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Synthesis of Cysteine-Rich Peptides by Native Chemical Ligation without Use of Exogenous Thiols

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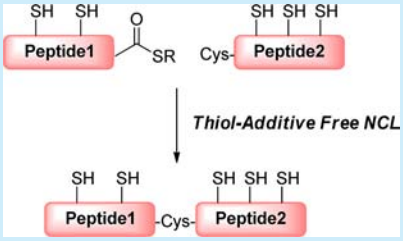
Supporting Information

ABSTRACT: Native chemical ligation (NCL) performed without resorting to the use of thiol additives was demonstrated to be an efficient and effective procedure for synthesizing Cys-rich peptides. This method using tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent facilitates the ligation reaction even at the Thr-Cys or Ile-Cys site and enables one-pot synthesis of Cys-rich peptides throughout NCL and oxidative folding.



Disulfide bond formation is a post-translational modification that plays an important role in the stabilization of the native conformation of peptides and proteins. Particular combinations with disulfide bonding in Cys-rich peptides are critical for expressing their intrinsic biological activities.[1] In particular, the venoms of snakes, scorpions, spiders, and marine snails have proven to be a cornucopia of biologically active Cys-rich peptides, which can be used not only as specific inhibitors for enzymes but also as subtype-specific ligands for receptors and ion channels.[2] The chemical peptide synthesis of such Cys-rich peptides holds much promise for (1) confirming the reported primary structures including disulfide structures, (2) obtaining much larger amounts of toxins than can be isolated from natural sources, (3) studying three-dimensional structures by CD and NMR to understand the molecular basis of subtype specificity, and (4) studying the development of analogues/therapeutics with activity and selectivity superior to the original natural product. In the course of synthesizing Cys-rich peptides, the success of the oxidative folding greatly depends on the purity of the starting reduced peptide. To obtain reduced peptides of high quality by solid-phase peptide synthesis (SPPS), the segment condensation strategy using native chemical ligation (NCL)[3] is one of the most promising procedures for excluding side products, i.e., terminated and truncated peptides arising during chain elongation on a solid support, because assembly of the entire molecule can be carried out with purified and well-characterized segments. In addition, Cys-rich peptides present a relatively large number of options for the ligation site of NCL (Xaa-Cys).[4] In general, peptide thioesters are straightforwardly prepared by SPPS in the form of less active peptide alkylthioesters because of their ease of preparation and handling during chain elongation and purification and are subsequently subjected to the NCL reaction in the presence of exogenous thiols, e.g., thiophenol (PhSH)[5] and 4-mercaptophenylacetic acid (MPAA)[6] in many cases, to promote the in situ formation of more active peptide arylthioesters. However, PhSH is characterized by an unpleasant odor and high toxicity, and MPAA in the practical peptide synthesis hampers the purification procedure using HPLC as it can frequently coelute with the ligated products, resulting in lower purification efficiency and isolated yields.[7] To avoid the problem associated with the use of MPAA, several measures have been reported.[8] Moreover, for proper progress of the subsequent oxidative folding reaction, special care has to be taken to prevent the ligated products from being contaminated by the thiols used for NCL. In the present study, we demonstrated the synthesis of Cys-rich peptides by NCL using the ordinary peptide alkylthioester (e.g., peptide-mercaptopropionyl-Leu-NH_2, also named peptide-MPAL) without resorting to the use of exogenous thiols. This enabled a one-pot approach to achieve a folded peptide throughout NCL and oxidative folding.

The rate of the ligation is greatly dependent on the nature of the *C*-terminal amino acid in the peptide thioester.[9] NCL performed at sterically hindered, branched *C*-terminal residues of peptide thioesters, such as Thr, Val, or Ile, often causes a significant reduction in transthioesterification with the Cys sulfhydryl group and results in a low yield even in the presence of a highly reactive catalyst, MPAA. Therefore, in order to demonstrate the utility of the thiol-free NCL we used it to synthesize protoxin I (ProTx-I), a 35-residue peptide having three disulfide bonds isolated from the venom of the tarantula *Thrixopelma pruriens*,[10] and its analogue [Ile^{14}]-ProTx-I by coupling between (1−14)-MPAL (N-segment) and the Cys^{15}-(16−35)-peptide (C-segment) at the Thr-Cys and Ile-Cys sites, respectively. In the NCL reaction of Cys-rich peptides, the excess amount of exogenous MPAA or PhSH is considered to be essential to reverse "nonproductive" side reactions, i.e., thiolactone formation with internal Cys residue(s) of the N-

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segment and transthioesterification with Cys not located on the *N*-terminus of the C-segment,[4] promoting the formation of active peptide arylthioesters. In addition, tris(2-carboxyethyl)-phosphine (TCEP) as a reducing agent has to be included in the NCL reaction mixture to prevent the formation of disulfide bonds, especially when using MPAA, as it has less reducing potential. On the other hand, Tam et al. pointed out that a trialkylphosphine activates the thioester as a phosphonium salt to accelerate the thiol-thioester exchange.[11] This prompted us to examine the effect of TCEP on the ligation rate by using a simple NCL system where both N- and C-segments of ProTx-I have their side chain thiols of Cys protected by the acetamidomethyl (Acm) group except for that located at the ligation site. Thus, in this system there was no chance for the formation of the "nonproductive" thioester intermediates described above.

The reaction between **1** (2.6 mM) and **2** (2.0 mM) was examined in the commonly used ligation buffer (100 mM Na_2HPO_4 + 6 M Gn·HCl, pH 7.8) containing sodium ascorbate (100 mM) by varying the concentration of TCEP (Figure 1). Sodium ascorbate was included to prevent the

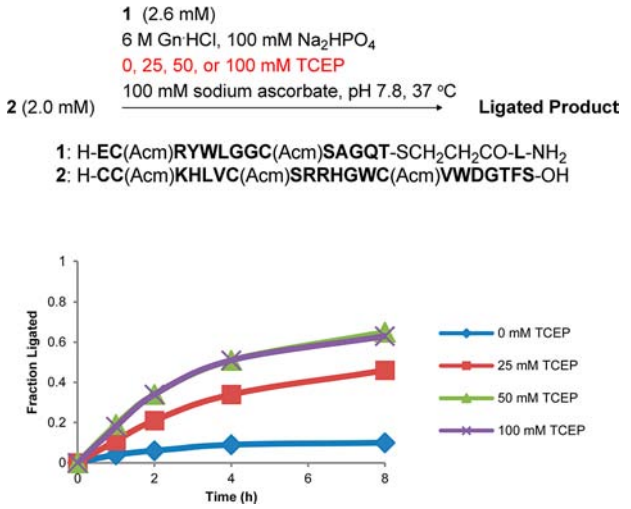


Figure 1. Progress of the NCL reaction between **1** and **2**: The extent of ligation was quantified by integration of the ligated product as a fraction of the sum of the unreacted C-segment peptide **2** and the ligated product by HPLC (220 nm).

TCEP-induced desulfurization both of the starting Cys-containing peptides and the ligated products.[12] The ligation rate increased as the concentration of TCEP increased with no difference being observed in the enhancing effect beyond 50 mM TCEP. This finding suggested that TCEP included in ligation media helps not only to maintain reducing environments but also to increase the reactivity of peptide thioesters. In view of this, we employed a ligation buffer containing more than 50 mM for additional examinations.

Next, we synthesized the ProTx-I molecule in reduced form by ligating the fully unprotected N-segment thioester **3** and C-segment **4** at the Thr-Cys site and compared the reaction profile obtained by the thiol-free NCL with those by the standard NCL using MPAA/PhSH (Figure 2).

Surprisingly, in terms of the ligation rate and yield, the thiol-free NCL was superior to NCL using PhSH and comparable to NCL using MPAA. Moreover, it is worth noting that the thiol-free NCL could be effectively accomplished even at the Ile-Cys

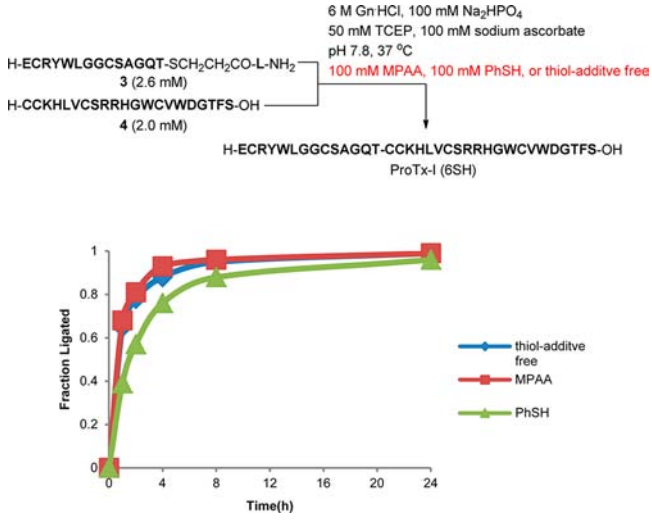
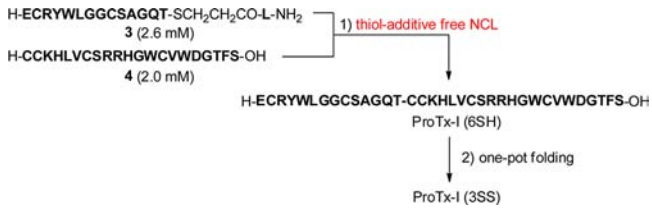


Figure 2. Effect of thiols on the NCL reaction between **3** and **4**: The extent of ligation was quantified by integration of the ligated product as a fraction of the sum of the unreacted C-segment peptide **4** and the ligated product by HPLC (220 nm).

site to produce the reduced [Ile[14]]-ProTx-I in a high yield (Figure S2, Supporting Information). Furthermore, the utility of this procedure was confirmed by synthesizing the reduced kurtoxin (63 amino acids)[13] and orexin A (33 amino acids)[14] ligated at Leu[26]-Cys[27] and Thr[11]-Cys[12], respectively, in the absence of thiol additives (Figures S4 and S6, Supporting Information). As for a one-pot approach throughout NCL and oxidative folding, the reduced ProTx-I thus obtained was subjected to the oxidative folding reaction by diluting the ligation mixture with 1 M NH_4OAc buffer (pH 7.8) containing 1 M Gn·HCl in the presence of reduced and oxidized gluthathione (GSH/GSSG). The reaction predominantly provided the folded ProTx-I under thermodynamic control (Scheme 1 and Figure 3). This freely oxidized product was confirmed to possess the native disulfide connectivity by a chemical procedure (see the Supporting Information).

Scheme 1. One-Pot Synthesis of ProTx-I[a]



[a]Details can be found in the Supporting Information.

The product was isolated and purified using RP-HPLC in 56% yield. From these results, we were able to demonstrate that thiol-free NCL in the presence of TCEP can be used for the synthesis of Cys-rich peptides, even when this is done at the Ile-Cys or Thr-Cys site, and it also enables one-pot synthesis throughout NCL and oxidative folding.

To grasp the scope and limitations of applying the present procedure for synthesizing Cys-rich peptides, the ligation efficiency was evaluated and compared by conducting the thiol-free NCL at the Thr[14]-Cys[15] site with combinations of the following N-/C-segments of ProTx-I (Figure 4): the coupling between (A) **1** and **2**, both having all Cys protected except for

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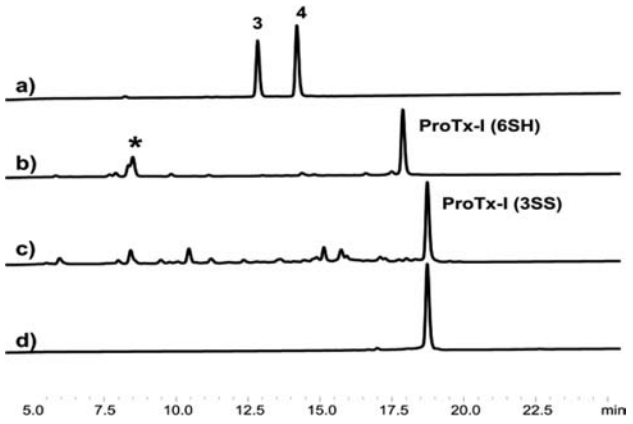

Figure 3. HPLC profiles of the reaction for one-pot synthesis of ProTx-I (3SS): (a) NCL (t = 0 h), (b) NCL (t = 20 h), (c) folding reaction (t = 24 h), (d) purified product. HPLC conditions: column, DAISO-PAK SP-120-5-ODS-BIO (4.6 × 150 mm); elution, 20−40% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm. *Thiolactone and hydrolysate of the N-segment.

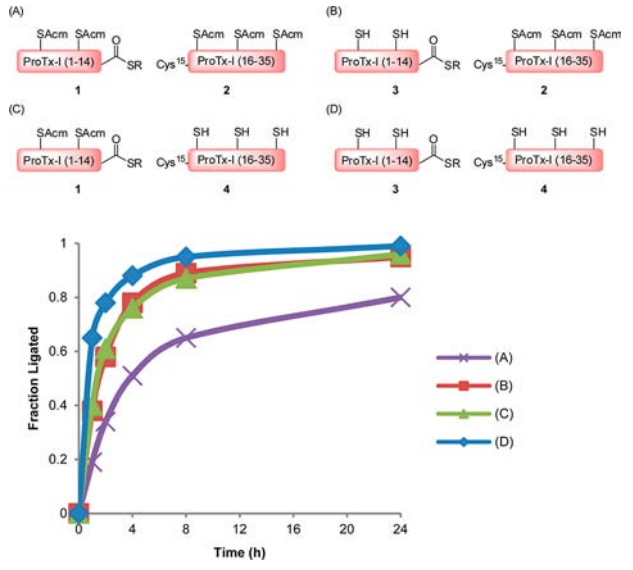

Figure 4. Progress of the thiol-free NCL between the N-segment thioester (2.6 mM) and C-segment (2.0 mM) of ProTx-I: (A) **1** + **2**, (B) **3** + **2**, (C) **1** + **4**, (D) **3** + **4**.

Cys[15], (B) **3** and **2**, the former having all Cys unprotected, (C) **1** and **4**, the latter having all Cys unprotected, and (D) **3** and **4**. In the case of A, the reaction could not be completed within 24 h, while in all other cases, the reactions approached completion. This clearly indicated that NCL could proceed without resorting to the use of thiols if either segment, the N- or C-one,[15] or both carried free thiol group(s) on Cys besides that on Cys[15]. Thus, we assumed that "nonproductive" thiolactone and thioester intermediates formed with the sulfhydryl group of Cys intra- and intermolecularly, respectively, could participate in the NCL reaction as reactive species to produce the ligated product. In fact, thiolactone and thioester intermediates generated upon the initiation of NCL without any thiol additive decreased as the respective ligated products increased in the case of B and C, respectively, in the HPLC detection. This finding could be clearly explained by the model experiment with **5** and **6** representing thiolactone and thioester intermediates, respectively (Figure 5).

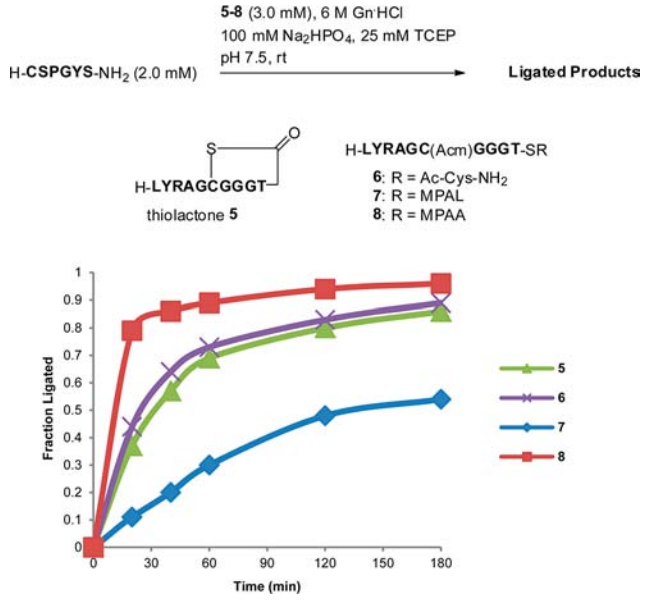

Figure 5. Progress of the NCL reaction with the model peptide thioesters **5**−**8**: The extent of ligation was quantified by integration of the ligated product as a fraction of the sum of the unreacted C-segment peptide and the ligated product by HPLC (220 nm).

These intermediates **5** and **6** were found to possess a higher reactivity than MPAL-thioester **7**, although they showed a slight reduction in the ligation rate when compared with MPAA-thioester **8**. It is considered that the pK_a of thiols incorporated in thioesters could be related to the transthioesterification potential; a thiol with lower pK_a should function as a favorable leaving group to promote the NCL reaction. Thus, ligation with **7** (pK_a of mercaptopropionic acid, >10) was substantially slower than that with **5** and **6** (pK_a of the side-chain thiol of Cys estimated from GSH, 8.7), and MPAA with a pK_a of 6.6 resulted in rapid ligation.[6,16] The pK_a of the side chain of Cys would be reflected by its surrounding environment since the Cys residue located in the active site of protein disulfide isomerase is known to show a low value (pK_a 6.7).[17] Therefore, if we are able to design a Cys-containing peptide with a lower pK_a of its side-chain thiol and to incorporate it into thioesters, this should offer a useful alternative to alkylthioesters for thiol-free NCL. It was formerly thought that the thiolactone and thioester intermediates generated during NCL stagnate the reaction progress. On this account, the addition of thiols such as MPAA or PhSH is recommended to reverse these "nonproductive" intermediates and, in turn, promote the formation of active arylthioester, especially in the case of Cys-rich peptides. However, our results clearly showed that these "nonproductive" intermediates can participate in NCL as reactive species. Finally, we concluded that thiol-free NCL in the presence of TCEP can support highly efficient synthesis of Cys-rich peptides.

In summary, we successfully synthesized Cys-rich peptides such as ProTx-I, [Ile[14]]-ProTx-I, kurtoxin, and orexin A by employing NCL without resorting to the use of thiol additives. Thiol-free NCL in the presence of TCEP facilitates the synthesis of Cys-rich peptides and also allows a one-pot procedure throughout NCL and oxidative folding. We are currently trying to develop a Cys-containing peptide with a side chain thiol having a lower pK_a for incorporation into thioesters

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to obtain a highly reactive alkylthioester applicable to thiol-free NCL.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, characterization, and spectroscopic and chromatographic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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