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A novel arrangement of Cys residues in a paralytic peptide of *Conus cancellatus* (jr. syn.: *Conus austini*), a worm-hunting snail from the Gulf of Mexico

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Abstract

The present study details the purification, the amino acid sequence determination, and a preliminary characterization of the biological effects in mice of a new conotoxin from the venom of *Conus cancellatus* (jr. syn.: *Conus austini*), a worm-hunting cone snail collected in the western Gulf of Mexico (Mexico). The 23-amino acid peptide, called as24a, is characterized by the sequence pattern CX₁CX₂CX₈CX₁CCX₅, which is, for conotoxins, a new arrangement of six cysteines (framework XXIV) that form three disulfide bridges. The primary structure (CKCPSCNFNDVTENCKCCIFRQP*; *, amidated C-terminus; calculated monoisotopic mass, 2,644.09 Da) was established by automated Edman degradation after reduction and alkylation, and MALDI-TOF and ESI mass spectrometry (monoisotopic mass, 2644.12/2644.08 Da). Upon intracranial injection in mice, the purified peptide provokes paralysis of the hind limbs and death with a dose of 240 pmoles (~0.635 ! g, ~24.9 ng/g). In addition, a post-translational variant of this peptide (as24b) was identified and determined to contain two hydroxyproline residues. These peptides may represent a novel conotoxin gene superfamily.

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Conflict of interest

The authors have no conflicting interests.

Contributors

MBA and AZ-C wrote the article. AZ-C, AF and CVFB performed the experiments. MBA and AZ-C analyzed the results. MBA, AZ-C, BMO, and EPHC ideated the project. EPHC coordinated the collection and taxonomical work.

All authors have read and approved the final article.

Keywords

Conus cancellatus, *Conus austini*; vermivorous; conotoxin; six Cys; paralysis

1. Introduction

Cone snails are a group of venomous marine gastropods that are found in tropical and subtropical waters [21, 35]. Venoms contained in the venom ducts of *Conus* species are delivered by harpoon-like hollow radular teeth to rapidly immobilize fish, molluscs, or worms. Each species has its own distinct complement of more than 100 venom peptides or “conotoxins”, and each cone snail species generally hunts only one kind of prey [32]. Most conotoxins studied have been obtained from species that prey on fishes or molluscs obtained from the shallow waters of the Indopacific region, where approximately 60% of the known ~500 species exists [10]. However, vermivorous cones represent more than 80 % [11] of the species whose feeding type is known.

Most venoms so far examined have yielded an array of low molecular weight peptides which typically have 7-40 amino acids and are highly constrained, generally by one to four disulfide bridges. Another feature of conotoxins is their unprecedented frequency of post-translational modifications, including proline hydroxylation, C-terminus amidation, tryptophan bromination, carboxylation of glutamate, as well as leucine and tryptophan isomerization [5].

Conotoxins are categorized into superfamilies based on the highly conserved signal sequences of their precursors, and they are further subdivided into families based on their receptor targets. The cysteine residues are arranged in distinct patterns, which define different structural frameworks or scaffolds [29]. Among the most studied conotoxins are those with six Cys residues, including the !-!!! !-!!! -, !- and the !-conotoxins [14, 38], and lesser known peptides, such as conotoxin VxVI [6]. In many cases, their mode of action has been elucidated by neurotoxicity assays in mammals, since many of these peptides elicit dictintive behavioral symptomatologies when injected directly into the central nervous system of mice [33].

Conotoxins bind with high affinity and specificity to ion channels and receptors, inhibiting their physiological functions [28], which makes them invaluable as tools for understanding channel structure and function as well as for developing pharmaceutical agents specific for these targets, thereby reducing side effects. [1, 17].

The present work reports on the isolation and characterization of two new conotoxins (named as24a and as24b) from *Conus cancellatus* (jr. syn.: *Conus austini*) (Rehder & Abbott, 1951) that possess a new cysteine framework. Peptide as24a elicits neurotoxic effects in mice, and peptide as24b contains two hydroxyproline residues.

2. Materials and methods

2.1. Materials

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) columns derivatized with octadecyl- (C18) and octyl- (C8) moieties were from Vydac (Hesperia CA). Reagents for N-terminal sequencing were from Applied Biosystems (Foster City CA). HPLC-grade acetonitrile (ACN) was purchased from Caledon (Georgetown, Ontario, Canada); 4-vinylpyridine from Sigma (St. Louis MO) was distilled under vacuum; trifluoroacetic acid (TFA) was from Fluka (Buchs, Switzerland).

2.2. Specimen collection

Adult *Conus cancellatus* (jr. syn.: *Conus austini*) specimens were collected by shrimping vessels and the research vessel (R/V) Justo Sierra at a depths of ~60-80 m along the coast of Tamaulipas, Mexico. The organisms were frozen in liquid nitrogen and stored at -70°C until they were used.

2.3. Venom fractionation

Venom ducts were dissected from the animals. A crude venom extract was obtained by homogenization of 10 venom ducts in 5 ml of extraction solution (40% (v/v) ACN containing 0.1% (v/v) TFA) at 4°C . The homogenate was centrifuged at $10,100 \times g$ at 4°C for 20 min, and the supernatant was lyophilized and stored at -20°C . The lyophilized whole venom was dissolved in deionized water containing 0.1% (v/v) TFA and then centrifuged at $10,100 \times g$ at 4°C for 20 min. Total protein was quantified by the Bradford method [4] using bovine serum albumin as standard (Protein Assay Kit; Bio-Rad, Hercules CA). For isolation of the peptides from the crude venom and all subsequent purification steps, solution A consisted of 0.1% (v/v) TFA in water, and solution B was 0.085% TFA (v/v) in 90% (v/v) ACN. Venom was loaded ! 0.3-1.0 mg at a time onto an analytical RP-HPLC C18 column (Vydac 218TP54; 4.6×250 mm, 5 ! m particle size) provided with a C18 guard column (Vydac 218GK54; 4.6×10 mm, 5 ! m particle size). Components were eluted at room temperature, first isocratically (5% solution B for 10 min), and then by a linear gradient (5-55% of solution B in 100 min) at a flow rate of 1 ml/min. The absorbance was monitored at 220 nm.

2.4. Toxin purification

One major and one minor fractions were further purified at room temperature. For the major fraction, the first step used the same analytical C18 column employed for fractionation of the venom, using an isocratic step (20% solution B for 20 min) followed by a gradient of 20-35% solution B in 60 min, at a flow rate of 1 ml/min. The second purification step involved an analytical C8 column (Vydac 208TP54; 4.6×250 mm, 5 ! m particle size) provided with a MetaGuard Nucleosil C8 column (0120-MG; 4.6×10 mm, 5 ! m particle size; Varian, Torrance CA), using an isocratic step (20% solution B for 10 min) followed by a gradient of 20-35% solution B in 60 min, at a flow rate of 1 ml/min. For the minor fraction, the purification step was performed with the same analytical C8 column mentioned above for the first fraction, using an isocratic step (20% solution B for 10 min) followed by a gradient of 20-35% solution B in 60 min, at a flow rate of 1 ml/min.

2.5. Molecular mass characterization

One sample of the major native peptide (! 200 pmol) was subjected to MALDI-TOF mass spectrometry on a Voyager DE Mass Spectrometer (Applied Biosystems) equipped with delayed ion extraction. Spectra were obtained in positive reflector mode using sinapinic acid as matrix.

Another sample of the major peptide purified from another crude extract was applied into a LC-MS system consisting of a micro flow Accela LC (Thermo-Fischer Co., San Jose CA) with "splitter" (1/20) coupled to a mass spectrometer Orbitrap Velos (Thermo-Fischer Co., San Jose CA) with nano-electrospray ionization (ESI) source.

Calibration of the mass spectrometer was carried out with the Calmix solution consisting of 10 different calibrants allowing measurements with accuracies better than 5 ppm (parts per million). The nano-flow chromatographic system was operated at 400 nanoliters/min using an isocratic gradient of 50% solvent B (solvent A, 0.1% acetic acid in water; solvent B, 0.1% acetic acid in acetonitrile) for 30 min. A micro-needle (New Objective, Woburn MA)

was used in the nano-electrospray ionization source operating with 2.0 kV of spray voltage. For Higher energy Collisional Dissociation (HCD) experiment the most intense charged ion was manually selected for fragmentation and the derived spectrum was manually analyzed.

2.6. Sequence determination

An aliquot (~1 nmol) of the major purified peptide was subjected to reduction and alkylation before sequencing. The peptide was dissolved in 100 μ l of 0.1 M Tris-HCl, pH 8.0, and 100 mg of guanidine hydrochloride was added and dissolved. Forty-five microliters of 50 mM dithiothreitol was added, and the mixture was incubated at 65 $^{\circ}$ C for 25 min under nitrogen. Subsequently, 4 μ l of 4-vinylpyridine were added, and the solution was incubated at room temperature for 16 h under nitrogen [37]. The sample was desalted at room temperature using an analytical C18 column eluted with an isocratic step (10% solution B for 20 min) followed by a linear gradient (10-40% buffer B in 30 min), with a flow rate of 1 ml/min. Amino acid sequencing of the pyridylethylated peptide was performed by automated Edman degradation using a Procise 491 Protein Sequencing System (Applied Biosystems).

Due to the small amount of material available, the minor purified peptide was sequenced without reduction and alkylation.

2.7. Preliminary biological characterization

Four-week old male mice (strain CD-1, 24-27 g body weight) were used. Control animals (n=3) were intracranially (i.c.) injected with 30 μ l of 0.9 % normal saline solution. Two doses (120 pmol/mouse (~0.317 μ g, ~12.4 ng/g), n=3, and 240 pmol/mouse (~0.635 μ g, ~24.9 ng/g), n=3) of the major purified peptide were dissolved in 30 μ l of 0.9% normal saline solution. The animals were continuously observed to detect any behavioral change or death, for up to 2 h post-injection [7].

2.8. Similarity search and sequence comparison

Basic Local Alignment Search Tool (BLAST) was employed to search the National Center for Biotechnology Information Non-redundant protein sequences database (all non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects) [27]. This database includes sequences determined at the protein level (PDB+SwissProt+PIR+PRF), and translations from nucleotide sequences (all non-redundant GenBank CDS translations excluding environmental samples from WGS projects). We employed the protein blast program (Search **protein** database using a **protein** query) with the blastp algorithm (protein-protein BLAST) (BLASTP 2.2.27, Feb-4-2013; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) [2]), using the default parameters, except that we limited our search to Organism = Conus (taxid:6490).

The ConoPrec tool, which is available on the ConoServer website [8, 20] was used to identify similar sequences recorded in the ConoServer database.

The ClustalW2 [22] server at EMBL-EBI [12] was employed for amino acid sequence alignment, employing the default settings.

3. Results

3.1. Venom fractionation and toxin purification

A major peak was manually collected by RP-HPLC from the venom crude extract (arrow in Fig. 1A). The elution positions of the peptide during its re-purifications on C18 and C8 columns are shown in Fig. 1B-C. A minor peak was also manually recovered from the crude extract (arrow and asterisk in Fig. 1A) and re-purified on a C8 column. During the re-

purification steps of both peptides manual collection was also employed, in order not to collect adjacent venom components (Fig. 1D).

3.2. Determination of molecular mass

The MALDI-TOF and ESI mass spectra of the major purified peptide are shown in Fig. 2. The MALDI-TOF monoisotopic m/z signal at 2,645.12 corresponds to a monoisotopic mass of 2,644.12 Da (Fig. 2A). The ESI mass spectrum shows m/z signals corresponding to the triply (882.69) and doubly charged (1323.54) ions. The isotopic resolution of the doubly charged ion (inset) indicates a monoisotopic mass of 2,644.08 Da (Fig. 2B).

3.3. Determination of amino acid sequence

For the major peak, both the HPLC (Fig. 1B-C) and mass spectrometry (Fig. 2) data were consistent with the presence of a single peptide. After reduction and pyridylethylation a single major derivative was obtained (data not shown).

Automatic sequencing of the pyridylethylated peptide for 27 cycles yielded a single sequence with 22 unambiguous amino acid residues and blank cycles starting at position 23: CKCPSCNFNDVTENCKCCIFRQ.

In comparing the theoretical monoisotopic mass of this sequence (2,548.02 Da for a free C-terminus; 2,547.04 Da for an amidated C-terminus) with the experimental monoisotopic mass (MALDI-TOF/ESI, 2,644.12/2,644.08 Da) a difference was observed: 96.10/96.06 Da if a free C-terminus is considered, 97.08/97.04 Da if an amidated C-terminus is supposed. This deficit indicated that either the C-terminal residue or a post-translational modification was not detected during the automated Edman degradation. The only amino acid residue and conotoxin post-translational modification with monoisotopic masses close enough to the difference found are, respectively, Pro (97.05 Da) and sulfination of Tyr (96.96 Da). The latter possibility was ruled out because the purified peptide does not contain Tyr residues. Thus, we tentatively concluded that a C-terminal Pro was not detected. The calculated monoisotopic mass for the putative, Pro-containing molecule is 2,645.07 Da for a free C-terminus, and 2644.09 Da for an amidated C-terminus. The experimental mass (2,644.12/2,644.08 Da) is very close (0.03/0.01 Da) to the theoretical mass of the amidated molecule. Thus, this concordance strongly suggests that the C-terminus of the purified peptide is amidated (Fig. 3, upper line). This inference was confirmed by the HCD fragmentation of the triply charged ion at m/z 882.69, which produced y ion series values corresponding to the theoretical values expected for an amidated C-terminal. The 115.062 Da value theoretically calculated for the first y ion series [(H₂O, 18.01 + H⁺, 1.008: 19.02 Da) + (96.042 Da, amidated Proline)] is in acceptable agreement with the experimental value obtained (115.09). The following values of the same ion series confirm the amidation and the correct sequence obtained previously by Edman degradation (Fig. 2C).

The sequencing of the native minor purified peptide for 25 cycles yielded a single sequence with 17 unambiguous amino acid residues and blank cycles at positions 1, 3, 6, 15, 17-18, and 24-25. Given its sequence identity with the major purified peptide, positions 1, 3, 6, 15, and 17-18 were assigned as Cys residues. Thus, the primary structure of this peptide is: CKCOSC NFNDVTENCKCCIFRQ?, where O represents 4-hydroxyproline, and ? indicates that the amidation of the C-terminus was not directly confirmed (Fig. 3, lower line).

3.4. Preliminary biological characterization of the major purified peptide

The i.c. injection, in 28-day old mice (n = 3), of the major purified peptide at 120 pmol/mouse (~0.317 ! g, ~12.4 ng/g) affected only one animal, causing a brief transitory

symptomatology involving paralysis and extension of hind limbs. However, a higher dose of 240 pmol/mouse (~0.635 ! g, ~24.9 ng/g) produced circular motion, urination and death of two animals, in addition to the symptoms elicited by the lower dose. Both control and test mice shared some symptoms, which can be attributed to the injection trauma, but none of the control animals displayed the symptoms observed only in three test mice (Table 1).

3.5. Similarity search and sequence comparison

The BLAST search revealed significant similarities (defined here as those “hits” having E-values < 2) with four M-conotoxins from three vermivorous species of the Indopacific (Mi3-D01 [GenBank: AEX60148], from *C. miles*; “cono peptide” [GenBank: AFI99276], from *C. characteristicus*; and Bt3-I03 [GenBank: AEX60048] and Bt3-I04 [GenBank: AEX60050], from *C. betulinus*). This search also displayed significant similarity with one M-conotoxin from a piscivorous species of the same region (S3-I05 [GenBank: AEX60054], from *C. striatus*) (Fig. 3B). We also found 97 sequences with less similarity: 1) the 20 sequences with $2 < E\text{-values} < 5$ were M-conotoxins, with one exception (an O-conotoxin); 2) the 77 sequences with E-values > 5 are mostly M-conotoxins, but there are also some O-, T-, alpha-, and I-conotoxins, among other *Conus* proteins.

The search by means of the ConoPrec tool yielded the six sequences closest to peptide as24a in the ConoServer database: Co3-R02 (GenBank: JF510548), Co3-H01 (GenBank: JF510546), and Co3-H03 (GenBank: JF510552), from *C. coronatus*; Cp3-R01 (GenBank: JF510547/ JF510550), from *C. capitaneus*; Vx3-R01 (GenBank: JF510549), from *C. vexillum*; and Eb3-H03 (GenBank: JF510620/JF510621), from *C. ebraeus* (Fig. 3B); all of them are expressed by vermivorous species of the Indopacific.

For comparison with peptide as24a, we only display the five most significant (E-values < 2) sequences (mature toxin region) found by BLAST, and the six sequences retrieved by ConoPrec (Fig. 3B).

Discussion

The venoms of *Conus* snails are complex mixtures of pharmacologically active peptides, most of which are disulfide-rich. This report continues the study of the components of the venom of *Conus cancellatus*, a vermivorous cone collected in the western Gulf of Mexico, Mexico, from which six- and four-Cys-containing peptides have been characterized by our group: an O-conotoxin [42] and two framework-XIV-conotoxins [43]; in these reports, we used the junior synonym (*Conus austini*) for this species.

The six Cys residues of the peptides purified and characterized in this work are arranged in a pattern (C-C-C-C-CC, where “-” represents one or more non-Cys residues) not previously reported for toxins of any *Conus* species. Conotoxins with six Cys residues have been characterized widely [8, 18-20], and the seven known patterns are frameworks II (CCC-C-C-C) [34], III (CC-C-C-CC) [36], IV (CC-C-C-C-C) [13], VI/VII (C-C-CC-C-C) [31], IX (C-C-C-C-C-C) [23], XVIII (C-C-CC-CC) [6], and XXIII (C-C-C-CC-C) [41] (Table 2). Therefore, based on the Cys frameworks recorded in the literature and the ConoServer database [8], we propose that the Cys framework of the peptides characterized in this work be assigned number XXIV. Thus, according to the nomenclature proposed by Olivera and Cruz [30], the names of the novel peptides are as24a and as24b.

The biological activity of many conotoxins has been evaluated by direct injection of a venom fraction into the central nervous system of mice; this method has successfully identified components active in vertebrates [16, 23, 33], even though the peptides were isolated from vermivorous cones, whose prey are invertebrates. Examples of conotoxins

from vermivorous *Conus* that are active in mice are: !-ImI and !-ImII, from *C. imperialis* (seizures and tremors) [26]; contryphan-Vn, of *C. ventricosus* (stiff tail syndrome) [25]; and conorfamide-Sr1, of *C. spurius* (hyperactivity syndrome) [24]. Although it is not possible to define circuit-specific effects in the CNS, some evidence provides experimental support for the proposal that the molecular targets of some conotoxins are specifically localized in both peripheral and central nervous system circuitry [33]. In this work, despite the low number of control and test animals used in the preliminary biological characterization (due to the availability of purified peptide), we found evidence suggesting that peptide as24a is biologically active, and for a peptide from a worm-hunting *Conus*, it seems to be very potent in the mammalian nervous system (! 5 pmol/g or 0.01 ! g/g), and to interact with important molecular targets in the central nervous system of the mouse (Table 1). This biological activity agrees with the notion that some receptors in the nervous system of polychaete worms (the natural prey of *C. cancellatus*) and vertebrates (mice) are moderately conserved. In fact, evidence from cloning of ionic channels and receptors suggests that these proteins evolved early in Eukaryota evolution and are present in all of the higher eukaryotic taxa [3].

In order to gain insight into the molecular target of peptide as24a we searched for the conotoxins most similar to it. We found that, despite having a different Cys framework, this peptide has local sequence similarities with some M-conotoxins, specifically ten M-1-conotoxins [9, 15] cloned from seven vermivorous species of the Indopacific, and one M-1-conotoxin cloned from a piscivore of the same region (see section 3.5). Unfortunately, no molecular target has been determined for any of these toxins, and thus, no putative molecular target could be tentatively proposed for peptide as24a. The difficulty in trying to identify the target of a conotoxin with a novel Cys framework was anticipated, and this issue has recently proven to be a challenging one, even employing a comprehensive experimental approach [39]. Recently, an M-1-conotoxin, It3a from *C. litteratus*, has been shown to enhance tetrodotoxin-sensitive sodium currents present in adult rat dorsal root ganglion neurons [40]; however, its sequence identity with peptide as24a (23%) is lower than those (27-40%) of the M-1-conotoxins found by the BLAST and ConoPrec similarity searches, and it is restricted to five Cys residues (Fig. 3B); thus, it does not seem feasible that peptide as24a targets neuronal sodium channels.

Although the target of conotoxin as24a has not been identified, this peptide is remarkable in two respects. First, it causes diverse symptoms and death in mice at a low dose (240 pmol/mouse or ~0.63 ! g/mouse). Second, it possesses a cysteine pattern clearly distinct from all those found in six-Cys-containing conopeptides studied previously, most of which occur in the venoms of species of the Indopacific region. Thus, *C. cancellatus* expresses a new pattern of cysteine residues that is probably a consequence of an evolutionary and ecological divergence related to the geographic separation from the Indopacific *Conus* species, and which might represent a novel gene superfamily; alternatively, the local sequence identity/similarity of this peptide with the C-termini of several M-1-conotoxins allows to speculate that peptides as24a/b might be members of a new branch of the M-superfamily. This suggests that *C. cancellatus* might contain more toxins with unusual arrangements of Cys residues; indeed, it expresses at least two toxins with framework-XIV [43], which is rather uncommon and has been found thus far only in a few species: two from the western Atlantic, 11 from the Indopacific, and one from the eastern Pacific [8].

The second peptide reported in this work, as24b, was obtained serendipitously while trying to purify more peptide as24a from a different batch of crude extract. In the conditions employed, it elutes slightly before peptide as24a, and this chromatographic behavior must be related to the presence of two additional hydroxyl groups (in the hydroxy-Pro residues) in comparison with this peptide. Although it was not possible to characterize its biological activity because of the small amount available after the purification and sequence

determination, its characterization revealed that post-translationally-modified peptides having the novel Cys- framework exist in the venom of *C. cancellatus*.

The characterization of more conotoxins from *C. cancellatus* and the determination of the gene superfamily and pharmacological family of peptides as24a and as24b will be of considerable interest and will help to establish the extent of conotoxin divergence in the species of the western Atlantic.

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Highlights

- Two six Cys-peptides (as24a and as24b) were purified from *Conus cancellatus* venom
- Peptides as24a and as24b have a novel arrangement of Cys residues
- Peptide as24a has paralytic and lethal activities
- Peptide as24b has two hydroxyproline residues
- Peptides as24a and as24b might represent a novel gene superfamily

Fig. 1. Purification of peptides as24a and as24b. Panel A) Fractionation of the venom extract (~0.3 mg total protein) on an analytical C18 column eluted with an isocratic step at 5% solution B for 10 min followed by a linear gradient from 5-55% solution B in 100 min, at 1 ml/min. HPLC solutions were: 0.1% (v/v) TFA in water (solution A), and 0.085% (v/v) TFA in 90% aqueous ACN (solution B). The absorbance was monitored at 220 nm. Panel B) The peak highlighted by an arrow in panel A was further purified on the same column, using an isocratic step at 20% solution B for 20 min, and a linear gradient from 20-35% solution B in 60 min, at 1 ml/min. Panel C) The peak shown in panel B was further purified on an analytical C8 column using an isocratic step at 20% solution B for 10 min, and a linear

gradient from 20-35% solution B in 60 min, at 1 ml/min. Panel D) The peak highlighted by an arrow and an asterisk in panel A was further purified on an analytical C8 column using an isocratic step at 20% solution B for 10 min, and a linear gradient from 20-35% solution B in 60 min, at 1 ml/min.

Fig. 2. Determination of the molecular mass of conotoxin as24a. Panel A) The MALDI-TOF monoisotopic m/z signal at 2,645.12 ($[M+H]^+$) corresponds to a monoisotopic mass of 2,644.12 Da. Panel B) The full scan mass spectrum obtained in an ESI-Orbitrap shows m/z signals corresponding to the triply ($[M+3H]^{3+}$, 882.69) and doubly charged ($[M+2H]^{2+}$, 1323.54) ions. The isotopic resolution ($R=60,000$) of the doubly charged ion (inset) indicates a monoisotopic mass of 2,644.08 Da. Panel C) HCD fragmentation spectrum showing the product ions derived from the triply charged precursor ion at m/z 882.69. The values of the main y ion series clearly demonstrate that the C-terminal of peptide as24a is amidated.

A

	5	10	15	20
as24a	<u>CK</u> <u>C</u> PS <u>C</u> NFNDVTEN <u>CK</u> <u>CC</u> IFRQP*			
as24b	<u>CK</u> <u>C</u> OS <u>C</u> NFNDVTEN <u>CK</u> <u>CC</u> IFRQO?			

B

Cp3-R01	--RC--CTWQECDGNCRCCQ----	16	31	v, IP
Co3-R02	--RC--CTWQECDGNCRCCQ----	16	31	v, IP
Vx3-R01	--RC--CTWQECDGNCRCCQ----	16	31	v, IP
Co3-H03	--RC--CNWQECDGNCHCCQ----	16	37	v, IP
Co3-H01	--RC--CTFSECDGNCHCCQ----	16	37	v, IP
Eb3-H03	---C--CRLRRCKTHCHCCVYRP-	18	27	v, IP/EP
Bt3-I04	QRRC--CVHPACHDDCICCIDR--	20	40	v, IP
Bt3-I03	QRRC--CVHPACHDDCICCITR--	20	40	v, IP
S3-I05	QRRC--CISPACHDDCICCITR--	20	40	p, IP
Mi3-D01	QQSC--CDIQWCYDNCDCCI----	18	33	v, IP
Ca-AFI99276	QRSC--CLKAWCEDDCRCCV----	18	27	v, IP
lt3a	-DEC--CEQWCDGACDCCS----	17	23	v, IP
as24a	-CKCPSCNFNDVTENCKCCIFRQP	23		
	* * . * *			

Fig. 3.

Panel A) Amino acid sequence of peptides as24a (upper line) and as24b (lower line) from *C. cancellatus*. Cys residues are displayed in bold font and underlined. For post-translational modifications, * and O represent, respectively, the amidation of the C-terminal residue, and 4-hydroxyproline residues, whereas ? indicates that the amidation of the C-terminus was not directly confirmed. Panel B) ClustalW2 alignment of peptide as24a (bold type) and 11 conotoxins similar to it: Cp3-R01 (*C. capitaneus*), Co3-R02, Co3-H03 and Co3-H01 (*C. coronatus*), Vx3-R01 (*C. vexillum*), Eb3-H03 (*C. ebraeus*), Bt3-I04 and Bt3-I03 (*C. betulinus*), S3-I05 (*C. striatus*), Mi3-D01 (*C. miles*), and Ca-AFI99276 (*C. characteristicus*). Amidations of the C-termini of as24a and Co3-H01 are not displayed. Identical residues and conserved substitutions with respect to toxin as24a are highlighted, respectively, in black and grey backgrounds. For all the sequences, asterisks and dots indicate, respectively, identical residues and semi-conserved substitutions. Figures from left to right are, respectively, number of residues in each peptide, and percent identity. v = vermivorous; p = piscivorous; IP = Indopacific; EP = eastern Pacific.

Table 1

Biological activity of peptide as24a from *Conus cancellatus* (jr. syn.: *C. austini*), assessed by intracranial injection in mice.

Normal saline solution (30 !l)	as24a* (120 pmol/30 !l: ~0.317 !g, ~12.4 ng/g)	as24a* (240 pmol/30 ~0.635 !g, ~24.9 ng/g)
0-10 min: unmoving, fast breathing, shrinking (contracted body). 10-50: min normal mice activity**	0-8 min: unmoving, fast breathing, shrinking (contracted body). In one mouse at 9 min: paralysis and extension of hindlimbs motion, (45 sec). Recovery by body beginning from 11 min. extension of Other two mice had normal later, activity** after 9 min. The other	0-12 min: unmoving, fast breathing, (contracted body). Two (at 15 min and 23 min) presented circular immediately followed paralysis with hindlimbs. One minute urination and death. mouse had no apparent behavioural distress and presented normal activity** after 13 min.

* Dissolved in normal saline solution.

** Sporadic scratching, grooming, and exploratory behaviour.

Table 2

Properties of six-Cys-containing conotoxins.*

Cysteine arrangement	Cys framework	Gene superfamily	Reference
CCC-C-C-C	II	A and M	[34]
CC-C-C-CC	III	M	[36]
CC-C-C-C-C	IV	A and M	[13]
C-C-CC-C-C	vi/vii	I1, I3, M, O1, O2, and O3	[31]
C-C-C-C-C-C	IX	M and P	[23]
C-C-CC-CC	XVIII	?	[6]
C-C-C-CC-C	XXIII	K	[41]
C-C-C-C-CC	XXIV	?	This work

* Information obtained from [8, 18-20].