Protein Synthesis

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A Synthetic Route to Human Insulin Using Isoacyl Peptides**

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Abstract: *The chemical synthesis of insulin has been a long-standing challenge, mainly because of the notorious hydrophobicity of the A chain and the complicated topology of this 51-mer peptide hormone consisting of two chains and three disulfide bonds. Reported herein is a new synthetic route utilizing the isoacyl peptide approach to address the hydrophobicity problems. The incorporation of isoacyl dipeptide segments into both A and B chains greatly improved their preparation and purification, and the RP-HPLC recovery of the chain ligation intermediates. The new route affords human insulin with a yield of 68% based on the starting purified A chain and an overall yield of 24% based on the substitution of the resin used for the preparation of A chain. To the best of our knowledge, this represents the most efficient route of human insulin chemical synthesis reported to date.*

Human insulin (**1**) consists of one A chain of 21 residues and one B chain of 30 residues, with its tertiary structure locked in place by two interchain disulfide bonds (CysA7-CysB7, CysA20-CysB19) and one intra-A-chain disulfide bond (CysA6-CysA11; Figure 1). Since its identification in

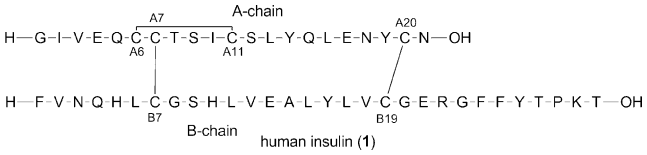


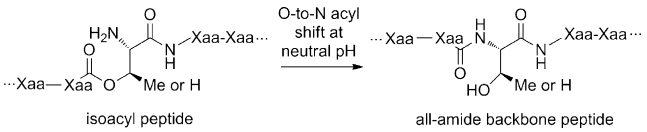
Figure 1. The sequence of human insulin (**1**).

the 1920s by Banting and colleagues,[1] insulin and its analogues have been serving as the key life-saving medicine for diabetic patients, and will undoubtedly continue to play this role until pancreas regeneration becomes clinically practical.[2] With respect to the production of insulin, recombinant DNA technology successfully generates human insulin and its analogues to supply the global diabetes market. However, the fermentation procedures suffer from the major limitation that generally only twenty genetically coded amino acids are allowed, and this becomes a significant constraint in discovery research. Conversely, the chemical synthesis of insulin expands the structural variations of each residue to unlimited possibilities, which could facilitate more thorough investigations into the chemistry, biology, and therapeutic applications of this important peptide hormone.[3]

Since the elucidation of its structure in the 1950s by Sanger and colleagues,[4] a number of synthetic routes to insulin have been reported.[3,5] These existing methods can be classified into two groups based on the strategy for forming the three native disulfide bonds: 1) those methods exploiting the inherent insulin protein structure to help direct disulfide formation using both a direct two-chain combination[5a–d] and an indirect method through a biomimetic single-chain insulin intermediate[5e–h] and, 2) those methods using differential thiol chemical reactivity to help direct disulfide bond formation.[5i–m] However, despite many years of research, existing routes still suffer from poor yield, which is generally less than 15% based on the starting peptides generated from solid-phase synthesis, and is even much lower based on the starting resin substitution.[3,5e,j]

The challenges of insulin chemical synthesis originate from the notorious hydrophobicity of the A chain, as it makes it very difficult to handle and purify,[5i,j] and the three disulfide bonds are challenging to form correctly in high yield. Both challenges need to be addressed when searching for a superior synthetic route. We prefer the directed disulfide bond-formation strategy, since it will theoretically be able to generate analogues with any possible mutation, a key advantage of chemical synthesis versus recombinant DNA technology. In contrast, it will be very challenging for the protein-folding strategy to produce analogues with mutations affecting the insulin folding process or its conformational stability.[6] In an earlier report, we described a concise one-pot procedure which avoided the purification of the A chain, B chain, and the intermediates to circumvent the hydrophobicity-associated issues, however, the yield of around 5% was less satisfactory.[5i] To continue perfecting the chemical synthesis of insulin, we herein report a new route utilizing the isoacyl peptide concept[7] which has greatly improved the overall efficiency of the synthesis.

The isoacyl peptide approach has been demonstrated to improve the biophysical properties of hydrophobic peptides to better facilitate their handling and purification (Scheme 1).[7] With the same purpose, we tested this method



Scheme 1. Conversion of an isoacyl peptide (purified under acidic conditions) into an all-amide backbone peptide mediated by an O-to-N acyl shift after the peptide is subjected to neutral pH conditions (Xaa: any amino acid).

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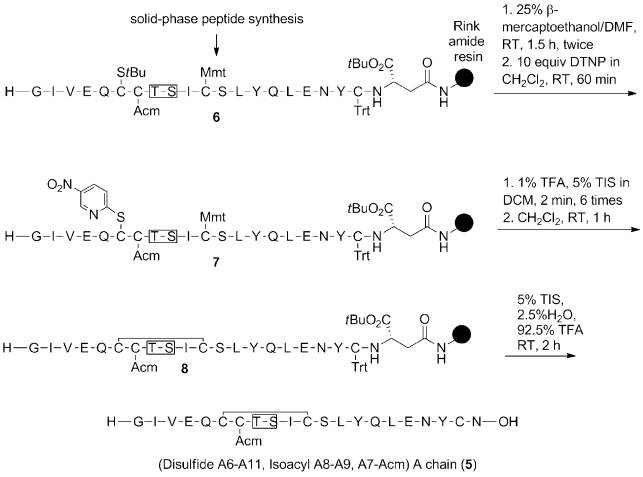
Table 1: The yield and purity of key A and B chains.

Peptide number	Peptide sequence[a]	Amount [mg][b]	Yield [%]	Purity [%][c]
3	H–G-I-V-E-Q-C(Acm)-C(Acm)-T-S-I-C(Acm)-S-L-Y-Q-L-E-N-Y-C-N–OH	110	42	85
4	H–G-I-V-E-Q-C(*t*Bu)-C(Acm)-T-S-I-C(*t*Bu)-S-L-Y-Q-L-E-N-Y-C-N–OH	231[d]	N/A	N/A
5	H–G-I-V-E-Q-C-C(Acm)-T-S-I-C-S-L-Y-Q-L-E-N-Y-C-N–OH	85	35	96
9	H–G-I-V-E-Q-C-C(Acm)-T-S-I-C-S-L-Y-Q-L-E-N-Y-C(Acm)-N–OH	60	24	96
10	H–F-V-N-Q-H-L-C(Acm)-G-S-H-L-V-E-A-L-Y-L-V-C(SNPy)-G-E-R-G-F-F-Y-T-P-K-T–OH	80	22	87
11	H–F-V-N-Q-H-L-C(Acm)-G-S-H-L-V-E-A-L-Y-L-V-C(SNPy)-G-E-R-G-F-F-Y-T-P-K-T–OH	125	35	98
12	H–F-V-N-Q-H-L-C(Acm)-G-S-H-L-V-E-A-L-Y-L-V-C(SNPy)-G-E-R-G-F-F-Y-T-P-K-T–OH	48	14	85

[a] Isoacyl segment is indicated by a box, Acm: acetamidomethyl, *t*Bu: *tert*-butyl, SNPy: (4-nitro-2-pyridinyl)thiol. [b] From 0.10 mmol scale synthesis. [c] Determined using C18 RP-HPLC at 220 nm. [d] Crude peptide.

on insulin at the three possible locations in the A and B chains: ThrA8-SerA9, GlyB8-SerB9, and TyrB26-ThrB27. The peptide [isoacyl A8-A9, (A6, A7, A11)-tri-Acm] A chain (**3**; Table 1 and Figure S1 in the Supporting Information) was prepared first and was found to be readily soluble in 0.05% TFA-containing aqueous acetonitrile, and was obtained with an excellent yield of 42% after purification using standard RP-HPLC conditions. Encouraged by this data, the [isoacyl A8-A9, (A6, A11)-di-*t*Bu, A20-Acm] A chain (**4**) was synthesized because the orthogonal Cys protection by *tert*-butyl groups could facilitate three stepwise disulfide formations.[5i,k] However, although unpurified **4** possessed good purity, the quick gelation and poor solubility of this peptide in 0.05% TFA-containing aqueous acetonitrile prohibited the purification by RP-HPLC (Table 1 and Figure S1).

The difference in solubility between **3** and **4** was presumably due to the additional hydrophobicity introduced by the (A6, A11)-di-*t*Bu protecting groups in **4**. Next, the (disulfide A6-A11, Isoacyl A8-A9, A7-Acm) A chain (**5**) was designed, wherein CysA7 was protected as Acm, and disulfide CysA6-CysA11 was preformed to avoid the additional hydrophobicity introduced by other Cys protecting groups. The A chain **5** would also concurrently enable a three-step disulfide bond-formation process. The synthesis of **5** started with loading Asp to the Rink amide resin through the β-carboxy group of Fmoc-Asp-O*t*Bu, which would be converted into the native Asn of residue A21 after resin cleavage (Scheme 2). The four Cys groups in the A chain were each protected with different protecting groups (CysA6-S*t*Bu, CysA7-Acm, Cys11-Mmt, Cys20-Trt). After all the residues were assembled by means of an automated peptide synthesizer, the resulting resin **6** was treated twice with 25% β-mercaptoethanol in DMF at room temperature for 1.5 hours to remove the *tert*-butylthiol moiety on CysA6. Subsequently the resin was mixed with DTNP (2,2′-dithiobis(5-nitropyridine))[8] in CH_2Cl_2 (dichloromethane) for 1 hour to activate CysA6 as SNPy [(4-nitro-2-pyridinyl)thiol] to afford **7**. The resin **7** was treated six times with CH_2Cl_2 containing 1% TFA and 5% TIS (triisopropylsilane) for 2 minutes to remove the Mmt (*p*-methoxytrityl) group on CysA11. The resulting free thiol of CysA11 quickly reacted with CysA6-SNPy in CH_2Cl_2 to form the disulfide A6-A11 to provide **8**.[9] Peptide cleavage from **8** was conducted with a cocktail of TFA, TIS, and H_2O,



Scheme 2. Synthesis of the isoacyl A chain **5** (isoacyl dipeptide segment is indicated by a box; only protections of Cys are shown on the resin bound peptides).

and the resulting **5** was found to be easily solubilized in 0.05% TFA-containing aqueous acetonitrile and readily purified by C18 RP-HPLC with an overall yield of 35% based on the substitution of the starting Rink amide resin (Table 1 and Figure S1). Interestingly, it was noted that the *tert*-butylthiol group on CysA6 of the resin-bound all-amide backbone A chain **S17**, having the same side-chain protection scheme as that for **6**, was completely inert to the treatment of 25% β-mercaptoethanol in DMF (see Figure S4 in the Supporting Information), presumably because of the aggregation of **S17** which prohibited access of the reagents to CysA6. This effect was likely eliminated by the isoacyl dipeptide segment ThrA8-SerA9 incorporated within **6**.

Understanding the kinetics of the O-to-N acyl shift of **5** is important for identifying the optimal reaction conditions for the A and B chain ligation reaction. To avoid disulfide scrambling induced by the free thiol-containing CysA20 of **5** at neutral or basic pH, the [disulfide A6-A11, Isoacyl A8-A9, (A7, A20)-di-Acm] A chain (**9**; Table 1 and Figure S1), having no free thiol, was prepared and its O-to-N acyl shift was evaluated at a pH range from 3.5 to 8.0. It was found that at pH 4.5 or below, no acyl shift took place within 4 hours,

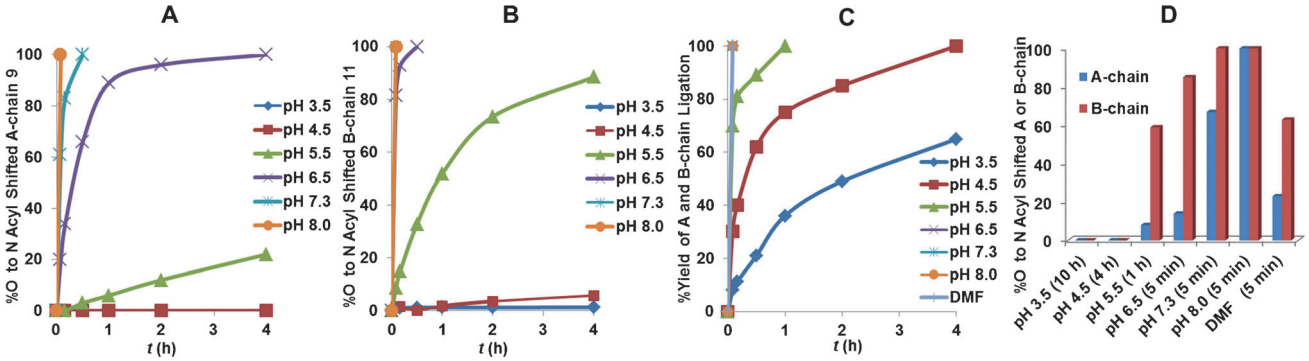


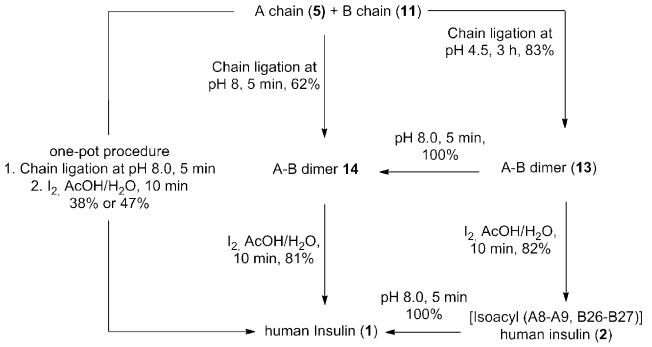
Figure 2. The kinetics of the O-to-N acyl shift for **9** (A) and **11** (B) under various pH conditions, and the formation of the A20–B19 disulfide bond using isoacyl peptides **5** and **11** (C and D). C: Ligation time course at different pH values in aqueous solution or in DMF (with 20 equiv DIEA). D: The percent of O-to-N shifted A and B chain in the newly formed A-B dimer at the time point when the A20–B19 ligation reaction was complete. The various times required to complete the ligation are in parenthesess (10 h for pH 3.5 is estimated based on yield at 4 h).

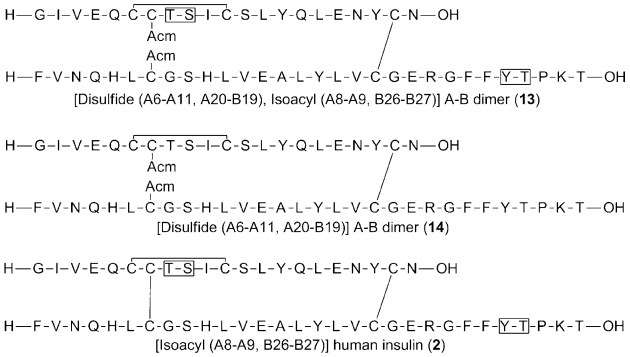
whereas the conversion was complete within 5 minutes at pH 8.0 or higher (Figure 2A and Figure S5, Scheme S1, and Table S2 in the Supporting Information).

With regard to B chain synthesis, the (B7-Acm, B19-SNPy) B chain (**10**; Table 1) was first prepared on ChemMatrix resin[10] with good crude purity after simultaneous resin cleavage and CysB19 activation using DTNP.[5i,11] The B chain **10** was obtained in 22% yield after purification by C18 RP-HPLC using 0.05% TFA-containing aqueous acetonitrile. In contrast, the isoacyl peptide (isoacyl B26-B27, B7-Acm, B19-SNPy) B chain (**11**) was obtained with 35% yield under the identical synthesis and purification conditions. The other isoacyl B chain (isoacyl B8-B9, B7-Acm, B19-SNPy) B chain (**12**) was also synthesized, however, it was found that crude **12** had lower purity as compared to **10** and **11**, and the yield after purification was only 14% (Table 1, and Figure 2 in the Supporting Information). The data for the crude purities and separation yields of **10**–**12** and all other isoacyl or all-amide backbone B chains evaluated (see Figure S2 and S3 in the Supporting Information) suggest that **11**, which contained isoacyl dipeptide TyrB26-ThrB27, was the best choice for B chain synthesis. The kinetics of the O-to-N acyl shift of **11** was evaluated under the same conditions as those used for **9**, and they indicated that almost no acyl shift took place within 4 hours at pH 4.5 or lower, while the conversion was complete within 5 minutes at pH 7.3 or higher (Figure 2B and Figure S6, Scheme S2, and Table S3 in the Supporting Information). Interestingly, it was found that **11** (isoacyl TyrB26-ThrB27) possessed faster O-to-N acyl shift kinetics than **9** (isoacyl ThrA8-SerA9). The slower kinetics of the O-to-N acyl shift of the isoacyl segment ThrA8-SerA9 may be a result of the conformational constraint in **9** imparted by the intrachain disulfide bond CysA6–CysA11.

The next step in the synthesis involved forming the interchain A20–B19 disulfide bond between **5** and **11**. Several reaction conditions were evaluated, including aqueous pH 3.5–8.0 in 6M urea as well an organic-solvent-based system of DMF using DIEA (*N*,*N*-Diisopropylethylamine) as a base (Figure 2C and Figure S7, Scheme S3, and Tables S4 and S5 in the Supporting Information). It was found that the ligation reaction finished within 5 minutes at pH 6.5 or higher and under the DMF conditions, but took 4 hours at pH 4.5. Since the O-to-N acyl shift occurred, in some cases, simultaneously with the chain ligation, the percentage of acyl-shifted A and B chains was determined at the time point when the chain ligation was complete (Figure 2D). Consistent with the kinetics of the O-to-N acyl shifts for **5** and **11**, it was found that no acyl shifts on either chain took place at pH 4.5 (4 h) or lower, whereas 100% of both chains were shifted at pH 8.0 (5 min). Acyl shifts occurred to different degrees of completion for the intermediate pH values and in DMF. Since having more than one product in the crude A-B dimer mixture makes the RP-HPLC purification unnecessarily complicated, and also considering the time needed to complete the acyl shift of the newly formed A-B dimer under each of the reaction conditions (Table S4), pH 4.5 (4 h) with no acyl shift and pH 8.0 (5 min) with a full acyl shift were chosen as two optimal ligation conditions for a preparative scale reaction to form the [disulfide (A6-A11, A20-B19), Isoacyl (A8-A9, B26-B27)] A-B dimer (**13**) and [disulfide (A6-A11, A20-B19)] A-B dimer (**14**), respectively (Scheme 3).

The stepwise approaches with two purification steps were first evaluated for the synthesis of human insulin (**1**). When conducting the chain ligation at pH 8 (6M urea, 0.2M NH_4HCO_3), **14** was obtained with 62% separation yield, and the subsequent iodine (I_2) oxidation of the purified **14** in acetic acid (AcOH) and H_2O generated **1** with an 81% separation yield, to afford an overall ligation/oxidation yield of 50% (Scheme 3 and Figure S10 in the Supporting Information). When conducting the chain ligation at pH 4.5 (6M urea, 0.2M NH_4OAc), the ligation reaction was completed after 3 hours and produced **13** with an 83% separation yield (Scheme 3, Figure 3, and Figure S11 in the Supporting Information). The dimer **13** could be fully converted into **14** for the I_2 oxidation by adjusting the pooled fractions, from RP-HPLC, with NH_4HCO_3 to pH 8 for 5 minutes. The direct I_2 oxidation of **13** produced the [isoacyl (A8-A9, B26-B27)] human insulin (**2**) with a yield of 82% after RP-HPLC purification and freeze-drying, and was fully converted into **1** by dissolving it in pH 8 aqueous acetonitrile for 5 minutes. The pooled RP-HPLC fractions of **2** could also be directly converted into **1** by adjusting the pH to 8 with NH_4HCO_3





Scheme 3. The isoacyl A- and B-chain-mediated synthetic routes to human insulin (**1**; Isoacyl dipeptide segment is indicated by a box). Overall ligation-oxidation yield of the two-step procedures: 68% by chain ligation at pH 4.5 (→**13**→**2**→**1** or →**13**→**14**→**1**) or, 50% by chain ligation at pH 8 (→**14**→**1**).

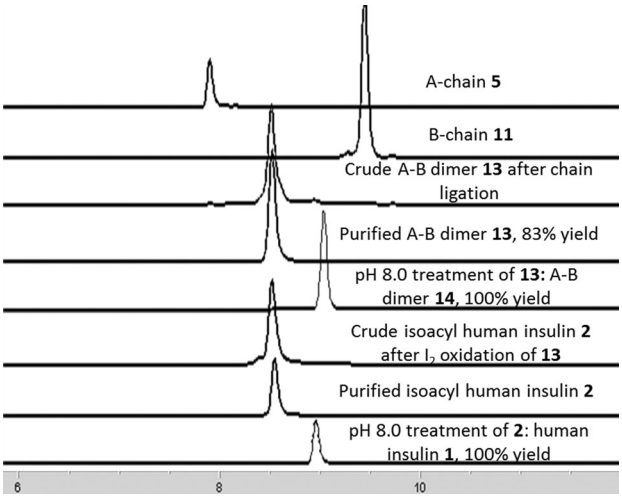


Figure 3. Preparation of human insulin (**1**) by a two-step method with chain ligation at pH 4.5 (HPLC traces at $\lambda = 220$ nm).

prior to freeze-drying. The overall ligation/oxidation yield for the stepwise route with chain ligation at pH 4.5 was 68%. It was found that **13** had a higher recovery yield than that of **14** from the RP-HPLC column, but that the yield of forming the A7–B7 disulfide bond of these two intermediates by I_2 oxidation was not differentiable.

Two one-pot methods were also tested for preparing **1**. The A–B chain ligation reaction was first attempted in 6 M urea and 0.2 M NH_4HCO_3 at pH 8, and the resulting solution containing the crude **14** was directly diluted with AcOH and treated with I_2 for 10 minutes to form the A7–B7 disulfide bond which afforded **1** with an overall ligation/oxidation yield of 47% after a single purification by RP-HPLC (Scheme 3 and Figure S8 in the Supporting Information). It should be noted that the mass corresponding to mono-iodinated insulin was identified with less than 10% of the desired **1** based on the signal intensity. The same one-pot procedure was also evaluated by conducting chain ligation in 8 M guanidine and 0.1 M Tris buffer at pH 8,[5i] and thus generated **1** in 38% yield but with no detectable iodinated insulin (Scheme 3 and Figure S9 in the Supporting Information).

The native disulfide bonding pattern of synthetic **1** was unambiguously confirmed by Glu-C (Staphylococcus aureus Protease V8) digestion of **1**, with comparisons to the authentic human insulin (Eli Lilly) and to all three possible disulfide isomers of the A(5–17)–B(1–13) fragments, which were generated by independent synthetic routes (see Figures S12 and S18 and Table S6 in the Supporting Information). The protein structure and biological function of synthetic **1** was also confirmed as being identical to the authentic material by comparing their CD spectra and binding affinities to the insulin receptor (Figure S19 and Table S7 in the Supporting Information).

In summary, the incorporation of isoacyl dipeptide segments into insulin A and B chains greatly improved the overall efficiency of insulin chemical synthesis. Incorporation of an isoacyl dipeptide (ThrA8–SerA9) into the A chain not only facilitated the purification of this peptide using standard C18 RP-HPLC conditions, but also enabled the on-resin formation of the A6–A11 disulfide bond. Incorporation of an isoacyl dipeptide (TyrB26-ThrB27) on the B chain also improved the yield after RP-HPLC purification by two- to three-fold compared to an all-amide backbone B chain. The disulfide ligation reaction linking the purified isoacyl A and isoacyl B chains at pH 4.5, with subsequent I_2 oxidation and the O-to-N acyl shift (or O-to-N acyl shift then I_2 oxidation), resulted in the generation of human insulin with a yield of 68% based on the starting A chain. The overall yield based on the substitution of the resin used for the synthesis of A chain was 24%. To the best of our knowledge, this is the highest reported yield of human insulin chemical synthesis. Lastly, we believe that this method could also be utilized for generating other disulfide-rich peptides where hydrophobicity/aggregation of the linear precursor significantly compromises the efficiency of the synthesis.

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