SUPPORTING INFORMATION

Backbone N-Heteroatom Substitution as a Strategy to Enhance Peptide Proteolytic Stability

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CONTENTS

SUPPLEMENTARY FIGURES

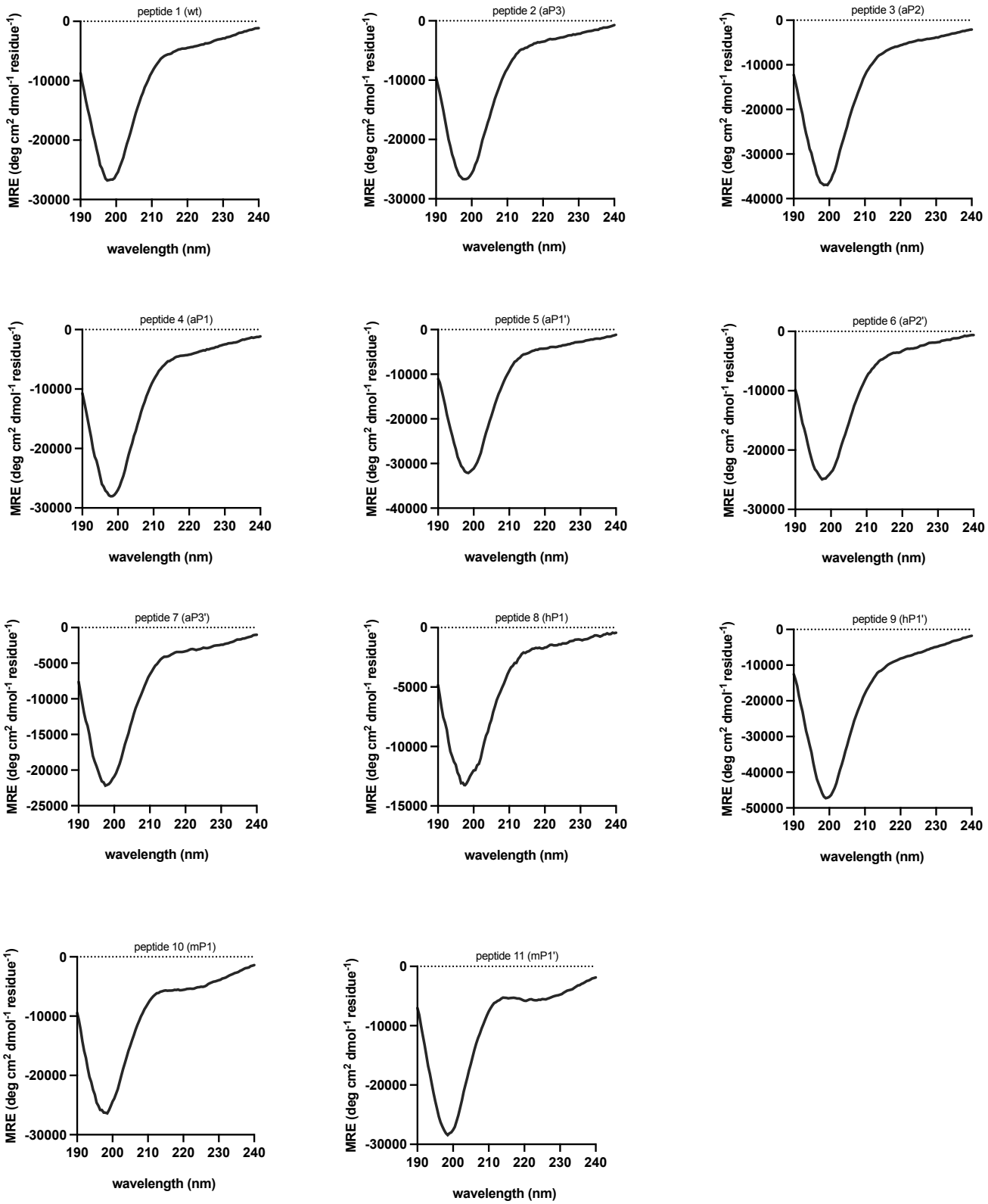


Figure S1. Far-UV CD wavelength scans (λ = 190–240 nm) of peptides **1**–**11** in 20 mM sodium phosphate buffer.

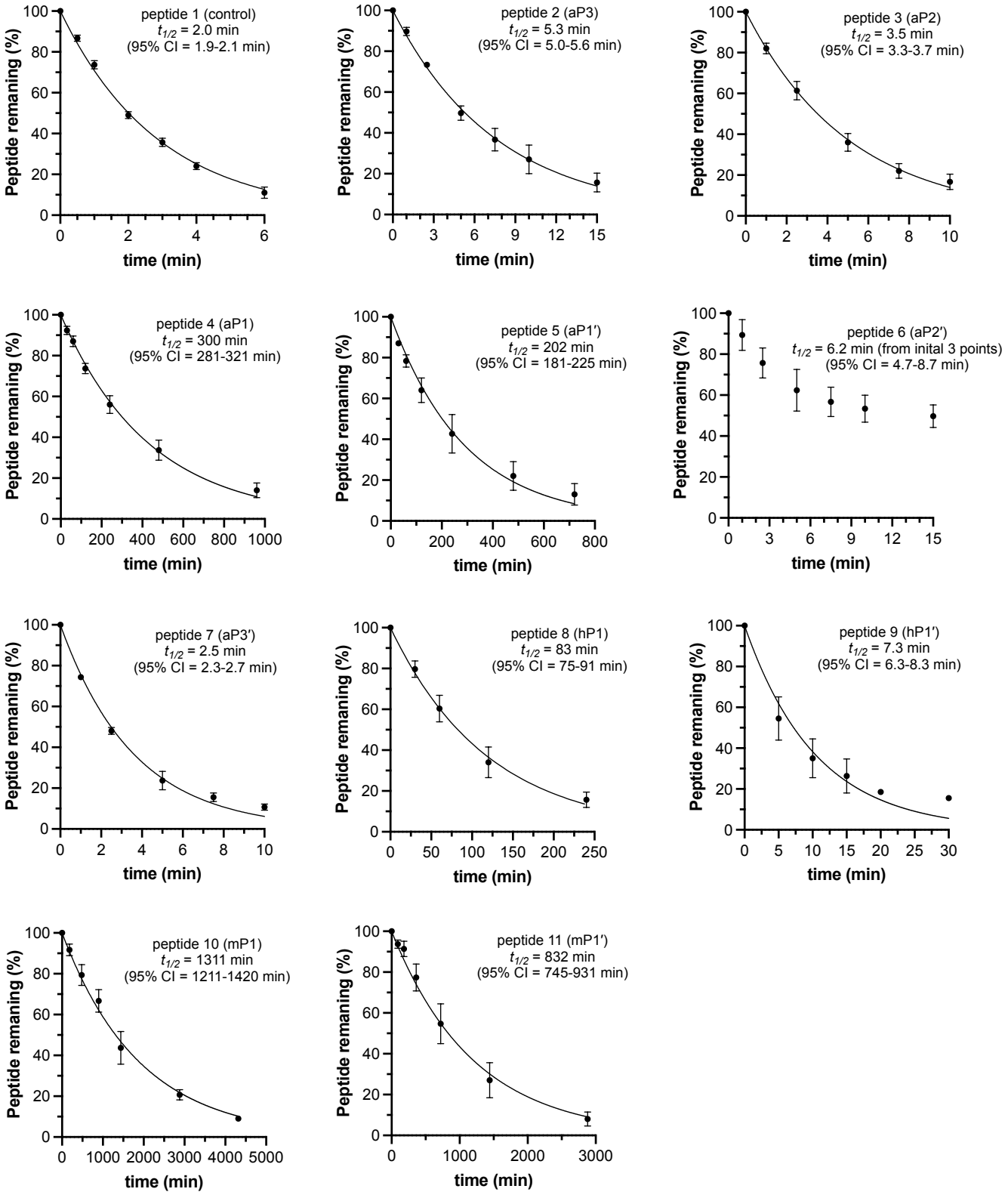


Figure S2. Individual plots of chymotrypsin-mediated degradation for peptides **1**–**11**, monitored by RP-HPLC (λ = 220 nm). Curves are derived from non-linear regression, and error bars represent SD for each data point (n = 3). 95% CI values are derived from curve fitting.

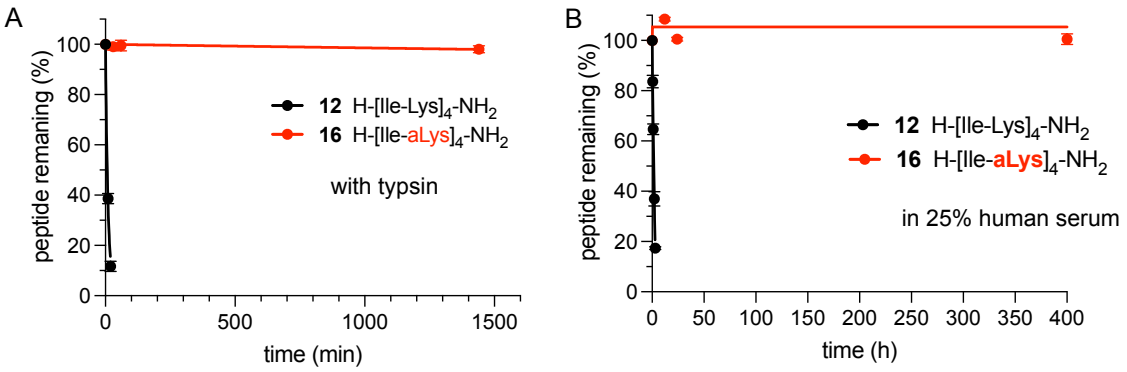


Figure S3. A) Stabilities of peptide **12**, tetra-NAP variant **16**, tetra-NMP variant **20** against trypsin, and B) stabilities of peptide **12** and **16** in 25% human serum, monitored by RP-HPLC (λ = 220 nm). Curves are derived from non-linear regression, and error bars represent SD for each data point (n = 3).

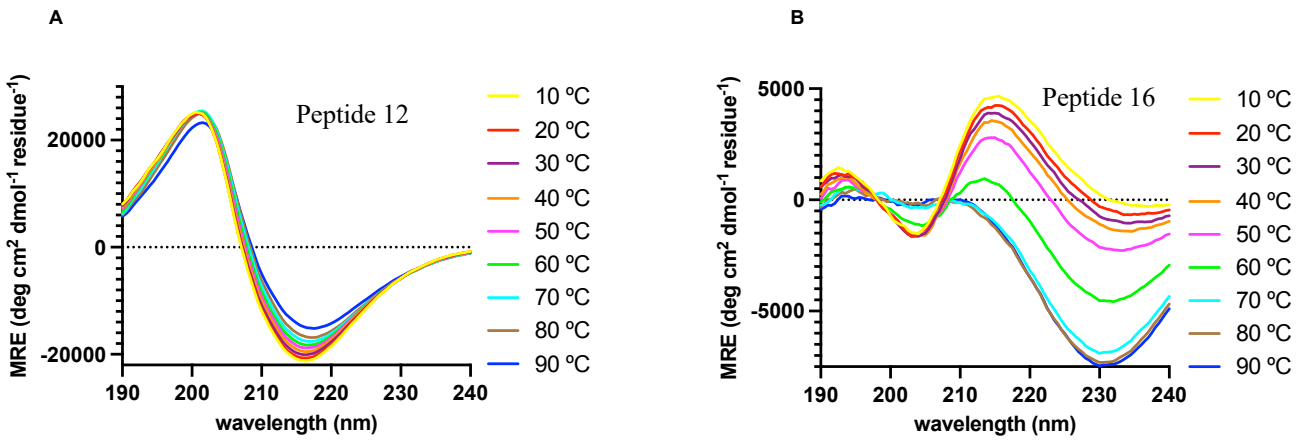
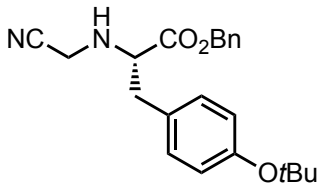


Figure S4. Far-UV CD wavelength scans (λ = 190–240 nm) of (A) peptide **12** and (B) peptide **16** in 25 mM aq SDS acquired by VT CD from 10 ºC to 90 ºC.

SYNTHETIC PROCEDURES

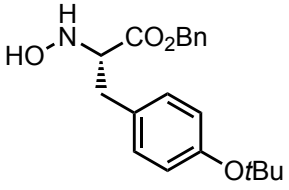
General notes. Commercial grade reagents and solvents were used without further purification except where noted. NMR spectra were recorded on a 400 MHz Bruker spectrometer. Proton chemical shifts are reported as δ values relative to residual signals from $CDCl_3$ or MeOD. Abbreviations for NMR signals are as follows: m = multiplet; q = quartet; t = triplet; d = doublet; s = singlet. Analytical HPLC chromatograms were acquired with a reverse-phase column (C18, 150 mm × 4.6 mm, 5 μm, 100 Å) using linear gradients of MeCN in H_2O (mobile phases modified with 0.1% formic acid) over 10 min, and spectra are provided for λ = 220 nm. Preparative HPLC purifications were performed with a reverse-phase column (C18, 250 mm x 21.2 mm, 5 μm, 100 Å) using linear gradients of MeCN in H_2O (mobile phases modified with 0.1% formic acid) over 30 min. HRMS spectra were acquired using a Bruker Impact II ESI-QTOF.

Solid-phase synthesis of N-aminated and N-methylated peptides. Automated solid-phase synthesis was carried out on a CEM Liberty Blue or a PurePep Chorus peptide synthesizer using ProTide Rink amide MBHA resin (100–200 mesh, 0.63 mmol/g). The following derivatives suitable for Fmoc SPPS were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH, Fmoc-(*N*-Me)Lys(Boc)-OH, Fmoc-(*N*-Me)Tyr(*t*Bu)-OH, H-a(Boc)Ala-OH, H-a(Boc)Lys(Boc)-OH, H-a(Boc)Ser(*t*Bu)-OH, and H-(Boc)aTyr(*t*Bu)-OH.[1] Fmoc-Ala-Cl, Fmoc-Lys(Boc)-Cl, Fmoc-Ser(*t*Bu)-Cl, Fmoc-Tyr(*t*Bu)-Cl, and Fmoc-Val-Cl were prepared by pretreating 10 equiv of their corresponding Fmoc-protected carboxylic acids (0.2 M in THF) with 10 equiv of Ghosez' reagent[2] for 1 min before addition to the resin. Fmoc deprotection steps were carried out by treating the resin with a solution of 20% (v/v) piperidine/DMF once at rt (5 min) and then at 50 °C (2 min). After Fmoc deprotection, the resin was washed with DMF (4×). Coupling of Fmoc-protected amino acids or α-hydrazino acids to primary amines was achieved using 10 equiv of DIC (1.0 M in DMF), 5 equiv of OxymaPure (1.0 M in DMF), and 5 equiv of the Fmoc-protected amino acids or α-hydrazino acids (0.2 M in DMF) at 50 °C (10 min). Couplings to α-hydrazino amides were achieved by washing the resin with DMF (3×) and then THF (3×), followed by the addition of the preformed solution of the acid chloride in THF (0.2 M, 10 equiv), and agitating at 50 °C for 1 h. After synthesis, the resin was washed with DCM (3×) and MeOH (3×) and dried under vacuum. Cleavage from the solid support and global deprotection was effected by incubating the dried resin in 5 mL of TFA:H_2O:TIPS:acetone (85:2.5:2.5:10) for 4 h. Note: for peptides **13**–**16,** a cleavage solution of HCl:HFIP:TIPS (1:98:1) was used to cleave the peptide from resin and globally deprotect. The resin was filtered, and the filtrate was collected in a 50 mL centrifuge tube. The resin was washed with the cleavage mixture (5 mL) and filtered, and the combined filtrate was evaporated under a stream of N_2. The concentrated filtrate was precipitated by the addition of cold Et_2O (45 mL, −20 °C). The mixture was centrifuged, and the supernatant was decanted. The pellet was dissolved in H_2O and purified by preparative RP-HPLC (C18, 250 mm × 21.2 mm, 5 μM, 100 Å) using a linear gradient of MeCN in H_2O (mobile phases modified with 0.1% formic acid) over 30 min and then lyophilized to afford the peptides as white powders.



S1

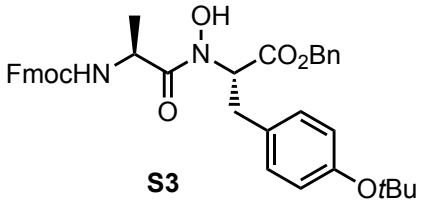
Benzyl (S)-3-(4-(*tert*-butoxy)phenyl)-2-((cyanomethyl)amino)propanoate) (S1). To a solution of H-Tyr(*t*Bu)-OBn (HCl salt, 1 equiv) in MeCN (0.05 M) and DIEA (2 equiv) was added bromoacetonitrile (1.2 equiv). The mixture was stirred for 18 h at 50 °C. Volatiles were removed under vacuum, and the crude material was partitioned between DCM and sat aq $NaHCO_3$. The aqueous layer was extracted with DCM. The organic layers were combined, dried over anhydrous Na_2SO_4, and concentrated. The reaction crude was purified by flash chromatography on a silica gel column (20% EtOAc/hexanes) to afford **S1** as a yellow oil (5.55 g, 79% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 – 7.28 (m, 5H), 7.06 – 6.98 (m, 2H), 6.92 – 6.86 (m, 2H), 5.15 (s, 2H), 3.70 (dd, *J* = 7.6, 5.6 Hz, 1H), 3.53 (d, *J* = 0.7 Hz, 2H), 3.04 (dd, *J* = 13.8, 5.6 Hz, 1H), 2.89 (dd, *J* = 13.8, 7.5 Hz, 1H), 1.91 (s, 1H), 1.33 (s, 9H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 172.8, 154.5, 135.2, 130.8, 129.7, 128.7, 128.6, 128.6, 124.3, 117.3, 78.5, 67.1, 61.2, 38.5, 36.0, 28.9. HRMS (ESI-TOF) *m/z* [M + Na]$^+$ calcd for $C_{22}H_{26}N_2NaO_3$ 389.1836, found 389.1840.



S2

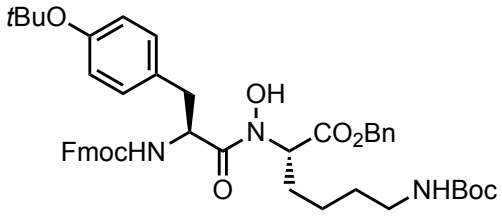
H-hTyr(*t*Bu)-OBn (S2). To a solution of **S1** (1 equiv) in DCM (0.1 M) at 0 °C was added 75% mCPBA (2 equiv) portion-wise over 20 min. The reaction was allowed to warm to rt and stirred for 1 h. A solution of sat aq $Na_2S_2O_3$ (30 mL) and sat aq $NaHCO_3$ (60 mL) was added, and the mixture was stirred for 30 min. H_2O was added and the mixture was extracted with DCM. The combined organic layers were dried over anhydrous Na_2SO_4, and concentrated. The resulting residue was dissolved in MeOH (0.2 M), and hydroxylamine hydrochloride (5 equiv) was added. The reaction was fitted with a reflux condenser and heated at 60 °C for 6 h using a heating mantle. The reaction mixture was concentrated, and the crude residue was taken up in DCM and sat aq $NaHCO_3$ and extracted with DCM. The organic layers were combined, dried over anhydrous Na_2SO_4, and concentrated. The reaction crude was purified by flash chromatography on a silica gel column (30% EtOAc/hexanes) to afford **S2** as a white solid (2.7 g, 52% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 – 7.20 (m, 5H), 7.07 – 6.97 (m, 2H), 6.93 – 6.82 (m, 2H), 5.15 (s, 2H), 3.93 (dd, *J* = 8.1, 6.0 Hz, 1H), 3.04 – 2.80 (m, 2H), 1.32 (s, 9H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 172.7, 154.4, 135.3, 130.9, 130.4, 129.6, 128.6, 128.6, 128.4, 128.4, 128.3, 124.3, 115.4, 78.5, 67.0, 66.2, 34.7, 28.8. HRMS (ESI-TOF) *m/z* [M + H]$^+$ calcd for $C_{20}H_{26}NO_4$ 344.1856, found 344.1860.

General procedure for the synthesis of benzyl ester dipeptides (S3–S5). A solution of Fmoc-Ala-OH, Fmoc-Tyr(*t*Bu)-OH, or Fmoc-Ile-OH (1.1 equiv) in DCM (0.05 M) was treated with Ghosez' reagent (1.1 equiv) and allowed to stir for 1 min at rt. This solution was then transferred to a solution of H-hTyr(*t*Bu)-OBn or H-hLys(Boc)-OBn[3] (1 equiv) in DCM (final concentration 0.05 M), and the reaction was allowed to stir for 1 h. The reaction mixture was diluted with DCM, washed with H_2O 4×, brine 4×, and the combined aqueous layer was extracted with more DCM. The organic layers were then combined, dried over Na_2SO_4, filtered, and concentrated.



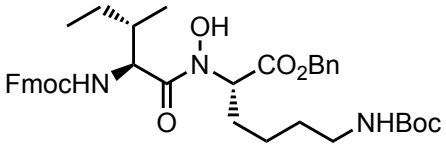
S3

Fmoc-Ala-hTyr(*t*Bu)-OBn (S3). The reaction crude was purified by flash chromatography on a silica gel column (20% EtOAc/hexanes), giving **S3** as a yellow foam (1.13 g, 67% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.6 Hz, 2H), 7.58 (d, *J* = 7.3 Hz, 2H), 7.35 (ddt, *J* = 25.7, 14.9, 7.3 Hz, 9H), 7.10 (d, *J* = 8.4 Hz, 2H), 6.85 (d, *J* = 8.4 Hz, 2H), 5.55 – 5.34 (m, 2H), 5.20 (s, 2H), 4.83 (q, *J* = 7.2 Hz, 1H), 4.44 – 4.06 (m, 3H), 3.40 – 3.07 (m, 2H), 1.34 – 1.27 (m, 3H), 1.23 (s, 9H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 171.9, 170.2, 156.0, 154.3, 143.8, 143.7, 141.3, 136.2, 135.0, 131.0, 129.9, 129.0, 128.7, 128.6, 128.5, 128.4, 127.8, 127.1, 126.9, 125.2, 125.1, 124.2, 120.0, 78.4, 67.6, 67.3, 59.5, 51.6, 47.0, 37.6, 34.1, 28.9. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{38}H_{41}N_2O_7$ 637.2908, found 637.2893.



S4

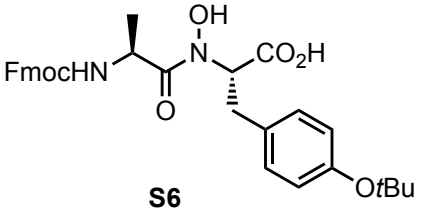
Fmoc-Tyr(*t*Bu)-hLys(Boc)-OBn (S4). The reaction crude was purified by flash chromatography on a silica gel column (30% EtOAc/hexanes), giving **S4** as a white foam (2.25 g, 85% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (s, 1H), 7.72 – 7.59 (m, 2H), 7.43 (d, *J* = 7.5 Hz, 2H), 7.34 – 7.14 (m, 9H), 7.05 – 6.94 (m, 2H), 6.84 – 6.70 (m, 2H), 5.50 (d, *J* = 7.3 Hz, 1H), 5.09 (dq, *J* = 8.1, 4.1 Hz, 4H), 4.31 – 4.13 (m, 1H), 4.13 – 3.94 (m, 2H), 3.11 (dd, *J* = 14.0, 5.1 Hz, 2H), 2.86 (dd, *J* = 13.6, 5.5 Hz, 1H), 2.64 (dd, *J* = 14.1, 8.2 Hz, 1H), 2.10 – 1.75 (m, 2H), 1.26 (d, *J* = 42.1 Hz, 22H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 173.2, 170.3, 157.1, 156.0, 154.1, 143.9, 143.8, 141.2, 135.1, 131.5, 129.9, 128.7, 128.5, 128.4, 127.7, 127.1, 125.2, 125.2, 124.1, 119.9, 119.9, 79.9, 78.3, 67.4, 67.1, 58.7, 52.4, 47.0, 36.8, 29.6, 28.8, 28.5, 26.5, 22.6. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{46}H_{56}N_3O_9$ 794.4011, found 794.3993.



S5

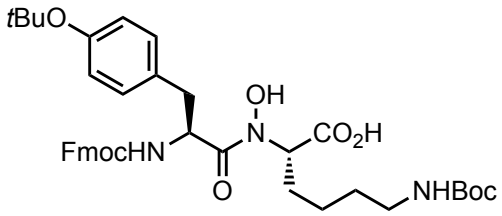
Fmoc-Ile-hLys(Boc)-OBn (S5). The reaction crude was purified by flash chromatography on a silica gel column (20% EtOAc/hexanes), giving **S5** as a white solid (3.25 g, 88% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (s, 1H), 7.80 – 7.70 (m, 2H), 7.58 (d, *J* = 7.5 Hz, 2H), 7.44 – 7.27 (m, 9H), 5.60 (d, *J* = 9.5 Hz, 1H), 5.25 – 5.12 (m, 3H), 4.84 (dd, *J* = 9.2, 5.7 Hz, 1H), 4.76 (s, 1H), 4.39 (dd, *J* = 10.4, 7.3 Hz, 1H), 4.32 – 4.16 (m, 2H), 3.16 (s, 1H), 3.02 – 2.84 (m, 1H), 2.16 – 1.81 (m, 2H), 1.40 (s, 15H), 1.20 – 1.05 (m, 1H), 0.97 (d, *J* = 6.8 Hz, 3H), 0.86 (t, *J* = 7.4 Hz, 3H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ 172.9, 170.5, 156.9, 156.7, 143.9, 143.8, 141.3, 135.2, 128.6, 128.5, 128.2, 127.7, 127.7, 127.1, 125.2, 125.1, 120.0, 120.0, 79.8, 77.3, 67.3, 67.2, 58.2, 55.4, 47.1, 36.5, 29.5, 28.5, 26.7, 23.9, 22.6, 15.9, 11.3. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{39}H_{50}N_3O_8$ 688.3592, found 688.3580.

General procedure for the synthesis of carboxylic acid dipeptides (S6–S8). To a round-bottom flask containing benzyl esters **S3–S5** (1 equiv) was added 0.2 equiv of 5% Pd/C followed by EtOAc (2 mL). The mixture was diluted with MeOH (0.1 M), and the flask was charged with H_2. After 3 h, the reaction was filtered through a pad of Celite, and the filtrate was concentrated *in vacuo*.



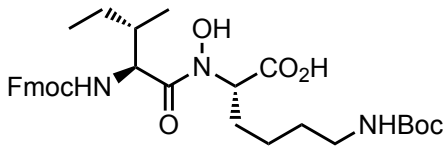
S6

Fmoc-Ala-hTyr(*t*Bu)-OH (S6).The reaction crude was purified by flash chromatography on a silica gel column (10% MeOH/DCM) to give **S6** as a pale orange foam (0.97 g, 97% yield). ^{1}H NMR (400 MHz, MeOD) δ 7.68 (d, *J* = 7.5 Hz, 2H), 7.54 (dd, *J* = 7.6, 4.2 Hz, 2H), 7.24 (dt, *J* = 33.3, 7.5 Hz, 4H), 7.06 (d, *J* = 8.4 Hz, 2H), 6.72 (dd, *J* = 8.5, 4.3 Hz, 2H), 5.07 (dd, *J* = 10.6, 4.5 Hz, 1H), 4.23 – 3.96 (m, 3H), 3.19 – 2.99 (m, 2H), 1.19 (d, *J* = 2.6 Hz, 3H), 1.08 (s, 9H). ^{13}C{^{1}H} NMR (101 MHz, MeOD) δ 174.2, 156.5, 153.5, 144.0, 143.8, 141.2, 141.1, 133.0, 129.1, 127.4, 126.8, 125.0, 124.9, 123.8, 119.5, 77.9, 66.6, 61.0, 32.8, 27.7, 16.3. HRMS (ESI-TOF) *m/z* [M + Na]$^+$ calcd for $C_{31}H_{34}N_2NaO_7$ 569.2258, found 569.2242.



S7

Fmoc-Tyr(*t*Bu)-hLys(Boc)-OH (S7).The reaction crude was purified by flash chromatography on a silica gel column (10% MeOH/DCM) to give **S7** as a pale yellow foam (0.90 g, 99% yield). ^{1}H NMR (400 MHz, MeOD) δ 7.80 (d, *J* = 7.6 Hz, 2H), 7.63 (d, *J* = 7.5 Hz, 2H), 7.46 – 7.10 (m, 6H), 6.97 – 6.78 (m, 2H), 5.08 – 5.03 (m, 2H), 4.33 – 4.20 (m, 1H), 4.20 – 4.03 (m, 2H), 3.31 (dd, *J* = 3.1 Hz, 1H), 3.04 (q, *J* = 6.7 Hz, 2H), 2.73 (dd, *J* = 14.0, 10.2 Hz, 1H), 2.04 – 1.91 (m, 2H), 1.59 – 1.20 (m, 22H). ^{13}C{^{1}H} NMR (101 MHz, MeOD) δ 174.0, 172.1, 157.1, 156.9, 153.7, 143.9, 143.8, 141.1, 141.1, 133.0, 129.6, 127.4, 126.8, 125.0, 124.9, 123.7, 119.5, 78.4, 78.1, 66.7, 58.7, 53.5, 46.9, 39.8, 35.7, 29.0, 27.8, 27.4, 26.9, 23.2. HRMS (ESI-TOF) *m/z* [M + H]$^+$ calcd for $C_{39}H_{50}N_3O_9$ 704.3542, found 704.3532.



S8

Fmoc-Ile-hLys(Boc)-OH (S8). The reaction crude was purified by flash chromatography on a silica gel column (10% MeOH/DCM) to give **S8** as a white solid (0.82 g, 95% yield). ^{1}H NMR (400 MHz, MeOD) δ 7.79 (d, *J* = 7.6 Hz, 2H), 7.75 – 7.58 (m, 2H), 7.40 (dt, *J* = 15.2, 7.5 Hz, 2H), 7.34 – 7.23 (m, 2H), 7.03 (d, *J* = 9.0 Hz, 1H), 5.03 (dt, *J* = 10.0, 5.0 Hz, 1H), 4.83 – 4.73 (m, 1H), 4.43 – 4.27 (m, 2H), 4.23 (t, *J* = 7.1 Hz, 1H), 3.07 – 2.89 (m, 2H), 2.07 – 1.81 (m, 3H), 1.65 – 1.08 (m, 15H), 1.01 (d, *J* = 6.8 Hz, 3H),

0.91 (t, J = 7.4 Hz, 3H).$^{13}C\{^1H\}$ NMR (101 MHz, MeOD) δ 173.6, 157.3, 157.1, 144.0, 143.8, 141.2, 129.5, 127.4, 126.8, 126.8, 124.9, 124.9, 119.5, 119.5, 78.4, 66.6, 58.3, 55.5, 47.0, 39.8, 36.1, 29.0, 27.4, 26.9, 23.7, 23.1, 14.8, 10.3. HRMS (ESI-TOF) m/z $[M + H]^+$ calcd for $C_{32}H_{44}N_3O_8$ 598.3123, found 598.3112

Synthesis of peptide hydroxamates for NHP ligation. For incorporation of N-hydroxylated residues, the peptide-resin (ProTide Rink amide MBHA resin (100–200 mesh, 0.63 mmol/g, 0.1 mmol scale) was transferred from the peptide synthesizer (using conditions previously mentioned) to a suitable vessel, washed with DCM (5 mL × 4), and dried under vacuum. Fmoc-protected dipeptides **S6** or **S7** (5 equiv, 0.2 M in DMF) were coupled manually using 5 equiv HATU (0.25 M in DMF), 10 equiv DIEA (1 M in DMF), at rt for 1 h, then the standard SPPS cycle was resumed up to the final Fmoc deprotection, and cleavage from the resin. Crude NHPs H_2N-AhYKSADSRVNST-NH_2 **(S9)** and H_2N-YhKSADSRVNST-NH_2 **(S10)** were utilized in the ligation reaction.

Synthesis of thioesters from peptide hydrazides for NHP ligation. Peptide thioesters were obtained from the corresponding peptide hydrazides prepared on Fmoc-hydrazine 2-chlorotrityl resin (0.71 mmol/g, 100–200 mesh, 0.1 mmol scale) following the standard Fmoc SPPS protocol described above. For conversion to the thioester, a 20 mM solution of the crude peptide hydrazide (1 equiv) in 6 M Gdn·HCl/PBS (pH 3) was cooled to −20 °C, and aq $NaNO_2$ (200 mM, 10 equiv) was added with vigorous stirring. After 20 min, MPAA (200 mM, 20 equiv in 6 M Gdn·HCl/PBS, pH 6) was introduced, and the mixture was warmed to rt and stirred for 2 h. The reaction mixture was diluted with 5% MeCN/H_2O (0.1% formic acid), centrifuged, and the supernatant was purified by RP-HPLC to obtain H_2N-VSADPSRVA-MPAA **(S11)** and H_2N-VSADPSRVAA-MPAA **(S12)**.

Synthesis of NHP P1 and P1' through ligation.[3] A solution of crude hydroxamates **S9** or **S10** (10 mM, 1.0 equivalent) was prepared in a 15 mL Falcon tube by dissolving the compound in a ligation buffer consisting of 6 M Gdn·HCl and aq PBS at pH 8.0. In a separate Falcon tube, a 13 mM **S11** or **S12** thioester solution (1.3 equiv) was prepared under the same buffer conditions. The two solutions were then combined to yield a final reaction mixture containing 5 mM peptide hydroxamate and 6.5 mM peptide thioester. This mixture was briefly sonicated for 1 minute to ensure homogeneity and then incubated at ambient temperature for 24 h. Following the incubation period, 10 µL aliquots were withdrawn and diluted into 190 µL of Milli-Q water for LC–MS analysis. If any residual hydroxamate was detected after complete consumption of the thioester, an additional portion of thioester powder was added to drive the reaction to completion.

Synthesis of peptide 21. For incorporation of N-hydroxy Lysine residues, the resin (ProTide Rink amide MBHA resin (100–200 mesh, 0.63 mmol/g, 0.1 mmol scale) was initially Fmoc deprotected with a solution of 20% (v/v) piperidine/DMF at rt for 10 min. Fmoc-protected dipeptide **S8** (5 equiv, 0.2 M in DMF) was coupled manually using 5 equiv HATU (0.25 M in DMF), 10 equiv DIEA (1 M in DMF), at rt for 1 h. After Fmoc deprotection, the resin was washed with DMF (4×) and DCM (4×), and the coupling of **S8** and deprotection were repeated for 3 additional cycles. After a final Fmoc deprotection, the resin was washed with DMF (3×), DCM (3×) and MeOH (3×) and dried under vacuum. Cleavage from solid support and global deprotection was effected by incubating the dried resin in 5 mL of TFA:H_2O:TIPS (95:2.5:2.5) for 2 h.

PEPTIDE STABILITY ASSAYS

Chymotrypsin stability of peptides. The stability of peptides (1-11) against bovine α-chymotrypsin (MilliporeSigma, product No. C4129, ≥40 units/mg protein) was determined by following a slightly modified procedure as reported by Horne's group.[4] Stock solutions of the peptide (200 μM) and bovine α-chymotrypsin (0.25 μM) were made in TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.5) and quantified by UV spectroscopy using ε_{280} = 1280 M^{-1} cm^{-1} and ε_{280} = 51,240 M^{-1} cm^{-1}, respectively. The reactions were initiated by treating the peptide (800 μL, 200 μM) with chymotrypsin (200 μL, 0.25 μM) at room temperature to a final sample concentration of 160 μM peptide and 50 nM chymotrypsin. At each time point, 100 μL of the sample was removed and treated with 2% TFA (10 μL, v/v in H_2O). The quenched samples were analyzed by analytical RP-HPLC (100 μL injections), and the remaining peptide was integrated at 220 nm. Degradation time courses were acquired with three replicate measurements at each time point. Nonlinear regression to a one-phase exponential decay model was performed using all replicate data points without averaging. Confidence intervals for fitted parameters were obtained from the regression, reflecting the variance of replicate measurements at each time point.

Trypsin stability of peptides. The stability of peptides (12, 16, and 20) against bovine trypsin (Sigma-Aldrich, product No. T1426, ≥10,000 BAEE units/mg protein) was determined by incubating stock solutions of the peptide (500 μL, 2 mg/mL in H_2O) and bovine trypsin (500 μL, 0.05 mg/mL in PBS). At each time point, 100 μL of the sample was removed and treated with 2% TFA (10 μL, v/v in H_2O). The quenched samples were analyzed by analytical RP-HPLC (100 μL injections), and the remaining peptide was integrated at 220 nm. Degradation time courses were acquired with three replicate measurements at each time point. Nonlinear regression to a one-phase exponential decay model was performed using all replicate data points without averaging. Confidence intervals for fitted parameters were obtained from the regression, reflecting the variance of replicate measurements at each time point.

Serum stability of peptides. The stability of peptides against human serum (MilliporeSigma, product No. H4522, from male plasma type AB) was determined by analytical RP-HPLC analysis. The reactions were initiated by treating a solution of the peptide (250 μL, 2.5 mM in H_2O, pre-warmed at 37 ºC) with 50% human serum (250 μL, v/v in H_2O, pre-warmed at 37 ºC). The sample mixtures were shaken at 200 rpm at 37 ºC. At each time point, 80 μL of sample was removed and treated with 10% trichloroacetic acid (20 μL, v/v in H_2O) and incubated at 4 ºC for 10 min. The samples were centrifuged at 10,000 rpm for 10 min at 4 ºC, and the supernatants were analyzed by analytical RP-HPLC (70 μL injections) using an initial isocratic gradient of 1% MeCN in H_2O (mobile phases modified with 0.1% formic acid) for 5 min followed by a linear gradient of 1–50% MeCN in H_2O (mobile phases modified with 0.1% formic acid) for 10 min. The remaining peptide was integrated at 220 nm and plotted as a function of time. Each data point represents the mean of 2–3 independent experiments.

BACTERIAL GROWTH INHIBITION ASSAYS

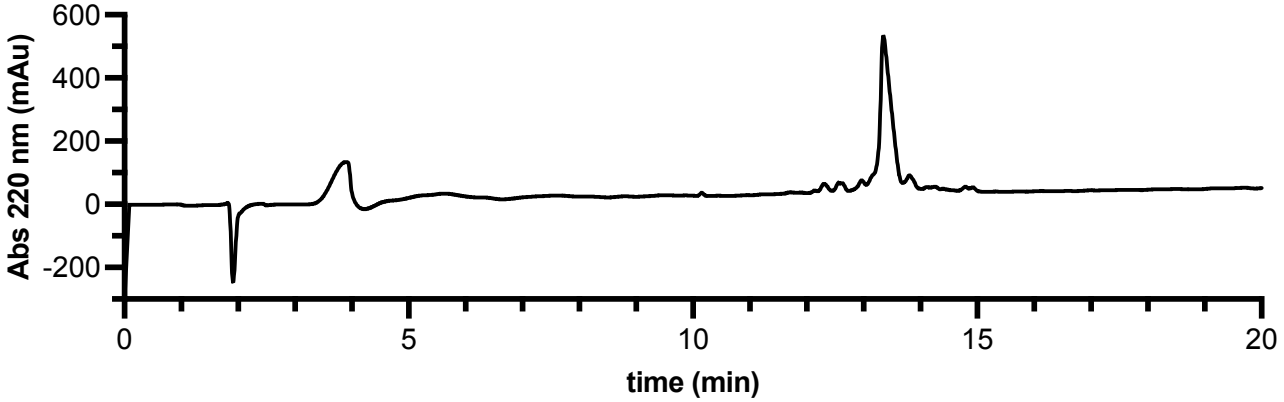
Minimum inhibitory concentrations (MICs) were quantified in broth-microdilution assays according to CLSI guidelines,[5] using a starting cell density of ~5×10^5 CFU/mL in cation-adjusted Mueller-Hinton II broth. Stock solutions of peptides were prepared in water. Assay plates were incubated for 16 h in an air atmosphere at 37 °C. The MIC was defined as the minimum concentration required to completely inhibit cell growth compared to the untreated control. The bacteria were obtained from ATCC (*S. epidermidis* 51625 and *E. coli* 25922 were used).

CIRCULAR DICHROISM STUDIES

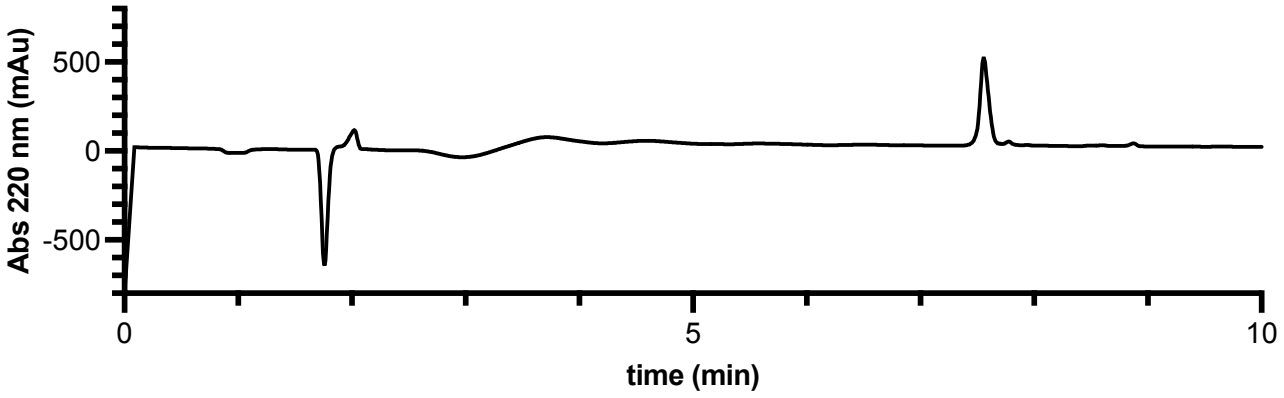
Stock solutions of the peptides were prepared by dissolving lyophilized powder in 20 mM sodium phosphate buffer (pH 6.9) to an approximate concentration of 40 μM for peptides 1–11 and 0.5 mg/mL in deionized water or 25 mM SDS for peptides 12 and 16. CD spectra were acquired using a JASCO J-1700 CD spectrometer in a 1 mm path length quartz cell with a 2 s detector integration time, 2 nm bandwidth, 0.5 nm data pitch, and a scan speed of 50 nm/min at 25 °C or the temperature reported for the thermal denaturation experiments. All ellipticity values are reported as the mean across 5 scans. Mean residue ellipticity at a given wavelength (MRE; deg cm^2 $dmol^{-1}$ $residue^{-1}$) was calculated based on the equation $MRE = \theta/(10 \times b \times C \times n)$, where θ is the ellipticity (mdeg), b is the path length (cm), C is the concentration of peptide (mol L^{-1}), and n is the total number of residues.

ANALYTICAL RP-HPLC SPECTRA

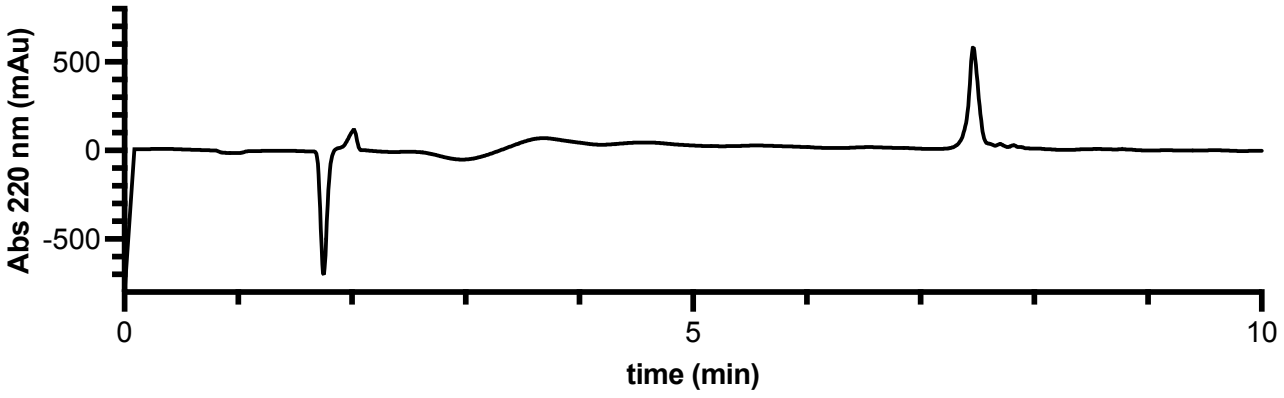
Peptide 1: Peptide 1 was obtained as a white powder (47 mg, 20%) after RP-HPLC purification using a linear gradient of 1–20% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 13.5 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{90}H_{151}N_{30}O_{33}$ 2180.1054 , found 2180.1053.



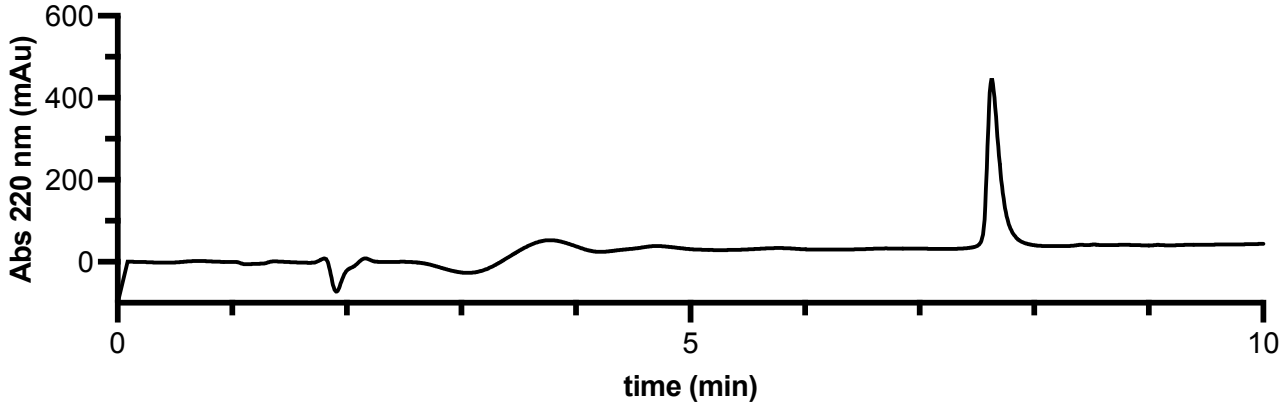
Peptide 2: Peptide 2 was obtained as a white powder (14 mg, 6%) after RP-HPLC purification using a linear gradient of 1–20% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 7.4 min. HRMS (ESI-TOF) *m/z* $[M + 3H]^+$ calcd for $C_{90}H_{154}N_{31}O_{33}$ 732.3770, found 732.3890.



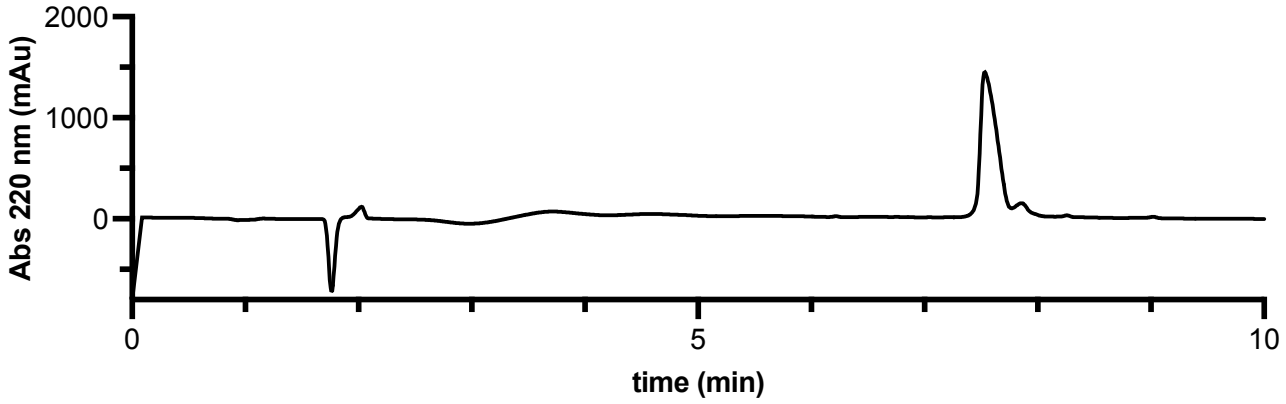
Peptide 3: Peptide 3 was obtained as a white powder (17 mg, 7%) after RP-HPLC purification using a linear gradient of 1–20% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 7.5 min. HRMS (ESI-TOF) *m/z* $[M + 2H]^+$ calcd for $C_{90}H_{153}N_{31}O_{33}$ 1098.0618, found 1098.0600.



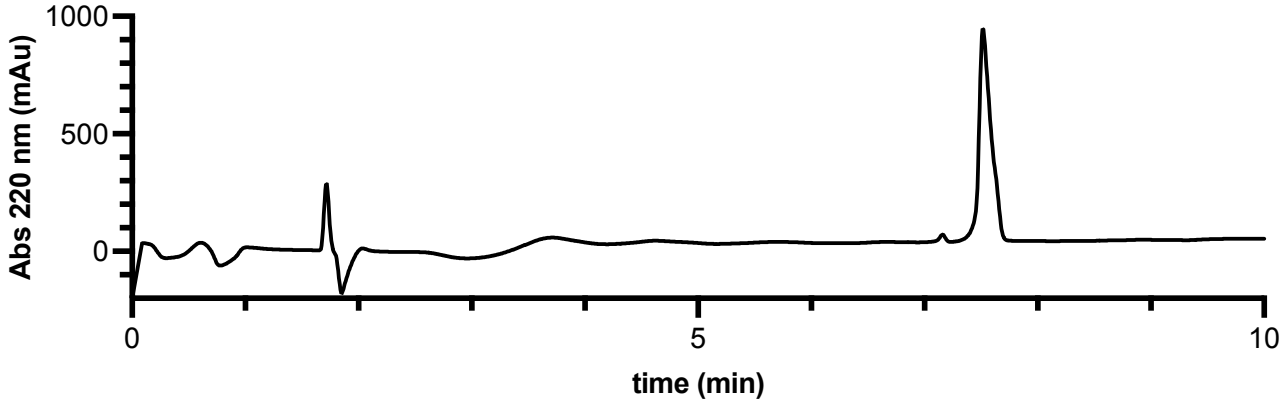
Peptide 4: Peptide 4 was obtained as a white powder (20 mg, 8%) after RP-HPLC purification using a linear gradient of 1–20% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 7.7 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{90}H_{152}N_{31}O_{33}$ 2195.1163, found 2195.1171.



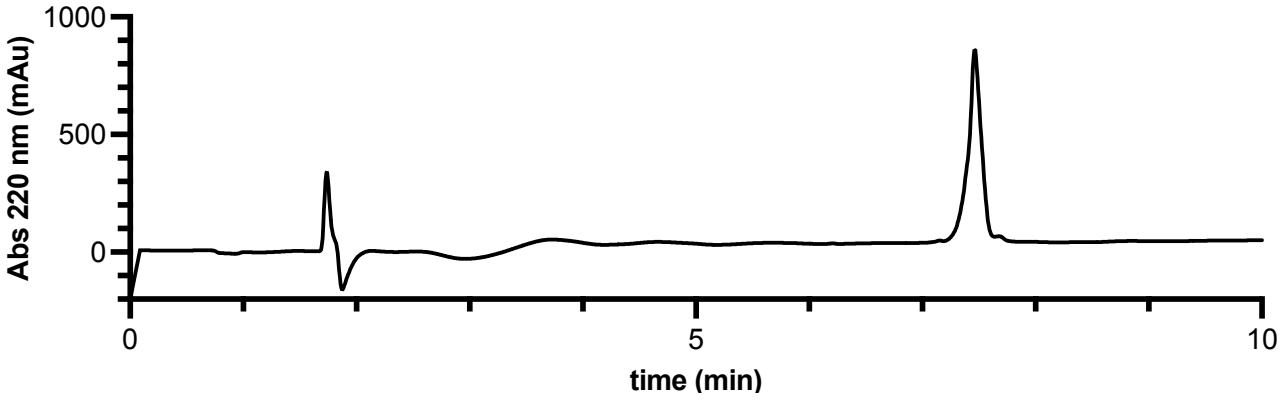
Peptide 5: Peptide 5 was obtained as a white powder (9.4 mg, 4%) after RP-HPLC purification using a linear gradient of 1–20% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 7.6 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{90}H_{152}N_{31}O_{33}$ 2195.1163, found 2195.1165.



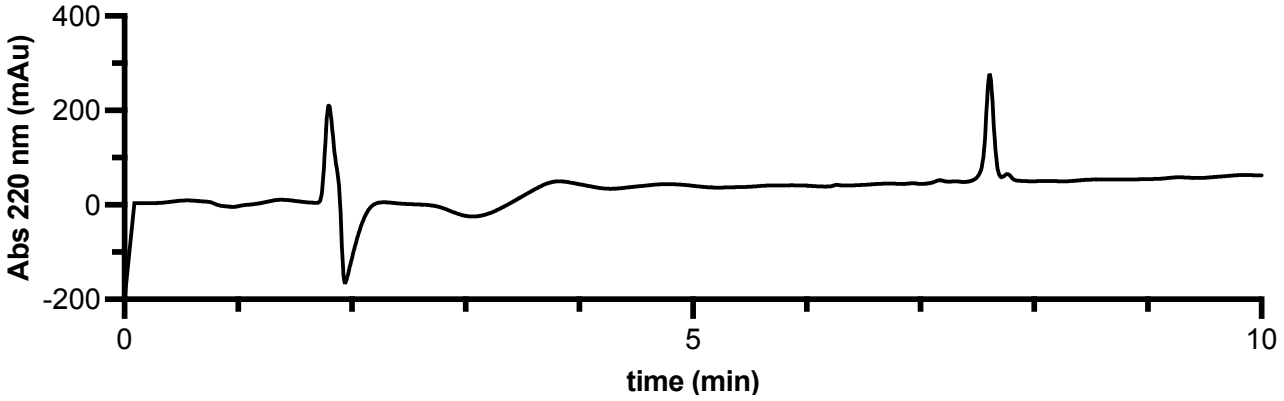
Peptide 6: Peptide 6 was obtained as a white powder (5 mg, 2%) after RP-HPLC purification using a linear gradient of 1–20% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 7.5 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{90}H_{152}N_{31}O_{33}$ 2195.1163, found 2195.1183.



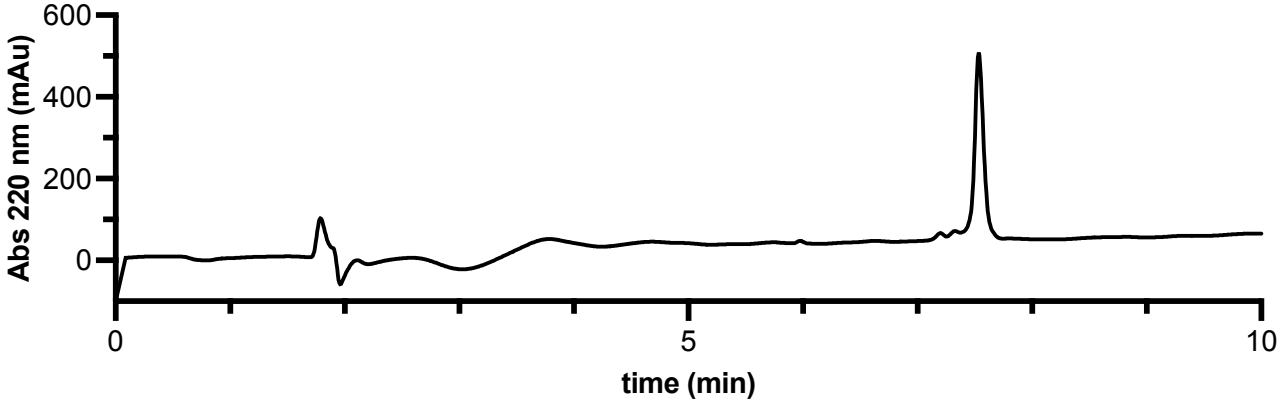
Peptide 7: Peptide 7 was obtained as a white powder (10 mg, 4%) after RP-HPLC purification using a linear gradient of 1–20% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 7.4 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{90}H_{152}N_{31}O_{33}$ 2195.1163, found 2195.1165.



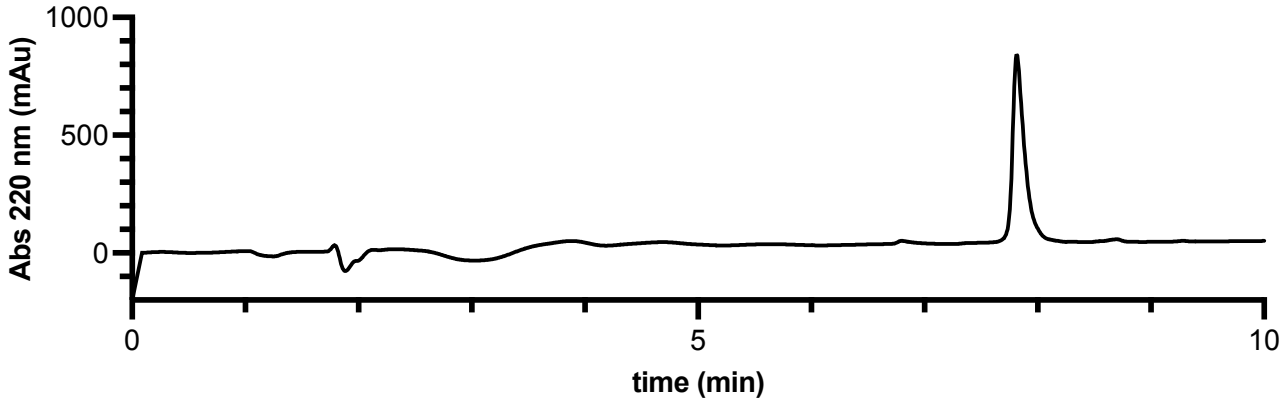
Peptide 8: The reaction went to completion after the addition of 1.5 equiv of thioester **S11** to a solution of crude **S9**. The crude reaction mixture was centrifuged, and the supernatant was purified by RP-HPLC using a 1–20% MeCN/H_2O gradient (with 0.1% formic acid) t_R = 7.6 min. The pure peptide was obtained in 27% isolated yield based on initial resin loading of **S9**. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{90}H_{151}N_{30}O_{34}$ 2196.1003, found 2196.1007.



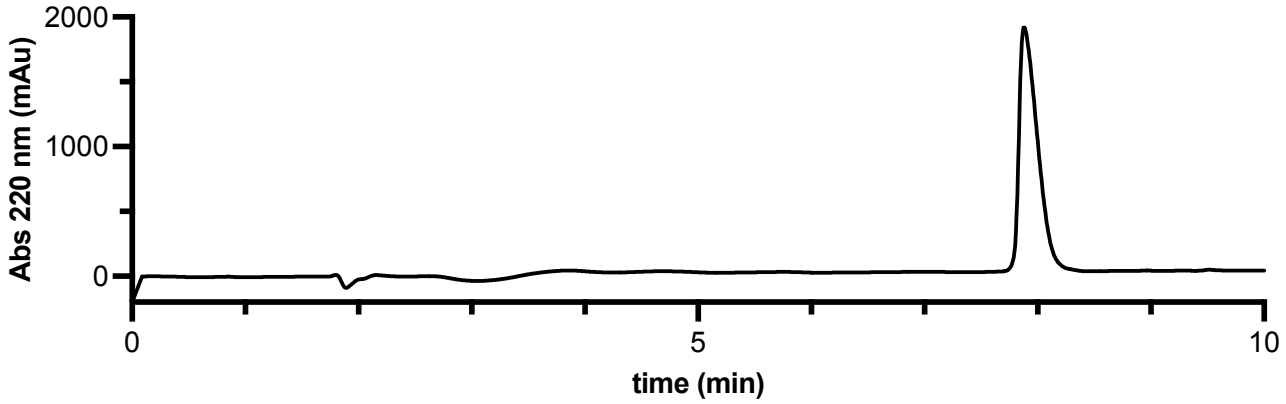
Peptide 9: The reaction went to completion after the addition of 1.3 equiv of thioester **S12** to a solution of crude **S10**. The crude reaction mixture was centrifuged, and the supernatant was purified by RP-HPLC using a 1–20% MeCN/H_2O gradient (with 0.1% formic acid) t_R = 7.5 min. The pure peptide was obtained in 23% isolated yield based on initial resin loading of **S10**. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{90}H_{151}N_{30}O_{34}$ 2196.1003, found 2196.0993.



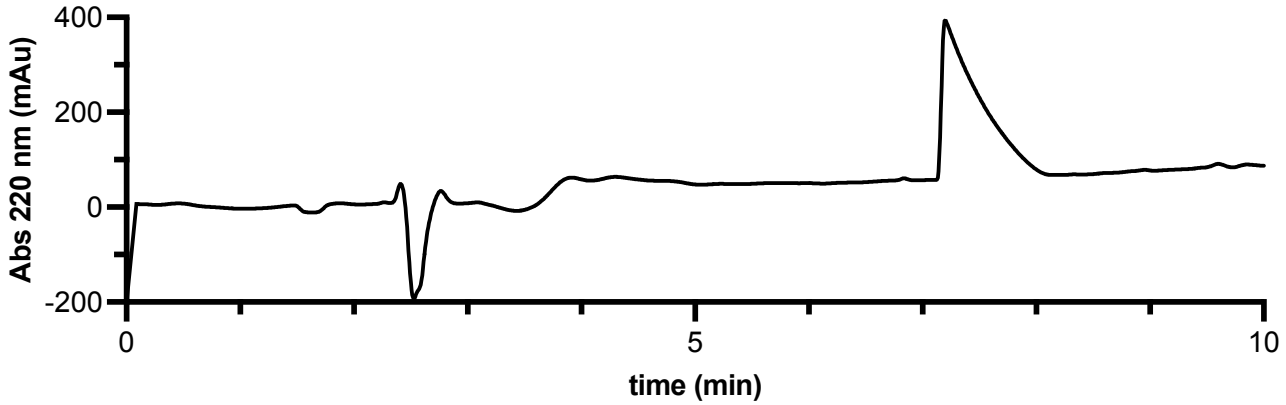
Peptide 10: Peptide 10 was obtained as a white powder (24 mg, 10%) after RP-HPLC purification using a linear gradient of 1–25% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 7.8 min. HRMS (ESI-TOF) *m/z* $[M + 3H]^+$ calcd for $C_{91}H_{155}N_{30}O_{33}$ 732.0452, found 732.0589.



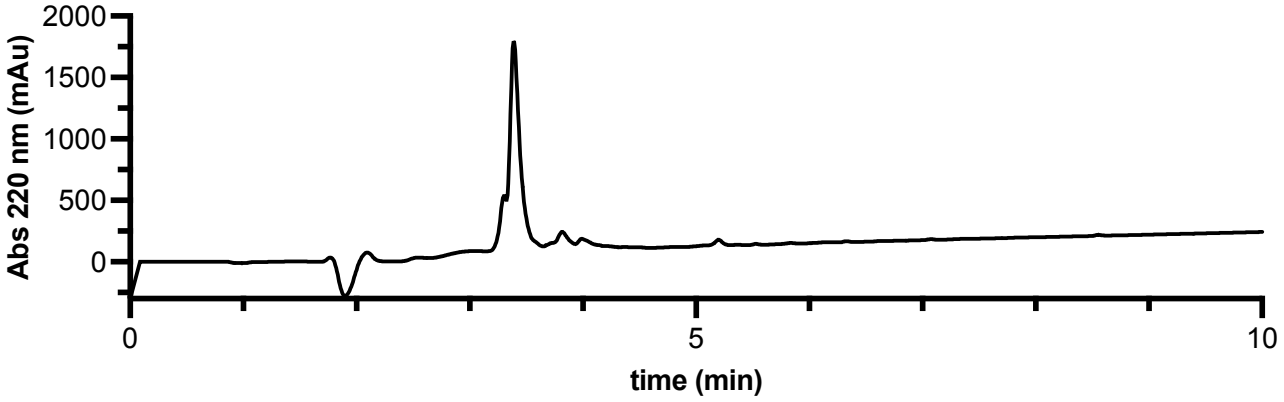
Peptide 11: Peptide 11 was obtained as a white powder (22 mg, 9%) after RP-HPLC purification using a linear gradient of 1–25% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 7.9 min. HRMS (ESI-TOF) *m/z* $[M + 3H]^+$ calcd for $C_{91}H_{155}N_{30}O_{33}$ 732.0452, found 732.0605.



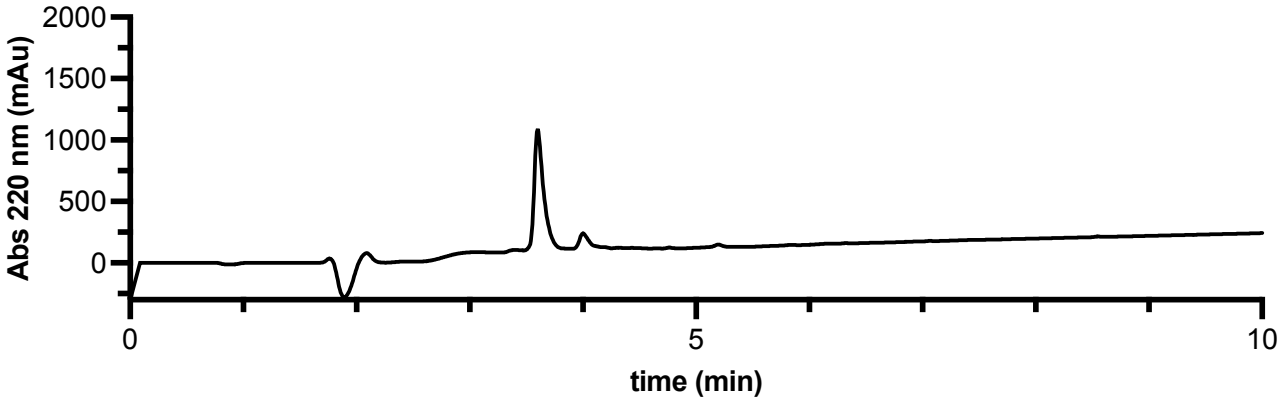
Peptide 12: Peptide 12 was obtained as a white powder (35 mg, 29%) after RP-HPLC purification using a linear gradient of 1–20% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 7.2 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{48}H_{96}N_{13}O_8$ 982.7499, found 982.7506.



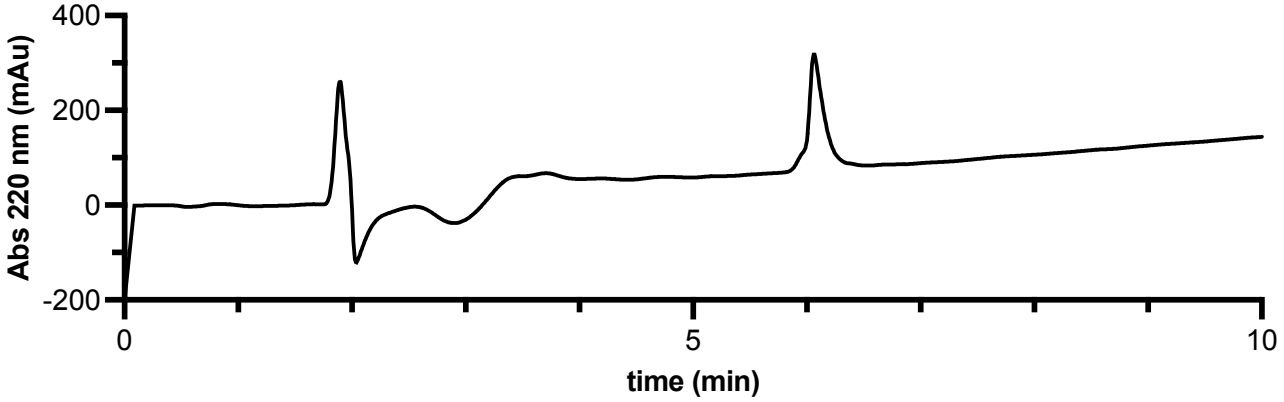
Peptide 13: Peptide 13 was obtained as a white powder (10 mg, 16%) after RP-HPLC purification using a linear gradient of 1–20% MeCN in H_2O (mobile phases modified with 0.1% formic acid). Analytical LC was taken at 5–95% MeCN in H_2O t_R = 3.4 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{48}H_{97}N_{14}O_8$ 997.7608, found 997.7587.



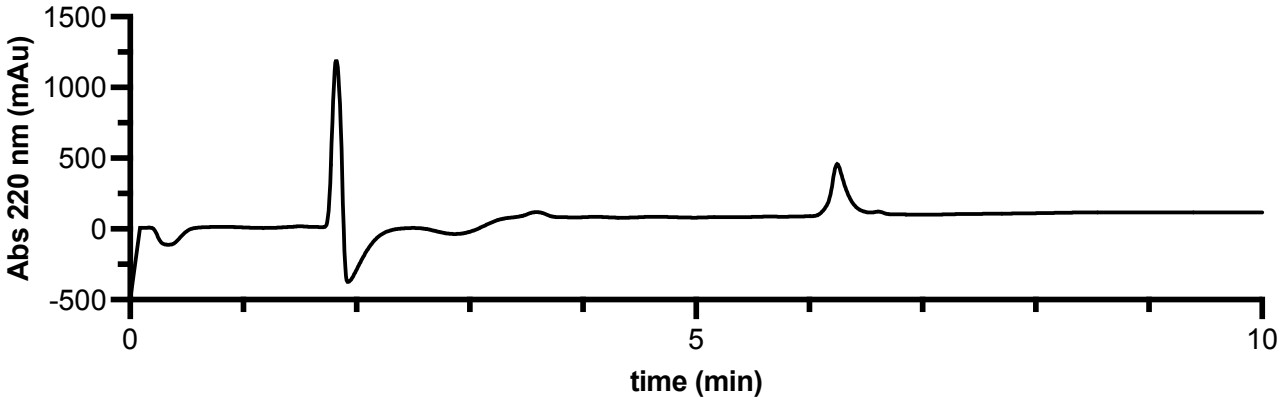
Peptide 14: Peptide 14 was obtained as a white powder (7 mg, 10%) after RP-HPLC purification using a linear gradient of 1–20% MeCN in H_2O (mobile phases modified with 0.1% formic acid). Analytical LC was taken at 5–95% MeCN in H_2O t_R = 3.6 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{48}H_{98}N_{15}O_8$ 1027.7717, found 1027.7763.



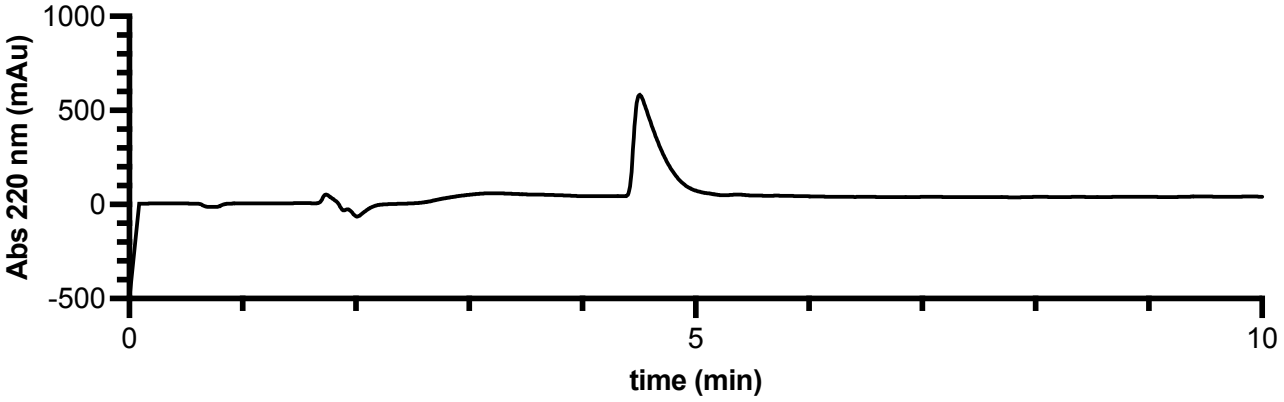
Peptide 15: Peptide 15 was obtained as a white powder (2.5 mg, 4%) after RP-HPLC purification using a linear gradient of 1–40% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 6.1 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{48}H_{99}N_{16}O_8$ 1027.7826, found 1027.7804.



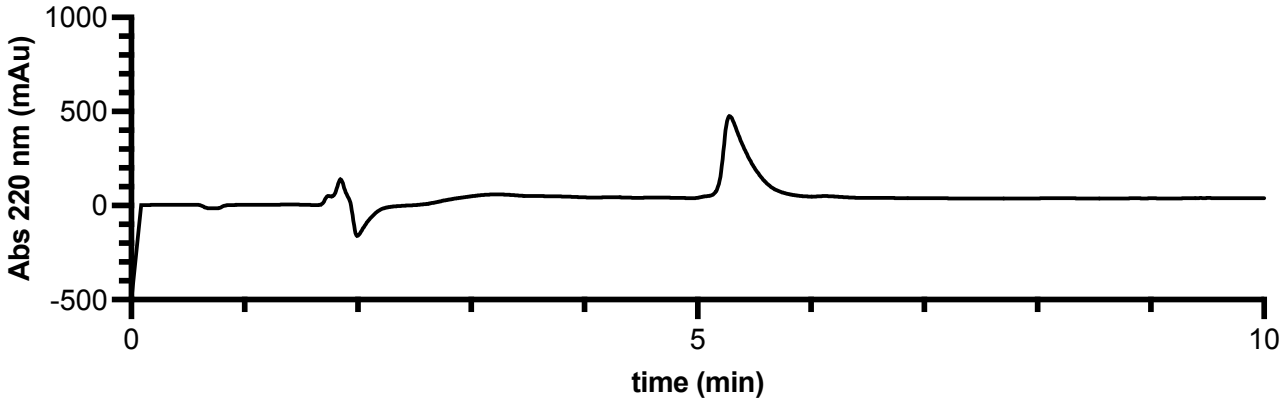
Peptide 16: Peptide 16 was obtained as a white powder (17 mg, 13%) after RP-HPLC purification using a linear gradient of 1–40% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 6.2 min. HRMS (ESI-TOF) *m/z* $[M + 2H]^+$ calcd for $C_{48}H_{100}N_{17}O_8$ 521.9004, found 521.9037.



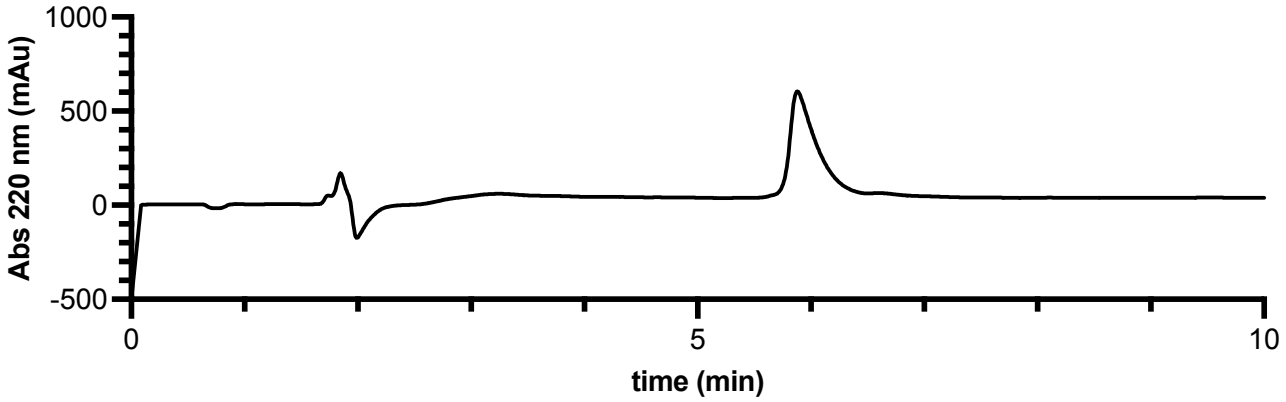
Peptide 17: Peptide 17 was obtained as a white powder (43 mg, 35%) after RP-HPLC purification using a linear gradient of 5–40% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 4.7 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{49}H_{98}N_{13}O_8$ 996.7656, found 996.7644.



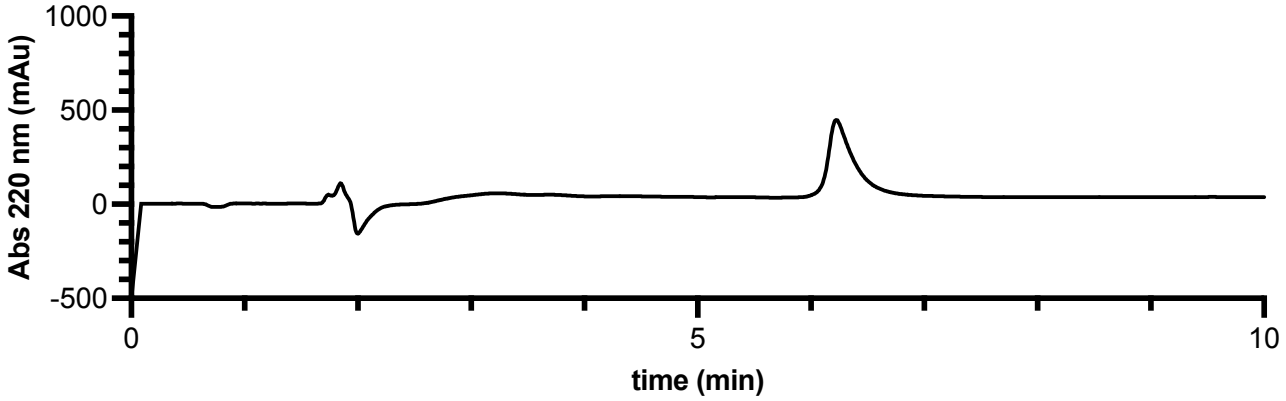
Peptide 18: Peptide 18 was obtained as a white powder (37 mg, 30%) after RP-HPLC purification using a linear gradient of 5–40% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 5.3 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{50}H_{100}N_{13}O_8$ 1010.7812, found 1010.7835.



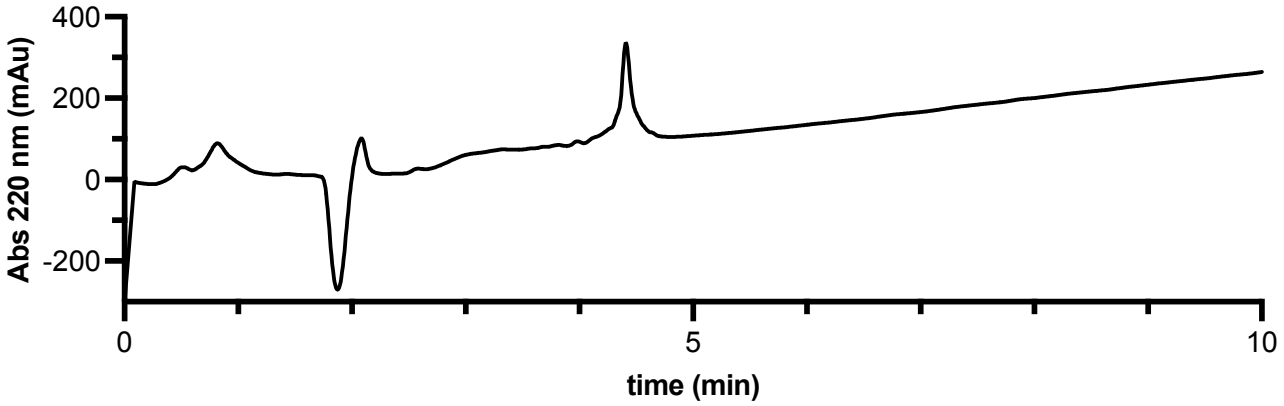
Peptide 19: Peptide 19 was obtained as a white powder (36 mg, 29%) after RP-HPLC purification using a linear gradient of 5–40% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 5.9 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{51}H_{102}N_{13}O_8$ 1024.7969, found 1024.7924.



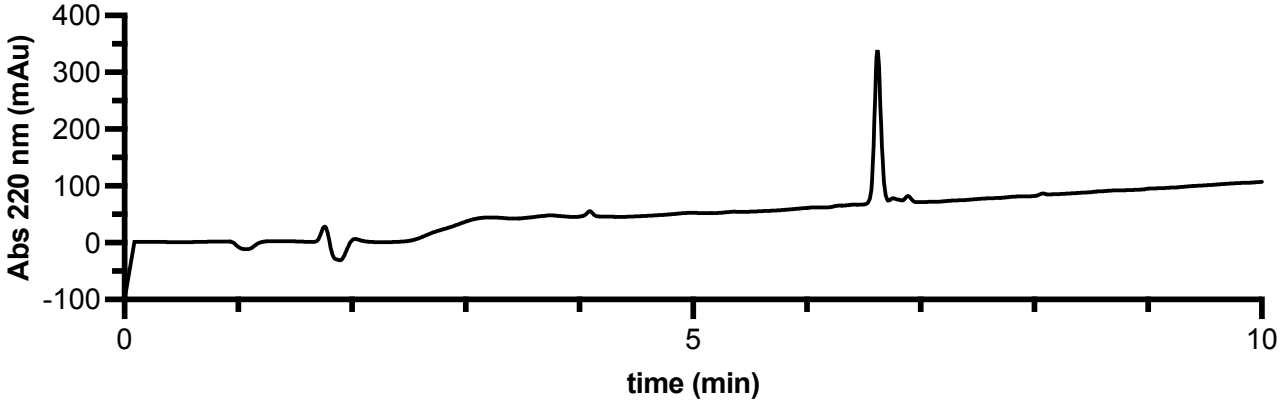
Peptide 20: Peptide 20 was obtained as a white powder (34 mg, 27%) after RP-HPLC purification using a linear gradient of 5–40% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 6.2 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{52}H_{104}N_{13}O_8$ 1038.8125, found 1038.8078.



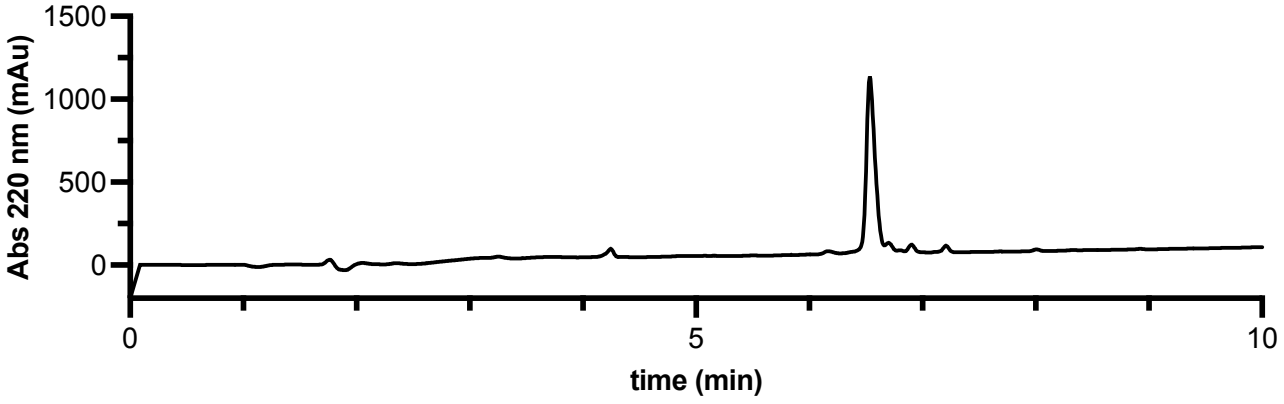
Peptide 21: Peptide 21 was obtained as a white powder (11 mg, 11%) after RP-HPLC purification using a linear gradient of a 5–60% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 4.4 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{48}H_{95}N_{13}O_{12}$ 1046.3670, found 1046.7376.



H_2N-VSADPSRVA-MPAA (S11). Thioester S11 was obtained as a white powder (58% two-step yield based on initial resin loading) after RP-HPLC purification using a linear gradient of 5–60% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 6.6 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{45}H_{71}N_{12}O_{15}S$ 1051.4877, found 1051.4883.



H_2N-VSADPSRVAA-MPAA (S12). Thioester S12 was obtained as a white powder (35% two-step yield based on initial resin loading) after RP-HPLC purification using a linear gradient of 5–60% MeCN in H_2O (mobile phases modified with 0.1% formic acid) t_R = 6.5 min. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{48}H_{76}N_{13}O_{16}S$ 1122.5248, found 1122.5284.



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