Definition of the M-Conotoxin Superfamily: Characterization of Novel Peptides from Molluscivorous *Conus* Venoms†

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ABSTRACT: Most of the >50 000 different pharmacologically active peptides in *Conus* venoms belong to a small number of gene superfamilies. In this work, the M-conotoxin superfamily is defined using both biochemical and molecular criteria. Novel excitatory peptides purified from the venoms of the molluscivorous species *Conus textile* and *Conus marmoreus* all have a characteristic pattern of Cys residues previously found in the μ-, κM-, and ψ-conotoxins (CC−C−C−CC). The new peptides are smaller (12−19 amino acids) than the μ-, κM-, and ψ-conotoxins (22−24 amino acids). One peptide, mr3a, was chemically synthesized in a biologically active form. Analysis of the disulfide bridges of a natural peptide tx3c from *C. textile* and synthetic peptide mr3a from *C. marmoreus* showed a novel pattern of disulfide connectivity, different from that previously established for the μ- and ψ-conotoxins. Thus, these peptides belong to a new group of structurally and pharmacologically distinct conotoxins that are particularly prominent in the venoms of mollusc-hunting *Conus* species. Analysis of cDNA clones encoding the novel peptides as well as those encoding μ-, κM-, and ψ-conotoxins revealed highly conserved amino acid residues in the precursor sequences; this conservation in both amino acid sequence and in the Cys pattern defines a gene superfamily, designated the M-conotoxin superfamily. The peptides characterized can be provisionally assigned to four distinct groups within the M-superfamily based on sequence similarity within and divergence between each group. A notable feature of the superfamily is that two distinct structural frameworks have been generated by changing the disulfide connectivity on an otherwise conserved Cys pattern.

Conotoxins are multiply disulfide cross-linked peptides found in the venoms of predatory gastropods belonging to the genus *Conus* (cone snails) (*1*, *2*). It has been estimated that >50 000 different peptides are present in these venoms, with the majority encoded by relatively few gene superfamilies. For every *Conus* peptide gene superfamily, the initial translation product is a conotoxin precursor that typically has three well-defined regions: the N-terminal signal sequence, a middle "pro" region, and at the C-terminal end, the "toxin region" that encodes the mature peptide, always in single copy. This canonical organization of conotoxin precursors was first established by Woodward et al. (*3*).

Although many mature conotoxins are 10−30 amino acids, the corresponding precursors from which they are proteolytically processed are generally 60−90 amino acids long. A particular conotoxin gene superfamily is characterized by a highly conserved signal sequence as well as a specific pattern of cysteine residues in the mature toxin region (the "Cys pattern"). These Cys residues form between two and five disulfide cross-links that define a structural framework that determines the backbone conformation of peptides that belong to that gene superfamily.

One discovery strategy for elucidating pharmacologically novel conotoxins has been to assess high-perfomance liquid chromatography (HPLC)[1]-separated fractions from crude venom extracts for biological activity using a simple assay chosen for its ability to detect a broad range of activities.

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[1] Abbreviations: ACN, acetonitrile; CZE, capillary zone electrophoresis; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; HAc, acetic acid; HFBA, heptafluorobutyric acid; HPLC, high-performance liquid chromatography; i.c., intracranial; i.p., intraperitoneal; LSI, liquid secondary ionization; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid.

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The traditional approach of our laboratories has been to perform intracranial injections in mice of lyophilized venom fractions (*1*, *4*). This allows the "tracking" of neurologically active venom components through multiple rounds of purification. A second strategy for discovering the sequences of novel conotoxins has been to derive cDNA libraries from venom duct material. Peptides can then be chemically synthesized and tested for biological activity.

In this paper, we describe the purification and characterization of novel peptides expressed in the venoms of two mollusc-hunting *Conus* species using a combination of the above technologies. These toxins elicited effects when assayed in mice that were generally excitatory and, upon biochemical characterization, proved to have a similar arrangement of Cys residues (CC- -C- -C- -CC- -), a Cys pattern previously found in conotoxins belonging to the μ, ψ, and κM families (*5*−*8*). All of these peptides also share considerable sequence identity in their precursor sequences; together with the conserved Cys pattern, this indicates that they all belong to the same gene superfamily, designated the M-conotoxin superfamily. However, there are characteristic differences between the new conotoxins and the M-superfamily peptides previously characterized. The new peptides are significantly smaller, and most unexpectedly, a novel pattern of disulfide connectivity was established. Thus, the peptides characterized belong to novel branches of the M-superfamily of conotoxins, at least two of which have different structural frameworks.

MATERIALS AND METHODS

Purification of Peptides from Venoms. Lyophilized *Conus textile* venom was extracted and fractionated with 1.1% acetic acid (HAc) on a Sephadex G-25 (110 × 2.5 cm) column and eluted with 1.1% HAc. An aliquot of pooled fractions was purified on a Vydac C_{18} semipreparative column and eluted using a system with two solvents, 0.1% trifluoroacetic acid (TFA) and solvent B_{60} [0.092% TFA in 60% acetonitrile (ACN)] with a flow rate of 3 mL/min. An aliquot of the bioactive peak was applied to a C_{18} analytical column and eluted using the same gradient at 1 mL/min. Peptides tx3a, tx3b, and tx3c were purified on the same column by elution with a gradient of ACN in 0.05% heptafluorobutyric acid (HFBA) at 1 mL/min.

Lyophilized crude venom from *Conus marmoreus* was extracted, placed in a Centriprep 30 microconcentrator, and centrifuged at 1500*g* for 8 h at 4 °C. The filtrate was purified on a Vydac C_{18} preparative column using a two-solvent gradient system (0.1% TFA and B_{60}) at a flow rate of 10 mL/min. For two subsequent steps, a Vydac C_{18} analytical column was used to purify peptide mr3a with the same two solvents described above, but a flow rate of 1 mL/min was used. Alternatively, lyophilized *C. marmoreus* venom was extracted and fractionated on a Sephadex G-25 column as described for *C. textile* venom. Fractions were pooled and applied to a Vydac C_{18} semipreparative column, eluted with a gradient of solvent B_{60} at a flow rate of 3 mL/min. The bioactive fraction was applied to a Vydac C_{18} analytical column and eluted using a gradient of ACN in 0.05% HFBA at 1 mL/min. The peptide mr3b was finally purified on the same column using a gradient of solvent B_{60} at 1 mL/min.

Reduction, Alkylation and Sequencing of Peptides. The peptides were reduced with dithiothreitol or β-mercaptoethanol, and cysteines were carboxymethylated or pyridylethylated as described previously (*9*−*11*). The alkylated peptides were purified with a Vydac C_{18} analytical column using the linear gradient of 0.1% TFA and solvent B_{60}. Alkylated peptide was sequenced by Edman degradation (*12*) using an Applied Biosystems 477A protein sequenator at the DNA/Peptide Facility of the University of Utah, courtesy of Dr. Robert Schackmann. Alternatively, alkylated peptide was sequenced using a Beckman 890D spinning cup sequencer, which contained 1 mg of polybrene that had been precycled through several steps of degradation.

Mass Spectrometry. Electrospray ionization (ESI) mass spectra were measured using an Esquire ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Liquid secondary ionization (LSI) mass spectra measurements were performed on a JEOL JMS-HX110 double-focusing mass spectrometer fitted with a Cs^+ gun, courtesy of Dr. Anthony Craig of the Salk Institute for Biological Studies, La Jolla, CA. An accelerating voltage of 10 kV and Cs^+ gun voltage between 25 and 30 kV were employed. The samples were added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix. Matrix-assisted laser desorption ionization (MALDI) mass spectra were obtained using a Voyager System 4271 at the University of Utah Mass Spectrometry Core Facility.

Bioassay. Biological activity was assayed by injection of peptides in mice as described previously (*13*). Aliquots of peptide samples were lyophilized and dissolved in normal saline solution (NSS). Swiss Webster mice were injected intracranially (i.c.) or intraperitoneally (i.p.) with 20 μL of the peptide solution using a 29-gauge needle. Goldfish (0.7−0.9 g) were injected i.p. with 5 μL of the peptide solution. Controls were similarly injected with NSS.

Disulfide Bridge Analysis. Peptide tx3c was purified from *C. textile* venom as previously described (*1*). The disulfide connectivity of the peptide was carried out by the partial reduction and alkylation method of Gray (*9*). The natural peptide tx3c (5 nmol) was reduced at pH 3 for 2 min with 10 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) at room temperature. Partially reduced peptides were sequentially alkylated with 4-vinylpyridine and iodoacetamide. The products were purified by HPLC using a C_{18} analytical column and a gradient of ACN in 0.1% TFA. The alkylated peptides were sequenced by the Edman degradation method.

The analysis of the disulfide connectivity of peptide mr3a was carried out using synthetic peptide prepared as described in the following section because the quantity of the natural peptide was limited. The analysis was similarly done using the method described for tx3c.

Peptide Synthesis. Peptide mr3a was synthesized by the solid-phase method on a Beckman 990 peptide synthesizer using standard Boc chemistry, starting with Boc-Val-CM resin (3 g, 0.6 mmol/g). Couplings were mediated either by diisopropylcarbodiimide and *N*-hydroxybenzotriazole in a mixture of dichloromethane and dimethyl sulfoxide (DMSO) or by TBTU and diisopropylethylamine in DMSO. Coupling time was 1 h, followed by acetylation using excess acetic anhydride in dichloromethane for 15 min. For each coupling, a 3-fold excess of protected amino acid was used based on the original substitution of the resin. The completed peptide resin was then cleaved and deprotected in 60 mL of anhydrous HF in the presence of 4 mL of anisole and 4 mL of methyl sulfide at 0 °C for 90 min. The crude peptide,

Table 1

A. Sequences and Behavioral Effects of Purified Conotoxins

peptide	sequence[a]	behavioral symptomatology used as an assay for purification
C. textile peptides		
tx3a	CCSWDVCDHPSCTCCG^	scratching, hyperactivity
tx3b	CCPPVACNMGCKPCC*	scratching, hyperactivity, circular motion
tx3c	CCRTCFGCTOCC*	scratching
C. marmoreus peptides		
mr3a	GCCGSFACRFGCVOCCV^	circular motion, barrel rolling
mr3b	SKQCCHLAACRFGCTOCCW^	scratching, hyperactivity

B. Comparison of Biological Effects of Synthetic and Natural Peptide mr3a[b]

peptide	dose (nmol/g body wt)	observed behavioral effects
synthetic mr3a	0.30	circular motion within ~1 min after injection; brief barrel rolling and convulsion within 1−2 min after injection
natural mr3a	0.30	circular motion within ~1 min after injection; brief barrel rolling and convulsion within ~1.5 min after injection
synthetic mr3a	0.74	barrel rolling and convulsions within ~1 min after injection; barrel rolling for a longer period within ~2 min after injection
natural mr3a	0.74	barrel rolling and convulsions within 0.5−1.0 min after injection; barrel rolling for a longer period within ~1.5−2.5 min after injection

C. Quantitation of Biological Effects of mr3a[c]

dose (nmol/g body wt)	mode of injection	observed behavioral effects
0.085	i.c.	scratching
0.17	i.c.	circular motion
0.34	i.c.	circular motion, barrel rolling, convulsion
0.85	i.c.	barrel rolling, convulsion, death
1.7	i.p.	none

[a] O, 4-*trans*-hydroxyproline; ^, C-terminal free acid; *, C-terminal amidation. [b] Varied doses of synthetic and natural peptide mr3a dissolved in NSS were injected i.c. into 10-day-old Swiss Webster mice weighing 6.6−6.9 g. Two mice were injected per dose. [c] Varied doses of synthetic mr3a dissolved in NSS were injected in 10-day-old Swiss Webster mice weighing 5.7−6.0 g. Three to four mice were injected per dose.

after HF removal and diethyl ether precipitation, was collected by filtration and dissolved in 400 mL of 0.5% TFA in 25% ACN. The pH of the crude peptide solution was adjusted to 4.5 with ammonium hydroxide, and the solution was then treated with 2.7 mmol of TCEP for 1 h at 50 °C and then overnight at room temperature. Some insoluble material was filtered off.

Prior to folding, the crude linear peptide was purified by preparative reverse-phase HPLC using a linear gradient of ACN (isocratic at 12% ACN for 10 min and then 12−36% ACN over 60 min) in 0.25 M triethylammonium phosphate at pH 2.25 (TEAP 2.25) in a Waters Associates Prep/LC System 500A at a flow rate of 10 mL/min. To the purified linear peptide in 1200 mL of 25% ACN/TEAP 2.25 buffer was added a 50% mixture of reduced/oxidized glutathione generated from 2.4 mmol of reduced glutathione in 15 mL of H_2O and 0.6 mmol of iodine in 15 mL of methanol. The pH of the mixture was adjusted to 7.2 with diluted ammonium hydroxide solution. The solution was purged with N_2 and then stirred overnight at room temperature.

The resulting cyclized peptide was purified by preparative reverse-phase HPLC using a linear gradient of ACN (isocratic at 15% ACN in TEAP 2.25 for 10 min and then 15−33% ACN in TEAP 2.25 over 90 min). Appropriate fractions were pooled and converted to the trifluoroacetate salt using a linear gradient of ACN in 0.1% TFA (isocratic at 21% ACN for 10 min and then 21−39% ACN over 60 min). The purity of the peptide was determined by both HPLC using a Vydac C_{18} column and capillary zone electrophoresis (CZE) on a Beckman P/ACE system 2050. CZE employed a field strength of 15 kV at 30 °C with a buffer of 15% ACN/85% 100 mM sodium phosphate at pH 2.5 on a Beckman CAP fused silica capillary. Amino acid analysis, after 4 M methanesulfonic acid hydrolysis for 24 h at 110 °C, was carried out on a Perkin−Elmer HPLC using *o*-phthalaldehyde postcolumn derivatization and fluorescence detection.

To determine if the correct synthetic mr3a was obtained, synthetic and natural peptides were separately applied into an analytical C_{18} column. The middle portions of the peak fractions were collected and combined. The combined sample was diluted, reapplied to the column, and eluted using the same gradient of ACN.

Identification and Sequencing of cDNA Clones. The cDNA clones encoding M-superfamily conotoxins from *C. textile* and *C. marmoreus*, as well as μ-, κM- and ψ-conotoxins from some fish-hunting *Conus* species, were determined using two methods.

Method I. Clones were selected from size-fractionated cDNA library constructed using mRNA obtained from *Conus* venom duct as described previously (*14*). The cDNA library was screened using a specific probe corresponding to the amino acids at the 5′ end or the signal sequence of the peptides. The oligonucleotide was end-labeled and hybridized, and a secondary screening by polymerase chain reaction (PCR) was performed on clones that hybridized to this probe as described earlier (*15*). Clones identified in the secondary screen were prepared for DNA sequencing by standard methods (*16*). The DNA sequence was determined according to the standard protocol for Sequenase version 2.0 DNA sequencing.

Method II. Clones were identified from size-fractionated cDNA libraries. First strand cDNA synthesis from RNA

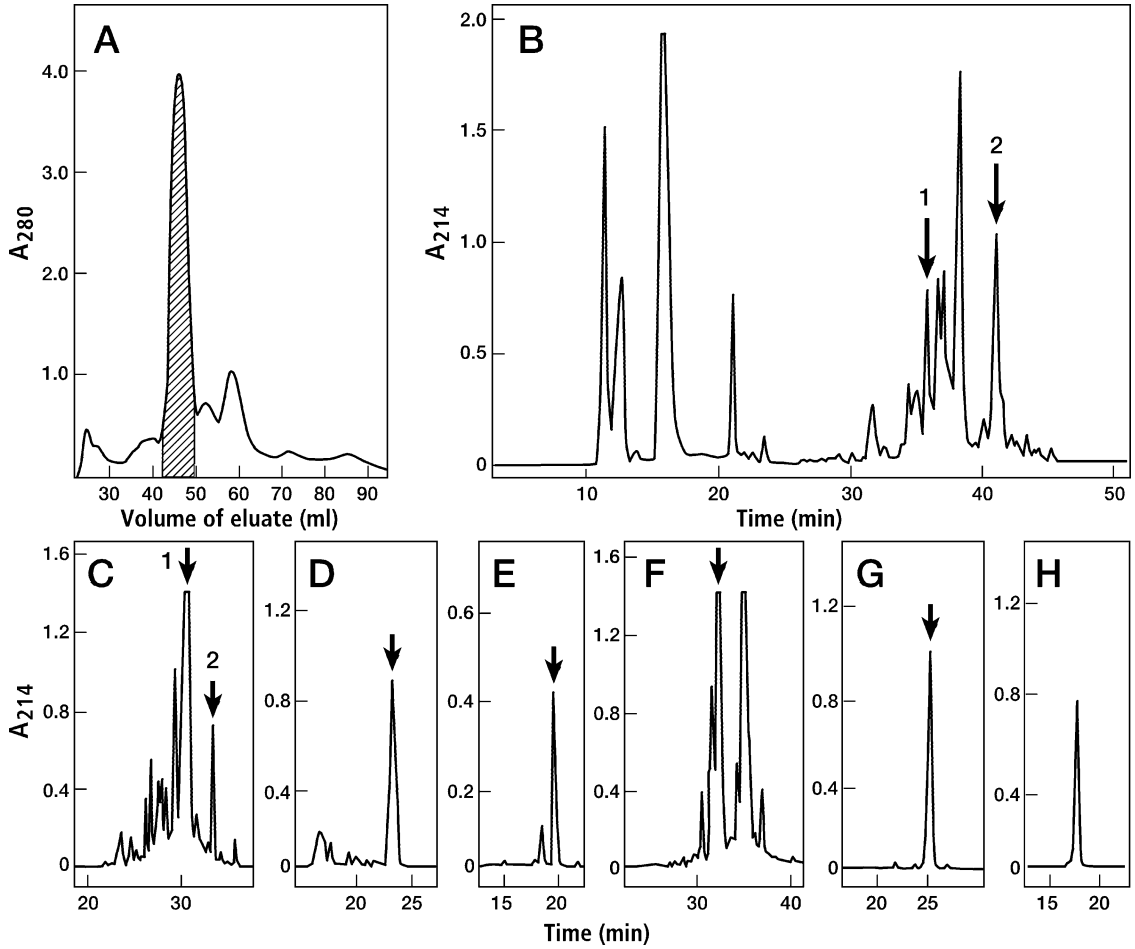


FIGURE 1: Purification of peptides tx3a, tx3b, and tx3c. (A) Lyophilized *C. textile* venom was extracted as described under the Materials and Methods. (B) An aliquot of pooled fractions from the shaded peak in A was run on a C_{18} semipreparative column eluted for 2 min at 10% B_{60} (60% ACN in 0.1% TFA), followed by a gradient of 10−100% B_{60} for 88 min with a flow rate of 3 mL/min. (C) An aliquot of the peak 1 fraction from B was run on a C_{18} analytical column and eluted for 2 min with 10% B_{60}, followed by a gradient of 10−60% B_{60} over 48 min at a flow rate of 1 mL/min. (D) Purification of tx3a. The peak 1 fraction from C was applied on the same analytical column and eluted for 2 min with 30% ACN in 0.05% HFBA, followed by a gradient of 30−60% ACN in 0.05% HFBA over 28 min. (E) Purification of tx3b. The peak 2 fraction from C was applied on the analytical column and eluted for 2 min with 30% ACN in 0.05% HFBA, followed by a gradient of 30−60% ACN in 0.05% HFBA over 28 min. (F) An aliquot of the peak 2 fraction from B was run on the analytical column and eluted for 2 min with 20% B_{60}, followed by a gradient of 10−70% B_{60} over 48 min. (G) Purification of tx3c. The fraction marked by an arrow in F was applied on the analytical column and eluted for 2 min with 30% ACN in 0.05% HFBA, followed by a gradient of 30−60% ACN in 0.05% HFBA over 28 min. (H) The fraction marked by an arrow in G was rerun on the analytical column eluted with a gradient of 30−60% B_{60} over 30 min.

isolated from venom ducts was primed with oligo(dT) linker ligated to a derivative of pGEM 3Zf(+). Extension products were size-fractionated by gel electrophoresis and transformed into MC1061 F′ laq. A cDNA library was constructed as described earlier (*14*). Clones were selected for sequencing based on the size of the cDNA determined by PCR amplification of the cDNA using the procedure described in Walker et al. (*17*). DNA templates for sequencing were prepared using QIAprep spin miniprep kit (Qiagen, Valencia, CA). Clones were sequenced using M13R and M13U primers and fluorescent sequencing at the Health Sciences Center Sequencing Facility, Eccles Institute of Human Genetics, University of Utah. Sequence analysis was done using SeqMan (DNASTAR, version 2.55, DNASTAR, Inc., Madison, WI).

RESULTS

Peptide Purification and Characterization. A number of venom fractions from the mollusc-hunting *Conus* species, *C. textile* and *C. marmoreus*, elicited excitatory symptomatology when injected i.c. into mice. These fractions were purified as described under the Materials and Methods, and the amino acid sequences of the purified peptides were determined. Table 1A gives a summary of the sequences of the peptides and the behavioral symptomatology used to follow the purification.

Peptides tx3a and tx3b were isolated from the venom of *C. textile* as shown in Figure 1. Intracranial injection of tx3a into 15-day-old mice caused scratching and hyperactivity. Peptide tx3b was purified from the same crude fraction from which tx3a was obtained but eluted later on subsequent HPLC run. It caused scratching, hyperactivity, and circular motion upon i.c. injection into 15-day-old mice. The sequences of the peptides are

tx3a CCSWDVCDHPSCTCCG

tx3b CCPPVACNMGCKPCC-NH_2

On the basis of ESIMS, peptide tx3a has a monoisotopic *m*/*z* 1712.5 (Figure 3A), consistent with the calculated value

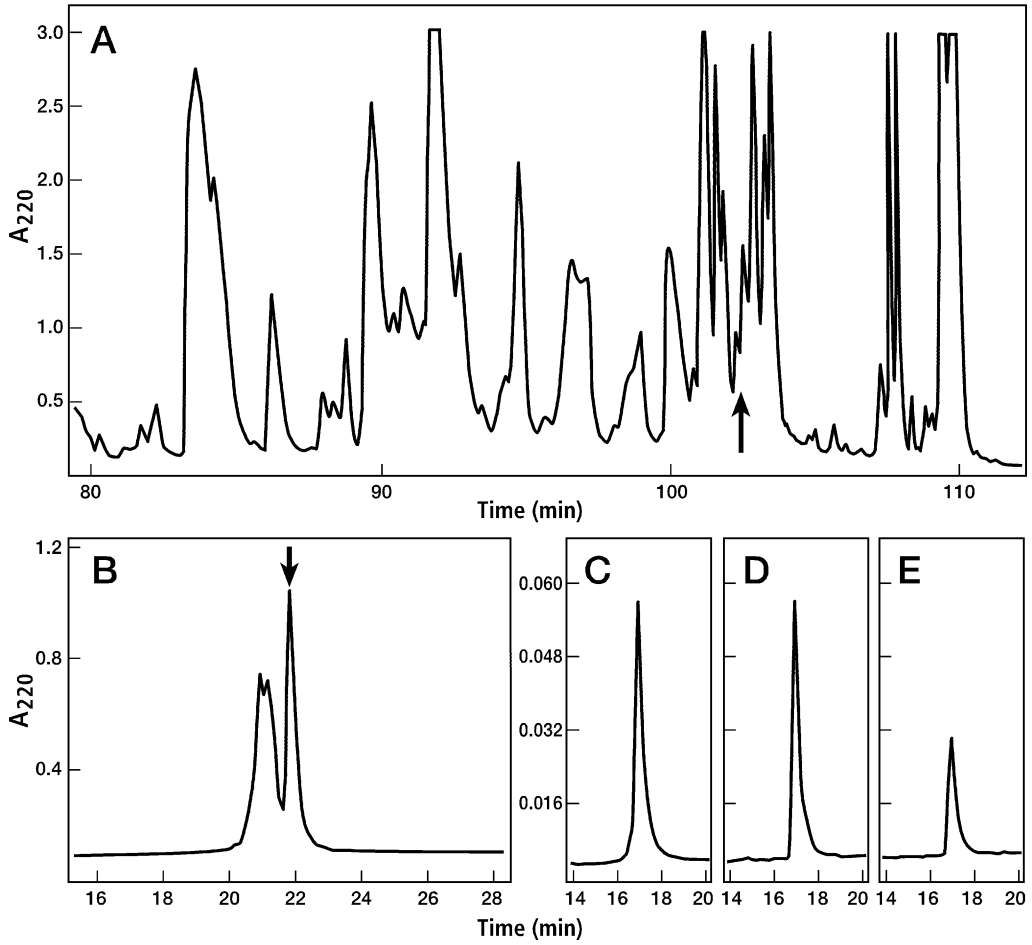


FIGURE 2: Purification of peptide mr3a. (A) Crude *C. marmoreus* venom was prepared as described under the Materials and Methods. The bioactive fraction was eluted with a gradient of 0−15% solvent B_{60}/15 min, then 15−39% B_{60}/72 min, then 39−65% B_{60}/15 min, and held at 100% B_{60} for 10 min at a flow rate of 10 mL/min. (B) The fraction marked by an arrow in A was purified by an isocratic run at 10% B_{60}/3 min and then a gradient of 10−50% B_{60}/7 min, followed by a gradient of 45−49% B_{60}/16 min and an isocratic run at 50% B_{60}/5 min. To compare native and synthetic peptides, their coelution under standard HPLC conditions was assessed. (C) An aliquot of the fraction indicated by the arrow in B was analyzed on an analytical HPLC column. (D) Synthetic peptide eluted at an identical position using the same HPLC conditions. (E) When aliquots of the natural and synthetic peak fractions in C and D were combined and rerun under identical HPLC conditions, the mixture eluted as a single peak.

(*m*/*z* 1712.47 Da) with a free carboxy terminus. For tx3b, the monoisotopic *m*/*z* 1521.4 obtained by ESIMS (Figure 3B) is consistent with the calculated *m*/*z* (1521.51 Da). Peptide tx3c was purified from *C. textile* venom, and the amino acid sequence was obtained as reported earlier (*1*). The sequence of the peptide is CCRTCFGCTOCC-NH_2 (*1*), consistent with the monoisotopic mass *m*/*z* 1305.31 obtained by LSIMS (Figure 3C) (calculated *m*/*z* 1305.38).

A crude fraction of *C. marmoreus* venom caused circular motion, barrel rolling, and convulsions in adult mice (20−23 g). The purification of mr3a from this fraction was monitored by following these symptoms upon i.c. injection in mice. In Figure 2A, the peak indicated by the arrow was the bioactive component, which was further purified as shown in parts B and C of Figure 2. LSIMS gave a monoisotopic mass *m*/*z* of 1721.6 (Figure 3D), consistent with the sequence assignment GCCGSFACRFGCVOCCV (O = 4-*trans*-hydroxyproline), and a free carboxy terminus (calculated monoisotopic *m*/*z* 1721.58 Da). In addition, the sequence of the peptide was directly confirmed by chemical synthesis, which will be detailed in the following section.

The purification of mr3b was similarly done as for peptides tx3a−c. When injected intracranially into 15-day-old mice, the purified peptide caused scratching and hyperactivity. ESIMS showed a monoisotopic mass *m*/*z* of 2126.9 (Figure 3E). The sequence of the purified peptide mr3b is SKQC-CHLAACRFGCTOCCW, which has a free carboxy terminus (calculated *m*/*z* 2126.79 Da).

Disulfide Bridge Analysis of Peptide tx3c. We performed a disulfide analysis on natural peptide tx3c (*1*). The analysis was carried out by a partial reduction and alkylation method as described earlier (*9*). Two major products (X and Y) were obtained, along with the native peptide (N) and a small amount of fully reduced peptide (R) (Figure 4A). Products X and Y were not completely separated even after further purification, and each contained small amounts of the other.

Alkylation of product X with 4-vinylpyridine gave one major and one minor product upon HPLC analysis (parts B and C of Figure 4). Sequencer analysis of the purified major product revealed clean labeling of Cys2 and Cys8, establishing one of the bridges (Figure 5A).

Alkylation of product Y gave a major and minor peak eluting at similar times as the peaks in product X but with

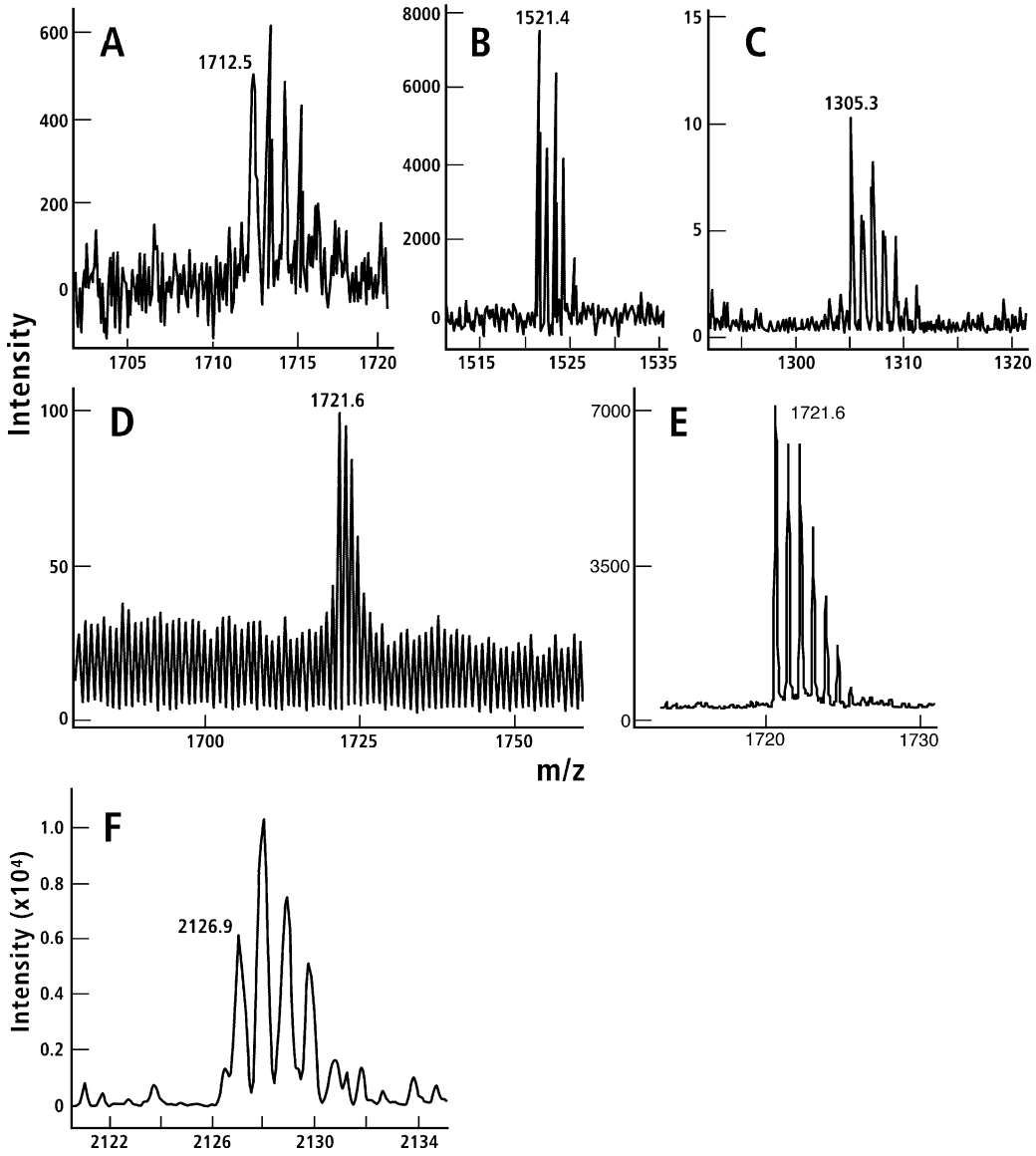


FIGURE 3: Mass spectra of purified peptides were obtained as described under the Materials and Methods. (A) ESIMS of tx3a. (B) ESIMS of tx3b. (C) LSIMS of tx3a. (D) LSIMS of mr3a. (E) MALDI of synthetic mr3a. (F) ESIMS of mr3b.

the relative magnitudes reversed. The major peak from Y was combined with the minor peak from X and sequenced. An ambiguous pattern was obtained: alkylation was present on Cys1, Cys8 (minor), and Cys12.

To resolve the ambiguity, a further sample of product Y was subjected to the very vigorous alkylation with iodoacetamide (*9*). After isolation of the carboxamidomethylpeptide by HPLC, it was fully reduced and alkylated with 4-vinylpyridine (parts D and E of Figure 4). Sequencer analysis of the purified product revealed clean labeling of Cys1 and Cys12 with iodoacetamide and labeling of the other four Cys residues with 4-vinylpyridine (parts B and C of Figure 5). It thus appears likely that the labeling of Cys8 in the prior experiment arose by attack of the free thiol of Cys12 on the Cys2-Cys8 bridge. The disulfide bridging pattern is thus established as

Characterization of Peptide mr3a. The most potent peptide, mr3a from *C. marmoreus* venom, was synthesized to make further biochemical and pharmacological characterization possible. The amounts of native peptide were insufficient to carry out an extensive biochemical/physiological characterization. Attempts to purify more natural peptide were complicated by the polymorphism observed in this peptide (see the following results).

The synthetic peptide was extensively characterized to ensure identity to the native material. Mass spectrometry analyses confirmed that the masses of the synthetic and natural peptides were the same (see parts D and E of Figure 3). Synthetic mr3a coeluted with the natural peptide from *C. marmoreus* upon HPLC analysis (parts C−E of Figure 2). A comparative bioassay in 10-day-old mice that was carried out at two doses (the amount of natural peptide available was limited) revealed the same biological activities for the natural and synthetic peptide mr3a (see Table 1B). Using synthetic mr3a, a more refined dose−response was carried out for 10-day-old mice, as shown in Table 1C. When mice were injected i.p., they did not show any symptoms at

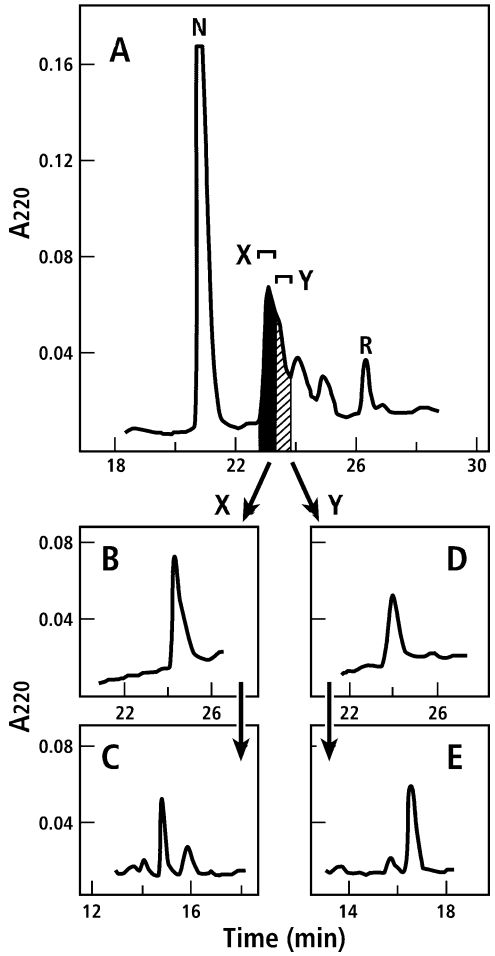


FIGURE 4: (A) HPLC elution of partially reduced tx3c as described in the Materials and Methods. The large initial peak N is unreduced peptide, and the small trailing peak R is fully reduced, as judged from control experiments. Between them is a series of peaks corresponding to partially reduced peptides. Products X and Y each have a single disulfide reduced. (B and C) Product X was alkylated with 4-vinylpyridine and repurified by HPLC. (D and E) Product Y was first alkylated with iodoacetamide and then fully reduced and alkylated with 4-vinylpyridine. Alkylated peptides were repurified by HPLC.

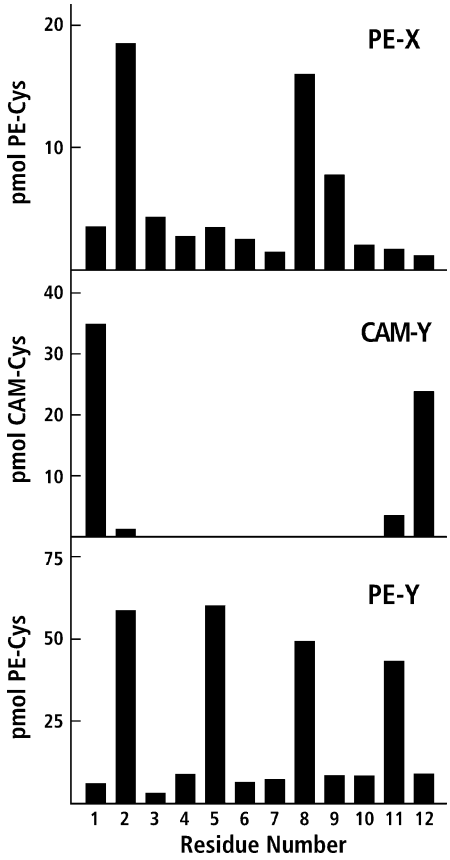
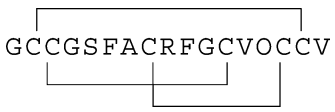


FIGURE 5: (A) Yields of pyridylethylcysteine (PE-Cys) at each step of sequencing of alkylated peptide X (PE-X). The pattern shows clearly that a single disulfide (Cys2-Cys8) had been opened. (B) Yields of carboxamidomethylcysteine (CAM-Cys) at each step of sequencing of alkylated peptide Y (CAM-Y). Clean labeling of cysteines 1 and 12 by iodoacetamide was obtained. (C) Yields of PE-Cys in the same analysis of peptide Y (PE-Y). The other four cysteines had been sequentially labeled with 4-vinylpyridine.

a dose of 1.7 nmol/g body wt. No effect was observed in goldfish injected i.p. at a dose of 2.5 nmol/g body wt.

The method of Gray (*9*) was used to determine the disulfide connectivity of synthetic mr3a, using a strategy similar to that described for tx3c. Partially reduced peptides were separated from the fully oxidized or reduced isoforms by HPLC, and reduced cysteines were sequentially alkylated with iodoacetamide and 4-vinylpyridine. Alkylated peptides were sequenced to identify the labeling pattern of cysteine residues; the HPLC and sequencer analyses of two partially reduced species (data not shown) were sufficient to unambiguously determine the disulfide connectivity of mr3a



Identification of M-Conotoxin Superfamily by cDNA Cloning. From cDNA libraries of *C. textile* and *C. marmoreus* (see the Materials and Methods), clones encoding peptides predicted to be identical or related to the purified natural peptides described above were identified. We used similar methods to identify clones encoding previously characterized peptides belonging to the μ-, κM-, and ψ-conotoxin families from fish-hunting *Conus* species. The sequences of the prepropeptides inferred from cDNA clones are aligned in Table 2. The high degree of identity and similarity in amino acid residues, particularly toward the N-terminal end of the open-reading frame, indicate that all of these peptides belong to the same gene superfamily. The shared sequence identities at the N-terminal region of the prepropeptide precursor, and the similar Cys pattern in the C-terminal mature toxin region (CC−C−C−CC) define members of what we designate as the M-conotoxin superfamily.

The cloning data defined 10 different cDNA clones from the two mollusc-hunting species (Tables 2 and 3). The peptides inferred from cDNA clones Tx3.2 and Tx3.3 correspond to peptides (tx3a and tx3b) purified from venom as described above; except that for tx3b the C-terminal Gly was processed, resulting in the C-terminal amidation of the mature peptide. Another clone (Tx3.4) encodes a scratcher peptide (tx3c) purified from *C. textile* venom (*1*), with the Pro at position 10 post-translationally modified to 4-*trans*-hydroxyproline and the C-terminal Gly processed to produce a C-terminal amide.

The peptides encoded by clones Mr3.3 and Mr3.4 are homologous with purified peptides mr3a and mr3b, respec-

Table 2: Comparison of cDNA Clones Encoding M-Superfamily Peptides[a]

Prepropeptide sequences determined from cDNA clones

```
                                  10        20        30        40        50
m-1 branch                                                                (1)

Tx3.1     MLKMGVVLFIFLVLFPLATLQLDADQPVERYAENKQLLNPDERREILLPALRKFCCDSN--WCHISD-CE---CCYG
Tx3.2     MLKMGVVLFIFLVLFPLATLQLDADQPVERYAENKQLLSPDERREIILHALGTRCCSWD--VCDHP-SCT---CCG

m-2 branch                                                                (2)

Tx3.3     MSKLGALLTICLLLFSLTAVPLDGDQHADQPAQRLQDRIPTEDHPLFDPNK--RCCPP--VACNM--GCKP--CCG
Tx3.4     MFKLGVLLTICLLLFSLNAVPLDGDQPADQPAERLLDDISFENNPFYDPAK--RCCR----TC-F--GCTP--CCG
Mr3.2     MSKLGILLTICLLLFPLTAVPLDGDQPADRPAERMQDDISSEHHPFFDPVK--RCCRL---SCGL--GCHP--CCG
Mr3.3     MSRLGVLLTICLLLFPLTAVPLDGDQPADRPAERLQDDISSEHHPHFDSGR--ECCGS--FACRF--GCVP--CCV
Mr3.4     MSKLGVLLTICLLLFPLTAVPLDGDQPADRPAERMQDDISSERHPFFDRSK--QCCHL--PACRF--GCTP--CCW
Mr3.6     MSKLGVLLTICLLLFALTAVPLDGDQPADRPAERMQDDISSERHPMFDAVR--DCCPL--PACPF--GCNP--CCG

m-3 branch                                                                (3)

Tx3.5     MSKLGVLLTICLLLFPLTALPLDGDQPADQAAERMQ----AEQHPLFDQKR--RCCKFP---CPD--SCRYL-CCG
Mr3.5     MSKLGVLLTICLLLFPLTALPLDGDQPADQRAERTQ----AEKHSLPDPRM--GCCPFP---CKT--SCTTL-CCG

m-4 branch                                                                (4)

G3.9      MSKLGVLLTICLLLFPLTALPMDGDEPANRPVERMQDNISSEQYPLFEKRR--DCCTP-PKKCKDRQ-CKPQRCCAGR
P3.7      MSKLGVLLTICLLLFPITALPMDGDQPADRLAERMQDNISSEEHPFEKRQR--LCCGF-PKSCRSRQ-CKPHRCCGR
R3.1      MSKLGVLLTICLLLFPLTALPMDGDQPVDRLAERMQDNISSEQHTFFEKRLP-SCCSLNLRLCPVPA-CKRNPCCTG
P3.8      MSKLGALLTICLLLFPITALLMDGDQPADRPAERMDYDISSEVHRLLERRHP-PCCMY-GR-CRRYPGCSSASCCQGG
```

[a] The number in parentheses indicates the number of amino acid residues between the fourth and fifth Cys residues of the C-terminal mature peptide. Conserved amino acid residues are shaded.

Table 3: Sequences of Purified or Predicted Mature Conotoxins[a]

peptide	clone	sequence[b]	species	reference
m-2 branch				
mr3a	(—)	GCCGSFACRFGCVOCCV^	*C. marmoreus*	this paper
(—)	Mr3.3	ECCGSFACRFGCVPCCV^	*C. marmoreus*	this paper
mr3b	(—)	SKQCCHLAACRFGCTOCCW^	*C. marmoreus*	this paper
(—)	Mr3.4	SKQCCHLPACRFGCTPCCW^	*C. marmoreus*	this paper
(—)	Mr3.2	CCRLSCGLGCHPCC*	*C. marmoreus*	this paper
(—)	Mr3.6	DCCPLPACPFGCNPCC*	*C. marmoreus*	this paper
tx3b	Tx3.3	CCPPVACNMGCKPCC*	*C. textile*	this paper
tx3c	Tx3.4	CCRTCFGCTOCC*	*C. textile*	*1*, this paper
m-1 branch				
tx3a^{+}	Tx3.2	CCSWDVCDHPSCTCCG^	*C. textile*	this paper
(—)	Tx3.1	FCCDSNWCHISDCECCY*	*C. textile*	this paper
m-3 branch				
(—)	Mr3.5	MGCCPFPCKTSCTTLCC*	*C. marmoreus*	this paper
(—)	Tx3.5	RCCKFPCPDSCRYLCC*	*C. textile*	this paper
m-4 branch				
μ-GIIIA	G3.9	RDCCTOOKKCKDRQCKOQRCCA*	*C. geographus*	*5*, this paper
μ-PIIIA	P3.7	ZRLCCGFOKSCRSRQCKOHRCC*	*C. purpurascens*	*6*, this paper
κM-RIIIK	R3.1	LOSCCSLNLRLCOVOACKRNOCCT*	*C. radiatus*	*8*, this paper
ψ-PIIIE	(—)	HOOCCLYGKCRRYOGCSSASCCQR*	*C. purpurascens*	*7*
(—)	P3.8	HOOCCMYGRCRRYOGCSSASCCQG*	*C. purpurascens*	this paper

[a] An unusual feature of peptide tx3a is that the C-terminal Gly residue has not been processed to a C-terminal amide, which is characteristic of conotoxins. In a separate purification, the amidated form was also isolated in a smaller amount (Enrique Baires, unpublished result). Thus, we have assumed that, for clones Mr3.2, Mr3.5, Mr3.6, Tx3.1 and Tx3.5, the normal process of C-terminal amidation, in which -X-Gly or -X-Gly-Arg is processed to a C-terminal amide (i.e., -X-NH_2), will occur, as in peptides tx3b and tx3c. Unhydroxylated Pro residues are indicated for peptide sequences inferred from cDNA clones (Mr3.2, Mr3.3, Mr3.4, Mr3.5, Mr3.6, and Tx3.5), where corresponding peptides have not been purified, although some of the Pro residues may possibly be hydroxylated. [b] O, 4-*trans*-hydroxyproline; Z, pyroglutamate; ^, C-terminal free acid; *, C-terminal amidation.

tively. As shown in Table 3, peptide mr3a is almost identical to that encoded by cDNA clone Mr3.3, except that the Glu at position 1 is replaced by Gly and the Pro at position 14 is post-translationally modified to 4-*trans*-hydroxyproline. Peptide mr3b is almost identical in sequence to that inferred from cDNA clone Mr3.4, with the Pro at position 8 substituted by Ala and the Pro at position 16 modified to 4-*trans*-hydroxyproline. The clones probably encode polymorphic variants of the peptides isolated from venom. The five other cDNA clones encode related open-reading frames; in all cases, the Cys pattern of the predicted mature conotoxins is CC−C−C−CC.

The peptide precursors can be tentatively grouped into four branches based on the precursor sequence similarity. The clustering based on the overall sequence similarity correlates with the number of amino acids between the fourth and fifth Cys residues in the primary sequence. Thus, at the C-terminal end of the mature peptide, a generic representation of all sequences is - -C(X_n)CC- -, where $n = 1$, 2, 3, or 4. The value of *n* corresponds to the grouping of peptides in Tables 2 and 3 and, as we discuss in the following section, is a useful criterion for provisionally subdividing the M-conotoxin superfamily into distinct branches.

DISCUSSION

Conotoxins with three disulfide cross-links exhibit four general Cys patterns (*18*). The O-superfamily conotoxins have the following pattern C−C−CC−C−C and include hydrophilic peptides such as the ω- and κ-conotoxins (which target voltage-gated calcium channels and voltage-gated potassium channels, respectively) (*19*, *20*) and very hydrophobic conotoxins, the δ- and μO-conotoxins (which inhibit inactivation and conductance of voltage-gated sodium channels, respectively) (*10*, *21*). Two distinct conotoxin families with three disulfide cross-links have the pattern CC−C−C−C−C: the αA-conotoxins (*22*), which are competitive nicotinic antagonists, and the κA-conotoxins, O-glycosylated peptides, which modulate the activity of voltage-gated ion channels (*23*); these belong to the A-superfamily [(*24*); R. Schoenfeld, R. Teichert et al., unpublished results]. The P-superfamily conotoxins have a third type of Cys pattern, C−C−C−C−C−C (*25*); the molecular targets of these peptides are unknown.

The first conotoxins characterized with three disulfide cross-links had the Cys pattern CC−C−C−CC. The data presented above establishes that all known *Conus* peptides sharing this arrangement of Cys residues in the mature toxin sequence also share considerable amino acid identity in the N-terminal signal sequence region of the precursors. Thus, they are all members of the same gene superfamily, which we designate the M-conotoxin superfamily.

Three distinct peptide families were previously characterized that belong to this group: the μ-, κM-, and ψ-conotoxins. These block voltage-gated sodium (μ-conotoxins) and potassium (κM-conotoxins) channels (*5*, *6*) and nicotinic acetylcholine receptors (ψ-conotoxins) (*7*), respectively; thus, their targets include members of both the voltage- and ligand-gated ion channel superfamilies. All of these conotoxin families have the same patterns of disulfide connectivity, Cys^1-Cys^4, Cys^2-Cys^5, and Cys^3-Cys^6. Furthermore, the peptides have a characteristic spacing between Cys residues and fit the consensus formula CC(X_{4-6})C(X_{4-5})C(X_4)CC. All μ-, κM-, and ψ-conotoxins characterized were from fish-hunting *Conus* species.

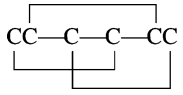
The initial focus of this study was excitatory conopeptides purified and characterized from the venoms of two mollusc-ivorous *Conus* species, *C. textile* and *C. marmoreus*. The biological activity of these peptides was assayed by i.c. injection into the mouse central nervous system. Surprisingly, all of these excitatory peptides had Cys patterns similar to the μ-, κM-, and ψ-conotoxins from piscivorous *Conus* venoms but were generally smaller.

A subsequent survey of cDNA clones derived from venom ducts of the same species (*C. textile* and *C. marmoreus*) revealed not only the precursor sequences of the peptides directly isolated from venom but also identified genes encoding additional conotoxins with the same Cys pattern as the peptides described above (CC- -C- -C- -CC). A total of 10 distinctive peptides with the Cys pattern were elucidated either by direct purification, molecular cloning, or a combination of both methods. It is notable that all mature peptides with the Cys pattern above also share a highly conserved signal sequence. Together, these data indicate that the 10 new peptides defined in this study belong to the same gene superfamily as the μ-, κM-, and ψ-conotoxins, the M-conotoxin superfamily.

The patterns of post-translational modifications observed in the nine peptides of the M-superfamily (see Table 3) that have been isolated from venoms deserve comment; in all nine cases, a corresponding cDNA clone was analyzed that either encodes the peptide or a presumed polymorphic variant of the peptide (with a single amino acid substitution in mr3a and mr3b and three amino acid substitutions for ψ-PIIIE). The degree of polymorphism observed is not unusual for polypeptide toxins found in venoms and has been observed in other conotoxin families (for example, see ref *26*). The more unusual post-translational modifications found in other *Conus* peptide superfamilies (for a review, see ref *27*) are absent in M-superfamily peptides. Two modifications were widespread: C-terminal amidation and proline hydroxylation. However, the M-superfamily peptides purified from mollusc-hunting *Conus* had a less predictable pattern; most Pro residues are hydroxylated, but in tx3a and tx3b, prolines were found to be unhydroxylated. Furthermore, unusually, the C-terminal - - -Cys-Gly sequence of tx3a was not processed to the predicted - - -Cys-NH_2 C-terminal end.

The M-superfamily peptides comprise a heterogeneous set. A major group of the peptides characterized in this study fit the following consensus pattern: CC(X_{2-4})C(X_{2-3})C(XP/O)-CC- -. A total of 6 of the 10 different clones defined from *C. textile* and *C. marmoreus* belong to this class, as do 4 of the 5 excitatory peptides purified from the venoms of these species. We will refer to the class of M-conopeptides that fit the consensus sequence above as the m-2 branch of the superfamily (see the discussion of this nomenclature below).

A most unexpected finding is that the two m-2 peptides analyzed have a different disulfide connectivity from the μ-, κM-, and ψ-conotoxins, even though they belong to the same gene superfamily and share the same Cys pattern when the primary sequence is examined. This is surprising given that the other conotoxins of the M-superfamily characterized so far, despite having diverse molecular targets, share the same disulfide connectivity. The disulfide connectivity for the μ-, κM-, and ψ-conotoxins is shown below



In contrast, the pattern that we have established for the m-2 peptides mr3a and tx3c is as follows:

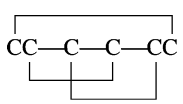


Table 4: Pairwise Divergence in the 30−50 Region of Precursor Sequences for M-Superfamily Peptides in Different Branches[a]

		m-1		m-2			m-3		m-4		
		Tx3.1	Tx3.2	Tx3.4	Mr3.3	Mr3.4	Tx3.5	Mr3.5	G3.9	R3.1	P3.8
m-1	Tx3.1	0									
	Tx3.2	3	0								
m-2	Tx3.4	17	17	0							
	Mr3.3	16	16	7	0						
	Mr3.4	15	15	9	5	0					
m-3	Tx3.5	17	17	14	12	11	0				
	Mr3.5	16	17	14	14	14	9	0			
m-4	G3.9	17	17	14	9	8	12	16	0		
	R3.1	16	16	13	9	7	12	14	5	0	
	P3.8	17	17	13	10	8	14	14	9	9	0

[a] The numbers given are the number of nonidentical amino acids between the two precursor sequences indicated, in positions 30−50 (as aligned and numbered in Table 2). The branches are specified for each set of peptides. Thus, intrabranch comparisons exhibit 3−9 amino acid differences, while interbranch comparisons exhibit 7−17 differences in the segment of the "pro" region analyzed. The degree of sequence similarity thus helps define members of a branch, as well as the relationship between branches. In contrast, there is a much greater sequence similarity among signal sequences (that defines members of the superfamily).

Although it seems highly likely that the disulfide connectivity of tx3c and mr3a is shared by the entire m-2 branch, this remains to be definitively established. The tx3c/mr3a disulfide connectivity pattern has also been established for a group of M-superfamily peptides from fish-hunting *Conus* venoms (J. M. McIntosh, J. Rivier and B. Olivera; data to be presented elsewhere) that do not belong to the m-2 branch. Thus, two different patterns of disulfide connectivity are likely to be broadly distributed within the M-conotoxin superfamily. An obvious question is the functional consequences of the shift in disulfide connectivity. The type of detailed structure/function study that would address this issue would be appropriate to initiate after the molecular targets of tx3c and mr3a have been identified.

One notable feature of the m-2 branch is the variety of such peptides characterized from venoms of both Indo-Pacific mollusc-hunting *Conus* species examined; because this is a large and successful clade of species (*28*), we can expect a very diverse set of m-2 peptides to be found in snail-hunting *Conus*. We note that the six peptides belonging to the m-2 branch of the M-superfamily are related to peptides previously elucidated from worm-hunting *Conus* venoms, specifically *C. quercinus* and *C. betulinus* (*29*, *30*). However, all of the m-2 peptides from *C. textile* and *C. marmoreus* have the following consensus sequence toward the C terminus: - - -ZGC(XP/O)CC- -, where Z is a large hydrophobic residue (F, L, or M) and X can be any amino acid. Although the peptides in the two worm-hunting venoms belong to the m-2 branch of the M-superfamily, they do not fit the more specific C-terminal consensus sequence given above for the six m-2 peptides from *C. textile* and *C. marmoreus*. Thus, although this branch of the M-superfamily appears to be found in both snail-hunting and worm-hunting *Conus*, there appears to be divergent features.

In addition to the m-2 peptides (that all share the motif −C(XO/P)CC−) and what we will refer to as the "m-4 peptide branch" (i.e., the μ-, κM-, and ψ-conotoxins, that share a C-terminal - -C(X)$_4$CC− motif), there are two other groups of peptides, with the C-terminal motifs - -C(X$_1$)CC− and - -C(X$_3$)CC−, respectively. These are less well-represented in the set of peptides described above; we provisionally refer to these as the m-1 and m-3 branches of the M-superfamily. These are represented by two sequences each, Tx3.1 and Tx3.2 (peptide tx3a from venom) for the m-1 branch and Tx3.5 and Mr3.5 for the m-3 branch, with both of the latter inferred from molecular cloning. Thus, the M-conotoxin superfamily peptides can be subdivided into distinct groups: the m-2 and m-4 branches (the latter, including the μ-, κM-, and ψ-conotoxins, clearly differ from each other in the different number of amino acids between the fourth and fifth Cys residues and in disulfide connectivity). Two additional putative groups, the m-1 and m-3 conotoxins, do not belong to the two branches above. In the m-1...m-4 nomenclature, the branch number refers to the number of amino acids between the fourth and fifth cysteine residues. A closer examination of the extent of amino acid identity of the sequences shown in Table 2 is summarized in Table 4. A segment of the prepropeptide region (amino acids 30−50 as aligned in Table 2) is shown in a pairwise comparison of peptides from the four proposed M-superfamily branches. The results suggest that the m-2 and m-4 branches are more closely related to each other than to either the m-1 and m-3 conopeptides and that the M-superfamily genes in mollusc-hunting cone snails that are likely to be homologues of the m-4 branch from piscivorous *Conus* encode the m-2 peptides.

It is noteworthy that considerable functional diversity exists, even for peptides within the same branch of the M-superfamily. Thus, the m-4 branch has peptides targeted to Na channels (encoded by the G3.9 gene), K channels (encoded by the R3.1 gene), and nicotinic receptors (encoded by the P3.8 gene). What the new results establish is that not only is there functional diversity, but there can be a divergence in the disulfide framework within a conotoxin superfamily to generate greater structural diversity than would have been expected from studies on the other previously characterized conotoxin superfamilies.

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