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Dual Kinetically Controlled Native Chemical Ligation Using a Combination of Sulfanylproline and Sulfanylethylanilide Peptide

Hao Ding, Akira Shigenaga, Kohei Sato, Ko Morishita, and Akira Otaka*

Institute of Health Biosciences and Graduate School of Pharmaceutical Sciences, The University of Tokushima, Shomachi, Tokushima 770-8505, Japan

aotaka@ph.tokushima-u.ac.jp

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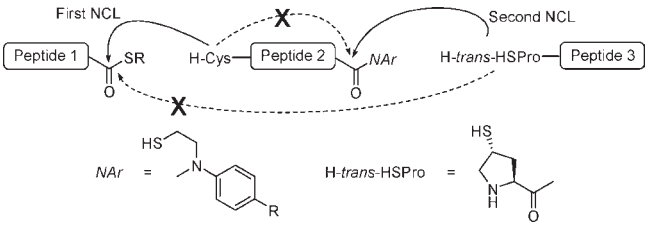
ABSTRACT



Dual kinetically controlled native chemical ligation using a newly developed sulfanylproline-mediated reaction in combination with an *N*-sulfanylethylanilide peptide was successfully applied to a previously unreported sequential coupling of peptide fragments added simultaneously to the reaction.

Native chemical ligation (NCL) has had a significant impact on protein/peptide chemistry.[1] The original NCL protocol requires two peptide segments: one is a thioester peptide and the other is an N-terminal cysteinyl peptide. Chemoselective reactions between the thioester and N-terminal cysteine, including successive $S-S$ and $S-N$ acyl transfers, yield the corresponding ligated product. One potential limitation of the original NCL is that the ligation site requires cysteine. The feasibility of NCLs at noncysteinyl sites has been extensively explored, including NCLs at alanine,[2,3] valine,[4] phenylalanine,[5] lysine,[6] leucine,[7,8] and threonine sites.[9] Essentially, such NCLs feature the original NCL-like reaction of β- or γ-sulfanylamino acid

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residues with a thioester, followed by desulfurization. Other options without the desulfurization reaction have also been reported.[10] Our efforts to synthesize a high-proline-content GM2-activator protein (GM2-AP)[11] have prompted us to examine NCL at the proline site. Very recently, Danishefsky and co-workers published the results of their research on a proline ligation involving an NCL-like reaction between *trans*-4-sulfanylproline (*trans*-HSPro) and a thioester precursor bearing a 2-ethyldithiophenyl ester moiety.[12] We were interested in studying ligation chemistry at a proline surrogate possessing a sulfanylated tether on 4-hydroxyproline (HOPro). In the course of this study, we prepared an ethoxythiocarbonylated HOPro as a requisite synthetic intermediate and fortuitously found that the *O*-ethoxythiocarbonyl group was smoothly transferred to the amino group in aqueous solution whereas such migration hardly occurred in an organic solvent (DMF in the presence of diisopropylethylamine (DIPEA)) (Figure 1). This finding encouraged us to attempt NCL at the proline site using HSPro. In this report, we will place special emphasis upon the utility of HSPro-mediated ligation with application to an unprecedented dual kinetically controlled reaction.

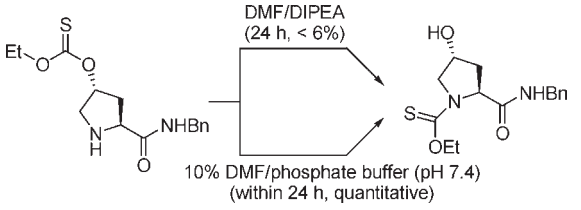
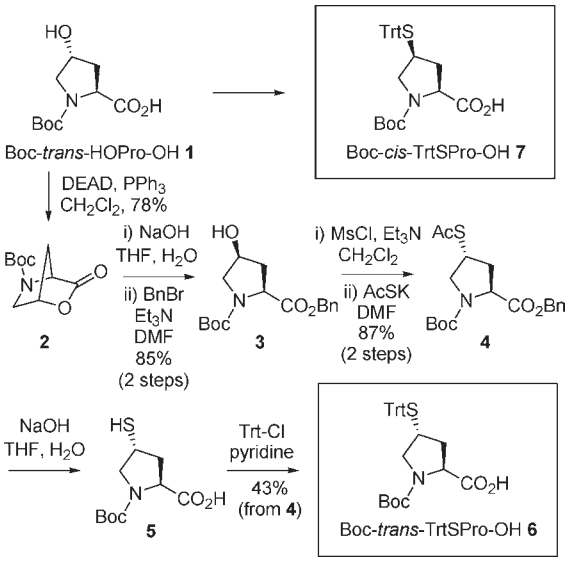


Figure 1. Unexpected *O*−*N* ethoxythiocarbonyl transfer.

The first step was the syntheses of the protected forms (**6** and **7**) of both possible diastereomers, i.e., the (4*R*)-sulfanylpyrrolidine-(2*S*)-carboxylic acid (H-*trans*-HSPro-OH) and its 4*S* diastereomer (H-*cis*-HSPro-OH), as proline surrogates, from Boc-*trans*-HOPro-OH (**1**), as shown in Scheme 1.[13] An intramolecular Mitsunobu reaction of **1**, followed by saponification and subsequent protection of the carboxy group, gave *cis*-hydroxyproline derivative **3**. The secondary hydroxyl group was subsequently converted to the *S*-acetyl group, with inversion of the configuration, by the action of methanesulfonyl chloride (MsCl) in the presence of Et_3N, followed by treatment with potassium thioacetate (AcSK). Alkaline hydrolysis of the ester **4**, followed by protection of the resulting sulfanyl group with triphenylmethyl chloride (Trt-Cl) in pyridine,

Scheme 1



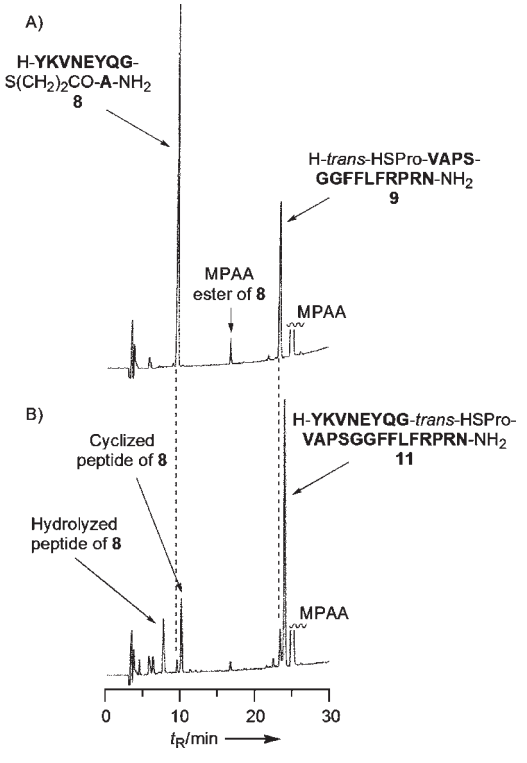


Figure 2. HPLC monitoring of *trans*-sulfanylproline (*trans*-HSPro)-mediated ligation for the synthesis of NMU. (A) Ligation (t = 0 min): thioester **8** (1.5 mM) and *trans*-HSPro peptide **9** (1.0 mM) were ligated. (B) Ligation (t = 14 h). HPLC conditions are described in the Supporting Information.

produced the desired *trans*-HSPro derivative **6**. An identical sequence of reactions to that used for conversion of **3** to **6** was applied to **1** to synthesize the *cis*-HSPro derivative **7**.

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With both diastereomers **6** and **7** available, we evaluated the applicability of the proline surrogates to NCL followed by desulfurization in the synthesis of rat neuromedin U[14] (NMU: H-**YKVNEYQGPVAPSGGFFLFRPRN**-NH_2), which consists of 23 amino acid residues. Initially, ligations were performed between the Gly8-Pro9 site by reactions of alkylthioester peptide **8**, corresponding to NMU (1−8), with N-terminal *trans*-HSPro peptide **9** or *cis*-HSPro peptide **10**, covering NMU (9−23). The alkylthioester (-SCH_2CH_2CO-**A**-NH_2) **8** was synthesized by Boc solid phase peptide synthesis (SPPS).[15] The HSPro-containing peptides **9** and **10** were prepared from Fmoc SPPS using **6** and **7**, respectively. Ligation of 1.5 equiv of **8** with *trans*-HSPro peptide **9** under standard NCL conditions (6 M guanidine hydrochloride (Gd·HCl)/0.1 M phosphate buffer (pH 7.4) in the presence of 30 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 30 mM 4-(carboxymethyl)thiophenol (MPAA)[16] at 37 °C) unambiguously proceeded to yield the corresponding ligation product **11** in 54% isolated yield along with a hydrolyzed product, cyclized product, and MPAA ester of **8** (Figure 2); this is because ligation of the *trans*-HSPro peptide with **8** proceeds more slowly than ligation of the N-terminal cysteinyl peptide.[17] The use of the *cis*-HSPro peptide **10** did not afford a detectable amount of the desired ligation product.[12] Compound **11** was then subjected to desulfurization[3d] with VA-044 in 6 M Gd·HCl/0.1 M phosphate buffer (pH 7.0) in the presence of 40 mM TCEP and 10 mM reduced-form glutathione (GSH) at 37 °C. Reaction for 20 h, followed by HPLC purification, gave the desired NMU in 70% isolated yield. These results clearly indicated that *trans*-HSPro-mediated ligation has a firmly established place among NCL protocols.

Next, we examined the difference in reactivities of **9** and its cysteinyl counterpart peptide **12** to the peptide thioester **8**. Reactions of **8** with 1 equiv of **9** or **12** in 6 M Gd·HCl/0.1 M phosphate buffer (pH 7.4) in the presence of 30 mM TCEP and 30 mM MPAA (or 50 mM sodium ascorbate[18]) at 37 °C were performed (Figure 3). Ligation of the cysteinyl peptide **12** in the presence of MPAA proceeded essentially to completion in 30 min, whereas the same ligation using *trans*-HSPro required more than 360 min to reach a plateau. Addition of MPAA to the reaction with **9** accelerated the ligation, even though the reaction was promoted to a lesser extent than in the case of the cysteinyl peptide.

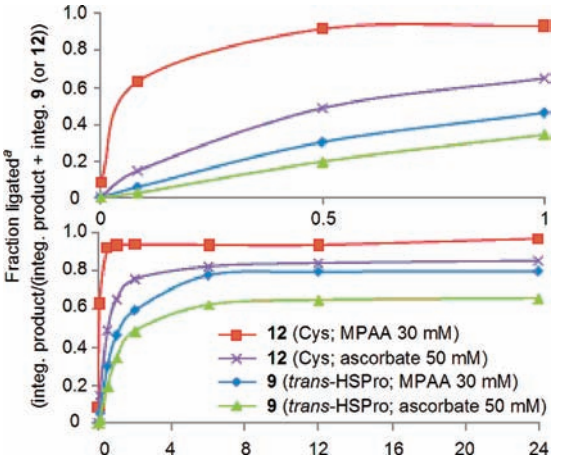

Figure 3. Examination of the difference in reactivities of the *trans*-HSPro and Cys-containing peptide (**9** and **12**) to alkylthioester peptide **8**. [a]The fraction ligated was determined by HPLC separation and integration of ligated product (integ. product) detected at 220 nm as a fraction of the sum of the unreacted sulfanyl peptide (integ. **9** or **12**) + integ. product. Top: reaction time range 0−1 h; bottom: reaction time range 0−24 h.

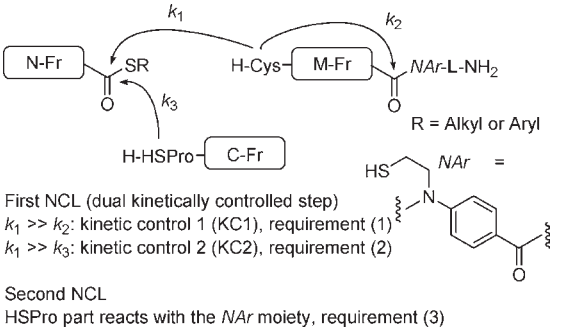

Figure 4. Requirements for the successful coupling of three peptide fragments mixed simultaneously.

Recently, we developed a kinetically controlled NCL[19] using *N*-sulfanylethylanilide (SEAlide) peptides,[20] which function as cryptic thioester peptides,[20b,21] in which excellent selectivity can be achieved. Based on the use of such SEAlide peptides, we expected that ordered couplings of three fragments added in one pot simultaneously should be possible. In this protocol, three peptide fragments (N-terminal fragment (Fr): N-Fr; middle Fr: M-Fr; C-terminal Fr: C-Fr) simultaneously present in the reaction are sequentially ligated in an N-to-C-directed manner. The

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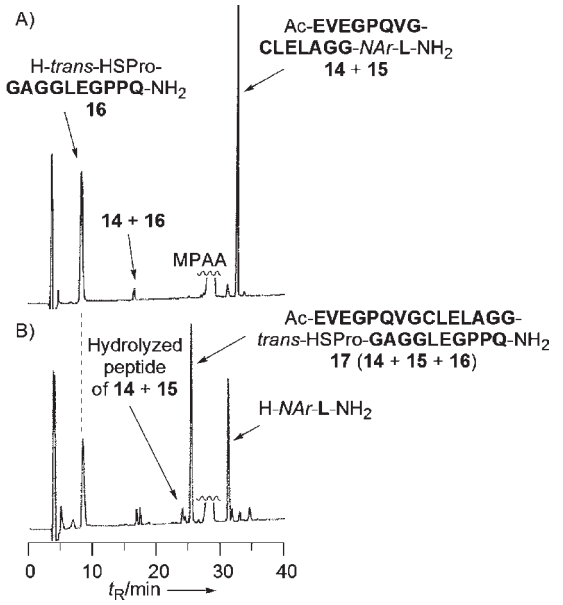

Figure 5. HPLC monitoring of ligations for the synthesis of the insulin C peptide. (A) First ligation (t = 2 h): Peptide fragments **14** (4 mM), **15** (4 mM), and **16** (8 mM) were simultaneously mixed. (B) Second ligation (t = 24 h): After 2 h of the first ligation, the second ligation was initiated by the addition of phosphate buffer. HPLC conditions are described in the Supporting Information.

anticipated reaction should succeed in yielding a correctly ordered peptide by satisfying all of the following requirements (Figure 4). (1) The cysteinyl residue in M-Fr intermolecularly reacts with the thioester in N-Fr, not with the intramolecular SEAlide moiety ($k_1 \gg k_2$: kinetic control 1 (KC1)). (2) Of the two sulfanyl amino acids, the thioester in N-Fr preferentially reacts with the cysteinyl residue in M-Fr ($k_1 \gg k_3$: kinetic control 2 (KC2)). (3) The *trans*-HSPro moiety in C-Fr reacts with the SEAlide unit under appropriate conditions.

Requirement (1) has already been confirmed to be satisfied by our recent investigation.[20b] With respect to requirement (2), the aforementioned experimental results (Figure 3) seem to meet this requirement. In order to confirm the possible involvement of the *trans*-HSPro in the SEAlide-mediated NCL, peptide **9** was subjected to a model NCL with a SEAlide peptide (H-**VQGSG**-*NAr*-**L**-NH_2 **13**) in 3 M Gd·HCl/0.5 M phosphate buffer (pH 7.4) in the presence of 30 mM TCEP and 30 mM MPAA at 37 °C. The reaction proceeded with an efficiency comparable to that observed in the NCL of **8** with **9**.

On the basis of these observations, we next sought to synthesize the bovine insulin C peptide (Ac-**EVEGPQVG ALELAGGPGAGGLEGPPQ**-NH_2) by establishing a dual kinetically controlled NCL (with simultaneous satisfaction of KC1 and KC2), along with the use of an HSPro-peptide in the SEAlide-mediated NCL (Figure 5). The three requisite peptide fragments, i.e., Ac-**EVEGPQVG**-SCH_2CH_2CO-**A**-NH_2 (**14**; 4 mM), H-**CLELAGG**-*NAr*-**L**-NH_2 (**15**; 4 mM), and H-*trans*-HSPro-**GAGGLEGPPQ**-NH_2 (**16**; 8 mM), were simultaneously mixed in 6 M Gd·HCl/0.2 M sodium 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonate (HEPPS) buffer (pH 7.8) in the presence of 100 mM TCEP and 50 mM MPAA at 37 °C. The first NCL between **14** and **15** proceeded under dual kinetic control to completion within 3 h to afford the ligated peptide **14** + **15**. Then the second ligation was initiated by the addition of 1 M phosphate buffer (pH 7.8)[20b,22] at 37 °C (final concentration: peptide **14** + **15** (ca. 2 mM) and **16** (4 mM) in 3 M Gd·HCl/0.1 M HEPPS/0.5 M phosphate, 50 mM TCEP, and 25 mM MPAA). The addition of phosphate and continuous reaction for 24 h allowed the SEAlide unit to function as a thioester, yielding the correctly ligated C peptide precursor **17** in 50% isolated yield. Peptide **17** was subsequently subjected to a desulfurization reaction[3d] identical to that employed for NMU to afford the bovine insulin C peptide in 78% isolated yield.

In conclusion, we achieved the synthesis of protected HSPro derivatives and developed an HSPro-mediated NCL reaction based on an unexpected $O-N$ thiocarbonyl transfer. A combination of the proline ligation and the SEAlide-peptide-mediated NCL allowed three peptide fragments to be correctly ligated in a dual kinetically controlled manner. Further extensions of the kinetic reaction are currently underway.

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Supporting Information Available. Experimental procedures and NMR spectra for key compounds. This material is available free of charge via the Internet at http://pubs.acs.org.