Supporting Information

Reagents. 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), N^{α}-Boc protected L-amino acids and Boc protected D-amino acids (Peptide Institute, Osaka, Japan), were obtained from Peptides International (Louisville, Kentucky). Side-chain protecting groups used were Arg(Tos), Asp(OcHex), Asn(Xan), Cys(4-CH_3Bzl), Glu(OcHex), His(DNP), Lys(2Cl-Z), Ser(Bzl), Thr(Bzl), Tyr(2Br-Z), Trp(Alloc). Boc-L-1,3-thiazolidine-4-carboxylic acid (Boc-Thz) was obtained from Bachem (Torrance, California). Boc-L-His(DNP), Boc-L-Trp and Boc-D-Trp were purchased from Chem-Impex International Inc (Wood Dale, Illiniois). Aminomethyl-resin (1.07mmol/g) was prepared from Biobeads S-X1 (BioRad, California).[1] Boc-L-Ala-OCH_2-phenylacetic acid was purchased from NeoMPS, Strasbourg, France. Fmoc Rink amide linker was purchased from Nova Biochem, Billerica, Massachusetts). N,N-Diisopropylethylamine (DIEA) was obtained from Applied Biosystems, Foster City, California). Diethyl ether, dichloromethane, N,N-dimethylformamide (DMF), HPLC-grade acetonitrile, and guanidine hydrochloride were purchased from Fisher Scientific, USA. Trifluoroacetic acid (TFA) was obtained from Halocarbon Products (New Jersey). HF was purchased from Matheson Trigas USA. All other reagents were purchased from Sigma-Aldrich, USA, and were of the purest grade available.

Peptide synthesis. Peptide segments were synthesized using manual 'in situ neutralization' Boc chemistry protocols for stepwise SPPS.[2] Peptide-thioesters were synthesized on trityl-SCH_2CO-Ala-OCH_2-Pam-resin[3] at a 0.2 mmol scale. After completion of the chain assembly and removal of the N^{α}Boc group, each peptide was cleaved from the resin and HF-labile side chain protecting groups removed by treatment at 0 °C for 1 h with anhydrous HF containing 5%v/v p-cresol as scavenger. After removal of HF by evaporation under reduced pressure, each crude peptide was

precipitated and washed with diethyl ether, then dissolved in 50% aqueous acetonitrile containing 0.1% TFA and lyophilized.

Peptides 1-23 and 24-46 were common to all the syntheses, and were prepared as peptide-thioesters on a Tyr(2Cl-Z)-SCH_2COAla-OCH_2Pam-resin.

Peptide segment Thz^{47}-Lys^{61}-thioester was synthesized on Lys(2Cl-Z)-SCH_2COAla-OCH_2Pam-resin, and peptides Cys^{62}-Lys-Ser-Gly-Lys^{66} and Cys^{62}-Lys-Ser-Gly-Lys-Lys^{67} were synthesized on Lys(2Cl-Z)OCH_2Pam-resin. Peptide Cys^{62}-Lys-Ser^{64}-$CONH_2$ was synthesized by Fmoc chemistry SPPS on Rink amide linker resin. Peptide segments Thz^{47}-Lys^{66}, Thz^{47}- Lys^{67}, Thz^{47}-Ser^{64}-$CONH_2$, and Thz^{47}-Cys^{62} were prepared by native chemical ligation reaction of Thz^{47}-Lys^{61}-thioester with the appropriate segment :Cys^{62}-Lys-Ser-Gly-Lys^{66}, Cys^{62}-Lys-Ser-Gly-Lys-Lys^{67}, Cys^{62}-Lys-Ser^{64}-$CONH_2$, or the amino acid Cys.

When we first prepared peptide segments Lys^{1}-Tyr^{23}-*$COSCH_2Ala$* and Thz^{24}-[$His(DNP)^{42}$]-Tyr^{46}-*$COSCH_2Ala$*, we had trouble purifying them by reverse phase HPLC: these peptide segments were observed to stick to the reverse phase support, and purification yields were low. To resolve this problem, peptide Lys^{1}-Tyr^{23}-*COSR* and peptide Thz^{24}-[$His(DNP)^{42}$]-Tyr^{46}-*COSR* were then prepared with an $–Arg_5$-Ala and $–Arg_4$-Ala tag, respectively, on the thioester leaving group at the C-terminus, in order to improve handling properties of the synthetic peptide segments[4] and to thus facilitate their purification.

During the synthesis of peptide Thz^{47}-Lys^{61}-*$COSCH_2Ala$*, at each Boc deprotection step a 30 second DCM wash was used before and after the TFA step, i.e. between the TFA and the DMF washes, in order to minimize the formation of aspartimide at the –Asn-Gly- sequence (the heat generated when TFA meets DMF accelerated the formation of aspartimide).[5]

Reverse phase HPLC and LC-MS analysis. Analytical reversed phase HPLC and LC-MS were performed using an Agilent 1100 series HPLC system equipped with an online MSD ion trap. The column used was a Phenomenex Aeris WIDEPORE 3.6 μm C4, 150 x 4.6 mm. Chromatographic separations were performed using a linear gradient of 5-45% acetonitrile (with 0.08% TFA) in water (with 0.1% TFA) over 40 min, with column temperature 40 °C. Flow rates were controlled at 0.9 mL/min. Peptide detection was based on UV absorption at 214 nm, and masses were obtained by online electrospray mass spectrometry.

Preparative HPLC. Peptide products from solid phase peptide synthesis or from ligation reactions were purified using a Phenomenex Jupiter 5.0 μm C4, 250 x 10.0 mm column at 40 °C. A shallow gradient of acetonitrile (with 0.08% TFA) versus water (with 0.1% TFA) was designed for each peptide based on its elution characteristics. Flow rates were controlled at 5 mL/min. Fractions containing the desired pure peptide were identified by analytical LC and mass spectrometry, then combined and lyophilized. LCMS data for the synthetic peptides are shown in Figure S1-S9.

Synthesis of full-length Ts3 polypeptide chains. In a typical procedure, exemplified by synthesis of the Ts3(1-64)-$CONH_2$ polypeptide, peptide Thz^{24}-[$His(DNP)^{42}$]-Tyr^{46}-$COSCH_2Arg4Ala$ (22.5 mg, 8.1 μmol, 4.0 mM) was dissolved in 2 mL buffer (6 M GuHCl, 0.2 M Na_2HPO_4, , pH 7.0) containing 0.2 M MESNa and the reaction was left at room temperature for 20 hours to remove the DNP group from the side chain of His^{42}. Peptide Cys^{47}-Ser^{64}-$CONH_2$ (21.8 mg, 11.3 μmol, 5.5 mM) and 4-mercaptophenylacetic acid (MPAA) (16.8 mg, 0.1 mmol, 50 mM) were added to the solution, and the pH was adjusted to 7.0 to start the first ligation reaction. The ligation reaction was left for 24 hours and DTT was added to reduce possible disulfide bonds. The N-terminal residue Thz^{24} was converted to Cys^{24} at pH 4 by addition of

$MeONH_2 \cdot HCl$ and solid phase extraction was then performed to remove excess peptide Cys^{47}-Ser^{64}-*$CONH_2$*. For the second ligation reaction, peptide Lys^{1}-Tyr^{23}-*$COSCH_2Arg5Ala$* (33.0 mg, 8.85 μmol, 2.4 mM) and the ~ 8 μmol (~2 mM) crude peptide Cys^{24}-Ser^{64}-*$CONH_2$* product from the first ligation reaction were dissolved in 4 mL buffer (6 M GuHCl, 0.2 M Na_2HPO_4, 50 mM MPAA, 20 mM TCEP hydrocholoride, pH 7.0). The reaction was left at room temperature for 21 hours before solid phase extraction was performed to recover the crude peptide Lys^{1}-Ser^{64}-*$CONH_2$* product. Analytical data for these ligation reactions is shown in the main text. The crude full length polypeptide was used directly for folding/disulfides formation.

Synthesis of the other Ts3 polypeptide candidates was carried out in the same fashion. Analytical LCMS data for synthesis of the polypeptides Ts3-Lys^{1}-Cys^{62}, Ts3-Lys^{1}-Lys^{66}, and Ts3-Lys^{1}-Lys^{67}, are shown in Figures S10-S12.

Synthesis of D-Ts3(1-64)-*$CONH_2$* protein. The peptide segments D-Lys^{1}-Tyr^{23}-*$COSCH_2Arg_5Ala$* and D-[$His(DNP)^{42}$]Thz^{47}-Lys^{61}-*$COSCH_2Arg_5Ala$* were synthesized by Boc chemistry 'in situ neutralization' SPPS. Peptide D-Thz^{47}-Lys^{61}-*$COSCH_2Ala$* was synthesized using a modified protocol: at each residue, a DCM wash was performed between the DMF wash before the TFA treatment to remove the Boc group and another DCM wash was performed after the TFA treatment, before the following DMF wash. Peptide D-$Cys^{62}$$LysSer^{64}$-*$CONH_2$* was synthesized by Fmoc chemistry SPPS on Rink amide linker resin. Analytical HPLC data for the synthesis by native chemical ligations of the D-Ts3(1-64)-*$CONH_2$* polypeptide chain is shown in Figure S14. After solid phase extraction and lyophilization, the crude D-Ts3(1-64)-*$CONH_2$* polypeptide was folded under the same conditions as were used for the L-protein toxins: 0.5 M L-arginine·HCl, 1mM EDTA, 0.1M Tris, 2 mM GSH, 1 mM GSSG, polypeptide 0.02 mg/mL, 20% DMSO, pH

8.5, room temperature (Figure S15). Analytical LCMS data for purified D-Ts3(1-64)-$CONH_2$ protein is shown in Figure S16.

Crystallization. Purified L-Ts3(1-64)-$CONH_2$ protein molecule was dissolved in water to a concentration of 10 mg/mL for the conventional protein crystallization trials. A racemic mixture of {L-Ts3(1-64)-$CONH_2$ toxin & D-Ts3(1-64)-$CONH_2$ protein} at 10 mg/mL (5 mg/mL of L-Ts3(1-64)-$CONH_2$ toxin & 5 mg/mL of D-Ts3(1-64)-$CONH_2$) was used for racemic protein crystallization trials. Each condition used 1 mL of well solution with hanging drops consisting of 1 µL well solution and 1 µL protein solution. The plates were kept in an incubator at 19 ºC.

X-ray diffraction data collection. For low temperature data collection, selected crystals were briefly transferred to the cryoprotectant (reservoir solution plus 20% (v/v) glycerol) and flash-frozen in liquid nitrogen. Atomic resolution X-ray diffraction data were collected at 100K at the Argonne National Laboratory (Advanced Photon Source, beamline 24ID-E) using 0.97 Å synchrotron radiation. Diffraction images were indexed, integrated, scaled, and merged with RAPD.[6] The reflection intensity statistics revealed that the molecule was crystallized in the centrosymmetric space group P1<bar>. The structure was solved by molecular replacement using the software Phaser[7] and refined using Phenix.[8] The final structure had an R-work/R-free of 0.26/0.30. Ramachandran plot analysis showed that 98.4% of the residues were in a favored region and 1.6% residues were in an allowed region. The Data collection and refinement statistics are shown in Table S1. Crystal structure data for racemic Ts3 protein has been deposited in the Protein Data Bank with PDB code 5CY0.

Preparation of Ts3 analogs for electrophysiology. All Ts3 analogs were diluted in the external solution (see Electrophysiology) containing 0.1% Bovine Serum Albumin (BSA). The concentrations of Ts3 analogs were obtained by NanoDrop spectroscopy at 280 OD.

Molecular biology. The alpha subunit and beta-1 subunit of rat skeletal muscle voltage-gated sodium channel ($rNa_v1.4$) were cloned into the pBSTA vector. For expression of $rNa_v1.4$, the cRNA of alpha and beta-1 subunit were transcribed in vitro using the T7 mMESSAGE cRNA kit (Ambion) and injected in a molar ratio 1:1 into Xenopus oocytes.[9] For the electrophysiological experiments, freshly isolated, defolliculated oocytes were injected with 1 ng of cRNA and kept in Standard Oocytes Saline (SOS) solution: 96mM NaCl, 2mM KCl, 1mM $MgCl_2$, 1.8mM $CaCl_2$, 10mM HEPES, 200mg/L sodium pyruvate, pH 7.4, for 1-2 days at 18℃.

Electrophysiology. The oocytes were mounted in a cut-open oocyte voltage clamp (COVC) set-up.[10] The external solution contained 57.5 mM nmethylglucamine (NMG)-methylsufonate (MS), 57.5mM Na-MS, 10mM HEPES, and 2mM Ca-MS, pH 7.4. The internal solution contained 103.5 mM NMG-MS, 11.5 mM Na-MS, 10mM HEPES, and 2 mM EGTA, pH 7.4. When Ts3 analogs were dissolved in these solutions, 0.1% BSA was added. When recording ionic currents in the presence of Ts3 analogs, these toxins were applied into both the upper chamber and the guard chamber of the COVC set-up. Before recording ionic currents, oocytes were voltage-clamped at -90 mV for 5min because Ts3 binds to $Na_v1.4$ in a state-dependent manner, i.e., it binds to the resting state at negative membrane potentials. The ionic currents were elicited by a pulse to -10 mV with 65 ms duration from holding at -90 mV. The inactivation decay of ionic currents, between 2ms and 65ms after applying a test pulse (time 0ms), was fitted to two exponentials. Ionic currents, sampled at 10 μs/point, were recorded with a PC44 board and digitized on a 16 bit A/D converter. Data acquisition and analysis programs were developed in house. Linear leak and membrane capacitive currents were subtracted using a P/6 protocol from a subtracting holding potential of -100 mV. All data were obtained at 20℃. Statistical

significance was determined using an unpaired t-test. Errors indicate standard error of means (SEM).

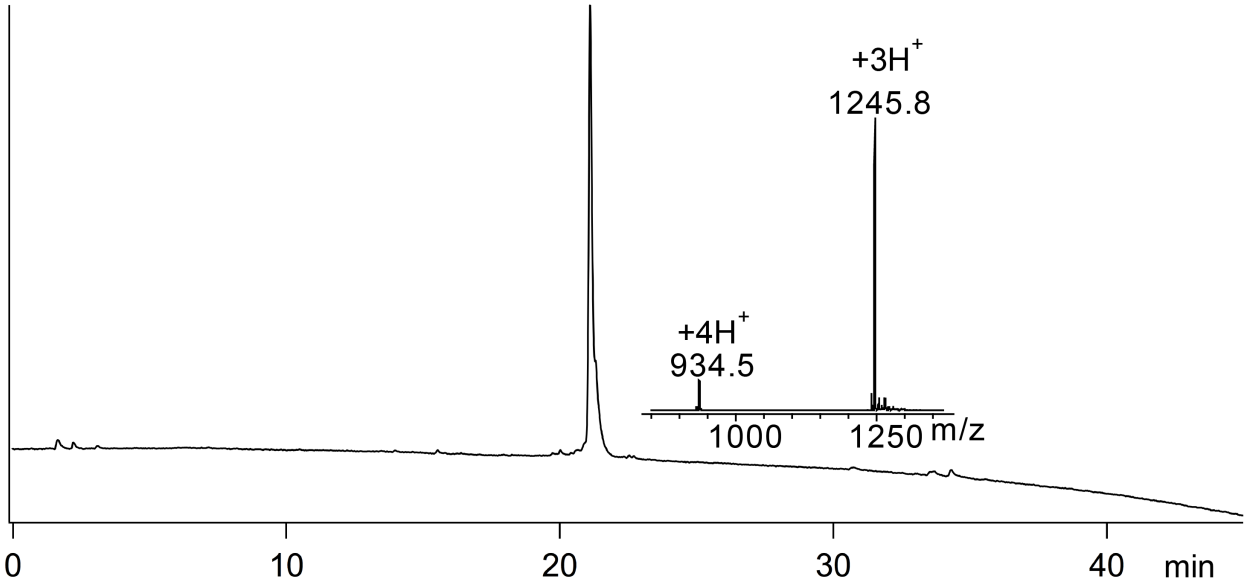

Figure S1. Purified peptide Lys[1]-Tyr[23]-*COSCH$_2$Arg5Ala*. Inset ESMS Calc. 3734.2 Da (av. isotope composition), Obsd. 3734.2 ± 0.2 Da. The MS data shown were collected across the entire UV absorbing peak in the chromatogram.

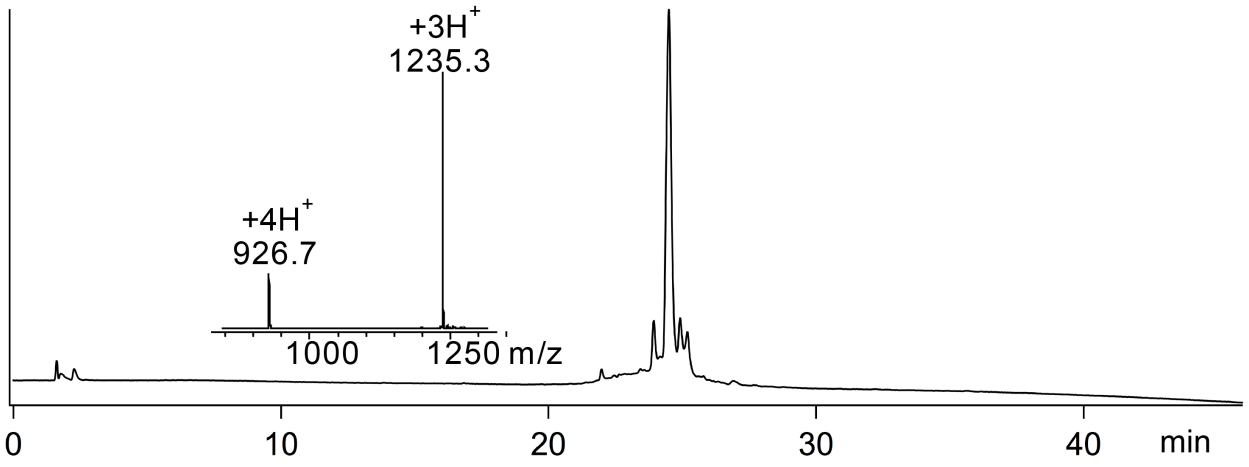

Figure S2. Purified peptide [His(DNP)[42]]Thz[24]-Tyr[46]-*COSCH$_2$Arg4Ala*. Inset ESMS Calc. 3703.3 Da (av. isotope composition), Obsd. 3702.9 ± 0.2 Da. The MS data shown were collected across the entire main UV absorbing peak in the chromatogram.

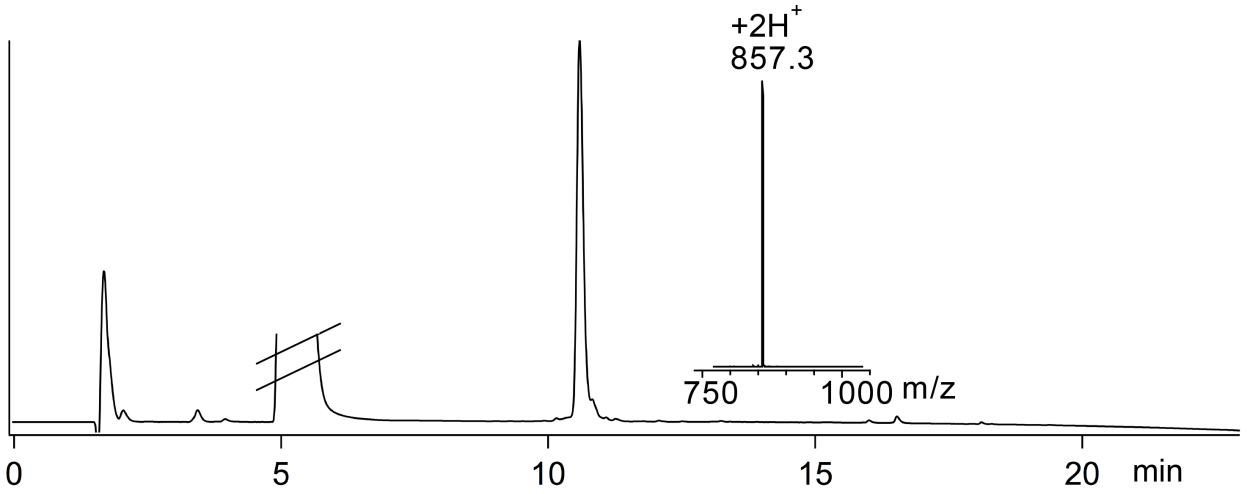

Figure S3. Purified peptide Cys^{47}-Cys^{62}. Inset ESMS Calc. 1712.9 Da (av. isotope composition), 1711.8 Da (mono. isotope composition), Obsd. 1712.6 ± 0.2 Da. The MS data shown were collected across the entire UV absorbing peak in the chromatogram.

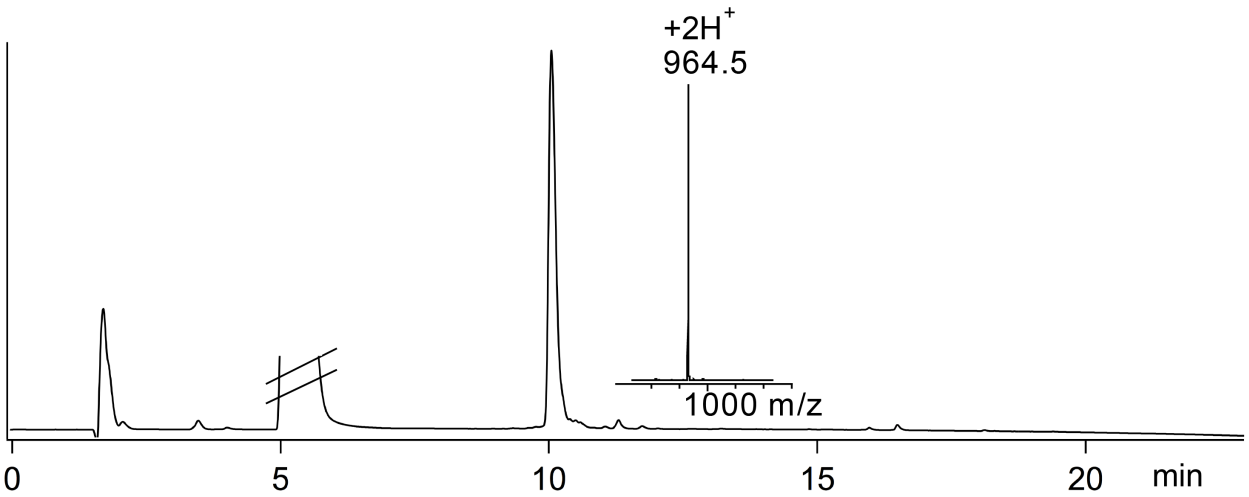

Figure S4. Purified peptide Cys^{47}-Ser^{64}-$CONH_2$. Inset ESMS Calc. 1927.2 Da (av. isotope composition), 1925.9 Da (mono. isotope composition), Obsd. 1927.0 ± 0.2 Da. The MS data shown were collected across the entire UV absorbing peak in the chromatogram.

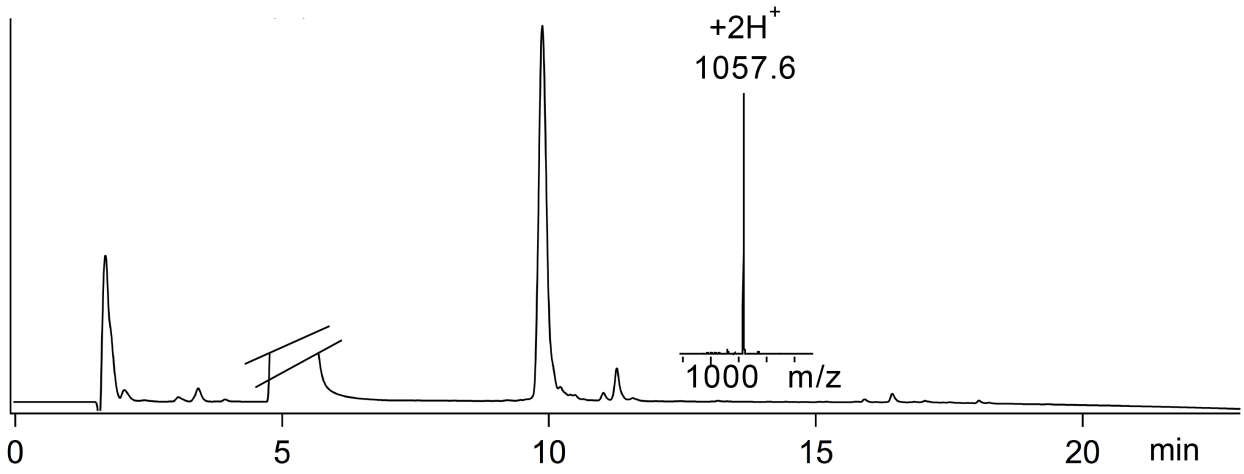

Figure S5. Purified peptide Cys^{47}-Lys^{66}. Inset ESMS Calc. 2113.4 Da (av. isotope composition), 2112.0 Da (mono. isotope composition), Obsd. 2113.2± 0.2 Da. The MS data shown were collected across the entire main UV absorbing peak in the chromatogram.

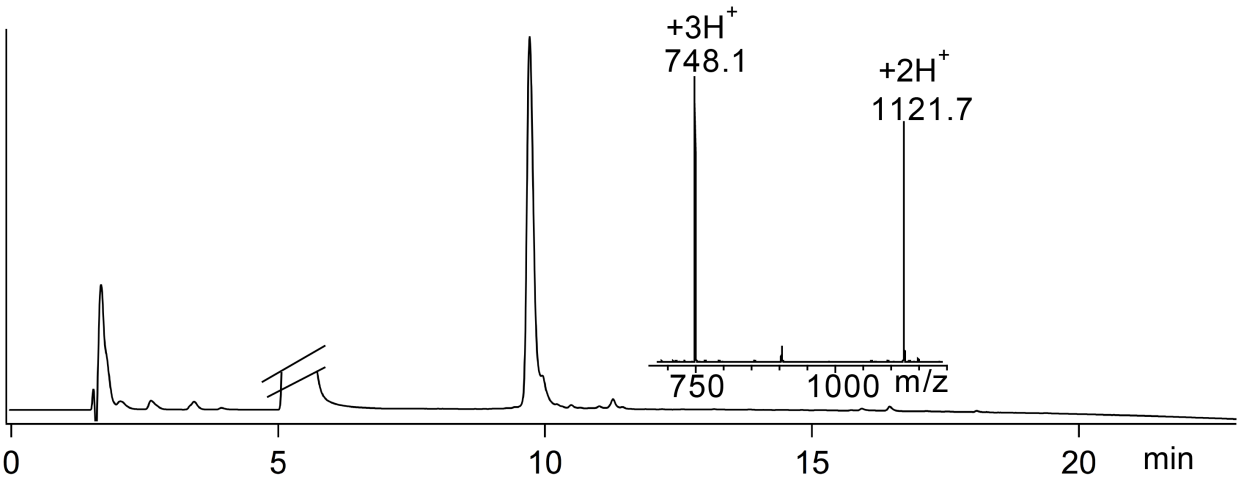

Figure S6. Purified peptide Cys^{47}-Lys^{67}. Inset ESMS Calc. 2241.6 Da (av. isotope composition), 2240.1 Da (mono. isotope composition), Obsd. 2241.4 ± 0.2 Da. The MS data shown were collected across the entire UV absorbing peak in the chromatogram.

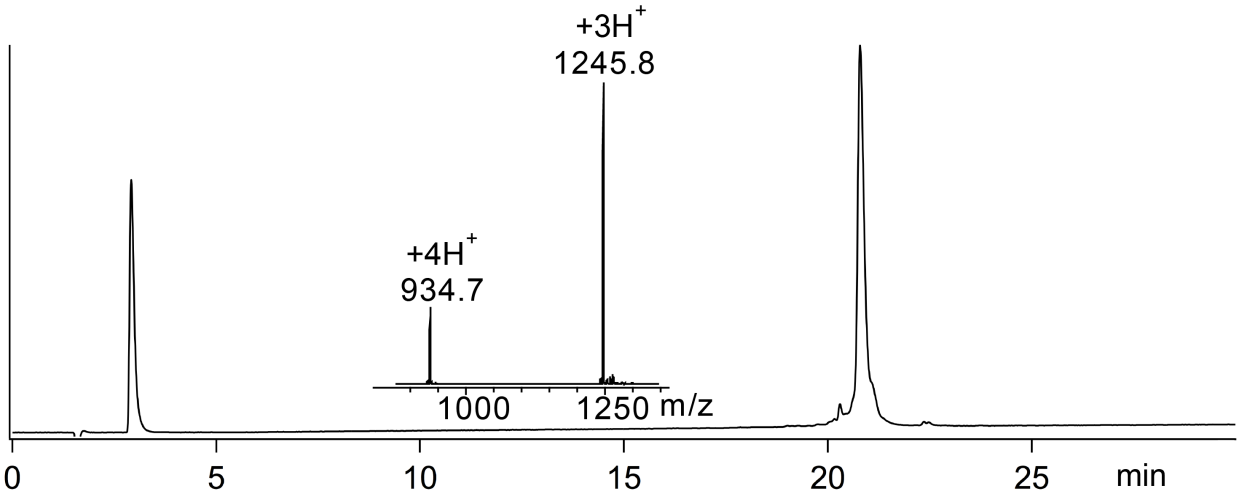

Figure S7. Purified peptide D-Lys[1]-Tyr[23]-*COSCH$_2$Arg5Ala*. Inset ESMS Calc. 3734.2 Da (av. isotope composition), Obsd. 3734.6 ± 0.2 Da. The MS data shown were collected across the entire main UV absorbing peak in the chromatogram.

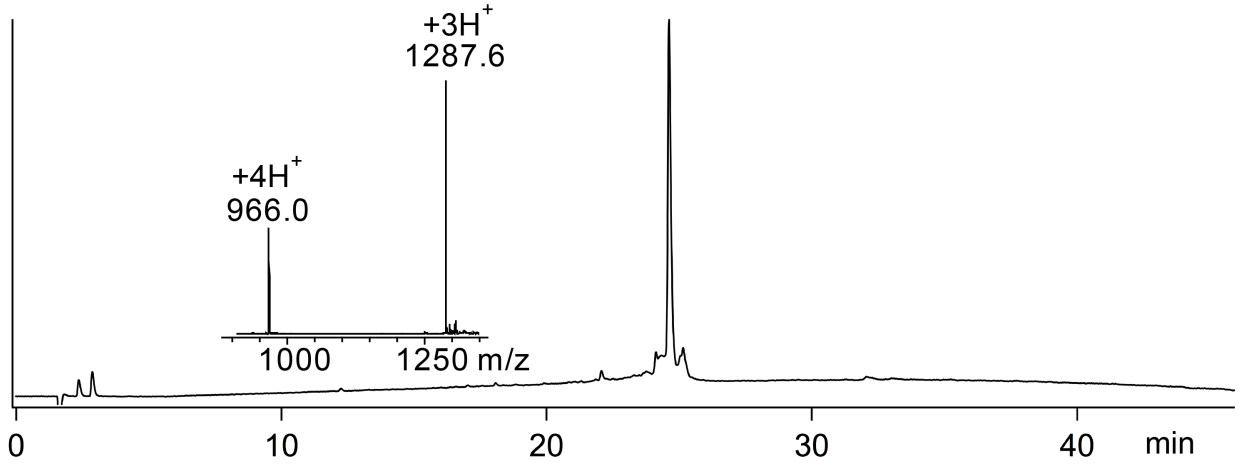

Figure S8. Purified peptide D-[His(DNP)[42]]Thz[24]-Tyr[46]-*COSCH$_2$Arg5Ala*. Inset ESMS Calc. 3859.5 Da (av. isotope composition), Obsd. 3859.9 ± 0.2 Da. The MS data shown were collected across the entire UV absorbing peak in the chromatogram.

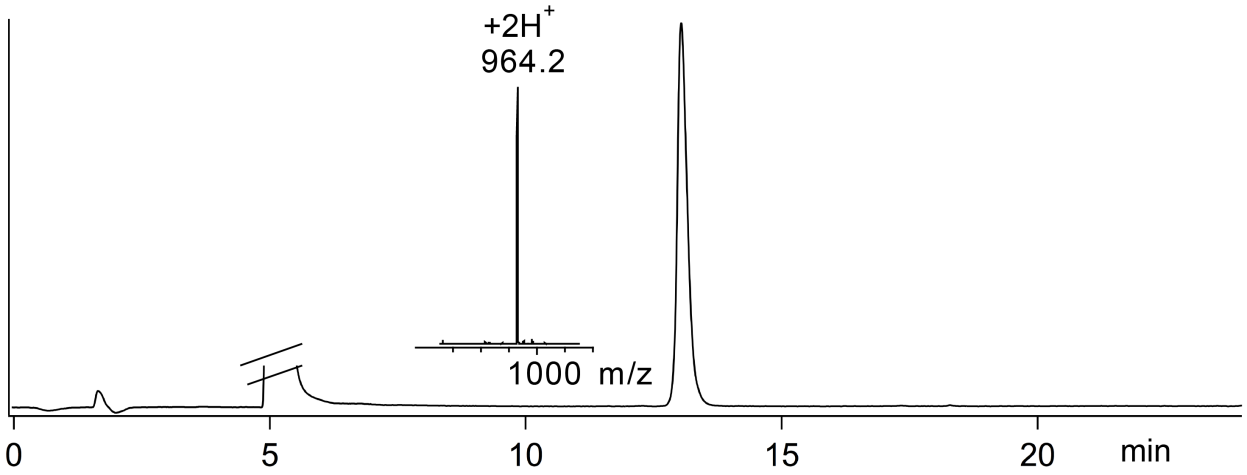

Figure S9. Purified peptide D-Cys^{47}-Ser^{64}-$CONH_2$. Inset ESMS Calc. 1927.2 Da (av. isotope composition), 1925.9 Da (mono. isotope composition), Obsd. 1926.4 ± 0.2 Da. The MS data shown were collected across the entire UV absorbing peak in the chromatogram.

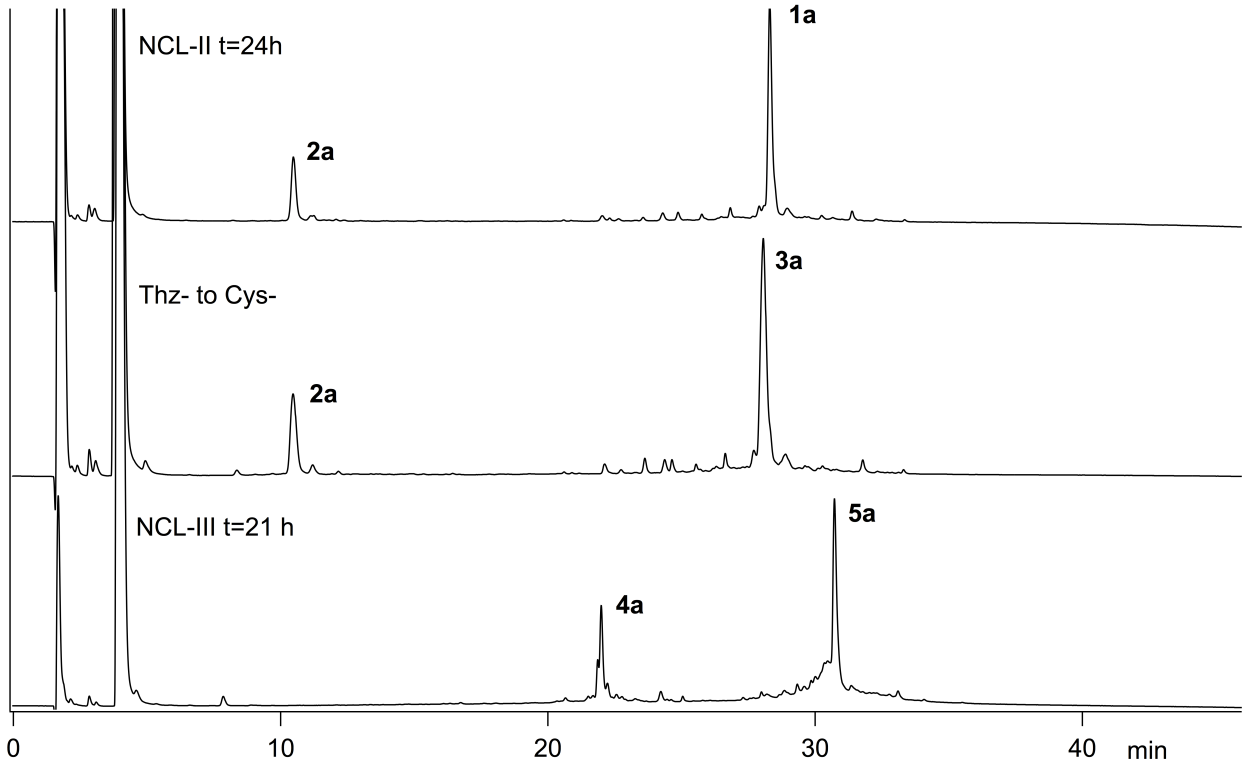

Figure S10. Analytical LCMS data for the synthesis of polypeptide Ts3-Lys^{1}-Cys^{62}. **1a** Thz^{24}-Cys^{62}; **2a** Cys^{47}-Cys^{62}; **3a** Cys^{24}-Cys^{62}; **4a** Lys^{1}-Tyr^{23}-*COS- thiolactone*; **5a** Lys^{1}-Cys^{62}.

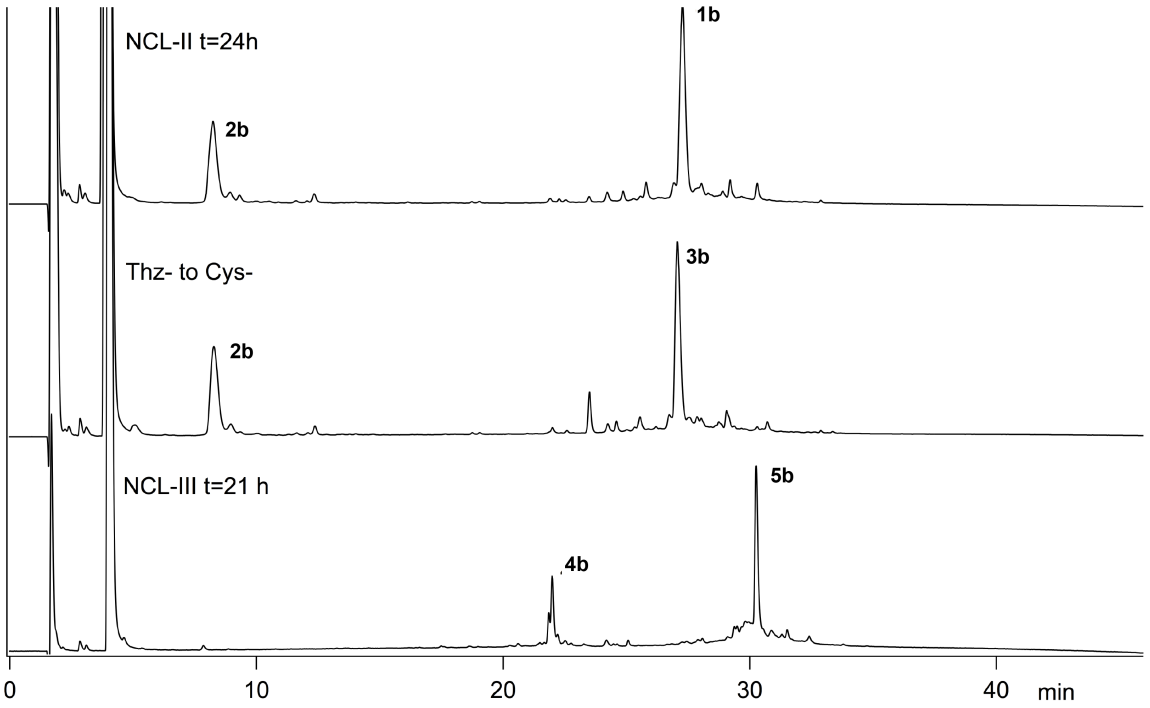

Figure S11. Analytical LCMS data for the synthesis of polypeptide Ts3-Lys^{1}-Lys^{66}. **1b** Thz^{24}-Lys^{66}; **2b** Cys^{47}- Lys^{66}; **3b** Cys^{24}- Lys^{66}; **4b** Lys^{1}-Tyr^{23}-*COS thiolactone*; **5b** Lys^{1}-Lys^{66}.

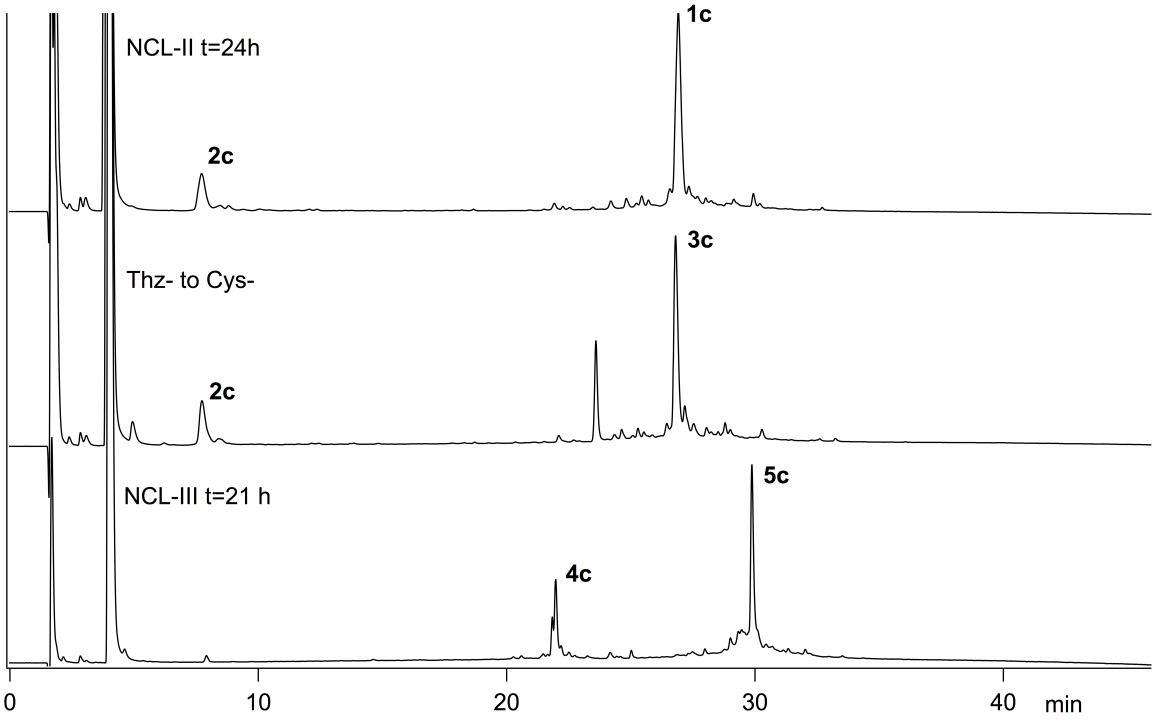

Figure S12. Analytical LCMS data for the synthesis of polypeptide Ts3-Lys^{1}-Lys^{67}. **1c** Thz^{24}-Lys^{67}; **2c** Cys^{47}- Lys^{67}; **3c** Cys^{24}- Lys^{67}; **4c** Lys^{1}-Tyr^{23}-*COS thiolactone*; **5c** Lys^{1}-Lys^{67}.

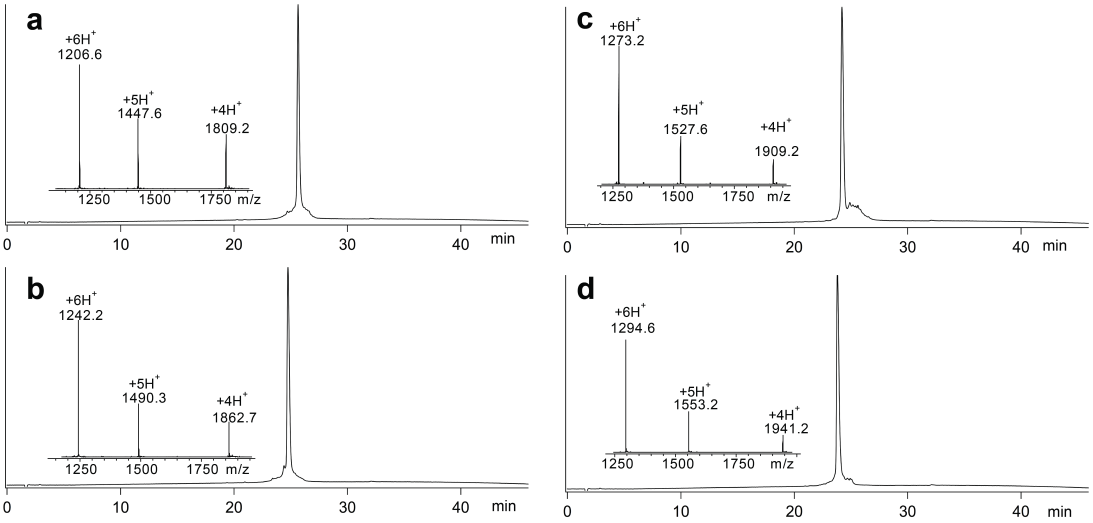

Figure S13. Analytical LCMS characterization of the candidate Ts3 synthetic proteins. a) Purified folded Ts3(1-62). [Inset] ESMS data. Mass: Obsd. 7232.0 ± 0.2 Da; Calc. 7232.1 Da (av. isotopes). b) Purified folded Ts3(1-64)-*CONH*$_2$. [Inset] ESMS data. Mass: Obsd. 7447.0 ± 0.2 Da; Calc. 7446.4 Da (av. isotopes). c) Purified folded Ts3(1-66). [Inset] ESMS data. Mass: Obsd. 7632.6 ± 0.2 Da; Calc. 7432.6 Da (av. isotopes). d) Purified folded Ts(1-67). [Inset] ESMS data. Mass: Obsd. 7761.1 ± 0.2 Da; Calc. 7760.8 Da (av. isotopes). Inset mass spectra were obtained by online electrospray mass spectrometry collected across the entire main UV absorbing peak in each chromatogram.

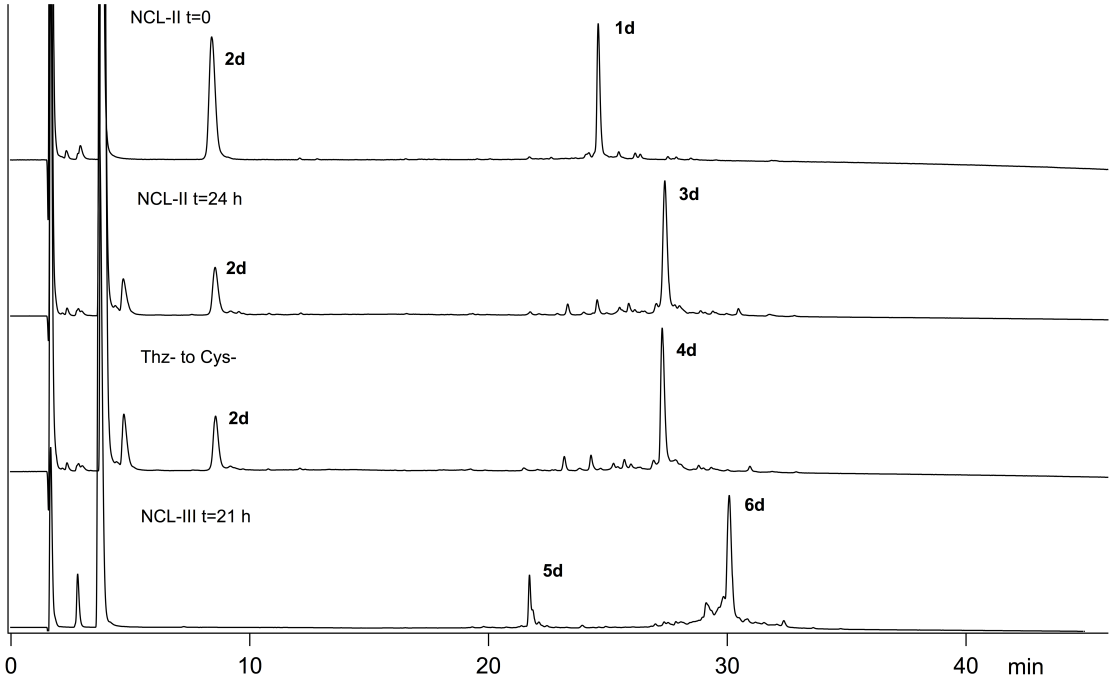

Figure S14. Analytical LCMS data for the synthesis of polypeptide D-Ts3(Lys^{1}-Ser^{64})-CONH$_2$. **1d** D-Thz^{24}-Tyr^{46}-*COSCH*$_2$*CH*$_2$*SO*$_3$ thioester; **2d** D-Cys^{47}-Ser^{64}-*CONH*$_2$; **3d** D-Thz^{24}-Ser^{64}-*CONH*$_2$; **4d** D-Cys^{24}-Ser^{64}-*CONH*$_2$; **5d** D-Lys^{1}-Tyr^{23}-*COS thiolactone*; **6d** D-Lys^{1}-Ser^{64}-*CONH*$_2$.

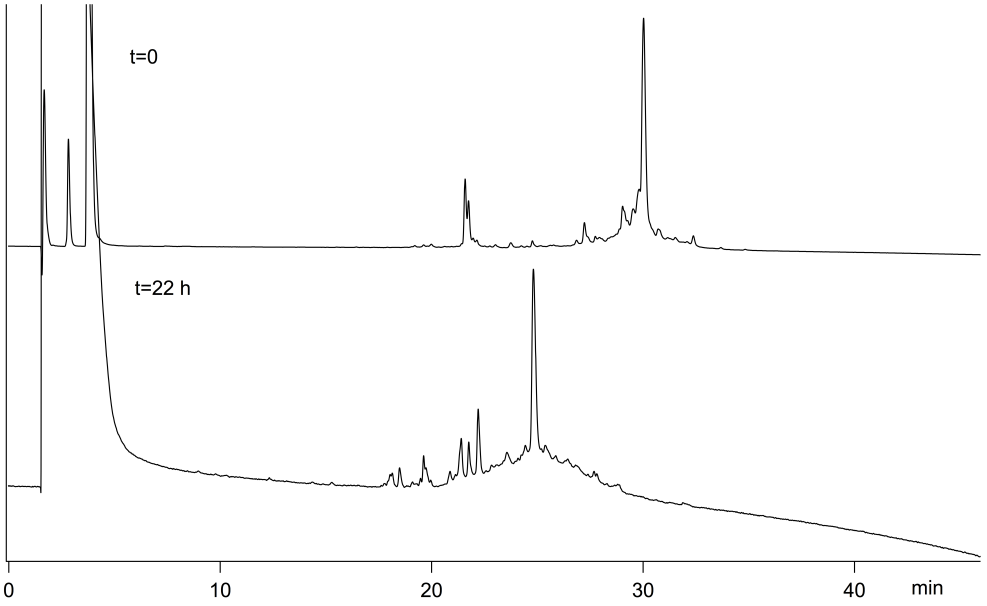

Figure S15. Folding of D-Ts3(1-64)-*CONH*$_2$ polypeptide. Folding condition: 0.5 M L-arginine·HCl, 1mM EDTA, 0.1 M Tris2 mM GSH, 1 mM GSSG, polypeptide 0.02 mg/mL, 20% DMSO, pH 8.5, room temperature.

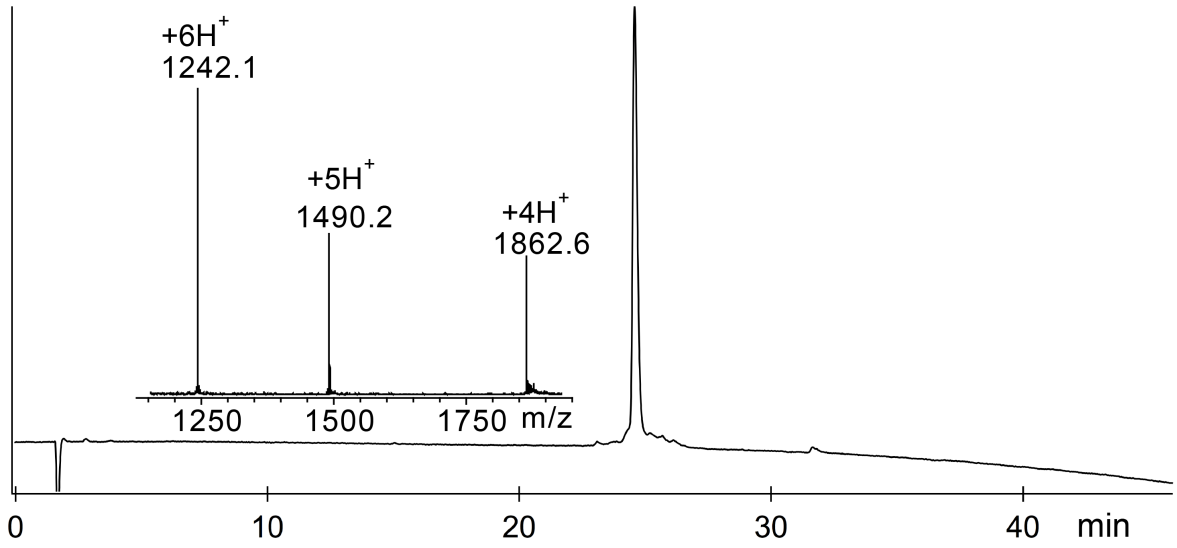

Figure S16. Purified folded D-Ts3(1-64)-*CONH*$_2$ protein. [Inset] ESMS data. Obsd. 7446.3 ± 0.2 Da; Calc. 7446.4 Da (av. isotopes). The MS data shown were collected across the entire UV absorbing peak in the chromatogram. LC-MS conditions were: Phenomenex Aeris WIDEPORE 3.6 µm C4, 150 x 4.6 mm column; linear gradient of 5-45% acetonitrile (with 0.08% TFA) in water (with 0.1% TFA) over 40 min, with column temperature 40 °C; flow rate was controlled at 0.9 mL/min with detection at 214 nm.

-KKDGYPVEYDN**C**AYI**C**WNYDNAY**C**DKL**C**KDKKADSGY**C**YWVHIL---**C**Y**C**YGLPDSEPTKTNGK**C**KS

MVRDAYIAKNYN**C**VYE**C**FR--DAY**C**NEL**C**TKNGASSGY**C**QWAGKYGNA**C**W**C**YALPDNVPIRVPGK**C**R

-VKDGYIVDDVN**C**TYF**C**GR--NAY**C**NEE**C**TKLKGESGY**C**QWASPYGNA**C**Y**C**YKLPDHVRTKGPGR**C**H

Figure S17. Sequence alignment, based on Cys residues, of Ts3 with LQH-α-IT, Aah2 (from top to bottom).

Table S1. X-ray data statistics of the *DL*-Ts3 racemic crystal structure

Data collection	
Space group	P1<bar>
Cell dimensions	
a, *b*, *c* (Å)	19.32, 30.41, 52.47
α, β, γ (°)	101.17, 91.26, 98.61
Resolution (Å)	18.2 – 1.93 (1.98 - 1.93)*
R_{sym} or R_{merge}	0.155 (0.635)
I / δI	4.4 (1.5)
Completeness (%)	92(92.4)
Redundancy	3.3(3.4)
Refinement	
Resolution (Å)	18.2 – 1.93 (1.98 - 1.93)
No. reflections	7937
R_{work} / R_{free}	0.26 / 0.30
No. atoms	
Protein	834
Ligand/ion	3
Water	67
B-factors (Å^2)	
Protein	29
Ligand	48
Water	38
R.m.s. deviations	
Bond lengths (Å)	0.007
Bond angles (°)	0.9

*Values in parentheses are for highest-resolution shell.

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