CHAPTER 2

Experimental Procedures

2.0 Experimental Procedures

This chapter describes the general experimental procedures used in this thesis. Specific details are mentioned in subsequent chapters.

2.1 Materials

9-Fluorenylmethoxy carbonyl (Fmoc) amino acids were obtained from Novabiochem AG (Switzerland) and Advanced ChemTech (Louisville, KY). The Fmoc amide handle crown was from Mimotopes (Australia). DCC, HOBt, HBTU, DIPEA and TFA were obtained from Sigma-Aldrich (USA) and Advanced ChemTech, USA. Solvents like DMF (purified and dried using molecular sieves), glacial acetic acid, DCM, chloroform, methanol, diethyl ether, isoamyl alcohol, isopropanol, DMSO, NMP and β-mercaptoethanol were purchased from either Merck (India) Limited, Qualigens Fine Chemicals (India) or Spectrochem (India). p-Hydroxymethylphenoxymethyl polystyrene (HMP) resin was obtained from Applied Biosystems (USA) while Fmoc-L-arginine 4-hydroxymethylphenoxy acetic acid (PAC (R)) and 4-hydroxymethylphenoxy acetic acid-polyethylene glycol-polystyrene (PAC-PEG-PS) resins were obtained from Millipore (USA). Piperidine was from Loba-Chemie (India). Most inorganic chemicals and salts like sodium chloride, mercuric acetate, EDTA, HEPES and potassium iodide were obtained from SD Fine Chemicals, India. All phospholipids were purchased from Avanti Polar Lipids (Alabama, USA). HCCA, CCCP, ONPG and TFE were obtained from Sigma (USA). Bacterial growth media chemicals were purchased from HiMedia Laboratories (India). FM 4-64 was obtained from Molecular Probes (USA). All other chemicals used were of the highest grade available.

2.2 Solid Phase Peptide Synthesis

Peptides were synthesized employing Fmoc chemistry by solid phase peptide synthesis strategy (Atherton and Sheppard, 1989). Fmoc is a base labile NH_2-protecting group, which can be easily removed by secondary amine bases like piperidine.

Peptides were synthesized either on Fmoc amide handle crown (6.9 μmol/pin), PAC-PEG-PS resin (0.18 mmol/g) or PAC-R resin (0.29 mmol/g). Fmoc amide handle crown are high-density polyethylene inert plastic with a derivatized surface to give solvent compatible polymer matrix. The amide handle [(R,S)-a-[1-(9H-fluorene-9-yl] methoxyformamide]-2,4-dimethoxy benzyl] phenoxy acetic acid (rink amide handle) is attached to the polymer matrix to produce peptides having an amidated C-terminus. PAC-PEG-PS resin contains 4-hydroxymethyl phenoxy acetic acid linker to polyethylene glycol graft polystyrene. A free acid at the C-terminus is produced after the cleavage of the peptide from the resin. HMP resin has a polystyrene base and the cleavage of peptide results in an acid C-terminus. Cleavage of peptides synthesized on PAC-R resin gives rise to free acid at the C-terminus.

2.2.1 Attachment of the First Amino Acid

In case of the peptides that were synthesized using Fmoc amide handle crown the first step was to remove the Fmoc group using 20% piperidine in DMF for 20 min. The crown was washed repeatedly with DMF to remove the piperidine completely. The crown was again washed twice with methanol and air dried for 30 min. Three-fold molar excess of HOBt, HBTU and six-fold molar excess of DIPEA were added to the three-fold excess Fmoc amino acid dissolved in DMF. The contents were mixed thoroughly by tapping and kept for 10 min for activation. Then the mix was added to the crown pin and kept for 2 h with occasional shaking. After 2 h the pin was washed twice with methanol and then twice with DMF. The reaction was repeated with two-fold molar excess of HOBt, HBTU, Fmoc amino acid and four-fold molar excess of DIPEA and kept for 1 h. The pin was washed twice with methanol and then twice with DMF to attach the next amino acid.

For peptides synthesized on PAC-PEG-PS and PAC-R resins, the first amino acid was attached by generating symmetric anhydride. Ten-fold molar excess of Fmoc amino acid was taken in a 10 mL round bottom flask and dissolved in minimum amount of DCM. If the amino acid did not dissolve, one or two drops of DMF were added. A ten-fold molar excess of dicyclohexylcarbodiimide (DCC) was then added to this solution. The mixture was stirred for 30 min after which a white precipitate was obtained. Then

DCM was evaporated completely in a rotavapor by using vacuum. The dry anhydride was redissolved in a minimum volume of DMF and added to the resin that was soaked overnight in DMF. DMAP (0.1 equivalents) dissolved in DMF was added to the resin amino acid mixture and the reaction was allowed to continue for three hours with thorough mixing. The resin was transferred to the synthesis vial and washed sequentially with DMF, isoamyl alcohol, glacial acetic acid, isoamyl alcohol and diethyl ether. Finally, the resin was dried and the degree of substitution was monitored by Fmoc estimation. If a low substitution value was obtained then the steps were repeated.

2.2.2 Determination of Amino Acid Substitution

Measurement of Fmoc released was used to determine the coupling efficiency after the attachment of Fmoc amino acid. It is reported that 1 mol of Fmoc in 3 mL of 20% piperidine in DMF gives an absorbance of 1.650 at 290 nm (Kawakami et al., 1999). About 3-4 mg of dry resin was treated with 3 mL of 20% piperidine in DMF for 20 min and the absorbance measured at 290 nm (A_{290}) was compared with the theoretical value to obtain percent substitution. In case of Fmoc amide pin, the entire pin was treated with 20% piperidine and then the Fmoc was estimated. Another convenient way to confirm the substitution of an amino acid at any point in synthesis procedure uses the absorbance of Fmoc at 301 nm (A_{301}) (ε=7800) (Meienhofer et al., 1979). In this procedure, the resin or pin was taken into a test tube or 1.5 mL microfuge and 20% piperidine in DMF was added. The substitution value was calculated using the formula:

Substitution value = (A_{301} x Vol (mL)) / (7800 x weight of resin in grams)

If the substitution was more than 80% further synthesis was continued otherwise recoupling of the same Fmoc amino acid was performed.

2.2.3 Subsequent Couplings of Amino Acids

In case of the peptides that were synthesized using Fmoc amide handle crown, the N-terminus protecting Fmoc was cleaved using 20% piperidine for 20 min and the resin was washed repeatedly with DMF to completely remove the piperidine. The resin was again washed twice with methanol and air dried for 30 min. Three-fold molar excess of HOBt, HBTU and six-fold molar excess of DIPEA were added to the three-fold excess

Fmoc amino acid dissolved in DMF. The contents were mixed thoroughly by tapping and kept for 10 min to activate Fmoc amino acids. Then the mix was added to the crown pin and kept for 2 h with occasional shaking. After 2 h the resin was washed twice with methanol and then twice with DMF. The reaction was repeated with two-fold molar excess of HOBt, HBTU, Fmoc amino acid and four-fold molar excess of DIPEA and kept for 1 h. The resin was washed twice with methanol and then twice with DMF before proceeding to deprotect the α-amino group and attaching the next amino acid.

For peptides synthesized on PAC-PEG-PS and PAC-R resins, 20% piperidine in DMF was added to the resin attached with the first amino acid and kept for 20 min. Resin was washed extensively with DMF to remove piperidine till the pH was neutral. Five-fold molar excess of HOBt and HBTU were added to the five-fold molar excess Fmoc amino acid dissolved in DMF. This mixture was added to the resin and after 5 min six-fold molar excess of DIPEA was added to this mixture. The reaction was carried out for 1 h with continuous shaking. This process was repeated again for 30 min with three-fold molar excess of amino acid. Next amino acid was attached by removing the Fmoc by using 20% piperidine in DMF and repeating the steps.

2.2.4 Cleavage, Deprotection and Precipitation of Synthesized Peptides

The synthesized peptides were cleaved and deprotected by using a mixture containing 82.5% trifluoroacetic acid (TFA), 5% phenol, 5% H_2O, 5% thioanisole, and 2.5% ethanedithiol for 12 to 15 h at room temperature (King et al., 1990). The volume of this mix varied according to the amount of resin that was deprotected. About 500 μL was used for 20-30 mg of resin. The cleavage mix was then separated from the resin and washed 5-6 times with cold diethyl ether to completely remove the scavengers. The precipitate was air dried to obtain crude peptide.

2.2.5 Purification of Peptides

The peptides were purified on a Hewlett-Packard 1100 series high-performance liquid chromatography instrument with a reversed-phase column. C4 or C18 Bio-Rad column were used for the purification of linear peptides whereas reversed-phase C8

Zorbax column was used for disulfide linked peptides. The solvents consisted of 0.1% TFA in H_2O as mobile phase A and 0.1% TFA in CH_3CN as mobile phase B in case of linear peptides while 0.1% TFA in H_2O as mobile phase A and 0.1% TFA in CH_3CN/Isopropanol (1:1) as mobile phase B was used for disulfide linked peptides. Elution of the peaks was monitored at 280 nm if tryptophan or tyrosine was present and at 214 nm in the absence of aromatic amino acids. The collected fractions were dried in a speed vacuum drier and stored until further use at 4 °C.

2.2.6 Estimation of Peptide Concentration

Purified peptides were dissolved in the appropriate solvents in which they were completely soluble. Except HNP-1, all the other peptides were dissolved in deionized water. The concentrations were determined by monitoring the absorbance of tryptophan or tyrosine at 280 nm using a Hitachi spectrophotometer. The molar extinction coefficient of tryptophan was taken as 5690 $Molar^{-1}$ cm^{-1} and 1280 $Molar^{-1}$ cm^{-1} for tyrosine. The volume of solvent was adjusted to get an absorbance in the range 0.05 to 0.1. For the peptides without tyrosine or tryptophan the following formula was used as described by Waddell (Waddell, 1956; Wolf, 1983): Concentration (μg/mL) = 144 (A_{215}-A_{225}), where A_{215} is the absorbance at 215 nm and A_{225} is the absorbance at 225 nm.

2.2.7 Peptide Characterization

MALDI-TOF Analysis: The purified peptides were characterized by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry on a Voyager DE STR mass spectrometer (Applied Biosystems) at the Proteomics Facility of the Centre for Cellular and Molecular Biology. Recrystallized HCCA was used to remove certain impurities that might interfere with the crystallization and mass spectral analysis. To recrystallize, 2.0 mg of HCCA was weighed into a microfuge tube and dissolved in 50 μL methanol. It was vortexed to completely dissolve the matrix and then centrifuged in a quick spin mini centrifuge (National Labnet company, USA) for 2-3 min. The clear supernatant was transferred into another microfuge tube and 10 μL of deionized water was added thrice (total of 30 μL) with vortexing to reprecipitate the matrix. The microfuge tube was centrifuged at 5000 rpm for 5 min at room temperature and the supernatant was

discarded. The pale yellow recrystallized HCCA was dissolved in acetonitrile/water (1:1) containing 0.1% TFA to obtain a final concentration of 10 mg/mL.

Sample Preparation for Mass Spectral Analysis: 1.0 μL of 2 pmoles/μL of peptide in acetonitrile/water (1:1) containing 0.1 % TFA was spotted on the grid and allowed to dry completely. This spot was overlaid with 0.8 μL of HCCA (10 mg/mL) and allowed to dry. The spectra were then recorded on a Voyager DE STR mass spectrometer (Applied Biosystems).

Characterization of Disulfide Connectivities: Synthetic peptides containing multiple disulfide connectivities were analyzed to assign disulfide linkages. A 1:50 (w/w) ratio of trypsin to peptide was used for the assay. The disulfide-linked peptides were incubated with trypsin at 37 °C for 18 h in 25 mM NH_4HCO_3. The digested peptide fragments were dried and redissolved in acetonitrile/water (1:1) containing 0.1% TFA and later analyzed by MALDI-TOF mass spectrometry.

Fluorescence Spectra: The Fluorescence spectra of the peptides were determined in aqueous medium at a concentration range of 1-5 μM. The excitation wavelength was set at 280 nm or 295 nm when Tyr was present and emission wavelength was scanned from 300-400 nm with an excitation/emission slit width of 5 nm/5 nm.

2.3 Bacterial Killing Assay

The bacterial strains used were *Escherichia coli* W 160-37, *Escherichia coli* MG 1655, *Staphylococcus aureus* (NCTC 8530), and *Pseudomonas aeruginosa* (NCTC 6750). Bactericidal activity was determined as follows. Mid-log-phase bacteria were obtained by growing bacteria in nutrient broth (HiMedia), washing them twice with 10 mM sodium phosphate buffer (pH 7.4), and then diluting them in the same buffer to give approximately 10^6 Colony forming unit (CFU)/mL. A final volume of 100 μL was used in sterile 96-well plates to examine the antibacterial activity of the peptides. After the incubation of bacteria with different concentrations of peptides for 2 h at 37 °C, 20 μL was plated on nutrient agar plates. The plates were incubated for 18 h at 37 °C, and the

colonies formed were counted. An incubation temperature of 30 °C was used for *S. aureus*. The lowest concentration of peptide at which there was complete killing was taken as the lethal concentration (LC). In the control experiments, cells were incubated with only buffer or 0.01% acetic acid (the solvent used to dissolve HNP-1). The values determined were the averages of three independent experiments.

2.4 Kinetics of Bacterial Killing

The kinetics of bacterial killing was determined against Gram-negative and Gram-positive bacteria. Mid-log-phase bacteria (10^6 CFU/mL) were incubated with LCs of peptides in 10 mM sodium phosphate buffer (pH 7.4) in a final volume of 100 μL. Aliquots of 20 μL were removed at fixed intervals and plated on nutrient agar plates. The CFU were counted after an incubation of 16 to 18 h at 37 °C. The values mentioned in the results are the averages of two independent experiments.

2.5 Salt Sensitivity of Peptides

Salts (NaCl, 100 mM; $MgCl_2$ and $CaCl_2$, 1 mM and 2 mM) were added to the incubation buffer to examine the effect of salt on the antibacterial activity of the peptides. The effect of salts was examined at the lethal concentrations of the peptides. *E. coli* MG 1655 (10^6 CFU/mL) was used to examine inhibition by $MgCl_2$ and $CaCl_2$. The values determined were the averages of three independent experiments. The variation was within ±5%.

2.6 Bacterial Outer Membrane Permeability Assay

The ability of peptides to permeabilize the outer membrane of *E. coli* MG 1655 cells was investigated using N-phenyl-1-N-naphthylamine (NPN) uptake assay (Sedgwick and Bragg, 1987; Loh et al., 1984). Bacterial cells at an optical density of 0.1 at 600 nm (OD_{600}) were suspended in 5 mM HEPES (pH 7.4) with 10 μM NPN. After 15 min of incubation, peptides were added and the fluorescence of NPN was monitored. The excitation wavelength was set at 350 nm and the emission wavelength was set at 420 nm. The experiment was carried out at 25°C.

2.7 Bacterial Inner Membrane Permeability Assay

The ability of peptides to permeabilize the inner membrane of bacteria was studied using *E. coli* GJ 2455 (*lacI⁻ lacZ*$^+$ strain derived from *E. coli* MG 1655 at Centre for Cellular and Molecular Biology), which constitutively expresses β-galactosidase in its cytoplasm. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) was used as the substrate for β-galactosidase. Late logarithmic-phase (OD_{600} of 0.5 to 0.6) cells were washed and diluted to an OD_{600} of 0.03 in 10 mM sodium phosphate buffer (pH 7.4) and 0.53 mM ONPG was added. After the addition of different peptide concentrations, OD was recorded every 5 min at 550 nm and 420 nm, and absorbance calculations [A_{420} - (1.75 X A_{550})] were taken as a measure of the β-galactosidase activity. The production of *o*-nitrophenol was monitored at 420 nm. The value 1.75 X A_{550} represents the light scattering by cell debris at 420 nm. Bacterial cells without any peptide were used as control.

2.8 Effect of Dissipation of Proton Motive Force on Antibacterial Activity

The effect of loss of proton motive force in bacteria due to the action of CCCP was examined as follows. Bacteria were preincubated with 20 μM CCCP for 20 min, followed by the addition of peptides at their LCs, with or without NaCl. The effect of incubation with 50 μM CCCP was also examined. CCCP at concentrations of 20 μM and 50 μM has been used for dissipation of the proton motive force in bacteria (Dibrov, 1991; Ohyama et al., 1992; Shafer et al., 1998). The bactericidal activity was determined as described earlier. The values determined were the averages of three independent experiments. The variation was within ±5%.

2.9 Lysis of Human Red Blood Cells

Human erythrocytes isolated from my blood were used to evaluate the hemolytic activity of the peptides. Erythrocytes were obtained by centrifuging (800 x *g*) heparinized blood and then were washed thrice with 5 mM HEPES (pH 7.4) containing 150 mM NaCl. Aliquots containing 10^7 red blood cells/mL were incubated in the presence of

different peptide concentrations in 0.5 ml microfuge tubes at a final volume of 100 μL for 30 min at 37 °C with gentle mixing. The tubes were centrifuged, and the absorbance of the supernatants was measured at 540 nm. Erythrocyte lysis occurring in deionized water was taken as maximal lysis.

2.10 Effect of Serum on Antimicrobial and Hemolytic Activity of Peptides

To study the effect of serum components on the antimicrobial activity and hemolytic activity of the peptides, serum was isolated from human blood after the removal of erythrocytes by centrifugation. Total protein was estimated using the Folin-Ciocalteu-Lowry method (Lowry et al., 1951). For both antimicrobial activity and hemolytic activity, serum at 1 mg/mL was included in the buffer and incubated for 15 min before the addition of peptides. Cells with only serum were used as control.

2.11 Circular Dichroism

A JASCO J-715 spectropolarimeter was used to record the spectra of peptides in water, trifluoroethanol (TFE), and 10 mM sodium dodecyl sulfate (SDS) at 25°C. A quartz cell with a 1 mm path length was used. Various instrument parameters used were as follows: Step resolution (0.5 nm), accumulation (8), band width (1 nm), slit width (500 μm), speed (20 nm/min), response (2 sec) and sensitivity (10 mdeg). Spectra were recorded in the far-UV region from 195 to 250 nm. Mean residue ellipticity was calculated using the formula:

$$[\theta]_{MRE} = Mr \times \theta_{observed}/100 \times l \times c$$

where '$[\theta]_{MRE}$' is the mean residue ellipticity, 'Mr' is mean residue weight (molecular weight/number of residues), '$\theta_{observed}$' is the observed ellipticity, 'l' is length in decimeter and 'c' is the concentration in mg/mL.

2.12 Fluorescence Microscopy

Membrane damage caused by peptides was visualized by fluorescence microscopy as follows: *E. coli* MG 1655 cells were stained using a lipophilic dye FM 4-

64 (N-(3-triethylammoniumpropyl)-4-(6-(4(diethylamino)phenylhexatrienyl) pyridinium dibromide) which preferentially stains the inner membrane (Fishov and Woldringh, 1999). FM 4-64 was added to a final concentration of approximately 3 μM. After 10 min, cells were washed once with 10 mM sodium phosphate buffer (pH 7.4) and diluted in the same buffer to give approximately 10^6 CFU/ml bacterial cells. A final volume of 100 μL was used for incubation with peptides at their lethal concentrations. After 10 min, 10 μL was layered on a glass slide and observed on a Zeiss Axioplan 2 imaging microscope under fluorescence optics using 100X oil immersion objective. Images were captured on Axioplan CCD camera using Axiovision (Version 3.1). The excitation filter was set at 510–560 nm and the emission filter at 590 nm.

2.13 Interaction of Peptides with Lipid Vesicles

Small unilamellar vesicles (SUVs) having different compositions of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl- 2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (POPG) were prepared by sonication (Vibra-Cell, 400 W, Sonics). A thin film of phospholipid was formed by evaporation of a solution of phospholipids in chloroform. The film was left overnight under vacuum for the complete removal of solvent. Hydration was carried out in 5 mM HEPES (pH 7.4). Subsequently the solution was sonicated to obtain SUVs. Binding of peptides to vesicles was assessed by monitoring the fluorescence of tryptophan. Peptides (1 μM) were titrated against increasing concentration of lipid vesicles. The excitation monochromator was set at 280 nm with 5 nm excitation and emission slit widths. The emission spectra were recorded from 300 to 400 nm. All the fluorescence measurements carried out in this study were performed in an F-4500 Hitachi fluorescence spectrophotometer. Quenching experiments using KI were carried out to determine accessibility of tryptophan. Increasing amounts of KI was added from a stock solution. The data were analyzed using the Stern-Volmer equation, $F_0/F = 1 + K_{sv}[Q]$, where F_0 and F represent the fluorescence intensities in the absence and presence of quencher [Q], respectively, and K_{sv} is the Stern-Volmer quenching constant. For the comparison of quenching data from different peptides, normalized

accessibility factor (naf) was calculated from the ratios of K_{sv} at a lipid to peptide ratio of 100:1 and K_{sv} for peptide in the absence of lipid.