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Direct Palladium-Mediated On-Resin Disulfide Formation from Allocam Protected Peptides

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Abstract

The synthesis of disulfide-containing polypeptides represents a long-standing challenge in peptide chemistry, and broadly applicable methods for the construction of disulfides are in constant demand. Few strategies exist for on-resin formation of disulfides directly from their protected counterparts. We present herein a novel strategy for the on-resin construction of disulfides directly from Allocam-protected cysteines. Our palladium-mediated approach is mild and uses readily available reagents, requiring no special equipment. No reduced peptide intermediates or *S*-allylated products are observed, and no residual palladium can be detected in the final products. The utility of this method is demonstrated through the synthesis of the C-carboxy analog of oxytocin.

Graphical abstract

We present a mild, convenient method for direct conversion of Allocam protected peptides to disulfide-containing protected peptides on solid support.

Introduction

Disulfide linkages improve the thermal, chemical, and enzymatic stability of polypeptides, as well as enforcing a defined tertiary structure.¹ Nature has capitalized on these properties, in combination with the diversity of amino acids, to produce vast libraries of stable, selective polypeptides with defined structures and biological activities.² Meanwhile, peptide-based pharmaceuticals are becoming increasingly important³ because of their greater specificity, potency and lower toxicity profile over traditional small molecule pharmaceuticals.^{3b,4} Additionally, new biological entities benefit from a higher regulatory approval rate than

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This manuscript is dedicated to Prof. Samuel J. Danishefsky in honor of his 80th birthday (March 2016).

traditional small molecule targets.^{3c} There are currently over 500 peptides in preclinical development, and 140 were in clinical trials as of 2015.^{3a} Several important single-disulfide containing pharmaceuticals⁵ are illustrated in Figure 1. Oxytocin (**1**)⁶ is used clinically to induce parturition and lactation in nursing mothers and to decrease bleeding postpartum.⁷ Desmopressin (**2**) is a vasopressin analog used in the treatment of nocturnal enuresis and blood clotting.⁸ Oxytocin and vasopressin are both implicated in social interactions in mammals.⁹ Lanreotide (**3**) is a somatostatin analog used in the treatment of acromegaly.¹⁰

The rapid preparation of disulfide-containing peptide targets and their analogs is currently limited by the lengthy nature of solution-phase folding protocols, which require resin cleavage and removal of all side chain protecting groups, purification of the reduced peptide, oxidative folding, and then a final purification. This approach is even more problematic for hydrophobic peptides or those with complex solubility profiles, where significant amounts of material can be lost during solution-phase manipulations.¹¹ Additionally, the need for multiple purifications increases costs. To address these challenges, we report herein a mild new single-step strategy for on-resin disulfide formation beginning from Allocam-protected peptides.

In addition to avoiding the aforementioned problems, an on-resin approach to disulfide formation is advantageous because it favors intramolecular disulfide formation.¹¹ Much effort has been devoted to the development of new cysteine protecting groups with the goal of improving the available toolbox for folding disulfide-rich polypeptides.¹² Although some of these groups can be removed while the peptide is on resin and in the presence of related protecting groups, several have practical limitations preventing their widespread use. For example, known photolabile groups are incompatible with piperidine,^{13a} and reductive removal of protecting groups can interfere with existing disulfide linkages, and sometimes cannot be removed at all.^{13b} Furthermore, the oxidation step is generally a separate operation that is conducted in the solution phase after resin cleavage and purification of the reduced peptide. The relatively few methods for *on-resin* disulfide formation¹⁴ are summarized in Scheme 1. A traditional approach to access disulfides on solid support (**5**) involves treatment with excess iodine, usually for peptides bearing trityl (Trt), acetamidomethyl (Acm), or 4-methoxytrityl (Mmt) protected cysteines (**4a**).^{15,12a} This is a convenient method that cannot always be employed because of issues with oxidation of sensitive amino acids (Met/Trp) or undesired cleavage from the popular TGT and CTC resins.¹⁶ On-resin oxidation of free thiols can be conducted using *N*-chlorosuccinimide¹⁷ or acidic DMSO (**4b**, HX= HCl or HOAc).^{12b} A variety of protecting groups (**4c**) can be cleaved by Tl(tfa)₃ to directly yield the disulfide containing product. This approach is not orthogonal to other cysteine protecting groups or acid-labile side chain protecting groups, and it will also induce cleavage from TGT and CTC resins.¹⁸ Benzyl derived protecting groups (**4d**) require a 2-step conversion to the disulfide. First, the benzyl group is removed with low concentrations of TFA (again causing incompatibility with Cys(Mmt), Cys(Trt), and CTC resins), then the free thiols are oxidized with CCl₄/TEA to give the corresponding disulfide bond. Historically, solution phase disulfide formation has yielded cleaner peptides than these on-resin approaches.¹¹ The final approach (**4e**) was developed to improve peptide purity when disulfide formation is conducted on the resin. This strategy entails 1) reductive

cleavage of an StBu group, 2) oxidation with 2,2'-dithiobis(5-nitropyridine) (DTNP) to form RS-SNP, 3) acidolysis of Mmt, and 4) microwave-assisted displacement of the SNP group.¹¹ This approach, while effective, is rather operationally intensive.

We endeavored to develop a single-step method for on-resin disulfide formation that would employ common reagents and have the potential to be orthogonal to other cysteine protecting groups as well as be compatible with all resins. In this context, we viewed the little-used allyloxycarbonyl-aminomethyl group (Allocam, **6**, Scheme 2A) as potentially enabling. From a mechanistic perspective, Allocam, which is cleaved with Pd(0) π -allyl conditions, should be orthogonal to most common cysteine protecting groups. A few key limitations have likely prevented the general use of Allocam and the related Fsam and Fnam (**9**) groups. These include acid lability, incomplete conversion due to catalyst poisoning, the use of Bu₃SnH as an allyl scavenger, and the observation of significant amounts of *S*-allylated side products.¹⁹ Additionally, a separate oxidation step was required to convert the initial deprotection products (**7**) to the desired disulfide (**8**). The acid stability of Allocam was improved via the installation of an electron-withdrawing group on the carbonate nitrogen (i.e. Fsam and Fnam, Scheme 2B).²⁰ However, this functional group often requires a subsequent acidic treatment to convert intermediate **10** to the free thiol (**11**). Again, a separate oxidation step is required to access the disulfide (**8**). For an on-resin approach, acid lability is not a significant concern. We envisioned that a re-investigation of the conditions for the cleavage of the Allocam group would enable *in situ* oxidation and potentially minimize *S*-allylation.

Results and Discussion

Although Fmoc-Cys(Allocam)-OH was known, its synthesis was not readily reproducible.¹⁹ Thus, we developed an alternative approach as described in Scheme 3. First, ammonia was bubbled through a solution of allyl chloroformate (**12**) to access the intermediate carbamate in 95% yield. Treatment with paraformaldehyde and Ba(OH)₂ generated hydroxymethyl carbamate **13** in 48% yield. Trimethylsilyl chloride activation of the hydroxymethyl group followed by addition of Fmoc-Cys(H)-O*t*Bu provided fully protected cysteine **14** in 70% yield. Finally, acidolysis of the *t*-Bu ester with 30% TFA produces Fmoc-Cys(Allocam)-OH (**15**) in 99% yield.²¹

With a convenient, scalable route to Fmoc-Cys(Allocam)-OH in hand, we began investigations to develop a new set of deprotection conditions that would affect both deprotection and disulfide formation in a single reaction. We envisioned that the reaction would proceed according to the mechanism in Scheme 4. If a Pd(II) pre-catalyst is employed, initial *in situ* reduction would convert Pd(II) to Pd(0). Coordination of **6** to **17** affords **18**, then oxidative addition leads to cationic π -allyl Pd(II) species **19** and intermediate **20**, which then decarboxylates, releases an equivalent of formimine, and liberates the thiol. π -Allyl Pd(II) intermediate **19** undergoes nucleophilic attack to turn over the catalyst (**21** then **17**) and release the allylated nucleophile. Meanwhile, the thiol would be oxidized *in situ* to the disulfide using a compatible oxidant.

For our investigations, we employed a model peptide derived from the first 9 amino acid residues of islet amyloid polypeptide (IAPP₁₋₉, **22**, Scheme 3), which has a native disulfide linkage.²² We selected this model because the residues between the two cysteines do not adopt any secondary structure, thus, the peptide should be neither particularly biased toward nor against disulfide formation. In the supplementary information of a 1993 report detailing cleavage conditions for an allyl aspartate, it was noted that a mixture of DMSO, AcOH, and NMM led to undesired oxidation of cysteine and produced disulfides as byproducts, presumably as a result of air oxidation.²³ We took these conditions as the starting point for our investigations. Thus, IAPP₁₋₉ was subjected to 5 equiv Pd(PPh₃)₄ in the presence of 3% NMM and 5% AcOH in a DMSO/THF solvent mixture (Table 1, **entry 1**). After 2 h, we observed a mixture of unreacted starting peptide **23**, desired disulfide **24**, and the mono-Allocam version of **23**. Increasing the amount of NMM increased the conversion to the disulfide, but ~1% mono-*S*-allylated product was observed along with the mono-Allocam side product (**entry 2**). With 7 equiv Pd(PPh₃)₄, the desired disulfide was the major product (80% conversion), but we also observed mono-Allocam, reduced, and mono- and bis-allylation products (**entry 3**). Switching the catalyst to Pd(dppf)Cl₂•CH₂Cl₂ completely shut down the reaction (**entry 4**). However, repeating this reaction in the presence of 20 equiv phenylsilane resulted in nearly complete conversion to the desired disulfide with no partially reacted products and no *S*-allylation (**entry 5**). By contrast, PhSiH₃ was not needed for Pd(OAc)₂. In the presence of 7 equiv Pd(OAc)₂, 10% NMM, and 5% AcOH in DMSO, we observed <5% starting material within 2 h as well as a small amount of mono-Allocam peptide (**entry 6**). Lowering the loading to 5 equiv and extending the reaction time to 4 h led to complete disulfide formation with no side products (**entry 7**). Further reducing the loading to only 1 equiv resulted in significantly reduced conversion and increased side product formation (**entry 8**). An increase to 1.5 equiv (**entry 9**) led to nearly complete conversion, which was improved to 100% conversion when less NMM was used (**entry 10**). These were taken as the optimized conditions for the NovaSynTGT resin. Because PEG resins have superior swelling properties relative to polystyrene resins,²⁴ we hypothesized that ChemMatrix resin would allow for lower loadings of Pd(OAc)₂. Unfortunately, upon exposure to 0.75 or 1.0 equiv Pd(OAc)₂, only 11 or 50% conversion was observed after 4 h (**entries 11, 12**). However, under the previously optimized conditions, complete conversion was observed after 2 h instead of 4h (**entry 13**). Overall, we were delighted with the low loadings of palladium needed for the transformation.²⁵ Rinsing the resin with a Pd scavenger prevents any detectable contamination of the cleaved products.²⁶ Under the optimized conditions, we did not observe partially deprotected or unoxidized products, and no *S*-allylated products were observed.²¹

Next, we employed these conditions for direct, on-resin disulfide formation in the synthesis of the neurotransmitter, oxytocin (**1**),^{6,27} a 9-mer peptide with one disulfide bond. We synthesized carboxy-oxytocin to facilitate analysis of the disulfide forming reaction. Fully protected peptide **25** was assembled by Fmoc-SPPS on a TGT-linked ChemMatrix resin (Scheme 5A). Under the optimized IAPP₁₋₉ conditions, ~80% conversion was observed after 6 h. Increased Pd(OAc)₂ loadings (3.0 equiv) facilitated complete conversion of peptide **25** to protected carboxy-oxytocin (**27**). The resulting folded peptide was cleaved from the

resin, the side chain protecting groups were removed, and it was purified by RP-HPLC (Scheme 5B) to afford carboxy-oxytocin (**27**) in 32% isolated yield.²¹

Conclusions

In summary, we present a novel Pd-mediated method to form disulfides on resin that proceeds directly from the protected peptide without loss of any acid-labile side chain protecting groups. This approach avoids the intermediacy of the reduced peptide, avoids solution-phase manipulations of the peptide, and minimizes the number of HPLC purifications needed to access the desired peptide. The reaction employs bench-stable reagents and requires no special equipment. No trace Pd or *S*-allylated products are observed. We have demonstrated the utility of this method in the solid phase synthesis and on-resin folding of the carboxy-terminated analog of the neurotransmitter oxytocin. Meanwhile, we anticipate that this method will be of broad utility in the synthesis of more complex multi-disulfide targets. Efforts to employ this reaction in such contexts are actively being pursued in our laboratory. Detailed investigations into the mechanistic nuances of this chemistry are ongoing and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Representative single-disulfide polypeptides.

Scheme 1.
Known methods for on-resin disulfide formation.

Scheme 2.

State-of-the-art for cleavage and oxidation of the Allocam (A) and the Fnam (B) group requires 2–3 steps.

Scheme 3.
Synthesis of Fmoc-Cys(Allocam)-OH.

Scheme 5.
On-resin synthesis of oxytocin.

Table 1

Optimization of deprotection/oxidation conditions.

entry	catalyst	equiv	additive (%v/v)		solvent	time (h)	23:	24:	other ^e
			NMM	AcOH					
1. ^b	Pd(PPh ₃) ₄	5.0	3%	5%	DMSO:THF (1:1)	2	57	34	9 ^f
2. ^b	Pd(PPh ₃) ₄	5.0	10%	5%	DMSO:THF (1:1)	2	11	71	18 ^{f,h}
3. ^b	Pd(PPh ₃) ₄	7.0	10%	5%	DMSO:THF (1:1)	2	1	80	19 ^{f,i}
4. ^b	Pd(dppf)Cl ₂	7.0	10%	5%	DMSO	2	100	0	0
5. ^{b,d}	Pd(dppf)Cl ₂	7.0	10%	5%	DMSO	2	2	98	0
6. ^b	Pd(OAc) ₂	7.0	10%	5%	DMSO	2	4	93	3 ^f
7. ^b	Pd(OAc) ₂	5.0	10%	5%	DMSO	4	0	100	0
8. ^b	Pd(OAc) ₂	1.0	10%	5%	DMSO	4	10	63	27 ^{f,g}
9. ^b	Pd(OAc) ₂	1.5	10%	5%	DMSO	4	4	96	0
10. ^b	Pd(OAc) ₂	1.5	3%	5%	DMSO	4	0	100	0
11. ^c	Pd(OAc) ₂	0.75	3%	5%	DMSO	4	89	11	0
12. ^c	Pd(OAc) ₂	1.0	3%	5%	DMSO	4	50	50	0
13. ^c	Pd(OAc) ₂	1.5	3%	5%	DMSO	2	0	100	0

^a Unless otherwise noted, reaction was performed using 10 mg of resin (initial loading 0.2 mmol/g) and 250 μ L total volume (all liquids). LCMS analysis is performed directly on crude peptide following removal of solvents from resin cleavage (1:1:3 TFE:AcOH:CH₂Cl₂).

^b NovaSyn PEG-PS copolymer resin with TGT linker, preloaded with Fmoc-Thr(OtBu).

^c Aminomethyl ChemMatrix Resin manually functionalized with TGT linker.

^d 20 Equiv PhSiH₃ added.

^e Ratio determined by integration of relevant peaks in mass spectra.

2 S-Allyls.

1 S-Allyl.

2 Free thiols.

1 Allocam remaining.

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