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Biomimetic Synthesis of Insulin Enabled by Oxime Ligation and Traceless "C-Peptide" Chemical Excision

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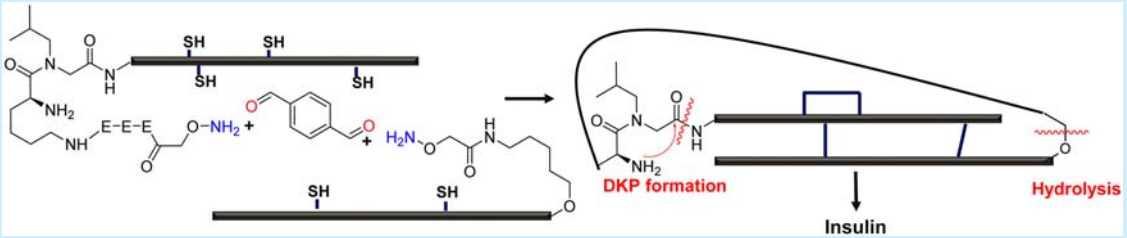
Supporting Information



ABSTRACT: For decades, insulin has represented a preeminent synthetic target. Recently introduced "biomimetic" strategies based on convertible single-chain precursors require incorporation of a chemical linker or a unique proteolytic site, which limits their practicality. In this approach the A- and B-chains are linked by two sequential oxime ligations followed by disulfide bond formation under redox conditions and linker excision by diketopiperazine (DKP) formation and ester hydrolysis, yielding native two-chain insulin. The method is expected to be applicable to any member of the insulin superfamily.

Human insulin is a heterodimeric peptide linked by one intrachain and two interchain disulfide bonds (Figure 1).[1] Since its discovery, insulin and structurally related

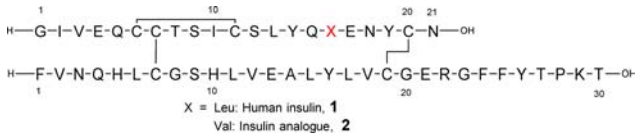


Figure 1. Sequences of human insulin (**1**) and insulin ValA16 analogue.

analogues have served as the most reliable medicines in the management of diabetes.[2] Whereas commercial insulins are currently produced by rDNA technology, chemical synthesis enables non-native diversity that may address insulin's remaining therapeutic limitations, such as glucose-responsive pharmacology.[3]

In the course of a half century, insulin chemical synthesis has advanced as each technical obstacle was successfully surmounted.[3b] The main factors that have compromised synthesis include the poor biophysical properties of the A-chain and the complexity of insulin's characteristic disulfide bond pattern. Chemical linkage of the A- and B-chains often offers high efficiency in disulfide bond formation, resembling the folding process of native proinsulin, which contains a connecting peptide.[3b] The prospect of an efficiently assembled and folded single-chain precursor that can be readily converted to the two-chain active form without enzyme catalysis is highly attractive.[1,3,4] In this regard, the recent reports of a single-chain insulin precursor tethered through an ester bond represent a landmark achievement. Kent and co-workers developed an elegant, purely chemical route to insulin lispro by linkage of the two chains through a side-chain ester bond between GluA4 and ThrB30, which was saponified after folding.[5] The sole limitation of this approach is the requirement of a glutamic acid and a threonine in sufficiently close proximity to form the interchain linkage, which precludes its application in other native insulin-like peptides.[5]

In this report, we present a convergent method for the assembly of a single-chain precursor that is sequentially ligated from independently synthesized A- and B-chains and then folded under redox conditions. Enzyme-free, traceless conversion is achieved through the formation of an internal diketopiperazine (DKP) followed by ester hydrolysis to excise the connecting peptide and liberate insulin.

In order to address the hydrophobicity of the A-chain, we utilized the previously reported isoacyl Thr-Ser dipeptide method of Liu at A8-A9.[6] An Fmoc/*t*Bu-based A-chain synthesis was initiated by acylation of ChemMatrix Rink amide resin with Fmoc-Asp-O*t*Bu to introduce the C-terminal Asn, and the remaining residues, including the isoacyl dipeptide Thr-Ser, were coupled by a standard automated solid-phase peptide synthesis (SPPS) protocol. The resin-bound A-chain **3** was bromoacetylated and subsequently aminated by treatment

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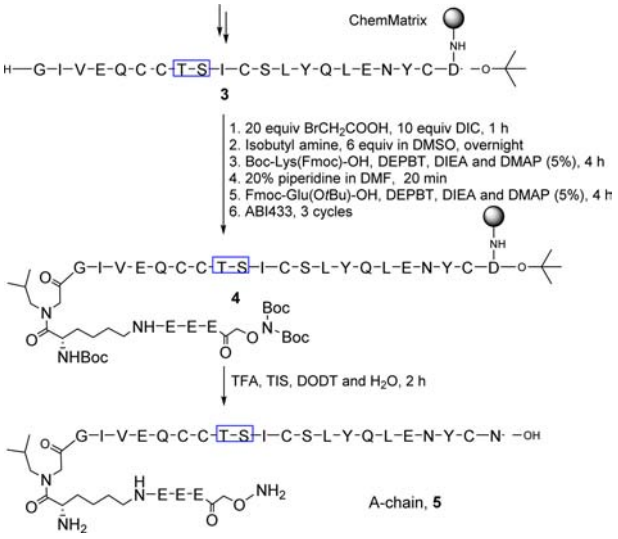
with isobutylamine in dimethyl sulfoxide (DMSO) overnight (Scheme 1). Boc-Lys(Fmoc)-OH was coupled in the presence of (3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one) (DEPBT) to generate the dipeptide adduct, which would subsequently function as the DKP trigger releasing the oxime linker from the A-chain. The three repeating glutamic acids and bis(Boc)aminoxyacetic acid were sequentially coupled to generate the resin-bound A-chain **4**. The glutamic acid tripeptide was incorporated in order to enhance the alkaline solubility of the single-chain insulin precursor in the course of disulfide folding.[7] Treatment of the protected peptidyl resin with a standard cocktail of trifluoracetic acid (TFA), triisopropylsilane (TIS), 2,2′-(ethylenedioxy)diethanethiol (DODT), and H_2O followed by reversed-phase high-performance liquid chromatography (RP-HPLC) purification provided A-chain **5** in 20% yield (Figures 2, S1, and S9).

Initial steps in the B-chain synthesis entailed coupling of Fmoc-Lys(Mtt)-OH to polystyrene Rink amide resin to generate peptidyl resin **6** followed by removal of the 4-methyltrityl (Mtt) group by treatment with 30% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in DCM (Scheme 2). Bis(Boc)-aminoxyacetic acid treatment followed by Fmoc removal and installation of 6-hydroxyhexanoic acid by DEPBT coupling produced the hydroxyl-terminal resin **7**. This was esterified with Fmoc-Thr(O*t*Bu)-OH under Mitsunobu conditions, yielding resin **8**. The remaining residues of the insulin B-chain, including the isoacyl dipeptide Tyr-Thr at B26-B27, were assembled by a standard automated SPPS protocol. Cleavage was conducted in a cocktail of TFA, TIS, DODT, and H_2O (Figure S2). The resulting aminooxy-containing B-chain was treated with terephthalaldehyde (10 equiv) in 0.1% TFA containing 70% aqueous acetonitrile (ACN) to generate the crude peptidyl aldehyde B-chain **10** by oxime ligation.[8] This reaction mixture was lyophilized, washed with diethyl ether to remove excess terephthalaldehyde, and purified by RP-HPLC to provide the B-chain aldehyde **10** in 15% yield (Figures 2, S3, and S10).

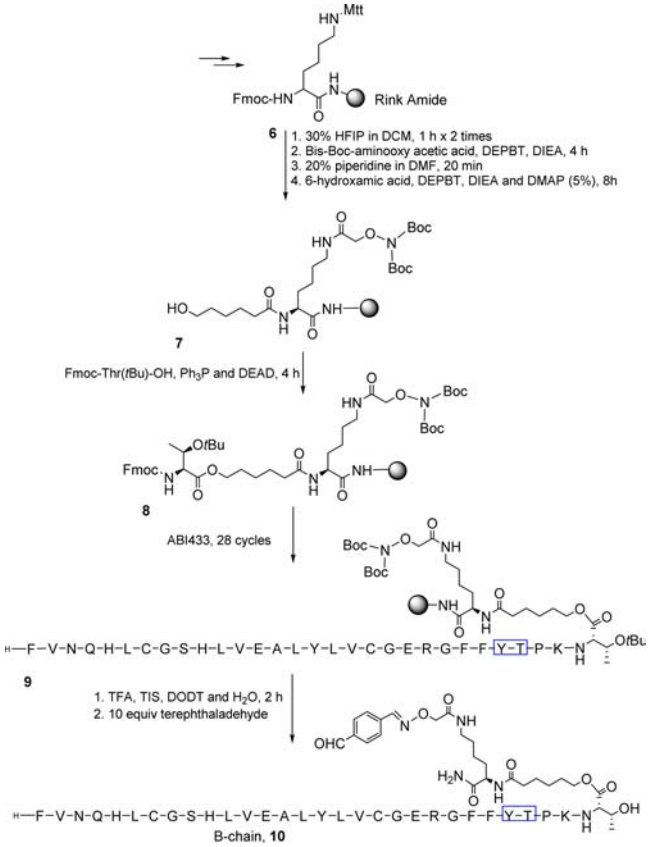
The ligation of insulin A-chain **5** and B-chain **10** was performed in 70% aqueous ACN containing 0.1% TFA and was complete within 2 h in nearly quantitative yield as determined by LC–MS (Scheme 3), with the solvent subsequently removed by freeze-drying. The resulting linear folding precursor **11** containing the glutamic acid tripeptide was freely soluble in alkaline folding buffer (20 mM Tris, 20 mM glycine, 2 mM cysteine, and 0.5 mM cystine, pH 8.0) (Scheme 3) and underwent complete disulfide bond formation after 5 min at 4 °C (Figure 2). Purification by RP-HPLC provided the single-chain insulin **12** in a combined yield of 45% for the ligation and folding steps (Figures 2, S4, S5, and S11)

Scheme 1. Synthesis of the Insulin A-Chain[a]



[a]The isoacyl segment is highlighted with a box.

Scheme 2. Synthesis of the Insulin B-Chain[a]



[a]The isoacyl segment is highlighted with a box.

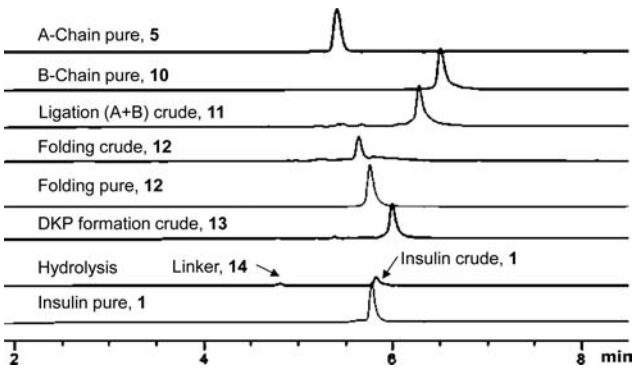


Figure 2. HPLC chromatograms characteristic of insulin synthesis (absorbance at λ = 220 nm). HPLC conditions: C8 column (Kinetex, 2.6 μm, 75 mm × 4.6 mm) with a linear gradient from 10% aqueous ACN (0.05% TFA) to 80% aqueous ACN (0.05% TFA) over 10 min at a flow rate of 1.0 mL/min.

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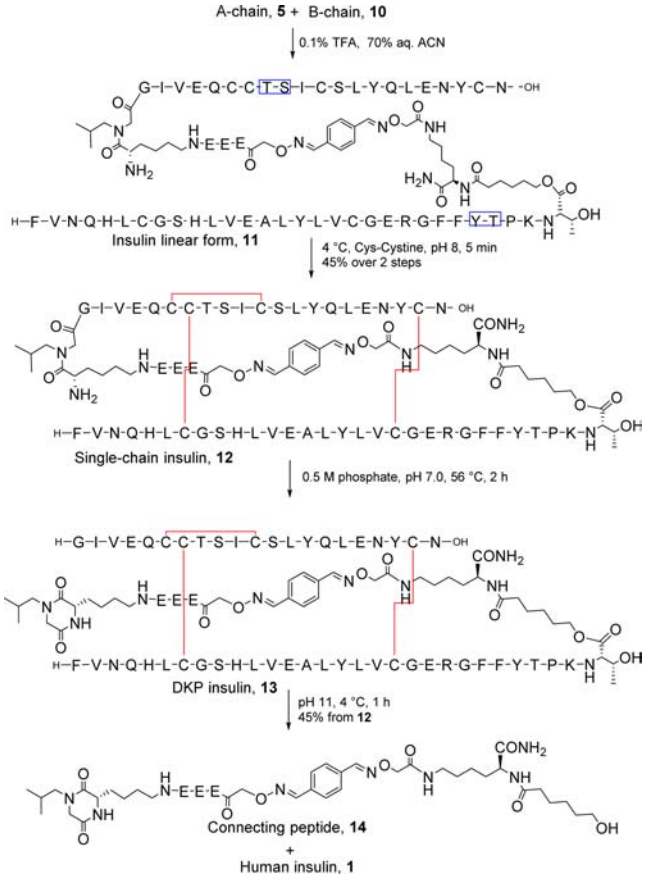
Scheme 3. Synthesis of Human Insulin[a]



[a]The isoacyl segment is highlighted with a box.

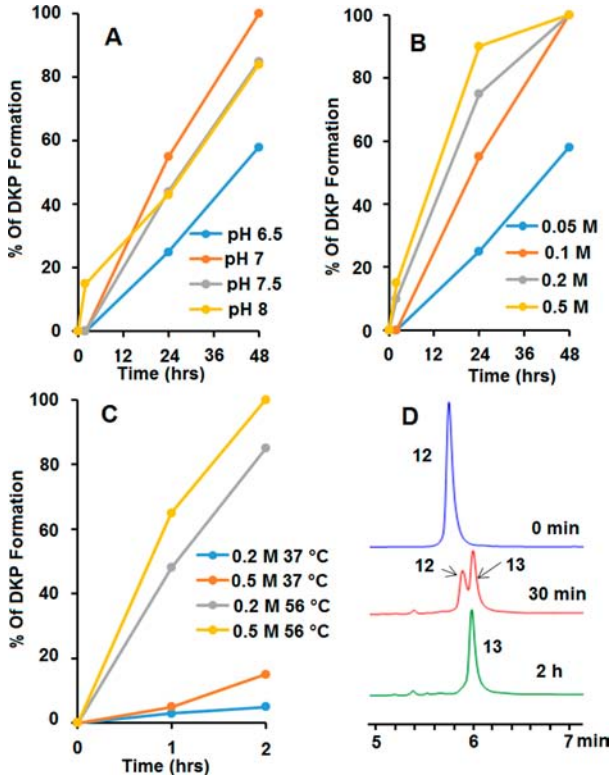


Figure 3. Kinetics of DKP formation of single-chain insulin **12**. (A) pH dependence at 37 °C. (B) Salt concentration dependence at 37 °C, pH 7. (C) Temperature dependence at pH 7. (D) HPLC traces for the conversion of single-chain insulin **12** to intermediate **13** via DKP formation.

We next explored the two-step chemical conversion of **12** to native human insulin **1** through excision of the connecting peptide via DKP formation and ester saponification (Scheme 3). Optimal conditions for the DKP formation were assessed by variation of the pH (6.0−8.0), salt concentration (0.1 to 0.5 M) and temperature (22, 37, and 56 °C) in a sodium phosphate buffer. It was determined that pH 7.0 and elevated salt concentration correlated with faster conversion. The reaction was found to be most sensitive to temperature, and the optimal conditions were set as 0.5 M phosphate buffer (pH 7.0) at 56 °C. Under these conditions, the DKP conversion was complete in 2 h (Figures 3 and S6). To the best of our knowledge, this represents the first application of a DKP-susceptible dipeptide tether in macromolecular synthesis. Following adjustment of the pH to 11, intermediate **13** was smoothly saponified after 1 h at 4 °C to afford human insulin **1** and the liberated connecting peptide **14** (Figures 2, S7, and S12). The yield of human insulin **1** after purification was 45%, which includes the DKP formation and saponification steps.

The overall yield of human insulin as presented in Figure 3 was 20% based on purified A-chain (or B-chain) or 3% when calculated from the initial resin substitution used in the yield-limiting B-chain synthesis. The native disulfide bond pattern was confirmed by Glu-C mapping in comparison to an authentic human insulin standard (Figures S8, S16, and S17). Assessment of in vitro biological activity was performed in an engineered cell assay where insulin receptor B tyrosine phosphorylation was measured. The synthetic human insulin activity was indistinguishable from that of the native hormone standard (Figure S18).

To confirm the scope and versatility of this route, we selected human insulin LeuA16Val, a historically problematic target for conventional biosynthesis because of poor folding efficiency and low thermodynamic stability.[9] Application of the method established above produced the ValA16 insulin analogue with folding efficiency and overall yield comparable to those of native human insulin **1**. This example provides additional validation for this approach as one that is general for insulin analogues, even those that have been historically challenging or of low biological activity (Figures S13−S15).

In conclusion, we have established a highly efficient, broadly applicable chemical route for the preparation of insulin and related analogues. This strategy employs isoacyl dipeptide surrogates to enhance the yield and biophysical handling of individual A- and B-chains as synthetic intermediates. The disulfide formation is intramolecular and enabled by a connecting peptide formed through two selective oxime ligations to produce an A−B single-chain precursor, which subsequently folds in near-quantitative yield. The final step is a novel two-stage transformation that employs DKP formation and ester saponification to release the connecting peptide, yielding the desired product. The present methodology eliminates the need for orthogonal protection of cysteine and subsequent deprotection with reagents such as iodine that compromise the use of oxidation-sensitive chemical functionality. Additionally, this method requires no enzymes and as such imposes no restriction to amino acids such as lysine or arginine that are required sites for Lys-C or trypsin-like

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proteolysis. Finally, the synthetic strategy is fully applicable to other heterodimeric peptides, as there is no requirement for any specific sequence to enable conversion to the heterodimer. With these improved features, we envision this method to be applicable to the broader insulin superfamily, where disulfide formation requires intramolecular assistance.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03876.

Solid-phase synthesis of the individual chains; ligation of the A- and B-chains; folding, DKP formation, and cleavage of the connecting peptide; LC−MS spectra of the key peptides and insulin receptor B tyrosine phosphorylation assay (PDF)

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Notes

The authors declare no competing financial interest.

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