Advanced Insulin Synthesis by One-pot/stepwise Disulfide Bond Formation Enabled by Acid-activated S-Protected Cysteine Sulfoxide in the Presence of Chloride Anion

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Dedication ((optional))

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Abstract: An advanced insulin synthesis is presented that utilizes one-pot/stepwise disulfide bond formation enabled by acid-activated S-protected cysteine sulfoxides in the presence of chloride anion. *S*-chlorocysteine generated from cysteine sulfoxides reacts with an S-protected cysteine to afford *S*-sulfenylsulfonium cation, which then furnishes the disulfide or reversely returns to the starting materials depending on the S-protection employed and the reaction conditions. Use of *S*-acetamidomethyl cysteine (Cys(Acm)) and its sulfoxide (Cys(Acm)(O)) selectively give the disulfide under weak acid conditions in the presence of $MgCl_2$ even if *S*-*p*-methoxybenzyl cysteine (Cys(MBzl)) and its sulfoxide (Cys(MBzl)(O)) are also present. In contrast, the *S*-MBzl pair yields the disulfide under more acidic conditions in the presence of a chloride anion source. These reaction conditions allowed a one-pot insulin synthesis. Additionally, lipidated insulin was prepared by a one-pot disulfide-bonding/lipidation sequence.

Introduction

A century has passed since the discovery of insulin in the 1920s, which clarified the physiological roles of insulin and resulted in a tremendous impact on the treatment of diabetes (Figure 1).

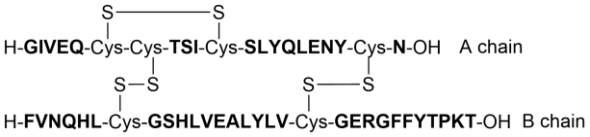


Figure 1. Sequence of human insulin, which was a milestone synthetic target in peptide chemistry.

Following determination of insulin's sequence, a myriad of attempts to synthesize insulin resulted in the continuous advance of peptide chemistry.[1-3] The chemical synthesis of insulin has remained a landmark that continues to define the state-of-the-art in peptide/protein chemistry. Insulin has two interchain disulfide bonds between the A and B chains and one intrachain linkage in the A chain. Furthermore, the A chain shows lower solubility in aqueous buffer, which leads to difficulty in manipulating the peptide. In this context, an efficient regioselective disulfide bond-forming reaction and solubilizing technology are still required to elevate the chemical synthesis of insulin.

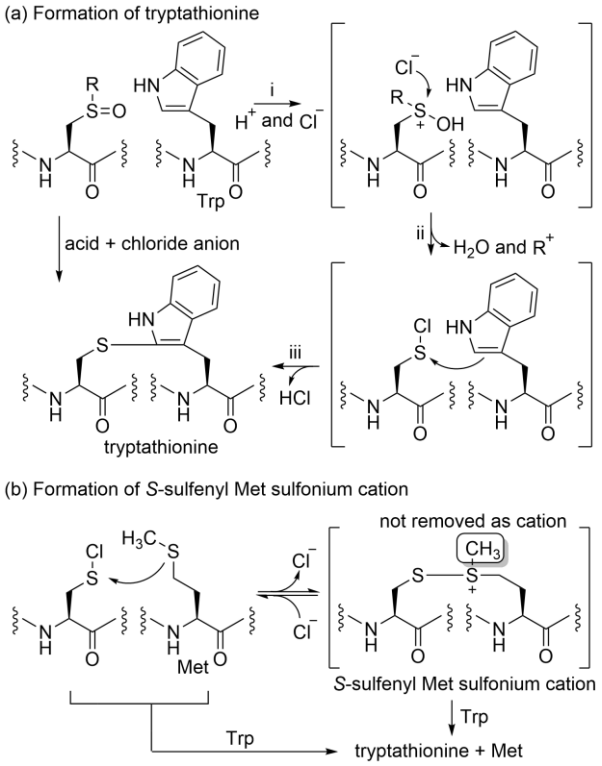


Figure 2. Involvement of *S*-chlorocysteine resulting from acid-activated S-protected cysteine sulfoxides in sulfenylation of tryptophan (Trp) (a) and methionine (Met) (b).

Our group has recently accomplished tryptophan (Trp)-selective C-H sulfenylation by utilizing an acid-activated S-protected cysteine sulfoxide[4] in the presence of chloride anion (Figure 2a).[5] This discovery provides insight into revising the mechanism involved in the sulfoxide-mediated disulfide bonding reaction previously reported by our group.[6] The Trp sulfenylation requires the formation of *S*-chlorocysteine[7,8] from S-protected cysteine sulfoxides aided by acid and chloride anion

with the resulting *S*-chlorocysteine participating in aromatic electrophilic substitution reaction (S_EAr) with the indole. The presence of Trp and methionine (Met) in peptides allows the *S*-chlorocysteine to react not only with Trp but also with Met in a Met-preferential manner. The reaction with Met reversibly gives *S*-sulfenylsulfonium Met cation due to the difficulty of releasing the methyl cation from the resulting sulfonium cation. Otherwise, the sulfonium cation could participate in the S_EAr with Trp as an electrophile (Figure 2b).[5b]

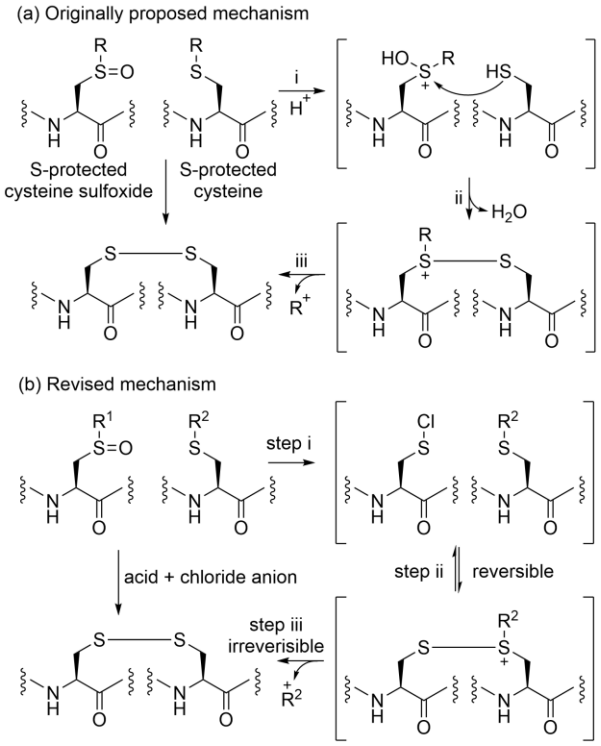


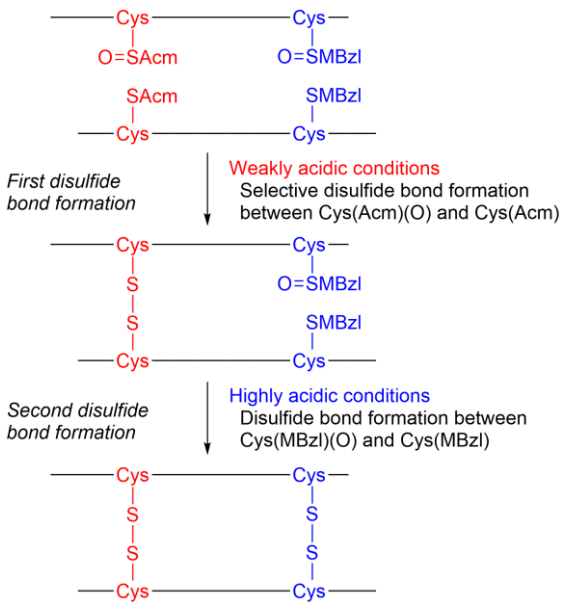
Figure 3. Reaction mechanism for S-protected cysteine sulfoxide-mediated disulfide formation: (a) originally proposed mechanism without involving *S*-chlorocysteine; (b) revised mechanism involving *S*-chlorocysteine.

In 1988, we reported a disulfide bond-forming reaction using acid-activated S-protected cysteine sulfoxides and proposed that nucleophilic attack of the free thiol group regenerated from a protected cysteine to an acid-activated sulfoxide gives the disulfide with the release of protection as a cation. (Figure 3a). Consequently, we and DiMarch's group have successfully applied this to discontinuous disulfide formation by synthesizing several disulfide-containing peptides.[6,9] However, stepwise disulfide bond formation using different sequential pairings of an S-protected cysteine sulfoxide and the corresponding S-protected cysteine has yet to be reported. Attempts based on the initially proposed mechanism failed to achieve the sequential disulfide formation.

The involvement of the Met sulfide in the *S*-chlorocysteine-mediated Trp-sulfenylation (Figure 2b) suggests that the reaction of *S*-chlorocysteine with an S-protected cysteine could also yield the corresponding *S*-sulfenylsulfonium cation. The resulting cation could then either be converted to the disulfide or revert back to starting materials depending on the ease of releasing an S-protecting group as a cation (Figure 3b, steps ii vs iii). This rationale encouraged us to synthesize insulin by utilizing S-protected cysteine sulfoxide-mediated stepwise disulfide bond formation. We anticipated that incorporating the sulfoxides into the A chain would also improve its solubility. We report herein an advanced insulin synthesis that uses acid-activated S-protected cysteine sulfoxides for one-pot/stepwise disulfide bond formation as well as a subsequent lipidation sequence.[10]

Results and Discussion

The conceptual guide to the strategy for synthesizing insulin arose from our previous study on peptide heterodimerization of Trp-peptides using a linker tagged with *S*-acetamidomethyl (Acm) and *S*-*p*-methoxybenzyl (MBzl) cysteine sulfoxides (Cys(Acm)(O) and Cys(MBzl)(O)).[11] We showed that Cys(Acm)(O) is converted to *S*-chlorocysteine under mildly acidic conditions (0.1% TFA either in AcOH or in the ionic liquid 1-butyl-1-methylpyrrolidinium trifluoromethanesulfonate (BMPy·OTf))[12] in the presence of magnesium chloride ($MgCl_2$), with coexisting Cys(MBzl)(O) remaining intact. In contrast, the formation of *S*-chlorocysteine from the Cys(MBzl)(O) occurs under more acidic conditions in the presence of guanidine hydrochloride (Gn·HCl). Additionally, the Cys(Acm)-derived sulfenylsulfonium cation, resulting from the reaction of *S*-halocysteine with Cys(Acm), releases the Acm group as a cation under mildly acidic or neutral conditions to lead to disulfide formation,[13,14] which probably may not be the case for the Cys(MBzl)-derived cation. Such a difference in the chemical behavior between Cys(Acm) and Cys(MBzl) derivatives allowed us to envision the strategy for stepwise interchain disulfide formations as shown in Scheme 1.



Scheme 1. Envisioned stepwise interchain disulfide formation using Cys(Acm)(O)/Cys(Acm) and Cys(MBzl)(O)/Cys(MBzl) pairs.

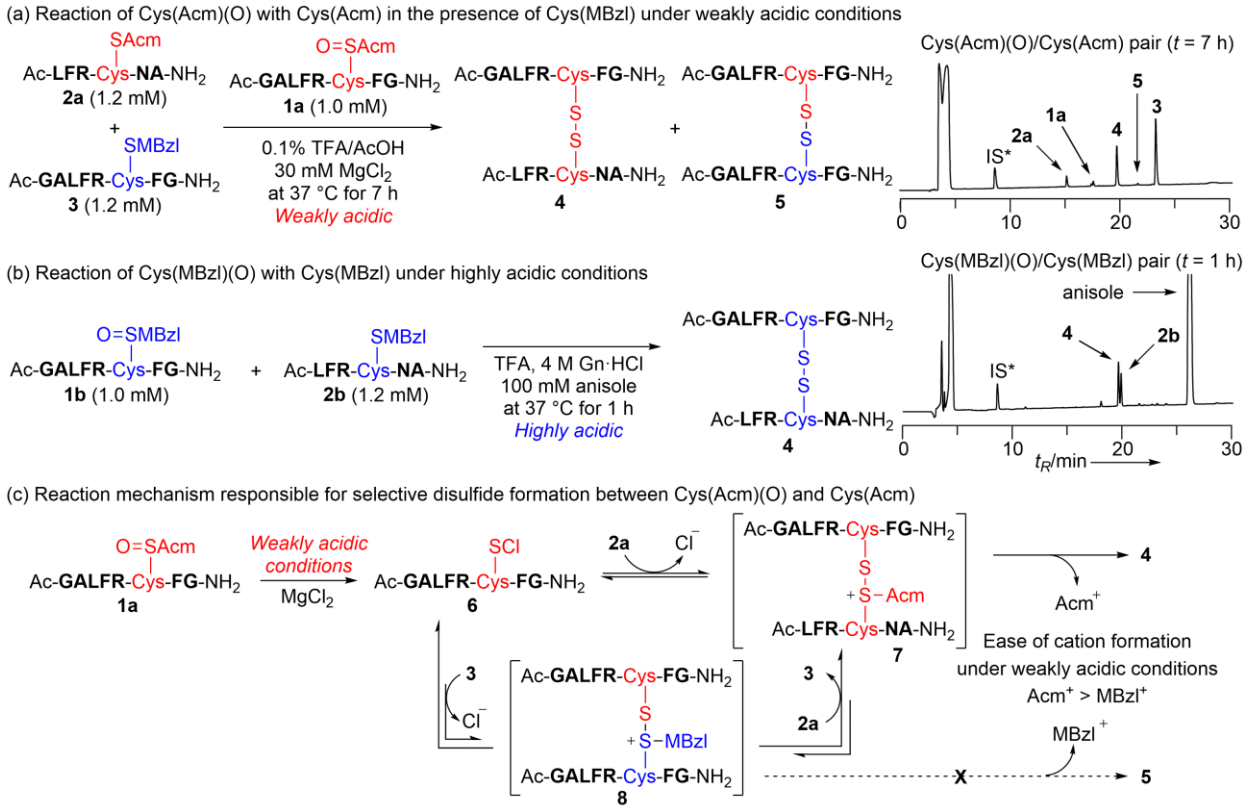


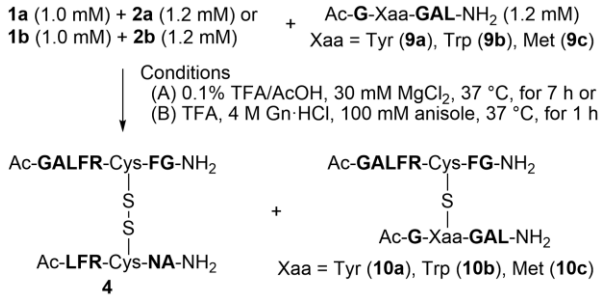
Figure 4. Evaluation of disulfide bond formation between S-protected cysteine sulfoxide and S-protected cysteine. (a) Selective disulfide-bonding reaction of Cys(Acm)(O) with Cys(Acm) in the presence of Cys(MBzl) under weakly acidic conditions. (b) Disulfide formation between Cys(MBzl)(O) and Cys(MBzl) under TFA conditions. (c) Plausible explanation for the selective disulfide formation between Cys(Acm)(O) and Cys(Acm) in the presence of Cys(MBzl) under weakly acidic conditions. Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 65% over 30 min. UV detection at 220 nm. *IS = internal standard: Phe.

Our initial evaluation of the proposed strategy began with an attempt at selective disulfide formation between the Cys(Acm)(O)/Cys(Acm)(O) pair in the presence of Cys(MBzl) under mildly acidic conditions (Figures 4a and S1). The reaction of a Cys(Acm)(O)-containing peptide (Ac-**GALFR**-Cys(Acm)(O)-**FG**-NH_2 (**1a**), 1.0 mM) with a Cys(Acm)-peptide (Ac-**LFR**-Cys(Acm)-**NA**-NH_2 (**2a**), 1.2 mM) or Cys(MBzl)-peptide (Ac-**GALFR**-Cys(MBzl)-**FG**-NH_2 (**3**), 1.2 mM) in 0.1% TFA/AcOH in the presence of 30 mM $MgCl_2$ at 37 °C for 7 h proceeded in a highly Cys(Acm)(O)/Cys(Acm)-selective manner to give the desired heterodisulfide peptide **4**. During the reaction, Cys(MBzl)-peptide **3** remained intact and significant quantities of possible peptide **5** from **1a** and **3** were not detected. This indicates that Cys(Acm)(O) can participate in disulfide formation with Cys(Acm) under mildly acidic conditions but not with Cys(MBzl). We next assessed the Cys(MBzl)(O)/Cys(MBzl) pair to determine whether disulfide formation would occur under more acidic conditions than that used for Cys(Acm)(O)/Cys(Acm) pair (Figures 4b and S2). The reaction of a Cys(MBzl)(O) peptide (Ac-**GALFR**-Cys(MBzl)(O)-**FG**-NH_2 (**1b**), 1.0 mM) with a Cys(MBzl) peptide (Ac-**LFR**-Cys(MBzl)-**NA**-NH_2 (**2b**), 1.2 mM) proceeded quantitatively in TFA with 4 M Gn·HCl to afford the desired heterodisulfide peptide **4**.

Regardless of whether the *S*-chlorocysteine **6** generated from Cys(Acm)(O) under mild acid conditions in the presence of $MgCl_2$ reacts in situ with Cys(Acm)- or Cys(MBzl)-peptide (**2a** or **3**), only the Cys(Acm)(O)/Cys(Acm) pair can give the disulfide. Two possible *S*-sulfenylsulfonium cations (**7** and **8**) could form from the reaction of *S*-chlorocysteine **6** with Cys(Acm) **2a** or Cys(MBzl) **3**. Under weakly acidic conditions, the S-Acm-derived cation **7** would become a disulfide with the Acm cation leaving. On the other hand, such mild conditions prevent the MBzl group from being removed from the sulfonium cation **8**, which reversibly reverts to Cys(MBzl) and *S*-chlorocysteine peptide (**3** and **6**) or turns to **7** by the reaction with **2a** (Figure 4c).

With the usefulness of the Acm(O)/Acm and MBzl(O)/MBzl pairs for selective disulfide formation tentatively confirmed, we examined the effect of transiently formed *S*-chlocysteine intermediate on Trp, Met, and Tyr (Table 1 and Figures S3 and S4). Each pair of Acm series (**1a** and **2a**) or MBzl series (**1b** and **2b**) was treated under disulfide-forming conditions suitable for the pairs in the presence of Tyr, Trp, or Met-containing peptide (Ac-**G**-Xaa-**GAL**-NH_2: Xaa = Tyr (**9a**), Trp (**9b**), Met (**9c**)), and the results are summarized in Table 1. Reactions using the Cys(Acm)(O)/Cys(Acm) pair under mild acidic conditions proceeded in almost completely disulfide selectivity (entries 1−3 and Figure S3). In contrast, selectivity in reactions of the MBzl pair were slightly decreased, but no significant side products, including peptides **10a** and **10c**, were observed on HPLC analysis of the reactions in the presence of **9a** and **9c**. Reaction of the Trp peptide **9b** was accompanied a small amount of **10b** and MBzl-modified **9b** (entries 4−6 and Figure S4).

Table 1. Examination of effect of *S*-chlorocysteine on Tyr, Trp and Met peptides.



entry	Peptide pair (protection pair)	Xaa (Peptide)	Reaction conditions[a]	Selectivity[b] (%)
1	**1a** + **2a** (Acm(O) + Acm)	Tyr (**9a**)	A	100
2	**1a** + **2a** (Acm(O) + Acm)	Trp (**9b**)	A	>98[c]
3	**1a** + **2a** (Acm(O) + Acm)	Met (**9c**)	A	100
4	**1b** + **2b** (MBzl(O) + MBzl)	Tyr (**9a**)	B	91
5	**1b** + **2b** (MBzl(O) + MBzl)	Trp (**9b**)	B	91
6	**1b** + **2b** (MBzl(O) + MBzl)	Met (**9c**)	B	94

[a] A mixture of S-protected Cys sulfoxide peptide (**1,** 1.0 mM) and S-protected Cys peptide (**2**, 1.2 mM) in the presence of Tyr, Trp, or Met peptide (**9**, 1.2 mM) was treated in 0.1% TFA/AcOH in the presence of $MgCl_2$ at 37 C° for 7 h or in TFA in the presence of 4 M Gn·HCl and 50 mM anisole at 37 C° for 1 h. [b] The selectivity ratio of the reactions of the sulfoxides with S-protected cysteines vs Tyr, Trp, or Met was estimated by comparing the reduction ratio of the starting sulfide peptides (**2a** or **2b**) (%) between in the presence and absence of peptides **9**. Each reduction ratio (%) was calculated using the equation (100-100*((integ. **2a** at 7 h (or **2b** at 1 h)/integ. IS)/(integ. **2a** (or **2b**) at 0 h/integ. IS)). The reduction ratio in the absence of peptides **9** was set to 100% and the selectivites were calculated, where integ. = integration of peak area of the UV absorption and IS = internal standard (Phe). [c] Negligible amount of 10b was detected on HPLC analysis.

Having established the fundamental reaction conditions for selective disulfide formation, we next challenged one-pot/stepwise regioselective disulfide formation using a sulfoxide peptide (Ac-**A**-Cys(Acm)(O)-**FRA**-Cys(MBzl)(O)-**K**-NH_2 (**11**)) and a sulfide peptide (Ac-**G**-Cys(Acm)-**LFR**-Cys(MBzl)-**FG**-NH_2 (**12**)) (Figures 5 and S5). Treatment of **11** (1 mM) with **12** (1.2 mM) in 0.1% TFA/AcOH in the presence of 30 mM $MgCl_2$ at 37 °C for 5 h allowed the regioselective intermolecular disulfide formation between the Cys(Acm)(O) and Cys(Acm) residues to give heterodimeric mono disulfide peptide **13** quantitatively. Upon addition of an equal volume of 4 M Gn·HCl−100 mM anisole in TFA to the first reaction mixture, the second disulfide formation occurred between the Cys(MBzl)(O) and Cys(MBzl) residues to afford the bis(disulfide) peptide **14** after 2 h reaction at 37 °C in 2 M Gn·HCl−50 mM anisole in TFA/AcOH (1:1).No detectable disulfide shuffling was observed. Tryptic digestion of **14** followed by peptide mapping indicated that the resulting **14** has the correct disulfide pairings (Figure S6). Based on the success of one-pot/stepwise regioselective disulfide formation using the model peptide, we undertook synthesis of human insulin using the stepwise disulfide formation protocol (Figure 6). This work began by assembling the protected peptide resin for A and B chains using 9-fluororenylmethyloxycarbonyl (Fmoc)-based solid-phase peptide synthesis (Fmoc SPPS). Chain assembly employed *S*-triphenylmethyl (Trt) group for CysA6 and CysA11, *S*-Acm for CysA7 and CysB7, and *S*-MBzl for CysA20 and CysB19. For the preparation of the hydrophobic A chain, an *O*-acyl isopeptide strategy[3g,15] was employed to address the solubility issue. The *O*-acyl dipeptide unit (Boc-SerA9(Fmoc-Thr(*t*Bu)A8)-OH) was incorporated into the growing peptide chain. The protected peptide resin for the A chain was treated with TFA−Et_3SiH (TES)−H_2O (95:2.5:2.5, (v/v)) at room temperature for 2 h to give the A chain isopeptide **15** with Cys(SH)6,11, Cys(Acm)7, Cys(MBzl)20. This was then subjected to the oxidation step with H_2O_2 in AcOH–citrate buffer–MeCN (5/4/1) (pH 3.0) to yield CysA6-CysA11 disulfide-bonding, Cys(Acm)(O)A7 and Cys(MBzl)(O)A20 isopeptide **16** in 11% isolated yield (Figures S7 and S8). Incubation of **16** in NH_4HCO_3 buffer (pH 8.0), followed by lyophilization, afforded the oxidized A chain **17** to complete the requisite stepwise disulfide formation. The B chain **18** containing Cys(Acm)7 and Cys(MBzl)19 was obtained by deprotection of the protected resin with TFA−TES−H_2O in 9% isolated yield (Figures S9).

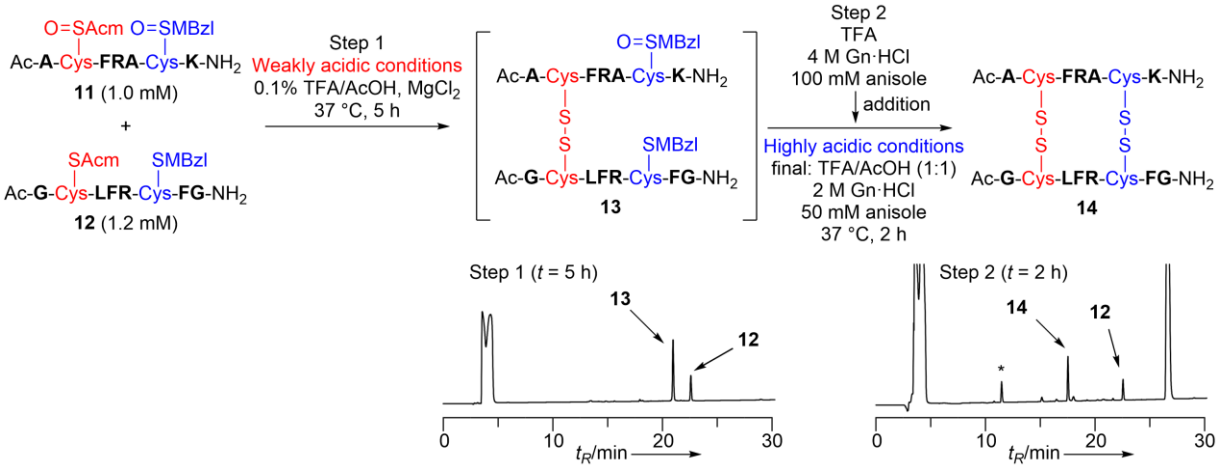


Figure 5. Examination of regioselective disulfide bond formation using model peptides. (a) HPLC examination of the disulfide-bonding reaction in the step 1. (b) HPLC examination of the disulfide-bonding reaction in the step 2. Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 65% over 30 min. UV detection at 220 nm.

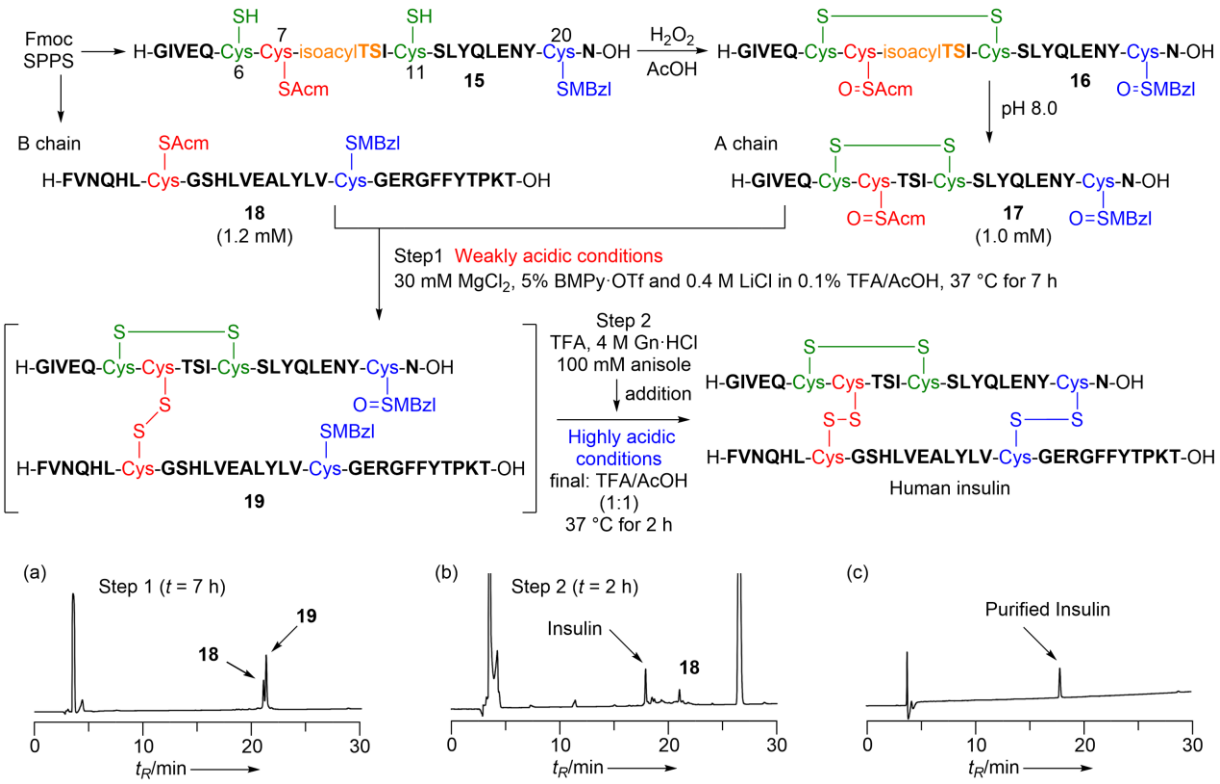


Figure 6. One-pot/stepwise disulfide formations for the synthesis of insulin and their HPLC analysis. (a) HPLC examination of the reaction in the step 1. (b) HPLC examination of the reaction in the step 2. (c) HPLC-purified insulin. Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 65% over 30 min. UV detection at 220 nm.

The resulting oxidized A chain **17** was then subjected to the first disulfide formation step induced by $MgCl_2$ under weakly acidic conditions, where the less soluble character of the A chain peptide necessitated the addition of an ionic liquid (BMPy·OTf) and LiCl as solubility-enhancing additives. The first disulfide formation between the oxidized A chain **17** (1.0 mM) and B chain **18** (1.2 mM) in 0.1% TFA/AcOH in the presence of 30 mM $MgCl_2$, 5% BMPy·OTf and 0.4 M LiCl at 37 °C for 7 h proceeded in a highly Cys(Acm)(O)A7/Cys(Acm)B7 selective manner between the A and B chains to yield heterodimeric mono disulfide peptide **19** (Figure 6a). Subsequent addition of 4 M Gn·HCl−100 mM anisole in TFA to the first reaction mixture, followed by reaction at 37 °C for 2 h, initiated the second disulfide formation between Cys(MBzl)(O)A19 and Cys(MBzl)B20 to give the desired insulin (Figure 6b). HPLC analyses of the reaction mixtures indicated that the stepwise disulfide-bonding steps proceeded in a highly regioselective manner to yield insulin efficiently. Finally, HPLC purification of the crude mixture gave homogeneous human insulin with an isolated yield of 21%, calculated based on oxidized A chain **17** (Figures 6c and S10). This result falls short of the best data (38 or 47%) obtained from a one-pot procedure that utilizes the well-established conventional disulfide-forming reactions.[3g] Peptide mappings of the digest of synthetic material or an authentic sample by endoproteinase Glu-C accorded well together and the disulfide pairing of fragments was also confirmed by the synthesis of possible three disulfide isomers (Figures S11−15). It is worth noting that the achieved stepwise disulfide formation relies on only a single oxidation step for the conversion of **15** to **16**. In contrast, conventional insulin synthesis requires three stepwise oxidative manipulations for disulfide formation. The application of the isopeptide **16** to stepwise disulfide formation could have overcome solubility issues of the oxidized A chain **17** that required the addition of the BMPy·OTf and LiCl, however no drastic improvement was observed. More importantly, the use of the isopeptide encountered a side reaction during the first disulfide-forming step (data not shown).

Having succeeded in the one-pot/stepwise synthesis of insulin using the acid-activated sulfoxide system, we next undertook a one-pot/stepwise disulfide formation-lipidation sequence for preparing a fatty acid-modified insulin. Lipidated insulins, such as insulin degludec[16] or insulin detemir,[17] have been widely used as long-acting basal insulin. These modified insulins have a fatty acid unit on their LysB29 residue. Therefore, we planned to replace the LysB29 with Trp, on which the acid-activated sulfoxide-mediated sulfenylation of the indole ring would permit incorporation of a fatty acid moiety.[10] Our initial attempt at constructing disulfide bonds of the TrpB29 insulin encountered problems, when during HPLC examination of the second-step reaction we observed co-elution of the desired peptide and anisole. To circumvent this, we performed a proof-of-concept experiment using a C-terminally TrpB31-amide-extended B chain (Figure 7). The requisite Trp31 B-chain **20** was prepared by Fmoc SPPS protocols (Figure S16). Reaction of **20** with **17** under the conditions identical to those employed for the one-pot synthesis of human insulin unambiguously afforded the desired TrpB31 insulin analogue **22** as a main component on HPLC analysis through the intermediacy **21** (Figures 7a and b). At this

stage, the insulin analogue was obtained in 16% isolated yield (Figures S17 and 18). Subsequent addition of a TFA solution of the Cys(Acm)(O)-modified fatty acid derivative **23** (Figure S19) into the reaction mixture gave the desired TrpB31-lipidated insulin 24 in a one-pot manner in 12% isolated yield (Figures S20 and 21).

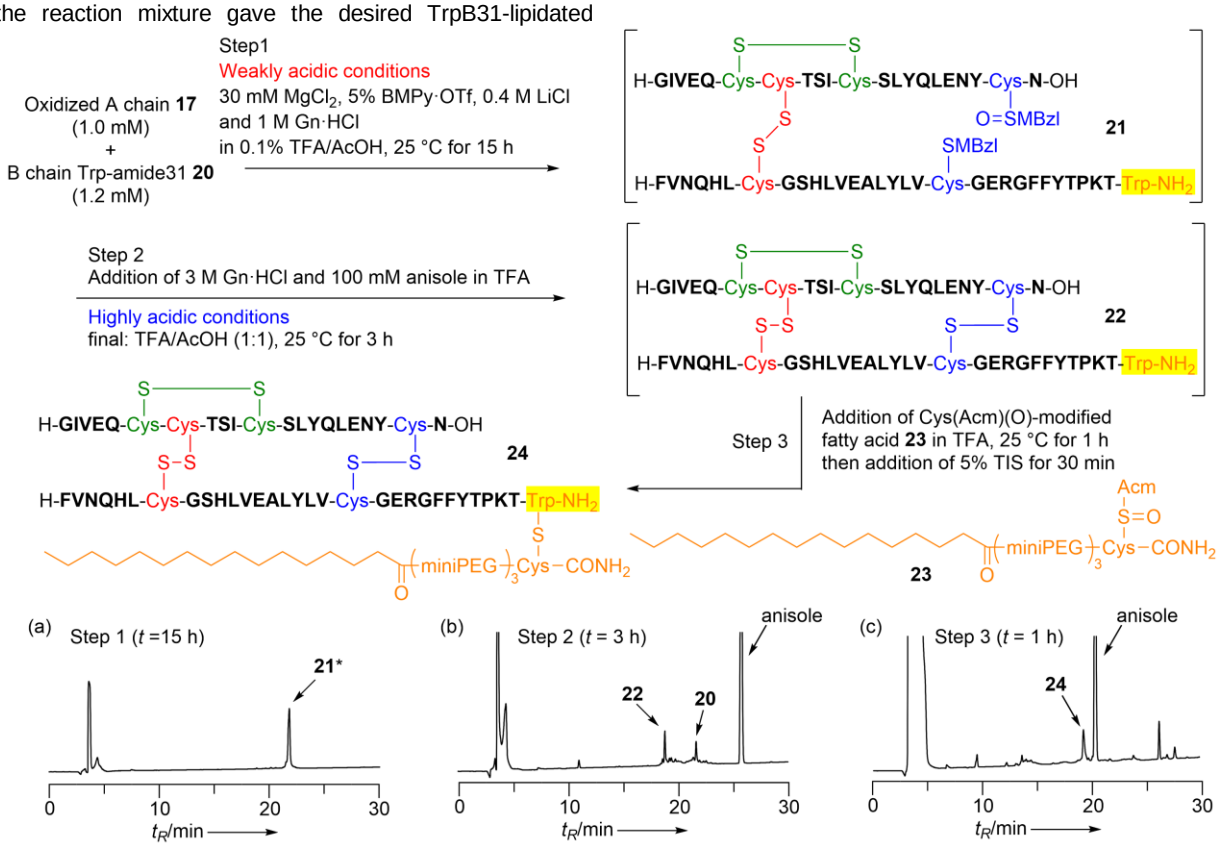


Figure 7. One-pot/stepwise disulfide formation and lipidation sequence for the synthesis of TrpB31-lipidated insulin (**24**) and their HPLC analyses of the reaction mixture. H-miniPEG-OH = H_2N-$(CH_2)_2$-O-$(CH_2)_2$-OCH_2CO_2H (a) HPLC examination of the reaction mixture in step 1. (b) HPLC examination of the reaction mixture in step 2. (c) HPLC examination of the reaction mixture in the step 3. Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 65% over 30 min for (a and b) and 5% to 95% over 30 min for (c). UV detection at 220 nm. *Remaining B chain peptide **20** was coeluted.

Conclusion

The sequential use of Cys(Acm)/Cys(Acm)(O) (first step) and Cys(MBzl)/Cys(MBzl)(O) (second step) pairs, with control of acidity in the presence of chloride anion, allowed one-pot/stepwise interchain disulfide formation. This led to the development of an unprecedented strategy for synthesizing insulin. The strategy features an acid-controllable redox reaction that occurs between an S-protected cysteine and the corresponding sulfoxide pair. Furthermore, conducting disulfide-bond formation and lipidation sequences utilizing an acid-activated cysteine sulfoxide permitted the one-pot synthesis of lipidated insulin. The methodology that we present herein should pave the way not only for insulin synthesis, but also for insulin-like peptides, including relaxins. Concerning the encountered side reaction in utilizing the isopeptide for disulfide formation, studies on exploring the cause and proposing potential solutions to overcome it will be presented in due course.

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Keywords: S-protected cysteine sulfoxide • step wise disulfide formation • insulin • *S*-chlorocysteine • peptide lipidation

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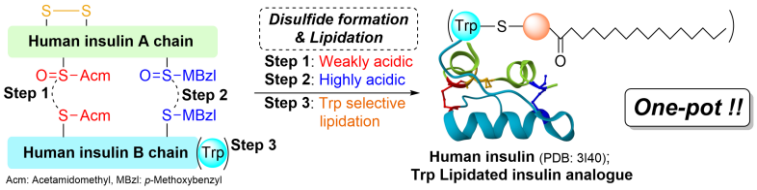
Entry for the Table of Contents



The reaction of S-protected cysteine and the corresponding cysteine sulfoxide with increasing acidity of the reaction allowed one-pot/stepwise disulfide formation for insulin synthesis. The stepwise disulfide bonding system employed is unprecedented. In addition, the sulfoxide-mediated protocol enabled one-pot synthesis of a lipidated insulin analogue.