Bromocontryphan: Post-Translational Bromination of Tryptophan†

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Received November 18, 1996⊗

ABSTRACT: We demonstrate that post-translational bromination of a tryptophan residue occurs in the biologically active octapeptide bromocontryphan, purified and characterized from *Conus radiatus* venom. Clones encoding bromocontryphan were identified from a cDNA library made from *C. radiatus* venom ducts. The mRNA sequence obtained predicts a prepropeptide which has the mature peptide sequence at the C-terminal end, with the L-6-bromotryptophan residue encoded by UGG, the Trp codon. These data provide the first direct evidence for post-translational bromination of a polypeptide which is translated through the normal cellular machinery. In addition to bromination, the peptide, which induces a "stiff tail" syndrome in mice, has several other modifications as shown by the sequence Gly-Cys-Hyp-D-Trp-Glu-Pro-L-6-Br-Trp-Cys-NH_2, in which Hyp = hydroxyproline. Asterisks indicate post-translational modifications (left to right): proteolytic cleavage at the N-terminus; hydroxylation of Pro^3; epimerization of Trp^4; bromination of Trp^7, and C-terminal amidation. Bromocontryphan appears to have the highest density of post-translational modifications known among gene-encoded polypeptides. The overall result is a molecule which closely resembles marine natural products produced through specialized biosynthetic pathways comprising many enzyme-catalyzed steps.

A firm boundary exists between gene-encoded polypeptides and natural products. The gene-encoded polypeptides use the normal cellular translation machinery and are made up of the standard twenty amino acids specified by the genetic code. In contrast, natural products are synthesized through biosynthetic pathways, usually involving multiple metabolic steps, each catalyzed by an enzyme. In this report, we describe the characterization of bromocontryphan, an octapeptide from a *Conus* venom that blurs the distinction between these two normally disparate categories; in many ways, bromocontryphan has the salient features typical of a marine natural product and yet, we show that it is in fact the direct translation product of a gene. In addition, the characterization of bromocontryphan has provided the first unequivocal evidence that bromination occurs as a post-translational modification of a normally-translated polypeptide chain.

Although the small, biologically active peptides present in the venoms of the predatory cone snails are directly translated from genes, many of these peptides are modified post-translationally (Olivera et al., 1990; Myers et al., 1993). The modifications include C-terminal amidation, hydroxylation of proline, γ-carboxylation of glutamate and the conversion of L-tryptophan to D-tryptophan (Jimenez et al., 1996). The peptide is initially translated from mRNA as a much larger prepropeptide precursor which is subsequently post-translationally modified and proteolytically processed to release the mature peptide from the C-terminal end of the precursor (Woodward et al., 1990; Colledge et al., 1992).

Bromocontryphan was purified and characterized from the venom of the deep-water Indo-Pacific species, *Conus radiatus*, a species believed to be piscivorous (fish-hunting). We show that for the small octapeptide characterized here, five distinct post-translational modifications take place during the maturation of the biologically-active pepide, including bromination of only one of the two L-tryptophan residue to L-6-bromotryptophan (Br-Trp). Although Br-Trp has been described previously in a number of peptidic structures (Zabriskie et al., 1986; Swersey et al., 1994), these are most likely not directly translated from genes. We have also found Br-Trp in two other peptides from *Conus* venoms which will be described in detail elsewhere (Craig et al., 1997).

MATERIALS AND METHODS

Reagents. Boc-Cys(Mob)-OH,[1] Boc-Glu(Chx)-OH, Boc-Trp-OH, Boc-D-Trp-OH, Bzl-Hypro-OH, and Boc-Gly-OH (Bachem, Torrance, CA); D,L-6-bromotryptophan (Biosynth AG, Staad, Switzerland).

† This work was supported by NIH Program Project No. GM48677, NIH1S1ORR-8425 (to A.G.C.), and a fellowship from DOST-ESEP, Philippines, to E.C.J.

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⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1997.

[1] Abbreviations: Boc, *tert*-butoxycarbonyl; CZE, capillary zone electrophoresis; Chx, cyclohexyl; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DIEA; diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; LSI, liquid secondary ionization; MALD, matrix assisted laser desorption; MBHA, 4-methylbenzhydrylamine; NMP, *N*-methylpyrrolidone; Mob, *S*-*p*-methoxybenzyl; MS, mass spectrometry; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TCEP, tris(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid; TMAC, tetramethylammonium chloride.

S0006-2960(96)02840-1 CCC: $14.00

Purification of the Peptide. Crude venom was extracted from venom ducts (Cruz et al., 1976), and the components were purified as previously described (Jimenez et al., 1996). The crude extract from venom ducts was applied into a C_{18} semi-preparative column (10 × 250 mm) and eluted with a linear gradient of acetonitrile in 0.085% TFA at 5 mL/min. Further purification of bioactive peaks was done on a C_{18} analytical column (4.6 × 250 mm) eluted with a gradient of acetonitrile in 0.085% TFA at 1 mL/min. The effluents were monitored at 220 nm. Peaks were collected, and aliquots were assayed for activity.

Bioassay. Biological activity was assayed by intracranial injection in mice (9 to 21 days old). Aliquots of peptide samples were lyophilized and dissolved in normal saline solution and then injected into mice using a 0.3 mL syringe with a 29-gauge needle. Each control mouse was injected with an equal volume of normal saline solution containing dissolved residue of lyophilized column buffer blanks. After injection, the mice were placed in cages for observation.

Peptide Sequencing. The purified peptide was reduced and alkylated as described by Shon et al. (1994) prior to sequencing by automated Edman degradation on an Applied Biosystems 477A Protein Sequencer with a 120A Analyzer (DNA/Peptide Facility, University of Utah).

Mass Spectrometry. Matrix assisted laser desorption mass spectra were measured using a Bruker REFLEX (Bruker Instruments Inc., Bremen, Germany) time-of-flight mass spectrometer fitted with a gridless reflection and N_2 laser and a 100 MHz digitizer. The sample (in 0.1% aqTFA) was applied with α-cyano-4-hydroxycinnamic acid and rinsed with water. Analysis was done with an accelerating voltage of +31 kV and a maximum reflector voltage of +30 kV. Under these conditions, the mass accuracy obtained was typically better than 200 ppm in the reflectron mode.

Liquid secondary ionization and matrix assisted laser desorption mass spectra were measured using a Jeol HX110 (Tokyo, Japan) double-focusing mass spectrometer operated at 10 kV accelerating voltage and a nominal resolution of 3000. The intact bromotryptophan sample was analyzed with MALD-MS and an electric field scan. The spectra were calibrated using peptide calibrants over a narrow mass range. The HPLC-purified product of carboxypeptidase hydrolysis was concentrated from 100 to 10 μL, mixed in a glycerol: 3-nitrobenzyl alcohol matrix (1:1), and analyzed with LSI-MS using a magnetic field scan. The LSI-MS spectra were calibrated using $[Cs(CsI)_n]^+$ cluster ions; the mass accuracy was typically better than 50 ppm.

Carboxypeptidase Y Hydrolysis. The reduced (TCEP) and alkylated (iodoacetamide) synthetic bromocontryphan (and analogs) were incubated with carboxypeptidase Y (1:5 enzyme:substrate) in 200 μL of 50 mM sodium acetate buffer (pH 5.5) at 22 °C for up to 7 days. The reaction was monitored with HPLC and MALD-MS after 1, 2, 4, and 7 day time intervals.

Peptide Synthesis and Purification. Bromocontryphan analogs (I) GCOWEPX*C-NH_2 and (II) GCOWEPX*C-NH_2 were synthesized using Boc-6-bromo-D,L-tryptophan generated from 6-bromo-D,L-tryptophan using standard procedures. The two diastereomers of peptide (I) were separated, (Ia) GCOWEPXC-NH_2 and (Ib) GCOWEPXC-NH_2, by HPLC, whereas the two diastereomers of peptide (II) were not separated and used as an approximate 50:50% mixture for assays. (O is hydroxyproline, W represents D-tryptophan, X* is 6-bromo-D,L-tryptophan, X is 6-bromo-L-tryptophan, and X is 6-bromo-D-tryptophan.)

The synthesis of peptide mixtures I and II was performed on MBHA resin (1.0 g, substitution 0.5 mequiv/g) using Boc-Cys(Mob), (D,L)-Fmoc-6-Br-Trp-OH, Boc-Hypro-OH, and Boc-Glu(Chx)-OH for the first four residues. The resin was split, and the fifth residue was added using Boc-Trp-OH or Boc-D-Trp-OH. Bzl-Hypro-OH, Boc-Cys(Mob)-OH, and Boc-Gly-OH were used to add the last three residues. The coupling steps were carried out in DCM with DIC as condensing agent. The Fmoc group of the bromotryptophan residue was removed with 20% MNP in piperidine for 2 × 10 min. The efficiency of each coupling reaction was checked by the Kaiser ninhydrin test. After removal of the final Boc protecting group, the peptide resin was washed with appropriate solvents and dried. The peptide resins (750 mg for the L-Trp-containing analog) were treated with 10 mL of HF in the presence of 1.0 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% aqueous TFA and 60% acetonitrile (3 × 25 mL). Both aqueous peptide solutions gave a strong Ellman's positive test for free thiol groups. The acetonitrile-containing aqueous extracts of the crude peptide were diluted to 1400 mL with 0.125 M ammonium acetate (pH 6.9) and 200 mL of acetonitrile was added as the solution turned clear. The reaction mixture was stirred slowly in a 2 L beaker for 36 hr until the Ellman test turned to negative. The pH of the solution was adjusted to 2.2 by adding TFA, and acetonitrile was removed by rotary evaporation.

The crude oxidized peptide solution (1000 mL) was filtered and loaded onto a Waters PrepLC/System 500A equipped with gradient controller, Waters model 450 variable wavelength detector, and Waters 1000 PrepPack cartridge chamber (65.5 × 320 mm) column, packed with Vydac C_{18} 15−20 μm particles; wavelength, 220 nm; AUFS, 2.0; flow, 100 mL/min; gradient, 0−50% in 100 min; buffer A, 0.1% aqueous TFA; buffer B, 60% acetonitrile in 0.1% aqTFA. The fractions (50−75 mL) were collected manually and analyzed by HPLC. Fractions with acceptable purity as determined by CZE analysis were pooled and lyophilized. The major component was collected and lyophilized (D-Trp^4; hydrophilic component, 3.0 mg; purity based on CZE 99.9%; hydrophobic component, 14.6 mg; purity based on CZE 92%; L-Trp^4; major component, 12.6 mg).

Comparison of Natural and Synthetic Peptides. The various bromocontryphan analogs were applied separately to a C_{18} analytical column and eluted at a flow rate of 1 mL/min with a gradient of ACN in 0.085% TFA. Aliquots of natural and synthetic bromocontryphan were co-injected and eluted under the same HPLC conditions.

Identification and Sequencing of a cDNA Clone Encoding Bromocontryphan. Bromocontryphan-encoding clones were selected from a size-fractionated cDNA library constructed using mRNA obtained from *C. radiatus* venom duct as previously described (Colledge, et al., 1992). The library was screened using a bromocontryphan-specific probe corresponding to the last six amino acids of the peptide (5′ CCR CAC CAN GGY TCC CA 3′, where R = A or G, N = A or C or G or T, and Y = C or T). The oligonucleotide was end-labeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase and hybridized to sets of duplicate nylon filters (Hybond-N,

Amersham) to which colonies of the *C. radiatus* cDNA library were bound. Hybridization was done for 48 h at 48 °C in 3 M TMAC, 0.1 M sodium phosphate, pH 6.8, 1.0 mM EDTA, 5× Denhardt's solution, 0.6% SDS, and 100 mg/mL salmon sperm DNA. After being washed at 50 °C in 3 M TMAC, 50 mM Tris pH 8.0, 0.2% SDS followed by a room temperature wash in 2× SSC, 0.1% SDS, positive colonies were identified by autoradiography.

A secondary screening by polymerase chain reaction was performed on 20 of 42 clones that hybridized to this probe. Putative bromotryptophan clones were amplified by PCR using the above probe as the first primer and a second primer corresponding to a portion of the plasmid vector upstream of the site into which the cDNA library was cloned (5′ GTT GTG TGG AAT TGT GAG CGG A 3′). The PCR reaction was carried out in a 10 μL sealed capillary using an Idaho Technology air thermocycler. Each reaction contained one colony, 0.5 μmol of each primer, and 0.5 unit of Taq polymerase (Boehringer Mannheim) in a buffer consisting of 50 mM Tris, pH 8.3, 250 μg/mL BSA, and 2 mM $MgCl_2$. Reaction conditions were as follows: 3 min of predenaturation at 94 °C followed by 45 cycles, through denaturation (94 °C, 5 s), annealing (50 °C, pulse), and elongation (72 °C, 15 s) steps.

Clones identified in the secondary screen were prepared for DNA sequencing as previously described (Monje et al., 1993). The nucleic acid sequence was determined according to the standard protocol for Sequenase version 2.0 DNA sequencing kit using the non-biotinylated vector primer and [^{35}S]dATP.

RESULTS

Purification and Characterization of Bromocontryphan. The purification and characterization of two D-amino acid-containing compounds, contryphan and des[Gly^1]contryphan, was previously described (Jimenez et al., 1996). We discovered a third fraction from *C. radiatus* venom which elicited the same general biological activity as the two previous peptides but which eluted later on an HPLC column. The purification of this peptide is shown in Figure 1. The basic biological assay used to follow the peptide during various purification steps was intracranial injection and scoring for the "stiff tail syndrome" previously described by Jimenez et al. (1996).

The amino acid sequence of the purified peptide was determined by standard methods; the results of the amino acid sequencing run revealed an octapeptide with the sequence GCOWEP_C. This is the same sequence as contryphan (GCOWEPWC-NH_2 where W is D-tryptophan) as previously reported (Jimenez et al., 1996), except that in position 7, no assignment could be made. Thus, the peptide was closely related to contryphan, and had similar activity when injected into mice. Since contryphan has an L-Trp at the homologous position, we considered the possibility that a modified Trp was present in position 7 of the new peptide.

MALD time-of-flight MS analysis of the purified fraction of bromocontryphan indicated partially resolved species at *m*/*z* 1068.8, 1070.5, and a separate species at *m*/*z* 1092.9. We interpreted the *m*/*z* 1070.5 and 1092.9 species as corresponding with intact $[M + H]^+$ and $[M + Na]^+$ molecular ions. MALD-MS analysis with a magnetic sector instrument of the purified peptide was also carried out, and

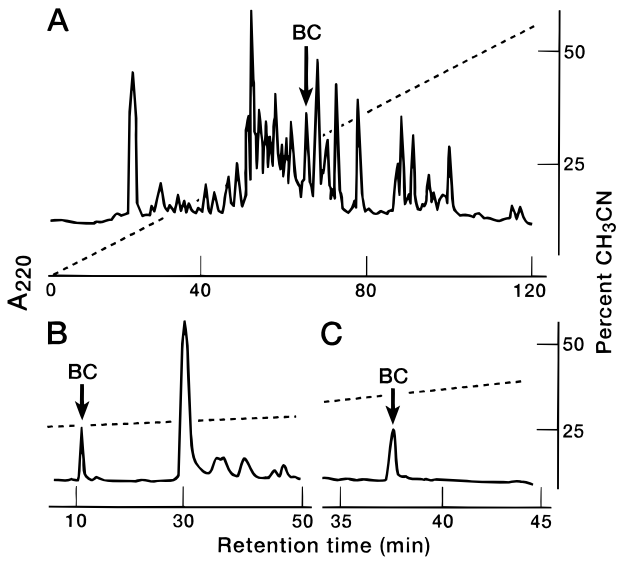

FIGURE 1: Purification of bromocontryphan. (A) Crude venom extract was applied on a C_{18} semi-preparative column and eluted at 5 mL/min with a gradient of 0.45% CH_3CN/min. (B) The bioactive fraction denoted "BC" was applied onto a C_{18} analytical column and eluted at 1 mL/min using a gradient of 0.09% CH_3CN/min. (C) The peak indicated by arrow in B was repurified at 1 mL/min with a gradient of 0.9% CH_3CN/min to obtain bromocontryphan.

relatively intense species were observed at *m*/*z* 1090.2, 1092.2, 1106.2, and 1108.2. The results with the higher resolution of the magnetic sector instrument were interpreted as the major isotopomers of the $[M + Na]^+$ and $[M + K]^+$ species, which would indicate an intact molecular mass of 1067.2 Da. These results indicate that the missing residue has a mass of 264 Da for the C-terminally amidated peptide or 265 Da for the free acid. The results are consistent with the missing residue in position 7 being bromotryptophan and the peptide being C-terminal amidated (the observed monoisotopic mass of *m*/*z* 1090.2 is consistent with the calculated monoisotopic $[M + Na]^+$ of 1090.25 Da). We note that both contryphan and des[Gly^1]contryphan are also C-terminally amidated. These data indicate that the sequence of the octapeptide is

Gly-Cys-Hyp-D-Trp-Glu-Pro-Br-Trp-Cys-NH_2

where Hyp is 4-*trans*-hydroxyproline.

However, these data do not reveal the location on tryptophan where the putative bromination takes place. Both the 5- and 6-bromotryptophan derivatives have previously been described in the literature. A number of preliminary studies indicated that *Conus* peptides do not contain the 5-bromotryptophan derivative (Craig et al., 1997). Thus, a contryphan homolog was synthesized with Trp^4 in the D-configuration (as in contryphan), and in position 7, D,L-6-bromotryptophan was incorporated. The synthetic peptides containing either L- or D-bromotryptophan could be resolved on HPLC and are referred to as the hydrophilic and hydrophobic components, respectively.

The more hydrophilic component of the synthetic material co-eluted with the native peptide (see Figure 2). Indeed, even when the peptides were reduced, there was co-elution of both native and synthetic peptide, further establishing the identity of the natural and synthetic material. Thus, we conclude that residue 7, which could not be assigned in amino acid sequencing, is in fact 6-bromotryptophan.

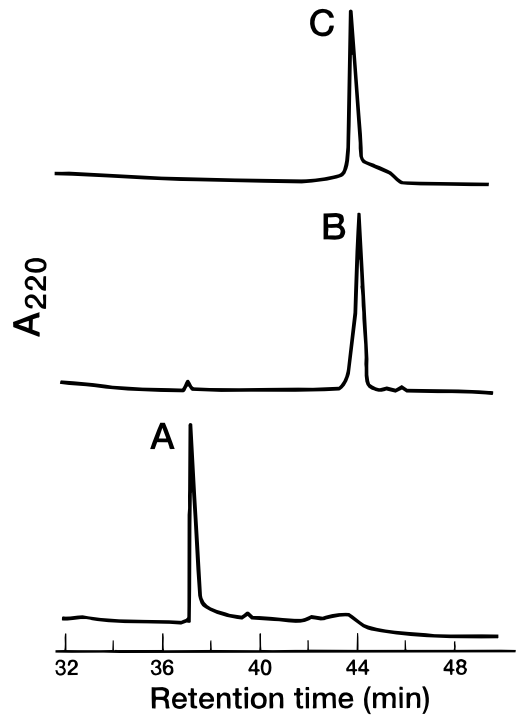

FIGURE 2: HPLC chromatogram showing co-elution of natural and synthetic bromocontryphan. (A) Natural and synthetic bromocontryphan, which has D-Trp[4] and 6-Br-Trp[7]; (B) synthetic [D-Br-Trp[7]]bromocontryphan; (C) synthetic [L-Trp[4], D,L-Br-Trp[7]] bromocontryphan. Elutions were done on a C_{18} analytical column and eluted at 1 mL/min using a gradient of 0.9% CH_3CN/min.

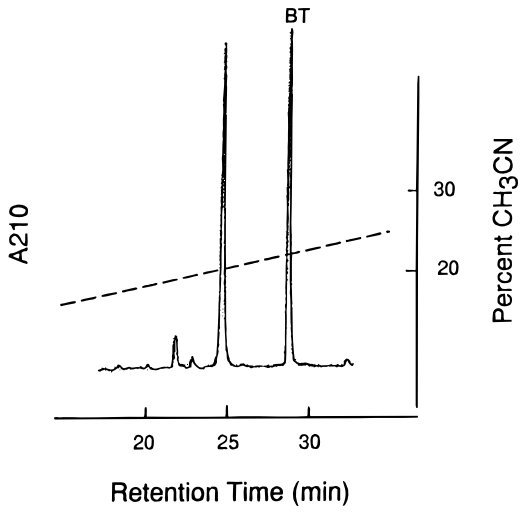

FIGURE 3: HPLC chromatogram of the synthetic hydrophilic peptide incubated with carboxypeptidase Y; the component BT (indicated) was identified as bromotryptophan with LSI-MS.

In order to confirm that the brominated residue was in the L- and not the D-configuration, a carboxypeptidase Y digest was carried out. This enzyme is not able to cleave D-amino acid residues at the carboxy terminus of a peptide. HPLC analysis of the enzyme incubations indicated no change in the retention time of the hydrophobic peptide, while the hydrophilic peptide was consumed and a number of new species were observed (see Figure 3). The fraction identified as BT in Figure 3, when isolated and measured with LSI-MS, revealed relatively intense species at *m*/*z* 283.0 and 285.0 (data not shown), consistent with the release of 6-bromotryptophan (the calculated mass for the $[M + H]^+$ of bromotryptophan amino acid is 283.1 Da). Since the hydrophilic peptide which was susceptible to the enzyme co-eluted with the natural peptide, we concluded that the bromotryptophan residue in bromocontryphan was in the L-configuration.

Biological Activity of Bromocontryphan. We designate the brominated peptide as bromocontryphan, since it clearly differs from contryphan by a single bromine moiety. We have examined the biological activity of bromocontryphan and compared it to contryphan, as well as to the D-Br-Trp[7] derivative of bromocontryphan. These results are shown in Table 1. The biological activity in mammalian systems is very similar to the spectrum of activity previously observed for both contryphan and des[Gly[1]]contryphan (Jimenez et al., 1996). However, for the analog with D-Br-Trp present in place of L-Br-Trp at position 7, no biological activity was observed at twice the active dose of bromocontryphan.

Identification and Sequencing of a cDNA Clone Encoding Bromocontryphan. The primary translation product for bromocontryphan was elucidated through a cDNA cloning strategy using methods previously described [see for example Woodward et al. (1990), Colledge et al. (1992), and Monje et al. (1993)]. The relevant clones were identified from a *C. radiatus* venom duct cDNA library that was prepared as described under Materials and Methods. Once the clones were isolated, both strands were sequenced.

The precursor sequence for bromocontryphan determined by this strategy from the *C. radiatus* venom duct library is shown in Table 2. The mature peptide is encoded at the extreme C-terminal end of the predicted prepropeptide precursor. As expected, the bromocontryphan residue is encoded in the mRNA by the codon for tryptophan.

Thus, we presume that the polypeptide precursor for bromocontryphan has a normal L-tryptophan after the primary translation event. A modification system then specifically brominates Trp[7] of bromocontryphan (most probably this modification takes place before the mature peptide is proteolytically cleaved from the precursor) to a 6-bromo-L-tryptophan residue.

DISCUSSION

The data in the Results section establish that bromocontryphan is the translation product of a gene. The peptide is first translated from mRNA as a 63-amino acid prepropeptide precursor, and proteolytic cleavage between residues 54 and 55 of the precursor yields a small C-terminal peptide. The biochemical characterization of contryphan from the venom of the radial cone revealed that at least four other post-translational modifications take place to yield the mature octapeptide. Two of these post-translational modifications are widespread in *Conus* peptides: the conversion of the C-terminal glycine of the precursor to an amide group, and the hydroxylation of a proline residue to hydroxyproline (Olivera et al., 1990; Myers et al., 1993). However, two of the post-translational modifications are novel: Trp[4] is epimerized from L-tryptophan to D-tryptophan (Jimenez et al., 1996), while Trp[7] is brominated to yield L-6-bromotryptophan.

The discovery of the bromination of Trp is specially significant. The biochemical results in combination with the cloning studies directly establish that a bromotryptophan residue in a polypeptide is encoded by a UGG codon in mRNA, the standard codon for tryptophan. Although several previous reports have indicated that brominated amino acids

Table 1: Biological Effects of Bromocontryphan and Analogs

peptide	dose[a] (nmol/g body wt)	observed effects
bromocontryphan	4−7	tail-raising, grooming, scratching, dragging of hind legs, circular motion, biting of hind paw
	10	grooming, scratching, circular motion, dragging of hind legs, barrel rolling
[D-Br-Trp[7]]bromocontryphan	15	none

[a] Aliquots of the peptides dissolved in normal saline solution were injected intracranially into mice as described under Materials and Methods.

Table 2: cDNA Sequence of Clone Encoding Contryphan/Bromocontryphan

(A) Contryphan/Bromocontryphan-Encoding Clone[a]

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                                        10
met gly lys leu thr ile leu val leu val ala ala val leu leu ser ala gln
ATG GGG AAA CTG ACA ATA CTG GTT CTT GII GCT GCT GTC CTG TTG TCG GCC CAG

     20                                                 30
val met val gln gly asp gly asp gln pro ala asp arg asn ala val pro arg
GTC ATG GTT CAA GGT GAC GGA GAT CAA CCT GCA GAT CGT AAT GCA GTG CCA AGA

                40                                      50
asp asp asn pro gly gly ala ser gly lys phe met  asn val leu arg arg
GAC GAT AAC CCA GGT GGA GCG AGT GGA AAG TTC ATG  AAT GTT CTA CGT CGG

                                        60
ser gly cys pro trp glu pro trp cys gly OPA
TCT GGA TGT CCG TGG GAA CCT TGG TGT GGC TGA
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(B) Post-Translational Modifications of Bromocontryphan[b]

bromocontryphan precursor C-terminal sequence: G C P W E P W C G ⇓

mature peptide: G C O W E P X C - NH_2

[a] cDNA clones from a *C. radiatus* venom duct cDNA library encoding bromocontryphan were identified and sequenced as discussed in Materials and Methods. The predicted amino acid sequence encoded in the longest open reading frame is given; the bold amino acids are those consistent with the sequence of the peptide directly purified and characterized from venom. The boxed sequence (amino acids 49−53) is a putative bromination signaling sequence referred to in the Discussion. [b] The presumed post-translational modification of precursor C-terminal sequences to the final mature peptides is shown. W = D-Trp, X = L-6-Br-Trp, O = hydroxyproline.

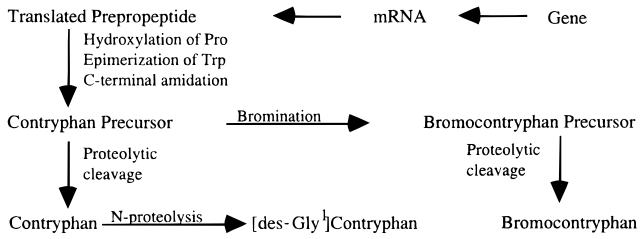
might be present in proteins (Welinder, 1972), this is the first unequivocal demonstration that bromination is a *bona fide* post-translational modification.

Mass spectrometry was critical for elucidating the presence of bromotryptophan in this peptide. The data strongly suggested that the tryptophan residue was brominated; the isotope distribution of the peptide was consistent with the mass distributions of the two major isotopes of bromine, a relatively subtle feature in the initial mass spectrometric data which was nevertheless difficult to rationalize other than by bromination. Since the non-brominated peptide had already been purified and characterized (Jimenez et al., 1996), the sequence homology and mass analysis were strongly suggestive that the unusual residue in the peptide was bromotryptophan. We conclude that the bromotryptophan residue is in the L-configuration from chemical synthesis and enzymatic criteria.

There clearly must be a selectivity that occurs with regard to the bromination reaction. Bromocontryphan is remarkable in that although it is only an octapeptide, it contains two tryptophan residues with different post-translational modifications. Trp^4 is post-translationally modified to D-tryptophan, while Trp^7 is modified to L-6-bromotryptophan.

The selectivity seen in bromination suggests that there may be a modification-signaling sequence to direct the tryptophan-brominating enzyme to a specific tryptophan residue. The sequence of a cDNA encoding another Br-Trp-containing peptide from *C. radiatus* has been determined (M. Watkins and D. Hillyard, unpublished results). No amino acid homology is seen between the mature peptides, but in the prepropeptide precursor, there is a 5-amino acid sequence, NV^L_IRR, which shows substantial sequence identity (this sequence is boxed in Table 2). It remains to be established whether or not this sequence actually plays a role in signaling bromination to the post-translational modification enzyme.

Scheme 1



The discovery of bromocontryphan, and the identification of an encoding cDNA for a precursor suggests that three related peptides are generated in *C. radiatus* venom ducts through the pathway in Scheme 1. We presume in this scheme that bromination precedes the final proteolytic cleavage.

Although cleavage of the precursor after the arginine pair should give rise to bromocontryphan or contryphan with an extra serine residue at the amino terminus, the serine-containing analogs have not been detected in *C. radiatus* venom, indicating the subsequent action of an exopeptidase. The presence of two non-brominated forms, contryphan and [des-Gly^1]contryphan, in native venom may be due to incomplete post-translational processing with the [des-Gly^1]

analog arising from a non-physiological *post-mortem* proteolysis. However, it is also possible that the presence of all three forms has true physiological significance; if this were so, it would indicate a complex biological role for these peptides *in vivo*.

Bromination reactions are much more commonly observed in biologically active compounds from marine organisms than in natural products of terrestrial origin; this is almost certainly because of the much greater accessibility of bromine in the marine environment (sea water is 1 mM Br^-) (Butler & Walker, 1993). A large number of brominated natural products are known, many of them containing a brominated indole group, likely derived from tryptophan (Davidson, 1993). At least some of these brominated natural products have some peptidic bonds (Zabriskie et al., 1986; Gulavita et al., 1992; Swersey et al., 1994), but there is no evidence that any of these have structural elements encoded by mRNA.

In many respects, bromocontryphan, with its cyclic structure and high density of modified amino acids, could well have been isolated as the result of a marine natural product screen. Bromocontryphan is well within the size range of marine natural products; furthermore, bromocontryphan, contryphan, and des[Gly^1]contryphan comprise a series of related structures reminiscent of the series of related natural products often seen in many marine organisms, e.g., eudistomins/eudistomidins of the tunicates *Eudistoma* sp. (Rinehart et al., 1987; Murata et al., 1991). Thus, the contryphan series in many ways represents an evolutionary initiative of making natural-product-like peptides, perhaps for biological purposes that overlap with why many marine organisms have evolved biosynthetic pathways for generating natural products. The key difference is that bromocontryphan and related peptides are all translated directly from genetic information, with modified amino acids put in place through post-translational modification of the polypeptide chain.

The discovery of this natural-product-like small peptide highlights a biological puzzle: if natural-product-like molecules can be generated by direct translation followed by a series of post-translational modifications, why is such a pathway not more routinely used in evolution? We suggest that the underlying biology may push this evolutionary initiative only in a specialized situation like the *Conus* venom system. Probably, the bulk of brominated marine natural products (and marine natural products in general) are meant to be defensive chemicals. This would therefore require that the compounds are able to elicit their biological effects even when ingested, a requirement that would be untenable for almost all peptides. However, in a situation where injection of venom into another animal routinely takes place, there would be no constraints in evolving peptides and peptide derivatives that resemble natural products but which are encoded by DNA and mRNA.

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BI962840P