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One-Pot Peptide Ligation−Oxidative Cyclization Protocol for the Preparation of Short-/Medium-Size Disulfide Cyclopeptides

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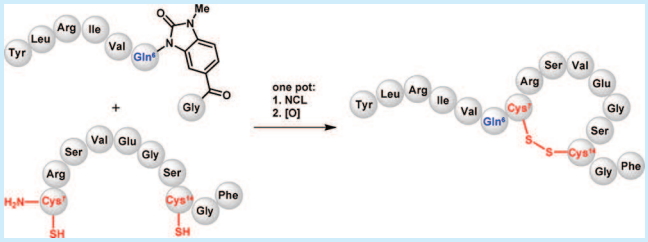
Supporting Information

ABSTRACT: Native chemical ligation (NCL) employing the *N*-methylbenzimidazolinone (MeNbz) linker readily provided the linear precursor of a 16-mer peptide that is difficult to obtain by stepwise solid-phase peptide synthesis. NCL and the workup conditions were improved toward a protocol that allows for quantitative removal of the 4-hydroxymercaptophenol additive and subsequent formation of the disulfide bridge in the NCL cocktail by oxidation in air, tolerated by the presence of tris(hydroxypropyl)phosphine.



Native chemical ligation (NCL) has become a standard method for the chemical synthesis of small proteins or large peptides.[1] Typically, the reaction proceeds between a peptide displaying a C-terminal thioester and an N-terminal Cys segment. The increasing utilization of the NCL has extended the ligation point to amino acids (aas) other than Cys, and now it is feasible to carry out NCL with any proteogenic amino acid (aa) by introducing a mercapto group in the β or γ position,[2] except for Gly, which requires the presence of *N*-auxiliaries.[3] Thus, combination of NCL/desulfurization is a powerful technique to access a wide variety of post-translational modifications or introduce site-specifically nonproteogenic aa's and engineer new functions in proteins.[4] Gratifyingly, several strategies including SEA-peptides, peptide hydrazides, or *N*-acylureas have been developed for the synthesis of peptide thioesters and thioester surrogates using standard Fmoc-SPPS techniques.[5]

NCL has potential advantages over segment condensation because it proceeds without epimerization and the solubility of unprotected segments in aqueous denaturating buffers, such as guanidine or urea, is usually better when compared with protected segments in organic solvents, enabling the ligation of large peptide fragments (>40-mer) with fast kinetics.[6] Despite these advantages, NCL is not a widely used alternative for the synthesis of short and medium-sized peptides, which are difficult to obtain with standard solid-phase techniques. Here, the cyclic peptide **1** was chosen as a model for the testing of a scalable NCL strategy for the preparation of difficult small peptides.

The cyclic peptide **1** (H-Tyr-Leu-Arg-lle-Val-Gln-Cys(&)-Arg-Ser-Val-Glu-Gly-Ser-Cys(&)-Gly-Phe-OH, where "&" denotes the disulfide interconnection[7]) is the 15-mer *C*-terminal sequence of human growth hormone (hGH) with one Tyr attached to its N-terminus.[8]

The linear precursor **2** is difficult to obtain by solid-phase peptide synthesis (SPPS). In the stepwise synthesis using standard peptide synthesis protocols (3 equiv Fmoc-aa, 3 equiv DIC/OxymaPure 1:1), the coupling of Arg^8 to Ser^9 was found to be practically ineffective. The use of a pseudoproline building block[9] at the position $Gly^{12}er^{13}$ renders a complex product mixture wherein the full sequence could be detected by MALDI-TOF. Segment condensation has been attempted between protected Tyr^1-Arg^8 and Ser^9-Phe^{16} segments but suffers from poor solubility of the segments as well as high levels of epimerization on Arg^8. The condensation between the protected segments Tyr^1-Gly^{12} and Ser^{13}-Phe^{16} is hampered by the low solubility of the Tyr^1-Gly^{12} fragment.[10]

Synthesis of peptide **1** by NCL requires two segments, the *C*-terminal Cys^7-Phe^{16} **3** with a Cys at the *N*-terminus and the *N*-terminal segment Tyr^1-Gln^6, where the *C*-terminus Gln should be activated as a thioester (Figure 1). The segment **3**

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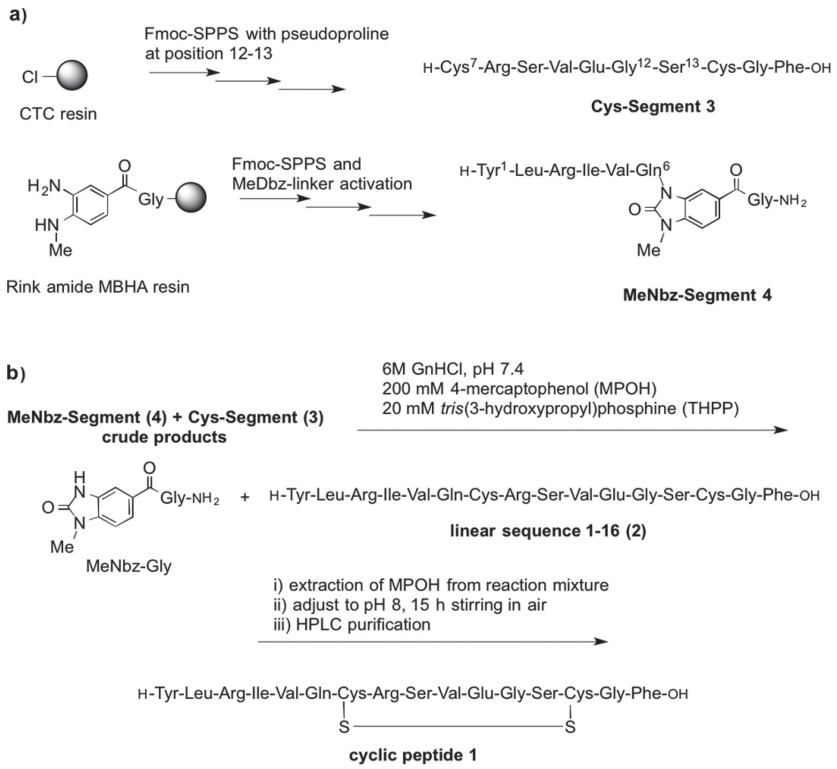

Figure 1. (a) SPPS of segments **3** and **4**; (b) streamed NCL and oxidative cyclization: NCL between fragments **3** and **4** (crude products) yields the linear precursor **2**. The subsequent steps are performed by manipulating the NCL reaction mixture.

was obtained by stepwise Fmoc-SPPS on CTC-resin using the commercial pseudoproline building block Fmoc-Gly-L-Ser[ψ-(Me,Me)Pro]-OH at position Gly[12]-Ser[13]. Coupling of the remainder the aa's was carried out using 3 equiv of aa and OxymaPure/DIC (1:1, 3 equiv of each) in a DMF/DCM mixture for 30 min to 1 h. The final detachment of the peptide from the resin was carried out with TFA/TIS/H_2O (95:2.5:2.5), which also removes the side-chain protecting groups, including the pseudoproline moiety, rendering the fully unprotected peptide containing two free Cys residues. The yield of segment **3** was on average between 40 and 50% with an analytical purity of 70−90% (HPLC trace at 220 nm). This peptide **3** was used without further purification. A decrease of resin functionalization was observed by checking the Fmoc-cleavage UV absorbance as well as with an internal reference.[11] This decrease was found to be constant rather than attributed to a certain coupling step. It was assumed to be caused by the slightly acidic conditions during the OxymaPure/DIC-mediated coupling steps. However, the more acid-stable trityl resin gave similar results.[12]

The *C*-terminus fragment **4** was prepared using the 3-Fmoc-amino-4-methylaminobenzoic acid (Fmoc-MeDbz) linker,[13] which is an improved version of the diaminobenzoic acid (Dbz) developed by Blanco-Canosa and Dawson.[14] The use of MeDbz does not produce regioisomers, and the additional Me group impedes undesired overacylation. Once the peptide chain has been built on the MeDbz, the reaction of the peptide linker with *p*-nitrophenyl chloroformate in CH_2Cl_2, followed by treatment with a base such as diisopropylethylamine (DIEA), render *N*-acyl-*N*′-methylbenzimidazolinone (MeNbz), a mildly activated species ready for generation of C-terminal arylthioesters by exchange with an external arylthiol. Fmoc-MeDbz can be purchased from commercially available sources or prepared by a simple procedure from commercial 4-fluoro-3-nitrobenzoic acid on a multigram scale.[13]

Since the formation of the aryl thioester is more efficient when it is carried out in solution after the cleavage of the unprotected peptide containing the MeNbz moiety, the Tyr[1]-Gln[6] MeNbz activated segment **4** was built up stepwise by SPPS on H-Gly-Rink amide MBHA resin functionalized with the MeDbz linker.[15] The Rink amide will us allow to obtain the readily activated segment with a final cleavage step. The Gly acts as spacer to facilitate the incorporation of the Fmoc-MeDbz onto the resin. The Fmoc-MeDbz was coupled on H-Gly-Rink amide MBHA resin using OxymaPure/DIC activation for 1.5 and 15 h. The acylation of the aniline linker function with the first aa (Fmoc-Gln(Trt)-OH) was ensured by four short couplings (15 min) with HATU/DIEA.[16] The rest of the sequence was assembled by standard OxymaPure/DIC activation of the corresponding Fmoc-aa's. Double couplings were required for the three last residues (Arg, Leu and Tyr). The Tyr[1] was introduced as Fmoc-Tyr(^{t}Bu)-OH, and then the Fmoc group was removed and the *N*-terminal amine was reprotected by reaction with Boc_2O/DMAP/DIEA before linker activation. The MeNbz formation proceeds in two steps: a short treatment with an excess of 4-nitrophenyl chloroformiate followed by cyclization to the acylurea with DIEA in DMF. H-YLRIVQ-MeNbz-Gly-NH_2 **4** was obtained in 90% average yield and purities between 52 and 68% (HPLC 220 nm). The lyophilized MeNbz-segment **4** can be stored for a long period of time in a freezer and purified by HPLC when needed. However, for NCL experiments the crude product was used.

NCL with the Nbz linker is typically performed in degassed 6 M guanidine hydrochloride (Gn·HCl)/200 mM 4-

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mercaptophenylacetic acid (MPAA)/20 mM tris(2-carboxyethyl)phosphine (TCEP)/200 mM phosphate buffer at a pH 6.8−7.4.[14,17] Under these conditions (peptide concentration 5 mM), the formation of the linear precursor **2** of the target peptide **1** was completed after 2 h. However, its isolation from the reaction mixture by HPLC was not straightforward because of the nearby eluting large amounts of the MPAA (40-fold excess respectively to peptide).[18] Within other effective thiol additives tested,[14a] it was found that the water-soluble 4-mercaptophenol (MPOH) could be completely removed by simple extraction with *tert*-butyl methyl ether (TBME) from the acidified (pH approximately 1) reaction mixture, resulting a much easier isolation of **2** by HPLC. Experiments at a 50 mg scale of crude **3** (83% HPLC at 220 nm) and **4** (54% HPLC at 220 nm) were performed. The isolated yields are between 25 and 30% after HPLC purification (the purities of the starting materials were not taken into account).

The final step in the synthesis of **1** is the oxidative formation of an intramolecular disulfide bond between the two Cys residues in the linear precursor **2**. Attempts to cyclize HPLC-purified lineal peptide **2** under aerobic conditions at basic pH (0.16 M NH_4HCO_3) led to extensive precipitation, probably due to aggregation or polymerization products, which could not be avoided by adding organic solvents (ACN, DMSO). It was sought to perform the oxidation step directly in the chaotropic (6 M Gn·HCl) NCL reaction mixture after extraction of the MPOH. However, the present phosphine (TCEP) additivés role is exactly to maintain thiols in their reduced form. Furthermore, TCEP is reported to undergo occasionally unwanted side reactions with peptides such as reduction of Cys to Ala.[19]

Alternatives for TCEP were investigated. Without any reducing additive, the yield of the NCL step dropped dramatically (7% isolated yield). Using DTT, side products (determined by HPLC UV integration) and a decrease in the yield of **2** were observed. Finally, tris(3-hydroxypropyl)-phosphine (THPP) showed at least the same efficiency as TCEP (30% isolated yield of the linear peptide). THPP is used to keep proteins and peptides in their reduced state.[20] It has been previously reported as reducing agent for sequential peptide ligations using controlled cysteinyl prolyl ester autoactivating units.[21]

THPP is oxidized to the corresponding phosphine oxide with a maximal conversion at pH 8, at which typically disulfide bonds are formed by stirring in air.[22] This property allows us to streamline NCL and oxidative cyclization into an efficient one-pot protocol. In a typical experiment, crude segment **3** (50 mg) together with MPOH (200 mM) and THPP (20 mM) was dissolved in degassed 6 M Gn·HCl/200 mM phosphate buffer (10 mL). The pH was brought to 7.3, and the solution was added to crude segment **4** (50 mg). It was stirred under N_2. After complete conversion, the solution was acidified to approximately pH 1, and the MPOH was extracted with TBME. The pH was adjusted to 8, and the solution was stirred for 15 h in air. The conversion of linear peptide to cyclic peptide could be monitored by UPLC at 220 nm (Figure 2). Semipreparative HPLC purification and lyophilization yielded 17% of **1** (the purities of the starting materials were not taken into account).

In summary, it was demonstrated on the example of the cyclic hGH fragment that NCL with the MeNbz linker can be advantageously applied to small difficult peptides. The combination of the thiol MPOH and THPP as reducing agent offers the option to save the isolation of the ligated peptide for further oxidative cyclization. Thus, oxidative cyclization can be carried out in the NCL reaction mixture after thiol extraction and THPP oxidation so that only one final purification step is needed. It is envisaged that this strategy could be further used for the synthesis of other short or middle-sized Cys containing peptides and, even, adapted for the NCL of proteins.

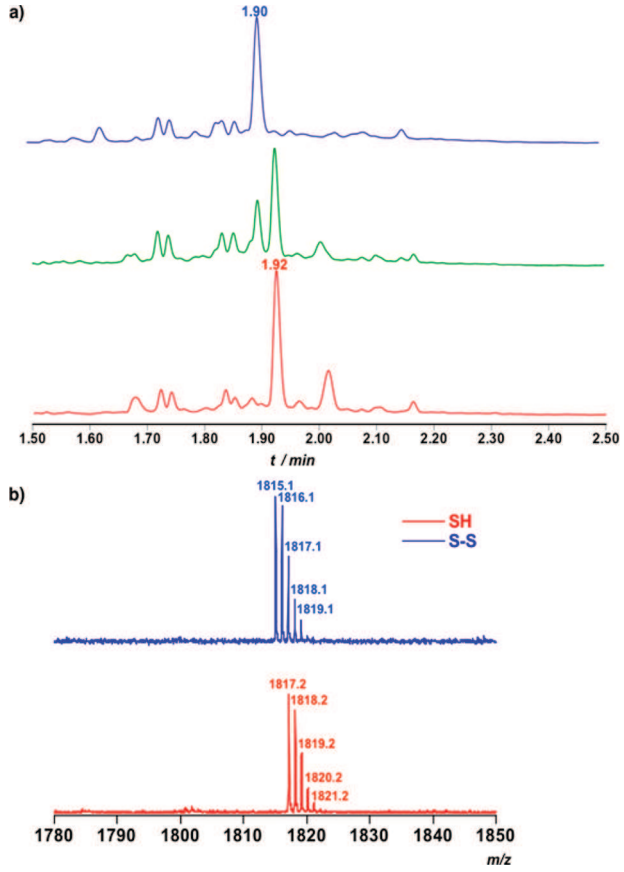

Figure 2. (a) UPLC at 220 nm directly after NCL (bottom, red: **2** at 1.92 min) and conversion of into **1** in the NCL cocktail after MPOH extraction and adjusting of pH to 8, in air, 4 h in air (middle, green), and overnight in air (top, blue: **1** at 1.90 min). (b) MALDI-TOF of the reduced peptide **2** (red, MW of main isotope calcd 1817.1, obsd 1817.2) and oxidized **1** (blue, MW of the main isotope calcd 1815.1, obsd 1815.1).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b01741.

Materials and methods corresponding to the solid-phase synthesis of the fragments. Experimental parts of the NCL and HPLC and MALDI-TOFF of the peptide intermediates and target peptide (PDF)

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Notes

The authors declare no competing financial interest.

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