Native Chemical Ligation

DOI: 10.1002/ange.201408078

Peptide *o*-Aminoanilides as Crypto-Thioesters for Protein Chemical Synthesis**

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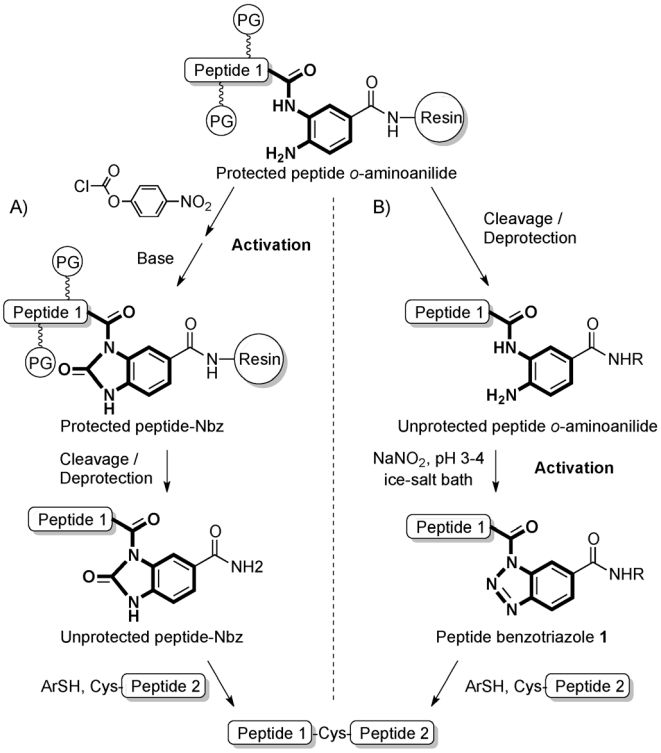
Abstract: *Fully unprotected peptide o-aminoanilides can be efficiently activated by $NaNO_2$ in aqueous solution to furnish peptide thioesters for use in native chemical ligation. This finding enables the convergent synthesis of proteins from readily synthesizable peptide o-aminoanilides as a new type of crypto-thioesters. The practicality of this approach is shown by the synthesis of histone H2B from five peptide segments. Purification or solubilization tags, which are sometimes needed to improve the efficiency of protein chemical synthesis, can be incorporated into the o-aminoanilide moiety, as demonstrated in the preparation of the cyclic protein lactocyclicin Q.*

Peptide thioesters are key intermediates in protein chemical synthesis.[1] Although the direct solid-phase peptide synthesis (SPPS) of peptide thioesters is possible by the use of *tert*-butoxycarbonyl (Boc) chemistry,[2] the preparation of peptide thioesters bearing acid-sensitive groups requires the 9-fluorenylmethyloxycarbonyl (Fmoc) method.[3] Among the Fmoc methods for thioester preparation, an efficient approach developed by Blanco-Canosa and Dawson involves the use of a 3,4-diaminobenzoic acid (Dbz) linker (Scheme 1 A).[4] Key to this approach is the on-resin transformation of an *o*-aminoanilide into an *N*-acylurea derivative upon treatment with *p*-nitrophenylchloroformate and a base. Subsequently, the resin-bound peptide *N*-acylurea can be deprotected and cleaved from the resin with trifluoroacetic acid (TFA). The resulting unprotected peptide *N*-acyl benzimidazolinone (peptide-Nbz) can undergo thiolysis in a neutral aqueous buffer to generate a peptide thioester for use in native chemical ligation (NCL).[5] As the Dbz-functionalized resins are commercially available,[6] this approach has been applied to the chemical synthesis of many proteins, such as ubiquitinated histone H2B,[7] exon 1 of the Huntingtin protein,[8] and tetraubiquitin.[9] However, as *p*-nitrophenylchloroformate is incompatible with many nucleophilic groups, the activation step must be conducted with full side-chain protection. It remains a challenge to activate peptide *o*-aminoanilides under protecting-group-free conditions so that peptide *o*-aminoanilides can be used with more flexibility for the chemical synthesis of proteins from multiple peptide segments.



Scheme 1. Two different methods for the activation of peptide *o*-aminoanilides: A) the method described by Blanco-Canosa and Dawson;[4] B) our method. PG = protecting group.

Herein, we describe a new strategy based on the $NaNO_2$-promoted activation of fully unprotected peptide *o*-aminoanilides to thioesters in an aqueous buffer (Scheme 1 B). This approach enables peptide *o*-aminoanilides to serve as a new class of peptide crypto-thioesters (i.e. masked peptide thioesters which, unless activated, are inert in NCL) that are useful in the condensation of multiple peptide segments.[10] In a few cases, the Fmoc-SPPS of peptide hydrazides was

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[**] This study was supported by the 863 Program of the Ministry of Science and Technology (2012AA02A700), the National Basic Research Program of China (973 Program, No. 2013CB932800), and the NSFC (No. 91313301).
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201408078.

reported to be difficult, and peptide *o*-aminoanilides had to be made.[11] Moreover, the carboxy group of the *o*-aminoanilide moiety provides an ideal site for the attachment of auxiliary groups, such as an Arg_6[12] or His_6[13] tag.

Our study started with the ligation of H-Leu-Tyr-Arg-Ala-Asn-Dbz-Ala-OH (**2a**) with H-Cys-Lys-Tyr-Ala-His-NH_2 (**3**; Figure 1). Peptide **2a** was synthesized by the procedure described by Blanco-Canosa and Dawson.[4] The two peptides (1.0:1.4) were dissolved in an aqueous buffer containing 6 M

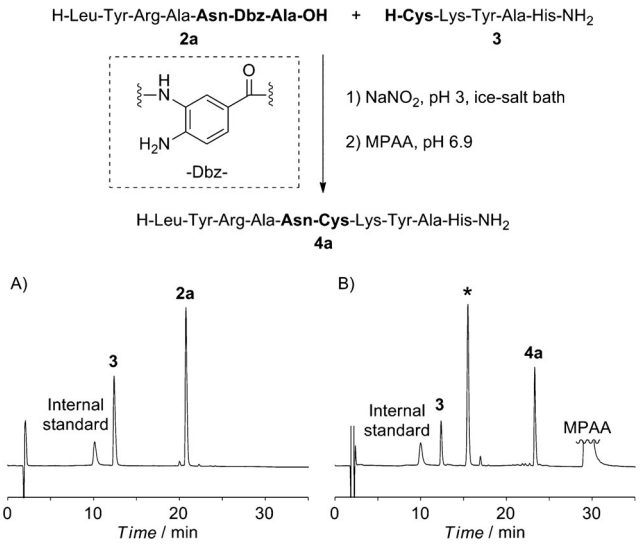


Figure 1. Reaction of **2a** with **3**. Analytical HPLC chromatograms ($\lambda = 214$ nm) are shown: A) before the addition of $NaNO_2$; B) after ligation for 1.5 h. Benzamide was used as an internal standard to measure conversion. The peak marked with an asterisk corresponds to the benzotriazole released in the reaction.

guanidine hydrochloride (Gn·HCl) and 0.2 M Na_2HPO_4 at pH 3. In an ice–salt bath at −10 °C, a solution of $NaNO_2$ (6 equiv) was added to activate **2a**. After 20 min, an aqueous solution of 4-mercaptophenylacetic acid (MPAA, 60 equiv) was added to the reaction mixture.[14] The pH value was then adjusted to 6.9 to initiate NCL at room temperature. The ligation proceeded smoothly to give **4a** with 80% conversion after 1.5 h. A similar conversion was observed when activation with $NaNO_2$ was conducted at pH 4. At pH 5, the activation was inefficient and led to less than 10% conversion.

To probe the scope of the method, we tested a number of model peptides with various C-terminal amino acid residues (Ala, Arg, Gln, Lys, Met, Ser, Tyr, Val). All of these ligations afforded the desired products with high conversion (78–90%), although the ligation at a sterically hindered site (i.e. Val) required a longer reaction time (Table 1). Previously, we could not produce C-terminal hydrazides of Asn, Asp, and Gln owing to the intramolecular cyclization that occurred when the peptide was released from the resin.[10b] In this study, we found that peptide *o*-aminoanilides with C-terminal Asn and Gln residues can be readily prepared by Fmoc-SPPS and can be smoothly ligated with Cys peptides. Nonetheless, Asp still cannot be used in the *o*-aminoanilide/$NaNO_2$ approach, because only hydrolysis occurred with a C-terminal Asp substrate (compound **2j**).[15]

Table 1: Scope of the ligation of peptide *o*-aminoanilides.[a]

H-Leu-Tyr-Arg-Ala-**Xaa-Dbz-Ala-OH** (**2a-2j**) + **H-Cys**-Lys-Tyr-Ala-His-NH_2 (**3**)

1) $NaNO_2$, pH 3, ice-salt bath; 2) MPAA, pH 6.9 → H-Leu-Tyr-Arg-Ala-**Xaa-Cys**-Lys-Tyr-Ala-His-NH_2 (**4a-4j**)

Entry	Xaa (**2**)	Ligation time [h]	Conversion [%][b]
1	Asn (**2a**)	1.5	80
2	Ala (**2b**)	1.5	78
3	Arg (**2c**)	1.5	90
4	Gln (**2d**)	1.5	84
5	Lys (**2e**)	1.5	89
6	Met (**2f**)	1.5	88
7	Ser (**2g**)	1.5	89
8	Tyr (**2h**)	1.5	85
9	Val (**2i**)	7.0	88
10	Asp (**2j**)	1.5	0[c]

[a] The reactions were performed in an aqueous buffer containing 6 M Gn·HCl and 0.2 M Na_2HPO_4. The activation step was conducted at pH 3 for 20 min in an ice–salt bath. The ligation step was performed at pH 6.9 at room temperature with MPAA. [b] Conversion was determined by HPLC with benzamide as the internal standard. [c] Only the hydrolysis product H-Leu-Tyr-Arg-Ala-Asp-OH was observed.

To examine the extent of racemization in the process, we compared **4b** produced from **2b** and **3** with authentic H-Leu-Tyr-Arg-Ala-(L-)Ala-Cys-Lys-Tyr-Ala-His-NH_2 and H-Leu-Tyr-Arg-Ala-(D-)Ala-Cys-Lys-Tyr-Ala-His-NH_2. HPLC analysis showed that the extent of racemization is less than 1%. This observation is similar to the situation with peptide hydrazides,[10b] thus confirming that $NaNO_2$ activation does not cause racemization at the C-terminal amino acid. Furthermore, in a ligation reaction of **2a** with **3**, we added free alanine (20 equiv). We did not observe any reaction between **2a** and alanine, thus confirming the chemoselectivity of the ligation.

LC–MS analysis of the reaction mixture after the activation of **2b** by $NaNO_2$ revealed the formation of a peptide benzotriazole **1**. Thus, we propose that activation/ligation of the *o*-aminoanilide proceeds through the mechanism depicted in Scheme 1. The $NaNO_2$-promoted conversion of an acyl *o*-aminoanilide into an acyl benzotriazole was observed previously.[16] Gilley and Kobayashi also showed that an acyl benzotriazole could be converted into a thioester by thiolysis.[17] Despite these previous observations, the present study established for the first time the practicality of using fully unprotected peptide *o*-aminoanilides for protein chemical synthesis.

As well as intermolecular ligation, we also tested the intramolecular ligation/cyclization of peptide *o*-aminoanilides in the synthesis of the cyclic peptide trypsin inhibitor SFTI-1[18] (Figure 2). The linear precursor **5** was prepared by Fmoc-SPPS. After the activation of **5** by $NaNO_2$ and the addition of MPAA, the cyclization reaction proceeded smoothly with concomitant formation of the disulfide bond to afford SFTI-1, which was isolated in 40% yield (Figure 2A). NMR spectroscopic structural analysis of the synthetic SFTI-1 (Figure 2B) showed an ordered β-hairpin structure consistent with previous reports.[18]

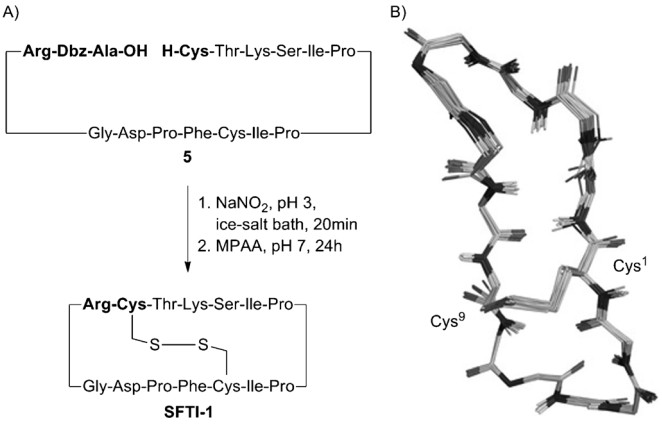
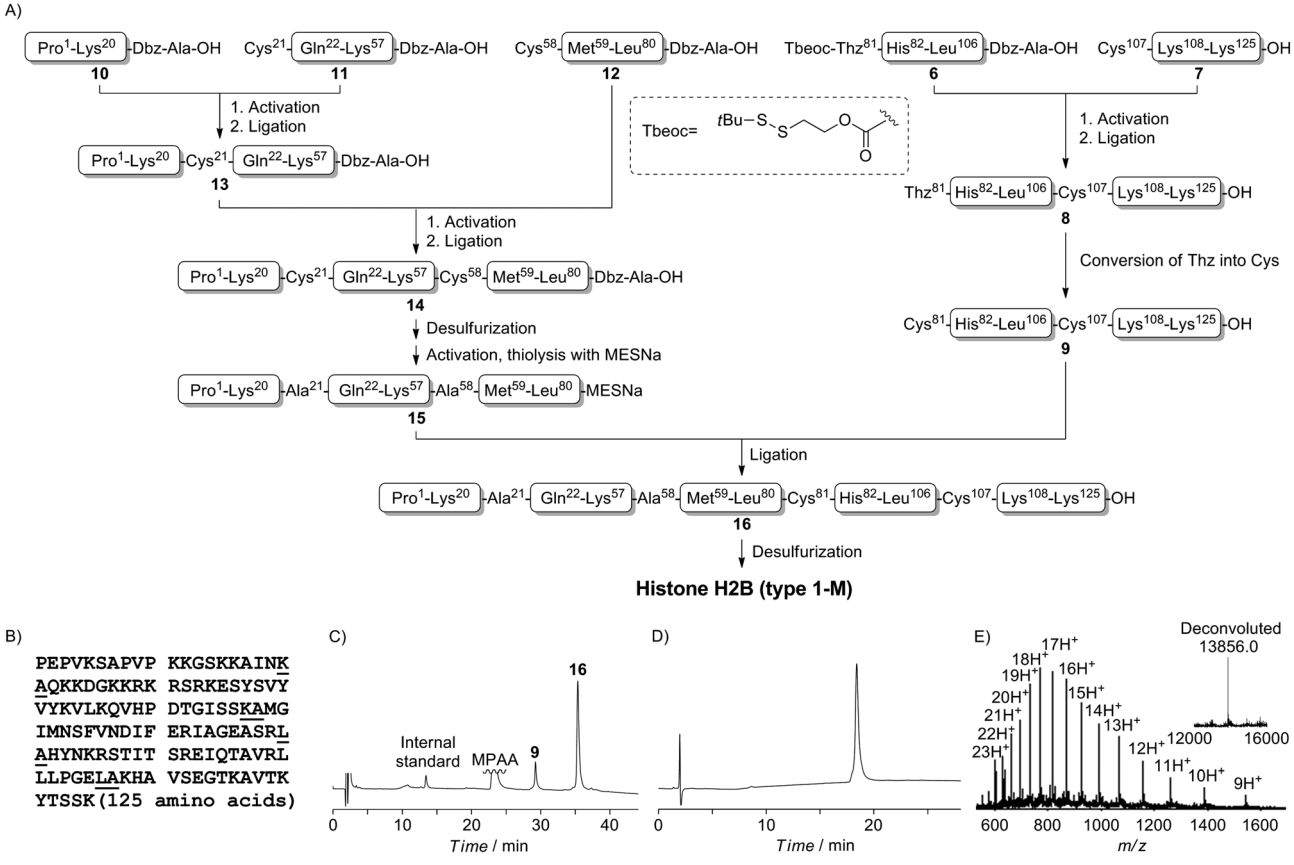


Figure 2. Synthesis of SFTI-1. A) Synthetic route. B) Structure determined by NMR spectroscopy.

In comparison to the on-resin *p*-nitrophenylchloroformate activation method described by Dawson, $NaNO_2$ activation under protecting-group-free conditions enables the use of peptide *o*-aminoanilides as peptide crypto-thioesters. This feature extends the utility of peptide *o*-aminoanilides in the convergent synthesis of proteins from multiple peptide segments. As a demonstration, we examined a convergent synthesis of histone H2B, which is one of the core histone proteins constituting the histone octamer in nucleosomes.[19,20] In our synthesis, we divided H2B into five segments with Ala[21], Ala[58], Ala[81], and Ala[107] temporarily mutated to Cys (Scheme 2 A).

The right half of H2B was made through the ligation of Tbeoc-Thz[81]-Leu[106]-Dbz-Ala-OH (**6**) with Cys[107]-Lys[125]-OH (**7**). Tbeoc-Thz was used to avoid the self-cyclization and oligomerization of **6**. HPLC monitoring showed that the ligation of **6** (activated in situ) with **7** was complete in 4 h. Treatment with TCEP (tris(2-carboxyethyl)phosphine) followed by methoxyamine hydrochloride[21] gave Cys[81]-Cys[107]-Lys[125]-OH (**9**), which was isolated in 53 % yield. The left half of H2B was made through sequential ligation of Pro[1]-Lys[20]-Dbz-Ala-OH (**10**), Cys[21]-Lys[57]-Dbz-Ala-OH (**11**), and Cys[58]-Leu[80]-Dbz-Ala-OH (**12**) in the N-to-C direction. In both ligations we observed some lactamization of the C-terminal Lys residue.[22] Nonetheless, Pro[1]-Cys[21]-Lys[57]-Dbz-Ala-OH (**13**) was obtained from **10** and **11** in 52 % yield, and Pro[1]-Cys[21,58]-Leu[80]-Dbz-Ala-OH (**14**) was obtained from **13** and **12** in 33 % yield. Remarkably, in both of the above ligation steps, the Dbz moiety in the Cys-peptide segment survived the reaction well and therefore could be used as a crypto-thioester for the next step.

To test the compatibility of the Dbz moiety with the free-radical-based desulfurization reaction,[23] we treated **14** with



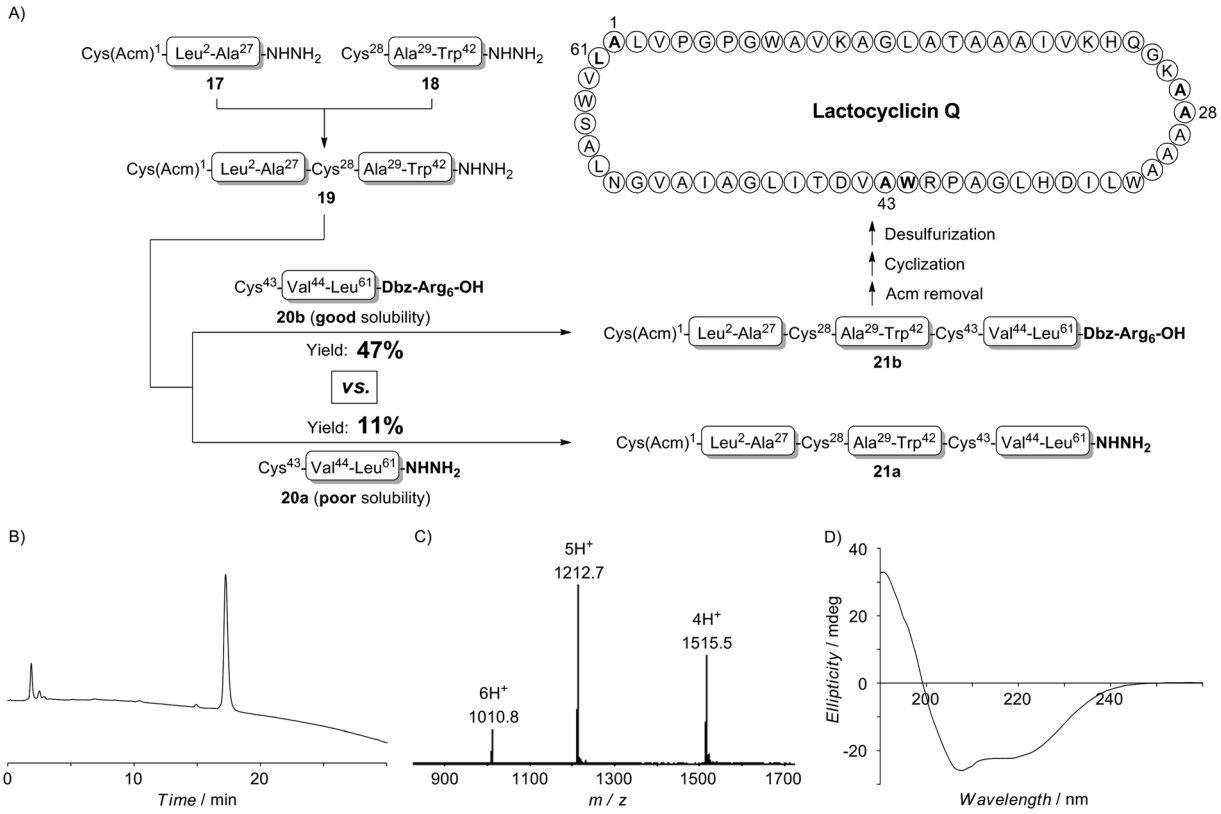
Scheme 2. Convergent synthesis of histone H2B. A) Synthetic route. Tbeoc = 2-(*tert*-butyldisulfanyl)ethyloxycarbonyl, MESNa = sodium 2-mercaptoethanesulfonate, Thz = L-thiazolidine-4-carboxylic acid. B) The amino acid sequence of histone H2B. C) HPLC chromatogram showing the complete consumption of **15** in the ligation between **15** and **9** at pH 6.8 (t = 5 h). Benzamide was added as an internal standard. D) Analytical HPLC chromatogram (λ = 214 nm) of purified histone H2B. E) Mass spectrum of histone H2B. The deconvoluted spectrum gave an observed mass of 13 856.0 Da (calcd: 13 857.9 Da, average isotopes).

TCEP, *t*BuSH, and 2,2′-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044). The desulfurized product was isolated in 79% yield with the Dbz moiety intact. We also tested the possibility of generating an isolated peptide thioester from the peptide *o*-aminoanilide by treating the product of the desulfurization of **14** with MESNa after $NaNO_2$ activation. The desired thioalkyl ester Pro^{1}-$Ala^{21,58}$-Leu^{80}-MESNa (**15**) was isolated in 75% yield. The final ligation between **15** and **9** was conducted at pH 6.8. After 5 h (Scheme 2C), the desired product **16** was obtained in 72% yield. The Cys^{81} and Cys^{107} residues of **16** were then converted back into Ala residues by free-radical-based desulfurization to give histone H2B in 79% yield (or an overall yield of the isolated product of 6%).

In comparison to the peptide hydrazide method, we observed an interesting advantage of the *o*-aminoanilide/$NaNO_2$ approach in our synthesis of the cyclic bacteriocin lactocyclicin Q:[24] Peptide *o*-aminoanilides can be readily modified with auxiliary groups, such as an Arg_6 or His_6 tag in the Dbz linker, whereas the corresponding hydrazides are structurally conservative, thus prohibiting any modification. As shown in Scheme 3, in our first synthetic route for lactocyclicin Q we used three peptide hydrazides: Cys-$(Acm)^1$-Ala^{27}-$NHNH_2$ (**17**), Cys^{28}-Trp^{42}-$NHNH_2$ (**18**), and Cys^{43}-Leu^{61}-$NHNH_2$ (**20a**). Ala^1, Ala^{28}, and Ala^{43} were temporarily mutated to Cys to enable the NCLs, whereas Cys^1 was protected as $Cys(Acm)^1$ to avoid the self-cyclization or oligomerization of **17**. The ligation of **17** with **18** proceeded smoothly to afford $Cys(Acm)^1$-Cys^{28}-Trp^{42}-$NHNH_2$ (**19**) in 55% yield. Unfortunately, in the ligation of **19** and **20a**, the latter segment was so poorly soluble that the yield only reached 11%.

To solve this problem, we designed Cys^{43}-Leu^{61}-Dbz-Arg_6-OH (**20b**), in which a solubilizing Arg_6 tag[12] was incorporated. The Arg_6 tag was assembled by SPPS prior to the coupling of Fmoc-Dbz-OH. Gratifyingly, **20b** showed remarkably higher solubility. No obvious precipitation was observed in the reaction with **19**. Consequently, the ligation product $Cys(Acm)^1$-$Cys^{28,43}$-Leu^{61}-Dbz-Arg_6-OH (**21b**) was obtained in 47% yield. After the removal of Acm by treatment with AgOAc (yield of the isolated product: 81%), intramolecular ligation by the $NaNO_2$ activation protocol afforded the cyclized product in 47% yield. Finally, free-radical-based desulfurization[23] furnished lactocyclicin Q in 73% yield upon isolation (or in 7% overall yield). The circular dichroism (CD) spectrum of synthetic lactocyclicin Q (Scheme 3D) exhibited double negative peaks in the 200–230 nm region that are consistent with a highly helical secondary structure,[25] as predicted on the basis of previous studies.[24]

In summary, fully unprotected peptide *o*-aminoanilides can be selectively and efficiently activated by $NaNO_2$ in an aqueous buffer and constitute a novel type of peptide cryptothioesters. The use of the *o*-aminoanilide/$NaNO_2$ method was



Scheme 3. Total synthesis of lactocyclicin Q. A) Synthetic route. The use of **20b** in place of **20a** led to a great increase in the yield of the second ligation step. Acm = acetamidomethyl. B) Analytical HPLC chromatogram ($\lambda = 214$ nm) of purified lactocyclicin Q. C) Mass spectrum of lactocyclicin Q. The spectrum gave an observed mass of 6058.4 Da (calcd: 6060.1 Da, average isotopes). D) CD spectrum of lactocyclicin Q in 50% trifluoroethanol in water (v/v).

demonstrated by the convergent synthesis of histone H2B from five peptide segments. We also showed in the total synthesis of the bacteriocin lactocyclicin Q that the *o*-aminoanilide/$NaNO_2$ method enables the incorporation of a solubilizing tag. The *o*-aminoanilide/$NaNO_2$ method overcomes the limitation of the peptide hydrazide method regarding ligation at Asn and Gln sites. As peptide *o*-aminoanilides have been reported to be more readily synthesizable than peptide hydrazides in some cases,[11] we envisage that the *o*-aminoanilide/$NaNO_2$ method will be very useful in chemical protein synthesis.

Received: August 8, 2014
Revised: November 18, 2014
Published online: December 4, 2014

Keywords: native chemical ligation · peptide *o*-aminoanilides · peptide thioesters · proteins · sodium nitrite

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