Peptide Ligation

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An Efficient Fmoc-SPPS Approach for the Generation of Thioester Peptide Precursors for Use in Native Chemical Ligation**

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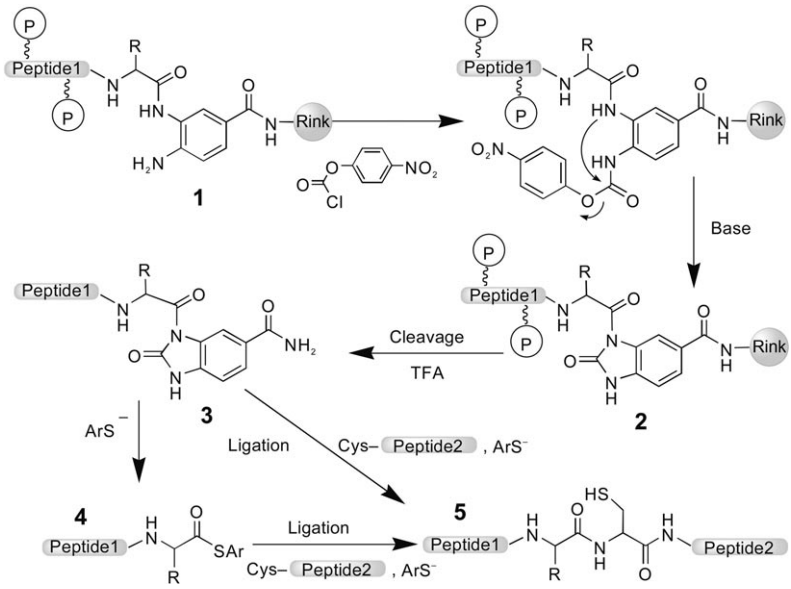
The straightforward C-terminal modification of peptides assembled on a solid support remains a significant challenge in peptide and protein chemistry. In particular, C-terminal thioester peptides are important intermediates for the generation of active esters, amides and hydrazides[1,2] and are an essential component of many synthetic strategies for protein synthesis.[3] Currently, the most effective approach for the synthesis of peptidyl thioesters is the in situ neutralization protocol for *tert*-butoxycarbonyl solid-phase peptide synthesis (Boc-SPPS)[4] using thioester linkers.[2,5] However, many laboratories use Fmoc-SPPS (Fmoc = 9-fluorenylmethyloxycarbonyl) exclusively and such protocols are favored when synthesizing glyco- and phosphopeptides. The thioester linkers used for Boc-SPPS have limited utility for Fmoc-SPPS due to the requirement for repeated Fmoc removal under basic conditions. Considerable effort has been applied to address this challenge[6] including optimized Fmoc deprotection cocktails,[7] thiol labile safety-catch linkers,[8] activation of protected peptides in solution,[9] and recently thioesters have been generated using O-to-S[10] or N-to-S[11] acyl transfer. Despite these notable advances, the synthesis of thioester peptides by Fmoc-SPPS remains significantly more challenging than the synthesis of the corresponding acid or amide peptide.

Herein, we describe an alternative approach for the Fmoc synthesis of peptides for use in native chemical ligation (NCL)[12] that is based on the formation of a C-terminal *N*-acylurea functionality. *N*-acylureas are mild acylating agents that have previously been explored in peptide synthesis by Pascal et al.[13,14] and for thioamide synthesis by Rapoport et al.[15] and Zacharie et al.[16] However, the utility of these groups as acylating agents has been limited due to the low reactivity of *N*-acylurea products towards aminolysis and the base lability of previous *N*-acylurea forming linkers.[13]

Inspired by this work, we have found that *o*-aminoanilides **1** are stable synthetic intermediates that can be efficiently transformed into an aromatic *N*-acylurea moiety **2**[14,16] following chain elongation (Scheme 1). Since this group has been previously described as an *N*-acyl-benzimidazolinone,[13–16] we propose the use of the abbreviation Nbz to indicate this leaving group. The resin-bound acylurea peptide **2** can be deprotected and cleaved from the resin with trifluoroacetic acid (TFA) using standard acid labile linkers (e.g. Rink or Wang linkers). The resulting mildly activated peptide-Nbz **3** is stable to the acidic conditions used for peptide handling and purification. However, in neutral aqueous buffers, the fully unprotected acylurea peptides undergo rapid thiolysis, enabling thioester peptide **4** to be generated before purification or in situ during a native chemical ligation. Importantly, the linker is a stable amide during chain assembly and the key activation step utilizes the most robust reaction in solid phase peptide synthesis: the acylation of an amine. As a result, the method is compatible with amino acid side chains and protecting groups commonly used in peptide synthesis.



Scheme 1. Synthetic strategy for Fmoc-SPPS of thioester peptides. Following SPPS, aminoanilide **1** undergoes specific acylation and cyclization to yield the resin-bound acylurea peptide **2**. Following cleavage and deprotection, peptide-Nbz **3** can undergo thiolysis to yield peptide thioester **4**, or direct ligation to yield the native peptide **5**.

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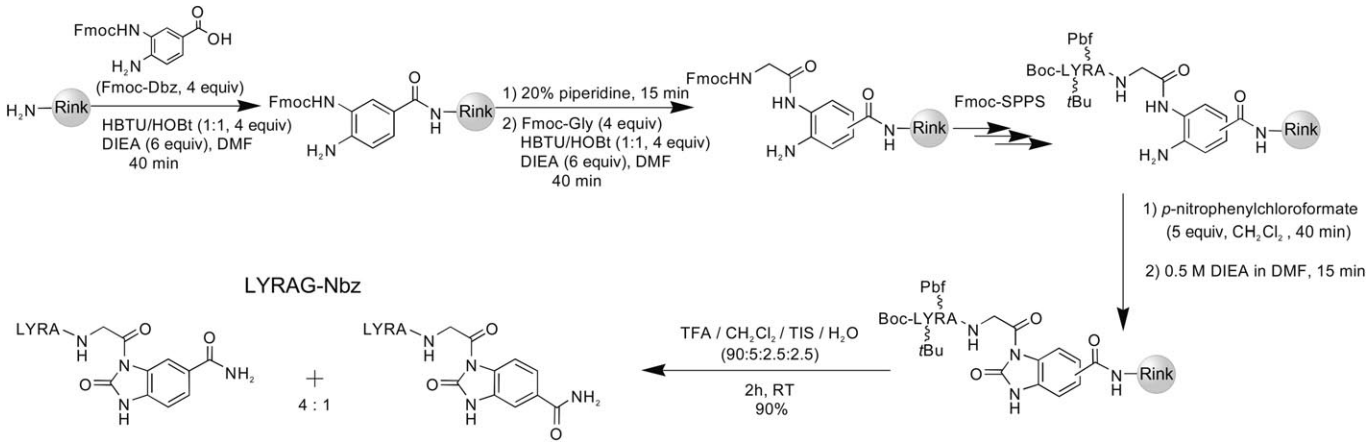

Scheme 2. Synthesis of LYRAG-Nbz. DIEA = *N*,*N*-diisopropylethylamine, HBTU = O-(benzotriazol-l-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, TIS = triisopropylsilane.

To test the method, we first synthesized the peptide LYRAG-Nbz (Scheme 2). Starting with Rink-PEG-PS resin (0.2 mmol g^{-1}, ABI; PEG = polyethylene glycol, PS = polystyrene, ABI = Applied Biosystems), 3-Fmoc-4-diaminobenzoic acid (Fmoc-Dbz) was coupled and the Fmoc group deprotected with 20% piperidine. Notably, after acylation, the free amino group in position 3 or 4 becomes hindered/deactivated and does not need further protection. Subsequent amino acids were coupled by standard Fmoc-SPPS with the N-terminal amino acid being introduced as Boc-Leu. Following chain assembly, the C-terminus was activated through acylation with *p*-nitrophenylchloroformate followed by the addition of base to promote intramolecular attack of the anilide to form the resin-bound benzimidazolinone. The peptide was deprotected and quantitatively cleaved from the Rink-PEG resin by treatment with TFA.

As shown in Figure 1 A, the desired peptide LYRAG-Nbz was obtained in high purity. No aminoanilide products were observed indicating that the conversion to the benzimidazolinone is quantitative. In this short peptide, the isomeric acylurea products are resolved by HPLC, indicating a 4:1 ratio. However, treatment of the peptide with 4-mercaptophenylacetic acid (MPAA) rapidly yields a single thioester product (LYRAG-SAr, Figure 1 B; Ar = aryl). Importantly, the thioester conversion occurs at pH 7.0 in under an hour, minimizing the likelihood of base mediated side reactions in susceptible peptides. In addition, since the benzimidazolinone is a poor nucleophile, the equilibrium lies fully towards the aryl-thioester product. These properties suggest that the Nbz peptides will be especially useful in the generation of aryl thioesters used in kinetically controlled and auxiliary-mediated ligation methods.[17]

The rapid conversion of LYRAG-Nbz to LYRAG-SAr suggested that acylurea peptides could be used directly in NCL without prior isolation of the thioester precursor. To test the in situ activation of the LYRAG peptide, we mixed LYRAG-Nbz (2 mM) with the N-terminal Cys peptide CRAFS (2.8 mM) at pH 7.0 in 6 M Gn·HCl Gn = guanidine, 200 mM Na_2HPO_4, 200 mM MPAA, 20 mM TCEP·HCl (TCEP = tris(carboxyethyl)phosphine).[18] As shown in Fig-

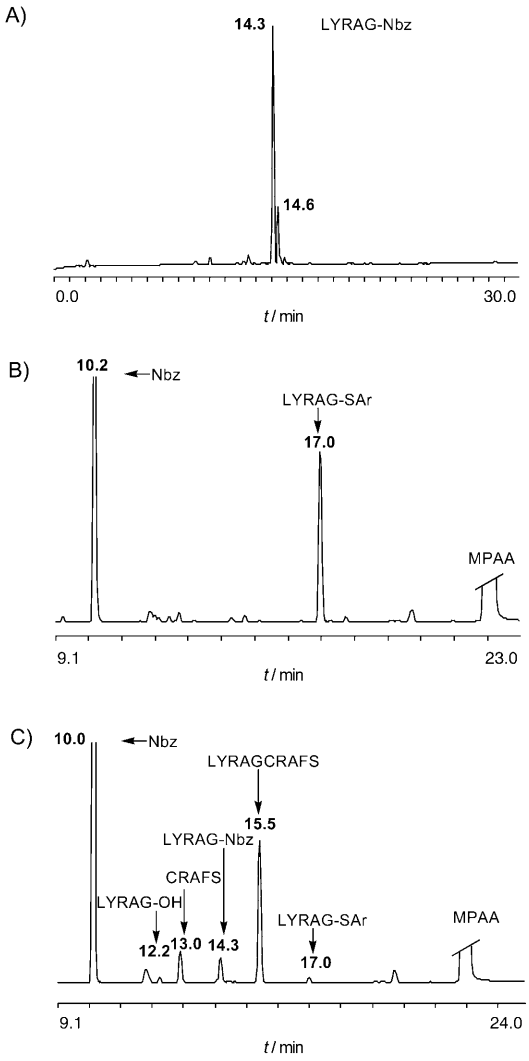

Figure 1. Synthesis, thioester exchange and ligation of LYRAG-Nbz. A) HPLC chromatogram of crude LYRAG-Nbz. B) Thiolysis of crude LYRAG-Nbz in 200 mM MPAA, pH 7.0 after 1 h. C) Ligation of CRAFS to LYRAG-Nbz after 50 min, pH 7.0.

ure 1 C, the ligation reaction proceeded efficiently, And under these mild conditions, the only side product stemmed from trace hydrolysis.[19] At intermediate time points, we found only trace amounts of the LYRAG-SAr peptide, suggesting that it was immediately converted into LYRAGCRAFS. These observations are consistent with aryl thioester formation being rate determining under these ligation conditions.

To probe the scope of the method, we synthesized a series of peptides with Ala, Phe, Tyr, Leu, Val, and Pro in the C-terminal position (Table 1). The loading on the Dbz linker was quantitative for each amino acid (see Supporting Information). Alternatively, 3-(Fmoc-Ala)-4-diaminobenzoic acid (Fmoc-Ala-Dbz-OH) was synthesized in order to obtain the peptide LYRGA-Nbz as a single isomer. We anticipate that the use of preformed Fmoc-aa-Dbz (aa = amino acid) linkers will be the most robust method for synthesis of Nbz peptides using preloaded Fmoc-aa-Dbz-resins in a similar manner to commercially available Boc-aa-Pam-resins and Fmoc-aa-Wang-resins.

Table 1: Peptide-Nbz synthesized by Fmoc-SPPS.

Entry	Peptide-Nbz	Purity [%][a]	Recovered Yield [%][b]
1	LYRAG-Nbz	95	90
2	LYRGA-Nbz	90	70
3	LARGF-Nbz	96	88
4	LARGY-Nbz	93	90
5	AYRGL-Nbz	92	87
6	LYRAP-Nbz	93	71
7	LYRGV-Nbz	94	67
8	(29aa) Rvg-Nbz	57	36

[a] Crude purity based on HPLC detection trace at 220 nm. [b] Non-optimized, the peptide is cleaved quantitatively from the Rink-PEG-PS resin.

Following chain assembly, the activation of the Dbz group with *p*-nitrophenylchloroformate and subsequent acidolytic cleavage was near quantitative in all cases including the hindered Val and Pro residues. Importantly, epimerization of the C-terminal amino acid was less than 2%.[20] One advantage of the acylurea method is that chain elongation occurs with a C-terminal amide linkage. As a result, no diketopiperazine formation was observed, even with the cyclization-prone LYRAP-Nbz peptide. Indeed, in previous work we observed significant diketopiperazine formation in the same peptide when using Boc-SPPS on a thioester resin.[5]

The six peptides were ligated with 2–3 mM CRAFS, pH 7.0, 25 °C, reaching completion in under 2 h except for Val (10 h) and Pro (24 h) residues considered to be poorly compatible with NCL.[5] On a preparative scale, 2.5 mM AYRGL-Nbz (7.5 mg, 1.0×10^{-2} mmol) and 3.0 mM CRAFS (7.0 mg, 1.2×10^{-2} mmol) formed the product AYRGL-CRAFS (10.9 mg, 9.5×10^{-3} mmol) in 95% yield of isolated product with no side reactions other than trace hydrolysis. From these studies, we anticipate that *N*-acylurea peptides will be versatile precursors to peptidyl thioesters.[2,5,21]

Since a primary utility of thioester peptides is in the synthesis of proteins and complex bioconjugates, it is essential for the *o*-aminoanilide linker to be compatible with the synthesis of large polypeptides using standard Fmoc-SPPS protocols. To test the robustness of the Dbz moiety, we synthesized a functionally diverse 29-amino-acid peptide derived from rabies virus glycoprotein (Rvg) that has recently been shown to be an effective carrier for cargo delivery into cells and through the blood–brain barrier.[22] Synthesis was performed on a 0.1 mmol scale using 4-fold excess (0.4 mmol) of amino acid and the activating mixture (HBTU/HOBt/DIEA (1:1:1.5)). The last residue (Tyr) was introduced as the side-chain-unprotected Boc-Tyr. After peptide elongation, an aliquot of peptide-resin (100 mg) was removed and *p*-nitrophenylchloroformate was added in CH_2Cl_2. The resulting peptidyl-carbamate was treated with 0.5 M DIEA/DMF (15 min), to afford the peptide-Nbz resin that was then cleaved with TFA. As shown in Figure 2, the peptide was obtained in high purity (57% by HPLC integration) and a yield of 36% was recovered after preparative HPLC. As with the LYRAG-Nbz model peptide, the Rvg-Nbz peptide efficiently exchanged with thiols to yield activated aryl thioesters (Supporting Information, pages S32–S34).[23] In addition, 2.0 mM of Rvg peptide (3.6 mg, 1.0×10^{-3} mmol) was ligated in situ with a second polypeptide, Tsr[24] (30 aa, 3.8 mg, 1.2×10^{-3} mmol, 2.4 mM) at pH 7.0 in 6 M Gn·HCl, 200 mM Na_2HPO_4, 200 mM MPAA, 20 mM TCEP·HCl to yield a 59 amino acid polypeptide (4.7 mg, 70%, Figure 3). After 2 h, no acylurea peptide remained. Significantly, these peptides contain the diverse functionality typical of complex peptides and proteins.

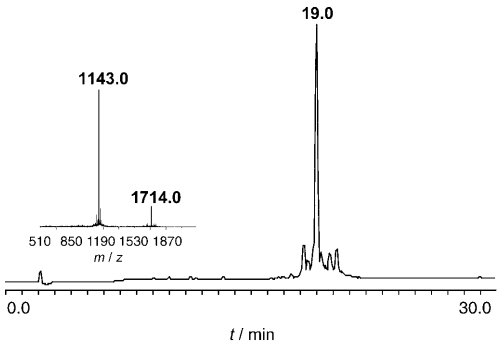

Figure 2. Rvg-Nbz HPLC and ESI mass (insert) of crude synthetic product. Mass observed: [*M*+H] 3426.0 Da, (calcd: [*M*+H] 3426.8 Da). Rvg sequence: YTIWMPENPRPGTPCDIFTNSRGKRASNG-Nbz.

These results suggest that *N*-acylurea activation will have broad utility in organic synthesis, including peptide and protein chemistry. These mildly activated peptides undergo rapid thiolysis to yield thioester peptides under standard NCL conditions. Importantly, activation of the Dbz moiety is compatible with both unhindered (Gly) and hindered (Val) C-terminal amino acids and is resistant to epimerization. In addition, the Dbz group is stable to standard Fmoc-SPPS coupling and deprotection protocols as demonstrated by the efficient synthesis of Rvg, a 29 aa rabies-derived peptide. Since the method relies exclusively on the acylation of amines and requires no additional protecting groups, it is compatible with standard side-chain protection strategies, linkers and

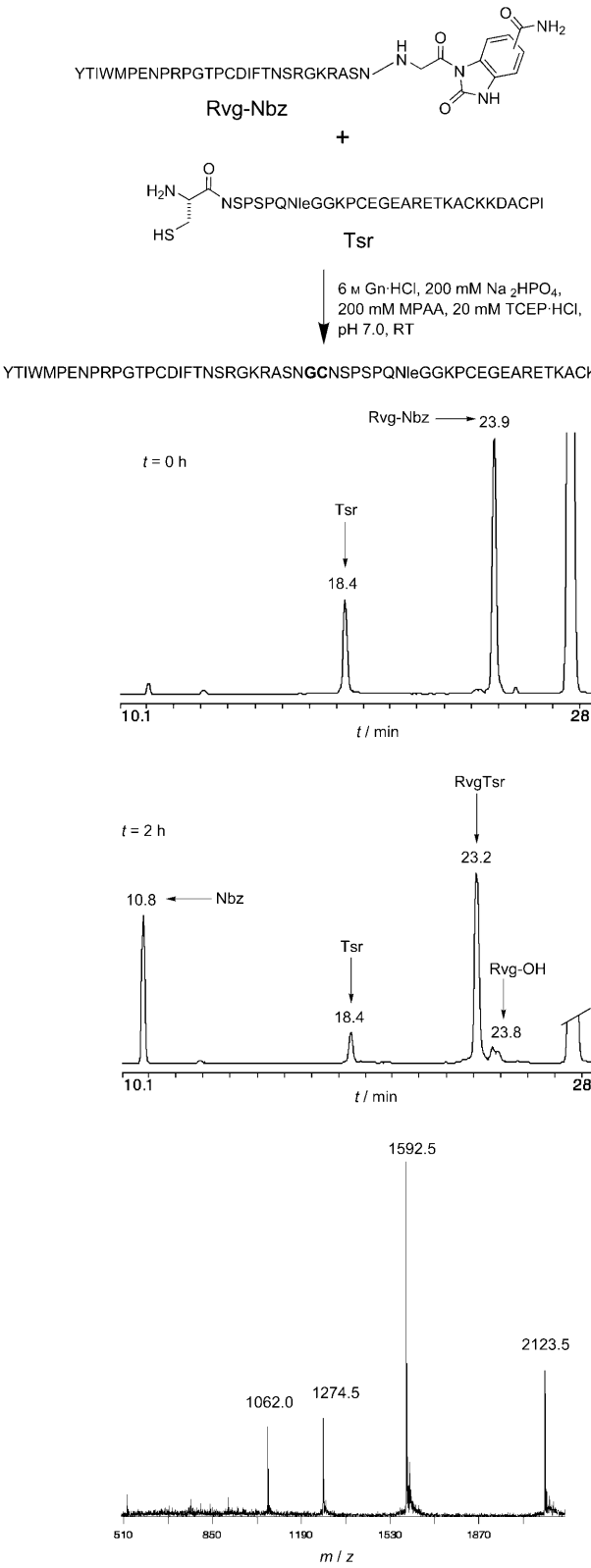

Figure 3. Ligation of Rvg-Nbz and Tsr (N-terminal Cys, 30 aa). HPLC traces are shown at 0 and 2 h. RvgTsr, mass observed: [*M*+H] 6367.0 Da, (calculated: 6367.3 Da).

resins and should also enable orthogonal protecting groups to be utilized. As a result, straightforward access to C-terminally activated Nbz peptides should widen the application of chemoselective ligation methods for the synthesis of complex post-translationally modified proteins and other macromolecules.

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[19] In the absence of thiol additives (MPAA), significant hydrolysis of the LYRAG-Nbz was observed (see the Supporting Information, pages S30–S32).
[20] As monitored by HPLC in comparison to C-terminally epimerized peptide LARG^{D}Y-Nbz (synthesized separately).
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[23] Note, both the Rvg-SAr and the cyclic thiolactone products were observed, highlighting the advantage of in situ thiolysis.
[24] Tsr is a 30-mer peptide fragment of Thrombospondin-1 anti-angiogenic protein. T. K. Tiefenbrunn, P. E. Dawson, unpublished results.

