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Immobilized *N*-Chlorosuccinimide as a Friendly Peptide Disulfide-Forming Reagent

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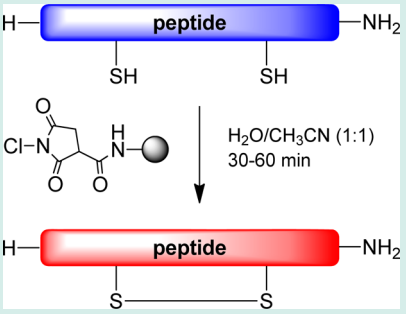
Supporting Information

ABSTRACT: A novel immobilized *N*-chlorosuccinimide resin was developed for peptide disulfide bond formation in combinatorial libraries. The resin is prepared in a simple two-step process from commercial starting materials. Disulfide formation is initiated by adding a peptide solution to the resin, and excess reagent is removed by a convenient filtration upon completion of disulfide formation. Completion of disulfide formation is rapid and clean, as demonstrated by the oxidation of a small nonapeptide library. This immobilized reagent allows a wider scope for the use of *N*-chlorosuccinimide-based disulfide formation in combinatorial chemistry.



KEYWORDS: *disulfide bond, disulfide formation, cysteine, thiol, NCS, oxidation, supported reagent, immobilized reagent*

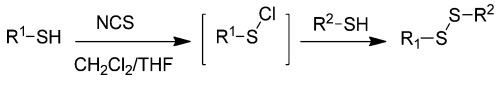
Disulfide bonds are key structural features of many conformationally constrained natural or synthetic peptides.[1] These bonds confer structural rigidity, which leads to peptides with strong binding and high selectivity.[2] In nature, this is illustrated by the highly potent disulfide-containing peptide toxins of cone snails, spiders, scorpions, and snakes.[3] Recently, disulfide-containing peptides have become available as therapeutics for a range of conditions, including the treatment of neuropathic pain with ziconotide and of chronic idiopathic constipation and irritable bowel syndrome with constipation in adults with linaclotide.[4−6] In addition, more than five disulfide-containing peptides are currently in various stages of clinical trials, thus indicating the increasing relevance of disulfide-containing therapeutics.[7]

New reagents and methods are needed to facilitate access to a wide range of disulfide-containing peptides. In this regard, we recently introduced *N*-chlorosuccinimide (NCS) as a highly efficient peptide disulfide-forming reagent. Initially, we used NCS to form a disulfide bond between Cys and trimethoxythiophenol to make the Cys protecting group *S*-Tmp.[8] The reaction between the thiol and NCS proceeds by the formation of a sulfenyl chloride, which is highly reactive toward other thiols. In this regard, we observed a rapid, clean, and efficient reaction (Scheme 1).

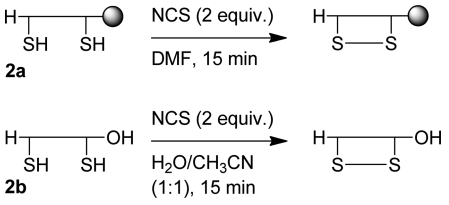
The efficiency of the mixed disulfide formation directed our attention to using NCS as an on-resin peptide disulfide-forming reagent. We reported efficient disulfide formation with a series of peptides within 15 min when using 2 equiv of NCS in DMF (Scheme 2a).[9] The formation of peptide disulfides in solution under aqueous conditions with NCS also proceeds with high efficiency (Scheme 2b).[10]

NCS was reported to be compatible with all amino acids except Trp and Met, which are prone to oxidation.[11] However, we showed that Trp is compatible with our conditions and Met can be used with on-resin disulfide formation by lowering the excess of NCS from 2.0 to 1.05 equiv. NCS can be used equally effectively in organic and aqueous media. These properties

Scheme 1. Mixed Disulfide Formation Mechanism Using NCS



Scheme 2. (a) On-Resin NCS Disulfide Formation and (b) Aqueous NCS Disulfide Formation in Solution



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allowed us to conclude that NCS is one of the most versatile peptide disulfide-forming reagents available. However, NCS is not entirely suitable for combinatorial libraries of disulfide-containing peptides. On-resin disulfide formation can be challenging because of the steric hindrance of reactions taking place within the resin matrix with protected peptides, and it frequently requires time-consuming optimization.[12] While peptide disulfide formation in solution with NCS is generally easier to perform, it requires preparative HPLC to remove the reagent after the reaction. To make NCS more amenable to combinatorial libraries of peptide disulfides, we chose to immobilize NCS on a suitable resin that can be removed after the reaction by a simple filtration.

The resin deemed most suitable for this purpose was the amphiphilic aminomethyl-ChemMatrix resin. ChemMatrix is a polyethylene glycol-based resin with excellent swelling properties in both organic and aqueous solvents.[13] These properties are crucial for an immobilized reagent as a resin with a high swelling facilitates the diffusion of the peptide into the resin matrix and consequently increases the reaction rate.[14] The primary amino group present on the resin allows for convenient anchoring of a reagent (Figure 1).

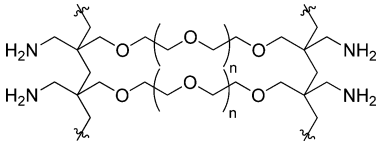
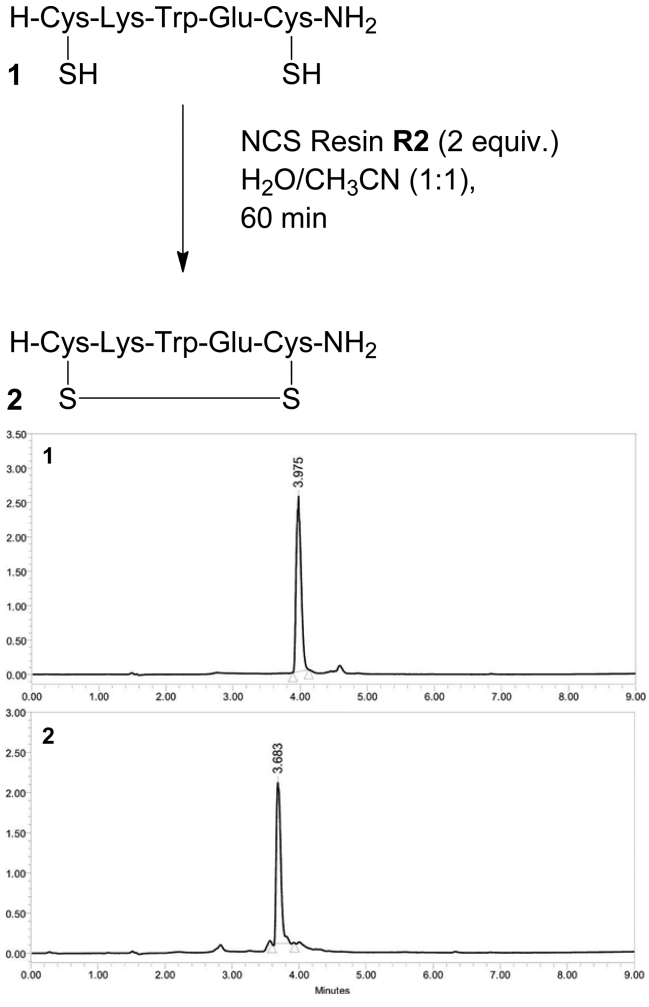


Figure 1. Aminomethyl-ChemMatrix resin structure.

Preparation of Immobilized NCS. Dioxo-pyrrolidine-3-carboxylic acid was immobilized on the aminomethyl-ChemMatrix resin using DIC. Thus, the carboxylic acid (2 equiv) was preactivated for 5 min using DIC (2 equiv) in DMF and subsequently reacted with the resin for 16 h at room temperature. After the reaction, the resin was washed and dried overnight in a vacuum oven. The increase in mass of the dry resin indicated a coupling efficiency of 99%. Following a procedure to prepare NCS and other N-chlorinated compounds, with minor modifications, the immobilized succinimide was chlorinated with trichloroisocyanuric acid (10 equiv) in a H_2O/AcOH mixture (2:1) for 6 h at room temperature (Scheme 3).[15] Upon completion of the reaction, the resin was washed and dried over a stream of nitrogen to give the immobilized NCS resin (**R2**). The resins were characterized by infrared (IR) spectroscopy (Supporting Information), and the loading of **R2** was assessed by UV spectroscopy (see below).

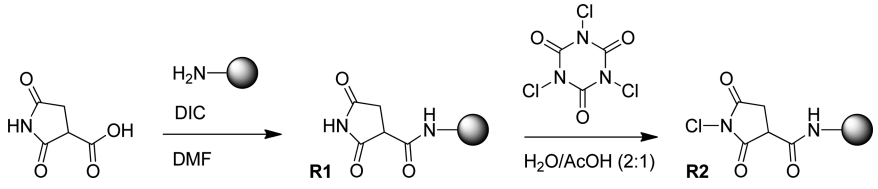
Peptide Disulfide Formation with Immobilized NCS. As the first model peptide, we chose a random pentapeptide (**1**) containing two Cys residues and three residues with different side chain functionalities, including oxidation sensitive Trp. The peptide was prepared on a Rink-Amide resin following general SPPS methods and subsequently cleaved from the resin (1 h, 95:2.5:2.5 TFA/TIS/H_2O). Peptide **1** was dissolved in a H_2O/CH_3CN mixture (1:1), a mixture suitable for a large proportion of deprotected peptides, and added to the immobilized NCS resin (2 equiv). The mixture was shaken for 60 min at room temperature and filtered to remove the resin. Characterization of the peptide by HPLC and liquid chromatography and mass spectrometry (LC–MS) showed complete formation of the disulfide (**2**) with high purity with all the starting material consumed (Scheme 4).

Scheme 4. Oxidation of Peptide 1 with NCS Resin



Following these experiments, a small library of biologically active nonapeptides based on vasopressin was prepared.[16] The linear peptides were synthesized on a Rink-Amide resin and subsequently cleaved. The peptides were oxidized with immobilized NCS using the same procedure that was described for peptide **1**. First, we oxidized vasopressin in H_2O, H_2O/CH_3CN (3:1), H_2O/CH_3CN (1:1), and H_2O/CH_3CN (1:3)

Scheme 3. Preparation of NCS Resin R2



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mixtures, and CH_3CN to determine the optimal solvent for oxidation with the NCS resin. The peptide solutions were added to the NCS resin (2 equiv). The mixture was shaken for 30−60 min at room temperature and filtered to remove the resin. Characterization of the peptides by HPLC and LC−MS showed that the best result was obtained with the H_2O/CH_3CN (1:1) mixture. We subsequently used this solvent mixture for the oxidation of the other library members. Analysis of the nonapeptides showed formation of the disulfides with high purity (Table 1).

Table 1. Oxidation of the Nonapeptide Library Using NCS Resin (2 equiv)

peptide	sequence	solvent	purity (%)
vasopressin	H-CYFQNCPRG-NH_2	H_2O	86
vasopressin	H-CYFQNCPRG-NH_2	H_2O/CH_3CN (3:1)	90
vasopressin	H-CYFQNCPRG-NH_2	H_2O/CH_3CN (1:1)	91
vasopressin	H-CYFQNCPRG-NH_2	H_2O/CH_3CN (1:3)	78
vasopressin	H-CYFQNCPRG-NH_2	CH_3CN (not soluble)	–
phenypressin	H-CFFQNCPRG-NH_2	H_2O/CH_3CN (1:1)	90
isotocin	H-CYISNCPIG-NH_2	H_2O/CH_3CN (1:1)	92
glumitocin	H-CYISNCPQG-NH_2	H_2O/CH_3CN (1:1)	86

In summary, immobilized NCS is an attractive and simple-to-use disulfide-forming reagent. Disulfide formation with high purity was achieved in a short time at room temperature, and the workup consists of a simple filtration. Given the simple protocol and straightforward removal of the immobilized reagent, we consider immobilized NCS to have great potential in combinatorial libraries of disulfide-containing peptides.

EXPERIMENTAL PROCEDURES

Analytical Methods. Analytical HPLC was conducted on a Waters instrument comprising a separation module (Waters 2695), an automatic injector, a photodiode array detector (Waters 2998), and a system controller (Empower login), with an Xbridge BEH130 C18 reversed-phase analytical column (4.6 mm × 100 mm, 3.5 μm). UV measurements were recorded at 254 and 220 nm, and linear gradients of acetonitrile (0.036% TFA) into water (0.045% TFA) over 8 min were used at a flow rate of 1.0 mL/min and a run time of 11 min.

LC−MS was conducted on a Waters Micromass ZQ spectrometer using a SunFire C18 analytical reversed-phase HPLC column (2.1 mm × 100 mm, 5 μm). Linear gradients of acetonitrile (0.07% formic acid) into water (0.1% formic acid) over 8 min were used at a flow rate of 1.0 mL/min and a run time of 11 min.

IR spectra were recorded in potassium bromide pellets on a Thermo Nicolet Nexus FT IR spectrometer. The sample was crushed using a mortar and pestle and mixed with KBr prior to being pressed into a pellet and subsequently analyzed.

UV measurements were recorded using a quartz cuvette in a H_2O/CH_3CN (1:3) mixture on a Shimadzu UV-2501PC UV−vis spectrometer at a wavelength of 300 nm.

Preparation of Immobilized NCS. Aminomethyl-ChemMatrix resin (3 g, 1.74 mmol, 1 equiv, 0.58 mmol/g) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min), a TFA/CH_2Cl_2 (1:99) mixture (5 × 1 min), CH_2Cl_2 (5 × 1 min), a DIPEA/CH_2Cl_2 (5:95) mixture (5 × 1 min), CH_2Cl_2 (5 × 1 min), and DMF (5 × 1 min). 2,5-Dioxo-pyrrolidine-3-carboxylic acid (0.75 g, 5.22 mmol, 3 equiv) was preactivated for 5 min using DIC (0.81 μL, 5.22 mmol, 3 equiv) in DMF. The preactivated mixture was added to the resin and the mixture shaken for 16 h at room temperature. The resin was washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min) and dried overnight in a vacuum oven at 40 °C. Resin **R1** was obtained with a 99% coupling efficiency (3.24 g of resin, 1.68 mmol).

Resin **1** (0.5 g, 0.29 mmol, 1 equiv) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min), DMF (5 × 1 min), and a H_2O/AcOH (2:1) mixture (5 × 1 min). Finely powdered trichloroisocyanuric acid (0.68 g, 2.9 mmol, 10 equiv) was added to a suspension of resin **1** in a H_2O/AcOH (2:1) mixture (20 mL), and the mixture was shaken for 6 h in the dark. The resin was washed with a H_2O/AcOH (2:1) mixture (5 × 1 min), DMF (5 × 1 min), and CH_2Cl_2 (5 × 1 min) and then dried under a stream of nitrogen. Immobilized NCS resin (**R2**) was obtained with a loading of 0.55 mmol/g (0.52 g).

Determination of the Loading of Immobilized NCS (R2). In our previous study, we observed the efficient reaction of 2,5-dimethoxythiophenol with another thiol to form a mixed disulfide.[8] In this case, we use the reaction between NCS and an excess of 2,5-dimethoxythiophenol to make a calibration curve to determine the loading of the resin based on the formation of 2,5-dimethoxyphenyl disulfide using UV spectroscopy at 300 nm.

Calibration Curve. For the 2,5-dimethoxythiophenol stock solution, 2,5-dimethoxythiophenol (68.1 mg, 400 μmol) was dissolved in 10 mL of a H_2O/CH_3CN (1:3) mixture in a volumetric flask. For the NCS stock solution, NCS (13.4 mg, 100 μmol) was dissolved in 10 mL of a H_2O/CH_3CN (1:3) mixture in a volumetric flask. For the reaction between 2,5-dimethoxythiophenol and NCS (100 μM), the 2,5-dimethoxythiophenol stock solution (100 μL), the NCS stock solution (100 μL), and the H_2O/CH_3CN (1:3) mixture (1 mL) were added to a 10 mL volumetric flask. The mixture was shaken for 30 min and subsequently diluted to 10 mL with a H_2O/CH_3CN (1:3) mixture. The absorbance was measured at 300 nm. The mixture was diluted to concentrations of 75, 50, 25, and 12.5 μM, and the absorbance (300 nm) was measured. This entire experiment was performed in triplicate, and a calibration curve was prepared (Figure 2).

Immobilized NCS resin (10 mg, maximum of 0.58 mmol/g) was placed in an Eppendorf cup, and the 2,5-dimethoxythiophenol stock solution (1 mL) was added. The mixture was shaken for 30 min; 100 μL of this mixture was diluted to 10 mL

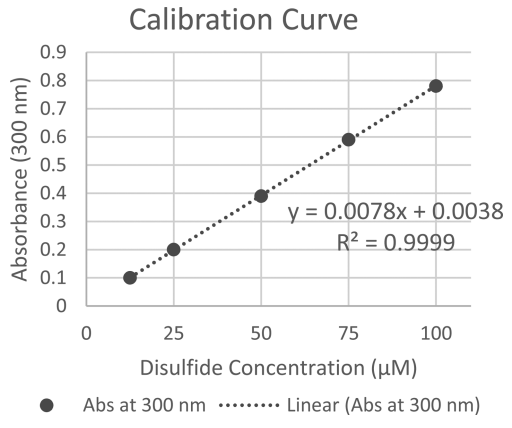

Figure 2. Calibration curve for the determination of resin loading.

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with a H_2O/CH_3CN (1:3) mixture (1 mL) in a volumetric flask, and the absorbance was measured at 300 nm [on the basis of the loading of the starting resin (0.58 mmol/g), the maximal concentration of disulfide would be 58 μM]. An absorbance of 0.43 absorbance unit was measured, which corresponds to a loading of 0.55 mmol/g.

General Procedure for Immobilized NCS Oxidation. The peptide (1 equiv) was dissolved in a H_2O/CH_3CN (1:1) mixture. The solution was added to immobilized NCS (**R2**) (2 equiv) and the mixture shaken for 30−60 min at room temperature. The mixture was filtered to remove the resin, and the filtrate was analyzed by HPLC and LC−MS.

■ ASSOCIATED CONTENT

Supporting Information

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

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AcOH, acetic acid; DMF, dimethylformamide; DIC, diisopropylcarbodiimide; HPLC, high-pressure liquid chromatography; NCS, *N*-chlorosuccinimide; TFA, trifluoroacetic acid; TIS, triisopropylsilane; *S*-Tmp, trimethoxyphenyl thio; SPPS, solid-phase peptide synthesis

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