Supporting Information

Secondary Amino Alcohols: Traceless Cleavable Linkers for Use in Affinity Capture and Release

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Abstract: Capture and release of peptides is often a critical operation in the pathway to discovering materials with novel functions. However, the best methods for efficient capture impede facile release. To overcome this challenge, we report secondary amino alcohol-based linkers for release of peptides after capture. These amino alcohols are based on serine (seramox) or isoserine (isoseramox) and can be incorporated into peptides during solid-phase peptide synthesis via reductive amination. Both linkers are quantitatively cleaved within minutes under NaIO₄ treatment. Cleavage of isoseramox produced a native peptide N-terminus. This linker also showed broad substrate compatibility: incorporation into a synthetic peptide library resulted in the identification of all sequences by nanoLC-MS/MS. The linkers are cell compatible; a cell-penetrating peptide that contained this linker was efficiently captured and identified after uptake into cells. These findings suggest secondary amino alcohol-based linkers might be suitable tools for peptide discovery platforms.

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Table of Contents

1. Supplementary figures .................................................................................................................. 3
2. General information ........................................................................................................................ 9
3. Synthesis of linkers ........................................................................................................................ 10
   a. Synthesis of the diol cleavable linker monomer ........................................................................ 10
   b. Synthesis of the isoserine monomer ......................................................................................... 10
   c. Synthesis of the dapamox monomer ........................................................................................ 12
   d. NMR spectra ............................................................................................................................. 13
4. Peptide synthesis ............................................................................................................................ 18
   a. General procedure for manual solid-phase peptide synthesis (SPPS) ................................... 18
   b. General procedure for semi-automated fast-flow solid-phase peptide synthesis (FF-SPPS) ................................................................................................................................. 18
   c. Procedure for split-and-pool peptide synthesis ................................................................... 18
   d. On-resin reductive aminations to incorporate cleavable linkers ............................................. 18
   e. On-resin incorporation of fluorophore onto peptides ............................................................ 18
   f. On resin removal of the isoseramox TBS protecting group .................................................. 18
   g. General procedure for cleavage .............................................................................................. 18
   h. General procedure for solid-phase extraction (SPE) purification .......................................... 18
   i. General procedure for Biotage purification .......................................................................... 18
   j. General procedure for HPLC purification ............................................................................. 19
   k. LC-MS spectra ......................................................................................................................... 19
5. Oxidative cleavage of linkers in a model peptide ....................................................................... 25
   a. Procedure for cleavage of peptides at 500 µM ..................................................................... 25
   b. Procedure for cleavage of 4b at 50 µM .................................................................................. 26
   c. Procedure for cleavage of 4c at 50 µM .................................................................................. 26
   d. Procedure for cleavage of peptides 4b and 4c at 10 µM ......................................................... 27
   e. Early procedure for cleavage of peptide 4d ........................................................................... 28
   f. Procedure for test cleavage of peptide 13 .............................................................................. 28
6. Recovery of a peptide library using (iso)seramox linkers .......................................................... 29
7. Study of complex peptide and protein substrates .................................................................... 38
   a. Structural analysis of miniprotein Z33 upon treatment with NaIO₄ ...................................... 38
   b. Comparison of seramox release method to SDS denaturing conditions after streptavidin capture ................................................................................................................................. 38
8. Recovery of penetratin from live cells ....................................................................................... 38
   a. Procedure for test pulldown of peptide 13 ........................................................................... 38
   b. Cell uptake and pulldown ....................................................................................................... 39
9. Fluorescent confocal imaging of peptide 15 in HeLa cells ....................................................... 40
10. Cell viability studies with periodate treatment ......................................................................... 41
11. Author Contributions .................................................................................................................. 41
12. References ................................................................................................................................. 41
1. Supplementary figures

**Figure S1.** Assessment of efficiency of reductive aminations of (iso)seramox linkers on resin. Both the (a) seramox linker and the (b) isoseramox linker were incorporated with >90% crude conversion by LC-MS. The primary byproducts observed were truncation of the peptide through incomplete reductive amination of either 1 or 8 followed by Boc-protection of the N-terminus (S1 and S4), forming S2 and S5 respectively.

**Figure S2.** Stability of model peptides (a) 4b and (b) 4c with oxidatively cleaved linkers in 1X PBS buffer (pH 7.5).
Figure S3. Cleavage of (a) seramox- and (b) isoseramox-containing model peptides at 50 µM concentration. Full conversion was observed after 1 h.
Figure S4. Cleavage of (a) seramox and (b) isoseramox-containing model peptides at 10 µM. Full conversion was observed after 3 h.
Figure S5. Studies into Dap-based diamine cleavable linkers (dapamox). The dapamox linker is derived from Boc-Dap(Fmoc)-OH (2,3-diaminopropionic Acid). The cleavage is much slower for diamine-based 4d than the (iso)seramox linkers (4b, 4c), and appears even slower than the diol linker (4a).

Figure S6. Periodate treatment does not disrupt a-helical structure. CD spectra of the helix-helix bundle miniprotein Z33 were recorded before (blue line) and after (orange line) periodate treatment (4 mM NaIO₄, 30 min). The treatment does not cause detectable structural alterations of the miniprotein.
Figure S7. Test cleavage of cell-penetrating peptide 13. Quantitative cleavage was observed after 10 minutes. Oxidative cleavage appears to be faster than Met oxidation, as only ~30% of 15 was observed. A mixture of aldehyde and oxime products of the other fragment are isolated under these quenching conditions.

Figure S8. Fluorescent imaging of peptide 15. Peptide 15 is diffuse in the cytosol of HeLa cells after a 1.5 h treatment at 10 µM. In addition to puncta, fluorescence is also evident in both the cytosol and nucleus of the HeLa cells.
Figure S9. LDH release from HeLa cells after NaIO₄ treatment. Periodate was not toxic to HeLa cells after 24-hour treatment at millimolar concentrations. Toxicity was measured by the release of the cytosolic lactate dehydrogenase (LDH) protein into the cell treatment medium using the Promega CytoTox 96™ Assay Kit and normalized to the LDH released from fully lysed cells.

8-hour treatment

24-hour treatment
2. General Information

**Chemicals:** Unless otherwise noted, all chemicals were obtained from commercial sources and used as received without further purification. Tetrahydrofuran (THF) and N,N-diisopropylethylamine (DIEA) were obtained from a Seca Solvent Purification System by Pure Process Technology.

**LC-MS analysis:** LC-MS chromatograms and associated mass spectra were acquired using an Agilent 6550 ESI-QToF mass spectrometer or an Agilent 6520 ESI-QToF mass spectrometer. Mobile phases used for LC-MS analysis are solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The following LC-MS methods were used:

- **Method A:** On 6550 MS; C4 Phenomenex Jupiter column (1 x 150 mm, 5 µm); LC conditions: 1% B from 0–2 minutes, linear ramp from 5% to 61% B from 2–12 minutes, 0.1 mL/min flow rate.
- **Method B:** On 6520 MS; Zorbax 300SB-C3 column (2.1 x 150 mm, 5 µm); LC conditions: 1% B from 0–2 min, linear ramp from 5% to 61% B from 2–12 min, linear ramp from 61% to 90% 11-12 min, 0.8 mL/min flow rate.
- **Method C:** On 6550 MS; C4 Phenomenex Biozen column (2.1 x 150 mm, 3.6 µM); LC conditions: 1% B from 0–2 minutes, linear ramp from 5% to 61% B from 2–12 minutes, 0.5 mL/min flow rate.

**NMR analysis:** NMR spectra were acquired on 400 or 500 MHz Bruker spectrometers at ambient temperature. Samples were prepared in chloroform-d (CDCl₃). ¹H NMR data are reported as chemical shifts with multiplicity, coupling constants (J) in Hz, and integrations. Proton chemical shifts are reported in ppm (δ) and referenced to residual solvent (CHCl₃, δ 7.26 ppm).¹ Multiplicity is reported as follows: singlet (s), broad singlet (bs), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), multiplet (m), and overlapping multiplets (comp). ¹³C NMR spectra were recorded on 500 (126) MHz Bruker spectrometers with complete proton decoupling at ambient temperature, unless otherwise noted. Carbon chemical shifts are reported in ppm (δ) and referenced to solvent (CDCl₃, δ 77.16 ppm).¹

**Nano-LC/MSMS analysis of peptide libraries:** Nano-LC/MSMS analysis of library bead mixtures was performed on Thermo Fisher Orbitrap Fusion Lumos Tribrid Mass Spectrometer coupled to Thermo Fisher EASY-nLC 1200 System equipped with Acclaim PepMap RSILC C18 column (250 mm x 75 µm ID, 2 µm 100Å silica). The standard nano-LC method was run at 40 °C and a flow rate of 300 nL/min with the following gradient: 1% of 80% acetonitrile in water with 0.1% formic acid added (solvent B') in water containing 2% methanol and 0.1% FA (solvent A') ramping linearly to 5% B' in A' over 2 minutes, followed by 5-61% B' in A' ramping linearly over 118 minutes, followed by 61-99% B' in A' ramping linearly over 5 minutes and finally 99% B' in A' for 5 minutes. MSMS acquisition over the course of the method duration was performed in a data-dependent style (Top N=15, z=2-10, intensity threshold = 10⁵) with a dynamic precursor exclusion for 30 seconds after each scan. CID and HCD fragmentation 9 spectra were acquired for every selected precursor ion. Orbitrap was used as a detection method for both primary (resolution=120000) and secondary (resolution=30000) mass spectra.

**Purification:** Normal-phase column chromatography was performed with a Biotage Selekt flash purification system, equipped with 25 g or 100 g Biotage Sfär Silica HC-D 20 µm columns, using an appropriate gradient of EtOAc/Hexanes. Reversed-phase column chromatography was performed with a Biotage Selekt flash purification system equipped with either 10 g Biotage SNAP Bio C18 20 µm columns or 25 g Biotage Sfär Bio C18 D 20 µm columns with an appropriate gradient of MeCN/H₂O (0.1% TFA). Reversed-phase preparative HPLC was performed using an Agilent mass directed purification system (1260 infinity LC and 6130 single quad MS), equipped with an Agilent Zorbax SB C3 column (9.4 x 250 mm, 5 µm), using an appropriate gradient of MeCN/H₂O (0.1% TFA).
3. Synthesis of linkers

a. Synthesis of the diol monomer

Scheme S1. Synthesis of diol linker precursor.

The synthesis of diol linker S9 was performed as previously reported.  

b. Synthesis of the isoserine monomer

Scheme S2. Synthesis of isoseramox linker precursor.

Fmoc-L-isoserine (6) was synthesized as previously reported, though it is also commercially available.

(9H-fluoren-9-yl)methyl (S)-(2-hydroxy-3-(methoxy(methyl)amino)-3-oxopropyl)carbamate (S10) was synthesized through modification of a previously reported procedure. A 250 mL round bottom flask was charged with 6 (1.0 g, 3.3 mmol, 1.0 equiv), dimethylhydroxylamine hydrochloride (1.3 g, 13 mmol, 4.0 equiv), and PyAOP (3.5 g, 6.7 mmol, 2.0 equiv). After addition of DMF (52 mL) and DIEA (1.7 mL, 10 mmol, 3.0 equiv), the reaction was stirred at room temperature for 18 h. After completion, the solution was diluted with EtOAc (500 mL), and then washed with 10% aq. HCl (250 mL x 1), and 5% aq. LiCl (250 mL x 2). The organics were dried over Na2SO4, filtered, and concentrated. The crude product was purified by automatic, normal-phase chromatography, eluting with the gradient of EtOAc/Hex shown below to yield pure product. Presumed isomerization of the amide bond was observed via NMR.

Yield: 1.2 g white solid, 99%

1H NMR (500 MHz, CDCl3) δ 7.76 (d, J = 7.5 Hz, 2H), 7.59 (dd, J = 7.5, 5.0 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.4 Hz, 2H), 5.27 (bs, 0.9H), 5.06 (bs, 0.1H)), 4.52 (bs, 1H), 4.43 – 4.29 (m, 2H), 4.22 (t, J = 7.2 Hz, 1H), 3.77 (s, 3H), 3.66 – 3.55 (m, 1H), 3.53 – 3.43 (m, 1H), 3.25 (s, 3H).

13C NMR (126 MHz, CDCl3) δ 172.5, 156.7, 144.1, 144.0, 141.4, 127.8, 127.2, 125.3, 125.2, 120.1, 68.4, 67.1, 61.7, 47.3, 44.3, 32.9.

HRMS (ESI-QToF) m/z: [M+H]+ calcd for C20H23N2O5 371.1607, found 371.1608.

IR (cm⁻¹, neat): 3333, 2940, 1706, 1652, 1524, 1448, 1370, 1242, 1147, 1122, 1079, 989, 759, 737.

TLC Rf (1:1 Hex/EtOAc) 0.24, visualized with UV light.

Biotage Trace:
(9H-fluoren-9-yl)methyl (S)-(2-((tert-butyl(dimethyl)silyl)oxy)-3-(methoxy(methyl)amino)-3-oxopropyl)carbamate (8) was synthesized through modification of a previously reported procedure. A 50 mL round bottom flask was charged with Weinreb amide 7 (0.26 g, 0.52 mmol, 1.0 equiv), followed by 6.0 mL THF. The solution was cooled to −78 °C and placed under an N₂ atmosphere. A 1 M solution of LiAlH₄ in THF (0.66 mL, 0.66 mmol, 1.3 equiv) was added dropwise to the solution. The reaction was stirred while allowing to warm to room temperature, at which EtOAc (50 mL) was added. The organics were washed with 1 M HCl (30 mL x 2) and sat. aq. NaCl (30 mL x 2). At this stage, the crude product could be used in subsequent reductive aminations without further purification (92% crude yield). The primary byproducts identified were starting aldehyde and minor amounts of over-reduction to the alcohol. Or, the organic layer could be dried over Na₂SO₄, filtered, and concentrated. The crude product was purified by automatic, normal-phase chromatography, eluting with the gradient of EIOAc/Hex shown below to yield pure product. Presumed isomerization of the amide bond was observed via NMR.

**Yield:** 0.16 g crude yield (92%); 80 mg pure, colorless oil, 46%.

**¹H NMR** (500 MHz, CDCl₃) δ 7.80 (d, J = 7.6 Hz, 2H), 7.62 – 7.53 (m, 2H), 7.47 – 7.36 (m, 2H), 7.35 – 7.28 (m, 2H), 5.04 (bs, 0.8H), 4.76 (bs, 0.2H), 4.51 (bs, 0.3H), 4.39 (d, J = 7.1 Hz, 1H), 4.21 (t, J = 7.0 Hz, 1H), 4.16 (t, J = 5.7 Hz, 0.8H), 3.85 (bs, 0.2H), 3.48 (t, J = 6.1 Hz, 1H), 3.29 (s, 0.2H), 0.94 (s, 9H), 0.12 (d, J = 16.3 Hz, 6H).

**¹³C NMR** (126 MHz, CDCl₃) δ 202.1, 156.4, 144.0, 143.9, 141.5, 127.9, 127.2 (2), 125.2, 125.1, 120.1, 76.5, 67.1, 47.3, 42.8, 29.8, 25.8, 18.3, −4.6, −4.9.

**HRMS** (ESI-QToF) m/z: [M–TBS+2H]+ calcd for C₉₄H₇₅NO₄Si 426.2101, found 426.2393.

**IR** (cm⁻¹, neat): 3446, 3350, 2928, 2965, 1703, 1515, 1449, 1251, 1103, 1005, 835, 777, 757, 738.

**TLC** Rf (2:1 Hex/EtOAc) 0.50, visualized with UV light.
c. Synthesis of the dapamox cleavable linker monomer

(9H-fluoren-9-yl)methyl tert-butyl (3-(methoxy(methyl)amino)-3-oxopropane-1,2-diyi)(S)-dicarbamate (S12). Boc-Dap(Fmoc)-OH (S11, 1.00 g, 2.3 mmol, 1.0 equiv), PyAOP (2.4 mg, 4.6 mmol, 2.0 equiv) and N,N-Dimethyloxyamine hydrochloride (0.91 g, 9.4 mmol, 4.0 equiv) were dissolved in DMF (50 mL). DIEA (1.2 mL, 7.0 mmol, 3 equiv) was added and the solution was stirred overnight at room temperature. EtOAc (500 mL) was added to the solution, which was subsequently washed with 1 M HCl (3 x 100 mL) and with 5% LiCl (1 x 100 mL). The organic layer was dried over Na2SO4, filtered, and concentrated. The crude material was purified by automatic, normal-phase chromatography, eluting with the gradient of EtOAc/Hex shown below to yield mostly pure product that was utilized in the next step without further purification.

Crude Yield: 923 mg (85 %), white solid. Presumed isomerization of the amide bond was observed via NMR.

1H NMR (500 MHz, CDCl3) δ 7.76 (d, J = 7.5 Hz, 2H), 7.59 (dd, J = 7.4, 4.0 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.4 Hz, 2H), 5.54 (bs, 1H), 5.24 (bs, 1H), 4.81 (bs, 1H), 4.35 (d, J = 6.8 Hz, 2H), 4.21 (t, J = 7.0 Hz, 1H), 3.79 (s, 3H), 3.61–3.46 (comp, 2H), 3.21 (s, 3H), 1.45 (s, 9H).

MS (ESI-QToF) m/z: [M–Boc+2H]+ calculated for C22H23N3O4 371.1845, found 371.1765.

TLC Rf (1:1 Hex/EtOAc) 0.38, visualized with UV light.

Weinreb amide S12 (0.41 g, 0.87 mmol, 1.0 equiv) was dissolved in dry THF (10 mL), and the flask was placed under an N2 atmosphere and cooled to –78°C. A freshly prepared 1 M solution of LiAlH4 in THF (1.1 mL, 1.1 mmol, 1.3 equiv) was added dropwise to the cooled solution. The reaction was stirred for 2h at –78°C, monitoring conversion by TLC. Upon completion, the reaction was quenched by the addition of 1 M HCl (10 mL), then warmed to room temperature. EtOAc (50 mL) was added, and the layers were separated. The organics were further washed with 1 M HCl (2 x 30 mL) and with sat. aq. NaCl (2 x 30 mL). The organic layer was dried over Na2SO4, filtered, and concentrated to yield mostly pure product that was utilized in the next step without further purification. 

Crude Yield: 283 mg (79%) mostly pure yellow solid.

1H NMR (500 MHz, CDCl3) δ 9.63 (s, 1H), 7.76 (d, J = 7.5 Hz, 2H), 7.56 (d, J = 7.4 Hz, 2H), 7.41 (t, J = 7.4 Hz, 2H), 7.32 (t, J = 7.4 Hz, 2H), 5.55 (bs, 1H), 5.16 (bs, 1H), 4.44–4.37 (m, 2H), 4.24 (bs, 1H), 4.19 (t, J = 6.6 Hz, 1H), 3.77 – 3.59 (comp, 2H), 1.46 (s, 9H).

MS (ESI-QToF) m/z: [M–Boc+2H]+ calculated for C18H19N2O3 311.1396, found 311.1396.

TLC Rf (1:1 Hex/EtOAc) 0.59, visualized with UV light.
d. NMR Spectra
4. Peptide synthesis

a. **General procedure for manual solid-phase peptide synthesis (SPPS).** ChemMatrix® Rink amide resin (loading 0.49 mmol/g, typical scale: 200 mg, 0.1 mmol) was loaded into a fritted syringe (12 mL), swollen in DMF (5 mL) for 5 minutes and then drained. Each Nα-Fmoc protected amino acid (1.0 mmol, 10 equiv) was dissolved in DMF containing 0.39 M HATU (2.5 mL). Immediately before the coupling DIEA (500 µL, 30 equiv) was added to the mixture to activate the amino acid. This solution after 15 seconds was added to the resin and reacted for 10 min, with occasional stirring. After completion of the coupling step, the syringe was drained, and the resin was washed with DMF (3 x 10 mL). Fmoc deprotection was performed by addition of piperidine (20% v/v in DMF, 5 mL), to the resin (1 x 1 min + 1 x 5 min), followed by draining and washing the resin with DMF (5 x 10 mL). Side chain protection was as follows: Asn(Trt), Asp(O′Bu), Cys(Trt), Gln(Trt), Glu(O′Bu), His(Trt), Lys(Boc), Ser(Bu), Thr(Bu), Trp(Boc), Tyr(Bu).

b. **General procedure for semi-automated fast-flow solid-phase peptide synthesis (FF-SPPS).** ChemMatrix® Rink amide resin (loading 0.49 mmol/g, typical scale: 200 mg, 0.1 mmol) was loaded into a stainless steel flow reactor as previously reported. The reactor was connected to a stainless steel preheat loop, primed with DMF, and maintained at 90 °C in a water bath. Activated amino acid (0.4M) was prepared by adding DIEA (500 µL, 30 equiv) to a solution of 1 mmol Fmoc-protected amino acid in 0.38M HATU in DMF, taken up by syringe and delivered to the reactor at 6 mL/min via a syringe pump. The resin was washed with DMF (20 mL, 20 mL/min), deprotected with piperidine (20% v/v in DMF, 6.7 mL, 20 mL/min), and washed again with DMF, via an HPLC pump. This process was repeated cyclically until the completion of the synthesis. The resin was then washed 3 times with CH₃Cl and dried under vacuum.

c. **Procedure for split-and-pool peptide synthesis.** Coupling and Fmoc deprotection steps were performed according to the stoichiometries and procedures described in Section 4a. Monosized Tentagel beads (90 µm, loading 0.26 mmol/g, 500 mg) were loaded into a fritted syringe, swollen in DMF and then coupled to Fmoc-Rink Amide linker. For variable positions the resin was suspended in DMF divided in equal aliquots, coupled to the respective Fmoc amino acid and then pooled before Fmoc deprotection.

d. **On-resin reductive aminations to incorporate cleavable linkers.** Resin containing 25 µmol peptide was washed with CH₂Cl₂. Fmoc-Gly-aldehyde (45 µmol, 13 mg, 1.8 equiv) or Fmoc-isoSer(TBS)-aldehyde (125 µmol, 53 mg, 5 equiv), respectively, was dissolved in CH₂Cl₂/MeOH (1:1, 0.5 mL) + 2 % CH₂COOH and added to the resin. After 30 minutes NaBH₄CN (500 µmol, 31.5 mg, 20 equiv.) was dissolved in CH₂Cl₂/MeOH (1:1, 0.5 mL) + 2 % CH₂COOH and added to the resin. Reductive amination was left for 60 minutes with occasional stirring, followed by draining and washing the resin with CH₂Cl₂ (5 x 2 mL). The resulting free secondary amine was protected by treating the resin with a solution of Boc₂O (500 µmol, 109 mg, 20 equiv.) and DIEA (1 mmol, 163 µL, 40 equiv.) in CH₂Cl₂ (1 mL) for 1 h at room temperature.

e. **On-resin incorporation of TAMRA into peptides.** After Fmoc removal, resin containing 15 µmol isoseramox- penetratin was washed with DMF and combined with 5-Carboxytetramethylrhodamine (5-TAMRA) (120 µM, 52 mg, 8 equiv) dissolved in DMF containing 0.39 M PYAOP (0.3 mL). Immediately before coupling, the mixture was activated with DIEA (60 µL, 30 equiv). The activated 5-TAMRA was then combined with the peptide resin and left for 60 minutes and room temperature with occasional stirring. The resin was then washed 3 times with DMF and 3 times with CH₂Cl₂ and dried under vacuum.

f. **On-resin removal of the isoseramox TBS protecting group.** The resin containing the peptide with the TBS protected isoseramox linker was washed with THF and then incubated with TBAF (1M in THF, 1 mL for 50 µmol) for 45 minutes, followed by CH₂Cl₂ washes.

g. **General procedure for cleavage.** Upon completion of the peptide synthesis, the resin was treated with a cleavage cocktail containing 94% TFA, 2.5% water, 2.5% 1,2-ethanediol (EDT) and 1% trisopropylsilane (TIPS) (v/v) at room temperature for 2 h. The TFA volume was then reduced under N₂ stream and cold diethyl ether (−80 °C) was added to precipitate the peptide. The resulting suspension was centrifuged at 4000 rpm for 3 min and the liquid was discarded. After repeating this step twice more, the pellet was dissolved in millipore water + 0.1% TFA and lyophilized.

h. **General procedure for solid-phase extraction (SPE) purification.** Peptides were desalted using C18 SPE cartridges according to general procedures from Agilent Sample Prep Solutions Bond Elut.

i. **General procedure for Biotage purification.** Samples purified using the Biotage Selekt flash purification system were dissolved in 10 mL 95.5 H₂O/MeCN (0.1% TFA) and then loaded onto either a 10 g Biotage SNAP Bio C18 20 µm column or 25 g Biotage Sfär Bio C18 D 20 µm column. Eluents were identified via UV/Vis, scanning from 200–400 nm. Mobile phases used for purifications are solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The follow methods were utilized:

   **Method A:** On 10 g SNAP Column; gradient conditions: 5% B for 1 CV, linear ramp from 5% to 35% B for 15 CV, linear ramp from 35% to 88% for 1 CV, 88% B for 2 CV, 25 mL/min flow rate.

   **Method B:** On 25 g Sfär Column; gradient conditions: 5% B for 1 CV, linear ramp from 5% to 55% B for 15 CV, linear ramp from 35% to 88% for 1 CV, 88% B for 2 CV, 40 mL/min flow rate.
j. **General procedure for HPLC purification.** The crude peptides were dissolved in 1% aqueous acetonitrile containing 0.1% TFA, filtered through a 0.22 μm nylon filter and purified by mass-directed semi-preparative reverse-phase HPLC. Solvent A was water with 0.1% TFA and Solvent B was acetonitrile with 0.1% TFA. A linear gradient that increased at a rate of 0.5% B/min was used. Most of the peptides were purified on an Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 μm. Extremely hydrophilic peptides, such as the cell-penetrating peptide sequences, were purified on an Agilent Zorbax SB C18 column: 9.4 x 250 mm, 5 μm. Pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

k. **LC-MS spectra.**

H₂N-Y-diol-LAGV-CONH₂ (4a)

**Synthesis:** Synthesized using manual SPPS.
**Purification:** Purified using general SPE Procedure.
**HRMS** (ESI-QtoF) m/z: [M+H]^+ calculated for C_{29}H_{48}N_{7}O_{9} 638.3514, found 638.3600 (Method A).

H₂N-Y-seramox-LAGV-CONH₂ (4b)

**Synthesis:** Synthesized using manual SPPS.
**Purification:** Using general Biotage Method B
**HRMS** (ESI-QtoF) m/z: [M+H]^+ calculated for C_{30}H_{51}N_{8}O_{8} 651.3830, found 651.3844 (Method A).
Synthesis: Synthesized using manual SPPS.

Purification: Using general Biotage Method A.

HRMS (ESI-QtoF) m/z: [M+H]^+ calculated for C_{28}H_{48}N_{7}O_{7} 594.3615, found 594.3643 (Method A).

Synthesis: Synthesized using manual SPPS.

Purification: Purified using general SPE Procedure.

HRMS (ESI-QtoF) m/z: [M+H]^+ calculated for C_{28}H_{49}N_{8}O_{6} 593.3775, found 594.3803 (Method A).

Synthesis: Synthesized using manual SPPS.

Purification: Used without further purification.

HRMS (ESI-QtoF) m/z: [M+7H]^+ calculated for C_{181}H_{293}N_{60}O_{57}S 605.3100, found 605.3142 (Method A; calculated exact mass = 4230.114).
**H₂N-YSNHALF-seramox-K(biotin)-CONH₂ (10a)**

**Synthesis:** Synthesized using manual SPPS.

**Purification:** Using general Biotage Method A.

**HRMS (ESI-QtoF) m/z:** [M+2H]+ calculated for C₆₂H₉₅N₁₇O₁₅S 674.8457, found 674.8687 (Method A; MW = 1348.59).

**H₂N-G-pS-YSNHALF-seramox-K(biotin)-CONH₂ (10b)**

**Synthesis:** Synthesized using manual SPPS.

**Purification:** Using general Biotage Method A.

**HRMS (ESI-QtoF) m/z:** [M+2H]+ calculated for C₆₇H₁₀₄N₁₉O₂₁PS 786.8556, found 786.8791 (Method A; MW = 1572.70).

**TAMRA-YSNHALF-seramox-K(biotin)-CONH₂ (10c)**

**Synthesis:** Synthesized using manual SPPS.

**Purification:** Using general Biotage Method A.

**HRMS (ESI-QtoF) m/z:** [M+3H]+ calculated for C₈₇H₁₁₆N₂₀O₂₁S 587.6328, found 587.6328 (Method A; MW = 1761.04).
**H₂N-YSHALFGSK(biotin)-CONH₂ (12a)**

**Synthesis:** Synthesized using manual SPPS.
**Purification:** Using general Biotage Method A.
**HRMS (ESI-QtoF) m/z:** [M+2H]+ calculated for C₇₂H₅₉N₁₇O₁₆S 681.8275, found 681.8562 (Method A; MW = 1361.6550).

**H₂N-G-pS-YSHALFGSK(biotin)-CONH₂ (12b)**

**Synthesis:** Synthesized using manual SPPS.
**Purification:** Using general Biotage Method A.
**HRMS (ESI-QtoF) m/z:** [M+2H]+ calculated for C₇₇H₁₀₂N₁₉O₂₂S 793.8453, found 793.8665 (Method A; MW = 1586.68).

**TAMRA-YSHALFGSK(biotin)-CONH₂ (12c)**

**Synthesis:** Synthesized using manual SPPS.
**Purification:** Using general Biotage Method A.
**HRMS (ESI-QtoF) m/z:** [M+3H]+ calculated for C₈₀H₁₁₄N₁₉O₂₀S 592.2658, found 592.2919 (Method A; MW = 1775.02).
**SUPPORTING INFORMATION**

**Biotin-PEG₄-amide-isoeramox-Grqikiwfqrrmkwwk-CONH₂ (13)**

**Synthesis:** Synthesized using manual flow SPPS

**Purification:** Using general preparative HPLC method (*Section 4i*)

**HRMS (ESI-QtoF) m/z: [M+3H]⁺ calculated for C₁₃₀H₂₁₇N₄₀O₂₈S₂ 950.2076, found 950.1591 (Method B; MW = 2847.60)**

**H₂N-Grqikiwfqrrmkwwk-CONH₂ (14)**

**Synthesis:** Synthesized using manual flow SPPS.

**Purification:** Using general preparative HPLC method (*Section 4*)

**HRMS (ESI-QtoF) m/z: [M+3H]⁺ calculated for C₁₅₆H₁₇₅N₃₆O₂₀S 768.1168, found 768.0897 (Method B; MW = 2301.83)**
**Synthesis:** Synthesized using manual flow SPPS

**Purification:** Using general preparative HPLC method (Section 4)

**HRMS (ESI-QtoF) m/z:** [M+3H]+ calculated for C_{134}H_{200}N_{39}O_{25}S 697.6383, found 697.6384 (Method C; MW = 2789.38)
5. Oxidative cleavage of linkers in a model peptide

a. Procedure for cleavage of peptides at 500 µM: Solutions of peptide (5.0 mM), NaIO₄ (10 mM), Na₂SO₃ (13 mM), and H₂N-OH•HCl (20 mM) were prepared in 1X PBS buffer (pH 7.5). A 200 µL Eppendorf tube was charged with 1X PBS buffer (16 µL, pH 7.5) and then peptide solution (2.0 µL, 1.0 equiv, 500 µM reaction concentration). In the dark, NaIO₄ solution (2.0 µL, 2.0 equiv, 1 mM reaction concentration) was added, and the reaction was left to sit for the allotted time listed below for each peptide (Table S1). Upon completion, the reaction was quenched with Na₂SO₃ solution (15 µL, 20 equiv) and H₂N-OH•HCl solution (25 µL, 50 equiv), and allowed to sit for the allotted time. The solution was diluted with 1:1 H₂O/MeCN (1% TFA), and analyzed via LC-MS.

[a] For isoseramox peptide (4b), no hydroxylamine was required; 25 µL of PBS buffer was added at this stage instead.

| Peptide               | Oxidation | Quench |
|-----------------------|-----------|--------|
| Diol Peptide (4a)     | 60 min    | 30 min |
| Seramox Peptide (4b)  | 5 min     | 30 min |
| Isoseramox peptide (4c)| 5 min   | 5 min  |

HRMS (ESI-QtoF) m/z: [M+H]+ calculated for C₄₈H₆₃N₆O₆ 429.2462, found 429.2462 (Method A).

HRMS (ESI-QtoF) m/z: [M+H]+ calculated for C₄₈H₆₃N₆O₆ 429.2464, found 429.2464 (Method A).

HRMS (ESI-QtoF) m/z: [M+H]+ calculated for C₄₈H₆₃N₆O₆ 358.2464, found 358.2463 (Method A).
b. Procedure for cleavage of 4b at 50 µM: Solutions of peptide (0.50 mM), NaIO₄ (1.0 mM), Na₂SO₃ (1.3 mM), and H₂N-OH-HCl (20 mM) were prepared in 1X PBS buffer (pH 7.5). A 200 µL Eppendorf tube was charged with 1X PBS buffer (15 µL, pH 7.5) and then peptide solution (10 µL, 0.1 equiv, 50 µM reaction concentration). In the dark, NaIO₄ solution (3.0 µL, 3.0 equiv, 150 µM reaction concentration) was added, and the reaction was left to sit for 60 min. Upon completion, the reaction was quenched with Na₂SO₃ solution (15 µL, 20 equiv) and H₂N-OH-HCl solution (25 µL, 500 equiv), and allowed to sit for 60 min. The solution was diluted with 1:1 H₂O/MeCN (1% TFA), and analyzed via LC-MS.

HRMS (ESI-QtoF) m/z: [M+H]⁺ calculated for C₁₈H₃₃N₆O₆ 429.2462, found 429.2463 (Method A).

4b (50 µM) + aldehyde

H₂N\textsuperscript{\textregistered}AGV\textsuperscript{\textregistered}CONH₂

5b

TIC

Counts vs. Acquisition Time (min)

10⁷

0

3 4 5 6 7 8 9 10 11 12 13

1 2 3

5b

c. Procedure for cleavage of 4c at 50 µM: Solutions of peptide (0.50 mM), NaIO₄ (1.0 mM), Na₂SO₃ (1.3 mM), and H₂N-OH-HCl (20 mM) were prepared in 1X PBS buffer (pH 7.5). A 200 µL Eppendorf tube was charged with 1X PBS buffer (30 µL, pH 7.5) and then peptide solution (4.0 µL, 1.0 equiv, 50 µM reaction concentration) was added, and the reaction was left to sit for 60 min. Upon completion, the reaction was quenched with Na₂SO₃ solution (30 µL, 20 equiv) and either 1X PBS buffer (50 µL) or H₂N-OH-HCl solution (50 µL, 500 equiv, interchangeably used with no discernable change) and allowed to sit for 20 min. The solution was diluted with 1:1 H₂O/MeCN (1% TFA), and analyzed via LC-MS.

HRMS (ESI-QtoF) m/z: [M+H]⁺ calculated for C₁₆H₃₂N₅O₄ 358.2454, found 358.2462 (Method A).

H₂N\textsuperscript{\textregistered}AGV\textsuperscript{\textregistered}CONH₂

5c

TIC

Counts vs. Acquisition Time (min)

10⁷

0

4 5 6 7 8 9 10 11 12

4c (50 µM)

4b - aldehyde

5b

4c

5c

H₂N\textsuperscript{\textregistered}AGV\textsuperscript{\textregistered}CONH₂

5c

TIC

Counts vs. Acquisition Time (min)

10⁷

0

4 4.5 5 5.5 6 6.5 7 7.5 8 8.5 9 9.5 10 10.5 11 11.5 12

4 5 6 7

5c

HRMS (ESI-QtoF) m/z: [M+H]⁺ calculated for C₁₆H₃₂N₅O₄ 358.2454, found 358.2462 (Method A).
d. Procedure for cleavage of peptides 4b and 4c at 10 µM: Solutions of peptide (0.50 mM), NaIO₄ (1.0 mM), Na₂SO₃ (1.3 mM), and H₂N-OH•HCl (20 mM) were prepared in 1X PBS buffer (pH 7.5). A 200 µL Eppendorf tube was charged with 1X PBS buffer (47.5 µL, pH 7.5) and then peptide solution (1.0 µL, 1.0 equiv, 10 µM reaction concentration). In the dark, NaIO₄ solution (1.5 µL, 3.0 equiv, 30 µM reaction concentration) was added, and the reaction was slowly for 3 h. Upon completion, the reaction was quenched with a solution of Na₂SO₃ (30 µL, 20 equiv), H₂N-OH•HCl (11.5 µL, 500 equiv), and 1X PBS buffer (6.0 µL) and allowed to sit for 1 h. The solution was diluted with 1:1 H₂O/MeCN (1% TFA), and analyzed via LC-MS.

[a] For isoseramox peptide (4c), no hydroxylamine was required; 17.5 µL of PBS buffer was added at this stage instead. This reagent would not harm the reaction and would simply quench the other aldehyde fragment.
**SUPPORTING INFORMATION**

e. Early procedure for cleavage of peptide 4d: Solutions of peptide (1.0 mM) in 1X PBS buffer (pH 9), and NaIO₄ (10 mM) and Na₂SO₃ (40 mM) in H₂O were prepared. To the peptide solution (500 µL, 1.0 equiv, 615 µM reaction concentration) was added NaIO₄ solution (150 µL, 3.8 equiv, 2.4 mM reaction concentration), and the solution was gently mixed in the dark. At each time point, an aliquot of the reaction (65 µL) was removed and quenched with sodium sulfite (15 µL, 12 equiv). The solution was diluted with 1:1 H₂O/MeCN (1% TFA), and analyzed via LC-MS.

\[
\text{HRMS (ESI-QtoF) m/z: [M+H]^+ calculated for C}_{16}\text{H}_{32}\text{N}_{5}\text{O}_{4} 358.2454, found 358.2510 (Method A).}
\]

f. Procedure for test cleavage of peptide 13. Solutions of peptide (1.0 mM), NaIO₄ (1.0 mM), Na₂SO₃ (1.3 mM), and H₂N-OH•HCl (20 mM) were prepared in 1X PBS buffer (pH 7.5). A 200 µL Eppendorf tube was charged with 1X PBS buffer (32 µL, pH 7.5) and then peptide solution (2.0 µL, 1.0 equiv, 50 µM reaction concentration). In the dark, NaIO₄ solution (6.0 µL, 3.0 equiv, 150 µM reaction concentration) was added, and the reaction was left to sit for 10 min. Upon completion, the reaction was quenched with Na₂SO₃ solution (30 µL, 20 equiv) and H₂N-OH•HCl solution (50 µL, 500 equiv) and allowed to sit for 1 h. The solution was diluted with 1:1 H₂O/MeCN (1% TFA), and analyzed via LC-MS.

\[
\text{HRMS (ESI-QtoF) m/z: [M+2H]^2+ calculated for C}_{106}\text{H}_{174}\text{N}_{36}\text{O}_{20}\text{S} 1151.6713, found 1151.9118 (Method B).}
\]

Oxidation of the methionine of 13 was slower than oxidative cleavage of the isoseramox linker. Three-hour reaction times were required to obtain >75% conversion to 14.
6. Recovery of a peptide library using (iso)seramox linkers

Library A: H-1234-LAYK (10 µM in 1X PBS)
Library B: Biotin-PEG4-iso-seramox-1234-LAYK (10 µM in 1X PBS)

With 1 = V, M, Y, W, D, Q, H, K; 2 = S, A, L; 3 = E, N, G; 4 = T, P, F

Library A (10 µM in 1X PBS, 4.3 µL, 43 pmol, 200 fmol per member) was added to 1 mg MyOne® streptavidin beads in 95 µL PBS buffer (1x, pH 7.5) and incubated for 1 h at 4 °C. The supernatant was collected, and the beads additionally washed with 6 M guanidine HCl (pH 6.8, 2 x 100 µL). The pooled supernatant and washing fractions were desalted by C18 ZipTip. 6 M guanidine HCl (pH 6.8, 5 µL) was added to the resulting solution (200 µL), which was then frozen, freeze dried and taken up in 20 µL Orbitrap solvent A. 5 µL of the resulting mixture (50 fmol per peptide) were used for nanoLC-MS/MS analysis.

Library B (10 µM in 1X PBS, 100 µL, 1 nmol) added to 1 mg MyOne® streptavidin beads (400 pmol/mg estimated capacity) and incubated for 1 h at 4 °C. The supernatant was removed, and the beads additionally washed with 6 M guanidine HCl (pH 6.8, 2 x 200 µL). The beads were then suspended in 100 µL PBS buffer (1x, pH 7.5) and treated with NaIO4 (1 mM in H2O, 2 µL) for 5 minutes at room temperature under exclusion of light. The oxidative cleavage was quenched with the addition of Na2SO3 (100 mM, 5 µL) and NH2OH (100 mM, 5 µL). The supernatant was collected, and the beads additionally washed with 6 M guanidine HCl (pH 6.8, 2 x 100 µL). The pooled supernatant and washing fractions were desalted by C18 ZipTip. 6 M guanidine HCl (pH 6.8, 5 µL) was added to the resulting solution (200 µL), which was then frozen, freeze dried and taken up in 40 µL Orbitrap solvent A. 5 µL of the resulting mixture (46 fmol per peptide considering approximately 20 % cleavage) were used for nanoLC-MS/MS analysis.

De novo sequencing with PEAKS and further filtering

De novo peptide sequencing was performed in PEAKS 8.5 from Bioinformatics Solutions Inc. (ON, Canada). Sequencing data obtained from the Orbitrap were refined as follows. HCD and CID scans were merged within a 0.2 minute and 10 ppm window, mass precursor correction was performed, and primary mass filtration was performed as appropriate. De novo sequencing was performed allowing 15 ppm assignment errors and 0.05 Da individual fragment mass errors. Methionine, histidine and tryptophan oxide were selected as variable PTMs. 15 candidate sequences were obtained for each preprocessed scan.

The complete list of peptide candidates was exported as .csv file and then filtered using a Python script. Briefly: sequences not matching library design rules (sequence length, correct monomers in every position) were eliminated from further consideration. Next, for scans with multiple remaining sequence candidates, a single peptide with highest ALC score per scan was retained, while the rest were excluded. After this, remaining sequences were labeled as “unique” and “non-unique” based on various criteria. The resulting final list of unique sequences was used for comparison between the reference library A and the cleaved library B.

Full lists of unique sequences for library A and library B after de novo sequencing and filtration

\[ m = \text{oxidized methionine} \]
\[ U = \text{C-terminal amide} \]
\[ ALC = \text{average level of confidence} \]

| PEPTIDE      | ALK (%) | M/Z       | Z  | RT | MASS     | PPM |
|--------------|---------|-----------|----|----|----------|-----|
| DAELAYKU     | 99      | 478.2478  | 2  | 47.78 | 954.4810 | 0.1 |
| DAELAYKU     | 92      | 453.2402  | 2  | 34.38 | 904.4654 | 0.6 |
| DAETLAYKU    | 95      | 455.237   | 2  | 32.83 | 908.4603 | -0.9|
| DAGFLAYKU    | 99      | 442.2365  | 2  | 47.25 | 882.4599 | -1.5|
| DAGPLAYKU    | 98      | 417.2286  | 2  | 34.82 | 832.4443 | -2  |
| DAGTLAYKU    | 92      | 419.2265  | 2  | 28.6  | 836.4392 | -0.9|
| DANFLAYKU    | 98      | 470.748   | 2  | 47.71 | 939.4814 | 0   |
| DANLAYKU     | 88      | 445.7397  | 2  | 38.09 | 889.4658 | -0.9|
| DANTLAYKU    | 88      | 447.7374  | 2  | 28.48 | 893.4607 | -0.5|
| DLEPLAYKU    | 99      | 474.263   | 2  | 46.72 | 946.5123 | -0.9|
| DLETLAYKU    | 99      | 476.2598  | 2  | 47.19 | 950.5073 | -2.3|
| DLGFLAYKU    | 99      | 463.2594  | 2  | 56.94 | 924.5069 | -2.9|
| DLGPLAYKU    | 97      | 438.252   | 2  | 46.85 | 874.4912 | -1.9|
| DLGTTLAYKU   | 99      | 440.2501  | 2  | 44.26 | 878.4861 | -0.5|
| DLNFLAYKU    | 86      | 491.7702  | 2  | 57.78 | 981.5283 | -2.5|
| DLNPLAYKU    | 98      | 466.7625  | 2  | 48.28 | 931.5127 | -2.5|
| DLNNTLAYKU   | 98      | 468.761   | 2  | 43.04 | 935.5076 | -0.2|

**SUPPORTING INFORMATION**
| SUPPORTING INFORMATION |
|-------------------------|
| DSEFLAYKU 99 486.2447 2 46.48 970.4760 -1.1 |
| DSEPLAYKU 94 461.2365 2 32.55 920.4603 -2 |
| DSETLAYKU 96 463.2343 2 29.79 924.4553 -1.3 |
| DSGFPLAYKU 99 450.2335 2 46.1 898.4548 -2.6 |
| DSGPLAYKU 99 425.2268 2 33.02 848.4392 -0.2 |
| DSGTLAYKU 98 427.2235 2 26.33 852.4341 -2 |
| DSNFLAYKU 99 478.7448 2 46.02 955.4763 -1.2 |
| DSNPLAYKU 89 453.7369 2 35.84 905.4606 -1.5 |
| DSNLPLAYKU 93 455.7341 2 26.75 909.4556 -2.2 |
| HAEFLAYKU 92 489.2633 2 35.73 976.5130 -0.9 |
| HAEPLAYKU 93 464.2558 2 24.3 926.4974 -0.4 |
| HAETLAYKU 90 466.2526 2 23.14 930.4923 -1.9 |
| HAGFLAYKU 93 453.2534 2 34 904.4919 0.3 |
| HAGPLAYKU 89 428.2455 2 23.67 854.4763 0.3 |
| HANFLAYKU 84 481.7629 2 35 961.5134 -2.2 |
| HANPLAYKU 86 456.756 2 25.63 911.4977 -0.4 |
| HLEFLAYKU 97 510.2868 2 46.22 1018.5600 -1 |
| HLEPLAYKU 91 485.2794 2 33.79 968.5443 -0.2 |
| HLETLAYKU 94 487.2768 2 30.83 972.5392 -0.2 |
| HLGFLAYKU 95 474.2764 2 44.25 946.5389 -0.7 |
| HLGPLAYKU 85 449.2677 2 32.3 896.5232 -2.5 |
| HLGLAYKU 89 451.266 2 28.97 900.5181 -0.8 |
| HLNFLAYKU 96 502.7873 2 45.34 1003.5600 -0.3 |
| HLNPPLAYKU 88 477.779 2 36.11 953.5447 -1.3 |
| HLNTLAYKU 89 479.7766 2 27.43 957.5396 -0.9 |
| HSEFLAYKU 94 497.2614 2 34.42 992.5079 0.4 |
| HSEPLAYKU 81 472.2534 2 23.55 942.4923 -0.1 |
| HSETLAYKU 92 474.25 2 22.68 946.4872 -1.8 |
| HSNFLAYKU 89 489.7611 2 32.96 977.5083 -0.6 |
| HSNPLAYKU 84 464.7532 2 24.72 927.4926 -0.8 |
| KAEFLAYKU 90 484.782 2 34.23 967.5491 0.3 |
| KAEPAYKU 91 459.7733 2 23.12 917.5334 -1.6 |
| KAETLAYKU 86 461.7709 2 22.03 921.5283 -1.2 |
| KAGFLAYKU 85 448.7701 2 31.19 895.5280 -2.5 |
| KAGPLAYKU 96 423.7626 2 22.79 845.5123 -1.8 |
| KANFLAYKU 86 477.2819 2 33.25 952.5494 -0.2 |
| KANPLAYKU 88 452.2743 2 23.85 902.5338 0.3 |
| KLEFLAYKU 99 505.8052 2 45.86 1009.5960 -0.1 |
| KLEPLAYKU 88 320.8664 3 32.38 959.5804 -3.1 |
| KLETLAYKU 89 482.7948 2 30.06 963.5753 -0.2 |
| KLFAGAYKU 95 313.5321 3 43.44 937.5749 -0.5 |
| KLNFLAYKU 98 498.3054 2 44.63 994.5964 -0.2 |
| KLNPLAYKU 91 473.2973 2 33.47 944.5807 -0.6 |
| KLNTLAYKU 86 475.2941 2 26.79 948.5756 -2.1 |
| KSEFLAYKU 97 492.7783 2 32.21 983.5440 -2 |
| KSEPLAYKU 85 467.7704 2 22.45 933.5283 -2.1 |
| KSGFLAYKU 84 456.7683 2 29.9 911.5229 -0.9 |
| KSGPLAYKU 83 288.1758 3 22.17 861.5072 -2 |
| Code          | Value1 | Value2 | Value3 | Value4 | Value5 |
|---------------|--------|--------|--------|--------|--------|
| mAEFLAYKU     | 98     | 494.252| 2      | 44.47  | 986.4895 | -0.1 |
| mAEPAYKU      | 84     | 461.2493| 2     | 32.44  | 920.4789 | 5.6 |
| mAETLAYKU     | 96     | 471.2411| 2     | 28.79  | 940.4688 | -1.1 |
| mAGFLAYKU     | 99     | 450.2432| 2     | 48.21  | 898.4734 | -1.7 |
| mAGPLAYKU     | 90     | 433.2337| 2     | 30.57  | 864.4528 | 0.1 |
| mAGTLAYKU     | 82     | 435.2308| 2     | 25.71  | 868.4477 | -0.7 |
| mANFLAYKU     | 98     | 486.7519| 2     | 44.58  | 971.4899 | -0.6 |
| mANPLAYKU     | 86     | 461.7444| 2     | 34.49  | 921.4742 | 0.1 |
| mANTLAYKU     | 97     | 463.7414| 2     | 25.88  | 925.4692 | -1 |
| mLFLAYKU      | 99     | 515.2745| 2     | 53.32  | 1028.537 | -2 |
| mLPLAYKU      | 91     | 490.2667| 2     | 42.56  | 978.5208 | -2.1 |
| mLETLAYKU     | 98     | 492.2643| 2     | 40.02  | 982.5157 | -1.8 |
| mLGFLAYKU     | 99     | 479.265 | 2     | 51.51  | 956.5153 | 0.2 |
| mLGPLAYKU     | 98     | 454.2568| 2     | 42.26  | 906.4997 | -0.7 |
| mLGTLAYKU     | 98     | 456.2533| 2     | 38.24  | 910.4946 | -2.8 |
| mLNFLAYKU     | 98     | 507.7743| 2     | 52.73  | 1013.537 | -2.8 |
| mLNPAYKU      | 89     | 482.7675| 2     | 44.33  | 963.5212 | -0.7 |
| mLNTLAYKU     | 96     | 484.7643| 2     | 36.53  | 967.5161 | -2.1 |
| mSEFLAYKU     | 99     | 502.2495| 2     | 43.28  | 1002.484 | 0 |
| mSEMPLAYKU    | 92     | 477.2416| 2     | 30.05  | 952.4688 | -0.1 |
| mSETLAYKU     | 96     | 479.2391| 2     | 28.17  | 956.4637 | 0 |
| mSGFLAYKU     | 99     | 466.2387| 2     | 42.2   | 930.4633 | -0.4 |
| mSGPLAYKU     | 91     | 441.2307| 2     | 29.31  | 880.4477 | -0.9 |
| mSGTLAYKU     | 85     | 435.2311| 2     | 30.06  | 868.4477 | 0 |
| mSNFLAYKU     | 98     | 494.7495| 2     | 42.9   | 987.4848 | -0.4 |
| mSNPLAYKU     | 90     | 469.7414| 2     | 32.83  | 937.4691 | -1 |
| mSNTLAYKU     | 97     | 471.7383| 2     | 25.15  | 941.4641 | -2.2 |
| QAFLAYKU      | 99     | 484.763 | 2     | 43.81  | 967.5127 | -1.3 |
| QAEPAYKU      | 98     | 459.7553| 2     | 29.8   | 917.4970 | -1 |
| QAETLAYKU     | 97     | 461.7529| 2     | 27.63  | 921.4919 | -0.8 |
| QAGFLAYKU     | 98     | 448.7521| 2     | 42.51  | 895.4916 | -2.1 |
| QAGPLAYKU     | 97     | 423.7445| 2     | 29.4   | 845.4759 | -1.6 |
| QAGTLAYKU     | 96     | 425.7421| 2     | 24.66  | 849.4708 | -1.5 |
| QANFLAYKU     | 98     | 477.2637| 2     | 43.55  | 952.5130 | -0.2 |
| QANPLAYKU     | 96     | 452.2555| 2     | 32.81  | 902.4974 | -1 |
| QANTLAYKU     | 95     | 454.2525| 2     | 24.83  | 906.4923 | -2 |
| QLEFLAYKU     | 99     | 505.7858| 2     | 53.2   | 1009.560 | -2.5 |
| QLEPLAYKU     | 98     | 480.7792| 2     | 42.11  | 959.5440 | -0.2 |
| QLETLAYKU     | 97     | 482.7766| 2     | 39.6   | 963.5389 | -0.3 |
| QLGFLAYKU     | 98     | 469.7756| 2     | 51.2   | 937.5385 | -1.9 |
| QLGTLAYKU     | 96     | 446.766 | 2     | 37.85  | 891.5178 | -0.4 |
| QLNFLAYKU     | 97     | 498.2859| 2     | 52.37  | 994.5600 | -2.8 |
| QLNPLAYKU     | 97     | 473.2788| 2     | 43.81  | 944.5443 | -1.4 |
| QLNTLAYKU     | 95     | 475.2763| 2     | 35.8   | 948.5392 | -1.3 |
| QSEFLAYKU     | 97     | 492.7611| 2     | 42.45  | 983.5076 | 0 |
| QSEPLAYKU     | 97     | 467.7528| 2     | 28.68  | 933.4919 | -1 |
| QSETLAYKU     | 96     | 469.7497| 2     | 26.86  | 937.4869 | -2.2 |
| QSGFLAYKU     | 96     | 456.7503| 2     | 41.25  | 911.4865 | -0.5 |
| SUPPORTING INFORMATION |
|-------------------------|
| **QSGPLAYKU** | 96 | 431.7424 | 2 | 28.16 | 861.4708 | -0.7 |
| **QSGTLAYKU** | 95 | 433.7394 | 2 | 23.3 | 865.4658 | -1.7 |
| **QSNFLAYKU** | 97 | 485.2612 | 2 | 41.7 | 968.5079 | -0.1 |
| **QSNPLAYKU** | 95 | 460.2522 | 2 | 31.11 | 918.4923 | -2.7 |
| **QSNTLAYKU** | 94 | 462.2508 | 2 | 23.94 | 922.4872 | -0.2 |
| **VAEFLAYKU** | 99 | 470.2674 | 2 | 47.2 | 938.5225 | -2.4 |
| **VAGFLAYKU** | 99 | 434.2546 | 2 | 46.01 | 866.5014 | -7.7 |
| **VAGPLAYKU** | 98 | 409.25 | 2 | 34.53 | 816.4858 | -0.4 |
| **VAGTLAYKU** | 98 | 411.2473 | 2 | 28.18 | 820.4807 | -0.7 |
| **VANFLAYKU** | 99 | 462.768 | 2 | 47.23 | 923.5229 | -1.5 |
| **VANPLAYKU** | 98 | 437.7607 | 2 | 39.45 | 873.5072 | -0.4 |
| **VANTLAYKU** | 99 | 439.7581 | 2 | 28.15 | 877.5021 | -0.6 |
| **VLEFLAYKU** | 99 | 491.2904 | 2 | 56.45 | 980.5695 | -3.3 |
| **VLEPLAYKU** | 99 | 466.2842 | 2 | 44.85 | 930.5538 | 0 |
| **VLETLAYKU** | 99 | 468.2805 | 2 | 42.59 | 934.5488 | -2.4 |
| **VLGFLAYKU** | 99 | 455.2807 | 2 | 54.2 | 908.5483 | -1.6 |
| **VLGPLAYKU** | 97 | 430.2731 | 2 | 44.07 | 858.5327 | -1.3 |
| **VLGTLAYKU** | 99 | 432.2707 | 2 | 40.52 | 862.5276 | -1 |
| **VLNFLAYKU** | 99 | 483.792 | 2 | 55.28 | 965.5698 | -0.3 |
| **VLNPLAYKU** | 99 | 458.7838 | 2 | 46.8 | 915.5541 | -1.2 |
| **VLNTLAYKU** | 99 | 460.7817 | 2 | 39.18 | 919.5491 | -0.3 |
| **VSEFLAYKU** | 99 | 478.2659 | 2 | 45.44 | 954.5175 | -0.2 |
| **VSEPLYKU** | 99 | 453.2576 | 2 | 34.75 | 904.5018 | -1.2 |
| **VSETLAYKU** | 99 | 455.2547 | 2 | 31.75 | 908.4967 | -2 |
| **VSGFLAYKU** | 99 | 442.2555 | 2 | 44.94 | 882.4963 | 0 |
| **VSGPLAYKU** | 98 | 417.2475 | 2 | 33.72 | 832.4807 | -0.3 |
| **VSGLAYKU** | 99 | 419.2448 | 2 | 27.54 | 836.4756 | -0.6 |
| **VSNFLAYKU** | 99 | 470.766 | 2 | 45.19 | 939.5178 | -0.3 |
| **VSNTPLAYKU** | 99 | 447.7552 | 2 | 27.46 | 893.4971 | -1.3 |
| **WAELAYKU** | 99 | 513.7727 | 2 | 56.37 | 1025.5330 | -2.5 |
| **WAEPAYKU** | 99 | 488.765 | 2 | 46.97 | 975.5178 | -2.3 |
| **WAETLAYKU** | 99 | 490.7634 | 2 | 45.38 | 979.5127 | -0.5 |
| **WAGFLAYKU** | 99 | 477.7634 | 2 | 55.34 | 953.5123 | 0 |
| **WAGPLAYKU** | 94 | 452.7544 | 2 | 47.09 | 903.4966 | -2.7 |
| **WAGTLAYKU** | 99 | 454.7529 | 2 | 42.94 | 907.4916 | -0.3 |
| **WANFLAYKU** | 99 | 506.2727 | 2 | 56.38 | 1010.5340 | -2.9 |
| **WANPLAYKU** | 99 | 481.2665 | 2 | 49.94 | 960.5181 | 0.4 |
| **WANTLAYKU** | 99 | 483.2632 | 2 | 42.73 | 964.5131 | -1.3 |
| **WLEFLAYKU** | 99 | 534.7966 | 2 | 66.05 | 1067.5800 | -1.6 |
| **WLEPLAYKU** | 99 | 509.7896 | 2 | 53.85 | 1017.5650 | -0.1 |
| **WLETLAYKU** | 99 | 511.7865 | 2 | 52.54 | 1021.5600 | -1.1 |
| **WLGFAYKU** | 98 | 498.7868 | 2 | 62.81 | 995.5593 | -0.1 |
| **WLGPLAYKU** | 98 | 473.7774 | 2 | 52.98 | 945.5436 | -3.4 |
| **WLGLAYKU** | 98 | 475.7751 | 2 | 50.53 | 949.5385 | -2.9 |
| **WLNFLAYKU** | 97 | 527.296 | 2 | 64.57 | 1052.5810 | -3.3 |
| **WLNPLAYKU** | 98 | 502.2892 | 2 | 55.82 | 1002.5650 | -1.1 |
| **WLNTLAYKU** | 99 | 504.2868 | 2 | 49.73 | 1006.5600 | -0.9 |
| **WSEFLAYKU** | 99 | 521.7711 | 2 | 53.52 | 1041.5280 | -0.7 |
| PEPTIDE     | ALC (%) | M/Z     | Z  | RT   | MASS     | PPM  |
|------------|---------|---------|----|------|----------|------|
| WSEPLAYKU  | 99      | 496.7629| 2  | 46.05| 991.5127 | -1.5 |
| WSETLAYKU  | 99      | 498.761 | 2  | 44.33| 995.5076 | -0.1 |
| WSGFLAYKU  | 99      | 485.7607| 2  | 53.79| 969.5072 | -0.3 |
| WSGPPLAYKU | 98      | 460.7523| 2  | 46.2 | 919.4916 | -1.8 |
| WSGTLAYKU  | 98      | 462.7503| 2  | 41.89| 923.4865 | -0.4 |
| WSNFLAYKU  | 99      | 514.2715| 2  | 53.77| 1026.5290| -0.3 |
| WSNTLAYKU  | 98      | 491.2614| 2  | 41.57| 980.5080 | 0.2  |
| YAELFLAYKU | 99      | 502.2655| 2  | 49.33| 1002.5180| -0.9 |
| YAEPLAYKU  | 98      | 477.2572| 2  | 39.94| 952.5018 | -1.9 |
| YAETLAYKU  | 99      | 479.2557| 2  | 37.31| 956.4967 | 0.2  |
| YAGFLAYKU  | 99      | 466.255 | 2  | 48.57| 930.4963 | -0.9 |
| YAGPPLAYKU | 98      | 441.2475| 2  | 39.4 | 880.4807 | -0.3 |
| YANFLAYKU  | 99      | 494.7666| 2  | 49.32| 987.5178 | -1.2 |
| YANPLAYKU  | 98      | 469.7581| 2  | 43.42| 937.5021 | -0.4 |
| YANTLAYKU  | 98      | 471.7566| 2  | 33.06| 941.4971 | -0.4 |
| YLEFLAYKU  | 99      | 523.2895| 2  | 58.58| 1044.5650| 0    |
| YLEPLAYKU  | 99      | 498.2804| 2  | 47.01| 994.5488 | -2.5 |
| YLTELAYKU  | 99      | 500.2791| 2  | 45.51| 998.5436 | -0.1 |
| YLGFLAYKU  | 99      | 487.2783| 2  | 55.9 | 972.5433 | -1.3 |
| YLGPLAYKU  | 96      | 462.2706| 2  | 46.55| 922.5276 | -1.1 |
| YLGTLAYKU  | 99      | 464.2687| 2  | 43.25| 926.5252 | 0.3  |
| YLNFLAYKU  | 99      | 515.7883| 2  | 57.32| 1029.5650| -2.6 |
| YLNPLAYKU  | 99      | 490.7813| 2  | 49   | 979.5491 | -1   |
| YLNTLAYKU  | 99      | 492.7776| 2  | 42.58| 983.5440 | -3.4 |
| YSEFLAYKU  | 99      | 510.2636| 2  | 47.5 | 1018.5130| 0.3  |
| YSEPLAYKU  | 99      | 485.2557| 2  | 38.98| 968.4967 | 0.1  |
| YSETLAYKU  | 99      | 487.2522| 2  | 36.63| 972.4916 | -1.9 |
| YSGFLAYKU  | 99      | 474.2529| 2  | 47.41| 946.4912 | 0    |
| YSGPLAYKU  | 99      | 449.2441| 2  | 38.47| 896.4756 | -2.2 |
| YSGTLAYKU  | 98      | 451.2417| 2  | 31.74| 900.4705 | -1.9 |
| YSNFLAYKU  | 99      | 502.7638| 2  | 47.42| 1003.5130| 0.3  |
| YSNPLAYKU  | 98      | 477.7548| 2  | 42.69| 953.4971 | -2.1 |
| YSNTLAYKU  | 99      | 479.7523| 2  | 31.8 | 957.4920 | -2.1 |

Table S2. Identity of peptides in library B
|    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|
| DLGFLAYKU | 98  | 463.2606 | 2 | 56.95 | 924.5069 | -0.2 |
| DLGPLAYKU | 97  | 438.2524 | 2 | 46.83 | 874.4912 | -1  |
| DLGTLAYKU | 98  | 440.2493 | 2 | 44.4  | 878.4861 | -2.4 |
| DLNFLAYKU | 96  | 491.7716 | 2 | 57.84 | 981.5283 | 0.3 |
| DLNPLAYKU | 96  | 466.7619 | 2 | 48.28 | 931.5127 | -3.6 |
| DLNTLAYKU | 97  | 468.7611 | 2 | 42.95 | 935.5076 | 0.1 |
| DSEFLAYKU | 99  | 486.2451 | 2 | 46.41 | 970.476  | -0.3 |
| DSEPLAYKU | 97  | 461.2378 | 2 | 32.9  | 920.4603 | 0.8 |
| DSETLAYKU | 93  | 463.2346 | 2 | 29.92 | 924.4553 | -0.7 |
| DSGFLAYKU | 99  | 450.234  | 2 | 45.97 | 898.4548 | -1.5 |
| DSGPLAYKU | 99  | 425.2269 | 2 | 33.01 | 848.4392 | 0   |
| DSGTLAYKU | 98  | 427.2243 | 2 | 26.3  | 852.4341 | 0   |
| DSNFLAYKU | 99  | 478.7449 | 2 | 45.97 | 955.4763 | -1.2 |
| DSNPLAYKU | 97  | 453.7376 | 2 | 35.95 | 905.4606 | 0   |
| DSNTLAYKU | 98  | 455.7353 | 2 | 26.82 | 909.4556 | 0.4 |
| HAELAYKU  | 89  | 489.2636 | 2 | 35.64 | 976.513  | -0.4 |
| HAEPLAYKU | 91  | 464.2557 | 2 | 24.38 | 926.4974 | -0.6 |
| HAEFLAYKU | 93  | 466.2534 | 2 | 23.19 | 930.4923 | -0.1 |
| HAGFLAYKU | 97  | 453.2531 | 2 | 33.38 | 904.4919 | -0.4 |
| HAGPLAYKU | 85  | 428.2454 | 2 | 23.59 | 854.4763 | 0   |
| HAGTLAYKU | 86  | 430.2427 | 2 | 20.35 | 858.4712 | -0.3 |
| HANFLAYKU | 82  | 481.7641 | 2 | 34.66 | 961.5134 | 0.3 |
| HANPLAYKU | 91  | 456.756  | 2 | 26.07 | 911.4977 | -0.2 |
| HANTLAYKU | 88  | 458.7536 | 2 | 20.72 | 915.4926 | 0   |
| HLEFLAYKU | 95  | 510.2868 | 2 | 46.23 | 1018.56  | -1  |
| HLEPLAYKU | 86  | 485.2795 | 2 | 33.64 | 968.5443 | 0.2 |
| HLETLAYKU | 93  | 487.2769 | 2 | 30.76 | 972.5392 | 0   |
| HLGFLAYKU | 90  | 474.2772 | 2 | 44.17 | 946.5389 | 1.1 |
| HLGPLAYKU | 87  | 299.8481 | 3 | 32.2  | 896.5232 | -0.8 |
| HLGTLAYKU | 89  | 451.2662 | 2 | 28.83 | 900.5181 | -0.4 |
| HLNFLAYKU | 92  | 502.7879 | 2 | 45.13 | 1003.56  | 1   |
| HLNPLAYKU | 87  | 477.7796 | 2 | 35.93 | 953.5447 | 0   |
| HLNTLAYKU | 83  | 479.7772 | 2 | 27.3  | 957.5396 | 0.2 |
| HSEFLAYKU | 90  | 497.2614 | 2 | 33.7  | 992.5079 | 0.2 |
| HSEPLAYKU | 81  | 315.1711 | 3 | 23.57 | 942.4923 | -1  |
| HSETLAYKU | 84  | 474.2505 | 2 | 22.61 | 946.4872 | -0.9 |
| HSGFLAYKU | 90  | 461.2507 | 2 | 31.79 | 920.4868 | 0.1 |
| HSGPLAYKU | 87  | 436.2428 | 2 | 23.02 | 870.4712 | -0.2 |
| HSNFLAYKU | 92  | 489.7614 | 2 | 32.7  | 977.5083 | -0.2 |
| HSNPLAYKU | 91  | 464.7538 | 2 | 25.02 | 927.4926 | 0.4 |
| KAEPLAYKU | 90  | 459.774  | 2 | 23.2  | 917.5334 | 0.2 |
| KAETLAYKU | 87  | 461.7714 | 2 | 22.03 | 921.5283 | -0.1 |
| KAGFLAYKU | 89  | 448.7711 | 2 | 31.14 | 895.528  | -0.4 |
| KAGPLAYKU | 84  | 423.7632 | 2 | 22.73 | 845.5123 | -0.5 |
| KANFLAYKU | 88  | 477.2817 | 2 | 32.46 | 952.5494 | -0.5 |
| KANPLAYKU | 90  | 452.2743 | 2 | 23.96 | 902.5338 | 0.2 |
| KLEFLAYKU | 99  | 505.8052 | 2 | 45.8  | 1009.596 | -0.1 |
| KLEPLAYKU | 86  | 320.8669 | 3 | 32.41 | 959.5804 | -1.5 |
| Substrates     | Replicate ID | 2  | 3  | 4  | 5  | 6  |
|---------------|--------------|----|----|----|----|----|
| KLEFLAYKU     | 84           | 482.7949 | 30.2 | 963.5753 | 0 |
| KLGFLAYKU     | 96           | 313.5321 | 43.33 | 937.5749 | -0.3 |
| KLGLFLAYKU    | 95           | 296.8604 | 31.45 | 887.5593 | 0 |
| KLGTFLAYKU    | 93           | 298.1918 | 27.99 | 891.5541 | -0.7 |
| KLETLAYKU     | 92           | 332.5392 | 44.64 | 994.5964 | -0.6 |
| KLNFLAYKU     | 89           | 315.8674 | 33.45 | 944.5807 | -0.3 |
| KNLFLAYKU     | 94           | 317.1992 | 26.96 | 948.5756 | 0.1 |
| KSEFLAYKU     | 95           | 492.7787 | 32.18 | 983.544 | -1.2 |
| KSGFLAYKU     | 82           | 456.7686 | 29.81 | 911.5229 | -0.4 |
| KSGPFLAYKU    | 86           | 431.7607 | 21.85 | 861.5072 | -0.4 |
| KSNFLAYKU     | 88           | 460.2718 | 23.35 | 918.5287 | 0.4 |
| mAFLAYKU      | 97           | 494.2516 | 44.59 | 986.4895 | -0.8 |
| mAEFLAYKU     | 96           | 469.2441 | 31.22 | 936.4739 | -0.3 |
| mAEFLAYKU     | 97           | 471.2415 | 28.79 | 940.4688 | -0.4 |
| mAGFLAYKU     | 98           | 458.2416 | 43.39 | 914.4684 | 0.2 |
| mAGFLAYKU     | 97           | 433.2336 | 30.73 | 864.4528 | -0.2 |
| mAGTLAYKU     | 94           | 435.231 | 25.57 | 868.4477 | -0.3 |
| mANFLAYKU     | 98           | 486.7511 | 44.51 | 971.4899 | -2.3 |
| mANFLAYKU     | 95           | 461.7444 | 34.48 | 921.4742 | -0.1 |
| mANTLAYKU     | 94           | 463.7447 | 25.82 | 925.4692 | 6 |
| mLFLAYKU      | 98           | 515.2755 | 53.43 | 1028.537 | -0.1 |
| mLFLAYKU      | 97           | 482.2692 | 46.99 | 962.5259 | -2.2 |
| mLETLAYKU     | 95           | 492.2648 | 39.96 | 982.5157 | -0.8 |
| mLFLAYKU      | 98           | 479.265 | 51.6 | 956.5153 | 0.2 |
| mLFLAYKU      | 87           | 454.2567 | 42.27 | 906.4997 | -0.9 |
| mLFLAYKU      | 97           | 456.2541 | 38.16 | 910.4946 | -1.1 |
| mLNFLAYKU     | 98           | 507.7757 | 52.75 | 1013.537 | 0.1 |
| mLNFLAYKU     | 95           | 482.7659 | 44.41 | 963.5212 | -4.1 |
| mLNFLAYKU     | 97           | 484.7655 | 36.8 | 967.5161 | 0.4 |
| mSFLAYKU      | 98           | 502.2497 | 43.31 | 1002.484 | 0.5 |
| mSFLAYKU      | 97           | 477.2414 | 30 | 952.4688 | -0.6 |
| mSFLAYKU      | 97           | 479.2392 | 34.85 | 956.4637 | 0.2 |
| mSFLAYKU      | 99           | 466.239 | 42.18 | 930.4633 | 0 |
| mSFLAYKU      | 97           | 441.2311 | 29.19 | 880.4477 | 0 |
| mSFLAYKU      | 98           | 443.2286 | 24.59 | 884.4426 | 0.1 |
| mSFLAYKU      | 98           | 494.7494 | 42.84 | 987.4848 | -0.6 |
| mSFLAYKU      | 97           | 469.7415 | 32.76 | 937.4691 | -0.7 |
| mSFLAYKU      | 97           | 471.7398 | 25.32 | 941.4641 | 1 |
| mSFLAYKU      | 97           | 484.7637 | 43.9 | 967.5127 | 0.1 |
| mSFLAYKU      | 96           | 459.7556 | 30.03 | 917.497 | -0.3 |
| mSFLAYKU      | 97           | 461.7533 | 27.77 | 921.4919 | 0 |
| mSFLAYKU      | 97           | 448.7532 | 42.56 | 895.4916 | 0.3 |
| mSFLAYKU      | 94           | 423.7452 | 29.43 | 845.4759 | -0.1 |
| mSFLAYKU      | 94           | 425.7425 | 24.71 | 849.4708 | -0.4 |
| mSFLAYKU      | 96           | 477.2637 | 43.54 | 952.513 | -0.2 |
| mSFLAYKU      | 95           | 452.2559 | 32.92 | 902.4974 | -0.2 |
| mSFLAYKU      | 94           | 454.2534 | 24.92 | 906.4923 | 0 |
| QLEFLAYKU     | 98           | 505.7871 | 53.38 | 1009.56 | 0 |
| SUPPORTING INFORMATION |
|------------------------|
| **QEPLAYKU** | 97 | 480.7787 | 2 | 42.26 | 959.544 | -1.3 |
| **QLETLAYKU** | 97 | 482.7766 | 2 | 39.69 | 963.5389 | -0.3 |
| **QLGFPLAYKU** | 97 | 469.7768 | 2 | 51.31 | 937.5385 | 0.6 |
| **QLGPLAYKU** | 96 | 444.7684 | 2 | 42.28 | 887.5229 | -0.7 |
| **QLGTLAYKU** | 95 | 446.7662 | 2 | 37.69 | 891.5178 | 0 |
| **QLNFLAYKU** | 96 | 498.2874 | 2 | 52.46 | 994.56 | 0.2 |
| **QLNPLAYKU** | 96 | 473.2796 | 2 | 43.86 | 944.5443 | 0.3 |
| **QLNLAYKU** | 92 | 475.2771 | 2 | 36.34 | 948.5392 | 0.5 |
| **QSEFLAYKU** | 96 | 492.7606 | 2 | 42.43 | 983.5076 | -1 |
| **QSEPLAYKU** | 94 | 467.7532 | 2 | 28.7 | 933.4919 | 0 |
| **QSETLAYKU** | 95 | 469.751 | 2 | 26.9 | 937.4869 | 0.6 |
| **QSGFLAYKU** | 94 | 456.7503 | 2 | 41.12 | 911.4868 | -0.6 |
| **QSGPLAYKU** | 94 | 431.7424 | 2 | 28.1 | 861.4708 | -0.7 |
| **QSGTLAYKU** | 92 | 433.7401 | 2 | 23.4 | 865.4658 | -0.1 |
| **QSNFLAYKU** | 96 | 485.261 | 2 | 41.55 | 968.5079 | -0.5 |
| **QSNPLAYKU** | 92 | 480.2533 | 2 | 31.15 | 918.4923 | -0.3 |
| **QSNTLAYKU** | 92 | 482.2508 | 2 | 24.08 | 922.4872 | -0.3 |
| **VAEFLAYKU** | 99 | 470.2677 | 2 | 47.25 | 938.5225 | -1.8 |
| **VAGFLAYKU** | 98 | 434.2543 | 2 | 46.16 | 866.5014 | -8.6 |
| **VAGPLAYKU** | 97 | 409.2502 | 2 | 34.58 | 816.4858 | 0 |
| **VAGTLAYKU** | 95 | 411.2474 | 2 | 28.21 | 820.4807 | -0.4 |
| **VANFLAYKU** | 98 | 462.7678 | 2 | 47.19 | 923.5229 | -2.1 |
| **VANPLAYKU** | 96 | 437.761 | 2 | 39.37 | 873.5072 | 0.3 |
| **VANTLAYKU** | 98 | 439.7582 | 2 | 28.16 | 877.5021 | -0.3 |
| **VLEFLAYKU** | 99 | 491.2917 | 2 | 56.56 | 980.5695 | -0.6 |
| **VLEPLAYKU** | 97 | 466.2845 | 2 | 44.91 | 930.5358 | 0.8 |
| **VLETAYKU** | 98 | 468.2814 | 2 | 42.79 | 934.5488 | -0.5 |
| **VLGFLAYKU** | 98 | 455.2812 | 2 | 54.28 | 908.5483 | -0.3 |
| **VLFPLAYKU** | 91 | 430.2732 | 2 | 44.23 | 858.5327 | -1.1 |
| **VLGLAYKU** | 97 | 432.2708 | 2 | 40.49 | 862.5276 | -0.8 |
| **VLNFLAYKU** | 98 | 483.7921 | 2 | 55.31 | 965.5698 | -0.2 |
| **VLNPLAYKU** | 97 | 458.7834 | 2 | 46.8 | 915.5541 | -2 |
| **VLNTLAYKU** | 98 | 460.7821 | 2 | 39.21 | 919.5491 | 0.6 |
| **VSEFLAYKU** | 99 | 478.2662 | 2 | 45.53 | 954.5175 | 0.5 |
| **VSEPPLAYKU** | 97 | 453.2534 | 2 | 34.76 | 904.5018 | -10.5 |
| **VSETLAYKU** | 98 | 455.2555 | 2 | 32.05 | 908.4967 | -0.4 |
| **VSGFLAYKU** | 99 | 442.2557 | 2 | 44.94 | 882.4963 | 0.5 |
| **VSGPLAYKU** | 99 | 417.2475 | 2 | 34.02 | 832.4807 | -0.3 |
| **VSGTLAYKU** | 97 | 419.2449 | 2 | 27.7 | 836.4756 | -0.3 |
| **VSNFLAYKU** | 99 | 470.7664 | 2 | 45.24 | 939.5178 | 0.6 |
| **VSNPLAYKU** | 97 | 445.7585 | 2 | 38.81 | 889.5021 | 0.3 |
| **VSNTLAYKU** | 98 | 447.7557 | 2 | 27.54 | 893.4971 | -0.3 |
| **WAELAYKU** | 99 | 513.7737 | 2 | 56.49 | 1025.533 | -0.6 |
| **WAELAYKU** | 97 | 488.7652 | 2 | 47.03 | 975.5178 | -1.9 |
| **WAETLAYKU** | 97 | 490.7641 | 2 | 45.37 | 979.5127 | 1 |
| **WAGPLAYKU** | 98 | 477.7635 | 2 | 55.29 | 953.5123 | 0.2 |
| **WAGPLAYKU** | 98 | 452.7547 | 2 | 47.08 | 903.4966 | -2 |
| **WAGTLAYKU** | 98 | 454.7531 | 2 | 42.98 | 907.4916 | 0 |
| SUPPORTING INFORMATION |
|-------------------------|
| **WANFLAYKU** | 98 | 506.2739 | 2 | 56.62 | 1010.534 | -0.5 |
| **WANPLAYKU** | 96 | 481.2661 | 2 | 50.23 | 960.5181 | -0.5 |
| **WANTLAYKU** | 98 | 483.2639 | 2 | 42.71 | 964.5131 | 0.2 |
| **WLEFLAYKU** | 99 | 534.7974 | 2 | 66.29 | 1067.58 | 0 |
| **WLEPLAYKU** | 97 | 509.7895 | 2 | 53.89 | 1017.565 | -0.3 |
| **WLETLAYKU** | 99 | 511.7871 | 2 | 52.7 | 1021.56 | 0 |
| **WLGFLAYKU** | 98 | 498.787 | 2 | 62.95 | 995.5593 | 0.3 |
| **WLGPLAYKU** | 97 | 473.7791 | 2 | 52.93 | 945.5436 | 0 |
| **WLGTLAYKU** | 97 | 475.7765 | 2 | 50.59 | 949.5385 | -0.1 |
| **WLNFLAYKU** | 98 | 527.2977 | 2 | 64.81 | 1052.581 | 0 |
| **WLNPLAYKU** | 97 | 502.2895 | 2 | 55.92 | 1002.565 | -0.6 |
| **WLNTLAYKU** | 98 | 504.287 | 2 | 49.75 | 1006.56 | -0.4 |
| **WSEFLAYKU** | 98 | 521.7715 | 2 | 53.66 | 1041.528 | 0.2 |
| **WSEPLAYKU** | 97 | 496.763 | 2 | 46.09 | 991.5127 | -1.3 |
| **WSETLAYKU** | 92 | 498.7597 | 2 | 44.45 | 995.5076 | -2.8 |
| **WSGFLAYKU** | 99 | 485.7607 | 2 | 53.8 | 969.5072 | -0.3 |
| **WSGPLAYKU** | 97 | 460.7523 | 2 | 46.07 | 919.4916 | -1.8 |
| **WSGTLAYKU** | 97 | 462.749 | 2 | 41.94 | 923.4865 | -3.3 |
| **WSNFLAYKU** | 99 | 514.2717 | 2 | 53.68 | 1026.529 | 0.1 |
| **WSNTLAYKU** | 96 | 491.2615 | 2 | 41.43 | 980.508 | 0.4 |
| **YAEFLAYKU** | 98 | 502.2656 | 2 | 49.48 | 1002.518 | -0.8 |
| **YAELAYKU** | 96 | 477.258 | 2 | 39.94 | 952.5018 | -0.3 |
| **YAEFLAYKU** | 98 | 479.2558 | 2 | 37.42 | 956.4967 | 0.3 |
| **YAGFLAYKU** | 96 | 466.2545 | 2 | 48.8 | 930.4963 | -2 |
| **YAGPLAYKU** | 98 | 441.2476 | 2 | 39.44 | 880.4807 | 0 |
| **YANFLAYKU** | 98 | 494.7658 | 2 | 49.44 | 987.5178 | -0.6 |
| **YANPLAYKU** | 97 | 469.7587 | 2 | 43.38 | 937.5021 | 0.8 |
| **YANLAYKU** | 98 | 471.7558 | 2 | 33.13 | 941.4971 | 0 |
| **YLEFLAYKU** | 99 | 523.2896 | 2 | 58.77 | 1044.565 | 0.1 |
| **YLEFLAYKU** | 97 | 498.2805 | 2 | 47.08 | 994.5488 | -2.4 |
| **YLETLAYKU** | 95 | 500.2796 | 2 | 45.5 | 998.5436 | 1 |
| **YLGFLAYKU** | 98 | 487.2878 | 2 | 55.99 | 972.5433 | -0.4 |
| **YLGPLAYKU** | 91 | 462.27 | 2 | 46.48 | 922.5276 | -0.2 |
| **YLTLAYKU** | 98 | 464.2667 | 2 | 43.21 | 926.5225 | 0.4 |
| **YLNFLAYKU** | 98 | 515.7897 | 2 | 57.48 | 1029.565 | 0.2 |
| **YLNPLAYKU** | 97 | 490.7808 | 2 | 49.03 | 979.5491 | -2.1 |
| **YLNTLAYKU** | 98 | 492.7789 | 2 | 42.56 | 983.544 | -0.7 |
| **YSEFLAYKU** | 99 | 510.2635 | 2 | 47.64 | 1018.512 | 0.2 |
| **YSEPLAYKU** | 98 | 485.2558 | 2 | 39.07 | 968.4967 | 0.3 |
| **YSETLAYKU** | 99 | 487.2532 | 2 | 36.9 | 972.4916 | 0.2 |
| **YSFLAYKU** | 99 | 474.2526 | 2 | 47.37 | 946.4912 | -0.7 |
| **YSGPLAYKU** | 96 | 449.2451 | 2 | 38.44 | 896.4756 | 0 |
| **YSGLAYKU** | 99 | 451.2423 | 2 | 31.89 | 900.4705 | -0.5 |
| **YSNFLAYKU** | 99 | 502.7626 | 2 | 47.32 | 1003.513 | -2.1 |
| **YSNPPLAYKU** | 98 | 477.7549 | 2 | 42.34 | 953.4971 | -1.8 |
| **YSNTLAYKU** | 98 | 479.7531 | 2 | 31.84 | 957.492 | -0.5 |
7. Study of complex peptide and protein substrates

a. Structural analysis of miniprotein Z33 upon treatment with NaIO4

The miniprotein Z33 (FNMQQRFYEALHDPNILNEERNAKISIRDDK) was synthesized by manual SPPS, cleaved from the resin and deprotected and desalted by SPE (Section 4). The resulting lyophilized powder was dissolved in 1x PBS (pH = 7.5, [Z33] = 0.5 mM). For the control CD spectrum (before NaIO4 treatment) 100 µL of the protein stock solution was diluted with 900 µL CD buffer (1x PBS containing 30% glycerol). For the CD spectrum (after NaIO4 treatment), 180 µL of the protein stock solution were treated with 20 µL of NaIO4 solution (40 mM, final concentration 4 mM) and incubated at room temperature for 30 minutes. After this time 100 µL of the periodate-treated protein solution were diluted with 900 µL CD buffer. CD spectra of both samples were recorded at 25 ºC at wavelength from 250 nm to 200 nm.

b. Comparison of seramox release method to SDS denaturing conditions after streptavidin capture

Peptides 10a–c and 12a–c were synthetized by manual SPPS, cleaved from the resin, deprotected, and purified via automatic reversed-phase chromatography (Section 4). Each peptide (50 µM 1x PBS) was immobilized on MyOne streptavidin beads (15 min, room temperature). The beads were washed with 6 M guanidine (500 µL x 1), followed by 1x PBS buffer (pH 7.5, 500 µL x 2).

Peptides 10a–c were subjected to periodate cleavage (100 µL, 0.5 mM, 10 min, room temperature). The reactions were quenched with a 50 µL solution of Na2SO3 (20 mM) and H2N-OH•HCl (20 mM). To the supernatant was added 6 M guanidine (50 µL), and the resulting solutions were desalted by C18 ZipTip (Section 6) and lyophilized.

Peptides 12a–c were subjected to SDS denaturing conditions (0.4% SDS, 20 mM biotin, 25 mM TRIS, 125 mM NaCl, pH 7.5, 95 ºC, 15 min). After cooling to room temperature, the supernatants were treated with 6 M guanidine (100 µL), and the resulting solutions were desalted by C18 ZipTip (Section 6) and lyophilized.

All samples were dissolved in 0.1% TFA (H2O/MeCN, 95:5) & analyzed by LC-MS (Method A). The results are presented below.

8. Recovery of penetratin from live cells

a. Procedure for test pulldown of peptide 13

Peptide 14: H-Grqikiwfqnrrmkwkk (10 µM in 1X PBS)
Peptide 13: Biotin-PEG4-isorseramox-Grqikiwfqnrrmkwkk (10 µM in 1X PBS)

Peptide 14 (10 µM in 1x PBS, 100 µL, 1 nmol) was added to 2 mg MyOne® streptavidin beads and incubated for 1 h at 4 ºC. The supernatant was collected, and the beads additionally washed with 6 M guanidine HCl (pH 6.8, 2 x 100 µL). The pooled supernatant and washing fractions were desalted by C18 ZipTip and analyzed directly by LC-MS.

Peptide 13 (10 µM in 1x PBS, 100 µL, 1 nmol) added to 2 mg MyOne® streptavidin beads (400 pmol/mg estimated capacity) and incubated for 1 h at 4 ºC. The supernatant was removed, and the beads additionally washed with 6 M guanidine HCl (pH 6.8, 2 x 200 µL). The beads were then suspended in 100 µL PBS buffer (1x, pH 7.5) and treated with NaIO4 (1 mM in H2O, 2 µL) for 5 minutes at room temperature under exclusion of light. The oxidative cleavage was quenched with the addition of Na2SO3 (100 mM, 5 µL) and NH2OH (100 mM, 5 µL) and incubated for 1 h. The supernatant was collected, and the beads additionally washed with 6 M guanidine HCl (pH 6.8, 2 x 100 µL). The pooled supernatant and washing fractions were desalted by C18 ZipTip and analyzed directly by LC-MS.
Peptide 1 was observed in various oxidation states (14\([\text{N}+\text{Bu}]\)), including the parent mass, the M+16, M+32, and M+48. Presumably this is the result of oxidation of the Met or Trp residues. The specific masses are shown below in Section 7b.

b. Cell uptake and pulldown

**Peptide 14**: H-Grkikwfqnrrmkwkk (10 \(\mu\)M in 1X PBS)

**Peptide 13**: Biotin-PEG4-isoseramox Grkikwfqnrrmkwkk (10 \(\mu\)M in 1X PBS)

HeLa cells (ATCC CCL-2) were maintained in MEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin at 37 °C and 5% CO₂. Sixteen hours prior to treatment, the cells were plated at a density of 250,000 cells per well in a 12-well plate in complete media. On the day of the experiment, 10 \(\mu\)M of peptide 13 was prepared in complete media and 1 mL of treatment media was added to the well. The plate was incubated for 1.5 h at 37 °C and 5% CO₂ before being washed three times with PBS and incubated with Trypsin-EDTA (0.25%) at 37 °C 5% CO₂ for 10 minutes. Once the cells had lifted, the plate was placed on ice and the trypsin was quenched with 100 \(\mu\)L soybean trypsin inhibitor (5 mg/mL) and bovine serum albumin (BSA, 1 mg/mL).

The cells were transferred to 1.5 mL microcentrifuge tubes and spun for 2 min. The supernatant was removed, and the cells were washed with 1 mL Buffer A (1X PBS, 1 mg/mL BSA) and spun again, and the supernatant was removed. 150 \(\mu\)L of lysis buffer (RIPA buffer) was added to the cell pellet, and the tube was heated to 95 °C for 15 minutes, cooled on ice briefly, and spun for 5 min. The pellet was washed with 350 \(\mu\)L Buffer A and the pooled supernatant was transferred to a new tube.

MyOne Streptavidin Dynabeads (1 mg) was transferred to a microcentrifuge tube, immobilized with a magnet stand, and washed three times with Buffer A. The supernatant isolated above was then added to the beads and rotated at 4 °C overnight. The following day, the beads were immobilized and washed twice with Buffer A (200 \(\mu\)L), twice with Buffer B (200 \(\mu\)L, 6M GuHCl pH 6.8 in 1X PBS), and twice with 1X PBS. The beads were then suspended in 100 \(\mu\)L PBS and treated with NaIO₄ (1 mM in H₂O, 2 \(\mu\)L) for 5 min at room temperature under exclusion of light. The reaction was quenched with the addition of Na₂SO₃ (100 mM, 5 \(\mu\)L) and NH₂OH (100 mM, 5 \(\mu\)L) and incubated at room temperature for 1 h. The supernatant was collected, and the beads additionally washed with Buffer B (2 \(\times\) 100 \(\mu\)L). Pooled supernatant was lyophilized and resuspended in water with 0.1% TFA, desalted by C18 ZipTip and analyzed directly by LC-MS. Isolated material was quantified by comparison to a standard curve of Peptide 14 generated by LC-MS.
Peptide 14 was observed in various oxidation states (14+[o]), including the parent mass, the M+16, M+32, and M+48. Presumably this is the result of oxidation of the Met or Trp residues. The specific masses utilized for the extracted ion chromatograph (EIC) are shown below in Table S2.

| Compound | mass | M+2H | M+3H | M+4H | M+5H | M+6H |
|----------|------|------|------|------|------|------|
| Exact mass | 2303.2 | 1152.6 | 768.7333 | 576.8 | 461.64 | 384.8667 |
| m+16      | 2319.2 | 1160.6 | 774.0667 | 580.8 | 464.84 | 387.5333 |
| m+32      | 2335.2 | 1168.6 | 779.4 | 584.8 | 468.04 | 390.2 |
| m+48      | 2351.2 | 1176.6 | 784.7333 | 588.8 | 471.24 | 392.8667 |

9. Fluorescent confocal imaging of peptide 15 in HeLa cells

To confirm that peptide 13 was extracted from within cells in the experiment described above, we analyzed the delivery of a fluorescent derivative (15) via live cell confocal imaging after identical treatment conditions. Resin containing isoseramox-penetratin was conjugated to the fluorophore TAMRA through manual SPPS to yield peptide 15 after cleavage and purification. HeLa cells (ATCC CCL-2) were maintained in MEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin at 37 °C and 5% CO₂. 36 hours prior to treatment, the cells were plated at a density of 5,000 cells per well in a 96-well 30mm glass-bottom plate in complete media. For cell treatment, the medium was removed, and fresh medium was added containing 10 µM 15. After 1.5 h incubation at 37 °C and 5% CO₂, the cells were washed with 1x PBS and the culture medium was replaced with fresh medium. The cells were then immediately imaged in the W.M. Keck microscopy facility on an RPI Spinning Disk Confocal microscope on brightfield and RFP setting (561 nm 100mW OPLS excitation laser, 605/70 nm emission). An example image is shown in Figure S8.

Peptide 18: TAMRA-isoseramox-Grqikiwfqnrmkwwk (10 µM in complete media)
SUPPORTING INFORMATION

10. Cell viability studies with periodate treatment

Cytotoxicity was measured with the CytoTox 96™ Non-Radioactive Cytotoxicity Assay Kit (Promega, WI, USA), which measures the level of the cytosolic enzyme lactate dehydrogenase (LDH) released into the culture medium after membrane rupture. HeLa cells (ATCC CCL-2) were maintained in MEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin at 37 °C and 5% CO₂. 24 hours before treatment, cells were plated in a flat-bottom 96-well plate at 8,000 cells per well. For cell treatment, the cell media was replaced with 100 µL MEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin containing the NaIO₄ at the indicated concentration in triplicate wells. The cells were incubated for 8 or 24 hours at 37 °C, 5% CO₂. 45 minutes before the treatment endpoint, one set of untreated wells were fully lysed with SDS Lysis Solution (supplied by manufacturer) to serve as a 100% LDH release control. After incubation, 50 µL of cell media was removed into a fresh 96-well plate and combined with equal volume of reconstituted Substrate Mix (supplied by manufacturer). The plate was incubated in the dark at room temperature for 30 minutes. 50 µL of Stop Solution (supplied by manufacturer) was added and the absorbance of each well at 490 nm was recorded.

The data is reported in Figure S9. The average 490 nm absorbance value of the PBS-treated cells was subtracted from all other values to create the 0% LDH release baseline, and all values were normalized to the absorbance of the SDS lysis control cells to produce a percent maximum LDH release plot. Stronger absorbance at 490 nm indicates increased LDH released into the cell culture supernatant, which in turn correlates with increase in cytotoxicity.

11. Author contributions

S.P., C.R.S., and B.L.P. conceived the study and prepared the manuscript. S.P., C.R.S., and A.M.S. performed the synthesis and experiments on model peptides and peptide library. C.E.F and C.K.S performed the synthesis and experiments for cell-based studies.

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