Supplementary Information

Chemical Synthesis of a Haemathrin Sulfoprotein Library Reveals Enhanced Thrombin Inhibition Following Tyrosine Sulfation

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Materials

Peptide grade dimethylformamide (DMF) was obtained from Labscan. HPLC grade acetonitrile (MeCN) was obtained from Merck or Sigma-Aldrich and supplemented with peptide-grade trifluoroacetic acid (TFA) or formic acid (FA) (Sigma-Aldrich) as specified. Ultra-pure (type 1) H_2O was used as supplied from a Merck Millipore Direct-Q 5 water purification system and supplemented with peptide-grade TFA or FA (Sigma-Aldrich) as specified. Amino acids, coupling reagents and resins for Fmoc-solid-phase peptide synthesis (Fmoc-SPPS) were obtained from either Novabiochem or GL Biochem. Fmoc-SPPS was performed in polypropylene syringes equipped with Teflon filters, obtained 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 vol% TFA in H_2O (Solvent A) and 0.1 vol% TFA in MeCN (Solvent B) in a linear gradient as indicated. The analysis of the chromatograms was conducted using Empower 3 Pro software (2010) and retention times (Rt, 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, 19 × 150 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 were purified using a mobile phase of 0.1 vol% TFA in H_2O (Solvent A) and 0.1 vol% TFA in MeCN (Solvent B) using the indicated linear gradient. After lyophilization, pure peptides and proteins were isolated as TFA salts.

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 (C18), 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 (C18) at a flow rate of 0.6 mL min^{-1}. Separations were performed using a mobile phase of 0.1 vol% FA in H_2O (Solvent A) and 0.1 vol% FA in MeCN (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 either positive or negative mode as indicated.

The building blocks, Boc-(β-SePMB)Asp-OH (**11**) and Boc-(γ-SePMB)Glu-OH (**10**), were synthesised as previously reported.[1]

General Methods

Preparative scale chromatography

Preparative reverse-phase HPLC (RP-HPLC) was performed on a Waters 2535 Quaternary Gradient system interfaced with a Waters 2489 UV/Vis Detector module operating at 230 nm and 280 nm and a Waters Fraction Collector III. A Waters Sunfire C18 OBD (100 Å, 5 μm, 19 x 150 mm) column was employed at a flow rate of 20 mL min^{-1} unless otherwise stated. A mobile phase composed of 0.1 vol% TFA in H_2O (Solvent A) and 0.1 vol% TFA in MeCN (Solvent B) was utilised operating with a linear gradient as specified. Chromatogram analyses were performed using Waters Empower 3 Pro software (2010).

UPLC-MS analysis

Reaction monitoring and identification of the haemathrin polypeptides was performed on a Shimadzu UPLC-MS system equipped with a Nexera X2 LC-30AD pump, a DGU-20A5R degassing unit and a Nexera X2 SPD-M30A diode array detector. A CTO-20A Column Oven was employed with a Waters C-18 BEH (130 Å, 1.7 μm, 2.1 x 50 mm) column operating at 0.60 mL min^{-1}. These components were interfaced with a CBM-20A Communications Bus Module alongside a Nexera X2 SIL-30AC Autosampler and a Shimadzu LCMS-2020 mass spectrometer operating in positive mode. Peptides were analyzed using a mobile phase of 0.1 vol% FA in H_2O and 0.1 vol% FA in MeCN operating with a linear gradient. Analyses of MS spectra were performed using Shimadzu LabSolutions software.

Analytical UPLC analysis

Purity of peptides and proteins was analyzed using a Waters Acquity UPLC system equipped with a Sample Manager FTN, Quaternary Solvent Manager (H-Class) and a PDA eλ detector (λ = 210 - 400 nm). Analyses were performed on a Waters Acquity UPLC C18 BEH (130 Å, 1.7 µm, 2.1 mm x 50 mm) column operating at 0.60 mL min^{-1}. Purity was determined by peak integration using Waters Empower Software.

High resolution Matrix-Assisted Laser Desorption Ionisation (MALDI) MS analysis

High resolution mass spectra were acquired for all final haemathrin analogues using either reflectron or linear mode on a Bruker (MA, USA) Autoflex™ Speed MALDI-TOF using either a matrix of saturated (sinapinnic acid specifications) α-cyano-4-hydroxycinnamic acid in 3:7 v/v MeCN:H_2O containing 0.1 vol% TFA (TA30), or 2′,4′,6′-trihydroxyacetophenone (THAP)/di-ammonium hydrogen citrate (18 mg/7 mg in 500 µL of TA30). Equal volumes of the sample in TA30 and the matrix were mixed well and spotted to a ground steel MALDI plate then allowed to dry. Data was acquired with Protein 1 calibrants (Bruker) and analyzed using Flex analysis (Bruker) software.

Solid-Phase Peptide Synthesis

Wang Resin Loading: The first residue was loaded to *p*-alkoxy-benzyl alcohol resin (Wang resin, 1.1 mmol g^{-1} loading) using the desired Fmoc-AA-OH (3 eq. relative to resin functionalisation), HBTU (3.0 eq.) and *i*Pr_2NEt (4.5 eq.) in DMF, followed by slow, dropwise addition of 4-dimethylaminopyridine (DMAP) (0.1 eq., dissolved in 500 µL of DMF) to the combined slurry of activated amino acid and resin. The resin was agitated gently over 16 h on an orbital shaker then drained and washed with DMF (5 x 5 mL). Unreacted alcohol groups were capped with a solution of Ac_2O (0.3 M) and *i*Pr_2NEt (0.3 M) in DMF (5 mL, 5 min), and then the resin washed thoroughly with DMF and dried with CH_2Cl_2.

Rink Amide Resin Loading: Rink amide resin (0.6 mmol g^{-1}) was swollen in CH_2Cl_2 for 30 min then washed with DMF (5 x 5 mL). The Fmoc group was removed using 20 vol% piperidine in DMF (2 x 5 mL, 3 min) followed by washing with DMF (5 x 5 mL), CH_2Cl_2 (5 x 5 mL) and DMF (5 x 5 mL). PyBOP (4 eq.) and *N*-methylmorpholine (NMM) (8 eq.) were added to Fmoc-AA-OH (4 eq.) in DMF (0.125 M relative to Fmoc-AA-OH) and the entirety of the mixture was added to the resin at room temperature

with agitation for 2 h. The resin was washed with DMF (5 x 5 mL), CH_2Cl_2 (5 x 5 mL) and DMF (5 x 5 mL) then capped with Ac_2O (0.125 M) and *i*Pr_2NEt (0.125 M) in DMF (5 mL). The resin was then washed with DMF (5 x 5 mL), CH_2Cl_2 (5 x 5 mL) and DMF (5 x 5 mL).

***Quantification of Resin Loading*:** A sample of resin (~20 mg) was treated with 20 vol% piperidine in DMF (2 x 3 mL, 3 min) and 50 μL of the combined deprotection solution was diluted to 10 mL with MeCN in a volumetric flask. The UV absorbance of the resulting piperidine-fulvene adduct was measured (λ = 301 nm, ε = 7800 M^{-1} cm^{-1}) to estimate the amount of amino acid loaded onto the resin.

Iterative peptide assembly (Fmoc-SPPS)

General Amino Acid Coupling: A solution of Fmoc-AA-OH (4 eq.), PyBOP (4 eq.) and NMM (8 eq.) in DMF (0.125 M of Fmoc-AA-OH) was added to the resin (200 μmol initial loading) and agitated at rt for 1 h. The resin was then washed with DMF (5 x 5 mL), CH_2Cl_2 (5 x 5 mL), and DMF (5 x 5 mL).

Capping: A solution of Ac_2O (0.125 M) and *i*Pr_2NEt (0.125 M) in DMF (5 mL) was added to the resin. After 3 min the resin was washed with DMF (5 x 5 mL), CH_2Cl_2 (5 x 5 mL), and DMF (5 x 5 mL).

Deprotection: The resin was treated with 20 vol% piperidine in DMF (2 x 3 mL, 3 min) and washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL) and DMF (5 × 3 mL).

Cleavage from Resin, Side Chain Deprotection and Work Up

A mixture of TFA:*i*Pr$_3$SiH:H$_2$O (90:5:5 v/v/v) was added to the resin. After 2 h, the resin was filtered and washed with an equal volume of TFA. The combined filtrates were concentrated under a stream of nitrogen to less than 1 mL. Et$_2$O (40 mL) was added and the solution cooled to -20 °C for 15 minutes. The precipitate was pelleted by centrifugation (4000 x g, 8 min) and the supernatant decanted. The remaining pellet was then allowed to dry under an inert atmosphere.

Microwave-Assisted Peptide Synthesis

Microwave-assisted peptide synthesis was performed on a CEM Liberty Blue® automated microwave peptide synthesiser (USA, NC). High temperature coupling used a 4 min coupling method: [2 min coupling (90 °C), 1 min deprotection (90 °C), 1 min associated with washes and liquid handling]. Deprotection solutions were supplemented with 0.1 M HOBt to suppress aspartimide formation. Each of the haemathrin *C*-terminal fragments were synthesised on a 500 μmol scale at 90 °C for the underlined residues (haemathrin-1: Boc-(γ-SePMB)Glu-OH (**10**)-RSGETDYDEY<u>EENENTPTPDPSAPTARPRLGRKNA</u>-OH, haemathrin-2: Boc-(β-SePMB)Asp-OH (**11**)-DYDEY<u>DADETTLSPDPDAPTARPRLGRKNA</u>-OH). The resin was then dried, split and the scale was reduced to 100 μmol to extend the different sTyr-containing peptides. Haemathrin analogues containing nP-protected sTyr were extended using a 40 °C coupling/deprotection method: [25 min coupling (40 °C), 6 min deprotection (3 min x 2, 40 °C), 1 min associated with washes and liquid handling]. Both methods used a 4-fold excess of Fmoc-AA-OH (0.125 M), Oxyma (0.125 M) and *N,N'*-diisopropylcarbodiimide (DIC) (0.125 M). Capping of unreacted *N*-terminal amines was achieved using *N*-acetylglycine, Oxyma and DIC at 5-fold molar excess using a similar coupling approach.

On-Resin Selenoesterification via a Side Chain Anchoring Strategy

Fmoc-Glu(OH)-OAll was loaded onto Rink amide resin followed by automated Fmoc-SPPS to obtain the desired peptide fragment, with Boc-Tyr(O*t*Bu)-OH as the *N*-terminal residue.[2] The allyl protecting group was removed by treating the resin with a solution of Pd(PPh$_3$)$_4$ (1 eq.) and PhSiH$_3$ (40 eq.) in dry CH$_2$Cl$_2$ (2 x 1 h) followed by washing with CH$_2$Cl$_2$ (10 x 5 mL), DMF (5 x 5 mL) and CH$_2$Cl$_2$ (5 x 5 mL). The *C*-terminal carboxylic acid was selenoesterified by adding a solution of diphenyl diselenide (DPDS) (30 eq.) and tri-*n*-butylphosphine (30 eq.) in DMF (2-3 mL for 25 μmol scale) to the resin at 0 °C. The resin was then shaken at 0 °C for 3 h, washed with DMF (5 x 5 mL) and CH$_2$Cl$_2$ (5 x 5 mL) then dried *in vacuo*. Subsequently,

the resin was subjected to the cleavage conditions described above to obtain the crude peptide which was purified by RP-HPLC to afford the pure selenoester.

Coupling of Selenylated Amino Acids and Fmoc-sTyr(SO_3nP)-OH (50 µmol scale)

Fmoc-Tyr(SO_3nP)-OH (**9**) (2 eq.) was coupled using 1-hydroxy-7-azabenzotriazole (HOAt) (13.6 mg, 100 µmol, 2 eq.) and DIC (15.6 µL, 100 µmol, 2 eq.) in DMF at 300 mM concentration for 5 hours. Boc-(γ-SePMB)-Glu-OH (**10**), and Boc-(β-SePMB)Asp-OH (**11**) (2 eq.) were coupled to the resin-bound peptide (50 mg, 100 µmol, 2 eq.) using HOAt (13.6 mg, 100 µmol, 2 eq.) and DIC (15.5 µL, 100 µmol, 2 eq.) in DMF at 100 mM concentration for 16 hours.

Note: *All the yields for the peptide fragments are calculated based on the initial resin loading.*

In situ Diselenide Formation [peptides with Boc-(γ-SePMB)Glu-OH (10)]

C-terminal diselenide fragments were generated directly from the resin-bound protected peptide by the addition of a 100-fold volume excess of TFA cleavage solution (TFA:*i*Pr_3SiH:H_2O:Gnd.HCl (87.5:5:5:2.5 v/v/v/w) (150 mg resin:15 ml TFA cleavage solution) containing 2,2'-dithiobis(5-nitropyridine) (DTNP) (10 eq.). This TFA cleavage solution was concentrated under a stream of nitrogen to less than 1 mL and the peptide precipitated with cold Et_2O then worked up as previously described. When on-resin peptide purity was insufficient to use the *in situ* diselenide formation approach, PMB-protected selenopeptides were cleaved from the resin, purified and then converted to diselenide dimers from dry peptide using an identical PMB deprotection solution [TFA:*i*Pr_3SiH:H_2O:Gnd.HCl (87.5:5:5:2.5 v/v/v/w)] containing DTNP (10 eq.).

PMB Deprotection using Oxidative Acidic Conditions [peptides with Boc-(β-SePMB)Asp-OH (11)]

The crude peptide bearing *N*-terminal (β-SePMB)Asp (obtained after acidolytic cleavage from resin) was dissolved in 20 vol% DMSO in a 3:1 v/v solution of TFA:6 M Gnd.HCl (0.1 M HEPES, pH 6.0; peptide concentration: ~ 7 mg mL^{-1}) and gently agitated on an orbital shaker then analyzed by LC-MS to ensure complete removal of the PMB protecting group. The crude mixture was diluted with 0.1 vol% TFA in H_2O and purified by RP-HPLC to afford the desired peptide diselenide dimer as a white powder after lyophilization.

Deprotection of the Neopentyl Sulfate Ester from sTyr-Containing Diselenides

sTyr-containing diselenides were dissolved to 2 mg ml^{-1} concentration in a solution of 6 M Gnd.HCl (100 mM Na_2HPO_4, pH 7.2), incubated at 50 °C and the reaction monitored at 30 min time points until the nP sulfate ester had been completely removed (typically 2 hours). In the case of the *C*-terminal fragments of haemathrin-1 and 2, the pH of the reaction mixture was noted to be ~ 6.5 during this transformation. *It is important to make sure that the pH is maintained above 5 to ensure retention of unprotected acid-labile sulfate esters.* The resultant sTyr-containing peptide diselenides were purified using RP-HPLC (Waters XBridge C18 column (300 Å, 5 μm, 10 x 250 mm); 10-40%B, 0.1 vol% TFA) and pure fractions pooled and lyophilized. HPLC fractions were collected on dry ice and immediately lyophilized after LC-MS analysis to avoid degradation of the acid-labile sTyr group.

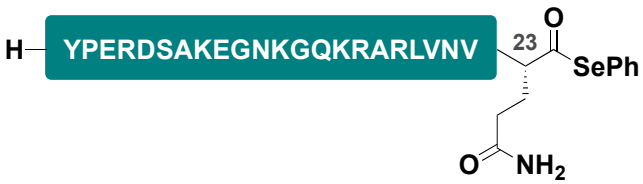
Note: Typically, unprotected sTyr residues are known to be acid-labile and peptides/proteins with these residues are analyzed and purified using FA buffers to avoid loss of sulfate esters. However, sulfated haemathrins (peptides and proteins) were found to be surprisingly stable under TFA buffer conditions. Hence, all of the sulfated peptides and proteins presented in this manuscript were isolated using TFA buffers, which offered better resolution during preparative HPLC purification compared to FA buffers.

General Procedure for Diselenide-Selenoester Ligation and Deselenization

Peptide selenoester (1.3 eq.) and peptide diselenide dimer bearing either *N*-terminal H-(γ-Se)Glu-OH or H-(β-Se)Asp-OH (0.5 eq. of the diselenide dimer, 1.0 eq. with respect to the monomer) were dissolved separately in 6 M Gnd.HCl buffer (100 mM Na_2HPO_4, pH 7.2) to a concentration of 13 mM with respect to the peptide selenoester and 5 mM with respect to the peptide diselenide dimer. The solutions were combined to give an overall concentration of 2.5 mM with respect to the selenopeptide dimer (5 mM with respect to the monomer), 6.5 mM with respect to the peptide selenoester and a final pH of the reaction adjusted to 6.2-6.5. Ligations were monitored by UPLC-MS and judged complete when the diselenide dimer fragment was fully consumed (after 5 min). The DPDS generated during ligation was extracted with hexane (5 x 500 μL) and after degassing, the ligation mixture was treated with an equal volume of deselenization solution (250 mM TCEP, 25 mM DTT, pH 5.0) and the resulting solution was incubated for 5 min at rt. Following complete deselenization (as judged by UPLC-MS), the reaction mixture was diluted with 1-2 mL of ligation buffer and then purified by semi-preparative RP-HPLC as described above. All peptides and proteins were isolated as white solids following lyophilization.

Syntheses of haemathrins-1 & -2

Haemathrin-1 N-terminal Selenoester (1-23) (28)



The *N*-terminal fragment of haemathrin-1 (residues 1-23, 55 µmol) (**28**) was prepared by loading Fmoc-Glu(OH)-OAll on Rink amide resin followed by automated Fmoc-SPPS as outlined in the general methods. After on-resin allyl ester deprotection and selenoesterification (see general methods), the peptide was subjected to acidolytic cleavage for 2 h at room temperature. The cleavage cocktail was removed under a stream of N_2 and the residue suspended in cold Et_2O and cooled to -20 °C. The precipitate was pelleted by centrifugation (4000 x g, 5 min) and the resulting supernatant was decanted, and the pellet allowed to air dry for 15 min. This pellet was then dissolved in 5 vol% MeCN in H_2O (0.1 vol% TFA) and purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) to yield the peptide selenoester **28** as a fluffy white solid after lyophilization (80.5 mg, 28.9 µmol, 53% yield).

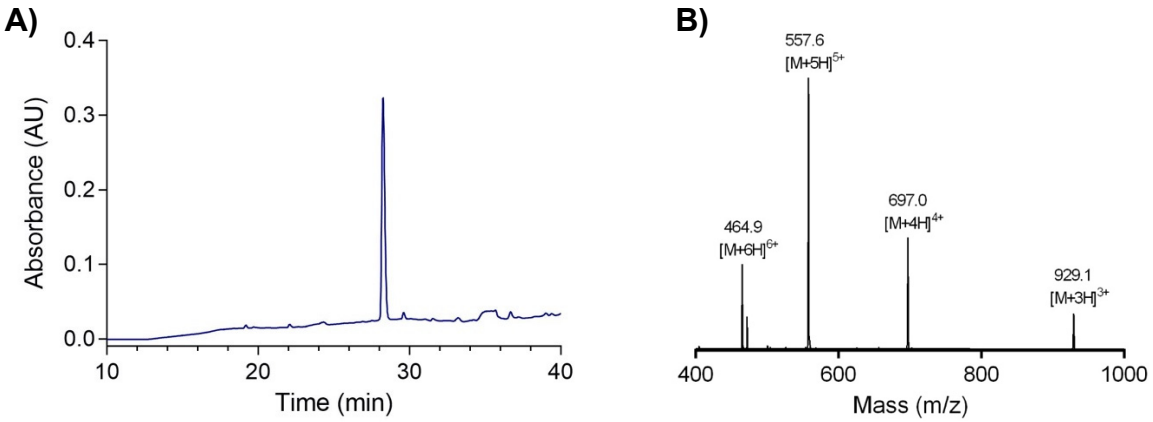
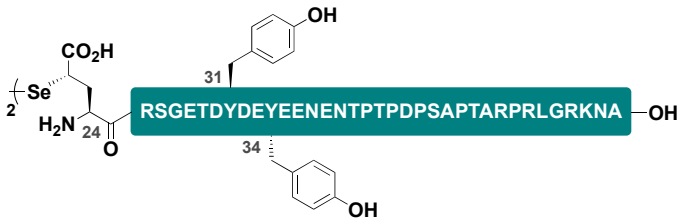


Figure S1. A) Analytical HPLC trace of purified haemathrin-1 (1-23) selenoester (**28**); R_t 28.25 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of purified haemathrin-1 (1-23) selenoester (**28**). Calculated Mass $[M+3H]^{3+}$: 928.7, $[M+4H]^{4+}$: 696.8, $[M+5H]^{5+}$: 557.6, $[M+6H]^{6+}$: 464.8; Mass Found (ESI+): $[M+3H]^{3+}$: 929.1, $[M+4H]^{4+}$: 697.0, $[M+5H]^{5+}$: 557.6, $[M+6H]^{6+}$: 464.9.

Haemathrin-1 C-terminal Diselenide Dimer (24-59) (20)



The *C*-terminal fragment of haemathrin-1 (residues 24-59) (**20**) was prepared on Wang resin (100 µmol) using automated Fmoc-SPPS (see general methods). Prior to the final coupling of Boc-(γ-SePMB)Glu-OH (**10**), the resin was dried and 12.5 µmol taken forward. After manual coupling of Boc-(γ-SePMB)Glu-OH (**10**) to the *N*-terminus (see general methods), the fully protected resin-bound peptide was cleaved and deprotected using a 100-fold volume excess (to weight of resin) of TFA/*i*Pr$_3$SiH/H$_2$O:Gnd.HCl (87.5:5:5:2.5 v/v/v/w) with the addition of DTNP (10 eq.) to remove the PMB group. The solution was agitated at room temperature for 1 h, the cleavage cocktail evaporated under a stream of N$_2$ and the residue precipitated with a 50-100-fold volume excess of cold Et$_2$O. After incubating the precipitate on ice for 30 min the suspension was precipitated by centrifugation (4000 x g, 5 min), the supernatant decanted, and the peptide pellet allowed to air dry for 10-15 min. The pellet was dissolved in 5 vol% MeCN in H$_2$O (0.1 vol% TFA) and purified *via* preparative RP-HPLC (5-35%B over 30 min, 0.1 vol% TFA) to yield the diselenide fragment **20** as a white solid after lyophilization (3.0 mg, 0.72 µmol (with respect to the monomer), 6% yield).

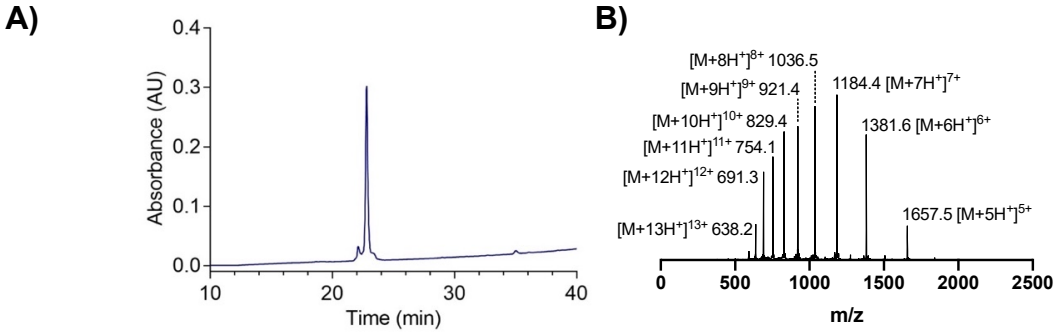
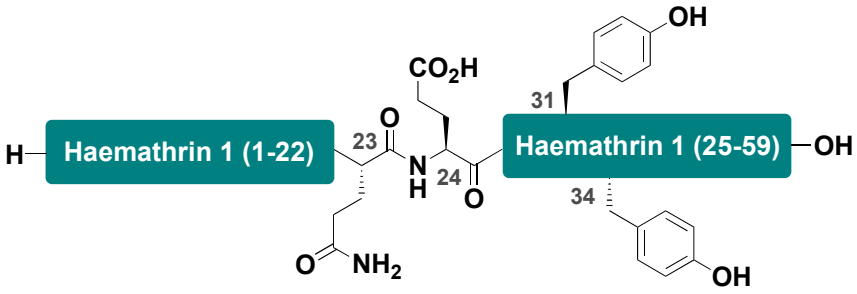


Figure S2. A) Analytical HPLC trace of purified haemathrin-1 (24-59) diselenide dimer (**20**); R$_t$ 22.80 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of purified haemathrin-1 (24-59) diselenide dimer (**20**). Calculated Mass $[M+5H]^{5+}$: 1657.9, $[M+6H]^{6+}$: 1381.7, $[M+7H]^{7+}$: 1184.5, $[M+8H]^{8+}$: 1036.6, $[M+9H]^{9+}$: 921.5, $[M+10H]^{10+}$: 829.4, $[M+11H]^{11+}$: 754.1, $[M+12H]^{12+}$: 691.4, $[M+13H]^{13+}$: 638.3,; Mass Found (ESI+): $[M+5H]^{5+}$: 1657.5, $[M+6H]^{6+}$: 1381.6, $[M+7H]^{7+}$: 1184.4, $[M+8H]^{8+}$: 1036.5, $[M+9H]^{9+}$: 921.4, $[M+10H]^{10+}$: 829.4, $[M+11H]^{11+}$: 754.1, $[M+12H]^{12+}$: 691.3, $[M+13H]^{13+}$: 638.2.

Haematrhrin-1 (1-59; unsulfated) (1)



The ligation of haemathrin-1 (24-59) diselenide dimer (**20**) (1.7 mg, 0.21 μmol) and haemathrin-1 (1-23) selenoester (**28**) (1.4 mg, 0.50 μmol) together with *in situ* deselenization was performed as outlined in the general methods section. Purification *via* preparative RP-HPLC (0 to 30%B over 40 min, 0.1 vol% TFA), followed by lyophilization afforded native haemathrin-1 (1-59) (**1**) as a fluffy white solid (1.5 mg, 0.22 μmol, 55% yield over 2 steps).

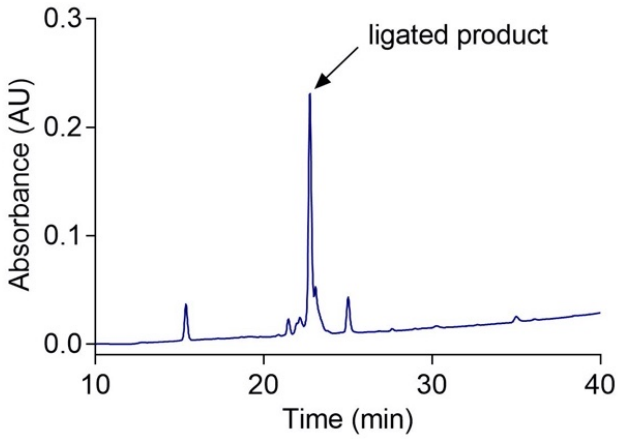


Figure S3. Analytical HPLC trace of crude haemathrin-1 (1-59) (**1**) post ligation-deselenization; R_t 22.75 min (0-50%B over 30 min, λ = 230 nm).

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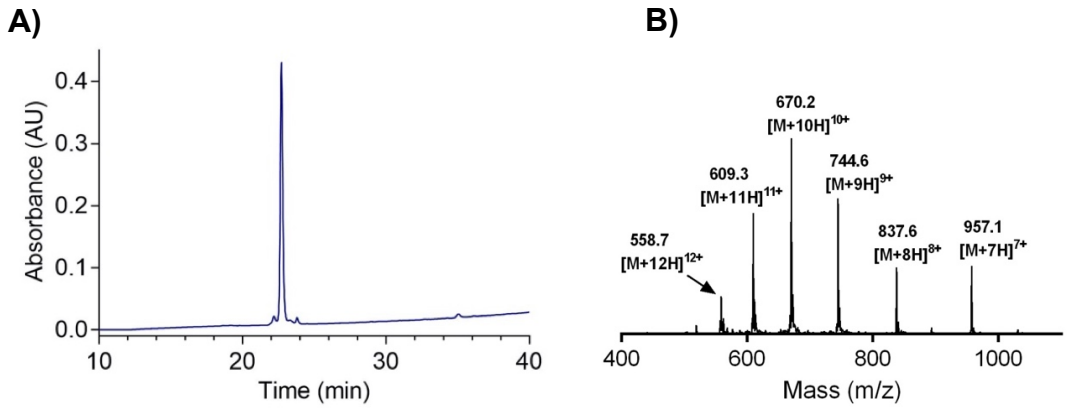


Figure S4. A) Analytical HPLC trace of purified haemathrin-1 (1-59) (**1**) R_t 22.72 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of purified haemathrin-1 (**1**). Calculated Mass $[M+7H]^{7+}$: 956.7, $[M+8H]^{8+}$: 837.3, $[M+9H]^{9+}$: 744.3, $[M+10H]^{10+}$: 670.0, $[M+11H]^{11+}$: 609.2, $[M+12H]^{12+}$: 558.5; Mass Found (ESI+): $[M+7H]^{7+}$: 957.1, $[M+8H]^{8+}$: 837.6, $[M+9H]^{9+}$: 744.6, $[M+10H]^{10+}$: 670.2, $[M+11H]^{11+}$: 609.3, $[M+12H]^{12+}$: 558.7.

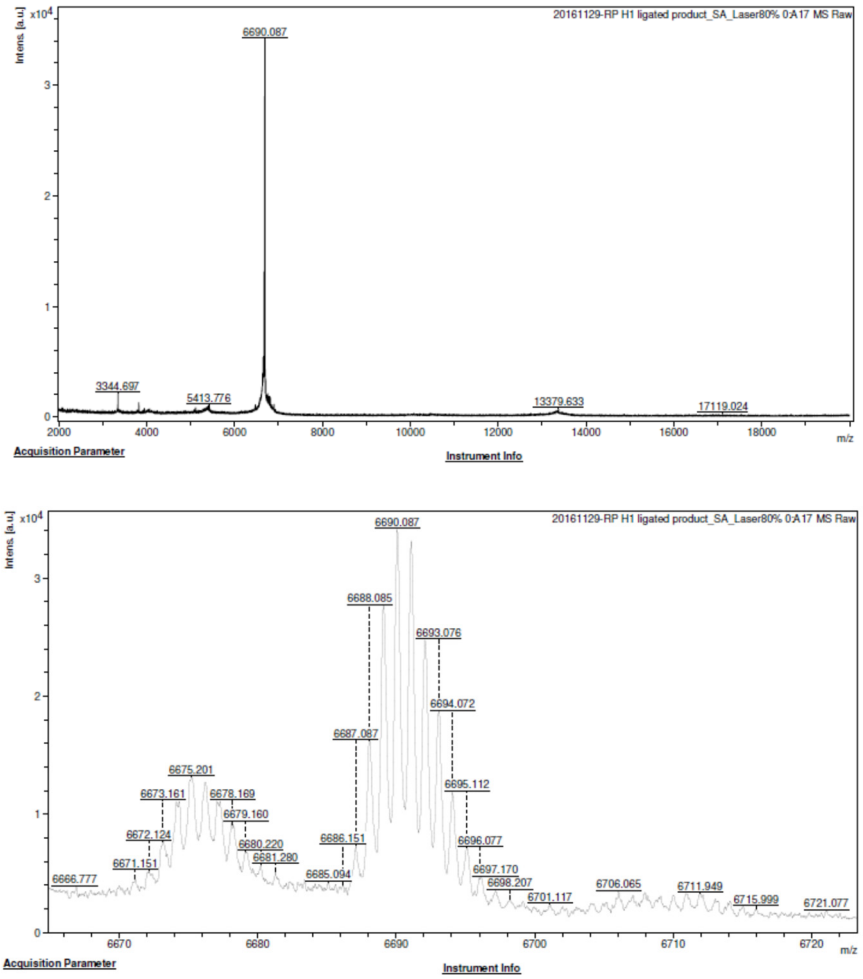
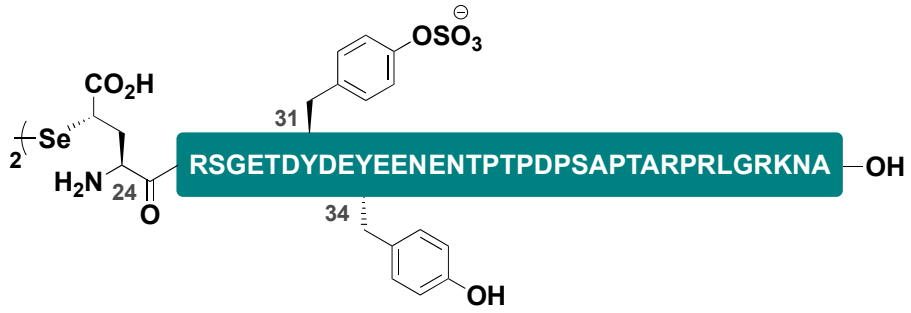


Figure S5. MALDI-TOF mass spectra of purified haemathrin-1 (1-59) (**1**) using sinapinic acid as a matrix; Calculated molecular weight: 6689.26; Observed most abundant isotope: 6690.08.

Haemathrin-1 C-terminal Diselenide Dimer (24-59; sY31) (21)



The monosulfated *C*-terminal fragment of haemathrin-1 (24-59; sY31) (**21**), was prepared on Wang resin (100 µmol) using automated Fmoc-SPPS as outlined in the general methods. 12.5 µmol of this dried resin was taken forward with the coupling of Boc-(γ-SePMB)Glu-OH (**10**), resin cleavage, deprotection and diselenide formation performed as described previously for the unsulfated haemathrin-1 *C*-terminal diselenide fragment (**20**). The crude Et_2O-precipitated pellet was then dissolved in 5 vol% MeCN in H_2O (0.1 vol% TFA), purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) and pure fractions were combined and lyophilized. The nP sulfate ester protecting groups were then removed by incubation of the peptide in ligation buffer (see general methods). The fully deprotected *C*-terminal fragment was then purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) to yield haemathrin-1 (24-59; sY31) (**21**) as a white solid after lyophilization (1.6 mg, 0.38 µmol (with respect to the monomer), 3% yield).

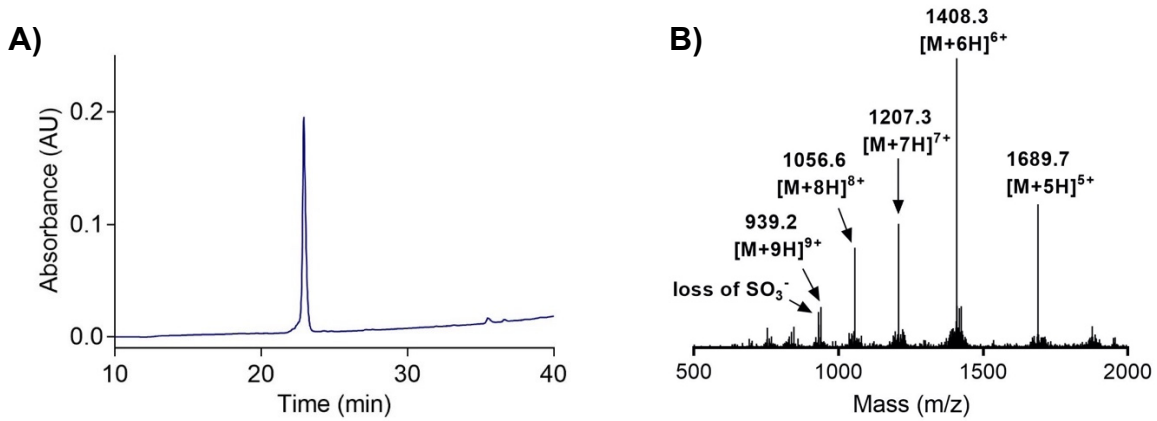
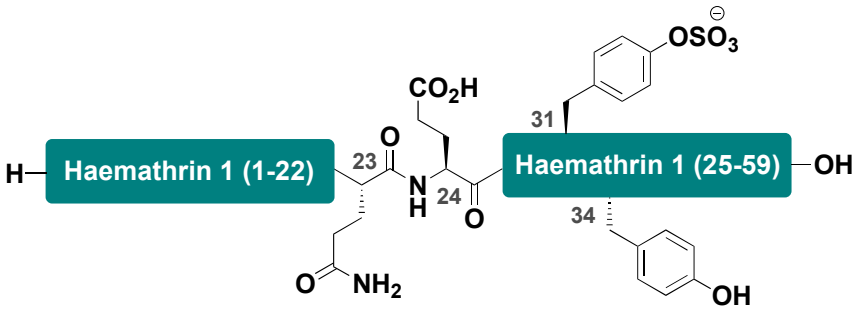


Figure S6. A) Analytical HPLC trace of purified haemathrin-1 (24-59; sY31) diselenide dimer (**21**); R_t 22.92 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of purified haemathrin-1 (24-59; sY31) (**21**). Calculated Mass $[M+5H]^{5+}$: 1689.9, $[M+6H]^{6+}$: 1408.4, $[M+7H]^{7+}$: 1207.4, $[M+8H]^{8+}$: 1056.6, $[M+9H]^{9+}$: 939.3; Mass Found $[M+5H]^{5+}$: 1689.7, $[M+6H]^{6+}$: 1408.3, $[M+7H]^{7+}$: 1207.3, $[M+8H]^{8+}$: 1056.6, $[M+9H]^{9+}$: 939.2.

Haematathrin-1 (1-59; sY31) (3)



The ligation of haematathrin-1 (24-59; sY31) diselenide dimer (**21**) (1.4 mg, 0.17 µmol) and haematathrin-1 (1-23) selenoester (**28**) (1.2 mg, 0.43 µmol) together with *in situ* deselenization was performed as outlined in the general methods section. Purification *via* preparative RP-HPLC (0-30%B over 40 min, 0.1 vol% TFA), followed by lyophilization afforded the monosulfated haematathrin-1 (1-59; sY31) (**3**) as a white solid (1.0 mg, 0.15 µmol, 44% yield over 2 steps).

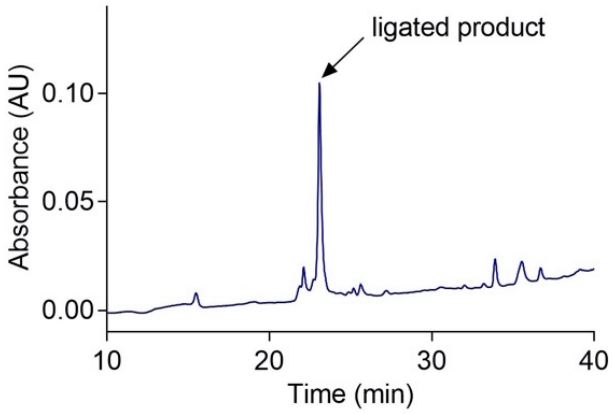


Figure S7. Analytical HPLC trace of crude haematathrin-1 (1-59; sY31) (**3**) post ligation-deselenization; R_t 23.08 min (0-50%B over 30 min, λ = 230 nm).

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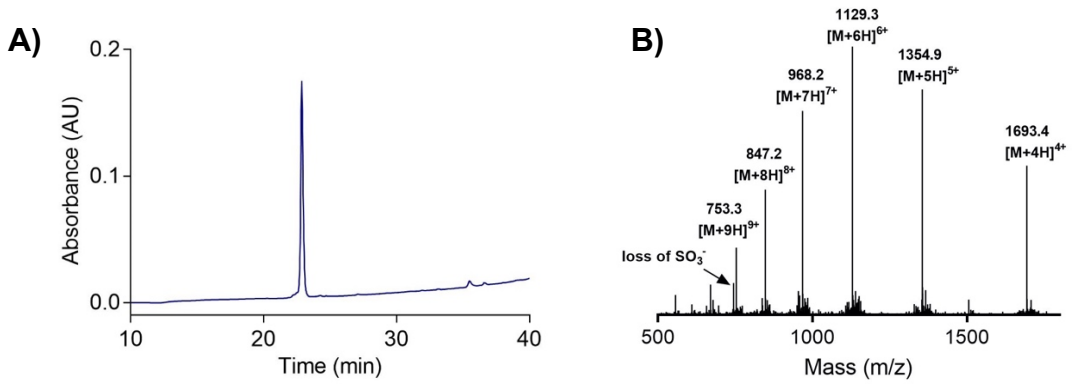


Figure S8. A) Analytical HPLC trace of purified haemathrin-1 (1-59; sY31) (**3**); R_t 22.87 min (0-50% B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of purified haemathrin-1 (1-59; sY31) (**3**). Calculated Mass $[M+4H]^{4+}$: 1693.6, $[M+5H]^{5+}$: 1355.0, $[M+6H]^{6+}$: 1129.4, $[M+7H]^{7+}$: 968.2, $[M+8H]^{8+}$: 847.3, $[M+9H]^{9+}$: 753.2; Mass Found (ESI+): $[M+4H]^{4+}$: 1693.4, $[M+5H]^{5+}$: 1354.9, $[M+6H]^{6+}$: 1129.3, $[M+7H]^{7+}$: 968.2, $[M+8H]^{8+}$: 847.2, $[M+9H]^{9+}$: 753.3.

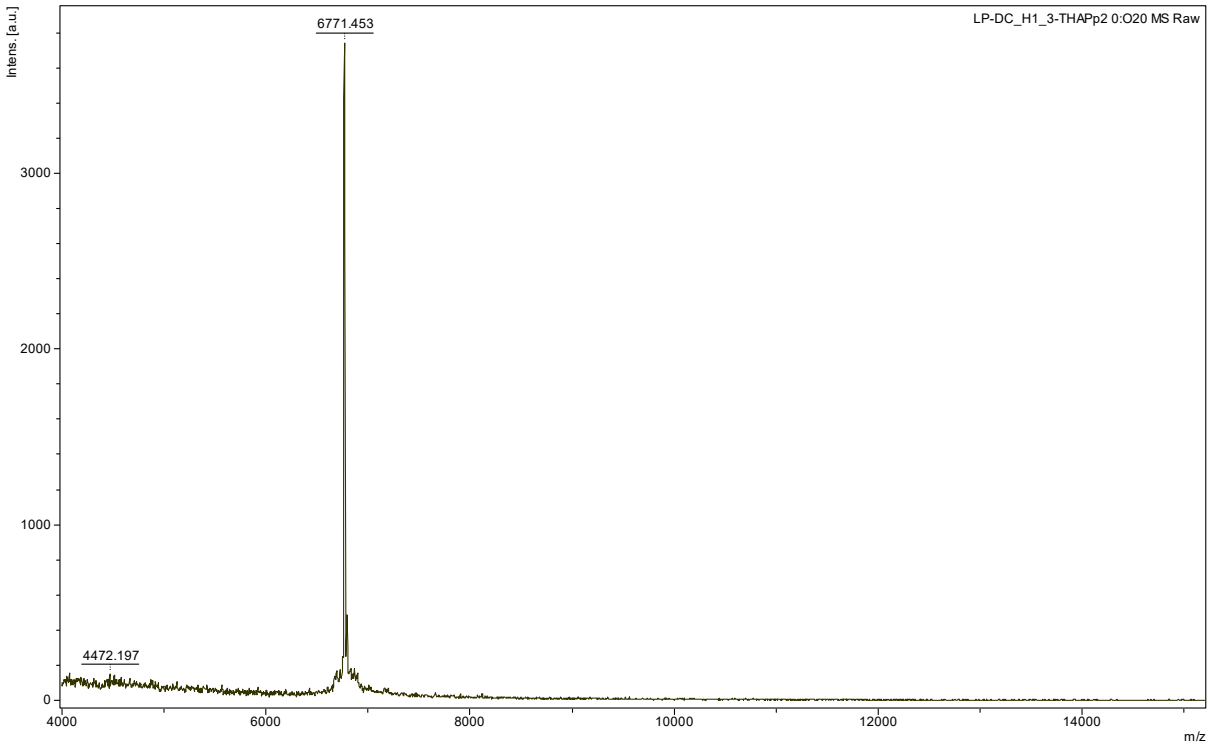
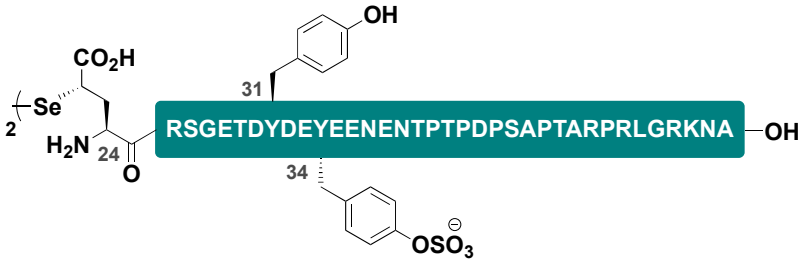


Figure S9. MALDI-TOF mass spectrum of haemathrin-1 (1-59; sY31) (**3**) using THAP/di-ammonium hydrogen citrate as a matrix; Calculated molecular weight: 6771.23; Observed most abundant isotope: 6771.45.

Haemathrin-1 C-terminal Diselenide Dimer (24-59; sY34) (22)



The singly sulfated *C*-terminal fragment of haemathrin-1 (24-59; sY34) (**22**), was prepared on Wang resin (100 μmol) using automated Fmoc-SPPS as outlined in the general methods. 12.5 μmol of this dried resin was taken forward with the coupling of Boc-(γ-SePMB)Glu-OH (**10**), resin cleavage, deprotection and diselenide formation performed as described previously for the unsulfated haemathrin-1 *C*-terminal diselenide fragment (**20**). The crude Et_2O-precipitated pellet was then dissolved in 5 vol% MeCN in H_2O (0.1 vol% TFA), purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) and pure fractions were combined and lyophilized. The lyophilized peptide was dissolved in ligation buffer (2 mg mL^{-1}, 6 M Gnd•HCl, 0.1 M Na_2HPO_4, pH 7.2) and incubated at 50 °C for 2 hours to remove the nP sulfate ester protecting groups. The fully deprotected *C*-terminal fragment was then purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) to yield the haemathrin-1 (24-59; sY34) (**22**) as a white solid after lyophilization (1.4 mg, 0.33 μmol (with respect to the monomer), 3% yield).

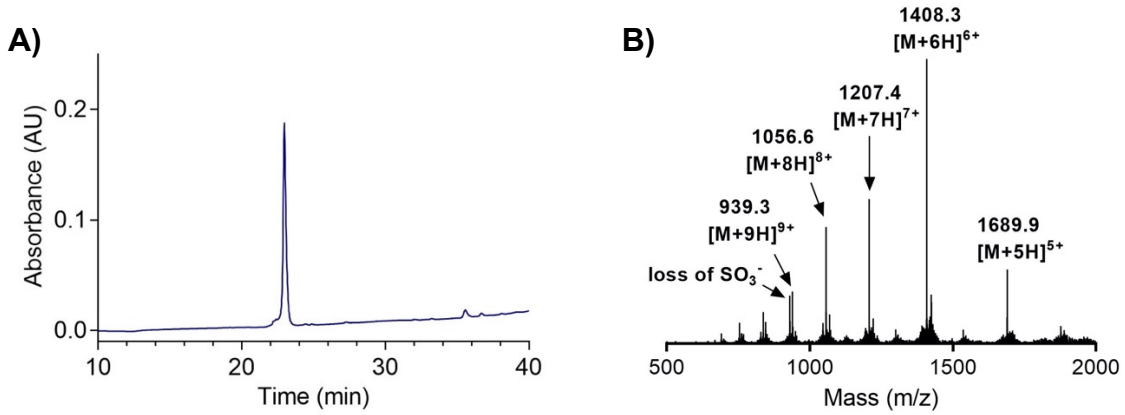
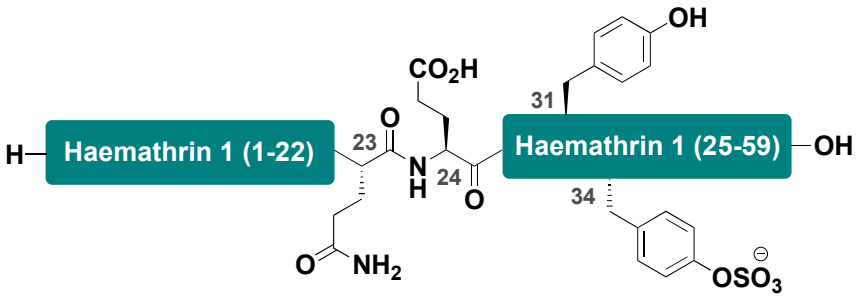


Figure S10. A) Analytical HPLC trace of purified haemathrin-1 (24-59; sY34) diselenide dimer (**22**); R_t 22.97 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of purified haemathrin-1 (24-59; sY34) (**22**). Calculated Mass $[M+5H]^{5+}$: 1689.9, $[M+6H]^{6+}$: 1408.4, $[M+7H]^{7+}$: 1207.4, $[M+8H]^{8+}$: 1056.6, $[M+9H]^{9+}$: 939.3; Mass Found $[M+5H]^{5+}$: 1689.9, $[M+6H]^{6+}$: 1408.3, $[M+7H]^{7+}$: 1207.4, $[M+8H]^{8+}$: 1056.6, $[M+9H]^{9+}$: 939.3.

Haematathrin-1 (1-59; sY34) (4)



The ligation of haemathrin-1 (24-59, sY34) diselenide dimer (**22**) (1.6 mg, 0.19 µmol) and haemathrin-1 (1-23) selenoester (**28**) (1.4 mg, 0.50 µmol) together with *in situ* deselenization was performed as outlined in the general methods section. Purification *via* preparative RP-HPLC (0-30%B over 40 min, 0.1 vol% TFA), followed by lyophilization afforded monosulfated haemathrin-1 (1-59; sY34) (**4**) as a white solid (1.0 mg, 0.15 µmol, 39% yield over 2 steps).

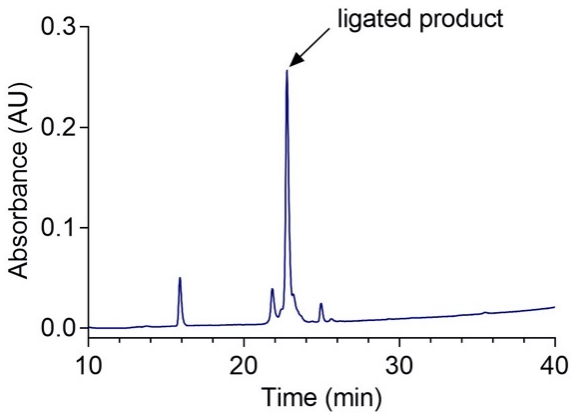


Figure S11. Analytical HPLC trace of crude haemathrin-1 (1-59; sY34) (**4**) post ligation-deselenization; R_t 22.76 min (0-50%B over 30 min, λ = 230 nm).

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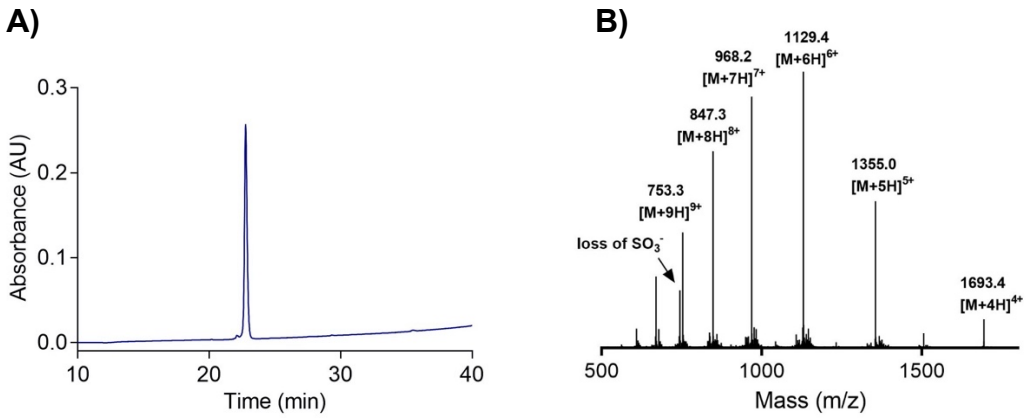


Figure S12. A) Analytical HPLC trace of purified haemathrin-1 (1-59; sY34) (**4**); R_t 22.78 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of purified haemathrin-1 (1-59; sY34) (**4**). Calculated Mass $[M+4H]^{4+}$: 1693.6, $[M+5H]^{5+}$: 1355.0, $[M+6H]^{6+}$: 1129.4, $[M+7H]^{7+}$: 968.2, $[M+8H]^{8+}$: 847.3, $[M+9H]^{9+}$: 753.2; Mass Found (ESI+): $[M+4H]^{4+}$: 1693.4, $[M+5H]^{5+}$: 1355.0, $[M+6H]^{6+}$: 1129.4, $[M+7H]^{7+}$: 968.2, $[M+8H]^{8+}$: 847.3, $[M+9H]^{9+}$: 753.3.

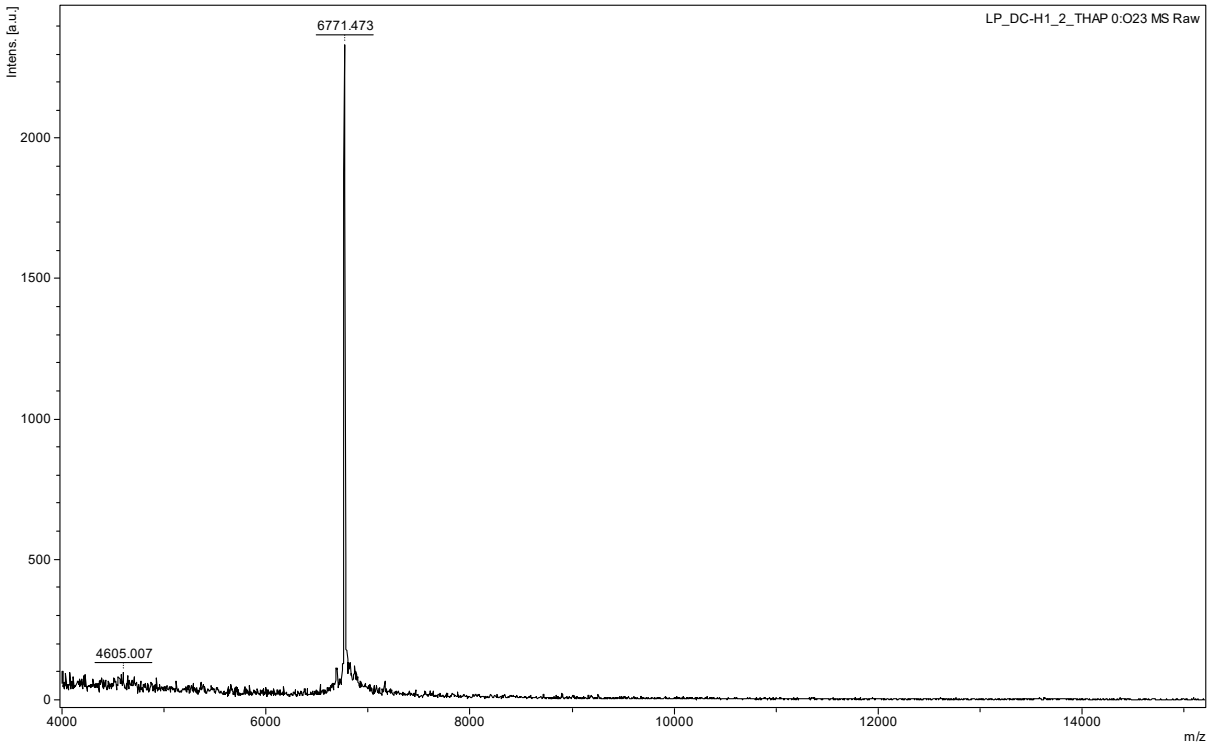
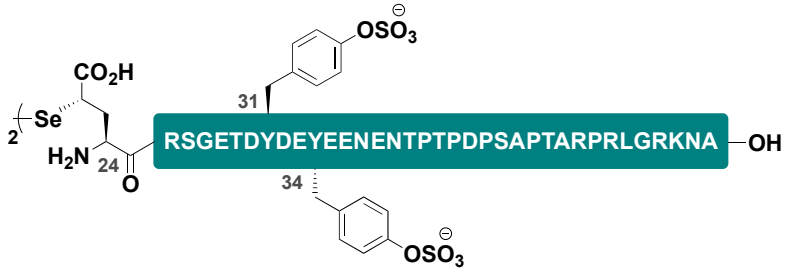


Figure S13. MALDI-TOF mass spectrum of purified haemathrin-1 (1-59; sY34) (**4**) using sinapinic acid as a matrix; Calculated molecular weight: 6771.23; Observed most abundant isotope: 6771.47.

Haemathrin-1 C-terminal Diselenide Dimer (24–59; sY31, sY34) (23)



The doubly sulfated *C*-terminal fragment of haemathrin-1 (24-59; sY31,sY34) (**23**) was prepared on Wang resin (100 μmol) using automated Fmoc-SPPS as outlined in the general methods. 12.5 μmol of this dried resin was taken forward with the coupling of Boc-(γ-SePMB)Glu-OH (**10**), resin cleavage, deprotection and diselenide formation performed as described previously for the unsulfated haemathrin-1 *C*-terminal diselenide fragment (**20**). The crude Et_2O-precipitated pellet was then dissolved in 5 vol% MeCN in H_2O (0.1 vol% TFA), purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) and pure fractions were combined and lyophilized. The nP sulfate ester protecting groups were then removed by incubation in ligation buffer as described previously. The fully deprotected *C*-terminal fragment was then purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) to yield the doubly sulfated haemathrin-1 (25-59; sY31, sY34) (**23**) as a white solid after lyophilization (1.6 mg, 0.37 μmol (with respect to the monomer), 3% yield).

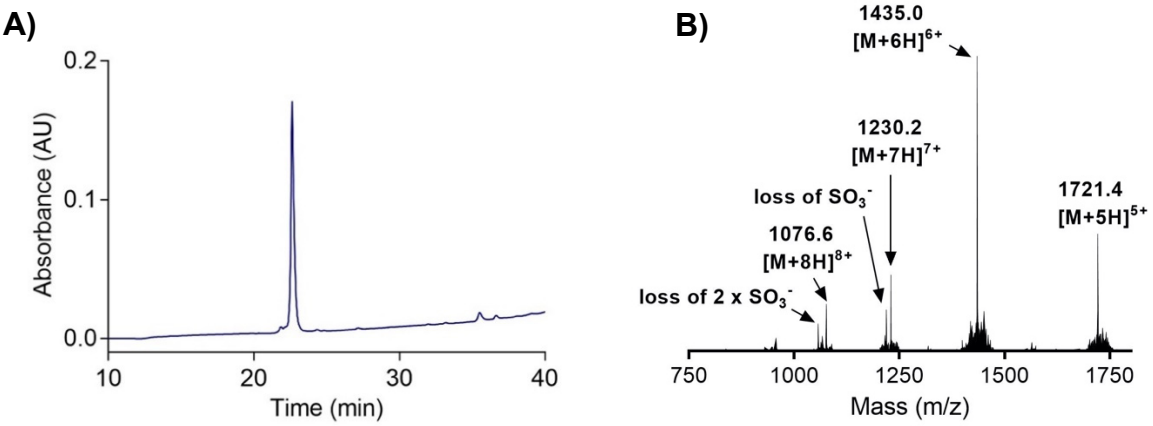
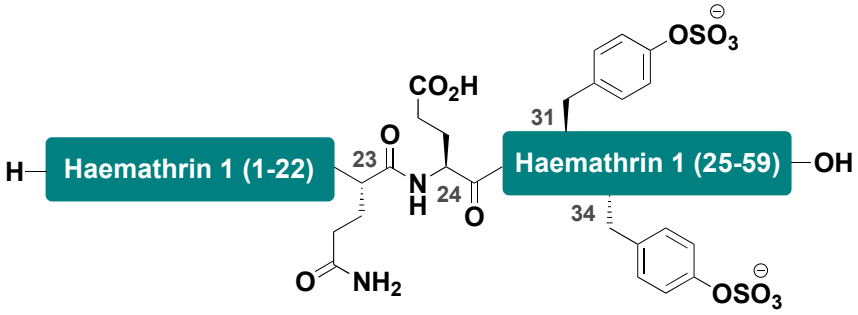


Figure S14. A) Analytical HPLC trace of purified haemathrin-1 (24-59; sY31, sY34) diselenide dimer (**23**); R_t 22.60 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of purified haemathrin-1 (24-59; sY31, sY34) (**23**). Calculated Mass $[M+5H]^{5+}$: 1721.9, $[M+6H]^{6+}$: 1435.1, $[M+7H]^{7+}$: 1230.2, $[M+8H]^{8+}$: 1076.6; Mass Found $[M+5H]^{5+}$: 1721.4, $[M+6H]^{6+}$: 1435.0, $[M+7H]^{7+}$: 1230.2, $[M+8H]^{8+}$: 1076.6.

Haematathrin-1 (1-59; sY31, sY34) (5)



The ligation of haematathrin-1 (24-59; sY31, sY34) diselenide dimer (**23**) (1.6 mg, 0.19 µmol) and haematathrin-1 (1-23) selenoester (**28**) (1.4 mg, 0.50 µmol) together with *in situ* deselenization was performed as outlined in the general methods section. Purification *via* preparative RP-HPLC (0-30%B over 40 min, 0.1 vol% TFA), followed by lyophilization afforded haematathrin-1 (1-59; sY31, sY34) (**5**) as a white solid (1.1 mg, 0.16 µmol, 43% yield over 2 steps).

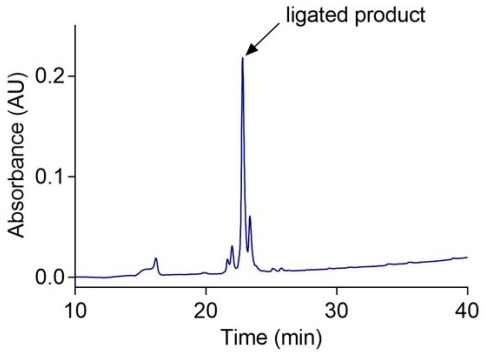


Figure S15. Analytical HPLC trace of crude haematathrin-1 (1-59; sY31, sY34) (**5**) post ligation-deselenization; R_t 22.80 min (0-50%B over 30 min, λ = 230 nm).

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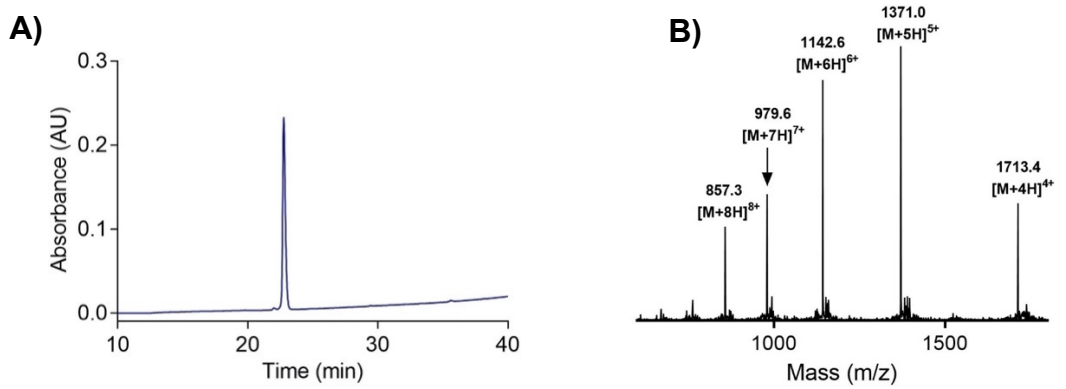


Figure S16. A) Analytical HPLC trace of purified haemathrin-1 (1-59; sY31, sY34) (**5**) R_t 22.77 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum of purified haemathrin-1 (1-59; sY31, sY34) (**5**). Calculated Mass $[M+4H]^{4+}$: 1713.6, $[M+5H]^{5+}$: 1371.1, $[M+6H]^{6+}$: 1142.7, $[M+7H]^{7+}$: 979.6, $[M+8H]^{8+}$: 857.3; Mass Found (ESI+): $[M+4H]^{4+}$: 1713.4, $[M+5H]^{5+}$: 1371.0, $[M+6H]^{6+}$: 1142.6, $[M+7H]^{7+}$: 979.6, $[M+8H]^{8+}$: 857.3.

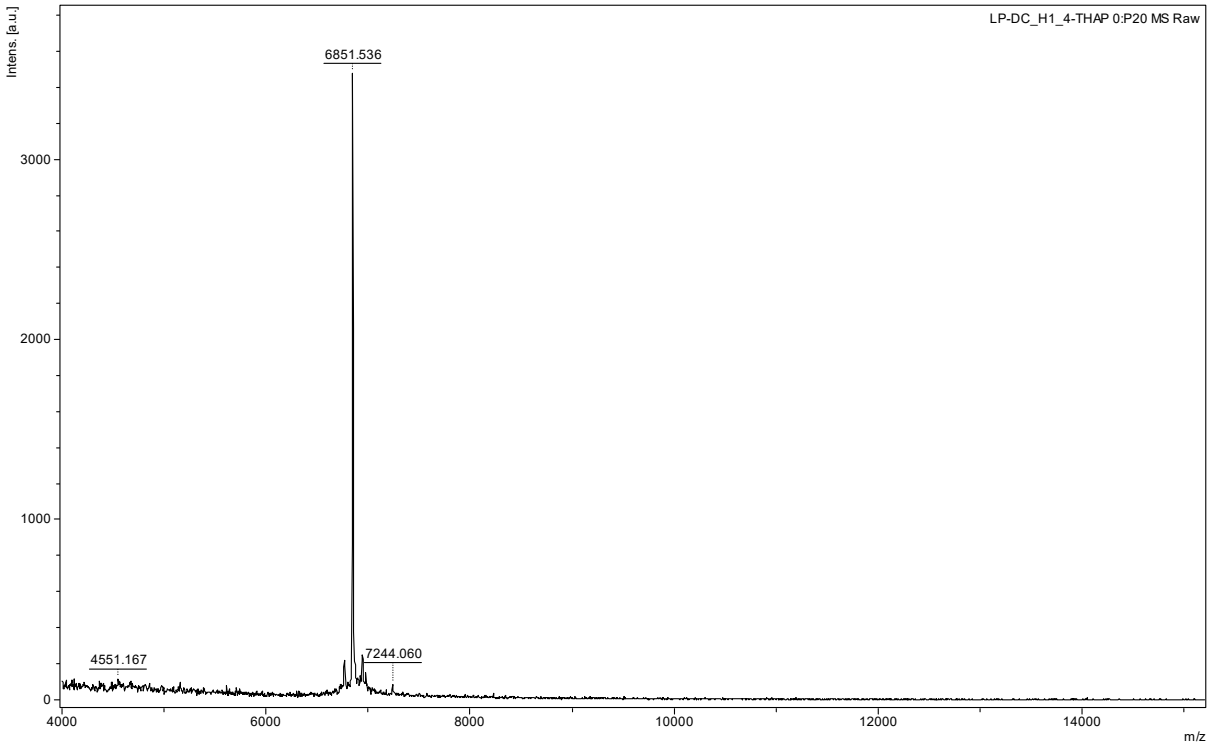
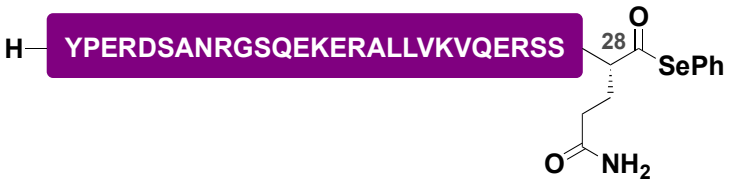


Figure S17. MALDI-TOF mass spectrum of haemathrin-1 (1-59; sY31, sY34) (**5**) using THAP/di-ammonium hydrogen citrate as a matrix; Calculated molecular weight: 6851.29; Observed most abundant isotope: 6851.54.

Haemathrin-2 N-terminal Selenoester (1-28) (29)



The *N*-terminal fragment of haemathrin-2 (1-28, 40 μmol) (**29**) was prepared by loading Fmoc-Glu(OH)-OAll on Rink amide ChemMatrix resin followed by automated Fmoc-SPPS as outlined in the general methods. After on-resin allyl deprotection and selenoesterification, the peptide was subjected to acidolytic cleavage for 2 h at room temperature. The cleavage cocktail was removed under a stream of N_2 and the residue suspended in cold Et_2O and cooled to -20 °C. The precipitate was pelleted by centrifugation (4000 x g, 5 min). The supernatant was decanted and the pellet allowed to air dry for 15 min. This pellet was then dissolved in 5 vol% MeCN in H_2O with 0.1 vol% TFA and purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) to yield haemathrin-2 (1-28) selenoester (**29**) as a white solid after lyophilization (36.7 mg, 10.8 μmol, 27% yield).

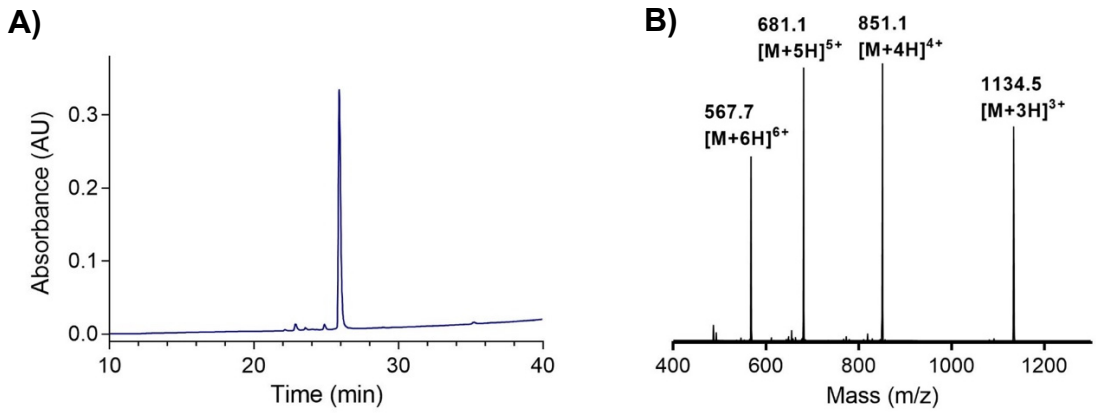
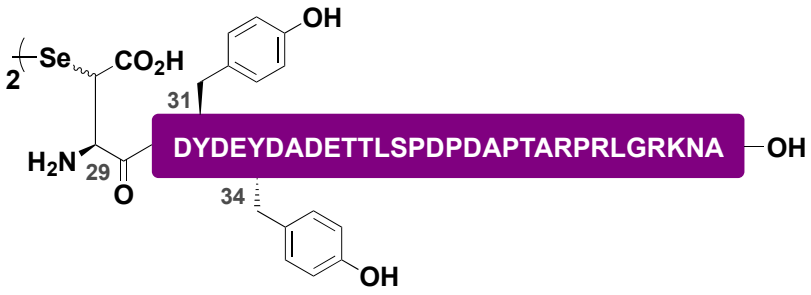


Figure S18. A) Analytical HPLC trace of purified haemathrin-2 (1-28) selenoester (**29**); R_t 25.89 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of purified haemathrin-2 (1-28) selenoester (**29**). Calculated Mass $[M+3H]^{3+}$: 1134.4, $[M+4H]^{4+}$: 851.1, $[M+5H]^{5+}$: 681.1, $[M+6H]^{6+}$: 567.7; Mass Found (ESI+): $[M+3H]^{3+}$: 1134.5, $[M+4H]^{4+}$: 851.1, $[M+5H]^{5+}$: 681.1 $[M+6H]^{6+}$: 567.7.

Haemathrin-2 C-terminal Diselenide Dimer (29–59) (24)



The *C*-terminal fragment of haemathrin-2 (29-59) (**24**) was prepared on Wang resin (100 µmol) using automated Fmoc-SPPS as outlined in the general methods. Prior to the final coupling of Boc-(β-SePMB)Asp-OH (**11**) the resin was dried and 12.5 µmol taken forward. After manual coupling of Boc-(β-SePMB)Asp-OH (**11**) to the *N*-terminus (see general methods), the fully protected resin-bound peptide was cleaved and deprotected using TFA/*i*Pr$_3$SiH/H$_2$O (90:5:5 v/v/v) as outlined in the general methods section. The crude peptide was then subjected to PMB deprotection using 20 vol% DMSO in a 3:1 solution of TFA:6 M Gnd.HCl (0.1 M HEPES, pH 6.0; peptide concentration: ~ 7 mg mL^{-1}) and purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) to yield the haemathrin-2 (29-59) diselenide dimer (**24**) as a white solid after lyophilization (1.9 mg, 0.54 µmol (with respect to the monomer), 4% yield).

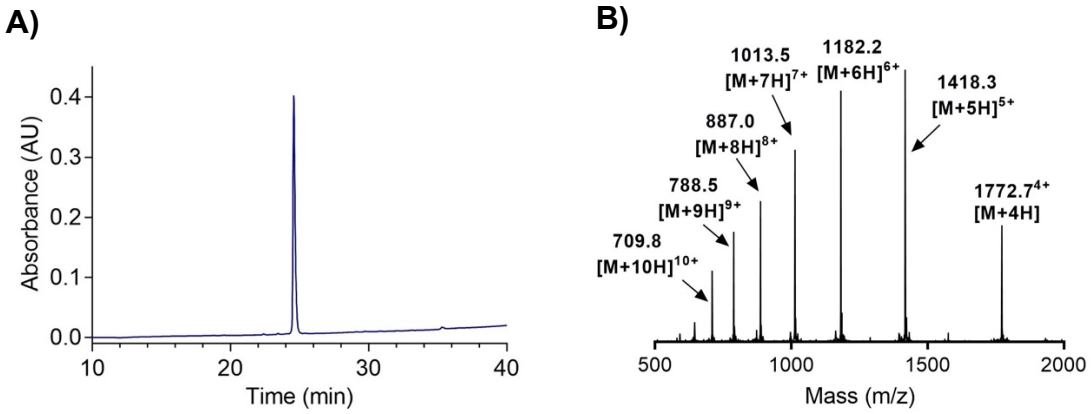
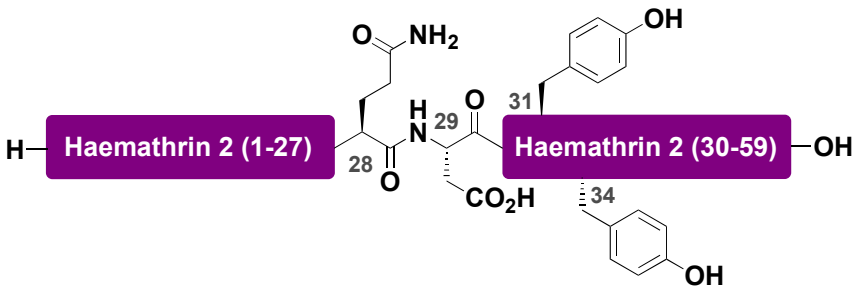


Figure S19. A) Analytical HPLC trace of purified haemathrin-2 (29-59) diselenide dimer (**24**); R$_t$ 24.58 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of purified haemathrin-2 (29-59) diselenide (**24**). Calculated Mass [M+4H]$^{4+}$: 1772.8, [M+5H]$^{5+}$: 1418.4, [M+6H]$^{6+}$: 1182.2, [M+7H]$^{7+}$: 1013.4, [M+8H]$^{8+}$: 886.9, [M+9H]$^{9+}$: 788.5, [M+10H]$^{10+}$: 709.7; Mass Found (ESI+): [M+4H]$^{4+}$: 1772.7, [M+5H]$^{5+}$: 1418.3, [M+6H]$^{6+}$: 1182.2, [M+7H]$^{7+}$: 1013.5, [M+8H]$^{8+}$: 887.0, [M+9H]$^{9+}$: 788.5, [M+10H]$^{10+}$: 709.8.

Haematathrin-2 (1-59, unsulfated) (2)



The ligation of haematathrin-2 (29-59) diselenide dimer (**24**) (1.4 mg, 0.20 μmol) and haematathrin-2 (1-28) selenoester (**29**) (1.8 mg, 0.53 μmol) together with *in situ* deselenization was performed as outlined in the general methods section. Purification *via* preparative RP-HPLC (0 to 30%B over 40 min, 0.1 vol% TFA), followed by lyophilization afforded the native haematathrin-2 (1-59) (**2**) as a white solid (1.2 mg, 0.18 μmol, 45% yield over 2 steps).

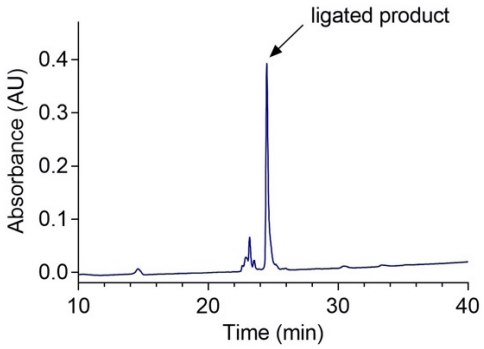


Figure S20. Analytical HPLC trace of crude haematathrin-2 (1-59) (**2**) post ligation-deselenization; R_t 24.51 min (0-50%B over 30 min, λ = 230 nm).

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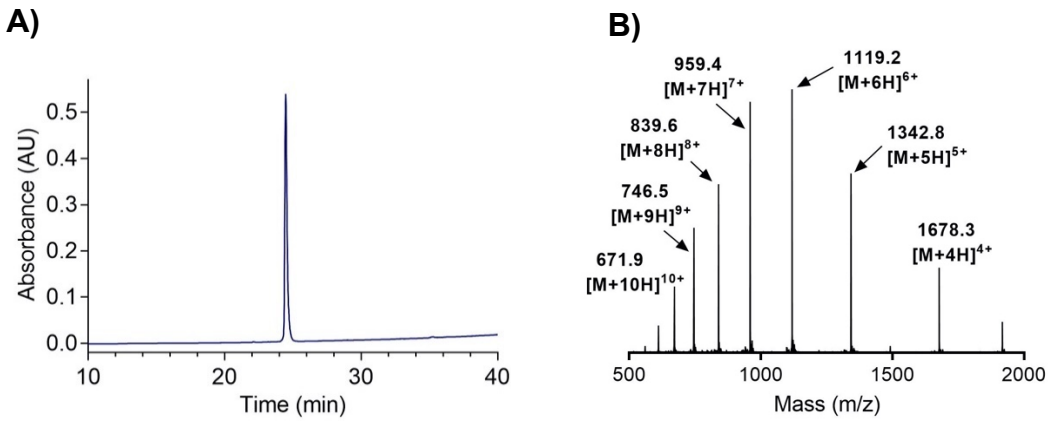


Figure S21. A) Analytical HPLC trace of purified haemathrin-2 (1-59) (**2**); R_t 24.48 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of purified haemathrin-2 (1-59) (**2**). Calculated Mass $[M+4H]^{4+}$: 1678.3, $[M+5H]^{5+}$: 1342.8, $[M+6H]^{6+}$: 1119.2, $[M+7H]^{7+}$: 959.5, $[M+8H]^{8+}$: 839.7, $[M+9H]^{9+}$: 746.5, $[M+10H]^{10+}$: 671.9; Mass Found (ESI+): $[M+4H]^{4+}$: 1678.3, $[M+5H]^{5+}$: 1342.8, $[M+6H]^{6+}$: 1119.2, $[M+7H]^{7+}$: 959.4, $[M+8H]^{8+}$: 839.6, $[M+9H]^{9+}$: 746.5, $[M+10H]^{10+}$: 671.9.

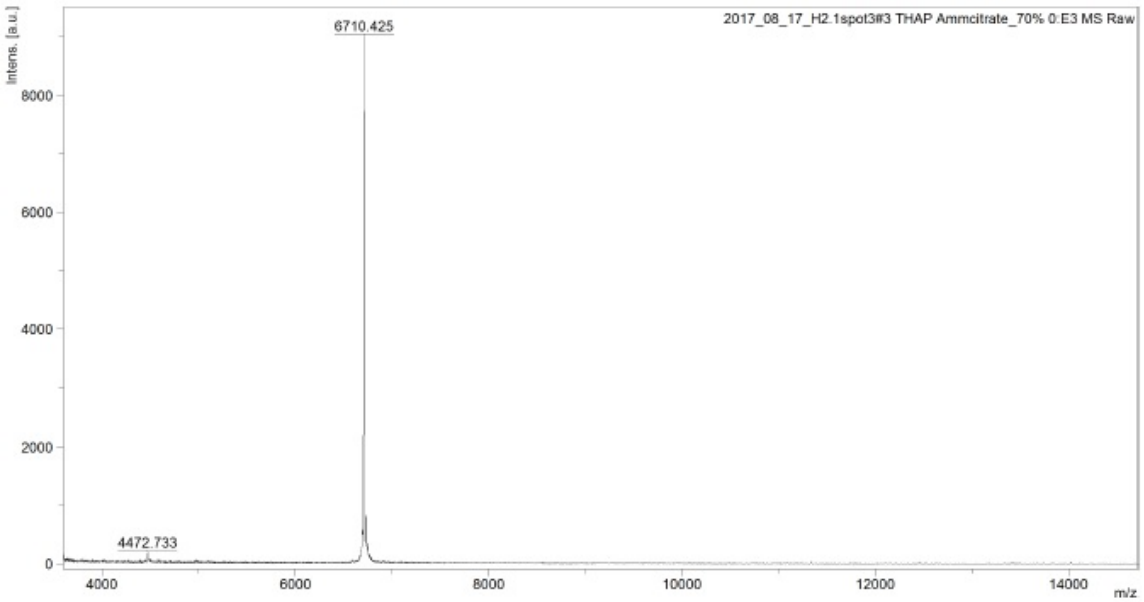
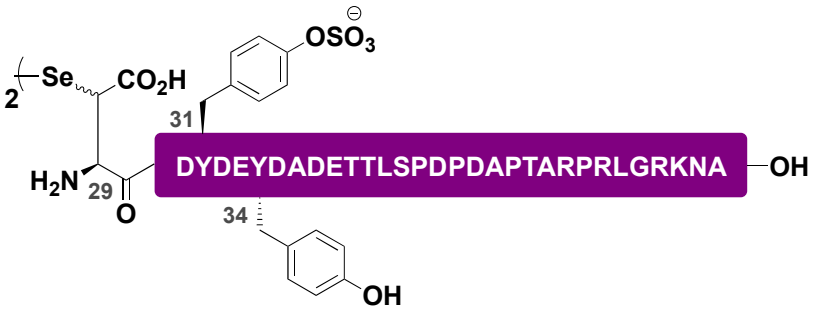


Figure S22. MALDI-TOF mass spectrum of haemathrin-2 (1-59) (**2**) using THAP/di-ammonium hydrogen citrate as a matrix; Calculated molecular weight: 6710.17; Observed most abundant isotope: 6710.43.

Haematathrin-2 C-terminal Diselenide Dimer (29-59; sY31) (25)



The singly sulfated *C*-terminal fragment of haemathrin-2 (24-59; sY31) (**25**) was prepared on Wang resin (100 μmol) using automated Fmoc-SPPS as outlined in the general methods. 12.5 μmol of this dried resin was taken forward with the coupling of Boc-(β-SePMB)Asp-OH (**11**), resin cleavage, deprotection and diselenide formation performed as described previously for the unsulfated haemathrin-2 *C*-terminal diselenide fragment (**24**). After PMB deprotection, the crude peptide was purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) and pure fractions were combined and lyophilized. The nP sulfate ester protecting groups were then removed by incubation in ligation buffer as described previously. The fully deprotected *C*-terminal fragment was then purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) to yield haemathrin-2 (24-59; sY31) diselenide dimer (**25**) as a white solid after lyophilization (1.5 mg, 0.41 μmol (with respect to the monomer), 3% yield).

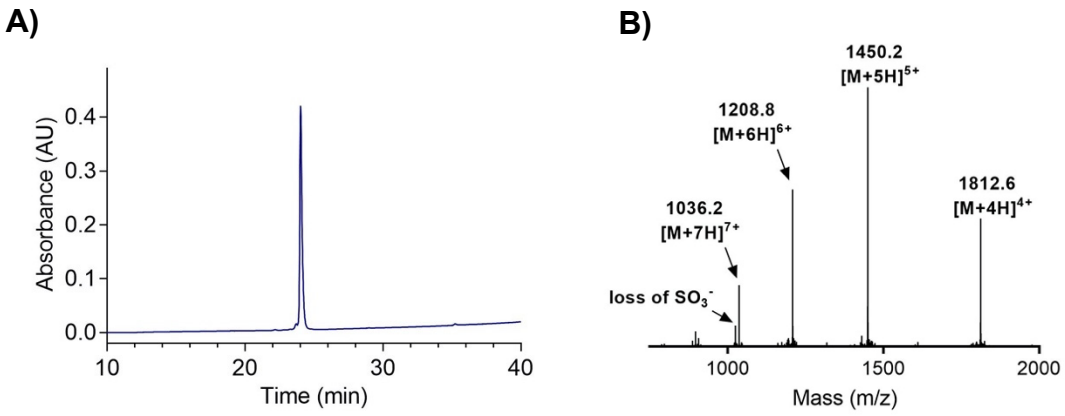
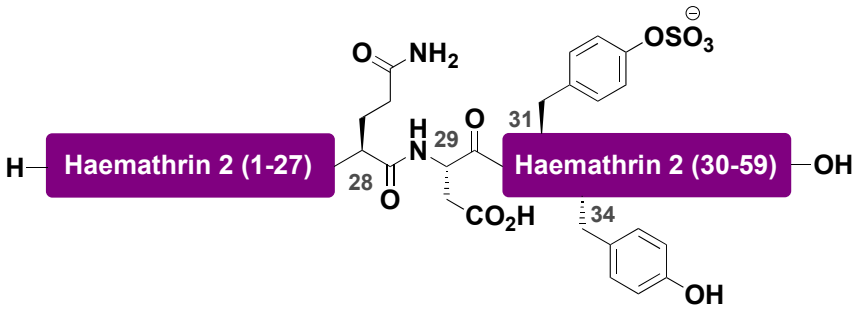


Figure S23. A) Analytical HPLC trace of purified haemathrin-2 (29-59; sY31) diselenide dimer (**25**); R_t 24.03 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of haemathrin-2 (29-59; sY31) diselenide dimer (**25**). Calculated Mass $[M+4H]^{4+}$: 1812.8, $[M+5H]^{5+}$: 1450.4, $[M+6H]^{6+}$: 1208.9, $[M+7H]^{7+}$: 1036.3; Mass Found (ESI+): $[M+4H]^{4+}$: 1812.6, $[M+5H]^{5+}$: 1450.2, $[M+6H]^{6+}$: 1208.8, $[M+7H]^{7+}$: 1036.2.

Haematathrin-2 (1-59; sY31) (6)



The ligation of haemathrin-2 (29-59; sY31) diselenide dimer (**25**) (1.5 mg, 0.21 μmol) and haemathrin-2 (1-23) selenoester (**29**) (1.8 mg, 0.53 μmol) together with *in situ* deselenization was performed as outlined in the general methods section. Purification *via* preparative RP-HPLC (0-30%B over 40 min, 0.1 vol% TFA), followed by lyophilization afforded the monosulfated haemathrin-2 (1-59; sY31) (**6**) as a white solid (1.4 mg, 0.21 μmol, 50% yield over 2 steps).

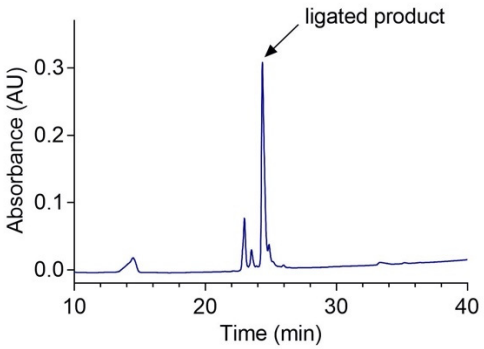


Figure S24. Analytical HPLC trace of crude haemathrin-2 (1-59; sY31) (**6**) post ligation-deselenization; R_t 24.36 min (0-50%B over 30 min, λ = 230 nm).

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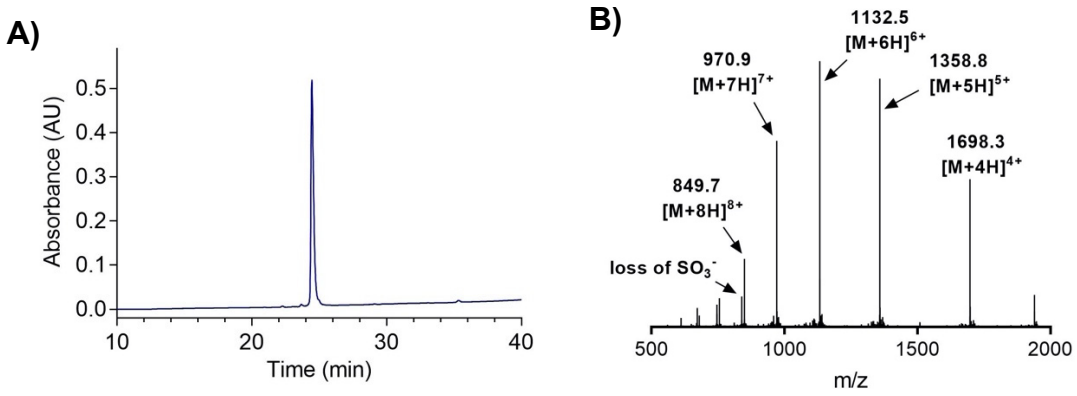


Figure S25. A) Analytical HPLC trace of purified haemathrin-2 (1-59; sY31) (**6**); R_t 24.45 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of haemathrin-2 (1-59; sY31) (**6**). Calculated Mass $[M+4H]^{4+}$: 1698.3, $[M+5H]^{5+}$: 1358.8, $[M+6H]^{6+}$: 1132.5, $[M+7H]^{7+}$: 970.9, $[M+8H]^{8+}$: 849.7; Mass Found (ESI+): $[M+4H]^{4+}$: 1698.3, $[M+5H]^{5+}$: 1358.8, $[M+6H]^{6+}$: 1132.5, $[M+7H]^{7+}$: 970.9, $[M+8H]^{8+}$: 849.7.

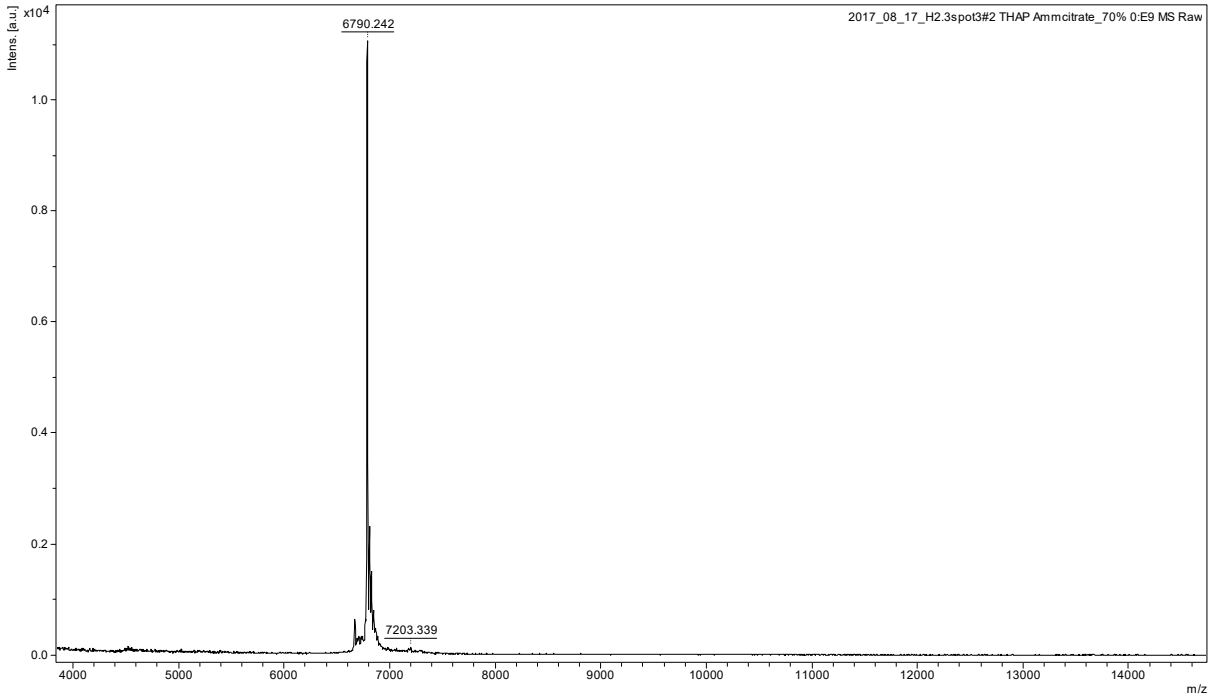
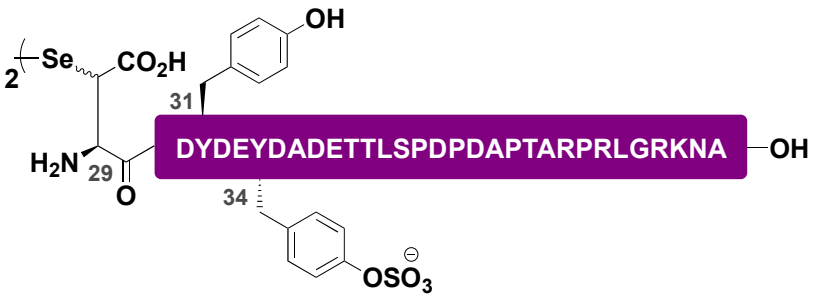


Figure S26. MALDI-TOF mass spectrum of haemathrin-2 (1-59; sY31) (**6**) using THAP/di-ammonium hydrogen citrate as a matrix; Calculated molecular weight: 6790.22; Observed most abundant isotope: 6790.24.

Haemathrin-2 C-terminal Diselenide Dimer (29-59; sY34) (26)



The singly sulfated *C*-terminal fragment of haemathrin-2 (29-59; sY34) (**26**), was prepared on Wang resin (100 µmol) using automated Fmoc-SPPS as outlined in the general methods. 12.5 µmol of this dried resin was taken forward with the coupling of Boc-(β-SePMB)Asp-OH (**11**), resin cleavage, deprotection and diselenide formation performed as described previously for the unsulfated haemathrin-2 *C*-terminal diselenide (**24**) fragment. After PMB deprotection, the crude peptide was purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) and pure fractions were combined and lyophilized. The lyophilized peptide was dissolved in ligation buffer (2 mg mL^{-1}, 6 M Gnd.HCl, 0.1 M Na_2HPO_4, pH 7.2) and incubated at 50 °C for 2 hours to remove the nP sulfate ester protecting groups. The fully deprotected *C*-terminal fragment was then purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) to yield haemathrin-2 (29-59; sY34) diselenide dimer (**26**) as a white solid after lyophilization (1.8 mg, 0.5 µmol (with respect to the monomer), 4% yield).

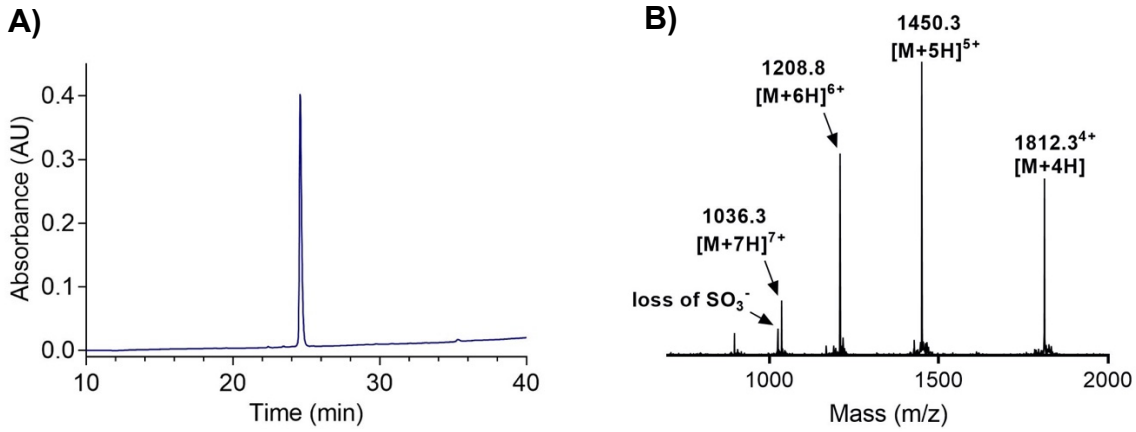
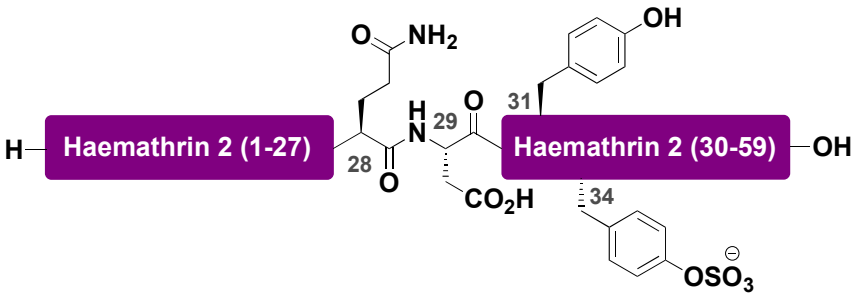


Figure S27. A) Analytical HPLC trace of purified haemathrin-2 (29-59; sY34) diselenide dimer (**26**); R_t 24.12 min (0-50%B over 30 min, λ = 230 nm). B) Mass spectrum (ESI+) of haemathrin-2 (29-59; sY34) diselenide dimer (**26**). Calculated Mass $[M+4H]^{4+}$: 1812.8, $[M+5H]^{5+}$: 1450.4, $[M+6H]^{6+}$: 1208.9, $[M+7H]^{7+}$: 1036.3; Mass Found (ESI+): $[M+4H]^{4+}$: 1812.3, $[M+5H]^{5+}$: 1450.3, $[M+6H]^{6+}$: 1208.8, $[M+7H]^{7+}$: 1036.3.

Haematathrin-2 (1-59; sY34) (7)



The ligation of haemathrin-2 (29-59; sY34) diselenide dimer (**26**) (1.4 mg, 0.19 μmol) and haemathrin-2 (1-28) selenoester (**29**) (1.8 mg, 0.53 μmol) together with *in situ* deselenization was performed as outlined in the general methods section. Purification *via* preparative RP-HPLC (0-30%B over 40 min, 0.1 vol% TFA), followed by lyophilization afforded haemathrin-2 (1-59; sY34) (**7**) as a white solid (1.2 mg, 0.18 μmol, 46% yield over 2 steps).

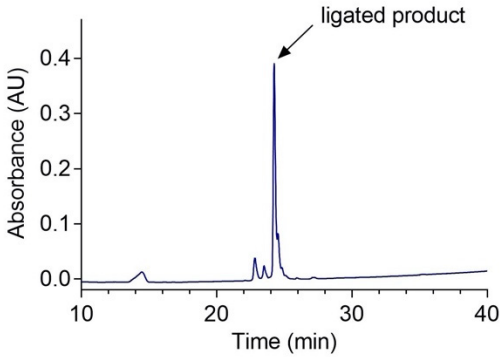


Figure S28. Analytical HPLC trace of crude haemathrin-2 (1-59; sY34) (**7**) post ligation-deselenization; R_t 24.25 min (0-50%B over 30 min, λ = 230 nm).

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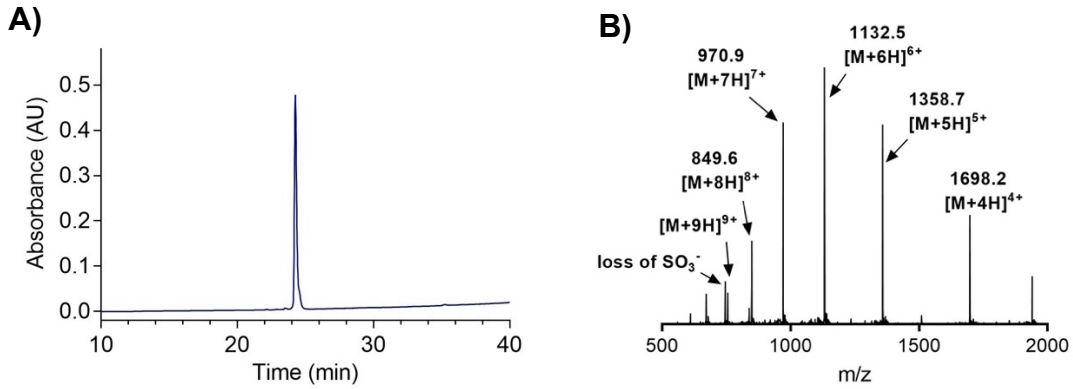


Figure S29. A) Analytical HPLC trace of purified haemathrin-2 (1-59; sY34) (**7**); R_t 24.26 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of haemathrin-2 (1-59; sY34) (**7**). Calculated Mass $[M+4H]^{4+}$: 1698.3, $[M+5H]^{5+}$: 1358.8, $[M+6H]^{6+}$: 1132.5, $[M+7H]^{7+}$: 970.9, $[M+8H]^{8+}$: 849.7; Mass Found (ESI+): $[M+4H]^{4+}$: 1698.2, $[M+5H]^{5+}$: 1358.7, $[M+6H]^{6+}$: 1132.5, $[M+7H]^{7+}$: 970.9, $[M+8H]^{8+}$: 849.6.

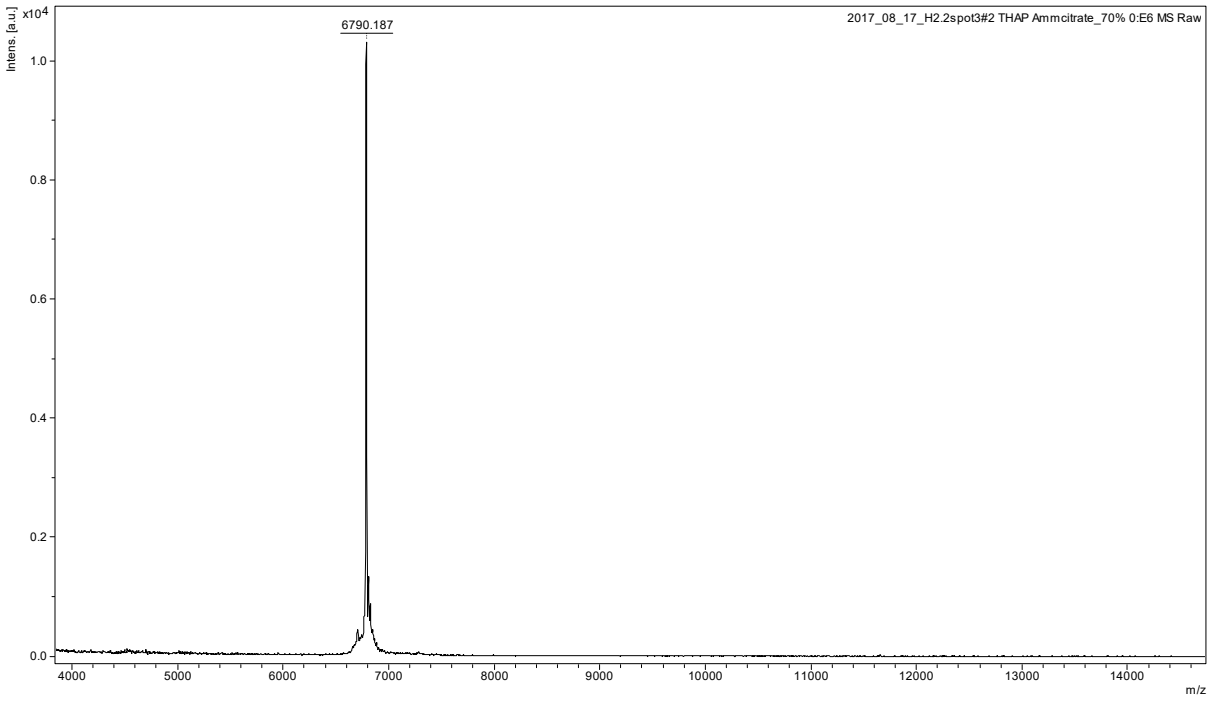
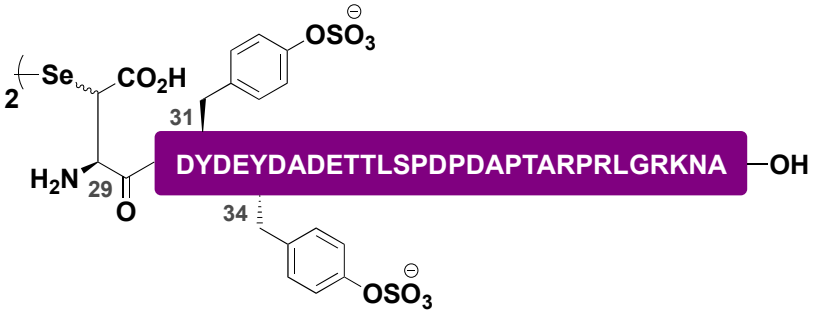


Figure S30. MALDI-TOF mass spectrum (linear positive mode) of haemathrin-2 (1-59; sY34) (**7**) using THAP/di-ammonium hydrogen citrate as a matrix; Calculated molecular weight: 6790.22; Observed most abundant isotope: 6790.19.

Haematathrin-2 C-terminal Diselenide Dimer (29-59; sY31, sY34) (27)



The doubly sulfated *C*-terminal fragment of haemathrin-2 (29-59; sY31, sY34) (**27**) was prepared on Wang resin (100 μmol) using automated Fmoc-SPPS as outlined in the general methods. 12.5 μmol of this dried resin was taken forward with the coupling of Boc-(β-SePMB)Asp-OH (**11**), resin cleavage, deprotection and diselenide formation performed as described previously for the unsulfated haemathrin-2 *C*-terminal diselenide (**24**) fragment. After PMB deprotection, the crude peptide was purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) and pure fractions were combined and lyophilized. The nP sulfate ester protecting groups were then removed by incubation in ligation buffer as described previously. The fully deprotected *C*-terminal fragment was then purified *via* preparative RP-HPLC (5-35%B over 40 min, 0.1 vol% TFA) to yield haemathrin-2 (29-59; sY31, sY34) diselenide dimer (**27**) as a white solid after lyophilization (1.5 mg, 0.4 μmol (with respect to the monomer), 3% yield).

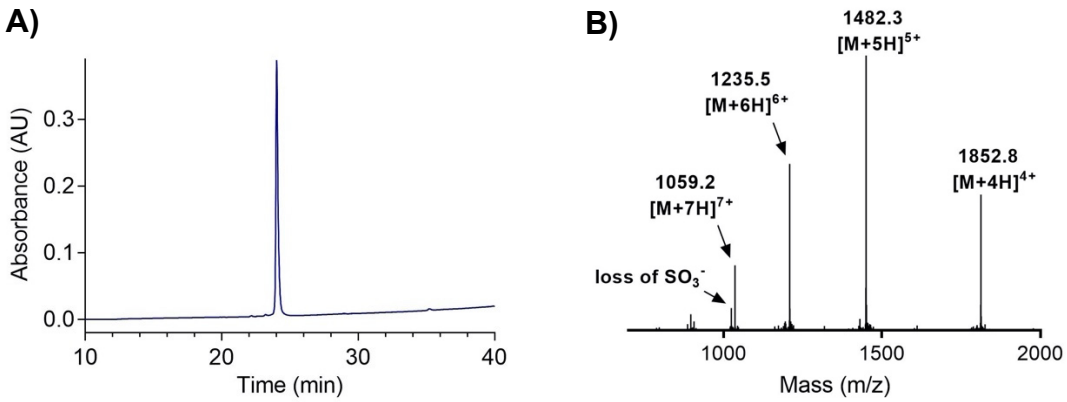
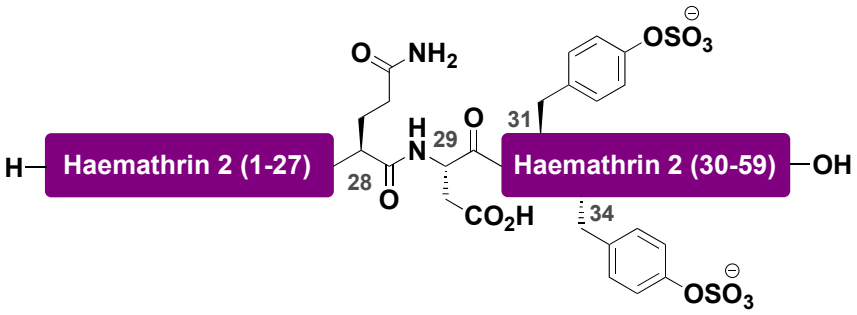


Figure S31. A) Analytical HPLC trace of purified haemathrin-2 (29-59; sY31, sY34) diselenide dimer (**27**); R_t 24.03 min (0-50%B over 30 min, λ = 230 nm). B) Mass spectrum (ESI+) of purified haemathrin-2 (29-59; sY31, sY34) diselenide (**27**). Calculated Mass $[M+4H]^{4+}$: 1852.9, $[M+5H]^{5+}$: 1482.5, $[M+6H]^{6+}$: 1235.6, $[M+7H]^{7+}$: 1059.2; Mass Found (ESI+): $[M+4H]^{4+}$: 1852.8, $[M+5H]^{5+}$: 1482.3, $[M+6H]^{6+}$: 1235.5, $[M+7H]^{7+}$: 1059.2.

Haematathrin-2 (1-59; sY31, sY34) (8)



The ligation of haemathrin-2 (24-59; sY31, sY34) diselenide dimer (**27**) (1.5 mg, 0.20 μmol) and haemathrin-2 (1-23) selenoester (**29**) (1.8 mg, 0.53 μmol) together with *in situ* deselenization was performed as outlined in the general methods section. Purification *via* preparative RP-HPLC (0-30%B over 40 min, 0.1 vol% TFA), followed by lyophilization afforded the doubly sulfated haemathrin-2 (1-59; sY31, sY34) (**8**) as a white solid (1.5 mg, 0.22 μmol, 54% yield over 2 steps).

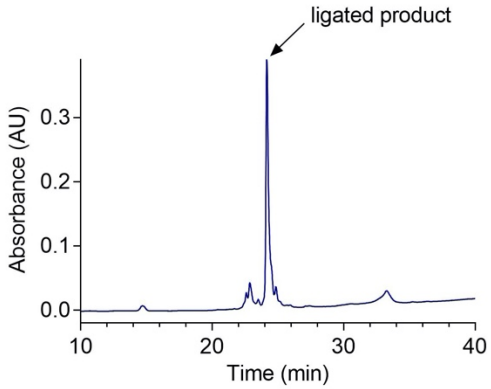


Figure S32. Analytical HPLC trace of crude haemathrin-2 (1–59; sY31, sY34) (**8**) post ligation-deselenization; R_t 24.17 min (0-50%B over 30 min, λ = 230 nm).

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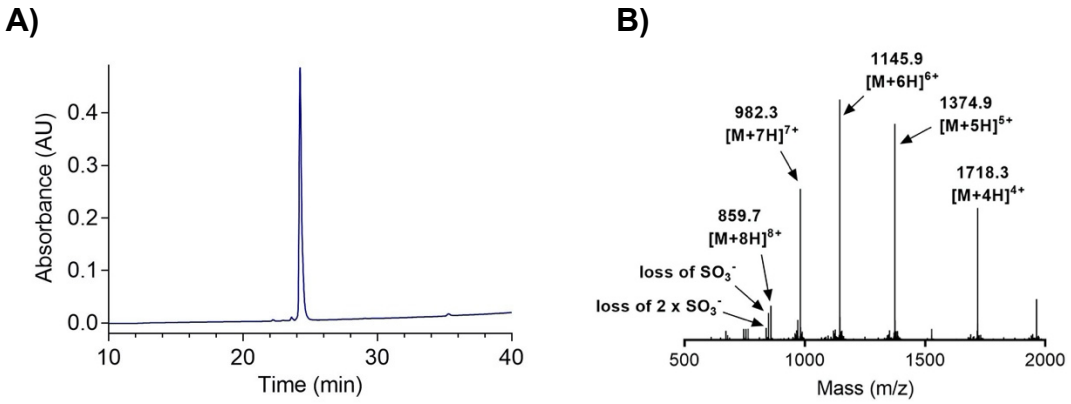

Figure S33. A) Analytical HPLC trace of purified haemathrin-2 (1–59; sY31, sY34) (**8**); R_t 24.24 min (0-50%B over 30 min, λ = 230 nm). **B)** Mass spectrum (ESI+) of purified haemathrin-2 (1-59; sY31, sY34) (**8**). Calculated Mass $[M+4H]^{4+}$: 1718.3, $[M+5H]^{5+}$: 1374.9, $[M+6H]^{6+}$: 1145.9, $[M+7H]^{7+}$: 982.3, $[M+8H]^{8+}$: 859.7; Mass Found (ESI+): $[M+4H]^{4+}$: 1718.3, $[M+5H]^{5+}$: 1374.9, $[M+6H]^{6+}$: 1145.9, $[M+7H]^{7+}$: 982.3, $[M+8H]^{8+}$: 859.7.

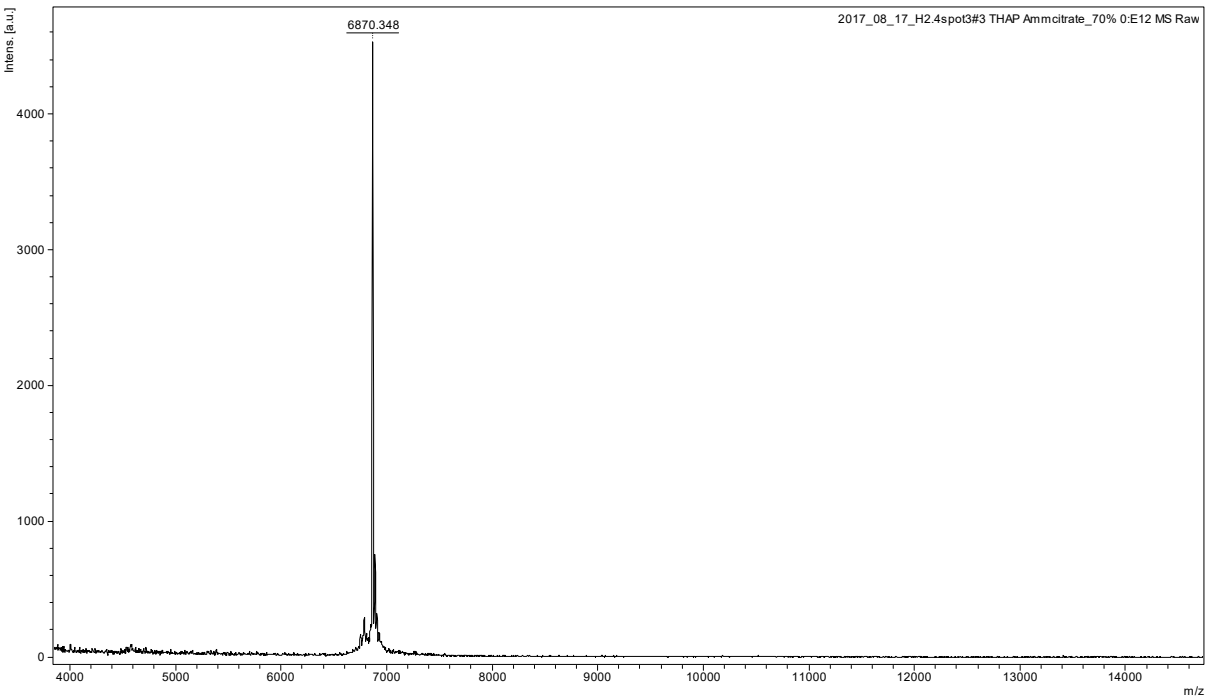

Figure S34. MALDI-TOF MS (linear positive mode) of haemathrin-2 (1–59; sY31, sY34) (**8**) using THAP/di-ammonium hydrogen citrate as a matrix; Calculated molecular weight: 6870.28; Observed most abundant isotope: 6870.35.

In vitro Inhibition of Human α- and γ-Thrombin

The inhibition of the amidolytic activity of human α- or γ-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 (**1**-**8**) was determined using a Direct Detect Infrared Spectrometer (Millipore). The inhibition constants (K_i) of all inhibitor variants were determined according to a tight-binding model by fitting the inhibited steady-state velocity data to the Morrison equation.[3] All reactions were carried out at 37 °C in 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mg mL^{-1} 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 R^2 value was between 0.989 and 0.999.

References

1. Mitchell, N. J., Sayers, J., Kulkarni, S. S., Clayton, D., Goldys, A. M., Ripoll-Rozada, J., Pereira, P. J. B., Chan, B., Radom, L., Payne, R. J. (2017) Accelerated Protein Synthesis via One-Pot Ligation-Deselenization Chemistry. *Chem 2*, 703–715.
2. Hanna, C. C., Kulkarni, S. S., Watson, E. E., Premdjee, B., Payne, R. J. (2017) Solid-phase synthesis of peptide selenoesters via a side-chain anchoring strategy. *Chem. Commun. 53*, 5424-5427.
3. Williams, J. W., Morrison, J. F. (1979) The kinetics of reversible tight-binding inhibition. *Methods Enzymol. 63*, 437-467.