Supplementary Information

Contents

1. General Information:

1.1 Chemical and Protein Materials

Insulin was purchased from Life Technologies unless otherwise specified. Peptides were synthesized via Fmoc solid phase peptide synthesis. N,N-Diisopropylethylamine (DIEA), triisopropylsilane, L-ascorbic acid, acetic acid (AcOH), iodine, piperidine, methanol (MeOH), urea and dichloromethane (DCM) were purchased from Sigma-Aldrich and used directly. Fmoc-protected amino acids and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) were purchased from Chem-Impex Int'l. Inc. Boc-Ser[Fmoc-Thr(tBu)] was purchased from AAPPTec. 2-Chlorotrityl chloride resin was purchased from ChemPep. Dimethylformamide (DMF), trifluoroacetic acid (TFA), acetonitrile (ACN) and ethyl ether were purchased from Fisher Scientific and used as supplied.

1.2 HPLC and LC/MS

All crude peptides were purified with a water/acetonitrile gradient in 0.1% TFA on an Agilent 1260 HPLC system. Fractions collected from HPLC were analyzed by LC/MS on a XBridge C18 5-μm (50 × 2.1 mm) column at 0.4 $mL.min^{-1}$ with a water/acetonitrile gradient in 0.1% formic acid on an Agilent 6120 Quadrupole LC/MS system. Fractions containing targeted product (based on LC/MS) were collected and lyophilized.

1.3 General RP-HPLC conditions

Method A: Individual chains were purified by a Preparative C18 (2) Column (Luna®, 5 μm, 250 x 21.2 mm) with a linear gradient from 25% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 $mL.min^{-1}$ for A chains and from 30% aqueous ACN (0.1% TFA) to 60% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 $mL.min^{-1}$ for B chains.
Method B: All A-B Dimer peptides were purified using a Preparative C18 (2) Column (Luna®, 5 μm, 250 x 21.2 mm) with a linear gradient from 25% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 $mL.min^{-1}$.
Method C: All final products were purified by a Preparative C18 (2) Column (Luna®, 5 μm, 250 x 21.2 mm) with a linear gradient from 20% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 $mL.min^{-1}$.

2. Peptide Synthesis

Peptides were synthesized via Fmoc solid phase peptide synthesis on a peptide synthesizer (Prelude; Protein Technologies, Inc). Automated peptide synthesis was carried out in a 45 mL reactor vial with the following protocols (for 0.05 mmol scale). For Fmoc deprotection: (i) 4.5 mL of 20% piperidine in DMF; (ii) mix 2 × 3 min (new solvent delivered for each mixing cycle). For amino acid coupling: (i) 1.5 mL of 0.2 M Fmoc-protected amino acid in DMF; (ii) 1.45 mL of 0.2 M HATU; (iii) 0.5 mL of 1.0 M DIPEA in DMF; and (iv) mix for 25 min at 25 ^{o}C. For DMF washing (performed between deprotection and coupling steps): (i) 4.5 mL of DMF; (ii) mix 45 s. Upon completion of the peptide chain, resins were washed with DCM and dried (using vacuum) for 30 min.

2.1 General procedure for A-chain

The syntheses of A-chains were conducted on 2-chlorotrityl chloride resin using a peptide synthesizer with a standard Fmoc/HATU/DIEA method. The resulting resin-bound A chain (0.1 mmol scale) was treated with 6.0 mL TFA solution containing 2.5% TIS, 2.5% H_2O at rt, with gentle shaking for 2.0 hours. The resin was filtered off and the filtrate was precipitated by cold ether (40 mL). The precipitate was collected by centrifugation then washed with cold ether (40 mL x 3), and vacuum dried and purified by preparative C18 column. 67 mg of **A2** (from 0.1 mmol starting resin) and 70 mg of **A3** (from 0.10 mmol starting resin) were obtained after lyophilization, with a yield of 24% for **A2** and 26% for **A3**.

2.2 General procedure for B chain

B-chain syntheses were conducted on 2-chlorotrityl chloride resin using a standard Fmoc/HATU/DIEA method. The first amino acids were synthesized manually. Cleavage was conducted by treating the resin (0.1 mmol scale) with 6.0 mL TFA solution that contained 2.5% TIS, 2.5% H_2O and 15 equivalents of DTDP at rt, with shaking for 2.5 hours. The resin was filtered off; and the filtrate was precipitated with cold ether (45 mL). The precipitate was collected by centrifugation, and then washed with cold ether (45 mL x 3). Crude B chain was dissolved in 0.05% TFA containing aqueous acetonitrile (ACN/H_2O: 50/50 vol/vol, 40 mL) and purified on a preparative C18 column. 87 mg of **B2** (from 0.10 mmol starting resin) was obtained with a yield of 24%; 91 mg of **B3** was obtained (from 0.10 mmol starting resin) with a yield of 25%; 78 mg of **B4** (from 0.10 mmol starting resin) was obtained with a yield of 21%.

2.3 General procedure for preparation of analogs by two-step method

A chain and B chain were mixed in 6 M urea, 0.2 M NH_4HCO_3 buffer (pH 7.5). The mixture was left at room temperature for 10 min. The resulting solution was purified on a preparative C18 column with a linear gradient from 25% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 mL/min. A-B dimer eluted at around 30 min.

The lyophilized powder A-B dimer was dissolved in 33% aqueous acetic acid, and treated with a freshly prepared solution of iodine in MeOH (10 mg/mL). The resulting solution was gently agitated at rt for 20 min before the addition of 1 M ascorbic acid until the iodine color (purple) disappeared. The crude was purified on a preparative C18 column with a linear gradient from 20% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 mL/min. The correctly folded analogs eluted as the earliest fraction by RP-HPLC at around 35 min.

2.4.1 Preparation of A22-B21-4SS-Ins:

A chain **A2** (31 mg) and B chain **B2** (52 mg) were mixed in 6 M urea, 0.2 M NH_4HCO_3 buffer (pH 7.5, 4.6 mL). The mixture was left at rt for 10 min. The resulting solution was purified by preparative C18 column with a linear gradient from 25% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 mL/min. 48 mg of dimer (**A2**+**B2**) was obtained with a yield of 68%.

The lyophilized powder A-B dimer (24 mg) was dissolved in 33% aqueous acetic acid (3.0 mL). It was treated with a freshly prepared solution of iodine in MeOH (10 mg/mL). The resulting solution

was gently agitated at rt for 20 min before the addition of 1 M ascorbic acid until the iodine color (purple) disappeared. The crude was purified by preparative column with a linear gradient from 20% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 mL/min. 2.5 mg of **A22-B21-4SS-Ins** was obtained with a yield of 11% from dimer (**A2**+**B2)**, and a yield of 7.4% over two steps based on **A2**.

2.4.2 Preparation of A22-B22-4SS-Ins:

A chain **A2** (30 mg) and B chain **B3** (48 mg) were mixed in 6 M urea, 0.2 M NH_4HCO_3 buffer (pH 7.5, 4.6 mL). The mixture was left at rt for 10 min. The resulting solution was purified by preparative column with a linear gradient from 25% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 mL/min. 40 mg of dimer (**A2**+**B3**) was obtained with a yield of 60 %.

The lyophilized powder A-B dimer (22 mg) was dissolved in 33% aqueous acetic acid (3.0 mL). It was treated with a freshly prepared solution of iodine in MeOH (10 mg/mL). The resulting solution was gently agitated at rt for 20 min before the addition of 1 M ascorbic acid until the iodine color (purple) disappeared. The crude was purified by preparative column with a linear gradient from 20% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 mL/min. 1.7 mg of **A22-B21-4SS-Ins** was obtained with a yield of 8.1% from dimer (**A2**+**B3**), and a yield of 4.9 % over two steps based on **A2**.

2.4.3 Preparation of A22-B23-4SS-Ins:

A chain **A2** (30 mg) and B chain **B4** (45 mg) were mixed in 6 M urea, 0.2 M NH_4HCO_3 buffer (pH 7.5, 4.6 mL). The mixture was left at rt for 10 min. The resulting solution was purified by preparative column with a linear gradient from 25% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 mL/min. 49mg of dimer (**A2**+**B4**) was obtained with a yield of 71%.

The lyophilized powder A-B dimer (25 mg) was dissolved in 33% aqueous acetic acid (3.0 mL). It was treated with a freshly prepared solution of iodine in MeOH (10 mg/mL). The resulting solution was gently agitated at rt for 20 min before the addition of 1 M ascorbic acid until the iodine color (purple) disappeared. The crude was purified by preparative column with a linear gradient from 20% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 mL/min. 2.9 mg of **A22-B23-4SS-Ins** was obtained with a yield of 13 % from dimer (**A2**+**B4**), and a yield of 9.1 % over two steps based on **A2**.

2.4.3 Preparation of A21-B22-4SS-Ins:

A chain **A3** (30 mg) and B chain **B3** (48 mg) were mixed in 6 M urea, 0.2 M NH_4HCO_3 buffer (pH 7.5, 4.6 mL). The mixture was left at rt for 10 min. The resulting solution was purified by preparative column with a linear gradient from 25% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 mL/min. 37 mg of dimer (**A3**+**B3**) was obtained with a yield of 55%.

The lyophilized powder A-B dimer (25 mg) was dissolved in 33% aqueous acetic acid (3.0 mL). It

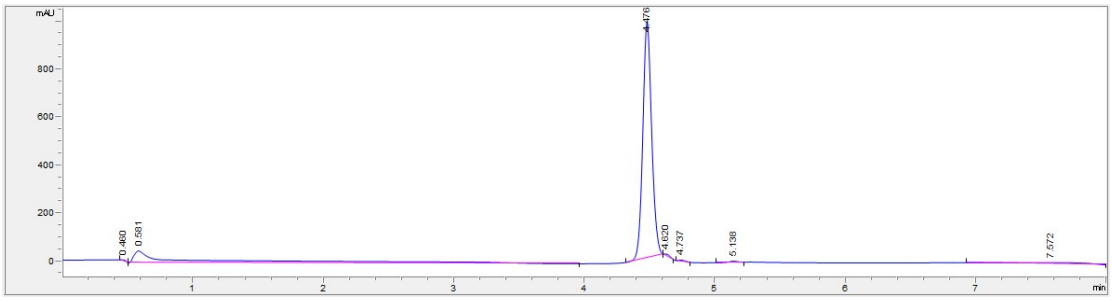
was treated with a freshly prepared solution of iodine in MeOH (10 mg/mL). The resulting solution was gently agitated at rt for 20 min before the addition of 1 M ascorbic acid until the iodine color (purple) disappeared. The crude was purified by preparative column with a linear gradient from 20% aqueous ACN (0.1% TFA) to 50% aqueous ACN (0.1% TFA) over 45 min at a flow rate of 5 mL/min. 2.7 mg of **A22-B23-4SS-Ins** was obtained with a yield of 11.6 % from dimer (**A3**+**B3**), and a yield of 6.4 % over two steps based on **A3**.

Table S1 The LC-MS characterization of the synthetic peptides

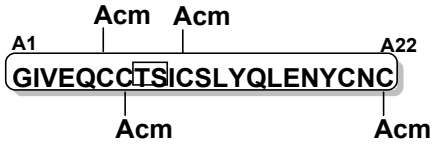
Peptide name	M.W.		Purity by RP-HPLC
	(calculated)	(observed)	
A2	2770.9	2771.1	97%
B2	3654.0	3654.9	97%
A2+B2	6314.8	6314.4	94%
A22-B21-4SS-Ins	5882.8	5882.4	98%
B3	3626.9	3627.5	98%
A2+B3	6287.8	6286.9	96%
A22-B22-4SS-Ins	5855.7	5855.1	99%
B4	3726.0	3726.9	95%
A2+B4	6386.9	6386.6	96%
A22-B23-4SS-Ins	5954.8	5954.2	97%
A3	2656.8	2656.5	98%
A3+B3	6173.7	6173.4	98%
A21-B22-4SS-Ins	5741.6	5740.9	99%

3. Copy of LC chromatogram, MS spectrum

LC for **A2:**

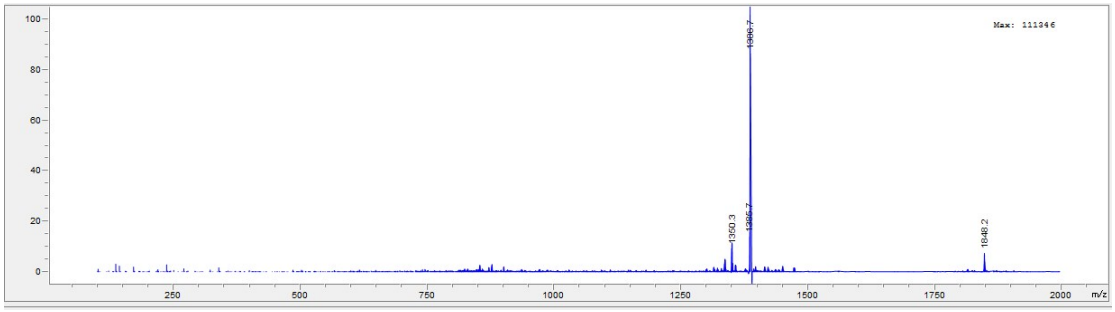


MS spectrum for **A2:**

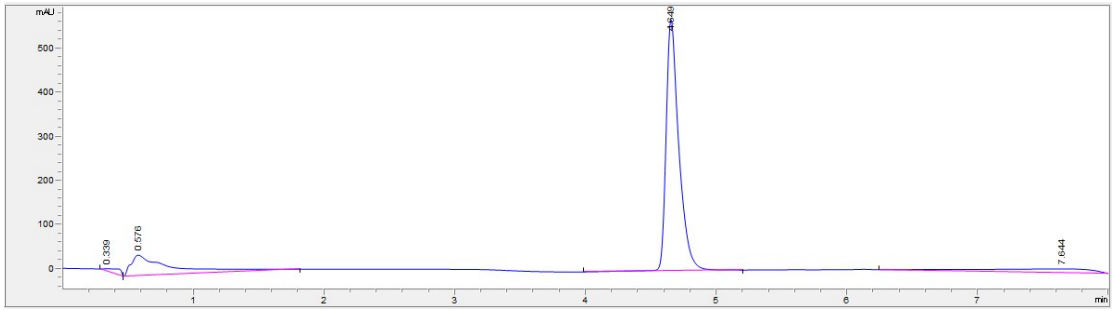


Expected Molecular weight: 2770.9

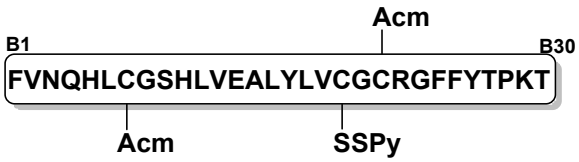
Observed Molecular weight: 2771.4([M]$^{+2}$ 1386.7)



LC for **B2**:

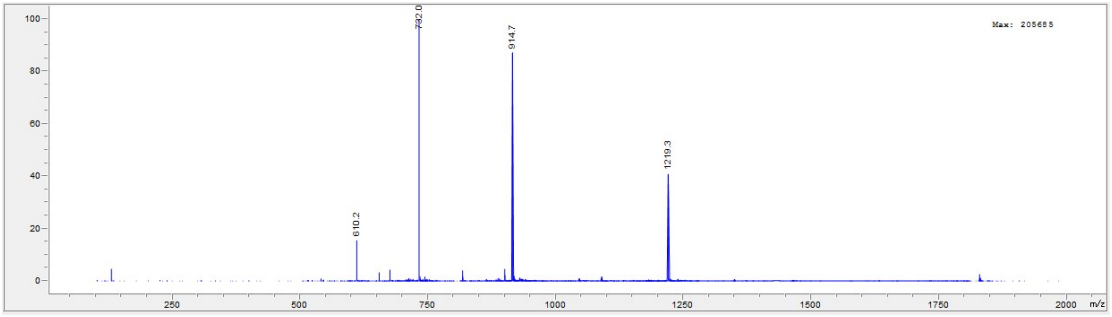


MS spectrum for **B2**:

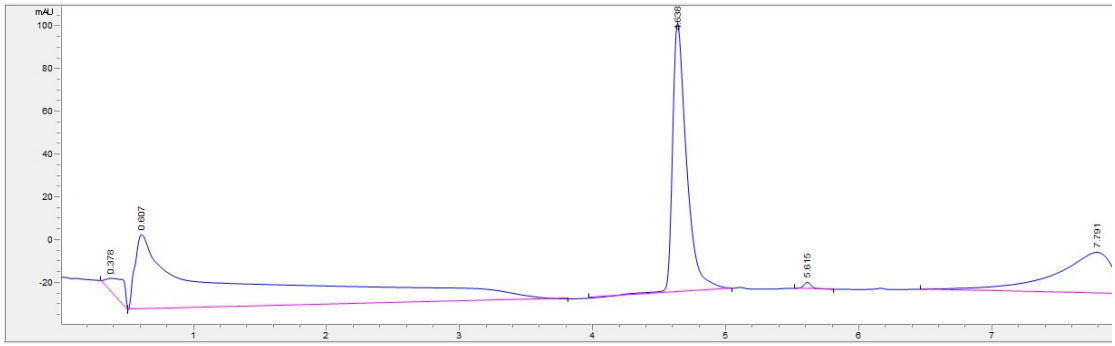


Expected Molecular weight: 3654.0

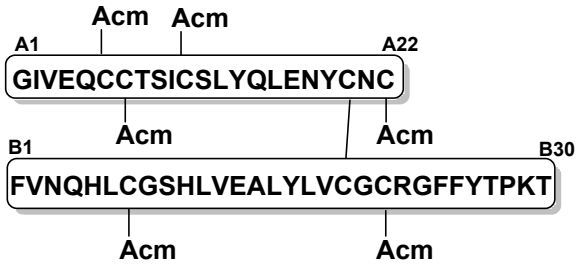
Observed Molecular weight: 3654.9 ($[M]^{+3}$1219.3; $[M]^{+4}$ 914.7; $[M]^{+5}$ 732.0)



LC for **A2**+**B2** comb:

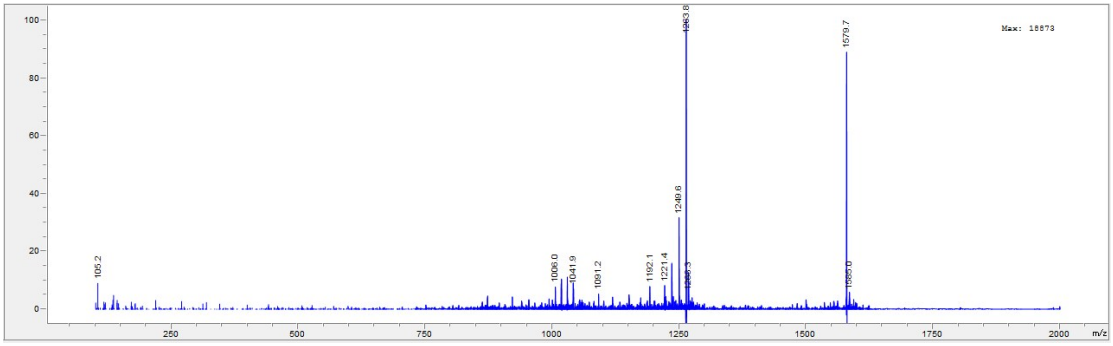


MS spectrum for **A2+B2** comb:

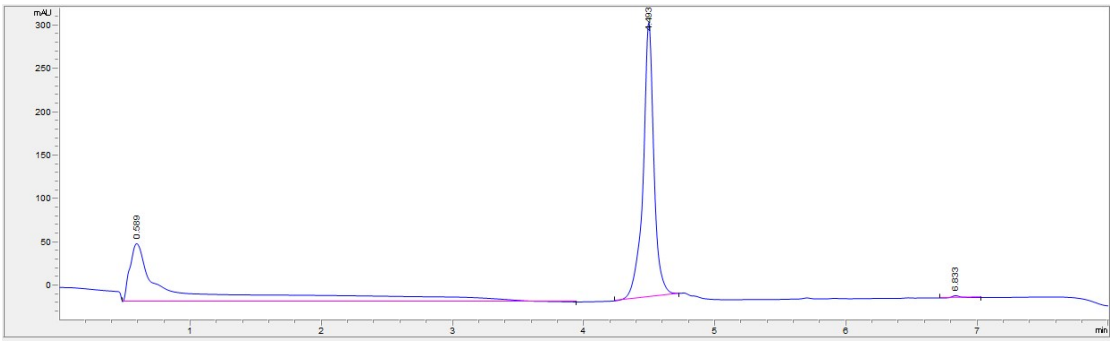


Expected Molecular weight: 6314.8

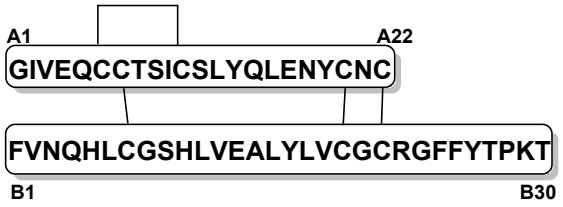
Observed Molecular weight: 6314.4 ($[M]^{+4}$ 1579.7; $[M]^{+5}$ 1263.8)



LC for **A22-B21-4SS-Ins:**

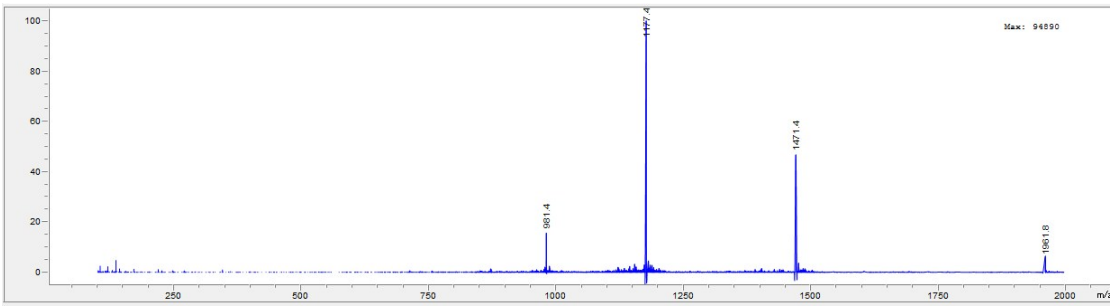


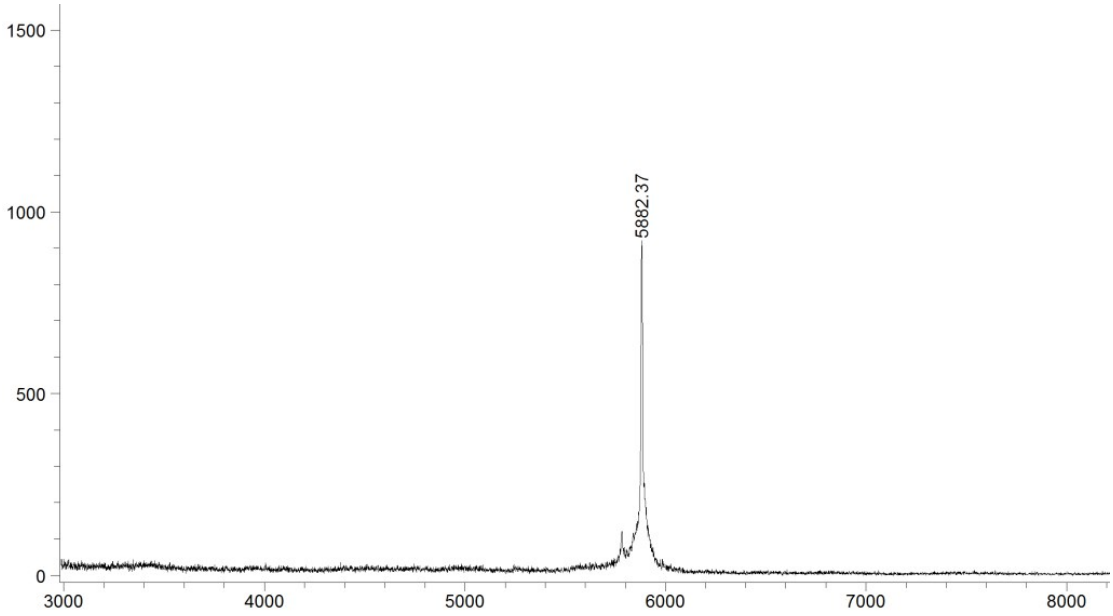
MS spectrum for **A22-B21-4SS-Ins:**:



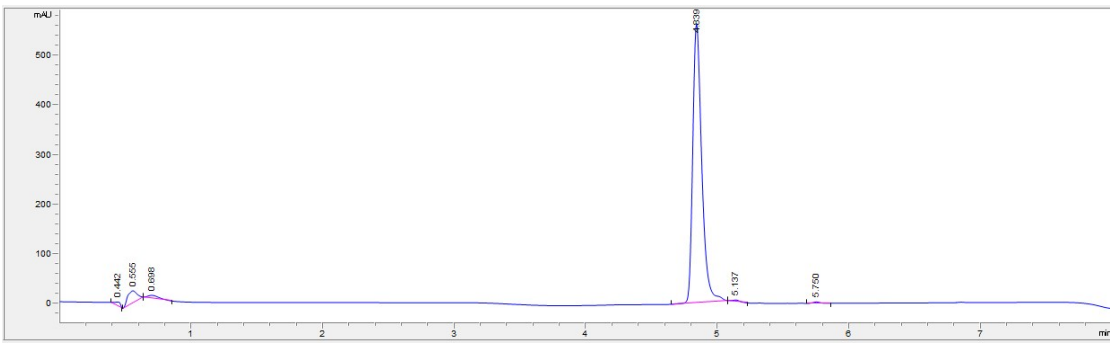
Expected Molecular weight: 5882.8

Observed Molecular weight: 5882.4 ($[M]^{+3}$ 1961.8; $[M]^{+4}$ 1471.4; $[M]^{+5}$ 1177.4; $[M]^{+6}$ 981.4)

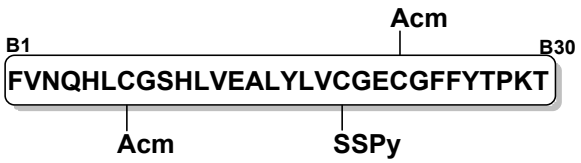




LC for **B3**:

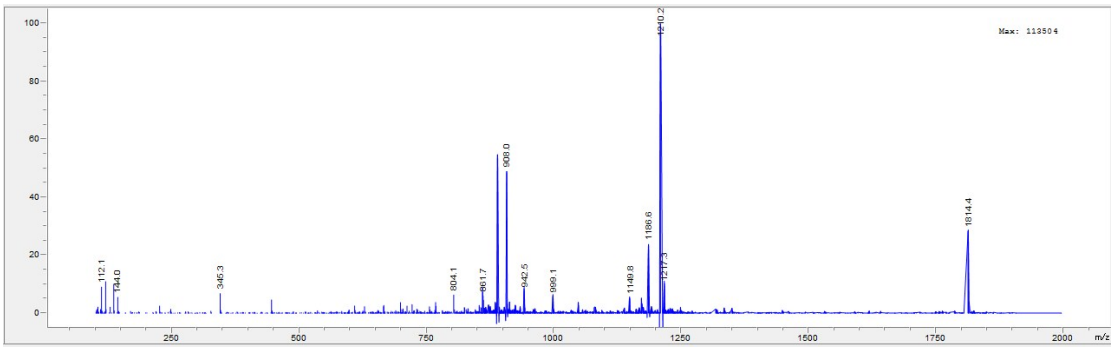


MS spectrum for **B3**:

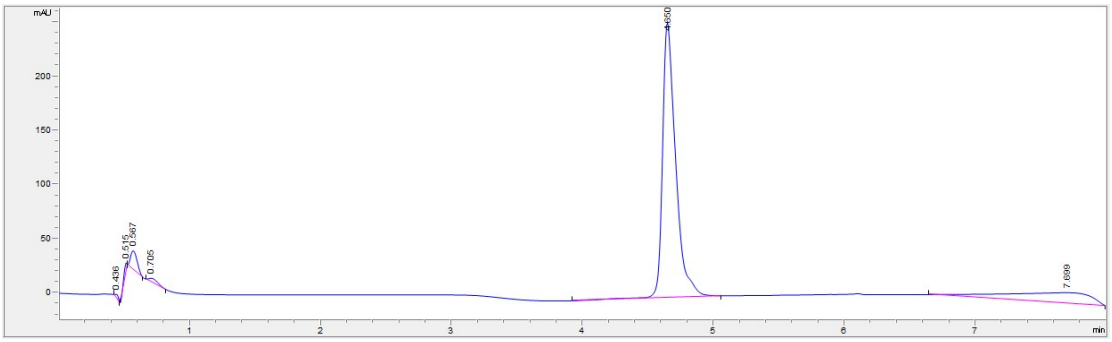


Expected Molecular weight: 3626.9

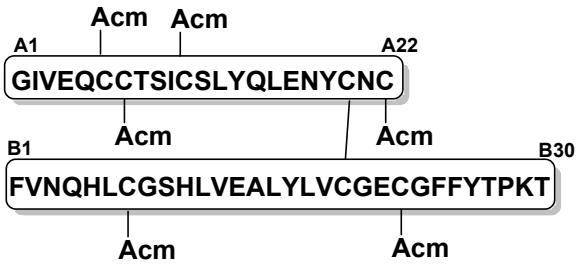
Observed Molecular weight: 3627.5 ($[M]^{+2}$ 1814.4; $[M]^{+3}$ 1210.2; $[M]^{+4}$ 908.0)



LC for **A2+B3** comb:

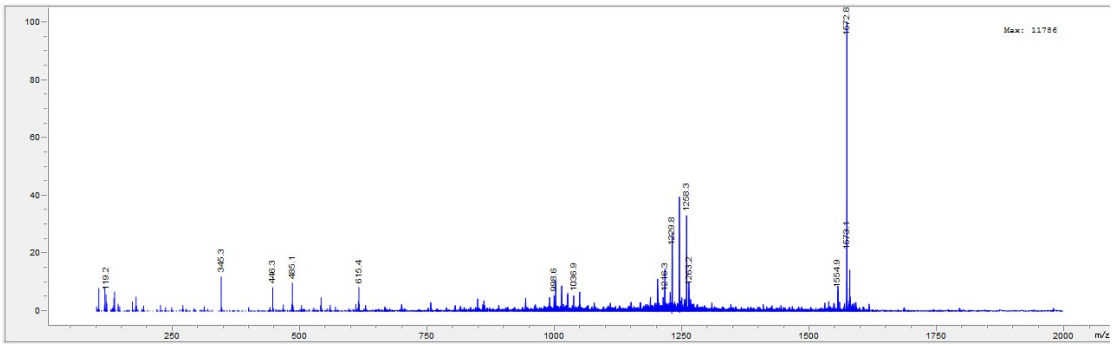


MS spectrum for **A2+B3** comb:

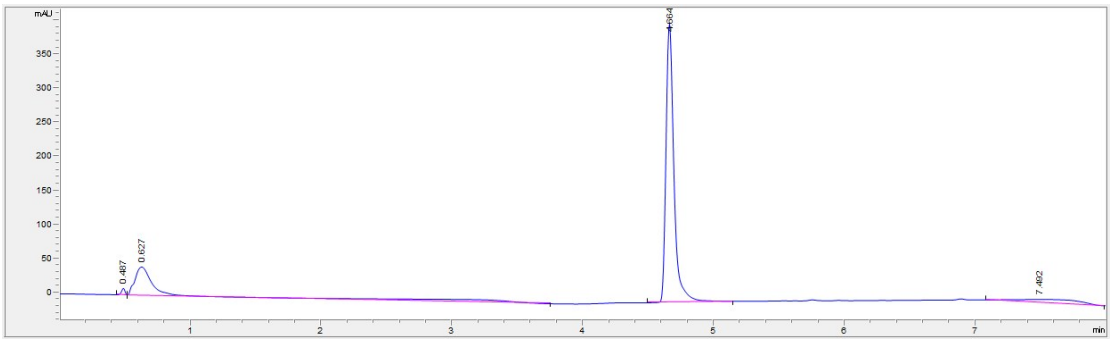


Expected Molecular weight: 6287.8

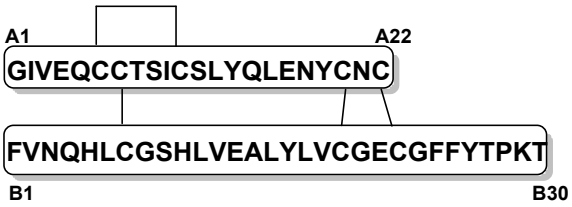
Observed Molecular weight: 6286.9 ([M]$^{+4}$ 1572.8; [M]$^{+4}$ 1258.3)



LC for **A22-B22-4SS-Ins**:

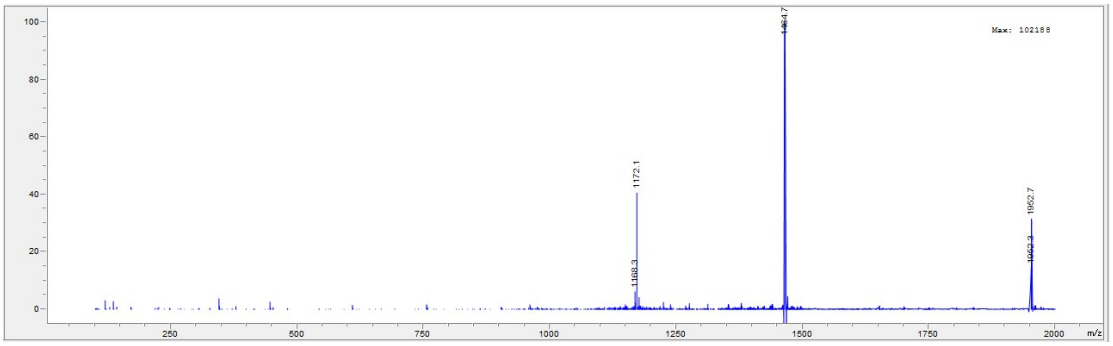


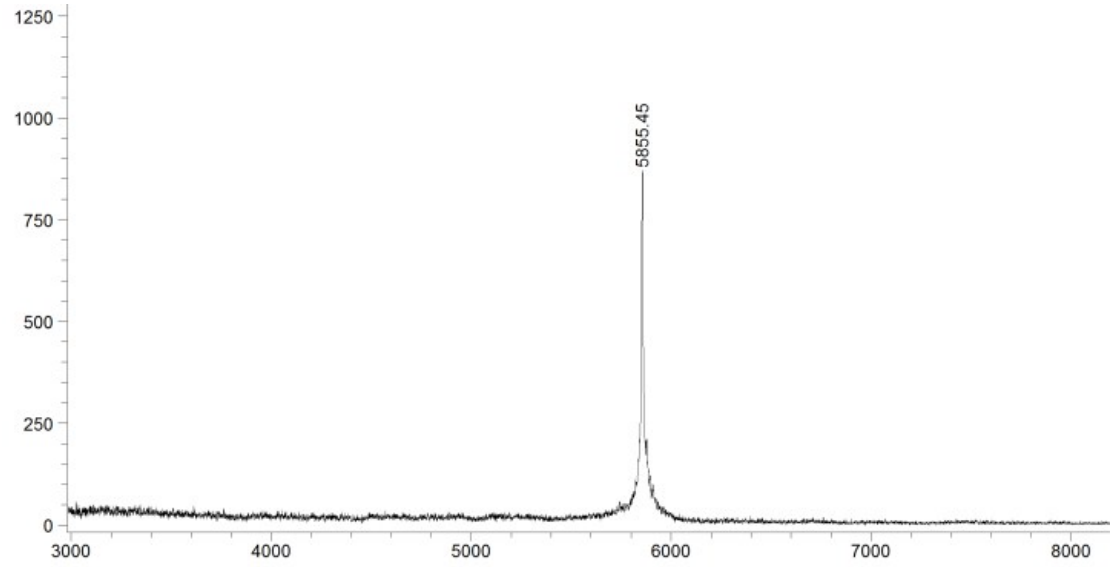
MS spectrum for **A22-B22-4SS-Ins**:



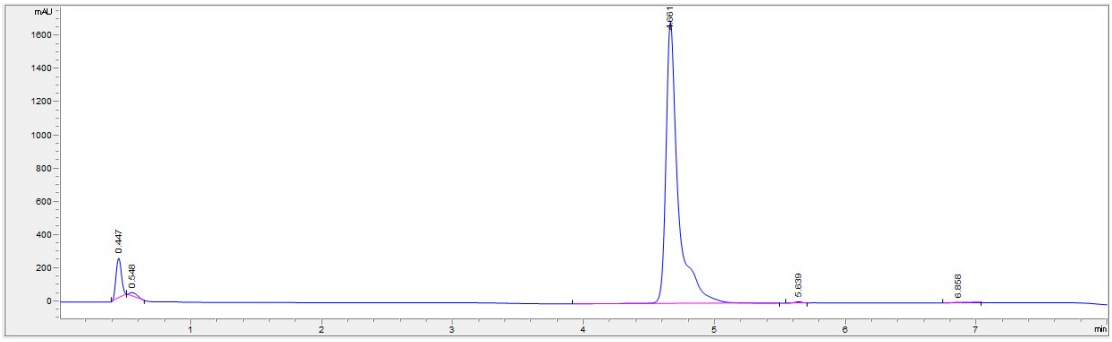
Expected Molecular weight: 5855.7

Observed Molecular weight: 5855.1([M]$^{+3}$ 1952.7; [M]$^{+4}$ 1464.7; [M]$^{+5}$ 1172.1)

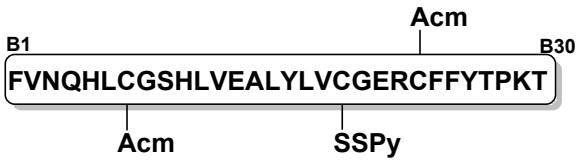




LC for **B4**:

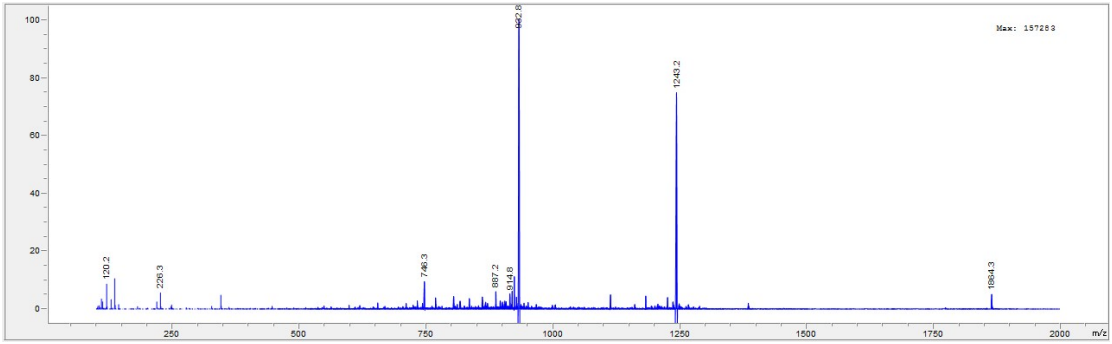


MS spectrum for **B4**:

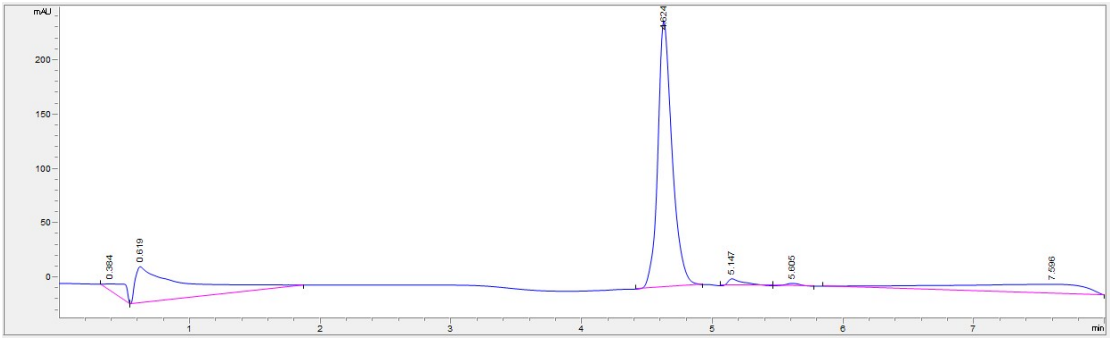


Expected Molecular weight: 3726.0

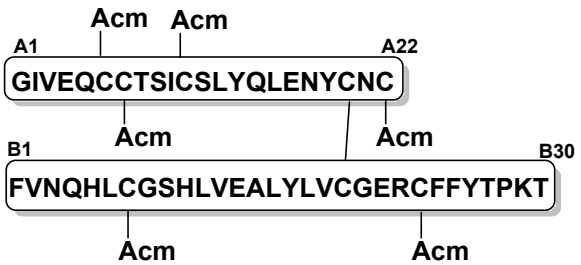
Observed Molecular weight: 3726.9 ($[M]^{+2}$ 1864.3; $[M]^{+3}$ 1243.3; $[M]^{+4}$ 932.8)



LC for **A2**+**B4** comb:

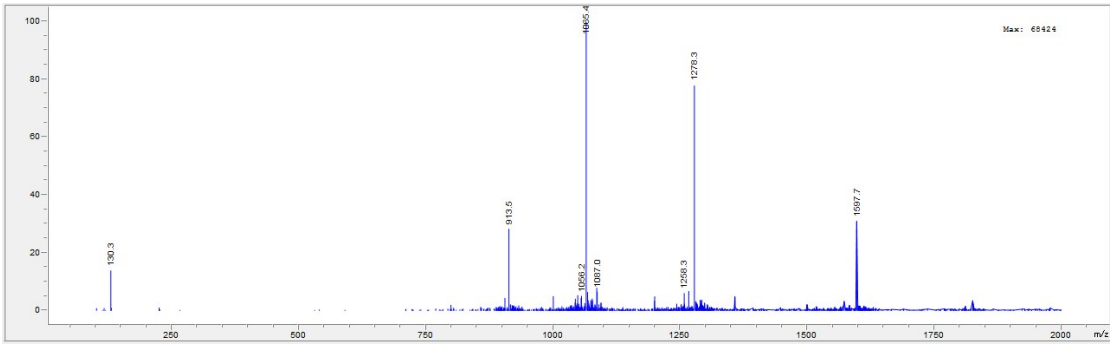


MS spectrum for **A2**+**B4** comb:

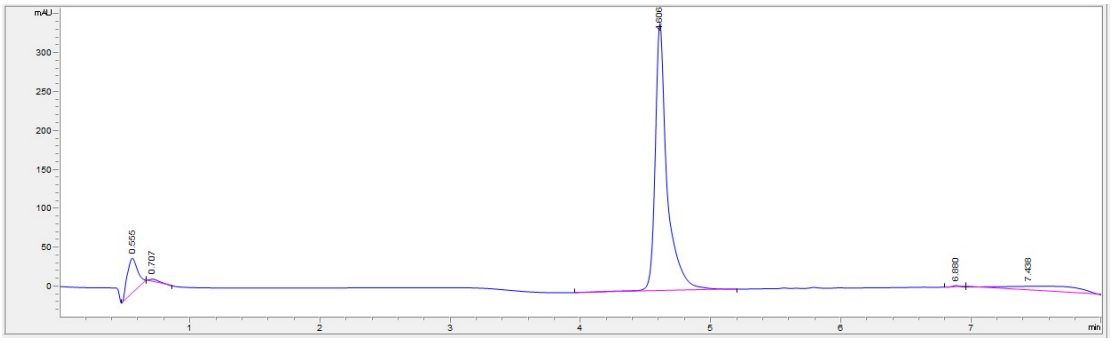


Expected Molecular weight: 6386.9

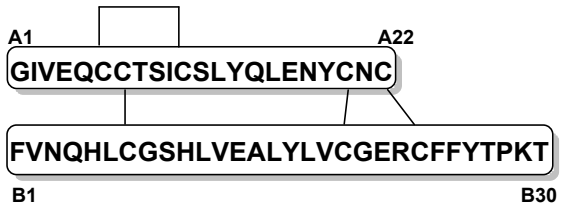
Observed Molecular weight: 6386.6 ([M]$^{+4}$ 1597.7; [M]$^{+5}$ 1278.3; [M]$^{+6}$ 1065.4; [M]$^{+7}$ 913.5)



LC for **A22-B23-4SS-Ins**:

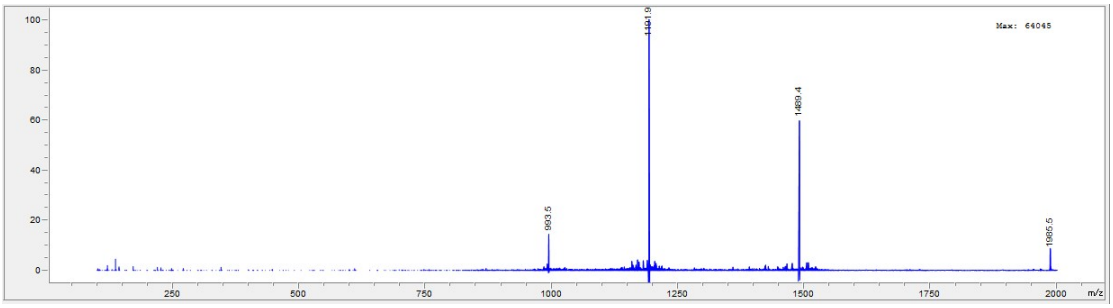


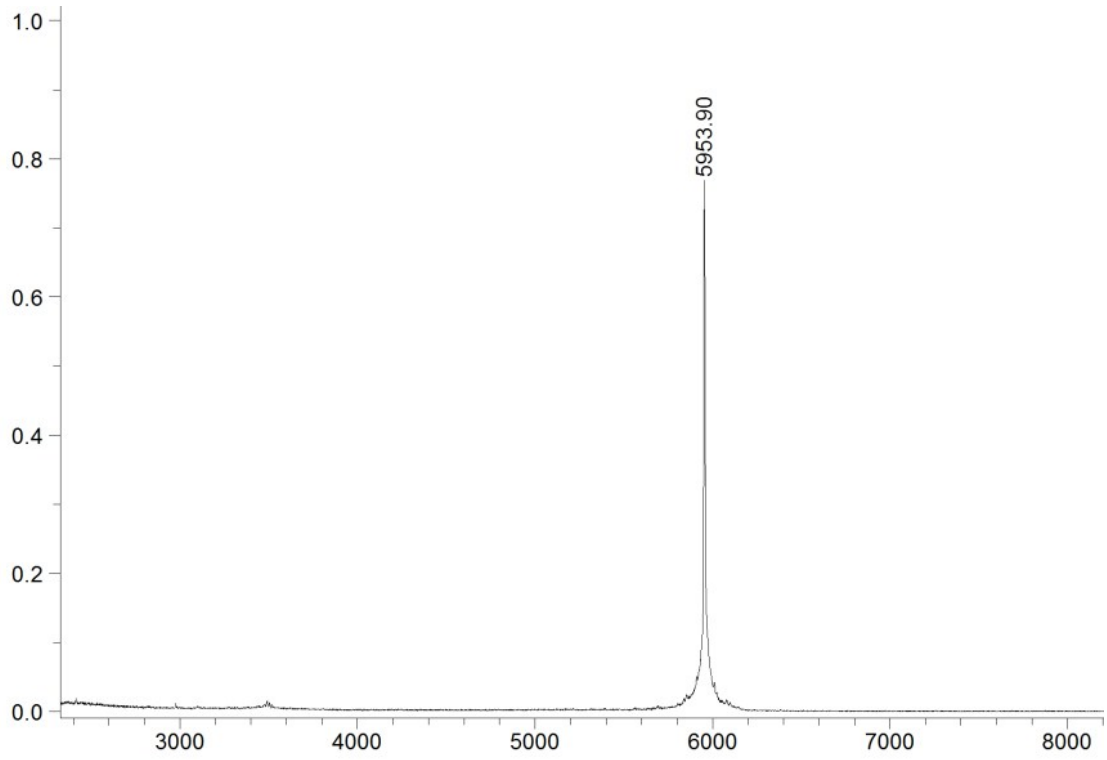
MS spectrum for **A22-B23-4SS-Ins**:



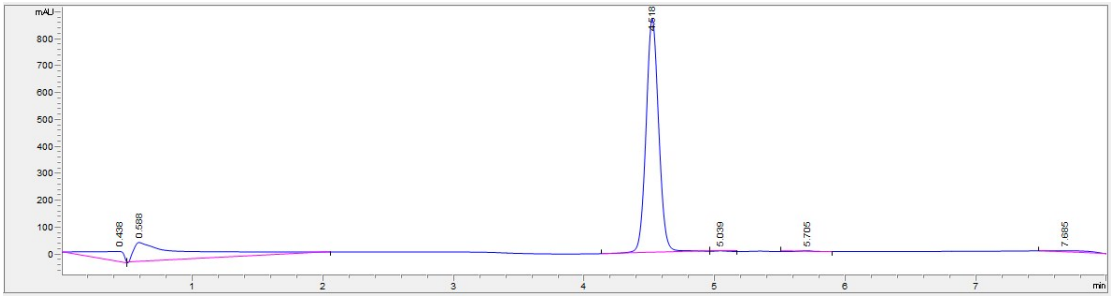
Expected Molecular weight: 5954.8

Observed Molecular weight: 5954.2 ([M]$^{+3}$ 1985.5; [M]$^{+4}$ 1489.4; [M]$^{+5}$ 1191.9; [M]$^{+6}$ 993.5)

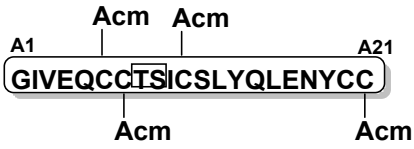




LC for **A3**:

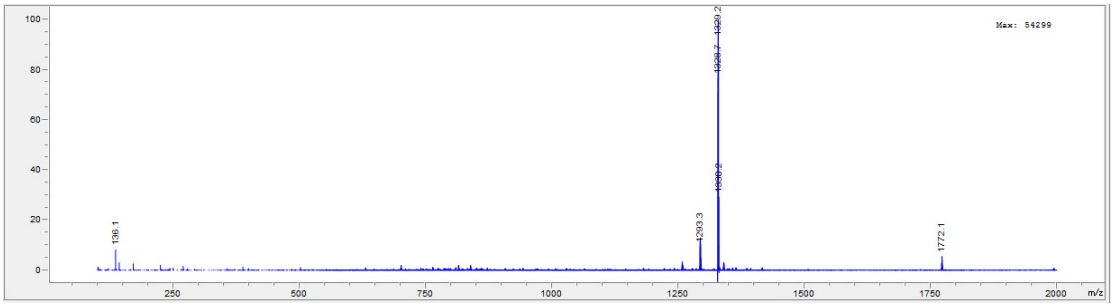


MS spectrum for **A3**:

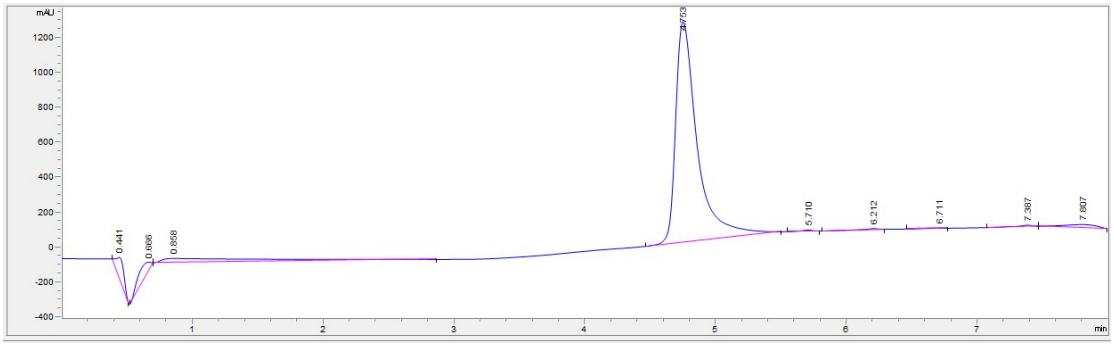


Expected Molecular weight: 2656.8

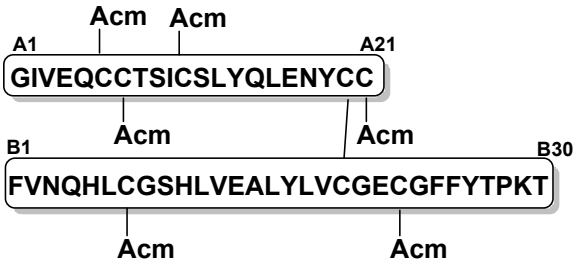
Observed Molecular weight: 2656.4 ([M]$^{+2}$ 1329.2)



LC for **A3**+**B3** Comb:

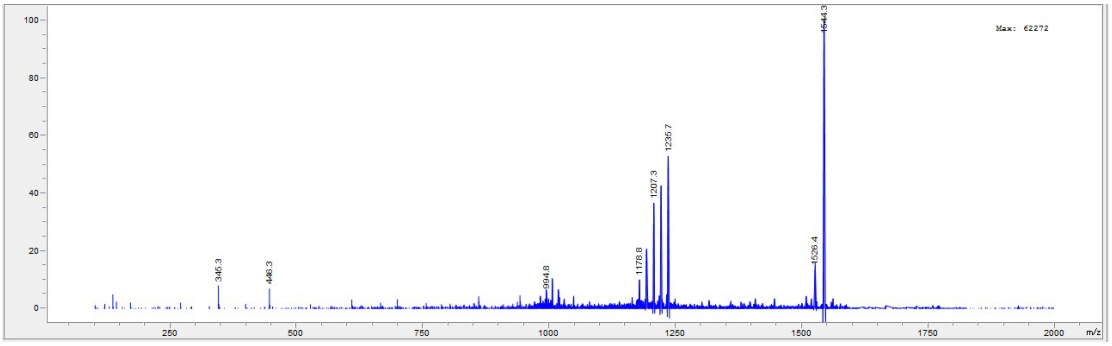


MS spectrum for **A3+B3** Comb:

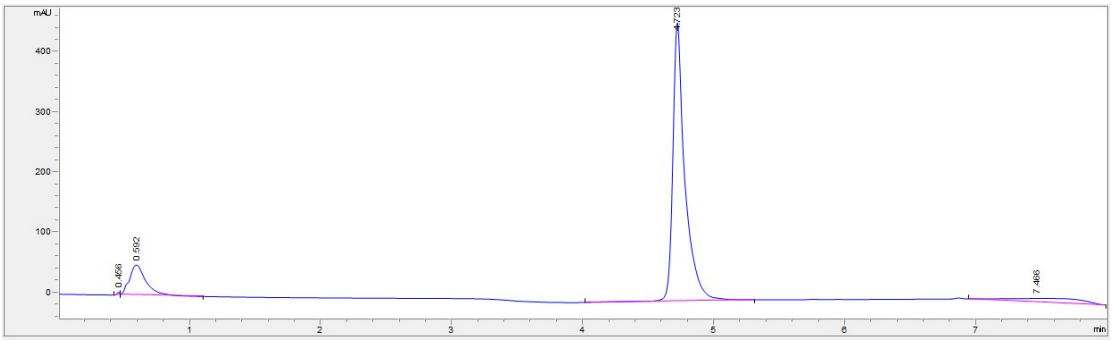


Expected Molecular weight: 6173.7

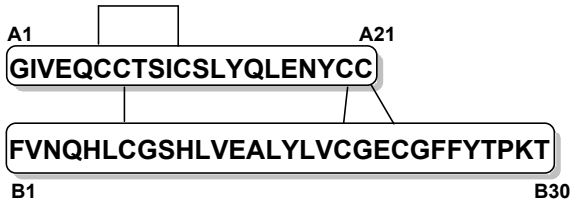
Observed Molecular weight: 6173.4([M]$^{+2}$ 1544.3; [M]$^{+3}$ 1235.7)



LC for **A21-B22-4SS-Ins**:

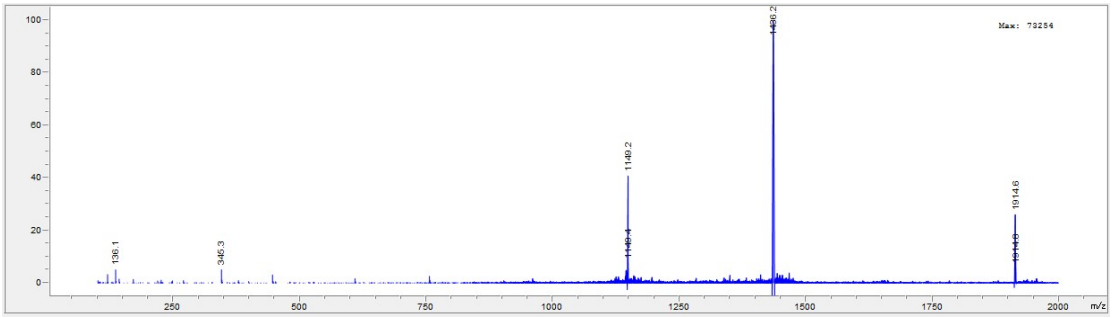


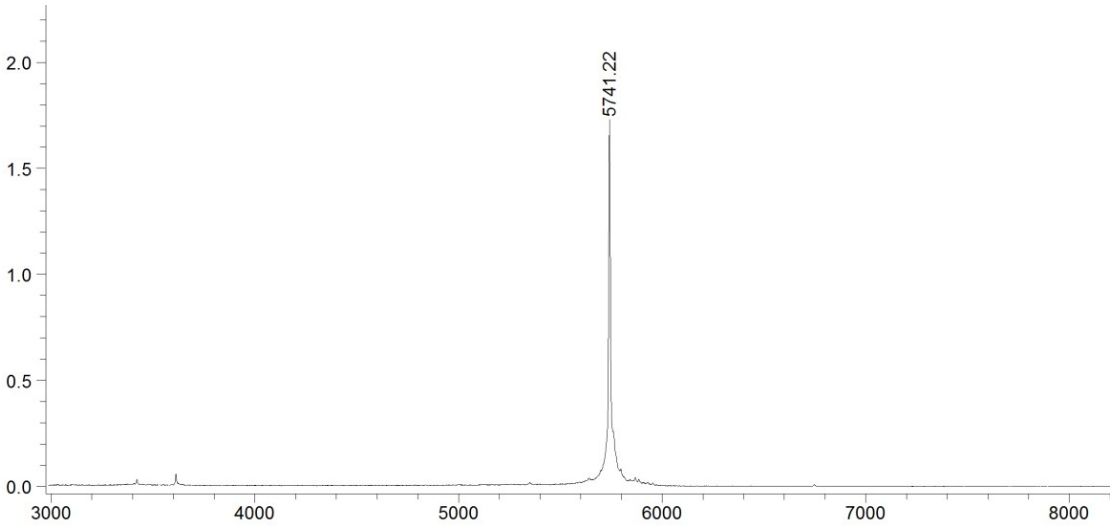
MS spectrum for **A21-B22-4SS-Ins**:



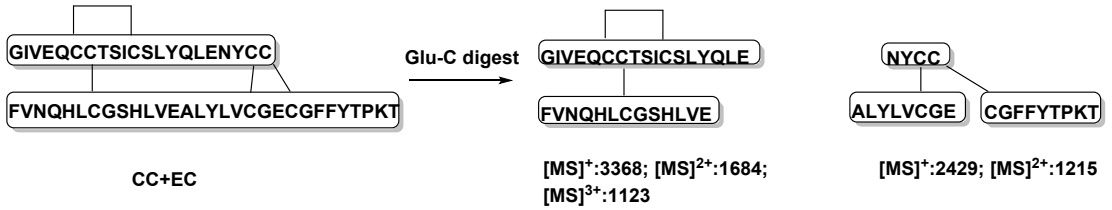
Expected Molecular weight: 5741.6

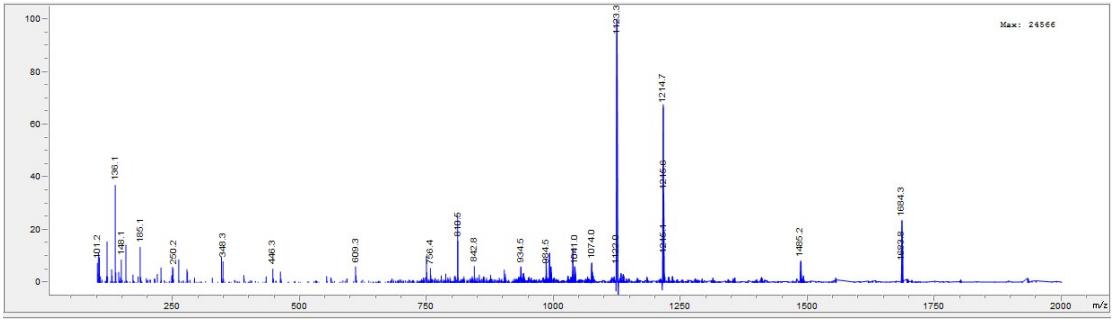
Observed Molecular weight: 5740.9 ([M]$^{+3}$ 1914.6; [M]$^{+4}$ 1436.2; [M]$^{+5}$ 1149.2)



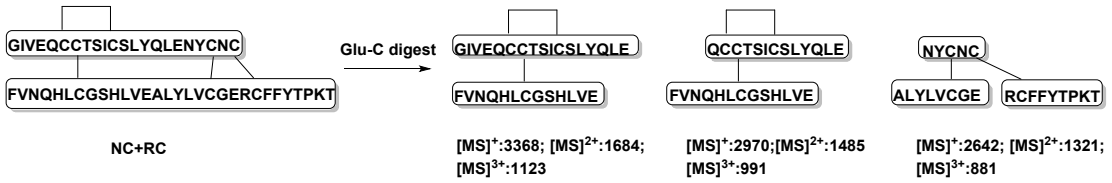


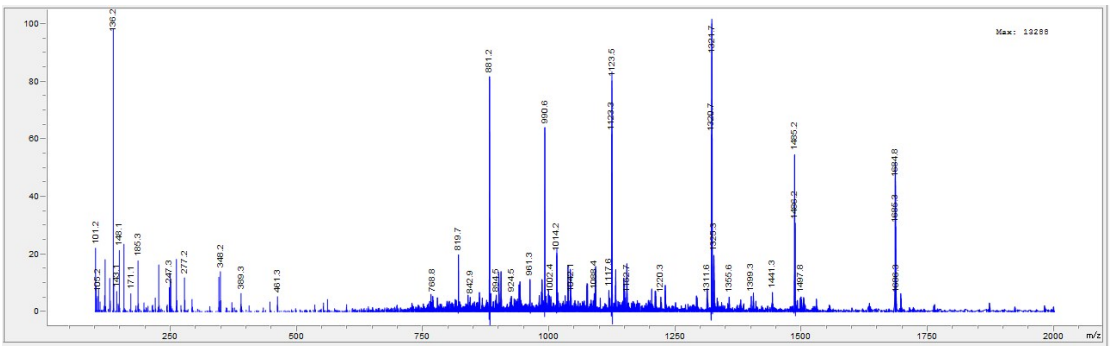
MS spectrum for glu-C digest of **A21-B22-4SS-Ins**





MS spectrum for glu-C digest of **A22-B23-4SS-Ins**:





4. Insulin activity and aggregation assays

Phospho-AKT (Ser 473) cell-based assay: pAkt Ser473 levels were measured in a mouse fibroblast cell line, NIH 3T3, overexpressing human insulin receptor isoform B (IR-B). The cell line was cultured in DMEM (Sigma Aldrich) with 10% fetal bovine serum (Gibco), 100 U/mL penicillin-streptomycin (Thermo Fisher Scientific) and 2 mg/mL puromycin (Thermofisher Scientific). For each assay, 40,000 cells per well and 100 µl per well, were plated in a 96-well plates with culture media containing 1% FBS. 20 hours later, 50 µl of SCS-Insulin or native insulin was pipetted into each well after the removal of the original media. After a 30-min treatment, the insulin solution was removed and the HTRF pAkt Ser473 kit (Cisbio, Massachusetts, USA) was used to measure the intracellular level of pAkt Ser473. Briefly, the cells were first treated with cell lysis buffer (50 µl per well) for 1 h under mild shaking. 16 µL of cell lysate was then added to 4 µL of detecting reagent in a white 384-well plate. After 4 h incubation, the plate was read in a Synergy Neo plate reader (BioTek, Vermont, USA) and the data processed according to the manufacturer's protocol

Insulin aggregation assay: The experiment was carried out according to a previously reported procedure by Webber et al.[1]. Insulin samples were dissolved in pH 7.4 PBS to a final concentration of 1 mg/mL. Samples were plated at 150 µL per well (n = 4/group) in a clear 96-well plate (Thermo Scientific Nunc) and sealed with optically clear and thermally stable seal. The plate was immediately placed into an Infinite M200 plate reader and shaken continuously at 37 °C. Absorbance readings at 540 nm were collected every 6 min for 100 h, and absorbance values were subsequently converted to transmittance.

Insulin tolerance test (ITT): Insulin tolerance test (ITT) was performed on normal chow fed, 12-15 weeks old mice. On the day of experiment, the mice were fasted for 4-6 hours with the food removed and new bedding in the cages. The food was withdrawn for the entire experimental duration. The body weights and basal blood glucose concentrations (using Glucometer Contour nextEZ, Ascensia Diabetes Care US, Inc.) were measured. Following the basal measurements, the mice received intraperitoneal injections of 0.75 U/Kg body weight of either human insulin (Novolin R, ReliOn) or SCS-Ins dissolved in saline. The blood glucose concentrations were measured at 10, 20, 30 and 60 min after injection.

5. X-ray Crystallography

One milligram of lyophilized insulin was dissolved in 25 uL of 0.01 M HCl and diluted to a final concentration of 10 mg/mL in a buffer solution of 10 mM Tris pH 8.0 and 0.6% (w/v) zinc acetate. This solution was spin filtered for 1 minute at a relative centrifugal field of 2,348 g using a 0.22 µm cellulose acetate filter. Analogs were subjected to an insulin-focused, in-house crystallization screen that was based on experimental conditions reported in the PDB. Sitting-drop vapor diffusion plates were prepared with drops comprising 1 µL precipitant and 1 µL insulin solution. Crystals of the A22C-B22C insulin appeared in less than 10 minutes in well condition 500 mM sodium citrate, 8.5% (v/v) acetone, and 0.1% (v/v) phenol. Their typical dimensions were approximately 175 x 175 x 20 µm. Crystals were transferred briefly to a mixture of 30% glycerol and 70% well solution, and cryo-cooled by plunging into liquid nitrogen. Diffraction data were collected at 100 K on beam line 9-2 at the Stanford Synchrotron Radiation Lightsource. Data integration and scaling were performed with XDS[2] to a resolution limit of 1.6 Å. Initial phase calculations were done in AutoSol[3] using the anomalous signal of the insulin-bound zinc atoms. Subsequent rounds of modeling and refinement were done with COOT[4] and PHENIX[5] to a final *R* factor of 0.163 and R_{free} of 0.196. Molecular graphics and RMSD calculations were performed with UCSF Chimera[6].

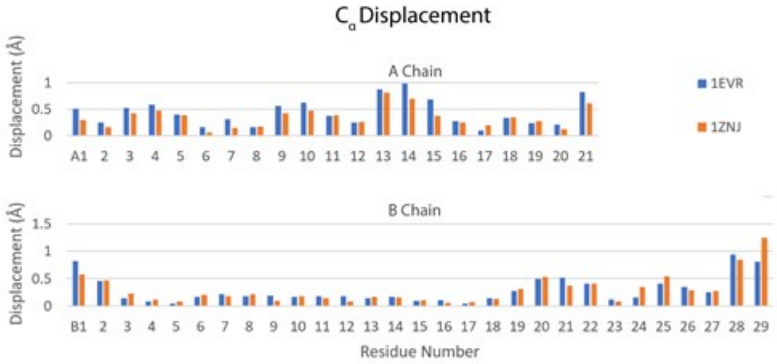


Figure S1. Structural similarity of A22-B22-4SS-Ins to native insulin. Cα displacement between A22-B22-4SS-Ins and two wild-type insulin structures (1EVR, blue; 1ZNJ, orange) following global overlay on the A chains and on the B chains.

Table S1. X-ray data collection and refinement statistics for A22-B22-4SS-Ins.

Wavelength (Å)	0.97946
Resolution range (Å)	35.6 - 1.6 (1.6182 - 1.6)
Space group	$P2_1$
Cell dimensions:	
a, b, c (Å)	46.84 61.55 60.63
α, β, γ (°)	90 111.57 90
No. total reflections	571356 (16608)
No. unique reflections	81415 (2146)
Redundancy	7.02 (6.3)
Completeness (%)	98.50 (76.0)

Mean $I/\sigma I$	20.39 (1.4)
No. atoms:	
Protein	2552
Ligands	49
Solvent	257
Average B-factor (Å^2)	35.1
R_{pim}	0.020 (0.318)
$CC_{1/2}$	1 (0.66)
Reflections used in refinement	81475 (17482)
Reflections used for R-free	8144 (1932)
R_{work}/R_{free}	0.163 / 0.196
R.M.S. deviations:	
Bond lengths (Å)	0.006
Bond angles (°)	0.833
Ramachandran outliers (%)	0.00
Ramachandran favored (%)	98.22
MolProbity score	1.32

Statistics for the highest resolution shell are shown in parenthesis.
Coordinates and diffraction data have been deposited in the Protein Data Bank and are available under accession code 6TYH

6. Isothermal Titration Calorimetry

To assess whether the addition of a disulfide bond near the insulin dimer interface affects the oligomerization of A22-B22-4SS-Ins, dissociation isothermal titration calorimetry (ITC) experiments were performed using human insulin and desoctapeptide insulin (DOI) as controls, essentially as described previously by Antolíková et al[7]. Lyophilized human insulin, DOI and A22-B22-4SS-Ins were reconstituted in 0.1 M glycine/HCl pH 2.5 to give a final volume of ~1 mL at an insulin concentration of 1 mM. The resulting insulin solutions were transferred to dialysis tubing and dialyzed against 300 mL of the glycine/HCl buffer for ~24 hours with three buffer exchanges. The final buffer exchange was performed least two hours before the insulins were recovered from the dialysis tubing. The concentration of the insulin analogs post-dialysis was determined by the absorbance at 280 nm using a NanoDrop 2000c spectrophotometer. ITC experiments were performed on a MicroCal iTC200 instrument, with each run comprising a total of twenty 2-uL injections with a 200-second interval. Human insulin was used as a dimer-forming positive control, and DOI was used as a non-dimerizing negative control. The first ITC experiments conducted on a given day were omitted due to large baseline shifts and high background signal. The ITC traces indicate that titration of A22-B22-4SS-Ins produced a similar heat effect to native insulin (Figure S2), presumably indicating the formation of A22-B22-4SS-Ins dimers. K_d values were calculated from the ITC data with Origin 7.0 (Table S2) using the updated dissociation model obtained from Malvern MicroCal. The apparent K_d for A22-B22-4SS-Ins and native insulin are very similar given the margin of error of these measurements.

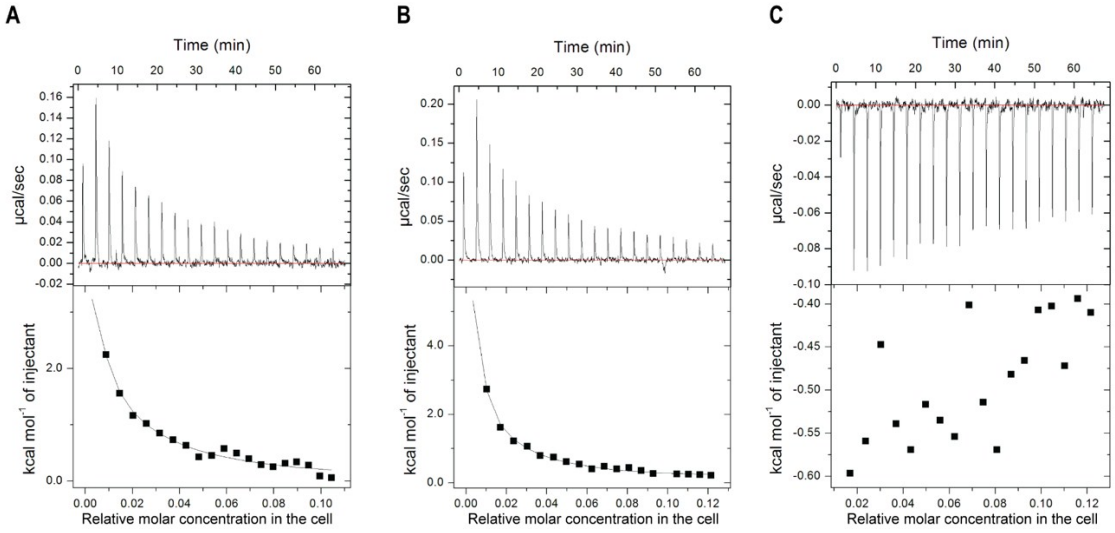


Figure S2. Raw and integrated ITC data. A) A22-B22-4SS-Ins, B) Human insulin, and C) Desoctapeptide insulin (DOI).

Table S2 – K_d values derived from ITC data

Insulin	c [mM]	*Kd* [uM]
Human insulin	0.71	26.3 ± 10.92
Human insulin	0.71	11.68 ± 7.71
Human insulin	0.71	13.13 ± 5.55
Human insulin	0.71	21.73 ± 8.21
A22-B22-4SS-Ins	0.61	34.8 ± 12.7
A22-B22-4SS-Ins	0.61	29.19 ± 13.7
A22-B22-4SS-Ins	0.61	30.92 ± 13.95
DOI	0.71	No heat effect
DOI	0.71	No heat effect

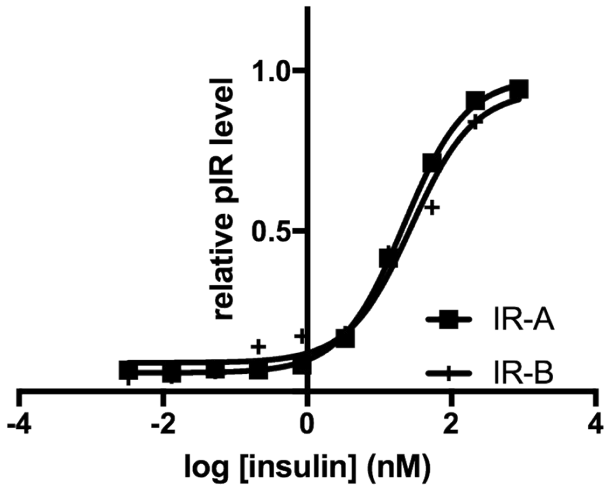


Figure S3. Measurement of A22-B22-4SS-Ins potency in IR-A or IR-B expressing 3T3 cells. The experimental condition is identical to the measurement using pAkt as an indication (Figure 2A). However, phosphorylation of insulin receptor was used in this assay (Cisbio, US). We observed no preference for A22-B22-4SS-Ins to differentiate IR-A vs IR-B.

REFERENCE

1. Webber, M.J., E.A. Appel, B. Vinciguerra, A.B. Cortinas, L.S. Thapa, S. Jhunjhunwala, L. Isaacs, R. Langer, and D.G. Anderson, *Supramolecular PEGylation of biopharmaceuticals.* Proc Natl Acad Sci U S A, 2016. **113**(50): p. 14189-14194.
2. Kabsch, W., *Xds.* Acta Crystallogr D Biol Crystallogr, 2010. **66**(Pt 2): p. 125-32.
3. Terwilliger, T.C., P.D. Adams, R.J. Read, A.J. McCoy, N.W. Moriarty, R.W. Grosse-Kunstleve, P.V. Afonine, P.H. Zwart, and L.W. Hung, *Decision-making in structure solution using Bayesian estimates of map quality: the PHENIX AutoSol wizard.* Acta Crystallogr D Biol Crystallogr, 2009. **65**(Pt 6): p. 582-601.
4. Emsley, P., B. Lohkamp, W.G. Scott, and K. Cowtan, *Features and development of Coot.* Acta Crystallogr D Biol Crystallogr, 2010. **66**(Pt 4): p. 486-501.
5. Adams, P.D., P.V. Afonine, G. Bunkoczi, V.B. Chen, I.W. Davis, N. Echols, J.J. Headd, L.W. Hung, G.J. Kapral, R.W. Grosse-Kunstleve, A.J. McCoy, N.W. Moriarty, R. Oeffner, R.J. Read, D.C. Richardson, J.S. Richardson, T.C. Terwilliger, and P.H. Zwart, *PHENIX: a comprehensive Python-based system for macromolecular structure solution.* Acta Crystallogr D Biol Crystallogr, 2010. **66**(Pt 2): p. 213-21.
6. Pettersen, E.F., T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, and T.E. Ferrin, *UCSF Chimera--a visualization system for exploratory research and analysis.* J Comput Chem, 2004. **25**(13): p. 1605-12.
7. Antolikova, E., L. Zakova, J.P. Turkenburg, C.J. Watson, I. Hanclova, M. Sanda, A. Cooper, T. Kraus, A.M. Brzozowski, and J. Jiracek, *Non-equivalent role of inter- and intramolecular hydrogen bonds in the insulin dimer interface.* J Biol Chem, 2011. **286**(42): p. 36968-77.