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Conantokin-P, an Unusual Conantokin with a Long Disulfide Loop

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Abstract

The conantokins are a family of *Conus* venom peptides (17-27AA) that are N-methyl D-aspartate (NMDA) receptor antagonists. Conantokins lack disulfide bridges (six out of seven previously characterized peptides are linear), but contain multiple residues of γ -carboxyglutamate. These post-translationally modified amino acids confer the largely helical structure of conantokins by coordinating divalent metal ions. Here, we report that a group of fish-hunting cone snails, *Conus purpurascens* and *Conus ermineus*, express a distinctive branch of the conantokin family in their venom ducts. Two novel conantokins, Conantokin-P (Con-P) and Conantokin-E (Con-E) are 24 AA long and contain five γ -carboxyglutamate residues. These two peptides are characterized by a long disulfide loop (12 amino acids including two Glu residues between the Cys residues). The oxidative folding studies of Con-P revealed that the formation of the disulfide bond proceeded significantly faster in the presence of Ca^{++} ions. Circular dichroism suggested that Con-P is less helical than other previously characterized conantokins. Con-P blocks NMDA receptors containing NR2B subunit with submicromolar potency. Furthermore, the subtype-selectivity for different NR2 subunits differs from that of the previously characterized conantokins. Our results suggest that different branches of the phylogenetic tree of cone snails have evolved distinct groups of conantokins, each with its own unique biochemical features.

1. Introduction

The conantokins are *Conus* venom components comprising the only peptide family known to specifically target N-methyl-D-aspartate (NMDA) receptors (Haack et al., 1990; Hammerland et al., 1992; Mena et al., 1990). There is a surprising diversity of conantokin and conantokin-like peptides from *Conus*, all of which are encoded by the same gene superfamily. Multiple residues of the unusual post-translationally modified amino acid, γ -carboxyglutamate (Glu) are present (McIntosh et al., 1984; Olivera et al., 1985). Most conantokins (six out of seven currently known) lack disulfide bonds; the Glu residues are the major determinant that confers

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the characteristic helical structure in the presence of divalent cations such as Ca^{++} (Chen et al., 1998). The importance of Ca^{++} chelation of Gla residues in stabilizing helical structure in polypeptides was first established for the Gla domains of mammalian blood-clotting factors (Sunnerhagen et al., 1996).

The first conantokin peptide, conantokin-G (con-G) was purified from the venom of *Conus geographus* over 20 years ago (McIntosh et al, 1984). In the ensuing decades, 3 additional conantokin peptides were characterized: Conantokin T (con-T) from the venom of *Conus tulipa* (Haack et al., 1990) conantokin R (con-R) from the venom of *Conus radiatus* (White et al., 2000) and Conantokin L (con-L) from the venom of *Conus lynceus* (Jimenez et al., 2002). Recently, three different conantokin peptides were identified and characterized from the venom of a single cone snail species, *Conus parius* (con-Pr1, con-Pr2 and con-Pr3) (Teichert et al., 2007). The conantokins from *Conus parius* were potent antagonists of the NMDA receptors containing NR2B subunit, but did not affect the activity of NR2A or NR2C containing receptor subtypes. Interestingly, some AA residues previously thought to be invariant in natural conantokins were found to be substituted in the *C. parius* conantokins.

Although the seven conantokin peptides previously purified from *Conus* venoms are quite diverse in AA sequence, the five species of *Conus* from which they were obtained belong to two well-known clades of fish-hunting, cone snails. Two of the species, *Conus geographus* and *Conus tulipa* are fish-hunting species that use a net strategy to capture their fish prey (Olivera 1999; Olivera et al., 1985), and belong to the subgenus *Gastroidium*. The three other species, *Conus radiatus*, *Conus lynceus* and *Conus parius* are believed to be piscivorous species, though little is known about how they actually capture fish. No detailed observations have been published describing how they envenomate their prey; all three species belong to the subgenus *Phasmoconus*.

In this report, we describe members of the conantokin family from two fish-hunting *Conus* species (see Figure 1) that do not belong in the same subgenera, *Conus purpurascens* (the purple cone) and *Conus ermineus* (the turtle cone). These are the only known fish-hunting *Conus* species from the tropical waters of the new world, and are believed to have evolved fish hunting independently (Imperial et al., 2007). *Conus ermineus* is a species widely distributed over the Atlantic Ocean, while *Conus purpurascens* is the only known piscivorous species in the Panamic biogeographic marine province. As shown in the phylogenetic tree in Figure 2, the two species are in quite a different branch within *Conus*, (the subgenus *Chelyconus*), from the other *Conus* species from which conantokins were previously characterized. The members of the conantokin family from these species have very distinctive AA sequence features, demonstrating that the conantokin family can have clade-specific characters. This study shows that the informed use of phylogenetics to systematically explore *Conus* peptide families (such as the conantokins) leads to the more efficient discovery of novel compounds (Espiritu et al., 2001; Olivera and Teichert, 2007).

2. Materials and Methods

2.1. Preparation of genomic DNA; characterization of clones encoding Conantokins

Genomic DNA was prepared from 50 mg of *Conus purpurascens* and *Conus ermineus* tissues using the Gentra PUREGENE DNA Isolation Kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's standard protocol. These genomic DNAs were used as templates for polymerase chain reaction (PCR) with oligonucleotides corresponding to conserved regions of the signal sequence and 3' UTR sequences of Conantokin prepropeptides. In the case of Conantokin-E, the 5' primer sequence binds downstream of the start codon, giving rise to sequence lacking the first eight residues of the signal sequence.

The resulting PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Indianapolis, IN) following the manufacturer's suggested protocol. The eluted DNA fragments were annealed to pAMP1 vector and the resulting products transformed into competent DH5a cells, using the CloneAmp pAMP System for Rapid Cloning of Amplification Products (Life Technologies/Gibco BRL, Grand Island, NY) following manufacturer's suggested protocols. The nucleic acid sequences of the resulting conantokin toxin-encoding clones were determined using standard Edman for Automated sequencing.

2.2. Peptide synthesis

The conantokin-P peptide was synthesized on solid support using ABI Model 430A peptide synthesizer by standard N-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry and activated pentafluorophenyl (Opfp) esters. The side chains of cysteines were protected with trityl group and gamma-carboxy glutamic acids were protected with tertiary butyl groups. Peptide was cleaved from the resin and simultaneous deprotection of side chains was achieved by agitating 20 mg of the resin in 1ml of reagent K {TFA/phenol/thioanisole/ water/ethanedithiol (82.5:5:5:5:2.5)} at room temperature. The mixture was filtered, precipitated using methyl-tert-butyl ether (MTBE) and pelleted by centrifugation. The pellet was repeatedly washed with ether and purified over a Vydac C₁₈ column (10mm × 250mm, 5 μm particle size) using ACN/H₂O/TFA solvent system. The flow rate was maintained at 3 ml min⁻¹ following a linear gradient of 10–40% ACN over 40 min. and the fractions were detected at 220 nm. The purified linear conantokin-P was subjected for oxidation using red-ox buffer oxidized/ reduced glutathione at pH 7.5. The folded conantokin-P was further purified using RP-HPLC over Vydac C₁₈ column (10mm × 250mm, 5 μm particle size) and characterized using mass spectrometry.

2.3. Oxidative Folding

Folding reactions were initiated by resuspending linear peptide into a 200 μl solution of folding buffer containing 0.1 M Tris-HCl (pH 7.5), 1 mM oxidized glutathione and 1 mM reduced glutathione. The reaction mixture also contains 1 mM EDTA or 10 mM CaCl₂ with the final peptide concentration of 20 μM. After an appropriate folding time, the reactions were quenched by acidification with formic acid (10% final concentration). The samples were separated by reversed-phase C₁₈ analytical HPLC over a linear gradient of 10–40% ACN in 40 minutes. The accumulation of folded peptide or decrease in linear peptide was calculated relative to other folding species by integrating the RP-HPLC peaks. The percentage of accumulation of folded peptide at particular time point was an average value obtained from three independent experiments. The linear peptide, folded peptide and the reaction intermediates were characterized by mass spectrometry. The experimental points were analyzed by prism software (GraphPad Software Inc., San Diego CA) and the rate of the reaction was calculated by single exponential fit.

2.4. Mass spectrometry

Electrospray Ionization (ESI) mass spectra were obtained using a Micromass Quattro II mass spectrometer at the Mass Spectrometry and Proteomic Core Facility of the University of Utah. The spectra were recorded over a mass range of 2000–8000 m/z. Nitrogen gas was used for nebulization. The data were analyzed using the MassLynx data analysis software.

2.5. Circular Dichroism

CD data were collected on an Aviv 62DS circular dichroism spectropolarimeter (University of Utah). All measurements were taken at room temperature in a 0.1 cm path length cuvette between 200 and 300 nm. Peptides were dissolved at 100 μM final concentration in 10mM

HEPES buffer, pH 7.0, with or without 2 mM CaCl₂. The molar ellipticity (θ) was calculated by using the following equation:

$$\theta = [100 * (\text{CD signal at 222 nm}) / (n - 1) * L * (\text{concentration of peptide in mM})],$$

where n = number of residues in the peptide; L = pathlength of the cuvette in cm, and the CD signal is in millidegrees.

The CD signal was adjusted by subtracting the CD signal for buffer solution alone from the CD signal for the solution containing peptide. Molar ellipticity of $-34642.20 \text{ degrees cm}^2 \text{ dmol}^{-1}$ was estimated to be a perfect α -helix (100% α -helix) (Chen et al., 1974). The percent helical conformation was calculated by assuming a linear relationship in comparison with 100% α -helix.

2.6. Heterologous expression of NMDA receptors in *Xenopus* oocytes

The rat NMDA receptor clones used were NR2A, NR2B, NR2C, NR2D, and NR1-3b; GenBank numbers AF001423, U11419, U08259, U08260, and U08266, respectively. All of the expression clones were driven by a T7 promoter and were used to make capped RNA (cRNA) for injection into the oocytes of *Xenopus laevis* frogs. The protocol for *Xenopus* oocyte harvesting was described previously in detail (Cartier et al., 1996). cRNA was prepared *in-vitro* using Ambion RNA transcription kits (Ambion, Inc.), according to manufacturer's protocols. To express NMDA receptors, 2–5 ng of cRNA for each subunit was injected per oocyte. All voltage-clamp electrophysiology was done using oocytes 1–6 days after injection.

2.7. Two-electrode voltage clamp electrophysiology

All oocytes were voltage clamped at -70 mV at room temperature. Oocytes were gravity perfused with Mg^{2+} -free ND96 buffer (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.2–7.5). Mg^{2+} was not included in the ND96 buffer because Mg^{2+} blocks NMDA receptors at the voltage potential used to clamp oocytes (-70 mV). To reduce nonspecific absorption of peptide, bovine serum albumin (BSA) was added to ND96 buffer at a final concentration of 0.1 mg/ml. To elicit current from oocytes expressing NMDA receptors, one-second pulses of gravity-perfused agonist solution were administered at intervals of 60s, 90s, or 120s, depending on the rate of receptor recovery from desensitization. Agonist solution was comprised of glutamate and co-agonist glycine suspended in Mg^{2+} -free ND96 buffer at final concentrations of 200 μM and 20 μM , respectively. With the exception of 5-minute equilibration periods, buffer was perfused continuously over the oocytes between agonist pulses. During the equilibration period, buffer flow was halted to create a static bath for application of either peptide (suspended in ND96 buffer at various concentrations), or control solution (ND96 buffer alone). The effect of a peptide on NMDA receptor-mediated current was determined by measuring the amplitude of the first agonist-elicited current pulse immediately following the 5-minute equilibration period as a percentage of the amplitude of the baseline current (agonist-elicited current immediately preceding 5-minute equilibration period). Data acquisition was automated by a virtual instrument made by Doju Yoshikami of the University of Utah. Concentration-response curves were generated using Prism software (GraphPad Software, Inc.), using the following equation, where nH is the Hill coefficient and IC_{50} is the concentration of peptide causing half-maximal block: $\% \text{ Response} = 100 / \{1 + ([\text{peptide}] / \text{IC}_{50})^{nH}\}$. A more detailed account of the voltage-clamp electrophysiology protocol used was described previously (Cartier et al., 1996).

3. Results

3.1. Analysis of clones encoding putative conantokins from *Conus purpurascens* and *Conus ermineus*

Two clones from *Conus purpurascens* and *Conus ermineus* encoding highly homologous peptide precursors that belong to the same gene superfamily as the conantokins were identified, as described in Materials and Methods. The predicted AA sequence of the open reading frames encoded by these clones is shown in Figure 3; for comparison, the precursor sequences for two previously characterized conantokins are also shown in the Figure. The striking similarity of the signal sequences and the pro regions of the four sequences in Figure 3 is diagnostic of genes belonging to the same superfamily.

Being members of the conantokin family, most of the glutamate residues in the *C. purpurascens* and *C. ermineus* sequences are predicted to be post-translationally modified to γ -carboxyglutamate, (except for Glu-2, which is never modified). The predicted post-translationally modified mature sequences are shown in Figure 3. Since all known Cys residues in peptides from *Conus* venoms are oxidized to form disulfide bonds, the predicted mature conantokin-P and conantokin-E peptides shown in the Figure differ significantly from other conantokins in the presence of a long disulfide loop (12AA between Cys residues). Furthermore, there are 2 Gla residues between the Cys residues. In the only other characterized conantokin with a disulfide bond (Con-R), the disulfide loop is much shorter (3AA between Cys residues) with no Gla residues within the Cys loop (see Table 1).

The sequences of the predicted *C. purpurascens* and *C. erminius* peptides (that are designated Conantokin-P (Con-P) and Conantokin-E (Con-E) respectively) are strikingly similar to each other. Thus, the conantokins in the subgenus *Chelyconus* (see Figure 2) have distinctive biochemical features compared to previously characterized conantokins.

3.2. Synthesis and folding of Conantokin-P

We chemically synthesized one of the two predicted peptides in Figure 3, conantokin-P. The peptide was constructed by solid phase peptide synthesis with γ -carboxyglutamate residues in all positions where there was a Glu codon in the corresponding mature toxin cDNA clone, except for Glu-2 as noted above. Disulfide bond formation was monitored using an oxidized/reduced glutathione redox buffer. Figure 4 shows representative HPLC profiles and the corresponding mass spectra. The presence of Gla residues in the sequence was verified by the observed loss of carboxyl groups detected using ESI-MS of conantokin-P.

The oxidative folding of conantokin-P was achieved using the redox buffer containing oxidized/reduced glutathione, the redox potential at which efficient disulfide formation occurs was optimal at an equimolar ratio of oxidized/reduced glutathione. The kinetics of the formation of the disulfide bond in conantokin-P was measured at pH 7.5, 25°C using either 10 mM Ca^{++} or 1 mM EDTA (to chelate any divalent cations) in the reaction mixture. The reaction was monitored at regular intervals; oxidative folding was initiated by adding the linear peptide into the redox buffer and the reaction quenched at particular time point by acidification using formic acid. The quenched reaction mixtures were separated using HPLC (see Methods). The extent of accumulation of folded peptide or the decrease in linear peptide was determined by integrating the chromatographic peaks in the background of other reaction intermediates.

Figure 5 represents HPLC profiles of the oxidative folding of conantokin-P at different time points, in the presence of Ca^{++} or in the absence of Ca^{++} (with EDTA). It is evident from these data that the reaction intermediates are more populated in the presence of EDTA compared to Ca^{++} during the oxidative folding. The two major reaction intermediates observed during the oxidative folding correspond to the mixed disulfides between one of the peptide thiols and

glutathione (the observed molecular mass of the intermediates is 3459.0 Da and 3458.8 Da). The very transient accumulation of mixed disulfides in the presence of Ca^{++} (compared to EDTA) at a given time point during the folding suggests a closer proximity of the thiol group in the presence of Ca^{++} .

The folding reaction was examined in the presence of Ca^{++} because of previous results with Gla-containing peptides in the P superfamily; it had been postulated in that prior work (Bulaj et al., 2003) that the presence of Gla residues within a disulfide loop can function to facilitate disulfide bond formation. For the disulfide bond in Con-P, the fairly large effect observed in Figure 6 suggests that a conformational change induced by Ca^{++} in conantokin-P facilitates disulfide bond formation. The rate constant (k_{on}) for accumulation of folded peptide, in the presence of EDTA was $19.0 \text{ min}^{-1} \text{ M}^{-1}$ and in the presence of calcium was $83.7 \text{ min}^{-1} \text{ M}^{-1}$. The fully oxidized synthetic peptide was used in the functional characterization of conantokin-P.

3.3. Circular Dichroism

Conantokin-P has the characteristic features of conantokins and functionally resembles Conantokin-G. Conantokin-G is known to adopt an α -helical confirmation in the presence of calcium and is nearly unstructured in the absence of calcium (Teichert et al., 2007). We carried out a structural comparison of conantokin-P and conantokin-G using circular dichroism as a probe. In contrast to conantokin-G, the helical propensity of conantokin-P is essentially the same in the presence or absence of calcium as shown in Figure 7. The estimated α -helical contents of conantokin-P is 44%, which is a significantly lower proportion of helix than con-G. The retention of helical structure in the absence of Ca^{++} is probably due to the presence of Lys-7; conantokins with Gla at this position (such as con-G) require Ca^{++} to stabilize their helical structure (Teichert et al., 2007).

3.4. Functional characterization of Conantokin-P

Many of the canonical amino acids that are characteristic of the conantokin family are conserved in Con-P, suggesting activity as a NMDA receptor antagonist. Nevertheless, because its sequence differs significantly from that of previously characterized conantokins (see Table 1), the activity of con-P on NMDA receptors was evaluated. Two-electrode voltage clamp electrophysiology was employed to assess the effect of the peptide on NMDA receptors heterologously expressed in *Xenopus* oocytes (see Experimental Procedures). Con-P was applied to oocytes expressing NR2B/NR1-3b in a static bath and allowed to equilibrate for 5 minutes. Following equilibration, a $3.3 \mu\text{M}$ concentration blocked nearly all of the current elicited from the first agonist pulse (Figure 8).

The NMDA receptor subtype selectivity of Con-P was evaluated using the same protocol as in Figure 8. Concentration-response curves were generated for four NMDA receptor subtypes, each containing a NR2 subunit (A–D) separately coexpressed with NR1-3b. Con-P blocked NR2B/NR1-3b receptor mediated current with the greatest potency, and the NR2A/NR1-3b subtype with moderate potency. The peptide blocked NR2C/NR1-3b and NR2D/NR2-3B with the least potency (Figure 9). The IC_{50} values for Con-P are shown in Table 2. For receptor subtypes containing NR1-3b, Con-P was least active on NR2C, and discriminated most strongly against NR2C and NR2D in favor of NR2B among all conantokins characterized thus far.

4. Discussion

In this work, we describe the discovery and characterization of a distinct branch of the conantokin family that is biochemically divergent from other conantokins. Conantokins-E and

-P both have a large disulfide loop, with two γ -carboxyglutamate residues within the loop, a feature not found in any previously characterized member of the family. The two peptides, which are highly similar in amino acid sequence, are likely to exhibit similar biological activity. This novel group of conantokins is phylogenetically delineated; the two closely-related but distinctive species of fish hunting *Conus* that are the source of the peptides are divergent from the other fish hunting cone snail species (see Figure 2) from which conantokins have previously been characterized.

It had previously been shown for other *Conus* peptide families that distinctive peptide toxins are present in *C. purpurascens* and *C. ermineus*. In the A superfamily, both *Conus purpurascens* and *Conus erminius* express the α A-conotoxins, a novel group of nicotinic antagonists (Hopkins et al., 1995; Jacobsen et al., 1997). The peptides in *Conus purpurascens* and *C. ermineus* venoms (e.g. α A-PIVA and α A-EIVA) although divergent in sequence, are more structurally similar to each other than to any other peptides in the large, well-characterized A-superfamily (Santos et al., 2004), which is distributed across the entire genus. The work presented here shows that the conantokin family peptides from these *Conus* species, predicted by the analysis of cDNA clones, are similarly distinctive when compared to other conantokins (see Figure 3).

We establish that despite the divergence in biochemical properties, conantokin-P is a *bona fide* conantokin; we have clearly demonstrated that like other conantokins, it is an antagonist of the NMDA receptor (although it more strongly discriminates against the NR2C and NR2D subtypes than do the other conantokins tested).

The presence of conantokins targeting NMDA receptors in three different clades of fish hunting cone snails was unexpected. It is easy to rationalize why venom components such as the α -conotoxins that inhibit the major postsynaptic receptor at the neuromuscular junction (McIntosh et al., 1994; McIntosh et al., 1999; Olivera, 1997), ω -conotoxins that inhibit presynaptic calcium channels (Hillyard et al., 1992; Terlau et al., 1996) or μ -conotoxins that target voltage gated sodium channels (Cruz et al., 1985; Terlau and Olivera, 2004) responsible for action potentials are generally found in the venoms of *Conus* species that prey on fish (Olivera and Cruz, 2001) — these targets are essential molecular components for neuromuscular transmission in all vertebrates, and their inhibition leads to paralysis. Why peptides that antagonize the NMDA receptor are broadly found among fish hunting cone snails is far less apparent.

In contrast to mammalian systems, where no clear demonstration of functional NMDA receptors outside the central nervous system has been reported, there are peripheral glutamatergic circuits in fish that are presumably the relevant physiological targets of conantokins and other peptides found in *Conus* venoms that affect glutamate receptors. One possibility is the lateral line circuitry of fish, used for water movement detection. The broad distribution of peptides that target NMDA receptors strongly suggests that one of the adaptations of cone snails that has evolved for effectively capturing fish is to target this, or some other yet undefined glutamatergic circuitry in the peripheral nervous systems of teleost fish.

The acceleration in the rate of disulfide bond formation in Conantokin-P when Ca^{++} is present is reminiscent of what was observed for the spasmodic peptides from *Conus textile* in the P-superfamily (Bulaj et al., 2003). It was established in that instance that the observed acceleration in disulfide bond formation was due to the presence of two γ -carboxyglutamate residues in the spasmodic peptide from *Conus textile*; the homologous peptide from *Conus gloriamarus*, nearly identical in sequence but lacking the two Gla residues did not exhibit an accelerated rate of disulfide formation in the presence of Ca^{++} . The data we present in Figure

5 is consistent with a wider role of γ -carboxyglutamate residues in the oxidative folding of conopeptides.

We note that the likely order of occurrence of these two post-translational modifications is consistent with this suggestion: the γ -glutamyl carboxylase enzyme is present in the *Conus* endoplasmic reticulum membrane, (Bandyopadhyay et al., 1998; Bandyopadhyay et al., 2002; Stanley et al., 1997) and would therefore be the first post-translational modification acting on a newly synthesized polypeptide chain destined for secretion; in contrast, the disulfide isomerase is present in the lumen of the endoplasmic reticulum (Bulaj et al., 2003; Bulaj and Olivera, 2008; Buczek et al., 2004). Thus, disulfide bond formation would be preceded by γ -carboxylation. Indeed, the synergistic interaction between the two post-translational events, γ -carboxylation of glutamate residues and the oxidation of Cys residues to form disulfide bonds has been suggested to be an evolutionarily ancient one that has been recapitulated in the *Conus* peptide system because of the high density of disulfide bonds in many short conopeptides. How widely distributed the role of γ -carboxyglutamate in folding will prove to be, remains to be investigated.

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Figure 1.

The seven species of *Conus* from which members of the conantokin family of peptides have been isolated and characterized. Top row, left to right: *Conus geographus* (Conantokin-G); *Conus tulipa* (Conantokin-T); *Conus radiatus* (Conantokin-R). Bottom row: *Conus erminius* (Conantokin-E — this work); *Conus purpurascens* (Conantokin-P — this work); *Conus parius* (Conantokins-Pr1, Pr2 and Pr3) and *Conus lynceus* (Conantokin-L). All of the shells shown were collected in the Philippines except for *Conus erminius* (Cape Verde Islands) and *Conus purpurascens* (Mexico).

Figure 2.

Phylogenetic tree of species shown in Figure 1. A phylogenetic tree was constructed as described under Methods; all of the species shown in Figure 1 are included in the tree, but 2 additional species (from which additional conantokins have been isolated), *Conus obscurus* and *Conus flavus* are included. As an outgroup, the worm-hunting species *Conus imperialis* was used. All of the other species are fish hunting. The asterisks denote the species from which the conantokins discussed in this article were isolated. The two specimens of *Conus erminius* were from the Caribbean and off West Africa respectively; the differences in sequence between them are too slight to give them any taxonomic significance. The subgenera to which each of the species belongs within *Conus* is indicated by the bold designation. Thus, the *Conus erminius/purpurascens* groups (***Chelyconus***) can confidently be separated into a different clade from all of the other fish hunters shown in the Figure.

Propeptide region comparison

Con-P	MQLYTYLYLLVPLVTFHLILSTGTLAHGGTLTERRSTDTTALKPEPVLLQKSDARSTDNDKDRLTQMKRILKKRGNKAR
Con-E	-----LLVPLVTFHLILGMGTLDHGGALTERRSADATALKPEPVLLQKSDARSTDNDKDRLTQMKRILKKRGNKAR
Con-G	MHLYTYLYLLVPLVTFHLILGTGTLDDGGALTERRSADATALKAEPVLLQKSAARSTDNDGKDRLTQMKRILKQRGNKAR
Con-L	MQLYTYLYLLVPLVTFHLILGTGTLDHGGALTERRSTDATALKPEPVLLQKSSARSTDNDGNDRLTQMKRILKKRGNKAR

Toxin region comparison

Con-P	GE $\gamma\gamma$ HSKYQ γ CLR γ IRVNKVQQ γ C
Con-E	GE $\gamma\gamma$ HSKYQ γ CLR γ IRVNNVQQ γ C
Con-G	GE $\gamma\gamma$ LQ γ NQ γ LIR γ KSN
Con-L	GE $\gamma\gamma$ VAKMAA γ IAR γ NAAN

Figure 3.

Similarity of conantokin-P and conantokin-E precursor sequences to previously characterized conantokins. The comparison of mature toxin region is also presented.

Figure 4. RP-HPLC profiles of a novel conantokin-P derived from *Conus purpurascence*. a) Linear conantokin-P, and b) Conantokin-P. Elution was done under the same linear gradient as mentioned in materials and methods. Inset shows the corresponding ESI-MS.

Figure 5.

Oxidative folding of conantokin-P in the presence of EDTA or in the presence calcium. A) RP-HPLC chromatograms of the oxidative folding pathways of conantokin-P in the presence of 1 mM EDTA and B) RP-HPLC chromatograms of the oxidative folding pathways of conantokin-P in the presence of 10 mM calcium.

a)

b)

Figure 6.

The kinetics of oxidative folding of conantokin-P in the presence of EDTA or in the presence of calcium. A) The accumulation of folded peptide or decrease in linear peptide was plotted against the regular intervals of time. The rate constants obtained by fitting the experimental points to single exponential fit was also depicted and B) Bar graph summarizing the half-time for accumulation of folded peptide or decrease in linear peptide during the oxidative folding.

Figure 7. Circular dichroism spectroscopy of a conantokin-P from *Conus purpurascence*. CD spectra of conantokin-P in the presence or absence of Ca^{++} ; shown is an averaged spectrum obtained from five independent scans ($n=5$).

Figure 8.

Activity of Conantokin-P on NR2B/NR1-3b NMDA receptor expressed in *Xenopus* oocytes. Current traces were obtained as described in Experimental Procedures. Baseline current was elicited in response to 1-second agonist pulse (200 μ M Glu, 20 μ M Gly) in freely perfusing ND96 buffer. Following baseline, buffer flow was halted, and either buffer control or Con-P was applied to static bath and allowed to equilibrate for 5 min. "Effect" is the first agonist-elicited current trace following application of either Con-P or buffer control. Left: effect of buffer control on agonist-elicited current. Right: effect of 3.3 μ M con-P on agonist-elicited current.

Figure 9.

NMDA receptor subtype selectivity of Conantokin-P. Concentration-response curves for Conantokin-P obtained on each NMDA receptor NR2 subtype separately coexpressed with NR1-3b. Various concentrations were tested against each subtype using the protocol described in Fig. 8 and in Experimental Procedures. Concentration-response curves were determined as described in Experimental procedures. (N=3 tests for each data point).

Table 1Comparison of previously characterized conantokins with predicted conantokin from *Conus purpurascens*.

Conus Species	Conantokin	Amino-Acid Sequence	Ref.
<i>C. purpurascens</i>	Con-P	GE $\gamma\gamma$ HS K YQ γ CLR γ IRV NKVQO γ C [*]	This work
<i>C. radiatus</i>	Con-R	GE $\gamma\gamma$ VA K MAA γ LAR γ NIAKGCKVNCYP [^]	White et al., 2000
<i>C. lynceus</i>	Con-L	GE $\gamma\gamma$ VA K MAA γ LAR γ DAVN [*]	Jiminez et al., 2002
<i>C. parius</i>	Con-Pr1	GE D γ YA γ GIR γ YQL I HGKI [^]	Teichert et al., 2007
<i>C. parius</i>	Con-Pr2	DE O γ YA γ AIR γ YQL K YGKI [^]	Teichert et al., 2007
<i>C. parius</i>	Con-Pr3	GE O γ VA K WA γ GLR γ KASSN [*]	Teichert et al., 2007
<i>C. tulipa</i>	Con-T	GE $\gamma\gamma$ YQ K ML γ NLR γ AEVKKNA [*]	Haack et al., 1990
<i>C. geographus</i>	Con-G	GE $\gamma\gamma$ LQ γ NQ γ LIR γ KSN [*]	McIntosh et al., 1984

O, 4-*trans*-hydroxyproline; γ , γ -carboxyglutamate;

* C-terminal amidation

[^] C-terminal free acid.

Cysteine residues are shown in bold font.

Table 2
Approximate IC₅₀ values for conantokins tested against various NR2 subunits.

Peptide	NR2A IC ₅₀ (μ M)	NR2B IC ₅₀ (μ M)	NR2C IC ₅₀ (μ M)	NR2D IC ₅₀ (μ M)	NR2D/NR2B IC ₅₀ Ratio	NR2C/NR2B IC ₅₀ Ratio
Con-P	2.3	0.3	$\gg 10$	$\gg 10$	> 10	$\gg 10$
Con-R	~ 1	1.0	7.3	$\gg 10$	$\gg 10$	7.3
Con-G	$\gg 10$	0.1	1	1	10	10
Con-Pr1	$\gg 10$	0.2	≥ 10	1	5	≥ 10
Con-Pr2	$\gg 10$	0.5	≥ 10	1	2	≥ 10
Con-Pr3	$\gg 10$	0.5	≥ 10	8	16	≥ 10

Con-P IC₅₀ values were obtained from the concentration-response curves in Fig. 6; data for Con-Pr1, Con-Pr2, Con-Pr3, and Con-G were obtained from Teichert et al., (2007); data for Con-R obtained using NR1-1b subunit in combination with NR2 subunits (White et al., 2000).