A Novel Conotoxin from *Conus delessertii* with Posttranslationally Modified Lysine Residues[†]

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ABSTRACT: A major peptide, de13a from the crude venom of *Conus delessertii* collected in the Yucatan Channel, Mexico, was purified. The peptide had a high content of posttranslationally modified amino acids, including 6-bromotryptophan and a nonstandard amino acid that proved to be 5-hydroxylysine. This is the first report of 5-hydroxylysine residues in conotoxins. The sequence analysis, together with cDNA cloning and a mass determination (monoisotopic mass of 3486.76 Da), established that the mature toxin has the sequence DCOTSCOTTCANGW̲ECCK̲GYOCVNK̲ACSGCTH*, where O is 4-hydroxyproline, W̲ 6-bromotryptophan, and K̲ 5-hydroxylysine, the asterisk represents the amidated C-terminus, and the calculated monoisotopic mass is 3487.09 Da. The eight Cys residues are arranged in a pattern (C-C-C-CC-C-C-C) not described previously in conotoxins. This arrangement, for which we propose the designation of framework #13 or XIII, differs from the ones (C-C-C-CC-CC-C-C and C-C-C-C-C-CC-C-C) present in other conotoxins which also contain eight Cys residues. This peptide thus defines a novel class of conotoxins, with a new posttranslational modification not previously found in other *Conus* peptide families.

Peptide toxins ("conopeptides") present in the venoms of marine cone snails (family Conidae, genus *Conus*) may be divided into two main structural groups: those with zero or one disulfide bridge and highly disulfide cross-linked peptides with two to five disulfide bridges, conventionally called "conotoxins". The mature toxins found in the venoms are processed from prepropeptide precursors produced by normal ribosomal translation by proteases and other posttranslational modification enzymes. Most of the >50000 different conotoxins belong to a relatively few major gene superfamilies (A, T, O, M, P, I, and S) (*1*); the peptides that belong to a particular superfamily share considerable sequence identity in their signal peptides, and have a specific arrangement of Cys residues within the mature toxin (the "Cys" pattern) (*1*).

A variety of posttranslational modifications have been found in *Conus* peptides. These include amidation of the C-terminus, epimerization to a D-amino acid, O-glycosylation of Ser and Thr, disulfide formation, γ-carboxylation of Glu, bromination of Trp, cyclization of Gln, sulfation of Tyr, and hydroxylation of Pro. These modifications increase the potential molecular diversity accessible to *Conus* peptides, and have been shown to be important for the activity of some of them (*2*, *3*).

In this work, we describe the purification and biochemical characterization of a novel peptide, de13a, from the venom of the worm-hunting snail *Conus delessertii*, collected in the Yucatan Channel, Mexico. This peptide has a posttranslationally modified amino acid residue (5-hydroxylysine, Hyl[1]) not previously described in conotoxins, and eight cysteine residues arranged in a pattern (C-C-C-CC-C-C-C) so far unknown within conotoxins. In addition, it contains an unusually high proportion of amino acid residues with hydroxylated side chains. Thus, this peptide is both the first example of a novel conotoxin superfamily and the first reported with Hyl.

EXPERIMENTAL PROCEDURES

Specimen Collection and Venom Extraction. Specimens of the worm-hunting snail *C. delessertii* were collected by fishing vessels in the Mexican Caribbean Sea off the Yucatan peninsula, and immediately frozen and stored at −70 °C until

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they were used. The venom from four specimens was obtained by dissection of the venom ducts that were homogenized in 5 mL of 0.5% (v/v) trifluoroacetic acid (TFA) in 50% aqueous acetonitrile (ACN). The homogenate was then centrifuged at 17000g for 30 min at 4 °C. The supernatant was kept at −70 °C; it contains the peptides and is termed the venom extract.

Peptide Purification by high-performance liquid chromatography (HPLC). The HPLC system consisted of a model 2800 solvent delivery system (Bio-Rad, Hercules, CA) equipped with a Bio-Dimension UV−vis monitor (Bio-Rad) and a model 7125-081 manual injector (Rheodyne, Cotati, CA). Venom extract was applied onto an analytical reversed-phase C_{18} HPLC column (Vydac, 218TP54, 4.6 mm × 250 mm, 5 μm particle size) provided with a guard column (Vydac, 218GK54, 4.6 mm × 10 mm, 5 μm particle size). The components of the extract were eluted at room temperature at a flow rate of 1 mL/min using a linear gradient from 5 to 95% solution B in 90 min. HPLC solutions were as follows: (A) 0.1% (v/v) TFA in water and (B) 0.085% (v/v) TFA in 90% aqueous ACN. The absorbance was monitored at 220 nm, and the chromatographic peaks were collected manually. The same column was employed for further purification using a linear gradient of solution B increasing by 0.2%/min.

Molecular Mass Determination. Positive ion matrix-assisted laser desorption ionization (MALDI) mass spectra were obtained in the reflector mode with a PerSeptive Biosystems Voyager-DE STR spectrometer (PerSeptive Biosystems/Global Medical Instruments, Clearwater, MN). Samples were dissolved in a 25% ACN/0.1% TFA mixture to give a concentration within the range of 2−20 pmol/μL. An aliquot of 1−2 μL of each sample was mixed with an equal volume of α-cyano-4-hydroxycinnamic acid, and another aliquot of the same size was mixed with an equal volume of cinnapinic acid. Both mixtures were scanned over the m/z range of 500−25000. A laser beam with a wavelength of 337 nm was used. The resulting ions were accelerated through the flight tube to the detector at 25 000 V. Data collection, which was conducted in the reflector mode, and analysis of the resulting data were carried out using version 5.0 of Biospectrometry Work Station in the Windows NT 4.0 operating system. The accuracy of the measurements was estimated to be within 0.01% of the mass on the reflector mode.

Sequence Determination. Disulfide bonds were reduced by incubating equal volumes of the eluted purified peptide fraction and 8 mM tris(2-carboxyethyl)phosphine in 0.25 M Tris-HCl (pH 8.0) for 20 min at room temperature. The reduced peptide was alkylated by adding 1 μL of 4-vinylpyridine per 100 μL of peptide solution. After incubation for 25 min in the dark, the solution was diluted to reduce the ACN concentration. The same column described above was employed to repurify the pyridylethylated peptide, using a linear gradient of solution B increasing by 0.33%/min.

The alkylated peptide was adsorbed onto a polybrene (Biobrene Plus; Applied Biosystems, Foster City, CA)-treated glass fiber filter, and the amino acid sequence was analyzed

by automated Edman degradation on a model 477A protein sequencer (Applied Biosystems), employing the pulsed-liquid method.

An aliquot of the purified native peptide was adsorbed onto a Biobrene-treated glass fiber filter, and the amino acid sequence was analyzed by automated Edman degradation on a Procise 491 protein sequencing system (Applied Biosystems), employing the pulsed-liquid method.

cDNA Cloning. The venom duct from one *C. delessertii* specimen was dissected, and total RNA was isolated by using RNAgents Total RNA Isolation System (Promega, Madison, WI) according to the supplier's instructions. First-strand cDNA synthesis was primed with random hexamers as follows: ∼1 μg of total RNA and 20 pmol of oligo-p(dN)$_6$ were added to a volume of 20 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dNTP mix, 50 units of RNase inhibitor, and ∼20 units of AMV reverse transcriptase (Roche Diagnostics Corp., Indianapolis, IN). The reaction mixture was incubated at 25 °C for 10 min and then at 42 °C for 60 min, followed by a 5 min incubation at 95 °C to inactivate the reverse transcriptase. The cDNA was amplified by Vent DNA polymerase (New England Biolabs, Inc., Beverly, MA) using reverse-transcription products as templates. The following primers were employed: De6.2.Up, 5′-GAYTGYCCNACNWSNTGYC-CNAC-3′, a degenerate oligonucleotide primer based on the Edman-determined amino-terminal region of the mature toxin; and oligo-p(dT)$_{22}$NN, where p(dT)$_{22}$ anneals to the polyA tail from the mRNA and NN anchors the primer to the border between the polyA tail and the 3′-UTR of mRNA. PCR was carried out as follows. To a total 100 μL reaction volume containing 1× ThermoPol Reaction Buffer [20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, and 0.1% Triton X-100] and 0.2 mM dNTP mix were added 5 μL of reverse transcription product and 20 pmol of each primer. The mixture was incubated in the thermal cycler at 95 °C for 5 min, and then 2 units of Vent DNA polymerase was added (Hot Start). The parameters for thermal cycling were as follows: 95 °C for 5 min, then 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s for 35 cycles, and then 72 °C for 5 min (GeneAmp PCR System 2400, Perkin-Elmer, Wellesley, MA). The PCR products were purified with an agarose gel/QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany) and blunt-end ligated with EcoRV-digested pBluescript II KS (+) vector (Stratagene, La Jolla, CA) using a 3:1 molar ratio of insert to vector. The ligation products were electrotransformed into electrocompetent DH5α *Escherichia coli* cells and plated on LB agar plates containing 200 μg/mL X-Gal/IPTG/Amp to select for recombinant colonies. Plasmid DNA was purified with a High Pure Plasmid Isolation Kit (Roche Diagnostics Corp.), and cloned DNA was sequenced in both directions with a 3100 DNA sequencer (Applied Biosystems) using the T3 and T7 primers flanking the cloning site of the vector.

Identification of 5-Hydroxy-L-lysine and 6-Bromo-L-tryptophan. Fifteen microliters of 2 pmol/μL Hyl [(5R)-5-hydroxy-L-lysine dihydrochloride monohydrate (Fluka, Buchs, Switzerland) and mixed DL- and DL-*allo*-δ-Hyl (Sigma, St. Louis, MO)] and bromo-L-tryptophan [6-bromo-L-tryptophan (Biosynth AG, Staad, Switzerland) and 5-bromo-DL-tryptophan (Aldrich, Milwaukee, WI)] dissolved in water were applied either separately or simultaneously to Biobrene Plus-

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[1] Abbreviations: Hyl, 5-hydroxylysine, HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; ACN, acetonitrile; TFA, trifluoroacetic acid.
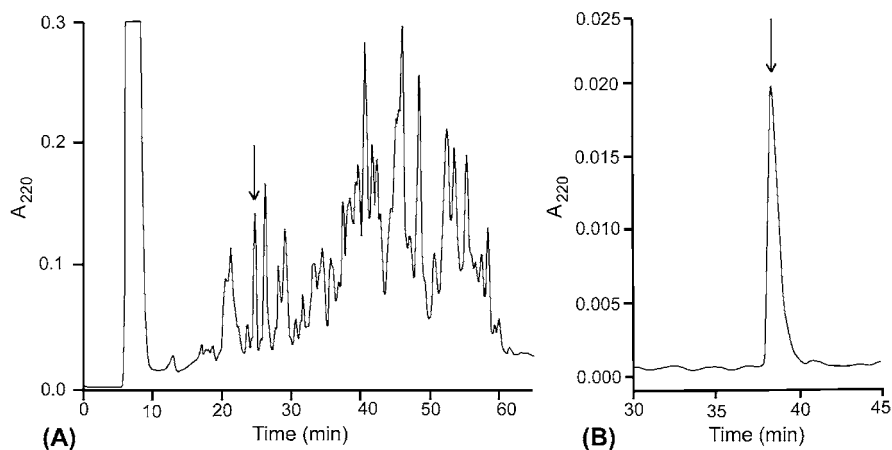
FIGURE 1: Purification of de13a by HPLC. (A) Fractionation of the extract from four venom ducts by an analytical Vydac C_{18} column eluted at room temperature with a linear gradient from 5 to 95% solution B over the course of 90 min at 1 mL/min. HPLC solutions were 0.1% (v/v) TFA in water (solution A) and 0.085% (v/v) TFA in 90% (v/v) aqueous ACN (solution B). The absorbance was monitored at 220 nm. (B) The peak highlighted by the arrow in panel A was further purified on the same column using a linear gradient starting with 10% solution B and increasing solution B by 0.2%/min at a rate of 1 mL/min. The peak highlighted by the arrow in panel B was further characterized.

treated glass fiber filters, and loaded into an Applied Biosystems Procise 491 protein sequencing system for analysis using the pulsed-liquid method.

RESULTS

Peptide Purification and Molecular Mass Determination. Crude venom obtained from the ducts of four *C. delessertii* specimens was fractionated by HPLC on an analytical C_{18} column (Figure 1A). Several absorbance peaks were observed, and one of the major peaks (arrow in Figure 1A) was further purified using the same column; this material was eluted as an apparently homogeneous component (arrow in Figure 1B). Two aliquots of the pure native peptide were employed for the determination of its molecular mass and amino acid sequence. Another portion was used for automatic sequencing after reduction and pyridylethylation of Cys residues.

MALDI mass analysis in the reflector mode yielded a monoisotopic $[M + H]^+$ signal at m/z 3487.76, which corresponds to a monoisotopic molecular mass of 3486.76 Da (Figure 2). A cluster of minor signals was also observed, with individual m/z values ∼16 units lower than those of the corresponding signal in the major cluster. These signals are probably due to a minor proportion of the peptide with a Pro residue at position 3, instead of 4-hydroxyproline (4-hydroxy-Pro) which is the major amino acid at this position (see Sequence Determination). This spectrum exhibited an unusual isotope ratio (see m/z signals at 3491.72 and 3493.73) which could be explained by the presence of a bromine atom (*4*). The bromination of Trp at position 6 of the indole ring has been reported in several conotoxins (*5−10*).

Sequence Determination. The homogeneous product obtained by reversed-phase HPLC on a C_{18} analytical column after reduction and pyridylethylation of the purified peptide (data not shown) was sequenced using standard Edman methods. Essentially, a single sequence of 32 amino acid residues was obtained; however, at position 3, a small amount of PTH-proline was observed in addition to PTH-4-hydroxy-Pro which was clearly the major derivative. At position 14, a very hydrophobic, nonstandard PTH derivative that eluted
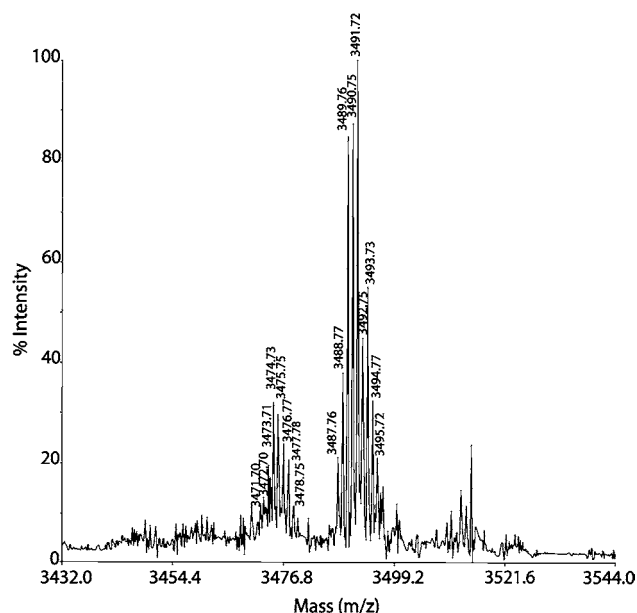

FIGURE 2: MALDI (reflector mode) mass spectrum of peptide de13a from the venom extract of *C. delessertii*.

after PTH-Leu (∼3 min, in the Procise sequencer) was observed; several posttranslationally modified hydrophobic amino acids could be responsible for the PTH derivative at this position: *p*-chloro-Phe, diiodo-Tyr, *N*-ϵ-methyl-Lys, *N*-ϵ-dimethyl-Lys (*11*), and 6-bromotryptophan (6-bromo-Trp) (E. C. Jimenez, Department of Biology, University of Utah, personal communication). Another nonstandard PTH derivative at positions 18 and 25 eluted slightly later than PTH-Val and just before diphenylthiourea (DPTU), and probably corresponds to PTH-Hyl (*11, 12*). At these positions, the peak height of this unknown derivative was lower than that of both the preceding and following residues, and this behavior has been reported for the Hyl residue at position 277 of recombinant human tissue plasminogen activator (*12*).

If one assumes that the eight Cys residues form four disulfide bridges and that positions 18 and 25 are occupied by Hyl residues, the calculated monoisotopic mass for the sequence determined chemically (except for position 14) is

A

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1              20              40              60
gattgcccgacgaggtgtccaacgacttgtgcgaacggctgggaatgctgcaaaggatac
 D  C  P  T  R  C  P  T  T  C  A  N  G  W  E  C  C  K  G  Y

         80             100             120
ccctgtgtcaacaaagcttgttctgggtgtacccatggcaaatagaacagtggtgttgta
 P  C  V  N  K  A  C  S  G  C  T  H  G  K  *

                140             160             180
tgacagtctgtcctgtcacagccaacttcttcgccttctcactagtcatcataaagccgt

                200             220             240
gtaggtcttgtttgatttgaaatagcgtagagaataaaacttgcaacgacaaagctaaaa

                260             280
aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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B

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            10            20            30
de13a    DCOTSCOTTCANGWECCKGYOCVNKACSGCTH*
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Experimental monoisotopic mass: 3,486.76 Da
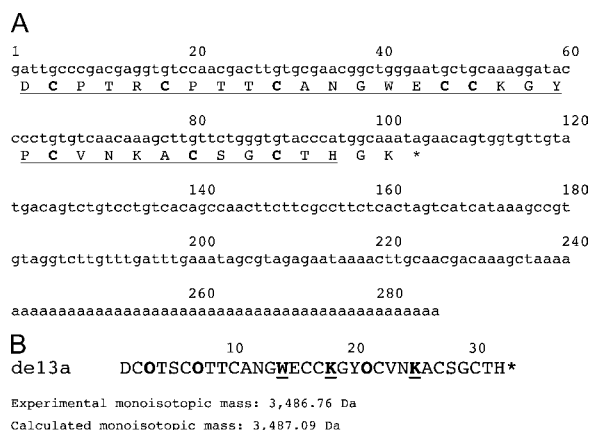Calculated monoisotopic mass: 3,487.09 Da

FIGURE 3: (A) Cloned cDNA sequence corresponding to the region encoding peptide de13a and the deduced amino acid sequence. The one-letter codes are employed for deoxyribonucleotides and standard amino acids. The residues present in the mature toxin are underlined. (B) Sequence of peptide de13a from *C. delessertii*. For posttranslational modifications, O represents hydroxyproline, W̲ 6-bromo-L-tryptophan, K̲ 5-hydroxy-L-lysine, and the asterisk the amidated C-terminus.

3224.08 Da for a free C-terminus, and 3223.10 Da for an amidated C-terminus. These theoretical values differ from the experimental monoisotopic mass (3486.76 Da) by 262.67 and 263.66 Da, respectively. Thus, the mass of the unknown residue at position 14 is close to the monoisotopic masses of monoglycosylated threonine (263.09 Da, if the carbohydrate moiety is a hexose residue) and bromotryptophan (263.99 Da). A very low signal for Thr was obtained at the position where contulakin-G contains a hexose-*N*-acetylhexosamine-Thr (*13*), and other types of glycosylated Thr residues have also been reported to deglycosylate with successive sequencing cycles (*11*). In the case of peptide de13a, not even a small increase in the signal for Thr was observed at position 14, which suggests that this position is not occupied by a glycosylated Thr, since some degree of deglycosylation would have been expected after the exposure to acid during 14 sequencing cycles.

Thus, the combined data from the mass spectrometry and chemical sequencing analyses suggest that position 14 is occupied by a bromotryptophan residue (probably 6-bromo-Trp), and are consistent with the presence of Hyl residues at positions 18 and 25.

cDNA Cloning. As a result of the cDNA cloning, the sequence of the toxin beyond the eighth amino acid position was confirmed and clarified at the C-terminus (Figure 3A). For position 5, the WSN codon from the oligonucleotide primer generated different sequences in several distinct clones, which prevented the confirmation of the fifth amino acid. However, during the chemical sequencing, this position was unambiguously identified as a Ser residue. All the sequenced clones showed a Trp residue at position 14, which was not observed during the direct Edman sequencing, supporting the potential posttranslational modification of this residue to bromotryptophan in the mature conotoxin, as suggested by the Edman sequence analysis and the mass spectrum. The cDNA cloning also showed the presence of Lys residues at positions 18 and 25, which were not observed during the direct chemical sequencing, consistent with posttranslational modification of these residues to hydroxylysine in the mature conotoxin, as suggested by the Edman
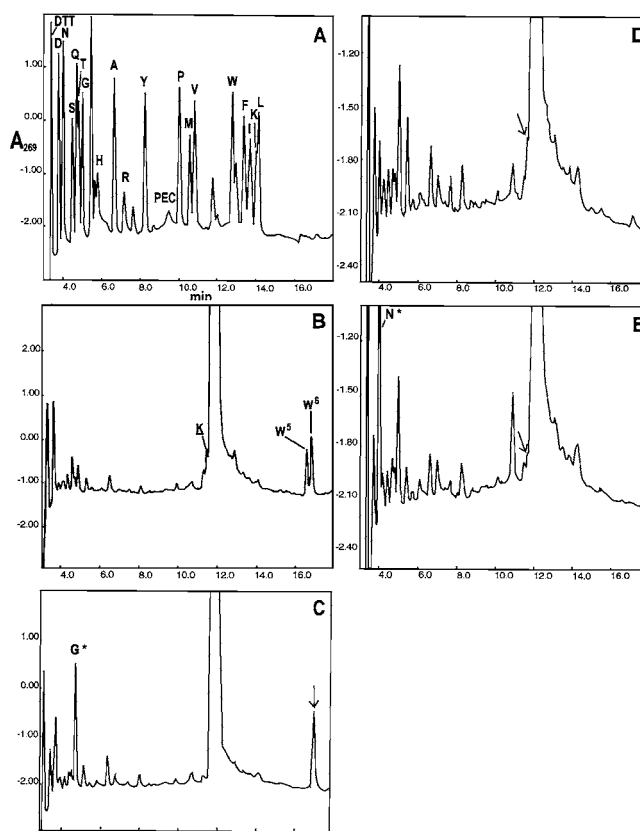

FIGURE 4: Identification of 6-bromo-L-tryptophan and 5-hydroxy-L-lysine in peptide de13a. (A) Chromatographic profile of a standard mixture of PTH derivatives (20 pmol each) of the 20 genetically encoded amino acids; the one-letter code is employed. (B) Standards of PTH-6-bromo-L-tryptophan (W[6]), PTH-5-bromo-DL-tryptophan (W[5]), and PTH-(5R)-5-hydroxy-L-lysine (K̲) generated and chromatographed simultaneously under the conditions of automatic sequencing (Procise 491 protein sequencing system). (C) PTH derivative (arrow) obtained at position 14 of native peptide de13a. (D) PTH derivative (arrow) obtained at position 18. (E) PTH derivative (arrow) obtained at position 25.

sequencing. The carboxy-terminal sequence of the encoded peptide was His-Gly-Lys, where the Gly residue followed by a basic residue (Lys) suggests the His residue at position 32 is a potential amidation site in the mature toxin (*14*). Therefore, the data from the mass spectrometry analysis (monoisotopic mass, 3486.76 Da), the chemical sequencing, and the cDNA cloning are consistent with a 32-residue, amidated mature toxin with one bromotryptophan and two hydroxylysine residues (theoretical monoisotopic mass, 3487.09 Da) (Figure 3B).

Identification of 5-Hydroxy-L-lysine and 6-Bromo-L-tryptophan. To identify the type of bromotryptophan residue and to confirm the Hyl residue suggested by the data from the mass spectrometry and Edman sequencing analyses and supported by the cDNA cloning, the PTH derivatives of Hyl and 5-bromo-DL- and 6-bromo-L-tryptophan were synthesized and compared to the nonstandard PTH amino acids observed at cycles 14, 18, and 25 during the automatic sequencing of peptide de13a. The hydrophobic derivative observed at position 14 (Figure 4C) had the same retention time as the standard of PTH-6-bromo-L-tryptophan (Figure 4B). PTH-6-bromo-Trp is more hydrophobic than any standard PTH-amino acid (Figure 4A) and that PTH-5-bromo-DL-tryptophan (Figure 4B). Thus, these results clearly indicate that the amino acid residue at position 14 is 6-bromo-L-tryptophan.

Table 1: Comparison of the Arrangement of Cys Residues of Peptide de13a with Those of the Other Eight Cys-Containing Conotoxins[a]

gene superfamily	cysteine arrangement	disulfide connectivity	framework	peptide scaffold	conotoxin families
I	C-C-CC-CC-C-C	?	XI	I-1	κI
?	C-C-C-C-CC-C-C	?	?	?	?
?	C-C-C-CC-C-C-C	?	XIII	?	? (this work)

[a] A hyphen denotes one or more non-Cys residues. Where known, disulfide connectivities, frameworks/scaffolds, and corresponding pharmacological families are also listed.

The unknown derivative at positions 18 and 25 (Figure 4D,E) had the same retention time as the standard of PTH-(5R)-5-hydroxy-L-lysine (Figure 4B). Thus, these results confirm that the amino acid residue at positions 18 and 25 is 5-hydroxy-L-lysine. Although it is likely that the 5-hydroxy-L-lysine is the same as that found in collagen (*15*) and styelin D (*16*), our data do not eliminate the possible presence of L-*allo*-Hyl [(5S)-Hyl]. The chromatographic conditions employed in the protein sequencer did not permit discrimination between PTH-(5R)-Hyl and any of the pairs of diastereoisomers present in the standard from Sigma (mixed DL- and DL-*allo*-δ-hydroxylysine).

DISCUSSION

Here we report the purification and characterization of a novel peptide, de13a, from the venom of the vermivorous species, *C. delessertii*, from the Yucatan Channel in the West Atlantic. On the basis of the amino acid sequencing data, we cloned a cDNA corresponding to the mature toxin, which confirmed the sequence and also predicted amidation of the C-terminus. This peptide has two novel features. (1) It contains a posttranslational modification not previously reported in conotoxins, the hydroxylation of lysine residues, and (2) its eight-Cys framework differs from any previously described in other conotoxins (*17*−*20*). Thus, the peptide defines a new conotoxin superfamily and a new Cys framework.

The peptide characterized here contains an unusually high proportion (37.5%) of amino acid residues with hydroxylated side chains. The most unusual, (5R)-5-Hyl (*21*, *22*), is a posttranslationally modified amino acid that occurs in small amounts in collagens, where it serves to form attachment sites for polysaccharides and to stabilize intermolecular cross-links (*23*). Since its isolation from gelatin (*15*), Hyl has also been found in collagen-like domains of a few proteins from several mammalian species, such as the C1q subcomponent of complement, acetylcholinesterase, mannose binding proteins, and conglutinin (*24*). It is also present in recombinant human tissue plasminogen activator and CD4 receptor (*12*), anglerfish (*Lophius americanus*) somatostatin-28 (*25*), mouse adiponectin (*26*), silkworm cecropin B (*27*), rat pulmonary surfactant-associated protein D precursor (*28*), sea squirt styelin D (*16*), and silaffin-1A(1) precursor from the marine diatom *Cylindrotheca fusiformis* (*29*). The cross-links formed from Hyl-derived aldehyde are more stable than those formed from Lys-derived aldehyde (*30*), and the Hyl residues are less susceptible to digestion by trypsin and lysyl endopeptidase (*31*). Hyl residues are generated by a hydroxylase enzyme complex that recognizes an Xaa-Lys-Gly consensus sequence available on the surface of the collagen molecule (*24*) or recombinant human tissue plasminogen activator and CD4 receptor (*12*). In some collagens, Hyl residues can be found in the short nonhelical regions at the termini of the α-chain, within the Xaa-Hyl-Ala and Xaa-Hyl-Ser sequences (*24*). We found that peptide de13a contains two Hyl residues at positions 18 and 25, within the Cys-Hyl-Gly and Asn-Hyl-Ala sequences, respectively. This location is consistent with other Hyl modification sites (*12*, *24*). We can only speculate about the role of this uncommon amino acid. The Hyl residues could provide additional sites for hydrogen bonds for binding the molecular target, or they may participate in O-glycosidic linkages as they do in collagens. At this point, there is no evidence for the existence of glycosylated variants of peptide de13a.

The other posttranslationally hydroxylated amino acid found in de13a is well-known in two structural proteins: *trans*-4-hydroxy-Pro occurs with a high frequency in collagens, the most abundant vertebrate proteins, and also in elastin at low frequency (*23*). In collagens, the hydroxyl group of *trans*-4-hydroxy-Pro participates in the stabilization of the structure of the triple helix by providing additional sites for hydrogen bond formation (*32*). Within conotoxins, hydroxylation of Pro to *trans*-4-hydroxy-Pro was first discovered in μ-conotoxin GIIIA (*33*); although a number of conotoxins have been shown to contain this modified residue, its role is unclear. For example, some underhydroxylated variants of μ-conotoxins GIIIA and GIIIB are biologically active (*34*). These residues could conceivably contribute sites for hydrogen bonds, as they are thought to do in collagens, for the interactions of conotoxins with molecular targets.

The other hydroxylated residues in de13a are standard amino acids (T, S, and Y). One example of the potential importance of hydroxylated residues is the case of ω-conotoxin GVIA, where the hydroxyl group of Tyr-13 is essential for binding to N-type Ca^{2+} channels and for biological activity on rat hippocampal neurons (*35*).

Peptide de13a also contains one tryptophan residue brominated at position 6 (6-Br-Trp), a posttranslational modification that occurs in relatively few *Conus* peptides, including the bromosleeper peptide (*5*), the bromoheptapeptide (*5*), bromocontryphan (*6*), σ-conotoxin GVIIIA (*7*), peptide tx5a/ε-TxIX (*8*, *9*), and the light sleeper peptide (*10*). These apparently unrelated peptides vary in size from 7 to 41 residues and contain from one to three brominated Trp residues. No specific function has been correlated with the presence of the 6-Br-Trp residue in any of these peptides so far. This posttranslationally modified amino acid was discovered in bovine neuropeptide B (*36*, *37*) and in sea squirt styelin D (*16*) after its elucidation in conotoxins.

Chemical synthesis and folding of the de13a peptide is in progress; with the high density of posttranslational modification, expression is not feasible. However, successful synthesis of biologically active de13a will be a formidable challenge

given the number of modified amino acids and disulfide bonds in the peptide. Chemical synthesis could confirm that the Hyl residues in de13a are the same as in collagen, and provide enough material for a thorough evaluation of the biological activity of the peptide. Successful synthesis would also be prerequisite to initiating structure−function studies for evaluating the role of the unusual posttranslational modifications found in this peptide.

Peptides with eight Cys residues had been isolated only from the venom of five species of cone snails: κ-BtX from *Conus betulinus* (*17*), ViTx from *Conus virgo* (*18*), r11a, r11b, r11c, r11d, and r11e from *Conus radiatus* (*19*), and peptides GlaMrII and GlaTxX from *Conus marmoreus* and *Conus textile*, respectively (*20*). The sequences of 13 additional peptides have been predicted from a cDNA library from the venom duct of *C. radiatus* (*19*). Some of these conotoxins have been shown to target Ca^{2+}- and voltage-sensitive BK channels (*17*), and Kv1.1 and Kv1.3 channels (*18*), and they have excitatory effects in frog cutaneus pectoris muscle and in the central nervous system of mice (*19*). Despite the target specificity, the distribution of Cys residues within the peptides from *C. betulinus*, *C. virgo*, and *C. radiatus* consists of two pairs of adjacent Cys residues flanked on each side by two nonadjacent Cys residues (C-C-CC-CC-C-C, where a hyphen denotes one or more non-Cys residues); this arrangement has been designated as framework #11 or XI in the previous nomenclature, and scaffold I-1 in the new nomenclature (*38*), and it defines the I superfamily of conotoxins (*19*) (Table 1). Peptides GlaMrII and GlaTxX contain their eight Cys residues in the pattern C-C-C-C-CC-C-C (*20*). The arrangement of the eight Cys residues of peptide de13a (C-C-C-CC-C-C-C) from the vermivorous species *C. delessertii* is clearly distinct from the one present in the peptides included in the I superfamily, and it differs from the one in peptides GlaMrII and GlaTxX. Thus, peptide de13a defines a novel conotoxin family with a new Cys framework, for which we propose the designation of framework #13 or XIII (Table 1). Although the molecular target and biological role of peptide de13a are yet to be determined, the new conotoxin family is already notable in having a previously unreported posttranslationally modified amino acid, 5-hydroxylysine.

ACKNOWLEDGMENT

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