Electronic Supplementary Information

Synthesis of Hydrophobic Insulin-based Peptides Using a Helping-Hand Strategy

Maria M. Disotuar, Mark E. Petersen, Jason M. Nogueira, Michael S. Kay and Danny Hung-Chieh Chou

Department of Biochemistry, University of Utah, Salt Lake City, UT 84112

Table of Contents

1. General Experimental Procedures and Materials

Chemicals: Fmoc protected amino acids were obtained from Protein Technologies Inc. Fmoc-*S*-acetamidomethyl-L-cysteine (Fmoc-L-Cys(Acm)-OH), Fmoc-*S*-*tert*-butylthio-L-cysteine (Fmoc-L-Cys(S*t*Bu)-OH), Fmoc-*S*-4methoxytrityl-L-cysteine (Fmoc-L-Cys(Mmt)-OH), Fmoc-L-aspartic acid-α-*tert*-butylester (Fmoc-L-Asp-OtBu), and 2,2'-Dithiodipyridine (DTDP) were obtained from Chem-Impex International Inc. Fmoc-N-amido-dPEG®2-acid was obtained from Quanta BioDesign, Ltd. 1-[Bis(dimethylamino)methylene]-1H-1,2,3 triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) and 2-chlorotrityl chloride resin were purchased from ChemPep ®. 2-Chlorotrityl Chloride Resin was obtained from ChemPep and H-Rink Amide ChemMatrix was provided by Biotage. Dimethylformamide (DMF), triflouroacetic acid (TFA), 1-methyl-2-pyrrolididone (NMP), acetonitrile (ACN) and ethyl ether were purchased from Fisher Scientific. Piperidine, triisopropylsilane (TIS), *N,N*-diisopropylethylamine (DIEA), dichloromethane (DCM), 2,2'-dithiobis (5-nitropyridine) (DTNP), 50% β-mercaptoethanol (BME), L-ascorbic acid, hydrazine, and 5,5-Dimethyl-1,3-cyclohexanedione were obtained from Sigma Aldrich. Native Insulin was obtained from Invitrogen Life Technologies.

2. Liquid Chromatography Mass Spectrometry (LC-MS) analysis

Characterization of peptides was performed by LC/MS on a Xbridge C18 5-µm (50 x 2.1 mm) column at 0.4 mL/min with a water/acetonitrile gradient in 0.1% formic acid on an Agilent 6120 Quadrupole LC/MS system. Some reactions and fractions were run on the same type of Agilent 6120 Quadrupole LC/MS system, however the fragmentor settings varied on the MS – 80 vs 200. Fractions collected from HPLC runs were also analyzed by LC/MS and fractions containing the targeted product were collected and lyophilized using a Labconco Freeze Dryer.

3. High Performance Liquid Chromatography (HPLC)

All samples were analyzed by the following conditions unless otherwise specified: Preparative reverse-phase HPLC of crude peptides was performed on Jupiter 5u C18 300 Å (250 x 10 mm) column at 3 mL/min with a water/acetonitrile (ACN) gradient in 0.1% TFA from 20% aqueous ACN to 95% aqueous ACN over 29 minutes on an Agilent 1260 HPLC system.

4. Synthesis and Characterization of Fmoc-Ddae-OH

To start, 0.5 g of N-Fmoc-amido-dPEG-acid (1.25 mmol), 193 mg dimedone (1.1 eq) and 263 mg EDC.HCl (1.1 eq) was dissolved in 1.4 ml CH_2Cl_2. The solution was cooled to 0ºC and then 15 mg (0.1 eq) of DMAP and 227 µl DIEA (1.1 eq) was added. After the addition, the reaction was allowed to warm up and stirred at RT for 23 hrs. The solution was diluted with 50 mL DCM and washed with 18 mL 1M aqueous HCl (3X), 18 mL 5% $NaHCO_3$ (3X), and 18 mL saturated NaCl (3X). The organic layer was dried over $MgSO_4$, filtered, and then then purified via flash column chromatography using an Isolera One(Biotage) using the following gradient: Hexane/EtOAc 65%-100% and holding at 100% to obtain 425 mg (65% yield) as a viscous oil. Fmoc-Ddae-OH linker was analyzed via NMR and matched to previously published 1H and 13C-NMR spectra by Jacobsen *et al*.[1]

5. General Peptide Synthesis

Chicken A chain: GIVEQCC<u>HN</u>TCSLYQLENYCN

Chicken B chain: AANQHLCGSHLVEALYLVCGERGFFYSPKA

Peptides were synthesized via Fmoc solid phase peptide synthesis on a commercial peptide synthesizer (Alstra; Biotage, Inc). H-Rink Amide ChemMatrix was used for Chicken A-chain and 2-chlorotrityl chloride resin was used for Chicken B-chain. The first residue on the A-chain (Asn) was coupled as Fmoc-Asp-O*t*Bu through its *beta*-carboxyl group. The first amino acid for the B-chain (Ala) was coupled manually to the 2-chlorotrityl resin using 5 equivalents DIEA in DCM and stirred for 1 hour. Upon completion, the resin was washed 3X DCM, 3X DMF, 3X DCM, followed by washing with 17:2:1 DCM: MeOH: DIEA and finally washed 3X DCM, 3X DMF, 3X DCM. After this the automated peptide synthesis was carried in a 10 mL reactor vial with the following protocols for 0.1 mmol scale for both chains. For Fmoc deprotection: (i) 4.5 ml of 20% piperidine in DMF; (ii) mix 2 x 3 min (new solvent delivered for each mix cycle). For amino acid coupling (i) 1.25 ml of 0.2 M Fmoc-protected amino acid in DMF; (ii) 1.225 ml of 0.2 M HATU in DMF; and (iii) mixed for 5 min at 75ºC (Cysteine/Histidine coupling: mix 10 min at 50ºC and Arginine was double coupled for 5 min at 75ºC). For DMF washing completed between the deprotection and coupling steps: (i) 4.5 mL of DMF; (ii) mix 45 seconds. Upon completion of the peptide chain resins were washed with DCM and dried under vacuum for 20 min.

Microcleavages were performed to confirm the mass of peptides **2**, **3**, and **5** – this was to confirm the synthesis of the A-chain, synthesis of A-chain with linker, and synthesis of A-chain with linker and tag respectively prior to disulfide bond formation. Briefly, 20 mg of resin was cleaved using a cocktail consisting of 92.5% TFA, 5% TIS and 2.5% H_2O (1 mL total) for 2 hours under gentle agitation on rotator. The resin was then filtered off and the filtrate was precipitated by cold ethyl ether (4 mL total) for 1 hour. Following precipitation, the peptide was spun down on a centrifuge for 5 minutes at 3000 RCF. The supernatant was removed, and the peptide pellet was subsequently washed with 5 mL of ethyl ether. The peptide was dried under vacuum for 1 hour and checked via LC/MS (all microcleavages were performed under the same conditions).

5.1 Coupling of Fmoc-Ddae-OH linker and try-lysine tag to A-chain

After synthesis of the A-chain (**2**) 25 µM resin (from 0.1 mmol starting resin) was used to couple 0.2 mmol of Fmoc-Ddae-OH to the N-terminal-Amino acid (Gly) in 0.1 M NMP for 4 hours (**3**). Upon completion resin was washed 3X DMF and 3X DCM. The resin coupled with Fmoc-Ddae-OH was reintroduced into the peptide synthesizer. The N-terminal Fmoc on the linker was removed and tri-lysine tag was introduced using the same standard coupling conditions as described above (**5**).

5.2 Deprotection, disulfide bond formation, and cleavage of A-chain with Fmoc-Ddae-OH and tri-lysine tag.

The deprotection and disulfide bond formation was completed in a continuous fashion. The resin-bound peptide was treated with 50% BME in DMF (4 mL total) twice for 2 hours under gentle agitation to remove the S*t*Bu protecting group on Cys^{A6}. Next the peptide was washed 3X DMF (5mL) and 3X DCM (5mL). The peptide was exposed to 10 equivalents DTNP (80 mg based on 25 µM scale) in 4 mL DCM for 1 hour under gentle agitation to activate this cysteine to SNPy [(4-nitro-pyridinyl) thiol]. The peptide was subsequently washed 3X DMF and 3X DCM. Next, the resin was exposed to 4 mL DCM cocktail consisting of 1% TFA and 5% triisopropylsilane (TIS) for 2 minutes per wash for 6 washes with gentle shaking to remove the Mmt protecting group on Cys^{A11}. The peptide was then incubated for 5 hours in 4mL DCM under gentle agitation to form the intramolecular disulfide bridge. Finally the peptide was washed 3X DMF and 3X DCM and left to dry for 30 minutes under vacuum.

The cleavage cocktail consisted of 92.5% TFA, 5% TIS and 2.5% H_2O (4 mL total) and was performed for 2 hours under gentle agitation on rotator. The resin was then filtered off and the filtrate was precipitated by cold ethyl ether (40 mL total) for 1 hour. Following precipitation, the peptide was spun down on a centrifuge for 10 minutes at 5000 RCF. The supernatant was removed and the peptide pellet was subsequently washed 2X with 15 mL of ethyl ether. The peptide was dried under vacuum for 2 hours and crude A-chain with Fmoc-Ddae-OH and tri-lysine tag was obtained to provide 58.5 mg (**8**). This was used without further purification.

5.3 Cleavage and Activation of B-chain

The cleavage cocktail, 95% TFA, 2.5% TIS and 2.5% H_2O (4 mL total) and 2X DTDP (400 mg -based on resin weight after synthesis) was performed for 2 hours under gentle agitation on rotator. The resin was then filtered off and the filtrate was precipitated by cold ethyl ether (40 mL total) for 1 hour. Following precipitation, the peptide was spun down on a centrifuge for 10 minutes at 5000 RCF. The supernatant was removed and the peptide pellet was subsequently washed 2X with 15 mL of ethyl ether. The peptide was dried under vacuum for 2 hours and crude B-chain was obtained to provide 170 mg (**10**), which was used without further purification.

6. Three-step combination protocol for removal of solubility tag and linker after formation of first intermolecular bridge.

Crude A-chain **8** (2.2 mg, .694 µmol) and crude B-chain **10** (2.6 mg, .763 µM) were mixed in 6 M urea containing 0.2 M NH_4HCO_3 (pH 8, 269 µL) buffer solution. This mixture was vortexed vigorously until the peptides were completely dissolved. The reaction was allowed to sit for 30 minutes before being treated with freshly prepared 1M ascorbic acid (40 µL) and the solution was diluted with H_2O (1.89 mL). The solution was then loaded onto the semi-preparative column and purified based on the conditions described above. The desired fraction eluted at 21 minutes and the fractions were pooled and lyophilized to obtain 1.5 mg the A-B dimer (**11**) with a yield of 33.1% based on A-chain **8**.

The Fmoc-Ddae-OH linker and solubility tag were subsequently removed using a hydrazine-based buffer (pH 7.5) similar to that previously described without the use of dithiothreitol (DTT).[1] Freshly prepared 5 mL stock of 2M hydrazine solution consisted of 2.85 g GnHCl, 0.5 mL of 1 M NaH_2PO_4, 1 ml of 10 M hydrazine in H_2O, 0.250 mL of 12 M HCl adjusted to pH 7.5. The peptide was first dissolved in phosphate buffer (46

μL) consisting of 6 M GnHCl and 100 mM phosphate buffer pH 7.5, and an equal volume (46 μL) of 2 M hydrazine pH 7.5 was added. The linker and solubility tag were removed within 30 minutes. This was subsequently diluted using 92 μL of 50% acetonitrile in H_2O and 1 mL 5% acetonitrile in H_2O and purified on a semi-preparative column as listed above. The desired fraction eluted at 21 minutes, and the pooled fractions were collected to obtain 1.2 mg of A-B dimer **12** with 89.1% yield. To form the second intermolecular bridge (Cys^{A7} and Cys^{B7}), the A-B dimer (1.2 mg, 0.205 μmol) without linker and tag was first dissolved in 29 μL AcOH and 114 μL H_2O and treated with freshly prepared I_2 (25 eq) in 465 μL AcOH for 15 minutes under gentle agitation. The oxidation was quenched with 93 μL of 1 M ascorbic acid and diluted in 2.44 mL of H_2O. This was purified using a semi-preparative column under purification conditions as described above and fractions were collected, pooled, and lyophilized to obtain .5 mg of final product **14** with 42.7% yield after HPLC purification. Starting from A-chain, we obtained an overall yield of 12.6%.

7. Three-step combination protocol for removal of solubility tag and linker after formation of second intermolecular bridge

To form the second intermolecular bridge, 2.6 mg (.399 μmol) of purified A-B dimer (**11**) was first dissolved in 52 μL AcOH and 208 μL H_2O and treated with freshly prepared I_2 (25 eq) in 906 μL AcOH for 20 minutes under gentle agitation. The oxidation was quenched with 181 μL of 1 M ascorbic acid and diluted in 2.72 mL of H_2O. This was purified using a semi-preparative column under purification conditions as described above. The desired fraction eluted at 23 minutes and fractions were collected, pooled, and lyophilized to obtain 1 mg of **13** with 39.3% yield after HPLC purification. The Fmoc-Ddae-OH linker and solubility tag were subsequently removed using a hydrazine-based buffer (pH 7.5) as described above. Freshly prepared 5 mL stock of 2M hydrazine solution consisted of 2.85 g GnHCl, 0.5 mL of 1 M NaH_2PO_4, 1 mL of 10M hydrazine in H_2O, 0.250 mL of 12 M HCl adjusted to pH 7.5. The peptide was first dissolved in phosphate buffer (76 μL) consisting of 6M GnHCl and 100 mM phosphate buffer pH 7.5, and an equivolume (76 μL) of 2 M hydrazine pH 7.5 was added. The linker and solubility tag were removed within 30 minutes. This was subsequently diluted using 152 μL of 50% acetonitrile in H_2O and .848 mL 5% acetonitrile in H_2O and purified on a semi-preparative column as listed above. The desired fraction eluted at 21 minutes and fractions were collected to obtain 0.8 mg of peptide **14** with 89.2% yield**.**

8. Cell-based Assay to Study Insulin Signaling Activation

To determine the extent of insulin signaling induced by chicken insulin compared to native insulin, 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 (Thermofisher 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 chicken 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.

9. Cell-based Assay to Induce Insulin Receptor Phosphorylation

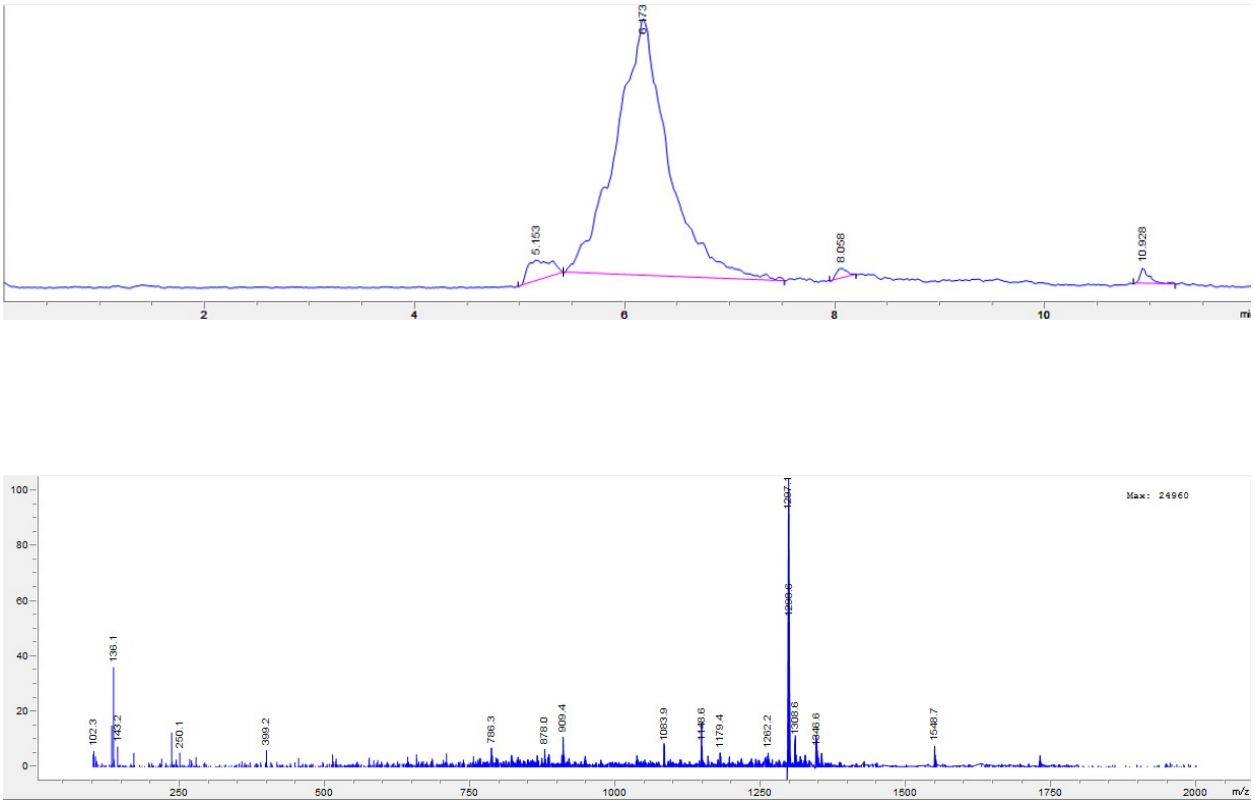
To detect endogenous levels of insulin receptor activation induced by chicken insulin compared to native insulin, phosphorylation on the receptor at Tyr 1150/1151 was measured. Phosphorylation was detected in a mouse fibroblast cell line, NIH 3T3, overexpressing human insulin receptor isoform B (IR-B). The cell line was cultured in DMEM (Sigma Aldrich) with 10% fetal bovine serum (Gibco), 100 U/mL penicillin-streptomycin (Thermofisher Scientific), 2 mg/mL puromycin (Thermofisher Scientific) and 1 mg/ml normocin (InvivoGen). For the assay, 40,000 cells per well and 100 µl per well, were plated in a 96-well plate with culture media containing 1% FBS. 20 hours later, 50 µl of chicken insulin or native insulin was pipetted into each well after the removal of the original media. After a 15-min treatment, the insulin solution was removed and the HTRF Phospho-IR beta (Tyr1150/1151) (Cisbio, Massachusetts, USA) was used to measure the activation of the insulin receptor only when phosphorylated at Tyr 1150/1151. 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 2-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.

10. Mass spectrometry data

Peptide	M/Z Expected (Da)	Observed M.W. (Da)	Method
2	2592.9	1297.1 [M/2]	ESI
3	3097.2	1549.2 [M/2]	ESI
5	3259.8	1631.3 [M/2 +H] 1087.4 [M/3+ H]	ESI
8	3169.7	1584.8 [M/2] 1057.5 [M/3+H]	ESI
10	3461.9	1731.3 [M/2] 1154.9 [M/3+H] 865.5 [M/4]	ESI
11	6520.4	1630.9 [M/4] 1305.1 [M/5+H] 1087.8 [M/6+H]	ESI
12	5854.6	1464.5 [M/4+H] 1171.8 [M/5 + H] 976.6 [M/6 +H]	ESI
13	6376.3	1275.2 [M/5] 1063.8 [M/6 +H] 912.0 [M/7 +H] 797.2 [M/8]	ESI
14	5710.4	1903.9 [M/3] 1428.6 [M/4 + H] 1143 [M/5] 952.8 [M/6 +H] 817.0 [M/7 +H]	ESI

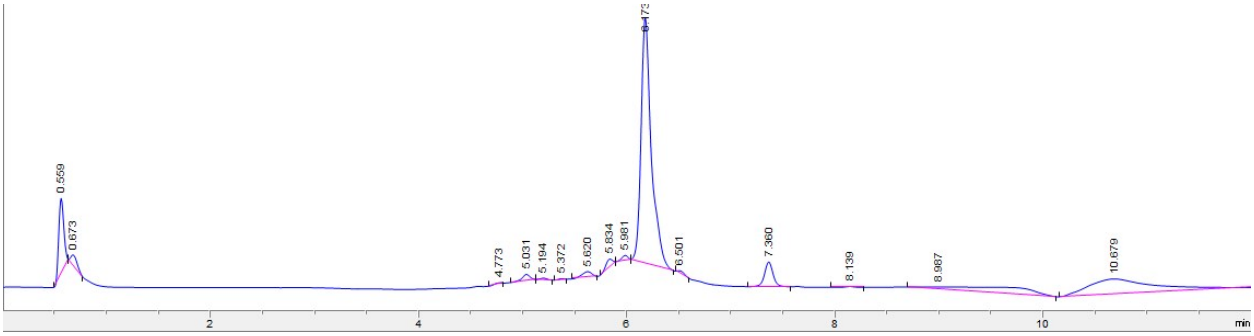
11. Supplementary Figure 1

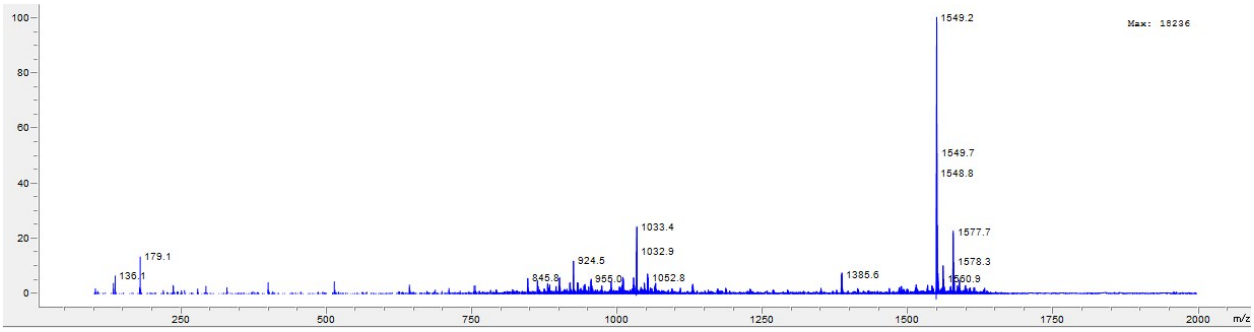
LC-Chromatogram (top) and MS-Spectrum (bottom) for crude peptide **2**.
Chicken insulin A-chain with Cys^{A6}- S*t*Bu and Cys^{A7}- Acm.



12. Supplementary Figure 2

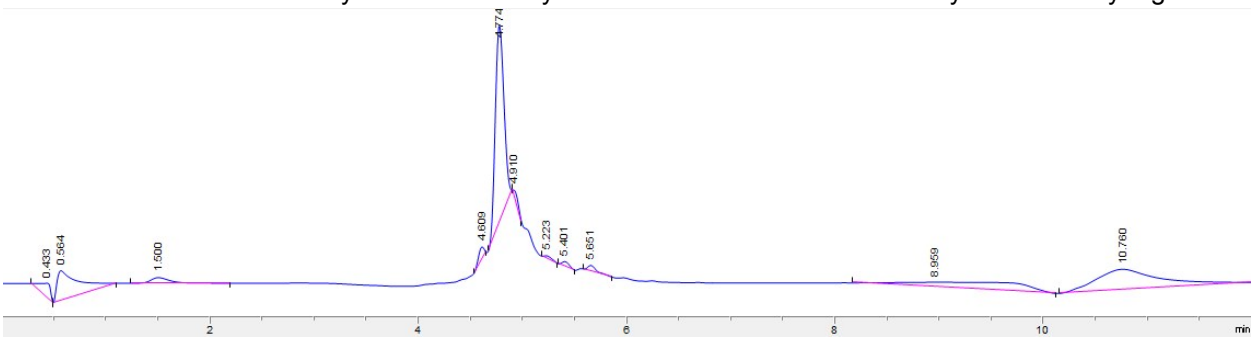
LC-Chromatogram (top) and MS-Spectrum (bottom) for crude peptide **3**.
Chicken Insulin A-chain w/ Cys^{A6}- S*t*Bu and Cys^{A7}- Acm and Fmoc-Ddae-Oh linker.

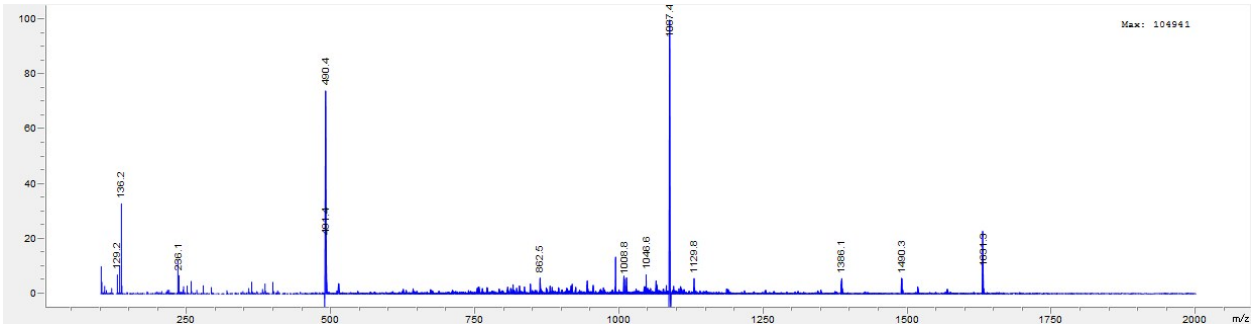




13. Supplementary Figure 3

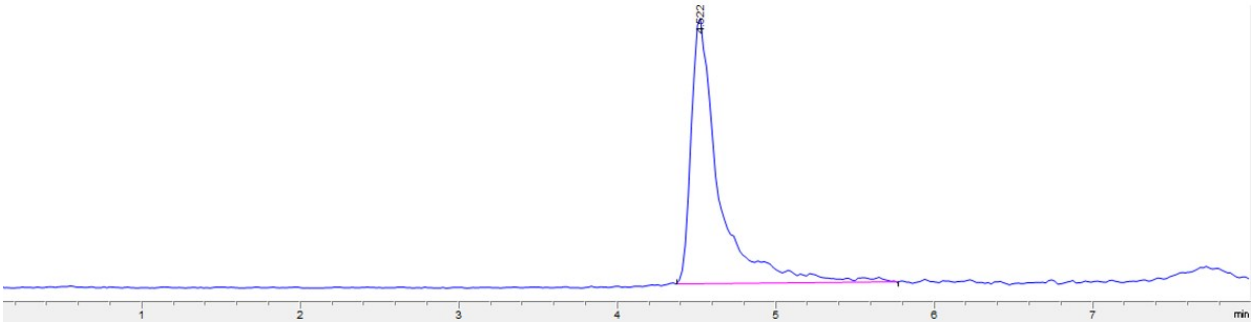
LC-Chromatogram (top) and MS-Spectrum (bottom) for crude peptide **5**.
Chicken insulin A-chain w/ Cys^{A6}- S*t*Bu and Cys^{A7}- Acm and Ddae linker and tri-lysine solubility tag.

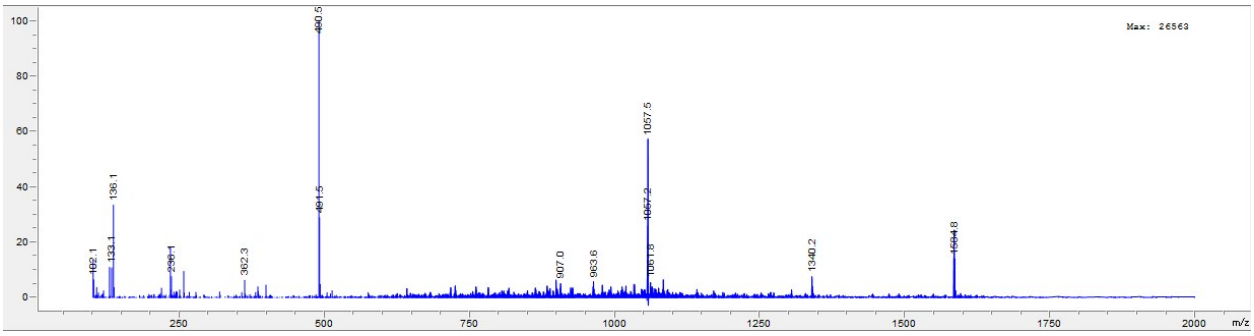




14. Supplementary Figure 4

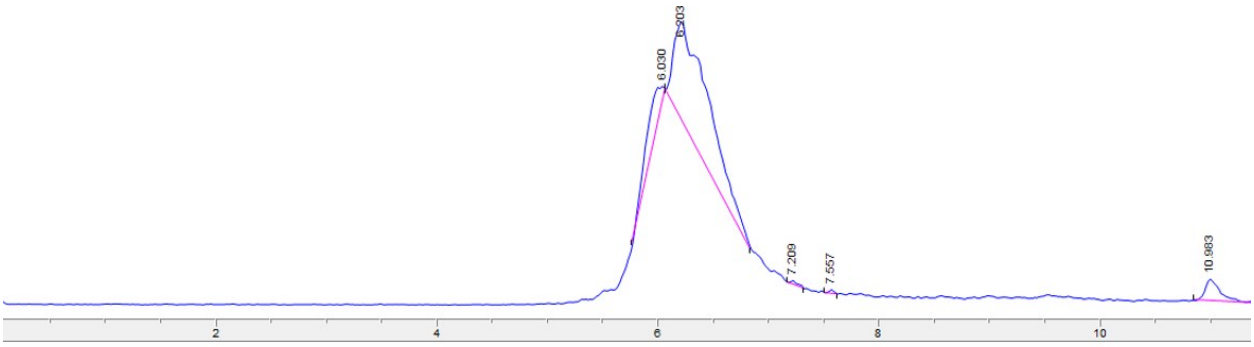
LC-Chromatogram (top) and MS-Spectrum (bottom) for crude peptide **8**.
Chicken insulin A-chain with Ddae linker and tri-lysine solubility tag after formation of intramolecular bridge between Cys^{A6}-Cys^{A11}.

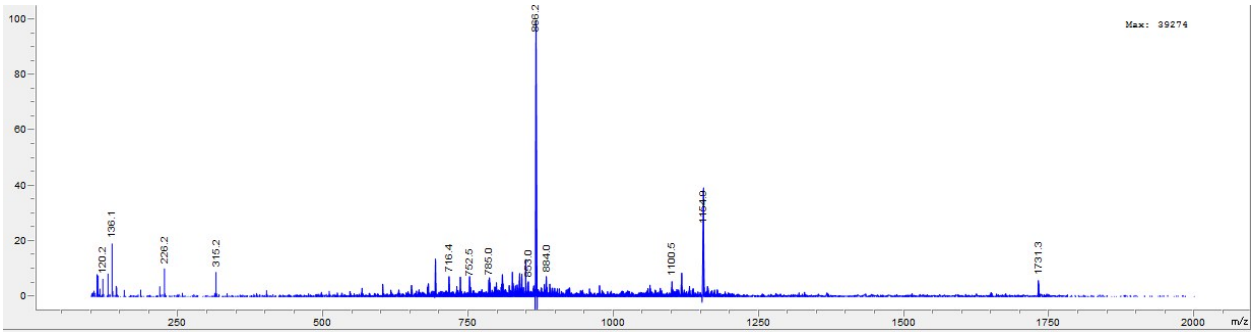




15. Supplementary Figure 5

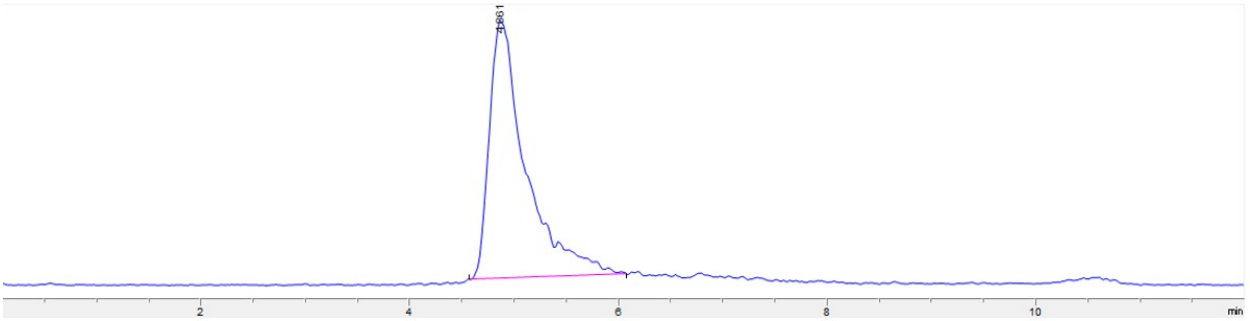
LC-Chromatogram (top) and MS-Spectrum (bottom) for crude peptide **10.** Chicken insulin B-chain.

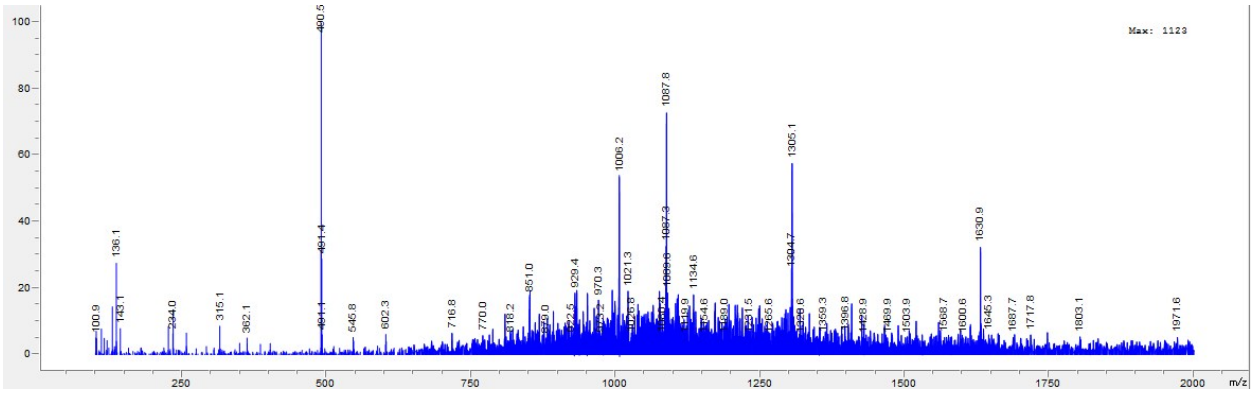




16. Supplementary Figure 6

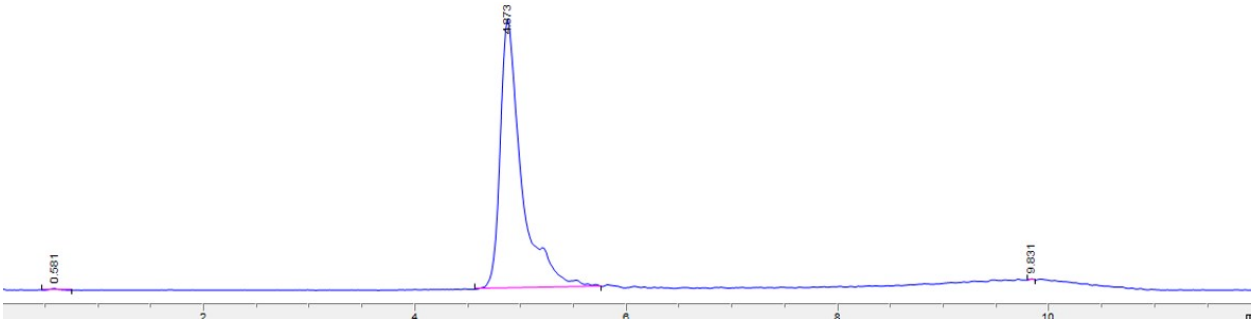
LC-Chromatogram (top) and MS-Spectrum (bottom) for purified peptide **11.**
Chicken insulin A-chain (with Ddae linker, tri-lysine solubility tag, and intramolecular bridge) and B-chain with first intermolecular bridge between Cys^{A20}-Cys^{B19}.

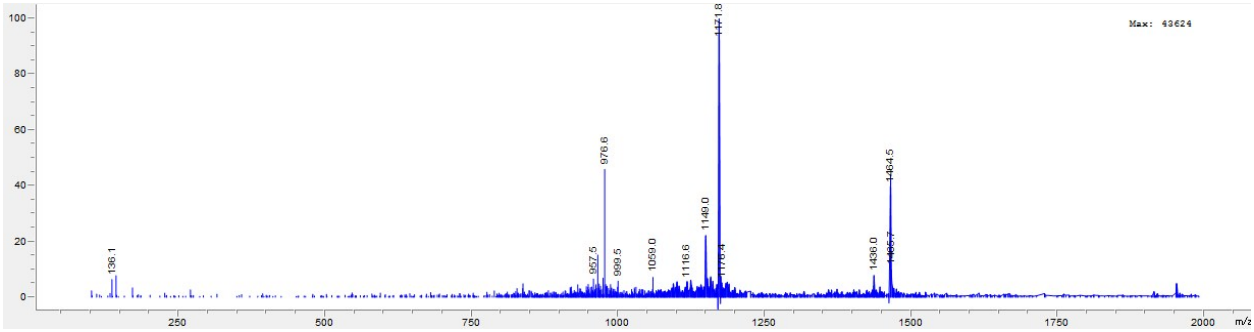




17. Supplementary Figure 7

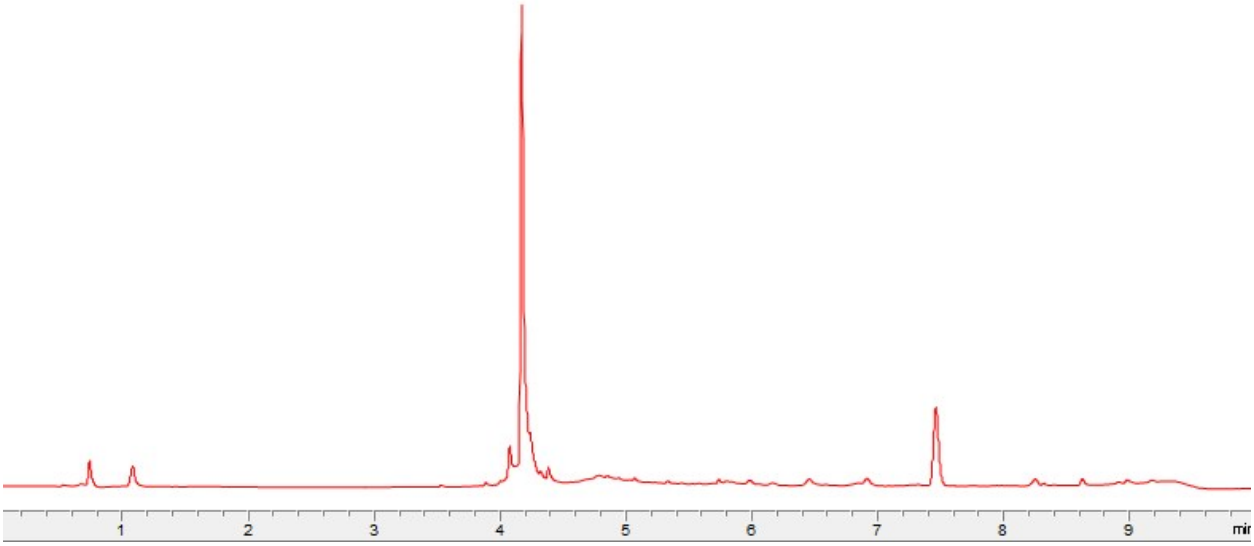
LC-Chromatogram (top) and MS-Spectrum (bottom) for purified peptide **12**.
Chicken insulin heterodimer with first intermolecular bridge after removal of Ddae linker and solubility tag.

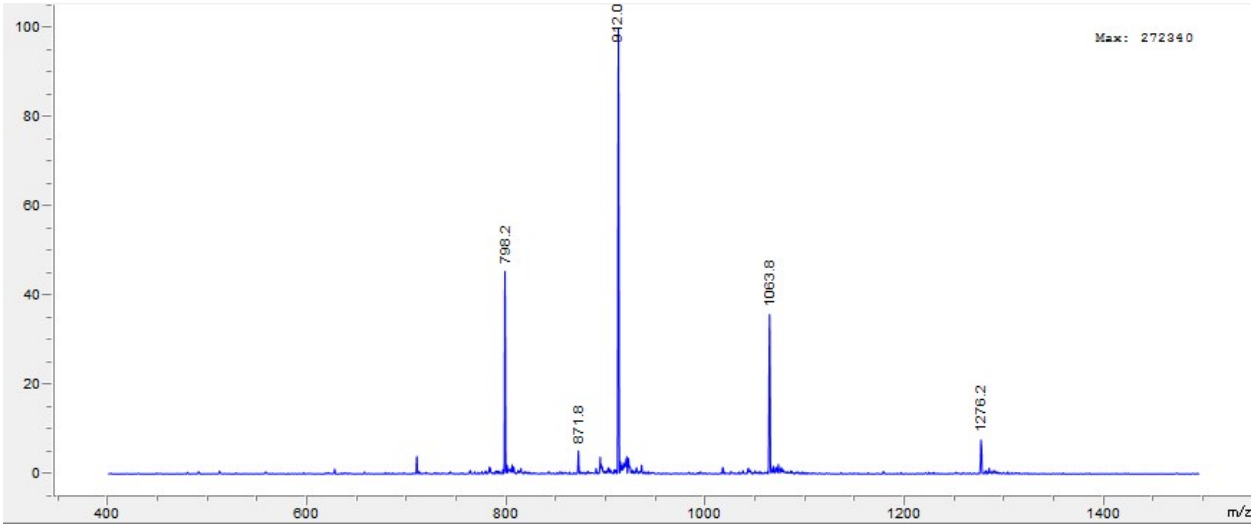




18. Supplementary Figure 8

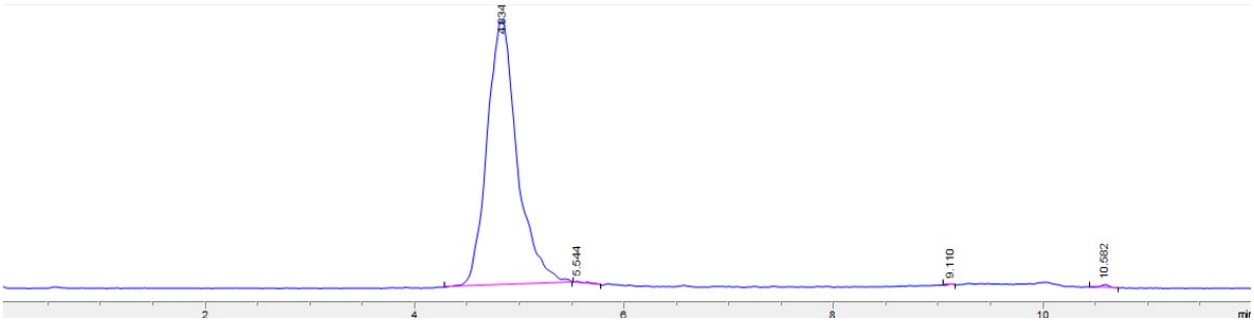
LC-Chromatogram (top) and MS-Spectrum (bottom) for purified peptide **13**.
Chicken insulin heterodimer after formation of the second intermolecular bridge between Cys^{A7}-$Cys^{B7}$with linker and solubility tag.

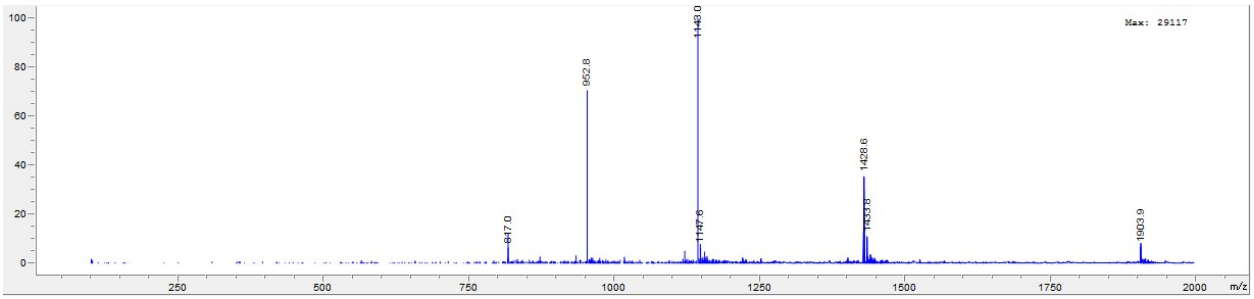




19. Supplementary Figure 9

LC-Chromatogram (top) and MS-Spectrum (bottom) for purified peptide **14**.
Formation of second intermolecular bridge (Cys^{A7} and Cys^{B7}) after removal of Ddae linker and solubility tag.





1. M. T. Jacobsen, M. E. Petersen, X. Ye, M. Galibert, G. H. Lorimer, V. Aucagne and M. S. Kay, *J Am Chem Soc*, 2016, **138**, 11775-11782.