Protein Synthesis

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Efficient Palladium-Assisted One-Pot Deprotection of (Acetamidomethyl)Cysteine Following Native Chemical Ligation and/or Desulfurization To Expedite Chemical Protein Synthesis

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Abstract: *The acetamidomethyl (Acm) moiety is a widely used cysteine protecting group for the chemical synthesis and semisynthesis of peptide and proteins. However, its removal is not straightforward and requires harsh reaction conditions and additional purification steps before and after the removal step, which extends the synthetic process and reduces the overall yield. To overcome these shortcomings, a method for rapid and efficient Acm removal using Pd^{II} complexes in aqueous medium is reported. We show, for the first time, the assembly of three peptide fragments in a one-pot fashion by native chemical ligation where the Acm moiety was used to protect the N-terminal Cys of the middle fragment. Importantly, an efficient synthesis of the ubiquitin-like protein UBL-5, which contains two native Cys residues, was accomplished through the one-pot operation of three key steps, namely ligation, desulfurization, and Acm deprotection, highlighting the great utility of the new approach in protein synthesis.*

Since the invention of modern organic synthesis for the preparation of natural product, peptides, and proteins, finding suitable protection and deprotection schemes for functional side chains has been a major goal. In peptide synthesis, for example, protecting the side chains of amino acids during solid phase peptide synthesis (SPPS) is essential for the success of the synthesis. Here the protecting groups have to be chosen carefully depending on which chemistry is used, that is, Boc- or Fmoc-SPPS, as well as the type of manipulations that are required after the cleavage and deprotection steps.[1] The latter aspect is particularly important for directing later bond formation processes and/or protecting specific side chains during solution chemistry.

One such group is the acetamidomethyl (Acm) moiety,[2] which is compatible with Fmoc- and Boc-SPPS and has been widely used for 1) regioselective disulfide bond formation in peptides and proteins,[3] 2) as a protecting group for N-terminal Cys in "middle fragments", which simultaneously bear an N-terminal Cys and a thioester and are used in protein synthesis by native chemical ligation (NCL),[4] and 3) to protect native Cys residue(s) when NCL is combined with desulfurization.[4d,5] Acm can also be used as an N-terminal Cys protecting group similar to the newly developed *para*-boronobenzyloxycarbonyl (Dobz) group[6] during the activation of a hydrazide into an acyl azide for thioester preparation where thiazolidine (Thz) is not suitable.[7] Despite its great utility and wide applicability, one of the drawbacks of the Acm protecting group is its removal, which often requires extended reaction times (6–15 h) and harsh conditions, such as the use of $Hg(OAc)_2$ in aqueous medium containing 50–90% acetic acid or the use of I_2 in 80–95% acetic acid.[3–5] Furthermore, when Acm is used as an orthogonal protecting group in peptide or protein synthesis or to protect native Cys from desulfurization, the polypeptide must first be isolated and then subjected to the removal conditions.[3–5] This also prevents one-pot ligation and/or desulfurization processes, necessitating another purification step after the removal. As a result, this often extends the synthetic process owing to the extra purification and lyophilization steps and reduces the overall yield.

Herein, we report that Pd complexes remove the Acm protecting group in peptides and proteins within minutes in aqueous medium and under NCL/desulfurization conditions. The utility of this approach is documented by the efficient synthesis of the ubiquitin-like protein UBL-5, which contains two native Cys residues. The synthesis was accomplished through the one-pot operation of three key steps, namely ligation, desulfurization, and Acm deprotection of the two protected native Cys residues, highlighting the great utility of the new approach in protein synthesis.

We have recently reported the use of Pd complexes for the removal of two different protecting groups of N-terminal Cys residues in middle fragments.[8] Specifically, we have shown the rapid removal of N-terminal thiazolidine (Thz) and propargyloxycarbonyl (Proc) protecting groups under NCL conditions, that is, 6 M guanidine hydrochloride (Gn·HCl) at pH 7, in the presence of 4-mercaptophenylacetic acid (MPAA) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) without prior isolation. We also demonstrated the utility of these conditions for the synthesis of synthetically challenging proteins.[8] In light of this, we wondered whether Pd complexes could also remove the Acm protecting group, considering that other transition metals (Ag and Hg) are generally used for this goal.[3a,e,4,5] The major difference of the use of Acm compared to Thz and Proc is the wider utility of the Acm group, which can be used not only for the protection of N-terminal Cys but also for Cys residues at any position in the peptide sequence.

Therefore, we initially examined Acm removal with the model peptide CLYRAGC(Acm)LYRAG (peptide 1) by

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exposing it to various removal conditions, including two different Pd^{II} complexes, PdCl$_2$ and [Pd(allyl)Cl]$_2$, in 6 M Gn·HCl, pH 7 at 37 °C. We tested this in the presence of 10 equiv of either one of these complexes, and we were pleased to observe quantitative removal of the Acm group within 5 min when using PdCl$_2$ followed by dithiothreitol (DTT) treatment to quench the reaction (Figure 1).

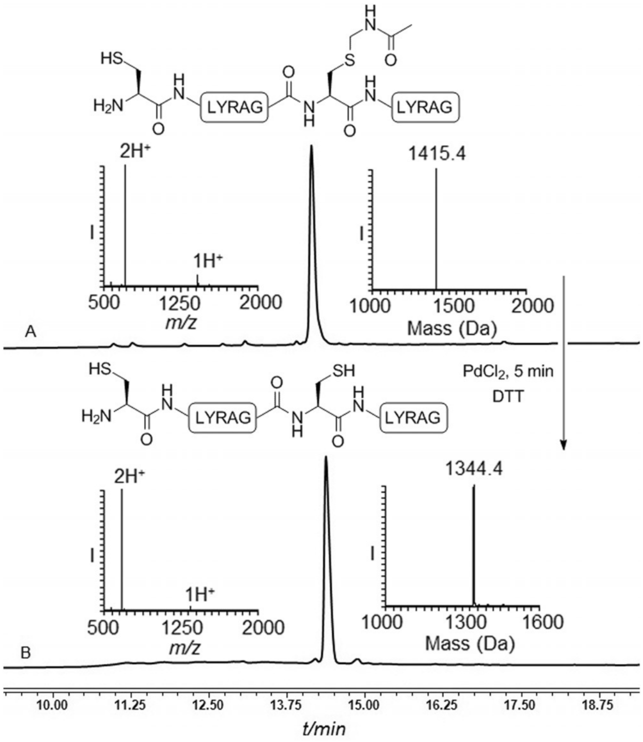

Figure 1. HPLC and mass-spectrometric analysis of A) purified Cys-LYRAGC(Acm)LYRAG (peptide 1; calcd: 1415.6 Da (average isotopes); found: 1415.4 ± 0.1 Da) and B) the same peptide after Acm removal (calcd: 1344.6 Da (average isotopes); found: 1344.4 ± 0.1 Da).

However, with [Pd(allyl)Cl]$_2$, we observed around 60 % conversion after 30 min (see the Supporting Information). This result implies that PdCl$_2$ is the more efficient reagent for the Acm removal in Gn·HCl. Notably, PdCl$_2$ dissolves in 6 M Gn·HCl more efficiently than in pure water, probably owing to coordination of the guanidinium group to the Pd^{II} center. It has been shown that guanidine derivatives are known to chelate Pd^{II} centers, and the formed complexes were found to be efficient catalysts for Suzuki cross-couplings with Pd^0.[9] To better understand the oxidation state of Pd required for efficient Acm removal, we performed the reaction of peptide 1 with Pd(dba)$_2$ or PdCl$_2$ in pure water. Both reagents were found to be similarly active, suggesting that both Pd species were suitable for effective Acm removal (see the Supporting Information). Based on the known strong chelation of Pd to a sulfur atom, we propose a mechanism that involves the coordination of Pd^0 or Pd^{II} species to the sulfur atom in Acm, thereby activating the C–S bond for nucleophilic attack of a water molecule, which leads to C–S bond cleavage and the release of a free thiol after DTT treatment (see the Supporting Information, Scheme S1).

We next studied the Acm removal under standard NCL conditions by ligating the model peptide 1 with a model thioester, LLYRAGLYRAG-MMP (MMP = methyl 3-mercaptopropionate; pepide 2) in the presence of 20 equiv of MPAA and 10 equiv of TCEP. After ligation, the Acm removal was accomplished quantitatively within 5 min in the presence of 50 equiv of PdCl$_2$ (Figure 2).

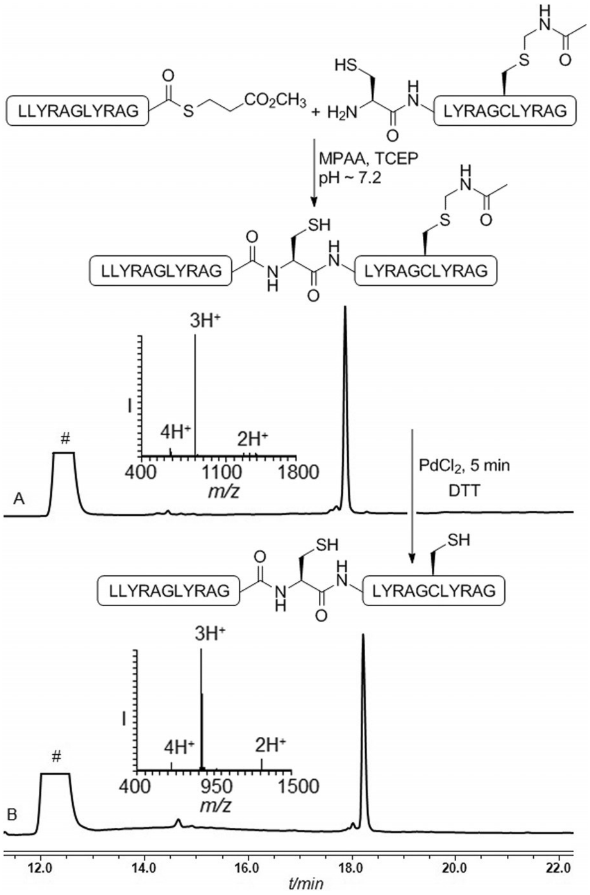

Figure 2. HPLC and mass-spectrometric analysis of A) the ligation product of peptides 1 and 2 (calcd: 2650.0 Da (average isotopes); found: 2650.2 ± 0.2 Da) and B) the same peptide after Acm removal (calcd: 2579.0 Da (average isotopes); found: 2578.8 ± 0.2 Da). Peptide 1: Cys-LYRAGC(Acm)LYRAG; peptide 2: LLYRAGLYRAG-MMP.

In our previous study with the Thz and Proc groups, we found that under the ligation conditions, the amount of Pd^{II} should be higher than the overall number of equivalents of MPAA and TCEP.[8] In this study, we wondered whether the amount of Pd^{II} could be reduced under NCL conditions while still achieving quantitative and rapid Acm removal. Reducing the amount of the Pd^{II} reagent has several advantages, including the possibility of performing consecutive ligations of three fragments in a one-pot fashion. We noticed that Davis and co-workers had reported that adding an excess of MgCl$_2$ to the reaction mixture facilitated cross-metathesis on proteins by preventing the non-productive chelation of the ruthenium catalyst to the side chains of the protein.[10]

Intrigued by these findings, we wondered whether the addition of an excess of Mg^{2+} to our Acm removal conditions could mask the ligation additives and thereby allow us to use a reduced amount of Pd^{II} relative to MPAA and TECP. In the search for more effective conditions, we synthesized a model peptide that contained Cys(Acm) in the N-terminal and an MMP thioester in the C-terminal position, Cys(Acm)-LYRAGLYRAG-MMP (peptide 3), and ligated this with the model peptide Cys-LYRAGLYRAG (peptide 4) in the presence of 20 equiv MPAA and 10 equiv TCEP. Subsequently, 50 equiv of $MgCl_2$ were added followed by the addition of various amounts of $PdCl_2$. To our delight, quantitative Acm removal was observed within one hour with only 15 equiv of $PdCl_2$ compared to 50 equiv without $MgCl_2$. With the optimized conditions in hand, we attempted to perform the next ligation on the Acm-free Cys and added 1.1 equiv of peptide 2. Under these conditions, the second ligation proceeded smoothly, and complete conversion of the starting materials was observed (Figure 3). Notably, a one-pot ligation of peptides with an Acm group at the N-terminal of the middle fragment had not been reported previously. In our previous work on Thz removal,[8] we could not achieve one-pot Thz deprotection and ligation owing to the presence of excess Pd in the reaction mixture. However, with the new conditions using $MgCl_2$, we achieved the efficient one-pot ligation of three peptide fragments (see the Supporting Information).

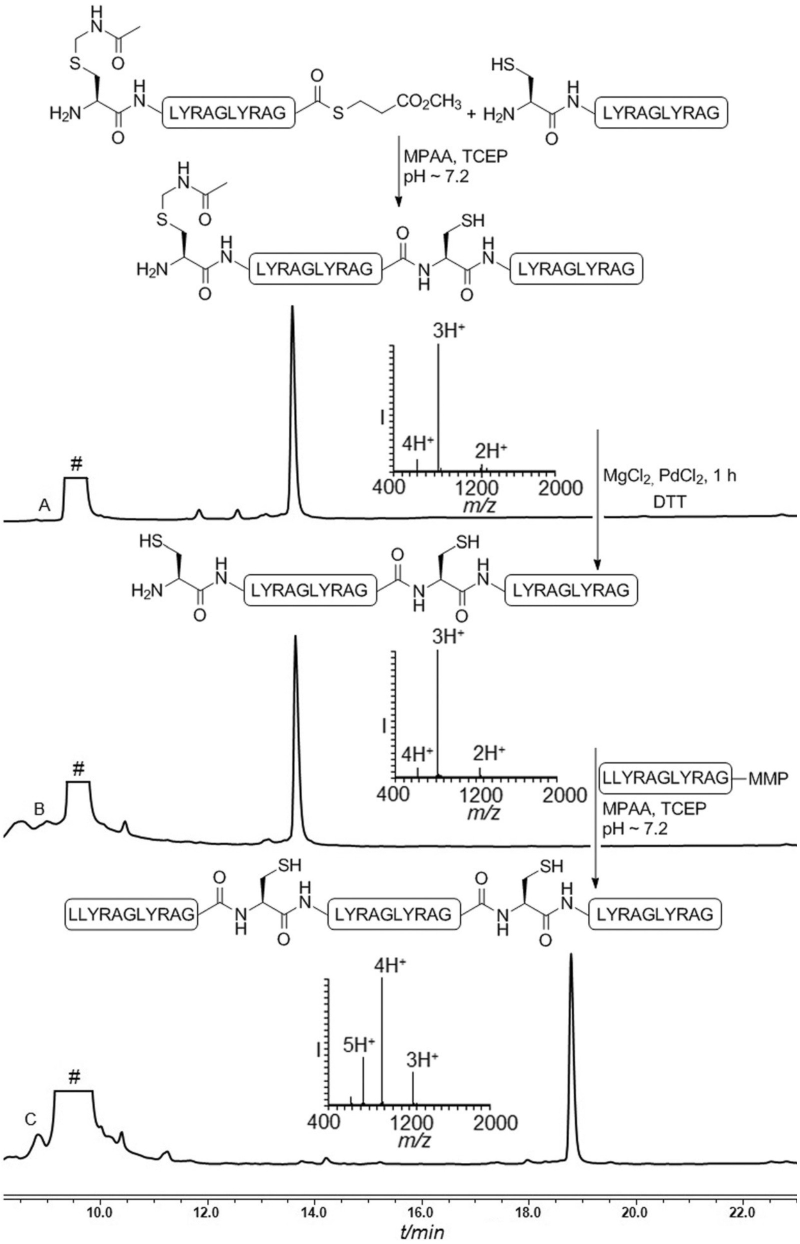

Figure 3. HPLC and mass-spectrometric analysis of A) the ligation product of peptides 3 and 4 (calcd: 2536.9 Da (average isotopes); found: 2536.9 ± 0.3 Da), B) the same ligation product after Acm removal (calcd: 2465.9 Da (average isotopes); found: 2465.6 ± 0.1 Da), and C) the one-pot ligation product of peptide 2 with the Acm-removed intermediate (calcd: 3700.4 Da; found: 3700.5 ± 0.5 Da). Peptide 2: LLYRAGLYRAG-MMP; peptide 3: Cys(Acm)-LYRAGLYRAG-MMP; peptide 4: Cys-LYRAGLYRAG.

Next, we focused on how our mild Acm removal in aqueous medium could be utilized to synthesize proteins containing native Cys residues by NCL and desulfurization. The Acm group was previously used to protect native Cys residue(s) when combining NCL with desulfurization.[5] However, the additional purification step that is required due to the previously used removal conditions prompted researchers to adopt different approaches to avoid this step.[11] For example, selenocysteine was utilized for ligation instead of Cys, which can be deselenized by using only TCEP without desulfurizing native unprotected Cys residues.[12] We reasoned that given the straightforward introduction of commercially available Cys(Acm) into peptide sequences and our new removal conditions, the limitations of using the Acm protecting group for such a goal could be overcome if the extra purification step could be avoided.

To test the feasibility of our strategy we chose to synthesize the human ubiquitin-like protein 5 (UBL-5), which contains two native cysteine residues in the N-terminal region (Cys6 and Cys18). The protein was divided into two fragments, UBL-5(1–32)-thioester (fragment 1) and Cys-UBL-5(34–76) (fragment 2). The two fragments were prepared in yields of approximately 50% using Fmoc-SPPS (see the Supporting Information). Fragment 1 was synthesized using the recently reported *ortho*-amino(methyl)aniline (MeDbz) linker.[13] After conversion of the activated *N*-acylurea moiety, the peptide was transformed into the MPAA thioester.

Our group has reported that activated MPAA thioesters can be ligated with Cys fragments without the need for additional MPAA as a ligation additive to enable ligation and desulfurization in a one-pot fashion.[14] Hence, we ligated the

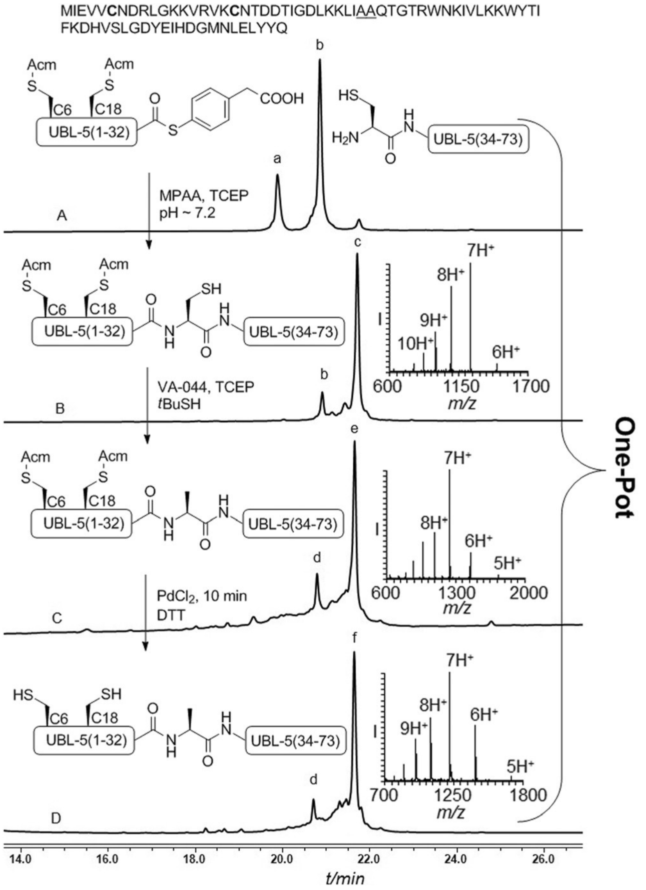

Figure 4. HPLC and mass-spectrometric analysis of the ligation of UBL-5 fragments 1 and 2 at A) $t=0$ (peak a: fragment 2; peak b: fragment 1) and B) after two hours. Peak b: residual fragment 1; peak c: the ligation product (calcd: 8701.9 Da (average isotopes); found: 8699.8 ± 0.4 Da). C) HPLC and mass-spectrometric analysis of the one-pot desulfurization. Peak d: desulfurized fragment 1; peak e: the desulfurized ligation product (calcd: 8669.9 Da (average isotopes); found: 8668.1 ± 0.3 Da). D) HPLC and mass-spectrometric analysis of the one-pot Acm removal. Peak f: Full-length UBL-5 after Acm removal (calcd: 8527.9 Da (average isotopes); found: 8526.2 ± 1.2 Da).

two UBL-5 fragments without the use of external MPAA and successfully desulfurized it in a one-pot fashion (Figure 4). After dialysis and without product isolation, quantitative Acm removal was achieved by adding 30 equiv of $PdCl_2$ for 15 min to afford the protein with two native cysteine residues, which was isolated in 36% overall yield over the three steps (Figure 5). The purified UBL-5 was analyzed by circular dichroism (CD) spectroscopy, and showed the expected CD signature of folded UBL-5 (Figure 5).

In summary, we have developed a novel and efficient method for Acm removal in aqueous medium and under NCL conditions by using Pd complexes. Moreover, for the first time, the one-pot ligation of three peptide fragments with Acm as the N-terminal Cys protecting group of the middle fragment was achieved. Using our conditions, the efficient synthesis of UBL-5, which bears two native Cys residues, was also achieved by conducting ligation, desulfurization, and Acm removal in a one-pot fashion. These advances in protein synthesis expand the toolbox for the synthesis and semisynthesis of proteins and should further facilitate the preparation of challenging targets for biochemical and biophysical studies.[15]

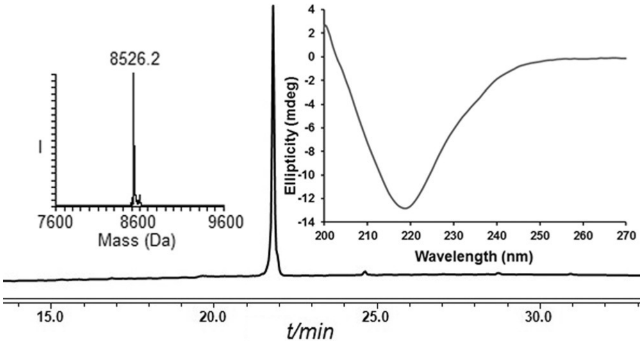

Figure 5. HPLC, mass-spectrometric, and CD analysis of purified full-length UBL-5.

The recently described impressive use of Pd complexes to decage protected Lys residues in proteins in cellular contexts[16] encourages us to suggest that this concept can also be extended to the Cys residue by using Cys(Acm). We envision the use of the well-developed methods for the incorporation of unnatural amino acids into proteins for the cellular expression of proteins containing Cys(Acm). This would then enable the caging of a catalytic Cys of a particular enzyme (e.g., protease), for example, which can then be decaged in temporally controlled fashion with the Pd complex to probe the function of these enzymes. We are currently expanding the usage of these methods and taking advantage of these new developments in chemical biology for investigating protein functions.

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