

Supporting information for

A simple and traceless solid phase method simplifies the assembly of large peptides and the access to challenging proteins

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1. General methods

Reagents and solvents

2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium fluorophosphate (HBTU) and *N*-Fmoc protected amino acids were obtained from Iris Biotech GmbH. Side-chain protecting groups used for the amino acids were Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Cys(*S**t*Bu)-OH or Fmoc-Cys(Trt)-OH. Synthesis of *bis*(2-sulfanylethyl)aminotriyl polystyrene (SEA PS) resin was carried out as described elsewhere.¹ Rink-PEG-PS resin (NovaSyn TGR) resin was obtained from Novabiochem. 4-Mercaptophenylacetic acid (MPAA), 3-mercaptopropionic acid (MPA), *tris*(2-carboxyethyl)phosphine hydrochloride (TCEP), triisopropylsilane (TIS), dimethyl sulfide (DMS), guanidine hydrochloride were purchased from Sigma-Aldrich. All other reagents were purchased from Acros Organics or Merck and were of the purest grade available.

Peptide synthesis grade *N,N*-dimethylformamide (DMF), dichloromethane (CH₂Cl₂), diethylether (Et₂O), acetonitrile (CH₃CN), heptane, LC-MS-grade acetonitrile (CH₃CN, 0.1% TFA), LC-MS-grade water (H₂O, 0.1% TFA), *N,N*-diisopropylethylamine (DIEA), acetic anhydride (Ac₂O) were purchased from Biosolve and Fisher-Chemical. Trifluoroacetic acid (TFA) was obtained from Biosolve. Water was purified with a Milli-Q Ultra Pure Water Purification System.

Analyses and purifications

Products were characterized by analytical LC-MS (Waters 2695 LC/ZQ 2000 quadrupole) on a reverse phase column XBridge BEH300 C18 (3.5 μm, 300 Å, 4.6 × 150 mm) at 30 °C using a linear gradient of 0-100% of eluent B in eluent A over 30 min at a flow rate of 1 mL/min (eluent A = 0.1% TFA in H₂O; eluent B = 0.1% TFA in CH₃CN/H₂O: 4/1 by vol) or on a Zorbax 300SB-C3 (3.5 μm, 300 Å, 4.6 × 150 mm) at 50 °C using a linear gradient of 0-50% of eluent D in eluent C over 30 min at a flow rate of 1 mL/min (eluent C = 0.1% formic acid in H₂O; eluent D = 0.1% formic acid in CH₃CN/H₂O: 4/1 by vol). The column eluate was monitored by UV at 215 nm and by evaporative light scattering (ELS, waters 2424). The peptide masses were measured by on-line LC-MS: Ionization mode: ES+, *m/z* range 350–2040, capillary voltage 3 kV, cone voltage 30 V, extractor voltage 3 V, RF lens 0.2 V, source temperature 120 °C, desolvation temperature 350 °C. Calculated masses were based on average isotope composition.

MALDI-TOF mass spectra were recorded with a Bruker Autoflex Speed using alpha cyano 4-hydroxycinnamic acid, sinapinic acid or 2,5-dihydroxybenzoic acid (DHB) as matrix. The observed *m/z* corresponded to the monoisotopic ions, unless otherwise stated.

Preparative reverse phase HPLC of crude peptides were performed with an preparative HPLC Waters system using a reverse phase column XBridge BEH300 Prep C18 (5 μm , 300 \AA , 10 \times 250 mm) and appropriate linear gradient of increasing concentration of eluent B in eluent A (flow rate of 6 mL/min). Selected fractions were then combined and lyophilized.

2. Synthesis of AcA peptides 4 on the solid phase (Scheme 1A, method A)

AcA peptides were produced by Fmoc-SPPS as described elsewhere.¹

Typical experimental procedures

Coupling of N-acetoacetyl group (AcA) on the solid phase

After elongation of the peptide on SEA PS resin and terminal Fmoc deprotection (0.25 mmol scale), diketen (10 equiv, 191.6 μL) or *N*-hydroxysuccinimidyl acetoacetate (10 equiv, 497.9 mg) and *N*-methylmorpholine (5 equiv, 137.4 μL) were dissolved in the minimal volume of DMF and the resulting solution was added to the peptidyl resin. After stirring 30 min, the resin was washed with DMF (3 \times 2 min). The completion of the acylation step was verified using the Kaiser test for primary amines. Then, resin was washed with DCM (3 \times 2 min), diethyl ether (3 \times 2 min), and dried *in vacuo*.

Final peptide deprotection and cleavage

Peptides were deprotected and cleaved from the resin using TFA/DMS/thioanisol/3,4-difluorothiophenol/water: 90/2.5/2.5/2.5/2.5 by vol for 1 h (10 mL for 0.10 mmol of peptidyl resin, 25 mL for 0.25 mmol of peptidyl resin). The crude peptides were precipitated in cold diethyl ether/heptane: 1/1 by vol (200 mL for 0.1 mmol of peptidyl resin, 500 mL for 0.25 mmol of peptidyl resin), solubilized in deionised water and lyophilized.

Oxidation (SEA^{on} \rightarrow SEA^{off}) and purification

The crude peptide (59.62 μmol) was dissolved in 50 mM MES buffer pH=6/DMSO 1/1 by vol (8.06 mL). The solution was stirred overnight at rt. The reaction medium was then diluted with eluent A (final volume 80 mL) and purified by RP-HPLC as described in the general procedure (eluent A = water containing 0.1% TFA, eluent B=acetonitrile in water 4/1 by vol containing 0.1% TFA, 30 $^{\circ}\text{C}$, detection at 215 nm).

Table S 1. Synthesis of AcA peptides **3a-e** (method 1).

peptide	scale (mmol)	crude yield	HPLC purified (yield)
4a	0.1 mmol	100 mg (74.7 %)	44.4 mg (33%)
4b	0.1 mmol	107.1 mg (79.2%)	46 mg (34%)
4c	0.1 mmol	107.4 mg (77%)	49.8 mg (35.8%)

4d	0.125 mmol	91.8 mg (46.3%)	17.9 mg (9%)
4e	0.25 mmol	141.3 mg (71.5%)	73.9 mg (37.5%)
4f	0.05 mmol	84.9 mg (44%)	

2.1 Characterization of *N*-acetoacetyl peptide 4a

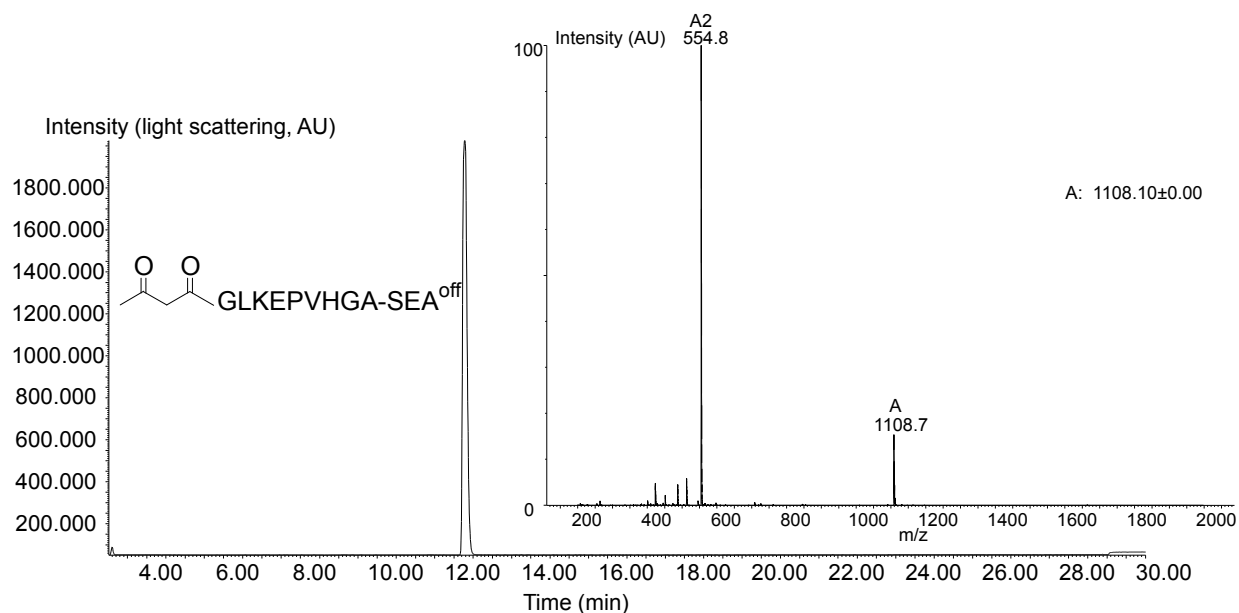


Figure S 1. LC-MS analysis of peptide **4a**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 μm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace. [M+H]⁺ m/z calcd. (monoisotopic) 1108.53, obs 1108.7.

Figure S 3. LC-MS analysis of peptide **4b**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 μm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace. [M+H]⁺ m/z calcd. (monoisotopic) 1122.54, obs 1122.6.

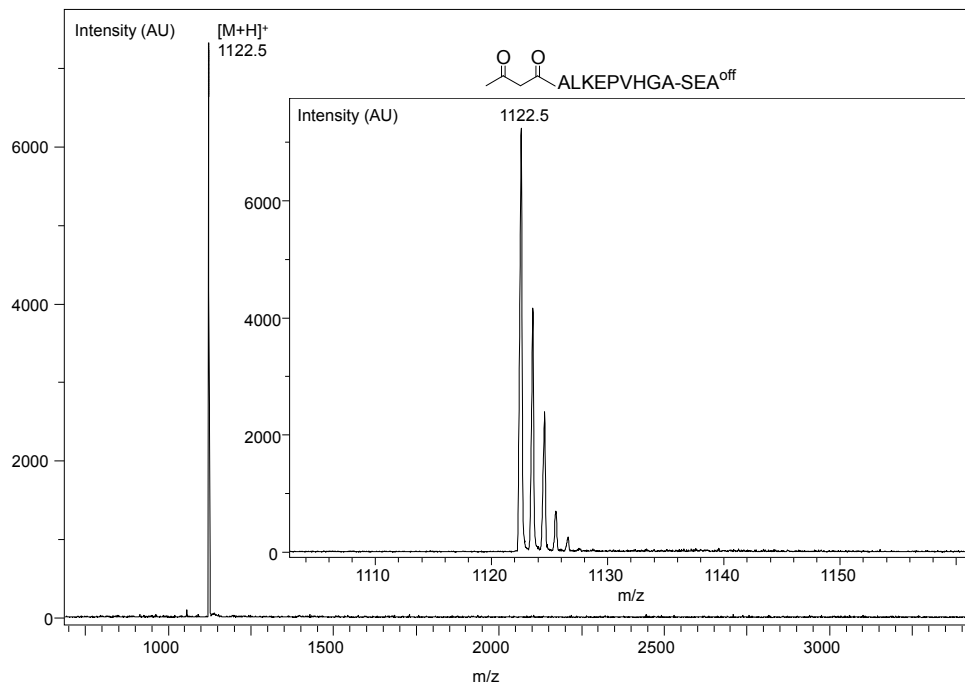


Figure S 4. MALDI-TOF analysis of peptide **4b**. Matrix 2,5-dihydroxybenzoic acid (DHB), positive detection mode, [M+H]⁺ calcd. (monoisotopic) 1122.54, found 1122.5.

2.3 Characterization of *N*-acetoacetyl peptide **4c**

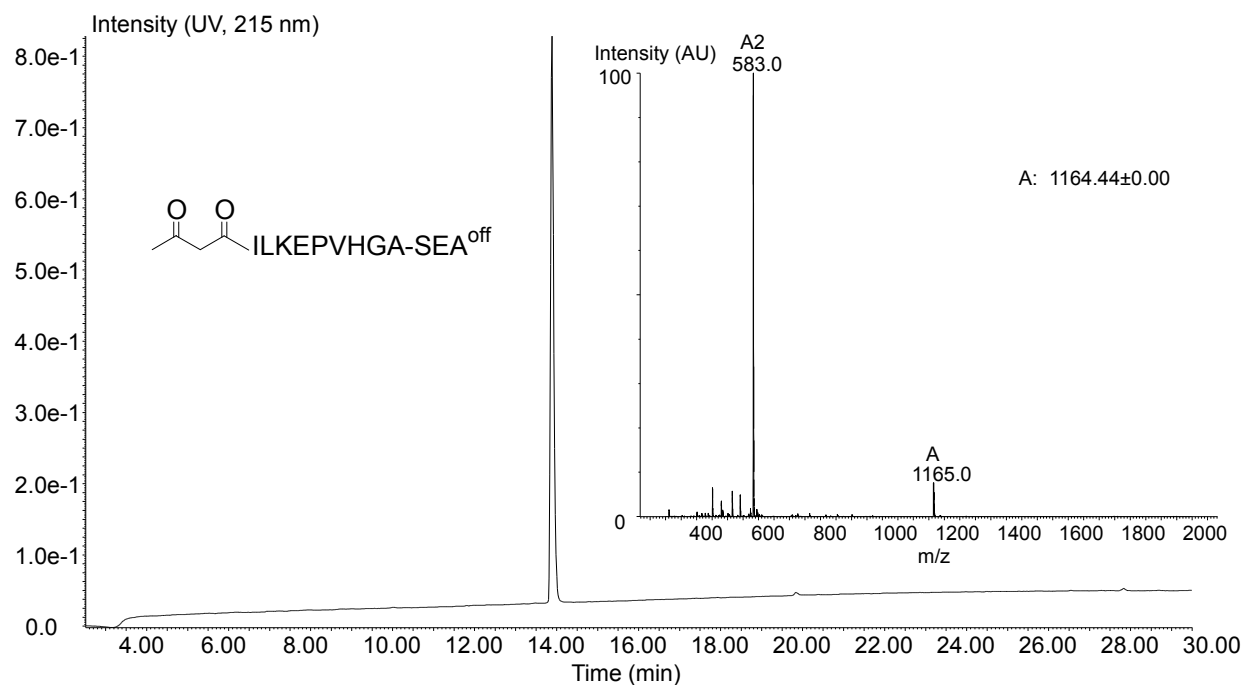


Figure S 5. LC-MS analysis of peptide **4c**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 μm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace. [M+H]⁺ m/z calcd. (monoisotopic) 1164.59, obs 1165.0.

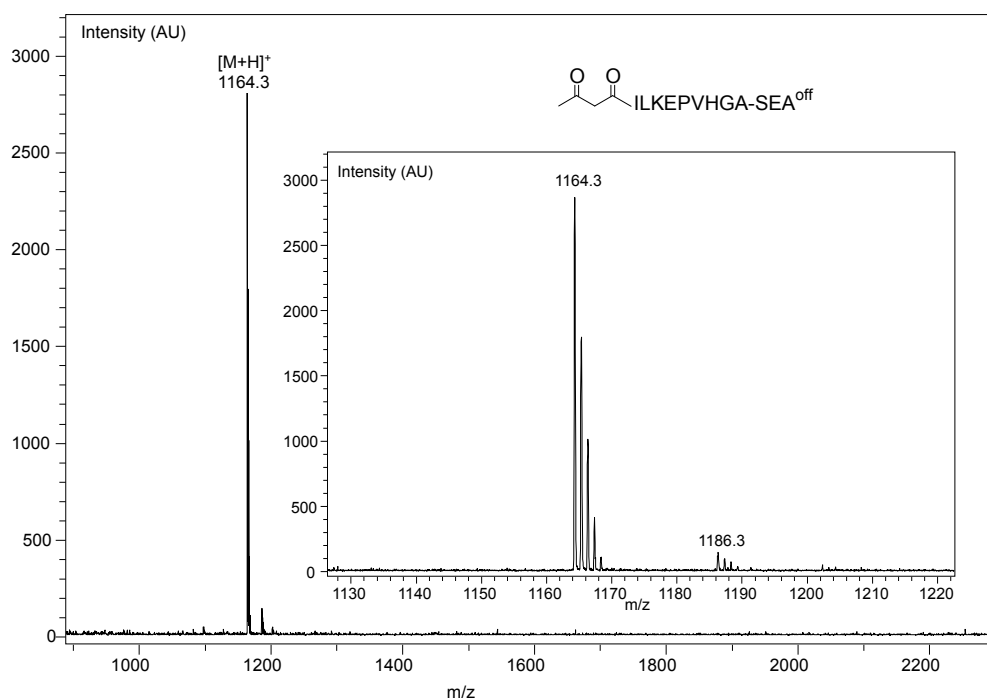


Figure S 6. MALDI-TOF analysis of peptide **4c**. Matrix 2,5-dihydroxybenzoic acid (DHB), positive detection mode, [M+H]⁺ calcd. (monoisotopic) 1164.59, found 1164.3.

2.4 Characterization of *N*-acetoacetyl peptide 4d

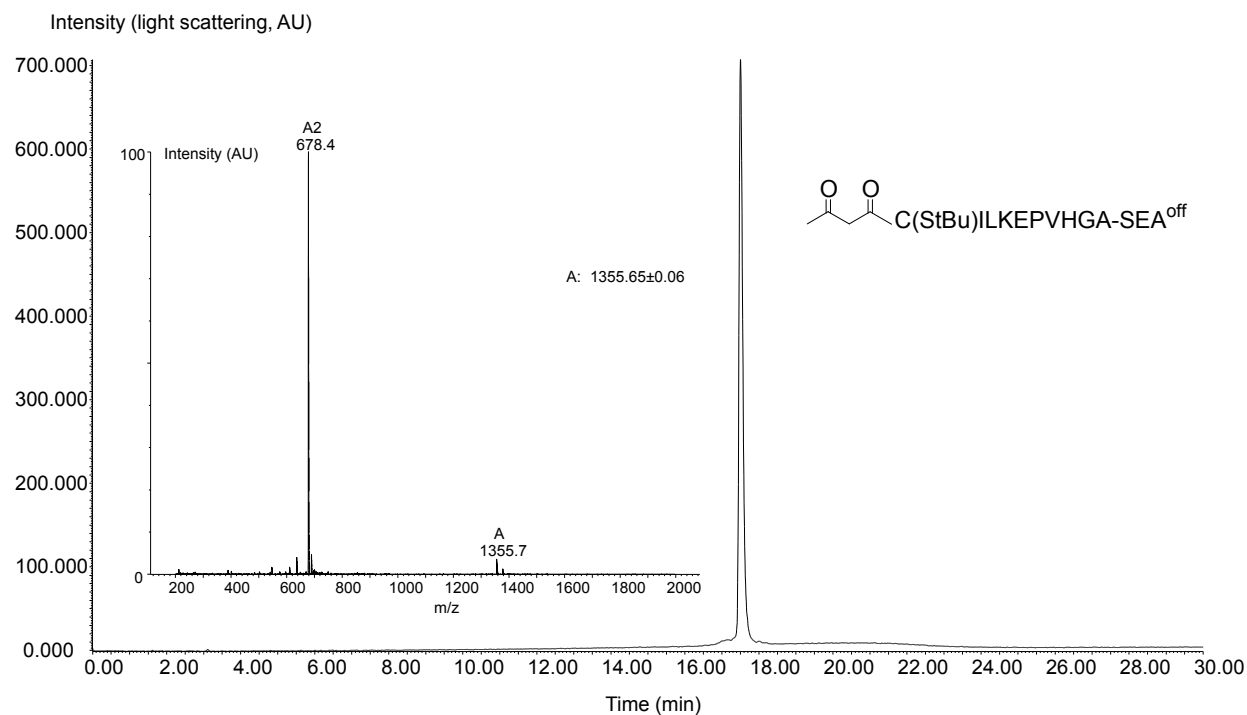


Figure S 7. LC-MS analysis of peptide **4d**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 μm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace. [M+H]⁺ m/z calcd. (monoisotopic) 1355.53, obs 1355.7.

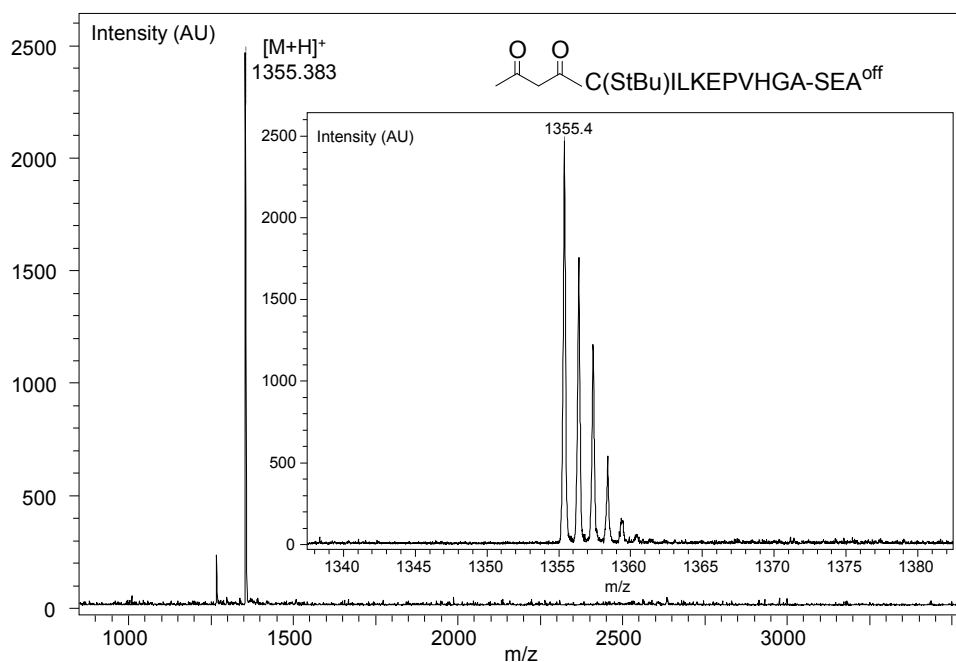


Figure S 8. MALDI-TOF analysis of peptide **4d**. Matrix 2,5-dihydroxybenzoic acid (DHB), positive detection mode, $[M+H]^+$ calcd. (monoisotopic) 1355.53, found 1355.4.

2.5 Characterization of N-acetoacetyl peptide 4e

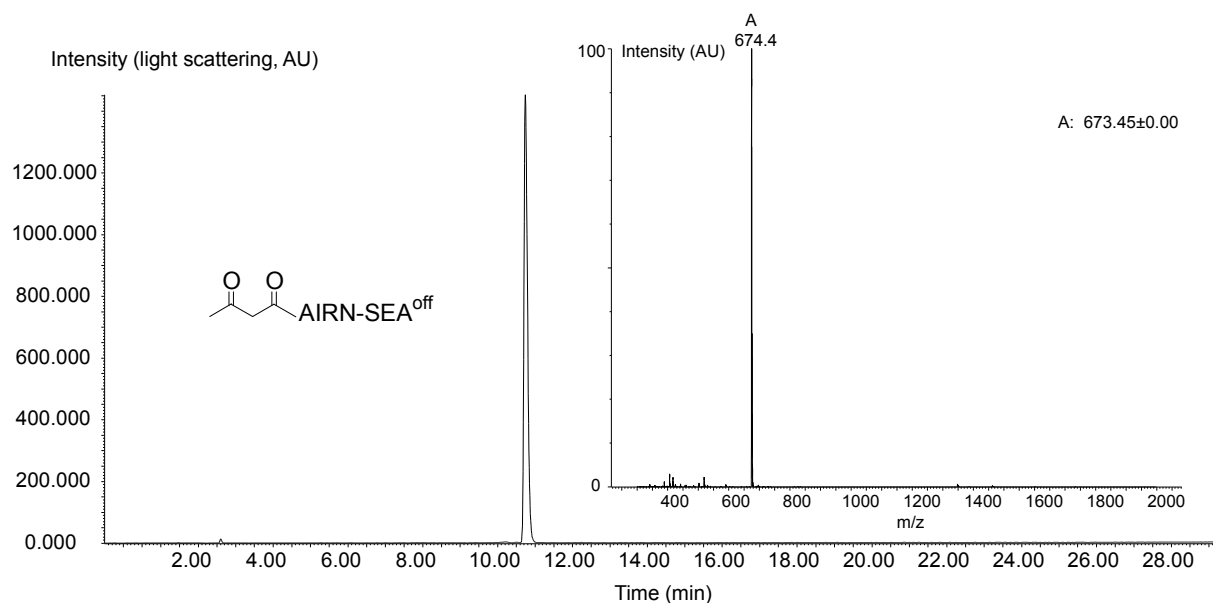


Figure S 9. LC-MS analysis of peptide **4e**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in $\text{CH}_3\text{CN}/\text{water}$: 4/1 by vol. C18 Xbridge BEH 300 Å 5 μm (4.6 \times 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace. $[M+H]^+$ m/z calcd. (monoisotopic) 674.31, obs 674.4.

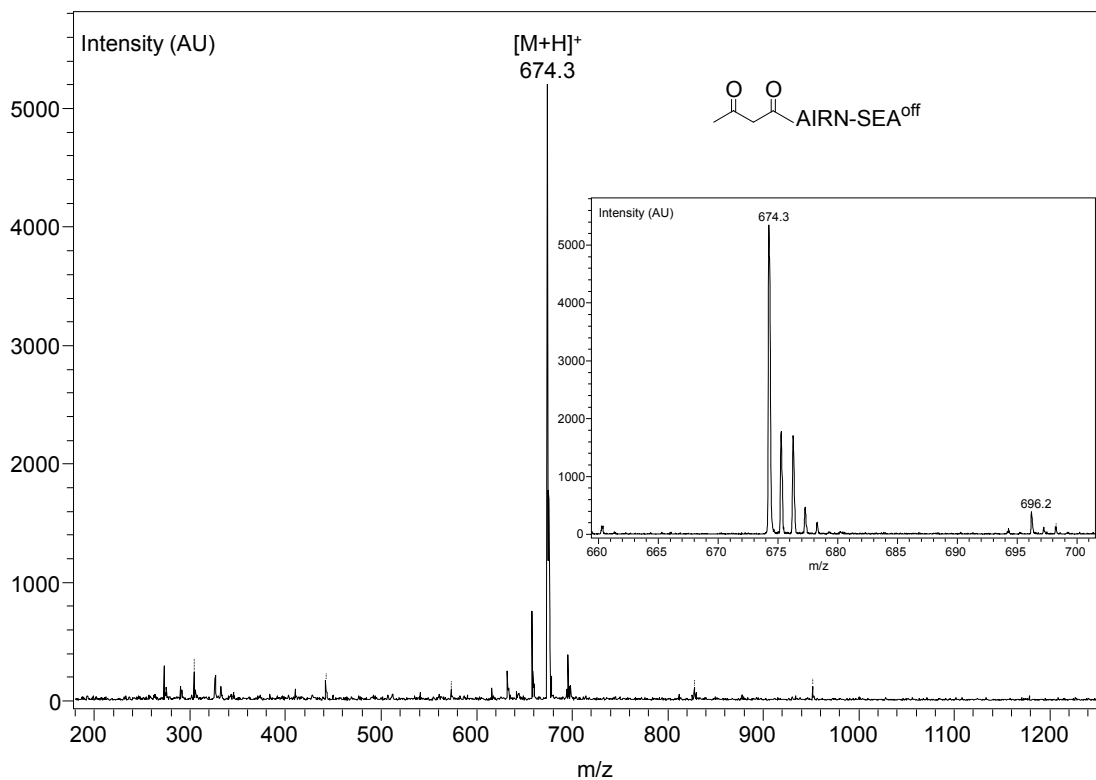
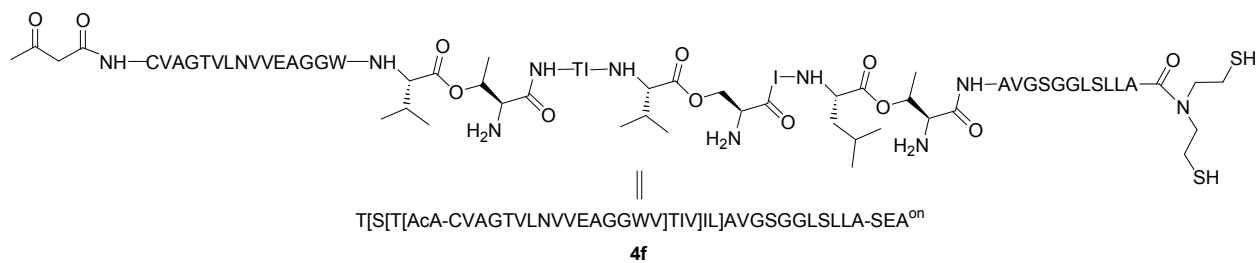


Figure S 10. MALDI-TOF analysis of peptide **4e**. Matrix 2,5-dihydroxybenzoic acid (DHB), positive detection mode, $[M+H]^+$ calcd. (monoisotopic) 674.31, found 674.3.

2.6 Characterization of *N*-acetoacetyl peptide **4f**



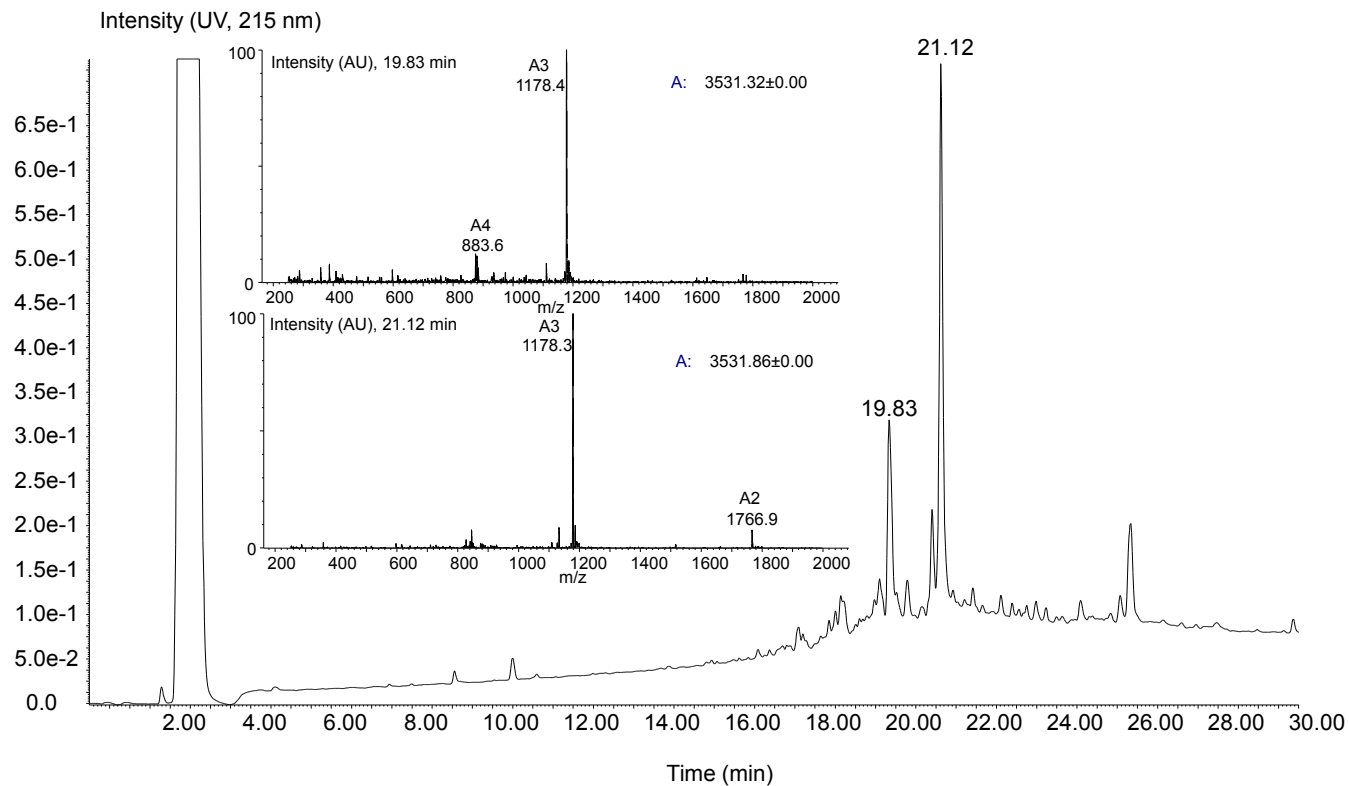
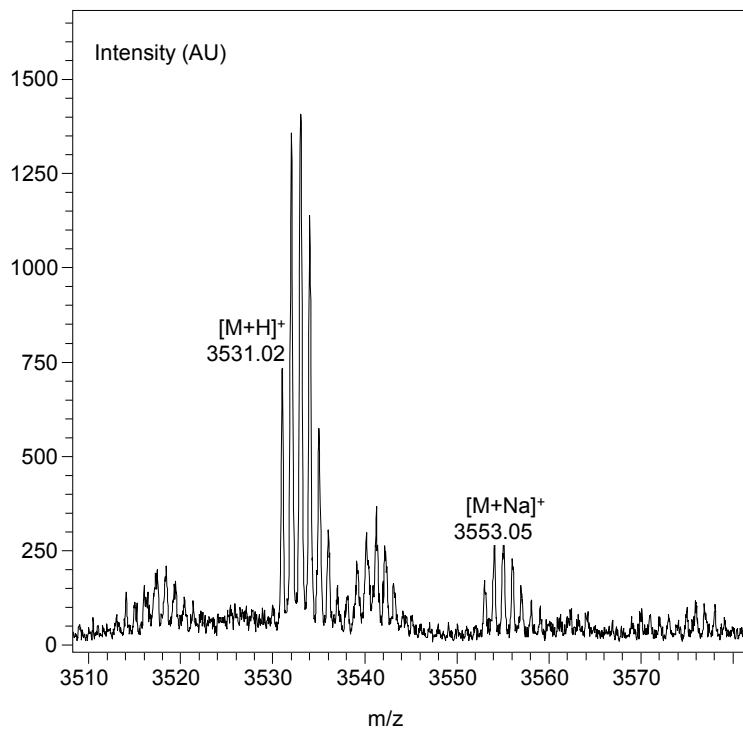
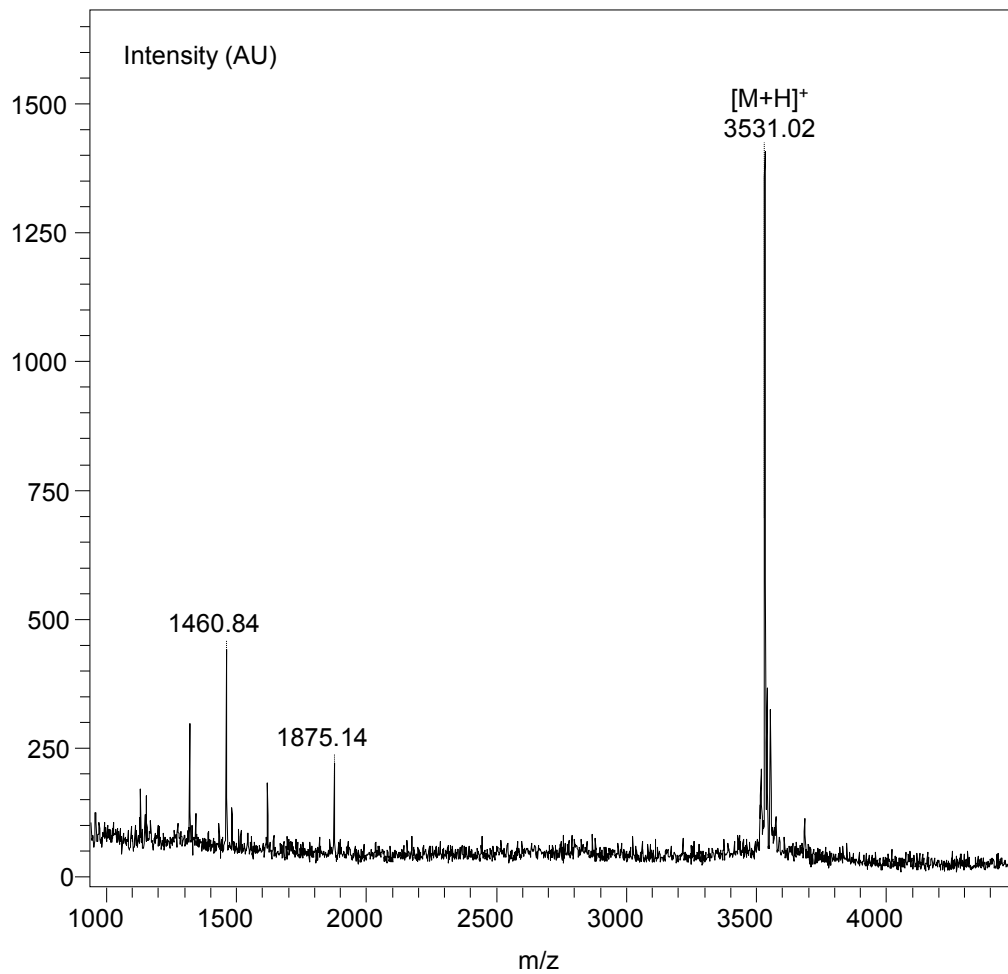


Figure S 11. LC-MS analysis of peptide **4f**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 µm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). The peak at 19.83 min corresponds to the thioester form of the SEA^{on} group. The peak at 21.12 min corresponds to the amide form of the SEA^{on} group. MS trace. [M+3H]³⁺ m/z calcd. (mean) 1178.42, obs 1178.3, [M+4H]⁴⁺ m/z calcd 884.06, obs 883.6.



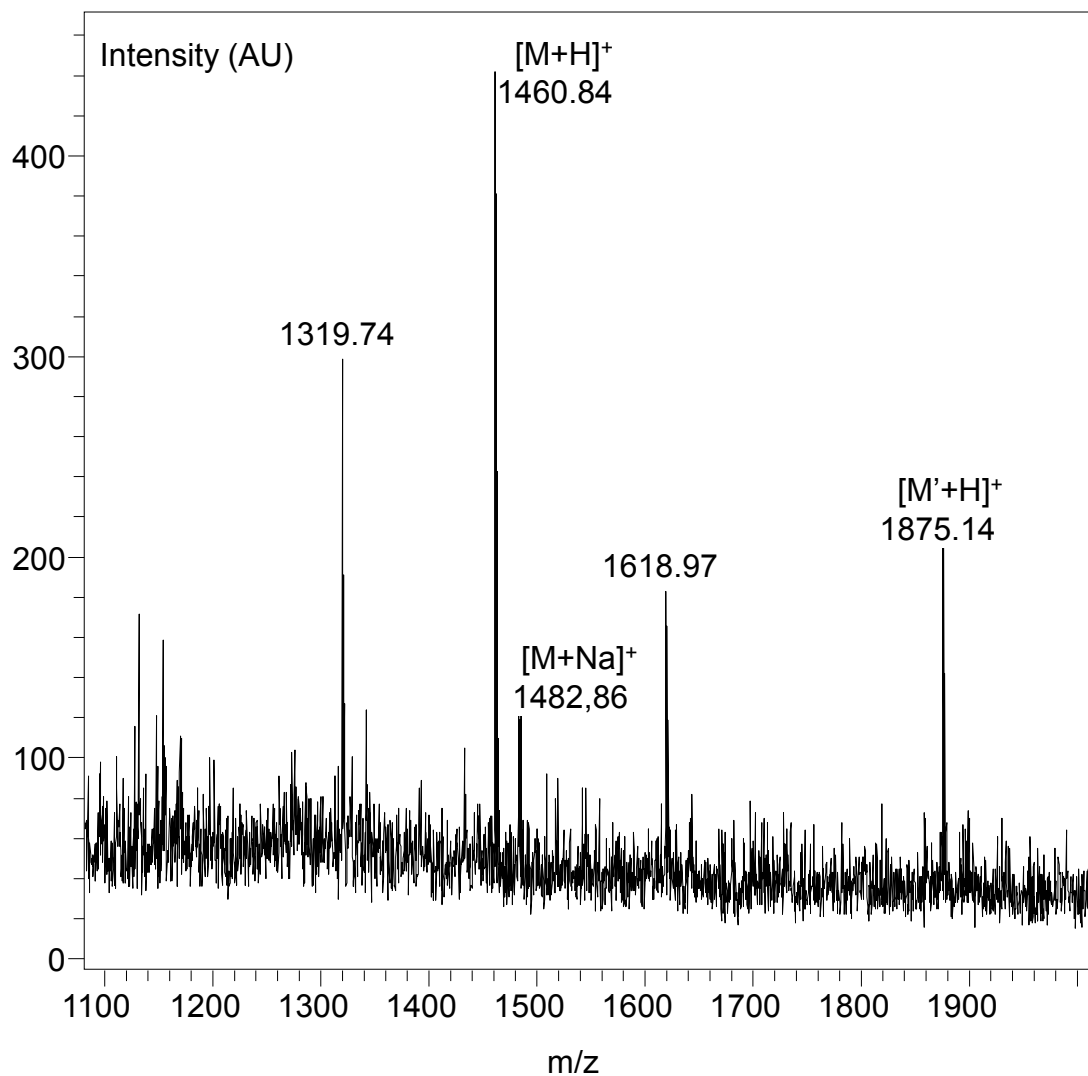


Figure S 12. MALDI-TOF analysis of peptide **4f**. Matrix 2,5-dihydroxybenzoic acid (DHB), positive detection mode, $[M+H]^+$ calcd. (monoisotopic) 3530.87, found 3531.02.

Figure S 12 shows that peptide **4f** decomposes during the ionization process according to a McLafferty mechanism. This is a typical signature of *O*-acyl isopeptides that has been discussed previously by us.¹ The formed enamine products hydrolyze into β -ketoamides as shown below.

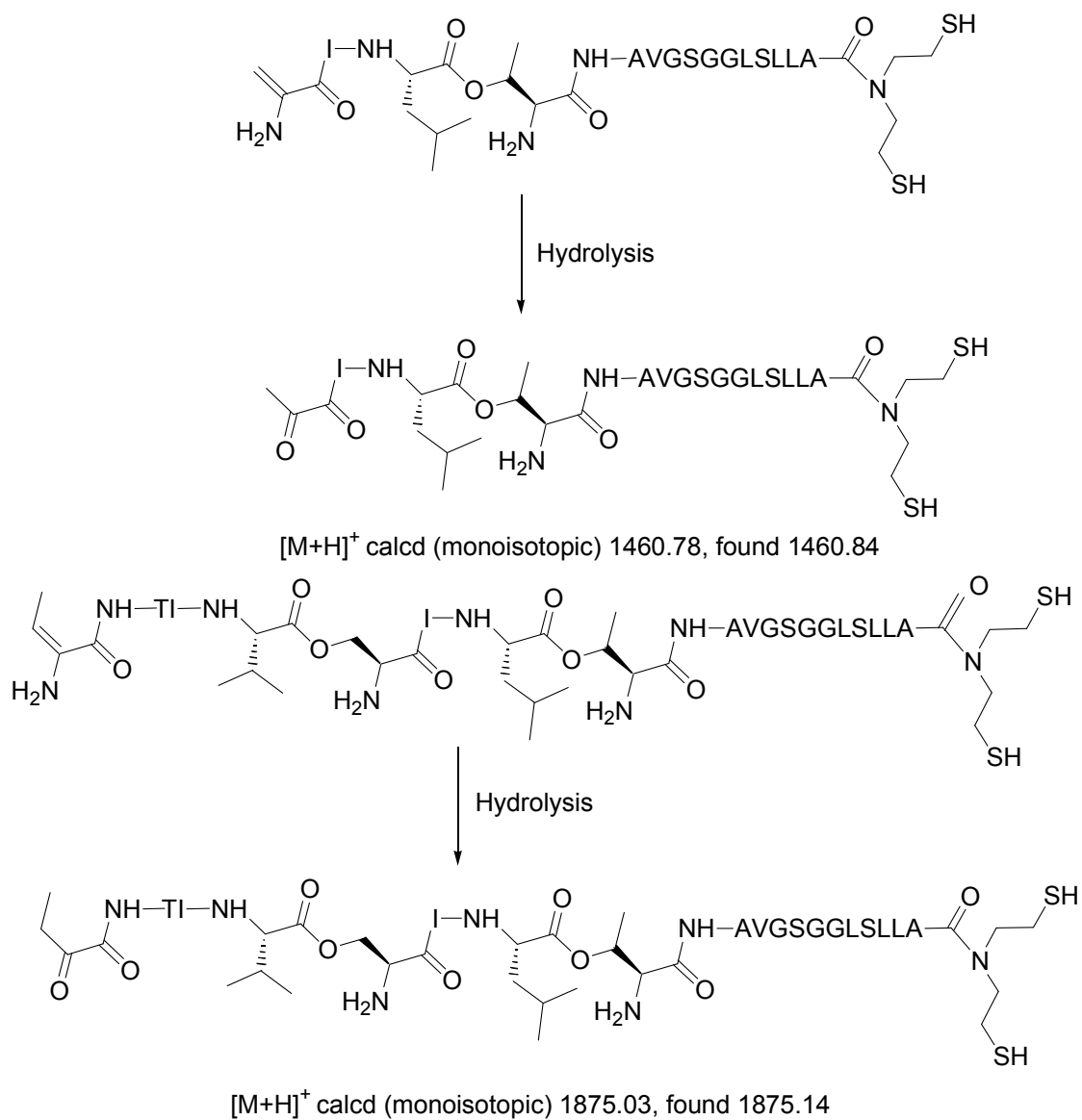


Figure S 13. *O*-acyl isopeptides decompose in the gas phase during the MALDI-TOF desorption/ionization process according to a McLafferty rearrangement. Ions presumably formed during the analysis of peptide **4f**.

2.7 Characterization of *N*-acetoacetyl peptide **8**

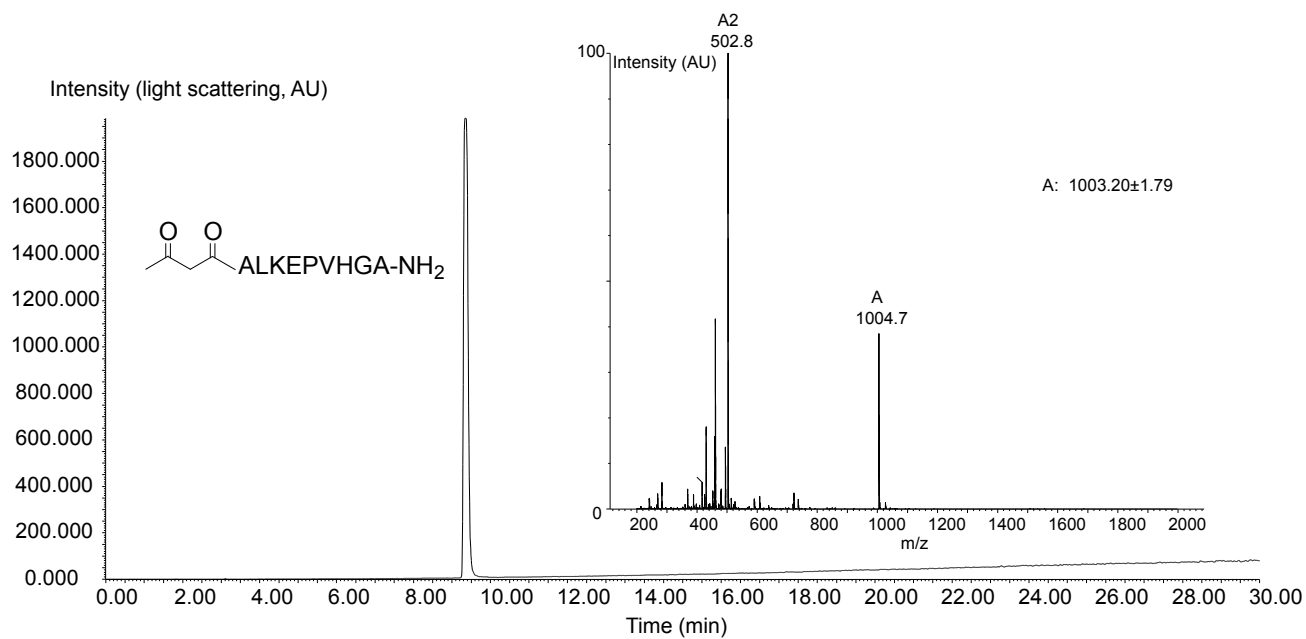


Figure S 14. LC-MS analysis of peptide **8**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 μm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace. [M+H]⁺ m/z calcd. (monoisotopic) 1004.55, obs. 1004.7.

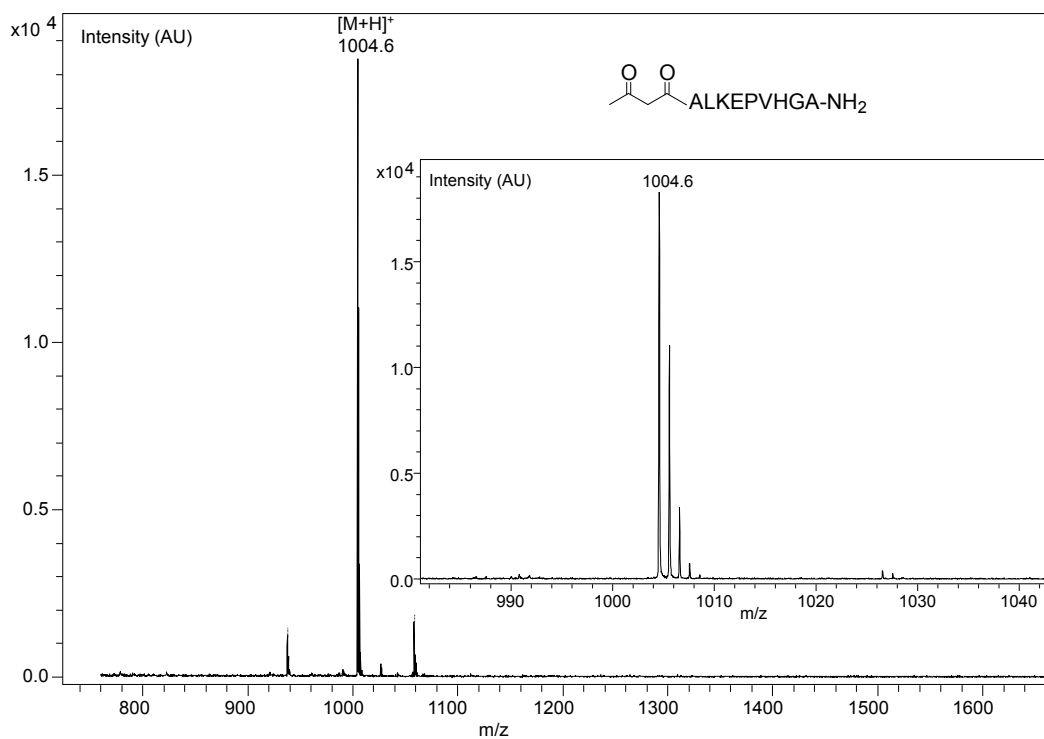


Figure S 15. MALDI-TOF analysis of peptide **8**. Matrix, 2,5-dihydroxybenzoic acid (DHB), positive detection mode, [M+H]⁺ calcd. (monoisotopic) 1004.55, found 1004.6.

3. Synthesis of *N*-acetoacetyl peptide 7 by NCL using acetoacetyl thioester 6 (Scheme 1, method B)

0.1 M phosphate buffer pH 7 was degassed during 30 minutes. 6 M Gn.HCl (2.86 g) was dissolved in this buffer (2.950 mL) to give a final volume of 5 mL and added on 4-mercaptophenylacetic acid (168.3 mg, ~0.2 M final concentration). 6 M NaOH (290 μ L) was added to adjust the pH to 7-7.5. Peptide H-C(StBu)IIGKGRSYKGTVSITKSGIK-SEA^{off} (19.8 mg, 6.43 μ mol) was dissolved in this solution (1.5 mL). This last solution was added to AcA-MPA (1.47 mg, 7.72 μ mol). The reaction was placed under inert atmosphere at 37°C overnight. The reaction mixture was then acidified with glacial acetic acid (400 μ L) and diluted with water (8 mL final volume). Extractions with diethyl ether were done to remove 4-mercaptophenylacetic acid before purification by RP-HPLC (eluent A = water containing 0.1% TFA, eluent B=acetonitrile in water 4/1 containing 0.1% TFA, 30°C, detection at 215 nm, 6 mL/min, 0 to 20% eluent B in 5 min, then 20 to 40% eluent B in 60 min, C18XBridge column) to give 11.9 mg of peptide 7 (62.4%).

3.1 Characterization of *N*-acetoacetyl peptide 7

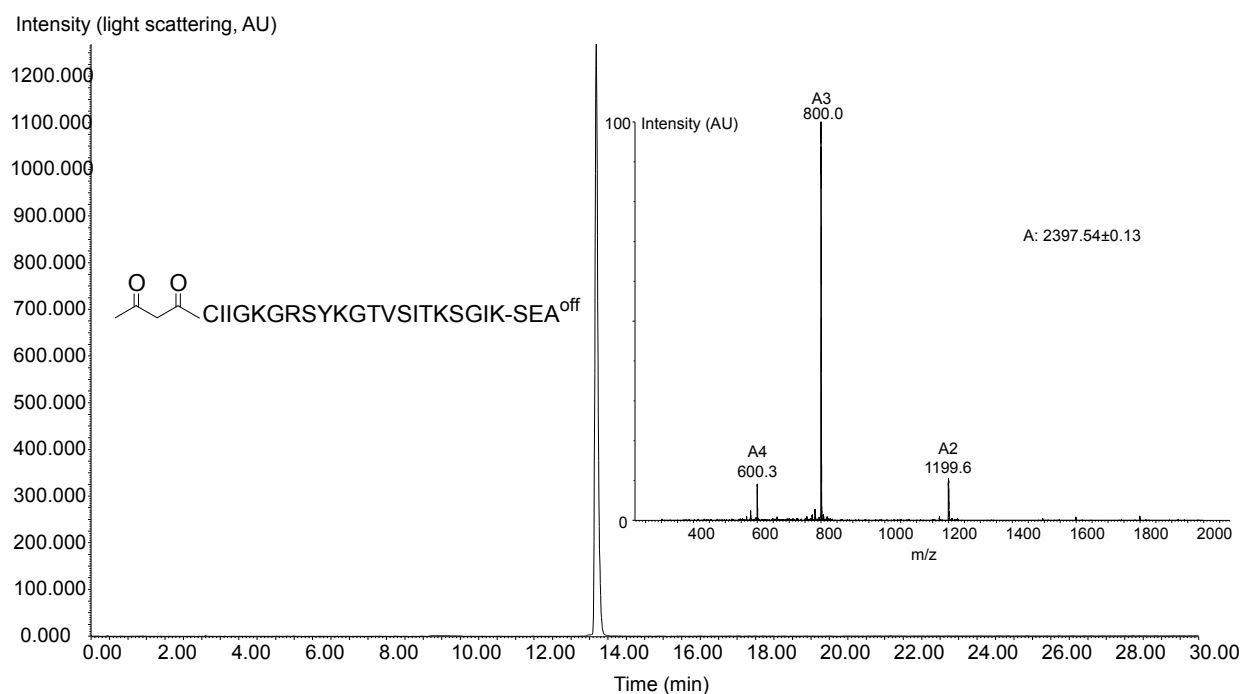


Figure S 16. LC-MS analysis of peptide 7. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 μ m (4.6 \times 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace: [M+2H]²⁺ m/z calcd (mean). 1199.98, obs. 1199.6, [M+3H]³⁺ m/z calcd. 800.32, obs.800.0, [M+4H]⁴⁺ m/z calcd.600.49, obs. 600.3.

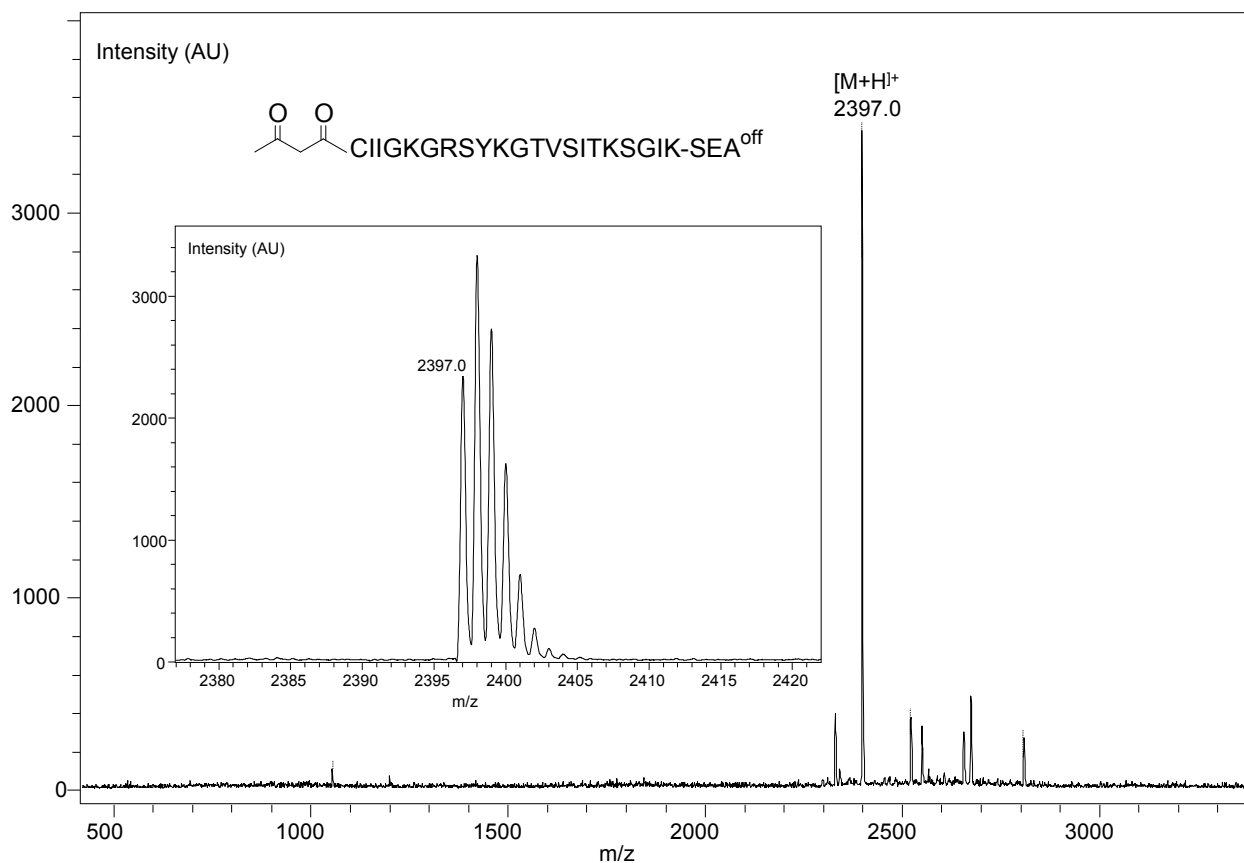


Figure S 17. MALDI-TOF analysis of peptide **7**. Matrix 2,5-dihydroxybenzoic acid (DHB), positive detection mode, $[M+H]^+$ calcd. (monoisotopic) 2397.28, found 2397.0.

4. Synthesis of AcAO peptide **9** and **10a-d**

*A typical protocol is illustrated with the synthesis of AcAO peptide **9***

Gn.HCl (573.5 mg) was dissolved in 0.1 M acetate buffer pH 4.4 (590 μ L). 6 M NaOH (3 μ L) was added to adjust the pH to 4.8. Then peptide AcA-ALKEPVHGA-NH₂ **8** (9.99 mg, 8.11 μ mol) was dissolved in this solution (final volume \sim 600 μ L). Aminoxyacetic acid (884 μ g, 8.11 μ mol) was dissolved in the same solution above (210.8 μ L) and added to the peptide solution. The measured pH was 4.18. The reaction was stirred at 37 $^{\circ}$ C. After 5 hours, the reaction mixture was diluted to 8 mL with eluent A and purified by RP-HPLC (preparative column XBridge BEH300 Prep C18, 5 μ m, 300 \AA , 10 \times 250 mm) to give 7.47 mg of AcAO peptide **9** (70.6%).

Table S 2. Synthesis of AcAO peptides **8** and **10a-d**.

starting AcA peptide (mass)	AcAO peptide (mass, isolated yield)
8 (9.99 mg)	9 (7.47 mg, 70.6%)
4a (10.64 mg)	10a (10.1 mg, 90.2%)
4b (29.9 mg)	10b (26.4 mg, 84%)
4c (10.22 mg)	10c (9.7 mg, 90.2%)
4d (6.41 mg)	10d (4.64 mg, 69.3%)

4.1 Characterization of AcAO peptide **9**

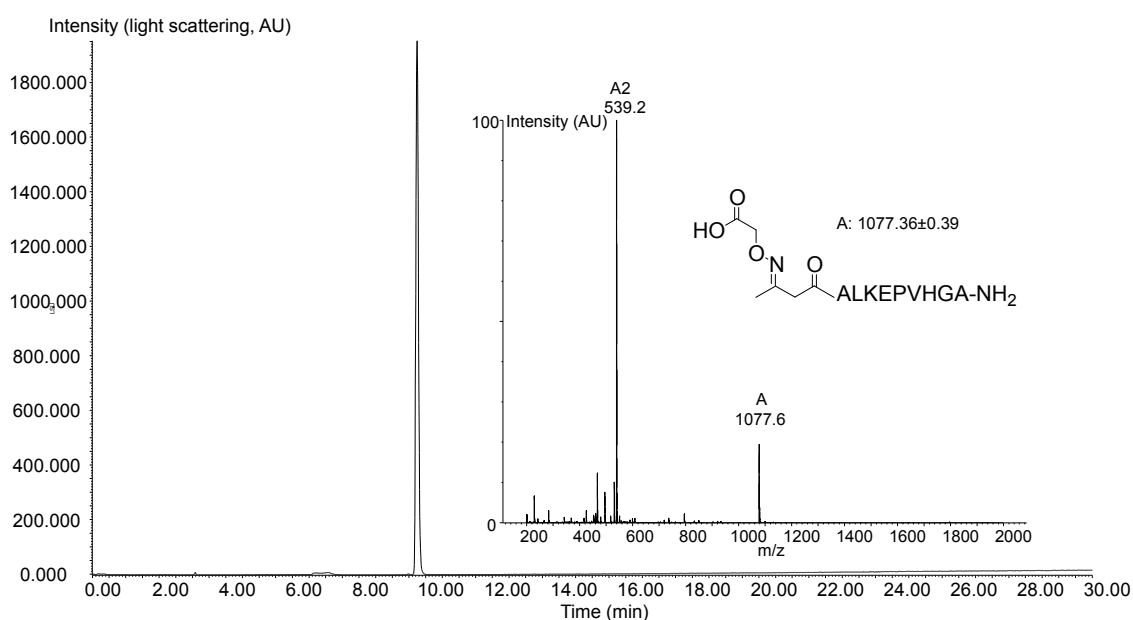


Figure S 18. LC-MS analysis of peptide **9**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 µm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace. [M+H]⁺ m/z calcd. (monoisotopic) 1077.57, obs 1077.6.

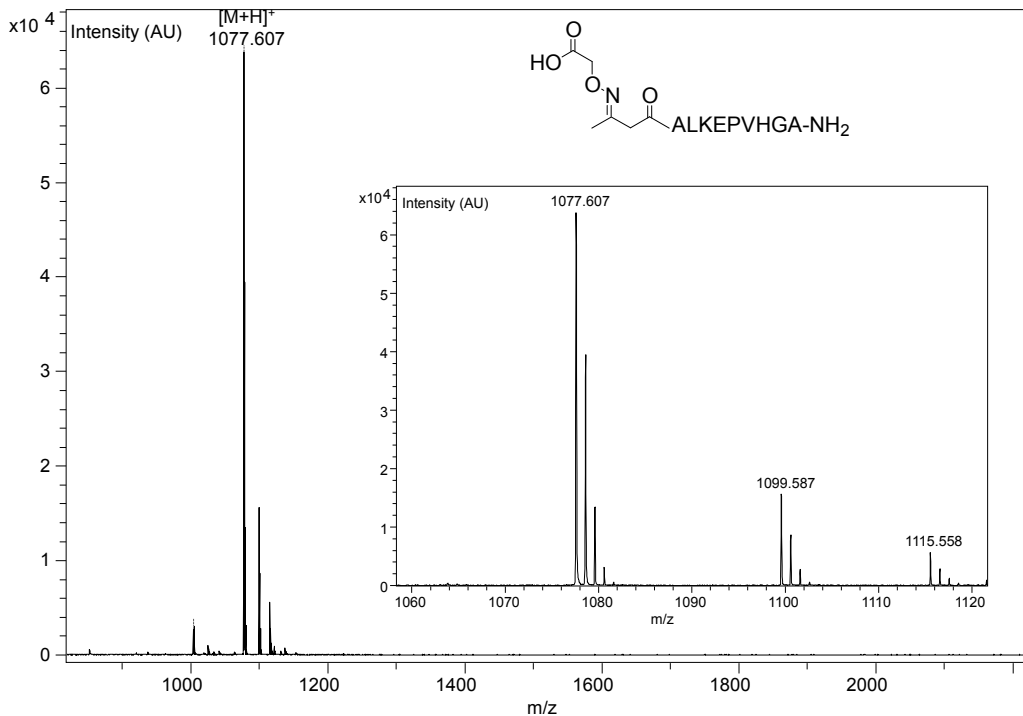


Figure S 19. MALDI-TOF analysis of peptide **9**. Matrix alpha cyano 4-hydroxycinnamic acid, positive detection mode, $[M+H]^+$ calcd. (monoisotopic) 1077.57, found 1077.6.

NMR analysis of AcAO peptide **9**

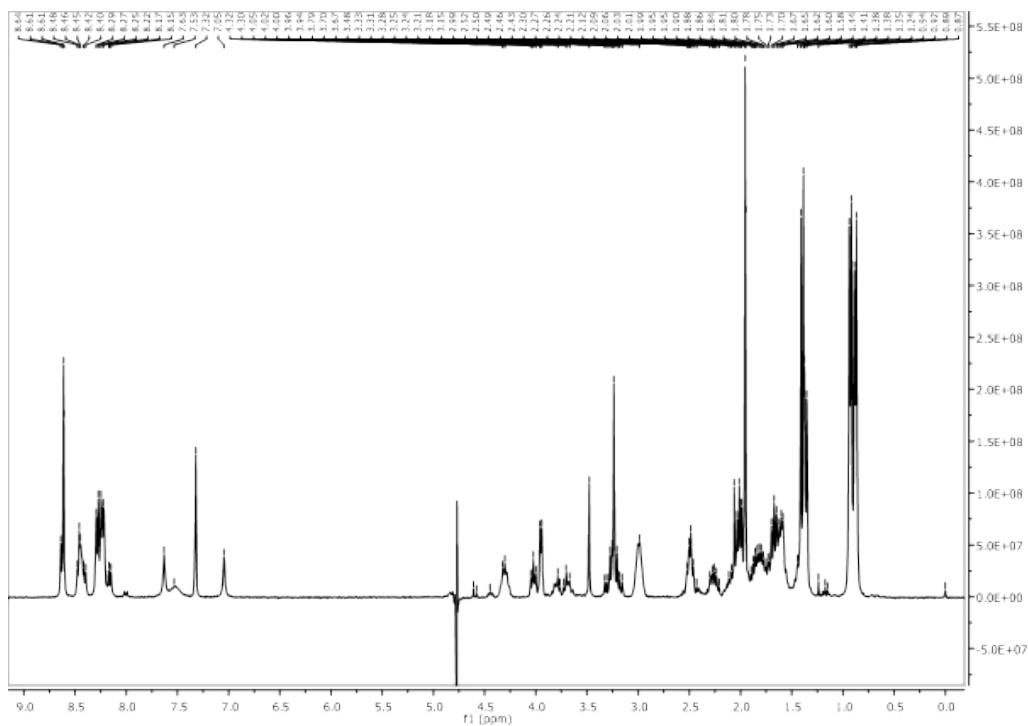


Figure S 20. ^1H NMR spectrum (300.0 MHz, $\text{H}_2\text{O}/\text{D}_2\text{O}$ 90/10 by vol) for AcAO peptide **9**.

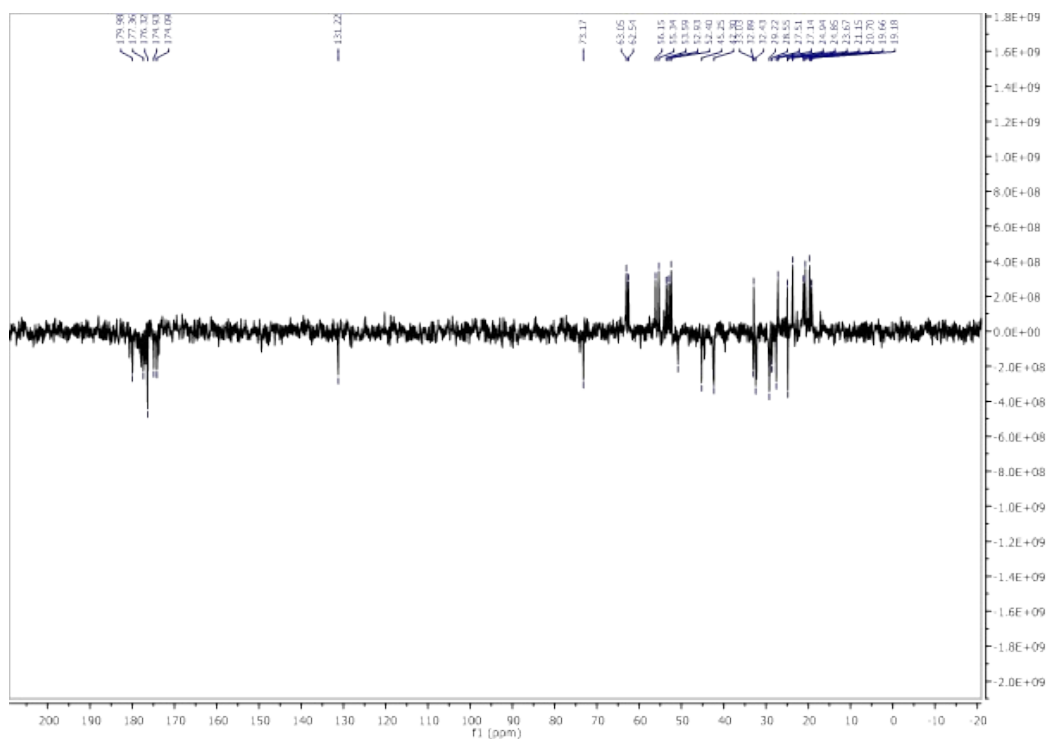


Figure S 21. ^{13}C NMR spectrum (75 MHz, $\text{H}_2\text{O}/\text{D}_2\text{O}$ 90/10 by vol) for AcAO peptide **9**.

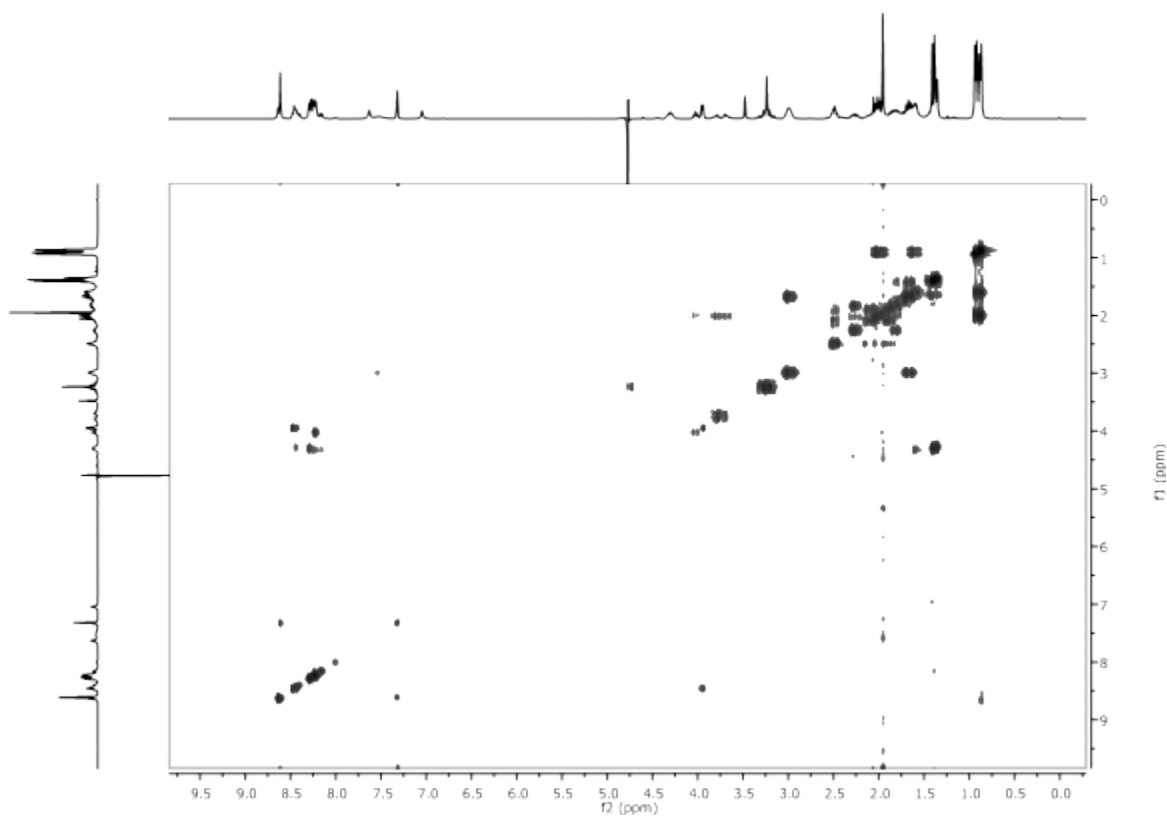
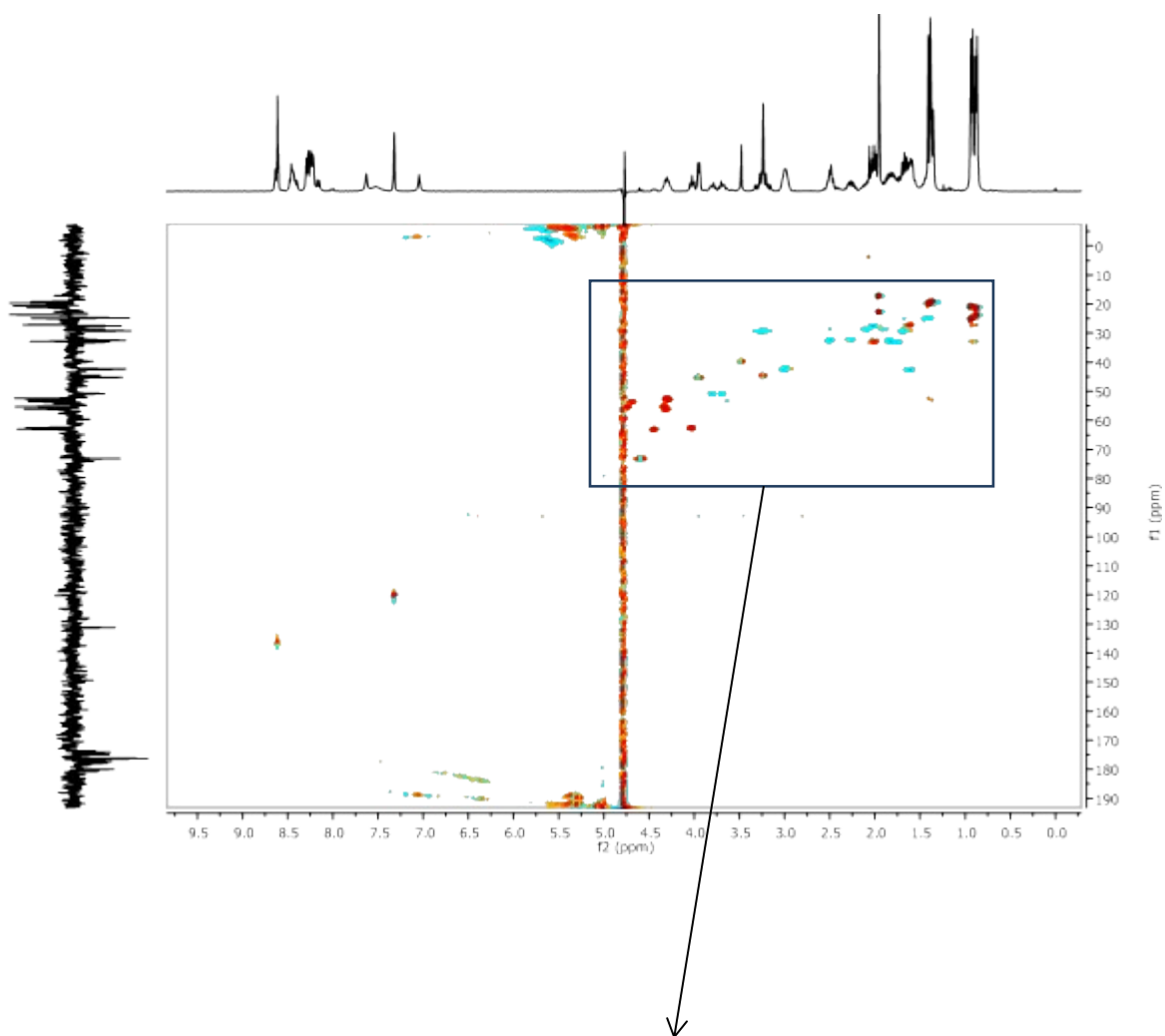


Figure S 22. ^1H - ^1H COSY spectrum ($\text{H}_2\text{O}/\text{D}_2\text{O}$ 90/10 by vol) for AcAO peptide 9.



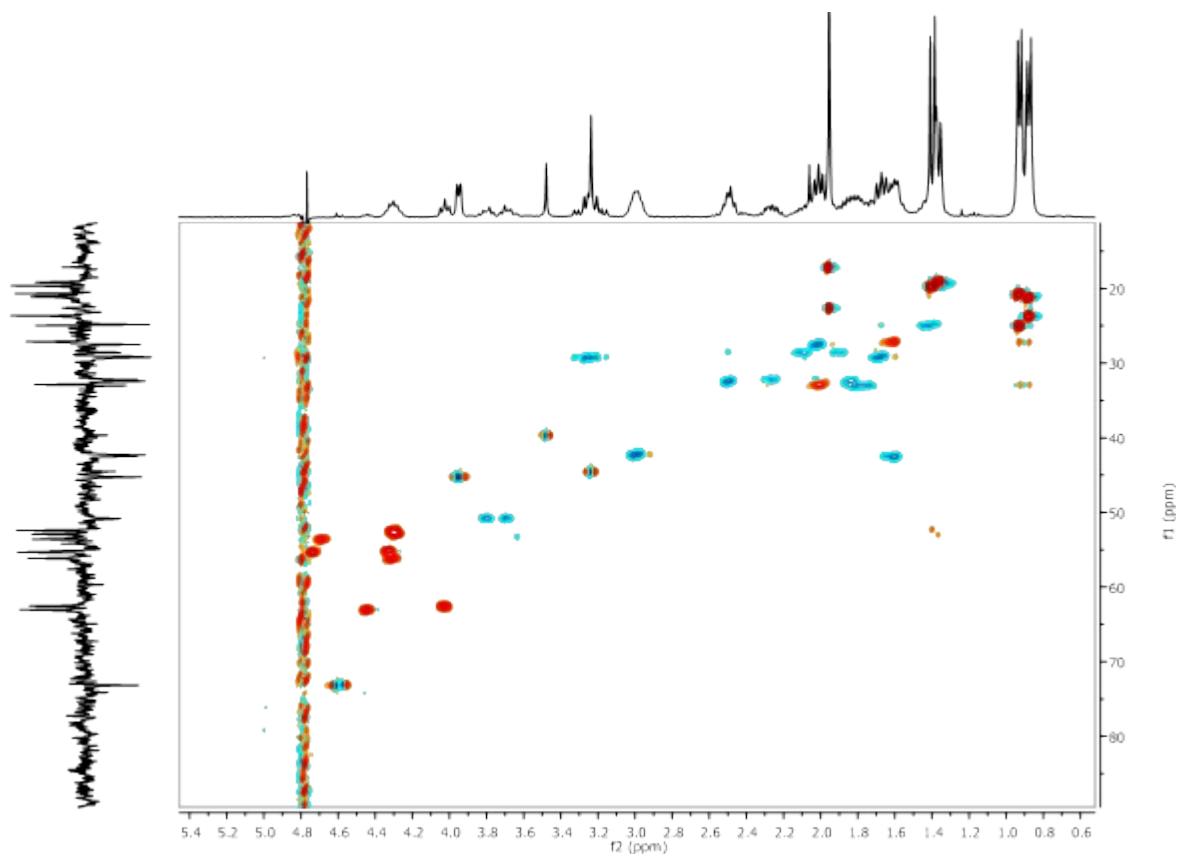


Figure S 23. ^1H - ^{13}C HSQC spectrum ($\text{H}_2\text{O}/\text{D}_2\text{O}$ 90/10 by vol) for AcAO peptide 9.

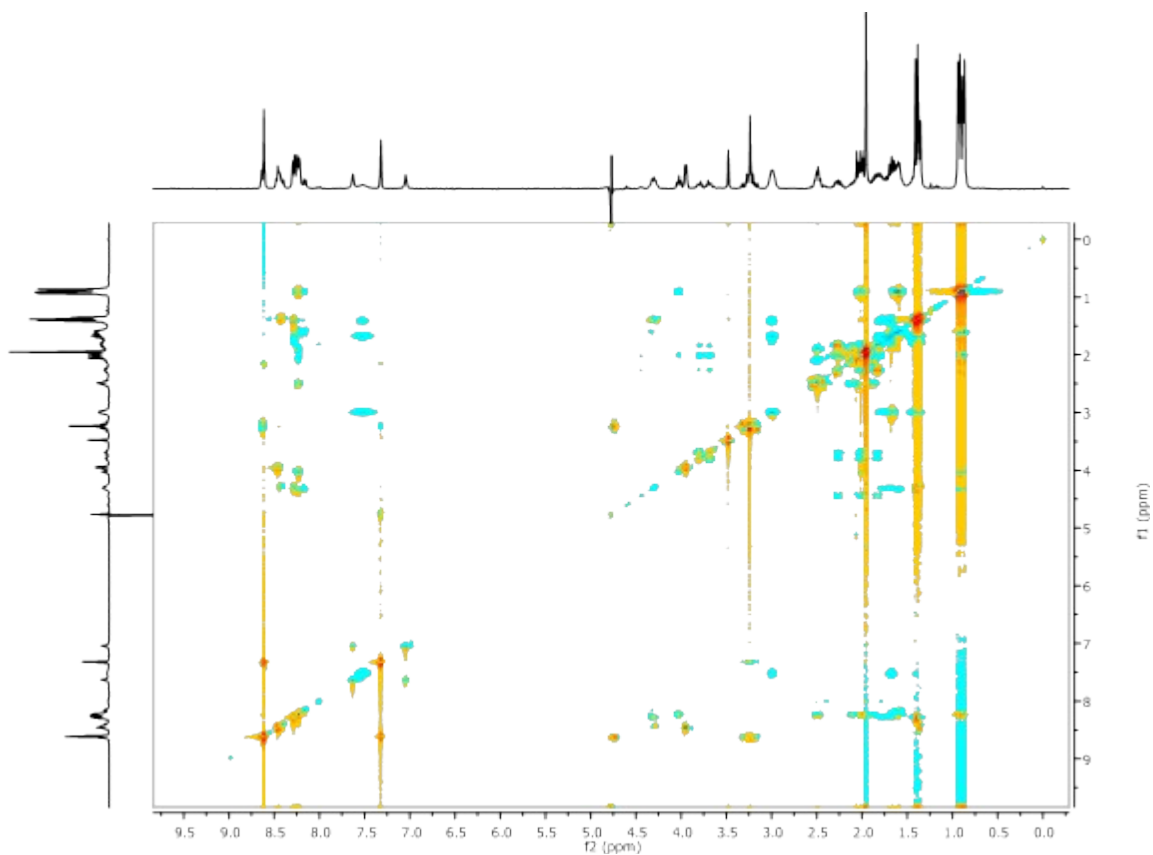


Figure S 24. ^1H - ^1H DIPSI spectrum (TOCSY) ($\text{H}_2\text{O}/\text{D}_2\text{O}$ 90/10 by vol) for AcAO peptide **9**.

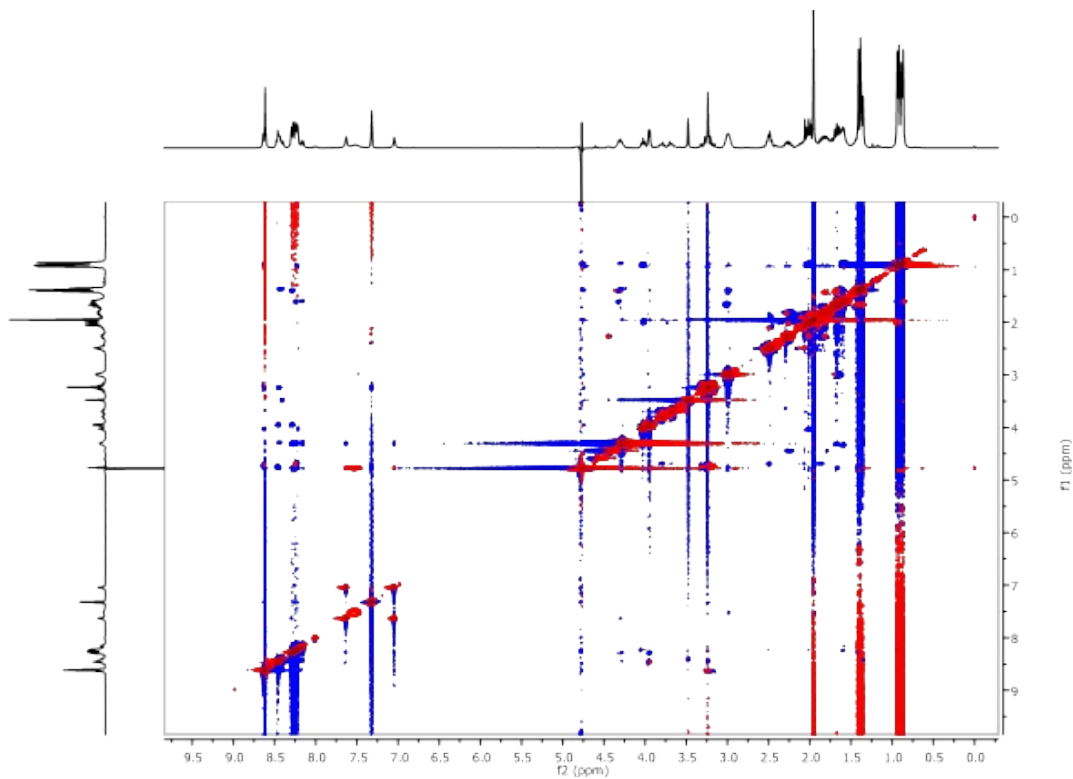


Figure S 25. ^1H - ^1H NOESY spectrum (TOCSY) ($\text{H}_2\text{O}/\text{D}_2\text{O}$ 90/10 by vol) for AcAO peptide **9**.

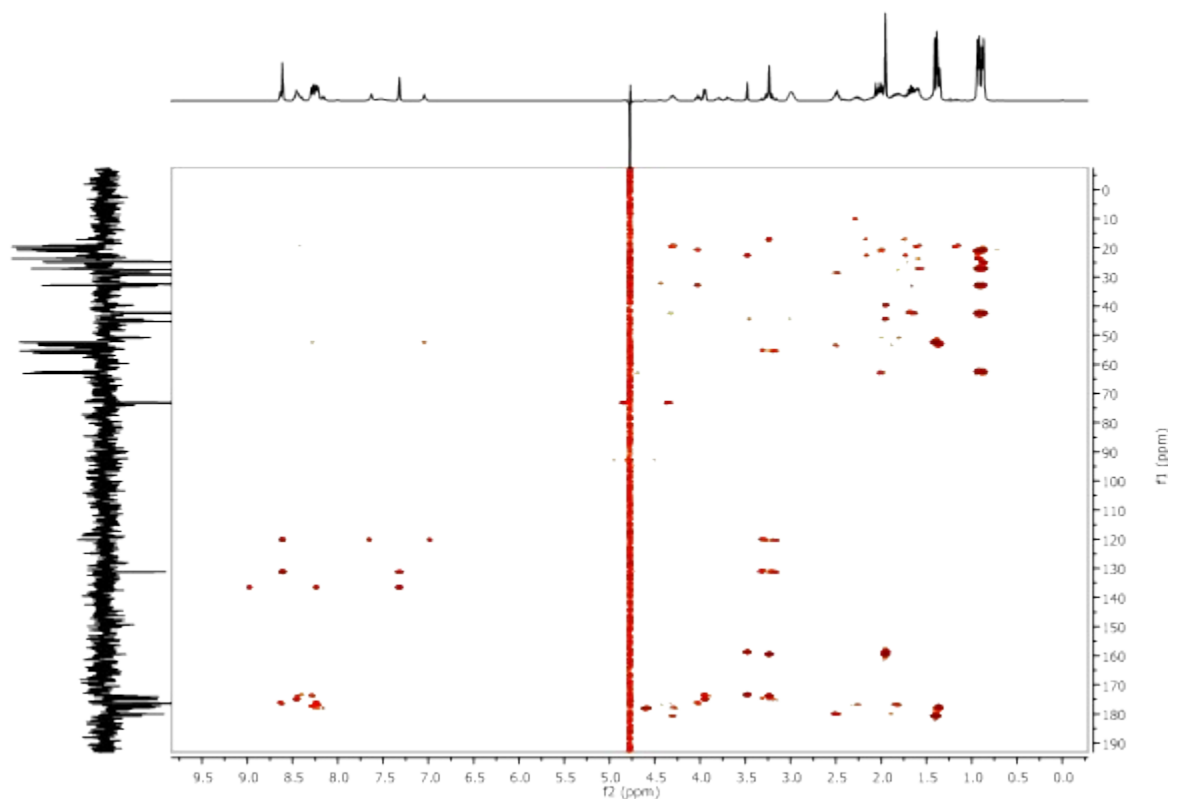


Figure S 26. ^1H - ^{13}C HMBS spectrum (TOCSY) ($\text{H}_2\text{O}/\text{D}_2\text{O}$ 90/10 by vol) for AcAO peptide **9**.

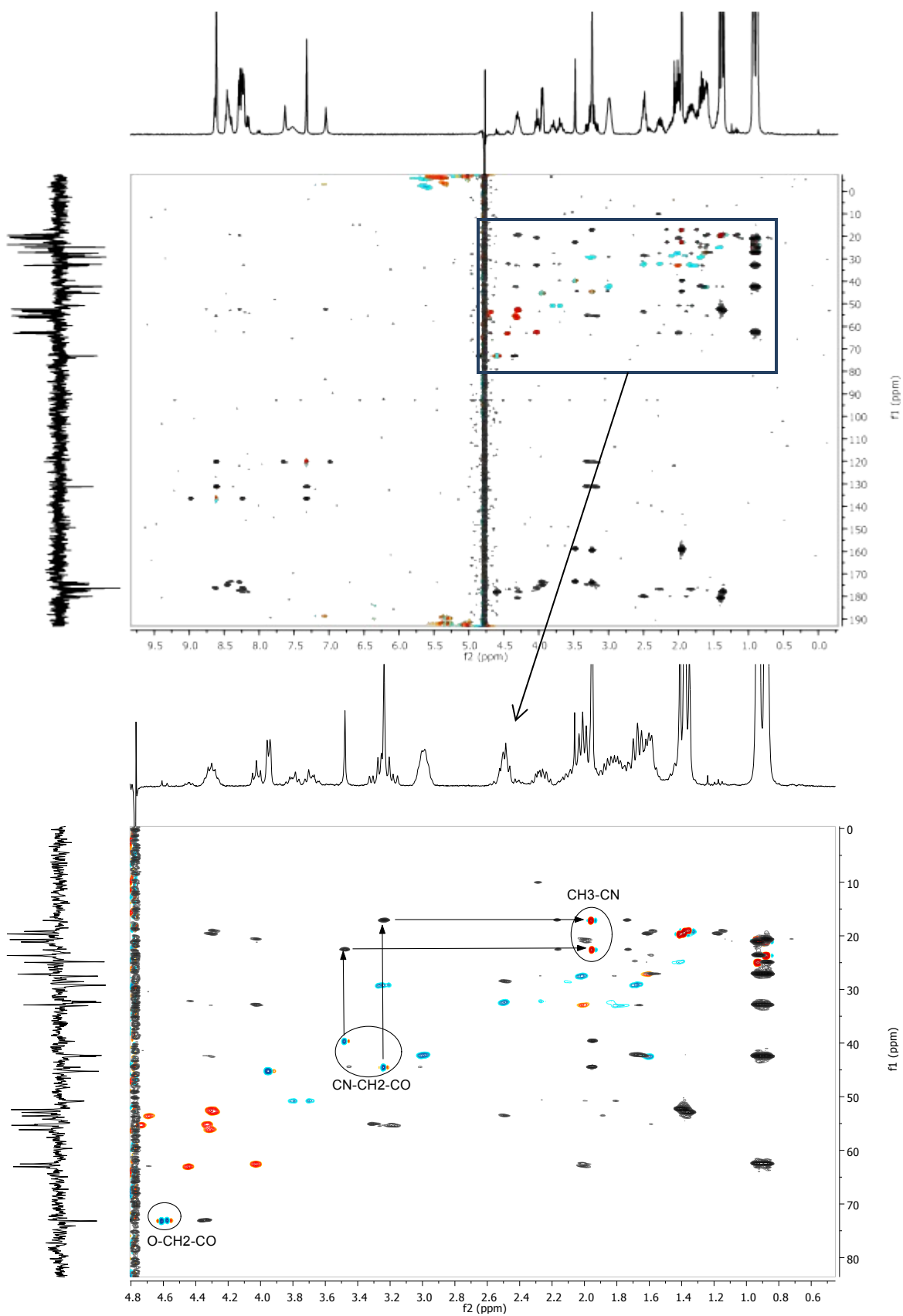


Figure S 27. HMBC/HSCQ spectrum (75 MHz, H₂O/D₂O 90/10 by vol) for AcAO peptide 9.

4.2 Characterization of AcAO peptide 10a

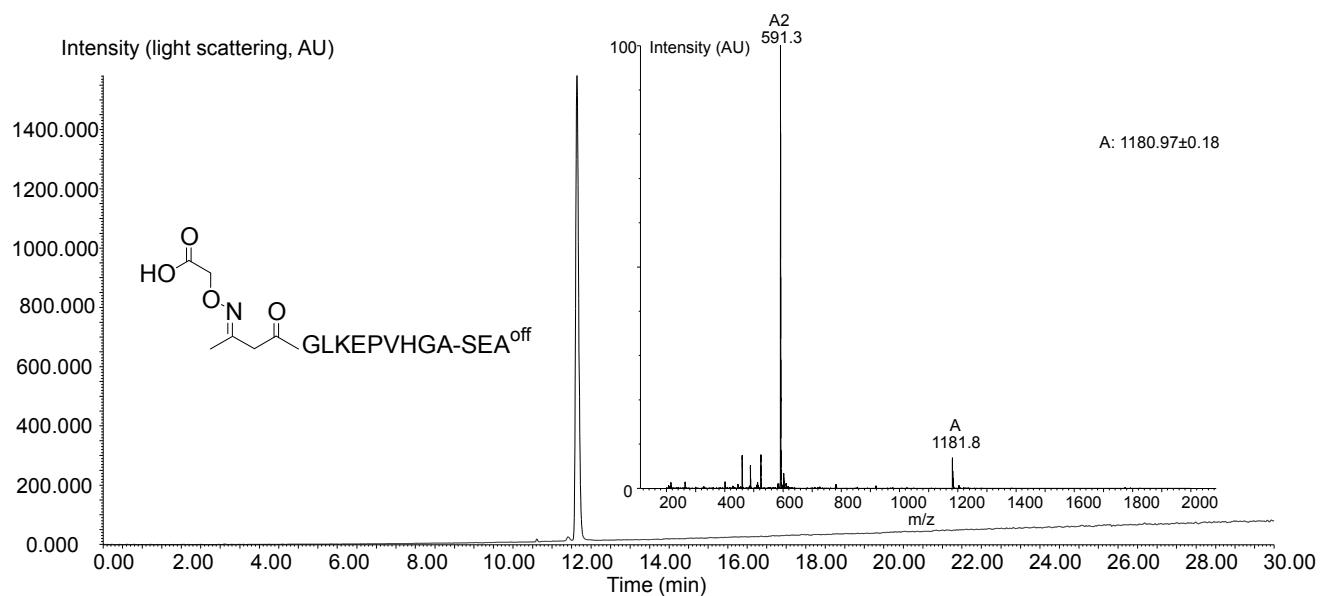


Figure S 28. LC-MS analysis of peptide **10a**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 μm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace. [M+H]⁺ m/z calcd. (monoisotopic) 1181.54, obs 1181.8.

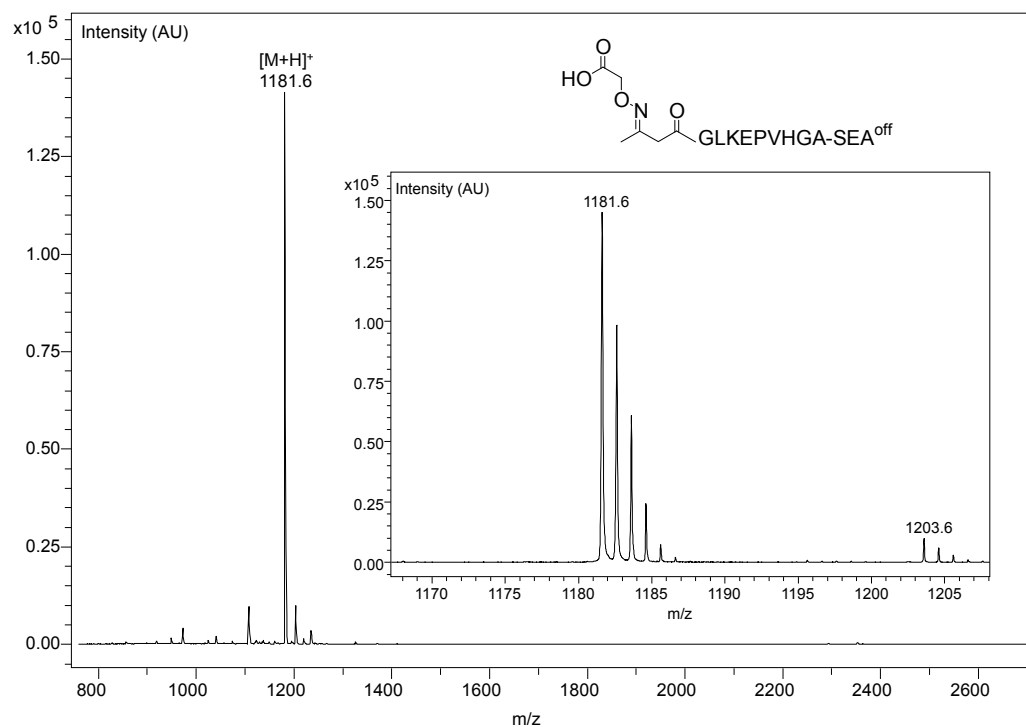


Figure S 29. MALDI-TOF analysis of peptide **10a**. Matrix alpha cyano 4-hydroxycinnamic acid, positive detection mode, $[M+H]^+$ calcd. (monoisotopic) 1181.54, found 1181.6.

4.3 Characterization of AcAO peptide **10b**

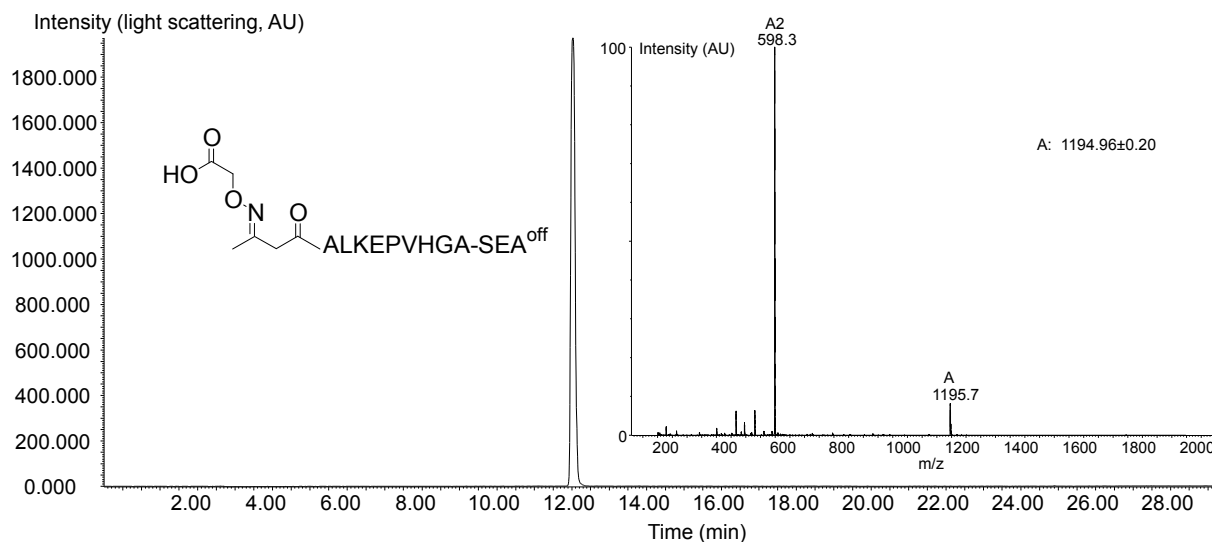


Figure S 30. LC-MS analysis of peptide **10b**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 μm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace. $[M+H]^+$ m/z calcd. (monoisotopic) 1195.56, obs 1195.7.

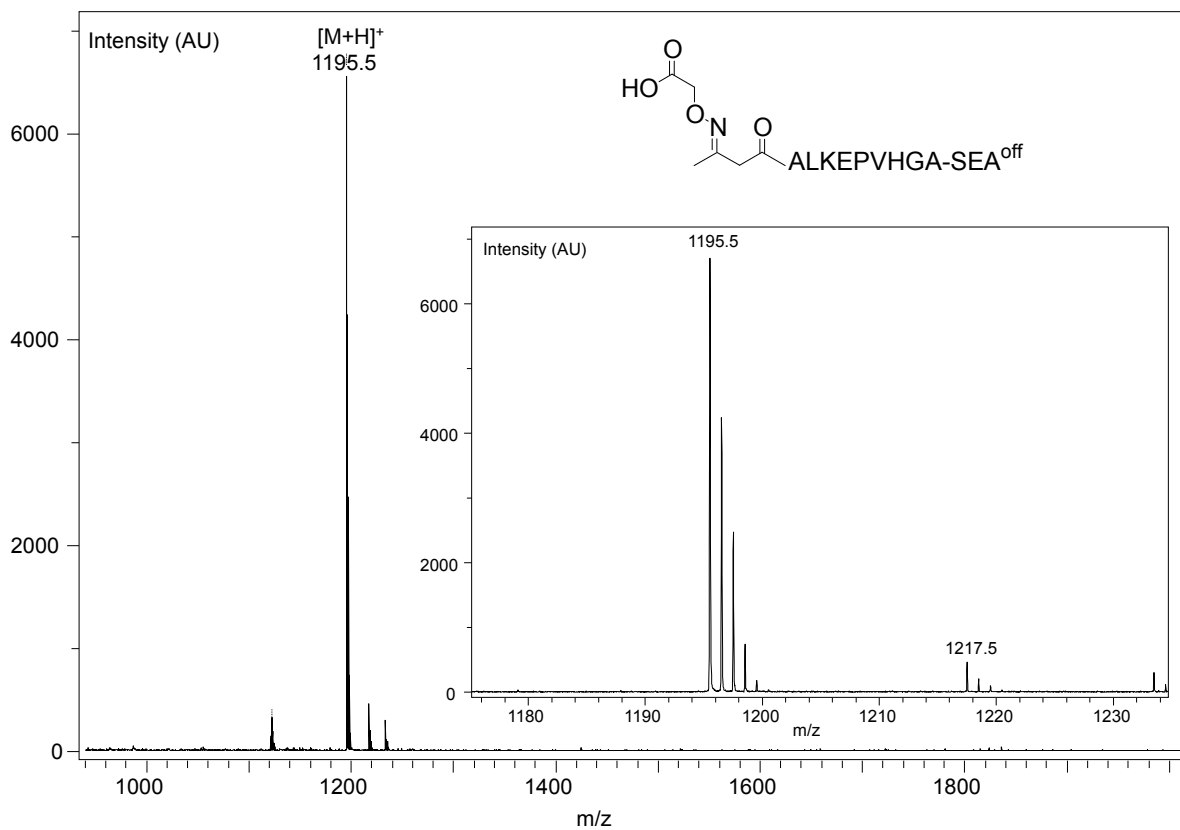


Figure S 31. MALDI-TOF analysis of peptide **10b**. Matrix alpha cyano 4-hydroxycinnamic acid, positive detection mode, $[M+H]^+$ calcd. (monoisotopic) 1195.56, found 1195.5.

4.4 Characterization of AcAO peptide **10c**

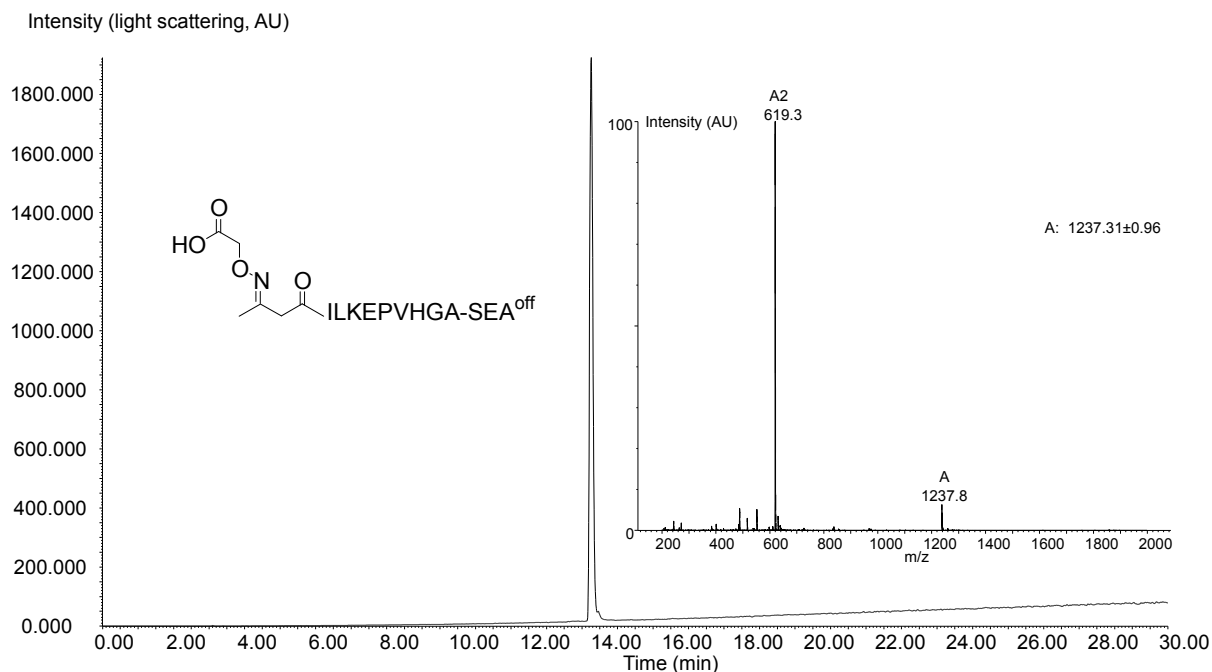


Figure S 32. LC-MS analysis of peptide **10c**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 µm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace. [M+H]⁺ m/z calcd. (monoisotopic) 1237.61, obs 1237.8.

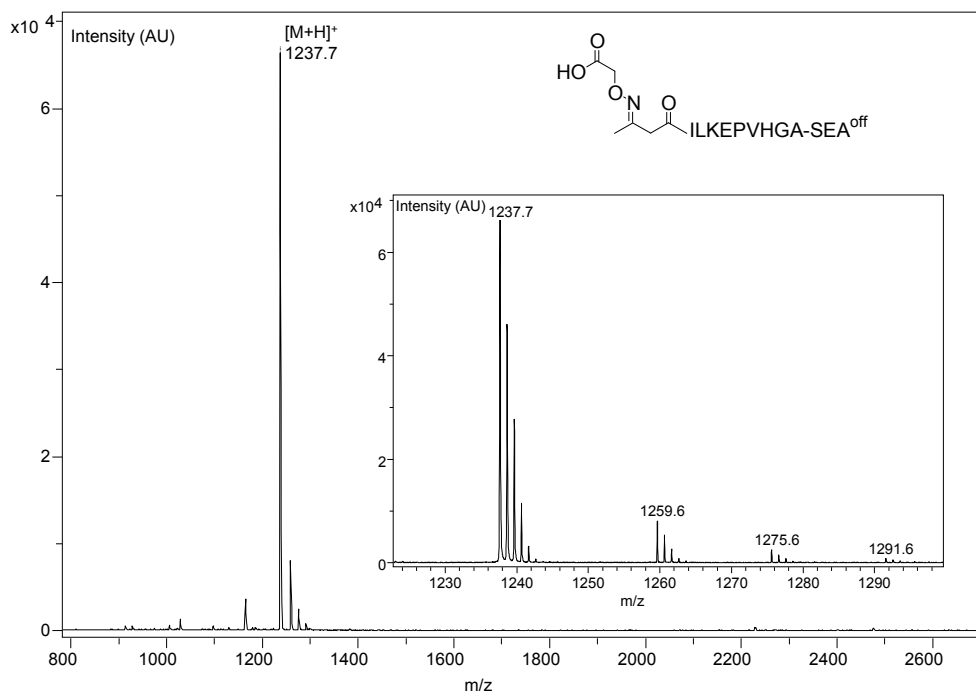


Figure S 33. MALDI-TOF analysis of peptide **10c**. Matrix alpha cyano 4-hydroxycinnamic acid, positive detection mode, [M+H]⁺ calcd. (monoisotopic) 1237.61, found 1237.7.

4.5 Characterization of AcAO peptide 10d

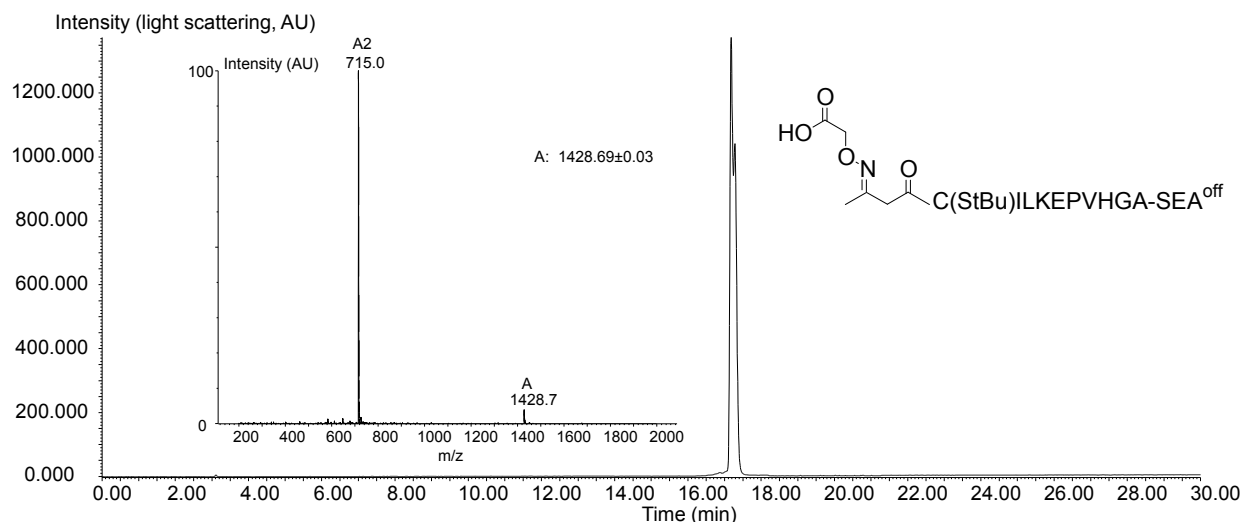


Figure S 34. LC-MS analysis of peptide **10d**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 μm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace. [M+H]⁺ m/z calcd. (monoisotopic) 1428.65, obs 1428.7.

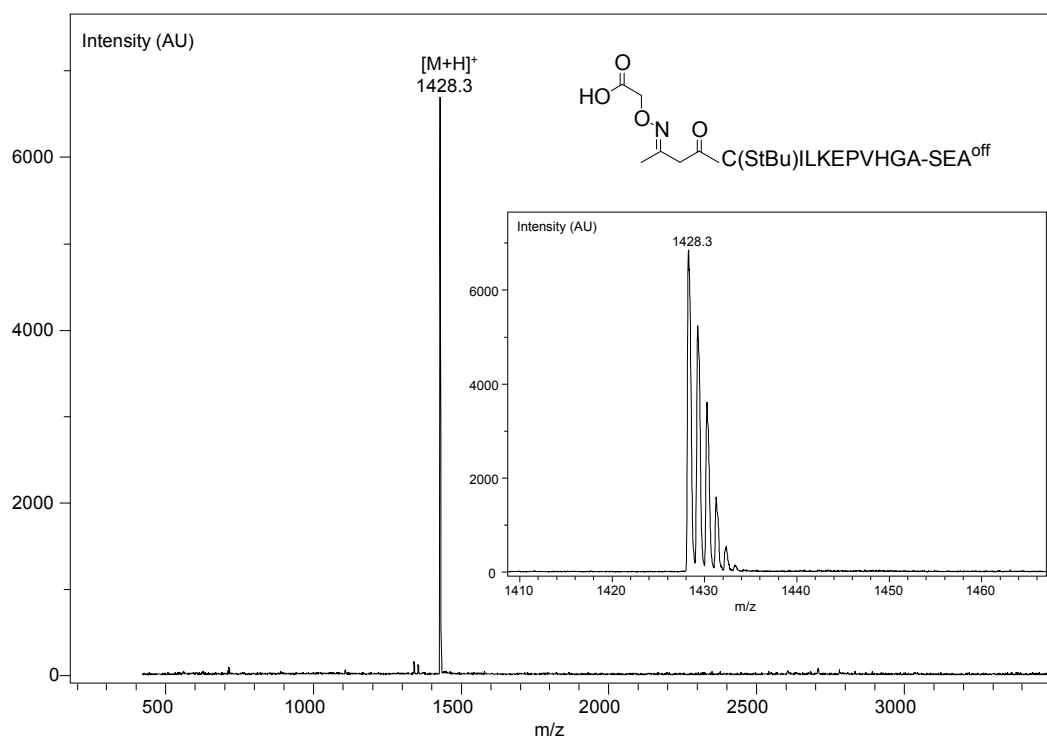


Figure S 35. MALDI-TOF analysis of peptide **10d**. Matrix alpha cyano 4-hydroxycinnamic acid, positive detection mode, [M+H]⁺ calcd. (monoisotopic) 1428.65, found 1428.3.

5. Synthesis of PEGA resin 11

5.1 Synthesis of Fmoc aminoxyacetic acid

In an ice-cooled round-bottomed flask, aminoxyacetic acid hydrochloride (500 mg, 4.58 mmol) was dissolved in water (20 mL). Sodium hydrogenocarbonate (1.15 g, 13.7 mmol) was dissolved in water (20 mL) and added to the aminoxyacetic acid solution. *N*-(9-Fluorenylmethoxycarbonyloxy)succinimide (1.70 g, 5.03 mmol) was dissolved in dioxane (60 mL) and added slowly to the above solution. After the addition, the ice-bath was removed and the reaction mixture was stirred at rt. After 4 hours, a first extraction with diethyl ether (100 mL) was performed. The aqueous phase (pH=8.4) was then acidified with 5 N HCl to pH 1 (a white precipitate forms). The mixture was extracted with ethyl acetate (3 × 50 mL). The combined organic phases were dried over anhydrous sodium sulfate during 30 min, filtered and evaporated to give 1.3 g (90.6%) of a yellow powder. Purification of the crude product (630 mg) by silica gel chromatography (DCM/AcOH 95/5 v/v by vol) yielded 330.6 mg (47.5%) of Fmoc aminoxyacetic acid.

5.2 Characterization of Fmoc aminoxyacetic acid

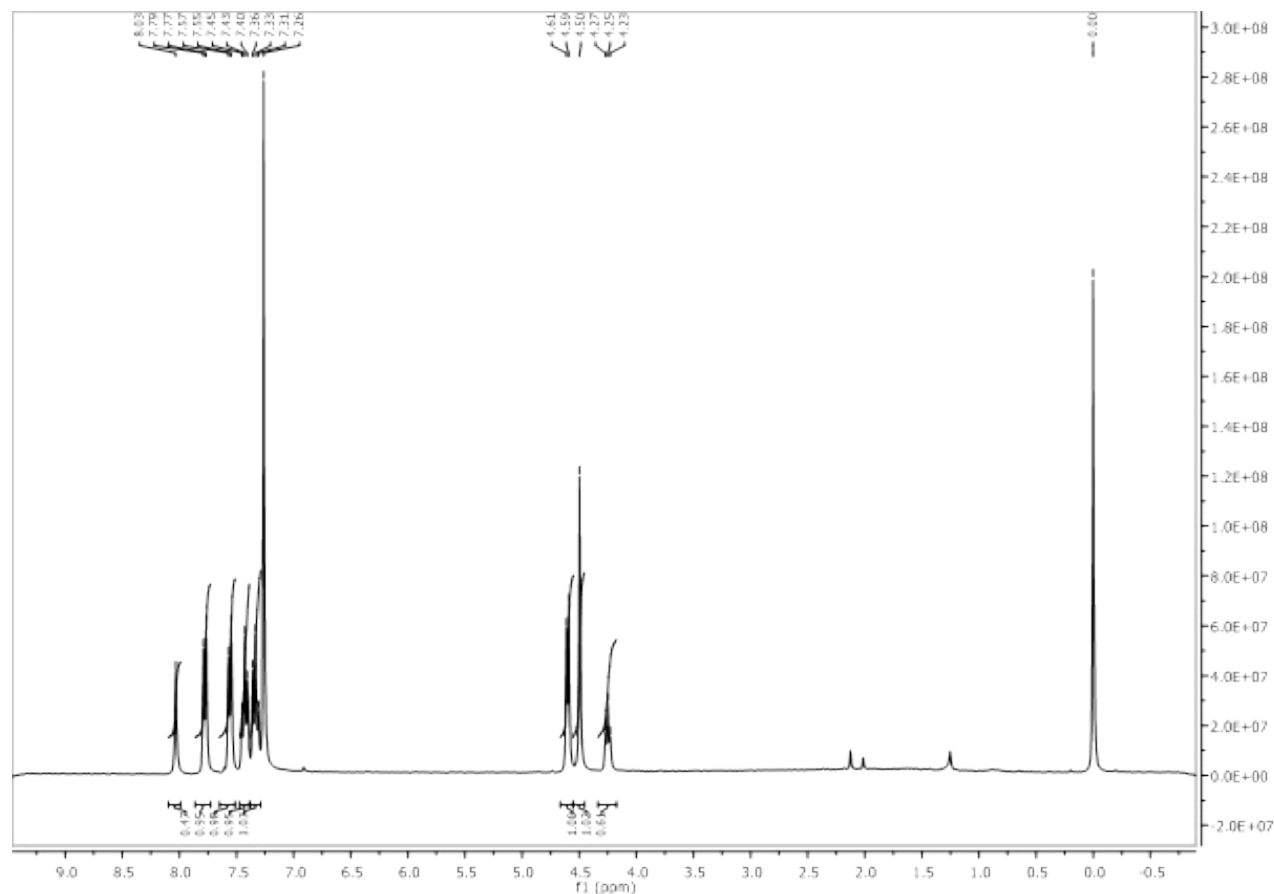


Figure S 36. ¹H NMR spectrum (300.0 MHz, CDCl₃) of Fmoc aminoxyacetic acid.

5.3 Preparation of resin 11

N-succinimidyl Fmoc aminoxyacetate

In a round-bottomed flask, Fmoc aminoxyacetic acid (93.94 mg, 0.299 mmol) and *N*-hydroxysuccinimide (34.79 mg, 0.302 mmol) were dissolved in dry dioxane/ethylacetate (1/1 v/v, 1 mL) and the reaction mixture was placed under inert atmosphere. The round-bottomed flask was placed on an ice bath and diisopropylcarbodiimide (47 μL, 0.3 mmol) was added dropwise. Then, the ice bath was removed and the reaction mixture was stirred at rt overnight. This solution was used directly in the next step.

Coupling of N-succinimidyl Fmoc aminoxyacetate to Rink PEGA resin

PEGA resin equipped with Rink linker (0.35 mmol/g, 24% in ethanol 1.19 g, 0.10 mmol) was washed with DMF (3 × 2 min). The solution of *N*-succinimidyl Fmoc aminoxyacetate (0.299 mmol) and *N*-methylmorpholine (16.5 μL, 0.15 mmol) were added to the resin. After 4 hours, the TNBS test showed the absence of free primary amino groups on the resin. Therefore, the solid support was washed with DMF (5 × 2 min), DCM (3 × 2 min) and absolute ethanol (3 × 2 min). The resin was stored swelled in absolute ethanol at 4°C. An aliquot was used to determine the loading of the resin by UV quantification (290 nm) of the dibenzofulvene-piperidine adduct produced by treating the resin with 20% piperidine in DMF (0.098 mmol/g in absolute ethanol).

6. Immobilization of AcA peptide 4b.

6.1 TFA cleavage leading to peptide 14b

The solid support prepared above (3.37 μmol) was washed with DMF (3 × 2 min). Fmoc protecting group was removed using 20% piperidine in DMF (2 × 2 min) to produce PEGA resin **11**. The solid support was washed with DMF (3 × 2 min), water (2 × 2 min) and 0.1 M sodium acetate buffer pH 4.4 (2 × 2 min). AcA peptide **4b** (5.012 mg, 3.711 μmol) was dissolved in 0.1 M sodium acetate buffer pH 4.4 (260 μL) and added to PEGA resin **11**. The disappearance of AcA peptide **4b** was monitored by RP-HPLC. After 48 h, the solid support was washed with water (4 × 2 min), DCM (3 × 2 min), diethyl ether (3 × 2 min) and dried under vacuum. The peptide was cleaved from the resin using TFA/water/thiophenol 95/2.5/2.5 for 1 h. The resin was drained and the cleavage repeated for 20 min using the same TFA cocktail. Precipitation of the peptide in diethylether/heptane 1/1 v/v furnished 1.7 mg of peptide **14b** (35.5%).

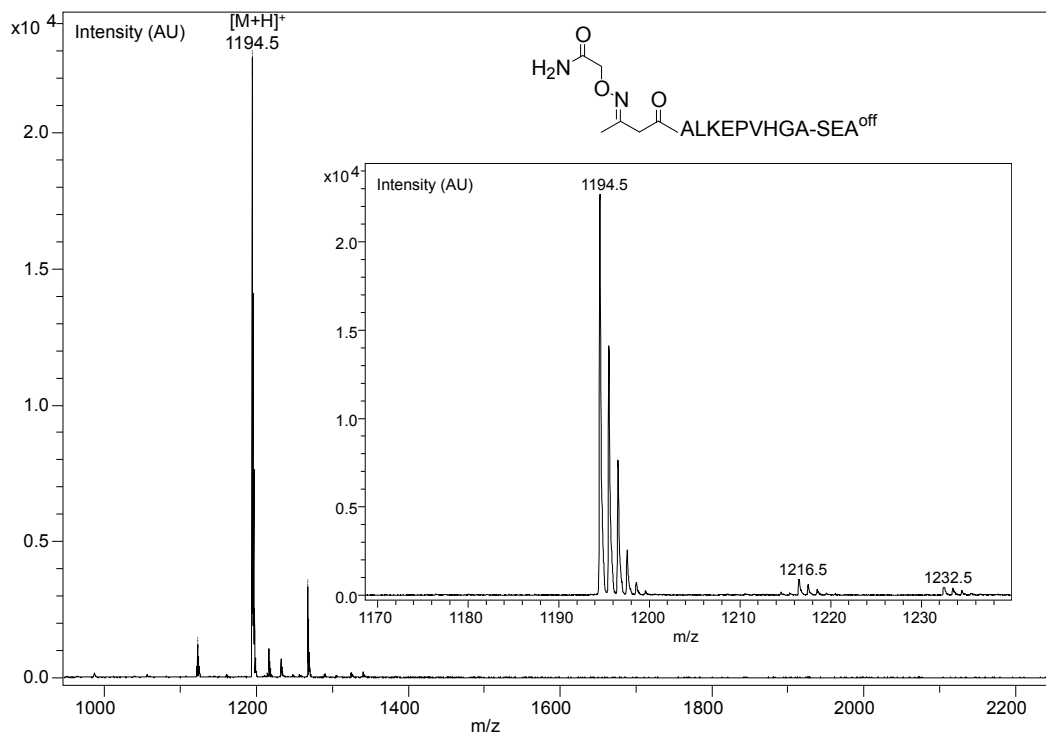


Figure S 38. MALDI-TOF analysis of peptide **14b**. Matrix alpha cyano 4-hydroxycinnaminic acid, positive detection mode, $[M+H]^+$ calcd. (monoisotopic) 1194.58, found 1194.5.

7. Kinetic studies of the transoximation/rearrangement sequence: effect of the pH in the presence of aniline

A typical procedure is described for 20% AcOH, 0.1 M hydroxylamine, 0.5 M aniline, pH=4.3
 Hydroxylamine (5.36 mg, 77.1 μmol) was dissolved in water (536.2 μL). Hydroxylamine solution (212.4 μL , 0.1 M final concentration), glacial acetic acid (61.12 μL , 20% final concentration) and aniline (13.94 μL , 0.5 M final concentration) were mixed together and the pH was adjusted to 4.3 by addition of 6 M NaOH (30 μL). This solution was added to oxime **10b** (435 μg , 0.31 μmol) and the reaction mixture was stirred at 37 $^{\circ}\text{C}$. The reaction was followed by RP-HPLC (column XBridge BEH300 C18 (3.5 μm , 300 \AA , 4.6 \times 150 mm) at 30 $^{\circ}\text{C}$ using a linear gradient of 0-100% of eluent B in eluent A over 30 min at a flow rate of 1 mL/min (eluent A = 0.1% TFA in H_2O ; eluent B = 0.1% TFA in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$: 4/1 by vol). An aliquot of the reaction mixture (10 μL) was diluted with 40 μL of eluent A. 40 μL of this solution was injected.

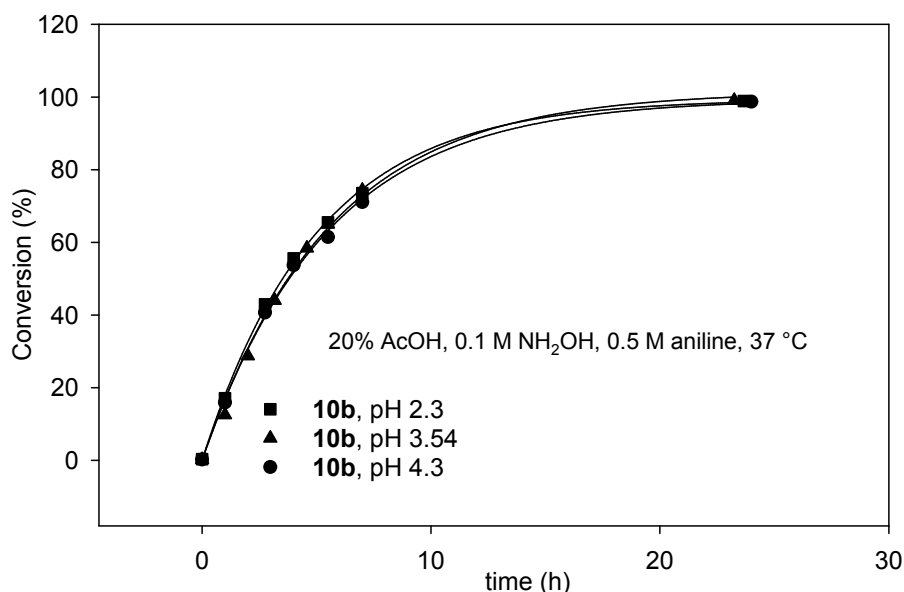


Figure S 39. Influence of the pH on the transoximation and cleavage reaction in presence of 0.5 M aniline, 0.1 M H_2NOH , 20% AcOH, 37 °C.

Conclusion: the pH has no significant effect on the rate of cleavage in the presence of aniline.

8. Optimization of the cleavage protocol to minimize the formation of hydroxamic acid derivatives from Gln and Asn

We used for this study peptide C(S*t*Bu)**Q**PWSSMIPHEHSFLPSSYRGKDL**Q**ENY-SEA^{off} which features two Gln residues.

8.1. Influence of the pH (cleavage method A at pH 4.3, 3.5 and 3.0)

Hydroxylamine (13.93 mg, 200,5 μmol) was dissolved in 20% AcOH (1.82 mL) and aniline (182.4 μL). Samples of this solution (1400 μL , 300 μL and 300 μL) were adjusted respectively at pH=3.03, pH=3.51 and pH=4.3 by addition of 6 M HCl (respectively 180 μL , 26 μL and 15 μL). These solutions (respectively 246.5 μL , 243.4 μL and 247.3 μL) were added to the peptide (respectively 1.014 mg, 1.001 mg and 1.017 mg) and the reaction mixtures were stirred at 45 °C under nitrogen. The appearance of +16 u side-products was monitored after 24 h by MALDI-TOF (matrix: sinapinic acid) and LC-MS (column XBridge BEH300 C18 (3.5 μm , 300 Å, 4.6 × 150 mm) at 30 °C using a linear gradient of 0-100% of eluent B in eluent A over 30 min at a flow rate of 1 mL/min (eluent A = 0.1% TFA in H_2O ; eluent B = 0.1% TFA in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$: 4/1 by vol).

Table S 3. Effect of the pH on the level of +16 u impurities.

MS technique	pH
--------------	----

	3.03	3.51	4.3
LC-ESI MS	10.3%	12.91%	19%
MALDI-TOF	4.2%	7.8%	9.9%

We conclude that lowering the pH from 4.3 to 3.0 results in a decrease in +16 u side-product formation.

8.2. Influence of additives: acetamide (cleavage method A at pH 3.0)

The above cleavage solution at pH 3.03 was used for this experiment. Acetamide (3.22 mg, 7.36 mg and 14.94 mg) was dissolved in the cleavage solution pH 3.03 (300 μ L) to test the effect of acetamide (respectively 0.18 M, 0.41 M and 0.84 M final concentrations) on the level of +16 u side-product formation. The peptide (1.004 mg, 1.038 mg and 1.005 mg) was dissolved in respectively 244.09 μ L, 252.4 μ L and 244.34 μ L of the above acetamide solutions and placed at 45 °C. The reaction was analyzed as described above after 24 h.

Table S 4. Effect of acetamide on the level of +16 u side-product formation (pH 3.03).

MS technique	acetamide concentration		
	0.18 M acetamide	0.41 M acetamide	0.84 M acetamide
LC-ESI MS	11.2%	16.2%	18.71%
MALDI-TOF	6.1%	8.6%	18.6%

Conclusion: addition of acetamide increases the amount of +16 u side-product formation.

8.3. Influence of aniline concentration on the rate of cleavage

A typical protocol is described using 20% AcOH, 0.1 M hydroxylamine and 2 M aniline at pH=3
 Hydroxylamine (5.18 mg, 74.5 μ mol) was dissolved in water (51.82 μ L). Hydroxylamine solution (22.6 μ L, 0.1 M final concentration), glacial acetic acid (65.05 μ L, 20% final concentration) and aniline (59.4 μ L, 2 M final concentration) were mixed together and the pH=3 adjusted after addition of 6 M HCl (90 μ L) and water (88.3 μ L). This solution was used to dissolve oxime **10b** (463 μ g, 0.32 μ mol). The reaction mixture was stirred at 45 °C. The reaction was monitored by RP-HPLC (column XBridge BEH300 C18 (3.5 μ m, 300 Å, 4.6 \times 150 mm) at 30 °C using a linear gradient of 0-100% of eluent B in eluent A over 30 min at a flow rate of 1 mL/min (eluent A = 0.1% TFA in H₂O; eluent B = 0.1% TFA in CH₃CN/H₂O: 4/1 by vol) by diluting 10 μ L of the reaction mixture in 40 μ L of eluent A and injecting 20 μ L.

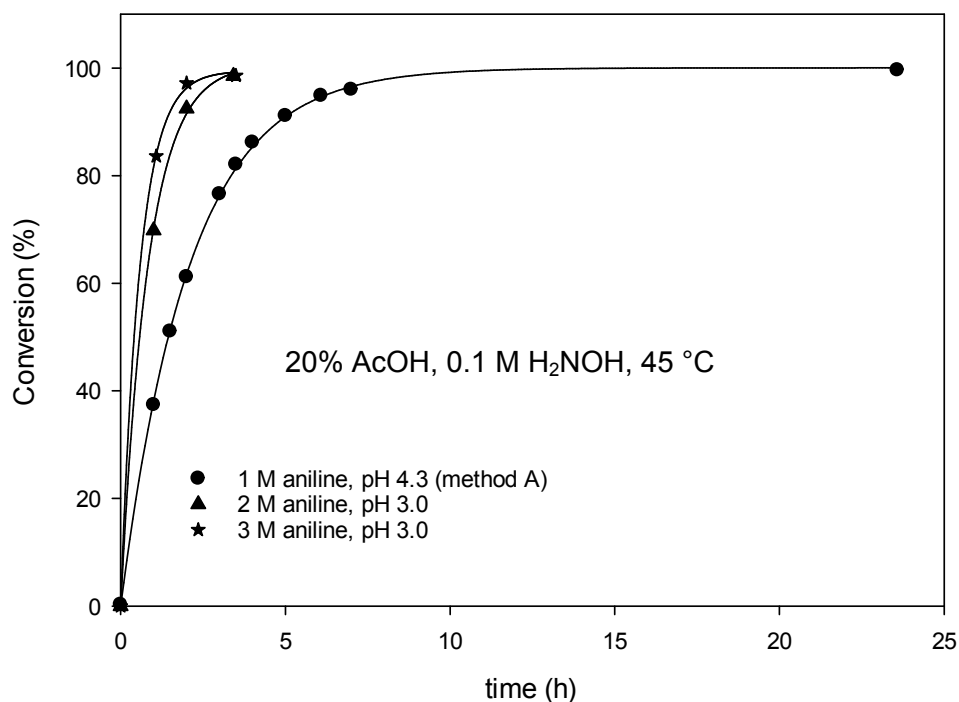


Figure S 40. Influence of aniline concentration on the rate of cleavage (peptide **10b** in solution).

Conclusion: the rate of cleavage is further increased by raising the concentration of aniline.

8.4. Influence of hydroxylamine concentration on the rate of cleavage

A typical protocol is described using 20% AcOH, 0.025 M hydroxylamine and 3 M aniline at pH=3

Hydroxylamine (2.69 mg, 38.7 μ mol) was dissolved in water (53.74 μ L). Hydroxylamine solution (11.45 μ L, 0.025 M final concentration), glacial acetic acid (65.9 μ L, 20% final concentration) and aniline (90.16 μ L, 3 M final concentration) were mixed together and the pH adjusted to 3.0 after addition of 6 M HCl (125 μ L) and water (37 μ L). This solution was added used to dissolve oxime **10b** (469 μ g, 0.33 μ mol). The reaction mixture was stirred at 45 °C. The reaction was monitored by RP-HPLC (column XBridge BEH300 C18 (3.5 μ m, 300 Å, 4.6 \times 150 mm) at 30 °C using a linear gradient of 0-100% of eluent B in eluent A over 30 min at a flow rate of 1 mL/min (eluent A = 0.1% TFA in H₂O; eluent B = 0.1% TFA in CH₃CN/H₂O: 4/1 by vol) by diluting 10 μ L of the reaction mixture in 40 μ L of eluent A and injecting 20 μ L.

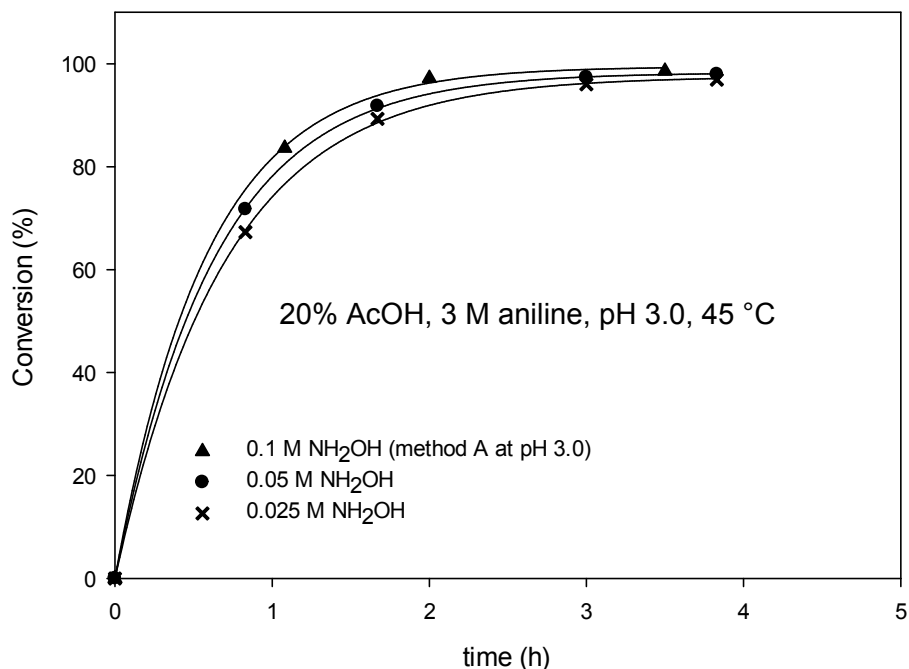


Figure S 41. Influence of hydroxylamine concentration on the rate of cleavage (peptide **10b** in solution).

9. Synthesis of ALKEPVHGA-SEA^{off} peptide **15b**

9.1 Cleavage method A

Oxime ligation on the solid support

Solid support **11** (5.55 μmol) was conditioned in 0.1 M sodium acetate buffer pH 4.4 (2×2 min) and drained. 2-hydroxy 5-methoxybenzoic acid (250 μg) was dissolved in 0.1 M sodium acetate buffer pH 4.4 (100 μL) and this solution was used to dissolve AcA peptide **4b** (5.13 mg, 3.79 μmol). The peptide solution was added to the solid support and the suspension was agitated at 37 $^{\circ}\text{C}$. The internal reference was used for monitoring the disappearance of AcA peptide **4b** in solution by RP-HPLC. Conditions: XBridge BEH300 C18 (3.5 μm , 300 \AA , 4.6 \times 150 mm), 30 $^{\circ}\text{C}$, linear gradient of 0-100% of eluent B in eluent A over 30 min at a flow rate of 1 mL/min (eluent A = 0.1% TFA in H_2O ; eluent B = 0.1% TFA in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$: 4/1 by vol).

Tandem transoximation/rearrangement reaction

After 48 h, the solid support was washed at room temperature with 0.1 M sodium acetate buffer pH 4.4 (3×2 min), water (4×2 min), 20% AcOH (2×2 min) and with the hydroxylamine solution (300 μL , 30 seconds, 0.1 M hydroxylamine, 1 M aniline, 6 M Gn.HCl, 20% AcOH, 14.9 mM 2-hydroxy 5-methoxybenzoic acid, pH 4.28).

Then, the solid support was drained and the hydroxylamine solution (300 μL) was added again to the beads which were agitated at 45 $^{\circ}\text{C}$. The release of the peptide **15b** in solution was quantified

by HPLC relative to the internal reference. Conditions: XBridge BEH300 C18 (3.5 μm , 300 \AA , 4.6 \times 150 mm), 30 $^{\circ}\text{C}$, 0-100% of eluent B in eluent A over 30 min at a flow rate of 1 mL/min (eluent A = 0.1% TFA in H_2O ; eluent B = 0.1% TFA in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$: 4/1 by vol).

After 30 hours, the solid support was drained, washed with 20% AcOH (5 \times 1 min, 3 mL) and water (5 \times 1 min). The combined supernatants (final volume = 12 mL) were purified by HPLC to produce 4.3 mg of peptide **15b** (82.2% for the two steps). Conditions: C18XBridge column, 0 to 10% eluent B in 5 min, then 10 to 50% eluent B in 120 min, eluent A = water containing 0.1% TFA, eluent B=acetonitrile in water 4/1 containing 0.1% TFA, rt, detection at 215 nm, 6 mL/min.

9.2 Characterization of peptide **15b** (cleavage method A)

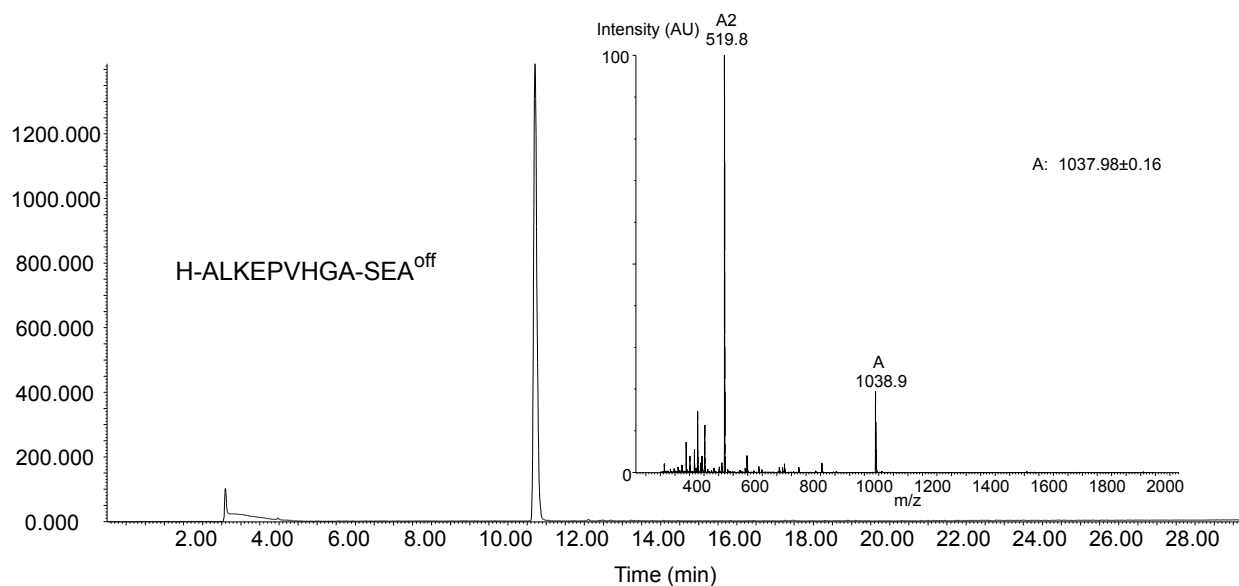


Figure S 42. LC-MS analysis of peptide **15b**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in $\text{CH}_3\text{CN}/\text{water}$: 4/1 by vol. C18 Xbridge BEH 300 \AA 5 μm (4.6 \times 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace. $[\text{M}+\text{H}]^+$ m/z calcd. (monoisotopic) 1038.52, obs 1038.9.

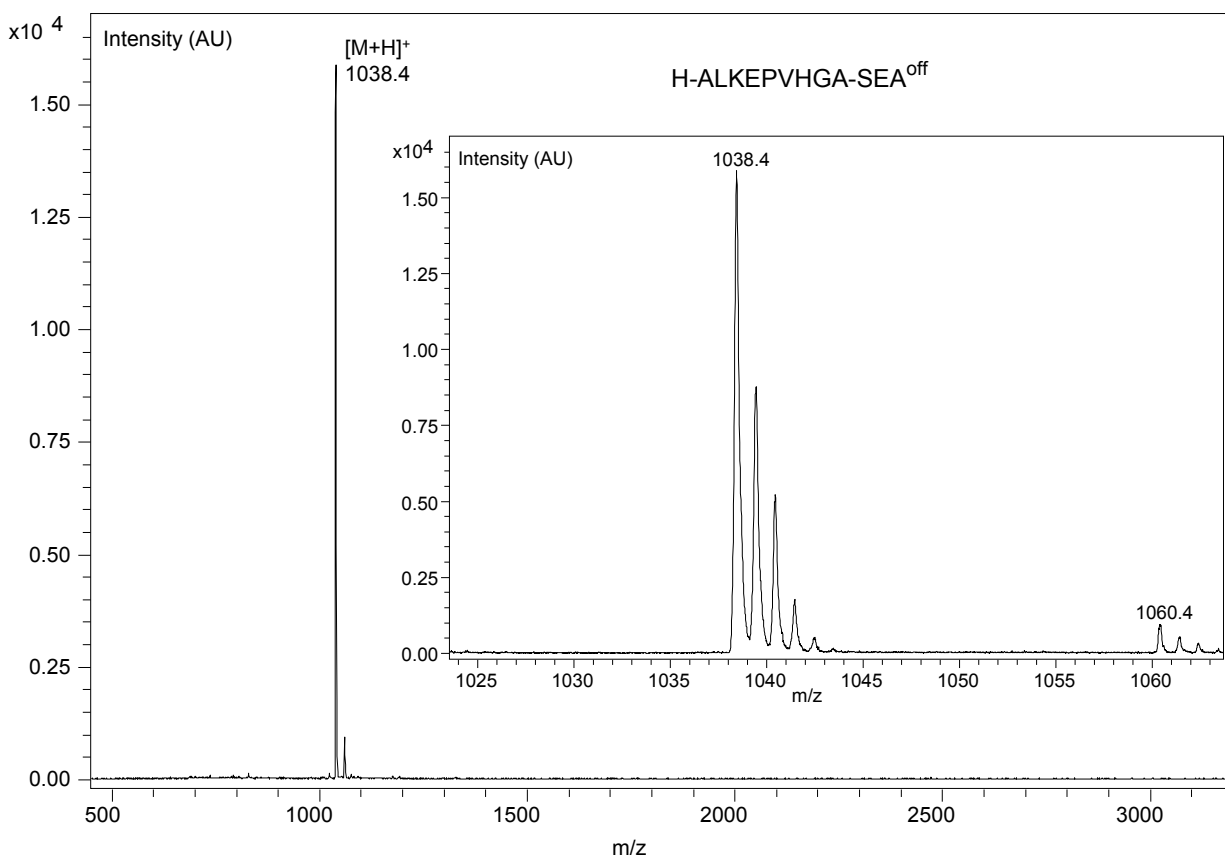


Figure S 43. MALDI-TOF analysis of peptide **15b**. Matrix alpha cyano 4-hydroxycinnamic acid, positive detection mode, $[M+H]^+$ calcd. (monoisotopic) 1038.52, found 1038.4.

9.3 Cleavage method B

AcA peptide **4b** (5.021 mg, 3.718 μmol) was dissolved in 0.1 M sodium acetate buffer pH 4.58 (100 μL containing 250 μg of 2-hydroxy 5-methoxybenzoic acid used as internal reference). This solution was added to solid support **11** (56.9 mg, 5.58 μmol). The oxime ligation on the solid support was monitored by RP-HPLC.

After completion of the ligation step, the solid support was washed with sodium acetate buffer pH 4.58 (3×2 min) and water (4×2 min), and with the cleavage solution (300 μL , 0.025 M hydroxylamine, 3 M aniline, 20% AcOH adjusted to pH 3.0). The solid support was then placed at 45 $^{\circ}\text{C}$ on a shaker. After 29 h, the solid support was drained and washed with buffer A (8×1 min, 5.7 mL). Purification by RP-HPLC (eluent A = water containing 0.1% TFA, eluent B=acetonitrile in water 4/1 containing 0.1% TFA, 30 $^{\circ}\text{C}$, detection C18XBridge column) afforded 3.2 mg of peptide **15b** (overall yield 63.7%).

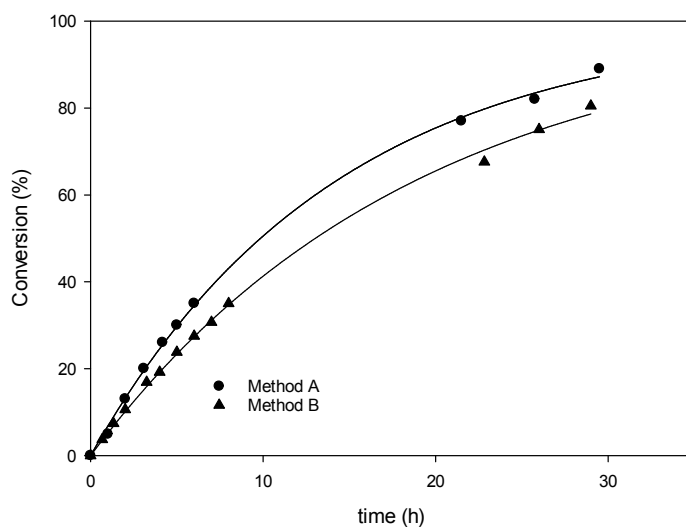


Figure S 44. Comparison between cleavage methods A and B for peptidyl resin **12b**. Cleavage method A: 20% AcOH, 0.1 M H₂NOH, 1 M aniline, pH 4.3, 45 °C; Cleavage method B: 20% AcOH, 0.025 M H₂NOH, 3 M aniline, pH 3.0, 45 °C.

9.4 Characterization of peptide **15b** (cleavage method B)

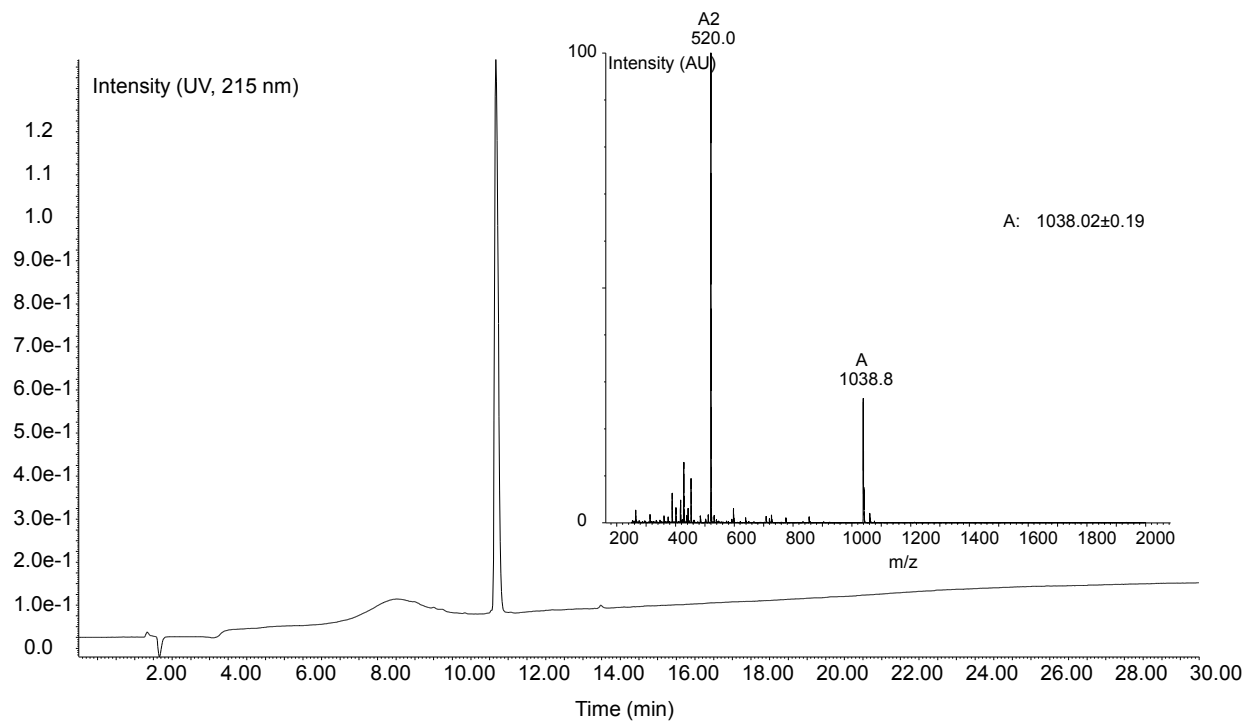


Figure S 45. LC-MS analysis of peptide **15b**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 µm (4.6 × 250 mm) column, gradient 0-100% B

in 30 min (1 mL/min, detection 215 nm). MS trace. $[M+H]^+$ m/z calcd. (monoisotopic) 1038.52, obs 1038.8.

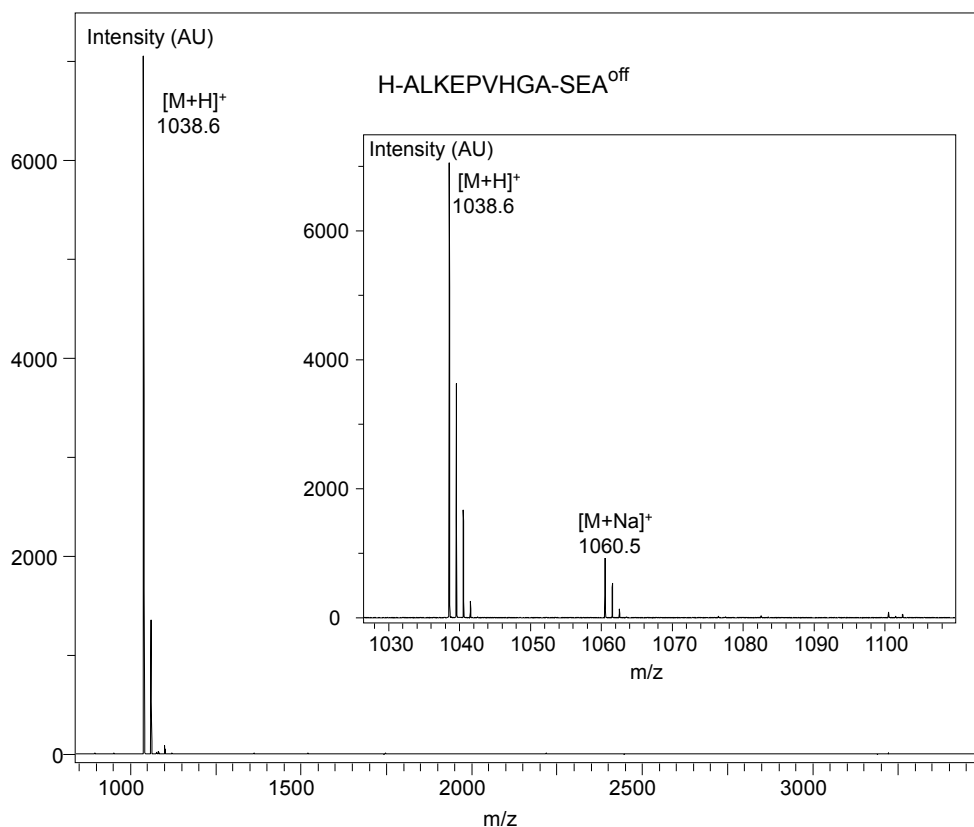


Figure S 46. MALDI-TOF analysis of peptide **15b**. Matrix alpha cyano 4-hydroxycinnamic acid, positive detection mode, $[M+H]^+$ calcd. (monoisotopic) 1038.52, found 1038.6.

10. Solid phase synthesis of peptide 17

10.1 Cleavage method A

Immobilization of AcA peptide 7

AcA peptide **7** (7.51 mg, 2.53 μ mol) was dissolved in 0.1 M sodium acetate buffer pH 4.58 (150 μ L containing 375 μ g of 2-hydroxy 5-methoxybenzoic acid used as internal reference) and reacted with solid support **11** (38.3 mg, 3.75 μ mol) as described before. The oxime ligation on the solid support was monitored by RP-HPLC. After 67 h, the solid support was washed with 0.1 M sodium acetate buffer pH 4.58 (3×2 min) and water (4×2 min).

Peptide elongation and cleavage

Peptide elongation was carried out using peptide segments and C(*St*Bu)QPWSSMIPHEHSFLPSSYRGKDLQENY-SEA^{off} and

CRNPRGEEGGPWCFTSNPEVRYEVC DIPQCSEVK(Biotin)-NH₂. The synthesis of the peptide segments as well as the method used for the elongation have been described elsewhere.²

At the end of the elongation, the solid support was washed with water (10 × 2 min), and quickly (30 seconds) with the cleavage solution (2 mL) at room temperature (0.1 M hydroxylamine, 1 M aniline, 6 M guanidinium hydrochloride, 20% AcOH, 2.24 mM internal reference 2-hydroxy 5-methoxybenzoic acid, pH 4.26). The cleavage solution was added again (2 mL) and the solid support was agitated at 45 °C. The release of the protein in solution was monitored by HPLC as described above.

After 29 hours, the solid support was drained and washed with 20% AcOH (5 × 1 min, 5 mL) and water (5 × 1 min, final volume = 20 mL). The supernatants were combined and extracted with diethyl ether to remove the internal reference prior to the HPLC purification (eluent A = water containing 0.1% TFA, eluent B=acetonitrile in water 4/1 containing 0.1% TFA, 30 °C, detection at 215 nm, 6 mL/min, 0 to 20% eluent B in 5 min, then 20 to 40% eluent B in 60 min, C18XBridge column). The purification furnished 7.9 mg of peptide **17** (overall yield 28%).

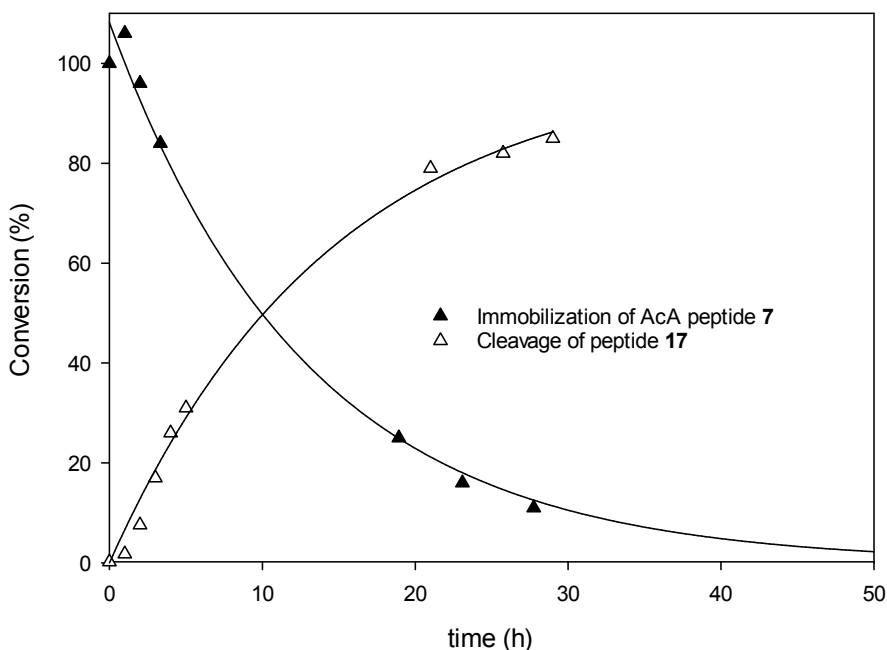


Figure S 47. Immobilization of AcA-peptide **7** and cleavage of peptide **17** as monitored by HPLC using 2-hydroxy 5-methoxybenzoic acid as an internal reference.

10.2 Characterization of peptide **17** (cleavage method A)

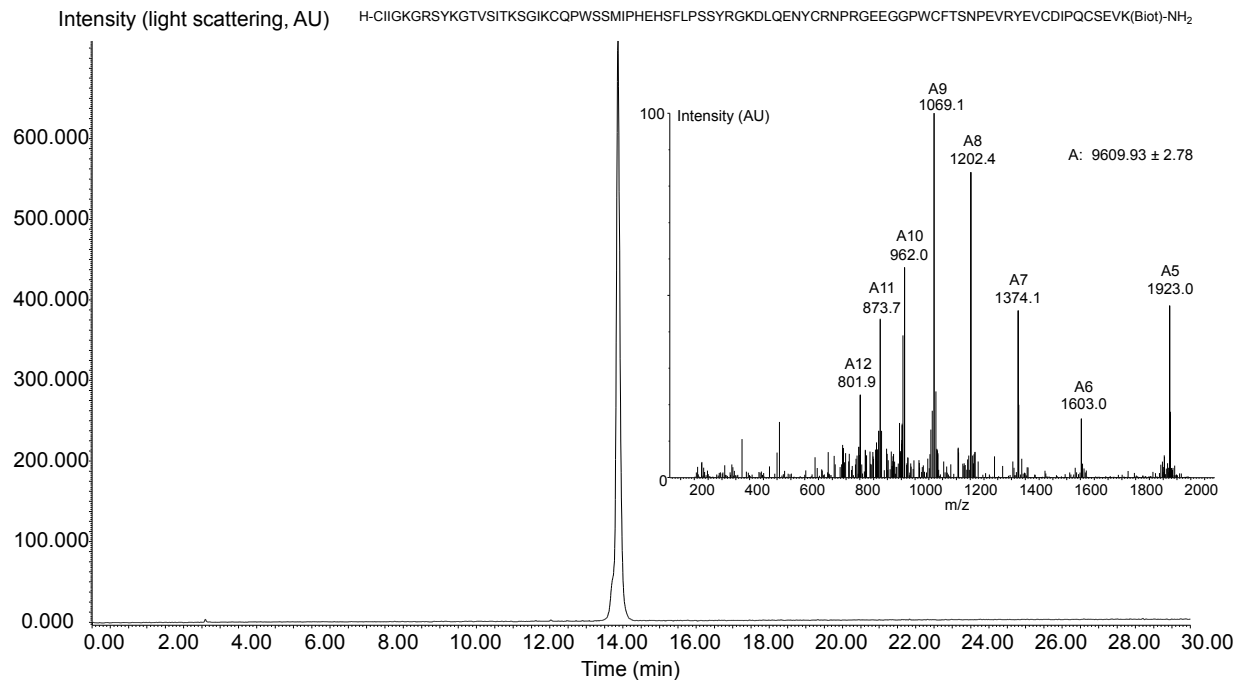


Figure S 48. LC-MS analysis of protein **17**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 µm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace: [M+5H]⁵⁺ m/z calcd. 1922.97, obs. 1923.0, [M+6H]⁶⁺ m/z calcd. 1602.64, obs. 1603.0, [M+7H]⁷⁺ m/z calcd. 1373.84, obs. 1374.1, [M+8H]⁸⁺ m/z calcd. 1202.23, obs. 1202.4, [M+9H]⁹⁺ m/z calcd. 1068.76, obs. 1069.1, [M+10H]¹⁰⁺ m/z calcd. 961.99, obs. 962.0, [M+11H]¹¹⁺ m/z calcd. 874.62, obs. 873.7, [M+12H]¹²⁺ m/z calcd. 801.82, obs. 801.9.

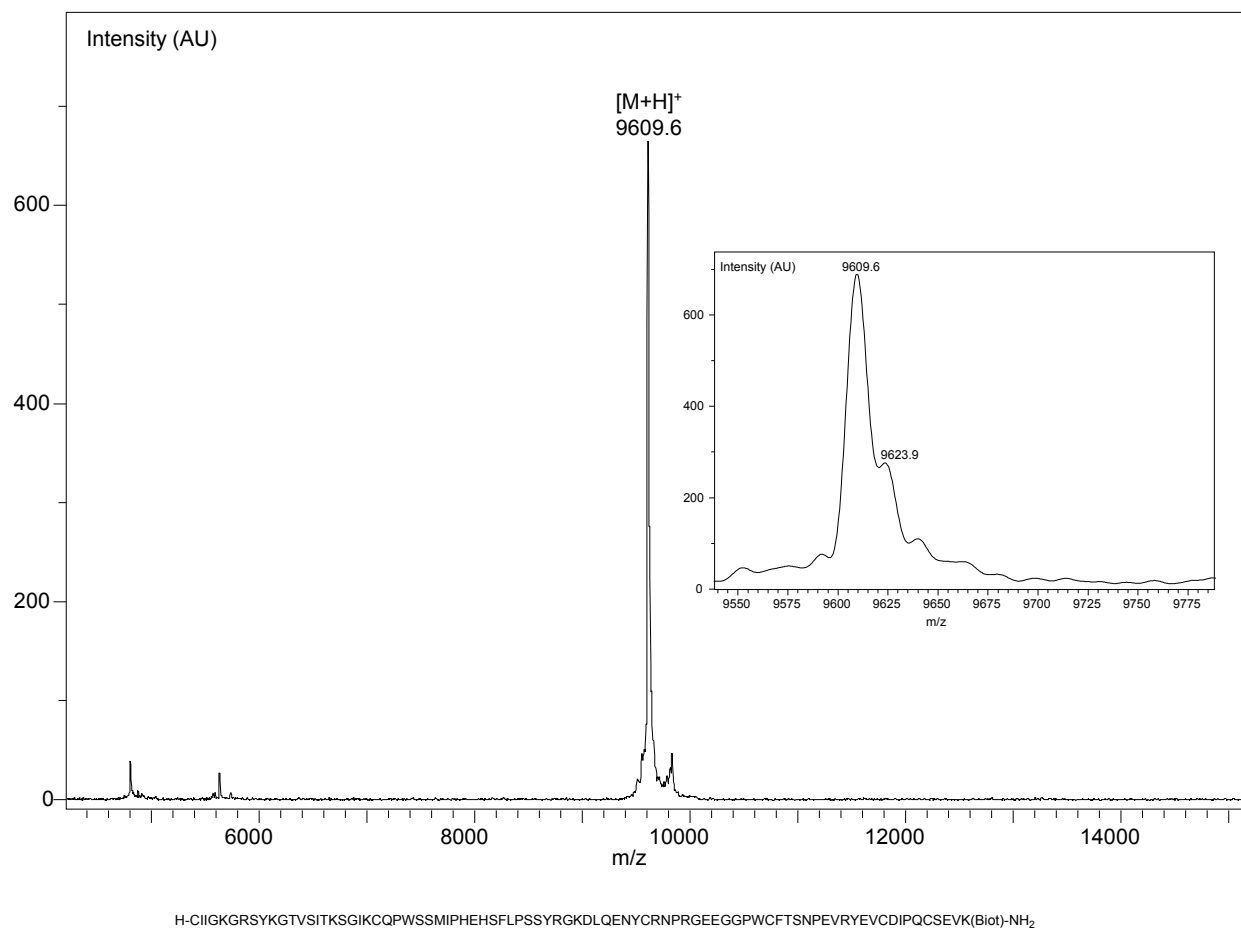


Figure S 49. MALDI-TOF analysis of protein **17**. Matrix sinapinic acid, positive detection mode, [M+H]⁺ calcd. (mean) 9610.86, found 9609.6.

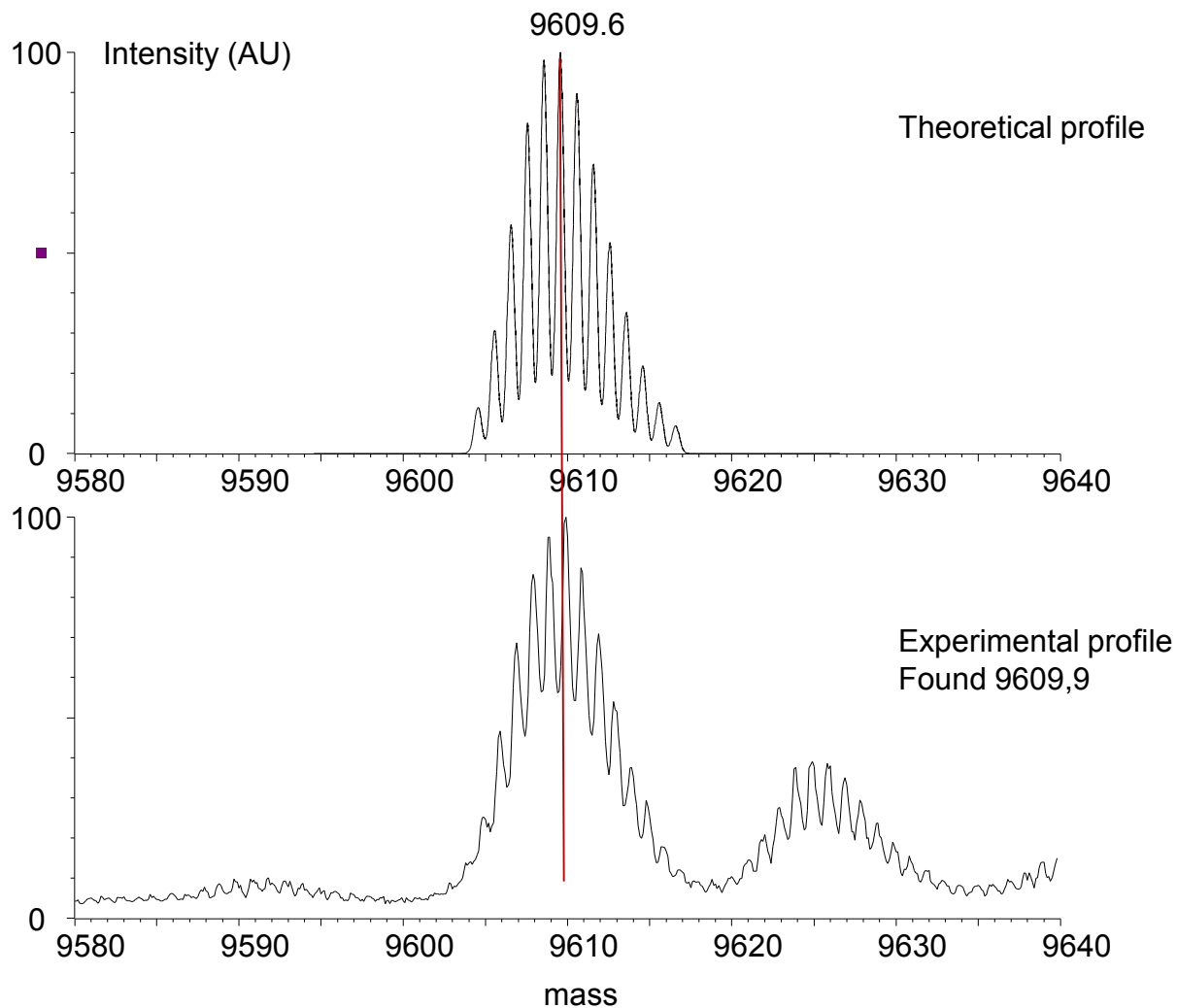


Figure S 50. High resolution ESI MS analysis for peptide **17**. Deconvoluted spectrum (bottom) and comparison with the theoretical profile (top).

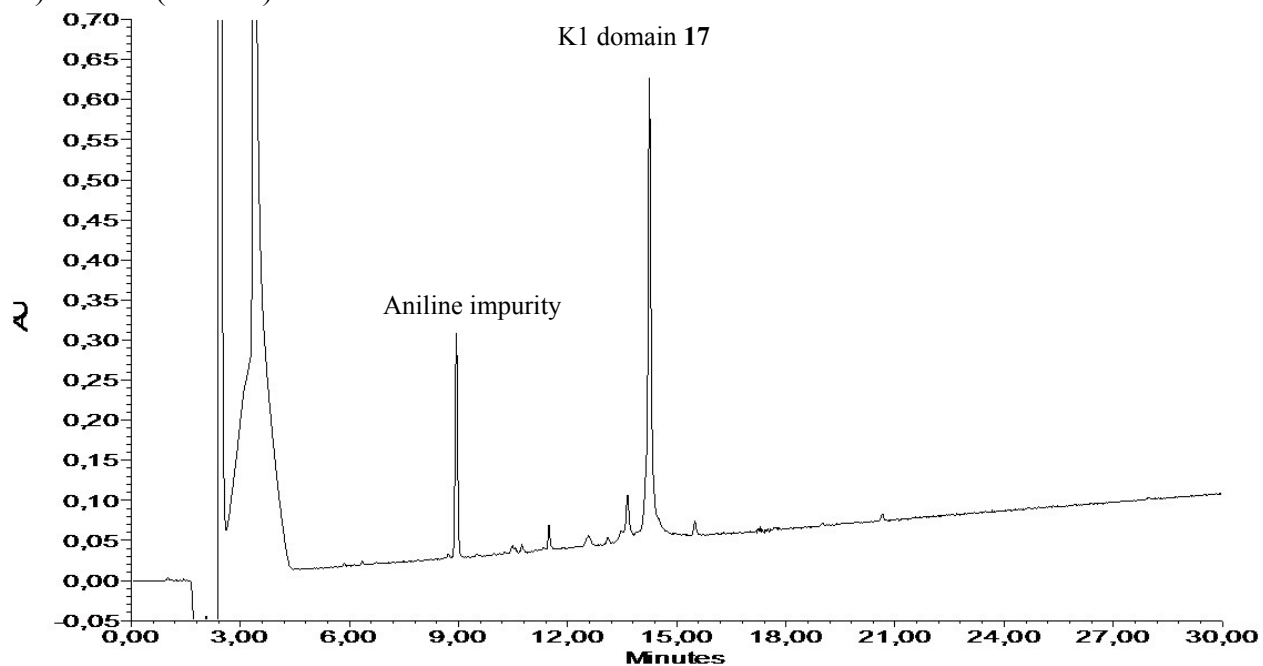
10.3 Cleavage method B

The peptidyl resin was prepared as described in the preceding section. At the end of the synthesis, the solid support was washed with water (10 × 2 min) and with the cleavage solution (2 mL, 0.025 M hydroxylamine, 3 M aniline, 20% AcOH adjusted to pH 3). The solid support was then placed at 45 °C on a shaker. After 4 h 20, the solid support was drained and the solution was frozen. A fresh solution (2 mL) was added to the solid support and the suspension was agitated for 5 h. The beads were drained and the supernatant frozen. The procedure was repeated twice after 14 h and 5 h 30.

At the end, the solid support was washed with eluent A (8×1 min, 4 mL). All the solutions were combined and purified by RP-HPLC (eluent A = water containing 0.1% TFA, eluent B=acetonitrile in water 4/1 containing 0.1% TFA, 30 °C, detection C18XBridge column) to afford 5.71 mg of peptide **17** (overall yield 21%).

10.4 Characterization of peptide **17** (cleavage method B)

A) Au (215 nm)



B)

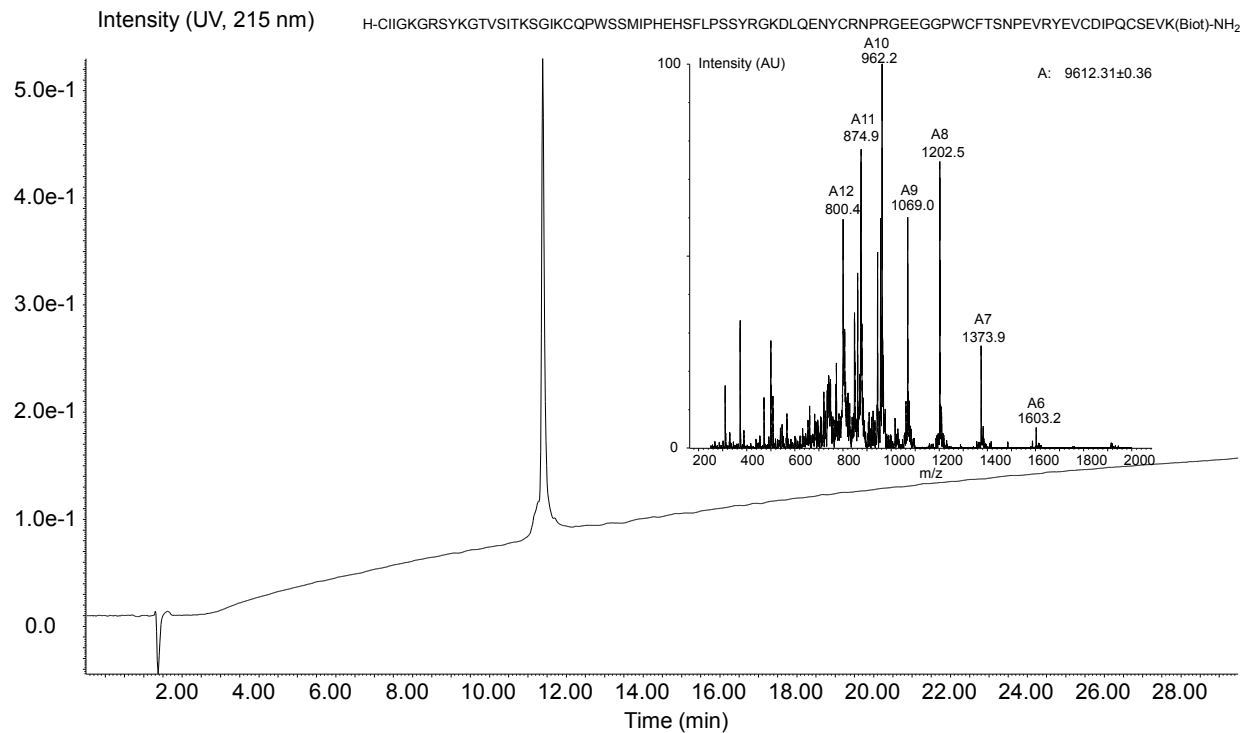


Figure S 51. LC-MS analysis of protein **17**. A) Crude protein **17** from cleavage solution (eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 XBridge BEH 300 Å 5 µm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm); B) purified protein **17**. LC trace, eluent C 0.10% FA in water, eluent D 0.10% FA in CH₃CN/water: 4/1 by vol. C3 Zorbax 300SB 3.5 µm (4.6 × 250 mm) column, gradient 0-100% D in 30 min (1 mL/min, detection 215 nm). MS trace: [M+6H]⁶⁺ m/z calcd (mean) 1602.6, obs. 1603.2, [M+7H]⁷⁺ m/z calcd. 1373.8, obs. 1373.9, [M+8H]⁸⁺ m/z calcd. 1202.2, obs. 1202.5, [M+9H]⁹⁺ m/z calcd. 1068.7, obs. 1069.0, [M+10H]¹⁰⁺ m/z calcd.961.99, obs.962.2, [M+11H]¹¹⁺ m/z calcd. 874.6, obs. 874.9, [M+12H]¹²⁺ m/z calcd. 801.8, obs. 800.4.

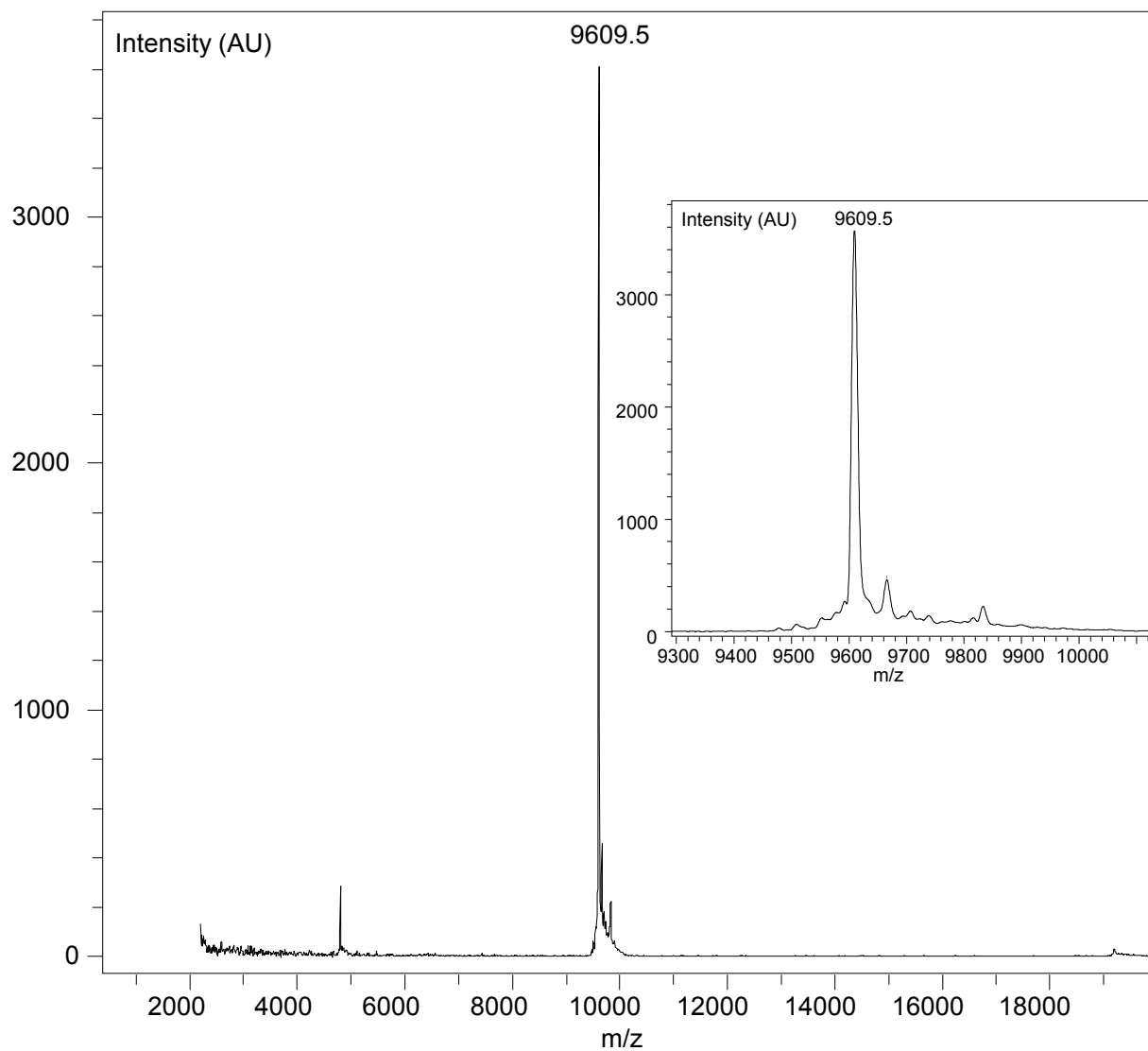


Figure S 52. MALDI-TOF analysis of protein **17**. Matrix sinapinic acid, positive detection mode, $[M+H]^+$ calcd. (mean) 9610.86, found 9609.5.

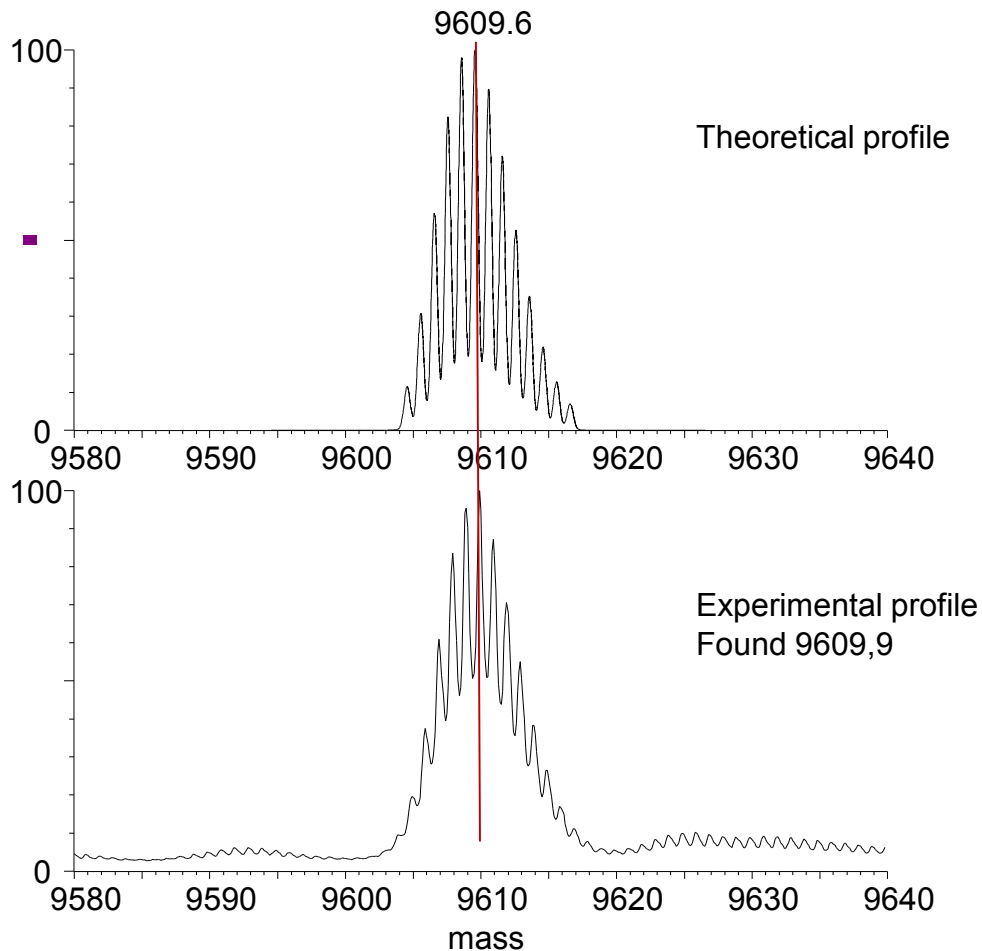


Figure S 53. Deconvoluted spectrum for peptide **17** and comparison with the theoretical profile.

11. Solid phase synthesis of peptide **18** (cleavage method A)

The protocol used for preparing peptide **17a** was applied to the synthesis of analog **18**. The starting peptide AcA-AIRN-SEA^{off} **4e** (1.99 mg, 2.52 μ mol) was dissolved in 0.1 M sodium acetate buffer pH 4.58 (100 μ L containing 250 μ g of 2-hydroxy 5-methoxybenzoic acid used as internal reference) and reacted with solid support **11** (38.3 mg, 3.75 μ mol). The oxime ligation on the solid support was followed as explained before by RP-HPLC.

After 22 h, the solid support was washed with 0.1 M sodium acetate buffer pH 4.58 (3 \times 2 min) and water (4 \times 2 min).

Peptide elongation was carried out using peptide segments C(S*t*Bu)IIGKGRSYKGTVSITKSGIK-SEA^{off}, C(S*t*Bu)QPWSSMIPHEHSFLPSSYRGKDLQENY-SEA^{off} and

CRNPRGEEGGPWCFTSNPEVRYEVC DIPCSEV-NH₂.

At the end of the synthesis, the solid support was washed with water (10 × 2 min) and quickly with the cleavage solution (30 seconds, 2 mL) at room temperature (0.1 M hydroxylamine, 1 M aniline, 6 M Gn.HCl, 20% AcOH, 2.15 mM 2-hydroxy 5-methoxybenzoic acid, pH 4.22). The solid support was drained and the cleavage solution was added again (2 mL). The suspension was then placed at 45 °C on a shaker.

After 29 hours, the solid support was drained. The solid support was washed with 20% AcOH (5 × 1 min, 5 mL), water (5 × 1 min, final volume = 20 mL). The supernatants were combined and extracted with diethyl ether to remove the internal reference prior to HPLC purification (eluent A = water containing 0.1% TFA, eluent B=acetonitrile in water 4/1 containing 0.1% TFA, 30 °C, detection at 215 nm, 6 mL/min, 0 to 20% eluent B in 5 min, then 20 to 40% eluent B in 60 min, C18XBridge column) which furnished 6.43 mg of peptide **18** (overall yield 23% starting from peptide segment **4e**).

11.1 Characterization of protein **18** (cleavage method A)

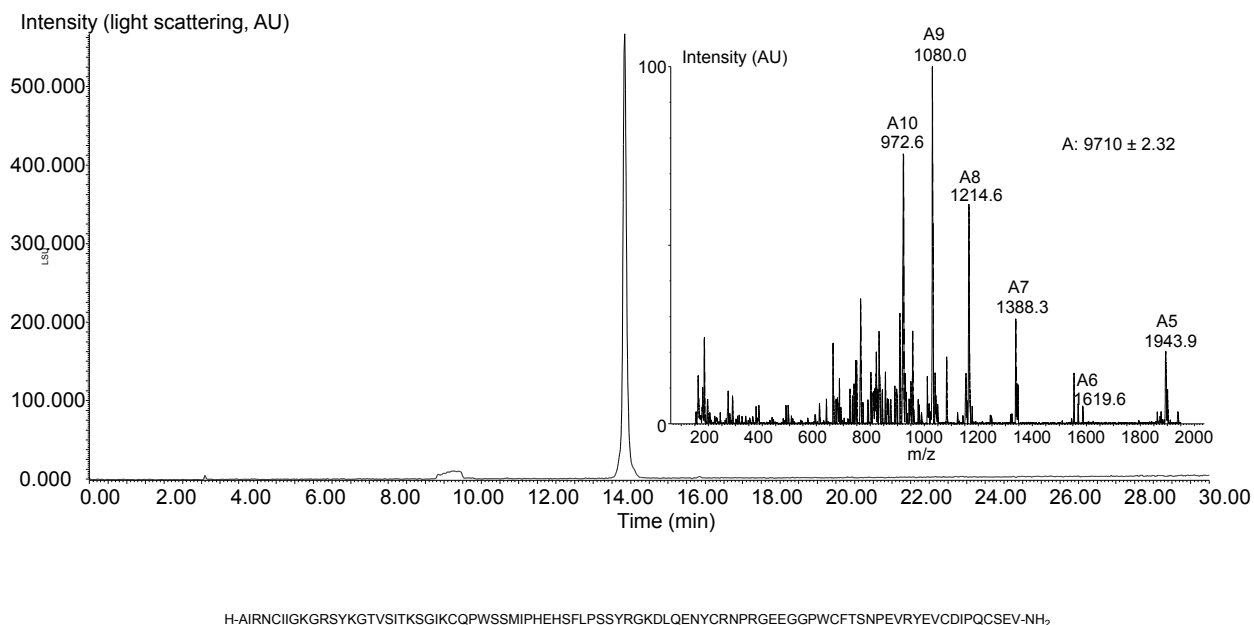


Figure S 54. LC-MS analysis of protein **18**. LC trace, eluent A 0.10% TFA in water, eluent B 0.10% TFA in CH₃CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 µm (4.6 × 250 mm) column, gradient 0-100% B in 30 min (1 mL/min, detection 215 nm). MS trace: [M+5H]⁵⁺ m/z (mean) calcd. 1942.98, obs. 1943.9, [M+6H]⁶⁺ m/z calcd. 1619.3, obs. 1619.6, [M+7H]⁷⁺ m/z calcd. 1388.1, obs. 1388.3, [M+8H]⁸⁺ m/z calcd. 1214.74, obs. 1214.6, [M+9H]⁹⁺ m/z calcd. 1079.88, obs. 1080.0, [M+10H]¹⁰⁺ m/z calcd. 971.99, obs. 972.6.

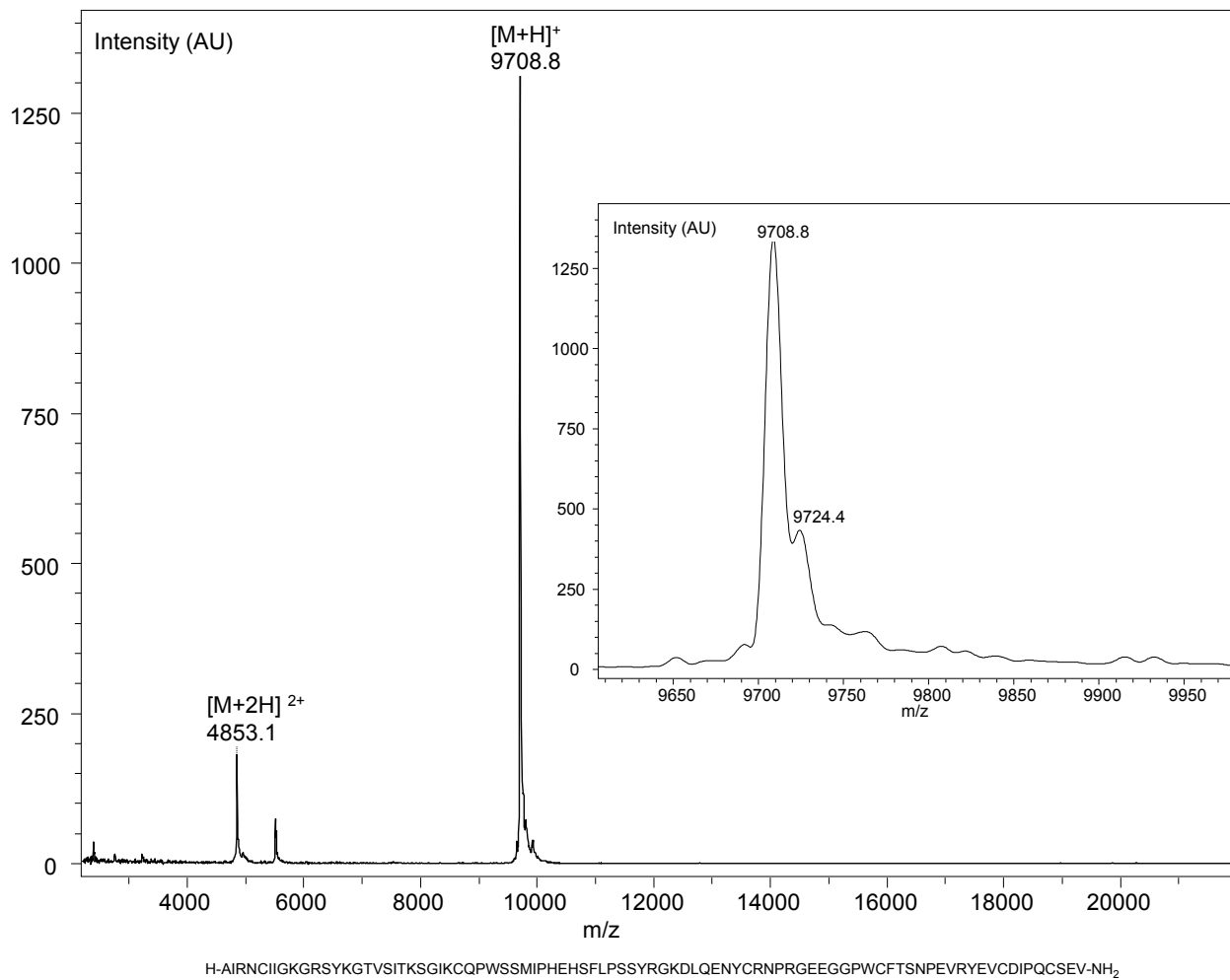


Figure S 55. MALDI-TOF analysis of protein **18**. Matrix sinapinic acid, positive detection mode, $[M+H]^+$ calcd. (mean) 9710.92 , found 9708.8.

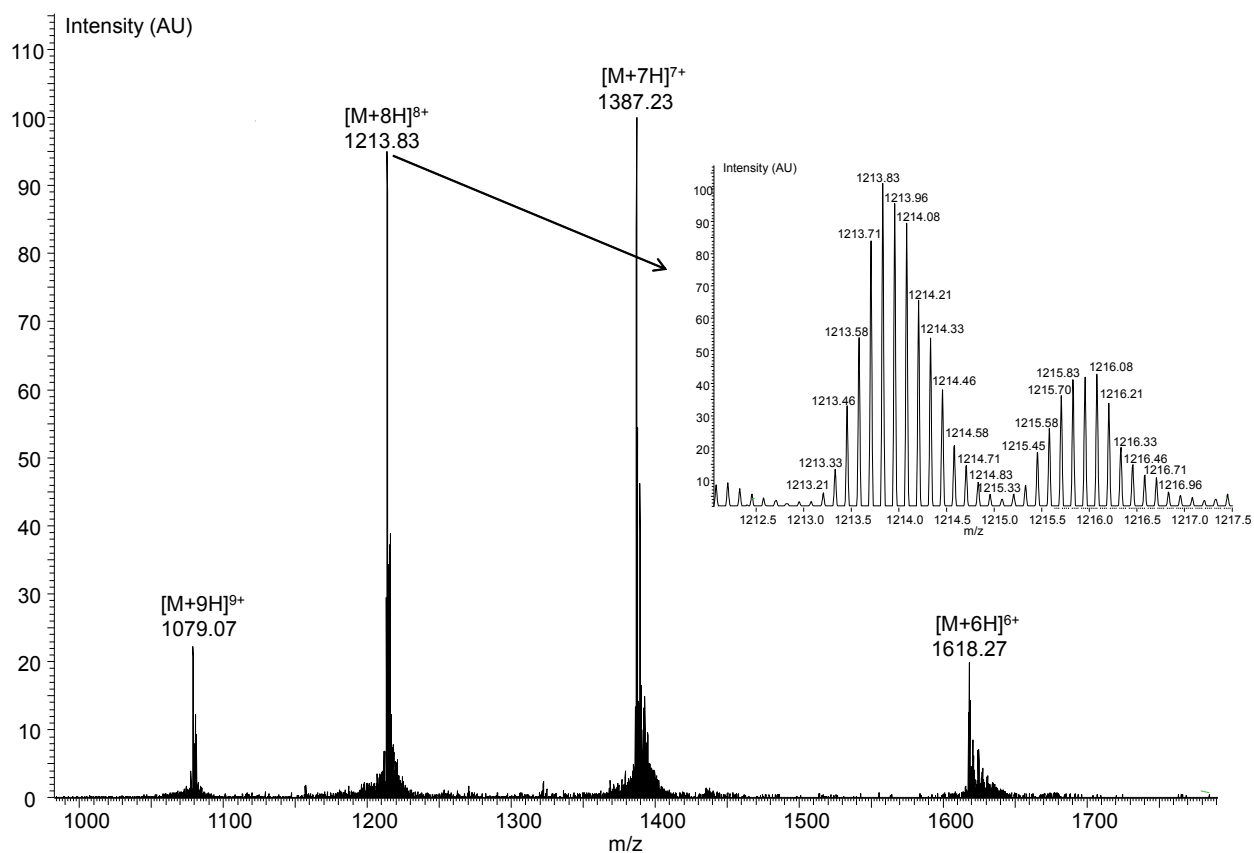


Figure S 56. ESI MS high resolution spectrum analysis of protein **18**. $[M+9H]^{9+}$ calcd. (monoisotopic) 1079.18, found 1079.07, $[M+8H]^{8+}$ calcd. 1213.95; found 1213.83, $[M+7H]^{7+}$ calcd. 1387.23, found 1387.23, $[M+6H]^{6+}$ calcd.1618.27, found 1618.27.

11.2 Folding of peptide **18** into HGF K1 domain **19**

Peptide **18** (5.85 mg, 0.52 μ mol) was dissolved in pH 7.4 10 mM sodium phosphate buffer containing 138 mM NaCl 2.7 mM KCl, 10% by vol of glycerol, 1 mM glutathione, 0.2 mM glutathione disulfide (total volume 17.3 mL).

The reaction mixture was placed at 4 $^{\circ}$ C. After 94 h, the reaction mixture was centrifuged at 4 $^{\circ}$ C at a relative centrifugal force (RCF) of 6000 during 15 minutes to eliminate the particles formed during the folding process.

The reaction mixture (~16.8 mL) was then concentrated by ultrafiltration (molecular weight cut-off of 3000, 12 $^{\circ}$ C, 12000 RCF). The solution was further dialysed 1 h then overnight at 4 $^{\circ}$ C in a dialysis cassette with a cut-off of 3500 against pH 7.4 10 mM sodium phosphate buffer containing 138 mM NaCl, 2.7 mM KCl, 10% by vol of glycerol (1 L). The volume was ~1.4 mL after dialysis.

The concentration of K1 domain **19** (144 μ M) was determined using BCA assay and BSA as a reference.

11.3 Characterization of HGF K1 domain **19**

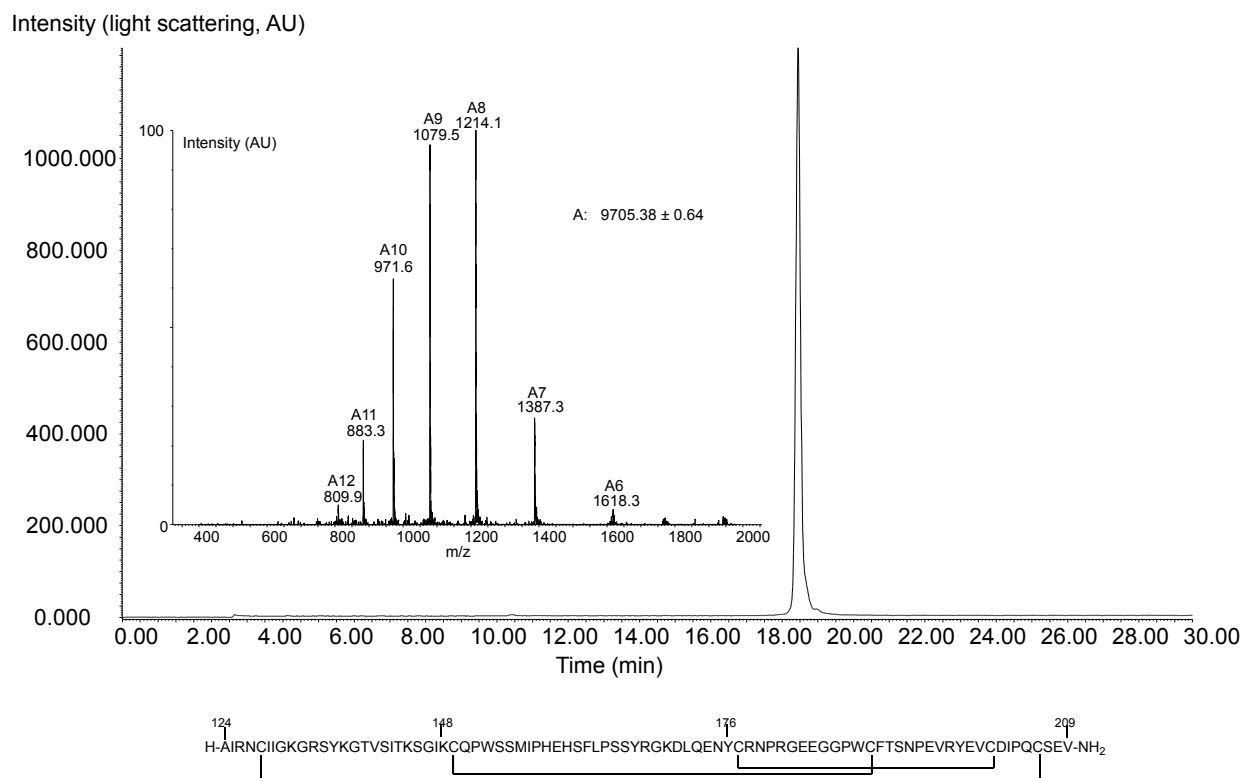


Figure S 57. LC-MS analysis of protein **19**. LC trace, eluent C 0.10% formic acid in water, eluent D 0.10% formic acid in $\text{CH}_3\text{CN}/\text{water}$: 4/1 by vol. Zorbax 300SB-C3 (3.5 μm , 300 \AA , 4.6 \times 150 mm) at 50 $^\circ\text{C}$, gradient 0-50% D in 30 min (1 mL/min, detection 215 nm). MS trace: $[\text{M}+6\text{H}]^{6+}$ m/z (mean) calcd. 1618.3, obs. 1618.3, $[\text{M}+7\text{H}]^{7+}$ m/z calcd. 1387.27, obs. 1387.3, $[\text{M}+8\text{H}]^{8+}$ m/z calcd. 1214.7, obs. 1214.1, $[\text{M}+9\text{H}]^{9+}$ m/z calcd. 1079.2, obs. 1079.5, $[\text{M}+10\text{H}]^{10+}$ m/z calcd. 971.4, obs. 971.6, $[\text{M}+11\text{H}]^{11+}$ m/z calcd. 883.2, obs. 883.3, $[\text{M}+12\text{H}]^{12+}$ m/z calcd. 809.7, obs. 809.9.

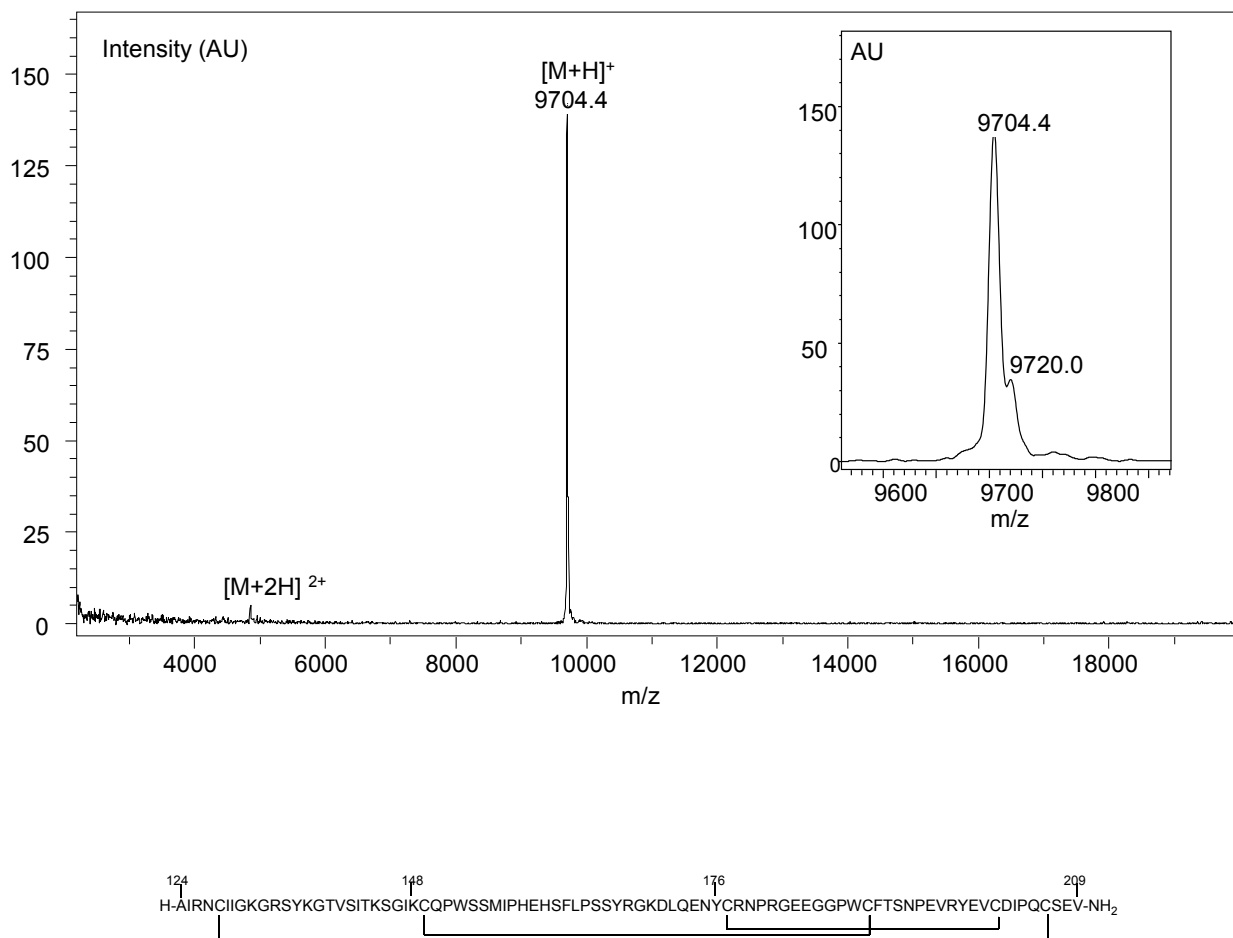


Figure S 58. MALDI-TOF analysis of K1 protein **19**. Matrix sinapinic acid, positive detection mode, $[M+H]^+$ calcd. (mean) 9704.92, found 9704.4.

11.4 Biological assays. K1 domain 19

MCF10A cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12 (1 :1), Gibco® Life Technologies®) with 20 ng/mL human recombinant Epidermal Growth Factor (Peprotech®), 100 ng/mL cholera toxin (Calbiochem®), 0.01 mg/mL insulin (Sigma Aldrich®), 500 ng/mL hydrocortisone (Calbiochem®) supplemented with 5% horse serum (Life Technologies®). All media were supplemented with 1% Zell Shield™ (Minerva Biolabs®)

Recombinant human HGF/SF was purchased from Peprotech® (Rocky Hill, CT, USA).

Cells were cultured in 6-well plates and starved overnight in 0.1% SVF medium. They were then lysed with 100 μ L RIPA buffer (20 mM HEPES, 1% NP40, 0.1% SDS, 5% glycerol, 142 mM KCl, 5 mM MgCl₂, 1 mM EDTA, pH 7.45) supplemented with phosphatase inhibitors (1/200

Phosphatase Inhibitor Cocktail 2 – Sigma® P5726) and protease inhibitors (1/400 Protease Inhibitor Cocktail – Sigma® P1860).

The total protein concentration was determined with the BCA Protein Assay Reagent Kit (Pierce®), and equal amounts of proteins were resolved on NuPAGE 4-12% Bis-Tris gels (Novex® by Life Technologies™). Separated proteins were transferred onto a polyvinyl difluoride membrane in Towbin buffer (10% methanol, 10% Tris-glycine 1X, 0.0025% SDS). The membrane was then equilibrated in blocking buffer (8 g casein/1L, PBS 1X, 0.2% Tween). Proteins were analyzed by western blotting with anti-phosphorylated MET at 1/1000 dilution from stock solution (Tyr 1234/1235, #3077 – Cell Signaling®) or anti-MET (3D4 - Life Technologies®). After incubation with the appropriate species-specific horseradish-peroxidase-conjugated secondary antibodies (anti-rabbit (#711-035-15), mouse (#115-035-146), or goat (#705-035-003) – Jackson ImmunoResearch Lab®), the antigen-antibody complexes were detected with a light-sensitive photographic film (CL-Xposure™ Film – Thermo Scientific®) after use of West Dura Extended Duration Substrate (SuperSignal®).

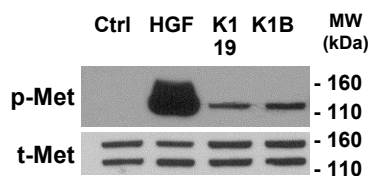
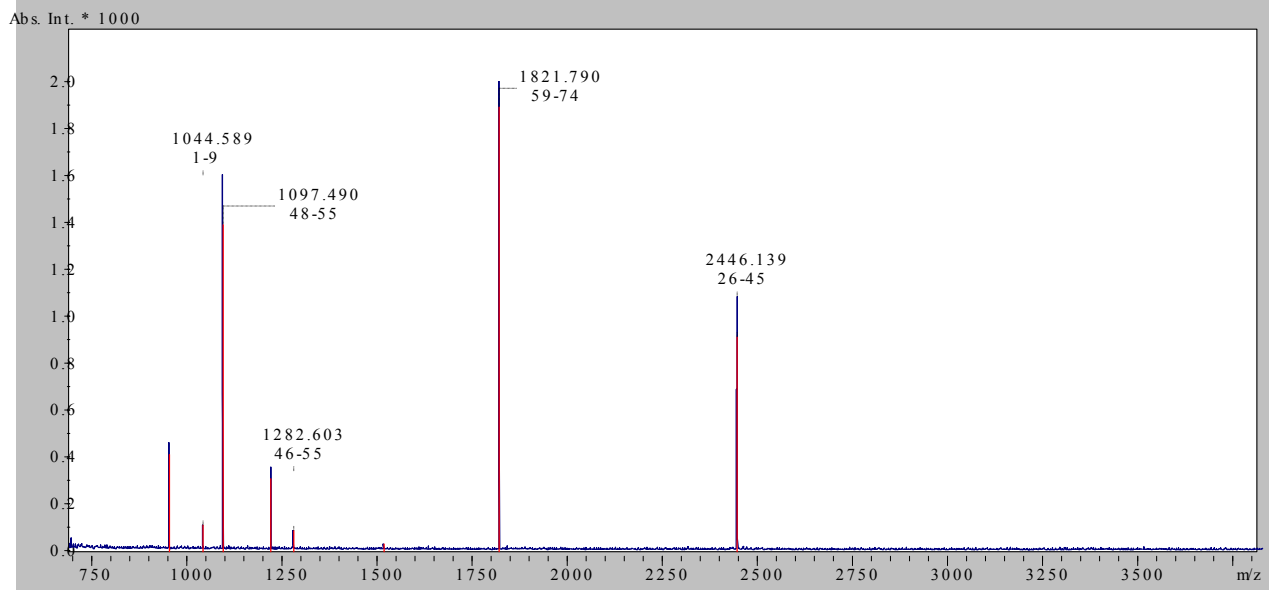


Figure S 59. MCF10A cells signaling analysis upon K1 stimulation. MCF10A cells were treated for 10 min with 500 pM mature HGF/SF (HGF), 1 μ M K1 domain **19** and 1 μ M K1B (see ref ³) used as a reference. Cell lysates were then analyzed by specific total MET or phospho-MET. Western blot. Ctrl: vehicle, MW: molecular weight.

11.5 Proteomic analysis of synthetic K1 domain 19

K1 domain **19** was dissolved in 25 mM ammonium bicarbonate pH 7.8 (1 mg/mL). This protein solution (10 μ g of protein) was mixed with DTT (10 μ g) and iodoacetamide (100 μ g). After 30 min, trypsin solution in 25 mM ammonium bicarbonate (100 ng/ μ L) was added on the above solution and let at 37 °C overnight. The solution was directly spotted on a MALDI plate and mixed with alpha cyano 4-hydroxycinnamic acid before analysis.



Tree hierarchy	Meas. M/z	Calc. MH+	▼ Ra...	P	Sequence
D:\Data\MALDI_2015\NAT...					
Unmatched					
Peaks					
peak 4	1222.60	-		-	
peak 6	1519.65	-		-	
peak 1	956.48	-		-	
Digest Results					
Modifications: Global: ...					
Search Parameter: Tit...					
peak 2	1044.59	1044.60	1 - 9	1	AIRNCIIGK 5: Carbamidomethyl (C)
peak 8	2446.14	2446.11	26 - 45	0	CQPWSSMIPHEHSFLPSSYR 1: Carbamidomethyl (C)
peak 5	1282.60	1282.58	46 - 55	1	GKDLQENYCR 9: Carbamidomethyl (C)
peak 3	1097.49	1097.47	48 - 55	0	DLQENYCR 7: Carbamidomethyl (C)
peak 7	1821.79	1821.79	59 - 74	0	GEEGPGWCFTSNPEVR 8: Carbamidomethyl (C)

Figure S 60. MALDI-TOF spectrum generated fragments after reduction, alkylation and trypsin cleavage of K1 domain 19.

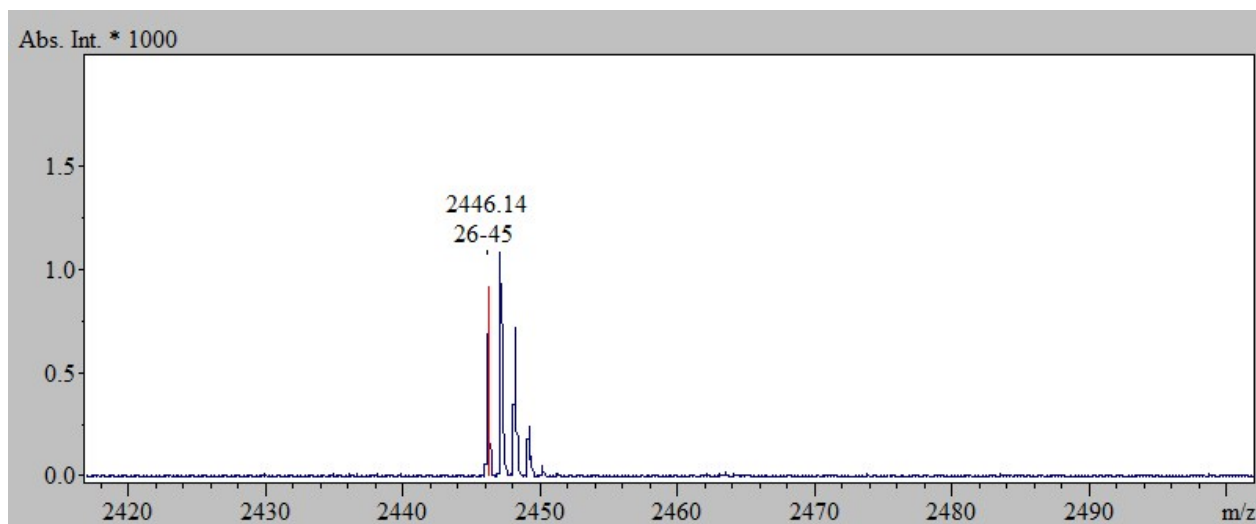


Figure S 61. MALDI-TOF spectrum generated fragments after reduction, alkylation and trypsin cleavage of K1 domain **19**. Focus on the ion at m/z 2446.14 which contains the unique Met residue found in K1 domain.

These data collected show that the +16 u modification is not located within the fragment 26-45 at m/z 2446.14 which contains the unique Met residue found in K1 domain **19** (no supplementary peak was observed at +16 u).

12. Total synthesis of biotinylated NK1 protein

12.1 One-pot synthesis of linear polypeptide NK1-B

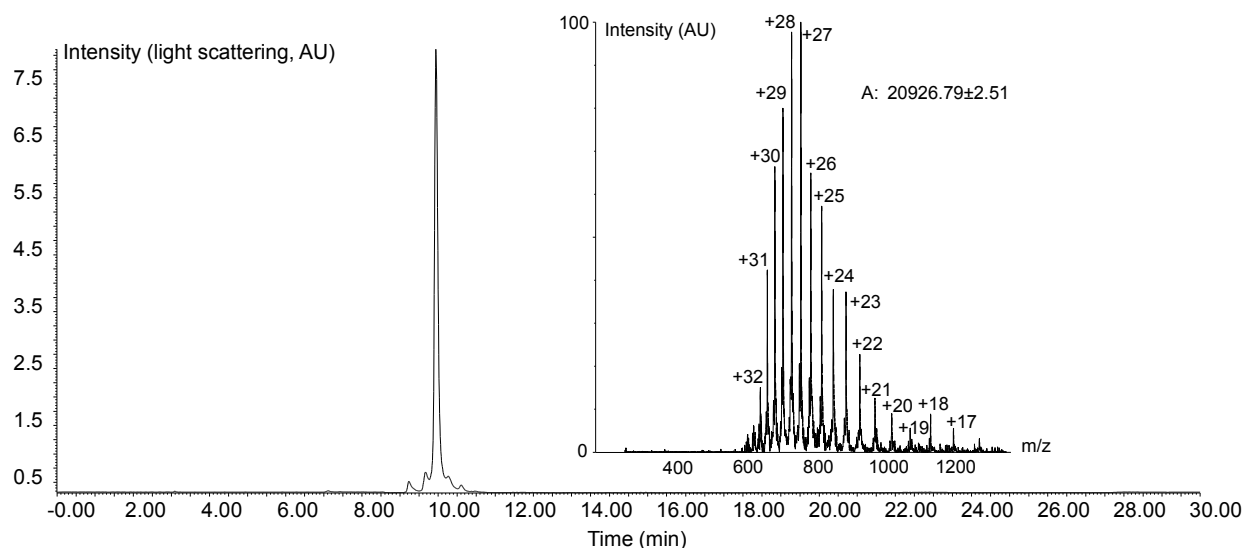
Gdn.HCl (57.6 mg) was dissolved in 0.1 M phosphate buffer (60 μ L) to give a final volume of 100 μ L (6 M final concentration) and this solution was added to 4-mercaptophenylacetic acid (3.39 mg, 0.2 M final concentration). 6 M NaOH (7 μ L) was added to adjust the pH to 6.98. Peptide **21** (1.07 mg, 0.22 μ mol) was dissolved in the above solution (45 μ L). After mixing, the solution was used to dissolve peptide **20** (2.25 mg, 0.22 μ mol). The reaction was placed under inert atmosphere at 37 $^{\circ}$ C overnight.

After 26 h, Gdn.HCl (57.8 mg) was dissolved in 0.1 M phosphate buffer (60 μ L) to give a final volume of 100 μ L (6 M final concentration) and added to *tris*(2-carboxyethyl)phosphine hydrochloride (5.78 mg, 0.2 M final concentration) and 4-mercaptophenylacetic acid (3.37 mg, 0.2 M final concentration). 6 M NaOH (13 μ L) was added to adjust the pH to 5.22. Peptide **17** (2.49 mg, 0.22 μ mol) was dissolved in this solution (45 μ L) and added to the above reaction mixture (final pH 5.48). The reaction was placed under inert atmosphere at 37 $^{\circ}$ C.

After 40 h, the reaction mixture was diluted in 6 M Gdn.HCl (2 mL) and acidified with glacial acetic acid (200 μ L). Three extractions with diethyl ether were done to remove 4-mercaptophenylacetic acid and the reaction mixture was further diluted with 6 M Gdn.HCl (2

mL) before RP-HPLC purification (eluent C = water containing 0.1% formic acid, eluent D=acetonitrile in water 4/1 containing 0.1% formic acid, 50°C, detection at 215 nm, 6 mL/min, 0 to 20% eluent D in 5 min, then 20 to 50% eluent D in 90 min, C3Zorbax column) which yielded 1.8 mg of protein NK1-B (35%).

A)



B)

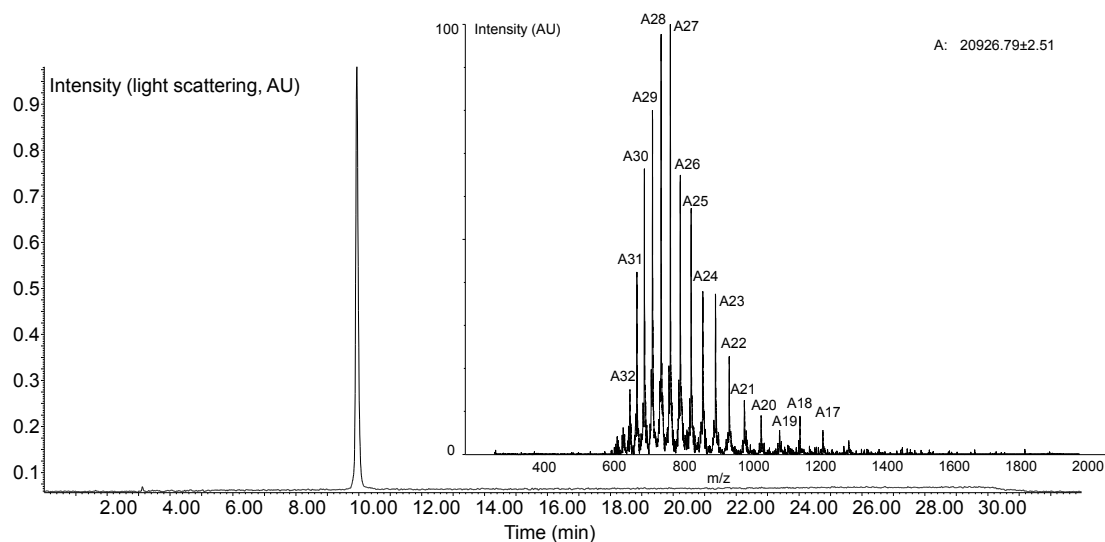


Figure S 62. LC-MS analysis of peptide **NK1-B**. A) Crude one-pot mixture after MPAA extraction. B) Purified **NK1-B** polypeptide. LC trace, eluent C 0.10% FA in water, eluent D 0.10% FA in CH₃CN by vol. C3 Zorbax 300SB 3.5 μm (4.6 × 250 mm) column, gradient 0-100% D in 30 min (1 mL/min, detection 215 nm). MS trace: M m/z calcd (mean) 20924.94, deconvoluted M 20926.79±2.51.

12.2 Folding of NK1-B polypeptide

NK1-B linear polypeptide (0.79 mg, 34.9 nmol) was dissolved in 10 mM PBS, 138 mM NaCl, 2.7 mM KCl buffer pH 7.4 containing 10 % by vol of glycerol, 1 mM reduced glutathione, 0.2 mM oxidized glutathione (1.176 mL). The reaction mixture was placed at 4°C.

The folding was monitored by LC-MS using a C3Zorbax 300 SB column (4.6 × 150 mm, 300 Å, 3.5 µm), 215 nm, 1 mL/min, 50°C, buffer C water containing 0.1 % formic acid, buffer D CH₃CN / water 4 / 1 containing 0.1 % formic acid, 0-100 % D in 30 min).

After 162 h, the reaction mixture was ultra-centrifuged at 6000 rcf and 12°C during 15 min to eliminate insoluble aggregates.

The mixture (876 µL) was then transferred in an ultrafiltration system (Vivaspin, 2 mL) with a cut-off of 3000 MWCO and ultrafiltered at 12000 rcf and 12°C during 45 min. Once concentrated (volume ~50 µL), the protein was diluted with 10 mM PBS, 138 mM NaCl, 2.7 mM KCl buffer pH 7.4 containing 10 % in vol of glycerol (100 µL) and then introduced in a dialysis cassette (cut-off of 3500 MWCO.)

Once dialysed twice (1 h then overnight) at 4°C against 10 mM PBS, 138 mM NaCl, 2.7 mM KCl buffer pH 7.4 containing 10 % in vol of glycerol (2 × 1 L), the volume was ~ 120 µL.

The concentration of the folded **NK1-B** protein was determined by measuring the absorbance at 280 nm with a Nanodrop system. Estimated concentration of **NK1-B** folded was 5.13 µM.

12.3 Biological activity

MCF10A or HeLA cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12 (1 :1), Gibco® Life Technologies®) with 20 ng/mL human recombinant Epidermal Growth Factor (Peprotech®), 100 ng/mL cholera toxin (Calbiochem®), 0.01 mg/mL insulin (Sigma Aldrich®), 500 ng/mL hydrocortisone (Calbiochem®) supplemented with 5% horse serum (Life Technologies®). All media were supplemented with 1% Zell Shield™ (Minerva Biolabs®)

Recombinant human HGF/SF was purchased from Peprotech® (Rocky Hill, CT, USA)

Cells were cultured in 6-well plates and starved overnight in 0,1% SVF medium. They were then lysed with 100 µL RIPA buffer (20 mM HEPES, 1% NP40, 0.1% SDS, 5% glycerol, 142 mM KCl, 5 mM MgCl₂, 1 mM EDTA, pH 7.45) supplemented with phosphatase inhibitors (1/200 Phosphatase Inhibitor Cocktail 2 – Sigma® P5726) and protease inhibitors (1/400 Protease Inhibitor Cocktail – Sigma® P1860).

The total protein concentration was determined with the BCA Protein Assay Reagent Kit (Pierce®), and equal amounts of proteins were resolved on NuPAGE 4-12% Bis-Tris gels (Novex® by Life Technologies™). Separated proteins were transferred onto a polyvinyl difluoride membrane in Towbin buffer (10% methanol, 10% Tris-glycine 1X, 0.0025% SDS). The membrane was then equilibrated in blocking buffer (8 g casein/1L, PBS 1X, 0.2% Tween). Proteins were analyzed by western blotting with anti-phosphorylated MET at 1/1000 dilution from stock solution (Tyr 1234/1235, #3077 – Cell Signaling®), anti-MET (3D4 - Life Technologies®), anti-phosphorylated-AKT (Ser473, #9271 - Cell Signaling®), anti-AKT (Cell

Signaling®), anti-phosphorylated-ERK (Thr202/Tyr204, #9106 - Cell Signaling®), anti-ERK2 (C14 – Santa Cruz®). After incubation with the appropriate species-specific horseradish-peroxidase-conjugated secondary antibodies (anti-rabbit (#711-035-15), mouse (#115-035-146), or goat (#705-035-003) – Jackson ImmunoResearch Lab®), the antigen-antibody complexes were detected with a light-sensitive photographic film (CL-Xposure™ Film – Thermo Scientific®) after use of West Dura Extended Duration Substrate (SuperSignal®).

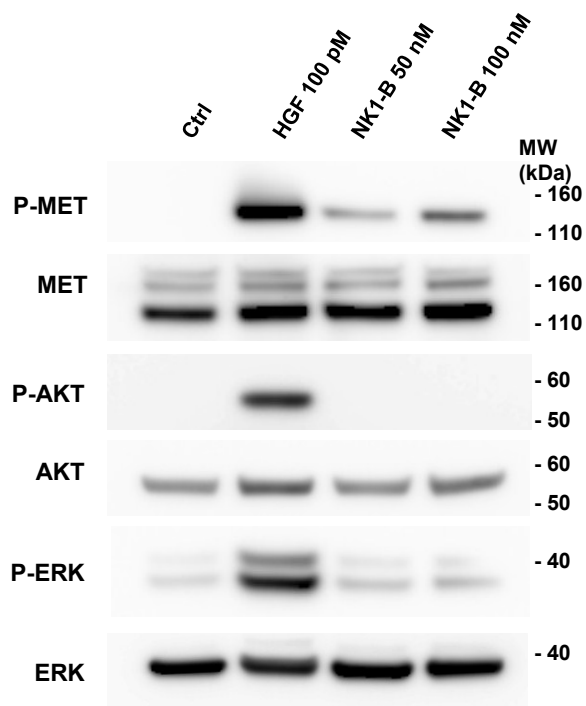


Figure S 63. HeLa cells signaling analysis upon NK1-B stimulation. HeLa cells were treated for 10 min with 500 pM mature HGF/SF (HGF), NK1-B protein (50 and 100 nM). Cell lysates were then analyzed by specific total or phosphor MET, Erk or Akt antibodies. Western blot. Ctrl: vehicle, MW: molecular weight.

13. Peptide purification by covalent capture

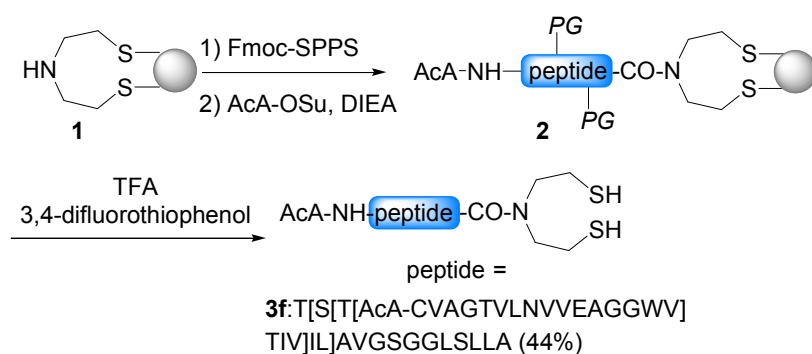
13.1 Introduction

Bifunctional linkers described in previous studies (see Table 1 in the main manuscript) enabled also the purification of peptides produced by SPPS by setting up a solid phase covalent capture/release approach.^{4,5} Despite remarkable achievements, the previous solid phase approaches could not be applied to the isolation of peptide thioesters and/or *O*-acyl isopeptides which do not tolerate aqueous basic conditions. Peptide thioesters are key

components in the synthesis of proteins using NCL, while the incorporation of *O*-acyl isodipeptide units in a peptide backbone is a popular method to improve significantly the solid phase peptide synthesis (SPPS)⁶⁻⁸ and the solubility of hydrophobic peptides in aqueous solution.⁹⁻¹¹

We were particularly interested in the development of a covalent capture/release purification method that would be compatible with peptide segments featuring a protected N-terminal Cys residue, internal *O*-acyl isodipeptide units and a C-terminal thioester group. A solid phase method enabling the purification of such peptide segments should facilitate considerably the preparation of hydrophobic proteins.

Peptide **3f** whose sequence is derived from AS-48 antimicrobial peptide was chosen as a model for this study. Preliminary attempts to produce the peptide with a natural backbone by classical Fmoc SPPS failed due to the high proportion of hydrophobic amino acids and the absence of charged residues in its sequence. The incorporation of three *O*-acyl isodipeptide units improved the synthesis and allowed the introduction of the AcA group after Fmoc SPPS (Scheme S 1).



Scheme S 1. Synthesis of peptide **3f**.

The purification of the crude peptide was carried out as shown in Scheme S 2. First, the AcA peptide **3f** was reacted with the aminooxyacetyl PEGA resin **11** at pH 3.0. At this pH, the *O*-acyl isopeptide units do not rearrange. This was confirmed by analysing the supernatant by HPLC which showed the stability of peptide **3f** in these conditions. Importantly, this analysis showed also the selective capture of both SEA^{on} amide (X_1) and thioester (X_2) forms (see Figure S 64 below). The beads were thoroughly washed with 6 M Gn.HCl (pH 3) to remove the sticky impurities and then cleaved in TFA to produce peptide **24**. In this peptide, the N-terminal Cys residue was protected by an *N*-acetoacetyloxime moiety while amide and thioester forms of the SEA group were present in equal proportion. The neutral amide form was further converted into the positively charged and hydrophilic thioester form by incubating the peptide in 6 M Gn.HCl pH 1.7. Peptide **25** which was equipped with a total of four positively charged amino groups and already separated from the main impurities by the solid phase capture step was further isolated by HPLC. Importantly, the structure of peptide **25** is confirmed by its MALDI-

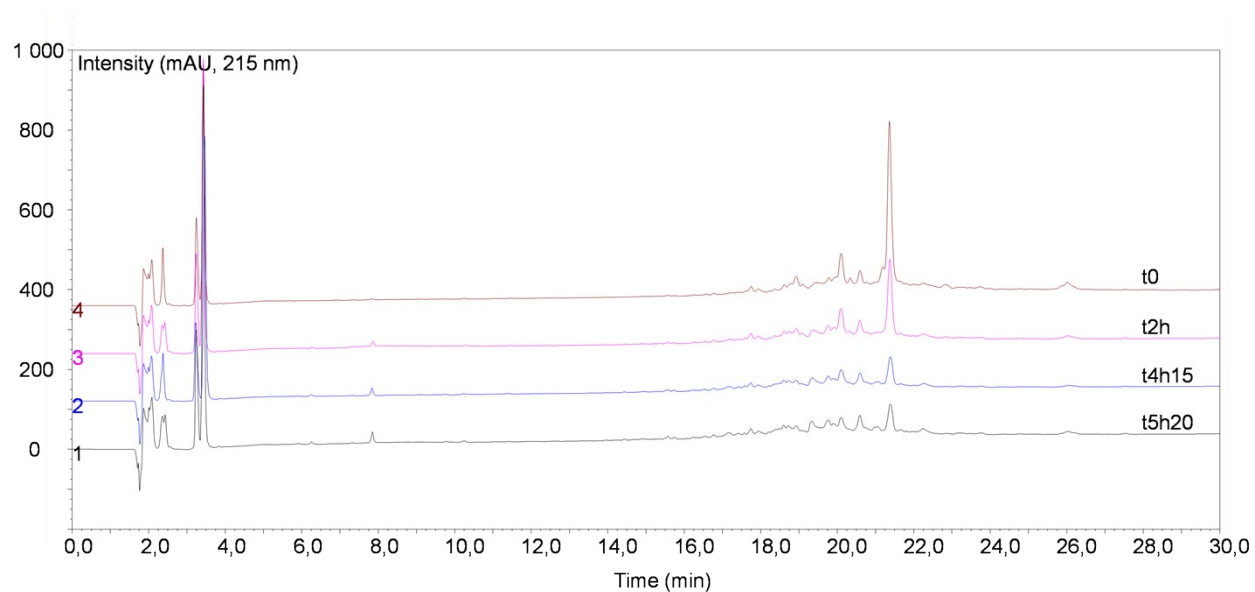


Figure S 64. Monitoring of the covalent capture of peptide **4f** (amide and thioester forms) by HPLC.

The monitoring of the covalent capture shows the selective capture of peptide **4f**, while impurities remained in solution.

After 5 h 20, the solid support was thoroughly washed with 6 M Gdn.HCl, 50 mM citrate buffer pH 2.97 (10×2 min) then eluent A (10×2 min). The solid support was further washed with methanol (2×2 min) and dried under vacuum (15 min).

TFA cleavage was performed twice with TFA/water/triisopropylsilane/thiophenol 90/2.5/5/2.5 by volume during 1 h ($2 \times 400 \mu\text{L}$). The cleaved peptide was precipitated in cold diethylether/heptane 1/1 by vol (8 mL), centrifuged, washed twice with cold diethylether/heptane 1/1 by vol and then dissolved in 6 M Gdn.HCl/eluent A pH 1.7. The peptide was immediately analyzed by LC-MS as shown in Figure S 64.

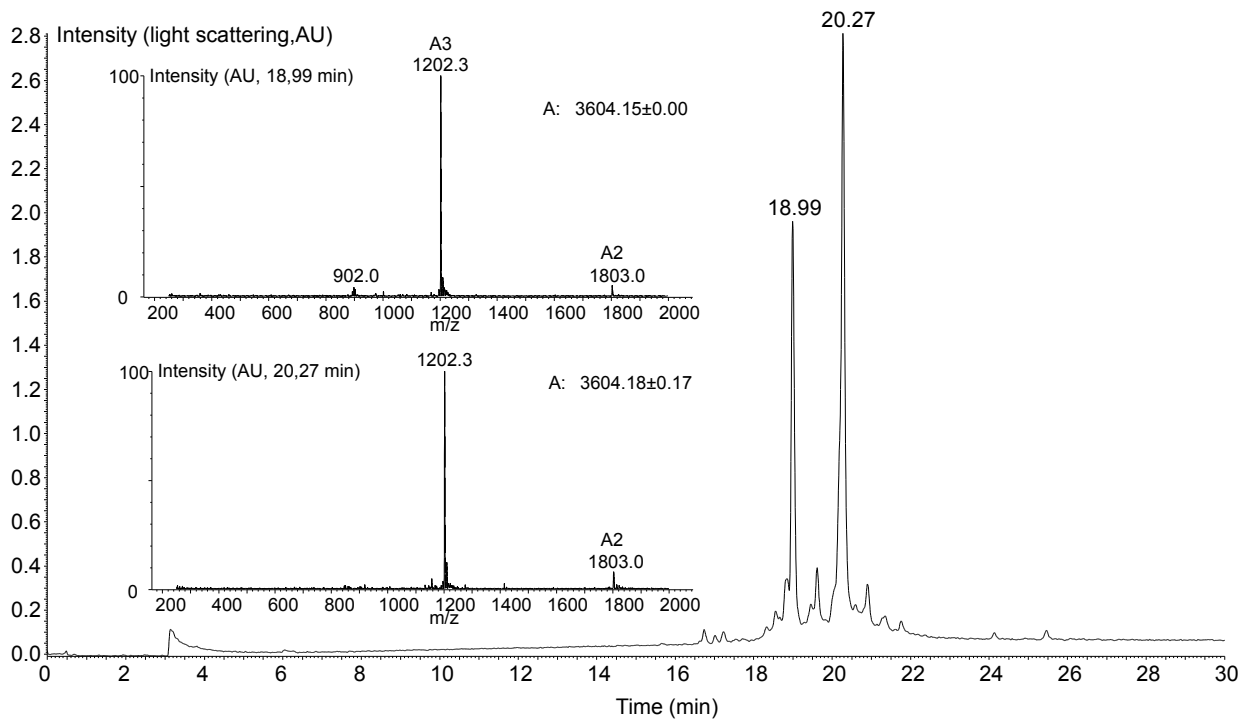


Figure S 65. LC-MS of peptide **24** obtained after TFA cleavage. Peak at 18.99 min, SEA^{on} thioester form $[M+2H]^{2+}$ m/z calcd. (mean) 1803.17, obs 1803.0, $[M+3H]^{3+}$ m/z calcd. 1202.4, obs. 1202.3, $[M+4H]^{4+}$ m/z calcd. 902.1, obs. 902.0; peak at 20.27 min, SEA^{on} amide form, $[M+2H]^{2+}$ m/z calcd. (mean) 1803.17, obs 1803.0, $[M+3H]^{3+}$ m/z calcd. 1202.4, obs. 1202.3.

In order to isolate a unique species, the SEA^{on} amide form was rearranged in SEA thioester which has the advantage of being more hydrophilic. For this, the peptide solution in 6 M Gdn.HCl/eluent A pH 1.7 was agitated at rt for one day. LC-MS analysis of the crude mixture showed is shown in Figure S 65 and shows the presence of the thioester form as the major species.

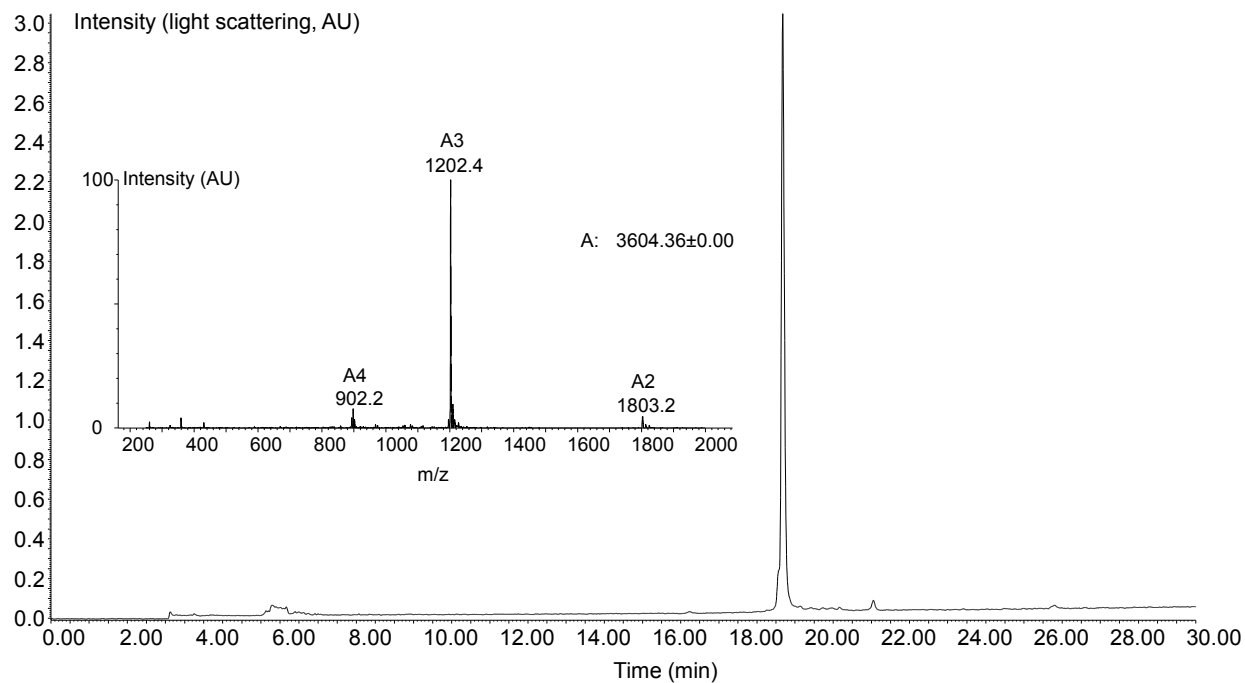


Figure S 67. LC MS of purified peptide **24**. MS trace: $[M+2H]^{2+}$ m/z calcd. 1803.17, obs. 1803.2, $[M+3H]^{3+}$ m/z calcd. 1202.4, obs. 1202.4, $[M+4H]^{4+}$ m/z calcd. 902.1, obs. 902.2.

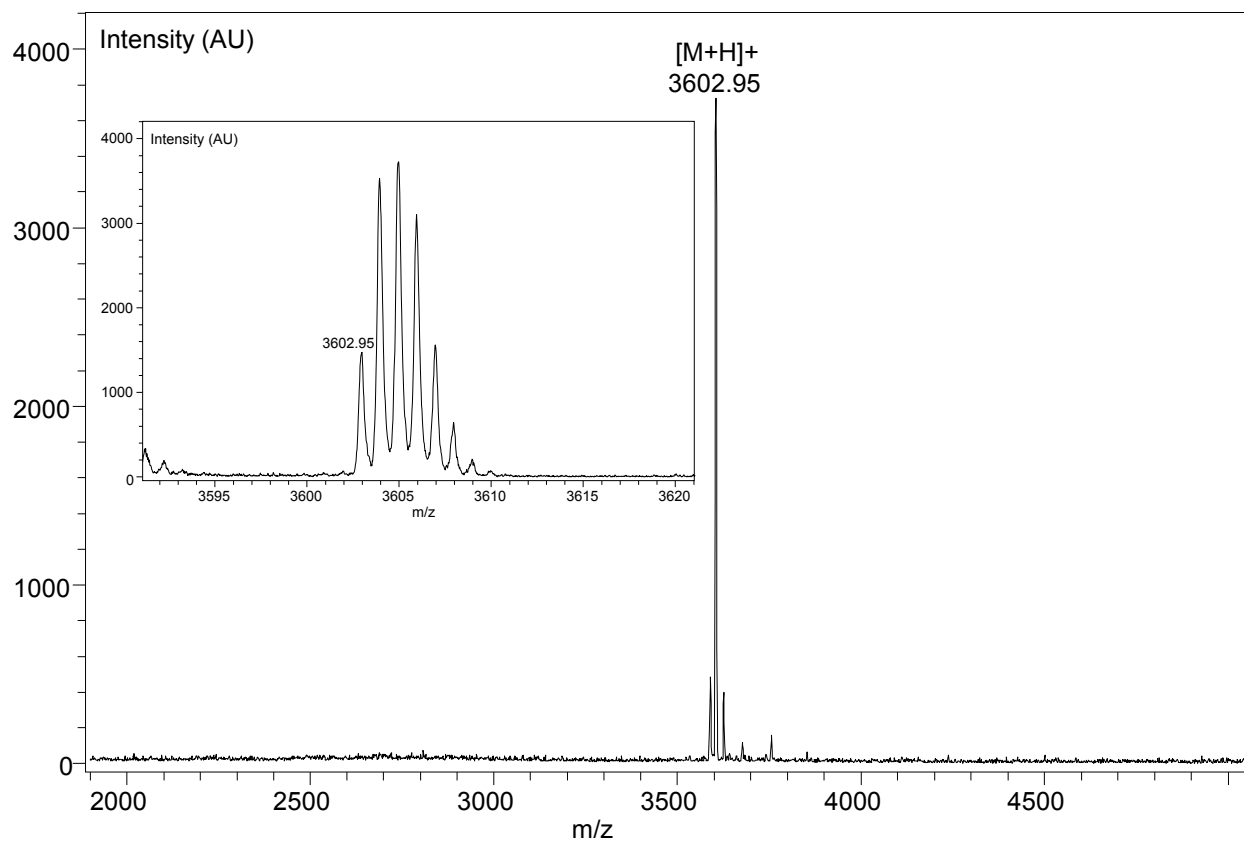


Figure S 68. MALDI-TOF analysis of peptide **25**. Matrix 2,5-dihydroxybenzoic acid (DHB), positive detection mode, $[M+H]^+$ calcd. (monoisotopic) 3602.9, found 3602.95.

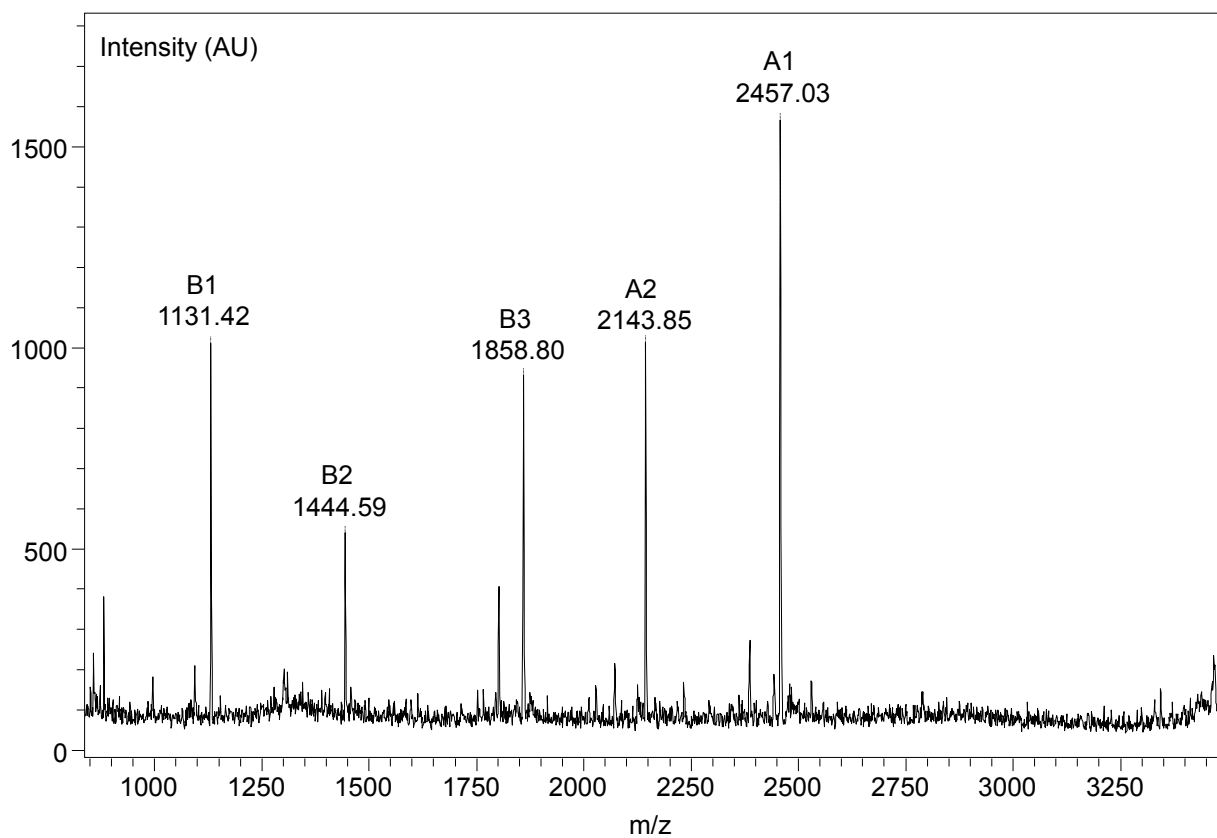


Figure S 69. MALDI-TOF analysis of peptide **25**. Matrix 2,5-dihydroxybenzoic acid (DHB), positive detection mode, focus on the ions produced by according a McLafferty rearrangement.

