The T-superfamily of Conotoxins*

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We report the discovery and initial characterization of the T-superfamily of conotoxins. Eight different T-superfamily peptides from five *Conus* species were identified; they share a consensus signal sequence, and a conserved arrangement of cysteine residues (- -CC- -CC-). T-superfamily peptides were found expressed in venom ducts of all major feeding types of *Conus*; the results suggest that the T-superfamily will be a large and diverse group of peptides, widely distributed in the 500 different *Conus* species. These peptides are likely to be functionally diverse; although the peptides are small (11–17 amino acids), their sequences are strikingly divergent, with different peptides of the superfamily exhibiting varying extents of post-translational modification. Of the three peptides tested for *in vivo* biological activity, only one was active on mice but all three had effects on fish. The peptides that have been extensively characterized are as follows: p5a, GCCP-KQMRCCTL*; tx5a, γCCγDGW$^+$CCT$^\$$AAO; and au5a, FC-CPFIRYCCW (where γ = γ-carboxyglutamate, W$^+$ = bromotryptophan, O = hydroxyproline, T$^\$$ = glycosylated threonine, and * = COOH-terminal amidation). We also demonstrate that the precursor of tx5a contains a functional γ-carboxylation recognition signal in the −1 to −20 propeptide region, consistent with the presence of γ-carboxyglutamate residues in this peptide.

Cone snails (genus *Conus*) are perhaps the most successful genus of marine invertebrates, with over 500 species, all of which are venomous (1, 2). These predatory marine snails have evolved a highly sophisticated neuropharmacological strategy based on small peptides (10–35 amino acids) in their venoms (3, 4). Most *Conus* peptides potently affect ion channel function; these are widely used pharmacological reagents in neuroscience, and several are being directly developed as diagnostic and therapeutic agents. Most *Conus* peptides are highly disulfide-rich; generically, *Conus* peptides with multiple disulfide cross-links have been referred to as conotoxins. It has become apparent in recent years that there are tens of thousands of different conotoxins in *Conus* venoms. Because of the remarkably rapid interspecific divergence of peptide sequences, each *Conus* species has its own distinct repertoire of between 50 and 200 different venom peptides (5).

A major simplification in understanding this complex array of *Conus* venom peptides is that most of the ~50,000 different molecular forms can be grouped into just a few superfamilies. Peptides in the same superfamily share both a conserved pattern of disulfide connectivity and a highly conserved signal sequence (when prepropeptide precursor sequences of the peptides are compared) (5, 6). Three large superfamilies of conotoxins are well characterized: the O-superfamily, comprising several distinct pharmacological families including the ω-, κ-, δ-, and μO-conotoxins (7); the A-superfamily, to which the α-conotoxins belong (8); and the M-superfamily, to which the μ-conotoxins belong. In this paper, we describe the T-superfamily, a previously uncharacterized group of *Conus* peptides that exhibit a novel disulfide pattern and share a conserved signal sequence.

The data presented in this report suggest that T-superfamily peptides are a major group of *Conus* peptides, and that considerable diversity will exist within the superfamily. Eight members of the T-superfamily have been identified in the venom ducts of four different cone snails, including fish-hunting, snail-hunting, and worm-hunting *Conus*. Although the molecular targets of T-superfamily peptides have not yet been identified, this report provides a clear roadmap for a systematic exploration of this diverse, yet coherent, group that may encompass ~1,000 distinct pharmacologically active peptides.

MATERIALS AND METHODS

Extraction and Fractionation of Crude Venom—The venom of *Conus purpurascens* was obtained by milking specimens maintained in aquaria as described previously (9). The collection from ~90 milkings (~0.5 ml) was diluted with 50 ml of 0.1% trifluoroacetic acid in water (buffer A) then fractionated on a Vydac C_{18} preparative column (22 mm × 25 cm, 15-μm particle size, 300-Å pore size, 20 ml/min flow rate). Venom components were eluted by a gradient of buffers consisting of 0.1% trifluoroacetic acid and 60% acetonitrile (CH_3CN) in 0.092% trifluoroacetic acid (buffer B60) or 90% acetonitrile in 0.08% trifluoroacetic acid (buffer B90). The absorbance at 220 nm was monitored, and fractions were collected at 30-s intervals.

Lyophilized *Conus aulicus* venom (550 mg) obtained from the Philippines was extracted with 40 ml of 40% CH_3CN in 0.5% trifluoroacetic acid. The suspension was homogenized at low speed with three strokes of a glass/Teflon homogenizer attached to a drill press and then centrifuged at 100,000 × *g* for 10 min. The supernatant was diluted with 10 volumes of 0.1% trifluoroacetic acid and then fractionated on a preparative C_{18} HPLC[1] column as described above.

Lyophilized *Conus textile* venom (400 mg) obtained from the Philip-

[1] The abbreviations used are: HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Fmoc, *N*-(9-fluorenyl)methoxycarboxyl; MALD, matrix-assisted laser desorption; ESI, electrospray; LSI, liquid secondary ionization; MS, mass spectroscopy; acm, *S*-acetamidomethyl.

pines was extracted sequentially with 10 ml each of 0%, 20%, 40%, and 60% CH$_3$CN. The mixture was sonicated for three 30-s periods while immersed in ice water, centrifuged at 5,000 × g for 5 min, and the combined supernatant was stored at −20 °C. The extract was fractionated in several runs on a Vydac C$_{18}$ semi-preparative column (10 × 250 mm, 5-μm particle size) and an analytical column (4.6 × 250 mm), both eluted with a 0 to 28% gradient of CH$_3$CN (0.45%/min) at a flow rate of 5 ml/min. Corresponding fractions were pooled for further purification.

Purification of the T-superfamily Peptides—The peptides of *C. purpurascens* (Fig. 1) and *C. textile* (Fig. 2) were purified from relevant fractions of the venom by analytical reverse phase chromatography (4.6 × 250 mm, 5 μm, 300 Å, Vydac C$_{18}$ or Microsorb MV). Gradient elution was done using the same buffer systems as for preparative columns. Peptides from *C. aulicus* (Fig. 5) were purified from preparative fractions using a sulfonic-based strong cation exchange-HPLC column (Vydac 400VHP575, 5 mm, 7.5 × 50 mm), followed by a run on a reverse-phase C$_{18}$ column. The strong cation exchange column was eluted by a gradient with limiting buffers consisting of 10 mM phosphate, pH 2.5, in 50% acetonitrile and 0.25 M NaCl, 10 mM phosphate, pH 2.5, in 50% acetonitrile. The active peak from this column was concentrated and desalted before application on the analytical C$_{18}$ column. Other details of the purification procedures are described in the legends of Figs. 1, 2, and 5.

Peptide Sequencing—Due to the limited amount of peptide p5a from *C. purpurascens*, it was sequenced on an ABI model 477A peptide sequencer without reduction and alkylation of potential cysteine residues. For determination of Cys residues in tx5a, au5a, and au5b, the peptides were reduced with dithiothreitol and alkylated with 4-vinylpyridine as described below. Approximately 20–80 pmol of the peptides were used. The alkylated peptides were sequenced by Edman degradation using an Applied Biosystems model 492 Sequenator (DNA/Peptide Facility, University of Utah). The 3-phenyl-2-thiohydantoin derivatives were identified by HPLC. Predicted masses for each sequence were verified by mass spectrometry, as described below.

Reduction and Alkylation of the Purified Peptide—The *C. textile* peptide (tx5a) and the *C. aulicus* peptides (au5a and au5b) were reduced with dithiothreitol and alkylated with 4-vinylpyridine. Prior to reduction, the peptide solution was adjusted to pH 8 with 0.5 M Tris base and 10 mM dithiothreitol was added. The solution was flushed with nitrogen gas, incubated at 65 °C for 15 min, and then cooled to room temperature. After adding 4-vinylpyridine (5 μl/ml of solution), the mixture was left in the dark at room temperature for 25 min. The mixture was diluted with 500 μl of 0.1% trifluoroacetic acid prior to purification of the reduced peptide on an analytical reverse-phase HPLC column.

Mass Spectrometry—Matrix-assisted laser desorption (MALD) (10) mass spectra were obtained using a Bruker REFLEX (Bruker Daltonics, Billerica, MA) time-of-flight (11) mass spectrometer. The sample (in 0.1% trifluoroacetic acid) was applied with α-cyano-4-hydroxycinnamic acid. Electrospray (ESI) mass spectra were obtained using an Esquire ion trap mass spectrometer (Bruker Daltonics). The HPLC-purified sample, collected in 0.1% trifluoroacetic acid and acetonitrile, was diluted with 1% acetic acid in methanol, transferred to a fused silica capillary, and infused at approximately 250 nl/min. Liquid secondary ionization (LSI) mass spectra were measured on a Jeol HX110 double focusing magnetic sector mass spectrometer (Jeol, Tokyo, Japan). The sample in a glycerol matrix was bombarded with high energy (25 keV) Cs$^+$ ions. The mass accuracy was typically better than 1000 ppm for the time-of-flight instrument, 200 ppm for the ion trap instrument, and 50 ppm for the magnetic sector instrument.

Asp-N Digestion—The tx5a peptide was digested with endoproteinase Asp-N as directed in the procedure provided by Roche Molecular Biochemicals. The lyophilized peptide was dissolved in 100 μl of 50 mM sodium phosphate buffer, pH 8.0. Endoproteinase Asp-N (1 or 5 μg of enzyme/20 μg of peptide) in 10 mM Tris-HCl, pH 7.5, was added and the mixture was incubated for 17 h at 37 °C. The reaction was stopped with 500 μl of 0.1% trifluoroacetic acid, and the digest was fractionated by HPLC with a linear gradient of 0.9% acetonitrile per ml/min. The intact masses of the digestion fragments were analyzed using MALD-MS and ESI-MS prior to chemical sequencing.

Chemical Synthesis—The peptides p5a and au5a were synthesized on Rink amide resin using Fmoc (*N*-(9-fluorenyl)methoxycarboxyl) chemistry and standard side chain protection except on the cysteine residues. For p5a synthesis, the first and third cysteine residues were *S*-trityl protected, while the second and fourth were protected with *S*-acetamidomethyl (acm) groups. Two possible disulfide-bonded forms of au5a were synthesized. In one isomer (S1), the first and third cysteine residues were *S*-trityl-protected, while the second and fourth were

protected with acm groups. In the other isomer (S2), the first and fourth cysteine residues were *S*-trityl-protected, whereas the second and third cysteine residues were acm-protected.

Peptides were removed from resin and precipitated as described previously (12, 13). A two-step oxidation protocol was used to selectively fold the peptide as detailed elsewhere (14, 15) with the modifications described below.

Following preparative purification of the linear peptide by HPLC with a 10–50% gradient of buffer B60, the appropriate HPLC fraction was added dropwise over several min to an equal volume of 20 mM K$_3$Fe(CN)$_6$ solution in 0.1 M Tris buffer, pH 7.7, and stirred for 30 min. An Alltech "extract-clean" syringe containing C$_{18}$ silica (1 g of silica, 100-μm particle size, 60-Å pore size) was wetted by gravity perfusion with buffer B60 for ~30 min followed by 1–2 min of perfusion with buffer A. The peptide oxidation mixture was diluted at least 2-fold with buffer A and passed through the silica under vacuum (flow rate ~50 ml/min). The silica was washed with ~1 liter of buffer A under vacuum, and peptide was then eluted by gravity perfusion with 20 ml of buffer B60. Removal of acm protection and closure of the final disulfide was done by oxidation with 5 mM iodine in 5% trifluoroacetic acid for 5 min. Fully oxidized peptide was purified by preparative HPLC using a 20–50% gradient of B60. Synthesis was confirmed by LSI-MS analysis and HPLC co-elution.

A second batch of au5a was synthesized on a 357 ACT Peptide Synthesizer (Advanced Chemtech, Louisville, KY) using standard Fmoc chemistry. The disulfide bonds were formed by a random folding strategy in the presence of 1 mM reduced and 0.5 mM oxidized glutathione (pH adjusted to 7.5). The major product (50% of the mixture) that had the desired folding pattern was purified by reverse phase HPLC and then lyophilized.

Biological Assay—Mice were injected intracranially or intraperitoneally with peptides in 15–20 μl of saline or with saline alone, and observed for behavioral changes. Siamese fighting fish were similarly injected with 10 μl of sample in the dorsal muscle and observed for suppression of reactions to self-observation following placement in front of a mirror. In control fish, behaviors typically include a "gill display" (downward extension of the gill flap); extension of dorsal, ventral, and pectoral fins; and, sometimes, agitated swimming and rubbing against the fish's reflection in the mirror. This behavior is similar to that produced by the presence of another fighting fish. The effect of peptide injection on gill display and extension of fins was used as a measure of activity.

A second hallmark symptom elicited by higher doses of T-superfamily peptides was an abnormal dorsal fin. These fish have long dorsal fins, which they can greatly extend in display, but on injection of T-superfamily peptides, the fins droop far below their usual resting position. Observing this symptom does not require putting a mirror in front of the fish.

Analysis of Tx5.1 and Gm5.1 Clones by the Expressed Sequence Tag Method—First-strand synthesis of complementary DNA was primed from oligo(dT) extension at the *Pst*I site of a linearized modified pUC13 plasmid using polyadenylated mRNA isolated from *C. textile* and *Conus gloriamaris* venom ducts. The products were size-fractionated by gel electrophoresis and used to transform *Escherichia coli* MC1061 to produce cDNA libraries (16). Expressed sequence tags were identified from single colonies randomly selected from Ampicillin-LB plates plated with *Conus* cDNA libraries (17). Insert sizes of the clones were analyzed by single colony PCR (18) with vector-specific oligonucleotides flanking the insert region (500 nM amount of each oligonucleotide, 2.5 mM MgCl$_2$, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 250 μg/ml bovine serum albumin, 125 μM amount of each dNTP, and 0.5 unit of *Taq* DNA polymerase). Reaction mixtures were amplified (50 cycles of 25 s at 94 °C, 25 s at 54 °C, 2 min at 72 °C) using a 1605 Air Thermo-Cycler™ (Idaho Technology, Idaho Falls, ID). Amplification products were analyzed by gel electrophoresis (1.5% agarose, 0.5× TBE buffer). Clones containing insert sizes larger than 400 base pairs in length were selected for sequencing. Templates were prepared using QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) and submitted for fluorescent sequencing primed by oligonucleotides M13R and subsequently M13U (19) at the Health Sciences Center Sequencing Facility, Eccles Institute of Human Genetics, University of Utah. All molecular biology techniques were as described by Sambrook *et al.* (20), unless otherwise specified. Sequence analysis was performed with SeqMan (DNASTAR, version 2.55; DNASTAR, Inc., Madison, WI) and Pileup (21).

Cloning of Tx5.2, P5.1, and Im5.1—The DNA sequence of clone Tx5.1 was analyzed, and the following oligonucleotide primers containing *Eco*RI endonuclease sites were designed to screen cDNA libraries of other species: first strand primer 1 (5'-GGA ATT CGG AAG CTG ACT

TABLE I
Nucleotide sequence and predicted translation product of cDNA Tx5.1

Asterisk represents post-translational amidation of the COOH terminus. A, the open reading frame and predicted amino acid sequence of cDNA Tx5.1. B, the prepropeptide of Tx5.1. The open arrow denotes predicted cleavage site of signal peptide, between Ala[22] and Gln[23]. The solid arrow indicates expected proteolytic processing site (between Arg[49] and Cys[50]) for the release of mature toxin product (Cys[50] to Gln[60]).

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A.
1/1                                        31/11
ATG TGC TGT CTC CCA GTG TTC GTC ATT CTT CTG CTG CTG ATT GCA TCT GCA CCT AGC GTT
 M   C   C   L   P   V   F   V   I   L   L   L   L   I   A   S   A   P   S   V
61/21                                      91/31
GAT GCC CAA CCG AAG ACC AAA GAT GAT GTG CCC CTG GCA CCT TTG CAC GAT AAT GCA AAG
 D   A   Q   P   K   T   K   D   D   V   P   L   A   P   L   H   D   N   A   K
121/41                                     151/51
AGT GCA CTA CAA CAT TTG AAC CAA CGC TGC TGC CAA ACA TTC TAT TGG TGC TGT GTT CAA
 S   A   L   Q   H   L   N   Q   R   C   C   Q   T   F   Y   W   C   C   V   Q
181/61
GGG AAA TGA
 G   K   *

B.
        10        20⇓⇓        30        40       ↓ 50        60
MCCLPVFVILLLLIASAPSVDAQPKTKDDVPLAPLHDNAKSALQHLNQRCCQTFYWCCVQ*
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ACA AGC AGA-3′) and second strand primer 2 (5′-GGA ATT CCA AAT GAT ATT ACT GAC-3′). The cDNA libraries were then screened using PCR. The reaction mixture contained standard PCR reagents and the following: 500 nM primer 1, 500 nM primer 2, 100 ng/µl cDNA library. Reaction mixtures were then amplified in thin-walled PCR tubes for 35 cycles at 94 °C for 25 s, 52 °C for 35 s, and 72 °C for 2 min using the Air Thermo-Cycler. PCR products were digested with *Eco*RI, ligated into Bluescript SK(−) vector (Stratagene, CA), and used to transform *E. coli* DH5α.

Cloning of Gm5.2—The cloning of Gm5.2 was identical to the above procedure except for the following alterations: oligonucleotide primer 3 (5′-AGC TCT AGA GGA AGC TGA CTA CAA GCA-3′) designed from 5′-untranslated region of Tx5.1, and oligonucleotide primer 4 (5′-CAC AAG CTT TAG GTC ATC CAG TTC C-3′) designed from the consensus 3′-untranslated region of several cloned fragments obtained through expressed sequence tag screening of a *C. textile* cDNA library, were used instead of primer 1 and primer 2. 100 ng/µl cDNA library was amplified as described above, and the amplicon was ligated into Bluescript SK(−) vector using blunt-end ligation.

γ-Glutamyl Carboxylase Assays—The peptide pro(−20 to −1).FLEEL-amide, PLSSLRDNLKRTIRTRLNIR. FLEEL-NH₂ (which contains the propeptide sequences −20 to −1 of Tx5.2 covalently linked to FLEEL-amide at the amino terminus) was synthesized by Dr. Bob Schackmann of the DNA/Peptide Facility, Huntsman Cancer Center (supported by Grant NCICA 42014), University of Utah. The identity of the peptide was verified by ESI-MS.

Partially purified γ-glutamyl carboxylase was prepared by the following procedure. Microsomes of *C. textile* were prepared as described by Stanley *et al.* (22). Microsomes were suspended in buffer containing 2.0 M NaCl, 0.1 M MOPS, pH 7.0, 0.8% CHAPS, 0.8% phosphatidyl choline, and incubated at 4 °C for 1 h, then centrifuged for 1 h at 125,000 × g. The supernatant containing γ-glutamyl carboxylase was adjusted to 66% saturation in ammonium sulfate. The enzyme recovered in the precipitate was dissolved in buffer containing 0.1 M NaCl, 0.025 M MOPS, pH 7.0, 0.1% CHAPS, 0.1% phosphatidylcholine, distributed into aliquots, quick frozen in liquid N₂, and stored at −80 °C. Fresh aliquots of enzyme were thawed individually for enzyme assays.

Carboxylase assays were performed using 0.5 µg of partially purified enzyme according to methods described by Stanley *et al.* (22). FLEEL and Tx5.2 pro(−20 to −1).FLEEL-amide were used as substrates in the carboxylase reaction. Experiments were done in triplicate, and the data were fitted to a single-site binding model and analyzed using Graph Pad Prism from GraphPad Software, Inc. (San Diego, CA).

Nomenclature—In this report, we adopt a nomenclature that is based on conventions used for naming ion channels, the likely molecular targets of these peptides. Putative sequences deduced from clones will be named as follows: 1) letters (one for fish-hunting species, two for non-fish-hunting species) designate the *Conus* species source of the clone, 2) a number represents the disulfide framework, and 3) a second number, separate by a decimal, indicates the order of clone identification. For example, P5.1 is from *C. purpurascens*, has disulfide framework "5" (the number assigned to the -CC- -CC- pattern), and is the first clone from the species with this framework. To distinguish clones from peptides that have been isolated from *Conus* venom, in the latter case all of the species letter(s) are small, the disulfide framework is represented by an arabic numeral and the order of discovery is indicated by a letter, starting with "a"; thus, clone P5.1 may encode peptide p5a isolated from venom. Likewise, the clones from *C. aulicus*, Au5.1 and Au5.2, correspond to the venom-purified peptides au5a and au5b.

T-superfamily peptide clones will be given the numerical designations 5.1, 5.2, etc., in the order in which they are discovered. The peptides purified from venom will be named as described previously (8); hence, au5a and au5b.

Finally, when a molecular target is assigned to a toxin, the name will be prefixed by the appropriate pre-existing or newly assigned Greek letter (ω- for calcium channels, α- for acetylcholine receptors, and so forth). Thus, if a member of the T-superfamily from *C. textile* is determined to have a novel physiological mechanism, it might be called τ-conotoxin TxVA (which might, for example, be encoded by clone Tx5.2; τ-TxVA would then permanently replace tx5a). This nomenclature gives information about physiological mechanism, structure, and the natural source from which the peptide was originally obtained.

We note that peptides of the conantokin family were referred to using "V" (conantokin-G was initially described as GV). Since this is not a multiply disulfide-bonded peptide, we now refer to all peptides of the conantokin family by a letter designation for the species: thus, conantokin-G, conantokin-R, etc. We will reserve the provisional designation "5" and the Roman numeral "V" for members of the T-superfamily having the cysteine arrangement - - -CC- - -CC- - -.

RESULTS

Discovery of a Novel Class of Conotoxin Clones—We have carried out a systematic characterization of clones present in cDNA libraries of *Conus* venom ducts. By analyzing different clones using an expressed sequence tag strategy, we identified different classes of cDNAs encoding *Conus* peptides. Although most of these fall into previously identified groups (such as the O-superfamily), a number of clones that were frequently encountered in some cDNA libraries do not belong to previously characterized superfamilies of *Conus* peptides.

One class of cDNAs encoded a novel group of *Conus* peptides characterized by a long 3′-untranslated region (~600 base pairs), which exhibited no sequence homology to conserved 3′-untranslated regions of characterized superfamilies. The prepropeptide precursor associated with this 3′-untranslated region predicted a small mature peptide with a unique pattern of four cysteine residues, - - -CC- - - - - -CC-. This novel class of cDNA clones was encountered at a frequency of over 20% in a cDNA library prepared from *C. textile* venom ducts. The sequence of the first such cDNA clone for which a complete open reading frame was deduced is shown in Table I. Like all *Conus* peptides, the predicted translation product has a prepropeptide organization, with a disulfide-rich mature conotoxin sequence present in a single copy at the COOH-terminal end; this clone is designated Tx5.1. Since the signal sequence encoded by clone Tx5.1 and the arrangement of Cys residues in the predicted

TABLE II
T-superfamily conotoxins

γ, γ-carboxyglutamate; W⁺, bromotryptophan; Tˢ, O-glycosylated threonine; *, COOH-terminal amidation. The cDNA sequences of mRNAs corresponding to the above prepropeptides have been deposited in the GenBank (Tx5.1, AF167164; Gm5.1, AF167165; Gm5.2, AF167166; Tx5.2, AF167167; P5.1, AF167168; Im5.1, AF167169).

	Complete prepropeptide
T-superfamily	
Tx5.1	**M**CCL**PVF**V**ILLLLLI**A**SAP**SVDAQPKTK**D**DVPLAPLHD**N**AKSALQHLNQR...**CC**QTFYW**CC**VQGK
Gm5.2	**M**RCL**PVF**V**ILLLLLI**A**SAP**SVDAQPKTK**D**DVPLAPLHD**N**IRSTLQTLRKK..V**CC**RPVQD**CC**SGK
P5.1	**M**RCL**PVF**V**ILLLLLI**P**SAP**CVDAHPKTK**D**DMPLASFHD**N**AKGTLQRFWKKR.G**CC**PKQMR**CC**TLG
Tx5.2	**M**RCF**PVF**I**ILLLLLI**A**SAP**CFDARTKTD**D**DVPLSSLRD**N**LKRTIRTRLNIR.E**CC**.EDGW**CC**TAAPLTGR
Gm5.1	**M**RYL**PVF**V**ILLLLLI**A**SIP**SDTVQLKTK**D**DMPLASFHG**N**GRRILRMLSNKR.L**CC**VTEDW**CC**EWW
Im5.1	**M**YCL**PVF**I**ILLLLLI**S**SAP**STPPQPRNK**D**RVHLISLLD**N**HKQILQR...DWNS**CC**GKNPG**CC**PWGK
Consensus	**M**RCL**PVF**V**ILLLLLI**A**SAP**SVDAQPKTK**D**DVPLASLHD**N**-K-LQ---------**CC**-----**CC**
O-superfamily	
ω-GVIA	MKLTCVVIVAVLLLTACQLITADDSRGTQKHRALGSTTELSLSTRCKSPGSSCSPTSYNCCRSCNPYTKRCYG
δ-TxVIA	MKLTCMMIVAVLFLTAWTFATADDPRNGLGNLFSNAHHEMKNPEASKLNKRWCKQSGEMCNLLDQNCCDGYCIVLVCT

	Mature toxin region		Mature toxin
Tx5.1	**CC**QTFYW**CC**VQGK		
Gm5.1	L**CC**VTEDW**CC**EWW		
Tx5.2	E**CC**EDGW**CC**TAAPLTGR	tx5a	γ**CC**γDGW⁺**CC**Tˢ AAO
Gm5.2	V**CC**RPVQD**CC**SGK		
P5.1	G**CC**PKQMR**CC**TLG	p5a	G**CC**PKQMR**CC**TL*
Im5.1	DWNS**CC**GKNPG**CC**PWGK		
		au5a	F**CC**PFIRY**CC**W
		au5b	F**CC**PVIRY**CC**W

mature tx5a peptide are novel, these define a novel group of *Conus* peptides (which we designate the *T-superfamily* of conotoxins).

Evidence That T-superfamily Peptides Are Broadly Distributed and Diverse in Conus—Given the apparent high frequency of clones encoding precursors belonging to this new superfamily of *Conus* peptides in *C. textile* venom, we used a PCR approach to identify related peptides in *C. textile* and other *Conus* cDNA libraries (see "Materials and Methods"). Peptides clearly belonging to the T-superfamily were identified from cDNA libraries made from venom ducts of *C. textile*, *C. gloriamaris*, *C. purpurascens*, and *Conus imperialis*. The deduced prepropeptide sequences of these peptides are shown in Table II.

These results strongly suggest that this new superfamily of *Conus* peptides will be widespread in the genus, since peptides belonging to the superfamily appear to be expressed in venom ducts of all major *Conus* feeding types (*C. purpurascens* is a fish-hunting species, *C. imperialis* specializes on polychaete worms, while *C. textile* and *C. gloriamaris* are snail-hunting *Conus* species). *C. textile* and *C. gloriamaris* each expressed two widely divergent peptide sequences belonging to the T-superfamily.

Purification and Characterization of p5a, a Peptide Belonging to the T-superfamily—In addition to a definition of the T-superfamily by cDNA cloning, three different peptides that clearly belong to the T-superfamily were directly isolated from venom. One of these was purified from venom obtained by milking *C. purpurascens* (9); the peptide from venom is clearly encoded by the cDNA clone P5.1 from a *C. purpurascens* cDNA library (Table II).

The purification of this T-superfamily peptide from *C. purpurascens* is shown in Fig. 1. Amino acid sequencing of the purified peptide and LSI-MS analysis (monoisotopic $[M + H]^+$ = 1337.5; calculated = 1337.54 Da) were consistent with the following sequence: Gly-Cys-Cys-Pro-Lys-Gln-Met-Arg-Cys-Cys-Thr-Leu-NH₂.

The sequence assignment was confirmed by synthesis of a peptide with the above sequence and specific disulfides (Cys[1]-Cys[3]; Cys[2]-Cys[4]). This synthetic peptide co-eluted with the natural material (see Fig. 1E). We give this peptide the provi-
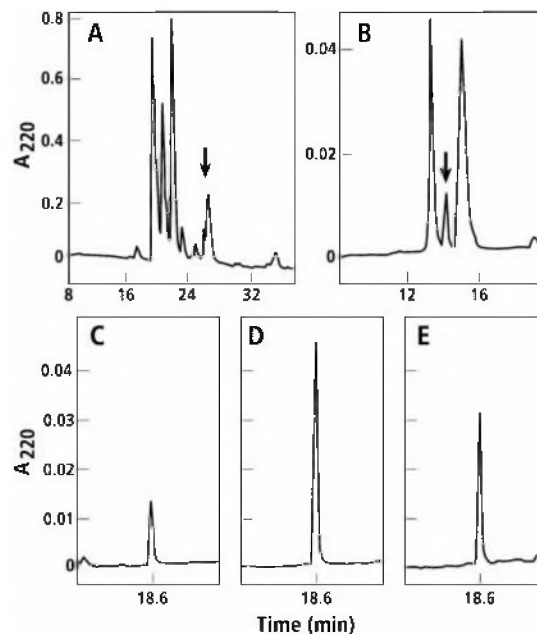

FIG. 1. **Purification of p5a.** *A*, the components of milked venom from *C. purpurascens* were fractionated by preparative RP C₁₈ HPLC column. Peptides were eluted using a linear gradient of 0–100% buffer B90 over 100 min. *B*, the fraction indicated in *A* was repurified by analytical HPLC using a gradient of 25–55% buffer B60 over 30 min. The *arrow* indicates the peak corresponding to p5a. *C–E*, co-elution of native p5a with synthetic material. Purified native peptide (*C*), synthetic peptide (*D*), and both combined (*E*) were chromatographed using a 20–50% gradient of buffer B60 over 30 min. In each case, a single homogeneous peak eluted at exactly 18.56 min.

sional designation p5a, which is the mature peptide encoded by clone P5.1. The COOH terminus is presumably processed by conventional mechanisms to yield the amidated COOH-terminal Leu residue.

The peptide showed no obvious symptomatology when injected intracranially or intraperitoneally into mice. However, when injected into male specimens of the Siamese fighting fish, *Betta splendens*, a clear deviation from normal behavior was observed. An immediate aggressive display is normally elicited
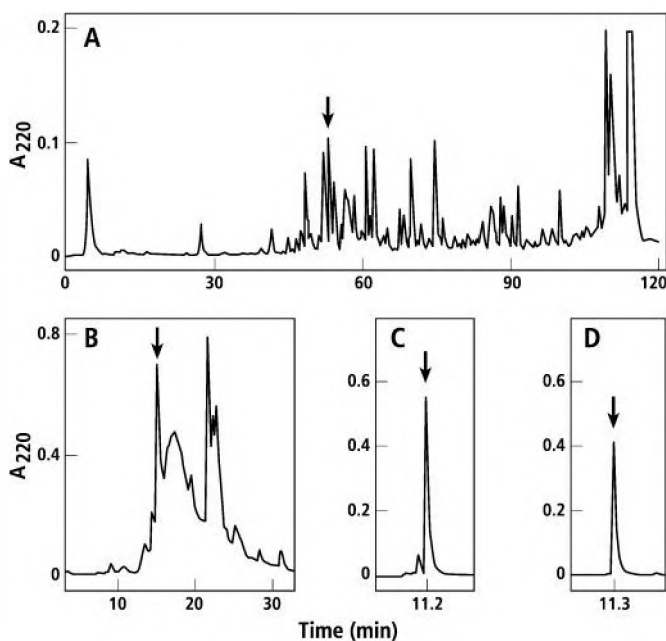
FIG. 2. **Purification of tx5a.** *A*, the panel shows a typical chromatogram of crude *C. textile* venom extract (500 μl) fractionated on a C_{18} Microsorb MV analytical column eluted with a linear gradient of 0.45% CH_3CN/min. *B*, the fraction marked by an *arrow* in *A* was pooled with similar fractions from other runs and chromatographed in the C_{18} analytical column using a gradient of 0.23% CH_3CN/min. *Panels C* and *D* are successive HPLC runs of the active peak (indicated by an *arrow*) using the same column and buffer gradient. The peptide eluted at 24.8% CH_3CN.

TABLE III
Sequence analysis of tx5a peptide

*, unassigned due to very low yield of phenylthiohydantoin derivatives. —, residues not found in the peptide isolated from *C. textile* venom.

Cycle	Residue	Yield	Residue predicted by clone Tx5.2
		pmol	
1	X	*	E
2	C	301.88	C
3	C	298.93	C
4	X	*	E
5	D	184.96	D
6	G	169.77	G
7	X	*	W
8	C	162.95	C
9	C	195.00	C
10	X	*	T
11	A	130.85	A
12	A	133.3	A
13	O	49.54	P
14	—	—	L
15	—	—	T

in these fish in response to their reflection when placed in a mirrored aquarium; injection of relatively high levels of the peptide suppressed this behavior (ED_{min} ~8 nmol).

Purification and Characterization of a Peptide Belonging to the T-superfamily of Conotoxins from C. textile Venom—A peptide that belongs to the T-superfamily was purified from *C. textile* venom using reversed phase HPLC, as shown in Fig. 2. This venom component caused hyperactivity and other excitatory behavior upon intracranially injection into mice. The purified material also potently affected fish (ED_{min} ~0.2 nmol using the *Betta* gill display assay).

The results of an Edman sequence analysis detected no phenylthiohydantoin derivatives; consequently, the peptide was reduced and alkylated, and the Edman sequence analysis of the modified peptide is shown in Table III. There are still four positions (1, 4, 7, and 10) that could not be assigned, but nine other residues could be identified unambiguously.

The sequence obtained closely corresponds to that predicted by clone Tx5.2 (see Tables II and III). However, the Glu residues predicted by the clone at positions 1 and 4 could not be assigned; examination of the Edman analysis revealed that a small yield of Glu was in fact detected in both of these cycles. This is a characteristic noted in previous Edman analyses of peptides containing γ-carboxyglutamate (Gla).

The two remaining blanks in the Edman sequence analysis, at positions 7 and 10, were predicted from the cDNA clone to be Trp and Thr, respectively. We have previously shown that Trp residues in *Conus* peptides can be modified to 6-bromotryptophan (which could account for the blank obtained for residue 7) (23). Recently, we demonstrated that in a novel peptide from *C. geographus*, contulakin-G, a threonine residue was *O*-glycosylated (24); an *O*-glycosylated threonine could account for the blank at position 10. Thus, γ-carboxylation of Glu^1 and Glu^4 to Gla, bromination of Trp^7 to 6-Br-Trp, and *O*-glycosylation of Thr^{10} would explain the Edman sequencing results shown in

Table III. We also note that no further phenylthiohydantoin derivatives were obtained in Edman steps beyond residue 13, despite the prediction from the nucleic acid sequence of clone Tx5.2 of two additional amino acid residues (see Table III).

The hypothesis that the peptide is post-translationally modified as proposed above is strongly supported by mass spectrometry data. ESI-MS analysis revealed a m/z 964.8 doubly charged negative species and several doubly charged positive species, *e.g.* m/z 862.4, 884.8, 904.7, 966.8 (resolved monoisotopomer m/z 965.7), and 985.7. We interpreted the m/z 966.8 species in the positive mode and the m/z 964.8 species in the negative mode as the $[M + 2H]^{2+}$ and $[M - 2H]^{2-}$, respectively, where molecule mass (M) is 1929.4 Da. The m/z 985.7 is consistent with $[M + H + K]^{2+}$, while m/z 884.8 and 904.7 were attributed to fragment ions involving loss of 162 Da (from m/z 966.8 and 985.7). The m/z 862.4 species is a separate form of the tx5a peptide in which only one Gla residue is present and the threonine residue incorporates the monosaccharide *N*-acetylhexosamine.

After reduction and alkylation of the sample with 4-vinylpyridine (to form the Cys(pyridylethyl) derivative, which has a residue mass of 208 Da) ESI-MS analysis revealed an intense m/z 804.0 positively charged species (*inset*, resolved mono-isotopomer of m/z 803.3) or a m/z 1203.5 negatively charged species (see Fig. 3), assigned as $[M_{R/A} + Fe]^{3+}$ and $[M_{R/A} + Fe - 5H]^{2-}$, respectively. The observed reduced and alkylated monoisotopic mass ($M_{R/A}$) of 2354.1 Da and the mass difference ($M_{R/A} - M$) of 424.7 is consistent with the presence of four cysteine residues. The ESI-MS/MS spectrum of the m/z 967 positively charged precursor resulted predominantly in loss of 162 Da (m/z 885.6), consistent with loss of a terminal hexose residue. In the ESI-MS/MS spectrum of the m/z 965 negatively charged precursor, loss of one or two molecules of CO_2 (m/z 942.8 and 920.1) predominated, indicative of two Gla residues. The MALD-MS analysis indicated the presence of both a Hex-HexNAc moiety and the Gla residues. Based on this evidence for the presence of a glycosylated residue, two Gla residues, a bromotryptophan residue, and the cDNA clone obtained, we proposed the sequence: Gla-Cys-Cys-Gla-Asp-Gly-Trp*-Cys-Cys-Thr†-Ala-Ala-Pro-OH, where Gla = γ-carboxyglutamatic acid, Trp* = bromotryptophan, and Thr† = Hex-HexNAc-Thr. The observed mass of the *C. textile* peptide (1929.4 Da) was consistent with the calculated mass (1929.42 Da). Comparison of the proposed sequence with the clone obtained indicates that the Leu-Thr dipeptide has been cleaved from the COOH terminus of the peptide.
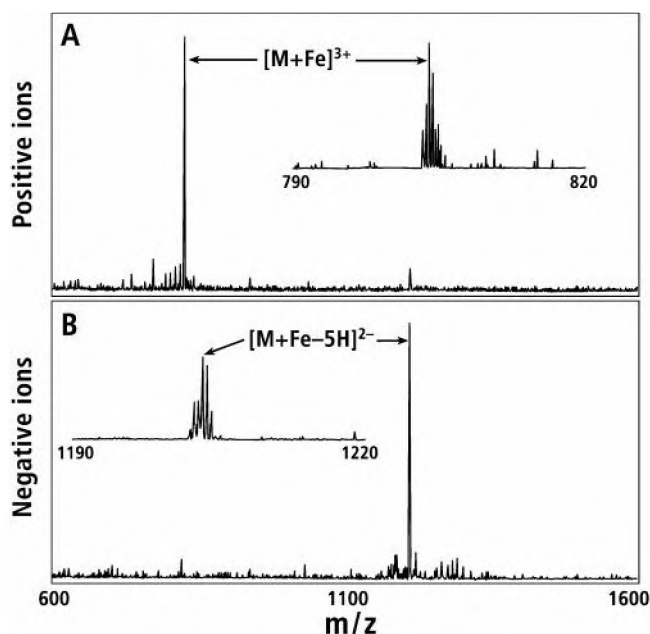
FIG. 3. **Electrospray ionization mass spectrum of the reduced and alkylated *C. textile* peptide (A) in the positive ionization mode (*inset*, the observed intact $[M + Fe]^{3+}$ resolved isotope distribution; observed monoisotopic M = 2354.1 (*m/z* 803.3), *cf.* calculated M = 2353.68 Da) and (*B*) the negative ionization mode (*inset*, the observed intact $[M + Fe - 5H]^{2-}$ resolved isotope distribution; observed monoisotopic M = 2353.8 (*m/z* 1202.3), *cf.* calculated M = 2353.68 Da).** The spectra were obtained as described under "Materials and Methods."

FIG. 4. **Enzymatic carboxylation of Tx5.2 pro(−20 to −1).FLEEL-NH$_2$ (▼) and FLEEL (■).** In the y axis, $^{14}CO_2$ incorporated into the substrates is expressed as percentage of maximal incorporation at the highest substrate concentration used in the experiment. The assay used partially purified carboxylase from *C. textile* microsomes as described under "Materials and Methods." The data were fitted to a single-site binding model.

Thus, in contrast to p5a, tx5a, the first T-superfamily peptide isolated and characterized from *C. textile* venom, exhibited a high degree of post-translational modification. The p5a peptide from *C. purpurascens* is unmodified except for amidation of the COOH terminus.

Evidence for a Functional γ-Carboxylation Recognition Sequence in the Tx5.2 Prepropeptide—The discovery that two glutamate residues in tx5a were γ-carboxylated suggested the presence of a γ-carboxylation recognition signal in the "pro" region of the precursor. Recently, it was established that the −1 to −20 region of the γ-carboxylated conantokins contains recognition signals that confer a higher affinity when present NH$_2$-terminal to a target sequence (25). However, there is no obvious sequence homology between the −1 to −20 regions of the conantokins and Tx5.2.

In order to test whether the *C. textile* Tx5.2 prepropeptide does indeed contain a γ-carboxylation recognition sequence in its −1 to −20 region, a peptide was synthesized with the −1 to −20 region from Tx5.2 attached to a standard γ-carboxylation target sequence, FLEEL. The γ-carboxylation of FLEEL was assessed in the presence and in the absence of the −1 to −20 region of Tx5.2.

As shown in Fig. 4, the presence of the −1 to −20 Tx5.2 region does indeed increase the affinity by over 2 orders of magnitude for the targeted FLEEL sequence. The estimated EC$_{50}$ values in the presence and absence of propeptide are 0.59 and 140 μM, respectively. It should be noted that maximum activity in the presence of saturating amounts of FLEEL was not achieved and so the EC$_{50}$ of 140 μM is probably a lower estimate. Thus, not only is γ-carboxyglutamate present in the mature peptide region, but a carboxylase recognition signal is present immediately NH$_2$-terminal to the targeted glutamate residues, in the Tx5.2 prepropeptide. There may also be recognition signals in the prepropeptide for bromination and *O*-glycosylation e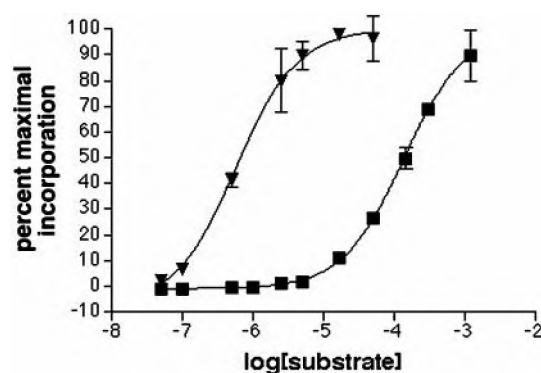nzymes. Thus, Tx5.2 and other members of the T-superfamily may provide good model substrates for studying post-translational modification of *Conus* peptides.

Purification of T-superfamily Peptides from C. aulicus Venom—Two peptides belonging to the T-superfamily were purified from *C. aulicus* venom as shown in Fig. 5. Edman sequencing of the two peptides showed that they had the Cys pattern of the T-superfamily. The two purified peptides, designated au5a and au5b, have the amino acid sequences shown in Table II.

The amino acid sequences were confirmed by LSI-MS (observed monoisotopic $[M + H]^+$ values for au5a and au5b are m/z 1436.6 and 1388.6, respectively; *cf* calculated values of 1436.5 and 1388.5 Da).

The au5a peptide was synthesized with directed disulfide formation. As shown in Fig. 5, only synthetic isomer S1 (1–3, 2–4 Cys bonding pattern) co-eluted with the native peptide.

No obvious symptomatology was elicited when 5 nmol of the au5a peptide was injected intracranially into mice. However, using the *Betta* gill display assay, the peptide was active at an ED$_{min}$ of ~0.2 nmol (Table IV).

A preliminary attempt to identify the molecular target of peptide au5A has been initiated. The peptide was iodinated at the tyrosine residue; the monoiodo-derivative was active (0.3 nmol of the monoiodo-derivative suppressed gill display). Since this derivative was biologically active, radiolabeled ^{125}I-au5a peptide was prepared for binding assays. These experiments were technically difficult, given the hydrophobicity of this peptide and high nonspecific binding background routinely observed. No measurable specific binding could be detected when either mouse brain or fish brain membranes were used. These results are consistent with either the molecular target of peptide au5a not being in neurons, or with rapid dissociation of radiolabeled peptide from the target receptor.

DISCUSSION

We describe the characterization of a novel group of peptides found in *Conus* venoms, designated the T-superfamily of conopeptides. Eight peptides belonging to this superfamily have been identified; three were isolated from venom and biochemically characterized, and two have been chemically synthesized (p5a from *C. purpurascens* and the au5a from *C. aulicus*). It seems probable that the members of this superfamily will be pharmacologically diverse, with a variety of different molecular targets (in much the same way that members of the O-superfamily target different sites on a diverse set of voltage-gated ion channels). Considering that the T-superfamily peptides identified so far fall into a size range of only 10–17 amino acids, the four *Conus* species examined express a remarkable

diversity of T-superfamily peptides.

The three peptides isolated from venom differ dramatically in the extent of post-translational modification found. In contrast to p5a and au5a, the tx5a peptide, which appears to be encoded by clone Tx5.2, has an exceptionally high density of post-translational modifications. The peptide contains two γ-carboxylated glutamate residues, one O-glycosylated threonine, one hydroxylated proline, and one brominated Trp. This is the first peptide in which these diverse modifications have been observed together, although each has been described previously in other *Conus* peptides. In addition, there may be an unusual proteolytic cleavage at the COOH terminus, although we cannot be absolutely certain whether this is physiological, a polymorphism, or an artifact of storage.

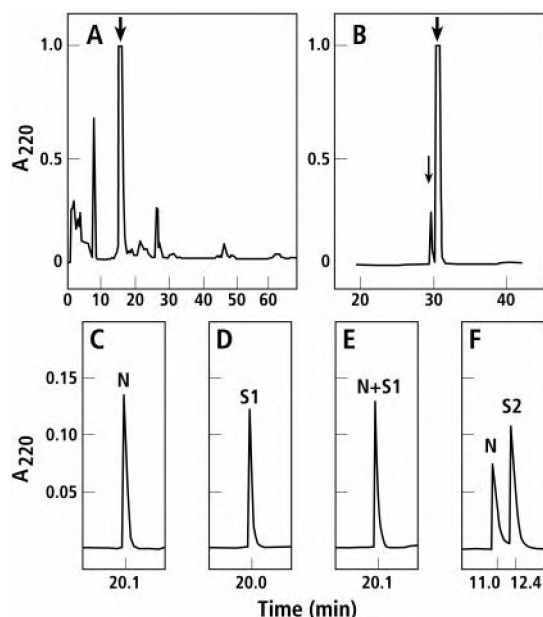Using a partially purified *C. textile* vitamin K-dependent

FIG. 5. **Purification of peptides from *C. aulicus* venom and comparison of natural au5a with synthetic peptides.** Lyophilized *C. aulicus* venom was fractionated by preparative HPLC. *A*, the peak that eluted at 15–16 min from the preparative column was fractionated on an strong cation exchange-HPLC column by elution with a gradient of 0–0.25 M NaCl in 10 mM phosphate 50% CH$_3$CN, pH 2.5, over 100 min. *B*, the peak indicated by an *arrow* in *A* was applied on an analytical RP C$_{18}$ column and eluted with a gradient of 0–90% CH$_3$CN in 0.1% trifluoroacetic acid over 60 min at 1 ml/h. The *broad arrow* indicates au5a and the *thin arrow*, au5b. *C–E*, separate analytical runs and co-elution of natural au5a (*N*) and synthetic au5a with a 1–3, 2–4 Cys bonding pattern (*S1*). The C$_{18}$ column was eluted with a gradient of 18–36% CH$_3$CN in 0.1% trifluoroacetic acid over 30 min. *F*, analytical run of a combination of natural au5a and synthetic peptide with au5a sequence but a 1–4, 2–3 Cys bonding pattern (*S2*). The gradient used was 33–39% CH$_3$CN in 0.1% trifluoroacetic acid over 30 min.

γ-glutamyl carboxylase, we demonstrated the presence of a recognition signal sequence in the −1 to −20 propeptide region of the tx5a precursor (deduced from clone Tx5.2). It is noteworthy that this Tx5.2 recognition sequence, which provides a >100-fold increase in apparent affinity for the carboxylase enzyme, shows no obvious sequence homology to the only other *Conus* peptide recognition sequence that has been functionally demonstrated, that of conantokin-G (25).

The characteristic signature of T-superfamily peptides is the presence of two pairs of cysteine residues; most T-superfamily peptides identified so far have five amino acids between the cysteines (except tx5a, which has four). For the two peptides that have been synthesized (p5a and au5a), directed synthesis of specific disulfide-bonded forms was carried out. The disulfide bonding pattern of the native peptides is Cys^1-Cys^3, and Cys^2-Cys^4. Since a conserved arrangement of cysteine residues generally implies a conserved disulfide configuration, it seems highly likely that the disulfide pattern of all T-superfamily peptides in Table II will be the same as the p5a and au5a peptides.

In this work, we have described eight different members of the T-superfamily of conotoxins; for two of these, both the cDNA clone and the actual venom peptide have been identified. Two of the peptides were isolated from venom, but corresponding clones have not yet been analyzed. For four of the peptides, an amino acid sequence can be predicted from the cDNA clone, but the extent of post-translational modification has not yet been specified. For some of these peptides, considerable post-translational modification may very well occur. Thus, in tx5a, Glu, Thr, and Trp residues are modified to γ-carboxyglutamate, O-glycosylated threonine, and 6-bromotryptophan, respectively. The same amino acids are present in the mature toxin region of clone Gm5.1; whether or not these will have similar modifications must be confirmed by characterizing the biologically active peptide from the venom of this species. We note that the most heavily modified peptide, tx5a, was the only one of the three T-superfamily conotoxins that was active in mice. This peptide may offer an unusual opportunity to evaluate the effects of different post-translational modifications on biological activity.

The identification of eight different T-superfamily conotoxins from our relatively small sample (five *Conus* species, approximately 1% of the genus) suggests that the T-superfamily will be large and diverse. These peptides are among the smallest of the multiply disulfide-bonded conotoxins, with four of the amino acids being highly conserved Cys residues. Except for the polymorphic variation in the au5a peptides, the amino acid sequences are remarkably divergent; several have very unusual distribution of amino acids (such as Gm5.1, with over 50% of the residues being Trp or Cys). We have demonstrated that the degree of post-translational modification of the small sample of

TABLE IV
Comparison of biological activity of naturally occurring T-superfamily conotoxins

| Toxin | Assay | | |
	Mice injected intracranially	Mice injected intraperitoneally	ED$_{min}$ for suppression of gill display in fighting fish[a]
p5a	No effect (~100 nmol)[b]	No effect (~100 nmol)[b]	8.2 ± 1.0 nmol (8)[d]
tx5a	Hyperactivity[c] and spasticity (at 0.5 nmol)	Not determined	0.20 ± 0.029 nmol Dorsal fins droop at ≥0.5 nmol (12)[d]
au5a	No effect (~5 nmol)[b]	No effect (~5 nmol)[b]	0.21 ± 0.037 nmol Dorsal fins droop at ≥0.5 nmol (14)[d]

[a] ED$_{min}$ is the minimum effective dose (± standard deviation). The activity was observed after intramuscular injection into 4–7 fish. Symptoms last for 4–5 min at the ED$_{min}$ and 16–20 min at 0.5 nmol of peptides tx5a and au5a.
[b] Maximum dose per animal.
[c] Hyperactivity symptoms include continuous running, jumping, and climbing in cages; this persists for >2 h. Of the six mice injected, one exhibited rigid extension of legs within several minutes of injection followed by death.
[d] Number of animals used.

peptides so far characterized from the T-superfamily also differs dramatically. Thus, there is every reason to expect many hundreds of different peptides belonging to the T-superfamily of conotoxins in *Conus* venoms. The work described in this report provides the defining characterization of this potentially large and diverse group of biologically active peptides.

Note Added in Proof—Recently, one of the peptides described above, t×5a (encoded by clone t×5.2), was also characterized by Rigby, A. C., Lucas-Meunier, E., Calume, D. E., Czerwizc, E., Hambe, B., Dahlquist, I., Fossier, T., Baux, G., Roepstorff, P., Baleja, J. D., Furie, B. C., Furie, B., and Stenflo, J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 5758–5763. Their ε-TxTX is identical to t×5a.

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Additions and Corrections

Vol. 274 (1999) 32613–32618

Functional characterization of human methylenetetrahydrofolate reductase in *Saccharomyces cerevisiae.*

Xiaoyin Shan, Liqun Wang, Roselle Hoffmaster, and Warren D. Kruger

Page 32615, Fig. 2, y axis: The unit for the activity should be per microgram (μg) not per milligram (mg). Thus, the y axis should be: activity (pmol/μg of extract/h) instead of activity (pmol/mg of extract/h).

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The T-superfamily of conotoxins.

Craig S. Walker, Douglas Steel, Richard B. Jacobsen, Marcelina B. Lirazan, Lourdes J. Cruz, David Hooper, Reshma Shetty, Richard C. DelaCruz, Jacob S. Nielsen, Li Ming Zhou, Pradip Bandyopadhyay, A. Grey Craig, and Baldomero M. Olivera

Page 30664: The following footnote was omitted:

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Functional analysis of the N-terminal CXXC metal-binding motifs in the human Menkes copper-transporting P-type ATPase expressed in cultured mammalian cells.

Ilia Voskoboinik, Daniel Strausak, Mark Greenough, Hilary Brooks, Michael Petris, Suzanne Smith, Julian F. Mercer, and James Camakaris

Page 22009, Table I: An arithmetical error occurred when converting units. Columns 2 and 4: pmol/min/mg should be nmol/min/mg. Footnotes a and c to this table: pmol of Cu/min/mg should be nmol of Cu/min/mg. The correct table is shown below.

TABLE I
Catalytic activities of the wild-type MNK and its metal binding site mutants

All constructs were transfected into CHO-K1 cells. 117, wild type MNK with all MBS intact; 114-1 and 114-2, clones with MBSs 1-3 mutated; 115, MBSs 4-6 mutated; 116-1 and 116-2, clones with MBSs 1-6 mutated. ^{64}Cu translocation into purified membrane vesicles was carried out as described under "Experimental Procedures." Expression of endogenous hamster MNK in EV cells was insignificant and did not permit correction relative to 117.

Cell line	Catalytic activity[a]	Normalized MNK expression[b]	Normalized catalytic activity[c]	Percent residual activity[d]
	nmol/min/mg		*nmol/min/mg*	
EV	0.028 ± 0.011	0.02	NA[e]	NA[e]
117	0.390 ± 0.067	1.0	0.390	100
114-1	0.347 ± 0.086	1.3	0.267	68
114-2	0.090 ± 0.044	0.3	0.300	77
115	0.069 ± 0.035	0.2	0.345	88
116-1	0.107 ± 0.031	0.5	0.214	55
116-2	0.082 ± 0.009	0.3	0.273	70

[a] nmol of Cu/min/mg of total protein in the presence of 2 μM Cu and 5 mM ATP.
[b] The levels of expression of MNK mutants relative to the level of MNK in 117 as compared by laser densitometry. The absolute amount of MNK could not be measured as there is no pure MNK available to use as a standard.
[c] Catalytic activity expressed in nmol of Cu/min/mg of total protein corrected for the relative levels of expression of MNK mutants.
[d] Catalytic activity expressed as % of activity of 117.
[e] NA, not applicable.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.