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One-Pot/Sequential Native Chemical Ligation Using Photocaged Crypto-thioester

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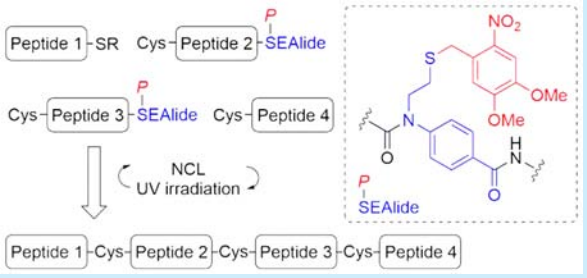
Supporting Information

ABSTRACT: A practical and efficient methodology for the chemical synthesis of peptides/proteins using a one-pot/sequential ligation is described. It features the use of photocleavable *S*-protection on an *N*-sulfanylethylaniline moiety. Removal of the *S*-protecting ligated materials under UV irradiation provides a readily usable mixture for subsequent native chemical ligation.



Protein chemical synthesis has great potential as a chemical means for elucidating a wide range of protein functions.[1] Native chemical ligation (NCL), which has been widely used for the synthesis of proteins, is among the most powerful methodologies.[2] NCL involves a chemoselective ligation between N-terminal cysteinyl and C-terminal thioester fragments through an initial transthioesterification, followed by a rapid intramolecular S → N acyl transfer, to generate a native peptide bond under mild conditions.

Recent and ever-increasing application of NCL to protein chemical synthesis has prompted the development of a one-pot/sequential NCL in a C−to−N- or N−to−C-directive manner. One-pot synthesis decreases the risk of product loss that would otherwise have resulted from HPLC purification following every round of NCL.[3] Generally, N-terminal cysteinyl thioesters, with an *N*-and/or *S*-protection on the cysteine, which are readily removed without affecting the progress of NCL, are used in the C−to−N-directive protocol.[4,5] Alternatively, Kent and co-workers developed the kinetically controlled N−to−C-directive NCL (KCL) protocol which exploits the difference in reactivity between alkyl thioester and aryl thioester fragments.[6] This research has encouraged research groups[7] including ours to develop other versions of the N−to−C protocol. In this context, we previously developed an *N*-sulfanylethylanilide (SEAlide) peptide **1** as a crypto-thioester for the N−to−C-directive one-pot/sequential three-fragment ligation.[8,9] In our one-pot protocol, N-terminal cysteinyl SEAlide peptide initially reacts with a thioester in the absence of a phosphate salt to afford the first ligation product; then the resulting SEAlide peptide in the reaction mixture is converted to a thioester form **2** and allowed to react with N-terminal cysteinyl peptide only by the addition of a phosphate salt (Figure 1).

However, use of the SEAlide peptide in a one-pot/sequential ligation involving more than three fragments has yet to be achieved.[10] With the intention of adding the SEAlide peptide to a multifragment ligation, we planned to use a photocleavable protecting group on the free thiol group of the SEAlide moiety. Recently, the 6-nitroveratryl (NV) group was reported to be a useful protecting group in peptide chemistry, because it can be readily removed by UV light under mild conditions.[11] Thus, we envisioned that the use of a photocleavable protective group on the SEAlide moiety should contribute the establishment of a sequential NCL without adding any reagents such as phosphate salts that modify the reactivity of SEAlide. That is, UV irradiation after each NCL step would allow sequential NCL to proceed in a one-pot manner as shown in Figure 2. Herein, we report a new one-pot/four-fragment sequential ligation method

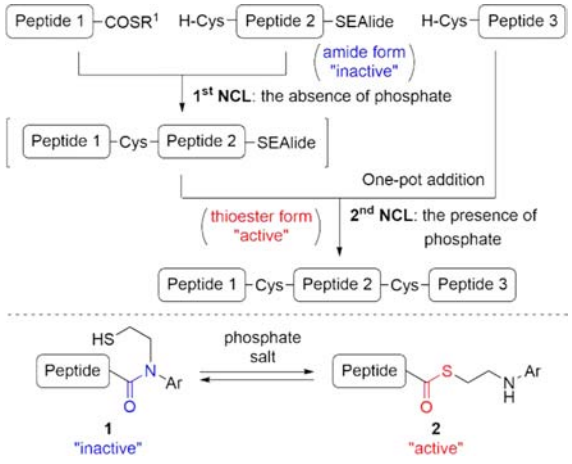


Figure 1. An outline of one-pot/sequential three-fragment NCL using SEAlide peptide.

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using a photocaged SEAlide peptide, which enables the synthesis of protein in the N-to-C direction.

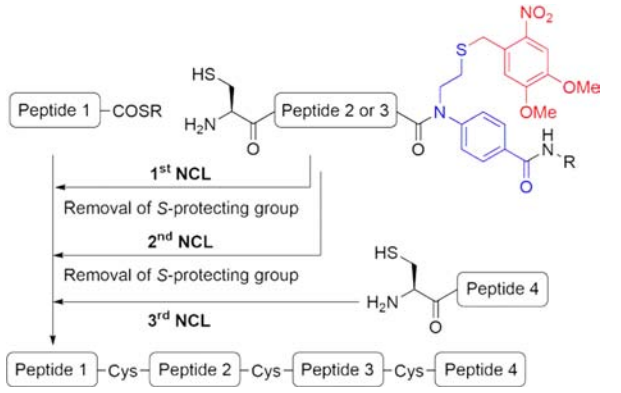
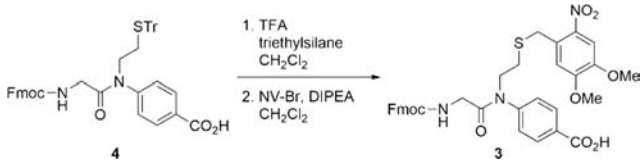


Figure 2. Strategy of peptide/protein synthesis using photocaged SEAlide peptide.

First, we synthesized the requisite photocaged SEAlide **3** as shown in Scheme 1. Deprotection of the trityl (Tr) group of

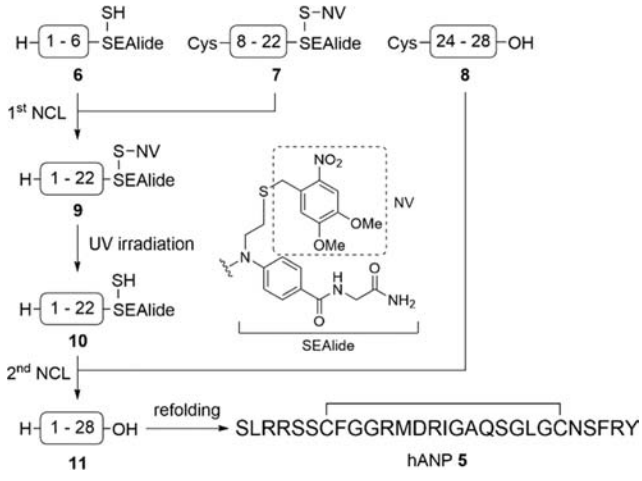
Scheme 1. Synthesis of the Caged SEAlide



Fmoc-Gly-SEAlide **4** with trifluoroacetic acid (TFA)-triethylsilane in CH_2Cl_2, followed by reprotection with 6-nitroveratryl bromide (NV-Br)-*N*,*N*-diisopropylethylamine (DIPEA), afforded the desired photocaged SEAlide **3** in 67% isolated yield over two steps. Then we attempted to synthesize hANP **5** using this photocaged SEAlide as a crypto-thioester moiety.

The three peptide fragments required for hANP synthesis, N-terminal **6**, middle **7**, and C-terminal **8**, were synthesized using a standard Fmoc solid-phase peptide synthesis (SPPS). Then the resulting fragments were used in a one-pot/sequential three-fragment ligation involving photoremoval of the NV group as shown in Scheme 2. The first ligation between N-

Scheme 2. Synthesis of hANP Using Sequential NCL



terminal **6** and middle **7** fragments under standard NCL conditions (3 M guanidine hydrochloride (Gn·HCl)−0.5 M sodium phosphate buffer (NaPB) (pH 7.0) in the presence of 2% (v/v) thiophenol at 37 °C) proceeded to give the corresponding ligated product **9**. Next we examined the removal of the NV group in a one-pot procedure. Direct UV irradiation of the first ligation mixture gave many byproducts due to the presence of a thiyl radical derived from thiophenol during photolysis. We speculated that the generation of the thiyl radical derived from thiophenol under photolysis conditions is responsible for generating these byproducts. Thus, we examined the extractive removal of thiophenol with Et_2O from the ligation mixture. Removal of thiophenol from the ligation mixture, followed by UV irradiation, resulted in the smooth progress of the reaction to yield the desired uncaged SEAlide peptide **10**. The resulting reaction mixture was directly brought to the second ligation with C-terminal segment **8** under standard conditions (3 M Gn·HCl−0.5 M NaPB (pH 7.0) in the presence of 2% (v/v) thiophenol at 37 °C) to yield a reduced form hANP **11** with high purity. Then removal of thiophenol, followed by dilution and subsequent addition of DMSO (10% (v/v)), allowed the oxidative folding of **11** to yield hANP **5** in 26% isolated yield after HPLC purification (Figure 3).

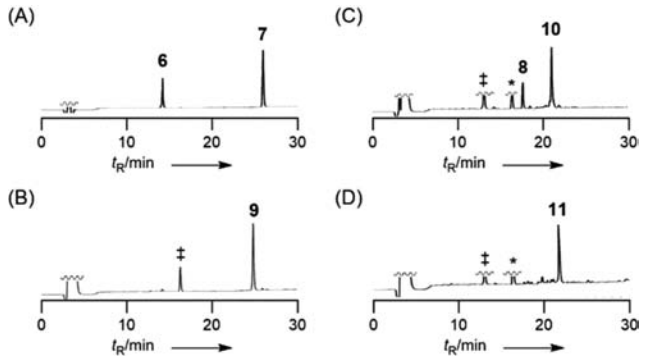


Figure 3. HPLC monitoring of one-pot/three-fragment ligation for the synthesis of hANP. (A) Ligation between **6** and **7** (t < 5 min). (B) Ligation between **6** and **7** (t = 15 h). (C) Ligation between **8** and **10** (t < 5 min). (D) Ligation between **8** and **10** (t = 18 h). ‡Released SEAlide unit. *Nonpeptidic compounds probably derived from photoremoval of the NV unit. HPLC conditions are described in the Supporting Information.

Next, we applied the photocaged SEAlide peptide to a one-pot/four-fragment sequential NCL for the synthesis of reduced form SNX-482 **12**. SNX-482, potent inhibitor of R-type Ca^{2+} channels isolated from the tarantula *Hysterocrates gigas*, contains 41 amino acids and three disulfide bonds.[12] The sequence of SNX-482 was divided in four fragments consisting of SEAlide **13**, photocaged SEAlide **14**, **15**, and N-terminal cysteinyl peptides **16** as shown in Scheme 3. All peptides were prepared by Fmoc SPPS where the isopeptide protocol was used for the preparation of **16** due to its insoluble nature.[13] The first NCL between peptide **13** and **14** under standard NCL conditions (3 M Gn·HCl−0.5 M NaPB (pH 7.0) in the presence of 2% (v/v) thiophenol at 37 °C) yielded a mixture containing ligated peptide **17** which was then subjected to UV irradiation to give peptide **18** after extractive removal of thiophenol. The second NCL for the synthesis of **19** was initiated by addition of fragment **16** and thiophenol to the reaction mixture containing

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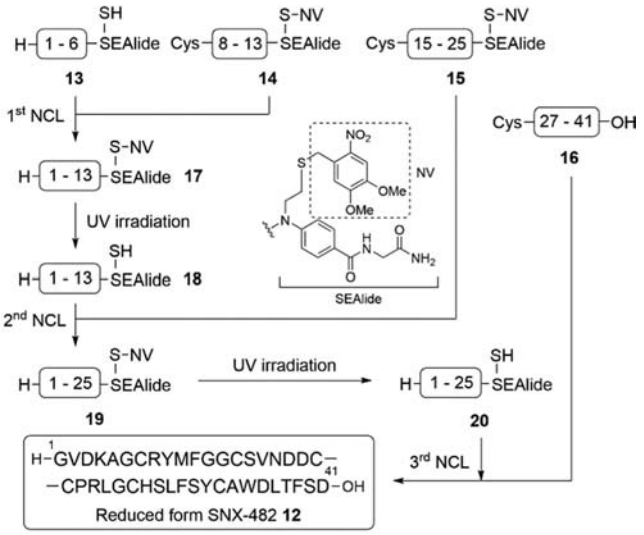
Scheme 3. Synthesis of Reduced Form SNX-482 Using N-to-C Directed Sequential NCL



18. After completion of the ligation, removal of thiophenol and subsequent UV irradiation gave the reaction mixture readily available for the next NCL step. The third NCL was performed in the presence of 80 mM (4-carboxymethyl)thiophenol (MPAA) and 60 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) by the addition of fragment **20** to the reaction mixture to yield the desired reduced form SNX-482 **12** in 24% yield after HPLC purification (Figure 4).

To further explore the potential utility of the photocaged SEAlide peptide, we attempted to synthesize reduced form SNX-482 **12** via a convergent route (Scheme 4). N-Terminally

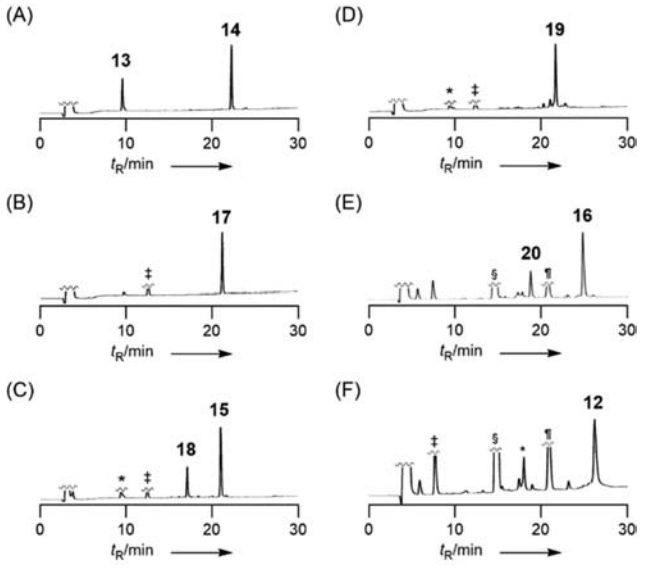
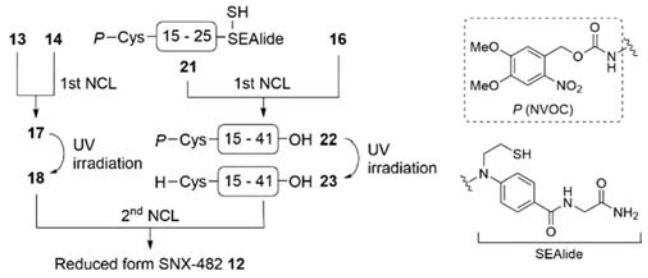


Figure 4. HPLC profiles of sequential ligation for the synthesis of reduced form SNX-482. (A) Ligation between **13** and **14** (t < 5 min). (B) Ligation between **13** and **14** (t = 23 h). (C) Ligation between **15** and **18** (t < 5 min). (D) Ligation between **15** and **18** (t = 20 h). (E) Ligation between **16** and **20** (t < 5 min). (F) Ligation between **16** and **20** (t = 25 h). ‡Released SEAlide unit. §MPAA. ¶Thiophenol. *Nonpeptidic compounds probably derived from photoremoval of the NV unit. HPLC conditions are described in the Supporting Information.

Scheme 4. Convergent Synthesis of Reduced Form SNX-482



6-nitroveratryloxycarbonyl (NVOC)-protected SEAlide peptide **21** was synthesized by standard Fmoc SPPS. NCL between **16** and **21** under standard NCL conditions (6 M Gn·HCl–0.2 M NaPB (pH 7.0) in the presence of 2% (v/v) thiophenol at 37 °C) gave a reaction mixture containing NVOC-protected ligated peptide **22**, which was then subjected to the extractive removal of thiophenol followed by UV irradiation for deprotection of NVOC group, to afford peptide fragment **23** required for the convergent assembly. The blending of reaction mixtures containing **23** or **18** in the presence of 80 mM MPAA and 60 mM TCEP·HCl enabled the convergent ligation to produce the desired reduced form SNX-482 **12** in 30% yield after HPLC purification (Figure 5).

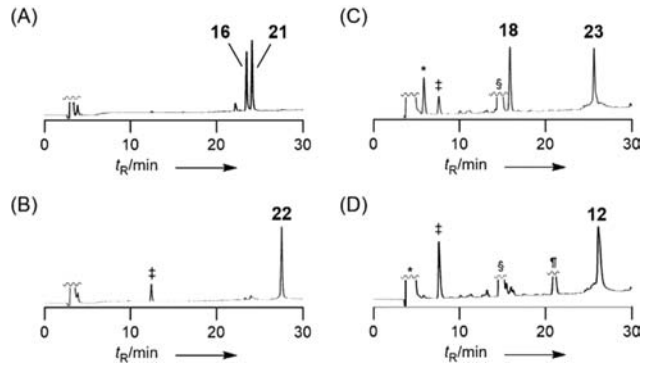


Figure 5. HPLC profiles for the convergent synthesis of reduced form SNX-482. (A) Ligation between **16** and **21** (t < 5 min). (B) Ligation between **16** and **21** (t = 43 h). (C) Ligation between **18** and **23** (t < 5 min). (D) Ligation between **18** and **23** (t = 43 h). ‡Released SEAlide unit. §MPAA. ¶Thiophenol. *Nonpeptidic compounds probably derived from photoremoval of the NV unit. HPLC conditions are described in the Supporting Information.

In summary, we successfully extended the usefulness of the SEAlide peptide by caging of the sulfanyl moiety with NV protection, which allowed the 41-residue SNX-482 to be synthesized by one-pot/sequential four-fragment ligation in an N–to–C-directive manner. Alternatively, the combination of the photocaged SEAlide and NVOC-protected N-terminal cysteine peptides enabled the convergent assembly of the four fragments in a one-pot synthesis. These successful results indicate that the photocaged SEAlide peptide can be employed in sequential and convergent approaches to peptide/protein synthesis.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b03661.

General procedures and additional HPLC, NMR, and MS data (PDF)

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Notes

The authors declare no competing financial interest.

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