PROGRESS REPORT
(2006-2007)

FOR

PROJECT PROPOSAL ENTITLED

PEPTIDE TOXINS OF THERAPEUTIC VALUE FROM INDIAN CONE SNAIL SPECIES

BT/PR4627/AAQ/03/222/204

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1. File number and title of the project:

BT/PR4627/AAQ/03/222/204

Peptide toxins of therapeutic value from Indian cone snail species

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3. **Dates of Sanction and Completion of the Project:**

31-3-2005 to 30-3-2008

4. **Total Approved project cost and duration: Rs. 96.52 lakh (3 years)**

5. **Year wise Grants released so far:**

Ist Year: Rs. 57.94 lakh
IInd year: Rs. 15.12 lakh

6. **Project Objectives as approved by DBT:**

1. Collection, identification and classification of Cone Snails from various identified locales in South Indian Coasts and if possible from Andaman, Nicobar and Lakshadweep at a later stage. Screening for biological activity and collection of venoms.
2. Isolation of active peptide toxins from venoms, their composition and sequence determination and ultimately three dimensional structure determination.
3. Elucidation of isolated conotoxin molecular mechanism of action by means of electrophysiological and biological experiments.
4. Identification of the target receptor system and in particular identification of putative targets in human genome for possible suggestions on therapeutic application.

5. Cloning and expression of peptides for large scale purification, confirmation of biological activity and for structure activity studies by amino acid substitution.
6. Complete *de novo* Chemical synthesis of peptides and spectroscopic and biological characterization
7. Folding and re-naturing studies wherever needed of both biosynthesized and chemically synthesized analogues
8. Specificity in different model systems with a view to assessing suitability for human trials in future.
9. Behavioral analysis by intra-cerebroventricular injection in mice Electrophysiological methods, including single-channel recording and whole-cell techniques, will be used. Drosophila NMJ recordings, Oocyte expressing specific channel proteins and a variety of Cell systems expressing specific receptor types and calcium sensitive flourophore proteins for assessing various channel and receptor effects will be used
10. Site-directed mutagenesis of the obtained recombinant peptides will be carried out for the design of new molecules with a highly specific and desirable mode of action.

7. Achievements/progress vis-à-vis above objectives.

Marine snails of genus *Conus* are dominant predators in the coral reef, feeding on narine worms, fishes and molluscs. The need of fast acting components for the success n their predation has resulted in the evolution of 'cabals' of potent and highly specific ieurotoxins to exploit prey niches. The ability of these toxins to distinguish between the losely related superfamily of ion channels and receptors in excitable cells has triggered onsiderable interest in the chemistry and neuropharmacology of conus peptides. The xquisite specificity of these toxins in mammalian systems that have pharmacologically iverse neuronal macromolecular receptors allows them to block the perception of pain in nimals, leaving normal sensory signals intact. Hence highly specific drugs targeted to euronal receptors with no side effects due to cross reactivity or habituation can be xpected to be discovered from these. We have initiated this multi-institutional project

with the goal of characterizing toxins from cone snails which are unique to the Indian coast. In view of the evolutionary strategies inherent in the development of toxin cabals in these animals, it is important to characterize natural peptide libraries to the highest degree of detail in the search for therapeutically important lead molecules. This report describes the work carried out in this project during the period **31-03-2006 to 31-06-2007**. We have undertaken a systematic approach to characterize the toxins, beginning with conus species collection and identification, purification and complete characterization of toxins in terms of structure and function using electrophysiological approaches. The results are briefly presented below.

8. Summary of the report 31-03-2005 to 31-03-2006.

Collection, identification and classification of 18 different cone snails from Indian coasts have been carried out. Peptide libraries from conus venom have been characterized using mass spectrometry to classify the peptides based on the molecular mass and disulfide patterns. It is readily demonstrated that each snail have a unique library of toxins. Purified peptides obtained from crude venom by RP-HPLC and subjected to primary structure determination, using 'de novo' sequencing by mass spectrometry. This approach has yielded ten novel conus peptide sequence derived from Indian marine snails. The proposed sequence has been further confirmed by synthesizing the corresponding peptide and comparing the fragmentation pattern of natural and synthetic peptides in some cases. Molecular biologically have also been employed to derive putative conotoxin sequences by the construction of cDNA libraries for conus venom. A proteomic approach involving global tryptic digestion of a venom library has been standerdized to confirm the presence of putative conotoxins sequences in the crude venom. Three putative conotoxins along with the posttranslational modifications have been identified using this methodology from C.achatinus. Electrophysiological experiments have been employed to identify the biological activity of conus peptides and three novel toxins are found to target voltage-gated ion channels. Specifically, Am2766 inhibits the inactivation of mammalian sodium channels, Mo1659 targets non-inactivating voltage dependent potassium channels and Lo959 targets high voltage activated calcium

channels. The 3D-structure of Am2766 has been determined using 2D-NMR experiments resulting a cysteine knot motif. Two distinct approaches has been developed to produce conus peptide sequence by Solid Phase Peptide Synthesis and expression of synthetic cDNA sequence using fusion protein construct.

1. **Isolation and purification of active peptide toxin components from the crude venom.**

(Dr. F.Benjamin, Dr. M.S.Ramasamy, Dr.S.S. Thakur, K. Hanumae gowd , Prof.K.S.Krishnan and Prof. P.Balaram)

Venom extracts from *Conus achatinus* have been subjected for purification using RP-HPLC (Figure 1a) and it is readily demonstrated that venom contains approximately 30 identifiable peptides. Figure 1b provides a summary of the deconvolution of the peptide library in terms of molecular mass, number of disulfide bonds and peptide hydrophobicity, as reflected in RP-HPLC retention times. An interesting feature of the *C.achatinus* venom is the presence of an intense HPLC peak corresponding to the linear peptides, namely Ac1423 and Ac1975.

a) b)

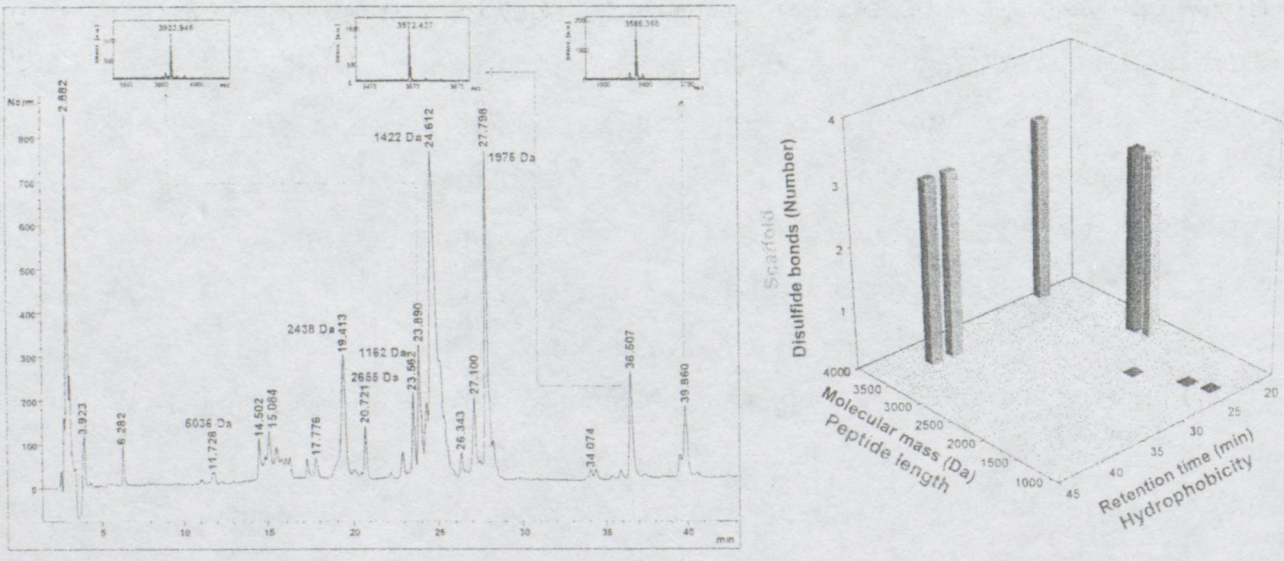


Figure 1 a) RP-HPLC profile of the crude venom from *C.achatinus* and b) graphical representation of the deconvoluted peptide library from *C.achatinus*

Further fractionation of crude venom derived from *C.araneosus, C.virgo,* and *C.monile* has been achieved, in order to obtain large quantity of purified peptides for detail structural and functional analysis.

2. Determination of primary structure of conotoxins.

(Dr.A.K.Mandal, Dr. M.S.Ramasamy, Dr.S.Sudarslal, Dr.S.Thakur, V.Sabareesh, and Prof. P.Balaram)

We have developed mass spectrometric *'de novo'* sequencing as the method of choice for the analysis of conus peptides. The use of both MALDI-MS/MS and ESI-MS/MS ion trap mass spectrometers, together with enzymatic cleavage and chemical modification procedures have been standardized. Figure 2 and Figure 3 illustrates typical

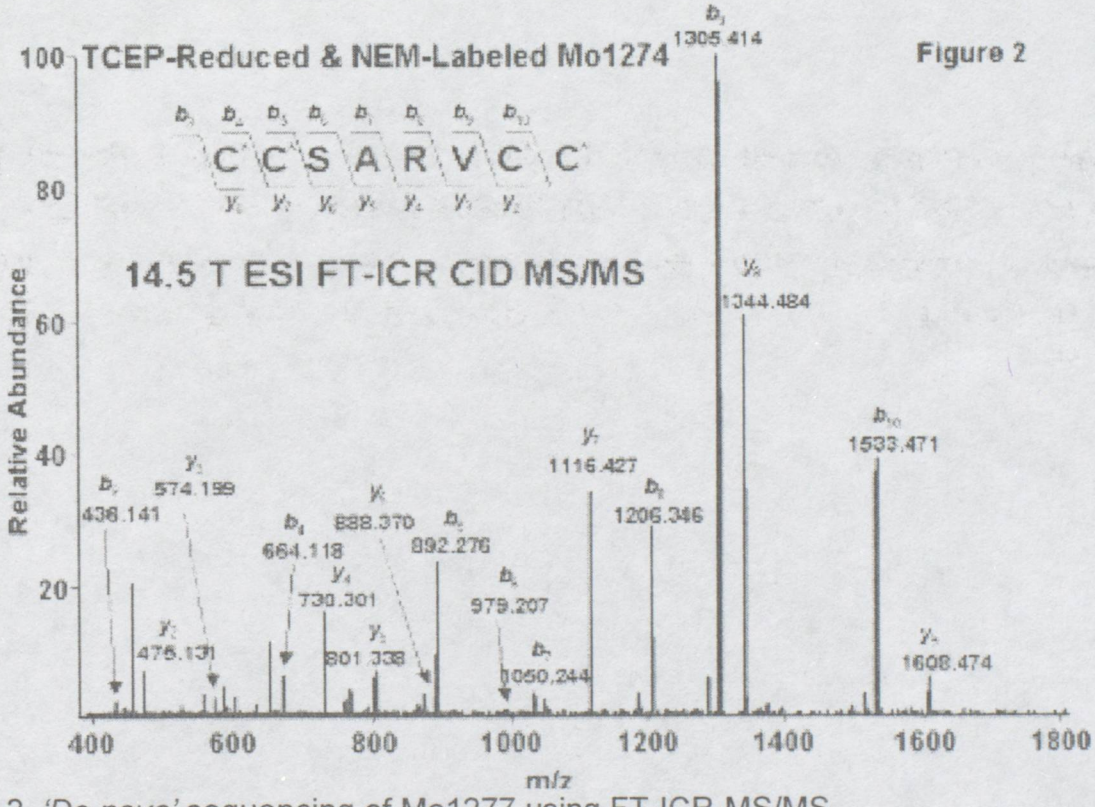


Figure 2. *'De novo'* sequencing of Mo1277 using FT-ICR-MS/MS

MALDI-MS/MS fragmentation pattern used in *'de novo'* sequencing. We have isolated and characterized a novel peptide, Mo1274, containing 11 amino acids, that shows the

same cysteine pattern, -CC-CC, and disulfide linkage as those of the T-1-family members. The complete sequence, GNWCCSARVCC, in which W denotes bromotryptophan, was derived from MS-based de novo sequencing. Disulfide connectivity of Mo1274, between cysteine pairs 1-3 and 2-4, was determined by mass spectrometry in combination with chemical derivatization employing tris(2-carboxyethyl)- phosphine, followed by differential alkylation with N-ethylmaleimide and iodoacetamide.

De novo mass spectrometric sequencing of two Conus peptides, Vi1359 and Vi1361, from the vermivorous cone snail *Conus virgo*, found off the southern Indian coast, is also achieved. The peptides, whose masses differ only by 2 Da, possess two disulfide bonds and an amidated C-terminus. Simple chemical modifications and enzymatic cleavage coupled with matrix assisted laser desorption ionization (MALDI) mass

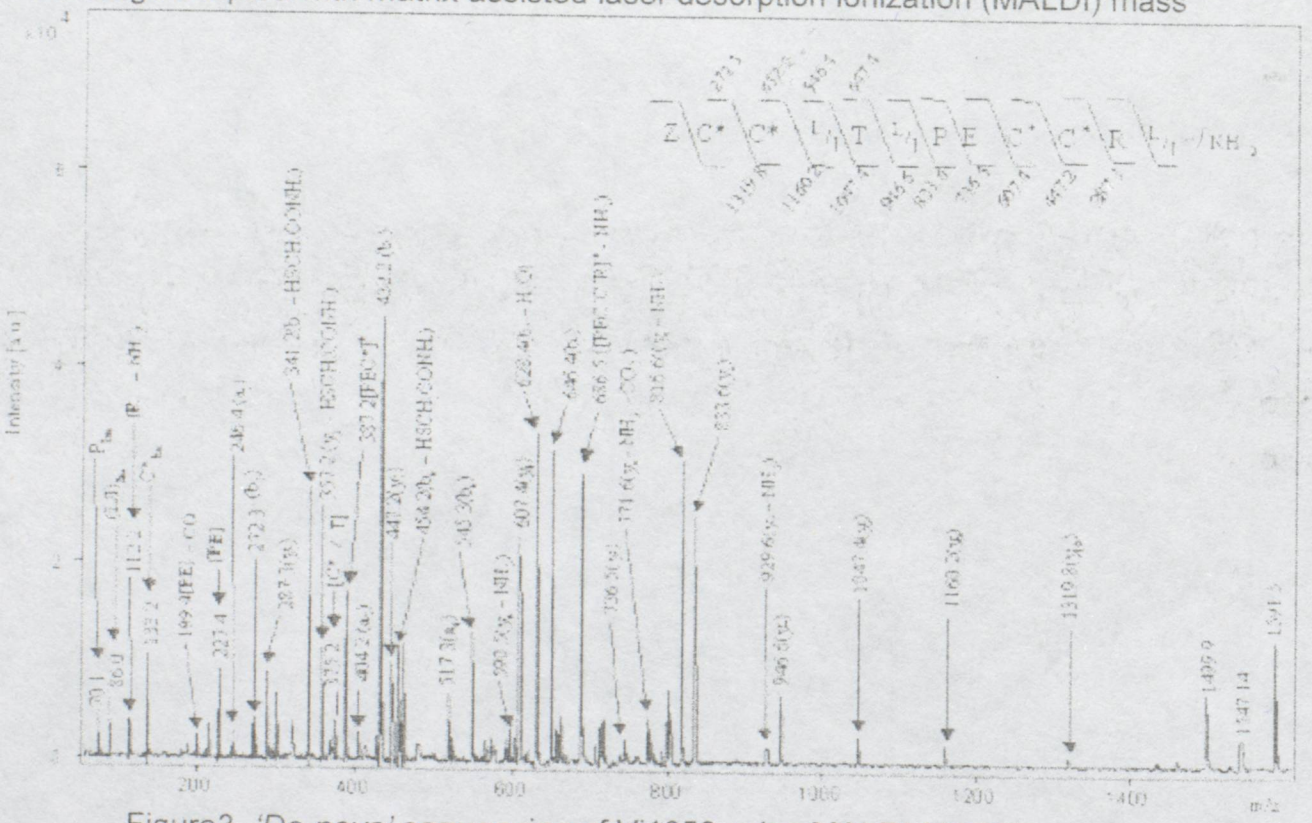


Figure3. *'De novo'* sequencing of Vi1359 using MALDI-MS/MS

spectrometric analysis aided in establishing the sequences of Vi1359, ZCCITIPECCRI-NH2, and Vi1361, ZCCPTMPECCRI-NH2, which differ only at residues 4 and 6 (Z = pyroglutamic acid). Differential cysteine labeling, in conjunction with MALDI-MS/MS,

permitted establishment of disulfide connectivity in both peptides as Cys2-Cys9 and Cys3-Cys10.

3. Sequence determination by mRNA isolation and cDNA sequencing.

(Dr.Kavitha, Kalyan K.Dewan, Prof.K.S.Krishnan and Prof.P.Balaram)

Molecular methods involving cDNA libraries construction for venom ducts mRNA and the prediction of primary structure is an effective means of determining putative conotoxin sequences. mRNA has been isolated from the venom ducts of *C.achatinus* , *C.monile* and *C.virgo*. An RT-PCR procedure used for the construction of cDNA libraries using sequence specific primers designed for O-superfamily and M-superfamily conotoxins. This strategy is based on the conservation of signal peptide sequence. The putative conotoxin derived from *C.virgo* is *QATERPCIWPWCRPPLMALCCAEYSITTAFWR*

4. Probing peptide libraries from C.achatinus using mass spectrometry and cDNA sequencing.

(K.Hanumae Gowd, Kalyan Dewan and Prof.P.Balaram)

Two independent approaches have been used in analyzing the venom peptide libraries of C.achatinus. MALDI-MS have been used to derive compositional information directly from crude venom, while cDNA library screening for specific families provides DNA sequences corresponding to conotoxin precursors. Crude venom samples have been probed by MALDI-MS using global reduction/alkylation procedures to identify multiple disulfide bonded peptides. The presence of large number of three disulfide containing peptides has been established in the crude venom of C.achatinus. A cDNA library was constructed using primers designed against the highly conserved signal sequence of the O-superfamily, which includes well characterized three disulfide containing conopeptides. The full-length cDNAs of five new O-superfamily conotoxins were cloned and sequenced using RT-PCR and 3'-RACE. The sequences of putative mature toxins have been inferred by identifying potential sites of proteolytic cleavage. The MALDI-MS spectra of crude venom have been carefully examined for the presence of peptides whose mass match with that computed for putative sequences derived from cDNA.

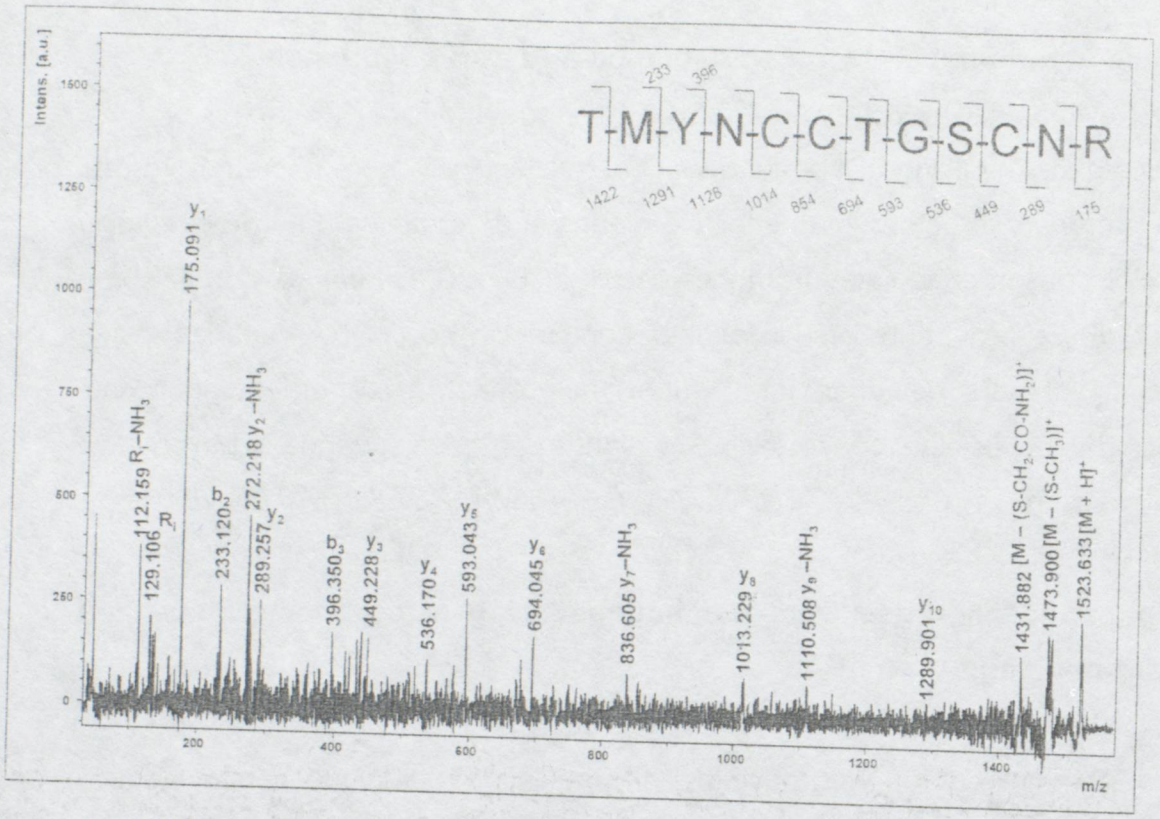


Figure 4. MALDI-MS/MS of a tryptic fragment (1523 Da) obtained by the global trypsin clevage of reduced/alkylated crude venom from C.achatinus. 1419 Da was selected for MS/MS based on the *in silico* tryptic clevage analysis of Ac6.2 (Inset shows the observed fragmentation pattern of 1523 Da).

Hypothetical posttranslational modifications have also been factored into the search for mature peptides. A strategy to carry out global tryptic digestion of venom libraries has been developed in order to use 'de novo' sequence determination of proteolytic peptides as a probe for the presence of mature toxins. Sequencing of proteolytic fragments by MALDI-MS/MS procedures permits the identification of venom components which corresponds to the peptide sequences derived from translating cDNA sequences. Figure 4 shows a representative example confirming the mass spectrometric sequencing of 12 residue (1523 Da) tryptic fragment derived from Ac 6.4. Identification of three putative δ-conotoxins and two ω-conotoxins are reported from cDNA analysis, while four of these are shown to be expressed in the venom. Figure 5 illustrates the putative conotoxins

derived from cDNA sequencing and the corresponding expressed toxins in the venom along with posttranslational modifications.

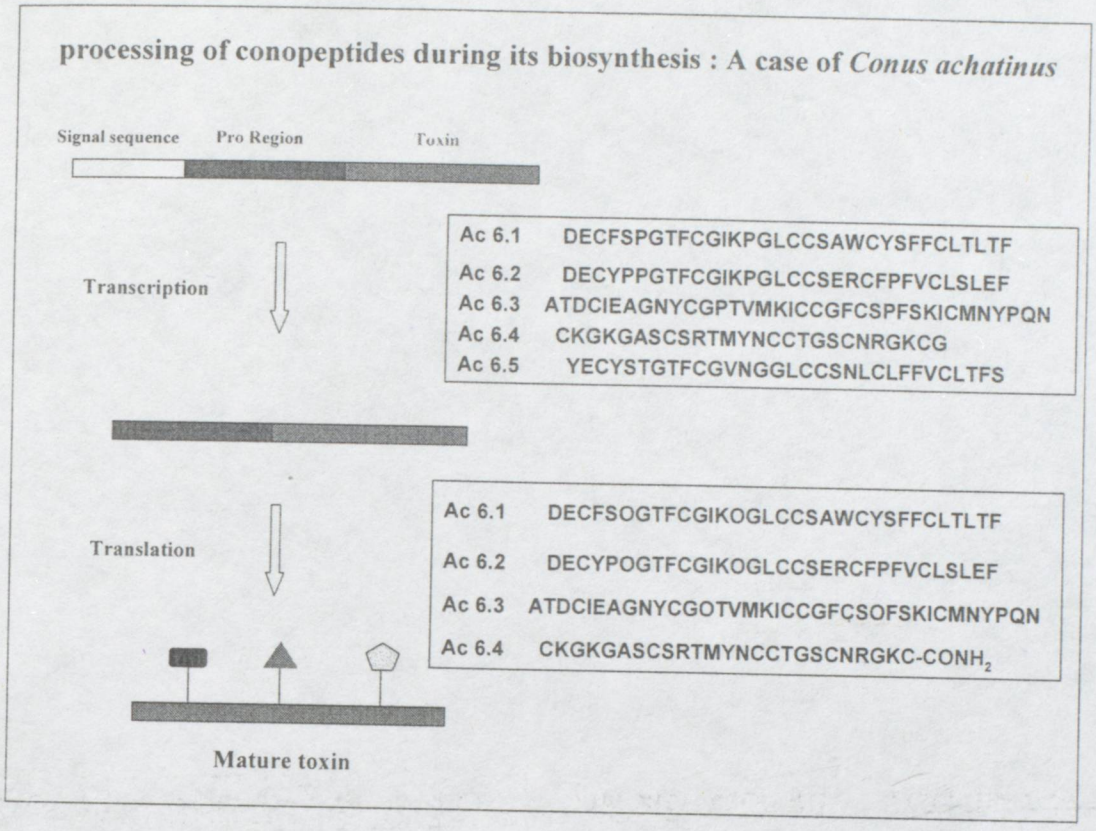


Figure 5 Pictorial representation of the biochemical synthesis of conotoxins. Putative conotoxins derived from cDNA sequencing and expressed toxins in the venom of *C.achatinus*.

5. Conotoxin three-dimensional structures.

(G. Senthil Kumar, K. Hanumae Gowd, Dr.S.Ragothama, Dr.S.P.Sarma and Prof.P.Balaram)

The three-dimensional (3D) NMR solution structure of Ar1447 and Lo 959 has been determined using 2D-NMR methods. The structure of Ar1447 consists of two type I β-turns between residues 2-5 and 7-10. Sequence specific resonance assignments were obtained by the simultaneous examination of DQF-COSY, TOCSY, ROESY and 1H-13C HSQC spectra. Stereospecific assignments of the methylene protons and $\chi 1$ torsion

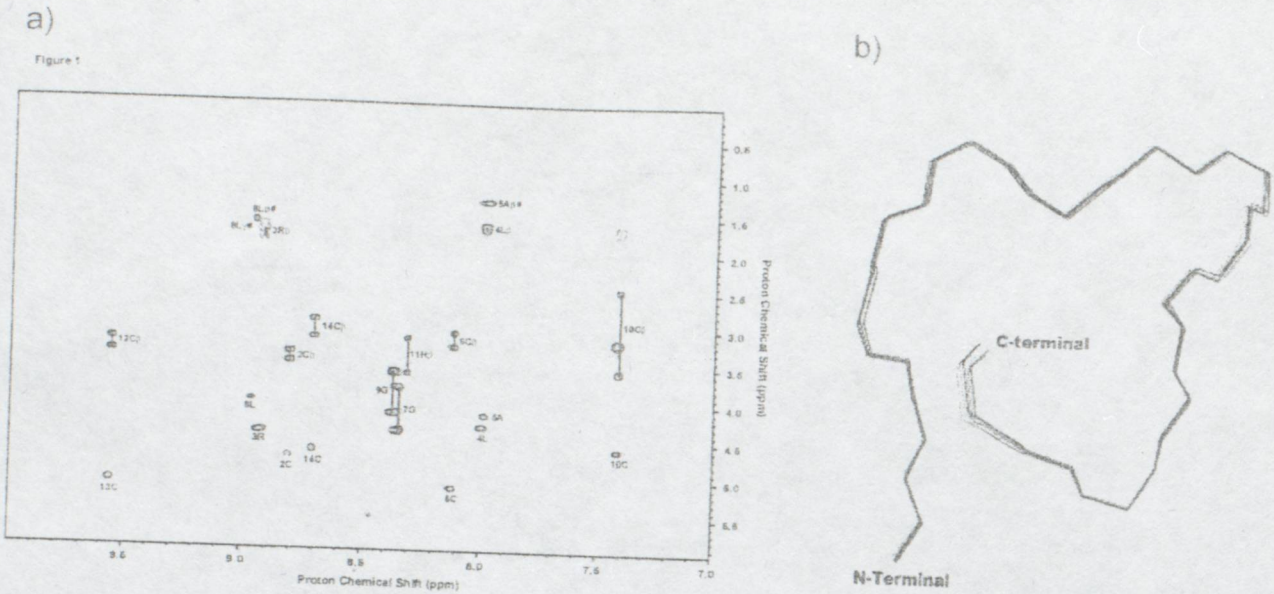


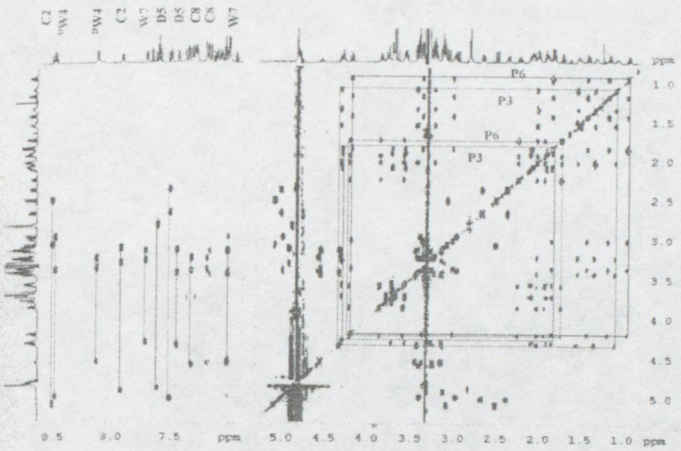
Figure 6 a) Shows the amide region of the TOCSY spectra and b) shows the superposition of 15 low energy structures on the backbone of N, Cα and C'

angle constraints for residues 2C, 3R, 4L, 6C, 10C, 14C were obtained from the ROESY spectra. The presence of N(i)-N(i+1), N(i+1)-N(i+2) and α(i)-N(i+2) between residues 8,9 and 10 indicates that residues 7 to 10 form a type I β-turn. Similarly residues 2-5 also form a type I β-turn. Disulfide connectivity in Ar1447 was established using mass spectrometry and NMR spectroscopy to be 1-6, 2-5 and 3-6 which has not been reported earlier. Figure 6a shows representative TOCSY spectra in the amide region and Figure 6b shows the energy minimized structure of Ar 1447. Ar1447 belong to the "Mini-M" class of conotoxins which contains less than 20 amino acids in their primary sequence.

Contryphans are a class of conopeptides that represent the smallest disulfide bridged peptide with unique fold represented as C-P/V-^{D}W/L-X-P-W-C. A novel contryphan-Lo (Lo959) was isolated from *Conus loroisii*. Its unusual biological activity, increase in calcium current (contrast to the known contryphan function) prompted for structural analysis by NMR. 2D-HSQC (1H-15N) in CD_3OH at natural abundance reveals two sets of peaks corresponds to two distinct conformers in equal amounts suggesting that contryphan-Lo exists in two distinct conformational states. Structural elucidation both the conformer of Lo959 was achieved using homo-/hetero-nuclear 2D-NMR

spectroscopy. Figure 7a shows representative TOCSY spectra and Figure 7b shows the key differences between the two conformer.

a) b)



• Following table highlights differences in *cis* and *trans* isomers

Sl. No.	*Cis* - isomer	*Trans* - isomer
1.	NOEs	
	C_2 $C^{\alpha}H - P_3$ $C^{\alpha}H$ - Strong (C_2 –P_3 *cis*) D_2 $C^{\alpha}H - P_6$ $C^{\delta}H_2$ - Strong (D_2 –P_6 *trans*)	C_2 $C^{\alpha}H - P_3$ $C^{\delta}H_2$ – Strong (C_2–P_3 *trans*) D_2 $C^{\alpha}H - P_6$ $C^{\delta}H_2$ – Strong (D_2 –P_6 *trans*)
2.	Other NOEs:	
	Other NOEs are typical of turn and extended segments	Other NOEs are typical of turn and extended segments
3.	^{13}C Chemical Shift:	
	$\Delta(\delta P_3\ C^{\beta} - \delta P_3\ C^{\gamma}) = (31.7 - 21.6) = 10.1$ ppm (large) $\Delta(\delta P_6\ C^{\beta} - \delta P_6\ C^{\gamma}) = (29.0 - 24.7) = 4.3$ ppm (small)	$\Delta(\delta P_3\ C^{\beta} - \delta P_3\ C^{\gamma}) = (28.1 - 25.1) = 3.1$ ppm (small) $\Delta(\delta P_6\ C^{\beta} - \delta P_6\ C^{\gamma}) = (28.9 - 23.9) = 5.0$ ppm (small)
4.	Structures differences: (visualized from model) Ring current shifts: (^1H Chemical shift)	Ring current shifts: (^1H Chemical shift)
	P3 $C^{\alpha}H$ and $C^{\beta}H$ – upfield shifted (1.32 & 1.68 ppm) Probably $^2W_1$ ring in the shielding	P6 $C^{\alpha}H$ and $C^{\beta}H$ – upfield shifted (0.94 & 1.18 ppm) Probably W7 ring shielding
5.	Temperature Coefficients:	
	All amides except D_2 – NH has high $d\delta / dT$	All amides except $^2W_1$ – NH has low $d\delta / dT$

Figure 7 a) 7000 MHz Partial TOCSY spectrum of Lo959 in CD_3OH/CD_3OD at 300K and b) Table highlights key features of two conformers arising due to Cis/trans isomerisation of the first proline (P-3) .

d_{NN} and $d_{\alpha N}$ NOE patterns along with CαH CSI (Chemical Shift Index) plot indicates the turn conformation in Lo959. This observation is further emphasized by CD data. Figure 8 shows an energy minimized model. It is clearly evident that the interconversion of two conformer under chromatographic time scale is due to cis/trans isomerisation about C_2-P_3 bond.

6. Folding studies of both biosynthesized and chemically synthesized analogues of conopeptides.

(K.Hanumae Gowd, G. Senthi Kumar, Dr. S.P.Sarma and Prof.P.Balaram)

The availability of small amount of natural conotoxins is a severe constraint for structural and functional analysis. We have therefore developed two independent approaches for the production of conus peptides. 1) Solid Phase Peptide Synthesis using an Fmoc(9-fluorenylmethyloxycarbonyl) strategy. 2) Expression of a synthetic cDNA fragment as a fusion protein with cytochrome-b5. These procedures require extensive experimentation in order to overcome the folding problem in the case of peptide with multiple disulfide bridges. Here we are demonstrating the synthesis and folding of a novel conopeptides

namely Am975, a single disulfide containing peptide and Vi1359, a two disulfide containing peptides.

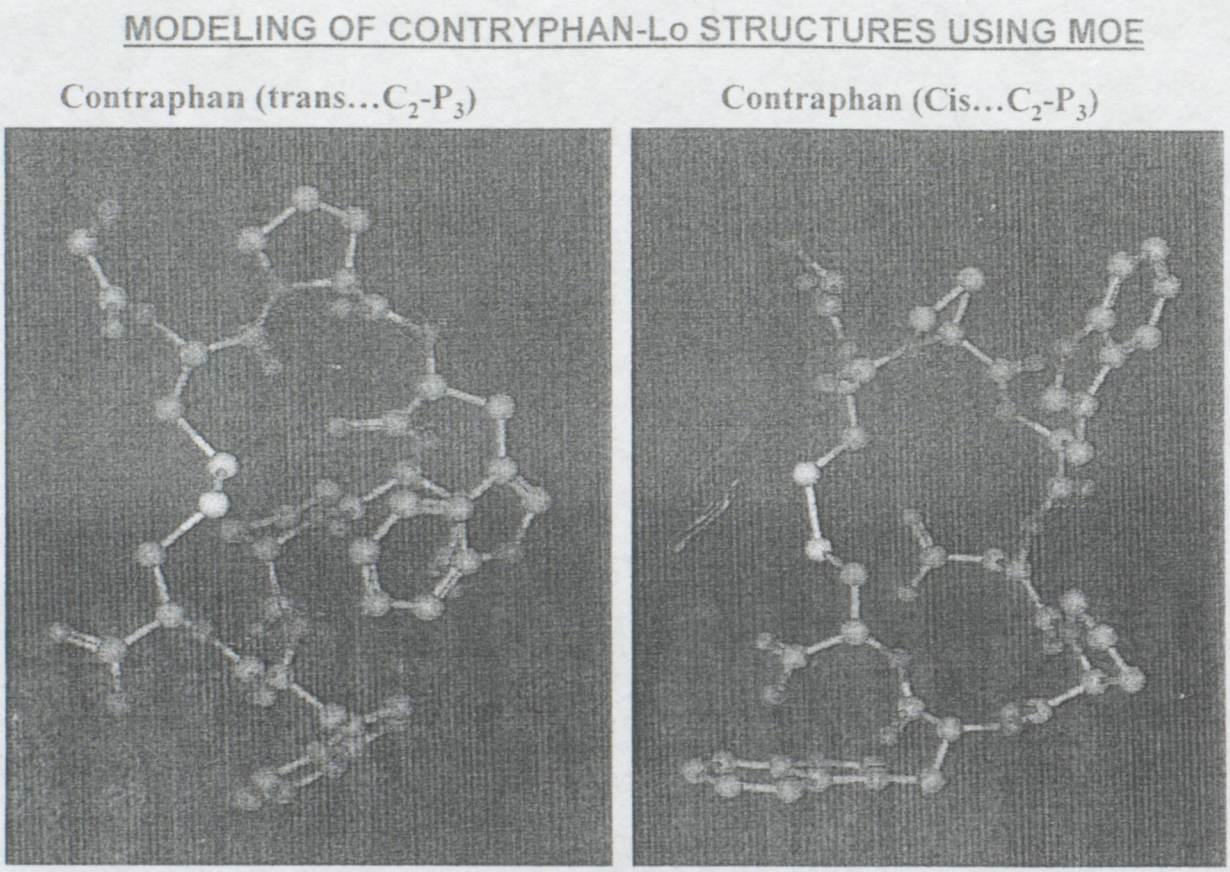


Figure 8. An energy minimized model of Lo959.

Am975 is a contryphan isolated from marine snail C.amadis; it contains a single disulfide loop with "D" configuration of tryptophan within the loop. Synthesis of Am 975 ($GCO^{D}WDPWC\text{-}NH_2$) was carried by solid phase synthesis methodology using Fmoc chemistry and pentafluorophenyl (Opfp) esters. The purified synthetic peptide was subjected to air oxidation in dilute solution (0.5mM) to minimize intermolecular disulfide bond formation. The folded synthetic Am975 was compared with natural peptide. Figure 9 illustrates the HPLC profiles of natural and synthetic Am975. It is clearly established the identity of natural and synthetic Am975. The two distinct HPLC fractions correspond to two slowly interconverting conformations. This is readily demonstrated by the fact that isolation and reinjection of a single component

(reinjection of peaks marked P.1 and P.2) results in the reappearance of both the HPLC peaks (Figure 3a inset). This slow conformational interconversion has been attributed to high activation barriers to rotation about X-Pro bonds, resulting from the constraints of disulfide bridge.

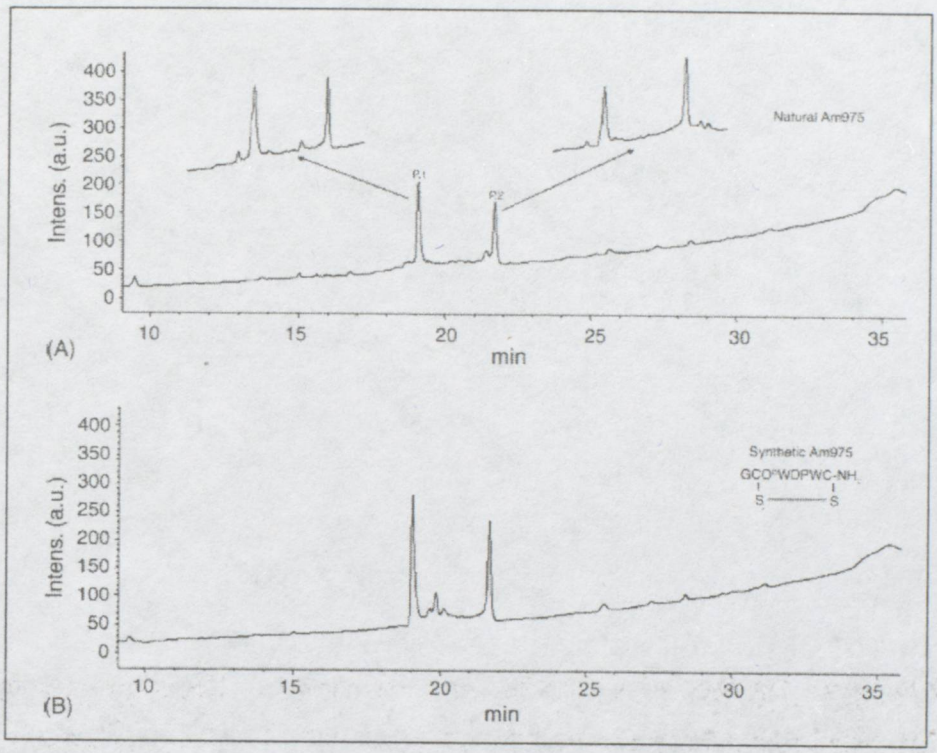


Figure 9. Comparison of chromatographic traces of (A) natural Am975 with that of (B) synthetic Am975 (inset) chromatographic traces obtained by reinjection of the fractions corresponding to P.1 and P.2

Vi1359 is a novel two disulfide containing conus peptide isolated from *C.virgo*, a unique feature of this peptide is the presence of pyroglutamic acid at the N-terminus, Vi1359 ZCCITIPECCRI-NH_2. Synthesis of this peptide has been achieved using solid phase peptide synthesis methodology and the coupling of pyroglutamic acid is affected by the *in situ* activation of its C-terminus using HBTU/DIPEA/HOBT as activation mixture. The purified reduced peptide was subjected for air oxidation by believing that native disulfide bonding will form predominantly.

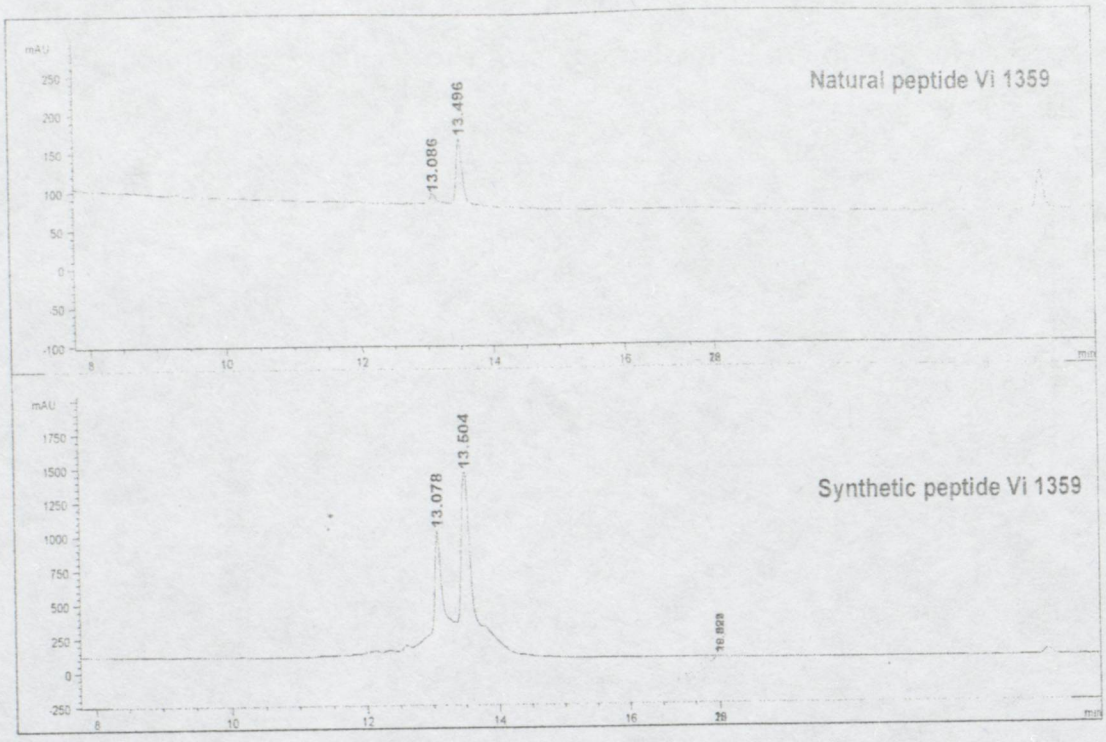


Figure 10. Comparison of RP-HPLC profile of natural and synthetic Vi1359

The disulfide paring of the folded peptide is found to be identical to the native peptide disulfide connectivity, i.e. 1-3 and 2-4. Figure 10 illustrates the comparison of RP-HPLC profiles of natural and synthetic Vi1359. The analgesic studies of the synthetic Vi1359 is found be identical to that of the natural peptide, further confirming the identity of the natural and synthetic Vi1359. Similarly synthesis and folding of a novel peptide Mo1277 has been achieved. The identity of natural and synthetic Mo1277 has been established using mass spectrometry and RP-HPLC. Synthesis and folding of Des(bromo) Mo1277, an analogue of Mo1277, also has been achieved in order to address the functional importance of tryptophan bromination.

7. Biological activity.

(P.Ramasamy, Dr.M.S.Ramasamy, Prof S.K.Sikdar and Prof.K.S.Krishnan)

Conotoxins target a wide range of receptors and channels in excitable cells. The biological activity of the purified conopeptides is identified by electrophysiology studies (Patch-clamp) using DRG neurons (or) CHO cells. Ion channel activity studies of Lo959

and Am 975 by electrophysiology studies using DRG neurons is briefly described. A two-pulse protocol was used to observe both the low-voltage activated (LVA) T-type Ca2+ current and the high voltage activated (HVA) Ca2+ current in relative isolation (19). The membrane potential was held at -100 mV. A 150 ms pre-pulse to - 40 mV activated the LVA / T-type Ca2+ current, which inactivated with time and remained inactivated for another 165 ms that was confirmed by an identical test

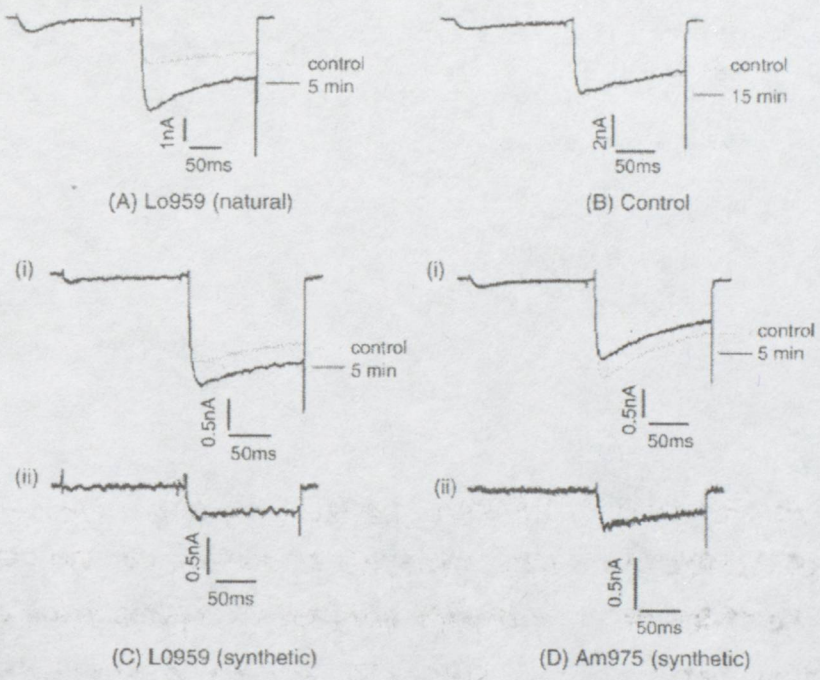


Figure 11 Selective modulation of the HVA-type Ca2+ current by the contryphans in medium sized DRG neurons.

pulse depolarization to - 40 mV, 10 ms after the pre-pulse (data not shown). A recovery time of 2s was allowed between the successive two-pulse protocols. However, when a similar two-pulse protocol was applied but with the test pulse depolarization to -10 mV, most of the T-type Ca2+ current remained inactivated, but evoked a high voltage-activated Ca2+ current in relative isolation. Both the natural and synthetic Am975 decreased the amplitude of high voltage-activated Ca2+ current with negligible effect on the low voltage-activated Ca2+ current. However, both the natural and synthetic Lo959 increased the amplitude of the high threshold Ca2+ current. Figure 11 summaries the

electrophysiology results of contryphans on DRG neurons. Similarity in the biological effects seen with the natural and synthetic contryphans on voltage-activated Ca2+ channels along with the similarity in the HPLC profiles confirms the homogeneity of the isolated contryphans and their sequences.

The analgesic activity of the peptides has also been demonstrated by the tail flick assay using mice as a model system. In this present study, the antinociceptive effect of the different Conus peptides has been studied by Tail flick analgesia meter. The results are summarized in the table and Figure 12 represents the analgesic studies of Vi1359.

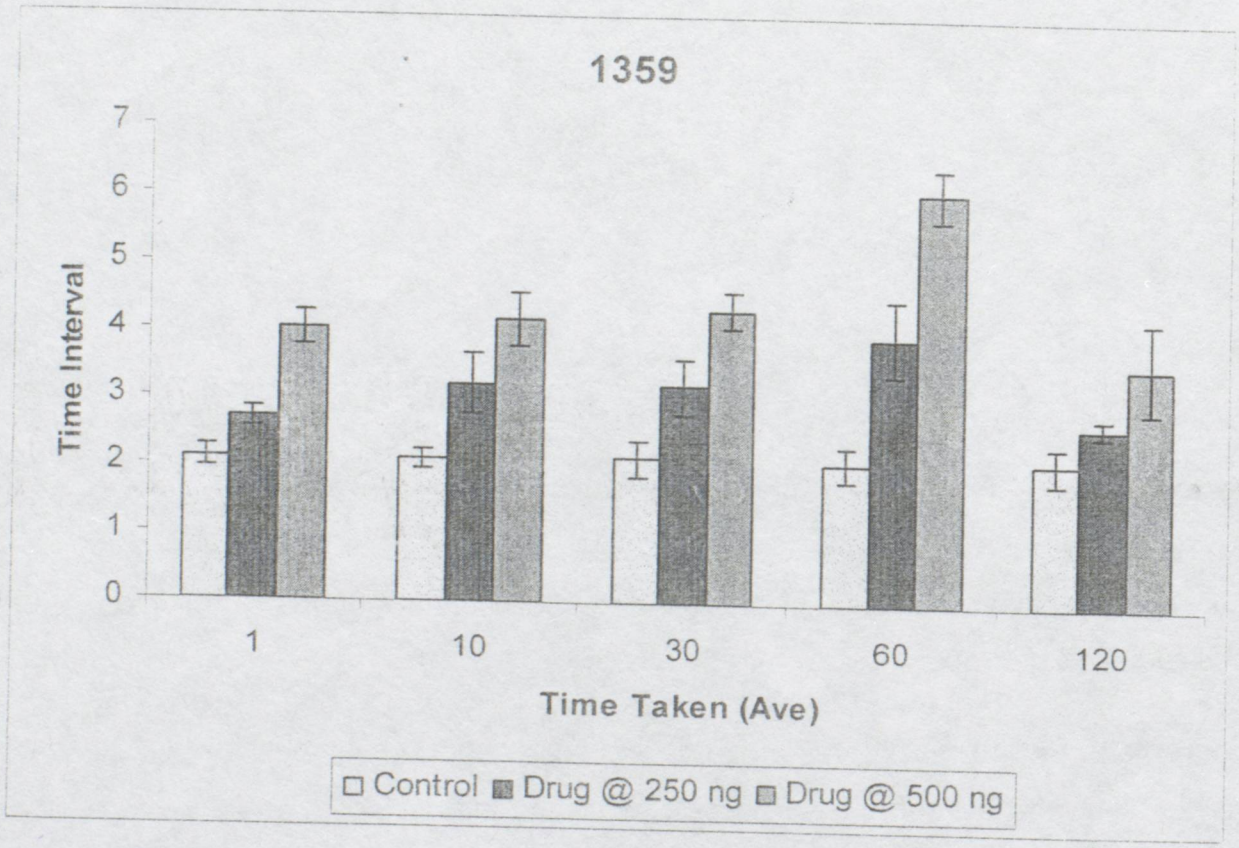


Figure 12. Statistical analysis of the latency period of the tail flick response as an index of antinociception (Vi1359).

Table 1. Summary of analgesic effect of conopeptides.

S. No	Peptide	Activity
1	Vi 1359	Antinociceptive , (analgesic) hypoactive
2	Ar 1447	Low antinociceptive analgesic, Excitory behavior, digging , scratching
3	Ar 1859	Antinociceptive, sleeper peptide
4	Ar 1813	Antinociceptive, Hypoactive
5	Lo959	Antinociceptive, Hypoactive

8. Deviation if any from the project objectives and reasons thereof.

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9. List of Patents Filed:

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10. List of Publications:

1. Characterization of contryphans from Conus loroisii and Conus amadis that target calcium channels.
   V. Sabareesh , K. Hanumae Gowd , P. Ramasamy , .Sudarslal, K.S. Krishnan, S.K. Sikdar , P. Balaram. Peptides. 2006 Nov;27(11):2647-54.

2. *'De novo'* sequencing and disulfide mapping of a bromotryptophan- containing

conotoxin by fourier transform ion cyclotron resonance mass spectrometry.
S. Sudarslal, C. Nilsson, M.R. Emmett, T.M. Schaub, K.Hanumae Gowd, S. S. Thakur, K.S. Krishnan, P. Balaram, Alan G. Marshall. *Anal Chem.* 2006 Dec 1; 78(23):8082-8.

3. Sequencing of T-Superfamily Conotoxins from Conus virgo: Pyroglutamic Acid Identification and Disulfide Arrangement by MALDI Mass Spectrometry.
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11. Leads developed and possible technology transfer/transferred.

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12. Summary of the progress.

Isolation and purification of active peptide toxin components are achieved using RP-HPLC from diverse cone snails. Purified peptides obtained from crude venom were subjected to primary structure determination, using *'de novo'* sequencing by mass spectrometry. The proposed sequence has been further confirmed by synthesizing the corresponding peptide and comparing the fragmentation pattern

of natural and synthetic peptides in some cases. Molecular biologically have also been employed to derive putative conotoxin sequences by the construction of cDNA libraries for conus venom. Peptide library of C.achatinus has been probed using mass spectrometry and cDNA sequencing. This procedure has yielded three putative δ-conotoxins and two ω-conotoxins from cDNA analysis, while four of these are shown to be expressed in the venom. The 3D-structure of Ar1447 and Lo959 has been determined using 2D-NMR experiments. Ar1447 found to contains two type 1 β-turn with disulfide connectivity between 1-6, 2-5 and 3-6 cysteine residues, which has not been reported earlier. Lo959 exist as a two conformers in solution due to cis/trans isomerisation about C_2-P_3 peptide bond. Synthesis and folding of novel conotoxins namely Lo959, Am975, Vi1359 and Mo1277 has been achieved using Solid Phase Peptide Synthesis and oxidative folding. Electrophysiological experiments have been employed to identify the biological activity of conus peptides particularly contryphans, Lo959 and Am975. Specifically, Am975 targets high voltage activated calcium channels and decreases calcium current. In contrast Lo959 targets high voltage activated calcium channels and increases calcium current. The antinociceptive effect of the different Conus peptides has been studied by Tail flick analgesia meter

13. Abbreviations of difficult words.