Supporting Information: General Experimental Details

^{1}H NMR spectra were recorded at 300 and 400 MHz, ^{13}C NMR spectra were recorded at 75 and 100 MHz respectively on a Bruker AMX instruments. Chemical shifts (δ) were reported in *ppm* and coupling constants (*J*) in Hz, signals were sharp unless stated as broad (br), s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet. Residual protic solvent, $CDCl_3$ (δ_H: 7.26, s) was used as the internal standard in ^{1}H-NMR spectra unless otherwise stated. Electrospray mass spectroscopy was carried out on a Waters Acquity SQD uPLC electrospray system with an applied voltage of 50-60V.

Solvent and reagents

All reagents and solvents were standard laboratory grade and used as supplied unless otherwise stated. Where a solvent was described as dry it was purchased as anhydrous grade. All organic extracts were dried over anhydrous magnesium sulfate prior to evaporation under reduced pressure.

Peptide synthesis

Peptide synthesis was carried out using Rink amide-MBHA (loading = 0.64 mmol/g) and Rink amide-MBHA-LL (loading = 0.34 mmol/g) resins for the production of peptide amides. All resins and Fmoc amino acids were purchased from Novabiochem. Semi-preparative HPLC was performed using a Phenomenex LUNA C_{18} column and a gradient of 5-60% acetonitrile containing 0.1% TFA over 45 minutes (flow rate of 4.0 mL/min). All other chemical reagents were obtained form Aldrich.

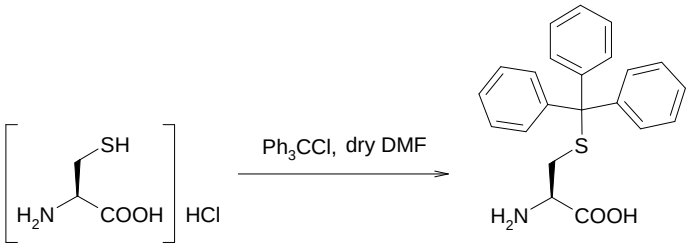
General peptide synthesis procedures.

Peptide C-terminal cysteinyl amides were prepared using standard procedures. Briefly, rink amide resin (0.05 mmol) was deprotected by exposure to 20 % piperidine in DMF. Fmoc-Cys(Trt)-OH (5 equiv) was coupled using HBTU/HOBt as coupling reagents in the presence of DIPEA (Fastmoc protocol). The coupling time was 2 h. The resin was then transferred to a reaction vessel of an Applied Biosystems 433A automated peptide synthesiser and peptide synthesis was continued in automated

fashion employing the Fastmoc protocol. The target peptide sequence was assembled and then cleaved with 95 % TFA, 2.5 % EDT, 2.5 % H_2O, for 4 h and precipitated from diethylether using well established procedures. For purification, crude, fully deprotected, and precipitated peptides was redissolved in 25 % aqueous MeCN and purified by semi-prep HPLC. The major peak was analysed by ESI-MS and was found to correspond to the desired product. This fraction was lyophilized and used in subsequent reactions.

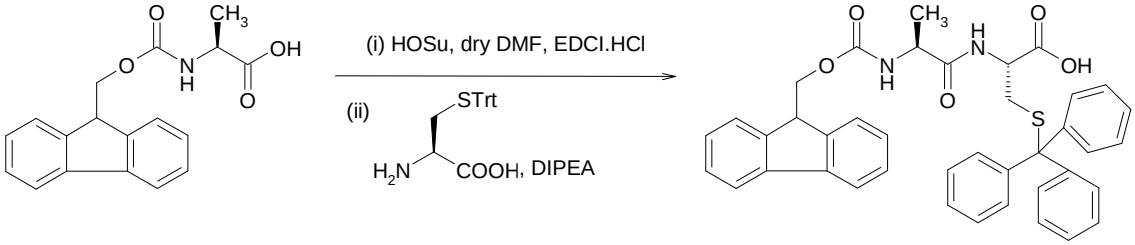
CPE peptide synthesis and Reactions:

H-(L)-Cys(Trt)-OH



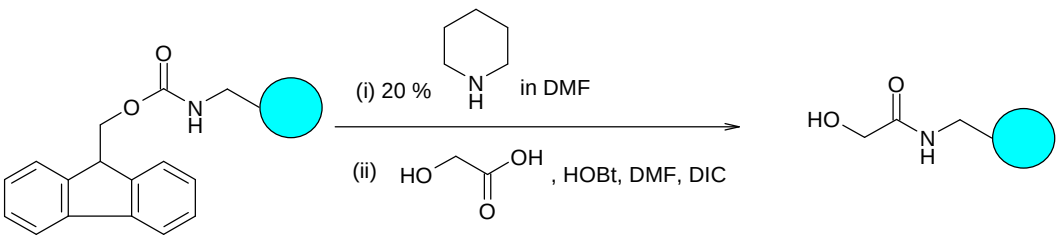
(L)-Cysteine hydrochloride (2.00 g, 12.69 mmol) and triphenylmethyl chloride (5.40 g, 19.37 mmol) were dissolved in anhydrous *N, N*-dimethylformamide (8.0 mL) and stirred under nitrogen for 24 h. Aqueous sodium acetate (10 % w/v) (70.0 mL) was added to the reaction mixture. The white precipitate that formed was filtered under vacuum and washed with water. The white precipitate was then stirred in acetone (50.0 mL) at 50 °C for 0.5 h. The resulting solution was cooled to room temperature, filtered with suction, washed with a small volume of acetone and dried under high vacuum to afford the protected amino acid (3.22 g, 70 %) as a white amorphous solid. R_f 0.1 (8:2 ethyl acetate/ methanol). ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD) δ (*ppm*) 7.19 (6 H, d, *J* = 7.5 Hz, Ph-**H** Trt); 7.05 (6 H, t, *J* = 7.0 Hz, *J* = 7.5 Hz, Ph-**H** Trt); 6.98 (3 H, t, *J* = 6.9 Hz, Ph-**H** Trt); 3.93 (1 H, br dt, C**H**$^{\alpha}$); 3.06 (1 H, br dd, C**H**$_2$); 3.00 (1 H, br dd, C**H**$_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ (*ppm*) 165.3 (**C**=O); 134.0 (Ph); 129.6 (Ph); 127.8 (Ph); 127.0 (Ph); 120.0 (**C**Ph_3); 77.2 (**C**$^{\alpha}$); 47.2 (**C**H_2). ESI+ MS (*m/z*) calculated for $C_{22}H_{21}O_2NS$ 363.13 found $[MH]^+$ 364.14.

Fmoc-Ala-Cys(Trt)-OH



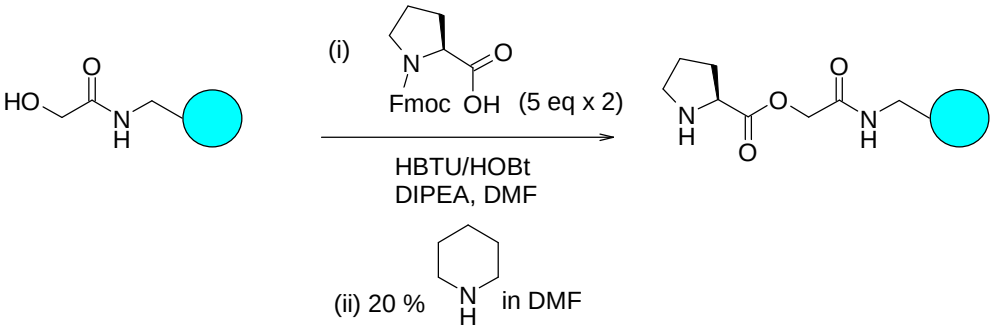
Fmoc-Ala-OH (623 mg, 2.00 mmol) and *N*-hydroxysuccinimide (230 mg, 2.00 mmol) were dissolved in anhydrous *N*, *N*-dimethylformamide (7.0 mL, 0.29 M). *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (383 mg, 2.00 mmol) was then added to the reaction mixture, which was then stirred under nitrogen for 24 h. H-Cys(Trt)-OH, (800 mg, 2.20 mmol) and diisopropylethylamine (348 µL) were then added to the reaction mixture and stirring continued for a further 6 h. The reaction was quenched with 1 M aqueous hydrochloric acid (2.0 mL) and the solution was concentrated *in vacuo*. Extraction ensued with ethyl acetate (50.0 mL), followed by washing with 1 M aqueous hydrochloric acid (2 × 10.0 mL) and brine (10.0 mL). The combined organic phase was dried with anhydrous magnesium sulfate, filtered and concentrated *in vacuo* to give the crude dipeptide. Purification via F.C.C. [(i) ethyl acetate; (ii) 8: 2 ethyl acetate/ methanol] afforded **6** (1.02 g, 78 %) as an off-white foam solid. R_f = 0.2 (8:2 ethyl acetate/ methanol). ^{1}H NMR (400 MHz, $CDCl_3$) δ (*ppm*) 8.65 (1 H, br s, CON**H** Fmoc-Ala); 8.12 (1 H, br s, CON**H** Ala-Cys); 7.72 (4 H, d, *J* = 4.9 Hz, Ph-**H** Fmoc); 7.51 (4 H, m, Ph-**H** Fmoc); 7.20 (15 H, m, Ph-**H** Trt); 4.33 (2 H, br s, C**H**$_2$); 3.83 (1 H, br dt, C**H**$^{\alpha}$ Cys); 3.07 (1 H, br dd, C**H**$_2$); 2.97 (1 H, br dd, C**H**$_2$); 2.77 (1 H, br s, C**H** Fmoc); 2.65 (1 H, br s, C**H**$^{\alpha}$ Ala); 1.24 (2 H, br dd, C**H**$_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ (*ppm*) 170.8 (**C**=O); 169.8 (**C**=O); 165.3 (**C**=O); 144.7 (Ph); 144.0 (Ph); 141.3 (Ph); 134.0 (Ph); 129.6 (Ph); 129.6 (Ph); 127.8 (Ph); 119.8 (**C**Ph$_3$); 77.2 (**C**$^{\alpha}$); 62.9 (**C**H$_2$); 63.0 (**C**$^{\alpha}$); 60.2 (**C**H Fmoc); 20.8 (**C**H$_3$); 14.1 (**C**H$_2$). ESI+ MS (*m/z*) calculated for $C_{40}H_{36}O_5N_2S$ 656.30 found $[MNa]^+$ 679.52.

HO-Gc-Rink amide resin



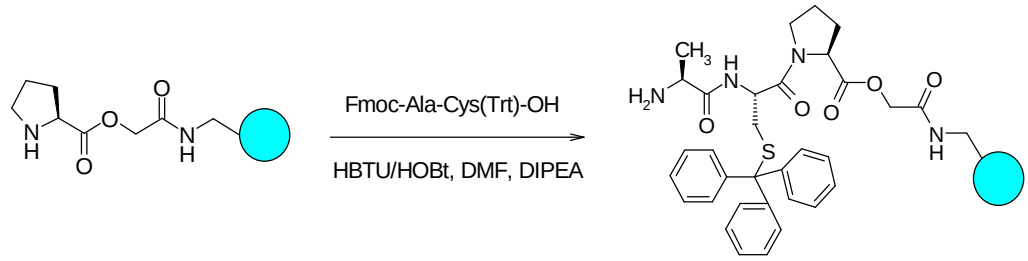
Fmoc-Rink amide AM resin (0.156 g, 0.10 mmol) was subjected to 20 % piperidine in DMF (v/v) (1.0 mL) for 5 min, and then washed exhaustively with *N*, *N*-dimethylformamide then dichloromethane. Glycolic acid (Gc) (38 mg, 0.50 mmol) and 1-hydroxy-1*H*-benzotriazole (HOBt) (40 mg, 0.50 mmol) were dissolved in anhydrous *N*, *N*-dimethylformamide (3.0 mL) and DIC (47 µL, 0.50 mmol) was added before being transferred to the dry resin. The reaction mixture was shaken at 410 rpm, at room temperature for 3 h, then filtered and washed with *N*, *N*-dimethylformamide (1.0 mL) and dichloromethane (2 × 1.0 mL).

H-Pro-Gc-Rink amide resin



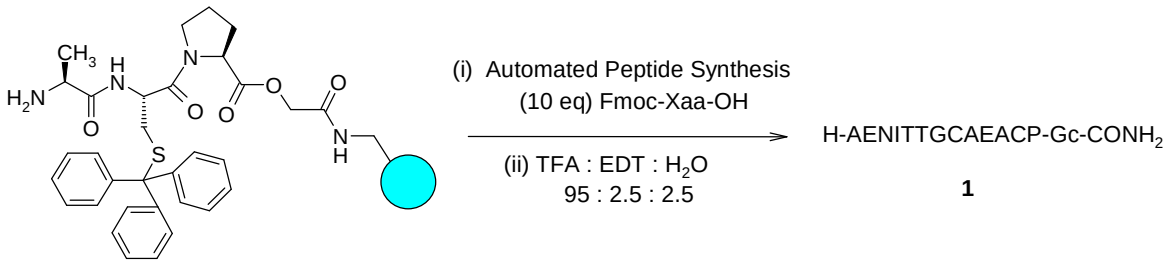
Fmoc-Pro-OH (0.169 g, 0.50 mmol) was then added to the resin bound glycolic acid, followed by HOBt/HBTU (1.1 mL, 0.45 M) and *N*, *N*-diisopropylethylamine (150 µL) in *N*, *N*-dimethylformamide (1.1 mL), the heterogeneous mixture was shaken for 4 h. The vessel was subsequently drained and washed with *N*, *N*-dimethylformamide (1.0 mL) and dichloromethane (2 × 1.0 mL), and the Fmoc-Pro-OH coupling procedure was repeated with shaking overnight. Fmoc-analysis was then carried out. This was achieved by measuring out (1) 2.0 mg of Fmoc-Pro-Gc-resin synthesised in 20 % piperidine in DMF (v/v) (3.0 mL), and (2) 2.1 mg of Fmoc-Pro-Gc-resin synthesised in 20 % piperidine in DMF (v/v) (3.0 mL). A blank solution of 20 % piperidine in DMF (v/v) (3.0 mL) was initially placed in the UV spectrophotometer and the absorbance taken as the reference value. Sequentially the two aforementioned solutions (1) and (2) were also placed in the photometer until a stable reading was taken. The absorbance at 290 nm for solution (1) was 1.972 and for (2) was 1.929. These values were used to determine a resin loading of 0.54 $mmolg^{-1}$ (85 %). Fmoc-Pro-Gc-Resin (0.0817 g, 0.05 mmol) was then deprotected with 20 % piperidine in DMF (v/v) (1.0 mL) for 5 min, and then washed exhaustively with *N*, *N*-dimethylformamide then dichloromethane.

H-Ala-Cys(Trt)-Pro-Gc-Resin



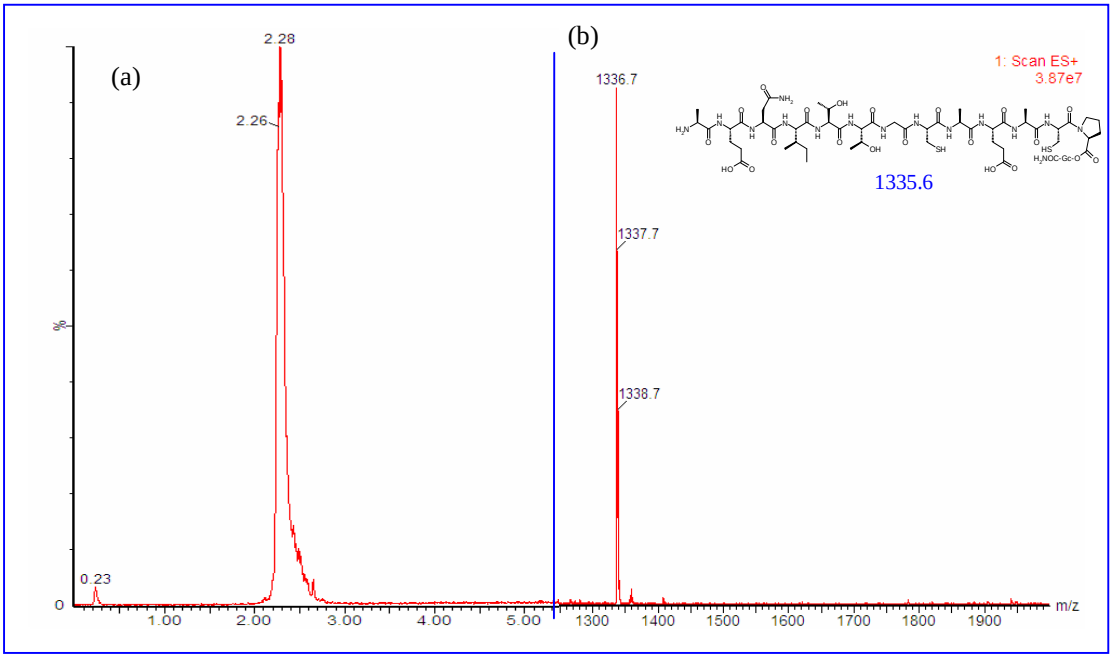
Fmoc-Ala-Cys(Trt)-OH dipeptide (0.161 g, 0.25 mmol) was then coupled to the resin using HOBt/HBTU (0.55 mL, 0.45 M), *N*, *N*-dimethylformamide (0.55 mL) and diisopropylethylamine (75 µL), and was shaken for 5 h. The resulting mixture was then filtered, washed with *N*, *N*-dimethylformamide (1.0 mL) then dichloromethane (2 × 1.0 mL). The coupling was observed by cleavage of an analytical sample: TFA solution [100 µL of trifluoroacetic acid: ethanedithiol: water (95: 2.5: 2.5)] was added to the resin-bound peptide (1.0 mg), which was left to stand at room temperature for 1h. The beads were then filtered and diethyl ether (1.0 mL) was added to the filtrate to initiate a white precipitate, which was then centrifuged. The diethyl ether was decanted and centrifugation was repeated with diethyl ether (1.0 mL) to afford a white solid. The solid was then dissolved in water for analysis. ESI+ LC-MS (m/z) calculated for Fmoc-ACP-Gc-$CONH_2$ 568.2, found $[MNa]^+$ 591.3. The remaining resin-bound peptide was then subjected to 20 % piperidine in DMF (v/v) (1.0 mL) for 8 min, filtered and washed with *N*, *N*-dimethylformamide (1.0 mL) then dichloromethane (2 × 1.0 mL) to afford the deprotected, resin-bound tripeptide, *H-Ala-Cys(Trt)-Pro-Gc-Resin*.

H-AENITTGCAEACP-Gc-$CONH_2$ = EPO(22-32)-CPE (1)



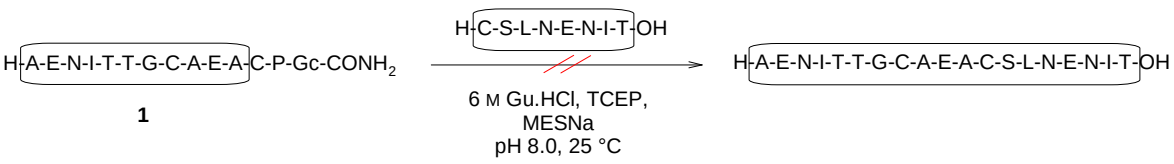
H-Ala-Cys(Trt)-Pro-Gc-Resin was transferred to an Applied Biosystems 433A reaction vessel and peptide elongation was performed in an automated fashion on an Applied Biosystems 433A automated synthesiser, using the Fastmoc protocol. Fmoc-Xaa-OH used (10 eq of each): Fmoc-A-OH, Fmoc-E(O^tBu)-OH, Fmoc-N(Trt)-OH,

Fmoc-I-OH, Fmoc-T(^{t}Bu)-OH, Fmoc-G-OH and Fmoc-C(Trt)-OH. The peptide was then cleaved from the solid-support by treatment with 95 % trifluoroacetic acid solution [3 mL (trifluoroacetic acid: ethanedithiol: water = 95: 2.5: 2.5)] for 4.5 h. The mixture was filtered, and finally rinsed with neat TFA (1.0 mL). Diethyl ether (20.0 mL) was added to the filtrate and centrifuged (15 min), this was then repeated with diethyl ether (20.0 mL). The crude peptide was a white precipitate and it was lyophilised from 1: 1 = acetonitrile: water, and then purified via semi-preparative reverse phase (RP)HPLC (gradient: 5 to 50 % acetonitrile in water, semi-preparative column, retention time = 23.0 min). The collections were lyophilised to afford *H-AENITTGCAEACP-Gc-CONH$_2$* (12.5 mg, 22 % calculated from resin loading) as a fluffy white solid. ESI+ LC-MS (m/z) calculated for H-AENITTGCAEACP-Gc-CONH$_2$ 1335.5, found $[MH]^+$ 1336.7.



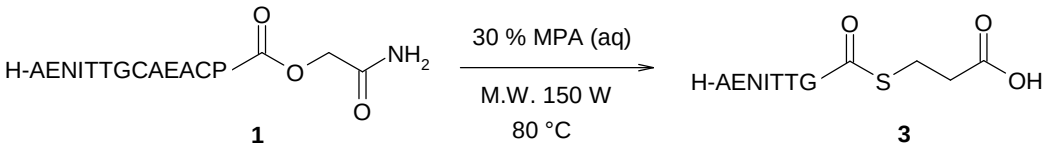
(a) TIC for EPO (22-32)CPE; (b) ESI$^+$ MS (*m/z*) $[MH]^+$ = 1336.7

Attempted CPE Ligation: EPO(22-40)



EPO(22-32)-CP-Gc-$CONH_2$, **1** (1.0 mg, 0.75 µmol) and EPO(33-40)-OH (1.0 mg, 1.12 µmol) were dissolved in 6 M guanidinium hydrochloride buffer (500 µL), adjusted to pH 8.0, before the addition of the thiol additive 1 M MESNa (25 µL, 50 mM final concentration) and reductant 1 M TCEP (5 µL,10 mM final concentration). The reactions were conducted in a thermomixer at 25 °C and monitored at regular intervals; from t = 1 to 122 h, via LC-MS. No ligation product was observed.

EPO(22-28)-$SCH_2CH_2CO_2H$ = EPO(22-28)-MPA (3)

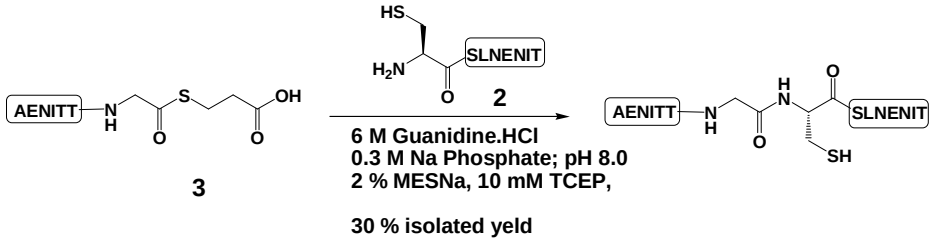


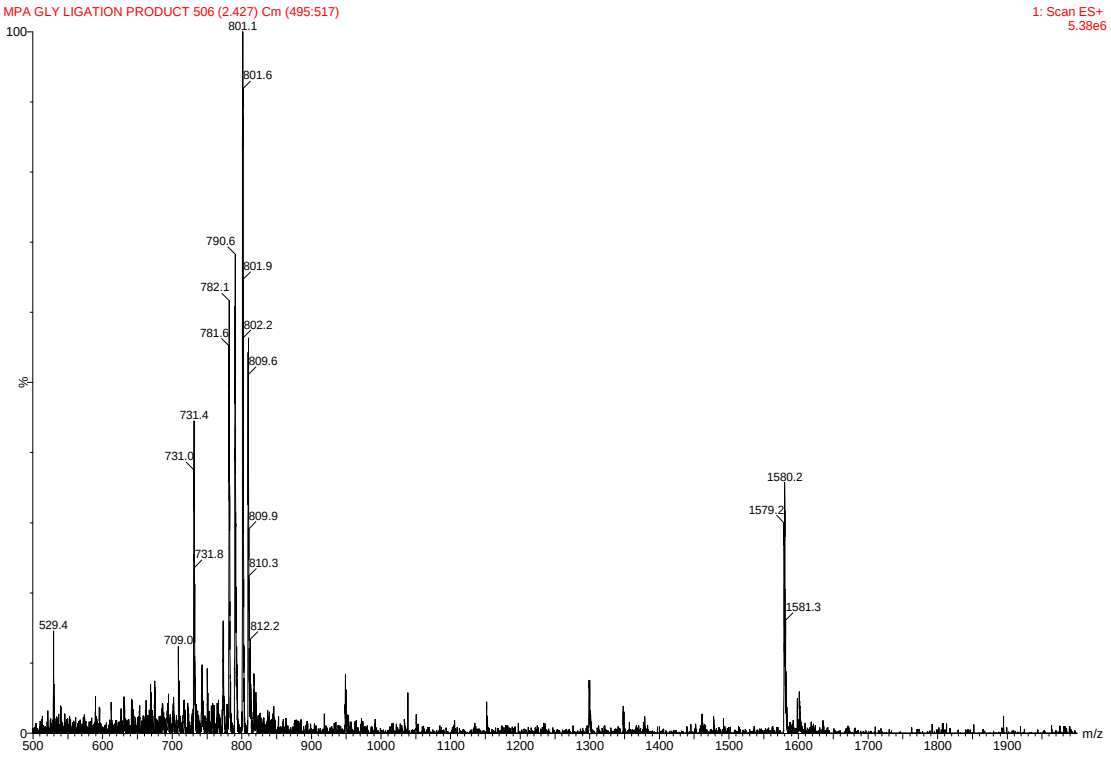
1 **3**

EPO(22-32)-CP-Gc-$CONH_2$, **1** (1.0 mg, 0.75 µmol) was dissolved in 3-mercaptopropionic acid (MPA) (150 µL) and water (350 µL) and placed in a microwave reactor at 80 °C, 150 W, for 4 h. LC-MS indicated the presence of starting material and hence the reaction was continued for a further 12 h. The reaction mixture was subsequently filtered and centrifuged and then purified via (RP)HPLC (gradient: 5 to 50 % acetonitrile in water, semi-preparative column). Fractions collected were lyophilised to afford **3**, *EPO(22-28)-MPA* (0.5 mg, 84 %) as a white solid. LC-MS (*m/z*) calculated for [M] 792.3, found $[MH]^+$ 793.4.

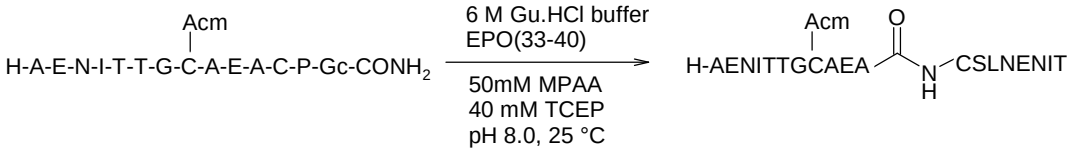
H-AENITTGCSLNENIT-NH_2 = Ligation between peptide **2** and **3**

The ligation was carried out as described above and the product isolated by reverse-phase HPLC:



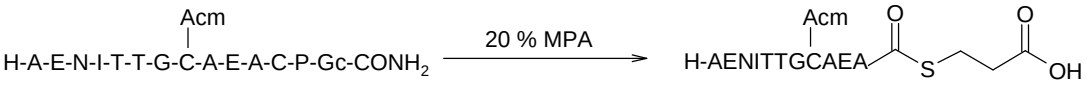


H-AENITTGC(Acm)AEACSLNENIT-OH = EPO(22-^{29}C(Acm)- 40)

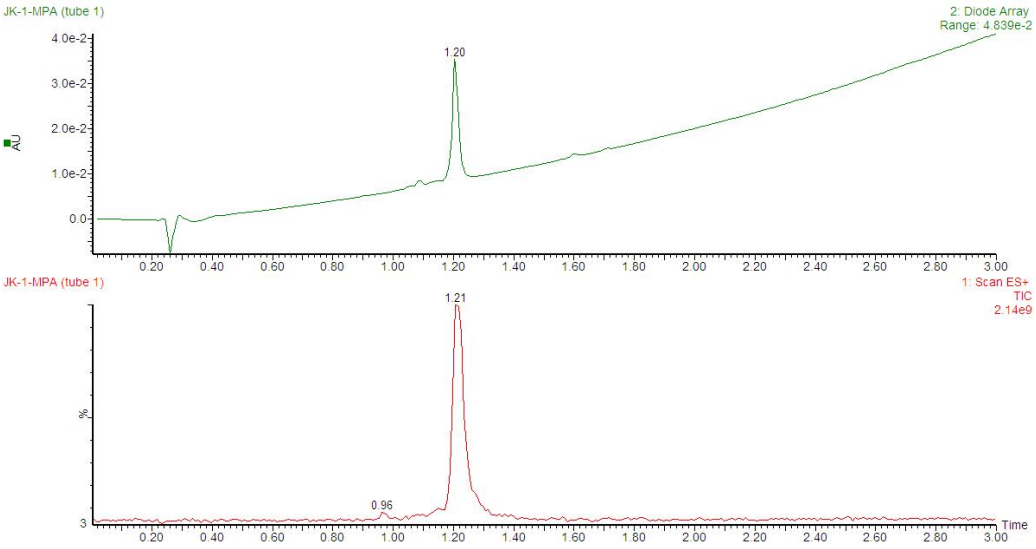


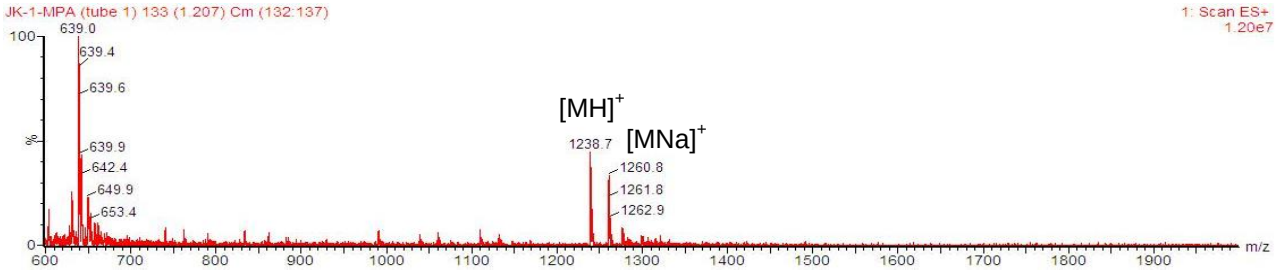
EPO(22-^{29}C(Acm)-32)-CPE (5.0 mg, 0.36 µmol) was dissolved in guanidinium hydrochloride buffer (6 M, pH 7) (500 µL), and added to EPO(33-40) (1.0 mg, 1.12 µmol). MPAA (1 M) (50 mM) and TCEP (1 M) (40 mM) were added to the reaction mixture, the pH was adjusted to 8.0, the reaction was carried out at 25 °C in a thermomixer, and it was continued until starting material was deemed undetectable by LC-MS to afford the sequence *EPO(22-^{29}C(Acm)- 40).* ESI+ LC-MS (*m/z*) calculated for $[MH_2]^+$ 1013.0 found $[MH_2]^+$ 1013.0.

Conversion of Gly-Cys(Acm)-**1** to Ala-MPA thioester



The peptide (1.0 mg, 0.75 µmol) was dissolved in 20 % v/v aqueous 3-mercaptopropionic acid (0.5 mL) and placed in an eppendorf thermomixer with shaking at 80 °C for 48 h. The reaction mixture was filtered and centrifuged and then purified via (RP)HPLC (gradient: 5 to 50 % acetonitrile in water, semi-preparative column). Fractions collected were lyophilised to afford the alanine thioester, *EPO(22-32)-MPA* as a white solid. LC-MS (*m/z*) calculated for [M] 1237.5, found $[MH]^+$ 1238.7.



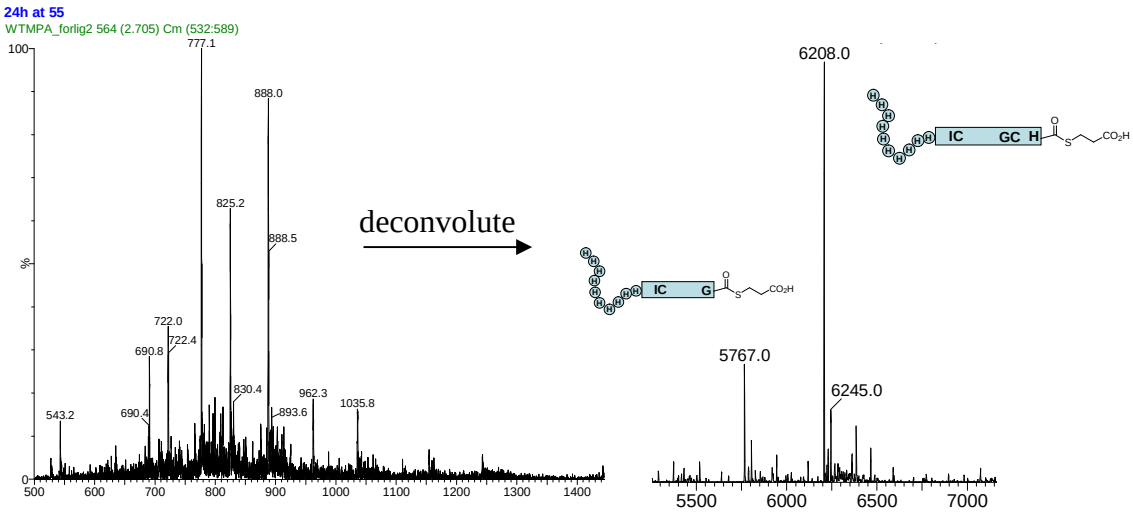


Protein cleavage reactions:

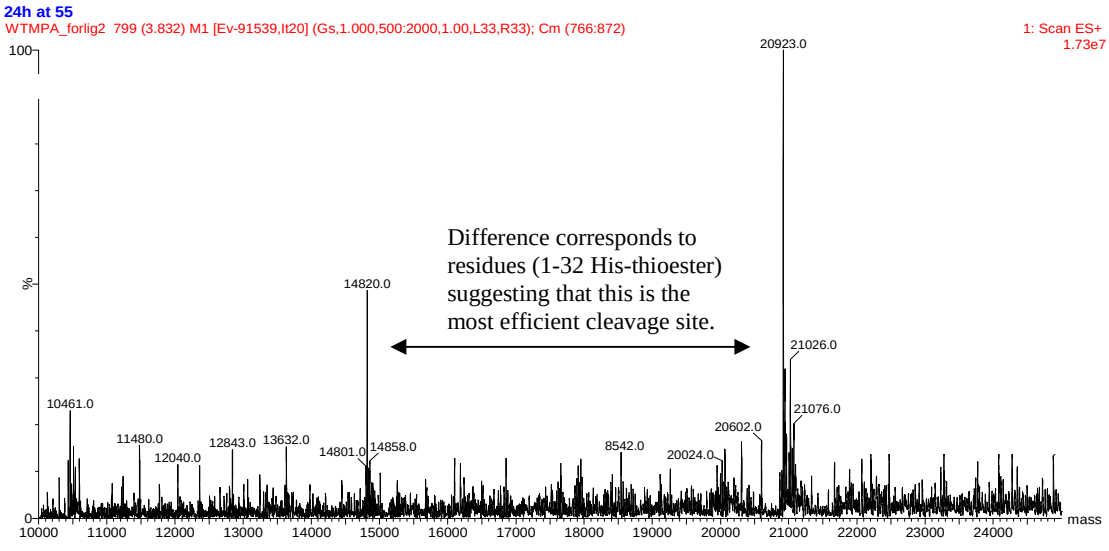
Precipitated protein samples prepared as previously described[1] (1-5 mg) were dissolved in 20 % v/v aqueous 3-mercaptopropionic acid (1.0 mL) and placed in an eppendorf thermomixer with shaking at 60 °C for 48 h. The reaction mixture was

filtered and then purified via (RP)HPLC (gradient: 5 to 50 % acetonitrile in water, semi-preparative column). Fractions at 31 and 32 mins were collected, combined and lyophilised to afford the mixture of His and Gly-MPA thioesters. (Sequences: GHHHHHHHHHHSSGHIEGRHMAPPRLICDSRVLERYLLEAKEAENITTG-MPA and GHHHHHHHHHHSSGHIEGRHMAPPRLICDSRVLERYLLEAKEAENITTGCAEH-MPA)

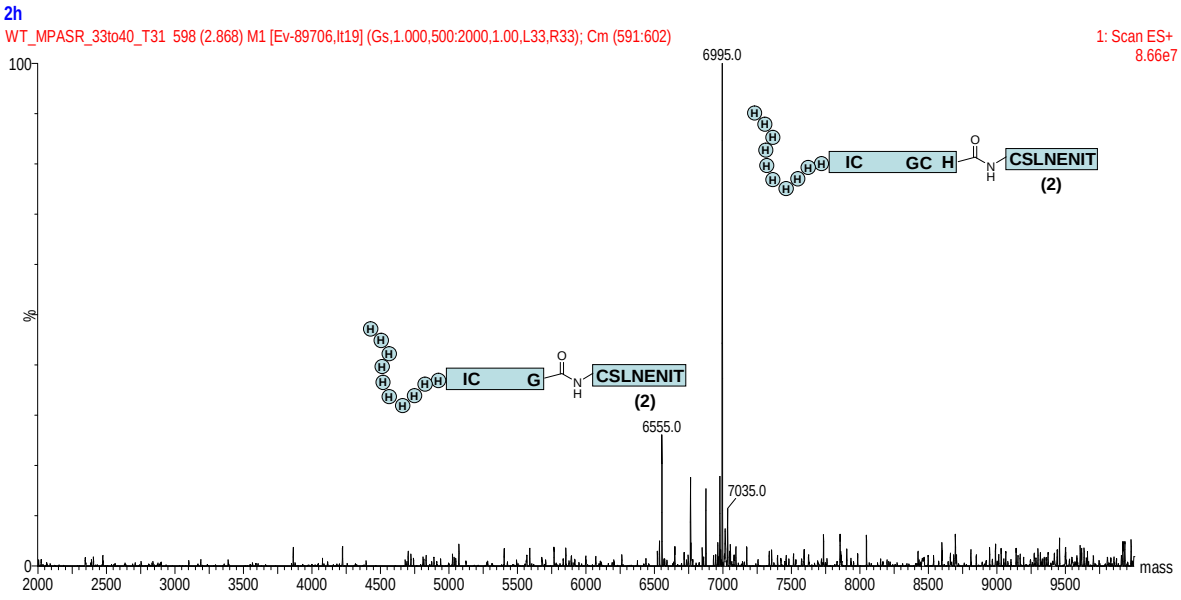
MS data for protein MPA reactions described in Figure 1:



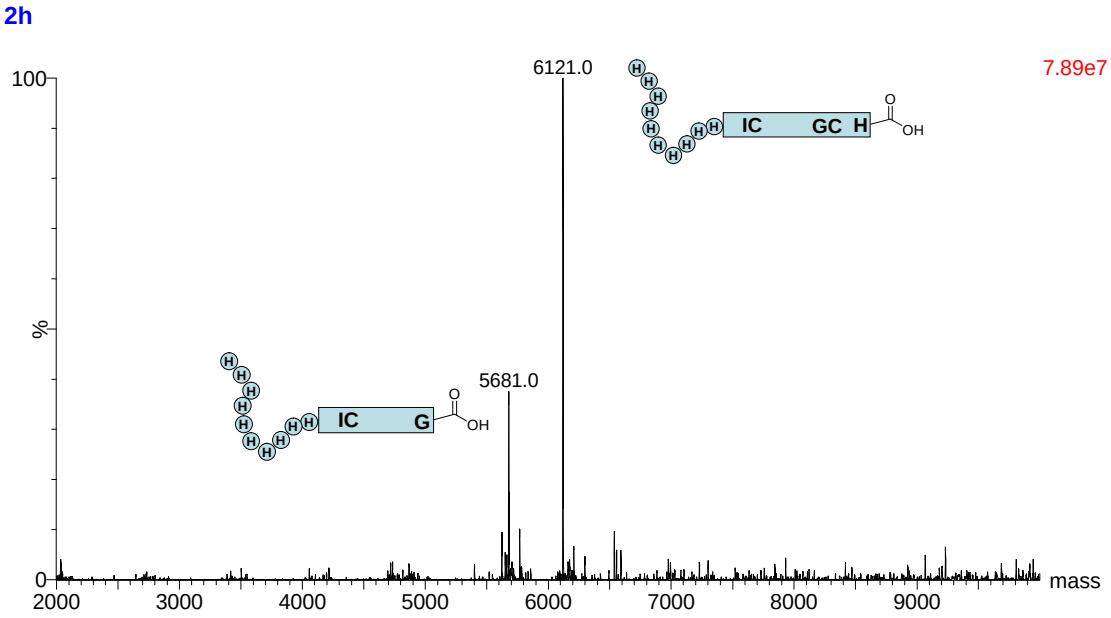
Analysis of the protein sample after MPA treatment for 24 h at 55 ^{o}C (below) showing fragmentation to yield EPO residues 33-166. MPA-mediated fragmentation is monitored by LC-MS and continued until the starting protein is consumed.



Ligation between mixture of peptides comprising His_{10}-EPO(1-28)-Gly MPA thioester and His_{10}-EPO(1-32)-His MPA thioester with peptide **2** (expected calculated masses = 6554.2 and 6994.7 respectively) :



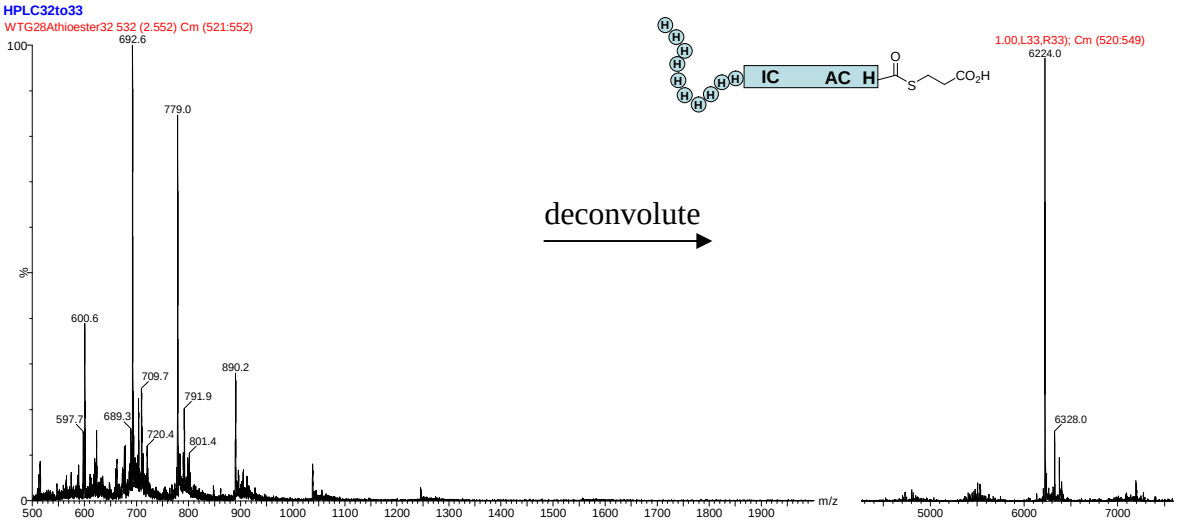
The ligation reaction also yields significant hydrolysis products in the presence of MPAA after 2 h:



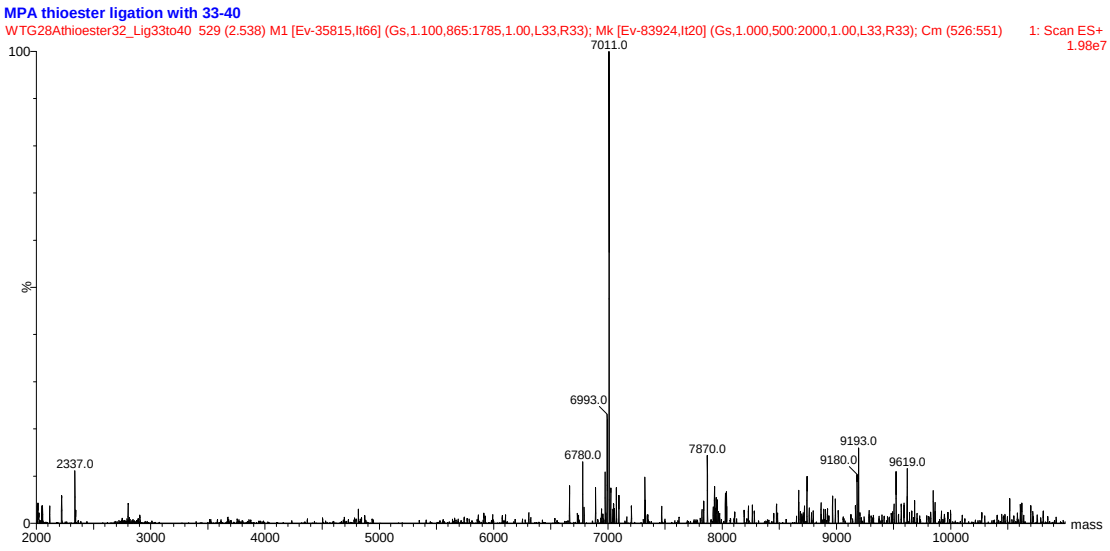
In Contrast when **The G28A** mutant was employed a far simpler spectrum was obtained for the MPA cleavage product:

Procedure: A Precipitated protein sample (6.1 mg) prepared as previously described[1] was dissolved in 15 % v/v aqueous 3-mercaptopropionic acid (1.0 mL) and placed in an eppendorf thermomixer with shaking at 60 °C for 48 h. The reaction mixture was allowed to cool and loaded directly onto a semi-preparative (RP)HPLC column (gradient: 5 to 50 % acetonitrile in water). Fractions eluting at 32 mins was collected and lyophilised to afford the MPA thioester as a white solid (0.5 mg, 28 %).

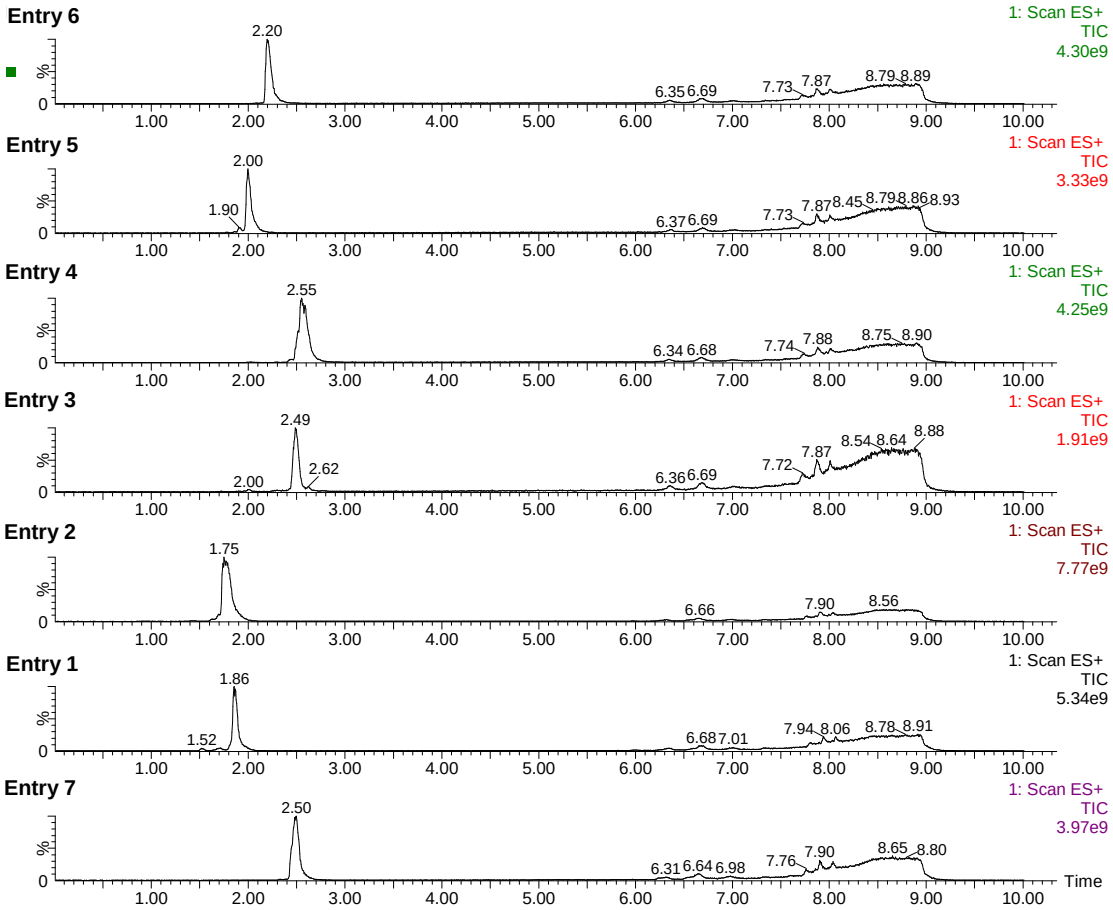
Sequence:GHHHHHHHHHHSSGHIEGRHMAPPRLICDSRVLERYLLEA KEAENITT**A**CAEH-MPA



Crude ligation product after ligation with model peptide **2**:

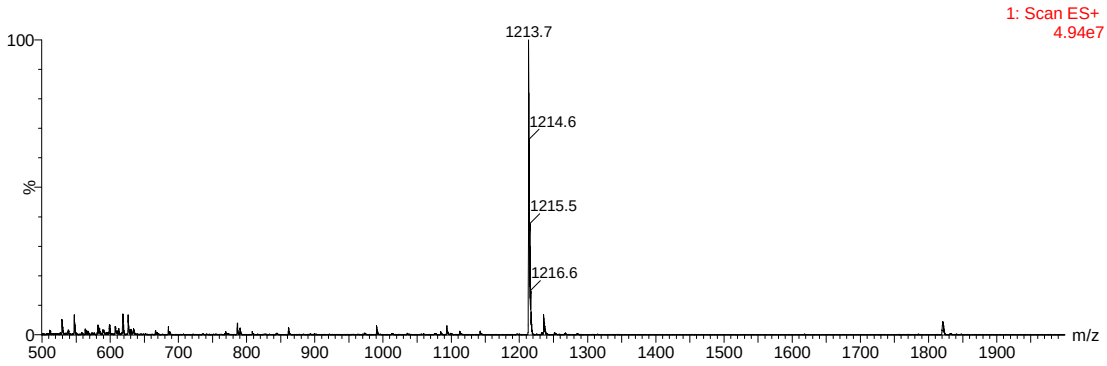


LC-MS data for the peptide precursors described in Table 1:

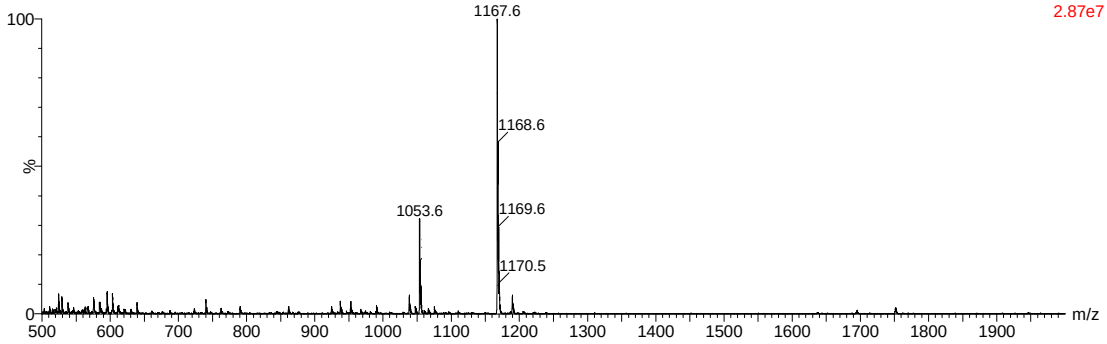


MS Data for peptide precursors described in Table 1:

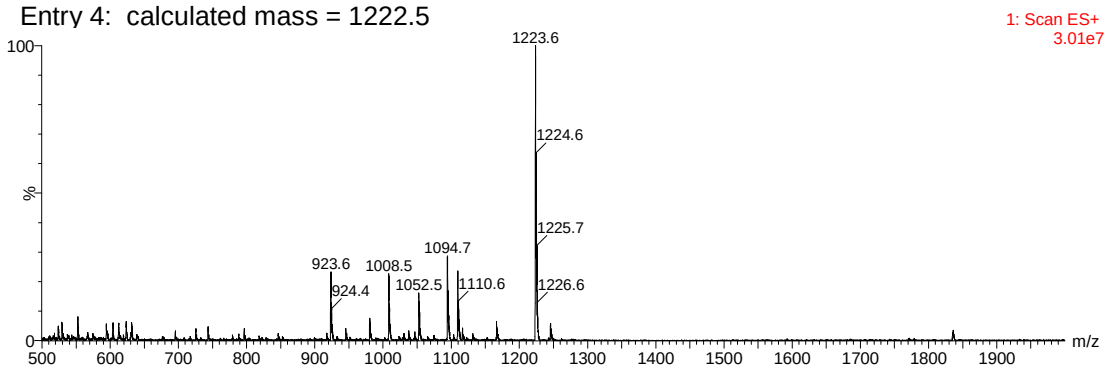
Entry 6: calculated mass = 1212.5



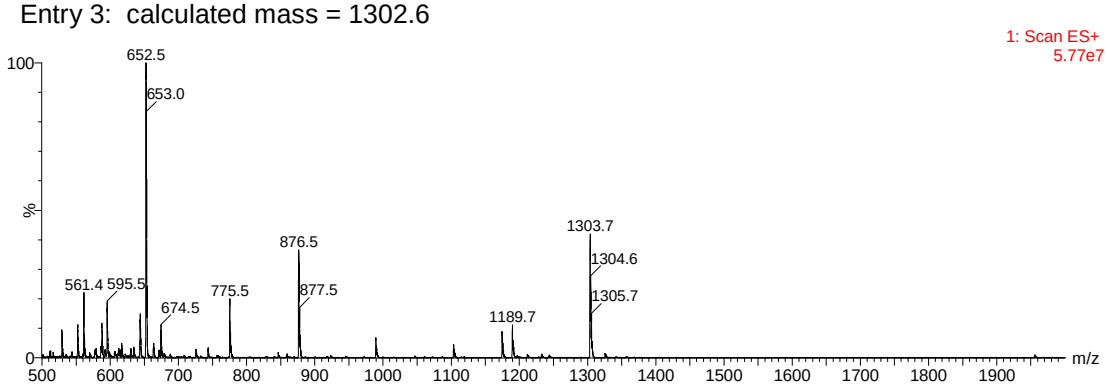
Entry 5: calculated mass = 1166.5



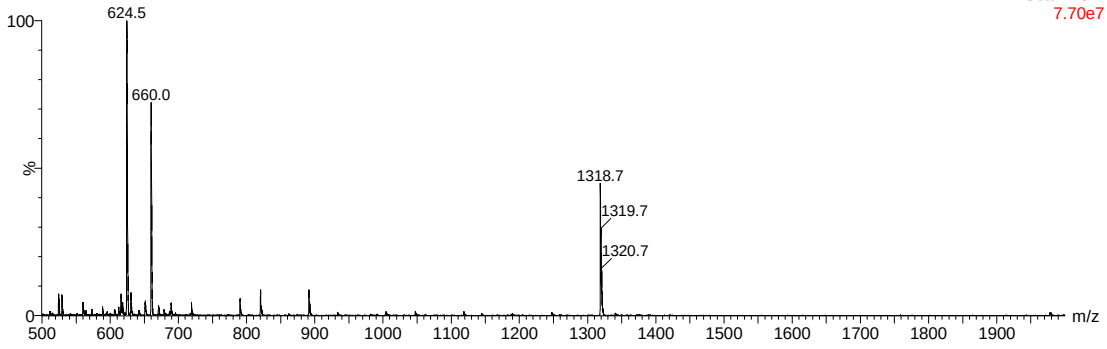
Entry 4: calculated mass = 1222.5

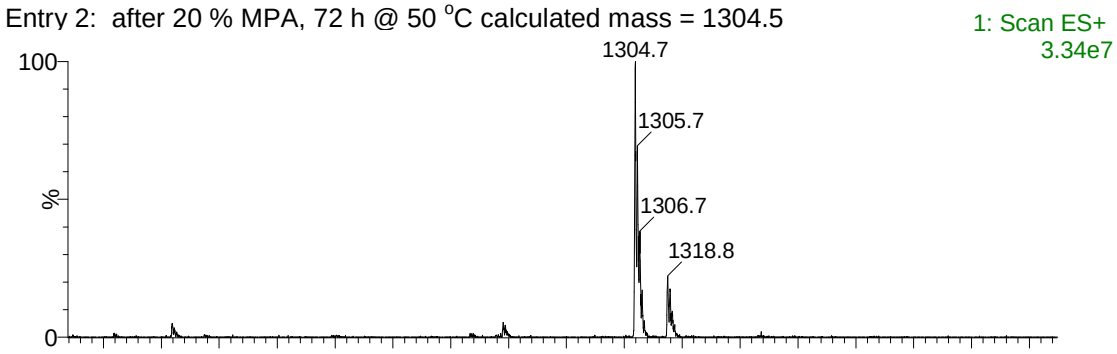


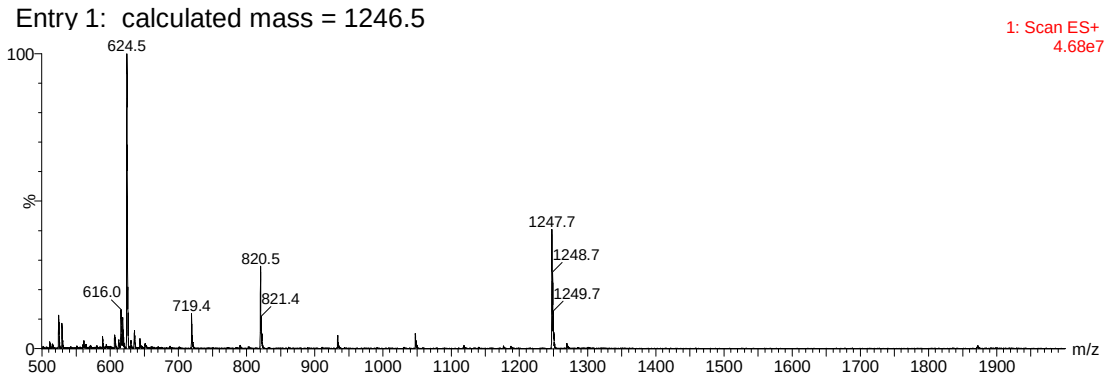
Entry 3: calculated mass = 1302.6



Entry 2: calculated mass = 1318.5





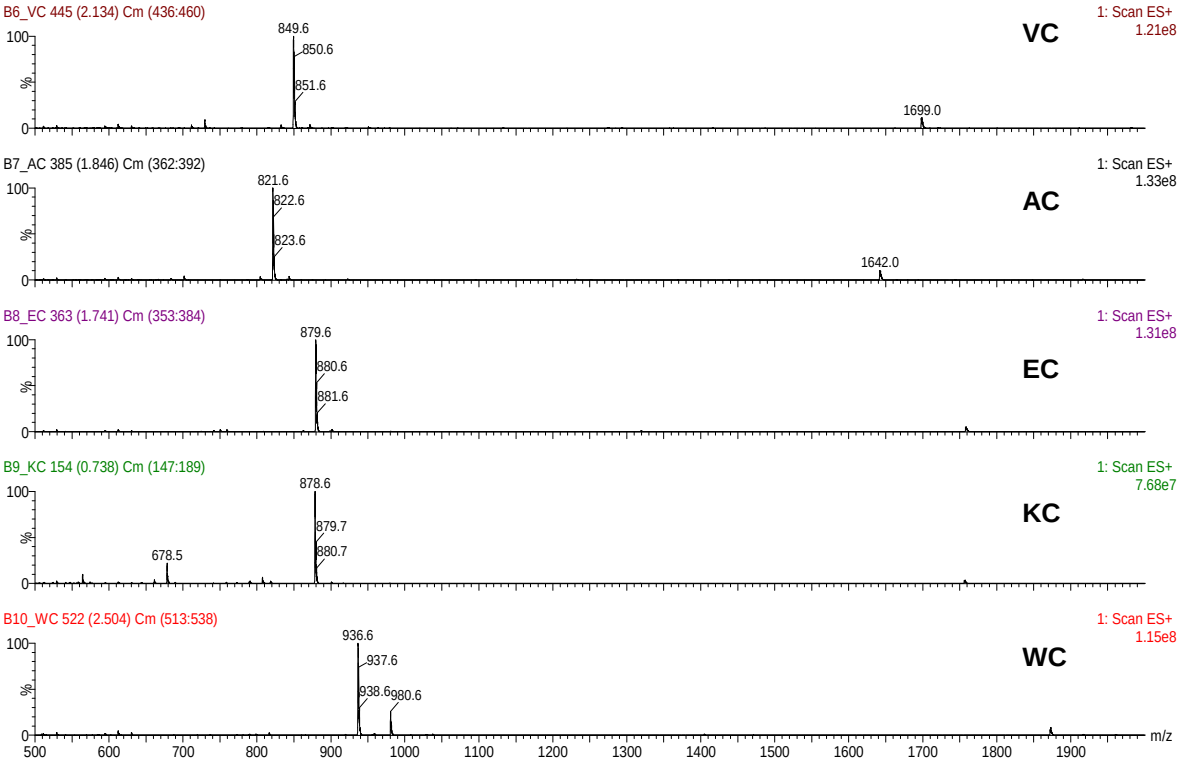


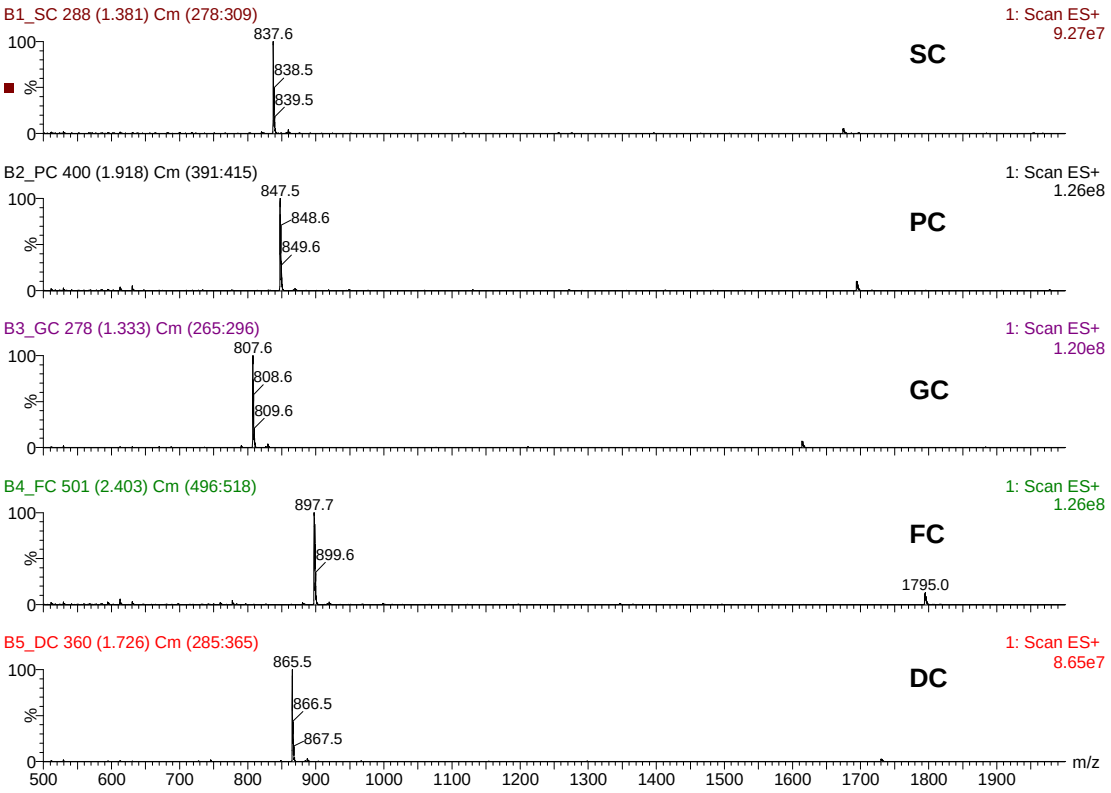
Synthesis of 10 model peptides described in Table 2 (below):

1 mmol Rink amide resin was first manually coupled to Fmoc-Cys(Trt)-OH, 5 mmol using HBTU/HOBt and DIPEA (the fastmoc protocol) in anhydrous DMF for 2h. The resin was dried then divided into 10 equal aliquots. To each of the aliquots was coupled suitably protected Fmoc amino acid (A, D, E, F, G, K, P, S, V, W), then manual synthesis was continued using 10 equivalents of each amino acid prepared as a 1 mmol stock solution and divided equally into each resin sample. At the end of the synthesis (Assembly of Boc-AENITTXC) the peptides were cleaved from the solid support by exposure to 95 % TFA, 2.5 % EDT, 2.5 % H_2O for 3.5 h. The crude peptides were dissolved in 1: 1 MeCN/H_2O and lyophilised. LC-MS analysis of each sample indicated the products were highly pure though in many cases (with the exception of FC, PC, KC, WC and VC containing peptides) two resolved peaks with identical masses were observed which were tentatively assigned as the *N*-peptide and the *S*-peptide. 9.1-12.5 mg of each sample was obtained corresponding to near quantitative yields so each peptide was not purified and used directly in MPA mediated thioesterification reactions.

For MPA thioesterification reactions a known peptide quantity (1.5-4 mg) was dispensed into a eppendorf tube and treated with 0.4 mL 20 % aqueous MPA. After 20 h at 80 ^{o}C the samples were allowed to cool to room temperature then centrifuged at maximum speed for 3 min to remove precipitated MPA prior to LC-MS analysis.

Mass spectral data for peptide precursors for experiments described in Table 2 with XC junction highlighted:



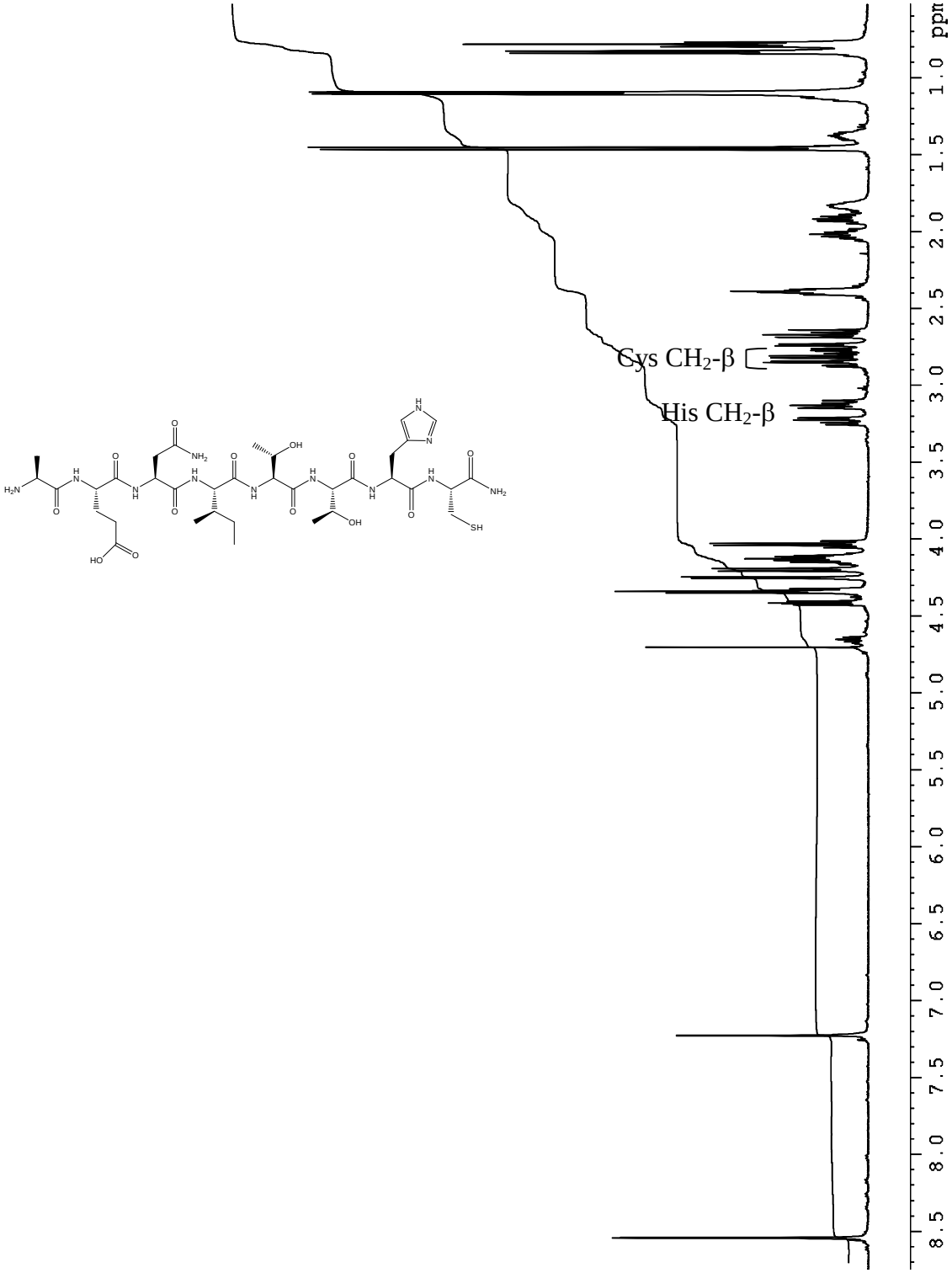


Thioester formation through MPA-mediated cleavage at **X**-C junctions at 80 °C.[a]

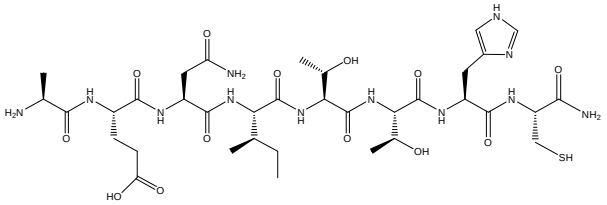
Entry	Peptide sequence	Cleavage extent (%)[b]	Thioester formation		Isolated yield (%)
			calc. m/z	obs. m/z	
1	AENITT**GC**-NH_2	85	793.3	793.5	33[e]
2	AENITT**AC**-NH_2	50	807.4	807.6	42, 40[f]
3	AENITT**PC**-NH_2	< 5	833.4	833.5	0, 49[f]
4	AENITT**EC**-NH_2	50	865.4	865.5	n.d.
5	AENITT**VC**-NH_2	< 5	835.4	835.6	0, 67[f]
6	AENITT**SC**-NH_2	50	823.4	911.5[c]	n.d.
7	AENITT**DC**-NH_2	> 90[d]	851.3	n/o	-

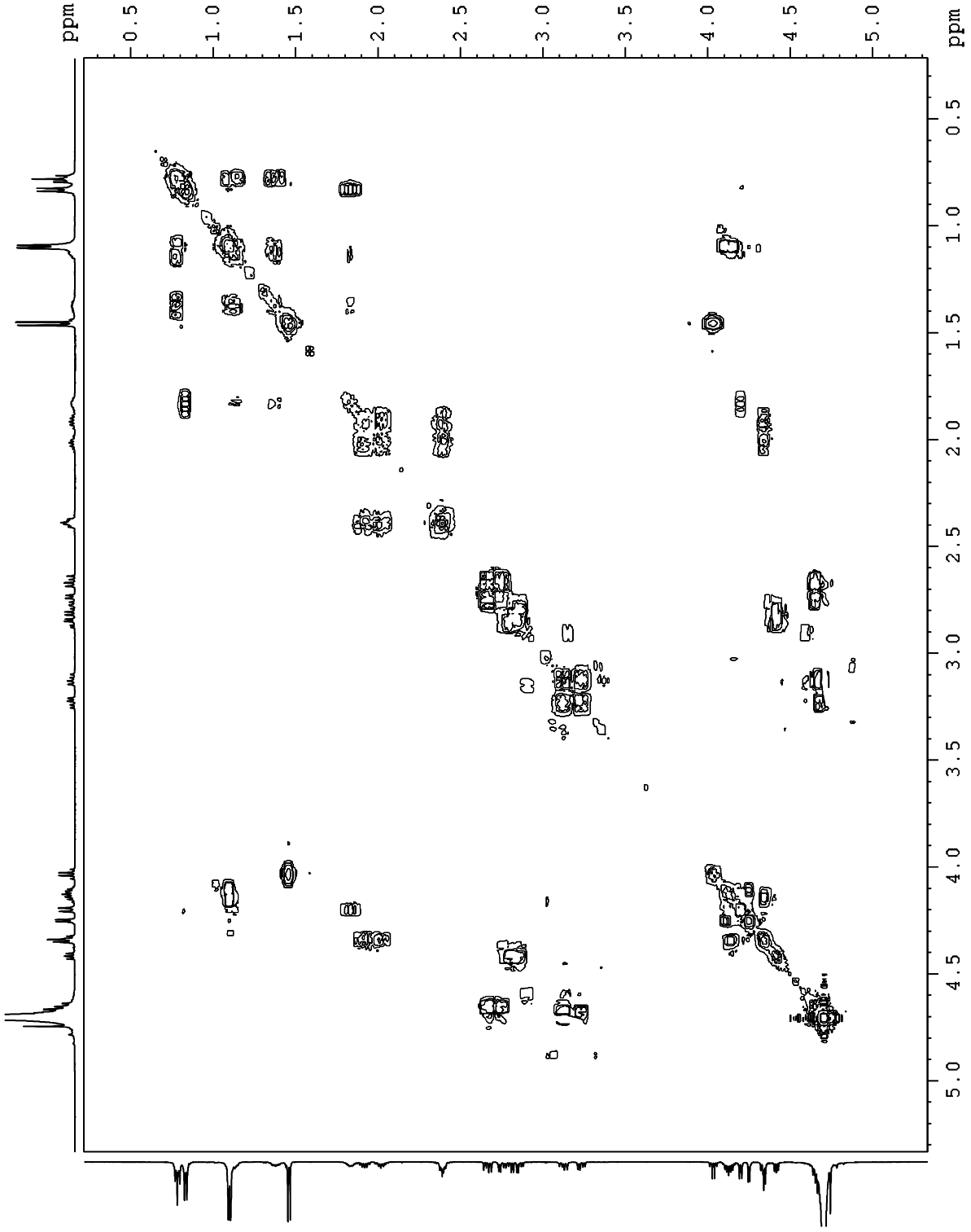
[a] data is not shown for FC, KC and WC junctions as product isolation proved difficult. [b]determined by LC-MS. [c] starting material and product appear further modified (esterified) with MPA. [d] No thioester observed. [e] considerable losses due to thioester hydrolysis under these conditions. [f] corresponds to the yield of recovered starting material.

^{1}H NMR analysis of model synthetic peptide H_2N-AENITT**HC**-NH_2

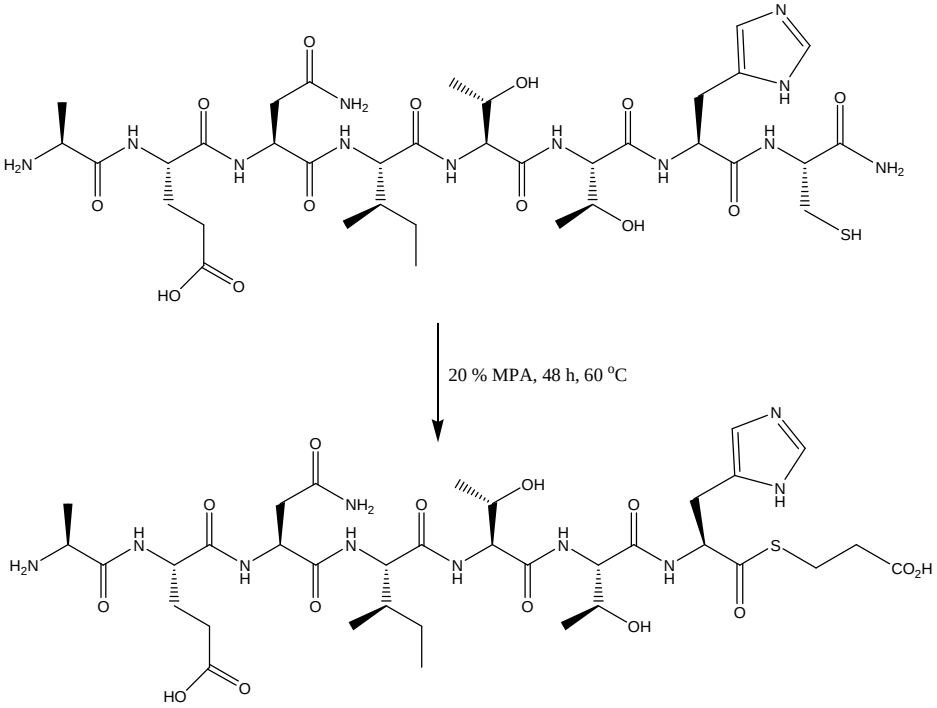


^{1}H NMR (2D-COSY) analysis of model synthetic peptide H_2N-AENITT**HC**-NH_2

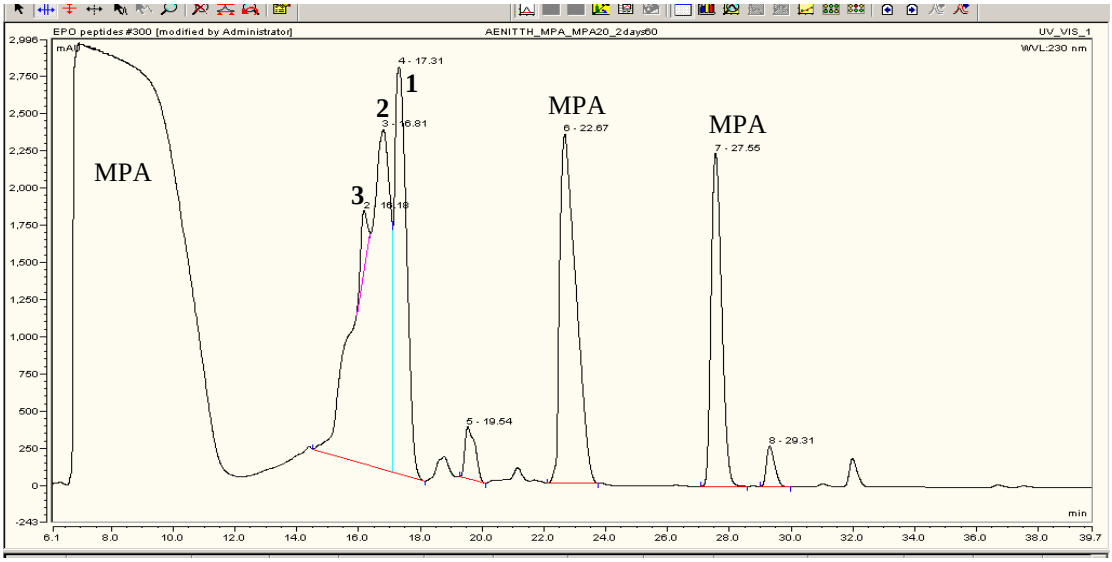




Conversion of H_2N-AENITT<u>HC</u>-NH_2 to H_2N-AENITT<u>H-MPA</u> thioester:



Procedure: 10 mg of purified H_2N-AENITTHC-NH_2 peptide was dissolved in 15 % aqueous MPA at 60 °C with shaking for 48 h. The reaction mixture was cooled to room temperature then loaded directly on to a semi-preparative HPLC-Column, from which three peptide derived product could be isolated:

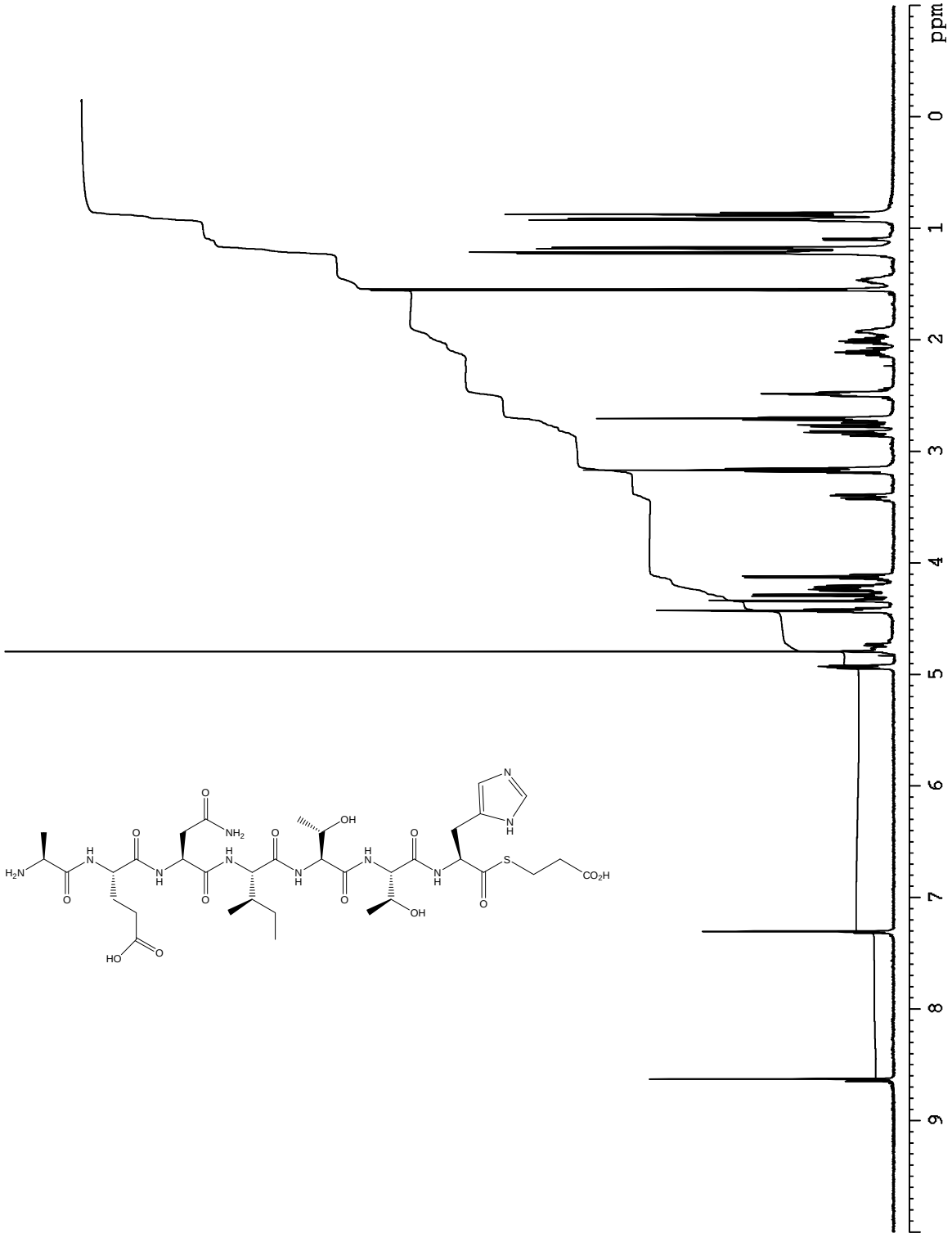


1 retention time = 17.3 min (AENITTH-MPA thioester product) (3 mg pure product isolated)

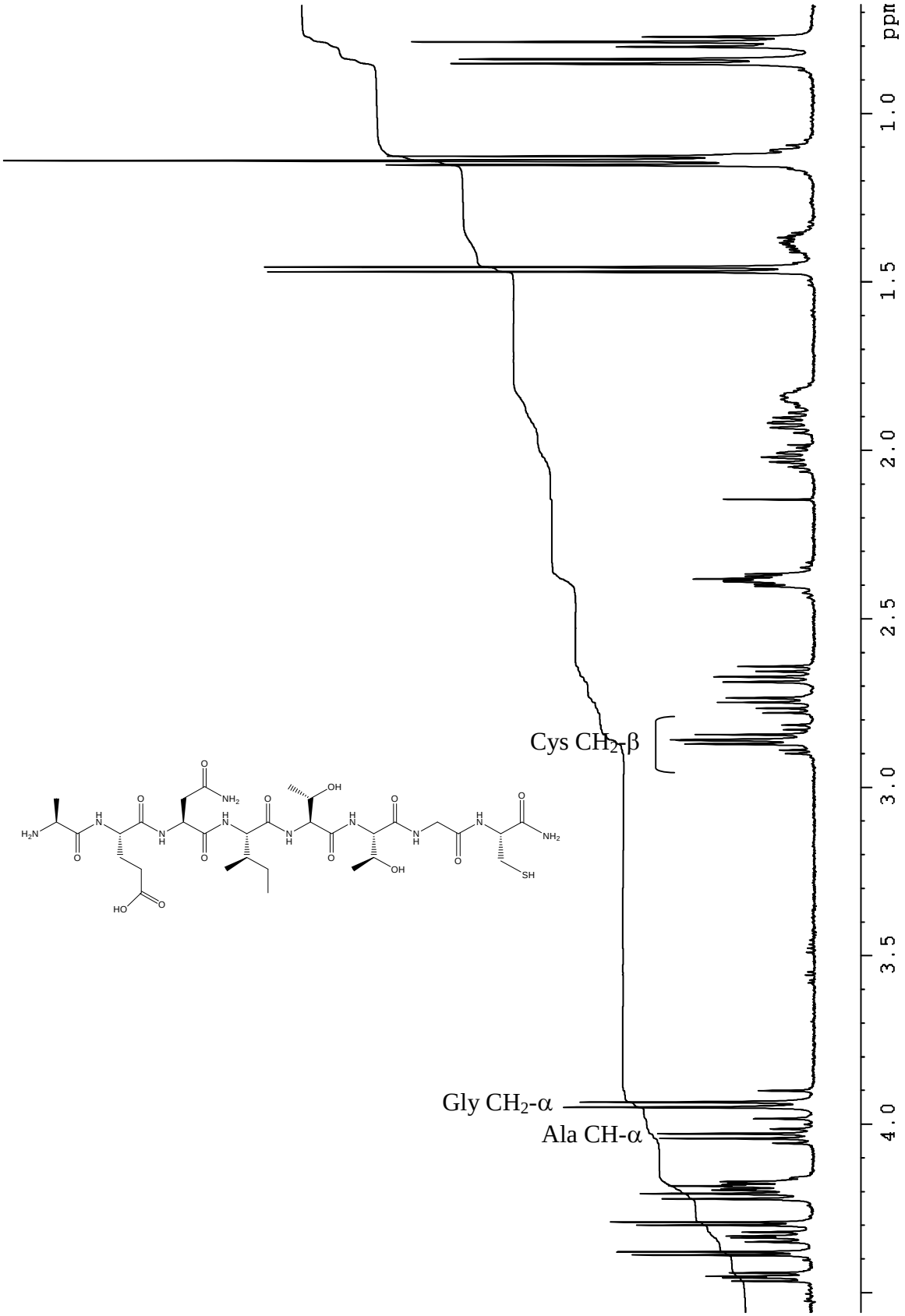
2 retention time = 16.8 min (AENITTHC starting material) (3.7 mg isolated as approximately 1:1 mixture of starting material and product.

3 retention time = 16.2 min (hydrolysed product) (2 mg isolated)

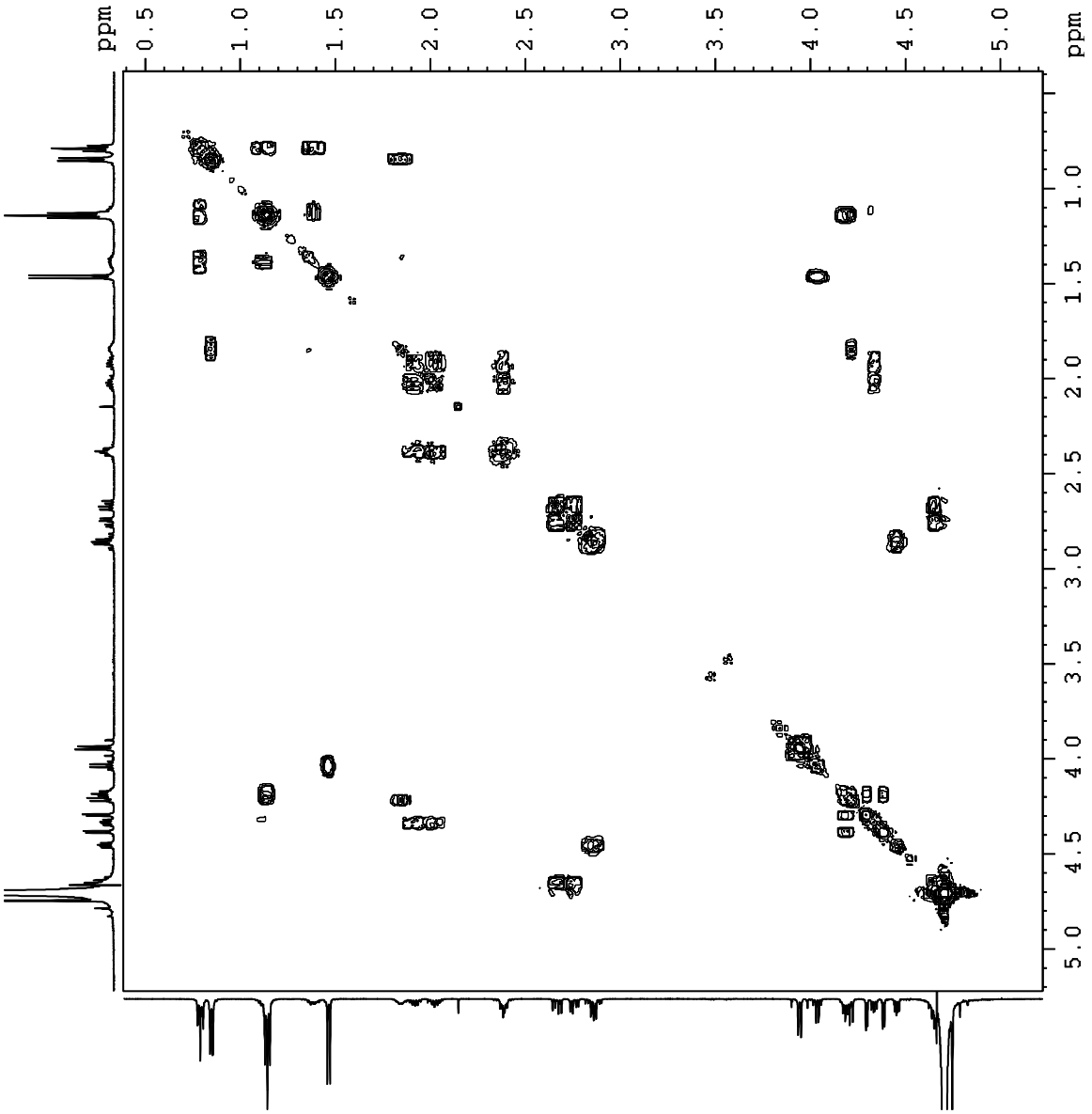
^{1}H NMR analysis of model synthetic peptide H_2N-AENITT**H-MPA** thioester



^{1}H NMR analysis of model synthetic peptide H_2N-AENITT**GC**-NH_2



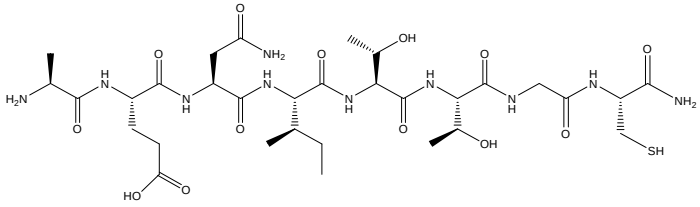
^{1}H NMR (2D-COSY) analysis of model synthetic peptide H_2N-AENITTGC-NH_2

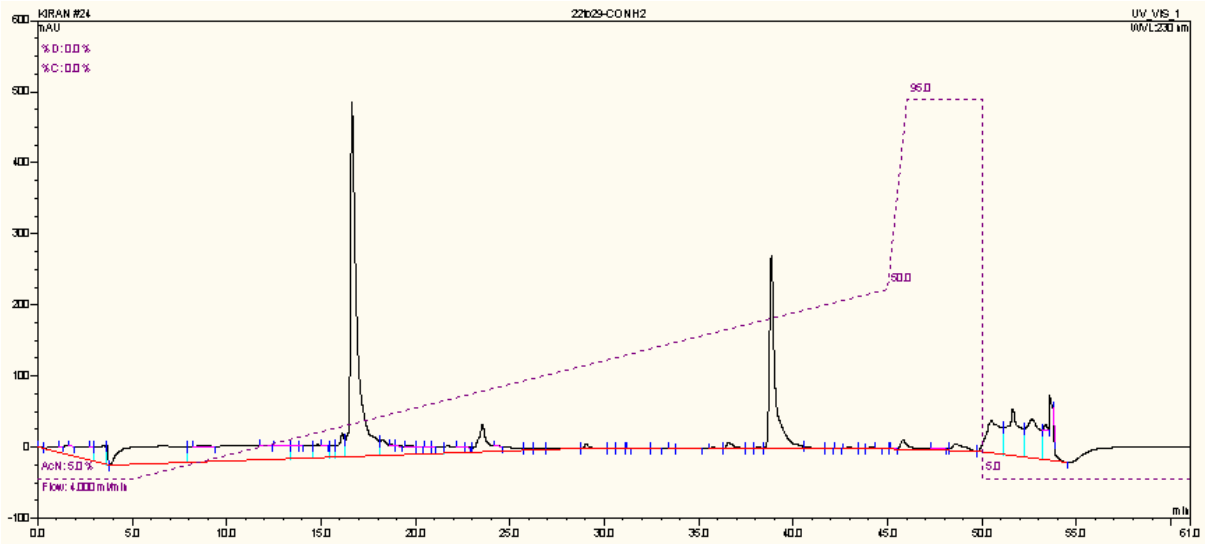


Conversion of H_2N-AENITTGC-NH_2 to H_2N-AENITTG-MPA thioester:

Representative HPLC data for MPA mediated thioesterification reaction:

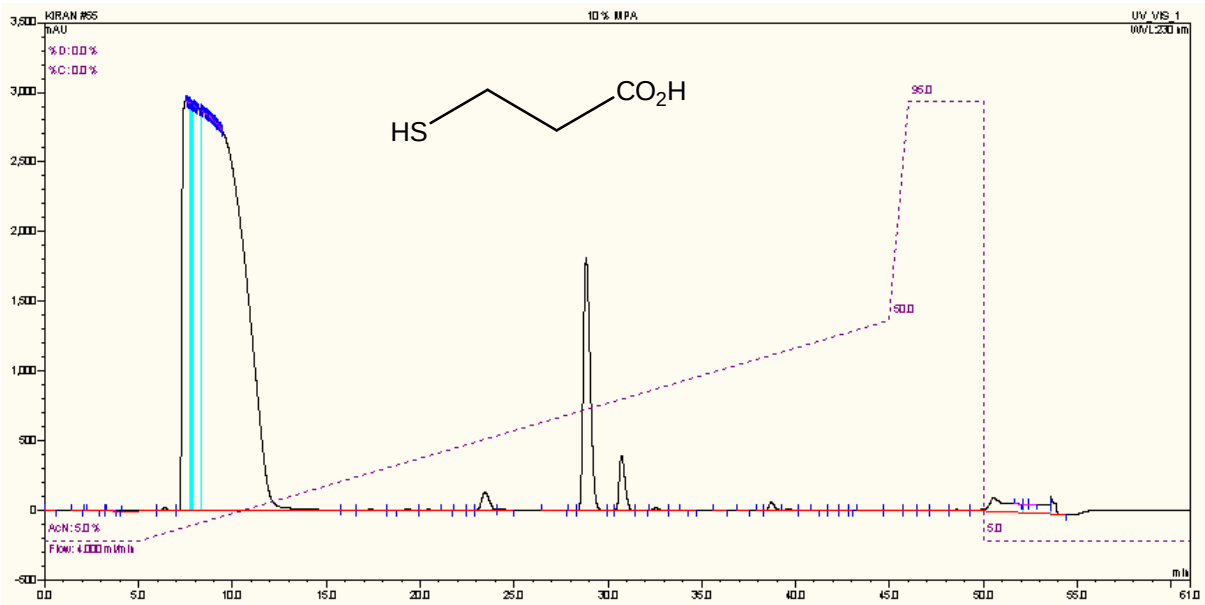
Purification of :



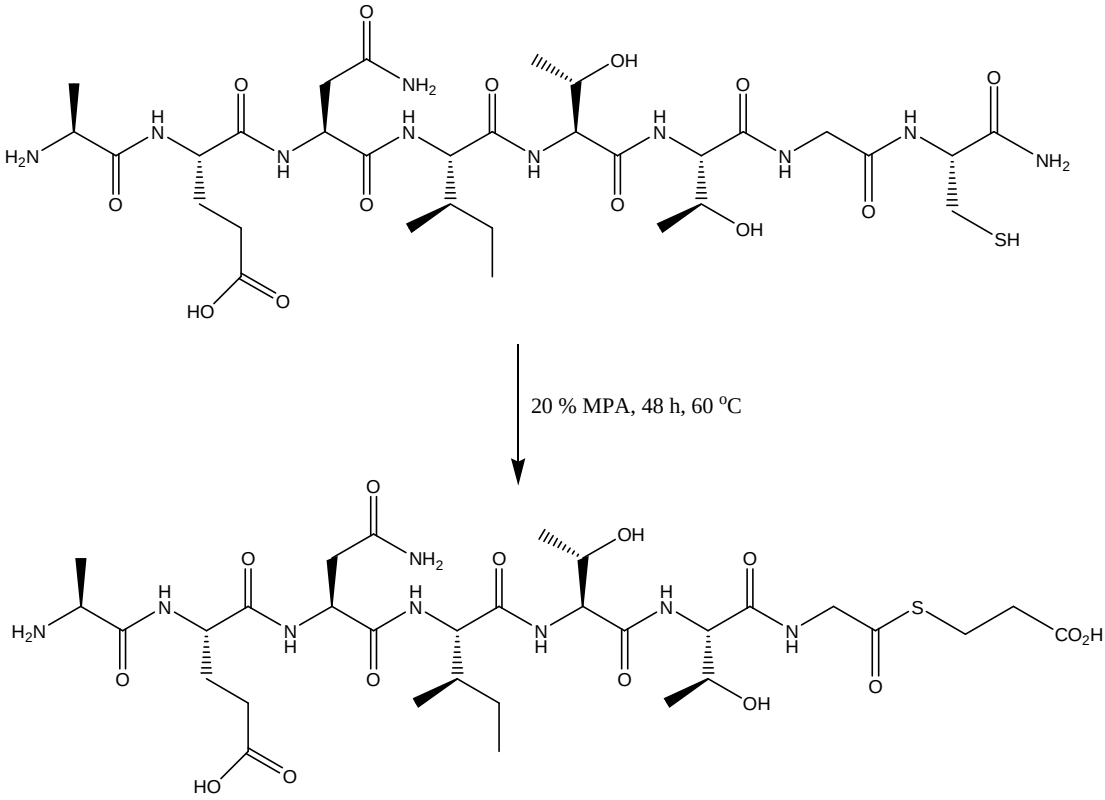


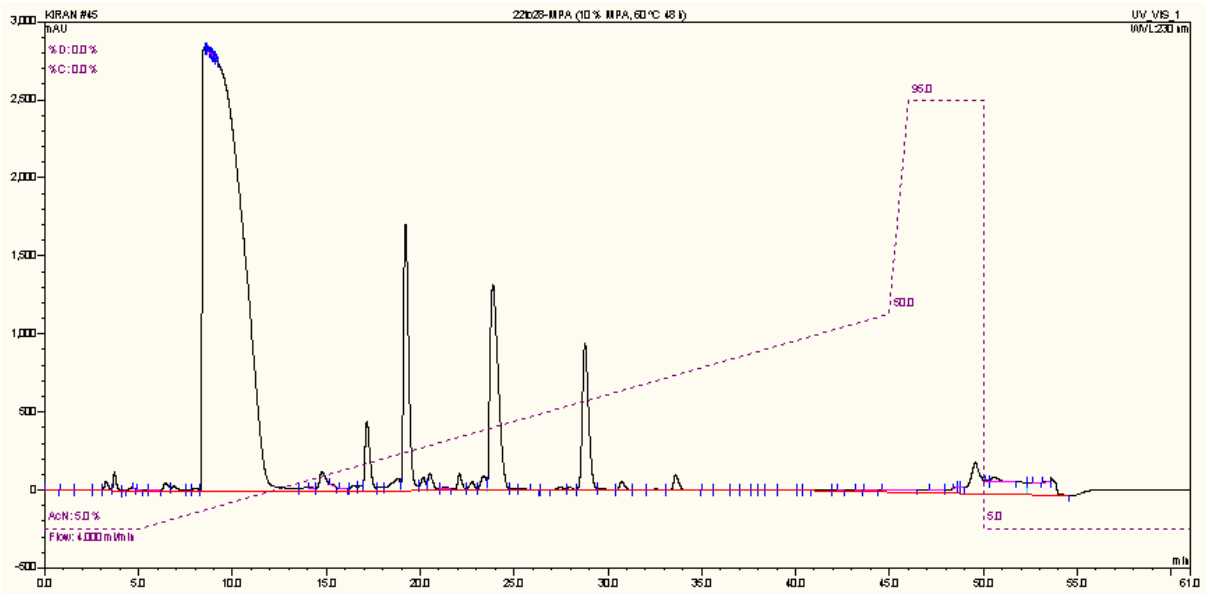
* H-AENITTGC-$CONH_2$, 5 to 50 % MeCN, 230 nm, retention time = 16.644 min

Identification of HPLC peaks derived from MPA



* <u>10 % aqueous MPA</u>, 5 to 50 % MeCN, 230 nm





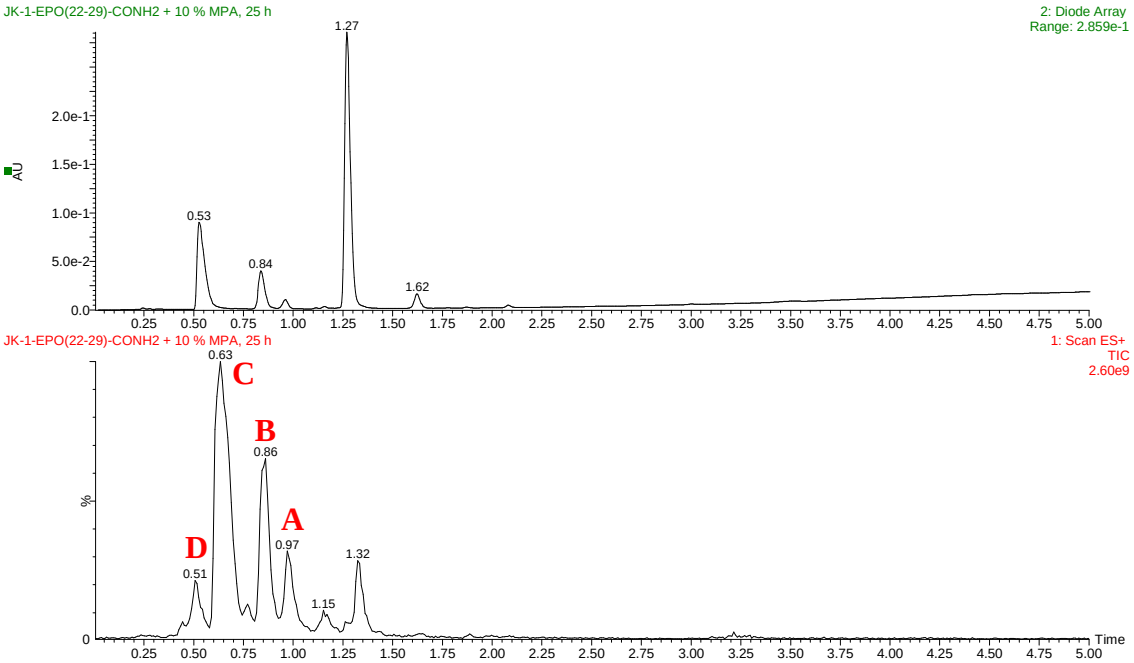
* Product = H-AENITTG-MPA, 5 to 50 % MeCN, retention time = 19.22 min

* SM = H-AENITTGC-$CONH_2$, 5 to 50 % MeCN, retention time = 17.17 min

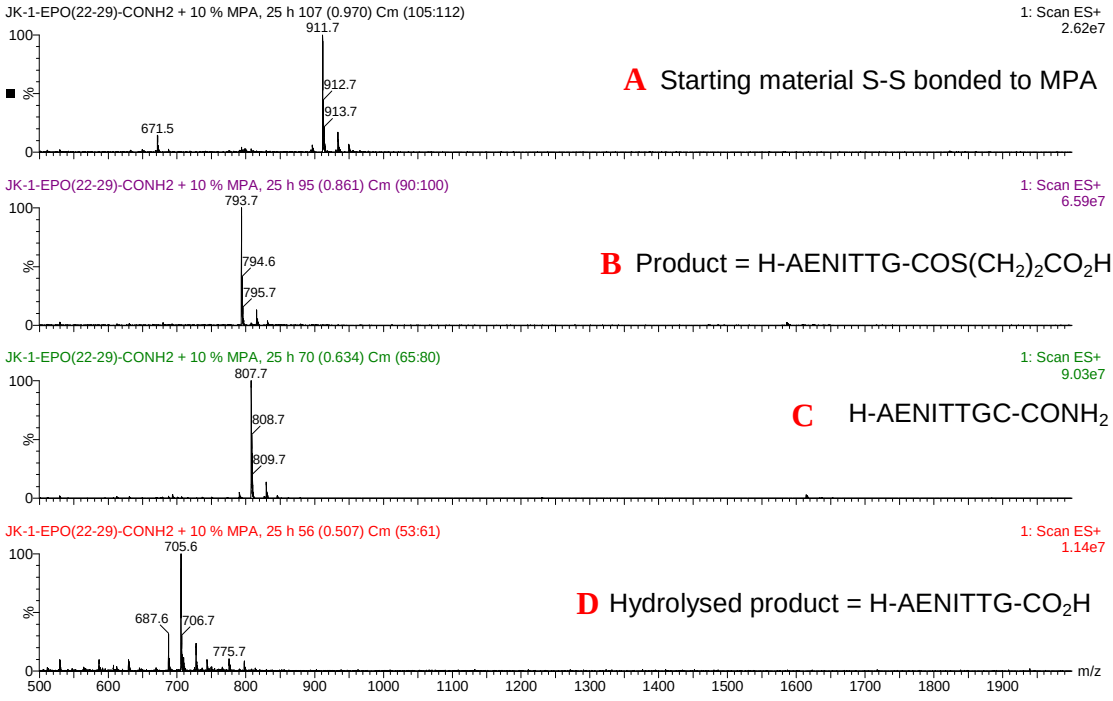
* Hydrolysed product = H-AENITTG-CO_2H, 5 to 50 % MeCN, retention time = 14.77 min

Representative LC-MS for the MPA mediated thioesterification reaction:

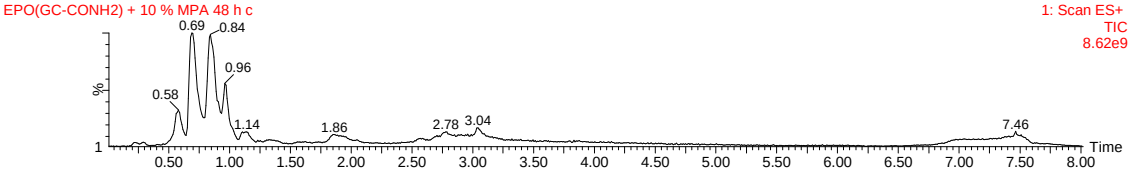
LC-MS analysis of the reaction (after 25)



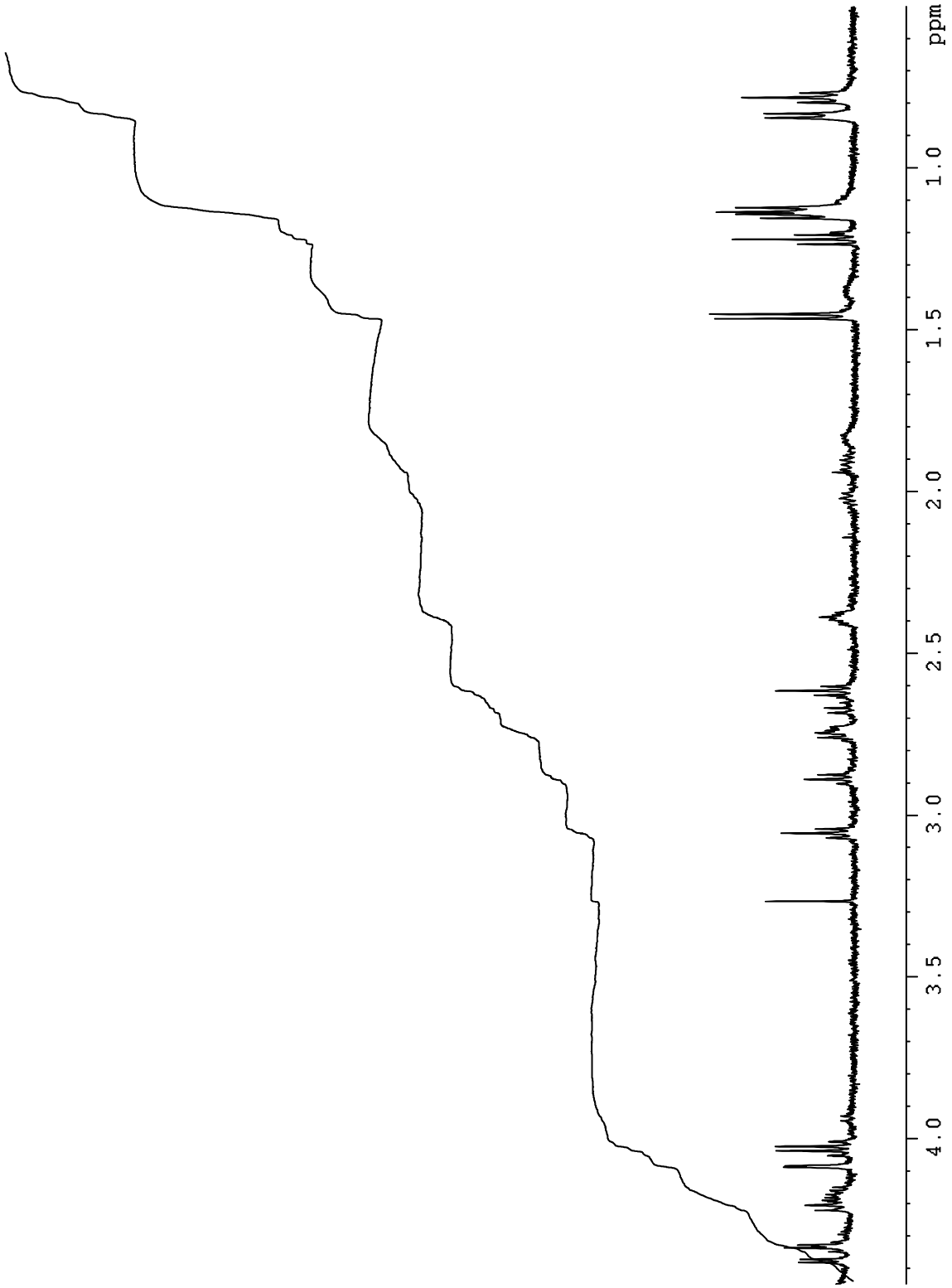
Assignment of TIC peaks **A-D**



TIC after 48 h

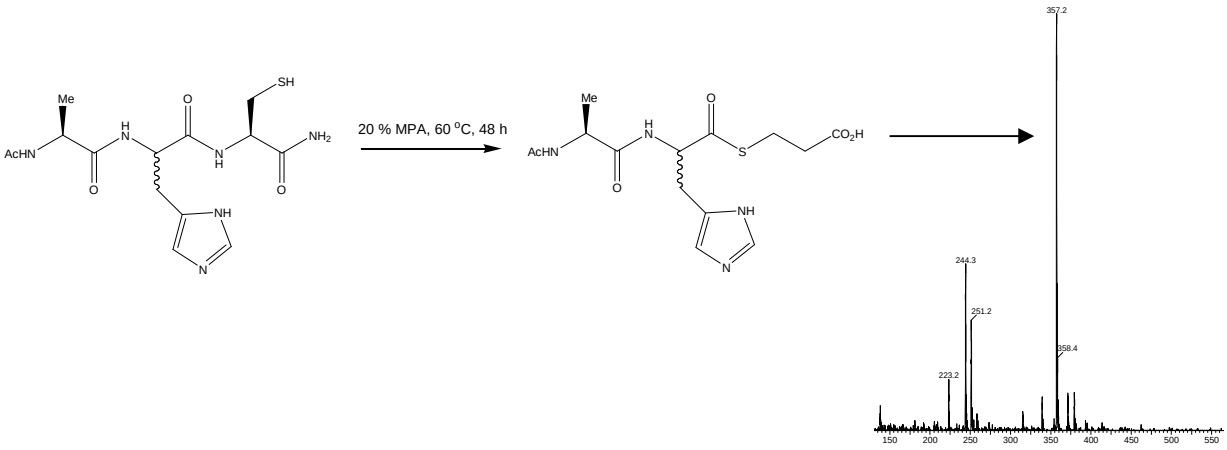


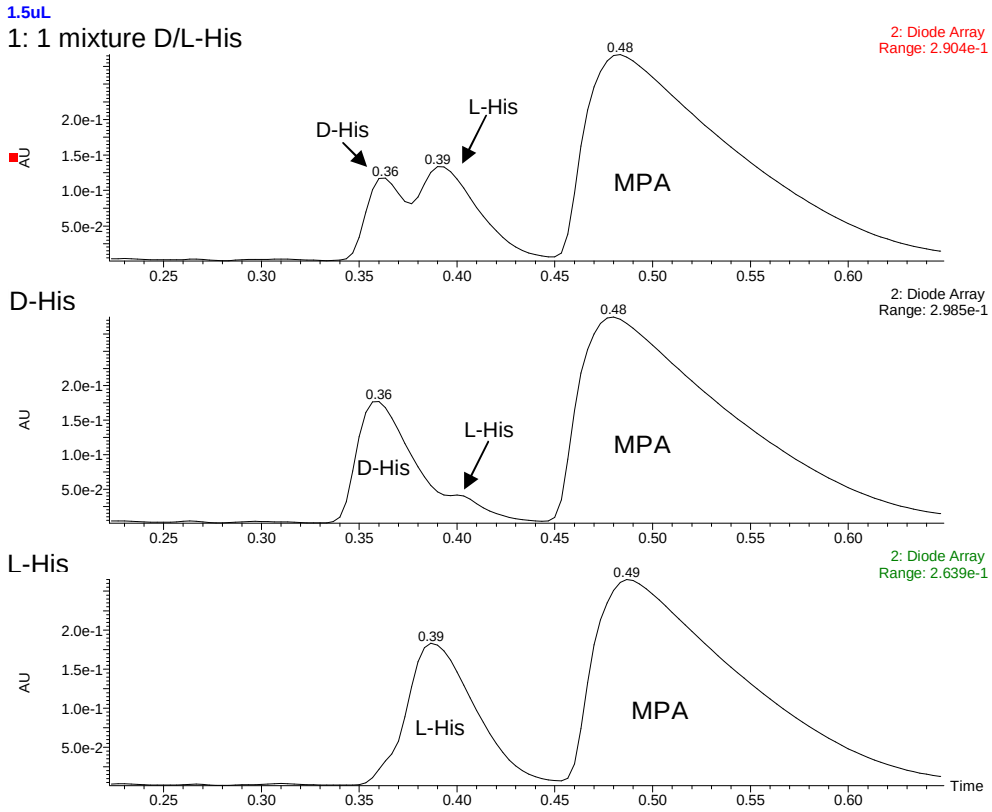
^{1}H NMR analysis of the isolated product (see semi-preparative HPLC trace above):



Synthesis of Ac-Ala-(L/D-)His-Cys-NH_2 for racemisation studies:

Synthesis of Ac-Ala-(*D*-)His-Cys-NH_2 and Ac-Ala-(*L*-)His-Cys-NH_2 was conducted on 0.05 mmol scale in manual fashion using HBTU/HOBt/DIPEA as described above: Upon cleavage and isolation it was clear that the hydrophilic nature of the peptides would make further purification difficult. Additionally, each peptide gave rise to two peptide species by LC-MS (tentatively assigned as the *N*-peptide and the *S*-peptide. Despite this operational difficulty, each peptide precursor gave rise to single thioester products (of identical mass) upon exposure to MPA with different retention times. Consequently ***three*** experiments were performed in parallel. The crude peptide samples were dissolved in 20 % v/v aqueous MPA. In two 1.5 mL eppendorf tubes 0.4 mL of each sample was heated at 60 ^{o}C for 48 h. In the third tube 0.2 mL of each sample was mixed and this sample was also subjected to heating at 60 ^{o}C in 20 % MPA for 48 h. Samples were analysed after 16 h and after 48 h by LC-MS:





After heating at 60 ^{o}C for 16 h the results (shown above) suggested that while the L-His containing sample appeared to have undergone little (if any) racemisation, the D-His containing peptide contained approximately 10 % of the L-His dipeptide. However, analysis of the peptide samples after 48 h showed no additional racemisation suggesting that the racemisation (of the D-His residue) had likely occurred during peptide synthesis and not during MPA treatment. The 1:1 mixture of each peptide precursor demonstrates how we would expect to observe a completely racemized starting material.

1) D. Macmillan, R. M. Bill, K. A. Sage, D. Fern, S. L. Flitsch, *Chem. Biol.* 2001, **8**, (2), 133.