CHAPTER II

MATERIALS AND METHODS
2.1 Source of chemicals

All the L-amino acids, DCC, HOBT, HBTU, TFE, DIPEA, TFA (TFA was used after distillation) were from Sigma Chemical Company, USA. TA and EDT were either from Fluka AG Chemical Corp, Switzerland or Pierce Chemical Company, USA. And 9-fluorenylmethoxycarbonyl (Fmoc) amino acids and 4-(hydroxymethyl) phenoxy acetamidomethyl resin (HMPA), KR, KA resins were obtained from Applied Biosystems (Foster City, CA) and Nova-Biochem, UK or from Pierce, USA. Solvents such as dichloromethane (DCM), glacial acetic acid (AcOH), diethyl ether, petroleum ether, isooamyalcohol were from E. Merck (India) Ltd, India. HPLC solvents such as methanol (MeOH), dimethylformamide (DMF) (DMF was purified by storing over molecular sieves of 4A°), acetonitrile (ACN), piperidine and NMP were from Qualigens Fine Chemicals, India. Most of the inorganic chemicals and salts such as sodium sulphate, sodium chloride and HEPES were purchased from SD Fine Chemicals, India. The growth media chemicals were from HiMedia Laboratories Pvt. Ltd, India. HPLC columns were from Hewlett-Packard or Bio-Rad Company.

7-(thienyl-2-acetamido)-3-(2-(4-N,N-dimethyl-aminophenylazo)-pyridiniummethyl)-3-cephem-4-carboxylic acid (PADAC) was purchased from Behring Diagnostics, La Jolla, CA. O-nitrophenyl-β-D-galactoside (ONPG) and trifluoroethanol (TFE) were obtained from Sigma, St. Louis, MO. Phosphilipids, Dansyl phosphatidyl ethanolamine and Cholesterol was purchased from Avanti polar lipids (Birmingham, Alabaster A.L,USA). OPA was purchased from Sigma (USA). 3,3’-dipropylthiocarbocyanine iodide (diS-C3-(5) cyanine dye) and N-phenyl naphthylamine (NPN) were purchased from Molecular Probes.

2.2 Peptide synthesis

All the peptides used in the studies were synthesized by Fmoc-chemistry protocols (Atherton and Sheppard, 1989). Fmoc is a base labile α-amino protecting group, which can be easily removed by secondary amine bases like piperidine. Amino
acids containing TFA-labile side chain protecting groups were used. Acetamidomethyl

group was used to protect the sulphahydryl side chain of cysteine.

The different solid support resins used for synthesis are:

**KR resin:** KR is a kieselguhr-based resin where 4- (2', 4'-dimethoxy phenyl-Fmoc
amino methyl) - phenoxy linker is attached via norleucine. Cleavage of the peptide would
result in an amidated C- terminus. The substitution of the resin was nearly 0.12 mmol/g.

**KA resin:** Cleavage of the peptide would result in an acid C-terminus. The substitution
of the resin was nearly 0.12 mmol/g.

**HMP resin:** HMP resin, a polystyrene based resin (supplied by ABI) with a substitution
of 0.83 mmol/g. Cleavage of the peptide would result in an acid C-terminus.

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**2.2.1 Attachment of first amino acid to the solid resin support:** Symmetrical
anhydride method on polyamide resin (Atherton *et al.*, 1989): 1 g of polyamide resin of
0.1 mmol/g substitution (Ultrasyn KA and/or KR) was placed in a clean, dry flask to
which about 20 ml of DMF was added and left with occasional swirling for about an
hour. The DMF was decanted off as much as possible. This process was repeated once
again. Fmoc-amino acid (1 mmol) was dissolved in dry DCM (3-5 ml). One or two drops
of DMF were added to complete the dissolution to which a solution of
dicyclohexylcarbodiimide (1 mmol, DCC) in dry DCM (1 mL) was added. The mixture
was stirred on a magnetic stirrer gently for 20 minutes at 0 °C by keeping the reaction
mixture free of moisture using a calcium chloride drying tube. The DCU formed was
filtered off in case of soluble anhydrides. For an insoluble anhydride, dry DMF was
added (2-3 mL), stirring was continued for a further 10 minutes and the mixture was
transferred to the resin flask. In case of soluble anhydride, the filtrate was collected in a
dry, clean flask and evaporated to dryness under reduced pressure using a rotary
evaporator. The flask was dried till DCM goes off completely which yielded as oil. The
residual oil was dissolved in a minimum of DMF (2-3 mL) and added to the resin after
decanting the DMF completely from the resin flask. DMAP (0.1 mmol) in DMF was
added to the resin-amino acid mixture. The flask was stoppered and the mixture was
allowed to stand at room temperature for one to three hours with occasional swirling. The
resin was filtered and washed sequentially with DMF, isoamyl alcohol, AcOH and
isoamyl alcohol (2 x 25 mL) and filtrate was discarded. The resin was washed thoroughly with anhydrous diethyl ether and dried in vacuum desiccators. The degree of attachment of the first amino acid residue was determined by Fmoc-estimation as mentioned in 2.2.2. The procedure was repeated to get optimal substitution i.e., when similar substitution values were obtained in consecutive determinations.

### 2.2.2 Monitoring the substitution of first amino acid:
The extent of substitution of the first amino acid was estimated as follows: About 4-5 mg of dry Fmoc-amino acid resin was weighed into two test tubes. Freshly prepared 20% piperidine in DMF (3 mL) was dispensed into them and agitated for 30 min. Untreated resin was used as blank. The absorbance of the supernatants was read at 290 nm. Absorbance of 1 μmole Fmoc is 1.650. The amount of Fmoc released shows the substitution of first amino acid.

### 2.2.3 Synthesis of peptide using semi-automatic peptide synthesizer:
Polyamide resin after the attachment of first amino acid was loaded in a glass column, connected to the peptide-synthesizer and the flow was set at to ~3.5 mL/min. Online UV detector connected to a plotter was used to monitor the total reaction cycles (washings, deprotection and acylation). The α-amino group was deprotected using a base reagent of 20% Piperidine/DMF mixture. All the washes were done using DMF. The couplings were done either in DMF or NMP for one hour. The amino acids were coupled as their esters using three to four fold excess of amino acid and HOBT: DCC in 1:1 equivalent. The synthetic cycles were repeated till a desired sequence was made and deprotected using the same base reagent mentioned above. The resin was removed from the column; resin was washed with DMF and ether. The resin was dried and peptide was obtained by acidolytic cleavage.

A similar protocol was also used for the synthesis of peptides wherein the couplings were done in DMF using AA: HBTU: HOBT: DIPEA in 1:1:1:2 molar equivalents using three fold excess of amino acids. This mixture was prepared just before coupling and loaded on the instrument and recycled for one hour. The couplings of hydrophobic amino acids were increased by 10 min.
2.2.4 Manual peptide synthesis: Synthesis using Fmoc chemistry were carried out in a Nalgene cryovial with dimension 1.2 cm X 4 cm and of volume 1.2 ml with HMP resin. Gentle stirring was done with magnetic stirrer. The first amino acid was attached to the resin by the symmetric anhydride procedure. Coupling of Fmoc amino acid mediated by HBTU/HOBT and DIEA was done for one hour. Subsequently, it was deprotected with 20% piperidine in DMF. Then it was washed thoroughly for complete removal of piperidine. Same procedure was repeated for adding other residues. At the end of the synthesis, resin was washed thoroughly with DMF, isoamyl alcohol and ether and air-dried.

2.3 Cleavage of peptides from the resin

Synthesized peptides were cleaved from the resin using about 5 mL of cleavage mixture containing TFA, crystalline phenol, EDT, thioanisole, H2O (v\v) 10: 0.75: 0.25: 0.5: 0.5 for 100 mg resin. After the cleavage mix was added to the resin, the reaction mix was kept at room temperature for 3 hrs. After the reaction time had elapsed, the mixture was filtered through a medium-porosity, fritted glass funnel into another flask to separate the peptide solution from resin support. Then cold diethyl ether was added to the mixture to precipitate the peptide. The precipitate was washed for 5 to 6 times with cold diethyl ether to remove scavengers completely. The precipitate was separated and dried in desiccator under vacuum. The dried crude peptide was redissolved and checked for purity on HPLC.

This protocol was successful for 13-residue peptide (crabrolin) containing one arginine (Mtr) residue. But for 24-residue peptides (brevinin 1E), incomplete deprotection of arginine (Mtr) was identified by mass spectrometry. For such long peptides overnight deprotection was carried out with cleavage mixture containing 2-5 mL TFA, C, TA, EDT in the ratio (v\v) 10: 1.5: 1.5: 0.75 of about for 100 mg resin.

2.4 Purification of peptides

All peptides synthesized by Fmoc-chemistry were directly purified by HPLC after cleavage from the polymer support. The peptides were purified by HPLC using
reversed phase column chromatography (analytical C-18 column or/and C-4 column). A proper gradient system was developed for good separation to the base line level by mixing two solvents, water and ACN containing 0.1% TFA. The eluted peptides were monitored at 280 or 210 nm and the corresponding peaks were collected simultaneously. In most of the cases, only a single peak was obtained as a major fraction, which was later confirmed as the right component by mass spectra.

2.5 Estimation of concentration of the peptides

The stock solutions of purified peptide were made either in water or in water-methanol mixtures. The concentration of the peptides were measured by UV absorbance using Hitachi spectrophotometer connected with a recorder at 280 nm using a $e$ of 5600 molar$^{-1}$ cm$^{-1}$ for tryptophan. The peptides which do not have tryptophan were either taken by weight or difference in absorption at 225 nm and 214 nm multiplied by 1.44 which gives $\mu$g/mL concentration (Wolf, 1983). Subsequently, the concentrations were also confirmed by amino acid analysis as mentioned below. The concentrations obtained by amino acid analysis were about 80-85% of the concentration observed by the UV absorbance monitored spectrophotometrically.

2.6 Characterization of synthetic peptides

2.6.1 Amino acid analysis: Amino acid analysis was done for purified peaks only. A concentration of about 10 nmoles of the samples was hydrolyzed with 100 $\mu$L of mixture of 6N HCl containing 0.1% phenol. The tubes were sealed under vacuum and hydrolyzed for 20-22 hours in a heating block set at 105-110°C. After which the tubes were opened, and the acid was evaporated under vacuum over NaOH and phosphorous pentoxide in a desiccator. The contents of the dried tubes were dissolved in loading buffer and analyzed on Pharmacia LKB-Alpha Plus amino acid analyser. The concentration and composition of amino acids for unknown samples were calculated by calibrating the instrument using a calibration mixture of amino acids of concentration 5 or 10 nmoles.
2.6.2 Peptide sequencing: The primary sequence was determined by sequencing the HPLC purified samples using 470A or 473A Applied Biosystems Protein Sequencer. The amino acids were identified by calibrating the instrument using PTH amino acids of 100 picomole concentration. Peptides in the concentration range of ~ 1 nmole were loaded as aqueous or in 0.1% TFA/water after confirming the condition of the instrument with sequence of protein β-lactoglobulin as standard.

2.6.3 Mass spectral analysis of peptides: HPLC purified peptides were analysed by MALDI-TOF mass spectrometry. Mass spectrometers from Kratos (IICT, Hyderabad) and Voyager DE-STR Perceptive Biosystems (Proteomics facility, CCMB) were used for analysis. Peptides were dissolved in 0.1% TFA in H₂O at a concentration of nearly 5 picomoles/μL. A 0.5 μL of sample was spotted on the MALDI plate and allowed to dry, followed by 0.5 μL of matrix which was allowed to dry. The mass to charge ratio obtained from MALDI-TOF was compared to theoretically calculated mass. Freshly prepared stock solution of 10 mg/mL recrystallized α-cyano hydroxy cinnamic acid (HCCA) was used as the matrix.

2.7 Disulphide bond formation

For peptides containing cysteine residues, actamidomethyl (Acm) protecting group was used to protect the SH group of cysteine. To deprotect Cys (Acm) group mercury (II) acetate was used (Veber et al., 1972) and disulphide bond formation was accomplished in 20% DMSO (Tam et al., 1991).

The following protocol was used for disulphide bond formation:

The peptide was dissolved in 30% acetic acid at the highest concentration possible depending on solubility of the peptide. Two equivalents of mercury (II) acetate for each equivalent of cysteine was added and the reaction was stirred for 1 hour. Then twenty equivalents of 2-mercaptoethanol was added to the reaction mixture. Stirring was done for another 1 hour until there was a gray precipitate of mercuric sulphide salt. The reaction mixture was centrifuged and the supernatant was loaded on a Sephadex G10
column to remove 2-mercaptoethanol using 30% acetic acid as eluting solvent. The peptide fraction was collected and speedvac concentrated and dissolved in 20% aqueous DMSO solution for formation of disulphide bonds as described by (Tam et al., 1991). The complete removal of Acm groups and disulphide bond formation of the peptide was confirmed by mass spectrometry. The MALDI mass spectral data for synthetic single disulphide peptides used for the studies are given in the Table 2.1 and 2.2.

2.8 Other experimental protocols

2.8.1 Antibacterial activity

2.8.1.1 Determination of Minimum inhibitory concentration (MIC): The antibacterial activities of the synthetic peptides were monitored by growth inhibition of microorganisms (sometimes this was confirmed by plating the cells at the MIC concentration and counting the viable cells). Minimum inhibitory concentration (MIC) was obtained by titrating different concentrations of peptide with about $10^6$ cells grown at mid-log phase. Cell cultures were revived from the plates inoculated with organisms in suitable media and were grown overnight. Next day, the overnight grown culture was subcultured to mid-log phase to an OD of 0.5 at 600 nm. This culture was diluted in media such that 1 mL contains $\sim 10^6$ cells. Peptides were prepared in autoclaved water or in DMSO-water mixture where the DMSO concentration did not exceed by 2%. Peptide solutions of corresponding volumes were pipetted in the tubes and freshly grown diluted cells i.e. $\sim 10^6$ in 1 mL of the media were added and incubated at 37°C for 6-9 h. After incubation the absorbance of the culture media at 600 nm was measured. Growth media (without inoculum or peptide) was used as controls. Melittin, the bee venom hemolytic peptide which also has antibacterial activity (Habermann, 1972; Dempsey, 1990) was included in the assay as control. The MICs obtained were an average of minimum of three assays carried out independently in duplicate or triplicate. Micro-organisms like Escherichia coli W160.37, Escherichia coli (MG1655) Staphylococcus aureus (ATCC 8530), Pseudomonas aeruginosa (NCTC 6751) were used for the assay.
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<th>S. No.</th>
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<th>Number of Residues</th>
<th>Sequence</th>
<th>Calculated</th>
<th>Observed</th>
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<tr>
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<td>24</td>
<td>FLPLLAGLCKITRKCAANFLPKIF-COOH</td>
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</table>

Table 2.1: MALDI mass spectra data on synthetic peptides
Table 2.2: MALDI mass spectra data on synthetic peptides

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Peptide</th>
<th>Number of Residues</th>
<th>Sequence</th>
<th>Calculated</th>
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</table>
2.8.1.2 Determination of Lethal concentration (LC): The LC of antibacterial activity of the peptides were examined in sterile 96-well plates in a final volume of 100 μL as follows: Bacteria were grown in nutrient broth to mid-log phase and diluted to 10^6 colony forming units (cfu)/mL in 10 mM sodium phosphate buffer (pH 7.4). Aliquots of 100 μL of diluted cultures were incubated with different concentrations of peptides (0.5-30 μM) for 2h at 37°C, and suitably diluted aliquots were plated on nutrient agar plates. After the plates were incubated at 37°C for 18 h, colonies formed were counted. The concentration of the peptides at which no viable colonies were formed was taken as the LC. The average of three independent experiments done in duplicate was determined for LC. Salts or divalent cations were included in the incubation buffer to determine their effect on antibacterial activity.

The chemical compositions of different growth media used for the assay are as follows:
Nutrient rich media: (Beef extract, 0.3%; Yeast extract, 0.2%; Bacto-peptone, 0.5%; NaCl, 0.5%). The above ingredients were mixed and autoclaved after adjusting the pH to 7.4. For agar plates, 1.7% agar was added to nutrient broth.
Minimal A: Media–A contains KH_2PO_4, 10.5 g/L; K_2HPO_4, 4.5 g/L; (NH_4)_2SO_4, 1.0 g/L; Sodium citrate, 0.5 g/L; MgSO_4.7H_2O, 0.1 g/L. Media–B contains L-arginine, 0.1 g/L; Glucose, 10.0 g/L. The pH of media was adjusted to 7.4. A and B were autoclaved independently and mixed 1:1 before using.

2.8.2 Hemolytic activity

2.8.2.1 Preparation of RBCs: The hemolytic activity of the synthetic peptides was determined using adult Wistar rat erythrocytes. The RBCs were prepared as follows. 2-3 mL of rat blood was drawn into 12 mL of heparinized 5 mM HEPES buffer pH 7.4 containing 150 mM NaCl, and centrifuged at 4000 rpm for 5 min. The cell pellet was washed three times with buffer and theuffy coat was removed. A working stock of RBCs was made by diluting the RBCs pellet (0.5-0.8 mL) to about 15 mL with the buffer. For standardization of the volume for lysis assay, different volumes (5-40 μL) of
diluted blood samples were drawn into 1 mL of water containing 0.1% of Triton X-100 and buffer with RBCs was used as blank. The solution was centrifuged at 4000 rpm, hemoglobin released in the supernatant was measured spectrophotometrically at 540 nm. The volume of RBCs was standardized so as the absorbance was about ~0.2 OD (about 2 x 10^6 cells/mL) range. This suitable volume was used for the hemolysis assays with peptides.

**2.8.2.2 Hemolysis with peptides:** Different volumes of each peptide were taken in 1.5 mL eppendorf tubes containing 5 mM HEPES buffer pH 7.4 containing 150 mM NaCl. To this, diluted blood suspension (volume as determined above) was added and total volume in the reaction tube was made upto 1 mL with the buffer. The tubes were incubated at 37°C for 30 min in a gentle shaking water bath. After incubation, the samples were centrifuged in a Kubota centrifuge at 4000 rpm for 5 min to remove the unhemolysed cells. The absorbance of the supernatant was measured at 540 nm. Buffer containing RBCs suspension was taken as buffer blank (A_0). The lysis obtained with 0.1% of Triton X-100 with RBCs was taken for 100% lysis control (A_{100}). The assays were carried out in duplicates and were repeated thrice. The average values were taken for plotting. The percentage hemolysis was calculated using the following equation:

\[
H\% = \frac{(A_{\text{sample}}-A_0) \times 100}{(A_{100}-A_0)}
\]

Where \(A_{100}\) and \(A_0\) are the absorbance of 100% and 0% hemolyzed cells respectively.

**2.8.3 Determination of secondary structures of peptides:** CD spectra were recorded on Jobin Yvon Dichrograph spectropolarimeter or on Jasco J-715 spectropolarimeter at room temperature. The calibration was done using d-camphor sulphonic acid. Spectra were recorded in 0.1 cm path length with a bandwidth of 1 nm and step resolution of 0.2 nm. An average of twelve scans per sample was recorded. Usually, the scans were recorded in the range from 195-250 nm. Proper blanks were recorded using peptide-free suspension and were subtracted from the corresponding sample spectra. The different media used for recording the spectra were 5 mM HEPES buffer pH 7.4, TFE and SDS micelles. The final spectra were plotted as mean residue molar ellipticity v/s wavelength.
2.8.4 Transmission Electron Microscopy (TEM): Bacteria were incubated with peptides at their LC as described above and centrifuged at 1500 g for 3 min. The pellet was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 3 h at 4°C. After fixation, the pellet was washed thrice with 0.1 M phosphate buffer. The samples were then post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 2 hrs. Fixed samples were washed thoroughly with phosphate buffer following which they were dehydrated through a series of acetone gradients. Dehydrated samples were passed through propylene oxide and infiltrated with epoxy resin overnight. Samples were then embedded in pure epoxy resin and dried at 60°C for 72 h. Sections were obtained using a Reichert Ultracut E Microtome and were stained with 2% uranyl acetate and Reynold’s lead citrate. Sections were observed in a JEOL 100 CX electron microscope at 80 KV. Control samples (without peptide) were made in the same manner.