REVIEW

D-amino acid-containing peptides in *Conus* venoms

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D-amino acids have been documented in relatively few gene products, including peptides found in *Conus* venoms. The present work provides an overview of various D-amino acid-containing *Conus* peptides (conopeptides or conotoxins), such as the contryphans, conomarphins, conophans, conomap and certain I_1-superfamily conotoxins. Characterization of their sequences and structures, as well as the known physiological targets are presented. Some contryphans and one I_1-superfamily conotoxin were found to bind specific ion channels, thus making them useful ligands to elucidate further the ion channel subtypes. *Conus* venom has notable diversity of peptides, of which the D-amino acid-containing peptides could serve as lead compounds or important scaffolds for the development of neuropharmacological agents. Future research will hopefully elucidate the specific physiological targets and mechanisms of action, as well as the exact biological roles in *Conus* venoms, of more members of these conopeptide families.

KEYWORDS

D-amino acid, *Conus* venom, conopeptide or conotoxin, contryphan, conomarphin, conophan, conomap, I_1-superfamily conotoxin

INTRODUCTION

Many organisms have evolutionarily adopted strategies for their survival. A remarkable strategy of some predators, particularly the cone snails (genus *Conus*), is their venom that is employed to immobilize prey. The venom provides a distinctive and abundant resource of molecular diversity as it is propelled by evolutionary pressure to enhance prey capture, protect the organism, or enable the organism to compete.

Figure 1 Shells of some *Conus* species from which D-amino acid-containing peptides have been characterized. Left to right: *Conus textile*, the textile cone or the cloth of gold cone; *Conus radiatus*, the radial cone; *Conus marmoreus*, the marbled cone.

The genus *Conus* comprises a large group of cone snails (Figure 1) with over 700 species that inhabit tropical and subtropical marine waters. They are classified as fish-hunting, mollusc-hunting and worm-hunting species in accordance with prey type. The venoms of these gastropod animals consist of a mixture of peptide neurotoxins, known as conopeptides or conotoxins, that they use for predation, defense and competition (Olivera 1997, Terlau and Olivera, 2004).

Conotoxins are generally small peptides with about 10-50 amino acid residues. They have diverse structures and biological functions as they target various membrane-bound receptors or ion channels in the nervous system. Their moderately small size, structural stability and target specificity render them the potential for development as neuropharmacological agents (Olivera 2006, Olivera and Teichert 2007, Teichert et al. 2009).

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Date Received: 10 April 2017
Date Revised: 15 June 2017
Date Accepted: 20 June 2017

Table 1: D-amino acid-containing peptides from various animal sources

Peptide	Sequence	Source	Reference
Dermorphin	YAFGYPS*	*P. sauvagei*	Monteccuchi et al. 1981
Met-deltorphin	YMFHLMD*	*P. sauvagei*	Kreil et al. 1989, Mor et al. 1989
Achatin-1	BFAD	*A. fulica*	Kamatani et al. 1989
Fulicin	FNEFV*	*A. fulica*	Ohta et al. 1991
Mytilus-FFRFamide	ALAGDHFFRF*	*M. edulis*	Fujisawa et al. 1992
ovCNP-39	LLHDHPNPRKYKPANKKGLSKGCFGLKLDRIGSTSGLGC	*O. anatinus*	de Plater et al. 1998 Torres et al. 2002
ω-Agatoxin TK	EDNCIAEDYGKCTWGGTKCCRGRPCRCSMIGTNCECTPRLIMEGLSFA	*A. aperta*	Kuwada et al. 1994

Underlined letters indicate D-amino acids. The asterisk stands for C-terminal amidation.

Several families of *Conus* peptides contain D-amino acids. Aside from the D-amino acids in conopeptides that are produced through the normal ribosomal pathway, D-amino acids have been reported in fairly few gene products (Table 1). Two 7-residue peptides, dermorphin and Met-deltorphin with D-Ala2 and D-Met2, respectively, were isolated from the frog *Phyllomedusa sauvagei* (Kreil et al. 1989, Monteccuchi et al. 1981, Mor et al. 1989). Isolated from the snail *Achatina fulica* were achatin-I and fulicin with four residues including D-Phe2 and five residues including D-Asn2, respectively (Kamatani et al. 1989, Ohta et al. 1991). Mytilus-FFRFamide, a 10-residue peptide from the mussel *Mytilus edulis*, has D-Leu2 (Fujisawa et al. 1992). The 39-residue C-type natriuretic peptide ovCNP-39 isolated from the platypus *Ornithorhynchus anatinus* has D-Leu2 (de Plater et al. 1998, Torres et al. 2002). The ω-agatoxin from the spider *Agelenopsis aperta* contains 48-residues with D-Ser46 (Kuwada et al. 1994). The crustacean hyperglycemic hormones, [D-Phe3]cHHA and [D-Phe3]cHHB from the lobster *Homarus americanus*, have D-Phe at position 3 of the 72-residue peptides. The corresponding isoforms, cHHA and cHHB, with L-Phe3 were also identified in the lobster (Soyez et al. 1994).

Unlike other post-translationally modified amino acids, a D-amino acid is not readily detectable by standard proteomic techniques, such as mass spectrometry and Edman sequencing. To be able to predict when the post-translational modification might arise, characterization and classification of different natural peptides containing D-amino acids is necessary.

This paper provides an overview of the various families of *Conus* peptides that contain D-amino acids. A description of their structural features and known target specificities is presented. These conopeptides are useful tools for neuroscience research and may have the potential for drug development.

THE L- TO D-ISOMERIZATION PROCESS

The isomerization of L- to D-amino acid is a post-translational modification that involves a change of chirality at the α-carbon. This process is catalyzed by an enzyme having aminoacyl-L/D-isomerase activity. Genes that potentilly code for the isomerase and related polypeptides have been found in mammalian and other vertebrate species (Jilek et al. 2012). A serine isomerase identified in the venom of the spider *Agelenopsis aperta* presented the first major evidence to describe how a multicellular organism is able to incorporate a D-amino acid into peptides. The enzyme has the capability to isomerize Ser, Ala, Cys and O-methylSer residues within the peptide chains, either way from the L- or the D-amino acid form. The peptides share a common recognition site Leu-Xaa-Phe-Ala. A two-base mechanism was indicated, in which abstraction of a proton from one face occurs simultaneously with the delivery by the conjugate acid of the second enzymic base from the opposite face (Heck et al. 1996).

Conversion of the chirality of a single amino acid residue within a peptide chain is indicated to be a supplementary mechanism resulting in structural and functional divergence of peptides. This occurrence has been studied at the cellular level in a neuroendocrine organ that generates a mixture of isoforms of the crustacean hyperglycemic hormone (Soyez et al. 1994, 2000). The amino acid isomerization was shown to arise in the perikarya of fully specialized neurosecretory cells, after propeptide cleavage, as a late step in the maturation of the hormone precursor (Soyez et al. 2000).

CONTRYPHANS

All known contryphans maintain five-residue intercysteine loop, with a D-amino acid within the loop. The contryphan structural motif embodies a conserved molecular framework whose main structural elements are the size of the intercysteine loop and the presence of a D-amino acid.

Sequence characterization of contryphans

The contryphan family appears widely distributed in venoms of fish-hunting *Conus* species (Table 2). The presence of D-amino acid in a normally translated peptide was initially discovered in venom of *Conus radiatus*. Characterization of this peptide that was later designated as contryphan-R revealed the following sequence: GCOWEPWC*, where W is D-Trp, O is 4-hydroxyproline (Jimenez et al. 1996). The presence of a D-Trp was confirmed by synthesis of contryphan-R, as well as mass spectrometry, HPLC coelution and bioassay of the natural and synthetic contryphan-R. Des[Gly1]contryphan (COWEPWC*) that appears to be a degradation product obtained by N-proteolysis of Gly in contryphan-R was also isolated from *Conus radiatus* venom (Jimenez et al. 1996). Bromocontryphan (GCOWEPXC*, where X is 6-bromotryptophan) was identified from a cDNA library prepared from *Conus radiatus* venom duct. It appears to be a post-translational product of contryphan-R in which Trp7 has been converted to 6-bromotryptophan (Jimenez et al. 1997). Putative contryphan-S (GCOWEPWC*) that is identical to contryphan-R was identified by expressed sequence tags generated from cDNA library prepared from venom duct of *Conus striatus* (Pi et al. 2006a). Contryphan-P was identified in *Conus purpurascens* venom duct by cDNA cloning method while contryphan-Sm was purified from *Conus stercusmuscarum* venom. The sequences of contryphan-P (GCOWDPWC*) and contryphan-Sm (GCOWQPWC*) differ from that of contryphan-R in the presence of Asp5 in contryphan-P and Gln5 in contryphan-Sm, instead of Glu5 (Jacobsen et al. 1998). Putative contryphan-Bu (COWSPWC*) found in *Conus bulatus* venom duct through transcriptome sequencing differs from des[Gly1]contryphan in the presence of Ser4 instead of Glu4 (Hu et al. 2011).

Further studies showed that contryphans occur broadly as well in mollusc-hunting *Conus* species (Table 2). The cDNA clones encoding contryphans from *Conus textile* venom were identified as contryphan R/Tx (GCOWEPWC*) that is identical to contryphan-R, and contryphan-Tx (GCOWQPYC*) in which Gln5 and Tyr7 are present instead of Glu5 and Trp7 in contryphan R/Tx (Jimenez et al. 2001). Contryphan-P/Am (Am975) (GCOWDPWC*) that is identical to contryphan-P was isolated from *Conus amadis* venom (Gowd et al. 2005,

Table 2: Contryphans

Peptide	Sequence	*Conus* species	Prey	Reference
Contryphan-R	GCOWEPWC*	*C. radiatus*	Fish	Jimenez et al. 1996
Des[Gly1]contryphan	COWEPWC*	*C. radiatus*	Fish	Jimenez et al. 1996
Bromocontryphan	GCOWEPXC*	*C. radiatus*	Fish	Jimenez et al. 1997
Contryphan-S	GCOWEPWC*	*C. striatus*	Fish	Pi et al. 2006a
Contryphan-P	GCOWDPWC*	*C. purpurascens*	Fish	Jacobsen et al. 1998
Contryphan-Sm	GCOWQPWC*	*C. stercusmuscarum*	Fish	Jacobsen et al. 1998
Contryphan-Bu	COWSPWC*	*C. bullatus*	Fish	Hu et al. 2011
Contryphan R/Tx	GCOWEPWC*	*C. textile*	Mollusc	Jimenez et al. 2001
Contryphan-Tx	GCOWQPYC*	*C. textile*	Mollusc	Jimenez et al. 2001
Contryphan-P/Am	GCOWDPWC*	*C. amadis*	Mollusc	Gowd et al. 2005
Glacontryphan-M	NγSγCPWHPWC*	*C. marmoreus*	Mollusc	Hansson et al. 2004
Contryphan Ar1313	ESγCPWHPWC*	*C. araneosus*	Mollusc	Vijayasarathy et al. 2017
Contryphan Ar1304	ESγCPWKPWC*	*C. araneosus*	Mollusc	Vijayasarathy et al. 2017
Contryphan Ar1175	SγCPWKPWC*	*C. araneosus*	Mollusc	Vijayasarathy et al. 2017
Contryphan Ar1131	SECPWKPWC*	*C. araneosus*	Mollusc	Vijayasarathy et al. 2017
Contryphan Ar1260	ESECPWKPWC*	*C. araneosus*	Mollusc	Vijayasarathy et al. 2017
Contryphan-Vc2	CRWTPVC*	*C. victoriae*	Mollusc	Robinson et al. 2014
Contryphan-Lt	GCOWEPWC*	*C. litteratus*	Worm	Pi et al. 2006b
Contryphan-Ca#	GCOWEPWC*	*C. caracteristicus*	Worm	Thakur and Balaram 2007
Contryphan-Le#	GCOWEPWC*	*C. leopardus*	Worm	Thakur and Balaram 2007
Contryphan Fr975	GCOWDPWC*	*C. frigidus*	Worm	Vijayasarathy et al. 2017
Contryphan Fr965	GCOWDSWC*	*C. frigidus*	Worm	Vijayasarathy et al. 2017
Contryphan-Be#	VVGCOWQPWC*	*C. betulinus*	Worm	Thakur and Balaram 2007
Contryphan-Ze#	VVGCOWQPWC*	*C. zeylanicus*	Worm	Thakur and Balaram 2007
Contryphan-Fi#	VVGCOWQPWC*	*C. figulinus*	Worm	Thakur and Balaram 2007
Contryphan fib	GCOWMPWC*	*C. figulinus*	Worm	Rajesh 2015
Contryphan fic	GCPWDPWC	*C. figulinus*	Worm	Rajesh 2015
Contryphan fid	CPWDPWC	*C. figulinus*	Worm	Rajesh 2015
Contryphan-Lo	GCPWDPWC*	*C. loroisii*	Worm	Gowd et al. 2005
Contryphan-Lo2#	NECPWQPWC*	*C. loroisii*	Worm	Vijayasarathy et al. 2017
Contryphan-Vn	GDCPWKPWC*	*C. ventricosus*	Worm	Massilia et al. 2001
Contryphan 72327	QSGCPWHPWC*	*C. pulicarius*	Worm	Lluisma et al. 2012
Leu-contryphan-P	GCVLLPWC	*C. purpurascens*	Fish	Jacobsen et al. 1999
Leu-contryphan-Tx	CVLYPWC*	*C. textile*	Mollusc	Jimenez et al. 2001
Contryphan-In	GCVLYPWC*	*C. inscriptus*	Worm	Gowd et al. 2005

O, 4-hydroxyproline; W, D-tryptophan; X, 6-bromotryptophan; L, D-leucine; γ, γ-carboxyglutamate; *, C-terminal amidation.
The two cysteine residues form a disulfide bond.
#The contryphans were provisionally designated with conventional two-letter symbols, if unnamed in the cited original work.

Sabareesh et al. 2006). Glacontryphan-M (NγSγCPWHPWC*, where γ is γ-carboxyglutamate) isolated from venom of *Conus marmoreus* has two γ-carboxyglutamate (Gla) residues outside the intercysteine loop, Pro instead of Hyp and basic His residue within the intercysteine loop (Hansson et al. 2004). At least five contryphans, namely, Ar1313 (ESγCPWHPWC*), Ar1304 (ESγCPWKPWC*), Ar1175 (SγCPWKPWC*), Ar1131 (SECPWKPWC*), and Ar1260 (ESECPWKPWC*), were detected in *Conus araneosus* by transcriptomic and mass spectrometric analyses. They have a high degree of homology to glacontryphan-M in which the N-terminal segment has Glu and/or Gla residues, and the intercysteine loop has His or Lys residue (Vijayasarathy et al. 2017). Contryphan-Vc2 (CRWTPVC*) was characterized from cDNA library generated from venom gland of *Conus victoriae*. It differs from most contryphans as it has Arg residue instead of Pro or Hyp, Thr instead of acidic or basic amino acid residue, and Val instead of another Trp in the intercysteine loop (Robinson et al. 2014).

Moreover, contryphans were discovered in worm-hunting *Conus* species (Table 2). By analyzing expressed sequence tags derived from *Conus litteratus* venom duct, contryphan-Lt (GCOWEPWC*) that is identical to contryphan-R/Tx was detected (Pi et al. 2006b). *Conus caracteristicus* and *Conus leopardus* contryphans (designated as contryphan-Ca and contryphan-Le, respectively) (GCOWEPWC*) that are identical to contryphan-R/Tx were detected by intact peptide fragmentation and rapid mass spectral analysis (Thakur and Balaram 2007). Contryphans Fr975 (GCOWDPWC*) and Fr965 (GCOWDSWC*) were discovered in *Conus frigidus* by transcriptomic and mass spectrometric methods. Fr975 is identical to contryphan-P/Am (Am975) while Fr965 has Ser6 instead of Pro6 (Vijayasarathy et al. 2017). Identical contryphans (VVGCOWQPWC*) detected by mass spectroscopy in *Conus frigidus*, *Conus betulinus* and *Conus zeylanicus* (designated as contryphans-Fi, Be and Ze, respectively) are homologous to contryphan-Sm but the N-terminal segment has two Val residues adjacent to the Gly residue (Thakur and Balaram 2007). *Conus figulinus* venom revealed contryphans-fib (GCOWMPWC*), fic (GCPWDPWC) and fid (CPWDPWC) that share homology with contryphan-P/Am, except that fib has Met5 instead of Asp5, contryphan-fic has Pro3 instead of Hyp3 and has no C-terminal amidation, while contryphan-fid appears to be a degradation product obtained by N-proteolysis of Gly in contryphan-fic (Rajesh 2015). Contryphan-Lo (Lo959) (GCPWDPWC*) isolated from venom of *Conus loroisii* differs from contryphan-P/Am in that Pro3 instead of Hyp3 is found in contryphan-Lo (Gowd et al. 2005, Sabareesh et al. 2006). With transcriptomic and mass spectrometric methods, contryphan (NECPWQPWC*) was

identified in *Conus loroisii* (designated as contryphan-Lo2). It shares homology with contryphan-Sm except that contryphan-Lo2 has Pro instead of Hyp and N-terminal segment Asn1Glu2 instead of Gly1 (Vijayasarathy et al. 2017). Contryphan-Vn (GDCPWKPWC*) purified from *Conus ventricosus* venom is homologous to Ar1304, Ar1175, Ar1131 and Ar1260 in the intercysteine loop sequence, including the presence of basic Lys residue (Massilia et al. 2001). From *Conus pulicarius*, contryphan 72327 (QSGCPWHPWC*) was identified by next-generation sequencing of venom duct transcriptome. The putative sequence within the intercysteine loop shares homology with glacontryphan-M and contryphan-Ar1313 but the N-terminal segment lacks acidic Glu and Gla residues (Lluisma et al. 2012).

A contryphan subfamily constitutes Leu-contryphans that contain D-Leu (Table 2). The presence of D-Leu in a conopeptide was first discovered in Leu-contryphan-P (GCVLLPWC, where L is D-Leu) purified from venom of *Conus purpurascens*. Leu-contryphan-P has a high density of hydrophobic amino acids in the intercysteine loop and lacks C-terminal amidation (Jacobsen et al. 1999). Leu-contryphan-Tx (CVLYPWC*) was isolated from venom of *Conus textile*. Compared to Leu-contryphan-P, Leu-contryphan-Tx lacks Gly1, has Tyr instead of Leu and is amidated at the C-terminus (Jimenez et al. 2001). Contryphan-In (In936) (GCVLYPWC*) isolated from *Conus insidious* venom differs from Leu-contryphan-Tx in the presence of Gly1 (Gowd et al. 2005, Sabareesh et al. 2006).

Structural characterization of contryphans

Several contryphans showed interconversion between conformational states. Contryphan-R and contryphan-Sm with Hyp3 and D-Trp4 exhibited two peaks under reverse-phase HPLC conditions, indicating interconversion between two distinct conformations. In contrast, [L-Trp4]contryphan-R and [L-Trp4]contryphan-Sm showed a single, broad peak that eluted later than the corresponding natural contryphans (Jacobsen et al. 1998). Contryphan-Tx similarly revealed two peaks under reverse-phase HPLC conditions (Jimenez et al. 2001). Contryphan-Am (Am 975) whose structure is identical to that of contryphan-P, as well as contryphan-Lo (Lo959) also showed conformational interconversion in reverse-phase HPLC conditions (Sabareesh et al. 2006). Leu-contryphan-P that has Val3 and D-Leu4 instead of Hyp3 and D-Trp4 exhibited only a single peak that eluted much later than the other contryphans (Jacobsen et al. 1999). Similarly, Leu-contryphan-Tx exhibited a single peak (Jimenez et al. 2001).

In contryphans, the peptide cyclization through a single disulfide bond, the isomerization from L to D form of an amino acid residue, and the presence of conserved Pro residue confer a stable and unique structure in solution. NMR and ultraviolet resonance Raman spectroscopy (UVRR) are particularly useful for examining the contryphans to elucidate the molecular motions of these peptides. NMR spectroscopy showed that contryphan-R, contryphan-Sm and contryphan-P are present in two forms in solution due to cis-trans isomerization about the Cys2-Hyp3 peptide bond (Pallaghy et al. 1999, Pallaghy et al. 2000). The structure of the major form of contryphan-R (with cis Cys2-Hyp3 peptide bond) has definite fold with non-hydrogen-bonded chain reversal from Gly1 to Glu5, type I β-turn from Glu5 to Cys8, and putative salt bridge between the N-terminal ammonium group and the Glu5 carboxyl group (Pallaghy et al. 1999). The structure of the major form of contryphan-Sm is similar to that of contryphan-R while the minor conformer (with trans Cys2-Hyp3 peptide bond) has hairpin structure with sheetlike hydrogen bonds and type II β-turn (Pallaghy et al. 2000). Compared to contryphan-R and contryphan-P, the cis-trans ratio was lowest for contryphan-Sm that has Gln5, in which the sidechain carboxylate is neutralized, indicating that an electrostatic interaction of the N-terminal ammonium group with the the carboxyl group in contryphan-R or contryphan-P stabilizes the cis conformer compared to the trans (Pallaghy et al. 1999, Pallaghy et al. 2000). In contryphan-Tx, UVRR spectroscopy showed a difference in the D-tryptophan dihedral angle for the cis and trans conformers (Jimenez et al. 2001).

NMR spectroscopy was done to examine the structure of glacontryphan-M with and without Ca^{2+}, due to the presence of two Gla residues in the N-terminal segment. The glacontryphan-M structure showed that Ca^{2+} binding stimulated structural perturbations in the N-terminal Gla2-Ser3 segment and intercysteine loop Cys11-Cys5-Pro6 segment. Due to the Ca^{2+} perturbations, the backbone of Gla2-Ser3 segment shifted from the aromatic sidechains of His8 and Trp10. Moreover, the alignment of the aromatic rings of D-Trp7 and His8 relative to that of Trp10 was changed (Grant et al. 2004).

The solution structure of contryphan-Vn determined by NMR spectroscopy and molecular dynamics (MD) simulation showed that the major conformer of contryphan-Vn has type IV β-turn from Gly1 to Lys6, with Pro4 mainly in the cis conformation, and type I β-turn from Lys6 to Cys9. Salt bridge between Asp2 and Lys6 and small hydrophobic region due to the nearness of the sidechains of Pro7 and Trp8 were observed (Eliseo et al. 2004). The structural characterization of [Trp8Ser]contryphan-Vn, an analog of contryphan-Vn, was done by NMR spectroscopy, fluorescence spectroscopy and MD simulation to identify the structural basis for the mechanism of cis-trans isomerization of Pro4. In [Trp8Ser]contryphan-Vn, Pro4 has almost equivalent amounts of cis and trans isomers. The isomers of [Trp8Ser]contryphan-Vn with Pro4 in cis and trans conformations showed structural differences as shown by the thermodynamic and kinetic parameters of the isomerization. The absence of salt bridge between the Asp2 and Lys6 in [Trp8Ser]contryphan-Vn may be due to the absence of the Trp8 sidechain leading to the inhibition of the electrostatic interaction (Nepravishta et al. 2014).

The effects of amino acid sequence on the cis-trans isomerization about Xxx-Pro bonds was investigated. Contryphan- Lo (Lo959) has two Pro residues while contryphan-In (In936) has only one Pro in the intercysteine loop. Isomerization about the Cys2-Pro3 bond in Lo959 and about the Tyr5-Pro6 bond in In936 were observed. The Tyr-Pro-Trp segment in In936 examined by MD simulations indicated that Tyr5 and Trp7 sidechain conformations are dependent on the conformation of the Xxx-Pro bond (Sonti et al. 2013).

Biological activities of contryphans

Contryphan-R elicited 'stiff-tail' syndrome and other excitatory symptoms based on mouse bioassay. [L-Trp4]contryphan-R showed similar biological activities (Jimenez et al. 1996). The characteristic symptoms were likewise observed in mice injected with contryphan-P, contryphan-Sm and contryphan-Tx (Jacobsen et al. 1998, Jimenez et al. 2001). Leu-contryphan-P and Leu-contryphan-Tx were less potent in eliciting these symptoms. The Leu-contryphan-containing peptides caused folding and drooping of the dorsal fins and passivity in Siamese fighting fish but contryphan-R and contryphan-Tx were less potent in inducing these symptoms (Jacobsen et al. 1999, Jimenez et al. 2001).

With NMR spectroscopy, contryphan-Vn revealed a Lys-Trp dyad that is similar to that found in voltage-gated K^+ channel inhibitors. Hence, contryphan-Vn was shown to regulate the

activity of voltage-gated and Ca^{2+}-dependent K^+ channels, with varied effects on invertebrate and vertebrate systems based on electrophysiological tests done using dorsal unpaired median neurons from cockroach nerve cord on rat fetal chromaffin cells (Massilia et al. 2003).

Electrophysiological tests using mouse pancreatic B-cells revealed that glacontryphan-M inhibited L-type voltage-gated Ca^{2+} channel activity in a Ca^{2+}-dependent mode. With fluorescence spectroscopy, it was shown that this peptide bound Ca^{2+} with a K_D of 0.63 mM (Hansson et al. 2004). The inhibition of L-type voltage-gated Ca^{2+} channel currents by glacontryphan-M required Ca^{2+} binding to N-terminal Gla residues, where probably His and Trp may become accessible to interact with the Ca^{2+} channel (Grant et al. 2004).

Electrophysiological experiments done using dorsal root ganglion neurons showed that contryphan-Am (Am975) and contryphan-Lo (Lo959) targeted high voltage-activated Ca^{2+} channels with different activities. Am975 inhibited while Lo959 enhanced the Ca^{2+} currents (Sabareesh et al. 2006).

For contryphan-Vc2, mouse assay revealed a depressive state instead of the hyperactive states usually observed in contryphans. Contryphan-Vc2 and [L-Trp3]contryphan-Vc2 showed comparable biological activities based on this assay. NMR and MD simulation analyses showed that Trp3 interacted with lipid membranes, suggesting membrane-mediated mechanism of action (Drane et al. 2017).

CONOMARPHINS

A 15-residue conomarphin with D-Phe13, purified from *Conus marmoreus* venom, was the first characterized peptide under the conomarphin family (Table 3) (Han et al. 2008). The cDNA-encoded conomarphin precursor was found to share homology with the signal peptide of the M-superfamily conotoxins indicating that conomarphin is under the same superfamily. NMR spectroscopy showed that conomarphin has well defined structure with tight loop in the middle and short 3^{10}-helix around the C terminus, whereas [L-Phe13]conomarphin has no loop (Han et al. 2008). NMR spectroscopy showed that [Hyp10Pro]conomarphin has type II β-turn instead of 3^{10}-helix around the C terminus. The compact loop region found in conomarphin is more open in [Hyp10Pro]conomarphin indicating that Hyp10 is crucial for the conomarphin structure (Huang and Du 2009).

Two short conomarphins were isolated from *Conus marmoreus* venom (Table 3). The peptides named conomarphin-14 and conomarphin-8 contain D-Phe13 and D-Phe7, respectively (Zhang et al. 2010). These peptides are presumably produced by post-translational proteolysis of conomarphin.

Putative conomarphin-Vc1 was identified from cDNA library prepared from venom gland of *Conus victoriae* (Table 3). The 13-residue peptide has D-Phe at position 11 (Robinson et al. 2014).

CONOPHANS

Peptides gld-V and gld-V' from *Conus gladiator* venom, and mus-V and mus-V' from *Conus mus* venom have been identified as conophans (Table 3). All these peptides contain eight amino acid residues with D-amino acids at position 6. Peptides gld-V and mus-V have D-Val while gld-V' and mus-V' have D-γ-hydroxyvaline (D-Hyv). Peptides gld-V' and mus-V', also known as γ-hydroxyconophans, are unusual because they have two hydroxylated residues. The Ser-D-Hyv-Trp segment is a structural motif that is stabilized by specific interactions between D-Hyv and its adjoining L-amino acids. These interactions inhibit peptide backbone scission usually initiated by γ-hydroxylated residues (Pisarewicz et al. 2005).

CONOMAP

Conomap-Vt (Conp-Vt), peptide with 14 amino acid residues including D-Phe2, was purified from *Conus vitulinus* venom (Table 3). The sequence has no homology with known conopeptides but shares homology with myoactive tetradecapeptide family members which are neuromodulators in annelids, insects and molluscs. Conp-Vt showed potent excitatory activity in snail tissue preparations, but nicotinic and muscarinic antagonists did not inhibit its effect. Tissue preparation treated with conp-Vt was responsive to acetylcholine, indicating that the contractions elicited by conp-Vt had non-cholinergic basis. Conp-Vt had greater biological activity than [L-Phe2]conp-Vt based on in vitro assay using snail tissue preparation (Dutertre et al. 2006).

I_1-SUPERFAMILY CONOTOXINS

Analysis of cDNA clones generated from *Conus radiatus* venom duct library revealed 18 encoded peptides, including several excitatory peptides that were purified from venom. These peptides were initially designated to be members of the I-superfamily. They have eight Cys residues arranged in a -C-C-CC-CC-C-C- pattern. Among these peptides are r11a, r11b and r11c (Table 4) (Jimenez et al. 2003).

Sequence and structural characterization of I_1-superfamily conotoxins

Peptides r11a, r11b and r11c were further investigated to elucidate their structures. Peptide r11a is a 46-residue conotoxin with D-Phe at position 44. Similarly, peptides r11b and r11c have D-Phe44 and D-Leu42, respectively, at the homologous loci. These peptides were later redefined to be under the I_1-superfamily (Buczek et al. 2005a, Buczek et al. 2005b).

Furthermore, putative I_1-superfamily conotoxins were determined by cDNA cloning method (Table 4). These include D-Leu-containing peptides R11.18 and F11.1 from *Conus radiatus* and *Conus figulinus*, respectively; D-Phe-containing peptides Fi11.6 and Fi11.8 from *Conus figulinus*; and D-Met-containing peptides S11.2 and M11.1 from *Conus striatus* and *Conus magus*, respectively (Buczek et al. 2005b, Buczek et al. 2008).

Table 3: Conomarphins, conophans and conomap

Peptide	Sequence	Superfamily/Family	*Conus* species	Reference
Conomarphin	DWEYHAHPKONSFWT	M	*C. marmoreus*	Han et al. 2008
Conomarphin-14	DWEYHAHPKONSFW	M	*C. marmoreus*	Zhang et al. 2010
Conomarphin-8	HPKONSFW	M	*C. marmoreus*	Zhang et al. 2010
Conomarphin-Vc1	AHHTHPNDNSFWT	M	*C. victoriae*	Robinson et al. 2014
Conophan gld-V	AOANSVWS	Conophan	*C. gladiator*	Pisarewicz et al. 2005
Conophan gld-V'	AOANSUWS	Conophan	*C. gladiator*	Pisarewicz et al. 2005
Conophan mus-V	SOANSVWS	Conophan	*C. musculus*	Pisarewicz et al. 2005
Conophan mus-V'	SOANSUWS	Conophan	*C. musculus*	Pisarewicz et al. 2005
Conp-Vt	AFVKGSAQRVAHGY*	Conomap	*C. vitulinus*	Dutertre et al. 2006

O, 4-hydroxyproline; F, D-phenylalanine; V, D-valine; U, D-γ-hydroxyvaline; *, C-terminal amidation

Table 3: I_1-superfamily conotoxins

Peptide	Sequence	*Conus* species	Reference
r11a (RXIA)	GOSFCKADEKOCEYHADCCNCCLSGICAOSTNWILPGCSTSSFFKI	*C. radiatus*	Jimenez et al. 2003, Buczek et al. 2005a
r11b	GOSFCKANGKOCSYHADCCNCCLSGICKOSTNVILPGCSTSSFFRI	*C. radiatus*	Jimenez et al. 2003, Buczek et al. 2005b
r11c	GOSFCKADEKOCKYHADCCNCCLGGICKOSTSWIGCSTNVFLT	*C. radiatus*	Jimenez et al. 2003, Buczek et al. 2005b
R11.18	GAVPCGKDGRQCRNHADCCNCCPFGTCAPSTNRILPGCSTGMFLTR	*C. radiatus*	Buczek et al. 2008
Fi11.1	GHVSCGKDGRACDYHADCCNCCLGGICKPSTSWIGCSTNVFLTR	*C. figulinus*	Buczek et al. 2008
Fi11.6	GCKKDRKPCSYHADCCNCCLSGICAPSTNWILPGCSTSSFFKI	*C. figulinus*	Buczek et al. 2008
Fi11.8	GPSSCKADEEPCEYHADCCNCCLSGICAPSTNWILPGCSTSSFFKI	*C. figulinus*	Buczek et al. 2008
S11.2	GCKKDRKPCSYQADCCNCCPIGTCAPSTNWILPGCSTGPFMAR	*C. striatus*	Buczek et al. 2008
M11.1	GAVPCGKDGRQCRNHADCCNCCPIGTCAPSTNWILPGCSTGQFMTR	*C. magus*	Buczek et al. 2008

O, 4-hydroxyproline; F, D-phenylanine; L, D-leucine; M, D-methionine

The solution structures of r11a and [L-Phe44]r11a were examined by NMR spectroscopy. The disulfide linkages were chemically determined as 5-19, 12-22, 18-27, and 21-38, indicating that r11a has an ICK structural motif with an additional disulfide 21-38. Aside from the first few residues, the structure is definite up to around Leu35. The ICK structural motif was confirmed and the C-terminal region including Phe44 was found to be disordered. Comparison of r11a and [L-Phe44]r11a indicated that the conversion from L- to D-Phe had slight effect on the structure (Buczek et al. 2007).

Biological activities of I_1-superfamily conotoxins

The isomerization from L- to D-Phe had a dramatic effect on the excitatory effects of r11a. The peptide was significantly more active than [L-Phe44]r11a in both in vivo and in vitro assays. Conotoxin r11a induced excitatory symptoms in mice and repetitive action potentials in frog motor axons. In an electrophysiological assay r11a was extremely potent, whereas [L-Phe44]r11a had no biological activity (Buczek et al. 2005a).

Mouse bioassay was used to determine the potencies of r11b, r11c and their corresponding L-amino acid-containing isomers. A 5-fold difference was observed in potencies of r11b and [L-Phe44]r11b based on comparison of the doses that induced hyperactivity symptoms. A 5-fold difference in potencies was similarly observed between r11c and the [L-Leu42]r11c based on comparison of the doses at which hyperactivity symptoms appeared (Buczek et al. 2005b).

Electrophysiological tests showed that r11a, r11b and r11c induced repetitive activity in frog motor nerve. The potencies of [L-Phe44]r11a and [L-Phe44]r11b were lower than the corresponding D-amino acid-containing isomers. In contrast, r11c and [L-Leu42]r11c were equally active in inducing repetitive activity on frog nerve-muscle preparation. Moreover, r11c (but not r11a or r11b) exhibited biological activity on skeletal muscle (Buczek et al. 2005b).

The physiological target of r11a (later designated as ι-RXIA upon identification of the target) is voltage-gated Na^+ channel $Na_V1.6$. The ι-RXIA served as an agonist by shifting the voltage dependence of activation of mouse $Na_V1.6$ expressed in *Xenopus* oocytes to more hyperpolarized level (Buczek et al. 2007). The ι-RXIA caused repetitive action potentials in mouse sciatic nerve. Electrophysiological experiments using rodent $Na_V1.1$ to $Na_V1.7$ with β1 subunit expressed in *Xenopus* oocytes and $Na_V1.8$ in dissociated mouse DRG neurons showed that ι-RXIA was active on $Na_V1.6 > Na_V 1.2 > Na_V1.7$, but was not active on the other Na^+ channel subtypes (Fiedler et al. 2008). The plot of k_{obs} vs. ι-RXIA concentration indicated a bimolecular reaction with k_d of ~ 3 μM that approaches the steady-state EC_{50} of ~ 2 μM. [L-Phe44] ι-RXIA had 2-fold faster off-rate and 2-fold lower affinity than ι-RXIA on $Na_V1.6$, and was not active on $Na_V1.2$. The efficacy of ι-RXIA was twice that of [L-Phe44] ι-RXIA at or near saturating peptide concentration (Fiedler et al. 2008).

CONCLUSION

The post-translational isomerization of L- to D-amino acid is a subtle modification not detectable by standard proteomic techniques. Prediction of the presence of D-amino acid entails examination of different D-amino acid-containing *Conus* peptides. The diversity of D-amino acid-containing conopeptides presents an opportunity for defining important parameters to elucidate this post-translational modification. In the case of contryphans and I_1-superfamily conotoxins characterized so far, accurate predictions of the occurrence of D-amino acids at common loci have become possible (Jimenez 2007, Buczek et al. 2008).

Conus venom is a complex mixture of more than100 peptides, and the composition varies among *Conus* species. Thus, more than 70,000 pharmacologically active compounds can be potentially explored. So far, an extremely small number of *Conus* peptides have been deeply characterized. Many of these peptides show significant selectivity in targeting specific subtypes of neurotransmitter receptors and ion channels. They have proven to be useful tools in neuroscience and that they have great potential for drug development. The importance of *Conus* venoms as 'gold mines' for the discovery of novel medications has been substantiated. Some conopeptides have actually reached clinical trials, one of which is currently in use as an antipain (Olivera 2006, Olivera and Teichert 2007, Teichert et al. 2009).

Conus venom has extraordinary diversity of peptides, of which the D-amino acid-containing peptides could as well serve as lead compounds for the development of therapeutics. Small D-amino acid-containing peptides, such as the contryphans, conomarphins, conophans and conomap, can serve as essential frameworks for drug development. They are more resistant to proteolysis, and do not have folding difficulties as the bigger disulfide-rich peptides. Numerous peptides that share homology with r11a (ι-RXIA) have been identified in *Conus* venoms, thus these peptides represent rich resources of potential Na^+ channel-targeting ligands. The I_1-superfamily conotoxins may be useful for further characterization of Na^+ channel subtypes.

Hopefully the D-amino acid-containing conopeptides will attract great interest in order to examine their specific

physiological targets and mechanisms of action. Their exact biological functions in *Conus* venoms could as well be investigated.

ACKNOWLEDGEMENTS

The support of UP Baguio is greatly appreciated.

CONFLICT OF INTEREST

The author declares no conflict of interest in relation to this work.

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