Novel Disulfide Formation Strategies in Peptide Synthesis

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NOVEL DISULFIDE FORMATION STRATEGIES IN PEPTIDE SYNTHESIS

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Thesis Doctoral

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Dirigida y revisada por:

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(Universitat de Barcelona)

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This work is dedicated to my mother who taught me the value of hard work & my father who was always there in difficult times

It doesn't matter how beautiful your theory is,
it doesn't matter how smart you are.
If it doesn't agree with experiment,
it's wrong.

Richard P. Feynman

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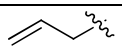
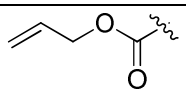
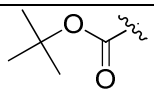
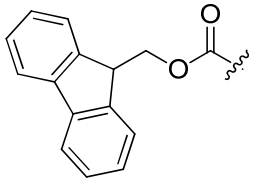
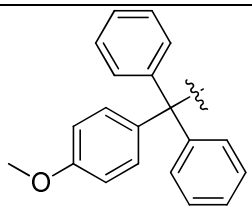
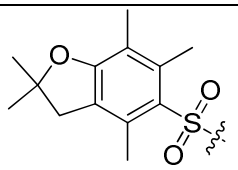
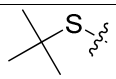
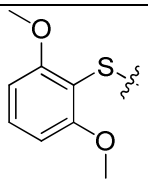
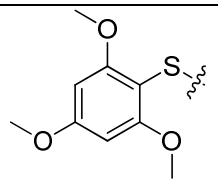
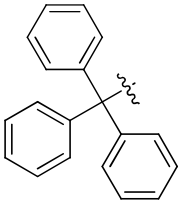
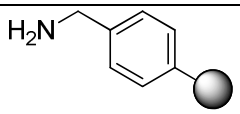
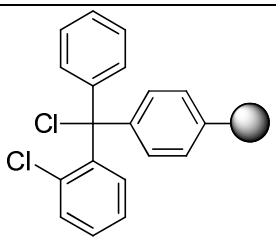
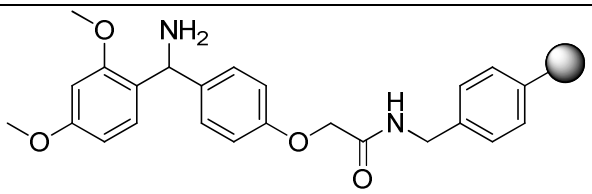
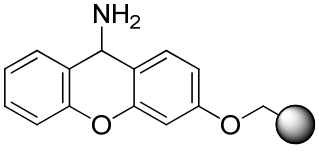
Annex 1. Peptide Chemistry Molecular Toolbox

Amino Acid Protecting Groups

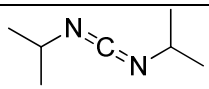
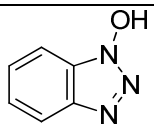
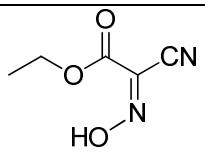
Abbreviation	Name	Structure
All	allyl	
Alloc	allyloxycarbonyl	
Boc	*tert*-butyloxycarbonyl	
Fmoc	9-fluorenylmethoxycarbonyl	
Mmt	4-methoxytrityl	
Pbf	2,2,4,5,7-pentamethyl-dihydrobenzofurane-5-sulfonyl	
*S*tBu	*tert*-butylthio	
S-Dmp	2,6-dimethoxyphenylthio	

S-Tmp	2,4,6-trimethoxyphenylthio	
Trt	trityl	

Resins

Name	Structure
Aminomethyl resin	
2-chlorotrityl chloride resin	
Rink amide resin	
Sieber amide resin	

Coupling Reagents and Additives

Abbreviation	Name	Structure
DIC	*N,N'*-diisopropylcarbodiimide	
HOBt	1-hydroxybenzotriazole	
Oxyma	Ethyl- 2-cyano-2-(hydroxyimino) acetate	

Annex 2. Abbreviations

Ac	acetyl
ACN	acetonitrile
Dap	2,3-diaminopropionic aid
DCM	dichloromethane
DIPEA	*N,N*-diisopropylethylamine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DTT	dithiothreitol
ESI	electron spray ionization
equiv	equivalent
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectrometry
LCMS	liquid chromatography mass spectrometry
MW	molecular weight
m/z	mass over charge ratio
NCS	*N*-chlorosuccinimide
NMM	*N*-methylmorpholine
NMR	nuclear magnetic resonance
PGA	penicillin G acylase
SPPS	solid-phase peptide synthesis
THF	tetrahydrofuran
TIS	triisopropylsilane
TLC	thin layer chromatography
t_R	retention time
UV	ultra violet
°C	degree Celsius
δ	chemical shift
J	coupling constant
ν	frequency

Annex 3. Publications and Conferences

Published

1 Tobias M. Postma, Matthieu Giraud and Fernando Albericio, Trimethoxyphenylthio as a Highly Labile Replacement for tert-Butylthio Cysteine Protection in Fmoc Solid Phase Synthesis. *Organic Letters* **2012,** *14* (21), 5468-5471

2 Tobias M. Postma and Fernando Albericio, *N*-Chlorosuccinimide, an Efficient Reagent for On-Resin Disulfide Formation in Solid-Phase Peptide Synthesis. *Organic Letters* **2013,** *15* (3), 616-619

3 Tobias M. Postma and Fernando Albericio, *N*-Chlorosuccinimide, an Efficient Peptide Disulfide Bond-Forming Reagent in Aqueous Solution. *RSC Advances* **2013,** *3* (34), 14277-14280

Publications Submitted

4 Tobias M. Postma and Fernando Albericio, Immobilized *N*-Chlorosuccinimide as a Friendly Peptide Disulfide Forming Reagent. (*Submitted*)

5 Tobias M. Postma and Fernando Albericio, Cysteine Pseudoprolines for Thiol Protection and Peptide Macrocyclization Enhancement. (*Submitted*)

6 Tobias M. Postma and Fernando Albericio, Novel Disulfide Formation Strategies in Peptide Synthesis. (*Submitted* – Minireview based on a modified version of the general introduction)

Conference Contributions

2013 – American Peptide Symposium - Waikoloa, Big Island, Hawaii - Oral presentation
Winner of the young investigator award for best presentation and was awarded a travel grant by the American Peptide Society.

2012 – European Peptide Symposium - Athens, Greece - Poster presentation

Thesis Outline

This thesis is a compendium of publications based on our research in peptide chemistry with a clear focus on the amino acid cysteine and its unique property to form disulfide bonds. This includes strategies to protect the thiol functionality and disulfide bond formation. The core of this manuscript is centered on the work we have published in peer-reviewed international scientific journals and on work we have recently submitted for publication in such journals. Tobias Maria Postma is the first author in all the chapters and the only experimentalist. All the experimental work and subsequent characterization described in this thesis was performed by Tobias Maria Postma alone. Only in **chapter 1** is there an additional author besides the thesis director Dr. Fernando Albericio, namely Dr. Matthieu Giraud from Lonza AG, who has contributed to this work by means of highly useful academic discussions on reducing agent-labile cysteine protecting groups. All chapters in this thesis are directly linked to the amino acid cysteine and the need for developing novel disulfide formation strategies to facilitate the formation of complex disulfide-rich peptides. The relationship of the chapters to one another and a very brief description of the contents of the chapters is described in this thesis outline.

Chapter 1 is based on our publication in Organic Letters which was the result of a fruitful collaboration with Dr. Matthieu Giraud from Lonza AG. This work stemmed from the need of finding a replacement for the difficult to remove *tert*-butylthio cysteine protecting group. This protecting group is very interesting in that it can be removed with mild reducing agents and is compatible with all other cysteine protecting groups. A novel highly reducing agent labile cysteine protecting group would allow the increased use of this type of protecting group and would aid in orthogonal strategies in the synthesis of disulfide-rich peptides. We introduced the protecting group 2,4,6-trimethoxyphenylthio as a replacement for *tert*-butylthio that could be removed in 5 minutes instead of many hours which was demonstrated in the synthesis of several peptides. The applicability of our protecting group 2,4,6-trimethoxyphenylthio in the synthesis of disulfide-rich peptides is illustrated by the commercialization of our protecting group by two companies.

Chapter 2 is based on our second publication in Organic Letters which was started by the observation that the mixed disulfide formation in the synthesis of 2,4,6-trimethoxyphenylthio protected cysteine, in **chapter 1**, was highly efficient with *N*-chlorosuccinimide (NCS). This observation lead to the hypothesis that NCS might be a good reagent for the on-resin disulfide formation of cysteine containing peptides. We

prepared peptides containing the protecting group developed in **chapter 1** and devised a simple protocol and tested it on several model peptides. Disulfide formation was clean and rapid in all cases and NCS was found to a very useful and versatile reagent. This publication was chosen to feature as a **SYNSTORY** in **SYNFORM** (2013/07) on trends in synthetic organic chemistry.

Chapter 3 is based on our third publication in RSC Advances and is the continuation of the work of **chapter 2**. Our results with NCS in the on-resin disulfide formation in **chapter 2** were very encouraging, which prompted us to continue with the development of NCS as a disulfide forming reagent. To broaden the scope of this reagent we investigated the use of NCS for disulfide formation in aqueous solution. The model peptides we used were found to be efficiently oxidized in aqueous solution and this proved a useful expansion to the applicability of NCS based disulfide formation.

Chapter 4 has been recently submitted for publication. This work was focused on developing NCS as a reagent for disulfide formation in combinatorial libraries because the methods employed in **chapters 2** and **3** were not suitable for this purpose. To remove the need for labourious removal of the oxidant following disulfide formation in combinatorial libraries we immobilized NCS to a suitable polymeric resin. The immobilized reagent simplified the workup by using a convenient filtration to remove the immobilized NCS. Disulfide formation using immobilized NCS was efficient and proved to be a suitable method for disulfide formation in a small combinatorial library.

Chapter 5 has been recently submitted for publication. We studied the use of cysteine pseudoproline dipeptides as building blocks in peptide synthesis where the pseudoproline acts simultaneously as a cysteine protecting group and macrocyclization enhancing moiety. Macrocyclization enhancement was not possible with the protecting group from **chapter 1** and following deprotection of the Cys pseudoproline methods from **chapters 2**, **3** or **4** can be used for disulfide formation. We observed unprecedented high acid lability of several cysteine pseudoprolines with deprotection times in the order of one to two hours. In addition, the cysteine pseudoprolines were found to enhance the macrocyclization of a peptide and thus shortened the reaction time significantly. This is a useful extension for the rarely used cysteine pseudoprolines.

Following these chapters there is a recapitulation of the conclusions from all preceding chapters which gives an overview of the results we obtained.

General Introduction

Importance of Disulfide Bonds

Cysteine (Cys) is unique among amino acids because of its capacity to form disulfide bonds in peptides and proteins.[1] A disulfide is a reversible covalent bond between the side chain thiols of two Cys residues. The disulfide bond causes conformational constraints, resulting in more rigid molecules that stabilize the peptide or protein fold.[2] Disulfide-rich peptides are prime examples of the attractive properties disulfide bonds can confer. For instance, peptides are highly prone to metabolic degradation via enzymatic digestion and typically have a short circulatory half-life in the range of a few minutes.[3] However, disulfides in disulfide-rich peptides can strongly increase metabolic and thermal stability. This is illustrated by the cyclotide Kalata B1, which contains three disulfide bonds. Kalata B1 is extracted from a plant by boiling in water, it is orally available, and it survives strongly proteolytic gastric juices.[4] This peptide becomes readily susceptible to degradation by several proteolytic enzymes upon reduction of the three disulfide bonds.

Cyclotides are disulfide-rich mini-proteins derived from plants. They contain between 28-37 residues with a head-to-tail cyclized backbone.[5-6] The main structural feature of cyclotides is the conserved Cys knot motif composed of three disulfide bonds, which, combined with the cyclized backbone, renders these molecules exceptionally stable. The therapeutic potential of cyclotides is vast because they show stability and a range of natural bioactivities, including antiviral and antimicrobial. They also serve as a scaffold on which to graft bioactive sequences.[7-10] The synthesis of cyclotides is readily performed with Boc SPPS, followed by cyclization and oxidative folding in solution.[11] The cyclization of the backbone is typically done with intramolecular native chemical ligation (NCL).[12] In this approach, two peptide fragments can be joined through the transthioesterification of the Cys thiol and a thioester, followed by an S-N acyl shift to form an amide bond.[13-15] The NCL technique has revolutionized the chemical synthesis of proteins and complex peptides and is an important application of Cys.[16]

The conformational stability of disulfide-rich peptides can lead to highly potent and selective binding of a disulfide-rich peptide to its target.[17] Disulfide-rich venom peptides from snakes, scorpions, cone snails, and spiders illustrate the diverse pharmacology of this class of molecules.[18-19] Conotoxin peptides are derived from cone snail venom. These peptides contain several disulfide bonds that have evolved into a

highly efficient and complex pharmacological cocktail, which the snail uses to immobilize prey via a harpoon-like radula.[20] Conotoxins act on neurological targets, such as ion channels and receptors. Given their wide diversity, selectivity, and potency, these molecules are of great interest from a therapeutic perspective.[21]

The three disulfide bond-containing ω–conotoxin ziconotide (Prialt) was introduced as the first conotoxin therapeutic for the treatment of acute and chronic pain (Figure 1a).[22] Linaclotide, another disulfide-rich peptide also with three disulfide bonds, is used for the treatment of chronic idiopathic constipation and irritable bowel syndrome with constipation in adults (Figure 1b).[23-24] Several disulfide-rich peptides are currently in clinical trials. This observation indicates the potential of disulfide-rich peptides as therapeutic agents.[20]

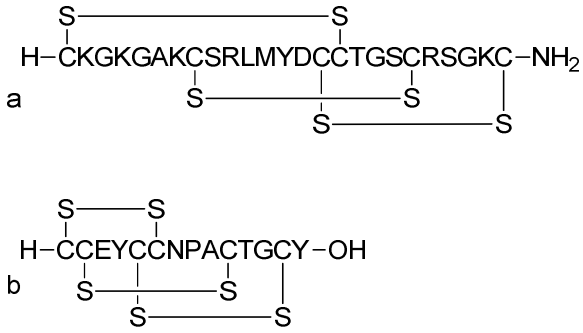


Figure 1: a) The sequence and disulfide connectivity of ziconotide and b) linaclotide

The importance of disulfide-containing peptides is not limited exclusively to therapeutic peptides. A wide range of applications involves the use of peptide disulfides, including bioconjugation,[25] β-sheet stabilization,[26] siRNA delivery,[27] increasing *in vivo* stability,[28] stabilization of peptide-based nanocarriers, and peptide backbone linkers.[29-30]

The increasing relevance of disulfide-rich peptides leads to the varied chemistry of peptide disulfide bond formation and the need for novel chemistries enabling the convenient and straightforward preparation of peptides with complex disulfide connectivity.[31]

Typically, three strategies can be used to synthesize disulfide-containing peptides: I) disulfides are prepared on solid-phase; II) a mixture of disulfide formation on the solid-phase followed by the subsequent disulfide formation in solution; and III) formation of all disulfides in solution.[32] With strategy I, the disulfide is formed on a resin with medium to low loading, and the kinetic phenomenon, referred to

as pseudo-dilution, favors intramolecular disulfide formation.[33] Disulfide-rich peptides can be regioselectively prepared using strategy I when appropriate orthogonal protection schemes are used. Strategy II can be used to form the first disulfide on the solid-phase, which restricts the conformational freedom, followed by formation of one or more disulfides in solution. As a result of the conformational restrictions conferred by the first disulfide, native conformations can be more easily obtained. Finally, strategy III, also called oxidative folding, involves the formation of all disulfides in solution and can work efficiently with natural peptide or protein sequences.[34] However, a mixture of peptides with distinct disulfide connectivities is frequently obtained and can thus be difficult to purify.[35]

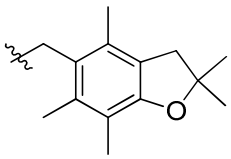
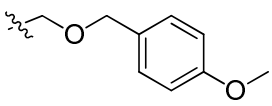
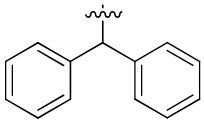
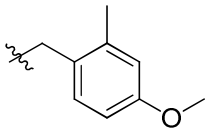
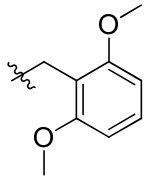
During the synthesis of Cys-containing peptides, care has to be taken to prevent or reduce side reactions at Cys.[36-37] This residue is prone to base-catalyzed racemization during coupling, and therefore coupling under neutral conditions using carbodiimide reagents is recommended.[38-39] Alkylation of the Cys thiol can be a problem during cleavage of the peptide from the resin with high concentrations of TFA.[40] In order to avoid such problems, cleavage time should be minimized in the presence of appropriate scavengers.

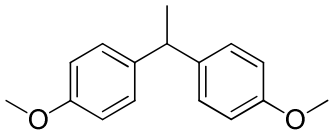
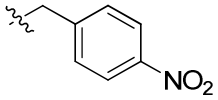
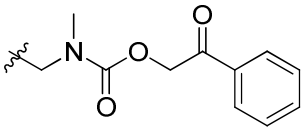
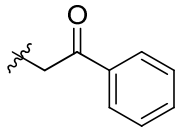
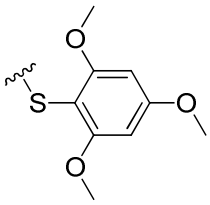
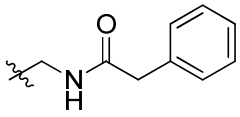
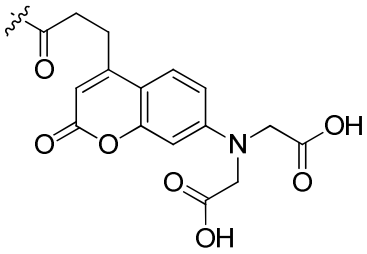
Disulfide Formation Strategies

The topic of this thesis is the development of novel disulfide formation strategies to facilitate the easier synthesis of complex disulfide-rich peptides. All following chapters will broach this subject and are linked to one another. In order to describe novel disulfide formation strategies we will first examine the current state of the art in the field op peptide chemistry in the following sections of the general introduction. Much has been written on disulfide formation strategies and to keep the work relevant to our research we will describe the strategies from 2006 until now and encompasses new methods with widely known reagents, novel reagents, new approaches, and the recently introduced Cys protecting groups. There are several excellent reviews of disulfide formation strategies, Cys chemistry, and protecting groups from 2006 and before.[32, 41-45]

Cys Protecting Groups

This section examines the novel Cys protecting groups that have emerged as a result of the need to synthesize increasingly complex peptides (Table 1). The applicability of several known Cys protecting groups has led to increased flexibility and milder deprotection conditions. Novel Cys protecting groups can introduce additional levels of orthogonality/compatibility, thus facilitating the regioselective preparation of complex peptides with a rich disulfide connectivity.

Cys Protecting Group	Deprotection Conditions	Stable to	References
Pbfm (1) 	**Acid-labile** 95% TFA/TES or I_2	Fmoc removal	[46]
MBom (2) 	**Acid-labile** 95% TFA	Fmoc removal	[47-48]
Dpm (3) 	**Acid-labile** 90-95% TFA	Fmoc removal	[48-50]
4MeO-2MeBn (4) 	**Acid-labile** 90-95% TFA	Fmoc removal	[49]
2,6diMeOBn (5) 	**Acid-labile** 90-95% TFA	Fmoc removal	[49]

Ddm (6) 	**Acid-labile** 10% TFA	Fmoc removal	[48-49]
pNB (7) 	**Reducing agent-labile** Zn/AcOH (in solution) or $SnCl_2$/HCl (on-resin)	Fmoc removal, HF, TFA	[51]
Pocam (8) 	**Reducing agent-labile** Zn/AcOH	Fmoc removal	[52]
Pac (9) 	**Reducing agent-labile** Zn/AcOH	Fmoc removal, TFA	[53]
***S*-Tmp (10)** 	**Reducing agent-labile** Thiols or trialkylphosphines	Fmoc removal	[54]
Phacm (11) 	**Enzyme-labile** Immobilized penicillin G acylase	Fmoc removal, TFA, HF	[55-56]
BCMACMOC (12) 	**Photo-labile** Light irradiation (λ 402 nm)	Fmoc removal (1% DBU/DMF, piperidine causes S-N acyl shift). TFA	[57]

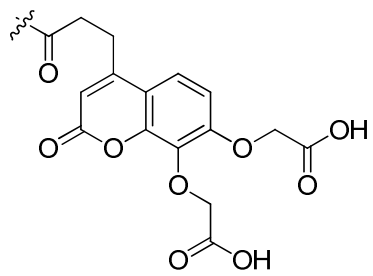
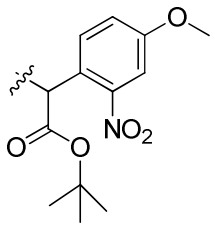
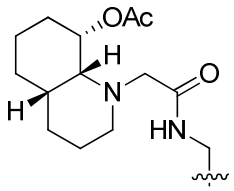
7,8BCMCMOC (13) 	**Photo-labile** Light irradiation (λ 325 nm)	Fmoc removal, TFA	[57]
C4MNB (14) 	**Photo-labile** Light irradiation (λ 325 nm)	Fmoc removal, TFA	[57]
Hqm (15) 	**Hydrazine-labile** 5% hydrazine (aq), I_2 or AgOAc	Fmoc removal, TFA, TFMSA (at 0°C)	[58]

Table 1: List of novel and recently reevaluated Cys protecting groups

Acid-labile Cys Protecting Groups

The acid-labile Cys protecting group 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-methyl (Pbfm) (**1**) was described as a replacement for Trt in Fmoc chemistry.[46] Trt-containing peptides are widely used and the protecting group is significantly hydrophobic. To reduce issues with regard to the hydrophobicity inherent to the Trt protecting group, Pbfm was introduced. This group can be easily removed with 1% TFA in the presence of 5% triethylsilane (TES) and also using a high TFA concentration in the total deprotection and cleavage from resin. The successful on-resin oxidative removal of Pbfm was performed using I_2 in various solvents with the model peptide oxytocin. Satisfactory results were obtained, thus demonstrating that this protecting group is a viable less hydrophobic alternative to Trt.

The 4-methoxybenzylozymethyl (MBom) (**2**) acid-labile Cys protecting group was introduced to suppress Cys racemization in Fmoc chemistry.[47] Cys is prone to racemization upon activation with phosphonium or uronium reagents, and carbodiimides are recommended as coupling reagents in order to reduce racemization.[39] However, carbodiimides are not equally efficient with respect to phosphonium and uronium reagents, and switching to another coupling system for Cys introduction is not practical, especially when using automated peptide synthesizers.[59] MBom has a similar acid-lability to Trt, and upon acidolysis it releases a molecule of highly reactive formaldehyde, which requires addition of the efficient formaldehyde scavenger methoxyamine hydrochloride.[60] The model peptide H-Gly-Cys-Phe-NH_2 was prepared with uronium activation and used to determine the levels of racemization with the protecting groups MBom, Trt and Acm. With conventional SPPS, the extent of racemization observed was as follows: MBom (0.4%), Trt (8.0%) and Acm (4.8%); microwave-assisted SPPS at 50°C: MBom (0.8%), Trt (10.9%) and Acm (8.8%); microwave-assisted SPPS at 80°C: MBom (1.3%), Trt (26.6%) and Acm (15.3). These results demonstrate the significant level of racemization suppression achieved with MBom in comparison to standard Cys protecting groups. The MBom protecting group has applications in peptide synthesis where Cys racemization poses a significant problem.

Diphenylmethyl (Dpm) (**3**), 4-methoxy2-methylbenzyl (4MeO-2MeBn) (**4**), 2,6-dimethoxybenzyl (2,6diMeOBn) (**5**) and 4,4'-dimethoxydiphenylmethyl (Ddm) (**6**) were recently introduced by our group as acid-labile Cys protecting groups.[49] We were searching for a replacement for the Mob group that is stable to low concentrations of TFA but readily removable with 95% TFA. The protecting groups Dpm, 4MeO-2MeBn, and 2,6diMeOBn were found appropriate for this purpose. Dpm was chosen as the most suitable due to the greatest cost effectiveness and ease of preparation. In contrast, Ddm was readily removed with 10% TFA and could be applied as a racemization suppressing replacement for the Trt protecting group.[48] Dpm was found to be fully compatible with the widely used acid-labile Cys protecting groups Trt and Mmt for use in Fmoc chemistry.[50] This was shown in an experiment with a peptide that contains three acid-labile protecting groups, namely Dpm, Trt and Mmt. The Dpm group was removed with the standard conditions used for total deprotection and cleavage from the resin (90-95% TFA + scavengers). Dpm was successfully used in the regioselective synthesis of the hinge fragment IgG1 and α-conotoxin ImI using either Mmt or Trt as compatible protecting groups. Racemization can be an issue with Cys protecting groups, and Dpm was compared with Trt using the model peptide H-Gly-Cys-Phe-NH_2.[48] The peptide was prepared with uronium activation, and the degree of racemization was determined. With conventional

SPPS, the extent of racemization was as follows: Dpm (1.2%) and Trt (8.0%); microwave-assisted SPPS at 50°C: Dpm (3.0%) and Trt (10.9%); and microwave-assisted SPPS at 80°C: Dpm (4.5%) and Trt (26.6%). These results show that Dpm can significantly reduce racemization compared to Trt and can be used as an alternative to Trt when Cys racemization is an issue. The commercially available Dpm group (Fmoc-Cys(Dpm)-OH) has applicability as an acid-labile protecting group that is fully compatible with Trt and Mmt. This is especially relevant given that Trt and Mmt are not fully compatible as acid-labile protecting groups and need diligent optimization to prevent overlap of the deprotection conditions. In addition, Dpm can be used to replace Trt because it reduces Cys racemization upon coupling.

As Cys racemization is inevitable during coupling, it is important to determine the extent of racemization with new Cys protecting groups. In a recent paper by Hibino et al., acid-labile Cys protecting groups MBom (**2**), Dpm (**3**), Ddm (**6**) and Trt were evaluated for their propensity to reduce Cys racemization.[48] The highest level of racemization was observed during coupling with uronium activation in the presence of base with Trt protected Cys (8%) (as described above). While the protecting groups MBom (**2**), Dpm (**3**) and Ddm (**6**) caused 0.4, 1.2 and 0.8% racemization respectively. When coupling with the recommended conditions for Cys, namely carbodiimide coupling without base, the levels of racemization are expected to be less. Taking this and the cost effectiveness into account, we recommend the use of the commercially available Dpm as the Cys protecting group of choice as it shows an acceptable low level of Cys racemization.

Reducing Agent-labile Cys Protecting Groups

The rarely used p-nitrobenzyl (pNB) (**7**) Cys protecting group was recently reevaluated for use in Boc chemistry.[51, 61] In contrast to Acm, pNB is completely stable to HF, thus making it a suitable alternative Cys protecting group for Boc-based peptide synthesis. The protecting group is removed using Zn/AcOH reduction in solution or $SnCl_2$/HCl reduction on-resin to give the p-aminobenzyl (pAB) protected Cys. Following reduction, the pAB group is oxidized using I_2 to give the disulfide. The stability of pNB to I_2 was tested, and no reaction was observed, thus indicating orthogonality to Acm. The protecting group pNB can be used in disulfide formation in conjunction with Boc chemistry and also in regioselective synthesis. However, its compatibility with other Cys protecting groups requires further research.

N-Methyl-phenacyloxycarbamidomethyl (Pocam) (**8**) was introduced as a new reducing agent-labile Cys protecting group for Fmoc chemistry.[52] The group was developed as a general Cys protecting group for use in conjunction with the condensation reactions of the thioester method.[62] Pocam is not acid-stable, and 4 h of TFA treatment at 4°C was required to achieve resin cleavage without significant Pocam removal. The protecting group is readily removed using Zn/AcOH in solution and is compatible with Acm. Orthogonality to Acm was demonstrated in a regioselective synthesis of SI α-conotoxin. Pocam is a Cys protecting group with limited applicability because of its instability to acid. Also, it is obsolete because of the Pocam replacement Pac, which was subsequently introduced by the same group.

Phenacyl (Pac) (**9**) was introduced as a reducing agent-labile Cys protecting group for general Fmoc SPPS and use in the condensation reaction of the thioester method.[53] The protecting group is stable to general Fmoc chemistry reaction conditions but shows some instability to strong acids (1 M TFMSA/TFA). Pac can be readily removed using Zn/AcOH in solution, and these conditions are compatible with Acm and Mob. There is partial lability to Acm and Mob deprotection conditions and therefore Pac must be removed before Acm or Mob. During peptide elongation, low yields were observed using DCC/HOBt. This result was partially attributed to imine formation; consequently, mildly basic coupling conditions using DIPEA are recommended. The protecting group was successfully applied to several peptides, including a regioselective synthesis of tachyplesin—a 17-residue peptide with 2 disulfide bonds—using Pac and Acm. Pac can be used as a compatible protecting group with Acm and Mob. However, some limitations need to be considered, such as the use of basic coupling conditions, which can promote Cys racemization.

The thiol-labile Cys protecting group trimethoxyphenylthio (*S*-Tmp) (**10**) was introduced by our group as a replacement for *tert*-butylthio (*S*tBu) for use in Fmoc chemistry.[54] The reducing agent-labile *S*tBu is orthogonal to all other Cys protecting groups.[63] Unfortunately, it is very difficult and sometimes impossible to remove *S*tBu using reducing agents.[64] *S*-Tmp can be easily removed in 5 min using a deprotection mixture containing dithiothreitol (DTT) (0.1 M NMM in 5% DTT/DMF). We used the *S*-Tmp protecting group to prepare several model peptides and oxytocin, and also for the regioselective synthesis of a two disulfide-containing SI α–conotoxin and the preparation of the 18-residue T22 peptide containing two disulfide bonds. *S*-Tmp is commercially available and given its ease of removal, it can be used in place of *S*tBu.

Enzyme-Labile Cys Protecting Group

Phenylacetamidomethyl (Phacm) (**11**) was pioneered by our group as an enzyme-labile Cys protecting group for both Fmoc and Boc SPPS.[65] Recently, the applicability of Phacm was broadened by exploring the versatility of immobilized enzyme biocatalysis for the deprotection of this protecting group.[55] The stability of Phacm to SPPS conditions and its lability profile are similar to those of Acm. In addition, Phacm can be removed by the enzyme penicillin G acylase (PGA) from *E. coli,* which offers mild deprotection conditions. Phacm can be removed with PGA immobilized on amino-acrylic resin. Immobilization of the enzyme allows capture of the supported PGA by a convenient filtration after deprotection, and the resin can be easily recycled. The biocatalytic deprotection conditions are mild, work under a broad range of conditions, prevent scrambling of disulfide bonds in disulfide-rich peptides, and are orthogonal to acid-labile protecting groups such as Trt. Disulfide formation can be initiated by addition of 10% DMSO to the deprotection mixture. Phacm deprotection with immobilized PGA is limited as it requires aqueous conditions, which can lead to solubility issues with certain peptide sequences. Phacm was used in the regioselective synthesis of the two disulfide-containing peptides T22 and RGD-4C. A more recent application of this Cys protecting group was in the synthesis of the marine natural product thiocoraline.[56] The commercial Cys protecing group Phacm is as an attractive alternative to Acm that does not require harsh deprotection conditions.

Photo-labile Protecting Groups

The related protecting groups 7-[bis(carboxymethyl)amino]coumarin-4-yl}methoxycarbonyl (BCMACMOC) (**12**), [7,8-Bis(carboxymethoxy)coumarin-4-yl]methoxycarbonyl (7,8BCMCMOC) (**13**) and R-carboxy-4-methoxy-2-nitrobenzyl (C4MNB) (**14**) were recently introduced as novel photo-labile Cys protecting groups.[57] They were used in a novel orthogonal scheme for peptide chemistry that employs chromatic orthogonality. Chromatic orthogonality is achieved when selective deprotection occurs by means of light irradiation with different wavelengths. Distinct wavelengths for photolysis of the protecting groups were observed for the protecting groups. For the photolysis of BCMACMOC, a wavelength of 402 nm was used, while for 7,8BCMCMOC and C4MNB irradiation with 325 nm initiated deprotection. Wavelength-selective deprotection was demonstrated using two model peptides in which either BCMACMOC and 7,8BCMCMOC, or C4MNB and BCMACMOC were used as chromatic orthogonal pairs. Efficient selective deprotection was observed in both cases, thus demonstrating the potential of

chromatic orthogonal schemes in peptide chemistry. However, coumarin-based protecting groups have a number of significant disadvantages, such as incompatibility with piperidine, a compound used for Fmoc deprotection. Piperidine causes an S-to-N Acyl shift in both BCMACMOC and 7,8BCMCMOC and requires 1% DBU/DMF for Fmoc removal. In addition, the N-terminus of the peptide requires acetylation prior to deprotection. These photo-labile protecting groups have demonstrated a novel orthogonal protection scheme that can be used in peptide chemistry. However, due to their drawbacks, they cannot be used in standard SPPS and thus require alternative protocols.

Hydrazine-labile Protecting Group

Hqm (1**5**), a hydroxyquinoline Acm derivative, is a hydrazine-labile Cys protecting group for use in Fmoc and Boc SPPS.[58] The Hqm protecting group is compatible with Acm, as it can be removed with 5% aqueous hydrazine at pH 8.5, to which Acm is stable (at pH 8.5 disulfide isomerization may occur and should be monitored). This was illustrated in the regioselective synthesis of human neutrophil defensin hNP2—a 29-residue peptide with three disulfide bonds—using the protecting groups Trt, Hqm and Acm. It should be noted that Acm deprotection reagents I_2 and AgOAc remove Hqm in about 30 min. Thus Hqm must be removed before Acm. Hqm is a versatile Cys protecting group that brings a new layer of orthogonality to disulfide formation strategies by using aqueous hydrazine as mild deprotection reagent.

Novel Disulfide Formation Strategies

The selenoxide reagent *trans*-3,4-dihydroxyselenolane oxide (DHS) was developed as a water-soluble disulfide formation reagent (Figure 2).[66]

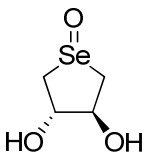
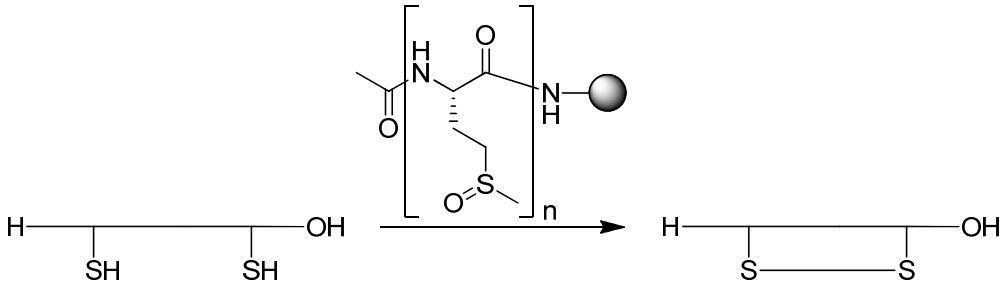


Figure 2: Structure of DHS

DHS is a strong and selective Cys oxidant that can be used to rapidly form disulfide bonds in proteins or peptides. The proposed oxidation mechanism follows a two-step process resulting in a disulfide bond and reduced DHS. DHS was used to study the folding pathway of bovine pancreatic ribonuclease A and described as a useful reagent for the determination of oxidative folding pathways in proteins.[67] Recently, DHS was successfully used for the oxidative folding of a three disulfide-containing recombinant hirudin analogue (CX-397) and the four disulfide-containing hen egg white lysozyme (HEL).[68-69] The oxidative folding was highly efficient and proceeded rapidly under aqueous conditions at neutral pH at room temperature. DHS is a strong stoichiometric oxidant for the rapid formation of disulfide bonds, and it has found applications in the study of oxidative folding in disulfide-rich substrates.

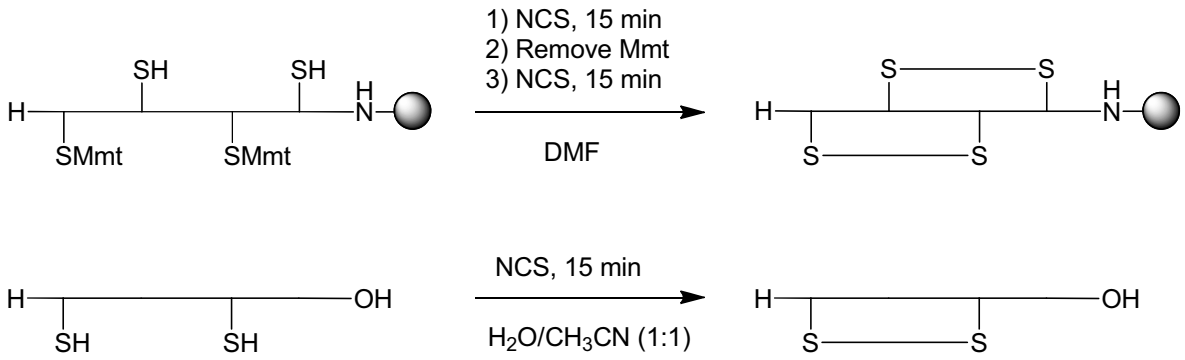
Supported methionine sulfoxide was described as a novel immobilized disulfide-forming reagent for Cys oxidation in solution or parallel peptide synthesis in solution.[70] The immobilized reagent can be readily prepared from the resin amino poly(ethyleneglycol)polyacrylamide (PEGA) by either consecutive Met couplings or oligomerization by using Met derived *N*-carboxyanhydrides and subsequent oxidation to Met sulfoxide using hydrogen peroxide (Scheme 1).



Scheme 1: Supported Met sulfoxide-mediated disulfide formation

Disulfide formation with supported Met sulfoxide proceeds in an analogous manner as DMSO oxidation. DMSO oxidation has one major drawback, namely the difficult removal of excess from the oxidized peptide. Supported Met sulfoxide does not have this disadvantage as the resin can be conveniently filtered off upon completion of disulfide formation. Several peptides were oxidized with immobilized Met sulfoxide, and efficient oxidations were reported using resins prepared by both N-carboxyanhydrides and with consecutive Met couplings. Efficiency of the supported Met sulfoxide was increased by longer Met sulfoxide chains, which raised the number of Met sulfoxide groups per gram of resin. Disulfide formation with immobilized Met sulfoxide was typically completed between 4 and 48 h using 5 equiv. of resin in an aqueous buffer. Supported Met sulfoxide has application as a disulfide-forming reagent that can be easily removed in general peptide synthesis and also in parallel peptide synthesis.

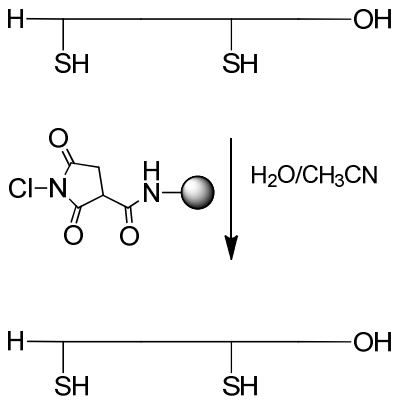
N-Chlorosuccinimide (NCS) was recently introduced by our group as a versatile and efficient disulfide-forming reagent for Fmoc SPPS.[71] Disulfide formation with NCS is efficient both on the solid-phase and aqueous solution.[72] The formation of disulfides was completed within 15 min, and the oxidized peptide was obtained in high purity. For on-resin disulfide formation, a solution of NCS (2 equiv.) in DMF is added to the resin and the mixture is shaken for 15 min. The excess reagent is easily removed by washing. NCS was found to be compatible with the oxidation prone Trp, and Met could be used by lowering the excess of NCS to 1.05 equiv. Additionally, the acid-labile Cys protecting groups Trt and Mmt were also found to be compatible. Several single disulfide-containing model peptides were efficiently oxidized with NCS. Subsequently, a regioselective synthesis of SI α–conotoxin using two consecutive on-resin NCS oxidations was performed (Scheme 2a).



Scheme 2: a) Regioselective on-resin NCS oxidation and b) NCS oxidation in solution

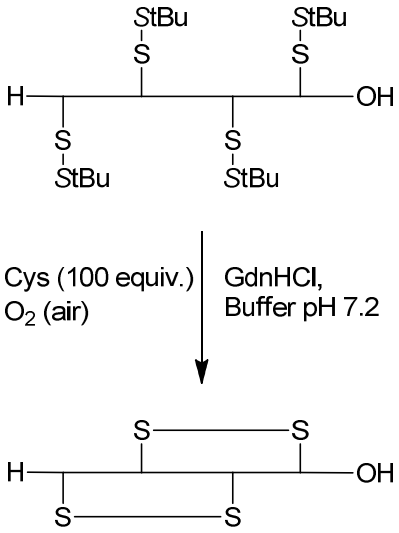
After on-resin NCS oxidation was established, the method was broadened in scope to include disulfide formation in aqueous solution. Excellent results were obtained using NCS under aqueous conditions with flexibility in the ratio of H_2O/CH_3CN (Scheme 2b). Typical conditions for disulfide formation were the addition of an NCS solution (2 equiv.) to the peptide under aqueous conditions, and disulfide formation was complete within 15 min. Thus, taking these results into account, NCS is currently one of the most versatile peptide disulfide-forming reagents available.

We continued with the development of NCS as a disulfide-forming reagent to broaden its applicability in combinatorial libraries.[73] NCS was immobilized on the versatile polyethylene glycol-based ChemMatrix resin. The immobilized reagent can be used in either organic or aqueous media and was found to efficiently form peptide disulfide bonds (Scheme 3). The immobilized reagent was readily removed by filtration upon completion of the reaction, thus making it applicable for the preparation of combinatorial libraries.



Scheme 3: Disulfide formation with immobilized NCS

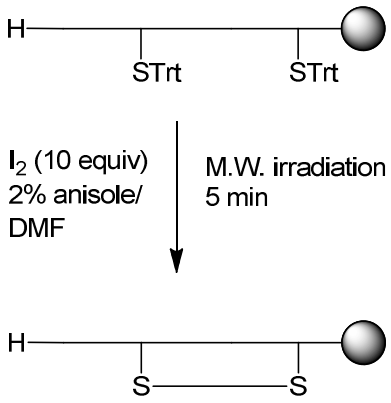
A useful new method for the oxidative folding of synthetic polypeptides was reported.[74] The method involves the use of *S*tBu-protected peptides in the presence of a large excess of Cys and chaotropic salts under buffered conditions (Scheme 4).



Scheme 4: Oxidative folding with *S*tBu-protected peptide

The formation of the folded disulfide-containing peptide is governed by a thiolate/disulfide exchange and leads to the thermodynamically favored product. To test the conditions, hu-TARC, a well-studied model peptide containing 71 residues and two disulfide bonds, was chosen. The peptide was oxidized using three methods: standard oxidative folding, and oxidative folding of the fully *S*-protected peptide (4x *S*tBu) and of the partially *S*-protected peptide (2x *S*tBu). Within 24 h, 77% conversion to the native disulfide was achieved with standard oxidative folding, with fully and partially *S*tbu-protected peptides showing equal or better performance. These strategies were successfully applied to the synthesis of a chemokine (hu-CCL1 24-96/I-309), large fragments of *P. falciparum*, and *P. berghei* circumsporozoite malaria protein. The oxidative folding using *S*tBu-protected synthetic peptides offers several advantages, such as the reduction of dimerization, as observed with the synthesis of the 102-residue fragment *Pf* CSP- (282–383), no premature oxidation of fully S-deprotected peptides, and fewer difficulties in the isolation of Cys-rich peptides. However, the *S*tBu protecting group is relatively stable and can be difficult to remove when sterically hindered by the sequence or fold.[64]

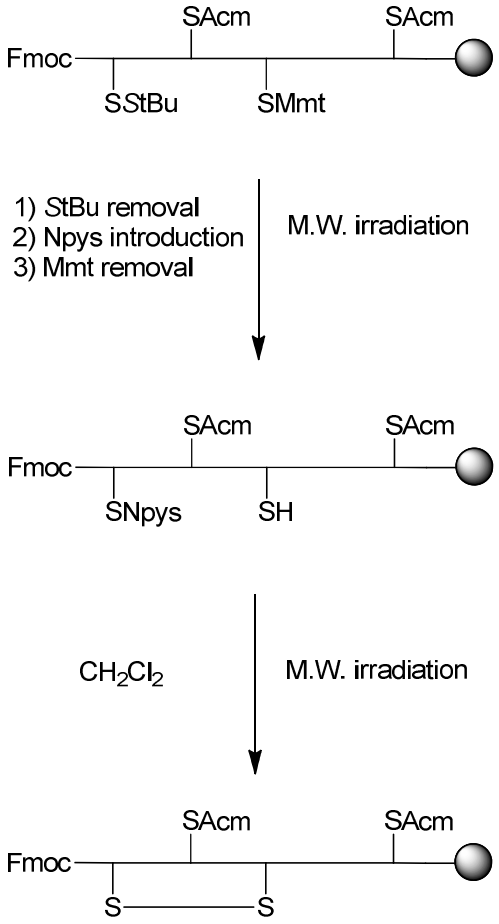
Microwave-assisted I_2 oxidation was reported as an efficient method for the formation of peptide disulfides.[75] In this simple procedure, a supported peptide with Trt-protected Cys is subjected to microwave assisted-oxidative disulfide formation. This method was successfully applied to the on-resin disulfide formation of the vasoactive cyclic peptide urotensin-II (Scheme 5).



Scheme 5: Microwave-assisted I_2 oxidation

In the microwave-assisted disulfide formation of urotensin-II, both the purity and the yield were superior to standard on-resin I_2 oxidation without microwave irradiation. However, microwave-assisted peptide synthesis with Cys-containing peptides can be hindered by increased Cys racemization. In this regard, alternative protocols are recommended if this becomes an issue.[48, 76] Quantitative disulfide formation was achieved in 5 min using this method. This observation demonstrates the applicability of this approach for the formation of disulfide-containing peptides. The current method seems to be limited to the formation of a single disulfide.

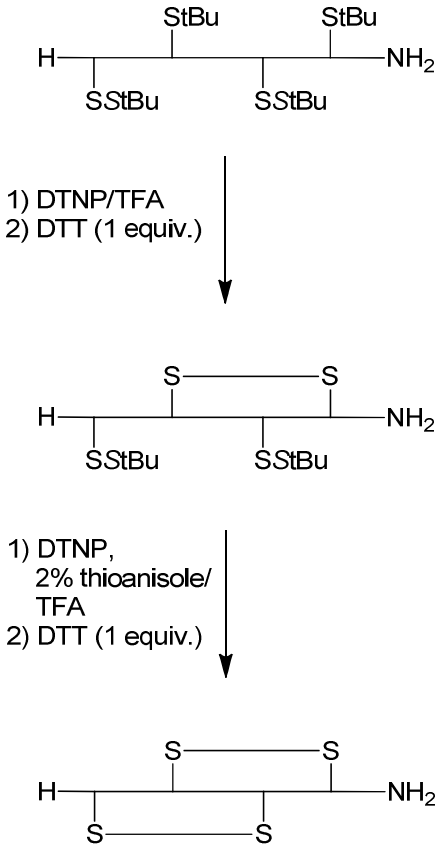
Highly efficient microwave-assisted on-resin disulfide formation was recently reported. In this case it used a displacement method to form the first intramolecular disulfide in the regioselective preparation of a two disulfide-containing peptide.[77] The displacement method requires the reaction of a free thiol with 2,2'-dithiobis(5-nitropyridine) (DTNP) to form an activated 5-nitropyridine sulfenyl (Npys) Cys. Subsequently, a highly acid-labile protecting group (Mmt) was removed and the resin was heated by microwave irradiation to form an intramolecular disulfide (Scheme 6).



Scheme 6: Microwave-assisted displacement method for disulfide formation

This method was successfully used in the synthesis of the two disulfide-containing α–conotoxin MII. The best results were obtained with the preparation of the first disulfide on-resin and the second disulfide in solution. The main advantages of this method are the high purity of the crude peptide and a significant reduction in the reaction time compared to performing the displacement on-resin at room temperature. The time required for completion of disulfide formation was dependent on whether the short or long disulfide loop was prepared first. The formation of the short loop was straightforward, taking 20 min at room temperature or 2 x 5 min with microwave heating. Conversely, the long loop required 3 days at room temperature or 6 x 5 min with microwave heating. The significant reduction in time required for disulfide formation illustrates the power of microwave heating using a displacement method. This method has application for the regioselective preparation of disulfide-rich peptides.

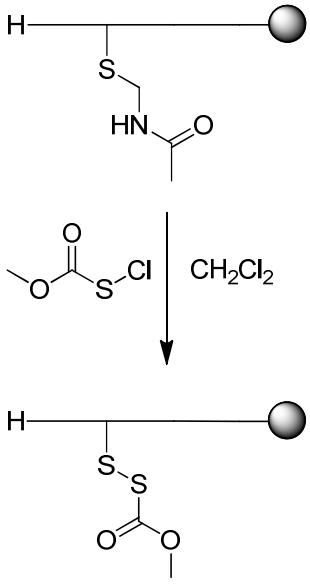
The formation of complex disulfide connectivities in the regioselective preparation of disulfide-rich peptides hinges on the compatibility of Cys protecting group pairs.[32] Novel methods for Cys protecting group removal can increase the utility of the protecting groups. A useful new method for the deprotection of several commercial Cys protecting groups using DTNP was recently described.[78] The deprotections were performed in TFA with or without the presence of thioanisole on the *bis*-protected model peptide oxytocin. The ease of deprotection in this system for the tested groups was Mob>tBu>StBu>Acm. The *S*tBu protecting group was notable in this case because thioanisole was needed to afford deprotection. Without thioanisole, this group was stable to DTNP. The bis-Npys protected peptide was observed upon removal of the Cys protecting groups with DTNP. A useful finding was that the addition of DTT (1 equiv.) led to nearly instantaneous quantitative disulfide formation. The applicability of the DTNP deprotection method was demonstrated in the regioselective synthesis of apamin. The choice of protecting groups was tBu and *S*tBu, and the first disulfide formation was initiated using DTNP/TFA to remove tBu in the absence of thioanisole. Following cyclization, StBu was removed by introducing thioanisole to commence *S*tBu removal (Scheme 7).



Scheme 7: Regioselective DTNP-mediated deprotection and oxidation of apamin

DTNP-mediated deprotection of several Cys protecting groups offers a mild alternative to the commonly used harsh or toxic reagents and has applicability in the synthesis of disulfide-containing peptides. A limitation of this approach is that in some cases the removal of both protecting groups in a bis-protected peptide can be slow, thus requiring an elevated temperature and increase in DTNP concentration.

A novel on-resin Cys protecting group transformation was recently reported for the Acm protecting group.[79] Acm is a widely used for direct I_2 oxidative disulfide formation. In addition, Acm can be removed using toxic heavy metal mercuric (II) salts. Acm can be converted to *S*-carbomethoxysulfenyl (Scm) in solution and can be reduced with DTT to the free thiol, and this mild method circumvents the need of toxic heavy metals.[80] The Scm group can also be used to form mixed-disulfides by the addition of a free thiol-containing peptide or small molecule. Solution-based Acm to Scm conversions are often difficult due to the difference in solubility of Acm- and Scm-containing peptides.[79] Such conditions require a large excess of Scm-Cl, which can sulfenylate both Trp and Tyr. To avoid these complications, an on-resin transformation of Acm to Scm was developed in which only a small excess of Scm-Cl was needed, which does not react with side-chain-protected Trp and Tyr (Scheme 8).



Scheme 8: On-resin Acm to Scm conversion

This transformation has some limitations, such as the possible reaction of the N-terminus with Scm-Cl to form acid-labile sulfenamides. It was found that reactions with immobilized Cys(Scm)-containing peptides

can be slow due to steric hindrance, and cleavage from the resin prior to Cys(Scm) reactions is recommended.

The widely used Acm protecting group is most often used for treatment with I_2 to afford oxidative deprotection and concomitant peptide disulfide formation.[81] During the removal of Acm, an excess of I_2 is used, and this needs to be rapidly quenched or absorbed in order to reduce side reactions following disulfide formation. A convenient one-step ether precipitation was recently introduced for simultaneous Acm removal, I_2 quenching, and peptide isolation. The peptide is oxidized in aqueous acetic acid (90-95%), and ice cold ether (9 equiv.) is added upon completion of the reaction. The mixture is cooled on dry ice for several minutes, centrifuged, and decanted to afford the crude peptide pellet. This method was successfully used on the nonapeptide vasopressin and human insulin-like peptide 3. Recovery rates of peptides using this method were comparable to those achieved with the conventional method, in which ascorbic acid is used as the quenching reagent. However, ether precipitation has the advantage that it requires fewer steps, is easy to apply, and has minimal side reactions.

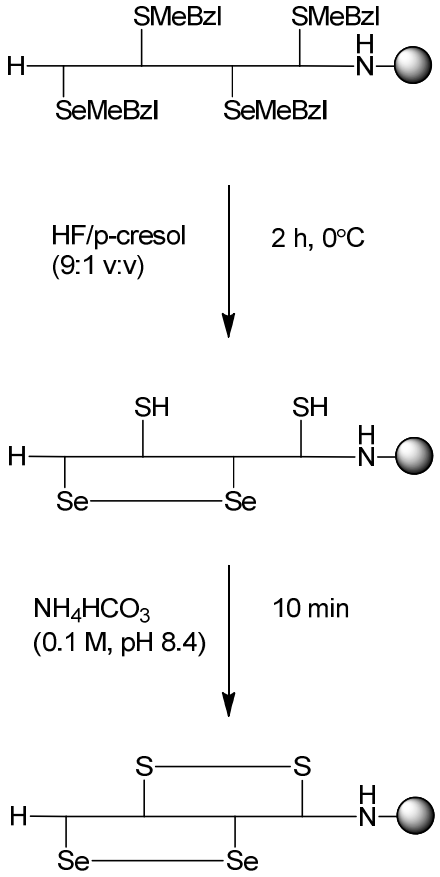
For large-scale I_2 oxidation of peptides, a new method was developed that uses a commercial anion exchange resin to quench the reaction.[82] Quenching of I_2 after the completion of disulfide formation is required in order to reduce side reactions associated with I_2 oxidation.[83] The commercial quaternary ammonium-based anion exchange resin Indion 830-S was described as an efficient I_2 quencher for use in large-scale disulfide formation. Standard I_2 quenching with ascorbic acid gave poorer results than quenching with the anion exchange resin. The quenching method is simple. Following disulfide formation, the resin is added and stirred for 30 min, followed by filtration of the resin. This method has applications mainly in the large-scale preparation of disulfide-containing peptides.

Selenocysteine and Disulfide Formation

The natural amino acid Sec, also referred to as the 21st proteinogenic amino acid, has gained popularity in peptide chemistry in recent years because of its close relation to Cys and its capacity to form diselenide bonds in an analogous fashion to disulfide bonds.[84] A comprehensive description of selenium chemistry and application in peptide chemistry is outside the scope of this review. However, due to the use of Sec in facilitating the formation of disulfide bonds, we will highlight a few notable recent examples.

The chemistry of selenium is similar to that of sulfur but with some clear distinctions such as the stronger acidity of the selenol group and a significantly lower redox potential of the diselenide bond in comparison to the disulfide bond.[85] The lower redox potential leads to the strongly favored formation of a diselenide instead of a mixed selenosulfide bond. This preferential diselenide formation has been exploited to direct disulfide formation in disulfide-rich peptides.[44]

An example of preferential diselenide formation was illustrated by the on-resin selenium-directed synthesis of conotoxin derivatives.[86] The linear sequences were synthesized via BOC SPPS using an HF-resistant SCAL linker with methylbenzyl (MeBzl)-protected Cys and Sec. Upon HF deprotection of the MeBzl protecting groups, the diselenide was spontaneously and quantitatively formed (Scheme 9). The subsequent oxidation of the Cys pair was rapid (<10 min at pH 8), demonstrating the utility of diselenide formation and direction of disulfide formation. An additional advantage of diselenide-directed disulfide formation is the preference to form the highly favored diselenide, thus making the determination of disulfide connectivity significantly less challenging. A useful property of Sec is that ^{77}Se can be used in NMR experiments to determine disulfide connectivity.[87] This is a clear advantage as determining disulfide connectivity can be difficult in certain peptides.

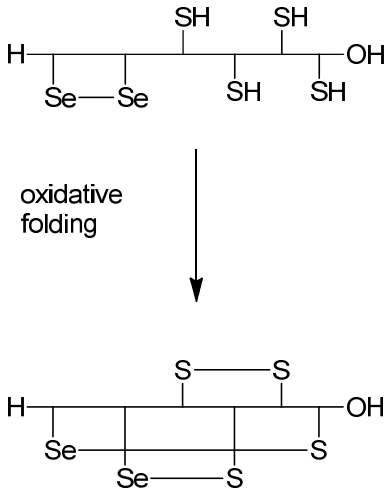


Scheme 9: On-resin Sec-directed disulfide formation

Intramolecular redox catalysis has recently emerged as an attractive and powerful method to greatly enhance the time to reach the native fold in disulfide-rich peptides and proteins. This method requires Sec as an intramolecular redox catalyst, by replacing a pair of Cys residues with Sec, to achieve the native fold by guiding the folding pathway to avoid kinetic traps.[88] Two very recent papers have demonstrated Sec redox catalysis in disulfide-rich peptides. The potential of this method in peptide chemistry has been briefly reviewed and highlighted by Craik.[89]

Selenium-based redox catalysis in peptide chemistry was demonstrated with the higher efficiency of selenoglutathione (GSeSeG) in comparison to glutathione (GSSG) as a redox auxiliary in the oxidative folding of bovine pancreatic trypsin inhibitor (BPTI).[90] This work led to the use of Sec as an intramolecular redox catalyst, which was applied to BPTI by the same group.[91] The most remarkable example used a

non-native diselenide connection to modulate the folding while avoiding kinetic traps, and finally a native connectivity was obtained with two less-favored Sec bonds (Scheme 10).



Scheme 10: Oxidative folding of BPTI using an intramolecular diselenide

A second example of this type of method was demonstrated by replacing a pair of Cys with Sec to guide disulfide formation in a conotoxin.[92] Native diselenide connectivity was used to enhance the folding speed over 130-fold compared to all-Cys containing conotoxins, without the need for additional reagents.

The increasing use of Sec in peptide chemistry and especially in combination with disulfide-rich peptides holds promise. The greater stability and reactivity of selenium makes it a useful tool in the peptide chemists' repertoire. Diselenide-containing peptides may offer therapeutic applications; however, it is yet to be determined whether diselenide bonds are biocompatible in vivo.

Perspectives on Disulfide Formation

Disulfide-rich peptides are becoming increasingly relevant as potential therapeutics and molecular probes. Constant development and innovation in Cys chemistry are crucial to speed up the synthesis and increase the yield of complex disulfide-containing peptides. Currently, the synthesis of disulfide-rich peptides is time-consuming, and new methods and reagents to accelerate this process will greatly benefit the preparation of peptides for drug research.

Natural combinatorial libraries show a remarkable hypervariability, illustrated by the great number of animal toxins. Such toxins provide us with millions of disulfide-rich toxin peptides. Unfortunately, the typical volume of venom is minute, and performing biological assays with the purified natural peptides is impractical and is highly time-consuming. Therefore, it is necessary to synthesize such compounds. Easy access to the synthetic preparation of such hypervariable sequences with control over disulfide connectivity would allow large-scale combinatorial library generation with which to tap into the enormous therapeutic potential of disulfide-rich peptides.

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Objectives

The overall objective of this thesis was to aid in the progression of the synthetic strategies to prepare complex disulfide-rich peptides.

- To study and develop a replacement for the difficult to remove cysteine protecting group *tert*-butylthio for use in regioselective synthesis of disulfide-rich peptides (chapter 1)

- To study and develop a novel on-resin disulfide formation strategy based on the reagent *N*-chlorosuccinimide (chapters 2)

- To study and develop a novel aqueous disulfide formation strategy based on the reagent *N*-chlorosuccinimide (chapters 3)

- To develop an immobilized derivative of *N*-chlorosuccinimide for the disulfide formation in combinatorial libraries (chapter 4)

- To study the rarely used cysteine pseudoproline dipeptide building blocks as protecting groups and their potential use as macrocyclization enhancing moieties (chapter 5)

Chapter 1

Trimethoxyphenylthio as a Highly Labile Replacement for *tert*-Butylthio Cysteine Protection in Fmoc Solid Phase Synthesis

Tobias M. Postma, Matthieu Giraud and Fernando Albericio

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Trimethoxyphenylthio as a Highly Labile Replacement for *tert*-Butylthio Cysteine Protection in Fmoc Solid Phase Synthesis

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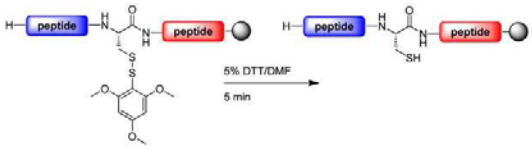
ABSTRACT



Trimethoxyphenylthio (*S*-Tmp) is described as a novel cysteine protecting group in Fmoc solid phase peptide synthesis replacing the difficult to remove *tert*-butylthio. *S*-Tmp and dimethoxyphenylthio (*S*-Dmp) were successfully used for cysteine protection in a variety of peptides. Moreover, both groups can be removed in 5 min with mild reducing agents. *S*-Tmp is recommended for cysteine protection, as it yields crude peptides of high purity.

Multiple disulfide containing peptides are ubiquitous in nature and therapeutically relevant because of their selective and potent bioactivities (e.g., conotoxins, cyclotides).[1,2] The synthesis of multiple disulfide containing peptides requires the use of orthogonal cysteine (Cys) protection strategies to ensure the correct disulfide connectivity.[3,4] Unfortunately, there is a lack of orthogonal cysteine protecting groups that can be used in routine SPPS under mild conditions.[5]

The concept of Cys protecting groups labile to mild reducing agents is highly promising due to orthogonality to other Cys protecting groups. The commercial Cys protecting group *tert*-butylthio (*S*tBu) is orthogonal to all other Cys protecting groups due to its mild deprotection conditions.[6] This protecting group can be removed with mild reducing agents (e.g., thiols or phosphines) and it is stable to piperidine, hence compatible with Fmoc/tBu peptide synthesis. However, owing to exceedingly long deprotection times (4–24 h), *S*tBu cannot be used in routine SPPS. As a result of the proximity of bulky protecting groups and sensitivity to certain amino acid sequences prone to folding, in some cases this protecting group has proven to be very difficult or even impossible to remove.[7,8] Thus, in previous studies on Linaclotide, a 14-residue peptide containing three disulfide bonds, we observed that *S*tBu groups on Cys2-Cys10 were not possible to remove.[7] Denis and Trifilieff used harsh conditions, heating a peptidyl resin to 135 °C for 24 h; however, they achieved only

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10.1021/ol3025499
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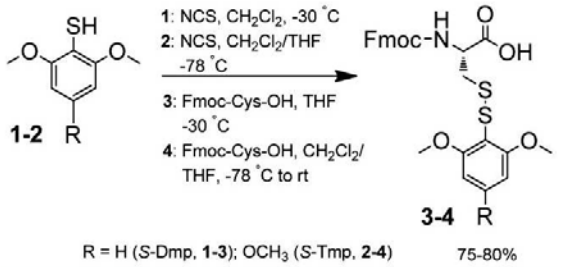
partial *S*tBu deprotection.[8] Additionally, reports of desulfurization of *S*tBu protected Cys to dehydroalanine, by means of prolonged exposure to reducing agents, illustrates the limitations of this protecting group.[9]

Given the importance of having a Cys protecting group removable by reducing agents to fulfill the orthogonal scheme for Cys and the significant limitations of *S*tBu, we have addressed novel reduction labile Cys protecting groups. As scaffolds for the preparation of mixed disulfides we initially studied phenyl and benzyl derivatives. The former were not stable to base while the latter were clearly not stable to acid (data not shown). The benzyl derivatives were discarded, and the phenyl group was modified to contain alkoxy groups on the 2,6 positions in order to study the balance between high lability to reducing agents and base stability (Scheme 1).

Scheme 1. Synthesis of Fmoc-Cys(*S*-Dmp)-OH (**3**) and Fmoc-Cys(*S*-Tmp)-OH (**4**)



The synthesis of Fmoc-Cys(*S*-Dmp)-OH and Fmoc-Cys(*S*-Tmp)-OH is shown in Scheme 1. 2,6-Dimethoxythiophenol was prepared following the literature, and 2,4,6-trimethoxythiophenol was synthesized with minor modifications of the same procedure.[10] The key reaction to the mixed disulfide containing Cys was inspired by a reaction used by Kraus and Jeon where *N*-chlorosuccinimide (NCS) reacts with thiophenol to form a highly reactive sulfenyl chloride.[11]

Fmoc-Cys(*S*-Dmp)-OH was prepared by forming a sulfenyl chloride from 2,6-dimethoxythiophenol. This sulfenyl chloride was subsequently added to a solution of Fmoc-Cys-OH, where nucleophilic attack on the sulfenyl chloride by Cys yields the mixed disulfide. The sulfenyl chloride of 2,4,6-trimethoxythiophenol was unstable at −30 °C and had to be formed in the presence of Fmoc-Cys-OH at −78 °C to obtain Fmoc-Cys(*S*-Tmp)-OH.

In order to compare the efficiency of *S*-Dmp and *S*-Tmp to *S*tBu, and SPPS compatibility, we prepared several model tripeptides (Figure 1). The model tripeptide Fmoc-Ala-Cys-Ala-NH_2 was already used in our laboratory to assess the Cys protecting group on resin. Cys residues

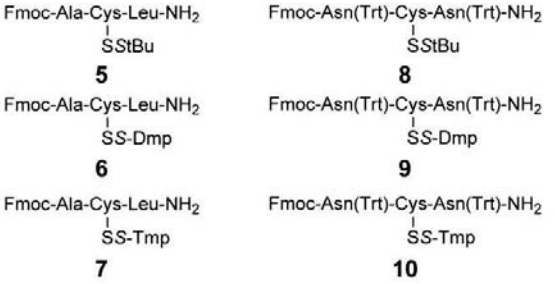


Figure 1. Tripeptides protected with *S*tBu, *S*-Dmp, and *S*-Tmp.

protected with *S*-Dmp, *S*-Tmp, and *S*tBu were incorporated in the model tripeptides by SPPS, using diisopropylcarbodiimide (DIC) and Oxyma Pure with 5 min of preactivation to prevent racemization of the Cys residue.[12]

Table 1. On-Resin Deprotection of Model Tripeptides[a]

peptidyl-resin	deprotection time (min) with BME	deprotection time (min) with DTT
5 (*S*tBu)	180	5% after 24 h
6 (*S*-Dmp)	5	5
7 (*S*-Tmp)	5	5
8 (*S*tBu)	360	2% after 24 h
9 (*S*-Dmp)	5	5
10 (*S*-Tmp)	5	5

[a] β-Mercaptoethanol (BME), Dithiothreitol (DTT). Deprotection conditions: 0.1 M NMM, BME (20%), or DTT (5%) in DMF.

The stability of the protecting groups to piperidine and trifluoroacetic acid (TFA), conditions used in routine SPPS, was evaluated. All protecting groups were found to be stable to 20% piperidine/DMF for 4 h, which is sufficient for routine applications. The peptides were cleaved from the resin with 95% TFA for 1 h at rt. The protecting groups were stable to these conditions.

Subsequently, the lability of these groups to reducing agents was studied. We found that the most efficient deprotection mixtures contained *N*-methylmorpholine (NMM) (0.1 M) and either the malodorous β-mercaptoethanol (BME) (20%) or nonmalodorous dithiothreitol (DTT) (5%) in DMF.[13] Both deprotection mixtures achieved quantitative removal of *S*-Dmp and *S*-Tmp from Fmoc-Ala-Cys(PG)-Leu-NH_2 model tripeptides in 5 min. In contrast, 3 h were required for quantitative *S*tBu removal using BME and practically no deprotection occurred with deprotection mixtures containing DTT (Table 1).

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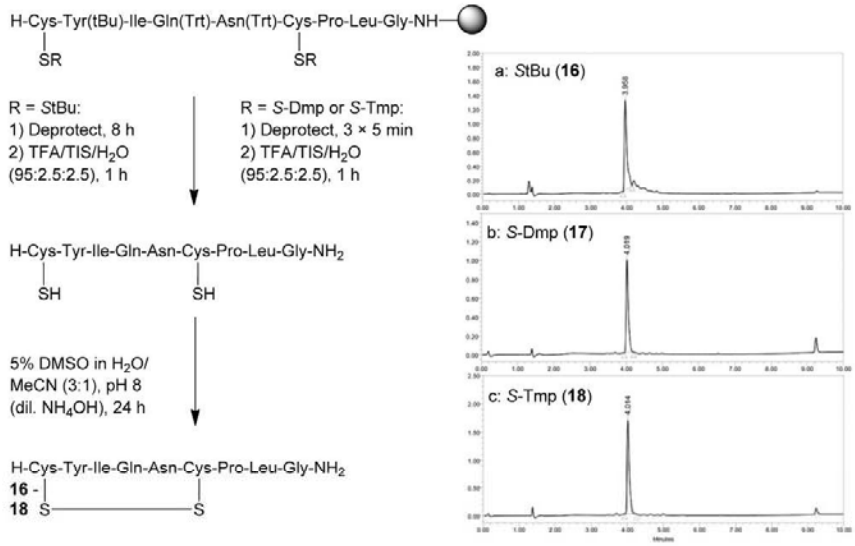
(13) No on-resin reduction was observed with TCEP in DMF and DMF/H_2O mixtures (low DMF solubility). We chose not to work with tributylphosphine to avoid possible desulfurization of Cys (ref 9).

Scheme 2. Oxytocin Synthesis from Linear Oxytocin Protected with *S*tBu (a), *S*-Dmp (b), and *S*-Tmp (c) on a Rink Amide AM Resin[a]



[a] *S*tBu removal: 0.1 M NMM in BME/DMF (1:4). *S*-Dmp/*S*-Tmp removal: 0.1 M NMM in DTT/DMF (5:95).

The deprotection efficiency for the removal of *S*tBu, *S*-Dmp, and *S*-Tmp, when flanked by bulky trityl (Trt) protecting groups, was studied. The model tripeptide Fmoc-Asn(Trt)-Cys(PG)-Asn(Trt)-NH_2 was used to determine whether bulky protecting groups influence deprotection times. The resin containing the peptides was treated with the BME deprotection mixture. The deprotection time for *S*tBu doubled, while those for *S*-Dmp and *S*-Tmp remained unchanged. On the other hand when the DTT mixture was used, both *S*-Dmp and *S*-Tmp were removed very gently in 5 min, while only 5% of *S*tBu was removed after 24 h. For subsequent on-resin deprotection, we applied 3 × 5 min treatments to ensure quantitative removal of *S*-Dmp and *S*-Tmp.

These promising results led us to examine a longer model peptide. For this purpose, we chose oxytocin,[14] an extensively studied nonapeptide containing two Cys residues and currently used as a drug to induce labor. A pair of Cys residues protected with *S*tBu, *S*-Dmp, or *S*-Tmp were incorporated into oxytocin. Treatment of the peptidyl-resin with 20% piperidine/DMF followed by cleavage with TFA/TIS/H_2O (95:2.5:2.5) led to the unexpected monodeprotection of *S*-Dmp (13%) and *S*-Tmp (8%). This process was not observed during the studies with the model tripeptides, and the mechanism of deprotection was unclear. However, we suspected that the monodeprotection was caused by a high concentration of TFA.

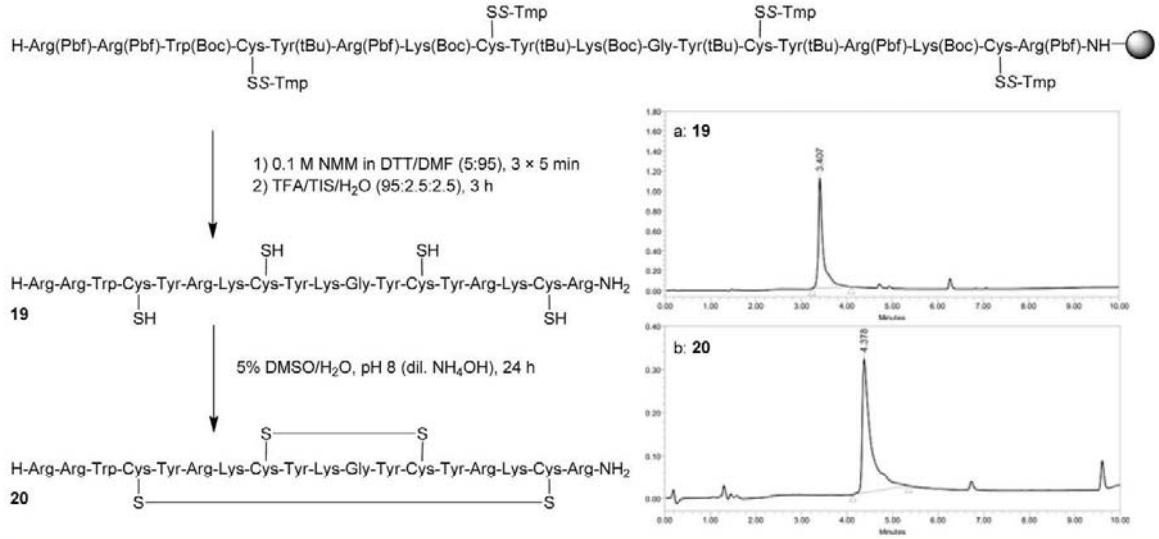
To confirm this notion, oxytocin was resynthesized with protecting group *S*-Dmp or *S*-Tmp on a Sieber Amide Resin. Previously, a Rink Amide resin was used. This support requires a cleavage mixture containing 95% TFA, whereas 1−5% TFA is sufficient to achieve peptide cleavage from Sieber Amide resin. The peptidyl-resin containing oxytocin protected with *S*-Dmp or *S*-Tmp was treated with 20% piperidine/DMF followed by cleavage with 1% TFA. Subsequent analysis showed no monodeprotection for either protecting groups, thereby corroborating the instability to high TFA concentrations. The partial instability of these protecting groups to high TFA concentrations is not detrimental for their use in peptide synthesis because in multiple disulfide containing peptides these groups are predominantly removed on-resin. Due to their deprotection mechanism, *S*-Dmp and *S*-Tmp must be removed first in orthogonal Cys protecting group strategies; otherwise the reducing agents required for deprotection would reduce the other disulfide bonds present. Hence, these protecting groups are removed on-resin prior to cleavage. Thus, in SPPS, the end of the synthesis involves removal of the Fmoc group, removal of the Cys protecting groups (*S*-Dmp/*S*-Tmp), and then either oxidation followed by cleavage or cleavage with subsequent oxidation in solution.

Linear oxytocin protected with *S*tBu, *S*-Dmp, or *S*-Tmp was deprotected on-resin (3 × 5 min, 0.1 M NMM in 5% DTT/DMF) for *S*-Dmp and *S*-Tmp, whereas 8 h (0.1 M NMM in 20% BME/DMF) were needed for *S*tBu deprotection (Scheme 2). The linear peptides were cleaved from the resin and oxidized in 5% DMSO in H_2O/CH_3CN (3:1) for 24 h. The peptides protected with *S*tBu, *S*-Dmp, and *S*-Tmp were obtained in 72%, 86%, and 94% purity after lyophilization. Given that the best results were obtained with *S*-Tmp, we focused on this protecting group.

To further demonstrate the applicability of *S*-Tmp, we synthesized the 18-residue T22 peptide containing 4 *S*-Tmp

(14) Viero, C.; Shibuya, I.; Kitamura, N.; Verkhratsky, A.; Fujihara, H.; Katoh, A.; Ueta, Y.; Zingg, H. H.; Chvatal, A.; Sykova, E.; Dayanithi, G. *CNS Neurosci. Ther.* **2010**, *16*, 138–156.

Scheme 3. Synthesis of T22 Using *S*-Tmp Protected Cys on a ChemMatrix Resin



protected Cys residues.[15] In a recent study we demonstrated that the T22 peptide folds into its native conformation by oxidative folding.[16] This peptide contains five arginine and three lysine residues, thus rendering it highly hydrophilic. We were unable to properly synthesize the peptide on a polystyrene based Rink Amide resin and required a hydrophilic polyethylene glycol based ChemMatrix resin for this purpose. Following peptide elongation, *S*-Tmp was removed on-resin (3 × 5 min, 0.1 M NMM, 5% DTT in DMF) and cleaved from the resin with TFA/TIS/H_2O (95:2.5:2.5) for 3 h to ensure complete deprotection of the five Pbf protecting groups (Scheme 3). The linear peptide was oxidized in solution using 5% DMSO in water at pH 8.0 for 24 h at rt and subsequently lyophilized to give crude T22 in 78% purity.

Both *S*-Dmp and *S*-Tmp are stable to base, SPPS coupling conditions, and a low concentration of TFA. In comparison to *S*tBu, *S*-Dmp and *S*-Tmp proved to be superior Cys protecting groups. They are labile to mild reducing agents and have rapid deprotection times of 5 min, which contrasts with the hours required for *S*tBu removal. No changes were observed in the deprotection times when the protecting group was flanked by bulky Trt groups. In concusion, we recommend the use of *S*-Tmp over *S*-Dmp because it showed greater stability, gave the purest product in the synthesis of oxytocin, and was successfully used in T22 synthesis.

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Supporting Information Available. Experimental details and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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Chapter 1

Supporting Information

Trimethoxyphenylthio as a Highly Labile Replacement for tert-Butylthio Cysteine Protection in Fmoc Solid Phase Synthesis

Tobias M. Postma, Matthieu Giraud and Fernando Albericio,

Organic Letters **2012**, *14*, 5468-5471

1-SI.1. General Procedures

Fmoc-amino acid derivatives, Fmoc-Rink Amide AM resin and 2-CTC resin were obtained from IRIS Biotech (Marktredwitz, Germany). Rink-Amide-Chemmatrix Low LOA was obtained from PCAS BioMatrix Inc. (Quebec, Canada). DIPEA, diisopropylcarbodiimide (DIC) and TFA were obtained from Aldrich (Milwaukee, USA). Oxyma was obtained from Luxembourg Industries Ltd. (Tel Aviv, Isreal). DMF, CH_2Cl_2, Et_2O, acetonitrile, DMSO and piperidine (HLPC grade) were obtained from SDS (Peypin, France). All reagents and solvents were used as received.

All non-aqueous reactions were performed under a nitrogen atmosphere using glassware that had been oven-dried overnight. Room temperature (rt) refers to ambient temperature. All temperatures below 0 °C are that of the external bath. Temperatures of 0 °C were produced and maintained with an ice/water bath. Temperatures below 0 °C were produced and maintained using an acetone/cardice bath.

Yields refer to spectroscopically and chromatographically pure compounds, except for peptides where yields refer to the area of the chromatographic product peak recorded at 220 nm. Reactions were monitored using thin layer chromatography (TLC) performed on commercially prepared aluminum plates pre-coated with Merck silica gel 60 F254 and visualized by quenching of UV fluorescence (ν_{max} = 254 nm) followed by staining with potassium permanganate. Flash column chromatography was carried out using SDS (Peypin, France) Silica (35-70 μm) under a positive pressure of compressed air.

Solid-phase syntheses were carried out manually in polypropylene syringed containing a polyethylene frit. Solvents and soluble reagents were removed by suction. Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min). Washings between deprotection and coupling were performed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Following the final coupling or deprotection the resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and dried under a stream of air.

Infrared (IR) spectra were recorded in potassium bromide pellets on a Thermo Nicolet Nexus FT IR spectrometer. Only absorption maxima (ν_{max}) of interest are reported in wavenumbers (cm^{-1}) with the following abbreviations: m, medium; s, strong; br, broad. Melting points were obtained on a Buchi B-540 melting point apparatus and are uncorrected.

Proton magnetic resonance (^{1}H NMR) spectra were recorded using a Varian Mercury-400 (400 MHz). Chemical shifts (δ_H) are quoted in parts per million (ppm) to the nearest 0.01 ppm downfield of trimethylsilane (δ_H = 0). Spectral data is recoded as follows: chemical shift, integration, multiplicity (s=singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad or a combination of these), coupling constants (*J*, measured in Hertz (Hz) and quoted to the nearest 0.1 Hz) and assignment. Carbon magnetic resonance (^{13}C NMR) spectra were recorded using a Varian Mercury-400 (100 MHz). Chemical shifts (δ_C) are quoted in parts per million (ppm) to the nearest 0.1 ppm downfield of trimethylsilane (δ_C = 0) and are referenced to the residual non-deuterated solvent peak as follows: $CDCL_3$, 77.36; δ_6-DMSO, 39.5 ppm.

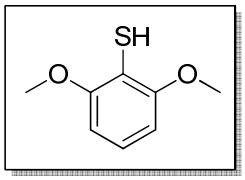
High resolution mass spectrometry (HRMS) measurements were recorded on Thermo Scientific LTQ-FT Ultra spectrometer. Mass values are quoted within the error limits of ±5 ppm mass units. ESI refers to the electrospray ionization technique.

Analytical high pressure liquid chromatography (HPLC) was carried out on a Waters instrument comprising a separation module (Waters 2695), automatic injector, photodiode array detector (Waters 2998) and 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^{-1} and a run time of 11 min.

LCMS was carried out 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^{-1} and a run time of 11 min.

1-SI.2. Characterization of Synthesized Compounds

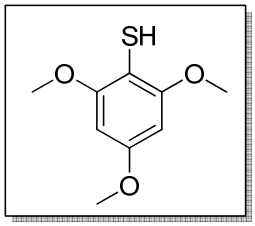
2,6-Dimethoxythiophenol (1)



To a mixture of 1,3-dimethoxybenzene (110 mL, 800 mmol) and n-butyllithium in hexane (500 mL, 800 mmol) at 0 °C was added a catalytic amount of *N,N,N′,N′*-tetramethylethylenediamine (1 mL, 6.7 mmol). The reaction mixture was allowed to warm to rt and stirred for 20 min to give a white suspension, to which was added dropwise a solution of elemental sulfur (23.1 g, 720 mmol) in toluene (600 mL, mild heating was needed to solubilize the sulfur in toluene). The reaction mixture was stirred at rt for 16 h and subsequently quenched by the addition of water (1 L). The aqueous layer was acidified with 1 M aqueous hydrochloric acid (800 mL) and the white precipitate was collected. The precipitate was recrystallized from methanol to yield the product as a crystalline while solid (102.14 g, 75%).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.06 (1H, t, *J* = 8.3 Hz, Ar C<u>H</u>), 6.56 (2H, d, *J* = 8.3 Hz, Ar C<u>H</u>), 4.04 (1H, s, Ar S<u>H</u>), 3.89 (6H, s, Ar OC<u>H</u>$_3$). **^{13}C NMR** (100 MHz, $CDCl_3$) δ_C 155.6, 125.2, 109.5, 104.0, 56.32. **IR** ν*max* (film/cm^{-1}) 2573 m, 1586 s, 1470 s, 1435 s, 1251 s. **mp** 83-84 °C. **LCMS** Observed $[M+H]^+$ 171.05 required $[M+H]^+$ 171.05. Data consistent with that reported in the literature.[1]

2,4,6-trimethoxythiophenol (2)

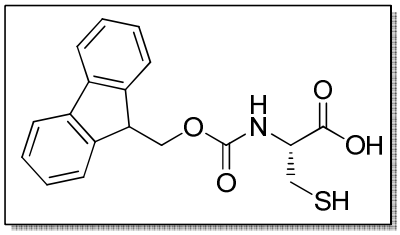


To a solution of 2,4,6-trimethoxybenzene (13.46, 80 mmol) in tetrahydrofuran (100 mL) was added n-butyllithium in hexane (50 mL, 80 mmol) at 0 °C followed by the addition of a catalytic amount of *N,N,N′,N′*-tetramethylethylenediamine (1 mL, 6.7 mmol). The reaction mixture was allowed to warm to rt

and stirred for 1 h to give an orange suspension, to which was added dropwise a solution of elemental sulfur (2.31 g, 72 mmol) in toluene (60 mL, mild heating was needed to solubilize the sulfur in toluene). The reaction mixture was stirred at rt for 6 h and subsequently quenched by the addition of water (100 mL). The aqueous layer was acidified with 1 M aqueous hydrochloric acid (80 mL), extracted with CH_2Cl_2 (3 × 50 mL), washed with water (3 × 50 mL), brine (50 mL) dried ($MgSO_4$) and concentrated under reduced pressure. The yellow oil was crystalized at -78 °C and recrystallized from hexanes containing a few drops of CH_2Cl_2 to yield the product as a crystalline yellow solid (10.80 g, 68%).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 6.18 (2H, s, Ar C<u>H</u>), 3.87 (6H, s, Ar OC<u>H</u>$_3$), 3.80 (3H, s, Ar OC<u>H</u>$_3$), 3.76 (1H, s, Ar S<u>H</u>). **^{13}C NMR** (100 MHz, $CDCl_3$) δ_C 158.9, 156.4, 100.0, 91.4, 56.2, 55.7. **IR** *νmax* (film/cm^{-1}) 2579 m, 1589 s, 1457 s, 1228 s, 1204 s. **mp** 55-56 °C. **LCMS** Observed $[M+H]^+$ 201.1 required $[M+H]^+$ 201.6. Data consistent with that reported in the literature.[2]

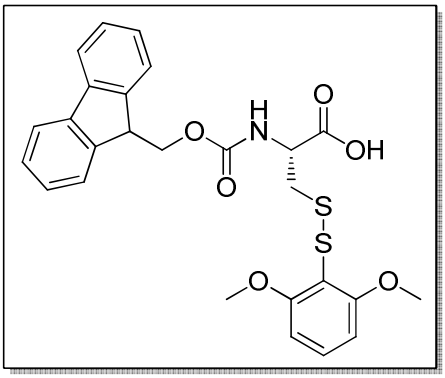
Fmoc-Cysteine-OH (3-1)



To a solution of Fmoc-Cys(Trt)-OH (20 g, 34.38 mmol) in CH_2Cl_2 (1360 mL) was added triisopropylsilane (40 mL, 19.5 mmol) followed by TFA (160 mL, 2.09 mol). The reaction mixture was stirred for 10 min at rt during which the bright orange solution turned colourless. The reaction mixture was concentrated under reduced pressure and co-evaporated with Et_2O to remove TFA. The residue was suspended in hexanes, centrifuged, the supernatant was discarded and the pellet was resuspended in hexanes (cycle repeated 5 ×). The pellet was dried under reduced pressure to yield the product as a white amorphous solid (11.80 g, 100%).

^{1}H NMR (400 MHz, δ_6-DMSO) δ_H 12.86 (1H, br s, CO_2<u>H</u>), 7.89 (1H, d, *J* = 7.5 Hz, Ar C<u>H</u>), 7.74 (2H, d, *J* = 7.3 Hz, Ar C<u>H</u>), 7.69 (1H, d, *J* = 8.3 Hz, C(=O)N<u>H</u>R), 7.42 (2H, t, *J* = 7.2 Hz, Ar C<u>H</u>), 7.33 (2H, td, *J* = 7.4, 1.0 Hz, Ar C<u>H</u>), 4.34 - 4.28 (2H, m, Fmoc CH_2), 4.24 (1H, t, *J* = 7.0 Hz, Fmoc C<u>H</u>), 4.13 (1H, td, *J* = 8.4, 4.4 Hz, α-C<u>H</u>), 2.94 – 2.85 (1H, m, β-C<u>H</u>$_2$), 2.78 – 2.67 (1H, m, β-C<u>H</u>$_2$). **^{13}C NMR** (100 MHz, $CDCl_3$) δ_C 171.9, 156.1, 143.8, 143.8, 140.7, 127.6, 127.1, 125.3, 120.1, 65.7, 56.6, 46.6, 25.4. **IR** *νmax* (film/cm^{-1}) 2569 m, 1718 s, 1518 m. **LCMS** observed $[M+H]^+$ 344.4 required $[M+H]^+$ 344.1.

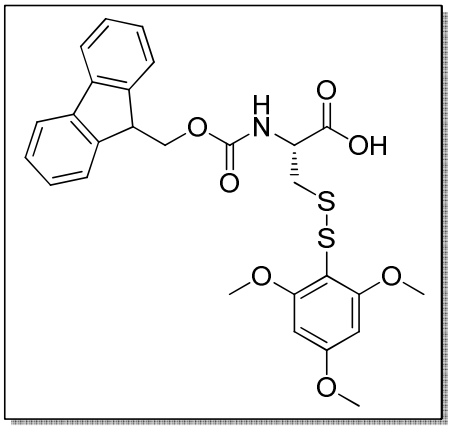
Fmoc-Cys(dimethoxyphenylthio)-OH (Fmoc-Cys(*S*-Dmp)-OH) (3)



To a suspension of *N*-chlorosuccinimide (2.80 g, 21 mmol) in CH_2Cl_2 (60 mL) at -30 °C under a nitrogen atmosphere, was added dropwise a solution of 2,6-dimethoxythiophenol (3.41 g, 20 mmol) in CH_2Cl_2 (20 mL). The reaction mixture was stirred for 30 min at -30 °C under the exclusion of light. The reaction mixture was cannulated dropwise to a solution of Fmoc-Cys-OH (6.87 g, 20 mmol) in THF (120 mL) at -30 °C under a nitrogen atmosphere, and stirred for a further 30 min at -30 °C under the exclusion of light. The reaction mixture was warmed to rt and diluted with CH_2Cl_2 (200 mL), washed with 2 M aqueous hydrochloric acid (3 × 200 mL), water (200 mL), brine (200 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by flash silica chromatography eluting with a gradient of CH_2Cl_2 : methanol (100:0 → 99:1) to yield the product as a white foam (7.88 g, 77%).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.75 (2H, d, *J* = 7.5 Hz, Ar C<u>H</u>), 7.61 (2H, dd, *J* = 7.0, 5.2 Hz, Ar C<u>H</u>), 7.39 (2H t, *J* = 7.4 Hz, Ar C<u>H</u>), 7.33 – 7.24 (3H, m, Ar C<u>H</u>), 6.54 (2H, d, *J* = 8.4 Hz, Ar C<u>H</u>) 6.03 (1H, d, *J* = 6.7 Hz, C(=O)N<u>H</u>R), 4.86 - 4.79 (1H, m, α-C<u>H</u>), 4.46 – 4.36 (2H, m, Fmoc C<u>H$_2$</u>), 4.23 (1H, t, *J* = 7.1 Hz, Fmoc C<u>H</u>), 3.85 (6H, s, Ar OC<u>H$_3$</u>), 3.37 (1H, dd, *J* = 14.2, 3.1 Hz, β-C<u>H$_2$</u>), 3.09 (1H, dd, *J* = 14.1, 8.3 Hz, β-C<u>H$_2$</u>). **^{13}C NMR** (100 MHz, $CDCl_3$) δ_C 175.3, 161.4, 156.4, 143.9, 141.4, 132.0, 127.8, 127.2, 125.3, 120.1, 112.1, 104.4, 67.5, 56.4, 54.5, 47.3, 38.9. **IR** ν*max* (film/cm^{-1}) 1722 s, 1579 s, 1519 m, 1252 s. **HRMS** (ESI+) *m/z* found $[M+H]^+$ 512.1197, $C_{26}H_{26}NO_6S_2^+$ required 512.1202.

Fmoc-Cys(trimethoxyphenylthio)-OH (Fmoc-Cys(*S*-Tmp)-OH) (4)



To a suspension of *N*-chlorosuccinimide (2.80 g, 21 mmol) in CH_2Cl_2 (30 mL) at -78 °C under a nitrogen atmosphere, was added dropwise a solution of 2,4,6-trimethoxythiophenol (4.01 g, 20 mmol) and Fmoc-Cys-OH (6.87 g, 20 mmol) in THF (40 mL) under the exclusion of light. The Reaction mixture was stirred at -78 °C for 1.5 h under the exclusion of light. The reaction mixture was warmed to rt and diluted with CH_2Cl_2 (200 mL), washed with 2 M aqueous hydrochloric acid (3 × 200 mL), water (200 mL), brine (200 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by flash silica chromatography eluting with a gradient of CH_2Cl_2 : methanol (100:0 → 99:1) to yield the product as a cream coloured foam (8.23 g, 76%).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.75 (2H, d, *J* = 7.5 Hz, Ar C<u>H</u>), 7.61 (2H, dd, *J* = 10.7, 4.0 Hz, Ar C<u>H</u>), 7.38 (2H, t, *J* = 7.4 Hz, Ar C<u>H</u>), 7.32 – 7.27 (2H, m, Ar C<u>H</u>), 6.10 (2H, s, Ar C<u>H</u>), 6.07 (1H, d, *J* = 6.9 Hz, C(=O)N<u>H</u>R), 4.87 – 4.78 (1H, m, α-C<u>H</u>), 4.45 – 4.35 (2H, m, Fmoc C<u>H$_2$</u>), 4.23 (1H, t, *J* = 7.2 Hz, Fmoc C<u>H</u>), 3.84 (6H, s, Ar OC<u>H$_3$</u>), 3.77 (3H, s, Ar OC<u>H$_3$</u>), 3.36 (1H, dd, *J* = 14.3, 3.5 Hz, β-C<u>H$_2$</u>), 3.08 (1H, dd, *J* = 14.3, 8.7 Hz, β-C<u>H$_2$</u>). **^{13}C NMR** (100 MHz, $CDCl_3$) δ_C 175.3, 163.5, 162.5, 156.6, 143.9, 141.4, 127.8, 127.3, 125.4, 120.1, 103.7, 91.3, 67.5, 56.3, 55.5, 54.5, 47.2, 38.5. **IR** *νmax* (film/cm^{-1}) 1721 s, 1590 s, 1521 m, 1227 s. **HRMS** (ESI+) *m/z* found $[M+H]^+$ 542.1304, $C_{27}H_{28}NO_7S_2^+$ required 542.1307.

1-SI.3. General Methods Peptide Synthesis

General Method 1: Peptide Synthesis

Fmoc-Rink-Amide AM resin (0.45 mmol/gram, 1 equiv.) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) followed by washing with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The protected Fmoc-amino acids (3 equiv.) were incorporated using DIC (3 equiv.) and Oxyma (3 equiv.) in DMF, as a coupling system, with 5 min preactivation for 1 h at rt. Washes between couplings and deprotections were performed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Completion of the coupling was monitored by the Kaiser test.[3] Following elongation a microcleavage was performed, 5 mg of dry resin was treated with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1) for analysis by HPLC and LCMS.

General Method 2a: Deprotection of disulfide containing protecting groups (non-malodorous)

The resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Deprotection was achieved by treatment with 0.1 M *N*-methylmorpholine in 5% dithiothreitol (DTT)/DMF (3 × 5 min) and subsequently washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min).

General Method 2b: Deprotection of disulfide containing protecting groups (malodorous)

The resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Deprotection was achieved by treatment with 0.1 M *N*-methylmorpholine in 20% β-mercaptoethanol/DMF (3 × 5 min) and subsequently washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min).

General Method 3: Disulfide Formation

The resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Deprotection of the disulfide containing protecting group was achieved with either general method 2a or 2b, unless otherwise specified. The peptide was cleaved from the resin using TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage

mixture was evaporated with argon, precipitated with Et_2O, centrifuged. The pellet was redissolved in 5% DMSO in H_2O/CH_3CN (3:1) (200 µL/mg resin) and the pH adjusted to 8.0 with dilute NH_4OH. The reaction mixture was shaken for 24 h at rt and subsequently lyophilized.

General Method 4: Microcleavage

Dry resin (5 mg) was treated with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1) for analysis by HPLC and LCMS.

1-SI.4. Peptide Synthesis

Fmoc-Ala-Cys(*S*tBu)-Leu-NH_2 (5)

Peptide **5** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (333.3 mg, 0.15 mmol, 0.45 mmol/gram). Microcleavage (5 mg resin) with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt and subsequent HPLC analysis found that peptide **5** was obtained in 98**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 7.9 min). **LCMS** observed $[M+H]^+$ 615.8 required $[M+H]^+$ 615.8.

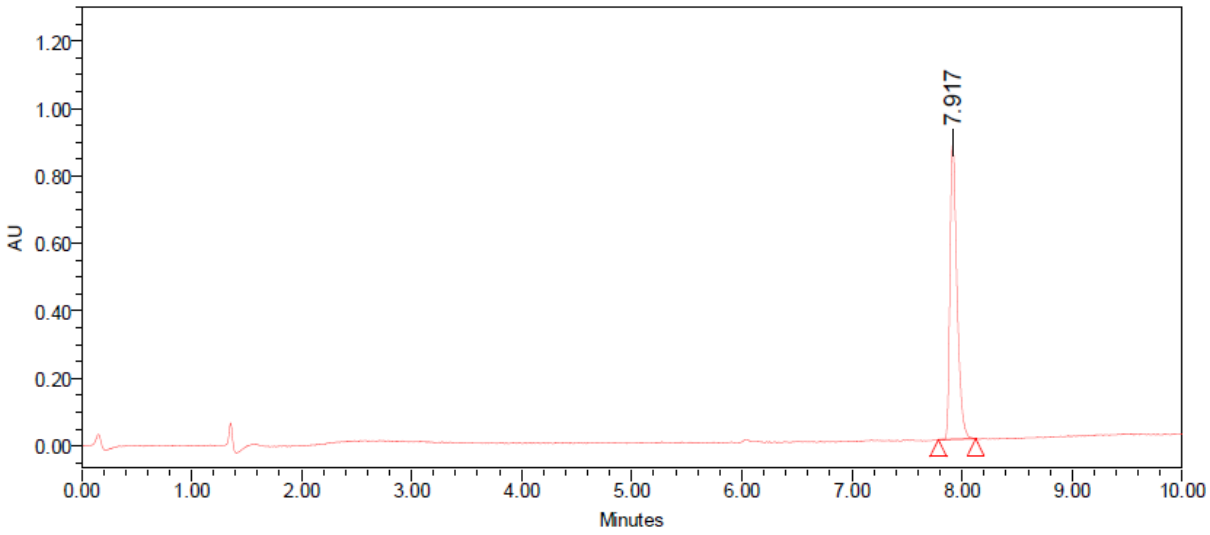


Figure S-1: HPLC chromatogram of peptide **5**

Fmoc-Ala-Cys(*S*-Dmp)-Leu-NH_2 (6)

Peptide **6** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (333.3 mg, 0.15 mmol, 0.45 mmol/gram). Microcleavage (5 mg resin) with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt and subsequent HPLC analysis found that peptide **6** was obtained in 97**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 7.6 min). **LCMS** observed $[M+H]^+$ 615.8 required $[M+H]^+$ 615.8.

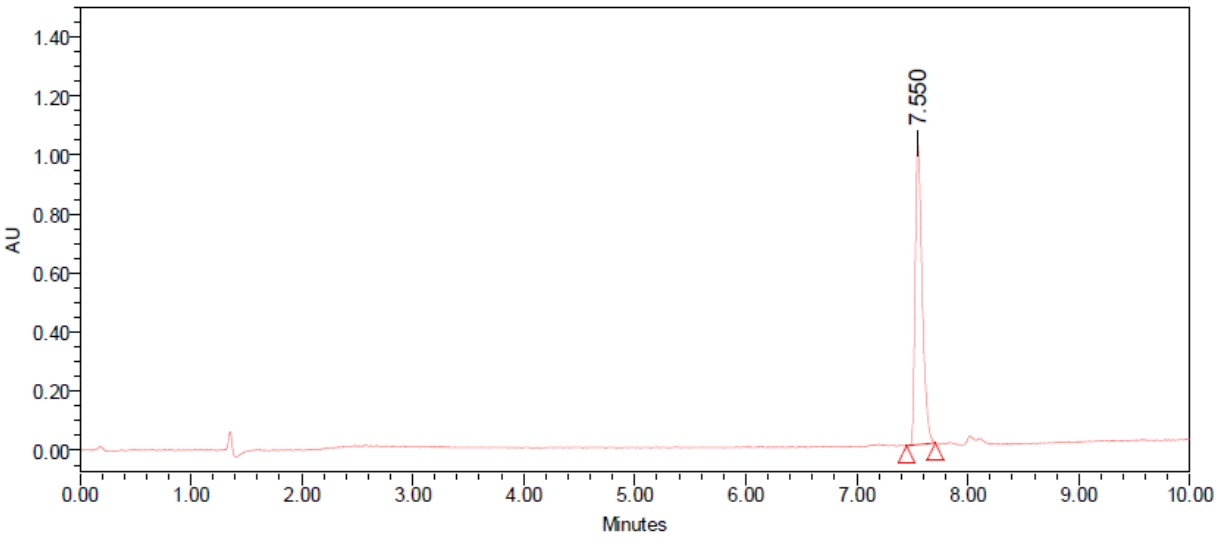


Figure S-2: HPLC chromatogram of peptide **6**

Fmoc-Ala-Cys(*S*-Tmp)-Leu-NH_2 (7)

Peptide **7** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (333.3 mg, 0.15 mmol, 0.45 mmol/gram). Microcleavage (5 mg resin) with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt and subsequent HPLC analysis found that peptide **7** was obtained in 94**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 7.6 min). **LCMS** observed $[M+H]^+$ 695.84 required $[M+H]^+$ 695.87.

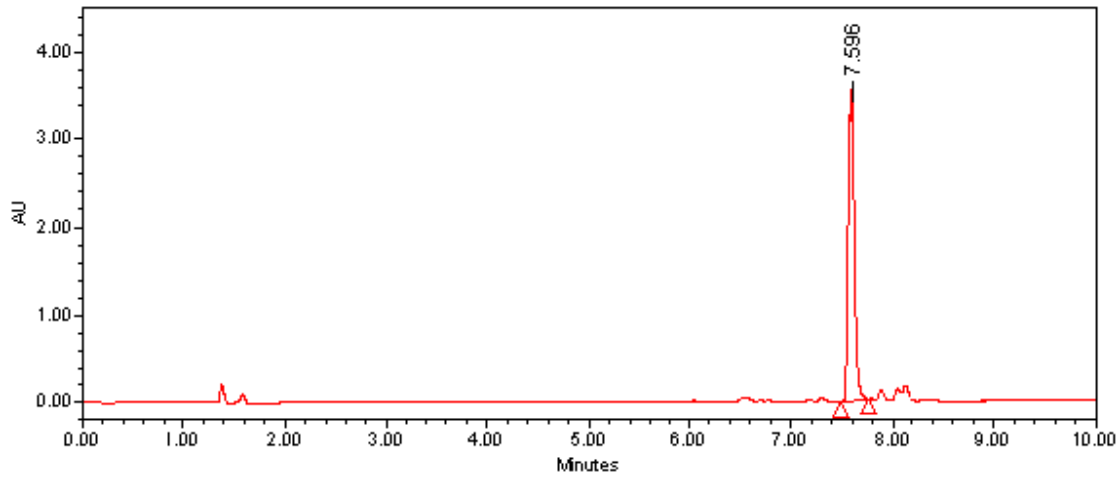


Figure S-3: HPLC chromatogram of peptide **7**

Fmoc-Asn(Trt)Cys(*S*-tBu)Asn(Trt)-NH_2 (8)

Peptide **8** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (333.3 mg, 0.15 mmol, 0.45 mmol/gram). Microcleavage (5 mg resin) with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt and subsequent HPLC analysis found that peptide **8** was obtained in 97**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 6.3 min). **LCMS** observed $[M+H]^+$ 659.7 required $[M+H]^+$ 659.8.

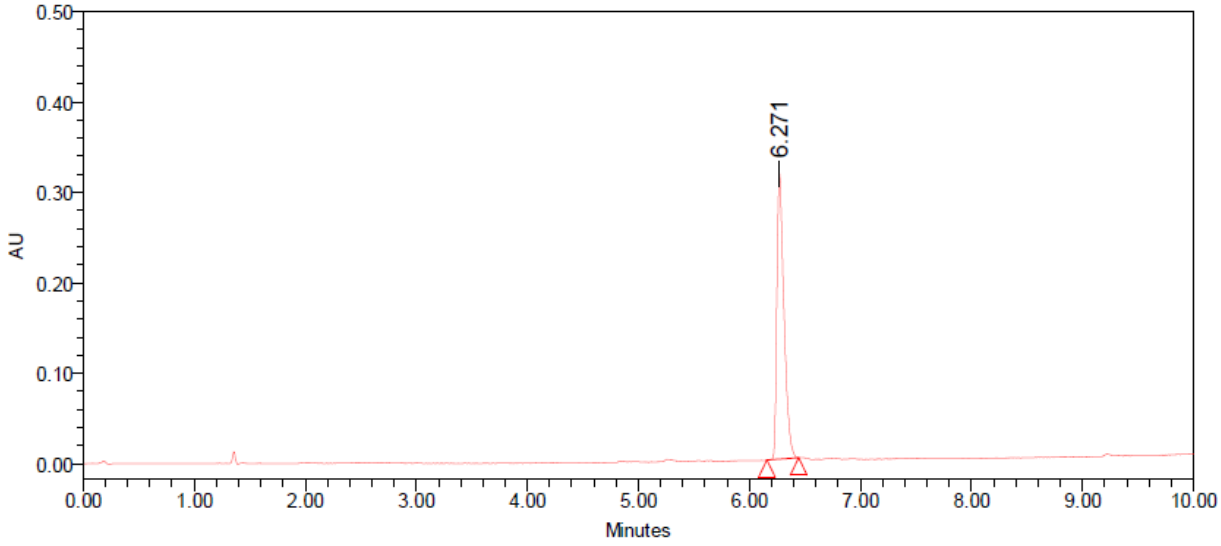


Figure S-4: HPLC chromatogram of peptide **8**

Fmoc-Asn(Trt)Cys(*S*-Dmp)Asn(Trt)-NH_2 (9)

Peptide **9** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (333.3 mg, 0.15 mmol, 0.45 mmol/gram). Microcleavage (5 mg resin) with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt and subsequent HPLC analysis found that peptide **9** was obtained in 99**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 6.1 min). **LCMS** observed $[M+H]^+$ 739.8 required $[M+H]^+$ 739.8.

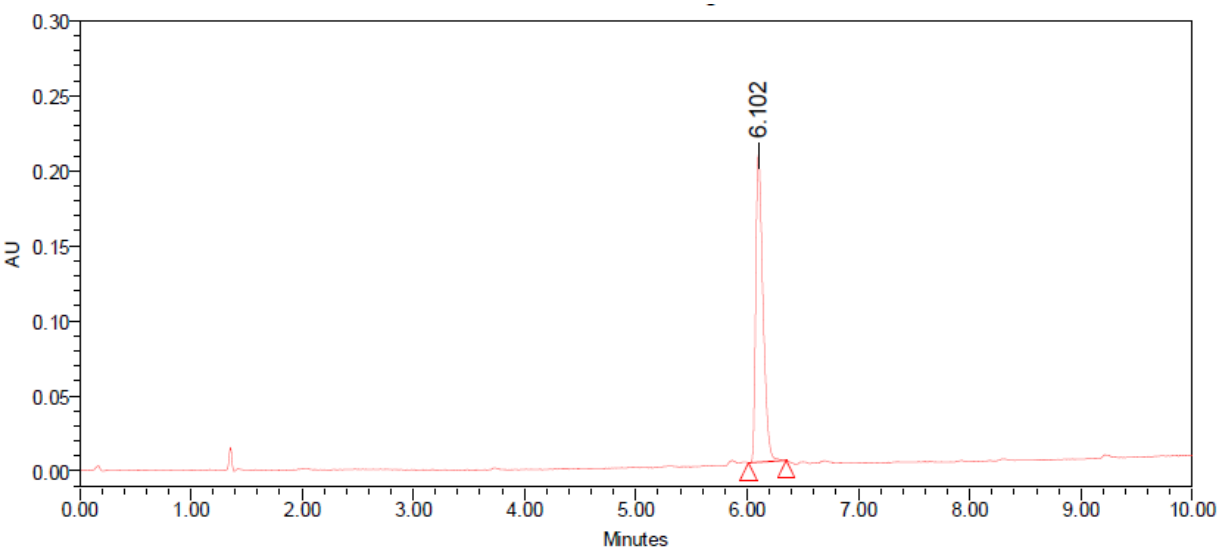


Figure S-5: HPLC chromatogram of peptide **9**

Fmoc-Asn(Trt)Cys(*S*-Tmp)Asn(Trt)-NH_2 (10)

Peptide **10** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (333.3 mg, 0.15 mmol, 0.45 mmol/gram). Microcleavage (5 mg resin) with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt and subsequent HPLC analysis found that peptide **10** was obtained in 92**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 6.2 min). **LCMS** observed $[M+H]^+$ 770.0 required $[M+H]^+$ 769.9.

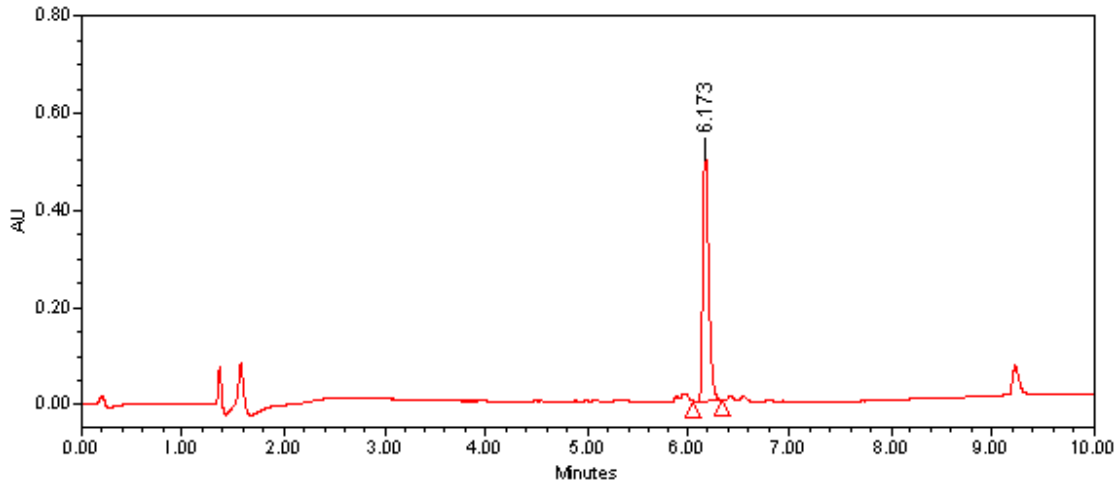


Figure S-6: HPLC chromatogram of peptide **10**

Thiol lability

Resin containing peptide **5**, **6**, **7**, **8**, **9** and **10** (50 mg, 22.5 μmol) were placed in separate solid phase reactors and washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). 0.1 M *N*-methylmorpholine in 20% β-mercaptoethanol /DMF (5 mL) was added to each vessel and aliquots (0.5 mL) containing dispersed resin were taken at t = 0, 5, 10, 15, 30 min; 1, 2, 3, 4, 5, 6, 8 and 24 h. The deprotection mixture was removed from the taken aliquot by suction and the resin washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and cleaved from the resin using TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1) for analysis by HPLC and LCMS.

Peptide (on-resin)	Deprotection Time (min) β-mercaptoethanol	Deprotection Time (min) dithiothreitol
5 Fmoc-Ala-Cys(*S*tBu)-Leu-NH_2	180	5% after 24 h
6 Fmoc-Ala-Cys(*S*-Dmp)-Leu-NH_2	5	5
7 Fmoc-Ala-Cys(*S*-Tmp)-Leu-NH_2	5	5
8 Fmoc-Asn(Trt)-Cys(*S*tBu)-Asn(Trt)-NH_2	360	2% after 24
9 Fmoc-Asn(Trt)-Cys(*S*-Dmp)-Asn(Trt)-NH_2	5	5
10 Fmoc-Asn(Trt)-Cys(*S*-Tmp)-Asn(Trt)-NH_2	5	5

Table 1: Deprotection times for model tripeptides protected with either *S*tBu, *S*-Dmp, *S*-Tmp

Base stability testing

Resin containing peptide **6** and **7** (50 mg, 22.5 μmol) were placed in separate solid phase reactors and washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). 20% Piperidine/DMF (5 mL) was added to each vessel and aliquots (0.5 mL) containing dispersed resin were taken at t = 0, 5, 10, 15, 30 min; 1, 2, 3, 4, 5, 6, 8 and 24 h. The deprotection mixture was removed from the taken aliquot by suction and the resin washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and cleaved from the resin using TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1) for analysis by HPLC and LCMS. Peptide **6-1** protected with *S*-Dmp was obtained in 97**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 4.6 min). **LCMS** observed $[M+H]^+$ 473.6 required $[M+H]^+$ 473.6.

Peptide **7-1** protected with *S*-Tmp was obtained in 98**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 4.8 min). **LCMS** observed $[M+H]^+$ 503.7 required $[M+H]^+$ 503.7.

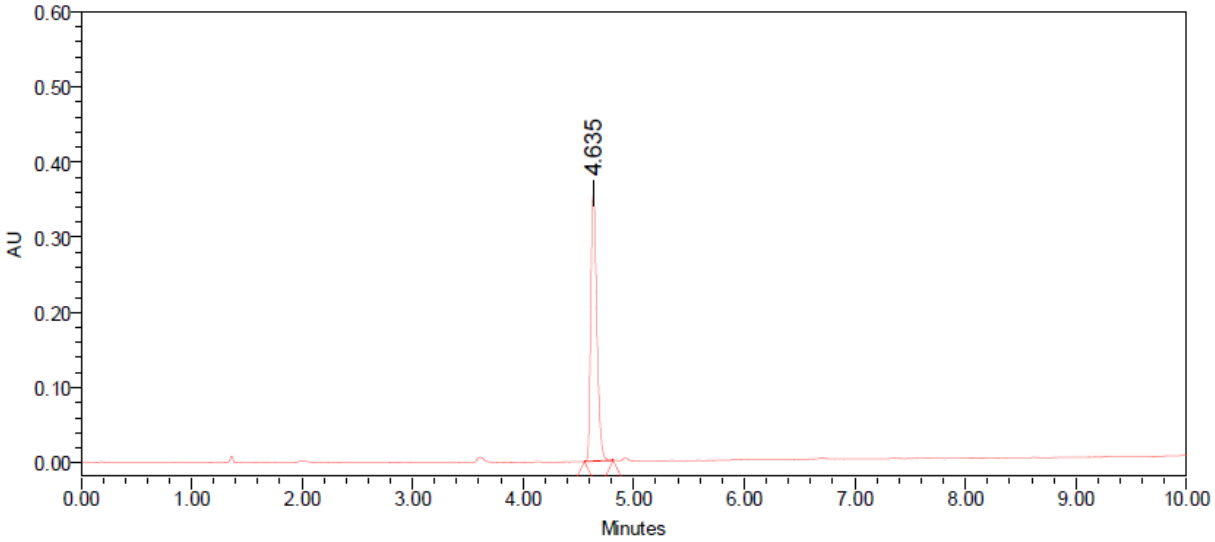


Figure S-7: HPLC chromatogram of peptide **6-1**

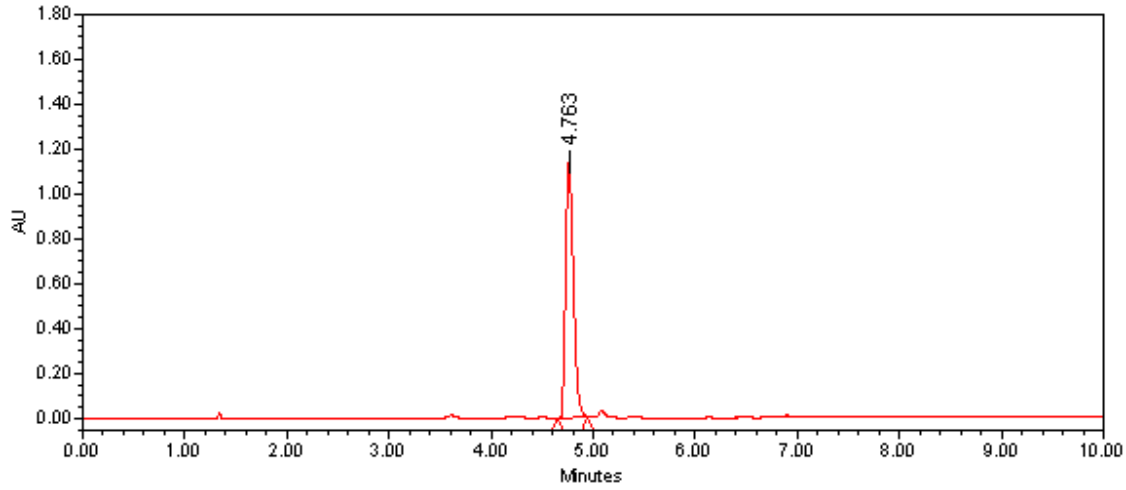
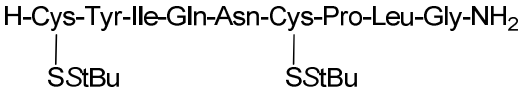


Figure S-8: HPLC chromatogram of peptide **7-1**

H-oxytocin(*S*tBu)$_2$-NH$_2$ (11)



Peptide **11** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (1.11 g, 0.5 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc followed by washing with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin) with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt and subsequent HPLC analysis found that peptide **11** was obtained in 97**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 5.4 min). **LCMS** observed $[M+H]^+$ 1186.8 required $[M+H]^+$ 1186.6.

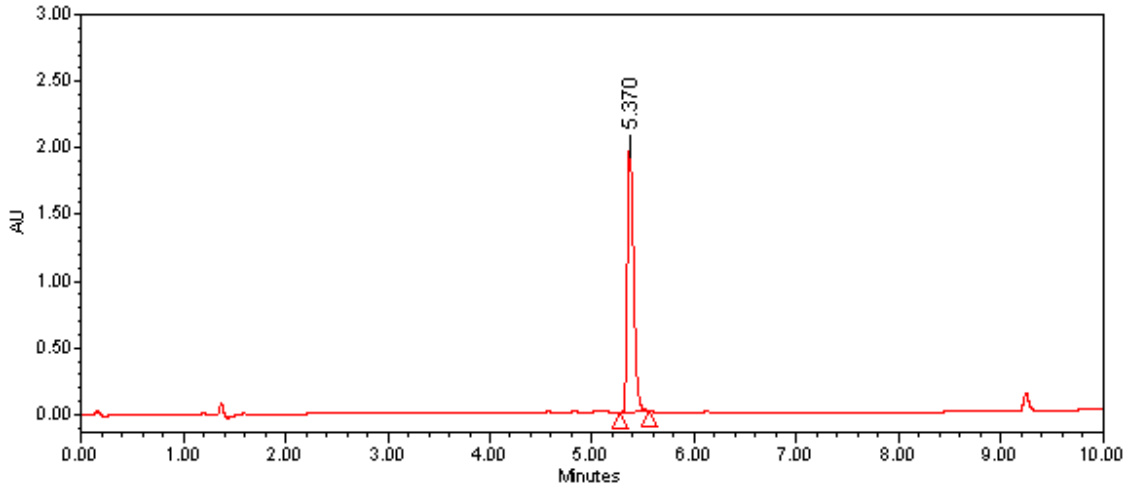
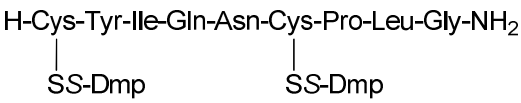


Figure S-9: HPLC chromatogram of peptide **11**

H-oxytocin(*S*-Dmp)$_2$-NH$_2$ (12)



Peptide **12** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (1.11 g, 0.5 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc followed by washing with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin) with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt and subsequent HPLC analysis found that peptide **12** was obtained in 80**%** purity (linear gradient from 5% to 100% acetonitrile over 8

min, t_R : 5.4 min). **LCMS** observed $[M+H]^+$ 1346.6 required $[M+H]^+$ 1346.8. Peptide **12** -1 *S*-DMP (13%) observed $[M+H]^+$ 1178.5 required $[M+H]^+$ 1178.4.

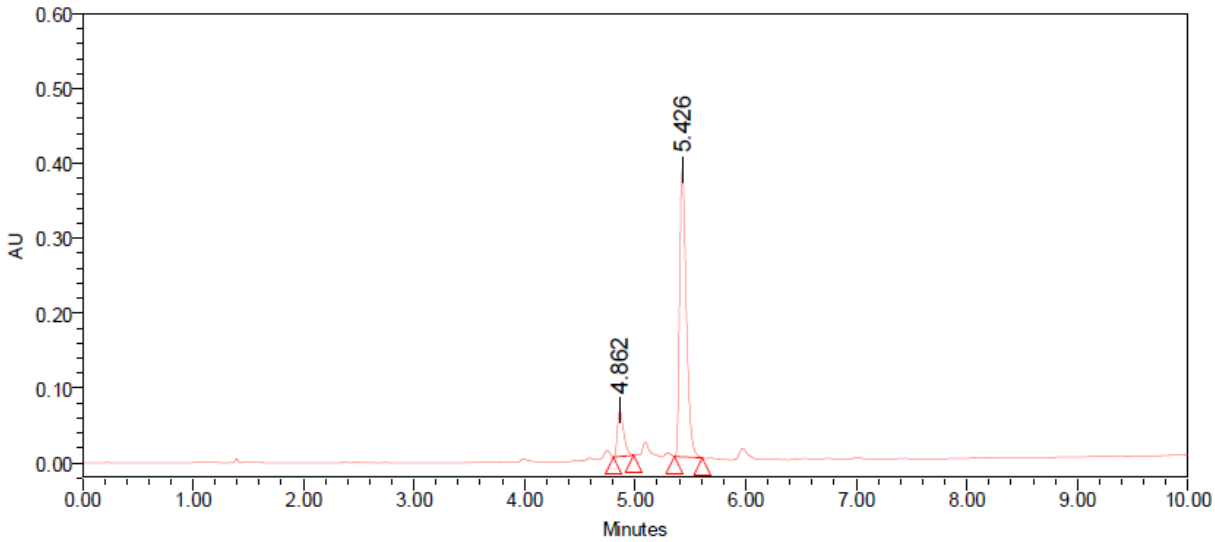
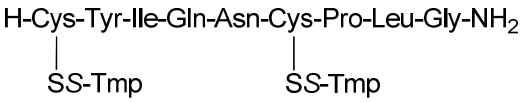


Figure S-10: HPLC chromatogram of peptide **12**

H-oxytocin(*S*-Tmp)$_2$-NH$_2$ (13)



Peptide **13** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (1.11 g, 0.5 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc followed by washing with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin) with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt and subsequent HPLC analysis found that peptide **13** was obtained in 89**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : min). **LCMS** observed $[M+H]^+$ 1406.9 required $[M+H]^+$ 1406.7. Peptide **13** -1 *S*-TMP (8%) observed $[M+H]^+$ 1208.3 required $[M+H]^+$ 1208.5.

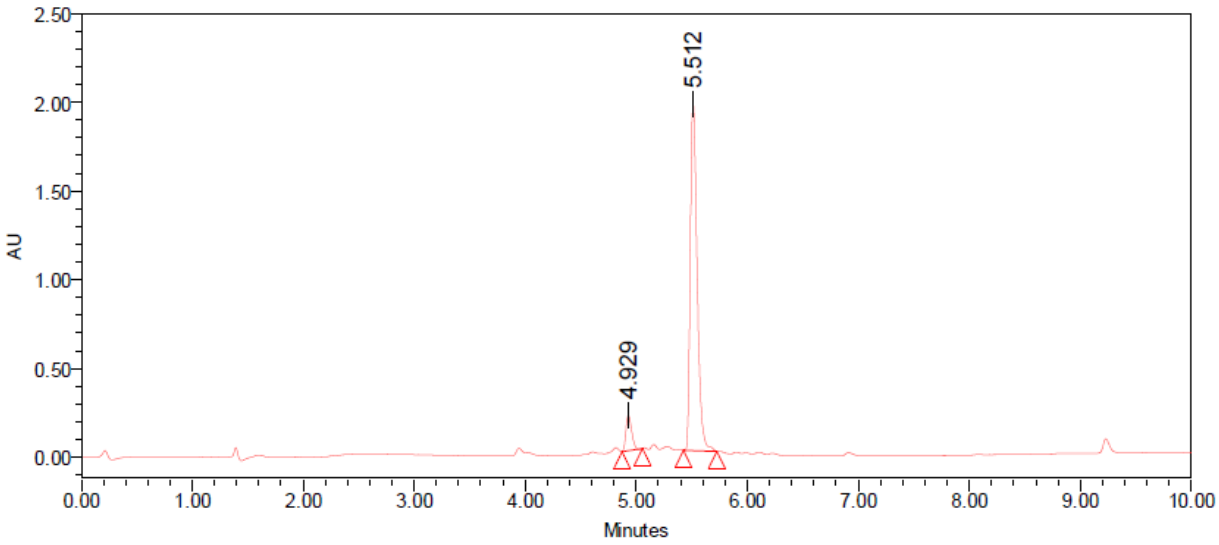
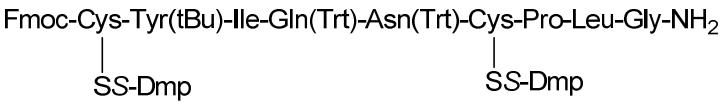


Figure S-11: HPLC chromatogram of peptide **13**

H-oxytocin(*S*-Dmp)$_2$-NH$_2$ (SIEBER AMIDE) (14)



Peptide **14** was synthesized according to General Method 1 using Sieber Amide resin (108.7 mg, 75 µmol, 0.69 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc followed by washing with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin) with TFA/CH_2Cl_2 (0.2:9.8) for 5 min at rt and subsequent HPLC analysis found that peptide **14** was obtained in 74**%** purity (linear gradient from 50% to 100% acetonitrile over 8 min, t_R : 7.2 min). **LCMS** observed $[M+H]^+$ 1887.7 required $[M+H]^+$ 1887.4.

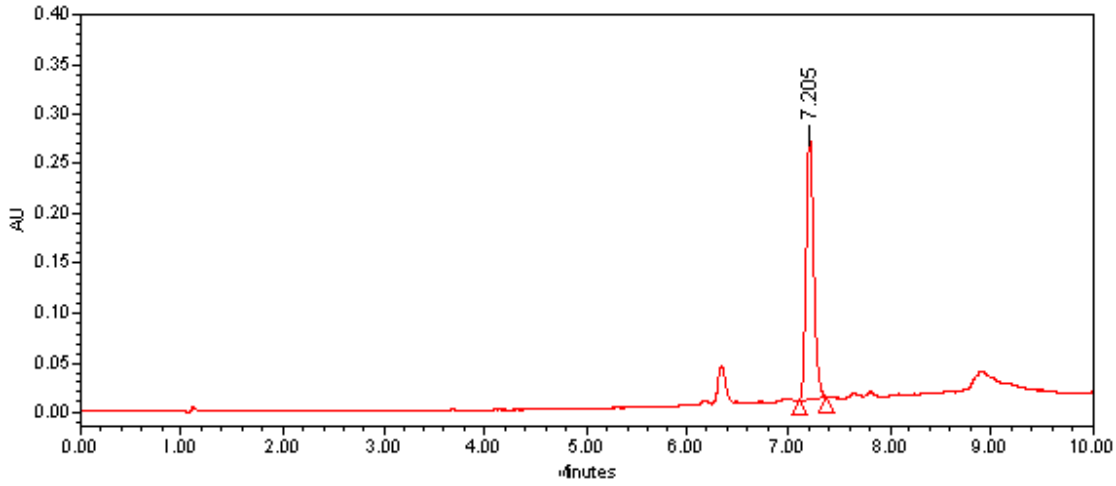
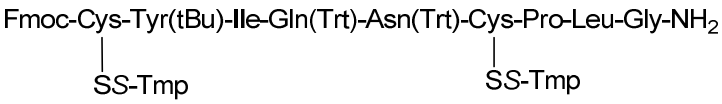


Figure S-12: HPLC chromatogram of peptide **14**

H-oxytocin(*S*-Tmp)$_2$-NH$_2$ (SIEBER AMIDE) (15)



Peptide **15** was synthesized according to General Method 1 using Sieber Amide resin (108.7 mg, 75 µmol, 0.69 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc followed by washing with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin) with TFA/CH_2Cl_2 (0.2:9.8) for 5 min at rt and subsequent HPLC analysis found that peptide **15** was obtained in 95**%** purity (linear gradient from 50% to 100% acetonitrile over 8 min, t_R : 7.2 min). **LCMS** observed $[M+H]^+$ 1947.9 required $[M+H]^+$1947.4.

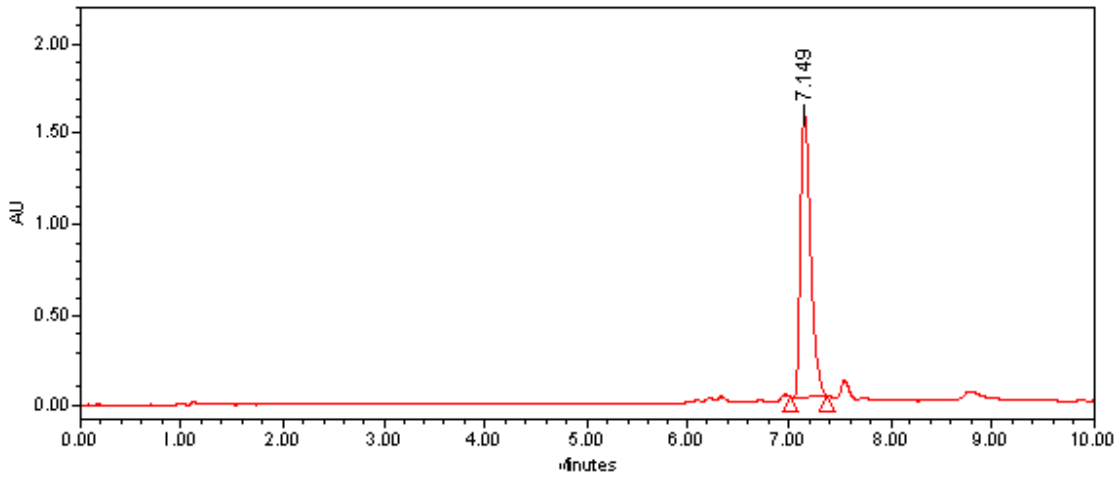
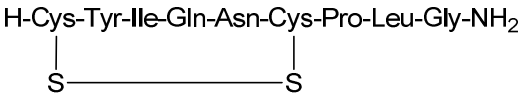


Figure S-13: HPLC chromatogram of peptide **15**

Oxidation of Oxytocin with *S*tBu protection (16)



Resin containing Fmoc-oxytocin(*S*tBu)$_2$-NH$_2$ (**11**) (5.0 mg, 2.25 µmol) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min), DMF (5 × 1 min). Fmoc removal was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) followed by washing with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The resin was treated with 0.1 M *N*-methylmorpholine in 20% β-mercaptoethanol / DMF (1 mL) for 8 h at rt to remove *S*tBu. The peptide was oxidized according to General Method 3. HPLC analysis found that peptide **16** was obtained in 72**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 4.0 min). **HRMS** (ESI+) *m/z* found $[M]^+$ 1006.4359, $C_{26}H_{26}NO_6S_2^+$ required 1006.4365.

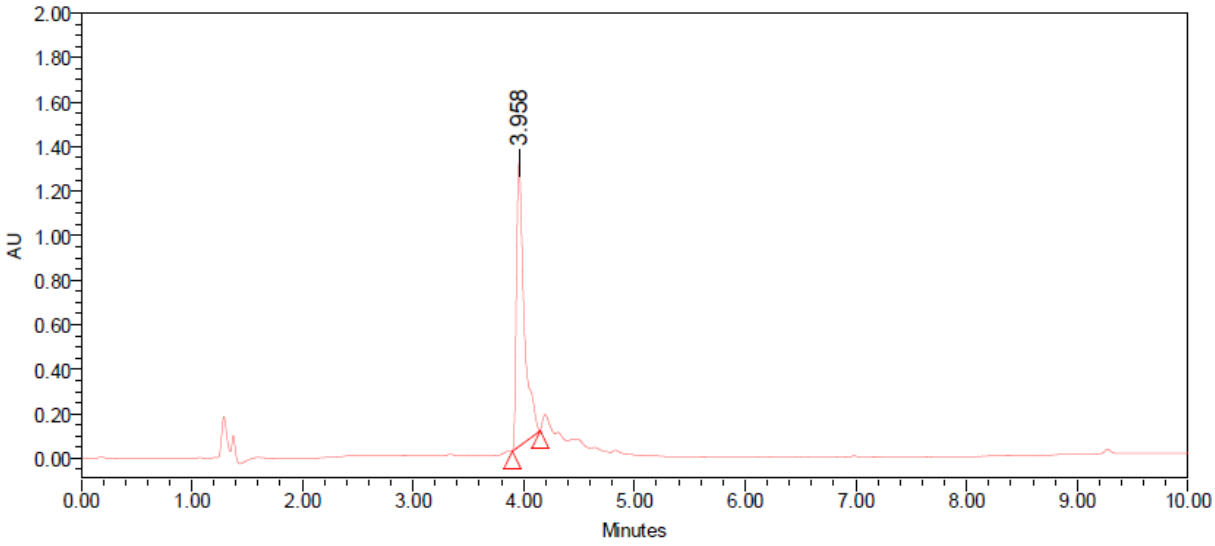
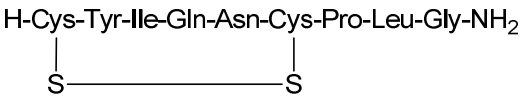


Figure S-14: HPLC chromatogram of peptide **16**

Oxidation of Oxytocin with *S*-Dmp protection (17)



Resin containing Fmoc-oxytocin(*S*-Dmp)$_2$-NH_2 (**12**) (5.0 mg, 2.25 µmol) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min), DMF (5 × 1 min). Fmoc removal was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) followed by washing with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The peptide was oxidized according to General Method 3 in combination with General Method 2a to remove *S*-Dmp. HPLC analysis found that peptide **17** was obtained in 86**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 4.0 min). **HRMS** (ESI+) *m/z* found $[M]^+$ 1006.4359, $C_{26}H_{26}NO_6S_2^+$ required 1006.4365.

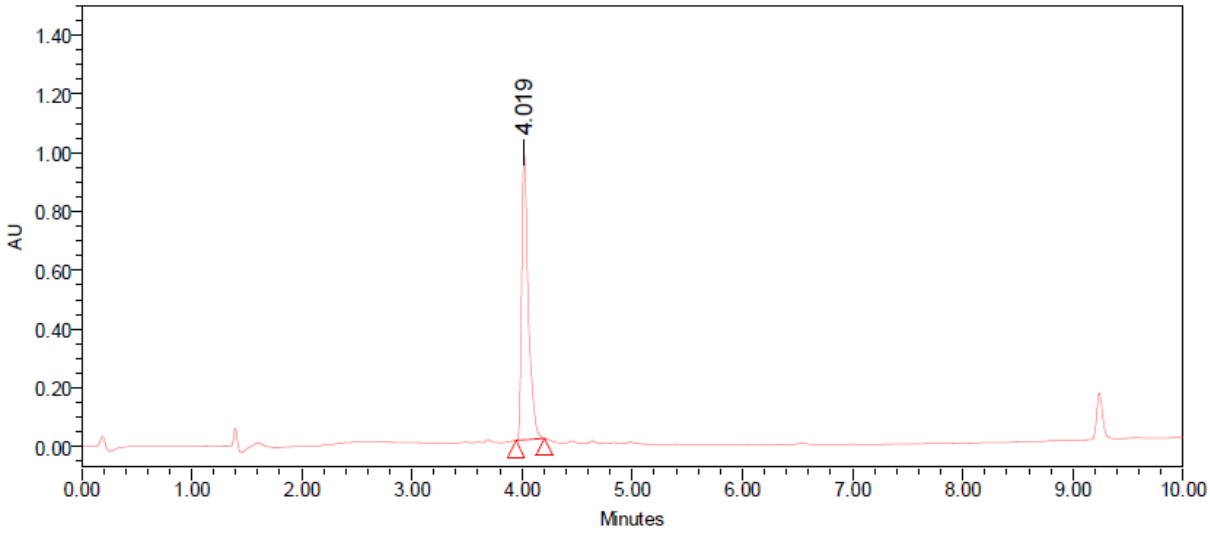
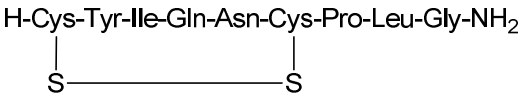


Figure S-15: HPLC chromatogram of peptide **17**

Oxidation of Oxytocin with *S*-Tmp protection (18)



Resin containing Fmoc-oxytocin(*S*-TMP)$_2$-NH_2 (**13**) (5.0 mg, 2.25 µmol) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min), DMF (5 × 1 min). Fmoc removal was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) followed by washing with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The peptide was oxidized according to General Method 3 in combination with General Method 2a to remove *S*-Tmp. HPLC analysis found that peptide **18** was obtained in 93**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 4.0 min). **HRMS** (ESI+) *m/z* found $[M]^+$ 1006.4361, $C_{26}H_{26}NO_6S_2^+$ required 1006.4365.

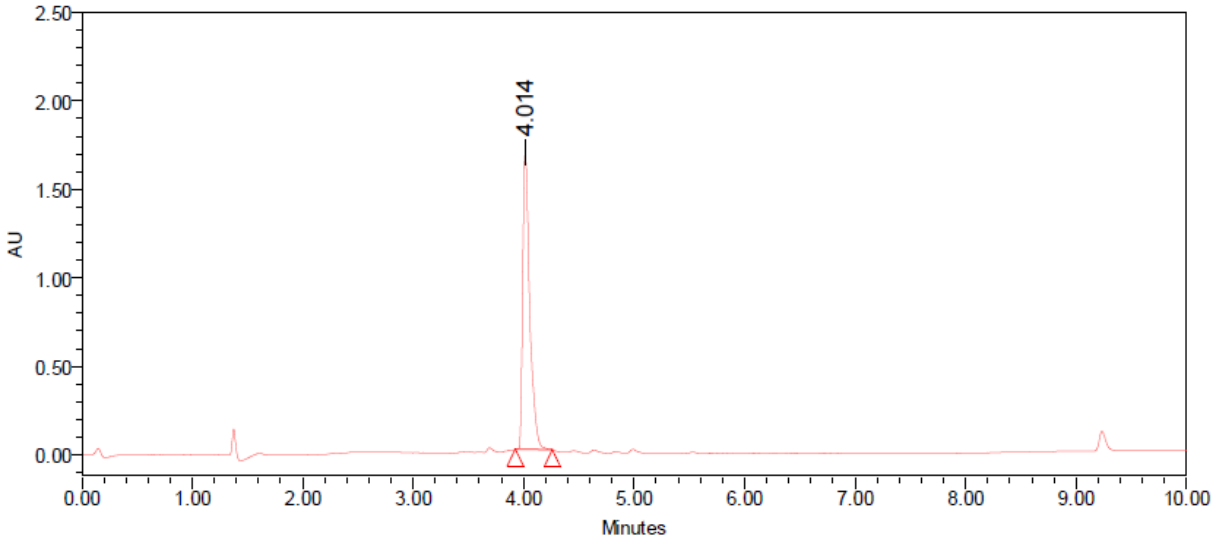
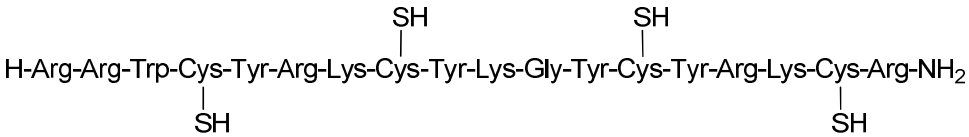


Figure S-16: HPLC chromatogram of peptide **18**

T22 Peptide Elongation Using *S*-Tmp Protection (19)



Rink-Amide-Chemmatrix Low LOA resin (0.71g, 1.15 mmol, 0.21 mmol/g) was washed with with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min), TFA/CH_2Cl_2 (1:99) (5 × 1 min), CH_2Cl_2 (3 × 1 min), DIPEA/CH_2Cl_2 (5:95) (5 × 1 min), CH_2Cl_2 (5 × 1 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) followed by washing with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). The protected Fmoc-amino acids (3 equiv.) were incorporated using DIC (3 equiv.) and Oxyma (3 equiv.) in DMF, as a coupling system, with 5 min preactivation for 1 h at rt. Washes between couplings and deprotections were performed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Completion of the coupling was monitored by the Kaiser test.[3] Following the elongation, *S*-Tmp was removed using General Method 2a. A microcleavage was performed with 5 mg of dry resin which was treated with TFA/TIS/H_2O (95:2.5:2.5) for 3 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O. HPLC analysis found that peptide **19** was obtained in 90**%** purity (linear gradient from 0% to 30% acetonitrile over 8 min, t_R : 3.4 min). **LCMS** observed $[(M+2H)/2]^{2+}$ 1246.7, $[(M+3H)/3]^{3+}$ 831.9, $[(M+4H)/2]^{4+}$ 624.2 required $[M+H]^+$ 2492.0.

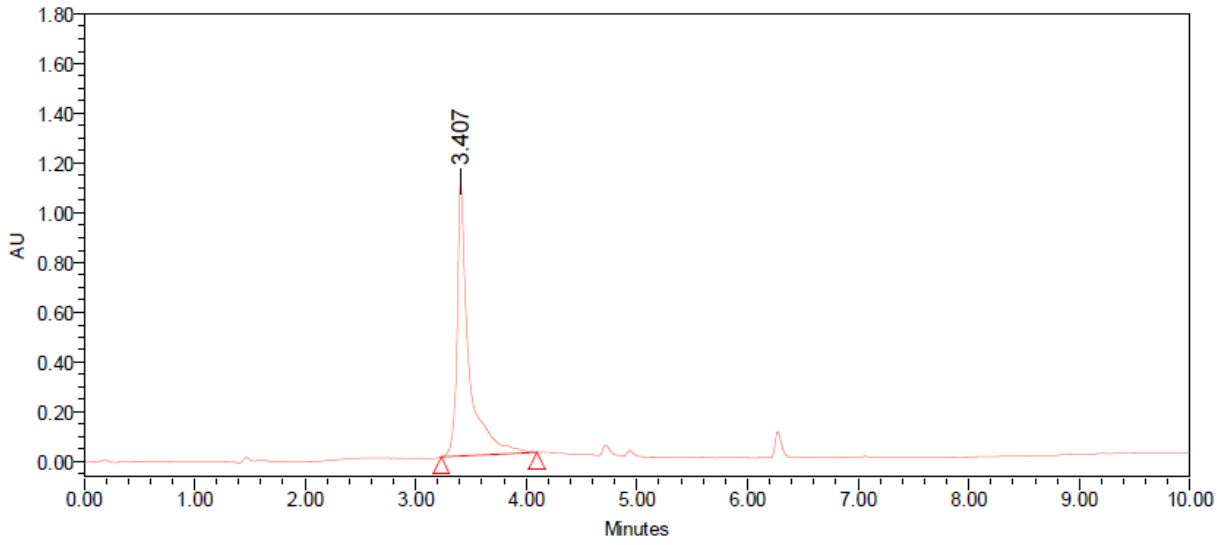
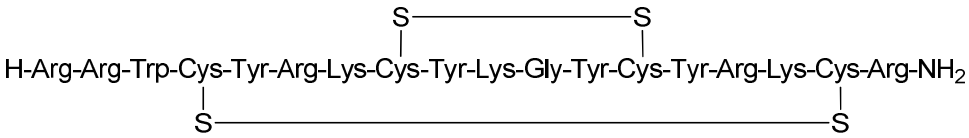


Figure S-17: HPLC chromatogram of peptide **19**

Oxidation of T22 (20)



The T22 peptide was cleaved from the resin (**19**) using TFA/TIS/H_2O (95:2.5:2.5) for 3 h at rt. The cleavage mixture was evaporated with argon, precipitated with Et_2O, centrifuged. The pellet was redissolved in DMSO/H_2O (0.05:0.95, 1mL) and the pH adjusted to 8.0 with dilute NH_4OH. The reaction mixture was shaken for 24 h at rt followed and subsequently lyophilized. HPLC analysis found that peptide **20** was obtained in 78**%** purity (linear gradient from 0% to 30% acetonitrile over 8 min, t_R : 4.4 min). **HRMS** (ESI+) *m/z* found $[M]^+$ 2485.1723, $C_{26}H_{26}NO_6S_2^+$ required 2485.1765.

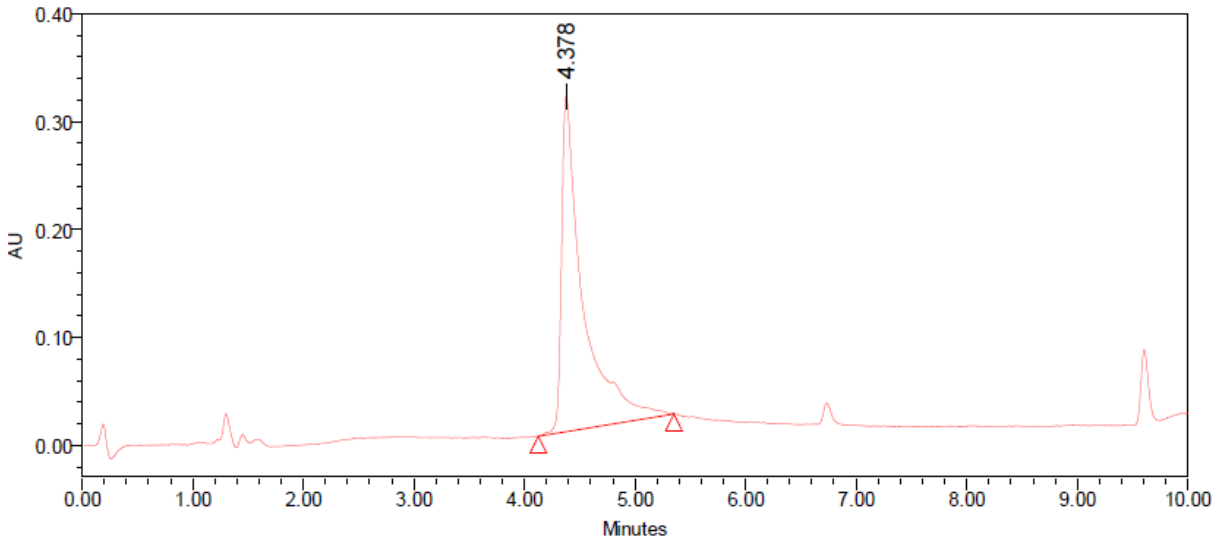


Figure S-1: HPLC chromatogram of peptide **20**

1-SI.5. References

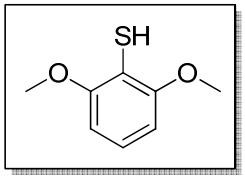
1. Wada, M.; Natsume, S.; Suzuki, S.; Uo, A.; Nakamura, M.; Hayase, S.; Erabi, T. *J. Organomet. Chem.* **1997**, 548, 223-227.

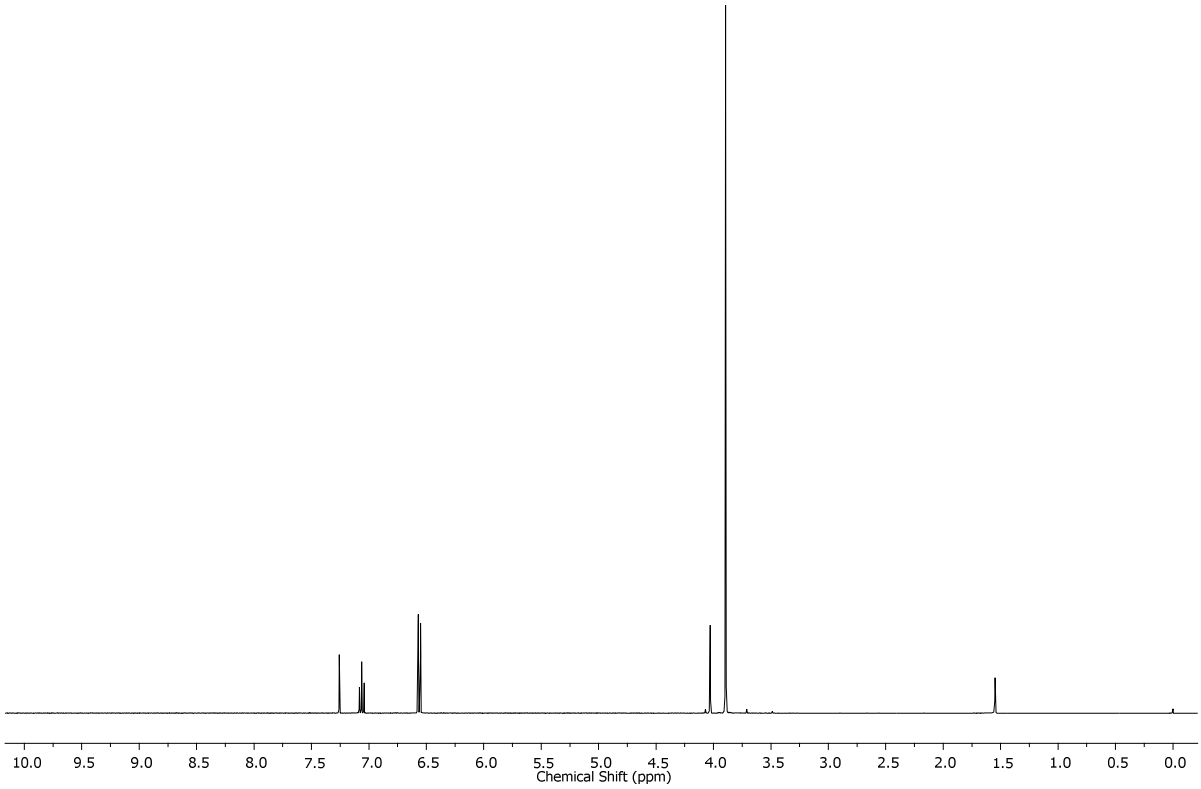
2. Bottino, F.; Fradullo, R.; Pappalardo, S. *J. Org. Chem.* **1981**, 46 (13), 2793–2795.

3. Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I., *Anal. Biochem.* **1970,** 34, 595-598.

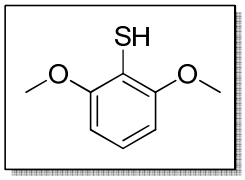
1-SI.6. ^{1}H-NMR and ^{13}C-NMR Spectra

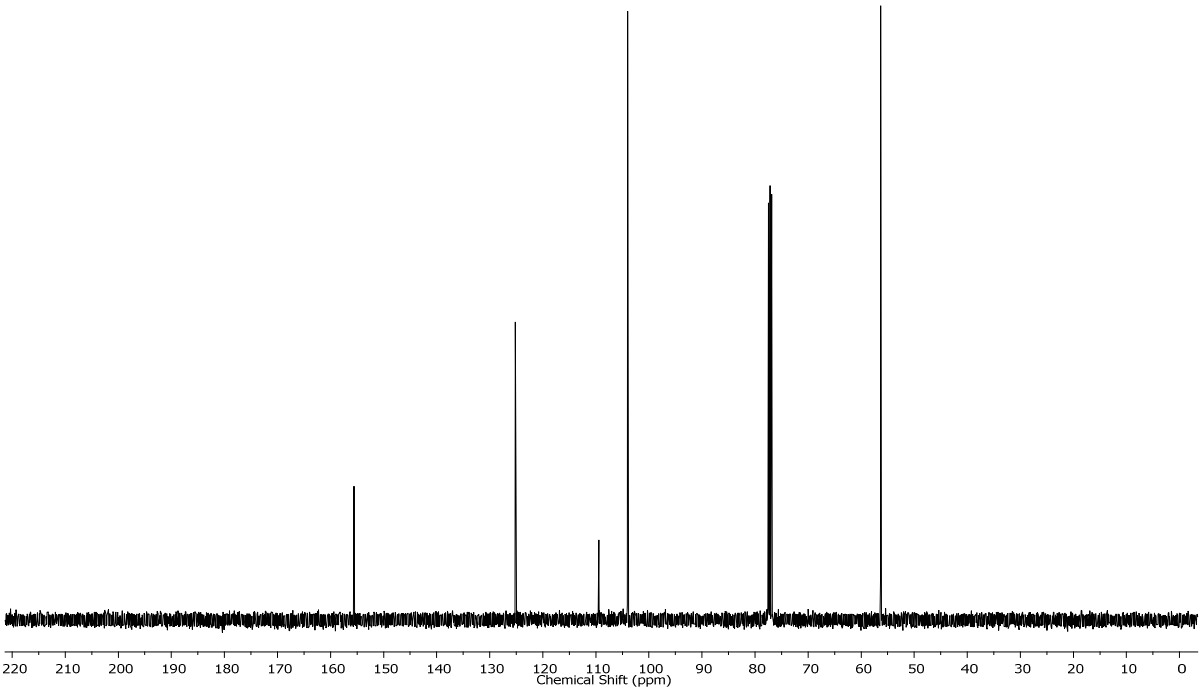
2,6-Dimethoxythiophenol (**1**)





2,6-Dimethoxythiophenol (**1**)





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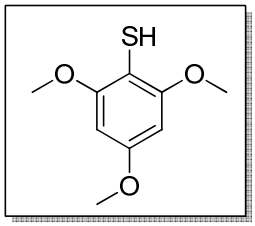

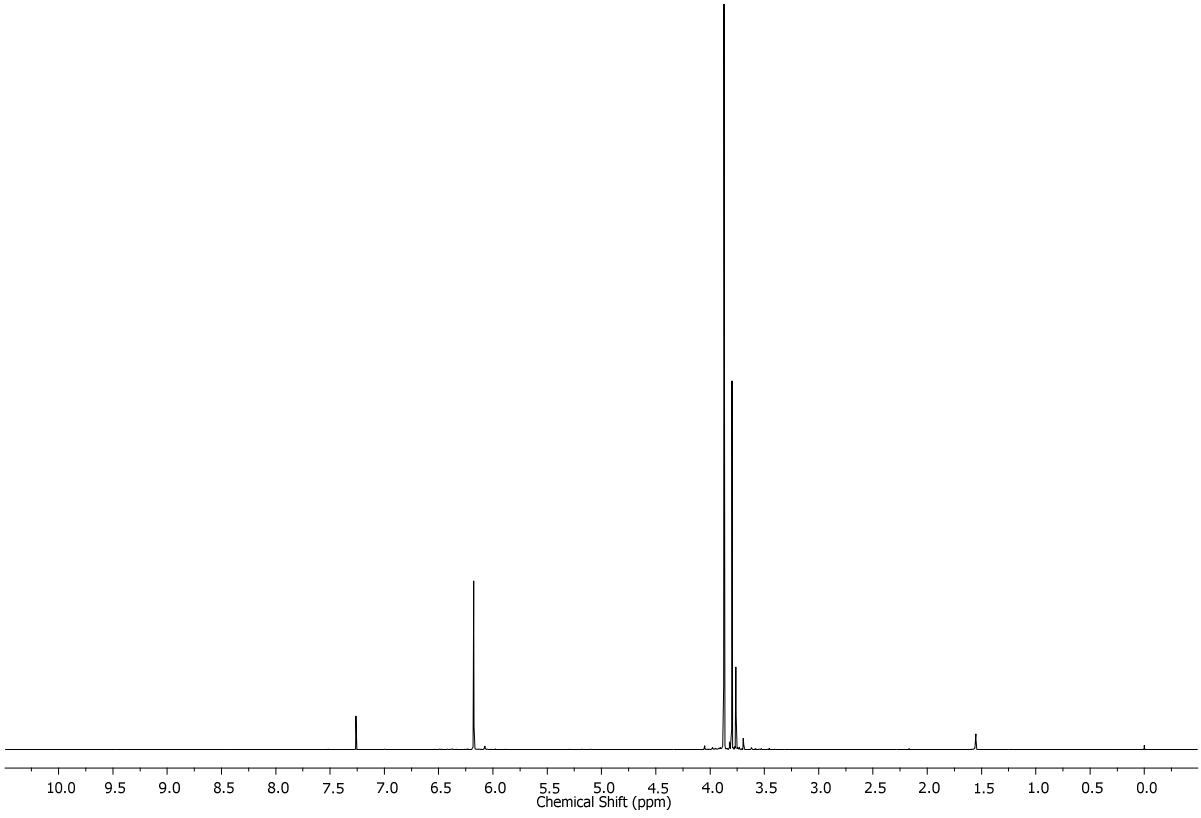

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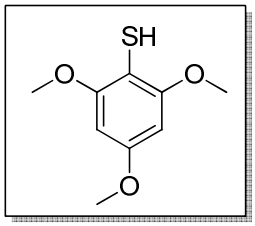

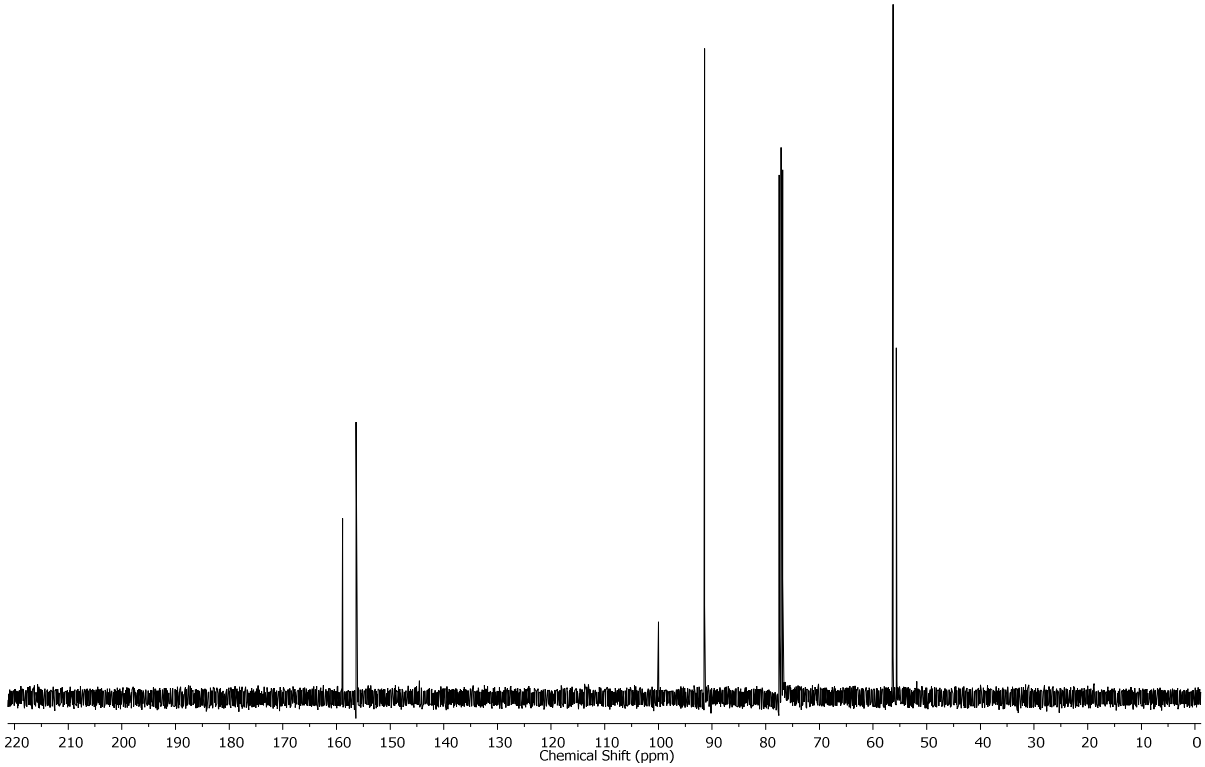

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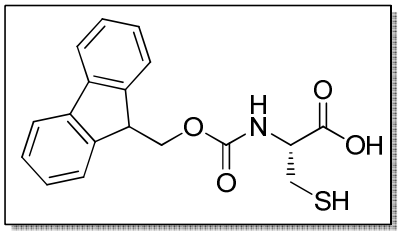
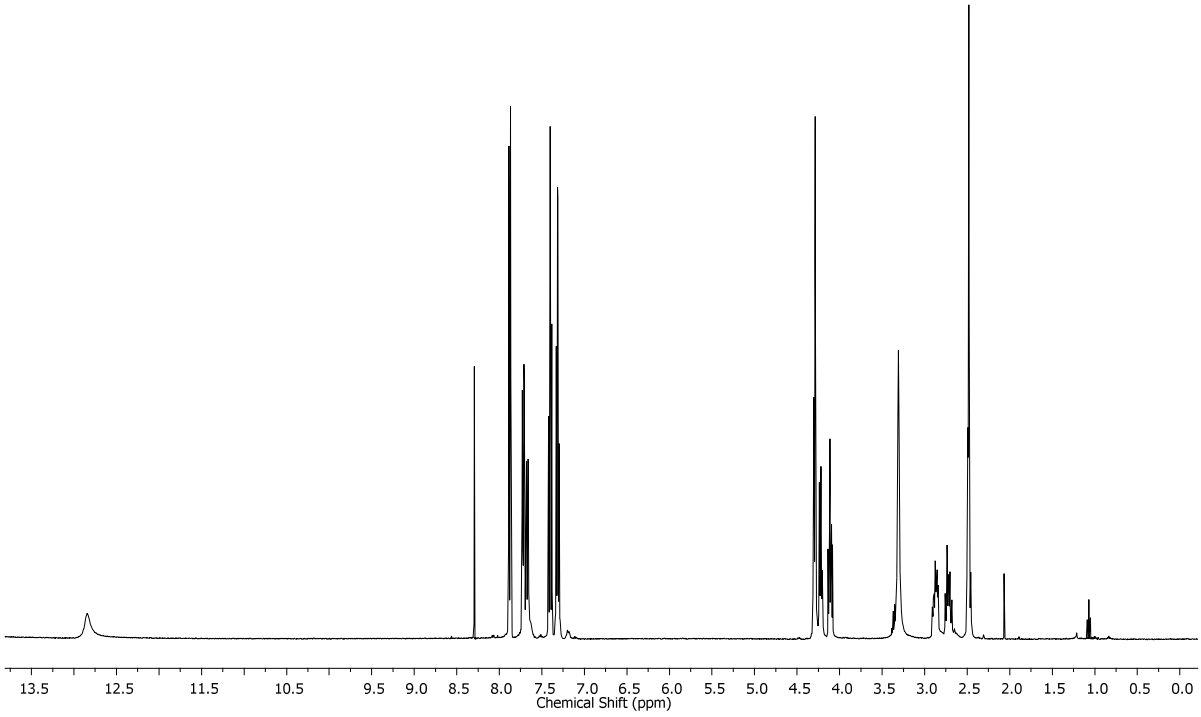
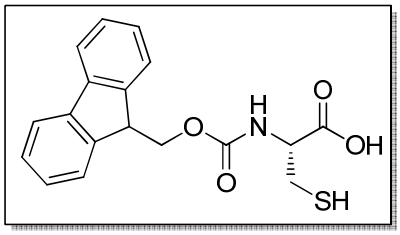
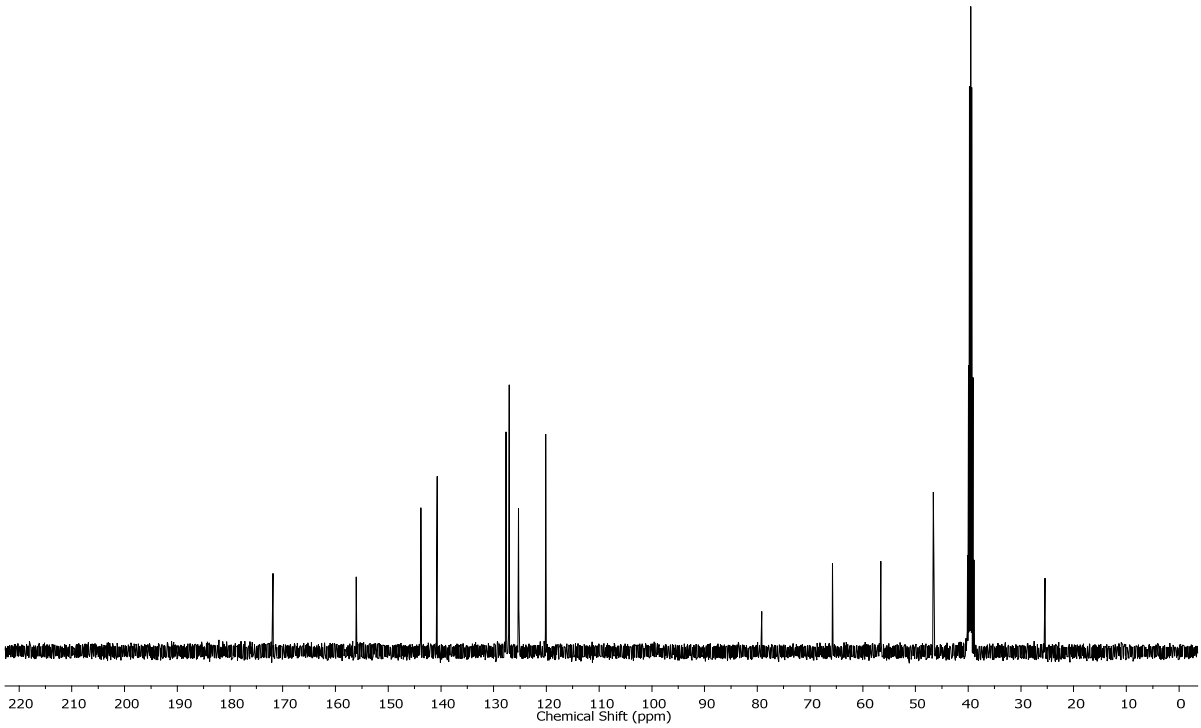




Fmoc-Cys-OH (3-1)





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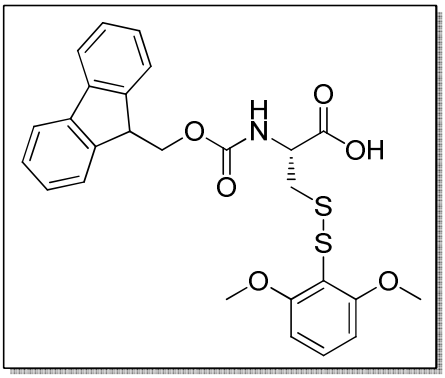

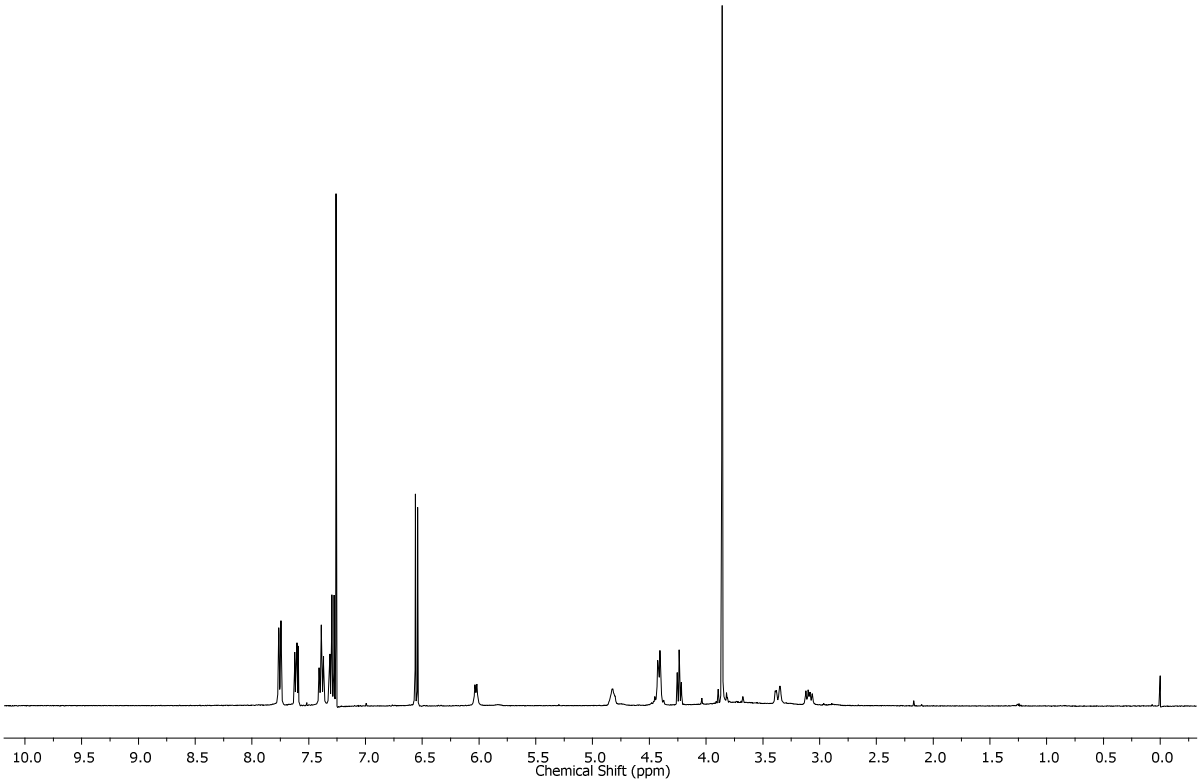

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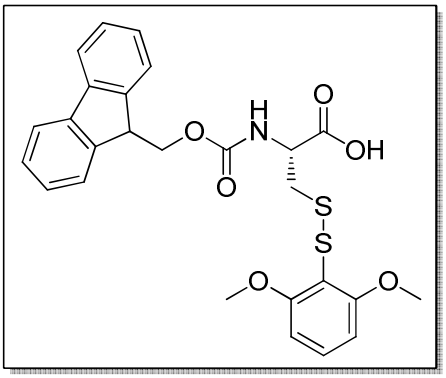

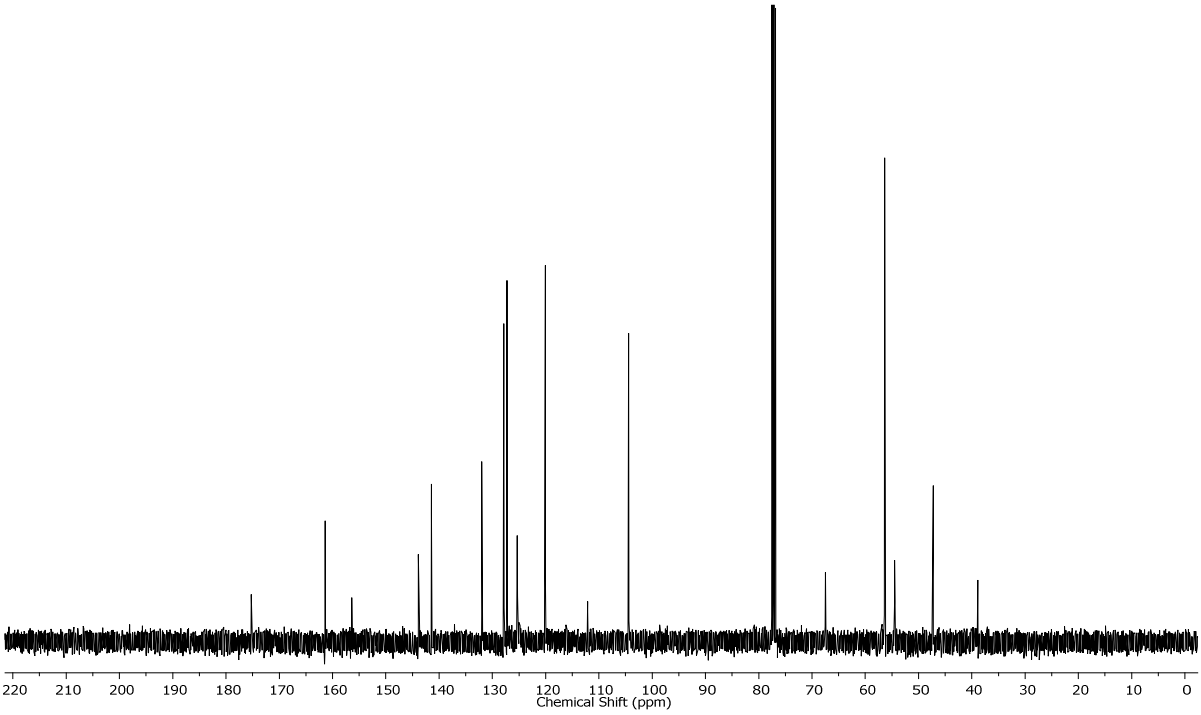

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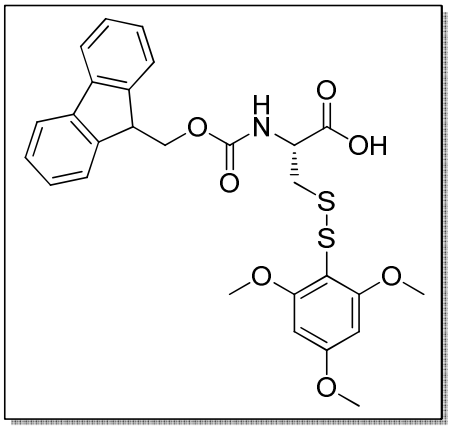
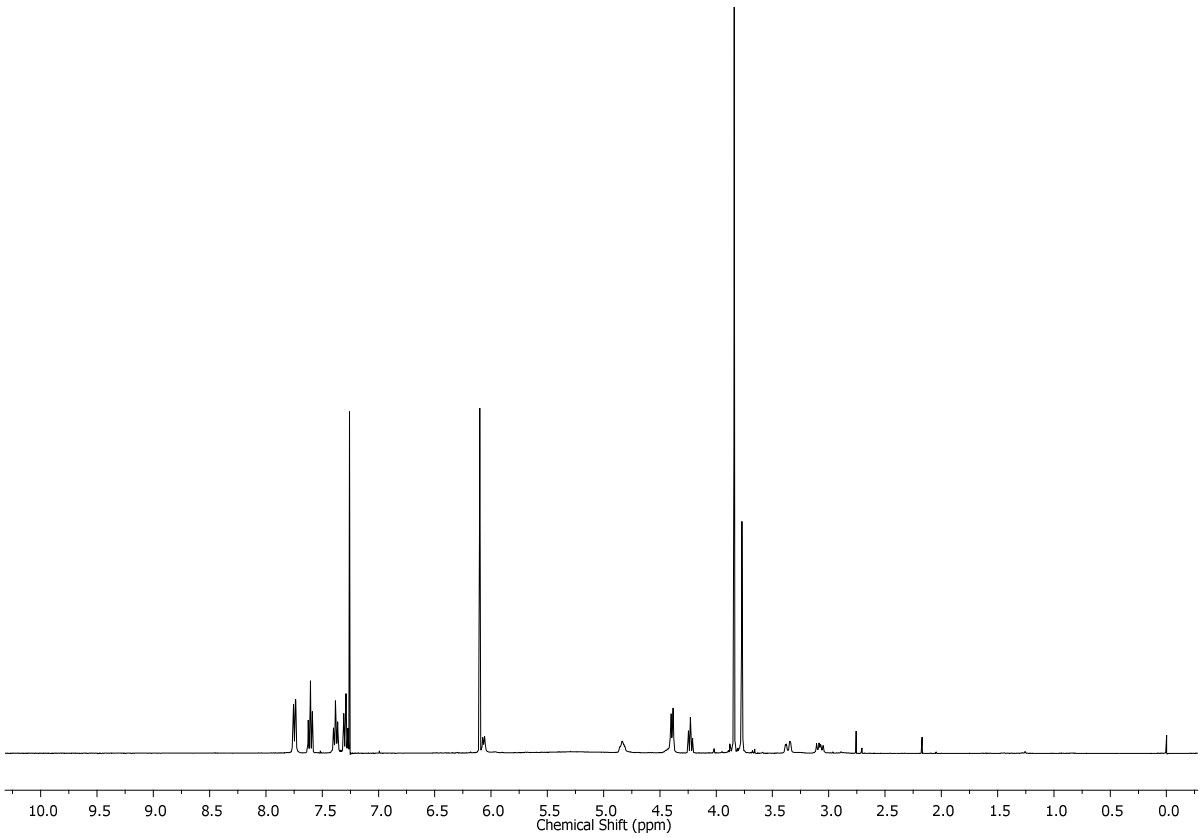
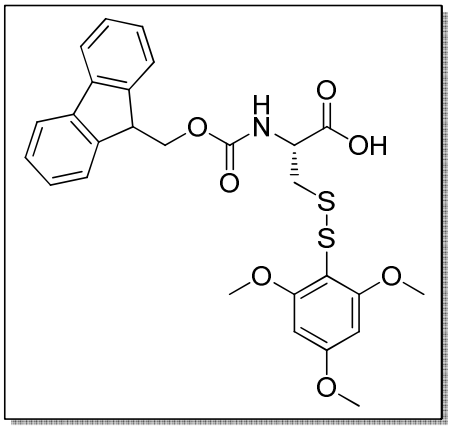
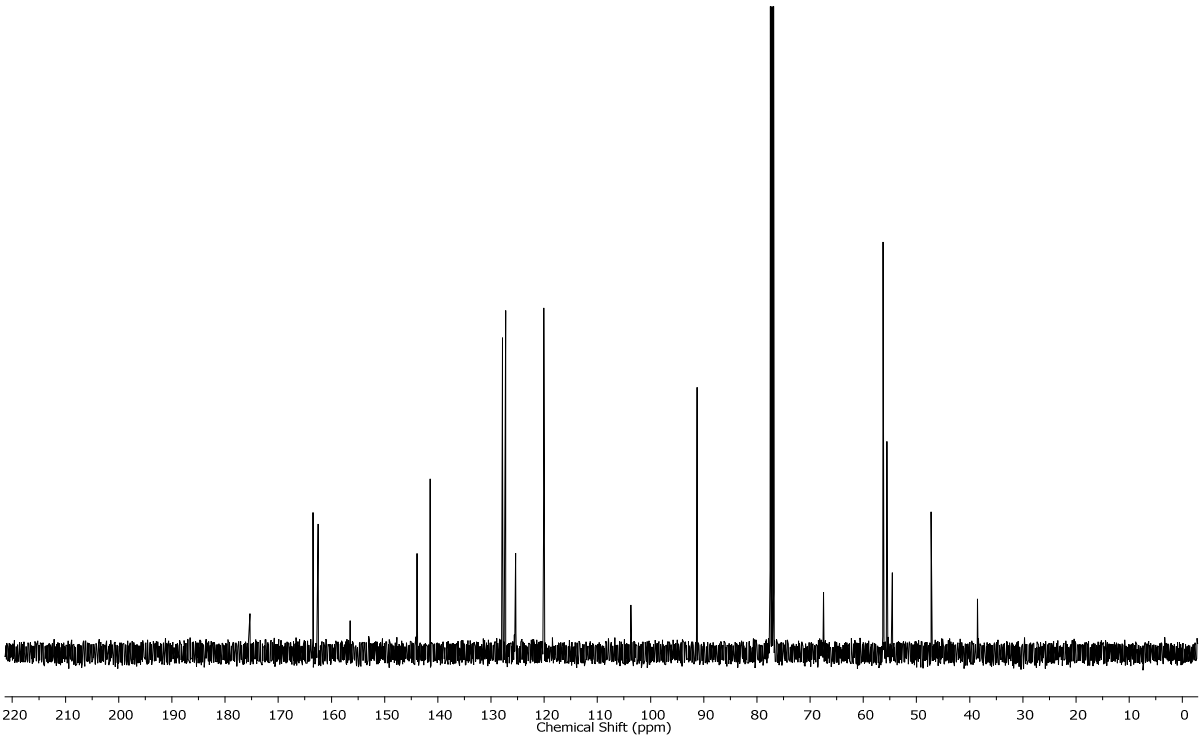




Fmoc-Cys(*S*-Tmp)-OH (4)





Chapter 2

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

Tobias M. Postma and Fernando Albericio

Organic Letters **2013**, *15*, 616-619

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

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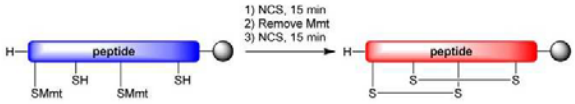
Received December 15, 2012

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

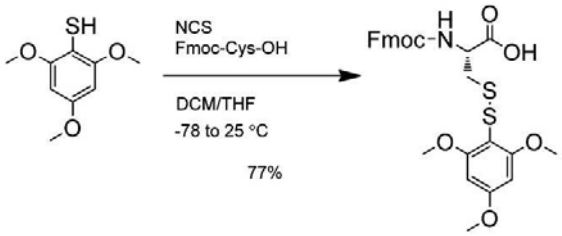
In view of the increasing number of multiple-disulfide-containing peptide drugs and the rising importance of this class of compounds, the challenge of preparing these molecules must be addressed. In this regard, the main challenge is the controlled formation of intramolecular disulfide bonds which assures 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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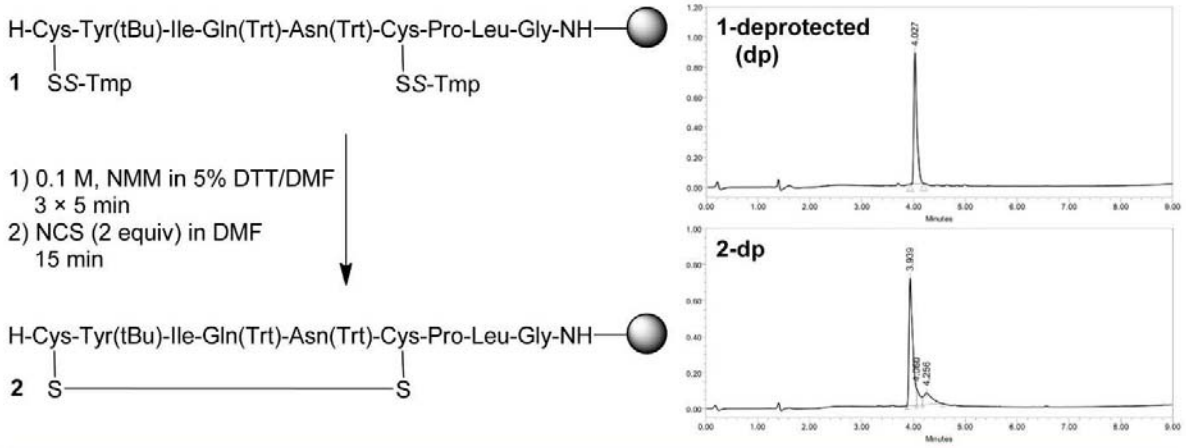
Scheme 2. On-Resin NCS Oxidation of Oxytocin



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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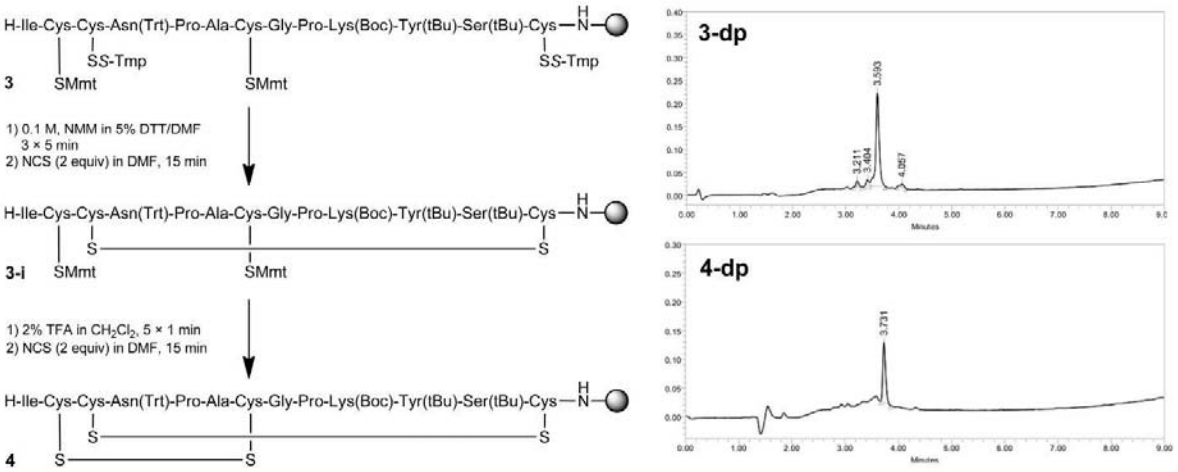
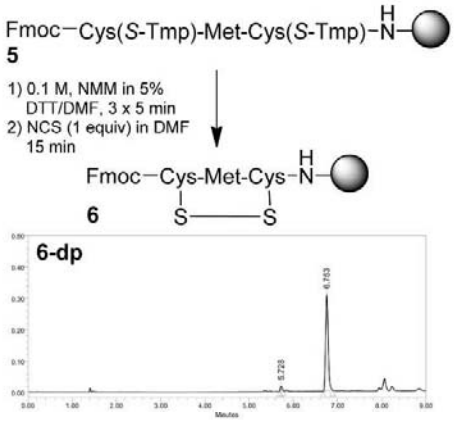
Scheme 3. Regioselective Synthesis of SI Conotoxin



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.

Chapter 2

Supporting Information

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

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Organic Letters **2013**, *15*, 616-619

2-SI.1. General Procedures

Fmoc-amino acid derivatives, Fmoc-Rink Amide AM resin and 2-CTC resin were obtained from IRIS Biotech (Marktredwitz, Germany). Fmoc-Cys(*S*-Tmp)-OH was prepared according to our previously published protocol.[1] Rink-Amide-Chemmatrix Low LOA was obtained from PCAS BioMatrix Inc. (Quebec, Canada). DIPEA, diisopropylcarbodiimide (DIC) and TFA were obtained from Aldrich (Milwaukee, USA). Oxyma was obtained from Luxembourg Industries Ltd. (Tel Aviv, Isreal). DMF, CH_2Cl_2, Et_2O, acetonitrile, DMSO and piperidine (HLPC grade) were obtained from SDS (Peypin, France). All reagents and solvents were used as received.

Room temperature (rt) refers to ambient temperature. Solid-phase syntheses were carried out manually in polypropylene syringed containing a polyethylene frit. Solvents and soluble reagents were removed by suction. Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min). Washings between deprotection and coupling were performed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Following the final coupling or deprotection the resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and dried under a stream of air. Yields for peptides refer to the area of the chromatographic product peak recorded at 220 nm.

High resolution mass spectrometry (HRMS) measurements were recorded on Thermo Scientific LTQ-FT Ultra spectrometer. Mass values are quoted within the error limits of ±5 ppm mass units. ESI refers to the electrospray ionization technique.

Analytical high pressure liquid chromatography (HPLC) was carried out on a Waters instrument comprising a separation module (Waters 2695), automatic injector, photodiode array detector (Waters 2998) and 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 $mL \cdot min^{-1}$ and a run time of 11 min.

LCMS was carried out 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 $mL \cdot min^{-1}$ and a run time of 11 min.

2-SI.2. General Methods Peptide Synthesis

General Method 1: Peptide Synthesis

Fmoc-Rink-Amide AM resin (0.45 mmol/gram, 1 equiv) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) followed by washing with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The protected Fmoc-amino acids (3 equiv) were incorporated using DIC (3 equiv.) and Oxyma (3 equiv) in DMF, as a coupling system, with 5 min preactivation for 1 h at rt. Washes between couplings and deprotections were performed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Completion of the coupling was monitored by the Kaiser test.[2] Following elongation a microcleavage was performed, 5 mg of dry resin was treated with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1) for analysis by HPLC and LCMS.

General Method 2: Deprotection of disulfide containing protecting groups

The resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Deprotection was achieved by treatment with 0.1 M *N*-methylmorpholine in 5% dithiothreitol (DTT)/DMF (3 × 5 min) and subsequently washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min).

General Method 3: Disulfide Formation

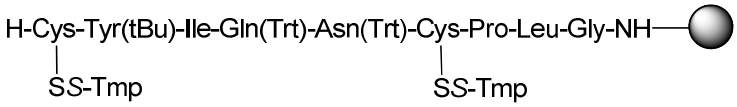
The resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Deprotection of the disulfide containing protecting group was achieved with either General Method 2, unless otherwise specified. The resin was treated with a solution of NCS (2 equiv) in DMF (100 µL/µmol peptide) and agitated for 15 min. The resin was washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min).

General Method 4: Microcleavage

Dry resin (5 mg) was treated with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1) for analysis by HPLC and LCMS.

2-SI.3. Peptide Synthesis

H-oxytocin(*S*-Tmp)$_2$-NH-resin (1)



Peptide **1** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (1.11 g, 0.5 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin): *S*-Tmp was removed using General Method 2, and cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **1-deprotected (dp)** was obtained in 96**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 4.0 min). **LCMS** observed $[M+H]^+$ 1010.3, required $[M+H]^+$ 1010.2.

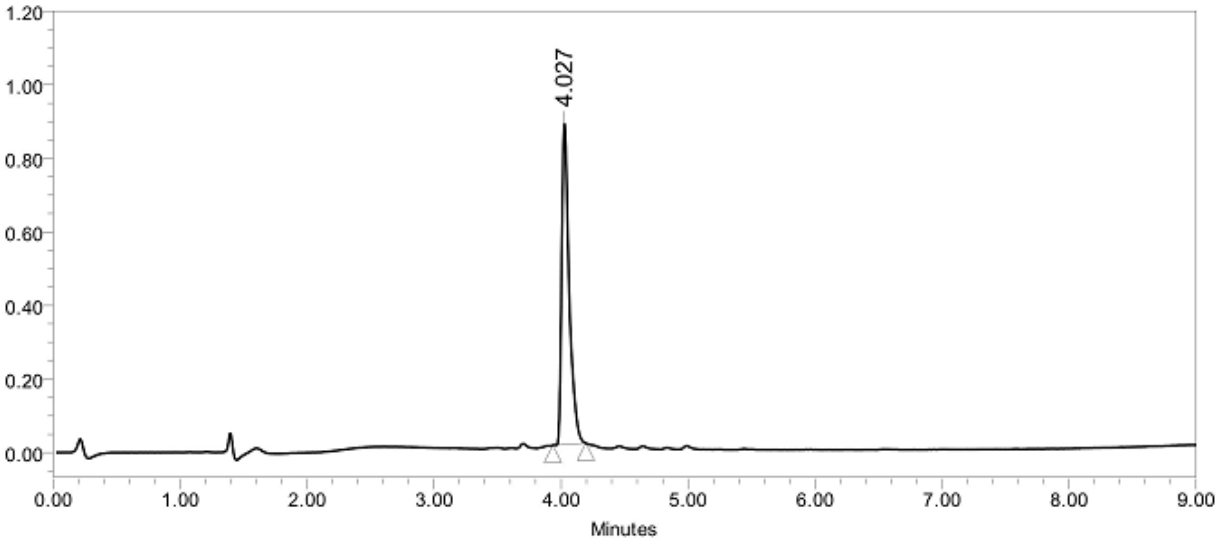
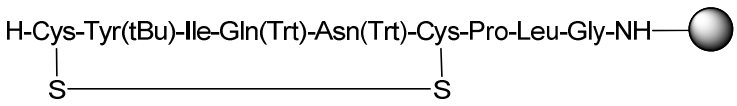


Figure S-1: HPLC chromatogram of peptide **1-dp**

H-oxytocin-NH-resin (2)



Peptide **2** was prepared by removing *S*-Tmp from the resin (10 mg, 5 µmol) containing peptide **1** according to General Method 2. The peptidyl resin was treated with NCS (2 equiv) following General Method 3. Cleavage of the peptide was achieved using TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1). HPLC analysis found that peptide **2-dp** was obtained in 75**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 3.9 min). **LCMS** observed $[M+H]^+$ 1007.4444, required 1007.4443.

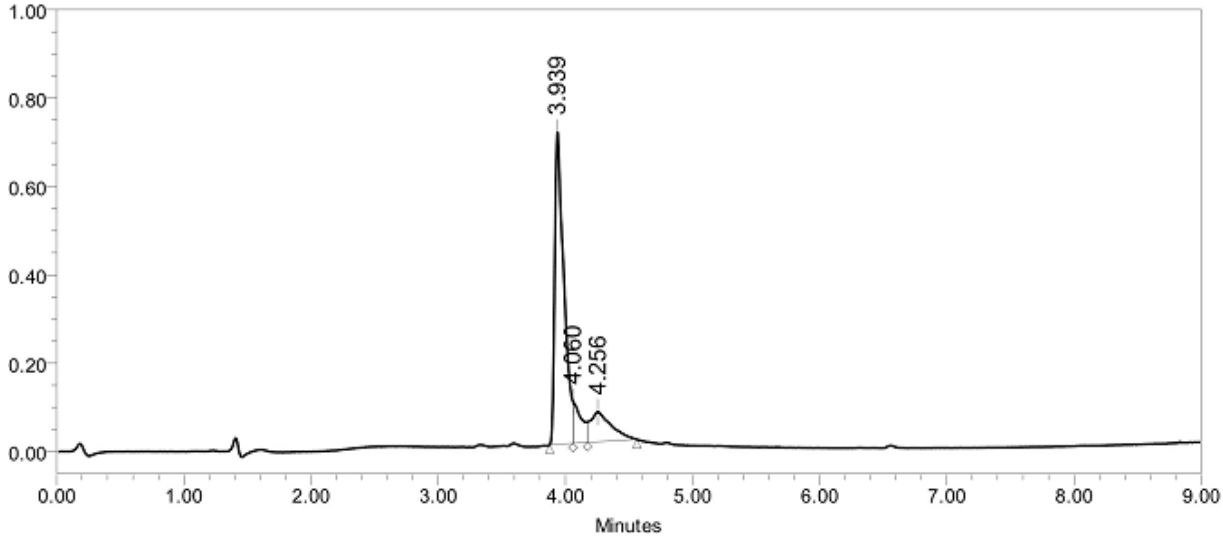
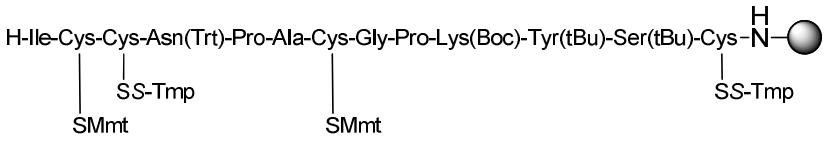


Figure S-2: HPLC chromatogram of peptide **2-dp**

H-SI conotoxin(Mmt)$_2$(*S*-Tmp)$_2$-NH-resin (3)



Linear SI conotoxin (**3**) was prepared according to General Method 1 using Fmoc-Rink-Amide AM resin (166.7 mg, 0.075 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin): *S*-Tmp was removed using General Method 2, and cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **3-dp** was

obtained in 87**%** purity (linear gradient from 0% to 30% acetonitrile over 8 min, t_R : 3.6 min). **HRMS** observed $[M+H]^+$ 1357.5535, required $[M+H]^+$ 1357.5520.

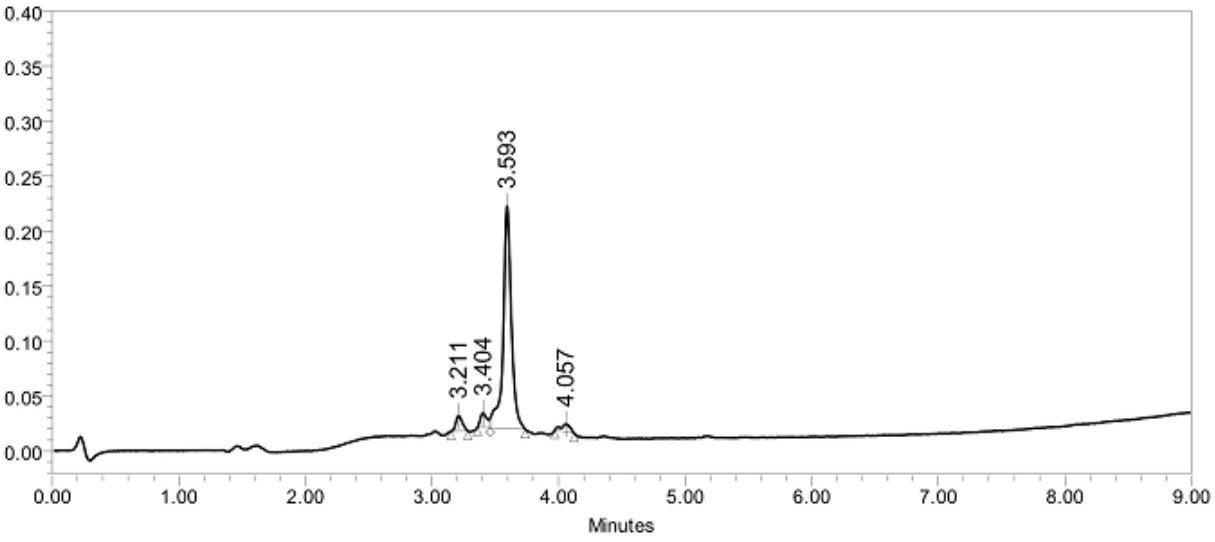
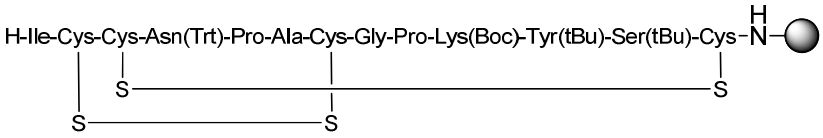


Figure S-3: HPLC chromatogram of peptide **3-dp**

H-SI_conotoxin-NH-resin (4)



Resin containing peptide **3** (10 mg, 5 µmol) was treated with a deprotection mixture to remove *S*-Tmp according to General Method 2. Subsequently, the resin was treated with NCS (2 equiv) to form the first disulfide (Cys^3-Cys^{13}) following General Method 3. Mmt was removed by treating the resin with 2% TFA/CH_2Cl_2 (5 × 2 min) and the resin washed with CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The resin was treated with NCS (2 equiv) to form the second disulfide (Cys^2-Cys^7) following General Method 3. HPLC analysis found that peptide **4-dp** was obtained in 70**%** purity (linear gradient from 0% to 30% acetonitrile over 8 min, t_R : 3.7 min). **HRMS** observed $[M+H]^+$ 1353.5233, required $[M+H]^+$ 1353.5210.

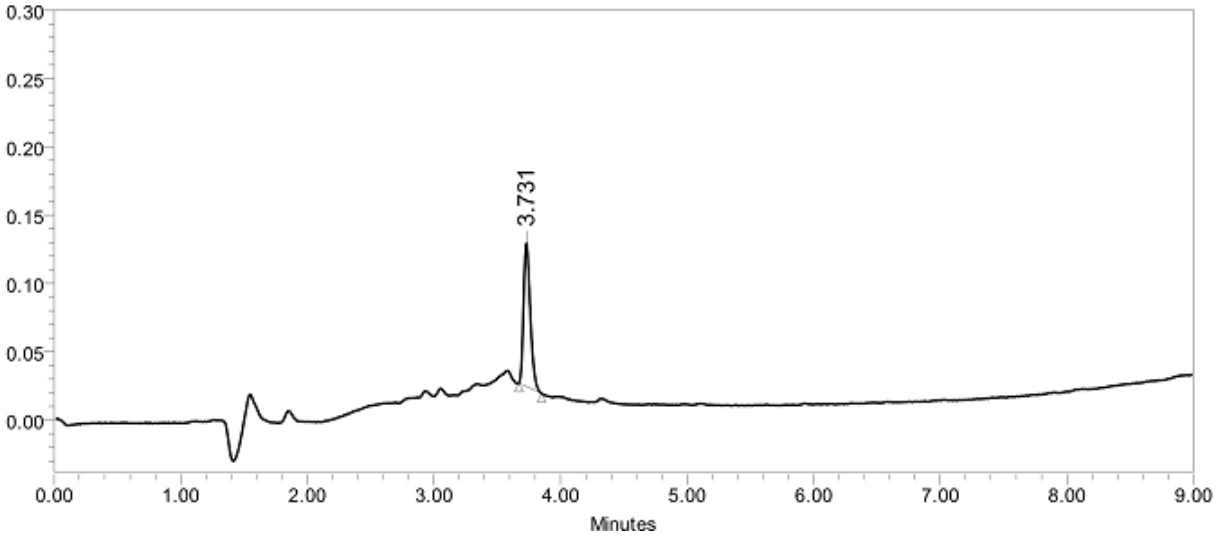


Figure S-4: HPLC chromatogram of peptide **4-dp**

Fmoc-Cys(*S*-Tmp)-Met-Cys(*S*-Tmp)-NH-resin (5)

Model tripeptide **5** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (55.6 mg, 0.025 mmol, 0.45 mmol/gram). Microcleavage (5 mg resin): *S*-Tmp was removed using General Method 2, and cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **5-dp** was obtained in 99**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 6.8 min). **LCMS** observed $[M+H]^+$ 577.2, required $[M+H]^+$ 577.2.

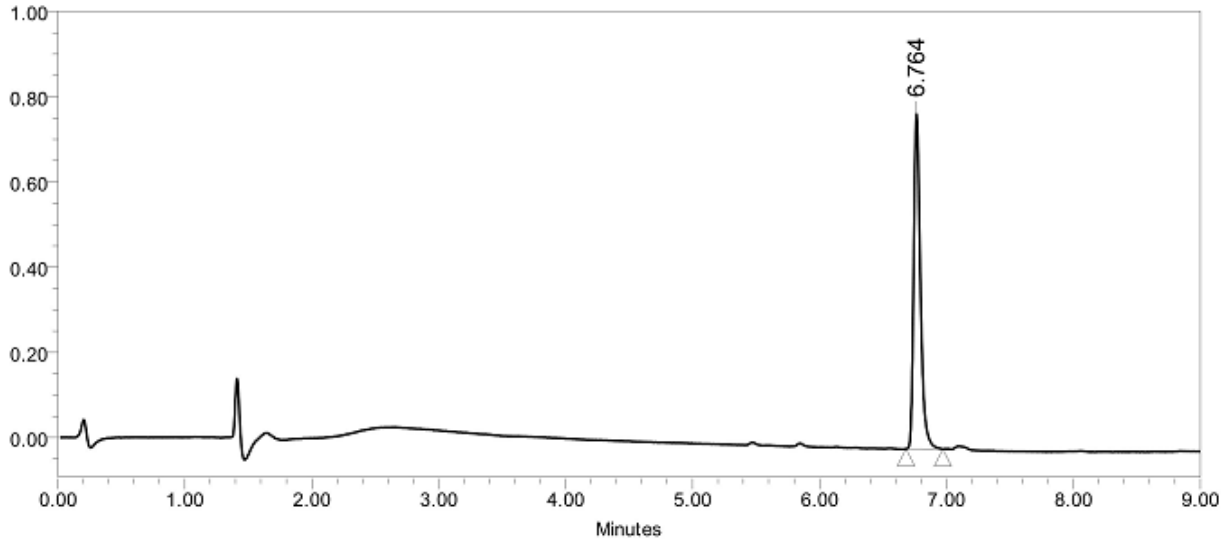
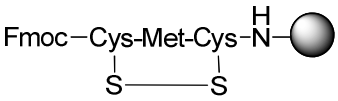


Figure S-5: HPLC chromatogram of peptide **5-dp**

Fmoc-Cys-Met-Cys-NH-resin (6)



The resin containing peptide **5** (10 mg, 5 µmol) was treated with a deprotection mixture to remove *S*-Tmp according to General Method 2. The resin was treated with a solution of NCS (1, 1.05 or 2 equiv) in DMF (100 µL/µmol or 200 µL/µmol peptide) and agitated for 15 min. The resin was washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min) and cleavage of the peptide was achieved using TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1). HPLC analysis showed that 1.05 equiv of NCS in DMF (200 µL/µmol peptide) gave the best result with less than 2% oxidized Met. Peptide **6-dp** was obtained in 78**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 6.8 min). **LCMS** observed $[M+H]^+$ 575.2, required $[M+H]^+$ 575.1. Peptide **6-dp** with oxidized Met (1.9%) (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 5.7 min). **LCMS** observed $[M+H]^+$ 591.2, required $[M+H]^+$ 591.7.

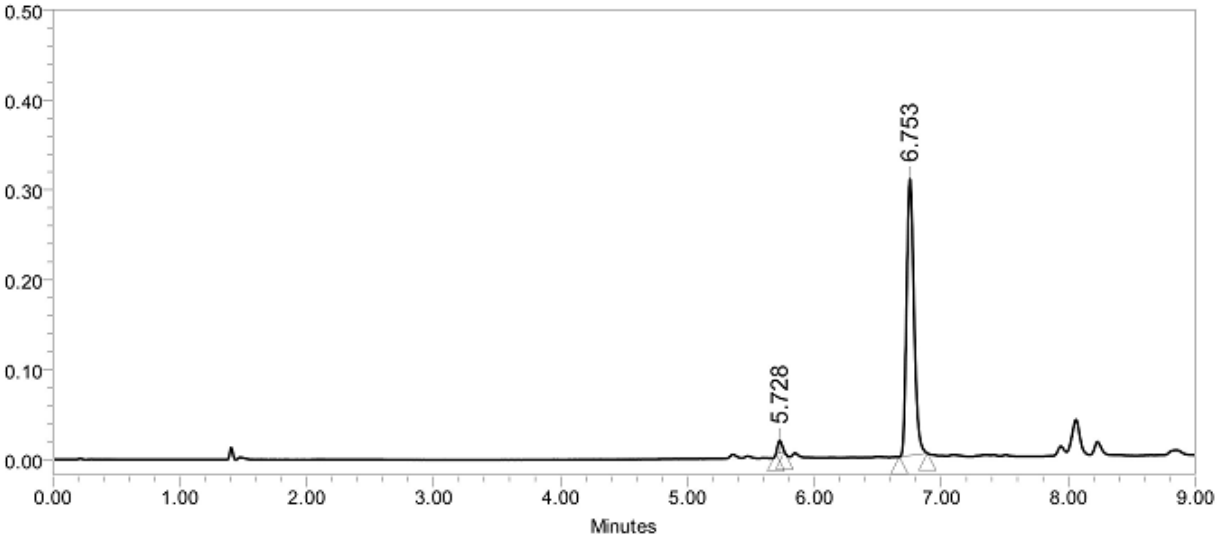
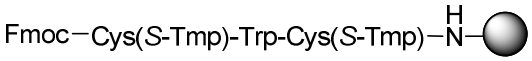


Figure S-6: HPLC chromatogram of peptide **6-dp**

Fmoc-Cys(*S*-Tmp)-Met-Cys(*S*-Tmp)-NH-resin (7)



Model tripeptide **7** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (55.6 mg, 0.025 mmol, 0.45 mmol/gram). Microcleavage (5 mg resin): *S*-Tmp was removed using General Method 2, and cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **7-dp** was obtained in 94**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 7.0 min). **LCMS** observed $[M+H]^+$ 632.1, required $[M+H]^+$ 632.2.

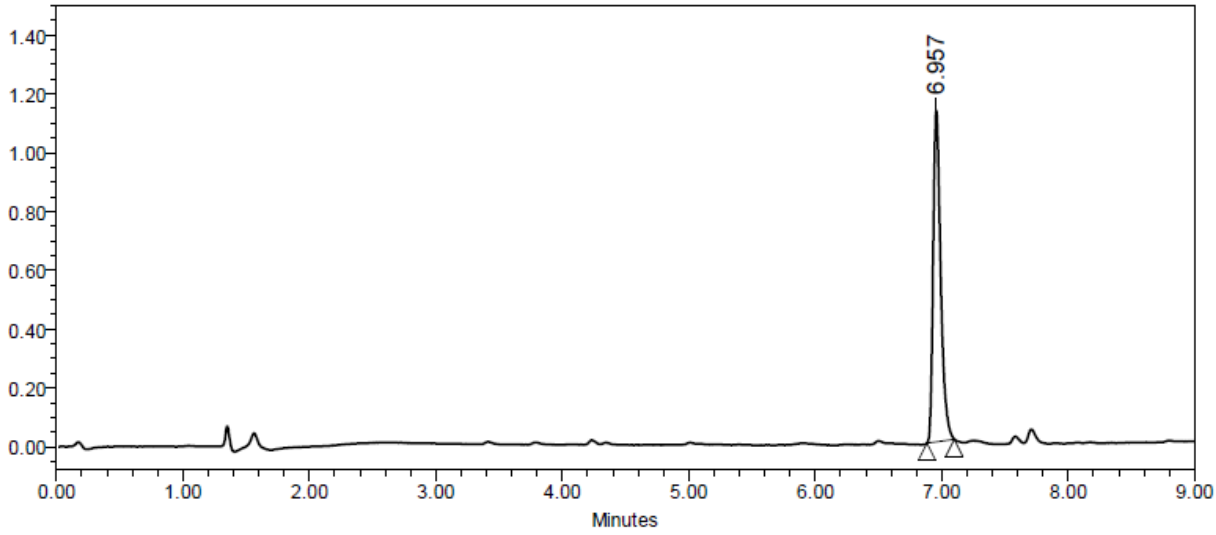
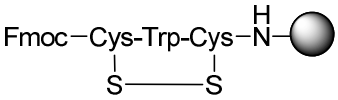


Figure S-7: HPLC chromatogram of peptide **7-dp**

Fmoc-Cys-Trp-Cys-NH-resin (8)



The resin containing peptide **7** (10 mg, 5 µmol) was treated with a deprotection mixture to remove *S*-Tmp according to General Method 2. The resin was treated with a solution of NCS (2 equiv) in DMF (100 µL/µmol) and agitated for 15 min. The resin was washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min) and cleavage of the peptide was achieved using TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1). Peptide **8-dp** was obtained in 91**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 7.0 min). **LCMS** observed $[M+H]^+$ 630.1, required $[M+H]^+$ 630.2.

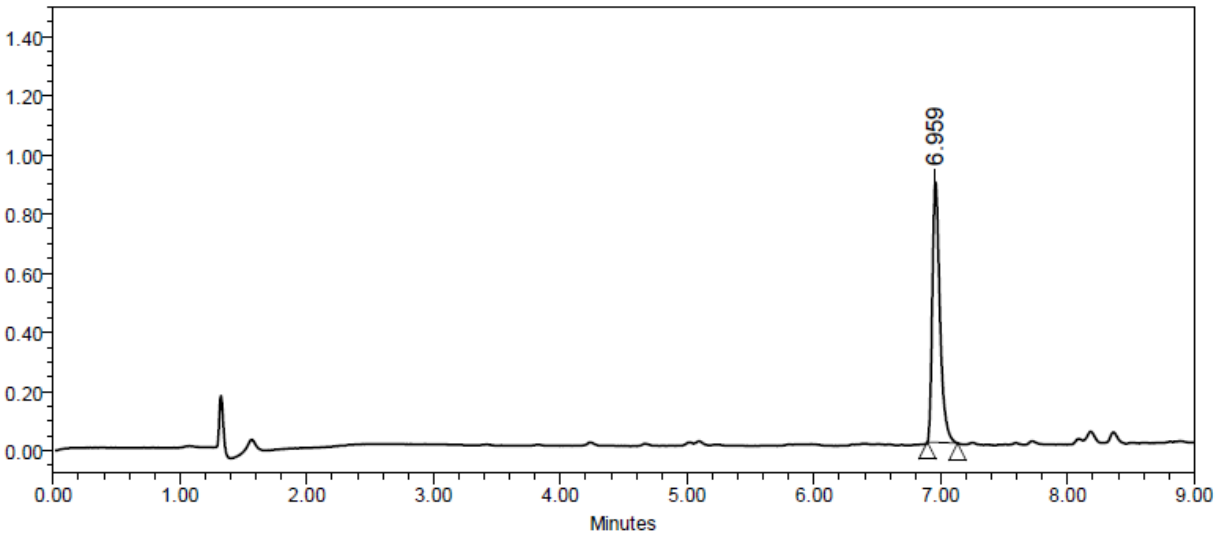


Figure S-8: HPLC chromatogram of peptide **8-dp**

2-SI.4. References

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Chapter 3

N-Chlorosuccinimide, an Efficient Peptide Disulfide Bond-Forming Reagent in Aqueous Solution

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RSC Advances **2013**, *3*, 14277-14280

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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

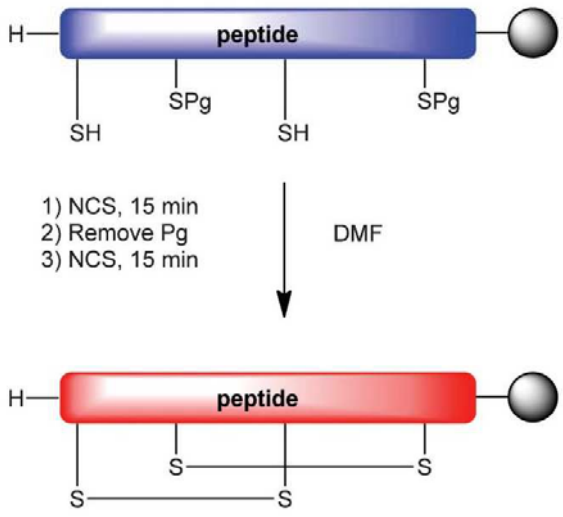

Fig. 1 Regioselective on-resin NCS oxidation.

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† Electronic supplementary information (ESI) available: Detailed experimental procedures, characterization, spectroscopic and chromatographic data. See DOI: 10.1039/c3ra43149e

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.

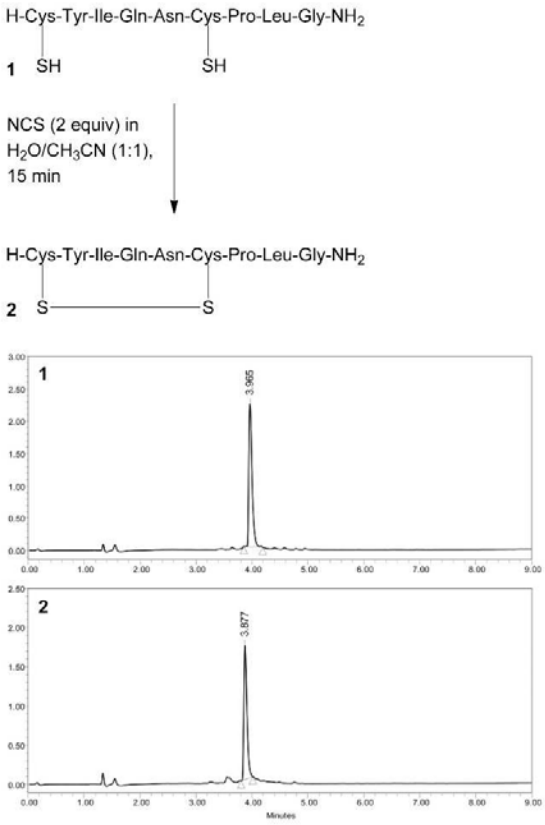
Our model system was based on the well-studied oxytocin, a nonapeptide currently used as a drug in obstetric medicine to facilitate childbirth.[12] Oxytocin contains one disulfide bond and a C-terminal amide. The peptide was synthesized on the solid-phase using standard Fmoc/*t*Bu chemistry on a Rink 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 temperature, lyophilized, and subsequently analyzed. Oxidized cyclic octreotate (**4**) was formed in high purity, and no linear octreotate was present (Scheme 2).



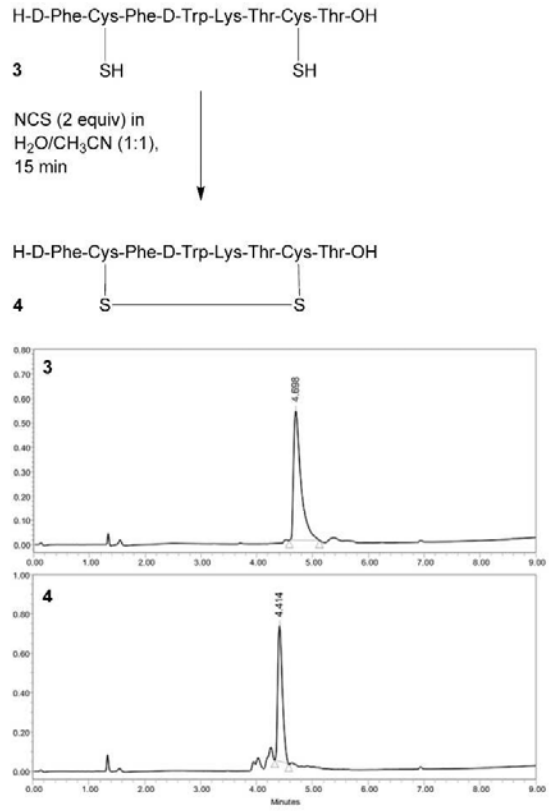
Scheme 1 Aqueous NCS oxidation of oxytocin.

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.

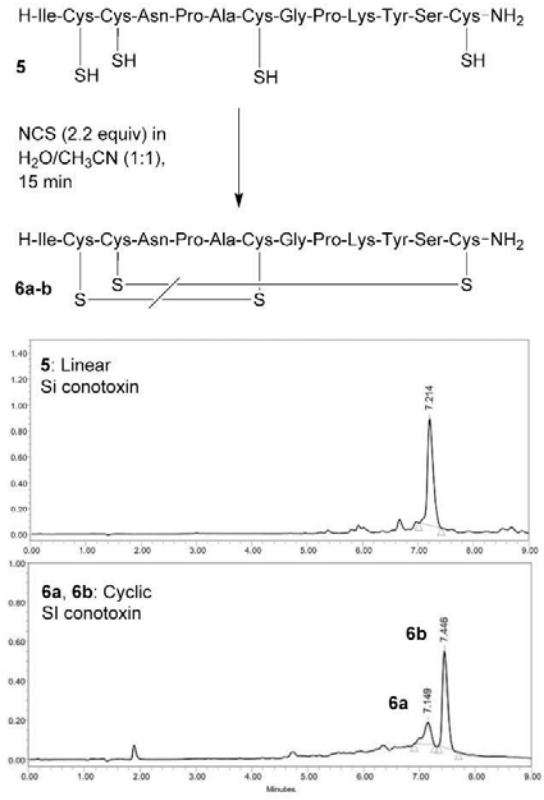


Fig. 2 HPLC chromatograms of linear and cyclic Si conotoxin.

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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Chapter 3

Supporting Information

N-Chlorosuccinimide, an Efficient Peptide Disulfide Bond-Forming Reagent in Aqueous Solution

Tobias M. Postma and Fernando Albericio,

RSC Advances **2013**, *3*, 14277-14280

3-SI.1. General Procedures

Fmoc-amino acid derivatives, Fmoc-Rink Amide AM resin and 2-CTC resin were obtained from IRIS Biotech (Marktredwitz, Germany). Fmoc-Cys(*S*-Tmp)-OH was prepared according to our previously published protocol.[1] Diisopropylcarbodiimide (DIC) and TFA were obtained from Aldrich (Milwaukee, USA). Oxyma was obtained from Luxembourg Industries Ltd. (Tel Aviv, Isreal). DMF, CH_2Cl_2, Et_2O, acetonitrile, DMSO and piperidine (HLPC grade) were obtained from SDS (Peypin, France). All reagents and solvents were used as received.

Room temperature (rt) refers to ambient temperature. Solid-phase syntheses were carried out manually in polypropylene syringed containing a polyethylene frit. Solvents and soluble reagents were removed by suction. Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min). Washings between deprotection and coupling were performed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Following the final coupling or deprotection the resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and dried under a stream of air. Yields for peptides refer to the area of the chromatographic product peak recorded at 220 nm.

High resolution mass spectrometry (HRMS) measurements were recorded on Thermo Scientific LTQ-FT Ultra spectrometer. Mass values are quoted within the error limits of ±5 ppm mass units. ESI refers to the electrospray ionization technique.

Analytical high pressure liquid chromatography (HPLC) was carried out on a Waters instrument comprising a separation module (Waters 2695), automatic injector, photodiode array detector (Waters 2998) and system controller (Empower login), with an SunFire C18 analytical reversed-phase HPLC column (2.1 mm × 100 mm, 5 µm) or 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^{-1} and a run time of 11 min.

LCMS was carried out 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^{-1} and a run time of 11 min.

3-SI.2. General Methods Peptide Synthesis

General Method 1: Peptide Synthesis

Fmoc-Rink-Amide AM resin (0.45 mmol/gram, 1 equiv.) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) followed by washing with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The protected Fmoc-amino acids (3 equiv.) were incorporated using DIC (3 equiv.) and Oxyma (3 equiv.) in DMF, as a coupling system, with 5 min preactivation for 1 h at rt. Washes between couplings and deprotections were performed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Completion of the coupling was monitored by the Kaiser test.[2] Following elongation a microcleavage was performed, 5 mg of dry resin was treated with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1) for analysis by HPLC and LCMS.

General Method 2: Deprotection of *S*-Tmp protecting groups

The resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Deprotection was achieved by treatment with 0.1 M *N*-methylmorpholine in 5% dithiothreitol (DTT)/DMF (3 × 5 min) and subsequently washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min).

General Method 3: Disulfide Formation

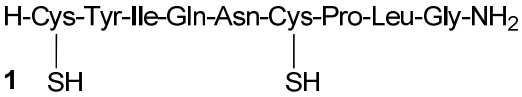
The fully deprotected peptide was dissolved in H_2O or H_2O/acetonitrile. A solution of NCS (1.0-2.2 equiv.) in H_2O or H_2O/acetonitrile was added and the mixture was shaken for 15 min at room temperature. Subsequently, the mixture was frozen in N_2 (l) and lyophilized.

General Method 4: Microcleavage

Dry resin (5 mg) was treated with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1) for analysis by HPLC and LCMS.

3-SI.3. Peptide Synthesis

H-oxytocin(2 x SH)-NH_2 (1)



Peptide **1** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (1.11 g, 0.5 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). The Cys protecting group *S*-Tmp was removed according to General Method 2. A microcleavage was performed according to General Method 4 and subsequent HPLC analysis found that peptide **1** was obtained in 97**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 4.0 min). **HRMS** observed $[M+H]^+$ 1009.4594, required $[M+H]^+$ 1009.4594.

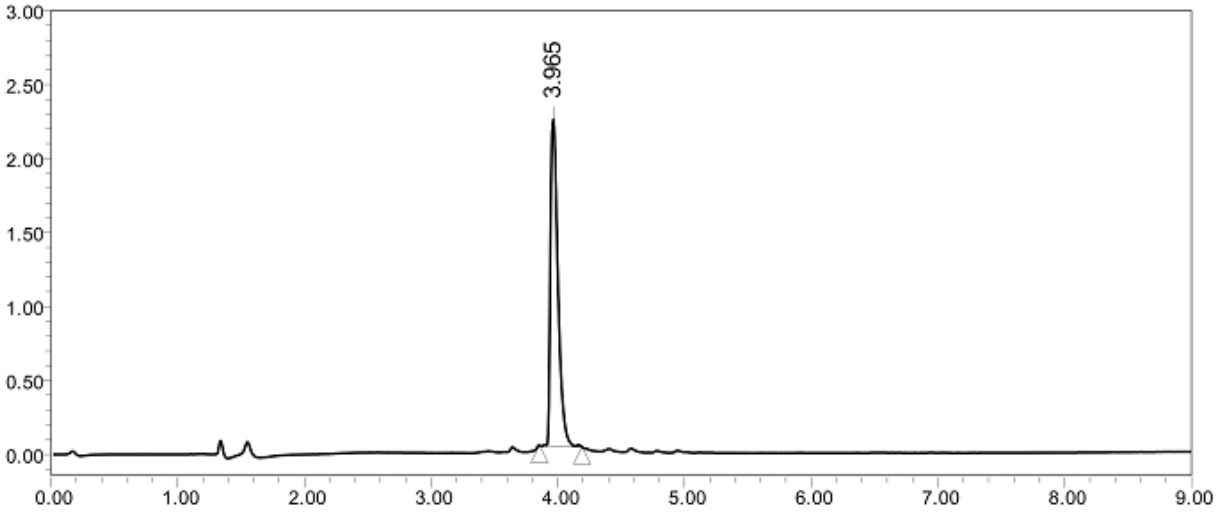
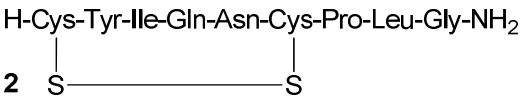


Figure S-1: HPLC chromatogram of peptide **1**

H-oxytocin-NH_2 (2)



Peptide **2** was prepared by removing *S*-Tmp from the resin containing peptide **1** (5 mg, 2.5 µmol) according to General Method 2, followed by cleavage according to General Method 4. The crude peptide was oxidized according to General Method 3 and subsequently analyzed by HPLC and LCMS. For the best example, HPLC analysis found that peptide **2-5** was obtained in 95**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 3.9 min). **HRMS** observed $[M+H]^+$ 1007.4437, required 1007.4443.

Oxytocin	H_2O/CH_3CN	NCS equiv.	Purity (%)
2-1	1:0	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

Table 1: Results of oxytocin NCS oxidation under different conditions

H-oxytocin-NH_2 (2-1)

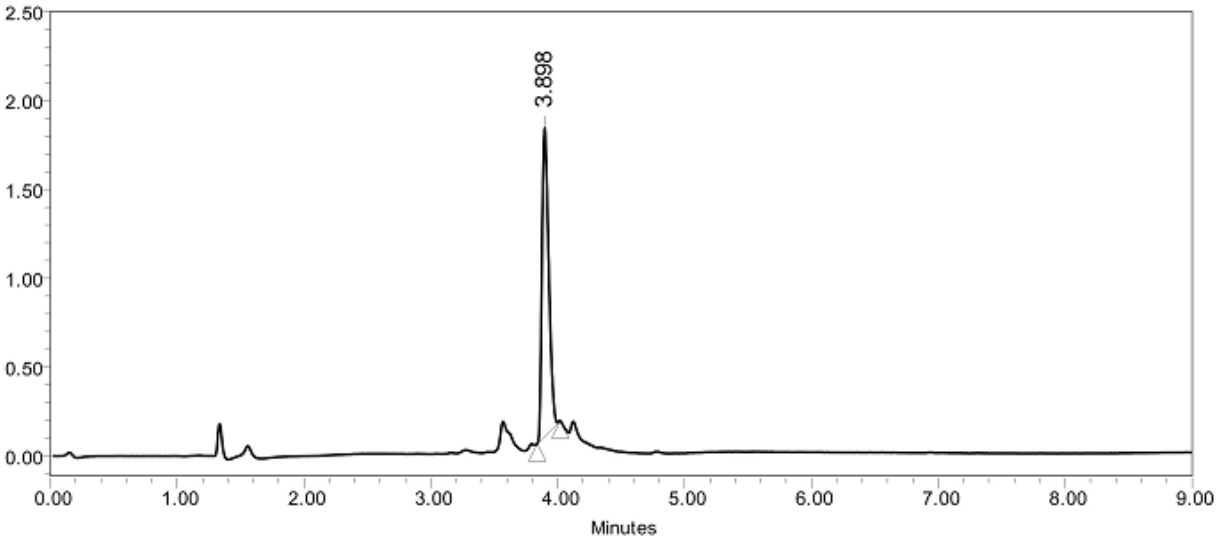


Figure S-2: HPLC chromatogram of peptide **2-1**

H-oxytocin-NH_2 (2-2)

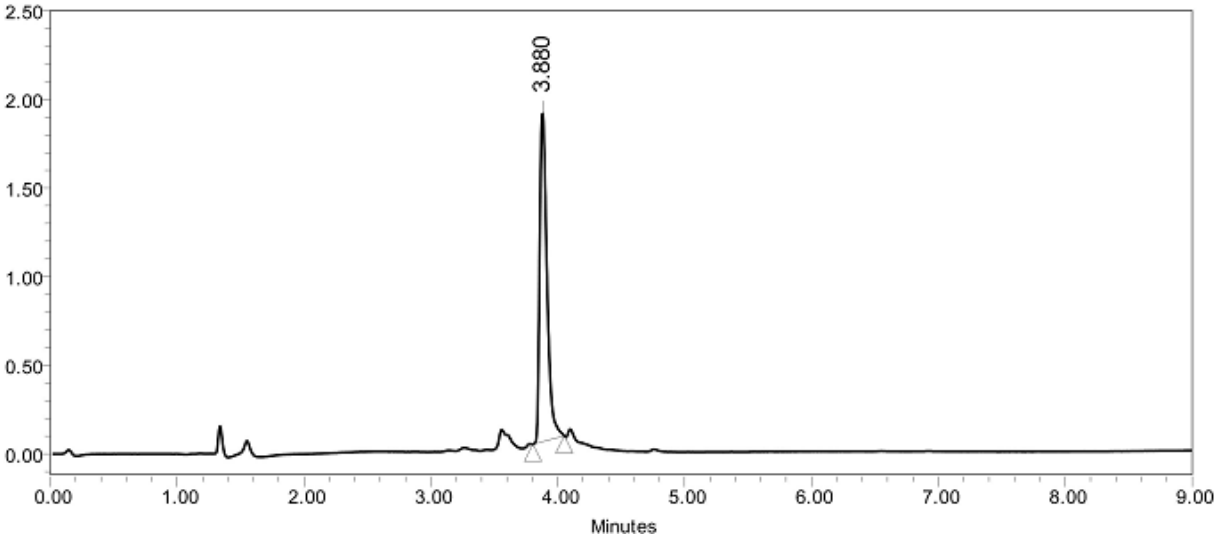


Figure S-3: HPLC chromatogram of peptide **2-2**

H-oxytocin-NH_2 (2-3)

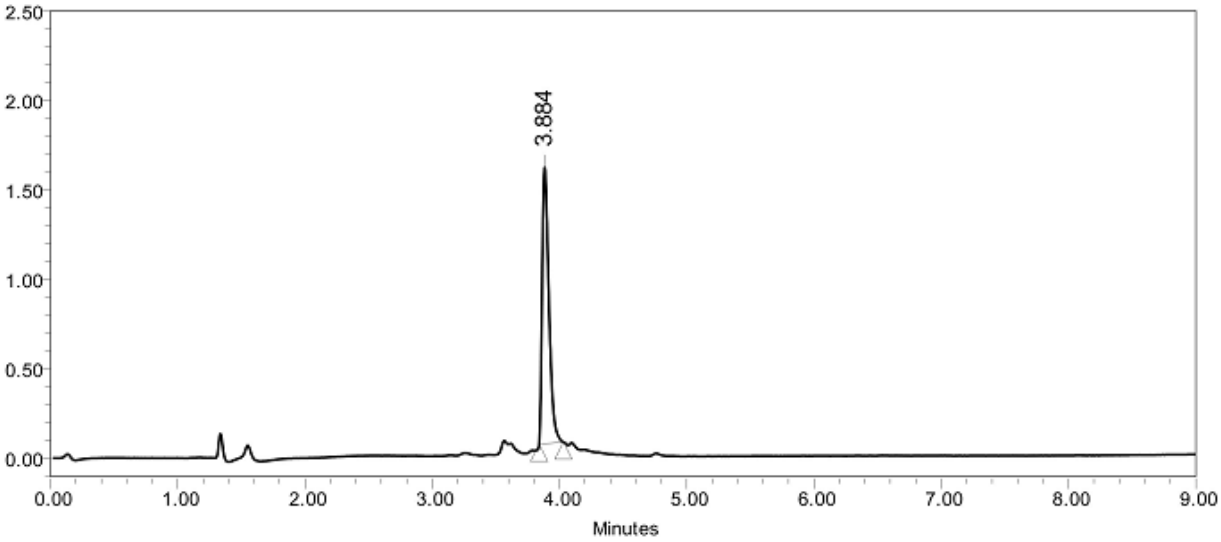


Figure S-4: HPLC chromatogram of peptide **2-3**

H-oxytocin-NH_2 (2-4)

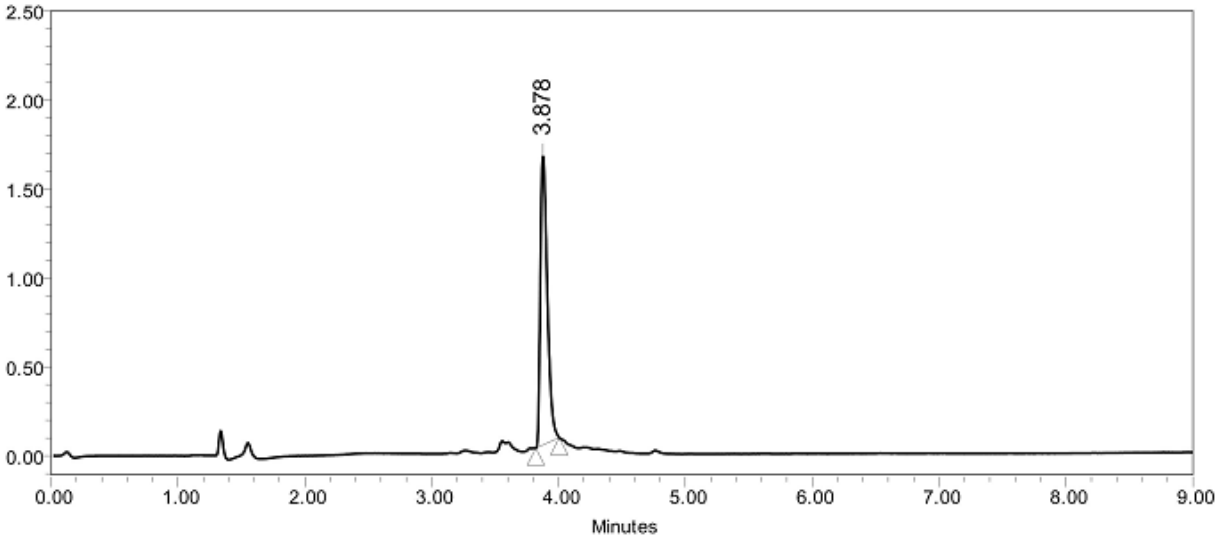


Figure S-5: HPLC chromatogram of peptide **2-4**

H-oxytocin-NH_2 (2-5)

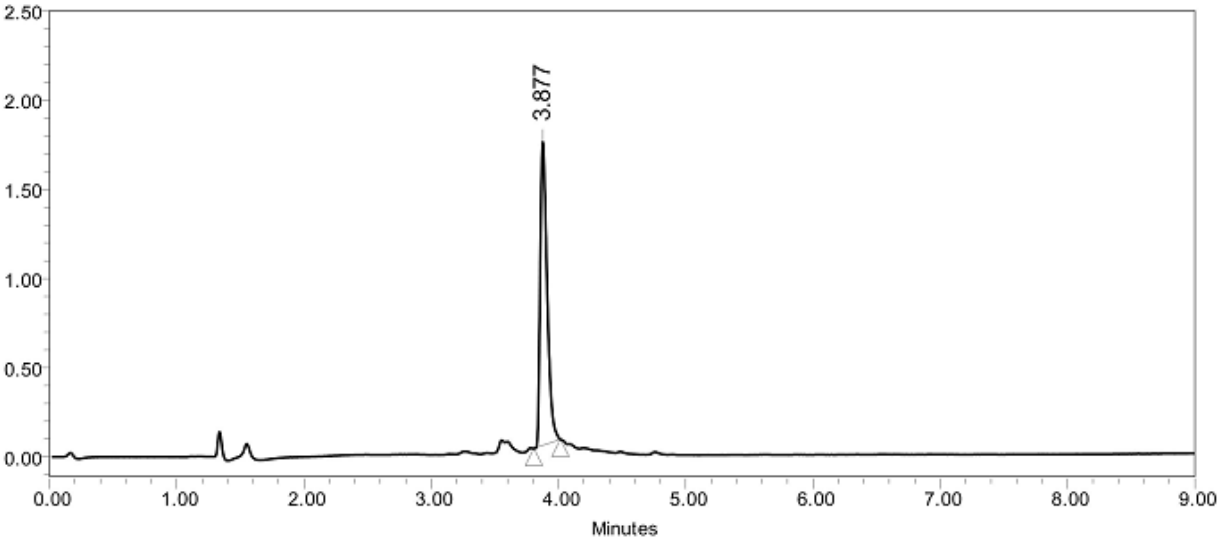


Figure S-6: HPLC chromatogram of peptide **2-5**

H-oxytocin-NH_2 (2-6)

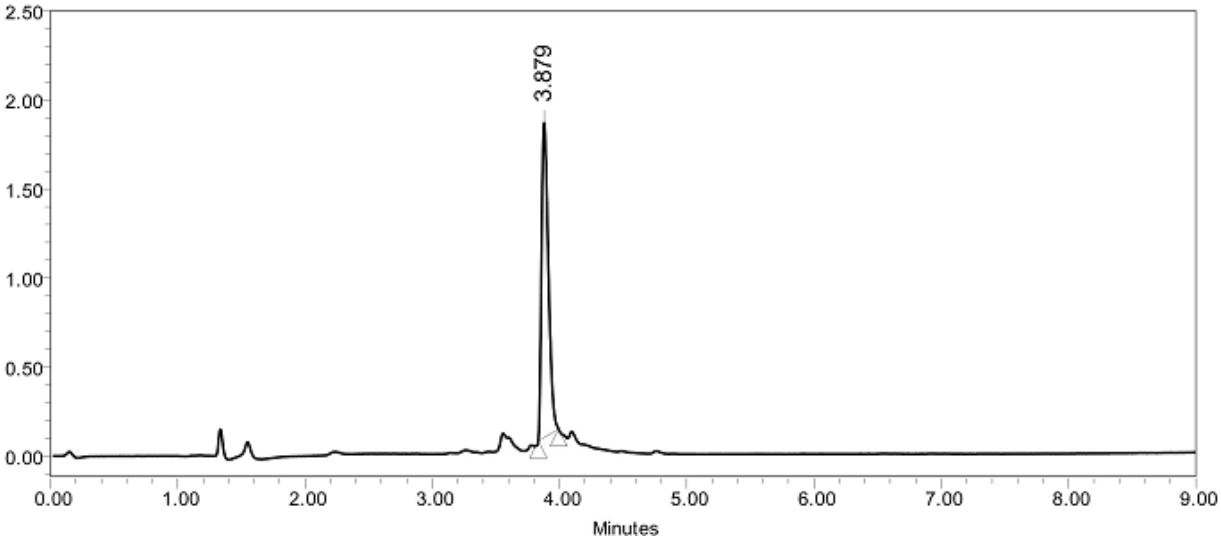


Figure S-7: HPLC chromatogram of peptide **2-6**

H-Octreotate(2 x SH)-OH (3)

H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-OH

3 SH SH

The 2-CTC resin (1g, 1.6 mmol, 1.6 mmol/g) was washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Fmoc-Thr(tBu)-OH (238.2 mg, 0.6 mmol) and DIPEA (0.865 ml, 5 mmol) in CH_2Cl_2 were added to the resin and the resin was shaken for 1 h at rt. The resin was capped by the addition of methanol (0.8 mL) and shaken for 10 min. The resin was washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min) and dried over air. The Fmoc loading was determined by treating a small portion of resin (5 mg) with piperidine-DMF (1:4) (1 mL) for 20 min and dilited to 25 mL in a volumetric flask. The solution was quantified by UV (290 nm) to give a loading of 0.48 mmol/g. The resin (156.3 mg, 0.075 mmol) containing Fmoc-Thr(tBu) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min) followed by peptide elongation according to General Method 1. Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). A microcleavage was performed according to General Method 4 and subsequent HPLC analysis found that peptide **3** was obtained in 94**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 4.7 min). **HRMS** observed $[M+H]^+$ 1035.4427, required $[M+H]^+$ 1035.4427.

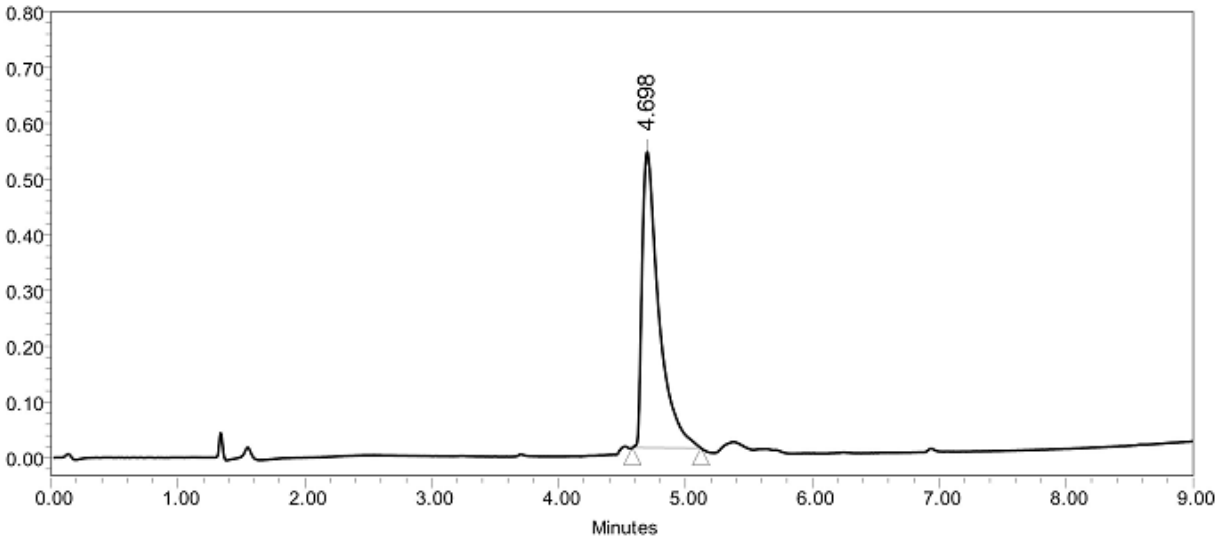
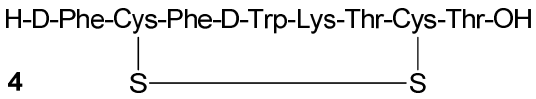


Figure S-8: HPLC chromatogram of peptide **3**

H-Octreotate-OH (4)



Peptide **4** was prepared by cleaving 5 mg of resin (**3**) according to General Method 4. The crude peptide was dissolved in H_2O/acetonitrile (1:1) and oxidized according to General Method 3 with NCS (2.0 equiv.) in H_2O/acetonitrile, lyophilized and subsequently analyzed. HPLC analysis found that peptide **4** was obtained in 84**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 4.4 min). **HRMS** observed [M] 1032.4198, required 1032.4197.

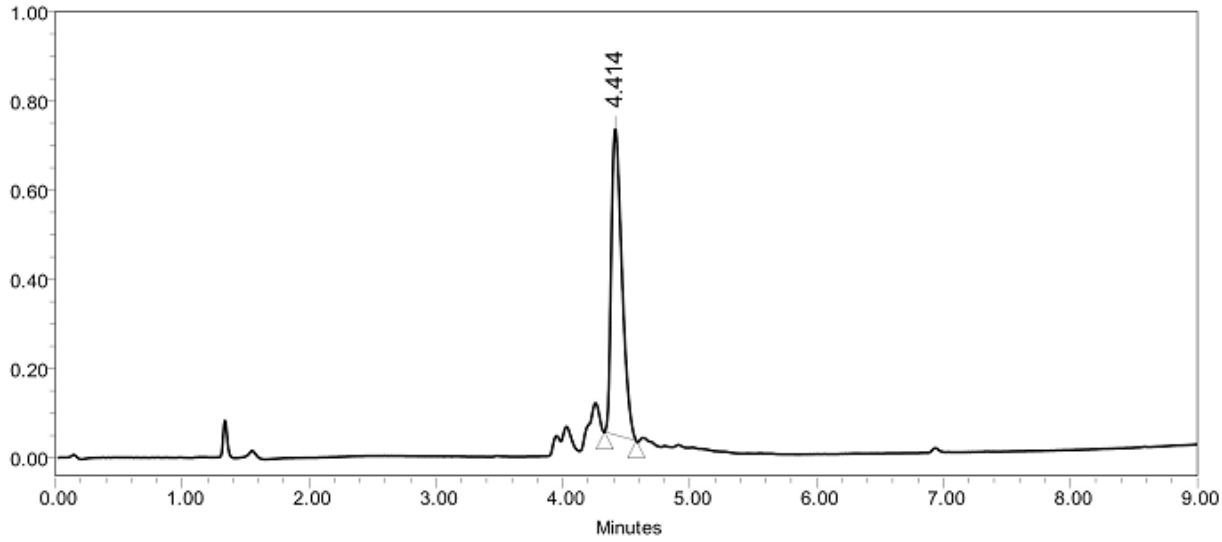
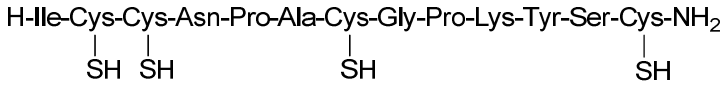


Figure S-9: HPLC chromatogram of peptide **4**

H-SI-Conotoxin(4 x SH)-NH_2 (5)



Linear SI conotoxin (**3**) was prepared according to General Method 1 using Fmoc-Rink-Amide AM resin (166.7 mg, 0.075 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). A microcleavage was performed according to General Method 4 and subsequent HPLC analysis found that peptide **5** was obtained in 85**%** purity (linear gradient from 0% to 30% acetonitrile over 8 min, t_R : 7.2 min). **HRMS** observed $[M+H]^+$ 1357.5492, required $[M+H]^+$ 1357.5520.

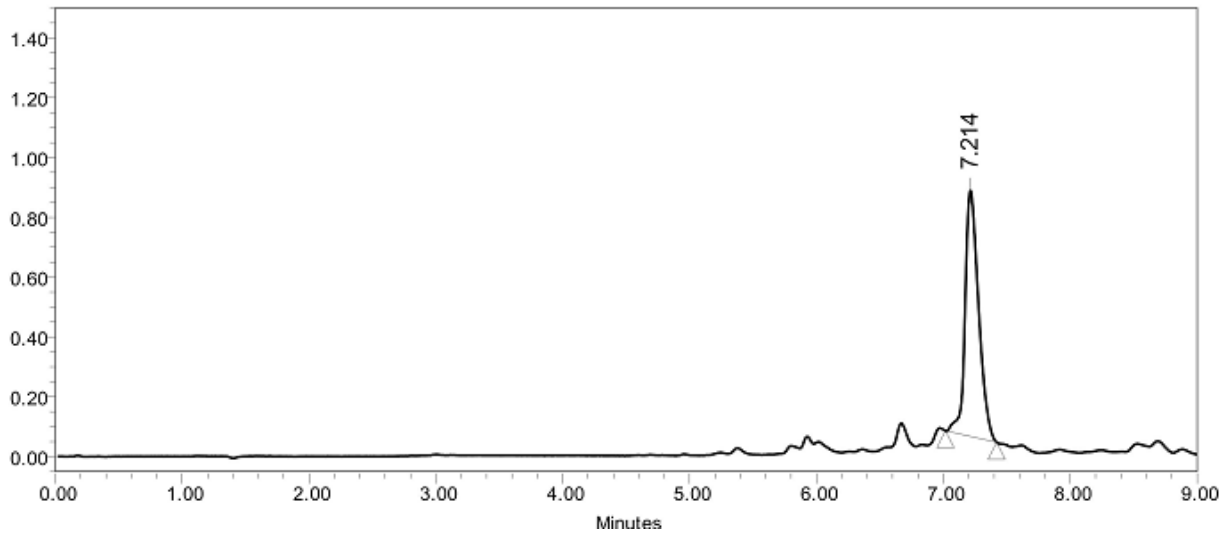


Figure S-10: HPLC chromatogram of peptide **5**

H-SI-Conotoxin-NH_2 (6)

Peptide **6** was prepared by cleaving 5 mg of resin (**5**) according to General Method 4. The crude peptide was dissolved in water and oxidized according to General Method 3 with NCS (2.2 equiv.) in H_2O/acetonitrile and subsequently analyzed. HPLC and LCMS analysis found that peptide **6** was obtained in two different disulfide connectivities in a ratio of 1:4 (linear gradient from 0% to 30% acetonitrile over 8 min, t_R : 7.2 and 7.5 min). **HRMS** observed $[M+H]^+$ 1353.5191, required $[M+H]^+$ 1353.5210.

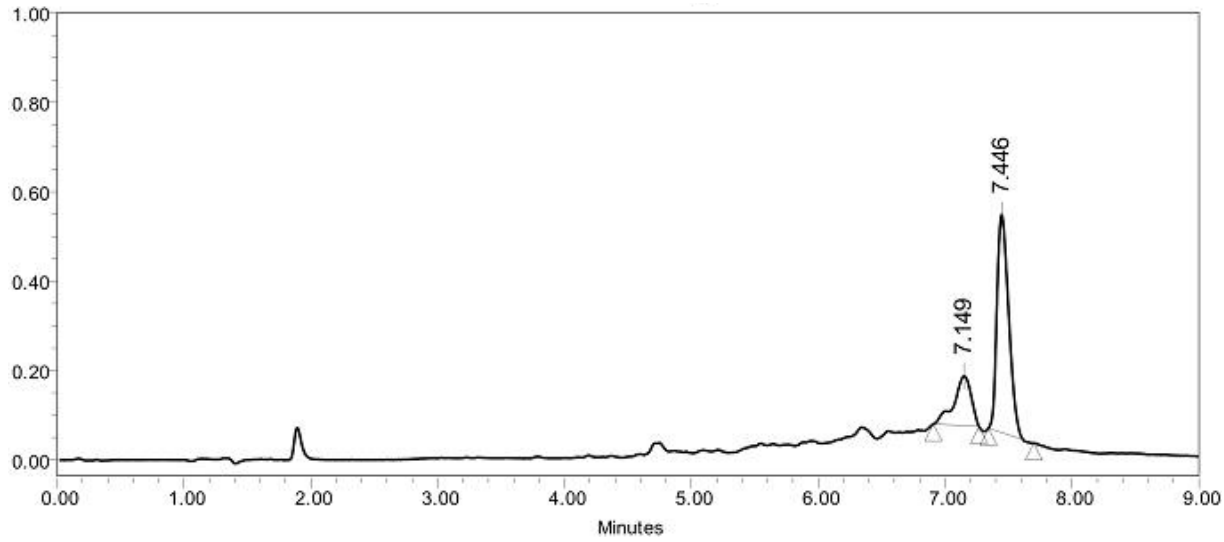


Figure S-11: HPLC chromatogram of peptide **6**

3-SI.4. References

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Chapter 4

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

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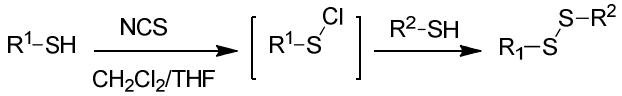
Abstract

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.

4.1 Introduction

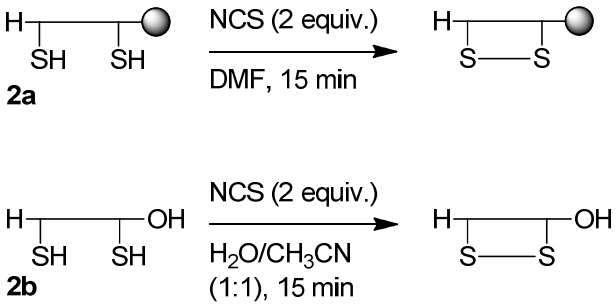
Disulfide bonds are key structural features of many conformationally constrained natural or synthetic peptides.[1] These bonds confer structural rigidity, which leads to peptides with strong binding and high selectivity.[2] In nature, this is illustrated by the highly potent disulfide-containing peptide 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, over 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 towards other thiols. In this regard, we observed a rapid, clean and efficient reaction (Scheme 1).



Scheme 1. Mixed disulfide-formation mechanism using NCS

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]



Scheme 2. a) On-resin NCS disulfide formation, b) Aqueous NCS disulfide formation in solution

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 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 amphyphilic 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 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).

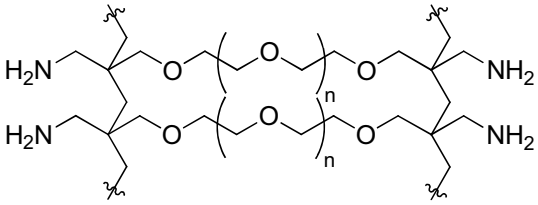
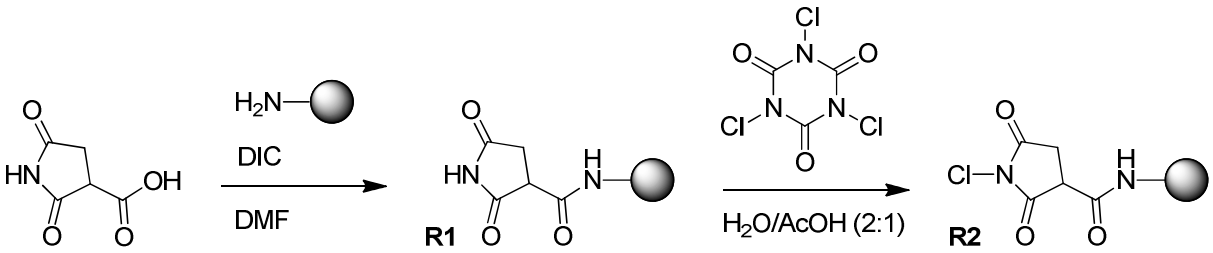


Figure 1. Aminomethyl-ChemMatrix resin structure

4.2 Results and Discussion

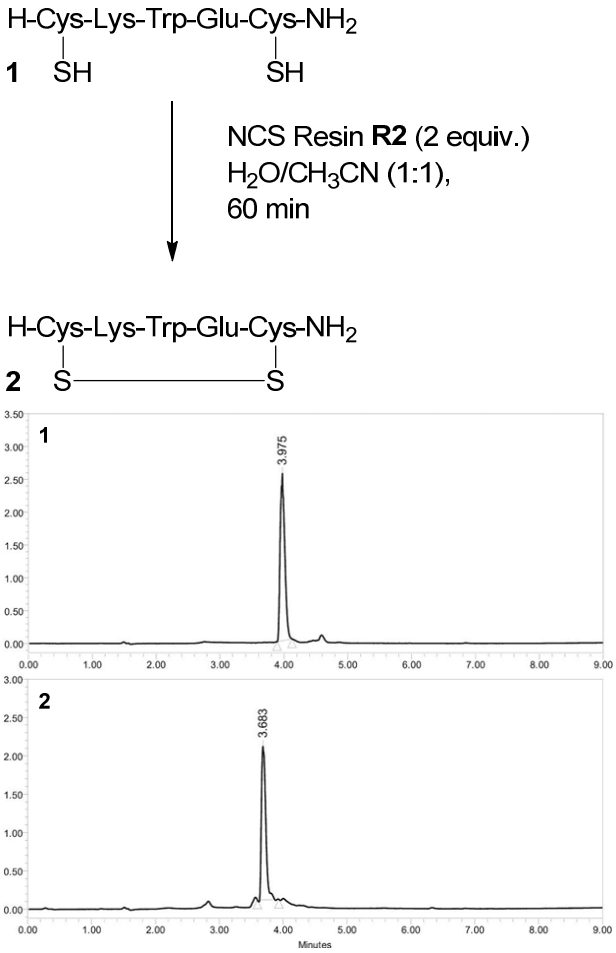
Preparation of Immobilized NCS. Dioxo-pyrrolidine-3-carboxylic acid was immobilized on the aminomethyl-ChemMatrix resin using diisopropylcarbodiimide (DIC). Thus, the carboxylic acid (2 equiv.) was pre-activated for 5 min using DIC (2 equiv.) in DMF and subsequently reacted with the resin for 16 h at room temperature. After the reaction, the resin was washed and dried overnight in a vacuum oven. The mass increase in 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 H_2O/AcOH (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 IR spectroscopy (Supplementary Information), and the loading of **R2** was determined by UV spectroscopy (see below).



Scheme 3. Preparation of NCS-resin **R2**

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, TFA/TIS/H_2O 95:2.5:2.5). Peptide **1** was dissolved in H_2O/CH_3CN (1:1), a solvent 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 LCMS showed complete formation of the disulfide (**2**) in 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 as described for peptide **1**. First, we oxidized vasopressin in H_2O, H_2O/CH_3CN (3:1), H_2O/CH_3CN (1:1), H_2O/CH_3CN (1:3) 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 LCMS showed that the best result was obtained with H_2O/CH_3CN (1:1). We subsequently used this solvent mixture for the oxidation of the other library members. Analysis of the nonapeptides showed formation of the disulfides in high purity (Table 1).

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

Table 1. Oxidation of nonapeptide library using NCS resin (2 equiv.)

4.3 Conclusions

In summary, immobilized NCS is an attractive and simple-to-use disulfide-forming reagent. Disulfide formation in 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.

4.4 Experimental Procedures

Analytical Methods. Analytical high pressure liquid chromatography (HPLC) was carried out on a Waters instrument comprising a separation module (Waters 2695), automatic injector, photodiode array detector (Waters 2998) and 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 $mL \cdot min^{-1}$ and a run time of 11 min.
LCMS was carried out 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 $mL \cdot min^{-1}$ and a run time of 11 min.

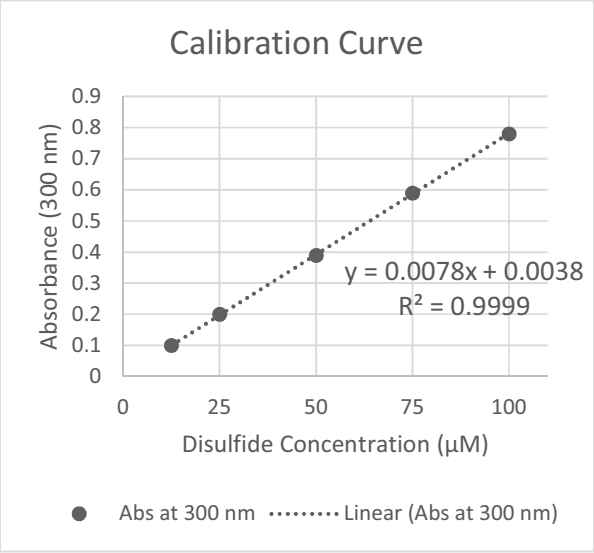
Infrared (IR) spectra were recorded in potassium bromide pellets on a Thermo Nicolet Nexus FT IR spectrometer. The sample was crushed using a mortar and pestle and mixed with KBr prior to pressing into a pellet and subsequently analyzed.
UV measurements were recorded using a quartz cuvette in H_20/CH_3CN (1:3) on a Shimadzu UV-2501PC UV-Vis spectrometer at a wavelength of 300nm.

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), TFA/CH_2Cl_2 (1:99) (5 × 1 min), CH_2Cl_2 (5 × 1 min), DIPEA/CH_2Cl_2 (5:95) (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 pre-activated for 5 min using DIC (0.81 µL, 5.22 mmol, 3 equiv.) in DMF. The pre-activated mixture was added to the resin and shaken for 16 h at room temperature. The resin was washed with DMF (5 × 1 min), 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 resin, 1.68 mmol).

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

Loading Determination 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. 2,5-Dimethoxythiophenol stock solution - 2,5-Dimethoxythiophenol (68.1 mg, 400 µmol) was dissolved in 10 mL H_2O/CH_3CN (1:3) in a volumetric flask. NCS stock solution - NCS (13.4 mg, 100 µmol) was dissolved in 10 mL H_2O/CH_3CN (1:3) in a volumetric flask. Reaction between 2,5-Dimethoxythiophenol and NCS (100 µM) - 2,5-Dimethoxythiophenol stock solution (100 µL), NCS stock solution (100 µL) and H_2O/CH_3CN (1:3, 1 mL) were added to a 10 mL volumetric flask. The mixture was shaken for 30 minutes and subsequently diluted to 10 mL with H_2O/CH_3CN (1:3). Absorbance was measured at 300 nm. The mixture was diluted to the following concentrations 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 (Graph 1).



Graph 1. Calibration curve for the determination of resin loading

Immobilized NCS Resin (10 mg, max 0.58 mmol/g) was placed in an Eppendorf cup and 2,5-dimethoxythiophenol stock solution (1 mL) was added. The mixture was shaken for 30 minutes. 100 µL of this mixture was diluted to 10 mL with H_2O/CH_3CN (1:3, 1 mL) in a volumetric flask, and absorbance was measured at 300 nm (based on the loading of the starting resin (0.58 mmol/g) the maximum concentration of disulfide would be 58 µM). An absorbance (AU) of 0.43 AU was measured, which corresponds to a loading of 0.55 mmol/g.

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

4.5 References

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Chapter 4

Supporting Information

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

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4-SI.1. General Procedures

Fmoc-amino acids and Fmoc-Rink Amide AM resin were obtained from IRIS Biotech (Marktredwitz, Germany). Aminomethyl-Chemmatrix was obtained from PCAS BioMatrix Inc. (Quebec, Canada). 2,5-Dioxo-pyrrolidine-3-carboxylic acid was obtained from CHESS GmBH (Mannheim, Germany). DIPEA, diisopropylcarbodiimide (DIC) and TFA were obtained from Aldrich (Milwaukee, USA). Oxyma Pure was obtained from Luxembourg Industries Ltd. (Tel Aviv, Isreal). DMF, CH_2Cl_2, Et_2O, acetonitrile, DMSO and piperidine (HLPC grade) were obtained from SDS (Peypin, France). All reagents and solvents were used as received.

Room temperature (rt) refers to ambient temperature. Solid-phase syntheses were carried out manually in polypropylene syringed containing a polyethylene frit. Solvents and soluble reagents were removed by suction. Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min). Washings between deprotection and coupling were performed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Following the final coupling or deprotection the resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and dried under a stream of air. Yields for peptides refer to the area of the chromatographic product peak recorded at 220 nm.

High resolution mass spectrometry (HRMS) measurements were recorded on Thermo Scientific LTQ-FT Ultra spectrometer. Mass values are quoted within the error limits of ±5 ppm mass units. ESI refers to the electrospray ionization technique.

Analytical high pressure liquid chromatography (HPLC) was carried out on a Waters instrument comprising a separation module (Waters 2695), automatic injector, photodiode array detector (Waters 2998) and 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 $mL \cdot min^{-1}$ and a run time of 11 min.

LCMS was carried out 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 $mL \cdot min^{-1}$ and a run time of 11 min.

Infrared (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 pressing into a pellet and subsequently analyzed.

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

4-SI.2. General Methods Peptide Synthesis

General Method 1: Peptide Synthesis on Rink Amide AM Resin

Fmoc-Rink-Amide AM resin (0.45 mmol/gram, 1 equiv) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) followed by washing with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The protected Fmoc-amino acids (3 equiv) were incorporated using DIC (3 equiv.) and Oxyma (3 equiv) in DMF, as a coupling system, with 5 min preactivation for 1 h at rt. Washes between couplings and deprotections were performed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Completion of the coupling was monitored by the Kaiser test.[1]

General Method 2: Microcleavage

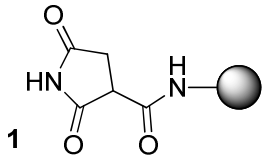
Dry resin (5 mg) was treated with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1) for analysis by HPLC and LCMS.

General Method 3: Disulfide Formation

The peptide was dissolved in H_2O/CH_3CN (1:1) and added to the immobilized resin. The mixture was shaken for 30-60 min at rt and subsequently filtered to remove the resin. The filtrate was analyzed by HPLC and LCMS

4-SI.3. Immobilized NCS Preparation

Immobilized Succinimide (R1)



1

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), TFA/CH_2Cl_2 (1:99) (5 × 1 min), CH_2Cl_2 (5 × 1 min), DIPEA/CH_2Cl_2 (5:95) (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 shaken for 16 h at rt. The resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and dried overnight in a vacuum oven at 40 °C. Resin **1** was obtained with a 99% coupling efficiency (3.24 g resin, 1.68 mmol). **FTIR** (KBr pellet) v*max* (cm^{-1}) 3452, 2873, 1641, 1468, 1351, 1105, 952.

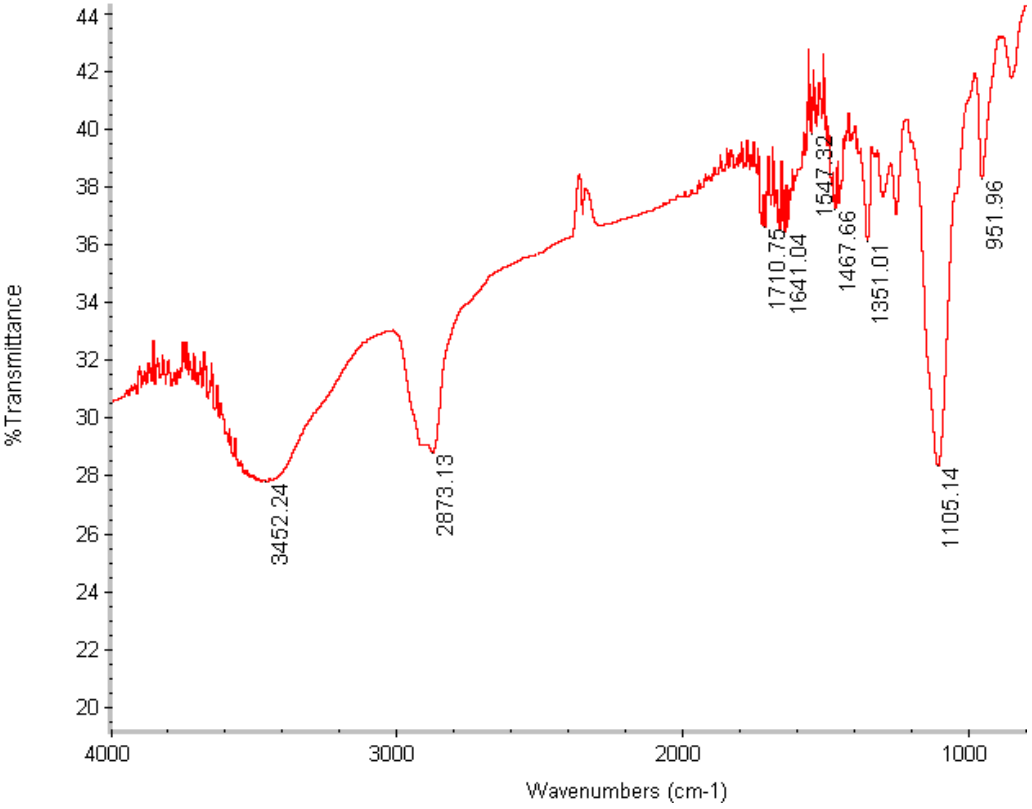
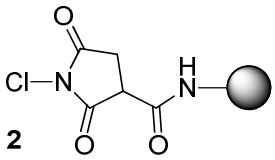


Figure 1: IR spectrum of Immobilized-Succinimide resin (**R1**)

Immobilized NCS (R2)



2

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

0.55 mmol/g, see below for loading determination (0.52 g resin). The resin was stored at -20°C. **FTIR** (KBr pellet) v*max* (cm^{-1}) 3491, 2872, 1664, 1459, 1351, 1109, 952.

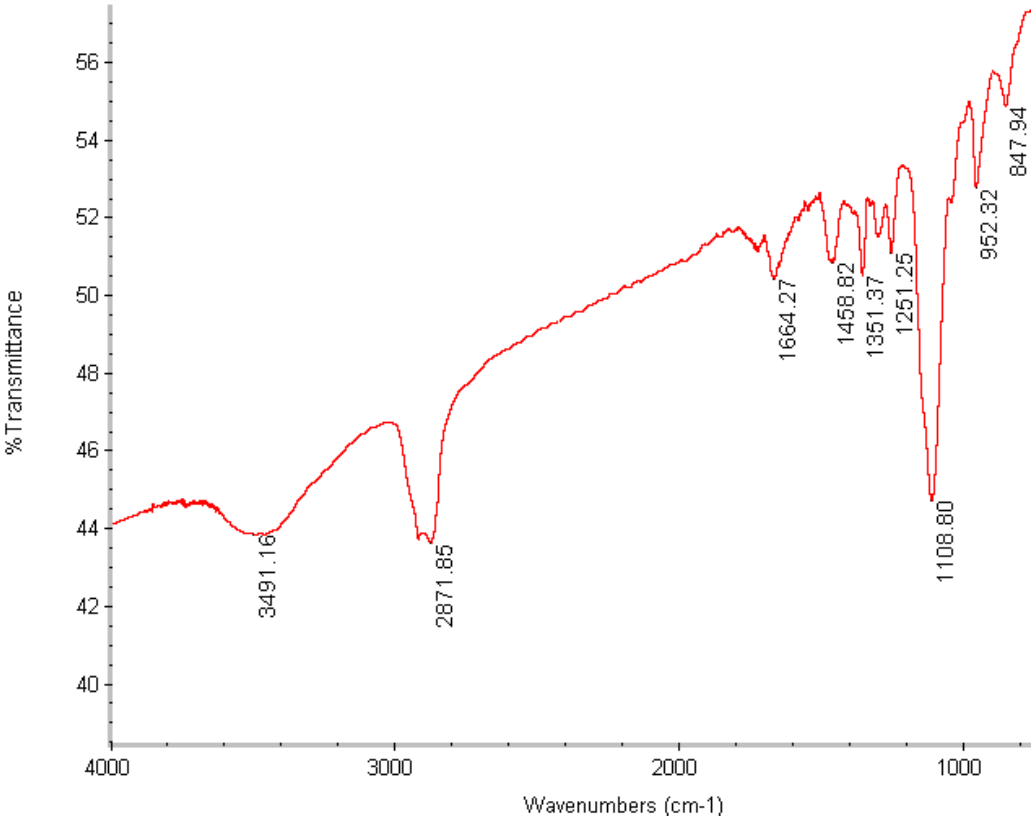


Figure 2: IR spectrum of Immobilized-NCS resin (**R2**)

Loading Determination 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.[2] 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.

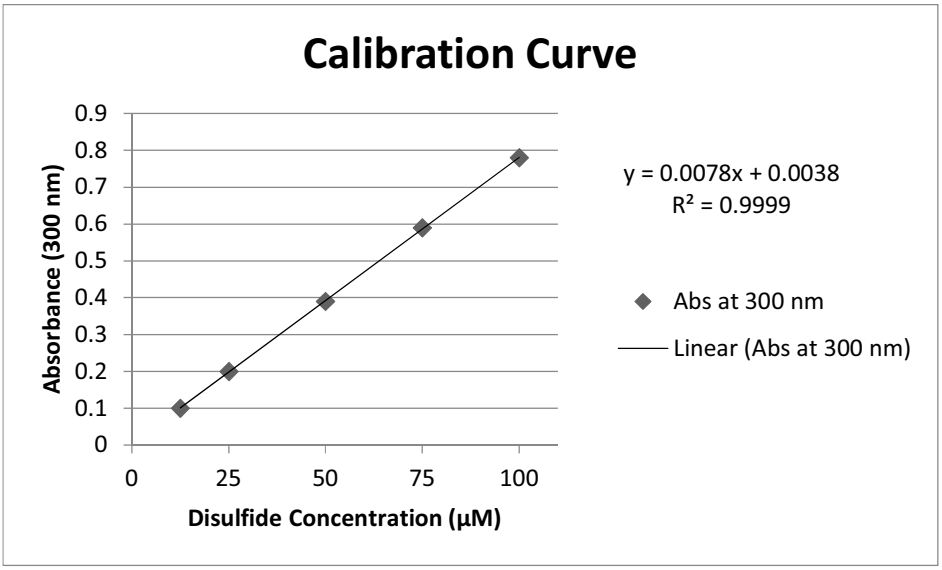
Calibration curve:

2,5-Dimethoxythiophenol stock solution - 2,5-Dimethoxythiophenol (68.1 mg, 400 µmol) was dissolved in 10 mL H_2O/CH_3CN (1:3) in a volumetric flask.

NCS stock solution - NCS (13.4 mg, 100 µmol) was dissolved in 10 mL H_2O/CH_3CN (1:3) in a volumetric flask.

Reaction between 2,5-Dimethoxythiophenol and NCS (100 µM) - To a 10 mL volumetric flask was added 2,5-Dimethoxythiophenol stock solution (100 µL), NCS stock solution (100 µL) and H_2O/CH_3CN (1:3, 1 mL). The mixture was shaken for 30 minutes and subsequently diluted to 10 mL with H_2O/CH_3CN (1:3). The absorbance was measured at 300 nm.

The mixture was diluted to the following concentrations 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 (Graph 1).



Graph 1: Calibration curve for determination of resin loading

Reaction of immobilized NCS with 2,5-dimethoxythiophenol

Immobilized NCS-Resin (10 mg, max 0.58 mmol/g) was placed in an Eppendorf cup and 2,5-dimethoxythiophenol stock solution (1 mL) was added. The mixture was shaken for 30 minutes. 100 µL of this mixture was diluted to 10 mL with H_2O/CH_3CN (1:3, 1 mL) in a volumetric flask and the absorbance was measured at 300 nm (based on the loading of the starting resin (0.58 mmol/g) the maximum concentration of disulfide would be 58 µM). An absorbance of 0.43 was measured and corresponds to a loading of 0.55 mmol/g.

4-SI.4. Peptide Synthesis

Peptide 1

Peptide **1** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (222.2 mg, 0.1 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin): Peptide **1** was cleaved from the resin according to General Method 2 and subsequent HPLC analysis found that peptide **1** was obtained in 97**%** purity (linear gradient from 0% to 100% acetonitrile over 8 min, t_R peptide **1** : 4.0 min). **LCMS** peptide **1** observed $[M+H]^+$ 667.3, required $[M+H]^+$ 667.3.

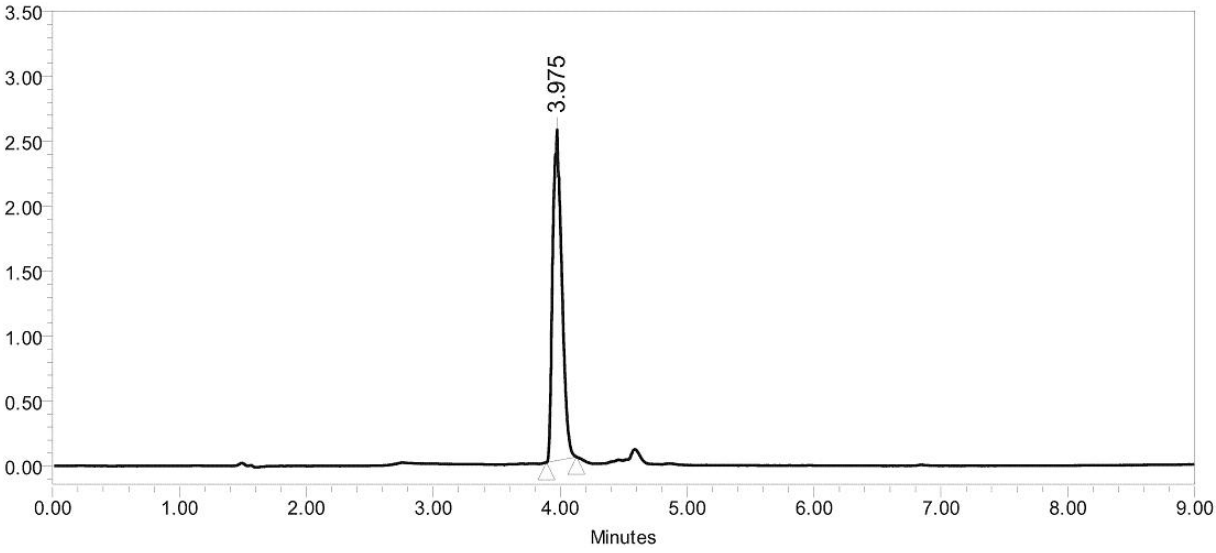


Figure S-1: HPLC chromatogram of peptide **1**

Peptide 2

Peptide **2** was prepared according to General Method 3 using resin containing peptide **1** (5 mg, 2.5 µmol, 1 equiv). Peptide **1** was cleaved from the resin according to general method 2 and dissolved in H_2O/CH_3CN

(1:1, 0.6 mL). The solution was added to immobilized-NCS **(R2)** (8.3 mg, 5 µmol, 2 equiv) and shaken for 60 min at rt. The mixture was filtered using a syringe filter and the filtrate was analyzed by HPLC. Peptide **2** was obtained in 91**%** purity (linear gradient from 0% to 100% acetonitrile over 8 min, t_R peptide **2** : 3.7 min). **LCMS** peptide **2** observed $[M+H]^+$ 665.3, required $[M+H]^+$ 665.3.

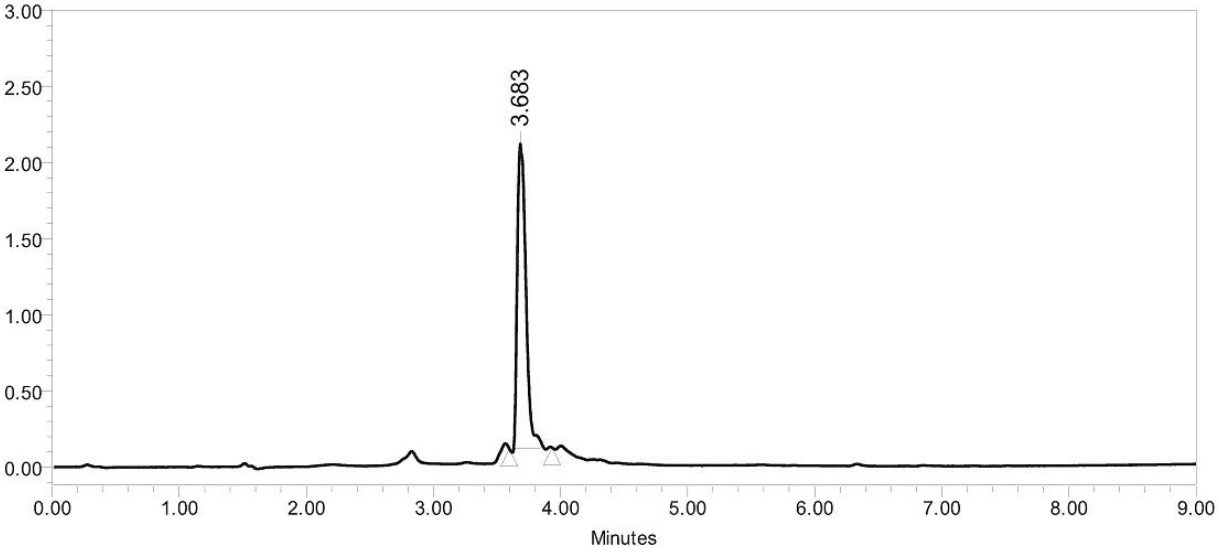
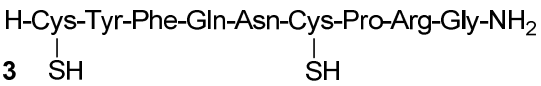


Figure S-2: HPLC chromatogram of peptide **2**

Vasopressin (3)



Peptide **3** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (222.2 mg, 0.1 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin): Peptide **3** was cleaved from the resin according to General Method 2 and subsequent HPLC analysis found that peptide **3** was obtained in 96**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R peptide **3** : 3.9 min). **LCMS** peptide **3** observed $[M+H]^+$ 1086.5, required $[M+H]^+$ 1086.5.

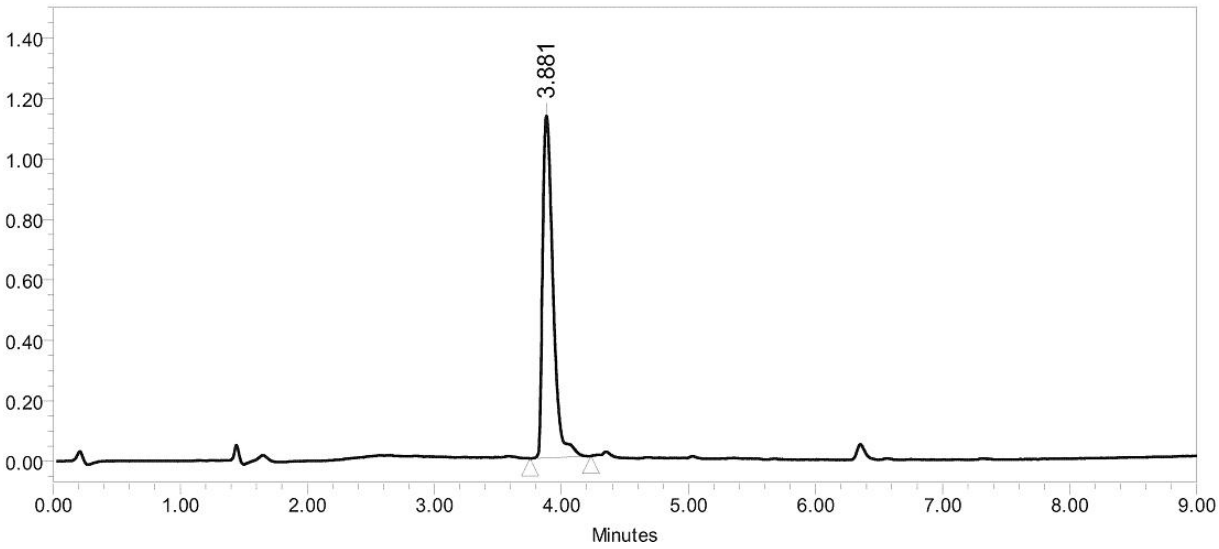
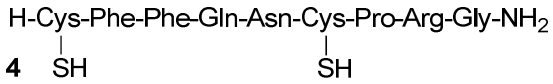


Figure S-3: HPLC chromatogram of peptide **3**

Phenypressin (4)



Peptide **4** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (222.2 mg, 0.1 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin): Peptide **4** was cleaved from the resin according to General Method 2 and subsequent HPLC analysis found that peptide **4** was obtained in 96**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R peptide **4** : 3.9 min). **LCMS** peptide **4** observed $[M+H]^+$ 1070.5, required $[M+H]^+$ 1070.5.

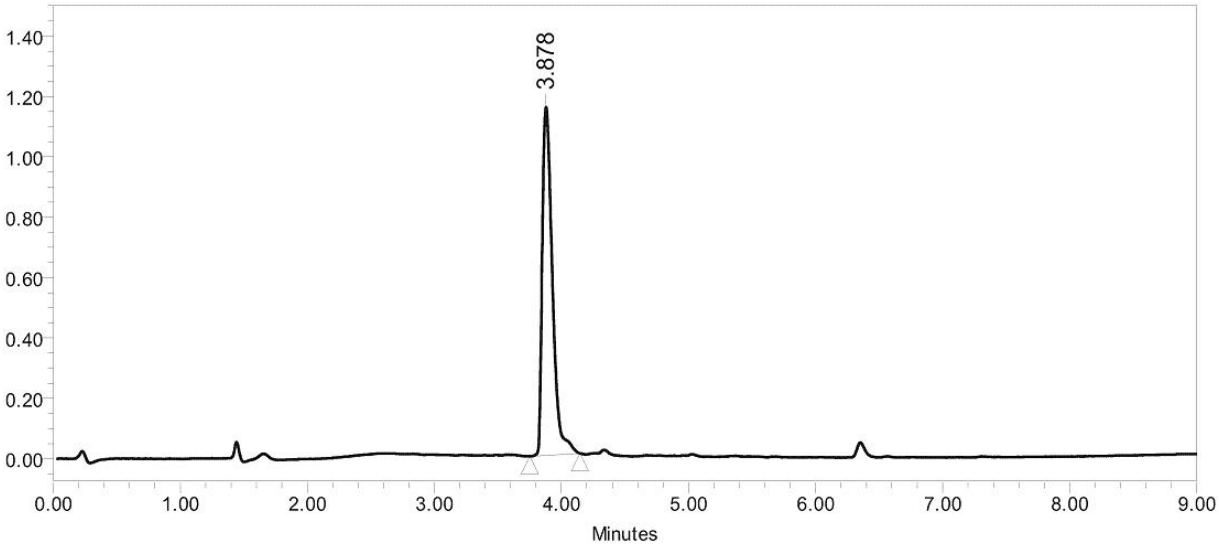
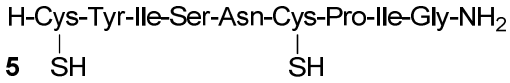


Figure S-4: HPLC chromatogram of peptide **4**

Isotocin (5)



Peptide **5** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (222.2 mg, 0.1 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin): Peptide **5** was cleaved from the resin according to General Method 2 and subsequent HPLC analysis found that peptide **5** was obtained in 94**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R peptide **5** : 3.9 min). **LCMS** peptide **5** observed $[M+H]^+$ 968.5, required $[M+H]^+$ 968.4.

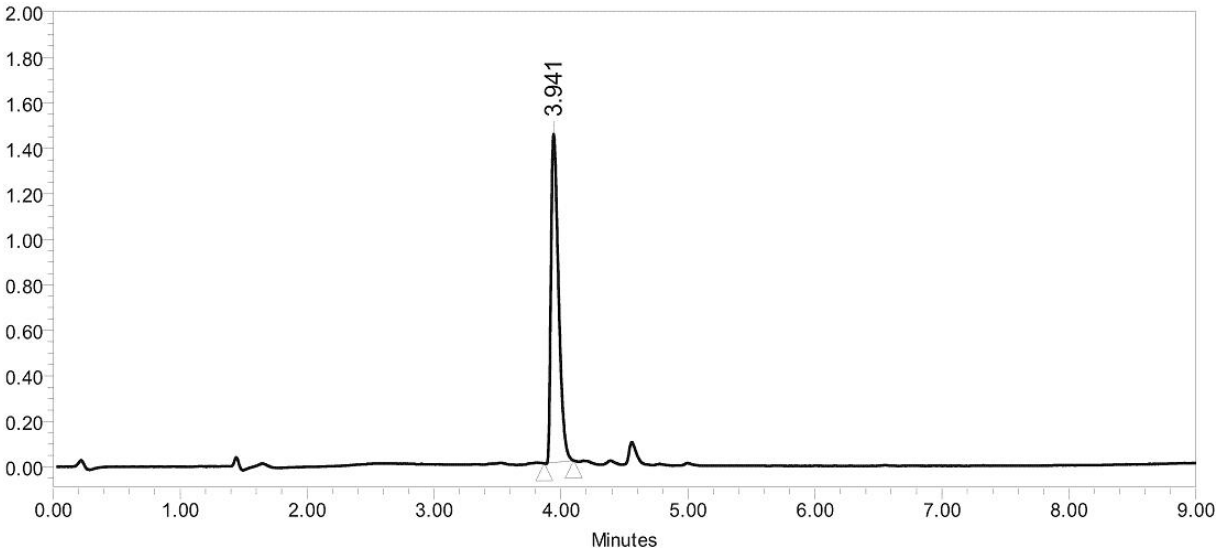
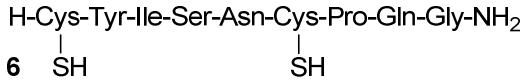


Figure S-5: HPLC chromatogram of peptide **5**

Glumitocin (6)



Peptide **6** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (222.2 mg, 0.1 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin): Peptide **6** was cleaved from the resin according to General Method 2 and subsequent HPLC analysis found that peptide **6** was obtained in 95**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R peptide **6** : 3.4 min). **LCMS** peptide **6** observed $[M+H]^+$ 983.4, required $[M+H]^+$ 983.4.

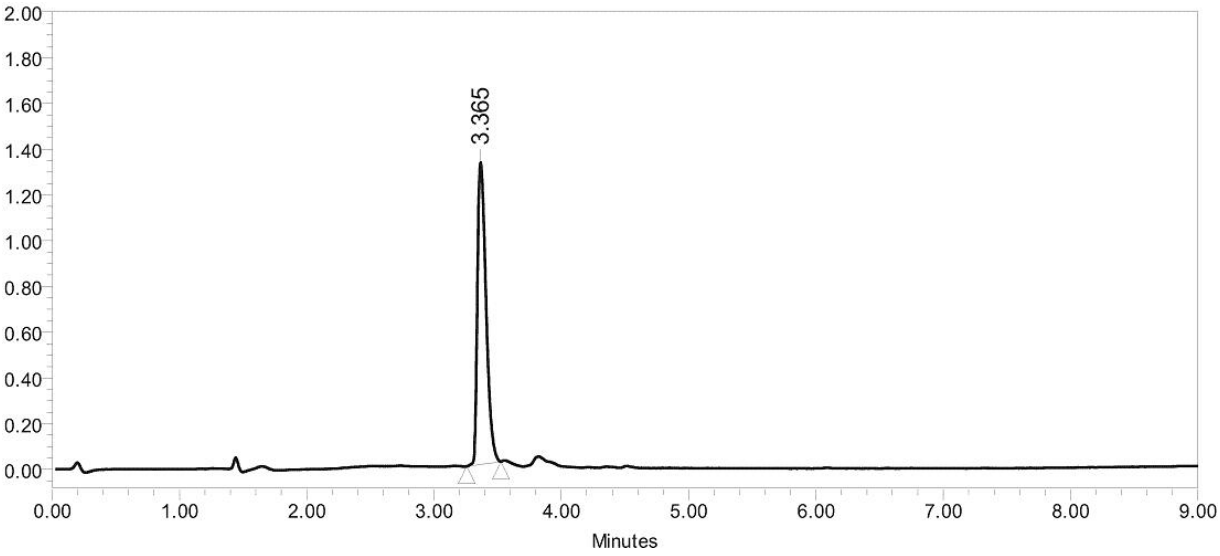
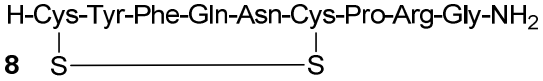


Figure S-6: HPLC chromatogram of peptide **6**

Oxidation of Vasopressin (8)



Peptide **8** was prepared according to General Method 3 using resin containing peptide **3** (5 mg, 2.5 µmol, 1 equiv). Peptide **3** was cleaved from the resin according to general method 2 and dissolved in H_2O/CH_3CN (1:1, 0.6 mL). The solution was added to immobilized-NCS **(R2)** (8.3 mg, 5 µmol, 2 equiv) and shaken for 30 min at rt. The mixture was filtered using a syringe filter and the filtrate was analyzed by HPLC. Peptide **8** was obtained in 91**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R peptide **8** : 3.6 min). **LCMS** peptide **8** observed $[M+H]^+$ 1084.5, required $[M+H]^+$ 1084.5.

The oxidation was repeated in several solvent mixtures to determine the optimal solvent mixture without changing the other parameters (Table 1). We chose to use H_2O/CH_3CN 1:1 as the best solvent.

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)	-

Table 1: Oxidation of nonapeptide library using NCS resin (2 equiv.)

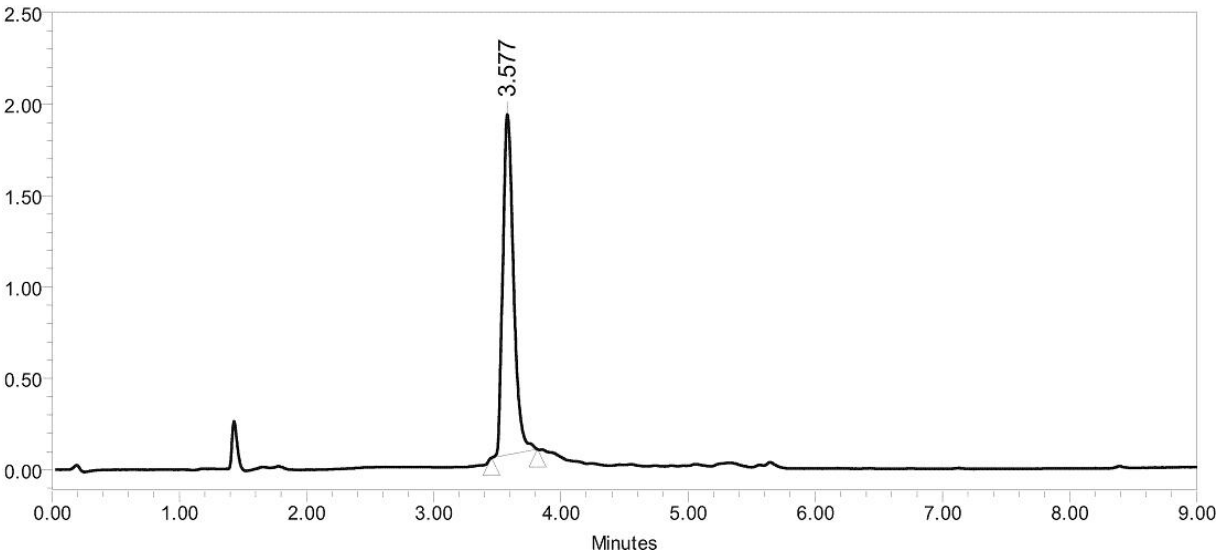
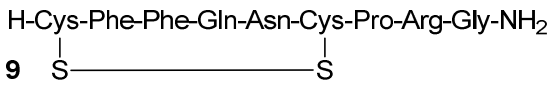


Figure S-8: HPLC chromatogram of peptide **8** (oxidized in H_2O/CH_3CN 1:1)

Oxidation of Phenypressin (9)



Peptide **9** was prepared according to General Method 3 using resin containing peptide **4** (5 mg, 2.5 µmol, 1 equiv). Peptide **4** was cleaved from the resin according to general method 2 and dissolved in H_2O/CH_3CN (1:1, 0.6 mL). The solution was added to immobilized-NCS **(R2)** (8.3 mg, 5 µmol, 2 equiv) and shaken for 60 min at rt. The mixture was filtered using a syringe filter and the filtrate was analyzed by HPLC. Peptide

9 was obtained in 90**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R peptide **9** : 4.0 min). **LCMS** peptide **9** observed $[M+H]^+$ 1068.6, required $[M+H]^+$ 1068.5.

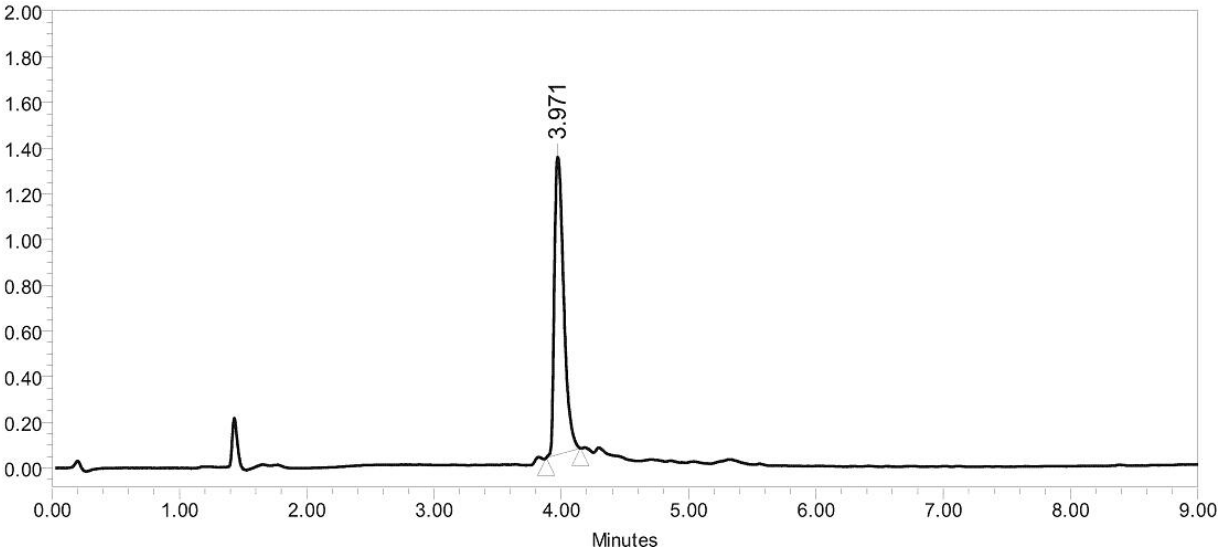
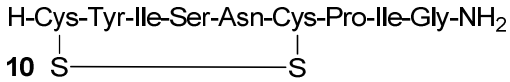


Figure S-9: HPLC chromatogram of peptide **9**

Oxidation of Isotocin (10)



Peptide **10** was prepared according to General Method 3 using resin containing peptide **5** (5 mg, 2.5 µmol, 1 equiv). Peptide **5** was cleaved from the resin according to general method 2 and dissolved in H_2O/CH_3CN (1:1, 0.6 mL). The solution was added to immobilized-NCS **(R2)** (8.3 mg, 5 µmol, 2 equiv) and shaken for 60 min at rt. The mixture was filtered using a syringe filter and the filtrate was analyzed by HPLC. Peptide **10** was obtained in 92**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R peptide **10** : 3.9 min). **LCMS** peptide **10** observed $[M+H]^+$ 966.5, required $[M+H]^+$ 966.4.

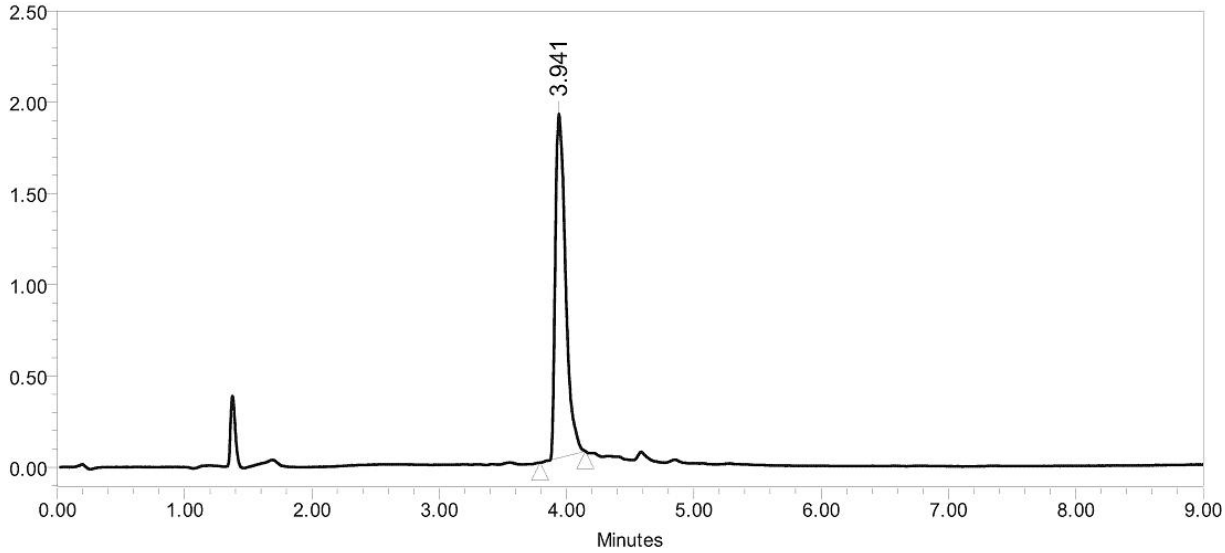
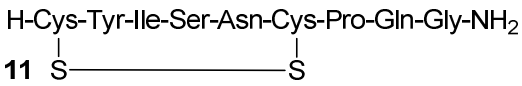


Figure S-10: HPLC chromatogram of peptide **10**

Oxidation of Glumitocin (11)



Peptide **11** was prepared according to General Method 3 using resin containing peptide **7** (5 mg, 2.5 µmol, 1 equiv). Peptide **6** was cleaved from the resin according to general method 2 and dissolved in H_2O/CH_3CN (1:1, 0.6 mL). The solution was added to immobilized-NCS **(R2)** (8.3 mg, 5 µmol, 2 equiv) and shaken for 60 min at rt. The mixture was filtered using a syringe filter and the filtrate was analyzed by HPLC. Peptide **11** was obtained in 86 **%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R peptide **11** : 3.5 min). **LCMS** peptide **11** observed $[M+H]^+$ 981.5, required $[M+H]^+$ 981.4.

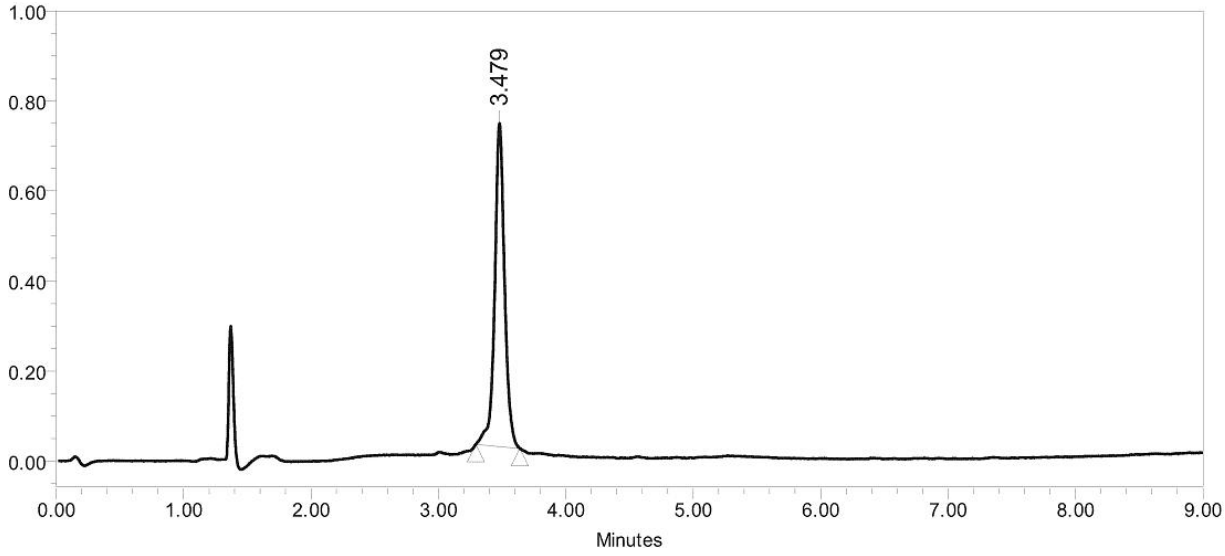


Figure S-11: HPLC chromatogram of peptide **11**

4-SI.5. References

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Chapter 5

Cysteine Pseudoprolines for Thiol Protection and Peptide Macrocyclization Enhancement

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Abstract

Contrary to other studies, here we describe cysteine (Cys) pseudoproline-containing peptides with short deprotection times in TFA. The deprotection times fell in the same range as other protecting groups commonly used in SPPS. Moreover, a novel application for Cys pseudoprolines is disclosed in which this protecting group acts as a peptide macrocyclization-enhancing moiety that considerably reduces reaction time.

5.1 Introduction

Since the introduction of solid-phase peptide synthesis (SPPS) and its subsequent maturation through the continuous refinement of reagents, linkers, resins and protocols, many complex peptides are readily accessible.[1] SPPS now allows the preparation of complex peptides on a large scale, thus facilitating the global commercialization of complex peptide drugs, such as prialt and ziconotide.[2-3] The prevalence of complex peptides is increasing, exemplified by the fact that several multiple disulfide-containing peptides are undergoing clinical trials.[4] However, there are still many obstacles to overcome in the synthesis of complex peptides, such as addressing difficult sequences and orthogonal protecting groups, and solving low coupling efficiencies and solubility issues. In this regards, the development of novel tools, protocols and techniques in peptide synthesis are required to tackle these difficulties. Developed by Mutter et al., pseudoproline dipeptide building blocks are a prime example of innovation in complex peptide synthesis.[5]

Pseudoproline dipeptides have become powerful tools for the synthesis of peptides containing difficult sequences.[6] Mechanistically, pseudoprolines act by disrupting the secondary structure and increase the solubilization of protected peptides. The disruption of secondary structure, such as β-sheet formation, is caused by the cisoid amide conformation of the oxazolidine- or thiazolidine- based pseudoproline rings, which introduce a “kink” in the backbone and disrupt backbone hydrogen bonding analogous to Pro (Figure 1).[7]

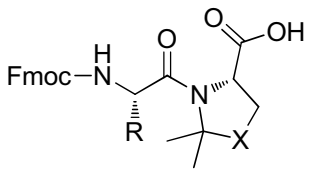


Figure 1: Structure of a pseudoproline dipeptide building block

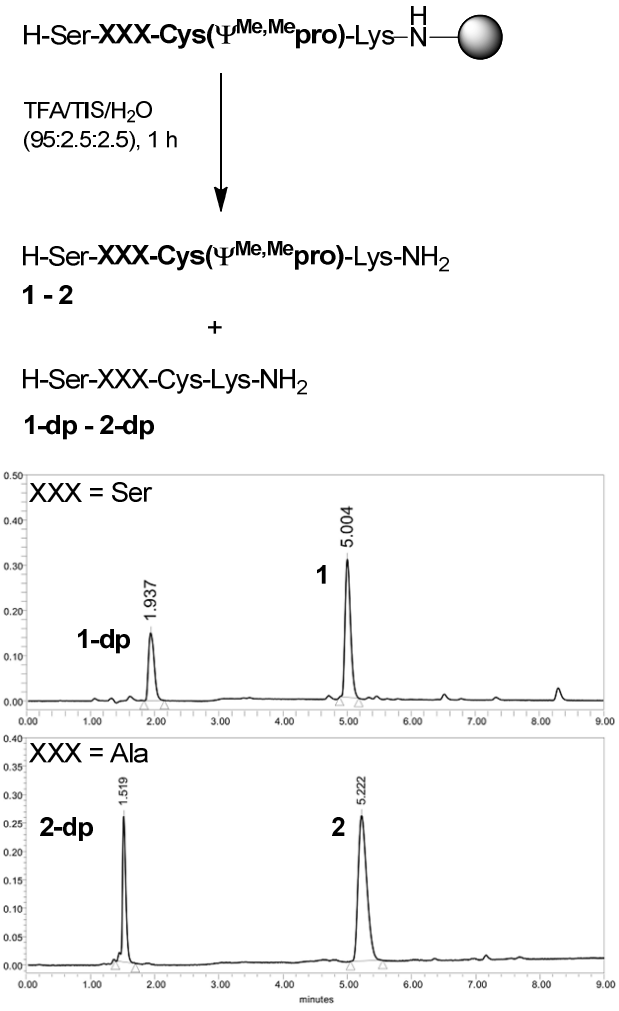
Oxazolidine-based dipeptides derived from Ser or Thr are extensively used and commercially available as Fmoc-protected dipeptides. Typically, the oxazolidine ring can be deprotected to the parent amino acid within several hours using TFA based cleavage cocktails, as illustrated in the synthesis of human amylin.[8] Conversely, thiazolidine based dipeptides have not gained widespread use because of their high stability to TFA. In the seminal paper by Mutter et al., deprotection times of 32 h were reported for the removal of Cys pseudoprolines in linear peptides.[5] This observation was confirmed in a recent publication, where treatment with TFA/TIS/H_2O (95:2.5:2.5) for 36 h was required to remove Cys pseudoproline in a linear peptide.[9] In addition, this publication claimed Cys pseudoproline removal in head-to-tail cyclic peptides takes 13 days in TFA/TIS/H_2O (95:2.5:2.5) and requires the use of harsh acids, such as neat trifluoromethanesulfonic acid, to achieve quantitative deprotection in a reasonable timeframe. Furthermore, our group has prepared head-to-tail cyclic peptides containing up to four Cys pseudoprolines.[10] In this case, we observed high stability to TFA, thereby confirming the long deprotection times for thiazolidine-based pseudoprolines in head-to-tail cyclic peptides.

Thus, the stability of Cys pseudoprolines is poorly understood and required further investigation. Moreover, Cys-based thiazolidine dipeptides show great potential as Cys protecting groups and as macrocyclization-enhancing moieties, a feature not previously exploited. To widen the applicability of Cys pseudoprolines, we initiated a study as part of our ongoing research into Cys protection.

Herein, we report on the remarkable Cys pseudoproline lability in the same timescale as oxazolidine-based pseudoprolines in a series of peptides. Moreover, we identify a novel use of Cys pseudoprolines as macrocyclization-enhancing moieties. In this regard, these moieties significantly reduced the coupling time required for complete peptide macrocyclization.

5.2 Results and Discussion

We first synthesized two random tetrapeptides using the commercial Cys pseudoproline building blocks Fmoc-Ser(tBu)-Cys($\psi^{Me,Me}$pro)-OH and Fmoc-Ala-Cys($\psi^{Me,Me}$pro)-OH. Model peptides **1** and **2** were prepared on a Rink Amide resin using standard SPPS protocols and were then cleaved from the resin using a 1 h treatment with TFA/TIS/H_2O (95:2.5:2.5). Chromatographic and spectroscopic analysis of peptide **1** revealed an unusual lability of the Cys pseudoproline to acidolysis (Scheme 1).

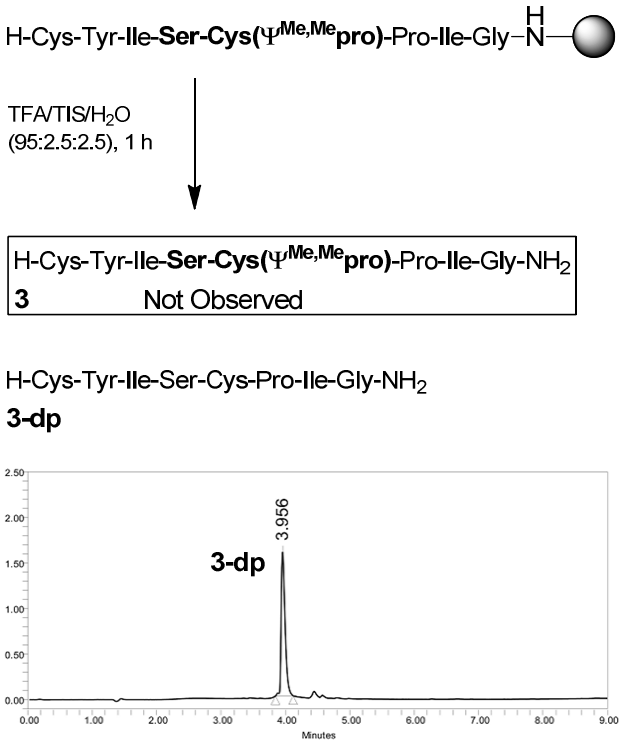


Scheme 1: Acidolysis of peptide **1** and **2** (1h TFA/TIS/H_2O (95:2.5:2.5))

Only 65% of the expected Cys pseudoproline was observed, and 35% of the peptide was fully deprotected to the parent Cys (**1-deprotected** (**dp**)). Analysis of peptide **2** revealed a similar lability to acidolysis with 85% of the Cys pseudoproline remaining after 1 h of TFA treatment. Complete Cys pseudoproline removal in peptide **1** required treatment with TFA/TIS/H_2O (95:2.5:2.5) for 4 h while peptide **2** required 6 h. These

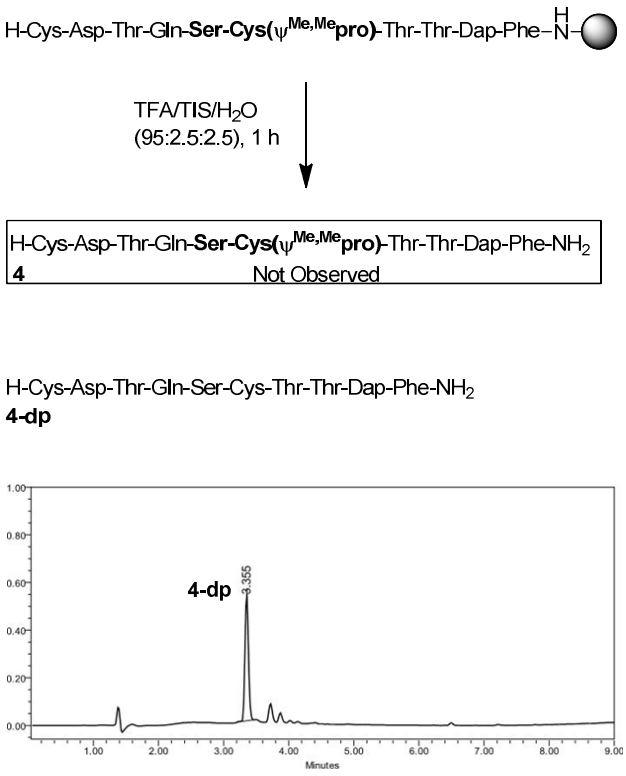
deprotection times are significantly shorter than the previously reported deprotection times of 32-36 h for Cys pseudoproline-containing linear peptides.

The next model peptide we chose was seritocin, a neurohypophysial peptide isolated from the African toad *Bufo regularis*.[11] The linear peptide was prepared using standard SPPS and subsequently cleaved from the resin. Analysis of the cleaved peptide revealed complete Cys pseudoproline removal after 1 h of TFA treatment (Scheme 2). This result contrasts with previously reported Cys pseudoproline deprotection times of 32-36 h using the same cleavage cocktail.



Scheme 2: Acidolysis of peptide **3** (1h TFA/TIS/H_2O (95:2.5:2.5))

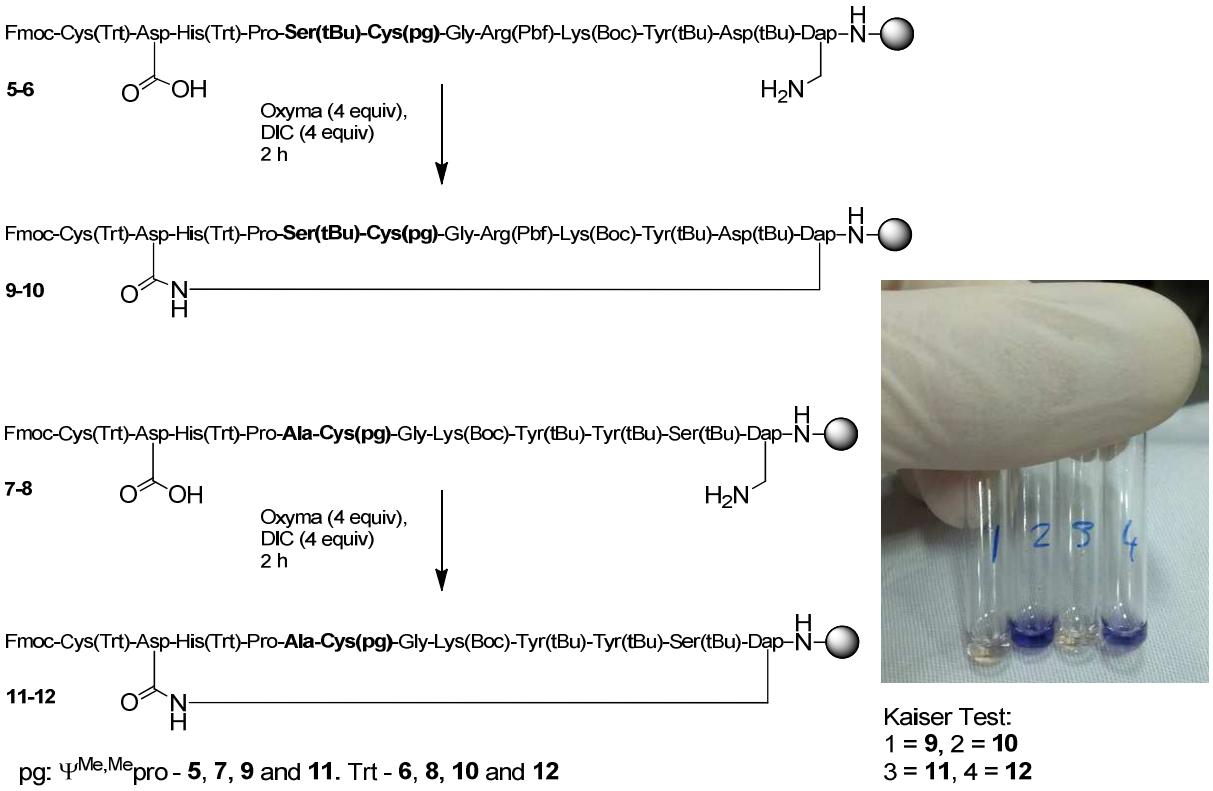
The rapid deprotection behavior of the Cys pseudoproline was again observed in a model peptide with a completely different sequence. Immobilized peptide **4** was treated with TFA for 1 h, and complete Cys pseudoproline removal was observed (Scheme 3). Our results strongly indicate that Cys pseudoproline removal is sequence-dependent. In addition, we noted that the deprotection times fell in the same range as for oxazolidine pseudoprolines, thereby making Cys pseudoprolines more applicable as they do not require harsh acids for deprotection.

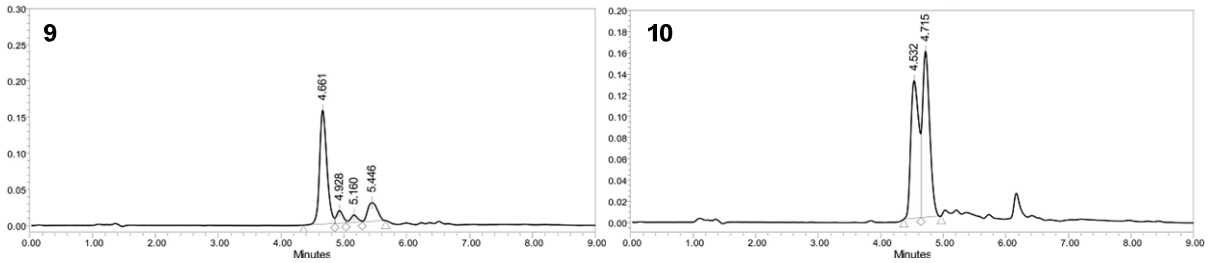


Scheme 3: Acidolysis of peptide **4** (1h TFA/TIS/H_2O (95:2.5:2.5))

After observing that Cys pseudoprolines were removed using short TFA treatments, we addressed the capacity of thiazolidine rings to enhance peptide macrocyclization. This experiment was based on the cisoid amide conformation of the thiazolidine ring, through which the "kink" in the backbone would bring the two reacting groups closer and consequently speed up the macrocyclization. We chose two peptides based on α–conotoxins (CnIB and A1.4) and replaced two of the four Cys residues with Allyl protected Asp and a diaminopropionic acid (Dap) residues.[12] The experiment required four peptides, two conotoxin derivatives with either a Ser-Cys or an Ala-Cys pseudoproline, and two peptides with standard protected amino acids. The Allyl protecting groups were removed using palladium catalysis and the on-resin macrocyclization was performed using diisopropylcarbodiimide (DIC) and OxymaPure as the coupling system. After 2 h the coupling was stopped. A Kaiser test indicated complete macrocyclization for the peptides containing the pseudoprolines (Scheme 4, vial 1 and 3). This observation contrasts with the incomplete macrocyclization detected for the peptides that did not contain Cys pseudoprolines (Scheme 4, vial 2 and 4). Analysis of the peptides after cleavage from the resin determined complete macrocyclization of those containing Cys pseudoprolines (**9** and **11**), while peptide **10** and peptide **12**,

which did not contain Cys pseudoprolines, showed 52% and 70% macrocyclization, respectively. These results show that the thiazolidine ring in Cys pseudoprolines enhance macrocyclization by significantly decreasing the coupling time.





Scheme 4: Macrocyclization experiments of peptides with and without pseudoprolines

Thiazolidine based Cys pseudoproline dipeptides have previously been described to be highly stable to TFA and require harsh acids for removal. As a result, these protecting groups are not widely used. We observed markedly different behavior to TFA, with deprotection times of 1 h for peptides **3** and **4** and

several hours for the other examples. Our findings show that Cys pseudoproline removal can be equally efficient as that of the widely used oxazolidine pseudoprolines.

5.3 Conclusions

Cys pseudoprolines were shown to enhance the on-resin macrocyclization efficiency of two conotoxin derivatives by significantly decreasing the coupling time required for completion of the reaction. Thus, we envisage that the combined use of Cys pseudoprolines and diphenylmethyl (Dmp) protection will render a pair of Cys protecting groups compatible with trimethoxyphenylthio (S-Tmp), trityl (Trt) or phenylacetamido (Phacm) protecting groups, thus amplifying the repertoire of Cys protecting groups for the synthesis of complex peptides. In summary, the short deprotection times and enhanced macrocyclization efficiency of Cys pseudoprolines significantly increase their applicability as Cys protecting groups.

5.4 References

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Chapter 5

Supporting Information

Cysteine Pseudoprolines for Thiol Protection and Peptide Macrocyclization Enhancement

Tobias M. Postma and Fernando Albericio

Manuscript submitted for publication

2014

5-SI.1. General Procedures

Fmoc-amino acids, Cys pseudoprolines and Fmoc-Rink Amide AM resin were obtained from IRIS Biotech (Marktredwitz, Germany). Rink-Amide-Chemmatrix Low LOA was obtained from PCAS BioMatrix Inc. (Quebec, Canada). DIPEA, diisopropylcarbodiimide (DIC) and TFA were obtained from Aldrich (Milwaukee, USA). Oxyma Pure was obtained from Luxembourg Industries Ltd. (Tel Aviv, Isreal). DMF, CH_2Cl_2, Et_2O, acetonitrile, DMSO and piperidine (HLPC grade) were obtained from SDS (Peypin, France). All reagents and solvents were used as received.

Room temperature (rt) refers to ambient temperature. Solid-phase syntheses were carried out manually in polypropylene syringed containing a polyethylene frit. Solvents and soluble reagents were removed by suction. Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min). Washings between deprotection and coupling were performed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Following the final coupling or deprotection the resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and dried under a stream of air. Yields for peptides refer to the area of the chromatographic product peak recorded at 220 nm.

High resolution mass spectrometry (HRMS) measurements were recorded on Thermo Scientific LTQ-FT Ultra spectrometer. Mass values are quoted within the error limits of ±5 ppm mass units. ESI refers to the electrospray ionization technique.

Analytical high pressure liquid chromatography (HPLC) was carried out on a Waters instrument comprising a separation module (Waters 2695), automatic injector, photodiode array detector (Waters 2998) and 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 $mL \cdot min^{-1}$ and a run time of 11 min.

LCMS was carried out 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 $mL \cdot min^{-1}$ and a run time of 11 min.

5-SI.2. General Methods Peptide Synthesis

General Method 1: Peptide Synthesis on Rink Amide AM Resin

Fmoc-Rink-Amide AM resin (0.45 mmol/gram, 1 equiv) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) followed by washing with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The protected Fmoc-amino acids (3 equiv) were incorporated using DIC (3 equiv.) and Oxyma (3 equiv) in DMF, as a coupling system, with 5 min preactivation for 1 h at rt. Washes between couplings and deprotections were performed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). Completion of the coupling was monitored by the Kaiser test.[1]

General Method 2: Peptide Synthesis on Rink Chemmatrix Resin

Rink-Amide-Chemmatrix Low LOA resin (0.53 mmol/g, 1 equiv) was washed with with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min), TFA/CH_2Cl_2 (1:99) (5 × 1 min), CH_2Cl_2 (3 × 1 min), DIPEA/CH_2Cl_2 (5:95) (5 × 1 min), CH_2Cl_2 (5 × 1 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) followed by washing with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). The protected Fmoc-amino acids (3 equiv.) were incorporated using DIC (3 equiv.) and Oxyma (3 equiv.) in DMF, as a coupling system, with 5 min preactivation for 1 h at rt. Washes between couplings and deprotections were performed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Completion of the coupling was monitored by the Kaiser test.[1]

General Method 3: Allyl Protection Removal

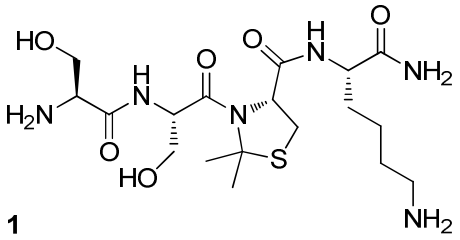
The resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min). The resin was suspended in dry CH_2Cl_2, phenylsilane (24 equiv) was added and the mixture was bubbled with N_2 for 10min. $Pd(PPh_3)_4$ (0.1 equiv) was added and the bubbled with N_2 for 10 min under the exclusion of light. The resin was washed with CH_2Cl_2 (5 × 1 min). This process was repeated twice.

General Method 4: Microcleavage

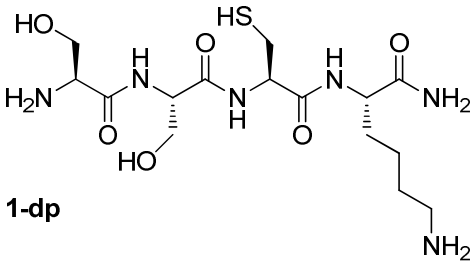
Dry resin (5 mg) was treated with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at rt. The cleavage mixture was evaporated with a stream of argon, precipitated with Et_2O, centrifuged and the pellet was redissolved in H_2O/CH_3CN (1:1) for analysis by HPLC and LCMS.

5-SI.3. Peptide Synthesis

H-Ser-Ser-Cys($\psi^{Me,Me}$pro)-Lys-NH_2 (1)



1



1-dp

Peptide **1** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (222.2 mg, 0.1 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin): *S*-Tmp was removed using General Method 2, and cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **1** was obtained in 65**%** purity and peptide **1-deprotected** (**dp**) 35% (linear gradient from 0% to 30% acetonitrile over 8 min, t_R peptide **1** : 5.0 min; peptide **1-dp**: 1.9 min). **LCMS** peptide **1** observed $[M+H]^+$ 463.2, required $[M+H]^+$ 463.2; peptide **1-dp** observed $[M+H]^+$ 423.2, required $[M+H]^+$ 423.2.

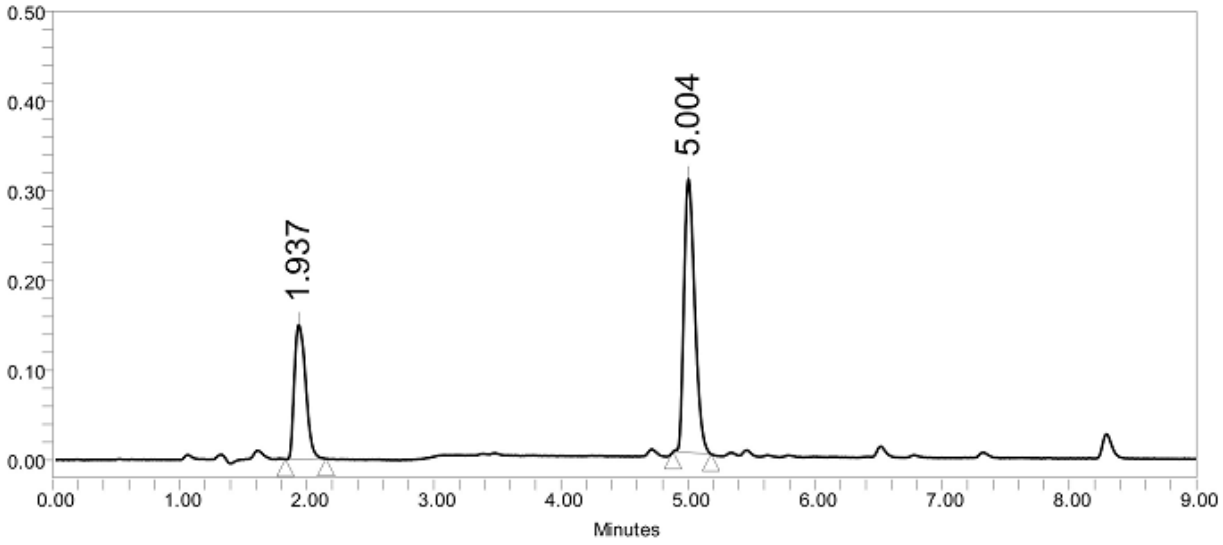
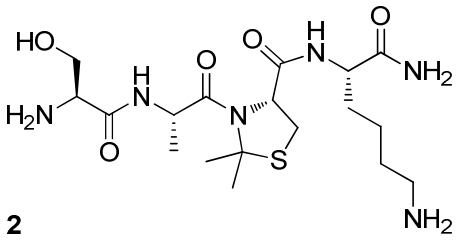
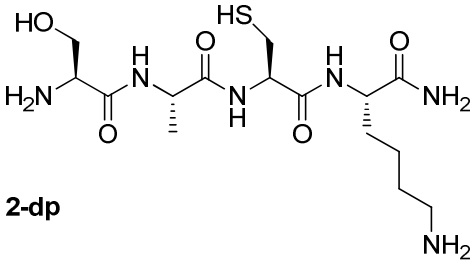


Figure S-1: HPLC chromatogram of peptide **1** and **1-dp**

H-Ser-Ala-Cys(ψ^{Me,Me}pro)-Lys-NH$_2$ (2)



2



2-dp

Peptide **2** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (222.2 mg, 0.1 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin): *S*-Tmp was removed using General Method 2, and cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **2** was obtained

in 85**%** purity and peptide **2-dp** 15% (linear gradient from 0% to 30% acetonitrile over 8 min, t_R peptide **2**: 5.2 min and peptide **2-dp** : 1.5 min). **LCMS** peptide **2** observed $[M+H]^+$ 447.3, required $[M+H]^+$ 447.2; peptide **2-dp** observed $[M+H]^+$ 407.2, required $[M+H]^+$ 407.2.

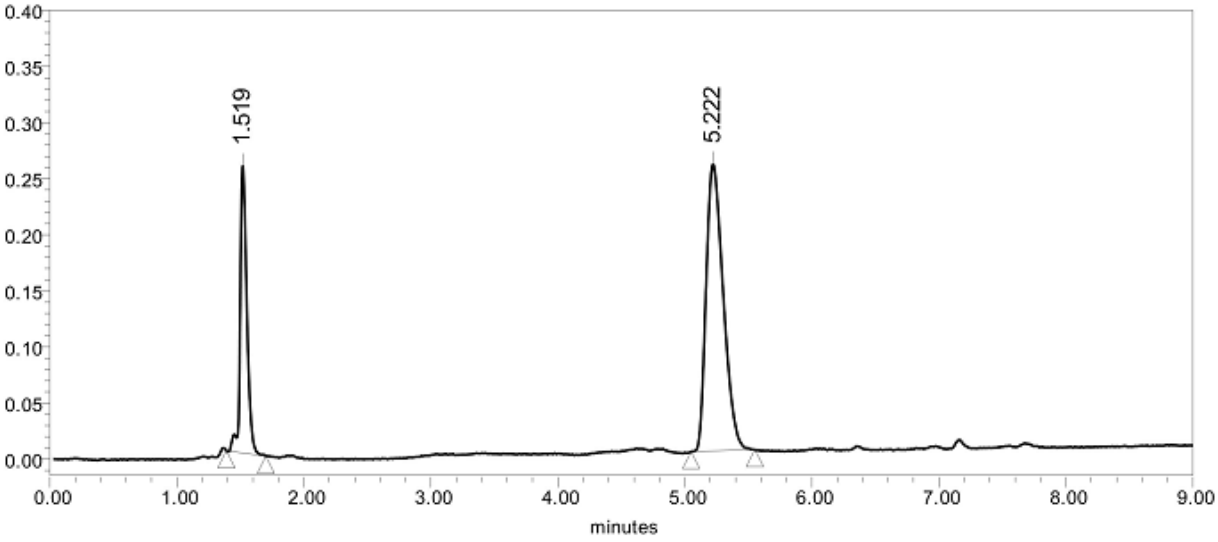
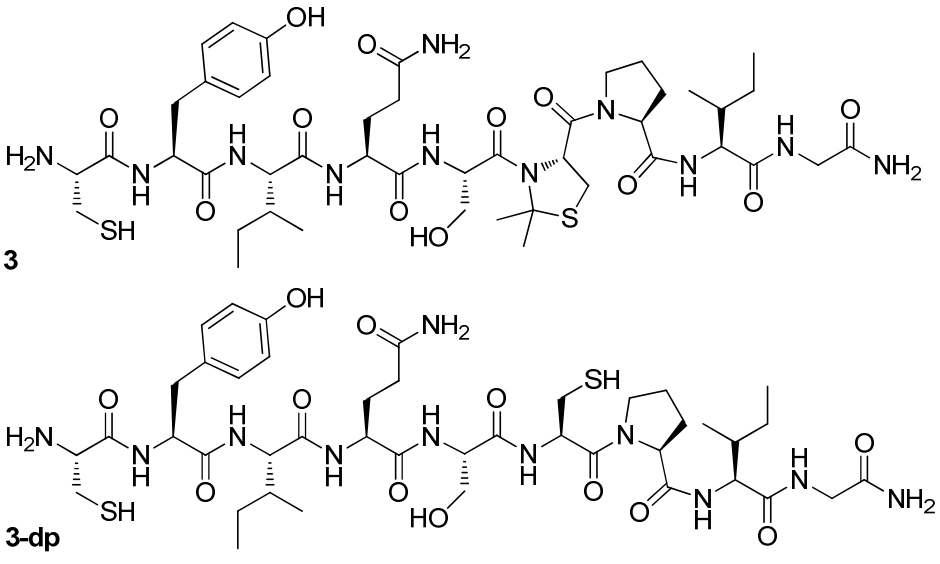


Figure S-2: HPLC chromatogram of peptide **2** and **2-dp**

Seritocin (3)



3

3-dp

Peptide **3** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (222.2 mg, 0.1 mmol, 0.45 mmol/gram). Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin): The peptide was cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **3-dp** was obtained in 98% purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 4.0 min). **LCMS** peptide **3** not observed; peptide **3-dp** observed $[M+H]^+$ 982.5, required $[M+H]^+$ 982.5.

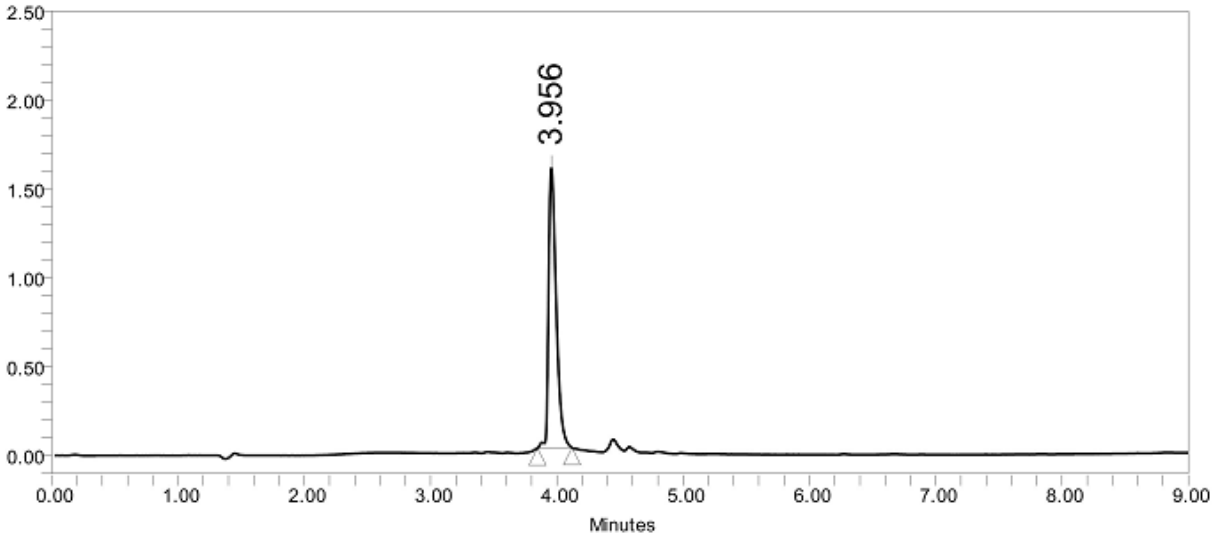
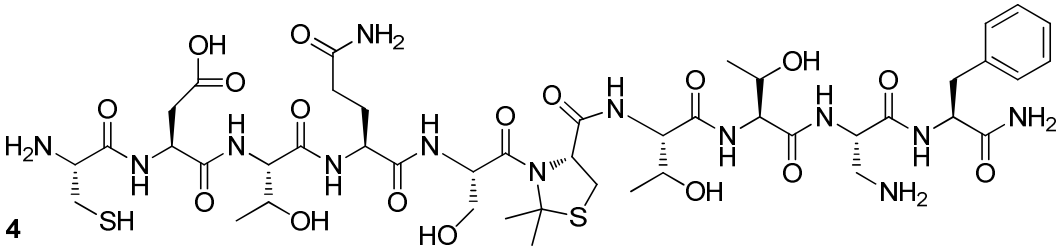
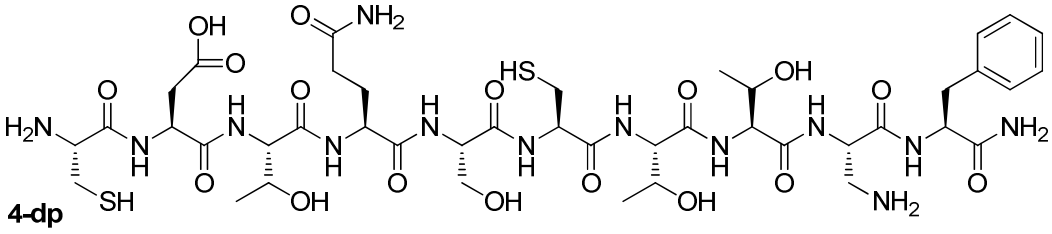


Figure S-3: HPLC chromatogram of peptide **3-dp**

Peptide (4)



4



4-dp

Peptide **4** was synthesized according to General Method 1 using Fmoc-Rink-Amide AM resin (222.2 mg, 0.1 mmol, 0.45 mmol/gram). The coupling of Fmoc-Ser-Cys($\psi^{Me,Me}$pro)-OH needed a recoupling to achieve complete conversion. Following peptide elongation, the resin was treated with 20% piperidine/DMF (1 × 1 min and 2 × 5 min) to remove Fmoc, and washed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Microcleavage (5 mg resin): The peptide was cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **4-dp** was obtained in 88**%** purity (linear gradient from 5% to 100% acetonitrile over 8 min, t_R : 3.4 min). **LCMS** peptide **4** not observed; peptide **4-dp** observed $[M+H]^+$ 1090.5, required $[M+H]^+$ 1090.4.

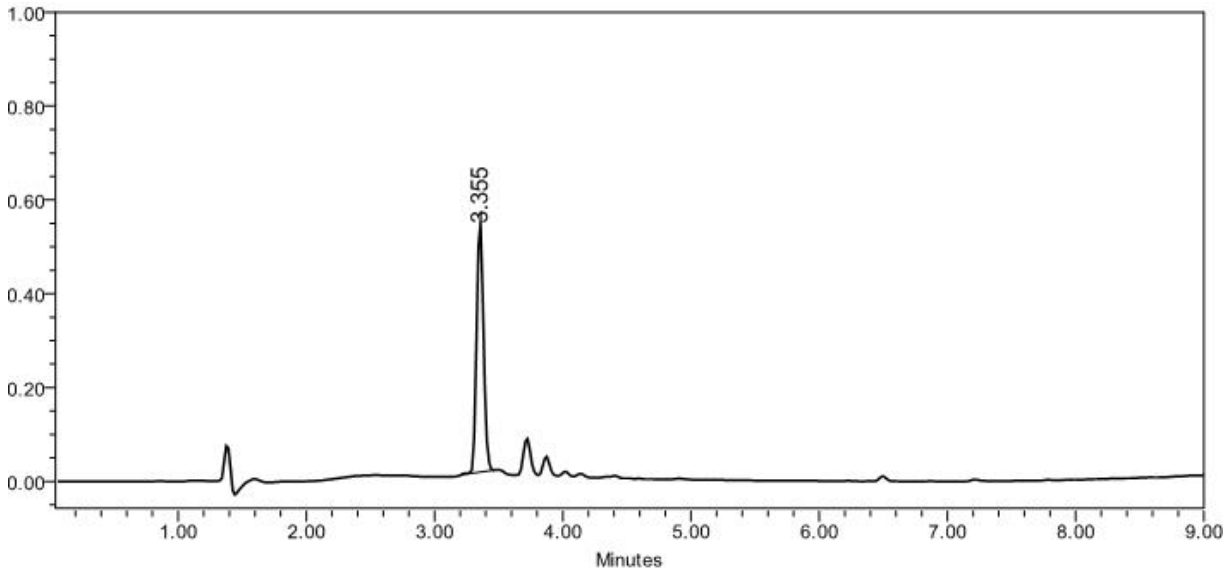
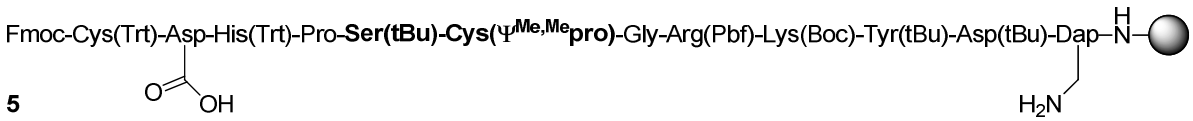


Figure S-4: HPLC chromatogram of peptide **4-dp**

Conotoxin Derivative (5)



Peptide **5** was synthesized according to General Method 2 using Rink-Amide-Chemmatrix Low LOA resin (188.7 mg, 0.1 mmol, 0.53 mmol/g). Fmoc-Ser-Cys($\psi^{Me,Me}$pro)-OH was coupled for 2 h to achieve complete conversion. Following peptide elongation, Allyl protection was removed according to General Method 4. Microcleavage (5 mg resin): The peptide was cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **5** was obtained in 35**%** purity and peptide **5-dp** 65% (linear gradient from 25% to 50% acetonitrile over 8 min, t_R peptide 5 : 4.9 min and peptide 5-dp : 4.3 min). **LCMS** peptide **5** observed $[M+H]^+$ 1590.8, required $[M+H]^+$ 1590.7 and peptide **5-dp** observed $[M+H]^+$ 1550.8, required $[M+H]^+$ 1550.6.

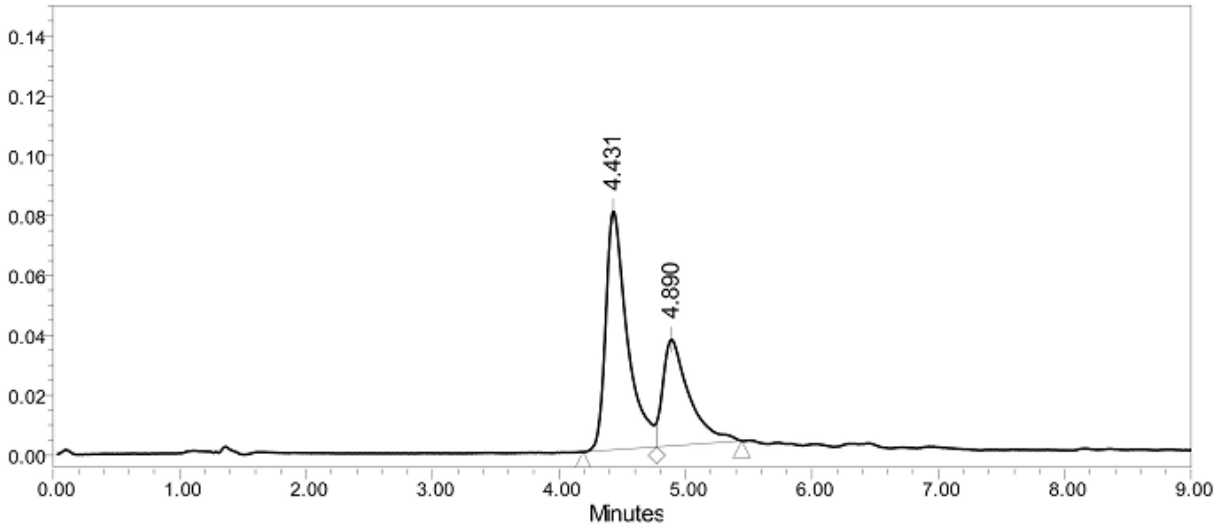
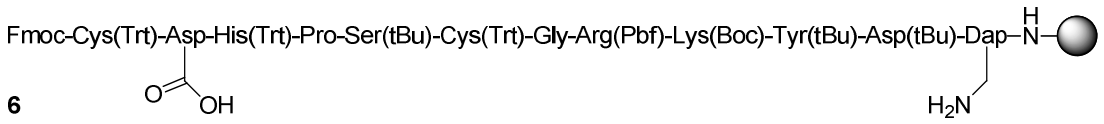


Figure S-5: HPLC chromatogram of peptide **5**

Conotoxin Derivative (6)



6

Peptide **6** was synthesized according to General Method 2 using Rink-Amide-Chemmatrix Low LOA resin (188.7 mg, 0.1 mmol, 0.53 mmol/g). Following peptide elongation, Allyl protection was removed according to General Method 4. Microcleavage (5 mg resin): The peptide was cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **6** was obtained in 97**%** purity (linear gradient from 25% to 50% acetonitrile over 8 min, t_R : 4.3 min). **LCMS** peptide **6** observed $[M+H]^+$ 1550.8, required $[M+H]^+$ 1550.6.

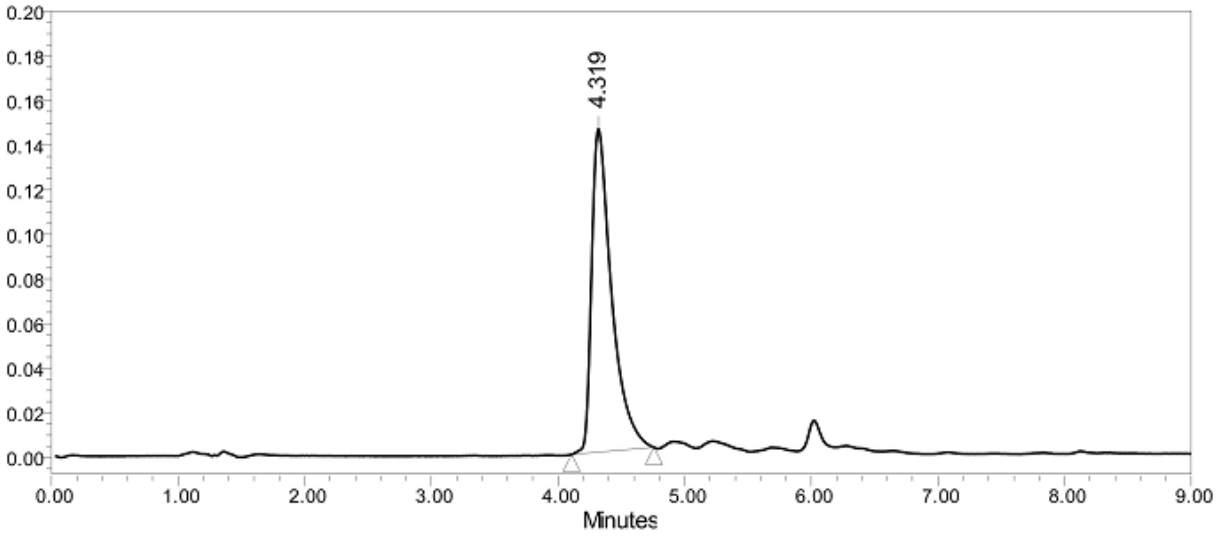
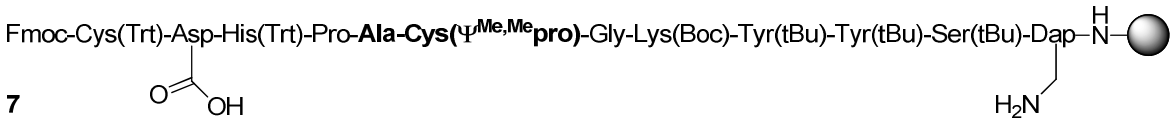


Figure S-6: HPLC chromatogram of peptide **6**

Conotoxin Derivative (7)



7

Peptide **7** was synthesized according to General Method 2 using Rink-Amide-Chemmatrix Low LOA resin (188.7 mg, 0.1 mmol, 0.53 mmol/g). Fmoc-Ala-Cys($\psi^{Me,Me}$pro)-OH was coupled for 2 h to achieve complete conversion. Following peptide elongation, Allyl protection was removed according to General Method 4. Microcleavage (5 mg resin): The peptide was cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **7** was obtained in 16**%** purity and peptide **7-dp** 84% (linear gradient from 25% to 50% acetonitrile over 8 min, t_R peptide **7** : 4.1 min and peptide **7-dp** : 3.7 min). **LCMS** peptide **7** observed $[M+H]^+$ 1627.8, required $[M+H]^+$ 1627.7 and peptide **7-dp** observed $[M+2H]^+$ 794.3, required $[M+2H]^+$ 794.3.

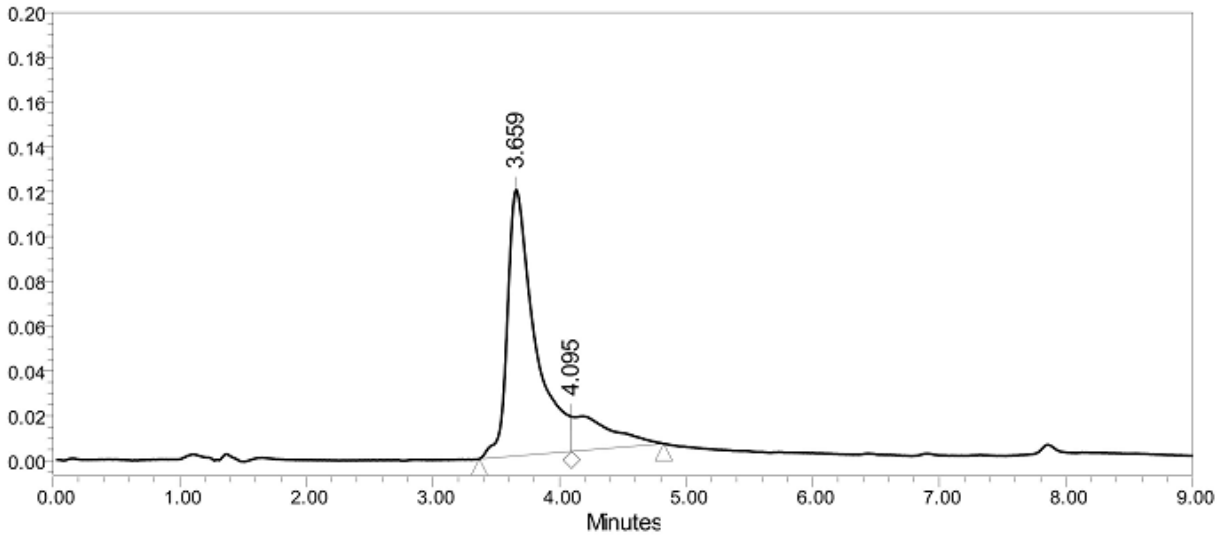
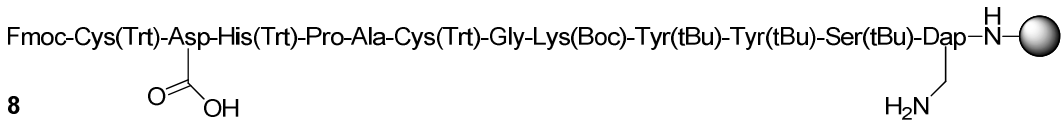


Figure S-7: HPLC chromatogram of peptide **7**

Conotoxin Derivative (8)



8

Peptide **8** was synthesized according to General Method 2 using Rink-Amide-Chemmatrix Low LOA resin (188.7 mg, 0.1 mmol, 0.53 mmol/g). Fmoc-Ser-Cys[Psi(Me,Me)Pro]-OH was coupled for 2 h to achieve complete conversion. Following peptide elongation, Allyl protection was removed according to General Method 4. Microcleavage (5 mg resin): The peptide was cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **8** was obtained in 98**%** purity (linear gradient from 25% to 50% acetonitrile over 8 min, t_R : 3.5 min). **LCMS** peptide **8** observed $[M+2H]^+$ 794.4, required $[M+2H]^+$ 794.3.

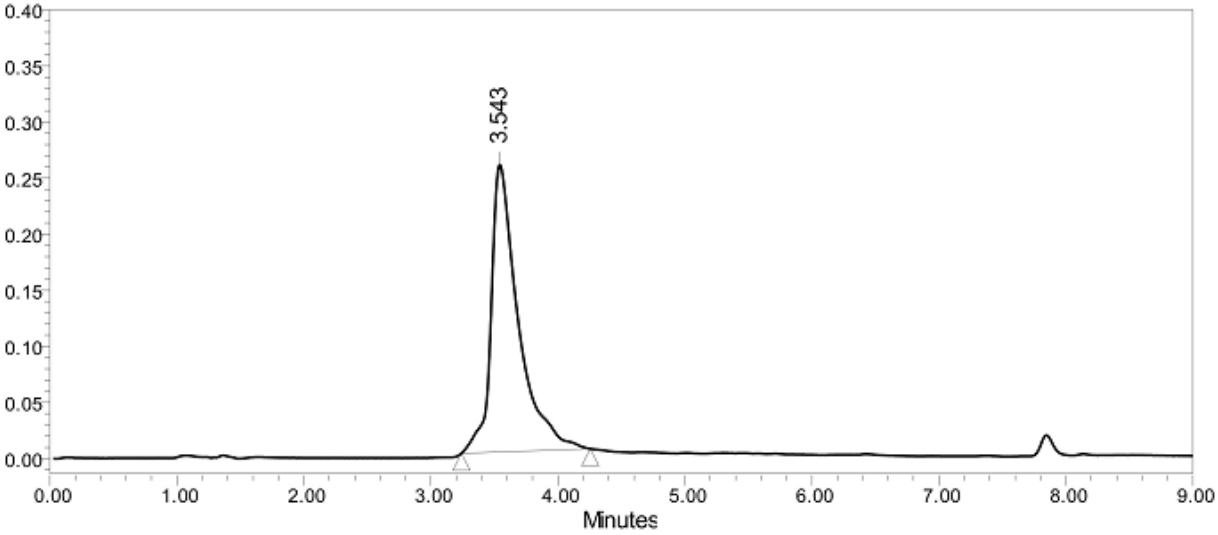
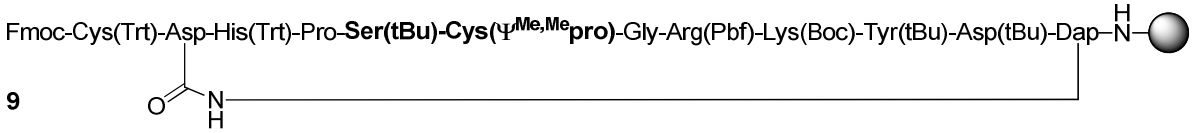


Figure S-8: HPLC chromatogram of peptide **8**

Macrocyclization of peptide 5 (9)



Peptide resin **5** (10 mg, 5.3 µmol) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The macrocyclization was performed using DIC (4 equiv.) and Oxyma Pure (4 equiv) in DMF (300 µl), as a coupling system, for 2 h at rt. The peptide was cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **9** was obtained in 18**%** purity and peptide **9-dp** was obtained in 77% (linear gradient from 25% to 50% acetonitrile over 8 min, t_R peptide **9** : 5.5 min and peptide **9-dp** : 4.7 min). **LCMS** peptide **9** observed $[M+H]^+$ 1609.8, required $[M+H]^+$ 1609.7; peptide **9-dp** observed $[M+H]^+$ 1569.4, required $[M+H]^+$ 1569.6.

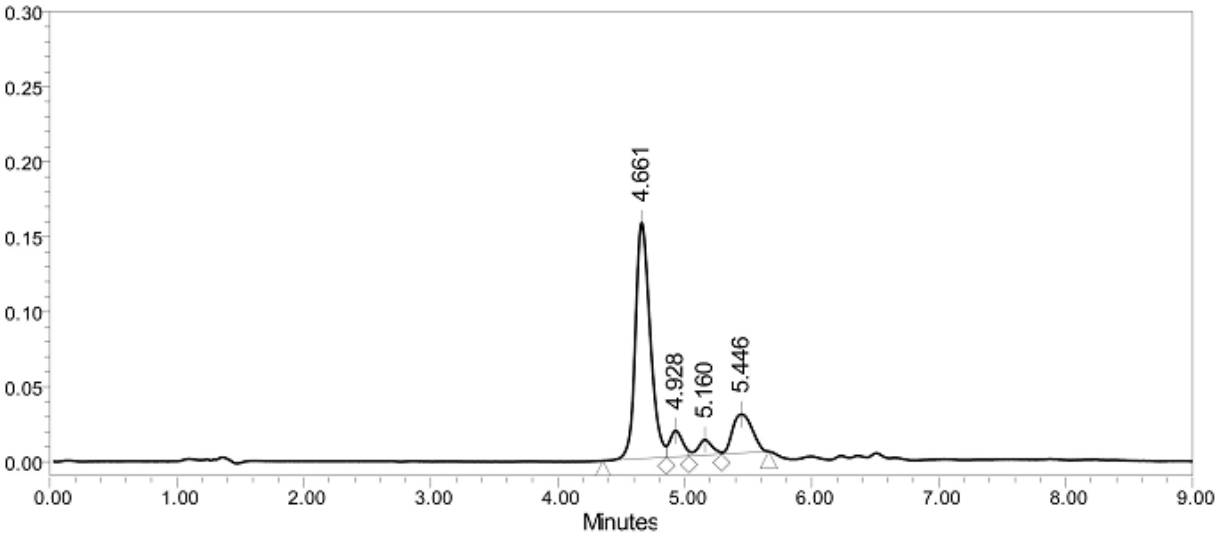
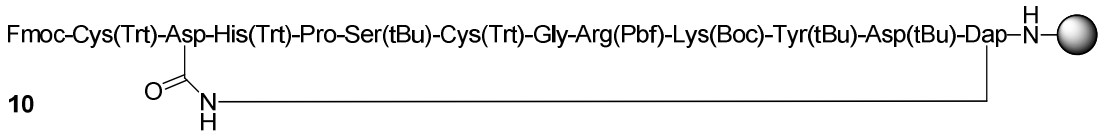


Figure S-9: HPLC chromatogram of peptide **9**

Macrocyclization of peptide 6 (10)



10

Peptide resin **6** (10 mg, 5.3 µmol) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The macrocyclization was performed using DIC (4 equiv.) and Oxyma Pure (4 equiv) in DMF (300 µl), as a coupling system, for 2 h at rt. The peptide was cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **10** was obtained in 45**%** purity (linear gradient from 25% to 50% acetonitrile over 8 min, t_R : 4.7 min). **LCMS** peptide **10** observed $[M+H]^+$ 1569.9, required $[M+H]^+$ 1569.6.

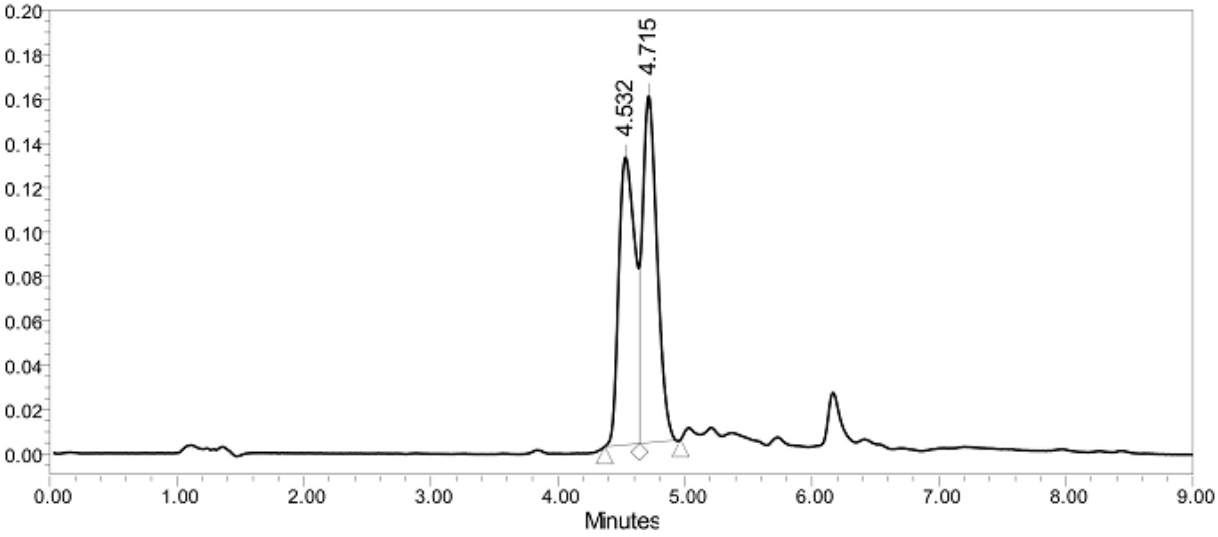
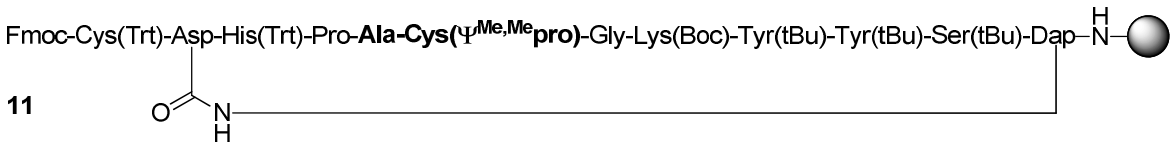


Figure S-10: HPLC chromatogram of peptide **10**

Macrocyclization of peptide 7 (11)



Peptide resin **7** (10 mg, 5.3 µmol) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The macrocyclization was performed using DIC (4 equiv.) and Oxyma Pure (4 equiv) in DMF (300 µl), as a coupling system, for 2 h at rt. The peptide was cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **11** was obtained in 14% purity and peptide **11-dp** 84% (linear gradient from 25% to 50% acetonitrile over 8 min, t_R peptide **11**: 3.8 min and peptide **11-dp** 3.5 min). LCMS peptide **11** observed $[M+H]^+$ 1572.9, required $[M+H]^+$ 1572.6; peptide **11-dp** observed $[M+H]^+$ 1532.8, required $[M+H]^+$ 1532.6.

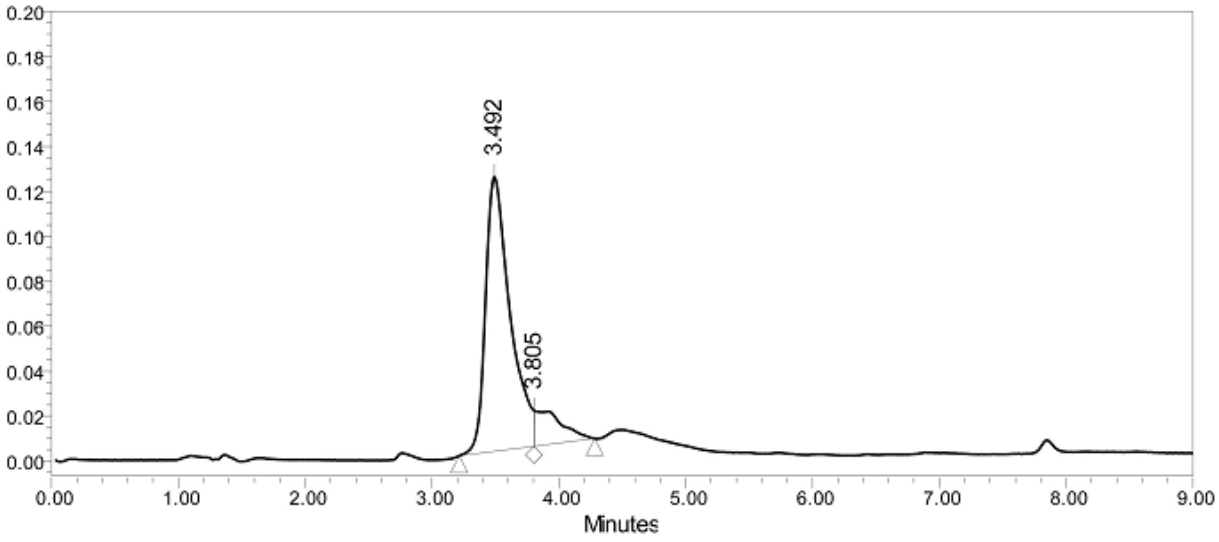
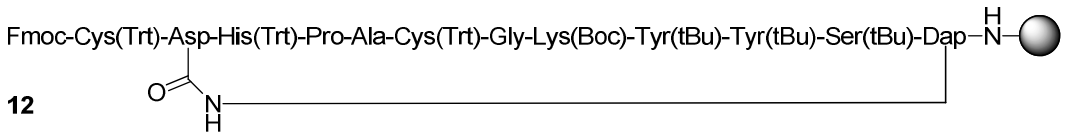


Figure S-11: HPLC chromatogram of peptide **11**

Macrocyclization of peptide 8 (12)



12

Peptide resin **8** (10 mg, 5.3 µmol) was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). The macrocyclization was performed using DIC (4 equiv.) and Oxyma Pure (4 equiv) in DMF (300 µl), as a coupling system, for 2 h at rt. The peptide was cleaved from the resin according to General Method 4 and subsequent HPLC analysis found that peptide **12** was obtained in 72**%** purity (linear gradient from 25% to 50% acetonitrile over 8 min, t_R : 3.5 min). LCMS peptide **12** observed $[M+H]^+$ 1532.8, required $[M+H]^+$ 1532.6.

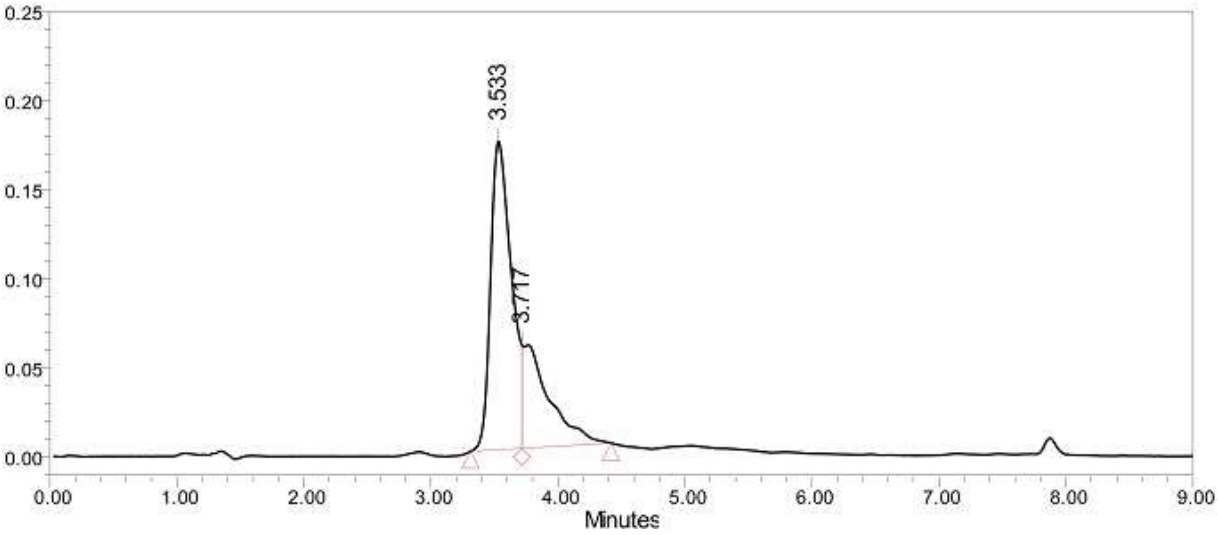


Figure S-12: HPLC chromatogram of peptide **12**

Kaiser test of Macrocyclization Experiments

After 2 h coupling a Kaiser test was performed. The peptides containing Cys pseudoproline groups had completed macrocyclization whereas the peptides with standard protection were not complete (Fig. S-13). Number 1 is peptide **9**, 2 is peptide **10**, 3 is peptide **11** and 4 is peptide **12**.

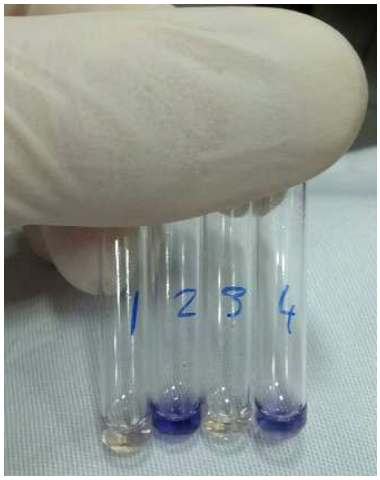


Figure S-13: Kaiser test of macrocyclization experiment

5-SI.4. References

1. Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I., *Anal. Biochem.* **1970,** 34, 595-598.

General Conclusions

Chapter 1

In this chapter we introduced the subject in a general introduction followed by a review of the current literature on disulfide formation strategies. The state-of-the-art in the field is described followed by a brief perspective of where the field is heading.

Chapter 2

The protecting groups *S*-Dmp and *S*-Tmp were introduced as suitable replacements for the difficult to remove StBu. The protecting groups are stable to base, SPPS coupling conditions, and a low concentration of TFA. The novel protecting groups can be removed with mild reducing agents within minutes, compared to many hours needed for *S*tBu removal. Steric hindrance when flanked with two bulky Trt groups did not change the deprotection time for *S*-Dmp and *S*-Tmp. In summary, we recommend the use of *S*-Tmp over *S*-Dmp because it showed greater stability, gave the purest product in the synthesis of oxytocin and was successfully used in T22 synthesis.

Chapter 3

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 one instead of two equivalents 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.

Chapter 4

The feasibility of using NCS for peptide disulfide bond formation under aqueous conditions was demonstrated in this chapter. 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.

Chapter 5

Immobilized NCS is an attractive and simple-to-use disulfide-forming reagent for use in combinatorial libraries. Disulfide formation in 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.

Chapter 6

Cys pseudoprolines were shown to enhance the on-resin macrocyclization efficiency of two conotoxin derivatives by significantly decreasing the coupling time required for completion of the reaction. Thus, we envisage that the combined use of Cys pseudoprolines and diphenylmethyl (Dmp) protection will render a pair of Cys protecting groups compatible with trimethoxyphenylthio (S-Tmp), trityl (Trt) or phenylacetamido (Phacm) protecting groups, thus amplifying the repertoire of Cys protecting groups for the synthesis of complex peptides. In summary, the short deprotection times and enhanced macrocyclization efficiency of Cys pseudoprolines significantly increase their applicability as Cys protecting groups.

Acknowledgements

After 3 years of working non-stop in the lab there are quite a lot of people to thank for their help, patience and guidance.

To start, I thank Fernando for accepting me for this PhD position. The guidance, academic support and mostly the freedom you gave me significantly increased grasp and skill in synthetic organic chemistry with a strong peptide flavour. Although, perhaps the flavour and odor were thiol related.

When starting about thiols I really ought to apologize to all the people in lab Pharmamar for being the cause of multiple thiol induced evacuations of the lab during my first year. I really hope that during the past years I have lost the title that Miriam gave me, the new king of smelly things!

My previous co-workers Albert and Nuria I would like to thank for telling me about the PhD position in Fernando's group and for recommending me to Fernando for the position.

I would like to thank Miriam for being a great help when I started in the lab. Also for your continued help with cysteine chemistry throughout the entire period! In a similar manner I would like to thank Marta for patiently helping me getting acquainted with all the analytical equipment in the lab. I shared some good times with the other members of Pharmamar so thank you all: Ivan, Gerardo, Pau, Hortensia and Ximena.

I had a great time with Vida and all the other members of the Marie Curie MemTide network. It was a real treat to visit all the member institutions all over Europe. It was educational to see research aspects on the interface of chemical engineering and synthetic organic chemistry branching both academic labs and industry.

In the big lab I thank Peter for being my only Dutch connection in Barcelona. Anna-Iris, it was great fun in Athens with lots of drinks and good conversation. See you in Holland! Markus, Hawaii was fun and was glad to see you there! I also thank all other members of the group of Fernando Albericio. I had a really enjoyable time in Barcelona and I wish you all luck with the progression of your careers!

For the funding I would like to thank the European Union for providing the generous funding which enabled me to do all the work. For coordinating the project and all the collaborators I thank Prof. Livingstone and Alan Ashton-Smith for putting in all the hard work.

The academic input from the annual meetings with Dr. Verdaguer, Dr. Delgado and Dr. Borrell was very useful and I thank them sincerely.

Resumen en Español

Capítulo 1 - Introducción

La cisteína (Cys) es un importante aminoácido que posee una propensión única en los péptidos y proteínas para formar enlaces disulfuro. [1] Un disulfuro es un enlace covalente reversible entre los tioles de cadena lateral de dos residuos de Cys. El enlace disulfuro provoca limitaciones conformacionales que dan lugar a moléculas más rígidas que normalmente estabiliza al péptido o proteína. [2] Los péptidos que poseen varios disulfuros son el paradigma de lo que los enlaces disulfuro pueden conferir a un péptido, sobre todo si lo comparamos con los péptidos sin enlaces disulfuro. Por ejemplo, los péptidos presentan gran propensidad a la degradación metabólica a través de la digestión enzimática y típicamente tienen una vida media corta, rango de unos pocos minutos, en la sangre. [3] Sin embargo, los péptidos ricos en disulfuro pueden incrementar drásticamente tanto su estabilidad metabólica como térmica. Esto queda perfectamente ilustrado en el ciclótido Kalata B1, que contiene tres enlaces disulfuro. Kalata B1, que se extrae de una planta meadiante agua en ebullición, presenta disponibilidad oral y sobrevive a los jugos gástricos fuertemente proteolíticos. [4] El mismo péptido es fácilmente susceptible a la degradación, después de la reducción de los 3 enlaces disulfuro mediante enzimas proteolíticas.

Los ciclótidos son mini-proteínas circulares (cabeza – cola del esqueleto cicladas) ricas en disulfuro que se encuentran en plantas y que contienen entre 28-37 residuos. [5, 6] La principal característica estructural de los ciclótidos es la conservación del motivo de cisteínas, compuesto por 3 enlaces disulfuro, y su combinación con el esqueleto ciclado que hace que estas moléculas sean excepcionalmente estables. El potencial terapéutico de ciclótidos es muy vasto debido a su estabilidad, a la variedad de bioactividades naturales, tales como actividades antivirales y antimicrobianos, y con un andamiaje para injertarle secuencias bioactivas. [7-10] La síntesis de los ciclótidos se realiza fácilmente con química en fase sólida Boc y oxidación conformacional en solución. [11] La ciclación de la cadena principal (esqueleto) se lleva a cabo típicamente mediante ligación química nativa intramolecular (NCL). [12] NCL es un método muy útil en el que dos fragmentos de péptidos se pueden unir a través de la transtioesterification del tiol de Cys y un tioéster seguido por una sustitución nucleófila sobre el acilo para formar un enlace amida. [13-15] La metodología NCL, que ha revolucionado la síntesis química de proteínas y de péptidos complejos, es una aplicación importante de Cys. [16]

La estabilidad conformacional de péptidos ricos en disulfuro puede conducir a una potente y selectiva unión del péptido a su diana. [17] Péptidos ricos en disulfuro del veneno de serpientes, escorpiones, arañas y caracoles cono, ilustran la diversa farmacología con alta selectividad y fuerte potencia de este tipo de moléculas. [18, 19] Conotoxinas son péptidos del veneno de caracoles cono que contienen varios enlaces disulfuro y que forman un cóctel farmacológico complejo altamente eficiente utilizado para la caza de otros organismos mediante un mecanismo basado en un arpón natural envenenado.[20] Las conotoxinas actúan sobre objetivos neurológicos tales como los receptores de canales iónicos y, debido a su amplia diversidad, selectividad y potencia, son muy interesantes desde el punto de vista terapéutico. [21]

La ω conotoxina ziconotida (Prialt), que contiene 3 puentes disulfuro, se introdujo como la primera conotoxina terapéutica para el tratamiento de dolor agudo y crónico asociado al cáncer (Figura 1a). [22] Linaclotida es otro péptido terapéutico, que contiene 3 enlaces disulfuro, que se usa para el tratamiento del estreñimiento idiopático crónico y síndrome de intestino irritable en adultos (Figura 1b). [23, 24] Actualmente, hay varios péptidos ricos en disulfuro en los ensayos clínicos que indica el potencial de estos péptidos como agentes terapéuticos. [20]

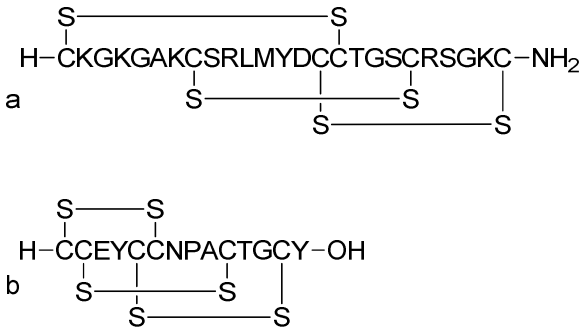


Figura 1: Secuencia y conectividad de disulfuros de a) Ziconotida, b) Linaclotida

La importancia de péptidos que contienen disulfuro no sólo se limita a péptidos terapéuticos. Hay una amplia gama de aplicaciones en las que se emplean péptidos con disulfuros para fines específicos tales como bioconjucation, [25]estabilización de β-hoja, [26] administración-liberación de siRNA, [27] aumento de la estabilidad *in vivo,* [28] estabilización de nanovehículos basados en péptidos, y enlazadores de cadena principal de péptidos. [29, 30]

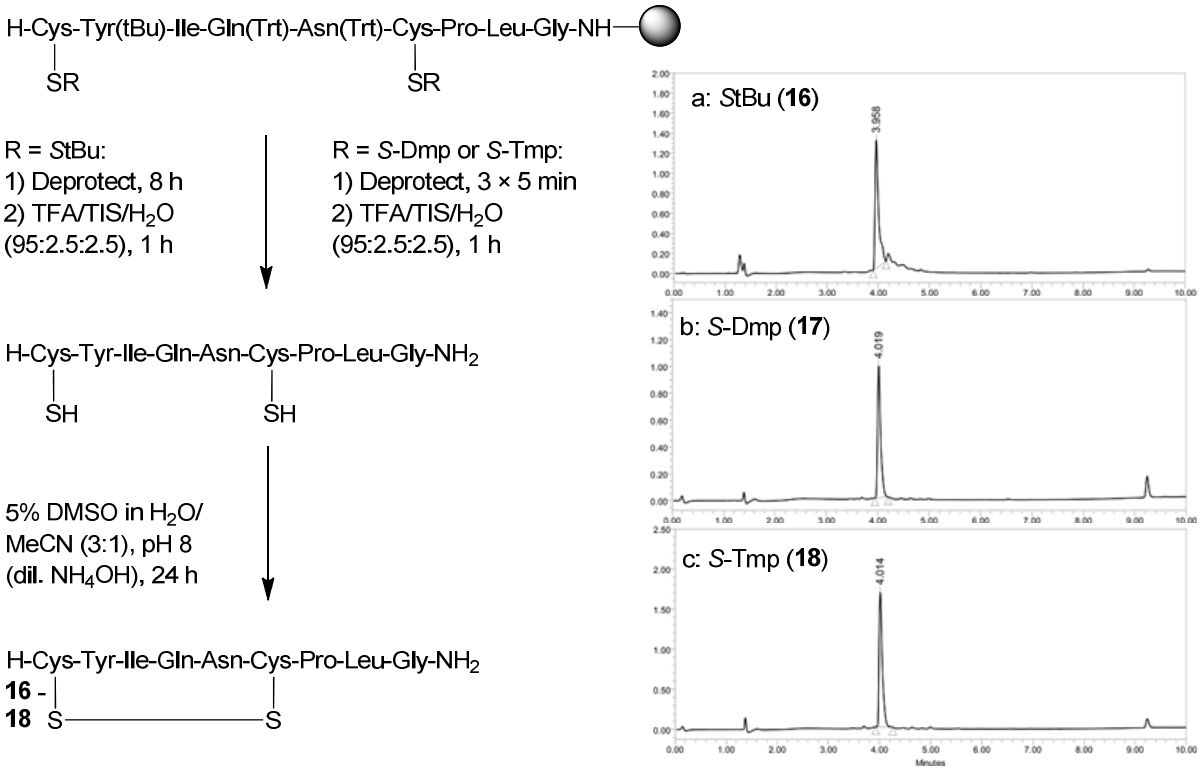
La importancia creciente de los péptidos ricos en disulfuro conduce a la necesidad de contar con una química variada para la formación de enlaces disulfuro y, por lo tanto, de nuevas estrategias químicas que permiten la preparación conveniente y fácil de péptidos con conectividades de disulfuro complejas. [31]

Por lo general, hay tres estrategias utilizadas para la síntesis de péptidos que contienen disulfuro: I) los disulfuros se preparan en la fase sólida; II) formación de algún disulfuro en fase sólida, seguido de la formación de un disulfuro posterior en solución; III) formación de todos los disulfuros en solución.[32] Para la estrategia I, el disulfuro se forma sobre una resina con una media a baja funcionalización, aprovechando el fenómeno cinético conocido como pseudo-dilución, que favorece la formación de un disulfuro intramolecular. [33] Péptidos ricos en disulfuro pueden ser regioselectivamente preparados utilizando esquemas de protección ortogonales. En la estrategia II se forma el primer disulfuro en la fase sólida, que restringe la libertad conformacional, seguido por la formación de uno o más disulfuros en solución. La formación de estos está teoricamente favorecid debido a las restricciones conformacionales impuestas por el primer disulfuro. Estrategia III, también llamado plegamiento oxidativo, se basa en la formación de todos los disulfuros en solución. Este método es muy eficiente con el péptido natural o con secuencias naturales de proteínas. [34] Sin embargo, con frecuencia una mezcla de péptidos con diferentes conectividades disulfuro se obtiene, lo que puede ser difícil de purificar. [35] En todos los casos, conformaciones nativas pueden ser más fácilmente obtenidas.

Durante la síntesis de péptidos que contienen Cys hay que tener cuidado para evitar o minimizar las reacciones secundarias en torno a la Cys. [36, 37] La Cys es propensa a la racemización catalizada por base durante el acoplamiento y, por lo tanto, el acoplamiento en condiciones neutras utilizando reactivos de carbodiimida se recomienda. [38, 39] Alquilación del tiol de la Cys puede ser un problema durante la escisión del péptido de la resina con altas concentraciones de TFA. [40] Con el fin de evitar estos problemas la escisión debe ser tan corta como sea posible en la presencia de los capturadores de carbocationes apropiados.

Capítulo 2

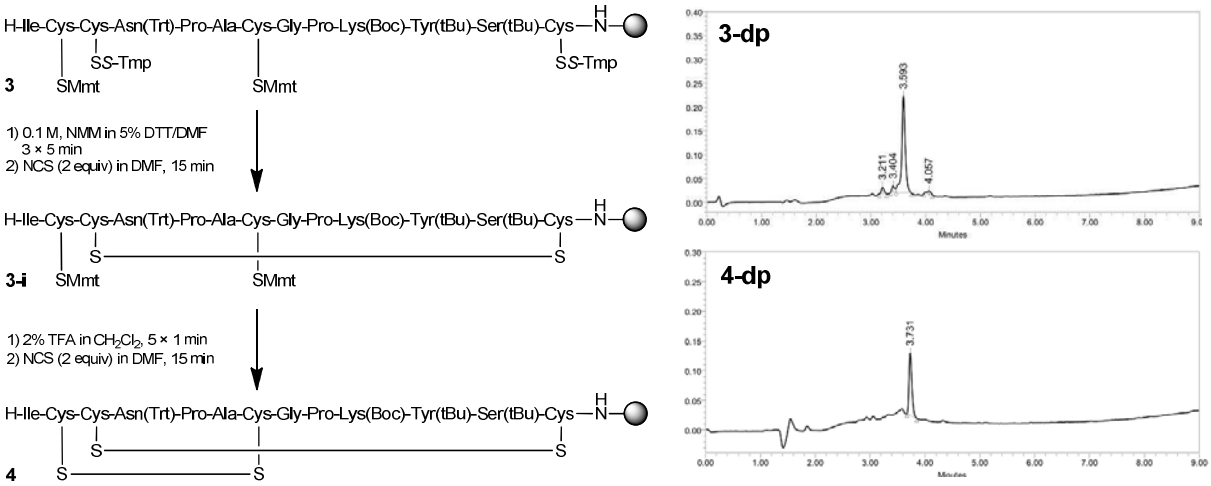
Se ha descrito el Trimetoxifeniltio (*S*-Tmp) como un nuevo protector de cisteína compatible con el grupo Fmoc para la síntesis de péptidos en fase sólida. El sustitución *S*-Tmp, puede sustituir *terc-butiltio* que es difícil de eliminar. *S*-Tmp y dimethoxyphenylthio (*S*-Dmp) se utilizaron con éxito para la protección de cisteína en una variedad de péptidos. Además, ambos grupos se pueden eliminar en 5 min con agentes reductores suaves (Esquema 1) *S*-Tmp se recomienda para la protección de cisteína ya que produce crudos peptídicos de alta pureza.



Esquema 1: Comparación de grupos protectores

Capítulo 3

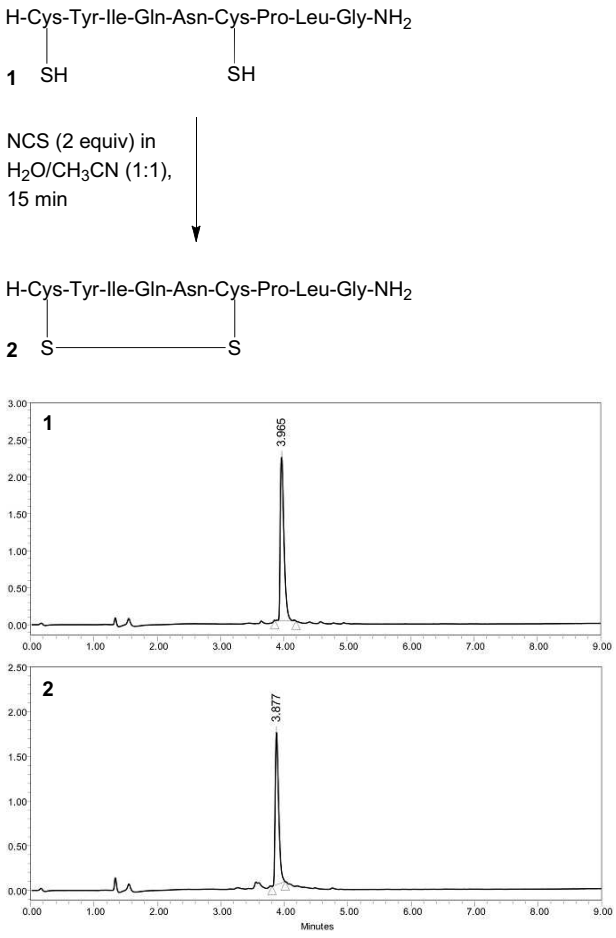
Se ha descrito la *N-clorosuccinimida* como un excelente reactivo para la formación de disulfuros sobre la resina. La formación de los enlaces disulfuro se completa en DMF en 15 minutos. Esta estrategia fue utilizada con éxito en la síntesis de la oxitocina, y una síntesis regioselectiva de un α-conotoxina (Esquema 2). Por otra parte, la formación de disulfuro con *N*-clorosuccinimida se encontró que era compatible con la presencia de metionina y triptófano que son propensos a la oxidación.



Esquema 2: La síntesis regioselectiva de conotoxina SI

Capítulo 4

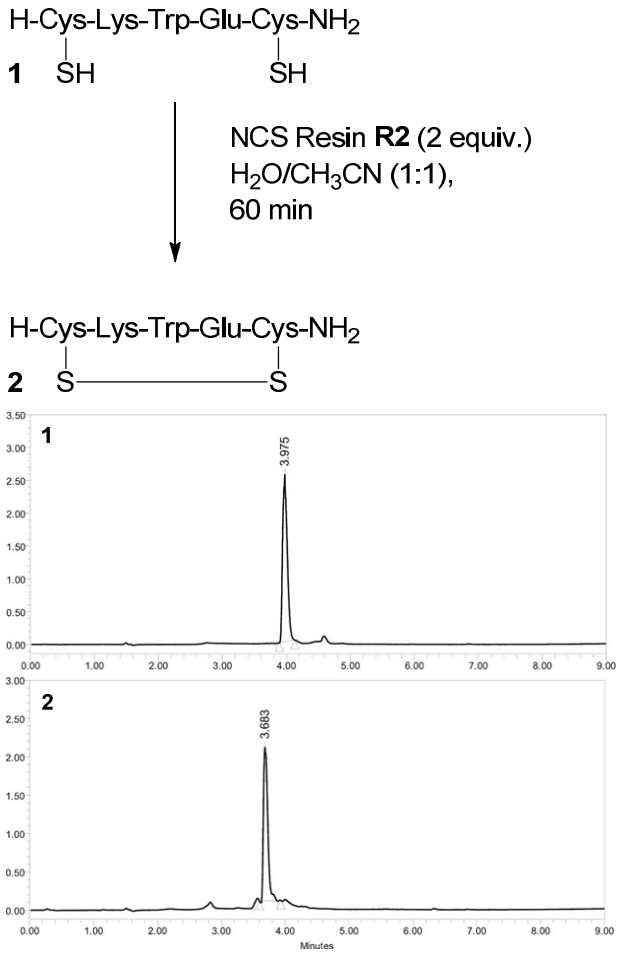
Se ha desarrollado usando *N*-clorosuccinimida un nuevo método para la formación eficiente de los enlaces disulfuro de péptidos en condiciones acuosas. La formación de enlace disulfuro es completa en 15 minutos con mezclas de disolventes que contienen agua y acetonitrilo (Esquema 3).



Esquema 3: Oxidación de la oxitocina usando NCS en medio acusoso

Capítulo 5

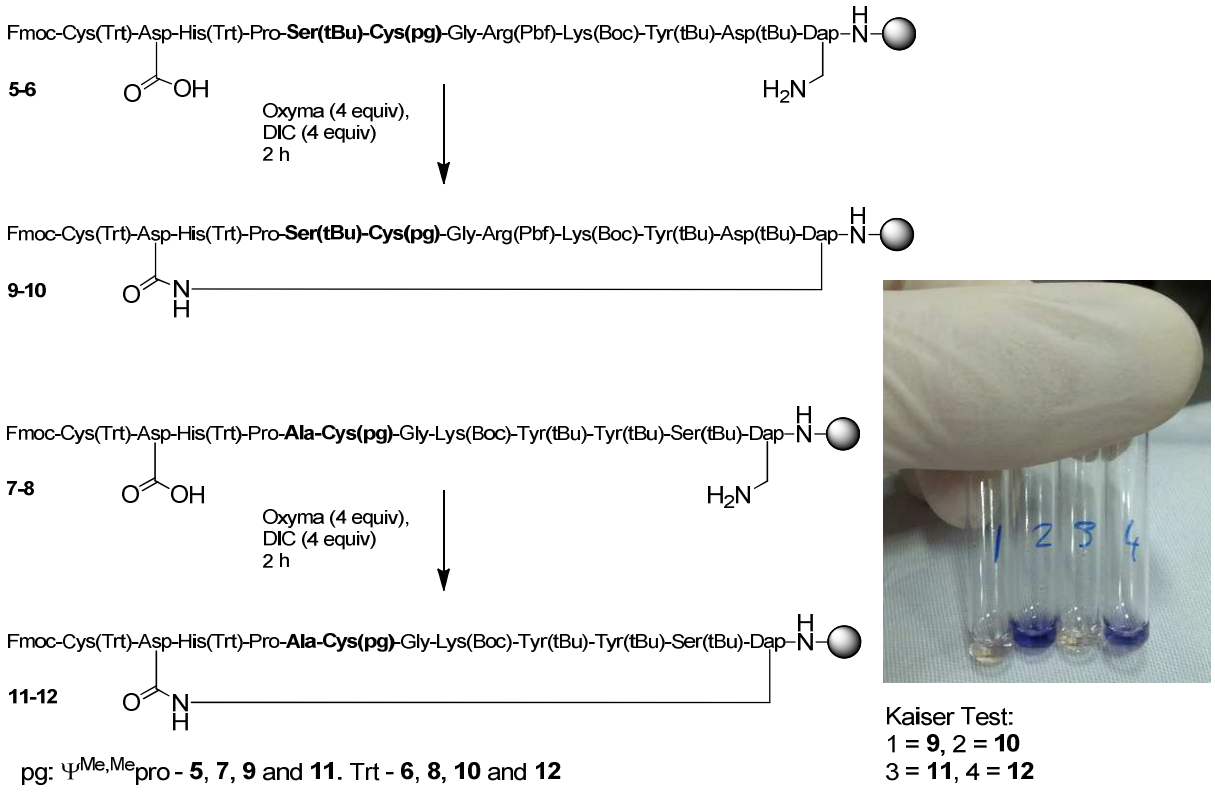
Se ha desarrollado una nueva resina inmovilizada de *N*-clorosuccinimida para la formación del enlace disulfuro de péptido, que ouede tener utilidad en la preparación de bibliotecas combinatorias. La resina se prepara mediante un sencillo proceso en dos etapa a partir de materiales de partida comerciales. La formación de disulfuro se inicia mediante la adición de una solución de péptido a la resina, y el exceso de reactivo se elimina por filtración conveniente tras la finalización de la formación de disulfuro (Esquema 5). La formación del disulfuro es rápida y limpia, como se demuestra por la oxidación de una pequeña biblioteca basada en un nonapéptido. Este reactivo inmovilizado permite expandir el uso *N*-clorosuccinimida para la formación de disulfuro.

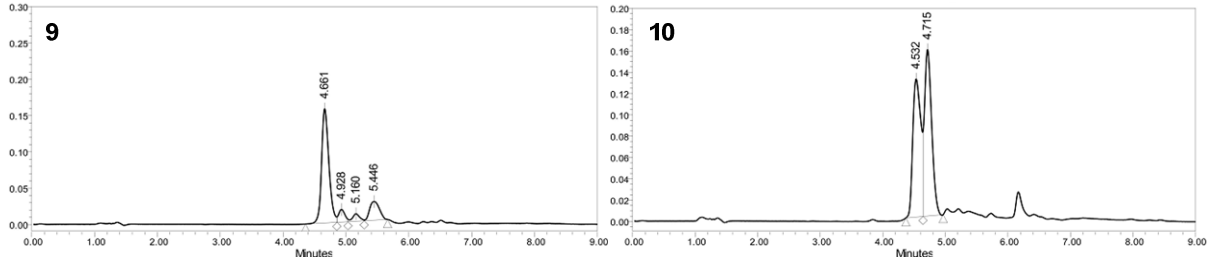


Esquema 5: La formación de disulfuro con NCS inmovilizados **(R2)**

Capítulo 6

A diferencia de otros estudios encontrados en la literatura, hemos comprobado que péptidos que contienen pseudoprolina de Cys se pueden desproteger mediante TFA por períodos no muy largos. Los tiempos de desprotección son del mismo rango que otros grupos protectores usados comúnmente en la SPPS. Por otra parte, hemos demostrado que la pseudoprolinas Cys (conformación cis) puede favorecer una macrociclación (Esquema 6).





Esquema 6: Mejora macrociclación pseudoprolina Cys

Conclusiones Generales

Capítulo 1

En este capítulo, hemos introducido el tema mediante una introducción general. Asmimismo, hemos realizado una revisión de la literatura actual sobre estrategias de formación de disulfuros. A parte de describir el estado de la técnica en el campo, se ha realizado una breve perspectiva de cómo evoluciona el campo.

Capítulo 2

Los grupos protectores *S*-Dmp y *S*-Tmp fueron introducidos como sustitutos ventajosos del *S*-tBu que difícil de eliminar. Los grupos protectores son estables a la base, condiciones de acoplamiento uilizadas en SPPS, y a una baja concentración de TFA. Los nuevos grupos protectores pueden eliminarse con agentes reductor suave en cuestión de minutos, en comparación con muchas horas necesarias para la eliminación de *S* tBu. Cuando existe impedimento estérico, por ejemplo Cys flanqueada con dos grupos Trt voluminosos, no cambió el tiempo de desprotección para los *S*-Dmp y *S*-Tmp. Se recomienda el uso de *S*-Tmp sobre *S*-DMP, ya que mostró una mayor estabilidad, y permitió obtener crudos peptídicos en el caso de se la oxitocina y de T22.

Capítulo 3

La NCS para la formación de disulfuro en resina ha demostrado ser versátil, eficaz y rápida para la síntesis de péptidos que contienen mono o multi disulfuros. Esto se ha mostrado en la oxidación de la oxitocina y la síntesis regioselectiva de conotoxina SI. En comparación con el yodo y el trifluoroacetato de talio (III), NCS es compatible con los grupos protectores sensibles al ácido Trt y Mmt, aumentando así su aplicabilidad. Por otra parte, NCS es compatible con péptidos que contengan Met (se debe utilizar un equivalente de NCS en vez de dos habituales. Además, este método basado en la NCS es compatible con péptidos que contienen Trp. Sobre la base de estas consideraciones, se ha llegado a la conclusión de que la NCS es el protocolo más ampliamente aplicable para la formación de disulfuro en resina.

Capítulo 4

Se ha demostrado la viabilidad de la utilización de NCS para la formación del enlace disulfuro en condiciones acuosas. La formación del enlace disulfuro es altamente eficiente enlace utilizando H_2O/acetonitrilo y con un ligero exceso de NCS. La formación de enlaces disulfuro con NCS en medio acuoso permite extender significativamente la aplicabilidad de la NCS, ya que no únicamente permite la formación de disulfuros en fase sólida, sino que permite un plegamiento oxidativo. Todo ello permite añadir una nueva herramienta al repertorio de fromación de disulfuros.

Capítulo 5

La NCS inmovilizada es una forma atractiva y fácil de usar este reactivo para la formación de disulfuros en las bibliotecas combinatorias. La formación de disulfuro tiene lugar con alta pureza, en cortos períodos de tiempo, a temperatura ambiente y con un tratamiento posterior basado en una sencilla filtración. Teniendo en cuenta todo ello, se considera que la NCS inmovilizada puede tener un gran potencial para la preparación de bibliotecas combinatorias de péptidos que contienen disulfuros.

Capítulo 6

Se la demsotrado que las pseudoprolinas de Cys pueden mejorar la eficiencia de macrociclación en la resina de dos derivados de conotoxina disminuyendo significativamente el tiempo de reacción requerido para completar la reacción. Por lo tanto, prevemos que el uso combinado de pseudoprolinas Cys y difenilmetilo protección (Dmp) se convertirá en un par de grupos protecctores de Cys, compatibles con trimetoxifeniltio (*S*-Tmp), tritilo (Trt) o fenilacetamido (Phacm) grupos protectores, y de sta manera se amplificará el repertorio de grupos protectores de Cys para la síntesis de péptidos complejs. En resumen, las pseudoprolinas de Cys (tiempos cortos de desprotección y una mayor eficiencia en la macrociclación) presentan una gran aplicabilidad como grupos protectores de Cys.

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