Review on the *o*-Aminoaniline Moiety in Peptide and Protein Chemistry

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Peptides and proteins are important functional biomolecules both inside and outside of living organisms. The ability to prepare various types of functionalized peptides and proteins is essential for understanding fundamental biological processes, such as protein folding and post-translational modifications (PTMs), and for developing new therapeutics for many diseases, such as cancers and neurodegenerative diseases. The o-aminoaniline moiety was first proposed for activation to a thioester precursor and used for native chemical ligation to prepare large peptides and proteins. In the past decade, the function of o-aminoaniline has been greatly expanded to facilitate the preparation of homogeneously modified peptide and protein samples, where the modifications can include cyclization, C-terminus diversification, etc. Many o-aminoaniline derivatives have also been developed to overcome the inherent limitations of previous versions. In this review, we attempt to summarize the recent developments of different o-aminoaniline derivatives, focusing on their application to the preparation of functional peptide and protein molecules.

1. Introduction

Peptides and proteins are important functional biomolecules both inside and outside of living organisms. The ability to prepare various types of functionalized peptides and proteins is essential for understanding fundamental biological processes, such as protein folding and post-translational modifications (PTMs),[1–7] and developing new therapeutics for various diseases. Nowadays, conventional recombinant expression technology provides rapid and convenient access to many protein molecules, but it is usually limited to natural amino acids. Site-specific modifications on side chains and the backbone of proteins remain challenging although some progress has been made using new strategies such as genetic codon expansion.[8,9] Chemical protein synthesis (CPS) gives atomic control over the composition of proteins and remains the best way to prepare homogeneously modified protein molecules. With the efforts from the past several decades, solid phase peptide synthesis (SPPS) is currently a routine for the preparation of peptides shorter than 50 residues, especially with the Fmoc (fluorenylmethyloxycarbonyl) deprotection chemistry and automation.[10] But the chemical synthesis of a protein molecule with medium to large sizes remains challenging. Many methods have been developed for putting peptide segments together to afford the full-length proteins and the native chemical ligation (NCL) developed by the S. B. Kent group[11] has found the most success. For a typical native chemical ligation, after each amino acid is attached to the solid resin following the peptide sequence, the peptide is cleaved off the resin and activated into a peptidyl thioester, which can react with another peptide bearing an N-terminal cysteine to generate a longer peptide with native peptide bond. A relatively large protein molecule can be obtained through multiple ligation steps, either in solution or on solid resins. There are more comprehensive reviews on either native chemical ligation or solid phase peptide synthesis that readers can reach to.[5,10,12–16]

The installation of a cysteine residue at the N-terminus of a peptide is relatively straightforward, but the preparation of peptide thioesters remains a significant challenge for native chemical ligation, particularly in the widely used Fmoc-SPPS. Over the past few decades, various strategies have been developed to address this,[17,18] including the use of hydrazides[19,20] and o-aminoaniline derivatives.[21–23] While the 3,4-diaminobenzoic acid (Dbz) linker was introduced over a decade ago as a tool for peptide thioester generation in Fmoc-SPPS, its application in chemical protein synthesis has been limited due to side reactions that complicate its use, especially for researchers unfamiliar with the method. Since its initial development, the o-aminoaniline moiety has been modified into multiple variants to overcome these challenges and has also been adapted for other applications, such as peptide cyclization and C-terminal diversification. However, selecting the suitable derivative of Dbz for a particular case is usually challenging, limiting its use to only the chemical protein synthesis field.

Here in this short review, we will focus on the development and application of o-aminoaniline moiety for their use in preparing functional peptide and protein molecules. We first discuss the development of Dbz and its modified variants for the preparation of peptidyl thioester used in native chemical ligation and protein preparation. This mainly

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covers the synthetic protein examples and the evolution of variants to overcome potential side reactions people observed. Later we introduce the expansion of o-aminoaniline moiety to peptide cyclization and C-terminus diversification. In the end, we provide a practical guide for the use of various o-aminoaniline linkers.

2. Development of *o*-Aminoaniline for Peptide Thioester Generation

NCL, in which a peptide thioester reacts with the N-terminal cysteine of another peptide to form a native peptide bond, is one of the most widely used strategies for protein chemical synthesis.[11,14] While introducing a cysteine residue at the N-terminus of a peptide is more accessible, generating a peptide thioester is challenging. Due to its milder deprotection conditions, Fmoc-SPPS is the preferred method for synthesizing short peptides. However, peptide thioesters are incompatible with the repeated piperidine treatments required in Fmoc-SPPS.

To address this issue, Dawson group developed Dbz as a stable precursor for generating peptide thioesters in Fmoc-SPPS.[21] Once protected on the 3-amino group with Fmoc, Dbz can be conveniently incorporated onto the resin as though it were an amino acid. After peptide elongation to the desired sequence, Dbz is activated on-resin via a two-step cyclization reaction (Figure 1, top pathway). First, the peptide is treated with 4-nitrophenyl chloroformate (4-NPCF) to generate a 4-nitrophenyl carbonyl intermediate. Cyclization to form N-acyl-benzimidazolinone (Nbz) is then achieved by adding Hünig's base, releasing 4-nitrophenol as a by-product. The unprotected peptidyl Nbz is subsequently converted in situ into a thioester upon the addition of an excess of external thiols, such as 4-mercaptophenylacetic acid (MPAA). The authors demonstrated the feasibility of the method by the ligation of a rabies virus glycoprotein derived peptide and Tsr peptide.

An alternative oxidative activation pathway was developed by Liu group to extend the use of Dbz to unprotected peptides (Figure 1, middle pathway).[22] In this approach, unprotected peptidyl Dbz is converted into benzotriazole (Bt) using sodium nitrite treatment at approximately −15 °C under acidic conditions (pH 2–3). Benzotriazole, a good leaving group, can then be displaced by external thiols like MESNA or MPAA, generating the corresponding thioester. The authors investigated the excellent stability of Dbz form under typical desulfurization and ligation conditions and supported its use for convergent ligation to prepare histone H2B. They further tested its use for the preparation of cyclic peptides: the cyclic sunflower trypsin inhibitor-1 (SFTI-1) and the poorly soluble cyclic bacteriocin lactocyclicin Q.

Ziyong Z. Hong received his PhD in Chemistry from The Ohio State University (2021). Currently, he is a postdoctoral research associate at the University of Wisconsin – Madison. His research interests focus on chemical biology tool development for efficient bio-macromolecule preparation, structural biology of O-GlcNAcylation, and peptide binder design for O-GlcNAc cycling enzymes.

3. *o*-Aminoaniline for Chemical Protein Synthesis

Numerous strategies have been developed to facilitate the preparation of peptide thioesters in Fmoc-SPPS, including safety-catch linkers,[24] peptide hydrazides,[19,20,25] and the bis(2-sulfanylethyl) amino (SEA) group,[26–29] among others. However, the advantages of the Dbz linker are particularly noteworthy. Firstly, mono-Fmoc-protected Dbz can be easily incorporated as an amino acid. Secondly, it can be activated into peptide thioesters via multiple pathways, offering flexibility to meet specific requirements. Thirdly, the Dbz form is stable under typical desulfurization and ligation conditions. Lastly, the extra functional group on the aromatic ring allows for auxiliary chemistry, such as the addition of soluble tags or reactive handles. Using Dbz chemistry, researchers have successfully synthesized proteins, including histones with extensive post-translational modifications (PTMs).

Histone proteins – including core histones H2A, H2B, H3, and H4, as well as linker histones H1s – are essential components of eukaryotic chromatin. The PTMs on histones regulate nucleosome and chromatin structure and function, thereby influencing gene expression.[30,31] The chemical synthesis of histones with site-specific PTMs provides molecular insights into how these modifications affect nucleosome and chromatin dynamics. Dbz chemistry has been instrumental in preparing such histone proteins, enabling detailed functional studies. For instance, the Brik group chemically synthesized H2A,[32] H2B,[33–35] and H3[36] with desired PTMs, comparing the efficiency of different synthetic strategies. Ottesen group synthesized H4 and the H3 variant CENP-A using hybrid phase ligation.[37] They further enhanced the yield and successfully achieved prepared linker histone H1.2 through a convergent hybrid phase ligation strategy leveraging the Dbz linker.[38]

Beyond histones, other proteins and domains have also been prepared using Dbz chemistry. Hartrampf group recently demonstrated the installation of a synthesis tag at the C-terminus of Dbz, simplifying the preparation of aggregation-prone peptides and proteins.[39] They successfully synthesized the transactivation domain of the oncogenic protein MYC. For more comprehensive reviews on chemical protein synthesis, please refer to additional sources.[3,6,40–42]

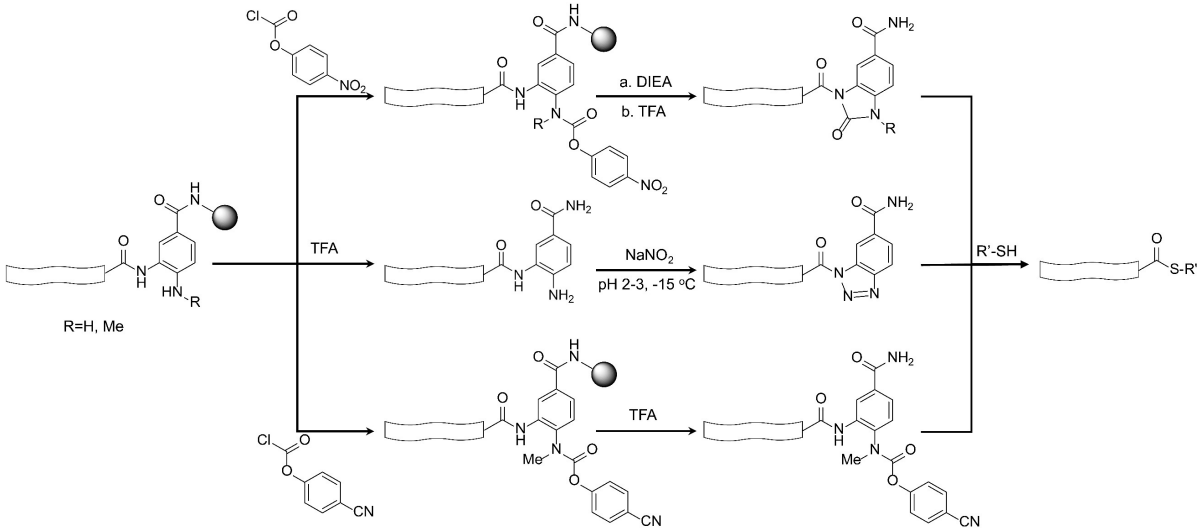

Figure 1. Activation Pathways of Dbz derivatives for thioesters used in native chemical ligation. Top: Nbz pathway. Middle: benzotriazole (Bt) pathway. Bottom: modified Nbz pathway.

4. Side Reactions and Derivatives of *o*-Aminoaniline Linkers

While the robustness of Dbz was demonstrated by some following studies, its application for chemical protein synthesis has been highly underrepresented, likely due to the side reactions observed and the different variants developed which complicate its use. Although the final activated thioester is not affected, a roughly 4:1 ratio of 3- and 4-isomer peptide products is often observed with Dbz, suggesting that both amines are accessible to acylation. While one amine of Dbz is acylated to elongate the peptide chain, the unprotected amine may also undergo acylation, resulting in doubly acylated side products that hinder conversion to Nbz (Figure 2, top panel). This issue is particularly noticeable in glycine-rich peptides and under conditions with excess base during coupling. Additionally, the free amine complicates acetyl capping – a standard step in SPPS to reduce impurities and simplify purification.

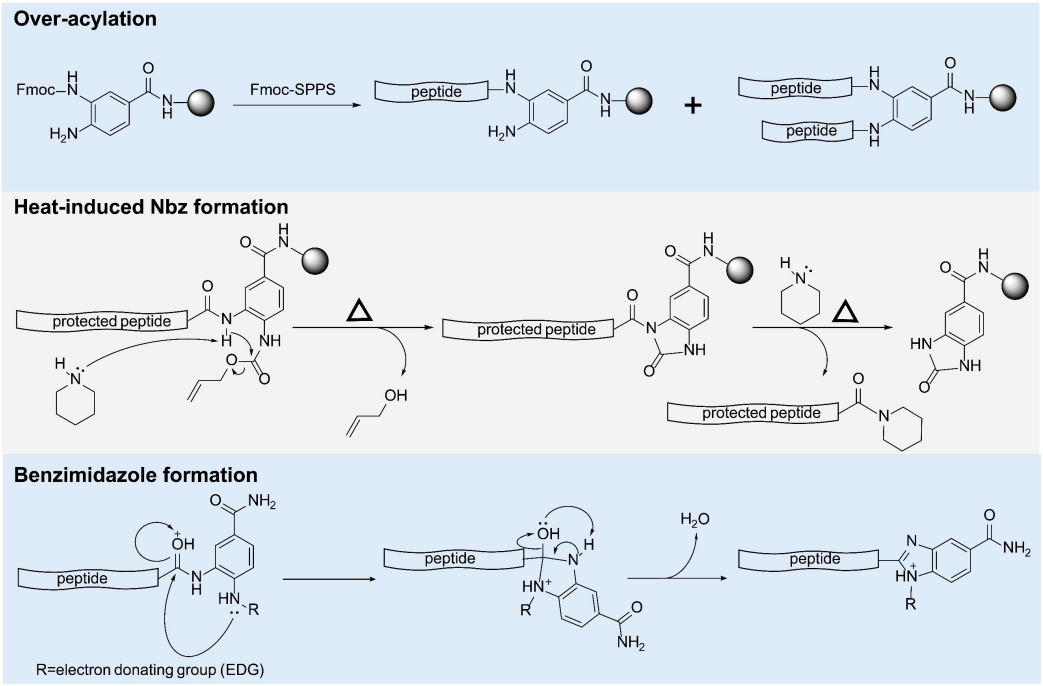

Figure 2. Side reactions on Dbz linkers. Top panel: over acylation happening on the second amine of Dbz. Middle panel: heat/microwave-induced Nbz formation during piperidine treatment. Bottom panel: acid-catalyzed formation of peptidic benzimidazole. The figure is adapted from ref. [38].

To overcome these challenges, a reversible protecting group, N-allyloxycarbonyl (Alloc), was initially introduced to mask the second amine.[43] The Alloc group can be easily installed and removed on-resin by allyl chloroformate and Tetrakis(triphenylphosphine)palladium(0), respectively. Propargyloxycarbonyl (Proc) protection of Dbz has also been used due to its efficient removal with palladium in aqueous solution. While Dbz is mainly used in Fmoc-SPPS, 2-chlorobenzyloxycarbonyl (2-ClZ) protected Dbz was developed to prevent over-acylation and enable use in Boc-SPPS.[44]

However, microwave-assisted peptide synthesis,[16,45] increasingly popular for its high efficiency, introduced new complications. Elevated temperatures (either through microwave or heating) caused Alloc(Dbz) to cyclize, forming peptidyl Nbz during piperidine-mediated Fmoc deprotection (Figure 2, middle panel).[38,46] Repeated piperidine treatments then led to premature peptide cleavage from the resin, significantly reducing yield. To address this, Yoshiya and co-workers developed Dbz(NO_2), a modified Dbz in which the 4-amino group is replaced with a nitro group.[46] This nitro group is reduced to an amine after peptide assembly to regenerate Dbz. However, this strategy is most practical for peptides with a C-terminal Gly due to the nitro group's presence.

Ottesen group has managed this issue by combining high-temperature coupling with room-temperature Fmoc deprotection. We previously attempted to Boc-protect Dbz by treating the Dbz-resin with $(Boc)_2O$ and DMAP, but this approach failed to yield only Nbz products. More recently, Mandal group developed Dbz(Boc) by using milder $(Boc)_2O$ and DIEA treatment.[47] Here, the 4-amino group is protected by a Boc group, which is widely used in peptide synthesis and is cleaved upon resin cleavage. However, this derivative cannot be activated on-resin to produce Nbz, meaning that unprotected peptidyl Dbz is converted to a thioester exclusively through the benzotriazole pathway.

To further address the over-acylation issue, a second-generation thioester precursor, 4-N-methyl-Dbz (MeDbz), was developed.[23] With the addition of a methyl group, the 4-amino group is effectively blocked, preventing doubly acylated side products and making it a popular alternative. However, MeDbz can only be activated via the MeNbz pathway. Additionally, acetylation of the methyl amine in MeDbz was observed when synthesizing peptides with C-terminal Gly using standard capping protocols (acetic anhydride and Hünig's base), which hinders further activation of MeDbz and leads to material waste. To resolve this, the Stockdill group developed a milder capping protocol using bulkier benzoic acid, which avoids acylation on the methyl amine.[48]

A new side reaction was identified during peptide synthesis with MeDbz: formation of peptidic benzimidazole upon peptide cleavage at the MeDbz stage (Figure 2, bottom panel). Dawson's lab investigated various electron-donating substitutions on the 4-amine of Dbz and developed a protocol to generate benzimidazole through a 24 hour TFA cocktail cleavage.[49] Our group also analyzed the conversion of MeDbz to benzimidazole, finding that this reaction proceeds readily in acidic aqueous solutions but can be suppressed by adjusting the pH to neutral or basic.[38] This reaction is sequence-dependent but can be driven to completion by extending the reaction time. Notably, benzimidazole has emerged as a promising core structure in the development of HDAC inhibitors.[50,51] While o-phenylenediamine has long been used for benzimidazole synthesis, this mild peptidic benzimidazole formation could be valuable for creating novel therapeutics targeting diseases associated with aberrant HDAC activity.

Incomplete conversion of MeDbz to MeNbz has also been observed in a sequence-dependent manner.[38] While the initial step – addition of 4-nitrophenyl chloroformate to generate 4-nitrophenyloxycarbonyl-o-MeDbz (p-NO_2-Phoc-MeDbz) – is consistently complete, cyclization to form MeNbz is inefficient in some peptide sequences. We previously attempted to improve this step by using elevated temperatures. Recently, Blanco-Canosa's group developed an alternative approach, using p-cyanophenyl chloroformate to create a stable p-CN-Phoc-MeDbz intermediate (Figure 1, bottom pathway).[52] This peptidic p-CN-Phoc-MeDbz cyclizes to MeNbz in a slightly basic aqueous solution and can be directly used in native chemical ligation with external thiols.

The core structure of the Dbz linker is o-aminoaniline, Hoheisel group directly used orthophenylene diamine with Wang resin to prepare peptidyl Dbz for thioester activation and following native chemical ligation.[53] Instead of deactivating one of the amines with a protecting group, Koh group attached o-phenylenediamine derivatives directly to 2-chlorotrityl chloride (CTC) resin via an amine group.[54] This approach prevents over-acylation at the amine, although peptidyl o-aminoanilide can only be activated through the benzotriazole pathway, likely due to the steric hindrance of the trityl group. Similarly, Blanco-Canosa's group used a PAL resin (4-(4-(aminomethyl)-3,5-dimethoxyphenoxy) butanoic/pentanoic acid) to link the o-phenylenediamine moiety via one amine. This method combines the advantages of both Dbz and MeDbz, allowing conversion through either the Nbz or benzotriazole pathways while avoiding over-acylation side products.[55]

These observed side reactions have inspired the development of new Dbz linker variants. Over the past decade, approximately nine different Dbz linker versions have been developed, each suited for specific applications (Figure 3), greatly expanding Dbz's utility in peptide and protein research. However, this diversity can complicate the selection process for researchers less familiar with Dbz chemistry. While our group previously provided a flowchart summarizing the selection of Dbz linkers,[38] we aim to include a more user-friendly guide at the end of this review.

5. *o*-Aminoaniline for Peptide C-Terminal Modifications

Modifications at the C-terminus can significantly impact a peptide's biophysical and biochemical properties. A common modification is the removal of the carboxylic acid's negative

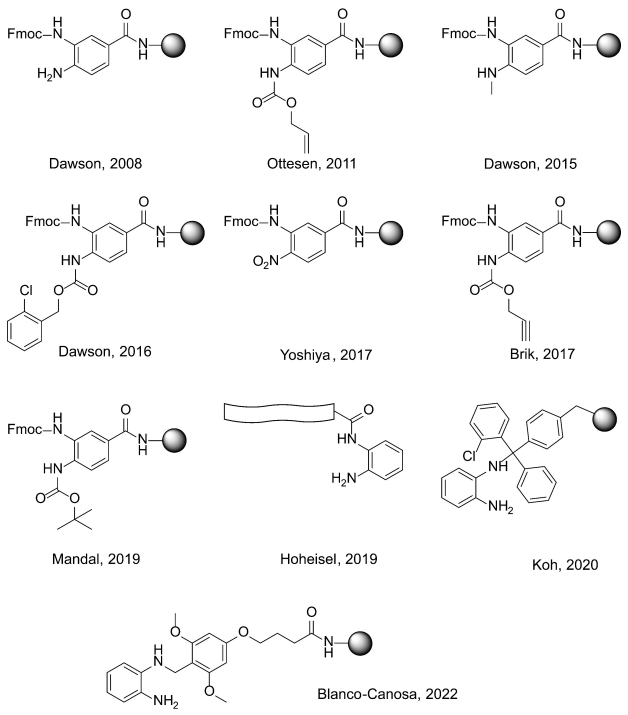

Figure 3. Dbz and its modified versions for solid phase peptide synthesis.

charge, often achieved through C-terminal amidation. This strategy is frequently used to create a neutral C-terminus, mimicking the environment of peptides derived from the middle of a protein. Other C-terminal modifications include esterification, thioesterification, and more. Dbz and its derivatives are particularly suited for post-synthesis C-terminal modifications, as they can be easily activated and substituted by various nucleophiles (Figure 4). For a more comprehensive discussion on peptide C-terminal modifications, please refer to other specialized resources.[56]

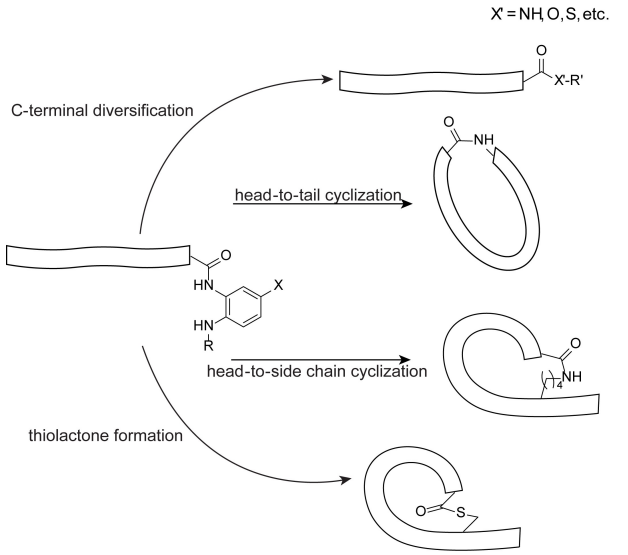

Figure 4. Application of Dbz linkers for preparation of C-terminal modified peptides and cyclic peptides.

Stockdill's group explored C-terminal diversification using MeDbz, where MeDbz is first converted to MeNbz, followed by treatment with different nucleophiles to yield carboxamides, alkyl and aryl amides, esters, hydrazides, hydroxamides, acids, and amino alcohols.[57–59] They compared C-terminal modifications in both protected and unprotected peptides. While protected peptides are well-suited for C-terminal modification, unprotected peptides with nucleophilic side chains or Pro or Gly residues require careful handling to prevent unintended cyclization.

Kao's group developed an on-resin activation method for Dbz by converting Dbz into Bt using isoamyl nitrite on-resin.[60] Cleavage and deprotection in the presence of nucleophiles then generated the desired C-terminal modifications. Recently, they extended this approach to create branched peptides via multiple lysine side chains and to synthesize peptidols through $NaBH_4$ reduction.[61,62]

6. *o*-Aminoaniline for Cyclic Peptide Synthesis

Cyclic peptides have garnered increasing attention in therapeutics and clinical applications due to their resistance to proteolysis, more rigid conformations, and larger binding interfaces with targets.[63] Successful synthesis of cyclic peptides is crucial for investigating their structure and functions. Peptide cyclization often involves the nucleophilic attack of a side chain or N-terminal amine on an activated carboxylic acid. Dbz and its derivatives are well-suited for this purpose. Among various cyclic peptide types, head-to-tail and head-to-side-chain cyclic peptides are the most commonly synthesized using Dbz and its derivatives (Figure 4). For a broader overview of peptide cyclization methods, please refer to additional sources.[64–66]

When native chemical ligation occurs within the same peptide, a homodetic cyclic peptide is formed. In 2013, Göransson group used the Dbz linker to synthesize cyclic peptides such as kalata B1 and sunflower trypsin inhibitor 1 (SFTI-1) through intramolecular native chemical ligation.[67] Adding external thiols like MPAA facilitates the intramolecular cyclization of purified peptidic Nbz with a C-terminal cysteine. This strategy has also been used to prepare other cyclic peptides, such as the lactadherin-mimicking peptide (cLac) and the cyclic citrullinated fibrinogen peptide, enabling functional and structural studies.[68–70] Given the general applicability of native chemical ligation, all modified Dbz derivatives should function similarly to the original Dbz linker and are not limited to the Nbz activation pathway.

Another Dbz linker strategy for peptide cyclization involves a direct nucleophilic attack by an amine or thiol on Dbz activated through either the Nbz or benzotriazole pathways, eliminating the need for external thiols to form a thioester intermediate. Oishi group synthesized a cysteine-free cyclic peptide, FC131, using a Dbz linker in both protected and unprotected forms.[71] The Dbz linker was first

activated into benzotriazole with nitrite, and cyclization proceeded efficiently through aminolysis by the N-terminal amine with catalysts like HOBt or HOAt. Albericio group employed the MeDbz linker to prepare cyclothiodepsipeptides on-resin.[72] After activating MeDbz to MeNbz, they deprotected a cysteine side chain to achieve cyclization via thiolysis, releasing thiolactone products from the resin.

Similarly, Olsen's, Stockdill's, and Albericio's groups have synthesized head-to-tail cyclic peptides using MeDbz in a one-pot protocol.[73–77] Here, thiolysis by the N-terminal cysteine cleaves the peptide from the resin, followed by an S-to-N shift to yield homodetic cyclic peptides. This method has been applied to various cyclic peptides, including SFTI-1, destoamide B, stellarin G, stylostatin 1, cyclonellin, and crotogossamide, demonstrating the robustness and versatility of this approach.

7. Summary and Outlook

Here we summarized the development of o-aminoaniline linkers for the synthesis of functional peptides and proteins, including site-specifically modified proteins, C-terminally modified peptides, and cyclic peptides. The multiple activation pathways available for o-aminoaniline linkers offer researchers flexible options for diverse applications. However, the potential side reactions associated with these linkers can complicate the selection process. To address this, we have provided a concise flowchart (Figure 5) to aid in choosing the optimal linker. The flowchart begins with the input of the peptide sequence, exemplified here with the histone H4 peptide sequence, SGRKGGKGLGKGG. In this example, (1) the peptide is glycine-rich, and a capping step is desirable to streamline synthesis; (2) the Fmoc-SPPS protocol is selected for its user-friendliness and accessibility; (3) microwave heating is chosen to accelerate the reaction and reduce synthesis time; and (4) we opt for the on-resin Nbz activation pathway, allowing direct use in native chemical ligation after purification. Based on these requirements, MeDbz is identified as the most suitable linker for this synthesis. We hope that this review will broaden the use of o-aminoaniline linkers in peptide and protein chemistry and serve as a straightforward yet effective guide for researchers outside the field.

List of Abbreviations

2-ClZ	2-chlorobenzyloxycarbonyl
4-NPCF	4-nitrophenyl chloroformate
Alloc	Allyloxycarbonyl
Boc	tert-butoxycarbonyl
Bt	Benzotriazole
CPS	Chemical protein synthesis
CTC	2-chlorotrityl chloride
Dbz	3,4-diaminobenzoic acid
DIEA	N,N-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
Fmoc	Fluorenylmethyloxycarbonyl
MeDbz	4-N-methyl-Dbz
MPAA	4-mercaptophenylacetic acid
Nbz	N-acyl-benzimidazolinone
NCL	Native chemical ligation
Proc	Propargyloxycarbonyl
PTMs	Post-translational modifications
SFTI-1	Sunflower trypsin inhibitor-1

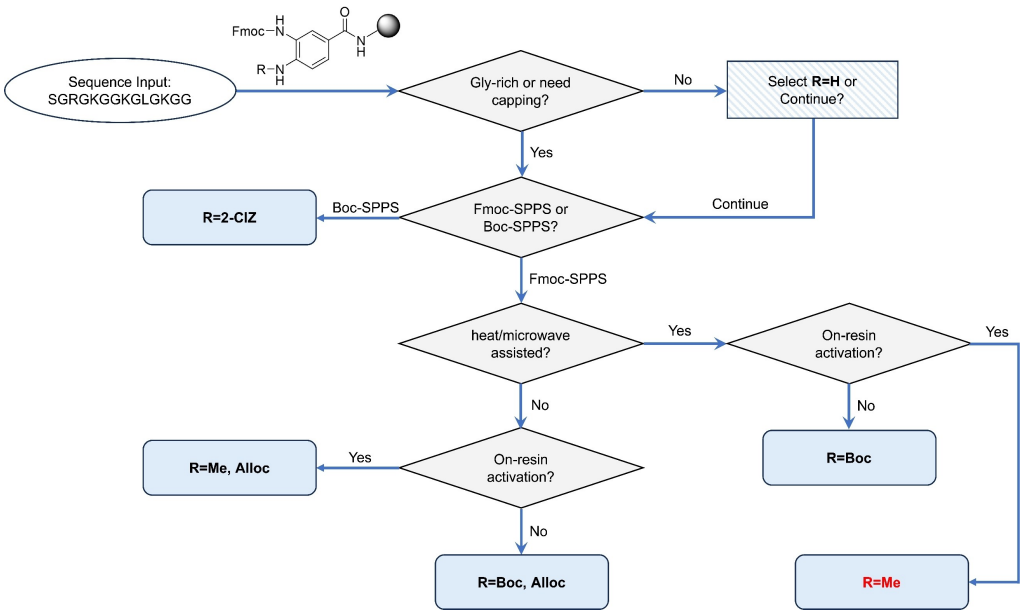

Figure 5. Selection of Dbz derivatives for peptide thioester generation. Assume that we need to synthesize the histone H4 peptide SGRKGGKGLGKGG activated into thioester through the Nbz pathway by microwave-assisted Fmoc-SPPS, the one highlighted in red represents the final choice. Oval: the input, rhombus: the decision tree, rectangle: decision tree or output choice, rounded rectangle: output choice. The figure is adapted from ref. [38].

SPPS Solid phase peptide synthesis
TFA Trifluoroacetic acid

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Conflict of Interests

The authors declare no conflict of interest.

Keywords: Solid phase peptide synthesis · Native chemical ligation · Cyclic peptides · Chemical protein synthesis · *o*-Aminoaniline

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