Protein Chemical Synthesis

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An Efficient One-Pot Four-Segment Condensation Method for Protein Chemical Synthesis**

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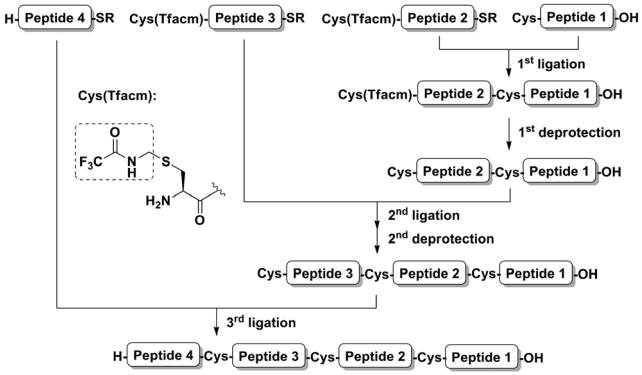
Abstract: *Successive peptide ligation using a one-pot method can improve the efficiency of protein chemical synthesis. Although one-pot three-segment ligation has enjoyed widespread application, a robust method for one-pot four-segment ligation had to date remained undeveloped. Herein we report a new one-pot multisegment peptide ligation method that can be used to condense up to four segments with operational simplicity and high efficiency. Its practicality is demonstrated by the one-pot four-segment synthesis of a plant protein, crambin, and a human chemokine, hCCL21.*

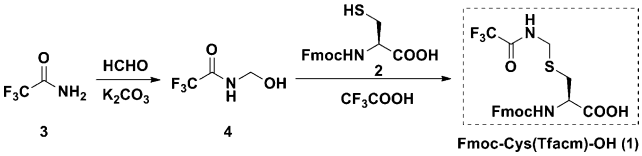
Protein chemical synthesis enables the generation of proteins with predesigned site-specific modifications for biological and pharmaceutical studies.[1] The synthesis can be usually achieved through chemoselective condensation of unprotected peptides by native chemical ligation.[2] An important recent advance is the development of one-pot ligation methods to improve the yield and decrease the time cost of protein synthesis, as a one-pot experiment can minimize the requirement to isolate and handle intermediate products.[3] Up to now the one-pot condensation of three peptide segments has been well studied.[4] The significance of this method has been demonstrated in the successful synthesis and studies of important proteins, such as hepatocyte growth factor[5] and ubiquitinated histone H2B.[6] It is envisaged that one-pot four-segment ligation will also be an important method for protein chemical synthesis.[7,8] Indeed, in the development of labeled chemokines we encountered the need to establish a one-pot four-segment method to prepare these proteins containing multiple cysteine (Cys) residues.

Herein we report a new one-pot ligation method for protein chemical synthesis that can handle up to four peptide segments (Scheme 1). This method uses Tfacm-protected Cys (Tfacm = trifluoroacetamidomethyl), which can be efficiently converted into its unprotected form by adjusting the pH value to 11.5. As no external nucleophilic or electrophilic reagent is added during the procedure, the reaction system in the one-pot experiment remains relatively clean and uncomplicated such that all of the peptide segments can be easily added in equal proportions. As a result of the clean reaction steps, the progress of the reaction was easily monitored and a high yield of the final product was obtained.



Scheme 1. One-pot four-segment ligation. Peptide thioesters are made from the corresponding hydrazides. Removal of Tfacm is achieved by adjustment of the pH value.

Our study began with the gram-scale preparation of Tfacm-protected Cys **1** suitable for Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase peptide synthesis (SPPS). As shown in Scheme 2, trifluoroacetamide (**3**) was reacted with formaldehyde to generate 2,2,2-trifluoro-*N*-(hydroxymethyl)-acetamide (**4**). Treatment of **4** with Fmoc-Cys-OH (**2**) afforded **1** which was isolated in an overall yield of 45%. With **1** in hand, we assembled Cys(Tfacm)-Ile-Ile-Ile-Pro-Gly-Ala-Thr-$NHNH_2$ (**5**) using standard Fmoc SPPS procedures.[9] After the final acidic cleavage of the product from the resin followed by separation using HPLC, **5** was successfully isolated in a good yield of 60%, as calculated from the resin loading.



Scheme 2. Synthesis of Fmoc-Cys(Tfacm)-OH.

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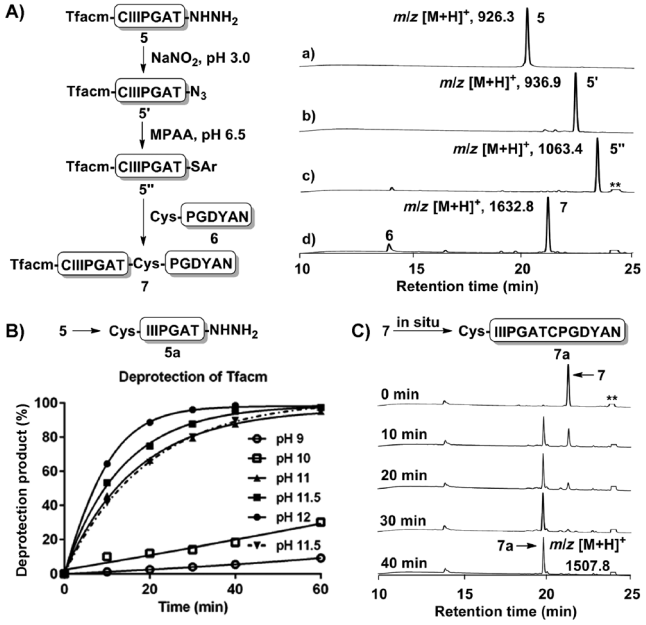

Figure 1. A) Ligation of **5** with **6** and monitoring of the reaction by analytical HPLC ($\lambda = 214$ nm): a) before addition of $NaNO_2$; b) after addition of $NaNO_2$ for 15 min, pH 3.0; c) after addition of MPAA and adjustment of the pH value to 6.5 for 10 min; d) after addition of **6** for 4 h, pH 6.5. B) Deprotection of Tfacm. The solid lines represented reactions at 37 °C and at pH 9 (○), 10 (□), 11 (▲), 11.5 (■), and 12 (●). The dotted line (▼) represented reaction at 25 °C and pH 11.5. C) Analytical HPLC traces for the removal of Tfacm from **7** by adjustment of the pH value to 11.5 at 37 °C. **: peak attributable to MPAA.

Next, we tried the ligation reaction between Tfacm-protected peptide hydrazide **5** and a model Cys-peptide, namely Cys-Pro-Gly-Asp-Tyr-Ala-Asn (**6**; Figure 1A). In aqueous phosphate buffer, **5** was cleanly oxidized by $NaNO_2$ at pH 3.0 and −15 °C for 15 min to generate a peptide azide **5′**. After addition of 4-mercaptophenylacetic acid (MPAA) and adjustment of the pH value to 6.5, **5′** was fully converted into a peptide thioester **5″**, which then reacted with **6** to afford the ligation product **7** in quantitative conversion within 4 h.

To remove Tfacm, **5** was dissolved in aqueous phosphate buffer (0.2 M) containing urea (8 M), MPAA (0.1 M), and TCEP (0.08 M; tris(2-carboxyethyl)phosphine). According to HPLC analysis (Figure 1B), deprotection at pH 9–11 was not efficient, whereas clean and rapid removal of Tfacm could be achieved at pH 11.5 and 12.

To test whether the deprotection involved racemization at the N-terminal cysteine residue, we compared **5a**, prepared by deprotection of **5** at pH 11.5 for 40 min, with Cys(D)-Ile-Ile-Ile-Pro-Gly-Ala-Thr-$NHNH_2$ (**5b**). The experiment showed that the extent of racemization was less than 1% (Supporting Information).

Next, we performed the deprotection reaction by adjusting the pH value in situ to 11.5 after the ligation between **5** and **6** was completed. As shown in Figure 1C, a quantitative conversion from **7** into **7a** was achieved within 40 min at 37 °C.

The above findings laid the foundation for the new one-pot ligation method. To test its efficiency, we first chose crambin as the synthetic target. Crambin is a 46-residue protein containing three disulfide bonds.[10] Crambin with a V15A mutation has been frequently used as a standard to test new methods for chemical protein synthesis.[3,4b,11] Our synthetic strategy is shown in Figure 2A, where [V15A]-crambin was divided into four peptide segments (**10**, **8**, **5‴**, and **6**). All the peptide thioesters are prepared through activation and thiolysis of the corresponding peptide hydrazides.[12] The one-pot experiment was carried out in the ligation buffer containing phosphate (0.2 M), urea (8 M), MPAA (0.2 M), and TCEP (0.16 M). The whole process was conducted at 37 °C and monitored by LC–MS (Figure 2).

The first ligation between **6** (1.0 equiv, 4 mM) and **5‴** (1.0 equiv, 4 mM) went to completion at pH 6.5 within 4 h to generate **7**. Then the pH value was raised to 11.5 for 40 min to remove Tfacm and produce **7a**. After the pH was adjusted back to 6.5, **8** (1.0 equiv, 2.7 mM) was added to the reaction to initiate the second ligation, which was finished within 2 h to generate **9**. The pH value was again raised to 11.5 for 40 min to convert **9** into **9a**, followed by readjustment of the pH value to 6.5. Finally, we added **10** (1.0 equiv, 2 mM) to the reaction. The last ligation went to completion within 20 h. HPLC analysis of the whole experiment showed that in each step of the one-pot process, the desired intermediate was cleanly produced as the major peak (Figure 2B). The one-pot experiment was completed within 30 h to produce the full-length peptide which was isolated in 40% yield.

Note that addition of TCEP significantly improved the reaction, as TCEP effectively prevented base-mediated elimination of disulfides to form dehydroalanine (Dha).[13] Indeed, in the absence of TCEP a byproduct with an increased mass of 134 Da was detected during the deprotection step, which could be attributed to the addition of MPAA to Dha (see the Supporting Information for details).

Synthetic [V15A]crambin was folded (Figure 2C) and then crystallized at 18 °C by the vapor-diffusion method. Its X-ray crystal structure solved at 2.1 Å resolution (Figure 2D,E) was fully consistent with the reported data.[14] To further verify that all amino acids remained intact in the one-pot process, we reduced the synthetic [V15A]crambin by dithiothreitol (DTT) and alkylated its cysteine residues with iodoacetamide. After digestion by trypsin, we obtained three peptide fragments which were analyzed by (LC–MS)/MS. The mass spectrum of the internal segment "SNFNAC*R" was shown in Figure 2F as a representative, which was identical to that expected from the user database (for more information see the Supporting Information; user database built by Proteome Discoverer 1.4 Software (Thermo Scientific) according to the sequence provided). Collectively, our X-ray crystal data and (LC–MS)/MS analysis confirmed that crambin prepared by the one-pot four-segment condensation method was correct at the atomic level.

To examine the use of the new one-pot method for the synthesis of more practically useful proteins, we chose human chemokine hCCL21 as another synthetic target. hCCL21 contains 111 residues and six cysteines. Through activation of G-protein-coupled receptor CCR7, hCCL21 recruits normal immune cells and metastasizes tumor cells to lymph nodes.[15] The details of hCCL21 biology have attracted increasing

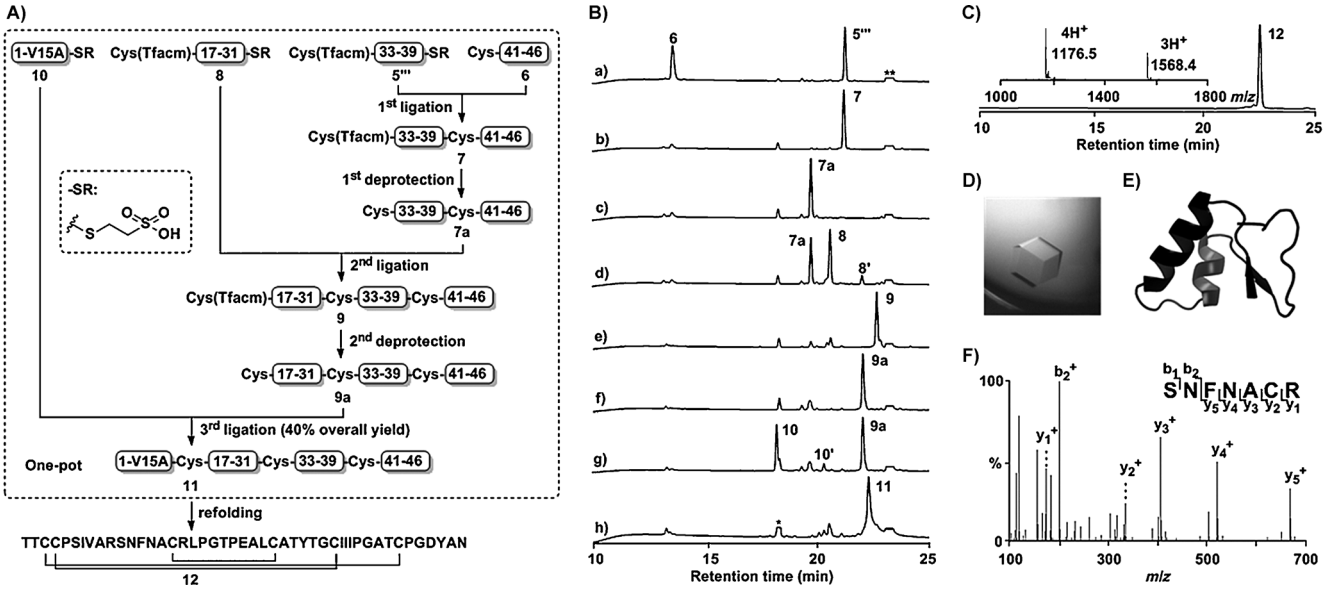

Figure 2. One-pot four-segment synthesis of crambin. A) Synthetic route. B) One-pot ligation monitored by analytical HPLC. The first ligation between **5''** and **6** at a) $t = 2$ min; b) $t = 4$ h; and c) after pH adjustment to 11.5 for 40 min. After addition of **8** at d) $t = 2$ min, pH 6.5; e) $t = 2$ h; and f) after pH adjustment to 11.5 for 40 min. After addition of **10** at g) $t = 2$ min, pH 6.5; h) $t = 20$ h. **8′** = MPAA thioester of **8. 10′** = MPAA thioester of **10**. C) Analytical HPLC trace and ESI-MS (inset) of purified folded crambin. Detected mass = (4702.1 ± 0.1) Da (calcd. 4702.4 Da, average isotopes). D) Crystals of crambin. E) Single-crystal X-ray structure of crambin. F) (LC–MS)/MS analysis of "SNFNAC*R". Cysteine was alkylated by iodoacetamide. *: not derived from peptide. **: MPAA.

research interest in terms of both fundamental studies and in the development of hCCL21-directed cancer therapies. The chemical synthesis of labeled hCCL21 carrying tailor-designed fluorophores or other biophysical probes will provide useful tools to elucidate its biological mechanism and dynamics.[16]

Chemokine hCCL21 was divided into four fragments (**13**–**16**). The one-pot four-segment ligation was conducted following the procedure outline in Figure 3A. First, **13** (1.0 equiv) and **14** (1.0 equiv) were dissolved in ligation buffer to reach a final concentration of 2.5 mM. After the first ligation was complete (within 2 h), the pH value was adjusted to 11.5 to remove the Tfacm protecting group from **17** to produce the desired product **18** within 40 min. After pH adjustment back to 6.5, **15** (1.0 equiv, 2.2 mM) was added to the reaction to start the second ligation, affording **19** within 4 h. Subsequently the pH value was again adjusted to 11.5 for 40 min to produce **20**. Finally, after the pH was changed to 6.5, **16** (1.0 equiv, 2 mM) was added to the reaction to undergo ligation with **20**. After 2 h, full-length hCCL21 (**21**) was produced as the major peak. Remarkably, the above one-pot synthesis took only 10 h in total. Analytical HPLC (Figure 3B) showed that all the reaction steps proceeded cleanly. A single final purification step was needed to isolate the desired product **21**, whose purity and identity was characterized by HPLC and MS analysis.

To test the robustness of the one-pot synthetic procedure, we repeated the process three times but on three different scales, to produce **21** in yields of 5, 20, or 50 mg (with yields of the isolated product of 41%, 45%, and 39%, respectively; see the Supporting Information). In a control experiment where HPLC purification was conducted after each ligation step, we isolated **21** in 13% yield (Supporting Information). This control experiment also took over one week to finish, showing that the one-pot method improved the efficiency in terms of both the yield and the time cost.

Full-length peptide **21** was folded to **22** in 50% yield (Figure 3C,D). SDS-PAGE analysis of synthetic hCCL21 **22** (shCCL21) and commercial recombinant hCCL21 (chCCL21) gave the same results (Figure 3E). According to the CD spectrum (Figure 3F), shCCL21 was about 40% disordered. This observation was consistent with a previous NMR study showing that hCCL21 contained an unstructured extended C terminus.[17] Finally, according to a trans-well migration assay using freshly isolated murine $CD8^+$ T lymphocytes, shCCL21 exhibited activity indistinguishable from that of chCCL21 (Figure 3G). At a lower concentration (100 ng mL^{-1}), both shCCL21 and chCCL21 were inert to T lymphocytes. At a higher concentration (500 ng mL^{-1}), both shCCL21 and chCCL21 exhibited good chemotactic activities to $CD8^+$ T lymphocytes.[18] All the data verified that bioactive hCCL21 was produced by the new one-pot ligation method.

In summary, by using the Tfacm group to protect the N-terminal cysteine residue we accomplished one-pot four-segment ligation with satisfactory operational simplicity and high efficiency. The practicality of the new one-pot ligation method was demonstrated in the synthesis of two proteins, namely, crambin and hCCL21. We anticipate that the new one-pot method will find applications in the development and production of small- to medium-sized protein reagents and post-translationally modified proteins that are needed in biomedical studies.

Keywords: native chemical ligation · peptides · protecting groups · protein chemical synthesis · solid-phase synthesis

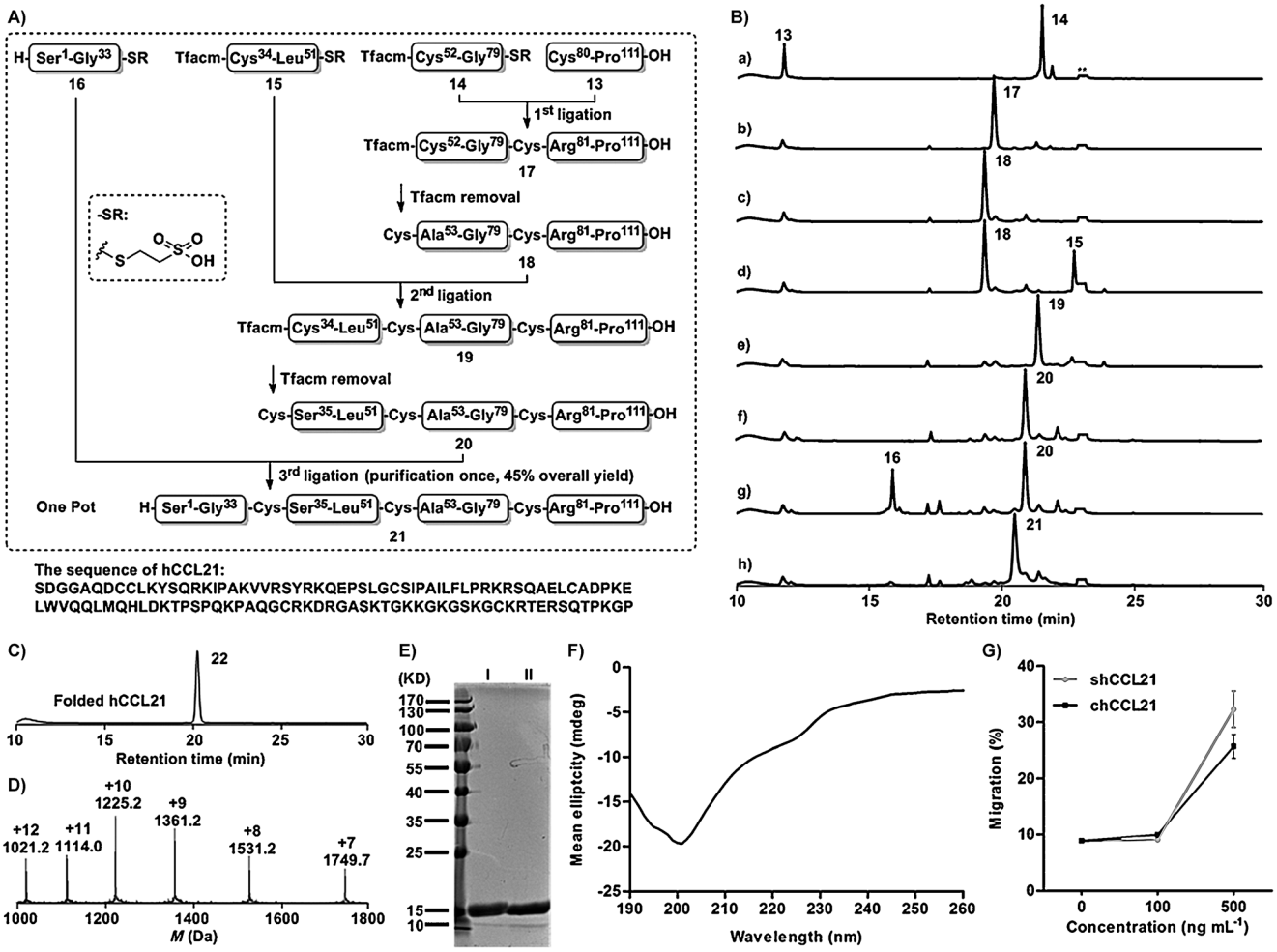

Figure 3. A one-pot four-segment ligation for the synthesis of hCCL21. A) Synthetic route. B) One-pot ligation monitored by analytical HPLC. The first ligation between **13** and **14** at a) $t = 2$ min; b) $t = 2$ h; and c) after pH adjustment to 11.5 for 40 min. The second ligation after **15** was added at d) $t = 2$ min, pH 6.5; e) $t = 4$ h; and f) after pH adjustment to 11.5 for 40 min. The third ligation after addition of **16** at g) $t = 2$ min, pH 6.5; h) $t = 2$ h. C) Analytical HPLC trace for purified folded hCCL21. D) Characterization of shCCL21 by mass spectrometry. Detected mass = 12242.0 ± 1.0 Da (calcd. 12242.4 Da, average isotopes). E) SDS-PAGE analysis of chCCL21 (I) and shCCL21 (II). F) CD spectrum of shCCL21 in water. G) Trans-well migration assay for chCCL21 and shCCL21 using murine $CD8^+$ T lymphocytes.

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