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N-chlorosuccinimide, an efficient peptide disulfide bond-forming reagent in aqueous solution†

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A novel method has been developed for the efficient formation of peptide disulfide bonds under aqueous conditions using *N*-chlorosuccinimide. Complete disulfide bond formation is achieved in 15 min with solvent mixtures containing water and acetonitrile.

Disulfide bonds are widespread in peptides and proteins where they strengthen conformation and lead to increased rigidity and proteolytic stability.[1–3] The number of disulfide-rich therapeutics currently available is increasing, such as the FDA-approved peptide drugs Prialt (ziconotide) and Linzess (linaclotide), both of which contain 3 disulfide bonds.[4–6] In order to prepare disulfide-rich peptides, efficient disulfide-forming reagents must be used that are compatible with all amino acids, and also preferably with the associated amino acid protecting groups. Many oxidants are used for disulfide bond formation in peptide chemistry (*e.g.* air, DMSO, iodine, thallium salts, mercury salts, and carbon tetrachloride).[7,8] However, given that there is no universal protocol for the formation of these bonds, this process is empirical and needs to be assessed on a case to case basis. Robust and widely applicable disulfide-forming reagents are required in order to quickly determine the conditions that will allow rapid and quantitative disulfide bond formation with minimal side-reactions.

Recently, we used *N*-chlorosuccinimide (NCS) to form a mixed disulfide between Fmoc-Cys-OH and trimethoxythiophenol under anhydrous conditions.[9] In comparison to most common oxidants for disulfide formation, NCS is an easy to handle, cheap, and shelf-stable crystalline solid that is soluble in both organic and aqueous solvent mixtures. NCS was found to be an efficient reagent for the formation of mixed cysteine disulfides in organic solvents. Subsequently, we reported NCS as a highly efficient on-resin disulfide-forming reagent for solid-phase peptide synthesis.[10] Quantitative disulfide formation was achieved in 15 min using 2 equiv. of NCS in DMF, which we demonstrated in the synthesis of several peptides, including an on-resin regioselective synthesis of an α-conotoxin using 2 sequential NCS oxidations (Fig. 1).

Shechter and co-workers reported that NCS is compatible with all amino acids except the oxidation-sensitive Trp and Met.[11] However, we found that under our conditions both Trp and Met are compatible with NCS.‡ This finding shows that all amino acids can be used with the NCS method on the solid-phase. In addition, NCS was found to be compatible with the amino acid protecting groups Trt and Mmt, while the common disulfide-forming oxidants such as iodine and thallium salts are not. These results allowed us to conclude that NCS is the most widely applicable disulfide-forming reagent for solid-phase peptide synthesis. However, solid-phase peptide synthesis has several disadvantages

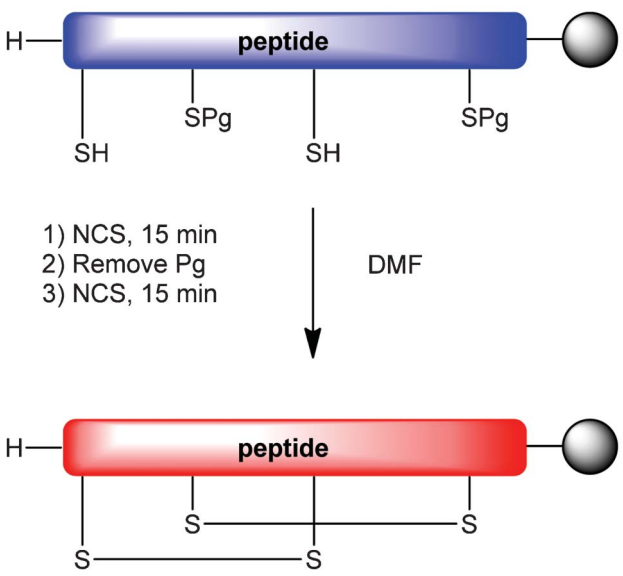

Fig. 1 Regioselective on-resin NCS oxidation.

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over solution phase such as: limited scalability and dilution, and generally slower reaction kinetics. For instance, when pseudo-dilution on the solid-phase is not sufficient and leads to intermolecular side-reactions, dilution is not possible and would necessitate resynthesis on a resin with a lower loading. To overcome these disadvantages with the solid-phase NCS method we initiated a study of NCS oxidation in solution. Herein, we report on the formation of peptide disulfide bonds in aqueous solution with NCS. This achievement significantly broadens the applicability of the NCS method.

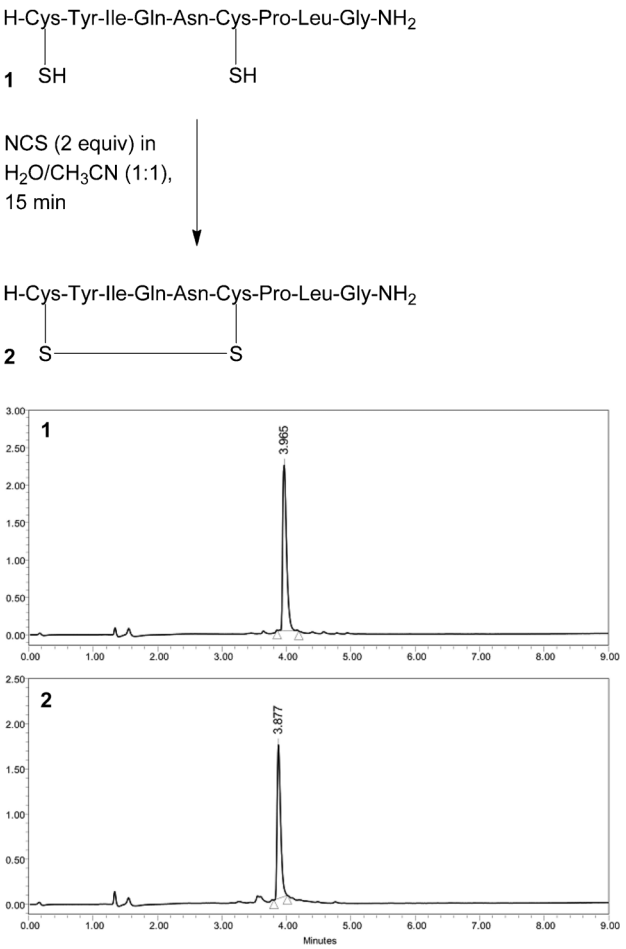
Our model system was based on the well-studied oxytocin, a nonapeptide currently used as a drug in obstetric medicine to facilitate childbirth.[12] Oxytocin contains one disulfide bond and a C-terminal amide. The peptide was synthesized on the solid-phase using standard Fmoc/*t*Bu chemistry on a Rink Amide resin. The reducing agent-labile protecting group *S*-Tmp was used for the protection of the cysteine thiol.[9] Prior to cleavage, *S*-Tmp was removed by treatment with dithiothreitol under mildly basic conditions. As a proof of concept for the NCS oxidation of oxytocin under aqueous conditions, experiments were set up using H_2O, and H_2O/acetonitrile mixtures. The fully deprotected peptide was dissolved in H_2O or H_2O/acetonitrile mixtures, and a solution containing 1.5 equiv. of NCS was added. The mixtures were shaken for 15 min at room temperature, lyophilized and subsequently analyzed by chromatographic and spectroscopic techniques. Upon analysis of the reactions, we found that in all cases oxidized oxytocin (**2**) was formed in high purity and all the starting material (**1**) was consumed (Scheme 1).

We next studied the effects of excess NCS on the efficiency of disulfide bond formation in H_2O/acetonitrile. The peptide was dissolved in H_2O/acetonitrile (1 : 1) and 1.1, 1.5 or 2 equiv. of NCS were added. This mixture was then shaken for 15 min, and lyophilized. In all cases, oxidized oxytocin was formed in high purity and no starting material was left (Table 1).

This observation shows that NCS can cleanly form peptide disulfide bonds in an aqueous solution under a wide variety of conditions. Moreover, we observed that an excess of NCS was not detrimental to the purity of the final peptide. The variety of aqueous solvent mixtures and NCS equiv. allows the tailoring of the reaction conditions to the peptide, thus ensuring optimal solubility while retaining highly efficient disulfide bond formation.

To further demonstrate the applicability of aqueous NCS disulfide bond formation, we synthesized the octapeptide octreotate. This compound is a somatostatin analog used for targeting somatostatin receptor-positive tumors, and for radionuclide treatment when conjugated to a radionuclide chelator.[13,14] Octreotate, contains 1 disulfide bond and 2 D-amino acids (D-Phe and D-Trp). The D-Trp-Lys sequence forms the key β-turn pharmacophore, which causes the high affinity of octreotate for somatostatin receptors.[15]

Octreotate was synthesized on a chlorotrityl resin using standard Fmoc/*t*Bu chemistry and subsequently cleaved from the resin. The deprotected peptide was dissolved in H_2O/acetonitrile (1 : 1), a solution containing 1.5 equiv. of NCS was added, and the mixture was shaken for 15 min at room



Scheme 1 Aqueous NCS oxidation of oxytocin.

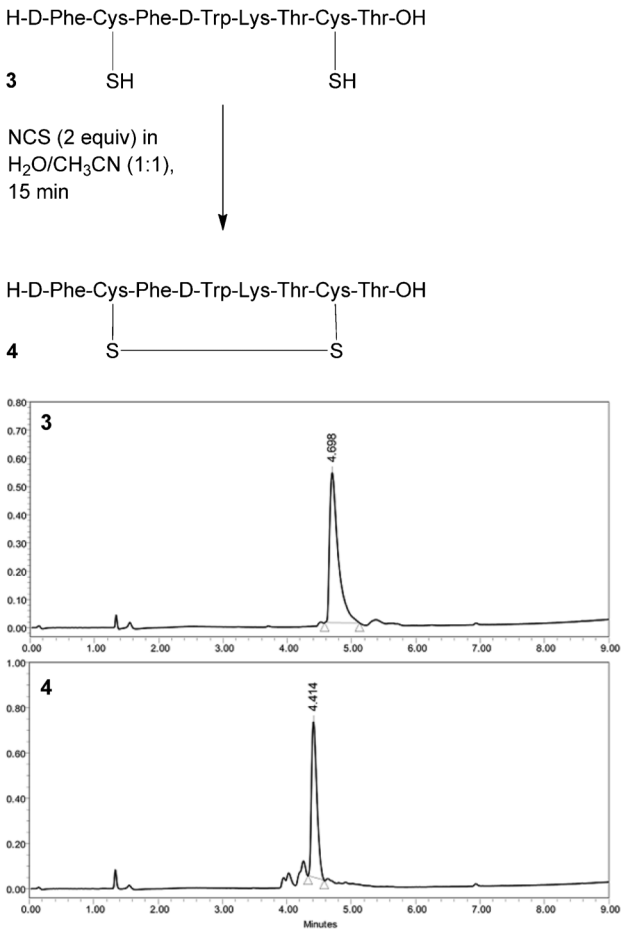
temperature, lyophilized, and subsequently analyzed. Oxidized cyclic octreotate (**4**) was formed in high purity, and no linear octreotate was present (Scheme 2).

As a final example, we prepared a linear α-conotoxin from a piscivorous conesnail. Oxidative folding of multiple disulfide-containing peptides is widely used for disulfide-rich peptides.[16,17] However, due to the multiple Cys residues present, several disulfide connectivities can arise, leading to complex mixtures of peptides. The desired disulfide connectivity can be achieved by careful tweaking of the oxidation conditions.[18] In this case, we

Table 1 Results of oxytocin NCS oxidation under distinct conditions

Oxytocin	H_2O/CH_3CN	NCS equiv.	Purity (%)[a]
1	n/a	n/a	98
2-1	H_2O	1.5	88
2-2	1 : 1	1.5	94
2-3	1 : 3	1.5	94
2-4	1 : 1	1.1	94
2-5	1 : 1	1.5	95
2-6	1 : 1	2.0	94

[a] Determined by the peak area of a HPLC chromatogram at 220 nm.



Scheme 2 Aqueous NCS oxidation of octreotate.

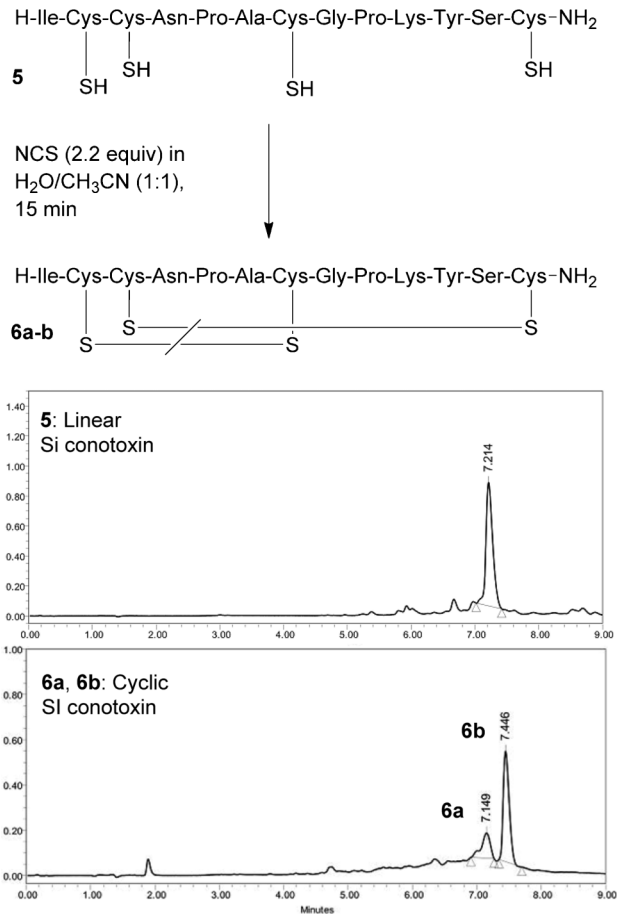


Fig. 2 HPLC chromatograms of linear and cyclic Si conotoxin.

prepared linear Si conotoxin, a peptide containing 13 residues with 4 Cys and a C-terminal amide.[19] The subsequent oxidative folding was performed to demonstrate the feasibility of NCS to fully oxidize disulfide-rich peptides under aqueous conditions, without optimizing the oxidation conditions to obtain a specific disulfide connectivity.

Si conotoxin was dissolved in H_2O/acetonitrile (1 : 1) and a solution containing 2.2 equiv. of NCS was added. The mixture was then shaken for 15 min and subsequently lyophilized. The linear peptide was completely consumed, and two major products were formed with the same mass in a ratio 1 : 4 (Fig. 2). The mass corresponds to the fully oxidized peptide and therefore two cyclic peptides with different disulfide connectivities were formed (**6a** and **6b**). This result shows that NCS can be used to efficiently oxidize disulfide-rich peptides in aqueous oxidative folding strategies. Moreover, NCS oxidation is completed in 15 min whereas air oxidation, the common approach in oxidative folding strategies, is time-consuming. Thus, aqueous NCS oxidation allows rapid screening of oxidation conditions.

Conclusions

Here we demonstrated the feasibility of using NCS for peptide disulfide bond formation under aqueous conditions. Highly efficient disulfide bond formation with NCS was achieved using H_2O or H_2O/acetonitrile mixtures and a slight excess of NCS. Aqueous NCS disulfide bond formation significantly extends the applicability of the NCS method because it overcomes the limitations of solid-phase disulfide formation and can be used in oxidative folding. Moreover, aqueous NCS oxidation is a powerful addition to the disulfide forming reagent repertoire.

Acknowledgements

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Notes and references

‡ Met was found not to be compatible with NCS in solution. When Met is present we recommend using NCS oxidation on the solid-phase.

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