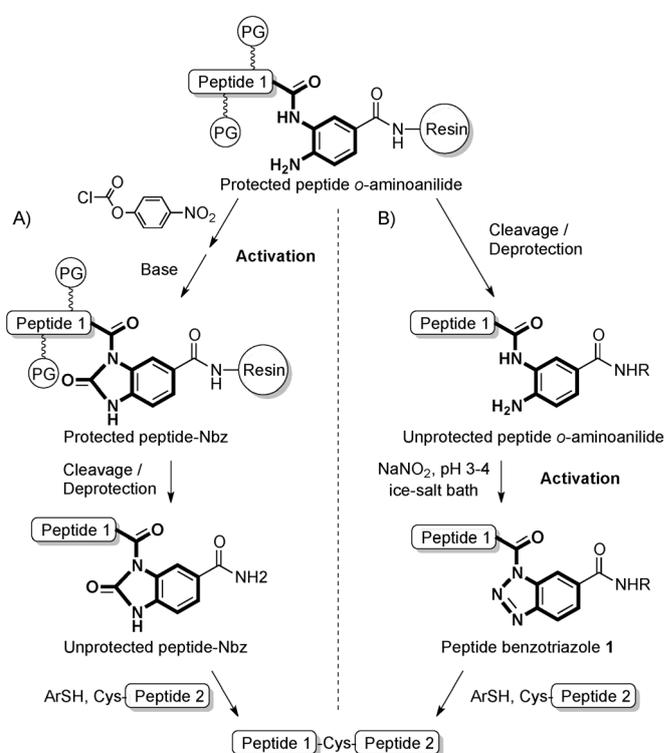


Peptide *o*-Aminoanilides as Crypto-Thioesters for Protein Chemical Synthesis**

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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-fluorenylmethoxycarbonyl (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



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.

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.

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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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 Arg₆^[12] or His₆^[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-NH₂ (**3**; Figure 1). Peptide **2a** was synthesized by the procedure described by Blanco-Canosa and Dawson.^[14] The two peptides (1.0:1.4) were dissolved in an aqueous buffer containing 6 M

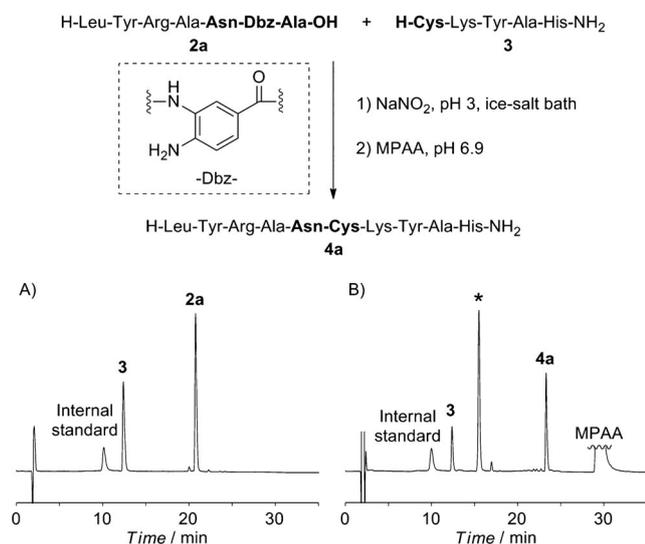


Figure 1. Reaction of **2a** with **3**. Analytical HPLC chromatograms ($\lambda = 214$ nm) are shown: A) before the addition of NaNO₂; B) after ligation for 1.5 h. Benzamide was used as an internal standard to measure conversion. The peak marked with an asterisk corresponds to the benzotriazole released in the reaction.

guanidine hydrochloride (Gn-HCl) and 0.2 M Na₂HPO₄ at pH 3. In an ice-salt bath at -10°C , a solution of NaNO₂ (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 NaNO₂ 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/NaNO₂ 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		+ H-Cys-Lys-Tyr-Ala-His-NH ₂	
2a-2j		3	
1) NaNO ₂ , pH 3, ice-salt bath		H-Leu-Tyr-Arg-Ala-Xaa-Cys-Lys-Tyr-Ala-His-NH ₂	
2) MPAA, pH 6.9		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 Na₂HPO₄. 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-NH₂ and H-Leu-Tyr-Arg-Ala-(D-)Ala-Cys-Lys-Tyr-Ala-His-NH₂. 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 NaNO₂ 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 NaNO₂ 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 NaNO₂-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 NaNO₂ 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]

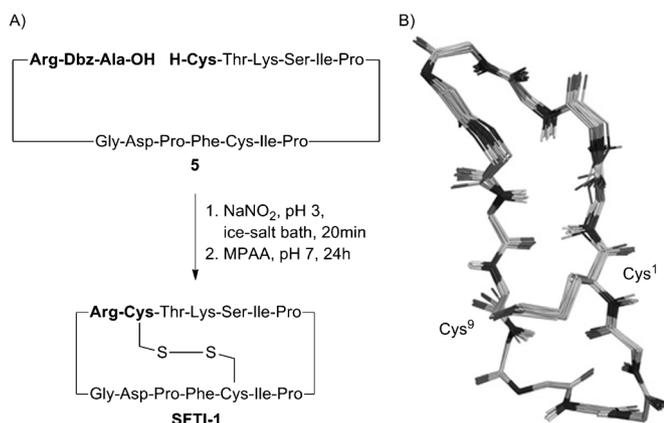


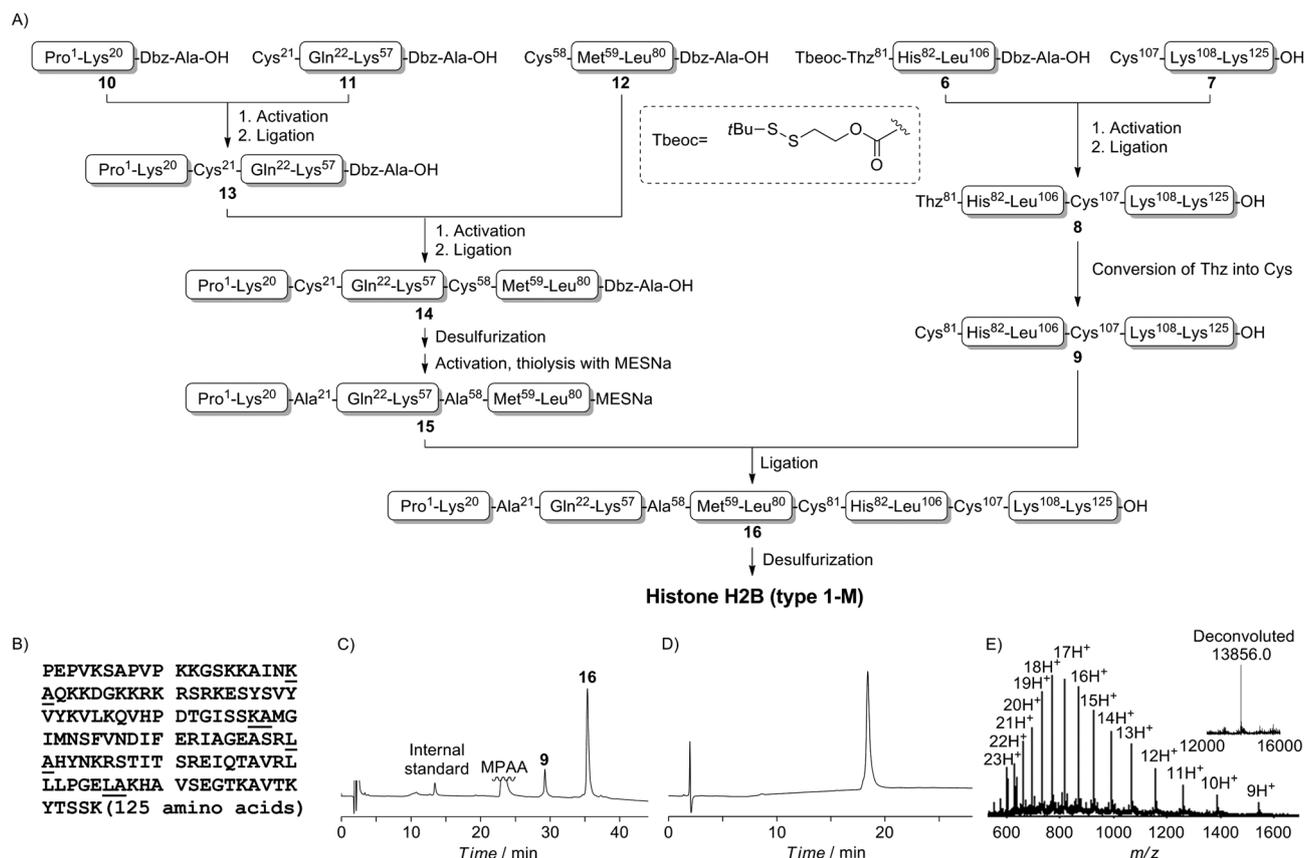
Figure 2. Synthesis of SFTI-1. A) Synthetic route. B) Structure determined by NMR spectroscopy.

In comparison to the on-resin *p*-nitrophenylchloroformate activation method described by Dawson, NaNO₂ 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 Ala²¹, Ala⁵⁸, Ala⁸¹, and Ala¹⁰⁷ temporarily mutated to Cys (Scheme 2 A).

The right half of H2B was made through the ligation of Tbeoc-Thz⁸¹-Leu¹⁰⁶-Dbz-Ala-OH (**6**) with Cys¹⁰⁷-Lys¹²⁵-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 Cys⁸¹-Cys¹⁰⁷-Lys¹²⁵-OH (**9**), which was isolated in 53 % yield. The left half of H2B was made through sequential ligation of Pro¹-Lys²⁰-Dbz-Ala-OH (**10**), Cys²¹-Lys⁵⁷-Dbz-Ala-OH (**11**), and Cys⁵⁸-Leu⁸⁰-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, Pro¹-Cys²¹-Lys⁵⁷-Dbz-Ala-OH (**13**) was obtained from **10** and **11** in 52 % yield, and Pro¹-Cys^{21,58}-Leu⁸⁰-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*-butyldisulfonyl)ethoxycarbonyl, 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 ($\lambda = 214$ 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).

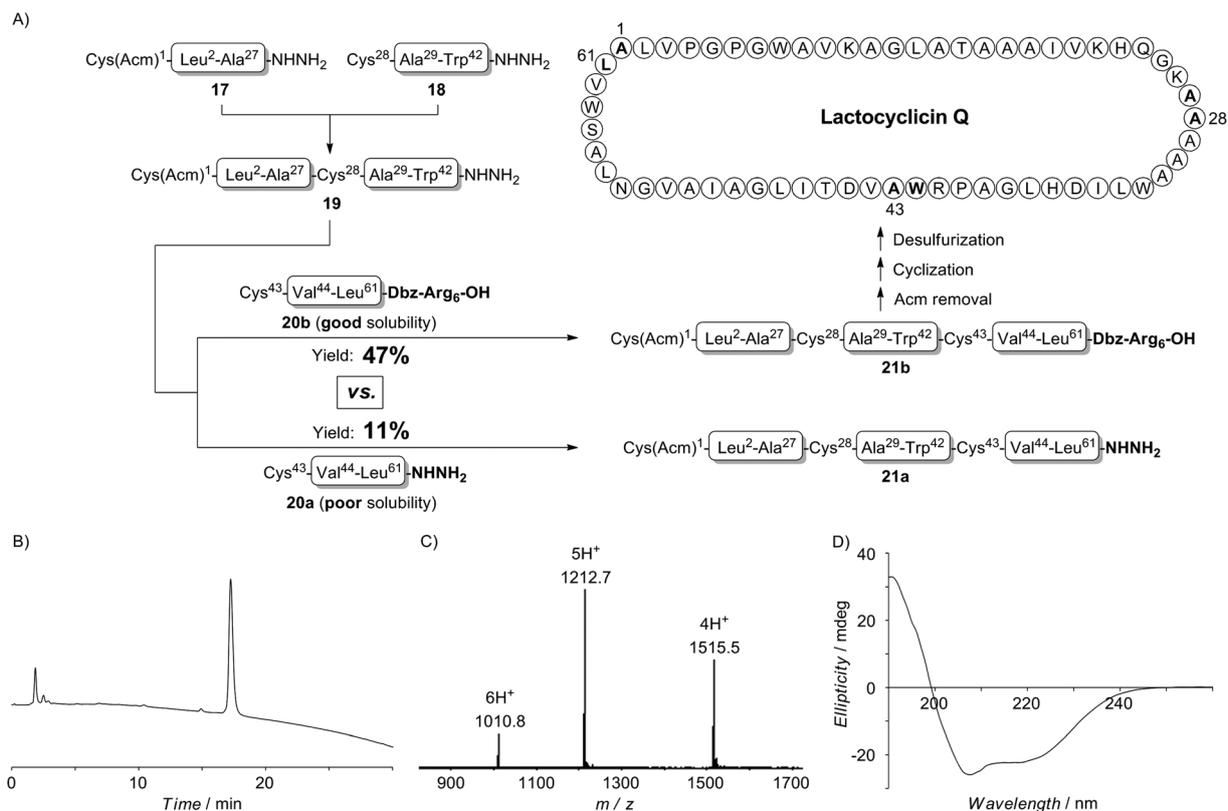
TCEP, *t*BuSH, and 2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044). The desulfurized product was isolated in 79% yield with the Dbz moiety intact. We also tested the possibility of generating an isolated peptide thioester from the peptide *o*-aminoanilide by treating the product of the desulfurization of **14** with MESNa after NaNO₂ activation. The desired thioalkyl ester Pro¹-Ala^{21,58}-Leu⁸⁰-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 Cys⁸¹ and Cys¹⁰⁷ 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/NaNO₂ approach in our synthesis of the cyclic bacteriocin lactocyclin Q:^[24] Peptide *o*-aminoanilides can be readily modified with auxiliary groups, such as an Arg₆ or His₆ 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 lactocyclin Q we used three peptide hydrazides: Cys-(Acm)¹-Ala²⁷-NHNH₂ (**17**), Cys²⁸-Trp⁴²-NHNH₂ (**18**), and Cys⁴³-Leu⁶¹-NHNH₂ (**20a**). Ala¹, Ala²⁸, and Ala⁴³ were temporarily mutated to Cys to enable the NCLs, whereas Cys¹ 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-Cys²⁸-Trp⁴²-NHNH₂ (**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 Cys⁴³-Leu⁶¹-Dbz-Arg₆-OH (**20b**), in which a solubilizing Arg₆ tag^[12] was incorporated. The Arg₆ 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}-Leu⁶¹-Dbz-Arg₆-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 NaNO₂ activation protocol afforded the cyclized product in 47% yield. Finally, free-radical-based desulfurization^[23] furnished lactocyclin Q in 73% yield upon isolation (or in 7% overall yield). The circular dichroism (CD) spectrum of synthetic lactocyclin 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 NaNO₂ in an aqueous buffer and constitute a novel type of peptide cryptothioesters. The use of the *o*-aminoanilide/NaNO₂ method was



Scheme 3. Total synthesis of lactocyclin 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 lactocyclin Q. C) Mass spectrum of lactocyclin Q. The spectrum gave an observed mass of 6058.4 Da (calcd: 6060.1 Da, average isotopes). D) CD spectrum of lactocyclin Q in 50% trifluoroethanol in water (v/v).

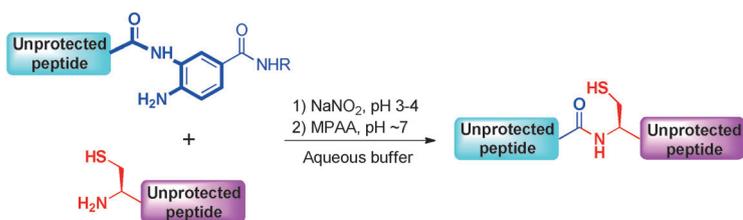
Communications



Native Chemical Ligation

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M. Yu, Z.-Y. Hong, L. Liu* — ■■■■—■■■■

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



Making it easier to make the link: The activation of fully unprotected peptide *o*-aminoanilides with NaNO_2 in an aqueous buffer enabled their use as peptide crypto-thioesters in native chemical ligation

(see scheme; MPAA = 4-mercapto-phenylacetic acid). The practicability and unique advantages of this method were demonstrated by the total chemical synthesis of histone H2B and lactocyclin Q.