Reversible Native Chemical Ligation:

a Facile Access to Dynamic Covalent Peptides

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Supporting Information

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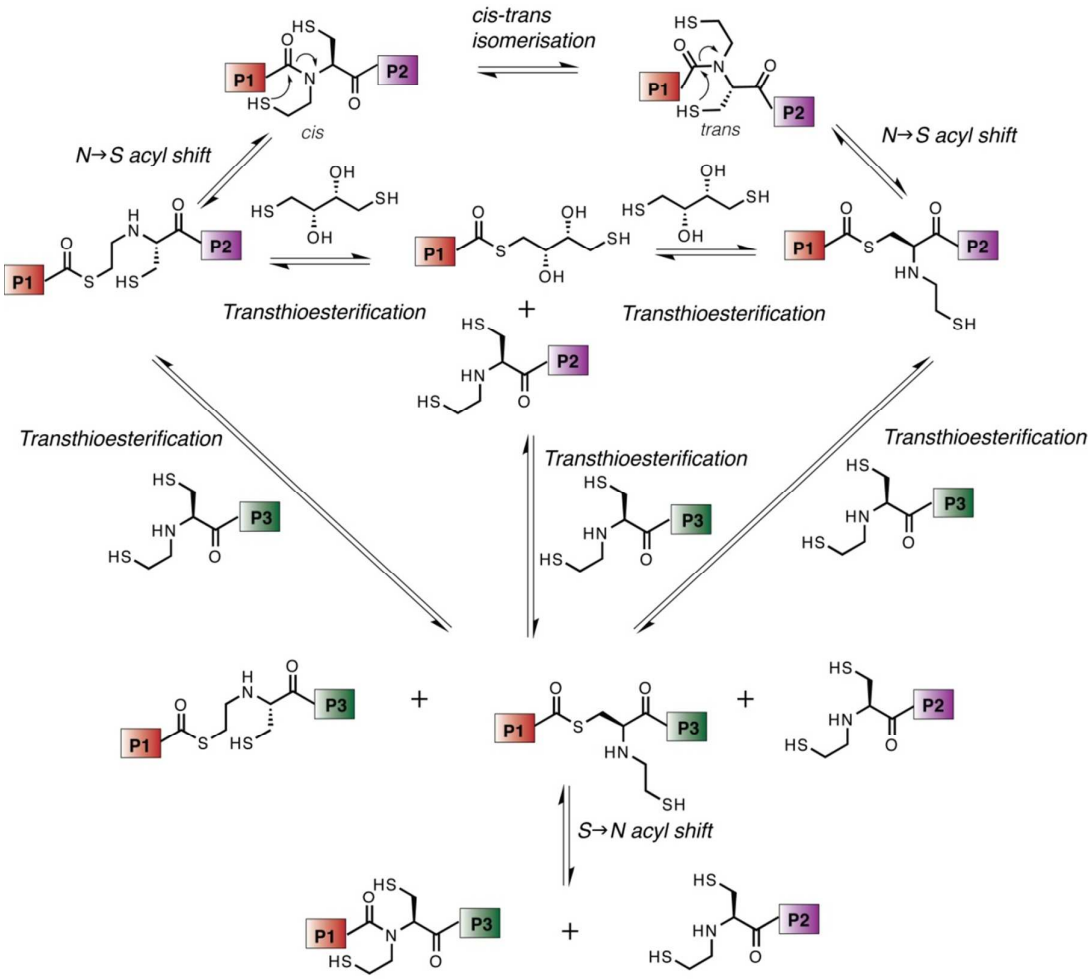
1. Detailed mechanisms involving N-(2-thioethyl)-cysteine



Scheme S1. Schematic representation of the cis-trans, N→S acyl shift, transthioesterification, and S→N acyl shift equilibria involved in the different possible pathways for peptides incorporating **Daa1**. The presence of two thiol groups on the particular case of **Daa1** systems allows the N→S acyl shift as well as the transthioesterification to occur at these two positions, resulting in 2 alternative pathways depending on which thiol is involved in the initial N→S acyl shift. The existence of these two thioesters is demonstrated experimentally in Fig. S10b.

2. Materials and methods

2.1 Generalities

Reagents and Analytical Instruments. All reagents and solvents were purchased from Sigma-Aldrich, Carlo Erba, Iris-Biotech, Alfa Aesar and Fisher Scientific at the highest commercial quality and used without further purification unless stated otherwise. Dry solvents were obtained using a double column *SolvTech* purification system. Peptide synthesis was performed on a microwave synthesizer (CEM Liberty 1™, Saclay, France) and on a parallel synthesizer (Heidolph® Synthesis 1). Peptides were purified by High-Performance Liquid Chromatography (HPLC) Waters AutoPurification® system, coupled to a Waters 2489 UV/Visible detector and Waters SQD 3100 electrospray mass detector. Purification was performed using 0.1% formic acid in water and 0.1% formic acid in methanol as the two mobile phases on either a preparative SunFire® Prep C18 QBD® 5 μm 19x150 mm column from Waters or a preparative XBridge® Prep C18 5 μm QBD® 19x150 mm column from Waters. The purity of the isolated peptides and the kinetics of the exchange reactions were monitored using a Waters ACQUITY UPLC® UltraPerformance Liquid Chromatography coupled to a SQD ACQUITY Electrospray mass detector (UPLC®-MS). The analysis were performed on an ACQUITY UPLC® HSS T3 1.8 μm 2.1x50 mm column from Waters using 0.1% formic acid in water and 0.1% formic acid in acetonitrile as the two mobile phases. The processing of the UPLC®-MS data (baseline subtraction and peak integration) was performed with the peak analyzer tool of OriginPro 9.0®. ^{1}H NMR spectra were recorded (as indicated) either on a *Bruker Avance 400* spectrometer at 400 MHz or on a *Bruker Avance 500* spectrometer at 500 MHz and ^{13}C NMR spectra either at 100 MHz or 125 MHz. The spectra were internally referenced to the CH_3- signals of *t*BuOH (1.24 ppm for ^{1}H NMR and 30.29 ppm for ^{13}C NMR)[1], the chemical shifts are given in ppm. Spin multiplicities are reported as a singlet (s), doublet (d), doublet of doublets (dd), triplet (t) and quartet (q) with coupling constants (J) given in Hz, or multiplet (m). Broad peaks are marked as br. When specified, ^{1}H resonances were assigned with the aid of additional information from 2D NMR proton correlation spectroscopy spectra (H-COSY). ESI-MS spectra were acquired using the *Waters SQD* Mass Detector directly connected to the output of an UPLC® or HPLC column.

Abbreviations. aa: amino acid; DBU: 1,8-Diazabicyclo[5.4.0]undec-7-ene; DCM: Dichloromethane; DIAD: Diisopropyl azodicarboxylate; DIEA: N,N-Diisopropylethylamine; DIPCDI: N,N'-Diisopropylcarbodiimide; DMF: N,N-Dimethylformamide; DTT: Dithiothreitol; Fmoc: 9-Fluorenylmethoxycarbonyl; HATU: 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; HBTU: N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate; MBHA: methylbenzhydrylamine; NMP: N-Methyl-2-pyrrolidone; Oxyma Pure®: Ethyl 2-cyano-2-(hydroxyimino)acetate; rt: room temperature; TBAF: Tetra-n-butylammonium fluoride; TCEP: *tris*(2-carboxyethyl)phosphine; THF: Tetrahydrofuran; TIS: Triisopropylsilane; TFA: Trifluoroacetic acid.

2.2 Syntheses

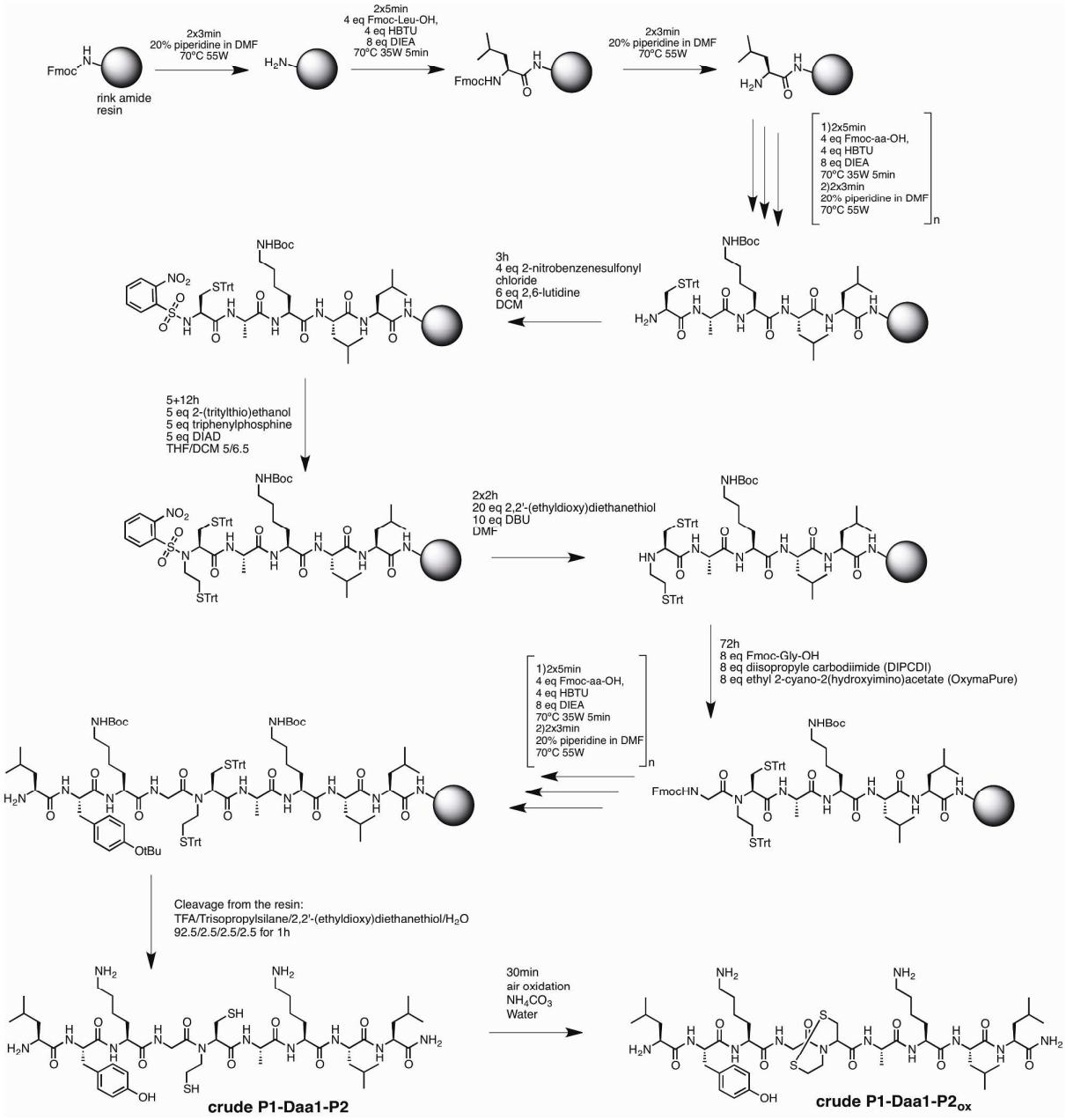
Standard solid phase peptide synthesis was performed using standard Fmoc/*tert*-butyl chemistry[2] and using Microwave assisted[3] solid phase peptide synthesis with a CEM Liberty 1™ microwave synthesizer starting with a Fmoc-Rink-amide MBHA resin (0.74 mmol/g). A typical synthesis was done on a 0.5 mmol scale. For each amino acid, double couplings were performed at 70 ºC and 35 W (microwave power) for 5 min (except for the coupling of Fmoc-Cys(Trt)-OH which is performed without microwave heating for 2 min and then at 50 ºC with 25 W for 4 min) using 4-fold molar excess of each Fmoc L-aa (10 mL of a 0.2 M solution in DMF), HBTU (4 mL of a 0.5 M solution in DMF) and DIEA (2 mL of a 2 M solution in NMP). Fmoc groups were deprotected with 2 successive treatments with 20 vol% piperidine solution in DMF (15 mL, 70 ºC for 3 min, 55 W).

2.2.1 Peptides incorporating the N-(2-thioethyl)-cysteine

A representative synthesis for this family of peptides is given in Scheme S2. The C-terminal peptide portion (-Cys-Ala-Lys-Leu-Leu-NH_2 **P2** or –Cys-Ala-Phe-Lys-Phe-NH_2 **P3**) of all peptides was synthesized using the standard Fmoc-solid phase peptide synthesis described above. The strategy used for the introduction of the N-(2-thioethyl) group on the cysteine was adapted from a protocol described by Hou *et al.* for the modification of primary amines on solid support.[4] After the standard deprotection of the Fmoc group at the terminal cysteine, the resin was transferred in a parallel synthesizer and the terminal amino group converted into a sulfonamide with a 2-nitrobenzenesulfonylchloride (4 eq) and 2,6-lutidine (6 eq) solution in DCM (10 mL) for 3 h. A Mitsunobu reaction was subsequently performed with 2-(tritylthio)ethanol[5] (5 eq), triphenylphosphine (5 eq), DIAD (5 eq) in a dry THF/dry DCM mixture (5 mL and 6.5 mL respectively) under argon atmosphere for 5 h; this reaction was then repeated once more for 12 hours. The amino group was then deprotected with 2,2'-(ethyldioxy)diethanethiol (20 eq) and DBU (10 eq) in 5 mL of DMF (2 x 2 h).
The following coupling, of Fmoc-Gly-OH, was performed using the Fmoc-aa (8 eq), DIPCDI (8 eq) and OxymaPure (8 eq) in DMF (5 mL) at rt under argon atmosphere for 72 h.

After each of these steps, a small amount of resin was submitted to a cleavage solution of TFA/TIS/2-2'(ethyldioxy)diethanethiol/H_2O: 92.5/2.5/2.5/2.5 vol% and the crude peptide isolated after precipitation in diethyl ether in order to check that the reaction went to completion using UPLC®-MS analysis.

For peptides **P1-Daa1-P2** and **P1-Daa1-P3** the remaining amino acids of the N-terminal peptide portion (Leu-Tyr-Lys) were then coupled using the standard Fmoc-solid phase peptide synthesis described above.



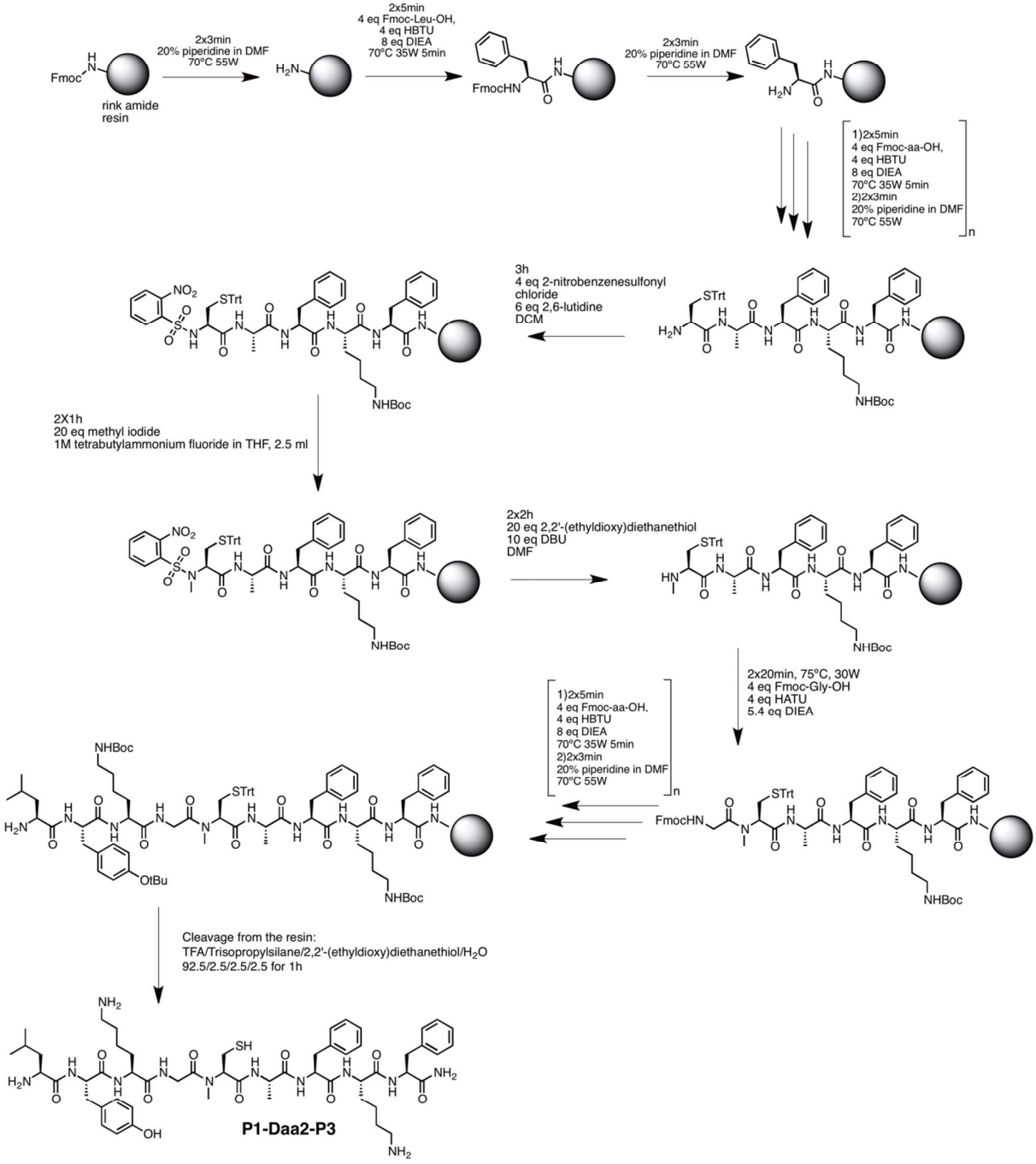
Scheme S2. Synthesis of **P1-Daa1-P2** and **P1-Daa1-P2$_{ox}$** incorporating a N-(2-thioethyl)-cysteine residue.

2.2.2 Peptides incorporating N-(methyl)-cysteine

A representative synthesis for this family of peptides is given in Scheme S3. The C-terminal peptide portion (-Cys-Ala-Lys-Leu-Leu-NH_2 **P2** or –Cys-Ala-Phe-Lys-Phe-NH_2 **P3**) of all peptides was synthesized using the standard Fmoc-solid phase peptide synthesis described above. The strategy used for the introduction of the N-(methyl) group on the cysteine was adapted from a protocol described by Erlich *et al.* for the modification of cysteine on solid support.[6] Once the coupling of the Fmoc-Cys(Trt)-OH was achieved and the Fmoc group removed, the resin was transferred in a parallel synthesizer for shacking. The terminal amino group was activated with a 2-nitrobenzenesulfonylchloride (4 eq) and 2,6-lutidine (6 eq) solution in DCM (10 mL) for 3h under argon. A methylation with methyl iodide (20 eq) and TBAF (1 M solution in THF, 2.5 mL) was then performed twice under argon (2 x 1 h). The amino group was then deprotected with 2-2'(ethyldioxy)diethanethiol (20 eq) and DBU (10 eq) in 5 mL of DMF (2 x 2 h).
The following coupling was performed in the CEM Liberty 1® microwave synthesizer using Fmoc-Gly-OH (4 eq), HATU (4 eq), and DIEA (5.4 eq) in DMF (8 mL) at 75 °C and 30 W (2 x 20 min).

After each of these steps, as small amount of resin was submitted to TFA/TIS/2-2'(ethyldioxy)diethanethiol/H_2O: 92.5/2.5/2.5/2.5 vol% and the crude peptide isolated after precipitation in diethyl ether in order to check that the reaction went to completion by UPLC®-MS analysis.

For peptides **P1-Daa2-P2** and **P1-Daa2-P3** the remaining amino acids of the N-terminal peptide portion (Leu-Tyr-Lys) were then coupled using the standard Fmoc-solid phase peptide synthesis described above.



Scheme S3.Synthesis of **P1-Daa2-P3** incorporating a N-(methyl)-cysteine residue.

2.2.3 Unmodified cysteine peptides for control experiments

These peptides were synthesized according to standard protocols for the Fmoc-solid phase peptide synthesis described above.

2.3 Final cleavages of the peptides from the resin and purification

The resin was suspended in a 10mL solution of TFA/TIS/2-2'(ethyldioxy)diethanethiol/H_2O: 92.5/2.5/2.5/2.5 vol%. After 1h, the resin was filtered and washed with TFA (5 mL). The combined filtrates were concentrated *in vacuo* and then added to cold diethylether (200 mL) to precipitate the crude peptides. The solids were separated by centrifugation, washed with cold diethylether (2 x 40 mL) and dried under argon to afford crude peptides.

Crude peptides were then purified by High-Performance Liquid Chromatography (HPLC) Waters AutoPurification® system, coupled to a Waters 2489 UV/Visible detector and Waters SQD 3100 electrospray mass detector. Purification was performed using 0.1 % formic acid in water (A) and 0.1 % formic acid in methanol (B) as the two mobile phases on either a preparative SunFire® Prep C18 QBD™ 5 μm 19x150 mm column from Waters or a preparative XBridge® Prep C18 5 μm QBD™ 19x150 mm column from Waters.

A typical gradient is given here:

Time (min)	Flow (mL/min)	%A	%B
0	24.5	95	5
0.95	24.5	95	5
25	24.5	5	95
33	24.5	5	95
34	24.5	95	5

For all peptides, fractions were automatically collected using the ESI-MS signal of the peptide as a trigger. Fractions were individually analyzed using Waters ACQUITY UPLC® Ultra Performance Liquid Chromatography coupled to a SQD ACQUITY Electrospray mass detector (UPLC®-MS). The analysis were performed on an ACQUITY UPLC® HSS T3 1.8 μm 2.1x50 mm column from Waters using 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile as the two mobile phases. The organic solvent (methanol or acetonitrile) was removed under reduced pressure from the combined fractions before lyophilization of the solution of the purified peptides.

2.3.1 Oxydation of N-(2-thioethyl)-cysteine

For the peptides incorporating N-(2-thioethyl)-cysteine, an additional oxidation step was performed on the crude products before injection in preparative HPLC. They were dissolved in milliQ H_2O (1 mL/50 mg), the pH was adjusted to 7-8 with $(NH_4)_2CO_3$ and compressed air was bubbled in the resulting solution for 30 min. Prior to purification, the pH was lowered to 4-5 with formic acid. This procedure was used to avoid complications afforded by the purification of these peptides in their corresponding reduced form, which can undergo oxidation during the purification process.

2.4 Protocols for exchange reactions

2.4.1 Control Exchange on Cysteine Native Peptides

A control exchange under identical conditions at pH 7 between the native peptides **P1-Cys-P2** and **Cys-P3** did not yield any detectable trace of exchange products **P1-Cys-P3** and **Cys-P2** after 4 weeks (Figure S1).

The 0.2 M phosphate buffer was prepared at pH 7: the amount of NaH_2PO_4 required to give a 200 mM solution in 20 mL of water (551 mg) was dissolved in 10 mL of water. The resulting acidic solution was titrated to pH 7 using 2 M NaOH and its volume adjusted to 20 mL to give the 0.2 M buffer solution at this pH. The buffer was degassed using freeze/thaw cycles and placed under argon prior to use.

Cys-P3 (formate salt) was dissolved in water to give a solution **A** (5 mM). **P1-Cys-P2** (diformate salt) was dissolved in water to give a solution **B** (5 mM). 250 μL of solution **A** were mixed with 250 μL of solution **B** and were immediately frozen and lyophilized. The lyophilized powder of the mixed peptides was diluted under argon with 500 μL of phosphate buffer (pH 7) containing 25 mM of DTT (5.8 mg in 1.5 mL).

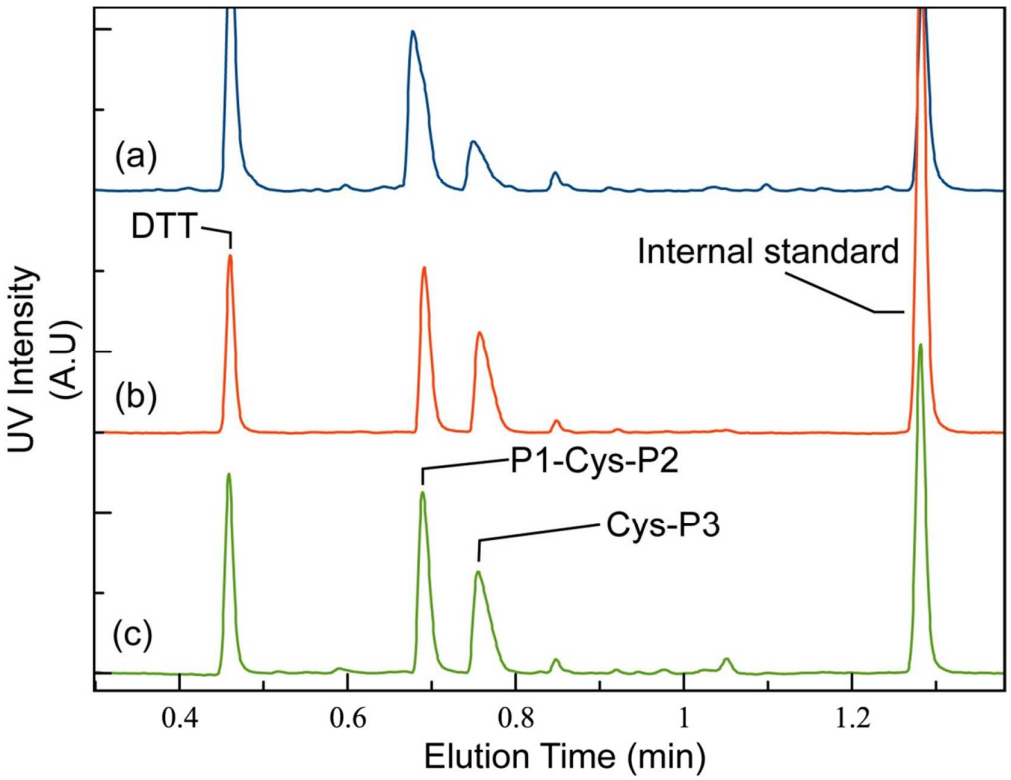


Figure S1. Reversed phase chromatograms (UPLC®) recorded with UV and MS detection of an exchange reaction between **P1-Cys-P2** and **Cys-P3** after 5 min (a), 7 days (b) and 4 weeks (c) setup at pH 7 as described above, and measured from an aliquot (25 μL) which was diluted with 175 μL of a dilute TCEP solution containing an internal UV standard (40 μL 3,5-dimethoxybenzoic acid solution (5 mM in a 0.2 M pH7 phosphate buffer) and 960 μL of a 10 mM TCEP*HCl solution in water).

2.4.2 Peptides incorporating N-(2-thioethyl)-cysteine

0.2 M phosphate buffers were prepared at pH 6, 7, 8 and 9: the amount of NaH_2PO_4 required to give a 200 mM solution in 20 mL of water (551 mg) was dissolved in 10 mL of water. The resulting acidic solution was titrated to pH 6, 7, 8, or 9 using 2 M NaOH and its volume adjusted to 20 mL to give 0.2 M buffer solutions at these pHs. Buffers were degassed using freeze/thaw cycles and placed under argon prior to use.
Daa1-P3 (formate salt) was dissolved in water to give a solution **A** (5 mM). **P1-Daa1-P2** (diformate salt) was dissolved in water to give a solution **B** (5 mM). 250 μL of solution **A** were mixed with 250 μL of solution **B** and diluted with a solution **C** of TCEP (10 mM, 7.1 mg in 2.5 mL of water neutralized with 8.4 mg of $NaHCO_3$, 2 eq., 250 μL) to give a solution **D**. This solution **D** was immediately frozen and lyophilized. The lyophilized powder of the mixed peptides was diluted under argon with 500 μL of phosphate buffer (pH 6, 7, 8 or 9) containing 25 mM of DTT (5.8 mg in 1.5 mL)

A control exchange was setup at pH 7 as described above but starting from **P1-Daa1-P3** and **Daa1-P2**. As expected this reaction led to the unambiguous formation of **P1-Daa1-P2** and **Daa1-P3** resulting from a dynamic native chemical ligation process at the Daa1 amino acid.

When aliquots of the reaction were diluted with water prior to UPLC®-MS analysis, the overlapping of the different peaks corresponding to the oxidized and reduced forms of the 4 peptides in equilibrium prevented a quantitative analysis of the reaction kinetics (Figure S2).

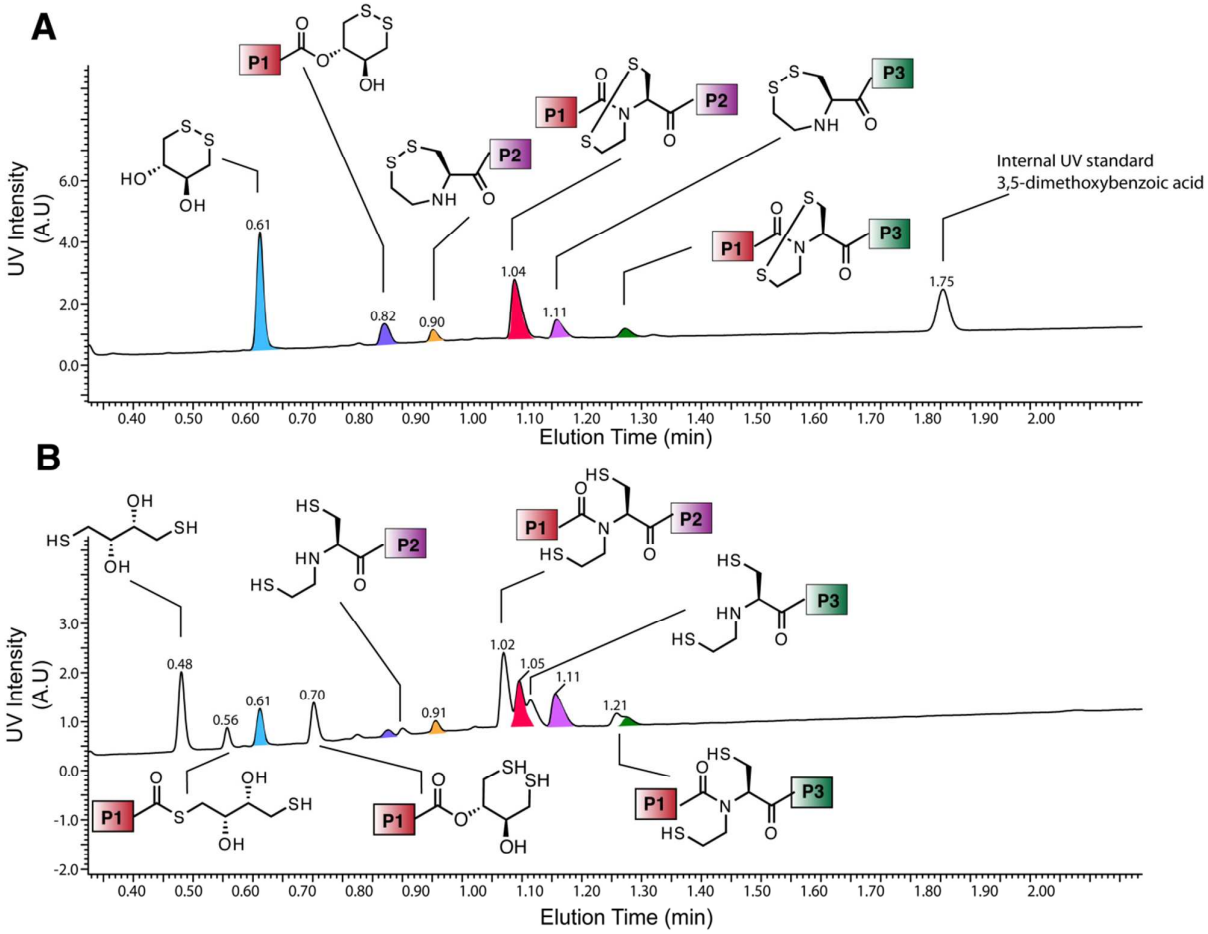


Figure S2. A Reversed phase chromatograms (UPLC®) recorded with UV and MS detection of an exchange reaction between **P1-Daa1-P2** and **Daa1-P3** setup as described in the experimental section and oxidation treatment of an aliquot (50 μL) with H_2O_2 by dilution with 350 μL of H_2O_2 containing 3,5-dimethoxybenzoic acid as an internal UV standard. **B** Reversed phase chromatograms (UPLC®) recorded with UV and MS detection of a sample of an exchange reaction between **P1-Daa1-P2** and **Daa1-P3** setup as described in the experimental section and diluted with 350 μL of H_2O. Peaks were assigned using the corresponding mass spectrum data. The peaks at 0.56 and 0.70 min showed similar response by MS detection with observed molecular weights of 616.43 and 616.51 Da respectively corresponding to the $(M+H)^+$ ion of the two possible isomers of **P1-DTT**.

In order to monitor these exchange reactions, for each time point an aliquot of the reaction (25 μL) was diluted with 175 μL of a dilute H_2O_2 solution containing an internal UV standard (23 μL H_2O_2 (30 % solution), 20 μL 3,5-dimethoxybenzoic acid solution (5 mM in a 0.2 M pH 7 phosphate buffer) and 957 μL water). The resulting solution was filtered and injected in the UPLC®-MS (2 x 2 μL injections).

The initial rates of the reactions were determined by plotting the product concentration vs. time graphics and by using the “tangent” tool of the “Origin” software at t = 0.

The precise attribution of the thioester and of the ester derivatives of **P1-DTT** (constitutional isomers) was made by trating the reaction mixture with hydroxylamine, which is selectively cleaving thioesters in presence of esters (Fig. S3a,b). The remaining signal was thus attributed to the ester form of **P1-DTT**. The same treatment with hydroxylamine was used after oxidation of the reaction mixture. The resistance of **P1-DTTox** to cleavage (Fig. S3c), together with the isotopic distribution observed by mass spectroscopy (Fig. S3d), allows the clear attribution of the structure of **P1-DTTox**, which corresponds to the ester with an intramolecular disulfure bond.

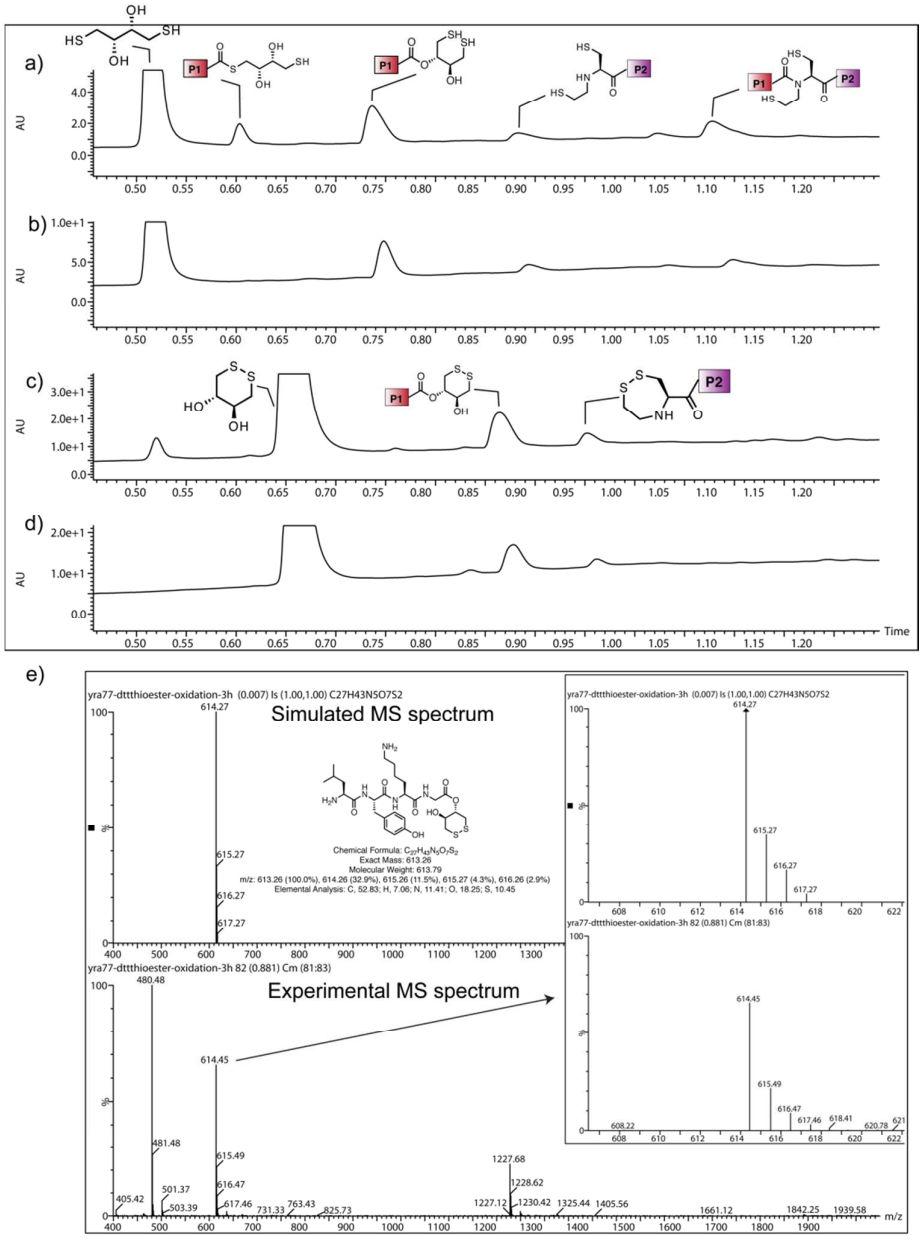


Figure S3. Reversed phase chromatograms (UPLC®) recorded with UV and MS detection (at t=0 and t=equilibrium) for exchange reaction (starting from **P1-Daa1-P2** and **Daa1-P3**) before (a) and after (b) treatment by hydroxylamine. The same protocol was performed but after oxidation of the mixture (c) and (d), in order to determine the structure of **P1-DTTox** which is represented in the figure and thanks to the comparison between theoretical and experimental mass spectra (e).

2.4.3 Peptides incorporating N-(methyl)-cysteine

0.2M phosphate buffers were prepared as described above at pH 6, 7 and 8. They were degassed and stored under Ar.

Daa2-P3 (formate salt) was dissolved in water to give a solution **A** (5 mM). **P1-Daa2-P2** (diformate salt) was dissolved in water to give a solution **B** (5 mM). 250 μL of solution **A** were mixed with 250 μL of solution **B** and were immediately frozen and lyophilized. The lyophilized powder of the mixed peptides was diluted under argon with 500 μL of phosphate buffer (pH 6, 7 or 8) containing 25 mM of DTT (5.8 mg in 1.5 mL).

In order to monitor these exchange reactions, for each time point an aliquot of the reaction (25 μL) was diluted with 175 μL of a dilute TCEP solution containing an internal UV standard (40 μL 3,5-dimethoxybenzoic acid solution (5 mM in a 0.2 M pH 7 phosphate buffer) and 960 μL of a 10 mM TCEP*HCl solution in water). The resulting solution was filtered and injected in the UPLC®-MS (2 x 2 μL injections).

Exchanged reaction in the presence of TCEP instead of DTT.

This reaction is performed with the same experimental protocol than the one above, but in the presence of 25 mM of TCEP. As shown in Fig. S4, no trace of exchanged product was obtained, however a large proportion of desulfurized starting material was detected after t=19.5 h. For desulfurization in presence of TCEP, see: Wang, Z.; Rejtar, T.; Zhou, Z. S.; Karger, B. L. Rapid. *Commun. Mass Spectrom*. **2010**, *24*, 267-275.

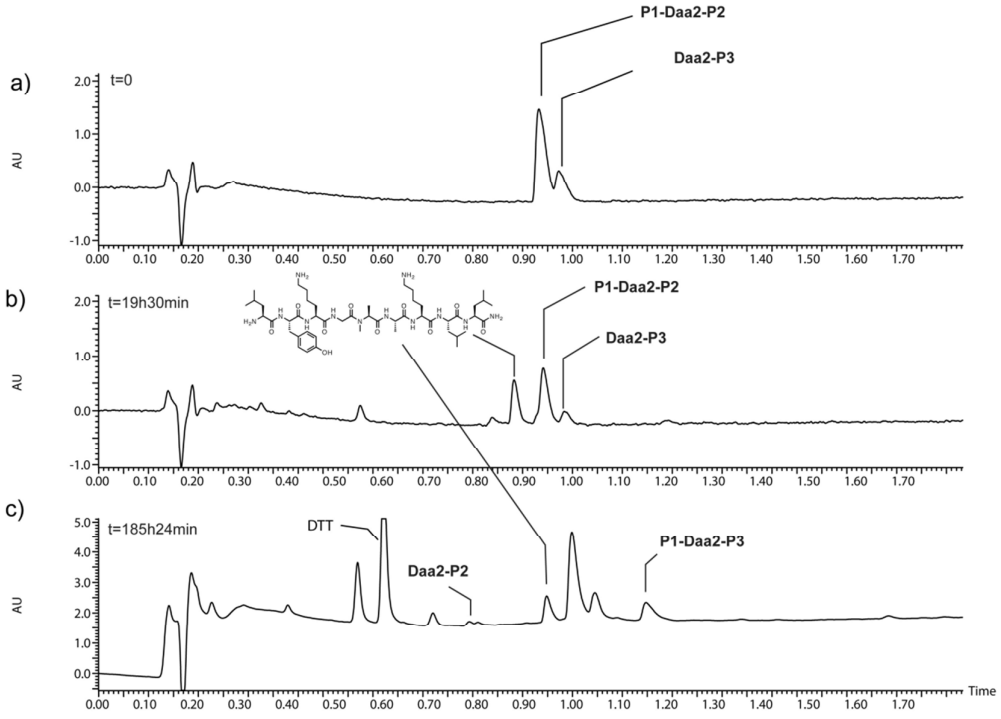


Figure S4. Reversed phase chromatograms (UPLC®) recorded with UV and MS detection ((a) t=0; (b) t=19.5 h) in the exchange reaction between **P1-Daa2-P2** and **Daa2-P3** in similar conditions of concentration and at pH 7 but using TCEP instead of DTT, showing the absence of exchanged product and the strong proportion of desulfurated starting material and additional non characterized byproducts. (c) Same exchange in a 1:1 mixture of DTT and TCEP showing both exchange products and desulfurated starting material.

2.4.4 α-Valine- N-(methyl)-cysteine modified peptides

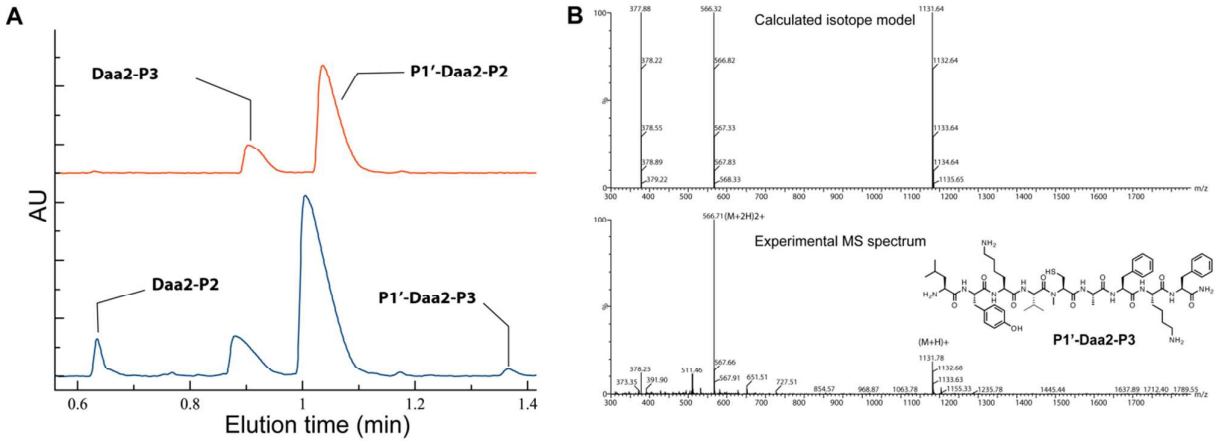


Figure S5. (a) Reversed phase chromatograms (UPLC®) recorded with UV and MS detection after 68 hours of the exchange reaction between **P1''-Daa2-P2** and **Daa2-P3**, at pH 8, concentration of 200 mM in buffer, 7.5 mM of starting materials, and in the presence of 0.6 %w/v of DTT, at 37 °C. No trace of hydrolysis byproduct was detected at that time of equilibration. (b) Corresponding mass spectra (calculated and experimentally obtained) for the exchange product **P1''-Daa2-P3**.

2.5 Calibration curves and error determinations

Calibration curves were established for peptides **P1-Daa1-P2**, **P1-Daa1-P3**, **P1-Daa2-P2**, and **P1-Daa2-P3** by injecting 2 μL of the peptides at known concentrations (62.50, 125.00, 156.25, 312.50, 500.00 and 625.00 μM) under conditions identical as the ones used for the dilution of the aliquots of the exchange reactions. After a manual baseline correction with the peak analyzer tool of OriginPro 9.0® the area of the peak corresponding to the peptide was then plotted against the concentration. Each standard point corresponds to an average of 3 replicates. A linear regression analysis then gave 4 linear calibration curves with R^2 between 0.99324 and 0.99924 that where then used to calculate the concentration of the peptides in the exchange mixtures (see below Figures S6-S9).

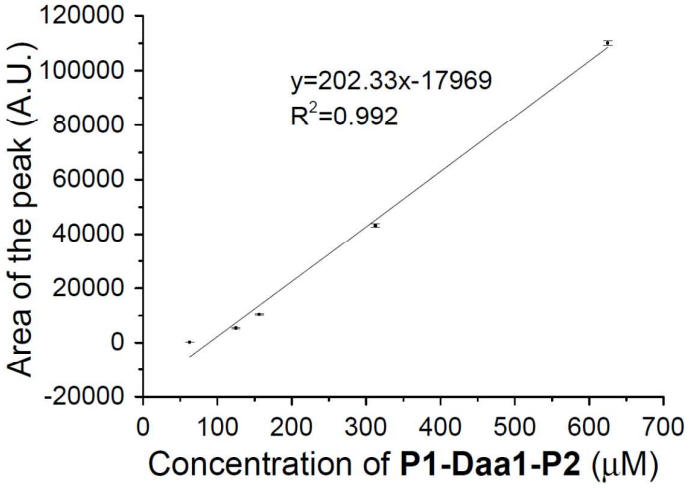


Figure S6. Calibration curve obtained by linear regression for **P1-Daa1-P2**.

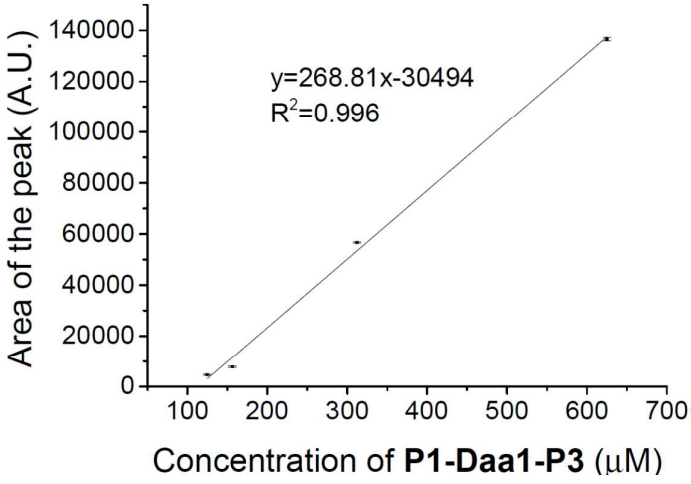


Figure S7. Calibration curve obtained by linear regression for **P1-Daa1-P3**.

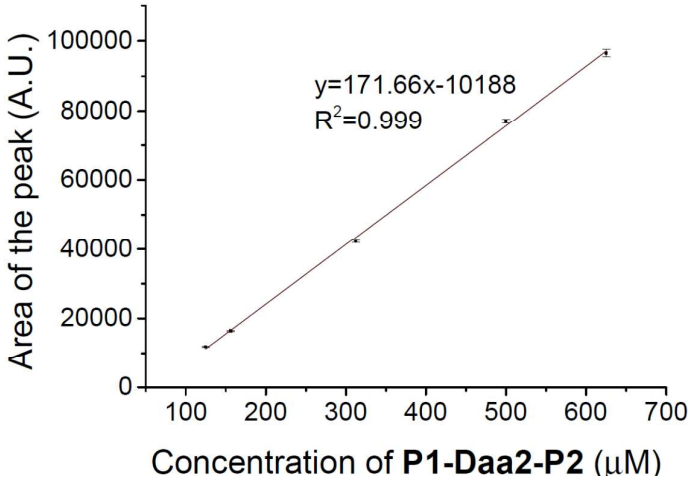


Figure S8. Calibration curve obtained by linear regression for **P1-Daa2-P2**.

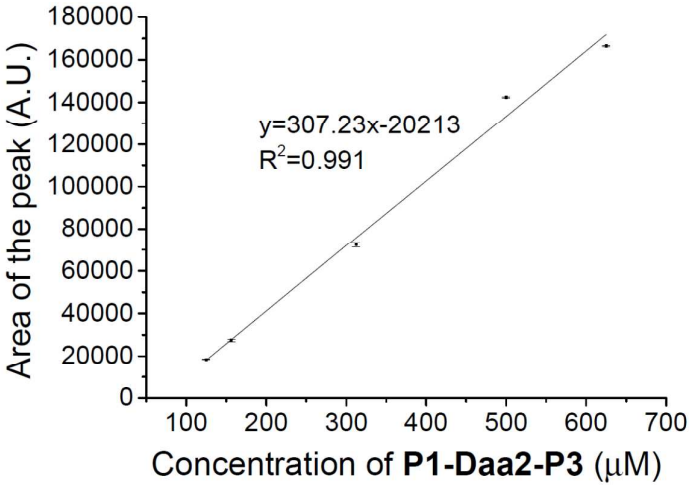


Figure S9. Calibration curve obtained by linear regression for **P1-Daa2-P3**.

The error on the injection volume inherent to the UPLC instrument (2 % accuracy for a 2 μL injection) was corrected by the use of an internal UV standard. Errors on the concentration were therefore estimated using the error on the dilution procedure required before injection onto the UPLC® column (25 μL of the reaction mixture + 175 μL of the standard solution containing either H_2O_2 or TCEP). The 50 and 100 μL Hamilton syringes used for the dilution are given with 1 % accuracy by the manufacturer. The error on a 25μL measurement on the 50 μL syringe is 2 %. The error on the 75 μL and 100 μL measurements on a 100 μL syringe are 1.3 % and 1 % respectively. This gives us a combined error E_{dil} of 2.6 % for the dilution procedure.

$$E_{dil} = \sqrt{2^2 + 1^2 + 1.3^2} = 2.6\%$$

The estimation of the error on the ratio $\boldsymbol{E_{ratio}}$ of the concentration given in Figures 2 and 3 of the main text was extrapolated using the following equation for each time point:

$$E_{ratio} = R\sqrt{\left(\frac{E_{C1}}{C1}\right)^2 + \left(\frac{E_{C1+C2}}{C1+C2}\right)^2}$$

Where ***C1*** and ***C2*** are the concentrations of the two peptides**,** ***R*** is the ratio between ***C1*** and ***C1+C2***. The errors $\boldsymbol{E_{C1}}$ and $\boldsymbol{E_{C2}}$ on the concentrations ***C1*** and ***C2*** were calculated according to the 2.6 % error on the dilution procedure (see above) and are therefore equal to ***C1*** x 0.026 and ***C2*** x 0.026 respectively. The estimation of the error $\boldsymbol{E_{C1+C2}}$ was extrapolated using the following equation at each time point.

$$E_{C1+C2} = \sqrt{{E_{C1}}^2 + {E_{C2}}^2}$$

The errors $\boldsymbol{E_{C1}}$ and $\boldsymbol{E_{C2}}$ on the concentration ***C1*** and ***C2*** were calculated according to the 2.6 % error on the dilution procedure (see above) and are therefore equal to ***C1***x0.026 and ***C2***x0.026 respectively.

3. Peptides characterizations

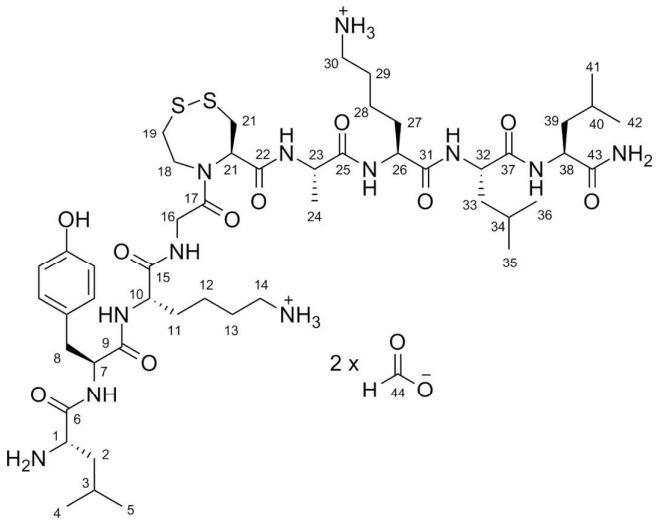
The purity of the isolated peptides and the kinetics of the exchange reactions were monitored using a Waters ACQUITY UPLC® UltraPerformance Liquid Chromatography coupled to a SQD ACQUITY Electrospray mass detector (UPLC-MS). The analysis were performed on an ACQUITY UPLC®HSS T3 1.8 μm 2.1x50 mm column from Waters using 0.1 % formic acid in water (eluent A) and 0.1 % formic acid in acetonitrile (eluent B) as the two mobile phases and the following gradient:

Time (min)	Flow (mL/min)	%A	%B
0	1	95	5
3.5	1	5	95
4	1	5	95
4.1	1	95	5
5.1	1	95	5

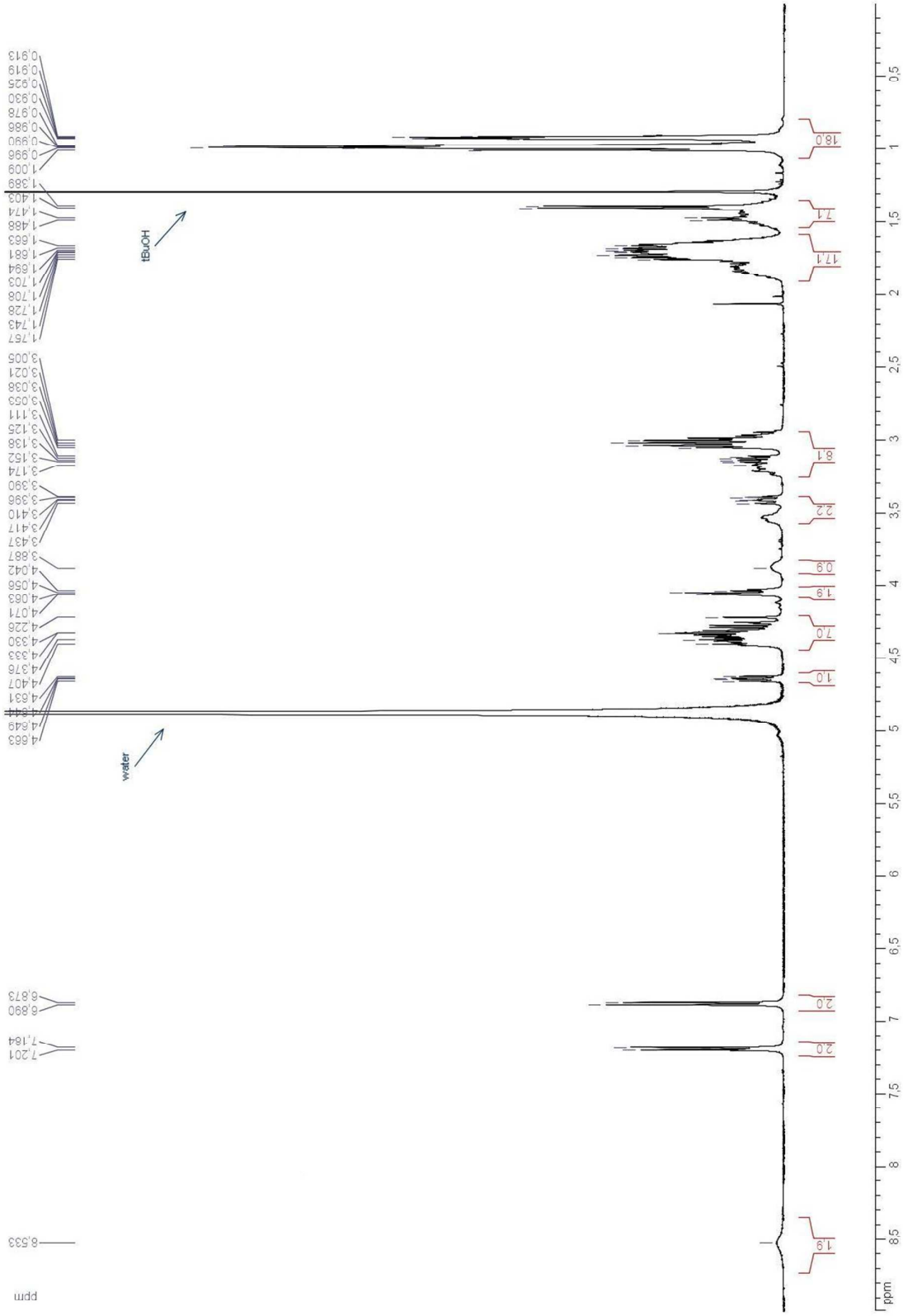
The processing of the UPLC® UV data (baseline subtraction and peak integration) was performed with the peak analyzer tool of OriginPro 9.0®.

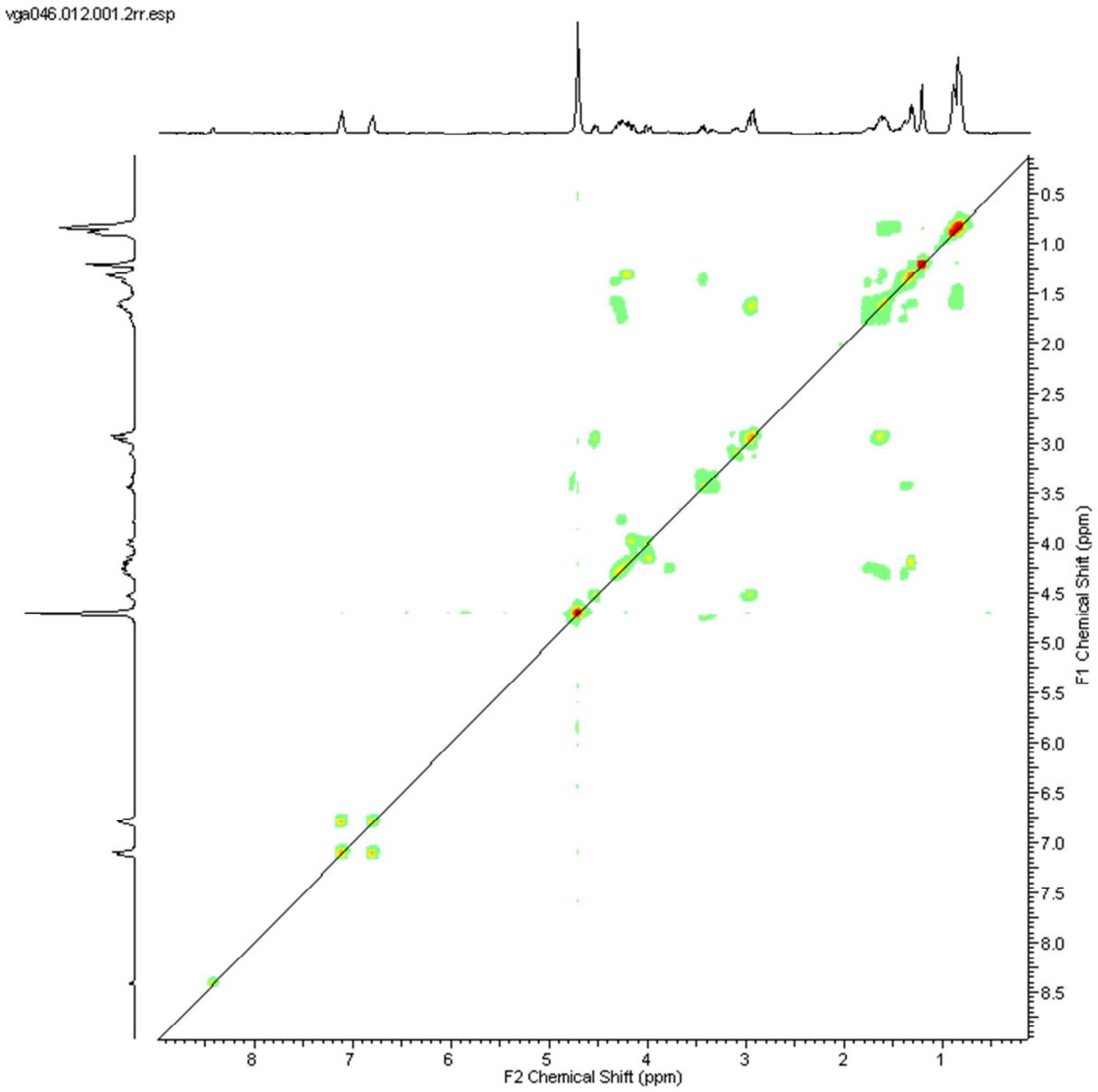
ESI-MS spectra were acquired using the Waters SQD Mass Detector directly connected to the output of an UPLC® or HPLC column.

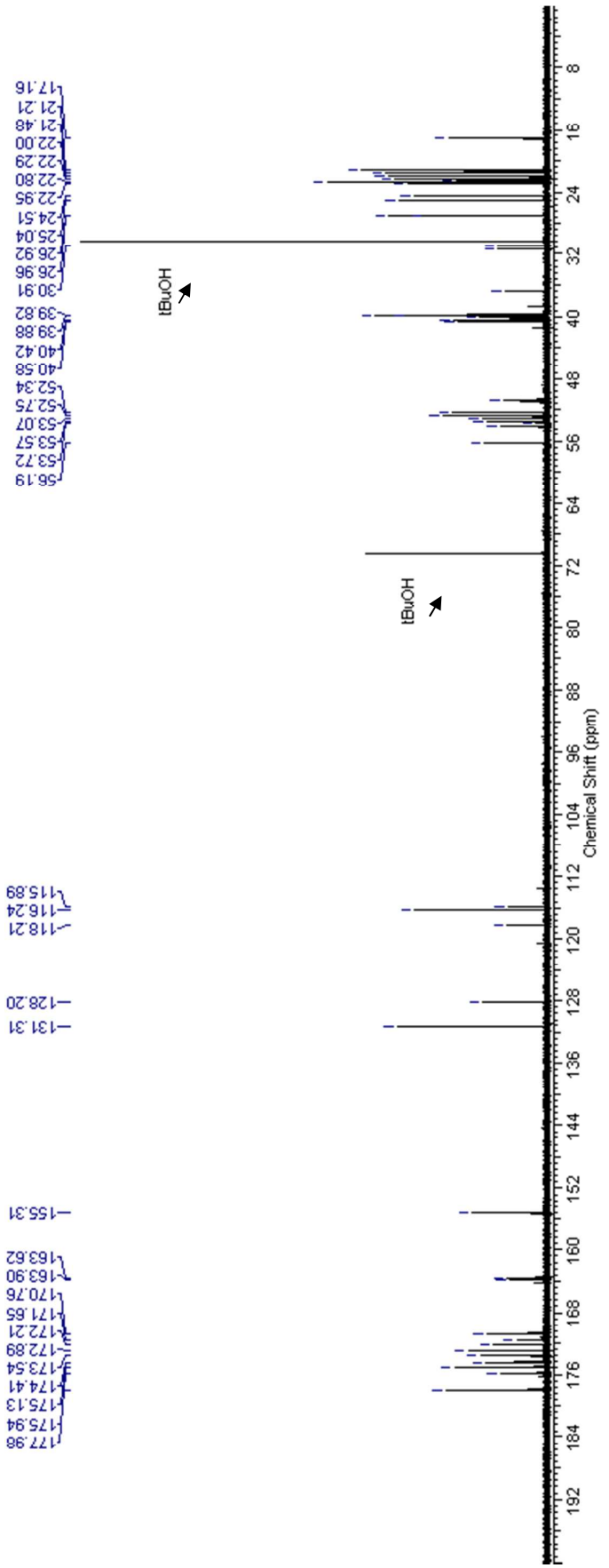
P1-Daa1-P2$_{ox}$



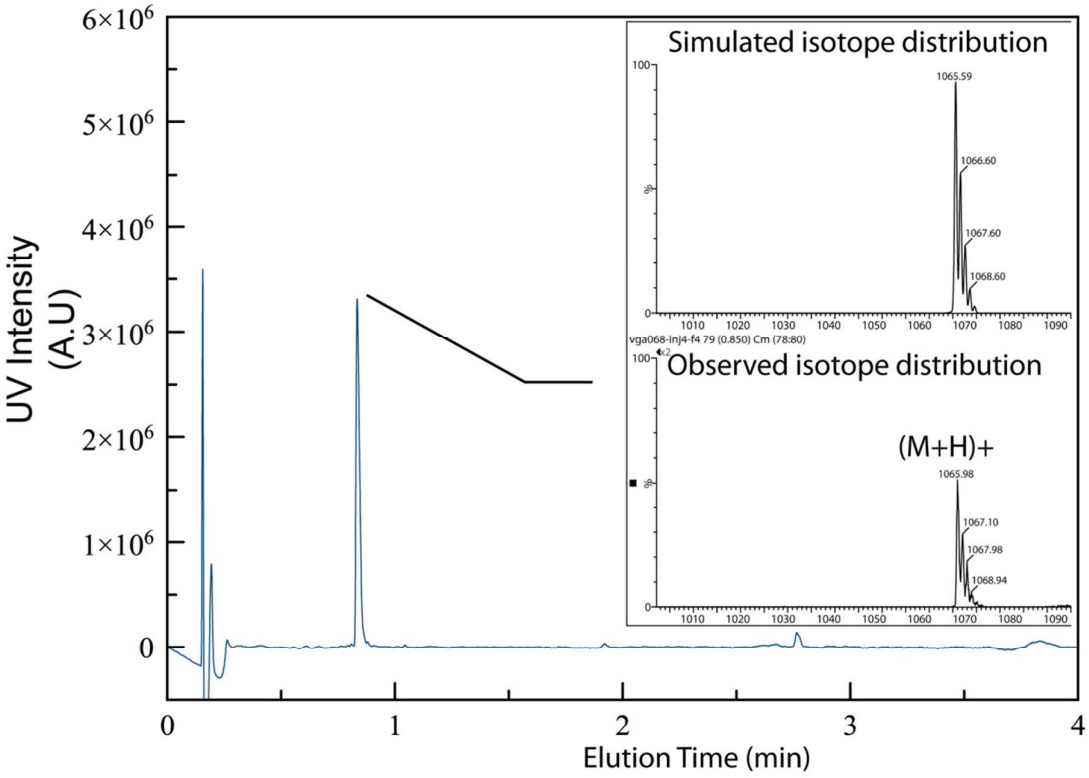
^{1}H NMR (500 MHz, D_2O): δ = 8.53 (bs, 2H, 2 H-C(44)), 7.14 (d, 2H, aromatic protons Tyr, *J* = 8.6 Hz), 6.83 (d, 2H, aromatic protons Tyr residue, *J* = 8.6 Hz), 4.59 (dd, 1H, *J* = 9.7 Hz, 6.8 Hz), 4.40-4.15 (m, 7H), 4.05-3.93 (m, 2H), 3.88-3.76 (m, 1H), 3.55-3.32 (m, 2H), 3.21-2.87 (m, 8H), 1.85-1.53 (m, 17H), 1.50-1.28 (m, 7H), 0.99-0.84 (m, 18H).
^{13}C NMR (125 MHz, D_2O): δ = 177.98, 175.94, 175.13, 174.41, 173.54, 172.89, 172.21, 171.65, 170.76, 163.90, 163.62, 155.31, 131.31, 128.20, 118.21, 116.24, 115.89, 56.19, 54.17, 53.72, 53.57, 53.07, 52.75, 52.34, 50.89, 40.58, 40.42, 40.16, 39.88, 39.82, 36.81, 31.33, 30.91, 26.96, 26.92, 25.04, 25.01, 24.51, 22.95, 22.80, 22.55, 22.29, 22.00, 21.48, 21.21, 17.16.


tBuOH
water
ppm

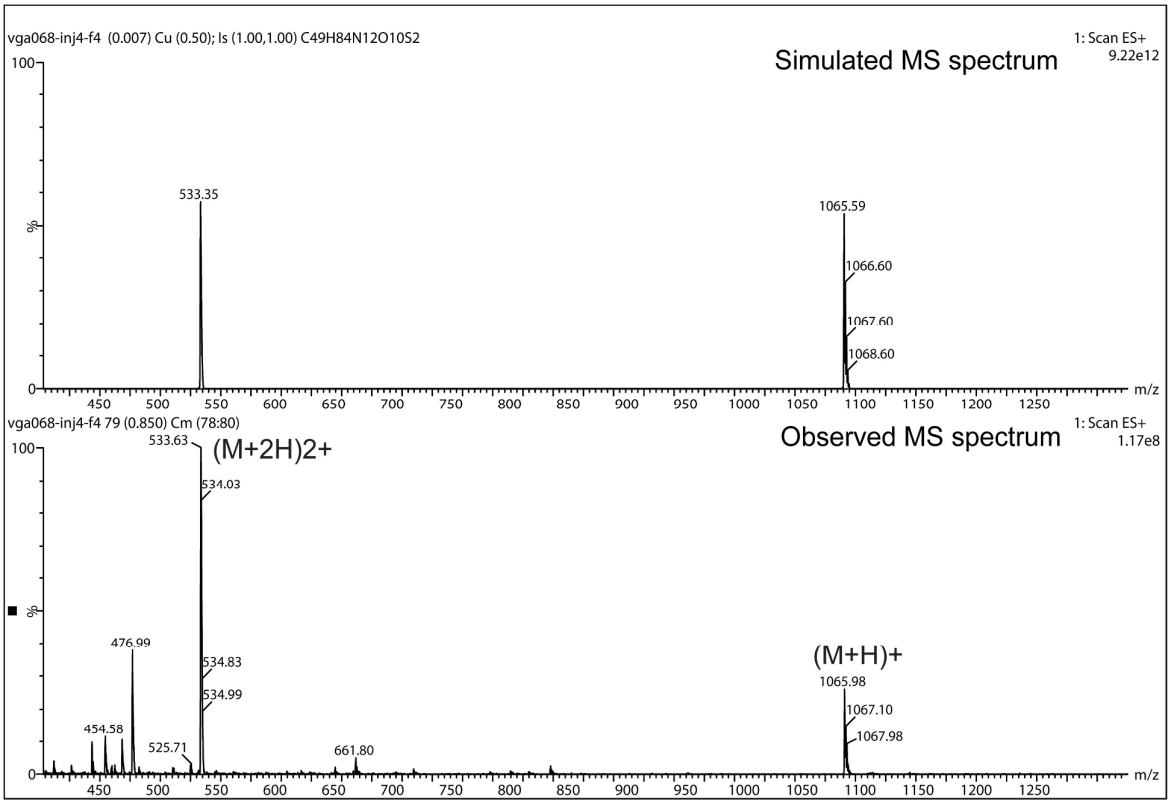

vga046.012.001.2rr.esp
F2 Chemical Shift (ppm)
F1 Chemical Shift (ppm)


17.16
21.21
21.48
22.00
22.29
22.80
22.95
24.51
25.04
26.92
26.96
30.91
39.82
39.88
40.42
40.58
52.34
52.75
53.07
53.57
53.72
56.19
tBuOH
tBuOH
115.89
116.24
118.21
128.20
131.31
155.31
163.62
163.90
170.76
171.65
172.21
172.89
173.54
174.41
175.13
175.94
177.98
8
16
24
32
40
48
56
64
72
80
88
96
104
112
120
128
136
144
152
160
168
176
184
192
Chemical Shift (ppm)

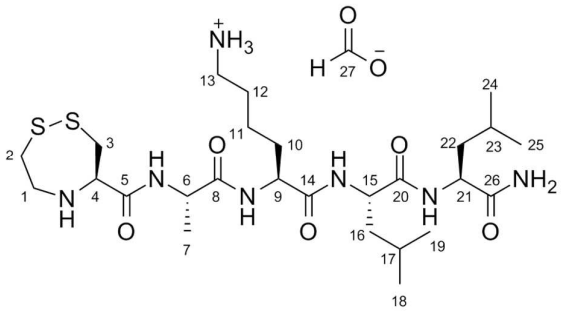
Analytical UPLC® chromatogram and associated MS (isotopic distribution) for purified peptide **P1-Daa1-P2ox**:



ESI-MS spectrum of the purified peptide **P1-Daa1-P2ox**:

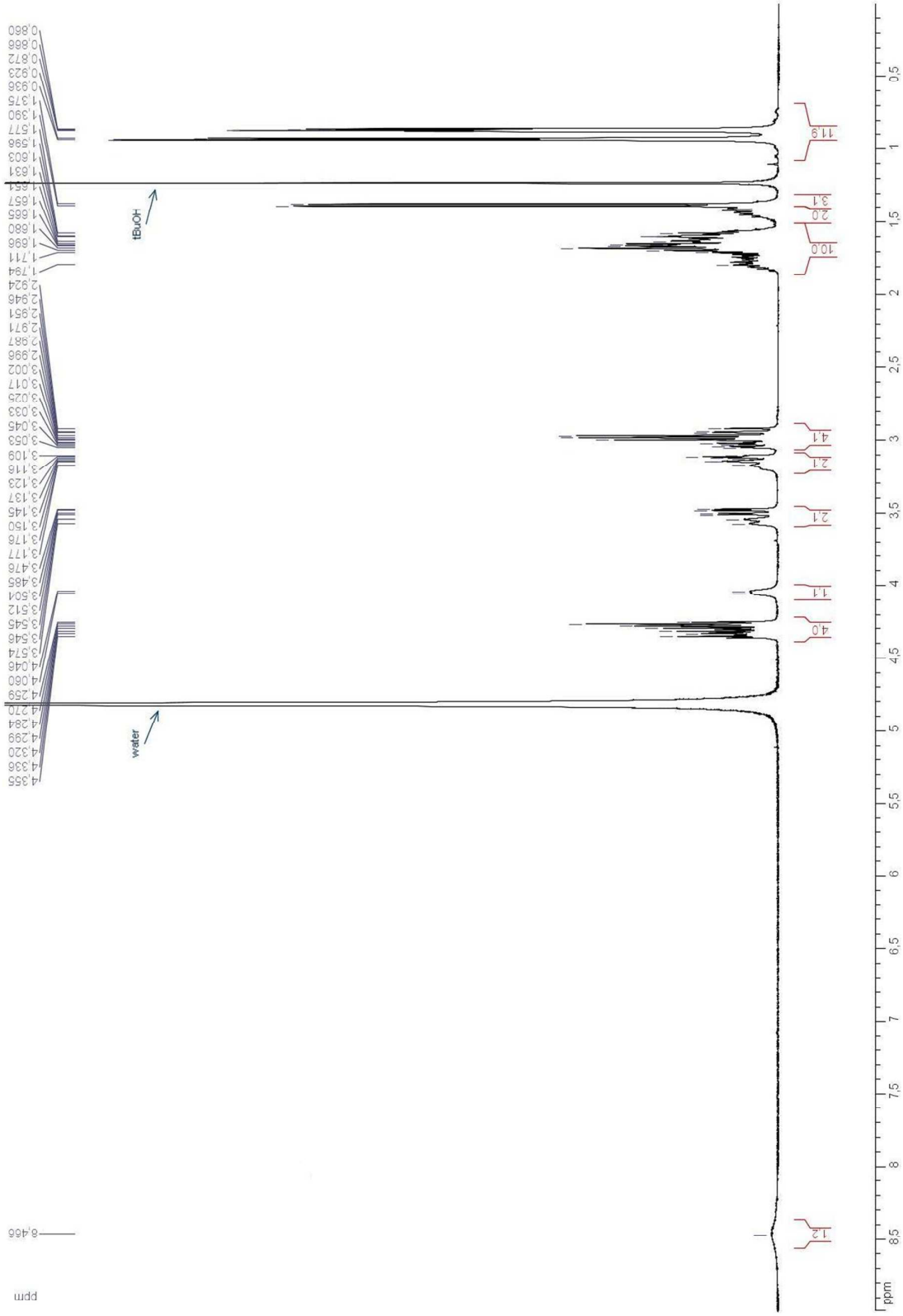


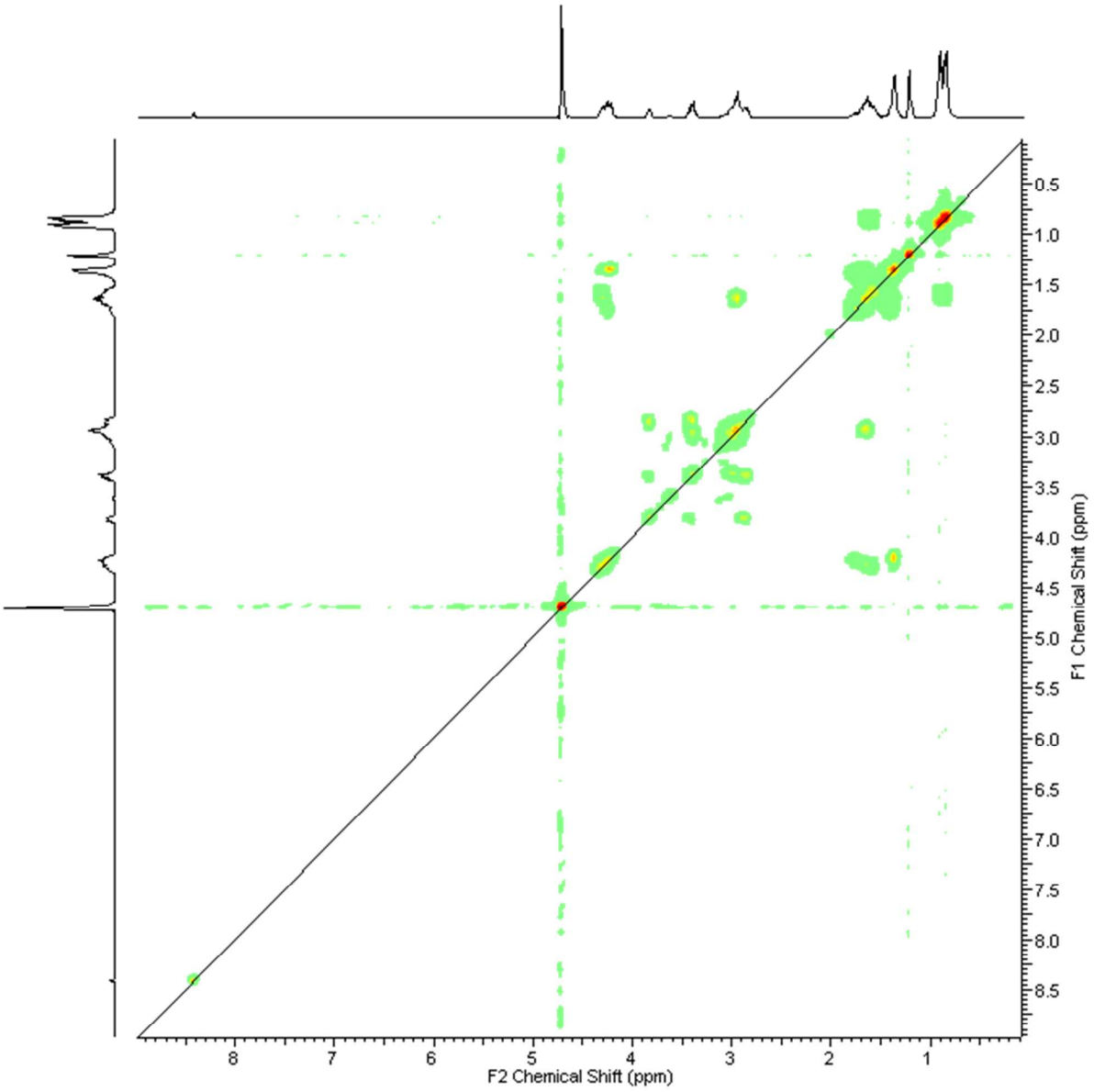
Daa1-P2$_{ox}$

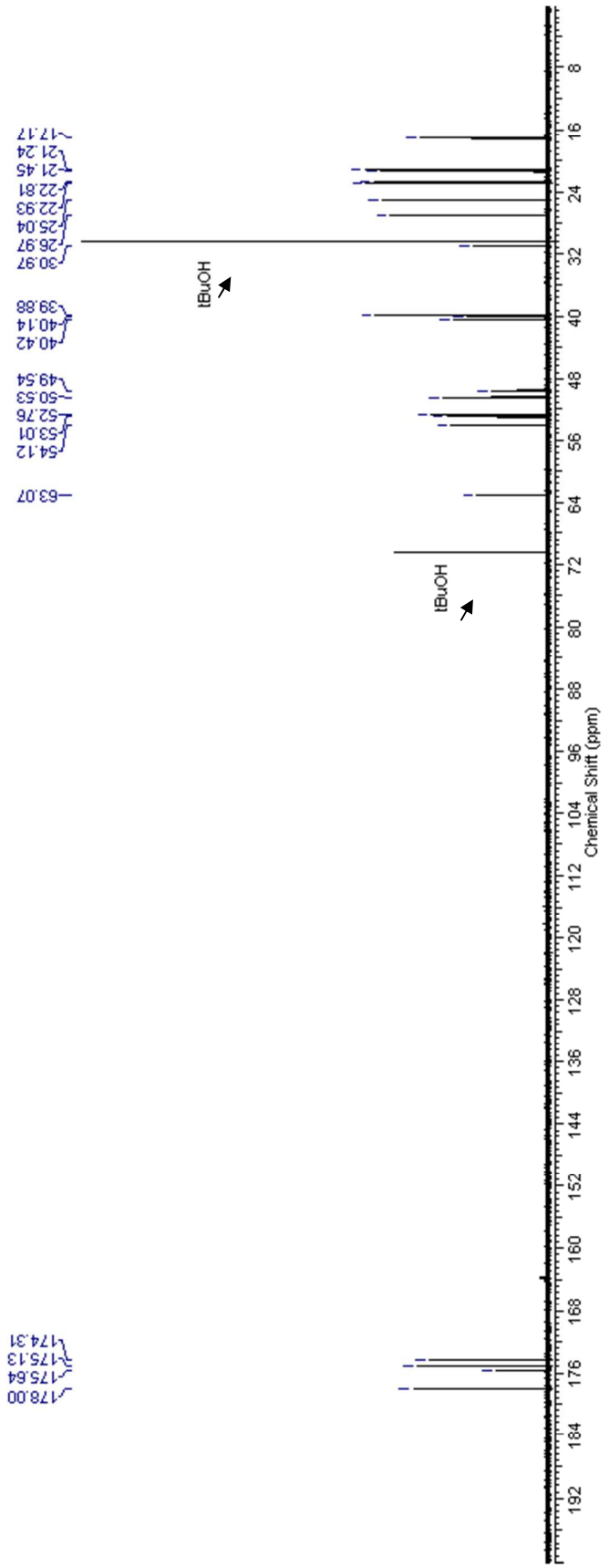


^{1}H NMR (500 MHz, D_2O): δ = 8.47 (bs, 1H, H-C(27)), 4.41-4.22 (m, 4H, H-C(6) + H-C(9) + H-C(15) + H-C(21)), 4.11-4.00 (m, 1H, H-C(4)), 3.62-3.45 (m, 2H, 1 H-C(1) + 1 H-C(3)), 3.23-3.09 (m, 2H), 3.07-2.89 (m, 4H), 1.86-1.51 (m, 10H, 2 H-C(11) + 2 H-C(12) + 2 H-C(16) + 1 H-C(17) + 2 H-C(22) + 1 H-C(23)), 1.51-1.40 (m, 2H, 2 H-C(10)), 1.40-1.31 (d, 3H, 3 H-C(7) J= 7.50), 1.08-0.69 (m, 12H, 3 H-C(18) + 3 H-C(19) + 3 H-C(24) + 3 H-C(25)).

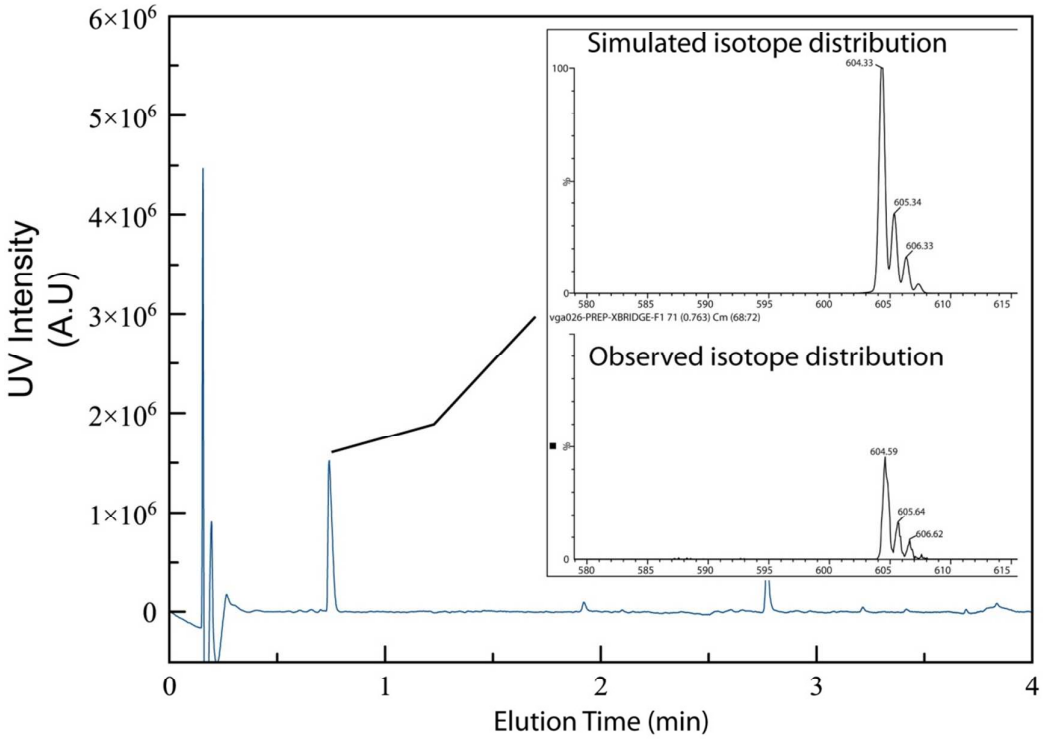
^{13}C NMR (125 MHz, D_2O): δ = 178.00, 175.64, 175.13, 174.31, 63.07, 54.12, 53.01, 52.76, 50.53, 49.54, 40.42, 40.14, 39.88, 30.97, 26.97, 25.04, 25.02, 22.93, 22.81, 22.76, 21.45, 21.24, 17.17.


tBuOH
water
11.9
3.1
2.0
10.0
4.1
2.1
2.1
1.1
4.0
1.2
ppm

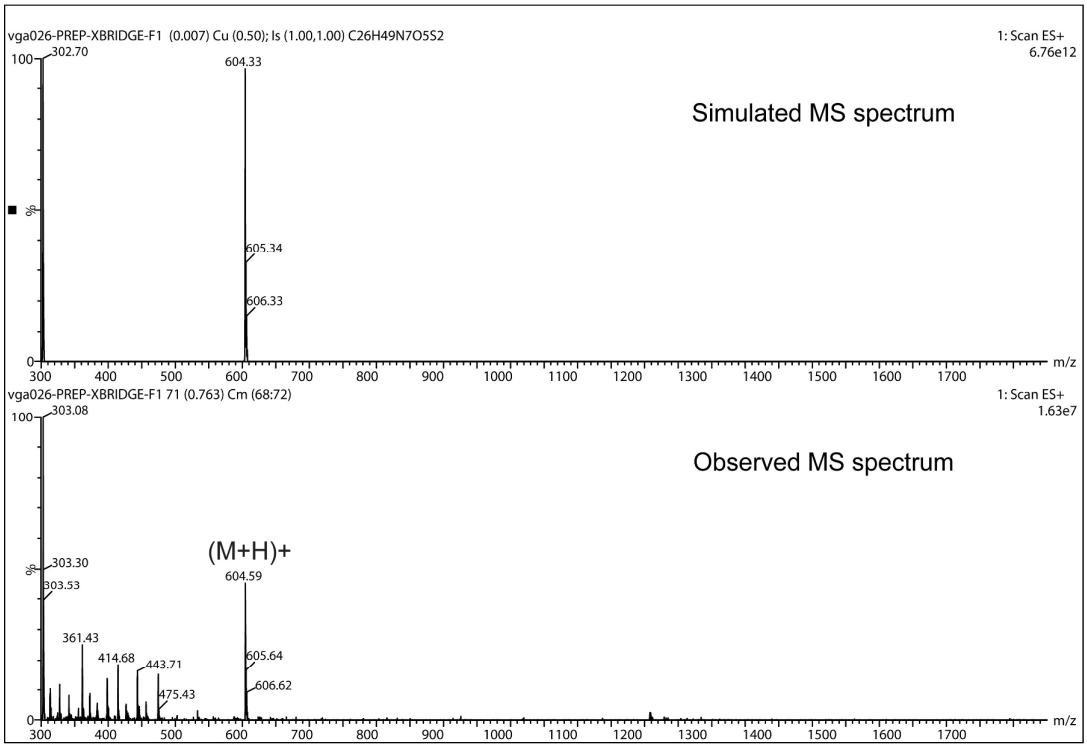

F2 Chemical Shift (ppm)
F1 Chemical Shift (ppm)


178.00
175.64
175.13
174.31
63.07
54.12
53.01
52.76
50.53
49.54
40.42
40.14
39.88
30.97
26.97
25.04
22.93
22.81
21.45
21.24
17.17
tBuOH
tBuOH
Chemical Shift (ppm)

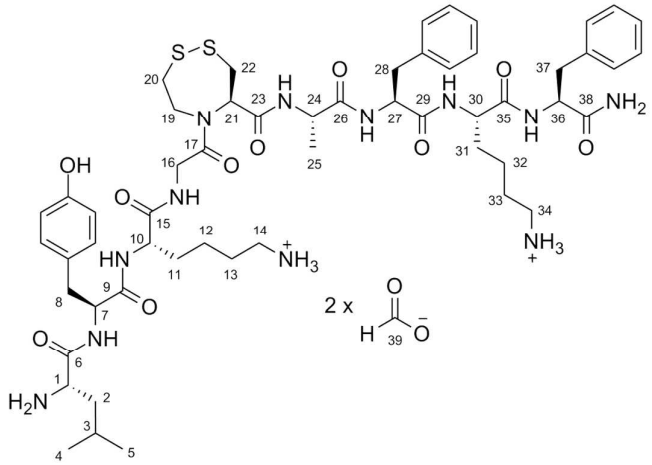
Analytical UPLC® chromatogram and associated MS (isotopic distribution) for purified peptide **Daa1-P2ox**:



ESI-MS spectrum of the purified peptide **Daa1-P2ox**:

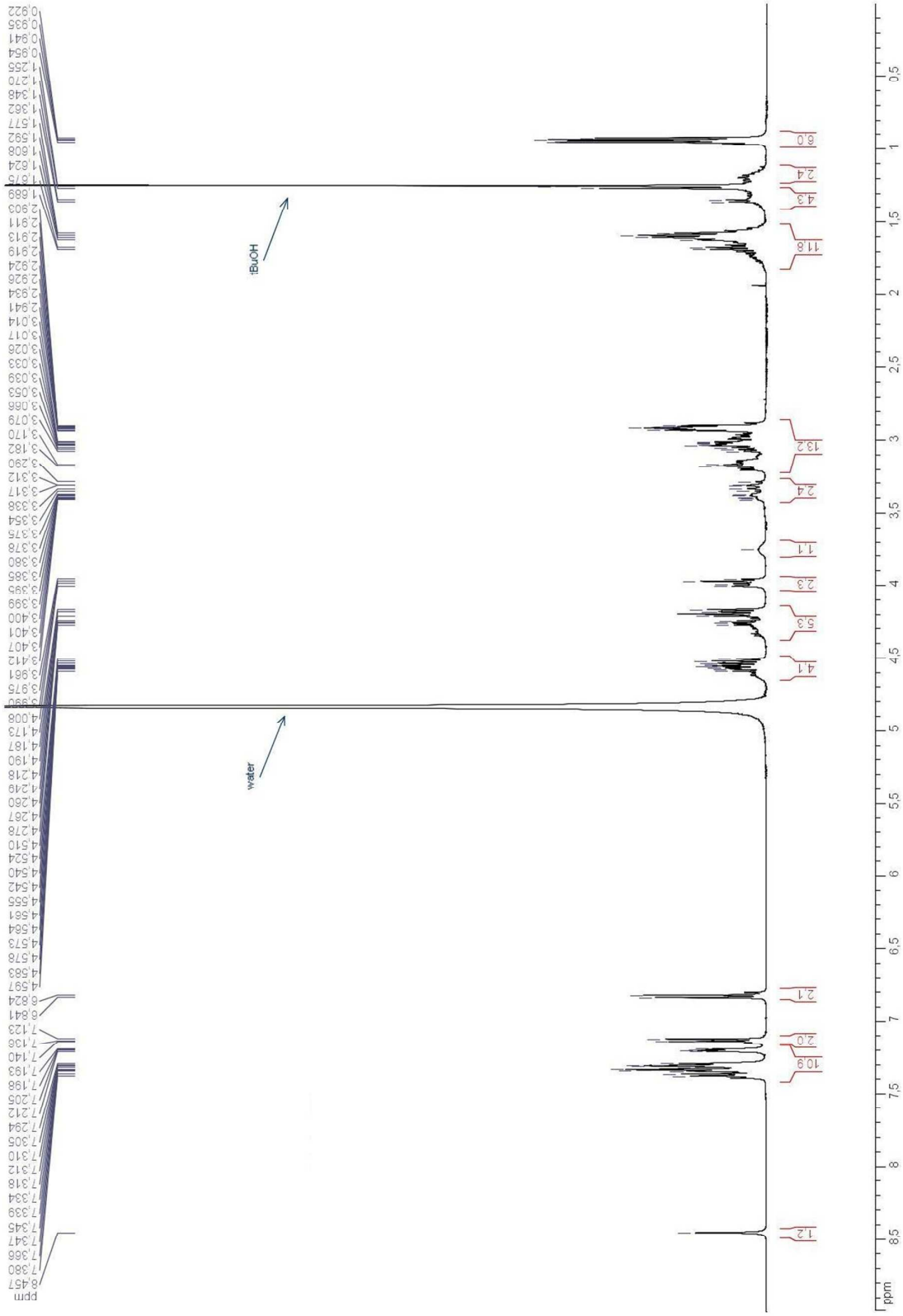


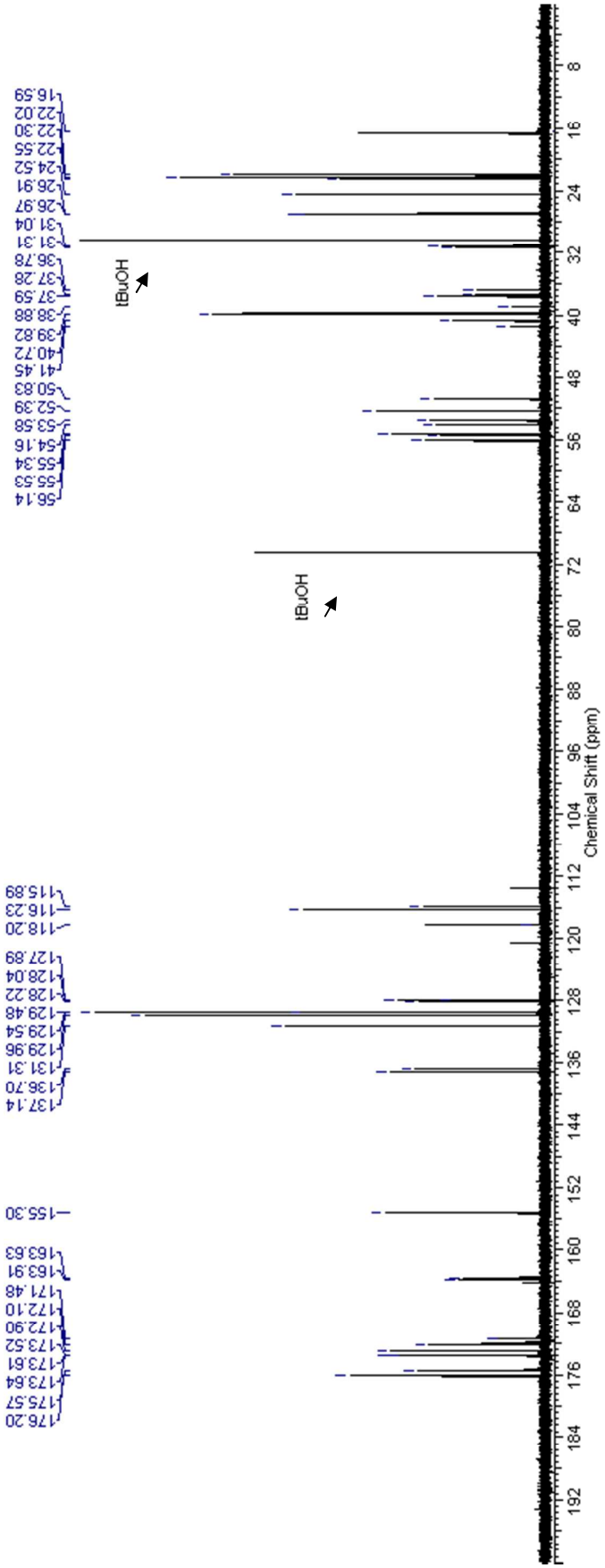
P1-Daa1-P3$_{ox}$



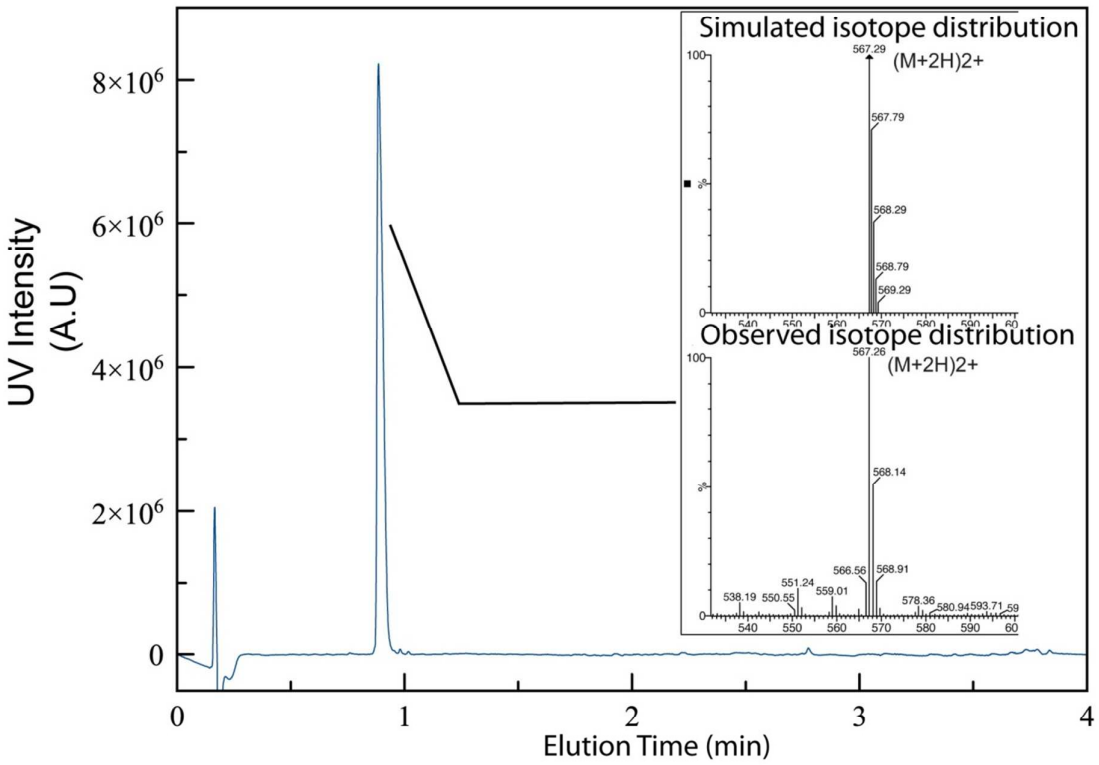
^{1}H NMR (500 MHz, D_2O): δ = 8.46 (s, 1H, 1 H-C(39)), 7.41-7.18 (m, 10H, aromatic protons Phe), 7.13 (d, 2H, 2 aromatic protons *J* = 8.6 Hz), 6.83 (d, 2H, 2 aromatic protons Tyr, *J* = 8.6 Hz), 4.67-4.50 (m, 4H), 4.37-4.16 (m, 5H), 4.04-3.95 (m, 2H), 3.81-3.69 (m, 1H), 3.43-3.27 (m, 2H), 3.23-2.87 (m, 13H), 1.82-1.50 (m, 12H), 1.41-1.27 (m, 4H), 1.22-1.11 (m, 2H), 0.99-0.88 (m, 3 H-C(4) + 3 H-C(5)).

^{13}C NMR (125 MHz, D_2O): δ = 176.20, 175.57, 173.64, 173.61, 173.52, 172.90, 172.10, 171.48, 163.91, 163.63, 155.30, 137.14, 136.70, 131.31, 129.96, 129.93, 129.54, 129.48, 128.22, 128.04, 127.89, 118.20, 116.23, 115.89, 56.14, 55.53, 55.34, 54.16, 53.58, 52.39, 50.83, 41.45, 40.72, 39.82, 39.79, 38.88, 37.59, 37.28, 36.78, 31.31, 31.04, 26.97, 26.91, 24.52, 22.55, 22.53, 22.30, 22.02, 16.59.

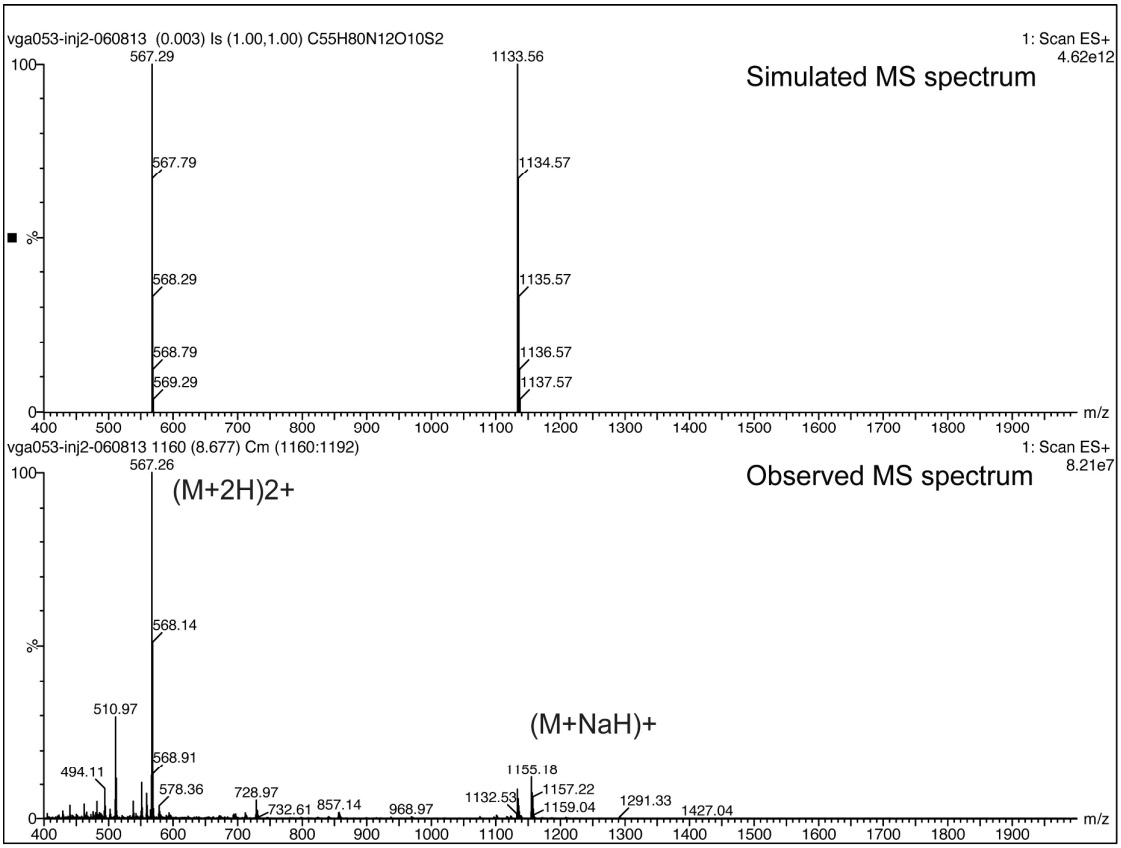

tBuOH
water
8.0
2.4
4.3
11.8
13.2
2.4
1.1
2.3
5.3
4.1
1.2
2.1
2.0
10.9
ppm


tBuOH
tBuOH
Chemical Shift (ppm)

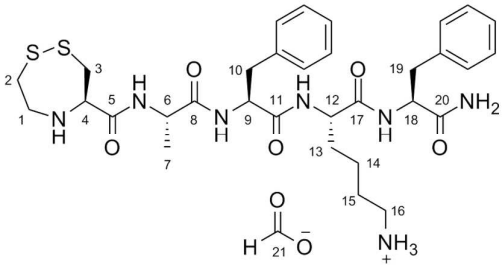
Analytical UPLC® chromatogram and associated MS (isotopic distribution) for purified peptide **P1-Daa1-P3ox**:



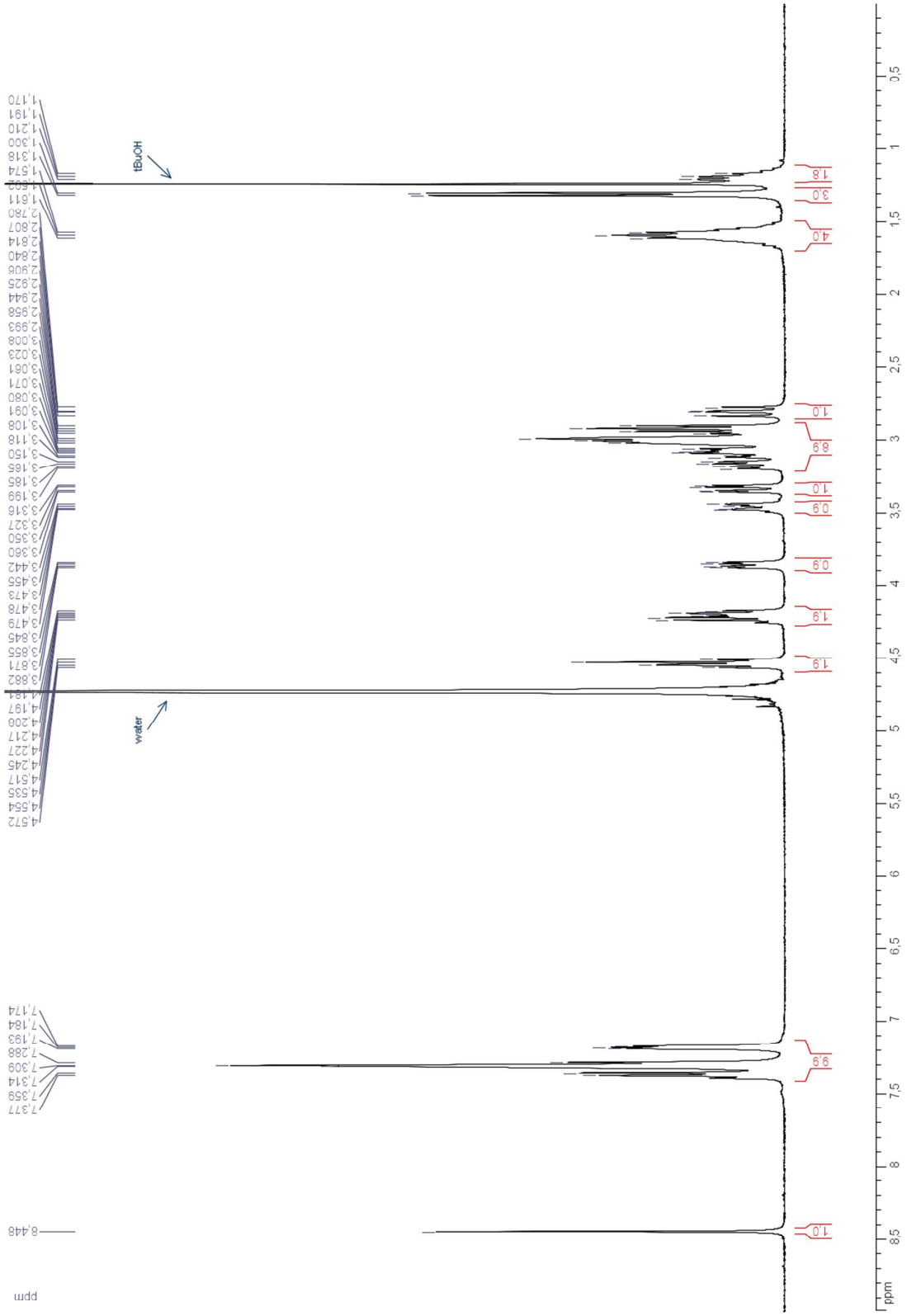
SI-MS spectrum of the purified peptide **P1-Daa1-P3ox**:

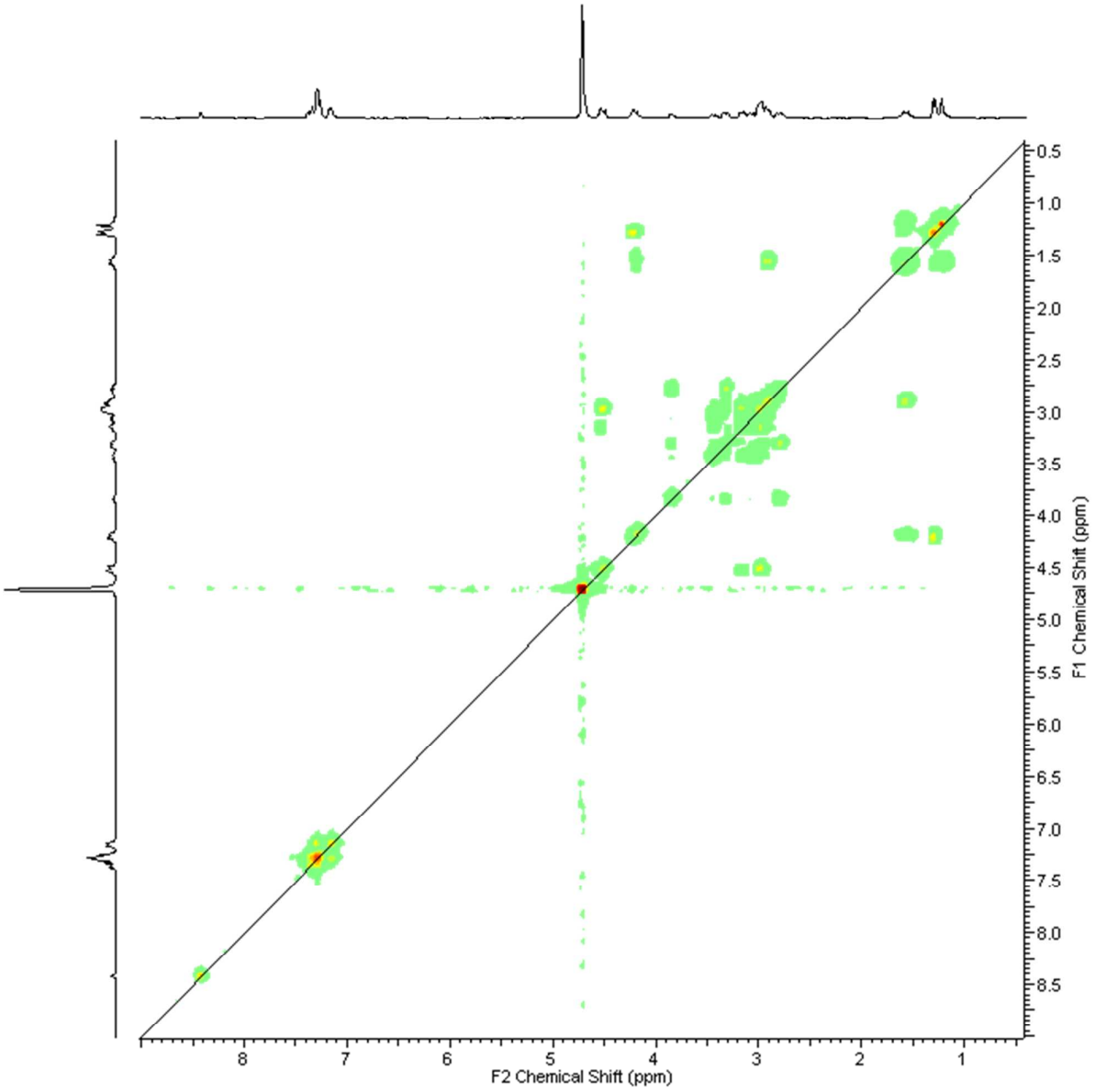


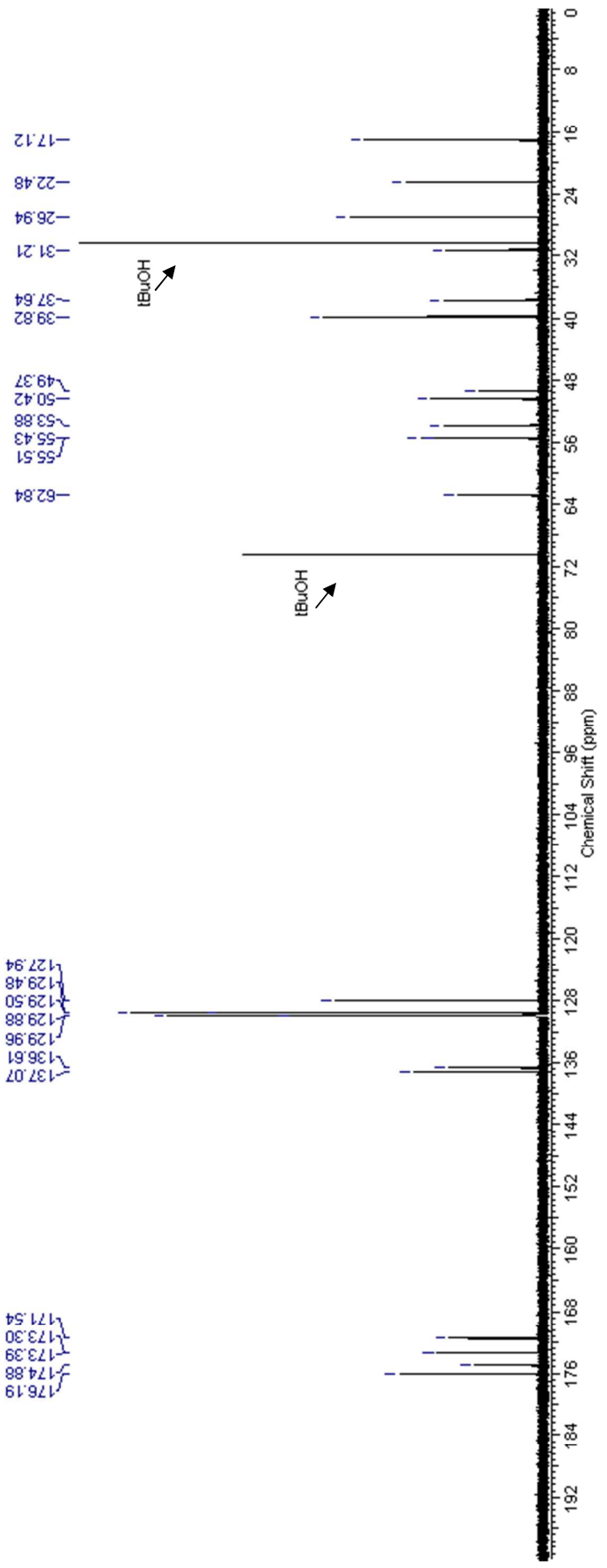
Daa1-P3$_{ox}$



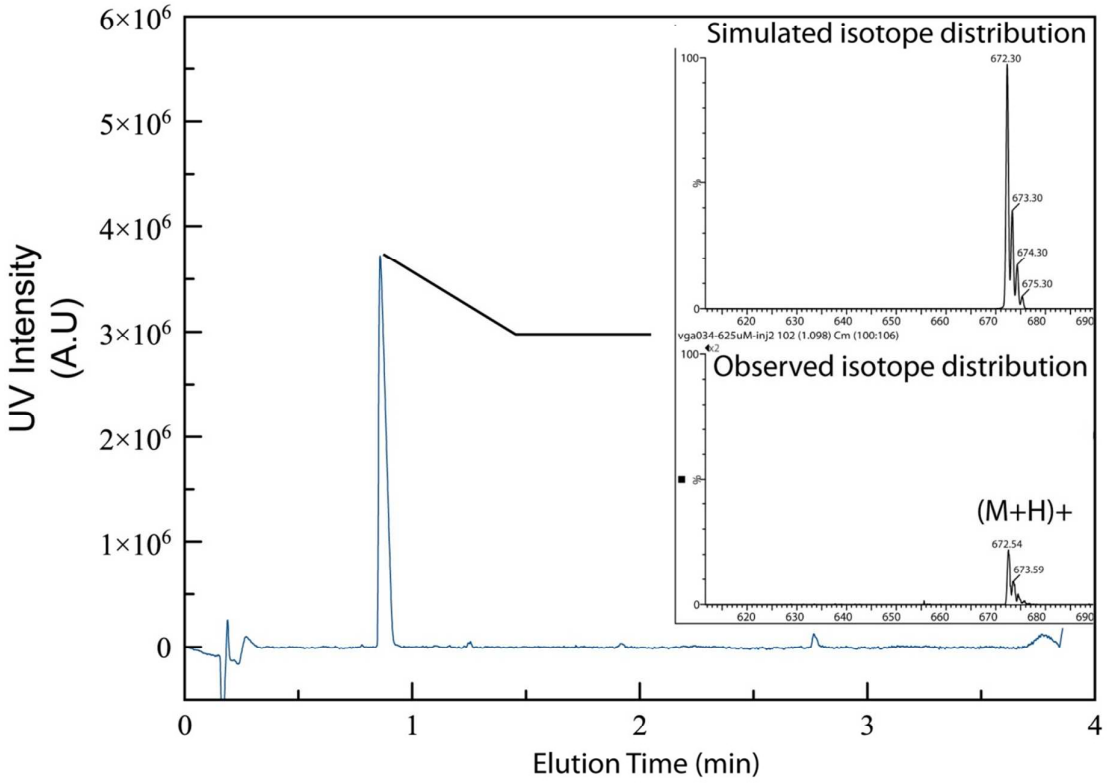
^{1}H NMR (400 MHz, D_2O): δ = 8.45 (s, 1H, H-C(21)), 7.42-7.15 (m, 10H, aromatic protons), 4.59-4.50 (m, 2H, H-C(9) + H-C(18)), 4.27-4.16 (m, 2H, H-C(6) + H-C(12)), 3.86 (dd, 1H, H-C(4) *J* = 10.75 Hz, 4.27 Hz), 3.52-3.42 (m, 1H, 1 H-C(1)), 3.34 (dd, 1H, 1 H-C(3) *J* = 13.48 Hz, 4.27 Hz), 3.22-2.88 (m, 9H, 1 H-C(1) + 2 H-C(2) +2 H-C(10) + 2 H-C(16) + 2 H-C(19)), 2.81 (dd, 1H, 1 H-C(3) *J* = 13.48 Hz, 10.75 Hz), 1.68-1.50 (m, 4H, 2 H-C(13) +2 H-C(15)), 1.31 (d, 3H, 3 H-C(7) *J* = 7.17 Hz), 1.26-1.14 (m, 2H, 2H-C(14)).
^{13}C NMR (125 MHz, D_2O): δ = 176.19, 174.88, 173.39, 173.30, 171.54, 137.07, 136.61, 129.96, 129.88, 129.50, 129.48, 127.94, 62.84, 55.51, 55.43, 53.88, 50.42, 49.37, 39.82, 37.67, 37.64, 31.21, 26.94, 22.48, 17.12.


tBuOH
water
ppm

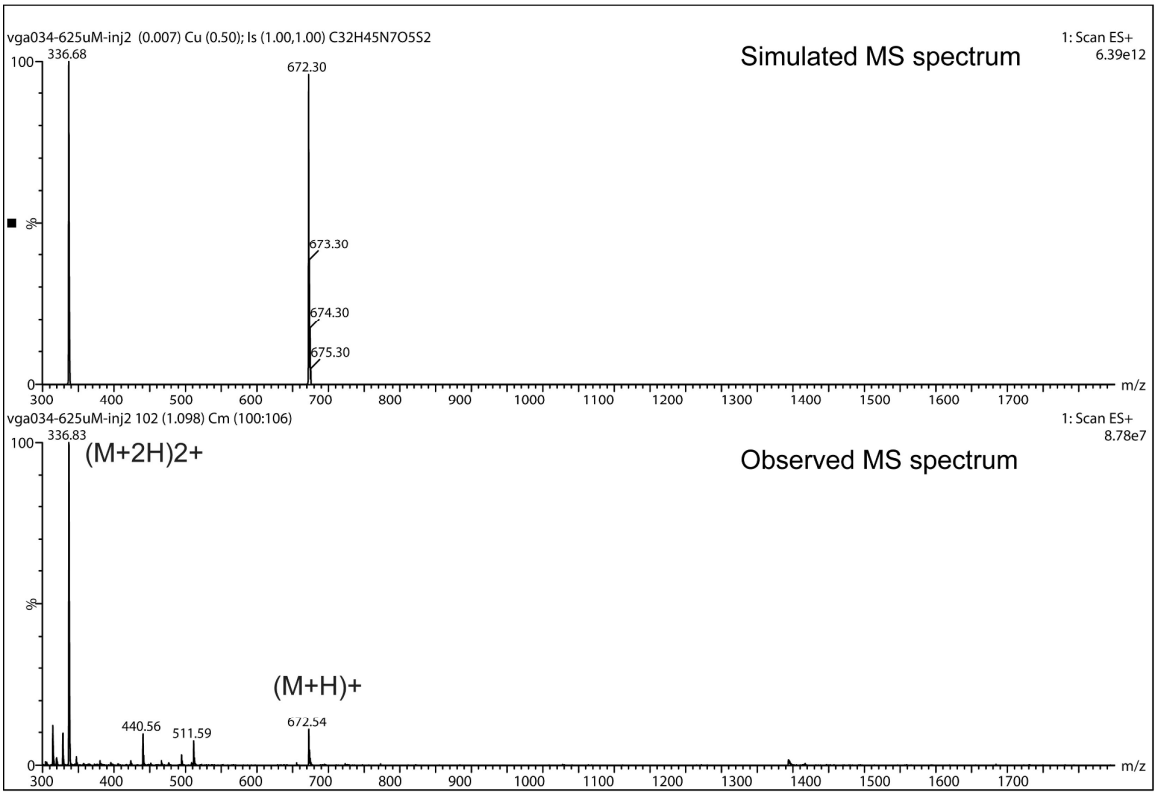

F2 Chemical Shift (ppm)
F1 Chemical Shift (ppm)


17.12
22.48
26.94
31.21
tBuOH
37.64
39.82
49.37
50.42
53.88
55.43
55.51
62.84
tBuOH
127.94
129.48
129.50
129.88
129.96
136.61
137.07
171.54
173.30
173.39
174.88
176.19
Chemical Shift (ppm)

Analytical UPLC® chromatogram and associated MS (isotopic distribution) for purified peptide **Daa1-P3ox**:

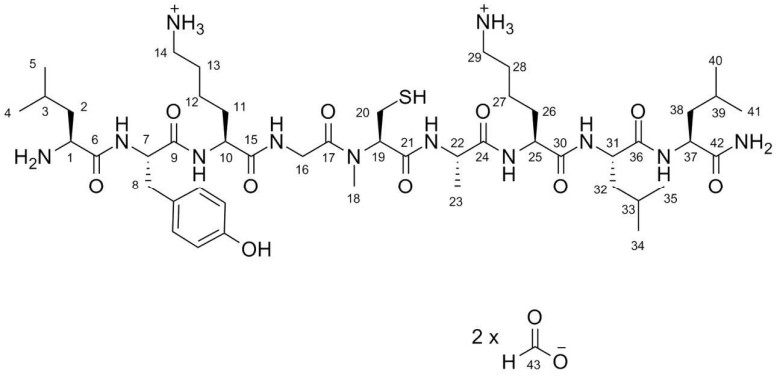


ESI-MS spectrum of the purified peptide **Daa1-P3ox**:

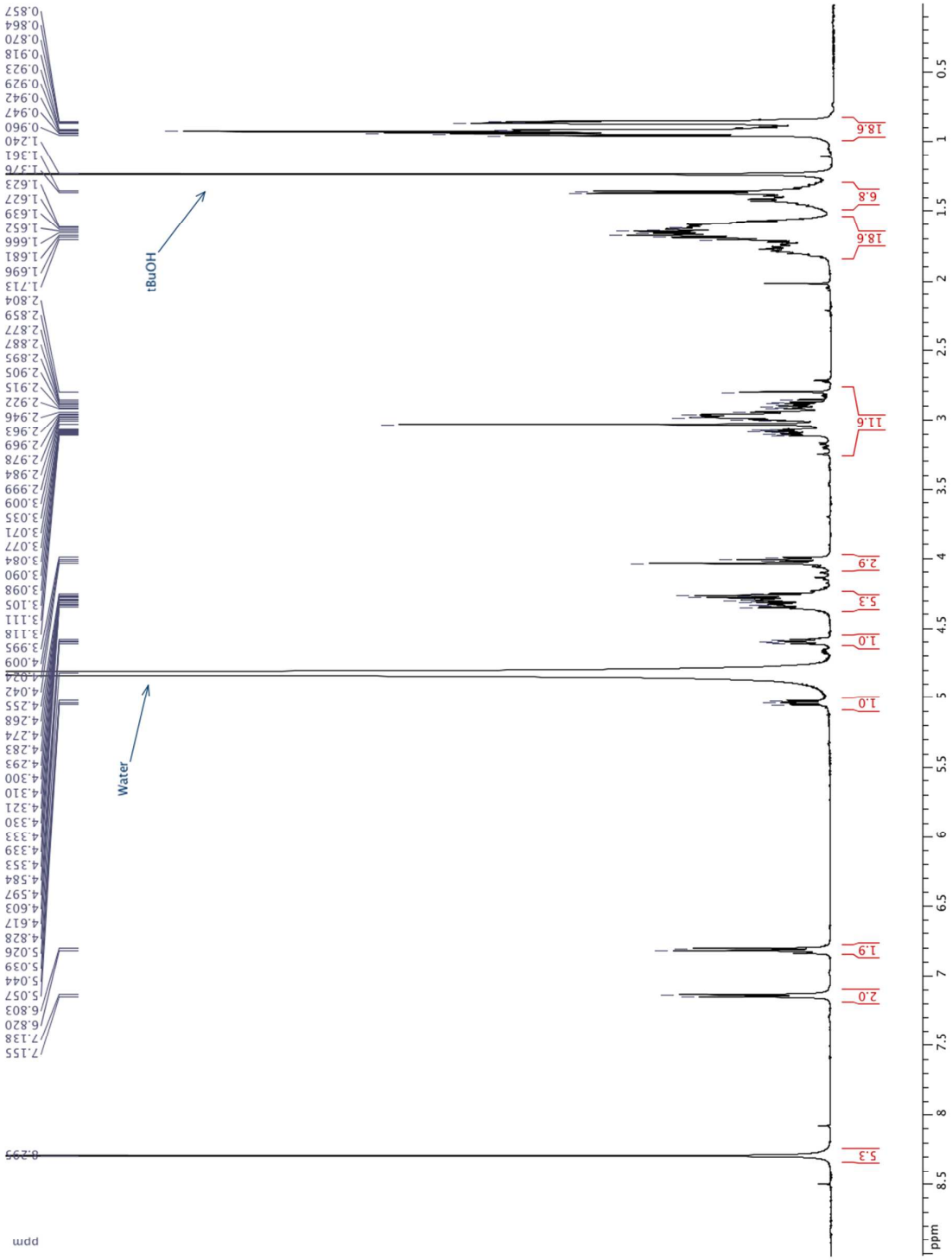


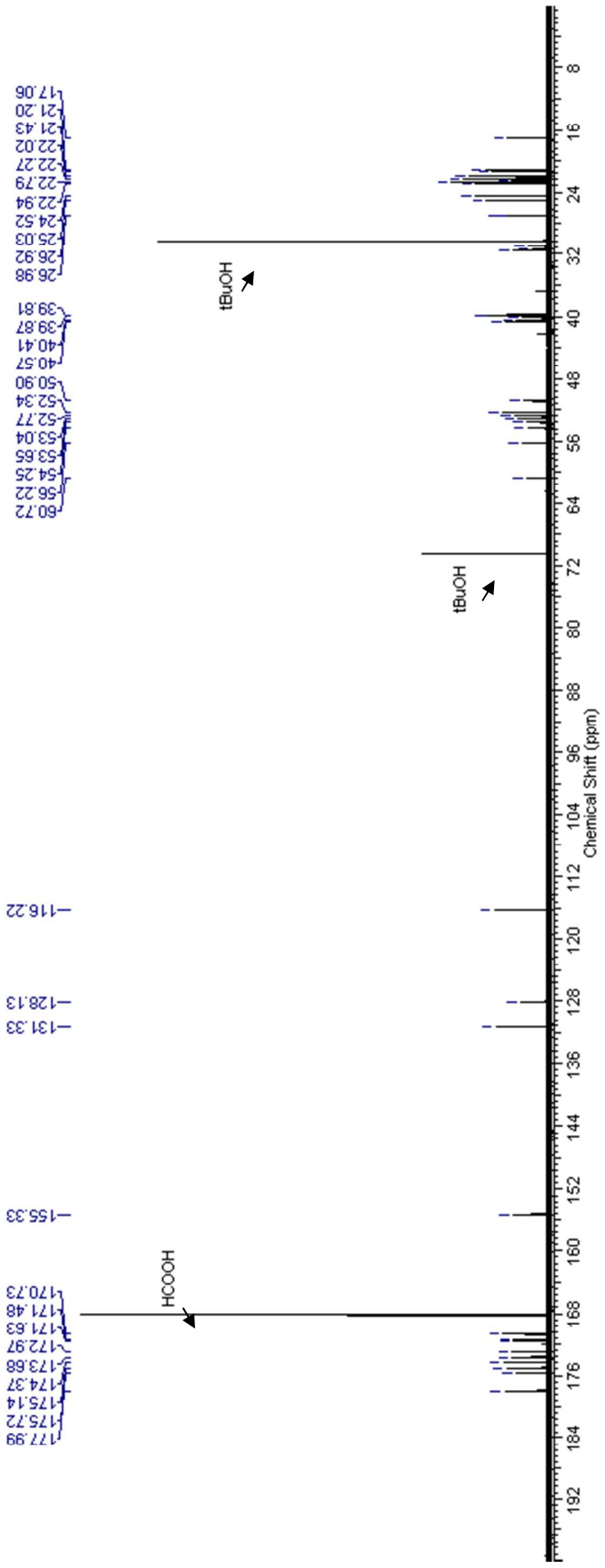
The following peptides where isolated in their reduced form. We therefore had to prevent oxidation into disulfides by lowering the pH of the solvents used for NMR by adding formic acid. In some cases oxidation still occurred, which results in additional peaks in the ^{1}H or ^{13}C NMR spectra, with however no relevance to purity as the later was established by analytical UPLC.

P1-Daa2-P2

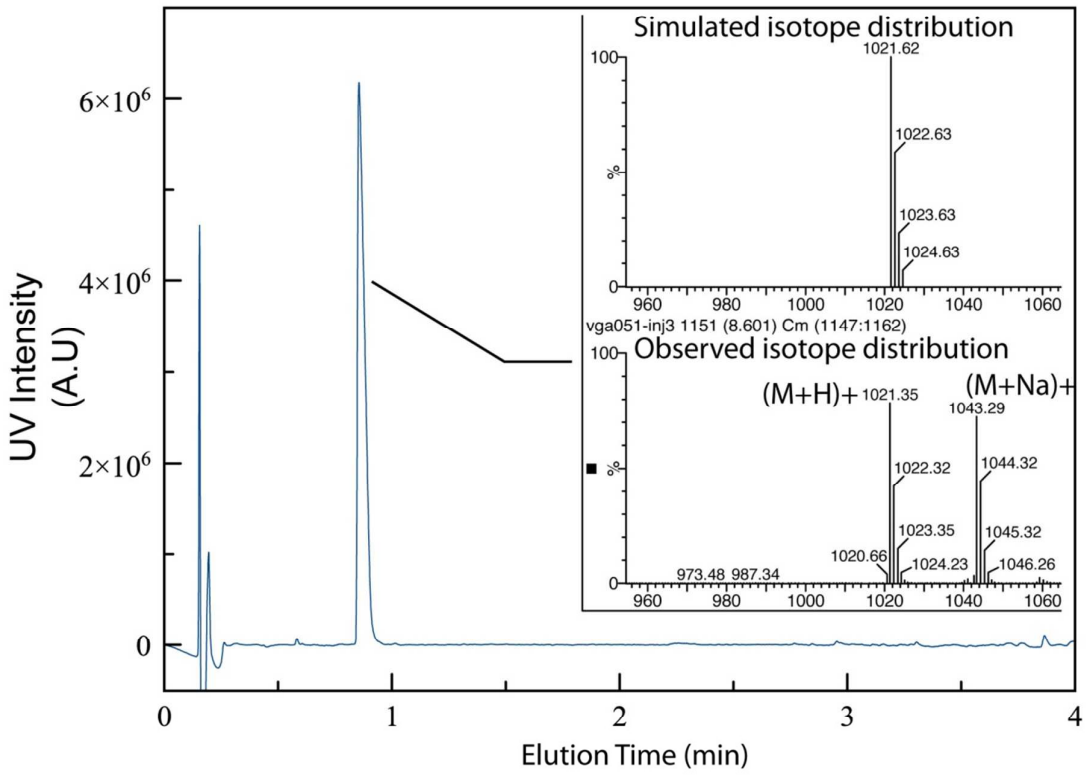


^{1}H NMR (500 MHz, D_2O): δ = 8.29 (s, excess formic acid to slow down oxidation), 7.15 (d, 2H, aromatic protons, *J* = 8.1 Hz), 6.81 (d, aromatic protons, *J* = 8.1 Hz), 5.04 (dd, 1H, *J* = 8.3 Hz, 6.4 Hz), 4.63-4.57 (m, 1H), 4.40-4.22 (m, 5H), 4.08-3.97 (m, 2H), 3.27-2.11 (m, 12H), 1.89-1.53 (m, 18H), 1.53-1.30 (m, 6H) 1.02-0.81 (m, 18H).
^{13}C NMR (125 MHz, D_2O): δ = 177.99, 175.72, 175.14, 174.37, 173.68, 172.97, 171.63, 171.48, 170.73, 155.33, 131.33, 128.13, 116.22, 60.72, 56.22, 54.25, 53.65, 53.04, 52.77, 52.34, 50.90, 40.57, 40.41, 40.16, 39.87, 39.81, 31.54, 31.29, 30.95, 26.98, 26.92, 25.03, 25.01, 24.52, 22.94, 22.79, 22.54, 22.27, 22.02, 21.43, 21.20, 17.06.

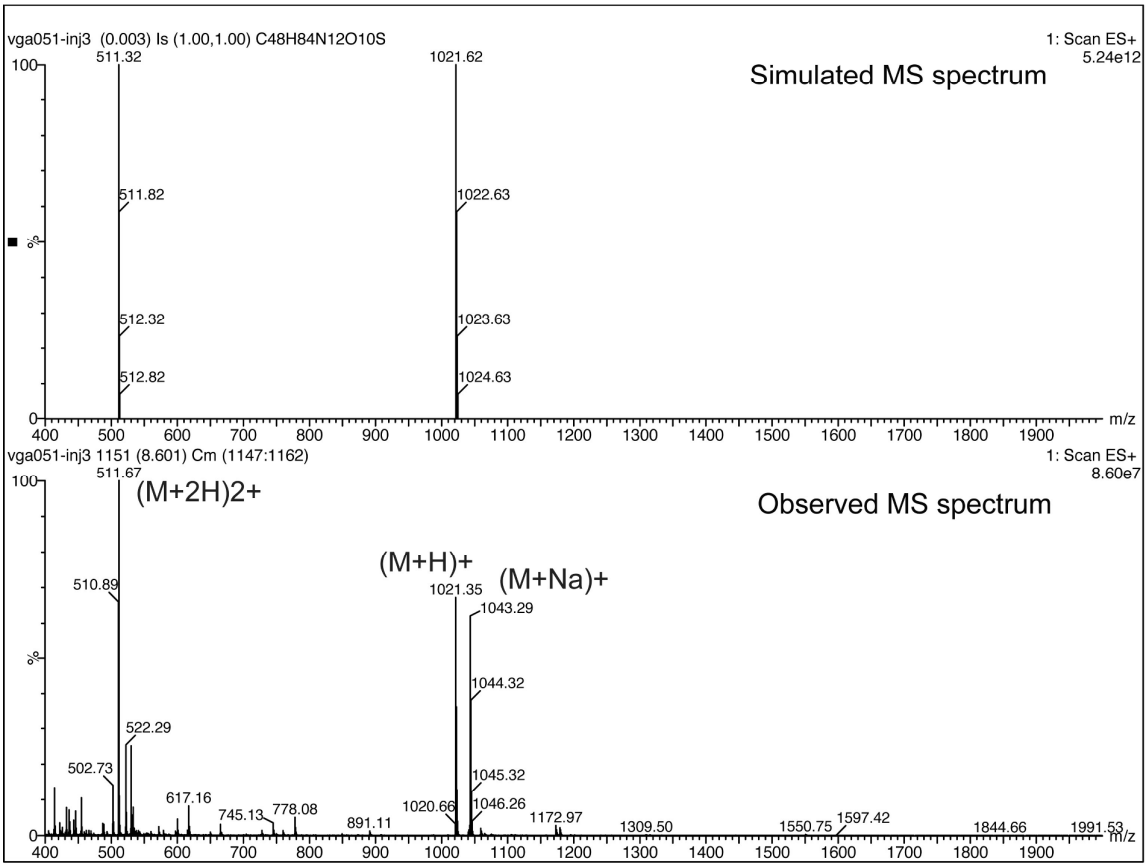

tBuOH
Water
18.6
6.8
18.6
11.6
2.9
5.3
1.0
1.0
1.9
2.0
5.3
ppm


17.06
21.20
21.43
22.02
22.27
22.79
22.94
24.52
25.03
26.92
26.98
39.81
39.87
40.41
40.57
50.90
52.34
52.77
53.04
53.65
54.25
56.22
60.72
tBuOH
tBuOH
116.22
128.13
131.33
155.33
HCOOH
170.73
171.48
171.63
172.97
173.68
174.37
175.14
175.72
177.99
8
16
24
32
40
48
56
64
72
80
88
96
104
112
120
128
136
144
152
160
168
176
184
192
Chemical Shift (ppm)

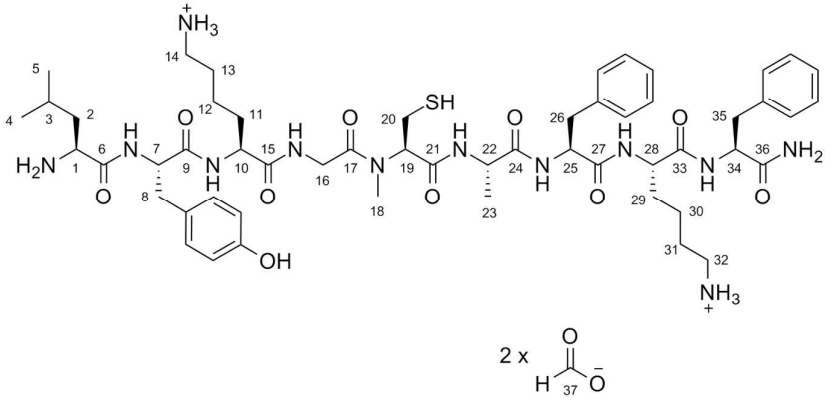
Analytical UPLC® chromatogram and associated MS (isotopic distribution) for purified peptide **P1-Daa2-P2**:



ESI-MS spectrum of the purified peptide **P1-Daa2-P2**:

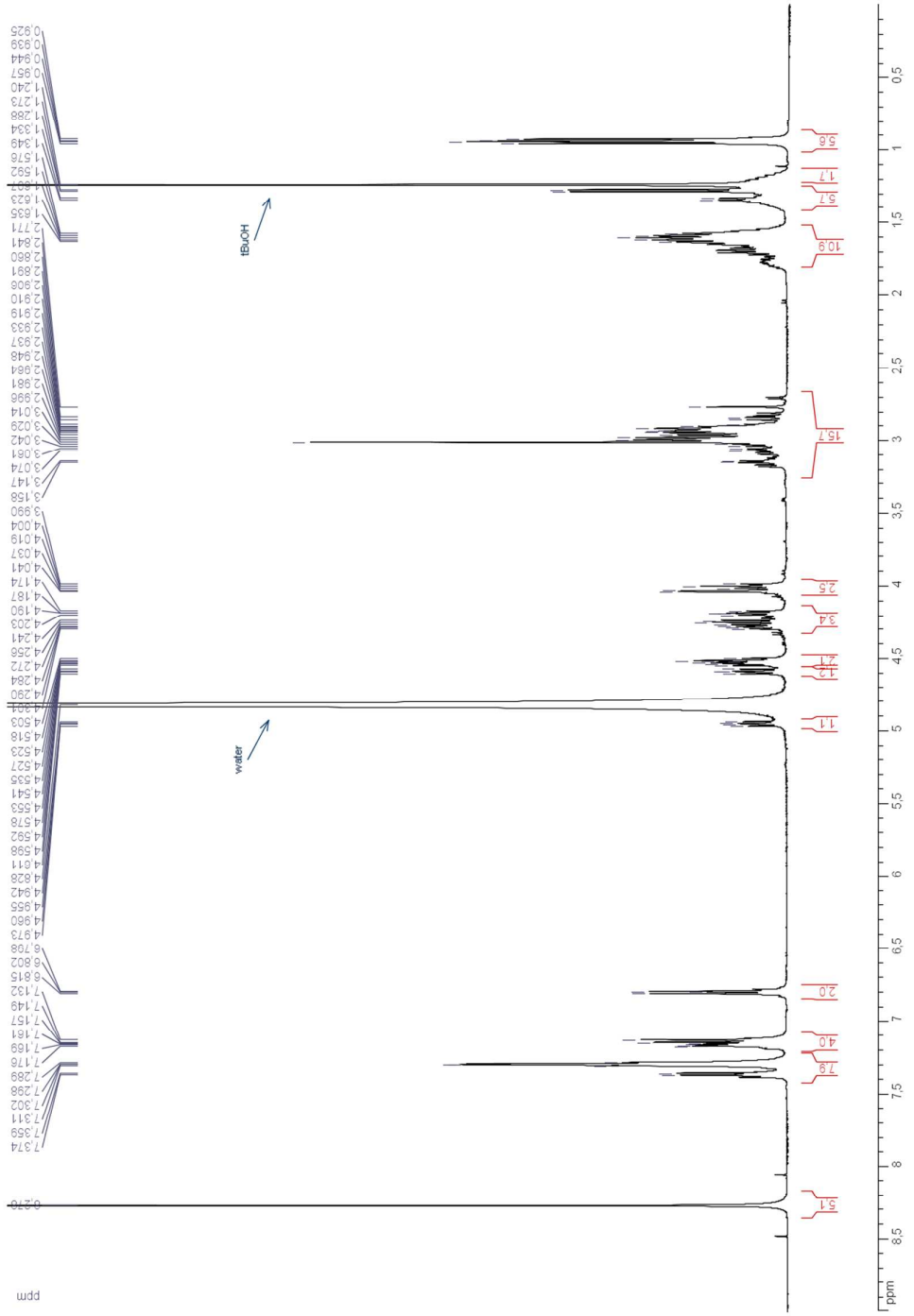


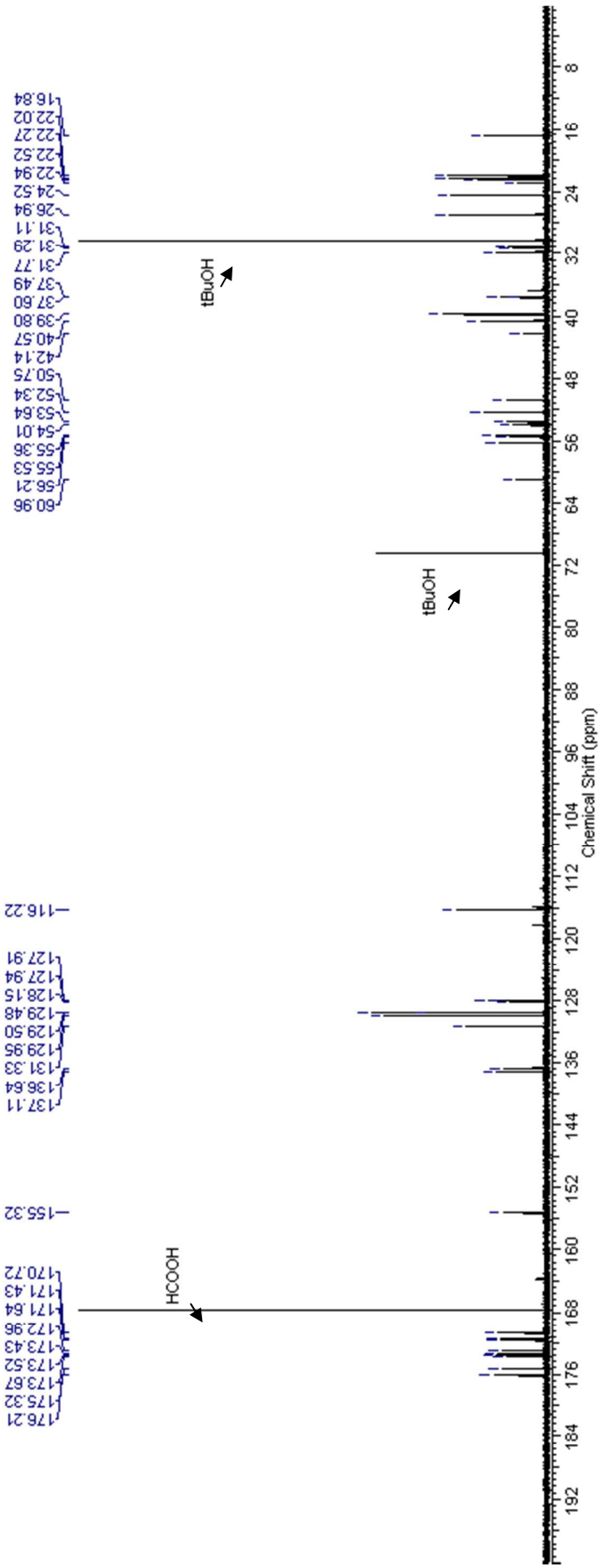
P1-Daa2-P3



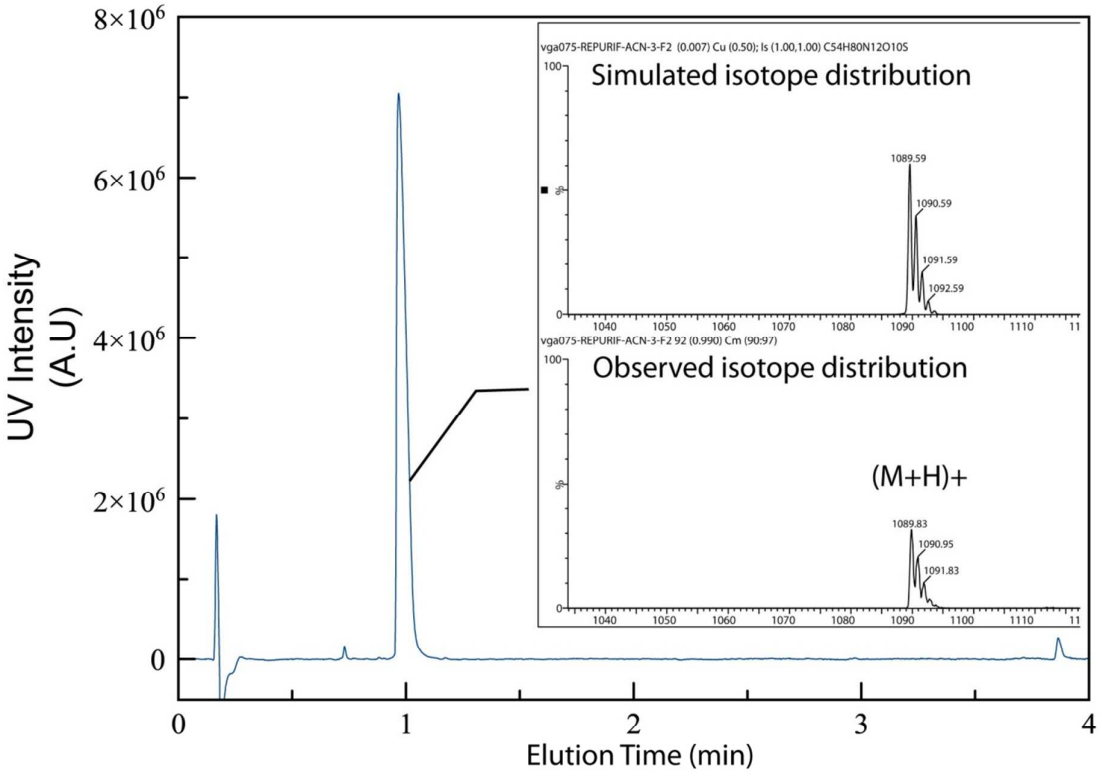
^{1}H NMR (500 MHz, D_2O): δ = 8.28 (s, excess formic acid to slow down oxidation), 7.41-7.22 (m, 8H, aromatic protons Phe), 7.21-7.08 (m, 4H, 2 aromatic protons Phe + 2 aromatic protons Tyr *J* = 8.6 Hz), 6.86-6.75 (m, 2H, aromatic protons Tyr *J* = 8.6 Hz), 4.96 (dd, 1H, *J* = 9.0 Hz, 6.3 Hz), 4.59 (dd, 1H, *J* = 9.9 Hz, 6.6 Hz), 4.57-4.48 (m, 2H), 4.39-4.14 (m, 3H), 4.11-3.94 (m, 2H), 3.23-2.68 (m, 16H), 1.85-1.47 (m, 10H), 1.46-1.11 (m, 8H), 1.03-0.86 (m, 6H, 3 H-C(4) + 3 H-C(5)).

^{13}C NMR (125 MHz, D_2O): δ = 176.21, 175.32, 173.67, 173.52, 173.43, 172.96, 171.64, 171.43, 170.72, 155.32, 137.11, 136.64, 131.33, 129.95, 129.91, 129.50, 129.48, 128.15, 127.94, 127.91, 116.22, 60.96, 56.21, 55.53, 55.36, 54.01, 53.64, 52.34, 50.75, 42.14, 40.57, 39.83, 39.80, 37.60, 37.49, 31.77, 31.29, 31.11, 26.94, 26.92, 24.52, 22.94, 22.54, 22.52, 22.27, 22.02, 16.84.

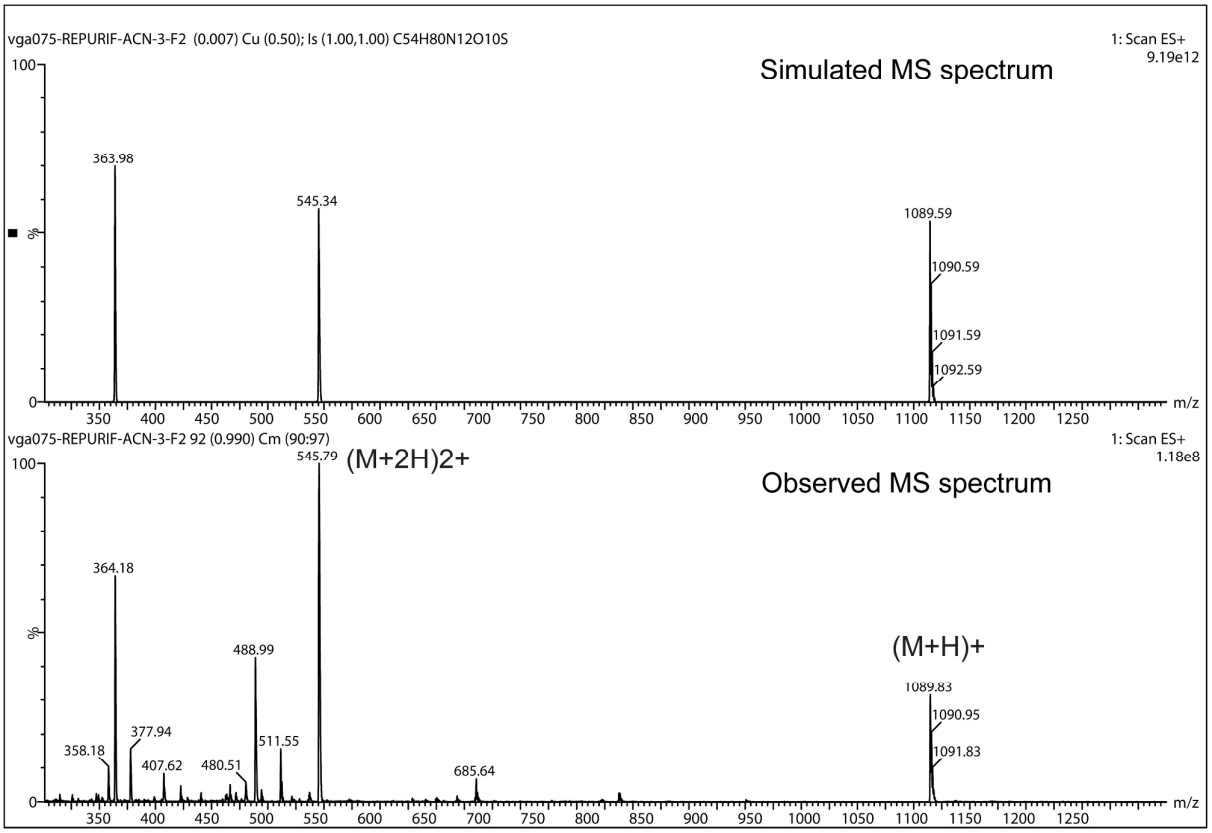



16.84
22.02
22.27
22.52
22.94
24.52
26.94
31.11
31.29
31.77
37.49
37.60
39.80
40.57
42.14
50.75
52.34
53.64
54.01
55.36
55.53
56.21
60.96
tBuOH
tBuOH
116.22
127.91
127.94
128.15
129.48
129.50
129.95
131.33
136.64
137.11
155.32
HCOOH
170.72
171.43
171.64
172.96
173.43
173.52
173.67
175.32
176.21
8
16
24
32
40
48
56
64
72
80
88
96
104
112
120
128
136
144
152
160
168
176
184
192
Chemical Shift (ppm)

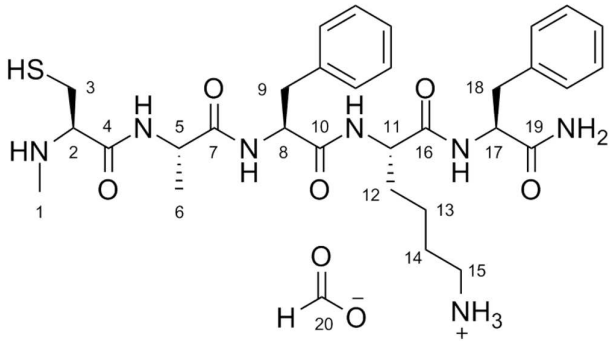
Analytical UPLC® chromatogram and associated MS (isotopic distribution) for purified peptide **P1-Daa2-P3**:



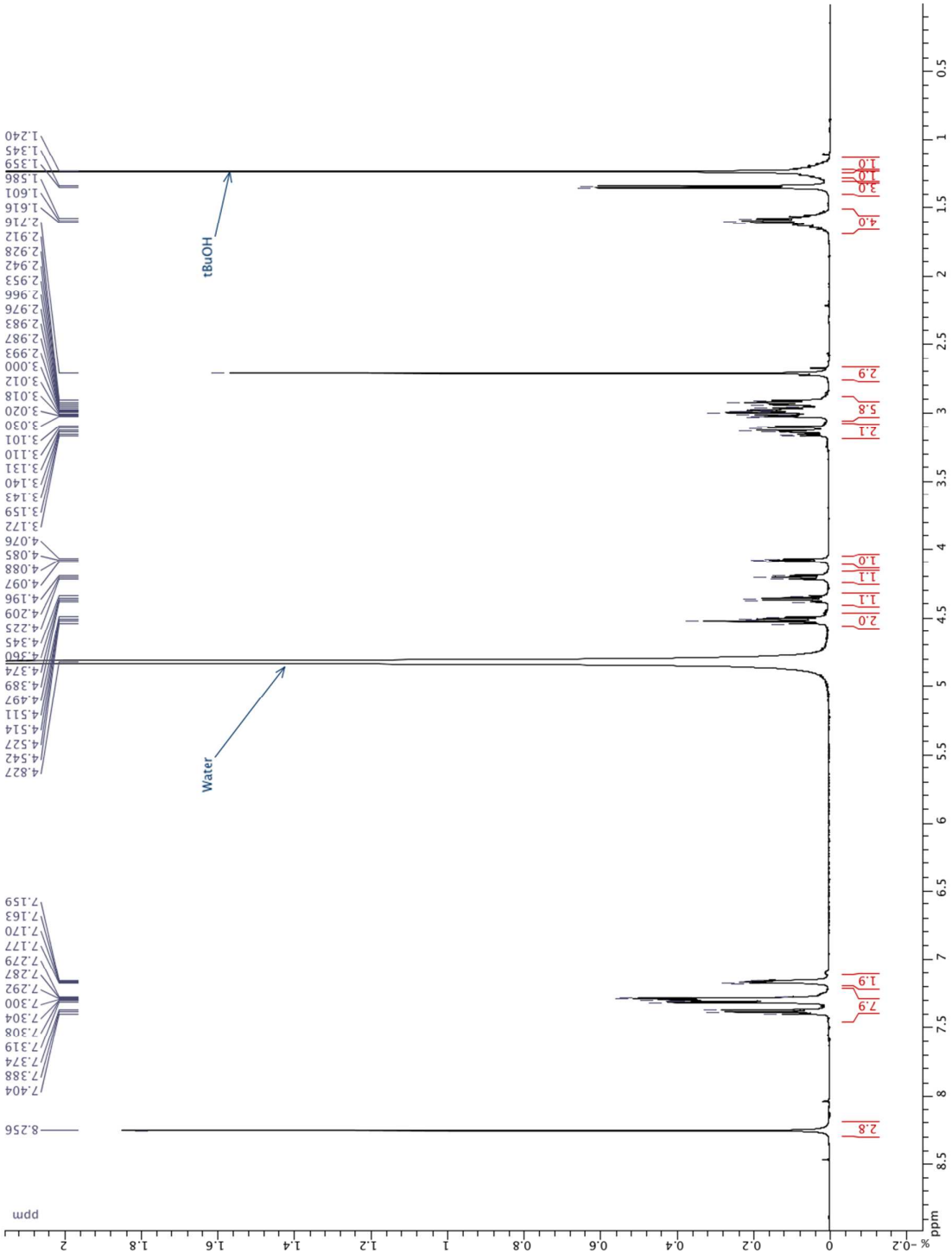
ESI-MS spectrum of the purified peptide **P1-Daa2-P3**:

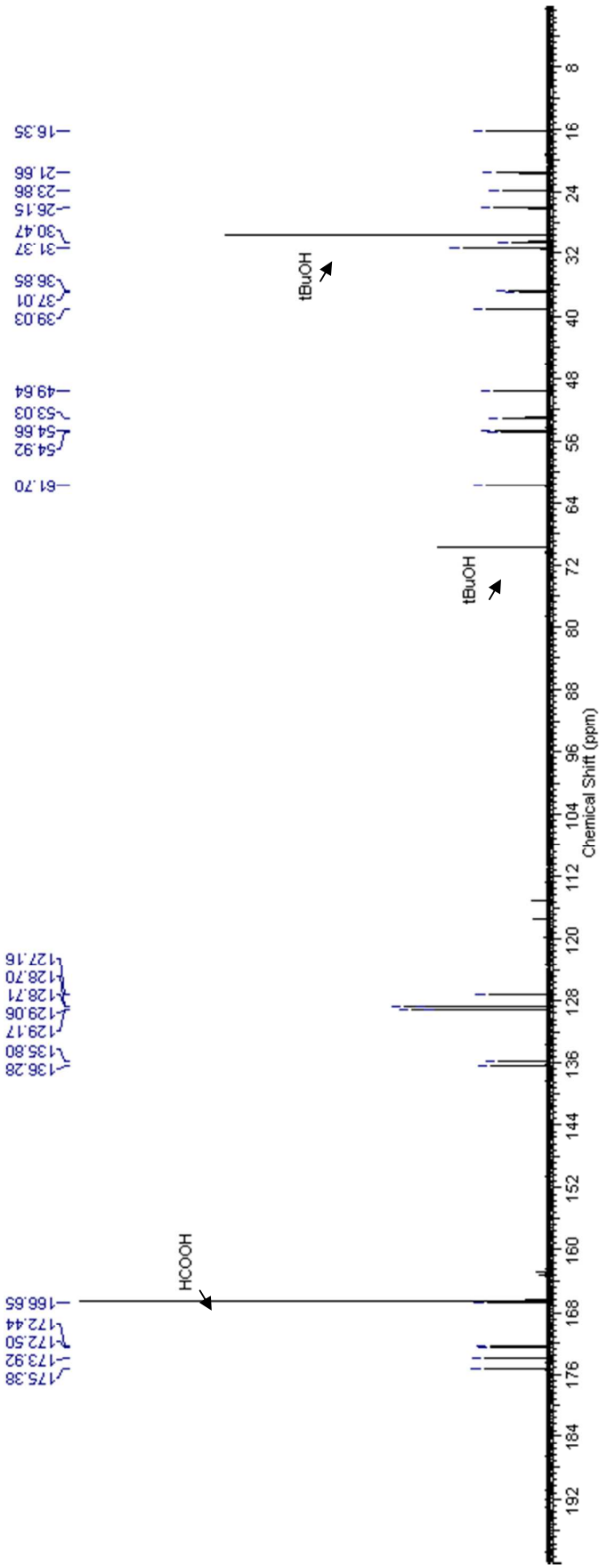


Daa2-P3

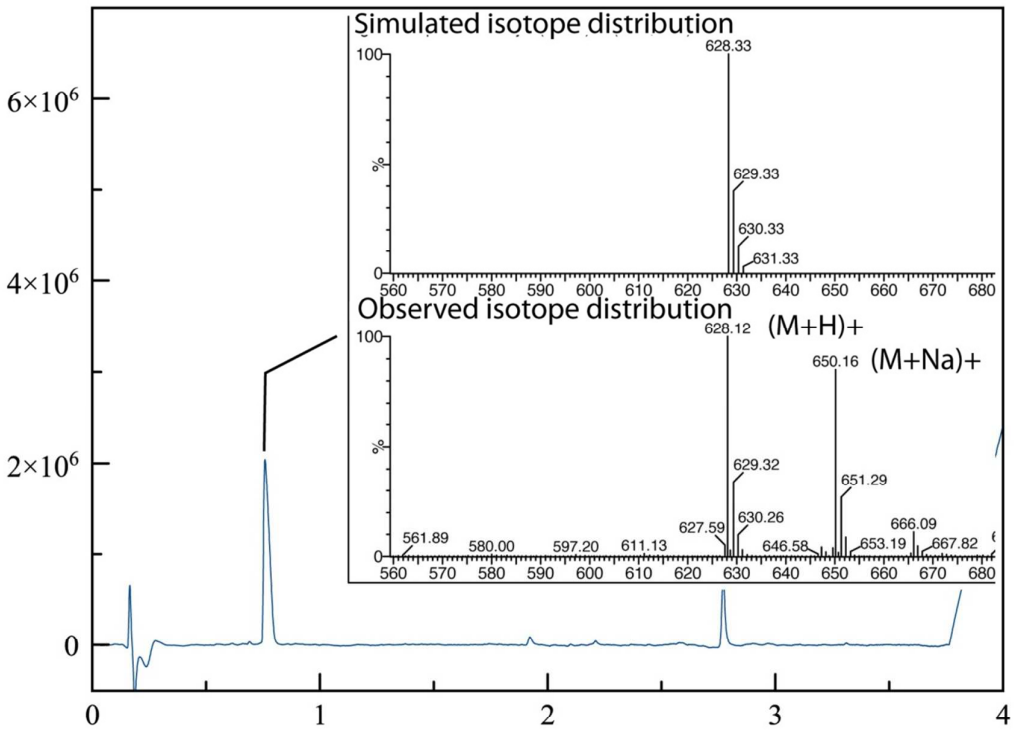


^{1}H NMR (500 MHz, D_2O): δ = 8.25 (s, 3H, excess formic acid to slow down oxidation), 7.43-7.13 (m, 10H, aromatic protons Phe), 4.56-4.49 (m, 2H, H-C(8) + H-C(17)), 4.36 (q, 1H, H-C(5) *J* = 7.0 Hz), 4.21 (dd, 1H, H-C(11), *J* = 7.5 Hz, 6.6 Hz), 4.09 (dd, 1H, H-C(2) *J* = 6.2 Hz, 4.6 Hz), 3.19-3.08 (m, 2H), 3.05-2.89 (m, 6H), 2.72 (s, 3H, 3 H-C(1)), 1.68 -1.52 (m, 4H), 1.35 (d, 3H, 3 H-C(6) *J* = 7.0 Hz), 1.30-0.15 (m, 2H).
^{13}C NMR (125 MHz, D_2O): 172.50, 172.44, 166.65, 136.28, 135.80, 129.17, 129.06, 128.71, 128.70, 127.16, 61.70, 54.92, 54.66, 53.03, 49.64, 39.03, 37.01, 36.85, 31.37, 30.47, 26.15, 23.86, 21.66, 16.35.

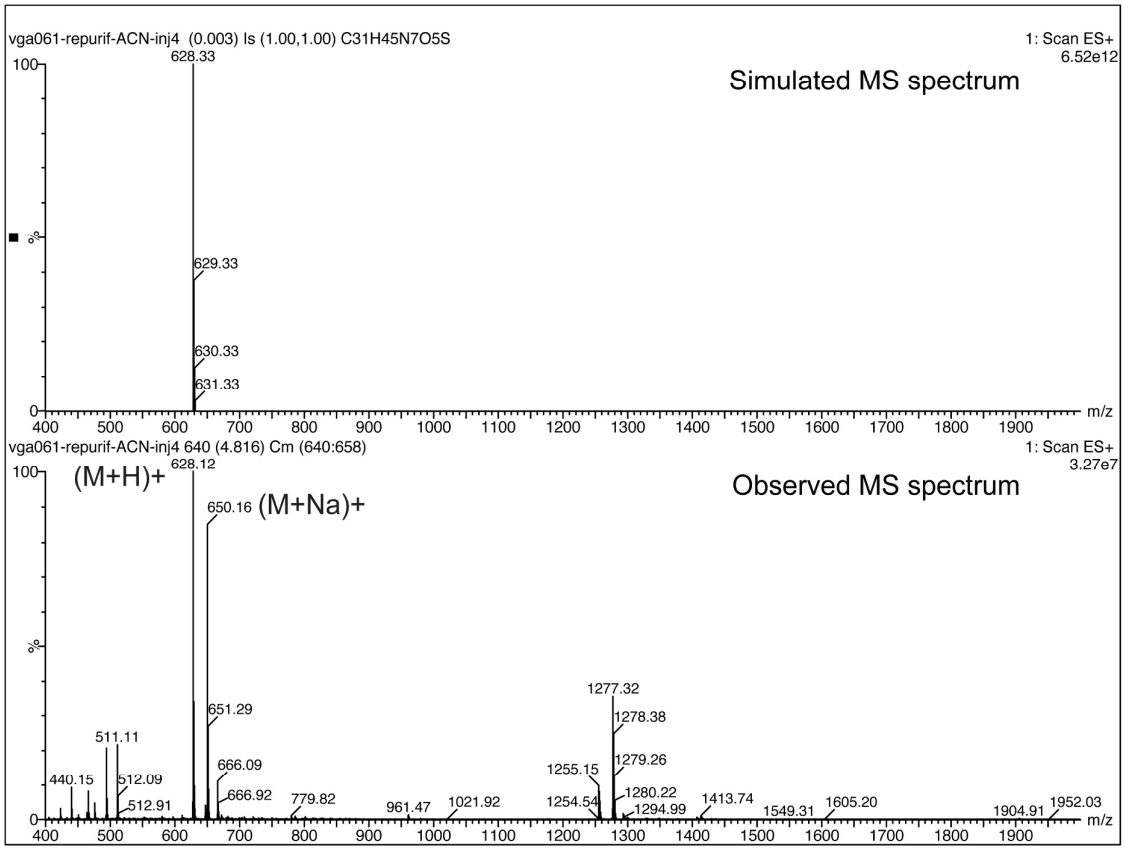

tBuOH
Water


16.35
21.66
23.86
26.15
30.47
31.37
36.85
37.01
39.03
49.64
53.03
54.66
54.92
61.70
127.16
128.70
128.71
129.06
129.17
135.80
136.28
166.65
172.44
172.50
173.92
175.38
tBuOH
tBuOH
HCOOH
8
16
24
32
40
48
56
64
72
80
88
96
104
112
120
128
136
144
152
160
168
176
184
192
Chemical Shift (ppm)

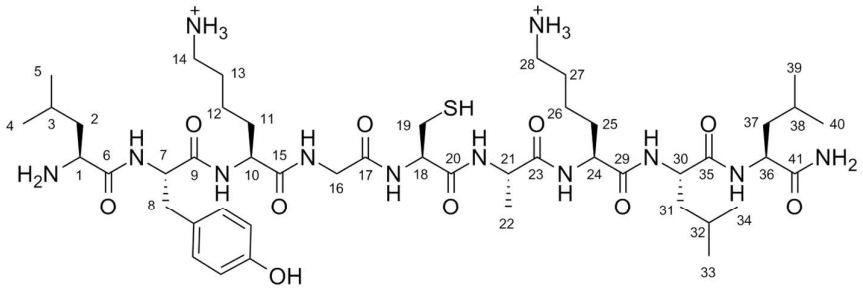
Analytical UPLC® chromatogram and associated MS (isotopic distribution) for purified peptide **Daa2-P3**:



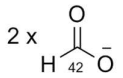
ESI-MS spectrum of the purified peptide **Daa2-P3**:



P1-Cys-P2

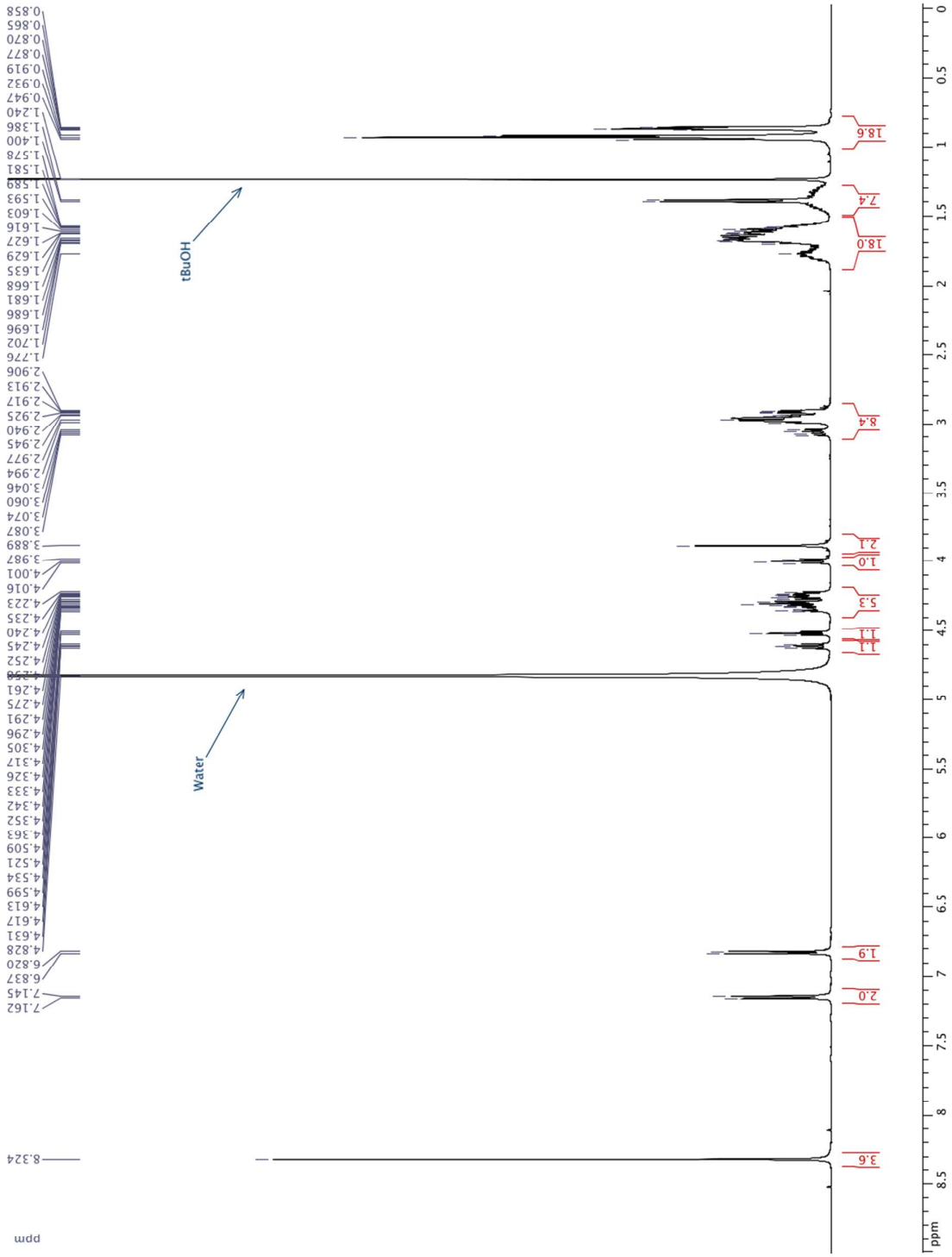


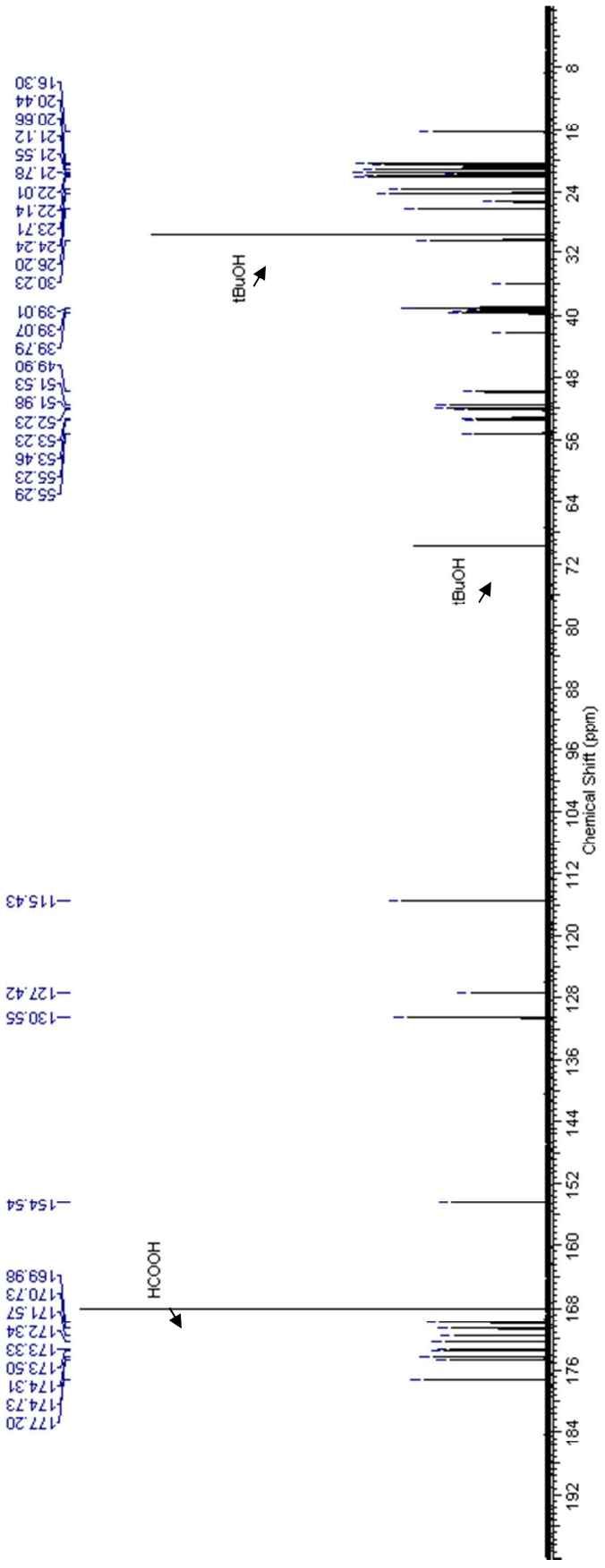
2 x



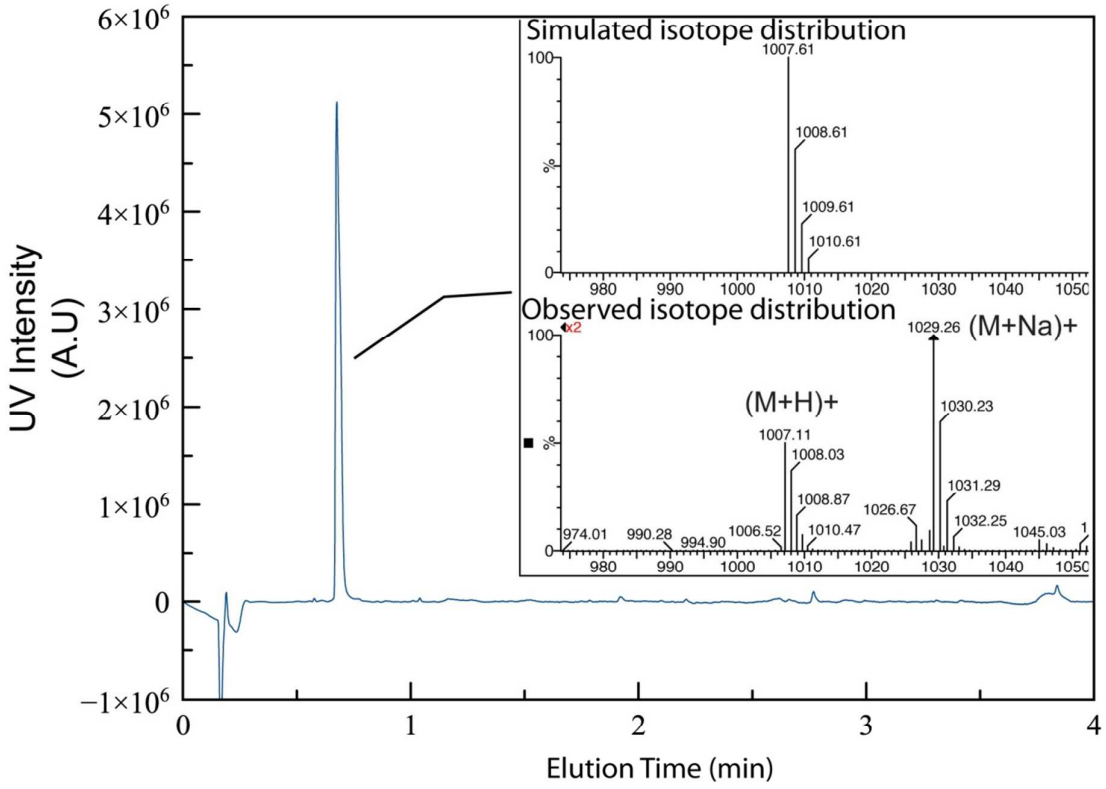
^{1}H NMR (500 MHz, D_2O): δ = 8.32 (s, excess formic acid to slow down oxidation), 7.15 (d, 2H aromatic protons Tyr, *J* = 8.0 Hz), 6.83 (d, 2H, aromatic protons Tyr, *J* = 8.0 Hz), 4.62 (dd, 1H, H-C(7) Tyr *J* = 8.9 Hz, 7.2 Hz), 4.52 (t, 1H, *J* = 6.5 Hz), 4.38-4.20 (m, 5H), 4.00 (t, 1H, *J* = 7.4 Hz), 3.89 (s, 2H, 2 H-C(16)), 3.10-2.86 (m, 8H), 1.86-1.52 (m, 18H), 1.50-1.27 (m, 7H), 0.97-0.82 (m 18H, 3 H-C(4) + 3 H-C(5) + 3 H-C(39) + 3 H-C(40)).

^{13}C NMR (125 MHz, D_2O): δ = 177.20, 174.73, 174.31, 173.50, 173.33, 172.34, 171.57, 170.73, 169.98, 154.54, 130.55, 127.42, 115.43, 55.29, 55.23, 53.46, 53.23, 52.23, 51.98, 51.53, 49.90, 42.19, 39.79, 39.62, 39.35, 39.07, 39.01, 35, 95, 30.23, 26.20, 26.18, 25.33, 24.24, 24.23, 23.71, 22.14, 22.01, 21.98, 21.78, 21.55, 21.12, 20.66, 20.44, 16.30.

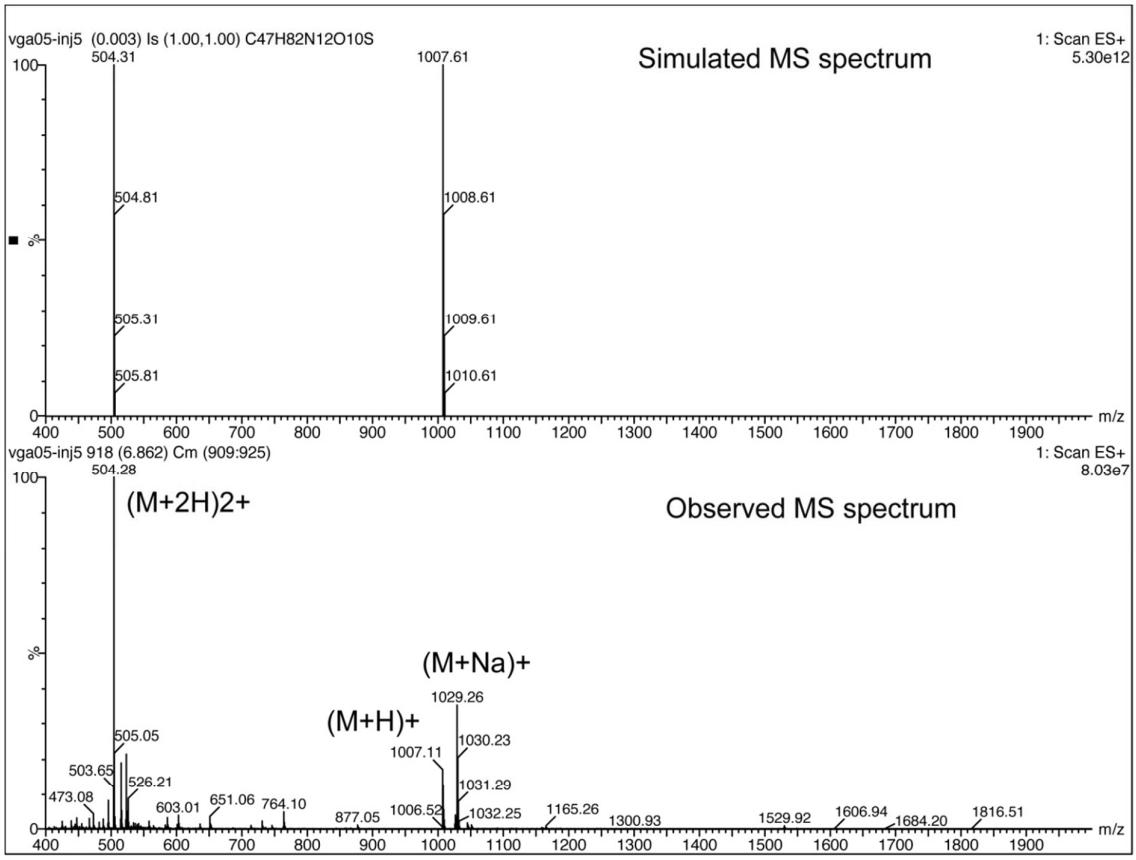

tBuOH
Water
18.6
7.4
18.0
8.4
2.1
1.0
5.3
1.1
1.1
1.9
2.0
3.6
ppm


HCOOH
tBuOH
tBuOH
Chemical Shift (ppm)

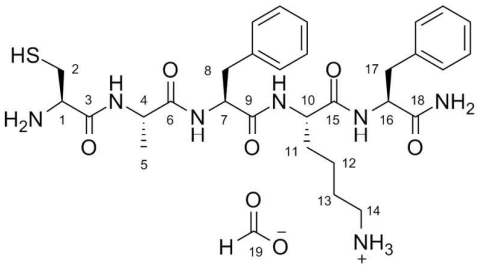
Analytical UPLC® chromatogram and associated MS (isotopic distribution) for purified peptide **P1-Cys-P2**:



ESI-MS spectrum of the purified peptide **P1-Cys-P2**:

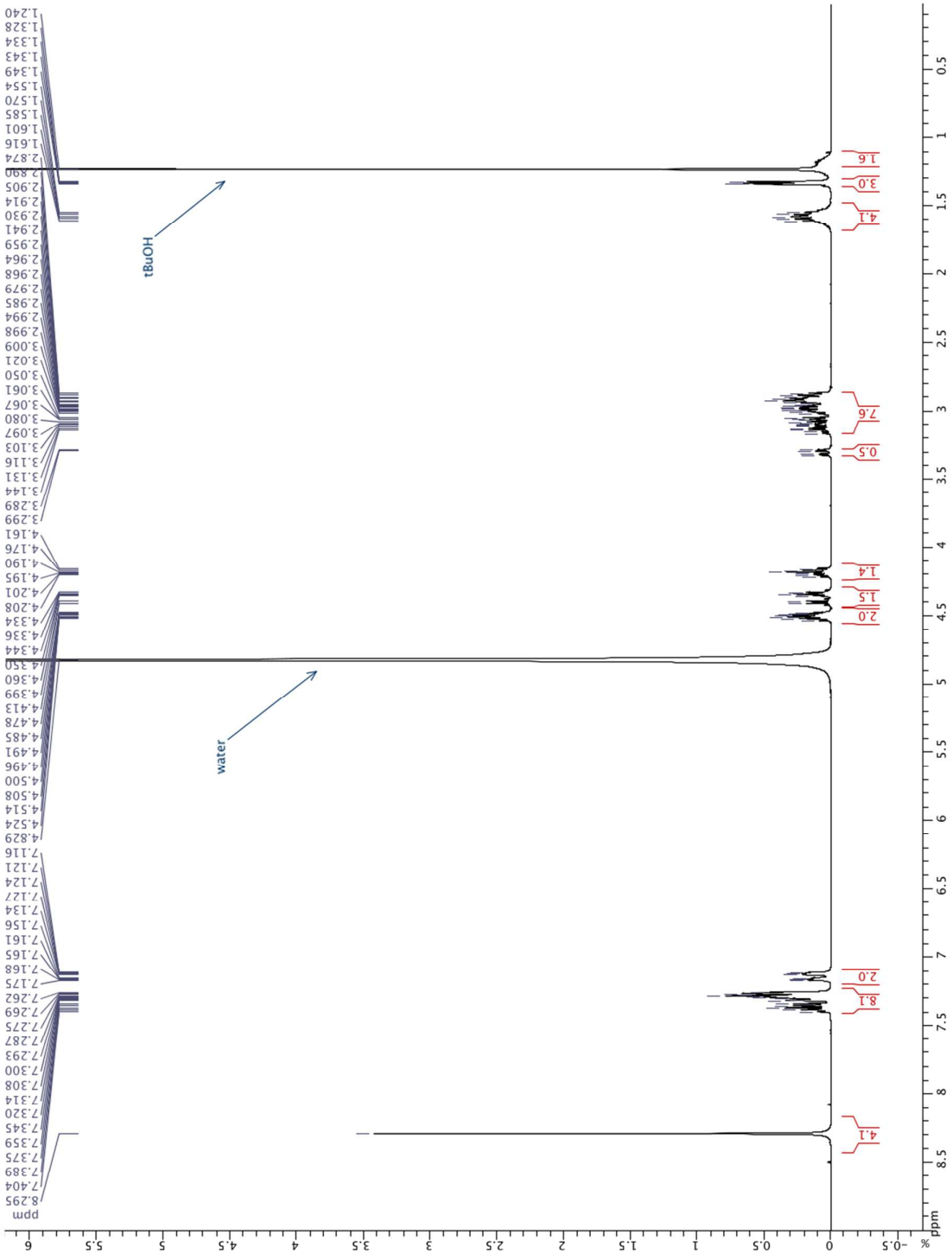


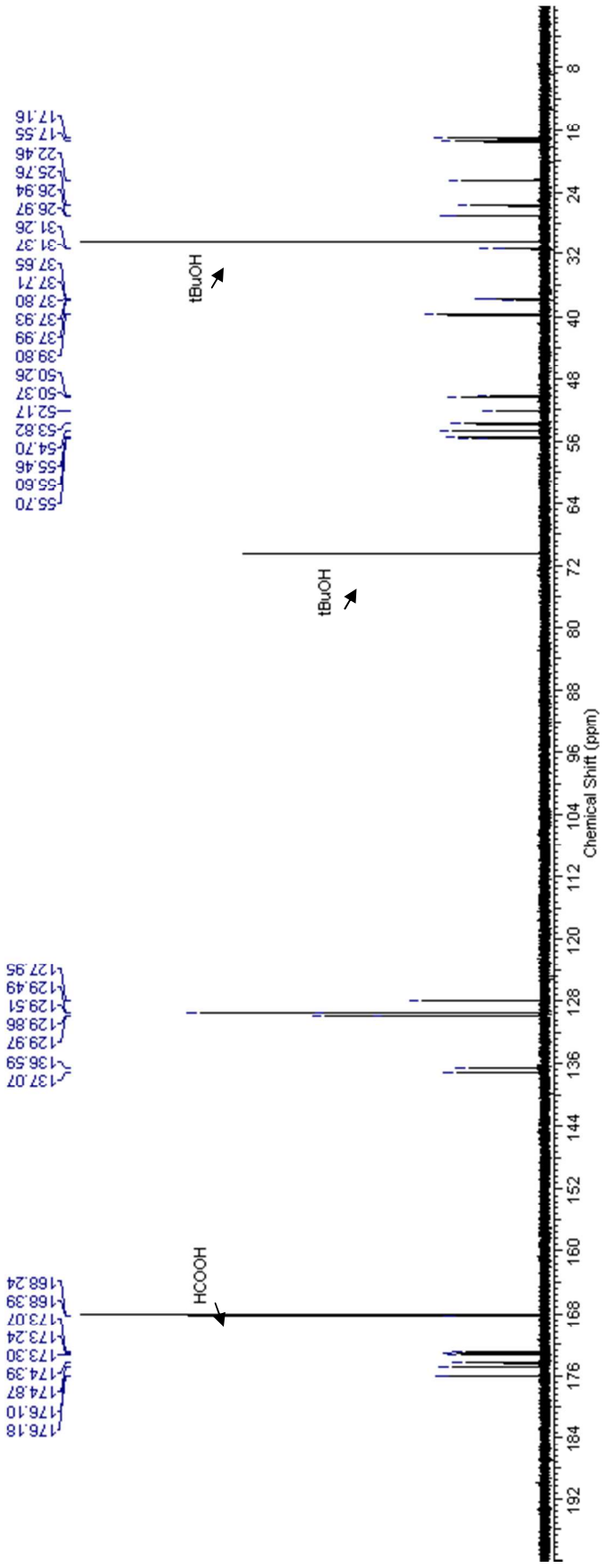
Cys-P3



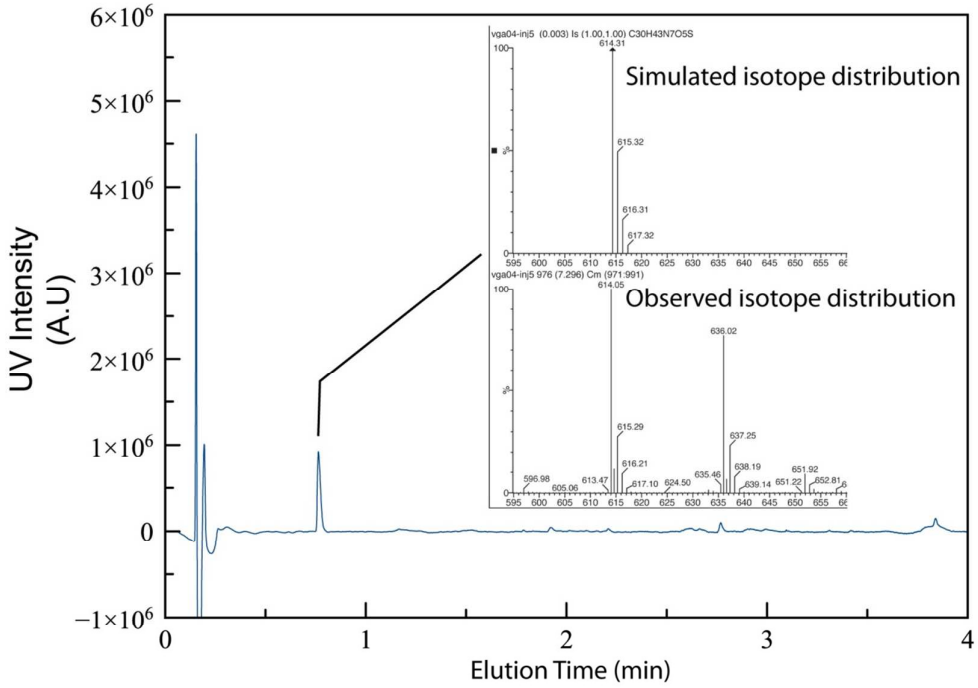
^{1}H NMR (500 MHz, D_2O): δ = 8.29 (s, excess formic acid to slow down oxidation), 7.43-7.10 (m, 10H, aromatic protons Phe), 4.56-4.45 (m, 2H, H-C(7) + H-C(16)), 4.44-4.31 (m, 1H), 4.25-4.13 (m, 1H), 3.36-3.27 (m, 0.5H), 3.19-2.85 (m, 7.5H), 1.68-1.49 (m, 4H), 1.37-1.31 (m, 3H, 3 H-C(5)), 1.28-1.11 (m, 2H).
^{13}C NMR (125 MHz, D_2O): δ = 173.20, 173.07, 168.39, 168.24, 137.07, 137.05, 136.61, 136.59, 129.95, 129.90, 129.86, 129.51, 129.49, 129.47, 127.95, 55.70, 55.60, 55.46, 55.44, 54.70, 53.84, 53.82, 52.17, 50.37, 50.26, 39.83, 39.80, 37.99, 37.93, 37.80, 37.71, 37.65, 31.37, 31.26, 26.97, 26.94, 25.76, 22.46, 22.43, 17.55, 17.16.

To slow down oxidation of this peptide during the NMR measurement we degassed the D_2O + *t*BuOH mixture and added an excess of formic acid to the sample. This didn't prevent oxidation completely and this compound is observed as a mixture of the reduced cysteine and disulfide peptides.

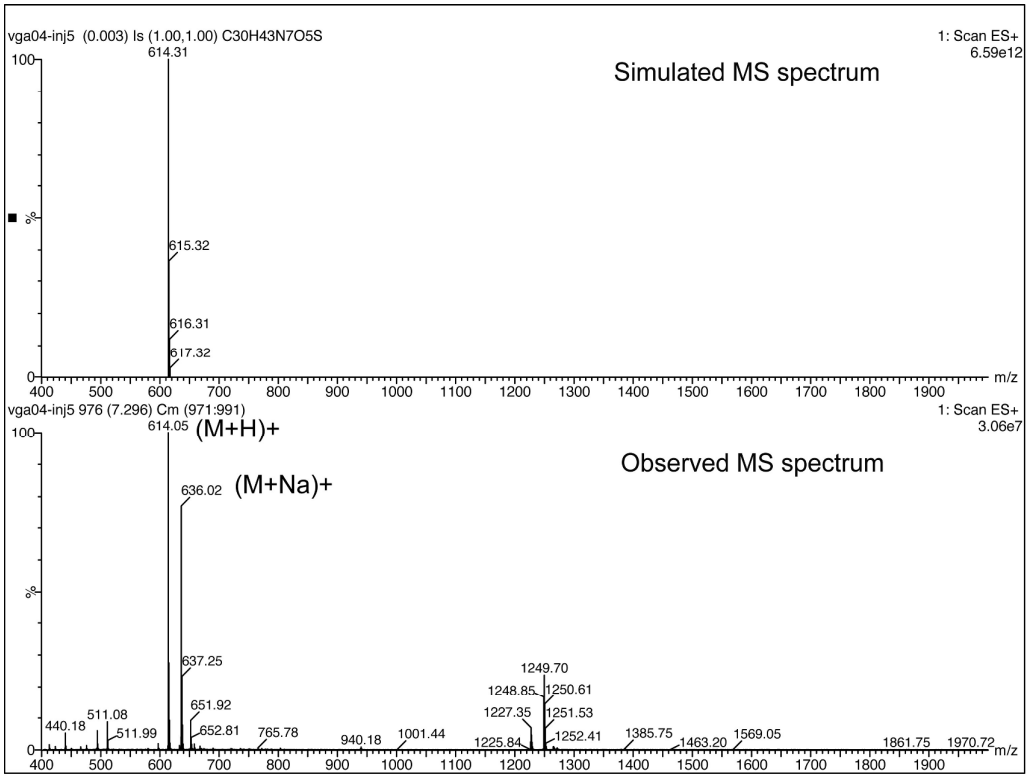

tBuOH
water
4.1
3.0
2.0
8.1
2.0
7.6
0.5
1.4
1.5
2.0
4.1
1.6
ppm


Chemical Shift (ppm)
tBuOH
tBuOH
HCOOH

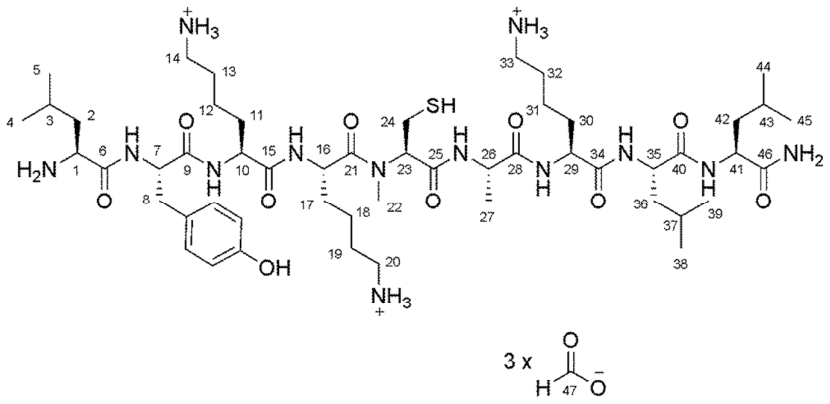
Analytical UPLC® chromatogram and associated MS (isotopic distribution) for purified peptide **Cys-P3**:



ESI-MS spectrum of the purified peptide **Cys-P3**:

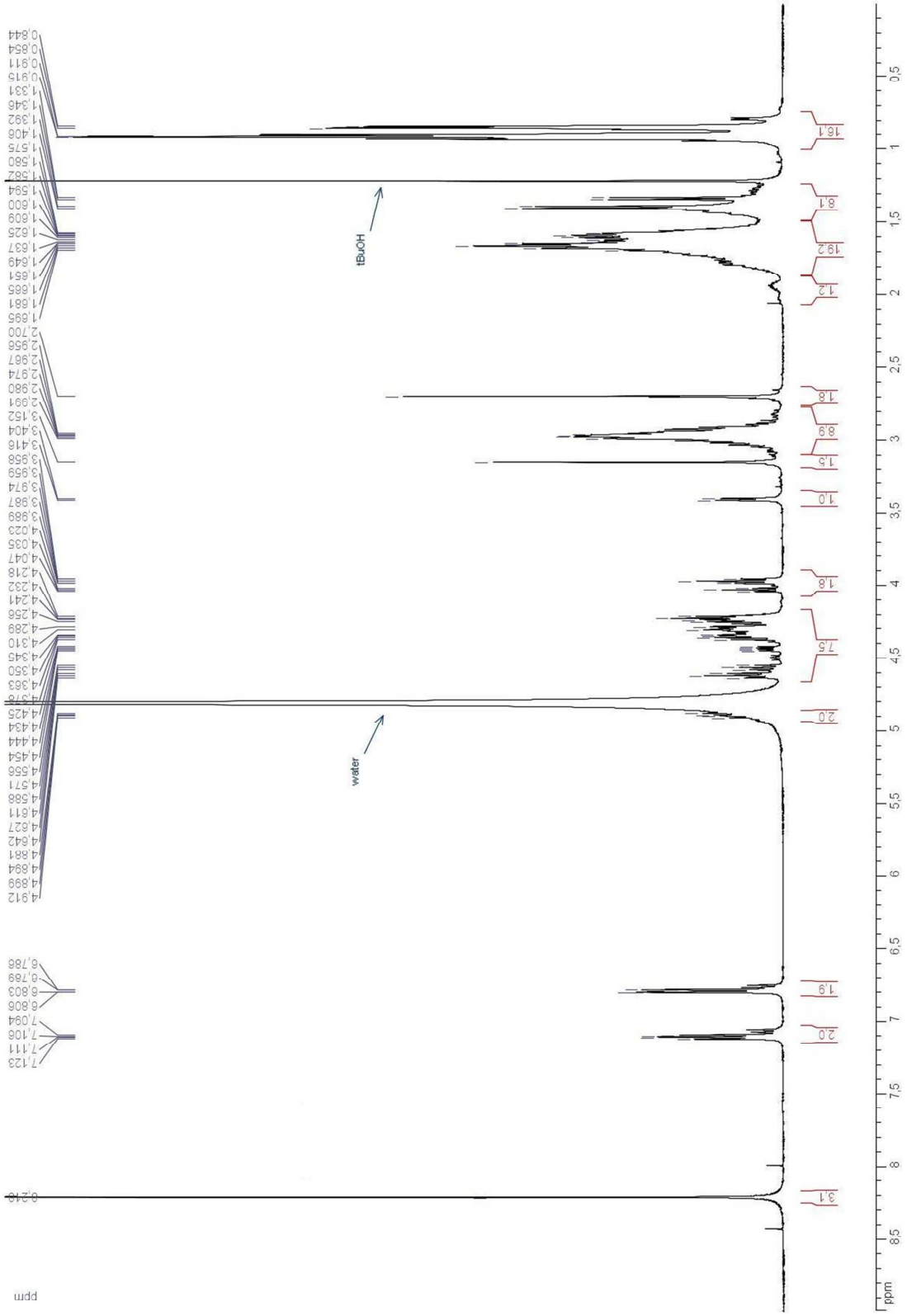


P1’-Daa2-P2



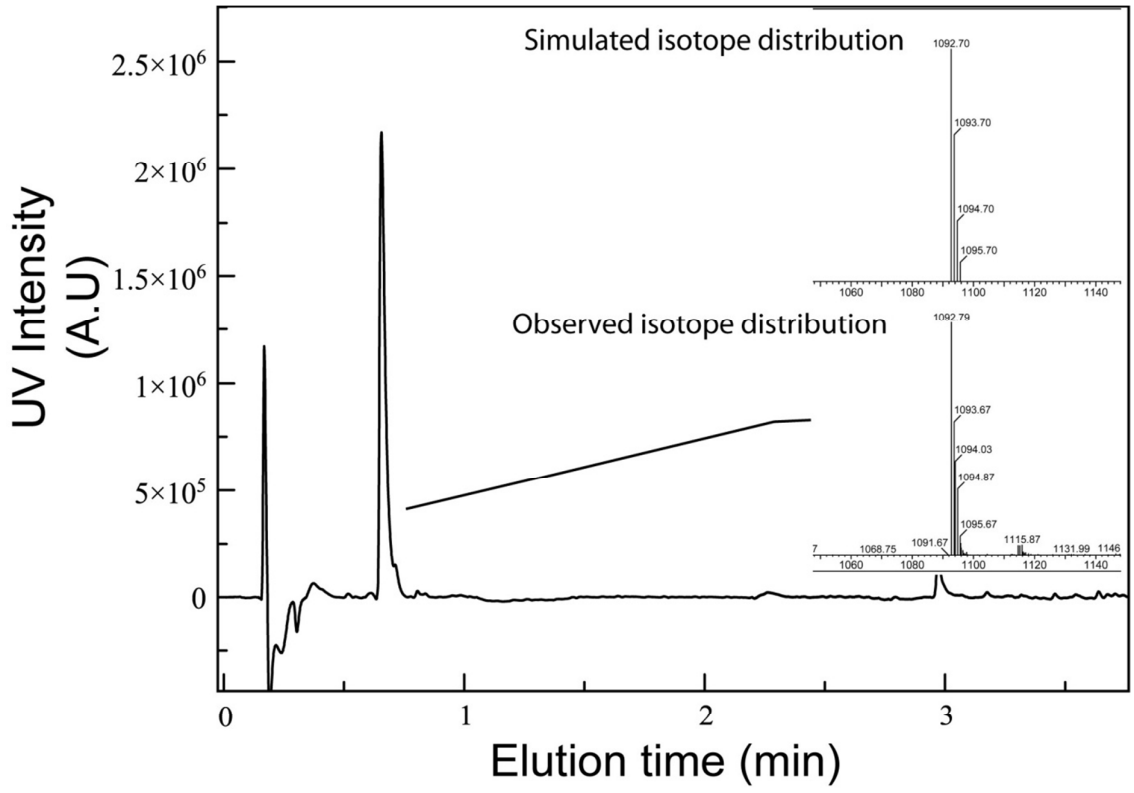
^{1}H NMR (500 MHz, D_2O): δ = 8.22 (s, 3H, 3 H-C(47)), 7.14-7.04 (m, 2H, aromatic protons Tyr), 6.83-6.73 (m, 2H, aromatic protons Tyr), 4.94-4.86 (m, 2H), 4.67-4.18 (m, 7.5H), 4.08-3.90 (m, 2H), 3.41 (d, 1H, *J* = 6.1 Hz), 3.20-3.10 (m, 1.5H), 3.10-2.78 (m, 9H), 2.77-2.64 (m, 2H), 2.07-1.87 (m, 1H), 1.87-1.50 (m, 19H), 1.49-1.24 (m, 8H), 1.00-0.74 (m, 16H).

^{13}C NMR (125 MHz, D_2O): δ = 177.96, 177.89, 175.58, 175.10, 174.95, 174.50, 174.29, 174.20, 174.16, 173.01, 172.67, 171.35, 170.78, 170.76, 167.00, 163.87, 163.59, 155.28, 155.27, 131.20, 131.17, 120.53, 118.21, 116.18, 116.11, 115.89, 113.57, 52.98, 52.75, 52.33, 40.57, 40.47, 40.44, 39.86, 39.81, 32.50, 27.28, 27.13, 27.01, 25.06, 25.02, 24.49, 22.96, 22.92, 22.89, 22.86, 22.83, 22.78, 22.59, 22.37, 22.35, 21.89, 21.60, 21.47, 21.32, 21.22, 17.49, 17.21.

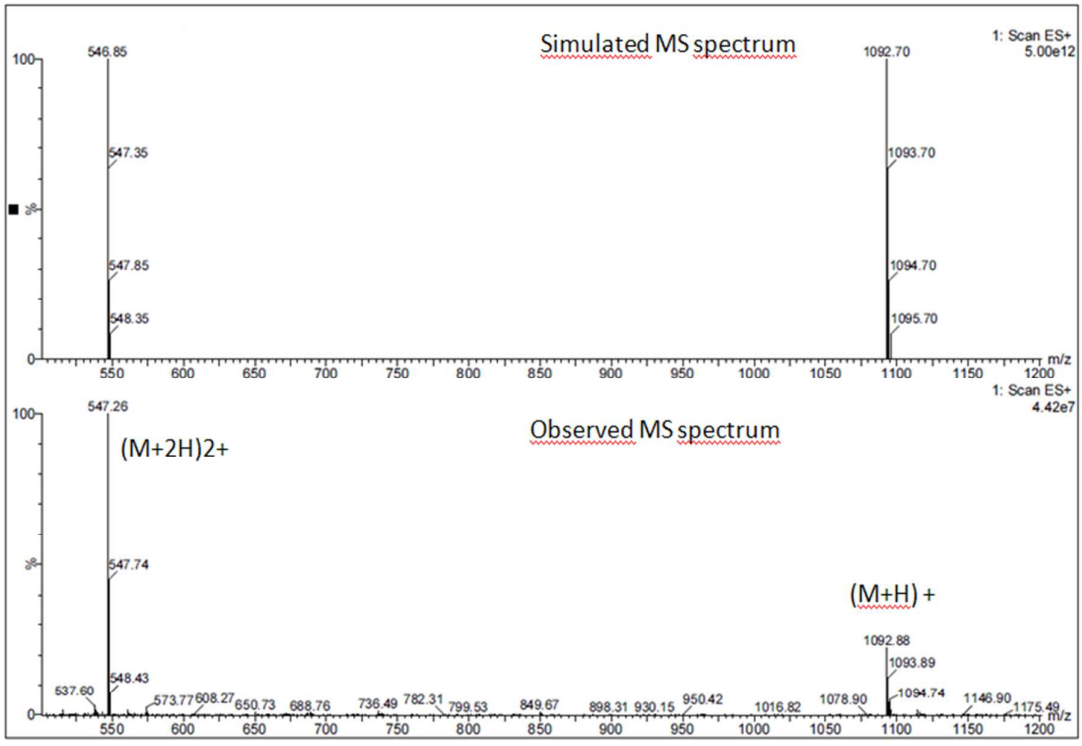

tBuOH
water
ppm

tBuOH
tBuOH
HCOOH
17.21
21.60
21.89
22.35
22.37
22.78
22.92
22.96
24.49
25.02
27.01
39.81
39.86
40.44
40.57
52.33
52.75
52.98
113.57
115.89
116.11
116.18
118.21
120.53
131.17
155.27
155.28
163.59
163.87
167.00
170.78
172.67
173.01
174.16
174.29
174.50
174.95
175.10
177.89
177.96

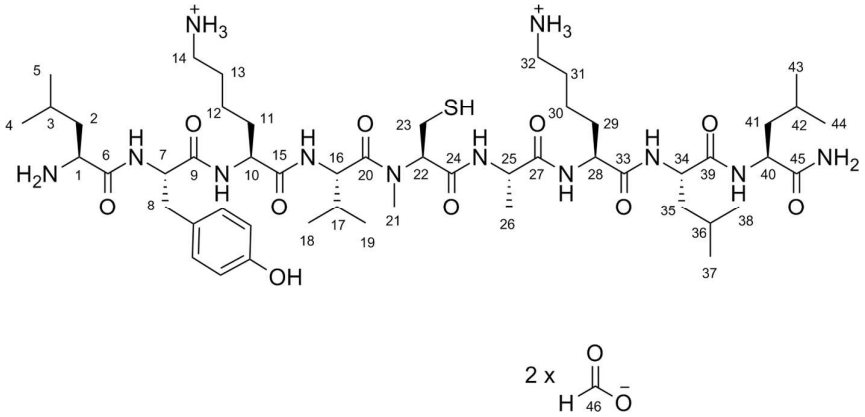
Analytical UPLC® chromatogram and associated MS (isotopic distribution) for purified peptide **P1'-Daa2-P2**:



ESI-MS spectrum of the purified peptide **P1'-Daa2-P2**:

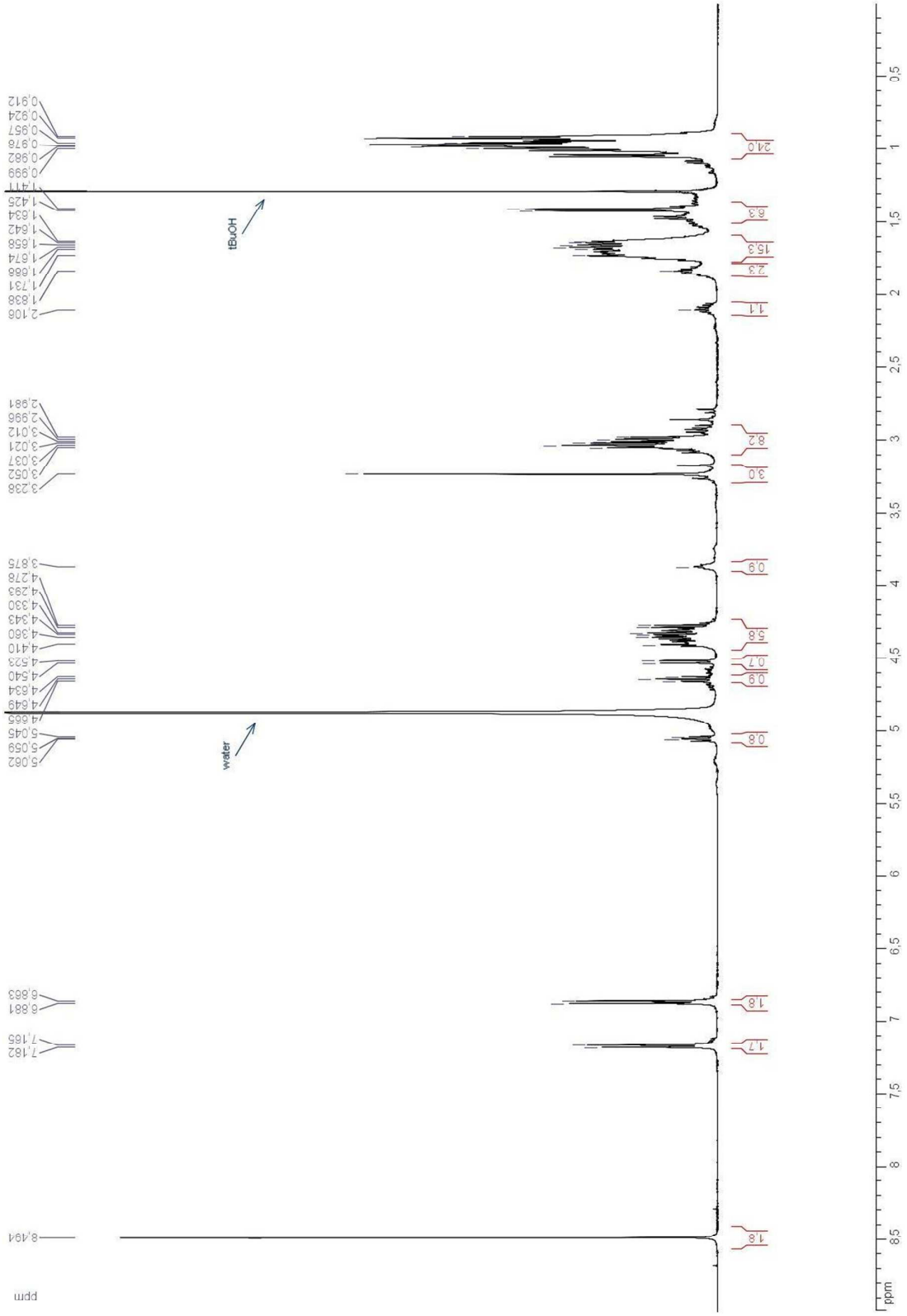


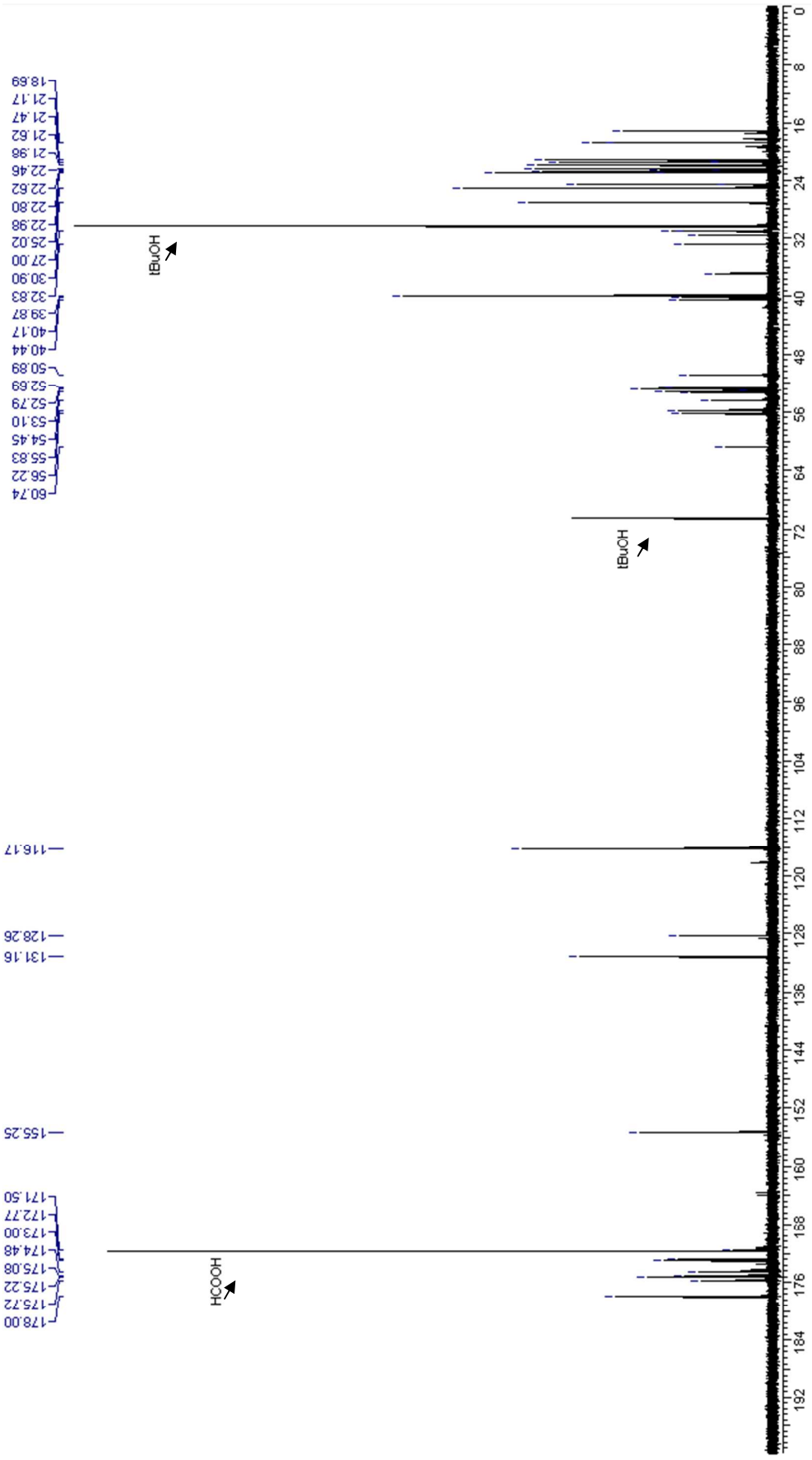
P1''-Daa2-P2



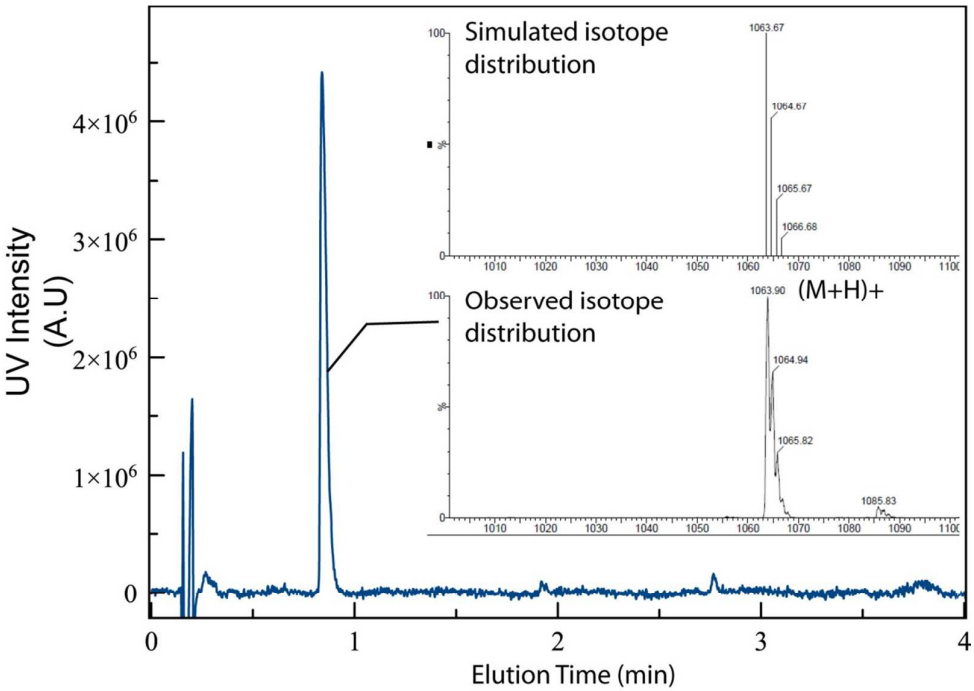
^{1}H NMR (500 MHz, D_2O): δ = 8.49 (s, 2H, 2 H-C(46)), 7.17 (d, 2H, aromatic protons Tyr, *J* = 8.6 Hz), 6.87 (d, 2H, aromatic protons Tyr, *J* = 8.6 Hz), 5.06 (dd, 1H, *J* = 8.6 Hz, 6.9 Hz), 4.67-4.62 (m, 1H), 4.53 (d, 1H, *J* = 8.5 Hz), 4.45-4.25 (m, 6H), 3.88 (t, 1H, *J* = 6.7 Hz), 3.24 (s, 3H, 3 H-C(21)), 3.10-2.91 (m, 8H), 2.14-2.05 (m, 1H), 1.87-1.79 (m, 2H), 1.78-1.59 (m, 15H), 1.51-1.36 (m, 6H, 3 H-C(26)), 1.08-0.89 (m, 24H, 3 H-C(4) + 3 H-C(5) + 3 H-C(18) + 3 H-C(19) + 3 H-C(37) + 3 H-C(38) + 3 H-C(43) + 3 H-C(44)).

^{13}C NMR (125 MHz, D_2O): δ = 178.00, 175.72, 175.22, 175.08, 174.48, 173.00, 172.77, 171.50, 155.25, 131.16, 128.26, 116.17, 60.74, 56.22, 55.83, 54.45, 53.10, 52.79, 52.69, 50.89, 40.44, 40.17, 39.87, 32.83, 30.90, 27.00, 25.02, 22.98, 22.80, 22.62, 22.46, 21.98, 21.62, 21.47, 21.17, 18.69.

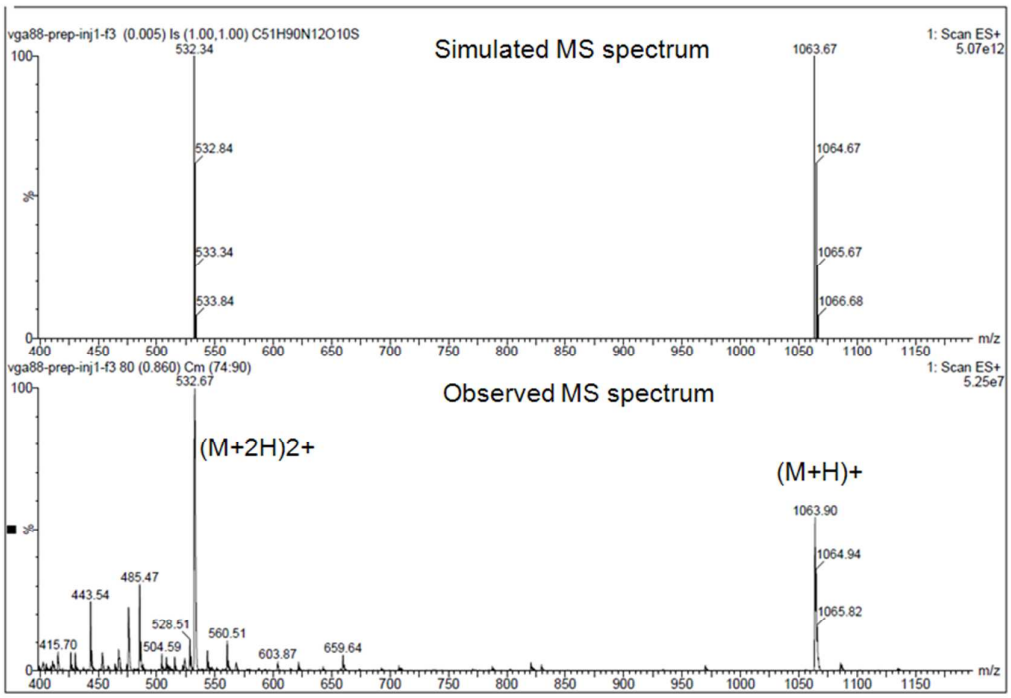

tBuOH
water
ppm


tBuOH
tBuOH
HCOOH
18.69
21.17
21.47
21.62
21.96
22.46
22.62
22.80
22.98
25.02
27.00
30.90
32.83
39.87
40.17
40.44
50.89
52.69
52.79
53.10
54.45
55.83
56.22
60.74
116.17
128.26
131.16
155.25
171.50
172.77
173.00
174.48
175.08
175.22
175.72
178.00

Analytical UPLC® chromatogram and associated MS (isotopic distribution) for purified peptide **P1''-Daa2-P2**:



ESI-MS spectrum of the purified peptide **P1''-Daa2-P2**:



4. Thioester intermediates characterization

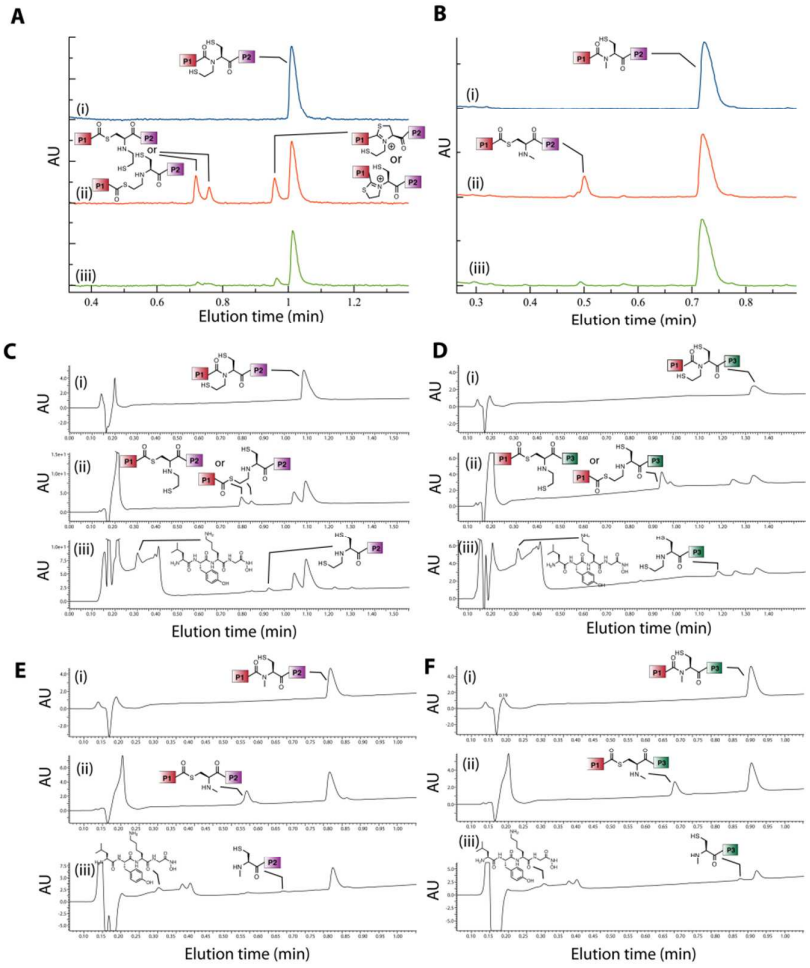


Figure S10. (a) Reversed phase chromatograms (UPLC®) recorded with UV and MS detection of **P1-Daa1-P2** (320 μM), at (i) under reductive condition in a solution of TCEP (28 mM) at pH 6; (ii) after 20 min in the same solution + 2.5% of TFA; (iii) after subsequent neutralization to pH 7 using $(NH_4)_2CO_3$ for 5 min. (b) Reversed phase chromatograms (UPLC®) recorded with UV and MS detection of **P1-Daa2-P2** (320 μM), at (i) under reductive condition in a solution of TCEP (28 mM) at pH 6; (ii) after 20 min in the same solution + 2.5 % of TFA; (iii) after subsequent neutralization to pH 7 using $(NH_4)_2CO_3$ for 5 min. (c) Same than in (a) for (i) and (ii); and with (iii) being the result after a 1 min treatment with 1M hydroxylamine solution at pH 5. (d) Reversed phase chromatograms (UPLC®) recorded with UV and MS detection of **P1-Daa1-P3** (320 μM), at (i) under reductive condition in a solution of TCEP (28 mM) at pH 6; (ii) after 120 min in the same solution + 2.5 % of TFA; and with (iii) being the result after a 5 min treatment with 1M hydroxylamine solution at pH=5. (e) Same than in (b) for (i) and (ii); and with (iii) being the result after a 5 min treatment with 1M hydroxylamine solution at pH 7. (f) Reversed phase chromatograms (UPLC®) recorded with UV and MS detection of **P1-Daa2-P3** (320 μM), at (i) under reductive condition in a solution of TCEP (28 mM) at pH 6; (ii) after 180 min in the same solution + 2.5 % of TFA; and with (iii) being the result after a 1 min treatment with 1 M hydroxylamine solution at pH 7.

n.b. The poor quality of the baseline before 0.45 min is due to the presence of hydroxylamine at high concentration in (c, iii); (d, iii); (e, iii); (f, iii).

5. Hydrolysis byproducts characterization and quantification

These byproducts were characterized by UPLC® chromatogram associated with MS in all the conditions studied.

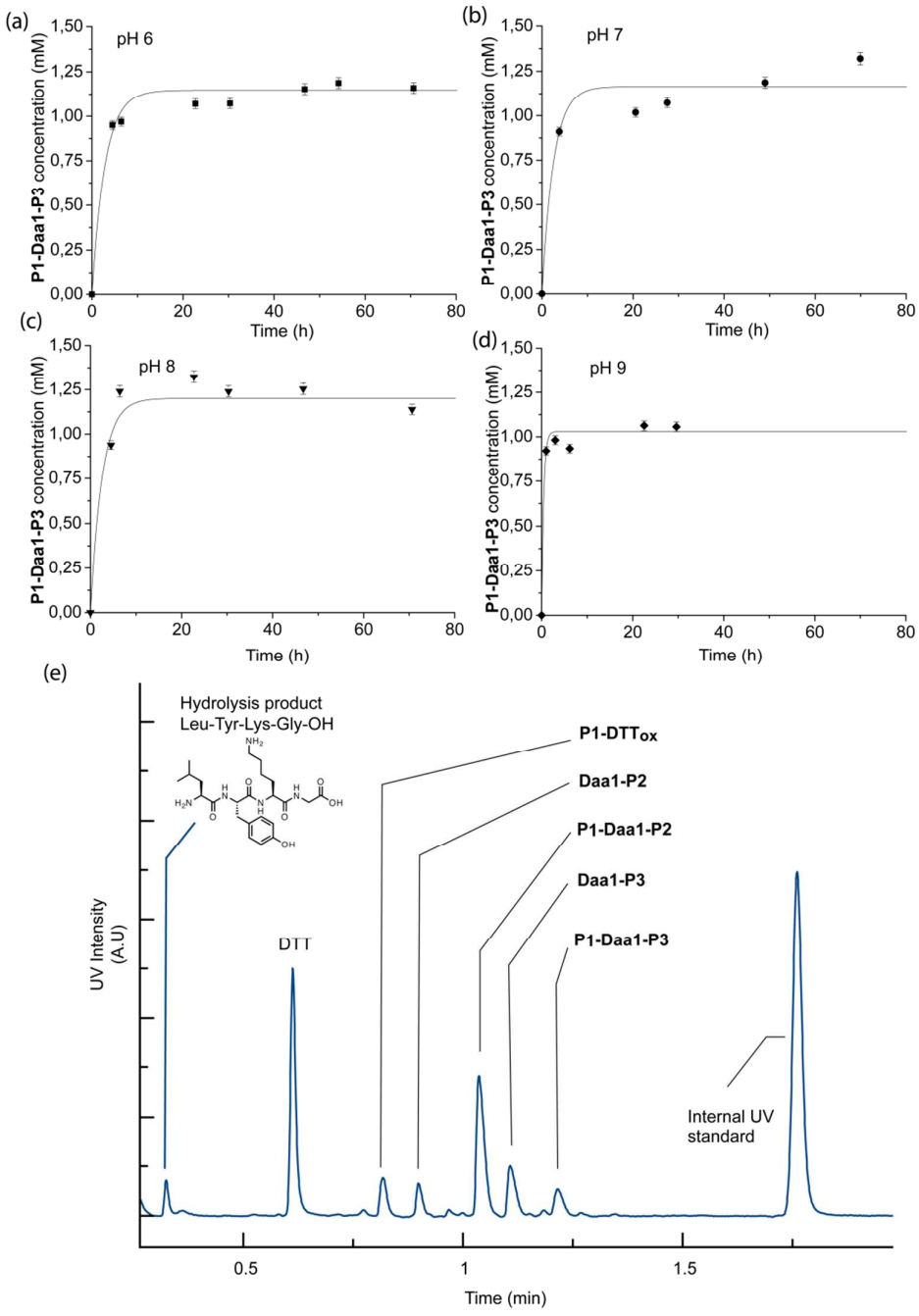


Figure S11. (a-d) Absolute concentration of exchanged peptide **P1-Daa1-P3** showing its stability, even long time after the thermodynamic equilibrium is reached, for all pH studied. Reversed phase chromatograms (UPLC®) recorded with UV and MS detection for the characterization of the hydrolysis byproducts at pH 7 (corresponding to plot (b)) and at time t = 28 h. This byproduct can be neglected in the timescale of the reaction.

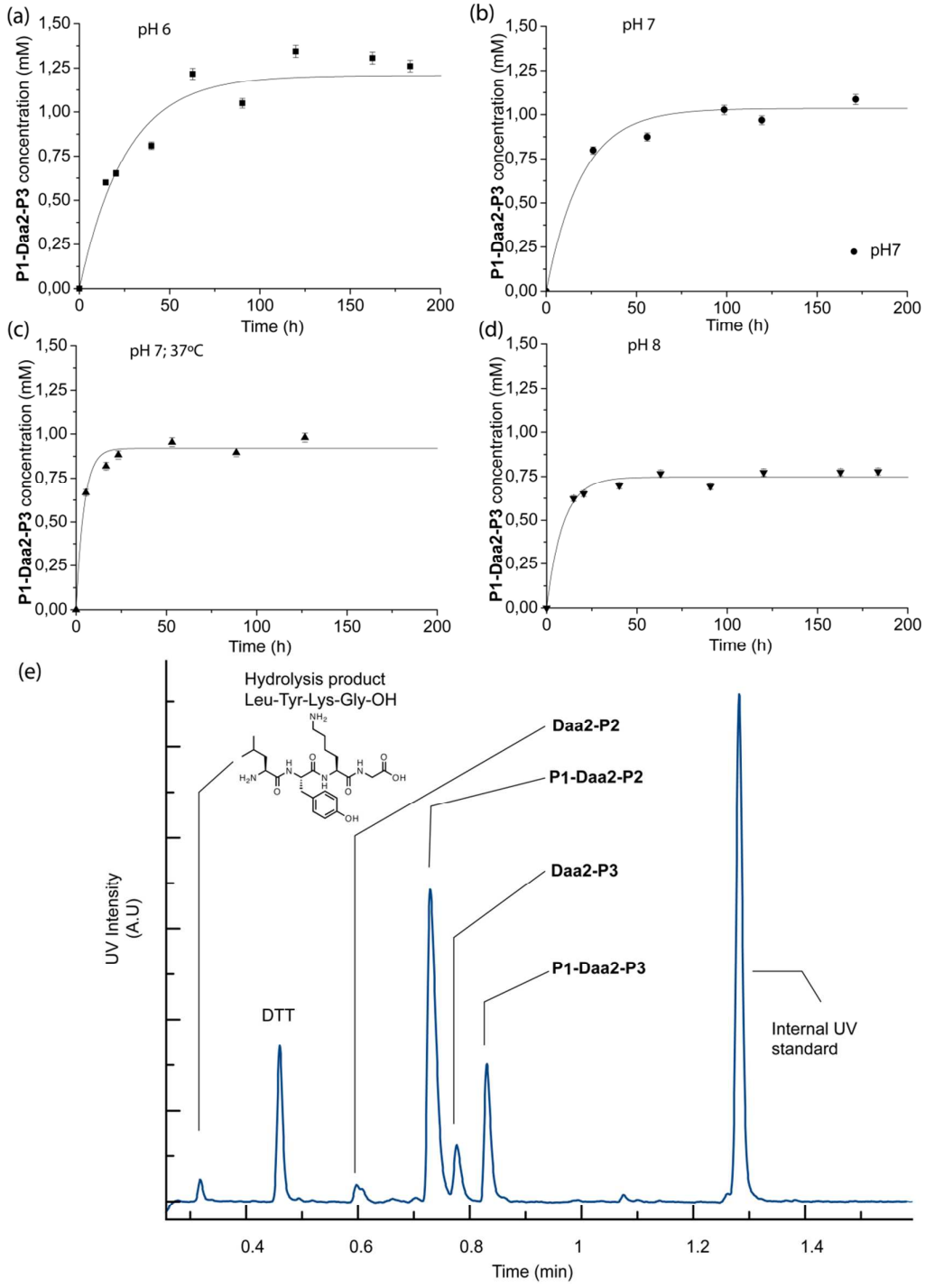


Figure S12. (a-d) Absolute concentration of exchanged peptide **P1-Daa2-P3** showing its stability, even long time after the thermodynamic equilibrium is reached, for all pH studied. Reversed phase chromatograms (UPLC®) recorded with UV and MS detection for the characterization of the hydrolysis byproducts at pH 7, 37 °C (corresponding to plot (c)) and at time t = 98 h. This byproduct can thus be neglected in the timescale of the reaction.

6. Exchange in the presence of 1,4-butanedithiol instead of DTT

These byproducts were characterized by UPLC® chromatogram associated with MS in all the conditions studied.

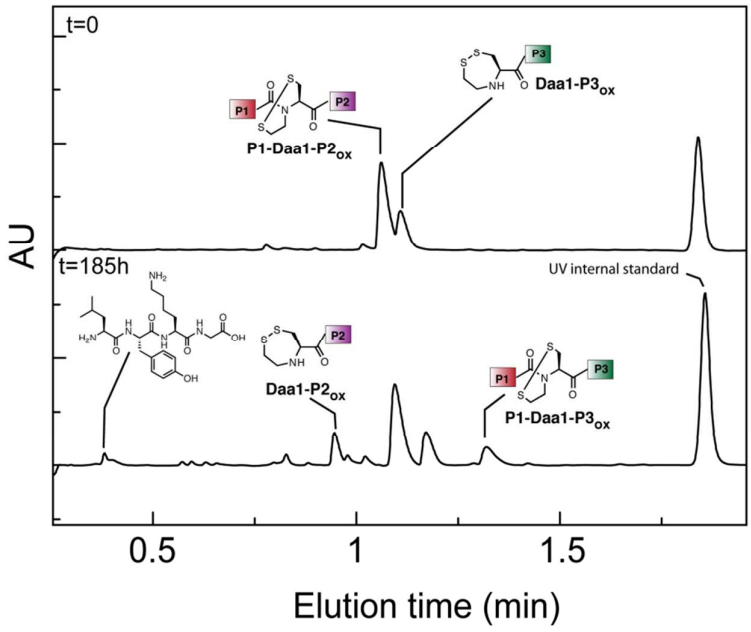


Figure S13. (a) Reversed phase chromatograms (UPLC®) recorded with UV and MS detection of the exchange reaction between **P1-Daa1-P**2 and **Daa1-P3** at pH 7, room temperature, and at t=0 (a) and t=185 h (b).

7. References

[1] H. E. Gottlieb, V. Kotlyar, A. Nudelman, *J. Org. Chem.* **1997**, *62*, 7512-7515.

[2] W. Chan, P. White, *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*, Oxford University Press, USA, **2000**.

[3] S. L. Pedersen, A. P. Tofteng, L. Malik, K. J. Jensen, *Chem. Soc. Rev.* **2012**, *41*, 1826-1844.

[4] W. Hou, X. Zhang, F. Li, C.-F. Liu, *Org. Lett.* **2011**, *13*, 386-389.

[5] W.-S. Yeo, D.-H. Min, R. W. Hsieh, G. L. Greene, M. Mrksich, *Angew. Chem. Int. Ed.* **2005**, *44*, 5480-5483.

[6] L. A. Erlich, K. S. A. Kumar, M. Haj-Yahya, P. E. Dawson, A. Brik, *Org. Biomol. Chem.* **2010**, *8*, 2392-2396.