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Palladium-Assisted Removal of a Solubilizing Tag from a Cys Side Chain To Facilitate Peptide and Protein Synthesis

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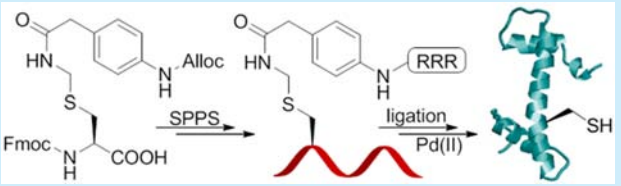
Supporting Information

ABSTRACT: Reversible attachment of solubilizing tags to hydrophobic peptides to facilitate their purification and ligation is an essential yet challenging task in chemical protein synthesis. The efficient palladium-assisted removal of the solubilizing tag linked to the Cys side chain is reported. The strategy was applied for the efficient preparation of histone protein H4 from two fragments via one-pot operation of ligation, removal of the solubilizing tag, and desulfurization.



Peptides are very important biomolecules because of their wide utility in different fields, such as in drug development, as functional biomaterials, and as probes for interrogating biological functions. Importantly, peptides are the building blocks for synthetic and semisynthetic proteins[1] where the fragments are linked together via chemoselective ligation strategies such as native chemical ligation (NCL).[2] Since the development of the revolutionary concept of solid-phase peptide synthesis (SPPS),[3] the field has been going through significant improvements from different aspects to increase the efficiency of peptide synthesis. However, researchers frequently encounter so-called difficult sequences such as peptides derived from membrane proteins and β-amyloid peptides, which are either difficult to synthesize and/or difficult to handle during the purification step after cleavage from the solid support.[4] In addition, in protein synthesis, dissecting the polypeptide to shorter fragments can lead in some cases to the generation of peptides that are difficult to solubilize compared to the full sequence.[5] Hence, ligation reactions become extremely difficult due to the low solubility of some of the fragments despite performing these reactions in denaturing solvents, such as in 6 M guanidinium hydrochloride buffer. As a result, several strategies have been developed to overcome these hurdles, for example, using organic solvents (e.g., DMF),[6] detergents,[7] pseudoproline dipeptides,[8] removable backbone modifications,[9] and solubilizing tags[5c] as well as the incorporation of *O*-acyl isopeptide unit.[10]

Although these methods have been proven to be useful in several studies, still they are not free from limitations. Notably, the use of solubilizing tags through the side chain of the amino acids is relatively still unexplored. Imperiali and co-workers used the *N*,*N*-dimethylethylenediamine (DMDA) group, attached it to the side chain of Asn via a photolabile group, and used it in the synthesis of a peptide derived from the human prion protein.[11] However, this method is restricted to Asn residues in the sequence and requires the solution-phase preparation of the Asn-DMDA analogue. The use of the same photolabile linker was extended to synthesize a lipid-anchored protein.[12] Danishefsky and co-workers demonstrated the attachment of an Arg residue through allylic ester or carbamate linkers to the side chains of Glu and Lys to assist the synthesis of peptide fragments derived from human erythropoietin.[13] However, this strategy does not allow the solid-phase attachment of multiple Arg residues on the same linker. Furthermore, removal of the allylic linker required the use of DMSO to solubilize the tetrakis(triphenylphosphine)-palladium(0) reagent. Development of an approach that enables straightforward installment and removal of the solubilizing tag to the side chain of amino acids is needed. Of great interest are linkers that are stable to SPPS, compatible to ligation approaches, and can be removed in fully aqueous media.

Recently, we reported palladium-assisted efficient removal of thiazolidine (Thz), propargyloxycarbonyl (Proc),[14] and acetamidomethyl (Acm)[15] protecting groups from Cys in the middle fragments and along the sequence under NCL and/or desulfurization conditions. During this work, we came across the phenylactamidomethyl (Phacm) Cys protecting group, which is an analogue of Acm.[16] We wondered if the phenyl ring in Phacm could be functionalized to attach solubilizing tags, which eventually could be efficiently removed by palladium (Scheme 1).

Accordingly, we successfully synthesized Alloc-Phacm linker (**1**) harboring an allyloxycarbonyl (Alloc) protected amine at the para position of the phenyl ring (Scheme 1). The synthetic strategy involved the Alloc protection of 4-aminophenylacetonitrile followed by acid-catalyzed hydrolysis of the nitrile functionality to the amide (Scheme 1). Subsequently, the amide was converted to the *N*-hydroxymethyl amide group, which was then reacted with Fmoc-Cys-OH in the presence of trifluoromethanesulfonic acid (TFMSA) and trifluoroacetic

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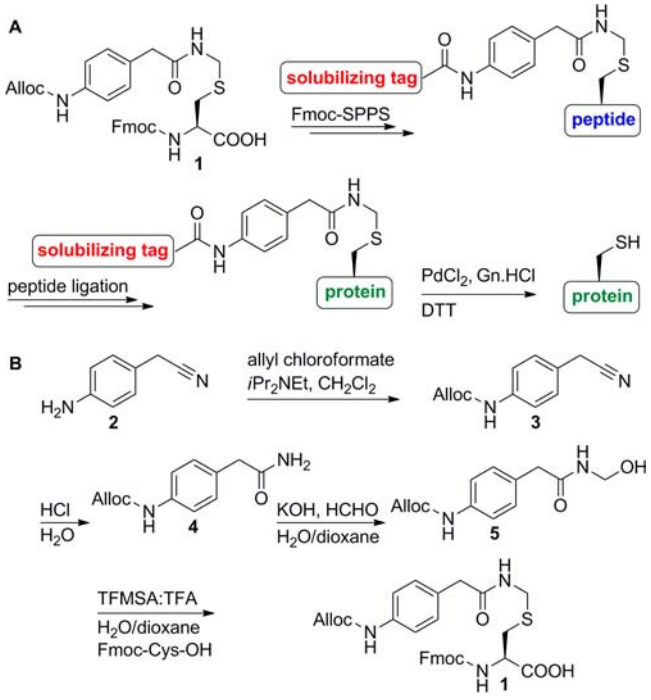
Scheme 1. (A) Attachment of Solubilizing Tag through Alloc-Phacm Linker to Peptides and Proteins as Well as Its Removal via Palladium. (B) Synthesis of Alloc-Phacm Linker 1



acid (TFA) in one-pot fashion to afford the Alloc-Phacm linker in ~35% yield for four steps (Scheme 1).

We then studied the feasibility of incorporating linker **1** in SPPS and the attachment of the solubilizing tag to the Cys residue in the model peptide A**C**LYRAGLYRAG (Figure 1). The Alloc-Phacm linker was successfully incorporated into peptide **1**, and the Alloc protecting group was removed using tetrakis(triphenylphosphine)palladium [$Pd(PPh_3)_4$] in the presence of phenylsilane. Importantly, the Phacm group was completely stable under these conditions. We then coupled Gly and Arg on the free amine and cleaved the peptide. Subsequently, the Alloc-Phacm tag was successfully removed, within 20 min, from the crude peptide in the presence of 10 equiv of $PdCl_2$ in 6 M guanidine hydrochloride (Gn·HCl), pH 7 (Figure 1). This demonstrated the reversibility of this tag, which opens new and promising applications in the synthesis of peptides with low solubility.

We then turned our attention to applying this strategy to facilitate the total chemical synthesis of histone protein H4. Recently, the synthesis of H4 has been accomplished by applying a hybrid phase ligation technique.[17] The total chemical synthesis of modified H4 in solution was also accomplished from three peptide fragments by applying one-pot ligation using *p*-boronobenzyloxycarbonyl (Dobz) as an N-terminal Cys protecting group.[18] However, the synthesis of the middle fragment required double coupling of all amino acids and suffered from low yield due to its low solubility.[18] Therefore, we decided to synthesize H4 from two fragments, i.e., Cys-H4(39−102) (fragment 1) and H4(1−37)-Nbz (Nbz is *N*-acylbenzimidazolinone) (fragment 2), and then perform NCL coupled with desulfurization (Scheme 2). To enable such an approach, the N-terminal Ala residue at position 38 in fragment 1 was temporarily mutated to Cys. Although the synthesis of

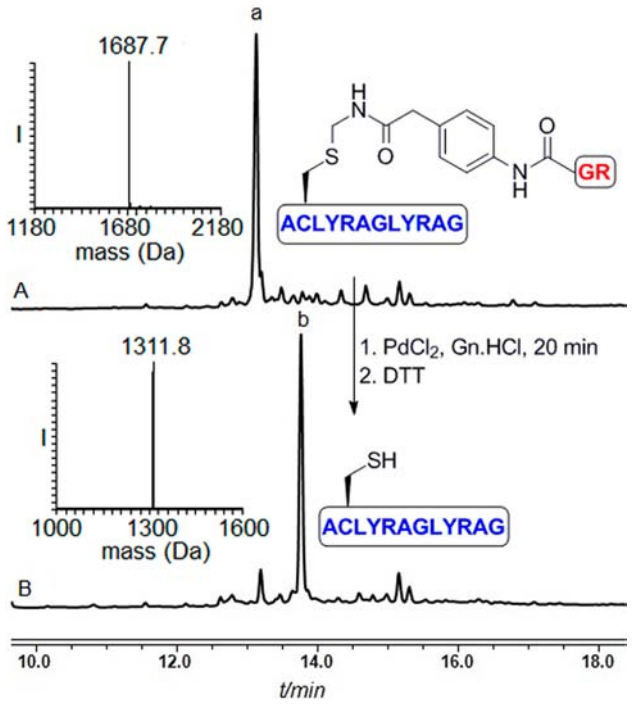
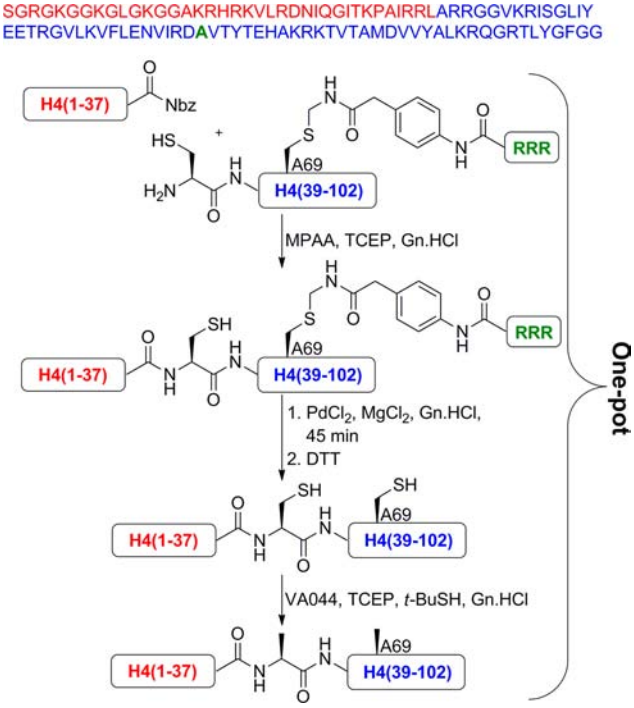


Figure 1. HPLC and mass analysis of (A) incorporation of Alloc-Phacm linker to model peptide **1** with the observed mass 1687.7 ± 0.1 Da (calcd 1687.8 Da); (B) removal of the linker from peptide **1** to give the unmodified peptide with the observed mass 1311.8 ± 0.1 Da (calcd 1312.5 Da). Peptide **1** = ACLYRAGLYRAG.

Scheme 2. Synthesis of Histone Protein H4 from Two Fragments through the One-Pot Operation of Ligation, Solubilizing Tag Removal, and Desulfurization[a]



[a]The modified Cys with the tag is shown in green.

fragment Cys-H4(39−102) was acceptable, the solubility of this peptide was very low, which interfered with the ligation step. Since the Cys-H4(39−102) fragment contains three Ala, we

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decided to switch Ala69 to Cys and incorporate the Alloc-Phacm linker at this position. This enabled coupling of three Arg residues on the amine functional group to increase the solubility of the fragment (Supporting Information (SI)). After the peptide chain elongation was completed, the Alloc group was removed and Arg residues were coupled straightforwardly on the Alloc-Phacm linker. Importantly, after removal of the Alloc-Phacm linker, the free Cys was converted to the native Ala through desulfurization, which demonstrated another advantage of this approach where the linker can be used at Ala and Cys sites or in principle with any thiolated amino acid designed originally for the ligation/desulfurization approach.[19]

Fragment 2 was synthesized on 3,4-diaminobenzoic acid (Dbz);[20] however, the cyclization to an activated *N*-acylurea moiety was unsuccessful under standard conditions in dichloromethane. Notably, the use of *N*,*N*-dimethylformamide resulted an efficient cyclization of Dbz and afforded the *N*-acylurea peptide with an additional mass of 28 Da due to formylation by the Vilsmeier–Haack reaction (SI).[21] Notably, upon thioesterification such additional mass will be eliminated to give the unmodified peptide.

Both fragments were obtained in good yield (~20–30%) and purity. Gratifyingly, fragment **1** was well soluble in a 50% acetonitrile–water mixture and behaved nicely during purification, in contrast to its unmodified version without the tag. On the other hand, the unmodified fragment **1** was difficult to solubilize even in Gn·HCl, resulting in low yield after purification (~10%) (SI). Having both fragments in hand, we proceeded with the ligation, which was performed in the presence of 20 equiv of 4-mercaptophenylacetic acid (MPAA) and 10 equiv of tris(2-carboxyethyl)phosphine hydrochloride (TCEP). Complete ligation of both starting materials was achieved within 4 h. Subsequently, the tag was removed quantitatively within 45 min upon addition of 50 equiv of $MgCl_2$ followed by 15 equiv of $PdCl_2$ (Figure 2). As described previously in our report, $MgCl_2$ was added to reduce the amount of Pd required for the tag removal.[15] Notably, when ligation was performed with unmodified fragment 1, full ligation was not achieved probably due to the low solubility of fragment **1** (SI).

Upon removal of the solubilizing tag linker, the reaction mixture was treated with DTT followed by centrifugation to separate the precipitate, and the supernatant solution was dialyzed overnight. Subsequently, radical-induced desulfurization was performed to give the full H4 in 20% overall isolated yield for the three steps, which were operated in a one-pot manner.[22] The purified H4 was analyzed by circular dichroism (CD) and showed the expected CD signature of folded H4 (SI). The protein was subjected to an inductively coupled plasma (ICP) experiment to determine the Pd content, and the amount of Pd detected was 0.07% of the protein.

In summary, we have developed a new palladium-assisted removable Cys/Ala side chain modification to incorporate solubilizing tags and facilitate the preparation of hydrophobic peptides and proteins. In principle, the strategy should be applied to other types of modifications, e.g., biotin, and in the preparation of other useful bioconjugates and might also find useful applications in cellular context and the general area of chemical biology.[23] These and others applications are currently being explored in our laboratory.

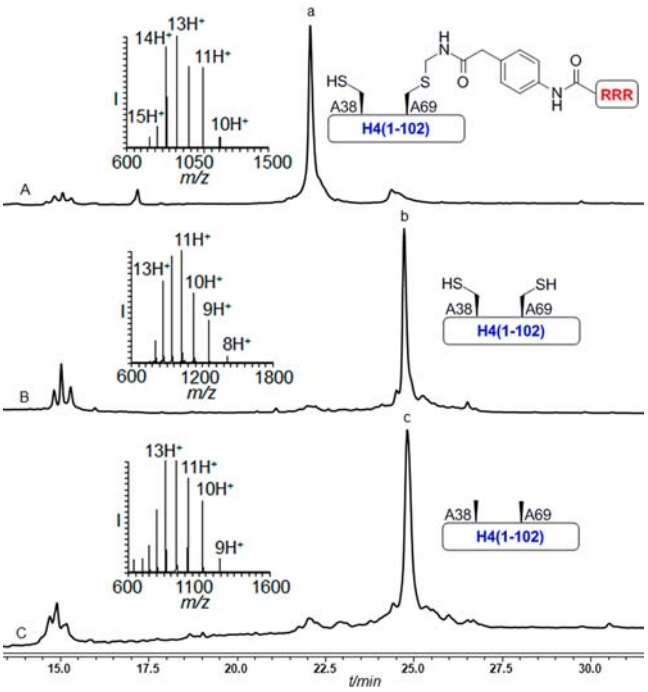

Figure 2. HPLC and mass analysis of (A) ligation of two fragments after 4 h to afford the full H4 containing solubilizing tag at Ala69 with the observed mass 11910.3 ± 0.8 Da (calcd 11911.8 Da); (B) solubilizing tag removal with the observed mass 11281.0 ± 0.2 Da (calcd 11281.2 Da); (C) desulfurization with the observed mass 11216.4 ± 0.8 Da (calcd 11217.1 Da).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b01442.

All experimental procedures, analytical data of synthetic small molecules, peptides and protein (PDF)

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Notes

The authors declare no competing financial interest.

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