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Synthesis and Characterization of an A6-A11 Methylene Thioacetal Human Insulin Analog with Enhanced Stability

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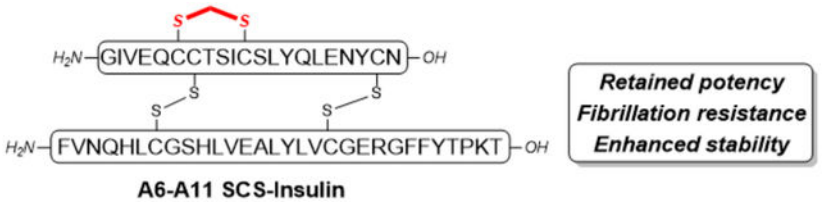
Abstract

Insulin has been a life-saving drug for millions of people with diabetes. However, several challenges exist which limit therapeutic benefits and reduce patient convenience. One key challenge is the fibrillation propensity, which necessitates refrigeration for storage. To address this limitation, we chemically synthesized and evaluated a methylene thioacetal human insulin analog (SCS-Ins). The synthesized SCS-Ins showed enhanced serum stability and aggregation resistance while retaining bioactivity compared with native insulin.

Graphical Abstract



INTRODUCTION

Insulin is a 51-amino acid peptide connected by two separate chains and three disulfide bonds (one intra-chain, A6-A11 and two inter-chain, A7-B7 & A20-B19).[1] Ever since its discovery in 1921, insulin has been the mainstay for the treatment of type 1 diabetes and late-stage type 2 diabetes.[2] Rapid- and long-acting insulin analogs have been developed over the past decades to achieve better blood glucose control.[3] However, these analogs still suffer

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

HPLC trace, LCMS characterization of intermediates and Glu-C digestion experiment. (PDF)

The authors declare no competing financial interest.

from the undesired properties of native insulin such as propensity of degradation, fibrillation, and disulfide scrambling, which makes them difficult for storage and transport, particularly in circumstances where refrigeration is challenging.[4] Therefore, the development of novel insulin analogs with enhanced stability and without the need of cold-chain delivery is highly desired.[5]

Due to this need, a number of strategies were reported recently to increase insulin stability. First, an additional disulfide bond between A10-B4 was introduced to native insulin to generate a four-disulfide analog, which demonstrates reduced aggregation propensity while preserving insulin bioactivity (Figure 1A).[6] Next, a single-chain insulin analog with a 6-residue linker connecting the C-terminal B chain and N-terminal A chain also led to reduced insulin fibrillation compared to native insulin (Figure 1B).[7] Furthermore, using *O*-mannosylation at B27 threonine, the Tan group reported an insulin analog with reduced proteolysis and self-association (Figure 1C).[8] The Tirrell group recently reported that mutation of B28 proline to a non-natural (4*S*)-hydroxyproline (Hzp) led to a stable analog with reduced aggregation through an additional hydrogen bond between Hzp and a backbone amide carbonyl group.[9]

Recently, disulfide mimetics have been introduced to insulin with a goal to prevent disulfide reduction, scrambling and further side reactions, and thus increase stability. However, the introduced disulfide mimetics may influence insulin bioactivity depending on the extent of the structural perturbation.[10] Among the reported studies, insulin analogs with A6-A11 thioether,[11] A6-A11 *cis*-dicarba[12] or A6-A11[13] & A7-B7[14] diselenide bond replacement preserved insulin bioactivity. On the other hand, replacements involving A7-B7 triazole[15] or A6-A11 *trans*-dicarba[12] resulted in reduced potency (Figure 1D). While these analogs have some desired properties, non-natural amino acids are required, which makes practical applications challenging. We are especially interested in bridging the disulfide bond with carbon linkers to increase peptide stability.[16] In this particular case, inserting a minimal linker into the disulfide bond is key to maintain the structural integrity and preserve the bioactivity of insulin. The Cramer group recently reported an elegant biocompatible solution phase synthesis of methylene thioacetal bond as a disulfide mimic and applied this method to the synthesis of SCS-oxytocin which shows enhanced stability and retained bioactivity.[17] In this brief article we present our chemical synthesis and characterization of an A6-A11 methylene thioacetal bridge replaced human insulin analog (Figure 1).

RESULTS AND DISCUSSION

The chemical synthesis of insulin and insulin analogs is still a daunting task even at the current stage largely due to the low yield in competing disulfide pairing and the notorious hydrophobicity of the A chain.[18] Several methods have been developed for improving the A chain solubility to facilitate synthesis.[19] We carried out our initial attempt by using an isoacyl peptide approach developed by Liu et al. (Scheme 1).[20] The partially protected A chain **S1** [isoacyl A8-A9, A7-*t*Bu, A20-Acm] was synthesized from 2-chlorotrityl chloride (2-CTC) resin. The free thiols in Cysteine A6 and A11 were intended to be converted into a methylene thioacetal. Following Cramer's protocol, the peptide was dissolved and treated with Tris(2-carbox-yethyl)phosphine hydrochloride (TCEP.HCl) and potassium carbonate

followed by trimethylamine (Et_3N) and diiodomethane. A white precipitate **S2** was generated; however, it is poorly soluble and chromatographically intractable (see supporting information for details). This result indicated that a simultaneous *O* to *N* acyl shift occurred during the reaction under basic condition, which removes the amine handle in the case of the isoacyl peptide, leading to reduced solubility.

To circumvent the labile nature of the isoacyl peptide under basic reaction conditions, we previously reported the use of Fmoc-Ddae-OH connecting N-terminal amine with a solubility tag through a hydrazine sensitive enamine bond.[21] While Fmoc-Ddae-OH as a "helping hand" successfully solved the problem, its viscous oil nature presents a technical challenge during chemical synthesis. Our collaborator, Kay group further developed a 2nd generation helping hand, Fmoc-Ddap-OH (*hh*), as white powder with the replacement of a PEG linker to a hexyl linker.[22] Our synthetic route using this helping hand approach towards the SCS-Ins is described in Scheme 2. First, the partially protected A chain [A7-Acm, A20-Mob] **A1** was synthesized on 2-CTC resin through Fmoc SPPS. The N-terminal Gly- at A1 position was treated with Fmoc-Ddap-OH (*hh*) in methylpyrrolidone (NMP) to form an enamine linked complex **A2**. Additional SPPS to install hexa-lysine sequence followed by cleavage generates highly soluble **A3**, a key intermediate to circumvent the hydrophobicity of insulin A chain. We next reacted **A3** with the Cramer's methylene thioacetal formation condition. As expected, **A3** showed greatly improved solubility in water and successfully yielded product **A4**, which also exhibited drastically increased solubility compared with **S2** (see supporting information for detail). We did not detect any hydrolysis product during the reaction, which further highlights the strength of this helping hand method. **A4** was then treated with a cocktail of TFA/TIPS/H_2O at 45 °C to remove the A20-Mob and yielded the corresponding A20-thiol product **A5** in 60% yield for two steps from purified **A3**.[23] Next, protected B chain [B7-Acm, B19-Trt] **B1** was generated through Fmoc-SPPS and activated with DTDP during acidic cleavage to give the [B7- Acm, B19-SPy] chain **B2**.[20]

With both SCS-A and B chains in hand, we then investigated the formation of the two inter-chain disulfide bonds. To this end, **A5** and **B2** were dissolved and stirred in a 6 M urea containing 0.2 M NH_4HCO_3 (pH 8.0) buffer solution. The reaction reached complete conversion of **A5** in 20 min and yielded a heterodimer **C1** in 40% yield based on **A5** after HPLC purification. Conversion of **C1** to **C2** (*hh*-tagged SCS-Ins) was smoothly achieved by treatment with the excess amount of iodine in AcOH and the A7-B7 disulfide bond was formed through an *in situ* Acm deprotection-disulfide formation manner. Again, we did not detect any de-tagged products during the two-step combination. To complete the synthesis, **C2** was treated with hydrazine to remove the helping hand tag. The product SCS-Ins **C3** was obtained in 82% yield after purification (Scheme 2). The observed mass of **C3** corresponded to the calculated mass with three crosslinks. The correct folding of two inter-chain disulfide bonds was further confirmed by proteinase Glu-C digestion and the fragments were characterized by LC-MS (Supporting information).[24]

The A6-A11 intra-chain disulfide bond plays a critical role in forming and stabilizing the receptor binding A chain N-terminal α-helix as a conformational constraint, which is important to facilitate the formation of two inter-chain disulfide bonds as well as the stability of the whole molecule.[25] This rationale is supported by previous folding studies of porcine

proinsulin and a recent study of reduced insulin A and B chain self-assembly experiment.[26] Therefore, to evaluate whether this methylene thioacetal replacement influences insulin properties, we performed a series of experiments to compare native insulin with SCS-Ins side-by-side. First, to determine if thioacetal linkage has an effect on the secondary structure, circular dichroism (CD) spectra were obtained (Figure 2A). SCS-Ins has the α-helical secondary structure consistent with native insulin, indicating that the secondary structure is similar between the two molecules. We further used a phospho-AKT (Ser 473) insulin signaling stimulation assay to evaluate insulin bioactivity (Figure 2B). SCS-Ins has 2-fold reduced bioactivity (EC_{50} 4.01 nM) than native insulin (EC_{50} 1.91 nM) suggesting that the thioacetal linkage does not greatly influence insulin activity as SCS-Ins is still comparably potent. This *in vitro* result is further strengthened by an insulin tolerance test (ITT) in mice. Briefly, equal amount of native insulin and SCS-Ins (0.75 U/Kg) was subcutaneously injected in C57BL/6 mice and blood glucose levels were measured. As shown in Figure 2C, the two insulin molecules have similar glucose lowering effect, suggesting a comparable *in vivo* activity.

We next performed various stability assays for both insulin molecules. We first evaluated their propensity for fibrillation using a stressed aging assay consisting of continuous agitation in pH 7.4 buffer at 37 °C.[27] As shown in Figure 2D, native insulin behaves similarly to previous reports as it aggregated in under 10 hours of shaking while SCS-Ins did not aggregate even after 100 hours of shaking. This result demonstrates that SCS-Ins has a superior effect over native insulin in preventing aggregation, a key factor for insulin formulation. It was reported that unfolding of the N-terminal helix of the A chain exposes a hydrophobic surface, which may lead to accelerated insulin fibrillation.[28] Fixing the A6-A11 disulfide bond using the unreducible methylene thioacetal in our case may stabilize the N-terminal helix of the A chain, leading to reduced insulin fibrillation. We argue that this may be a valuable strategy for future efforts to achieve stable insulin analogs. Next, both native insulin and SCS-Ins were exposed to human serum at 37 °C. SCS-Ins has enhanced serum stability compared to native insulin (Figure 2E). The SCS-Ins was digested to 70% whereas native insulin was digested to 40% after being incubated for 24 hours. Similarly, SCS-Ins also showed increased thermal stability compared with native insulin when both were incubated in a PBS (pH 7.4) at 60 °C for 4 days (Figure 2F). Taken together, the SCS-Ins preserves the biological activity of native insulin and demonstrates superior aggregation, serum and thermal stability over native insulin.

CONCLUSIONS

In conclusion, we have completed the chemical synthesis of the A6-A11 methylene thioacetal human insulin analog (SCS- Ins). A successful application of the second generation “helping hand” linking a hexa-lysine moiety at the N-terminal A chain enabled a solubility increase and the tolerance of harsh basic condition used to form the target methylene thioacetal. Along with our previous work on using this helping hand strategy to synthesize chicken insulin, we believe that this approach is a valuable and generally applicable tool for the chemical synthesis of peptides or proteins with high hydrophobicity. Furthermore, SCS-Ins not only retained bioactivity both *in vitro* and *in vivo* but also exhibited enhanced stability, indicating that methylene thioacetal could serve as an ideal

disulfide surrogate for insulin modification. On the basis of current research, modifications of other clinically used insulin analogs and attempts to synthesize the inter-chain methylene thioacetal replaced insulin analogs are currently underway.

EXPERIMENTAL SECTION

Chemicals.

All commercially available chemicals were purchased and used directly without further purification.

Animals.

All animal experiments were performed in accordance with NIH guide for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee (IACUC) of University of Utah.

Automated solid phase peptide synthesis.

Peptides were synthesized via Fmoc solid phase peptide synthesis on a commercial peptide synthesizer (Syzo I). The first amino acid was coupled manually to the 2-CTC resin (for 0.1 mmol peptide; 250 mg resin; 0.77 mmol/g) using 0.12 mmol of Fmoc-L-Asn(trt)-OH or Fmoc-L-Thr(tBu)-OH and 0.2 mmol of DIEA in 3.5 mL DCM and stirred at 25 °C for 1.5 h. The resin then was washed DMF and then DCM, followed by capping with a cocktail of DCM: MeOH: DIEA = 17:2:1 (3 min; 5 rounds) and finally washed DMF to get amino acid bonded resin (final loading = 0.4 mmol/g). Then the automated peptide synthesis was carried out in a 10 mL reactor vial with the following protocols for 0.1 mmol scale of both chains. Fmoc deprotection: 20% piperidine in DMF; Amino acid coupling: Fmoc protected amino acid (5.0 eq.), HATU (5.0 eq.), and DIEA (10.0 eq.) were used for the coupling steps.

Coupling of Fmoc-Ddap-OH linker.

For 0.1 mmol scale A chain bonded resin, 1.5 eq. of Fmoc-Ddap-OH dissolved in NMP (3 mL) was added and the total coupling required > 4 h agitation on rotator.[22]

Cleavage and Activation.

For Mob containing A chain resins, the cleavage was conducted using a cocktail consistent of 85% TFA, 5% TIS, 5% H_2O and 5% thioanisole for 2 h at room temperature under gentle agitation on rotator. For B chain resins, the cleavage were conducted using a cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O containing 5.0 eq. of DTDP for 2.5 h at room temperature. The resin was then filtered off and the filtrate was precipitated by cold ethyl ether (40 mL) and cooled at −20 °C for 25 min. Following precipitation, the peptide was spun down on a centrifuge for 10 minutes at 3000 rcf. The supernatant was removed and the peptide pellet was subsequently washed 2x with 30 mL of cold ethyl ether. The peptide was dried under vacuum for 1 h before HPLC purification.

LC-MS analysis.

Characterization of peptides was performed by LC/MS on a Phenomenex Luna® 5 μm Phenyl-Hexyl (50 × 2 mm) 100 Å column at 0.4 mL/min with a water/acetonitrile gradient in 0.1% formic acid on an Agilent 6120 Quadrupole LC/MS system.

HPLC purification.

Preparative RP-HPLC of crude peptides was performed on Jupiter 5 μm C18 300 Å (250 × 10 mm) column at 3 mL/min with a water/acetonitrile gradient in 0.1% TFA from 5% aqueous ACN to 65% aqueous ACN over 45 minutes on an Agilent 1260 HPLC system.

Purity check.

Purity of SCS-Ins and native insulin samples for biological testing were determined to be >95% by analytical RP-HPLC performed on C18 column: Phenomenex, 150 mm × 4.6 mm, 100 Å, 5 μm at a flow rate 0.4 mL/min.

Synthesis of S2.

To a stirred solution of **S1** (3.5 mg) in H_2O (220 μL) and ACN (80 μL) was added a premixed solution of TCEP.HCl (780 μg, 2.0 eq.) and K_2CO_3 (760 μg, 4.0 eq.) in H_2O (150 μL) in a dropwise manner. After stirring at room temperature for 1.5 h, Et_3N (1.9 μL, 10.0 eq.) in THF (36 μL) was added to the mixture and followed with CH_2I_2 (1.0 μL, 6.0 eq.) in THF (36 μL). The mixture was then allowed to react shielded from light till a complete conversion of the starting material monitored by LCMS. The reaction was then quenched with 10% AcOH to a final pH of 6.5 then purified by HPLC. ESI M/Z (Da): Calc 1262.5 $[M+2]^{+2}$; Found 1262.1 $[M+2]^{+2}$.

Synthesis of A4.

The synthesis was conducted using standard condition in **S2** synthesis with the replacement of **A3** as substrate. ESI M/Z (Da): Calc 1202.6 $[M+3]^{+3}$, 1803.5 $[M+2]^{+2}$; Found 1202.7 $[M+3]^{+3}$, 1803.7 $[M+2]^{+2}$.

Synthesis of A5.

Purified **A4** was dissolved and stirred at 45 °C in a cocktail (4 mL) consistent of 95% TFA, 2.5% TIS and 2.5% H_2O. The reaction was monitored by LCMS till a complete consumption of A4 was reached (about 4 h). Then cold ethyl ether (40 mL) was added and the participated crude **A5** was centrifuged and washed with cold ether (15 mL × 3). The collected crude **A5** was dried in vacuum and purified by HPLC. Two-step yield was 60% based on **A3**. ESI M/Z (Da): Calc 1162.7 $[M+3]^{+3}$, 1743.6 $[M+2]^{+2}$; Found 1162.4 $[M+3]^{+3}$, 1742.3 $[M+2]^{+2}$.

Synthesis of C1.

Purified **A5** (3.0 mg, 1.0 eq.) and **B2** (3.7 mg, 1.2 eq.) was dissolved in a 0.2 M NH_4HCO_3 buffer containing 6 M urea (700 μL, pH 8.0) and vigorously stirred for 20 min at 25 °C. The reaction was detected by LCMS. When **A5** was complete consumed, the reaction was diluted with 0.1% TFA ddH_2O to 2 mL and the mixture was purified by HPLC. 40% yield

based on **A5**. ESI M/Z (Da): Calc 1165.1 $[M+6]^{+6}$, 1397.9 $[M+5]^{+5}$, 1747.1 $[M+4]^{+4}$; Found 1165.0 $[M+6]^{+6}$, 1397.6 $[M+5]^{+5}$, 1746.7 $[M+4]^{+4}$.

Synthesis of C2.

To a stirred solution of **C1** (7.0 mg, 1 eq.) in H_2O containing 20% AcOH (900 μL) was added dropwise with freshly prepared I_2 solution in AcOH (25 eq, 100 μL, 0.1 M) and the mixture was allowed to stir at 25 °C for 5 min. The oxidation was quenched with addition of 1 M ascorbic acid till decay of dark red color to colorless. Then the mixture was diluted with H_2O and purified by HPLC. ESI M/Z (Da): Calc 978.4 $[M+7]^{+7}$, 1141.3 $[M+6]^{+6}$, 1369.4 $[M+5]^{+5}$, 1711.5 $[M+4]^{+4}$; Found 978.2 $[M+7]^{+7}$, 1141.0 $[M+6]^{+6}$, 1368.8 $[M+5]^{+5}$, 1711.9 $[M+4]^{+4}$.

Synthesis of C3 (SCS-Ins).

Purified **C2** (4.0 mg) was dissolved and stirred in 500 μL of a 100 mM PBS (500 μL) consisting of 6 M Gn.HCl (pH 7.5) followed by addition of 100 mM PBS containing 2 M hydrazine pH 7.5 (500 μL). The linker and solubility tag were removed (about 30 min) and monitored by LCMS. Then the mixture was subsequently diluted with H_2O and purified by HPLC. 82% yield; 6.9 % overall yield based on **A3**. Purity >95%. ESI M/Z (Da): Calc 971.3 $[M+6]^{+6}$, 1165.3 $[M+5]^{+5}$, 1456.4 $[M+4]^{+4}$, 1941.6 $[M+3]^{+3}$; Found 971.1 $[M+6]^{+6}$, 1165.2 $[M+5]^{+5}$, 1456.2 $[M+4]^{+4}$, 1941.8 $[M+3]^{+3}$. MALDI-MS (Da): Calc 5821.6; Found 5821.4.

Circular dichroism spectroscopy.

Circular dichroism spectroscopy was performed on an Applied Photophysics Chirascan Plus instrument. 0.2 mg/mL PBS (pH 7.4) solutions of both SCS-Ins and native insulin were prepared, and experiments were performed at 25 °C across a wavelength range from 200–250 nm. CD spectra were obtained with a step of 0.5 nm, 0.5 s per point, and the average of five scans with an averaged against five scan with PBS baseline subtracted.

Phospho-AKT (Ser 473) cell-based assay.

We followed a standard phosphorylation assay previously described.[21b] The 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 containing 10% fetal bovine serum, 100 U/mL penicillin-streptomycin and 2.0 mg/mL puromycin. 100 μl (about 40,000 cells) per well were plated in a 96-well plates for each assay with culture media containing 1% FBS. 20 hours later, 50 μl of SCS-Ins or native insulin series dilution (0.003 to 860 nM) were pipetted into each well after the removal of the original media following 30-min incubation. Then the insulin solution was removed and the intracellular level of pAkt Ser473 was measured by HTRF pAkt Ser473 kit (Cisbio, Massachusetts, USA). 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 4h incubation, plate was read in a Synergy Neo plate reader and the data processed according to the manufacturer's protocol. Each point is consisted of 4 replicates from two independent experiments.

In vitro human serum stability assay.

Each insulin sample was dissolved in a PBS (1,0 mg/mL, pH 7.4) and 100 μL of the dissolved solution was added into 900 μL of human serum from human male AB plasma (Sigma-Aldrich, sterile filtered, defrosted only once and pre-centrifuged 15 min at 13,000 rpm to remove lipid) at a final insulin concentration of 0.1 mg/mL. Tha mixture was then incubated at 37 °C water bath and individual 100 μL of the solution was taken up at certain, pre-determined time points (0, 1, 2, 4, 8, 24 h) and treated with 300 μL ACN containing 10% H_2O and cooled on ice for 20 mins. The suspension was centrifuged at 13,000 rpm for 10 min at room temperature. Then 20 μL of supernatant was taken up and dissolved in 15 μL of 0.1% TFA containing H_2O to make the HPLC sample. The sample was analyzed by RP-HPLC (injection volume = 30 μL; column: Phenomenex, 150 mm × 4.6 mm, 100 Å, μm) with a linear gradient of 5–50% B over 9 min (A: H_2O + 0.1% TFA, B: ACN + 0.1% TFA; 0.4 mL/min flow rate). Peptide peak areas were integrated and the % of peptide left, compared to the initial was graphed against the time. The serum stability experiments were repeated 3 times for both SCS-Ins native insulin.

In vitro thermal stability assay.

Each insulin sample dissolved in PBS (0.1 mg/mL, pH 7.4). 200 μL of the dissolved solution was incubated at 60 °C (water bath) and individual 30 μL of the solution was taken up at day points (0, 1, 2, 3, 4 day) was analyzed by HPLC (column: Phenomenex, 150 mm × mm, 100 Å, 5 μm) with a linear gradient of 5–50% B over 8 min (0.4 mL/min flow rate). Peptide peak areas were integrated and the % of peptide left, compared to the initial was graphed against the time. The thermal stability experiments were repeated 3 times for both SCS-Ins and native insulin.

Insulin fibrillation assay.

We followed a standard method as previously described.[27] Insulin samples were dissolved in pH 7.4 PBS to a final concentration of 1.0 mg/mL. Samples were plated at 150 μL per well (n=4/group) in a clear 96-well plate and sealed with optically clear and thermally stable seal. 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 was performed on normal chow fed, 12–15 weeks' old C57BL/6 mice with both genders. 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 withdrawn for the entire experimental duration. The body weights and basal blood glucose concentrations (using Glu cometer Contour next EZ, Ascensia Diabetes Care US, Inc.) were measured and divided into 2 groups (n=4/group). Following the basal measurements, the mice received ip injections 0.75 U/Kg body weight of either human insulin (Novolin ReliOn) or SCS-Ins dissolved in saline. The blood glucose concentrations were measured at 10, 20, 30 and 60 min after injection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

Acm	acetamidomethyl
ACN	acetonitrile
2-CTC	2-chlorotrityl chloride
DCM	dichloromethane
DIEA	diisopropylethylamine
DTDP	2,2'-Dithiodipyridine
FA	formic acid
HATU	*O*-Benzotriazole-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate
Ins	insulin
ip	intraperitoneal
Mob	4-methoxybenzyl
NMP	methylpyrrolidone
RP	reversed phase
SD	standard deviation
SPPS	solid phase peptide synthesis
TCEP.HCl	Tris(2-carboxy-ethyl)phosphine hydrochloride
TFA	trifluoroacetic acid
TIPS	triisopropylsilane

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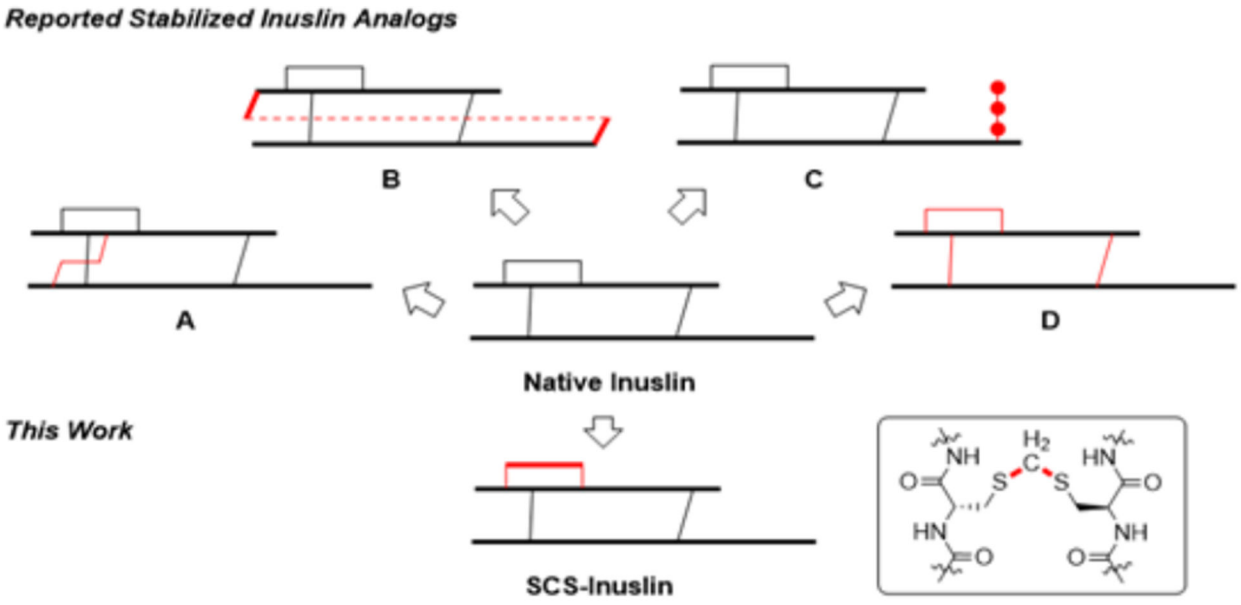


Figure 1.

Top: Previously reported strategies to stabilize insulin. Bottom: The present work to introduce a methylene thioacetal to replace A6-A11 disulfide bond.

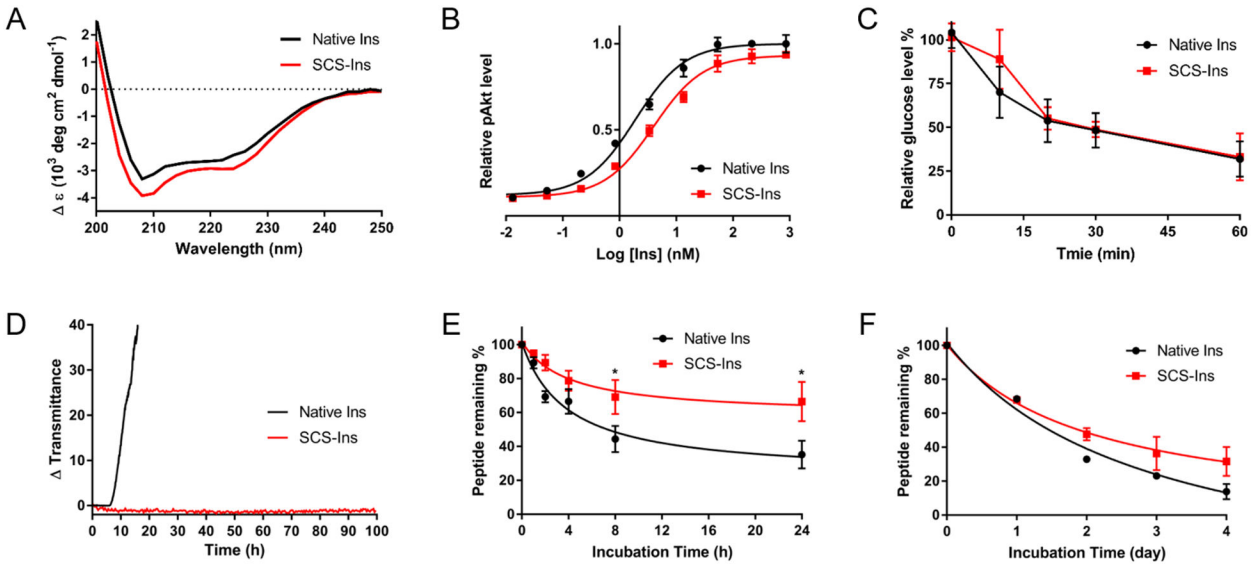
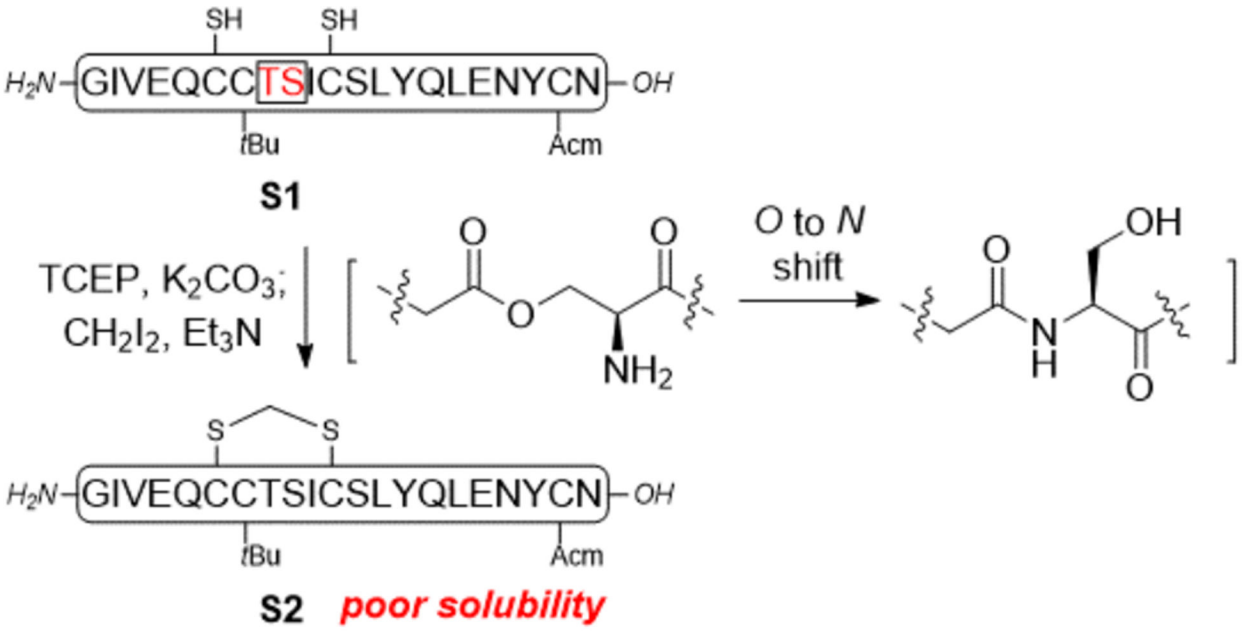


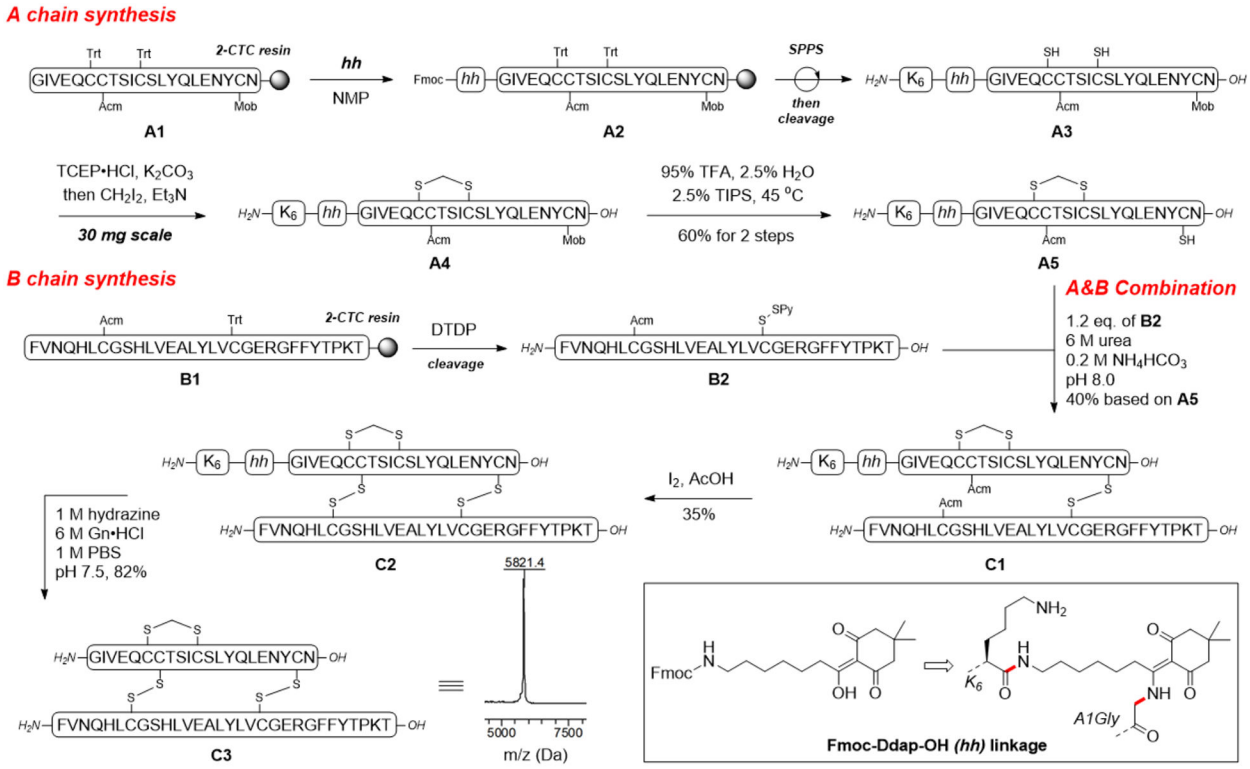
Fig. 2.

Characteristics of SCS-Ins in comparison with native Insulin. A) Circular dichroism spectra measured at 25 °C with a concentration of 0.2 mg/mL in pH 7.4 PBS. B) Human insulin signaling activation (Akt phosphorylation) assay. Data represent the average of 4 independent measurements and error bar represents SD. calc EC_{50} (95% CI): [Native Insulin = 1.91 (1.36–2.69) nM; SCS-Ins = 4.01 (2.91–5.53) nM]. C) Insulin tolerance test in mice. Data represent average of 4 male and female mice and error bar represents SD. D) Kinetic profiling of fibrillation, monitoring change in transmittance (i.e., increase in turbidity) at 1.0 mg/mL under continuous agitation at 37 °C in pH 7.4 PBS. E) Serum stability comparison at 37 °C at 0.1 mg/mL insulin concentration in human serum. Data represent 3 replicates at each time point and error bar represents SD. F) Thermal stability assay at 60 °C with 0.1 mg/mL insulin in pH 7.4 PBS. Data represent 3 replicates at each data point and error bar represents SD. 95% CI = 95% confidence intervals; SD = standard deviation; *p<0.05, Student t (unpaired) test.



Scheme 1.

Initial attempt to synthesize the SCS-Insulin A chain using an isoacyl peptide strategy



Scheme 2.

Chemical synthesis of SCS-Insulin enabled by N-terminal helping hand strategy