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Palladium Mediated Rapid Deprotection of N-Terminal Cysteine under Native Chemical Ligation Conditions for the Efficient Preparation of Synthetically Challenging Proteins

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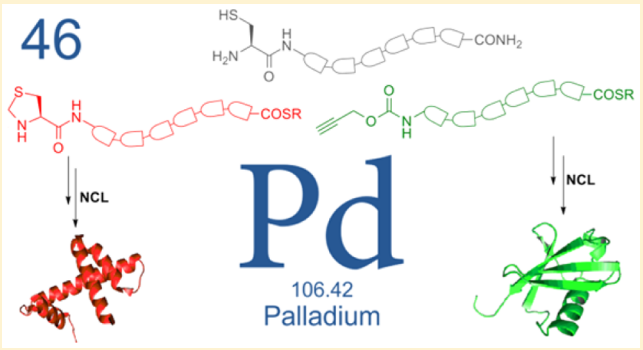
Supporting Information

ABSTRACT: Facilitating the process of chemical protein synthesis is an important goal in order to enable the efficient preparation of large and novel protein analogues. Native chemical ligation, which is widely used in the synthesis and semisynthesis of proteins, has been going through several developments to expedite the synthetic process and to obtain the target protein in high yield. A key aspect of this approach is the utilization of protecting groups for the N-terminal Cys in the middle fragments, which bear simultaneously the two reactive groups, i.e., N-terminal Cys and C-terminal thioester. Despite important progress in this area, as has been demonstrated in the use of thiazolidine protecting group in the synthesis of over 100 proteins, finding optimal protecting group(s) remains a challenge. For example, the thiazolidine removal step is very slow (>8 h), and in some cases the applied conditions lead to undesired side reactions. Here we show that water-soluble palladium(II) complexes are excellent reagents for the effective unmasking of thiazolidine, enabling its complete removal within 15 min under native chemical ligation conditions. Moreover, palladium is also able to rapidly remove propargyloxycarbonyl-protecting group from the N-terminal Cys in a similar efficiency. The utility of the new removal conditions for both protecting groups is exemplified in the rapid and efficient synthesis of Lys34-ubiquitinated H2B and for the first time neddlyated peptides derived from cullin1. The current approach expands the use of palladium in protein chemistry and should significantly facilitate the chemical and semisynthesis of synthetically challenging proteins from multiple fragments.



INTRODUCTION

The chemical protein synthesis field is witnessing important progresses both at the application level and method developments. The latter is particularly important in order to streamline the synthetic process and enables facile access of large and novel protein analogues. The most effective peptide ligation methods are those that based on installing unique reactive functionalities at the C- and N-termini of unprotected peptides.[1] Despite important advancements being made in these directions,[2] native chemical ligation (NCL) remains the method of choice when attempting the ligation of unprotected peptides in aqueous media. NCL utilizes N-terminal thiol modified amino acid (e.g., Cys) from the first peptide and C-terminal thioester functionality from a second peptide, which chemoselectively react to form an amide bond at the ligation junction.[3]

The synthesis of medium to large proteins (>120 amino acids) often requires three or more peptide fragments wherein the middle fragment(s) must bear both the N-terminal thiol modified amino acid and the thioester functionality.[4] As a result, the N-terminal thiol modified amino acid must be protected to avoid peptide polymerization and/or cyclization. Several protecting groups have been used for this goal including methylsulfonylethyloxycarbonyl amine (Msc),[5] acetamidomethyl (Acm),[6] and the thiazolidine protecting group (Thz).[7] Despite this, protein chemists continue to search for an optimal protecting group for the middle fragments and new ones have been recently developed such as *p*-boronobenzyloxycarbonyl group (Dobz),[8] *N*-acetoacetyl group (AcA),[9] 4-(dimethylamino) phenacyloxycarbonyl group (Mapoc)[10] and azide.[11] Despite these efforts, the protecting groups suffer either from the use of harsh removal conditions (e.g., $Hg(OAc)_2$ for Acm, pH 13−14 for Msc),[5,6] requirement of peptide isolation prior to the removal step (e.g., Acm) and/or special preparations (e.g., Mapoc).[10]

Since its first demonstration in chemical protein synthesis by Kent and co-workers,[7a] Thz is considered the protecting group of choice for many laboratories when attempting protein synthesis from three or more fragments. Remarkably, Thz has been used in the synthesis of over hundred different proteins, such as HIV-1 protease and its analogues,[4c,12] native and modified alpha-synuclein,[13] human parathyroid hormone-related protein (hPTHrP),[14] different forms of erythropoietin

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(EPO),[4b,15] several ubiquitinated proteins[13b,c,16] and modified histones.[17] The wide utility of this protecting group is mainly due to its removal conditions, which is achieved by adjusting the pH of the ligation mixture to ∼4 in the presence of a large excess of methoxylamine. This is in addition to the commercial availability of the thiazolidine-protected form of Cys and its straightforward installation in thiol modified amino acids.[17c,18] However, the deprotection step is slow and requires long period of reaction (>8 h), which might lead to accumulation of side reactions and slow down the synthetic process of the final target. In the case of peptide thioester bearing C-terminal Gly residue, methoxylamine has been reported to react with the thioester peptide when one-pot ligation is employed.[17b,19] In addition, it has been reported during the oxidative switching of hydrazide peptide, that the Thz was found to be unstable.[20] Hence, a more facile deprotection of the Thz under NCL conditions is desired. In addition, a new protecting group, which can be orthogonal or complementary to the Thz and removed efficiently under NCL conditions in one-pot manner, could also find important applications in protein synthesis.

Here we report that water-soluble palladium(II) complexes are excellent reagents for the effective unmasking of Thz, enabling its complete removal within 15 min under NCL conditions. Moreover, these complexes are able to rapidly remove propargyloxycarbonyl group (Proc) from the N-terminus Cys of the middle fragment peptides. The utility of these novel conditions for Thz and Proc removal from the N-terminal Cys of the middle fragment is exemplified in the rapid and efficient synthesis of Lys34-ubiquitinated H2B and for the first time neddlyated peptides derived from cullin1.

RESULTS AND DISCUSSION

The use of palladium in protein chemistry has been documented in various elegant studies such as chemical protein modification based on aryl/alkenyl halides[21] and other coupling partners, e.g., alkenes (Mizoroki−Heck),[22] boronic acids (Suzuki−Miyaura),[23] and alkynes (Sonogashira).[24] Recently, it has been shown that 2-dicyclohexylphosphino-29,69-diisopropoxybiphenyl aryl palladium(II) ((RuPhos)Pd(Ar)X), (X = counterion) complexes can be used for selective and rapid Cys modification in proteins.[25] In addition, we were intrigued by the recent use of Pd(0) and Pd(II) complexes to convert chemically caged Lys analogue to free ε-amine from the Proc-protected moiety, which was used for masking the cellular activity of a Lys residue of an intact protein.[26] We wondered if such a protecting group can be also used to mask the N-terminal Cys in the middle fragments during protein synthesis.

To begin examine the utility of the Proc-protecting group, we initially prepared eleven-mer peptide, Proc-Cys-LYRAGLYRAG (peptide-1 = LYRAGLYRAG), wherein the Proc-Cys was prepared in solution and coupled to the N-terminus of the peptide by using standard Fmoc-SPPS (Supporting Information). Alternatively, the Proc protecting group could be also incorporated on solid support by reacting the free N-terminus with propargyl chlorofromate. Peptides with excellent crude purities were obtained demonstrating that the Proc protecting group is stable to Fmoc-SPPS. Next, we examined the Proc removal from the N-terminal Cys by exposing the peptide to 20 equiv allylpalladium chloride dimer $[Pd(allyl)Cl]_2$ reagent (in 6 M Gnd.HCl, pH ∼ 7.2, 37 °C) followed by the addition of 1,4-dithiothreitol (DTT) to quench the Pd species as well as to release any bound Pd to the peptide. This led to a complete conversion of the Proc-Cys-peptide-1 to Cys-peptide-1, within

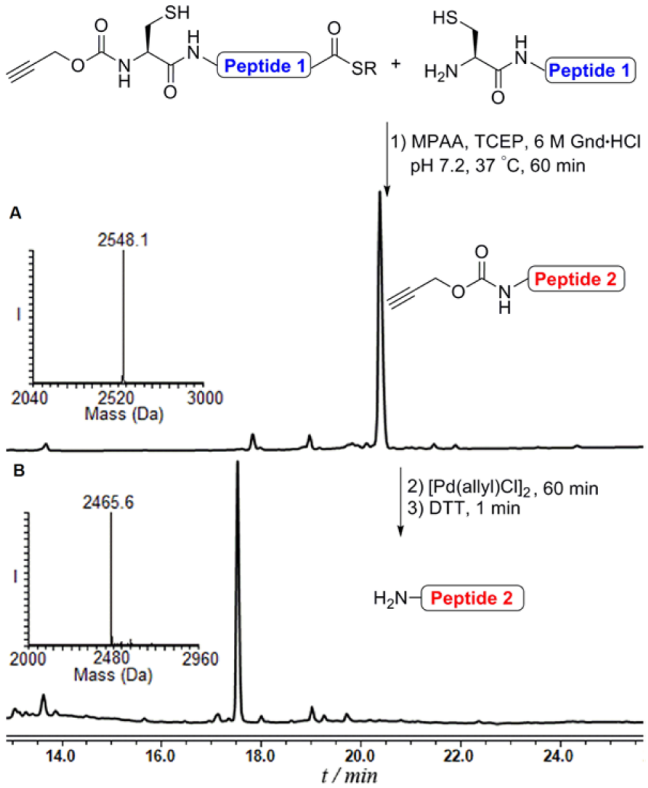

Figure 1. (A) Analytical HPLC and mass analysis of ligated proc-peptide-2 with the observed mass 2548.1 Da ± 0.6 Da, calcd 2548.9 Da, (average isotopes). (B) Analytical HPLC and mass analysis of deptotected peptide-2 with the observed mass 2465.6 Da ± 0.1 Da, calcd 2465.9 Da, (average isotopes). Peptide-1: LYRAGLYRAG, Peptide-2: CLYRAGLYRAGCLYRAGLYRAG. R = Methyl 3-mercaptopropionate.

1 h. Under these conditions no racemization of the N-terminal Cys was observed (Supporting Information).

We then examined if the Proc protecting group can be removed under NCL conditions in particular in the presence of the ligation additives (i.e., phosphine and thiols) as well as the high concentration of guanidine, which is known to chelate Pd(II) in aqueous media and affect its reactivity.[27] For this, two model peptides were prepared, where the first having N-terminal Cys, Cys-peptide-1 and the other have both N-terminal Proc-Cys and C-terminal thioester functionality Proc-Cys-peptide-1-SR, (R = Methyl 3-mercaptopropionate). The two fragments were ligated under NCL conditions in the presence of 20 equiv of 4-mercaptophenylacetic acid (MPAA) and 10 equiv of Tris(2-carboxyethyl)phosphine (TCEP).[28] Upon completion of the ligation reaction and without isolation or changing the reaction conditions, 20 equiv of $[Pd(allyl)Cl]_2$ was added to the reaction mixture. However, under these conditions the reaction was extremely slow, presumably due to the presence of MPAA and TCEP, which could interfere with the metal reactivity. Notably, with the addition of extra 20 equiv of $[Pd(allyl)Cl]_2$, a quantitative deprotection of the ligation product was observed within 60 min, which was confirmed by HPLC-MS analysis (Figure 1).

Encouraged by these results, we then aimed to test the applicability of this protected group in protein synthesis and examined it in the total chemical synthesis of ubiquitinated H2B at Lys 34 (H2BK34Ub). This mark has been proposed to stimulate H3K79 and K4 methylation in vivo and in vitro

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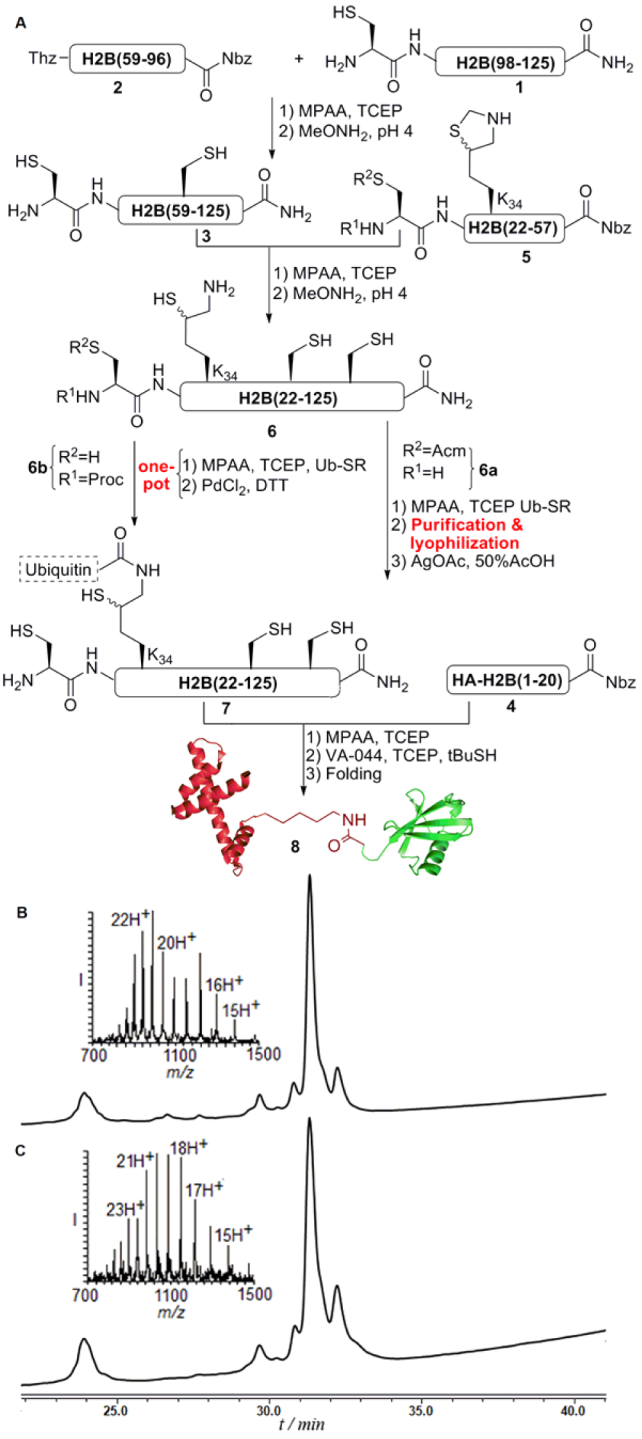


Figure 2. (A) Synthesis of HA-H2BK34Ub: (B) Analytical HPLC and mass analysis of ligated product Proc-H2B(22−125)K34Ub with the observed mass 20709.5 Da ± 3.2 Da, calcd 20714.5 Da, (average isotopes). (C) Analytical HPLC and mass analysis of deptotected H2B(22−125)K34Ub with the observed mass 20631.0 ± 3.0 Da, calcd 20631.5 Da, (average isotopes). Nbz = *N*-acyl-benzimidazolinone.

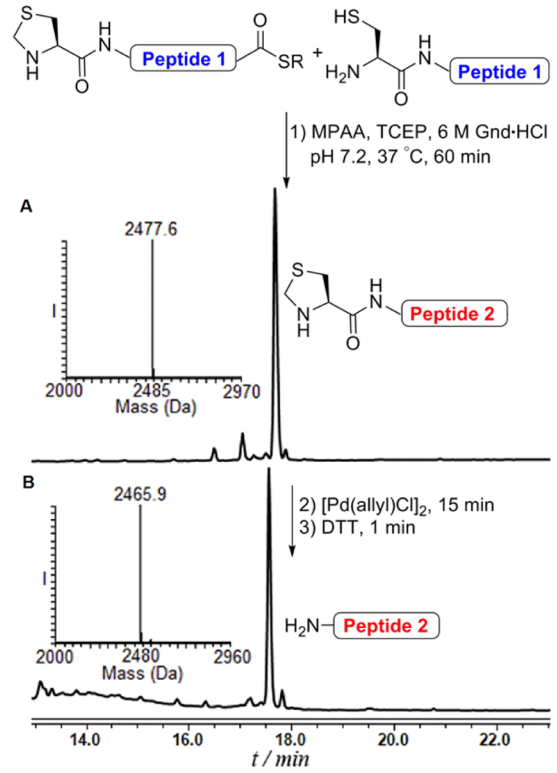
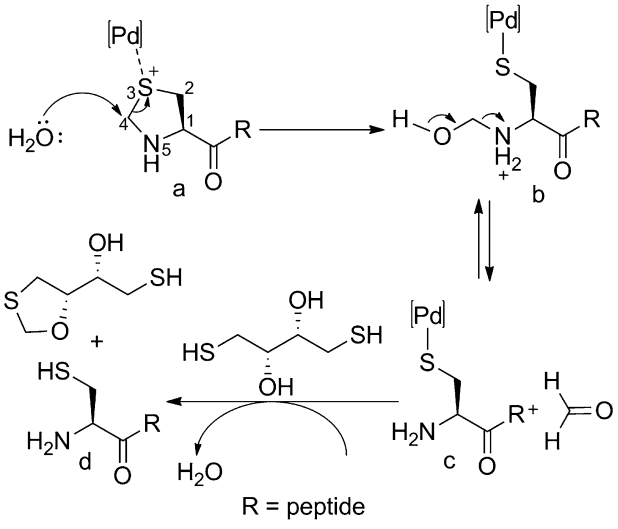


Figure 3. (A) Analytical HPLC and mass analysis of ligated Thz-peptide-2 with the observed mass 2477.6 Da ± 0.6 Da, calcd 2477.9 Da, (average isotopes). (B) Analytical HPLC and mass analysis of deptotected peptide 2 with the observed mass 2465.9 Da ± 0.3 Da, calcd 2465.9 Da, (average isotopes). Peptide-1: LYRAGLYRAG, Peptide-2: CLYRAGLYRAGCLYRAGLYRAG. R = Methyl 3-mercaptopropionate.

Scheme 1. Proposed Mechanism for Pd-Assisted Thz Conversion to a Free Cys



through trans-tail regulation; however, the effect of H2BK34Ub on the regulation of chromatin structure and the interacting proteins still unclear.[29] The synthesis of this challenging target was recently reported by our group and was used to learn about the interacting proteins with ubiquitinated nucleosome.[17a,30] However, our approach suffered from multiple purification steps, which prolonged the time of synthesis and negatively affected the total yield.[17a,b] More efficient synthesis of this ubiquitinated histone will assist in dissecting the role of ubiquitination at this site in chromatin context.

The previous synthesis of H2BK34Ub depends on using Acm for the N-terminal Cys as an orthogonal protecting group of δ-mercaptolysine in fragment **5** (Figure 2).[17a,b]

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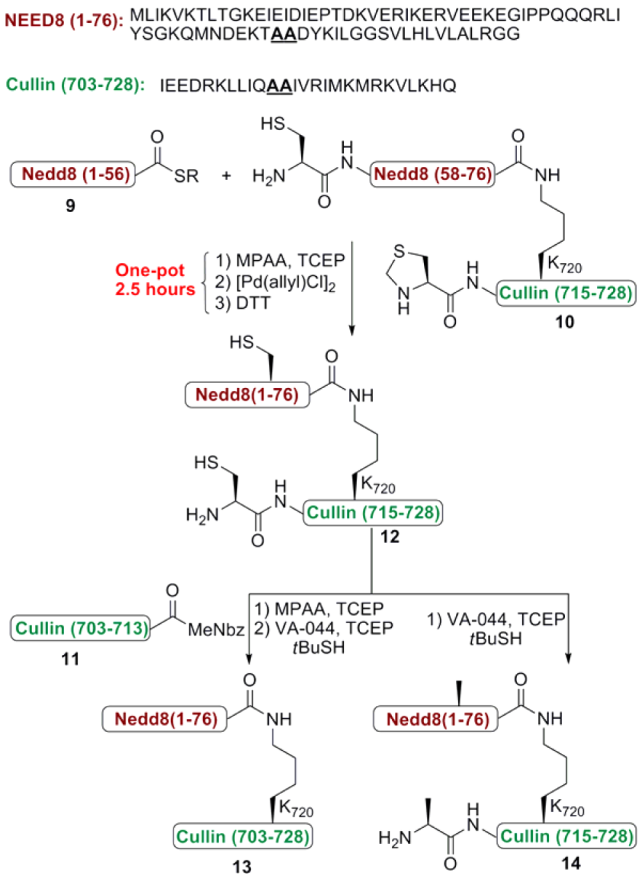
Scheme 2[a]



[a]Top: Sequence of NEDD8 (1-76) with highlighted ligation site. Sequence of Cullin (703-728) with highlighted ligation site. Bottom: Synthesis of neddlyated cullin (703-728) and neddylated cullin (714-728). R = Methyl 3-mercaptopropionate. MeNbz = N-acyl-*N*-methylacylurea.

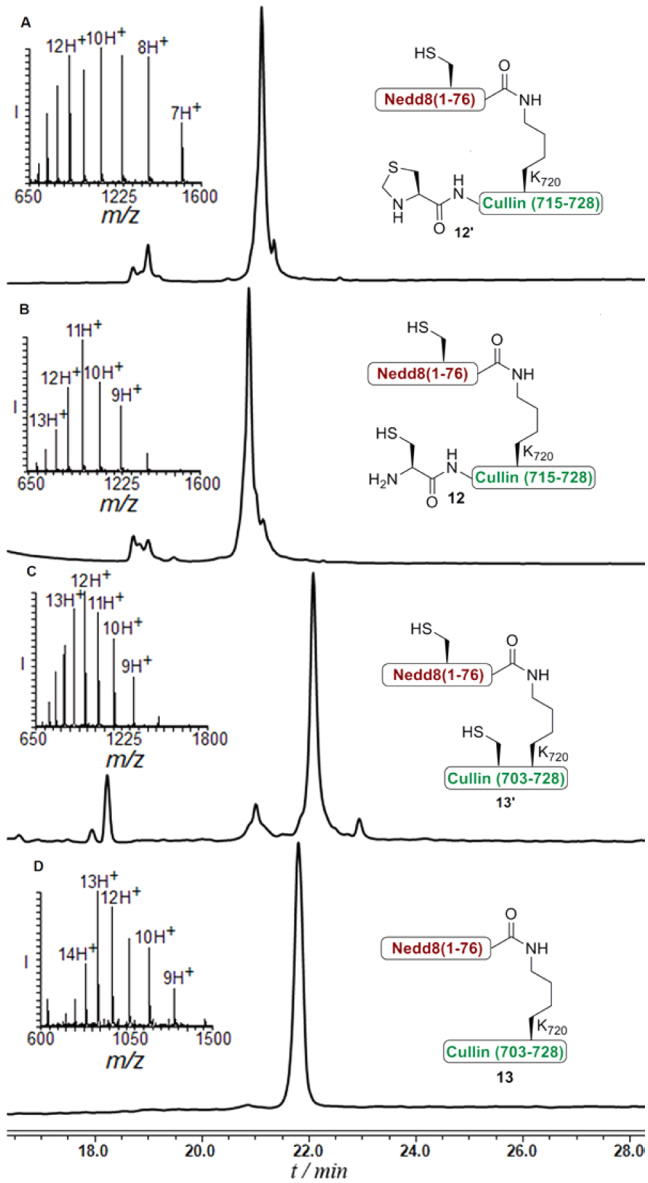


Figure 4. (A) Analytical HPLC and mass analysis of ligated product of **9** and **10** with the observed mass 10449.5 Da ± 0.6 Da, calcd 10450.4 Da, (average isotopes). (B) Analytical HPLC and mass analysis of deptotected peptide **12** with the observed mass 10437.8 ± 1.1 Da, calcd 10438.4 Da, (average isotopes). (C) Analytical HPLC and mass analysis of final ligated product with the observed mass 11747.7 Da ± 0.4 Da, calcd 11748.0 Da, (average isotopes). (D) Analytical HPLC and mass analysis of final desulfurized product **13** with the observed mass 11682.9 Da ± 0.4 Da, calcd 11683.7 Da, (average isotopes).

After ubiquitination of Lys34, the ligation product **6** had to be isolated to enable Acm removal using $Ag(OAc)_2$ in 50% acetic acid (Figure 2). New synthesis based on Proc-Cys as an orthogonal protecting group should in principle overcome this additional purification step where the removal of the Proc can be achieved directly after ligation. The key synthetic intermediate **6** was achieved from one-pot ligation of three fragments employed also methoxylamine treatment. Notably the Proc protecting group was stable during the removal of Thz protecting group of the thiolysine using methoxylamine. Purified **6b** was ligated with Ub-thioester in the presence of MPAA and TCEP for 3 h followed by the addition of $[Pd(allyl)Cl]_2$ for 1 h. The desired product **7** was observed, however, along with unidentified product (~50%) having the additional mass of +32 Da, which did not react further in the ligation reaction presumably due to capping of the free Cys via unclear mechanism associated with the Pd complex. To try to overcome this, we retuned back to our model peptides and examined various Pd(II) complexes such as $PdCl_2$ and $Pd(OAc)_2$. In this case, $PdCl_2$ gave much better results with a quantitative conversion of the ligated product Proc-peptide-2 (peptide-2 = CLYRAGLYRAGCLYRAGLYRAG) to the free form (NH_2-peptide-2) within 5 min (Supporting Information), prompting us to test these conditions in the H2BK34Ub synthesis. We were very pleased to find that with $PdCl_2$ the removal of the Proc was very rapid (5−10 min) and no +32 Da adduct was observed to give (H2B(22−125)K34Ub) **7** in 40%. Ligation with fragment **4** followed by desulfurization, as we have previously reported, gave the desired product **8**, H2BK34Ub, in ~5% overall yield, which is ~25% increase in the yield compared to the previous synthesis, (Figure 2).

The previous observations in which the Thz group was not stable during the oxidative switching of Thz-peptide-$NHNH_2$ to Thz-peptide-thioester via the acyl azide intermediate[20] prompted us to examine the stability of Proc-Cys under these conditions. For this, we prepared five-model peptides based on Proc-Cys-peptide-1-$NHNH_2$ with different C-terminal amino acids. The Proc group in all these peptides exhibited high

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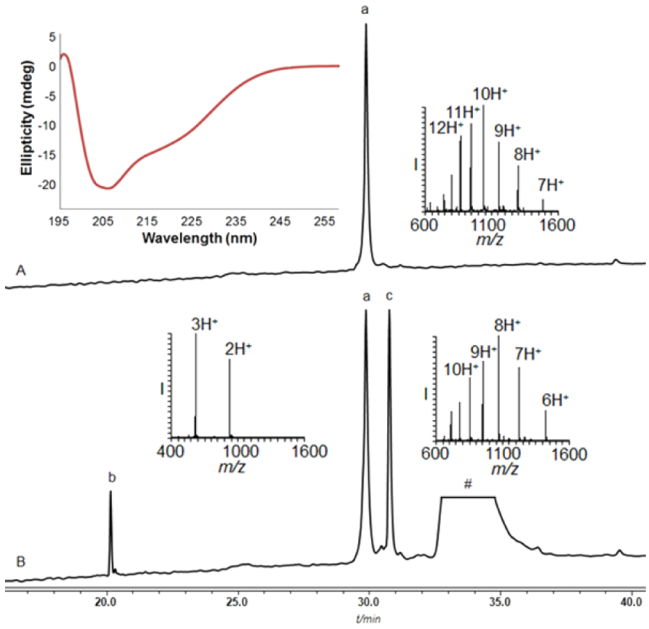

Figure 5. Analytical HPLC and mass data of (A) purified protein **14** with the observed mass 10373.8 ± 0.2, calcd 10374.3 (average isotopes) and CD spectra of **14**; (B) after UCHL3 treatment where peak b corresponds to the cleaved peptide fragment from cullin sequence with the observed mass 1849.6 ± 0.1, calcd 1850.4 (average isotopes), peak c corresponds to cleaved NEDD8 with the observed mass 8541.8 ± 0.2, calcd 8540.9 (average isotopes). # is bovine serum albumin (BSA).

stability under the switching conditions of the C-terminal hydrazide to thioester. Further, we validated that such a thioester reacts quantitatively in NCL. These results make the Proc group a better choice than the Thz for some synthetic applications involving NCL (Supporting Information).

During our work with Pd complexes in one of the model systems we observed also removal of the Thz protecting group, which promoted us to study further this reaction using various Pd complexes, realizing the potential use of this approach in protein synthesis.[31] For this, two eleven-mer peptides were prepared; the Cys-peptide-1 and the other having both N-terminal Thz and C-terminal thioester, Thz-peptide-1-SR. The two peptides were ligated under NCL conditions in the presence of MPAA and TCEP. Upon completion of the ligation reaction and without isolation, $[Pd(allyl)Cl]_2$ was added to the ligation mixture followed by DTT to give within 15 min, quantitatively the ligated product with the free Cys, (Figure 3). When using the $PdCl_2$ complex for the Thz removal the reaction was slower and took around 60 min for completion. Under these conditions no racemization of the N-terminal Cys was observed (Supporting Information). Notably, when the Thz protected peptide-1 was exposed to the same Pd complex, however without MPAA and TCEP, the removal was extremely slow and showed only 40% removal after 4 h. When adding MPAA or TCEP, separately, 50−70% of the deprotected peptide was obtained within 30 min, compared to a complete removal within 15 min when both TCEP and MPPA are present. This indicates that both ligation additives, which are essential part of the NCL conditions, are important for more efficient removal probably by chelating/reducing Pd(II) to Pd(0).[32]

To further shed light on this, we carried out the removal without TCEP and MPAA but in the presence of sodium ascorbate, which is known to reduce Pd(II) to Pd(0).[26] We observed significant improvement in the Thz removal where ∼45% of the deprotected peptide was obtained within 30 min, supporting that Pd(0) might be the more reactive species in the removal step. Efforts to directly work with Pd(0) complexes, e.g., $Pd(dba)_2$ (dba = dibenzylideneacetone), were not successful due to low solubility of this complex in the aqueous media and under different conditions. Interestingly, in the case of the Proc removal via $PdCl_2$ we did not observe difference in the removal, with or without the ligation additive, i.e., MPAA and TCEP. This probably due to the different mechanisms involved in the removal of the two different protecting groups. One has to note that in the described conditions for the removal of the two protecting groups with the different Pd complexes, it is unclear yet what is the composition of the reactive Pd complex(s) due to various possibilities of chelation of the ligation additives to the Pd species(s). The depropargylation mechanism was proposed to proceed via Pd(0) or/and Pd(II) complexes involving Pd(0)-allenylpalladium intermediate or Pd(II) hydration mechanism.[26] On the other hand, we propose a mechanism for Thz removal where the Pd complex activates the sulfur atom of the Thz (a), (Scheme 1), which initiates the ring opening by the nucleophilic attack of a water molecule to form carbinolamine intermediate (b). This intermediate spontaneously collapses to release free Cys and formaldehyde (c). The latter can be trapped by DTT,[7b] which is added in large excess to quench the reaction (Scheme 1).

Recent efforts from different laboratories[33] expanded NCL to selenocysteine (Sec)[34] in particular the use of Thz protected Sec to form selenazolidine (Sez).[35] To examine if our new approach could convert Sez to a free Sec, we prepared peptide-1, having N-terminal Sez and C-terminal thioester functionality, Sez-peptide-1-SR. This peptide was ligated under NCL conditions in the presence of MPAA with, Cys-peptide-1. Upon completion of the ligation reaction, $[Pd(allyl)Cl]_2$ or $PdCl_2$ was added to the ligation mixture followed by DTT. Interestingly, despite rapid disappearance of the starting material we did not observe the desired product or the dimer oxidized product, which is often observed in Sec based ligation. Rather unidentified products were obtained despite variations in the removal conditions. This might be due to differences in the reactivity of Sec compared to Cys, where it could after removal behave differently with Pd under these conditions.

To further explore the utility of the new removal conditions of Thz in the synthesis of challenging proteins, we undertook the synthesis of neddlyated peptides derived from cullin1.[36] Neddlyation by the small Ub like protein NEDD8 (neural precursor cell expressed developmentally downregulated protein 8) has been mainly characterized as activating the largest E3-ligases family, known as the cullin-Ring ligases. In this process, the target of neddlyation is the cullin1 from the cullins family having the conserved cullin1 domain. Despite its large sequence homology to Ub, NEDD8 expression is known to be very low and chemical biology tools based on NEDD8 still lacking. This is in contrary to Ub where various conjugates and analogues are available and have become extremely useful in studying ubiquitination and deubiquitination.[37] Hence generating a toolbox based on NEDD8 could be highly valuable for the research community to study neddylation and denddylation processes. To begin this journey, we tested whether neddlyated peptides can be synthesized where Pd can facilitate the rapid assembly of these conjugates. To do so, we

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decided to neddlyate 26-mer derived from cullin1 using a similar approach for ubiquitinated peptides based on orthogonal protection[38] of the neddlyated Lys (Lys720 of cullin) from the peptide target, (Scheme 2).

For this synthesis we noted the presence of Ala57 in NEDD8, which can be used as a ligation junction after the synthesis of the cullin1 peptide having the isopeptide bond linked to the C-terminal 19 amino acids of NEDD8. On the other hand, the complementary part of NEDD8 can be prepared as a thioester. We also decided to switch Ala714 from the cullin peptide to Thz, which after full assembly of the neddlyated peptide can be opened with $[Pd(allyl)Cl]_2$ to enable further elongation of the cullin1 peptide or modification by other groups such as a fluorescence tag for uses in monitoring the reversible reaction. Hence, peptide **10** was prepared directly on solid support to give, after purification, the purified peptide in 50% yield, (Supporting Information). The complementary part of NEDD8 peptide **9**, and peptide **11** were prepared using the *o*-amino(methyl)aniline (MeDbz) linker[39] recently developed by the Dawson group to give these peptides in 40−50% yield, (Supporting Information). Having these peptides in hand, NCL was applied between **9** and **10** in the presence of MPAA and TCEP for 2 h, followed by the addition of $[Pd(allyl)Cl]_2$ for 15 min. The reaction was quenched by DTT to give, after purification, peptide **12** in 45% isolated yield. Peptide **12** was ligated with fragment **11**, followed by radical desulfurization[40] to give, after purification, the neddlyated cullin (703−728) peptide **13** in 30% yield, (Figure 4).

Circular dichroism spectrum (CD) of neddlyated cullin (714−728), **14** exhibited the expected CD signature of the NEDD8 (Figure 5A). We also studied the hydrolysis of neddylated cullin (714−728) using ubiquitin C-terminal hydrolase isozyme 3 (UCH-L3). This enzyme is known to process both Ub and NEDD8 from their precursors, where the initial gene products made from Ub or NEDD8 has a carboxyl-terminal extension and the removal of these extensions is necessary before Ub or NEDD8 becomes a substrate for conjugation.[41] Yet, it is still unclear if UCH-L3 can remove NEDD from neddlyated proteins linked via an isopeptide bond.[36a] In our study UCH-L3 was also able to remove NEDD8 from the 15-mer peptide, where ∼42% hydrolysis was obtained after 30 min incubation with the enzyme (Figure 5). These results suggest that longer substrates linked to NEDD8 via an isopeptide bond might be processed by UCH-L3.

SUMMARY

We have discovered new deprotection method for the well-utilized Thz protecting group based on Pd complexes. Moreover, for the first time we extended the use of the Proc protecting group in chemical protein synthesis and showed its compatibility to peptide synthesis of the middle fragments. Importantly, the removal of both protecting groups can be achieved under NCL conditions without prior peptide isolation and is compatible with the 20 natural amino acids. The deprotection step for both groups is rapid and can be achieved within minutes. These conditions can be applied in the synthesis of complex proteins as exemplified in the case of ubiquitinated H2B and for the first synthesis of neddlyated peptides. The ability to prepare neddylated peptides in high efficiency opens new opportunities in studying this system, which we are currently exploring. We believe that the current approach will find important applications in protein synthesis and facilitate the efficient chemical preparation of complex proteins.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b13580.

All experimental procedures, analytical data of synthetic small molecule peptides and proteins, enzymatic hydrolysis studies. (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Schnolzer, M.; Kent, S. B. H. *Science* **1992**, *256*, 221.

(2) (a) Bode, J. W.; Fox, R. M.; Baucom, K. D. *Angew. Chem., Int. Ed.* **2006**, *45*, 1248. (b) Zhang, Y.; Xu, C.; Lam, H. Y.; Lee, C. L.; Li, X. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 6657. (c) Raj, M.; Wu, H.; Blosser, S. L.; Vittoria, M. A.; Arora, P. S. *J. Am. Chem. Soc.* **2015**, *137*, 6932.

(3) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. *Science* **1994**, *266*, 776.

(4) (a) Weinstock, M. T.; Jacobsen, M. T.; Kay, M. S. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 11679. (b) Wang, P.; Dong, S.; Shieh, J. H.; Peguero, E.; Hendrickson, R.; Moore, M. A.; Danishefsky, S. J. *Science* **2013**, *342*, 1357. (c) Torbeev, V. Y.; Kent, S. B. *Angew. Chem., Int. Ed.* **2007**, *46*, 1667. (d) Sato, K.; Shigenaga, A.; Kitakaze, K.; Sakamoto, K.; Tsuji, D.; Itoh, K.; Otaka, A. *Angew. Chem., Int. Ed.* **2013**, *52*, 7855. (e) Kumar, K. S.; Bavikar, S. N.; Spasser, L.; Moyal, T.; Ohayon, S.; Brik, A. *Angew. Chem., Int. Ed.* **2011**, *50*, 6137.

(5) Hackeng, T. M.; Griffin, J. H.; Dawson, P. E. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 10068.

(6) Canne, L. E.; Botti, P.; Simon, R. J.; Chen, Y. J.; Dennis, E. A.; Kent, S. B. H. *J. Am. Chem. Soc.* **1999**, *121*, 8720.

(7) (a) Bang, D.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2004**, *43*, 2534. (b) Villain, M.; Vizzavona, J.; Rose, K. *Chem. Biol.* **2001**, *8*, 673.

(8) Li, J. B.; Li, Y. Y.; He, Q. Q.; Li, Y. M.; Li, H. T.; Liu, L. *Org. Biomol. Chem.* **2014**, *12*, 5435.

(9) Boll, E.; Ebran, J. P.; Drobecq, H.; El-Mahdi, O.; Raibaut, L.; Ollivier, N.; Melnyk, O. *Org. Lett.* **2015**, *17*, 130.

(10) Ueda, S.; Fujita, M.; Tamamura, H.; Fujii, N.; Otaka, A. *ChemBioChem* **2005**, *6*, 1983.

(11) Pan, M.; He, Y.; Wen, M.; Wu, F. M.; Sun, D. M.; Li, S. J.; Zhang, L. H.; Li, Y. M.; Tian, C. L. *Chem. Commun.* **2014**, *50*, 5837.

(12) (a) Torbeev, V. Y.; Raghuraman, H.; Hamelberg, D.; Tonelli, M.; Westler, W. M.; Perozo, E.; Kent, S. B. H. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 20982. (b) Torbeev, V. Y.; Kent, S. B. H. *Org. Biomol. Chem.* **2012**, *10*, 5887.

(13) (a) Fauvet, B.; Butterfield, S. M.; Fuks, J.; Brik, A.; Lashuel, H. A. *Chem. Commun.* **2013**, *49*, 9254. (b) Haj-Yahya, M.; Fauvet, B.; Herman-Bachinsky, Y.; Hejjaoui, M.; Bavikar, S. N.; Karthikeyan, S. V.; Ciechanover, A.; Lashuel, H. A.; Brik, A. *Proc. Natl. Acad. Sci. U. S. A.*

DOI: 10.1021/jacs.5b13580
J. Am. Chem. Soc. 2016, 138, 5069−5075

2013, *110*, 17726. (c) Hejjaoui, M.; Haj-Yahya, M.; Kumar, K. S. A.; Brik, A.; Lashuel, H. A. *Angew. Chem., Int. Ed.* **2011**, *50*, 405.
(14) Shang, S. Y.; Tan, Z. P.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 5986.
(15) (a) Liu, S. H.; Pentelute, B. L.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2012**, *51*, 993. (b) Murakami, M.; Okamoto, R.; Izumi, M.; Kajihara, Y. *Angew. Chem., Int. Ed.* **2012**, *51*, 3567.
(16) Hemantha, H. P.; Bavikar, S. N.; Herman-Bachinsky, Y.; Haj-Yahya, N.; Bondalapati, S.; Ciechanover, A.; Brik, A. *J. Am. Chem. Soc.* **2014**, *136*, 2665.
(17) (a) Siman, P.; Karthikeyan, S. V.; Nikolov, M.; Fischle, W.; Brik, A. *Angew. Chem., Int. Ed.* **2013**, *52*, 8059. (b) Seenaiah, M.; Jbara, M.; Mali, S. M.; Brik, A. *Angew. Chem., Int. Ed.* **2015**, *54*, 12374. (c) Fierz, B.; Kilic, S.; Hieb, A. R.; Luger, K.; Muir, T. W. *J. Am. Chem. Soc.* **2012**, *134*, 19548.
(18) (a) Haj-Yahya, M.; Kumar, K. S. A.; Erlich, L. A.; Brik, A. *Biopolymers* **2010**, *94*, 504. (b) Sayers, J.; Thompson, R. E.; Perry, K. J.; Malins, L. R.; Payne, R. J. *Org. Lett.* **2015**, *17*, 4902. (c) Creech, G. S.; Paresi, C.; Li, Y. M.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 2891.
(19) (a) Johnson, E. C. B.; Malito, E.; Shen, Y. Q.; Rich, D.; Tang, W. J.; Kent, S. B. H. *J. Am. Chem. Soc.* **2007**, *129*, 11480. (b) Piontek, C.; Silva, D. V.; Heinlein, C.; Pohner, C.; Mezzato, S.; Ring, P.; Martin, A.; Schmid, F. X.; Unverzagt, C. *Angew. Chem., Int. Ed.* **2009**, *48*, 1941.
(20) Fang, G. M.; Wang, J. X.; Liu, L. *Angew. Chem., Int. Ed.* **2012**, *51*, 10347.
(21) (a) Tilley, S. D.; Francis, M. B. *J. Am. Chem. Soc.* **2006**, *128*, 1080. (b) Li, J.; Chen, P. R. *ChemBioChem* **2012**, *13*, 1728. (c) Spicer, C. D.; Davis, B. G. *Nat. Commun.* **2014**, *5*, 4740.
(22) Kodama, K.; Fukuzawa, S.; Nakayama, H.; Kigawa, T.; Sakamoto, K.; Yabuki, T.; Matsuda, N.; Shirouzu, M.; Takio, K.; Tachibana, K.; Yokoyama, S. *ChemBioChem* **2006**, *7*, 134.
(23) (a) Chalker, J. M.; Wood, C. S.; Davis, B. G. *J. Am. Chem. Soc.* **2009**, *131*, 16346. (b) Ojida, A.; Tsutsumi, H.; Kasagi, N.; Hamachi, I. *Tetrahedron Lett.* **2005**, *46*, 3301.
(24) (a) Kodama, K.; Fukuzawa, S.; Nakayama, H.; Sakamoto, K.; Kigawa, T.; Yabuki, T.; Matsuda, N.; Shirouzu, M.; Takio, K.; Yokoyama, S.; Tachibana, K. *ChemBioChem* **2007**, *8*, 232. (b) Li, N.; Lim, R. K. V.; Edwardraja, S.; Lin, Q. *J. Am. Chem. Soc.* **2011**, *133*, 15316.
(25) Vinogradova, E. V.; Zhang, C.; Spokoyny, A. M.; Pentelute, B. L.; Buchwald, S. L. *Nature* **2015**, *526*, 687.
(26) Li, J.; Yu, J. T.; Zhao, J. Y.; Wang, J.; Zheng, S. Q.; Lin, S. X.; Chen, L.; Yang, M. Y.; Jia, S.; Zhang, X. Y.; Chen, P. R. *Nat. Chem.* **2014**, *6*, 352.
(27) Li, S. H.; Lin, Y. J.; Cao, J. G.; Zhang, S. B. *J. Org. Chem.* **2007**, *72*, 4067.
(28) Johnson, E. C. B.; Kent, S. B. H. *J. Am. Chem. Soc.* **2006**, *128*, 6640.
(29) Wu, L. P.; Zee, B. M.; Wang, Y. M.; Garcia, B. A.; Dou, Y. L. *Mol. Cell* **2011**, *43*, 132.
(30) Shema-Yaacoby, E.; Nikolov, M.; Haj-Yahya, M.; Siman, P.; Allemand, E.; Yamaguchi, Y.; Muchardt, C.; Urlaub, H.; Brik, A.; Oren, M.; Fischle, W. *Cell Rep.* **2013**, *4*, 601.
(31) Kent and coworkers reported that during the synthesis of neoglycopeptides applying click chemistry reaction in presence of Cu(I), the Thz was removed. The authors did not investigate this reaction further, probably due to the difficulties in working with Cu(I) in the context of proteins; for more details, see this reference. Lee, D. J.; Mandal, K.; Harris, P. W. R.; Brimble, M. A.; Kent, S. B. H. *Org. Lett.* **2009**, *11*, 5270.
(32) Amatore, C.; Jutand, A.; Thuilliez, A. *Organometallics* **2001**, *20*, 3241.
(33) (a) Metanis, N.; Keinan, E.; Dawson, P. E. *Angew. Chem., Int. Ed.* **2010**, *49*, 7049. (b) Dery, S.; Reddy, P. S.; Dery, L.; Mousa, R.; Dardashti, R. N.; Metanis, N. *Chem. Sci.* **2015**, *6*, 6207. (c) Malins, L. R.; Mitchell, N. J.; McGowan, S.; Payne, R. J. *Angew. Chem., Int. Ed.* **2015**, *54*, 12716. (d) Mitchell, N. J.; Malins, L. R.; Liu, X. Y.; Thompson, R. E.; Chan, B.; Radom, L.; Payne, R. J. *J. Am. Chem. Soc.* **2015**, *137*, 14011.
(34) Malins, L. R.; Mitchell, N. J.; Payne, R. J. *J. Pept. Sci.* **2014**, *20*, 64.
(35) Reddy, P. S.; Dery, S.; Metanis, N. *Angew. Chem., Int. Ed.* **2016**, *55*, 992.
(36) (a) Enchev, R. I.; Schulman, B. A.; Peter, M. *Nat. Rev. Mol. Cell Biol.* **2015**, *16*, 30. (b) Pan, Z. Q.; Kentsis, A.; Dias, D. C.; Yamoah, K.; Wu, K. *Oncogene* **2004**, *23*, 1985.
(37) (a) Spasser, L.; Brik, A. *Angew. Chem., Int. Ed.* **2012**, *51*, 6840. (b) Weller, C. E.; Pilkerton, M. E.; Chatterjee, C. *Biopolymers* **2014**, *101*, 144. (c) Abeywardana, T.; Pratt, M. R. *ChemBioChem* **2014**, *15*, 1547. (d) Pham, G. H.; Strieter, E. R. *Curr. Opin. Chem. Biol.* **2015**, *28*, 57.
(38) Kumar, K. S. A.; Spasser, L.; Ohayon, S.; Erlich, L. A.; Brik, A. *Bioconjugate Chem.* **2011**, *22*, 137.
(39) Blanco-Canosa, J. B.; Nardone, B.; Albericio, F.; Dawson, P. E. *J. Am. Chem. Soc.* **2015**, *137*, 7197.
(40) Wan, Q.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2007**, *46*, 9248.
(41) (a) Wada, H.; Kito, K.; Caskey, L. S.; Yeh, E. T. H.; Kamitani, T. *Biochem. Biophys. Res. Commun.* **1998**, *251*, 688. (b) Linghu, B.; Callis, J.; Goebl, M. G. *Eukaryotic Cell* **2002**, *1*, 491.

DOI: 10.1021/jacs.5b13580
J. Am. Chem. Soc. 2016, 138, 5069−5075