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Native Chemical Ligation Strategy to Overcome Side Reactions during Fmoc-Based Synthesis of C-Terminal Cysteine-Containing Peptides

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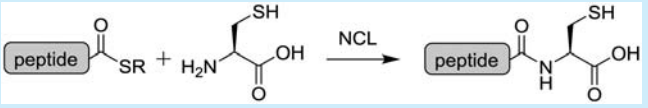
Supporting Information

ABSTRACT: The Fmoc-based solid phase synthesis of C-terminal cysteine-containing peptides is problematic, due to side reactions provoked by the pronounced acidity of the Cα proton of cysteine esters. We herein describe a general strategy consisting of the postsynthetic introduction of the C-terminal Cys through a key chemoselective native chemical ligation reaction with *N*-Hnb-Cys peptide crypto-thioesters. This method was successfully applied to the demanding peptide sequences of two natural products of biological interest, giving remarkably high overall yields compared to that of a state of the art strategy.



C-Terminal cysteines are frequent in the sequences of disulfide-rich peptide natural products of biological or therapeutic interest. However, the efficacy of their Fmoc-based solid phase peptide synthesis (SPPS) is impaired by the pronounced acidity of the Cα proton of Cys esters. Epimerization can occur not only when forming the ester bond to immobilize Cys on a solid support but also during the repeated piperidine treatments used for Fmoc deprotection during SPPS elongation.[1] A second commonly encountered side-reaction is the formation of 3-(*N*-piperidinyl)alanine[2] arising from piperidine-mediated β-elimination followed by Michael-type addition to the resulting dehydroalanine (Figure 1B). Contamination by these side-products becomes particularly problematic when synthesizing long peptide sequences. Several methodologies have been developed in the past years to try to overcome these limitations: use of a 2-chloro-trityl linker,[1] side-chain anchoring strategies,[3] or *S*-protecting groups alternative to the classical *S*-trityl protection[4] were shown to dramatically slow down the kinetics of the two parasite reactions. Alternatively, introduction of the Cys as an orthoester derivative[5] can totally suppress the formation of the two side-products, but the latter strategy is hampered by the need of a post-SPPS base-catalyzed hydrolysis of the orthoester.

We recently faced these problems during the SPPS of the reduced form (**1**) of the disulfide-rich miniprotein AhPDF1.1b,[6] an intriguing 51 amino acid plant defensin that, besides classical antifungal properties, is implied in zinc tolerance of *Arabidopsis halleri*.[7] Even when using a trityl linker, more than 30% epimerization (relative to **1**) was observed in the crude target peptide,[8] which considerably complicated its purification and led to a disappointing overall 7% isolated yield.

To improve this yield, we thought to design a general strategy based on postsynthetic introduction of the C-terminal Cys through native chemical ligation (NCL)[9,10] with a peptide thioester (Figure 1A). Because of its C-terminal Pro-Cys, the peptide sequence of AhPDF1.1b, peptide **1** constitutes a highly challenging example: indeed, NCL using proline thioesters is known to lead to the slowest kinetics among the 20 proteinogenic amino acids.[11] Moreover, a side-product arising from the deletion of the C-terminal Xaa-Pro dipeptide through diketopiperazine formation has been recently reported.[12]

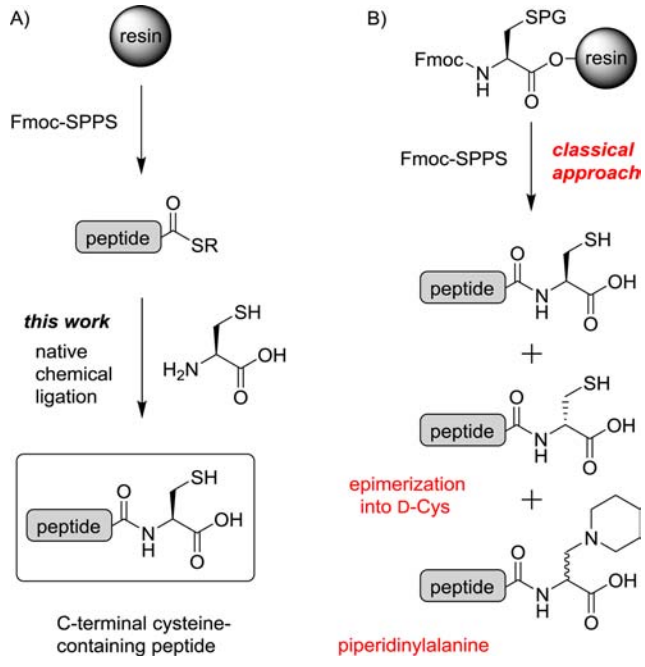


Figure 1. General NCL-based approach for the postsynthetic introduction of the C-terminal cysteine on synthetic peptides (A) versus classical Fmoc SPPS (B). PG: protective group.

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Key compounds for NCL-based synthetic approaches are peptide Cα thioesters, but their Fmoc-based synthesis is not straightforward using available tools.[13] We recently introduced a simple, inexpensive, and fully automated methodology for the synthesis of peptide crypto-thioesters[14] based on a C-terminal *N*-(2-hydroxy-5-nitrobenzyl)cysteine (*N*-Hnb-Cys) thioesterification device: such peptides are efficient thioester surrogates for NCL.[15] The key phenol group of this device plays a dual role: accelerating the intramolecular $N \rightarrow S$ acyl shift-based conversion into a reactive thioester during NCL by a biomimetic acid−base catalysis mechanism and also assisting the acylation of the secondary amine of the device to introduce the C-terminal residue of the sequence through an $O \rightarrow N$ acyl shift.

The main limitation we identified so far for this otherwise quite general strategy relies on the low yields observed under standard SPPS conditions for the coupling of the *N*-Hnb-Cys device with Pro as well as the sterically hindered Ile, Val, and Thr. Although multiple couplings or microwave heating of the coupling reaction can overcome this problem,[15] the present application to a C-terminal Pro-containing crypto-thioester prompted us to further explore the underlying reasons for this slow coupling. We clearly showed that, at least in the case of Pro, the N-acylation process is limited by a slow $O \rightarrow N$ acyl shift (Supporting Information (SI), pp S9−S15) as it has been previously speculated for a related $O \rightarrow N$ shift-assisted N-acylation process.[16] This finding is supported by a simple experiment: if a single 30 min coupling at room temperature of Fmoc-Pro-OH using HCTU leads to only 20% N-acylation, elimination of the reagents by thorough washing of the resin followed by a 24 h incubation shows a much greater 61% yield. A simple 5-fold coupling without further incubation leads to a satisfying 50% yield.

The initial amount of aminomethyl resin can be conveniently increased to achieve the expected synthetic scale, knowing that the capped Ac-(Hnb)Cys(S*t*Bu)-Gly-NH_2 coproduct is readily eliminated during ether precipitation of the peptide crypto-thioester after TFA-mediated cleavage. Therefore, robust conditions were optimized to ensure quantitative capping to avoid contamination with C-terminally truncated peptide crypto-thioesters.

On the basis of these results, automated SPPS of an *N*-Hnb-Cys crypto-thioester corresponding to the [1−50] AhPDF1.1b sequence (**2**) proceeded with a good 55% elongation yield, determined by UV titration of the fluorenylmethylpiperidine byproduct from the last Fmoc group removal, (Arg^2) as compared to the first one (Pro^{50}) (AhPDF1.1b numbering; see Scheme 1). The crude mixture was pure enough to be directly engaged in the next step without any chromatographic purification (see SI, p S25).

For analytical purposes, the NCL conditions were first optimized using an HPLC-purified sampled of **2**: we performed all of the optimization processes (Scheme 1) employing a rather low 1 mM concentration of **2**, representative of NCL with demanding peptides in terms of limited solubility. We started with using a large excess of cysteine (100 mM) in conjunction with the classical arylthiol catalyst 4-mercaptophenylacetic acid (MPAA, 100 mM) in a pH 7.2 phosphate buffer containing 6 M guanidinium chloride (Gu·HCl) and 50 mM of the classically used *tris*-(2-carboxyethyl)phosphine (TCEP) reducing agent. Under these conditions, S*t*Bu-protected **2** was nearly instantaneously converted into latent thioester **3**, ready for *in situ* $N \rightarrow S$ acyl shift-based conversion into a NCL-reactive thioester. As anticipated from the C-terminal Pro residue, the reaction was sluggish at room temperature,[11,12] leading to low 3% and 12% conversion after 6 and 24 h, respectively (Table 1). In the latter case, careful LC-HRMS analysis of the reaction mixture showed the formation of a minor coproduct (5% relative to the expected product **1**) with a +1 Da increase in mass, which we attributed to aspartimide formation at the ^{18}Asn-Gly site, followed by hydrolysis into Asp-Gly and/or isoAsp-Gly (**5**).[17] Asn-Gly sequences are well known to be prone to aspartimide-mediated degradation,[17,18] being a general problem for the stability of

Scheme 1. Application to the Reduced Form of AhPDF1.1b 1 of an NCL-Based Approach for the Post-synthetic Introduction of the C-Terminal Cysteine

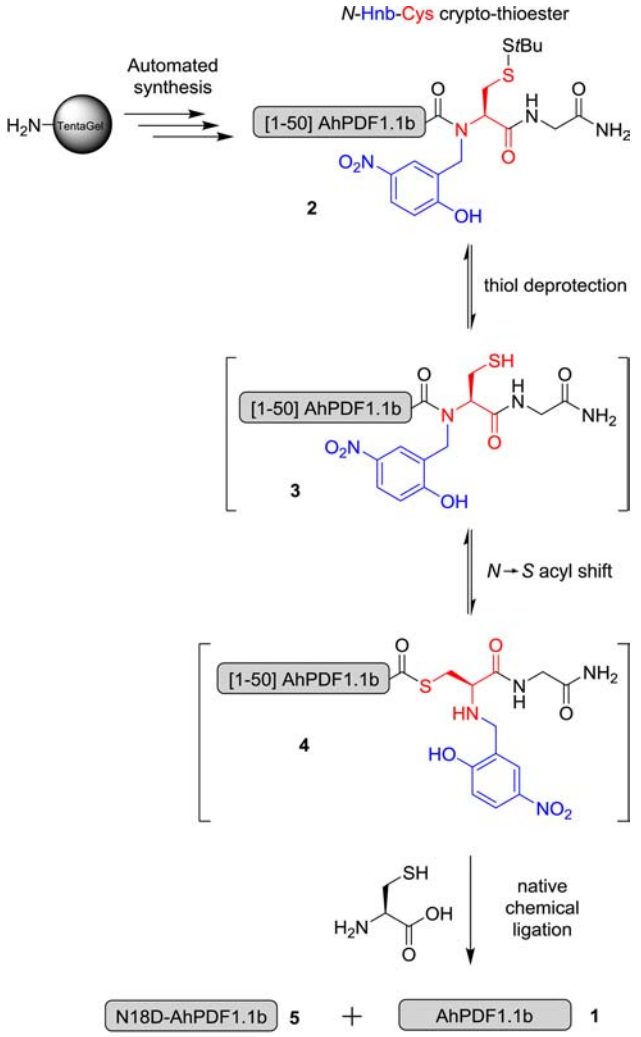

Table 1. Optimization of the NCL Conditions

temp (°C)	pH	time (h)	NCL yield[a,b] (%)	ratio **1**/**5**	yield[b] in **1** (%)
20	7.2	6	4	nd	nd
20	7.2	24	14	95:5	13
37	7.2	24	42	81:19	34
50	7.2	24	60	41:59	25
50	5.8	24	50	93:7	47
50	5.8	48	>95	87:13	83

[a]NCL yields correspond to the formations of both **1** and **4**; not isolated. [b]Yields determined by integrations of the HPLC peaks corresponding to compounds **1**, **3**, and **5**. nd: not determined.

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synthetic peptides or recombinant proteins.[19] Further identification of a −17 Da coproduct (3.5% relative to **1**) and traces (<0.1%) of [19−51]AhPDF1.1b attributed to the aspartimide-associated cleavage of the Asn-Gly bond[20] are additional evidence for the ^{18}Asn-Gly aspartimide hypothesis. As expected, heating of the ligation mixture at 37 or 50 °C[12a] led to improved NCL yields (40% and 58%, respectively) but also to a dramatic enhancement of the kinetics of formation of coproduct **5** (19% and 59% relative to **1**, respectively). Such a marked dependence on temperature has previously been observed for aspartimide formation at Asn-Gly sites.[17b,20] This unanticipated difficulty was solved by performing NCL at a lower pH, taking our inspiration from the observed dramatic pH dependence on the base-catalyzed aspartimide-mediated degradation process.[21] Note that if the optimal pH for NCL using a model *N*-Hnb-Cys peptide was previously evaluated to be around 6.5, the reaction was shown to also perform with fast kinetics at lower pH.[15]

Conducting the ligation of **2** with cysteine at pH 5.8 for either 24 or 48 h led to a much cleaner reaction (coproduct **5** limited to 7% and 13%, respectively) and a 80% yield in **1** in the latter case, an excellent yield when considering the specific limitations arising from the highly demanding AhPDF1.1b sequence. Further careful LC-MS analysis of the crude ligation mixture starting from crude **2** showed about 5% aspartimide formation (−17 Da) together with 6% hydrolysis of the crypto-thioester into [1−50]AhPDF1.1b.[22] The surprising relatively high amount of hydrolysis compared to our model studies[15] was attributed to the C-terminal proline-containing sequence of crypto-thioester **2**. No traces of deletion of the ^{49}Tyr-Pro dipeptide were observed in any tested conditions.[12]

Further highlighting the efficiency of our strategy, NCL on the crude SPPS mixture led to a much improved chromatographic profile as compared with that of classical Fmoc-SPPS using a trityl linker (Figure 2). HPLC purification furnished the pure reduced form of AhPDF1.1b **1** in a good 13.4% overall yield, which is about double the yield initially obtained, in spite of the sequence-specific aspartimide problems we encountered. Despite a close elution in RP-HPLC of **5** with **1**, less than 2% contamination was observed in the purified material.

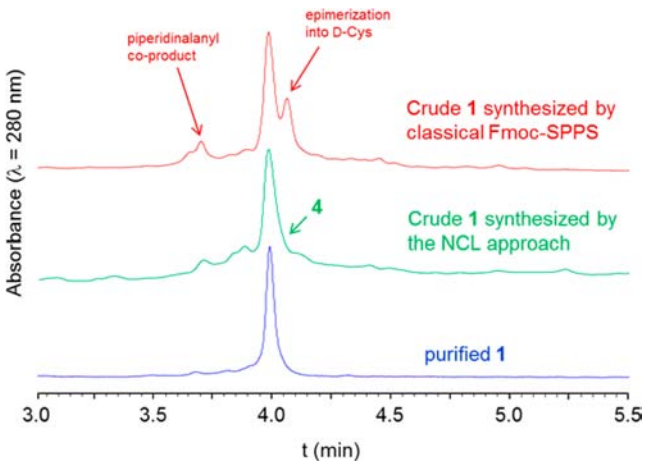

Figure 2. Comparative RP-HPLC analyses of AhPDF1.1b **1**. Red trace: crude peptide obtained with a classical Fmoc-SPPS strategy using a trityl linker. Green trace: crude peptide from the NCL-based approach for the postsynthetic introduction of the C-terminal cysteine. Blue trace: HPLC-purified **1**.

In order to illustrate the general scope of our strategy, we finally applied the protocol optimized for AhPDF1.1b to another related defensin from *A. hallieri*, AhPDF1.5,[6,23] without trying to adapt it, even if the sequence implies a much more kinetically favorable NCL at serine. The SPPS of [1−50]AhPDF1.5 crypto-thioester (**6**) proceeded in a good 56% elongation yield (SI, p S33). As expected, NCL was much faster (ligation completed in less than 2 h), and the overall process including NCL and purification starting from the crude peptide crypto-thioester led to isolation of the pure (>95%) reduced form of AhPDF1.5 (**7**) in an excellent 16% overall yield (SI, p S35). Note that, in this second case, less than 2% thioester hydrolysis was observed, supporting our hypothesis from a C-terminal Pro-specific and somewhat limited side reaction in the first example.

In conclusion, we herein introduced a simple and general NCL-based strategy for the high-yielding synthesis of C-terminal Cys-containing peptides, which allows bypassing the problematic side-reactions associated with these valuable peptide sequences. The work described here conveniently makes use of the facile synthesis of peptide crypto-thioesters through the *N*-Hnb-Cys technology but could probably be generalized to other popular Fmoc-based strategies for thioester synthesis. Work is ongoing to generate a diverse library of naturally occurring C-terminal Cys-containing plant defensins and analogues, related to our primary target AhPDF1.1b, to help decipher the unique zinc-tolerance properties induced by this molecule through further structural, bioinorganic, and biological studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b03612.

Materials and methods, compound characterization, and RP-HPLC chromatograms, and their analyses; and detailed studies on the mechanism of the N-acylation of the device (PDF)

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Notes

The authors declare no competing financial interest.

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(22) Traces of nonacetylated truncated peptides containing an N-terminal Cys residue were also observed in the LC/MS analysis of the mixture (see Supporting Information, p S30). These coproducts were attributed to in situ *N*→*S* shift at Xaa-Cys sites followed by trans-thioesterification at high temperature, as reported by Macmillan et al. See ref 19 and refs cited therein.
(23) AhPDF1.5 sequence: ^{1}ZLCKRESETWSGRCVNDYQCRDH CINNDRGNDGYCAGGYPWYRGCFCFFS^{51}C, with Z = pyroglutamic acid (41% homology with AhPDF1.1b sequence).

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