Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Replacement of the CysA7-CysB7 disulfide bond with a 1,2,3-triazole linker causes unfolding in insulin glargine

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Supplementary Information

Contents

General Details

All materials were purchased as reagent grade and used without further purification. *O*-(6-Chlorobenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HCTU) 4-[(R,S)-a-[1-(9H-Fluoren-9-yl)-methoxy-formamido]-2,4-dimethoxybenzyl-phenoxyacetic acid (RINK), 1-hydroxybenzotriazole (HOBt), 4-hydroxymethylbenzoic acid (HMBA) and Fmoc-amino acids were purchased from GL Biochem (Shanghai, China). These amino acids were supplied with side-chain protecting groups as follows: Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Trt)-OH or Fmoc-Cys(Acm)-OH, Fmoc-Gln(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH and Fmoc-Tyr(tBu)-OH. Fmoc -L-β-azidoalanine and Fmoc-L-ε-azidonorleucine were purchased from IRIS Biotech (Marktredwitz, Germany), and Fmoc-L-propargylglycine from Chem-Impex (Wood Dale, IL). *N,N*-Diisopropylamine (iPr$_2$NEt), 4-methylmorpholine (NMM), *N,N'*-diisopropylcarbodiimide (DIC), piperidine, 3,6-dioxa-1,8-octanedithiol (DODT), 1-methyl-2-pyrrolidinone (NMP), tris(benzyltriazolylmethyl)amine (TBTA) and triisopropylamine (iPr$_3$SiH) were purchased from Sigma-Aldrich (Sydney, Australia), and 2,2'-dipyridyldisulfide (DPDS) from Fluka (Switzerland). *N,N'*-dimethylformamide (DMF) and acetonitrile (MeCN) were obtained from Scharlau, Spain). Trifluoroacetic acid was supplied by Halocarbon (River Edge, NJ) and dimethylsulfoxide (DMSO) by Romil Ltd (Cambridge UK). Aminomethyl ChemMatrix® resin, was purchased from PCAS Biomatrix Inc. (St-Jean-sur-Richelieu, Quebec) and preloaded Fmoc-Arg(Pbf)-HMB-Tentagel resin was obtained from Rapp Polymere (Tübingen, Germany).

Fmoc SPPS was performed on a 0.1 mmol scale using a Liberty Microwave-assisted Peptide Synthesiser (CEM Corp., Mathews, NC). The couplings were carried out on a 0.1 mmol equivalents of resin using the protocols recommended by CEM. Typically, the Fmoc-amino acid (0.2M in DMF, 5 equiv.), HCTU (0.45 M in DMF, 0.45 equiv.) and iPr$_2$Net (2 M in NMP, 10 equiv.) were added in sequence to the resin, which was then irradiated for 5 minutes at 25 W, allowing a maximum temperature of 72°C. Following the coupling step, the Fmoc protecting group was removed by 20% v/v piperidine in DMF (30 seconds followed by a second treatment for 3min at 62 W and 75°C maximum temperature. Manual couplings were performed in a similar manner but at room temperature in a sintered glass reaction vessel, with a coupling time of one hour, during which the vessel was agitated gently, followed by deprotection with 20% v/v piperidine in DMF for 10 min., also at room temperature. On completion of the peptide sequences the product was cleaved from resin by incubation in a mixture of TFA/EDT/H$_2$O/iPr$_3$SiH (94/2.5/2.5/1 v/v/v/v) for two hours at room temperature. The TFA was then drained into chilled diethyl ether and the precipitated peptide pelleted by centrifugation, washed (ether), dissolved in 50% aqueous MeCN and lyophilized in preparation for analysis and purification.

Both analytical and semi-preparative reverse-phase, high-performance liquid chromatography (RP-HPLC) was carried out on a Dionex Ultimate U3000 system using the columns, flow and gradients indicated in the synthesis section. The solvent system employed 0.1% TFA in water as eluent A and 0.1% TFA in acetonitrile as eluent B with simultaneous detection at 210, 225, 254 and 280 nm. ESI mass spectra were obtained using a Hewlett-Packard 1100MSD mass spectrometer operating in the positive mode.

Synthesis of Peptide 5

Using manual peptide coupling conditions a 0.3 mmol sample of resin was derivatised first with Fmoc-RINK linker and subsequently with five lysine(Boc) residues. In a separate flask HMBA (92 mg, 0.6 mmol) and HOBt (93 mg, 0.6 mmol) were combined and dissolved in DMF (3 mL) and the solution transferred to the resin. Neat DIC (93 μL, 0.6 mmol) was added and the mixture agitated for one hour. After draining and washing the resin, the process was repeated twice more, after which the resin was treated with a mixture of DMF (2 mL) and 1 M aqueous NaOH (2 mL) for 5 minutes, drained and washed well with 1:1 water/DMF (5 x 10 mL) and finally DMF (5 x 5 mL). An Fmoc-Gly residue was then appended by adding solid Fmoc-Gly-OH (892 mg, 3 mmol) and 4-dimethylaminopyridine (37 mg, 0.3 mmol) to the resin followed by sufficient DMF to just dissolve the solids and give a free-flowing suspension of resin (approx. 3 mL). Neat DIC was added and the mixture agitated at RT for three hours before being drained and washed with DMF to afford Fmoc-Gly-HMBA-[Lys(Boc)]$_5$-RINK-ChemMatrix resin.

Microwave-enhanced SPPS was then used to synthesise peptide **5** sequence (the A-chain), incorporating Cys(Trt) residues at A6 and A11, propargyl glycine at position A7 and Cys(Acm) at

A20. On cleavage from resin this afforded crude **5** (120 mg). A quantity of this material (ca. 30 mg) was dissolved in water to 1 mg/mL and a series 4 mL (4 mg) aliquots purified on a Phenomenex Gemini C18 (5µ 110Å, 10 x 250mm) column, generating a linear gradient 10%B to 40%B over 30 min at a flow of 5 mL/min. The main peak of each run was collected, pooled with those of the other runs and lyophilised to give pure **5** (13 mg) **(Figure S1)**; *m/z* (ESI-MS) 1582.3 ($[M + 2H]^{2+}$ requires 1581.8).

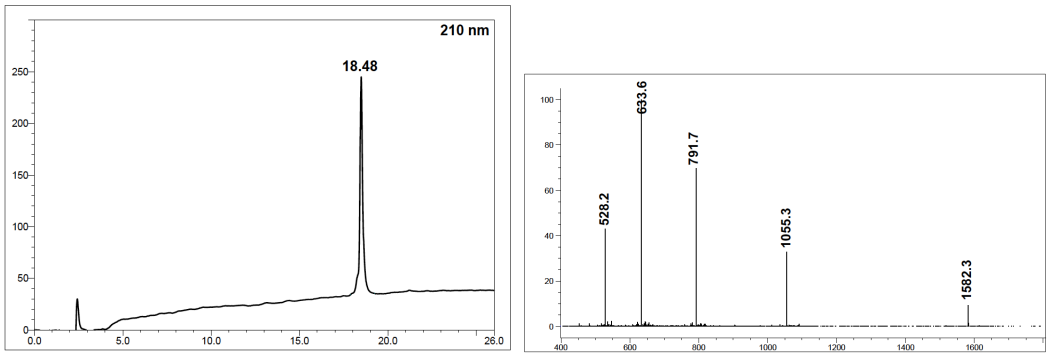

Figure S1 RP-HPLC and ESI-MS traces of purified peptide **5**; column: Phenomenex Gemini C18 (5µ 110Å, 4.6 x 150mm); gradient: 0-1min, 1%B then 1%B to 51%B over 25 min., eluting at 1mL/min., R_t 18.48 min.

Synthesis of Peptide 6

A solution of DPDS (1.0 mg, 4.5 µmol) in methanol (0.4 mL) was added in one portion to a solution of peptide **5** (12 mg, 3.8 µmol) in water (12 mL) and after stirring continuously for 30 minutes, HPLC analysis showed complete conversion to the disulfide (Figure S2). 4 mL aliquots purified on a Phenomenex Gemini C18 (5µ 110Å, 10 x 250mm) column, generating a linear gradient 10%B to 40%B over 30 min at a flow of 5 mL/min. The main peak of each run was collected, pooled and then lyophilised to give pure **6** (10.5 mg); *m/z* (ESI-MS) 1581.0 ($[M + 2H]^{2+}$ requires 1580.8).

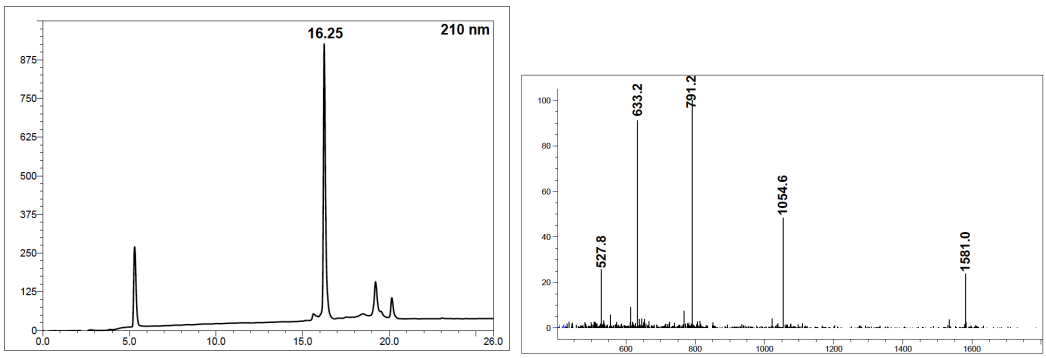

Figure S2 RP-HPLC and ESI-MS traces showing conversion to disulfide **6**; column: Phenomenex Gemini C18 (5µ 110Å, 4.6 x 150mm); gradient: 0-1min, 1%B then 1%B to 51%B over 25 min., eluting at 1mL/min., R_t 16.25 min.

Synthesis of Peptide 7

Microwave-enhanced synthesis of the B-chain peptide **7** was carried out on a 0.1 mmol scale using the described methodology, incorporating a Cys(Acm) residue at position B19 and an azido-alanine residue at position B7. Cleavage from resin afforded 240 mg of crude product. 50 mg of this material was suspended in MeCN (0.5 mL) and water (0.5 mL) added to dissolve the suspension. This was diluted further with water (4 mL) to give a 10 mg/mL solution, 1 mL aliquots of which were purified on a Phenomenex Gemini C18 (5µ 110Å, 10 x 250 mm) column with a solvent flow of 5 mL/min and generating the following series of gradient steps: 0-1 min, 10%B; 1-3min, 25%B; 3-25 min, 35%B. From each run the main peak was collected and pooled and this pool lyophilised to give **7** (12 mg) (Figure S3); *m/z* (ESI-MS) 1273.8 ($[M + 3H]^{3+}$ requires 1274.3).

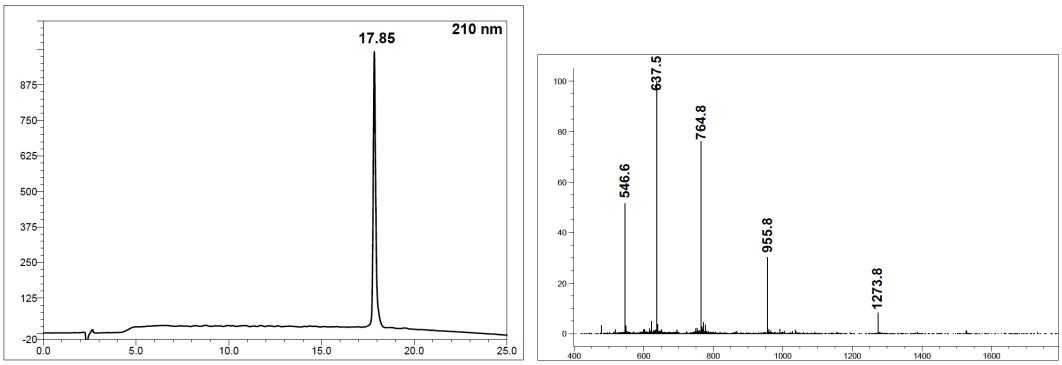

Figure S3 RP-HPLC and ESI-MS traces of purified peptide **7**; column: Dionex Acclaim C18 (4.6 x 150mm); gradient: 0-1min, 1%B then 1%B to 61%B over 25 min., eluting at 1mL/min.; R_t 17.85 min.

Synthesis of Peptide **8**

Synthesis of the B-chain peptide **8** was carried out on a 0.1 mmol scale using the same procedure as for peptide **7**, except that an L-ε-azidonorleucine residue was incorporated at position B7. Cleavage from resin afforded 250 mg of crude product. 30 mg of this material was suspended in MeCN (0.6 mL) and the suspension progressively diluted with water (4.4 mL) to give a 6 mg/mL solution, 1 mL aliquots of which were purified on a Phenomenex Gemini C18 (5µ 110Å, 10 x 250 mm) column with a solvent flow of 5 mL/min and generating the following series of gradient steps: 0-1 min, 10%B; 1-3min, 25%B; 3-25 min, 35%B. From each run the main peak was collected and pooled and this pool lyophilised to give **8** (7 mg) (Figure S4); *m/z* (ESI-MS) 1288.8 ($[M + 3H]^{3+}$ requires 1288.3).

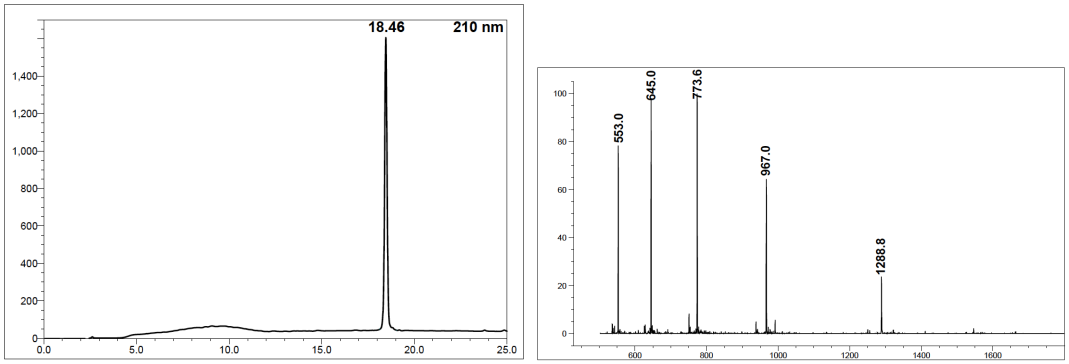

Figure S4 RP-HPLC and ESI-MS traces of purified peptide **8**; column: Dionex Acclaim C18 (4.6 x 150mm); gradient: 0-1min, 1%B then 1%B to 61%B over 25 min., eluting at 1mL/min.; R_t 18.46 min.

Synthesis of Peptide 9

A-chain peptide **6** (2.3 mg, 0.73 µmol), B-chain peptide **7** (2.7 mg, 0.71 µmol) and TBTA (0.5 mg, 1 µmol) were combined and dissolved in degassed DSMO (110 µL). An aqueous 0.25 M solution of $CuSO_4$ (11.6 µL, 2.9 µmol) was added followed by aqueous 0.25 M sodium ascorbate (11.6 µL, 2.9 µmol), causing the blue solution to become colourless. The mixture was agitated for 2 min. at 75°C, cooled and diluted to 1 mL with water and purified on a Phenomenex Gemini C18 (5µ 110Å, 10 x 250 mm) column with a solvent flow of 5 mL/min and generating the following series of gradient steps: 0-1 min, 10%B; 1-3min, 20%B; 3-25 min, 35%B to afford peptide **9** (3.8 mg) (Figure S5); *m/z* (ESI-MS) 1746.4 ($[M + 4H]^{4+}$ requires 1745.9).

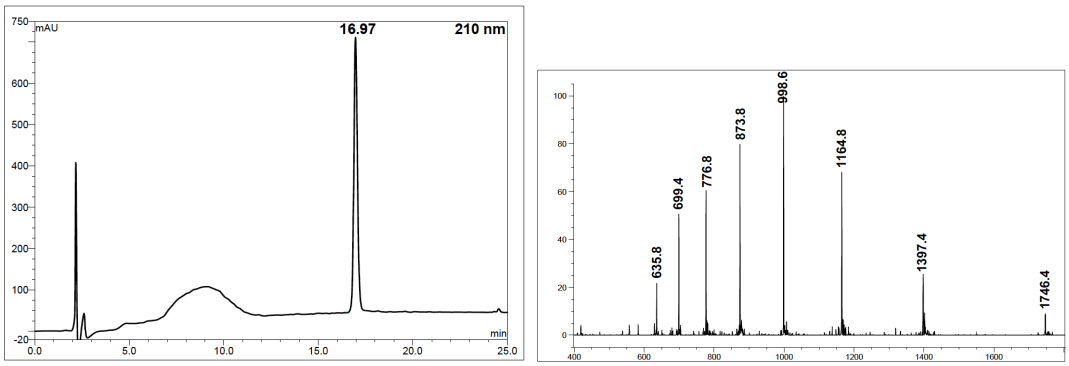

Figure S5 RP-HPLC and ESI-MS traces of purified peptide **9**; column: Dionex Acclaim C18 (4.6 x 150mm); gradient: 0-1min, 1%B then 1%B to 61%B over 25 min., eluting at 1ml/min; R_t 16.97 min.

Synthesis of Peptide 10

A-chain peptide **6** (1.7 mg, 0.54 µmol), B-chain peptide **8** (2.1 mg, 0.54 µmol) and TBTA (0.4 mg, 0.7 µmol) were combined and dissolved in degassed DSMO (80 µL). An aqueous 0.25 M solution of $CuSO_4$ (8 µL, 2 µmol) was added followed by aqueous 0.1 M sodium ascorbate (20 µL, 2 µmol), causing the blue solution to become colourless. The mixture was agitated for 2 min. at 80°C, cooled and diluted to 1 mL with water and purified on a Phenomenex Gemini C18 (5µ 110Å, 10 x 250 mm) column with a solvent flow of 5 mL/min and generating the following series of gradient steps: 0-1 min, 10%B; 1-3min, 25%B; 3-25 min, 45%B to afford peptide **10** (2.6 mg) (Figure S6); *m/z* (ESI-MS) 1757.2 ($[M + 4H]^{4+}$ requires 1756.4).

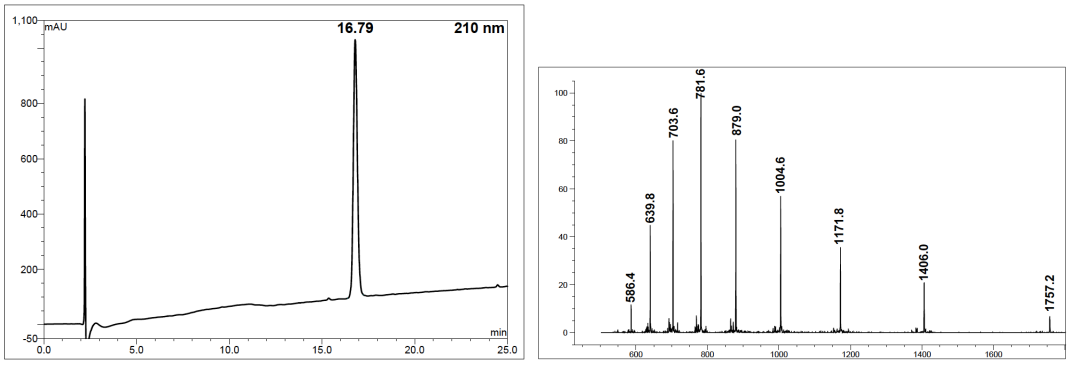

Figure S6 RP-HPLC and ESI-MS traces of purified peptide **10**; column: Dionex Acclaim C18 (4.6 x 150mm); gradient: 0-1min, 1%B then 1%B to 61%B over 25 min., eluting at 1mL/min.; R_t 16.79 min.

Synthesis of Peptide 11

The bis-Acm peptide **9** (1 mg, 0.15 µmol) was dissolved in chilled 80% acetic acid in water (1 mL) and cooled on ice-water. Iodine (20 µL of a 0.5 M solution in MeOH, 10 µmol) was added and the resulting mixture incubated at 0°C for 50 min. with periodic agitation. The reaction was quenched by addition of sodium ascorbate (20 µL of a 0.5 M solution in water, 10 µmol) was added, causing the colour to fade to pale brown, and the mixture diluted with water (2 mL). The resulting clear, entirely colourless solution was purified on a Phenomenex Gemini C18 (5µ 110Å, 10 x 250mm) column with a solvent flow of 5 mL/min and generating the following series of gradient steps: 0-1 min, 10%B; 1-3min, 25%B; 3-25%B, 35%B to afford peptide **11** (1.0 mg) (Figure S7); *m/z* (ESI-MS) 1710.4 ($[M + 4H]^{4+}$ requires 1709.6).

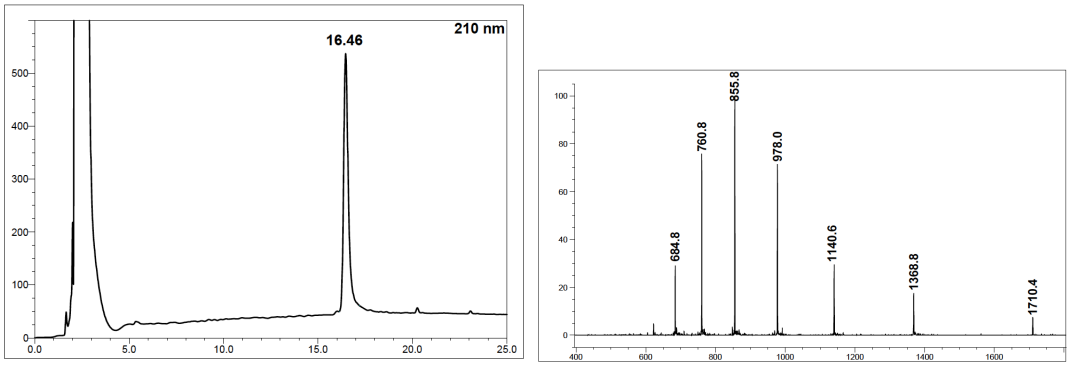

Figure S7 RP-HPLC and ESI-MS traces of purified peptide **11**; column: Dionex Acclaim C18 (4.6 x 150mm); gradient: 0-1min, 1%B then 1%B to 61%B over 25 min., eluting at 1mL/min.; R_t 16.46 min.

Synthesis of Peptide 12

The bis-Acm peptide **10** (4 mg, 0.57 µmol) was dissolved in chilled 80% acetic acid in water (3.3 mL) and cooled on ice-water. Iodine (80 µL of a 0.5 M solution in MeOH, 40 µmol) was added and the resulting mixture incubated at 0°C for 50 min. with periodic agitation. The reaction was quenched by addition of sodium ascorbate (400 µL of a 0.1 M solution in water, 40 µmol) and the mixture diluted with water (6.3 mL). The entire solution was passed through a Phenomenex Gemini C18 (5µ 110Å, 10 x 250mm) column equilibrated in 10%B at a flow of 5 mL/min and the eluent then switched to 41%B to strip the absorbed product in one portion. This fraction was lyophilised to afford peptide **12** (3.7 mg) (Figure S8); *m/z* (ESI-MS) 1721.0 ($[M + 4H]^{4+}$ requires 1720.3).

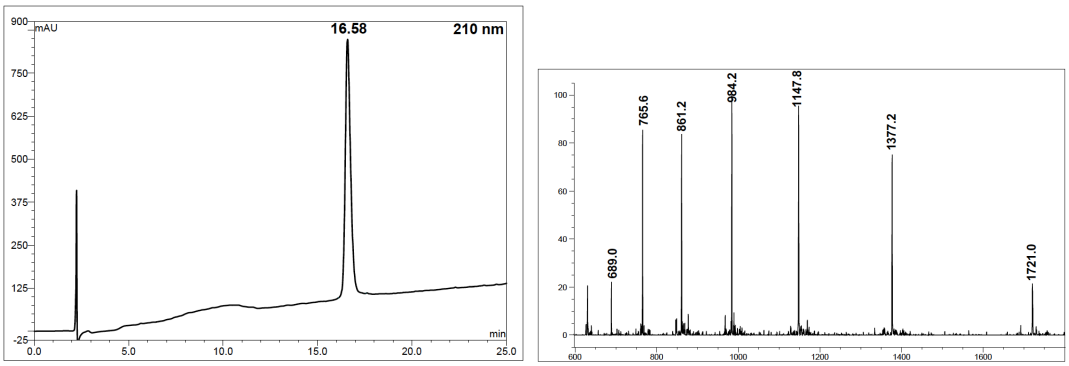

Figure S8 RP-HPLC and ESI-MS traces of purified peptide **12**; column: Dionex Acclaim C18 (4.6 x 150mm); gradient: 0-1min, 1%B then 1%B to 61%B over 25 min., eluting at 1mL/min.; R_t 16.58 min.

Synthesis of Peptide 3

A sample of Peptide **11** (1.0 mg, 0.16 µmol) was dissolved in water (100 µL) and cooled on ice-water. An ice-cooled solution of aqueous 0.2 M NaOH (100 µL) was added, the mixture incubated at 0°C for 10 min and then acidified with neat TFA (2 µL). After diluting with water (800 µL) the product was purified (Figure S9**)** in one portion using a Phenomenex Jupiter Proteo C12 (4µ 90Å, 10 x 250mm) column, generating a linear gradient 1%B to 61%B over 30 min at a flow of 5 mL/min. The main peak was collected and lyophilised to give **3** (1 mg); *m/z* (ESI-MS) 1517.0 ($[M + 4H]^{4+}$ requires 1516.5).

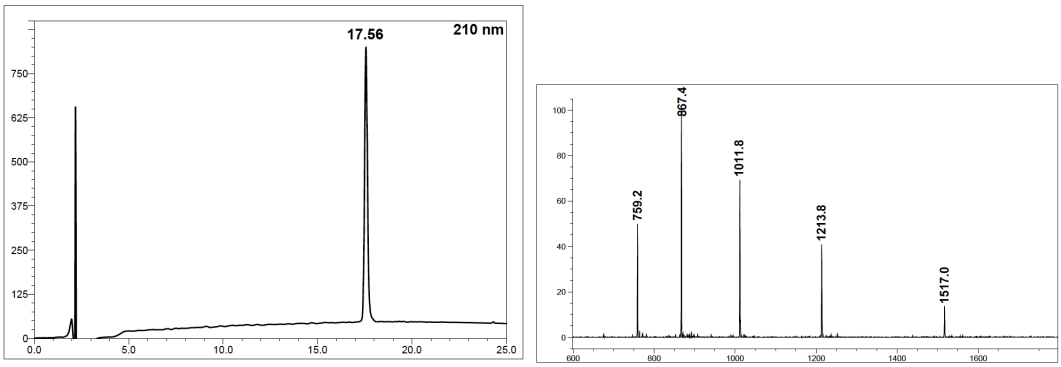

Figure S9 RP-HPLC and ESI-MS traces of purified peptide **3**; column: Dionex Acclaim C18 (4.6 x 150mm); gradient: 0-1min, 1%B then 1%B to 61%B over 25 min., eluting at 1mL/min.; R_t 17.56 min.

Synthesis of Peptide 4

The same procedure as for peptide **3** was employed, affording peptide **4** (Figure S10); *m/z* (ESI-MS) 1527.6 ($[M + 4H]^{4+}$ requires 1527.0).

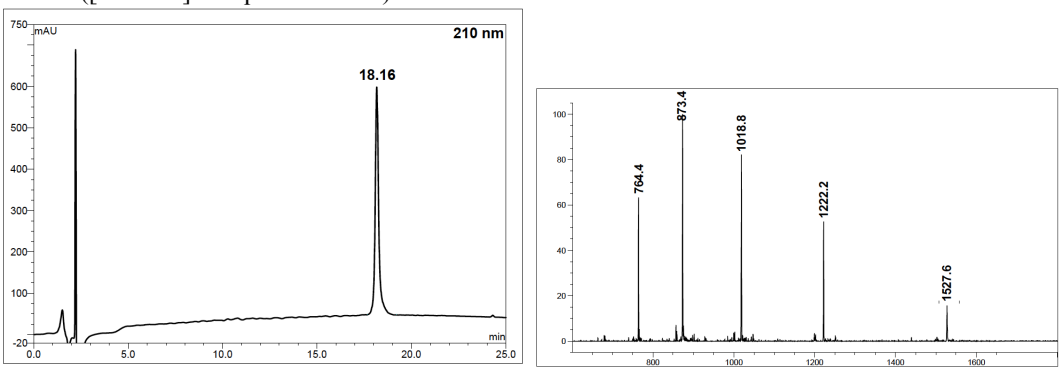

Figure S10 RP-HPLC and ESI-MS traces of purified peptide **4**; column: Dionex Acclaim C18 (4.6 x 150mm); gradient: 0-1min, 1%B then 1%B to 61%B over 25 min., eluting at 1mL/min.; R_t 18.16 min.

Circular Dichroism Spectra

Insulin Glargine and analogues **3** and **4** were each dissolved in 1 mM aqueous HCl to approximately 0.2-0.5 mg/mL and the exact protein concentration determined[15] by measuring the absorbance at 276 nm. The circular dichroism measurements were made using an Applied Photophysics PiStar-180 spectrapolarimeter at room temperature. Spectra were recorded from 250 to190 nm using a cell with 0.1-cm path length. Data are expressed as molar ellipticity with a protein concentration adjusted to 0.2 mg/mL.

Peptide Formulation and Dosing Methods

A 20 mL aqueous solution comprising *m*-cresol (25 mM), $ZnCl_2$ (0.22 mM), Glycerol (200 mM) and citric acid (4 mM) was prepared and NaOH (0.4 mL of a 0.2 M aqueous solution) added to give a buffer of pH 4.0, which was passed through a 0.2 micron filter prior to use.

Intraperitoneal (IP) insulin tolerance tests (ITTs) were performed in male FVB/N mice approximately 85 days old. All experiments were approved by the Animal Ethics Committee of the University of Auckland, and performed in accordance with the Animal Welfare Act 1999. All peptides were prepared in the above buffer at 100mU/μL (one IU = 45 μg) and diluted in sterile saline for delivery by IP injection at time =0. The dose was 1mU/g for all tests; Lantus (insulin glargine) purified from commercial preparation n=3; blank no peptide control n=3; analogue **3** n=4 and analogue **4** n=4. Repeated measures ANOVA was used to compare the four datasets with Dunnett's Multiple Comparisons Test performed *post hoc* vs Blank.