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-C-.). 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 superfamly 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, γCCγDGWW'CCT'AAO; and au5a, FCCPFIRYCC where γ = γ-carboxyglutamate, W = bro-motryptophan, 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 prepropeptide region, consistent with the presence of γ-carboxyglutamate residues in this peptide.

Conus 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 superfamly 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 α-, 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 superfamly. 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 C4 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 with limiting buffers consisting of 0.1% trifluoroacetic acid and 60% acetonitrile (CH3CN) 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% CH3CN 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 C18 HPLC1 column as described above.

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

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1 The abbreviations used are: HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; CHAPS, 3-(3-cholamidopropyl)dime-thylammonio)-1-propanesulfonic acid; Fmoc, N-(9-fluorenylmethoxycarbonyl); 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% CH₃CN. 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 C₄ semi-preparative column (10 × 250 mm, 5 µm, 300 A, Vydac, or Microsorb-MG). Purification 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 400HVP575, 5 mm, 7.5 × 50 mm), followed by a run on a reverse-phase C₃₈ 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 C₃₈ 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. textile, sequencing was done on an ABI 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 9 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 µmol 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 (Brucker 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 (9) capillary, and infused at approximately 250 nl/min. Liquid secondary ion trap mass spectrometer (Bruker Daltonics). The HPLC-purified samples were also analyzed by MALD on a Vickers Cyano-4-hydroxycinnamic acid matrix. The peptide was then eluted by gravity perfusion with 20 ml of buffer 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 Fmoc standard 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 that they can greatly extend in display, and 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 by oligo(dT) extension at the 3′site of a linearized modified pUC13 plasmid isolated from Conus textile and Conus gloriamarissus venoms. 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 nt amount of each oligonucleotide, 2.5 mM MgCl₂, 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) Cloning of Tx5.2, P5.1, and Im5.1—The DNA sequence of clone Tx5.1 was analyzed, and the following oligonucleotide primers containing EcoRI endonuclease sites were designed to screen cDNA libraries of other species: first strand primer 1 (5'-GGA ATT CGG AAG CTG ACT) 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.
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 Ala66 and Gln62. The solid arrow indicates expected proteolytic processing site (between Arg49 and Cys50) for the release of mature toxin product (Cys50 to Gln60).
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 C. 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-Glu-Met-Cys-Acu-Cys-Cys-Thr-Leu-NH2.

The sequence assignment was confirmed by synthesis of a peptide with the above sequence and specific disulfides (Cys1-Cys2; Cys2-Cys4). This synthetic peptide co-eluted with the natural material (see Fig. 1E). We give this peptide the provisional 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.
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 \( (E_{\text{yield}} - 8 \text{ nmol}) \).

**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 \( (E_{\text{yield}} - 0.2 \text{ nmol using the Betta gall display assay}) \).

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 \( (1, 4, 7, \text{ and } 10) \) 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 \( \gamma \)-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, \( \gamma \)-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 \([M + H + K]^+\), 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 \( m/z \) 864.2 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(pyrididyethyl) 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 \([M_{\text{RA}} + \text{Fe}]^+\) and \([M_{\text{RA}} + \text{Fe} - 5H]^2-\), respectively. The observed reduced and alkylated monoisotopic mass \( (M_{\text{RA}}) \) of 2354.1 Da. The mass difference \( (M_{\text{RA}} - M) \) 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 = \( \gamma \)-carboxyglutamic acid, Trp\(^*\) = bromotryptophan, and Thr\(^*\) = Hex-HexNAc-Thr. The observed mass of the C. textile peptide \( (1929.4 \text{ Da}) \) was consistent with the calculated mass \( (1929.42 \text{ Da}) \). 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.

**Table III**

Sequence analysis of ts5a peptide

| Cycle | Residue | Yield | Residue predicted by clone Tx5.2 |
|-------|---------|-------|---------------------------------|
| 1     | X       | E     |                                 |
| 2     | C       | 301.88| E                               |
| 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       |       |                                 |
| 11    | A       | 130.85| A                               |
| 12    | A       | 133.3 | A                               |
| 13    | O       | 49.54 | P                               |
| 14    | —       | —     | L                               |
| 15    | —       | —     | T                               |
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 NH2-terminal to a target sequence (25). However, 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 NH2-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 enzymes. 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_{50} 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 125I-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 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 Cys1-Cys3, and Cys2-Cys4. 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-carboxylated glutamate, 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 peptides

![FIG. 5. Purification of peptides from C. aulicus venom and comparison of natural au5a with synthetic peptides.](image)

**TABLE IV**

| Assay                  | Mice injected intracranially | Mice injected intraperitoneally | ED<sub>min</sub> for suppression of gill display in fighting fish<sup>a</sup> |
|------------------------|------------------------------|---------------------------------|----------------------------------------------------------------------------|
| p5a                    | No effect (~100 nmol)<sup>b</sup> | No effect (~100 nmol)<sup>b</sup> | 8.2 ± 1.0 nmol (8)<sup>c</sup>                                               |
| tx5a                   | Hyperactivity<sup>c</sup> and spasticity (at 0.5 nmol) | Not determined                   | Dorsal fins droop at ≥0.5 nmol (12)<sup>c</sup>                               |
| au5a                   | No effect (~5 nmol)<sup>b</sup> | No effect (~5 nmol)<sup>b</sup> | 0.21 ± 0.037 nmol                                                             |

<sup>a</sup> ED<sub>min</sub> 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<sub>min</sub> and 16–20 min at 0.5 nmol of peptides tx5a and au5a.

<sup>b</sup> Maximum dose per animal.

<sup>c</sup> 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.

<sup>d</sup> 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, t35a (encoded by clone t35.2), was also characterized by Rigby, A. C., Lucas-Meunier, E., Calume, D. E., Czerwizie, E., Hambe, B., Dahliquist, 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 e-TxTX is identical to t35a.

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