Protein Synthesis

International Edition: DOI: 10.1002/anie.201900988
German Edition: DOI: 10.1002/ange.201900988

Palladium-Mediated Direct Disulfide Bond Formation in Proteins Containing S-Acetamidomethyl-cysteine under Aqueous Conditions

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Abstract: *One of the applied synthetic strategies for correct disulfide bond formation relies on the use of orthogonal Cys protecting groups. This approach requires purification before and after the deprotection steps, which prolongs the entire synthetic process and lowers the yield of the reaction. A major challenge in using this approach is to be able to apply one-pot synthesis under mild conditions and aqueous media. In this study, we report the development of an approach for rapid disulfide bond formation by employing palladium chemistry and S-acetamidomethyl-cysteine [Cys(Acm)]. Oxidation of Cys(Acm) to the corresponding disulfide bond is achieved within minutes in a one-pot operation by applying palladium and diethyldithiocarbamate. The utility of this reaction was demonstrated by the synthesis of the peptide oxytocin and the first total chemical synthesis of the protein thioredoxin-1. Our investigation revealed a critical role of the Acm protecting group in the disulfide bond formation, apparently due to the generation of a disulfiram in the reaction pathway, which significantly assists the oxidation step.*

Disulfide bridges are often evolutionarily conserved motifs, and they play an important role in the stabilization of the native conformation of various peptides and proteins, as well as in the catalysis of known enzymes.[1] Therefore, when attempting the synthesis of these macromolecules, the correct connectivity of the disulfide bonds must be formed in order to obtain functional molecules.[2] Chemical protein synthesis[3] is a useful method to prepare complex protein analogues with high homogeneity and in workable quantities.[4] Nevertheless, the generation of proteins bearing disulfide bonds remains a challenging task despite success in some interesting cases (e.g., erythropoietin).[1,2,5]

Two main synthetic strategies are often applied for the formation of disulfide bridges in peptides and proteins.[1,6] The first one relies on subjecting the reduced peptide to freely oxidative folding conditions using a buffer containing redox reagents (e.g., Cys/Cystine) to form the correct connectivity under thermodynamic control.[5a,b,d,7] The main limitations of this approach are the slow rate of the reaction (hours–days) and the requirement for careful optimization of the desired ratio between the redox reagents for each system.[6,8]

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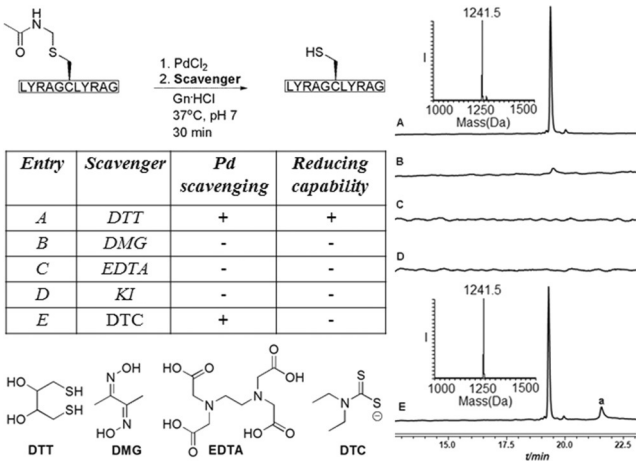
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201900988.

The second approach is based on regioselective disulfide bond formation through stepwise oxidation by employing various orthogonal Cys protecting groups (PGs) such as the S-acetamidomethyl (Acm) and the *tert*-butyl (*t*-butyl).[5c,8] Although this approach has enabled the chemical syntheses of various interesting peptides and proteins (e.g., insulin),[2,5c,6,9] the harsh removal conditions for the Cys PGs significantly reduce the efficiency of this approach.[8,10] For example, a widely used method to convert two Cys(Acm) residues into the corresponding disulfide is based on iodine oxidation in acetic acid,[11] however, over-oxidation of the thiol functionality to sulfonic acid could occur, and other side reactions have been reported when Tyr, Trp, and Met are present.[12]

Herein, we report the development of a new approach for rapid disulfide bond formation by employing Pd chemistry and Cys(Acm) and demonstrate its utility in various systems. The oxidation of Cys(Acm) to the corresponding disulfide bond was achieved in a highly efficient manner in one pot by applying Pd and diethyldithiocarbamate (DTC). We also report on initial mechanistic studies of this transformation, which revealed an important role of the Acm PG in this process.

We recently discovered that Pd^{II} complexes have unprecedented reactivity for the efficient deprotection of several important side-chain PGs.[13] In particular, we have reported the use of $PdCl_2$ under native chemical ligation (NCL) conditions for rapid Cys(Acm) deprotection. This step was followed by NCL coupled with desulfurization in a one-pot manner as demonstrated in the total chemical synthesis of various proteins.[13a,e,h] In order to efficiently recover the peptide, we used excess dithiothreitol (DTT) to scavenge the free Pd and Pd bound to the peptide side chains.[13a,e,h] However, DTT is a reducing agent,[14] which cannot be used when in situ oxidation of Cys residues is desired. Therefore, finding an appropriate Pd scavenger as an alternative to DTT an the essential step for performing one-pot Cys deprotection coupled with disulfide formation.

For this purpose we prepared the model peptide **1** (LYRAGC(Acm)LYRAG) and treated it with 10 equiv $PdCl_2$ for 5 min for Acm removal[13a] followed by incubation with various potential Pd chelators that cannot reduce disulfide bonds. This list includes 1) DTT as a reference reaction[13a,e,h] 2) dimethylglyoxime (DMG),[15] 3) ethylenediaminetetraacetic acid (EDTA),[16] 4) potassium iodide (KI),[17] and 5) DTC.[18] The reactions were analyzed using HPLC and mass analysis, and we found that the peptide was not recovered upon incubation for 25 min with DMG, EDTA, or KI (Figure 1 B–D), probably due to inability to release the thiolate group from Pd. However, we were pleased to observe



Entry	Scavenger	Pd scavenging	Reducing capability
A	DTT	+	+
B	DMG	-	-
C	EDTA	-	-
D	KI	-	-
E	DTC	+	-

Figure 1. Analytical HPLC and mass analysis of the model peptide **1** treated with 10 equiv $PdCl_2$ at 37 °C, pH 7 in 6 M Gn·HCl followed by quenching with various scavengers. A) After 5 min treatment with $PdCl_2$ and 25 min incubation with 25 equiv DTT: the main peak corresponds to unmasked peptide **1** (observed mass: 1241.5 ± 0.1 Da, calcd: 1241.4 Da, average isotopes). B–D) After 5 min treatment with $PdCl_2$, peptide **1** was incubated for 25 min with 25 equiv DMG (B), EDTA (C), KI (D), or DTC (E). The main peak corresponds to unmasked peptide **1** (observed mass: 1241.5 ± 0.1 Da, calcd: 1241.4 Da, average isotopes). Peak (a) corresponds to dimerized peptide **1** after deprotection (observed mass: 2480.6 ± 0.1 Da, calcd: 2481.0 Da, average isotopes).

quantitative recovery of our model peptide when using DTC as a scavenger similar to DTT (Figure 1 A, E).

We then attempted to examine the use of Pd and DTC in one-pot Acm removal and Cys oxidation. We chose as a model system the natural peptide hormone oxytocin, which consists of 9 amino acids (AAs) with two native Cys residues that form a disulfide bridge for the active structure.[19] Our strategy was to make the peptide synthetically with two Cys(Acm) residues and test Acm removal followed by quenching with DTC and in situ oxidation. After preparing the oxytocin peptide with Cys(Acm) using Fmoc solid-phase peptide synthesis (Fmoc-SPPS), we performed Acm removal using 10 equiv of $PdCl_2$ for 5 min[13a] followed by quenching the reaction with 25 equiv DTC for 25 min at pH 7, 37 °C (Figure S4 B in the Supporting Information). The reaction was then monitored by HPLC–MS analysis, which indicated the formation of two products, namely the oxidized (15 %) and reduced(85 %) forms of oxytocin. After overnight incubation under these conditions the oxidation reaction was completed without the requirement for isolation and oxidation. A control experiment with DTT as the scavenger clearly showed no oxidation even after overnight treatment (Figure S4 D).

At this stage we wondered whether changes in the reaction conditions could affect the oxidation rate. Therefore, we initially focused our attention in examining the effect of pH (6–8), which did not show a major effect (Figure S5). We then decided to examine the effect of the DTC amount on the rate of disulfide bond formation. We treated the protected oxytocin with 10 equiv $PdCl_2$ followed by quenching the reactions with increasing amounts of DTC up to 100 equiv

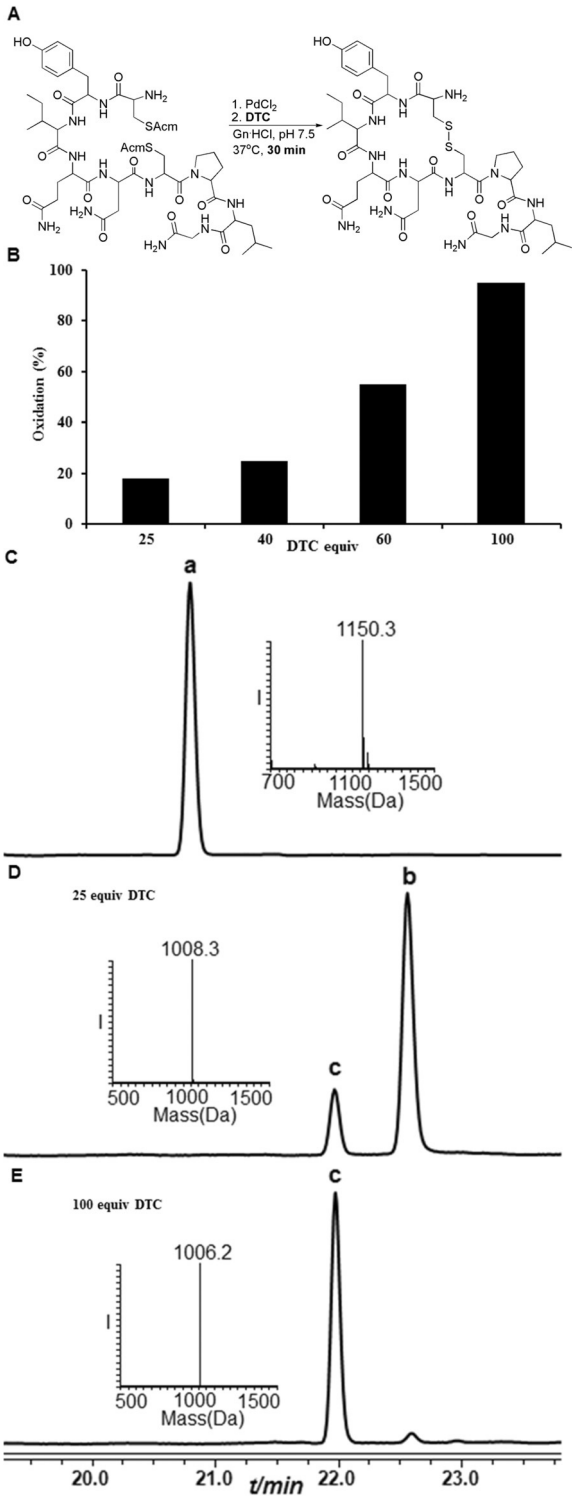


Figure 2. Optimization of one-pot Acm removal and disulfide bond formation in oxytocin. A) Schematic presentation of Acm removal by $PdCl_2$ followed by treatment with DTC. B) The effect of DTC amount on disulfide formation after 25 min. C) Analytical HPLC and mass analysis of purified protected oxytocin (peak (a), observed mass: 1150.3 ± 0.1 Da, calcd: 1151.2 Da, average isotopes). E, D) Reaction of protected oxytocin with 10 equiv $PdCl_2$ for 5 min followed by quenching for 25 min with 25 equiv of DTC (D) or 100 equiv of DTC (E). Peak (b) corresponds to the reduced unmasked oxytocin (observed mass: 1108.3 ± 0.1 Da, calcd: 1109.2 Da, average isotopes). Peak (c) corresponds to the oxidized unmasked oxytocin (observed mass: 1106.2 ± 0.1 Da, calcd: 1107.2 Da, average isotopes).

Interestingly, the rate of the oxidation significantly accelerated with increasing DTC amount. The oxidation was completed within 30 min when 100 equiv of DTC was used in comparison to requirement for overnight treatment with 25 equiv DTC (Figure 2).

To test the applicability of our new strategy for the total chemical synthesis of proteins containing native disulfide bonds, we chose as a target the *E. coli* thioredoxin-1 (Trx-1) enzyme. The sequence of Trx-1 consists of 109 AAs and bears a pair of Cys residues in positions 33 and 36, which form a disulfide bond as part of the catalytic machinery.[20] Trx-1 acts as thiol disulfide oxidoreductase and catalyzes the reduction of disulfides in various proteins.[20b] Trx-1 is an interesting challenge in terms of synthesis due to the fact that it contains two native Cys residues in non-strategical positions for NCL. Therefore, the native Ala in position 57 has to be mutated into Cys in order to perform NCL. Subsequently, this Cys must be converted back into Ala after the NCL step through desulfurization.[21] Thus, the two native Cys residues have to be protected with Acm to keep them intact during the desulfurization step.[22] We envisioned here the use of our new approach to enable one-pot ligation/desulfurization[23] followed by Acm removal and disulfide bond formation without the need for multiple purification steps and the accompanying loss of material.

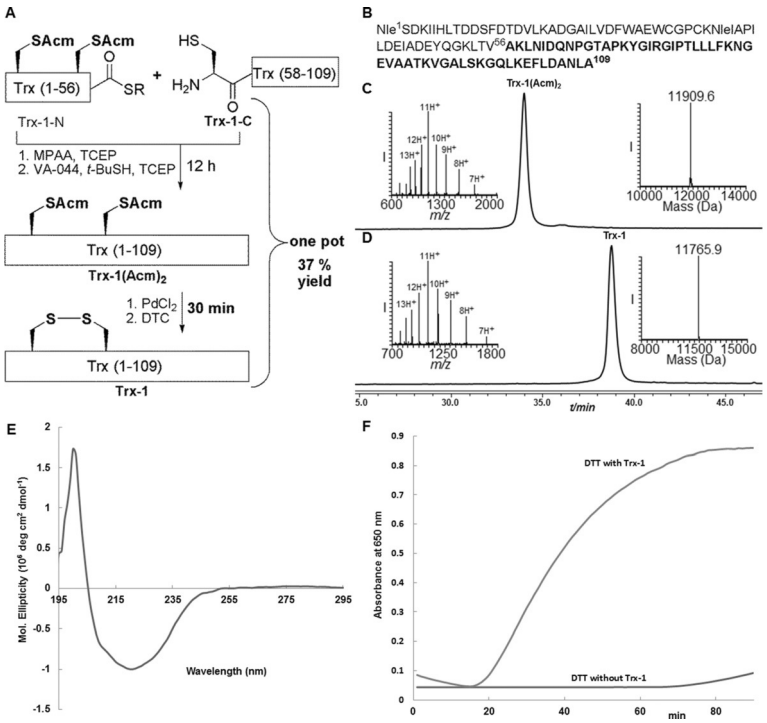


Figure 3. Total chemical synthesis of Trx-1. A) Our approach to Trx-1 synthesis. B) The Trx-1 sequence. C) Analytical HPLC and mass analysis of purified Trx-1(Acm)$_2$ (observed mass: 11 909.6 ± 0.4 Da, calcd: 11 911.5 Da, average isotopes) after one-pot ligation and desulfurization. D) Analysis of purified full-length oxidized Trx-1 (observed mass: 11 765.9 ± 0.3 Da, calcd: 11 767.5 Da, average isotopes). E) CD analysis of the Trx-1 final product. F) Activity assay for the reduction of bovine insulin catalyzed by the chemically synthesized Trx-1 with and without DTT. The turbidity change of the reaction mixture was analyzed by plotting the absorbance at 650 nm versus time.

We divided the Trx-1 sequence into two segments, Trx-1(1–56)-thioester (Trx-1-N) and Cys-Trx-1(58–109) (Trx-1-C). Trx-1-C was synthesized on a Rink amide resin, while Trx-1-N was synthesized on a diaminobenzoic acid (Dbz) linker[24] (Figures S7–8). Subsequently, we ligated Trx-1-C and Trx-1-N using 4-mercaptophenylacetic acid (MPAA) and tris(2-carboxyethyl)phosphine (TCEP) as additives in 6 M Gn·HCl, pH 7.3 at 37 °C.[25] The ligation was completed within 10 h and the product was dialyzed against 6 M Gn·HCl to enable one-pot desulfurization, which was completed within 2 h (Figure 3A–C and Figure S9). The reaction mixture was then dialyzed again, treated with 10 equiv $PdCl_2$ for 5 min, and quenched with 100 equiv DTC for 25 min to afford the full-length Trx-1 protein with the disulfide bond between Cys 33 and Cys 36 in one pot and 37 % overall yield of isolated product (Figure 3D). Circular dichroism (CD) spectroscopy analysis of the purified oxidized Trx-1 exhibited the expected CD signature of the folded protein[26] (Figure 3E). In addition, we confirmed the catalytic activity of the synthetic Trx-1 using an established Trx-1 activity assay[27] (Figure 3F).

At this stage, we were puzzled about the effect of DTC combined with Pd on the rate of oxidation and were curious to examine whether the enhancement of the oxidation reaction could occur in the case of free and reduced Cys residues. Therefore, we synthesized the oxytocin peptide with unprotected and reduced Cys residues. Subsequently, the peptide was exposed to 10 equiv of Pd and 100 equiv of DTC and the reaction was monitored by analytical HPLC–MS. To our surprise, after 30 min of incubation at 37 °C, only 10 % oxidized oxytocin was obtained compared to full conversion when the two Cys residues were masked with Acm (Figure 4B). The oxidation was completed only after overnight incubation. These findings inspired us to further investigate the necessity of Acm for enhancing the oxidation reaction. To examine this, we prepared another oxytocin variant bearing unprotected N-terminal Cys while Cys 6 was protected with the Acm. The peptide was treated under the same conditions as before and 80 % oxidation was observed after 30 min, with the reaction being completed within 2 h (Figure 4C). Together these results indicate that the deprotection step to remove Acm under our conditions plays an important role in the oxidation step. Notably, it has been proposed that disulfiram (DSF), which is the oxidized form of DTC, is able to induce glutathione (GSH) oxidation, probably through disulfide-bond exchange.[28] In a different study, a complex of Cu^{II} and DTC as a ligand was reported to promote GSH oxidation, possibly via a copper redox cycle.[29] Although the exact mechanism is unclear, the generation of DSF through a redox cycle of $Cu^{II}(DTC)_4$ to $Cu^{I}(DTC)_2$ has been proposed.[29]

These previous findings prompted us to examine whether DSF, which could be formed via a redox cycle involving Pd, could also be a key element in the oxidation step in our

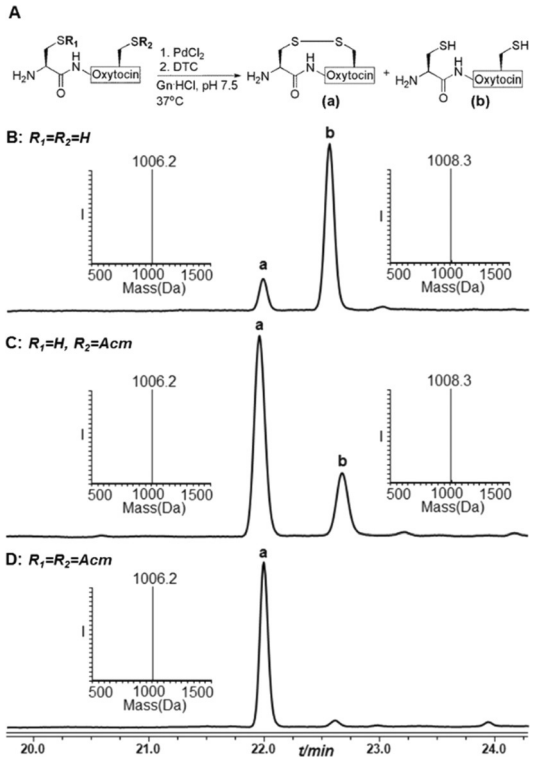
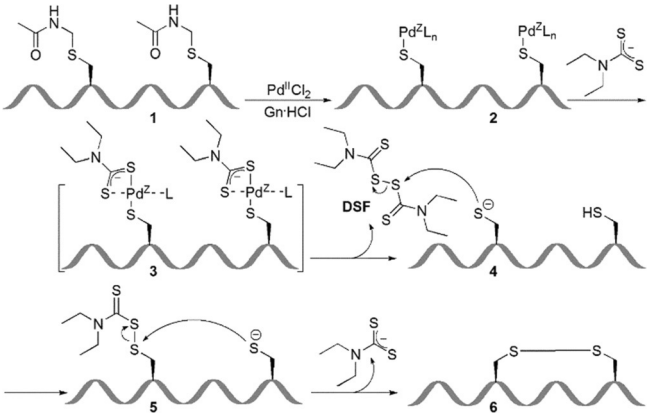


Figure 4. The effect of Acm on the Pd/DTC mediated disulfide bonds formation. A) Reaction of different analogues of oxytocin with $PdCl_2$ followed by treatment with DTC in 6 M Gn·HCl, pH 7.5, 37 °C. Oxytocin analogues were incubated with 10 equiv $PdCl_2$ for 5 min followed by quenching with 100 equiv DTC for additional 25 min. B) The result with unprotected oxytocin; peak (a) corresponds to the oxidized oxytocin (observed mass: 1106.2 ± 0.1 Da, calcd: 1107.2 Da, average isotopes). Peak (b) corresponds to the reduced and unmasked oxytocin (observed mass: 1108.3 ± 0.1 Da, calcd: 1109.2 Da, average isotopes). C) The result with an oxytocin analogue bearing one Cys(Acm). D) The result with oxytocin bearing two Cys(Acm) residues.

reaction. To examine this idea, we first exposed the unprotected oxytocin to our optimized conditions in the presence of commercially available DSF (2 equiv). Complete oxidation was observed within 5 min compared to overnight treatment in the absence of DSF. When this peptide was incubated with DSF only and without Pd and DTC, the same results were obtained. In addition, when oxytocin bearing two Cys(Acm) residues was treated with 10 equiv of $PdCl_2$, 25 equiv of DTC, and 2 equiv of DSF, we observed Cys deprotection and oxidation in situ within 10 min, compared to 30 min when using 100 equiv DTC and Pd. These results support the possible formation of DSF during the Acm deprotection step and a critical role of DSF in the rapid disulfide bond formation. Notably, external addition of acetamidomethyl alcohol (2 equiv), which is one of the products of the deprotection step, [13a] to the reaction mixture had no effect on the oxidation rate. To further support the requirement for Cys(Acm) for the generation of DSF assisted by Pd, we treated unprotected oxytocin peptide with an external peptide containing Cys(Acm) in the presence of $PdCl_2$ and DTC, which led to complete disulfide bond formation within 1.5 h compared to overnight treatment in the absence of Cys(Acm) (Figure S14).

Based on these observations, we propose a mechanism whereby after the rapid Acm deprotection step, the Pd bound to the unprotected thiolate undergoes complexation with the added DTC. Such a complex could contain Pd and a DTC ligand on each Cys of the unprotected peptide. Indeed, we were able to isolate such an intermediate in the case of Trx-1 and characterize it by mass spectrometry, and it matches the mass of the Trx-1 in addition to two Pd atoms and two DTC ligands (**3**, Scheme 1). Moreover, this isolated intermediate in



Scheme 1. Proposed mechanism for disulfide bond formation via Pd-mediated Acm removal and DSF formation.

presence of excess Pd and DTC was converted into the oxidized Trx-1 product within 30 min (Supporting Information, Figure S12). Therefore, we also propose that such an intermediate in presence of excess DTC collapses rapidly to the free peptide (**4**, Scheme 1) and generates DSF, similar to the Cu^{II} case, through an as yet unclear mechanism.[29] Subsequently, the free Cys residues react with DSF, which facilitates rapid disulfide bond formation (Scheme 1) to form a stable disulfide bond that DTC cannot further reduce.

Due to the quantitative appearance of free thiols immediately after adding the DTC to the Pd-bound peptide (formation of **4**, Scheme 1) as we detected by HPLC–MS analysis (Figure S11), it seems that the generation of DSF after the removal of Acm is a rate-limiting step. Hence, the additional 25 min that was required to obtain the product is likely the time that is needed to generate enough DSF for the rapid oxidation step. We also observed a clear correlation between the amount of DSF and the oxidation rate, since the addition of 1 equiv of DSF led to a relatively slower oxidation of oxytocin, which was completed within 45 min compared to 5 min with 2 equiv of DSF. This is also consistent with our study on the oxytocin peptide bearing one Cys(Acm), where the oxidation step was slower and required 2 h compared to 30 min in the case of oxytocin with two Cys(Acm) residues (Figure 4). Based on these results, we developed an optimized protocol in which Cys(Acm) deprotection is performing through treatment with 10 equiv $PdCl_2$ for 5 min followed by the addition of 25 equiv DTC and 2 equiv DSF for another 5 min to furnish the desired oxidized product (Figure S15).

In summary, we have established for first time the use of Pd chemistry in the context of disulfide bridge formation

within peptides and proteins under mild and aqueous conditions. Our results revealed unprecedented highly efficient oxidation of Cys(Acm) residues to the corresponding disulfide mediated by Pd and DTC and the method was found to be compatible with the one-pot total chemical synthesis of proteins as exemplified by Trx-1. In this process, the generation of DSF appears to be the key reagent for rapid disulfide bond formation. Our study also shows the first utilization of DSF as an efficient and mild reagent for rapid disulfide bond formation in peptide and protein synthesis under aqueous media. In light of our recent findings regarding the on-demand deprotection of various Cys PGs with Pd,[13h] this approach has the potential to be further expanded to generate multiple disulfide bonds in peptides and proteins in a regioselective manner with one-pot operation. This exciting prospect is currently under investigation in our laboratory.

Acknowledgements

A. Brik holds The Jordan and Irene Tark Academic Chair. H. S. is supported in part at the Technion by a Technion-Guangdong Fellowship.

Conflict of interest

The authors declare no conflict of interest.

Keywords: desulfurization · disulfide bonds · palladium · protein synthesis · S-acetamidomethyl-cysteine

How to cite: *Angew. Chem. Int. Ed.* **2019**, *58*, 5729 – 5733
Angew. Chem. **2019**, *131*, 5785 – 5789

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Manuscript received: January 24, 2019
Accepted manuscript online: March 4, 2019
Version of record online: March 20, 2019

