Supporting information for:

Phosphine addition to dehydroalanine for peptide modification

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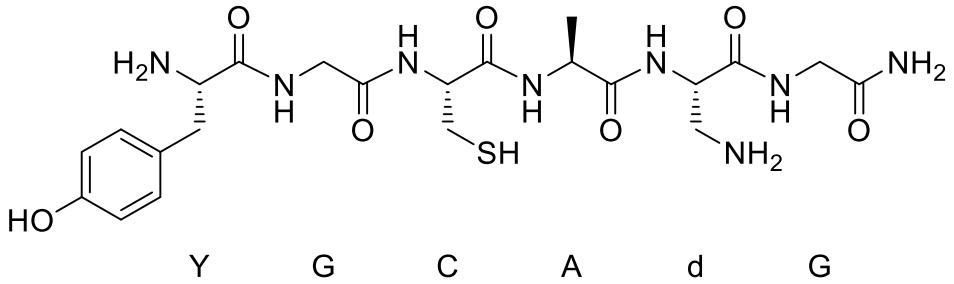
General Information

Chemicals were purchased from Sigma Aldrich, Alfa Aesar or GLbiochem and used without further purification. HPLC-grade solvents were purchased from Biosolve BV Netherlands or VWR chemicals. Nisin A was obtained from dr. Eefjan Breukink (Utrecht University) as a dry powder with estimated 90% purity. DBAA and DBV were synthesised following the literature [REF]. MALDI-TOF-MS spectra were measured on an UltrafleXtreme system (Bruker). Analytic LC-MS was performed on an automated HPLC system (Shimadzu LC-20) equipped with UV/VIS detector at 214/280 nm on a C18 column (particle size: 5 µm, 150x4.6 mm) at a flow rate of 1 $mL.min^{-1}$ using a linear gradient of buffer A (0.1% Formic acid in CH_3CN/H_2O 5:95 v/v) and buffer B (0.1% Formic acid in CH_3CN/H_2O 95:5 v/v) from 10-70% B over 30 min and connected to a Bruker daltonics micrOTOF, or alternatively was performed with an Agilent 1260 II Infinity LC system using an Agilent poroshell-120 EC-C18 column (particle size: 2.7 µm, 100x4.6 mm) at a flow rate of 0.6 $mL.min^{-1}$ using a linear gradient of buffer A (0.1% Formic acid in H_2O) and buffer B (0.1% Formic acid in CH_3CN) from 5-95% B over 10 or 22 min and connected to Agilent InfinityLab LC/MSD XT. Preparative RP-HPLC was performed on an automated preparative HPLC system equipped with a UV/VIS detector at 214/280nm using a C18 column (particle size: 10 µm, 250x22 mm) at a flow rate of 12.5 $mL.min^{-1}$ using a linear gradient of buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) and buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) from 10-70% B over 60 min. The concentration was calculated through the UV absorbance at 280 nm measured on Nanodrop (BioRad) and with calculated extinction coefficients. ^{1}H and ^{13}C NMR spectra were recorded on Varian 400 MHz, Bruker 500 MHz or Bruker 600 MHz systems.

General peptide synthesis.

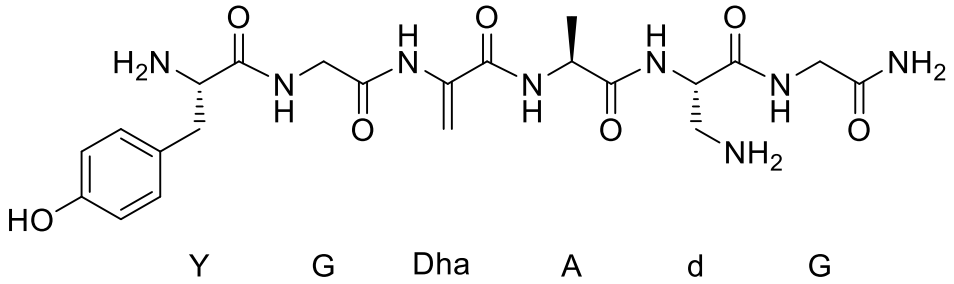
Peptides were synthesized by automated Fmoc solid phase synthesis on a Biotage Syro II or Syro I with tentagel S-RAM resin from Rapp polymere. Peptide couplings were performed using Fmoc-protected amino acids (4 equiv), HOBt (4 equiv), HBTU (4 equiv) and DIPEA (8 equiv) for 40 min twice, and deprotection was by two 10 min treatments with 20% piperidine, all in DMF. Upon completion of SPPS, each peptide was treated with a cleavage cocktail (TFA/H_2O/TIPS/EDT 90:5:2.5:2.5) before precipitation with at least 10 volume equivalents of cold diethyl ether, and the resulting pellet washed thrice with the same solvent. This pellet was then dissolved in 1-2 mL DMSO for HPLC purification.

Synthesis of peptides for NMR characterization

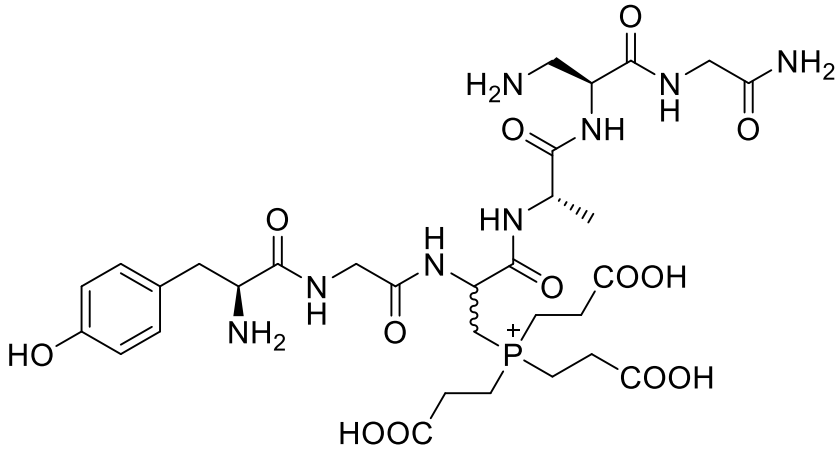


Peptide for the NMR experiments (**P1**) was synthesized on solid phase peptide synthesis at 50 µmol scale following the general method above (multiple syntheses, batchwise). HPLC-purified peptide was dissolved

in D_2O for ^1H, ^{13}C, COSY, HSQC and HMBC experiments. Afterwards, peptide was lyophilised and re-dissolved in 9:1 H_2O : D_2O mixture for TOCY analysis. ^1H NMR (600 MHz, D_2O) δ 7.07 (d, J = 8.4 Hz, 2H), 6.78 (d, J = 8.4 Hz, 2H), 4.65 (t, J = 7.4, 5.9Hz, 1H), 4.44 (t, J = 6.1 Hz, 1H), 4.24 (q, J = 7.2 Hz, 1H), 4.15 (t, J = 7.2 Hz, 1H), 3.87 (ABq, J = 21.7, 16.4Hz, 4H), 3.43 (dd, J = 13.4, 5.7 Hz, 1H), 3.20 (dd, J = 13.4, 7.6 Hz, 1H), 3.05 (dd, J = 30.8, 14.3, 7.2 Hz,2H), 2.86 – 2.77 (m, 2H), 1.32 (d, J = 7.2 Hz, 3H) ppm. ^{13}C NMR (151 MHz, D_2O) δ 175.37 (s), 173.62 (s), 171.85 (s), 170.65 (s), 170.34 (s), 169.86 (s), 155.11 (s), 130.79 (s), 125.41 (s), 115.81 (s), 69.54 (s), 55.21 (s), 54.48 (s), 50.55 (s), 50.10 (s), 42.06 (s), 39.78 (s), 35.95 (s), 25.34 (s), 16.01 (s) ppm.



P1 was converted to Dha-peptide 1 (**P2**) through a DBV-mediated elimination of Cys. Starting material (100 µmol) was dissolved in 3 mL of degassed 400 mM sodium phosphate buffer pH 8.5. 5 eq. of DBV (500 µmol, 136.5 mg) were added and the reaction mixture was stirred at 42 °C under N_2 atmosphere overnight. The reaction was monitored on LC-MS. 1 mL of brine was subsequently added and the majority of DBV removed as an immiscible neat liquid. The remaining aqueous layer was purified by HPLC. Purified peptide was dissolved in D_2O for ^1H, ^{13}C, COSY, HSQC and HMBC experiments. Afterwards, peptide was lyophilised and re-dissolved in 9:1 H_2O : D_2O mixture for TOCSY analysis. ^1H NMR (600 MHz, D_2O) δ 6.77 (d, J = 8.4 Hz, 2H), 5.58 (d, J = 12.0 Hz, 2H), 4.26 (dq, J = 28.4, 7.2 Hz, 1H), 4.18 – 4.14 (m, 1H), 4.12 (t, J = 5.5 Hz, 1H), 3.88 (ABq, 2H), 3.68 – 3.61 (m, 1H), 3.10 – 3.00 (m, 1H), 1.33 (dd, J = 26.6, 7.3 Hz, 2H) ppm; ^{13}C NMR (151 MHz, D_2O) δ 175.83, 175.56, 170.40, 169.92, 169.79, 169.77, 155.13, 134.57, 130.79, 125.33, 115.82, 112.71, 112.38, 54.44, 52.67, 50.56, 50.41, 50.29, 42.58, 42.03, 39.67, 39.48, 35.91, 15.84. LC-MS (microTOF, Bruker) calculated $[M+H]^+$: 521.2; observed $[M+H]^+$: 521.3.



P2 was converted to TCEP-peptide (**P3**) directly after NMR analysis. **P2** was transferred from the NMR tube to 3 mL of MilliQ H_2O. A stock TCEP solution was prepared by dissolving TCEP (500 µmol, 5 eq.) in 1 mL of 500 µmol NaHCO3. The TCEP solution was added to the peptide and the reaction stirred overnight with monitoring by TLC and LC/MS. The product was purified by preparatory HPLC. Purified peptide was

dissolved in D_2O for ^1H, ^{13}C, COSY, HSQC and HMBC experiments. Afterwards, peptide was lyophilised and re-dissolved in 9:1 H_2O : D_2O mixture for TOCSY analysis. ^1H NMR (600 MHz, D_2O) δ 7.11 (t, J = 7.7 Hz, 2H), 6.82 (dd, J = 8.3, 1.6 Hz, 2H), 4.94 – 4.84 (m, 1H), 4.33 – 4.09 (m, 3H), 3.97 – 3.78 (m, 4H), 3.72 – 3.58 (m, 2H), 3.16 – 3.08 (m, 2H), 3.02 – 2.95 (m, 1H), 2.79 – 2.69 (m, 6H), 2.68 – 2.64 (m, 1H), 2.64 – 2.52 (m, 6H), 1.39 – 1.27 (m, 3H) ppm. ^{31}P NMR (202 MHz, D_2O) δ 35.18, 35.07 ppm. ^{13}C NMR (151 MHz, D_2O) δ 175.59, 175.52, 174.62, 174.61, 174.53, 174.51, 173.35, 170.95, 170.82, 170.29, 170.08, 170.06, 170.05, 170.02, 170.00, 169.97, 163.29, 163.06, 162.82, 162.59, 155.13, 131.72, 125.37, 119.20, 117.26, 115.80, 115.33, 54.42, 54.40, 52.68, 52.61, 50.35, 50.27, 47.28, 47.26, 47.22, 47.20, 42.56, 42.44, 42.02, 42.00, 41.94, 39.46, 39.43, 35.88, 25.96, 25.71, 25.68, 16.33, 16.27, 15.09, 14.77, 14.75, 13.81. LC-MS (microTOF, Bruker) calculated M^+: 771.3; observed M^+: 771.3.

General procedure for “one-pot” phosphine addition

All of the phosphine addition reactions were carried out as detailed here unless otherwise specified. The Cysteine elimination reaction mixture contained 2.5 mM DBAA or DBV and 50 mM sodium phosphate buffer at pH 8.0, with peptide at 100 μM. The reaction mixture was incubated at 42 ^0C for 2 hrs, which typically gave close to full conversion. After that, phosphine (5 mM) was added to the reaction mixture without further purification. For the testing the phosphine scope, **P4** was used as model peptide. For the less water soluble tri(2-furyl)phosphine, a stock was prepared in DMF at 50 mM and on dilution into the reaction mixture no precipitation was observed. To quantify the yield and conversion of phosphine addition, samples were analyzed by LC-MS with 93 μM Tyr as internal standard.

Kinetic Study of TCEP Addition Reaction

The kinetics of the addition reaction with 1 mM **P2** and 3 mM TCEP in 50 mM pH 8.0 sodium phosphate buffer at room temperature was monitored by HLPC using 250 μM Trp as internal standard. The reaction was quenched with 1 % H_2O_2 at different time points (15 s, 30 s, 45 s, 60 s, 2 min, 4 min, 7 min, 12 min, 20 min, 30 min, 60 min). For a t=0 sample, water was added instead of TCEP. The experiments were repeated for a total of 3 times. The samples were analyzed by LC-MS with a linear gradient of 5-95% buffer A (see general information for details) over 22 min. The data were analyzed by GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

Stability test of TCEP product

The stability of peptide **P3** at 100 μM concentration in water was tested under different conditions as detailed in Figures S1-S10. The reaction mixture was kept at each of the different conditions for 5 hrs or 24 hrs before injection on LC-MS. For pH 1 and pH 14 conditions, the samples were quenched with NaOH or HCl solution, respectively, before analysis. 25 μM of tryptophan was used as internal standard for all conditions. The conversion rate was calculated based on the area under the peak from UV absorbance at 280 nm. Due to the overlap of **P3** and H_2O_2, the recovery rate was calculated by peak area of an extracted ion chromatogram for the M^+ peaks.

Nisin A modification with TCEP

Nisin A was obtained from dr. Eefjan Breukink (Utrecht University) in the form of a dry powder with ~90%

purity. The peptide was solubilised in water with 0.1% AcOH to make a stock with a concentration of 0.5 M. A 250 µL reaction mixture was prepared containing 5 mM of the Nisin A stock and 5 eq. of TCEP with 10 mM phosphate buffer at pH 8.0, and incubated at at a temperature of 42 °C. For analysis at each time point (1, 3 and 5 hours), 20 µl of the reaction mixture was added to 20 µl of buffer A (95% MilliQ H_2O, 5% ACN, 0.1% FA) and centrifuged for 5 min at 3300 x g. The supernatant was measured on LC-MS (micrOTOF, Bruker).

Peptide modification following *in vitro* translation and in mRNA display

A DNA template encoding the test peptide MKYSHCGFLTKENLYFQGSGSGS was assembled from primers by extension PCR, purified by precipitation from 0.3 M NaCl in 70% ethanol, and then dissolved in water. Coupled transcription and translation reactions were carried out using a custom reconstituted recombinant system (PURE) at 2.5 µL scale per experiment with 100 nM input DNA at 37 °C for 30 min, as detailed previously.[1] Following translation, elimination was carried out by addition of sodium phosphate buffer (pH 8.5) to 40 mM and DBAA to 5 mM before incubation at 42 °C for 1.5 hours. Phosphine addition was carried out in tandem with elimination by addition of 5 mM phosphine. Samples were analysed by pre-purification through C18 extraction in microtip format, washing with 5% acetonitrile in water and eluting in 80% (both with 0.1% acetic acid) before spotting on MALDI plates with 50% saturated α-CHCA in the elution solvent.

Peptides in mRNA display format were prepared by using puromycin-ligated mRNA as input at 1.2 µM. Following translation, ribosomes were disrupted by addition of EDTA to 10 mM and then the mRNA copied to cDNA by reverse transcription at 42 °C for one hour. Displayed peptides were purified by passing through a column of Sephadex G-25 fine in PBS-T (pH 8.5) of bed volume ~0.7 mL. In tandem with phosphine modification as above, peptide cyclisation was carried out by addition of disuccinimidyl glutarate at 0.8 mM (repeated for a total of two additions of 45 min reaction each). Following another gel filtration purification, the peptide was cleaved by incubation with 5 units AcTEV protease (Thermo Fisher) at 25 °C for 2 hours and then analysed by MALDI-TOF-MS as above.

Peptide capture by TCEP immobilized agarose gel

Peptide **P7** (100 µM) was used for the capture test. The DBAA elimination followed the procedure described before. Then 20 µL of Pierce™ Immobilized TCEP Disulfide Reducing Gel (Thermo Scientific™ cat: 77712) was added into 20 µL of peptide solutions. The mixture slurry was allowed to stir at room temperature overnight (14 hours). After that, the mixture was centrifuged for 3min at 15000g and the supernatant analyzed by LC-MS.

HPLC traces of Stability Tests

pH 1

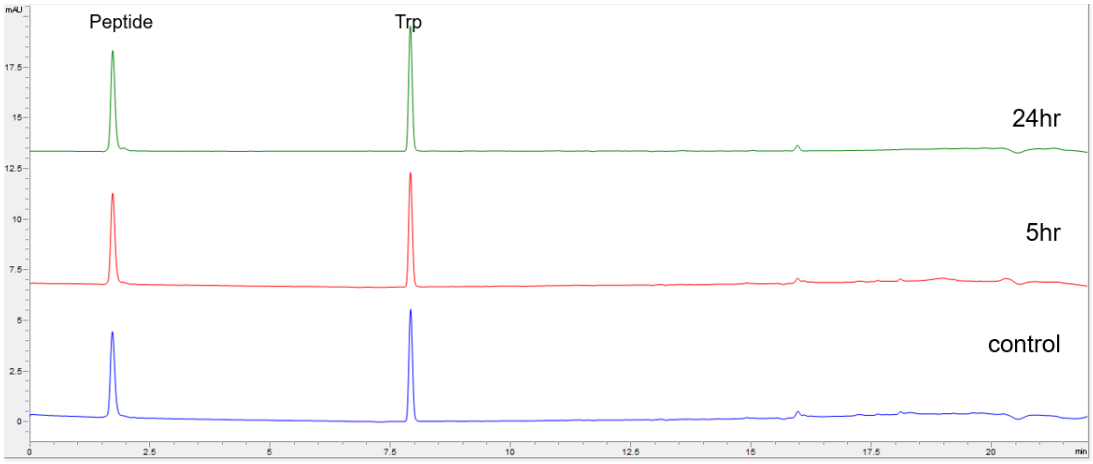


Figure S1. A280 trace showing stability of peptide **P3** at pH ~1 (HCl 100 mM).

pH 14

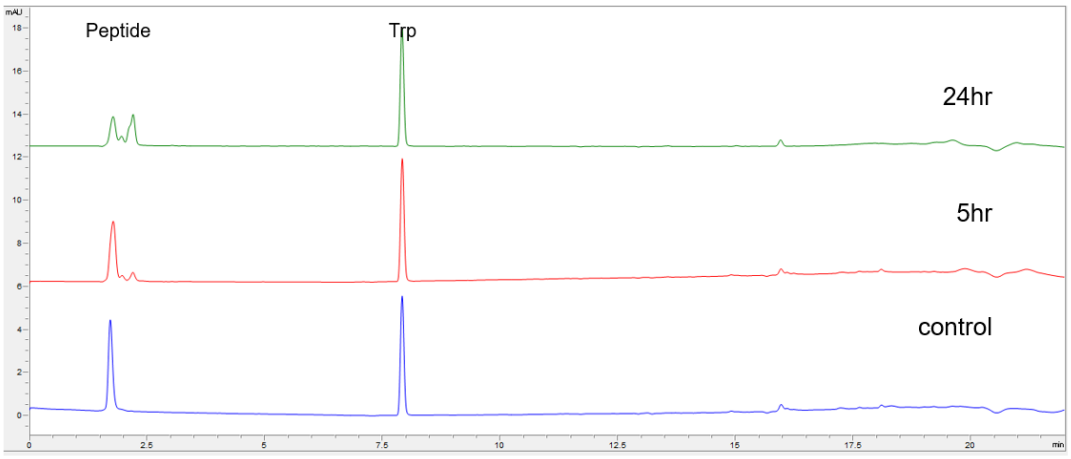


Figure S2. A280 trace showing stability of peptide **P3** at pH ~14 (KOH 100 mM).

60 ^{0}C

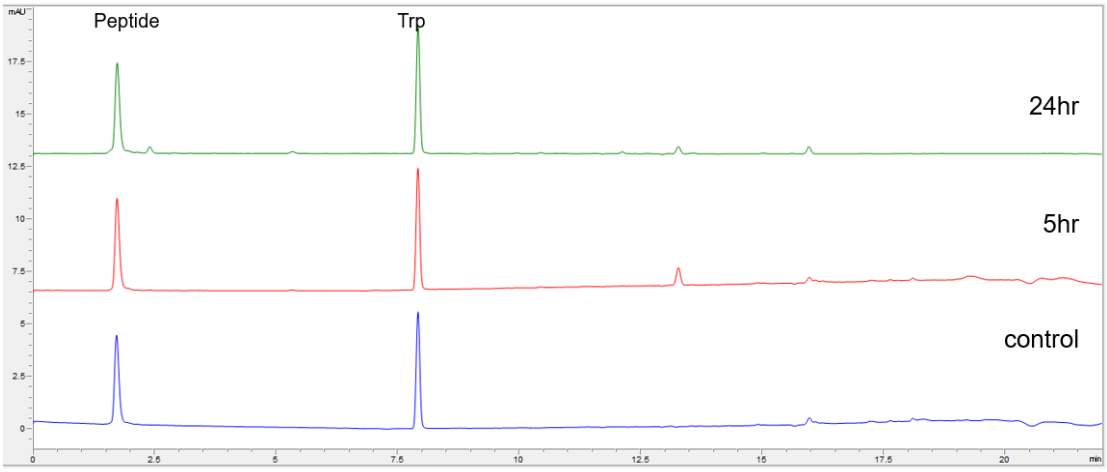


Figure S3. A280 trace showing stability of peptide **P3** at 60 ^{0}C.

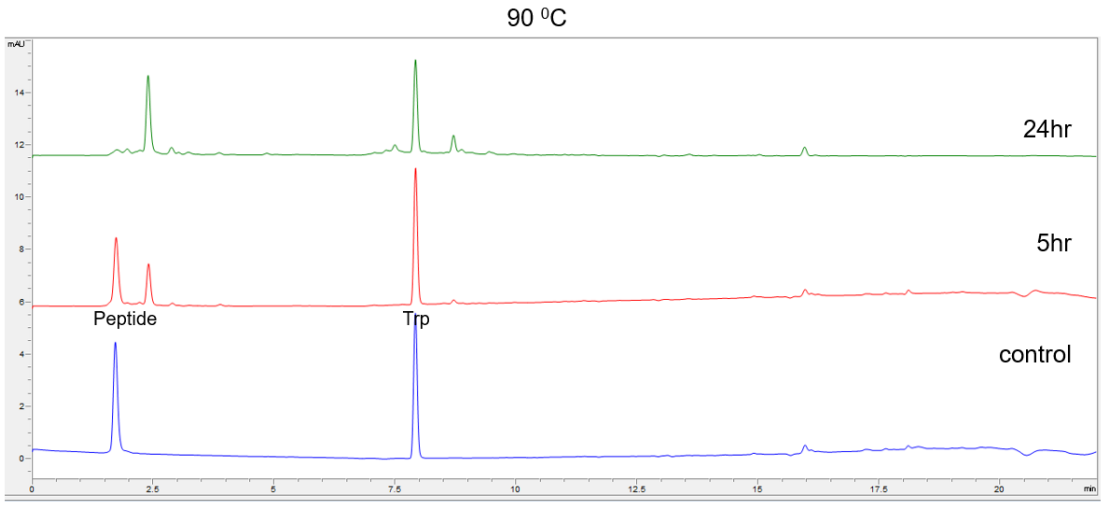


Figure S4. A280 trace showing stability of peptide **P3** at 90 ^{0}C.

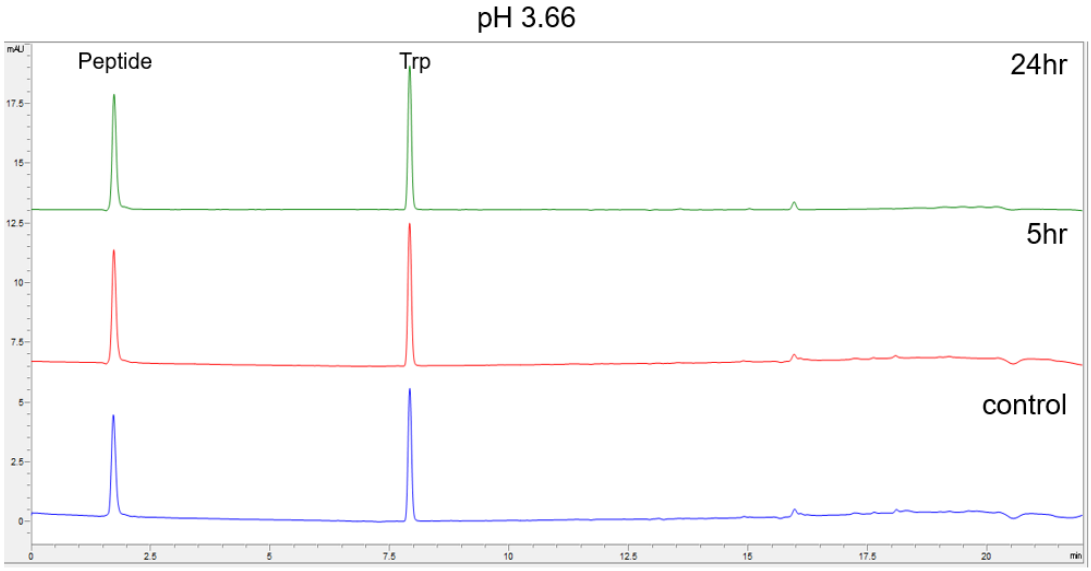


Figure S5. A280 trace showing stability of peptide **P3** at pH 3.66 (acetate 50 mM).

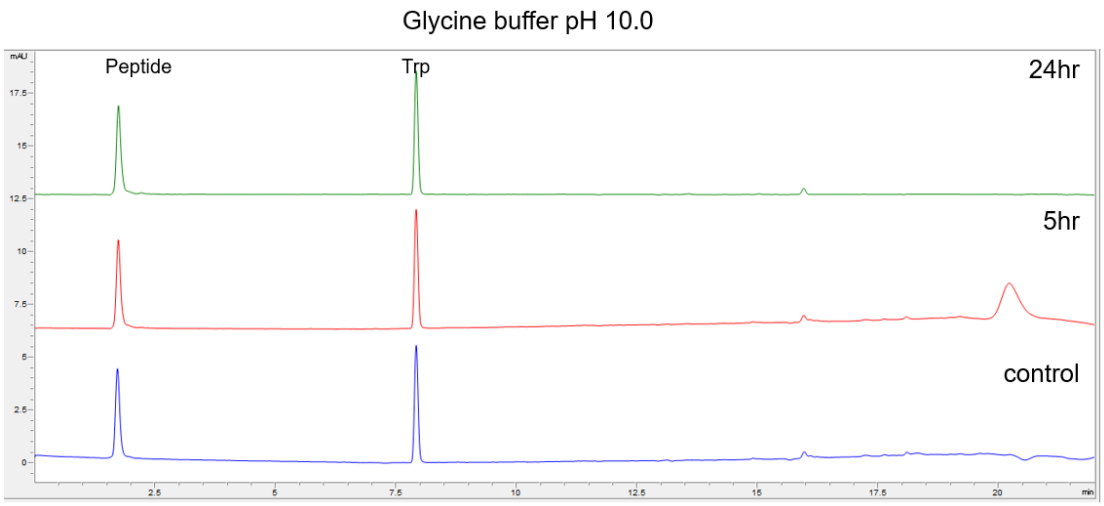


Figure S6. A280 trace showing stability of peptide **P3** at pH 10.0 (Glycine 50 mM).

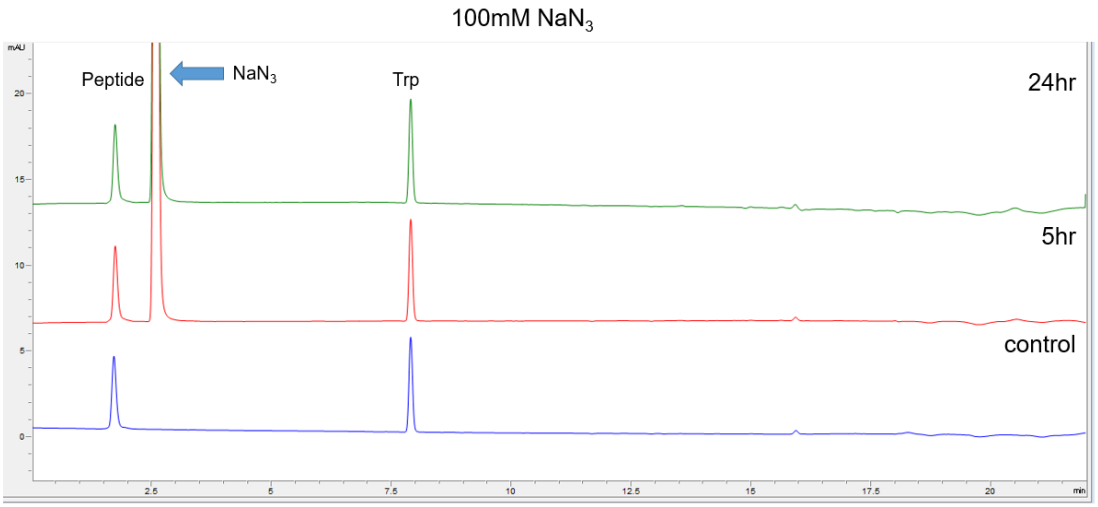


Figure S7. A280 trace showing stability of peptide **P3** in 100 mM NaN_3.

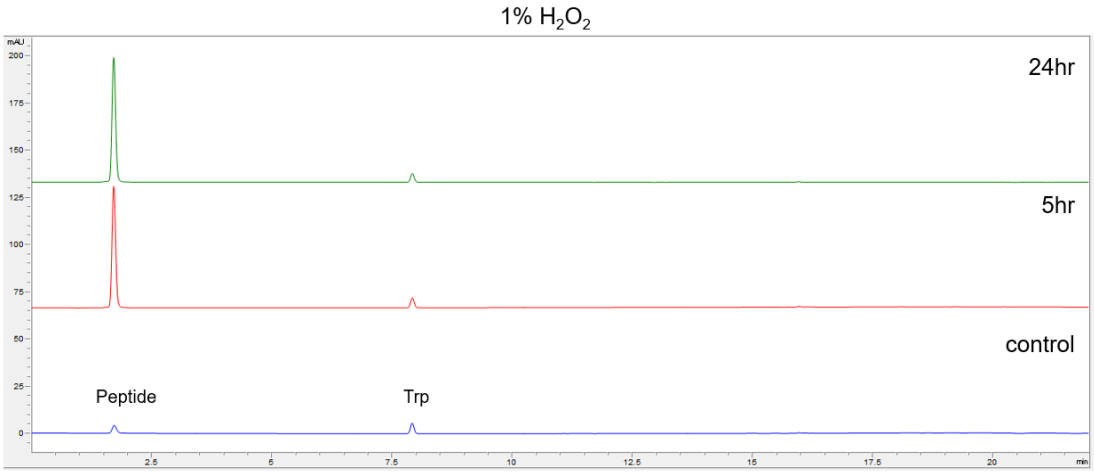


Figure S8. A280 trace showing stability of peptide **P3** in 1% H_2O_2.

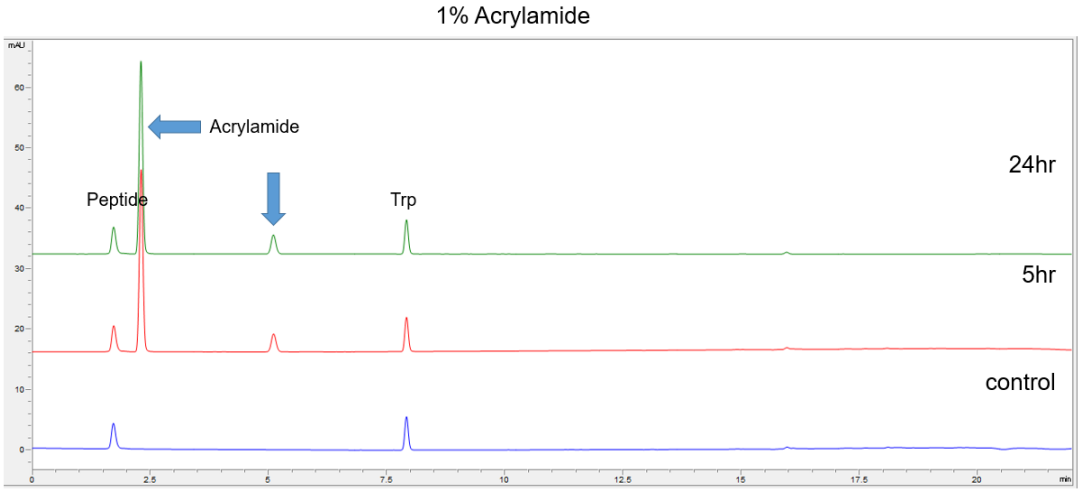


Figure S9. A280 trace showing stability of peptide **P3** in 1% Acrylamide.

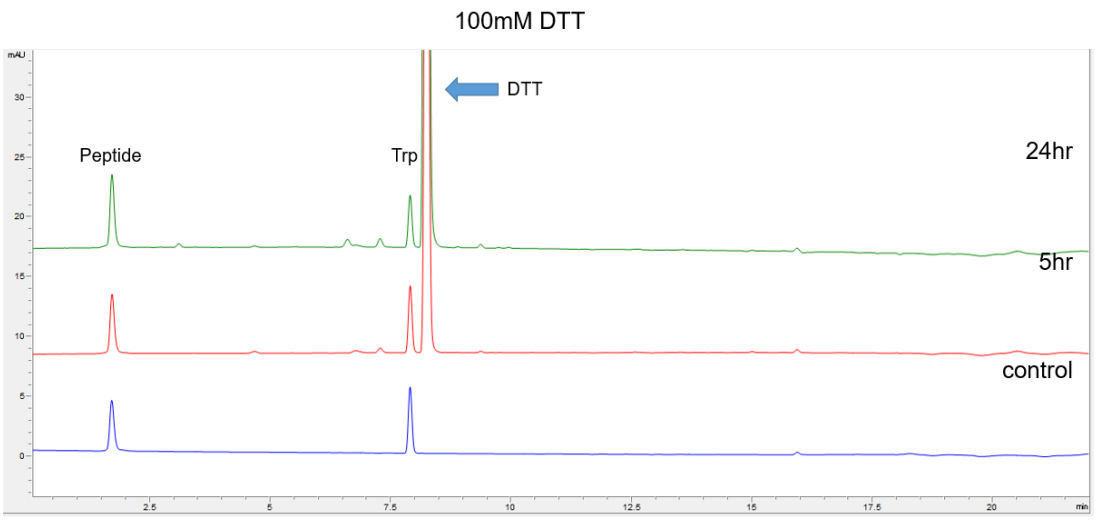


Figure S10. A280 trace showing stability of peptide **P3** in 100 mM DTT.

HPLC traces of peptide and phosphine scope

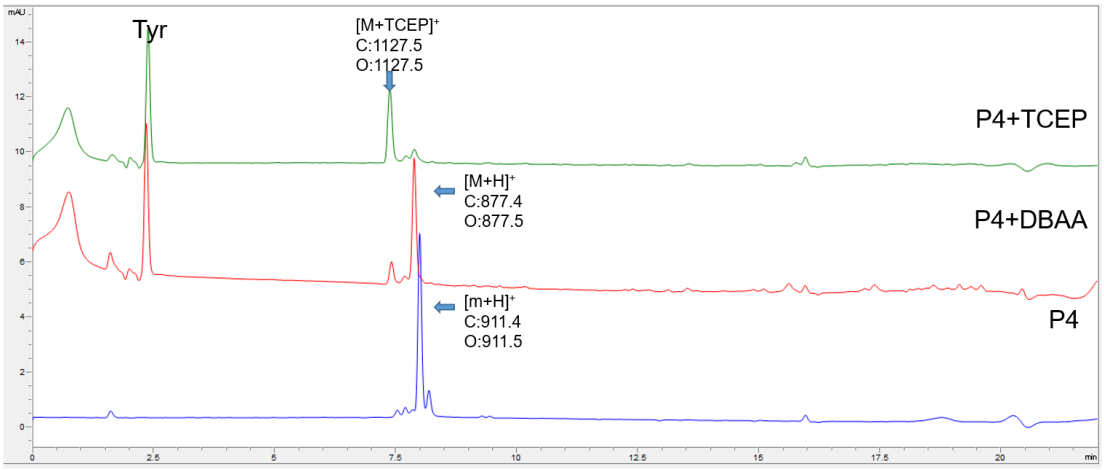


Figure T280 trace showing TCEP addition to peptide **P4**.

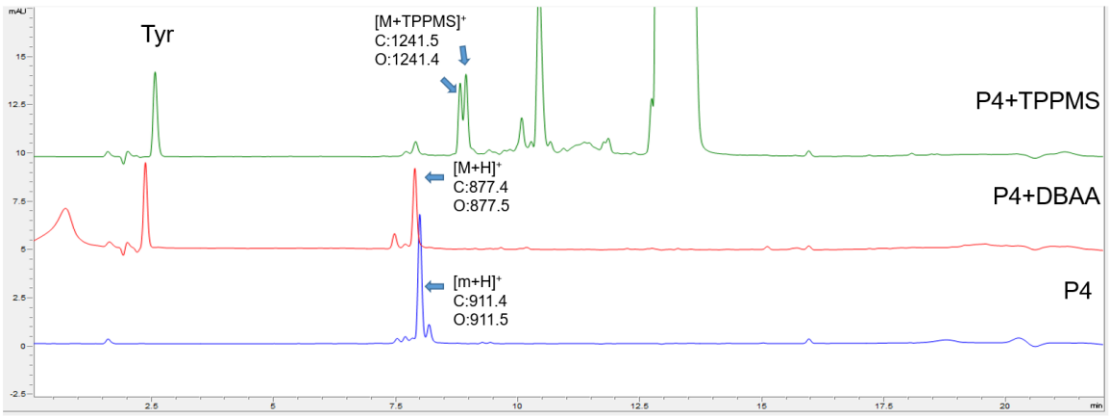


Figure S12. A280 trace showing TPPMS addition to peptide **P4**.

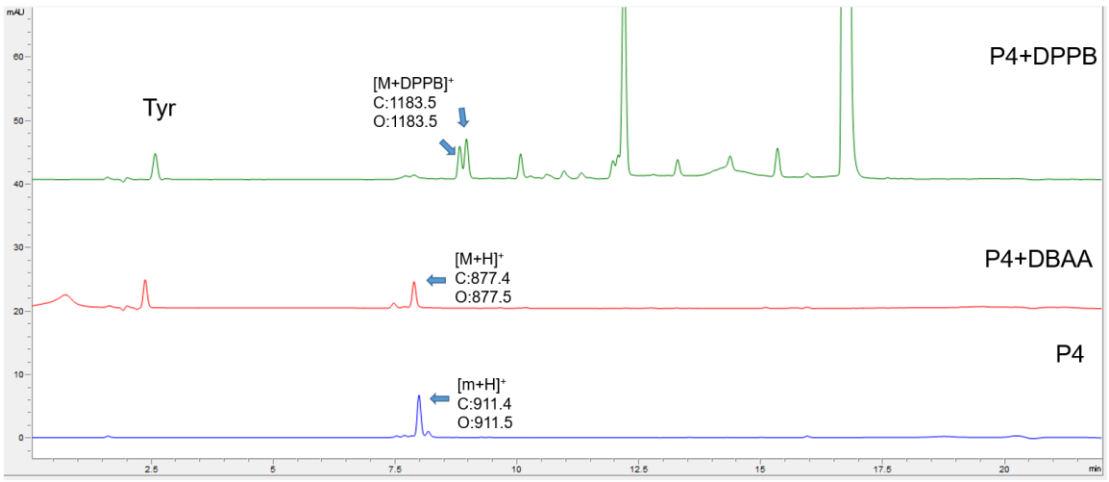


Figure S13. A280 trace showing DPPB addition to peptide **P4**.

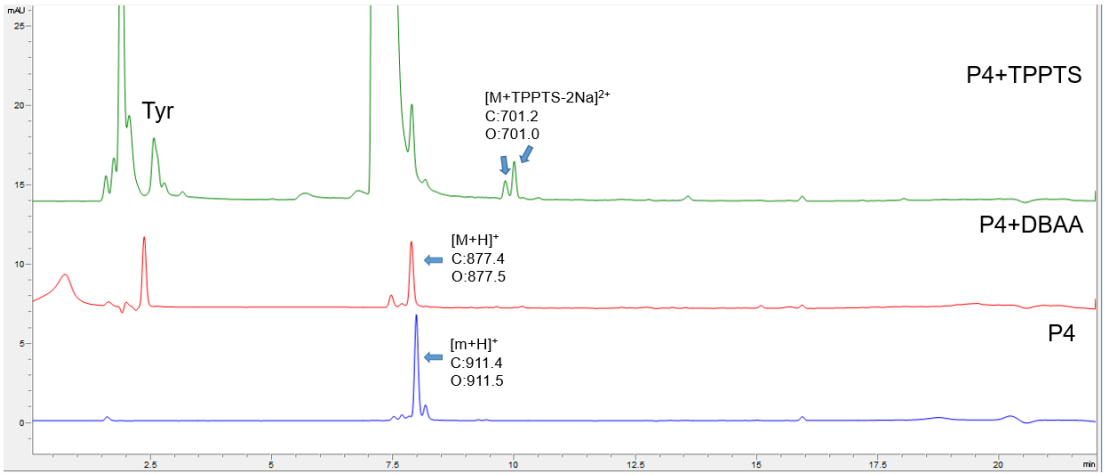


Figure S14. A280 trace showing TPPTS addition to peptide **P4**.

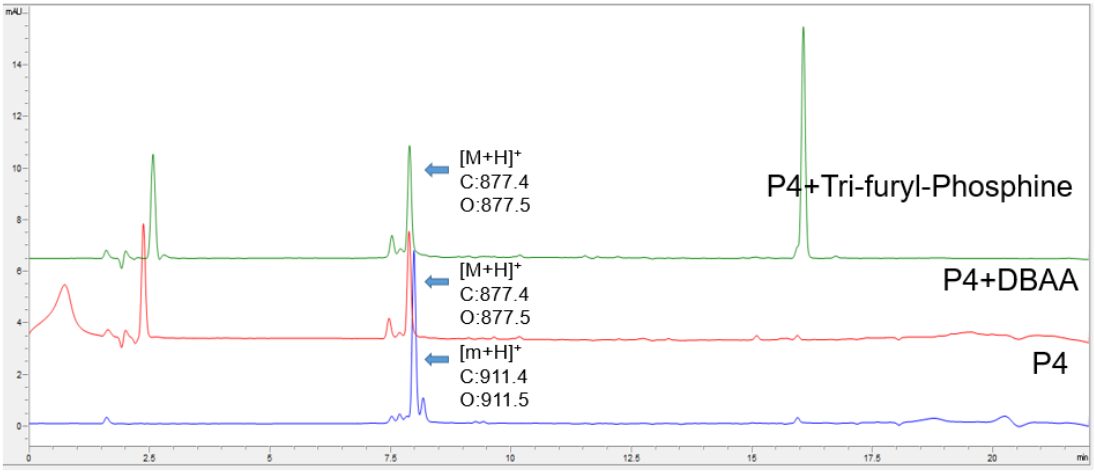
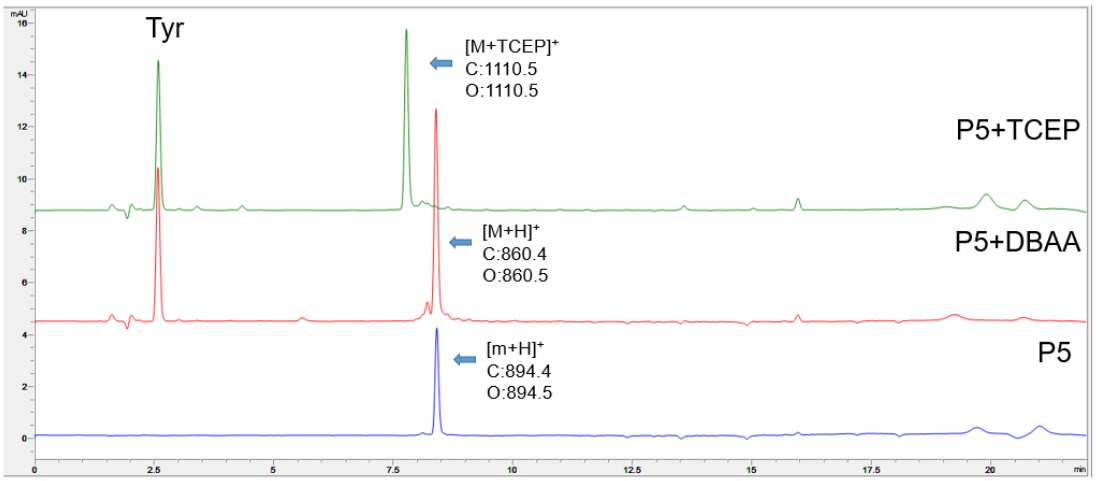


Figure S15. A280 trace showing tri(2-furyl)phosphine addition to peptide **P4** (no reaction found).

A.



B.

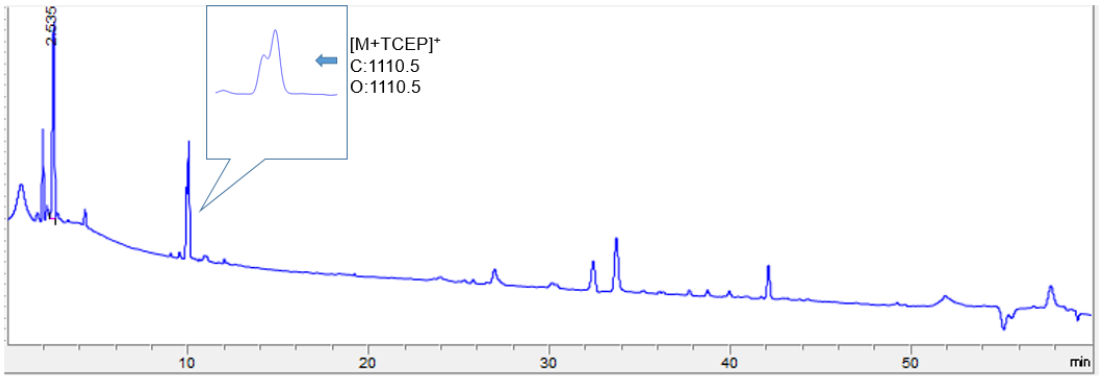
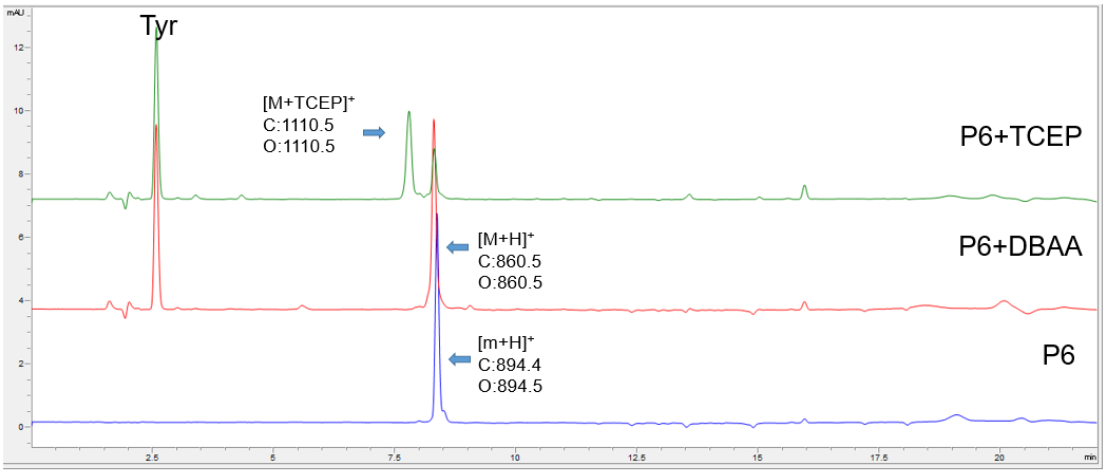


Figure S16. **A**. A280 trace showing TCEP addition to **P5**; **B**. Analysis with an extended 60 min program to reveal diastereomer peaks (inset)

A



B

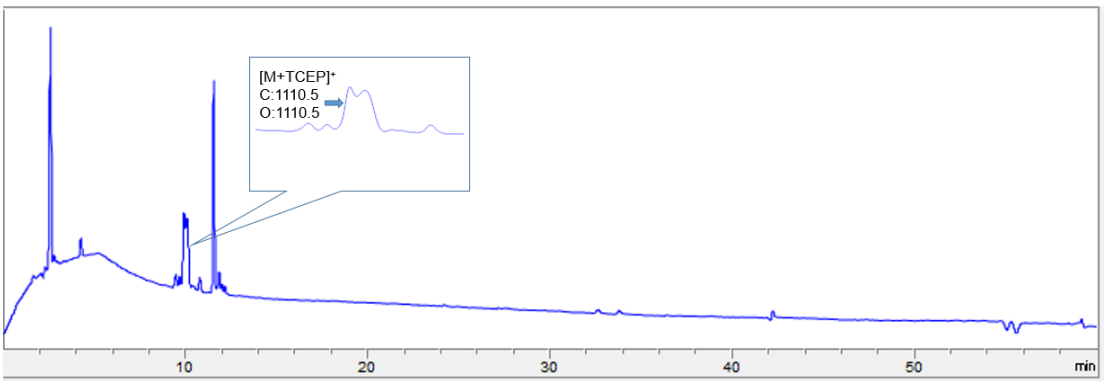


Figure S17. **A**. A280 trace showing TCEP addition to **P6**; **B**. Analysis with an extended 60 min program to reveal diastereomer peaks (inset).

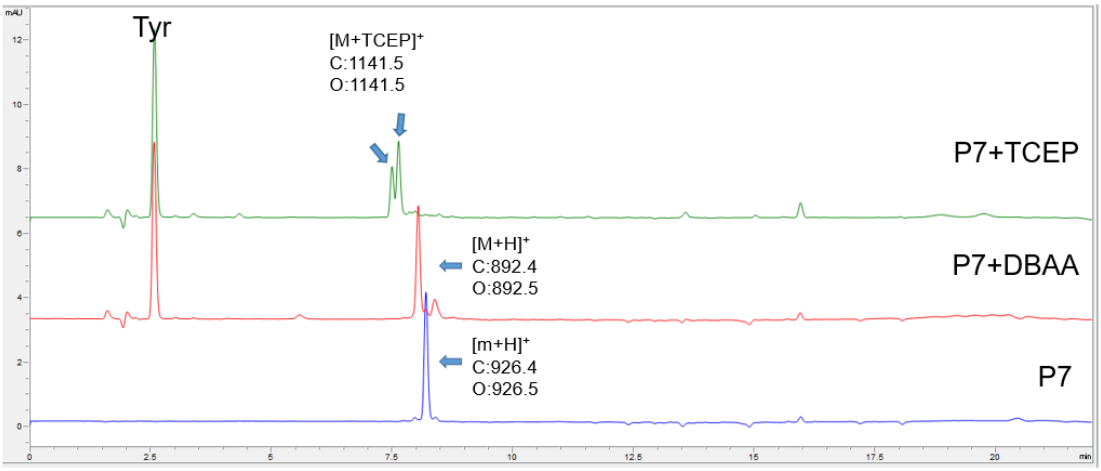


Figure S18. A280 trace showing TCEP addition to **P7**.

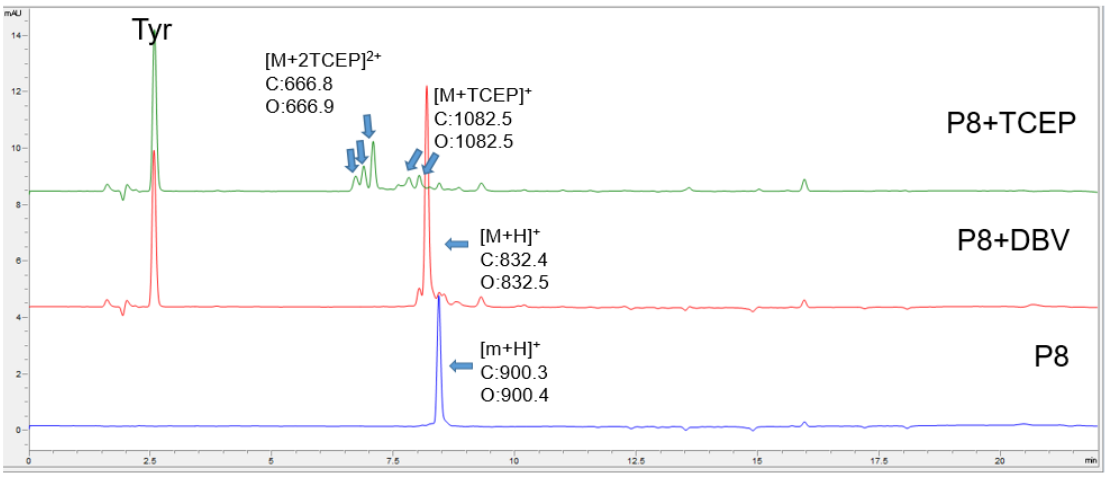


Figure S19. A280 trace showing TCEP addition to **P8**. The synthesis followed the general protocol except that the cysteine elimination was carried out with 5 mM DBV (instead of DBAA) and TCEP addition was carried out with 10 mM TCEP (instead of 5 mM), to account for the two cysteine positions.

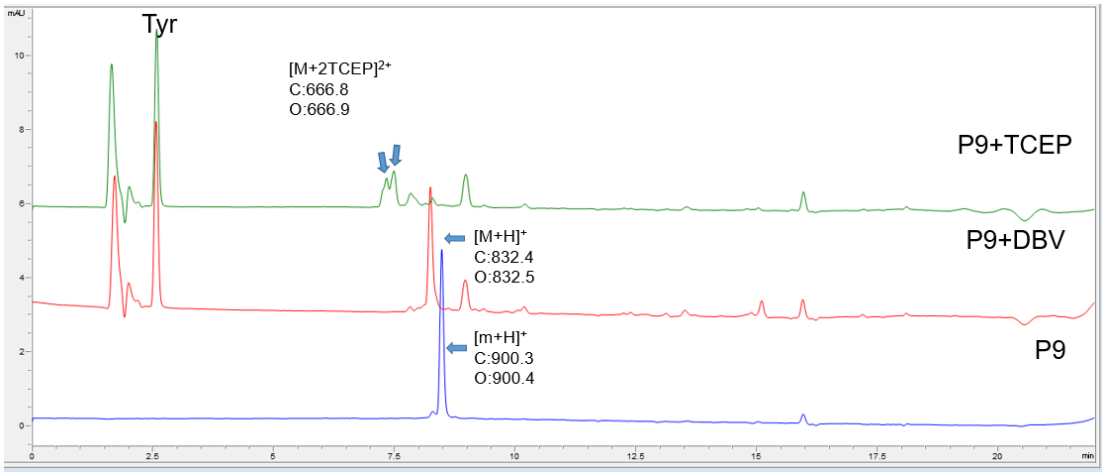


Figure S20. A280 trace showing TCEP addition to **P9**. The synthesis followed the general protocol except that the cysteine elimination was carried out with 10 mM DBV (instead of DBAA) and TCEP addition was carried out with 20 mM TCEP (instead of 5 mM), to account for the two cysteine positions.

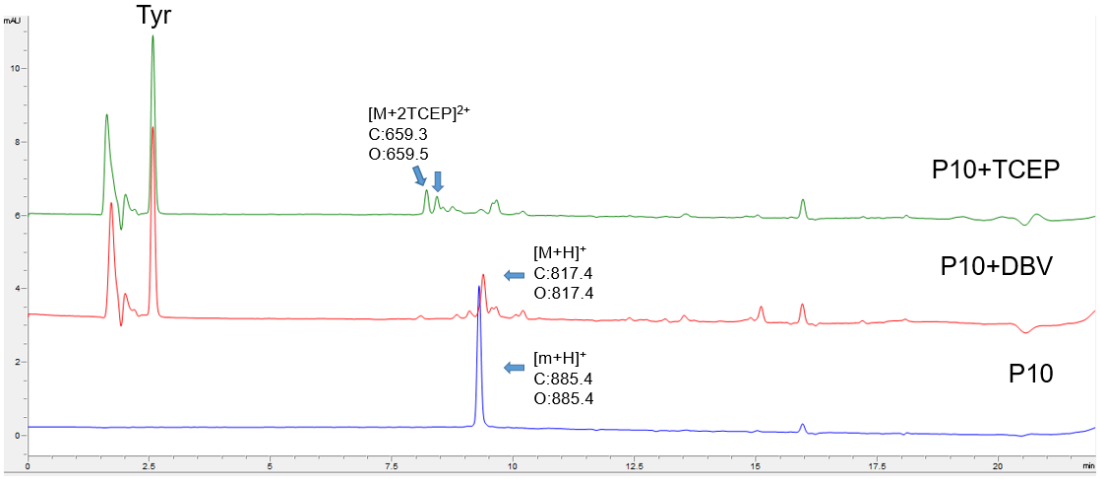


Figure S21. A280 trace showing TCEP addition to **P10**. The synthesis followed the general protocol except that the cysteine elimination was carried out with 10 mM DBV (instead of DBAA) and TCEP addition was carried out with 20 mM TCEP (instead of 5 mM), to account for the two cysteine positions.

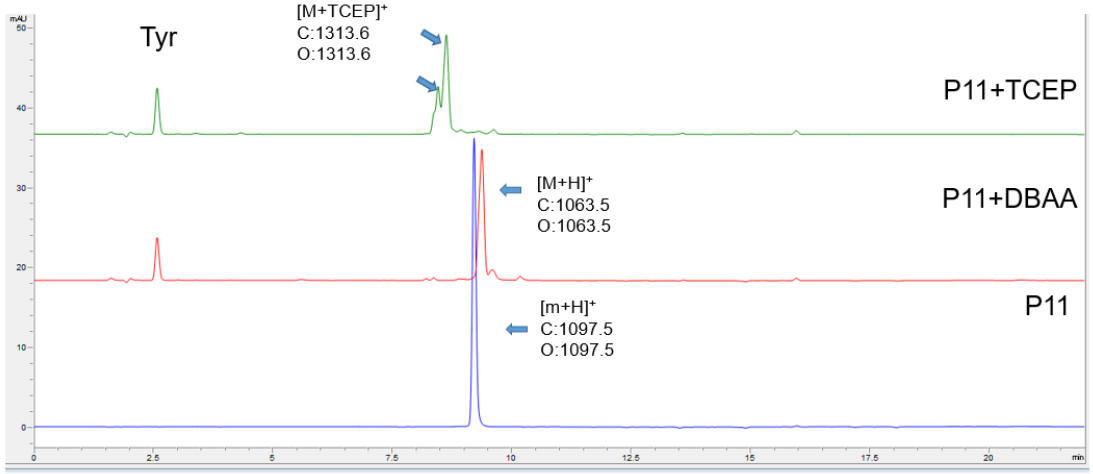


Figure S22. A280 trace showing TCEP addition to **P11**.

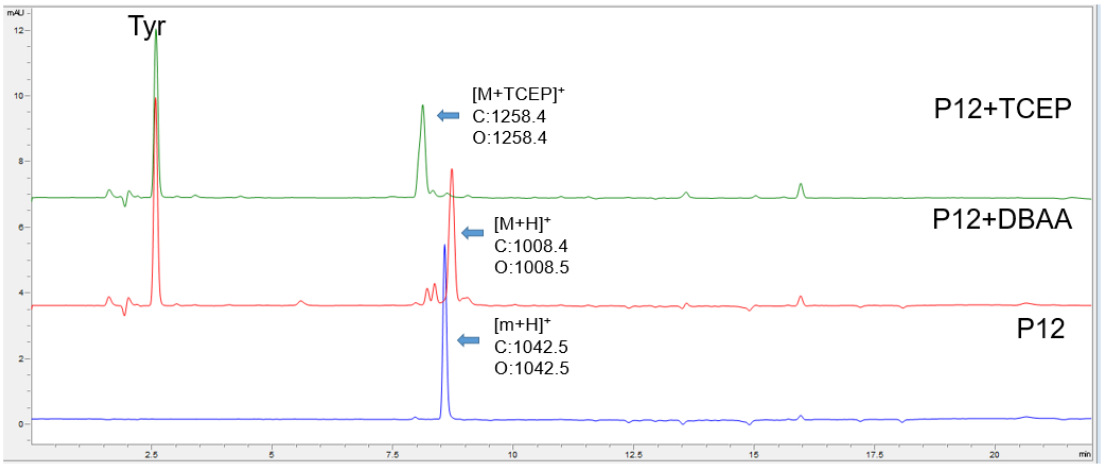


Figure S23. A280 trace showing TCEP addition to **P12**.

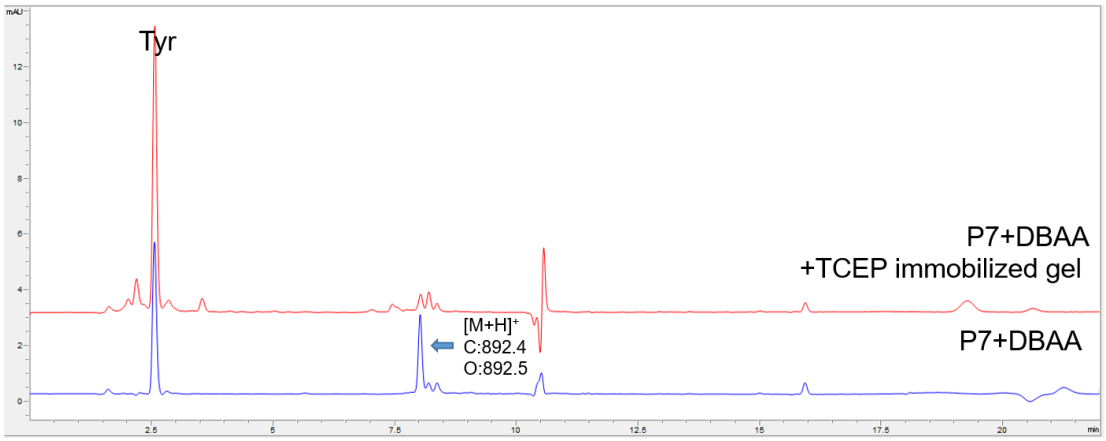


Figure S24. A280 trace showing depletion of **P7** by addition to TCEP immobilized agarose gel with 91% efficiency, referenced to tyrosine internal standard.

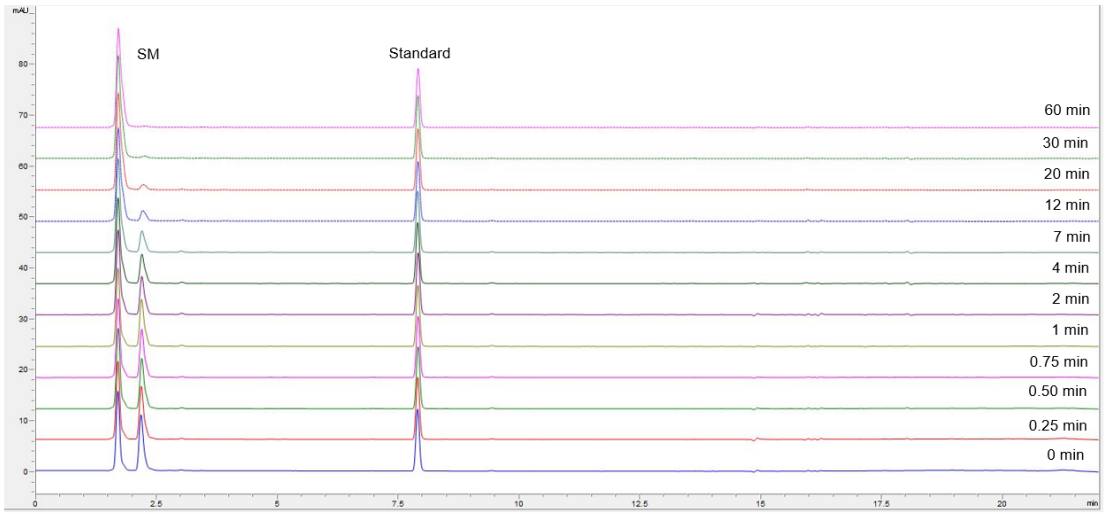


Figure S25. Example A280 trace showing the kinetic study of **P2** reaction. The **P3** peak was overlapped by the H_2O_2 peak with UV detection, but product was visible in an extracted ion chromatogram.

NMR Spectra

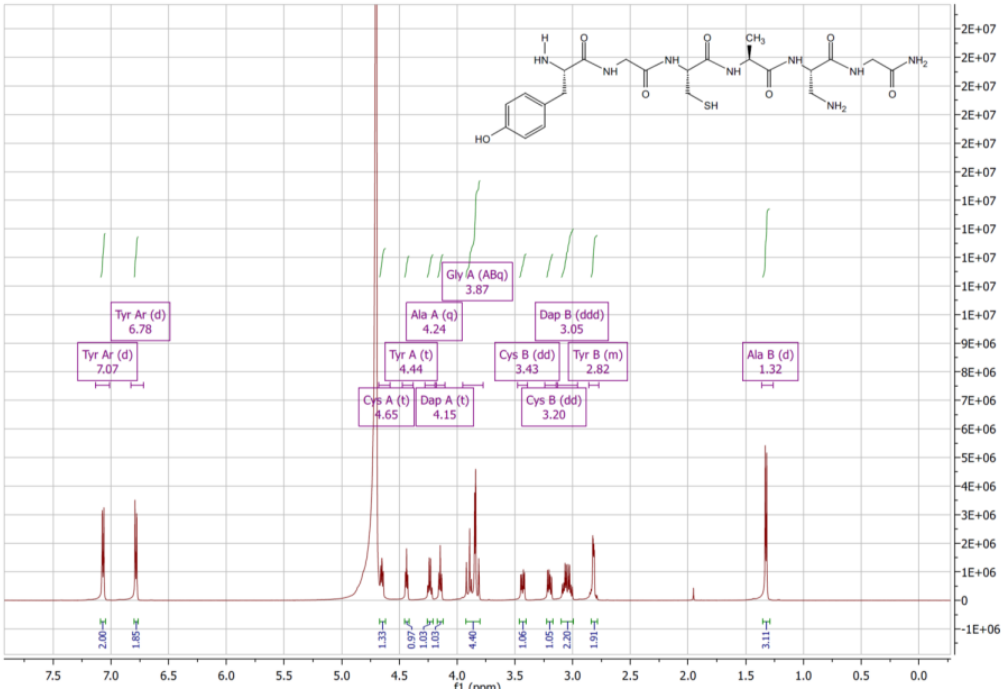


Figure S26. ^{1}H-NMR spectrum of **P1**

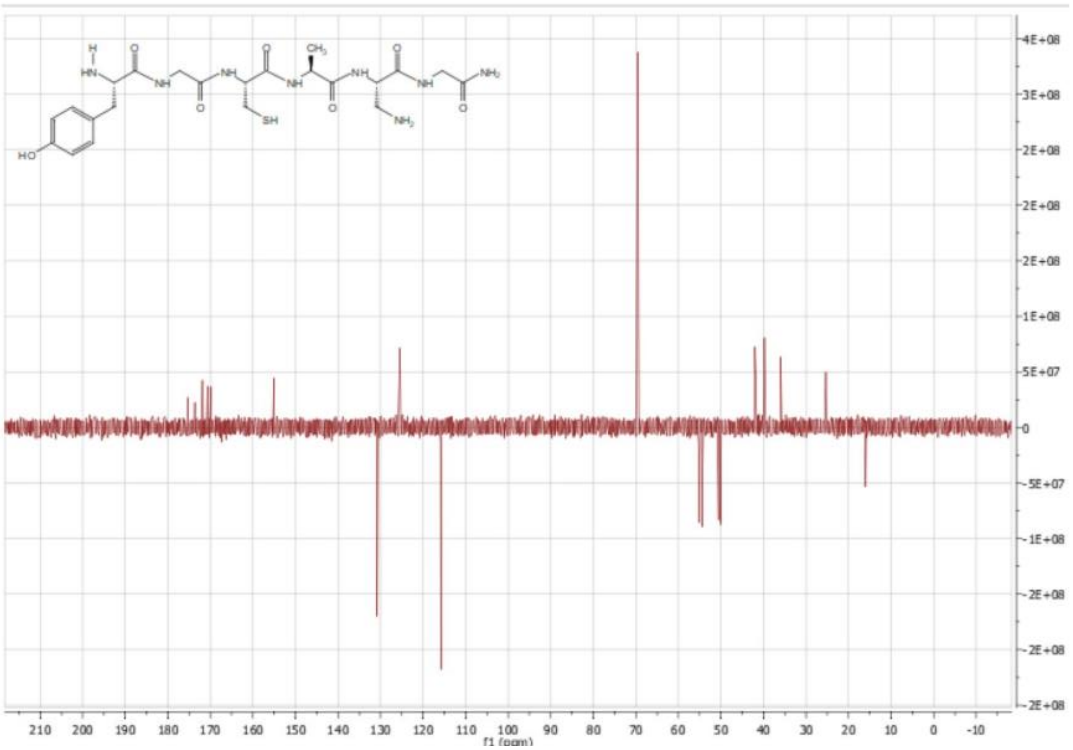


Figure S27. ^{13}C-NMR spectrum of **P1**

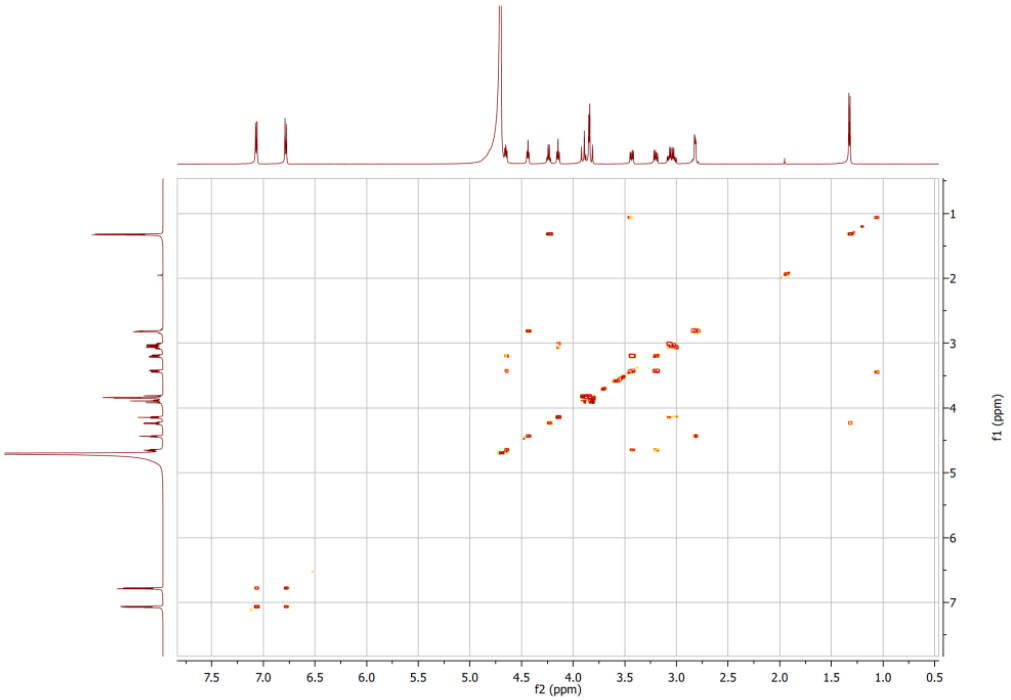


Figure S28. COSY spectrum of **P1**

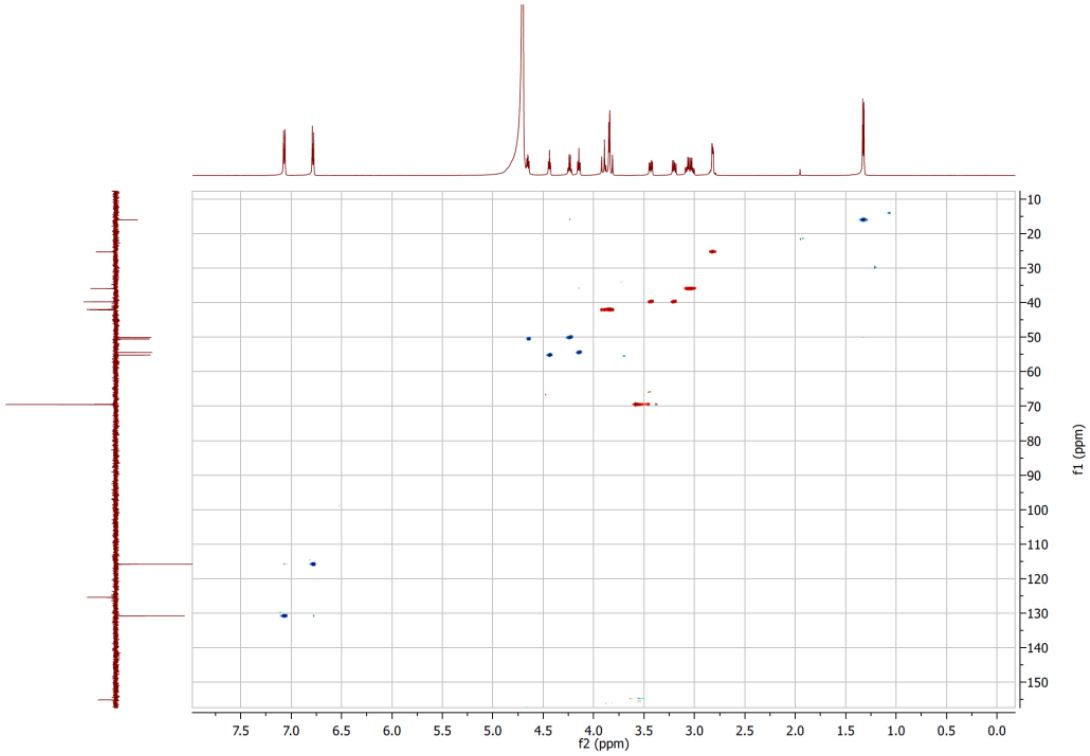


Figure S29. HSQC spectrum of **P1**

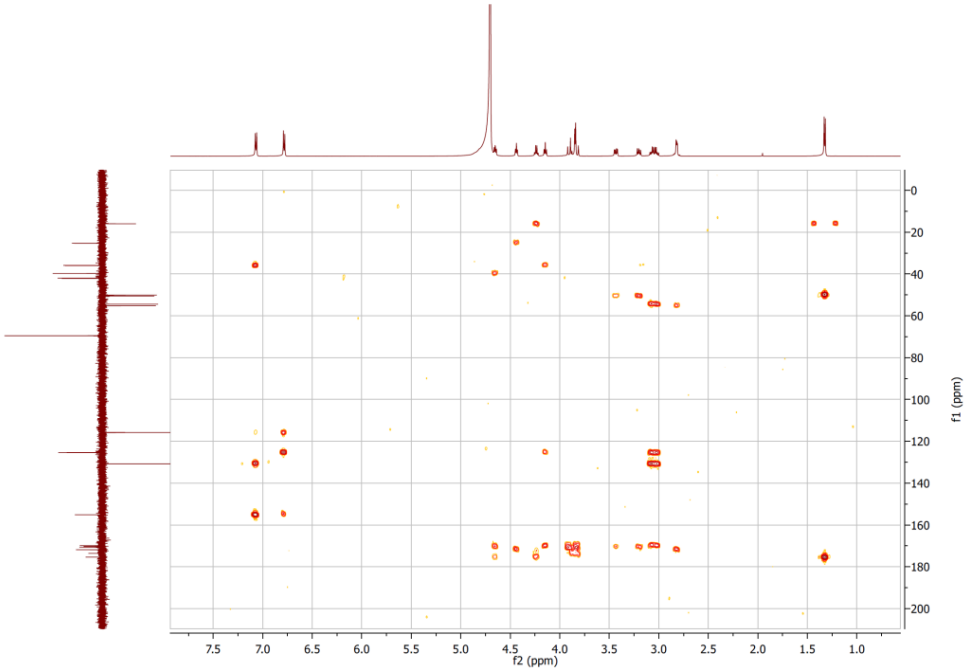


Figure S30. HMBC spectrum of **P1**

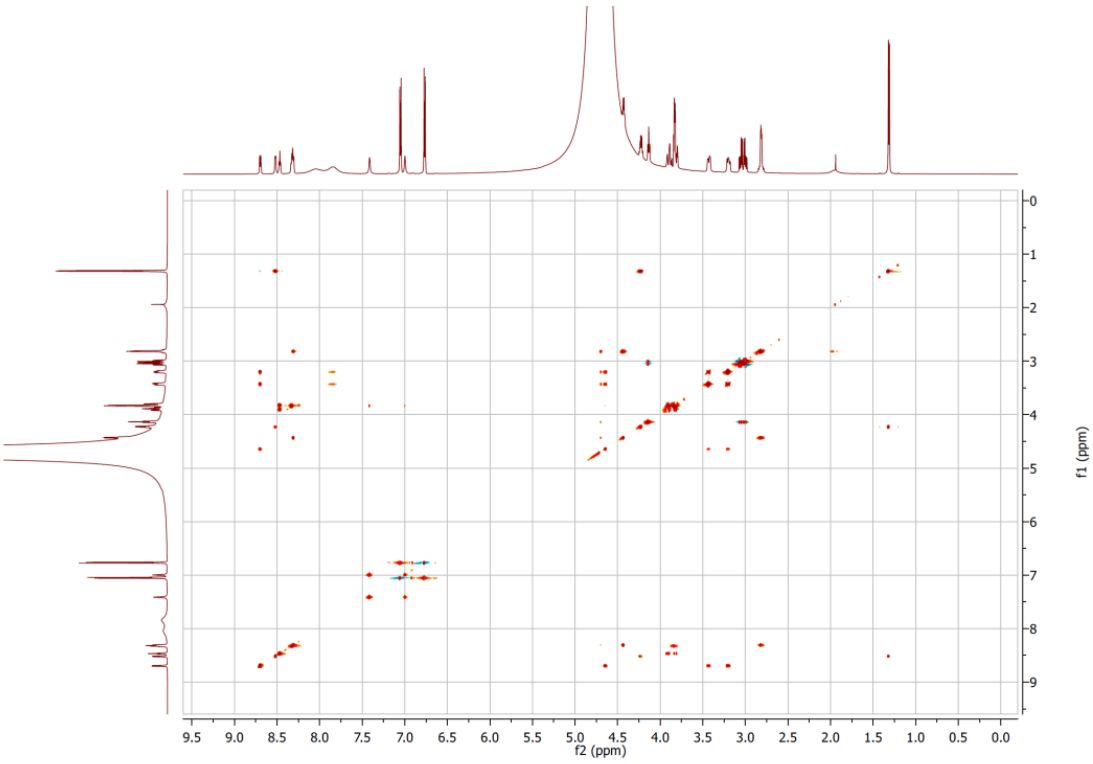


Figure S31. TOCSY spectrum of **P1**

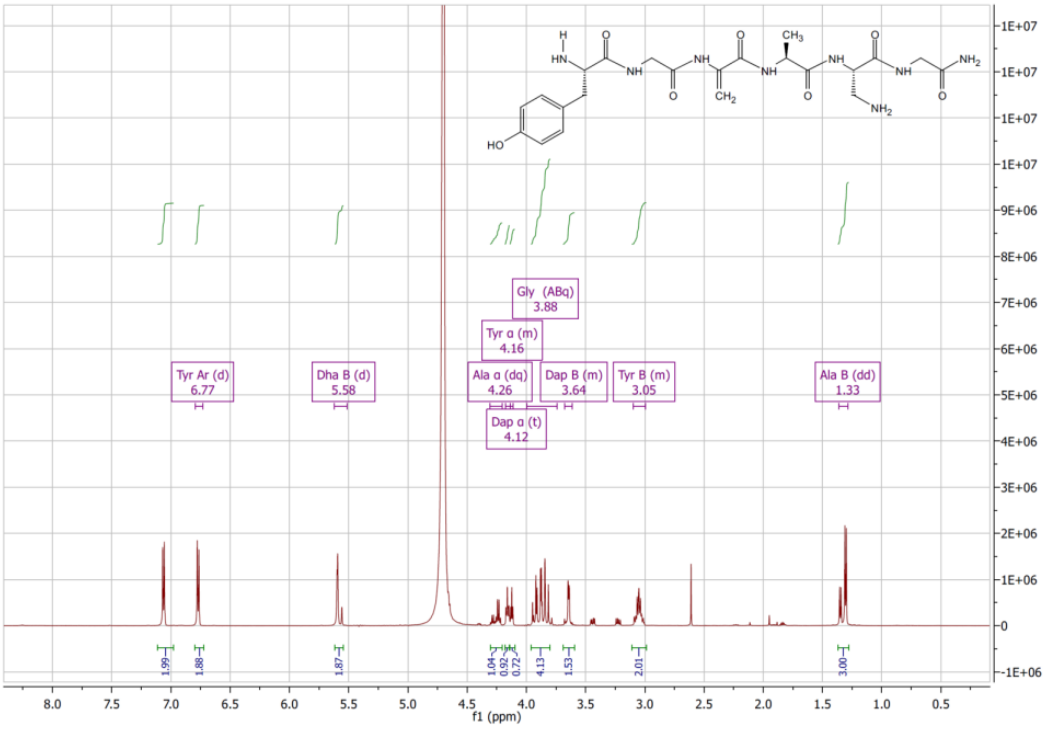


Figure S32. ^{1}H-NMR spectrum of **P2**

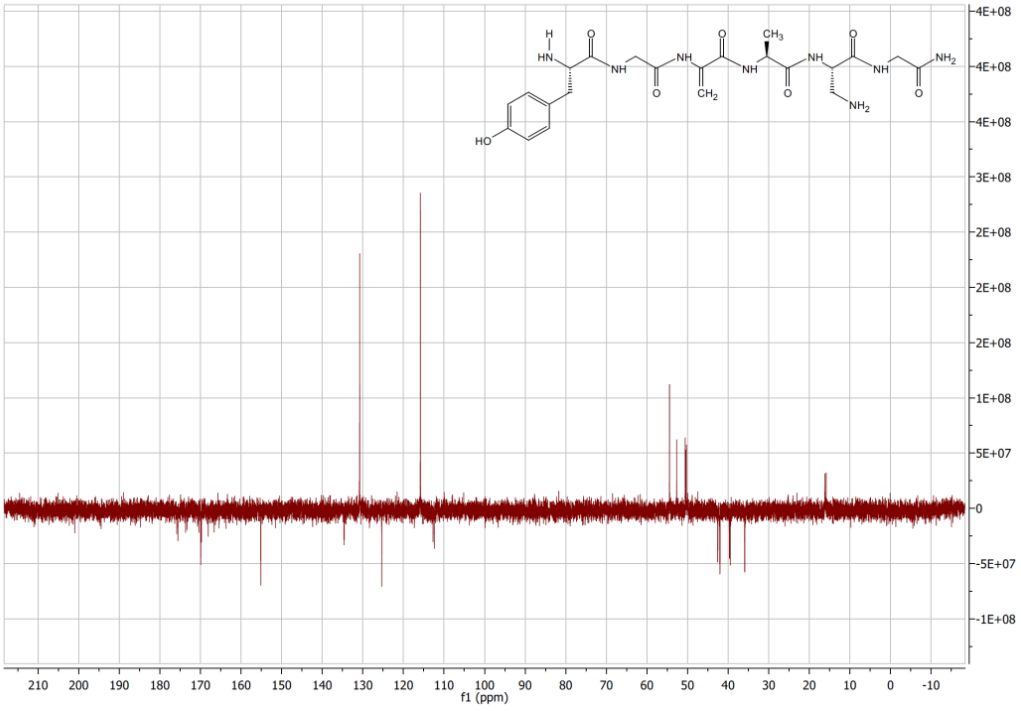


Figure S33. ^{13}C-NMR spectrum of **P2**

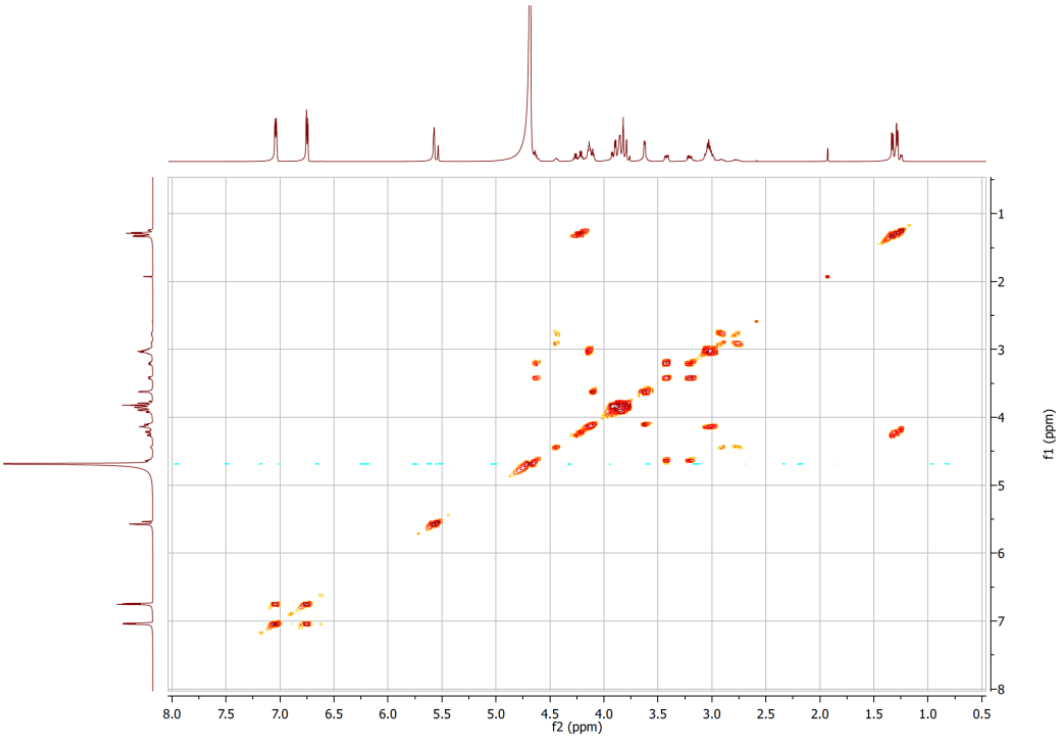


Figure S34. COSY spectrum of **P2**

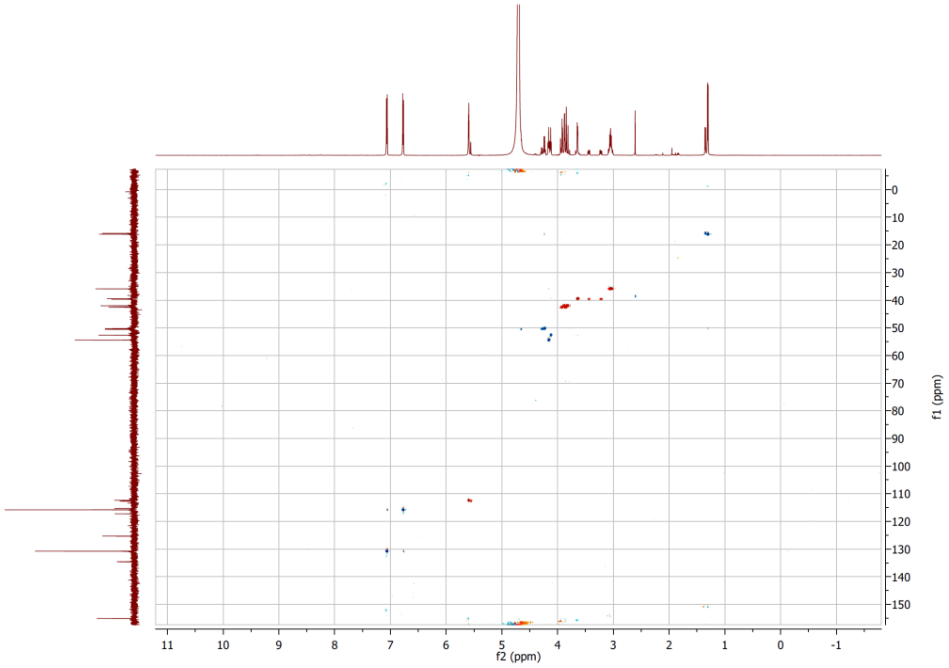


Figure S35. HSQC spectrum of **P2**

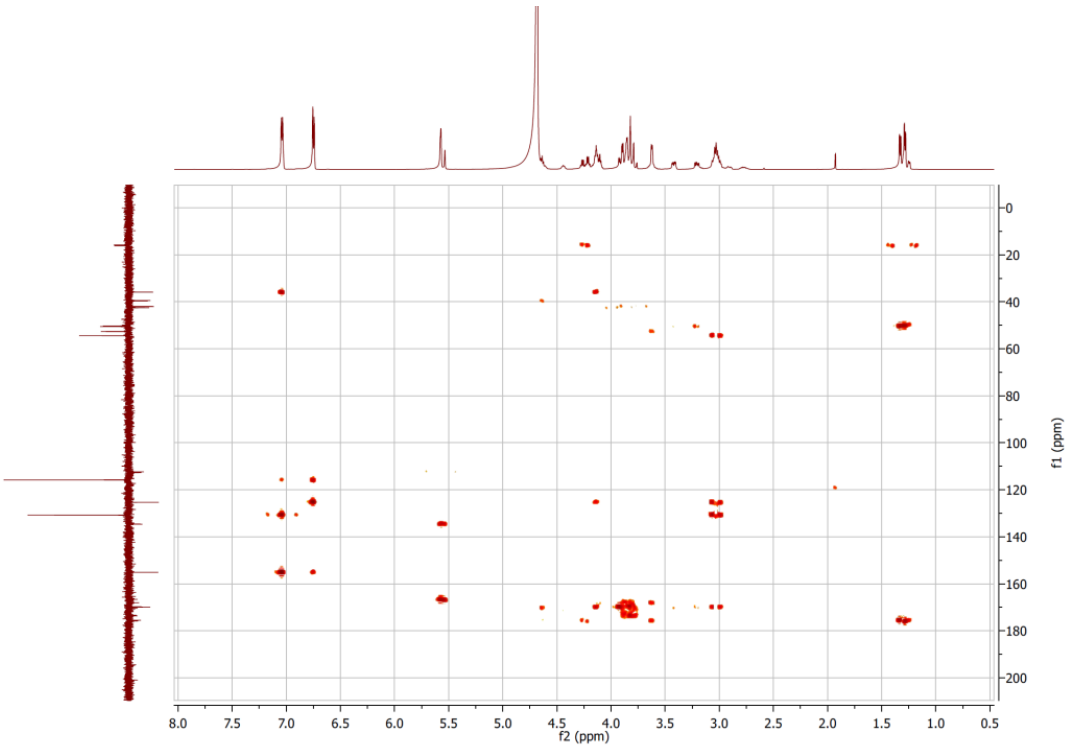


Figure S36. HMBC spectrum of **P2**

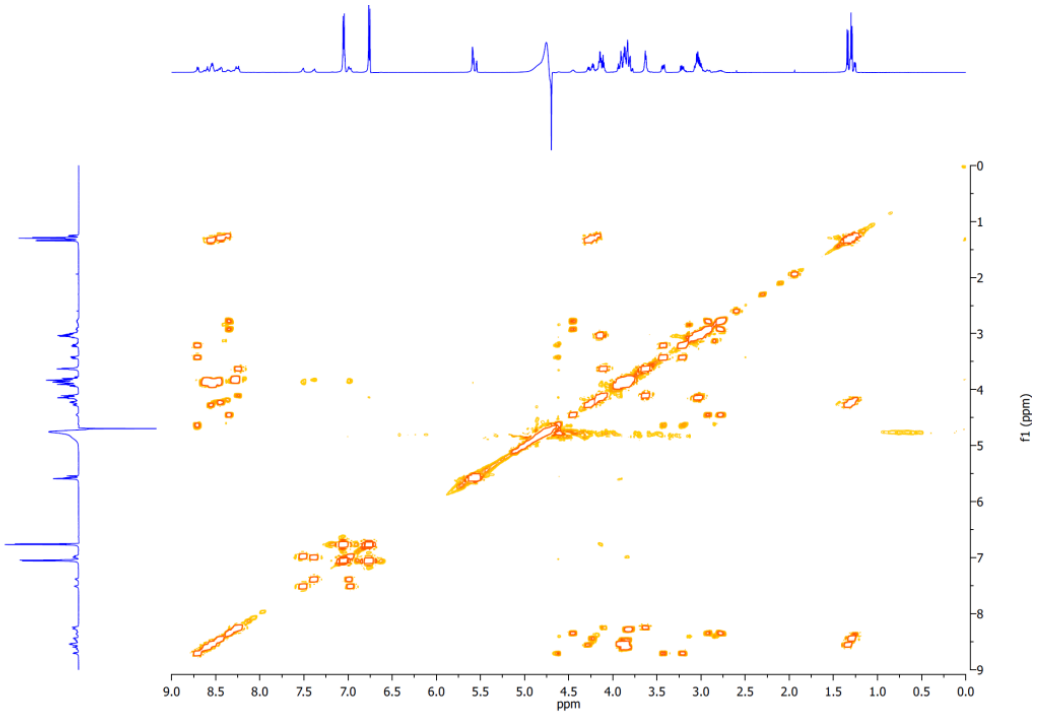


Figure S37. TOCSY spectrum of **P2**

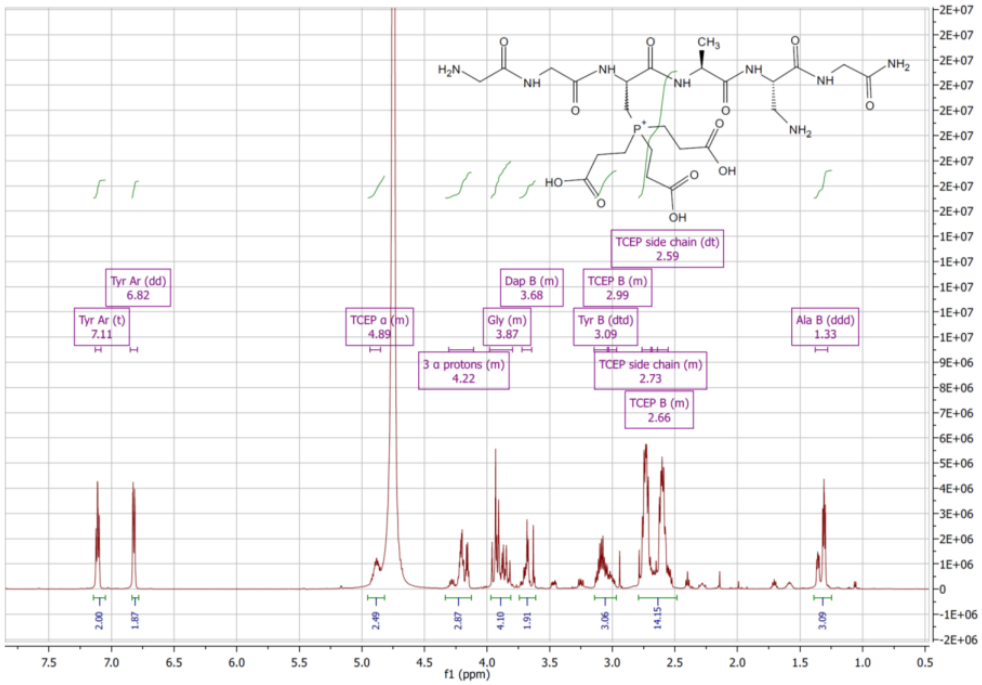


Figure S38. ^{1}H-NMR spectrum of **P3**

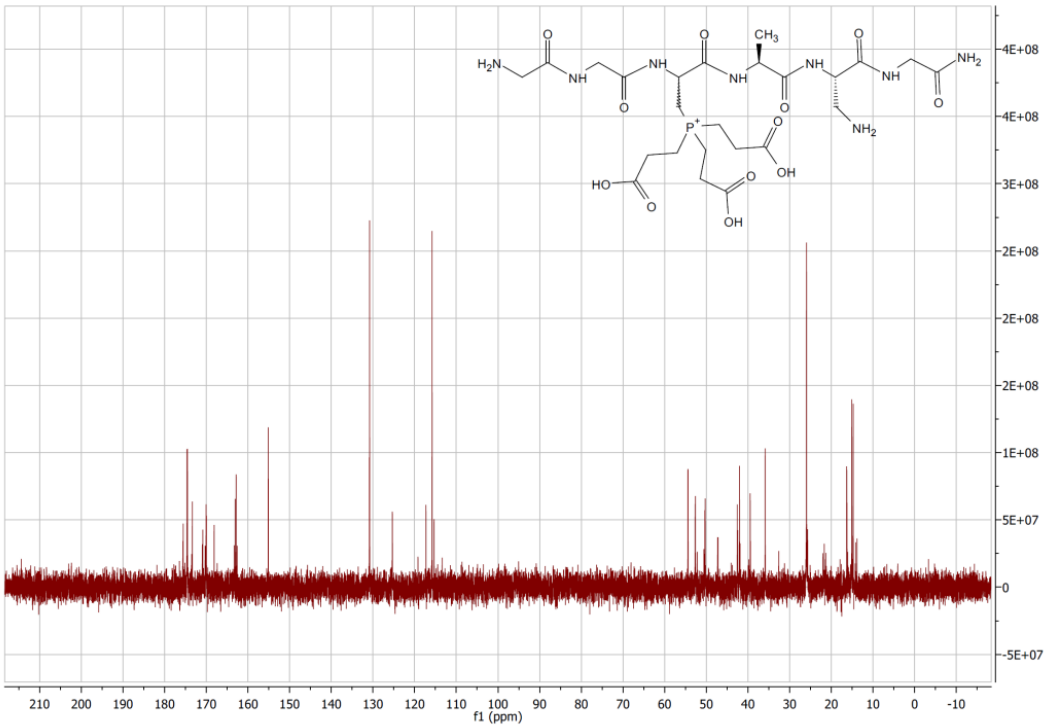


Figure S39. ^{13}C-NMR spectrum of **P3**

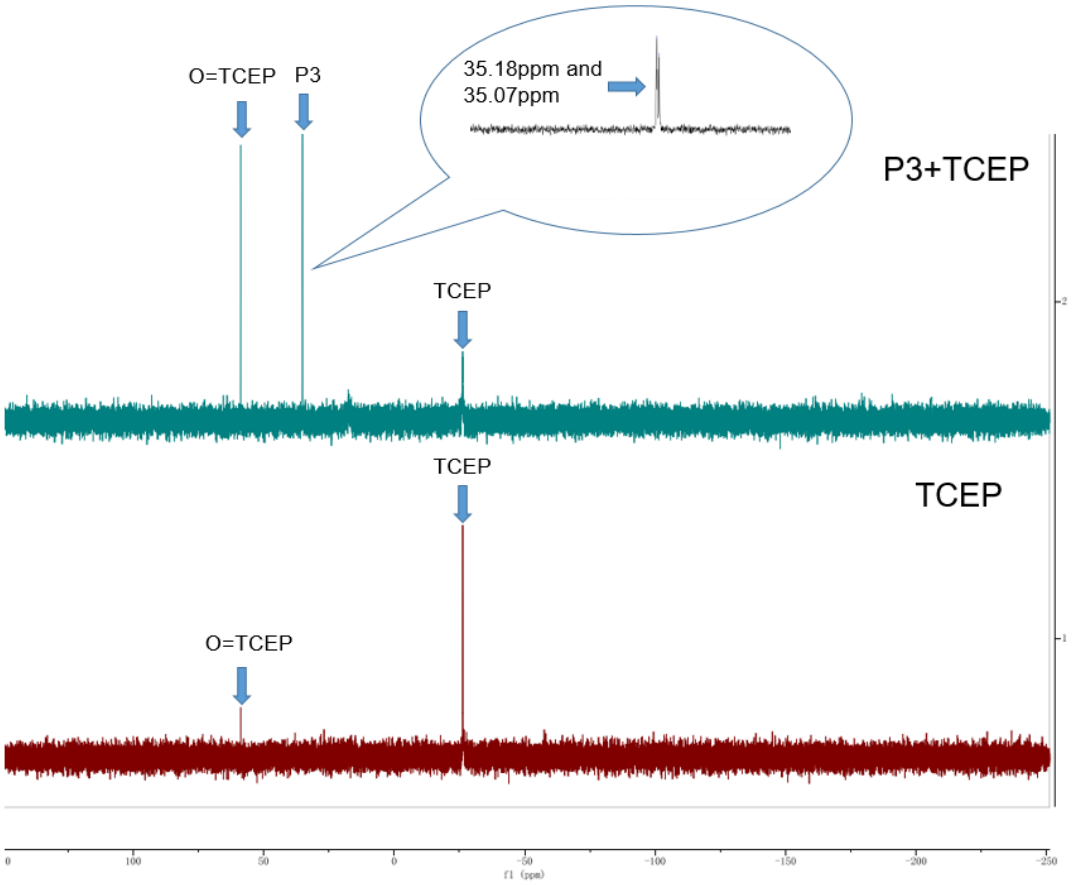


Figure S40. ^{31}P-NMR spectrum of unpurified **P3** and TCEP (expansion in inset to show diastereomers)

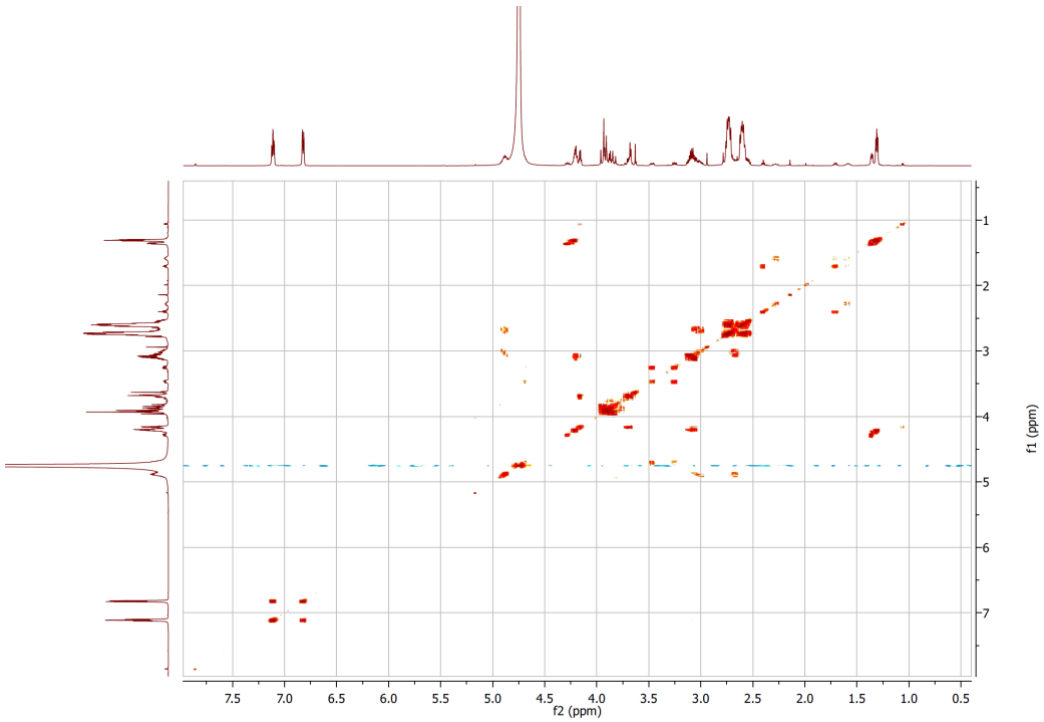


Figure S41. COSY spectrum of **P3**

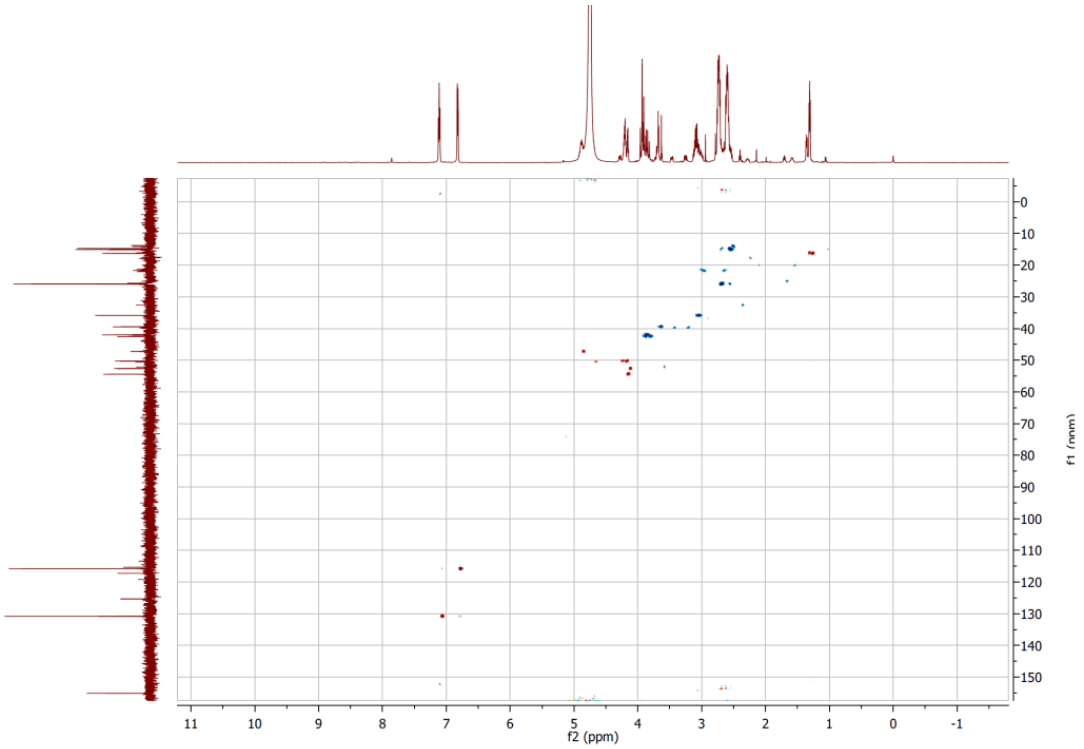


Figure S42. HSQC spectrum of **P3**

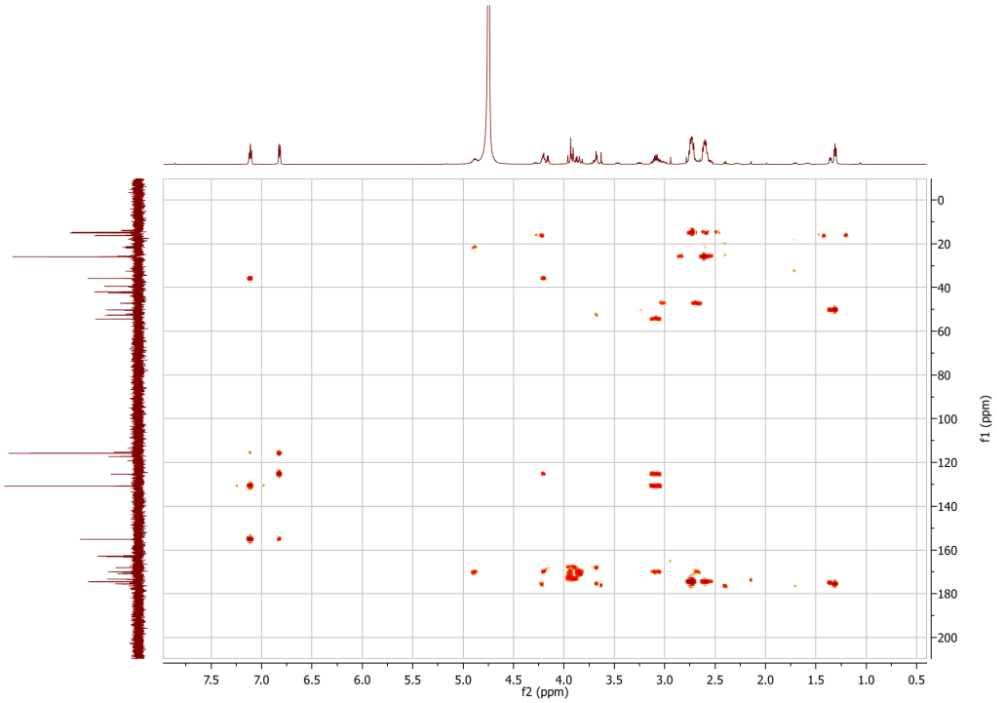


Figure S43. HMBC spectrum of **P3**

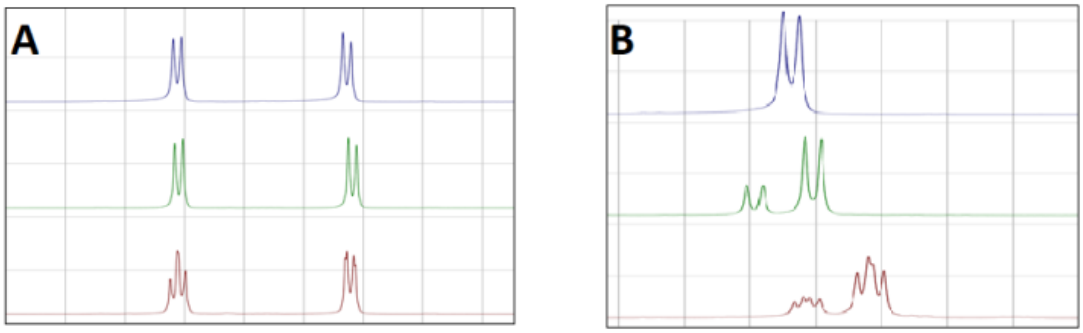


Figure S44. ^{1}H-NMR expansions of **P1** (blue, upper), **P2** (green, mid) and **P3** (red, lower) showing the tyrosine aromatic peaks (A) and the alanine methyl peaks (B), emphasising splitting from both conformers (**P2** and **P3**) and diastereomers (**P3**)

References

1. Jongkees, S. A. K.; Umemoto, S.; Suga, H., Linker-free incorporation of carbohydrates into in vitro displayed macrocyclic peptides. *Chemical Science* **2017,** *8*, 1474-1481.