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RESEARCH

Multiple Bromotryptophan and γ-carboxyglutamate Residues in a *Conus* Peptide

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A novel peptide was purified from *Conus textile* venom which caused hyperactivity in mice. The 31-amino acid peptide has six residues with unusual post-translational modifications: four γ-carboxyglutamates and two brominated tryptophan residues. This peptide, which we have designated the dibromorunning peptide, is the first known gene product with multiple bromotryptophan residues. We discuss the apparent non-random association of γ-carboxyglutamate and bromotryptophan in *Conus* peptides.

Key words: *Conotoxin, bromotryptophan, γ-carboxyglutamate, hyperactivity, Conus textile, post-translational modification*

The predatory cone snails use a peptide-based neuropharmacological strategy to capture prey, defend against predators and interact with competitors (Olivera 1997). Approximately 100 different peptides can be found in the venom of each *Conus* species. Furthermore, the peptides made by one species are a distinctive set, different from the peptides in any other venom. Since there are approximately 500 different species of cone snails, ca. 50,000 different peptides are likely present in the venoms of the living *Conus*. Only a tiny fraction of these have been characterized, even superficially.

The conopeptides are mostly small (8 - 35 amino acids in length) and multiply disulfide-bonded (Olivera 1997, Olivera et al. 1990). One of the hallmark characteristics of a significant proportion of *Conus* peptides is the unusual post-translational modifications found, some previously undescribed. Among the most distinctive are the γ-carboxylation of glutamate residues to yield γ-carboxyglutamate (Gla) (McIntosh et al. 1984) and the bromination of tryptophan residues to 6-bromotryptophan (Trp) (Craig et al. 1997; Jimenez et al. 1997). Although these post-translational modifications are unusual even among *Conus* peptides, γ-carboxyglutamate and bromotryptophan were found together in two previously characterized

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Abbreviations: DTT, dithiothreitol; ESI, electrospray ionization; Gla, γ-carboxyglutamate; HPLC, high performance liquid chromatography; Hyp, hydroxyproline; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; Trp, bromotryptophan.

peptides. The first was the bromosleeper peptide from *Conus radiatus* (Craig et al. 1997). More recently, a peptide belonging to a new superfamily of *Conus* toxins, tx5a of the T-superfamily, was found to have both 6-bromotryptophan and γ-carboxyglutamate (as well as an O-glycosylated threonine residue) (Rigby et al. 1999, Walker et al. 1999).

In this report, we describe a novel peptide from *Conus textile* venom which is the third to have both these two unusual post-translational modifications. This peptide that we refer to as dibromorunning peptide or tx7a has three disulfide linkages, four Gla residues and, uniquely, two residues of bromotryptophan.

Methods

Purification of the peptide

Lyophilized *Conus textile* venom from the Philippines (200 mg) was extracted sequentially with 10 mL each of 0%, 20%, 40% and 60% acetonitrile. The mixture was sonicated for 30 s periods over ice, centrifuged at 5,000 x g for 5 min and the combined supernatant was stored at -20° C. Aliquots of the extract were fractionated by HPLC chromatography on a C_{18} Microsorb MV analytical column or a Vydac C_{18} semi-preparative column as previously described (Walker et al. 1999). The columns were eluted with a 0.45%/min gradient of acetonitrile (CH_3CN) in 0.1% trifluoroacetic acid (TFA) at 1 mL/min for the analytical column or 5 mL/min for the semi-preparative column (Fig. 1). Corresponding peaks from several runs were pooled and peptide tx7a was purified from the pool by two consecutive runs on the analytical column eluted with 0.23%/min of CH_3CN in 0.1% TFA. The final purification was a run on the same column eluted isocratically at 38% CH_3CN in 0.1% TFA.

Bioassay

The biological activity of HPLC fractions was determined by intracranial (i.c.) injection into 9- to 15-day-old mice. Fractions were lyophilized then dissolved in normal saline solution before injection using an ultrafine insulin syringe. The 26-gauge needle was allowed to penetrate the cranium to ~1.0-1.5 mm depth. Control mice were injected with equal volume (15-20 µL) of normal saline solution containing dissolved residue (if any) of lyophilized column buffer. After injection, the mice were returned to their cages and observed for any behavioral symptoms. The time at which the mouse first showed abnormal behavior was also recorded. The dose used was ~0.3 to 0.5 nmole per mouse. A higher dose was not tried due to a limited amount of sample.

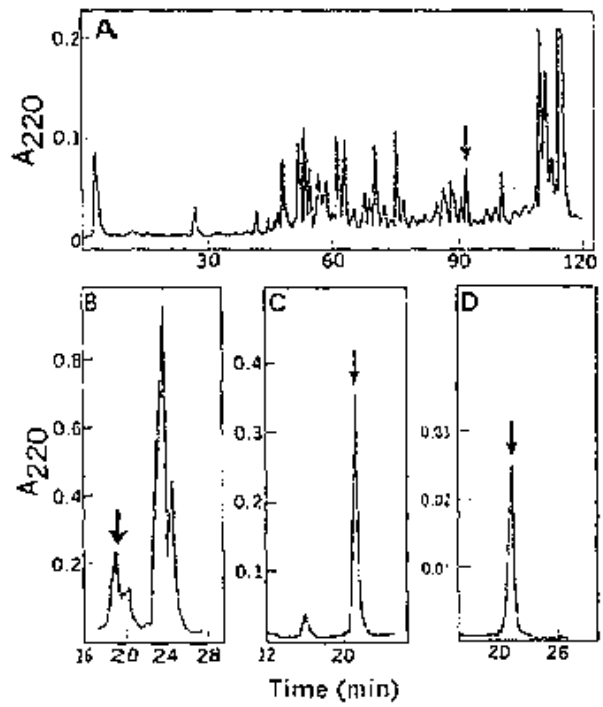

Figure 1. Purification of the dibromorunning peptide, tx7a, from *C. textile* venom. (A) HPLC chromatogram of an aliquot of the crude venom extract obtained as described under Methods. (B) A pool of fractions corresponding to the bioactive peak indicated by arrow in Panel A was applied to an analytical C_{18} Microsorb MV column, and then eluted with a 0.23%/min gradient of 36-48% acetonitrile. (C) The bioactive fraction in Panel B was repurified using the same conditions in B. (D) Isocratic elution of the pure peptide with 38% acetonitrile. The bioactive peak in panels B through D is indicated by an arrow.

Reduction and alkylation

The purified peptide was reduced with dithiothreitol (DTT) and alkylated with 4-vinylpyridine as follows. The peptide solution was adjusted to pH 8 with 0.5 M TRIS before DTT was added to a concentration of 10 mM. The solution was flushed with N_2, incubated at 65° C for 15 min then cooled to room temperature. After adding 4-vinylpyridine (5 µL per mL of solution), the mixture was left in the dark at room temperature for 25 min. The mixture was diluted with 0.5 mL of 0.1%

TFA prior to purification of the reduced peptide by HPLC with the C_{18} Microsorb MV analytical column.

Digestion of the peptide

Approximately 1.5 nmol of peptide was digested with endoproteinase AspN (Boeringer Mannheim). The lyophilized peptide was dissolved in 0.1mL of 50 mM sodium phosphate, pH 8. Endoproteinase AspN (1 mg enzyme per 20 mg peptide) in 10 mM TRIS Cl, pH 7.5, was added, and the reaction incubated for 17 h at 37° C. To stop the reaction, 0.1% TFA (0.5 mL) was added, and the digest was fractionated by HPLC with a C_{18} Microsorb MV analytical column eluted with a linear gradient of 0.9% acetonitrile per mL/min. The intact mass of the digestion fragments were analyzed by ESIMS prior to chemical sequencing.

Mass spectrometry

Electrospray ionization (ESI) mass spectra were measured using an Esquire ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). The HPLC-purified sample, collected in 0.1% TFA and acetonitrile was diluted in methanol-1% acetic acid, transferred to a fused silica capillary and infused at approximately 500 nL/min. The mass accuracy of the ion trap instrument was typically better than 200 ppm.

Peptide sequencing

The peptide was reduced with DTT and alkylated with 4-vinylpyridine. Approximately 10 to 100 pmol of alkylated peptide and N-terminal digestion fraction fragments were sequenced by Edman degradation with an Applied Biosystem Model 492 Sequenator (DNA Peptide Facility, University of Utah). The 3-phenol-2-thiohydantoin derivatives were identified by HPLC. Predicted masses for each sequence were routinely verified by mass spectrometry.

Identification and characterization of a cDNA

The Expressed Sequence Tag (EST) method previously described (Walker et al. 1999) was used to identify a cDNA clone encoding tx7a. Templates were prepared using a QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and submitted for fluorescent sequencing primed with nucleotides, M13R and subsequently M13U (Messing 1983) at the Health Sciences Center Sequencing Facility, Eccles Institute for Human Genetics, University of Utah. All molecular biology techniques were as described in Sambrook, Fritsch and Maniatis (Sambrook et al. 1989).

Results

Purification of the "dibromorunning" peptide

A fraction of *Conus textile* venom which caused hyperactivity in mice was purified as described under Methods. The peptide induced rapid running in mice upon intracranial injection. The purification of the peptide is shown in Fig. 1.

Table 1. Sequence analysis of tx7a.

Cycle	Residue	Yield (pmol)	Residue Predicted by Clone
1	G	77.9	G
2	M	94.4	M
3	X	–	W
4	G	53.1	G
5	X	–	E
6	C	48.0	C
7	K	25.1	K
8	D	33.4	D
9	G	39.0	G
10	L	43.7	L
11	T	22.5	T
12	T	27.5	T
13	C	29.0	C
14	L	29.9	L
15	A	22.1	A
16	O	34.0	P
17	S	10.3	S
18	X	–	E
19	C	19.0	C
20	C	26.0	C
21	S	8.6	S
22	X	–	E
23	D	9.5	D
24	C	12.3	C
25	X	–	E
26	G	14.8	G
27	S	5.3	S
28	C	8.2	C
29	T	4.4	T
30	M	4.1	M
31	X	–	W

– yield not determined

Chemical sequence analysis of the peptide was carried out for 32 Edman cycles. As shown in Table I, the sequence Gly-Met-Xxx-Gly-Xxx-Cys-Lys-Asp-

Gly-Leu-Thr-Thr-Cys-Leu-Ala-Hyp-Ser-Xxx-Cys-Cys-Ser-Xxx-Asp-Cys-Xxx-Gly-Ser-Cys-Thr-Met could be assigned up to the 30th cycle. In cycles 3, 5, 18, 22 and 25 (indicated by Xxx) no standard amino acid was obtained at the expected levels. However, in positions 5, 18, 22 and 25, a small amount of glutamate was detected, which we have noticed is a 'tell-tale' sign that is characteristic of the presence of a γ-carboxyglutamate residue. This suggested that four of the unassigned cycles were in fact γ-carboxyglutamate residues.

In order to identify the residue at position 3, the peptide was treated with AspN and the N-terminal heptapeptide fragment analyzed with electrospray ionization (ESI) mass spectrometry (MS). In the positive ESI mass spectrum, intense doublets were observed at m/z 1037.1 / 1039.1 and 1053.1 / 1055.1 (Fig. 2), while in the negative ESI mass spectrum, intense doublets were observed at m/z 1035.1 / 1037.1 (Fig. 2 inset) and 1051.1 / 1053.1 (data not shown). The observed mass in the positive ionization mode (m/z 1037.1) was consistent with the calculated monoisotpic mass (1037.29 Da) for the $[M+H]^+$ of the peptide H-Gly-Met-Trp-Gly-Gla-Cys*-Lys-OH, where Trp is 6-bromotryptophan and Cys* is pyridylethylcysteine. Furthermore, the mass spectra of the peptide had the characteristic doublet suggestive of the presence of bromine, indicating that there might be a 6-bromotryptophan residue in the N-terminal fragment. Thus the observed mass from the N-terminal fragment peptide fits with the assumption that the absence of PTHs at cycle positions 3 and 5 was due to the presence of 6-bromotryptophan and γ-carboxy-glutamate, respectively. The second set of doublets observed in both the positive (Fig. 2) and negative ionization mode are attributed to oxidation of the methionine residue to methionine sulfoxide. This evidence strongly suggests that within the first thirty amino acids of the sequence.

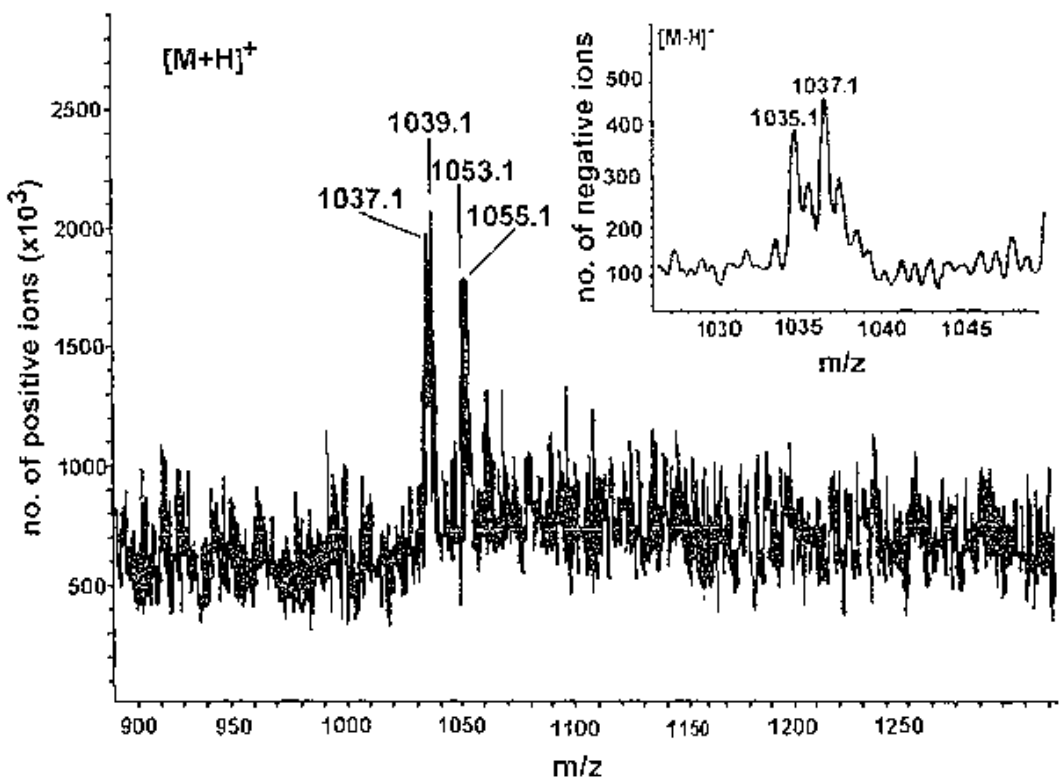

Figure 2. ESI mass spectrum, measured in the positive mode, of the N-terminal heptapeptide fragment of tx7a obtained by AspN-treatment. Inset shows the expanded region of the negative ESI mass spectrum.

the five unassigned residues were 6-bromotryptophan at position 3, and γ-carboxyglutamates at positions 5, 18, 22 and 25.

The ESI mass spectrum of the intact peptide contained fragments at m/z 1226.4, 1231.7, 1244.2, 1249.3, 1261.8, 1266.8, 1279.7 and 1284.9 (positive ionization mode) which we interpret as $[M+3H]^{3+}$, $[M_{ox}+3H]^{3+}$, $[M+Fe]^{3+}$, $[M_{ox}+Fe]^{3+}$, $[M+Fe-3H]^{3+}$, $[M_{ox}+Fe-3H]^{3+}$, $[M+2Fe-6H]^{3+}$ and $[M_{ox}+2Fe-6H]^{3+}$ (where M_{ox} indicates the methionine sulfoxide species; Fig. 3). The inset in Fig. 3 shows the resolved isotope distribution superimposed on the normal spectrum, the spacing between the isotopomers in each of the different species indicated the charge state (+3). In the negative ionization mode the spectrum contains intense species at m/z 1224.2, 1227.8, 1242.2, 1247.5, 1259.7, 1265.3, 1227.6 and 1282.6 which we interpret as $[M-3H]^{3-}$, $[M_{ox}-3H]^{3-}$, $[M+Fe-6H]^{3-}$, $[M_{ox}+Fe-6H]^{3-}$, $[M+2Fe-9H]^{3-}$, $[M_{ox}+2Fe-9H]^{3-}$, $[M+3Fe-12H]^{3-}$ and $[M_{ox}+3Fe-12H]^{3-}$. A close correspondence is observed between the species observed in the positive and negative ESI spectra (Fig. 4A and 4B). A mass difference of 6 Da (m/z 2 for triply-charged ions) is consistent with a difference of six protons between the positive and negative species, which supports the interpretation given above. The MS/MS spectrum measured in the negative ionization mode of the m/z 1242.2 species contained intense fragment ions at m/z 1182.7, 1197.2, 1211.8 and 1226.3 corresponding with the loss of one to four molecules of CO_2 (consistent with the presence of four γ-carboxyglutamate residues), but no other fragment ions (spectrum not shown).

However, the observed average mass (M) of the intact peptide of 3676.2 Da was greater than the mass predicted for the 30 AA sequence obtained by the Edman analysis (3412.45 Da). The mass difference (≈264 Da) between the observed ESIMS and the calculated mass for the thirty residues (including the Trp at position 3 and four Gla residues) was consistent with an additional residue of 6-bromotryptophan. This result suggested an additional Trp residue at the C-

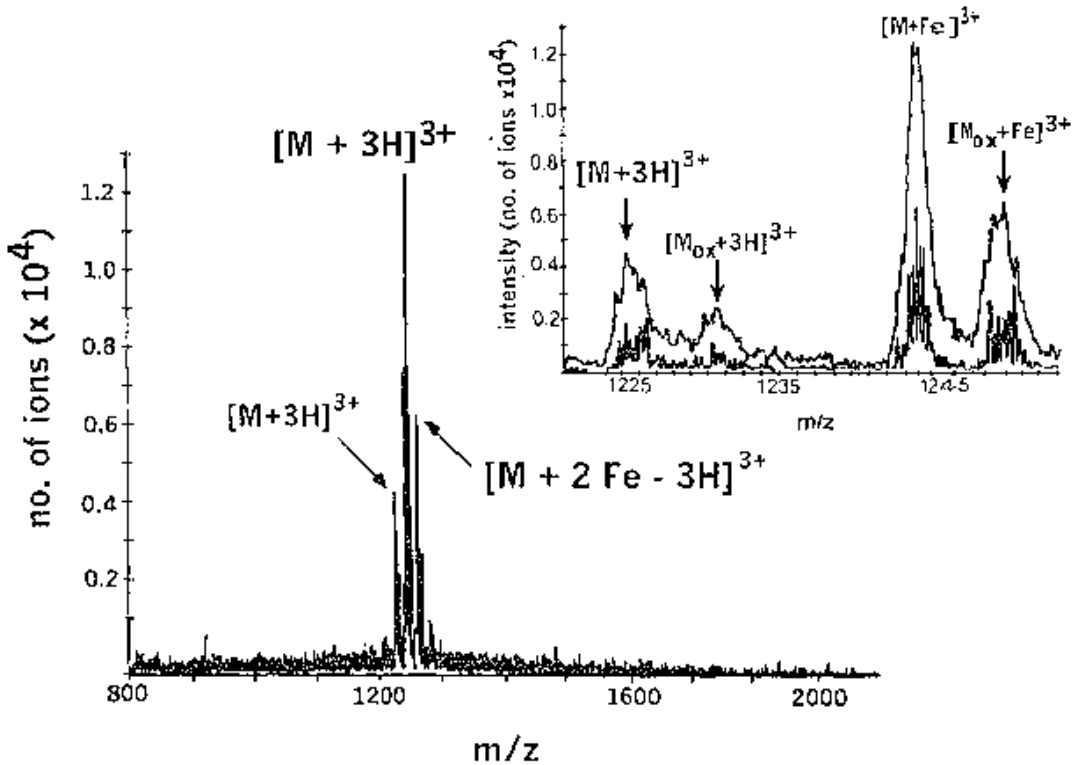

Figure 3. ESI mass spectrum, measured in the positive mode, of intact 1x7a. Inset shows an expanded range measured in both normal and maximum-resolution settings.

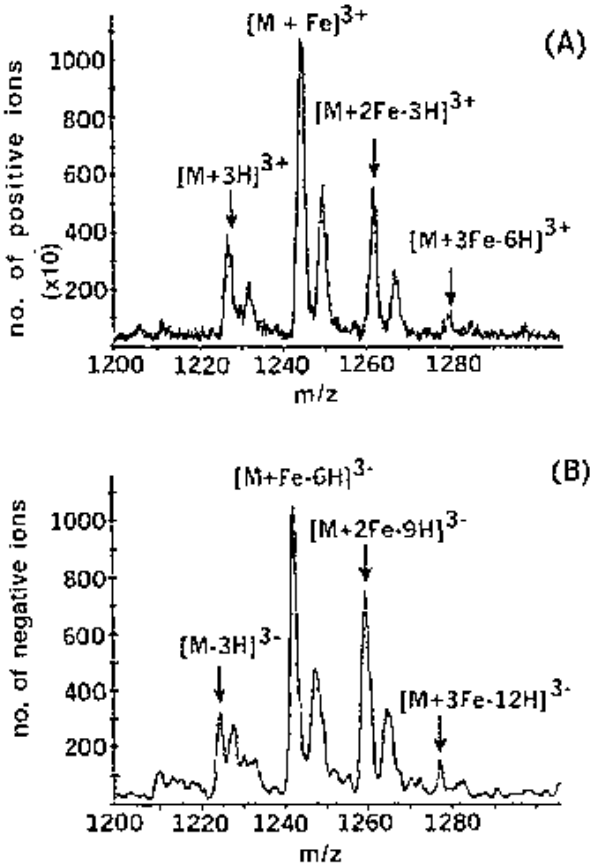

Figure 4. ESI mass spectra of intact tx7a measured in both the positive (A) and negative (B) ionization modes. The close correspondence between the species observed in A and B and the mass shift of 6 Da was used to assign the species.

terminal end (which would not have yielded an assignable residue under the conditions of Edman analysis utilized).

We have previously determined that the γ-carboxyglutamate-containing conotoxin tx5a can retain a single ferric cation and lose five protons to form a negatively-charged species (Walker et al. 1999; A.G. Craig, J.P. Spier, S. Rosamilia, V. Fursey, M.B. Lirazan, submitted to J. Amer. Soc. Mass Spectrom, in press). The close correspondence between the positive and negative ESI spectra and the mass difference of 6 Da between corresponding positive and negative species (m/z 2 for triply-charged ions) listed above strongly supports the assignment given and therefore the retention of three ferric cations by the molecule and the loss of up to 12 protons to form negatively charged species. Since only six acidic residues are present in this peptide (4 γ-carboxyglutamates and 2 aspartates) which could give up a maximum of 10 protons, protons must be removed from two of the six hydroxy amino acids present in the peptide. The MS/MS spectrum in which four molecules of CO_2 are lost without loss of a ferric cation and the absence of other high mass fragment ions in the MS/MS spectrum are consistent with our previous findings for the ferric-cationized conotoxin tx5a.

Cloning

A cDNA clone, Tx7.1, encoding the peptide was obtained using PCR as described under Methods; the amino acid sequence predicted by the cDNA clone is shown in Table I. Consistent with the postulate that there was a 6-bromotryptophan residue at position 3, a Trp codon was found. The sequence deduced from the clone was also consistent with γ-carboxyglutamate at positions 5, 18, 22 and 25, since Glu codons were found at these positions. Most importantly, the clone did predict a C-terminal tryptophan codon, consistent with 6-bromotryptophan as the C-terminal amino acid.

These results lead to the following sequence assignment for the peptide:

GMWGγCKDGLTTCLAOSγCCSγDCγGSCTMW
(where W = Trp and γ = Gla)

This is the first peptide known to contain more than one Trp residue. Injection of six mice at doses between 50 to 80 pmol/g of the purified peptide elicited continuous running for more than an hour in all of the animals. We refer to the peptide as the "dibromorunning peptide." In conformation with the nomenclature proposed by McIntosh et al. (McIntosh et al. 1999), the peptide is given the provisional technical designation tx7a, encoded by the cDNA clone Tx7.1.

Discussion

We characterized a 31-amino acid peptide with three disulfide bonds that causes a characteristic rapid-running hyperactivity when injected i.c. into mice. The presence of two residues of 6-bromotryptophan, and four residues of γ-carboxyglutamate in the peptide is especially noteworthy. Together, the cysteines involved in disulfide bonding, the γ-carboxyglutamate residues and the 6-bromotryptophan residues account for 12 of

the 31 amino acids in the peptide. Like the bromosleeper peptide, it has a disulfide pattern that superficially, at least, resembles that of the O-superfamily. However, additional cloning data (R. Shetty and C. Walker, unpublished results) indicate that this peptide is not a member of the O-superfamily, since its signal sequence differs from that of other O-superfamily members (even though the arrangement of cysteines appears to be homologous to the O-superfamily). Further characterization of the peptide and an identification of the role of γ-carboxyglutamate and 6-bromotryptophan residues awaits chemical synthesis and structure/function studies.

The finding that two unusual post-translational modifications, bromination of tryptophan and γ-carboxylation of glutamate residues occur together in three unrelated *Conus* peptides strongly suggests that the combination of these two post-translational modifications yields a functional motif important for the neuropharmacological activity of these peptides. As is typical of *Conus* peptides, their structures and symptoms elicited are very diverse. Thus, although the present peptide, tx7a, has a cysteine framework similar to that of the bromosleeper peptide previously characterized (rather than to that of conotoxin tx5a from the same venom, *Conus textile*), this peptide makes mice hyperactive when injected intracranially while as previously reported, the bromosleeper peptide induces a sleep-like state. Although both tx7a and tx5a elicit excitatory symptomatology, they appear to be completely unrelated structurally. Thus, the subset of conotoxins which have both 6-bromotryptophan and γ-carboxyglutamate are a diverse, highly potent and intriguing group of *Conus* venom peptides.

The only conopeptide with 6-bromotryptophan whose function is known is ω-conotoxin from *Conus geographus* which targets the $5HT_3$ receptor; it was postulated that the single residue of Trp might interact with the serotonin (5HT) binding site (England et al. 1998). In principle, one rationale for bromotryptophan in conopeptides is that all Trp-containing peptides could be targeted to 5HT binding sites on the diverse types of serotonin receptors. The discovery of a peptide with two Trp residues makes this explanation less likely, and suggests that the brominated residues have other mechanistic roles.

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