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

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  Pca-Cys-Gly-Gln-Ala-Trp*-Cys-NH₂
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and

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  Trp*-Ala-Thr-Ile-Asp-Gla-Cys-Gla-Gla-Thr-Cys-Aan-Val-Thr-Phɛ-
  1  5  10  15
  Lys-Thr-Cys-Gly-Hyp-Hyp-Gly-Asp-Trp-Gln-Cys-Val-Gla-Ala-
  16  20  25  30  35  31  33
  Cys-Pro-Val-OH
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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 proteinogenic amino acids.

These modified amino acids include amino acid conjugates where the side chain is linked to a glycocalix, 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'-triiodothyronine or T₃)¹, 3,5,3'-triiodothyronine (T₃), and 3,3'-diiodothyronine. The presence of free T₃ and T₄ 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 proteinogenic 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 Hayashi (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 (10–11) and amino acids identified are marked "advertisement" in accordance with 18 U.S.C, Section 1754 solely to indicate this fact.

¹ The abbreviations used are: T₄, 3,5,3’-triiodothyronine; T₃, 3,5,3’-triiodothyronine; Boc, tert-butyloxycarbonyl; Cys(Mob), S-carboxamidomethylcysteine; Cys(Cam), S-carboxyamidomethylcysteine; Cys(Mob), S-carboxamidomethylcysteine; Cys(Cam), S-carboxamidomethylcysteine; CZE, capillary zone electrophoresis; DCM dichloromethane; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; Gln(Xnt), N-α-tetramethyluronium tetrafluoroborate; TCEP, tris-(2-carboxyethyl)phosphine. Amino acids are indicated by the standard three letter abbreviations unless otherwise indicated.
apparatus consisted of HPXL pumps and either a Dynamax model UV 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 bromohexapeptide 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 17 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 million units 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 bromohexapeptide was reduced with 10 μM 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 μM 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 bromohexapeptide 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 10-kV accelerating volt-

**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 Vydac 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 μl/min. The HPLC
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 as the coupling reagent, with the Boc protection strategy in DCM with the exception of the introduction of the Fmoc group with 20% piperidine in NMP. Four-fold excess of amino acid derivatives were used in the coupling reactions with the exception of the N₆-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 50 ml of ether (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 C₃₄ (5 μm, 300 Å particle size) and a second extraction with 0.1% aqueous trifluoroacetic acid and 60% acetonitrile (3 × 50 ml) and loaded in 0.5 ml portions to 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%). LSU-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(OBz)-OH and Boc-Ala-OH (3 equivalents) in DCM. The N-terminal residue N₆-bromotryptophan was introduced as the N-Fmoc-N₆-bromotryptophan derivative (2 equivalents). The coupling was mediated with DIK/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 protected 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 × 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 L-6-bromo-tryptophan 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 permission 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. LSU-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 C₃₄ (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 L- or D-isomers of 6-bromotryptophan) were redissolved and alkylated. The HPLC-purified reduced and alkylated peptides were lyopholized and redissolved in 250 mM Tris base, pH 8.5, and incubated with a-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 endoprotease 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 C₃₄ 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.

RESULTS

Bromopeptide—Two peptides containing L-6-bromotryptophan were isolated from the venom of predatory Conus species. The first was a bromopeptide 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. 3A). Several relatively intense species were observed with 2 Da separation at m/z 853.9 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. 3A).
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 \( \text{NH}_3 \) from the protonated molecule ion. The presence of the doublets was characteristic of a molecule containing a single \(^79\text{Br}\) or \(^81\text{Br}\) 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. 3B) separated by 16 Da. We interpreted these species as \([M'+Na]^+\) and \([M'+K]^+\) of the reduced and alkylated peptide \((M' = 968 \text{ Da})\). The shift in mass after reduction and alkylation \((M' - M = 968 - 852 = 116 \text{ Da})\) was consistent with the presence of two cysteine residues. Other species observed in Fig. 3B were interpreted as fragment ions \((e.g., m/z \ 788\) corresponds with the post source decay \( b_6 \) fragment ion).\(^2\) The post-source decay FAST spectrum of the reduced and alkylated product, measured at low, medium, and high laser power fluences, are shown in Fig. 3C. In Fig. 3C, the position of the because this ion has undergone metastable decomposition after exiting the source. In Fig. 3C, the FAST spectra are calibrated to compensate for the reduced energy of fragment ions, and this procedure results in a more accurate mass measurement \((m/z \ 793)\).

\(^2\) In Fig. 3B, the time-of-flight of the \( b_6 \) fragment ion and therefore the observed \( m/z \ 788 \) is slightly lower than the calculated mass \((782.2 \text{ Da})\).
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 (b6, b5, and b4) 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-NH₂ (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. 3A).

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. 4A), 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. 4B). 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. 4C), 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
Identification of Bromotryptophan in Conus Peptide Toxins

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 bromohexampeptide is not active in this assay.

| Dose (pmol/g body weight) | 9-Day-old mice | 17-Day-old mice | 22-Day-old mice |
|--------------------------|----------------|-----------------|-----------------|
| 10–15                    | Slept for ~1 h | —               | —               |
| 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                      | —             | —               | Lethargic/drowsy after ~12 min, slept for ~3 h |

*— not tested.

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 Conus radiatus venom (peak BS of Fig. 5A). 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 Conus radiatus venom (peak BS of Fig. 5A). 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.

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 phenylthiohydantoin 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, 9, 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 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 (cf. calculated monoisotopic [M + H]+ of 568.177 Da for the sequence W*ATL, where W* = bromotryptophan).

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 bromohexampeptide with the racemic mixture of D- and L-5-bromotryptophan. This peptide did not co-elute on HPLC with the natural bromohexampeptide (data not shown).

Effects on young mice: The bromosleeper peptide was purified from marine products, in order to unambiguously resolve the question (data not shown).

The results of 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 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 bromohexampeptide (see Fig. 3A, 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–C6H12 (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
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 amino acid residues in Conus peptides is a well-characterized phenomenon which inhibits the N-terminal fragment containing a 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.

6-bromotryptophan, a hydrophobic and a hydrophilic peptide isomer were purified (see Fig. 7A). 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. 7B). 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. 7C), 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).
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TABLE II
Marine compounds containing a brominated indole group

| Origin            | Species                        | Compound                                   | Peptidic | Ref. |
|-------------------|--------------------------------|--------------------------------------------|----------|-----|
| Sponge            | Pachymatisma johnstoni and     | d-6-Bromohypaphorine                      | No       | 6   |
|                   | Aplysia sp.                    |                                            | 51, 52   |     |
| Sponge            | Smenospongia aurea             | 6-Bromoaoplyisopin                        | 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            | Dromacidi sp. and Hexadella sp.| (±)-Dromacidi and drmacidom B             | No       | 6   |
|                   |                                |                                            | 56–58    |     |
| Bryozoa           | Flustra foliacea               | Flustramine, flustrabromine, and           | No       | 6   |
|                   |                                | flustrarine B                              | 59–61    |     |
| Sponge            | Jaspiis sp.                    | d-N-Methyl-2-bromotryptophan               | Yes      | 2   |
|                   |                                |                                            | 47       |     |
| Spongle           | Lissoclinum sp.                | Lissoclina C                               | No       | 6   |
|                   |                                |                                            | 62       |     |
| Tunicate          | Eusynastyela misakiensis       | Eusynastyelamine                           | Yes      | 6   |
|                   |                                |                                            | 48       |     |
| Shellfish         | Babyonia japonica             | Surugatokin and neosurugatokin            | No       | 6   |
|                   |                                |                                            | 63, 64   |     |
| Tunicate          | Halcocyste roreti              | Halocymes A and B                          | Yes      | 6   |
|                   |                                |                                            | 49       |     |
| Tunicate          | Eudistoma ollivaceum, Ritterella sigillinaeides, Eudistoma fragrum, Eudistoma glauces Dilemnum candidum | 6-Bromotryptamine, 2,2-bis(6-bromo-3-indolyl)ethylamine, and 2,5-bis(6-bromo-3-indolyl)pyraperazine | No | 6 | 71 |
| Sponge            | Smenospongia echna             | 5,6-Dibromo-N,N-dimethyltryptamine         | No       | 5, 6|
|                   |                                |                                            | 72       |     |
| Acorn worms       | Enteropneustes                 | 6-Bromo-3-chlorindole                      | No       | 6   |
|                   |                                |                                            | 73       |     |
| Algae             | Larencia bronniartii           | 2,3,6-Tetramonindole                      | No       | 2, 3, 5, 6|
|                   |                                |                                            | 74       |     |
| Sponge            | Ictrochota sp.                | Methyl-3-(6-bromomindol-3-yl)prop-2-enoate | No       | 2   |
|                   |                                |                                            | 75       |     |
| Mollusk           | Muriceidea proso branchial     | 6,6'-Dibromoiindigo                       | No       | 6   |
|                   |                                |                                            | 76       |     |

* 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, MS sequence experiment provided sequence information encompassing the modified amino acid. As a result, we can conclude 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, 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. After synthesis of the peptides, it has been possible to resolve and thereby purify each 6-bromotryptophan-containing peptide, bromocontryphan, has been determined.4 The mature peptide purified from venom is co-eluted 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 (45,144) 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 jaspadime (47), eusynetylamine (48), halocynamine A, and halocynamine 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.
The occurrence of l-6-bromotryptophan in Conus peptide toxins 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.

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