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N-Chlorosuccinimide, an Efficient Reagent for On-Resin Disulfide Formation in Solid-Phase Peptide Synthesis

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ABSTRACT

***N*-Chlorosuccinimide is described as a widely applicable on-resin disulfide-forming reagent. Disulfide bond formation was completed within 15 min in DMF. This strategy was successfully used in the synthesis of oxytocin and a regioselective synthesis of an α-conotoxin. Moreover, disulfide formation with *N*-chlorosuccinimide was found to be compatible with oxidation-prone methionine and tryptophan.**

The increasing number of peptide therapeutics in the market reflects the importance and potential of these molecules.[1] However, the main drawback of peptide therapeutics is their limited stability in biological fluids as a result of proteolytic enzymes.[2] In nature, many biomolecules, such as enzymes, hormones, toxins, and growth factors, contain disulfide bonds.[3,4] These bonds constrain the conformation of a peptide or protein and increase stability to proteolysis. This strategy has been adopted in peptide chemistry to prepare natural products and to increase the biostability of therapeutic peptides, where disulfide bonds are often used to enhance resistance to proteolysis, thus effectively increasing circulation times.[5] Moreover, disulfide bonds confer conformational rigidity, which can increase binding affinity by favoring entropic molecular recognition.

There are a growing number of multiple-disulfide containing peptide drugs for the treatment of various diseases. The most recent addition is the FDA-approved linaclotide (Linzess) for the treatment of chronic idiopathic constipation and irritable bowel syndrome with constipation in adults.[6] Linaclotide is an orally available 14-residue peptide that contains three disulfide bonds.[7] These structures confer sufficient stability to resist proteolytic cleavage in the gastrointestinal tract. Another example of a multiple disulfide containing peptide drug is ziconotide (Prialt), a ω-conotoxin with three disulfide bonds, used for the treatment of chronic pain.[8] Currently, at least five conotoxins or conotoxin-based molecules, all of which contain multiple disulfide bonds, are being tested in various stages of clinical

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trials (CGX-1007, CGX1160, CGX-1051, ACV1, and Xen2174).[9]

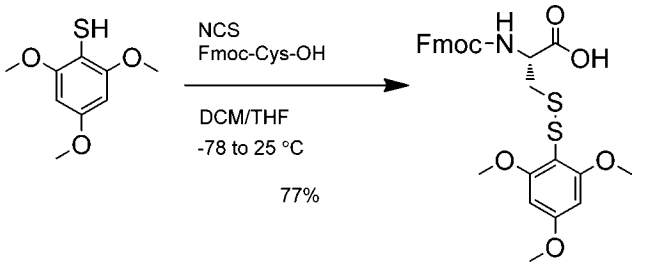
In view of the increasing number of multiple-disulfide-containing peptide drugs and the rising importance of this class of compounds, the challenge of preparing these molecules must be addressed. In this regard, the main challenge is the controlled formation of intramolecular disulfide bonds which assures the correct disulfide connectivity.[10,11] Two main strategies are available for introducing sequential disulfide bonds into a peptide: (i) the peptide is cleaved from the solid support and subsequently oxidized in solution or (ii) the peptide is oxidized directly on the solid support.[12] The former typically uses air or DMSO oxidation under mildly basic conditions to form the disulfide bond. Unfortunately, oxidation in solution must be performed under high dilution; otherwise intermolecular reactions, which form dimers or oligomers, are favored over intramolecular cyclization. Furthermore, the process is time-consuming as each sequential step requires purification and lyophilization.

On the other hand, strategy ii is much quicker because of simple purification by convenient filtration of excess reagents from the resin. Furthermore, the intramolecular reaction is favored as a result of the pseudodilution effect, a kinetic phenomenon that mimics high dilution in a microporous environment through which it preferentially selects intramolecular disulfide formation over intermolecular side reactions.[13] Owing to pseudodilution, the reactions can be performed under higher concentration, thus leading to smaller reaction volumes. Various protocols can be used to prepare disulfide bonds on resin using diverse oxidation reagents, including mercury salts, thalium salts, iodine, carbon tetrachloride, and DMSO/air oxidation.[14] However, the use of these reagents is hindered by their high toxicity, capacity for scrambling disulfide bonds and incompatibility with sensitive residues such as methionine (Met) and tryptophan (Trp).

In the case of multiple-disulfide-containing peptides, a very attractive synthetic strategy is through an orthogonal Cys protection scheme with disulfide pairing in both solid-phase and solution. Thus, the first pair of Cys residues is deprotected and subsequently oxidized on the solid support. The second disulfide bond is usually prepared in a separate oxidation step after cleavage or alternatively during cleavage. Quantitative oxidation of all Cys residues is difficult, and if the formation of disulfide bonds does not run to completion, scrambling of the bonds, by disulfide exchange, may occur.[14] With the exception of iodine, all of the aforementioned reagents take many hours to oxidize the unprotected Cys, thus allowing side reactions to occur. Iodine-mediated oxidation is much quicker than the other methods because of the high reactivity of this reagent. However, iodine often leads to side reactions, disulfide bond scrambling, incompatibility with Trt protecting groups, and sensitive amino acids.[15]

Generally, solution-based methods yield purer peptides; however, the entire procedure is significantly more time-consuming. Conversely, solid-phase peptide synthesis lends itself well to high-throughput synthesis, and efficient on-resin disulfide formation allows convenient access to diverse disulfide-containing peptides through automation. Given the rising importance of multiple-disulfide-containing peptides and the challenges involved in the preparation thereof, we initiated a study of novel oxidizing reagents to fill the gaps in the current repertoire of disulfide forming reagents. The aim of this work was to find a novel on-resin oxidation method that leads to rapid quantitative oxidation with minimal side reactions for the synthesis of multiple-disulfide containing peptides.

Scheme 1. Synthesis of Fmoc-Cys(*S*-Tmp)-OH



Recently, we introduced the novel reducing agent labile Cys protecting group trimethoxyphenylthio (*S*-Tmp).[16] During the synthesis of the monomer Fmoc-Cys(*S*-Tmp)-OH we used *N*-chlorosuccinimide (NCS) to prepare a mixed disulfide between Fmoc-Cys-OH and 2,4,6-trimethoxythiophenol (Scheme 1). NCS was compatible with Cys, and the reaction between the sulfhydryl moiety and NCS proceeded rapidly to form a highly reactive sulfenyl chloride. NCS is known to be compatible with all amino acids except Met and Trp.[17] In Trp-containing peptides, the tryptophanyl peptide bond is highly labile to cleavage by NCS. The rapid and clean formation of the mixed disulfide in the synthesis of Fmoc-Cys(*S*-Tmp)-OH and compatibility with amino acids spurred our study of the on-resin formation of peptide disulfides with NCS.

A widely studied model peptide was needed as a proof of concept. For this purpose, we chose oxytocin, a nonapeptide

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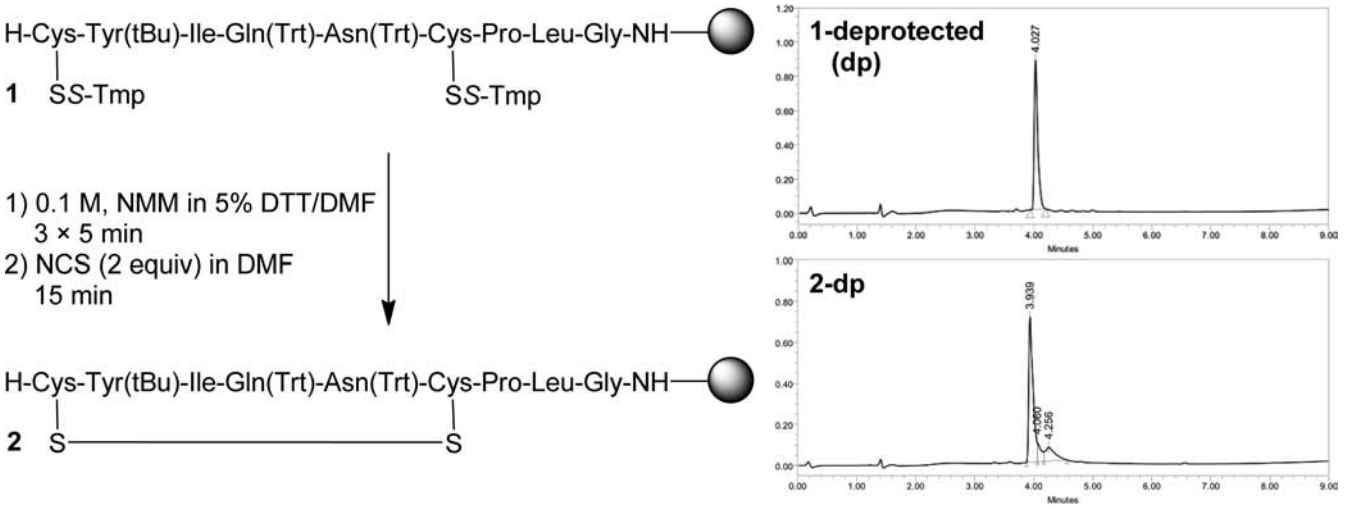
Scheme 2. On-Resin NCS Oxidation of Oxytocin



currently used as a drug to induce labor.[18] Oxytocin was prepared on a Rink Amide resin with *S*-Tmp-protected Cys residues (Scheme 2). The *S*-Tmp-protecting groups were removed with a dithiothreitol (DTT)-containing deprotection mixture, and the peptidyl resin was subsequently treated with either 1, 1.5, 2, or 3 equiv of NCS in DMF for 15 min. Following disulfide formation, the peptidyl resin was washed and treated with a cleavage mixture to release the peptide from the resin. Chromatographic and spectroscopic analysis showed clean and quantitative formation of the disulfide with minimal dimerization. The purest product was obtained with 2 equiv of NCS. Consequently, we used these conditions in subsequent experiments when referring to the NCS method. In comparison, Shih studied the formation of disulfides with *N*-halosuccinimides.[19] The study focused on on-resin disulfide formation with *N*-iodosuccinimide from protected Cys (4-MeOBzl, 4-MeBzl, and Acm) and typical reaction times of 1−2 h. In the case of oxytocin low yields were reported (∼1%). NCS gave the best results in the formation of cystine from Cys but was not further investigated.

To demonstrate the applicability of the NCS method with multiple-disulfide-containing peptides, we applied this protocol on a regioselective synthesis of an α-conotoxin. The SI α-conotoxin is a 13-residue peptide from a piscivorous cone snail, and it contains two disulfide bonds and a C-terminal amide.[20] The linear peptide was prepared on a Rink amide resin with orthogonal Cys protection (Scheme 3). 4-Methoxytrityl (Mmt) protection was used for Cys^2-Cys^7 and *S*-Tmp protection for Cys^3-Cys^{13}. S-Tmp was removed with a deprotection mixture containing DTT, and subsequent analysis of a microcleavage showed the linear peptide in high purity. The first disulfide was formed with the NCS method, the resin was treated with a dilute TFA solution to remove Mmt, and the second disulfide bond was formed using the NCS method. Peptide cleavage was achieved by treatment of the peptidyl resin with TFA/TIS/H_2O (95:2.5:2.5) for 1 h. Chromatographic and spectroscopic analysis of the peptide confirmed the formation of SI conotoxin in 70% purity. The regioselective synthesis of the SI conotoxin illustrates the versatility and efficiency of the NCS method in the preparation of multiple-disulfide containing peptides. In addition, it demonstrates the compatibility of NCS with peptides containing Trt and Mmt protecting groups, which are incompatible with reagents such as iodine and thallium(III) trifluoroacetate.

Iodine, thallium(III) trifluoroacetate, and other conventional oxidation reagents are not compatible with Met-containing peptides because of the formation of Met-*S*-oxide.[21] We prepared a model tripeptide (Fmoc-Cys-Met-Cys-NH_2) to determine whether Met can be used under the reaction conditions applied in the NCS method. Both 1 and 2 equiv of NCS in DMF for 15 min were used, and the crude chromatograms were compared.

An excess of 2 equiv of NCS led to the formation of 25% oxidized Met, while 1 equiv produced less than 2% (Scheme 4). However, with 1 equiv of NCS the peptide was not quantitatively oxidized and a slight excess of 1.05 equiv of NCS achieved quantitative disulfide formation with less than 2% Met oxidation. These observations indicate that Met containing peptides can be used in the NCS method with a stoichiometric amount or a slight excess of NCS, which keeps Met oxidation at acceptable low levels.

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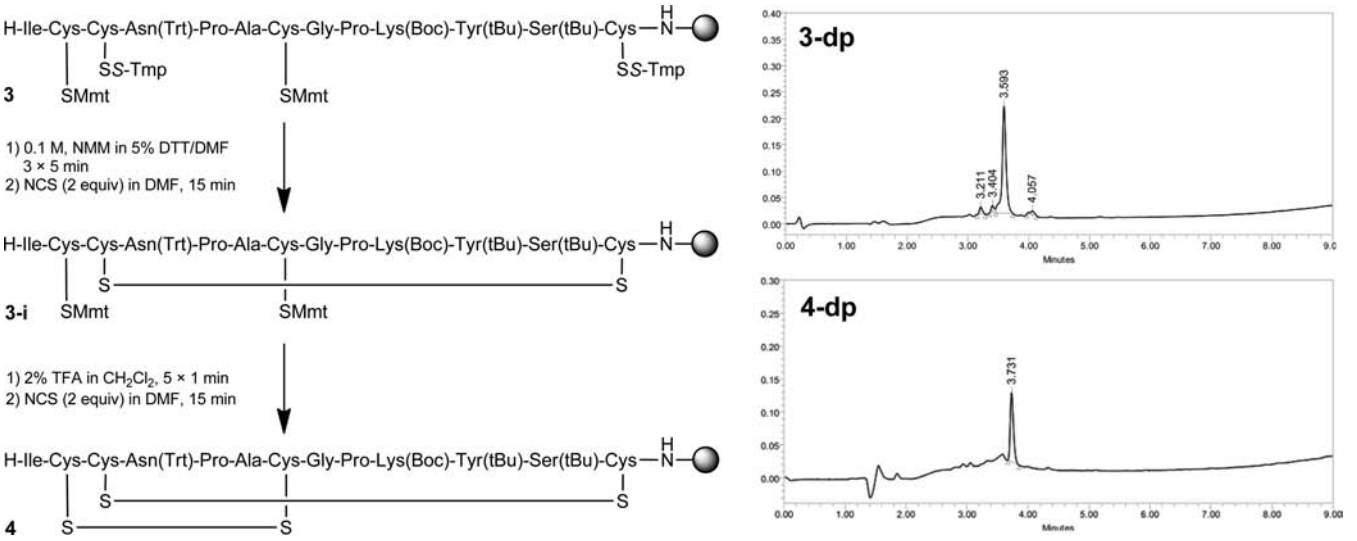
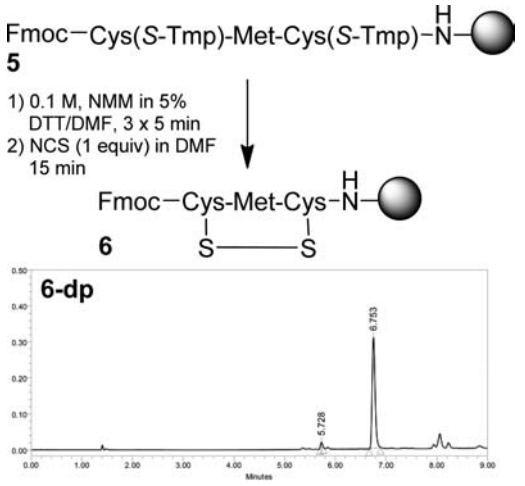
Scheme 3. Regioselective Synthesis of SI Conotoxin



Scheme 4. Met Compatibility with NCS



The experiment in Scheme 4 was repeated with Trp, instead of Met, in order to determine whether Trp is compatible with the NCS method. Trp was expected to be less sensitive toward oxidation, and therefore, we used 2 equiv of NCS for 15 min. The disulfide was formed without major side reactions, and no tryptophanyl peptide bond cleavage was observed showing that Boc-protected Trp is compatible with the NCS method without modification.

In conclusion, the use of NCS for on-resin disulfide formation has proven to be a versatile, efficient, and rapid technique for the synthesis of mono- and multiple-disulfide-containing peptides. This was shown in the oxidation of oxytocin and the regioselective synthesis of SI conotoxin. In comparison to iodine and thallium(III) trifluoroacetate, NCS is compatible with the sensitive Trt and Mmt protecting groups, thus increasing the applicability of the NCS method. Moreover, NCS is compatible with Met-containing peptides when 1 instead of 2 equiv of NCS is used. Additionally, Trp-containing peptides were found to be compatible with the NCS method without modification. On the basis of these considerations, we conclude that the NCS method is the most widely applicable protocol for on-resin disulfide formation in peptides.

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Supporting Information Available. Detailed experimental procedures, characterization, and spectroscopic and chromatographic data. This material is available free of charge via the Internet at http://pubs.acs.org

The authors declare no competing financial interest.