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Boc-SPPS: Compatible Linker for the Synthesis of Peptide *o*-Aminoanilides

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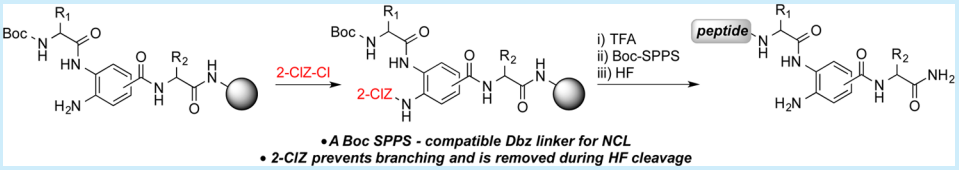
Supporting Information



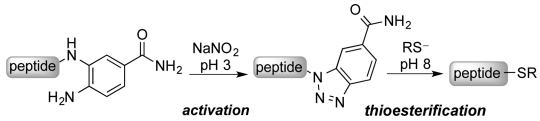
ABSTRACT: A protection strategy is described for the efficient synthesis of peptide *o*-aminoanilides using *in situ* neutralization protocols for Boc-SPPS. On-resin protection of Boc-protected aminoacyl *o*-aminoanilides is achieved with 2-chlorobenzyl chloroformate. Activation through a peptidyl-benzotriazole intermediate allows for facile conversion to peptide-thioesters for use in native chemical ligation. In addition to providing a robust alternative to established thioester resins, as a latent thioester, the peptide *o*-aminoanilide has broad utility in convergent ligation strategies.

The chemical synthesis of proteins allows for complete control over their covalent structure and enables the introduction of various natural and unnatural modifications with atomic precision.[1,2] Additionally, chemical synthesis enables the preparation of mirror-image proteins and proteins with non-natural architectures.[3−5] Native chemical ligation (NCL) is a versatile method that is commonly used in chemical protein synthesis for the chemoselective ligation of unprotected peptide fragments in solution.[6] NCL proceeds through the selective but reversible reaction between a peptide with an N-terminal cysteine and a peptide-thioester. As a result, the generation of C-terminal thioester peptides is critical for the implementation of NCL and related synthetic approaches.

Peptide thioesters can be synthesized directly by solid phase peptide synthesis (SPPS) using the Boc/Bzl protection strategy.[7,8] Peptide-thioester[9] and peptide-thioacid[10] linkers have been used in the synthesis of a wide range of protein molecules;[11] however, the sensitivity of the thioester moiety to nucleophiles requires the use of partially protected peptide intermediates. For instance, His(Dnp) and Trp(For) cannot be removed in the presence of the thioester and typically remain protected following HF cleavage.[12] Similarly, the use of the Fmoc group for orthogonal side chain protection is limited due to the nucleophilic nature of piperidine.[13] A base stable linker that could be converted to a thioester following chain elongation would provide a general solution to these synthetic limitations.

The 3,4-diaminobenzoyl (Dbz) group has been developed as a linker for the Fmoc-SPPS of thioester peptides.[14,15] Following chain elongation, the linker can be treated with nitrophenyl-chloroformate to generate an *N*-acylbenzimadazolinone (Nbz) intermediate[16,17] that can be readily converted into a thioester peptide.[14] Alternatively, *o*-aminoanilide peptides can be obtained by cleaving the Dbz linker without modification. Subsequent treatment of the unprotected peptide with sodium nitrite yields an acyl benzotriazole (Bt) intermediate[18−20] that can be intercepted by a thiol to generate C-terminal thioester peptides[21] (Scheme 1) in an analogous manner to the transfer active ester approach of Ramage.[22,23] The utility of acylbenzotriazole peptides has been extensively explored by Katrizky and applied to the synthesis of peptide thioesters.[24,25]

Scheme 1. Peptide-Dbz Conversion to Peptide-Thioester via Acylbenzotriazole Intermediate[21]



The Dbz linker was originally developed for Fmoc-SPPS protocols using neutral to acidic coupling conditions.[15] In our hands, the Dbz linker is not suitable for the synthesis of long peptides using the in situ neutralization protocols for Boc SPPS, pioneered by Schnölzer et al.,[8] since the use of excess base to neutralize residual TFA leads to branched intermediates[26] (Scheme 2, top). In order to facilitate a more robust route for peptide *o*-aminoanilides by Boc-SPPS, we have developed a solid phase Dbz protection strategy that is compatible with basic *in situ* neutralization coupling protocols (Scheme 2, bottom).

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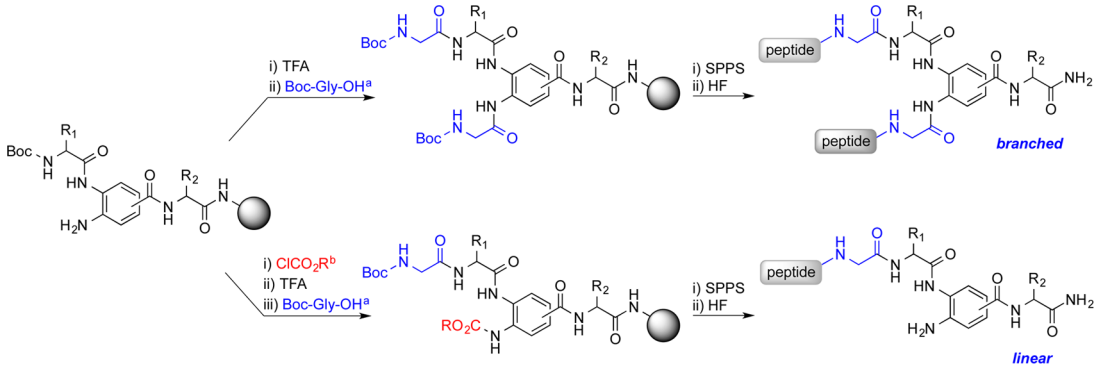
Scheme 2. Use of the Dbz Linker in SPPS Using Boc *in Situ* Neutralization with and without a Free Amine



[a]Conditions: Boc-Gly-OH (5 equiv), HATU (5 equiv), DIEA (7.5 equiv), DMF (0.4 M), 15 min, 23 °C. [b]10% (v/v) $ClCO_2R/CH_2Cl_2$ (10 equiv), 16 h, 23 °C.

To illustrate the limitations of the first generation Dbz linker, a model glycine rich peptide Phe-Gly_5-Dbz-Lys-NH_2 was synthesized using excess base during coupling.[26,15] We recommend incorporating a Lys residue at the C-terminus (ultimately part of the leaving group) to maintain the known acid lability of α-amino acids from MBHA resin[27] and also to provide improved handling and ionization properties. The peptide was synthesized using a common variant of the Boc *in situ* neutralization protocol, which employs excess base (5 equiv of amino acid, 5 equiv of HATU, 7.5 equiv of DIEA).[8] As may have been expected, without prior protection of the free aromatic amine, a high degree of branched peptides **1a**–**1d** was obtained, while minimal amounts (>10%) of the desired product **2** were observed (Figure 1).

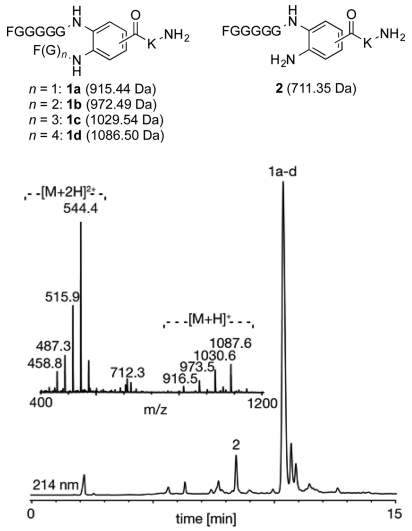


Figure 1. Synthesis of FGGGGG-Dbz-K-NH_2 using the Boc in situ neutralization protocol without Dbz protection. Observed masses of branched peptides: **1a**, 915.5 ± 0.1 Da; **1b**, 972.5 ± 0.1 Da; **1c**, 1029.7 ± 0.1 Da; **1d**, 1086.6 ± 0.2 Da. Calculated monoisotopic masses: **1a**, 915.44 Da; **1b**, 972.49 Da; **1c**, 1029.54 Da; **1d**, 1086.50 Da.

To avoid the formation of branched side products, carbamate protection of the Dbz linker has been demonstrated.[26] To obtain Dbz-peptides after cleavage, a protecting group that is TFA stable and HF labile was evaluated. The 2-ClZ group, which is commonly used for Lys side chain protection, was expected to have these properties. However, because 2-ClZ-anilides are HF-labile, we sought to validate our approach with the analogous HF-stable ethyl carbamate. Boc-Gly-Dbz-resin was treated with 10% (v/v) ethyl chloroformate in DCM for 16 h before a polyglycine model peptide was synthesized. The protected chloroformate was then cleaved by HF to yield **3**. Analysis of the crude reaction by MS showed that only negligible amounts of branched peptide were detected, with the main mass corresponding to that of the desired product (calculated, 783.4 Da; observed, 783.5 ± 0.1 Da) (Figure 2).

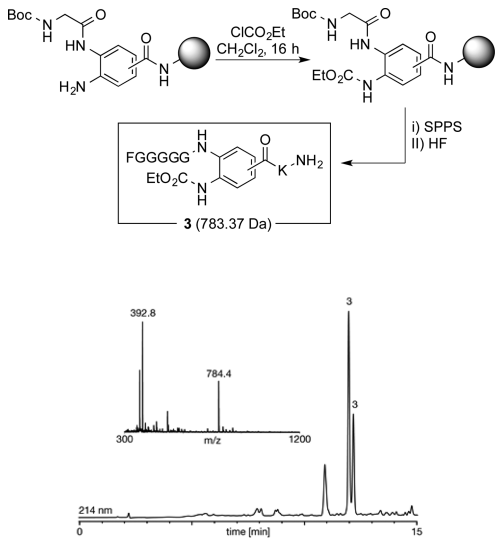


Figure 2. Use of ethyl chloroformate protected Dbz for the synthesis of a linear polyglycine peptide. Observed mass of **3**, 783.5 ± 0.1 Da; calculated monoisotopic mass, 783.4 Da.

Based on the results from the HF stable ethyl carbamate, the HF labile 2-ClZ was installed by treatment of Boc-Gly-Dbz-resin with 2-chlorobenzyl chloroformate, and the desired non-branched model peptide **2** was formed as the main synthetic product (Figure 3). The clean product obtained from 2-ClZ protected Dbz is in stark contrast to that obtained from the Dbz linker and suggests that carbamate protection could be a general solution for the synthesis of *o*-aminoanilide thioester precursor peptides by means of Boc in situ neutralization SPPS.

In order to demonstrate the broader applicability of this approach, we synthesized peptide sequences of practical relevance. The most common His protecting group for thioester peptides is dinitrophenyl (Dnp).[7,28] The alternative His(Bom)

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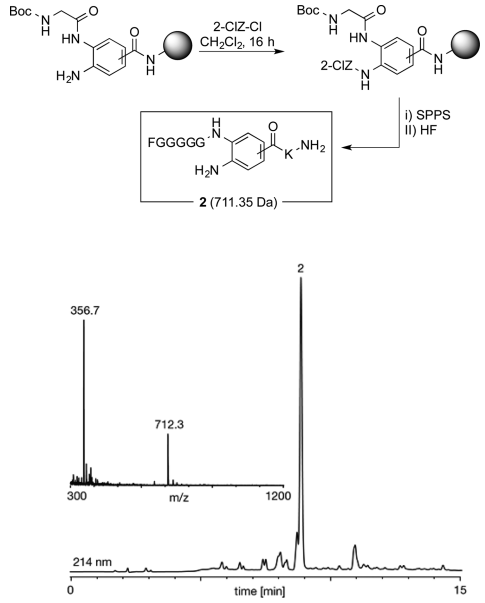


Figure 3. Use of 2-ClZ protected Dbz for the synthesis of a linear polyglycine peptide. Observed mass of **2**, 711.3 ± 0.1 Da; calculated monoisotopic mass, 711.35 Da.

releases formaldehyde and is therefore not commonly used in peptides for NCL.[29] Because on-resin Dnp deprotection is not compatible with thioester linkers, this side chain protecting group remains on the peptide until ligation.[7] Tryptophan is frequently used without any side chain protection for the same reason, or alternatively, the formyl group is removed post-ligation.[30] Therefore, we synthesized peptide **4** (WHGGG-Dbz-K), using Boc-Trp(For)-OH and Boc-His(Dnp)-OH. Peptide cleavage off the resin was successfully performed before and after deprotection to generate partially protected peptide **4a** or fully unprotected peptide **4b** (Figures S1 and S2). This illustrates that the Dbz thioester precursor enables the standard usage of both of these amino acids in the synthesis of *o*-aminoanilide peptides because the linker is stable to on-resin deprotection of His(Dnp) (20% (v/v) *β*-mercaptoethanol, 10% (v/v) DIEA in DMF, 23 °C) and Trp(For) (10% (v/v) piperidine in DMF, 0 °C).[8,12]

Biotin or other side chain modifications during Boc SPPS are commonly introduced through the incorporation of Boc-Lys(Fmoc)-OH.[31] This approach utilizes the robust deprotection and orthogonality of the Fmoc protecting group. The stability of the *o*-aminoanilide linker to piperidine should enable the routine use of Boc-Lys(Fmoc)-OH (Scheme 3). Indeed, we were pleased to find that FK(Biotin)GGG-Dbz-K (**5**) could be obtained using our protocol (Figure S3). HPLC and MS analysis showed that this biotinylated model peptide was obtained in high purity (calculated, 951.14 Da; observed, 951.6 Da ± 0.1 Da). As this is a general approach for Lys side chain modifications, it is not limited to biotinylation and could be applied to other modifications (dyes, fluorescent probes, etc.) or for the synthesis of branched peptide[32] or dendrimer thioesters.[33,34]

Hepcidin is a regulator of iron metabolism,[35] containing eight cysteines that are oxidized in the native conformation.[36] We sought to synthesize this peptide with a site for side chain modification at residue 12 using side chain Fmoc protection and NCL. The peptide DTHFPICIFCCK(N^{ϵ}-biotin)-Dbz-K-NH_2 (**6**) was prepared on MBHA resin,[27] cleaved with HF, and purified by HPLC (Figure 4). Activation to the peptidyl-

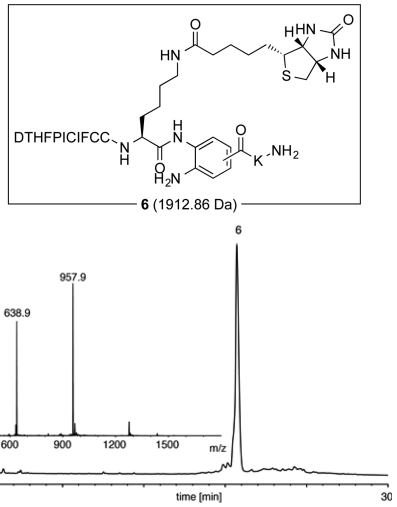
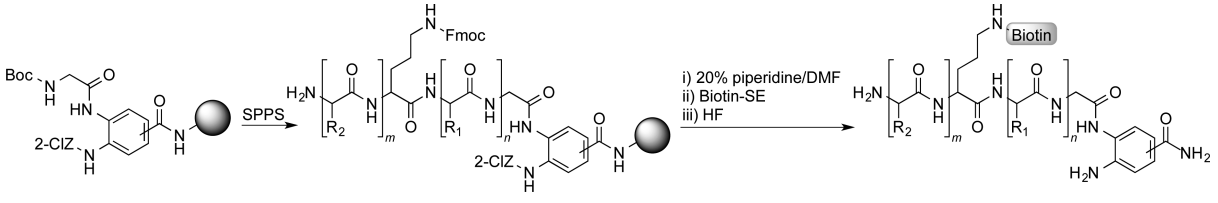


Figure 4. HPLC and MS traces of DTHFPICIFCCK(N^{ϵ}-biotin)-Dbz-K-NH_2 (**6**).

benzotriazole was achieved by incubation of 10 mM peptide in 6 M GuHCl, 100 mM NaH_2PO_4, 50 mM sodium nitrite, final pH 3, at −15 °C, for 5 min. Direct conversion to the thioester proceeded upon adding one volume of 200 mM Mesna in 6 M GuHCl and 200 mM Na_2HPO_4 (pH 8) adjusted to a final pH of 7, for 5 min, 23 °C.[21] The peptide thioester **7** was purified immediately by preparative HPLC (53% recovered yield, Figure S4). DTHFPICIFCCK(N^{ϵ}-Biotin)-Mesna (**7**) was then ligated with the N-terminal peptide CCHRSKCGMCCKT (**8**) in 6 M GuHCl, 200 mM phosphate buffer (final pH 7) and 100 mM mercaptophenylacetic acid.[37] After 2 h, the peptides were fully reduced by the addition of 10 equiv TCEP and the product (**9**) was purified by HPLC, 80% conversion based on limiting peptide **7** (Figure 5).

Finally, to demonstrate the utility of this approach in the synthesis of longer peptides, a 27 amino acid fragment of the SH2 domain (**10**) was prepared on a 0.1 mmol scale to yield 405 mg of peptide resin, representing 76% of the expected weight gain (Figure S5). The resin was cleaved to yield 245 mg of crude

Scheme 3. Strategy for the Use of Boc-Lys(Fmoc)-OH in the Synthesis of Biotinylated *o*-Aminoanilide Peptides, Demonstrating the Compatibility of Fmoc-Protective Groups with Boc-SPPS



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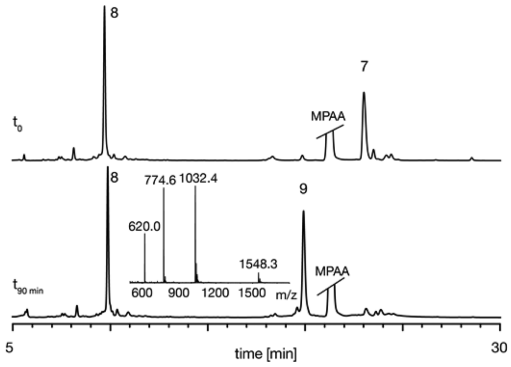

Figure 5. HPLC traces of the ligation between thioester peptide **7** and Cys-peptide **8** at 0 and 90 min. MS of purified ligation product **9** is also shown.

peptide **10**, 74% recovered yield, suggesting that the linker does not reduce the loading of the resin and is stable to Boc-SPPS.

In summary, a solid phase protection strategy has been developed that allows for the synthesis of thioester precursor *o*-aminoanilide peptides. The strategy is compatible with Boc *in situ* neutralization chemistry and nucleophile-labile protecting groups, and we expect that the standard linkers and resins previously developed for Boc-SPPS can also be used. Moreover, the resulting *o*-aminoanilide peptides are compatible with convergent ligation strategies[30] and allow for N to C terminal sequential peptide ligation.[21,38] The 2-ClZ-Dbz linker enables efficient synthesis of highly reactive aryl thioesters that can be used directly for NCL or of highly stable alkyl thioesters that can be stored long-term.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b03111.

Experimental procedures and HPLC and MS data of new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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