The Folding of Cysteine-rich Peptides is Depending on the Medium – Introduction of Ionic Liquids for the Synthesis of Conopeptides

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For my lovely family and for you,
with gratitude

Curiosity is always first in a problem that waits to be solved.

Galileo Galilei (15.02.1564 – 08.01.1642)

Zusammenfassung

Die vorliegende Dissertation befasst sich mit Untersuchungen zu Struktur und Aktivität ausgewählter Cystein-reicher Peptide. Dazu wurden effiziente Synthesestrategien entwickelt, die ausreichende Mengen der entsprechenden Substanzen liefern. Die Anwendung neuer Medien, d.h. von Ionischen Flüssigkeiten (ILs), für Reaktionen, in denen Cysteinreste miteinander reagieren, wurde erstmalig sowohl für die Oxidative Faltung (Bildung von Disulfidbindungen zwischen zwei Cysteinen) als auch für die Native Chemische Ligation (Bildung einer Peptidbindung zwischen zwei Segmenten) beschrieben. Darüber hinaus wurden NMR-Spektroskopie und Bioaktivitätsmessungen, wie z.B. ein Herzschlagtest und elektrophysiologische Messungen zur Aufklärung der dreidimensionalen Struktur und der spezifischen biologischen Aktivität der untersuchten Peptide herangezogen.

Conopeptide, die aus dem Gift der Kegelschnecken isoliert wurden, gehören zur Klasse der Neuropeptide. Sie unterscheiden sich in Struktur und Anzahl an Disulfidbindungen. Ihre Selektivität und Affinität gegenüber Ionenkanälen und Rezeptoren im Nervensystem macht Conopeptide zu potentiellen Zielen für die Medikamentenentwicklung. Das Kegelschneckengift enthält eine Mischung aus vielen verschiedenen Cystein-reichen Peptiden. Die Isolierung einer einzelnen peptidischen Komponente aus diesem Gift führt meist zu nur sehr geringen Mengen, die oftmals für die vollständige Charakterisierung und Aktivitätsuntersuchungen nicht ausreichen. Daher stellt die chemische Synthese dieser Komponenten prinzipiell eine Lösung dieses Problems dar.

Allerdings besteht eine große Herausforderung bei der chemischen Synthese dieser Verbindungen darin, die spezifische Verknüpfung der Thiolfunktionen der Cysteinreste für die Ausbildung der nativen bioaktiven Konformation zu erzielen. Weiterhin ist die Bildung der Disulfidbindungen der limitierende Schritt für hohe Ausbeuten bei der Synthese dieser Peptide. Ein wichtiges Ziel dieser Arbeit war daher die Optimierung bereits existierender synthetischer Methoden für die Herstellung Cystein-reicher Peptide. Darüber hinaus waren die Zusammenhänge zwischen den spezifischen Strukturen der Conopeptide CCAP-vil, μ-SIIIA und μ-PIIIA und ihren Aktivitäten ein weiterer Schwerpunkt, der im Rahmen dieser Arbeit untersucht werden sollte. Bei der anschließenden analytischen und strukturellen Charakterisierung dieser Peptide lag der Fokus sowohl auf der Untersuchung der linearen Vorstufen (reduzierte Peptide, freie Thiolfunktionen) als auch auf den gefalteten Peptiden (oxidierte Peptide, Disulfid-verbrückt).

Weiterhin sollte eine neue Methode entwickelt werden, die den Zugang zu Cystein-haltigen Peptiden unter umweltfreundlichen Bedingungen für die Bildung von Disulfidbrücken und die Synthese von Peptiden ausgehend von zwei Segmenten ermöglicht. Für diese Untersuchungen wurden Ionische Flüssigkeiten, darunter $[C_2mim][OAc]$, $[C_4mim][OAc]$, $[C_2mim][OTs]$, $[C_2mim][Et_2PO_2]$, und $[C_2mim][N(CN)_2]$ ausgewählt, die hauptsächlich in der Art ihrer Anionen variieren.

Für die Synthese Cystein-reicher Peptide (oxidative Faltung) in Ionischen Flüssigkeiten wurden zahlreiche positive Effekte im Vergleich zu konventionellen Methoden beobachtet. Die hydrophoben Peptide (δ-EVIA, δ-SVIE) waren in Ionischen Flüssigkeiten im Vergleich zu Pufferlösungen, die meist bei konventionellen Methoden zum Einsatz kommen, besser löslich. Daher war der Zusatz organischer Lösungsmittel nicht notwendig. Für alle untersuchten Peptide war das Ausmaß an Nebenreaktionen, wie z. B. Dimerisierung, Oligomerisierung und die Bildung fehlgefalteter Produkte, in Gegenwart Ionischer Flüssigkeiten deutlich niedriger. Weiterhin konnte auf die Zugabe zusätzlicher Reagenzien (wie organische Lösungsmittel, Redoxreagenzien, z.B. reduziertes/oxidiertes Glutathion) für eine effektive oxidative Faltung verzichtet werden. Die Ausbeuten und Effektivität der Bildung von Disulfidbrücken in unterschiedlichen ILs variierten für Conopeptide stark in Abhängigkeit von ihrer Aminosäuresequenz, ihrem Gehalt an Disulfidbindungen, ihrer Hydrophobizität und offensichtlich auch in Abhängigkeit von der Basizität der Anionen der verwendeten IL. Das neutral geladene Dekapeptid CCAP-vil, das nur eine Disulfidbindung enthält, wurde in $[C_2mim][OTs]$ am effizientesten oxidiert. Stark positiv geladene Conotoxine, wie z. B. α-GI, μ-PIIIA, μ-SIIIA, δ-EVIA, δ-SVIE, die zwei oder drei Disulfidbindungen enthalten, konnten in $[C_2mim][OAc]$ sehr effektiv oxidiert werden.

Die zweite Reaktion, die im Rahmen dieser Arbeit untersucht werden sollte, ist die Native Chemische Ligation (NCL) eines 36er mit einem 30er Peptidsegment. Für die Bildung des 66er Peptides Tridegin aus *Haementeria ghilianii* konnten in $[C_2mim][OAc]$ die besten Ergebnisse erzielt werden, auch ohne Zugabe weiterer Reagenzien (Thiophenol und Benzylmercaptan) zur Reaktionsmischung, wie sie typischerweise bei der konventionellen Methode erfolgt. Ferner konnte die Oxidative Faltung in $[C_2mim][OAc]$ für dieses Peptid mit sechs Cysteinresten erfolgreich demonstriert werden.

Die vollständige analytische Charakterisierung der Conopeptide wurde mittels chemisch-analytischer Verfahren und Methoden der Strukturbestimmung durchgeführt. Für die Unterscheidung zwischen reduzierter und oxidierter Form des Conopeptids CCAP-vil aus *Conus villepinii* kamen verschiedene Methoden, wie MALDI-TOF Massenspektrometrie,

Raman-Spektroskopie, Ellman's Test und NMR-Spektroskopie zur Anwendung. Die dreidimensionale Struktur, die mittels NMR Spektroskopie bestimmt wurde, zeigt eine starre Struktur des Peptidrückgrates, die an einen Typ(I)-β-turn erinnert und durch eine Disulfidbrücke stabilisiert wird. Bioaktivitätsstudien mittels eines Herzschlagtests an Zebrafisch- (*Danio rerio*) Embryonen ergab eine Abnahme der Herzfrequenz um 15% für das Conopeptid CCAP-vil, was auf einen herzschlagsenkenden Effekt hinweist.

Weiterhin konnte die dreidimensionale Struktur des 22er Conotoxins μ-SIIIA aus *Conus striatus* (sechs Cysteine) durch den Vergleich seines ^{1}H-NMR Spektrums mit NMR-Strukturdaten anderer Gruppen (Schroeder *et al*., 2008; Yao *et al*., 2008), bestätigt werden. Um die Spezifität von μ-SIIIA auf molekularer Ebene für verschiedene Na_V-Kanal-Subtypen zu bestimmen, wurden systematische Untersuchungen mittels der „whole cell patch clamp"-Methode durchgeführt. μ-SIIIA blockiert partiell und langsam Ratten (r)-$Na_V1.4$ Kanäle mit einem IC_{50}-Wert von 0.56 ± 0.29 μM. Allerdings war die Blockierung nicht vollständig und eine Restspannung von 10% mit einer dazugehörigen, reduzierten Einzelkanalleitfähigkeit bei hohen Peptid-Konzentrationen blieb erhalten. Bei einer Konzentration von 10 μM blockiert μ-SIIIA $rNa_V1.2$-, $rNa_V1.4$-, humane (h)-$Na_V1.4$- und Maus (m)-$Na_V1.6$-Kanäle wirksam, während $hNa_V1.7$-Kanäle nur zu 58.1 ± 5.0% inhibiert werden. $hNa_V1.5$, $rNa_V1.8$ und $hNa_V1.8$ waren unempfindlich gegenüber μ-SIIIA. Durch die Untersuchung von Domänenchimären von $Na_V1.4$ und $Na_V1.5$ konnte das wichtigste μ-SIIIA-Spezifitätsepitop in der Domäne 2 und eine zweite untergeordnete Interaktionsstelle für die μ-SIIIA-Bindung in der Domäne 1 der Kanäle lokalisiert werden. Weiterhin wurden drei Einzelmutationen im $rNa_V1.4$-Kanälen durch den Austausch mit Aminosäureresten von $Na_V1.7$ (A728N, S729D und N732T) und ihrer entgegengesetzten Mutationen von $Na_V1.7$ (N889A) durchgeführt. Dadurch konnte erstmals eine für die μ-SIIIA-Selektivität essentielle Position gefunden werden (N889 in $Na_V1.7$ und A728 in $Na_V1.4$).

Abschließend wurde ein drittes Peptid aus der Familie der μ-Conotoxine, μ-PIIIA aus *Conus purpurascens*, näher untersucht. Dieses Peptid bildet, im Gegensatz zur Oxidativen Faltung in $[C_2mim][OAc]$, in wässriger Lösung (konventionelle Methode) eine Mischung verschiedener Isomere. Drei dieser entstandenen Isomere konnten in ausreichenden Mengen für die Bestimmung ihrer dreidimensionalen Struktur mittels Lösungs-NMR-Spektroskopie isoliert werden. Weiterhin konnte der Einfluss der Disulfidverbrückung auf ihre Aktivität aufgezeigt werden. Die Untersuchungen ergaben für zwei Isomere eine unterschiedliche Disulfidverbrückung (Isomer 1: Cys4-Cys21, Cys5-Cys22, Cys11-Cys16 und Isomer 2: Cys4-Cys16, Cys5-Cys21, Cys11-Cys22). In beiden Fällen liegt eine starre Peptidstruktur vor. Das

zweite Isomer konnte der Struktur zugeordnet werden, die zuvor bereits durch Nielsen *et al.* (Nielsen *et al*., 2002) als native Struktur vorgeschlagen wurde. Ein drittes, vollständig oxidiertes Isomer war für eine Strukturbestimmung mittels NMR zu flexibel und weist vermutlich die folgende Disulfidverbrückung auf: Cys4-Cys5, Cys11-Cys16, Cys21-Cys22. Überraschenderweise zeigten alle Isomere eine Affinität im nanomolaren Bereich gegenüber dem spannungsgesteuerten $Na_V1.4$. Im Gegensatz zu bereits veröffentlichten Daten, zeigt Isomer 1 mit der Disulfidverbrückung Cys4-Cys21, Cys5-Cys22, Cys11-Cys16 eine größere Aktivität als Isomer 2, dem vermutlich die native Faltung zugrunde liegt.

In der vorliegenden Arbeit wurden alternative Protokolle für die Oxidative Faltung und die Native Chemische Ligation Cystein-reicher Peptide entwickelt, in denen bei Raumtemperatur flüssige ILs als Reaktionsmedium eingesetzt werden. Diese Methode kann als entscheidende Verbesserung für die Herstellung Cystein-reicher Peptide in größeren Mengen und besseren Ausbeuten betrachtet werden, als dies bisher mittels konventioneller Methoden möglich ist. Zudem ist in vielen Fällen der Wirkungsmechanismus wichtiger Cystein-haltiger Peptide, z.B. Conotoxine, mit ihren Interaktions-partners noch nicht vollständig verstanden. Es konnte gezeigt werden, dass die Kombination verschiedener analytischer Verfahren mit Methoden der Strukturaufklärung einen wichtigen Beitrag für das Verständnis der Struktur-Aktivitätsbeziehungen leisten kann. Weiterhin konnte gezeigt werden, dass die Struktur der Toxine sehr stark mit ihrer biologischen Aktivität korreliert, womit die NMR-Spektroskopie eine Schlüsselmethode für die Bereitstellung wichtiger dynamischer und struktureller Informationen für die relevanten Targets im Hinblick auf die Medikamentenentwicklung darstellt. Die Ergebnisse dieser Arbeit bilden somit eine Grundlage für weitere Untersuchungen auf dem Gebiet der Synthese Cystein-reicher Peptide und der Bestimmung ihrer Struktur und Bioaktivität.

Summary

The present thesis is dedicated to the investigation of structure and activity of cysteine-rich peptides. For that purpose, efficient synthetic strategies were established to obtain sufficient amount of a respective cysteine-rich product. The application of new media, i.e. ionic liquids (ILs), for reactions including cysteine residues, such as oxidative folding (disulfide bond formation between two cysteine residues) and native chemical ligation (formation of a peptide bond between two segments), is described for the first time. Furthermore, solution NMR spectroscopy and bioactivity methods, such as heart beat assays and electrophysiological measurements, were applied to reveal the three-dimensional structure and the specific biological activity of cysteine-rich peptides, respectively.

Conopeptides, isolated from the venom of cone snails, belong to a class of neuropeptides. They possess diverse structures and contain different numbers of disulfide bonds. Moreover, their selectivity and affinity for ion channels and receptors in the nervous system make them potential targets for drug development. Cone snail venom contains a mixture of cysteine-rich peptides. The isolation of a single peptidic component usually results in an insufficient amount for structural characterisation and activity investigations. The chemical synthesis of these peptides can solve this particular problem. However, the challenge of the chemical synthesis is faced with a specific connection of cysteine thiol-groups to form the bioactive native conformation. The optimisation of existing methods is required, because disulfide-bond formation is the limiting step in high yield synthesis of such peptides and thus, was one specific aim of this thesis. Moreover, correlations between the specific structures of conopeptides, such as CCAP-vil, μ-SIIIA, μ-PIIIA, and their activities were the major focus of this work. The complete analytical and structural characterisation was performed for these peptides, with emphasis on both, the linear precursors (in reduced state, containing thiol groups) and the folded (disulfide bridged) peptides.

Furthermore, a new approach was established in order to simplify the access to the class of peptides containing cysteines in their structure, providing environmentally friendly conditions for cysteine bridge formation as well as the synthesis of peptides from two segments forming a native peptide bond. Ionic liquids, primarily differing in the type of anion were chosen for these studies, namely $[C_2mim][OAc]$, $[C_4mim][OAc]$, $[C_2mim][OTs]$, $[C_2mim][Et_2PO_2]$, and $[C_2mim][N(CN)_2]$. Various positive effects on the

synthesis of cysteine-rich peptides in ionic liquids compared to conventional methods were observed. The hydrophobic peptides (δ-EVIA, δ-SVIE) were better soluble in ionic liquids in comparison to buffer solutions usually applied in conventional methods. Therefore, there was no need to apply organic solvents. The extent of side reactions, such as dimerisation, oligomerisation or formation of misfolded products, was considerably lower for all investigated conopeptides. Moreover, no other additional reagents (such as organic solvents, redox reagents (e.g. glutathione reduced or oxidised)) were required to perform effective oxidative folding. The yields and efficiency of disulfide bond formation in various ILs differed strongly for conopeptides that varied in the amino acid sequence, disulfide bond content and hydrophobicity, and obviously depended on the hydrogen bond basicity of the IL anion. The almost neutral decapeptide CCAP-vil, containing only one disulfide-bond, was successfully oxidised in [C_2mim][OTs]. However, the highly positively charged conotoxins α-GI, μ-PIIIA, μ-SIIIA, δ-EVIA, δ-SVIE, containing two or three disulfide bonds, were efficiently oxidised in [C_2mim][OAc].

The second reaction to be investigated, the native chemical ligation of 36-mer and 30-mer peptide fragments, succeeded for the 66-mer reaction product, i.e. the natural product tridegin from *Haementeria ghilianii*, in [C_2mim][OAc] – even without addition of other reagents (thiophenole and benzyl mercaptan) to the reaction mixture typically used in conventional methods. Also, oxidative folding of this peptide with six cysteine residues was successfully carried out in [C_2mim][OAc] indicating the perspective application of ILs for both NCL and oxidative folding in a consecutive way for such peptides.

The complete characterisation of the conopeptide CCAP-vil from *Conus villepinii* was performed with chemical methods and techniques of structure elucidation. Various approaches to discriminate between the reduced and oxidised forms of this peptide, such as MALDI-TOF mass spectrometry including derivatisation with iodoacetamide, Raman spectroscopy, Ellman's test and finally NMR spectroscopy, were applied. The three-dimensional structure, obtained by solution NMR spectroscopy, was assigned as a rigid backbone conformation stabilised by one disulfide bridge reminiscent of a type(I) β-turn. The bioactivity studies using a heart-beat assay with *Danio rerio* (zebra fish) embryos of CCAP-vil revealed a 15% decrease of the heart rate, indicating a cardio decelerating effect.

Secondly, the three-dimensional structure of 22-mer conotoxin μ-SIIIA from *Conus striatus*, containing six cysteine residues, was confirmed by comparison of the ^{1}H-NMR spectrum with published data reported earlier by other groups (Schroeder, *et al*., 2008, Yao *et al*.,

2008). This peptide was systematically investigated using the whole cell patch clamp method todetermine the specificity of μ-SIIIA for various Na_V channel subtypes on a molecular level. μ-SIIIA slowly and partially blocked rat-$Na_V1.4$ channels with an apparent IC_{50} of 0.56 ± 0.29 μM. However, the block was not complete, i.e. a residual current component of about 10% with a correspondingly reduced single-channel conductance was left at high concentrations. At 10 μM μ-SIIIA potently blocked rat-$Na_V1.2$, rat-$Na_V1.4$, human-$Na_V1.4$, and mouse-$Na_V1.6$ channels, while human-$Na_V1.7$ channels were only inhibited by 58.1 ± 5.0%. Human-$Na_V1.5$, rat-$Na_V1.8$, and human-$Na_V1.8$ channels were insensitive. Using domain chimeras of $Na_V1.4$ and $Na_V1.5$, the major μ-SIIIA specificity epitope was located in domain-2 of the channels, and a second μ-SIIIA interaction site with a minor contribution to μ-SIIIA binding was identified in domain-1. Furthermore, three single-site mutations in the background of rat-$Na_V1.4$ (A728N, S729D, and N732T) by insertion of the respective residues of human-$Na_V1.7$ as well as a reverse mutation in the background of $Na_V1.7$ (N889A) were performed. As a result an essential position for μ-SIIIA selectivity was resolved (N889 in $Na_V1.7$ and A728 in $Na_V1.4$).

Finally, a third peptide derived from the μ-conotoxin family, μ-PIIIA from *Conus purpurascens*, was investigated in more detail. It was observed that this peptide, in contrast to the use of [C_2mim][OAc], yields a mixture of different isomers upon oxidative folding in aqueous solution (conventional methods). Sufficient amounts for three of these structural isomers were isolated to elucidate their three-dimensional structures by solution NMR spectroscopy. Furthermore, the impact of disulfide-connectivities on the structure-activity relationship of these μ-PIIIA isomers was highlighted. The investigations revealed at least two different connectivities of the cysteine residues, namely 1) Cys4-Cys21, Cys5-Cys22, Cys11-Cys16, and 2) Cys4-Cys16, Cys5-Cys21, Cys11-Cys22, forming a rigid structure. The second isomer was allocated to the proposed native structure reported by Nielsen *et al.* (Nielsen, *et al*., 2002). A third isolated completely oxidised isomer was too flexible for NMR studies and therefore suggested to possess cysteine connectivities as follows: 3) Cys4-Cys5, Cys11-Cys16, Cys21-Cys22. Surprisingly, all structural isomers showed an activity to the skeletal muscle voltage-gated sodium channel $Na_V1.4$ in nanomolar range. Inconsistently with already published data, the isomer bridged according to cysteine connectivity 1) displayed a higher affinity than suggested natively folded isomer 2).

In the present thesis two alternative protocols for oxidative folding and native chemical ligation of cysteine-rich peptides based on the use of room temperature ionic liquids as reaction media were developed. This method tends to be key for access to the production of

cysteine-rich peptides in higher amounts and better yields than by conventional methods. Besides, in many cases the mechanism of action of relevant cysteine-containing peptides, such as conotoxins, with their targets is not completely understood. A combination of several analytical and structure determination methods was shown to be beneficial with respect to progress in the elucidation of structure-activity-relationships. It was demonstrated that the toxin structure strongly correlates with the corresponding biological activity and insofar, NMR spectroscopy is a key method which provides appropriate information about the structure and dynamics of potential drug leads. Thus, results of this work provide the basis for further investigation in the field of synthesis of cysteine-rich peptides, their structure and bioactivity determination.

Table of Contents

Explanatory Note

This cumulative thesis contains five full papers. The author of this thesis is the first author of four represented original manuscripts. In addition, the author is one of the inventors of a pending Patent (HA10-33). Two papers have already been published in international scientific Journals: *Journal of Peptide Science* and *Peptides*. Another paper has been submitted to: *Chemical Communications*. Two further manuscripts are in preparation for: *Journal of Neuropharmacology* and *Structure*, respectively.
All of these articles are closely connected with the specific aims of this thesis: *Investigation of structure-activity relationships of cysteine-rich peptides from the venom of different conus species and introduction of a new methodology for peptide oxidation and native chemical ligation (NCL) in biocompatible ionic liquids.*

The **first article**, entitled: *A room temperature ionic liquid as convenient solvent for the oxidative folding of conopeptides*, introduces for the first time the use of ionic liquids as reaction media for oxidative folding of cysteine-rich peptides (representatives of μ- and δ-conotoxins). In this article suitable ionic liquids were disclosed for efficient oxidative folding of different conopeptides containing six cysteines in their structure and differing in physico-chemical properties, such as hydrophobicity. The hydrophobic conotoxins (e.g. δ-EVIA, δ-SVIE) and hydrophilic conotoxins (μ-PIIIA and μ-SIIIA) were successfully oxidised in ionic liquids consisting of a substituted imidazolium cation (1-ehyl-3-methylimidazolium) and acetate as anion. The favoured formation of the native conformation together with minimised formation of by-products in comparison to conventional methods is described in this article.

The **second article**, entitled: *An unusual peptide from Conus villepinii: synthesis, solution structure, and cardioactivity,* describes a new cardioinhibitory decapeptide (CCAP-vil), from *Conus villepinii*, with two cysteine residues. This peptide was the subject of interest in the present study because of its previously described cardioinhibitory effect on *Drosophila melanogaster* larvae. This is the first peptide isolated from the venom of a marine organism known to possess such an effect. A biological test system using zebrafish embryos was established and realised through direct injection of peptide in the heart of the 35 hpf embryos and leaving them in the peptide solution to confirm activity: the cardioinhibitory effect. The straightforward and efficient method of synthesis, purification, characterisation and structure determina-

tion of this peptide were the main aims of this project. Ionic liquids were applied for oxidative folding of CCAP-vil, revealing the 1-ehyl-3-methylimidazolium tosylate as the most efficient ionic liuiqid. The solution NMR structure was determined by applying different homonuclear (COSY, ROESY, NOESY, TOCSY) and heteronuclear (DQF-COSY) methods, the structure is represented in this article and in the BRMB database.

In the **third article**, entitled: *New insights into the application of ionic liquids as reaction media for peptide oxidation and fragment ligation*, describes two reactions involving cysteine residues performed in ionic liquids, oxidative folding and native chemical ligation. Differences in the efficiency of both reactions performed in a set of ionic liquids containing mainly different anions in the structure, namely [C_2mim][OAc], [C_4mim][OAc], [C_2mim][OTs], [C_2mim][Et_2PO_2], and [C_2mim][$N(CN)_2$], were observed. The structure, net charge and primary sequence of conopeptides containing different numbers of cysteine residues had also an effect on the reaction rate and yields. Additionally, for the first time the reaction of native chemical ligation on the example of a peptide 66-amino acid in length was performed in ionic liquids. Different conditions of native chemical ligation were investigated compared to conventional methods. The observed efficiencies of ionic liquids for the formation of the desired product were vaying depending on the type if IL and consequences were described.

The **fourth article**, *Molecular determinant for the subtype specificity of μ-conotoxin SIIIA targeting neuronal voltage-gated sodium channels*, describes the elucidation of a molecular determinant for selectivity and specificity of the μ-conotoxin SIIIA with three disulfide bonds from *Conus striatus*. This peptide was synthesised chemically and oxidative folding was performed by a conventional method. NMR spectroscopy data were compared to already reported data. Systematical electrophysiological investigations using domain chimeras and individual site mutations of corresponding channels revealed an essential amino acid on the channel surface for the μ-SIIIA specificity.

The **fifth article**, *Structurally diverse disulfide-connected isomers of neurotoxin μ-PIIIA block skeletal muscle sodium channel $Na_V1.4$*, is focused on the structure elucidation of μ-PIIIA conotoxin structural isomers, formed during oxidative folding with conventional methods. These isomers were purified in sufficient amounts allowing the three-dimensional NMR structure elucidation of the main three isomers. One of these isomers represented the structure of the suggested native peptide synthesised by another research group. The electrophysiologi-

cal experiments on the skeletal muscle $Na_V1.4$ were performed for the three peptide isomers. Surprisingly, one of the isomers possessed a well-defined structure and demonstrated a higher affinity than the suggested native isomer. Another isomer revealed a random-coil structure with lower biological activity.

1 Introduction

"... At the beginning of the 21st century, the field of synthetic peptide chemistry is in the early stages of a renaissance. Several factors contribute to this rebirth: the discovery of new classes of diverse and potent peptide natural products, such as the conotoxins and the cyclotides; a resurgence of interest in peptides for use as human therapeutics; and the use of synthetic peptides in the total chemical synthesis and semisynthesis of proteins... Some of the most important challenges currently facing the worldwide biomedical research community, such as vancomycin resistance in pathogenic bacteria, will place extreme demands on innovative synthetic peptide science." S. Kent, from Chicago University[1]

1.1 Natural peptidic toxins

A large variety of toxins belongs to the potent natural compounds mentioned above. Peptidic toxins are present in plants, animals or microbes, for example snakes, spiders, scorpions, jellyfishes and cone snails (Figure 1). They apply their venom in the purpose of feeding or defence, immediately paralysing or killing their prey. These effects were investigated by scientists for a long time, have been done with respect to their potential in the development of drugs such as analgesics and anaesthetics or in different kinds of therapy treatment: epilepsy, chronic pain, hypertension and hypotension or different kinds of tumour (see also chapter 1.7).[2, 3] Components of these venoms possess a high selectivity and affinity for diverse biological targets, such as transmembrane receptors, ion channels, or transporters.[4, 5] Furthermore, it was found that numerous peptide toxins contain a rather high number of cysteine (Cys, C) residues in their structure that are specifically connected to stabilise the bioactive conformation of these peptides (chapter 1.4).

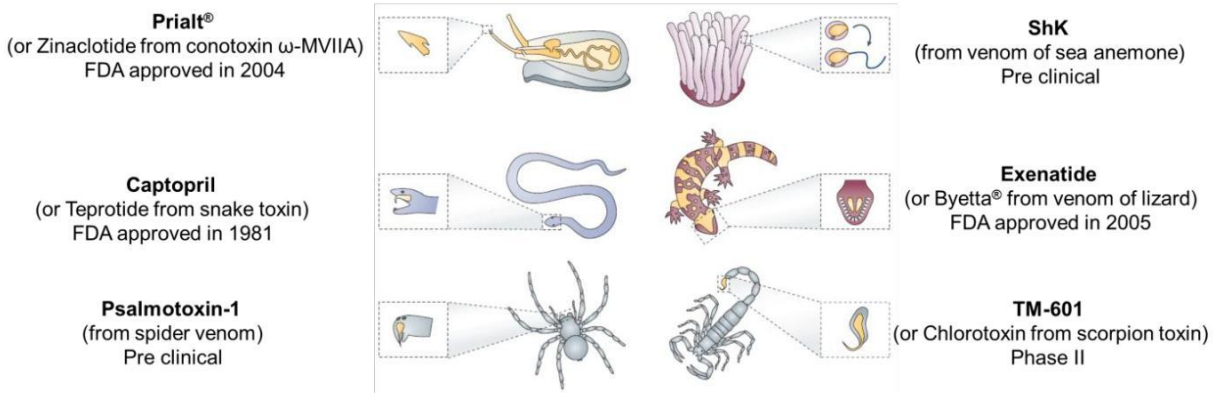

Figure 1. Venom of cone snails, anemones, spiders, snakes and scorpions with therapeutical potential and examples of the peptides FDA approved, in clinical or in pre-clinical stages. Modified from 4

The isolation of the venom from natural material usually results in very small amounts of the venom mixture. The collection of the venom from more than thousand animals is required to obtain enough substance to perform all kinds of structure and activity investigations. There are "farms" for growing toxic organisms aiming at the isolation of venoms for research and commercial purposes. For example "Venom Suppliers PTY LTD" (Tanunda, South Australia) investigates and sells venom from snakes. Usually, the naturally isolated product is used for the determination of the pharmacologically relevant compounds and primary sequence of these peptides. Afterwards, these peptides are to be made artificially through chemical synthesis or recombinant production, synthesised and produced in high purity and amounts for complete analytical, structural and bioactivity investigations.[6] Some of those chemically synthesised peptides already became drug leads and FDA approved medicals, e.g. Prialt® (or Zinaclotide, derived from conotoxin ω-MVIIA), Captopril (or Teprotide, derived from snake toxin) and Exenatide (or Byetta®, derived from the venom of lizard) (chapter 1.7).

The way to produce such drug leads is long and challenging due to the different steps of the investigation process:

- Isolation of the natural venom
- Determination of the potential peptides in the venom followed by their sequencing
- Chemical synthesis or/and recombinant production
- Structure-activity relationship studies

Pharmacologically potent conopeptides, which are referred by S. Kent[1] in the citation above, were in the focus of the present studies.

1.2 Conopeptides and their classification

Conopeptides are neuropeptides isolated from marine cone snails of the genus *Conus*. There are more than 500 species known thus far. They are representing the richest diverse class of hunting gastropods, especially marine invertebrates. The venom of each species comprises from 50 to 200 different components which are representing a cocktail of diverse peptides with very specific activity (chapter 1.5). That means that more than 50.000 pharmacologically relevant compounds are hidden in these unique species.[7]

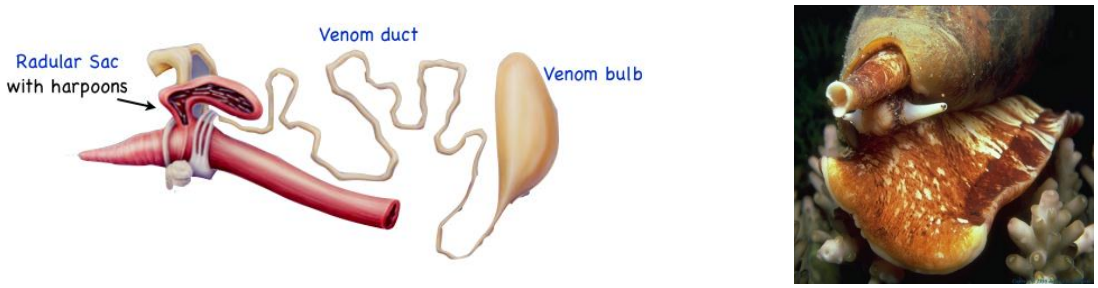

Figure 2. An example of a cone snail (right)[8] and construction of it venom apparatus (left).[9]

Cone snails utilise their venom either to capture prey or to defend themselves. Their prey spectrum is broad, and the strategies of hunting vary.[10] According to their prey preferences, they are categorized into

a) fish eating cone snails, which are called priscivorous,
b) worm hunting (vermivorous),
c) mollusc eating (molluscivorous).[18, 19]

The construction of the venom apparatus is depicted in Figure 2. Barbed and disposable harpoon-like teeth (called toxoglossin radula) are stored in the radula sack. Over 50 teeth can be stored in the radula sack. The teeth are hollow, but loaded with toxic venom. The venom is produced in the venom duct and ejected by contraction of the muscular venom bulb. The one tooth moves through the pharynx into the proboscis and is ready to be injected into the intended prey. After the venom is injected, the prey is immediately paralysed or killed by the venom. Finally, the cone snail regenerates and is ready for further hunting with a new harpoon loaded with deadly venom.[9]

Around 40 years ago the interest in cone snail venom has been raised after accidences with humans who collected cone snail species. This confirms that the venom has an effect on humans, too. A. J. Kohn investigated the hunter-prey relationship of cone snails describing the specificity of the venomous apparatus of cone snails.[11-13] Afterwards, R. Endean and coworkers discovered that cone snail venoms differ from each other and contain components with therapeutic potential that is not observed to the same extent in other known venoms.[14, 15]

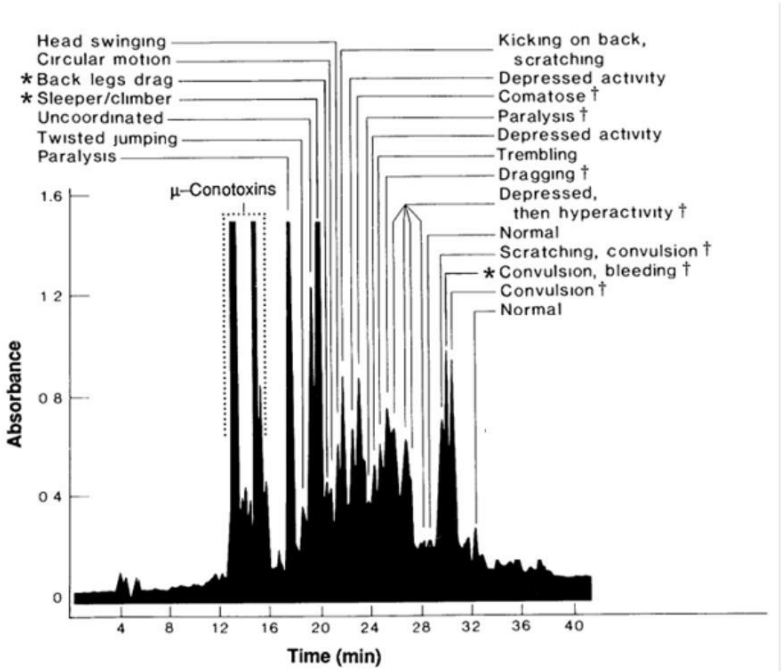

Figure 3. HPLC elution profile of the *Conus geographus* venom with the effects caused by injection of ~0.1-2 nmol intracranially into mice. † A lethal effect was observed at least for one injected mouse. Modified from 9

The first attempts to characterise the venom of cone snails was reported by I. Spence *et al.*.[16] They purified the venom of *Conus geographus*, and tried to determine the amino acid composition of the peptides in this venom.[16]

The first laboratory techniques for venom isolation and purification, however, were established in the laboratory of B. M. Olivera.[17, 18] Their research was focused on the investigation of the venom from fish-hunting species *Conus geographus*, *Conus magus* and *Conus striatus*. The *Conus geographus* venom composition is represented in Figure 3. The eluted fractions were collected and injected intracranially into experimental mice and different symptoms including paralysis, death or inadequate behaviour were observed. It was recognized that the major part of the isolated products represent well-structured cysteine-rich peptides affecting the function of ion channels. Later these peptides were named conotoxins.

Meanwhile, the *Conoserver* database contains data of approximately 3660 peptides, yet only 131 three-dimensional structures, from 87 different species.[19] Most peptides isolated from the venom of cone snails are described to represent cysteine-rich peptides of 10-40 amino acids in length. However, conopeptides with more than 60 amino acids have also been mentioned.[6] Conopeptides are shorter than peptides isolated from the venom of spiders, snakes or scorpions, that usually consist of 40 to 100 amino acids.[9]

To systematically classify all known conopeptides, Norton & Olivera categorised them according to their Cys patterns and number of Cys residues in their sequence. Based on this

principle conopeptides are separated into two main groups. The first group represents peptides with more than two cysteines, while the second group are peptides with a single or no cysteines. The latter are called non-disulfide-rich peptides (Figure 4).[20]

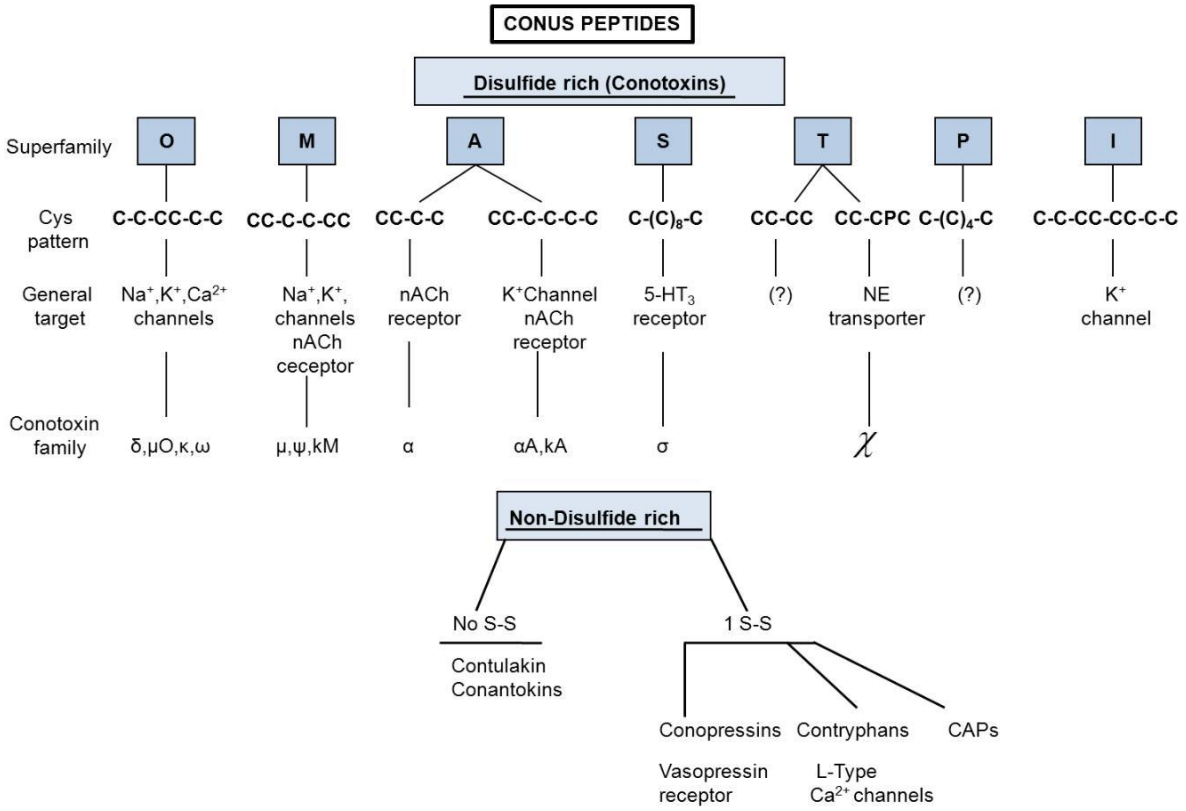

Figure 4. Classification of peptides isolated from marine cone snails. Seven major superfamilies of conotoxins are represented. Cysteine patterns are shown, where "-" between C represents the amino acids between the Cys residues, so called loops. Abbreviations: nACh – nicotinic acetylcholine receptors, NE – norepinephrine, CAP – cardioactive peptides. Modified from 20

For example, conantokins belong to the group of non-disulfide-rich-peptides. These are linear peptides, usually 17-27 amino acids in length, and inhibit N-methyl-D-aspartate receptors.[21, 22] Other representatives of this group are contryphans. They contain only a single disulfide bond and affect the L-type of Ca^{2+} channels.

The second vast diverse group, which is called conotoxins, is divided in genetic superfamilies. In Figure 4 seven major superfamilies are depicted. However, a total number of twelve superfamilies are currently known.[19] Each superfamily is distinguished by the specific disulfide-bridge framework and number of Cys residues. Furthermore, each superfamily contains several families that are specified by their pharmacological activity. For instance, the O-superfamily contains peptides with six Cys, all representatives possess the same Cys-framework (**C**-**C**-**CC**-**C**-C-motif). This superfamily contains δ-, μO-, ω- and κ- families. Representatives of the O-superfamily are specified to target voltage-gated ion channels (Na^+,

K^+ and Ca^{2+}). The differences in cysteine connectivities, i.e., which cysteines are connected to form disulfide-bridge, length of the loops, and amino acid sequence between the cysteine residues are responsible for their specific biological activity. Members of one family from different *Conus* species possess homologous Cys-frameworks but show extreme sequence hypervariability in the number and kind of amino acids included in the loop regions. For example, representatives of the μ-family have the same cysteine connectivity (I-IV, II-V, III-VI), but the resulting structures, target-affinity and -selectivity are very different from each other. This means that based on few differences in primary amino acid sequence the peptide μ-PIIIA in contrast to μ-GIIIA discriminates between neuronal and muscle TTX-sensitive voltage-gated Na^+ channels.[23]

Conotoxins are usually identified by designation comprising the first letter of the corresponding *Conus* name (for example *Conus ermineus* – starts with E), followed by a Roman number specifying the cysteine-framework and finally the letter depicting the order of discovery. For example, δ-EVIA is derived from *Conus ermineus*, belongs to δ-family of conotoxins, possess number VI of cysteine-frameworks, and is the first member of this series discovered.

To summarize, representatives of the one superfamily of conotoxins have various structures and different amino acid sequences and hence, they differ in target-selectivity and target-affinity. For that reason, investigation of the correlation between structure and bioactivity is very important for the determination of the therapeutic potential of conopeptides. These aspects are described in the next chapters.

1.3 Synthesis of conopeptides

1.3.1 Synthetic and recombinant strategies for conopeptide synthesis

Only very small amounts of pharmacologically relevant conopeptides can be isolated from cone snail venom. Therefore, one needs to establish new efficient methods of conotoxin production to obtain sufficient material substance for a complete characterisation of the pharmacologically promising components. Two artificial methods, more precisely solid phase peptide synthesis (SPPS) and recombinant production of conopeptides, provide an access to artificial conotoxins.[6] Nevertheless, the production of venom peptides represents a sophisticated technical and synthetical challenge due to the large number of structurally important disulfide

bonds. The following chapter gives a short overview about the various synthetic strategies of conopeptide synthesis.

Regardless of the method of production, the problem of generating the native disulfide-bond architecture must be addressed as a toxin with three, four, or five disulfide bonds is theoretically capable of forming 15, 105, or 945 different disulfide-bond isomers, respectively.

Very long cysteine-rich peptides can be synthesised by combination of SPPS with native chemical ligation (NCL). NCL can be used to synthesise oligopeptides with sequences of up to 200 amino acids what generally difficult if only using SPPS. The rationale behind NCL is that the yield of synthetic peptide can be significantly improved by dividing the peptide into two or more easily synthesised fragments that can subsequently be joined (Figure 5). Owing to their high cysteine content, disulfide-rich peptide toxins are ideal candidates for NCL.[24, 25] NCL affords the connection of two peptide segments, where the N-terminal peptide as thioester reacts with the cysteine of the C-terminal fragment. Followed by formation of a thioester connected intermediate and a S→N acyl-shift a peptide bond is formed N-terminal to the Cys residue.[24, 26] Thiol-rearrangement is the rate limiting step. The reaction can be accelerated by addition of other thiol additives to the reaction mixture due to generation of a better leaving group in a thiol exchange reaction.[27]

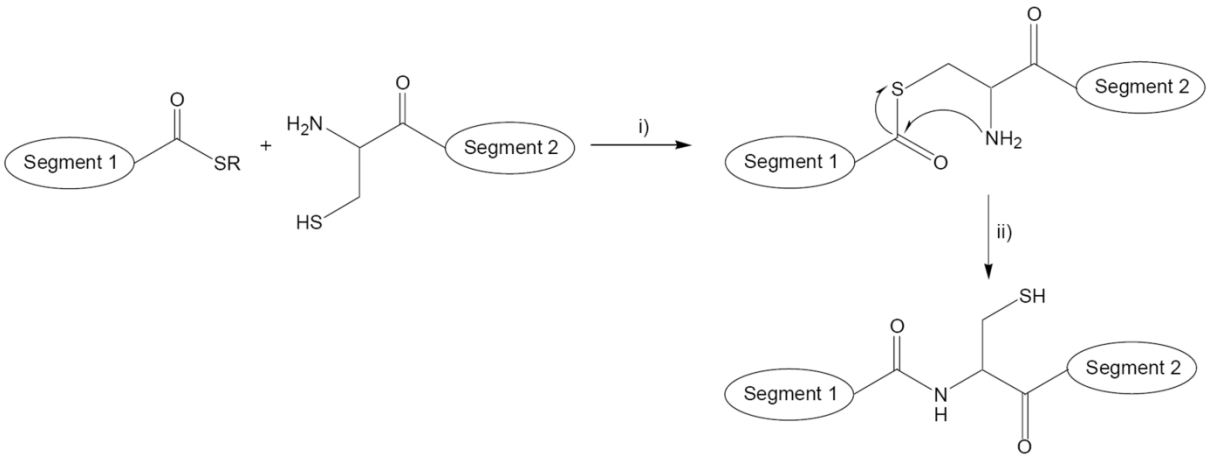

Figure 5. Schematic representation of native chemical ligation (NCL) performed to connect two peptide segments: N-terminal segment, segment 1 (with thioester at C-terminus) and C-terminal segment, segment 2 (with cysteine at N-terminus), forming a peptide bond between the segments.[24, 26]

The NCL of cysteine-rich peptides is faced with the challenge of subsequent oxidative folding, dimerisation or oligomerisation. These undesired side reactions may occur affecting the reaction of the Cys with the thioester in the ligation step. To minimise these reactions additive such as tris(2-carboxyethyl)phosphin (TCEP), is added to inhibit the formation of unwanted disulfide bonds.[28-30] For short or medium-sized conotoxins, NCL is not required.

Here, SPPS followed by peptide oxidation is a general accepted strategy; yet, the oxidative folding is the yield-limiting step. Since the latter aspect is the most challenging one in conotoxin synthesis, the next chapter describes the different approaches in more detail.

Toxins can also be obtained by recombinant methods using different expression systems, e.g. *Escherichia coli* bacteria[31], insect cells[32] or yeast[33]. The recombinant procedures easily enable the incorporation of full isotope (^{13}C and/or ^{15}N) enriched amino acids for NMR spectroscopy analysis and mutations for SAR studies.[34] However, the recombinant production is more cost intensive and time consuming compared to chemical synthesis. Additionally, the introduction of highly diverse posttranslational modifications in conopeptides is limited and thus disadvantageous for this way of toxin generation.

1.3.2 Oxidative folding of cysteine-rich peptides

Cysteine is a very important amino acid since many naturally occurring peptides contain intramolecular disulfide bonds that stabilise the biologically active conformation.[35-38] Due to highly reactive thiol functions cysteine-containing peptides have always been one of the most challenging compounds with respect to establishing highly efficient synthetic strategies.[39]
Disulfide bonds can be formed at various steps of the synthesis according to different methods. These methods can be divided into two categories, depending on the kinetics of the disulfide bond formation:

- spontaneous oxidation (self-folding strategy)
- controlled oxidation (orthogonal protecting group strategy)

Usually, the self-folding method is performed in solution and the orthogonal protecting group method on a polymer support (resin) (Figure 6).[40-43]Also, the combination of both methods is described.[44, 45]

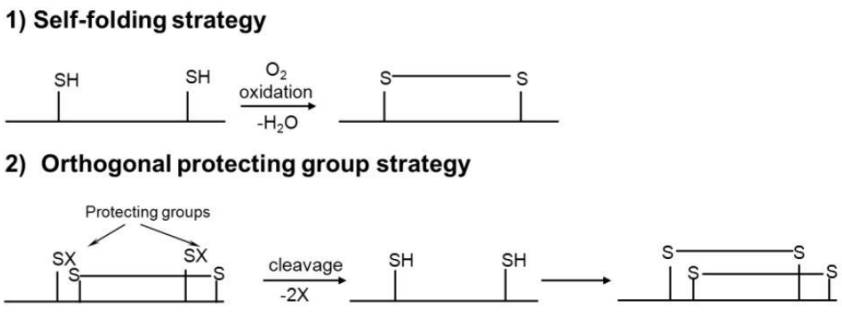

Figure 6. Oxidative folding strategies.

For oxidative folding in solution various side reactions can occur during disulfide bond formation as depicted in Figure 7, such as dimerisation, oligomerisation, or intramolecular mispairing (scrambling), resulting in formation of non-native structures, pharmacological inactivity and low yields of the desired toxin.

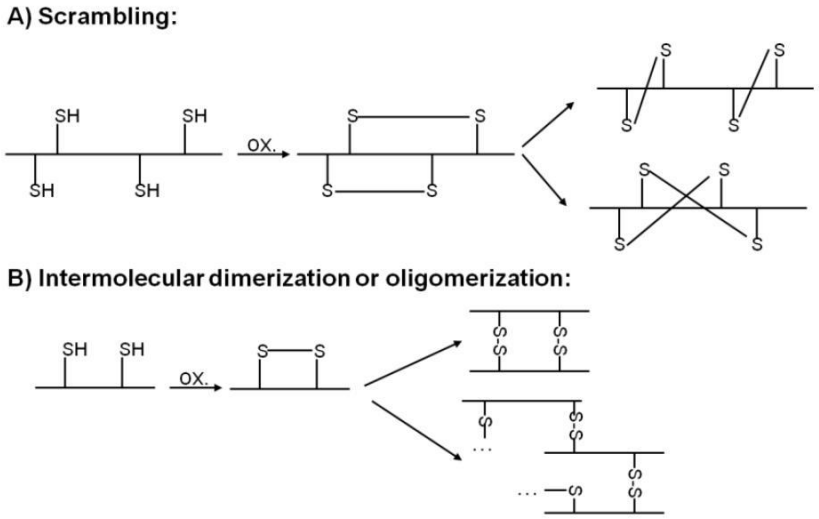

Figure 7. Side reactions that may occur during disulfide bond formation.

The oligomerisation can occur in peptides containing at least two Cys residues. However, this can be avoided through high dilution of the respective peptide in the reaction mixture. But the situation becomes more difficult if the peptide contains more than six Cys in the sequence. The probability of isomer (N) formation during thiol group (2n) oxidation forming (j) disulfide bonds increases dramatically according to equation (1).[46]

$$N_{2n}^{j} = \frac{(2n)!}{2^{j}(2n-2j)!\,j!} \qquad (1)$$

This means that the number of possible isomers increases for peptides with six, eight and ten Cys, from 15 to 105 and 945 isomers, respectively.

For oxidative folding on a polymer support by the orthogonal protecting group strategy, the thiol-groups can be connected directly during SPPS after the peptide sequence has been assembled on the polymer support. However, the most important limiting factor of this method is that thiol groups have to be protected with selectively cleavable protecting groups orthogonal to the protection of the peptide backbone and with respect to the different cleaving conditions for each disulfide bridge. This strategy is not compatible with solution methods and usually results in a low yield of oxidised peptide.[39]

For these reasons, spontaneous oxidative folding in solution is the primarily applied method enabled by air oxidation in general. Usually, this reaction is carried out in water/buffer solution and/or with addition of organic solvents (OS) at pH 7.5 - 8.5 at very low concentra-

tions (10^{-3} to 10^{-5} M) of the peptide. Reagents, which assist the oxidative folding process, can be added to the reaction mixture, e.g. glutathione in its oxidised (GSSG) and reduced (GSH) form or cysteamine/cystamine. Further reagents that minimise aggregation, e.g. urea or guanidinium hydrochloride can also be added.[47, 48] Ionic strength and temperature of the reaction mixture were reported to have a great influence on the reaction rate, too.[49] In case of oxidising highly hydrophobic peptides, the addition of OS is recommended and often required. However, peptides with a high potential of aggregation can only be synthesised in low yields. Hence, the optimisation of oxidative folding conditions for these peptides is necessary.

With the exception of oxygen as oxidising agent for the oxidative folding step, other chemicals were used, e.g. H_2O_2, iodine/ethyl iodide[50] or thallium-trifluoroacetate.[51] These reactions are usually performed in water or in a water/OS mixture. The problem with these methods is the undesired oxidation of sensitive amino acids, e.g. methionine or tryptophan. Furthermore, the oxidative folding with thallium compounds is generally omitted due to their toxicity, and, in addition, the quantitative separation of the peptide from these substances is very difficult.

Another method, which is widely used, is the oxidation in aqueous solution containing dimethylsulfoxide (DMSO).[52] This method has been recommended because of the possible variation of the pH value over a wide range.[53] However, again a reaction mixture with high DMSO concentrations is difficult with respect to further processing, including purification steps.

Oxidative folding with azodicarbonic acid[54] or chlorosilane/sulfoxide mixture[39, 55] has been published for peptides with only two Cys residues, however, it seems not appropriate for peptides with more than one disulfide bond.

In summary, the major drawbacks of the peptide oxidation in all conventional methods in solution can be recapitulated to the following aspects:

a) High dilution of reaction solution,
b) very long reaction times,
c) inadequate solubility of hydrophobic peptides in buffer solutions (and necessity of OS addition),
d) difficulties in controlling the oxidation (performing of the reactions at low temperatures (4°C) to slow down the oxidation process),
e) addition of various other additives,

limiting production of conotoxins in industrial scale.

The combination of oxidation in solution and on the solid support (polymer resins) was also described to be successful using CLEAR-OX. This is a polymer support where the Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB) is coupled to the CLEAR-

resin.[56-60] This method was successfully applied for toxins including up to six cysteines and is compatible with the presence of sensitive amino acids in the peptide structure. The comparison of the CLEAR-OX oxidation with the method of DMSO-solution oxidation was recently described, illustrating the advantageous oxidative folding of toxins by the CLEAR-OX method.[45]

Following all described investigations, the method of "integrated oxidative folding" has been developed. Here, the substitution of one disulfide bond by a diselenide bridge combined with the incorporation of $^{15}N/^{13}C$ labeled Cys is introduced. This strategy helps to simplify the time consuming structure determination by NMR spectroscopy.[61, 62] However, the application of the non-natural selectively protected selenocysteine derivatives and labeled amino acids makes this method very cost intensive.

In conclusion, for peptides with a high content of cysteine it is rather difficult to find an optimal strategy for efficient oxidative folding. Over the past years many research groups were working on the establishment of methods to enable an easier access to cysteine-rich peptides, yet this still represents a major task in the field of peptide synthesis.

1.4 Spatial structure of conopeptides

In this chapter a short overview concerning the structural characteristics of conopeptides, such as cystine knot structures and numerous posttranslational modifications is described. The determination of their spatial structure is essential for further structure-activity relationship (SAR) studies and determination of interactions between the potential drugs and their targets.

1.4.1 Cystine connectivities and cystine knot motif

The unique selectivity of conopeptides for several receptors and ion channels is attributed to the specificity of their structures. The large content of Cys amino acids in the structure is essential for the toxin is biological function. The formation of disulfide bridges is responsible for the stabilisation of a distinct structure, forming the rigid secondary structure of the respective peptide. The peptides of the same superfamily possess a conserved number of disulfide bridges, but the loops between these bridges vary extremely (Figure 8 B). Conotoxins form different structures depending on their Cys-connectivities. The peptides from O, P and I superfamilies possess a structural fold which is called inhibitory cystine-knot (ICK) structural motif, e.g. ω-conotoxin MVIIA (Figure 9). The cystine knots are also characteristic for a

variety of other peptides derived from natural sources: plants, fungi, insects or spiders. This structure is defined by the embedded ring formed by two disulfide bonds connecting back-bone segments, penetrated by the third disulfide bond (Figure 8 A).[63]

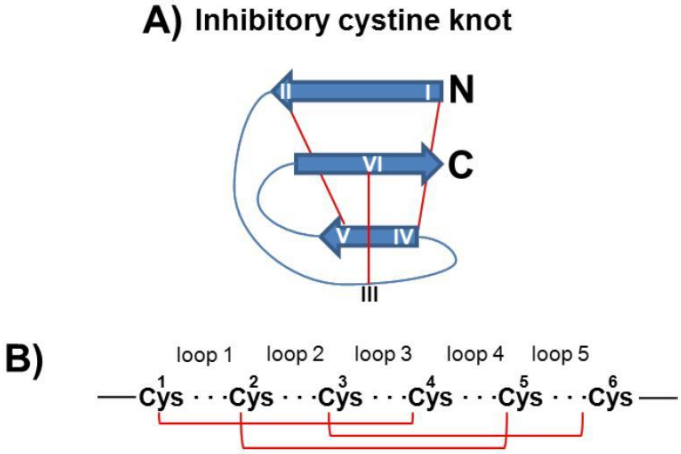

Figure 8. Schematic representation of the inhibitory cystine knot structural motif, where cysteines are connected in the order 1-4, 2-5, 3-6 (A, B), showing that this type of cysteine connectivity forms a triple-stranded β-sheet. Cysteines 1-4 and 2-5 in ICK are forming a ring which is penetrated by the 3-6 cysteine bridge (A).[64] The amino acids between the Cys residues are called loops, and Cys residues are numbered within the peptide sequence from N- to C-terminus (B).

The characteristic feature of an ICK is the cysteine connectivity: Cys(1-4), Cys(2-5) and Cys(3-6). Typical for ICK is the antiparallel β-sheet structure and a short 3_{10}-helix, which is held together by the three intramolecular disulfide bonds. The size of the loops between the corresponding Cys residues is quite similar among such peptides. However, not all conotoxins possessing the same cysteine connectivity form an ICK. For example, representatives of the M superfamily, e.g. μ-conotoxin GIIIB, do not form to ICK (Figure 9). This can be explained by the differences in the loop size of these peptides. That is different to those forming the cystine knot. For peptides that form the ICK motif the consensus sequence was determined: $CX_{3-7}CX_{3-8}CX_{0-7}CX_{1-4}CX_{4-13}C$.[63]

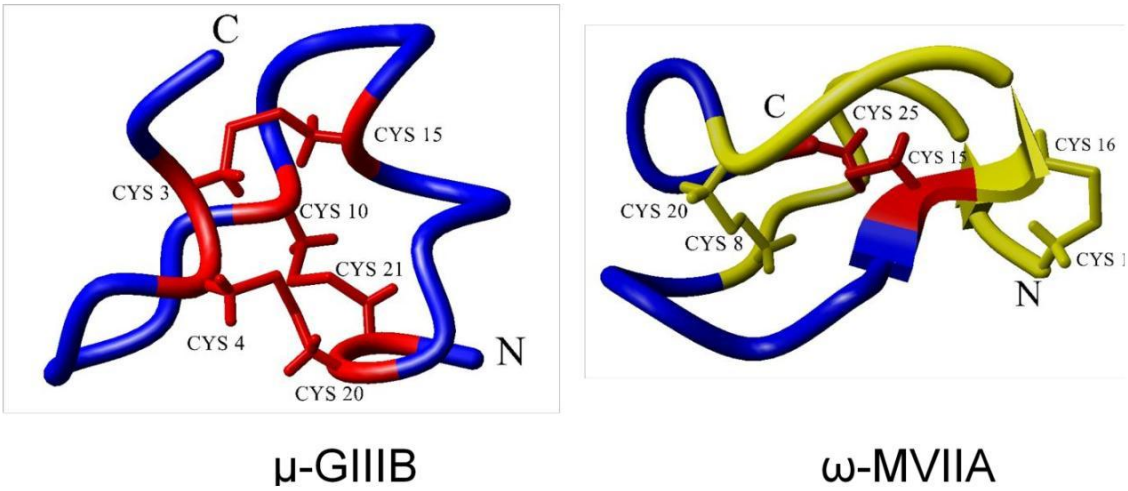

Figure 9. NMR structures of μ-GIIIB (left, PDB entry 1GIB)[65] and ω-MVIIA (right, PDB entry 1DW5)[66] conotoxins with disulfide connectivities. ω-MVIIA forms a cysteine knot. This knotting shows the disulfide bridge Cys15-Cys25 penetrating the macrocycle formed by the two other disulfide bridges Cys1-Cys16 and Cys8-Cys20 The structures were generated using Yasara.[67]

1.4.2 Posttranslational modifications of conopeptides

A characteristic feature of conopeptides is not only their disulfide bridges, but also numerous posttranslational modifications found in their structure. These modifications include: hydroxylation of proline, amidation of the C-terminus, cyclisation of N-terminal glutamate to pyroglutamic acid, carboxylation of glutamic acid, bromination of glutamic acid, isomerisation of tryptophan (Trp, W), sulfation of tyrosine, O-glucosylation (Figure 10).[2, 10] There are indications that cone snails express an enzyme important for these modifications in the venom duct.[10, 68] The functional importance of these modifications is still not fully understood, yet a lack of structure/function information for many of these modifications prevents a better understanding of their role in receptor selectivity and specificity.[2]

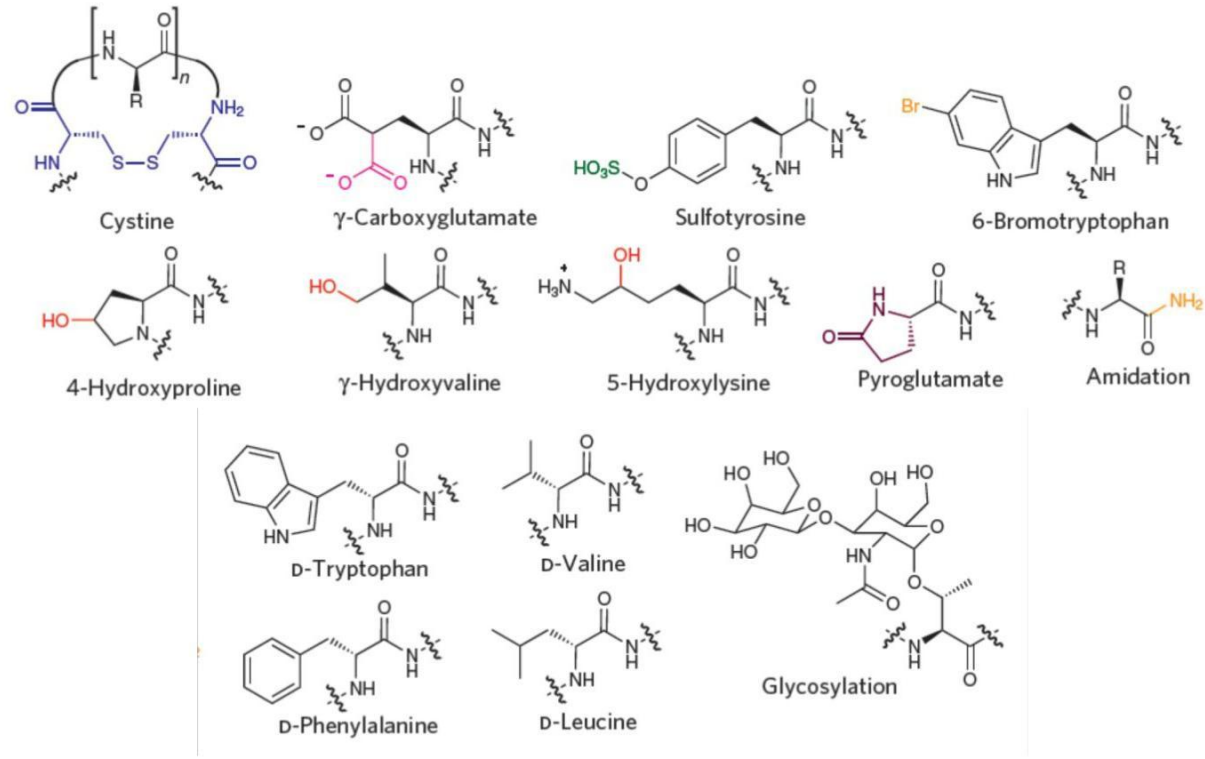

Figure 10. Posttranslational modifications found in conopeptides. Modified from 69

1.4.3 Structure determination using NMR spectroscopy

In 1946 Felix Bloch and Edward Mills Purcell independently found first evidences of nuclear resonance signals. Since that time, NMR spectroscopy became one of the most important spectroscopic methods. NMR is mainly used by biologists, physicists and chemists for structural investigations during the past years. NMR is also important for physicians as a diagnostic tool.[70] The structure determination of large proteins is generally performed by X-ray crystallography. Since small peptides are difficult to crystallise, NMR spectroscopy became also a general tool in solving three-dimensional (3D) structures of cysteine-rich peptides like conotoxins.[34] The limiting factor of the NMR analysis of isolated natural substances is the small amount that can be derived from natural venom. Nowadays research groups solved these kinds of problem using chemical synthesis of conotoxins in milligram scale to perform notable characterisation.

There are two approaches to perform NMR structure analysis:

1) homonuclear-based approach; It relies on the data obtained from hydrogen nuclei (protons),[71]

2) heteronuclear-based approach; This approach uses the data from hydrogen, carbon and nitrogen.[34]

The second approach requires isotope labelling of the peptide with ^{13}C and/or ^{15}N. Through the new synthetic approaches this labelling can be easily incorporated during the chemical synthesis or recombinant production. However, this approach is more cost intensive compared to the homonuclear-based one.

For the first approach mentioned above the complete proton resonance assignment is required. The distribution of ^{1}H resonances for the functional groups in peptides and proteins is shown in Figure 11.

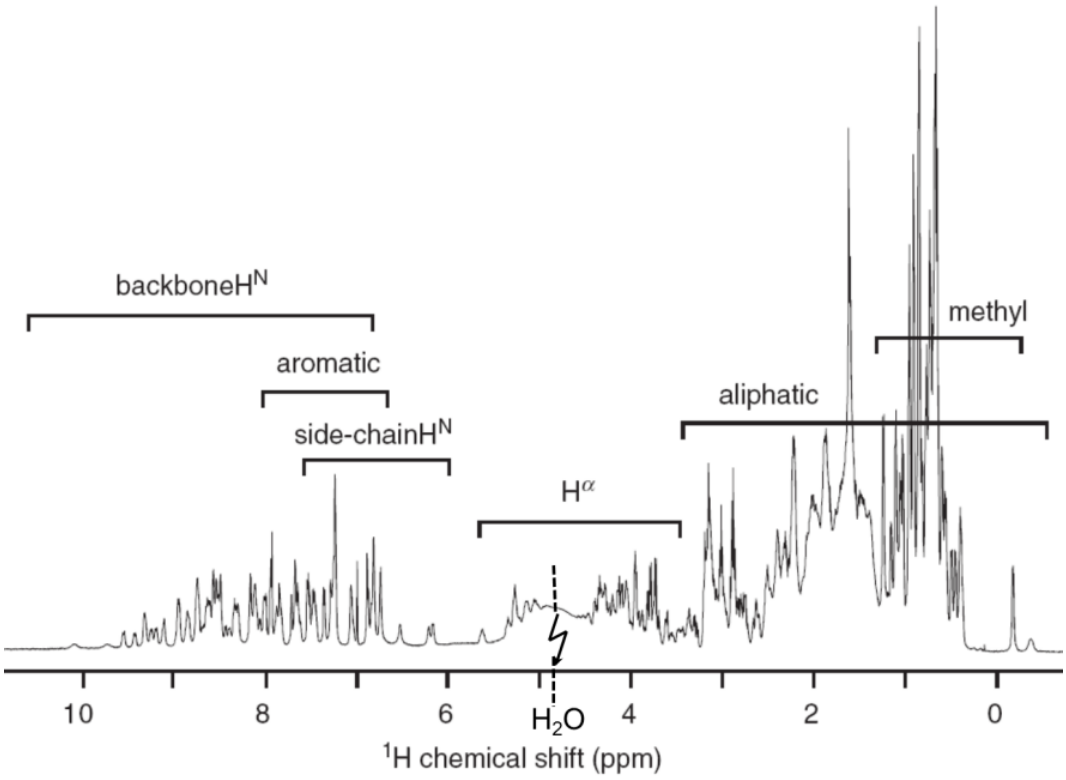

Figure 11. Chemical shift range of different types of ^{1}H resonances on the exemplified for ubiquitin (in H_2O).[72]

In order to assign the proton resonances and to determine the 3D structure of a peptide or protein the combination of the following two-dimensional (2D) experiments is benificial: [^{1}H, ^{1}H]-NOESY, [^{1}H, ^{1}H]-TOCSY, [^{1}H, ^{1}H]-ROESY, [^{1}H, ^{1}H]-DQF-COSY and [^{1}H, ^{13}C]-HSQC at natural abundance as shown in Figure 12.

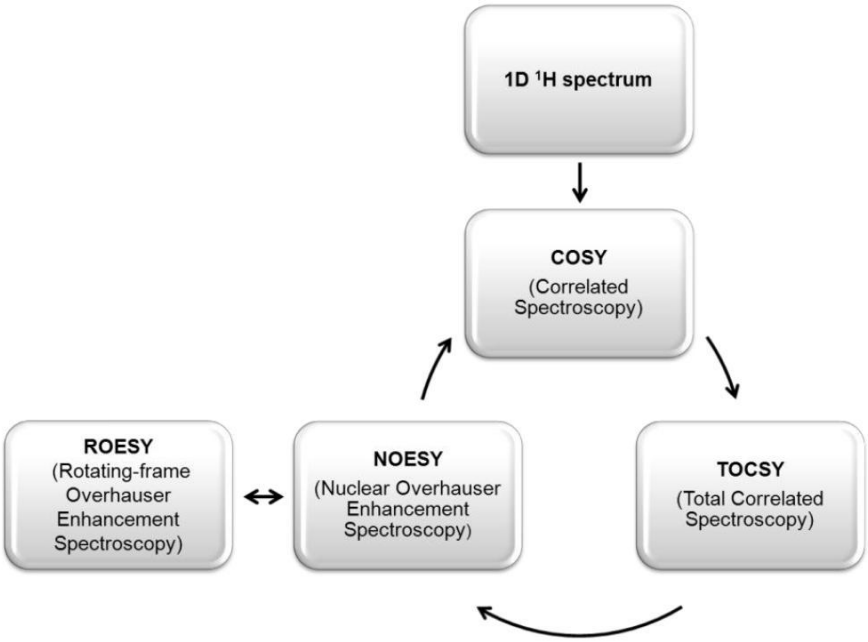

Figure 12. Schematic representation of the NMR spectroscopy methods required for the 3D structure determination of peptides and proteins.

Through the combination of these NMR spectroscopic methods the following information can be obtained:

- through-bond interactions (*via* scalar couplings)
- through-space interactions (*via* dipolar couplings)
- chemical environment (*via* isotropic chemical shifts).[72]

2D spectroscopy methods are shortly described below.

COSY

- coherence transfer between coupled spins
- correlations between pairs of protons separated by three bonds
- $^1H^N$ – $^1H^\alpha$ cross peaks (the backbone fingerprint)
- $^1H^\alpha$– $^1H^\beta$ cross peaks (crowded)

TOCSY

- cross peaks are generated between all resonances within a spin system
- magnetisation is transferred through several couplings

[^{1}H,^{13}C]-HSQC at natural abundance

- cross peaks between heteroatom (^{13}C) and directly bound proton
- cross-check of resonance assignment
- can also be used to determine intermolecular interactions, for example between the toxin and its target protein.

For the ^{1}H resonance assignment the "**sequential assignment strategy"** is usually applied.[72] A combination of 2D NMR experiments is used to connect amino acid spin systems. For that purpose, the sequence of a peptide can be reproduced with the help of cross-peaks of backbone protons (of the peptide bond: nitrogen proton H^{N1} to carbon proton $H^{\alpha 1}$ of the definite amino acid and from the $H^{\alpha 1}$ and H^{N2} of the next amino acid).[72] The complete peptide sequence can be assigned through the so called "sequential walk" (Figure 13).

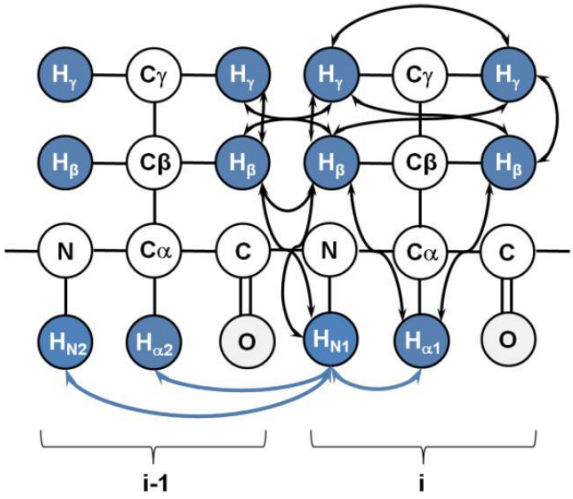

Figure 13. Schematic representation of the principles of a "sequential walk" (blue arrows) and NOE magnetisation exchange between hydrogens (blue and black arrows). Modified from 73

Since **NOE**-experiment (**N**uclear **O**verhauser **E**nhancement) allows the detection of cross peaks between two protons separated by less than 6 Å, conformational details of molecules in solution can be deduced.[72]

The NOE-effect is based on the increase of the signal intensity of ^{1}H, when the resonance of an adjacent nucleus (mostly ^{1}H) is saturated (e.g. ^{1}H decoupling during ^{13}C detection) and causes a polarisation transfer during the so called mixing period between both adjacent nuclei. The NOE-effect is proportional to r^{-6} and is detectable up to a distance of 6 Å between two coupled protons. The magnetic interactions described above are causing the cross peaks in a 2D NOE experiment. In contrast to the NOE spectroscopy the ROE (**R**otating-frame **O**ver-

hauser **E**nhancement) spectroscopy uses an additional "spinlock" technique and is more practicable for smaller peptides between 1000-2000 g/mol.

The through-space correlations of hydrogen atoms obtained by [^{1}H, ^{1}H]-NOE or [^{1}H, ^{1}H]-ROE experiments, lead to detailed information about the 3D structure of complex biological molecules. Therefore, the cross peaks are integrated and translated into distance constraints. It should be noticed that spectral noise can distort the real information for the distances and the exact data cannot be obtained. For that reason only the cross peaks with distances of the upper limit 2.8, 3.6 or rather 5.5 Å are taken into account.

To summarise, various methods are known to determine the 3D structure of peptides and proteins in solution. However, the time for data acquisition and analysis is still taking months to obtain 3D structure of cysteine-rich peptides like conotoxins. Some researchers are, therefore, calling this approach a bottleneck of the venom-based drug design.[34]

1.5 Targets and function of conopeptides

1.5.1 Ion channels as targets for conopeptides

The potent neurotoxins target selectively different types of ion channels and receptors.[9, 18] Depending on the kind of their targets, toxins are divided into two major groups:

1) conopeptides targeting voltage-gated ion channels (VGC)[74],
2) conopeptides targeting ligand-gated ion channels.[7]

Ion channels are large protein complexes in the biological membranes and play a key function in excitation, i.e. generation and propagation of action potentials, control of neurotransmitter release etc..[7] VGC are activated in response to changes in the transmembrane voltage. These transmembrane proteins possess selectivity for definite cations like Na^+, K^+ and Ca^{2+}, and are termed Na_V, K_V and Ca_V channels, respectively.[75] VGC include several subunits. For K_V channels, four subunits form a functional channel and are arranged around the central pore that permits the transport of ions through the membrane. However, Na_V and Ca_V channels are complexes of one large pore-forming subunit and smaller subunits with regulatory function. The pore-forming subunit has a pseudotetrameric structure, i.e. it is composed of four homologous domains arranged in a clockwise orientation around a central pore.[76]

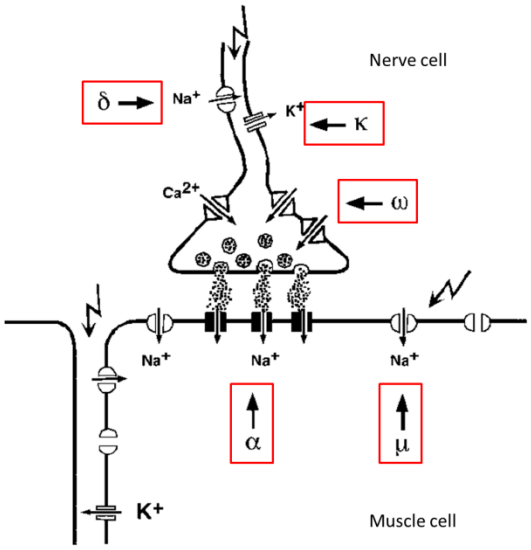

Figure 14. Schematic view of the presynaptic nerve terminal represented with conopeptide targets. δ – conotoxins inhibit inactivation of Na_V channels, κ –conotoxins interact with K_V, ω-conotoxins block presynaptic Ca_V channels; α-conotoxins block nicotinic acetylcholine receptors; μ- and μO-conotoxins block Na_V channels. Modified from 77

VGC are interacting with different families of conopeptides (Figure 14):

- μ-conotoxins block voltage-dependent sodium channels.[78]
- δ-conotoxins delay or inhibit the fast inactivation of Na_V channels.[79] They are supposed to cause an intense pain. The effect is comparable with the electrical shock, as a result of targeting of a definite Na_V channel subtype.
- μO-conotoxins are structurally similar to δ-conotoxins but they inhibit Na_V channels as μ-conotoxins do although by a different molecular mechanism.[74]
- κ-conotoxins inhibit potassium channels.[80] Their physiological effect is similar to δ-conotoxins.
- ω-conotoxins inhibit N-type Ca_V channels.[81] Their effect is to inhibit the release of the neurotransmitter acetylcholine at the presynaptic nerve terminal, therefore preventing the pain transmission signal to the brain.

The group of conopeptides which interact with the ligand-gated ion channels is poorly investigated in comparison to the first group. All known conopeptides of this group are known to target the muscle-type nicotinic receptors.

Some families of peptides are interacting with ligand-gated ion channels:

- ψ-conotoxins are known as non-competitive antagonists of the nicotinic receptors. One of its representatives is ψ-PIIIE.

- αA-conotoxins, e.g. αA-PIVA and αA-EIVA, are known as competitive nicotinic receptor antagonists.
- α-conotoxins are targeting diverse nicotinic receptor subtypes.
- σ-conotoxin targets 5-hydroxytryptamine ($5HT_3$) channel or ligand-gated Na^+ and K^+ channels. They play an important role in learning, memory or addiction. The only representative of this family is σ-GIIIA.
- Conantokins are targeting glutamate receptors. They are known as antagonists of a subclass of glutamate receptors – the N-methyl D-aspartate (NMDA) receptors. One representative of this family is conantokin-G, now in clinical trials as a potent anticonvulsant.

The next section gives a brief overview of the structure of the voltage-gated sodium channels (VGSC) and some examples of toxins derived from venom of cone snails that target these channels.

1.5.2 Voltage-gated sodium channels

To understand how conopeptides are interacting with their target (primarily ion channels), the mode of function and the structure of their specific target need to be fully understood. Therefore, a short introduction about the structure and mode of action of ion channels is given below.

VGSCs are composed of a large α-subunit (approximately 260 kDa) and one or more β-subunits. Nowadays, nine subtypes of Na_V channels ($Na_{1.1}$-$Na_{1.9}$) are known and their functional characteristics has already been determined (Figure 15).[82] The subtypes of VGSCs are pharmacologically distinguished by their ability to be blocked by tetrodotoxin (TTX), a toxin that was isolated from the puffer fish.[83] It was found to bind to the ion selective pore of some Na_V channel subtypes. That is why Na_V channels are divided into TTX-sensitive (TTX-s), blocked by nanomolar concentrations of TTX and TTX-resistant (TTX-r) Na_V channels, that are resistant to micromolar concentrations of TTX. $Na_V1.1$-4, $Na_V1.6$, and $Na_V1.7$ belong to TTX-s, while $Na_V1.5$, $Na_V1.8$, $Na_V1.9$ belong to TTX-r channel subtypes.[84]

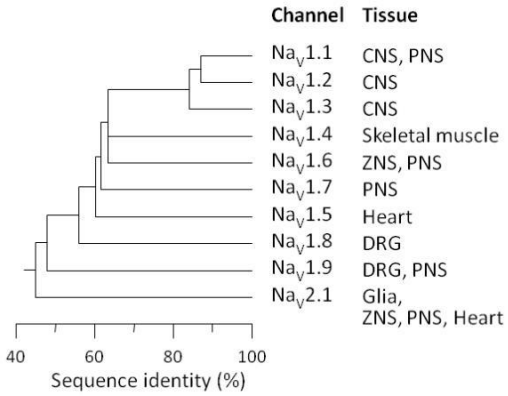

Figure 15. Subtypes of Na_V channels and their distribution in the human tissue and sequence homology.[82]

The working principle of Na_V channels is represented in Figure 16. Upon the membrane depolarisation Na_V channels change their conformation from closed to open; and Na^+ ions can pass through the channel pore. From the open state Na_V channels can be either inactivated by further conformational changes or they deactivate by returning to the closed state.[85]

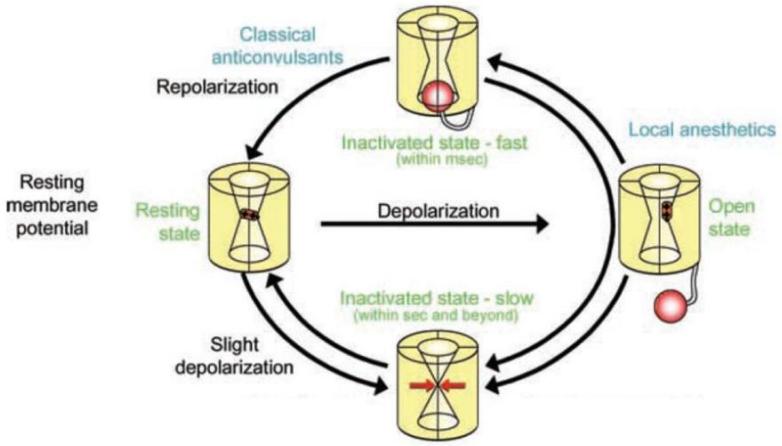

Figure 16. Schematic working mechanism of voltage-gated sodium channels. At the resting potential sodium channels are closed and can be opened by depolarization of the membrane. From this state channels can close from the inside of the neuron and go into the fast inactivated state. When the membrane potential returns to its baseline the sodium channel goes back to its resting state. Under conditions of slight prolonged depolarization and repetitive neuronal activity the sodium channel can go into the slow inactivated state by closing the pore from the inside. Drugs can either block the open channel (e.g., local anaesthetics), or enhance fast inactivation (classical anticonvulsants) or enhance slow inactivation.[86]

Each Na_V subtype is composed of a pore-forming α-subunit and one or two auxiliary β-subunits. The α-subunit comprises four structurally homologous domains (D1-D4) (Figure 17). Each domain consists of six transmembrane segments connected together by small extracellular loops. The large loops connecting S5 and S6 segments are so called pore loops which harbour the Na^+-selective filter. Epitopes in the pore loops are found to be essential for μ-conotoxin binding.[87, 88] The S4 segments of each domain contain positive-charged amino

acids at every third position and were shown to function as voltage sensors.[89, 90] The so called inactivation gate (IFM) is formed by the intracellular loop between D3 and D4 domains of the α-subunit (Figure 16). It has an ability to inactivate the channel after it has been opened. The structure of this loop has been recently resolved, showing that IFM is formed from a rigid α-helical region leading by two turns.[91]

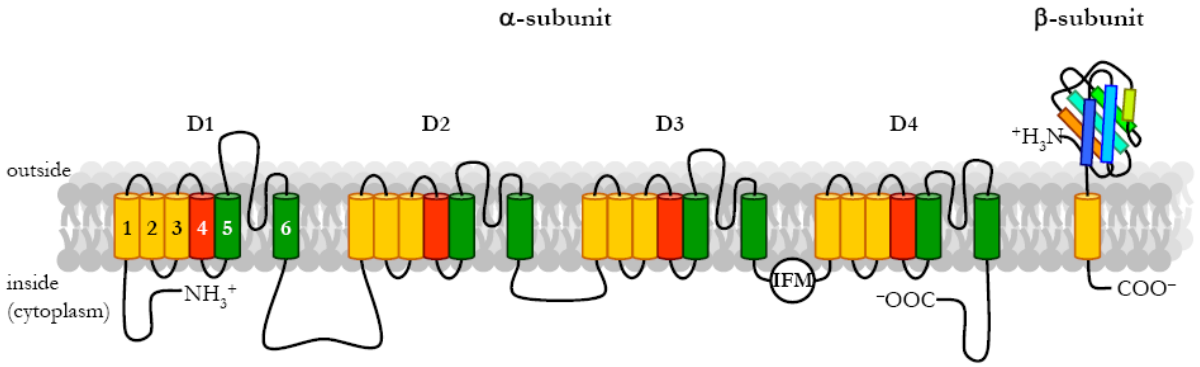

Figure 17. Topology model of the VGSC α- and β-subunits. Four homologous domains consisting of six transmembrane seqments (S1-S6) are connected together. The voltage sensor S4, and pore regions (forming a selectivity filter) S5-S6.[74]

As described above, three types of conotoxins target Na_V channels, explicitly μ-, μO-, and δ-conotoxins.[74] μO-conotoxins are reported to bind to receptor site 4, which involves the voltage sensor in domain D2.[92] Finally, δ-conotoxins are described to bind to receptor site six (voltage sensor in domain D4).[74] The differences in the binding sites and the structures of those peptides as well as the specificity to discriminate between the Na_V channel subtypes makes them important research tools. Moreover, these conotoxins possess therapeutic potential, e.g. due to inhibition if the pain-relevant Na_V channels like $Na_V1.7$ or $Na_V1.8$. The application of μO-conotoxins against pain is reported as prospective for this family of conotoxins.[93, 94] μ-conotoxins possess analgesic potential, too; e.g. μ-SIIIA conotoxin blocks $Na_V1.7$ channels. However, the μ-SIIIA mechanism of channel block as well as the subtype specificity is not fully understood. Furthermore, systematic investigations on the mechanism of pore block and subtype specificity are challenging, because the toxin-target-interaction is complex and, e.g. μ-SIIIA, potentially involves all four channel domains.

Therefore, combining the methods starting from production of sufficient amounts of well characterised conotoxins, through their chemical and structural characterisation, to systematic electrophysiological measurements will help to reveal the mechanism of action of conotoxins and to design subtype specific channel blockers.[84]

1.6 Structure-activity relationship studies

The specific binding properties of conopepides to their corresponding target provide an excellent tool to reveal the structure and mode of function of their target. Due to the investigation of the surface topology of conopeptides the possible topology of the binding site of the target protein can be determined.[6] Therefore, these investigations play an important role to reveal a more detailed image about the general working principles of ion channels and interactions with their ligands on a molecular level.[95]

Up to now, only little structural information of the target proteins is available. In 2003, Roderick MacKinnon was awarded with the Nobel Prize in Chemistry for his research in solving the structure and working principles of potassium ion channels (KcsA). An important part of this work was based on investigations of toxins isolated from scorpion venom, which selectively binds to a specific potassium ion channels.[96] By analysing the interactions of these toxins with the potassium channel first structural information about these channels were gained. Currently, probing peptide based toxins and ion channels became an appropriate tool to understand ligand-receptor-interactions. Interestingly data obtained by solid-state NMR revealed the binding of a toxin peptide (kaliotoxin) to the target KcsA-Kv1.3 and conveys to the conclusion that conformational changes occur for toxin as well as the target.[97] These results show that the structural dynamics also have to be considered during the toxin-target interactions. Recently, binding mechanism of voltage-sensitive potassium channel and toxin **κ**-conotoxin PVIIA has been proposed.[80] Later on, the structure of acetylcholine receptor (Ach) was published by Unwin *et al*..[98]

Since very little information is available about the structure of Na^+ channels, scientists started to perform molecular modeling of ligands and the Na^+ channels by replacing structural unknown parts of it with homologous regions of KscA channel.[95, 99, 100] Some efforts have been made to localize the interaction site of μ-conotoxin GIIIA.[87] Due to the mutations of amino acid residues of the peptide and target protein (VGSC Na_V 1.4.), it was suggested that the toxin binds to the outer vestibule of the channel. This method provides an opportunity to design small molecules with different mutations to understand why some of these peptides reveal selectivity and others do not. Moreover, μ-conotoxin GIIIA was used to determine the clockwise orientation of Na_V channel domains.[101]

1.7 Therapeutic potential of conopeptides

Since many decades, the development of drugs with high affinity and specificity occupied minds of scientists. A drug displaying desired therapeutically effects only, interacting with only desired target and causing no secondary effects, is a dream of all pharmacologists. This potential, however, was described for conotoxins in a unique way.

The first substance from marine venom which is now used clinically is the synthetic version of ω-conotoxin MVIIA from *Conus magus*. It is applied as an analgesic for the treatment of chronic pain under the name Ziconotide.[102] Another name for this drug is Prialt™ or "**Pri**mary **Alt**ernative to morphine". It is known to be more potent than morphine and in comparison to morphine, it does not cause addiction and tolerance.[103] It affects the N-type of Ca_V channels which are found primarily at presynaptic terminals and are involved in neurotransmitter release. Here, ω-MVIIA blocks the pain transmission by binding and blocking Ca_V channels and thus, is used in the intrathecal pain treatment. It was found, that ω-MVIIA binds to the outer vestibule of the Ca2.2. ion-conducting pore.[104] Other conotoxins of the ω-family were found to possess drug potential as well. For example, ω-CVID, known as AM336 or AMRAD is supposed to be the most selective inhibitor of N-type Ca_V channels over the P/Q-type and is therefore tested in preclinical studies for being applied in treatment of morphine resistant pain.[104]

The question arises, why morphine causes tolerance, while ω-conotoxins do not? The mechanisms leading to tolerance are poorly understood. However, different research groups try to answer this question. To understand this phenomenon, the mechanism of pain transmission should be introduced, depicted in Figure 18. This mechanism is described as follows: When the electrical signal coming down the nerve and reaching the end of the nerve, Ca_V channels open up and calcium influx is initiated. This causes neurotransmitter to be released, which binds to the receptors of the ligand-gated sodium channels on the postsynaptic membrane. Now, the sodium channels open and Na^+-ions are passing through. The pain or signal is now transmitted to the receiving cell.

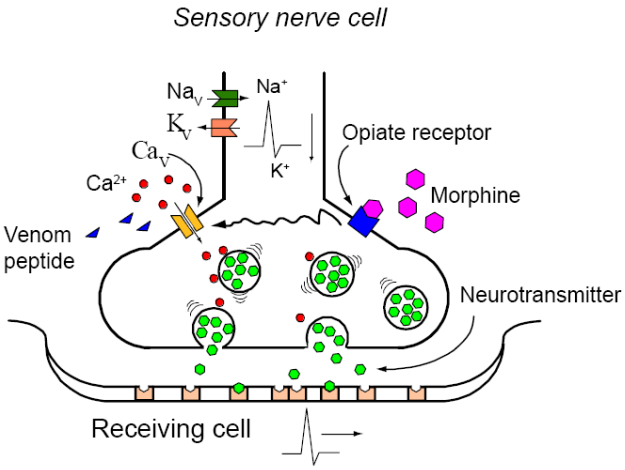

Figure 18. Schematic view of the presynaptic nerve terminal representing the mode of action of ω-conotoxins vs. morphine.[104]

The treatment with ω-MVIIA results in the blockage of calcium influx and thus, the signal cannot be trigged to the receiving cell, the pain signal is blocked. If morphine is used for pain treatment, the activation of the μ-opioid receptor leads to the inhibition of the N-type of Ca_V channel current by interacting with the pore-forming α1 subunit *via* the G-protein coupled receptor (GPCR) by changing channel gating and by altering ion permeation.[104] This inhibition of the N-type of Ca_V channel contributes to morphine analgesia and uncoupling of the opioid receptor and G-protein may underlie morphine tolerance development. In comparison, ω-MVIIA binds to Ca_V-channels without affecting GPCRs and is still effective in small doses after a long time of the pain treatment.[104, 105]

Not only ω-conotoxins possess a high specificity and affinity for their targets. In Table 1 different classes of conopeptides are summarised according to their targets and therapeutic potential. For example, μ-conotoxins have a therapeutic potential as analgesics, because of their ability to block VGSCs which are involved in pain perception, e.g. $Na_V1.7$. Understanding their mechanism of subtype specificity in detail would it would be prerequisite for their further structure optimisation, i.e. introduction of non-peptidic mimetics, described by Norton *et. al.*.[106] Such mimetics would constitute lead compounds in the development of new therapeutics for the treatment of pain.[106]

However, there are two problems which limit the use of conotoxins in medicine:

1) Rapid hydrolytic degradation in the body.
2) Limited oral intake. Therefore, they are used for injection into the spinal cord (intrathecal).

Nevertheless, some research groups reported a therapeutic potential of backbone- or side-chain cyclic peptides with higher *in vivo* stability and enhanced bioactivity.[107-110] They added

to the N-, C-terminus of α-conotoxin Vc1.1 (16 amino acids in length and 4 cysteines) the peptide sequence GGAGG (the length is around 10 Å) forming cyclic peptide. The peptide was mentioned to be more stable against hydrolysis and has been described to have a potential for oral intake.[110]

Table 1. Classes of conopeptides, their targets and therapeutic potential. modified from 3

Peptide families	Targets of action	Therapeutic potential
Conopeptides without or with one disulfide bridge		
Conatokins	NMDA receptors	Epilepsy, pain, stroke, Parkinson's disease
Contulakin-G	Neurotensin receptors	Pain and CNS disorders
Conopressin	Vasopressin receptors	Blood pressure regulation
Conotoxins		
α-family	Antagonists of neuronal and skeletal muscle nAChR	Anxiety, Parkinson's disease, pain, muscle relaxants
μ-family	Skeletal muscle sodium channels	Neuromuscular block, pain
μO-family	voltage-gated sodium channels	Analgesics
κ-family	Potassium channels	Hypertension, arrhythmia, asthma
ω-family	Calcium channels	Stroke, pain

To sum up, this section shows that a vast diversity of conopeptides target different types of transmembrane proteins and receptors with high selectivity and affinity. Some representatives of marine drugs have found their application in medicine, and the scientific works of many research groups are going further to optimize their application in the field of new drug development.

Overview of manuscripts

1. **Miloslavina, A. A.**, Leipold E., Kijas M., Stark A., Heinemann S. H., Imhof D. A room temperature ionic liquid as convenient solvent for the oxidative folding of conopeptides (2008) *J. Pept. Sci.*, 15(2), 72-77

2. **Miloslavina, A. A.**, Ebert, C., Tietze, D., Ohlenschläger, O., Englert, C., Görlach, M., Imhof, D., An Unusual Peptide from *Conus villepinii*: synthesis, solution structure, and cardioactivity (2010) *Peptides*, 31, 1292-1300

3. **Miloslavina, A. A.**, Kühl, T., A., Neugebauer, U., Popp, J., Stark, A., Imhof, D., New insights into the application of ionic liquids as reaction media for peptide oxidation and fragment ligation (2010) *Chem. Commun* (submitted)

4. Leipold, E., Markgraf, R., **Miloslavina A. A.**, Kijas M., Schirmeyer, J., Imhof D., Heinemann S. H., Molecular determinant for the subtype specificity of μ-conotoxin SIIIA targeting neuronal voltage-gated sodium channels (2010) in preparation for J. Neuro-pharmacol.

5. **Miloslavina, A. A.**, Tietze, D., Ohlenschläger, O., Ullrich, F., Leipold, E., Heinemann, S. H., Görlach, M., Imhof, D., Structurally diverse disulfide-connected isomers of neurotoxin μ-PIIIA block skeletal muscle sodium channel $Na_V1.4$, in preparation for Structure

Work not included in the thesis:

6. **Miloslavina A. A.**, Stark A., Leipold E., Heinemann S. H., Imhof D., An alternative way for conopeptide formation, Peptides 2008, Proceedings of 30th European Peptide Symposium (30th EPS), Helsinki (Finland), 178-179

7. Imhof, D., Stark, A, **Miloslavina, A. A.**, Kühl, T., Verfahren zur Herstellung von Cysteinhaltigen Peptiden, Patent (submitted)

8. **Miloslavina, A. A.**, Imhof, D., Ionic liquids are solvents of choice for peptide preparation and characterization, Review, in preparation for Curr. Pharm. Design

Poster Presentations

Miloslavina A. A., Stark A., Leipold E., Heinemann S. H., Imhof D., An alternative way for conopeptide formation, 30th European Peptide Symposium (30th EPS), Helsinki, Finland (31 - 05/08/2008)

4. Workshop Biochromatographie Hochauflösende Chromatographie von Proteinen, Peptiden & Kohlenhydrate, Halle, Germany (24/04/2009)

Miloslavina A. A., Stark A., Leipold E., Heinemann S. H., Imhof D., An alternative way for conopeptide formation, Workshop Ionic Liquids, Jena (11 – 12/08/2009)

Miloslavina A. A., Kunze C., Stark A., Imhof D., Disulfide bridge formation in biocompatible ionic liquids, 18 Nachwuchswissenschaftler-Symposium Bioorganische Chemie, Hannover (28 – 30/09/2009)

Miloslavina A. A., Ohlenschläger O., Imhof D., Structure-activity relationship of cystein-rich peptides (conopeptides), NMR AMPERE Sommer School, Wierzba, Poland,
POSTER AWARD (20-26/06/10)

Miloslavina A. A., Stark A., Imhof D., Bioactive cysteine-rich peptides are interesting targets for oxidative folding and native chemical ligation in ionic liquids, 31th European Peptide Symposium, Copenhagen, Denmark (5-9/10/2010)

Oral Presentations

Specific Binding, Transport and Cellular Uptake: Peptide-tagged Carriers for Diagnostic and Therapeutic Purposes, Mini-Symposium zum Forschungsschwerpunkt “Innovative Materialien & Technologien”, Jena (16/07/2009)

Cone Snail Toxins: Peptides with Therapeutic Potential, CMB Seminar, Beutenberg Campus, Jena (17/11/2009)

Documentation of authorship

This section contains an individual author's contribution to each publication in order to document which part of work has been performed by the author of this thesis. In case of collaborations, authors were assigned into groups to simplify an overview.

1. A room temperature ionic liquid as convenient solvent for the oxidative folding of conopeptides by Alesia A. Miloslavina, Enrico Leipold, Michael Kijas, Annegret Stark, Stefan H. Heinemann, Diana Imhof *Journal of Peptide Science* **2008**, 15(2), 72-77

A. A. Miloslavina: synthesis and characterisation of peptides, establishment and performance of oxidation in ILs, analysis and evaluation of the data, preparation of manuscript

E. Leipold, M. Kijas, S. H. Heinemann: electrophysiological investigation of μ-SIIIA

A. Stark: provision of ionic liquids, helpful discussions in the field of green chemistry

D. Imhof: supervision, preparation and correction of manuscript

2. An unusual peptide from *Conus villepinii*: synthesis, solution structure, and cardioactivity by Alesia A. Miloslavina, Christina Ebert, Daniel Tietze, Oliver Ohlenschläger, Christoph Englert, Matthias Görlach, Diana Imhof, *Peptides* **2010**, 31, 1292-1300

A. A. Miloslavina: synthesis and characterisation of all peptides, establishment and performance of oxidation in ILs, structure determination of CCAP-vil by solution NMR, zebrafish bioexperiments, analysis and evaluation of the data, preparation of manuscript

C. Ebert, C. Englert: support by zebrafish experiments

D. Tietze: support by assignment of chemical resonances of CCAP-vil NMR structure

O. Ohlenschläger, M. Görlach: performing of NMR measurements, structure analysis

D. Imhof: supervision, preparation and correction of manuscript

3. New insights into the application of ionic liquids as reaction media for peptide oxidation and fragment ligation by Alesia A. Miloslavina, Toni Kühl, Ute Neugebauer, Jurgen Popp, Torsten Steinmetzer, Annegret Stark, Diana Imhof, *Chemical Communications* **2010** (submitted)

A. A. Miloslavina: synthesis and characterisation of conotoxins, performance of oxidation in ILs, analysis and evaluation of the data, preparation of manuscript

T. Kühl: synthesis, chemical ligation and oxidation of Tridegin, contribution to the manuscript

U. Neugebauer, J. Popp: measurement and evaluation of Raman spectra

A. Stark: provision of ionic liquids, helpful discussions in the field of green chemistry

T. Steinmetzer: measurement of inhibitor activity of Tridegin with a Factir XIIIa assay

D. Imhof: supervision, preparation and correction of manuscript

4. Molecular determinant for the subtype specificity of μ-conotoxin SIIIA targeting neuronal voltage-gated sodium channels, by Enrico Leipold, Rene Markgraf, Alesia A. Miloslavina, Michael Kijas, Jana Schirmeyer, Diana Imhof, Stefan H. Heinemann, in preparation for *Journal of Neuropharmacology*, **2010**

A. A. Miloslavina: synthetic and experimental work on the conotoxin, NMR characterisation of μ-SIIIA and comparison to published data, characterisation of μ-SIIIA

E. Leipold, René Markgraf, M, Kijas, Schirmeyer, J.: electrophysiological measurements, results interpretation and manuscript writing

D. Imhof: supervision in peptide chemistry work

S. H. Heinemann: supervision and writing of manuscript

5. Structurally diverse disulfide-connected isomers of neurotoxin μ-PIIIA block skeletal muscle sodium channel NaV1.4, by Alesia A. Miloslavina, Daniel Tietze, Oliver Ohlenschläger, Florian Ullrich, Enrico Leipold, Andre Mischo, Stefan H. Heinemann, Matthias Görlach, Diana Imhof, in preparation for *Structure* **2010**

A. A. Miloslavina: synthesis, characterisation and NMR structure determination of conotoxin μ-PIIIA and its structural isomers, interpretation of results, preparation of manuscript

D. Tietze: support by NMR spectroscopy cross-peak assignments of μ-PIIIA and its structural isomers

O. Ohlenschläger, M. Görlach: support by measurements of NMR spectra and structure analysis

F. Ullrich, E. Leipold, S. H. Heinemann: electrophysiology measurements and interpretation of biological activity results

A. Mischo: support by CD experiments

D. Imhof: supervision, preparation and correction of manuscript

I hereby certify that the information in this documentation of authorship is true and correct and the contribution of the thesis author in the research work published in manuscripts is as stated above.

_____

PD Dr. D. Imhof

2 Manuscripts

A Room Temperature Ionic Liquid as Convenient Solvent for the Oxidative Folding of Conopeptides

Miloslavina, A. A., Leipold E., Kijas M., Stark A., Heinemann S. H., Imhof D.[*]

Journal of Peptide Science **2008**, 15 (2), 72-77

An Unusual Peptide from *Conus villepinii*: Synthesis, Solution Structure, and Cardioactivity

Miloslavina, A. A., Ebert, C., Tietze, D., Ohlenschläger, O., Englert, C., Görlach, M., Imhof, D.[*]

Peptides **2010**, 31, 1292-1300

New Insights into the Application of Ionic Liquids as Reaction Media for Peptide Oxidation and Fragment Ligation

Miloslavina, A. A., Kühl, T., Neugebauer, U., Popp, J., Steinmetzer, T., Stark, A., Imhof, D.[*]

Chemical Communications **2010** (submitted)

Molecular Determinant for the Subtype Specificity of μ-conotoxin SIIIA Targeting Neuronal Voltage-gated Sodium Channels

*Leipold, E., Markgraf, R., Miloslavina A. A., Kijas M., Schirmeyer, J., Imhof D., Heinemann S. H.**

2010, in preparation for Journal of Neuropharmacology

Structurally Diverse Disulfide-connected Isomers of Neurotoxin μ-PIIIA Block Skeletal Muscle Sodium Channel $Na_V1.4$

*Miloslavina, A. A., Tietze, D., Ohlenschläger, O., Ullrich, F., Leipold, E., Heinemann, S. H., Görlach, M., Imhof, D.**

2010, in preparation for Structure

3 Discussion

3.1 Ionic liquids in peptide synthesis

Ionic liquids (ILs) or "molten salts" were established as new generation solvents in Green Chemistry. Today they found their application also in peptide chemistry, i.e. in the synthesis[111] and the characterisation of peptides[112]. With respect to synthesis, for example, it was first demonstrated that a cyclic bioactive pentapeptide (sansalvamide A) was efficiently generated in an IL, even in the case of sterically hindered, barely soluble amino acids.[113]

In comparison to conventional solvents, ILs consist of an anion and a cation and thus possess a dual nature character. By manipulating the properties of anions, e.g. halogenides, tetrafluoroborate or hexafluorophosphate, and cations, such as imidazolium or pyridinium, it is plausible to design ILs with desired properties, e.g. hydrophilicity or hydrophobicity, solubility, or for special reaction conditions, e.g. to tune the reaction rate, modify the protein selectivity, and increase the substrate solubility.[114,115]

Recently, various reports about the mechanism and forces causing IL-solute interactions have been published. Weingärtner *et al.* and He *et al.* investigated the effects of IL ions on peptide and protein properties such as folding, stability and activity reporting a higher stability and activity in ILs.[116, 117]

By means of ^{1}H-NMR it was possible to detect the interaction of IL anions, e.g. acetate, diethylphosphate, chloride, methanesulfonate, ethylsulfate, tetrafluoroborate, bis(trifluoromethanesulfonyl)amide, with OH-groups of cellulose as the solute, indicating that the acetate had the highest hydrogen bond interaction.[118] The probability of anion-OH-anion complex formation was discussed as well with the cation forming the outer shell of the solvation layer.[118] Additionally, very good solubility of biopolymers, such as cellulose or silk in ILs was reported.[119, 120]

The effects of ILs on the reaction process and interactions with the solute were described to be affected by the anion rather than the cation, however, this is not the case if the solute is negatively charged.[121, 122] This tendency has been explained by the hydrogen bond accepting ability and dipolarity/polarisability effects of the respective ion pair.[123] Investigations of structure stabilisation and activity of proteins in ILs were explained by the Hofmeister series.[124] The protein stabilisation and activity was observed if using chaotropic cations and kosmotropic anions according to the order depicted in Figure 19. Chaotropic cations are large, low charged and weakly hydrated ions, while kosmotropic anions are small, highly charged and

strongly hydrated anions. The ion kosmotropicity could be quantified by many thermodynamic parameters, among them e.g. viscosity *B*-coefficients. Thus, kosmotropic ions usually show higher viscosity than chaotropic ones.

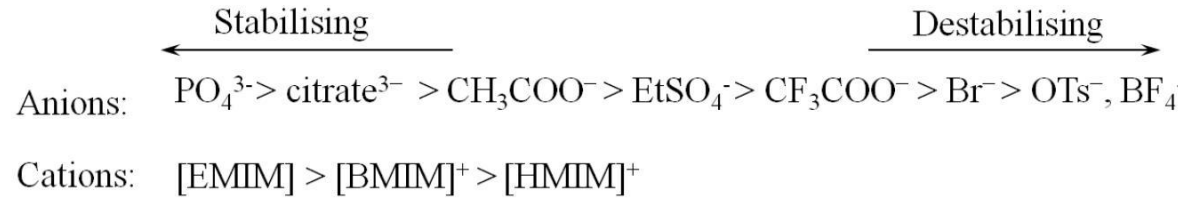

Figure 19. The Hofmeister series of ion effects on protein stability. Abbreviations of ILs are described in the Appendix Figure A 1. Modified from 125

However, this behavior is described in the absence of water and when no cation-protein complexes can be formed in the reaction mixture. Moreover, impact of other factors on protein activity and stability should be mentioned, such as pH or IL impurities. Also, the hydrophobic properties of the solute play an important role in the choice of solvent with the aim to create a suitable environment and thus to support the peptide/protein folding.

Finally, it needs to be mentioned that increased yields of desired oxidised product ω-conotoxin MVIC were obtained upon addition of ammonium salts with anions such as SO_4^{2-}, OAc^-, Cl^- to the oxidative folding buffer solution (conventional methods).[49]

Based on the knowledge obtained from these previous studies, biocompatible ILs which are liquid at room temperature and possess low viscosity were selected as solvents for cysteine-rich peptides (reduced form) that have to be further processed in order to obtain the bioactive counterparts (oxidised form) of conopeptides and miniprotein. The oxidative folding process for cysteine-rich peptides in ILs has not been described previously.

3.1.1 Oxidative folding of cysteine-rich peptides in ionic liquids

Following investigations for selected conopeptides CCAP-vil, α-GI, μ-SIIIA, μ-PIIIA, δ-EVIA, δ-SVIE (**1-6**), and miniprotein tridegin (**7**) a Factor XIIIa inhibitor[126] (overview given in Table 2) were included in the studies to examine the impact of peptide chain length, amino acid composition, cysteine content, disulfide connectivity, and physicochemical properties on the yield and the efficiency of the folding process. Afterwards, the peptides were subjected to oxidative folding in a set of ILs ([C_2mim][OAc], [C_4mim][OAc], [C_2mim][OTs], [C_2mim][DEP], and [C_2mim][$N(CN)_2$], full names are represented in Appendix Figure A 1) that differ mainly in the composition of the anion and thus their pK_a values.

Table 2. Representation of model peptides varying in cysteine content, amino acid sequence and length used for the investigations.

Nr.	Peptide	Sequence	Cysteine pattern	AA
	Peptide with <u>one</u> disulfide-bridge:			
1	CCAP-vil	PFCNSFGCYN-NH_2	C-C	10
	Peptide with <u>two</u> disulfide-bridges:			
2	α-GI	E**C**C**C**NPA**C**GRHYS**C**-NH_2	CC-C-C	13
	Peptides with <u>three</u> disulfide-bridges:			
3	μ-SIIIA	ZN**C**C**C**NGG**C**SSKW**C**RDHAR**C**C-NH_2	CC-C-C-CC	20
4	μ-PIIIA	ZRL**C**C**C**GFOKS**C**RSRQ**C**KOHR**C**C-NH_2	CC-C-C-CC	22
5	δ-EVIA	DD**C**IKOYGF**C**SLPILKNGL**C**C**C**SGA**C**VGV**C**ADL-NH_2	C-C-CC-C-C	32
6	δ-SVIE	DG**C**SSGGTF**C**GIHOGL**C**C**C**SEF**C**FLW**C**ITFID-NH_2	C-C-CC-C-C	31
7	Tridegin	KLLP**C**LQWHQGIPNPR**C**W**C**GADLE**C**AQDQY**C**AF IPQ**C**RPRSELIKPMDDIYQRPVEFPNLPLKPRE-NH_2*	C-C-C-C-C-C	66

NH_2 – amidated C-terminus, O - 4-Hydroxyproline, Z – pyroglutamic acid, cysteine connectivities are shown with solid lines.
*Cysteine connectivities are not resolved yet

The complete transformation of the reduced precursor into the disulfide-bridged peptide was characterised by various methods: MALDI-TOF mass spectrometry including derivatisation with iodoacetamide, Raman spectroscopy, Ellman's test and finally NMR spectroscopy for suitable candidates. Correct folding was additionally confirmed by investigating the biological activity of these peptides, especially of candidates that are not structurally characterised yet, e.g. tridegin (**7**). For the characterisation of the oxidised peptide, purification and separation of the product from the IL reaction mixture was required. Because of the small volumes of the reaction mixture (1 mg of peptide per 40-100 μl of IL) in comparison to conventional methods (1 mg of peptide per 17 ml of buffer solution), this step could be easily solved by dilution of the reaction mixture and further RP-HPLC purification.

Raman spectroscopy was used as a new method to observe the completion of the folding process. In contrast to NMR analysis of the rather complex peptides, it is a fast and convenient method to derive the required information, i.e. Cys-rich peptide conversion to the disulfide-bridged counterpart. During oxidative folding usually an increase of the S-S stretching mode is detectable at 485-550 cm^{-1},[127] while at the same time the S-H stretching mode at 2525-2580 cm^{-1} disappears.[128] The disulfide stretching mode region has been assigned to three possible conformations depending on the configuration of the C-S-S-C moiety.[127] The most stable *gauche-gauche-gauche* conformation of the S-S bonds in the region ν_{SS} 499-508 cm^{-1} was observed for all investigated peptides. In the case of peptide **4**, a shoulder next

to S-S stretching mode in Raman spectrum was observed that corresponds to the possible presence of an additional conformation. This so called minor conformation was detected in solution NMR experiments according to earlier studies of Nielsen *et al*..[23]

The results derived from oxidative folding by two conventional methods, i.e. oxidation in buffer solution containing redox active agents like glutathione (reduced/oxidised form)[47, 129] or reduced glutathione/cystamine were compared to the new approach using ionic liquids.[130] According to present findings, the oxidative folding results were not significantly different for the two conventional methods (buffer conditions) used. However, a variety of unwanted side-products occurred to a rather high extent. Contrary to hydrophilic peptides **1**–**4** a major drawback of oxidising peptides such as **5**–**6** is their low solubility in aqueous solutions. Thus, beside the fact that high dilution (10^{-4}-10^{-5} M) of such peptides prevents intermolecular oligomerisation in this medium, their concentration is already rather low compared to other representatives. That is why the use of organic solvents, such as isopropanol or methanol, was required to obtain a sufficient solubility of hydrophobic peptides in conventional systems.

The oxidative folding reaction for investigated peptides **1**-**7** was carried out without any other additives, and the water content of the ILs varied from 0 - 4%. Since the oxidative folding in ILs is carried out on air, the surface of the reaction mixture should be maximised in order to provide a maximum uptake of atmospheric oxygen to oxidise the peptide.

As a result, yields and efficiency of oxidative folding differed strongly depending on the IL anion for investigated peptides **1**-**7**. For decapeptide **1** the results for disulfide bond formation were completely different from those obtained for the conotoxins **2**-**6** or miniprotein **7**. The efficiency of oxidative folding in ILs for **1** was found to decrease in the following order: $[C_2mim][OTs] > [C_2mim][DEP] > [C_2mim][OAc] > [C_2mim][N(CN)_2]$.[131] This order follows the increasing pK_a of the corresponding anions, with except of ion $[N(CN)_2]^-$. The inverse tendency to the Hofmeister series for CCAP-vil is probably due to the peptide's small molecular weight and almost neutral net charge. Results show that the p-toluenesulfonate anion with a lower pK_a value compared to other anions yield > 90% of the oxidised peptide **1**. Moreover, if increasing the length of the cation alkyl substituent from ethyl to butyl, but using the same anion (acetate), the yield of the oxidised product decreased from ~ 60% to approximately 47%, i.e. the effectiveness of the oxidation reaction decreased from $[C_2mim][OAc]$ to $[C_4mim][OAc]$ confirming the previous findings.[125]

Interestingly, the oxidised product was formed to a higher extent in $[C_2mim][OAc]$ for other peptides (**2**-**7**), and therefore followed the principle of kosmotropic anion and chaotropic cation as described above. Moreover, the formation of properly folded species was improved

in comparison to conventional methods and literature data.[132] The yields among μ-conotoxins **3** and **4** varied between 60% - 70% and 25% for δ-conotoxins **5** and **6** in [C_2mim][OAc], while conventional methods yielded only 16% - 50% for μ-conotoxins and <10% for δ-conotoxins. Additionally, the expected cyclic toxins were not formed in [C_2mim][OTs] and [C_2mim][DEP], probably due to the combination of two facts: first, high positive net charge of conotoxins and secondly, chaotropic properties of these anions. Moreover, the same behaviour as found for peptide **1** was observed for conotoxins **2**-**6** with respect to an increasing length of the side chain substituent in the imidazolium-derived cation, the yields decreased in the following order: [C_2mim][OAc] > [C_4mim][OAc].

For oligopeptide **7** it was possible to increase the yield of the desired product through optimisation of the oxidation procedure using ILs as well. Conversion of the reduced precursor into the fully oxidised tridegin gave satisfactorily high yields (80-90%) in [C_2mim][OAc] compared to the yield obtained for the conventional method (20-45%). However, in comparison to the oxidation of conotoxins **2**-**6** in ILs with diethylphosphate, tosylate and dicyanamide a mixture of partially oxidised (one and two disulfide bridges) and fully oxidised peptide **7** was observed.

To summarise, depending on the net charge, primary amino acid sequence and the hydrophobicity of the peptide the efficiency of folding in the IL selected differed strongly. In contrast to the conotoxins (μ-SIIIA, μ-PIIIA, δ-EVIA, δ-SVIE) that showed best results if using [C_2mim][OAc] as reaction medium, optimal conversion of linear CCAP-vil was observed in [C_2mim][OTs]. The efficiency of oxidative folding decreased with the increase of the imidazolium side chain length from ethyl to butyl, but using the same anion (acetate). Differences in oxidative folding efficiency for the small peptide with only one disulfide bond compared to peptides containing three disulfide bonds may be explained by the differences in primary amino acid sequence and their net charge. The large number of positively charged residues in conotoxins **3**-**6**,[132] on the one hand and almost neutral net charge of CCAP-vil, on the other hand obviously leads to different ways of interaction with the respective ions of the IL. Thus the ILs which favour the oxidative folding of investigated peptides can be divided into two groups: a) ILs supporting oxidative folding of rather small peptides with neutral charge and b) ILs that favour the oxidative folding for positively charged peptides and oligopeptides.

Finally, it was demonstrated that the choice of the IL anion is important for oxidative folding of cysteine-rich peptides. Indeed, it is evident that the primary amino acid sequence has an impact on the interaction with the respective ionic liquid and thus the outcome of the

reaction. It is hypothesised that stabilisation of a preformed secondary structure occurs in the ILs that include peptide molecules in their network and provide an ideal environment for individual folding rather than coiling, aggregation and oligomerisation caused by intermolecular interactions (Figure 20). This in turn leads to better yields of the desired, correctly folded product.

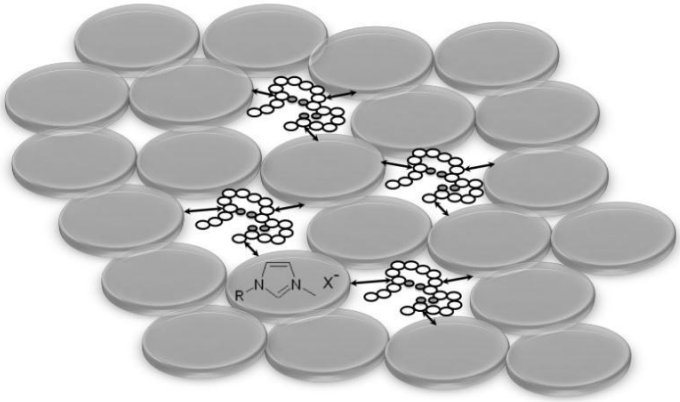

Figure 20. Hypothesis: Peptides are included in the IL network as molecules individually leading to correct disulfide bond formation and stabilisation of the bioactive conformation.

Yields, however, depend on both, the IL constitution as well as the peptide sequence and its net charge, i.e. caused by the interaction of the IL ions with the respective peptide, providing a proton activity as well as hydrogen bonding donor and acceptor sites.

The major advantages of the oxidative folding in ionic liquids were observed for peptides investigated in this thesis, namely CCAP-vil, α-GI, μ-SIIIA, μ-PIIIA, δ-EVIA, δ-SVIE and the oligopeptide tridegin:

1) ILs are able to dissolve a significant higher amount of both hydrophilic and hydrophobic peptides in comparison to conventionally used solvents with a final peptide concentration of 3-20 mM (10-25 mg/ml).
2) Oxidation succeeds without addition of redox agents or limitation to low temperatures, but on air at room temperature.
3) Possibility to perform oxidative folding in small volumes (1 mg of peptide in 40-100 μl of IL) simplifying the purification of the oxidation product.
4) Formation of by-products (misfolded peptides) is prevented.

The described properties, i.e. improved solubilisation and stabilisation of the secondary structure, have been reported previously for biocompatible ILs such as 1-butyl-3-methylimidazolium dihydrogenphosphate and *N*-butyl-*N*-methylpyrrolidinium dihydrogenphosphate using cytochrome c as model protein.[133] These effects on peptides, more precisely the oxidative folding process of cysteine-rich peptides in ILs have not been described previously.

3.1.2 Native chemical ligation of cysteine-rich peptides in ionic liquids

To test whether native chemical ligation (NCL) occurs in ILs, the 66-mer anticoagulant Factor XIIIa inhibitor tridegin (**7**) was used as a model peptide.

The first attempt to perform a ligation reaction, in particular a protease-catalysed ligation, in an ionic liquid was highlighted by Bordusa *et al.* earlier.[134] The reaction was performed in buffer with addition of IL [mmim][Me_2PO_4] (1,3-dimethylimidazolium dimethylphosphate). Some positive effects were observed during this reaction in comparison to conventional methods: the complete suppression of proteolytic side reactions (proteolytic hydrolysis, decrease of competing hydrolysis activity of proteases to acyl donor esters), high turnover rates, protease stability, good solubility of the reactants and products of the reaction. The latter fact was also observed for hydrophobic conotoxins, which showed good solubility in ILs.

According to this knowledge and the finding that the two segments of tridegin (1: N-terminal with 36 amino acids, 2: 30 amino acids as C-terminal amide), which were used to synthesise the 66-mer tridegin, were not equally well soluble in the buffer systems (Na_2HPO_4 or NH_4HCO_3, +/- 6 M guanidine HCl) commonly used in NCL. ILs were tested for this reaction type, too.[24, 26] Segment 2 of tridegin could easily be dissolved in concentrations up to 2 mM in aqueous systems, while the N-terminal peptide was almost insoluble at 400 µM. However, in ILs segment solubility was significantly improved (≥ 2 mM). For NCL studies we applied the same ionic liquids as for oxidative folding, namely [C_2mim][OAc], [C_4mim][OAc], [C_2mim][OTs], [C_2mim][DEP], and [C_2mim][$N(CN)_2$]. Furthermore, segment ligation with and without additives (e.g. thiols using individual components or mixtures of thiophenol (4% v/v) and benzyl mercaptan (4% v/v)) was examined[27, 29, 30, 135] and compared to the conventional methods described in chapter 1.3 (Figure 21).

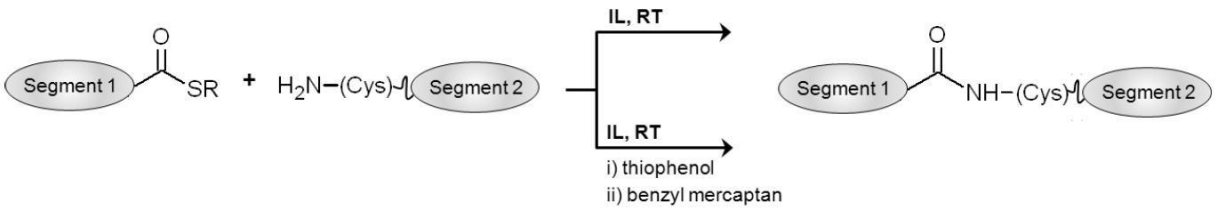

Figure 21. Schematic illustration of native chemical ligation of two segments, where the N-terminal segment 1 is a thioester and the C-terminal segment 2 contains a Cys residue at the first position, in ionic liquid with or without additives.

The completion of the ligation reaction was proved by several analytical methods, including Edman degradation and MALDI-TOF mass spectrometry. It was found that ionic liquids with acetate as the anion ([C_2mim][OAc] and [C_4mim][OAc]) displayed higher efficiency in experiments carried out either in the presence or absence of thiols than any other IL tested. In comparison to ligation with thiols, the product was observed in [C_2mim][DEP], too. However, the oxidised product retention time in RP-HPLC overlapped with retention times of the partially oxidised (with one or two disulfide-bonds) by-products, and it was impossible to achieve baseline separation of both peaks.

The proposed approaches might be very useful for the synthesis of small disulfide-bridged proteins, since both reactions – native chemical ligation as well as oxidative folding – can be carried out consecutively in the same medium, i.e. the respective IL.

3.1.3 Conclusions and outlook for the use of ILs in peptide synthesis

In this thesis, alternative protocols for oxidative folding and native chemical ligation of cysteine-rich peptides based on the use of room temperature ionic liquids as reaction media were developed. These are first examples of ILs found to influence the folding tendencies of mono- and multiple disulfide-bridged peptides.

The major advantages of the oxidative folding and NCL in IL are a good solubility of hydrophobic peptides in ILs, the possibility to perform reactions in high concentrations and small volumes, without addition of any redox agents or additives. Furthermore, the side reactions are minimised and reaction conditions are improved.

Further investigations should be focused on the effect of reaction conditions, e.g. water content of the ILs and temperature, on the reaction rate and outcome of the desired product. Preliminary results show a high stability of the peptides at high temperatures. However, the water content seems to have a negative effect on the oxidative folding of peptides, what describes an opposite effect of water on the peptide structure in comparison to protein structure. This behavior should be confirmed for peptides differing in peptide chain length, amino acid composition, cysteine content, disulfide connectivity, and physicochemical properties. Moreover, methods to purify and separate the oxidised product from the IL reaction mixture could be optimised in future investigations, e.g. dialysis or phase separation can be applied.

The high efficiency of the synthesis of cysteine-rich peptides and small proteins in ILs can significantly promote industrial production and application of these candidates possessing therapeutic potential and thus, has an impact on future developments in this field of research. The proposed approaches might be very useful for the synthesis of cysteine-rich peptides and miniproteins, since both reactions – native chemical ligation as well as oxidative folding – can be carried out consecutively in the same medium, i.e. the respective IL.

3.2 SAR-studies of cysteine-rich conopeptides

A very important and challenging topic in conopeptide research is structure-activity relationship (SAR) studies aiming at the disclosure of the molecular basis of toxin-target-interactions. The determination of the three-dimensional (3D) structures of the peptides plays a key role in the understanding of the function and therapeutic potential of conopeptides. Due to the small size of conopeptides the preferred technique to determine the structure of native toxins is NMR spectroscopy. In addition, X-ray or solid-state NMR studies allow the determination of the complexes of conopeptides with their binding partners resulting in a better understanding of the mechanism of action. For example, crystal structure of acetylcholine binding protein (AChBP) in complex with α-conotoxin PnIA (A10L D14K) has been determined.[136] Solid-state NMR studies revealed that for binding of the peptide kaliotoxin to its target KcsA-Kv1.3, a conformational change occurs for the toxin as well as the target.[97] Additionally, combining solution and solid-state NMR techniques allows the study of the dynamics and structure of the membrane proteins.[137, 138]

In order to provide sufficient information about a respective conopeptide for further SAR-studies, the solution structure of selected conopeptides, e.g. CCAP-vil, μ-SIIIA and μ-PIIIA has been determined.

3.2.1 CCAP-vil: characterisation, structure determination and bioactivity investigations

The first cysteine-containing peptide, whose structure and biological activity were investigated, was the decapeptide CCAP-vil with one disulfide bond. CCAP-vil derived from the cone snail *Conus villepinii* was suggested to belong to the family of cardioactive peptides displaying an opposite effect to the cardioaccelerating neuropeptide CCAP.[139] The objective

of this study was to find an optimised synthetic procedure to yield CCAP-vil in a sufficient amount and purity to enable structural studies and to explore the extent to which CCAP-vil affects the heart rate of a living organism.

One of the first challenges of CCAP-vil characterisation was the overlapping retention times of reduced peptide and oxidised product in RP-HPLC analysis. To distinguish the reduced and oxidised substances clearly, other methods were used such as capillary electrophoresis (CE), iodoacetamide-derivatisation followed by MALDI-TOF MS analysis[140], Ellman's test and Raman spectroscopy. Raman spectroscopy measurements were included to confirm the transition of the reduced peptide into the oxidised state, too. These measurements again revealed a *gauche-gauche-gauche* conformation of the S-S bond with ν_{SS} at 503 cm^{-1} as the most stable conformation of CCAP-vil. The same tendency as found for CCAP-vil was described for snake neurotoxins.[141] Thus, this finding correlates with the fact that upon oxidation the S-S bond stabilises the secondary structure of peptides.[142]

The NMR solution structure of CCAP-vil was determined using a combination of 2D NMR experiments: [^{1}H, ^{1}H]-NOESY, [^{1}H, ^{1}H]-TOCSY, [^{1}H, ^{1}H]-ROESY, [^{1}H, ^{1}H]-DQF-COSY (Figure 12), and [^{1}H, ^{13}C]-HSQC at natural abundance introduced in the chapter 1.4.3. The ^{1}H resonance assignments were performed using the "sequential assignment strategy" proposed by K. Wüthrich.[72] The above mentioned 2D NMR experiments were applied to connect amino acid spin system. For that purpose the cross-peaks were used to reproduce the sequence of the peptide with the help of coupling of backbone protons (of the peptide bond: nitrogen proton H^{N1} to carbon proton $H^{\alpha 1}$ of the definite amino acid and from $H^{\alpha 1}$ and H^{N2} of the next amino acid). The chemical shift dispersion of backbone ^{1}H resonances provided the first indication of a well-defined structure of CCAP-vil in solution. This is illustrated in the NOESY spectrum shown in Appendix Figure A 3 and A 4. The complete peptide sequence was assigned through the so called "sequential walk" depicted in the Figures Appendix A 2 and. The conopeptide CCAP-vil contains two asparagines residues (Asn), which were not visible in the H^NH^α cross-peaks part of the 2D-NOESY spectrum in the Appendix Figure A3 and therefore interrupt the sequential H_α–NH_{i+1} connectivities.

The NMR studies were consistent with the expectations for the C-S-S-C moiety obtained from Raman spectroscopy. Torsion angles of -75°, -69° and 95° for the disulfide bridge explain the wave numbers observed and indicative for a *gauche-gauche-gauche* conformation. In solution, CCAP-vil forms a cyclic conformation reminiscent of a type (I) β-turn. However, no hydrogen bonds stabilising this turn conformation could be deduced from the calculated structures. Typically, β-turn structures were also observed for other disulfide-bridged pep-

tides, e.g. CCAP[143], vasopressin[144] and oxytocin[145]. Comparison of the 3D structures of CCAP-vil (BMRB code: 21022) and CCAP (1V46) are represented in the Appendix Figure A 5. In the case of CCAP, a type(IV) β-turn was identified between Ala^5 and Gly^8 residues in water and between Cys^3 and Phe^6 in a micellar medium.[143] However, two molecular structures of CCAP are accessible thus far (PDB entries 1V46 and 1Y49). These NMR structures of CCAP from *Drosophila melanogaster* were determined in DMSO and DMSO/water (1:1), respectively. Since oxytocin (PDB code 1NPO) and vasopressin (1YF4) were suggested to be similar to CCAP, the pairwise superimposition of the heavy backbone atoms of their cyclic moieties in comparison to CCAP-vil was assessed in order to unravel a potential structural similarity for intended structure-activity relationship studies. However, superimposition of CCAP (1V46) and CCAP (1Y49) (cyclic residues Cys^3-Thr^7, Cys^9) with CCAP-vil (Cys^3-Cys^8) yields no significant similarity for the backbone structure. The corresponding root-mean-square-distance (r.m.s.d.) values are 1.50 Å and 1.34 Å, respectively. High deviations in backbone conformations are also observed between oxytocin (Cys^1-Tyr^2, Asn^5-Cys^6; 1NPO) and CCAP-vil (Cys^3-Asn^4, Gly^7-Cys^8) as well as vasopressin (Cys^1-Cys^6; 1YF4) and CCAP-vil (Cys^3-Cys^8) with r.m.s.d. values of 1.39 Å and 1.46 Å, respectively.

Secondly, the interest was focused on the cardiovascular role of CCAP-vil compared to CCAP. In the present study, zebrafish embryos were used to examine cardioactivity of the peptides. The response of zebrafish embryos after exposure to CCAP-vil at 0.6 mM after 4 h (up to 24 h) was significantly different from that elicited by exposure to CCAP at the same concentration and after the same time period. The assays revealed that the conopeptide CCAP-vil decreases heart rate by approximately 15%, while no effect was found for the insect neuropeptide CCAP. Surprisingly, the majority of the embryos died at concentrations higher than 1 mM of CCAP-vil indicating a severe impact on embryonic development or homeostasis. The activity found for CCAP-vil in *Danio rerio* is not as significant as demonstrated for *Drosophila melanogaster larvae*, where a 38% decrease of heart rate was determined.[139] CCAP, in contrast, increases heart rate by about 50%. In general, the primary reported effect of CCAP on isolated or semi-isolated hearts, e.g. of *Carcinus maenas* and *Orconectes limosus*,[146, 147] is its chronotropic influence. However, others were unable to demonstrate a significant effect of CCAP on heart rate *in vivo*, e.g. in intact *Cancer*.[148] The authors suggested that CCAP induces species-specific effects on crustacean heart muscle and is not a physiologically important cardiac neurohormone in the cancrid genera. This was corroborated by the fact that the pericardial organs of *Cancer maenas* contain concentrations of CCAP 6.75 times greater than those of *Cancer pagurus*. In addition, despite the similarity to CCAP in

disulfide-bridge connectivity and amino acid sequence two other peptides, M-CCAP1 and M-CCAP2, from the CNS of *Helix pomatia* show an excitory effect in the snail *Lymnaea stagnalis* and a rhythmic activity (fictive feeding) of about 67% of the assay used, while CCAP displays only a slight membrane depolarisation.[149] This indicates that minor structural differences between the mentioned cardioactive peptides might cause diverse activities in both, *in vitro* and *in vivo* experiments. CCAP was also described to be included in ecdysis control behaviour in the neuroendocrine cascade in insects.[150] It has been shown that there is a dramatic release of the peptide into the hemolymph just prior to ecdysis of the crab, *Carcinus maenas*, and the crayfish, *Orconectes limosus*.[151] Other studies show that CCAP acts as an adipokinetic hormone releasing factor and neurohormonally stimulating visceral muscle, as a neurotransmitter/neuromodulator on the insect gut.[152] CCAP-vil was suggested to bind to a target protein that causes systolic Ca^{2+} release via ryanodine receptor (RyR), resulting in a cardiotonic effect.[139] Also, cell permeability was suggested for CCAP-vil in comparison to CCAP. To prove these suggestions in the present investigations, fluorescein-labelled peptides were used in order to address whether the differences regarding heart activity might be caused by a different membrane permeability of CCAP vs. CCAP-vil. However, the results were inconclusive, probably because the label significantly changed and probably even dominated the physicochemical properties of the peptides.

3.2.2 Molecular determinant for μ-SIIIA subtype specificity

Na_V channels play a pivotal role in neuronal excitability; they are specifically targeted by μ-conotoxins from the venom of marine cone snails. These peptide toxins were described to bind to the outer vestibule of the channel pore thereby completely blocking ion conduction through Na_V channels. μ- SIIIA from *Conus striatus* is a very interesting candidate among representatives of the μ-family of conotoxins. It was shown to be a potent inhibitor of neuronal sodium channels and to display analgesic effects in mice and therefore has a therapeutic potential in pain treatment, albeit the molecular targets are not unambiguously known. To investigate systematically the mechanism of μ-SIIIA ion channel selectivity and specificity was the aim of the present research. For this purpose, the synthesis of reduced precursor followed by conventional oxidative folding[71] was first performed to obtain μ-SIIIA to ultimately determine the peptide structure and perform bioactivity studies. Since the 3D NMR structure of conotoxin μ-SIIIA was already published (BMRB entry 20025) the ^1H NMR spectrum was recorded for the peptide prepared in this thesis to compare the chemical shifts

with the published dataset.[105,153] In Appendix Figure A 6 amide and aromatic region of the ^{1}H NMR spectrum is shown confirming the published data.

The recombinant Na_V channels used for electrophysiological experiments were expressed in mammalian cells with the whole-cell patch-clamp method to elucidate the molecular determinants for μ-SIIIA subtype specificity on the channel surface. μ-SIIIA slowly and partially blocked rat-$Na_V1.4$ channels with an apparent IC_{50} of 0.56 ± 0.29 μM; the block was not complete, leaving at high concentration a residual current component of about 10% with a correspondingly reduced single-channel conductance, in comparison to μ-KIIIA that was reported to completely block the ion conduction of neuronal VGSCs.[154] At 10 μM, μ-SIIIA potently blocked rat-$Na_V1.2$ and rat-$Na_V1.4$, consistent with previous studies on TTX-competition and expression in *Xenopus* oocytes.[105, 153] Likewise it was found a reduced activity on human-$Na_V1.7$ channels and no activity on human-$Na_V1.5$, correlating with published data.[153] However, human-$Na_V1.8$ and rat- $Na_V1.8$ were insensitive to 10 μM of μ-SIIIA contrary to published μ-SIIIA activity on TTX-resistant channels in rat DRG neurons.[155] Thus, according to our results, μ-SIIIA is not a potent and specific blocker for mammalian TTX-resistant channels as proposed by Wang *et al.*.[155]

Furthermore, to find out the molecular determinants for μ-SIIIA specificity, domain chimeras between $Na_V1.4$ and $Na_V1.5$ were employed. The domain-2 and a minor contribution of domain-1 were found to be essential for μ-SIIIA binding. The latter was largely accounted by the alteration in the TTX binding site (Tyr401 for $Na_V1.4$, Cys373 for $Na_V1.5$, and Ser357 for $Na_V1.8$). Incorporation of domain-2 pore loops of all tested channel isoforms into rat-$Na_V1.4$ conferred the μ-SIIIA phenotype of the respective donor channels highlighting the importance of the domain-2 pore loop as the major determinant for μ-SIIIA subtype specificity.

Finally, three sites were mutated individually in the background of rat-$Na_V1.4$ inserting the respective residues of human-$Na_V1.7$, i.e. A728N, S729D and N732T. Results show that residue Asn889 in rat-$Na_V1.4$ seemed to be a crucial position for high sensitivity of μ-SIIIA. The results of the reversed experiment, insertion of the equivalent mutation N889A in the background of human-$Na_V1.7$, confirmed the importance of this position also for $Na_V1.7$.

Finally these results may pave the way for the rational design of selective Na_V channel antagonists for research and medical applications.

3.2.3 Conventional oxidation methods reveal different structural isomers of neurotoxin μ-PIIIA

Since the published structure of synthetic μ-PIIIA is defined as the native toxin fold and no other biologically active isomers of μ-PIIIA were reported, results of this study highlight structurally diverse isomers formed by conventional methods of oxidative folding that possess bioactivity.

The oxidative folding conditions used were slightly different to those published by Nielsen *et al.*.[23] It was noticed that reaction products were already formed after five minutes using glutathione as redox agent and further extension of the reaction time did not significantly change the composition of the reaction mixture regarding formation of μ-PIIIA-isomers as depicted in Appendix Figure A10. From this reaction mixture three isomers of μ-PIIIA were isolated in sufficient amounts and high purity. The formation of different μ-PIIIA isomers was reported earlier suggesting the formation of misfolded products or not fully oxidised peptides.[78, 132] As proved by Ellman's test and MALDI-TOF mass spectrometry all three isomers were completely oxidised and disulfide-bridged. However, the short reaction times for oxidative folding of μ-PIIIA suggest a spontaneous collapse mechanism as was first proposed by Fuller *et. al.*.[132] The same mechanism was observed for SmIIIA and SIIIA (our observations), but not for RIIIK and GIIIA of the same family of conotoxins.[132]

Members of the μ-family of conotoxins represent a very interesting class of peptidic toxins that are able to discriminate between Na_V channel subtypes. Therefore, they can help to elucidate the working mechanism of their targets, as well as being used as therapeutics, e.g. as analgesics or for pain treatment. μ-PIIIA was found to inhibit TTX-s Na_V channels, but had only little effect on TTX-r Na_V channels. To understand the reasons of μ-PIIIA selectivity, SAR studies may provide an insight and also contribute to the development of highly specific probes for TTX-sensitive Na_V channels. According to the standard way of determination of the native conotoxin structure and previous reports that only the native conformation possesses a biological activity[156] μ-PIIIA isomers were screened for their ability to inhibit Na^+ current of $Na_V1.4$ channels in whole-cell patch-clamp experiments. Surprisingly, all isomers were biologically active, i.e. all blocked $Na_V1.4$ mediated currents, although with different efficiencies. The activity decreased in the following order: μ-PIIIA-1 > μ-PIIIA-2 > μ-PIIIA-3. It is the first time this effect was observed for peptides with three disulfide bridges.

To explain the results of bioactivity studies and to elucidate which isomer represents the suggested native fold it was essential to perform structural analysis of isomers formed under

the oxidative folding conditions used. The first solution NMR structure of synthetic μ-PIIIA has been reported by Nielsen *et.al*..[23] This structure was defined as native structure according to its biological activity on DRG neurons. Bioactivity of additional isomers of μ-PIIIA has not been reported yet. Furthermore, structural information about natural μ-PIIIA from cone snail venom has not been determined up to now, too. Moreover, the published μ-PIIIA data of proton shifts in water was not complete, especially in the regions of cysteine residues which are essential to determine the disulfide-bridge connectivities.[23] Therefore, in this work the resonance assignment for three peptide isomers was necessary (Appendix Figure A 8 and A 9). The results show that two of three isomers, namely μ-PIIIA-1 and μ-PIIIA-2 possess a rigid structure, but different cysteine connectivities Cys4-Cys21, Cys5-22, Cys11-Cys16 and Cys4-Cys16, Cys5-Cys21, Cys11-Cys22 respectively (Appendix Figure A11 a, b). The third isomer μ-PIIIA-3 was supposed to be too flexible due to the low distribution of proton resonances according to 2D experiments and suggested to possess disulfide bond connectivities corresponding to this flexibility, namely Cys4-Cys5, Cys11-Cys16, Cys21-Cys22 (Appendix Figure A11 c). The second isomer μ-PIIIA-2 indicates the cysteine framework typical for μ-conotoxins. Comparison of μ-PIIIA-1 and μ-PIIIA-2 structures resulted in obvious structural differences of these isomers with a backbone (residues 16-22) r.m.s.d. value of 1.752 Å (Appendix Figure A12). Moreover, the imposition of the suggested native μ-PIIIA (PDB database entry:1r9i) with μ-PIIIA-2 (backbone atoms 3-11) revealed a 0.67Å r.m.s.d. value (Appendix Figure A13). However, the deviations within the overall structures can occur through the different measurement conditions of NMR spectra (i.e. water and acetonitrile).

3.2.4 Conclusions and outlook for structure-activity studies of conopeptides

In this thesis several conopeptides were chemically and structurally characterised. First, structure and activity studies were performed for a new member of cardioactive peptides CCAP-vil, which possesses cardioinhibitory properties. This peptide shows a high sequence similarity to cardioacceleratory peptides such as CCAP or M-CCAP1. However, the biological effects are opposite, i.e. the structure of these peptides is essential for their function and thus, there are differences in bioactivity. Indeed, comparison of 3D solution structures of CCAP-vil and CCAP reveals no significant similarity for the backbone structure (Appendix Figure A 5). Since CCAP is a hormone, it was described to follow a membrane associated pathway and bind to a G-protein coupled receptor (GPCR).[143] However, CCAP-vil was suggested to bind to a target protein that causes systolic Ca^{2+} release via ryanodine receptor

(RyR).[139] However, very little information is available about the mechanism of these peptides with their targets. Thus, to determine the differences in the biological effect, binding studies to the suggested target proteins should be performed in future work.

Secondly, the 22-mer conotoxin μ-SIIIA from *Conus striatus* containing six cysteines, was systematically investigated using the whole-cell patch clamp method to determine the specificity vs. various Na_V channel subtypes on a molecular level. μ-SIIIA did block rat-$Na_V1.4$ channels in micromolar concentrations, yet, the block was not complete. Thus, in future investigations it would be interesting to elucidate the reason of this behavior, i.e. to reveal conformational changes of the Na_V channel upon toxin binding.

Thirdly, SAR-studies were performed for conotoxin μ-PIIIA from *Conus purpurascens*. Oxidative folding using conventional methods reproducibly revealed a mixture of stable isomers which occurred in different concentrations each in the reaction mixture. They were formed in a 1:2:2 ratios according to RP-HPLC data. Three major isomers were characterised in more detail. All three isomers were completely oxidised, i.e. disulfide-bridged. Surprisingly, biological activity studies using the $Na_V1.4$ channels revealed a reduced channel response for all three isomers in nanomolar range. However, the activity decreased in the following order: μ-PIIIA-1 > μ-PIIIA-2 > μ-PIIIA-3. Solution NMR spectroscopy analysis for μ-PIIIA1 and μ-PIIIA-2 resulted in well-defined structures with differences in disulfide bond connectivities. For μ-PIIIA-1, the following disulfide bonds Cys4-Cys21, Cys5-Cys22, Cys11-Cys16 were detected, while for μ-PIIIA-2 the disulfide bonds are Cys4-Cys16, Cys5-Cys21, Cys11-Cys22. However, μ-PIIIA-3 showed high structural flexibility in the 2D NMR experiments. Therefore, the 3D structure of μ-PIIIA-3 could not be determined, but suggested to form Cys4-Cys5, Cys11-Cys16, Cys21-Cys22 disulfie-bonds. However, for the first time structural isomers differing in disulfide bond connectivity for a peptide with three disulfide bonds were identified to possess biological activity. These results indicate that structural differences, in particular the disulfide bond connectivities, have an impact on the biological activity. Therefore, it seems to be possible to tune the affinity to their targets and to elucidate their working mechanism using the structures of such different isomers as templates. Future studies should be focused firstly on the structural characterisation of the toxin μ-PIIIA and secondly, on the subtype specificity of the biologically active isomers.

It was shown herein that NMR spectroscopy studies of cysteine-rich peptides are essential, since the three-dimensional structure of toxins provides information about the structural differences of peptides which are similar in primary amino acid sequence, but differ in biological activity and on the molecular level. Differences in regions of flexibility can be observed

adding another dimension of information. Moreover, NMR relaxation studies could highlight the regions of the structure undergoing conformational exchange that may have implications for binding to a biological target. The NMR methods applied to resolve the 3D structure of CCAP-vil, μ-SIIIA and μ-PIIIA structural isomers represent a basis for more complex structural studies of other cysteine-rich peptides, e.g. δ-EVIA and δ-SVIE.

4 Abbreviations

For long peptide sequences the one letter code is used with N-terminus on the left side of the sequence, and C-terminus of the right side. The abbreviation of amino acids is given according to the recommendation of the Nomenclature Committee of IUB (NC-IUB) and the IUPAC-IUB Joint Commission or Biochemical Nomenclature (JCBN).[157] All amino acids used in these studies were of L-configuration.

Abbreviation	*Name*
3D	three-dimensional
Cys or C	cysteine
DMSO	dimethyl sulfoxide
DQF-COSY	double quantum filtered correlated spectroscopy
FDA	food and drug administration
GSH	glutathione reduced
GSSG	glutathione oxidised
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
ICK	inhibitory cysteine knot
ICK	inhibitory cystine-knot
IL	ionic liquid
MS	mass spectrometry
NCL	native chemical ligation
NMDA	N-methyl D-aspartate
NMR	nuclear magnetic resonance
SPPS	solid phase peptide synthesis
NOE	nuclear overhauser enhancement
NOESY	nuclear overhauser and enhancement spectroscopy
OS	organic solvent
r.m.s.d.	root-mean-square-distance
ROE	rotating-frame overhauser enhancement
ROESY	rotating-frame nuclear overhauser enhancement spectroscopy

RP	reversed-phase
SAR	structure-activity relationship
SAR	structure-activity relationship
TCEP	tris(2-carboxyethyl) cysteine
DTT	dithiothreitol
TFA	trifluoro acetic acid
TLC	thin layer chromatography
TOCSY	total correlated spectroscopy
TTX	tetrodotoxin
VGSC	voltage-gated sodium channels

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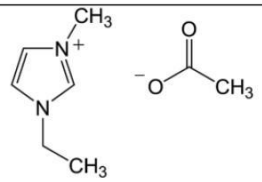
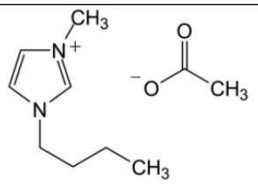
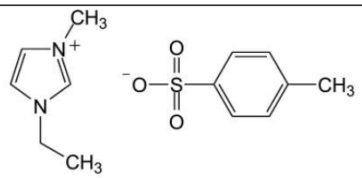
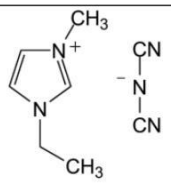
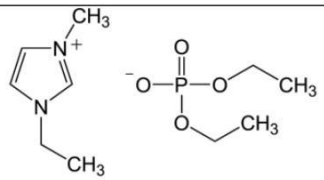
Jena, den 9. Dezember 2010

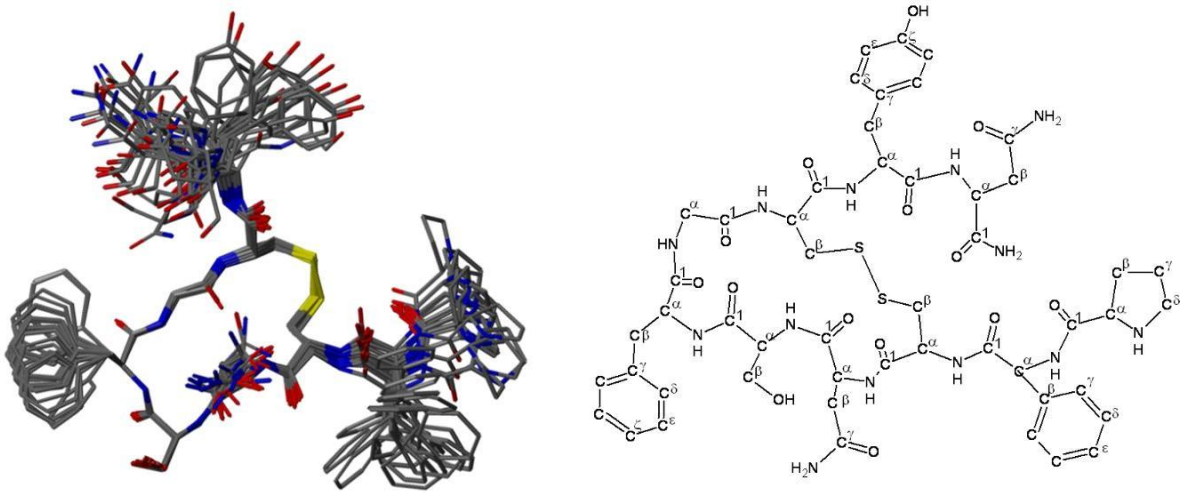
Alesia Miloslavina

6 Appendix

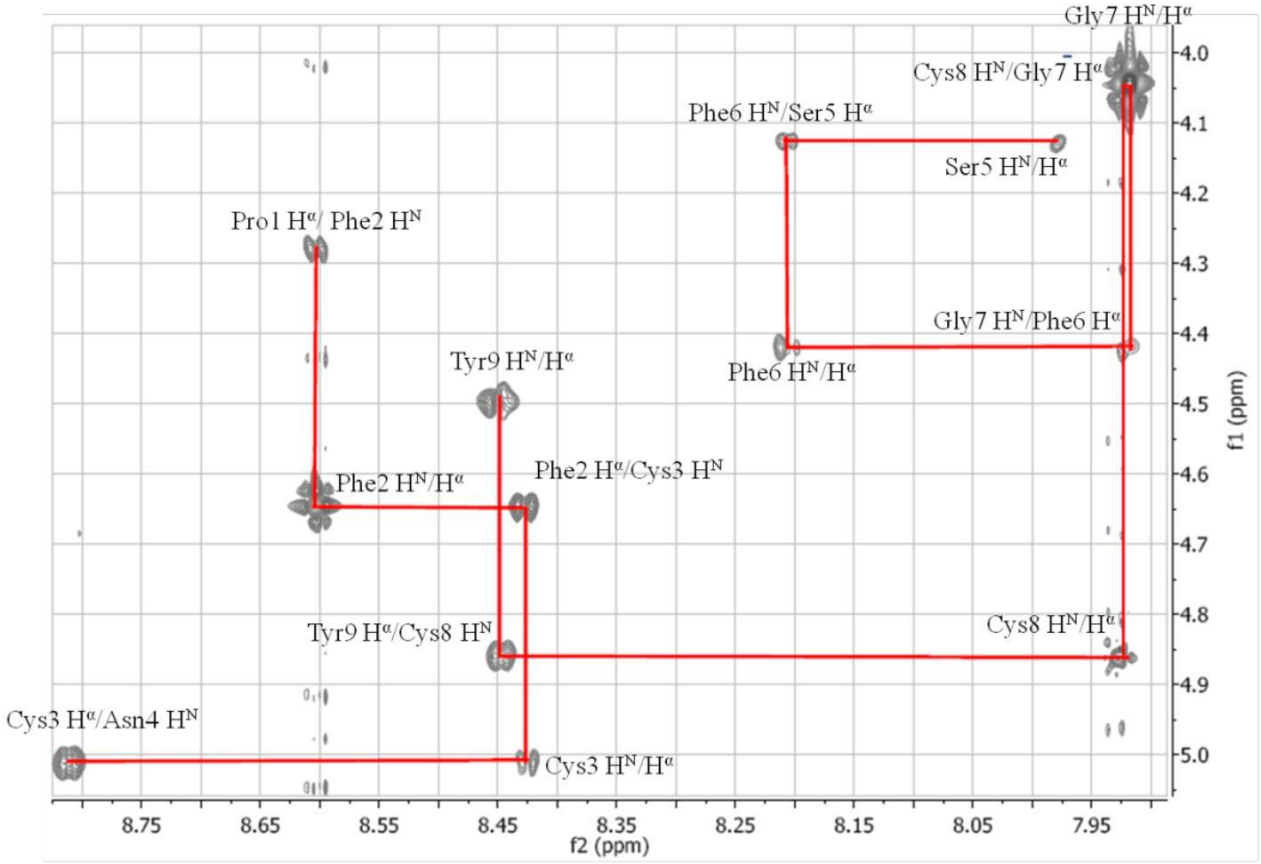
A 1. The name, structure and abbreviations of the ionic liquids used in the studies.

IL	Structure	Name
$[C_2mim][OAc]$		1-Ethyl-3-methylimidazolium acetate
$[C_4mim][OAc]$		1-Butyl-3-methylimidazolium acetate
$[C_2mim][OTs]$		1-Ethyl-3-methylimidazolium toluenesulfonate
$[C_2mim][N(CN)_2]$		1-Ethyl-3-methylimidazolium aminocyanide
$[C_2mim][DEP]$		1-Ethyl-3-methylimidazolium diethylphosphate

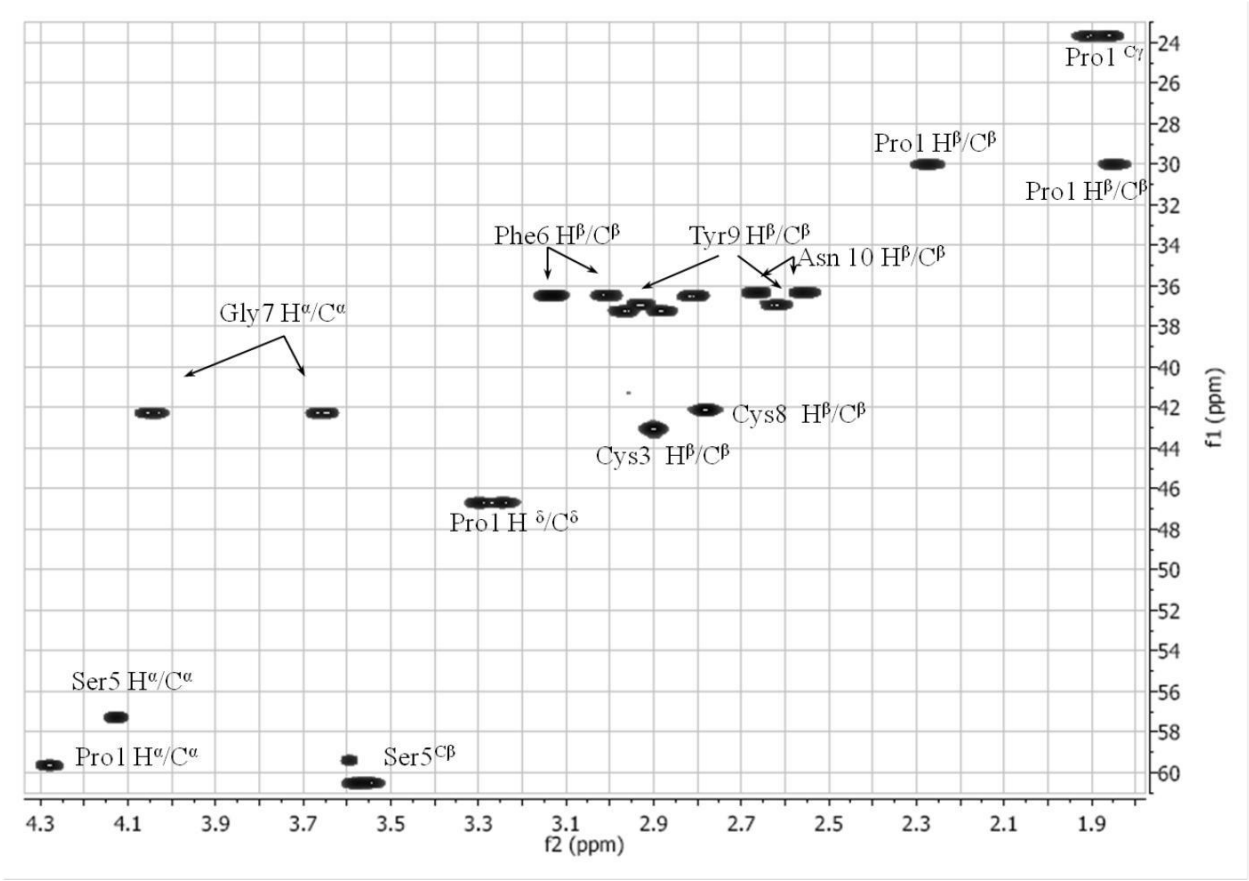
A 2. The solution NMR structure of the CCAP-vil in 90 % H_2O / 10 % D_2O at RT (left) and an atom indication used for cross peak assignment (right).



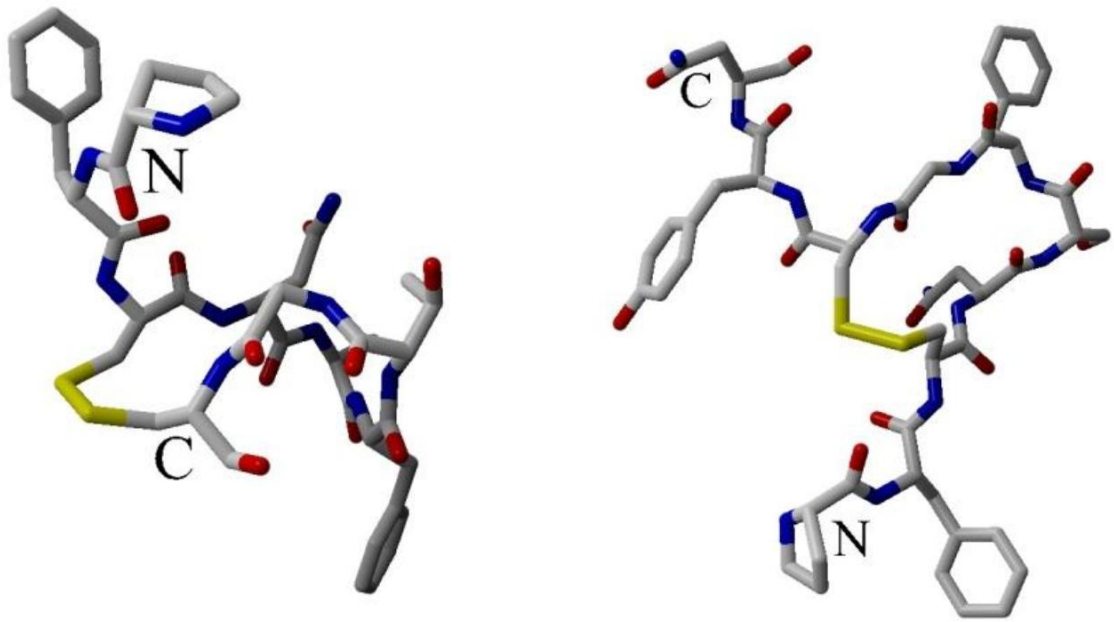
A 3. Cut-out of the 2D-NOESY spectrum with corresponding H^NH^α cross peaks of the CCAP-vil backbone assignment, recorded on a Bruker Advance III spectrometer with a proton frequency of 750 MHz in 90 % H_2O / 10 % D_2O at RT.



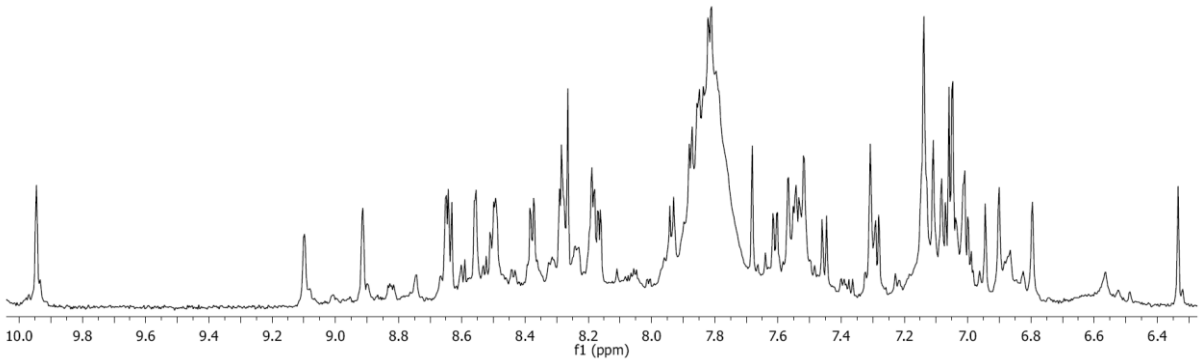
A 4. [^{1}H,^{13}C]-HSQC spectrum of the CCAP-vil with assigned cross peaks, recorded on a Bruker Advance III spectrometer with a proton frequency of 750 MHz in 90 % H_2O / 10 % D_2O at RT.



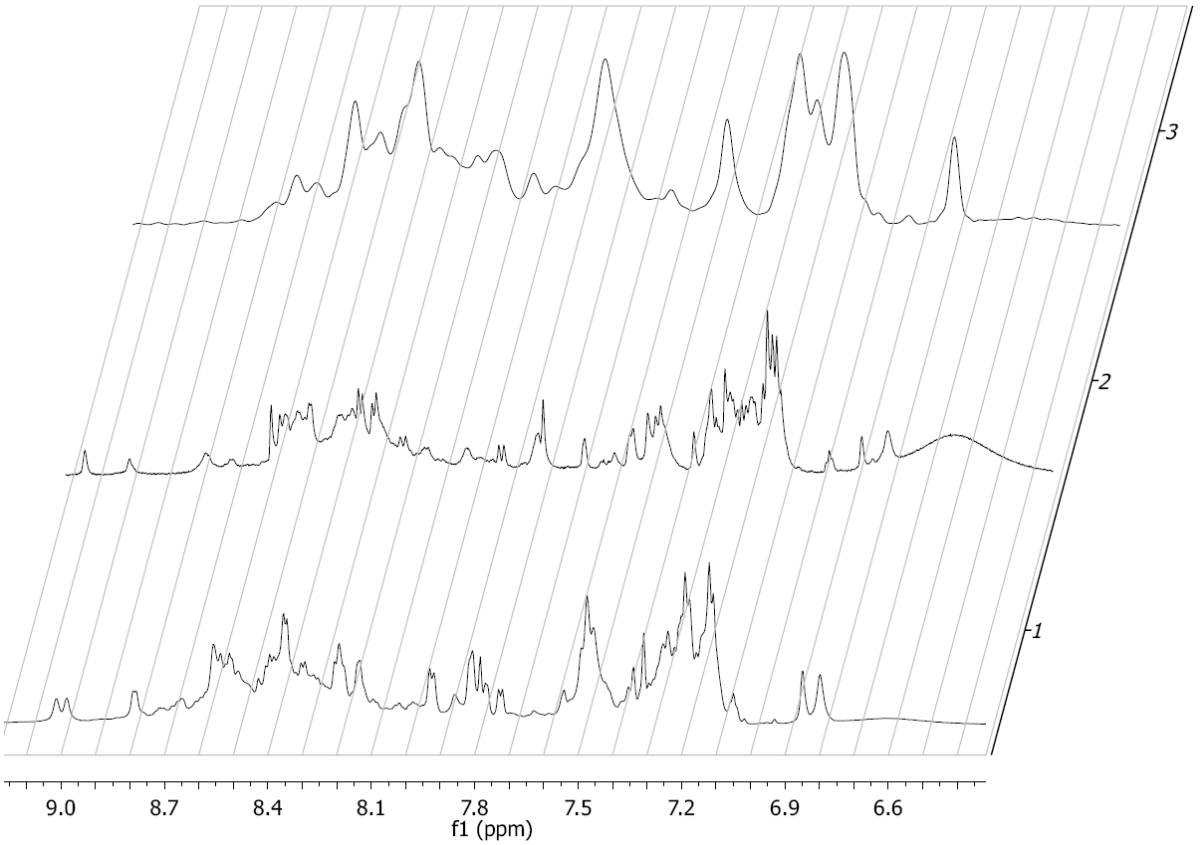
A 5. NMR structure comparison of the CCAP (1V46) and the CCAP-vil (BMRB code: 21022) proceeded with Yasara program.



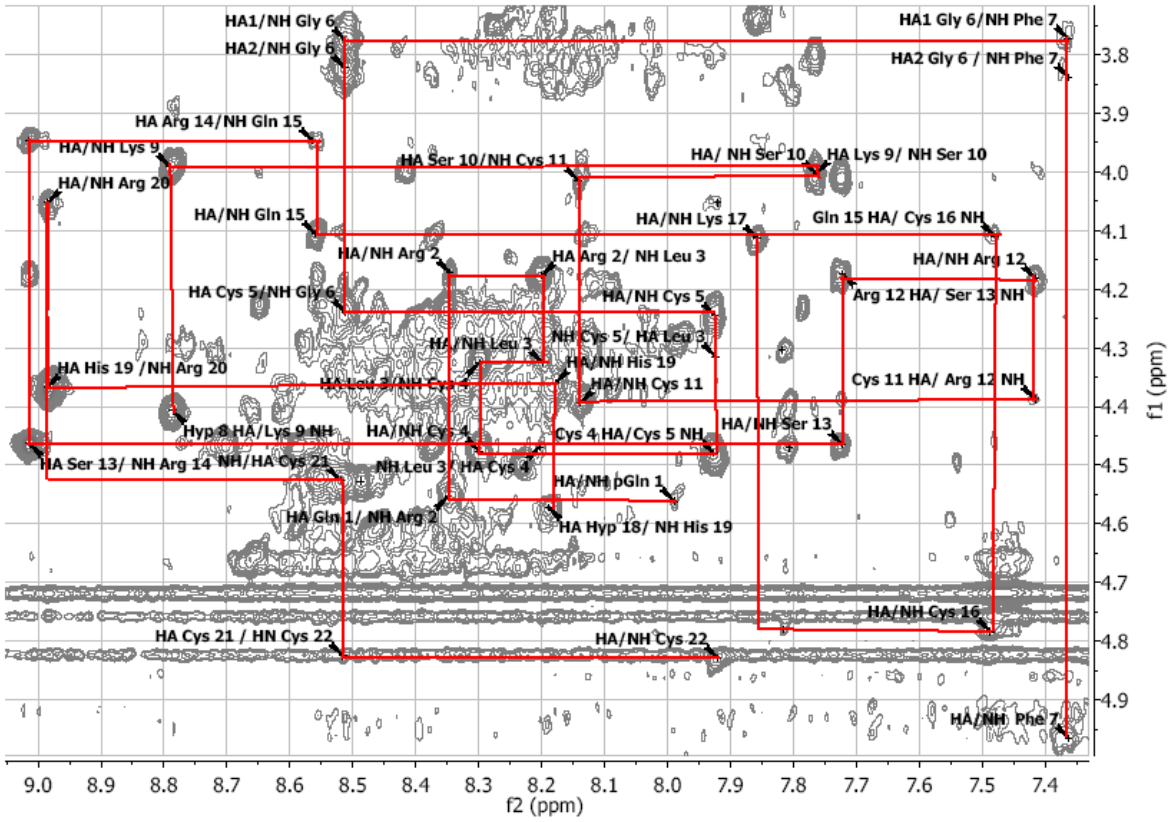
A 6. Expanded view of amide and aromatic region of ^{1}H spectrum of µ-SIIIA, recorded on a Bruker Advance III spectrometer with a proton frequency of 750 MHz.



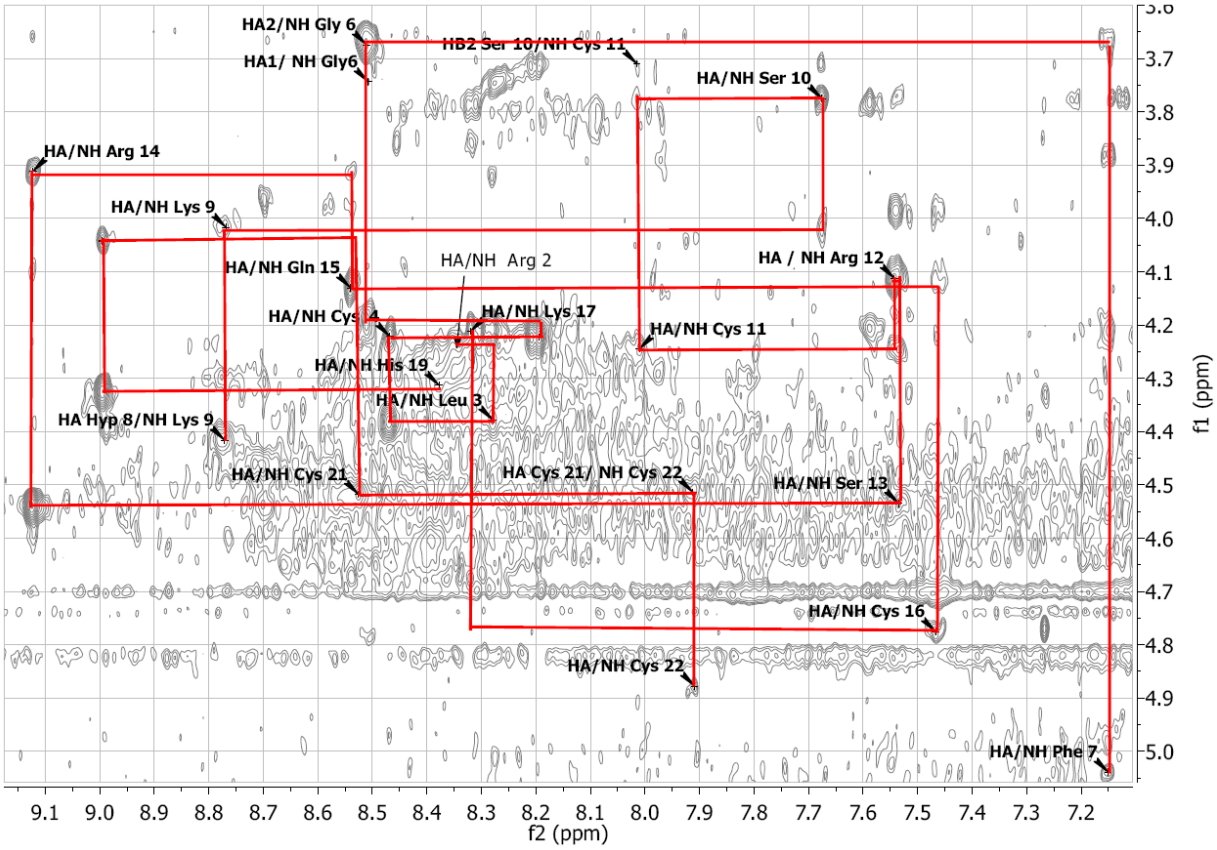
A 7. Stacked ^{1}H NMR spectra of amide and aromatic region of structural isomers of µ-PIIIA: 1 –isomer with cysteine connectivities Cys4-Cys21, Cys5-Cys22, Cys11-Cys16; 2- with cysteine connectivities Cys4-Cys16. Cys5-Cys21, Cys11-Cys22; 3- with suggested cysteine connectivities Cys4-Cys5, Cys11-Cys16, Cys21-Cys22 recorded on a Bruker Advance III spectrometer with a proton frequency of 600 MHz, in 90 % H_2O / 10 % D_2O at 293 K.



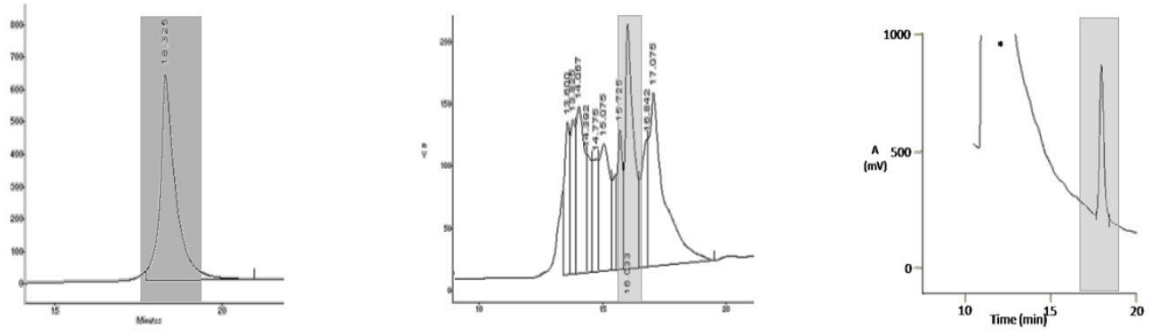
A 8. [^{1}H,^{1}H]-NOESY spectrum representing cross-peaks and "sequential walk" of the µ-PIIIA (native structure) backbone amino acids, recorded on a Bruker Advance III spectrometer with a proton frequency of 600 MHz in 90 % H_2O / 10 % D_2O at 293 K.



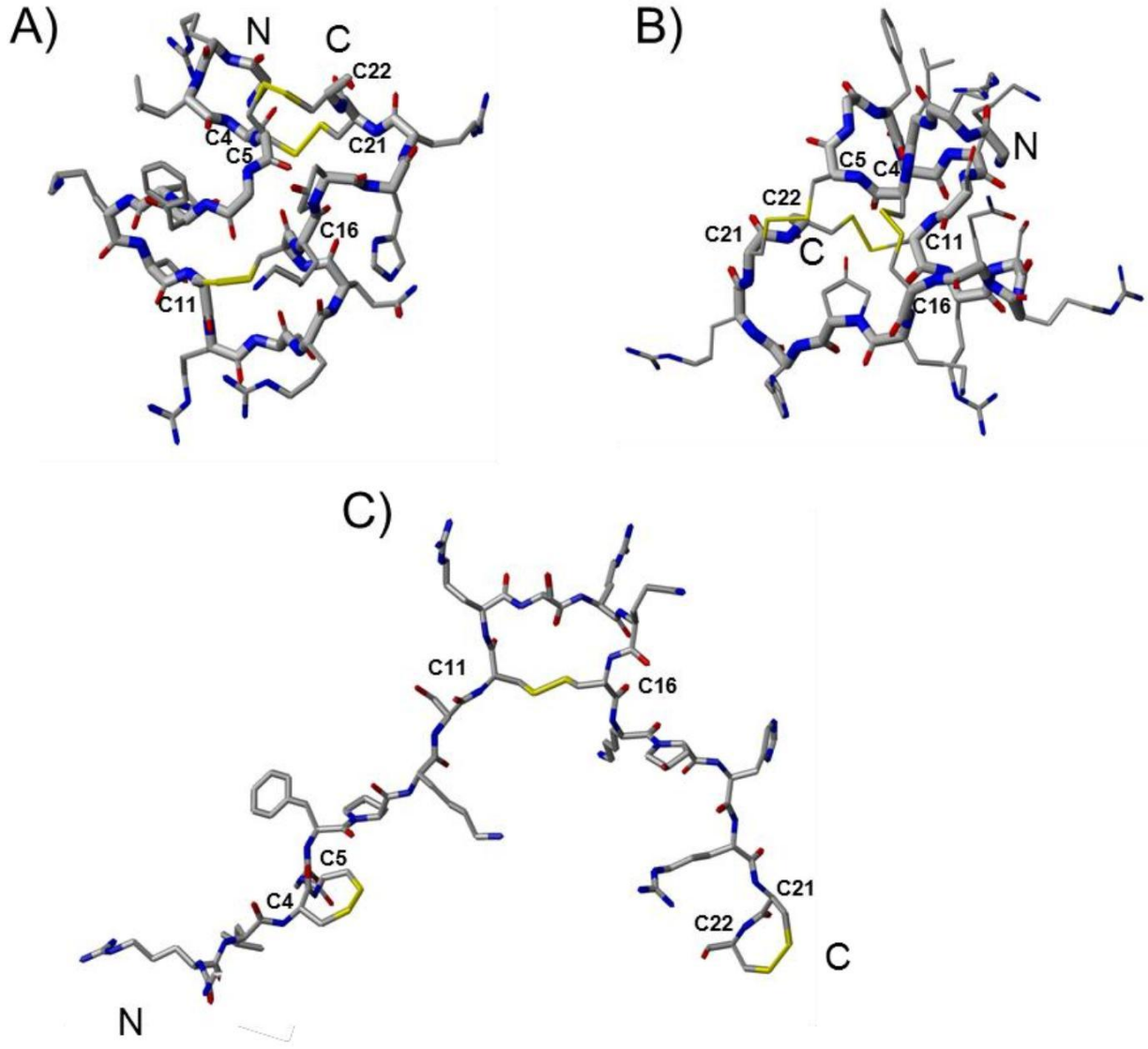
A 9. [^{1}H,^{1}H]-NOESY spectrum representing cross-peaks of the structural isomer of µ-PIIIA with cysteine connectivities …, recorded on a Bruker Advance III spectrometer with a proton frequency of 600MHz in 90 % H_2O / 10 % D_2O at 293 K.



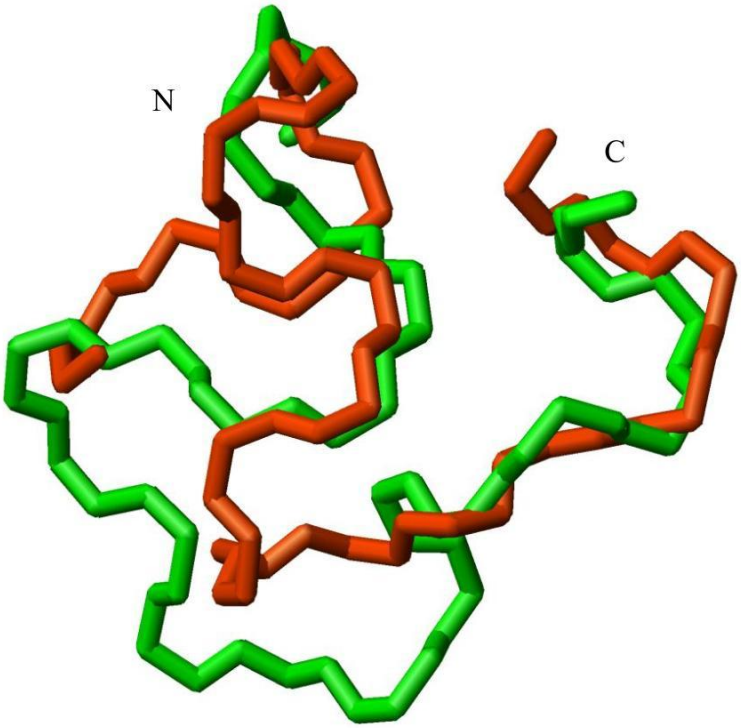
A 10. Elution profiles of neuropeptide conotoxin (μ-PIIIA): linear (left), oxidized by conventional method (middle) and in IL (right). The active conformation shaded. RP HPLC method: 10-40% of eluent B (acetonitrile/0.1% TFA), eluent A (water/0.1.% TFA). Modified from 130



A 11 Three dimensional NMR structures of μ-PIIIA1 (A), μ-PIIIA2 (B), and μ-PIIIA3 (C). The N-terminal pGlu residue side chain is not shown on structures, because calculations were performed with Glu residue. The figure was prepared using MOLMOL.



A 12 Superposition of three dimensional NMR backbone distribution for the µ-PIIIA1 (green) and the µ-PIIIA2 (red). Backbone atoms 16-22 r.m.s.d. 1.752 Å.



A 13 Superposition of three dimensional NMR backbone distribution for the µ-PIIIA2 (orange) and µ-PIIIA (1r9i PDB database). Backbone atoms 3-11 r.m.s.d. 0.67Å.