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A room temperature ionic liquid as convenient solvent for the oxidative folding of conopeptides

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We report the first example of conopeptide oxidation performed in a biocompatible ionic liquid, 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]), which enables the efficient formation of both hydrophilic and poorly water-soluble conotoxins compared with conventional methods. Moreover, the method features a high-concentration approach ultimately leading to higher yields at reduced separation effort.

Supporting information may be found in the online version of this article

Keywords: conotoxins; cysteine-rich peptides; oxidative folding; ionic liquids

During the last decade, the interest in environmentally friendly reaction processes, for example, the use of ecoefficient solvents for organic syntheses, and in particular for oxidation reactions with air, has increased immensely. Ionic liquids (ILs), which are widely used in this respect, exhibit unconventional solvent properties due to unique features such as non-flammability, non-combustibility, almost no vapour pressure, high thermal stability and high ionic conductivity [1–4]. These properties result from the composition of cations, such as imidazolium or pyridinium, and anions, for example, halogenides, tetrafluoroborate, or hexafluorophosphate. Usually, viscosity and polarity of ILs can be tuned by modifying their structure, for example, by changing the length of the side chain of the imidazole ring and the size of the cation [5].

Solvation of substances by ILs occurs by ions only. Currently, ionic liquid–solute interactions have been investigated in more detail. It was found that solvation is due to the formation of hydrogen bonds between the anions of the imidazolium salt and the hydroxyl groups of the solute. Nevertheless, the function of the cation is still not fully understood [1,6–8]. Besides, several studies have been driven to describe the interaction of ILs with water [9,10] or organic solvents [7] using simulation studies, NMR or fluorescence spectroscopy. In the first case, ILs are able to aggregate in aqueous solution forming micelles. This provides the opportunity to consider ILs as a new class of surfactants, which could be used in catalysis [11].

Distinct ILs exist in the liquid state at room temperature and they have thus gained attention for a variety of applications [2,4]. Beside their use in classical organic syntheses, biocompatible ILs have emerged as solvents for polar substances such as carbohydrates, oligonucleotides or amino acids, peptides, and proteins [12–14]. These approaches range from using ILs as solvents for enzymatic catalysis [5,15,16], for selective extraction of double-stranded DNA [17] or carbohydrates from natural sources [6], as matrices for the identification of proteins with MALDI MS [18], as reaction media for oxidative processes [19] to their application in peptide synthesis [20]. In the latter case, it was demonstrated that dipeptide formation can be performed efficiently in ILs, even in the case of sterically hindered, barely soluble amino acids. This finding focused our attention on the use of ILs as solvents for rather hydrophobic cysteine-rich peptides (reduced form) that have to be further processed to obtain the bioactive counterparts (oxidised form) which are called conotoxins.

Conotoxins are toxic peptides produced by marine cone snails for capturing prey, self-defence and competitor deterrence [21]. They are not only valuable research tools but also bear a therapeutic potential, for example, as analgesics or antinociceptive agents [22]. The repertoire of conotoxins is divided into so called superfamilies according to the number and pattern of intramolecular disulfide bridges. Superfamilies are further subdivided into target-specific toxin families. Three families of functionally different conotoxins target voltage-gated sodium channels (Na_V channels), all with three intramolecular disulfide bridges. μ-Conotoxins belong to the M-superfamily (CC-C-C-CC) and inhibit Na_V channels by blocking the channel pore [23–25]. The μO- and δ-conotoxins belong to the O-superfamily of conopeptides and harbour the cysteine pattern C-C-CC-C-C [25]. While μO-conotoxins reduce the activity of Na_V channels by preventing their voltage-dependent activation [26,27], δ-conotoxins gain Na_V channel activity by slowing down channel

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inactivation [28]. Due to their pharmacological properties, for example, target specificity, these peptides represent ideal tools to study the structure and functions of ion channel proteins.

In recent years, tremendous efforts have been undertaken with respect to general applicable chemical syntheses and oxidative folding procedures for the formation of native disulfide crosslinks in conopeptides. The synthesis of such multiple disulfide-bridged peptides can be achieved either by direct oxidative folding or by orthogonal protection strategies [29]. However, in our experience, due to aspects such as the number of reaction steps required, the overall yield of reaction product and formation of by-products (e.g. misfolded peptides), the direct oxidative folding of the non-protected linear precursor is preferred for conopeptides. The key to successful synthesis and isolation certainly is the efficient and correct folding of the reduced peptide [30–34].

In order to validate whether oxidation of conopeptide precursors in ionic liquids can be developed to a more general application, conotoxins mainly differing in their chemical properties, in particular hydrophobicity, were selected for this study. The linear precursors of 20-mer μ-SIIIA, 22-mer μ-PIIIA, 32-mer δ-EVIA and 31-mer δ-SVIE have been prepared by SPPS and characterised appropriately (Table 1). The peptides were synthesised according to a standard Fmoc-protocol using TentaGel R RAM resin with a low loading capacity (0.19 mmol/g). Due to a high potential of aggregation and incomplete coupling reactions during chain prolongation of hydrophobic δ-EVIA and δ-SVIE, tetramethylfluoroformamidinium hexafluorophosphate (TFFH) has been used as coupling reagent, while the assembly of hydrophilic peptide sequences μ-SIIIA and μ-PIIIA was performed with the commonly used reagents HBTU and HOBt. All crude linear precursors were purified by semi-preparative reversed-phase HPLC prior to the investigation of oxidative folding. The quality of the peptides used for these studies is exemplified in Figure 1(A).

The purified reduced peptides were subjected to direct oxidative folding conditions as described previously by several groups [31–33]. Intensive studies with δ-EVIA (data not shown) using a variety of known protocols and investigating the influence of different parameters, for example, the addition of organic solvents and detergents, led us to focus on two different procedures only. The reason for that can primarily be attributed to aspects such as the yield of oxidised product, the formation of side products and the resolution between compound peaks for HPLC separations. In a first attempt, a method reported by DeLaCruz *et al*. [31] using glutathion (reduced/oxidised form) was employed for the oxidation reaction (method I, see Supporting Information). Compared with this and other methods, the second approach (method II, see Supporting Information) employing methanol (30–40%) as additive and a mixture of GSH and cystamine as redox agents was slightly more effective in our hands. In general, however, our results indicate that for the four peptides direct oxidative folding was not significantly changed under the two different buffer conditions used. The formation of the oxidation products exemplified for conopeptides μ-SIIIA and δ-EVIA is depicted in Figure 1(B). Although the desired oxidised peptides were formed, a variety of unwanted side products occurred to a rather high extent. Contrary to hydrophilic μ-SIIIA or μ-PIIIA, a major drawback of oxidising peptides such as δ-EVIA and δ-SVIE is their low solubility in aqueous solutions. Thus, beside the fact that high dilution of such peptides prevents intermolecular oligomerisation in this medium, their concentration is already rather low compared with other representatives.

In analogy to the knowledge obtained about the solubility of other biopolymers, such as cellulose or silk in ionic liquids [35,36], a set of ILs with rather nucleophilic anions capable of breaking the strong network of hydrogen bonds were selected and tested for the ability to dissolve hydrophobic peptides and subsequent oxidative folding without the need for redox-active agents. As opposed to the dissolution of cellulose, which allows for a processing temperature of 100 °C without chain degradation, the choice of ILs for the problem at hand is further limited to ILs with a melting point $<$20 °C and low viscosity, due to the thermal lability of peptides in general. Hence, although 1-ethyl-3-methylimidazolium tosylate ([C_2mim][TOS]), is capable of dissolving hydrophobic δ-EVIA, the viscosity at room temperature proved to be too high for efficient stirring. Likewise, the very efficient solvent for cellulose, 1-butyl-3-methylimidazolium chloride, is a solid at room temperature. One of the few alternatives with non-toxic anions is [C_2mim][OAc], which indeed proved to be the IL of choice for the dissolution and conversion of the linear peptides described herein (method III, see Supporting Information). In a general experiment, 4 mg of linear peptide (10–15 mM depending on the peptide sequence) was dissolved in 100 µl of [C_2mim][OAc] (60 equiv.) and gently stirred at room temperature. After 30 min, 20 µl of 0.1% TFA/water and 60 µl of 0.1% TFA/acetonitrile were added, and the crude reaction mixture was purified by RP-HPLC.

Figures 2 and 3 illustrate the efficiency of the IL for oxidative folding of several conotoxins, highlighting three advantages of this protocol. First, [C_2mim][OAc] is able to dissolve a significant amount (final concentrations 10–15 mM) of either peptide, in particular of the rather hydrophobic representatives δ-EVIA and δ-SVIE. Second, it is obvious that the pre-formed secondary structure of the peptides is stabilised. Hence, the formation of the correctly folded product is favoured and the amount of misfolded peptides reduced, as opposed to oxidative folding

Table 1. Analytical characterization of the reduced and oxidised conopeptides described

Peptide	Sequence	t_R(red) (min)	t_R(ox) (min)	MW(red) $(g/mol)_{theor}$	MW(red)[a] $(g/mol)_{found}$	MW(ox) $(g/mol)_{theor}$	MW(ox)[a] $(g/mol)_{found}$	Yield[e] (%) (A, B)	Yield[e] (%) (C)
μ-SIIIA	ZN**C**C̲NGGCSSKW**C**RDHARC̲C-NH_2	25.9[b]	17.3[b]	2214.5	2215.9	2208.5	2209.1	10–15	25
μ-PIIIA	ZRL**C**C̲GFOKSCRSRQ**C**KOHRC̲C-NH_2	23.6[b]	17.8[b]	2608.1	2608.7	2602.1	2603.5	10–15	25
δ-EVIA	DD**C**IKOYGFC̲SLPILKNGL**C**CSGAC̲VGVCADL-NH_2	15.7[c]	18.4[c]	3296.0	3296.8	3290.0	3291.2	8–10	20
δ-SVIE	DG**C**SSGGTFC̲GIHOGL**C**CSEFC̲FLWCITFID-NH_2	19.0[c]	23.8[c]	3315.8	3354.8[d]	3309.8	3311.1	n.d.	n.d.

[a] $[M + H]^+$.
[b] 0–40% eluent B in 40 min.
[c] 25–27% eluent B in 30 min, eluent B: 0.1% TFA in acetonitrile.
[d] $[M + K]^+$; disulfide bridging patterns of the respective cysteine residues are highlighted (bold, underlined, italics).
[e] Oxidation yields were obtained from HPLC analyses, taking into account the results of amino acid analyses. n.d., not determined.

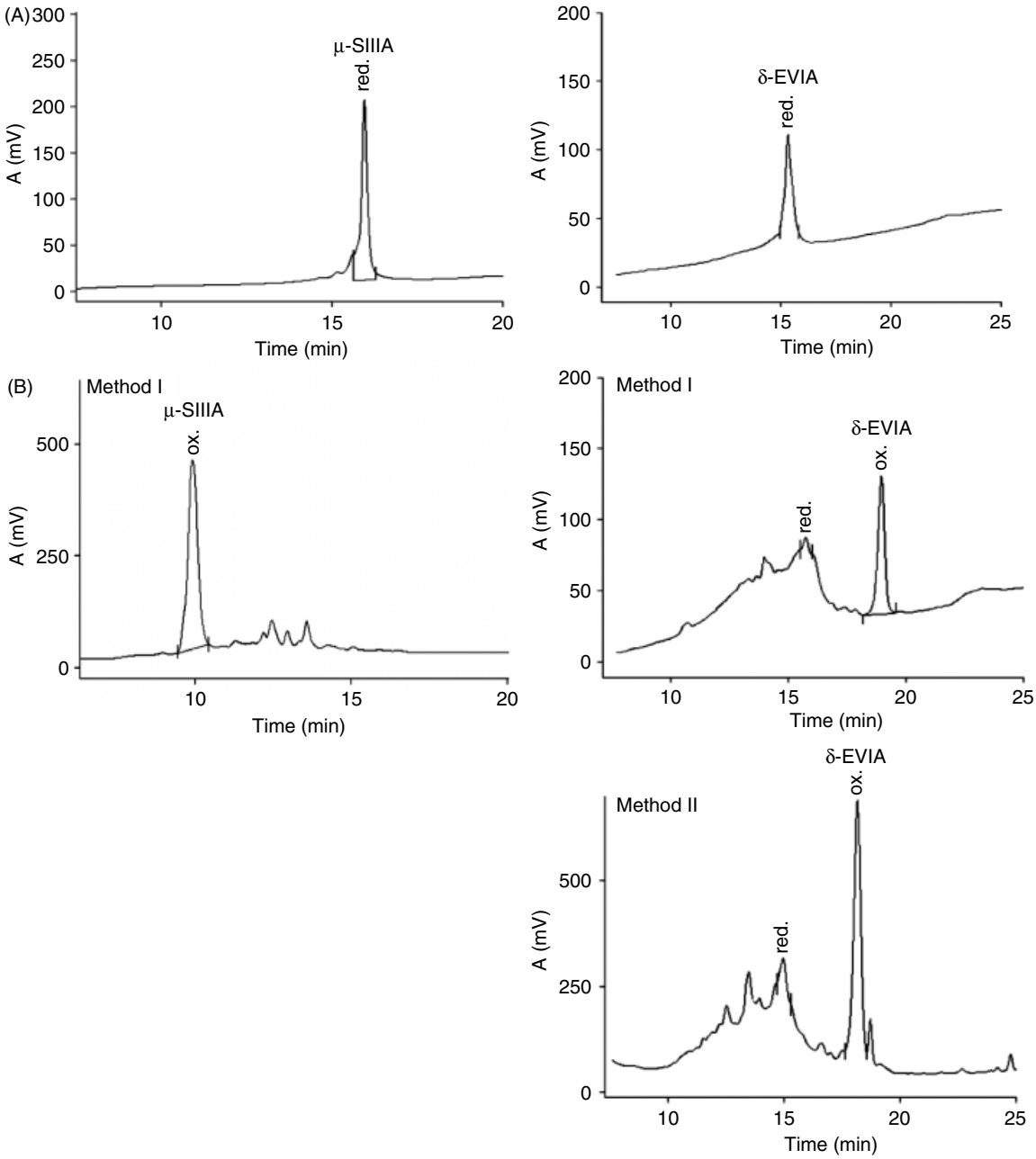

Figure 1. RP-HPLC chromatograms at 220 nm for (A) purified, linear precursor peptides: μ-SIIIA (left) and δ-EVIA (right), and for (B) crude oxidised peptides (methods I and II, see Supporting Information): μ-SIIIA (left) and δ-EVIA (right and bottom).

in conventional redox buffer systems. These properties, that is, 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 [13]. However, to the best of our knowledge, the oxidation of peptides in ILs has not been described yet. Third, the use of the IL [C_2mim][OAc] gives the opportunity to perform the oxidative folding in small reaction volumes simplifying the purification of the oxidation product.

As example we illustrate in Figure 4 the biological activity of the μ-conotoxin SIIIA. Voltage-gated sodium channels of rat skeletal muscle were expressed in a mammalian cell line and resulting currents were measured as described previously [26]. μ-SIIIA at a concentration of 10 μM blocked these ion channels by 96.9 ± 2.4% with a mono-exponential onset characterized by a time constant of 120 ± 14 s ($n = 8$).

In summary, a novel method for the solubilisation of hydrophobic peptides and for oxidation of cysteine-rich peptides with air was presented which allows an increase of three orders of magnitude in concentration compared with conventionally used buffer solutions. Furthermore, the addition of redox-active agents to bring about the oxidative folding was found to be superfluous. The reaction time was reduced and the desired oxidised peptide was formed selectively. This report demonstrates the impact of ILs for the oxidative folding of peptides with air. The nature of the anion seems to be the most important factor with respect to solubilisation of conopeptides. [C_2mim][OAc] is compatible with

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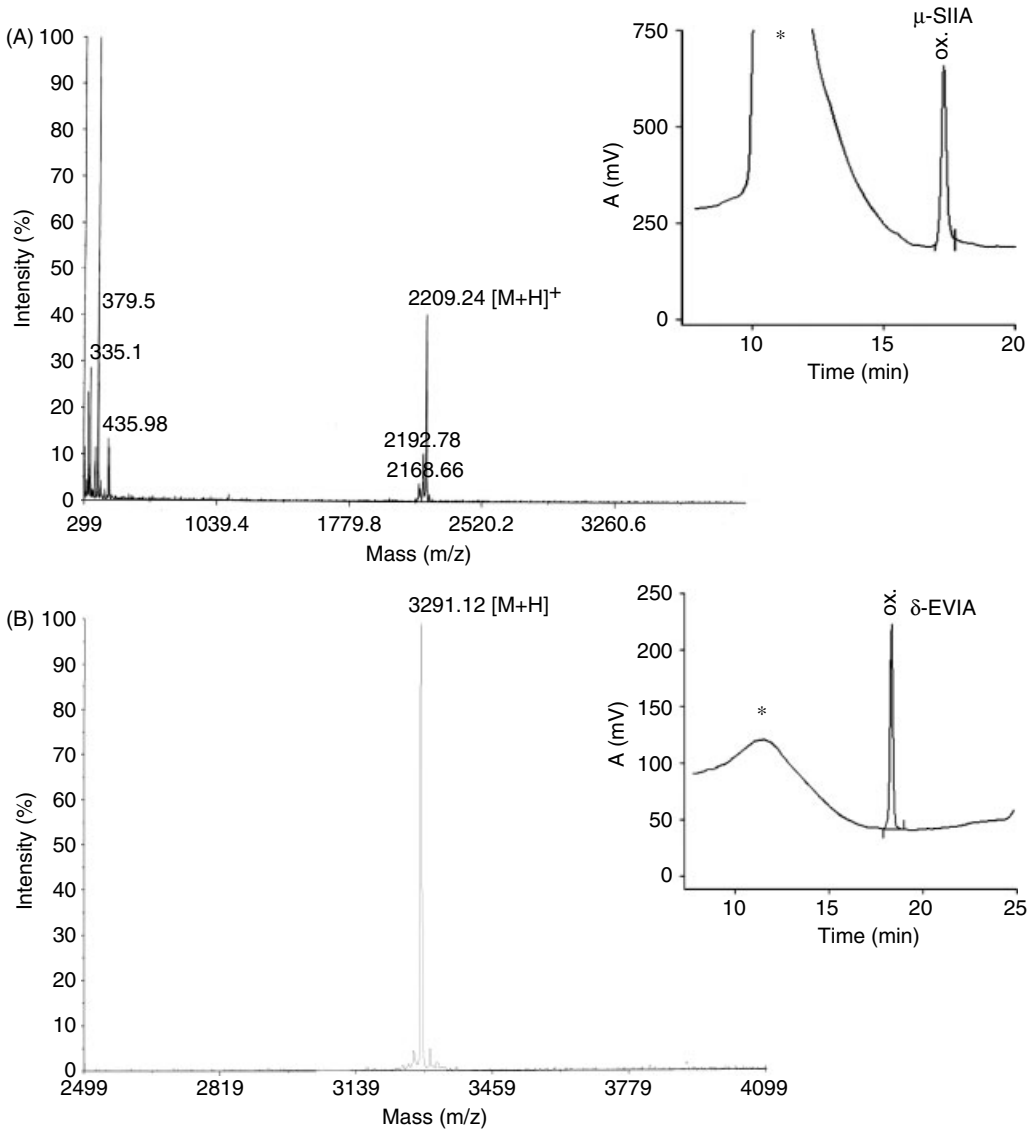

Figure 2. MALDI-TOF mass spectra and RP-HPLC chromatograms at 220 nm for crude oxidised peptides formed in [C_2mim][OAc] (method III, see Supporting Information): (A) μ-SIIIA and (B) δ-EVIA. *Peak from the ionic liquid [C_2mim][OAc].

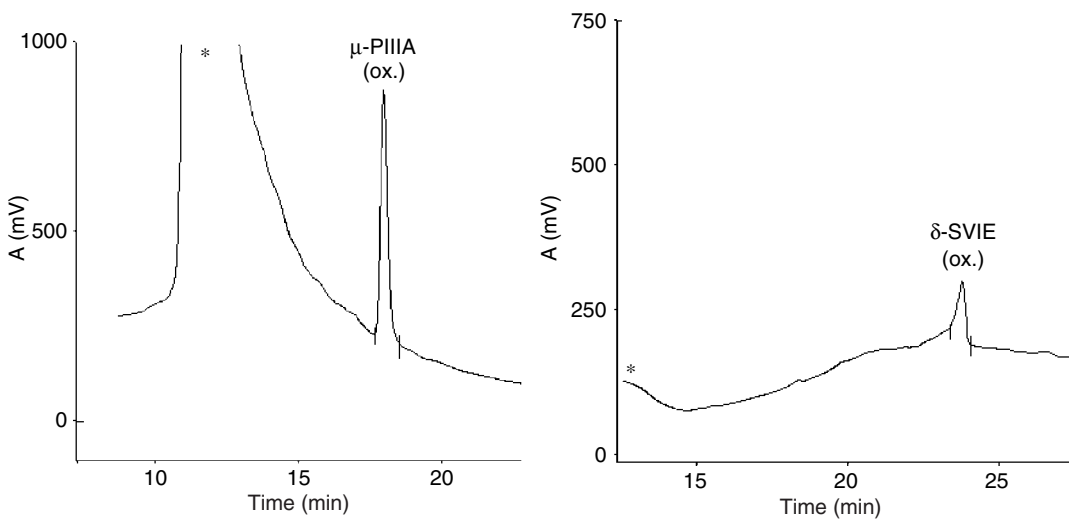

Figure 3. RP-HPLC elution profiles of crude reaction mixtures of conopeptides μ-PIIIA and δ-SVIE obtained after 30-min incubation time in [C_2mim][OAc] (method III, see Supporting Information). *Peak from the ionic liquid [C_2mim][OAc].

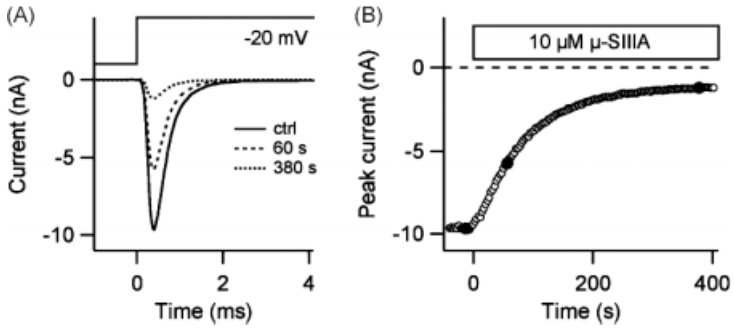

Figure 4. Block of rat skeletal muscle sodium channel $Na_V1.4$ by μ-SIIIA (oxidised according to method III, HPLC-purified). (A) Sodium channel $Na_V1.4$ was expressed in HEK 293 cells and currents were recorded in the whole cell configuration of the patch–clamp method. Repetitive depolarisations from −120 to −20 mV were applied to elicit sodium current. Sample current traces before (ctrl) and 60 and 380 s after extracellular application of 10 µM μ-SIIIA are shown. (B) Time course of peak current reduction obtained by 10 µM μ-SIIIA. Filled symbols indicate data from traces shown in (A).

different peptide structures and concentrations as shown for several conotoxins in this study. It should therefore be applicable for other peptides and reactions with peptides, too. Optimisation may depend on the nature of the amino acid sequence and length as well as the number of cysteine residues.

Further studies on the impact of the IL structure, the effect of water concentration, and a detailed structural and electrophysiological investigation of the conotoxins described herein are the focus of our current work, and will be presented elsewhere. In addition, future work will probe the role of the cation in assisting solubilisation and oxidative folding of cysteine-rich peptides.

Supporting information

Supporting information may be found in the online version of this article.

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