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## Discovery of a new subclass of $\alpha$ -conotoxins in the venom of *Conus australis*



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### ABSTRACT

Cone snails (*Conus* sp.) are poisonous animals that can be found in all oceans where they developed a venomous strategy to prey or to defend. The venom of these species contains an undeniable source of unique and potent pharmacologically active compounds. Their peptide compounds, called conotoxins, are not only interesting for the development of new pharmaceutical ligands, but they are also useful for studying their broad spectrum of targets. One conotoxin family in particular, the  $\alpha$ -conotoxins, acts on nicotinic acetylcholine receptors (nAChRs) which dysfunctions play important roles in pathologies such as epilepsy, myasthenic syndromes, schizophrenia, Parkinson's disease and Alzheimer's disease.

Here we define a new subclass of the  $\alpha$ -conotoxin family. We purified the venom of a yet unexplored cone snail species, i.e. *Conus australis*, and we isolated a 16-amino acid peptide named  $\alpha$ -conotoxin AusIA. The peptide has the typical  $\alpha$ -conotoxin CC-X<sub>m</sub>-C-X<sub>n</sub>-C framework, but both loops (*m/n*) contain 5 amino acids, which has never been described before. Using conventional electrophysiology we investigated the response of synthetically made globular (I–III, II–IV) and ribbon (I–IV, II–III) AusIA to different nicotinic acetylcholine receptors. The  $\alpha_7$  nAChR was the only receptor found to be blocked with a similar potency by both peptide-configurations. This suggests that both  $\alpha_5/5$  conotoxin isomers might be present in the venom gland of *C. australis*. NMR spectroscopy showed that no secondary structures define the peptides' three-dimensional topology. Moreover, the ribbon configuration, which is generally considered to be non-native, is more stable than the globular isomer. Accordingly, our findings show relevancy concerning the  $\alpha$ -conotoxin classification which might be helpful in the design of novel therapeutic compounds.

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## 1. Introduction

Venoms from cone snails (genus *Conus*) offer a unique and extensive source of chemical diversity as they are driven by evolutionary pressure to improve prey capture and/or protection of the species. These snails are

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considered as specialized predators that have developed a sophisticated peptide chemistry and neuropharmacology by producing a structural and functional variety of neurotoxins. Therefore, the *Conus* venom gland is recognized as an emerging source of peptide-based therapeutics (Lewis et al., 2012). Their ability to use a diverse array of small disulfide-bridged peptides, called conopeptides or conotoxins, makes them very unique (Fry et al., 2009). Up to date, only 0.1% out of potentially 500,000 venom components has been functionally and structurally investigated. The largest group of characterized *Conus* sp. peptides is the family of  $\alpha$ -conotoxins (belonging to the A-superfamily), that are selective antagonists of the muscle and neuronal subtype nicotinic acetylcholine receptors (nAChRs) (McIntosh et al., 1999). They act at the nAChR acetylcholine binding site as competitive antagonists and are among the smallest of the conopeptides (12–20 amino acid residues) (McIntosh et al., 1999; Terlau and Olivera, 2004). Alpha-conotoxins have a characteristic CC-X<sub>m</sub>-C-X<sub>n</sub>-C framework and these four cysteines can yield three possible disulfide connectivities: globular (I–III, II–IV), ribbon (I–IV, II–III) and beads (I–II, III–IV). However, naturally appearing  $\alpha$ -conotoxins typically exhibit the globular conformation (Janes, 2005). The number of residues included within the two loops (*m,n*) of  $\alpha$ -conotoxins is the basis for the division into several structural subgroups (*m/n*: 3/5, 3/6, 4/2, 4/3, 4/4, 4/5, 4/6, 4/7, 4/8, 5/2 and 5/8) (Akondi et al., 2014). The most commonly reported framework is the 4/7 subgroup (Terlau and Olivera, 2004).

Nicotinic acetylcholine receptors, being a member of ligand-gated cationic channels, mediate fast synaptic transmission. They are broadly distributed throughout the peripheral and central nervous systems of both primitive and evolutionarily advanced organisms (Gotti and Clementi, 2004). Consequently, nAChR dysfunctions are involved in a number of central nervous system (CNS) disorders such as epilepsy, Alzheimer's disease, Parkinson's disease, schizophrenia, nicotine addiction, pain, cancer etc. (Albuquerque et al., 2009; Hurst et al., 2013; Improgo et al., 2010; Tapper et al., 2004).

In mammals there are 16 different nAChR subunits: 9  $\alpha$ -subunits ( $\alpha_{1-7}$ ,  $\alpha_9$  and  $\alpha_{10}$ ), 4  $\beta$ -subunits ( $\beta_{1-4}$ ), as well as  $\gamma$ ,  $\delta$  and  $\epsilon$  subunits. Five of these subunits combine to form muscle nAChR subtypes ( $\alpha_1\beta_1\gamma\delta$  and  $\alpha_1\beta_1\delta\epsilon$ ) which are found exclusively at neuromuscular junctions, whereas the other subunits ( $\alpha_2$ – $\alpha_{10}$ ,  $\beta_2$ – $\beta_4$ ) assemble in numerous homomeric (exclusively  $\alpha$ -subunits) or heteromeric ( $\alpha$ - and  $\beta$ -subunits) neuronal nAChR subtypes (Albuquerque et al., 2009). The assembly of different pentamers forms a complex variety of nAChR subtypes with different pharmacological and biophysical properties (Grishin et al., 2010; Hurst et al., 2013; Indurthi et al., 2014).

Here we report the isolation of a novel 16-amino acid  $\alpha$ -conotoxin from the venom of the vermivorous marine snail *Conus australis* and its electrophysiological screening against six different nAChRs. To the best of our knowledge this is the first conotoxin to be characterized from this species found in the Indian Ocean near Tamil Nadu, India. The peptide, called AusIA belongs to a new subclass of the  $\alpha$ -conotoxin family (i.e.  $\alpha_5/5$ ) which has never been described before. We investigated the effect of globular

(I–III, II–IV) and ribbon (I–IV, II–III) AusIA on different nAChRs from which  $\alpha_7$  nAChR was the only receptor found to be blocked with a similar potency by both peptide-configurations. NMR spectroscopy shows that no secondary structures define the peptides' three-dimensional topology. Moreover, the ribbon configuration, which is considered to be non-native, is more stable than the globular isomer.

## 2. Materials and methods

### 2.1. Cone snail specimens and venom extraction

Specimens of *C. australis* (identified by Holten in 1802 and classified by Tucker and Tenorio (Tucker and Tenorio, 2009)) were collected from the Indian Ocean near Tamil Nadu, India. The venomous apparatuses (venom bulbs and venom ducts) were extracted from the specimens as previously described (Olivera et al., 1985). The collected tissue was preserved in RNAlater solution (Ambion) and stored at  $-20$  °C. The venomous apparatuses were used for peptide/protein extraction with 50% acetonitrile (ACN)/water.

### 2.2. Peptide fractionation and purification

Sample fractionation occurred by reversed-phase high performance liquid chromatography (RP-HPLC, Gilson, Middleton, WI, USA). Two steps were followed for the separation of the venom compounds. In the first step, the lyophilized crude venom powder was solubilized into 50% acetonitrile (ACN)/water and aliquots were loaded on a Gel filtration Superdex™ Peptide 10/300 GL column with 50% ACN/water as mobile phase (flow rate 0.5 ml/min) to separate the peptides and proteins based on their size. Three sample collections were made that were stored overnight at  $-80$  °C, freeze-dried and finally solubilized in 0.1% trifluoroacetic acid (TFA)/H<sub>2</sub>O. For the second step, an analytical Vydac C<sub>18</sub> column (218MS54, 4.6 × 250 mm, 5- $\mu$ m particle size; Grace, Deerfield, IL) with a two solvent system was used: (A) 0.1% trifluoroacetic acid (TFA)/H<sub>2</sub>O and (B) 0.085% TFA/ACN. The sample was eluted at a constant flow rate of 1 ml/min with a 0–80% gradient of solvent B over 90 min (1% ACN per minute after 10 min of solvent A). The HPLC column elutes were monitored by a UV/VIS-155 detector (Gilson) scanning both 214 nm and 280 nm.

### 2.3. Peptide sequencing

Isolated AusIA was collected and freeze-dried for direct peptide sequencing and molecular mass analysis (MALDI-TOF). A Protein Sequencer PPSQ-31A/33A (Shimadzu, Japan) was used to determine the amino acid sequence of the separated compound. In this Edman degradation method, the sample was loaded onto a polybrene-pretreated, precycled glass fibre disk, and was Edman sequenced for 24 residue cycles.

### 2.4. Peptide synthesis and folding

AusIA(g) (globular) and AusIA(r) (ribbon) were synthesized by GenicBio Limited (Shanghai, China). Formation of

the two disulfide bridges was carried out by adopting the selective protection and deprotection strategy *in vitro*. The resulting bicyclic peptides were subsequently purified by HPLC and analysed with LC-MS (Waters ZQ2000, USA), then freeze-dried and stored at  $-20\text{ }^{\circ}\text{C}$  until use. Prior to functional characterization, the purity of the synthetic peptides was checked and a chromatographical characterization was undertaken by RP-HPLC. On the basis hereof, a careful comparison of the retention time with the one of the native material was done. The material needed for NMR spectroscopy was purified using RP-HPLC.

### 2.5. Functional characterization

Complementary DNA encoding the nAChR-channels was subcloned into the corresponding vector: human  $\alpha_3$ /pcDNA3(XbaI), human  $\alpha_4$ /pGEM-HE(NheI), chick  $\alpha_7$ /pBlueScript(NotI), human  $\beta_2$ /pSP64(PvuII), human  $\beta_4$ /pcDNA3(XbaI), rat  $\alpha_1$ /pSP0oD(Sall), rat  $\beta_1$ /pSP0oD(Sall), rat  $\gamma$ /pSP0oD(Sall), rat  $\delta$ /pSP0oD(Sall), rat  $\epsilon$ /pSP0oD(Sall). The linearized plasmids - respective restriction enzymes are indicated in parentheses - were transcribed using the T7 ( $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_7$ ,  $\beta_4$ ) or the SP6 ( $\beta_2$ ,  $\alpha_1$ ,  $\beta_1$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ) mMACHINE-mMACHINE transcription kit (Ambion, Austin, TX).

The harvesting of stage V-VI oocytes from anaesthetized female *Xenopus laevis* frogs was previously described (Van Der Haegen et al., 2011). Oocytes were injected with 50–70 nl of cRNA at a concentration of 1–3 ng/nl using a micro-injector (Drummond Scientific, Broomall, PA). The oocytes were incubated in a ND96 solution containing: 96 mM NaCl, 2 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 2 mM  $\text{MgCl}_2$  and 5 mM HEPES (pH 7.4), supplemented with 1.25 ml/l gentamicin and 90 mg/l theophylline. Oocytes were stored for 1–5 days at  $16\text{ }^{\circ}\text{C}$  until sufficient expression of nAChRs was achieved.

Whole-cell currents from oocytes were recorded at room temperature ( $18\text{--}22\text{ }^{\circ}\text{C}$ ) by the two-electrode voltage clamp technique using a GeneClamp 500 amplifier (Molecular Devices, Sunnyvale, CA, USA) controlled by a pClamp data acquisition system (Molecular Devices, Sunnyvale, CA, USA). Oocytes were placed in a bath containing ND96 solution. Voltage and current electrodes were filled with 3 M KCl, and the resistances of both electrodes were between 0.5 and 1.5 M $\Omega$ . The elicited currents were sampled at 100 Hz and filtered at 50 Hz using a four-pole, low pass Bessel filter. To eliminate the effect of the voltage drop across the bath grounding electrode, the bath potential was actively controlled by a two-electrode bath clamp. During recordings, oocytes were continuously perfused with ND96 at a rate of 2 ml/min, with the conopeptides applied during 30 s before ACh was added. ACh (200  $\mu\text{M}$  for  $\alpha_7$ , 70  $\mu\text{M}$  for  $\alpha_3\beta_4$ , 50  $\mu\text{M}$  for  $\alpha_4\beta_2$  and  $\alpha_4\beta_4$ , 10  $\mu\text{M}$  for  $\alpha_1\beta_1\gamma\delta$  and  $\alpha_1\beta_1\delta\epsilon$ ) was applied for 2 s at 2 ml/min, with 30 s washout periods between different ACh applications and 200 s after toxin application. The percentage response or percentage inhibition was obtained by averaging the peak amplitude of at least three control responses (two directly before exposure to the peptide and one after 200 s washout). The synthetic peptides were dissolved in ND96 according to the desired

concentrations. These peptide concentrations were controlled using spectrophotometry.

Whole-cell current traces were evoked from a holding potential of  $-90\text{ mV}$ . Concentration-response curves were constructed by application of different toxin concentrations to the nAChR-expressing oocytes. The percentage of nAChR-blockade was plotted against the logarithm of the applied concentrations and fitted with the Hill equation:  $y = 100/[1 + (\text{IC}_{50}/[\text{toxin}])^h]$ , where  $y$  is the amplitude of the toxin-induced effect,  $\text{IC}_{50}$  is the toxin concentration at half maximal efficacy,  $[\text{toxin}]$  is the toxin concentration and  $h$  is the Hill coefficient.

Comparison of two sample means was made using a paired Student's  $t$  test ( $p < 0.05$ ). All data are presented as mean  $\pm$  standard error (S.E.M) of at least 3 independent experiments ( $n \geq 3$ ). All data was analysed using pClamp Clampfit 10.0 (Molecular Devices<sup>®</sup>, Downingtown, PA) and Origin 7.5 software (Originlab<sup>®</sup>, Northampton, MA).

### 2.6. NMR spectroscopy

Initially temperature ( $5\text{--}30\text{ }^{\circ}\text{C}$ ) and pH ( $3.8\text{--}6.0$ ) dependent 1D proton spectra were recorded for both purified peptide samples (AusIA(g) and AusIA(r)), with 2–3 mM peptide solution (200  $\mu\text{L}$ ) in  $\text{H}_2\text{O}:\text{D}_2\text{O}$  on a 500 MHz (equipped with a 5 mm TCI HCP Z-gradient probe) and 600 MHz (equipped with a 5 mm TCI HCN Z-gradient cryoprobe) Bruker Avance II NMR spectrometer. Detailed two-dimensional (2D) NMR spectra for AusIA(r) were recorded with a 3 mM solution (200  $\mu\text{L}$ , pH 4.0) of the folded peptide in  $\text{H}_2\text{O}:\text{D}_2\text{O}$  (9:1) mixture at  $5\text{ }^{\circ}\text{C}$  on 600 MHz NMR spectrometer. All spectra were processed by using Topspin 2.1 (Bruker Biospin) and analysed with the program CARRA (version 1.8.4) (Keller, 2004).

In the 1D and 2D spectra the water signal was suppressed by using excitation sculpting with gradients (Hwang and Shaka, 1995). Two-dimensional NOESY spectra (mixing time 200 and 300 ms) were recorded with a sweep width of 7210 Hz in both dimensions, 64 scans, 2048 data points in  $t_2$ , and 1024 free induction decays (FIDs) in  $t_1$ . Two-dimensional total correlation spectroscopy (2D-TOCSY) (Shaka et al., 1988) in 90%  $\text{H}_2\text{O}$  was recorded with DIPSI2 sequence for mixing (mixing time 80 ms). A double quantum-filtered correlation spectrum (DQF-COSY) (Derome and Williamson, 1990) in  $\text{H}_2\text{O}$  was acquired using excitation sculpting with gradients for water suppression with a sweep width of 7210 Hz in both dimensions, 64 scans, 2048 data points in  $t_2$ , and 1024 FIDs in  $t_1$ . In the processing of two-dimensional spectra, data were apodized with a shifted sine-bell square function in both dimensions.

Natural abundance  $^1\text{H}$ ,  $^{13}\text{C}$  heteronuclear single quantum correlation ( $^1\text{H}\text{--}^{13}\text{C}$  HSQC) spectrum was recorded with sensitivity enhancement and gradient coherence selection, optimized for selection of aliphatic CH groups ( $J_{\text{CH}} = 135\text{ Hz}$ ) using 64 scans, 1024/2048 complex data points, and 12072/7210 Hz spectral widths in  $t_1$  and  $t_2$  respectively. For the selection of aromatic CH groups 170 Hz was used for  $J_{\text{CH}}$  along with 64 scans, and 128/2048 complex data points.

### 3. Results

#### 3.1. Isolation of a novel $\alpha$ -conotoxin in *C. australis* venom

N-terminal Edman degradation of the purified active peptide revealed a novel 16-residue  $\alpha$ -conotoxin, called AusIA, with the sequence SCCARNPACRHNHPCV and a molecular mass of 1763.90 Da, determined by MALDI-TOF (4800 Analyzer, Applied Biosystems, USA). This is in perfect agreement with the mass of the synthetic peptide (1764.03 Da) which has been determined by LC-MS and the theoretically calculated mass of 1764.03 Da (online Peptide Mass Calculator–Peptide Protein Research Ltd.). To the best of our knowledge, AusIA is the first conotoxin isolated and pharmacologically characterized from *C. australis*, a worm hunting cone snail species commonly found in the Indian Ocean in Tamil Nadu, India. The RP-HPLC purification of *C. australis* venom is shown in Fig. 1. The fraction containing AusIA is indicated with an arrow (see Fig. 1). Remarkably, AusIA has 5 amino acids in both *m* and *n* loops. This classifies the peptide as an  $\alpha 5/5$  conotoxin which, to the best of our knowledge, has never been described before.

#### 3.2. Alpha-conotoxin AusIA isomers

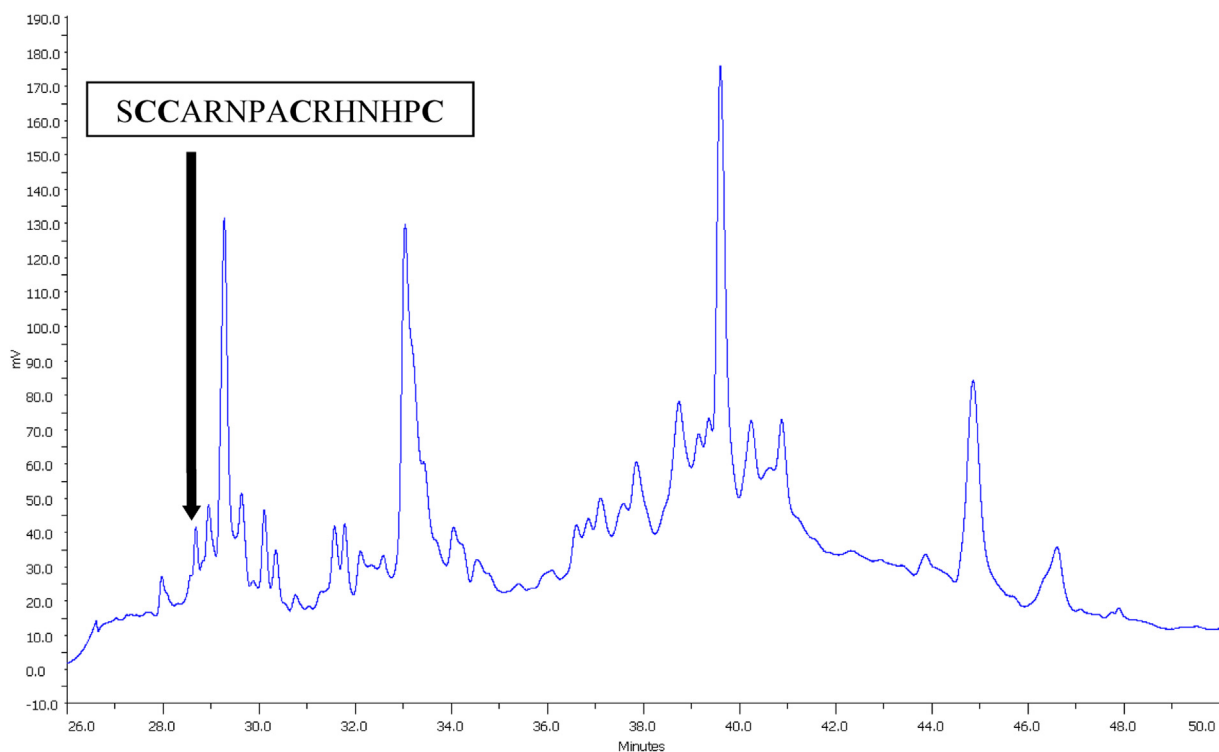
Typically,  $\alpha$ -conotoxins have a globular configuration (CysI–CysIII; CysII–CysIV). Therefore this configuration is assumed to be the native one (Janes, 2005). We decided to investigate both globular (I–III, II–IV) and ribbon (I–IV, II–III) isomers of AusIA, which we named  $\alpha$ -conotoxins

AusIA(g) and AusIA(r) respectively. The potency and selectivity of both isomers at neuronal and muscle subtype nAChRs was determined by examining its effect on ACh-evoked currents mediated by different nAChR subunit combinations expressed in *Xenopus* oocytes. Both peptides, AusIA(g) and AusIA(r) (10  $\mu$ M), only inhibited ACh-evoked current amplitude mediated by  $\alpha_7$  nAChRs (40% and 51% respectively; Figs. 2 and 3). No remarkable effect was seen at the muscle subtype nAChRs  $\alpha_1\beta_1\gamma\delta$  and  $\alpha_1\beta_1\delta\epsilon$  as well as at neuronal subtype nAChRs  $\alpha_3\beta_4$ ,  $\alpha_4\beta_2$ , and  $\alpha_4\beta_4$  (Figs. 2 and 3). Concentration-response curves for AusIA(g) and AusIA(r) inhibition of ACh-evoked currents at the  $\alpha_7$  nAChR revealed an  $IC_{50}$  value of  $11.68 \pm 1.53$   $\mu$ M and  $9.67 \pm 0.01$   $\mu$ M respectively. Hill coefficients were 1.85 and 5.20 for AusIA(g) and AusIA(r) respectively (Fig. 4).

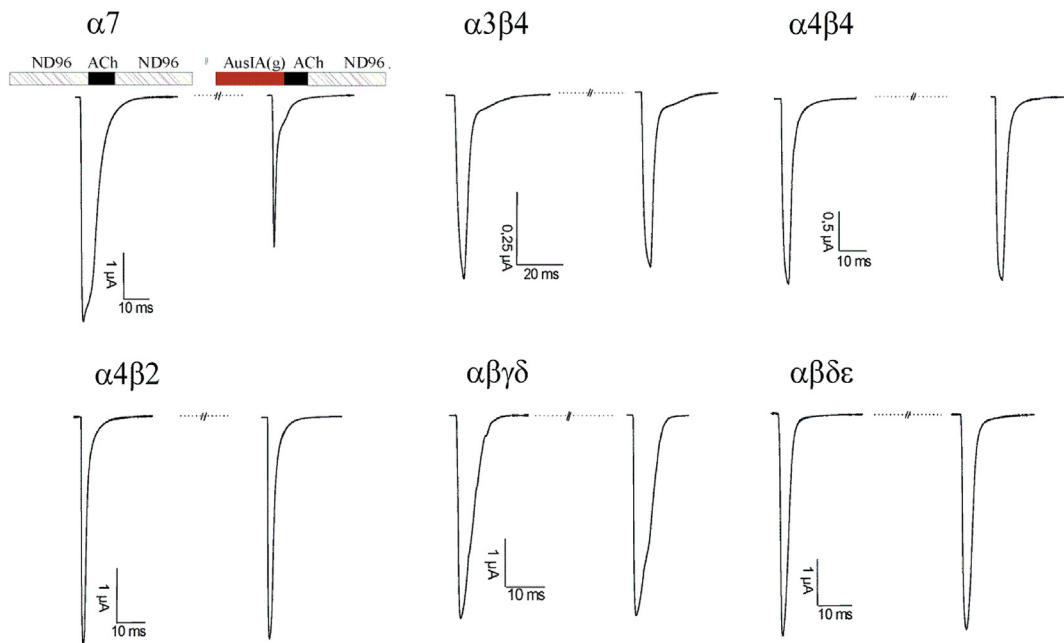
#### 3.3. NMR spectroscopy

In order to investigate the structural properties of AusIA(g) and AusIA(r), we carried out solution state NMR. The proton NMR spectra of the first peptide, AusIA(g), appeared severely broad in the tested screening conditions (Fig. 5A). These broad spectra indicate that at least two or more conformers of AusIA(g) exist in an intermediate rate of exchange. It is therefore unlikely that AusIA(g) has any uniquely folded structure and consequently, no detailed NMR investigation could be performed.

In contrast, the spectra of AusIA(r) were more promising. In lower pH, one set of major NMR signals appeared (Fig. 5B) together with a minor form (Fig. 6). Detailed two-



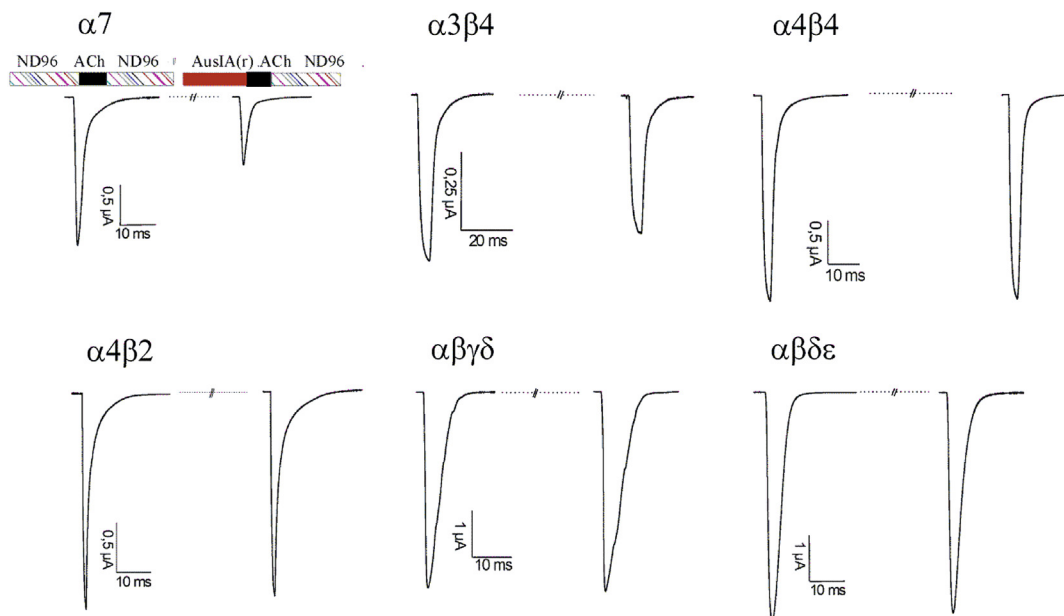
**Fig. 1.** C18 reversed phase HPLC profile of the third gel filtration fraction of *C. australis* venom. The arrow indicates the peptide described in this work, namely AusIA.



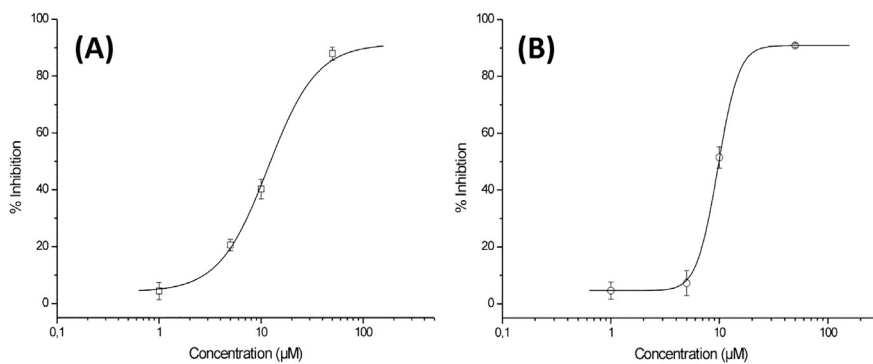
**Fig. 2. Electrophysiological profile of AusIA(g).** During recordings, oocytes were continuously perfused with ND96 at a rate of 2 ml/min, with 10  $\mu$ M AusIA(g) applied during 30 s before ACh was added. ACh (200  $\mu$ M for  $\alpha_7$ , 70  $\mu$ M for  $\alpha_3\beta_4$ , 50  $\mu$ M for  $\alpha_4\beta_2$  and  $\alpha_4\beta_4$ , 10  $\mu$ M for  $\alpha_1\beta_1\gamma\delta$  and  $\alpha_1\beta_1\delta\epsilon$ ) was applied for 2 s at 2 ml/min, with 30 s washout periods between different ACh applications and 200 s after toxin application.

dimensional NMR experiments were performed for AusIA(r) at pH 4.0. This was done following the standard procedures as outlined by Wüthrich and co-workers (Wüthrich, 1986).

The coupling constant ( $^3J_{\text{HNH}\alpha}$ ) values of AusIA(r) are indicated in Fig. 5B. All values are between 5 and 9 Hz which indicates that the angle averages across the Phi dihedrals and points to some flexibility in the peptide



**Fig. 3. Electrophysiological profile of AusIA(r).** During recordings, oocytes were continuously perfused with ND96 at a rate of 2 ml/min, with 10  $\mu$ M AusIA(r) applied during 30 s before ACh was added. ACh (200  $\mu$ M for  $\alpha_7$ , 70  $\mu$ M for  $\alpha_3\beta_4$ , 50  $\mu$ M for  $\alpha_4\beta_2$  and  $\alpha_4\beta_4$ , 10  $\mu$ M for  $\alpha_1\beta_1\gamma\delta$  and  $\alpha_1\beta_1\delta\epsilon$ ) was applied for 2 s at 2 ml/min, with 30 s washout periods between different ACh applications and 200 s after toxin application.



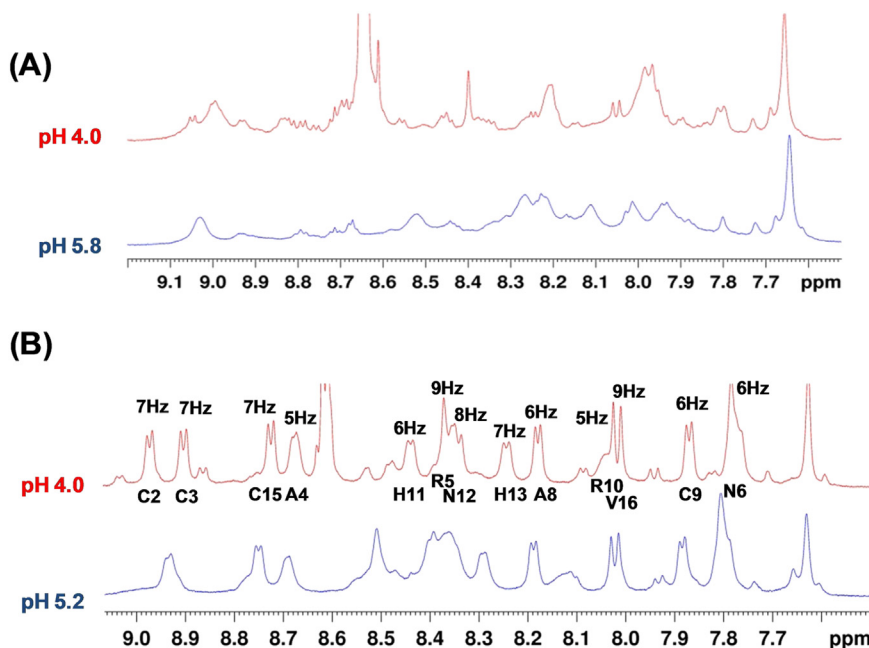
**Fig. 4.** Concentration–response curve on  $\alpha_7$  nAChR. Different concentration of AusIA(g) ( $\square$ , **A**), AusIA(r) ( $\circ$ , **B**) were tested on  $\alpha_7$  nAChRs. The corresponding  $IC_{50}$  values are  $11.68 \pm 1.53 \mu\text{M}$  and  $9.67 \pm 0.01 \mu\text{M}$  respectively. Currents were obtained as described under “Materials and Methods”. The percentage of block was plotted against the logarithm of the tested concentrations. The results were fit with the Hill equation and the Hill coefficients were 1.85 and 5.20 for AusIA(g) and AusIA(r) respectively.

backbone. Amino acid residues flanked by Pro<sup>7</sup> and Asn<sup>12</sup> show coupling values around 6 Hz, which can be an indication of an equilibrium between a helix and a coil formation. For the backbone amide protons, the temperature shift coefficients show an average value of 4.2 ppb/K ( $\Delta\delta/\Delta T$ ) per residue (Fig. 7) [lit. values for shielded and exposed residues are  $<3$  ppb/K and  $>5$  ppb/K respectively (Raghothama, 2010)]. Interestingly, amino acid residues between Ala<sup>4</sup> and His<sup>13</sup> have temperature shift coefficients mostly  $\leq 3$  ppb/K. This may indicate the existence of a quasi-stable helical region in the middle of the peptide, since these amide protons are not highly exposed to the water molecules in the solvent. However, the absence of

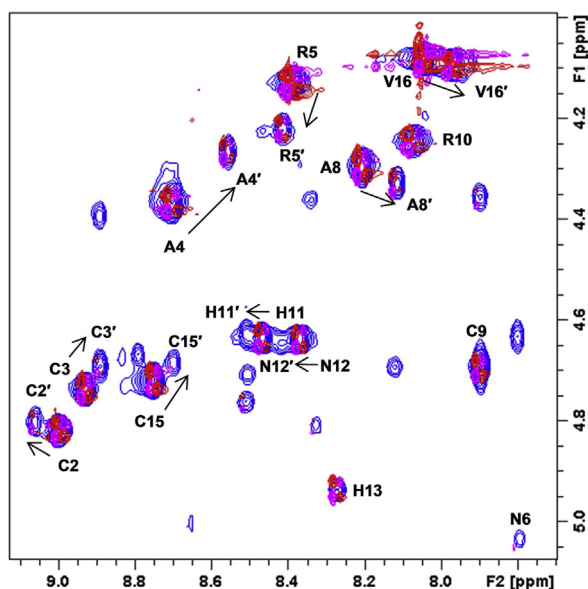
any medium range NOE confirms that AusIA(r) is devoid of any stable secondary structural unit such as  $\alpha$ -helices or  $\beta$ -turns. This is in contrast with most other 3D structures of the  $\alpha$ -conotoxin family (Chi et al., 2004; Park et al., 2001).

#### 4. Discussion

$\alpha$ -Conotoxins are defined as a family of cysteine-rich peptides having the typical CC-C-C framework and acting as competitive antagonists of nicotinic acetylcholine receptors (Dutertre and Lewis, 2006). In general, there are two different nAChR subtypes: neuronal and muscle. The neuronal  $\alpha_7$  nAChRs have been investigated thoroughly



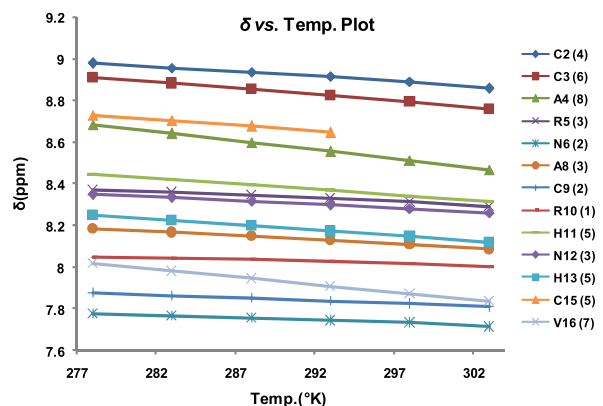
**Fig. 5.** Proton NMR spectra of AusIA(g) (**A**) and AusIA(r) (**B**) demonstrating structural flexibility in the solution state. Backbone amide proton region of the one-dimensional spectrum, recorded at 5 °C is shown at higher pH (in blue) and lower pH (in red). Amide proton signals of AusIA(r) at pH 4.0 are labelled with their residue labels and the corresponding  $^3J_{\text{HNHz}}$  coupling (Hz) values are provided on top of the peaks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 6.** Overlay of the “fingerprint” region of TOCSY (in blue) and DQF-COSY (in red and pink) spectra of AusIA(r). Cross-peaks arising from the major form and the exchange peaks from the minor form are marked with their corresponding residue numbers without and with a prime ('), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Couturier et al., 1990), because these nAChRs have important functions in the brain, including regions involved in learning and memory, the hippocampus and the cerebral cortex (Breese et al., 1997; Rubboli et al., 1994; Wevers et al., 1994). Consequently,  $\alpha_7$  nAChR dysfunctions are associated with a variety of severe pathologies such as certain types of epilepsy, myasthenic syndromes, schizophrenia, Parkinson's and Alzheimer's diseases (Gotti and Clementi, 2004; Lebbe et al., 2014b; Sacco et al., 2004; Steinlein and Bertrand, 2010).

As mentioned earlier, the number of residues included within the two loops ( $m/n$ ) of  $\alpha$ -conotoxins is the basis for



**Fig. 7.** Chemical shift ( $\delta$  in ppm) vs. temperature ( $^{\circ}$ K) plot for the backbone amide proton of different residues of AusIA(r). Temperature shift coefficients ( $\Delta\delta/\Delta T$  in ppb/ $^{\circ}$ K) are mentioned within parentheses beside the residue labels.

the division into several structural subgroups ( $m/n$ : 3/5, 3/6, 4/2 4/3, 4/4, 4/5, 4/6, 4/7, 4/8, 5/2 and 5/8) (Akondi et al., 2014; Lebbe et al., 2014b). The loop size is believed to roughly correlate with the pharmacological target selectivity. In general,  $\alpha$ -conotoxins with a 3/5 framework are isolated from fish-hunting snails and are mainly active toward fish and/or mammalian neuromuscular nAChRs, whereas conotoxins from the 4/3, 4/4, 4/5, 4/6 or 4/7 classes primarily interact with mammalian neuronal nAChRs (Terlau and Olivera, 2004). Exceptions do exist, since there are also 4/7 and 4/4 conotoxins that affect the muscular nAChR subtypes. The pharmacophore of these neuronal nAChR targeting  $\alpha$ -conotoxins has been investigated in detail. It is composed of a conserved hydrophobic patch in the first loop which determines binding, and a more variable second loop, which administers selectivity through pairwise interactions with different nAChRs subunits (Dutertre et al., 2005). In this study, we revealed the amino acid composition from a new  $\alpha 5/5$  conotoxin from *C. australis*, called AusIA, of which both loops ( $m/n$ ) consist of 5 amino acids. This is exceptional since, as far as we are aware, no such peptide has been described. The first loop of AusIA, having two Ala amino acids, is more hydrophobic than the second loop which contains following hydrophilic amino acids: one Arg, two His and one Asn. Moreover, AusIA has a Pro at position 7 which is conserved among all  $\alpha$ -conotoxins. As is visualized in Fig. 8, also amino acids Ala<sup>8</sup> and Pro<sup>14</sup> of AusIA are regularly found in other  $\alpha$ -conotoxins. AusIA shows the highest percentage of similarity (67%) with Pu1.3, an  $\alpha 4/5$  conotoxin of *Conus pulicarius*, a worm-hunting cone snail species as well (Yuan et al., 2007). Other  $\alpha$ -conotoxins showing a moderate percentage of

AnIB (41%)	GG - CC - SHPACAANNQDYC - - - - 17
GIC (44%)	G - CC - SHPACAGNNQHIC - - - - 16
MII (44%)	G - CC - SNPVCHLEHSNLC - - - - 16
PnIA (44%)	G - CC - SLPPCAANNPDYC - - - - 16
TxIA (44%)	G - CC - SRPPCIANNPDLIC - - - - 16
Vc1.1 (44%)	G - CC - SDPRCNYDHPEIC - - - - 16
Epl (44%)	G - CC - SDPRCNMNNPDYC - - - - 16
AriB (45%)	- DECC - SNPACRVNNPHVC - RRR - 20
OmlA (47%)	G - CC - SHPACVNNNPHIC - G - - 17
TxIB (50%)	G - CC - SDPPCRNKHDPDLIC - - - - 16
RegIIA (50%)	G - CC - SHPACVNNNPHIC - - - - 16
Lp1.10 (53%)	- NDCC - HNA PCRNNHPGIC - - - - 17
PuSG1.2 (53%)	- DDCC - PDPACRQNHPEICPS - - - 19
PelA (56%)	G - CC - SHPACSVNHPPELC - - - - 16
Lo1a (61%)	- EGCC - SNPACRTNHPPEVC - - - - 18
Qc1.5 (62%)	G - CC - SNPACSVNHPPELC - - - - 16
Pu1.3 (67%)	- LSCCA - DPACKHT - PG - C - - - 15
AusIA	- - SCCARNPACRRNHP - CV - - - 16

**Fig. 8.** Alignment of 16  $\alpha$ -conotoxins with AusIA, sorted by percentage of identity (indicated in the left column) with AusIA. References from top to bottom: AnIB (*C. anemone*, (Loughnan et al., 2004)), GIC (*C. geographus*, (McIntosh et al., 2002)), MII (*C. magus*, (Cartier et al., 1996)), PnIA (*C. pen-naceus*, (Luo et al., 1999)), TxIA (*C. textile*, (Dutertre et al., 2007)), Vc1.1 (*C. victoriae*, (Sandall et al., 2003)), Epl (*C. episcopates*, (Loughnan et al., 1998)), AriA (*C. arenatus*, (Whiteaker et al., 2007)), OmlA (*C. omaria*, (Talley et al., 2006)), TxIB (*C. textile*, (Luo et al., 2013a)), RegIIA (*C. regius*, (Franco et al., 2012)), Lp1.10 (*C. leopardus*, (Yuan et al., 2007)), PuSG1.2 (*C. pulicarius*, (Kaes et al., 2012)), PelA (*C. pergrandis*, (McIntosh et al., 2005)), Lo1a (*C. longurionis*, (Lebbe et al., 2014b)), Qc1.5 (*C. quercinus*, (Yuan et al., 2007)), Pu1.3 (*C. pulicarius*, (Yuan et al., 2007)), AusIA (*C. australis*, this work).

similarity with AusIA are Qc1.5 (62%; *Conus quercinus*) (Yuan et al., 2007), Lo1a (61%; *Conus longurionis*) (Lebbe et al., 2014a) and PeIA (56%; *Conus pergrandis*) (McIntosh et al., 2005). The activity of Qc1.5 has not yet been determined while Lo1a targets mainly  $\alpha_7$  nAChRs and PeIA targets  $\alpha_9\alpha_{10}$ ,  $\alpha_3\beta_2$ ,  $\alpha_3\beta_4$ ,  $\alpha_6/\alpha_3\beta_2\beta_3$  and  $\alpha_6/\alpha_3\beta_4$  nAChRs. It appears that Arg<sup>5</sup> and the C-terminal Val<sup>16</sup> of AusIA are rarely observed in other  $\alpha$ -conotoxins at these particular positions.

In this study we investigated both globular (I–III, II–IV) and ribbon (I–IV, II–III) AusIA isomers. Once the activity of AusIA was determined electrophysiologically, we employed NMR-based techniques to elucidate the structure of this novel peptide. The NMR data revealed that both peptides, AusIA(g) and AusIA(r), adopt very flexible structures without having a unique folded structure, lacking any stable  $\alpha$ -helical and/or  $\beta$ -turn units. While AusIA(g) appears to exist in an intermediate rate of exchange between at least two conformers, AusIA(r) shows to have a major conformer (Fig. 6).

These results are remarkable in several points of view. First, the similar IC<sub>50</sub> values ( $11.68 \pm 1.53 \mu\text{M}$  and  $9.67 \pm 0.01 \mu\text{M}$  for AusIA(g) and AusIA(r) respectively) cite to a similarity in potency. This demonstrates that the Cys-connectivity is less important for the peptides' potency. The most important value is arguably the basis structure having a typical hydrophobic patch determining binding, which has been demonstrated before (Chi et al., 2004; Dutertre et al., 2005; Luo et al., 2013a, 2013b)). Previous work of Khoo et al. (2012) showed that both peptide isomers of  $\mu$ -conotoxin KIIIA (-CC-C-CC-) are functional peptides, raising questions about which, if either, is the 'native' disulfide pattern in the venom of *Conus kinoshitai* (Khoo et al., 2012). Other work by Safavi-Hemami et al. (2012) provided evidence of the presence of both globular and ribbon forms of  $\alpha$ -conotoxin Iml in the venom of *Conus imperialis* (Safavi-Hemami et al., 2012). Additionally, non-native disulfide isomers have also been reported for other  $\alpha$ -conotoxins such as GI (*Conus geographus*) and AulB (*Conus aulicus*) (Dutton et al., 2002; Gehrman et al., 1998). Therefore, one might believe that here too both isomers are present in the venom gland of *C. australis*. However, chromatographically we could not demonstrate this unequivocally because the RP-HPLC retention times are almost identical for both synthetically synthesized isomers (data not shown). Secondly, from both peptides the ribbon isomer is slightly more potent, but significantly more stable in solution than the globular isomer. The higher potency of the ribbon isomer has been described for one other  $\alpha$ -conotoxin only, namely AulB (*C. aulicus*) which selectively blocks the  $\alpha_3\beta_4$  nAChR subtype (Luo et al., 1998).

In an attempt to correlate the structure–function relationship, it appears that other  $\alpha$ -conotoxins such as Lo1a of the  $\alpha_4/7$  conotoxin family, form a robust folded structure, and consequently show higher affinity binding and inhibition of the receptor (Lo1a has an IC<sub>50</sub> value of  $3.24 \pm 0.70 \mu\text{M}$  on  $\alpha_7$  nAChRs) (Lebbe et al., 2014a). Being not structured, both peptides, AusIA(g) and AusIA(r), demonstrate weak inhibition compared to that of Lo1a for the same receptor. One might speculate that the 'real' target is another receptor or ion channel, or else, that different

combinations of  $\alpha$  or  $\beta$  nAChR subunits might lead to a variation in potency. In this context,  $\alpha$ -conotoxin Vc1.1 is an interesting example. Synthetic Vc1.1 (ACV1) was initially shown to potently block neuronal ( $\alpha_3$ ,  $\alpha_5$ ,  $\alpha_7$  and  $\beta_4$  nAChR subunits) versus muscle nAChRs (Livett et al., 2006). Therefore it was selected for testing in pain models revealing Vc1.1 as the first  $\alpha$ -conotoxin exhibiting efficacy in these models (Sandall et al., 2003; Satkunanathan et al., 2005). Later, Vincler et al. showed that Vc1.1 is a potent antagonist of  $\alpha_9\alpha_{10}$  nAChRs which potentially contributes to its analgesic effect (Vincler et al., 2006).

Another explanation might be the impaired affinity-selectivity of  $\alpha_5/5$  conotoxins, possibly providing an answer for their rare biological existence. It is also tantalizing to speculate that the superior family of toxins like  $\alpha_4/7$  and others with well-defined structure–function properties may have been evolved and the rare  $\alpha_5/5$  family became extinct in some *Conus* species. Finally, further characterization is needed in order to know if AusIA can displace  $\alpha$ -bungarotoxin from  $\alpha_7$  nAChRs, and as such to see if an overlapping binding site is shared or not. Moreover for this would indicate whether or not AusIA shows affinity for the AChBP.

In summary, we defined a new subclass of the  $\alpha$ -conotoxin family. We isolated an  $\alpha_5/5$  conotoxin, called AusIA, from the venom of *C. australis*, from which both globular and ribbon isomers demonstrated a similar potency on  $\alpha_7$  nAChRs. Therefore, our study broadens the  $\alpha$ -conotoxin subfamily classification which might be useful regarding the design of novel (therapeutic) compounds.

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## Conflict of interest

The authors declare that there are no conflicts of interest.

## Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.toxicol.2014.08.074>.



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