Electronic Supplementary Material (ESI) for Chemical Communications.

Synthesis and incorporation of an advanced lipid peroxidation end-product building block into collagen mimetic peptides

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Supporting information

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General information

All reagents were used as supplied from commercial sources. Solvents for RP-HPLC were purchased as HPLC grade and used without further purification. Analytical thin-layer chromatography (TLC) was carried out using Kieselgel F_{254} 200 μm (Merck) silica plates. The compounds were then visualised by ultraviolet fluorescence. Column chromatography was performed using Kieselgel F_{254} S 63-100 μm silica gel with the indicated eluent. Infrared (IR) spectra were recorded on a Perkin Elmer Spectrum 100 infrared spectrometer and reported in wavenumbers (ν, cm^{-1}). High resolution mass spectra (HRMS) were recorded on a Bruker micrOTOFQ mass spectrometer.

Nuclear magnetic resonance (NMR) spectra were recorded at room temperature on Bruker AVANCE 300 spectrometer (^1H, 300 MHz; ^{13}C, 75 MHz). The multiplicities of ^1H signals are designated by the following abbreviations: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad; dd doublet of doublets; dt = doublet of triplets; dm = doublet of multiplets. All coupling constants J = are reported in Hertz. All ^{13}C NMR spectra were acquired using broadband decoupled mode, and assignments were determined using DEPT sequences.

Chromatographic separations were performed using a Thermo Scientific Dionex Ultimate 3000 UHPLC using a XTerra® MS C18 (5 μ; 4.6 x 150 mm) column and a linear gradient of 1-61% B over 20 min (*ca*. 3% B per min) at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*).

Semi-preparative RP-HPLC was performed on a Waters 600 System using a semi-preparative column (XTerra® Prep. C18, 300 mm × 19 mm; 10, Waters) at a flow rate of 10 mL min^{-1} using a shallow linear gradient, where solvent A was 0.1% TFA in H_2O and B was 0.1% TFA in acetonitrile.

LCMS spectra were acquired on either an Agilent Technologies 1120 Compact LC equipped with a Hewlett-Packard 1100 MSD mass spectrometer or an Agilent Technologies 1260 Infinity LC equipped with an Agilent Technologies 6120 Quadrupole mass spectrometer. An analytical column (Agilent C3, 150 mm x 3.0 mm, 3.5 μm) was used at a flow rate of 0.3 mL min^{-1} using a linear gradient of 5% B to 65% B over 20 min, where solvent A was 0.1% formic acid in H_2O and B was 0.1% formic acid in acetonitrile.

Procedures for Building Block Syntheses

1. Synthesis of building blocks

To a solution of Fmoc-Lys(Boc)-OH **1** (4 g, 8.53 mmol) in dichloromethane (20 mL) was added trifluoroacetic acid (20 mL) portionwise at room temperature and the mixture stirred for 1 h. The reaction was concentrated *in vacuo* to afford amine **2**. To a solution of crude amine **2** in dichloromethane (60 mL) was added trifluoroacetic acid (200 μL) and acrolein (11.4 mL, 170 mmol) and the solution heated under reflux for 1 h. The reaction was concentrated *in vacuo* and purified by flash column chromatography on silica gel (methanol then methanol:acetic acid 4:1 *v*/*v*) to afford MP-lysine **6** (0.6 g, 16% over two steps from compound **1**) as a brown oil. An analytical sample was prepared by purification of compound **6** by RP-HPLC using a XTerra®Prep MS C18 (Waters, 10 μ 19 × 300 mm) at a flow rate of 8.0 mL/min. Buffer A:. H_2O containing 0.1% TFA (*v*/*v*), Buffer B: acetonitrile containing 0.1% TFA (*v*/*v*) as an eluent, followed by lyophilization of the product.

$[\alpha]_D^{22}$ -5.83 (*c* 0.01 in CH_3OH); $\boldsymbol{\nu_{max}}$ (neat)/cm^{-1} 3324, 2949, 2837, 1648, 1449, 1406, 1203, 1112; $\boldsymbol{\delta_H}$ (300 MHz; $CDCl_3$:CD_3OD, 9:1 *v*/*v*) 8.79-8.72 (m, 2H, Ar-*H*), 8.16 (d, *J* = 7.95 Hz, 1H, Ar-*H*), 7.89-7.82 (m, 1H, Ar-*H*), 7.76 (d, *J* = 7.61 Hz, 2H, Ar-*H*), 7.60 (t, *J* = 6.33 Hz, 2H, Ar-*H*), 7.40 (t, *J* = 7.12 Hz, 2H, Ar-*H*), 7.30 (dt, J_1 = 1.08 J_2 = 7.45 Hz, 2H, Ar-*H*), 4.62-4.53 (m, 2H, C*H*$_2$NAr), 4.41-4.29 (m, 2H, Fmoc-C*H*$_2$), 4.28-4.17 (m, 2H, α-C*H* and Fmoc-C*H*), 2.54 (s, 3H, Nar-C*H*$_3$), 2.06-1.77 (m, 4H, β-C*H*$_2$ and C*H*$_2$CH$_2$NAr), 1.51-1.40 (m, 2H, C*H*$_2$CH$_2$CH$_2$NAr); $\boldsymbol{\delta_C}$ (75 MHz; $CDCl_3$:CD_3OD, 9:1 *v*/*v*) 173.8 (quat., *C*OOH), 156.5 (quat., *C*ONH), 145.6 (CH, Ar*C*H), 143.9 (CH, Ar*C*H), 143.7 (quat., Ar), 143.6 (quat., Ar), 141.7 (CH, Ar*C*H), 141.1 (quat., Ar), 139.9 (quat., Ar), 127.68 (CH, Ar*C*H), 127.65 (*CH,* Ar*C*H), 127.04 (CH, Ar*C*H), 127.01 (CH, Ar*C*H), 125.02 (CH, Ar*C*H), 124.9 (CH, Ar*C*H), 119.8 (CH, Ar*C*H), 66.8 (CH_2, Fmoc-*C*H_2), 61.4 (CH_2, *C*H_2NAr), 53.1 (CH, α-*C*H), 46.9 (CH, Fmoc-*C*H), 31.0 (CH_2, β-*C*H_2), 30.5 (CH_2, *C*H_2CH$_2$NAr), 21.5 (CH_2, *C*H_2CH$_2$CH$_2$NAr), 18.1 (CH_3, NAr-*C*H_3); HRMS (ESI): calcd. for [$C_{27}H_{29}N_2O_4$] requires 445.2122; M^+ found 445.2118.

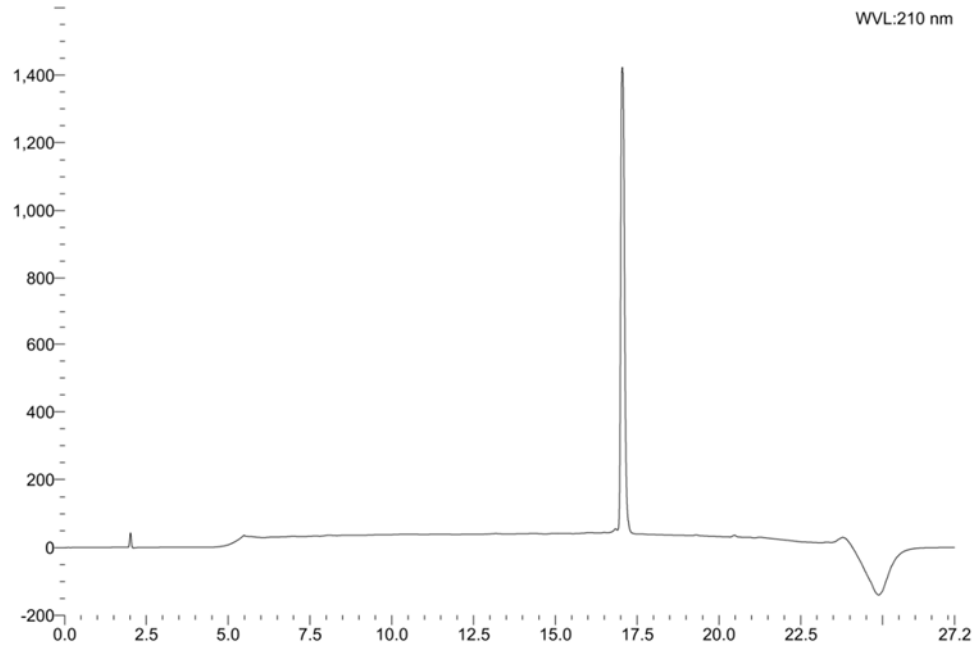


Figure SI-1: HPLC chromatogram of compound **6**; XTerra® MS C18 (5 µ; 4.6 x 150 mm) and a linear gradient of 1-61% B over 20 min (*ca*. 3% B per min) at a flow rate of 1.0 mL/min. Buffer A: H_2O containing 0.1% TFA (*v/v*); Buffer B: acetonitrile containing 0.1% TFA (*v/v*). Expected compound **6** eluted at 17min.

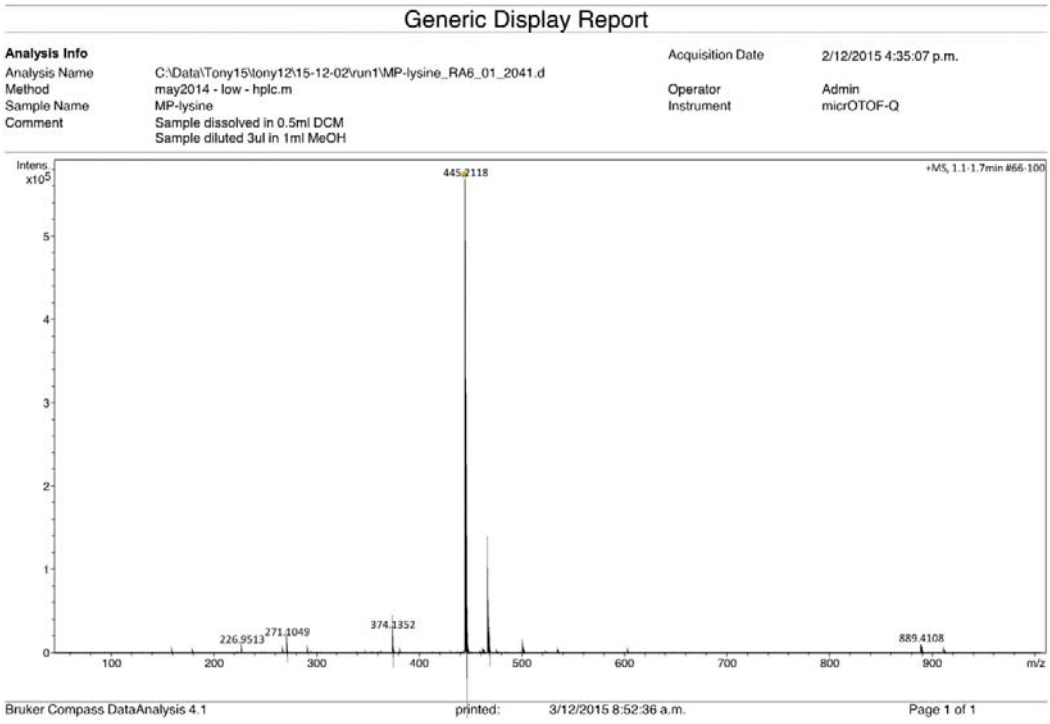


Figure SI-2: HRMS profile of compound **6.**

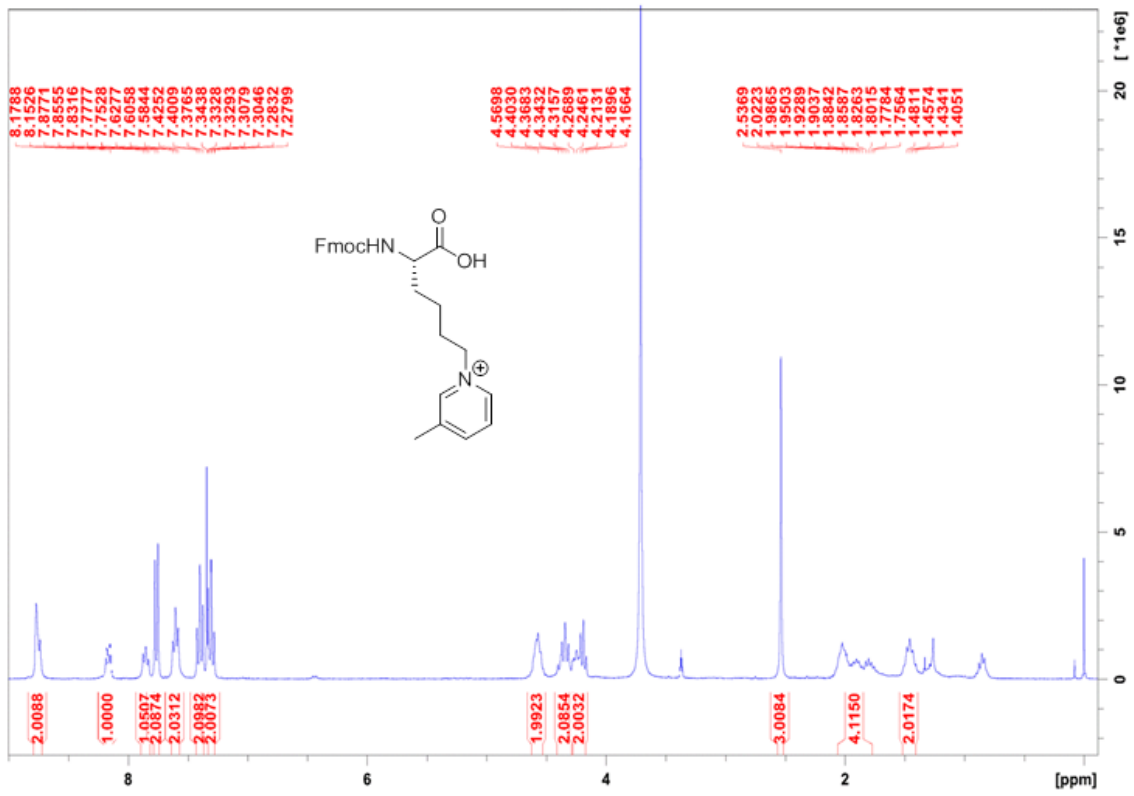


Figure SI-3: ^{1}H NMR spectrum of compound **6**

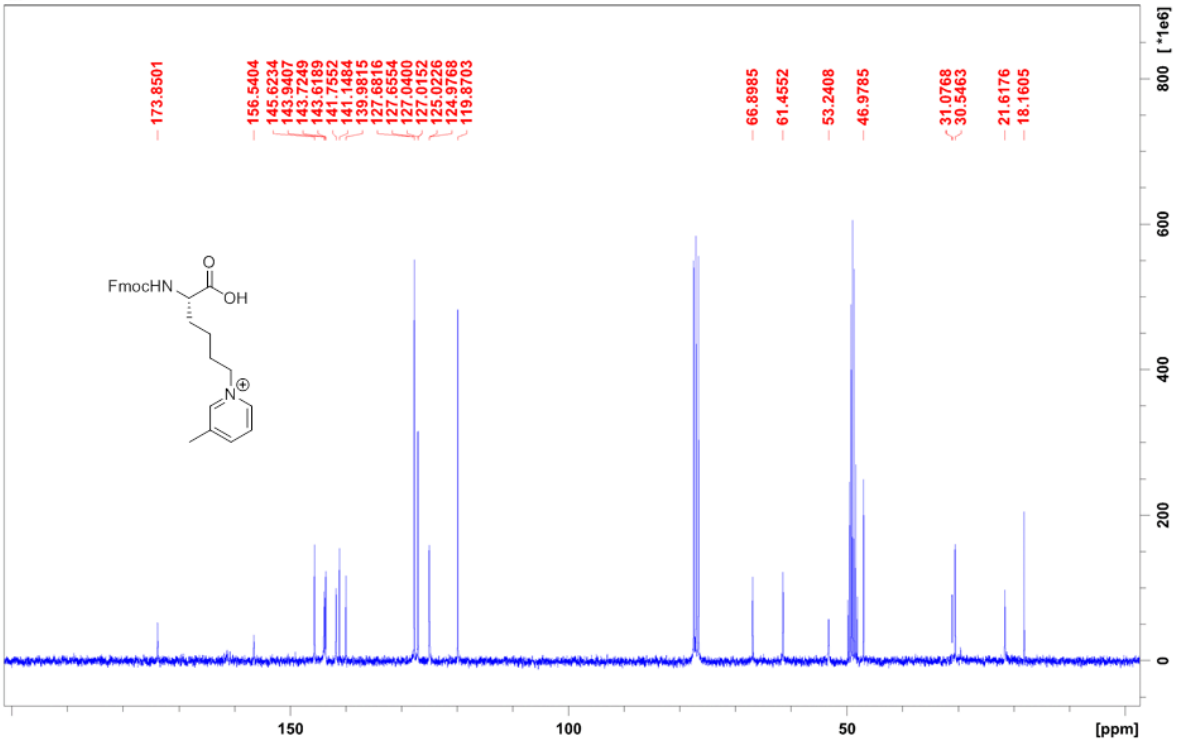


Figure SI-4: ^{13}C NMR spectrum of compound **6**

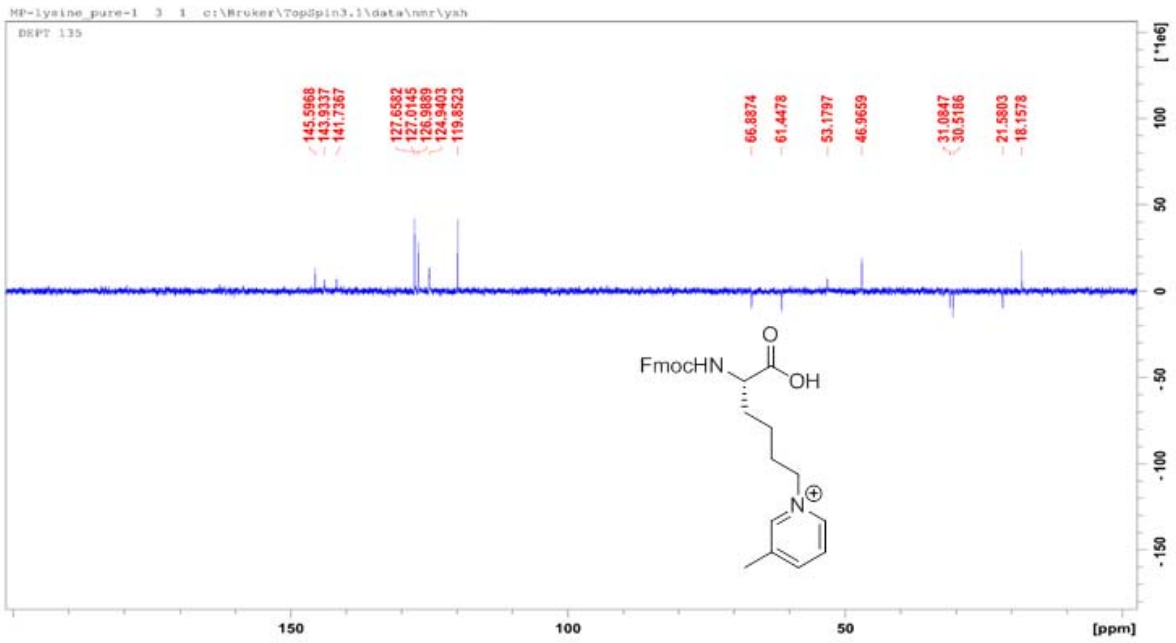


Figure SI-5: DEPT spectrum of compound **6.**

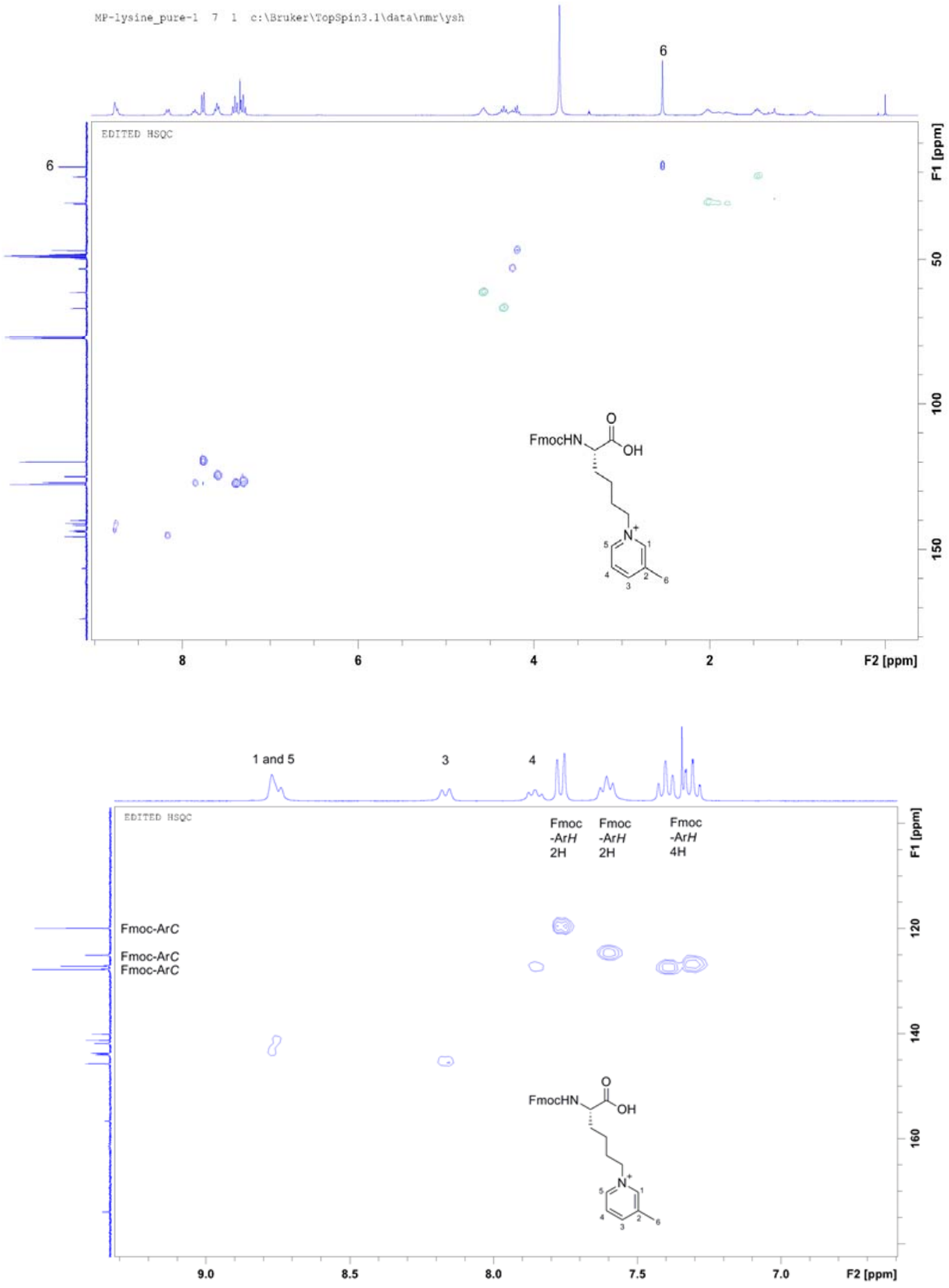


Figure SI-6: HSQC spectrum of compound **6.**

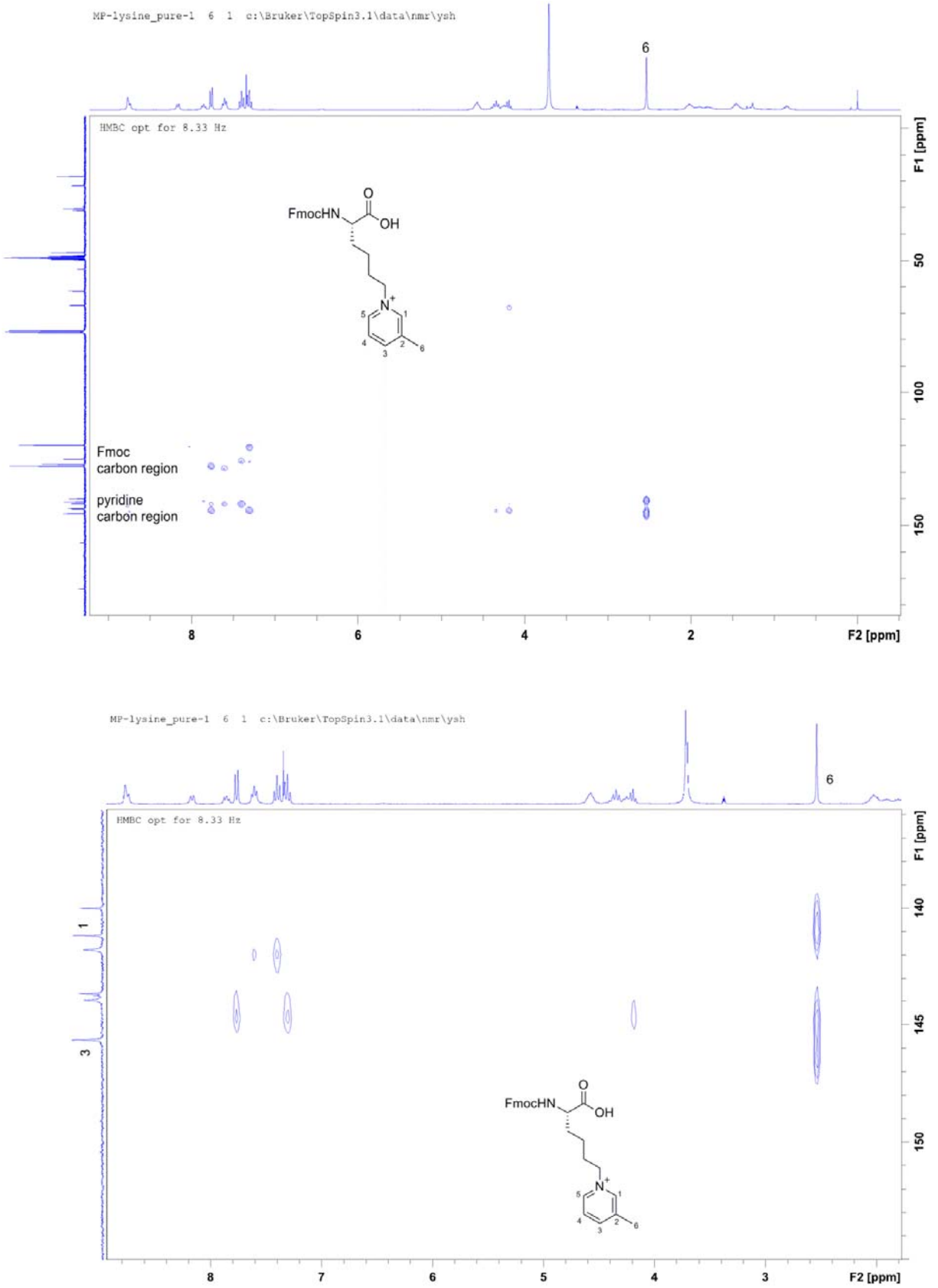
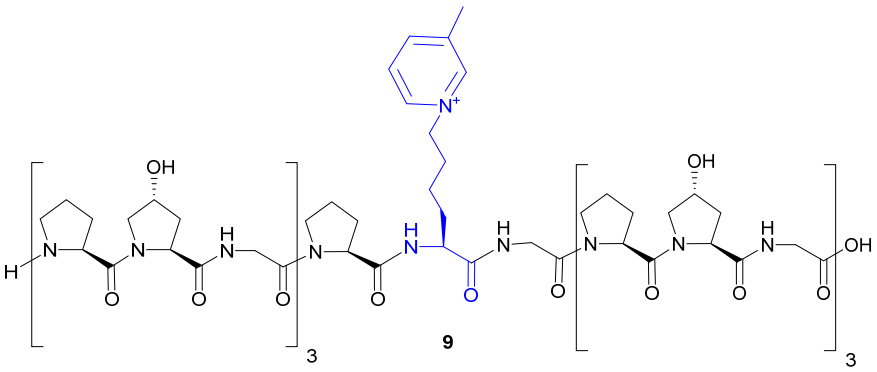


Figure SI-7: HMBC spectrum of compound **6.**

Procedures for peptide synthesis

MP-lysine CMP (11)

Aminomethyl polystyrene resin (117.6 mg, 0.1 mmol, loading = 0.85 mmol/g) was swollen in DMF for 10 min. The resin was shaken with Fmoc-Gly-HMPP-linker (2 eq.) and N, N'-diisopropylcarbodiimide (DIC) (2 eq.) for 3 h in 1:9 DMF/DCM (v/v) (complete by Kaiser test). Solid phase peptide synthesis was carried out using Biotage Alstra Peptide synthesiser (Biotage AB, Uppsala, Sweden) on 0.1 mmol scale using the Fmoc/tBu strategy. The Fmoc group was deprotected with 20% v/v piperidine in DMF for 30 seconds followed by 3 min using a microwave power of 60 W for both deprotections. The maximum temperature for both deprotections was set at 70 °C. Unless noted, coupling of individual amino acids was performed with 5 equivalents of Fmoc protected amino acid in DMF (0.2 M), 4.9 equivalents of *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) in DMF (0.45 M) and 10 equivalents of *N*-Methylmorpholine (NMM) (2 M). Couplings were performed for 5 min at 25 W at a maximum temperature of 75 °C to achieve peptide **11** (Scheme 2). To the resin-bound peptide **11** was added a mixture of MP-lysine building block (5 eq), HATU (4eq) and iPr_2EtN (10eq) in DMF (2mL). The reaction mixture was agitated at room temperature for 1 h, after which the resin was filtered and washed with DMF (3 x 5 mL). The resulting peptide **12** was treated with a solution of 20% v/v piperidine in DMF (2 mL) and the mixture agitated at room temperature for 10 min, filtered and repeated once for a further 10 min. The resin was filtered and washed with DMF (3 x 5 mL). The resulting peptide was elongated using microwave SPPS, HATU/ iPr_2EtN for coupling and 20% v/v piperidine in DMF for Fmoc removal to obtain desired resin-bound peptide **13.** The peptide was cleaved from the resin using TFA: TIPS: H_2O (95:2.5:2.5, v/v/v) at room temperature for 2 h. The crude peptide was precipitated with cold diethyl ether, isolated by centrifugation, washed in cold diethyl ether, dissolved in 1:1 (v/v) MeCN/H_2O containing 0.1% TFA and lyophilised, and analysed by LC-MS. Purification by semi-preparative RP-HPLC afford peptide **9** (29.8 mg, yield:15.1%). ESI-MS: $[M+H]^{2+}$ calc 989.9, obs 990.6. Deconvolution yields a mass of 1979.06 ±0.48 Da. Calculated mass 1979.95 Da.



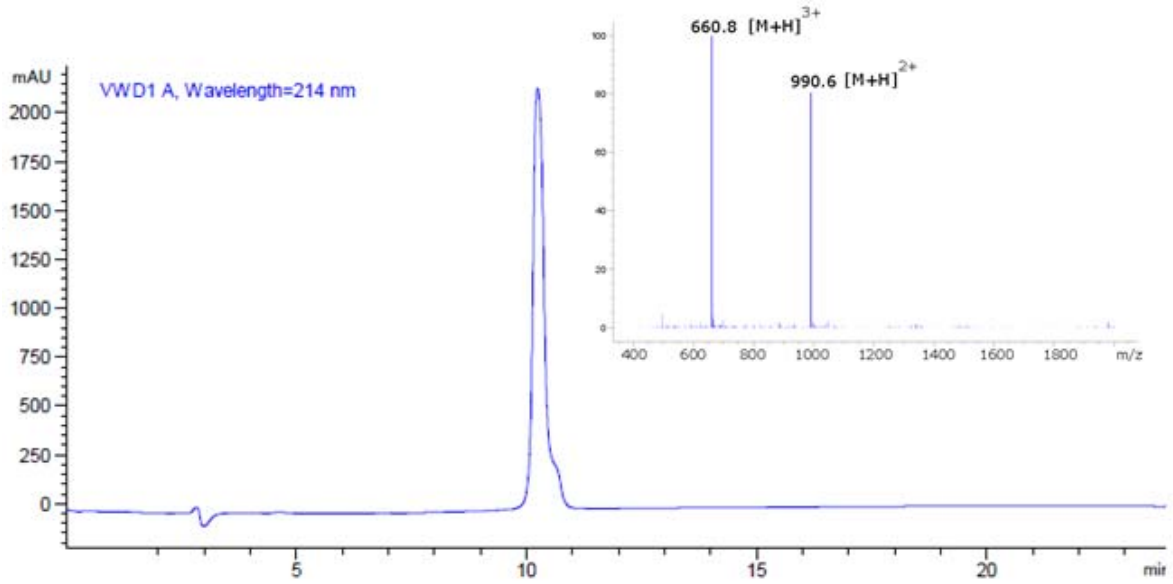
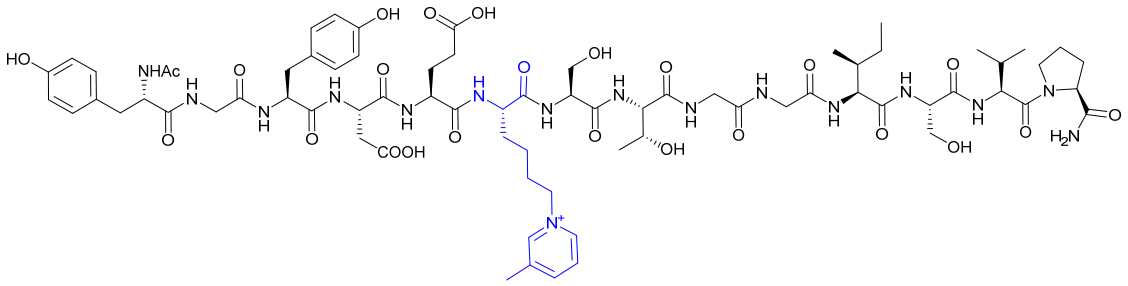


Figure SI-8: LC-MS profile of pure peptide **9** (ca. 98% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax-C3, (150 mm x 3.0 mm, 3.5 µm), linear gradient of 5% B to 65% B (ca. 3% B/min), 0.3 mL/min. m/z $[M+H]^{2+}$ calcd: 989.9; found: 990.6. Expected compound **9** eluted at 10 min.

MP-lysine CTP (14)

To Aminomethyl polystyrene resin (117.6 mg, 0.1mmol, loading = 0.85mmol/g) pre-swollen in DMF (2 mL, 10 min), was added a solution of Rink amide linker (4eq), DIC (4 eq) and 6-chloro-1-hydroxy-1*H*-benzotriazole (6-Cl-HOBt) (4 eq.) in DMF. The reaction mixture was agitated at room temperature for 3 h. The resin was filtered and washed with DMF (2 x 5 mL). SPPS was performed manually using the Fmoc/tBu strategy on 0.1 mmol scale. The Fmoc group was deprotected with 20% piperidine in DMF for 5 then 10 minutes at room temperature. Fmoc amino acids (4 eq) were coupled with HATU (3.9 eq) and iPr_2EtN (10 eq) in DMF (2mL) for 30 min at room temperature to afford peptide **16** (Scheme 3). Fmoc-MP-lysine (5 eq), was then coupled to the resin-bound peptide **16** using HATU (4 eq) and iPr_2EtN (10 eq) in DMF (2 mL) for 1h, after which the resin was filtered and washed with DMF (3 x 5 mL). The resulting peptide **17** was treated with a solution of 5% piperazine/0.1 M Cl- HOBt in DMF (2 mL) and the mixture agitated at room temperature for 5 minutes, filtered and repeated once for a further 10 min. The resin was filtered and washed with DMF (3 x 5 mL). The resultant resin bond peptide was subjected to further SPPS using HATU (3.9 eq) and iPr_2EtN (10 eq) in DMF (2 ml) for coupling and 5% piperazine/0.1 M Cl- HOBt in DMF (2 mL) for Fmoc removal. After removal of the final Fmoc group, the amine was acetylated with Ac_2O in the presence of iPr_2EtN at room temperature for 20 min. The resin was filtered, washed in DMF and the peptide cleaved from the resin with TFA: TIPS: H2O (95:2.5:2.5, v/v/v). The resultant peptide was precipitated with cold diethyl ether, isolated by centrifugation, washed in cold diethyl ether, dissolved in 1:1 (v/v) $MeCN/H_2O$ containing 0.1% TFA and lyophilised, and analysed by LC-MS and purified by semi-preparative RP-HPLC to afford peptide **15** (9 mg, yield: 5.5%). ESI-MS: $[M+H]^{2+}$ calc 795.8, obs 795.4. Deconvolution yields a mass of 1589 ±0.35 Da. Calculated mass 1589.75 Da.



15

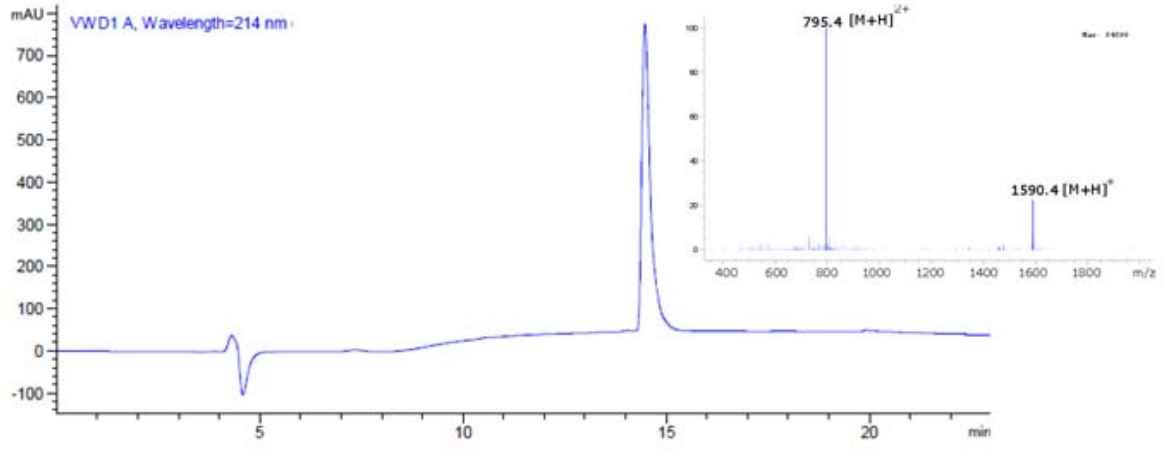


Figure SI-9: LC-MS profile of pure peptide **15** (ca. 97% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax-C3, (150 mm x 3.0 mm, 3.5 µm), linear gradient of 5% B to 65% B (ca. 3% B/min), 0.3 mL/min. m/z $[M+H]^{2+}$ calcd: 795.8; found: 795.4.Expected compound **15** eluted at 15 min.

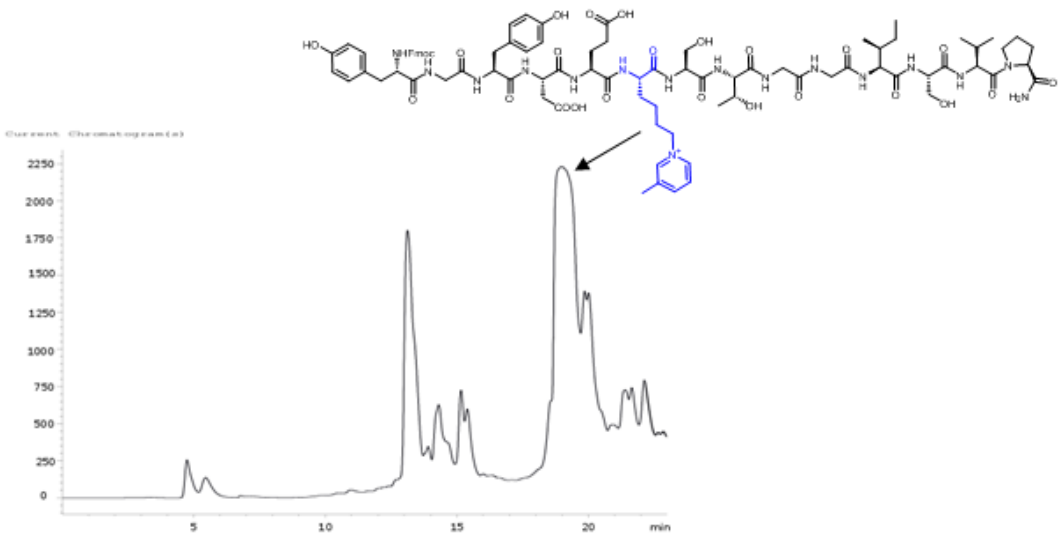


Figure SI-10: HPLC profile of crude MP-lysine modified CTP when 20% piperidine in DMF was used as the Fmoc-deprotecting agent. The profile shows 18% yield as judged by peak area of HPLC at 210 nm; Agilent Zorbax-C3, (150 mm x 3.0 mm, 3.5 µm), linear gradient of 5% B to 65% B (ca. 3% B/min), 0.3 mL/min. m/z $[M+H]^{2+}$ calcd: 795.8; found: 795.4.

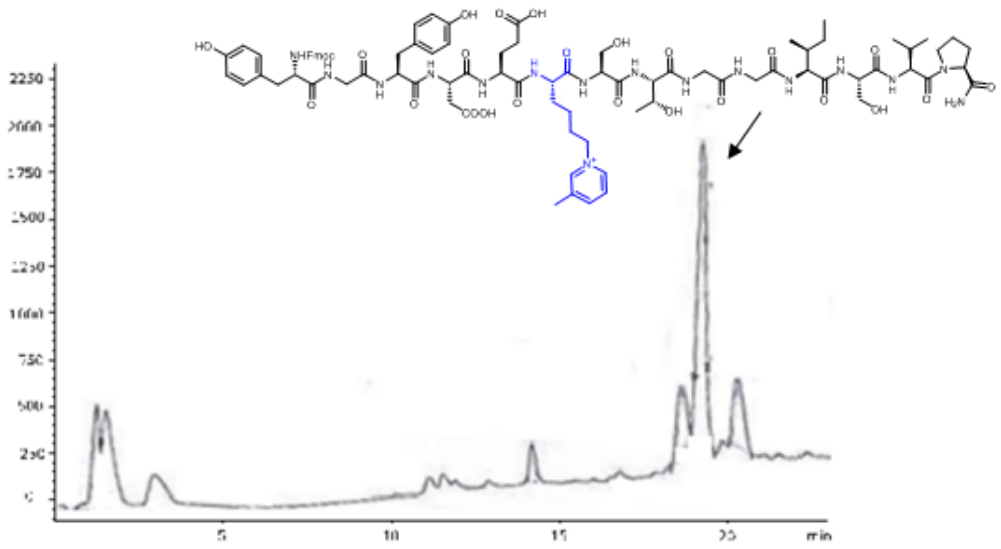


Figure SI-11: HPLC profile of crude MP-lysine modified CTP, when 5% piperazine/0.1 M Cl-HOBt in was used as the Fmoc-deprotecting agent. The profile shows 45% yield as judged by peak area of HPLC at 210 nm; Agilent Zorbax-C3, (150 mm x 3.0 mm, 3.5 µm), linear gradient of 5% B to 65% B (ca. 3% B/min), 0.3 mL/min. m/z $[M+H]^{2+}$ calcd: 795.8; found: 795.4.

Procedure for Bovine Trypsin Digest

Bovine trypsin (0.3 mg, type XI, 9090 units/mg, Sigma) was dissolved in H_2O (1 mL), 3.3 µL (9 units) of this solution diluted to 1 mL using Tris buffer (pH 8.0) and incubated at 37 °C for 30 min. Peptide 15 (0.21 µmol) was added in one portion and 50 µl aliquots removed every minute, quenched with 1 M HCl (50 µl), and analyzed by analytical RP-HPLC (Dionex ASI-100) at 210 nm using a Luna C18 column (3µ; 150 x 3 mm; Phenomenex) at 0.3 mL/min using a linear gradient of 5% B to 65% B over 20 min.

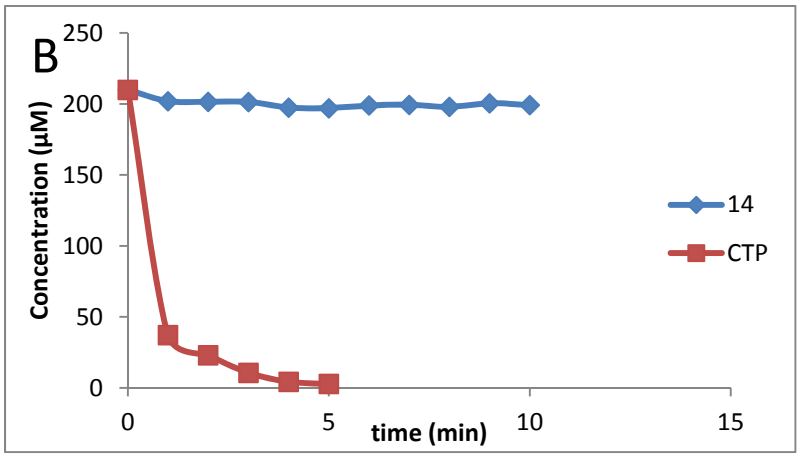


Figure SI-12: Concentrations of MP-lysine modified CTP **15** and native CTP **14** during the trypsin digest, extrapolated from HPLC.

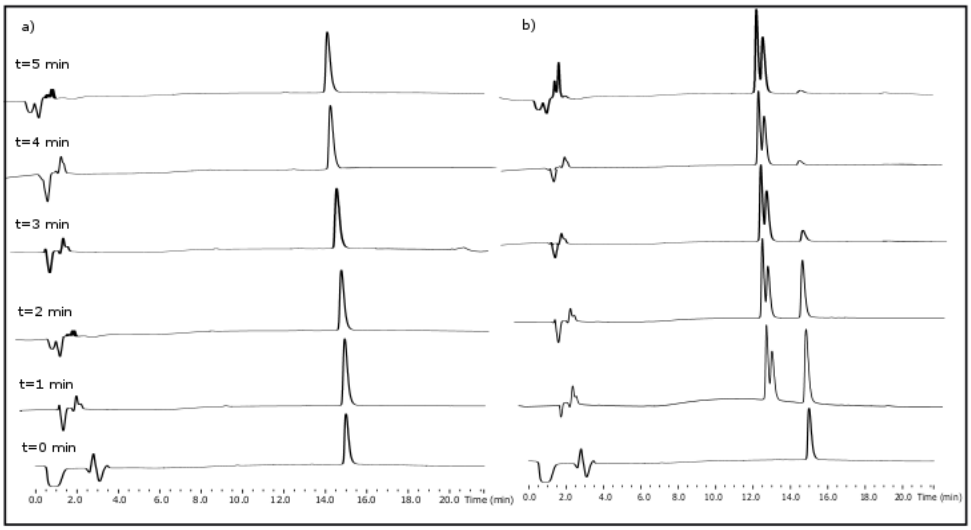


Figure SI-13: Trypsin digestion of collagen telopeptides. HPLC elution profiles during the incubation of peptides a) MP-lysine modified CTP **15** and b) native CTP **14** at various time with trypsin.

Circular Dichroism Measurements

A Chirascan (Applied Photophysics Ltd, Leatherhead, UK) was used for CD measurements.CD spectra were recorded on peptide solutions at 0.2 mM in 50 mM AcOH that had been incubated at 5 °C for a minimum of 24 h. Each CD spectrum measurement represents the average of five scans. Baseline spectra were collected with buffer alone and then subtracted from the raw peptide spectral data. Melting experiments were performed from 6 to 70 ºC, monitoring at 224 nm (Quartz cells were used with path lengths of 5 mm), and the first derivative of the thermal unfolding curve was taken to determine the melting temperature of the sample. The molar residual ellipticity (MRE) is calculated from the measured ellipticity using the equation:

$$[\theta] = \frac{\theta \times m}{c \times l \times n_r}$$

Where θ is the ellipticity in millidegrees, m is the molecular weight in g mol^{-1}, c is the concentration in mg ml^{-1}, l is the path length of the cuvette in cm and n_r is the number of amino acids in the peptide.

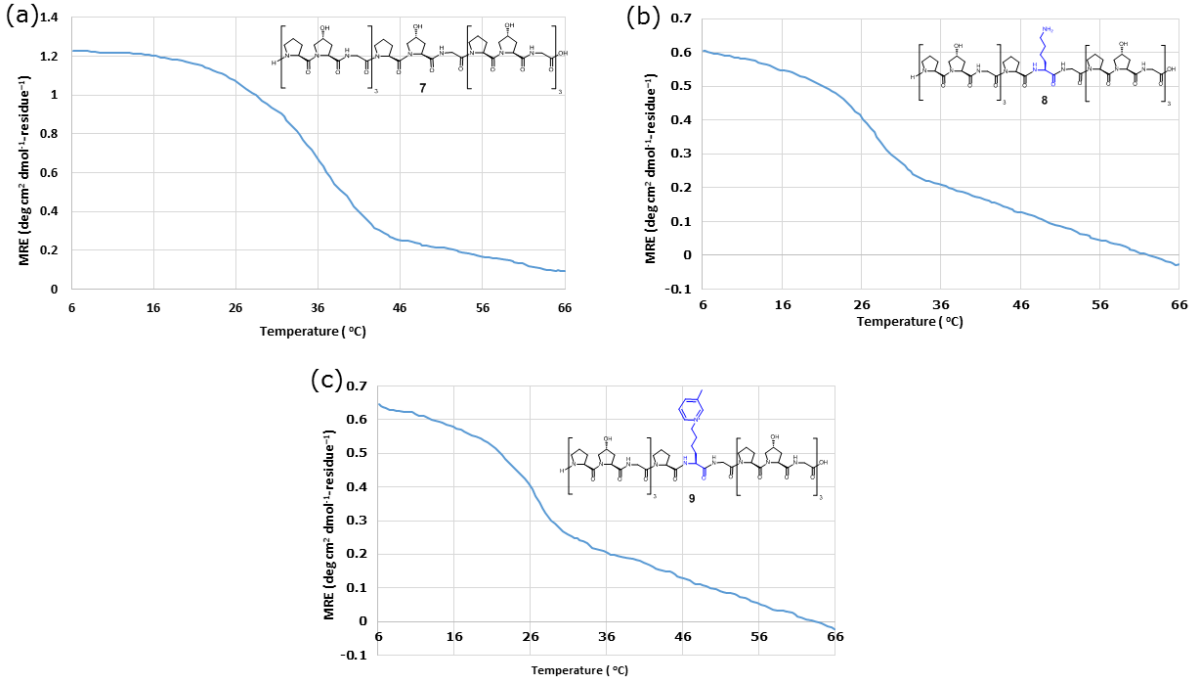


Figure SI-14: CD thermal transition curves for a) CMP **7**, b) CMP-K **8** and c) peptide **9** measured in 50 mM aqueous AcOH (0.2 mM peptide).