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Short communication

Identification of short single disulfide-containing contryphans from the venom of cone snails using de novo mass spectrometry-based sequencing methods

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ABSTRACT

We identified 12 short single disulfide-containing conopeptides from the venom of *Conus coronatus*, *C. leopardus*, *C. lividus* and *C. zonatus*. Interestingly, we detected the shortest contryphan sequence thus far characterized which contains only six amino acid residues. We also identified three distinct contryphan sequences of *C. lividus* without any proline residues and one sequence with an unusual post-translational modification (bromination of tryptophan). Furthermore, we characterized venom peptides of *C. zonatus* for the first time.

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The venom of predatory marine cone snails mainly contains a diverse array of unique bioactive peptides commonly referred to as conopeptides (Peng et al., 2016). Conopeptides are disulfide-rich neurotoxic peptides with 10–30 residues (Wu et al., 2016). It has been reported that there are over 100,000 conopeptides existing in approximately 700 species of cone snails (Daly and Craik, 2009). These peptides have proven to be valuable pharmacological probes and potential drugs because of their high specificity and affinity for important ion channels, receptors and transporters of the nervous system. Based on the sequence similarities of signal peptides in the precursors, conopeptides are currently classified into 26 gene superfamilies (A, B1, B2, B3, C, D, E, F, G, H, I1, I2, I3, J, K, L, M, N, O1, O2, O3, P, S, T, V and Y) (Kaas et al., 2010; Puillandre et al., 2012; Peng et al., 2016). While the widely studied conopeptides contain multiple disulfide linkages, venoms of cone snails also include a major class of peptides termed contryphans. Contryphans constitute a group of conopeptides that possess a single disulphide loop and are rich in unusual post-translational modifications that include tryptophan bromination, amidation of the C-terminal residue, leucine, and tryptophan isomerization, proline hydroxylation and glutamic acid γ-carboxylation (Jimenez et al., 1997; Craig et al., 1999; Hansson et al., 2004; Buczek et al., 2005; Thakur and

Abbreviations: NMDA, *N*-methyl-D-aspartate; TCEP, Tris (2-carboxyethyl) phosphine (Pierce Scientific, United States); NEM, *N*-Methylmaleimide (Sigma-Aldrich, United States); CCA, Alpha-cyano-4-hydroxycinnamic acid; TFA, Trifluoroacetic acid (Merck, India); Phe, Hydrophobic phenyl alanine; HPLC, High Performance Liquid Chromatography; ESI, Electro Spray Ionization; LC-ESI-MS, Liquid Chromatography - Electro Spray Ionization - Mass Spectrometry; LC-MS, Liquid Chromatography - Mass Spectrometry; MALDI-TOF, Matrix Assisted Laser Desorption Ionization - Time of Flight; CID, Collision Induced Dissociation; ETD, Electron Transfer Dissociation.

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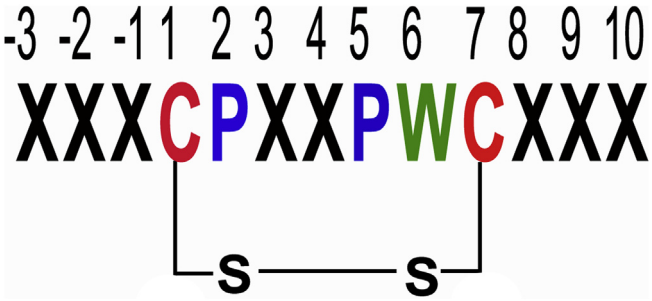

Fig. 1. Conserved sequence motif of contryphan. C-cysteine, P-proline, W-tryptophan.

Balaram, 2007; Vijayasarathy et al., 2016). The characteristic feature of contryphan sequences (Fig. 1) determined thus far is the presence of a tryptophan residue and one or more proline residues in the five-residue segment between the disulfide-bonded Cys residues (Jimenez et al., 1996; Jacobsen et al., 1998; Vijayasarathy et al., 2016). Contryphans exist in solution as a mixture of two conformers due to cis-trans isomerization around the N-terminal Cys–Pro peptide bond (Pallaghy et al., 2000; Vijayasarathy et al., 2016). The molecular target of most of the contryphan peptides is yet to be determined. However, a few are known including contryphan-Vn which is a Ca^{2+}-dependent K^{+} channel modulator (Massilia et al., 2001), while glacontryphan-M is an L-type calcium channel blocker (Hansson et al., 2004). A recent study on the physiological effect of contryphan Vc2 in mice showed limited movement, splaying of hind limbs, dragging of hind limbs and flattening of the lower body during movement. The study also suggested the significance of Trp3 in bioactivity (Drane et al., 2017).

In this study, we describe twelve short single disulfide-containing conopeptides from the venom of *Conus leopardus*, *C. coronatus*, *C. lividus* and *C. zonatus*. We documented for the first time the occurrence of a short amino acid sequence with six residues in contryphans and three contryphan sequences lacking proline residues from *C. lividus*. We also observed an unusual post-translational bromination of tryptophan in a contryphan sequence which is only the second report of such occurrence in contryphans. In addition, we also report a short conopeptide sequence with six amino acid residues from *C. leopardus*. We also characterized the venom peptides of *C. zonatus* for the first time.

Specimens of *Conus leopardus*, *C. coronatus*, *C. lividus* and *C. zonatus* were collected from south Andaman, Andaman and Nicobar Islands and were identified following standard keys (Röckel et al., 1995; Franklin et al., 2009). The venom ducts of one to three individuals of each species were dissected and stored in 50% HPLC grade acetonitrile. Samples were finely ground with 50% HPLC grade acetonitrile and the extract was filtered through Whatman No.1 filter paper. The filtrate was concentrated using Heidolph Laborota 4000 rotary vacuum evaporator (Germany), lyophilized using Labconco Freezone Freeze Dry System 4.5 (United States) and stored at −20 °C until further processing. ESI mass spectra were recorded on Esquire 3000-plus mass spectrometer (Bruker Daltonics, Bremen, Germany) attached to an Agilent 1100 series HPLC system. The samples were subjected to mass spectrometry either by direct injection or through an HPLC using a C18 analytical column (Agilent Zorbax, 150 mm × 4.6 mm, 5 μm particle size) and eluted using a binary gradient of water (0.1% TFA): acetonitrile (0.1% TFA) at a flow rate of 0.2 mL min^{-1}. Data were detected within the mass to charge ratio (*m/z*) range 100–2000 (Mandal et al., 2007; Rajesh, 2015; Franklin and Rajesh, 2015). The MALDI-MS and MS/MS data were performed on an Ultraflex TOF/TOF (Bruker Daltonics, Bremen, Germany) mass spectrometer when necessary, operated in positive ion mode, in a 90 ns time delay and a 25 kV accelerating voltage.

LC-ESI-MS analysis was performed to identify the number of peptide components in the venom. The lyophilized venom was dissolved in 2 mL of methanol and filtered through a 0.2 μM filter. The filtered extract (stock solution) was used for mass spectrometric analysis. Global reduction and alkylation of peptides were analyzed to identify the number of disulfide rich conopeptides and to establish the number of disulfides in each peptide (Thakur and Balaram, 2007; Rajesh, 2015; Franklin and Rajesh, 2015). Acetylation of reduced and alkylated peptides were performed to identify free amino terminus and/or lysine residues in the amino acid sequence. The acetylation reaction enabled us to distinguish lysine and glutamine residues (Mandal et al., 2007). Esterification of reduced and alkylated peptides was carried out to determine amidation of the peptide (Rajesh, 2015; Franklin and Rajesh, 2015). The C-terminal amidation is a common post-translational modification of bioactive peptides. Therefore, it is essential to determine the amino acid sequence of a peptide to establish whether or not the C-terminus is amidated. Auto MS(n) experiments (CID and ETD fragmentation) were performed on reduced and alkylated peptide molecules. The chemically modified peptides were chromatographically separated based on their polarity using a reverse phase C18 column. Peptides eluting from the column were fragmented using nitrogen gas (CID fragmentation) and by collision of these peptides with thermal excited electrons in a methane atmosphere (ETD fragmentation). The daughter ion spectra were analyzed to derive amino acid sequences of the peptides (Rajesh, 2015; Franklin and Rajesh, 2015).

Peptides were sequenced based on the fragmentation of the chemically modified parent ions. Peptide sequences were achieved manually by analyzing the daughter ions generated from the fragmented spectrum (Supplemental Figs. 1–12). In total, 12 short single disulfide-containing conopeptides from the venom of *Conus leopardus*, *C. coronatus*, *C. lividus* and *C. zonatus* were sequenced (Table 1). The seven single disulfide-containing conopeptides sequences that were determined from *C. leopardus*, *C. lividus* and *C. zonatus* ranged in size from six to nine amino acids. The peptide Le685 isolated from *C. leopardus* with *m/z* of 685 (CFJSDC) was identified as the second cone snail venom peptide sequenced that has only six residues reported to date. Previously, only one peptide from *C. emaciatus* with *m/z* of 765.3 (CPFWIC) of M-superfamily has been known to have six amino acid residues (Zhou et al., 2013). The peptides Zo763 from *C. zonatus* with *m/z* of 763 (SPCPFOC; O = hydroxyproline, Hyp) and Zo747 with *m/z* of 747 (Delta mass-16D) (SPCPFPC) differed at one residue due to a post-translationally modified hydroxyl proline. This is the first report of conopeptide sequences from *C. zonatus*.

Five contryphan sequences isolated from *C. coronatus* and

Table 1
Conopeptide sequences determined from four species of cone snails. W-D-tryptophan or L-tryptophan, W̲-bromotryptophan, J-leucine/isoleucine, O-hydroxyproline, E-glutamic acid and S-serine, *- C-terminal amidation.

Source	Label	Monoisotopic mass	Average mass	Sequence
Conus leopardus	Le685	684.2	684.7	CFJSDC
	Le755	755.2	755.8	CFJSDCA
	Le851	851.3	851.9	CFJSDCAP*
	Le925	925.3	926.0	CFJSDCAOG
Conus zonatus	Zo763	763.3	763.9	SPCPFOC*
	Zo747	747.2	747.8	SPCPFPC*
Conus coronatus	Co838	837.3	838.0	CWFPWC*
	Co854	853.2	854.0	CWFOWC*
Conus lividus	Li1044	1043.2	1044.1	GCEWVSW̲C*
	Li965	965.3	966.1	GCEWVSW̲C*
	Li981	981.3	982.0	GCEWDSWC*
	Li834	834.3	835.0	CYRPPVC

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