THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 8, Issue of February 21, pp. 4689–4698, 1997
Printed in U.S.A.

A Novel Post-translational Modification Involving Bromination of Tryptophan

IDENTIFICATION OF THE RESIDUE, L-6-BROMOTRYPTOPHAN, IN PEPTIDES FROM *Conus imperialis* AND *Conus radiatus* VENOM*

(Received for publication, October 1, 1996, and in revised form, December 9, 1996)

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We report a novel post-translational modification involving halogenation of tryptophan in peptides recovered from the venom of carnivorous marine cone snails (*Conus*). The residue, L-6-bromotryptophan, was identified in the sequence of a heptapeptide, isolated from *Conus imperialis*, a worm-hunting cone. This peptide does not elicit gross behavioral symptoms when injected centrally or peripherally in mice. L-6-Bromotryptophan was also identified in a 33-amino acid peptide from *Conus radiatus*; this peptide has been shown to induce a sleep-like state in mice of all ages and is referred to as bromosleeper peptide. The sequences of the two peptides

Pca-Cys-Gly-Gln-Ala-Trp*-Cys-NH_2

SEQUENCE 1

and

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1                5                   10                  15
Trp*-Ala-Thr-Ile-Asp-Gla-Cys-Gla-Gla-Thr-Cys-Asn-Val-Thr-Phe-
16               20                  25                  30
Lys -Thr-Cys-Cys-Gly-Hyp-Hyp-Gly-Asp-Trp-Gln-Cys-Val-Gla-Ala-
31      33
Cys -Pro-Val-OH
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SEQUENCE 2

were determined using a combination of mass spectrometry, amino acid, and chemical sequence analyses, where Pca = pyroglutamic acid, Hyp = hydroxyproline, Gla = γ-carboxyglutamate, and Trp* = L-6-bromotryptophan. The precise structure and stereochemistry of the modified residue were determined as L-6-bromotryptophan by synthesis, co-elution, and enzymatic hydrolysis experiments. To our knowledge this is the first documentation of tryptophan residues in peptides/proteins being modified in a eukaryotic system and the first report of halogenation of tryptophan *in vivo*.

Polypeptides encoded by genes are primarily made up of the 20 common amino acids that are directly translated using the genetic code. However, many of these amino acids can be further modified post-translationally to yield a set of additional amino acids that contribute to the function of the mature protein. Together the 20 primary amino acids and these "secondary" amino acids which are found in proteins comprise the set of proteinogenous amino acids.

These modified amino acids include amino acid conjugates where the side chain is linked to a glycosyl, phosphate, or sulfate group and amino acids such as 5-hydroxylysine, 4-hydroxyproline, and γ-carboxyglutamate. One notable set of modified amino acids are halogenated derivatives of tyrosine and histidine. Naturally occurring halogenated tyrosine and histidine residues have previously been identified from proteins (1–4). A particularly well documented example of *in vivo* post-translational halogenation of an amino acid residue in a protein is afforded by thyroglobulin (5). After iodination of several tyrosine residues, selective cleavages release the iodinated thyroid hormones thyroxine (3,5,3′,5′-tetraiodothyronine or T_4)[1]; 3,5,3′-triiodothyronine (T_3), and 3,3′-di-iodothyronine. The presence of free T_3 and T_4 has also been shown in protochordates, suggesting a primitive thyroid function exists in tunicates (2).

Of the 20 common amino acids, alanine, glycine, isoleucine, leucine, and valine, which lack side chain functional groups, have not been implicated in post-translational modifications (6). In 1907 it was proposed that hydroxytryptophan was a proteinogenous amino acid (7), but this proposal has not been verified (1), and chemical oxidation of tryptophan is likely to be responsible for the observed modification. More recently, Takai and Hayaishi (8) have described tryptophan side chain oxidases in *Pseudomonas*, while McIntire *et al.* (9) have described a redox co-factor which is covalently linked to 2 tryptophan residues in *Methylobacterium extorquens* and *Thiobacillus versutus*. In this report, we establish, using accepted criteria (10–

* This work was supported by National Institutes of Health Grant MH53631 and a Scientist Development Award for Clinicians K20 MH00929 (to J. M. M.), National Institutes of Health Grant GM 48677 (to B. M. O.), National Institutes of Health-National Center for Research Resources Shared Instrument Grant 1S10RR-8425 (to A. G. C.), a fellowship from Department of Science and Technology-ESEP, Philippines (to E. C. J.), and conducted in part by the Foundation for Medical Research (A. G. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: T_4, 3,5,3′,5′-tetraiodothyronine; T_3, 3,5,3′-tri-iodothyronine; Boc, *tert*-butoxycarbonyl; Cys(Cam), *S*-carboxyamidomethylcysteine; Cys(Mob), *S*-*p*-methoxybenzyl-L-cysteine; CZE, capillary zone electrophoresis; DCM dichloromethane; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; Gln(Xnt), N^{γ}-xanthyl-L-glutamine; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; LSI, liquid secondary ionization; MALD, matrix-assisted laser desorption; MS, mass spectrometry; NMP, *N*-methylpyrrolidone; TBTU, *O*-(benzotriazol-1-yl)*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TCEP, tris-(2-carboxyethyl)phosphine. Amino acids are indicated by the standard three letter abbreviations unless otherwise indicated.

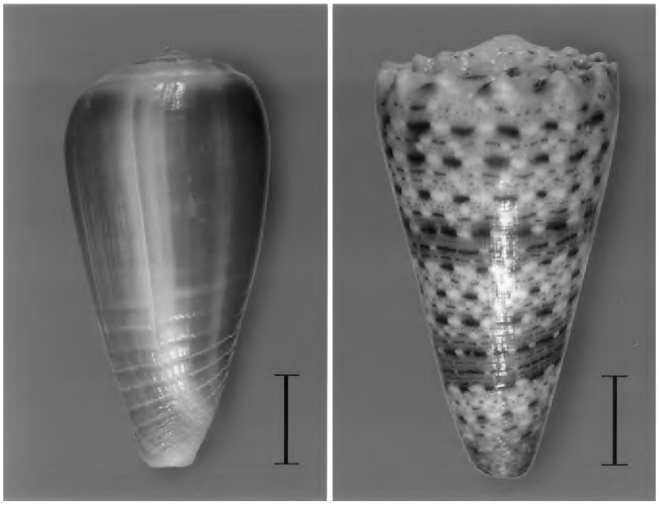

FIG. 1. **Shells of *C. imperialis* and *C. radiatus* collected in the Philippines.** The two brominated peptides described in this report were isolated from the venom duct of these species of carnivorous marine snails. *C. imperialis* hunts marine worms, whereas *C. radiatus* feeds on vertebrate fish. Reported shell lengths are 40–105 mm (*imperialis*) and 40–77 mm (*radiatus*).

12), that 6-bromotryptophan is a secondary amino acid and establish its presence in two peptides purified from the venoms of two *Conus* species, the imperial cone, *Conus imperialis*, and the radial cone, *Conus radiatus* (Fig. 1).

Most conotoxins are small, highly constrained and specialized polypeptides present in the venom produced by marine cone snails (*Conus*) (13). A distinctive characteristic of the conotoxins is the high percentage of cysteine residues mediating disulfide bridges in many of the peptides. The disulfide bridges are responsible for the highly constrained conformations of these toxins enabling the molecules to block a variety of different receptors with high binding affinity and selectivity. These peptides also have high levels of other modified amino acids including hydroxy proline and γ-carboxyglutamic acid (14). To this list we now add 6-bromotryptophan.

EXPERIMENTAL PROCEDURES

Reagents

The following reagents were used: DIC (Chem-Impex International, Wood Dale, IL); DCM (Fisher); Boc-Ala-OH, Boc-Cys(Mob)-OH, Boc-Gln(Xnt)-OH, Boc-Gly-OH, pGlu-OH, Boc-Thr(OBzl)-OH, and Boc-Ala-OH (Bachem, Torrance, CA); DL-6-bromotryptophan (Biosynth A.G., Staad, Switzerland); DL-5-bromotryptophan (synthesized as described elsewhere (15)); acetyl chloride (99+%), anisole, ascorbic acid, hexyl alcohol (98%), HOBt, iodine, and piperidine (Aldrich); DIEA (Fisher); TBTU and *N*-methylpyrrolidone (Advanced Chemtech, Louisville, KY); HF (Matheson Gas Products, Cucamonga, CA); trifluoroacetic acid (Halocarbon Products Corp., River Edge, NJ); ether, acetonitrile, and acetic acid (Mallincrodt Baker Inc., Paris, KY); methanol (Burdick and Jackson Division, Muskegon, MI); TCEP (synthesized by the method of Burns *et al.* (16)); iodoacetamide, EDTA, guanidine hydrochloride, Hepes, sodium carbonate, 4-vinyl pyridine, Tris-HCl, and Tris base (Sigma); β-mercaptoethanol (Pierce); α-chymotrypsin, aminopeptidase-M, carboxypeptidase-Y, endoproteinase Lys-C, endoproteinase Asp-N, and pyroglutamate aminopeptidase (Boehringer Mannheim).

Crude Venom

The crude venom was obtained from *C. imperialis* and *C. radiatus* by dissection of the venom duct gland and was stored at −70 °C.

Peptide Purification

Pooled venom was diluted with 0.1% aqueous trifluoroacetic acid and injected onto a reverse phase high performance liquid chromatography (HPLC) using a Vydak C_{18} column (10 × 250 mm, 5 μm particle size, Rainin Instruments, Woburn, MA) and eluted with a linear gradient of acetonitrile in 0.085% trifluoroacetic acid at 5 mL/min. The HPLC apparatus consisted of HPXL pumps and either a Dynamax model UVI or UV-DII detector (Rainin Instruments). Subsequent purification steps utilized a C_{18} Microsorb or C_{18} Vydac column, 4.6 × 250 mm, 5 μm particle size (Rainin Instruments). The effluents were monitored at 220 nm. Peaks were collected, and aliquots were assayed for activity.

Bioassays

Biological activity of both the heptapeptide and the bromosleeper peptide was assayed by intracranial and intraperitoneal injection in mice (9–22 days old) as described previously (17). Aliquots of peptide samples were lyophilized and suspended in normal saline solution, then injected into mice using a 0.3-ml syringe with a 29-gauge needle. Twenty-microliter volumes were injected intracranially, and 50-μl volumes were injected intraperitoneally. Each control mouse was injected with an equal volume of normal saline solution containing dissolved residue of lyophilized column buffer. After injections, the mice were placed in cages for observation.

Enzyme Hydrolysis

PAP Hydrolysis—The bromoheptapeptide was incubated in a 0.1 M Na_2CO_3 solution with pyroglutamate aminopeptidase (1:1, enzyme:substrate) at 37 °C for 17 h.

Endoproteinase Lys-C Hydrolysis—Fragments of the pyridylethylated sleeper peptide were generated by digestion with endoproteinase Lys-C. The peptide was incubated with the enzyme in a total volume of 50 μl (25 mM sodium bicarbonate buffer, pH 8.5, 10% acetonitrile) at 37 °C for 16 h. The reaction was stopped by addition of 25 μl of acetonitrile and 5 μl of 10% trifluoroacetic acid and the digest purified with HPLC.

Endoproteinase Asp-N Hydrolysis—Fragments of the pyridylethylated sleeper peptide were generated by digestion with endoproteinase Asp-N. The peptide was incubated with the enzyme in a total volume of 50 μl (25 mM potassium phosphate buffer, pH 8, 10% acetonitrile) at 37 °C for 19 h. The reaction was stopped by addition of 25 μl of acetonitrile and 5 μl of 10% trifluoroacetic acid and the digest purified with HPLC.

Aminopeptidase-M Hydrolysis—The synthetic 1–4 bromosleeper N-terminal fragment (100 pmol) was incubated in 100 μl of 100 mM Hepes solution with 20 milliunits of aminopeptidase-M at 24 °C and the reaction monitored with HPLC at 24-h and 4-day time points.

Chemical Modifications

Esterification—Esterification of the N-terminal (1–4) fragment of the bromosleeper peptide with acidic hexanol, prepared from acetyl chloride and hexyl alcohol, was carried out as outlined elsewhere (18).

Reduction and Alkylation—The bromoheptapeptide was reduced with 10 mM TCEP in 200 mM sodium acetate buffer, pH 5, and alkylated with iodoacetamide in 30 mM Tris base, pH 10, containing 2 mM EDTA at room temperature as described elsewhere (19). The bromosleeper peptide was reduced and alkylated by either of two methods. In method 1, the peptide was dissolved in 250 mM Tris-HCl, 6 M guanidine hydrochloride, and 2 mM EDTA, pH 7.5, reduced with a 10% solution of β-mercaptoethanol in deionized H_2O at room temperature and alkylated in the dark with a 20% solution of 4-vinyl pyridine in ethanol. With method 2 the peptide was reduced with TCEP and alkylated with 4-vinyl pyridine as described previously (20).

Chemical Sequence and Amino Acid Analyses

Automated chemical sequence analysis was performed on a 477A Protein Sequencer (Applied Biosystems, Foster City, CA). Amino acid analysis was carried out using pre-column derivatization. Approximately 500 pmol of the bromoheptapeptide was sealed under vacuum with conc. HCl, hydrolyzed at 110 °C for 24 h, lyophilized and then derivatized with *o*-phthalaldehyde. The derivatized amino acids were then analyzed with HPLC.

Mass Spectrometry

Matrix-assisted laser desorption (MALD) (21) mass spectra were measured using a "Bruker REFLEX" (Bruker Instruments Inc., Bremen, Germany) time-of-flight (22) mass spectrometer fitted with a gridless reflectron and a N_2 laser and a 100-MHz digitizer. An accelerating voltage of +31 kV and a reflector voltage of between 1.16 and 30 kV were employed for the post source decay FAST (23) measurements. The sample (in 0.1% aqueous trifluoroacetic acid) was applied with α-cyano-4-hydroxycinnamic acid. Liquid secondary ionization (LSI) (24) mass spectra were measured using a Jeol HX110 (Jeol, Tokyo, Japan) double focusing mass spectrometer operated at 10-kV accelerating volt-

age. The sample (in 0.1% aqueous trifluoroacetic acid and 25% acetonitrile) was mixed in a glycerol, 3-nitrobenzyl alcohol matrix (1:1) and analyzed with either magnetic or electric field scans. The spectra were calibrated using $[Cs(CsI)_n]+$ cluster ions. The LSIMS spectra were measured at nominal resolutions of either 1000 or 3000. Electrospray mass spectra were measured using an LCQ (Finnigan MAT, San Jose, CA) ion trap mass spectrometer. The samples (in 0.1% aqueous trifluoroacetic acid) were analyzed by direct infusion with (bromoheptapeptide) or without (N-terminal 1–4 fragment of the bromosleeper peptide) the addition of 20% acetic acid (6:1) solution. The mass accuracy was typically better than: 1000 ppm for the time-of-flight instrument in the linear mode, 200 ppm for the time-of-flight instrument in the reflectron mode and the ion trap instrument, and 50 ppm for the double focusing mass spectrometer. We note that when analyzing Gla-containing peptides with time-of-flight mass spectrometers, decarboxylation can significantly reduce the mass accuracy below these levels (25).

Synthesis of the Bromoheptapeptide

The synthesis was carried out manually on a 4-methylbenzhydrylamine resin using DIC as coupling reagent with the Boc protection strategy in DCM with the exception of the introduction of the Fmoc (9-fluorenylmethoxycarbonyl)-DL-6-bromotryptophan or Fmoc-DL-5-bromotryptophan. The 4-methylbenzhydrylamine resin was prepared at The Salk Institute, with a substitution of 0.5 mmol/g. The DL-6-bromotryptophan and DL-5-bromotryptophan were derivatized to Fmoc-DL-6-bromotryptophan and Fmoc-DL-5-bromotryptophan, respectively. In the case of the Fmoc-DL-6-bromotryptophan-mediated coupling, HOBt/DIEA was used with TBTU in *N*-methylpyrrolidone (NMP) followed by the deprotection of the Fmoc group with 20% piperidine in NMP. Fourfold excess of amino acid derivatives were used in the coupling reactions with the exception of the DL-6-bromotryptophan where a 10% excess was used. The efficiency of the coupling reactions was checked using the Kaiser-ninhydrin test. The dried peptide-resin (1.28 g) was treated with 15 ml of HF in the presence of 1.5 ml of anisole at 0 °C for 1.5 h. After the evaporation of HF, a first extraction was carried out with ether (3 × 100 ml) and a second extraction with 0.1% aqueous trifluoroacetic acid and 60% acetonitrile (3 × 50 ml). Purification of the crude reduced product on analytical HPLC indicated two major components whose measured mass (MALD-MS) were consistent with the calculated peptide mass. The extract of the crude peptide (approximately 150 ml) was diluted with 500 ml of acetic acid, mixed vigorously, and titrated with iodine in methanol (1%) until the color of the solution remained amber for 10 min. The excess of iodine was eliminated with a 5% ascorbic acid solution. The volume was reduced by evaporation to 70 ml and the residue diluted with 430 ml of triethylamine phosphate, pH 2.25, buffer. Acetonitrile was added (approximately 20 ml) until the opalescent liquid turned clear.

The crude bromoheptapeptide solution was clarified by the addition of acetonitrile (50 ml) and loaded on a 45 × 320-mm column packed with Vydac C_{18} 15–20-μm particles and eluted with a Waters PrepLC/System 500A equipped with gradient controller, Waters model 450 variable wavelength detector and Waters 1000 PrepPack cartridge chamber in 0.1% aqueous trifluoroacetic acid, using a gradient of 60% acetonitrile in 0.1% aqueous trifluoroacetic acid. Fractions (50 ml) were collected manually. Two components were isolated, one of them at approximately 16% acetonitrile, the other at approximately 22% acetonitrile. The collected fractions of the first component (1300 ml) were diluted with the same volume of 0.1% aqueous trifluoroacetic acid and reloaded onto the equilibrated preparative HPLC column. One major component was collected at approximately 22% acetonitrile and lyophilized (34.8 mg, purity based on HPLC 99%, based on CZE 97%). The collected fractions of the second component (700 ml) were purified as above. The major component was collected at approximately 26% acetonitrile and lyophilized (28.9 mg, purity based on HPLC 99%, based on CZE 97%). LSI-MS measurement of both components resulted in an intense species at m/z 852.2 consistent with the calculated monoisotopic $[M + H]^+$ mass of 852.17 Da.

Synthesis of Bromosleeper N-terminal (1–4) Fragment

The synthesis was initiated on Boc-Ile-CM-resin (300 mg), prepared at The Salk Institute (1% cross-link, 200–400 mesh, substitution 0.71 mmol/g). The coupling reactions were carried out manually using the Boc/trifluoroacetic acid protocol with DIC in the case of coupling Boc-Thr(OBzl)-OH and Boc-Ala-OH (3 equivalents) in DCM. The N-terminal residue DL-6-bromotryptophan was introduced as the N-Fmoc-DL-6-bromotryptophan derivative (2 equivalents). The coupling was mediated with DIEA/HOBt, and TBTU was used as a condensing agent in NMP. Each coupling reaction was completed within 2 h and checked using the Kaiser-ninhydrin test. The N-terminal Fmoc group was removed with 20% piperidine/NMP in 2 × 10 min. The weight of the washed and dried resin was 390 mg (weight gain 90 mg). The dried deprotected peptide-resin (390 mg) was treated with 4 ml of HF in the presence of 0.4 ml of anisole at 0 °C for 1.5 h. After the evaporation of HF, a first extraction was carried out with ether 3 × 25 ml) and a second extraction with 0.1% trifluoroacetic acid and 60% acetonitrile (3 × 15 ml) in water. The fingerprint of the crude product on analytical HPLC indicated two major components (diastereomer of the L- or D-6-bromotryptophan tetrapeptide) with observed masses (MALDI-MS) which were consistent with the calculated peptide mass.

The purification of bromosleeper N-terminal (1–4) fragment involved evaporation of 25% of the aqueous extract (rotavap 38 °C water bath). The solid residue was redissolved in 3.0 ml of 0.1% aqueous trifluoroacetic acid, 20% acetonitrile buffer and loaded in 0.5 ml portions to a semipreparative column. The fractions were collected manually and checked by analytical HPLC. The first pooled and lyophilized fractions resulted in an 8.8-mg peptide that was 99.9% pure by CZE, and the second fraction resulted in a 7.8-mg pure optical isomer tetrapeptide with 98% purity by CZE. LSI-MS measurement of both fractions resulted in an intense species at m/z 568.2, which compared closely with calculated $[M + H]^+$ monoisotopic mass of 568.18 Da.

Peptide Characterization

Co-elution of Natural and Synthetic Bromoheptapeptide—Reverse phase HPLC co-elution experiments of natural and synthetic bromoheptapeptide were carried out with a Vydac C_{18} (5 μm, 300 Å particle size) 2.1 × 150-mm column at a flow rate of 200 μl/min. Volatile buffers (0.055% aqueous trifluoroacetic acid and acetonitrile) were used and the effluent was monitored at 210, 254, and 280 nm with a HP1090 (Hewlett-Packard, Wilmington, DE).

Determination of L- Versus D-6-Bromotryptophan-containing Bromoheptapeptide—The two purified peptides (corresponding with either the D- or L-isomers of 6-bromotryptophan) were reduced and alkylated. The HPLC-purified reduced and alkylated peptides were lyophilized and redissolved in 250 mM Tris base, pH 8.5, and incubated with α-chymotrypsin (1:40, 1:10, and 1:5; enzyme:substrate) for up to 9 days. The progress of the reaction was monitored with HPLC and MALD-MS.

Co-elution of Natural and Synthetic Bromosleeper Peptide N-terminal (1–4) Fragment—Approximately equal amounts of the endoproteinase Asp-N-digested N-terminal fragment of the bromosleeper peptide and the synthetic peptide Trp*-Ala-Thr-Ile-OH were mixed (where Trp* = bromotryptophan). The mixture was applied onto a C_{18} analytical column and eluted at 1 ml/min using a gradient of acetonitrile in 0.085% trifluoroacetic acid. A similar amount of the synthetic peptide was analyzed on HPLC under the same conditions.

Determination of L- Versus D-6-Bromotryptophan in the Bromosleeper Peptide N-terminal (1–4) Fragment—An aliquot (10 μl) of each synthetic peptide (1 mM) was lyophilized, redissolved in 100 mM Hepes, pH 7.5, and incubated with aminopeptidase-M (1:40; enzyme:substrate) for up to 4 days. The progress of the reaction was monitored with HPLC. The product of the aminopeptidase-M reaction was co-injected with the racemic mixture of 6-bromotryptophan or with the racemic mixture of 5-bromotryptophan.

RESULTS

Bromoheptapeptide—Two peptides containing L-6-bromotryptophan were isolated from the venom of predatory *Conus* species. The first was a bromoheptapeptide from *C. imperialis*, purified from a side fraction isolated during the purification of another unrelated peptide α-conotoxin IMI (26) (see Fig. 2). Our interest in this sample was initially stimulated after scanning fractions present in the crude venom for novel peptides based on the intact mass (27), rather than biological activity. MALD-MS analysis of the purified fraction indicated species at m/z 853, 855, 875, and 879 (data not shown). In order to obtain higher mass resolution without sacrificing sensitivity, this sample was analyzed with electrospray ionization and an ion trap mass spectrometer (see Fig. 3*A*). Several relatively intense species were observed with 2-Da separation at m/z 835.9 and 837.9, m/z 853.1 and 855.1, and m/z 875.2 and 877.2, which are referred to as doublets. These doublets could be clearly resolved using a zoom scan (*e.g.* m/z 853.1 and 855.1; see Fig.

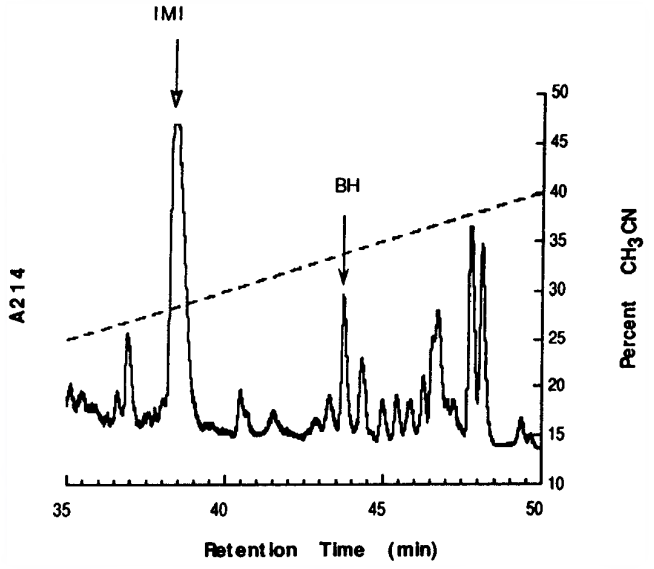

FIG. 2. **Purification of the bromoheptapeptide.** Crude venom extract was applied on a C_{18} analytical column and eluted at 1 ml/min with a gradient of 1.0% CH_3CN/min. The *solid arrow* labeled *BH* indicates the fraction containing the bromoheptapeptide, which was subsequently re-applied and eluted at 0.22% CH_3CN/min. The *open arrow* labeled *IMI* indicates the retention time of α-conotoxin IMI previously reported to be present in *C. imperialis* venom (26).

3*A*, *inset*). The separation of approximately 22 Da between *m/z* 853.1 and 875.2 (and also between *m/z* 855.1 and 877.2) suggested competitive $[M + H]^+$ and $[M + Na]^+$ formation (M = 852). Accordingly, the *m/z* 835.9 and 837.9 doublets were attributed to loss of NH_3 from the protonated molecule ion. The presence of the doublets was characteristic of a molecule containing a single ^{79}Br or ^{81}Br atom. This pattern of isotopomers is quite distinctive, since these isotopes are separated by 2 Da and have approximately equal natural abundance, and this characteristic doublet was observed from both the synthetic 5- and 6-bromotryptophan amino acids (data not shown). Accurate mass analysis using LSI on a magnetic sector instrument was used to determine masses of *m/z* 853.19 and 855.20 for the $[M + H]^+$ doublets (data not shown). It was deduced that the peptide was N-terminally blocked with pyroglutamic acid from initial chemical sequencing measurements and the shift in chromatographic retention time following treatment with pyroglutamate aminopeptidase. Automated Edman degradation analysis after pyroglutamate aminopeptidase treatment resulted in the sequence: Cys-Gly-Gln-Ala-*X*-Cys, where *X* indicates a blank cycle. Amino acid analysis confirmed the presence of 1 mole ratio of glycine and alanine to 2 mole ratios of glutamate and cysteine. A major component of the hydrolysate eluted 0.7 min after the expected retention time of derivatized tryptophan, but 2.2 min before the expected retention time of derivatized arginine. After reduction and alkylation of the sample with iodoacetamide (to form the Cys(Cam) derivative which has a residue mass of 160 Da) MALD-MS analysis revealed two sets of doublets at *m/z* 991 and 993 and *m/z* 1007 and 1009 (see Fig. 3*B*) separated by 16 Da. We interpreted these species as the $[M' + Na]^+$ and $[M' + K]^+$ of the reduced and alkylated peptide (M′ = 968 Da). The shift in mass after reduction and alkylation (M′ − M = 968−852 = 116 Da) was consistent with the presence of two cysteine residues. Other species observed in Fig. 3*B* were interpreted as fragment ions (*e.g.* *m/z* 788 corresponds with the post source decay b_6 fragment ion).[2] The post-

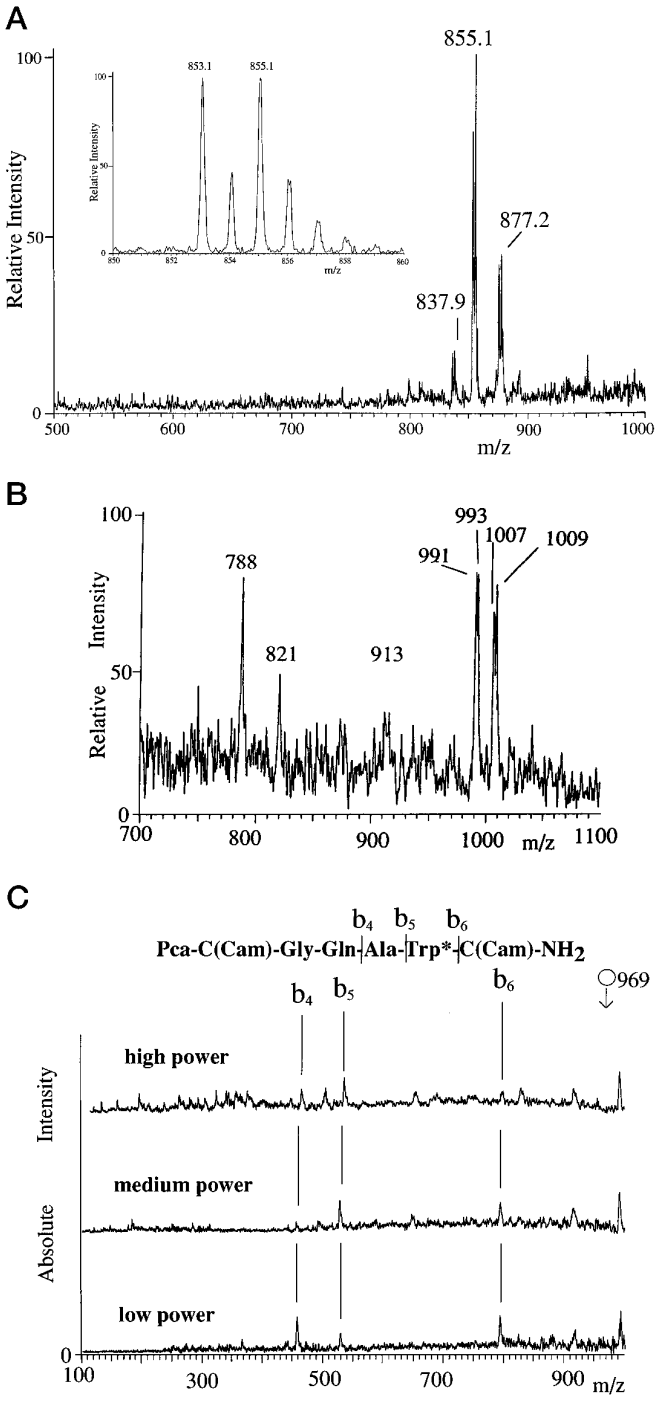

FIG. 3. *A*, electrospray ionization MS of natural bromoheptapeptide between 500 and 1000 Da (*inset* shows the zoom scan between 850 and 860 Da) showing a doublet of peaks at *m/z* 853.1 and 855.1 (*cf.* calculated monoisotopic $[M + H]^+$ of 853.18 Da for the sequence ZCGQAW*C-NH_2, where Z = pyroglutamic acid and W* = bromotryptophan); *B*, MALD-MS of natural reduced and alkylated bromoheptapeptide between 700 and 1100 Da; *C*, MALD post-source delay-MS of reduced and alkylated bromoheptapeptide between 100 and 1000 Da measured at low, medium, and high laser power fluences.

source decay FAST spectrum of the reduced and alkylated product, measured at low, medium, and high laser power fluences, are shown in Fig. 3*C*. In Fig. 3*C*, the position of the

[2] In Fig. 3*B*, the time-of-flight of the b_6 fragment ion and therefore the observed *m/z* 788 is slightly lower than the calculated mass (792.2 Da), because this ion has undergone metastable decomposition after exiting the source. In Fig. 3*C*, the FAST spectra are calibrated to compensate for the reduced energy of fragment ions, and this procedure results in a more accurate mass measurement (*m/z* 793).

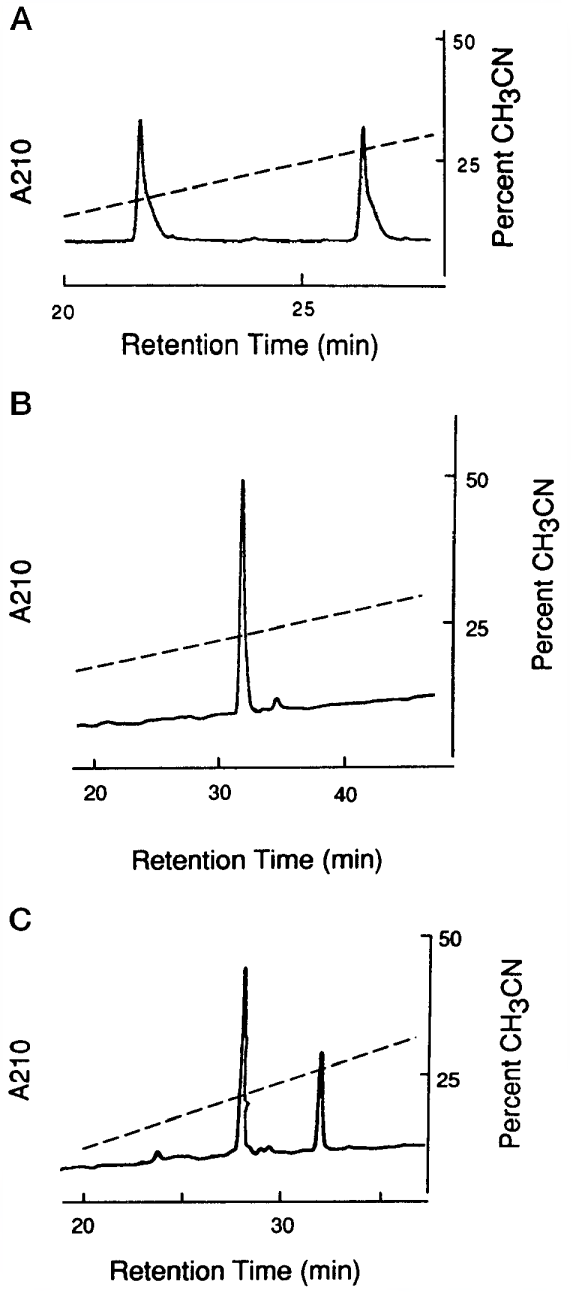

FIG. 4. **HPLC chromatogram of synthetic bromoheptapeptide when purified on C_{18} reverse phase showing a hydrophilic and a hydrophobic component (*A*), co-elution (C_{18} reverse phase) of hydrophilic component of synthetic bromoheptapeptide and natural bromoheptapeptide (*B*), and purification (C_{18} reverse phase) of the products formed from the incubation of the hydrophilic component of the synthetic bromoheptapeptide with α-chymotrypsin (*C*).**

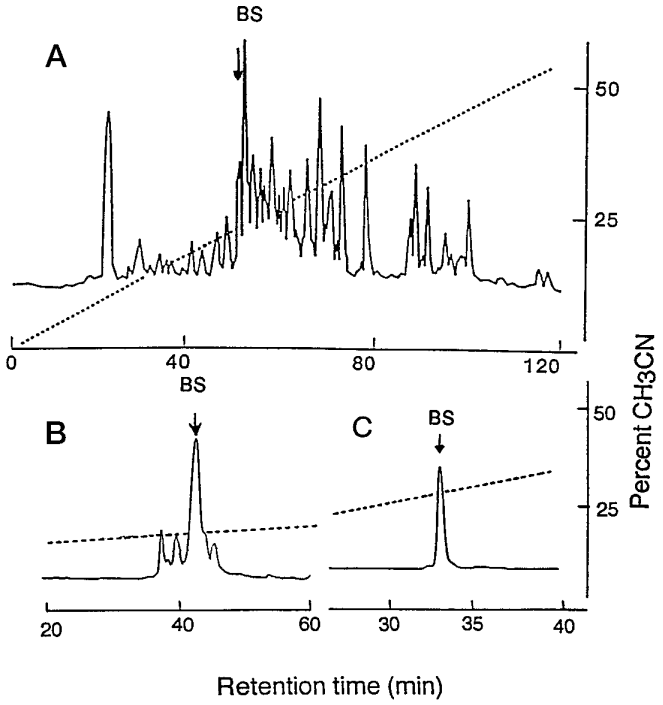

FIG. 5. **Purification of the bromosleeper peptide.** *A*, crude venom extract was applied on a C_{18} semipreparative column and eluted at 5 ml/min with a gradient of 0.45% CH_3CN/min. *B*, the bioactive fraction denoted "*BS*" was applied onto a C_{18} analytical column and eluted at 1 ml/min using a gradient of 0.18% CH_3CN/min. *C*, the peak indicated by the *arrow* in *B* was repurified at 1 ml/min with a gradient of 0.9% CH_3CN/min to obtain the bromosleeper peptide.

monoisotopic $[M' + H]^+ = 969$ is indicated, although only the cationized intact ions were observed. Three fragment ions, which were observed above the background noise level in the low power spectrum at m/z 793.1, 528.1, and 456.6, were also observed at varying intensities at medium and high laser power. Based on the mass differences between the parent and the successive fragments (176.9, 265.0, and 71.5 Da), we interpreted these ions as corresponding to N-terminal fragments differing in mass by Cys(Cam), *X*, and Ala where *X* = 265 Da. We conclude that these three fragment ions (b_6, b_5, and b_4) encompass the blank observed in the chemical sequence data. Based on these results we deduced that the 6th amino acid, was hydrophobic, absorbed intensely at 280 nm, and had a mass of 265 Da. We proposed the sequence: Pca-Cys-Gly-Gln-Ala-Trp*-Cys-NH_2 (where Pca = pyroglutamic acid and Trp* = bromotryptophan). The calculated $[M + H]^+$ for the monoisotopic mass of the bromoheptapeptide (853.176 Da) was consistent with the measured m/z of 853.19 (see Fig. 3*A*).

Assignment of bromotryptophan was confirmed by total chemical synthesis of the bromoheptapeptide. The synthesis of bromoheptapeptide using a racemic mixture of the D and L forms of 6-bromotryptophan resulted in two diastereomeric peptides, one containing the D configuration 6-bromotryptophan at position 6, while the other containing the L configuration 6-bromotryptophan at position 6. HPLC purification of the crude synthesis product indicated the presence of a hydrophobic and a hydrophilic species (see Fig. 4*A*), both of which contained the characteristic doublet at m/z 853.2 and 855.2 consistent with the native bromoheptapeptide based on LSI-MS analysis (data not shown). The separation of the peptides containing a D *versus* an L amino acid by reverse phase HPLC exploits structural differences that attend chiral inversion at the Cα atom. The hydrophilic isomer co-eluted with the natural peptide on HPLC (see Fig. 4*B*). The susceptibility of the modified tryptophan residue toward enzymatic hydrolysis after reduction and alkylation of the cysteine residues was determined by incubation of the synthetic peptide with α-chymotrypsin. The hydrophilic isomer was observed to hydrolyze when incubated with a 1:10 enzyme to substrate ratio within 2 days (see Fig. 4*C*), whereas the hydrophobic isomer underwent no hydrolysis at this enzyme to substrate ratio when incubated for 9 days (data not shown). The intact mass and isotopomer distribution for the chymotrypsin-hydrolyzed fragment analyzed with LSI-MS corresponded with the calculated mass for the reduced and alkylated 1–6 bromoheptapeptide fragment (Pca-Cys(Cam)-Gly-Gln-Ala-Trp*-OH; calculated monoisotopic mass for the $[M + H]^+$ is 810.19 Da, observed m/z 810.2

TABLE I
Biological effects of the bromosleeper peptide

Varied doses of purified peptide dissolved in normal saline were injected intracranially into mice. Two to three mice were used per dose. Note that the bromoheptapeptide is not active in this assay.

Dose	Observed effects		
	9-Day-old mice	17-Day-old mice	22-Day-old mice
pmol/g body weight			
10–15	Slept for ~1 h	—[a]	—
40–50	Slept for >10 h	Lethargic/drowsy after ~15 min	Lethargic/drowsy after ~19 min
70	Slept for >24 h	Lethargic/drowsy after ~11 min, slept for ~4 h	—
100	—	—[a]	Lethargic/drowsy after ~12 min, slept for ~3 h

[a] —, not tested.

(data not shown)).

Although bromine is typically incorporated at position 6 in marine products, in order to unambiguously resolve the question of the stereochemistry of this modified amino acid, we synthesized the bromoheptapeptide with the racemic mixture of D- and L-5-bromotryptophan. This peptide did not co-elute on HPLC with the natural bromoheptapeptide (data not shown).

Insufficient native bromoheptapeptide was available for testing biological activity. Synthetic bromoheptapeptide was assayed for biological activity by injecting 20 nmol of peptide either intracranially or intraperitoneally into mice. No gross behavioral changes in activity were observed, and this peptide was not further tested for biological activity.

Bromosleeper Peptide—Concurrent with the investigation of the bromoheptapeptide we also characterized a novel "sleeper" peptide, which induces a sleep-like state in mice; this peptide was purified from *C. radiatus* venom (*peak BS* of Fig. 5*A*). In the subsequent purification steps using an analytical HPLC column, the sleeper activity coincided with the major peaks shown in Fig. 5, *B* and *C*.

A family of peptides which elicit a sleep-like state in mice, the conantokins, have been described previously from *Conus* venoms. The peptide isolated from *C. radiatus* was very different from the conantokins with respect to activity profile; whereas the conantokins cause sleep when injected intracranially in very young mice (<2 weeks old) and hyperactivity in older mice (>3 weeks old), the new peptide induced sleep in mice of both age groups. A summary of the *in vivo* effect of the purified peptide is shown in Table I. It is obviously more potent in 9-day-old mice than in the older mice; the effective dose is lower, the onset time is shorter, and the duration of sleep is longer in younger mice.

MALD-MS analysis of the purified fraction measured in the linear mode indicated a broad species at *m/z* 3884 in contrast to the measurement in the reflectron mode of *m/z* 3729. LSI-MS analysis of the same sample indicated a species at *m/z* 3916.4 (data not shown). These results suggested that the peptide contained a number of Gla residues (25). Chemical sequencing after reduction and pyridylethylation of the cysteine residues gave no regular phenylthiohydantoid derivative of an amino acid at cycle one but an intense signal for alanine at cycle two followed by normal repetitive recovery. Weak signals were also observed at positions 6, 8, and 9. Endoproteinase Asp-N hydrolysis of the bromosleeper peptide generated three major fractions, which when submitted to chemical sequence analysis revealed the following sequences: (i) *X*-Ala-Thr-Ile; (ii) Asp-*X*-Cys-*X*-*X*-Thr-Cys-Asn-Val-Thr-Phe-Lys-Thr-Cys-Cys-Gly-Hyp-Hyp-Gly, and (iii) Asp-Trp-Gln-Cys-Val-*X*-Ala-Cys-Pro-Val; where *X* indicates a blank cycle. Endoproteinase Lys-C hydrolysis generated two fragments that after mass spectrometry and chemical sequence analysis could be used to deduce the connectivity of the three Asp-N fragments as (i) 1–4, (ii) 5–23, and (iii) 24–33. Based on mass spectrometric analysis of the Asp-N fragments 5–23 and 24–33, the weak

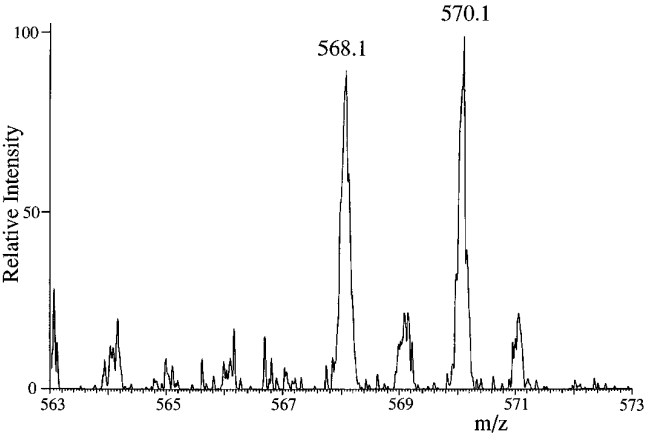

FIG. 6. **Electrospray ionization MS zoom scan of the N-terminal 1–4 fragment of the natural bromosleeper peptide between 563 and 573 Da showing a doublet of peaks at *m/z* 568.1 and 570.1 (*cf.* calculated monoisotopic [M + H]$^+$ of 568.177 Da for the sequence W*ATI, where W* = bromotryptophan).**

signals at 6, 8, 9, and 29 were interpreted as Gla residues. The accurate mass of the 24–33 fragment indicated that the C terminus of the peptide was in the free acid form (data not shown). We noted a strong UV absorption at 280 nm for both the intact peptide and the N-terminal fragment characteristic of an indole group. Although it was reasonable to expect such absorption from the intact peptide that contains tryptophan, in the case of the 1–4 fragment this adsorption was attributed to the residue in position 1. Electrospray ionization ion trap mass spectral analysis of the N-terminal 1–4 fragment revealed a weak (but constant) signal which when accumulated over several minutes resulted in a doublet of peaks at *m/z* 568.1 and 570.1; the zoom scan of which is shown in Fig. 6. LSI-MS analysis of the N-terminal 1–4 fragment (after esterification with hexanol to optimize the sensitivity of LSI analysis; resulting in addition of 84 Da) also resulted in a weak signal where a doublet at *m/z* 652.27 and 654.26 could be observed which was not present when the matrix alone was analyzed (data not shown). We noted the similarity between the isotopomer intensities of the doublet observed in Fig. 6 and that observed for the bromoheptapeptide (see Fig. 3*A*, *inset*) and the synthetic bromotryptophan amino acids. The properties of the modified amino acid were consistent with the presence of bromotryptophan; *e.g.* the observed *m/z* of 652.27 was consistent with the calculated monoisotopic mass for the [M + H]$^+$ of the 1–4 fragment Trp*-A-T-I-C_6H_{12} (652.271 Da). We therefore proposed the sequence Trp*-Ala-Thr-Ile-Asp-Gla-Cys-Gla-Gla-Thr-Cys-Asn-Val-Thr-Phe-Lys-Thr-Cys-Cys-Gly-Hyp-Hyp-Gly-Asp-Trp-Gln-Cys-Val-Gla-Ala-Cys-Pro-Val-OH (where Hyp = hydroxyproline, Gla = γ-carboxy glutamate, and Trp* = bromotryptophan).

After synthesis of the bromosleeper peptide 1–4 N-terminal fragment with a racemic mixture of the D and L forms of

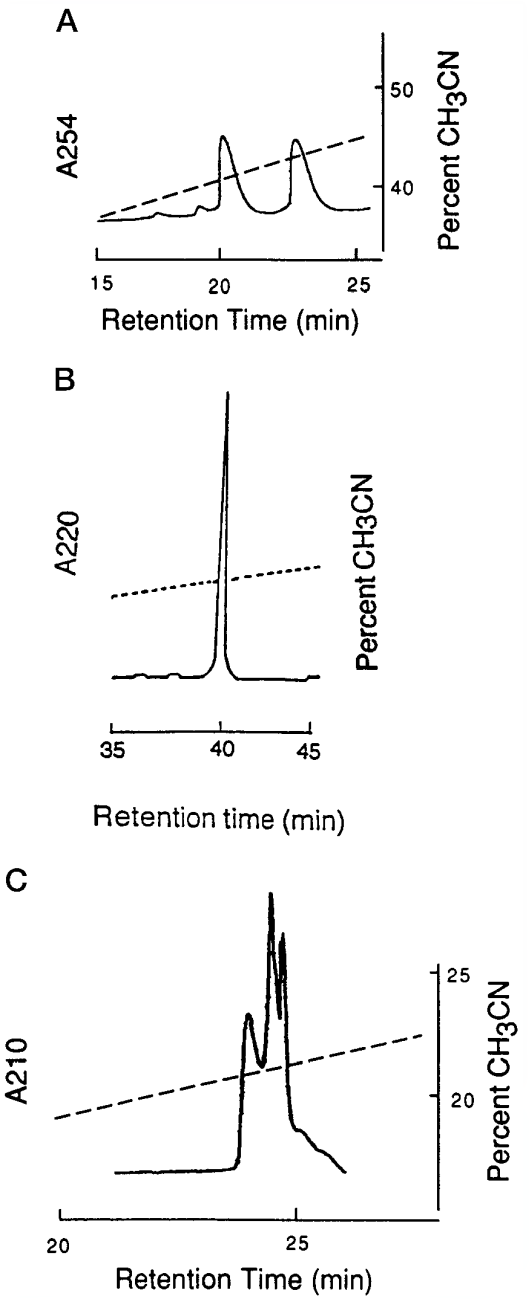

FIG. 7. **HPLC chromatogram (C_{18} reverse phase) of synthetic bromosleeper N-terminal 1–4 fragment containing a racemic mixture of the D and L forms of 6-bromotryptophan showing hydrophilic and hydrophobic components (*A*), co-elution (C_{18} reverse phase) of hydrophilic component of synthetic and natural N-terminal fragment (*B*), and purification (C_{18} reverse phase) of the products formed from the incubation of the hydrophilic component of the synthetic N-terminal fragment with aminopeptidase M (*C*).**

6-bromotryptophan, a hydrophobic and a hydrophilic peptide isomer were purified (see Fig. 7*A*). LSI-MS analysis of both the hydrophobic and the hydrophilic peptide isomer resulted in mass spectra consistent with that of the native 1–4 bromosleeper fragment (data not shown). The hydrophilic isomer was found to co-elute with the natural peptide on HPLC (see Fig. 7*B*). The susceptibility of the bromotryptophan residue toward enzymatic hydrolysis was determined by incubation of the synthetic peptide with aminopeptidase-M. The hydrophilic isomer was observed to hydrolyze within 24 h (see Fig. 7*C*), whereas the hydrophobic isomer underwent no hydrolysis under the same conditions or when incubated for 4 days. The bromotryptophan amino acid released from the hydrophilic isomer co-eluted on HPLC with the racemic mixture of 6-bromotryptophan but did not co-elute with the racemic mixture of 5-bromotryptophan (data not shown).

DISCUSSION

We report the purification of two peptides from venoms of two species of cone snails that are divergent from each other, both in their morphology and the feeding biology. The two peptides from these disparate venoms are not homologous to each other, but unexpectedly, we have found that they share one novel biochemical feature, the presence of L-6-bromotryptophan.

The first peptide characterized was a heptapeptide isolated from the venom of the imperial cone, *C. imperialis*, which hunts polychaete worms. The bromoheptapeptide exhibited no gross peripheral or central symptoms when injected into mice; its biological activity in marine annelid systems has yet to be assessed. The bromoheptapeptide incorporates three post-translational modifications other than bromotryptophan (pyroglutamic acid, disulfide bridge, and amidated C terminus). We know of only a few other peptides that contain pyroglutamic acid, a disulfide bridge, and an amidated C terminus (*e.g.* tunicate gonadotropin releasing hormone II from *Cheylosoma productum* (28)), emphasizing the highly modified nature of the bromoheptapeptide.

In contrast to the worm-hunting *C. imperialis*, the radial cone, *C. radiatus,* is a deep water Indo-Pacific fish-hunting cone snail. The 33-residue bromosleeper peptide isolated from *C. radiatus* venom induces a sleep-like state when injected centrally into mice, symptomatology reminiscent of the biological effects of the conantokins, a well characterized *Conus* peptide family which inhibits the *N*-methyl-D-aspartic acid receptors (29–33). However, the bromosleeper peptide differs from the conantokins in that it induces a sleep-like state in mice of all ages (conantokins cause sleep in young mice under 2 weeks of age; mice older than 3 weeks exhibit a climbing syndrome). For the bromosleeper peptide, no climbing syndrome is observed at any age.

The number of post-translational modifications in the bromosleeper peptide is also noteworthy: in addition to having bromotryptophan, the bromosleeper peptide has three disulfide bridges, 4 residues of γ-carboxyglutamate (formed by the vitamin K-dependent post-translational carboxylation of glutamate residues) and 2 hydroxyproline residues. Furthermore, there clearly is selectivity with respect to the bromination reaction; only Trp (1) is brominated, while Trp (25) is unmodified. This selectivity in bromination suggests a Trp-bromination signaling site to direct the modification enzyme to a specific tryptophan residue.

Evidence for L-6-Bromotryptophan as a Secondary Amino Acid—To our knowledge, L-6-bromotryptophan has neither been reported among the numerous peptides previously characterized from a variety of species of *Conus* venoms nor from any other natural peptides/proteins. Thus, the discovery of this amino acid residue in *Conus* peptides both establishes L-6-bromotryptophan as a proteinogenous amino acid and unequivocally demonstrates that tryptophan can be a precursor for a secondary amino acid in eukaryotic systems.

In order to minimize the erroneous labeling of modified amino acids as proteinogenous, guidelines with specific criteria have been proposed (10) for acceptance of new proteinogenous amino acids. Unfortunately, these guidelines are not always infallible and have not circumvented the inappropriate acceptance and subsequent withdrawal of residues such as β-hydroxyglutamic acid and norleucine as proteinogenous amino acids. The submission and subsequent withdrawal of modified

TABLE II
Marine compounds containing a brominated indole group

Origin	Species	Compound	Peptidic	#[a]	Ref.
Sponge	*Pachymatisma johnstoni* and *Aplysina* sp.	D-6-Bromohypaphorine	No	6	51, 52
Sponge	*Smenospongia aurea*	6-Bromoaplysinopsin	No	6	53
Sponge	*Hamacantha* sp.	Hamacanthins A and B	No	6	54
Sponge	*Gellius* sp. and *Orina* sp.	Gellusines A and B	No	6	55
Sponge	*Dragmacidin* sp. and *Hexadella* sp.	(±)-Dragmacidin and dragmacidon B	No	6	56–58
Bryozoa	*Flustra foliacea*	Flustramine, flustrabromine, and flustrarine B	No	6	59–61
Sponge	*Jaspis* sp.	D-*N*-Methyl-2-bromotryptophan	Yes	2	47
Sponge	*Lissoclinum* sp.	Lissoclins C	No	6	62
Tunicate	*Eusynstyela misakiensis*	Eusynstyelamide	Yes	6	48
Shellfish	*Babylonia japonica*	Surugatoxin and neosurugatoxin	No	6	63, 64
Tunicate	*Halocynthia roretzi*	Halocyamines A and B	Yes	6	49
Tunicate	*Eudistoma olivaceum*, *Ritterella sigillinoides*, *Eudistoma fragrum*, *Eudistoma glaucus*	Eudistomines, eudistomin K sulfoxide, woodinine, and eudistomidins	No	5, 6, 7	65–70
Tunicate	*Didemnum candidum*	6-Bromotryptamine, 2,2-bis(6′-bromo-3′-indolyl)ethylamine, and 2,5-bis(6′-bromo-3′-indolyl)piperazine	No	6	71
Sponge	*Smenospongia echina*	5,6-Dibromo-*N*,*N*-dimethyltryptamine	No	5, 6	72
Acorn worms	*Enteropneusta*	6-Bromo-3-chloroindole	No	6	73
Algae	*Lärencia brongniartii*	2,3,5,6-Tetrabromindole	No	2, 3, 5, 6	74
Sponge	*Iotrochota* sp.	Methyl-3-(6-bromoindol-3-yl)prop-2-enoate	No	6	75
Mollusk	*Muriceidea prosobranchial*	6,6′-Dibromoindigo	No	6	76

[a] Position of bromine substitution.

amino acids as proteinogenous highlights the difficulties that may be encountered in determining the nature of modified amino acids. MS techniques have been used previously to reduce possible ambiguities in the determination of a new amino acid (12). In contrast to the proteinogenous amino acids that were identified after using acid or base hydrolysis to liberate the amino acid, orthogonal techniques for sequence determination of peptides (34, 35) have allowed the unambiguous identification of the bromotryptophan amino acid residue. In particular, the MS sequence experiment provided sequence information encompassing the modified amino acid. As a result, we can exclude the possibility of hydrolysis introducing some chemical modification.

Previously proposed criteria (11), which have been fulfilled for classification of this modified amino acid as proteinogenous, are as follows.

(i) The complete sequences of two peptides that contain this post-translationally modified amino acid have been characterized. In addition, the sequence of a third novel bromotryptophan-containing peptide, bromocontryphan,[3] has been determined.

(ii) The complete synthesis of the bromoheptapeptide and the synthesis of the N-terminal tetrapeptide fragment of the bromosleeper peptide have been carried out using a racemic mixture of 6-bromotryptophan. After synthesis of the peptides, it has been possible to resolve and thereby purify each 6-bromotryptophan-containing peptide (see Figs. 4*A* and 7*A*). The purified synthetic hydrophilic isomer of the bromoheptapeptide has been shown to co-elute with the natural bromoheptapeptide (see Fig. 4*B*). The purified synthetic hydrophilic isomer of the N-terminal 1–4 fragment of the bromosleeper peptide has been shown to co-elute with the natural bromosleeper peptide 1–4 fragment (see Fig. 7*B*).

(iii) The purified hydrophilic synthetic isomer of the bromoheptapeptide has been identified as the L conformation on the basis of the ability of α-chymotrypsin to cleave the peptide (see Fig. 4*C*). The purified synthetic hydrophilic isomer of the N-terminal 1–4 fragment of the bromosleeper peptide has been identified as the L conformation on the basis of the ability of aminopeptidase-M to cleave the N-terminal residue from the tetrapeptide (see Fig. 7*C*), and the fragment was observed to co-elute with the racemic mixture of 6-bromotryptophan under conditions that distinguish this amino acid from 5-bromotryptophan.

It is also noteworthy that the sequence of a cDNA clone encoding the bromosleeper peptide from *C. radiatus* has been determined.[4] The mature peptide purified from venom is encoded at the extreme C-terminal end of the predicted prepropeptide precursor. The bromotryptophan residue is encoded in the mRNA by the codon for tryptophan. We presume that the polypeptide precursor for the bromosleeper peptide has a normal L-tryptophan after the primary translation event. A modification system then specifically brominates Trp (1) of the bromosleeper peptide (most probably this modification takes place before the mature peptide is proteolytically cleaved from the precursor) resulting in a L-6-bromotryptophan residue.

Brominated Conus Peptides in the General Context of Brominated Amino Acids and Natural Products—A wide variety of haloperoxidases are known that can brominate tyrosine residues in humans and animals, including myeloperoxidases and lactoperoxidases (4). In addition, bromoperoxidases have been isolated from several species of algae (4,36).

Post-translationally iodinated (1, 37–40, 41), chlorinated (42–44), and brominated (45,1 44) tyrosine residues have been isolated from a variety of marine specimens. Although it has been suggested that there must be other halogenated amino acid residues than tyrosine in sponges, none have been positively identified.

The bromination of tryptophan or its derivatives appears to occur widely in marine organisms. Brominated indole derivatives have been characterized in a wide variety of marine natural products over a broad phylogenetic range (from marine algae to vertebrate systems). The remarkable diversity of marine natural products containing indole derivatives is briefly summarized in Table II (see Ref. 46 for other brominated marine products). Of the brominated compounds isolated from marine products, only jaspamide (47), eusynstyelamide (48), halocyamine A, and halocyamine B (49) contain brominated

[3] Jimenez, E. C., Craig, A. G., Watkins, M., Hillyard, D. R., Gray, W. R., Gulyas, J., Rivier, J. E., Cruz, L. J., and Olivera, B. M. (1997) *Biochemistry*, in press.

[4] M. Watkins and D. Hillyard, personal communication.

indole groups within a peptidic framework. Jaspamide is a metabolite of mixed peptide polyketide biosynthetic origin which has been proposed as a cyclic depsipeptide made up of 3 amino acids and an octyl ketide group (47). Eusynstyelamide is an extremely polar molecule isolated after methanol extraction which has been proposed to be a highly modified peptide dimer where each monomer is made up of two modified amino acids and a guanidinyl-amide group (41). Halocyamine A and B are modified peptidic structures where both (A and B) contain three residues, 2 of which are common amino acids (49). The structure of the L-6-bromotryptophan amino acid observed in the bromoheptapeptide and the bromosleeper differs from the D-*N*-methyl-2-bromotryptophan in jaspinamide, the bromoindoyl group in eusynstyelamide and the modified bromodidehydrotryptamine found in the halocyamines. However, the major difference is that neither jaspinamide, eusynstyelamide, nor the halocyamines are purely peptidic structures. Although the cyclic structure of jaspinamide does incorporate one other common amino acid (alanine), it is better described as a hybrid polyketide/polypeptide. Eusynstyelamide is also not a true peptide structure, and although a case can be made that it incorporates a modified arginine amino acid and modified tryptophan, the structure is more correctly a bromoindoyl-succinic acid-guanidinyl-amide. The halocyamines A and B are closest in structure to peptidic, containing histidyl, threonyl, glycy, and dihydroxy phenylalanyl residues linked at the C terminus to the 6-bromo-8,9-didehydrotryptamine group. However, the bromodidehydrotryptamine is a C-terminal modification extending a tripeptide rather than a fourth amino acid. Thus, neither jaspinamide, eusynstyelamide, nor the halocyamines are the result of mRNA transcription, and therefore the resulting modified bromoindole groups present in these molecules cannot be considered as proteinogenous amino acids. With the exception of D-*N*-methyl-2-bromotryptophan, all of the molecules listed in Table II involve bromine substitution at position 6 (and in some cases at additional sites) of the indole ring. On the basis of these observations a general rule for incorporation of bromine at position 6 in marine organisms has been proposed (50).

Some of the great diversity of marine natural products containing brominated indole derivatives are likely to be defensive compounds with strong pharmacological activity, particularly those from sessile marine organisms which rely strongly on their chemical defenses. Thus, although bromination has been reported from a very diverse group of biologically active compounds, it has not previously been demonstrated that a brominated tryptophan occurs in a regular polypeptide structure. However, given that the cone snails have developed an amazing brew of potent antagonists, it is perhaps not surprising that as marine organisms, they have recruited bromination as a means of increasing their peptidic repertoire. In effect, they may have recruited bromination in much the same way as other marine organisms have used bromination for generating pharmacologically active natural products.

Thus, if the cone snails have indeed evolved bromination of tryptophan as a novel adaptation in their venom ducts, it would not be difficult to see potential intermediate steps in the generation of such a system. The broad distribution of brominated natural products in the marine environment presumably arises through a broad spectrum of bromo- and lactoperoxidases in marine organisms. If a bromoperoxidase were recruited to become sequence-specific, it would become a post-translational modification enzyme, only catalyzing the bromination of specific tryptophan residues in a polypeptide structure, such as apparently occurs for the bromosleeper peptide. Thus the occurrence of L-6-bromotryptophan in *Conus* peptides could be due to an adaptation involving the recruitment of an enzymatic system already generally distributed among marine organisms; the cone snails have used this more general bromination chemistry evolved in marine ecosystems in a specialized way in their venom ducts.

Acknowledgments—We are grateful to Ferring Research Institute (San Diego, CA) for access to the electrospray mass spectrometer.

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