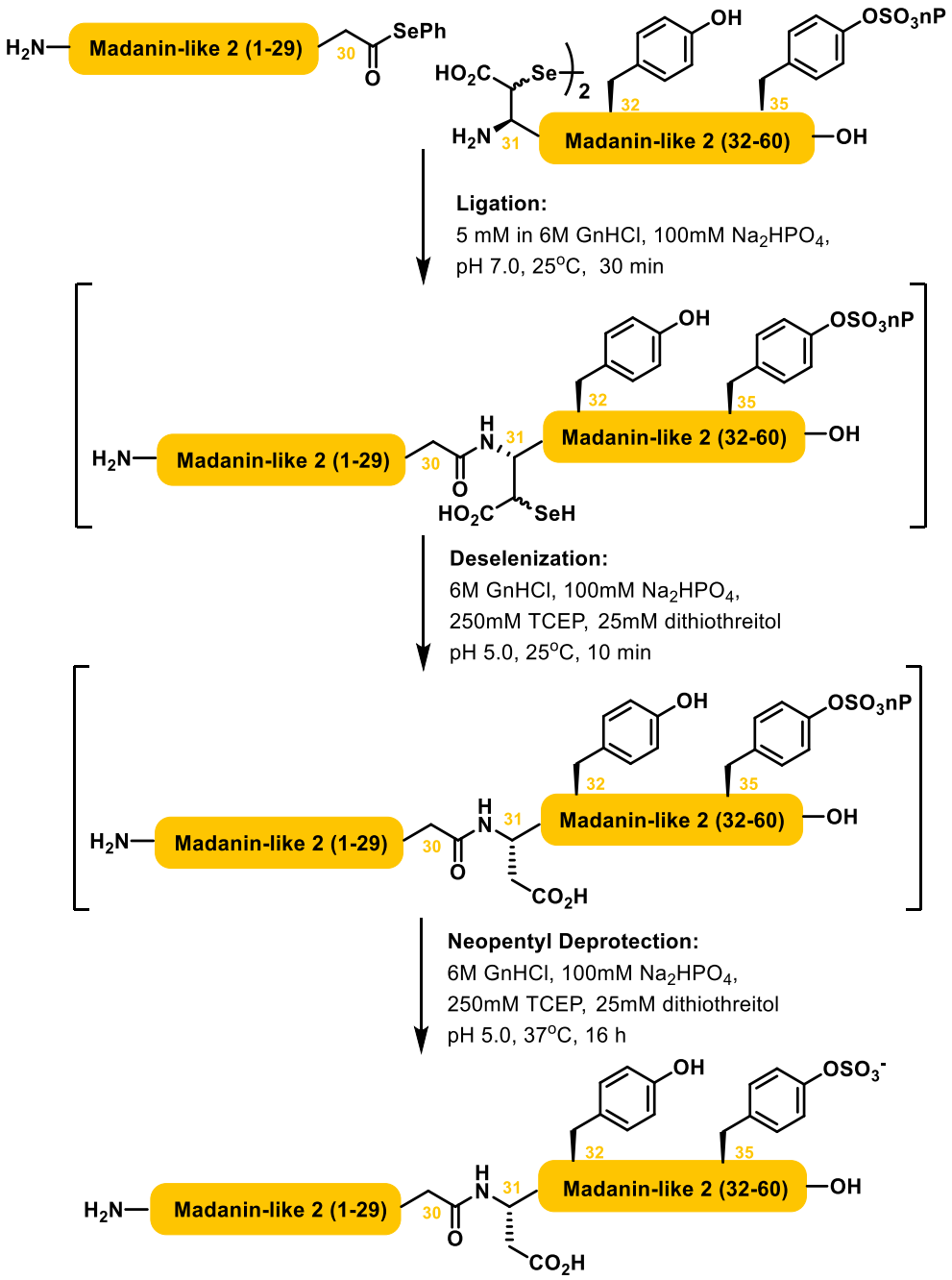


Figure S82: Synthesis of **MadL2 Y35** via DSL-deselenization.

The one-pot peptide ligation of **MadL2 (1-30)** selenoester (1.55 mg, 0.44 µmol) and **MadL2 Y35 (31-60)** diselenide (1.3 mg, 0.37 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0 to 30% B over 50 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **MadL2 Y35** as a white solid (1.3 mg, 55%). Analytical HPLC (purified final product): R_t 3.86 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1727.8 $[M+5H]^{5+}$: 1382.4 $[M+6H]^{6+}$: 1152.2 $[M+7H]^{7+}$: 987.7 $[M+8H]^{8+}$: 864.4 Mass Found (ESI) 1728.7 $[M+4H]^{4+}$, 1383.2 $[M+5H]^{5+}$, 1152.9 $[M+6H]^{6+}$, 988.3 $[M+7H]^{7+}$, 864.9 $[M+8H]^{8+}$. High Res (ESI+): calcd for $C_{285}H_{438}N_{85}O_{114}S$: $[M+8H]^{8+}$, 864.89440 Found: 864.89513 $[M+8H]^{8+}$. Difference: 0.9 ppm.

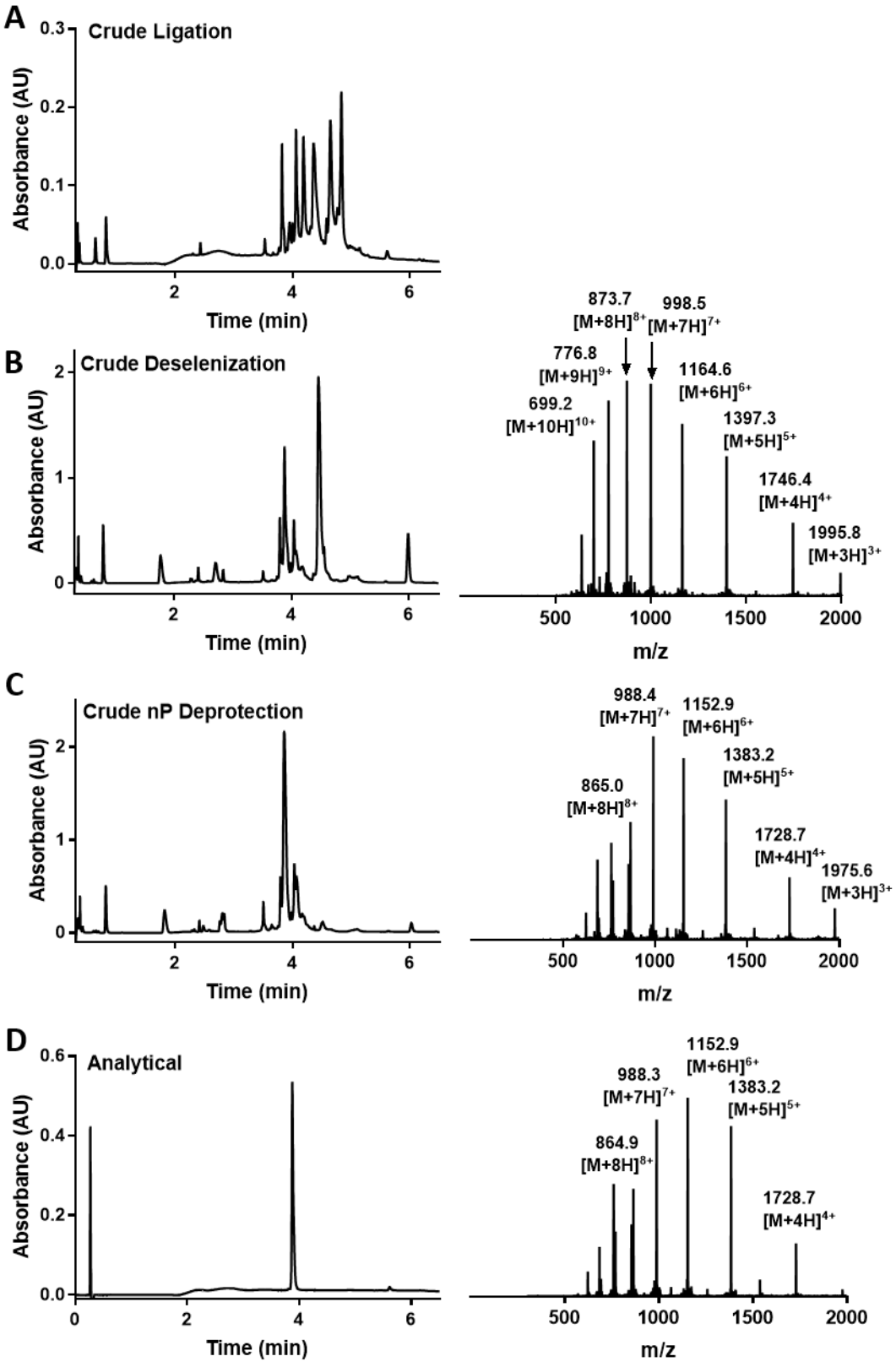


Figure S83: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **MadL2 Y35**.

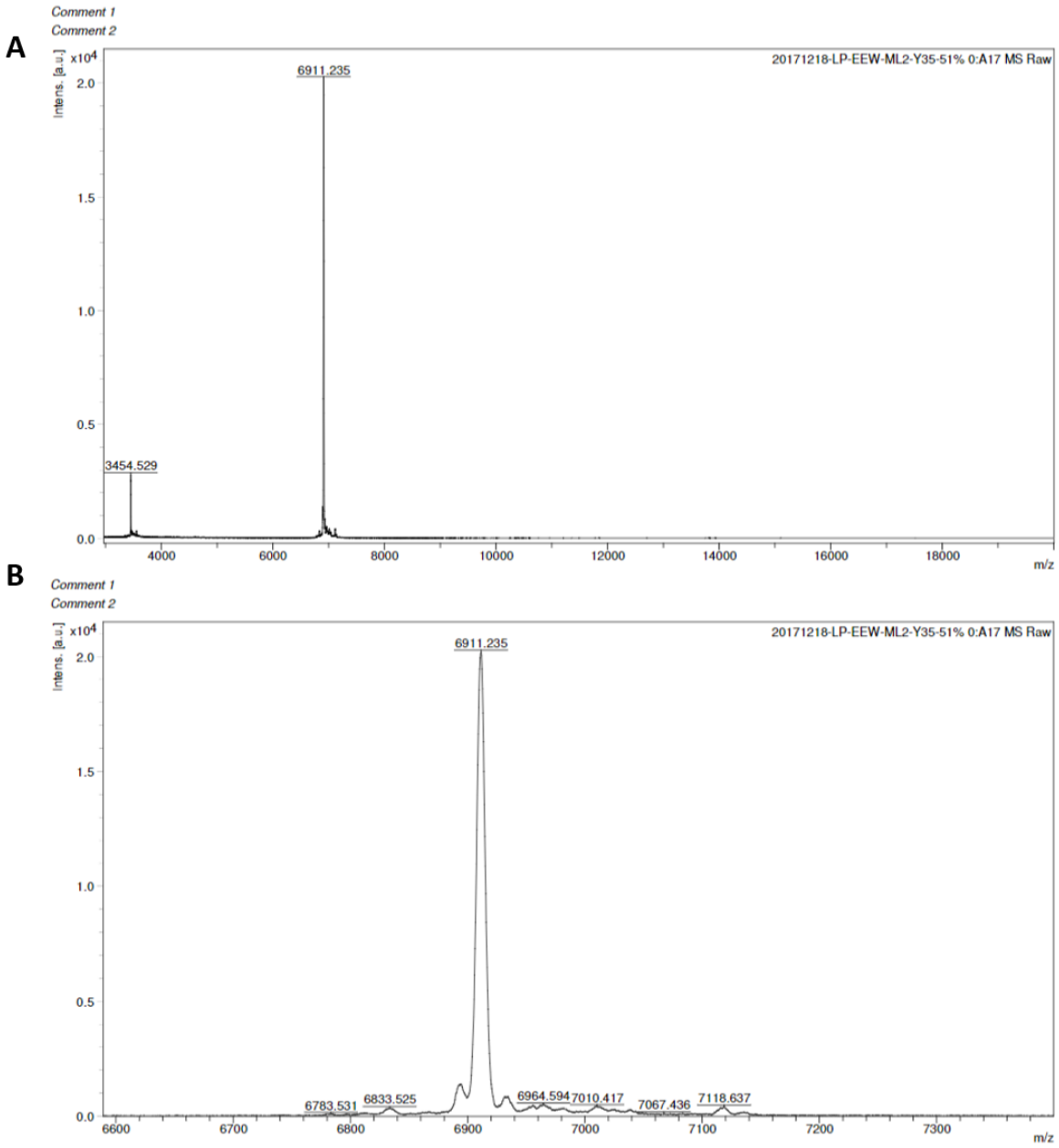
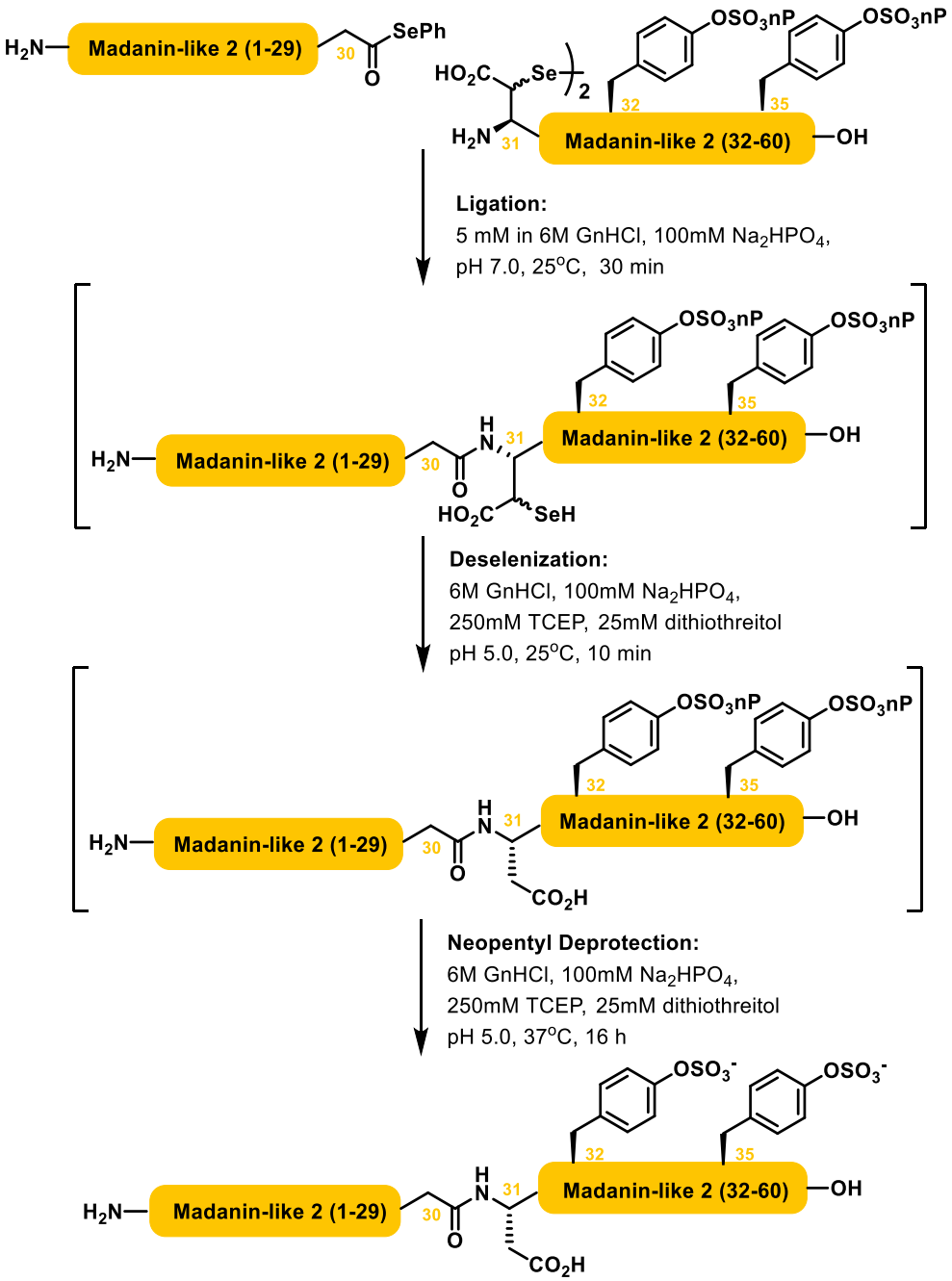


Figure S84: MALDI-TOF mass spectrum of **MadL2 Y35** (*A*) 4000-2000 m/z and (*B*) 6600-7300 m/z.

MadL2 DS



Scheme S85: Synthesis of **MadL2 DS** via DSL-deselenization.

The one-pot peptide ligation of **MadL2 (1-30)** selenoester (2.7 mg, 0.77 µmol) and **MadL2 DS (31-60)** diselenide (2.0 mg, 0.52 µmol) followed by *in situ* deselenization was performed according to the general procedure, replacing 10% of the buffer volume with DMF. Purification via preparative HPLC (0 to 40% B over 40 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **MadL2 DS** as a white solid (1.99 mg, 55%). Analytical HPLC (purified final product): R_t 3.84 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1747.5 $[M+5H]^{5+}$: 1398.2 $[M+6H]^{6+}$: 1165.4, $[M+7H]^{7+}$: 999.0, $[M+8H]^{8+}$: 874.3, $[M+9H]^{9+}$: 777.2 Mass Found (ESI) 1748.9 $[M+4H]^{4+}$, 1399.2 $[M+5H]^{5+}$, 1166.2 $[M+6H]^{6+}$, 999.8 $[M+7H]^{7+}$, 874.9 $[M+8H]^{8+}$, 777.9 $[M+9H]^{9+}$. High Res (ESI+): calcd for $C_{285}H_{437}N_{85}O_{117}S_2$: $[M+8H]^{8+}$, 874.89037. Found: 874.88897 $[M+8H]^{8+}$. Difference: 1.6 ppm.

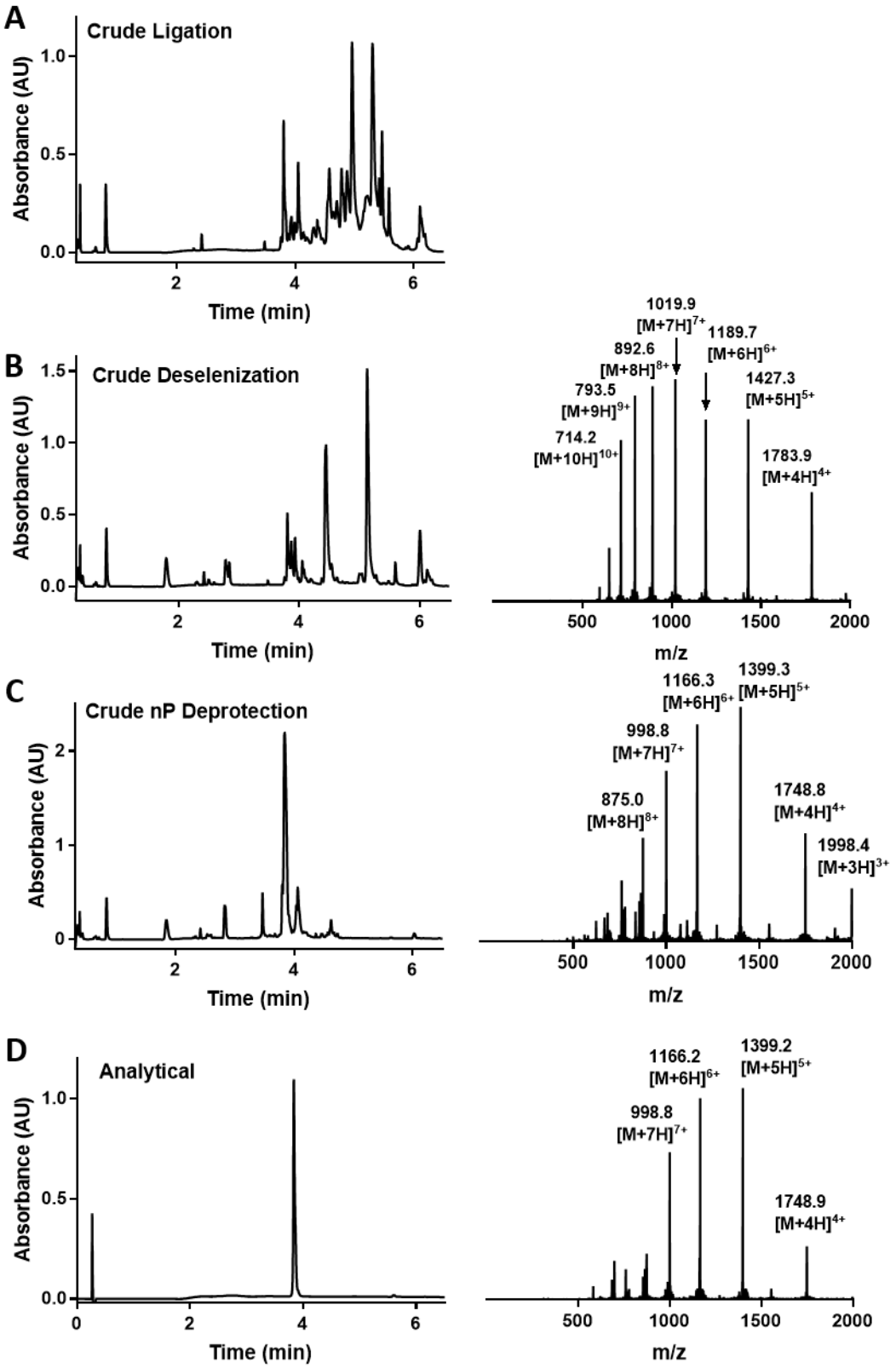


Figure S86: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **MadL2 DS**.

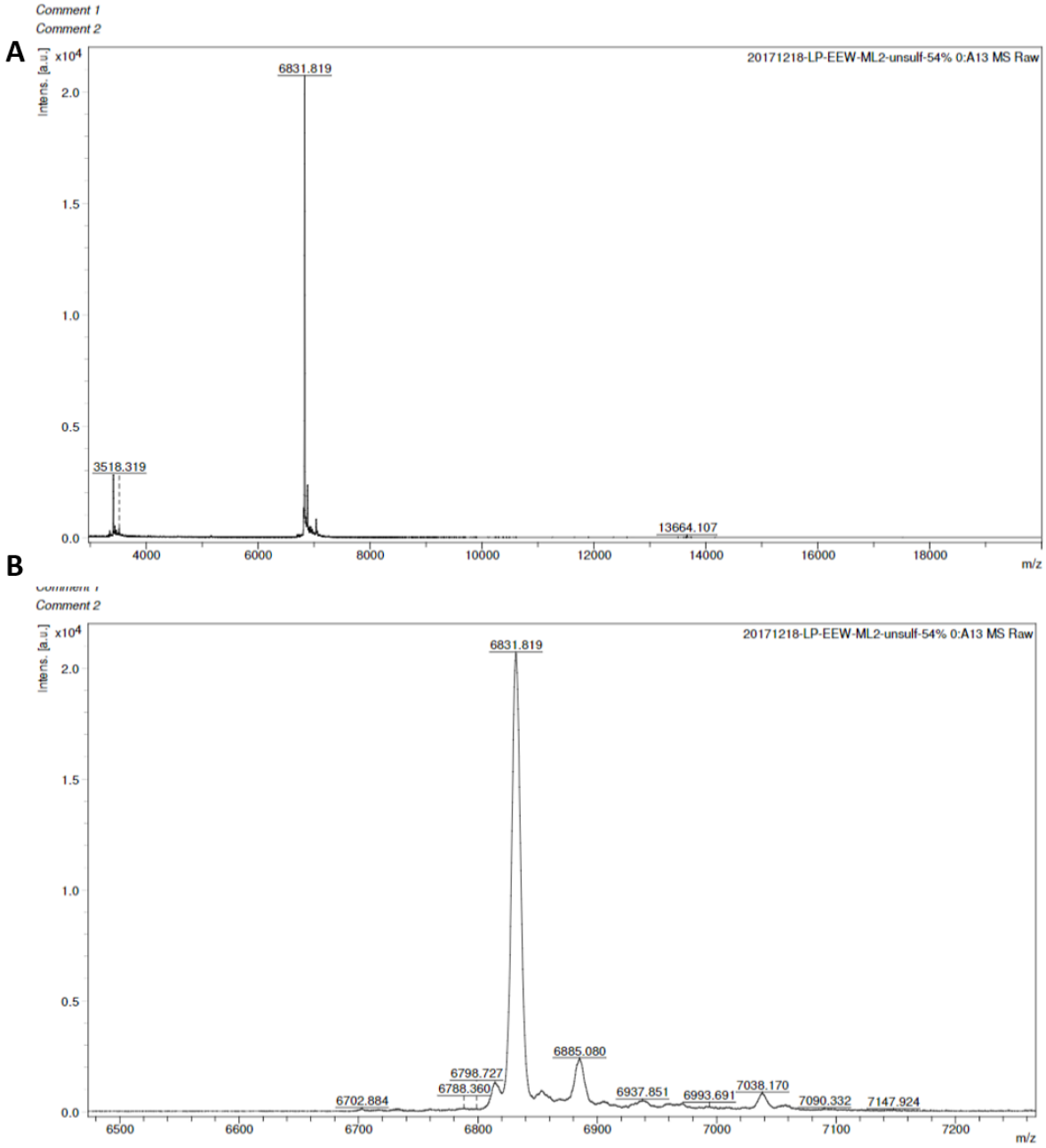


Figure S87: MALDI-TOF mass spectrum of **MadL2 DS** *(A)* 4000-20000 m/z and *(B)* 6600-7400 m/z.

And82 Un

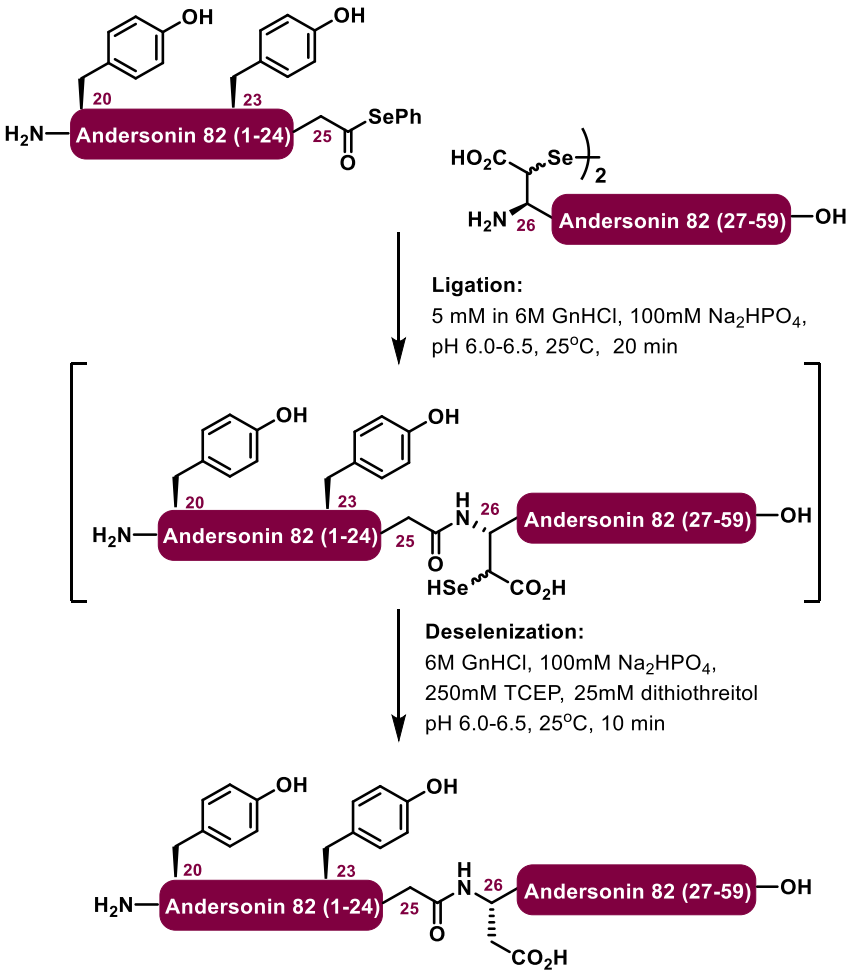


Figure S88: Synthesis of **And82 Un** via DSL-deselenization.

The one-pot peptide ligation of **And82 Un (1-25)** selenoester (2.7 mg, 0.86 µmol) and **And82 (26-59)** diselenide (2.0 mg, 0.58 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0 to 40% B over 50 min, 0.1% v/v TFA) followed by lyophilization afforded final protein **And82 Un** as a white solid (1.65 mg, 44% yield). Analytical HPLC (purified final product): R_t 4.60 (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1593.5, $[M+5H]^{5+}$: 1275.0, $[M+6H]^{6+}$: 1062.7 $[M+7H]^{7+}$: 911.0, $[M+8H]^{8+}$: 797.2, $[M+9H]^{9+}$: 708.8, $[M+10H]^{10+}$: 638.0, Mass Found (ESI) 1595.4 $[M+4H]^{4+}$, 1276.5 $[M+5H]^{5+}$, 1063.9 $[M+6H]^{6+}$, 911.9 $[M+7H]^{7+}$, 798.0 $[M+8H]^{8+}$, 709.4 $[M+9H]^{9+}$, 638.6 $[M+10H]^{10+}$. High Res (ESI+): calcd for $C_{263}H_{405}N_{85}O_{101}$: $[M+6H]^{6+}$, 1063.16171. Found: 1063.16168 $[M+6H]^{6+}$. Difference: less than 0.1 ppm.

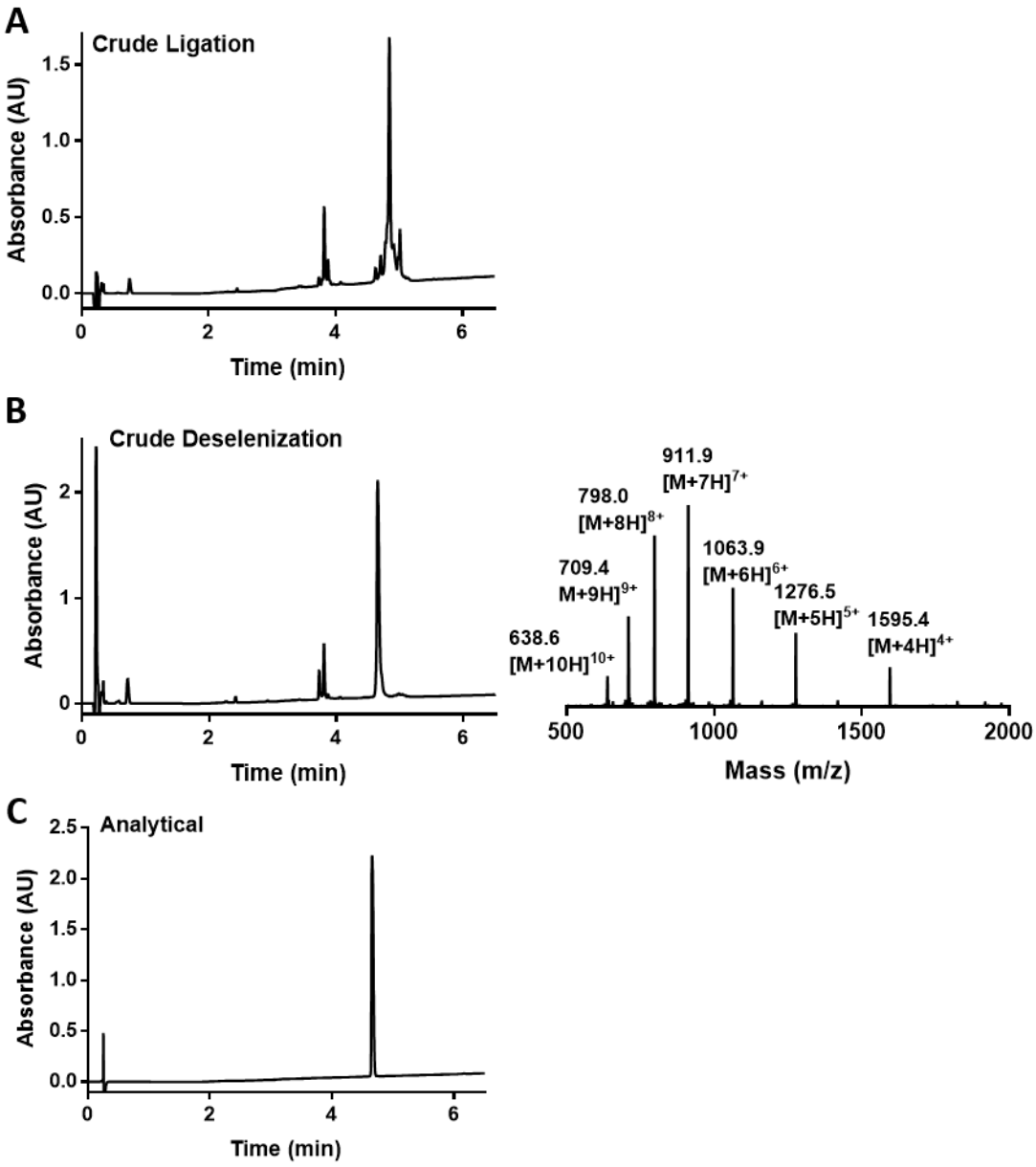


Figure S89: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, and (*C*) purified **And82 Un**.

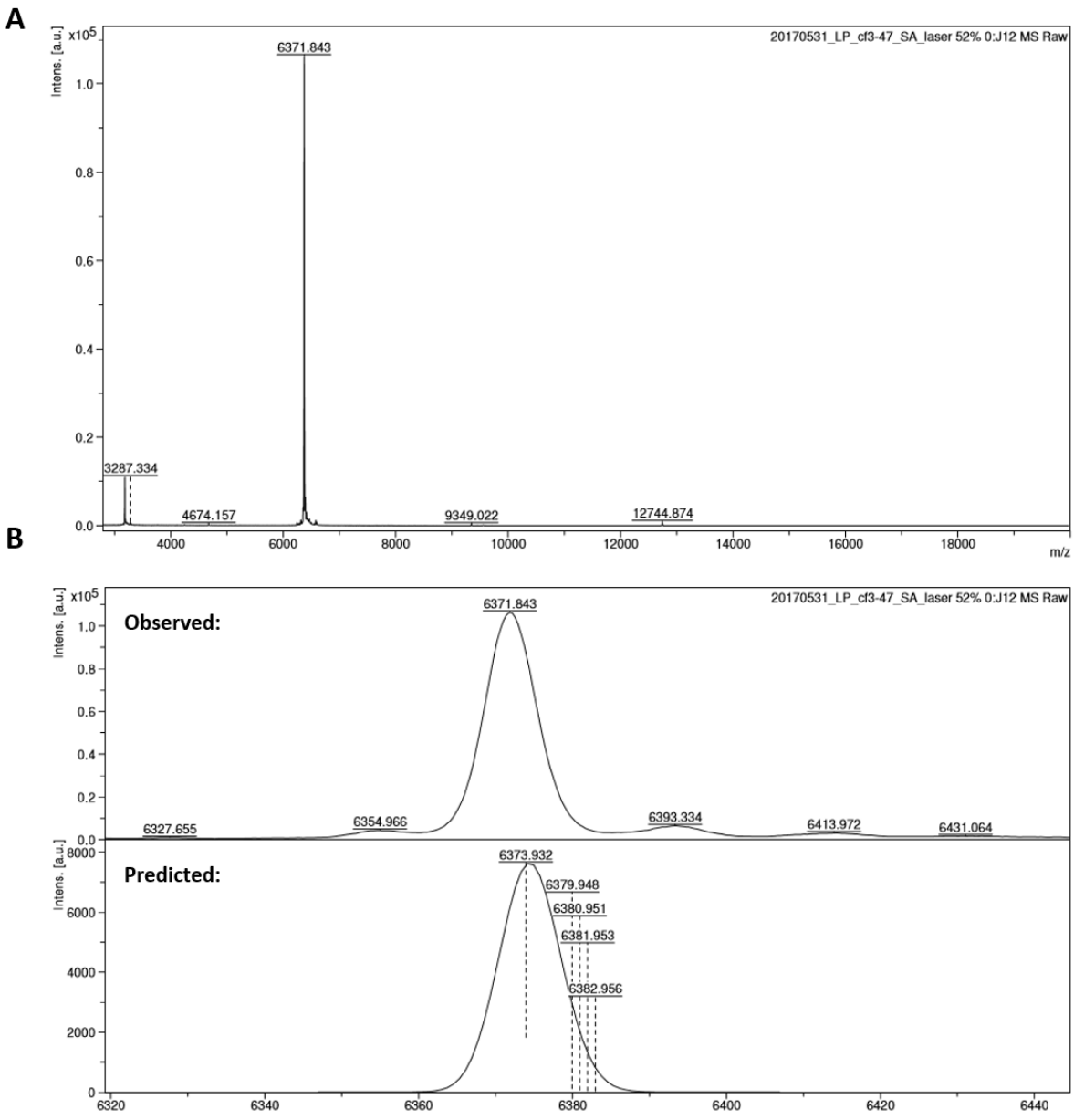


Figure S90: MALDI-TOF mass spectrum of **And82 Un** (*A*) 4000-20000 m/z and (*B*) 6320-6440 m/z.

And82 Y20

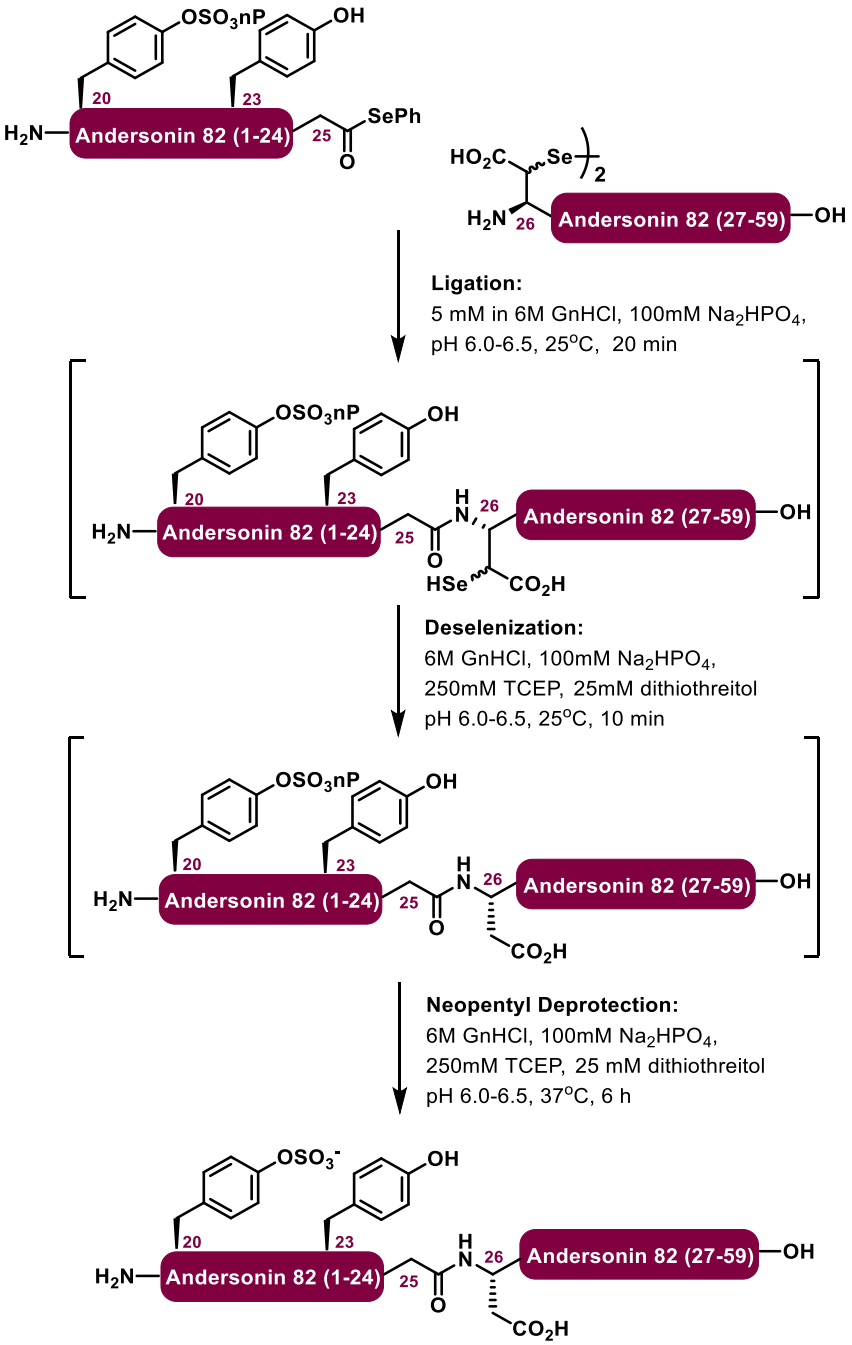


Figure S91: Synthesis of **And82 Y20** via DSL-deselenization.

The one-pot peptide ligation of **And82 Y20 (1-25)** selenoester (2.82 mg, 0.86 µmol) and **And82 (26-59)** diselenide (2.0 mg, 0.58 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0 to 40% B over 50 min, 0.1% v/v Formic acid) followed by lyophilization afforded **And82 Y30** as a white solid (1.97 mg, 53%). Analytical HPLC (purified final product): R_t 4.08 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1613.5, $[M+5H]^{5+}$: 1291.0, $[M+6H]^{6+}$: 1076.0, $[M+7H]^{7+}$: 922.4, $[M+8H]^{8+}$: 807.2, Mass Found (ESI) 1614.3 $[M+4H]^{4+}$, 1291.7 $[M+5H]^{5+}$, 1076.5 $[M+6H]^{6+}$, 911.3/922.8 ($-SO_3$ as a mass spec artefact) $[M+7H]^{7+}$, 797.6/807.6 ($-SO_3$ as a mass spec artefact) $[M+8H]^{8+}$. High Res (ESI+): calcd for $C_{263}H_{405}N_{85}O_{104}S$: $[M+7H]^{7+}$, 922.99088. Found: 922.99296 $[M+7H]^{7+}$. Difference: 1.6 ppm.

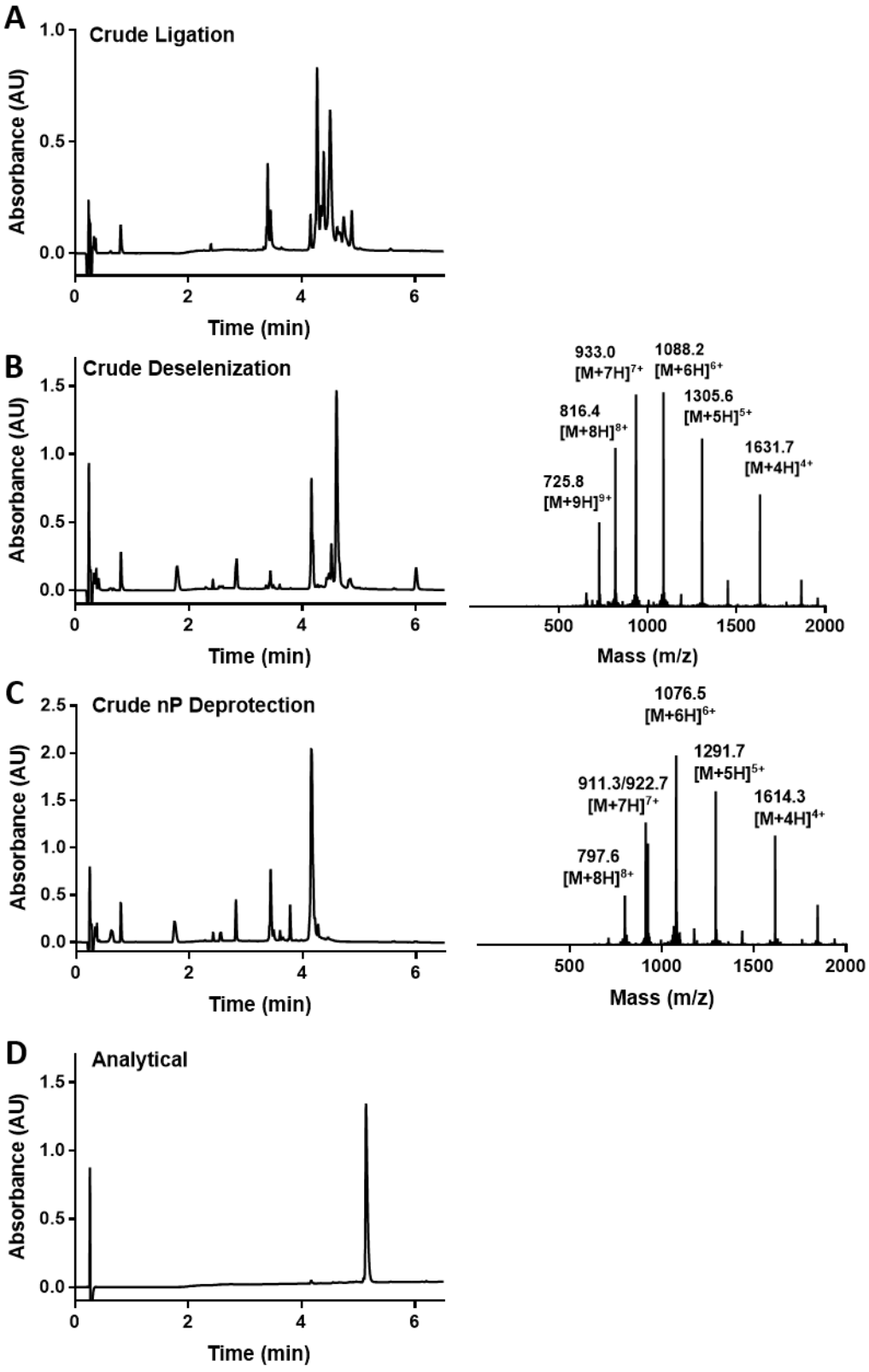


Figure S92: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **And82 Y20**.

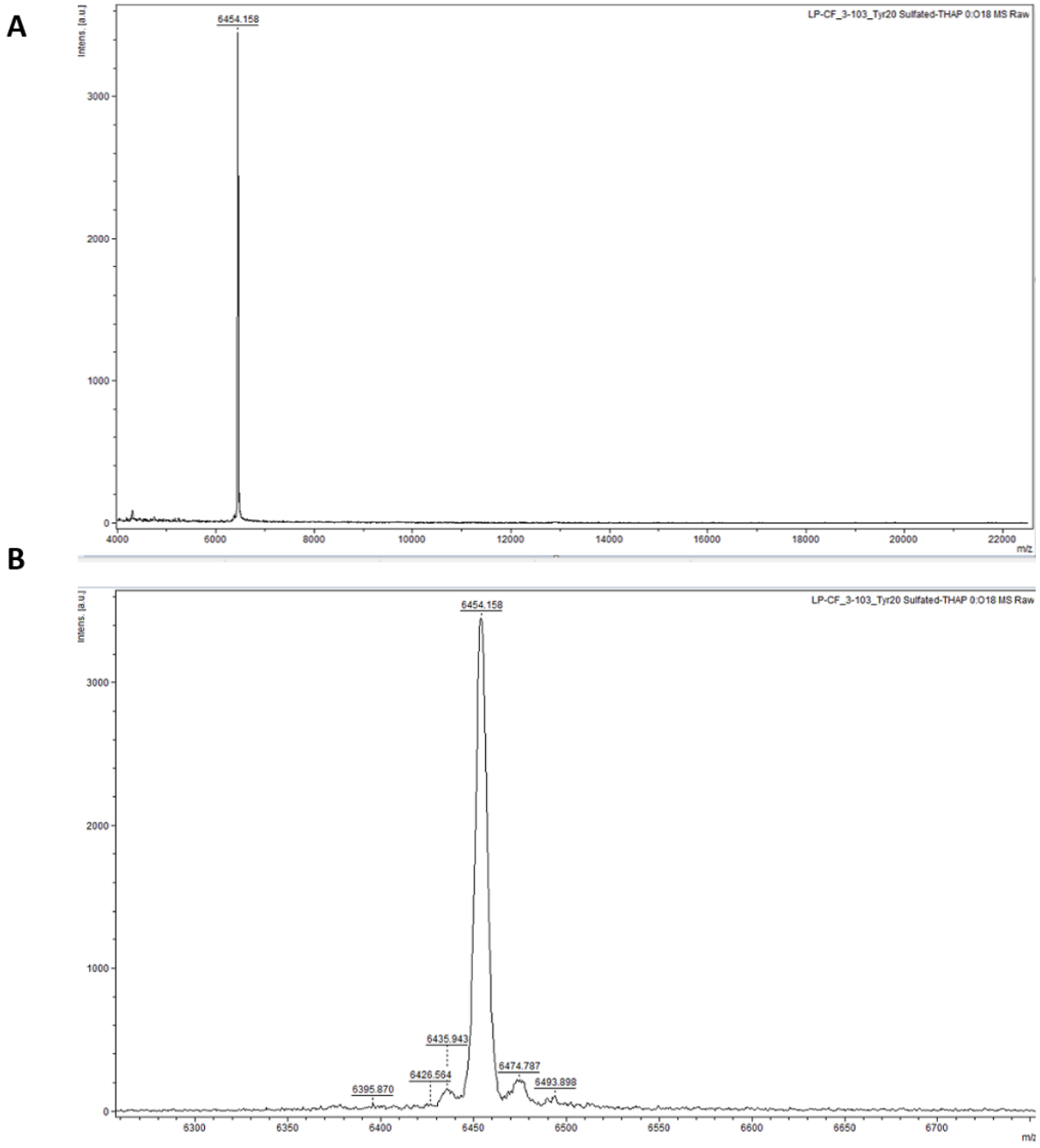


Figure S93: MALDI-TOF mass spectrum of **And82 Y20** (*A*) 4000-22000 m/z and (*B*) 6300-6700 m/z.

And82 Y23

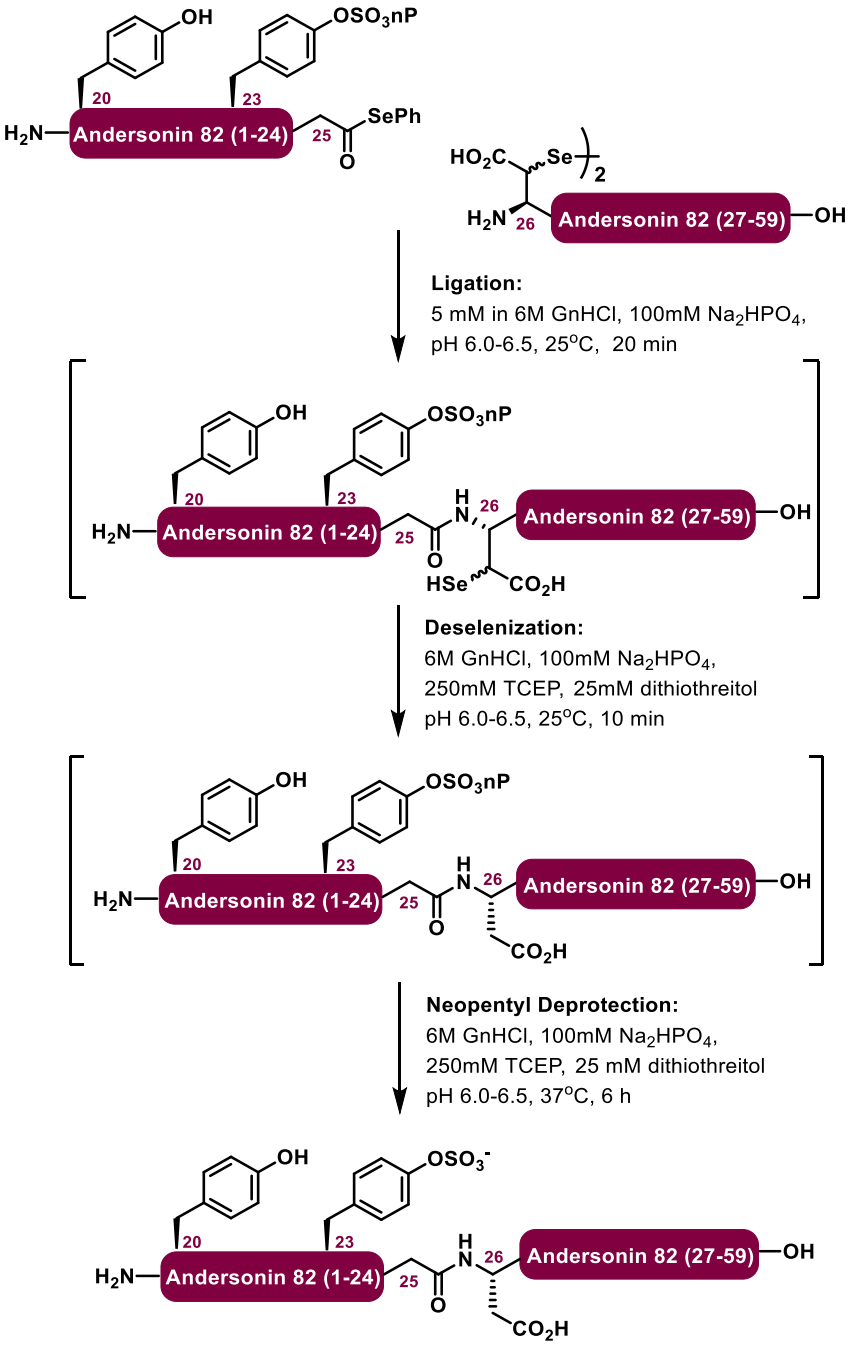


Figure S94: Synthesis of **And82 Y23** via DSL-deselenization.

The one-pot peptide ligation of **And82 Y23 (1-25)** selenoester (2.82 mg, 0.86 µmol) and **And82 (26-59)** diselenide (2.0 mg, 0.58 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0 to 40% B over 50 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **And82 Y23** as a white solid (1.89 mg, 51%). Analytical HPLC (purified final product): R_t 4.07 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1613.5, $[M+5H]^{5+}$: 1291.0, $[M+6H]^{6+}$: 1076.0, $[M+7H]^{7+}$: 922.4, $[M+8H]^{8+}$: 807.2, Mass Found (ESI) 1614.4 $[M+4H]^{4+}$, 1291.7 $[M+5H]^{5+}$, 1076.5 $[M+6H]^{6+}$, 922.8 $[M+7H]^{7+}$, 807.6 $[M+8H]^{8+}$. High Res (ESI+): calcd for $C_{263}H_{405}N_{85}O_{104}S$: $[M+7H]^{7+}$, 922.84759. Found: 922.84926 $[M+7H]^{7+}$. Difference: 1.9 ppm.

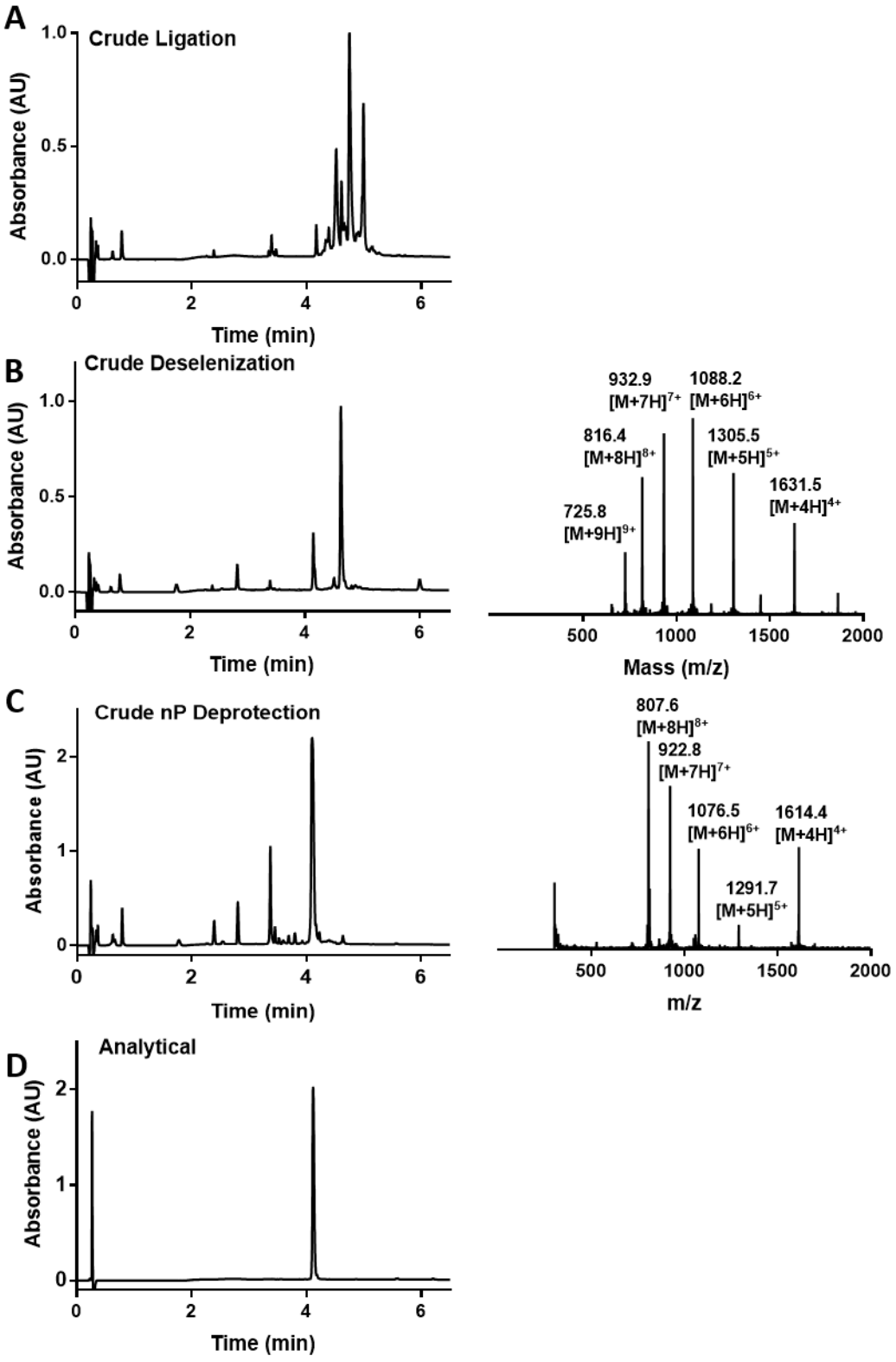


Figure S95: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **And82 Y23**.

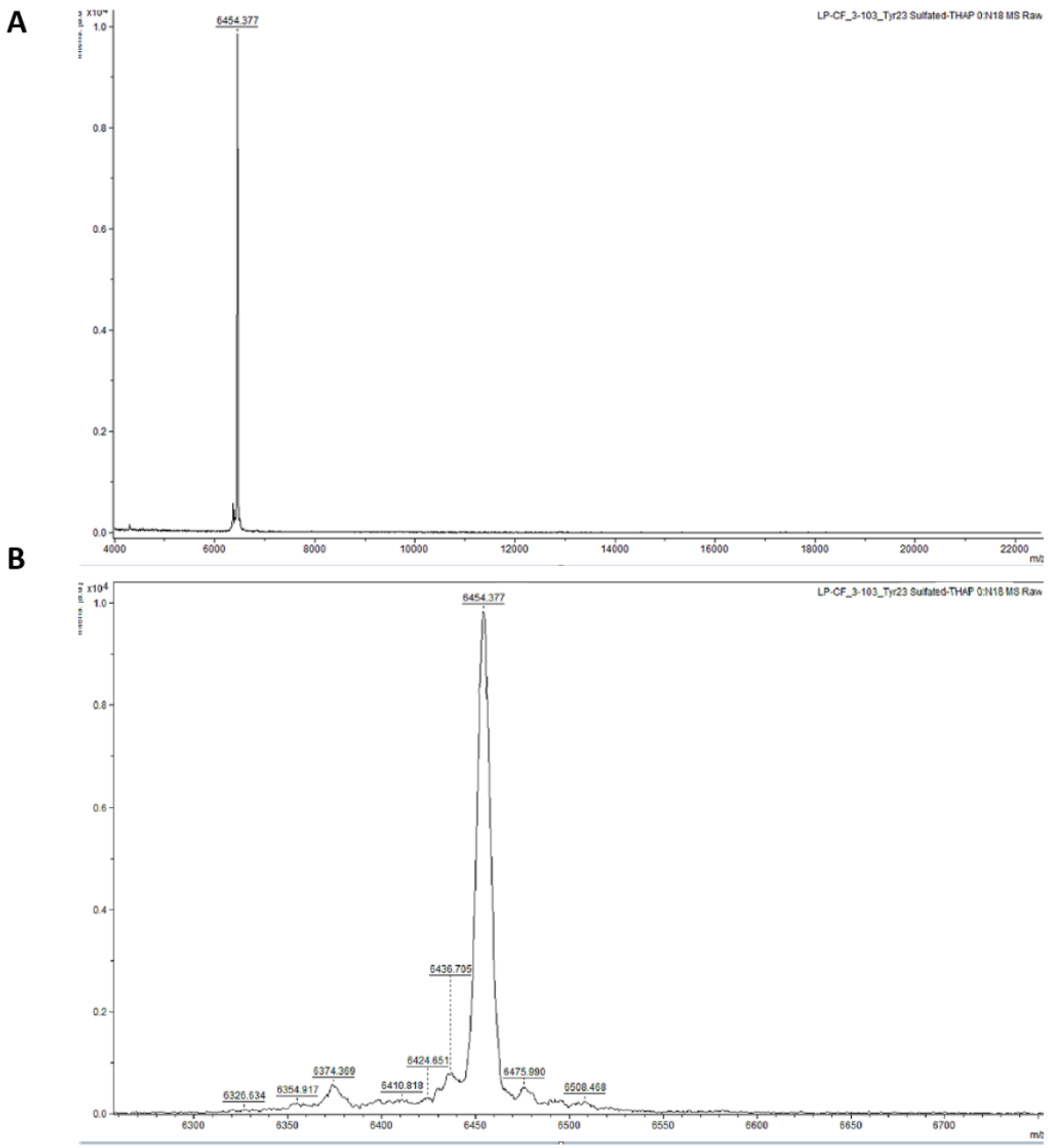


Figure S96: MALDI-TOF mass spectrum of **And82 Y23** (*A*) 4000-22000 m/z and (*B*) 6300-6700 m/z.

And82 DS

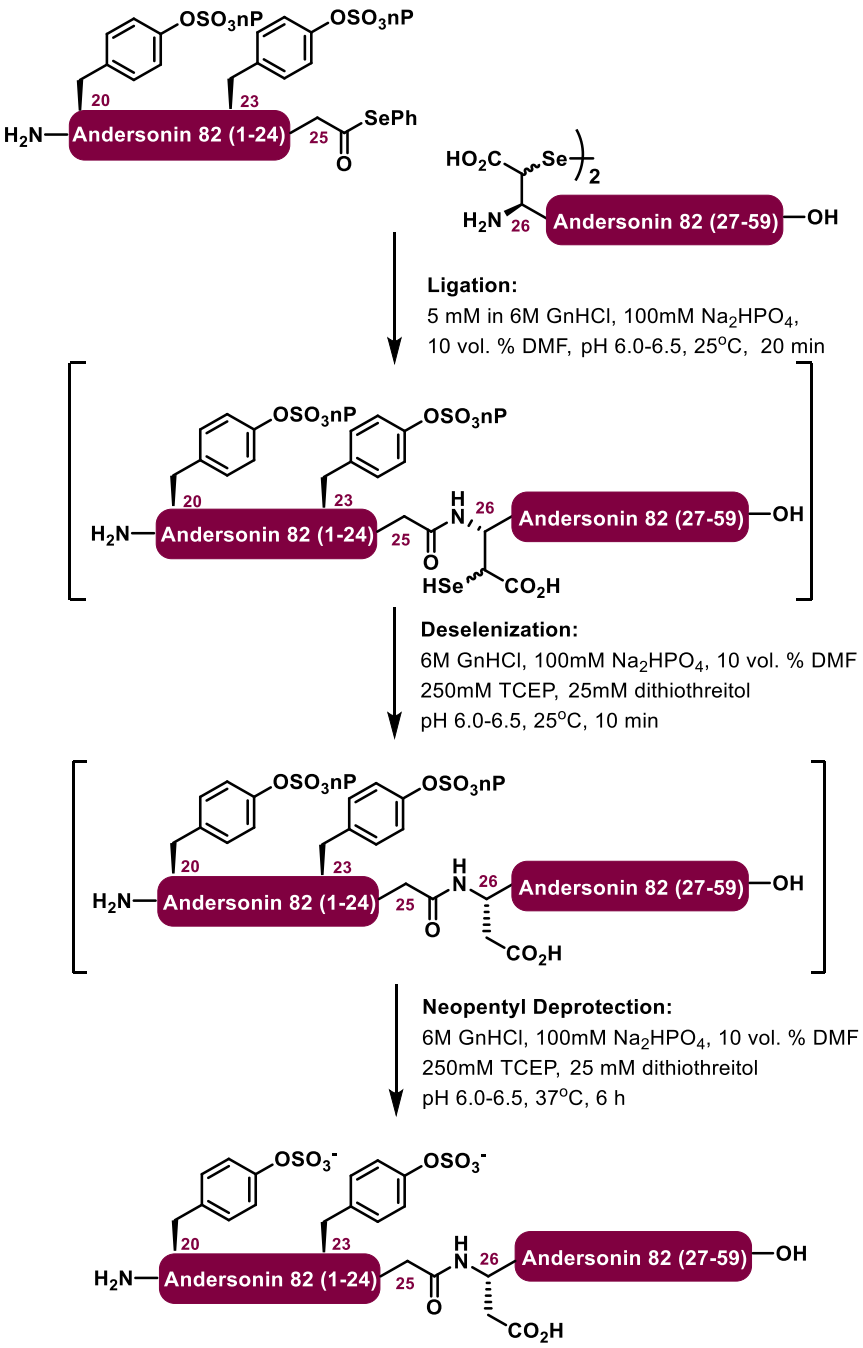


Figure S97: Synthesis of **And82 DS** via DSL-deselenization.

The one-pot peptide ligation of **And82 DS (1-25)** selenoester (2.97 mg, 0.86 µmol) and **And82 (26-59)** diselenide (2.0 mg, 0.58 µmol) followed by *in situ* deselenization was performed according to the general procedure, replacing 10% of the buffer volume with DMF. Purification via preparative HPLC (0 to 40% B over 40 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **And82 DS** as a white solid (1.34 mg, 35%). Analytical HPLC (purified final product): R_t 4.06 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1633.5, $[M+5H]^{5+}$: 1307.0, $[M+6H]^{6+}$: 1089.3, $[M+7H]^{7+}$: 933.9, $[M+8H]^{8+}$: 817.2, Mass Found (ESI) 1634.4 $[M+4H]^{4+}$, 1307.7 $[M+5H]^{5+}$, 1089.8 $[M+6H]^{6+}$, 934.3 $[M+7H]^{7+}$. High Res (ESI+): calcd for $C_{263}H_{405}N_{85}O_{107}S_2$: $[M+7H]^{5+}$, 934.26999. Found: 934.27085 $[M+5H]^{5+}$. Difference: 0.8 ppm.

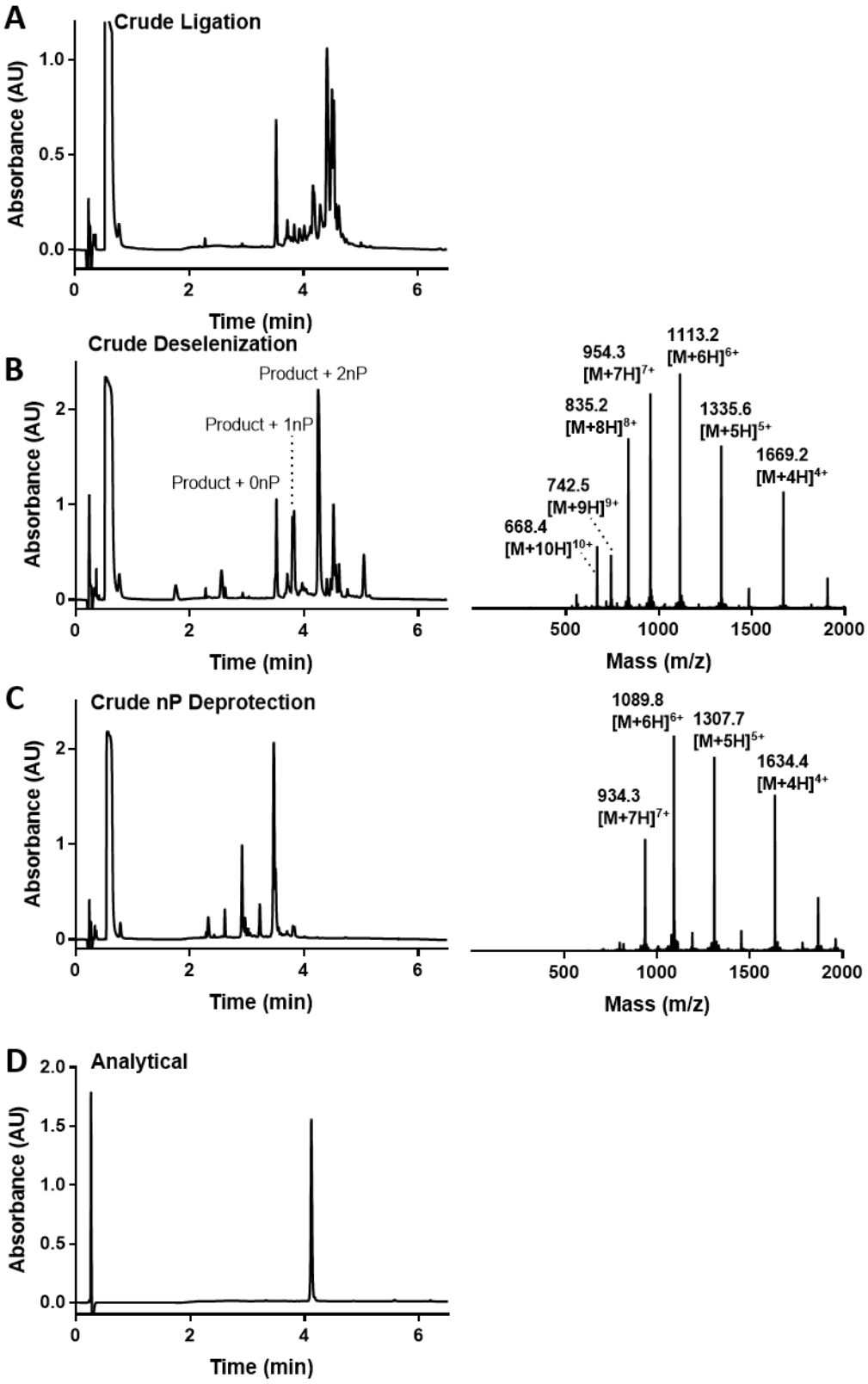


Figure S98: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **And82 DS**.

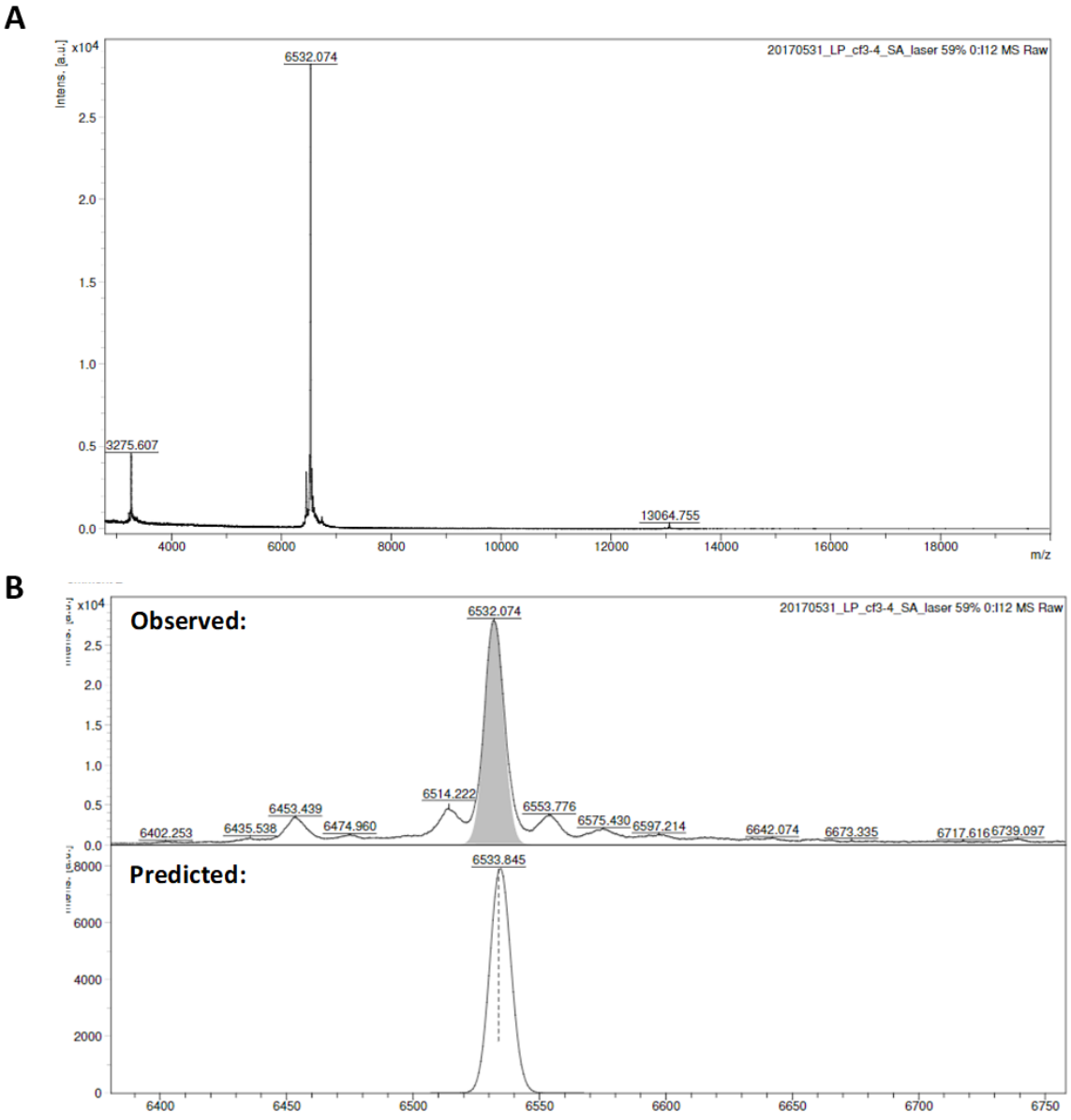


Figure S99: MALDI-TOF mass spectrum of **And82 DS** (*A*) 4000-20000 m/z and (*B*) 6400-6750 m/z.

And310 Un

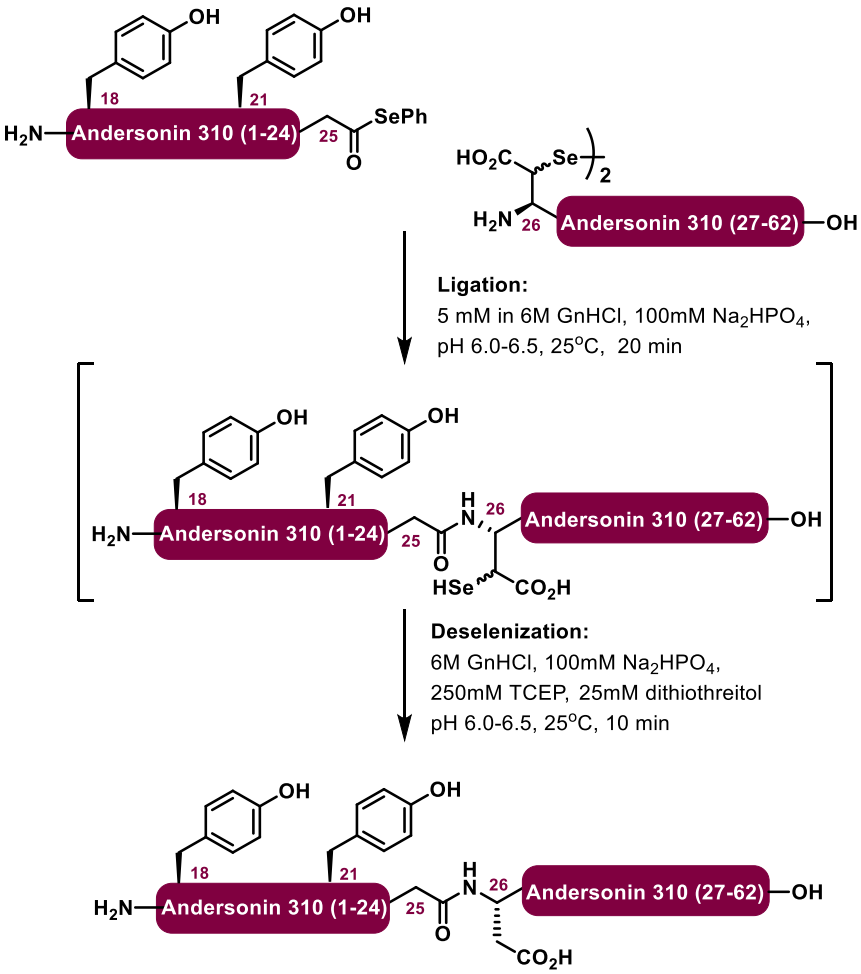


Figure S100: Synthesis of **And310 Un** via DSL-deselenization.

The one-pot peptide ligation of **And310 Un (1-25)** selenoester (2.4 mg, 0.77 µmol) and **And310 (26-62)** diselenide (2.0 mg, 0.51 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0 to 40% B over 40 min, 0.1% v/v TFA) followed by lyophilization afforded final protein **And310 Un** as a white solid (2.2 mg, 64%). Analytical HPLC (purified final product): R_t 4.00 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+6H]^{6+}$: 1131.8 $[M+7H]^{7+}$: 970.3 $[M+8H]^{8+}$: 849.1 $[M+9H]^{9+}$: 754.9 $[M+10H]^{10+}$: 679.5 Mass Found (ESI) 1132.1 $[M+6H]^{6+}$, 970.7 $[M+7H]^{7+}$, 849.3 $[M+8H]^{8+}$, 755.1 $[M+9H]^{9+}$, 679.6 $[M+10H]^{10+}$. High Res (ESI+): calcd for $C_{288}H_{450}N_{88}O_{103}$: $[M+8H]^{8+}$, 849.03419. Found: 849.03475 $[M+5H]^{5+}$. Difference: 0.8 ppm.

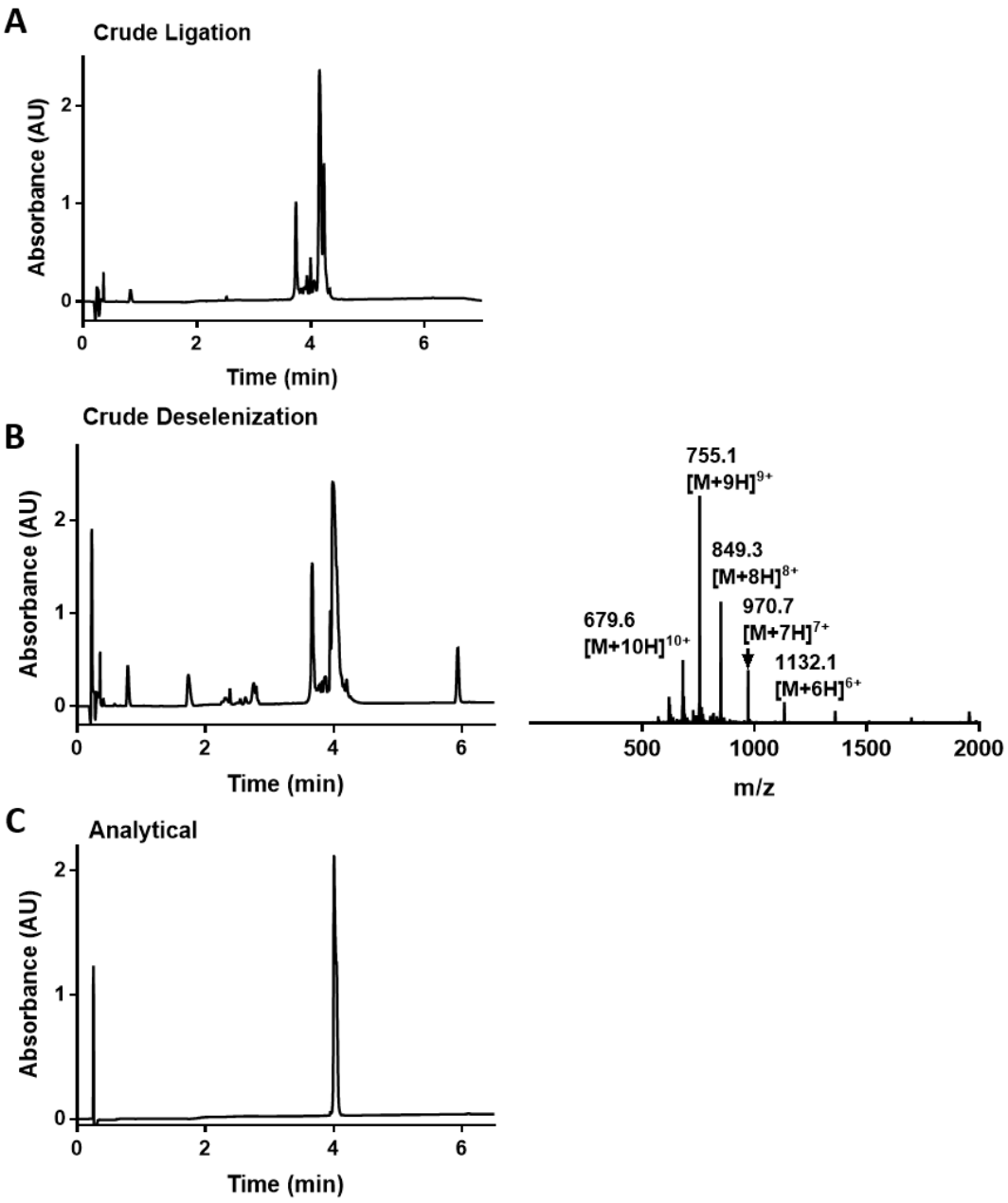


Figure S101: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **And310 Un**.

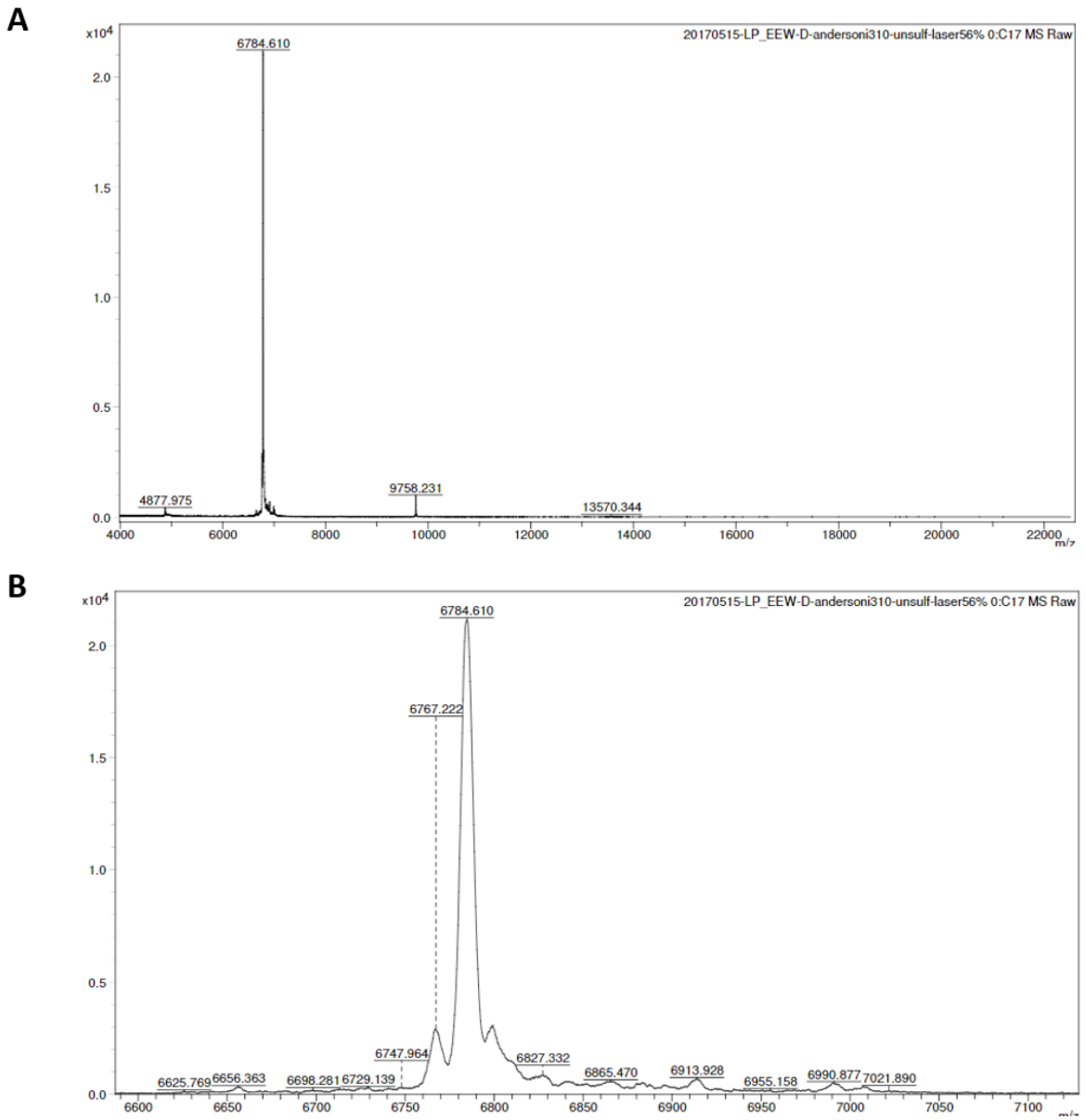


Figure S102: MALDI-TOF mass spectrum of **And310 Un** (*A*) 4000-22000 m/z and (*B*) 6600-7100 m/z.

And310 Y18

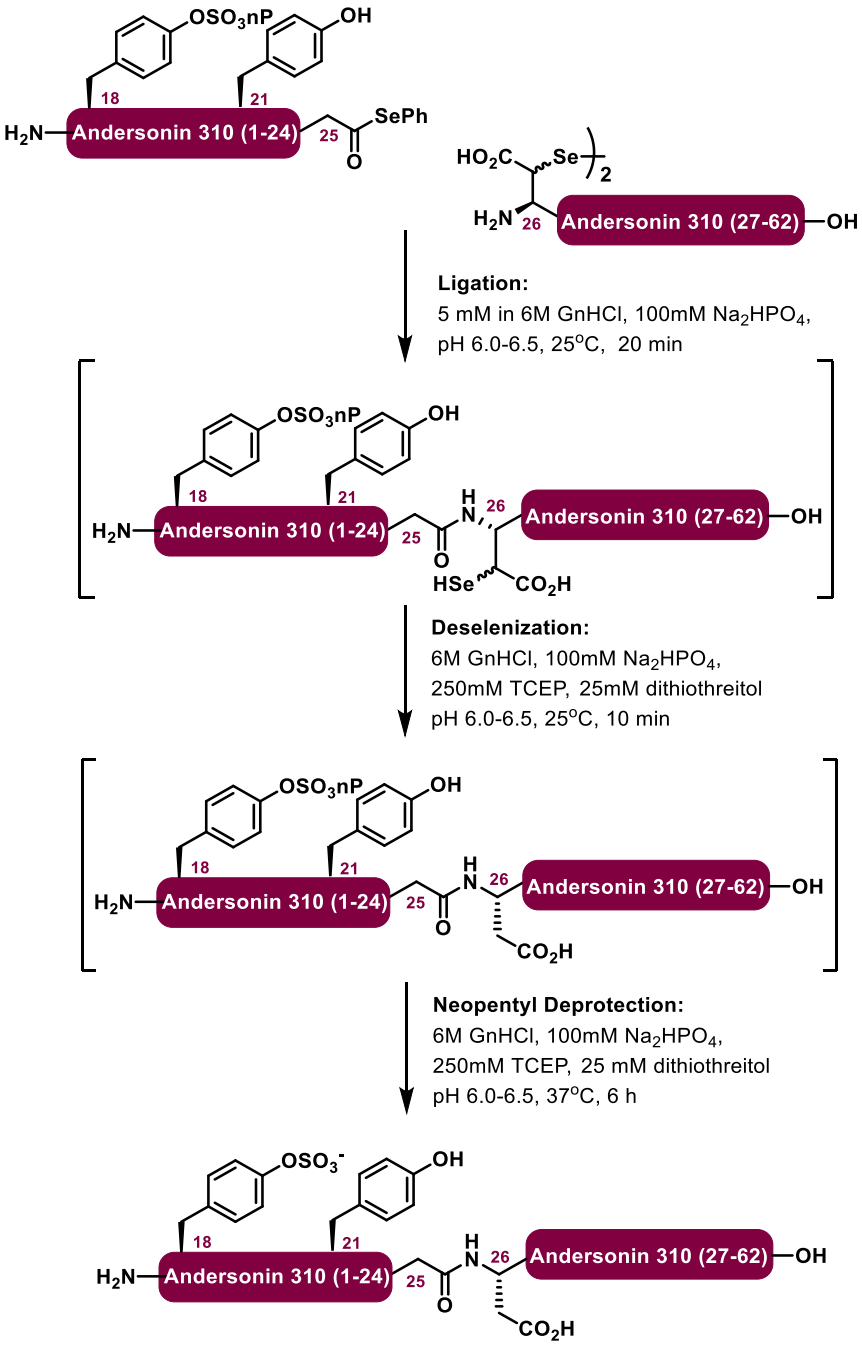


Figure S103: Synthesis of **And310 Y18** via DSL-deselenization.

The one-pot peptide ligation of **And310 Y18 (1-25)** selenoester (2.4 mg, 0.73 µmol) and **And310 (26-62)** diselenide (1.9 mg, 0.49 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0 to 40% B over 40 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **And310 Y18** as a white solid (1.6 mg, 50%). Analytical HPLC (purified final product): R_t 4.01 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+5H]^{5+}$: 1373.2 $[M+6H]^{6+}$: 1144.5, $[M+7H]^{7+}$: 981.2 $[M+8H]^{8+}$: 858.7 $[M+9H]^{9+}$: 763.4 $[M+10H]^{10+}$: 687.2 Mass Found (ESI) 1374.8 $[M+5H]^{5+}$, 1143.7 $[M+6H]^{6+}$, 982.1 $[M+7H]^{7+}$, 859.4 $[M+8H]^{8+}$, 764.0 $[M+9H]^{9+}$, 687.7 $[M+10H]^{10+}$. High Res (ESI+): calcd for $C_{288}H_{448}N_{88}O_{106}S$: $[M+6H]^{6+}$, 1145.03575. Found: 1145.03652 $[M+5H]^{5+}$. Difference: 0.6 ppm.

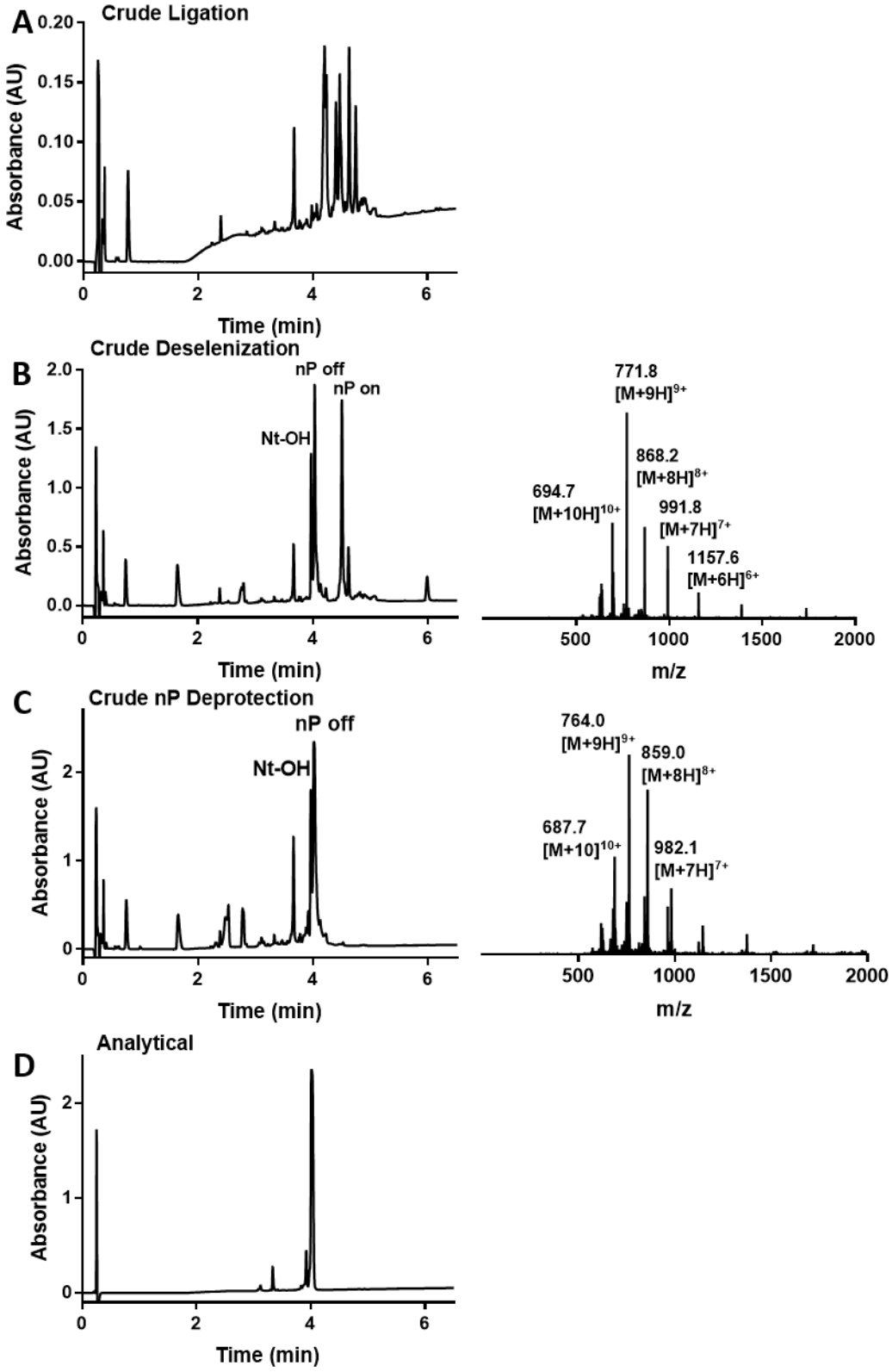


Figure S104: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **And310 Y18**.

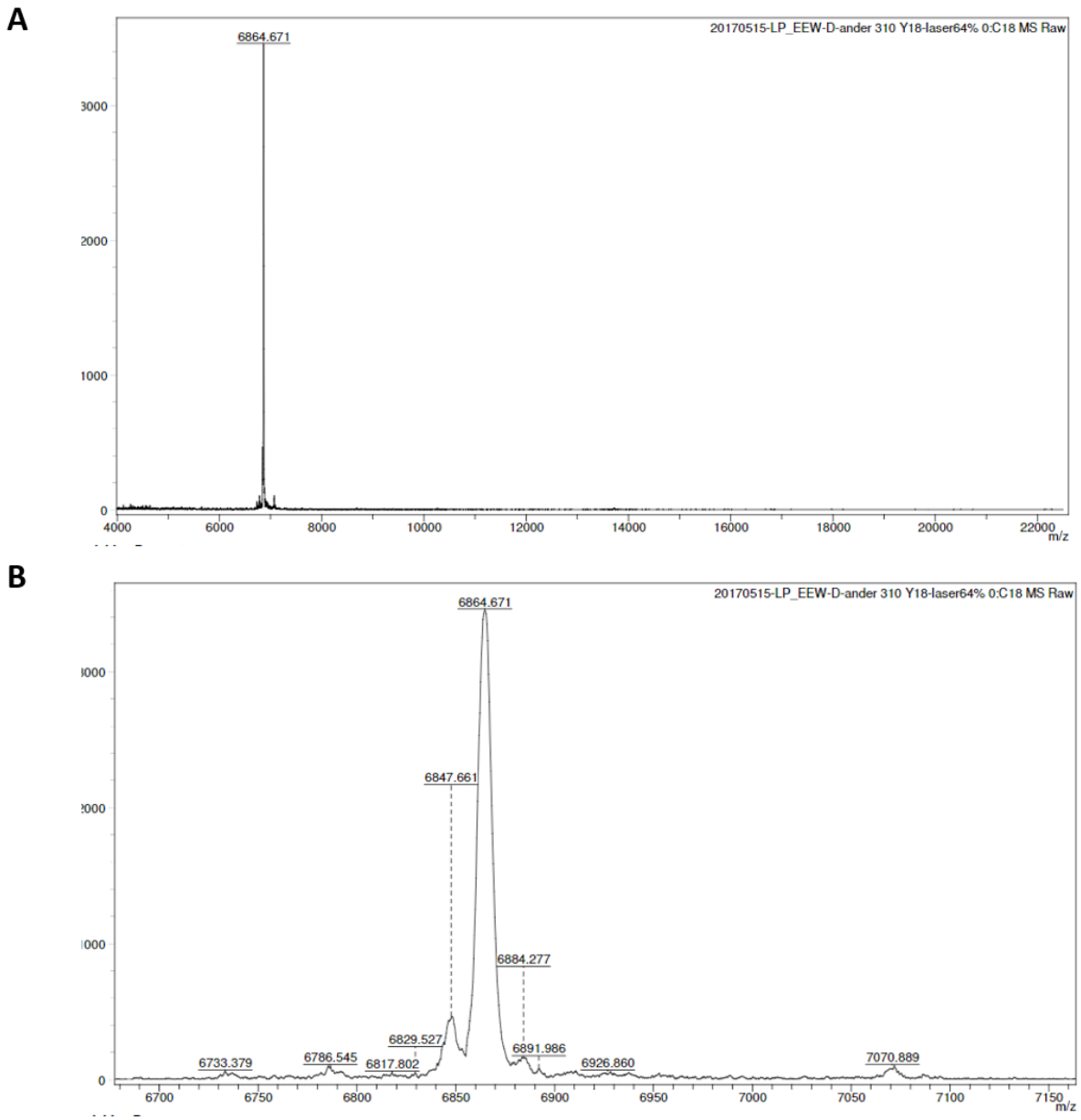


Figure S105: MALDI-TOF mass spectrum of **And310 Y18** (*A*) 4000-22000 m/z and (*B*) 6700-7150 m/z.

And310 Y21

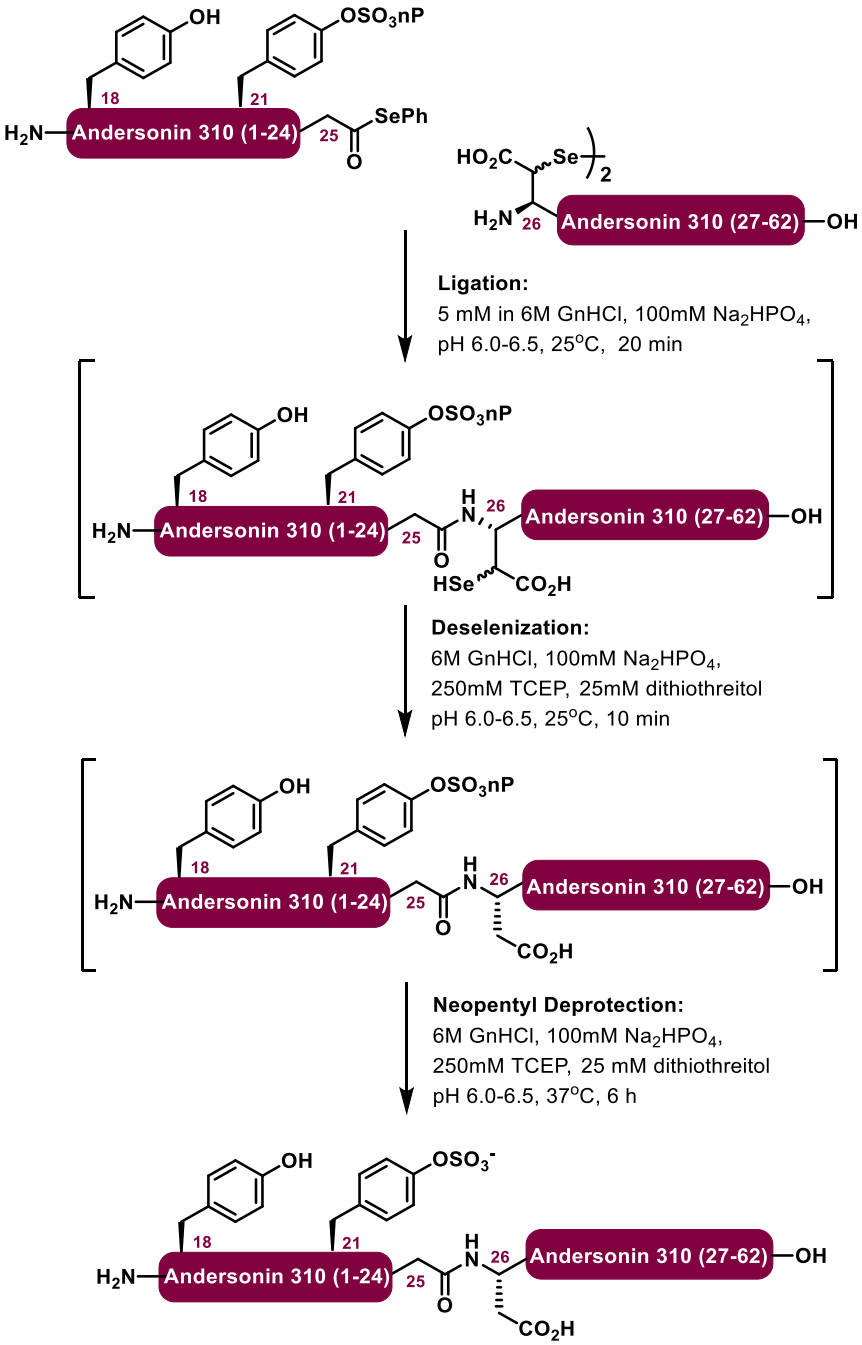


Figure S106: Synthesis of **And310 Y21** via DSL-deselenization.

The one-pot peptide ligation of **And310 Y21 (1-25)** selenoester (1.9 mg, 0.58 µmol) and **And310 (26-62)** diselenide (1.5 mg, 0.40 µmol) followed by *in situ* deselenization was performed according to the general procedure. Purification via preparative HPLC (0 to 40% B over 40 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **And310 Y21** as a white solid (1.4 mg, 51%). Analytical HPLC (purified final product): R_t 3.98 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+5H]^{5+}$: 1374.2, $[M+6H]^{6+}$: 1145.2, $[M+7H]^{7+}$: 981.7, $[M+8H]^{8+}$: 859.1 $[M+9H]^{9+}$: 763.8 $[M+10H]^{10+}$: 687.5 Mass Found (ESI) 1374.9 $[M+5H]^{5+}$, 1145.9 $[M+6H]^{6+}$, 982.3 $[M+7H]^{7+}$, 859.6 $[M+8H]^{8+}$, 764.1 $[M+9H]^{9+}$, 687.8 $[M+10H]^{10+}$. High Res (ESI+): calcd for $C_{288}H_{449}N_{88}O_{106}S$: $[M+7H]^{7+}$, 981.74640. Found: 981.74628 $[M+5H]^{5+}$. Difference: 1.2 ppm.

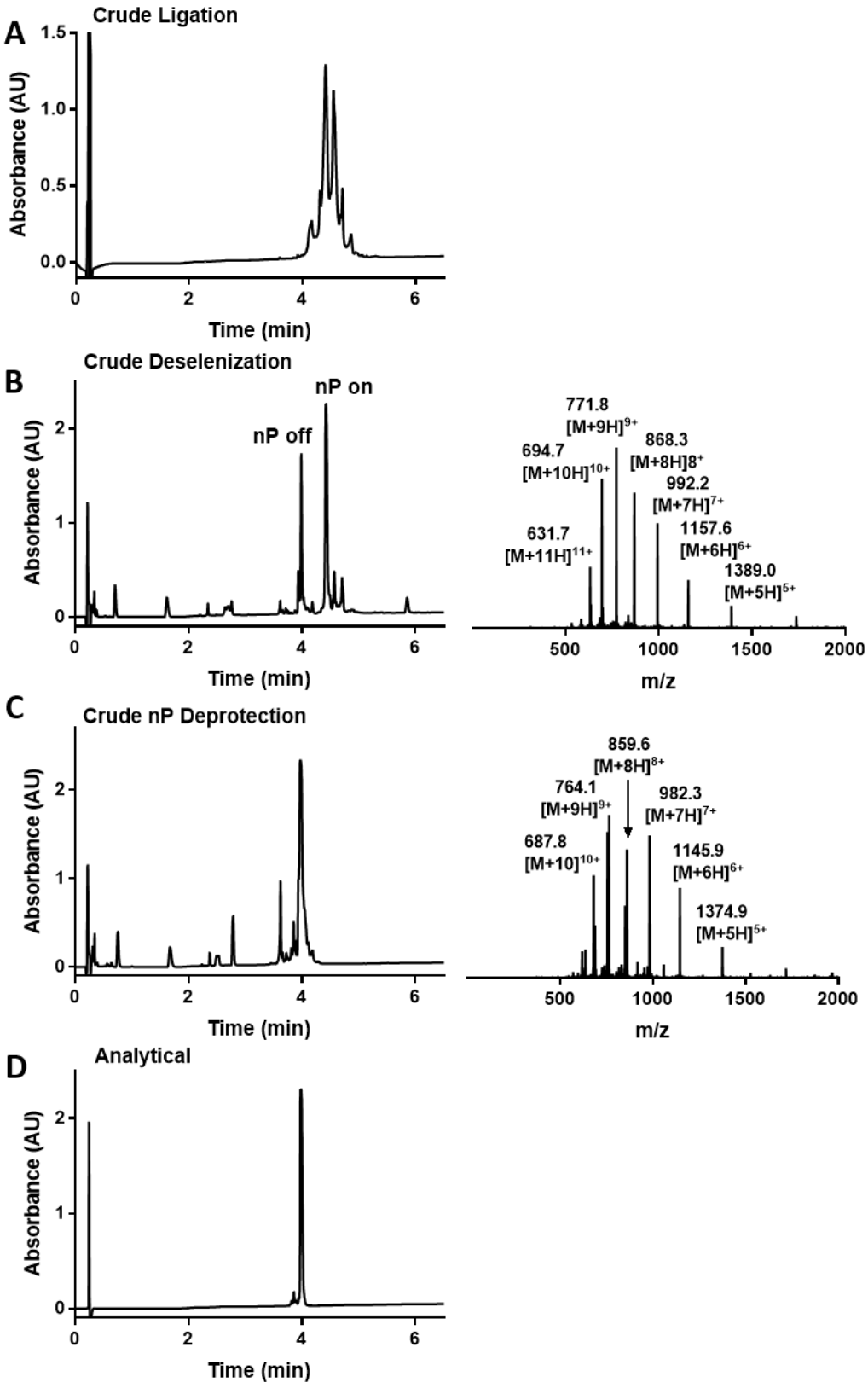
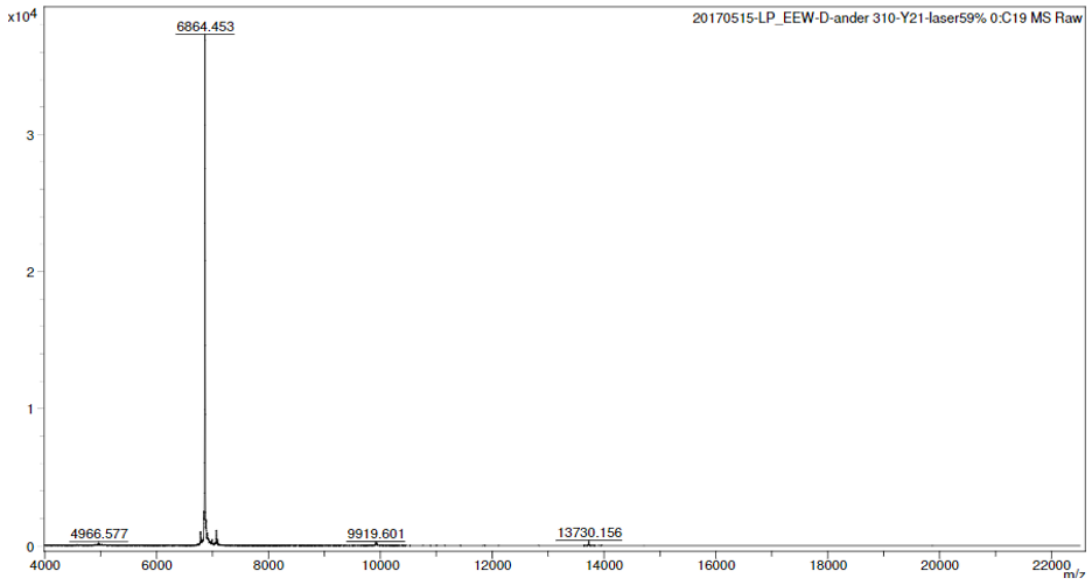


Figure S107: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **And310 Y21**.

A



B

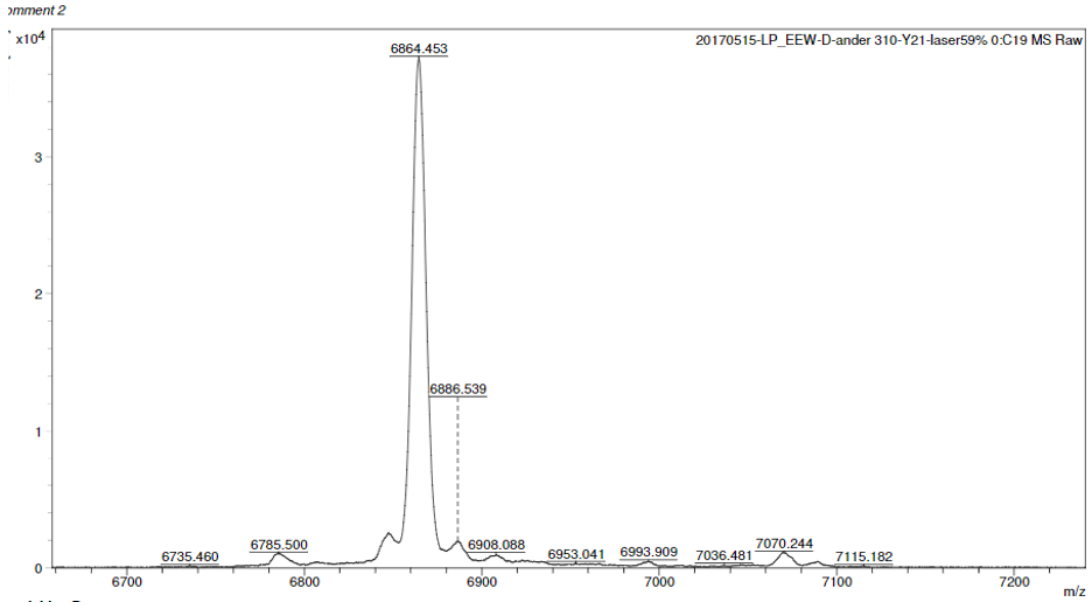


Figure S108: MALDI-TOF mass spectrum of **And310 Y21** (*A*) 4000-22000 m/z and (*B*) 6700-7200 m/z.

And310 DS

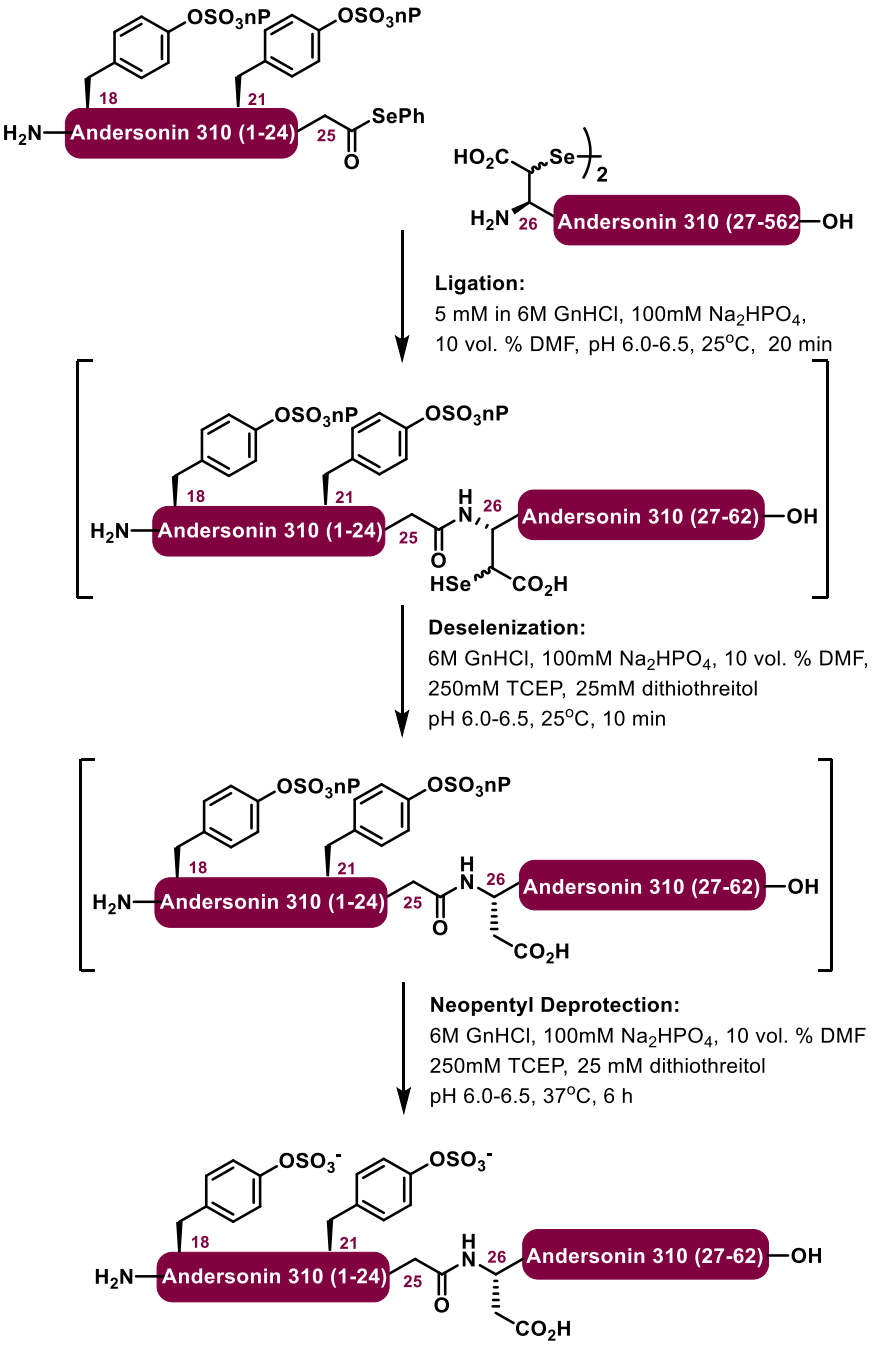


Figure S109: Synthesis of **And310 DS** via DSL-deselenization.

The one-pot peptide ligation of **And310 DS (1-25)** selenoester (1.4 mg, 0.39 µmol) and **And310 (26-62)** diselenide (1.0 mg, 0.26 µmol) followed by *in situ* deselenization was performed according to the general procedure, replacing 10% of the buffer volume with DMF. Purification via preparative HPLC (0 to 40% B over 40 min, 0.1% v/v Formic acid) followed by lyophilization afforded final protein **And310 DS** as a white solid (1.1 mg, 63%). Analytical HPLC (purified final product): R_t 3.99 min (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm); Calculated Mass $[M+5H]^{5+}$: 1390.7 $[M+6H]^{6+}$: 1158.5, $[M+7H]^{7+}$: 993.1 $[M+8H]^{8+}$: 869.1 $[M+9H]^{9+}$: 772.7 Mass Found (ESI) 1391.0 $[M+5H]^{5+}$, 1159.3 $[M+6H]^{6+}$, 993.8 $[M+7H]^{7+}$, 869.6 $[M+8H]^{8+}$, 772.6 $[M+9H]^{9+}$. High Res (ESI+): calcd for $C_{288}H_{450}N_{88}O_{109}S_2$: $[M+8H]^{8+}$, 869.14854. Found: 869.14965 $[M+8H]^{8+}$. Difference: 1.4 ppm.

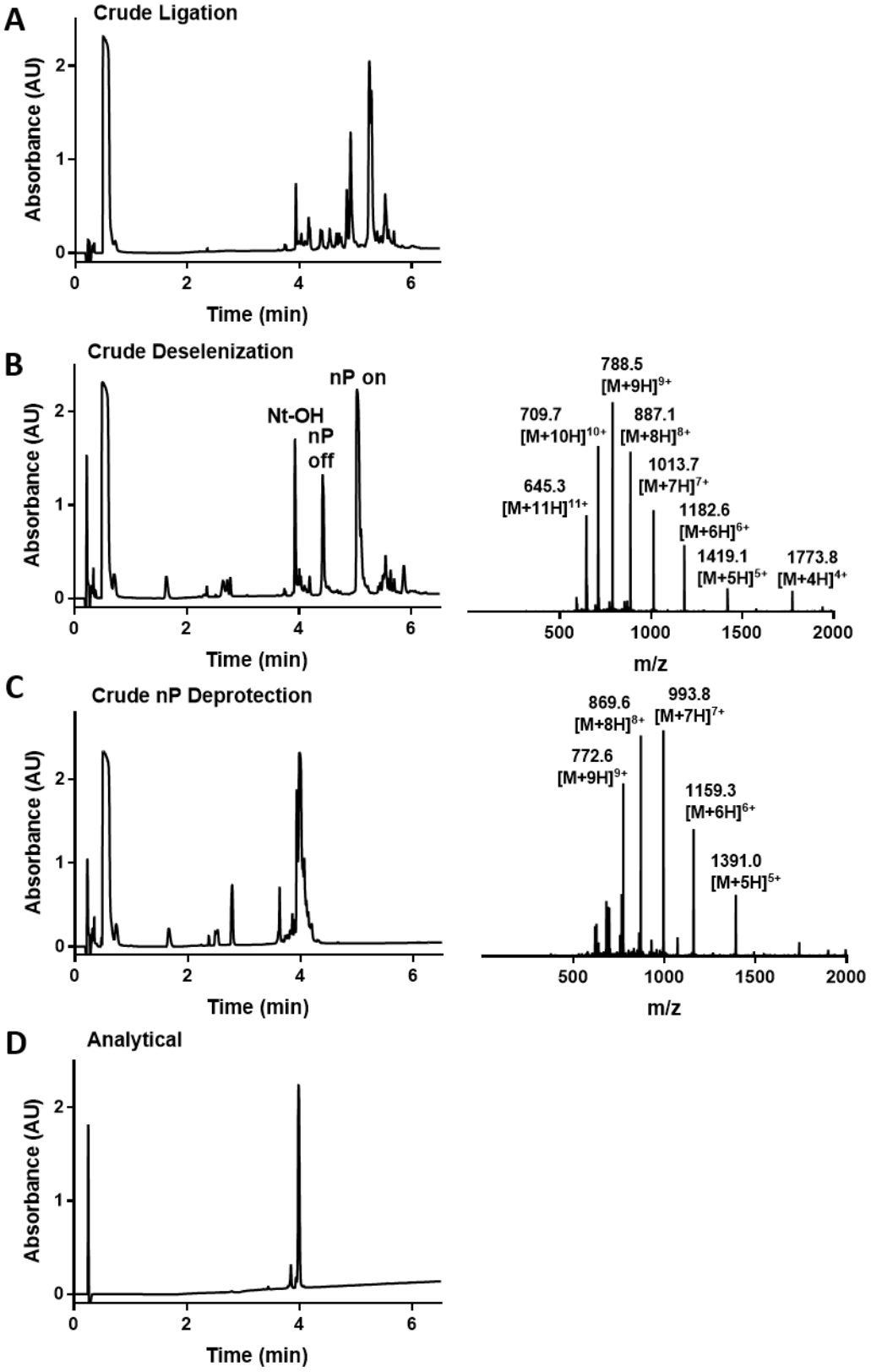


Figure S110: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **And310 DS**.

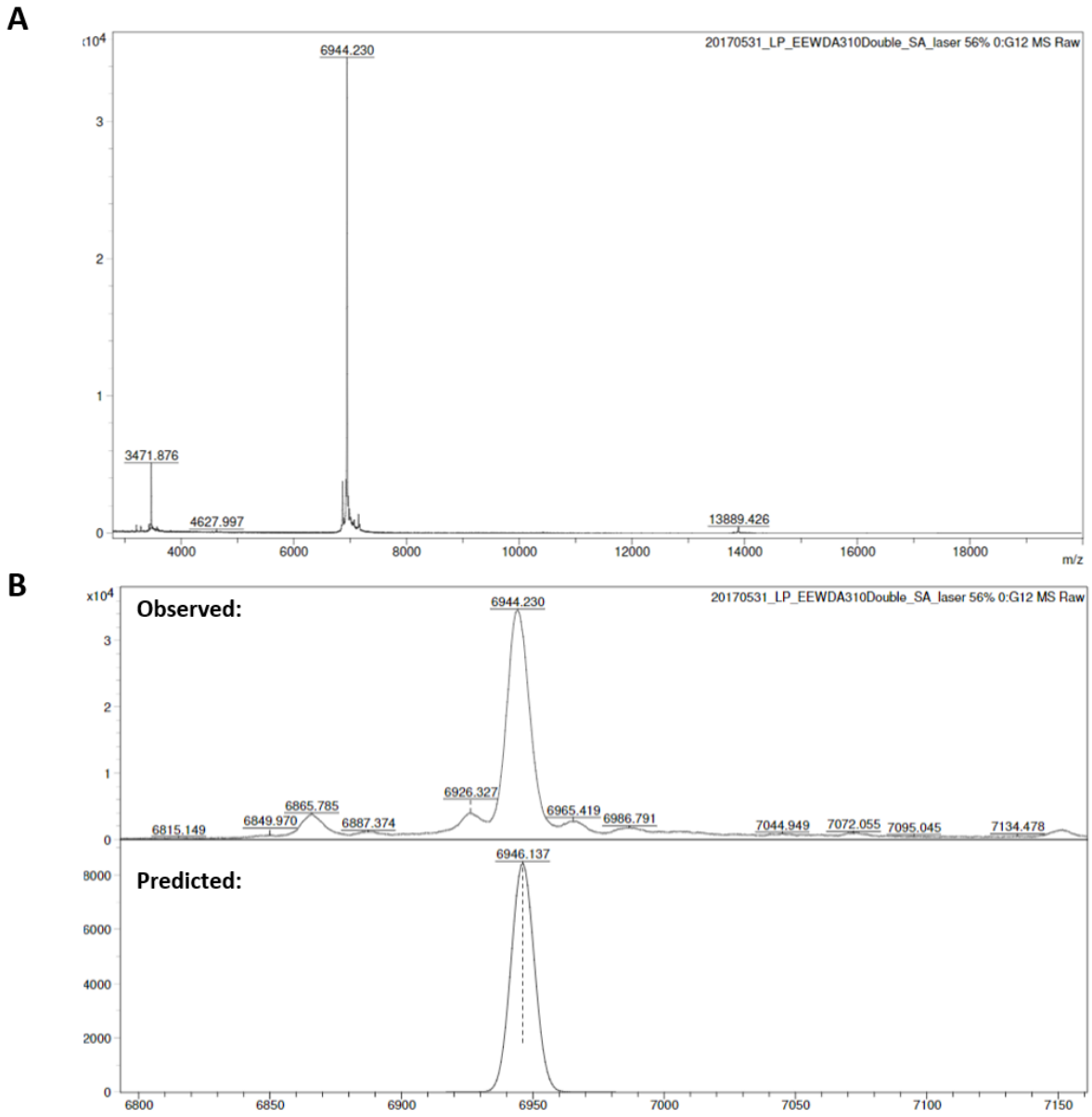


Figure S111: MALDI-TOF mass spectrum of **And310 DS** (*A*) 4000-2000 m/z and (*B*) 6800-7150 m/z.

In vitro Inhibition of Human α- and γ-Thrombin and Mouse α-Thrombin

The inhibition of the amidolytic activity of human α- or γ-thrombin or of mouse α-thrombin (Haematologic Technologies) was followed spectrophotometrically using Tos-Gly-Pro-Arg-p-nitroanilide (Chromozym TH; Roche) as chromogenic substrate. Inhibition assays were performed using 0.2 nM enzyme, 100 µM substrate, and increasing concentrations of inhibitor. The concentration of each inhibitor variant was determined using a Direct Detect Infrared Spectrometer (Millipore). The inhibition constants (K_i) of all inhibitor variants (except for the double methylated andersonin variants) were determined according to a tight-binding model by fitting the inhibited steady-state velocity data to the Morrison equation (2). All reactions were carried out at 37 °C in 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mg/mL BSA in 96-well microtiter plates. Reaction progress was monitored at 405 nm for 1–2 h on a Synergy2 Multimode microplate reader (BioTek). Dose–response curves were used to determine the K_i values using Prism 8.0 (GraphPad Software). For each inhibitor, at least two independent experiments with duplicate reactions were performed, together with control reactions in the absence of enzyme. For all curves, the goodness of fitting parameter R^2 was between 0.989 and 0.999.

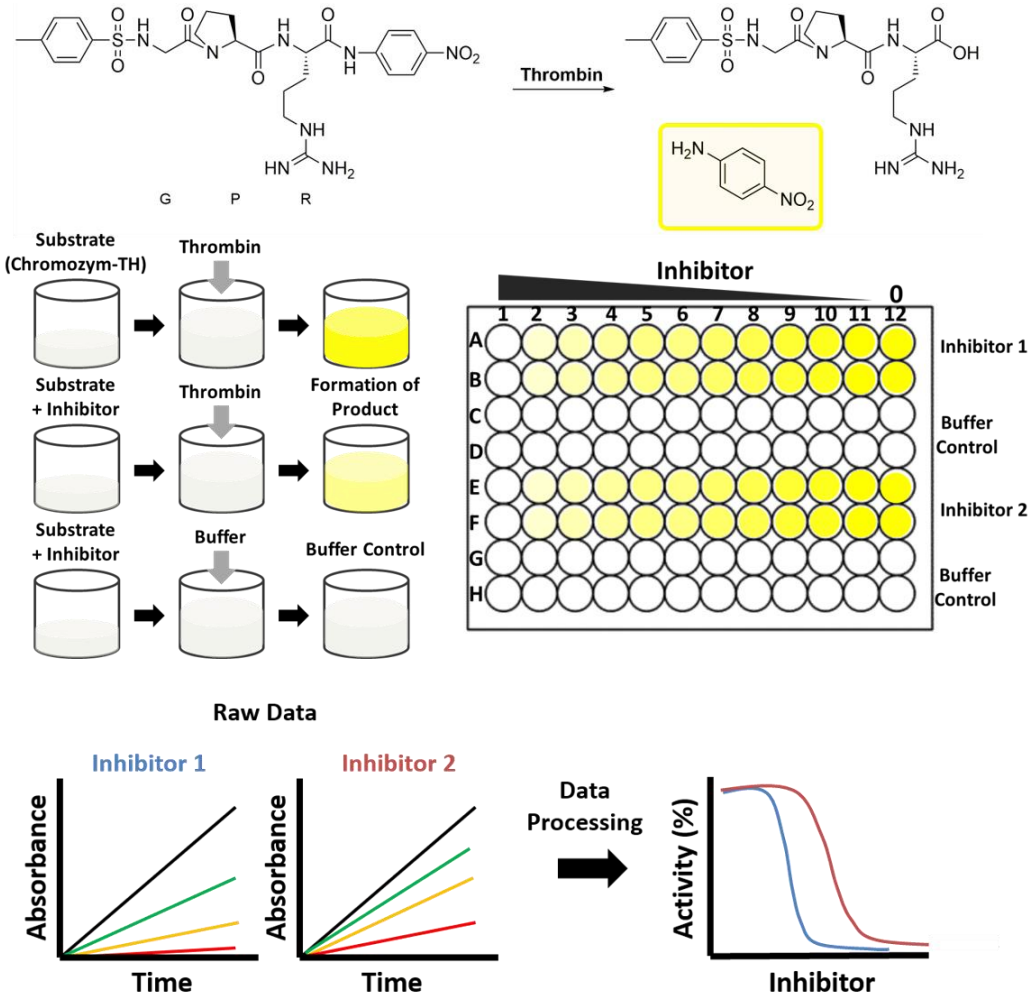


Figure S112: Schematic representation of the *in vitro* thrombin inhibition assay described above.

Thrombin-Time (TT) Assay

The anticoagulant activities of the tick-derived thrombin inhibitors were determined by measuring their ability to prolong clotting of human plasma. Human plasma (800 µl) was mixed with 200 µl of inhibitor (1, 2, 10 and 20 nM final concentration in 20 mM Tris-HCl pH 8.0, 50 mM NaCl), and TT was measured at a commercial diagnostics laboratory (BMAC – Análises Clínicas, Portugal) following standard protocols.

Activated Partial Thromboplastin Time (aPTT) Assays

Animals: Male C57Bl/6 mice weighed 25-30 grams were used for harvesting mouse plasma. C57Bl/6 mice were all sourced from Australian BioResources. All procedures involving the use of mice were approved by the University of Sydney Animal Ethics Committee (Project # 2017/1197). All animals were housed in specific pathogen free (SPF) facility in accordance with the Australian code of practice for the care and use of animals for scientific purposes and maintained on a 12/12 h light/dark cycle and allowed free access to food and water. Animals were fully anaesthetized with Lethabarb (~100mg/kg) before blood collection.

aPTT assay: To test the potency of the direct thrombin inhibitors to thrombin inhibition via exosite II and active site each compound was added to human (*in vitro*) and mouse (*in vitro*) normal plasma at concentrations of 0, 0.4, 0.8, 1.6, 2, 3.2, 4.8, 6.4, 8, 9.6, 12 and 15 µg/mL prior to performing the activated partial thromboplastin time (aPTT) assay.

Mouse and human blood was collected into sodium citrate (3.8%) containing syringes, and the citrated blood was centrifuged at 4 °C, 13,400 rpm for 15 min to obtain plasma. Individual healthy human plasma was used without pooling whereas mouse plasma was pooled before aPTT measurement. aPTT was measured by using an aPTT kit according to the manufacturer's instructions (Siemens Healthineers). aPTT was performed to measure the intrinsic activation pathway of the coagulation system and the activation was initiated by addition of ellagic acid (1.0×10^{-4} M) with the presence of purified soy bean phosphatides and calcium chloride (0.025 mol/L) to the plasma.

More specifically, all reagents (ACTIN FS & calcium chloride) and fresh plasma were pre-warmed to 37 °C before aPTT measurement. Initially, 25 µL of aPTT-ACTIN FS solution was added, mixed well with 25 µL plasma in a 96-well plate and incubated for 3 min at 37 °C. Next, 25 µL of the kit calcium chloride solution was added to the mixture to initiate the reaction. The time taken for the mixture to turn from transparent to opaque/white web-like precipitate as an indication of fibrin formation was recorded after addition of calcium chloride. The entire aPTT measurement was performed in duplicate and fully automated by the microplate-reader equipped with automated injectors (CLARIOstar, BMG Labtech). The dose response effects (extension of clotting time) of the thrombin inhibitor candidates on aPTT were plotted and compared.

Thrombin Inhibition Data

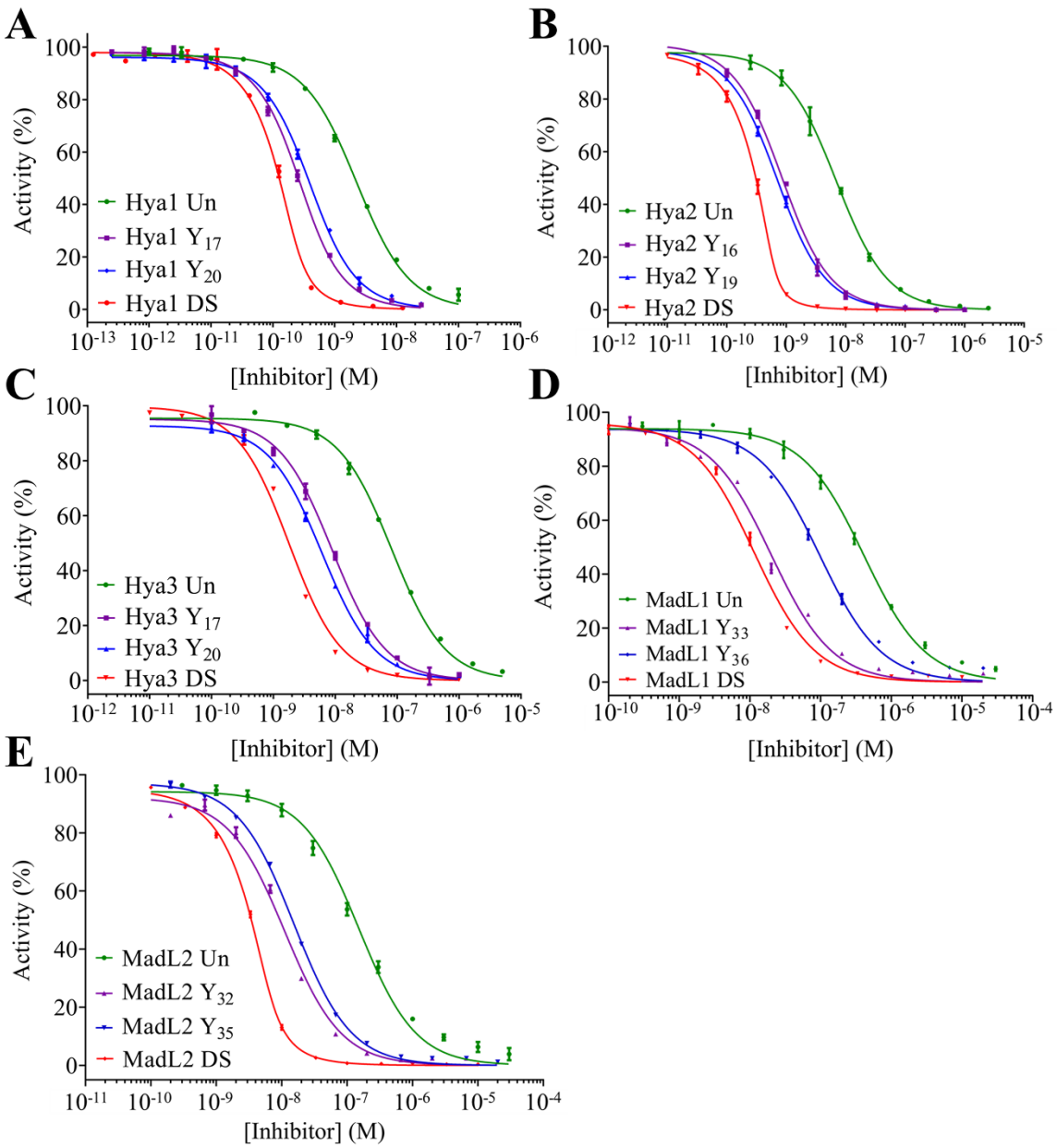


Figure S113: Tyrosine sulfation strongly modulates the anti-thrombin activity of Hyalomin and Madanin-like inhibitors *in vitro*. Dose–response curves for the inhibition of the amidolytic activity of human α-thrombin (0.2 nM) toward Chromozym-TH (100 μM) by increasing concentrations of synthetic unsulfated (green curves), monosulfated (blue and purple curves) or disulfated (red curves) sulfoforms in an amidolytic assay. (*A*) Hyalomin 1. (*B*) Hyalomin 2. (*C*) Hyalomin 3. (*D*) Madanin-like 1. (*E*) Madanin-like 2. Errors bars represent SEM values.

Table S1: Inhibition constants (K_i) for Hyalomin and Madanin-like sulfoforms, determined by fitting the inhibited steady-state velocity data to the Morrison model. The given K_i values ± SEM are representative of two independent experiments. Also shown are the thrombin times (TTs) in seconds, measuring the clotting time of human plasma in the presence of different concentrations of synthetic sulfoforms (at 1, 2, 10 and 20 nM). In the absence of inhibitor, the TT was 19.5 s (mean of four replicates). Errors are depicted as standard deviations of the mean from two independent measurements.

		Thrombin Time (seconds)			
Inhibitor	**K_i**	**1 nM**	**2 nM**	**10 nM**	**20 nM**
Hya1 Un	388.8 ± 16.7 pM; R^2 = 0.998	22 ± 0	22.5 ± 0.71	24 ± 0	
Hya1 Y17	30.29 ± 1.31 pM; R^2 = 0.999	22.5 ± 0.71	23 ± 0	48.5 ± 3.54	
Hya1 Y20	53.09 ± 2.13 pM; R^2 = 0.999	23 ± 0	23 ± 0	44 ± 12.73	
Hya1 DS	5.39 ± 0.63 pM; R^2 = 0.998	23 ± 0	24 ± 0	70.5 ± 4.95	
Hya2 Un	1236 ± 59.6 pM; R^2 = 0.998		22 ± 0	22.5 ± 0.71	25.5 ± 2.12
Hya2 Y16	133.6 ± 7.7 pM; R^2 = 0.997		22.5 ± 0.71	27 ± 0	34 ± 0
Hya2 Y19	109.8 ± 3.4 pM; R^2 = 0.999		23.5 ± 0.71	45 ± 0	89.5 ± 20.51
Hya2 DS	5.4 ± 0.8 pM; R^2 = 0.999		23 ± 0	49.5 ± 2.12	135.5 ± 16.26
Hya3 Un	14.71 ± 0.63 nM; R^2 = 0.998		22.5 ± 0.71	24 ± 0	26.5 ± 0.71
Hya3 Y17	1.58 ± 0.08 nM; R^2 = 0.997		23 ± 0	27 ± 1.41	31 ± 1.41
Hya3 Y20	1.08 ± 0.05 nM; R^2 = 0.998		24 ± 0	26.5 ± 0.71	31.5 ± 2.12
Hya3 DS	0.304 ± 0.019 nM; R^2 = 0.996		23 ± 0	31.5 ± 0.71	46 ± 1.41
MadL1 Un	73.84 ± 4.17 nM; R^2 = 0.996		19 ± 0	22.5 ± 0.71	20 ± 0
MadL1 Y33	3.59 ± 0.28 nM; R^2 = 0.993		20 ± 0	22.5 ± 0.71	29.5 ± 0.71
MadL1 Y36	17.60 ± 1.09 nM; R^2 = 0.995		20 ± 0	24 ± 0	28 ± 0
MadL1 DS	2.06 ± 0.12 nM; R^2 = 0.996		22.5 ± 0.71	24.5 ± 0.71	30 ± 0
MadL2 Un	27.22 ± 2.23 nM; R^2 = 0.992		20 ± 0	20 ± 0	19 ± 0
MadL2 Y32	1.99 ± 0.14 nM; R^2 = 0.995		20 ± 0	23.5 ± 0.71	28 ± 0
MadL2 Y35	2.77 ± 0.09 nM; R^2 = 0.999		19.5 ± 0.71	22.5 ± 0.71	25.5 ± 0.71
MadL 2 DS	0.156 ± 0.02 nM; R^2 = 0.999		21 ± 0	34.5 ± 0.71	56.5 ± 0.71
		Control 19.5 ± 0.58			

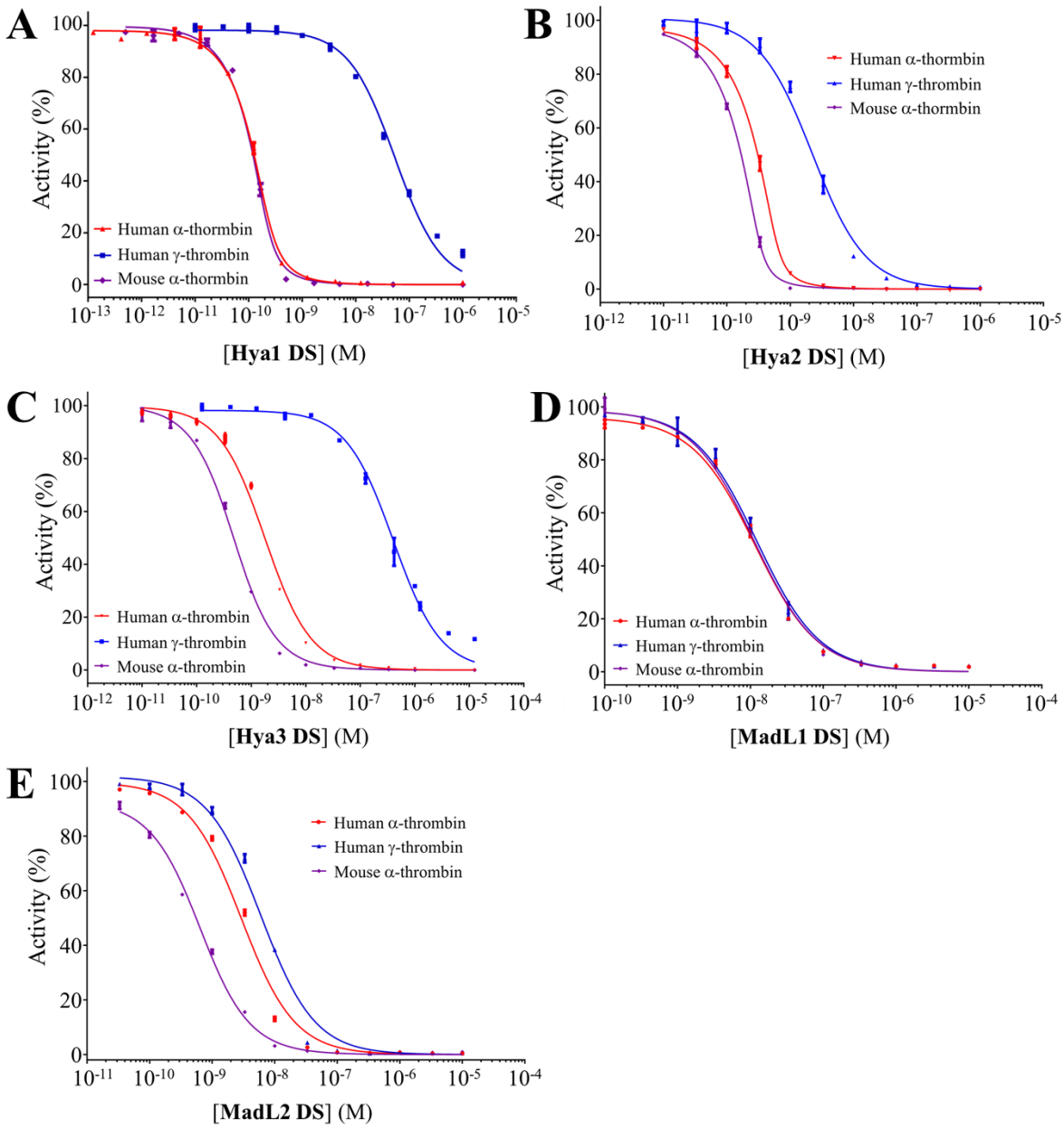


Figure S114: Dose-response curves for the inhibition of human α- and γ-thrombin (red and blue, respectively) and mouse α-thrombin (purple) activity by increasing concentrations of synthetic disulfated variants. (*A*) Hyalomin 1 (**Hya1 DS**). (*B*) Hyalomin 2 (**Hya2 DS**). (*C*) Hyalomin 3 (**Hya3 DS**). (*D*) Madanin-like 1 (**MadL1 DS**). (*E*) madanin-like 2 (**MadL2 DS**).

Table S2: Inhibition constants (K_i) for disulfated sulfoforms, determined by fitting the inhibited steady-state velocity data to the Morrison model. The given K_i values ± SEM are representative of two independent experiments.

	K_i		
Inhibitor	**Human α-thrombin**	**Human γ-thrombin**	**Mouse α-thrombin**
Hya1 DS	5.39 ± 0.63 pM; R^2 = 0.998	14.6 ± 0.98 nM; R^2 = 0.991	1.46 ± 0.21 pM; R^2 = 0.997
Hya2 DS	5.4 ± 0.8 pM; R^2 = 0.999	580.7 ± 43.4 pM; R^2 = 0.995	1.05 ± 0.16 pM; R^2 = 0.999
Hya3 DS	304.4 ± 19.61 pM; R^2 = 0.996	109.2 ± 7.87 nM; R^2 = 0.989	26.16 ± 1.40 pM; R^2 = 0.998
MadL1 DS	2.06 ± 0.12 nM; R^2 = 0.996	3.30 ± 0.21 nM; R^2 = 0.996	0.778 ± 0.035 nM; R^2 = 0.998
MadL2 DS	156.3 ± 17.2 pM; R^2 = 0.999	430.9 ± 80.8 pM; R^2 = 0.999	37.44 ± 2.06 pM; R^2 = 0.997

Assembly of Chimeric Thrombin Inhibitors

Hya1/3 DS

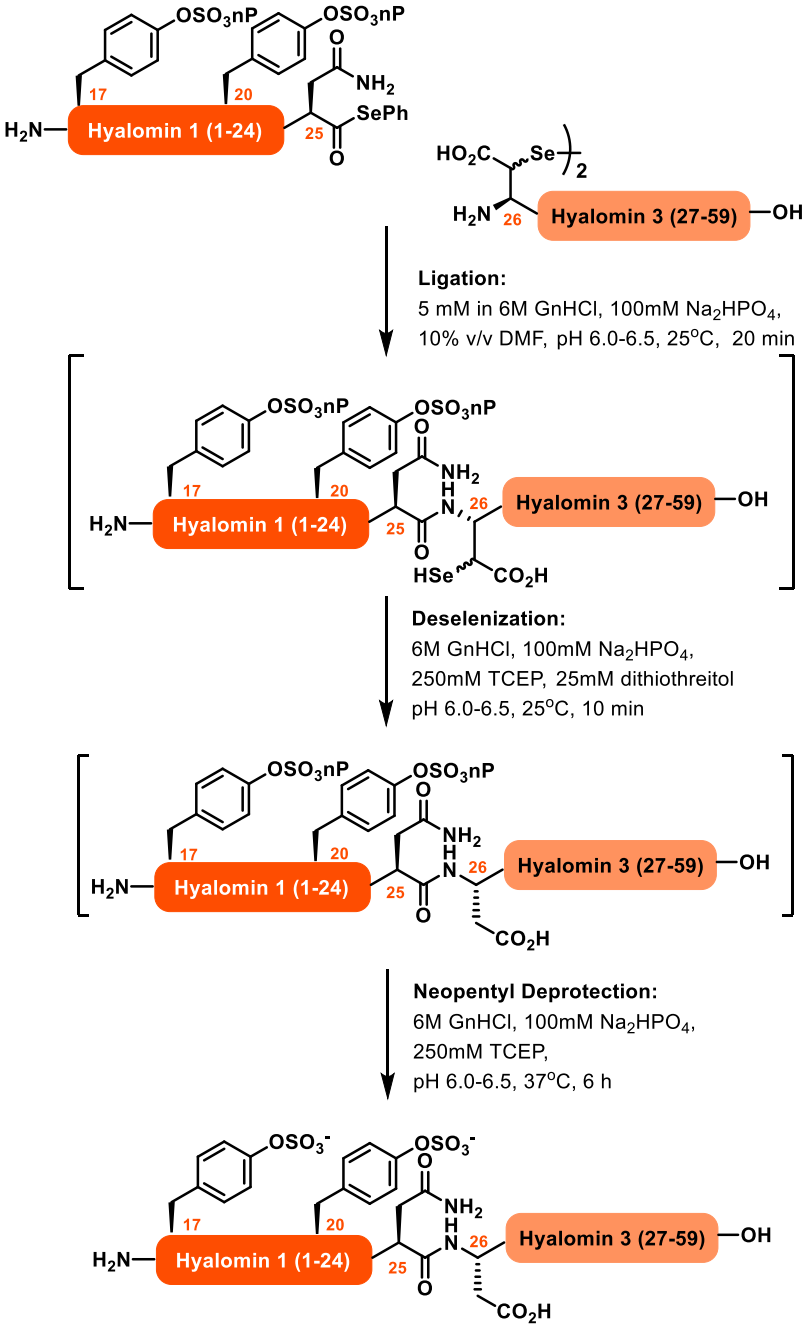


Figure S115: Synthesis of **Hya1/3 DS** via DSL-deselenization.

The one-pot peptide ligation of **Hya1 DS (1-25)** selenoester (3.2 mg, 0.97 µmol) and **Hya3 (26-59)** diselenide (2.2 mg, 0.65 µmol) followed by *in situ* deselenization was performed according to the general procedure, replacing 10% of the buffer volume with DMF. Neopentyl deprotection was the performed as described in the general procedure, followed by purification *via* preparative HPLC (0 to 40% B ov 40 min, 0.1% FA) followed by lyophilisation afforded final protein **Hya1/3 DS** as a white solid (1.8 mg, 45%). Analytical HPLC (purified final product): R_t 4.08 min (0 to 50% B ov 5 min, 0.1% TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1582.8 $[M+5H]^{5+}$: 1266.4 $[M+6H]^{6+}$: 1055.5 Mass Found (ESI) 1582.4 $[M+4H]^{4+}$, 1266.1 $[M+5H]^{5+}$, 1055.0 $[M+6H]^{6+}$. High Res (ESI+): calcd for $C_{252}H_{383}N_{75}O_{112}S_2$: $[M+5H]^{5+}$, 1269.52879. Found: 1269.52991 $[M+5H]^{5+}$. Difference: 0.6 ppm.

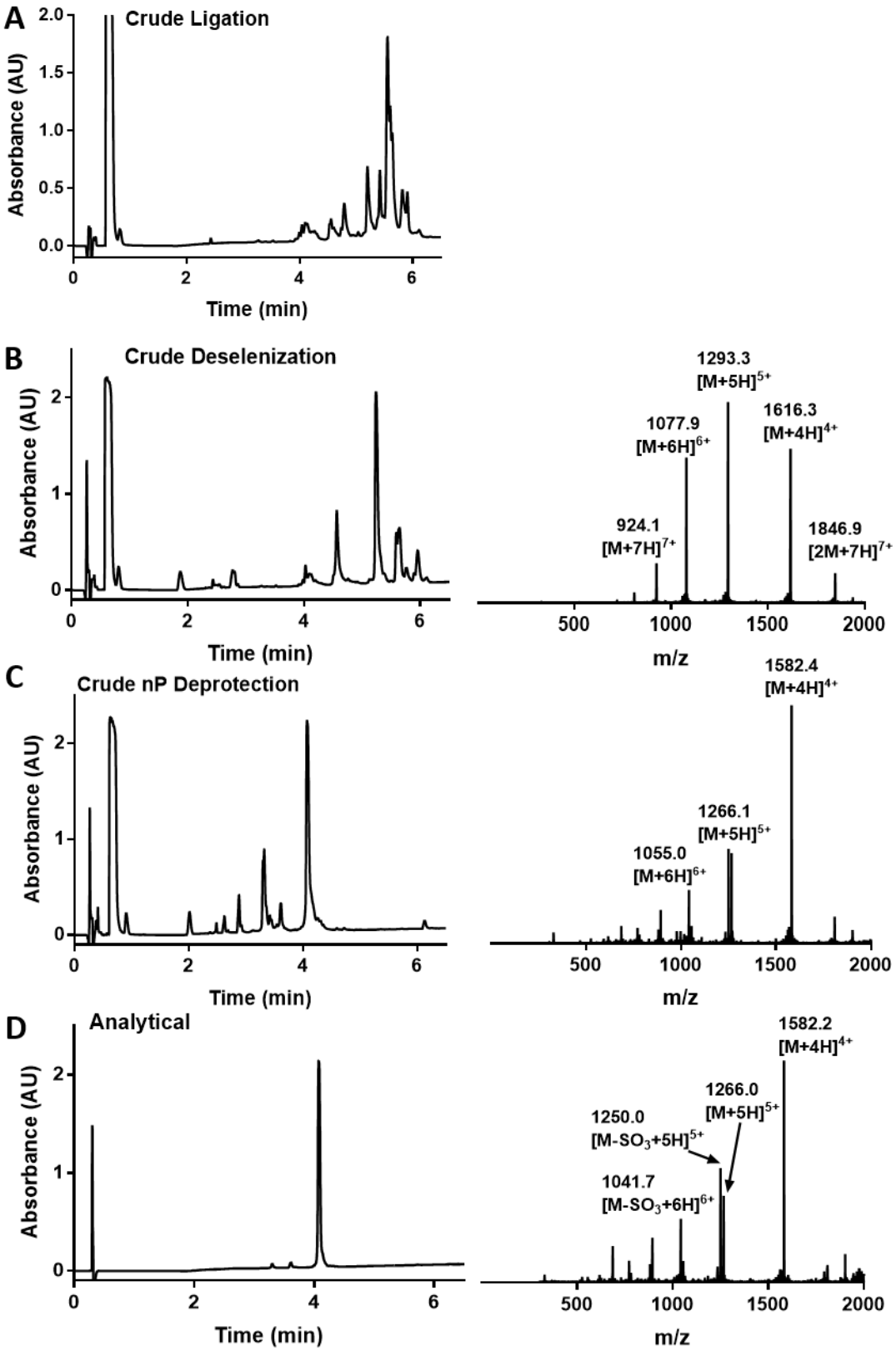


Figure S116: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **Hya1/3 DS**.

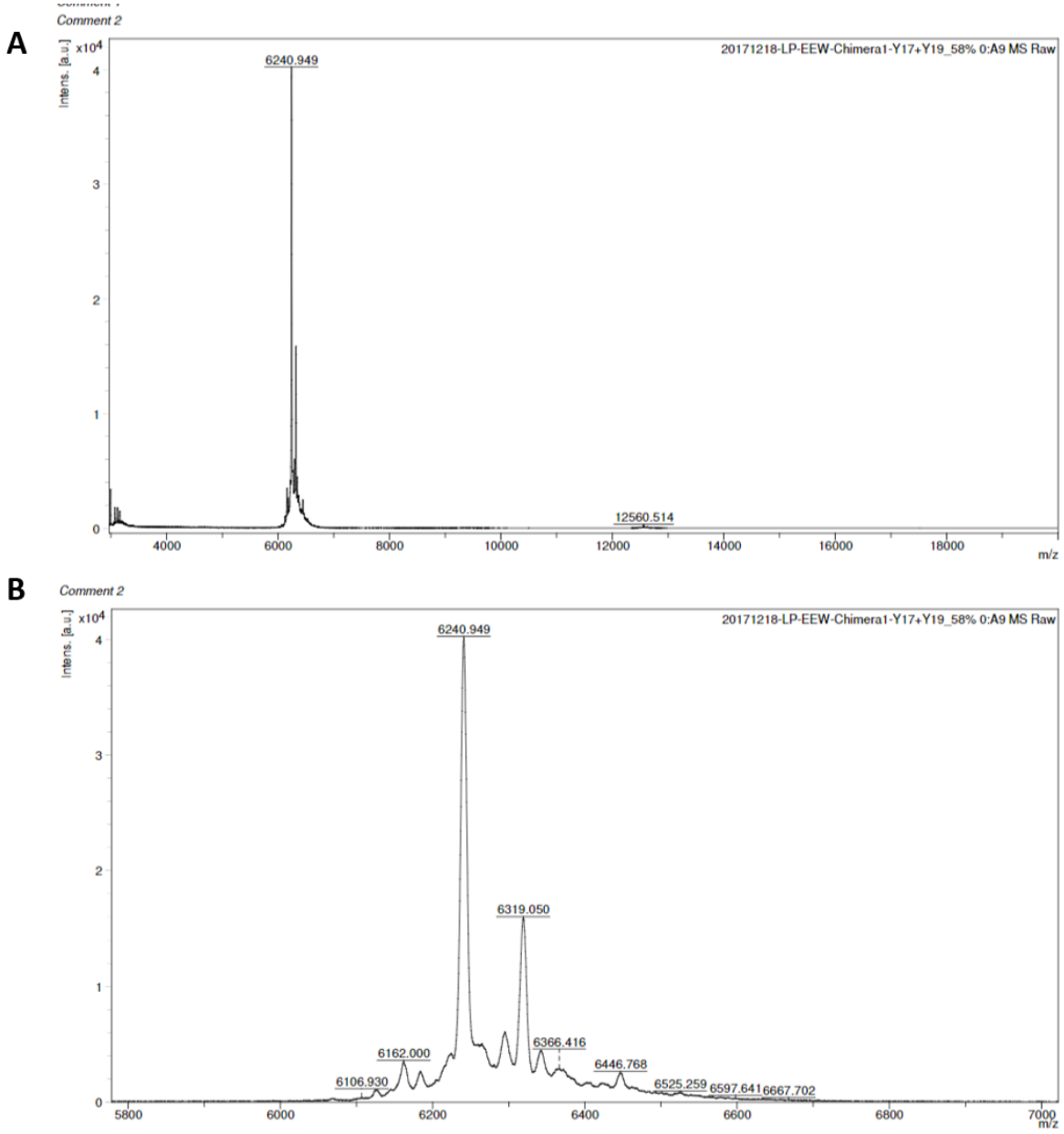


Figure S117: MALDI-TOF mass spectrum of **Hya1/3 DS** *(A)* 4000-20000 m/z and *(B)* 5800-7000 m/z. NB: In this case artefactual loss of the two sulfate esters were observed due to the acidity of the matrix.

Hya3/1 DS

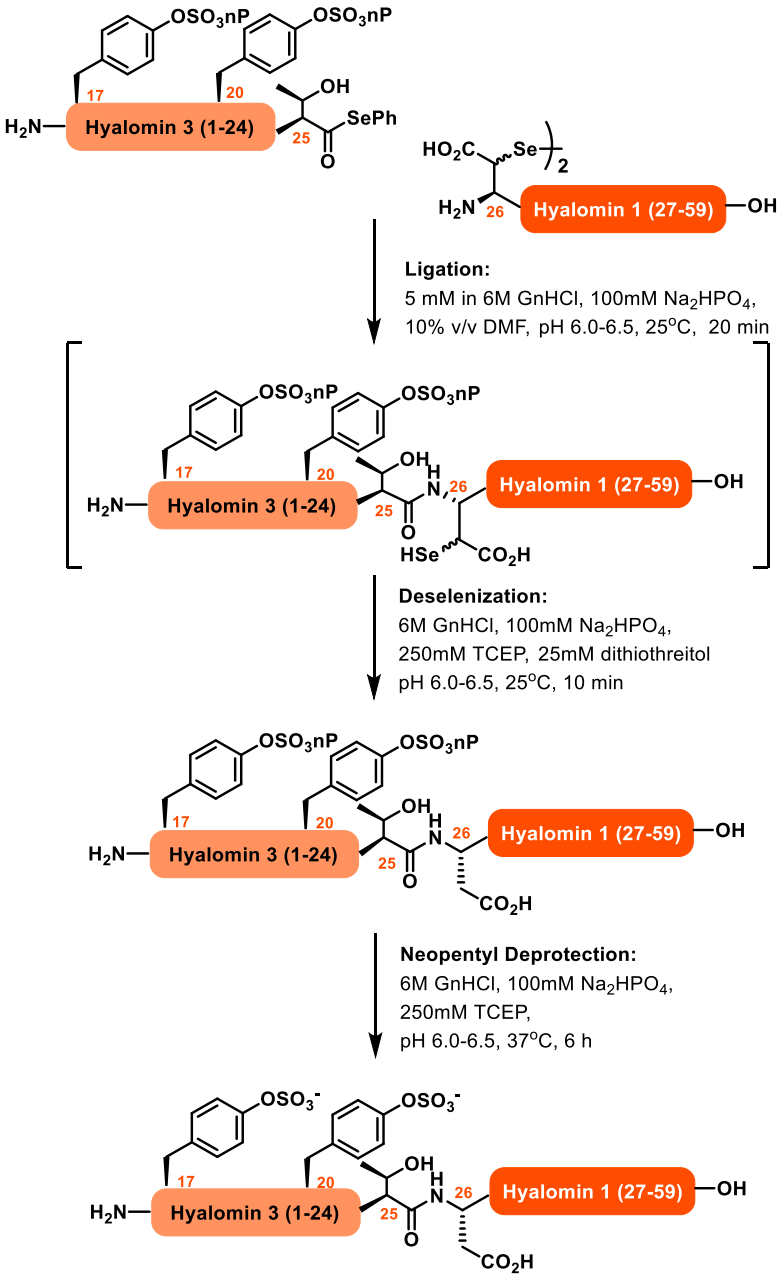


Figure S118: Synthesis of **Hya3/1 DS** via DSL-deselenization.

The one-pot peptide ligation of **Hya3 DS (1-25)** selenoester (2.5 mg, 0.78 µmol) and **Hya1 (26-59)** diselenide (1.4 mg, 0.39 µmol) followed by *in situ* deselenisation was performed according to the general procedure, replacing 10% of the buffer volume with DMF. Purification *via* preparative HPLC (0 to 40% B ov 40 min, 0.1% TFA) followed by lyophilisation afforded the neopentyl protected protein **Hya3/1 DS** as a white solid (1.6 mg, 64 %). Neopentyl deprotection was the performed as described in the general procedure, followed by purification *via* preparative HPLC (0 to 40% B ov 40 min, 0.1% formic acid) followed by lyophilisation afforded final protein **Hya3/1 DS** as a white solid (0.66 mg, 41%) (27% yield overall). Analytical HPLC (purified final product): R_t 4.25 min (0 to 50% B ov 5 min, 0.1% TFA, λ= 214 nm); Calculated Mass $[M+4H]^{4+}$: 1615.2 $[M+5H]^{5+}$: 1292.3 $[M+6H]^{6+}$: 1077.1 $[M+7H]^{7+}$: 923.4 Mass Found (ESI) 1615.6 $[M+4H]^{4+}$, 1292.6 $[M+5H]^{5+}$, 1077.3 $[M+6H]^{6+}$, 923.9 $[M+7H]^{7+}$. High Res (ESI+): calcd for $C_{262}H_{410}N_{80}O_{107}S_2$: $[M+6H]^{6+}$, 1077.32022. Found: 1077.32085 $[M+5H]^{5+}$. Difference: 0.6 ppm.

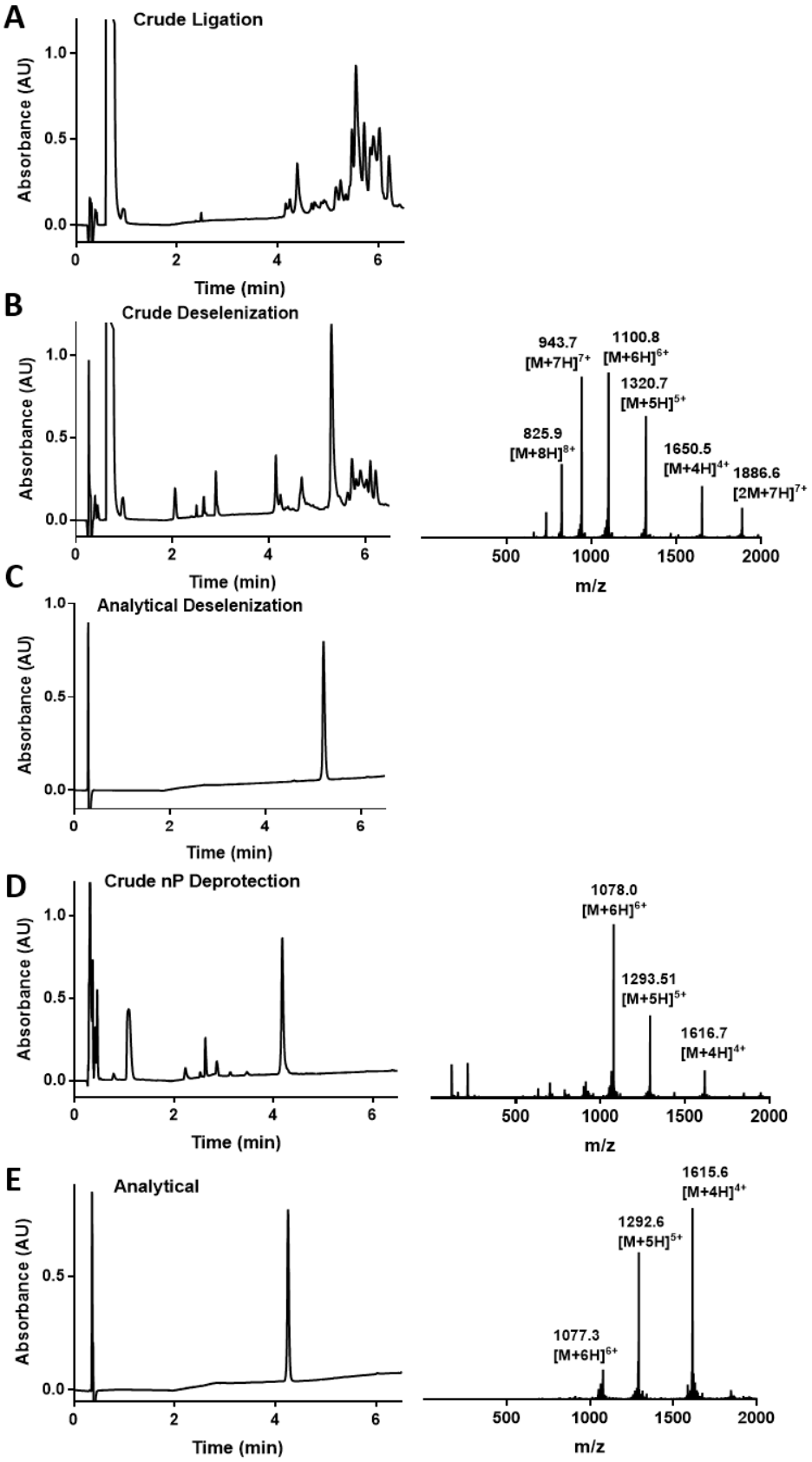


Figure S119: Analytical HPLC trace (0 to 50% B over 5 min, 0.1% v/v TFA, λ= 214 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) analytical deselenization, (*D*) crude nP deprotection and (*E*) purified **Hya3/1 DS**.

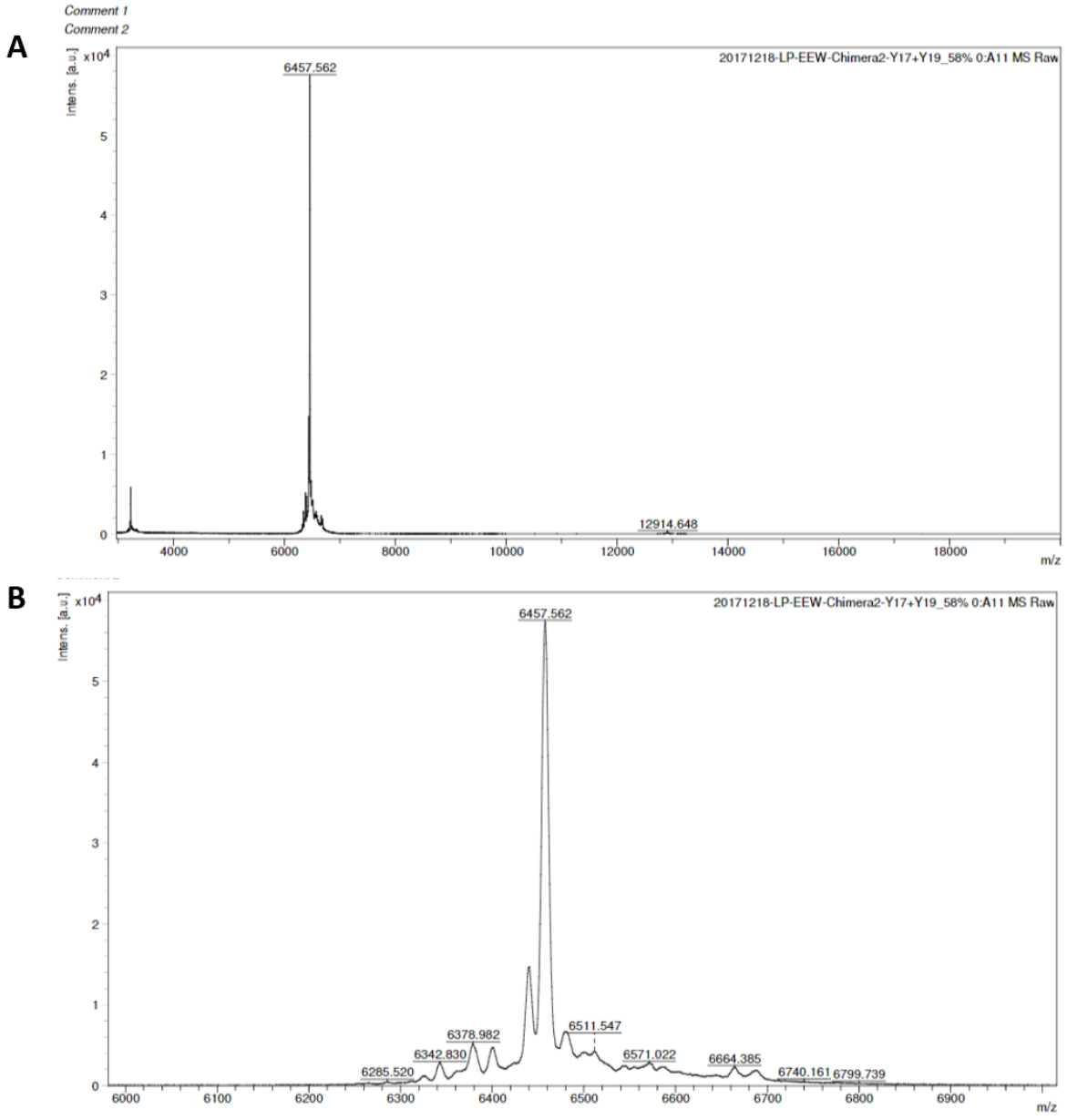


Figure S120: MALDI-TOF mass spectrum of **Hya3/1 DS** *(A)* 4000-20000 m/z and *(B)* 6000-6900 m/z.

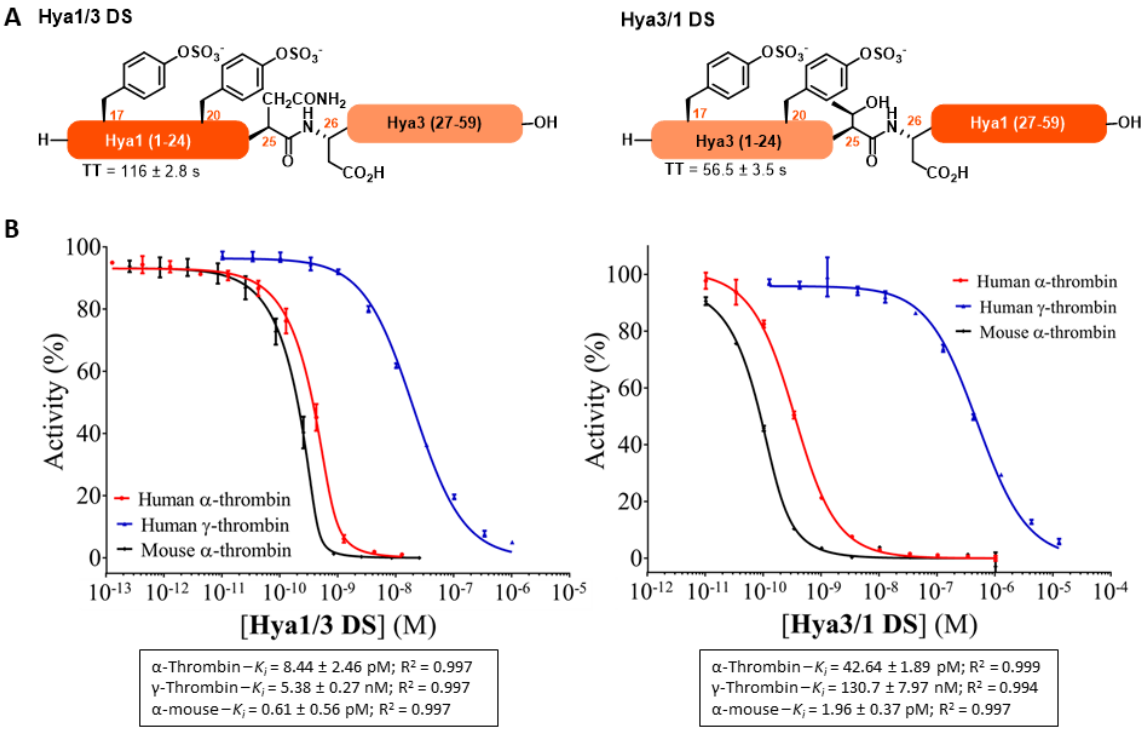


Figure S121: (*A*) Structure, TT and (*B*) thrombin inhibitory activity against human α- and γ-thrombin, and mouse α-thrombin of chimeric proteins **Hya1/3 DS** (left) and **Hya3/1 DS** (right).

Inhibition Kinetics of And310 Variants

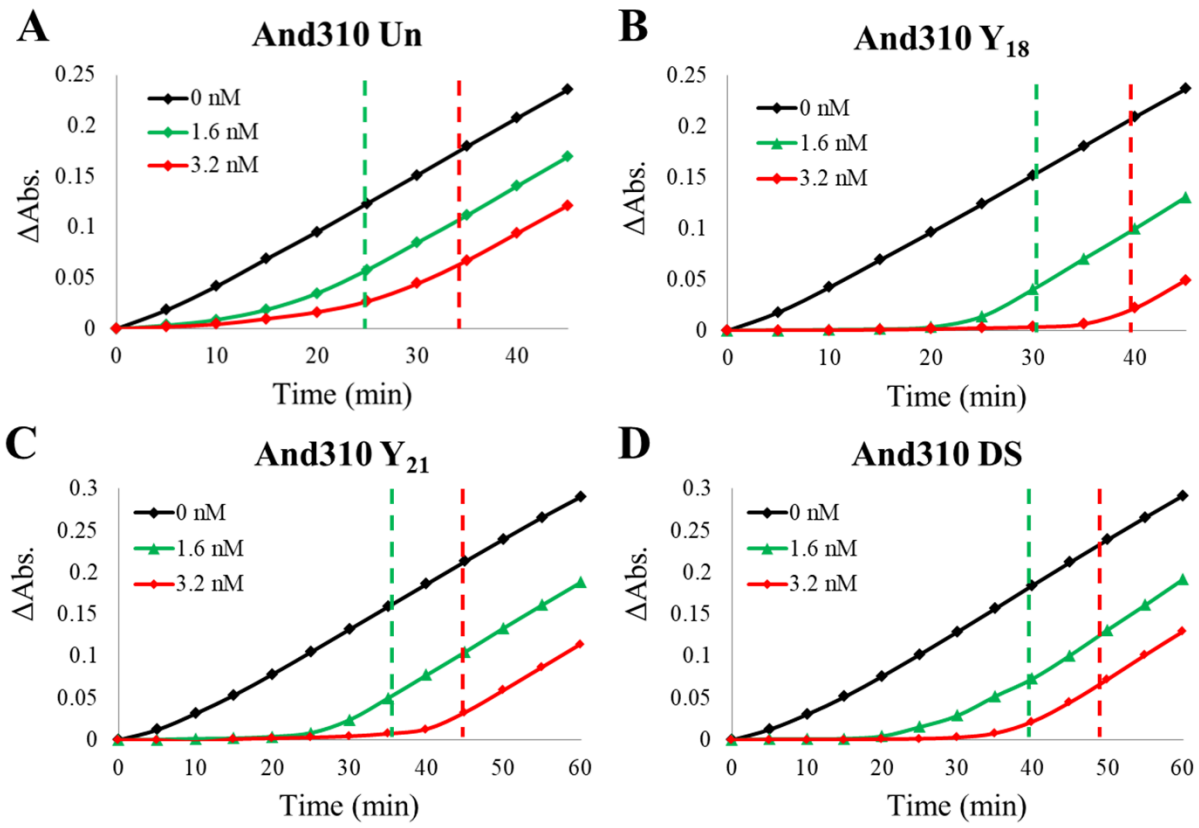


Figure S122: Representative progress curves of thrombin activity. Human α-thrombin (0.2 nM) in the presence of Chromozym-TH (100 µM) and (*A*) **And310 Un**, (*B*) **And310 Y_{18}**, (*C*) **And310 Y_{21}**, and (*D*) **And310 DS**. Dashed lines represent the time from which the slope is the same to the one in the absence of inhibitor. Concentrations of the inhibitor variants tested are specified in the insets.

Mass-Spectrometry Analysis of Thrombolytic Digestion of And82 Un and And310 Un

And82 Un (160 µM) and **And310 Un** (150 µM) were incubated in 20mM Tris-HCl pH 8, 150mM NaCl with human α-thrombin (14.5 µM) for 90 min at room temperature. After that, cleavage products were identified by nanoLC-MS on an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific). Samples were pre-concentrated in a C18 trap column cartridge with a mobile phase of 2% (v/v) MeCN, 0.1% formic acid (v/v) at 10 µL/min. After 3 min loading, the trap column was switched in-line to a 15 cm C18 column at 300 nL/min. Separation was generated by mixing A: 0.1% (v/v) formic acid, and B: 80% (v/v) MeCN, with the following gradient: 22 min (2.5% B to 50% B) and 5 min (50% B to 95% B). Data acquisition was controlled by Xcalibur 4.0 and Tune 2.8 software (Thermo Scientific). The mass spectrometer was operated in positive acquisition mode at *m/z* 150 - 2000 scan range at 140k resolution (*m/z* 200), AGC target 3e6, maximum injection time 200 ms and lock mass at *m/z* 445.12003. ESI spray voltage was 1.9 kV.

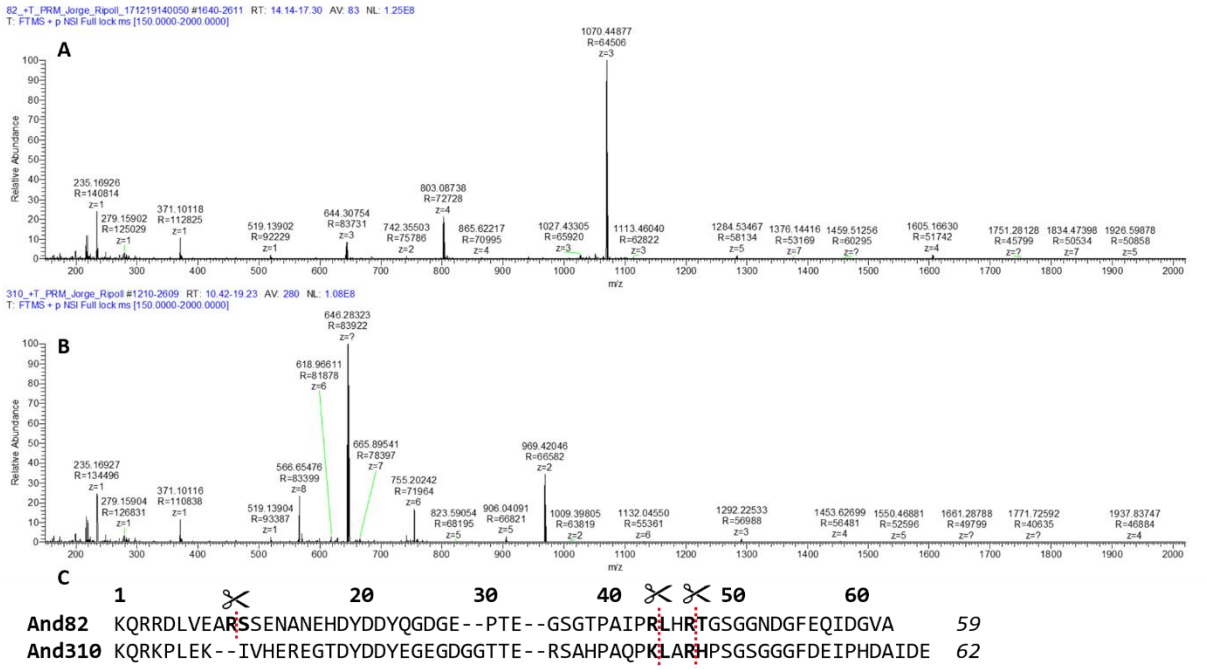


Fig. S123: Mass spectra of (*A*) **And82 Un** and (*B*) **And310 Un** after incubation with thrombin. (*C*) Amino acid sequence alignment of And82 and And310 showing the identified sites of proteolytic cleavage by thrombin (red dashed lines).

Kinetics of And310 Thrombolytic Cleavage

Thrombolytic cleavage of the respective And310 variants was carried out as follows: the And310 variant (60 nM) was dissolved in 50 mM Tris-HCl pH 8, 50 mM NaCl to which was added human alpha-thrombin (2 nM final concentration). The thrombolytic cleavage (at 37 °C) was monitored at 10 min intervals on a Waters Acquity UPLC system using a Waters Acquity UPLC BEH 1.7 μm 2.1 x 50 mm column (C-18) at a flow rate of 0.6 mL min^{-1} [mobile phase composed of 0.1% v/v TFA in H_2O (Solvent A) and 0.1% v/v TFA in MeCN (Solvent B)]. Gradient: 0 to 60% B over 5 min. The samples were incubated at 37ºC using the system sample oven between injections and were monitored over 90 min, with additional time points taken at 2, 4, 8 and 16 h, where appropriate. At the conclusion of the kinetic study, the resulting peaks were identified by UPLC-MS.

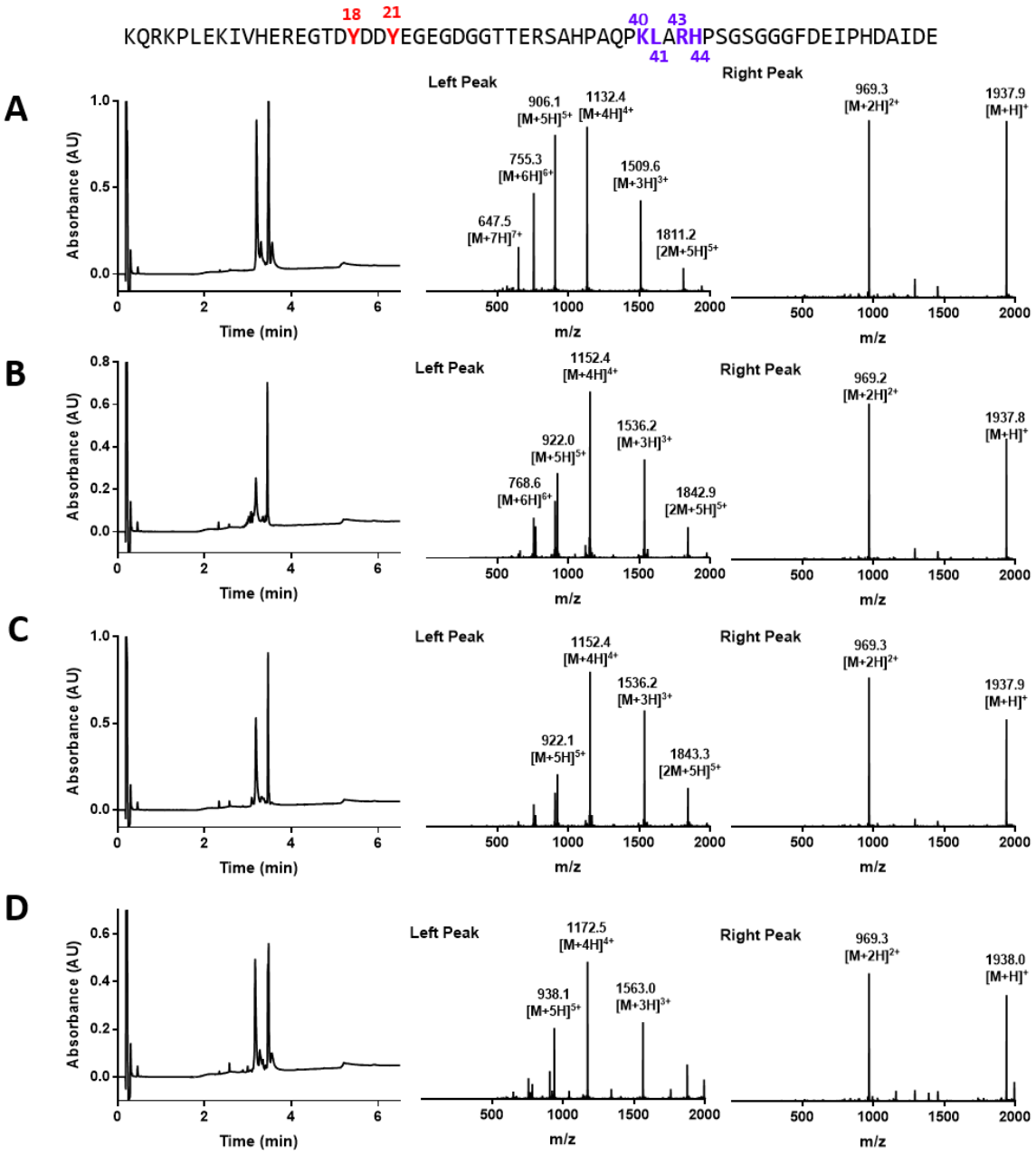


Figure S124: Analytical traces and mass spectra of thrombolytic cleavage of And310 sulfoforms. (*A*) **And310 Un**, (*B*) **And310 Y18**, (*C*) **And310 Y21** and (*D*) **And310 DS**. Predicted masses: K1 to K40 = 4523.1 (unsulfated), 4603.1 (monosulfated), 4683.1 (double sulfated); H44 to E62 = 1935.8.

Table S3: Thrombin times for andersonin sulfoforms. The thrombin times (TT) in seconds, measure the clotting time of human plasma in the presence of different concentrations of synthetic andersonin sulfoforms (at 2, 10 and 20 nM). In the absence of inhibitor, the TT was 19.5 s (mean of four replicates). Errors are depicted as standard deviations of the mean from two independent measurements.

	Thrombin Time (seconds)		
Inhibitor	**2 nM**	**10 nM**	**20 nM**
And310 Un	20 ± 0	23.5 ± 0.71	27.5 ± 0.71
And310 Y18	20 ± 0	33.5 ± 2.12	68.5 ± 2.12
And310 Y21	20.5 ± 0.71	33 ± 1.41	65 ± 2.83
And310 DS	20 ± 0	36.5 ± 0.71	69.5 ± 4.95
And82 Un	20 ± 0	22 ± 0	25.5 ± 0.71
And82 Y20	21 ± 0	30 ± 1.41	54.5 ± 0.71
And82 Y23	21 ± 0	31.5 ± 2.12	57.5 ± 3.54
And82 DS	21 ± 0	35.5 ± 0.71	73.5 ± 0.71
	Control 19.5 ± 0.58		

Synthesis of And310 Methylated Variants

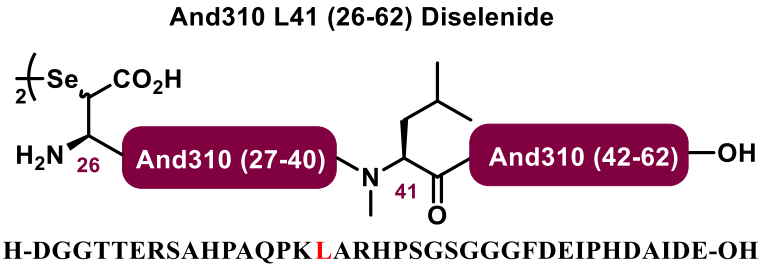
General Protocol for On-Resin Methylation of Histidine

A procedure for the on-resin methylation of histidine was adapted from that reported by Biron *et al.*(3) Briefly, the peptide N-terminus was protected as an *o*-NBS group using *o*-NBS-Cl (4 eq.) and collidine (10 eq.) in NMP (0.1 M) which was added to the resin and allowed to shake at room temperature for 15 min. N-methylation was then achieved via a Mitsunobu reaction so as to prevent methylation on the imidazole ring of histidine. After washing (5x NMP, 5x dry THF), the resin was treated with Ph_3P (5 eq.) and MeOH (10 eq.) in dry THF (0.2 M) for 1 min prior to the addition of DIAD (5 eq.) in THF (0.2M), with this complete reaction cocktail incubated with the resin for a further 10 min at room temperature, with shaking. Finally, after washing (5x THF), the *o*-NBS group was removed by treating twice with β-mercaptoethanol (10 eq.) and DBU (5 eq.) in NMP (0.1 M) for 5 min at room temperature.

Coupling of Amino Acids Following NMe Amino Acids

Coupling to secondary amines resulting from NMe sites was mediated by HATU (4 eq.) and DIPEA (8 eq.) in DMF (0.1 M) using 4 eq. of the appropriate amino acids. The reaction was allowed to proceed at room temperature with shaking for 2 h and then repeated with a fresh reaction cocktail for a further 2 h to ensure complete reaction.

Synthesis of N-Methylated Andersonin 310 Fragments



And310 L41 (26-62) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (25 µmol). Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 hours at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 40% B over 40 min, 0.1% TFA) afforded **And310 L41 (26-62)** diselenide as a fluffy white solid after lyophilization (4.5 mg, 4.6% yield).

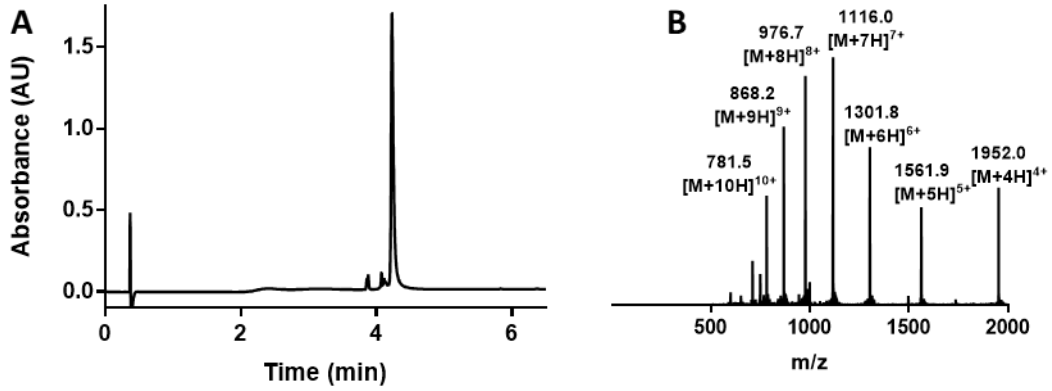
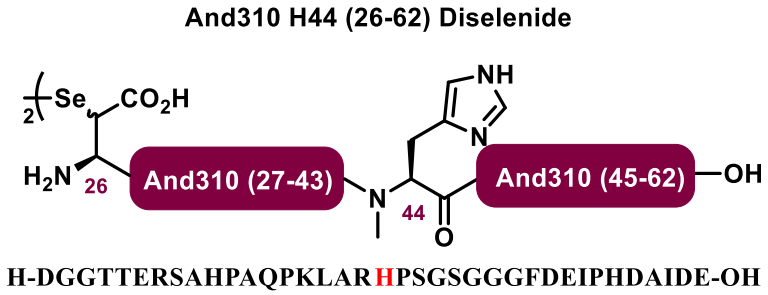


Figure S125: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **And310 L41 (26-62)** diselenide Analytical HPLC Rt 4.23 min (0 to 50% over 5 min, 0.1% TFA, λ = 214 nm); Calculated Mass (as the diselenide) $[M+4H]^{4+}$: 1951.9 $[M+5H]^{5+}$: 1561.7 $[M+6H]^{6+}$: 1301.6 $[M+7H]^{7+}$: 1115.8 $[M+8H]^{8+}$: 976.4 $[M+9H]^{9+}$: 868.0 $[M+10H]^{10+}$: 781.3 Mass Found (ESI) 1952.0 $[M+4H]^{4+}$, 1561.9 $[M+5H]^{5+}$, 1301.8 $[M+6H]^{6+}$, 1116.0 $[M+7H]^{7+}$, 976.7 $[M+8H]^{8+}$, 868.2 $[M+9H]^{9+}$, 781.5 $[M+10H]^{10+}$.



And310 H44 (26-62) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (25 µmol). On-resin methylation of Histidine and coupling of the subsequent Arginine residue were conducted as described above. Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 hours at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 40% B over 40 min, 0.1% TFA) afforded **And310 H44 (26-62)** diselenide as a fluffy white solid after lyophilization (3.5 mg, 3.6% yield).

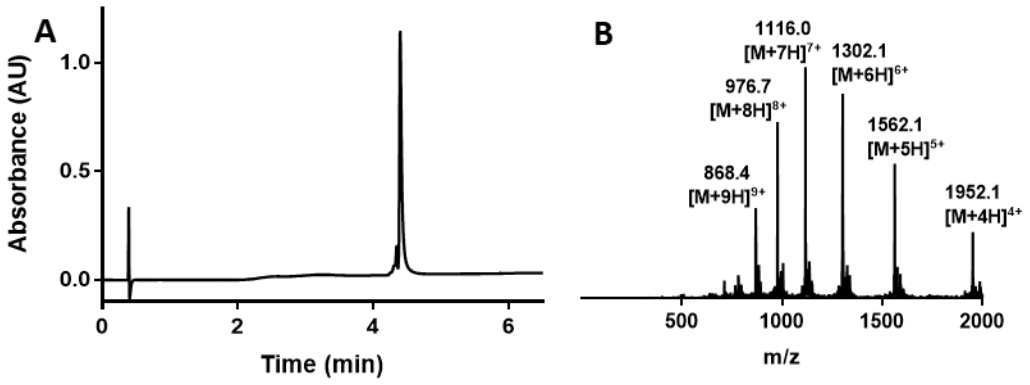
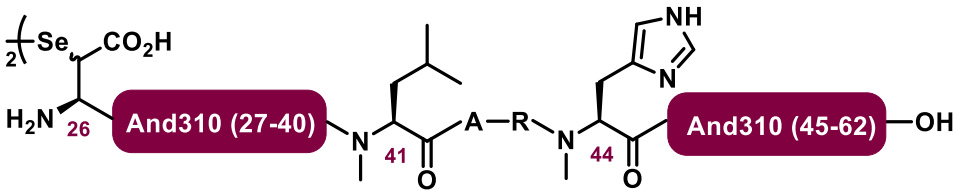


Figure S126: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **And310 H44 (26-62)** diselenide. Analytical HPLC Rt 4.40 min (0 to 50% over 5 min, 0.1% TFA, λ = 214 nm); Calculated Mass (as the diselenide) $[M+4H]^{4+}$: 1951.9 $[M+5H]^{5+}$: 1561.7 $[M+6H]^{6+}$: 1301.6 $[M+7H]^{7+}$: 1115.8 $[M+8H]^{8+}$: 976.4 $[M+9H]^{9+}$: 868.0. Mass Found (ESI) 1952.1 $[M+4H]^{4+}$, 1562.1 $[M+5H]^{5+}$, 1302.1 $[M+6H]^{6+}$, 1116.0 $[M+7H]^{7+}$, 976.7 $[M+8H]^{8+}$, 868.4 $[M+9H]^{9+}$.

And310 L41 H44 (26-62) Diselenide



H-DGGTTERSAHPAQPKLARHPSGSGGGFDEIPHDAIDE-OH

And310 L41 H44 (26-62) diselenide was prepared according to Fmoc-strategy SPPS, through a combination of automated and manual synthesis, on 2-chlorotrityl chloride resin (25 µmol). On-resin methylation of Histidine and coupling of the subsequent Arginine residue were conducted as described above. Removal of all of the acid labile protecting groups was achieved via treatment of the crude peptide selenoester with a solution of TFA/iPr$_3$SiH/H$_2$O (90:5:5 v/v/v, 4 mL) for 2 hours at room temperature. The deprotection solution was then concentrated under nitrogen flow and the crude peptide from ice-cold diethyl ether. The PMB group was then removed as described above. Purification of the crude peptide by preparative RP-HPLC (0 to 40% B over 40 min, 0.1% TFA) afforded **And310 L41 H44 (26-62)** diselenide as a fluffy white solid after lyophilization (6.1 mg, 6.2% yield).

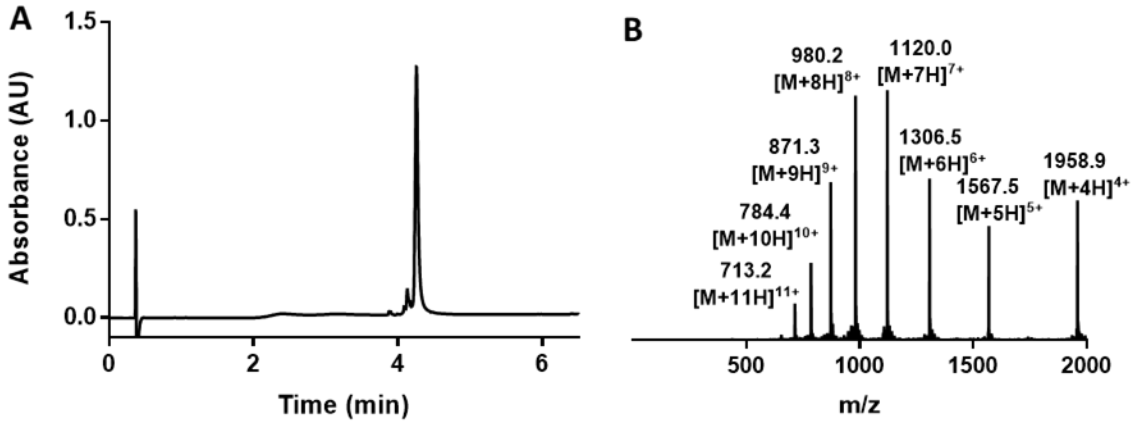


Figure S127: Analytical HPLC trace (*A*) and mass spectrum (*B*) of purified **And310 L41 H44 (26-62)** diselenide. Analytical HPLC Rt 4.25 min (0 to 50% over 5 min, 0.1% TFA, λ = 214 nm); Calculated Mass (as the diselenide) $[M+4H]^{4+}$: 1958.9 $[M+5H]^{5+}$: 1567.3 $[M+6H]^{6+}$: 1306.3 $[M+7H]^{7+}$: 1119.8 $[M+8H]^{8+}$: 979.9 $[M+9H]^{9+}$: 871.2 $[M+10H]^{10+}$: 784.1 $[M+11H]^{11+}$: 712.9 Mass Found (ESI) 1958.9 $[M+4H]^{4+}$, 1567.5 $[M+5H]^{5+}$, 1306.5 $[M+6H]^{6+}$, 1120.0 $[M+7H]^{7+}$, 980.2 $[M+8H]^{8+}$, 871.3 $[M+9H]^{9+}$, 784.4 $[M+10H]^{10+}$, 713.2 $[M+11H]^{11+}$.

Assembly of Methylated Andersonin 310 Variants

And310 DS L41

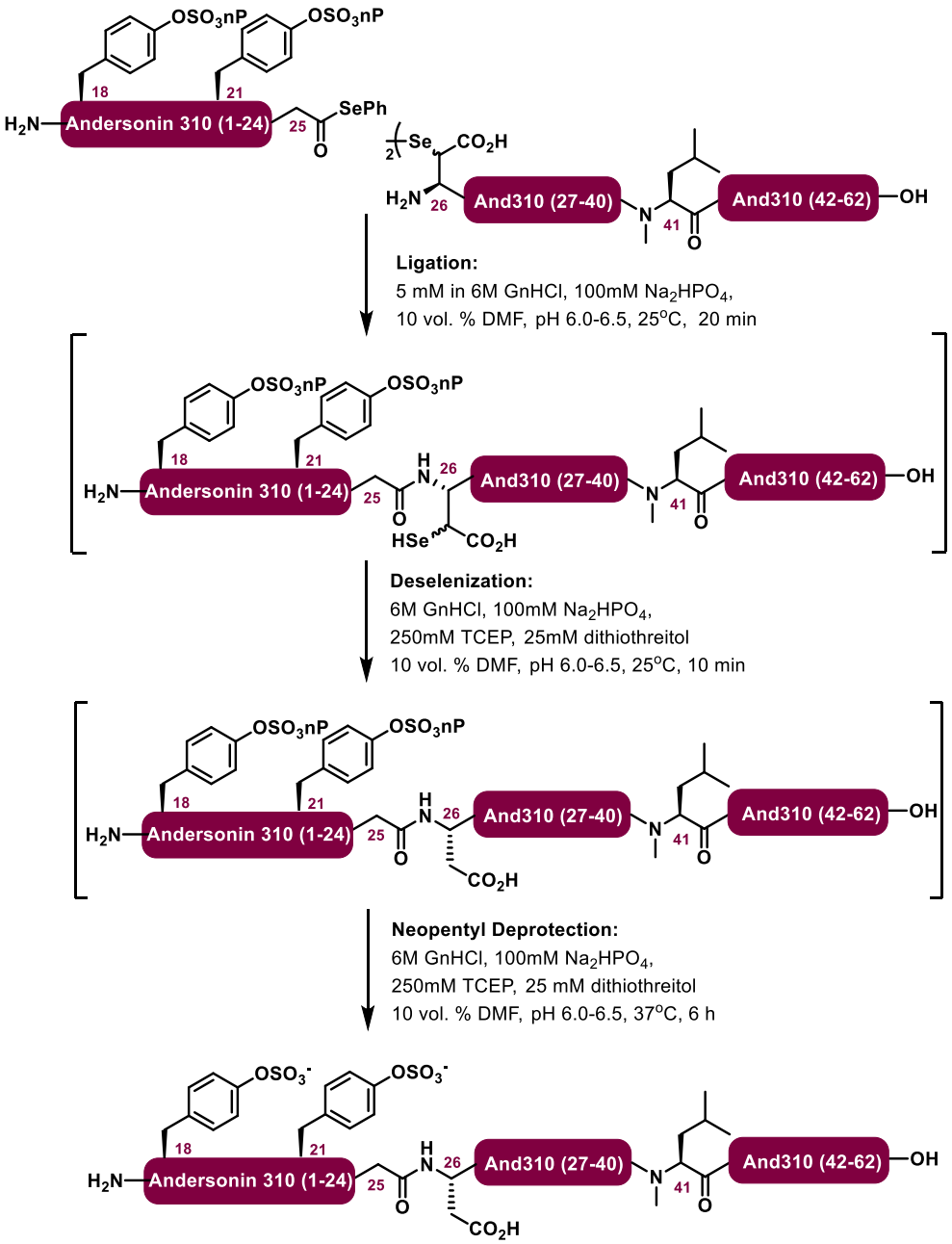


Figure S128: Synthesis of **And310 DS NMeLeu41** via DSL-deselenization.

The one-pot peptide ligation of **And310 DS (1-25)** selenoester (2.9 mg, 0.86 µmol) and **And310 L41 (26-62)** diselenide (1.7 mg, 0.43 µmol) followed by *in situ* deselenization was performed according to the general procedure, replacing 10% of the buffer volume with DMF. Purification *via* preparative HPLC (15 to 35% B ov 40 min, 0.1% formic acid) followed by lyophilization afforded final protein **And310 DS L41** as a white solid (1.5 mg, 50%). Analytical HPLC (purified final product): R_t 3.59 min (0 to 60% B ov 5 min, 0.1% TFA, λ= 215 nm); Calculated Mass $[M+4H]^{4+}$: 1740.5 $[M+5H]^{5+}$: 1392.6 $[M+6H]^{6+}$: 1160.7, $[M+7H]^{7+}$: 995.0 $[M+8H]^{8+}$: 870.8 $[M+9H]^{9+}$: 774.2 Mass Found (ESI) 1740.8 $[M+4H]^{4+}$, 1392.8 $[M+5H]^{5+}$, 1160.8 $[M+6H]^{6+}$, 995.2 $[M+7H]^{7+}$, 870.9 $[M+8H]^{8+}$, 774.3 $[M+9H]^{9+}$. High Res (ESI+): calcd for $C_{289}H_{444}N_{88}O_{109}S_2$: $[M+8H]^{8+}$, 870.90040. Found: 870.90100 $[M+8H]^{8+}$. Difference: 0.7 ppm.

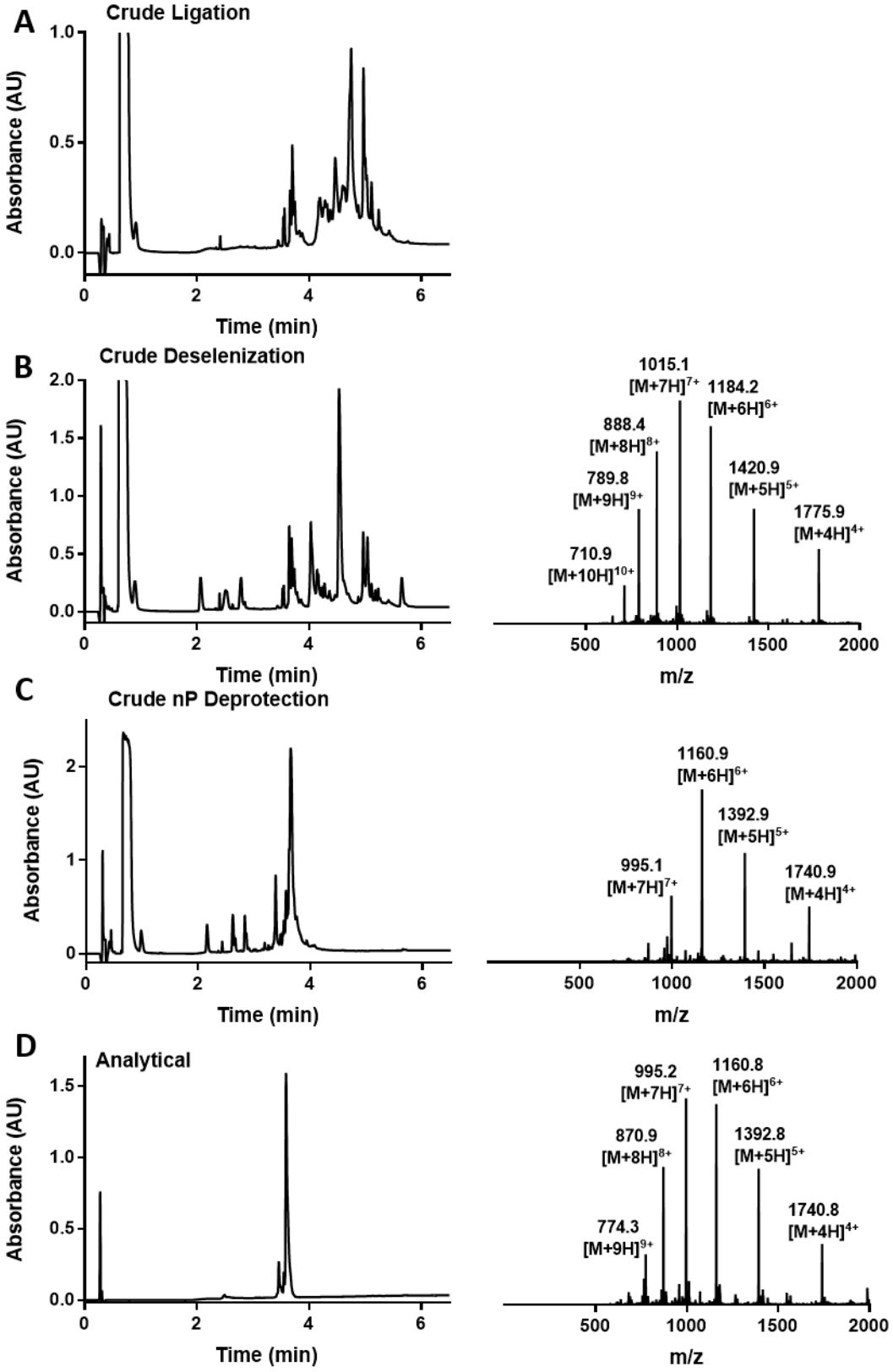


Figure S129: Analytical HPLC trace (0 to 60% B ov 5 min, 0.1% TFA, λ= 215 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **And310 DS L41**.

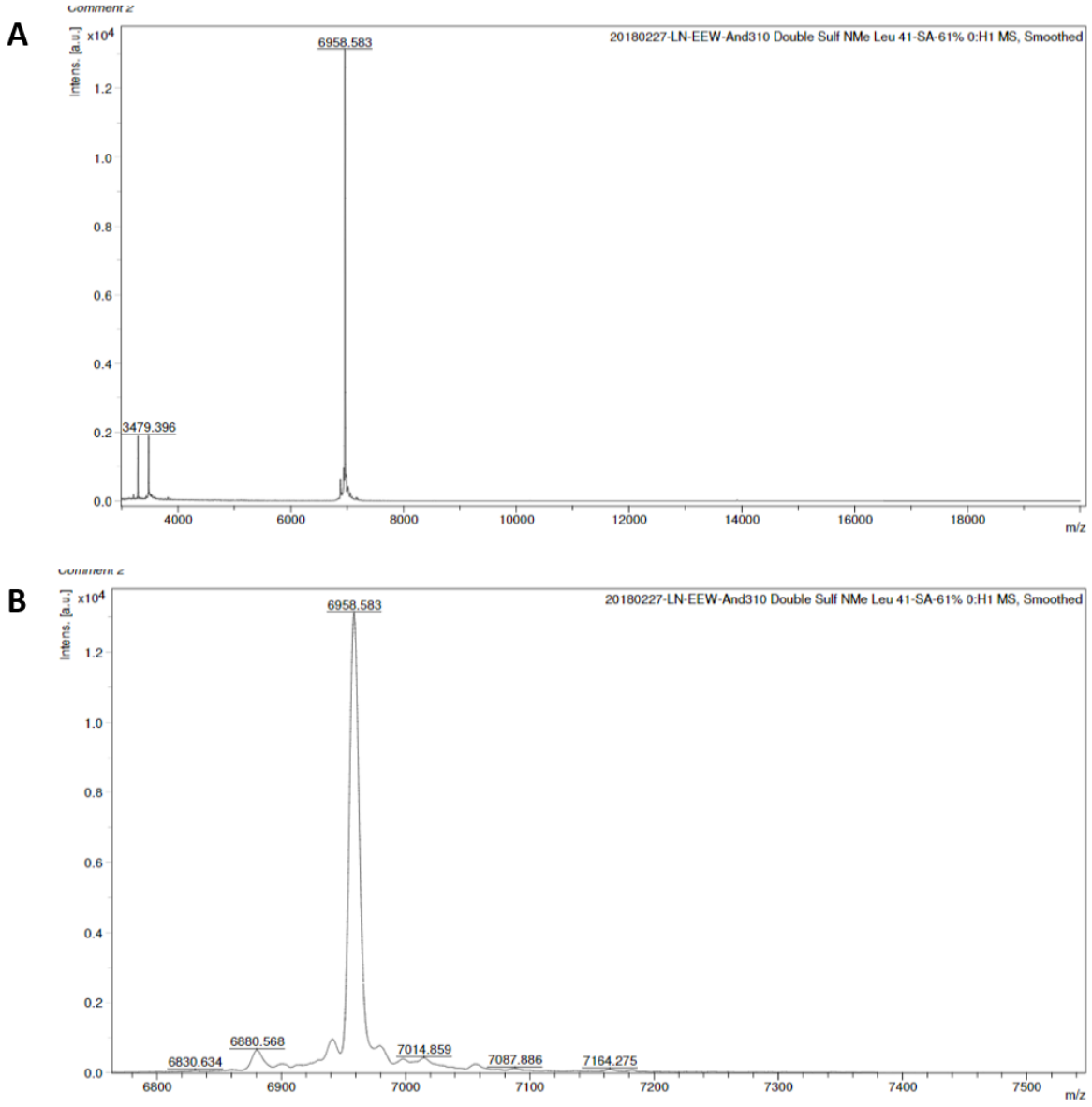


Figure S130: MALDI-TOF mass spectrum of **And310 DS L41** (*A*) 4000-20000 m/z and (*B*) 6800-7500 m/z.

And310 DS H44

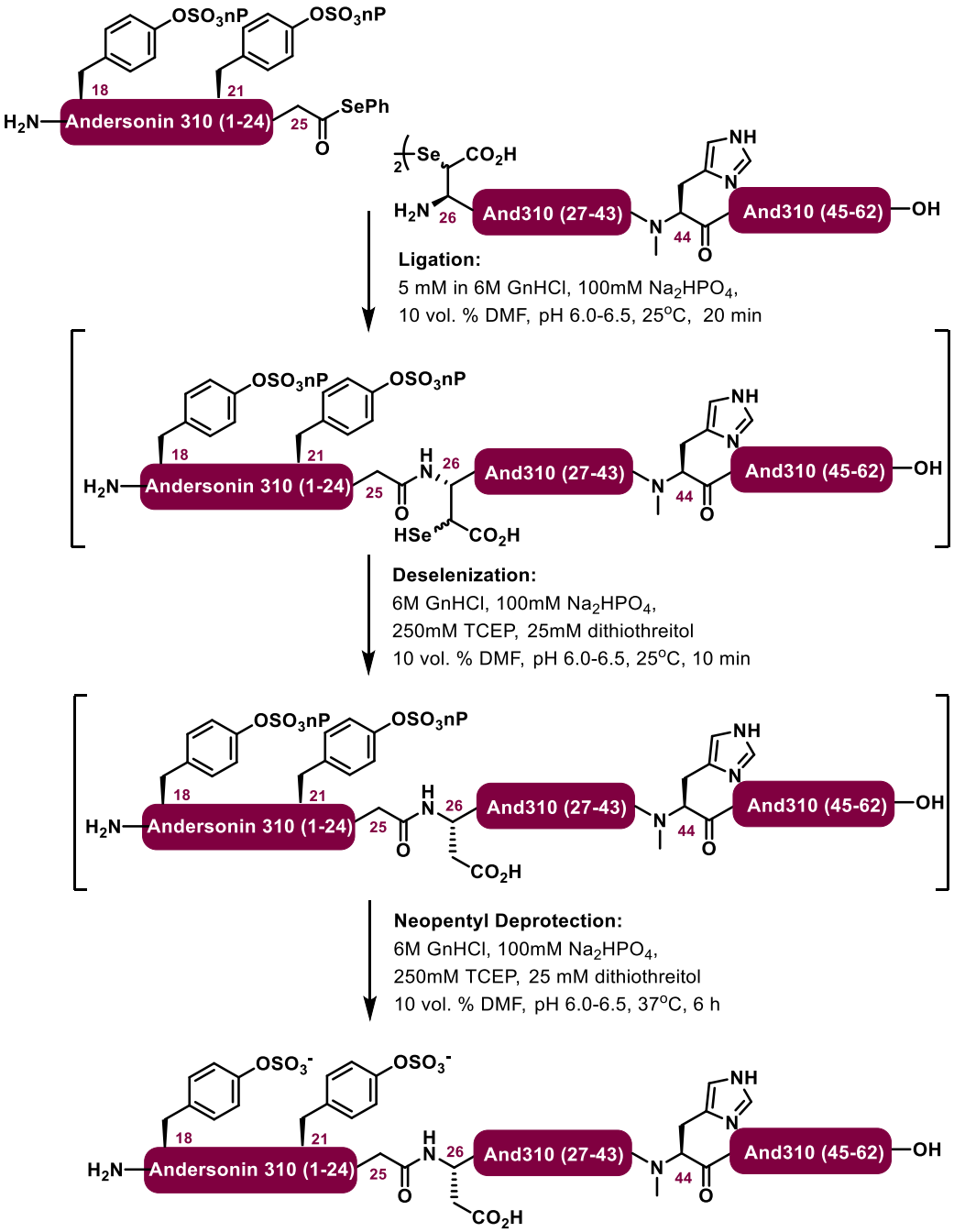


Figure S131: Synthesis of **And310 DS H44** via DSL-deselenization.

The one-pot peptide ligation of **And310 DS (1-25)** selenoester (2.7 mg, 0.78 µmol) and **And310 H44 (26-62)** diselenide (1.5 mg, 0.39 µmol) followed by *in situ* deselenization was performed according to the general procedure, replacing 10% of the buffer volume with DMF. Purification via preparative HPLC (0 to 40% B ov 40 min, 0.1% formic acid) followed by lyophilization afforded final protein **And310 DS H44** as a white solid (1.1 mg, 41%). Analytical HPLC (purified final product): R_t 3.61 min (0 to 60% B over 5 min, 0.1% TFA, λ= 215 nm); Calculated Mass $[M+4H]^{4+}$: 1740.5 $[M+5H]^{5+}$: 1392.6 $[M+6H]^{6+}$: 1160.7, $[M+7H]^{7+}$: 995.0 $[M+8H]^{8+}$: 870.8 $[M+9H]^{9+}$: 774.1 Mass Found (ESI) 1740.9 $[M+4H]^{4+}$, 1392.7 $[M+5H]^{5+}$, 1160.8 $[M+6H]^{6+}$, 995.1 $[M+7H]^{7+}$, 870.9 $[M+8H]^{8+}$, 774.2 $[M+9H]^{9+}$. High Res (ESI+): calcd for $C_{289}H_{444}N_{88}O_{109}S_2$: $[M+8H]^{8+}$, 870.90040. Found: 870.90101 $[M+8H]^{8+}$. Difference: 0.8 ppm.

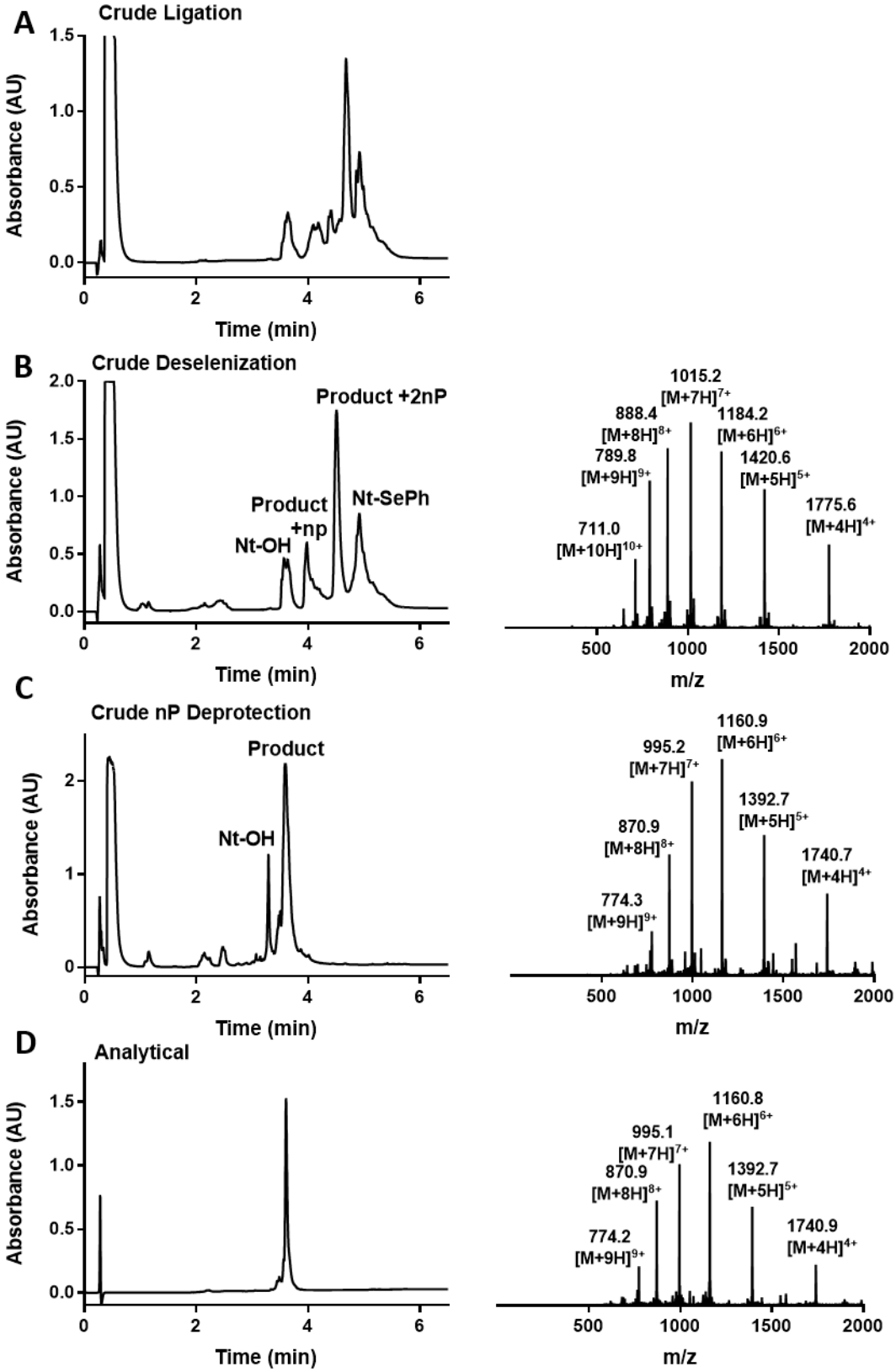


Figure S132: Analytical HPLC trace (0 to 60% B over 5 min, 0.1% TFA, λ= 215 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **And310 DS H44**.

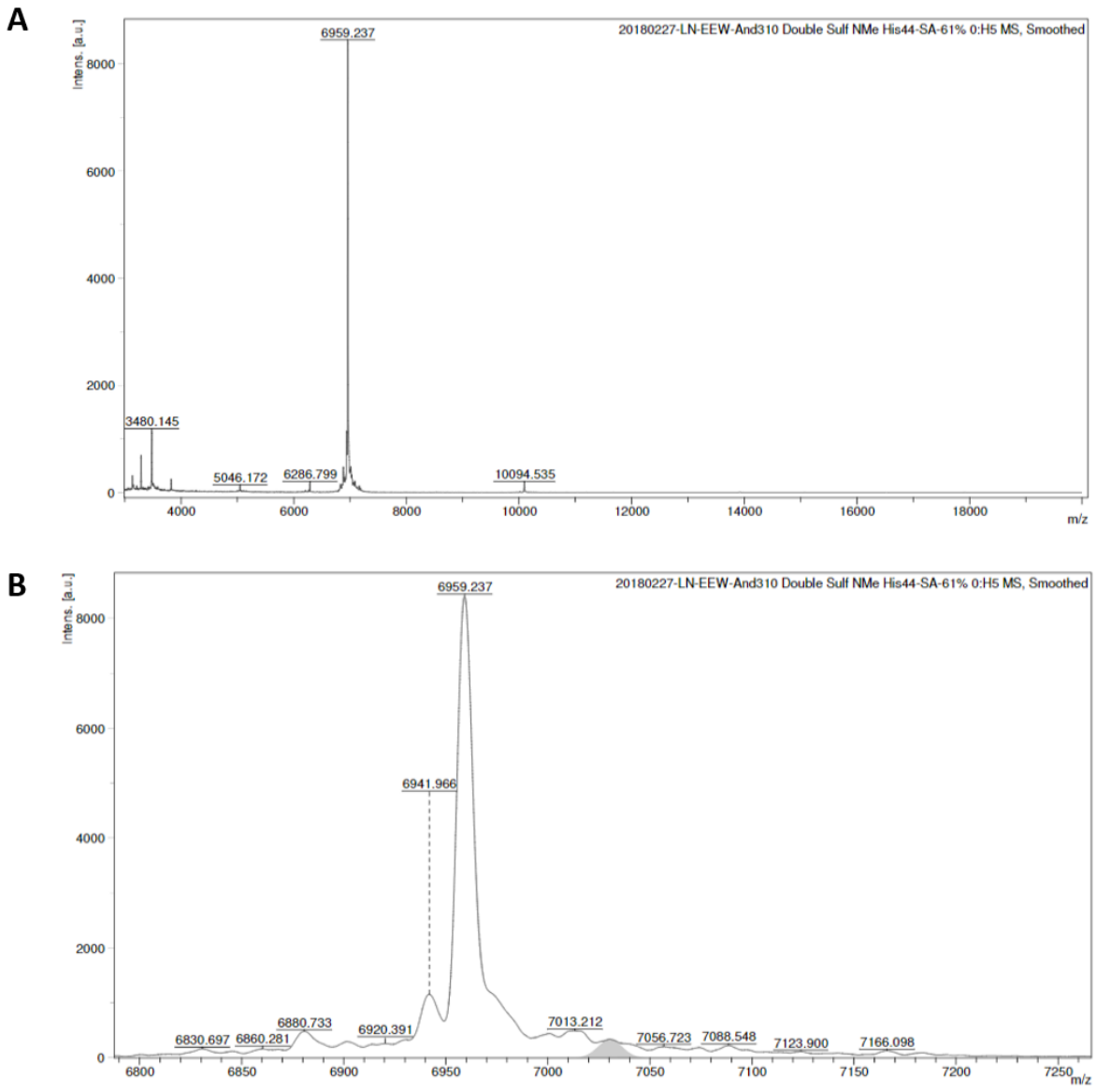


Figure S133: MALDI-TOF mass spectrum of **And310 DS H44** (*A*) 4000-20000 m/z and (*B*) 6800-7250 m/z.

And310 DS L41 H44

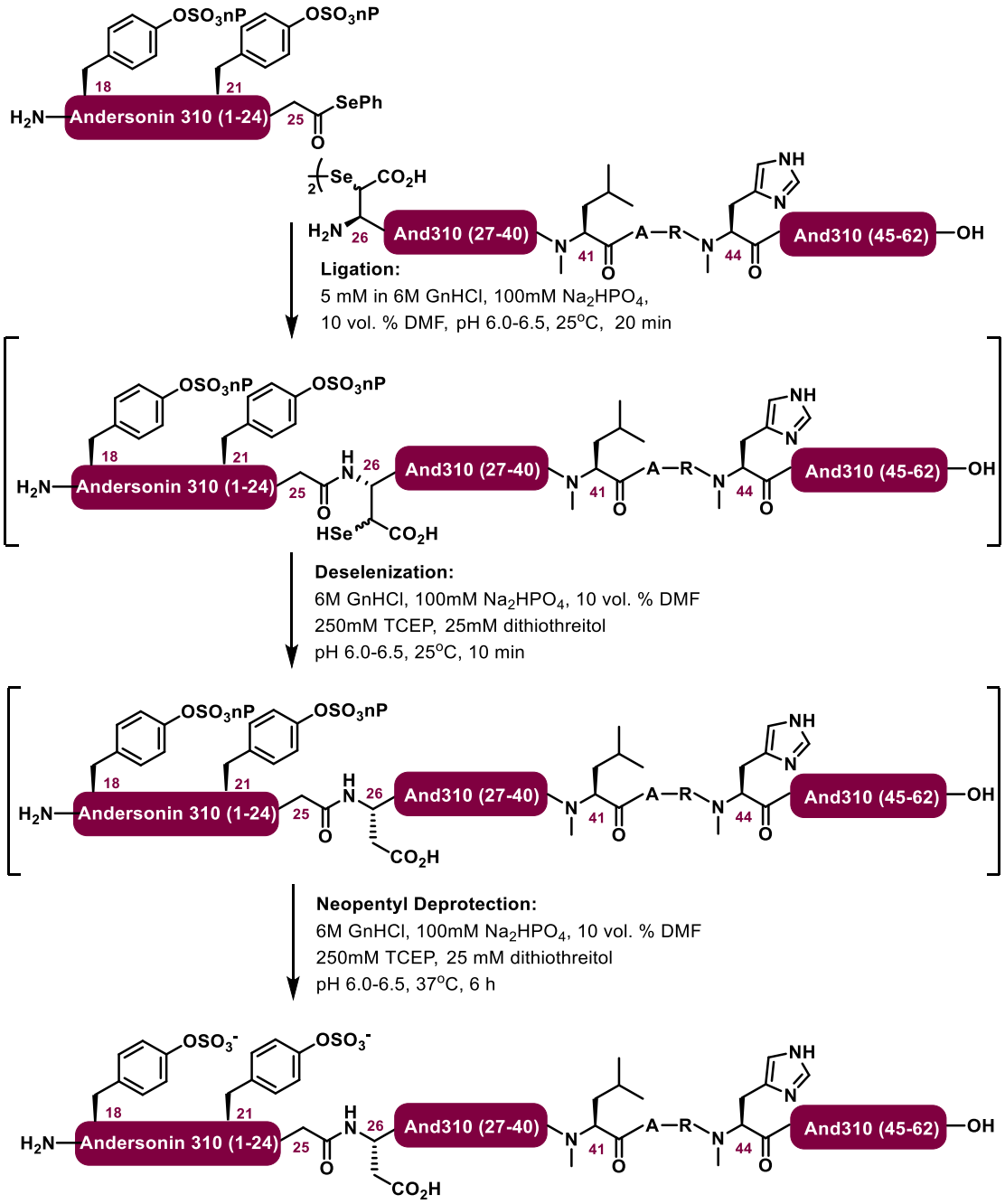


Figure S134: Synthesis of **And310 DS L41 H44** via DSL-deselenization.

The one-pot peptide ligation of **And310 DS (1-25)** selenoester (2.7 mg, 0.76 µmol) and **And310 L41 H44 (26-62)** diselenide (1.5 mg, 0.38 µmol) followed by *in situ* deselenization was performed according to the general procedure, replacing 10% of the buffer volume with DMF. Purification *via* preparative HPLC (15 to 35% B ov 40 min, 0.1% FA) followed by lyophilization afforded final protein **And310 DS L41 H44** as a white solid (1.2 mg, 45%). Analytical HPLC (purified final product): R_t 3.58 min (0 to 60% B over 5 min, 0.1% TFA, λ= 215 nm); Calculated Mass $[M+4H]^{4+}$: 1744.3 $[M+5H]^{5+}$: 1395.7 $[M+6H]^{6+}$: 1163.2, $[M+7H]^{7+}$: 997.2 $[M+8H]^{8+}$: 872.7 $[M+9H]^{9+}$: 775.8 Mass Found (ESI) 1744.4 $[M+4H]^{4+}$, 1395.6 $[M+5H]^{5+}$, 1163.2 $[M+6H]^{6+}$, 997.1 $[M+7H]^{7+}$, 872.7 $[M+8H]^{8+}$, 775.8 $[M+9H]^{9+}$. High Res (ESI+): calcd for $C_{290}H_{446}N_{88}O_{109}S_2$: $[M+8H]^{8+}$, 872.65236. Found: 872.65057 $[M+8H]^{8+}$. Difference: 1.7 ppm.

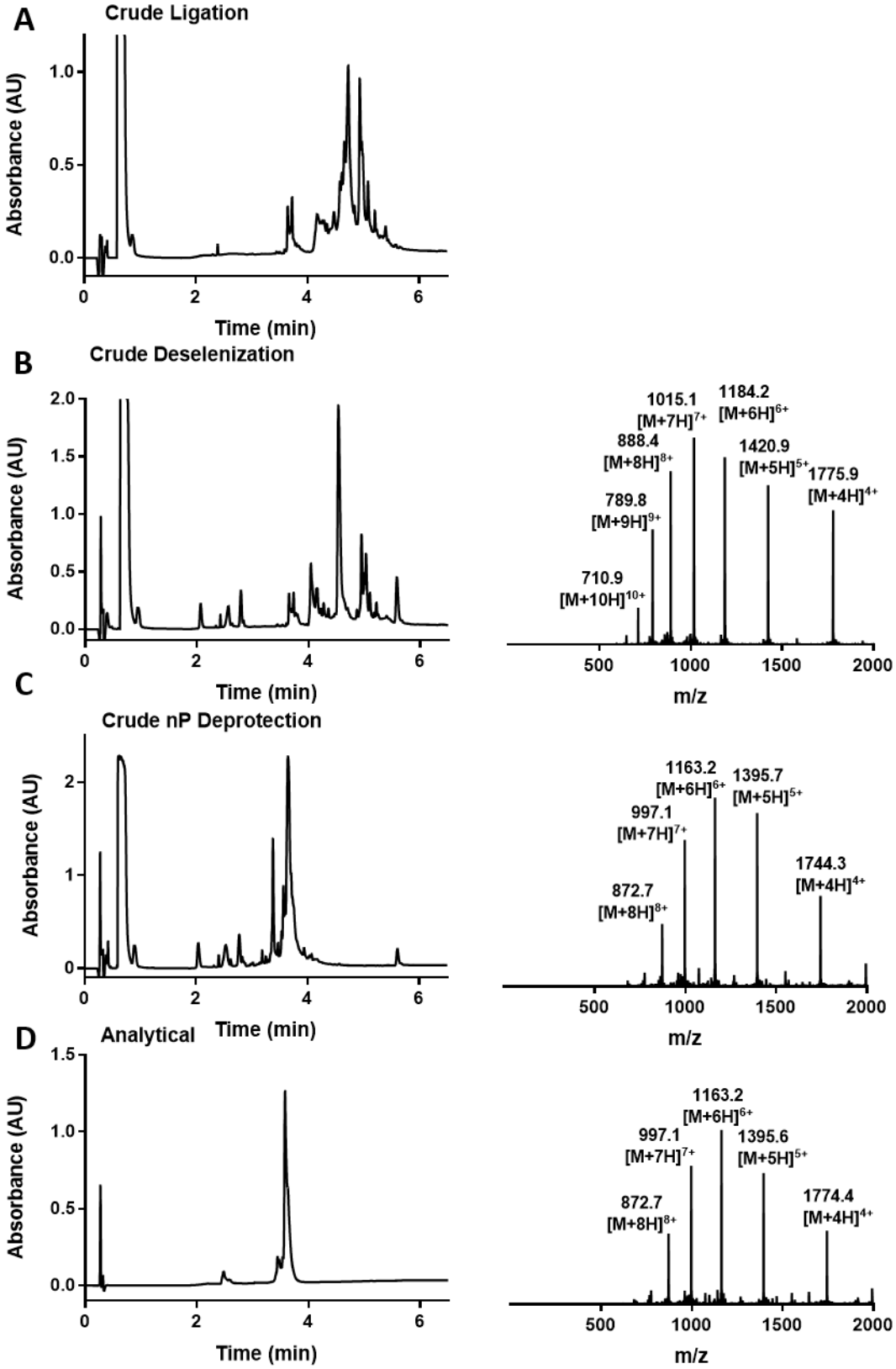


Figure S135: Analytical HPLC trace (0 to 60% B over 5 min, 0.1% TFA, λ= 215 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization, (*C*) crude nP deprotection and (*D*) purified **And310 DS L41 H44**.

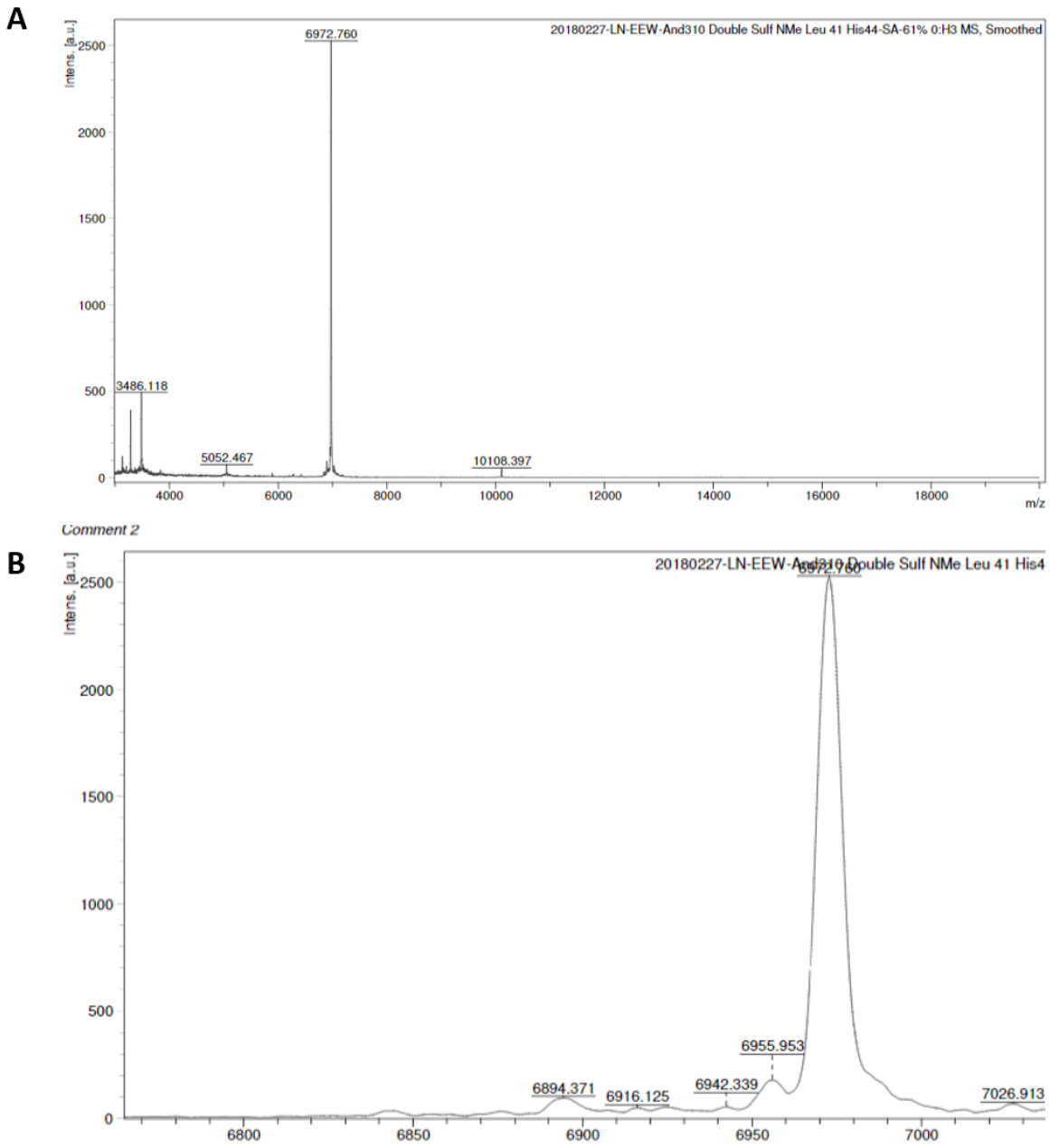


Figure S136: MALDI-TOF mass spectrum of **And310 DS L41 H44** (*A*) 4000-20000 m/z and (*B*) 6800-7000 m/z.

And310 Un L41 H44

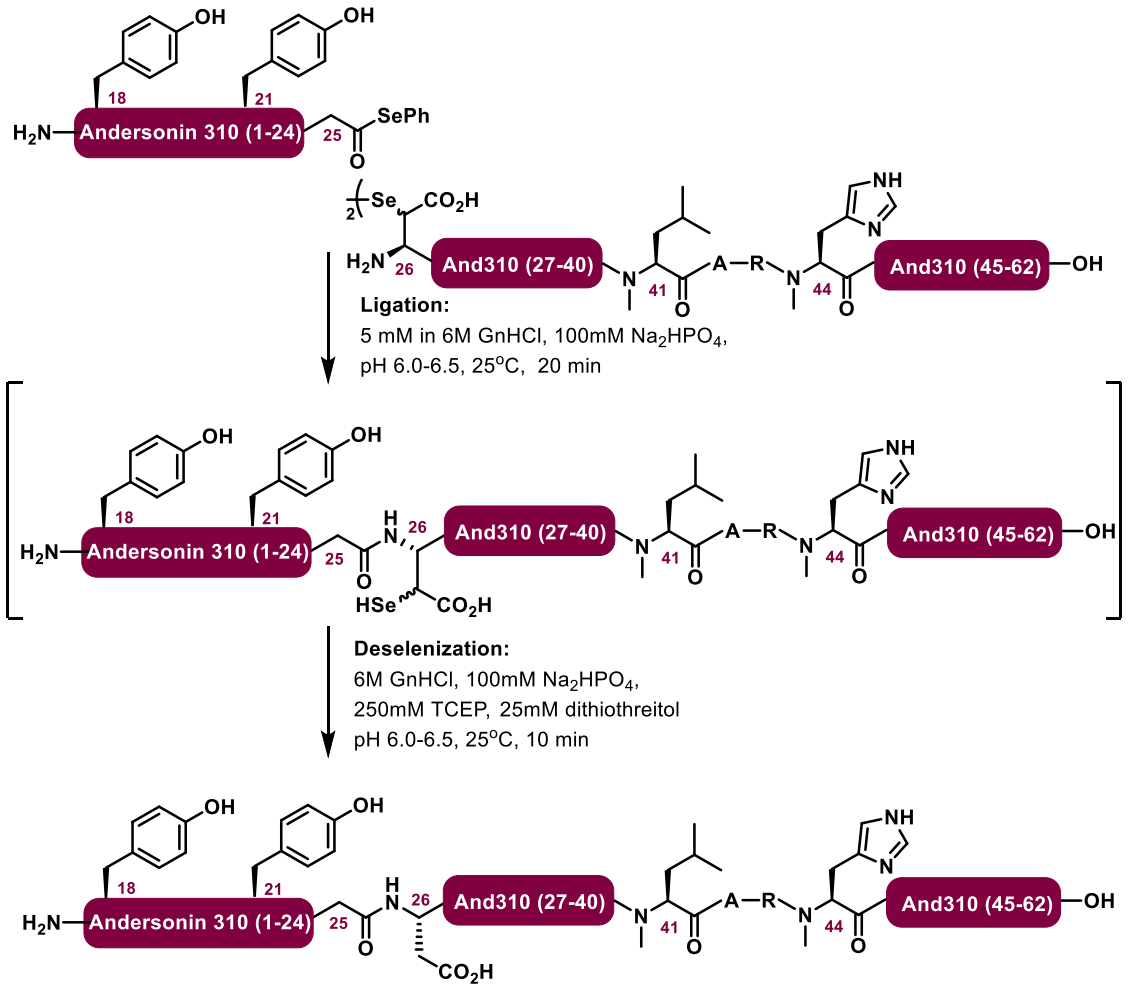


Figure S137: Synthesis of **And310 Un L41 H44** via DSL-deselenization.

The one-pot peptide ligation of **And310 Un (1-25)** selenoester (2.9 mg, 0.92 µmol) and **And310 L41 H44 (26-62)** diselenide (1.8 mg, 0.46 µmol) followed by *in situ* deselenization was performed according to the general procedure followed by purification *via* preparative HPLC (0 to 40% B ov 40 min, 0.1% TFA) and lyophilization to afford final protein **And310 Un L41 H44** as a white solid (1.3 mg, 42%). Analytical HPLC (purified final product): R_t 3.41 min (0 to 60% B ov 5 min, 0.1% TFA, λ = 214 nm); Calculated Mass $[M+4H]^{4+}$: 1704.3, $[M+5H]^{5+}$: 1363.7, $[M+6H]^{6+}$: 1136.6, $[M+7H]^{7+}$: 974.3, $[M+8H]^{8+}$: 852.7; Mass Found (ESI) 1704.1 $[M+4H]^{4+}$, 1363.4 $[M+5H]^{5+}$, 1136.4 $[M+6H]^{6+}$, 974.2 $[M+7H]^{7+}$, 852.5 $[M+8H]^{8+}$. High Res (ESI+): calcd for $C_{290}H_{446}N_{88}O_{103}$: $[M+8H]^{8+}$, 852.53791. Found: 852.53804 $[M+8H]^{8+}$. Difference: 0.8 ppm.

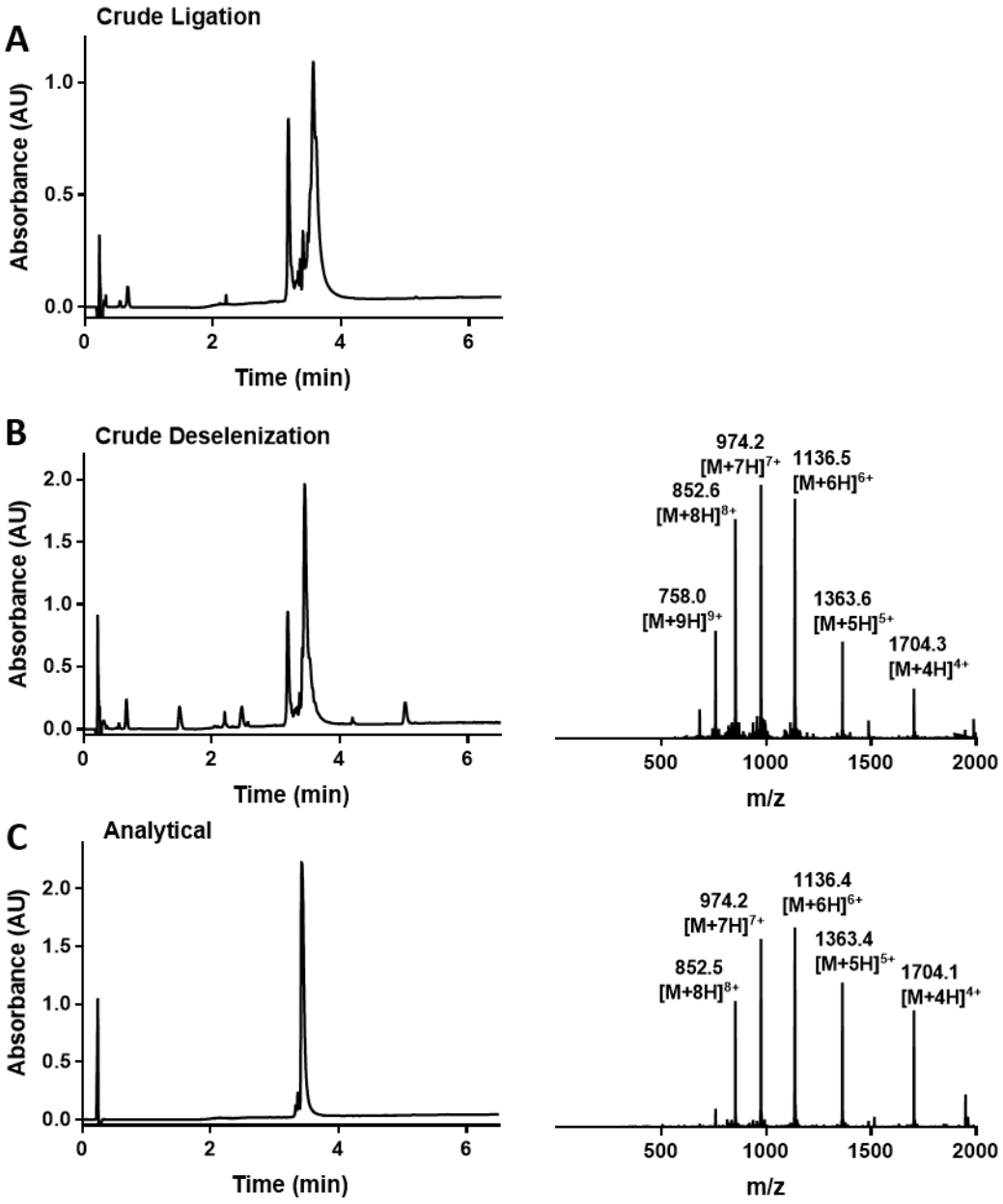


Figure S138: Analytical HPLC trace (0 to 60% B over 5 min, 0.1% TFA, λ= 215 nm) and mass spectrum of (*A*) crude ligation, (*B*) crude deselenization and (*C*) purified **And310 Un L41 H44**.

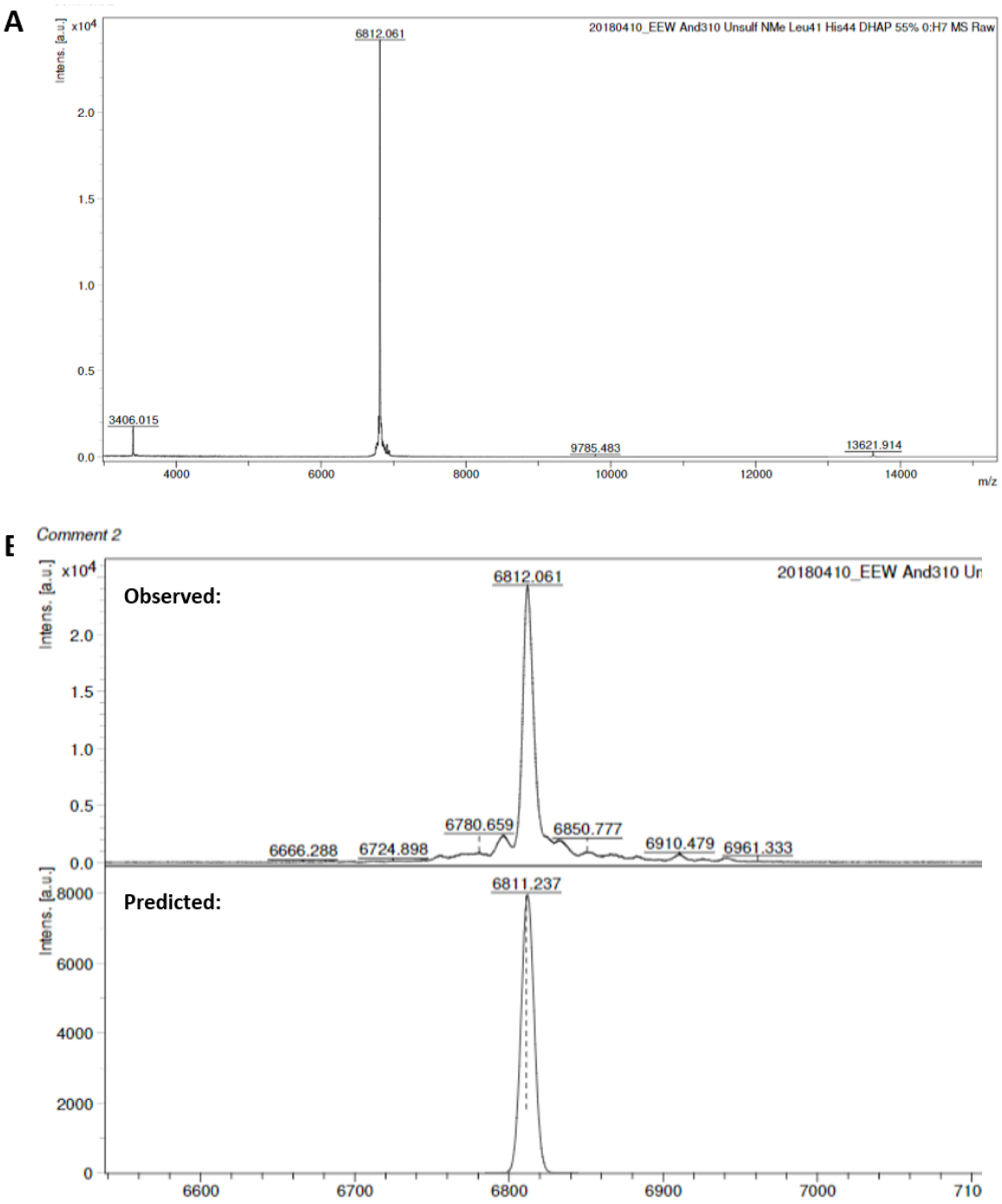


Figure S139: MALDI-TOF mass spectrum of **And310 Un L41 H44** (*A*) 4000-14000 m/z and (*B*) 6600-7100 m/z.

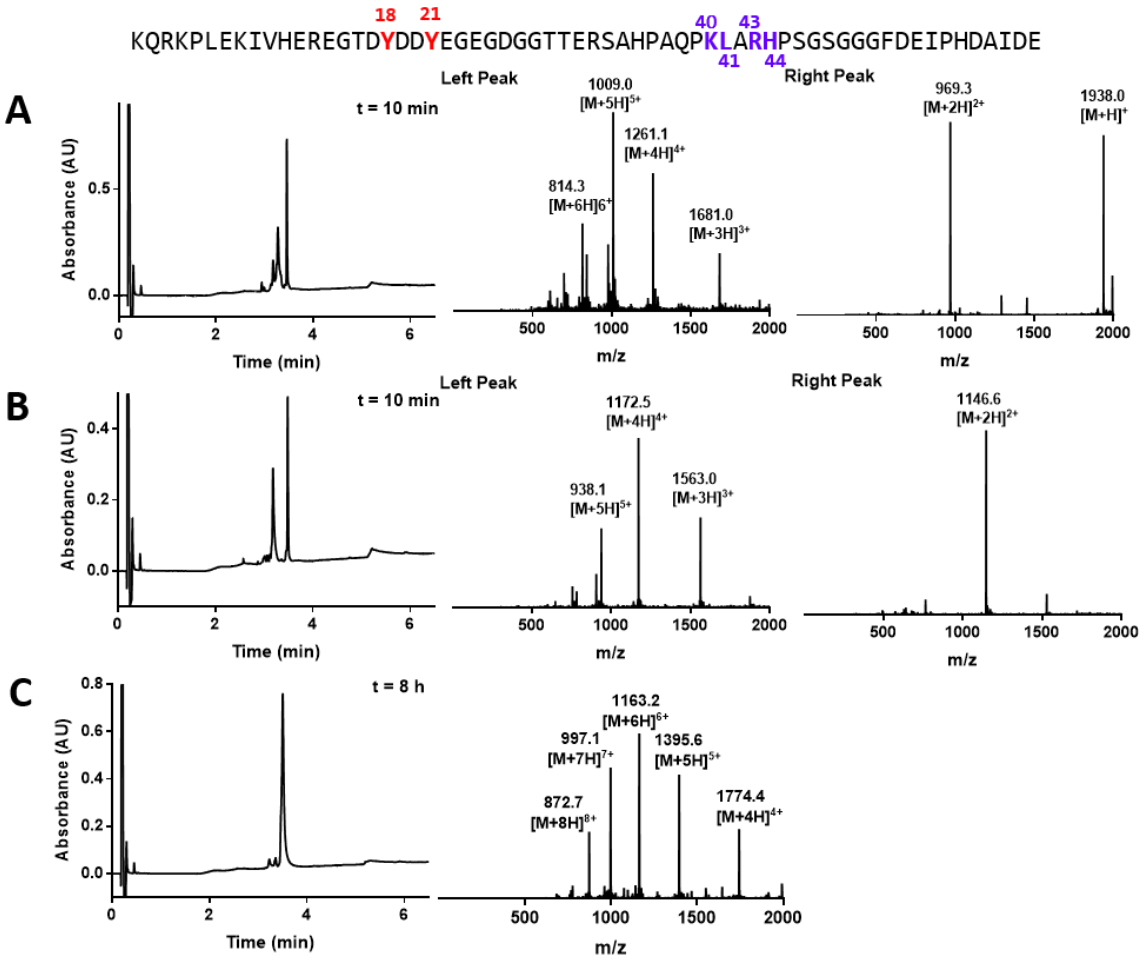


Figure S140: Analytical traces and mass spectra of thrombolytic cleavage of differentially methylated And310 variants. (*A*) **And310 DS L41**; (*B*) **And310 DS H44** and (*C*) **And310 DS L41 H44**. Conditions: 2 nM human α-thrombin, 60 nM inhibitor in 50 mM Tris-HCl pH 8, 50 mM NaCl for 10 min or 8 h as specified at 37 ºC. Predicted masses: K1 to K40 = 4683.1 (double sulfated); K1 to R43 (NMe-Leu41 double sulfated) = 5037.3; L41 to E62 (NMe-His44) = 2290.1; H44 to E62 = 1935.8; K1 to R32 (unsulfated) = 3706.7; S33 to E62 (NMe-Leu41 NMe-His44) = 3120.5

Inhibition Kinetics of Doubly Methylated And310 Variants

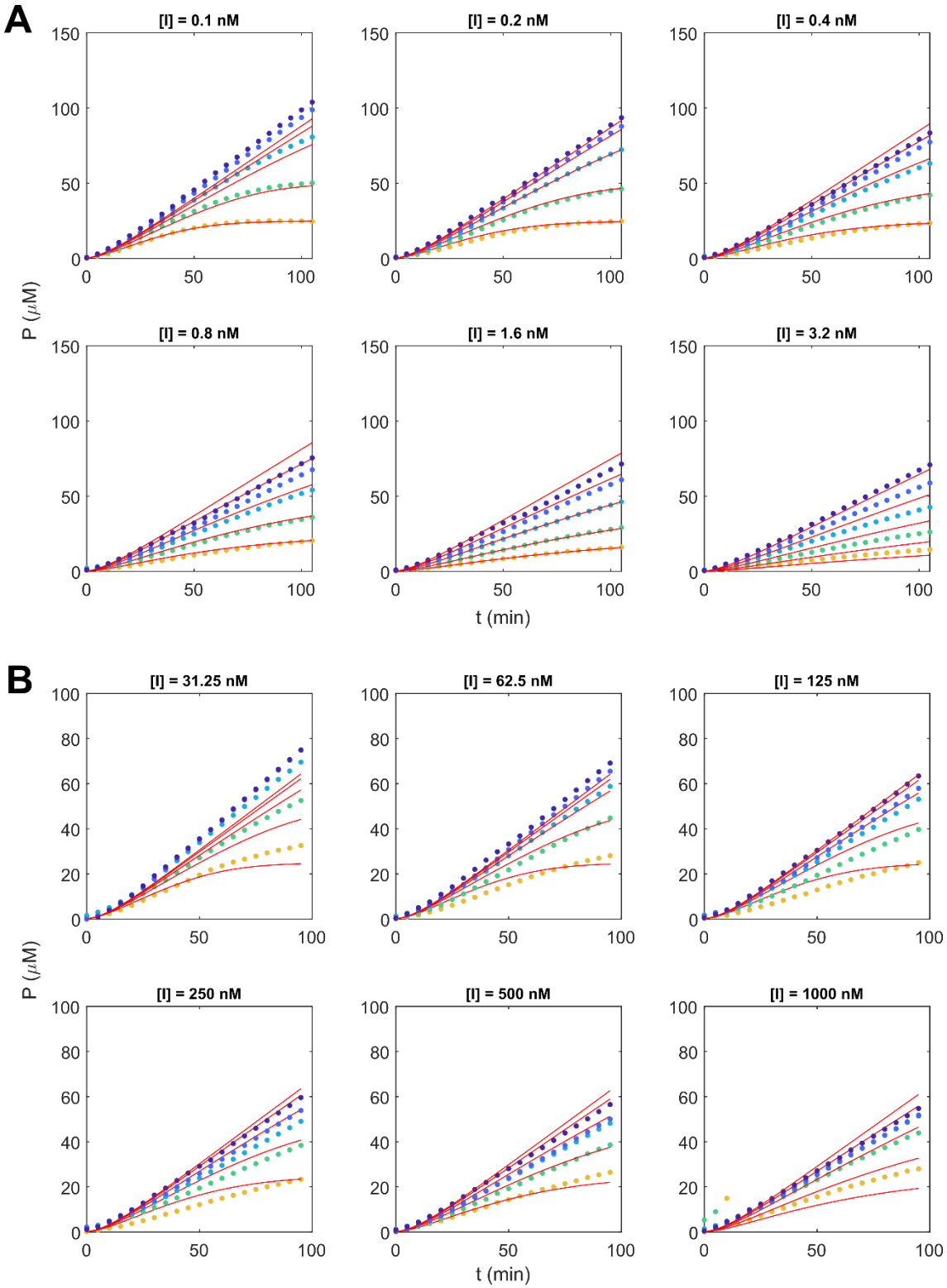
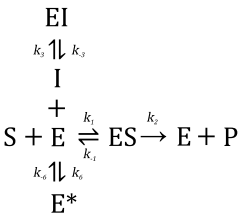


Figure S141: Kinetic analysis of the inhibitory activity of sulfated and unsulfated homologues of the doubly methylated variants of **And310** against human α-thrombin for substrate concentrations of (symbols from top to bottom) 400, 200, 100, 50 and 25 μM. Reaction product concentration measured over time (symbols) and numerical fit to the mechanism described in Figure S142 for (*A*) **And310 DS L41 H44** and (*B*) **And310 Un L41 H44**. The data plotted correspond to a representative experiment.

A



B

$$\frac{d[S]}{dt} = k_{-1}[ES] - k_1[E][S]$$

$$\frac{d[P]}{dt} = k_2[ES]$$

$$\frac{d[E]}{dt} = (k_{-1} + k_2)[ES] - k_1[E][S] + k_{-6}[E^*] - k_6[E] + k_{-3}[EI] - k_3[E][I]$$

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_{-1} + k_2)[ES]$$

$$\frac{d[E^*]}{dt} = k_6[E] - k_{-6}[E^*]$$

$$\frac{d[EI]}{dt} = k_3[E][I] - k_{-3}[EI]$$

$$\frac{d[I]}{dt} = -k_3[E][I] + k_{-3}[EI]$$

C

	And310 DS L41 H44	And310 Un L41 H44
k_1 ($\mu M^{-1}min^{-1}$)	$4.55 \times 10^3 \pm 2.02 \times 10^4$	$9.30 \times 10^3 \pm 1.04 \times 10^6$
k_{-1} (min^{-1})	$8.66 \times 10^3 \pm 6.93 \times 10^4$	$1.00 \times 10^4 \pm 1.70 \times 10^6$
k_2 (min^{-1})	$6.75 \times 10^3 \pm 89.7$	$5.17 \times 10^3 \pm 95.0$
k_6 (min^{-1})	0.219 ± 0.080	0.546 ± 0.195
k_{-6} (min^{-1})	0.0805 ± 0.0096	0.0892 ± 0.098
k_3 ($\mu M^{-1}min^{-1}$)	$2.11 \times 10^5 \pm 1.08 \times 10^4$	863 ± 308
k_{-3} (min^{-1})	15.6 ± 2.75	65.7 ± 15.0
K_I (nM)	0.0738 ± 0.0136	76.2 ± 32.2

Figure S142: Slow-onset inhibition due to enzyme isomerization. (*A*) Schematic mechanism, where E and E* represent the non-isomerized and isomerized enzyme, respectively; S, I and P represent substrate, inhibitor and product, respectively; ES and EI represent the enzyme-substrate and enzyme-inhibitor complexes, respectively. (*B*) System of ordinary differential equations describing the proposed mechanism; square brackets represent molar concentrations. The solver ode15s of MATLAB R2018a (Mathworks) was used to numerically solve this system in two steps: (1) the equilibrium concentrations of E and E* are obtained using as total [E] = 0.30 nM and [S] = [I] = 0 (pre-mixing conditions); (2) the system is solved using as initial conditions the concentrations of S and I corresponding to each assay and the halved equilibrium concentrations of E and E* (reaction start conditions). (*C*) Fitting results obtained using function lsqcurvefit of MATLAB R2018a (Mathworks).

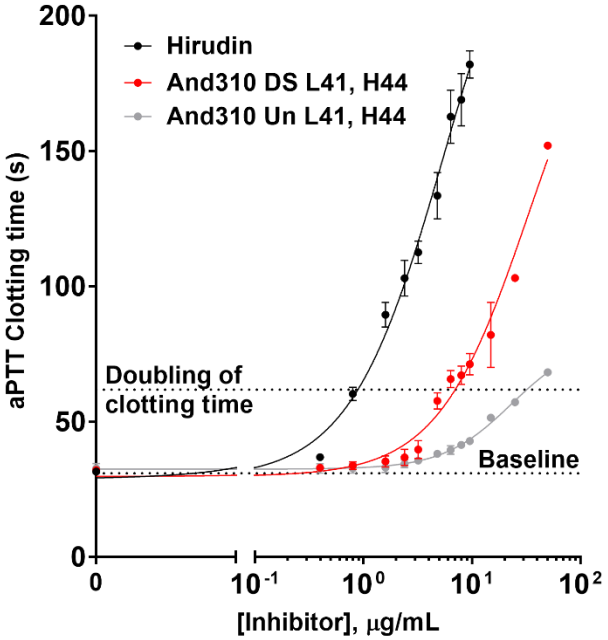


Figure S143: Human (*in vitro*) aPTT dose response curves of backbone methylated And310 analogues **And310 DS L41, H44** and **And310 Un L41, H44**, compared to hirudin. Data points represent mean ± SEM of 3-4 independent plasma samples for aPTT assay of each inhibitor. NB: Inhibitor concentrations are plotted on a log scale.

References:

1. Hackenberger CPR (2006) The reduction of oxidized methionine residues in peptide thioesters with NH4I–Me2S. *Org Biomol Chem* 4:2291-2295.
2. Williams JW & Morrison JF (1979) [17] The kinetics of reversible tight-binding inhibition. *Methods in Enzymology* (Academic Press) 63:437-467.
3. Biron E, Chatterjee J, & Kessler H (2006) Optimized selective N-methylation of peptides on solid support. *J Pept Sci* 12:213-219.