

Supporting information

One-pot oxime ligation from peptides bearing thiazolidine and aminoxyacetyl groups

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General information

All Fmoc protected amino acids and peptide synthesis reagents were purchased from Novabiochem (Merck Millipore, Darmstadt, Germany). Other chemical reagents and solvents are from Carlo Erba Reagents (Val de Reuil, France). Boc-Aoa-OH (2-(*tert*-butyloxycarbonylaminoxy)acetic acid) is from Iris Biotech GmbH. Peptides were synthesized in solid phase using Fmoc chemistry on microwave assisted CEM-Liberty One synthesizer. HPLC analysis was performed on a Shimadzu Prominence LC-20AD HPLC using a Phenomenex Luna C₁₈ column (5 μm, 4.6 × 250 mm) with dual UV detection at 214 nm and 254 nm and a linear mobile phases A-B gradient at a flow rate of 1 mL/min. Crude peptides purification performed on a Shimadzu semi-preparative HPLC system using an Alltima C₁₈ column (5 μm, 10 × 250 mm) at a flow rate of 2 mL/min. The purity of the peptides was attested by analytical HPLC, and the purified peptides were further characterized by MALDI-TOF mass spectrometry on an Applied Biosystems/MDS SCIEX 4800 MALDI TOF Analyzer with α-cyano-4-hydroxycinnamic acid as matrix.

HPLC mobile phase A: aqueous solution containing 0.1 % TFA, named Solution A

HPLC mobile phase B: aqueous 70% acetonitrile solution containing 0.1 % TFA, named Solution B

pH 4.5 buffer: 500mM ammonium acetate, pH was adjusted with acetic acid

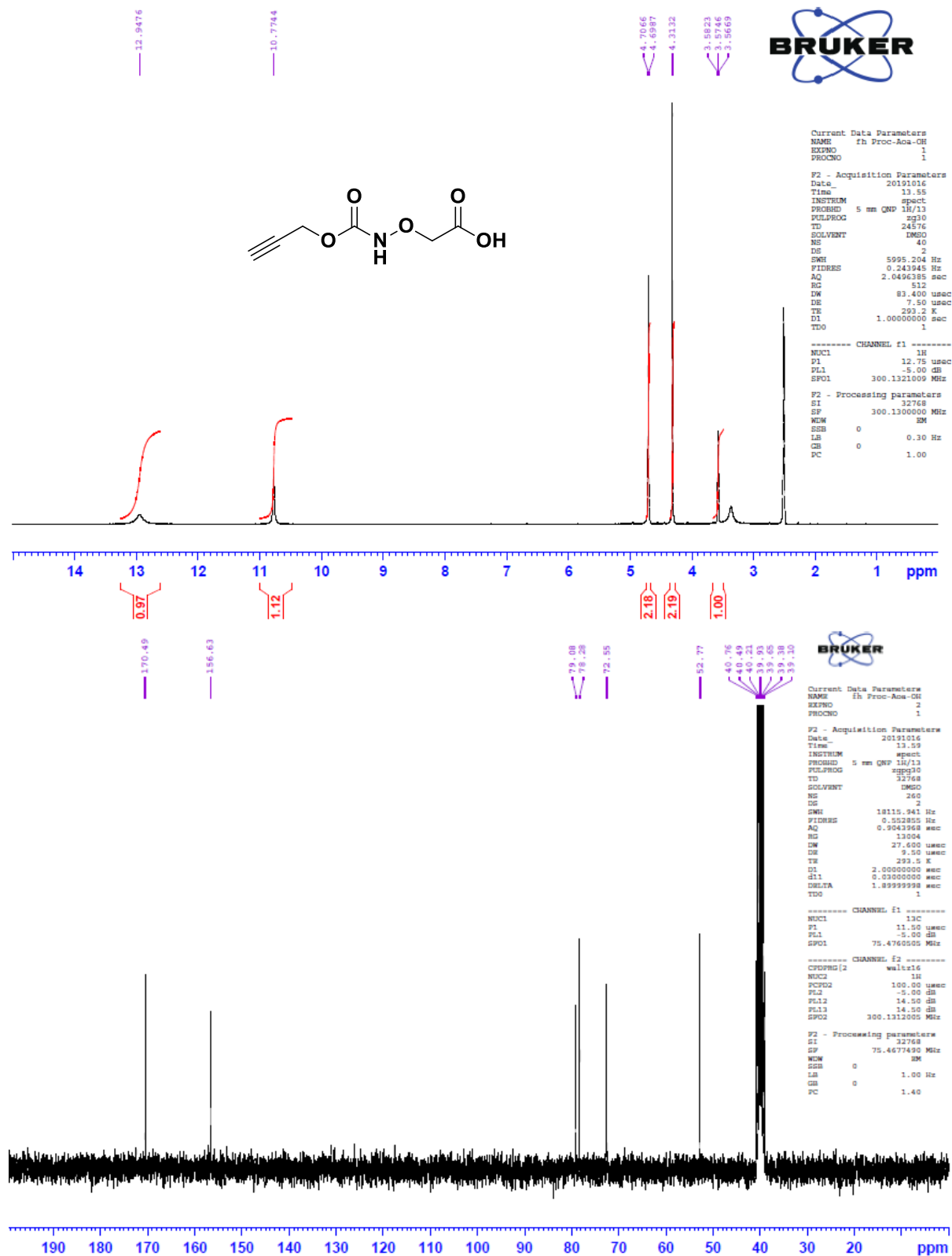
pH 7 buffer: Phosphate-buffered saline (PBS) 1x

Synthesis of Proc-Aoa-OH

2-(Propagylloxycarbonylaminoxy)acetic acid (Proc-Aoa-OH)

(Aminoxy)acetic acid hydrochloride (Aoa•0.5 HCl, MW109.3, 1.1 g, 10 mmol) was dissolved in 10 mL water, cooled in ice-water bath. Solid KHCO₃ (MW100.1, 2.7 g, 30 mmol) was added in small portions. Propagyl chloroformate (MW 118.52, d 1.215 g/ml, 0.98 mL, 10 mmol) in 10 mL dioxane was added dropwise, the mixture was stirred from 0°C to room temperature overnight. Dioxane was then evaporated, aqueous residue washed with ethyl ether (2*10 mL) and acidified with 12 N HCl to pH 1. Saturated the aqueous phase with solid NaCl, extracted with chloroform (3*50 mL). Organic extract was dried with MgSO₄ and evaporated to dryness then crystallized in small volume of dichloromethane to give 1.07 g white waxy solid (yield 61.6 %). The low yield is due to the high hydrophilic property of Proc-Aoa-OH.

$^1\text{H-NMR}$ (DMSO- d_6 , δ ppm): 12.95 (s, 1H, CO_2H); 10.77 (s, 1H, NH); 4.70 (d, $^4J = 2.3$ Hz, 2H, CH_2); 4.31 (s, 2H, CH_2); 3.57 (t, $^4J = 2.3$ Hz, 1H, CH). $^{13}\text{C-NMR}$ (DMSO- d_6 , δ ppm): 170.5, 156.6, 79.1, 78.3, 72.5, 52.7.



Solid-phase peptide synthesis

Peptide synthesis

All peptides were synthesized on CEM Liberty One peptide synthesizer, using Pepdrive standard procedures.

Rink Amide MBHA resin* (0.52 mmol/g, 100-200 mesh) was used in small scale (0.1 mmol). Fmoc-protected amino acids were used in 0.5 mmol (5.0 eq.) to assure complete coupling. A solution of 20% piperidine in N,N-dimethylformamide (DMF) was used for Fmoc deprotection. DIC (diisopropylcarbodiimide) and Oxymapure® were used as coupling reagents (5.0 eq each). All amino acids were coupled by single coupling, except arginine which was coupled by double-coupling cycle and cysteine was coupled with weaker microwave radiation, following the Pepdrive guidance.

*model peptides **5**, **6** were synthesized using pre-loaded Fmoc-Lys(Boc)-Wang resin

Compound	Sequence	Mass found (Da)
Peptide 1	Thz-Ahx-C*PSDSTRKGGRC*GRRL-NH ₂	2234
Peptide 2	CHO-(CO)-Ahx-C*PSDSTRKGGRC*GRRL-NH ₂	2175
Peptide 3	Proc-Aoa-RQIKIWFQNRRMKWKK-NH ₂	2400
Peptide 4	Aoa-RQIKIWFQNRRMKWKK-NH ₂	2318
Peptide 5	Thz-GYRMHK-OH	906
Peptide 6	Aoa-GYRMHK-OH	864

Resin cleavage and peptide isolation

After the complete peptide synthesis, the peptide resin was washed with dichloromethane (3x) and dried under vacuum. Peptide side chain deprotection and resin cleavage were triggered in a cleavage cocktail comprised of either 94/2.5/2.5/1 (v/v) TFA/H₂O/Ethanedithiol/TIPS for peptide bearing cysteine residues (Peptide **1**) or 95/2.5/2.5 (v/v) TFA/H₂O/TIPS for others (Peptide **3**, **4**, **5**, **6**). (TFA: trifluoroacetic acid; TIPS: triisopropylsilane). The cleavage mixture was gently agitated by rotation for 3 h. The reaction mixture was then drained into diethyl ether and centrifuged at 4000 rpm at 4 °C (15min). The supernatant was carefully removed, the pellet was subsequently resuspended in ether and centrifuged. This ether wash process was repeated three times. The precipitated peptide pellet was finally dissolved in water and lyophilized.

Thz-Ahx-C*PSDSTRKGGRC*GRRL-NH₂ (Peptide 1)

The linear peptide containing Ahx spacer (6-aminohexanoic acid) was synthesized and purified according to the above procedure using racemic Boc-Thz-OH as the last amino acid. Resin cleavage and deprotection adapted to cysteine residues, purification (gradient: 5-45% B in 50 min) and lyophilization afforded the peptide as a fluffy white powder; MALDI-TOFF: Found $[M+H]^+$ 2132 Da for C₈₃H₁₅₀N₃₆O₂₄S₃; HPLC: Rt 12.6 min (gradient: 10-100% B in 30 min).

Linear peptide was then cyclized. 1,2-Bis(bromomethyl)benzene (50 mM solution in acetonitrile, 1.1 eq) was added to the linear peptide solution in Tris buffer (pH 7.6)/acetonitrile (v/v 2/1). The mixture was stirred at room temperature and the reaction monitored by HPLC. Cyclization was complete after one hour. Reaction mixture was then quenched with 20% acetic acid and lyophilized. The crude peptide was purified using 5-50% B in 50 min gradient to afford Peptide **1** (51% yield). MALDI-TOFF: Found $[M+H]^+$ 2234 Da for C₉₁H₁₅₆N₃₆O₂₄S₃; HPLC: Rt 13.4 min (gradient: 10-100% B in 30 min).

CHO-(CO)-Ahx-C*PSDSTRKGGRC*GRRL-NH₂ (Peptide 2)

During Thz deprotection assays, Peptide **2** was isolated and characterized using HPLC and mass spectrometry. MALDI-TOFF: found $[M+H]^+$ 2175 Da for C₈₉H₁₅₂N₃₅O₂₅S₂; HPLC: Rt 13.6 min (gradient: 5-100% B in 30 min)

Proc-Aoa-RQIKIWFQNRRMKWKK-NH₂ (Peptide 3)

Peptide **3** was synthesized and purified according to the above procedure using Proc-Aoa-OH as the last amino acid. Resin cleavage and deprotection, purification (gradient: 25-65% B in 50 min) and lyophilization afforded the peptide as a fluffy white powder. MALDI-TOFF: Found $[M+H]^+$ 2400 Da; C₁₁₀H₁₇₄N₃₆O₂₃S; HPLC: Rt 11.9 min (gradient: 30-100% B in 30 min).

Aoa- RQIKIWFQNRRMKWKK-NH₂ (Peptide 4)

Peptide **4** was synthesized and purified according to the above procedure using Boc-Aoa-OH as the last amino acid. Resin cleavage, purification (gradient: 25-65% B in 50 min) and lyophilization afforded the peptide as a fluffy white powder. MALDI-TOFF: Found $[M+H]^+$

2318 Da for $C_{106}H_{172}N_{36}O_{21}S$; HPLC: Rt 16.1 min (gradient: 10-100 % B in 30 min). We observed a by-product formed during Peptide 4 purification step which was identified as the acetoxime form of Peptide 4 (Oxime 2, MALDI-TOFF: Found $[M+H]^+$ 2358 Da for $C_{109}H_{176}N_{36}O_{21}S$).

Thz-GYRMHK-OH (model peptide, Peptide 5)

Peptide 5 was synthesized and purified according to the above procedure using racemic Boc-Thz-OH as the last amino acid. Resin cleavage, purification (gradient: 5-45% B in 50 min) and lyophilization afforded the peptide as a fluffy white powder. MALDI-TOFF: found $[M+H]^+$ 906 Da for $C_{38}H_{58}N_{12}O_{10}S_2$; HPLC: 2 peaks, Rt 13.5 + 13.7 min (Gradient : 10-60% B in 30 min). In this shorter peptide, the two isomers resulted from racemic thiazolidine residue can be seen on the HPLC spectrum.

Aoa-GYRMHK-OH (model peptide, Peptide 6)

Peptide 6 was synthesized and purified according to the above procedure using Boc-Aoa-OH as the last amino acid. Resin cleavage, purification (gradient: 5-45% B in 50 min) and lyophilization afforded the peptide as a fluffy white powder. MALDI-TOFF: found $[M+H]^+$ 864 Da for $C_{36}H_{57}N_{13}O_{10}S$; HPLC: Rt 11.3 min (gradient: 10-60% B in 30 min).

Metal assisted oxime ligation protocol

$PdCl_2$ 1.25mM (720 μ L, 0.90 mmol) was added to a reaction mixture comprised of peptide 1 (2.0 mg, 0.89 mmol) and peptide 3 (2.3 mg, 0.96 mmol) in 200 μ L of solution A. Reaction was monitored with HPLC then quenched with 50eq of DTT after an overnight stirring to afford 2.1 mg of purified **oxime 1** (53% yield). MALDI-TOFF: found $[M+H]^+$ 4473 Da for $C_{195}H_{321}N_{71}O_{45}S_3$; HPLC: Rt 7.9 min (gradient: 30-100% B in 20 min)

Metal-free oxime conjugation assay using model peptides

Similar equivalent of peptides **5** (Thz-GYRMHK) and **6** (Aoa-GYRMHK) were mixed at different conditions indicated in the table. % of oxime conversion was calculated from the HPLC peak areas of Thz-peptide and oxime conjugate (Oxime **3**) as: $100 * (\text{oxime area}) / (\text{oxime area} + \text{Peptide 5 area})$ MALDI-TOFF : Found $[M+H]^+$ 1692 Da for $C_{72}H_{107}N_{23}O_{21}S_2$

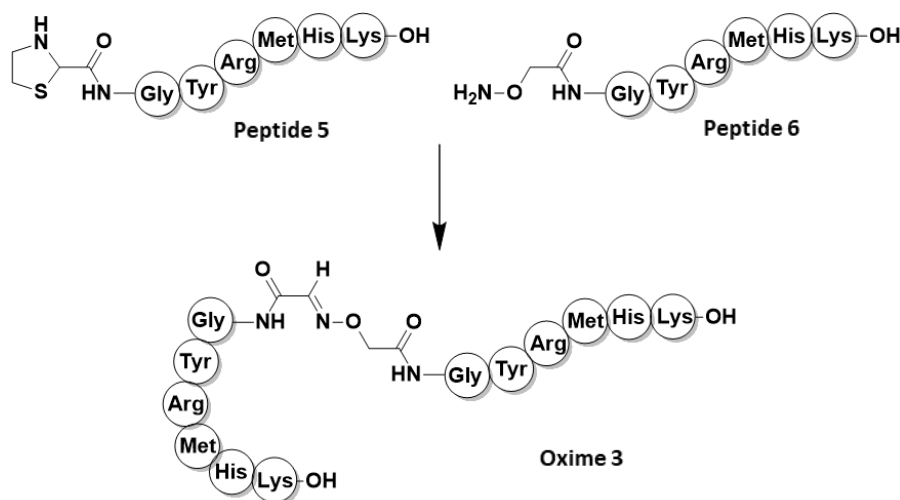
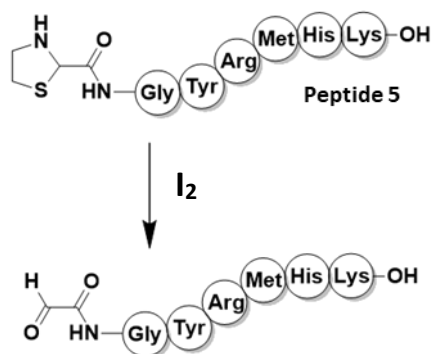


Table S1.

Entry	Buffer	pH	T (°C)	% oxime 24h	% oxime 48h
1	AcONH ₄ 500mM	4.5	37	75	87
2	AcONH ₄ 500mM + 10mM aniline	4.5	37	65	89
3	HPLC mobile phase B	2	25	25	44
4	HPLC mobile phase B	2	37	85	>95
5	PBS 1X	7.2	37	-	1

As ammonium acetate buffer at pH 4.5 acts as a standard condition in oxime ligation, and the thioproline (ThP) uncaging by methoxyamine has been conducted at 37°C, we firstly used these combined conditions. pH 2 or pH 4.5 buffer and 37°C are preferable for this conjugation.

Iodine-mediated Thz opening using model peptide at pH 2, 4.5 and 7



Peptide **5** (Thz-GYRMHK) was incubated with iodine in solution A, pH 4.5 ammonium acetate buffer and PBS 1x as indicated in the table. Thz uncaging was monitored after 15 min treatment, overnight reaction did not progress in the case of 0.2 – 0.6 eq of iodine. % of Thz uncaging was calculated on the HPLC peak areas of Thz-peptide and CHO-peptide as: $100 \cdot (\text{CHO-peptide}) / (\text{CHO-peptide} + \text{Thz-peptide area})$.

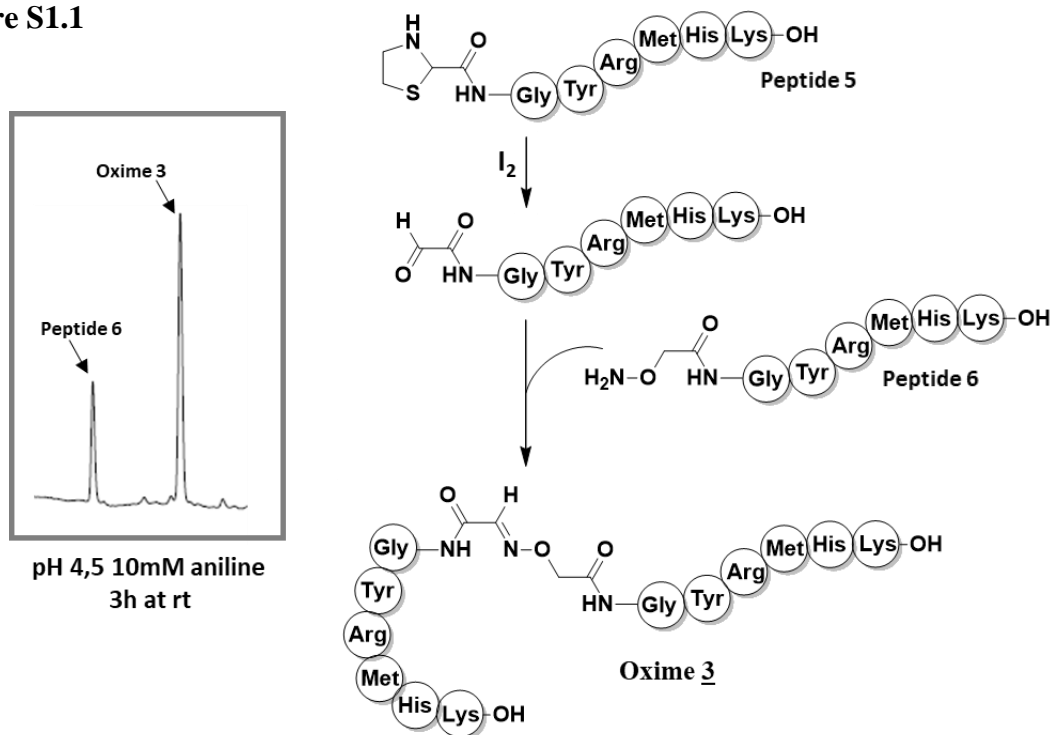
Table S2.

Entry	Buffer	I ₂ (eq)			
		0.2	0.4	0.6	1.0
1	Solution A	18	36	65	96
2	AcONH ₄ 500mM	24	49	84	100
3	PBS 1x	29	52	87	100

Iodine-mediated Thz opening followed by oxime ligation at pH 4.5 and 7.

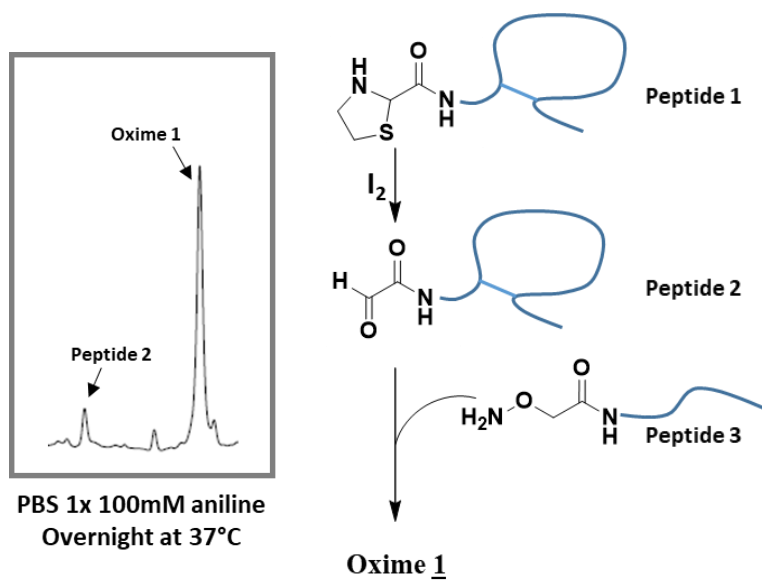
Peptide **5** (Thz-GYRMHK) (1.0 eq) was incubated with 1.0 eq iodine using pH 4.5 10mM aniline buffer. After 15 min, iodine was quenched by sodium thiosulfate (1.2 eq) and peptide **6** (Aoa-GYRMHK) (1.5 eq) was added to perform oxime ligation under HPLC monitoring.

Figure S1.1



Peptide **1** (1.0 eq) was likewise incubated with 1.0 eq iodine. After 15 min, iodine was quenched by sodium thiosulfate (1.2 eq) and peptide **3** (1.0 eq) was added to perform oxime ligation under HPLC monitoring. Contrary to pH 4.5 ligation described in the manuscript, pH 7 buffer requires an overnight moderate heat and aniline to perform the oxime coupling.

Figure S1.2



Transoximation assays using model peptides

Peptide **5** (Thz-GYRMHK) and **Oxime 4** (acetoxime of Peptide **6** Aoa-GYRMHK obtained by acetone addition to Peptide **6** followed by lyophilization) were mixed at different conditions indicated in the table S3. In the table S3, transoximation into **Oxime 3** was monitored by HPLC and % of transoximation was calculated from the HPLC peak areas of Peptide **5** and **Oxime 3** conjugate as: $100 * (\text{Oxime 3 area}) / (\text{Oxime 3 area} + \text{Peptide 5 area} + \text{CHO-Peptide area})$

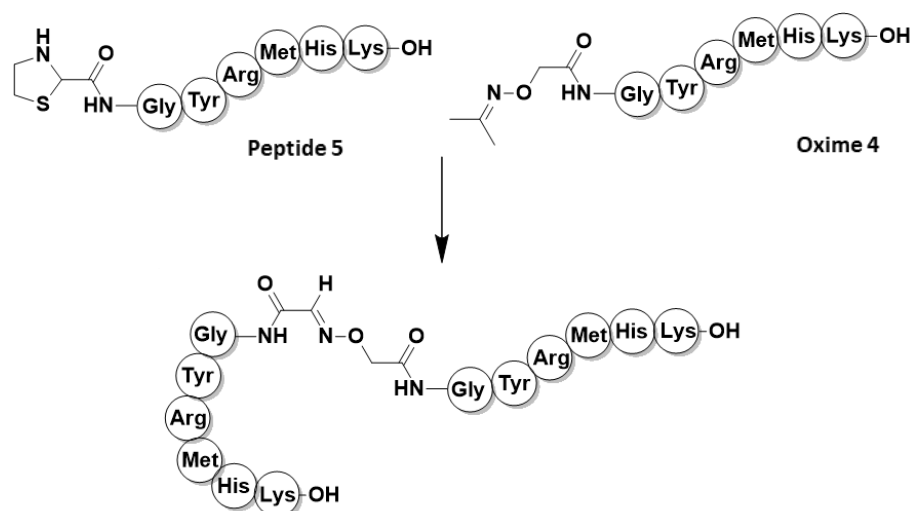


Table S3.1 Transoximation in solution A

Entry	T (°C)	% transoximation 24h	% transoximation 48h
1	rt	4	9
2	37	40	60
3	37 (open vial)	55	71

Transoximation at 37°C without sealing the vial gave better results compared to sealed vial at 37 °C and 25 °C experiments.

Table S3.2 Transoximation in pH 4.5 buffer at 37°C

Entry	Buffer	% transoximation 24h
1	Solution A	41
2	AcONH ₄ 500mM	11
3	AcONH ₄ 500mM + 10mM aniline	40

To extend our results to conventional oxime buffer, transoximation was performed at 37°C in

pH 4.5 buffer with or without aniline. pH 4.5 buffer containing 10mM aniline exhibited similar results as pH 2 solution A.

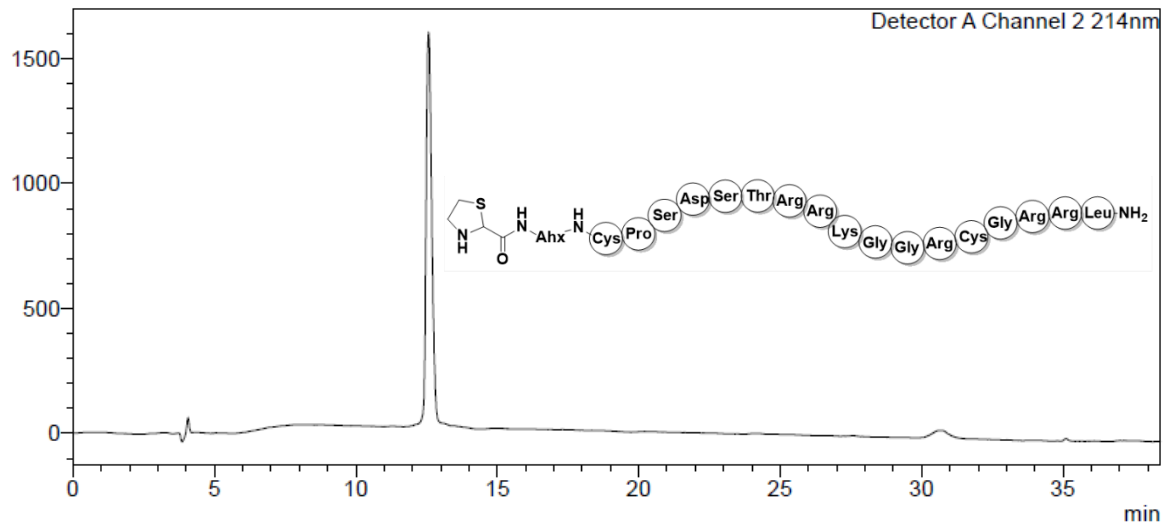
Table S3.3 Transoximation in pH 4.5 buffer at 37°C using different amounts of Oxime 4

Entry	acetoxime (eq)	% transoximation 24h
1	2	37
2	3	41
3	5	42

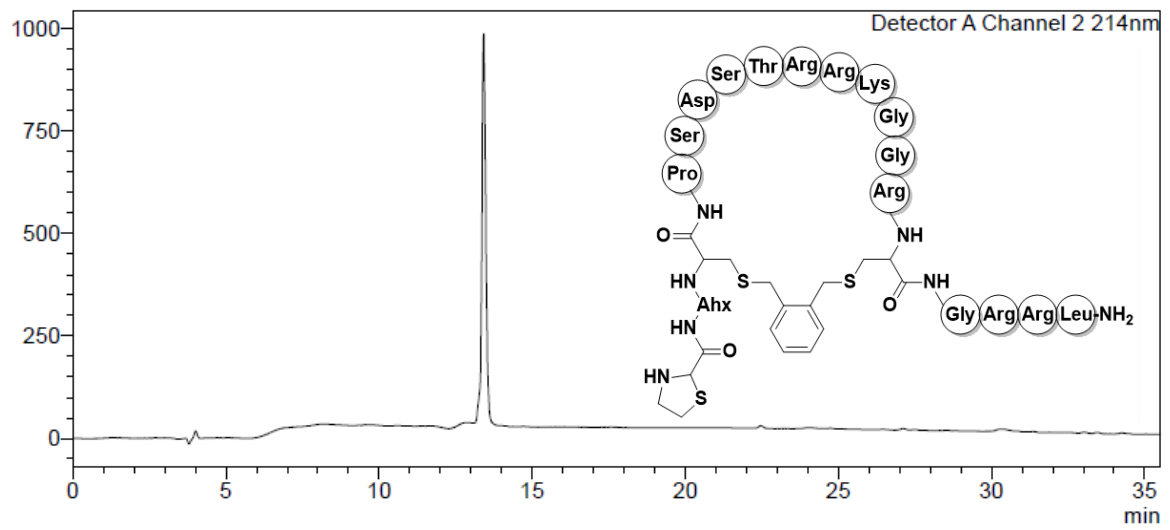
Peptide **5** (Thz-GYRMHK) was mixed with several equivalent **Oxime 4** (acetoxime of Peptide **6** Aoa-GYRMHK) were mixed in pH 4.5 buffer containing aniline at 37°C. Higher amount of acetoxime did not significantly increase the transoximation rate.

HPLC profiles

mV **Linear precursor of peptide 1**

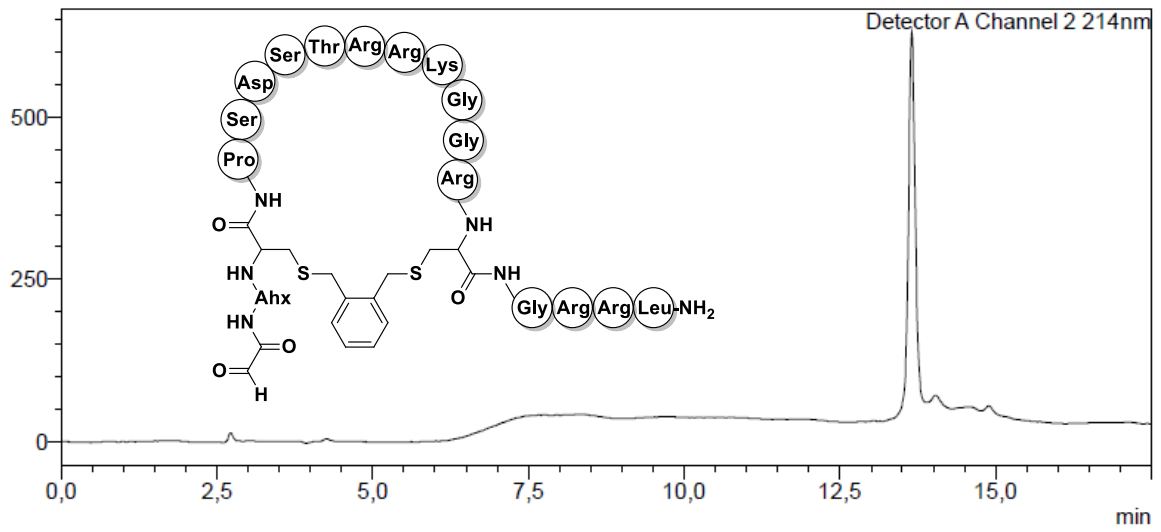


mV **Peptide 1**



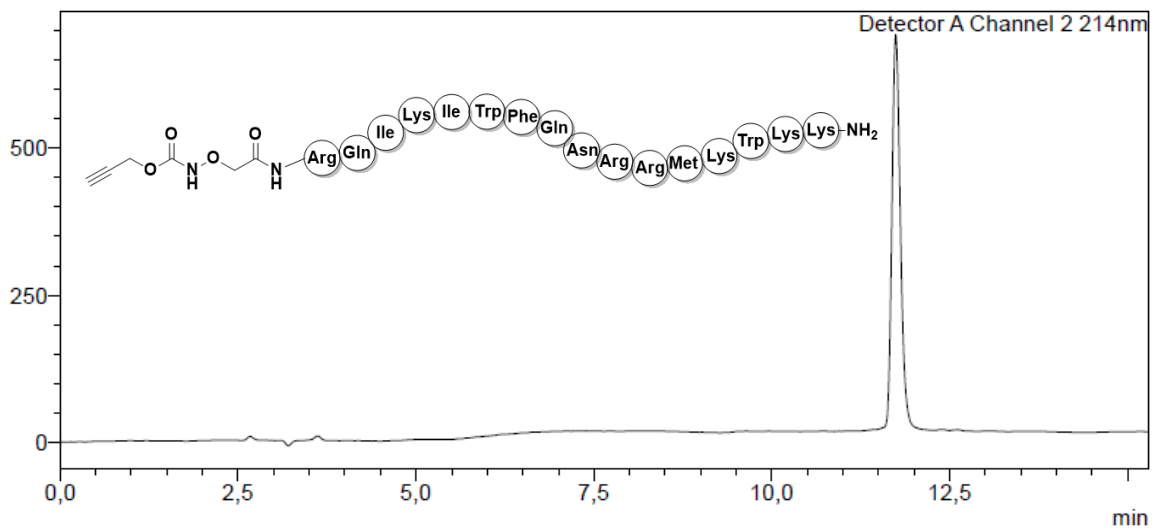
mV

Peptide 2



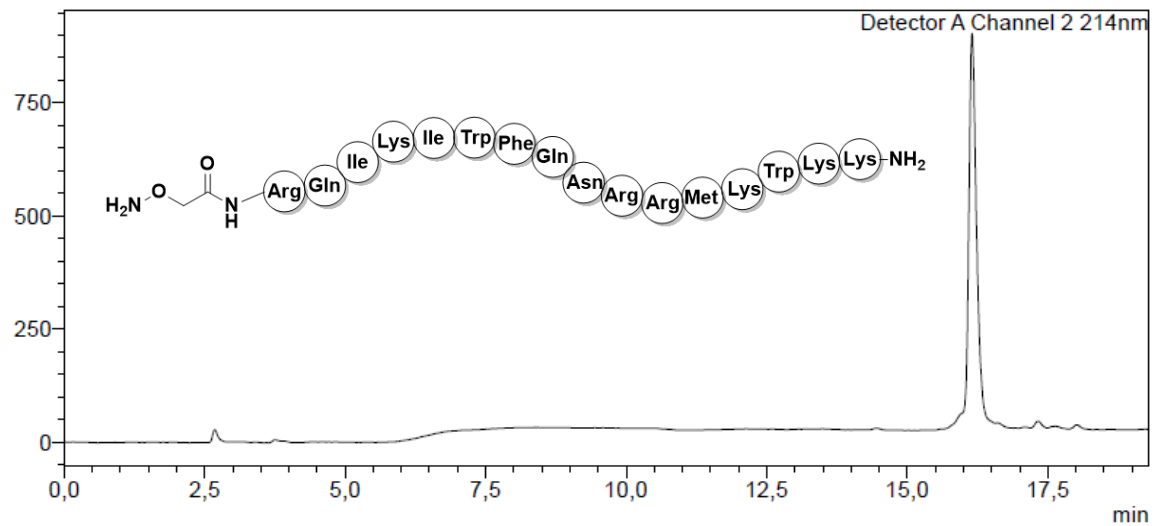
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Peptide 3

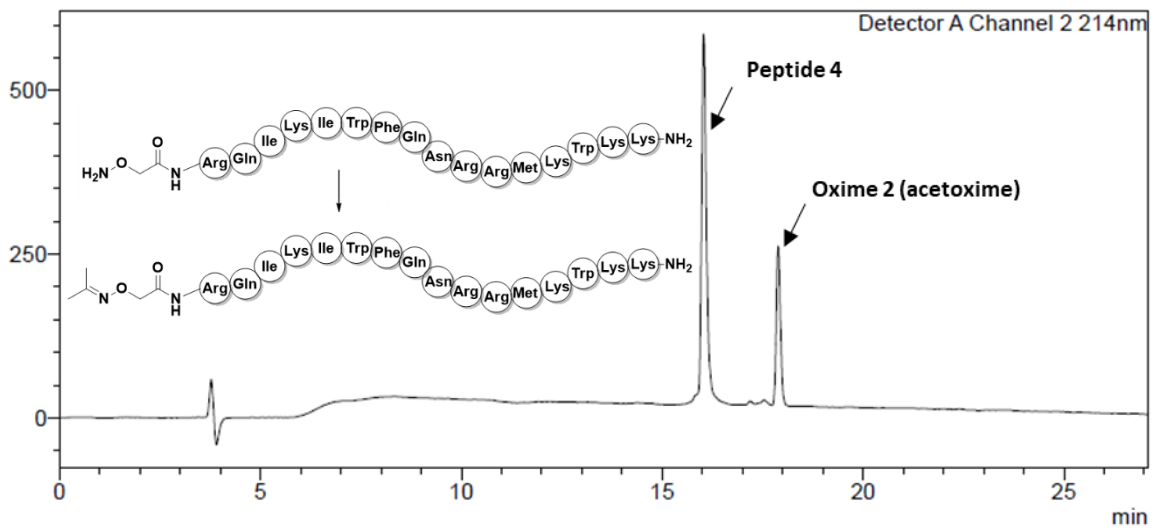


mV

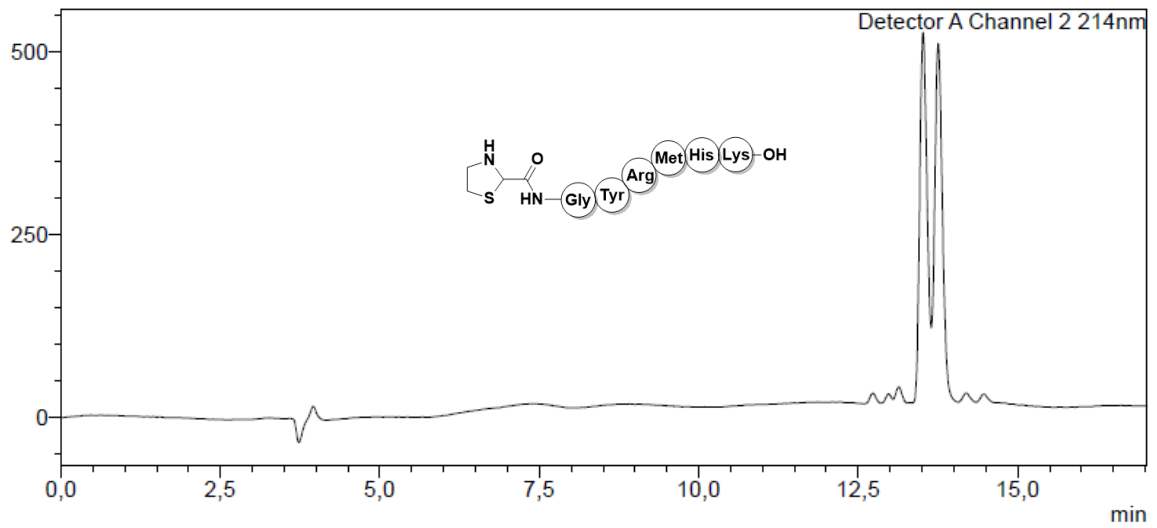
Peptide 4



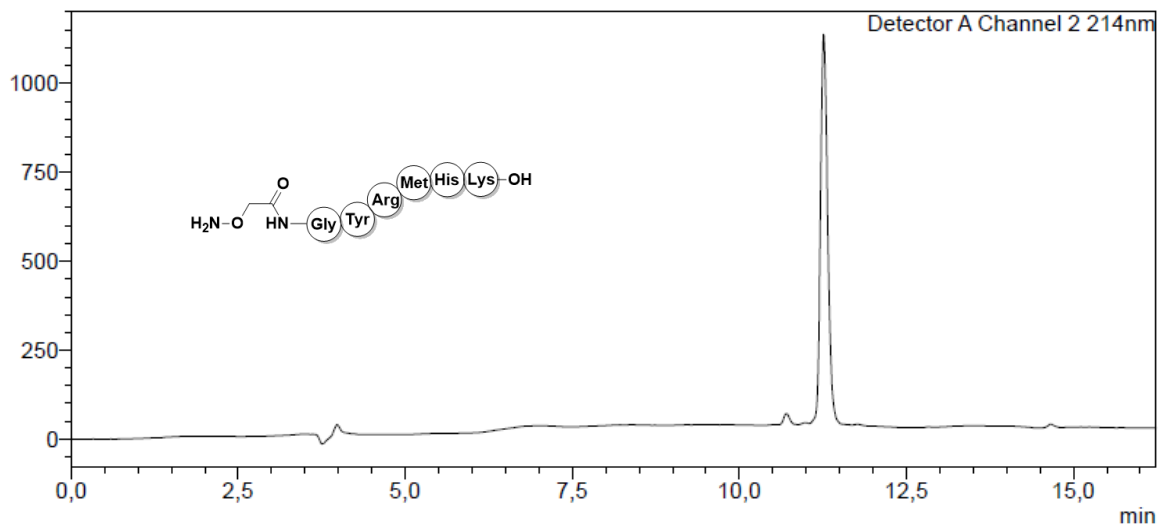
mV **Peptide 4 and acetoxime by-product**



mV **Peptide 5**

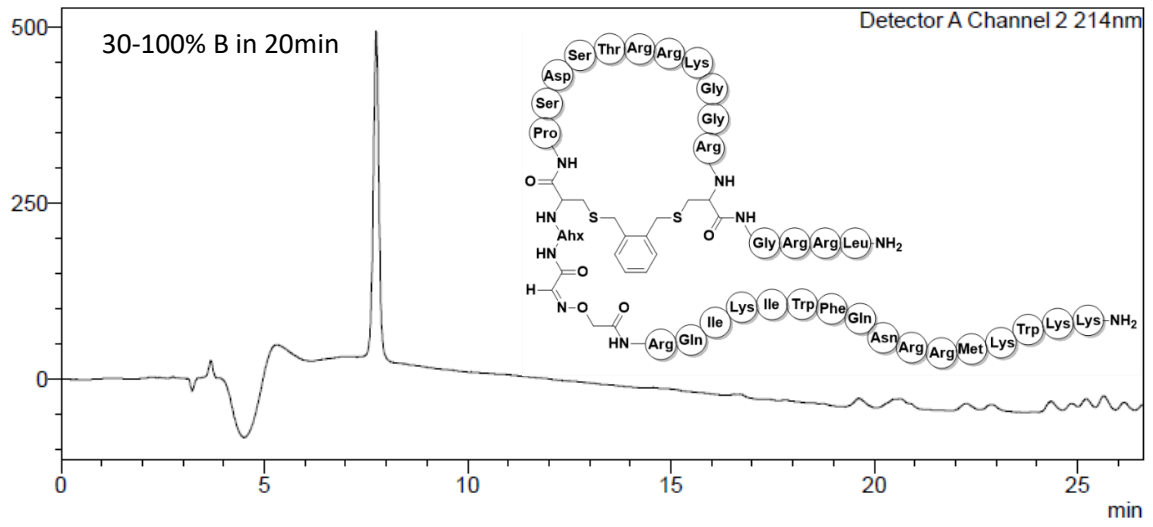


mV **Peptide 6**



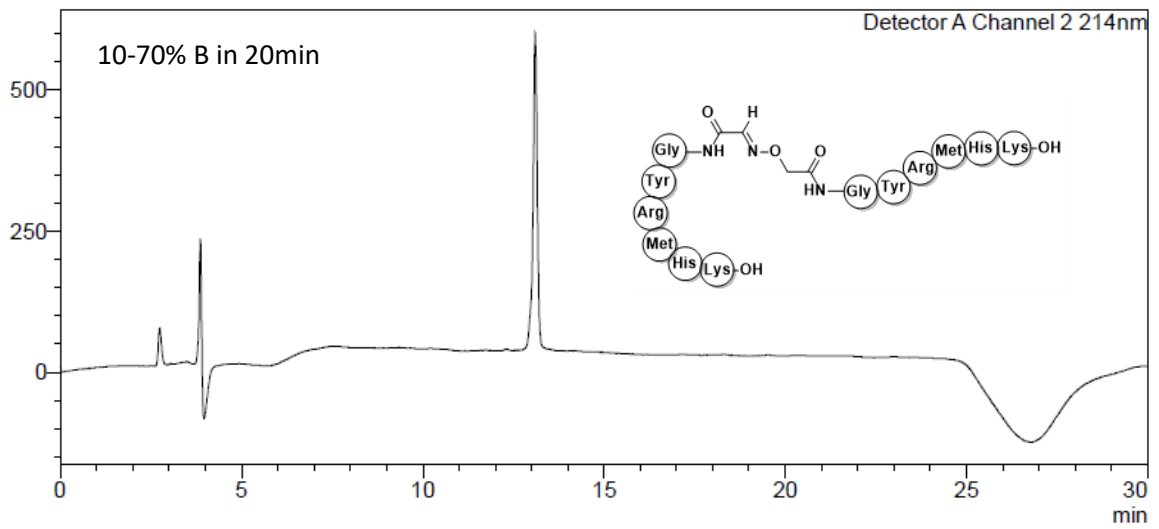
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Oxime 1

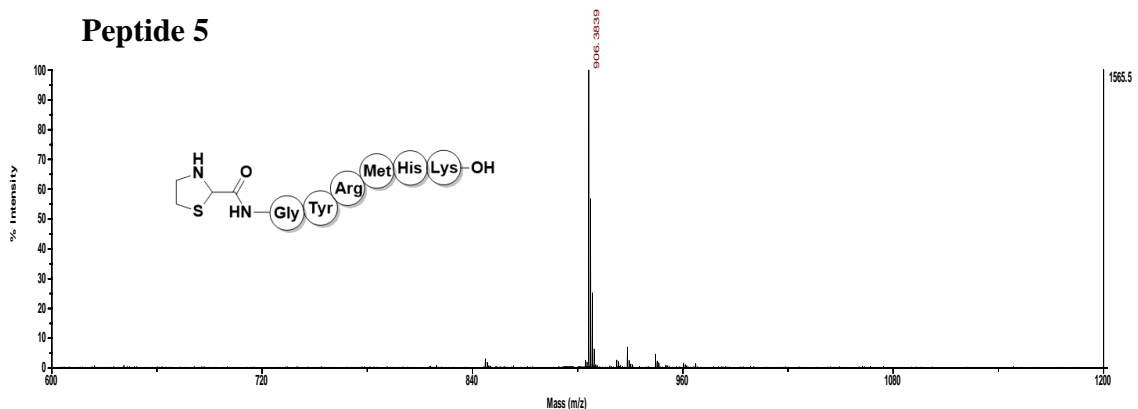
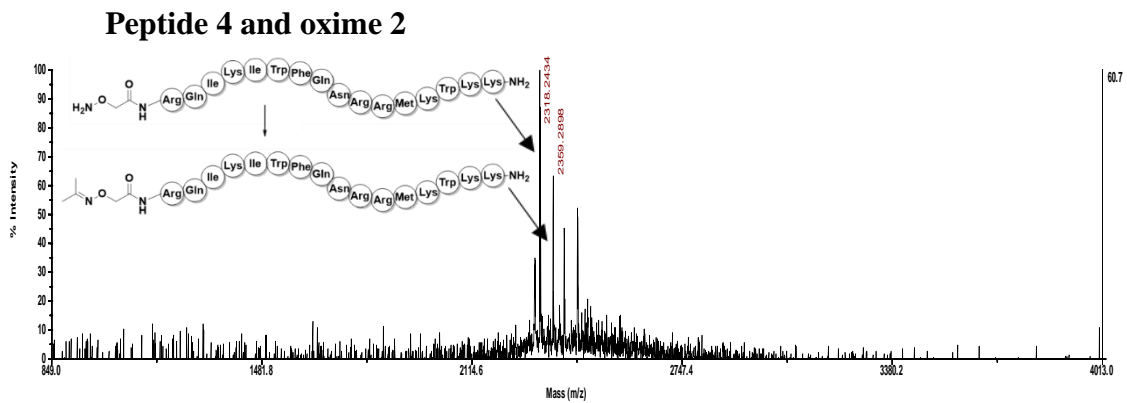
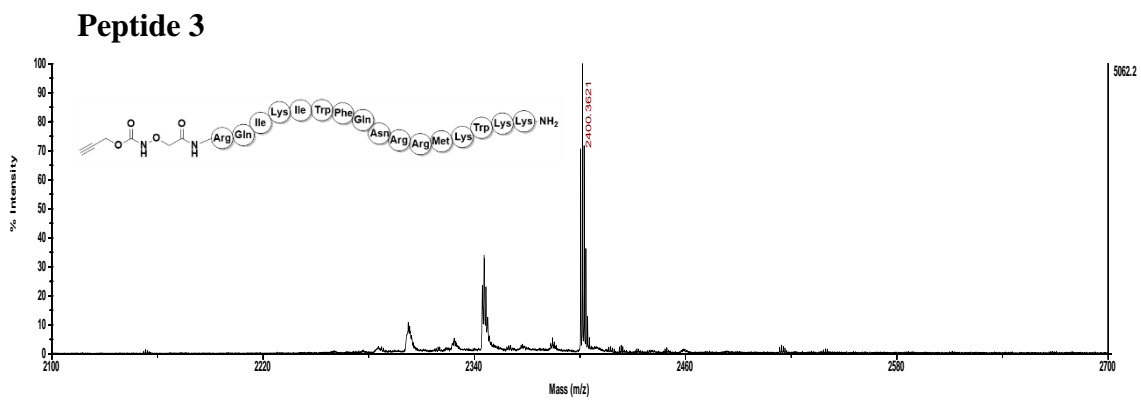
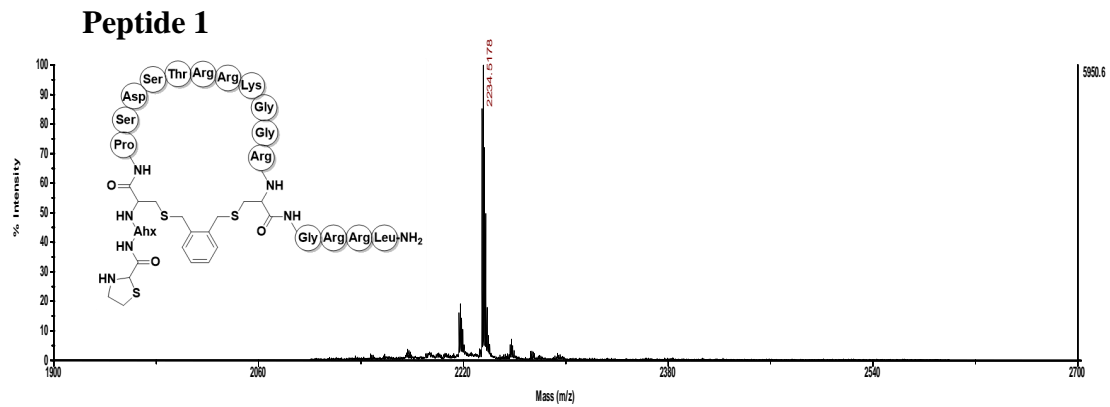


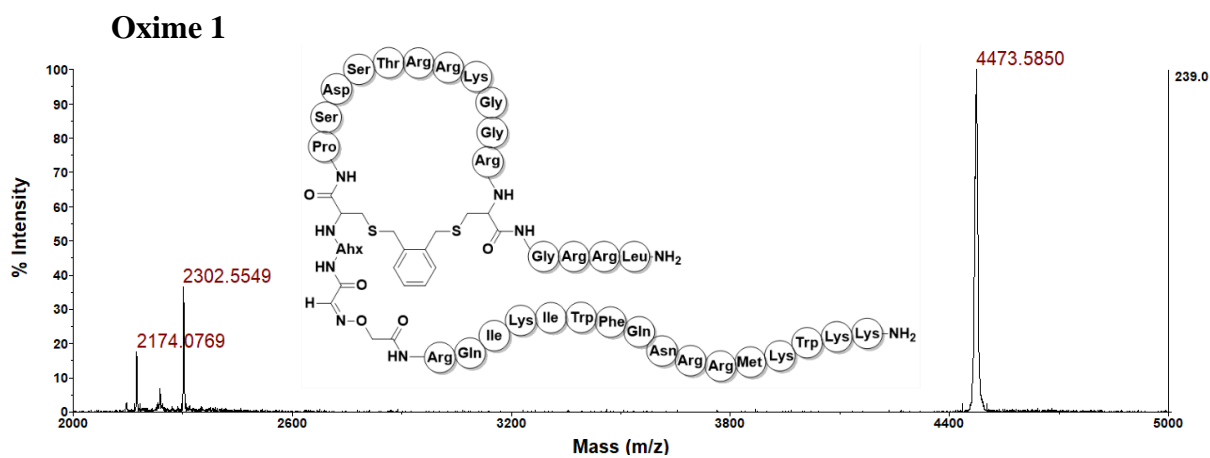
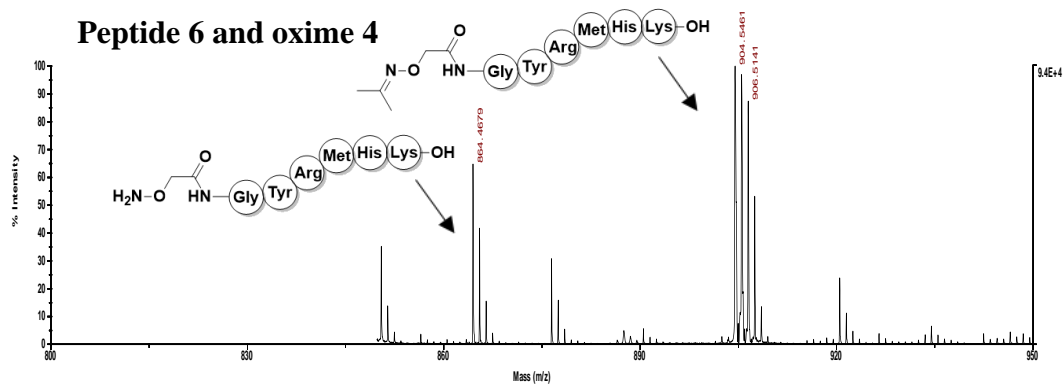
mV

Oxime 3

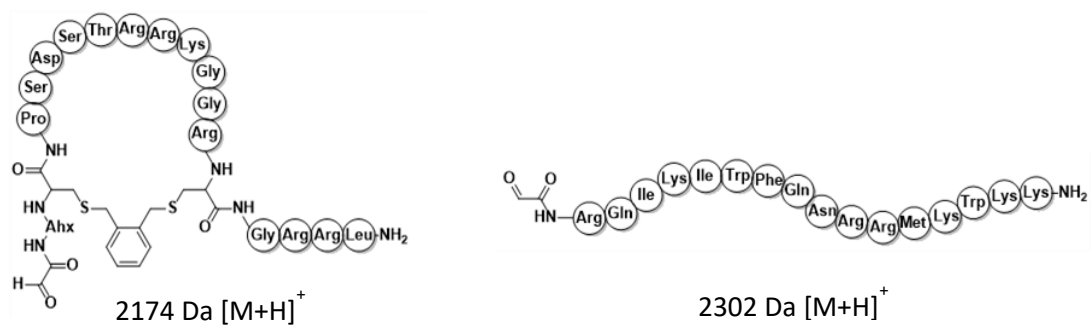


MALDI-TOFF spectra





Fragments attribution was guided with previous work from Hardouin *et al.* (2011) *Rapid communications in mass spectrometry: RCM*, 25(14), 2106.



Oxime 3

