A Novel Conotoxin Framework with a Helix−Loop−Helix (Cs α/α) Fold †

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A Novel Conotoxin Framework with a Helix−Loop−Helix (Cs α/α) Fold†

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ABSTRACT: Venomous predatory animals, such as snakes, spiders, scorpions, sea anemones, and cone snails, produce a variety of highly stable cystine-constrained peptide scaffolds as part of their neurochemical strategy for capturing prey. Here we report a new family of four-cystine, three-loop conotoxins (designated framework 14). Three peptides of this family (flf14a−c) were isolated from the venom of *Conus floridanus floridensis*, and one (vil14a) was isolated from the venom of *Conus villepinii*, two worm-hunting Western Atlantic cone snail species. The primary structure for these peptides was determined using Edman degradation sequencing, and their cystine pairing was assessed by limited hydrolysis with a combination of CNBr and chymotrypsin under nonreducing, nonalkylating conditions in combination with MALDI-TOF MS analysis of the resulting peptidic fragments. CD spectra and nanoNMR spectroscopy of these conotoxins directly isolated from the cone snails revealed a highly helical secondary structure for the four conotoxins. Sequence-specific nanoNMR analysis at room temperature revealed a well-defined helix−loop−helix tertiary structure that resembles that of the Cs α/α scorpion toxins *κ*-hefutoxin, *κ*-KTx1.3, and Om-toxins, which adopt a stable three-dimensional fold where the two α-helices are linked by the two disulfide bridges. One of these conotoxins (vil14a) has a Lys/Tyr dyad, separated by approximately 6Å, which is a conserved structural feature in K^+ channel blockers. The presence of this framework in scorpions and in cone snails indicates a common molecular imprint in the venom of apparently unrelated predatory animals and suggests a common ancestral genetic origin.

Animals that utilize venom to capture prey, such as snakes, spiders, scorpions, sea anemones, and cone snails, produce a plethora of cystine-stabilized peptidic scaffolds that target specifically ion channels and neuronal receptors as part of their neurochemical strategy for predation. Among them, cone snails, a genus (*Conus* ssp.) of marine gastropods that can prey upon fish (piscivorous), mollusks (molluscivorous), and worms (vermivorous), contain in their venom an extraordinarily complex and diverse mixture of small neuroactive peptides (conopeptides) that specifically target ion channels and neuronal receptors (*1*). Conopeptides are important tools for investigating ion channel and receptor function and have great potential pharmacological applications (*2*, *3*). They can be classified into two major groups: (1) conotoxins, which contain two or more disulfide bonds, and (2) those with only a single disulfide bridge or none at all, which are designated with trivial names such as contryphans, conantokins, contulakins, conorfamides, conophans, and *γ*-hydroxyconophans. Conotoxins are grouped into various superfamilies (O, M, A, S, T, P, and I), each with highly conserved signal sequences in their precursor proteins and a characteristic cystine arrangement in the mature peptides. Within the superfamilies, conotoxins are further classified into families according to their pharmacological targets, which include voltage-gated ion channels (Na^+, K^+, and Ca^{2+}), ligand-gated ion channels (nAChR and 5-HT_3R), receptors (neurotensin type 1, α1 adrenergic, NMDA, RFamide, and vassopressin), and neurotransmitter transporters (NE) (*1*, *2*).

Conopeptides inherently contain high degrees of modified amino acids (usually combinations of them), such as cystines, hydroxyproline, *γ*-carboxyglutamate, Br-Trp, D-Trp, D-Leu (*4*−*6*), D-Phe (*7*), D-Ile (*8*), D-Met (*8*), D-*γ*-hydroxyvaline (*9*), pyro-Glu, glycosylated Ser/Thr, and sulfated Tyr (*4*−*6*). These modifications confer conopeptides with unique stability and exquisite specificity toward neuronal targets (*4*, *10*, *11*), enabling cone snails to capture prey.

The precise composition of cone snail venom is species-specific (*11*−*13*), and it is the product of 55 million years of evolutionary refinement that has yielded a complex library of more than 100 000 neuroactive conopeptides, as this genus comprises more than 1000 species distributed in the tropical and subtropical areas of the Atlantic, Indian, and Pacific oceans (*14*). Only a small fraction of this immense conopeptide library has been analyzed to date (<0.2%), and many novel conopeptide frameworks are yet to be discovered. Most conopeptides that have been isolated and characterized are from *Conus* species found in the Indo-Pacific region. As part of our efforts toward the analysis and characterization of conopeptides isolated from cone snail species from the

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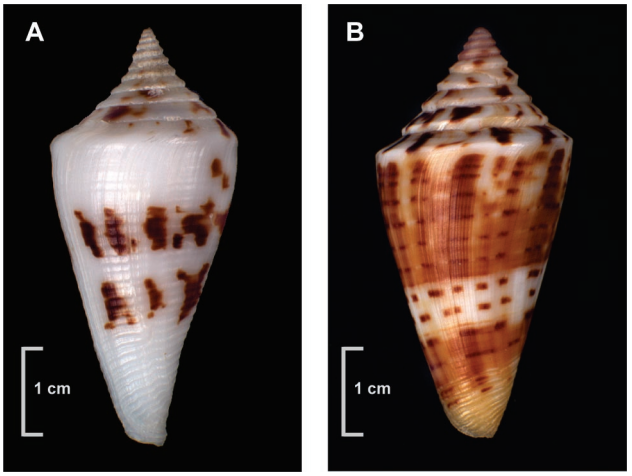


FIGURE 1: Shells of *C. villepinii* (A) and *C. floridanus floridensis* (B).

Americas, we proceeded with the isolation and structural analysis of conopeptides from *Conus floridanus floridensis* and *Conus villepinii*, which are widespread worm-hunting cone snail species of the Western Atlantic Ocean (Figure 1). *C. villepinii* is a deep-water species (>100 m), and its habitat ranges from the Florida coastline and the Gulf of Mexico to Brazil. *Conus floridanus* is also a Western Atlantic species whose range varies from Florida to the Yucatan Peninsula; however, unlike *C. villepinii*, *C. floridanus* inhabits shallow sandy areas (*14*). Four variants of *C. floridanus* exist: *C. floridanus floridensis*, *Conus floridanus buryae*, *Conus floridanus patglicksteinae*, and *Conus floridanus yucatanensis* (*14*); these variants of *C. floridanus* are biogeographical subspecies that show differences in their venom composition. To date, no peptides have been isolated from the venom of these Western Atlantic *Conus* species.

Here we present the results from the isolation and structural characterization of a novel four-cystine, three-loop conotoxin framework (framework 14) from *C. villepinii* (vil14a) and *C. floridanus floridensis* (flf14a−c). These new conotoxins are 27-residue polypeptide chains with a 1−4/2−3 cystine pairing. NanoNMR analysis revealed a well-defined helix−loop−helix three-dimensional fold where the two α-helices are linked by the two disulfide bridges. This tertiary structure resembles that of the Cs α/α[1] toxins recently found in scorpion venom that targets the potassium channels (*15*−*17*).

MATERIALS AND METHODS

Specimen Collection. Specimens of *C. villepinii* (30−80 mm) were collected off the Florida Keys (Marathon Key, FL) using a Capetown dredge deployed from the oceanic research vessels R/V Suncoaster and R/V Bellows at depths ranging from 100 to 200 m. Additional snails were collected using the Johnson-Sea-Link deep submersible vehicle operated from the R/V Seward Johnson and working at a depth of 200 m at the same location indicated above. Cone snails were collected using a suction device attached to a robotic arm of the DSV. Specimens of *C. floridanus floridensis* (20−45 mm) were collected along the southwest coast of Florida in sandy areas at low tides. All snails were kept in aquaria prior to transportation to the lab, where there were dissected and immediately frozen at −80 °C.

Crude Venom Extraction. Venom ducts dissected from either 63 specimens of *C. villepinii* or 47 specimens of *C. floridanus floridensis* were homogenized in 0.1% TFA at 4 °C. Whole extracts were centrifuged at 10000*g* for 20 min, at 4 °C, and the resulting pellets were washed three times with 0.1% TFA and recentrifuged under identical conditions. The supernatants containing the soluble peptides were pooled, lyophilized, and stored at −80 °C until further use.

Peptide Purification. Crude venom was initially fractionated by SE-HPLC on a Pharmacia Superdex-30 column (2.5 cm × 100 cm) equilibrated and eluted with 0.1 M NH_4HCO_3 using a flow rate of 1.5 mL/min. Chromatographic fractions were monitored at λ values of 220, 250, and 280 nm. Additional purification of peptide-containing peaks was achieved by RP-HPLC on a C18 semipreparative column (Vydac, 218TP510, 10 mm × 250 mm; 5 μm particle diameter; 300 Å pore size) equipped with a C18 guard column (Upchurch Scientific, AC-43 4.6 mm) at a flow rate of 3.5 mL/min. Further peptide purification was carried out by rechromatographing fractions on an analytical C18 column (Vydac, 238TP54, 4.6 mm × 250 mm; 5 μm particle diameter; 300 Å pore size), with a flow rate of 1 mL/min. For semipreparative and analytical RP-HPLC separation, the buffers were 0.1% TFA (buffer A) and 0.1% TFA in 60% acetonitrile (buffer B). Peptides were eluted with an incremental linear gradient of 1% B/min. Absorbances were monitored at λ values of 220 and 280 nm. All HPLC fractions were manually collected, lyophilized, and kept at −40 °C prior to further use.

Reduction and Alkylation of Cysteyl Residues. Reduction and alkylation of cystine groups were carried out as previously described (*18*) with slight modifications. An aliquot of each peptide (~1 pmol) was dried, redissolved in 0.1 M Tris-HCl (pH 6.2), 5 mM EDTA, and 0.1% sodium azide, and reduced with 6 mM DTT. Following incubation at 60 °C for 30 min, peptides were alkylated in a final volume of 15 μL with 20 mM IAM and 2 μL of NH_4OH (pH 10.5), at room temperature, for 1 h, in the dark. The reduced and alkylated peptides were purified using a Zip Tip (C18, size P10, Millipore).

Peptide Sequencing. Alkylated peptides were adsorbed onto Biobrene-treated glass fiber filters, and amino acid sequences were determined by Edman degradation using an Applied Biosystems Procise model 491A sequencer. The concentration of the peptides was determined by using the calibrated intensities of the first five PTH-amino acid residues on samples that were not reduced and alkylated.

Molecular Mass Determination. Positive ion MALDI-TOF mass spectrometry was carried out on an Applied Biosystems Voyager-DE STR spectrometer. Samples were dissolved in 0.1% TFA and 50% acetonitrile and applied on an α-cyano-4-hydroxycinnamic acid matrix. Spectra were obtained in

[1] Abbreviations: DSV, deep submersible vehicle; TFA, trifluoroacetic acid; SE, size exclusion; HPLC, high-performance liquid chromatography; RP, reversed phase; DTT, dithiothreitol; IAM, iodoacetamide; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; CNBr, cyanogen bromide; PFG, pulse field gradient; RF, radio frequency; TSP, 3-(trimethylsilyl)propionic acid-d_4, sodium salt; WET, water suppression enhanced through T1 effects; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlated spectroscopy; BLAST, basic local alignment search tool; Cs β/β, cystine-stabilized β/β; Cs α/α, cystine-stabilized α/α.

the linear and reflector mode using Calmix 1 and Calmix 2 (Applied Biosystems) as external calibration standards.

Disulfide Connectivity Analysis. Approximately 1 nmol of each lyophylized conopeptide was digested with a combination of CNBr and α-chymotrypsin prepared free of autolysis products and low-molecular weight contaminants. In this case, peptides were not previously reduced and alkylated to keep their disulfide bonds intact. Lysis of the peptides with CNBr (Acros Organics) was performed using a CNBr:peptide mass ratio of 2:1 (*19*), in 20 μL of 70% formic acid. The peptidic sample was incubated for 20 h, at room temperature, in the dark, and the reaction was terminated by diluting the mixture with ~5 volumes of H_2O followed by removal of the excess free reagents by lyophilization with a Speed Vac concentrator. Subsequently, the sample was further digested with chymotrypsin (Sigma-Aldrich), using a 1:20 ratio of chymotrypsin to substrate, in 20 μL of 0.1 M NH_4HCO_3 (pH 8.0), for 4 h, at 37 °C (*20*). Final digests were directly analyzed by MALDI-TOF mass spectrometry.

Circular Dichroism Spectroscopy. All circular dichroism (CD) measurements were carried out with a JASCO J-810 spectropolarimeter instrument using a 200 μL solution of each peptide in HPLC water (final concentration of 3 μM; determined by normalized UV at a λ value of 280 nm) in a quartz cell. Spectra were recorded over a 190−250 nm range (λ) at 25 °C using an average of 10 scans (scan speed of 100 nm/min). Peptide helical content was estimated using the method proposed by Baldwin et al. (*21*).

NMR Spectroscopy. NMR spectra were acquired on a Varian Inova 500 MHz instrument equipped with PFG, 3xRF channels and waveform generators. Nanomolar quantities of the native conopeptides directly isolated from the venom (32 nmol of vil14a, 0.1 nmol of flf14a, 3 nmol of flf14b, and 0.6 nmol of flf14c) were dissolved in 40 μL of water with 10% D_2O (used for locking purposes) and 4 nmol of TSP and placed in 1.7 mm NMR tubes (Wilmad WG-1364-1.7). The pH was adjusted using 0.01 M solutions of HCl and NaOH and a Thermo micro-pH probe. Spectra were obtained using a Varian *g*HCN (generation 5) high-performance 3 mm probe (pw90 = 3 μs, at the upper limit of the linear range of the RF amplifier) with a 1.7 mm capillary adaptor (Wilmad V-GFK-10/1.7). NMR experiments were conducted at pH 3.60 and at different temperatures (0, 10, and 25 °C) to achieve the best chemical shift dispersion possible to aid the sequence-specific assignments. For one-dimensional (1D) NMR experiments, the water signal was suppressed by using either WET (*22*) or presaturation. In addition to the concentrations determined from sequencing, peptide concentrations were also evaluated by integrating the NMR signals of selected methyl groups and using the known concentration of TSP as an internal standard (*23*) or the signal of selected methyl groups from peptides with known concentrations as external standards. For two-dimensional (2D) experiments, water suppression was carried out using WATERGATE (*wg*) (*24*) in combination with 3919 purge pulses with flipback (*25*), which were implemented in the TOCSY and NOESY pulse sequences. The *wg*TOCSY and *wg*NOESY experiments were used to obtain information about sequence-specific assignments and the secondary structure of the framework 14 conotoxins (*26*). All 2D NMR spectra were recorded in the phase sensitive mode using the States−Haberkorn method (*27*) with a spectral width of 6000 Hz and 2K data points. For the *wg*TOCSY experiment, 160 scans for each of the 96 FIDs were acquired with relaxation delay of 1.7 s and a mixing time of 120 ms. The 2D *wg*NOESY spectra of the vil14 and flf14 conopeptides were recorded using 256 scans for each of the 128 FIDs acquired with a 1.7 s relaxation delay and a mixing time of 200 ms. All 2D NMR data were processed using VNMR 6.1C (Varian NMR Instruments) on Sun Blade 150 workstations. FIDs were apodized with a shifted sine bell window function and linearly predicted to 1K points in t_1 and zero-filled to 2K × 2K data matrices. The data were baseline corrected in F_2 by applying a polynomial function. NOESY cross-peaks were assigned and classified according to their intensities as strong, medium, or weak with the aid of their volume integrals. Sequence-specific assignments off all proton resonances were carried out using standard biomolecular NMR procedures (*26*).

Molecular Model of vil14a. Molecular models were built by comparative modeling methods (*28*) based on the NMR structure of the κ-hefutoxin (PDB entry 1HP9) and the Om-toxins (PDB entries 1WQC, 1WQD, and 1WQE) as templates and using Modeler (version 8.0). Briefly, conotoxin sequences were aligned according to the standard routine in the program using the PDB entries as templates (Table 1). A set of 20 model structures was built accordingly (*29*). We selected the structure of better target Modeler energy. The final structure was evaluated for its agreement with the NOE NMR data. Molecular graphics were created using UCSF Chimera beta version 1 (*30*).

Nomenclature. In this publication, we adopt a nomenclature of three letters to designate *Conus* species, because the one- or two-letter nomenclature currently in place will not be enough to describe the large number of different non-fish-hunting species, especially those with similar first letter names. We decided to use the three letters "vil" to name the peptides from *C. villepinii* and "flf" for peptides from *C. floridanus floridensis.* Arabic numbers were used to represent the disulfide framework; since 13 has already been assigned to a new eight-cystine, six-loop conotoxin family (*31*), the framework described in this paper will become framework 14. The letter after the framework number indicates the order of elution on SE-HPLC.

RESULTS

Peptide Purification. Crude venom from *C. villepinii* (113 mg) and crude venom *C. floridanus floridensis* (97 mg) were initially fractionated using SE-HPLC on the Superdex 30 column (Figure 2). The flf14 peptides were separated by the Superdex-30 column despite their similar size. This is not entirely unexpected, since this column is also known to partition analytes by hydrophobic interactions (*32*). The SE fractions identified by the arrows in Figure 2 were rechromatographed on a semipreparative C18 column (Figure 3). Purification to single components was ultimately achieved using an analytical C18 column (Figure 3, insets).

Reduction and/or Alkylation and Peptide Sequence Determination. The purified peaks were subjected to reduction with DTT and alkylation with iodoacetamide. Mass spectrometry of the reduced and/or carboxymethylated peptides and of native peptides showed a mass difference consistent with the presence of four cysteine residues for each peptide.

Table 1: Amino Acid Sequences of Peptides flf14a, flf14b, flf14c, and vil14a[a]

Peptide	Sequence	Source	Ref
flf14a	WDVNDCIHFCLIGVVERSYTECHTMCT	*Conus floridanus floridensis*	This work
flf14b	WDVNDCIHFCLIGVVGRSYTECHTMCT	*Conus floridanus floridensis*	This work
flf14c	WDAYDCIQFCMRPEMRHTYAQCLSICT	*Conus floridanus floridensis*	This work
vil14a	GGLGRCIYNCMNSGGGLSFIQCKTMCY	*Conus villepinii*	This work
κ-Hefutoxin 1	G--HACYRNCWRE--GNDEETCKERC	*Heterometrus fulvipes* (Scorpion)	(*15*)
κ-Hefutoxin 2	G--HACYRNCWRE--GNDEETCKERCG	*Heterometrus fulvipes* (Scorpion)	(*15*)
κ-KTx1.3	G--F-CYRSCWKA--GHDEETCKKECS	*Heterometrus spinifer* (Scorpion)	(*16*)
OmTX1	---DPCYEVCLQQHGNV--KECEEACKHPVE	*Opisthacanthus madagascarienses* (Scorpion)	(*17*)
OmTx2	---DPCYEVCLQQHGNV--KECEEACKHPVEY	*Opisthacanthus madagascarienses* (Scorpion)	(*17*)
OmTx3	N--DPCYEVCLQHTGNV--KACEEACQ	*Opisthacanthus madagascarienses* (Scorpion)	(*17*)
OmTx4	---DPCYEVCLQQHGNV--KECEEACKHP	*Opisthacanthus madagascarienses* (Scorpion)	(*17*)
MBP-1	RSGRGECRRQCLRRHEGQPWETQECMRRCRRRG	*Zea mays* (Corn)	(*39*)
Tachyplesin	---KWCFRVCYRGI-------CYRRCR	*Tachypleus tridentatus* (Horsehoe crab)	(*48*)
Gomesin	----ZCRRLCYKQR-------CVTYCRGR	*Acanthoscurria gomesiana* (Spider)	(*49*)

[a] Sequences are aligned for maximal identity, and identical residues are shown with bold type. The flf14 and vil14 conotoxins were also aligned with several four-Cys, three-loop peptide sequences. Peptides with the functional Tyr/Lys dyad for K^+ channel binding are indicated with Y (red) and K (blue) colored labels.

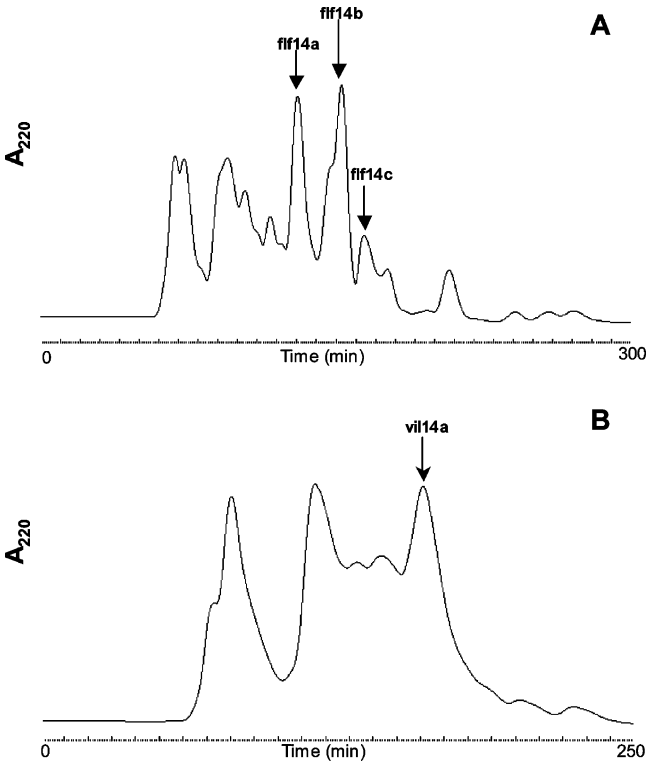


FIGURE 2: Purification of peptides by HPLC-Superdex 30. The column was eluted with 0.1 M NH_4HCO_3 at a flow rate of 1.5 mL/min. (A) Fractionation of the venom of *C. floridanus floridensis*. Peptides flf14a, f1f14b, and f1f14c were purified from the peaks identified by the arrows. (B) Separation of the venom of *C. villepinii*. Peptide vil14a was purified from the peak marked with an arrow.

The reduced and/or alkylated conotoxins were sequenced to completion by Edman degradation. The sequences of the flf14 and vil14 conotoxins are shown in Table 1. Peptides flf14a and flf14b differed by only one amino acid residue. All of these conotoxins contained 27 residues, and the four cysteines were separated by three loops of conserved size (loop 1 has three amino acids, loop 2 has 11 amino acids, and loop 3 has three amino acids). This is a novel arrangement of Cys residues within conotoxin families that we have designated framework 14. Additionally, a BLAST search (*33*) of the databases (Swissprot/EMBL, PIR, PDB, and nrdb95) did not show any significant sequence homology to reported proteins and peptides. Several four-cystine, three-loop sequences (Table 1), including peptides from scorpion venom, κ-hefutoxins (*15*), κ-KTx1.3 (*16*), and Om-toxins (*17*), were found to have loop spacing similar to that of the new conotoxins described here.

Mass Spectrometry of Purified Peptides. Mass spectrometry carried out using MALDI-TOF in the reflector mode (M/ΔM resolution ~ 10 000) yielded the following monoisotopic molecular ions: 3170.2 Da for flf14a, 3098.0 Da for flf14b, 3280.4 Da for flf14c, and 2872.5 for vil14a. Mass analysis of the reduced and/or S-acetamide peptides and the native peptides showed a mass difference consistent with the presence of four cysteine residues in each peptide. The masses obtained for the peptides were in agreement with the calculated theoretical monoisotopic values determined for the assigned sequences and indicated that the peptides were not amidated at the C-terminus. The calculated molecular masses were obtained using Protein Prospector (*34*).

Disulfide Connectivity Analysis. The presence of four cysteine residues in these sequences suggested the existence of two disulfide bridges. Because of the limited quantities of native conotoxins available after their purification, the disulfide connectivity was established by cleaving the peptide

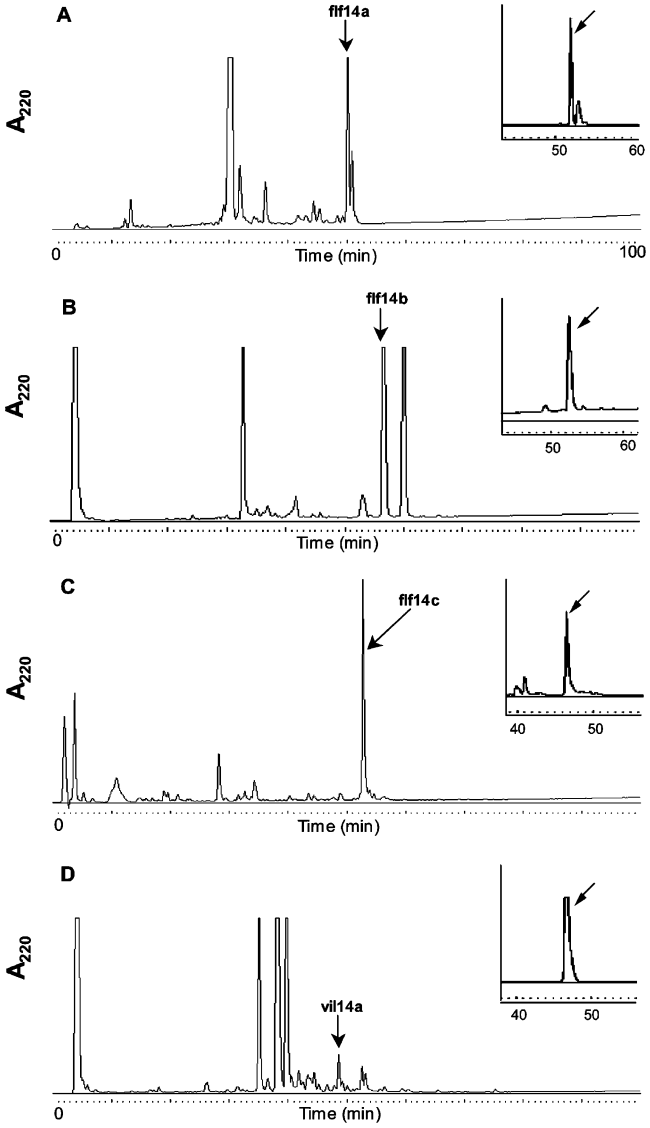


FIGURE 3: Purification of peptides flf14a (A), flf14b (B), flf14c (C), and vil14a (D). Fractions marked with the arrows in Figure 2 were applied to a Vydac C18 semipreparative column and eluted with a linear gradient of 1% buffer B increase per minute for 100 min at a flow rate of 3.5 mL/min. The peaks highlighted with the arrows were further purified on an analytical Vydac C18 column using the same gradient for 100 min at 1 mL/min (insets). For both semipreparative and analytical RP-HPLC, the buffers were 0.1% TFA (buffer A) and 0.1% TFA in 60% acetonitrile (buffer B).

bonds between the loops of the native peptides (not reduced) to obtain fragments maintaining all disulfide bonds. The molecular masses of these fragments were determined by MALDI-TOF mass spectrometry, which only requires femtomole quantities of the sample. Three disulfide pairing patterns can be possible for these conotoxins, and they can be distinguished using a combination of CNBr and chymotrypsin cleavage patterns (Figure 4). Peptides flf14a, flf14b, and vil14a contain a Met residue in the third loop which could be readily cleaved by CNBr. Further hydrolysis was then achieved by using chymotrypsin, which cleaves after aromatic residues Phe, Trp, and Tyr. Disulfide pairing of flf14c was not assessed as it lacks a Met residue in loop 3. MALDI-TOF mass spectra following treatment of each peptide with the combination of CNBr and chymotrypsin revealed that the disulfide pattern for these three peptides is C6−C26 and C10−C22 (Figure 5). No fragments represent-

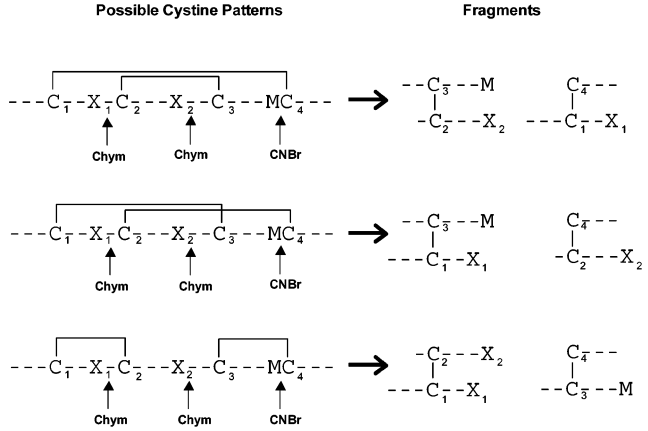


FIGURE 4: Schematic representation of peptides digested with cyanogen bromide (CNBr) and chymotrypsin (Chym). Peptide fragments and molecular weight will depend on the disulfide pattern. X_1 and X_2 are either Phe, Trp, or Tyr.

ing other possible disulfide patterns were found. As expected, some fragments containing the Met showed a $MH^+ - 30$ Da fragment that corresponded to a homoserine lactone formed as a consequence of the CNBr treatment (*35*). Since vil14a contains two Met residues, a loss of 60 Da was evidenced in the corresponding hydrolytic fragments of this peptide. A product of dehydration ($MH^+ - 18$ Da) was obtained in the fragment DVNDCIHF bonded to CT. Dehydration has been reported for residues such as Ser, Asp, Glu, and Thr (*34*). Other MS peaks that were observed represented intermediate products of digestion.

Circular Dichroism Spectra. CD spectra of flf14a, flf14b, flf14c, and vil14a are shown overlaid in Figure 6. Similar overall spectra were obtained for the four peptides with a maximum positive ellipticity at 195 nm (λ) and a minimum with negative ellipticity at 208 nm (λ). Conotoxin vil14a shows another minimum at 225 nm (λ). Analyses performed using the method proposed by Baldwin (*21*) indicate that these peptides are predominantly α-helical, which is in good agreement with data derived from NMR spectroscopy.

NMR Spectroscopy. We were able to obtain NMR spectra (1D and 2D) of nanomolar quantities (nanoNMR) of the flf14 and vil14 conotoxins directly isolated from the venom of the cone snails. In all cases, 2D *wg*TOCSY spectra were acquired; 2D *wg*NOESY spectra with spectral quality suitable for structural determination (sufficient signal-to-noise ratio) were obtained for flf14b and vil14a. Figure 7 shows *wg*NOESY spectra of flf14b (A) and vil14a (B) at 25 °C. Despite their small size (27 residues), a large number of NOE (>300) cross-correlations were found for these peptides. Spectra at lower temperatures (0 and 10 °C, data not shown) resulted in even higher number of NOE cross-correlations. The flf14b and vil14a conotoxins have very well-defined structure in solution at room temperature and in agreement with the results of CD results (Figure 6). Sequence-specific assignments for all protons (except Gly1) in vil14a were achieved using the nanoNMR data (Table S1). This allowed us to generate the NOE-based secondary structure connectivity map shown in Figure 8A. 2D *wg*NOESY spectra revealed a high α-helical content for all four peptides. For vil14a, sequential NN(i, $i + 1$) and αN(i, $i + 1$) NOEs in conjunction with midrange NN(i, $i + 3$), αN(i, $i + 3$), and αβ(i, $i + 3$) indicate two α-helical segments between residues 5−12 and

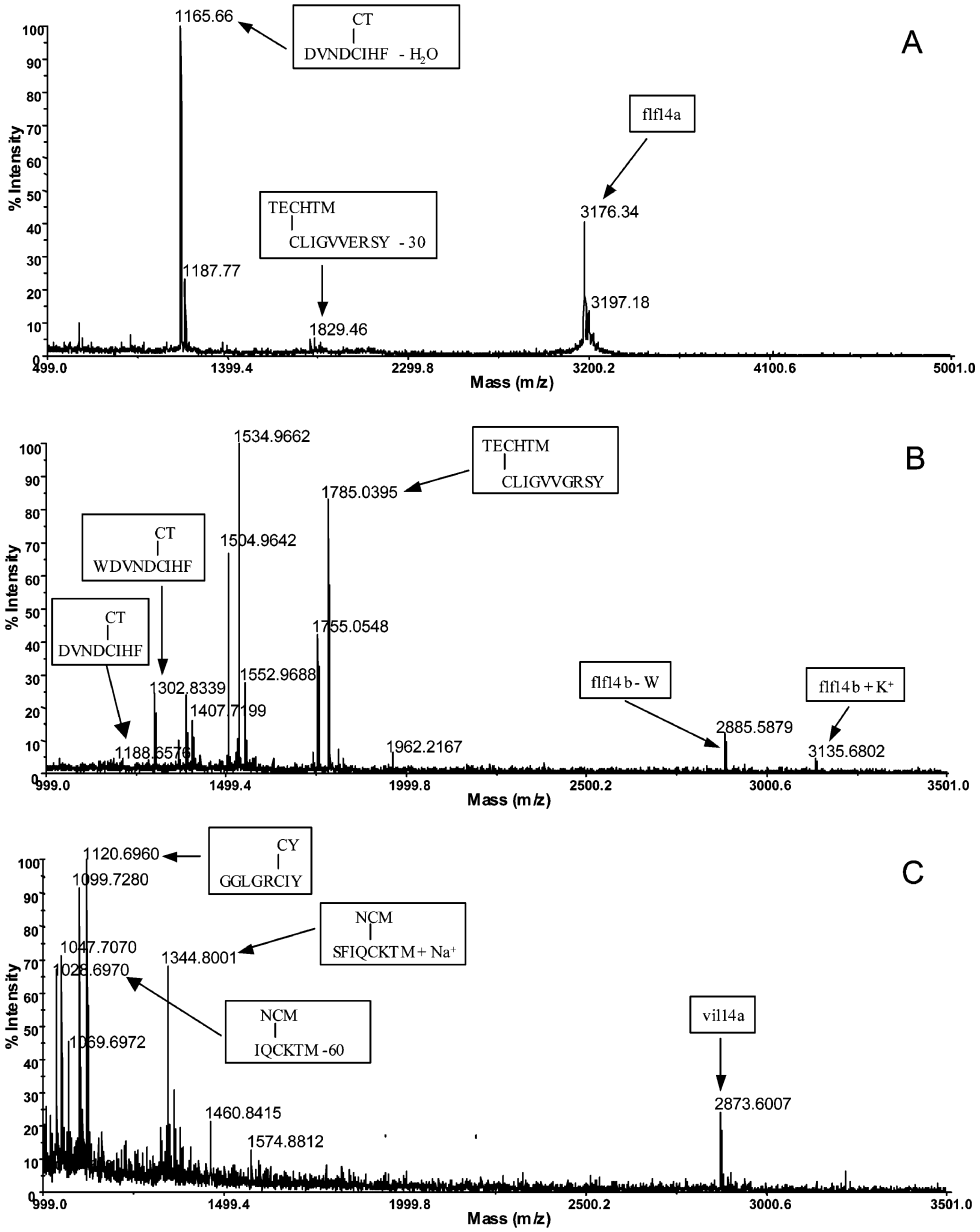


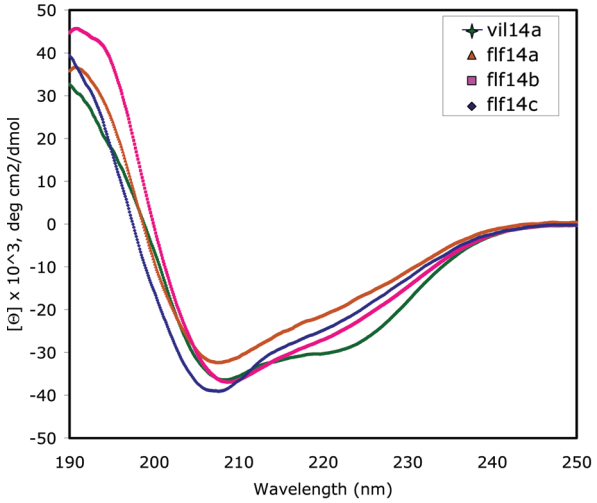
FIGURE 5: MALDI-TOF mass spectra (reflector mode) of peptides flf14a (A), flf14b (B), and vil14a (C) after treatment with CNBr and/or chymotrypsin. Arrows highlight some molecular weights, and the corresponding fragments are shown. Monoisotopic molecular weights were compared with the calculated molecular weights.

19−26 separated by the Gly-rich (with few NOEs) segment (residues 13−18). The CSI plot (*36*) of vil14a (Figure 8B) indicated negative values for the same segments of residues 5−12 and 19−26, whereas the rest of amino acids showed positive or near-zero values.

Molecular Model of vil14a. A model of the four-Cys, three-loop sequence (1−4, 2−3 Cys pairing) with a helix−loop−helix fold found in vil14a (Figure 8C) was built using the *κ*-hefutoxin (*15*) and Om-toxins (*17*) three-dimensional (3D) structures as templates. Slight structural differences are found among these template structures; however, we selected the *κ*-hefutoxin as the template for the model of vill14a shown in Figure 8C, as the *κ*-hefutoxin-based model provided the lowest Modeler target energy.

DISCUSSION

Here we detail the isolation of four novel *Conus* peptides from the venom of *C. villepinii* and *C. floridanus floridensis* that reveal a new conotoxin framework that highly differs from those of all known conotoxins. This new framework is characterized by a four-Cys, three-loop sequence with a conserved ^{1}Cys-X$_3$-^{2}Cys-X$_{11}$-^{3}Cys-X$_3$-^{4}Cys loop spacing and 1−4, 2−3 Cys pairing. We have termed this new arrangement framework 14. The only posttranslational modifications found in all these of 27-residue framework 14 conotoxins are cystine bridges. Unlike other four-Cys conotoxins, vil14 and flfl14 conotoxins are not amidated at the C-terminal end. While these peptides have the same framework, the inter-cystine amino acid sequences can be quite variable. Besides the cystines, the only conserved residue in all these four conotoxins is Ile7. flf14a differs from flf14b in residue 16 (E in flf14a vs G in flf14b); therefore, there are three distinct framework 14 conotoxins that show different degrees of sequence variability. The N-terminal tail and loop 1 are homologous among the flf14 conotoxins. The central loop shows the greatest degree of variability among these three



flf14a	flf14b	flf14c	vil14a
α: 0.54	α: 0.69	α: 0.63	α: 0.81

FIGURE 6: CD spectra of native peptides flf14a, flf14b, flf14c, and vil14a in H_2O at 25 °C. The table below shows the relative α-helix content for each peptide.

variants. Loop 3 is conserved between flf14a/b and vill14a, but different for flf14c. This sequence variability is typical within conotoxin families, as interspecies sequence divergence, even in closely related *Conus* species (*37*), is usually observed. This new conotoxin framework was found in two different *Conus* species that exist in unrelated environments, as *C. villepinii* is restricted to deep-water habitats (>100 m), whereas *C. floridanus* is restricted to shallow water (<10 m). However, it has been recently suggested that *C. floridanus* and *C. villepinii* are in the same taxonomical clade (*38*).

It is likely that this new conotoxin framework defines a new conotoxin gene superfamily, as in most cases determined so far, new peptidic frameworks in *Conus* venom have defined new superfamilies. This is particularly true in this case, since the distribution of cysteine residues within the sequence is quite unique for *Conus*. It will be necessary to compare the mRNA/cDNA sequences that encode the precursor proteins of these conotoxins and evaluate if these precursors share a highly conserved and unique signal sequence that would define a gene superfamily to this new framework. However, this putative new superfamily would not be unique to *Conus* as other organisms (*39*), particularly scorpions (*15*−*17*), are capable of expressing this peptide framework.

Four other families of four-Cys conotoxins have been described, α-conotoxins (*40*) and ρ-conotoxins (*41*), which belong to the A superfamily, and ϵ-conotoxins (*42*) and χ-conotoxins (*43*), which are members of the T superfamily. α-, ϵ-, and ρ-conotoxins have a ^{1}Cys-^{3}Cys/^{2}Cys-^{4}Cys connectivity. By way of contrast, χ-conotoxins have ^{1}Cys-^{4}Cys/^{2}Cys-^{3}Cys connectivity. We obtained a ^{1}Cys-^{4}Cys/^{2}Cys-^{3}Cys pairing for the new framework 14 conotoxins (the same connectivity as χ-conotoxins) by digestion of three of these novel conotoxins with CNBr and chymotrypsin. Unlike all other four-Cys conotoxins, the flf14 and vil14 conotoxins have all four cystine residues spaced out by loops (no vicinal Cys residues); this allowed the use of specific peptide bond cleavage between the loops, thus releasing fragments that would reveal the Cys pairing. The advantage of this method is that it requires smaller quantities of sample and it is less laborious, as the cleavage products can be directly detected by MALDI-TOF MS.

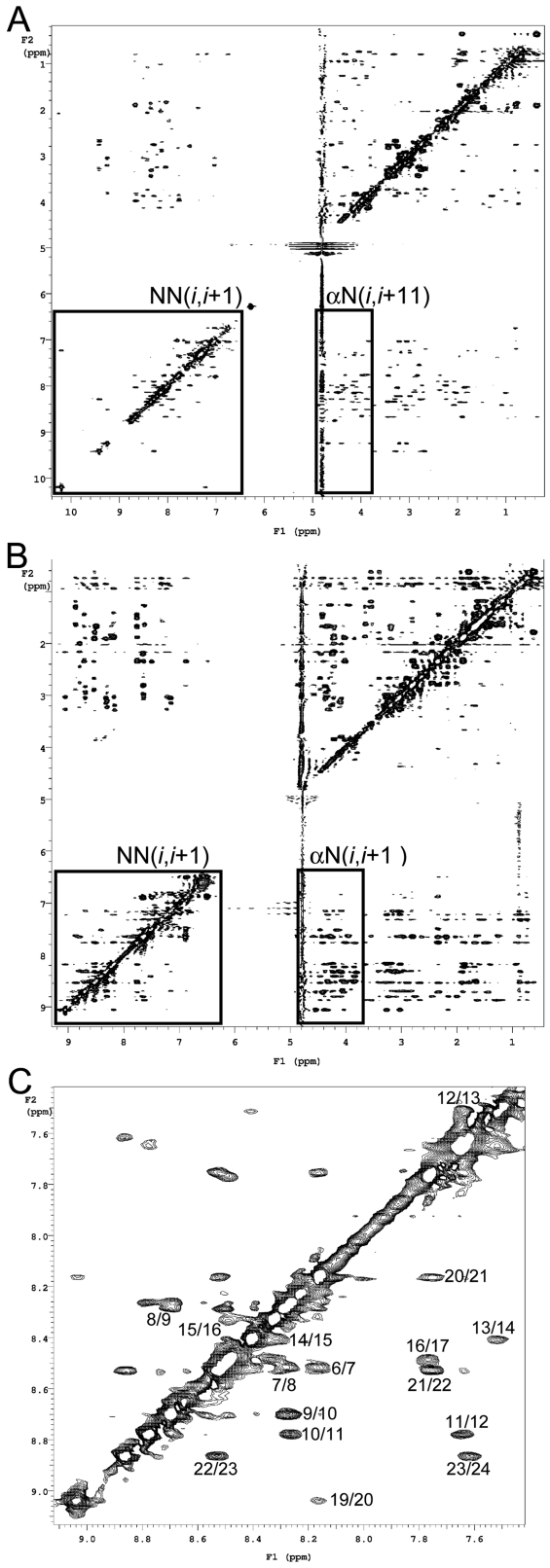


FIGURE 7: 2D NOESY spectra of flf14b (A) and vil14a (B) recorded at 25 °C using a 1.7 mm NMR tube in 3 mM gHCN probe. Water suppression was achieved using Watergate (*25*). Regions containing the NN(i, i + 1) and αN(i, i + 1) correlations are outlined with rectangular boxes. (C) NN(i, i + 1) sequence-specific assignments of vil14a.

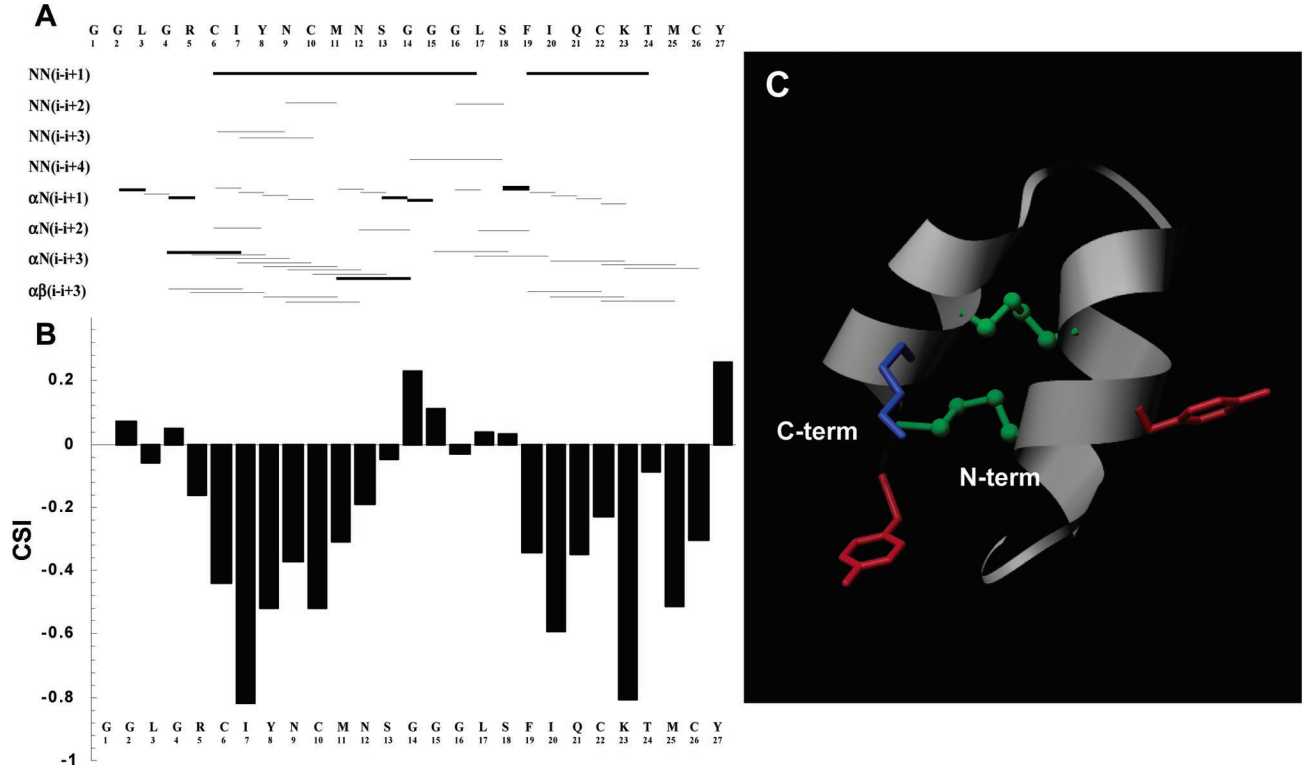


FIGURE 8: (A) Sequential NOEs of vil14a (sequence shown at the top). The relative strengths of the sequential NOEs are indicated by thickness of the bars. Two α-helical segments were found between residues 6−12 and 18−26. (B) A proton chemical shift index (CSI) indicates the presence of two helical segments in the peptide separated by a loop. (C) Model of the lowest-energy structure depicting the backbone ribbon fold and disulfide bridges of vil14a (green). Lys23 is colored blue, along with two proximal Tyr residues (8 and 27), which are colored red.

CD spectroscopy revealed a predominant α-helical content for all flf14 and vil14 conotoxins. This is an unusual structural feature, as most conotoxins are nonhelical. Measurements of the molar ellipticity at 222 nm (λ) provided us with the relative helical contents of these framework 14 conotoxins (*21*). This approach yields good estimations of the relative helical content of these peptides, provided that contributions from tertiary structure and/or disulfide bonding are equivalent within a set of peptides (*44*). CD spectra for all flf14 conotoxins were similar with one minimum of ellipticity between 207 and 209 nm (λ). The CD spectrum of vil14a was slightly different with two distinct minima (λ = 209 and 222 nm). These slight differences in the CD spectra of the framework 14 conotoxins are consistent with slight structural differences in solution as observed in the three different NMR structures of Om-toxins.

Other four-Cys, three-loop peptides with ^{1}Cys-^{4}Cys/^{2}Cys-^{3}Cys pairing have been described. Notably, the κ-hefutoxins (*15*), κ-KTx1.3 (*16*), and the Om-toxins (*17*), a family of K^+ channel-binding scorpion toxins, share the same framework as these newly described conotoxins. However, prior to the discovery of this framework in the scorpion toxins, a sequence with similar characteristics was described for MBP-1, an antibiotic peptide present in the seed of *Zea mays* (*39*) (Cys pairing was not determined for MBP-1). All of these peptides, including the flf14 and vil14 conotoxins, share this conserved framework where loops 1 and 3 invariably have three amino acids and loop 2 varies from 7 to 11 residues (Table 1). These peptides are highly α-helical according to their CD spectra, and it has been determined by NMR that the κ-hefutoxin and the Om-toxins form a helix−loop−helix cystine-stabilized (Cs α/α) fold, where the two helices are linked by the two disulfide bridges.

NanoNMR analysis was used to address the details of the structural fold found in the flf14b and vil14a conotoxins; 3−32 nmol (9−90 μg) isolated directly from the cone snails were sufficient to yield spectra suitable for the structural analysis of these peptides. By way of contrast, 7 μmol (19 mg) of synthetic peptide were utilized to determine the structure of the κ-hefutoxin. Similar synthetic quantities (∼1 μmol) were used for the structural determination of the Om-toxins. Despite the severe resonance overlap, particularly of HN protons, sequence-specific assignments of all proton resonances were achieved for the vil14 conotoxin, allowing us to assess the structural features found in this new conotoxin fold. The 2D *wg*NOESY spectra of the flf14 and vil14 conotoxins at room temperature showed very well defined structures in solution, in a way that is reminiscent of the one observed in larger tightly folded globular proteins. The NMR structures of four other disulfide-bonded conotoxins (α, ϵ, ρ, and χ) have been determined (*41*, *42*, *45*). However, these determinations have been carried out at lower temperatures and with a relatively low number of NOE constraints per residue. Highly structured frameworks have been observed in three disulfide-bonded conotoxins belonging to the O and M superfamilies, where a cystine knot with a triple-stranded β-sheet is the prevalent structural motif within these tightly compact scaffolds (*46*). This is not the case for peptides flf14a, flf14b, flf14c, and vil14a, since here we have two disulfide bonds and all four cystine residues spaced by loops of at least three amino acids. Instead, two α-helical distinct segments, residues 5−12 and 19−26, are

clearly identified by their NOE connectivities and their chemical shift indexes. This is in good agreement with the results of CD spectra, which revealed a predominantly α-helical secondary structure for these four new conotoxins. Most conotoxin frameworks described to date are nonhelical, with the exception of the α- and ρ-conotoxins, which have a short helical segment, and some M superfamily conotoxins (*47*).

The flf14 and vil14 conotoxins have the same helix−loop−helix Cs α/α fold first described for the κ-hefutoxins from the scorpion *Heterometrus fulvipes* (*15*). The κ-hefutoxins block the voltage-gated K^+ channels, Kv1.2 and Kv1.3, and also slow the activation kinetics of the Kv1.3 currents. Other related scorpion toxins, the κ-KTx1.3 from *Heterometrus spinifer* (*16*) and the Om-toxins from *Opisthacanthus madagascarienses* (*17*), share this same fold, and they show differential targeting of the K^+ channels. In addition to the Cys residues, the κ-hefutoxins have several amino acids in common with the vil14 and flf14 conotoxins. Examination of existing four-Cys frameworks with a ^{1}Cys-^{4}Cys/^{2}Cys-^{3}Cys pairing reveals several related nonhelical toxins such as the χ-conotoxins from *Conus marmoreus*, which target the noradrenaline transporter (*41*); Tachyplesin, a peptide with antimicrobial properties isolated from the horseshoe crab *Tachypleus tridentatus* (*48*); and Gomesin, also an antimicrobial peptide from hemocytes of the spider *Acanthoscurria gomesiana* (*49*). However, their 3D fold is quite different from that of the framework 14 conotoxins.

Comparison of the loop size of the vill14 and flf14 conotoxins with Cs α/α scorpion toxins shows that the first and third loops have the same three-amino acid spacing (Table 1); the second loop of κ-hefutoxins is two residues shorter. Even shorter spacing of the central loops can be found in the Tachyplesin and Gomesin (3/5/3); however, these peptides are not helical, and they exhibit a Cs β/β fold. Therefore, the length of the central loop is critical in determining the overall fold of these motifs.

Structural analysis of our nanoNMR data confirmed that the flf14 and vil14 conotoxins have the same three-dimensional fold as the κ-hefutoxins and Om-toxins. It is quite remarkable that we found a new conotoxin framework in two *Conus* worm-hunting species that resemble so closely these newly discovered Cs α/α K^+ channel-binding scorpion toxins. Just as for other potassium channel toxins, the vil14 conotoxin has a functional Lys/Tyr (or Phe) dyad separated by 6.0 ± 1.0 Å (*50*). This is consistent with the model built for the vil14a conotoxin, based on the NMR structure of the κ-hefutoxin. This model is in agreement with our current nanoNMR data, and only small variations with the nanoNMR-based 3D structure of the vil14a (currently in progress) are expected. Furthermore, the Cs α/α scorpion toxins exhibit only small structural variations among themselves (*17*). The location of Lys23 (in blue in Figure 8C) indicates two proximal Tyr residues (Y8 and Y27). Therefore, two potential dyad pairs can be considered for the K^+ channel binding properties of vil14a. This dyad was found in structurally unrelated potassium channel-blocking toxins from scorpions (*51*), sea anemones (*52*) and in the κ-conotoxins (*53*−*55*). The vil14a conotoxin has a putative functional dyad in residues Tyr8 and Lys23 or Tyr27 and Lys23. Given that the κ-hefutoxins and Om-toxins bear the dyad and clearly are structurally related to the framework 14 conotoxins, we can extend the dyad binding mode to the new framework 14 conotoxins. We have carried out experiments that indicate that vil14a indeed blocks K^+ channels in PC12 cells; these results will be reported elsewhere. However, the sequences of flf14 conotoxins do not have Lys residues, and no apparent dyad is contained in these conotoxins. This is also the case with OmTx3, the only Om-toxin (Table 1) that lacks the functional dyad; however, it is the best blocker of the Om-toxin set. It has been proposed that other positively charged residues may mimic K^+ ions entering the pore, occluding the ion pathway (*56*). Furthermore, alternate modes of binding to the K^+ channels, which excludes the aromatic amino acid as being critical for binding (*57*), or that completely exclude the functional dyad, have been suggested (*58*). The binding determinants and the differential targeting of these conotoxins are currently being addressed with the appropriate electrophysiological experiments.

The commonalities of these new framework 14 conotoxins with the recently described Cs α/α K^+ channel binding toxins found in scorpions are an indication of a shared molecular imprint in these unrelated predatory venomous animals. While the venom of these animals is a rich source of Cys-constrained neuroactive peptides, the detailed frameworks, the number of Cys residues, the arrangement of Cys with the sequence, loop sizes, the Cys pairing, and the length of the polypeptide chains generally differ. Scorpion toxins are larger, with more Cys bridges, and their frameworks are different from the ones found in conotoxin families and superfamilies. In fact, the gene arrangements are different as toxin precursors are encoded by three exons in cone snails as opposed to two exons in scorpions (*59*). However, all of these toxins are probably related by common ancestral genes which were initially found in scorpions. Ancient scorpions of aquatic origin are estimated to have appeared 400 million years ago, whereas cone snails date to 55 million years ago according to the fossil record (*59*). While *Conus* venom toxins are considered molecularly more diverse, as they are capable of a combinatorial plethora of posttranslational modifications and express linear families of conopeptides, it is unclear how much commonality there is between *Conus* peptides and peptides found in other organisms since only a small fraction of either the *Conus* peptide library or the scorpion toxins library has been characterized. Another conotoxin framework, specifically the classical O superfamily conotoxin C-C-CC-C-C array, has been found in spiders (*60*) and sea sponges (*61*) and within the viral genomes (*62*). However, in the case of the fll14 and vil14 conotoxins, cone snails are utilizing the same biochemical strategy as scorpions in targeting ion channels through this particular Cs α/α framework to capture their prey. Further structural and functional studies of these conotoxins will provide a better understanding of the usefulness of this new scaffold in molecular engineering and neuronal targeting and could lead to the design of neuropharmacological agents that target specific receptors or ion channels.

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SUPPORTING INFORMATION AVAILABLE

^{1}H NMR chemical shift assignments for vil14a. The sequences of the framework 14 conotoxins have been deposited with the UniProt Knowledgebase with accession numbers P84704 for conotoxin vil14a, P84705 for conotoxin flf14a, P84706 for conotoxin flf14b and P84707 for conotoxin flf14c. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Terlau, H., and Olivera, B. M. (2004) *Conus* venoms: A rich source of novel ion channel-targeted peptides, *Physiol. Rev. 84*, 41−68.
2. McIntosh, J. M., and Jones, R. M. (2001) Cone venom: From accidental stings to deliberate injection, *Toxicon 39*, 1447−1451.
3. Mari, F., and Fields, G. B. (2003) Conopeptides: Unique pharmacological agents that challenge current peptide methodologies, *Chim. Oggi 21*, 43−48.
4. Craig, A. G., Bandyopadhyay, P., and Olivera, B. M. (1999) Post-translationally modified neuropeptides from *Conus* venoms, *Eur. J. Biochem. 264*, 271−275.
5. Craig, A. G., Norberg, T., Griffin, D., Hoeger, C., Akhtar, M., Schmidt, K., Low, W., Dykert, J., Richelson, E., Navarro, V., Mazella, J., Watkins, M., Hillyard, D., Imperial, J., Cruz, L. J., and Olivera, B. M. (1999) Contulakin-G, an O-glycosylated invertebrate neurotensin, *J. Biol. Chem. 274*, 13752−13759.
6. Craig, A. G., Park, M., Fischer, W. H., Kang, J., Compain, P., and Piller, F. (2001) Enzymatic glycosylation of contulakin-G, a glycopeptide isolated from *Conus* venom, with a mammalian ppGalNAc-transferase, *Toxicon 39*, 809−815.
7. Buczek, O., Yoshikami, D., Bulaj, G., Jimenez, E. C., and Olivera, B. M. (2005) Post-translational amino acid isomerization: A functionally important D-amino acid in an excitatory peptide, *J. Biol. Chem. 280*, 4247−4253.
8. Franco A., Pisarewicz, K., Moller, C., Mora D., Fields G. B., Mari, F. (2005) Hyperhydroxylation: A new strategy for neuronal targeting by venomous marine molluscs, in *Marine Molecular Biotechnology: Mollusca* (gastropoda) (Cimino, G., Ed.) Springer, New York (in press).
9. Pisarewicz, K., Mora, D., Pflueger, F. C., Fields, G. B., and Mari, F. (2005) Polypeptide chains containing D-γ-hydroxyvaline, *J. Am. Chem. Soc. 127*, 6207−6215.
10. McIntosh, J. M., Olivera, B. M., and Cruz, L. J. (1999) *Conus* peptides as probes for ion channels, *Methods Enzymol. 294*, 605−624.
11. Myers, R. A., Cruz, L. J., Rivier, J. E., and Olivera, B. M. (1993) *Conus* peptides as chemical probes for receptors and ion channels, *Chem. Rev. 93*, 1923−1936.
12. Olivera, B. M. (2002) *Conus* venom peptides: Reflections from the biology of clades and species, *Annu. Rev. Ecol. Syst. 33*, 25−47.
13. Newcomb, R., and Miljanich, G. (2002) Neurotoxins of cone snail venoms, *Handb. Neurotoxicol. 1*, 617−651.
14. Filmer, R. M. (2001) A catalogue of nomenclature and taxonomy in the living, *Conidae 1758−1998*, Backhuys Publisher, Leiden, The Netherlands.
15. Srinivasan, K. N., Sivaraja, V., Huys, I., Sasaki, T., Cheng, B., Kumar, T. K. S., Sato, K., Tytgat, J., Yu, C., San, B. C. C., Ranganathan, S., Bowie, H. J., Kini, R. M., and Gopalakrishnakone, P. (2002) κ-Hefutoxin-1, a novel toxin from the scorpion *Heterometrus fulvipes* with unique structure and function: Importance of the functional diad in potassium channel selectivity, *J. Biol. Chem. 277*, 30040−30047.
16. Nirthanan, S., Pil, J., Abdel-Mottaleb, Y., Sugahara, Y., Gopalakrishnakone, P., Joseph, J. S., Sato, K., and Tytgat, J. (2005) Assignment of voltage-gated potassium channel blocking activity to κ-Ktx1.3, a non-toxic homologue of κ-hefutoxin-1, from *Heterometrus spinifer* venom, *Biochem. Pharmacol. 69*, 669−678.
17. Chagot, B., Pimentel, C., Dai, L., Pil, J., Tytgat, J., Nakajima, T., Corzo, G., Darbon, H., and Ferrat, G. (2005) An unusual fold for potassium channel blockers: NMR structure of three toxins from the scorpion *Opisthacanthus madagascariensis*, *Biochem. J. 388*, 263−271.
18. Yen, T. Y., Yan, H., and Macher, B. A. (2002) Characterizing closely spaced, complex disulfide bond patterns in peptides and proteins by liquid chromatography/electrospray ionization tandem mass spectrometry, *J. Mass Spectrom. 37*, 15−30.
19. Simpson, R. J. (2002) *Proteins and proteomics: A laboratory manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.
20. Sudarslal, S., Majumdar, S., Ramasamy, P., Dhawan, R., Pal, P. P., Ramaswami, M., Lala, A. K., Sikdar, S. K., Sarma, S. P., Krishnan, K. S., and Balaram, P. (2003) Sodium channel modulating activity in a δ-conotoxin from an Indian marine snail, *FEBS Lett. 553*, 209−212.
21. Scholtz, J. M., Hong, Q., York, E. J., Stewart, J. M., and Baldwin, R. L. (1991) Parameters of helix-coil transition theory for alanine-based peptides of varying chain lengths in water, *Biopolymers 31*, 1463−1470.
22. Smallcombe, S. H., Patt, S. L., and Keifer, P. A. (1995) WET solvent suppression and its applications to LC-NMR and high-resolution NMR spectroscopy, *J. Magn. Reson. 117*, 295−303.
23. Larive, C. K., Jayawickrama, D., and Orfi, L. (1997) Quantitative analysis of peptides with NMR spectroscopy, *Appl. Spectrosc. 51*, 1531−1536.
24. Piotto, M., Saudek, V., and Sklenar, V. (1992) Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions, *J. Biomol. NMR 2*, 661−665.
25. Price, W. S. (1999) Water signal suppression in NMR spectroscopy, *Annu. Rep. NMR Spectrosc. 38*, 289−354.
26. Wuthrich, K. (1986) *NMR of proteins and nucleic acids*, Wiley & Sons, New York.
27. States, D. J., Haberkorn, R. A., and Ruben, D. J. (1982) A two-dimensional nuclear Overhauser experiment with pure absorption phase in 4 quadrants, *J. Magn. Reson. 48*, 286−292.
28. Fiser, A. S., and Sali, A. (2003) MODELLER: Generation and refinement of homology-based protein structure models, *Methods Enzymol. 374*, 463−493.
29. John, B., and Sali, A. (2003) Comparative protein structure modeling by iterative alignment, model building and model assessment, *Nucleic Acids Res. 31*, 3982−3992.
30. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera: A visualization system for exploratory research and analysis, *J. Comput. Chem. 25*, 1605−1612.
31. Aguilar, M. B., Lopez-Vera, E., Ortiz, E., Becerril, B., Possani, L. D., Olivera, B. M., and de la Cotera, E. P. H. (2005) A novel conotoxin from *Conus delessertii* with posttranslationally modified lysing residues, *Biochemistry 44*, 11130−11136.
32. Aird, S. D. (2004) Chromatographic behavior of *Bothrops erythromelas* phospholipase and other venom constituents on Superdex 75, *Prep. Biochem. Biotechnol. 34*, 345−364.
33. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J. H., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped blast and psi-blast: A new generation of protein database search programs, *Nucleic Acids Res. 25*, 3389−3402.
34. Clauser, K. R., Baker, P., and Burlingame, A. L. (1999) Role of accurate mass measurement ($\pm$ 10 ppm) in protein identification strategies employing MS or MS/MS and database searching, *Anal. Chem. 71*, 2871−2882.
35. Zhang, X. Y., Dillen, L., Vanhoutte, K., VanDongen, W., Esmans, E., and Claeys, M. (1996) Characterization of unstable intermediates and oxidized products formed during cyanogen bromide cleavage of peptides and proteins by electrospray mass spectrometry, *Anal. Chem. 68*, 3422−3430.
36. Wishart, D. S., Sykes, B. D., and Richards, F. M. (1992) The chemical-shift index: A fast and simple method for the assignment of protein secondary structure through NMR-spectroscopy, *Biochemistry 31*, 1647−1651.
37. Woodward, S. R., Cruz, L. J., Olivera, B. M., and Hillyard, D. R. (1990) Constant and hypervariable regions in conotoxin propeptides, *EMBO J. 9*, 1015−1020.
38. Duda, T. F., and Rolan, E. (2005) Explosive radiation of Cape Verde *Conus*, a marine species flock, *Mol. Ecol. 14*, 267−272.
39. Duvick, J. P., Rood, T., Rao, A. G., and Marshak, D. R. (1992) Purification and characterization of a novel antimicrobial peptide from maize (*Zea mays*) kernels, *J. Biol. Chem. 267*, 18814−18820.
40. McIntosh, J. M., Santos, A. D., and Olivera, B. M. (1999) *Conus* peptides targeted to specific nicotinic acetylcholine receptor subtypes, *Annu. Rev. Biochem. 68*, 59−88.

41. Sharpe, I. A., Gehrmann, J., Loughnan, M. L., Thomas, L., Adams, D. A., Atkins, A., Palant, E., Craik, D. J., Adams, D. J., Alewood, P. F., and Lewis, R. J. (2001) Two new classes of conopeptides inhibit the α1-adrenoceptor and noradrenaline transporter, *Nat. Neurosci. 4*, 902−907.
42. Rigby, A. C., Lucas-Meunier, E., Kalume, D. E., Czerwiec, E., Hambe, B., Dahlqvist, I., Fossier, P., Baux, G., Roepstorff, P., Baleja, J. D., Furie, B. C., Furie, B., and Stenflo, J. (1999) A conotoxin from *Conus textile* with unusual posttranslational modifications reduces presynaptic Ca^{2+} influx, *Proc. Natl. Acad. Sci. U.S.A. 96*, 5758−5763.
43. McIntosh, J. M., Corpuz, G. O., Layer, R. T., Garrett, J. E., Wagstaff, J. D., Bulaj, G., Vyazovkina, A., Yoshikami, D., Cruz, L. J., and Olivera, B. M. (2000) Isolation and characterization of a novel *Conus* peptide with apparent antinociceptive activity, *J. Biol. Chem. 275*, 32391−32397.
44. Forood, B., Feliciano, E. J., and Nambiar, K. P. (1993) Stabilization of α-helical structures in short peptides via end capping, *Proc. Natl. Acad. Sci. U.S.A. 90*, 838−842.
45. Millard, E. L., Daly, N. L., and Craik, D. J. (2004) Structure−activity relationships of α-conotoxins targeting neuronal nicotinic acetylcholine receptors, *Eur. J. Biochem. 271*, 2320−2326.
46. Craik, D. J., Daly, N. L., and Waine, C. (2001) The cystine knot motif in toxins and implications for drug design, *Toxicon 39*, 43−60.
47. Hill, J. M., Alewood, P. F., and Craik, D. J. (1996) Three-dimensional solution structure of μ-conotoxin GIIIB, a specific blocker of skeletal muscle sodium channels, *Biochemistry 35*, 8824−8835.
48. Kawano, K., Yoneya, T., Miyata, T., Yoshikawa, K., Tokunaga, F., Terada, Y., and Iwanaga, S. (1990) Antimicrobial peptide, Tachyplesin-I, isolated from hemocytes of the horseshoe-crab (*Tachypleus tridentatus*): NMR determination of the β-sheet structure, *J. Biol. Chem. 265*, 15365−15367.
49. Silva, P. I., Daffre, S., and Bulet, P. (2000) Isolation and characterization of Gomesin, an 18-residue cysteine-rich defense peptide from the spider *Acanthoscurria gomesiana* hemocytes with sequence similarities to horseshoe crab antimicrobial peptides of the Tachyplesin family, *J. Biol. Chem. 275*, 33464−33470.
50. Dauplais, M., Lecoq, A., Song, J. X., Cotton, J., Jamin, N., Gilquin, B., Roumestand, C., Vita, C., deMedeiros, C. L. C., Rowan, E. G., Harvey, A. L., and Menez, A. (1997) On the convergent evolution of animal toxins: Conservation of a dyad of functional residues in potassium channel-blocking toxins with unrelated structures, *J. Biol. Chem. 272*, 4302−4309.
51. Miller, C., Moczydlowski, E., Latorre, R., and Phillips, M. (1985) Charybdotoxin, a protein inhibitor of single Ca^{2+}-activated K^+ channels from mammalian skeletal-muscle, *Nature 313*, 316−318.
52. Castaneda, O., Sotolongo, V., Amor, A. M., Stocklin, R., Anderson, A. J., Harvey, A. L., Engstrom, A., Wernstedt, C., and Karlsson, E. (1995) Characterization of a potassium channel toxin from the Caribbean sea-anemone *Stichodactyla helianthus*, *Toxicon 33*, 603−613.
53. Jacobsen, R. B., Koch, E. D., Lange-Malecki, B., Stocker, M., Verhey, J., Van Wagoner, R. M., Vyazovkina, A., Olivera, B. M., and Terlau, H. (2000) Single amino acid substitutions in κ-conotoxin PVIIA disrupt interaction with the shaker K^+ channel, *J. Biol. Chem. 275*, 24639−24644.
54. Savarin, P., Guenneugues, M., Gilquin, B., Lamthanh, H., Gasparini, S., Zinn-Justin, S., and Menez, A. (1998) Three-dimensional structure of κ-conotoxin PVIIA, a novel potassium channel-blocking toxin from cone snails, *Biochemistry 37*, 5407−5416.
55. Terlau, H., Shon, K. J., Grilley, M., Stocker, M., Stuhmer, W., and Olivera, B. M. (1996) Strategy for rapid immobilization of prey by a fish-hunting marine snail, *Nature 381*, 148−151.
56. Goldstein, S. A. N., and Miller, C. (1993) Mechanism of charybdotoxin block of a voltage-gated K^+ channel, *Biophys. J. 65*, 1613−1619.
57. Mouhat, S., Mosbah, A., Visan, V., Wulff, H., Delepierre, M., Darbon, H., Grissmer, S., De Waard, M., and Sabatier, J. M. (2004) The 'functional' dyad of scorpion toxin pi1 is not itself a prerequisite for toxin binding to the voltage-gated Kv1.2 potassium channels, *Biochem. J. 377*, 25−36.
58. Al-Sabi, A., Lennartz, D., Ferber, M., Gulyas, J., Rivier, J. E. F., Olivera, B. M., Carlomagno, T., and Terlau, H. (2004) κM-conotoxin RIIIK, structural and functional novelty in a K^+ channel antagonist, *Biochemistry 43*, 8625−8635.
59. Sollod, B. L., Wilson, D., Zhaxybayeva, O., Gogarten, J. P., Drinkwater, R., and King, G. F. (2005) Were arachnids the first to use combinatorial peptide libraries? *Peptides 26*, 131−139.
60. Balaji, R. A., Sasaki, T., Gopalakrishnakone, P., Sato, K., Kini, R. M., and Bay, B. H. (2000) Purification, structure determination and synthesis of covalitoxin-II, a short insect-specific neurotoxic peptide from the venom of the *Coremiocnemis validus* (Singapore tarantula), *FEBS Lett. 474*, 208−212.
61. Fusetani, N. (2004) Enzyme inhibitors from Japanese marine sponges, XI International Symposium on Marine Natural Products, Sorrento, Italy.
62. Eldridge, R., Li, Y. H., and Miller, L. K. (1992) Characterization of a baculovirus gene encoding a small conotoxin-like polypeptide, *J. Virol. 66*, 6563−6571.

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