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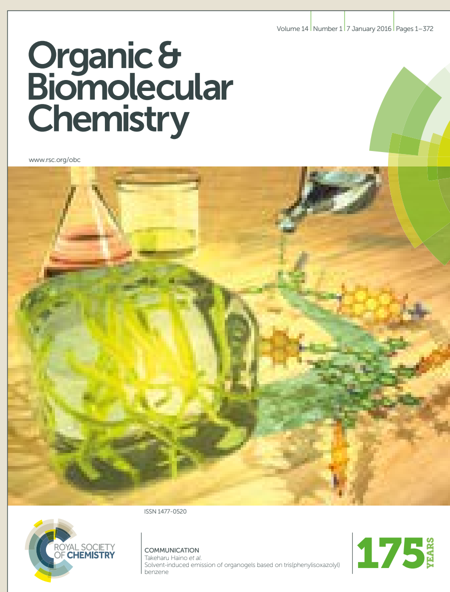

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Robust Synthesis of C-terminal Cysteine-Containing Peptide Acids through A Peptide Hydrazide-Based Strategy

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A new robust strategy was reported for epimerization-free synthesis of C-terminal Cys-containing peptide acids through mercaptoethanol-mediated hydrolysis of peptide thioesters made in situ from peptide hydrazides. This simple-to-operate and highly efficient method avoids the use of derivatization reagents for resin modification, thus providing a practical avenue for the preparation of C-terminal Cys-containing peptide acids.

C-terminal Cys carboxylates are frequently present in the sequences of disulfide-rich bioactive peptides, such as somatostatin, conotoxins, and neocyclosimides.[1] These C-terminal Cys peptides are highly important drug candidates or scaffold templates in many studies[1] as well as in our own researches on the structural engineering of disulfide-rich bioactive peptides.[2] The C-terminal Cys residue of these peptides can form an intramolecular disulfide bond with internal Cys residues, which are indispensible for stabilizing their bioactivity-related conformations. However, owing to the pronounced acidity of the Cα proton of Cys esters, Fmoc-based solid-phase synthesis (SPPS) of C-terminal Cys-containing peptide acids usually encounters severe side reactions. Epimerization at the C-terminal Cys can occur not only in the ester bond forming reaction during anchoring of C-terminal Cys onto a solid support, but also in the repeated piperidine treatment during Fmoc SPPS.[3] The second side reaction is the piperidine-induced β-elimination of the C-terminal Cys, again due to the relatively high acidity of the Cα proton of cysteine esters. The resulting dehydroalanine moiety can react with piperine by Michael-type addition to form 3-(N-piperidinyl)alanine.[4] During the synthesis of peptide sequences, contaminants from the C-terminal Cys-related side reactions can make purification difficult and greatly lower the final yield of the desired peptides.

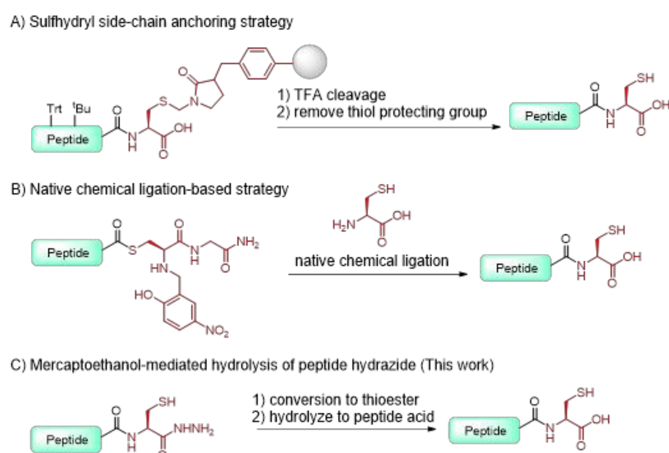

Fig. 1. Recent methods for synthesis of C-terminal Cys peptide acids.

Given the importance of the C-terminal Cys bioactive peptides, considerable recent efforts have been made to develop approaches for the synthesis of C-terminal Cys-containing peptide acids (**Fig. 1**). These include: 1) the use of Cys side-chain anchoring strategy, 2) introduction of Cys as an orthoester derivative, and 3) NCL (native chemical ligation) based strategy.[5] Despite these advances, new methods are still needed for more robust synthesis of the C-terminal Cys-containing peptide acids due to various practical reasons. For instance, when using the side-chain anchoring strategy, Cys derivatives (such as Cys orthoester) are not commercially available and require multiple-step chemical synthesis.[5a-5d] In the previous NCL based strategy, the reported intermediates were structurally complex crypto-thioester peptides that need to be made from special resins.[5e] Thus, during our own studies on the pharmaceutically relevant disulfide-rich peptides, we sought to develop a more robust and simple approach for the preparation of C-terminal Cys-containing peptide acids.

Herein, we report a new, robust strategy using unprotected Cys peptide hydrazides as precursors to synthesize C-terminal

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Cys peptide acids (**Fig. 1C**).[6] This method shows three important advantages: First, the C-terminal Cys hydrazide peptide can be readily made through Fmoc SPPS without sophisticated resin modification. Second, the acidity of the Cα proton of the Cys hydrazide is much weaker than that of the corresponding ester, so that side reactions of epimerization and β-elimination at the C-terminal Cys can be effectively avoided.[5] Finally, through sequential treatment with $NaNO_2$ and mercaptoethanol, the C-terminal Cys peptide hydrazide can be smoothly converted into C-terminal Cys peptide mercaptoethanol-thioester, and subsequently hydrolyzed to C-terminal Cys peptide acid rapidly under mild conditions. The efficiency and practical utility of the new method has been demonstrated by the preparation of Riparin 1.1b, somatostatin, and α-conotoxin Vc1.1.

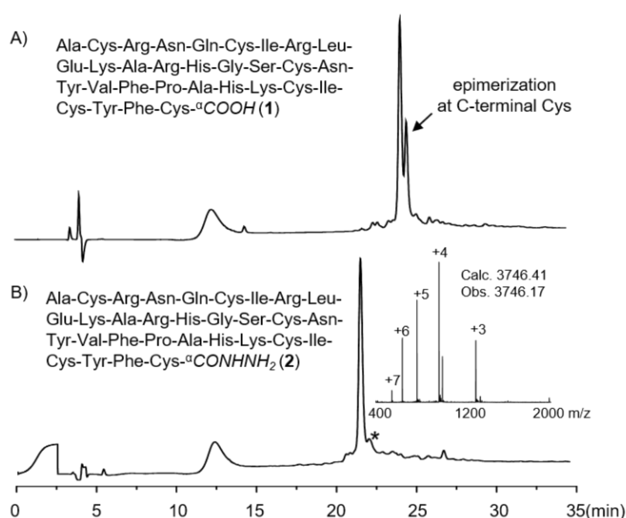

Fig. 2. Analytic HPLC traces (210 nm) of A) crude C-AhPDF 1.1b acid (**1**) by using trityl(2-Cl) chloride resin; B) crude C-AhPDF 1.1b hydrazide (**2**) by using hydrazine-trityl(2-Cl) resin, * denotes the epimer of hydrazide **2**.

The difficulty in synthesizing C-terminal Cys peptide acids can be primarily attributed to the enhanced acidity of the Cα proton of Cys esters, which renders C-terminal Cys moiety prone to racemization and β-elimination during repeated piperidine treatment.[3,4] Notably, the acidity of Cα proton of Cys hydrazide is significantly lower than that of the Cys ester, and thus the undesirable side reactions could be suppressed during the Fmoc SPPS of C-terminal Cys peptide hydrazides. With this notion in mind, we began our study with synthesis of a 31 amino acid C-terminal Cys peptide, namely C-AhPDF1.1b, taken from C-terminus of a 51 amino acid plant defensin.[7] Previous work has shown that even using 2-chloro-trityl resin, more than 30% epimerization at C-terminal Cys occours during Fmoc SPPS of C-AhPDF1.1b (**1**). To make comparison, we were interested in whether Fmoc SPPS of the C-terminal Cys peptide hydrazide can avoid the C-terminal Cys racemization. We first synthesized **1** by using 2-chloro-trityl resin and microwave method. Consistent with that previously reported, HPLC analysis of the crude C-AhPDF1.1b acid showed about 30% epimerization at C-terminal Cys site (**Fig. 2A**), making the purification step difficult. Satisfyingly, when using hydrazine-2-chloro-trityl resin, the epimer of C-AhPDF1.1b hydrazide (**2**) is less than 3% (**Fig. 2B**), indicating that Fmoc SPPS of C-Cys hydrazide peptide does avoid racemization of the C-terminal Cys site. Of note, the hydrazine-2-chloro-trityl resin was readily prepared by hydrazinolysis of 2-chloro-trityl resin.[8]

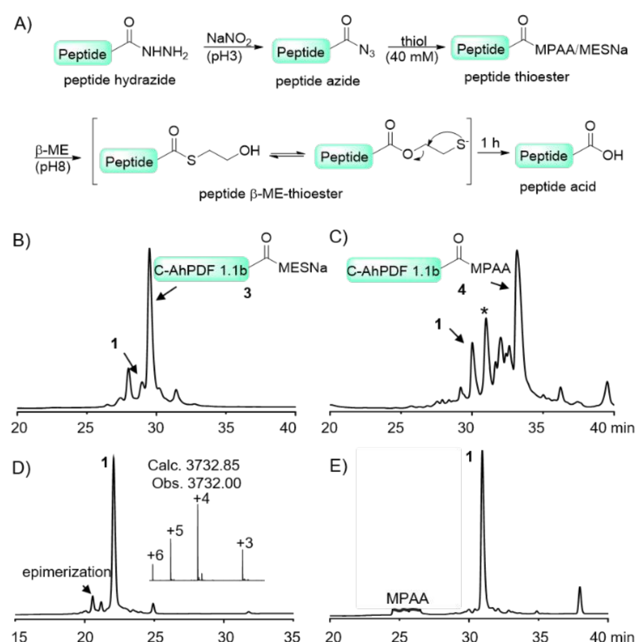

Fig. 3. Mercaptoethanol-mediated hydrolysis of C-AhPDF 1.1b thioester. A) general route for conversion of peptide hydrazide to peptide acid; B) C-AhPDF 1.1b MESNa-thioester (**3**) at pH 8 for 1 h; C) C-AhPDF 1.1b MPAA-thioester (**4**) at pH 8 for 2.5 h, * denotes internal thiolactones formed by intramolecular thiol-thioester exchange; D) C-AhPDF 1.1b mercaptoethanol-thioester (**5**) at pH 8 for 1 h; E) mercaptoethanol-mediated hydrolysis of C-AhPDF 1.1b MPAA-thioester (**4**) (pH 8, 400 mM mercaptoethanol, 1h).

Next, we investigated the conversion of C-AhPDF1.1b hydrazide (**2**) to C-AhPDF1.1b acid (**1**). This process involves the formation of a C-AhPDF1.1b thioester from C-AhPDF1.1b hydrazide and subsequent hydrolysis of C-AhPDF1.1b thioester. At pH 3 and under -10 °C, C-AhPDF1.1b hydrazide (**2**, 1 equiv) reacts with $NaNO_2$ (10 equiv) to form C-AhPDF1.1 azide, which is then smoothly converted to C-AhPDF1.1b thioester after the addition of thiol. Then we conducted the hydrolysis of peptide thioester under mild (pH 8) conditions.[9] When using sodium 2-mercaptoethanesulflnate (MESNa, 200 equiv), the conversion of the C-AhPDF1.1b MESNa-thioester (**3**) to the C-AhPDF1.1b acid (**1**) was less than 7% after 1 hour (**Fig. 3B**). When using 4-carboxymethyl)thiophenol (MPAA, 200 equiv), the hydrolysis yield of C-AhPDF1.1b MPAA-thioester (**4**) was less than 19% (**Fig. 3C**). For the MPAA conditions, we observed a byproduct peptide caused by the aminolysis of the C-AhPDF1.1b MPAA-thioester (**4**) by guanidine (**Fig. S5**). Collectively, our results indicated that direct conversion of regular peptide thioesters to peptide acids was not pratical even under basic conditions.

At this point, we noticed an important finding by Kent and co-workers, that in the presence of 2-mercaptoethanol, a peptide thioester can be quantitatively and rapidly converted to the corresponding peptide acid.[10] Thus we studied the mercaptoethanol-mediated hydrolysis of peptide thioester.

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After treating the C-AhPDF1.1b hydrazide (**2**, 1 equiv) with $NaNO_2$, 200 equivalents of mercaptoethanol were added and the pH value was adjusted to 8. To our joy, the resulting C-AhPDF1.1b mercaptoethanol-thioester (**5**) was almost quantitatively converted to the C-AhPDF1.1b acid (**1**) in 1 hour (**Fig. 3D**). Intriguingly, we observed about 5% epimerization at C-terminal Cys site during this process. We hypothesized that the racemization of C-terminal Cys site may be due to the limited reaction rate of the conversion of C-AhPDF1.1b hydrazide to C-AhPDF1.1b mercaptoethanol-thioester by 2-mercaptoethanol. One possible way to overcome this problem is to increase the rate of the formation of the C-AhPDF1.1b thioester. Thus we used MPAA (20 equiv) instead of mercaptoethanol in the peptide thioester forming step because aryl thiols are more reactive than alkyl thiols under weakly acidic conditions. To help the hydrolysis of the C-AhPDF1.1b MPAA-thioester (**4**), mercaptoethanol (200 equiv) was added and the pH of the solution was adjusted to 8. Delightedly, the combined use of MPAA and mercaptoethanol completely avoided the epimerization at C-terminal Cys, providing a satisfying 98% yield in C-AhPDF1.1b acid (**1**) formation (**Fig. 3E**). Note that the equivalent of MPAA should be at least 10 times higher than the amount of peptide hydrazide, otherwise the C-terminal acyl azides can undergo a Curtius rearrangement to form peptide isocyanates, as reported by Pentelute and co-workers.[11] Taken together, we demonstrated that the C-terminal Cys-containing peptide hydrazide can be used as substrates for the efficient preparation of the C-terminal Cys peptide acid.

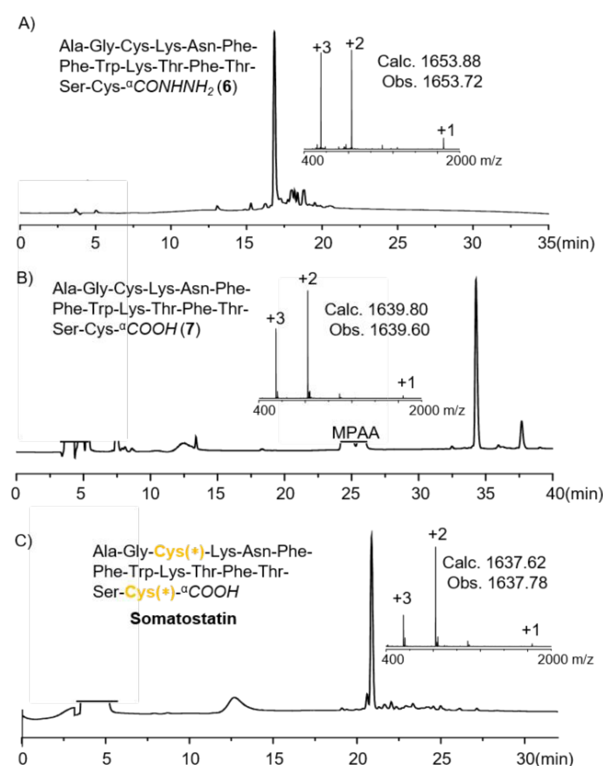

Fig. 4. Analytic HPLC traces (210 nm) of A) crude somatostatin hydrazide (**6**); B) somatostatin acid (**7**) after mercaptoethanol-mediated hydrolysis of **6**; C) correctly folded somatostatin after I_2-treatment, with a disulfide bond formed between ³Cys(*) and ¹⁴Cys(*).

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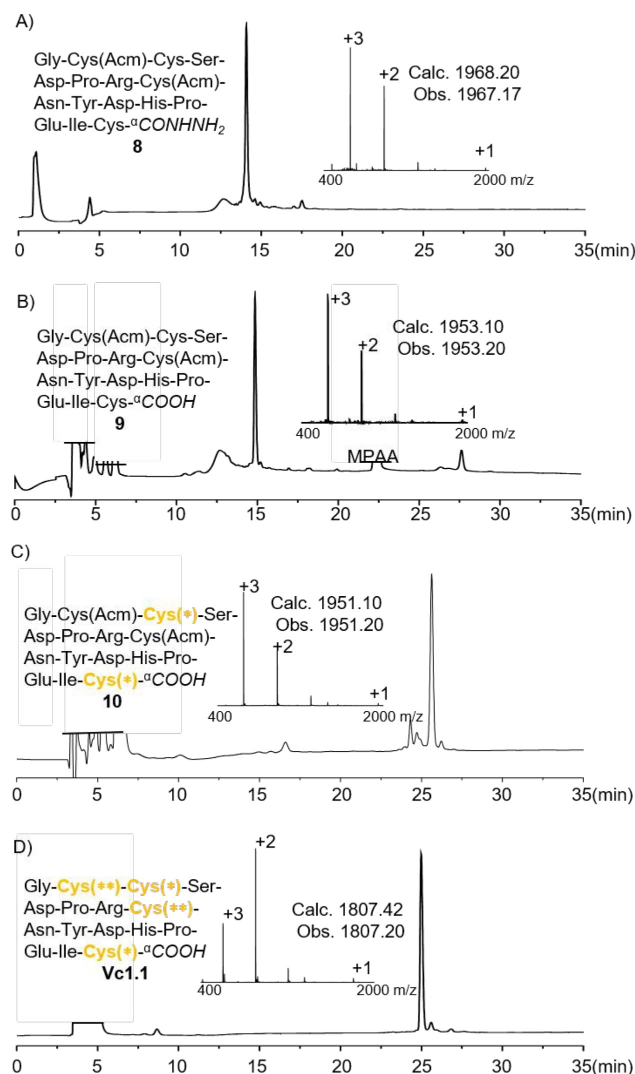

Fig. 5. Analytic HPLC traces (210 nm) of A) crude Vc1.1 hydrazide (**8**); B) Vc1.1 acid (**9**) after mercaptoethanol-mediated hydrolysis of **8**; C) Vc1.1 **10** under the redox conditions of GSH/GSSG, with a disulfide bond formed between ³Cys(*) and ¹⁶Cys(*); D) correctly folded Vc1.1 after I_2-treatment, with a disulfide bond formed between ²Cys(**) and ⁸Cys(**).

With the optimized conditions in hand, we next tested the applicability of the new method for synthesis of somatostatin. This 14 amino acid C-terminal Cys-containing peptide hormone is used to regulate the endocrine system and has clinical use in inhibiting various tumours.[12] By using hydrazine-trityl(2-Cl) resin, we obtained the somatostain hydrazide (**6**) with a HPLC purity higher than 75% (**Fig. 4**). We did not observe any byproducts from epimerization and β-elimination. At pH 3, **6** was oxidized by $NaNO_2$ and subsequently reacted with MPAA to form the somatostatin MPAA-thioester. Excessive mercaptoethanol were immediately added, and the pH value was adjusted to 8. To our delight, the HPLC yield of the unfolded somatostatin acid (**7**) reaches up to 98%. By the assistance of I_2, two Cys residues formed an intramolecular disulfide bond,

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providing the correctly folded somatostain with an isolated yield of 68% (**Fig. 4**).

Furthermore, by using the same strategy, we also obtained the host-defense skin peptide Riparin 1.1b containing one disulfide bond (**Fig. S12**).[13] As expected, chemical synthesis of Riparin 1.1b was smooth. Riparin 1.1b hydrazide was readily prepared from the hydrazine-trityl(2-Cl) resin in an isolated yield of 73%. Riparin 1.1b hydrazide was cleanly converted to Riparin 1.1b acid in a high isolated yield (71%) through the $NaNO_2$/MPAA/mercaptoethanol treatment. Under the GSH/GSSG condition, Riparin 1.1b peptide acid was efficiently folded into the desired Riparin 1.1b with an isolated yield of 65%.

Finally, we tested the utility of the new method for synthesis of α-conotoxin Vc1.1 containing two pairs of disulfide bonds. This 16 amino acid peptide can alleviate neuropathinc pain in several rat models.[14] The Vc1.1 hydrazide (**8**) was readily prepared by Fmoc-based solid phase synthesis using hydrazine-trityl(2-Cl) resin with an isolated yield of 68% (**Fig. 5**). After sequential treatment with $NaNO_2$, MPAA and mercaptoethanol, this polypeptide hydrazide was almost quantitatively converted to the unfolded Vc1.1 acid (**9**). Under the redox conditions of GSH/GSSG, a disulfide bond was formed between Cys3 and Cys16, affording **10** in a high analytical yield (82%). Upon addition of I_2, the side chain Acm-protecting group of Cys2 and Cys8 was removed, followed by the formation of the second disulfide bond (**Fig. 5**). The desired Vc 1.1 was isolated in 70% yield.

To summarize, we have developed a new robust strategy for the synthesis of C-terminal Cys-containing peptide acids. The peptide hydrazide-based strategy has the following advantages: 1) C-terminal Cys hydrazide peptides can be readily prepared by using low-cost hydrazine-trityl(2-Cl) resin; 2) Fmoc SPPS of C-terminal Cys hydrazide peptides does not show any side reactions of epimerization and β-elimination at the C-terminal Cys; 3) through the $NaNO_2$/MPAA/mercaptoethanol treatment, C-terminal hydrazide peptides can be cleanly converted to the desired C-terminal Cys peptide acids. The utility of this method has been demonstrated in the synthesis of C-AhPDF1.1b, somatostatin, Riparian 1.1b, and α-conotoxin Vc1.1 acid. Taken together, the new method offers an efficient and practical strategy for the preparation of C-terminal Cys peptide acids. Use of the new method for the development of peptide therapeutics and diagnositics is undergoing in our laboratory and will be reported in due course.

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Conflicts of interest

There are no conflicts to declare.

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