Published in final edited form as:

Toxicol. 2008 July ; 52(1): 139–145. doi:10.1016/j.toxicol.2008.05.014.

Purification and characterization of a novel excitatory peptide from *Conus distans* venom that defines a novel gene superfamily of conotoxins

Ping Chen^{a,*}, James E. Garrett^b, Maren Watkins^c, and Baldomero M. Olivera^a

^aDepartment of Biology, 257 South, 1400 East, University of Utah, Salt Lake City, UT 84112, USA

^bCognetix, Inc., Salt lake City, Utah

^cDepartment of Pathology, 417 Wakara Way, Suite 3323, University of Utah, Salt Lake City, Utah 84108, USA

Abstract

An excitatory peptide, di16a, with 49 amino acids and ten cysteine residues was purified and characterized from the venom of *Conus distans*. Five AA residues were modified: one γ -carboxyglutamate (Gla), and four hydroxyproline (Hyp) residues. A cDNA clone encoding the precursor for the peptide was characterized; the peptide has a novel cysteine framework and a distinctive signal sequence that differs from any other conotoxin superfamily. The peptide was chemically synthesized and folded, and synthetic and native materials were shown to co-elute. Injection of the synthetic peptide causes a hyperexcitable phenotype in mice greater than three weeks of age at lower doses, and lethargy at higher doses. The peptide defines both a previously-uncharacterized gene superfamily of conopeptides, and a new Cys pattern with three vicinal Cys residues.

Keywords

Conus venom; *Conus* peptide; Conotoxin; γ -Carboxyglutamate; Hydroxyproline; *Conus distans*

1. Introduction

Each of the ~700 species of cone snails (*Conus*) has evolved its own repertoire of 100–200 different peptide toxins (conotoxins) (Terlau and Olivera 2004, Olivera 2006). The conotoxins are initially translated as prepropeptide precursors (Woodward et al. 1990). The organization of the prepropeptide includes a highly conserved N-terminal signal sequence that defines the superfamilies to which the conotoxins belong, as well as an intervening pro region that contain recognition signals for post-translational modification enzymes (see, for

*To whom correspondence should be addressed: Department of Biology, University of Utah, 257 South 1400 East, Salt Lake City, Utah, 84112. Telephone: (801) 581-8370 Fax: (801) 585-5010 pc15@utah.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

example, (Bandyopadhyay et al. 1998). The C-terminal mature toxin region encodes the functional gene product injected into a target animal to accomplish a specific physiological mission (Woodward et al. 1990, Olivera 2006). The majority of the conotoxins characterized to date are small peptides of 10–30 amino acids with a high frequency of cysteine residues that form disulfide bonds that make the toxins structurally constrained; the cysteine arrangement and connectivity are also indicative of the peptide superfamily to which a conotoxin belongs.

So far, 15 different Cys patterns have been identified, most of which define conotoxin superfamilies. Each superfamily comprises several families, each targeting a specific group of ion channels, receptors or transporters (Terlau and Olivera 2004). For example, δ -conotoxins from the O superfamily slow down the inactivation of Na channels (Fainzilber et al. 1994, Shon et al. 1995, Leipold et al. 2005). κ M-conotoxins from the M superfamily are specific K channel inhibitors (Ferber et al. 2003, Ferber et al. 2004), and α -conotoxins from the A superfamily inhibit different subtypes of nicotinic acetylcholine receptors (McIntosh et al. 1994, Cartier et al. 1996, Whiteaker et al. 2007).

This report describes the purification and characterization of a novel peptide, di16a, from the venom of the vermivorous *Conus* species, *Conus distans* (Fig. 1). Compared to typical conotoxins with 2–3 disulfide bonds and 10–30 amino acids, di16a is a much larger peptide, comprising 49 residues and 5 disulfide bonds. While some other conotoxins also contain 10 cysteines, such as σ -GVIIIA in the S-superfamily that targets the 5HT3 receptor and α S-RVIIIA that targets ACh receptors (England et al. 1998, Teichert et al. 2005), di16a has a quite different cysteine pattern, including 3 cysteine residues adjacent to each other. The characterization of di16a defines a novel family of conotoxins with a new Cys pattern (class 16).

2. Materials and Methods

Purification of di16a by reverse-phase HPLC

A crude venom extract was prepared from *Conus distans* as described previously (Jimenez et al. 1996). The venom extract was applied on a preparative Vydac C18 HPLC column (22mm $\times$ 250mm), and eluted using a gradient of 4–65% B90 (90% ACN with 0.1% TFA) at 2%ACN/min, followed by the gradient of 65–80% B90 for 4.3 minutes, followed by the gradient of 80–100% B90 for 2 minutes (Fig. 2A). The flow rate for the preparative column was 20mL/min. An analytical Vydac C18 HPLC column (4.6 mm $\times$ 250mm) with a gradient of 8–18% B90 at 0.2%B90/min at 1mL/min was used for subsequent fractionation (Fig. 2B). The same column, the same flow rate, and the gradient of 5–20% B90 at 0.5%B90/min were used for further purification of di16a (Fig. 2C) and for the coelution of the native and the synthetic peptides.

Mass spectrometry

ESI-MS spectra were measured on a Bruker Esquire 3000 Plus instrument performed at the Salk Institute for Biological Studies (San Diego, CA). All spectra were recorded in a positive ion mode. Alternatively, MALDI mass spectra were obtained through the Mass Spectrometry and Proteomic Core Facility of the University of Utah.

Peptide sequencing

The purified peptide was reduced with DTT and alkylated with 4-vinylpyridine as described previously (Gray 1993). The alkylated peptide was purified using HPLC and sequenced and analyzed using a PE ABI model 492 precise sequencer and PTH analyzer at the DNA/Peptide facility of University of Utah.

Peptide synthesis and confirmation

Linear peptide was synthesized by standard Fmoc(N-(9-fluorenyl)methoxycarbonyl) chemistry using an ABI Model 40A Peptide Synthesizer at the DNA/Peptide Core facility of the University of Utah. The peptide was folded by oxidation in the presence of a combination of oxidized and reduced glutathione (1mM GSSG, 2mM GSH, 0.1 M Tris-HCl, and 0.01 M EDTA at pH 7.5). The oxidation was allowed to progress at room temperature for 8 h. The homogeneity of the synthetic peptide was verified by MALDI mass spectrometry.

3'- and 5'-RACE

3'- and 5'-RACE were used to clone the cDNA of di16a from the fresh venom ducts of *C. distans* as described (Clontech Co.). Three PCR primers are designed according to the peptide and cDNA sequences as underlined in Fig. 5. The PCR products were sequenced. The whole cDNA sequence of di16a was generated by combining the two fragments amplified by 3'- and 5'-RACE. The signal sequence and the "pro" region were predicted using SignalP 3.0 server.

Biological Assays

The lyophilized peptide was dissolved in normal saline solution and injected using a 29-gauge insulin syringe. Swiss Webster mice (2 weeks and 3 weeks old or older) were injected intracranial (*i.c.*) with 20 μ L of the peptide solution. Control animals were similarly injected with normal saline solution.

3. Results

Peptide purification and characterization

Venom was extracted from specimens of *Conus distans* collected in the Philippines (see Figure 1) as described previously (Jimenez et al. 1996). The venom ducts were dissected and venom removed from the ducts. An initial fractionation of *C. distans* venom was carried out (Figure 2A). The fraction containing di16a was further subjected to subsequent fractionation to purify the peptide (designated di16a, see following sections) (Figure 2B, 2C). The apparently homogenous purified peptide was analyzed by ESI mass spectrometry; a monoisotopic molecular weight of 5065.33 Da was obtained (Figure 3). The peak of 5021.44 Da is result from the decarboxylation of a carboxyglutamate (Gla or γ) residue when MALDI was carried out.

The amino acid sequence of the di16a peptide (Figure 3) was determined by standard Edman sequencing (see Methods). The peptide has 49 amino acids, including 10 cys residues; a notable feature is that three Cys residues are adjacent to each other in the primary sequence.

The amino acid sequence has a preponderance of hydroxylated AA: 7 Thr, 6 Ser and 4 Hyp. The AA sequence yields a predicted mass of 5065.5 Da, which is consistent with the actual experimental mass value 5065.33 Da.

Peptide synthesis; biological activity

The di16a peptide was synthesized and folded as described in Materials and Methods. The homogeneity of the peptides was confirmed by MALDI mass spectrometry (see Figure 3). Coelution of the synthetic and native toxins using an analytical Vydac C18 column resulted in a single symmetric peak (Figure 4), suggesting identity of the synthetic and native peptides.

Mice at different ages were injected *i.c.* with synthetic di16a. At a dose of 1.4~3 nmol/g, di16a elicited hyperactivity in mice 3 weeks old or older. At a higher dose (4 nmol/g), mice exhibited hyperactivity followed by slight lethargy; an even higher dose (6 nmol/g), elicited lethargy in the injected mice. However, di16a does not elicit any apparent phenotypic effects in 2 week-old mice (see Table 1). Thus, the symptomatology elicited by injection of the peptide is age-dependent.

A cDNA clone encoding the precursor structure of di16a

A cDNA clone encoding the di16a peptide precursor was obtained by 3'- and 5'-RACE. The amino acid sequence deduced from the cDNA sequence (Figure 5) revealed that di16a was initially translated as a prepropeptide of 98 amino acids with an organization similar to previously characterized precursors of other disulfide-rich peptide toxins from *Conus* venoms (Woodward et al. 1990). A signal peptide of 19 residues at the N-terminus is followed by a "pro" region of 30 residues, with the expected mature toxin sequence at the C-terminus. The precursor sequence has no apparent similarity to previously characterized *Conus* venom prepropeptides.

4. Discussion

Conus distans venom has not been analyzed extensively; to date, only two polypeptide toxins, 25.5 kDa and 24 kDa, respectively, were isolated and identified, but their peptide sequences were not reported (Saxena et al. 1992, Partoens et al. 1996). These two venom components were reported to affect the uptake of Ca in bovine chromaffin cells; the 25.5 kDa-component increases the initial rate of Ca uptake but it does not inhibit the stimulation-evoked uptake, whereas the 24 kDa-component produces the complementary effects. A combination of the two peptides inhibits noradrenaline release in rat hippocampus.

In this report, a novel *Conus* peptide, di16a, was purified and characterized from the venom of *C. distans*. Its amino acid sequence, including post-translational modifications, and sequence of the corresponding prepropeptide precursor, were established by a combination of Edman sequencing, 3'- and 5'-RACE amplification, mass spectrometric analysis and chemical synthesis. The biological activity elicited by the peptide was characterized by *i.c.* injection in mice; the symptomatology observed is age-dependent and is elicited in mice that are over three weeks of age.

The AA sequence of di16a differs significantly from all other previously characterized *Conus* peptides. One unique feature is that the mature toxin comprises 49 amino acid residues (in contrast to 10–30 AA residues in most other conopeptides); however, like most conotoxins, it has a high density of disulfide cross-links. It is among the longest *Conus* peptides whose amino acid sequences have been determined; while it is comparable in length to konkunitzin-S (Bayrhuber et al. 2005, Dy et al. 2006, Imperial et al. 2007), a striking difference is that di16a is much more disulfide rich. The 10 cysteine residues presumably form intramolecular disulfide bonds in a novel framework: XCX₅CX₄CX₆CCCX₅CX₉CXCX₆CX₂. Two other conotoxins containing 10 cysteines have reported, σ-GVIII A and αS-RVIII A; these both belong to the S-superfamily (England et al. 1998, Teichert et al. 2005). Di16a contains three adjacent cysteine residues; this is the first time that a peptide with such an arrangement of 10 Cys residues has been reported and, thus, defines a novel Cys pattern. In addition, the signal sequence of di16a lacks any apparent homology to any other conotoxin signal sequence, indicating that di16a is a member of a new superfamily of *Conus* peptides.

The peptide contains two types of post-translational modifications, one carboxyglutamate (γ or Gla) and four hydroxyprolines (O or Hyp). The presence of Gla suggests the presence of a γ-carboxylase recognition sequence (γ-CRS) that directs the vitamin K-dependent carboxylase to recognize and catalyze the carboxylation of the single glutamate residue in the mature peptide region (Brown et al. 2005, Bandyopadhyay 2008). The γ-CRS of many other Gla-containing peptides generally resides on their propeptide region –20 to –1. This region generally contains multiple basic residues and leucine residues (Bandyopadhyay et al. 1998). Like other γ-CRS containing propeptides, the –20 to –1 region of the propeptide of di16a displays a marked preference for basic residues (Isoelectric point=12.3, Peptide property calculator, software at www.innovagen.com) and leucine, suggesting the presence of γ-CRS.

Hydroxyproline residues are a commonly occurring post-translationally modified amino acid in conotoxins. In other conotoxins, hydroxyproline plays a role in either folding and/or functional efficacy (Buczek et al. 2005). It is notable that there is one Pro residue that is not hydroxylated, raising the question of how the post-translational modification enzyme recognizes which Pro residues to modify (and which not to modify).

The peptide elicits hyperactivity in mice in an age-dependent manner. Injected mice 3 weeks old or older showed hyperactivity when exposed to a loud sound at the lower doses tested (1.4–3 nmol/g). At a higher dosage of 4 nmol/g, however, hyperactivity and lethargy occurred alternatively in the tested mice. At an even higher dosage of 5–6 nmol, the mice became lethargic. On the other hand, similar injections in the mice 2 weeks old did not elicit any apparent abnormal behaviors. Another conopeptide that has age-dependent activity is conantokin-G, which is 17 AA long and contains no Cys residues (McIntosh et al. 1984). In contrast to di16a, intracranial injections of conantokin-G into 2-week-old mice resulted in a sleeper syndrome, which did not occur when mice between 3 and 4 weeks old were injected (Rivier et al. 1987), but some lethargic and hyperactive behaviors were noted in older mice following conantokin-G injection. Conantokin-G is a highly selective NMDA receptor inhibitor (Mena et al. 1990, Hammerland et al. 1992), and the age-related symptomatology of

this peptide is likely indicative of developmental changes in circuitry containing the NMDA receptors in the mouse brain. Like conantokin-G, the age-related symptoms observed for di16a are suggestive of developmental changes of the specific molecular targets of this peptide. However, when 100 μ M of di16a was tested on the rat NMDA receptor, as well as mouse glutamate receptor, rat K_V1.1 channel, human K_V1.2 through K_V1.6 channels, rat Na_V1.2 and Na_V1.4 channels, and rat Ach receptor α 9/ α 10 as described previously (Imperial et al. 2006, Walker et al. 2006, Buczek et al. 2007, Teichert et al. 2007, Zhang et al. 2007, Whiteaker et al. 2008), no apparent activities were observed (not illustrated). The molecular identity of the target of di16a remains undetermined and needs to be elucidated.

Acknowledgments

This work was supported by National Institutes of Health Program Project GM 48677. We thank Dr. Michael Tamkun for sharing the rat K_V1.1 clone. We thank Dr. Minmin Zhang for testing Na channels, Cheryl Dowell for testing Ach receptor, Vernen Twede for testing NMDA receptor, and Dr. Graig Walker for testing glutamate receptor. We thank Dr. Grzegorz Bulaj for helpful discussion.

References

- Bandyopadhyay, P. Vitamins and Hormones. Elsevier Inc; 2008. Vitamin K-Dependent gamma-Glutamylcarboxylation: an Ancient Posttranslational modification. In press
- Bandyopadhyay PK, Colledge CJ, Walker CS, Zhou LM, Hillyard DR, Olivera BM. Conantokin-G precursor and its role in γ -carboxylation by a vitamin K-dependent carboxylase from a *Conus* snail. J Biol Chem. 1998; 273:5447–5450. [PubMed: 9488665]
- Bayrhuber M, Vijayan V, Ferber M, Graf R, Korukottu J, Imperial J, Garrett JE, Olivera BM, terlau H, Zweckstetter M, Becker S. Conkunitzin-S1 is the first member of a new Kunitz-type neurotoxin family – structural and functional characterization. J Biol Chem. 2005; 180:21246–21255.
- Brown MA, Begley GS, Czerwicz E, Stenberg LM, Jacobs M, Kalume DE, Roepstorff P, Stenflo J, Furie BC, Furie B. Precursors of novel Gla-containing conotoxins contain a carboxy-terminal recognition site that directs gamma-carboxylation. Biochemistry. 2005; 44:9150–9159. [PubMed: 15966739]
- Buczek O, Bulaj G, Olivera BM. Conotoxins and the posttranslational modification of secreted gene products. Cell Mol Life Sci. 2005; 62:3067–3079. [PubMed: 16314929]
- Buczek O, Wei D, Babon JJ, Yang X, Fiedler B, Chen P, Yoshikami D, Olivera BM, Bulaj G, Norton RS. Structure and sodium channel activity of an excitatory II-superfamily conotoxin. Biochemistry. 2007; 46:9929–9940. [PubMed: 17696362]
- Cartier GE, Yoshikami D, Gray WR, Luo S, Olivera BM, McIntosh JM. A new α -conotoxin which targets α 3 β 2 nicotinic acetylcholine receptors. J Biol Chem. 1996; 271:7522–7528. [PubMed: 8631783]
- Dy CY, Buczek P, Imperial JS, Bulaj G, Horvath MP. Structure of conkunitzin-S1, a neurotoxin and Kunitz-fold disulfide variant from cone snail. Acta Crystallogr D Biol Crystallogr. 2006; 62:980–990. [PubMed: 16929098]
- England LJ, Imperial J, Jacobsen R, Craig AG, Gulyas J, Akhtar M, Rivier J, Julius D, Olivera BM. Inactivation of a serotonin-gated ion channel by a polypeptide toxin from marine snails. Science. 1998; 281:575–578. [PubMed: 9677203]
- Fainzilber M, Kofman O, Zlotkin E, Gordon D. A new neurotoxin receptor site on sodium channels is identified by a conotoxin that affects sodium channel inactivation in molluscs and acts as an antagonist in rat brain. J Biol Chem. 1994; 269:2574–2580. [PubMed: 8300586]
- Ferber M, Al-Sabi A, Stocker M, Olivera BM, Terlau H. Identification of a mammalian target of κ M-conotoxin RIIIK. Toxicon. 2004; 43:915–921. [PubMed: 15208025]
- Ferber M, Sporning A, Jeserich G, DeLa Cruz R, Watkins M, Olivera BM, Terlau H. A novel *Conus* peptide ligand for K⁺ channels. J Biol Chem. 2003; 278:2177–2183. [PubMed: 12399472]

- Gray WR. Disulfide structures of highly bridged peptides: a new strategy for analysis. *Protein Sci.* 1993; 2:1732–1748. [PubMed: 8251945]
- Hammerland LG, Olivera BM, Yoshikami D. Conantokin-G selectively inhibits N-methyl-D-aspartate-induced currents in *Xenopus* oocytes injected with mouse brain mRNA. *Eur J Pharmacol.* 1992; 226:239–244. [PubMed: 1358659]
- Imperial J, Silver N, Olivera BM, Bandyopadhyay P, Sporning A, Ferber M, Terlau H. Using chemistry to reconstruct evolution: on the origins of fish-hunting in venomous cone snails. *Proceedings of The American Philosophical Society.* 2007; 151:185–200.
- Imperial JS, Bansal PS, Alewood PF, Daly NL, Craik DJ, Sporning A, Terlau H, Lopez-Vera E, Bandyopadhyay PK, Olivera BM. A novel conotoxin inhibitor of Kv1.6 channel and nAChR subtypes defines a new superfamily of conotoxins. *Biochemistry.* 2006; 45:8331–8340. [PubMed: 16819832]
- Jimenez EC, Olivera BM, Gray WR, Cruz LJ. Contryphan is a D-tryptophan-containing *Conus* peptide. *J Biol Chem.* 1996; 281:28002–28005. [PubMed: 8910408]
- Leipold E, Hansel A, Olivera BM, Terlau H, Heinemann SH. Molecular interaction of delta-conotoxins with voltage-gated sodium channels. *FEBS Lett.* 2005; 579:3881–3884. [PubMed: 15990094]
- McIntosh JM, Olivera BM, Cruz LJ, Gray WR. γ -Carboxyglutamate in a neuroactive toxin. *J Biol Chem.* 1984; 259:14343–14346. [PubMed: 6501296]
- McIntosh JM, Yoshikami D, Mahe E, Nielsen DB, Rivier JE, Gray WR, Olivera BM. A nicotinic acetylcholine receptor ligand of unique specificity, α -conotoxin ImI. *J Biol Chem.* 1994; 269:16733–16739. [PubMed: 8206995]
- Mena EE, Gullak MF, Pagnozzi MJ, Richter KE, Rivier J, Cruz LJ, Olivera BM. Conantokin-G: a novel peptide antagonist to the N-methyl-D-aspartic acid (NMDA) receptor. *Neurosci Lett.* 1990; 118:241–244. [PubMed: 2177176]
- Olivera BM. *Conus* peptides: biodiversity-based discovery and exogenomics. *J Biol Chem.* 2006; 281:31173–31177. [PubMed: 16905531]
- Partoens P, Wang JM, Coen EP, Vauquelin G, De Potter WP. Two polypeptide toxins with opposite effects on calcium uptake in bovine chromaffin cells: isolation from the venom of the marine snail *Conus distans*. *Neurochem Int.* 1996; 28:619–624. [PubMed: 8792344]
- Rivier J, Galyean R, Simon L, Cruz LJ, Olivera BM, Gray WR. Total synthesis and further characterization of the γ -carboxyglutamate-containing “sleeper” peptide from *Conus geographus* venom. *Biochemistry.* 1987; 26:8508–8512. [PubMed: 3442672]
- Saxena VK, Partoens P, de Block J, Coen EP, Vauquelin G, De Potter WP. Inhibition of evoked neurotransmitter release from rat hippocampus by a polypeptide toxin isolated from the marine snail *Conus distans*. *Neurochem Int.* 1992; 20:69–74. [PubMed: 1304320]
- Shon K, Grilley MM, Marsh M, Yoshikami D, Hall AR, Kurz B, Gray WR, Imperial JS, Hillyard DR, Olivera BM. Purification, characterization and cloning of the lockjaw peptide from *Conus purpurascens* venom. *Biochemistry.* 1995; 34:4913–4918. [PubMed: 7711013]
- Teichert RW, Jimenez EC, Olivera BM. α S-Conotoxin RVIIIA: a structurally unique conotoxin that broadly targets nicotinic acetylcholine receptors. *Biochemistry.* 2005; 44:7897–7902. [PubMed: 15910004]
- Teichert RW, Jimenez EC, Twede V, Watkins M, Hollmann M, Bulaj G, Olivera BM. Novel conantokins from *Conus parius* venom are specific antagonists of N-methyl-D-aspartate receptors. *J Biol Chem.* 2007; 282:36905–36913. [PubMed: 17962189]
- Terlau H, Olivera BM. *Conus* venoms: a rich source of novel ion channel-targeted peptides. *Physiol Rev.* 2004; 84:41–68. [PubMed: 14715910]
- Walker CS, Brockie PJ, Madsen DM, Francis MM, Zheng Y, Koduri S, Mellem JE, Strutz-Seeböhm N, Maricq AV. Reconstitution of invertebrate glutamate receptor function depends on stargazin-like proteins. *Proc Natl Acad Sci U S A.* 2006; 103:10781–10786. [PubMed: 16818877]
- Whiteaker P, Christensen S, Yoshikami D, Dowell C, Watkins M, Gulyas J, Rivier J, Olivera BM, McIntosh JM. Discovery, synthesis, and structure activity of a highly selective α 7 nicotinic acetylcholine receptor antagonist. *Biochemistry.* 2007; 46:6628–6638. [PubMed: 17497892]

- Whiteaker P, Marks MJ, Christensen S, Dowell C, Collins AC, McIntosh JM. Synthesis and characterization of [¹²⁵I]{α}-conotoxin ArIB[V11L;V16A], a selective {α}7 nAChR antagonist. *J Pharmacol Exp Ther*. 2008
- Woodward SR, Cruz LJ, Olivera BM, Hillyard DR. Constant and hypervariable regions in conotoxin propeptides. *EMBO J*. 1990; 1:1015–1020. [PubMed: 1691090]
- Zhang MM, Green BR, Catlin P, Fiedler B, Azam L, Chadwick A, Terlau H, McArthur JR, French RJ, Gulyas J, Rivier JE, Smith BJ, Norton RS, Olivera BM, Yoshikami D, Bulaj G. Structure/function characterization of micro-conotoxin KIIIA, an analgesic, nearly irreversible blocker of mammalian neuronal sodium channels. *J Biol Chem*. 2007; 282:30699–30706. [PubMed: 17724025]

Figure 1. The shell of *Conus distans*

Conus distans is a relatively large *Conus* species; the specimen shown, collected in the Philippines is 110mm in length.

Figure 2. Purification of di16a from the crude venom of *C. distans*

The arrow in each HPLC run indicates the location of fractions containing the peptide di16a.

(A) The venom extract was chromatographed in a preparative Vydac C18 column eluted with the gradient described under the Materials and Methods. (B) The peak containing the fraction indicated by an arrow in (A) was subfractionated using an analytical Vydac C18 column under conditions described in Materials and Methods. (C) The peak indicated by an arrow in (B) was applied to an analytical Vydac C18 column and eluted using the gradient specified in Materials and Methods.

GCDOTDGCQTTC γ TDTGCCCKPNFTCQISNSGTKSCSCSGQOSDCOV

Figure 3. Mass spectrometry of the di16a peptide

The sequence of di16a (O= 4-transhydroxyproline; γ = carboxyglutamate) obtained by standard Edman methods is shown. The sequence obtained is consistent with the mass determined for the major peak. Measurements were carried out as described under Materials and Methods.

Figure 4. Co-elution of the native and synthetic di16a

(A) Native peptide (0.5 nmol) was applied on the analytical Vydac C18 column with a gradient of 4.5–22.5% ACN/40min at 1ml/min. The same conditions were used for the synthetic folded peptide (B). (C) Co-elution was carried out by mixing native and synthetic peptides on the analytical Vydac C18 column, using the same gradient and flow rate.

A. Sequence of a cDNA clone

```

1  AGCTACATC AGATCTTCAC CTGCCGACAG ACGTGTCTCC TGTTCCTCTCC
51  ATCTCCGTCT ACCTGCTTGT CTGCATACCT ACCTGTGCCT GTGCGCATAC
101 CTACCTGTGC TTGTCCGGCC ATCGTCTCAC TGCAGACTTG AACAGAATTG
151 AAAGGCAGCC AAG ATG TCC ACA CTG GGA ATA CTG CTG CCG ATC
      M   S   T   L   G   I   L   L   P   I
193 GCT CTT CTG TTG CTT CTT GCC AAC CCT GCA GAA AAT GGA GAT
      A   L   L   L   P   L   A   N   P   A   E   N   G   D
235 GGT CAA GCG ATG CCA AGA ACC CGC AAC CTC CGT TCA TTG AGC
      G   Q   A   M   P   R   T   R   N   L   R   S   L   S
277 TTT GGG CGT ACC CTC AGG CGG CTT GAG AAA CGA GGT TGT GAT
      F   G   R   T   L   R   R   L   E   K   R   G   C   D
319 CCC ACT GAC GGC TGT CAG ACA ACT GTG TGC GAA ACG GAT ACT
      P   T   D   G   C   Q   T   T   V   C   E   T   D   T
361 GGC CCC TGC TGC TGC AAG CCC AAT TTT ACC TGC CAA ATC TCG
      G   P   C   C   C   K   P   N   F   T   C   Q   I   S
      Primer 1
403 AAC AGT GGT ACG AAG TCA TGT AGC TGT TCG GGT CAG CCT TCT
      N   S   G   T   K   S   C   S   C   S   G   Q   P   S
445 GAT TGT CCG GTG TAA GCGAACCACA AAGACACAAC CTTGATCAAC
      D   C   P   V   -   Primer 3           Primer 2
490 ACCGTAATTT GCTTCTAAAT AAAACAGAGA CACAGAAAAA AAAAAA

```

B. Predicted precursor sequence:

(After post-transcriptional modification but before proteolytic cleavage indicated by arrow)

↓

MSTLGILLPIALLLPLANPAENGDGQAMPRTNLRSLSFGRTLRRLEKRG**GCD**OTDGCQTTVC γ TDTG**QCCCK**
PNFTCQISNSGTKSCSG**QOSDCOV**

Figure 5. Sequence of di16a precursor

Top: the cDNA sequence and the corresponding predicted amino acid sequence encoded in the Di16.1 cDNA clone. The sequences used for 3', 5'-RACE primers are underlined.

Bottom: The peptide precursor sequence. The sequence in bold represents the mature toxin. The sequence in normal type is the signal sequence, with the pro region in italics. The amino acid residues underlined represent the posttranslational modifications (O= 4-transhydroxylated proline, γ =carboxylglutamate).

Table 1

Mouse bioassay for the peptide di16a, following i.c. administration^a

Dose	Mice at 2 weeks old	Mice of 3 weeks old or older
~0.7nmol/g	No detectable effects	No detectable effects
1.4~3nmol/g	No detectable effects	1–2 min after injection, hyperactive, hypersensitive to stimuli; mice move fast or jump when exposed to a loud sound or touch; normal behavior 5–10 min after injection at the lower dose, recovered after 20–30 min after injection at the higher doses.
~4nmol/g	No detectable effects	Hyperactive after injection for 10min, followed by slight lethargy for a few minutes; mice recover 30–60min after injection.
5~6nmol/g	No detectable effects	Immediate lethargy following injection; rear legs weak and gasp for air; 10 min after injection, move slowly occasionally but generally lethargic, unresponsive to stimuli like sound and touch; recover 30–60 min after injection.

^a 1–4 mice per dose were used in these experiments.