# Synthesis and Amylin Receptor Activity of Glycomimetics of Pramlintide using Click Chemistry

Lauren R. Yule,[a,b,c] Rebekah L. Bower,[a] Harveen Kaur,[b,c] Renata Kowalczyk,[a,b,c] Debbie L. Hay,[a,c] Margaret A. Brimble.[*a,b,c]

[a]*The School of Biological Sciences, University of Auckland, 3 Symonds St, Auckland 1010, New Zealand. E-mail: dl.hay@auckland.ac.nz*

[b]*The School of Chemical Sciences, University of Auckland, 23 Symonds St, Auckland 1010, New Zealand. E-mail: m.brimble@auckland.ac.nz*

[c]*Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Private Bag 92019, Auckland 1010, New Zealand.*

## 1. Materials

All reagents were purchased as reagent grade and used without further purification. *O*-(6-Chlorobenzotriazol-1-yl)-*N,N,N*',*N*'-tetramethyluronium hexafluorophosphate (HCTU), *O*-(7-azabenzotriazol-1-yl)-*N,N,N*',*N*'-tetramethyluronium hexafluorophosphate (HATU), *N*-(9-fluorenylmethoxycarbonyloxy) succinimide (FmocOSu), 4-[(*R,S*)-α-[1-(9*H*-fluoren-9-yl)]-methoxycarbonylamino]-2,4-dimethoxy]phenoxyacetic acid (Fmoc-Rink amide linker) **19** and Fmoc-amino acids were purchased from GL Biochem (Shanghai, China). Fmoc-amino acids were supplied with the following side-chain protection: Fmoc-Tyr(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Lys(Boc)-OH.

Fmoc-Ser(*t*Bu)-Ser($\Psi^{Me,Me}$pro)-OH **21** was purchased from Aapptec (Louisville, Kentucky). *N,N*-Diisopropylethylamine ($i$Pr$_2$NEt), 2,4,6-collidine, piperidine, *N,N'*-diisopropylcarbodiimide (DIC), 3,6-dioxa-1,8-octane-dithiol (DODT), triisopropylsilane ($i$Pr$_3$SiH), 1-methyl-2-pyrrolidinone (NMP), 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt), ninhydrin, phenol, potassium cyanide (KCN), methanol (MeOH), ethanol (EtOH), diethyl ether (Et$_2$O), hydrazine hydrate (NH$_2$NH$_2$·1.5 H$_2$O), and copper(II) sulphate pentahydrate (CuSO$_4$·5 H$_2$O) were purchased from Sigma-Aldrich (St. Louis, Missouri). Dichloromethane (CH$_2$Cl$_2$), disodium hydrogen phosphate (Na$_2$HPO$_4$), magnesium sulphate (MgSO$_4$), sodium bicarbonate (NaHCO$_3$), sodium carbonate (Na$_2$CO$_3$), ethyl acetate (EtOAc) and hexane were purchased from ECP limited (Auckland, New Zealand). Hydrochloric acid (HCl), sodium hydroxide (NaOH), *N,N*-dimethylformamide (DMF) (synthesis grade), and acetonitrile (MeCN), were purchased from Scharlau (Barcelona, Spain). Dimethyl sulfoxide (DMSO) was purchased from Romil Limited (Cambridge, United Kingdom). Tris(2-carboxethyl)-phosphine hydrochloride (TCEP·HCl) and L-propargylglycine (L-Pra) were purchased from AK Scientific (Union City, California). Tetrahydrofuran (THF) was purchased from Avantor Performance Materials (Centre Valley, Pennsylvania). Guanidinium chloride (Gu·HCl) was purchased from MP Biomedicals (Santa Ana, California). Trifluoroacetic acid (TFA) was purchased from Halocarbon (River Edge, New Jersey).

Aminomethyl polystyrene resin **18** (AMPS)[1] and Fmoc-propargylglycine **20** (Fmoc-L-Pra-OH)[2] were synthesised following literature procedures. 2-acetamido-2-deoxy-β-D-glucopyranosyl azide (β-GlcNAcN$_3$) **22** and $N^4$-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-$N^2$-(9-fluorenylmethylcarbonyl)asparagine (Fmoc-Asn(GlcNAc(OAc)$_3$)-OH) **23**[3] were supplied from Professor Antony Fairbanks from the University of Canterbury, New Zealand.

## 2. General procedure for peptide synthesis

Peptides were synthesised by automated 9-fluorenylmethoxycarbonyl solid phase peptide synthesis (Fmoc-SPPS) using either a microwave enhanced Biotage® initiator + alstra or a room temperature (rt) Tribute™ peptide synthesiser on a 0.1 mmol scale.

Using the Tribute™ peptide synthesiser, all amino acid couplings were performed as single coupling cycles. Protected amino acids were incorporated using Fmoc-AA-OH (5.0 eq., 0.5 M), HCTU (4.5 eq., 0.45 M) and *i*$Pr_2$NEt (10 eq., 2 M) in DMF, for 45 min. Fmoc-[Asn[GlcNAc$(OAc)_3$]-OH **23** (2 eq.), Fmoc-L-Pra-OH **20** (2 eq.) and Fmoc-Ser(*t*Bu)-Ser($\Psi^{Me,Me}$pro)-OH (2 eq.) **21** were coupled for 1.5 h at room temperature in the presence of HATU (1.9 eq.) and collidine (6 eq.) in DMF. The Fmoc group was removed using 20% piperidine in DMF (2 x 5 min).

Using the Biotage® initiator + alstra peptide synthesiser, all amino acid couplings were performed as single coupling cycles, with the exception of Fmoc-Arg(Pbf)-OH, Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH where a doubling coupling cycle was performed. Protected amino acids were incorporated using Fmoc-AA-OH (5.0 eq., 0.2 M), HCTU (4.5 eq., 0.23 M) and *i*$Pr_2$NEt (10 eq., 2 M) in DMF, for 5 min at a maximum temperature of 75 °C and at 25 W, except Fmoc-Arg(Pbf)-OH which was coupled for 25 min at rt followed by a second coupling for 3 min at a maximum temperature of 72 °C and at 25 W, and Fmoc-His(Trt)-OH and Fmoc-Cys(Trt)-OH which were coupled for 10 min at rt followed by a second coupling for 5 min at a maximum temperature of 47 °C and at 25 W. Fmoc-Pra-OH **20** (2 eq.), and Fmoc-Ser(*t*Bu)-Ser($\Psi^{Me,Me}$pro)-OH (2 eq.) **21** and Fmoc-Asn[GlcNAc$(OAc)_3$]-OH **23**[3] (2 eq.) were coupled for 15 min at a maximum temperature of 75 °C and at 25 W in the presence of HATU (1.9 eq.) and collidine (6 eq.). The Fmoc group was removed using 20% piperidine in DMF (2 x 3 min at a maximum temperature of 70 °C and at 62 W).

Peptides were cleaved from the resin by treatment with trifluoroacetic acid/triisopropylsilane/water/3,6-dioxa-1,8-octanedithiol (TFA/TIS/$H_2O$/DODT) (*v*/*v*/*v*/*v*; 94/1/2.5/2.5) for 2.5 h at rt The crude peptides were precipitated and triturated with cold diethyl ether (40 mL), isolated (centrifugation), and dissolved in MeCN/$H_2O$ (1:1) containing 0.1% TFA and lyophilised.

## 3. General procedure for purification and analysis

Analytical reverse phase high-performance liquid chromatography (RP-HPLC) was performed on a Dionex ultimate 3000 using the following columns: Vydac Diphenyl 300 Å, 3 µm, 4.6 mm x 250 mm, Agilent Zorbax 300SB-C3, 3.0 mm x 150 mm; 3.5 µm, Agilent Zorbax 300SB-C3, 4.6 mm x 150 mm; 5 µm. Analytical liquid-chromatography-mass spectrometry (LCMS) was performed on an Agilent Technologies 1120 Compact LC connected to a HP Series 1100 MSD spectrometer using an Agilent Zorbax 300SB-C3, 3.0 mm x 150 mm; 3.5 µm column. Semi-preparative reverse phase high-performance liquid chromatography (RP-HPLC) was performed using either a Waters 600E System with a Waters 2487 dual wavelength absorbance detector or a Dionex Ultimate 3000 using the following columns: Phenomenex Gemini $C_{18}$ 110 Å, 10.0 mm x 250 mm; 5 µm (5 mL/min) or Vydac Diphenyl 300 Å, 10.0 mm x 250mm; 5 µm (5 mL/min). A linear gradient of 0.1% trifluoroacetic acid/water (A) and 0.1% trifluoroacetic acid/acetonitrile (B) was used with detection at 210 nm. Gradient systems used for semi-preparative RP-HPLC were adjusted according to the elution and peak profiles obtained from the analytical RP-HPLC chromatograms, and are specified in the experimental procedures section.

## 4. General procedure for disulfide bond formation (Cys-2/Cys-7) for pramlintide 1 and analogues 5-7

The final peptide concentration used for each disulfide bond formation reaction was 3 mM in a mixture of Gu·HCl (6 M) and $Na_2HPO_4$ buffer (adjusted to the final concentration of 0.2 M). The mixture was then agitated at rt for 2 h. The crude product was lyophilised and purified by RP-HPLC using conditions as specified in general procedures section 3.

## 5. General procedure for Cu(I) mediated azide-alkyne cycloaddition "click" reaction with simultaneous disulfide bond formation (Cys-2/Cys-7) for analogues 2-4 and 10-11

The final peptide concentration used for each "click" reaction was 3 mM in a solution of Gu·HCl (6 M) and $Na_2HPO_4$ buffer (adjusted to the final concentration of 0.2 M). The final concentrations of TCEP·HCl and $CuSO_4{\cdot}5H_2O$ were adjusted to 20 mM.

0.5 M Stock solutions of TCEP·HCl and $CuSO_4{\cdot}5\ H_2O$ were prepared. 100 µl of the 0.5 M stock solution of TCEP·HCl was basified to pH 7 using solid NaOH. The required volume of 0.5 M $CuSO_4{\cdot}5\ H_2O$ (6.7 eq.) was added to the required volume of 0.5 M, pH 7 TCEP·HCl (6.7 eq.), mixed, and shaken for 1 min until a blue precipitate formed. Partial disappearance of the blue precipitate was observed over the next 2-3 min. The peptide containing a propargylglycine residue (1.0 eq.) was dissolved in the deoxygenated (Ar, 30 min) buffer solution of Gu·HCl and $Na_2HPO_4$. The cloudy mixture of $CuSO_4{\cdot}5\ H_2O$/ TCEP·HCl was then added to the peptide solution portion-wise to further solubilise the precipitate. The mixture was then incubated for 30 min at 60 °C after which time a clear, faint blue solution was obtained. $GlcNAcN_3$ **22** (6 eq.) was then added to the mixture and the solution was purged with Ar, which was then subjected to microwave irradiation for 2 h at 60 °C and 20 W and the reaction progress was monitored by RP-HPLC. The crude product was diluted ($H_2O$, 1 mL), acidified to pH 1 (TFA), lyophilised, and purified by RP-HPLC using conditions as specified in general procedures section 3.

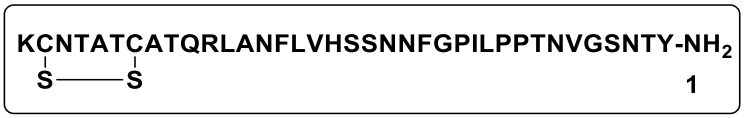
## 6. General procedure for acetate removal with simultaneous disulfide bond formation (Cys-2/Cys-7) for analogues 8-13

The crude acetate protected glycopeptide was dissolved in 5% $NH_2NH_2{\cdot}1.5\ H_2O$ in DMSO to reach a final concentration of 3 mg/ml. The mixture was agitated at rt for 3 h and DMSO was removed by RP-HPLC using an isocratic method of 80% B for 15 minutes. The crude peptide was then lyophilised, and purified by RP-HPLC using conditions specified in general procedures 3.

## 7. General procedure for measuring the agonist effect of pramlintide 1 and pramlintide analogues 2-13 at the $AMY_{1(a)}$ receptor

Pramlintide **1** and glycopeptides **2**-**13** were screened at the $AMY_{1(a)}$ receptor. Pramlintide **1** was included as a control in each experiment. Cos 7 cells were transiently transfected with the necessary receptor components, and cyclic AMP production was measured according to our published methods.[4,5,6] The $hCT_{(a)}$ construct used was the insert negative $hCT_{(a)}$ receptor with leucine at the polymorphic amino acid position 447 and an N-terminal hemagglutinin tag in pcDNA 3.1 vector (from Professor Patrick Sexton, Monash Institute of Pharmaceutical Sciences, Monash, Australia). Human RAMP1 with an N-terminal myc tag in a pcDNA3 vector was used (from Steven Foord, GlaxoSmithKline). Peptides **1**-**13** were weighed out and stock solutions made at 1 mM or 10 mM (diluted in sterile water), on the basis of peptide weight. 80% peptide content was assumed and taken into account in these calculations. All peptide stock solutions were stored in siliconised or Lobind (Eppendorf, Hamburg, Germany) microcentrifuge tubes at -30 °C in 2-6 µL aliquots to minimise freeze-thaw cycles.

## Synthesis of Pramlintide 1




Automated Fmoc-SPPS using Tribute™ rt peptide synthesiser was used for the synthesis of the linear pramlintide **14**, which was followed by resin cleavage using the conditions outlined in general procedure 2 to afford crude reduced **14** as a white solid (172 mg, 19% yield based on 43% purity by LCMS), (Figure S 1). The crude linear peptide **14** (9.62 mg, 2.43 × $10^{-3}$ mmol) was dissolved in a mixture of 6 M Gu·HCl (0.81 mL) and $Na_2HPO_4$ (20 mg, 0.16 mmol) to form a disulfide bond between Cys-2 and Cys-7 according to general procedure 4 (Figure S 2) to afford crude pramlintide **1**.

The crude pramlintide **1** was purified by semi-preparative RP-HPLC using Dionex Ultimate 3000 on a Vydac Diphenyl column, using a gradient of 0%B to 13%B over 13 min (*ca.* 1% B/min) then 13%B to 60%B over 313 min (*ca.* 0.15%B/min). Fractions were collected at 0.5 min intervals and analysed by ESI-MS and RP-HPLC. Fractions identified with the correct m/z were combined and lyophilised to afford the *title compound* **1** as a white amorphous solid (2.75 mg, 67% yield, 96% purity); $R_t$ 30.06 min; *m/z* (ESI-MS) 987.7 ([M+4H]$^{4+}$ requires 988.4), Figure S 3.

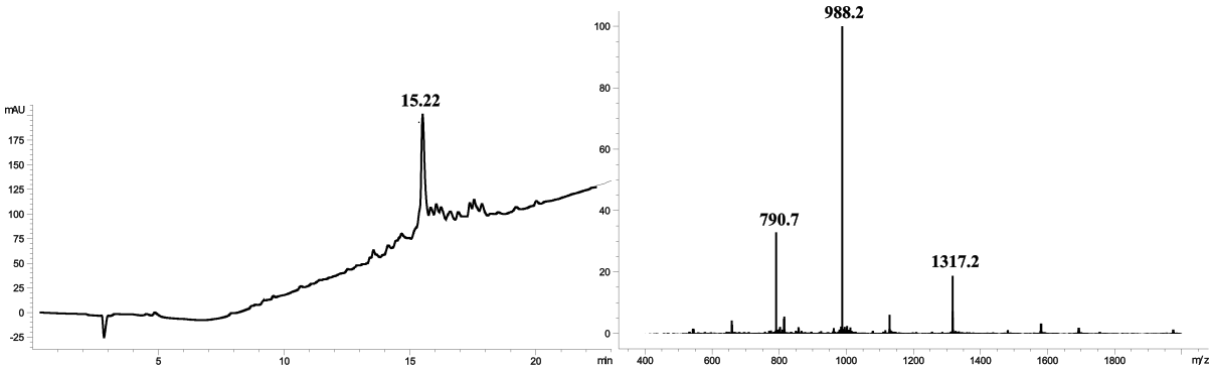



**Figure S 1** LCMS profile of crude linear pramlintide **14 (***ca* 43% as analysed by peak area of RP-HPLC at 214 nm); linear gradient of 5%B to 65%B over 20 min (*ca.* 3%B/min) at 40 °C, 0.3 mL/min.

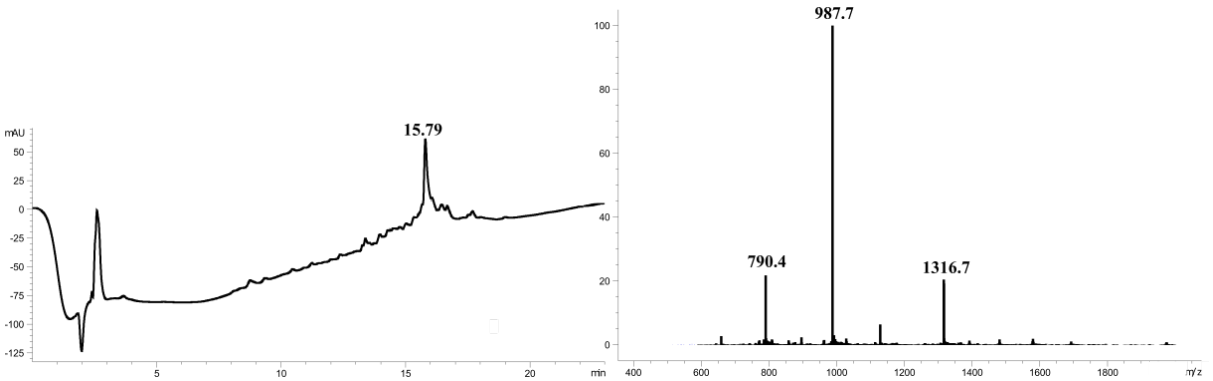



**Figure S 2** LCMS profile of crude pramlintide **1**; linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

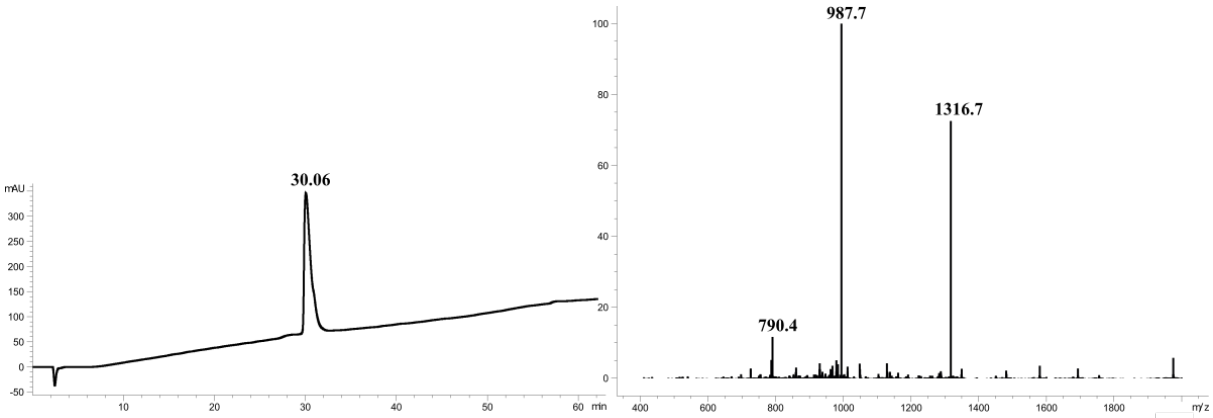
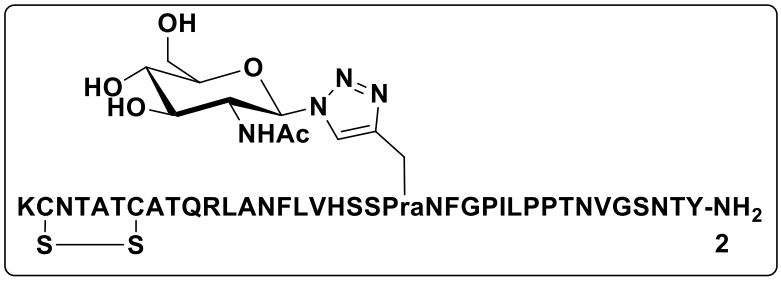



**Figure S 3** LCMS profile of pure pramlintide **1** (97%); linear gradient of 5%B to 65%B over 60 min, (*ca*. 1%B/min) at 40 °C, 0.3 mL/min.

## Synthesis of pramlintide analogue 2




Automated Fmoc-SPPS using Tribute$^{TM}$ rt peptide synthesiser was used for the synthesis of reduced pramlintide precursor **15**, which was followed by resin cleavage using the conditions outlined in general procedure 2 to afford crude reduced pramlintide analogue **15** as a white solid (170 mg, 29% yield based on 68% purity by LCMS) (Figure S 4). The crude linear peptide **15** (20 mg, $4.7 \times 10^{-3}$ mmol) underwent a Cu(I) mediated cycloaddition reaction and simultaneous disulfide bond formation (between Cys-2 and Cys-7) with $GlcNAcN_3$ **22** (7.45 mg, $3.1 \times 10^{-2}$ mmol) according to general procedure 5 (Figure S 5). This reaction was carried out using 0.5 M TCEP·HCl (68 µL, $3.4 \times 10^{-2}$ mmol, pH 7), 0.5 M $CuSO_4 \cdot 5H_2O$ (68 µL, $3.4 \times 10^{-2}$ mmol) and $Na_2HPO_4$ (48.3 mg, 0.34 mmol) in 6 M Gu·HCl (1.56 mL) to afford crude pramlintide analogue **2**.

The crude pramlintide analogue **2** was purified by semi-preparative RP-HPLC using Dionex Ultimate 3000 on a Diphenyl Vydac column, using a gradient of 0%B to 13%B over 13 min (*ca.* 1%B/min) then 13%B to 60%B over 313 min (*ca.* 0.15%B/min). This afforded the *title compound* **2** as a white amorphous solid (3.3 mg, 23% yield, 95% purity); $R_t$ 15.23 min; *m/z* (ESI-MS) 1045.0 ($[M+4H]^{4+}$ requires 1045.1), Figure S 6.

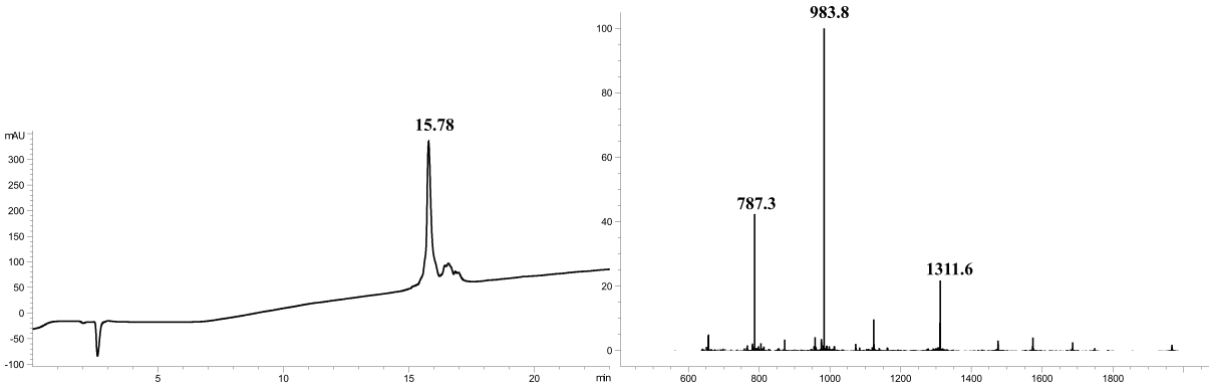



**Figure S 4** LCMS profile of crude linear pramlintide analogue **15** (*ca* 68% as analysed by peak area of RP-HPLC at 214 nm); linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

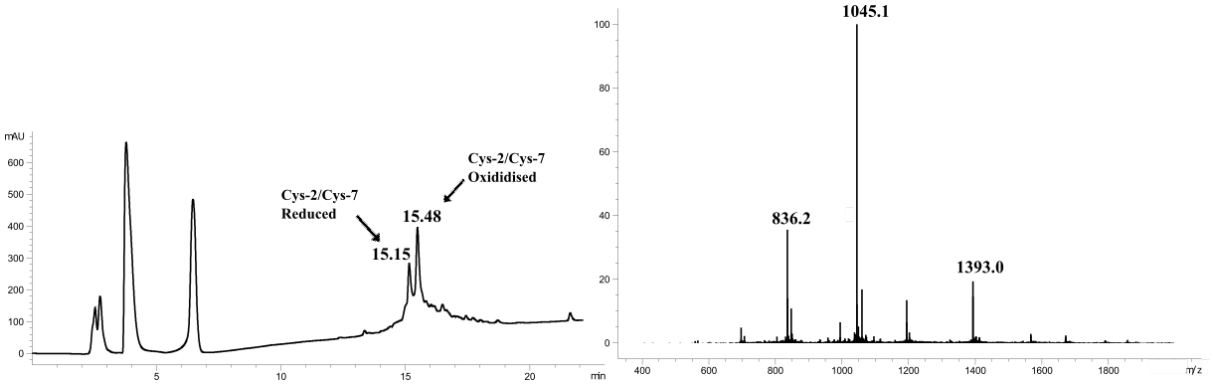



**Figure S 5:** LCMS profile of crude pramlintide analogue **2**; linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

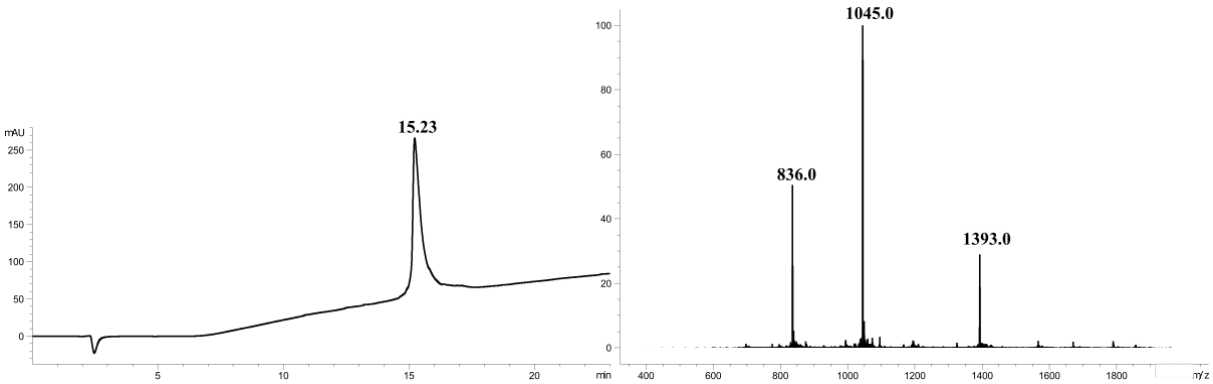
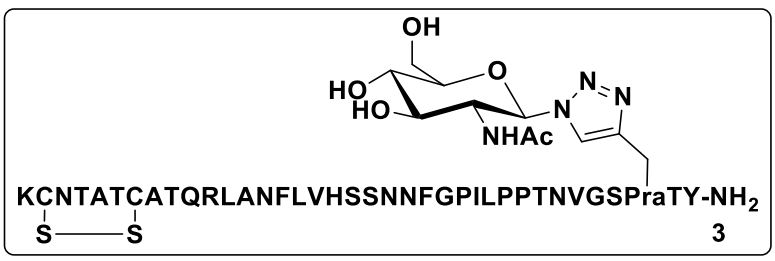



**Figure S 6:** LCMS profile of pure pramlintide analogue **2** (*95%*); linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

## Synthesis of pramlintide analogue 3



Automated Fmoc-SPPS using Tribute$^{TM}$ rt peptide synthesiser was used for the synthesis of reduced pramlintide precursor **16** which was followed by resin cleavage using the conditions outlined in general procedure 2 to afford crude reduced pramlintide analogue **16** as a white solid (222 mg, 30% yield based on 53% purity by LCMS) (Figure S 7). The crude linear peptide **16** (20 mg, $4.7 \times 10^{-3}$ mmol) underwent a Cu(I) mediated cycloaddition reaction and simultaneous disulfide bond formation (between Cys-2 and Cys-7) with $GlcNAcN_3$ **22** (7.45 mg, $3.1 \times 10^{-2}$ mmol) according to general procedure 5 (Figure S 8). This reaction was carried out using 0.5 M TCEP·HCl (68 µL, $3.4 \times 10^{-2}$ mmol, pH 7), 0.5 M $CuSO_4 \cdot 5H_2O$ (68 µL, $3.4 \times 10^{-2}$ mmol) and $Na_2HPO_4$ (48.3 mg, 0.34 mmol) in 6 M Gu·HCl (1.56 mL) to afford crude pramlintide analogue **3**.

The crude pramlintide analogue **3** was purified by semi-preparative RP-HPLC using Dionex Ultimate 3000 on a Diphenyl Vydac column, using a gradient of 0%B to 13%B over 13 min (*ca.* 1%B/min) then 13%B to 60%B over 313 min (*ca.* 0.15%B/min). This afforded the *title compound* **3** as a white amorphous solid (1.1 mg, 10% yield, 97% purity); $R_t$ 39.88 min; *m/z* (ESI-MS) 1045.1 ($[M+4H]^{4+}$ requires 1045.1), Figure S 9.

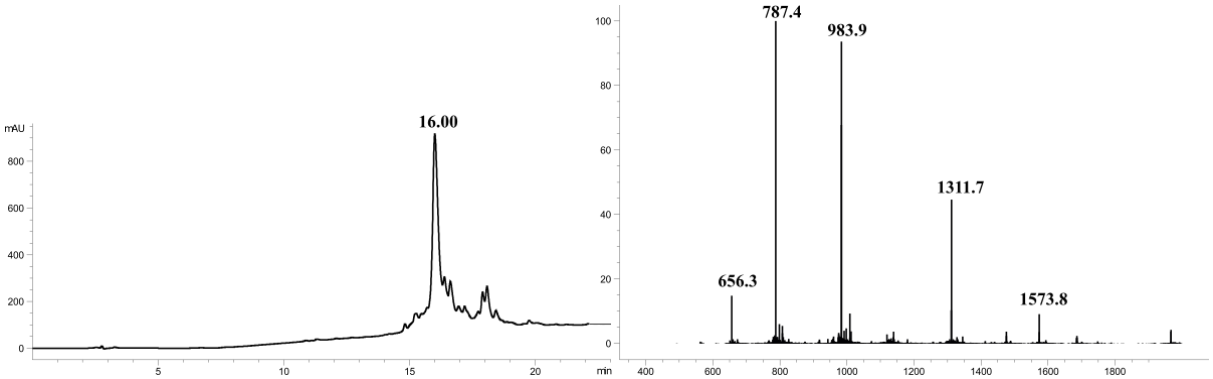



**Figure S 7** LCMS profile of crude linear pramlintide analogue **16** (*ca* 53% as analysed by peak area of RP-HPLC at 214 nm); linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

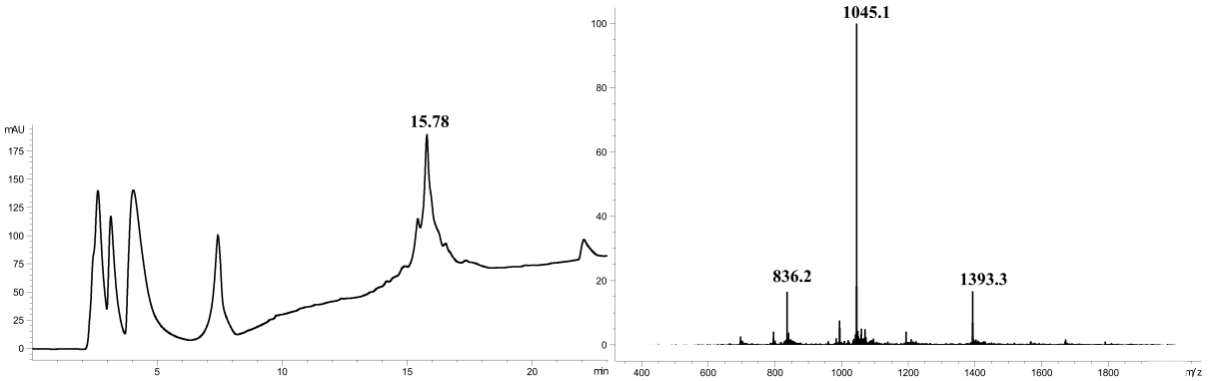



**Figure S 8** LCMS profile of crude pramlintide analogue **3**; linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

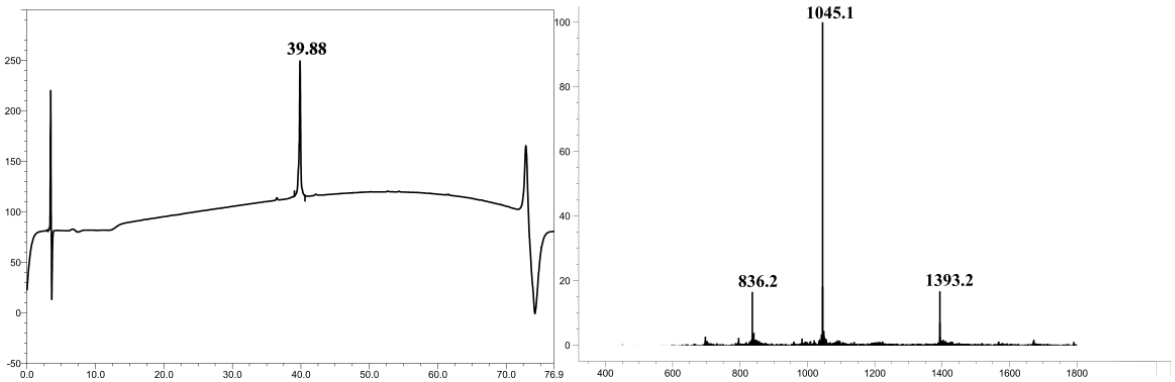
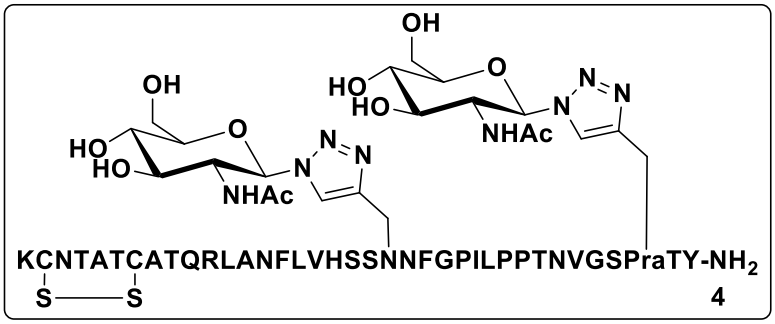



**Figure S 9:** Analytical RP-HPLC and ESI-MS profile of pure pramlintide analogue **3** (97%); linear gradient of 5%B to 65%B over 60 min (*ca.* 1%B/min) at 40 °C, 1 mL/min.

## Synthesis of pramlintide analogue 4




Automated Fmoc-SPPS using Tribute$^{TM}$ rt peptide synthesiser was used for the synthesis of linear pramlintide analogue **17** which was followed by resin cleavage using the conditions outlined in general procedure 2 to afford crude linear pramlintide **17** as a white solid (156 mg, 19% yield based on 48% purity by LCMS) (Figure S 10). The crude linear peptide **17** (20 mg, 5.1 × $10^{-3}$ mmol) underwent a Cu(I) mediated cycloaddition reaction and simultaneous disulfide bond formation (between Cys-2 and Cys-7) with $GlcNAcN_3$ **22** (7.55 mg, 3.1 × $10^{-2}$ mmol) according to general procedure 5 (Figure S 11). This reaction was carried out using 0.5 M TCEP·HCl (136 µL, 6.8 × $10^{-2}$ mmol, pH 7), 0.5 M $CuSO_4{\cdot}5H_2O$ (136 µL, 6.8 × $10^{-2}$ mmol) and $Na_2HPO_4$ (48.3 mg, 0.34 mmol) in 6 M Gu·HCl (1.43 mL) to afford the crude pramlintide analogue **4**.

The crude pramlintide analogue **4** was purified by semi-preparative RP-HPLC using Dionex Ultimate 3000 on a Diphenyl Vydac column, using a gradient of 0%B to 13%B over 13 min (*ca.* 1%B/min) then 13%B to 60%B over 313 min (*ca.* 0.15%B/min). This afforded the *title compound* **4** as a white amorphous solid (2.6 mg, 24% yield, 96% purity); $R_t$ 15.32 min; *m/z* (ESI-MS) 1101.8 ($[M+4H]^{4+}$ requires 1101.8), Figure S 12.

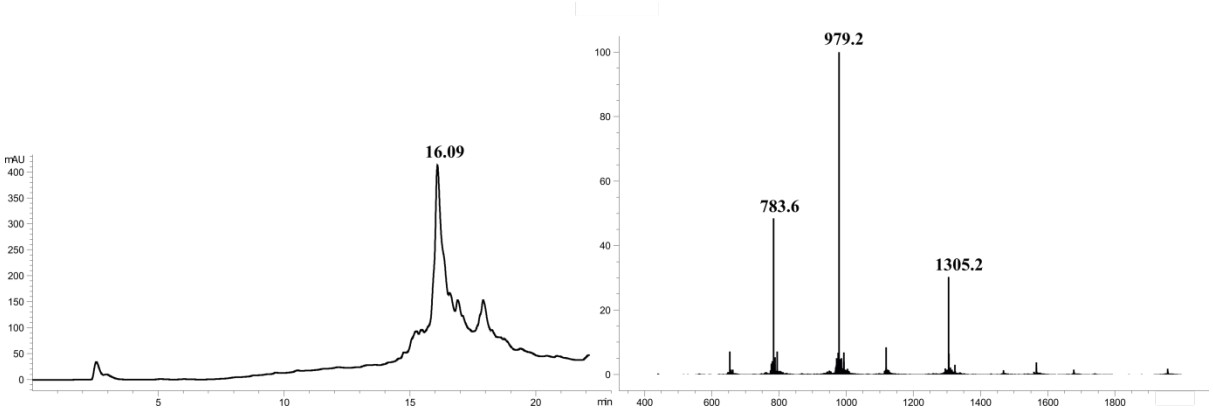



**Figure S 10** LCMS profile of crude linear pramlintide analogue **17** (*ca* 48% as analysed by peak area of RP-HPLC at 214 nm); linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

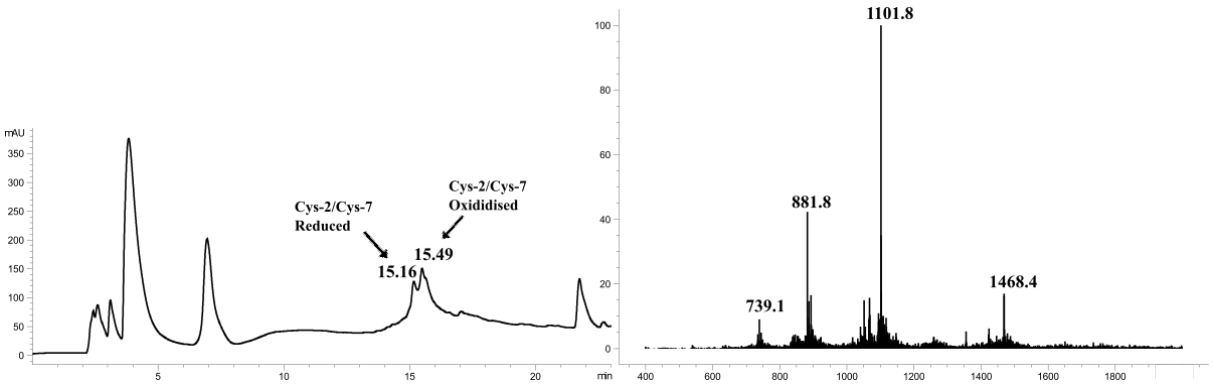



**Figure S 11:** LCMS profile of crude pramlintide analogue **4**; linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

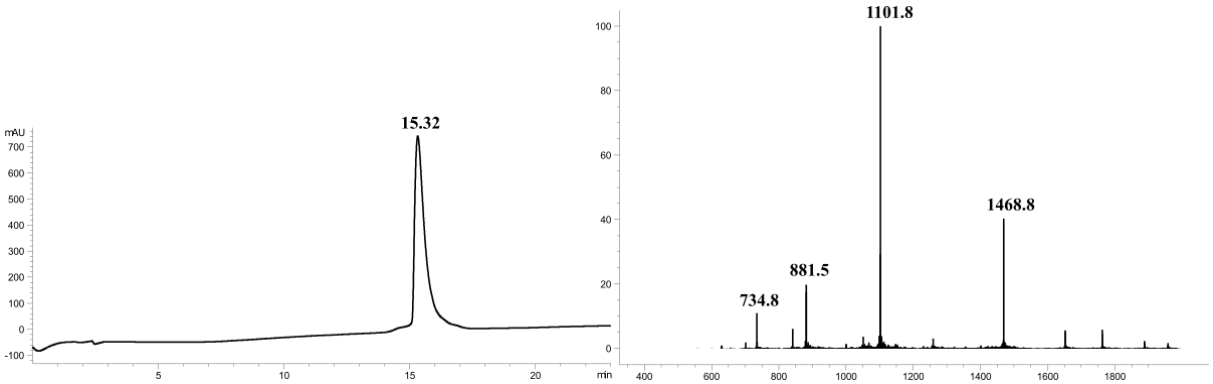
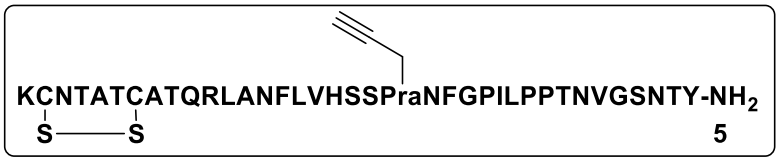



**Figure S 12** LCMS profile of pure pramlintide analogue **4** (*98%*); linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

## Synthesis of pramlintide analogue 5



The crude linear peptide **15** (see synthesis of pramlintide analogue **2**, Fig S 4) (20 mg, 4.7 × $10^{-3}$ mmol) was dissolved in a solution of 6 M Gu·HCl (1.56 mL) and $Na_2HPO_4$ (44 mg, 0.31 mmol), to form a disulfide bond between Cys-2 and Cys-7 according to general procedure 4 to afford crude pramlintide analogue **5** (Figure S 13).

The crude pramlintide analogue **5** was purified by semi-preparative RP-HPLC using Dionex Ultimate 3000 on a Diphenyl Vydac column using a gradient of 0%B to 14%B over 14 min (*ca.* 1%B/min) then 14%B to 60%B over 307 min (*ca.* 0.15%B/min). Fractions were collected at 0.5 min intervals and analysed by ESI-MS and RP-HPLC. Fractions with the correct m/z were combined and lyophilised to afford the *title compound* **5** as a white amorphous solid (3.6 mg, 26% yield, 99% purity); $R_t$ 16.28 min; *m/z* (ESI-MS) 983.5 ($[M+4H]^{4+}$ requires 983.6), Figure S 14.

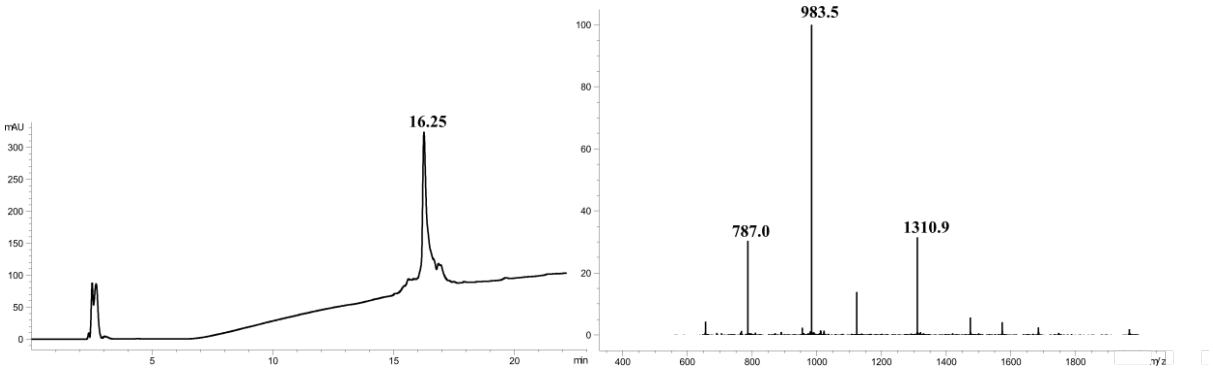



**Figure S 13** LCMS profile of crude pramlintide analogue **5**; linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

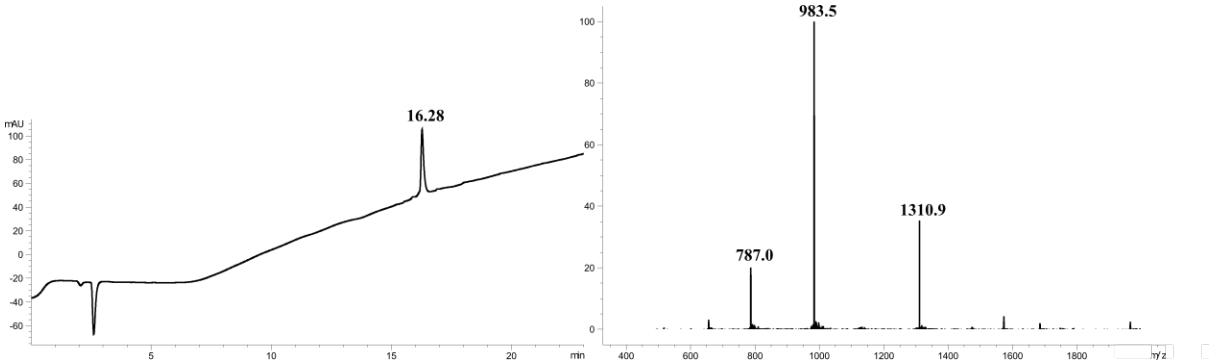



**Figure S 14:** LCMS profile of pure pramlintide analogue **5** (98%); linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

## Synthesis of pramlintide analogue 6


The crude linear peptide **16** (see synthesis of pramlintide analogue **3**, Fig S 7) (20 mg, 4.7 × $10^{-3}$ mmol) was dissolved in a solution of 6 M Gu·HCl (1.56 mL) and $Na_2HPO_4$ (44 mg, 0.31 mmol), to form a disulfide bond between Cys-2 and Cys-7 according to general procedure 4 to afford crude pramlintide analogue **6** (Figure S 15).

The crude pramlintide analogue **6** was purified by semi-preparative RP-HPLC using Waters 600E System on a Gemini $C_{18}$ column, using a gradient of 0%B to 20%B over 20 min (*ca.* 1%B/min) then 20%B to 60%B over 267 min (*ca.* 0.15%B/min).

This afforded the *title compound* **6** as a white amorphous solid (3.5 mg, 33% yield, 96% purity); $R_t$ 16.10 min; *m/z* (ESI-MS) 983.5 ($[M+4H]^{4+}$ requires 983.6), Figure S 16.

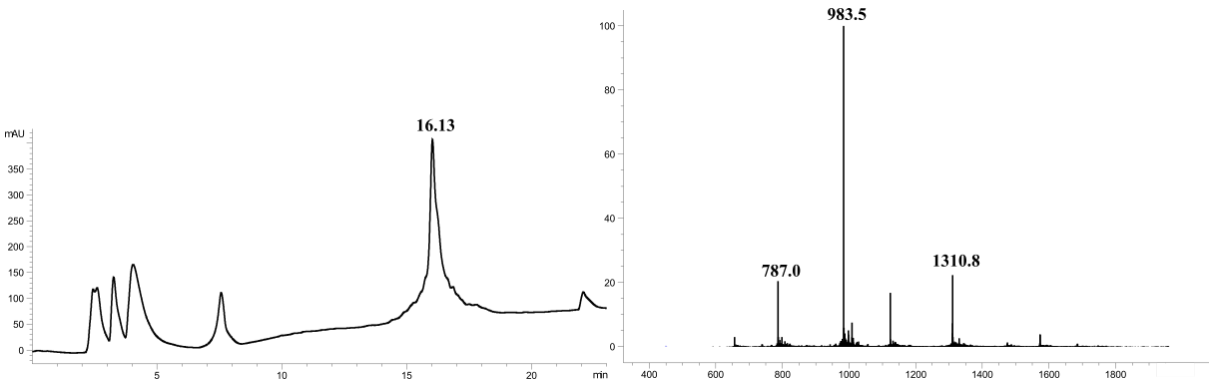



**Figure S 15** LCMS profile of crude pramlintide analogue **6**; linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

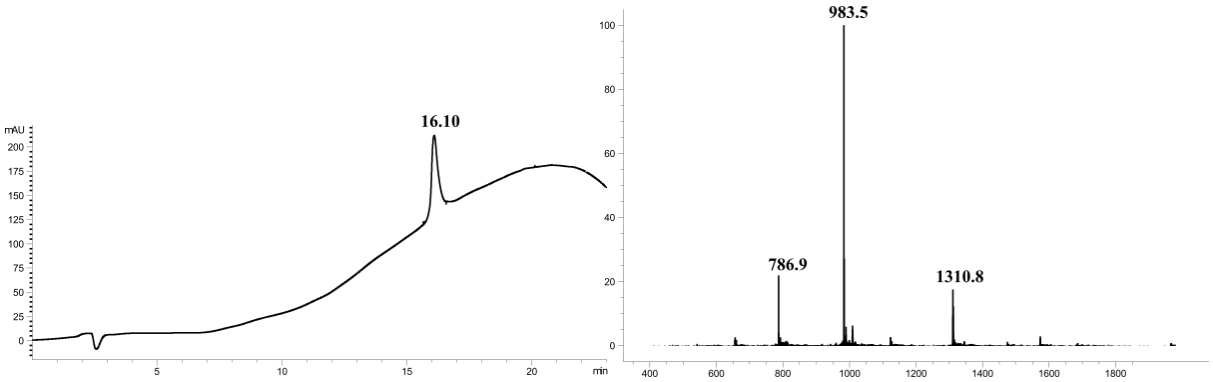
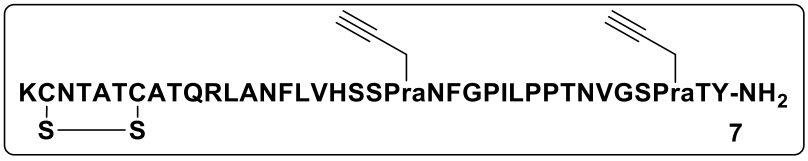



**Figure S 16** LCMS profile of pure pramlintide analogue **6** (*96%*); linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

## Synthesis of pramlintide analogue 7




The crude linear peptide **17** (see synthesis of pramlintide analogue **4**, Fig S 10) (20 mg, $5.1 \times 10^{-3}$ mmol) was dissolved in a solution of 6 M Gu·HCl (1.70 mL) and $Na_2HPO_4$ (48 mg, 0.34 mmol), to form a disulfide bond between Cys-2 and Cys-7 according to general procedure 4 to afford crude pramlintide analogue **7** (Figure S 17).

The crude pramlintide analogue **7** was purified by semi-preparative RP-HPLC using Dionex Ultimate 3000 on a Gemini $C_{18}$ column, using a gradient of 0%B to 23%B over 23 min (*ca.* 1%B/min) then 23%B to 60%B over 247 min (*ca.* 0.15%B/min). This afforded the *title compound* **7** as a white amorphous solid (6.90 mg, 8% yield, 99% purity); $R_t$ 34.97 min; *m/z* (ESI-MS) 978.5 ($[M+4H]^{4+}$ requires 978.9), Figure S 18.

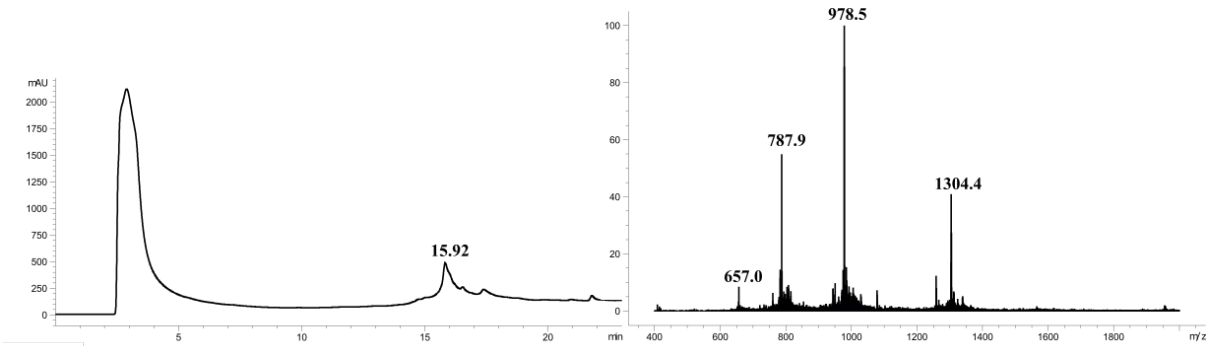



**Figure S 17** LCMS profile of crude pramlintide analogue **7**; linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

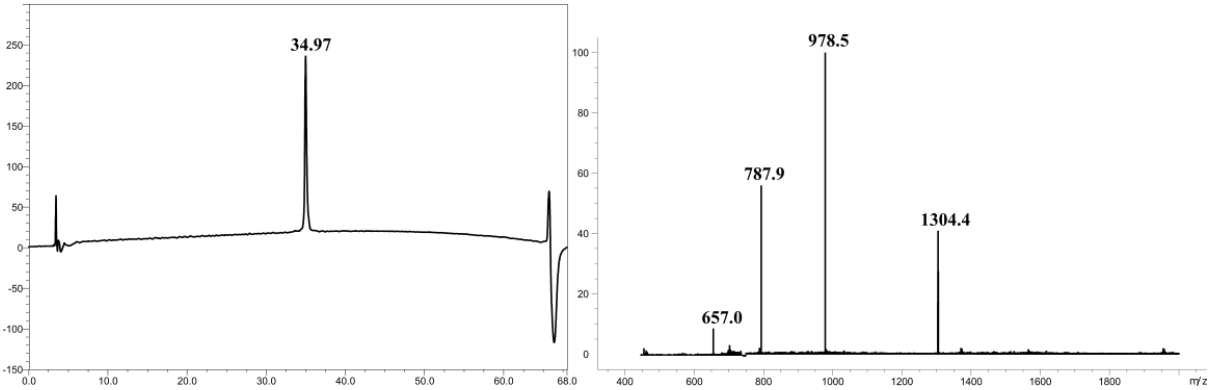
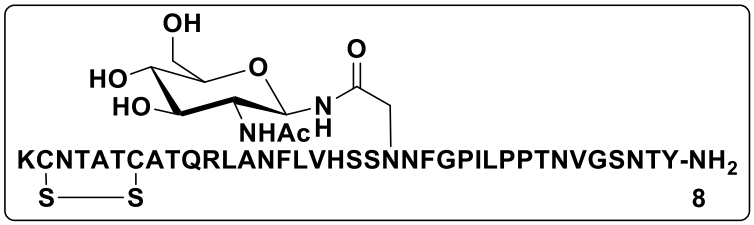



**Figure S 18:** Analytical RP-HPLC and ESI-MS profile of pure pramlintide analogue **7** (99%); 5%B to 65%B over 60 min (*ca.* 1%B/min) at 40 °C, 1 mL/min.

## Synthesis of pramlintide analogue 8




Automated Fmoc-SPPS using Tribute$^{TM}$ rt peptide synthesiser was used for the synthesis of the crude reduced and acetate protected pramlintide analogue **24** which was followed by resin cleavage using the conditions as outlined in the general procedure 2 to afford crude reduced and acetate protected pramlintide **24** as a white solid (186 mg, 45% yield based on 45% purity by LCMS) (Figure S 19). Acetate protecting groups were removed from peptide **24** (30 mg, $7.22 \times 10^{-3}$ mmol) along with simultaneous disulfide bond formation between Cys-2 and Cys-7 using conditions described in general procedure 6 using $NH_2NH_2 \cdot 1.5\ H_2O$ (0.5 mL) in DMSO (9.5 mL), to afford crude pramlintide analogue **8** (Figure S 20).

The crude pramlintide analogue **8** was purified by semi-preparative RP-HPLC using Dionex Ultimate 3000 on a Gemini $C_{18}$ column, using a gradient of 0%B to 25%B over 25 min (*ca.* 1%B/min) then 25%B to 60%B over 233 min (*ca.* 0.15%B/min). This afforded the *title compound* **8** as a white amorphous solid (5.5 mg, 42% yield, 99% purity); $R_t$ 30.17 min; *m/z* (ESI-MS) 1038.9 ($[M+4H]^{4+}$ requires 1039.1), Figure S 21.

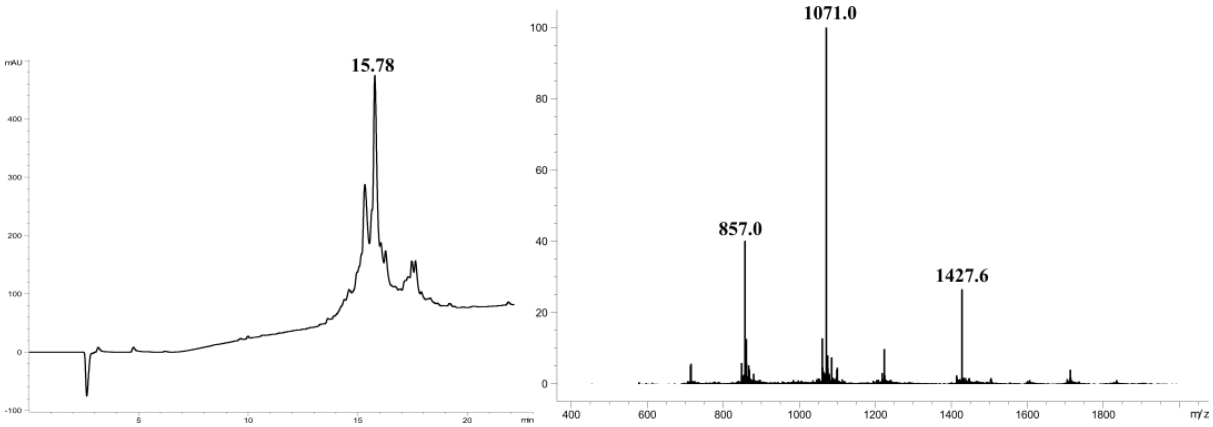



**Figure S 19:** LCMS profile of crude linear and acetate protected pramlintide analogue **24** (*ca* 45% as analysed by peak area of RP-HPLC at 214 nm); linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

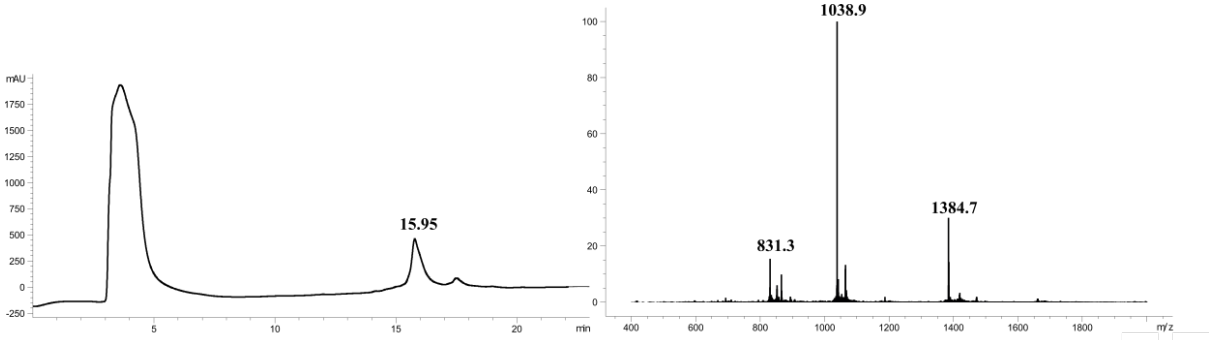



**Figure S 20:** LCMS profile of crude pramlintide analogue **8**; linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

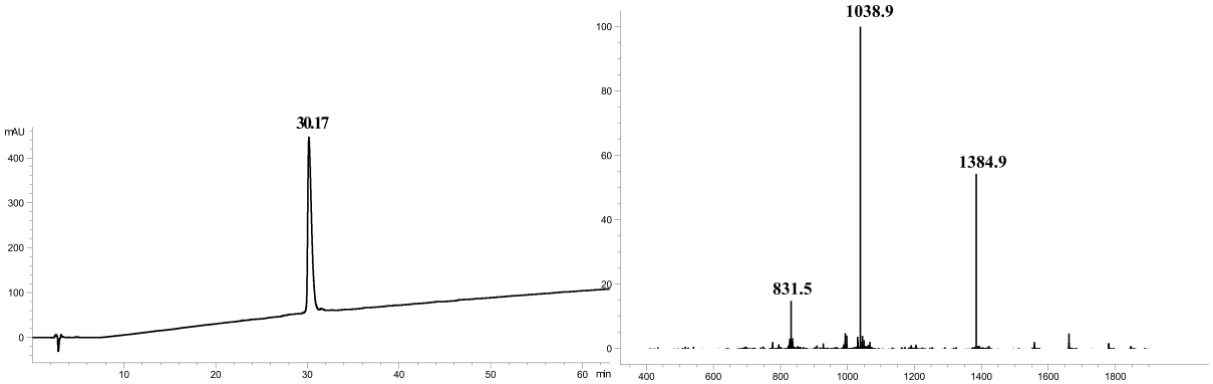
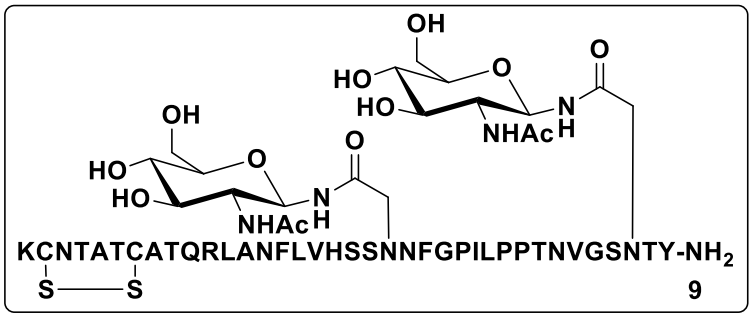



**Figure S 21:** LCMS profile of pure pramlintide analogue **8** (*98*%); linear gradient of 5%B to 65%B over 60 min, (*ca*. 1%B/min) at 40 °C, 0.3 mL/min.

## Synthesis of pramlintide analogue 9




Automated Fmoc-SPPS using Biotage® microwave enhanced peptide synthesiser was used for the synthesis of the crude reduced and acetate protected pramlintide **25** which was followed by resin cleavage using the conditions as outlined in general procedure 2 to afford crude reduced and acetate protected pramlintide **25** as a white solid (256 mg, 23% yield based on 41% purity of fully acetate protected product by LCMS) (Figure S 22). Acetate protecting groups were removed from peptide **25** (32 mg, $7.34 \times 10^{-3}$ mmol) along with simultaneous disulfide bond formation between Cys-2 and Cys-7 using conditions described in general procedure 6 using $NH_2NH_2 \cdot 1.5\ H_2O$ (0.5 mL) in DMSO (9.5 mL) to afford crude pramlintide analogue **9** (Figure S 23).

The crude pramlintide analogue **9** was purified by semi-preparative RP-HPLC using Dionex Ultimate 3000 on a Gemini $C_{18}$ column, using a gradient of 0%B to 25%B over 25 min (*ca.* 1%B/min) then 25%B to 60%B over 233 min (*ca.* 0.15%B/min). This afforded the *title compound* **9** as a white amorphous solid (4.5 mg, 36% yield, 98% purity); $R_t$ 29.80 min; *m/z* (ESI-MS) 1089.8 ($[M+4H]^{4+}$ requires 1089.8), Figure S 24.

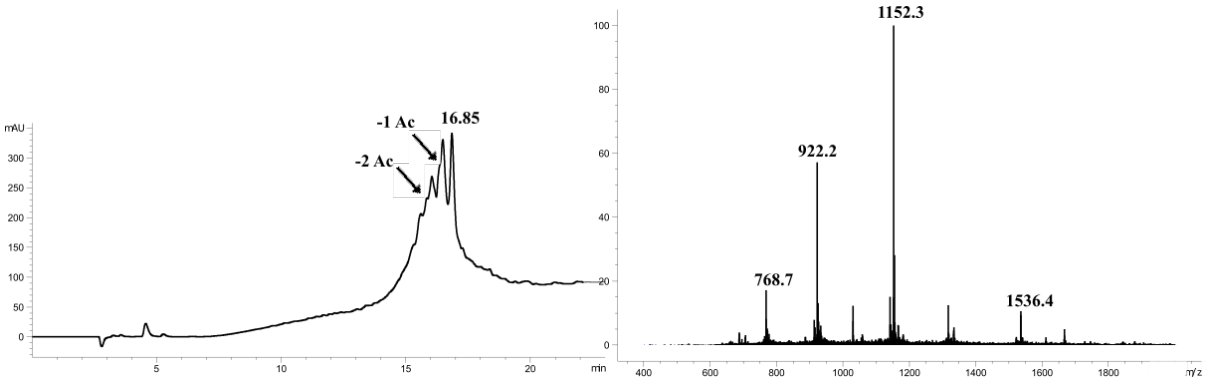



**Figure S 22:** LCMS profile of crude linear and acetate protected pramlintide analogue **25**; linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

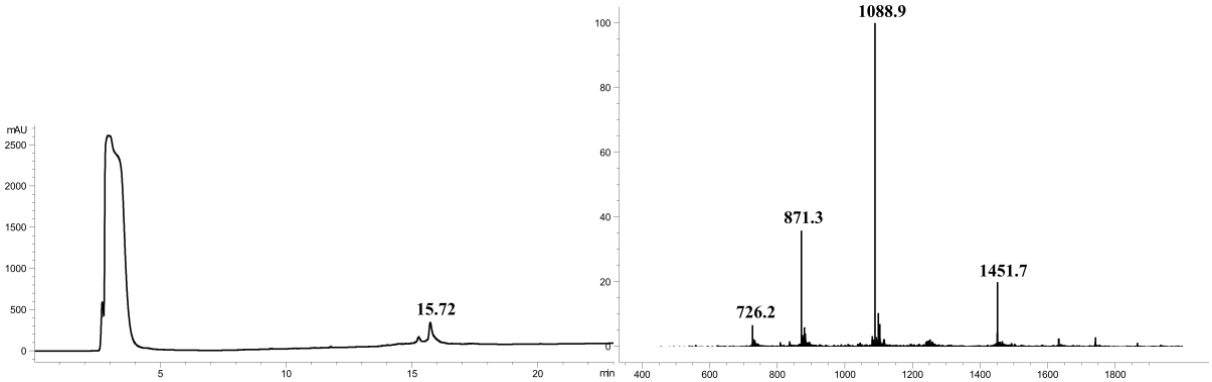


**Figure S 23:** LCMS profile of crude pramlintide analogue **9**; linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

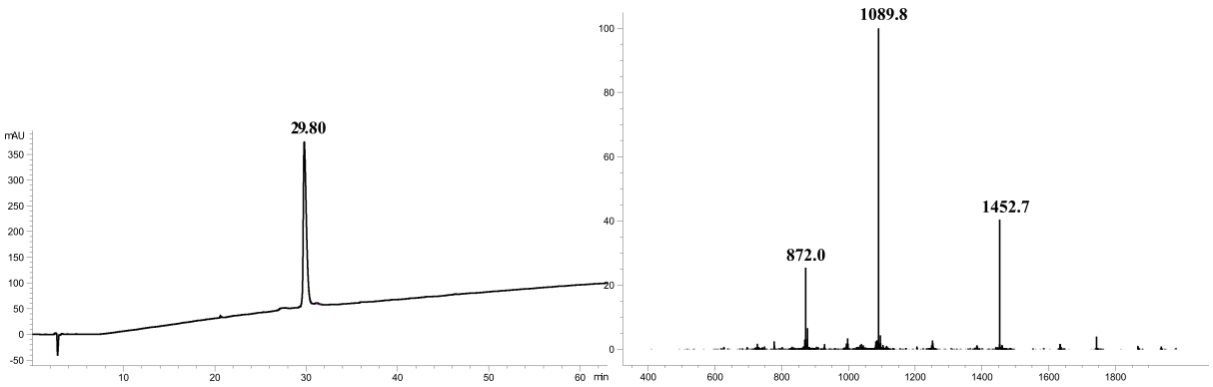
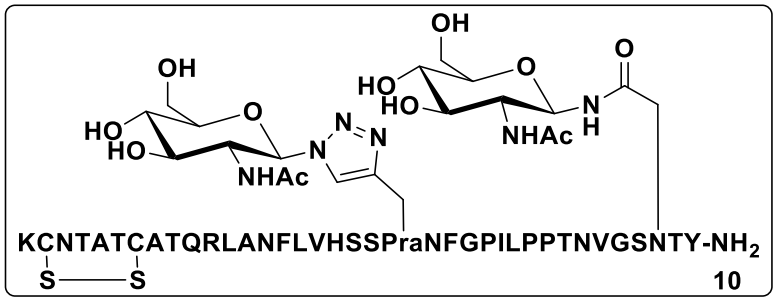



**Figure S 24:** LCMS profile of pure pramlintide analogue **9** (*98*%); linear gradient of 5%B to 65%B over 60 min, (*ca*. 1%B/min) at 40 °C, 0.3 mL/min.

## Synthesis of pramlintide analogue 10




Crude pramlintide **12** (20 mg, $4.8 \times 10^{-3}$ mmol) underwent a Cu(I) mediated cycloaddition reaction (between Cys-2 and Cys-7) with $GlcNAcN_3$ **22** (7.15 mg, $2.9 \times 10^{-2}$ mmol) according to general procedure 5 (Figure S 25). This reaction was carried out using 0.5 M TCEP·HCl (64 µL, $3.2 \times 10^{-2}$ mmol), 0.5 M $CuSO_4 \cdot 5H_2O$ (64 µL, $3.2 \times 10^{-2}$ mmol) and $Na_2HPO_4$ (45.7 mg, 0.32 mmol) in 6 M Gu·HCl (1.48 mL) to afford crude pramlintide analogue **10**.

The crude peptide **10** was then purified by semi-preparative RP-HPLC using Dionex Ultimate 3000 on a Gemini $C_{18}$ column, using a gradient of 0%B to 15%B over 15 min (*ca.* 1%B/min) then 15%B to 60%B over 300 min (*ca.* 0.15%B/min). This afforded the *title compound* **10** as a white amorphous solid (2.8 mg, 25% yield, 99% purity). $R_t$ 38.43 min; *m/z* (ESI-MS) 1095.8 ($[M+4H]^{4+}$ requires 1095.8), Figure S 26.

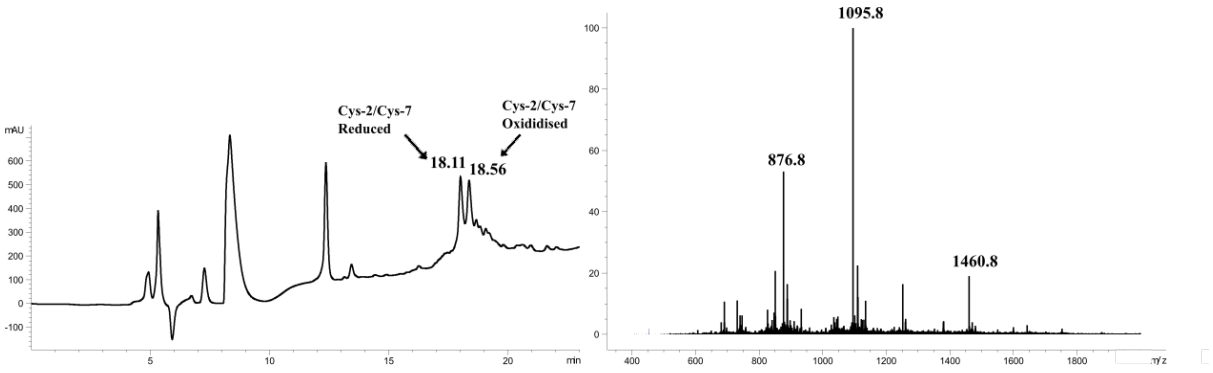


**Figure S 25:** LCMS profile of crude pramlintide analogue **10;** linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

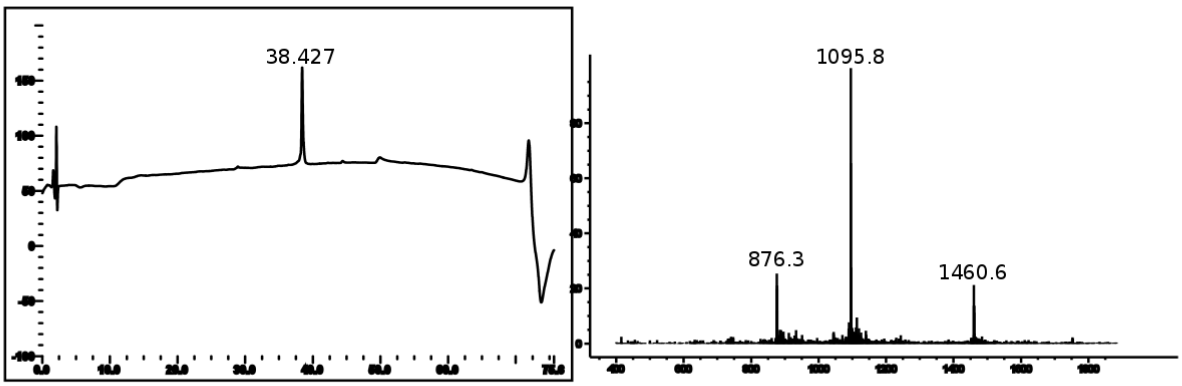
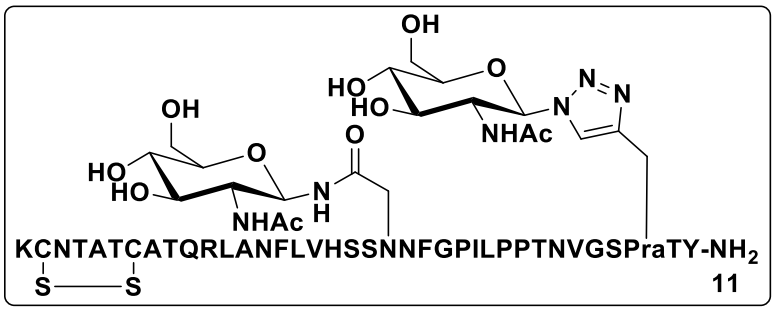



**Figure S 26:** Analytical RP-HPLC and ESI-MS profile of pure pramlintide analogue **10** (96%); 5%B to 65%B over 63 min (*ca.* 1%B/min) at 40 °C , 1 mL/min.

## Synthesis of pramlintide analogue 11




Crude pramlintide analogue **13** (20 mg, 4.8 × $10^{-3}$ mmol) underwent a Cu(I) mediated cycloaddition reaction (between Cys-2 and Cys-7) with $GlcNAcN_3$ **22** (7.15 mg, 2.9 × $10^{-2}$ mmol) according to general procedure 5 (Figure S 27). This reaction was carried out using 0.5 M TCEP·HCl (64 µL, 3.2 × $10^{-2}$ mmol), 0.5 M $CuSO_4 \cdot 5H_2O$ (64 µL, 3.2 × $10^{-2}$ mmol) and $Na_2HPO_4$ (45.7 mg, 0.32 mmol) in 6 M Gu·HCl (1.48 mL) to afford crude pramlintide analogue **11**.

The crude pramlintide analogue **11** was then purified by semi-preparative RP-HPLC using Dionex Ultimate 3000 on a Gemini $C_{18}$ column, using a gradient of 0%B to 15%B over 15 min (*ca.* 1%B/min) then 15%B to 60%B over 300 min (*ca.* 0.15%B/min). This afforded the *title compound* **11** as a white amorphous solid (3.1 mg, 28% yield, 99% purity). $R_t$ 26.52 min; *m/z* (ESI-MS) 1095.8 $[M+4H]^{4+}$ ($[M+4H]^{4+}$ requires 1096.0), Figure S 28.

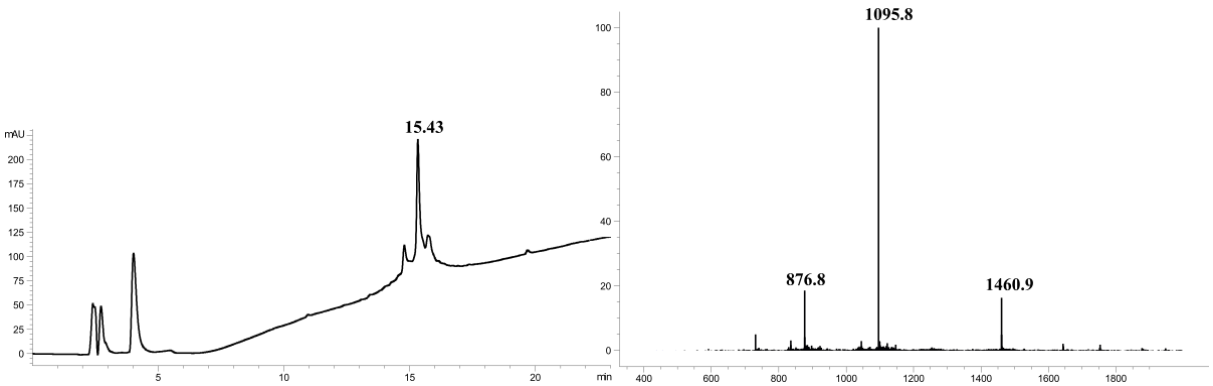



**Figure S 27:** LCMS profile of crude pramlintide analogue **11**; linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

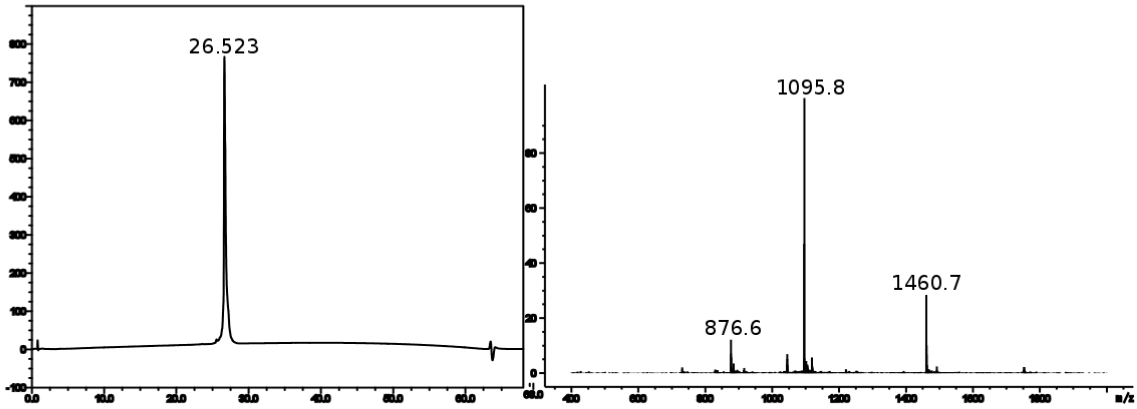
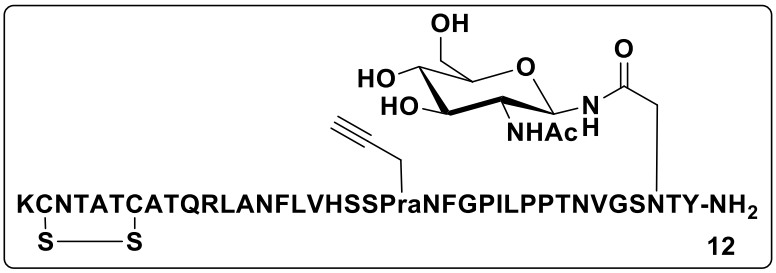



**Figure S 28:** Analytical RP-HPLC and ESI-MS profile of pure pramlintide analogue **11** (99%); 5%B to 65%B over 63 min (*ca.* 1%B/min) at 40 °C, 1 mL/min, using Agilent Zorbax 300SB-C3, 4.6 mm x 150 mm, 5 µm column.

## Synthesis of pramlintide analogue 12




Automated Fmoc-SPPS using Tribute$^{TM}$ rt peptide synthesiser was used for the synthesis of the crude reduced and acetate protected pramlintide **26** which was followed by resin cleavage using the conditions as outlined in the general procedure 2 to afford crude reduced and acetate protected pramlintide **26** as a white solid (220 mg, 27% yield based on 52% purity by LCMS) (Figure S 29). Acetate protecting groups were removed from peptide **26** (21 mg, 4.7 × $10^{-3}$ mmol) along with simultaneous disulfide bond formation between Cys-2 and Cys-7 using conditions described in general procedure 6 using $NH_2NH_2$·1.5 $H_2O$ (0.88 mL) in DMSO (16.7 mL) to afford crude pramlintide analogue **12**.

The crude pramlintide analogue **12** was then purified by semi-preparative RP-HPLC using Dionex ultimate 3000 on a Gemini $C_{18}$ column, using a gradient of 0%B to 15%B over 15 min (*ca.* 1%B/min) then 15%B to 60%B over 300 min (*ca.*

0.15%B/min). This afforded the *title compound* **12** as a white amorphous solid (3.8 mg, 36% yield, 99% purity). $R_t$ 32.82 min; *m/z* (ESI-MS) 1034.2 ($[M+4H]^{4+}$ requires 1034.4), Figure S 30.

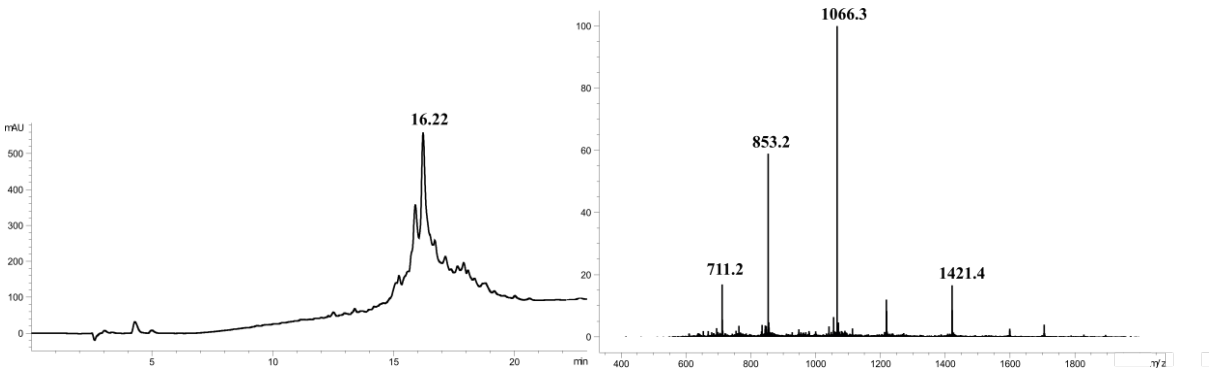



**Figure S 29:** LCMS profile of crude pramlintide analogue **26** (*ca* 52% as analysed by peak area of RP-HPLC at 214 nm); linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

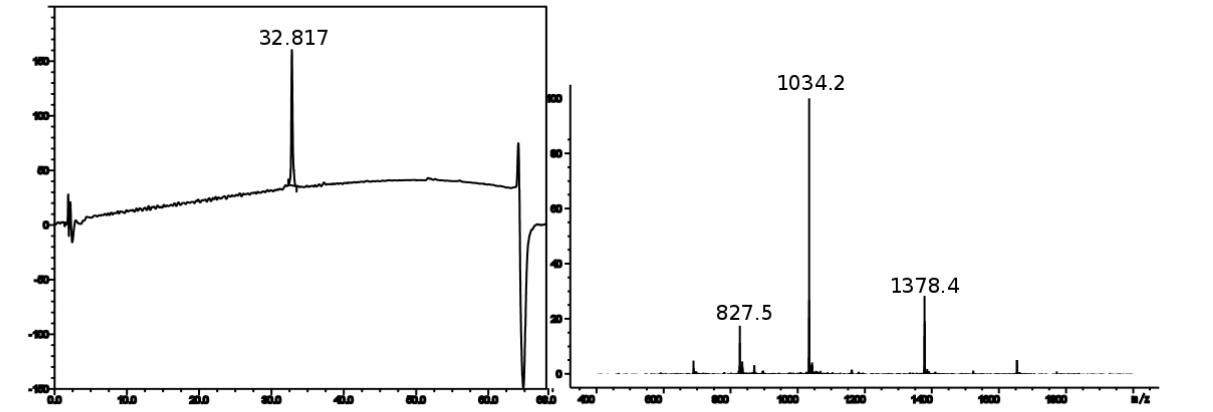
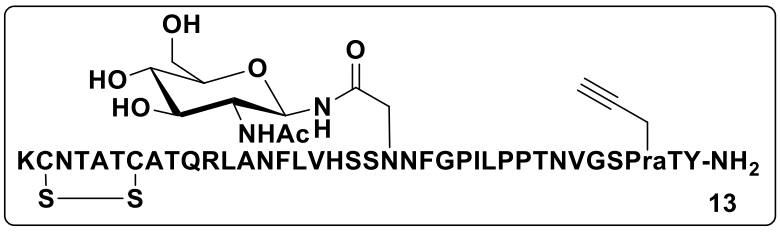



**Figure S 30:** Analytical RP-HPLC and ESI-MS profile of pure pramlintide analogue **12** (98%); 5%B to 65%B over 60 min (*ca.* 1%B/min) at 40 °C, 1 mL/min.

## Synthesis of pramlintide analogue 13




Automated Fmoc-SPPS using Biotage® microwave enhanced peptide synthesiser was used for the synthesis of the crude reduced and acetate protected pramlintide **27** which was followed by resin cleavage using the conditions as outlined in the general procedure 2 to afford crude reduced and acetate protected pramlintide **27** (240 mg, 30% yield based on 41% purity of fully acetate protected product by LCMS) (Figure S 31). Acetate protecting groups were removed from peptide **27** (22 mg, $4.9 \times 10^{-3}$ mmol) along with simultaneous disulfide bond formation between Cys-2 and Cys-7 using conditions

described in general procedure 6 using $NH_2NH_2 \cdot 1.5\ H_2O$ (0.88 mL) in DMSO (16.7 mL) to afford crude pramlintide analogue **13** (Figure S 32).

The crude pramlintide analogue **13** was then purified by semi-preparative RP-HPLC using Dionex Ultimate 3000 on a Gemini $C_{18}$ column, using a gradient of 0%B to 15%B over 15 min (*ca.* 1%B/min) then 15%B to 60%B over 300 min (*ca.* 0.15%B/min). This afforded the *title compound* **13** as a white amorphous solid (4.1 mg, 36% yield, 99% purity). $R_t$ 31.68 min; *m/z* (ESI-MS) 1034.0 ($[M+4H]^{4+}$ requires 1034.4), Figure S 33.

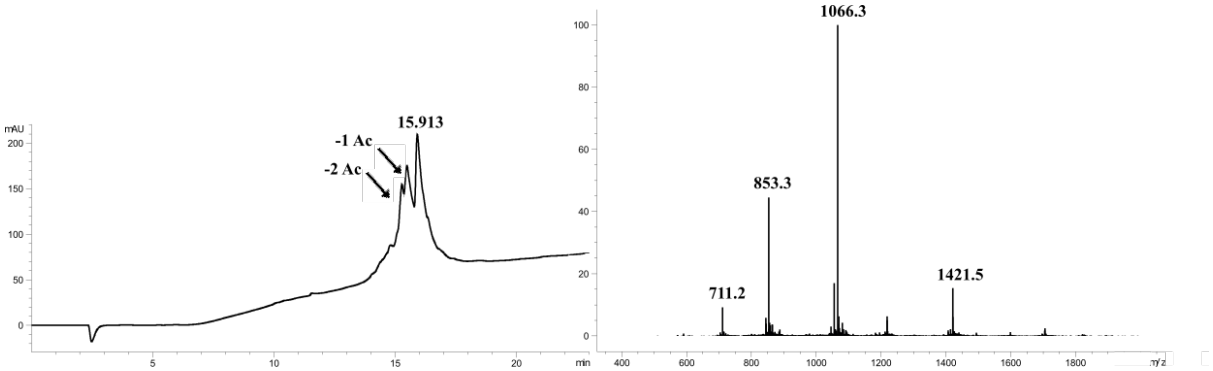



**Figure S 29:** LCMS profile of crude pramlintide analogue **31**; linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

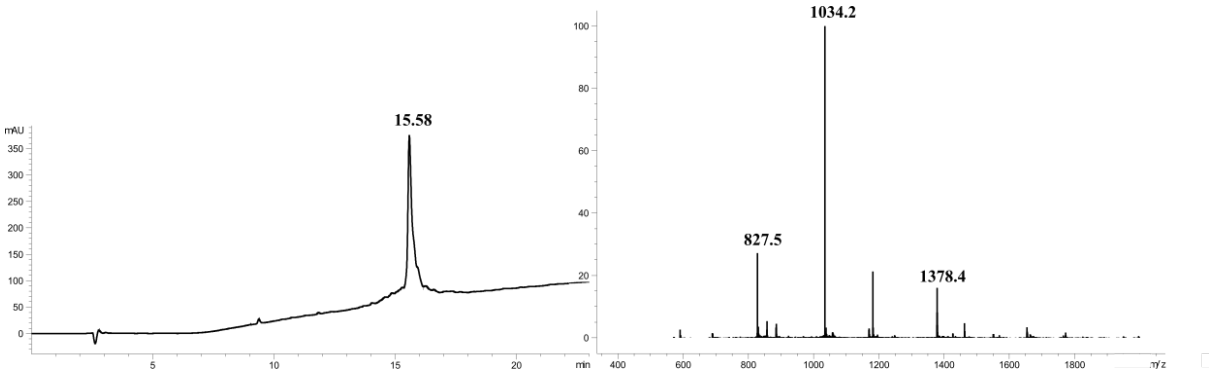



**Figure S 30:** LCMS profile of crude pramlintide analogue **32;** linear gradient of 5%B to 65%B over 20 min, (*ca*. 3%B/min) at 40 °C, 0.3 mL/min.

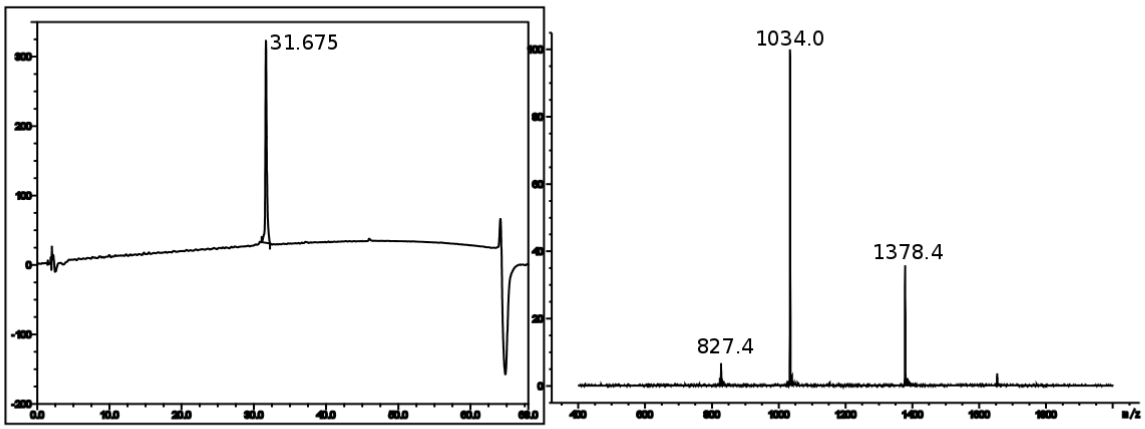



**Figure S 31:** Analytical RP-HPLC and ESI-MS profile of pure pramlintide analogue **33** (99%); 5%B to 65%B over 63 min (*ca.* 1%B/min) at 40 °C , 1 mL/min.

## References


1. P. W. R. Harris, S. H. Yang and M. A. Brimble, *Tetrahedron Lett.*, 2011, **52**, 6024-6026.
2. K. J. Jensen, M. Meldal and K. Bock, *J. Chem. Soc., Perkin Trans. 1*, 1993, 2119-2129.
3. T. Inazu, *Synlett*, 1993, **11**, 869-870.
4. R. J. Bailey and D. L. Hay, *Peptides*, 2006, **27**, 1367-1375.
5. J. J. Gingell, T. Qi, R. J. Bailey and D. L. Hay, *Peptides*, 2010, **31**, 1400-1404.
6. J. J. Gingell, E. R. Burns and D. L. Hay, *Endocrinology*, 2014, **155**, 21-26.