University of Alberta

Synthesis and SAR studies of Antimicrobial Peptide Leucocin A

By

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Dedication

I would like to dedicate this thesis to
my parents Venkateswarlu and Rama Devi
who have never failed to give me moral and financial support
and to my brother Baswanth Maharshi for his love, and encouragement.

Abstract

In this study, we report the synthesis of a potent antimicrobial peptide Leucocin A (LeuA) using two solid phase peptide synthesis methods. One of the methods, native chemical ligation, gave high yield (12.5%) of 37-residue LeuA and can be utilized in the synthesis of LeuA to perform structure-activity relationship (SAR) studies. Three analogues (**1**-**3**) of LeuA were designed and synthesized to explore the SAR in the N-terminal domain of LeuA. In the analogues, N-terminal β-sheet residues Cys9-Ser15 of the native peptide were replaced with shorter β-turn motifs. Such replacement abolished the antimicrobial activity in all the analogues. Circular dichroism spectroscopy suggested that only analogue **1** adopted similar folding as LeuA. Therefore, **1** was able to competitively inhibit the activity of native LeuA. However, analysis of the secondary structure of **1** using molecular dynamics simulations suggested lack of β-sheet formation in the N-terminal region compared to LeuA emphasizing the role of proper folding and sequence in the activity of class IIa bacteriocins.

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List of Abbreviations

aa	Amino acid
AMP	Antimicrobial peptide
BOP	Benzotriazol-1-yloxy-tris (dimethylamino)-phosphonium hexafluorophosphate
CD	Circular dichroism
CDC	Centre for Disease Control
Cl^-	Chloride ion
DCM	Dichloromethane
ddH_2O	Double distilled water
DIC	1, 3-diisopropylcarbodiimide
DMF	*N*,*N*-dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPC	Dynamic poly conjugates
FDA	Food and drug administration
Fmoc	9-Fluorenylmethoxycarbonyle
GROMACS	GROningen MAchine for Chemical Simulations
HBTU	*O*-Benzotriazole-*N*,*N*,*N*,*N*-tetramethyl-uronium hexafluorophosphate
HOBt	N-Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
IPA	Isopropyl alcohol
iPr2EtN	N,N-diisopropylethylamine
K	Kelvin
L	Liter
LAB	Lactic acid bacteria
LINCS	Linear Constraint Solver
M	Molar
MALDI	Matrix-assisted laser desorption/ionization

MD	Molecular dynamic simulations
MDR	Multi Drug Resistance
mg	Milligram
MIC	Minimum Inhibitory Concentration
Min	Minute
mL	Milliliter
mM	Milimolar
MS	Mass spectrometry
Na^+	Sodium ion
ng	Nanogram (10^{-9} gram)
nm	Nanometer
NMM	N-methylmorpholine
NMP	1-Methyl-2-pyrrolidinone
NMR	Nuclear magnetic resonance
ns	Nanosecond
OD	Optical density
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PBS	Phosphate buffer saline
PDB	Protein Data Bank
PyBop	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RNA	Ribonucleic acid
SPPS	Solid phase peptide synthesis
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
TMB	3,3',5,5'-Tetramethylbenzidine
TOF	Time of flight
UV	Ultraviolet
Vis	Visible
VMD	Virtual Molecular Dynamics

WHO	World Health Organization

Amino acids Abbreviations

Ala (A)	Alanine
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic acid
Cys (C)	Cysteine
Gln (Q)	Glutamine
Glu (E)	Glutamic acid
Gly (G)	Glycine
Leu (L)	Leucine
Lys (K)	Lysine
Phe (F)	Phenylalanine
Pro (P)	Proline
Tyr (Y)	Tyrosine
Val (V)	Valine
Nle(X)	Norleucine
β-Ala (Z)	β−Alanine

Chapter 1 Introduction

In recent days, food safety has become an important concern globally. Several antibiotics are being developed to treat food borne diseases like Listeriosis. In 2010, in the United States alone, contaminated food caused approximately 1,000 reported disease outbreaks and an estimated 48 million illnesses, 128,000 hospitalizations, and 3,000 deaths.[1] Due to rapid development of multiple-drug-resistance in bacterial strains, there is a high necessity for the development of new drug entities which can be used as potential antibiotics. Antimicrobial peptides are currently being investigated as new potential antibiotics to treat multiple drug-resistances in bacteria because of high potency, low incidence of developing drug-resistance and low toxicity.[2,3] In this study, I focused my research on antimicrobial peptides which can treat Listeriosis, a dangerous food borne disease caused by *Listeria monocytogenes*.

1.1 Listeriosis

Listeriosis is a potentially fatal food borne infection in humans caused by gram positive pathogenic bacteria *Listeria monocytogenes* (**Figure 1.1**) that occurs ubiquitously in nature.[4] It has a low incidence rate when compared to other food borne diseases caused by *E.coli* and *Staphylococcus* but has highest mortality rate (30%-40%).[5] First description of listeriosis can be found in 1926 as the cause of an outbreak in guinea pigs and rabbits.[4] The first listeriosis outbreak in humans was identified in maritime provinces in Canada with a high case-fatality rate [6], since then several outbreaks were reported worldwide.[4] Two forms of listeriosis have been

observed, invasive and non-invasive. Clinical manifestations in case of non-invasive form include febrile gastroenteritis, severe sepsis, meningitis, rhombencephalitis, perinatal infections, and abortions in case of invasive form.[7]

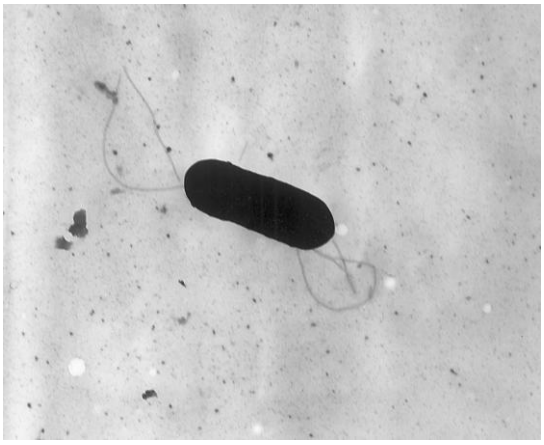


Figure 1.1 Scanning Electron Micrograph of *Listeria Monocytogenes*

The best treatment for listeriosis available to date is the synergistic action of an amino penicillin (amoxicillin or ampicillin) and gentamicin. Amino penicillin can be replaced with trimethoprim in case of intolerance of beta-lactams.[8,9] Several studies have recently been reported with an increased rate of resistance to one or several clinically relevant antibiotics in environmental isolates.[10-14] Morvan *et al* [15] extensively studied the prevalence of resistance in a large collection of 4,816 clinical *L. monocytogenes* strains isolated between 1989 and 2007 and studied the temporal evolution of susceptibility to 23 different antibiotics (**Table 1.1**). Their study revealed that among the 4,668 clinical L. monocytogenes strains tested, 61 (1.27%) strains were resistant to at least one clinically relevant antibiotic and two isolates showed multi drug resistance.

Table 1.1 Resistance to antibiotics of *L. monocytogenes* strains isolated from humans between 1989 and 2007. (Adapted from reference [15])

Antibiotic	No. of resistant strains	Pulsotype	MIC or MIC range (µg/ml)	Resistance mechanism or gene
Trimethoprim	1	1	1,024	*dfrD*
Tetracycline	34	15	16-128	*tet*(M) *int*-Tn
Minocycline			8-16	*tet*(M) *int*-Tn
Erythromycin	1	1	256	Putative chromosomal mutation
Streptomycin	2	2	256	Putative ribosomal Mutation
Chloramphenicol	1	1	48	*cat*
Ciprofloxacin	20	14	6->32	*lde*

Due to the development of antibiotic resistance in Listeria species, there is a need for new potential anti-listerial drugs. Another challenge in treatment of listeriosis is poor diagnosis of the disease because of the lack of fast and reliable diagnostic tools. The common symptoms include fever and muscle aches and vomiting, which are common in most of the bacterial infections. Thus, reliable early detection plays a key role in listeriosis treatment.

1.1 Bacteriocins

What are Bacteriocins? Bacteriocins are ribosomally synthesized, membrane-permeabilizing, antimicrobial peptides (AMPs), and are widely distributed in nature. It has been identified that 30-99% of bacteria can produce at least one bacteriocin.[16] The first description of bacteriocins as antibiotics was

reported 80 years ago. The use of bacteriocins as food preservatives was first reported in 1950.[17] First description of a proteinaceous antimicrobial substance was described in 1933 [18] and later, in 1947, it was named as nisin. Nisin was first approved for marketing in England in 1953 and after, it has been approved in 48 countries to be used as food preservative and still being used as food preservative in dairy products.[19]

Applications of Bacteriocins: Bacteriocins produced by lactic acid bacteria (LAB) are considered food grade and can be used as preservatives in the biopreservation of meat, dairy products, canned food, fish, alcoholic beverages, salads, egg products, high-moisture bakery products, and fermented vegetables, to prevent various food borne diseases.[19] At present, nisin in the form of Nisaplin (Danisco) and pediocin PA1/AcH in the form of ALTA 2431 (Quest) have been approved for use as food preservatives. In 2010, Micocin®, a bacteriocin derived from *Carnobacterium maltaromaticum UAL307* was approved for sale in Canada.[20] Bacteriocins can also be used as antibiotics to treat various infectious diseases [21] and as antiviral agents. [22,23] The use of lantibiotics (bacteriocins containing lanthionine ring in their strucutre) in treating infections caused by S. pneumonia, [24] and methicillin resistant *Staphylococcus aureus* (MRSA), [25] and in preventing tooth decay and gingivitis has been successfully demonstrated in initial *in vivo* trials with animal models.[26]

Classification: There are several classifications of bacteriocins described in literature based on their source, spectrum of activity, molecular weight and chemical

nature.[19,27,28] Classification based on the chemical nature is described here [27]. Primarily, bacteriocins can be divided into four distinct categories: lanthionine ring-containing bacteriocins (lantibiotics, class I), non lanthionine ring-containing bacteriocins (class II) [19], and protein bacteriocins with molecular masses of >30 KDa. It has been proposed that circular bacteriocins produced by LAB should be included in a separate category as class IV.[29]

1.1.1 Lanthionine ring-containing bacteriocins (Lantibiotics, class I)

Lantibiotics are post-translationally modified peptides which contain lanthionine ring in their structure.[30] Lanthionine ring formation reaction in lantibiotics is an enzyme catalyzed two step reaction: dehydration of serine residues followed by ring formation.[19]

Lantibiotics can be subdivided into two types based on their structure: type A, elongated molecules with molecular mass <4 kDa and type B, globular molecules with molecular mass ranging from 1.8 to 2.1 kDa.[27] Elongated lantibiotics (for example, nisin) are amphiphilic in nature and act by formation of pores in the target cell wall followed by efflux of cell contents, while globular lantibiotics (for example, actagardin) act by inhibiting bacterial enzymes.[31]

Table 1.2 Classification of Bacteriocins basing on their chemistry. (Adapted from reference [27])

Category	Characteristics	Subclass	Examples
Class I	Lantibiotics (containing lanthionine)	***Type A***	Nisin
		Type B	Mersacidin
Class II	Non modified heat-stable bacteriocins containing peptides with molecular masses of <10 kDa	***Subclass IIa***	Leucocin A Pediocin
		Subclass IIb	Plantaricin EF Plantaricin JK
		Subclass IIc	Lactococcin A
Class III	Protein bacteriocins with molecular masses of >30 kDa	-	Helveticin

1.1.2 Non-lanthionine containing bacteriocins (class II)

Class II bacteriocins are non-lanthionine containing, non post transitionally modified, heat-stable cationic peptides with molecular masses of <10 kDa. These peptides can be used to treat food borne infections caused by *Listeria monocytogenes* in nanomolar concentrations.[32,33] Class II bacteriocins are further subdivided into sub classes such as, class IIa (also called as Pediocin-like bacteriocins) and class IIb (two-peptide bacteriocins) based on their structure.[19] Class IIa bacteriocins are narrow spectrum AMPs, but show high potency against *Listeria monocytogenes*, a gram-positive causative organism for fatal listeriosis in humans. Number of amino

acid residues in class IIa bacteriocins ranges from 37 aa (leucocin A and mesentericin Y105) to 48 aa (carnobacteriocin B2 and enterocin SE-K4). Sequence alignment and molecular modelling studies of class IIa bacteriocins reveals important structural features that include YGNGV sequence and a conserved disulphide bridge at the N-terminus [34] (**Figure 1.2**). N-terminal sequence of this class shows high homology [35,36] and is believed to be involved in nonspecific binding to the target cell surface.[37,38] Whereas, the C-terminal region, which is located after the hinge region, shows little homology but their sequences allow formation of a hydrophobic amphipathic α-helix [39], which plays a key role in antimicrobial

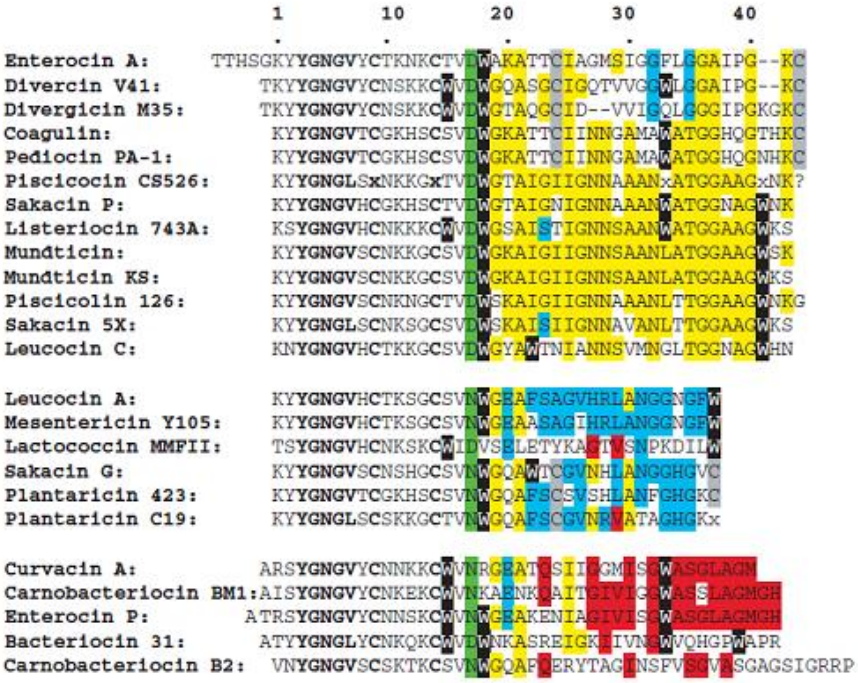


Figure 1.2 Sequence alignment of class IIa bacteriocins (pediocin-like AMPs) highlighting the conserved YGNGV sequence and cysteine residues (depicted as bold letters) in the N-terminus, tryptophan residues (black boxes), and less conserved residues in the C-terminus of the peptides (yellow, blue, gray, green, and red boxes). (Adapted from reference [33])

specificity and activity of these peptides.[40-43]

Three-dimensional (3D) structures of several class IIa bacteriocins such as leucocin A,[44] carnobacteriocin B2,[45] sakacin P,[39] and curvacin A,[46] have been elucidated by NMR spectroscopy (**Figure1.3**). The solution structure in membrane mimicking solvents like 2,2,2-Trifluoroethanol (TFE) shows that class IIa bacteriocins consist of an N-terminal β-sheet-like domain which is structurally stabilized by the conserved disulfide bridge.[33] The C-terminal amino acid sequence of class IIa bacteriocins consists of one or two α-helices, often ending with a structurally extended C-terminal tail.[33] In the C-terminal domain, class IIa bacteriocins, such as sakacin G, plantaricin 423, pediocin PA-1/AcH, divercin V41, and enterocin A, contains two disulfide bridges which plays an important role in stabilizing their 3D structure.[33,39]

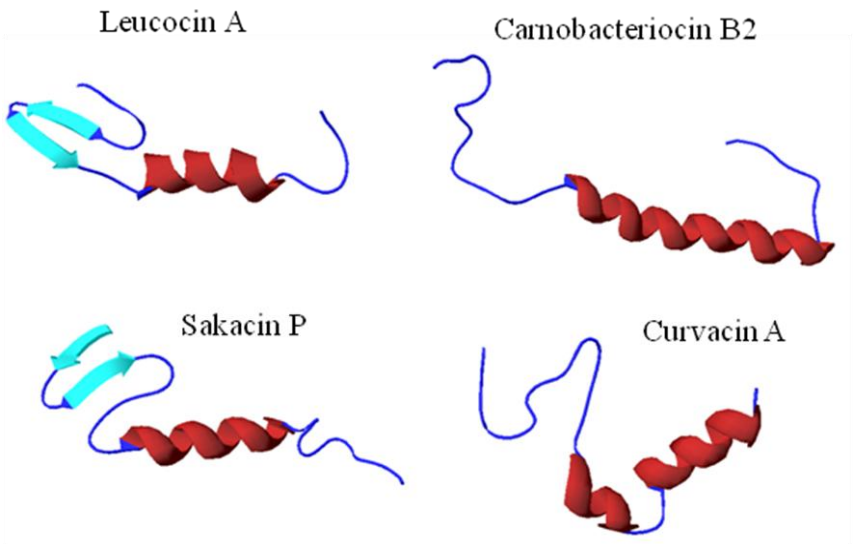


Figure1.3 Diagrammatic representation of the 3D NMR solution structures of four class IIa bacteriocins namely, leucocin A,[44] carnobacteriocin B2, sakacin P,[39] and curvacin A.[46]

Class IIb bacteriocins (for example, plantaricin EF) are also called as two-peptide bacteriocins, because of the synergistic activity of two peptides. A peptide from this class will show activity only in the presence of the complimentary peptide from the same type of bacteriocin or a homologous two-peptide bacteriocin. For instance, in order to show the activity of plantaricin EF, two peptides plantaricin E and F should be present.[47,48] Class IIb peptides show very low or no activity when tested individually.[19,49] Molecular dynamic simulation studies [50] and gene encoding studies of these peptides showed that the synergistic action of these two peptides is due to the fact that they interact with each other and form as a single antibacterial unit rather than individually act on the target cell membrane.[49] Two-peptide bacteriocins act by permeabilizing the target cell wall by forming pores which allows the efflux of essential ions out of the cell.[51]

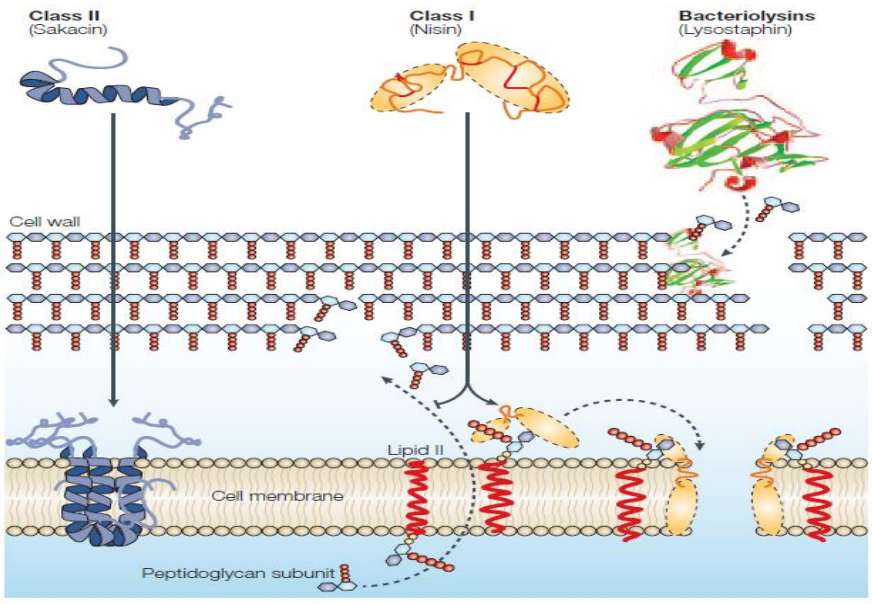


Figure 1.4 Mechanism of action of bacteriocins. (Adapted from reference [19])

Among class IIa bacteriocins, Leucocin A is a promising AMP with very high potency (nano molar) against *Listeria monocytogenes* which is the organism responsible for listeriosis in humans.

1.1.3 Leucocin A (Class IIa bacteriocin)

LeucocinA (LeuA) is a 37 aa cationic membrane permeabilizing class IIa bacteriocin derived from *Leuconostoc gelidium*. LeuA is highly potent against a variety of food pathogens, including *Listeria monocytogenes* in nanomolar concentrations.[27] Net charge of LeuA is +2 at neutral pH.[35]

NMR solution structure: Three dimensional solution structure of LeuA was first reported by Fregeau *et al.* [44] (**Figure 1.5**). This study revealed that LeuA is potentially unstructured in water but in membrane mimicking solvents like TFE and dynamic poly conjugates (DPC) micelles, it shows defined three-dimensional conformations. Fleury *et al.* [52] conducted a series of experiments to elucidate amino acid sequencing and structure-activity relationship studies (SAR) of LeuA. All the above studies suggested that structure of LeuA can be divided into two

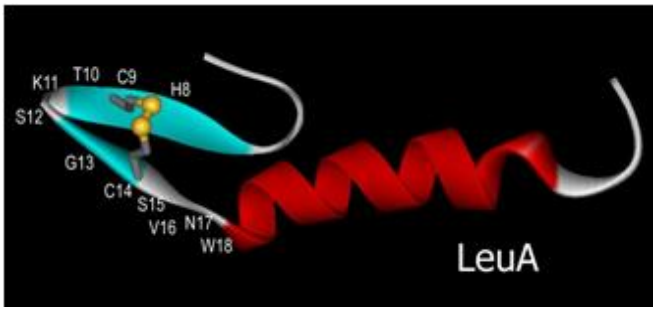


Figure 1.5 NMR solution structure of native LeuA (PDB code: 1CW6) in 90% TFE.[44]

regions: (i) a hydrophilic N-terminal region containing conserved YGNGV sequence that forms a three-stranded antiparallel β-sheet (residues 2-16) supported by a conserved disulfide bridge formed between the cysteines 9 and 14. (ii) amphiphilic/hydrophobic C-terminal region (residue 20 onwards) that forms an amphipathic α-helix.[33] However, with little homology sequence with other class IIa bacteriocins, the extended C-terminal tail forms a hairpin-like structure by folding back on to the central α-helix (**Figure 1.5**). There is a formation of flexible hinge between the N-terminal β-sheet and the hairpin like C-terminal region, which allows the relative movement of two domains to each other.[39]

Mechanism of action: Site directed *in vitro* mutagenesis studies has revealed that the N-terminal β-sheet region binds to the target cell membrane through electrostatic interactions, [37,38] and the hydrophobic C-terminal α-helix region will penetrates into the target cell membrane (**Figure 1.6**) which ultimately leads to the efflux of cell contents.[38,42,43,53] Recent site directed *in vitro* mutagenesis studies proved that the C-terminal region, which is present after the hinge region, is a major determinant in the target specificity of LeuA.[33,41] These results have been further confirmed by Fimland *et al*[54] by altering C-terminal region with another class IIa bacteriocin, in which, the target specificity always comes from the peptide from which the C-terminal region is derived.

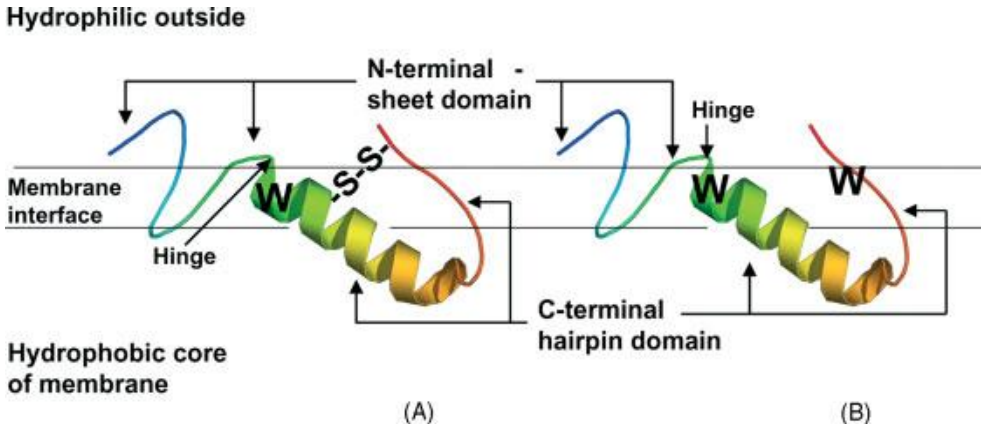


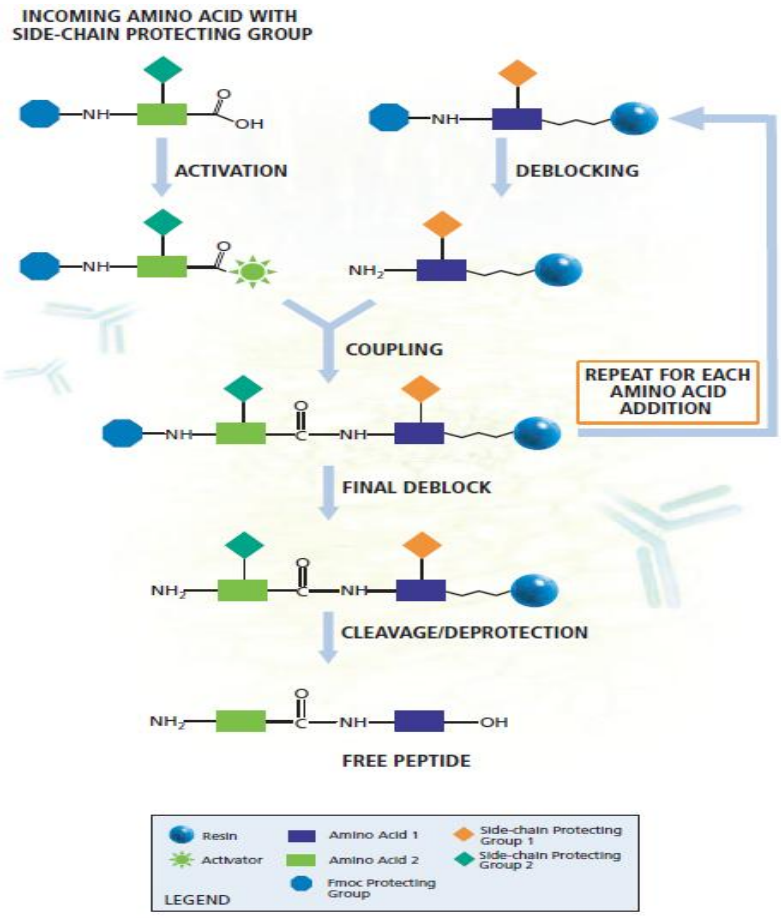
Figure 1.6 A diagrammatic representation of orientation of pediocin-like AMPs in the biological membrane. A pediocin-like AMP, in which the C-terminal hairpin structure is stabilized by a disulfide bridge (A), and an interface-localized tryptophan residue (B), near the C-terminal end of the AMP. Tryptophan residues (represented as W) are responsible for the localization of the peptide in the membrane-water interface and the disulfide bridge (represented as –S–S–). (Adapted from reference [36]).

It is now believed that the C-terminal region of the LeuA will bind to the receptor on the target cell membrane called membrane-bound mannose phosphotransferase (Mpt) system permease.[55-58] Comparative two-dimensional gel analysis conducted by Gravesen *et al* [58] revealed that the MptA subunit of the protein was absent in leucocin A-resistant mutants derived from leuA sensitive listerial strains, while heterologous expression of the MptC subunit of the same protein in an leuA insensitive strain resulted in the sensitive strain to several class IIa bacteriocins. From these studies, it has been suggested that, the MptC subunit of the Mpt protein functions as a receptor or docking site for class IIa bacteriocins.[58] For all the proposed mechanisms by which type IIa bacteriocins shows antimicrobial activity, it confirms that the folded conformation of the peptide is a key element.[59-61]

1.2 Native Chemical Ligation

The vast use of potent AMPs such as class IIa bacteriocins as antibiotics, food preservatives, pathogen sensors, to name a few, is slow due to the lack of availability of large quantities of these peptides. Availability of these peptides and their analogues in pure forms can allow detailed SAR studies and elucidation of their mechanism of action. In this regard, several lead researchers are trying to search for methods for chemical synthesis of peptides and proteins as they are difficult to isolate in large amounts from natural sources in homogenous form.

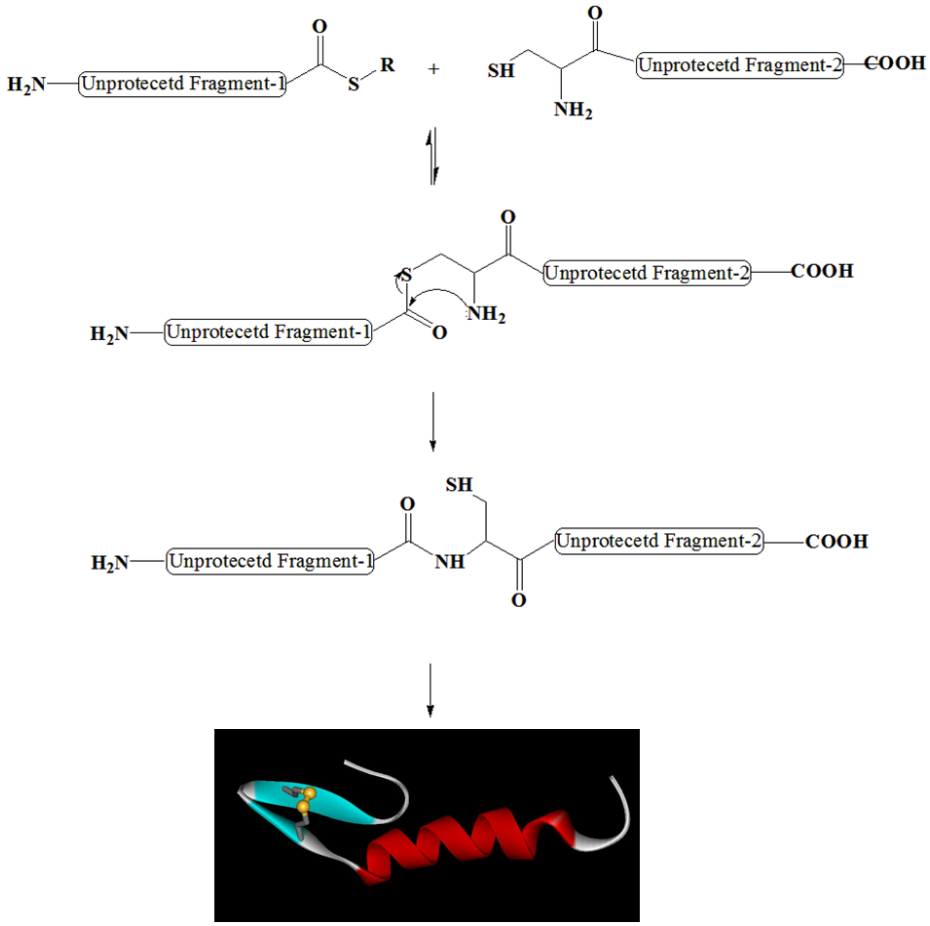
Robert Bruce Merrifield, a noble prize winner in chemistry (1984), in 1963, proposed a versatile method for synthesis of peptides on solid support called as solid phase peptide synthesis (SPPS).[62] In this method amino acid monomers can be attached on the solid support (resin) in a step wise fashion (**Scheme 1.1**).



Scheme 1.1 General protocol for solid phase peptide synthesis (SPPS) based on Fmoc-chemistry (adapted from Sigma-Aldrich custom peptide brochure)

SPPS has some advantages over liquid phase peptide synthesis, such as higher yields, low racemisation and ease of handling. However, it has limitations especially when synthesizing long peptides more than 30 amino acids. To overcome limitations of the stepwise SPPS, Dawson *et al* [62] proposed a new technique for synthesizing proteins called native chemical ligation (NCL). In this technique, peptides are synthesized in the form of fragments and then these fragments are subjected to ligation reaction to yield a single ligated peptide (**Scheme 1.2**), whereas in stepwise SPPS, the total peptide sequence is synthesized continuously (**Scheme 1.1**).[63] NCL technique has several advantages over stepwise SPPS: (i) grants

complete control over the peptide molecule, (ii) high purity, (iii) higher yields and low racemisation, (iv) low aggregation/truncation of peptide, and.(v) can synthesize larger peptides. In order to perform NCL reaction, one fragment should be of N-terminal cysteine and second fragment should be C-terminal thioester.[62] General protocol for NCL is described in **Scheme 1.2**.



Scheme 1.2 General protocol for native chemical ligation.[10]

Mechanism: NCL reaction involves ligation of two unprotected peptide fragments, where the highly reactive thioester rapidly exchanges with thiol moiety of

cysteine, followed by S→N transfer via a favourable five membered transition state to form an irreversible native peptide bond. Internal cysteines, if any, present in the sequence will not interact with the thioester, only cysteine present at the N-terminus will react hence it is called as chemo selective reaction.[64]

Advantages of NCL method: NCL method has been used to synthesize a variety of peptides, proteins and enzymes [63] and with this method (i), it is straightforward to incorporate unlimited number of unnatural / non coded amino acids in the peptide molecule,[63] (ii) virtually any conceivable covalent modification can be introduced anywhere in the peptide molecule. Such modifications are impossible when peptides are synthesized by the ribosomal machinery,[63] (iii) Synthesis of peptides by NCL allows site specific fluorescent tagging of peptides without affecting their biological activity,[65] (iv) Using NCL, peptides can be synthesized in higher purity ≥ 95% and higher yields (>10 mg). This allows the researcher to study SAR of the peptide and elucidation of three dimensional structure of the peptide by X-ray crystallography or by NMR spectroscopy.[63,66]

1.3 Thesis Proposal

1.3.1 Thesis Rationale

Several studies have been performed to study the importance of different structural motifs of LeuA (α-helix and β-sheet) to engender their role and importance in interaction with target bacterial cell membrane in order to show antibacterial activity. This includes the specific target recognition, receptor specificity and perturbation or translocation through the cell membrane. However, the exact role of

the specific domains of LeuA in target specificity remains unclear. Hydrophobic amphipathic α-helix present in the C-terminal region of LeuA seems to be essential for penetration of the peptide into the target cell membrane. Extensive structural analyses of various domains of LeuA are necessary in order to reveal the SAR of these domains in their function and potency.

1.3.2 Hypotheses

Chemical synthesis of LeuA using NCL will give higher yields and higher purities of the peptide compared to stepwise SPPS.

Identification of certain structural motifs (secondary structure) present in class IIa bacteriocin Leucocin A which play specific role in peptide binding and insertion into the target cell membranes to display potent antimicrobial activity.

1.3.3 Thesis Objectives

To verify above hypotheses, first we developed a chemical synthesis for LeuA using NCL method. Next, a number of analogues of LeuA were designed, synthesized, and evaluated for SAR studies. Accordingly, the first objective of this thesis was to develop a chemical synthesis method for obtaining LeuA in high purity and high yields. The second objective was to study the structure activity relationship (SAR) of the N-terminal domain of LeuA by designing and synthesizing analogues of Leucocin A.

- Efforts towards the synthesis of LeuA using two methods, stepwise SPPS and NCL, are described in Chapter 2.

- Design and synthesis of LeuA analogues by replacing the N-terminal β-sheet residues Cys9-Ser15 of the native peptide with shorter β-turn motifs are described in Chapter 3. The analogues are compared to the native peptide to elucidate SAR among these peptides.

- General conclusions from this study as well as future prospects of the results are described in Chapter 4.

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Chapter 2 Synthesis of LeuA Using Stepwise SPPS and Native Chemical Ligation

2.1 Background

Due to rapid development of multiple-drug-resistance in bacterial strains, there is a high necessity for the development of new drug entities which act by different mechanisms. Bacteriocins are ribosomally synthesized potent antimicrobial peptides and are being investigated as new potential antibiotics to treat bacterial multiple-drug-resistance.[1,2] Bacteriocins are derived from lactic acid bacteria as a part of their self defence mechanism to destroy competing microorganisms.[3] Class IIa bacteriocins (typically 37–48 residues) are useful for food preservation because of their activity against a variety of important food pathogens, including *Listeria monocytogenes* in the nanomolar range.[2] Leucocin A (LeuA), a potent class IIa bacteriocin, is a 37 aa cationic antimicrobial peptide with net charge of +2 (**Figure 2.1**).

Figure 2.1 Amino acid sequence of LeuA. C-terminal residues (Trp 18 – Gly 32) that form amphipathic α-helix (red) are highlighted.

The C-terminal domain of LeuA consists of hydrophobic residues, which render its synthesis very difficult. During solid phase peptide synthesis (SPPS), after coupling about 25 amino acid residues on the solid support, the synthesis of LeuA becomes very difficult. The elongated peptide on the solid support tends to aggregate leading to the appearance of truncated peptides.

2.2 Objective

The objective of this study is to develop a solid phase synthesis method for LeuA to obtain the peptide in high yield and with high purity. Two synthetic protocols were used, (i) stepwise SPPS and (ii) native chemical ligation (NCL) in order to achieve the above goal. The peptide obtained from the two methods is compared in terms of yield, purity, solution conformation, and antimicrobial activity.

2.3 Results and Discussion

2.3.1 Stepwise SPPS of LeuA (1-37)

Synthesis of 37-residue LeuA was first attempted by stepwise SPPS using standard Fmoc-chemistry on automated peptide synthesizer. Fmoc protected amino acids (2 eq, 0.4 mmol) were activated by diisopropylcarbodiimide/1-L-hydroxybenzotriazole (DIC/HOBt) activation protocol, and were coupled in stepwise fashion to the 2-chlorotrityl chloride resin (0.2 mmol) on a custom modified MPS 357 automated peptide synthesizer. The optimized Fmoc-chemistry described previously demonstrated to increase assembly of amino acid residues on to the resin.[4] The peptide chain assembly reaction was monitored by standard ninhydrin assay [5] and by performing test cleavage after each 5 residues till 20 residues and each 3 residues thereafter using 50% trifluoro acetic acid (TFA) in dichloromethane (DCM).

After completion of 37-residue chain assembly, crude peptide was deprotected and cleaved from the resin using 50% TFA in DCM, and purified on a semi-preparative RP-HPLC column. **Figure 2.2**a shows the HPLC chromatogram of

the complete crude peptide sample. The crude sample contains a number of other truncated peptides.

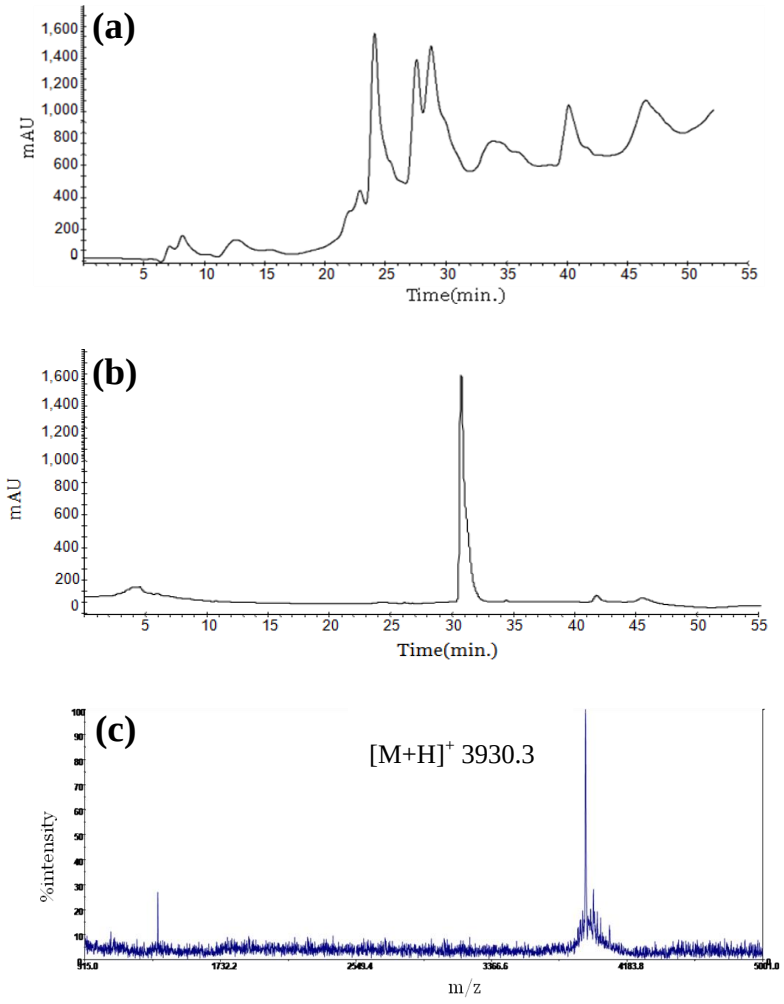


Figure 2.2 Characterization of LeuA synthesized by stepwise SPPS using RP-HPLC and MALDI-TOF. Chromatogram of crude peptide (a), purified peptide (b) and MALDI-TOF spectrum of pure peptide (c). The gradient used on RP-HPLC was 25-95% isopropanol/water in 45 min at a flow rate of 1.0 mL/min. The peptide elutes at 31 min. The MALDI-TOF of the peptide shows the molecular ion $[M+H]^+$ at 3930.3 Da.

Fractions (peaks) were analyzed using MALDI-TOF mass spectrometry, and the peak corresponding to the correct mass for LeuA with the two cysteine (Cys9 and Cys14) thiols in reduced SH form was collected. The desired unoxidized peak of

correct mass that eluted at 31 minutes was pooled and lyophilized. The pure peptide was characterized by RP-HPLC (**Figure 2.2**b) and MALDI-TOF mass spectrometry (**Figure 2.2**c). The observed mass of the reduced peptide $[M+H]^+$ was 3930.3 (calculated $[M+H]^+$ 3930.7 Da). Typically, 47 mg (yield ~ 6%) of the reduced peptide was obtained after purification from a 0.2 mmole scale synthesis.

2.3.2 Peptide Folding and Purification

Oxidative folding and disulfide bond formation between Cys9 and Cys14 was carried out by dissolving the unprotected peptide in reduced form in Tris buffer (pH 8.4) containing 6 M guanidine hydrochloride (GuHCl) to a final concentration of 0.1 mg/mL. It was found that at pH lower than 7.5, folding reaction was significantly reduced. This might be due to protonation of sulpha hydryl groups of Cys.[10]

The folding reaction (oxidation) was monitored using multiple techniques such as Ellman test[11], RP-HPLC and MALDI-TOF mass spectrometry, and was found to be complete after 48 hr. The pure oxidised peptide (LeuA) was collected using HPLC, followed by characterization by RP-HPLC (**Figure 2.3**a) and MALDI-TOF mass spectrometry (**Figure 2.3**b). Typical yield after the oxidation was calculated to be 39 mg (~ 5%). It is worth mentioning that stepwise SPPS of LeuA has been reported previously using a polyamide/kieselguhr resin (overall yield 16%).[6] Also, synthesis of several unnatural analogues of LeuA, such as *ent*-LeuA and carba-LeuA and diallyl-LeuA has been reported.[7-9] The overall yield for *ent*-LeuA was 6% using Wang resin as a solid support.[8]

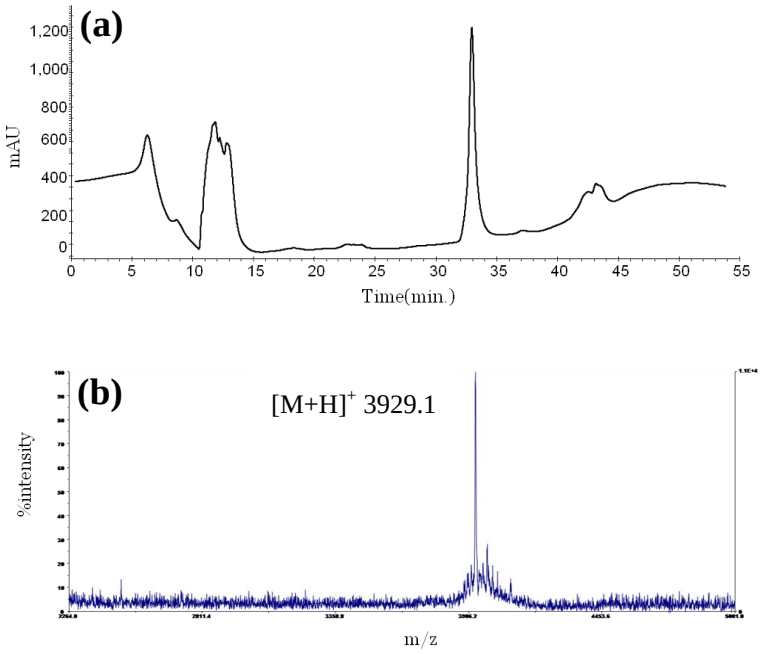
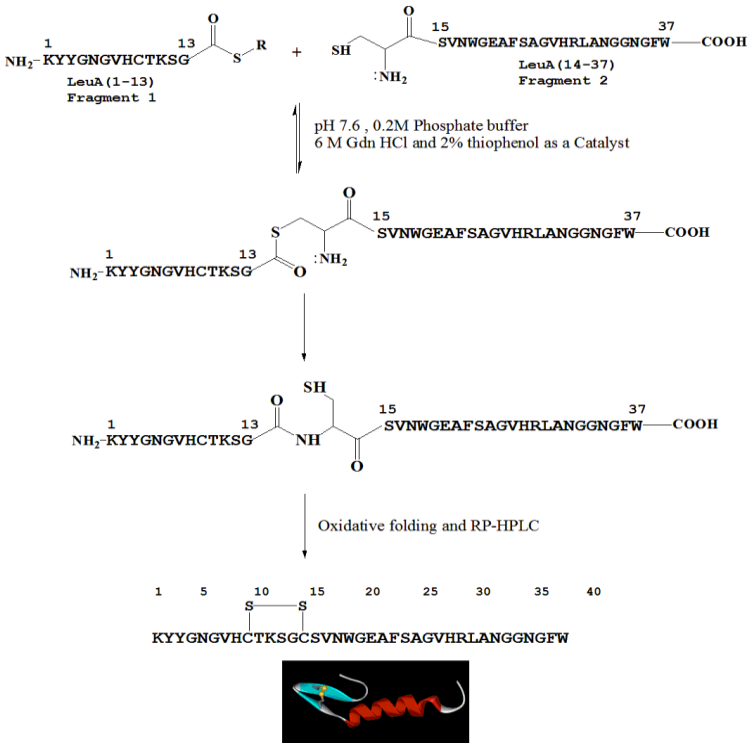


Figure 2.3 Characterization of LeuA (37aa) after oxidative folding using RP-HPLC (a) and MALDI-TOF mass spectrometry (b). The gradient used on RP-HPLC was 25-95% isopropanol/water in 45 min at a flow rate of 1.0 mL/min. The peptide elutes at 33 min. The MALDI-TOF of the peptide shows the molecular ion $[M+H]^+$ at 3929.1 Da (calculated $[M+H]^+$ 3928.7 Da).

2.3.3 Synthesis of LeuA (1-37) by Native Chemical Ligation

Native chemical ligation (NCL) is a technique which allows synthesis of two smaller fragments that are ligated to give the complete product. As mentioned before (Section 1.2), this method has several advantages over stepwise SPPS. In order to perform NCL reaction, the complete sequence is divided into two fragments where the first fragment should have C-terminal thioester and the other fragment should have N-terminal cysteine residue. In general, the fragments are chosen based on the presence of cysteine residue somewhere in the middle of the sequence. However this is not a prerequisite as other residues, such as Alanine, can also be chosen at the fragmentation point.[12] After NCL was first reported by Dawson et al, [13]a number of

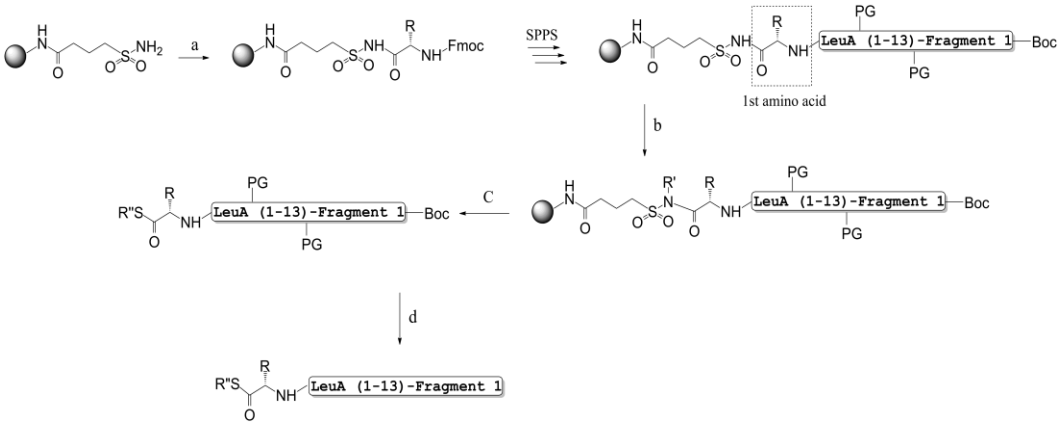
peptides, protein, and enzymes have been synthesized using this strategy.[14] LeuA was synthesized by NCL following the protocol shown in **Scheme 2.1**. First, the NCL reaction is initiated by a chemo selective nucleophilic attack of the thiol of Cys (N-terminal) of unprotected peptide fragment-2 on the C-terminal thioester moiety of unprotected peptide fragment-1. This reaction is followed by rapid spontaneous rearrangement, through an S-N acyl shift, giving rise to a ligated product. For LeuA, Cys14 was chosen as the potential ligation site giving rise to two fragments, namely, 13-residue fragment-1 (1-13) and 24-residue fragment-2 (14-37).



Scheme 2.1 Protocol for synthesis of LeuA using NCL.

Synthesis of Fragment 1. Fragment 1, LeuA (1-13), with the C-terminal thioester was synthesized in stepwise fashion using sulfonamide safety catch linker resin (**Scheme 2.2**). Two types of resins were tested to obtain fragment 1 in high

yield. These resins were 4-sulfamylbutyryl AM resin (loading 0.09 mmol/g) and 4-sulfamylbutyryl AM NovaGel resin (loading 0.67 mmol/g). After coupling of the first amino acid Fmoc-Gly (activation with PyBOP) manually to the resin, the remaining peptide chain assembly was carried out on automated synthesizer following the protocol described previously.[15,16] Coupling reaction was monitored using ninhydrin test.[5]



Scheme 2.2 Protocol for synthesis of thioester fragment (LeuA 1-13). (a) Fmoc-Gly, PyBOP, iPr_2EtN; (b) TMS-CHN_2 or iodoacetonitrile (R' = CH_3); (c) HSR''/NaSPh; (d) TFA, TIS and water.(PG-protecting groups)

After complete chain assembly, activation of the sulfonamide with iodoacetonitrile and displacement with the thiol nucleophile ethyl-3-mercaptopropionate produced the protected thioester peptide. The sulfonamide activation can be carried out using iodoacetonitrile or TMS-diazomethane activation.[16] TMS-diazomethane activation was found to give higher yields when compared to iodoacetonitrile activation.[16,17] LeuA 1-13 thioester (Fragment 1) was obtained after deprotection of side chain protecting groups (PG) using a mixture containing TFA, TIS and water. Typical yield of the HPLC purified fragment-1

synthesized using 4-sulfamylbutyryl AM resin was 91 mg (30% based on 0.2 mmol scale) and 55.3 mg (18% based on 0.2 mmol scale) using 4-sulfamylbutyryl AM NovaGel resin. Higher yield was observed with 4-sulfamylbutyryl AM resin followed by TMS-diazomethane activation, whereas, NovaGel resin gave lower yield of the thioester peptide. NovaGel resin was difficult to work with due to sticky nature of the resin. Crude peptide (**Figure 2.4**a) was purified using semi-preparative RP-HPLC to give pure fragment-1 which was characterized by RP-HPLC (**Figure 2.4**b) and MALDI-TOF mass spectrometry (**Figure 2.4**c). The mass $[M+H]^+$ of the fragment was 1530.3 (calculated $[M+H]^+$ 1530.1 Da, average isotope composition).

Synthesis of Fragment 2. The 24 residue fragment 2, LeuA (14-37), was synthesized on 2-chloro trityl chloride resin (0.2 mmole, 1.0% DVB cross-linked, loading 0.9 mmole/g) using automated MPS 357 synthesizer. All the amino acids were coupled as described before. After complete peptide chain assembly, crude peptide was cleaved from the resin using 50% TFA in DCM and purified using semi-preparative RP-HPLC (**Figure 2.5**a). Fractions of desired mass 2536.6 $[M+H]^+$ (calculated $[M+H]^+$ 2536.1 Da, average isotope composition) that eluted at 23 minutes were pooled and lyophilized.

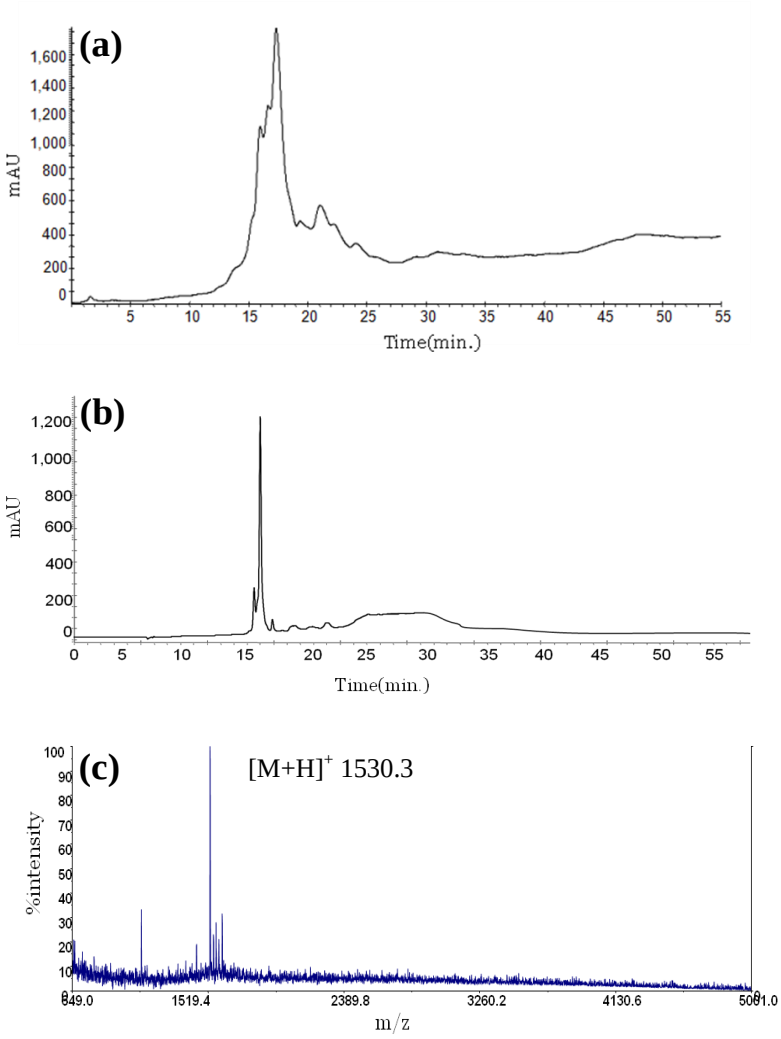


Figure 2.4 Characterization of synthetic peptide Fragment-1 (1-13) using RP-HPLC and MALDI-TOF. Chromatogram of crude peptide (a), purified peptide (b) and MALDI-TOF mass spectra of pure peptide (c). The gradient used on RP-HPLC was 25-95% isopropanol/water in 45 minutes at a flow rate of 1.0 mL/min. The peptide elutes at 16 min. The MALDI-TOF of the peptide shows the molecular ion $[M+H]^+$ at 1530.3 Da (calculated $[M+H]^+$ 1530.1 Da).

Pure fragment 2 was characterized using RP-HPLC (**Figure 2.5**b) and MALDI-TOF mass spectrometry (**Figure 2.5**c). A typical yield of purified fragment 2 LeuA (14-37) on a 0.2 mmole scale was 152 mg (~ 30%).

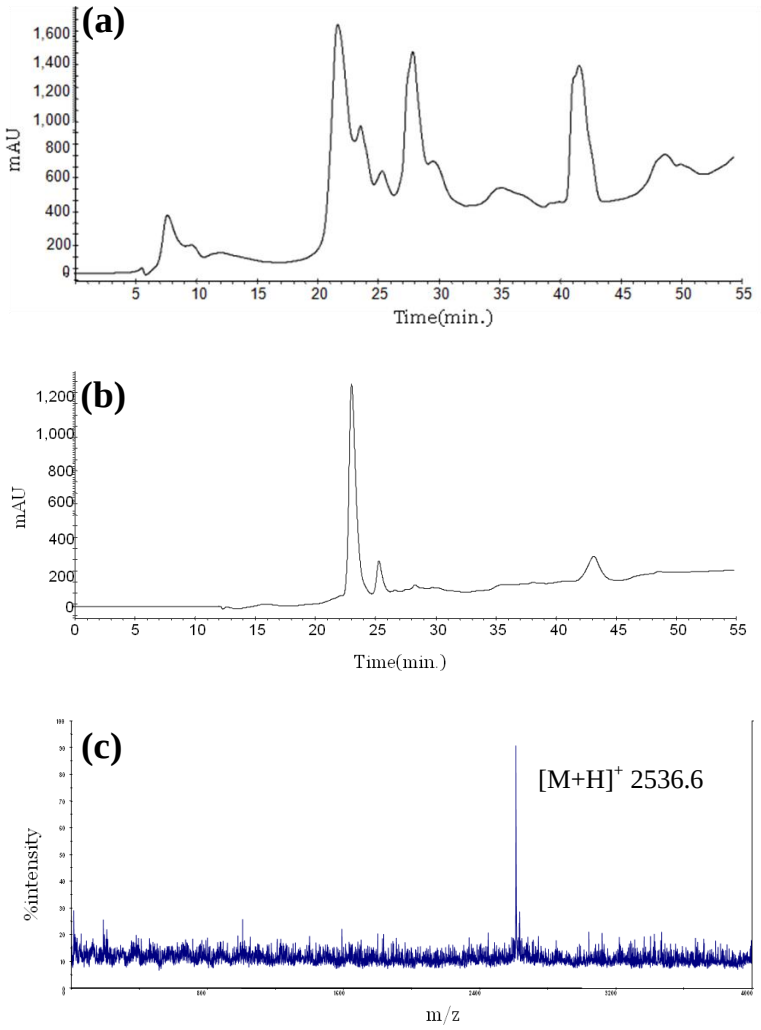


Figure 2.5 Characterization of synthetic peptide Fragment-2 (14-37) using RP-HPLC and MALDI-TOF. Chromatogram of crude peptide (a), purified peptide (b) and MALDI-TOF mass spectra of pure peptide (c). The gradient used on RP-HPLC was 25-95% isopropanol/water in 45 minutes at a flow rate of 1.0 mL/min. The peptide elutes at 23 min. The MALDI-TOF of the peptide shows the molecular ion $[M+H]^+$ at 2536.6 Da.

Chemical Ligation of Fragments. The ligation of the two fragments, fragment 1 thioester (0.016 mmol) and fragment 2 (0.018 mmol), was carried out in phosphate buffer (pH 7.6) containing 6 M GnHCl and 2% thiophenol as catalyst. The reaction was monitored at different time intervals (0, 12 h, 24 h) by RP-HPLC. As shown in **Figure 2.6**, the ligation was completed in 24 h, after which the resultant

ligated product (reduced peptide) was purified using preparative RP-HPLC. During the chemical ligation reaction, 3-mercaptopropionic acid ethyl ester served as a reducing agent to prevent the formation of both intermolecular and intramolecular disulfide bonds between Cys9 and Cys14.

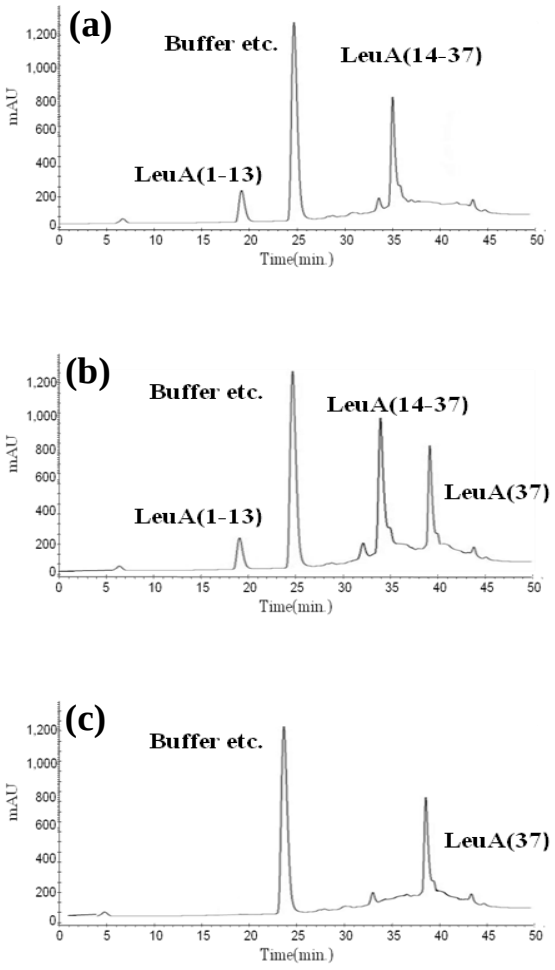


Figure 2.6 Monitoring of NCL reaction using semi preparative RP-HPLC. NCL reaction at 0hr (a), 12 hrs (b), 24 hrs (c)

Furthermore, the presence of thiophenol with excess of 3-mercaptopropionic acid ethyl ester significantly enhanced the ligation reaction rate through nucleophilic

catalysis.[18] The pure peptide was characterized by RP-HPLC and MALDI-TOF mass spectrometry (**Figure 2.7**). The mass $[M+H]^+$ of the peptide was found to be 3930.5 (calculated $[M+H]^+$3930.7, average isotope composition). The yield of the pure peptide in the reduced form was 48% (34.5 mg).

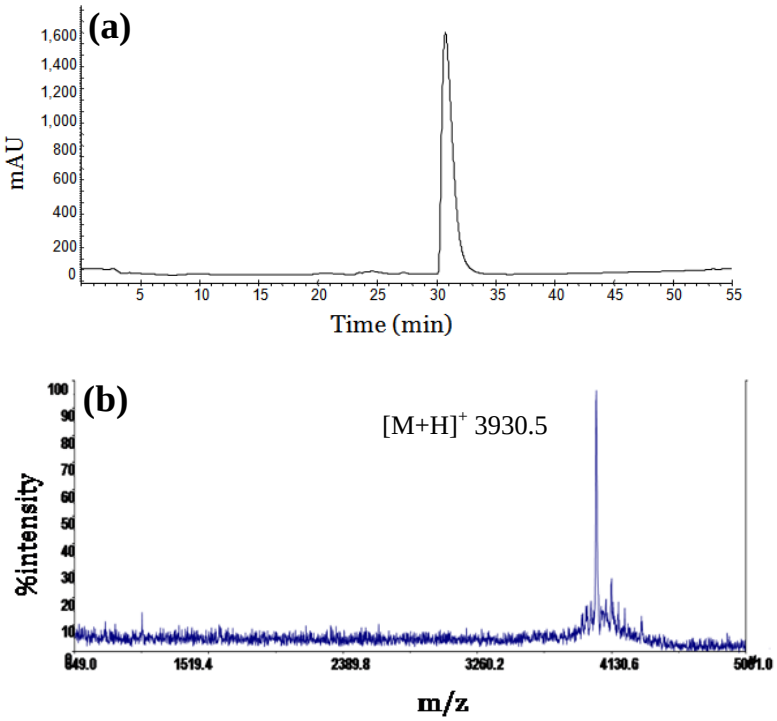


Figure 2.7 Characterization of synthetic peptide LeuA (1-37) using RP-HPLC and MALDI-TOF. Chromatogram of pure peptide (a), and MALDI-TOF mass spectra of pure peptide (b). The gradient used on RP-HPLC was 25-95% isopropanol/water in 45 minutes at a flow rate of 1.0 mL/min. The peptide elutes at 36 min. The MALDI-TOF of the peptide shows the molecular ion $[M+H]^+$ at 3930.5 Da.

The yield of the chemical ligation reaction to give pure peptide in the reduced form was 48% (34.5 mg). The overall yield of the purified reduced LeuA was 0.032 mmol (15.9%) on a 0.2 mmol scale synthesis.

2.3.4 Peptide Folding and Purification

Oxidative folding and disulfide bond formation of the reduced peptide (34.5 mg) was carried out as described for the stepwise SPPS.

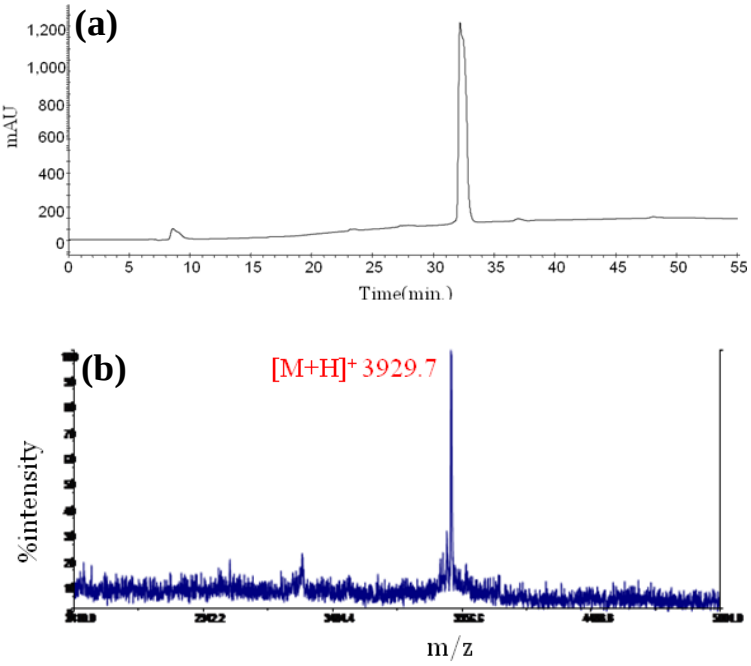


Figure 2.8 Characterization of synthetic peptide LeuA (1-37) after oxidation using RP-HPLC and MALDI-TOF. Chromatogram of pure peptide (a), and MALDI-TOF mass spectra of pure peptide (b). The gradient used on RP-HPLC was 25-95% isopropanol/water in 45 minutes at a flow rate of 1.0 mL/min. The peptide elutes at 32 min. The MALDI-TOF of the peptide shows the molecular ion $[M+H]^+$ at 3929.5 Da.

The peptide was purified and characterized (**Figure 2.8**) to give 27 mg of synthetic Leu A and the overall yield was 12.5% based on 0.2 mmol scale synthesis.

2.3.5 Structural Analysis

To determine if the synthetic LeuA peptides are correctly folded, we compared the circular dichroism (CD) spectra of the peptides with that of the wild type LeuA. CD spectra allow quick analysis of the folding of peptides and proteins in solution. In general, class IIa bacteriocin are unstructured in water but in membrane mimicking solvents like 2,2,2-trifluoroethanol (TFE), and DPC micelles, substantial secondary structure is induced.[19,20] An amphipathic α-helical structure in the C-terminal region is found to be conserved in this class of peptides. It is proposed that the folded conformation is required for specific interaction with the mannose phosphotransferase (PTS) system receptor protein on the target bacterial cell surface.[21]

The CD spectra of the two synthetic peptides, SPPS LeuA and NCL Leu A, in 90% TFE (pH 2.5) were collected with a final peptide concentration of 200 μM. The folding pattern and the secondary structures of synthetic LeuA peptides were compared to that of wild type LeuA.[19] As shown in **Figure 2.9**, the CD spectra shows similar peaks as observed previously for wild type LeuA[19] at 206 nm (θ = -10.5 x 10^3) and 220 nm (θ = -6.0 x 10^3) suggesting similar folding pattern. CD of NCL LeuA shows a negative band at 207 nm (θ = -11.0 x 10^3) and a negative shoulder near 220 nm (θ = -8.6 x 10^3) indicating the formation of significant α-helical structure.[22] The CD of SPPS LeuA also shows the two characteristic peaks

(207 and 220 nm), however, the intensity (θ = -6.6 x 10^3 and -5.1 x 10^3, respectively) was much less compared to NCL LeuA and wild type LeuA.

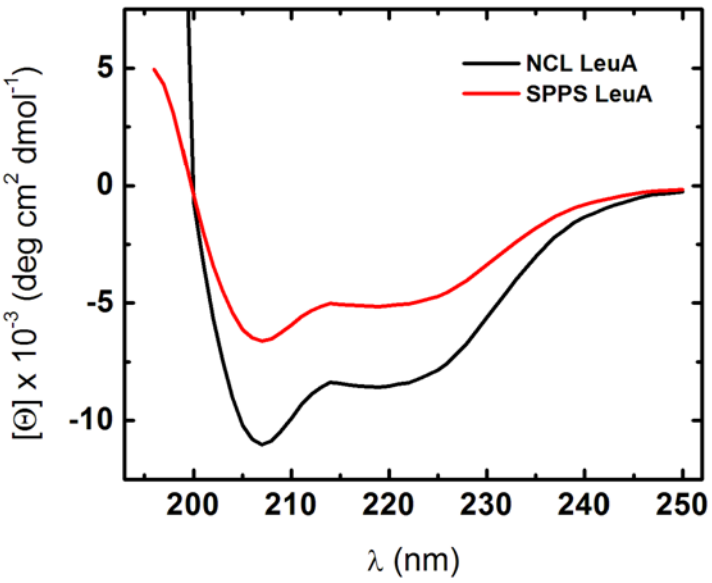


Figure 2.9 CD spectra of LeuA synthesized by NCL in 90% TFE (0.1% TFA final concentration, pH 2.5) at 200 μM concentration.

2.3.6 Analysis of Antimicrobial Activity

Finally, the structure–function analysis and evaluation of the correct folding of the synthetic peptides was done by antimicrobial activity assay. Synthetic SPPS LeuA and NCL LeuA were tested for antimicrobial activity against two indicator strains namely, *Carnobacterium divergens* and *Listeria monocytogenes* using the liguid growth inhibition assay. As shown in **Figure 2.10** both the peptides displayed potent activity against the indicator strains.

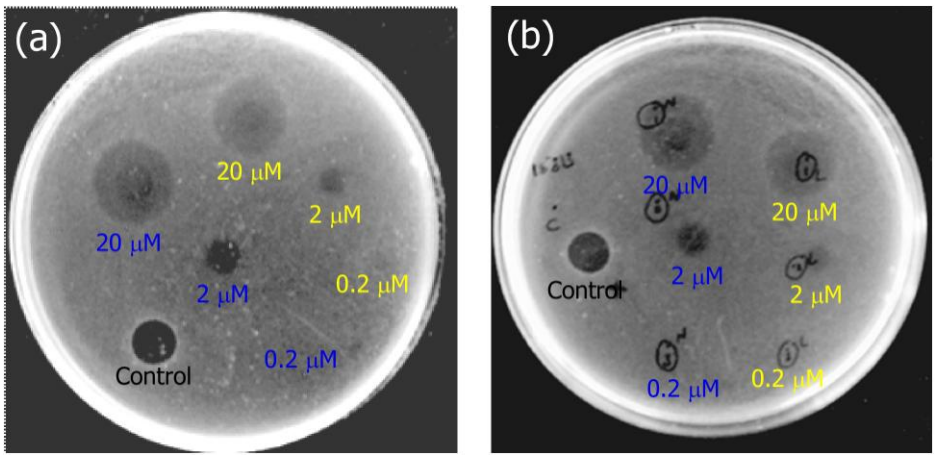


Figure 2.10 Spot on lawn antimicrobial activity assay of LeuA synthesized using SPPS (concentration labelled in yellow) and NCL (blue) aganist indicator strains (a) *Carnobacterium divergens* and (b) *Listeria monocytogenes*. Wild type LeuA was used as a positive control.

Next, the minimum inhibitory concentration (MIC_{50}) of the peptides were determined using the microtitre plate assay (**Figure 2.11**). MIC values of synthetic LeuA(**Table 2.1**) were found to be comparable to that of natural LeuA described previously.[9,19] MIC values of synthetic LeuA by both methods showed slight difference. LeuA synthesized by NCL showed lower MIC (37-39 nM) than the LeuA synthesized using stepwise SPPS (43-47 nM). The low activity of the SPPS LeuA could be due to the increased racemisation caused by longer exposure of peptide to piperidine during the stepwise SPPS.

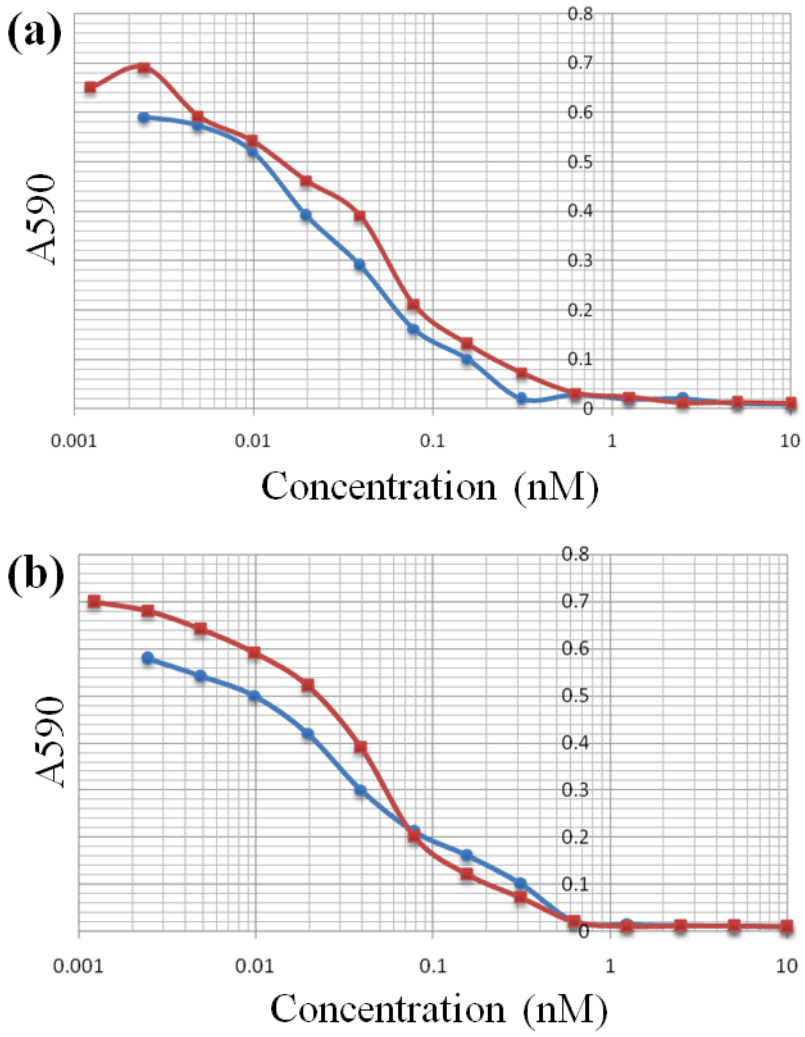


Figure 2.11 MIC_{50} assay of LeuA synthesized by stepwise SPPS (red) and NCL (blue) against *Listeria monocytogenes* (a) and *Carnobacterium divergens* (b).

Table 2.1 Comparision of MICs of synthetic LeuA

Peptide	MIC[a] (nM)	
	Listeria monocytogenes	*Carnobacterium divergens*
Natural LeuA	35	35
LeuA by SPPS	43± 0.6	47± 0.3
LeuA by NCL	37 ± 0.4	39 ± 0.2

[a]MIC value is the peptide concentration that inhibited the growth by 50%. Data are represented as means ± standard errors of the means. The values are results of at least three independent measurements.

2.4 Summary

In this study, the synthesis of a highly potent 37-residue antimicrobial peptide LeuA was explored by two solid phase synthesis methods. Leu A was synthesized by (i) stepwise SPPS and (ii) native chemical ligation (NCL). Stepwise SPPS used amino acid monomer (37 amino acids) addition in a stepwise manner till the complete peptide was assembled, whereas, NCL involved ligation of two small fragments (13 and 24 amino acids) synthesized by SPPS to give the complete peptide. The synthetic peptides obtained by the two methods were extensively characterized and were found to display potent antimicrobial activity similar to the wild type LeuA. The NCL method gave higher overall yield (12.5%) compared to the stepwise SPPS (5%) method due to the less number of truncation peptides in the former method. The CD spectra of NCL LeuA resembled more closely the CD of wild type LeuA suggesting similar folding in solution. Thus, among the two synthetic approaches, NCL proved to be a better method for synthesizing LeuA.

Based on the results presented, it is now possible to rapidly synthesize LeuA in large quantities (12.5% based on 0.2mmol scale) to perform studies such as, comprehensive structure activity relationship (SAR), and design of diagnostic devices that can detect the presence of listeriosis causing pathogens with high precision.

2.5 Experimental Section

2.5.1 General

Solvents and reagents: Fmoc-protected-L-amino acids were procured from Novobiochem Inc and Aapptec Inc (Louisville, KY). Sulfonamide safety catch linker resins namely, 4-Sulfamylbutyryl AM resin (loading 0.9 mmol/g, 222 mg) and 4-Sulfamylbutyryl AM NovaGel resin (loading 0.67 mmol/g, 298 mg), were purchased from Novabiochem Inc. Coupling agent HBTU was obtained from Anaspec Inc, whereas, L-hydroxybenzotriazole (HOBt) was obtained from ChemImpex Inc. Thiophenol sodium salt, NMM, DIC, piperidine, DIPEA, TFA and NMP were purchased from Sigma Aldrich. TMS-diazomethane was obtained from Alfa Aesar and 3-mercaptopropionic acid ethyl ester was obtained from TCI America. Acetic anhydride was purchased from Fluka chemicals. Solvents DCM, DMF and IPA were of HPLC grade and were obtained from Caledon Ltd.

Equipment: Stepwise SPPS was performed using Fmoc chemistry on an automated peptide synthesizer robot MPS 357 (Advanced Chemtech Inc., USA). Crude peptides were purified by preparative reverse phase-HPLC, carried out on the same solvent delivery module using an Agilent C8 column (7 µm, 21.2 X 250mm,

flow rate 4 ml/min). Further purification and isolation of oxidized peptides was carried out on semi preparative reverse-phase HPLC using Vydac C18 column (5 µm, 20 X 250mm, flow rate 1ml/min). Solvent A for HPLC was water, containing 0.05%TFA; solvent B was isopropanol containing 0.05% TFA. Analytical reverse-phase HPLC was carried out on Varian Prostar 210 solvent delivery system equipped with Varian Prostar 325 detector, using a Vydac C18 column (5 µm, 4.6 X 250 mm, flow rate 1 ml/min). Matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF MS) data were acquired on an Applied biosystems sciex voyager elite MALDI-TOF (Foster City, CA) using α-Cyano-4-hydroxycinnamic acid matrix, purchased from Sigma Aldrich. CD spectra of peptides were obtained using Olis CD spectrophotometer (Georgia, USA) in a quartz cell with 0.02 cm path length over 190-250 nm, Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada. MIC assay values were recorded spectrophotometrically at 600 nm using a micro plate reader (TECAN, Männedorf, Switzerland).

2.5.2 Synthesis of LeuA using SPPS

Synthesis of LeuA was carried out on a 0.2 mmol scale of 2-Chlorotrityl chloride resin (1.0% DVB cross-linked, loading 0.9 mmol/g) by following automated Fmoc-SPPS method (Appendix A.1) described previously.[8] The first amino acid was added manually as follows, the resin was weighed (222 mg) and transferred to reaction vessel with a frit at the bottom, and washed with IPA, DCM and DMF. The resin was washed and swelled in DMF (5 mL) for 30 min. After swelling, excess of DMF was removed and Fmoc-Trp(Boc)-OH (0 4 eq, 0.8 mmol, 842.5 mg), DIPEA (5 eq, 1 mmol, 174 µL) in DMF (2 mL) were added to the swollen resin and agitated for 24

hrs at room temperature. After the coupling step, the resin was washed with IPA, DCM and DMF. After completion of coupling step for the first amino acid tryptophan, reaction was monitored using ninhydrin test. After confirming the coupling, fmoc removal step was carried out using freshly prepared 20% piperidine in DMF (2 x 8 min, 4 mL each). The resin was vacuum dried and transferred to the automated peptide synthesizer robot. The rest of the 36 amino acid residues were added using automated peptide synthesizer robot MPS 357 (detailed protocol see Appendix). Coupling of Fmoc protected amino acid residues was carried out using coupling agents DIC and HOBt. Acid labile side chain protections used were: Asn(Trt), Asp(tBu), Cys(Trt), Glu(tBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc), and Tyr(tBu). Synthesis protocol was optimized as follows, Single coupling, 1h each, was employed until 10 amino acid residues. For amino acid residues 10-20, double coupling, 1.5h each, was employed and from 20 to 37 amino acid residues, double coupling, 2h each was carried out to ensure effective coupling reaction. Each Fmoc deprotection step (using freshly prepared 20% piperidine in DMF) was carried out for two times, 6 minutes each, until 25 amino acid residues. From 25 to 37 residues, longer deprotection times, 8 minutes each, was employed. During the entire synthesis, coupling reactions were monitored constantly using ninhydrin test and by performing test cleavage after coupling of each five amino acids till 20 residues and each two residues till 37 residues. Ninhydrin test was carried out by taking a small amount of resin in a glass tube and by adding each 200 µL of 5% ninhydrin in ethanol, 80% phenol in ethanol and potassium cyanide solution (KCN). The tube was heated for 1-2 min at 100^0C, the presence of resin-

bound free amine is indicated by blue resin beads. Test cleavage was performed by taking a small amount of resin from the synthesizer and adding a mixture of 50% TFA and DCM followed by shaking for 30 min at room temperature. The cleavage mixture with the peptide was concentrated in the vacuum. The sample was then analysed by MALDI-TOF mass spectrometry. After complete peptide assembly, the peptide sequence was released from resin with concomitant removal of acid-labile side chain protecting groups by adding a mixture of 50% TFA and DCM (~ 7 mL) followed by shaking for 30 min at room temperature. The filtrate from the cleavage reactions was combined with TFA washes (3 x 2 min, 1 mL), and concentrated in vacuum. Crude peptide present in the cleavage mixture was precipitated by adding cold anhydrous diethyl ether (~15 mL). After triturating for 2 min, the peptide was collected by centrifugating the sample mixture. The crude peptide was dissolved in 20% aqueous acetonitrile and purified on a semi-preparative VYDAC C18 reversed-phase HPLC column (1 x 25 cm, 5 μM) using an isopropanol/water gradient in the presence of 0.05% TFA. The gradient used was 25-95% isopropanol/water in 45 minutes at a flow rate of 1.0 mL/min (**Table 2.2**). Pure peptide was characterized by analytical reversed-phase HPLC and MALDI-TOF mass spectrometry. The purity of the peptide was verified to be ≥95% by reverse-phase chromatography. The pure lyophilized linear peptide was stored at -20 $^{\circ}$C under nitrogen, to proceed for the oxidative folding reaction.

Table 2.2 Gradient system used for purification of peptides using RP-HPLC

Time(min)	%A (water)	%B (IPA)
5	75	25
35	30	70
40	5	95
45	5	95
50	75	25
55	75	25

2.5.3 Oxidative folding and disulfide bond formation

Oxidative folding and disulphide bond formation between Cys 9 and Cys 14 was achieved by air oxidation in freshly degassed 50 mM Tris buffer (pH 8.4). Linear LeuA in reduced form (41 mg, 10.25 µmol) was dissolved in folding buffer solution at a concentration of 1mg/mL, 20% DMSO was added to the buffer to enhance peptide solubility and oxidation. The solution was gently stirred in an open-air flask for 48 h. The folding reaction was monitored using Ellman test [11] and MALDI-TOF mass spectrometry (Figure 2.5). MALDI-TOF spectrum showing [M-2] ion peak indicated loss of two protons of free sulpha hydryl (-SH) groups and formation of the disulfide bond within the peptide. Fractions showing oxidized peptides based on HPLC-MS analysis were pooled and lyophilized. MALDI-TOF calculated for oxidized LeuA, $[M + H]^+$ 3928.7; found $[M + H]^+$ 3929.1; overall yield 5%.

2.5.4 Synthesis of LeuA using NCL

2.5.4.1 Synthesis of LeuA (1-13) (Fragment 1)

Fragment-1(1-13 amino acid residues) was synthesized using Fmoc chemistry on sulfonamide safety catch linker resin to introduce a thioester at the C-terminal as described below.

Protected C-terminal thioester was synthesized independently on both 4-Sulfamylbutyryl AM resin (loading 0. 9 mmol/g, 222.2 mg) and 4-Sulfamylbutyryl AM NovaGel resin (loading 0.67 mmol/g, 298 mg) using protocol described by Ingenito *et al*.,[16]. The first amino acid glycine (5 eq., 1 mmol, 594.6 mg) was coupled manually to the resin using coupling agents PyBoP / iPr_2EtN. First of all, the resin was weighed and transferred to the reaction vessel and washed thoroughly with DCM, IPA, and DMF. Swelling of the resin was carried out by adding of DMF (4 mL) to the reaction vessel followed by gentle agitation for 30 min. at room temperature. Then the whole instrument setup with the reaction vessel was moved to freezer for 45 min in order to maintain a temperature of -20 ^{0}C. After 45 minutes, excess of DMF was removed and iPr_2EtN (130 μL, 0.75 mmol) and Fmoc-glycine (5 eq., 1 mmol, 594.6 mg) were added to the reaction vessel and the reaction vessel was agitated for 20 min. at -20^{0}C. After 20 min, PyBop (234mg, 0.450mmol) was added to the reaction mixture as a solid and the reaction vessel was agitated at -20 ^{0}C for 8 hr followed by 8 hr at room temperature. Adjusting these conditions gives the maximum loading for the first amino acid. Increased coupling times anymore will increase the level of racemisation.[15] Double coupling was carried out in order to maintain efficient loading of amino acid on to the resin. Fmoc deprotection was

carried out using freshly prepared 20% piperidine in DMF. After deprotection of the first amino acid, rest of the amino acids were coupled using N,N'-Diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) activation protocol on automated peptide synthesizer MPS 357 (Advanced Chemtech Inc., USA), using the standard amino-acid side chain functionality.Single coupling, 1h each, was employed until 5 amino acid residues. For amino acid residues 6-13, double coupling, 2h each, was employed. Each Fmoc deprotection step (using freshly prepared 20% piperidine in DMF) was carried out for two times, 6 min each, until 6 amino acid residues. From 6 to 13residues, increased deprotection times, 8 minutes each, were employed. During the entire synthesis, coupling reactions were monitored constantly using ninhydrin test. After completion of synthesis, the full length peptide was released from resin with concomitant removal of acid-labile side chain protecting groups using the protocol described below (**Scheme 2.2**).

Activation of safety catch linker resin: The peptidyl resin (0.2mmol) was washed with anhydrous NMP (3 x 5ml) and swelled in N-methyl pyrrolidinone (NMP ~5ml) for 30minutes. Excess of NMP was removed by vacuum filtration. DIPEA (10 equiv, 2 mmol, 340 µL) and iodoacetonitrile (8 equiv, 1.6 mmol, 1.6 mL) were added to the reaction vessel containing swollen resin, and the reaction vessel was protected from light. The reaction mixture was agitated for 24 h at room temperature. The activated resin was washed with anhydrous NMP (3 x 5mL) and DCM (3 x 5mL) and dried in vacuum.

Displacement of protected peptide fragment from resin: The activated safety catch resin (0.2mmole) was swollen in anhydrous DMF (~5mL, 30min), ethyl-3-

mercaptopropionate (50 eq., 650µL) and sodium thiophenol (0.5 eq., 35 mg) were then added to final concentration of 1 M. The reaction mixture was agitated for 24h at room temperature. The resin was filtered and washed with anhydrous DMF (3 x 2mL). The combined filtrate and washings were collected in flask, vacuum concentrated and washed with cold anhydrous diethyl ether to remove traces of mercaptans.

Removal of N terminal Boc and side chain protecting groups: To remove the amino terminal side chain protective groups, the peptide fragment was treated with cleavage cocktail of (7.5ml TFA 99%, triisopropylsilane 150µl and water 150µl) at room temperature for 2.5 hrs. The solution was evaporated in vacuum and the residual oil was treated with cold anhydrous ether (~15ml). The resulting precipitate was collected by centrifugation and freeze dried. Precipitate was dissolved in 20% acetonitrile/water and purified by semi-preparative HPLC.

2.5.4.2 Synthesis of LeuA (14-37) (Fragment 2)

Synthesis of LeuA (amino acid residues 14-27) was carried out on a 0.2-mmol scale of 2-Chlorotrityl chloride resin (1.0% DVB cross-linked) by following the standard Fmoc solid-phase peptide chemistry using the same method described in section 2.5.2 on Automated peptide synthesizer robot MPS 357 (Advanced Chemtech Inc., USA). Activation of amino acid residues was carried out by using DIC/HOBt according to the manufacturer's protocol. Amino acid side chain functionality was protected using acid labile side chain protections as follows: Asn(Trt), Asp(tBu), Cys(Trt), Glu(tBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc), and Tyr(tBu). For amino acid residues 14-20, single coupling protocol was employed for 1.5h each

and for amino acid residues 20-27, double coupling protocol 2h each was employed. Fmoc deprotection was carried out by using 20% piperidine in DMF for two times, 6minutes each. Coupling reaction was monitored constantly by performing test cleavage by shaking small amount of resin with 50% TFA in DCM for 30minutes and by using MALDI-TOF mass spectrometry. After completion of full synthesis, the peptide fragment was cleaved from the resin by stirring the resin in the solution of 50% TFA in DCM in presence of triisopropylsilane as scavenger for 45minutes. The filtrate containing crude peptide was concentrated in vacuum. Crude peptide was precipitated by adding cold diethyl ether (~15 ml), precipitate was freeze dried. Precipitate was dissolved in 20% acetonitrile/water and purified by semi-preparative RP-HPLC. MALDI-TOF calcd for fragment 2, $[M + H]^+$ 2536.1; found $[M + H]^+$ 2536.6; overall yield 30%.

2.5.4.3 Native Chemical Ligation

The chemical ligation of unprotected linear synthetic peptide fragments 1 and 2 was carried out by following the protocol described earlier in literature [23]. Fragment 1 (1-13)-thioester (27.5 mg, 18 μM) and fragment 2(13-27)-N-terminal cysteine (41.8 mg, 16.5 μM) were dissolved in freshly degassed 0.2M sodium phosphate buffer containing 6 M guanidine HCL, pH 7.2. Ligation reaction started by addition of thiophenol (35 μL) to the reaction mixture. The solution was stirred for 24hr under argon at room temperature and monitored by semi preparative HPLC and MALDI-TOF MS. The ligation reaction was completed after 24hr. After 24hrs the ligated product was formed as a precipitate. The precipitate was collected, freeze dried and purified by RP-HPLC and characterization. MALDI-TOF calculated for

reduced LeuA, $[M + H]^+$ 3930.7; found $[M + H]^+$ 3930.5. The pure peptide was subjected to oxidative folding (disulfide formation) as described above.

2.5.5 CD Spectroscopy

The ability of synthesized LeuA to adopt an ordered secondary structure after oxidative folding was evaluated by CD spectra obtained using a Olis CD spectrophotometer (GA,USA) in a thermally controlled quartz cell with 0.02 cm path length over 190-250 nm. Peptide sample with a concentration of 780 μg/mL were prepared in 90% TFE containing 0.1% aqueous TFA (pH~2.5). The baseline scan was performed by taking the buffer alone and subtracted from experimental readings. Data was collected every 0.05 nm and were average of 6 scans. The normalized CD data was expressed in units of molar ellipticity (deg cm^2 $dmol^{-1}$) and plotted versus the wavelength.

2.5.6 Antimicrobial Assay

Two bacterial strains, namely, *Carnobacterium divergens* UAL9 (grown in APT broth at 25^0C), *Listeria monocytogenes* ATCC 15313 (TSYBE, 37^0C) were used as indicators for the antimicrobial activity assay. All indicator strains were obtained from the culture collection of CanBiocin Inc. (Edmonton, Alberta, Canada). Synthetic LeuA was dissolved in methanol and its concentration was determined by measuring the A_{280} according to standard procedures found in the literature.[24] Antimicrobial activity assay was performed using the spot-on-lawn method.[7] Minimal inhibitory concentration (MIC) of LeuA synthesized by both stepwise SPPS and NCL was also determined using a micro titre plate assay as described previously

[4,25] using the indicator strains *Listeria monocytogenes* and *Listeria innocua*. Culture media, peptide (2 fold dilution), and the indicator strain were added to a final volume of 200µL in each micro well. The minimal inhibitory concentration (MIC) was defined as the concentration of LeuA that inhibited the growth of the indicator strain by 50%. Plates were incubated overnight at the temperature of 37 ^{0}C and the optical density (OD) values were recorded spectrophotometrically at 590 nm using a micro plate reader (TECAN, Männedorf, Switzerland) and the absorbance data was plotted against concentration of the peptide ($\log_{10}$). The data collected was of at least three times repetition of the experiment. Standard error was calculated using the formula $\sigma_x = \sigma/\sqrt{N}$ whereas σ– standard deviation, N–number of readings obtained.

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Chapter 3 Design and Synthesis of LeuA analogues

3.1 Background

Leucocin A (LeuA) is a 37 residue class IIa bacteriocin from *Leuconostoc gelidium* (**Table 3.1**). A series of experiments conducted by Fleury *et al.* [1] to elucidate SAR of LeuA show that C-terminal tryptophan and N-terminal YGNGV sequences are essential for activity. The N-terminal of LeuA (residues 2-16) consists of a three strand anti-parallel β-sheet which is stabilized by the disulphide bond between residues 9 and 14 (Figure 3.1a).[2] The conserved disulphide bond between two cysteines maintains the correct geometry of other residues in the sequence and this geometry is essential for its activity.[3] Studies conducted by Derksen *et al.* [3] confirmed that the disulphide bond only contributes to maintain correct geometry in the molecule but does not bind to the receptor on the bacterial cell membrane. The authors substituted the disulphide bond with carbocyclic rings without losing activity while maintaining flexibility and geometry in the synthetic LeuA. More recently it was shown that the replacement of the two Cys residues in the N-terminal region of LeuA with hydrophobic residues such as Phe, Norvaline, AllylGly yields a fully active analogue.[4] On the other hand, substitution of the Cys with Ser residues in LeuA made the peptide inactive. These results suggests that although the disulfide bond is conserved among class IIa bacteriocins, it can be replaced with residues which can induce similar geometry in the molecule without losing its antibacterial activity.

3.2 Hypothesis

We hypothesized that analogues of LeuA can be designed by replacing a portion of the N-terminal β-sheet region (Cys9-Ser15) with a smaller β-turn. To validate that, in this study we have designed and synthesized three analogues (**1**-**3**) of LeuA (**Table 3.1**) by manipulating the N-terminal region of the wild type LeuA to study the structure-activity relationships. The activity of the analogues was evaluated by spot-on-lawn method using two indicator strains, *Listeria innocua* and *Carnobacterium divergens*, and the three analogues did not display any activity. Solution conformation study using CD spectroscopy suggested that only analogue **1** adopts similar folding as LeuA, and **1** was able to competitively inhibit the activity of native LeuA. Finally, MD simulations were used to study the conformation of analogue **1** that showed lack of folding in the N-terminal region compared to LeuA emphasizing the role of proper folding in the activity of class IIa bacteriocins.

Table 3.1 Amino acid sequences of native LeuA and peptide analogues studied herein.

Peptide	Sequence	Charge	No. of residues
LeuA	KYYGNGVH**CTKSGCS**VNWGEAFSAGVHRLANGGNGFW	+2	37
1	KYYGNGVH--K**P**---VNWGEAFSAGVHRLANGGNGFW	+2	32
2	KYYGNGVH--KSG---NWGEAFSAGVHRLANGGNGFW	+2	32
3	KYYGNGVH--K**PNG**---WGEAFSAGVHRLANGGNGFW	+2	32

3.3 Objective

The objective of the current study is to evaluate the structure activity relationship of the N-terminal region of LeuA.

3.4 Results and Discussion

3.4.1 Design and Synthesis of LeuA Analogues

Three analogues of LeuA were designed by replacing the N-terminal β-strand residues Cys9-Asn17 with smaller β-turn sequences (**Figure 3.1**). The analogues were 32-residue long, five residues less compared to the wild type LeuA (Table 3.1). The charged residue Lys11 was not deleted to maintain the positive charge of the N-terminal region. The β-strand residues Cys9-Thr10 and Gly13-Ser15 were deleted in analogue **1**, and S12P mutation was introduced to increase the conformational stability of the β-turn. Proline residue is often found in turns presumably due to its unique restricted O angle which is entropically favourable at certain turn positions.[5] In analogue **2**, the β-turn sequence KSG from the wild type LeuA was maintained whereas, in analogue **3**, the β-turn was substituted with another β-turn motif which is a conserved tripeptide sequence Pro-Asx-Gly.[6]

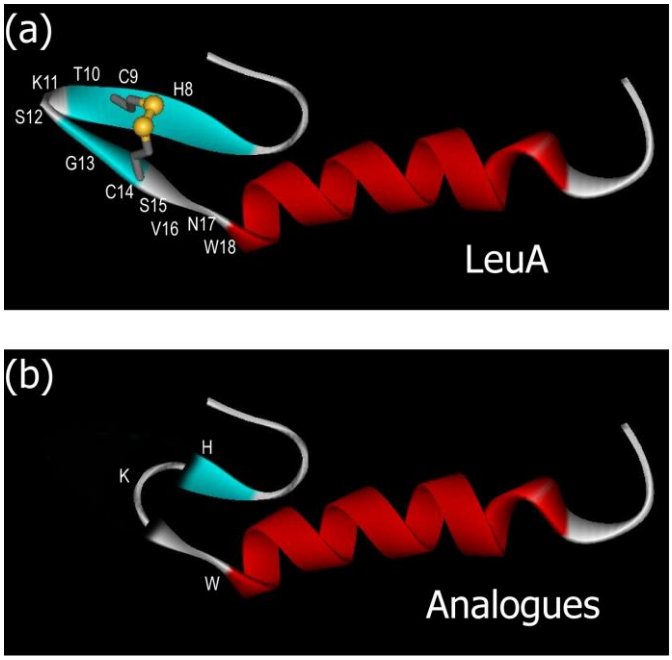


Figure 3.1 (a) NMR solution structure of native LeuA (PDB code: 1CW6) in 90% TFE. (b) Representation of the LeuA analogues studied here depicting the deletion of portion of N-terminal β-sheet region.

Peptide analogues **1**-**3** were synthesized on 2-chlorotrityl chloride resin using standard Fmoc solid-phase peptide synthesis (**Figure 3.2**) as described previously by Kaur *et al.*[7]. DIC and HOBt were used as coupling agents. After the cleavage of the peptides from the resin, crude peptides were purified on a semi-preparative reversed-phase HPLC column by a gradient elution method using isopropanol and water as solvents. The mass of the crude and pure peptides were confirmed by MALDI-TOF mass spectrometry (**Figure 3.2**). Purity of the peptides was confirmed by reversed-phase HPLC and mass spectrometric analysis.

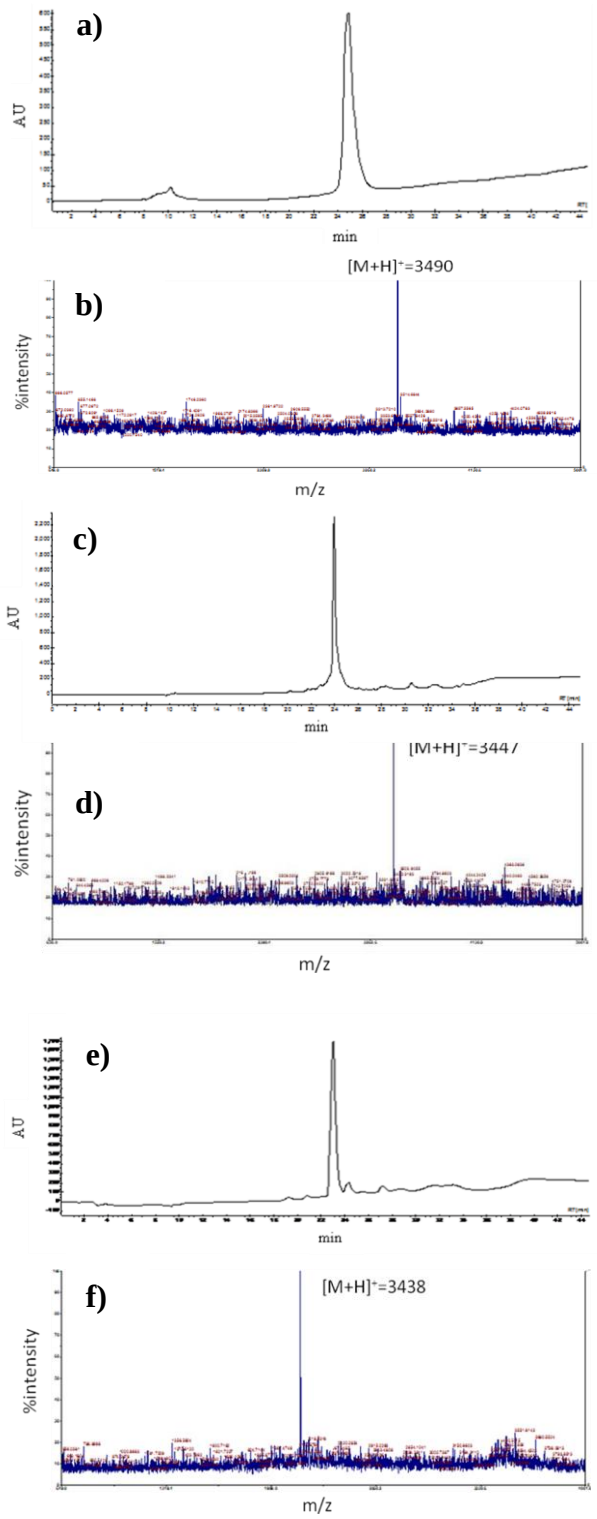


Figure 3.2 RP-HPLC chromatographs and MALDI-TOF spectra for LeuA analogues **1 (a,b)**, **2 (c,d)** and **3 (e,f).**

Pure peptides were obtained with an overall yield of 15-20% and purity greater than equal to 95%.

3.4.2 Structure - Activity Relationship

3.4.2.1 CD Spectroscopy

CD spectroscopy was used to compare the secondary structure of the synthetic LeuA analogues with the native LeuA. The spectra for all the three analogues were obtained in TFE and water (9:1) containing 0.1% TFA (pH~2.5). Class IIa bacteriocins such as LeuA are completely unstructured in water, but form well-defined secondary structures in membrane mimicking solvents like TFE.[2] Among the three analogues, significant helical structure was induced only in analogue **1**, as indicated by the appearance of a distinct negative band at 208 nm (θ = -11.4 x 10^3), and a negative shoulder near 220 nm (**Figure 3.3**). The other two analogues (**2** and **3**) were not structured as there were no characteristic peaks present in the CD spectra. The CD spectrum of **1** was similar to that of the native LeuA suggesting similar folding in the two peptides.

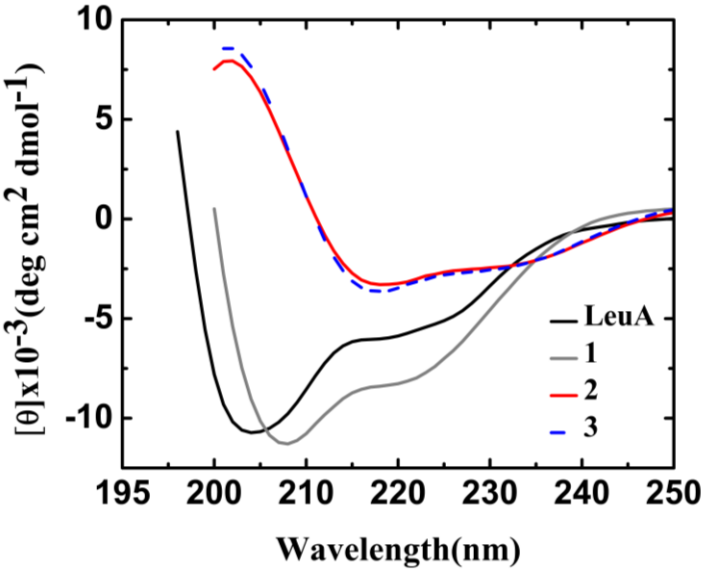
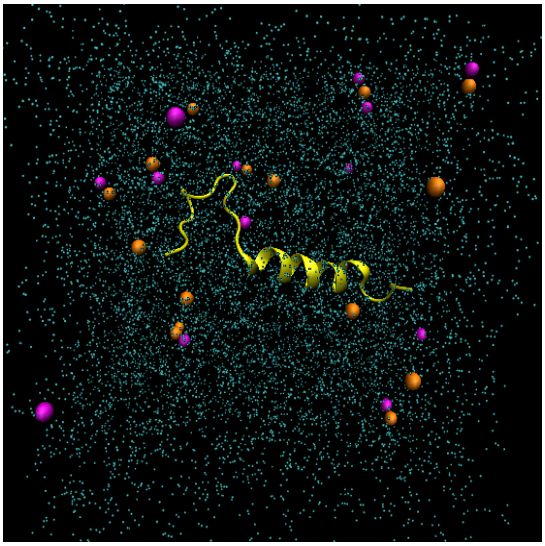


Figure 3.3 CD spectra of peptide analogues **1**, **2**, **3** and native LeuA in 90% TFE (0.1% TFA final concentration, pH 2.5) at 200 μM concentration.

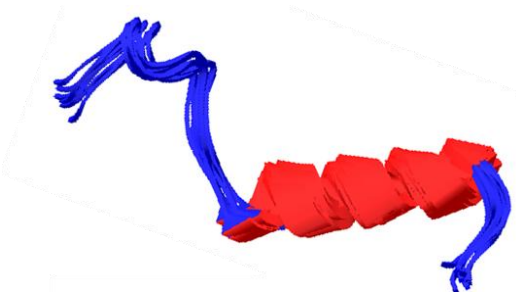
3.4.2.2 Homology modeling and MD Simulations

As the CD spectra of analogue **1** resembled that of the native LeuA, the secondary structure of **1** was further explored using homology modeling followed by MD simulations. Homology model of analogue **1** was constructed as described previously.[7] The PDB structure of native LeuA (PDB code-1CW6) was used as a template to create the homology model using "magic fit" tool in the VMD software.[8] Homology model was used to study the secondary structure dynamics of **1** in TFE by conducting MD simulations. Simulation system was constructed by placing the homology model in the middle of a cubic box **(Figure 3.4**a). The box was filled with solvent followed by MD simulations at two different temperatures, 298 and 310 K. Snapshots were extracted after the simulations at each nanosecond and analysed using VMD software.[8] As shown in

(a)



(b)



(c)

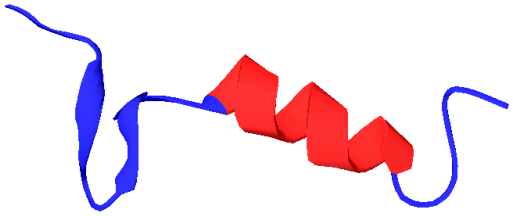


Figure 3.4 (a) Initial setup of simulation box with box size X, Y and Z = 6 nm. TFE is rendered in blue, peptide **1** in yellow ribbon, and Na^+ and Cl^- ions rendered in magenta and blue, respectively. (b) Snapshots of peptide **1** (taken from 9-10 ns) superimposed on each other, extracted from simulation at 298 K. The superimposed images show that the peptide conformation was stable with an N-terminal coil and C-terminal helix. (c) Snapshot of peptide **1** extracted (~ 9 ns) from simulation at 313 K

Figure 3.4b, analogue **1** formed a stable structure with a C-terminal α-helix and the N-terminal region mainly folded into a coiled structure. The N-terminal region of **1** was very different from the native LeuA that has a well defined anti-parallel β-sheet structure. Interestingly at elevated temperature (310 K), peptide **1** showed the appearance of an N-terminal β-sheet (**Figure 3.4**c), however, this conformation was present only in a few snapshots toward the end of the simulation (~ 9-10 ns).

These results suggest that analogue **1** has C-terminal region that is folded more like native LeuA, however, the N-terminal region displays marked differences from the native peptide.

3.4.2.3 Antimicrobial Activity

The activity spectra of the LeuA analogues **1**-**3** were evaluated against two indicator strains, namely, *Listeria innocua* and *Carnobacterium divergens.* All analogues were tested using broth assay as described previously.[3] Synthetic leuA displayed potent activity against *L. innocua* and *C. divergens* with MIC values of 37 nM and 39 nM respectively. These MIC were similar to the values reported by Derksen *et al.* [3] and Kaur *et al.* [7]. However, none of the analogues (**1**-**3**) displayed any activity up to 200 μM concentration (**Table 3.2**).

Table 3.2 Antibacterial activity of synthetic LeuA (NCL) and analogues **1-3**.

Peptides	MIC[a] (μM) of Peptides	
	Listeria innocua	*Carnobacterium divergens*
Leu A	+++	+++
1	-	-
2	-	-
3	-	-

[a]MIC value is the peptide concentration that inhibited the growth by 50%. -, no activity detected up to 200 μM. Data are represented as means ± standard errors of the means. The values are results of at least three independent measurements.

3.4.2.4 Competitive Binding Assay

Next, the ability of analogue **1** to inhibit the antimicrobial activity of the wild-type LeuA was explored using competitive binding assay. As shown in Figure 3.4, the presence of analogue **1** led to significant inhibition of LeuA activity against *C. divergens* in a dose-dependent manner.

These results suggest that the C-terminal helical region in the peptide analogue **2** may play an important role in determining cell specificity or mediating specific interaction with the target cell membrane receptor. The N-terminal sequence in analogue **1** that lacks the β-sheet folding seems to be the reason for absence of any activity. Chen *et al*. previously showed using N-terminal pediocin PA-1 fragments that electrostatic interaction direct peptide binding to the target membrane.[9] In our analogue design (**1**-**3**), the charge of the N-terminal region was maintained (Table 3.1). Lack of antimicrobial activity in analogues suggesting that proper folding and/or complete sequence is essential for peptide bactericidal activity. It is important

to note that in class IIa bacteriocins like divercin V41, peptide fragment from the C-terminal domain (from residue 18 to 43) displays activity.[10] On the other hand, a change in the N-terminal domain in the native sequence (such as in analogue **1**) while keeping the remaining sequence intact causes loss of activity. Likewise, fragment from C-terminal region of pediocin PA-1 (from residue 20 to 34) did not display activity but were able to specifically inhibit the bactericidal activity of 44-residue pediocin PA-1.[11] A 15-mer fragment derived from the N-terminal of class IIa bacteriocin enterocin displayed antimicrobial activity, however, smaller fragments from the N-terminal were inactive.[12]

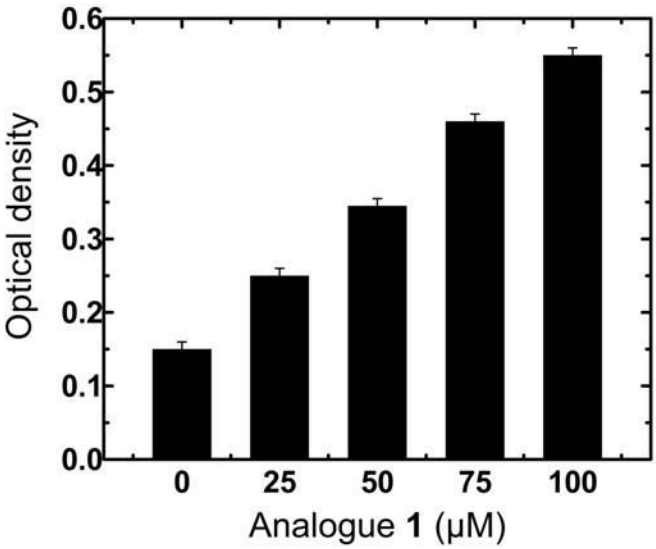


Figure 3.5 Dose dependent decrease in antimicrobial activity of LeuA in the presence of analogue **1** against *C.divergens* (UAL9). Antimicrobial activity is expressed as optical density at 600 nm ± standard deviation.

3.5 Summary

In conclusion, the analogues studied here support earlier investigations that the C-terminal region is required for specificity and dictates the antimicrobial profile, whereas, the N-terminal sequence is important for activity. Replacement of few N-

terminal residues with conservative substitutions is allowed as shown previously,[4] however, the complete N-terminal domain is required for activity.

3.6 Experimental Section

3.6.1 Materials and Equipment

Analogues were synthesized using 2-chlorotritylchloride resin (loading 1.05 mmol/g) on automated peptide synthesizer, and Fmoc protected amino acids purchased from Novobiochem. Coupling agent HBTU was obtained from Anaspec Inc, whereas, L-hydroxybenzotriazole (HOBt) was obtained from ChemImpex Inc. NMM, DIC, piperidine, DIPEA, TFA and NMP were purchased from Sigma Aldrich. Acetic anhydride was purchased from Fluka chemicals. Solvents DCM, DMF and IPA were of HPLC grade and were obtained from Caledon Ltd.

3.6.2 Peptide Synthesis and Purification

Synthesis of peptide analogues **1**-**3** was carried out on a 0.2-mmol scale of chlorotrityl resin (loading 1.05 mmol/g, 190 mg) following the standard Fmoc solid-phase peptide chemistry with acid labile side chain protections (t-Bu, Boc, Trt) as described previously.[13] Peptides were synthesized using MPS 357 automated peptide synthesizer robot (Advanced Chemtech Inc., USA). Fmoc protected L-amino acids were used, and the side chain protections used were Asn(Trt), Asp(tBu), Cys(Acm), Cys(Trt), Glu(tBu), His(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc), and Tyr(tBu). To ensure efficient coupling of amino acids to the resin, single coupling (1.5 hrs each), was employed till 10 residues. To assist the coupling of difficult residues (Asn, Ser), double coupling was employed. For all residues after position 10,

increased coupling times were employed (2 hrs each). Fmoc deprotection was carried out using freshly prepared 20% piperidine in DMF. Coupling reactions were monitored constantly by performing test cleavage after coupling of each five amino acids till 20 residues and each two residues till 32 residues. Test cleavage was performed by adding a mixture of 50% TFA and DCM to a small amount of resin followed by shaking for 30 min at room temperature. The complete peptide sequences were released from support with concomitant removal of acid-labile side chain protecting groups using the same procedure as used for the test cleavages. The filtrate from the cleavage reactions was combined with TFA washes (3 x 2 min, 1 mL), and concentrated in vacuum. Cold diethyl ether (~15 mL) was added to precipitate the crude cleaved peptide. After trituration for 2 min, the peptides were collected and the crude peptides were dissolved in 20% aqueous isopropanol and purified on a semi-preparative VYDAC C8 reversed-phase HPLC column (1 x 25 cm, 5 μm) using an isopropanol/water gradient in the presence of 0.05% TFA. The gradient used was 25-95% isopropanol/water in 45 minutes at a flow rate of 1.0 mL/min. Pure peptides were characterized by analytical reversed-phase HPLC and MALDI-TOF mass spectrometry. The purity of the peptides was verified to be ≥95% by reverse-phase chromatography (Figures S1-S3, supporting information). The pure lyophilized peptide analogues were stored at -20 $^{\circ}$C under nitrogen.

3.6.3 CD Spectroscopy

CD spectra of LeuA analogues **1-3** were obtained using a Olis spectrophotometer (GA, USA) in a quartz cell with 0.02 cm path length over 190-

250 nm. Peptide samples with a concentration of 35 μg/mL were prepared in 90% TFE containing 0.1% aqueous TFA (pH~2.5). Data were collected every 0.05 nm and were average of 6 scans. Results were expressed in units of molar ellipticity per residue (deg cm^2 $dmol^{-1}$) and plotted versus the wavelength.

3.6.4 Molecular Dynamic Simulations

MD simulation of peptide analogue **1** was conducted using GROMACS as described previously. Search for a sequence similar to the sequence of analogues **1** was carried out by using both BLAST [14] and FASTA [15] sequence alignment tools. Analogue **1** displayed high sequence identity with LeuA (PDB code 1CW6) for which the three dimensional NMR structure in TFE has been reported previously.[2] Homology model of **1** was constructed using VMD-software.[8] The homology model was created by taking PDB structure of native LeuA as a template and by using magic fit tool in the VMD software. The homology model was chosen as the starting structure for further MD simulations.

GROMACS molecular dynamics simulation package [16,17] (GROMOS96 force field) was used to perform simulation studies. Simulations were conducted in the NPT ensemble at 298 or 313 K using periodic boundary conditions (Appendix A.2). Weak coupling of the peptide to a solvent bath of constant temperature was maintained using the Berendsen thermostat [18] and pressure was controlled using the Berendsen algorithm at 1 bar with a coupling constant 4 ps, using a compressibility of liquid TFE of 1.22×10^{-4} and 1.34×10^{-4} kJ^{-1} mol nm^3 at 298 and 313 K, respectively.[19] The electrostatic and van der Waals interactions were truncated at a

cutoff distance of 1.2 nm. The integration time step was 2 fs, and the coordinates and velocities were saved every 2 ps. The LINCS algorithm was used to restrain all bond lengths. A cubic box (6 nm × 6 nm × 6 nm) was constructed and used to run the simulation.[20] LeuA forms stable secondary structure in membrane environment mimicking solvents, so 2, 2, 2-trifluoroethanol (TFE) was used to solvate the box. Parameters for TFE solvent were prepared according to Fioroni *et al*.[19] LeuA analogue **1** was placed in the centre of the box by replacing the TFE molecules equivalent to the peptide size. The N-terminal was positively charged (NH_3^+) and C-terminal was considered to be negatively charged (COO^-). In the sequence, residues Asp, Arg, Glu and Lys were charged and His residues were kept neutral. Sufficient counter ions (Na^+, Cl^-) were added to make the system electro neutral and to provide a final concentration of ~ 100 mM. Energy within the system was minimized before MD simulation using 200 steps of the steepest descent energy minimization method in order to relax any steric conflicts generated during the set-up. The equilibration of the LeuA-TFE system was achieved by performing a 3 ns MD run with positional restrain on the peptide molecule. Following this, a full MD run of 10 ns was performed without any restraints at 298 K followed by at 310 K. Snapshots of the peptides were extracted from 1-10 ns. Simulations were analyzed using various GROMACS post processing routines.[16] Swiss - Pdb Viewer [21] and VMD [8] softwares were used to visualize, analyse and superimpose structures.

3.6.5 Antimicrobial activity

Synthetic LeuA (synthesized previously) and LeuA analogues **1-3** were dissolved in methanol individually and their concentrations were determined by

measuring the A_{280} according to standard procedures found in the literature.[22] Peptide stock solutions with a concentration of 200 µM were prepared and serial dilutions were made to obtain the concentrations of 100 µM, 50 µM and 10 µM. Antimicrobial activity assay was performed using the spot-on-lawn method. The indicator strains used were *Listeria innocua* and *Carnobacterium divergens* (UAL9). The minimum inhibitory concentration (MIC) of LeuA was determined using microtiter plate method as described previously.[23] MIC was defined as the peptide concentration that inhibited the growth by 50%.

3.6.6 Competitive Binding Experiment

Competition experiments were performed similarly where a 200 uL volume of culture medium, wild-type LeuA (slightly above its MIC), a range of concentrations of peptide fragments at 2-fold dilutions, and the indicator strain (*C. divergens* UAL9, diluted 1:100) were added to each well of the microtiter plate. The microtiter plate culture was incubated overnight at 25 $^{\circ}$C, after which the growth of the indicator strain was measured spectrophotometrically at 600 nm using microtitre plate reader (TECAN, Männedorf, Switzerland).

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Chapter 4 General Conclusions and Future Directions

4.1 General Conclusions

Our efforts toward the design, synthesis, and SAR studies of Leucocin A analogues, as well as, synthesis of LeuA using stepwise SPPS and NCL were discussed in this thesis.

Due to changing food habits in the modern world, there has been an increasing number of food borne diseases reported every year.[1] One of the effective practices to prevent these food borne diseases is the use of preservatives in foods. Due to the toxicity and adverse effects of conventional food preservatives, antimicrobial peptides from lactic acid bacteria that target food pathogens without toxic or other adverse effects have great potential as food preservatives as well as therapeutics.[2] Chapter 2 describes the synthesis of LeuA using both stepwise SPPS and NCL. LeuA was successfully synthesized using stepwise SPPS and NCL in good yields (up to 12.5%) and high purity (≥ 95%). Synthesis of LeuA using NCL gave full control over the entire peptide molecule.[3] Characterisation of synthetic LeuA was carried out using RP-HPLC and MALDI-TOF mass spectrometry. Solution conformation of synthetic LeuA was carried using CD spectroscopy showing that the secondary structure of the peptide is similar to that of native LeuA.[4] Furthermore, antimicrobial studies of synthetic LeuA were carried out against two bacterial strains including the *Listeria monocytogenes* which is the causative organism for listeriosis in humans. The MIC values of synthetic LeuA are comparable to that of native LeuA described in literature.[4,5] Thus, a method for

preparing large amount of LeuA has been developed and will allow synthesis of LeuA and analogues for comprehensive SAR studies. Another major advantage of NCL method for synthesis of analogues is that it avoids unnecessary repeated synthesis of the unaltered sequences.

In Chapter 3, SAR of N-terminal region of LeuA were studied by designing and synthesizing three analogues (**1**-**3**) of Leucocin A by replacing the N-terminal β-sheet residues Cys9-Ser15 of the native peptide with shorter β-turn motifs.[5-7] All the three analogues (**1**-**3**) were synthesized using stepwise SPPS and characterized using RP-HPLC and MALDI-TOF mass spectrometry. The anti microbial activity of the analogues was evaluated by spot-on-lawn method using two indicator strains, *Listeria innocua* and *Carnobacterium divergens*, and the three analogues did not display any activity.[5,8] The solution confirmation of the three analogues using CD spectroscopy confirmed that only analogue **1** adopts similar folding as native LeuA among all three analogues. In addition, the ability of the analogue **1** to competitively inhibit the activity of native LeuA was explored using competitive binding assay.[8] Analogue **1** competitively inhibited the activity of LeuA in a dose dependent manner. Furthermore, the secondary structure of analogue **1** was explored using MD simulations.[4] Analogue **1** showed a stable C-terminal α-helix, whereas the N-terminal region mainly folded into a coiled structure. Interestingly at elevated temperature (310 K), analogue **1** showed the appearance of an N-terminal β-sheet. These results suggest that analogue **1** has C-terminal region that is folded more like

native LeuA, however, the N-terminal region displays distinctive difference from the native LeuA.

By analysing these results, it is evident that the C-terminal helical region in the peptide plays an important role in determining cell specificity and the N-terminal sequence in LeuA plays an important role in binding to the receptor on the bacterial cell surface.

4.2 Future Directions

Based on the synthetic strategy developed in the present work, a number of analogues of class IIa bacteriocin such as LeuA can be synthesized. One area that needs to be explored is the synthesis of proteolytically stable analogues of LeuA to increase the half life of such peptides. Proteolytically stable analogues of LeuA can be designed by substituting some of the amino acids with β-amino acids.[9,10] As shown in **Figure 4.1**, terminal residues such as Lys1 and Leu26 as well as the central hinge region residue Val16 can be replaced with β-amino acids. Such replacements will not change the 3D structure of the peptide analogues and will maintain the activity.

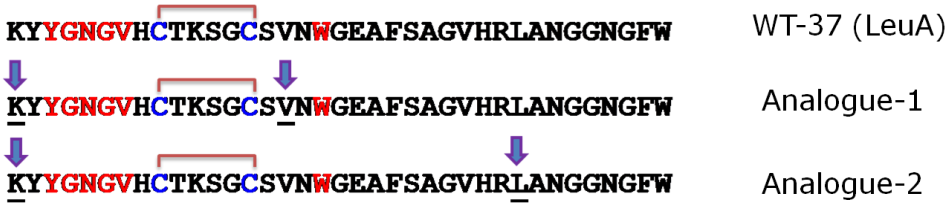

Figure 4.1 Design of analogues of LeuA by substituting α-amino acid residues with β-amino acid residues.

Class IIa bacteriocins such as LeuA are very potent but display a narrow spectrum activity spectrum. However, this allows their use in the development of very sensitive sensor for pathogens like *Listeria monocytogenes*. Therefore, one application of LeuA currently being explored is development of a robust and portable biosensor for the detection of listeriosis pathogen in clinical samples for effective diagnosis, food samples and biological samples for contamination. Designing biosensor can be achieved by immobilizing LeuA on its gold microelectrodes (Figure 4.2). This can be achieved in LeuA by adding a C-terminal cysteine residue as described previously.[11] This C-terminal cysteine acts as a linker between LeuA and gold surface. Because of the high sensitivity of LeuA towards *Listeria monocytogene* , this bacteria will attach to the immobilized peptide (Figure 4.2). Attachment of the bacterium to the immobilized peptide can be monitored by electrical signal readout setup. Such modification like incorporation of C-terminal cysteine or incorporation of β-alanine as linker in LeuA can be effectively achieved using NCL.

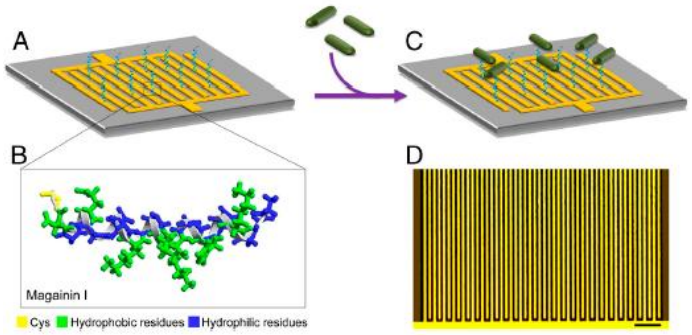

Figure 4.2 (A) Immobilisation of AMP Magainin on a microelectrode array.(B) Magnified image of Maganin. (C) Detection and binding of bacteria by Maganin. (D) Image of microelectrode array. (Adapted from reference [11])

4.3 References

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Appendix

A.1. Protocol Used to Synthesize LeuA on Automated Peptide Synthesizer MPS 357

FOR AMINO ACIDS 2-11:-

1. Fill CV with 100 ml of DMF
2. Mix for 20.0 minute(s) on speed 5
3. Empty collection vessel
4. Fill CV with 2.0ml of AA
5. Fill CV with 2.0ml of DIC
6. Mix both for 60.0 minute(s) on speed 5
7. Empty collection vessel
8. Fill CV with 5.0 ml of DMF
9. Mix both for 2.0 minute(s) on speed 5
10. Empty collection vessel
11. Fill CV with 2.0ml of AA
12. Fill CV with 2.0ml of DIC
13. Mix both for 60.0 minute(s) on speed 5
14. Empty collection vessel
15. Fill CV with 5.0 ml of DMF
16. Mix both for 2.0 minute(s) on speed 5
17. Empty collection vessel
18. Fill CV with 5.0 ml of DCM
19. Mix both for 2.0 minute(s) on speed 5
20. Empty collection vessel
21. Fill CV with 5.0 ml of DMF
22. Mix both for 2.0 minute(s) on speed 5
23. Empty collection vessel
24. Fill CV with 3.0 ml of PIP
25. Fill CV with 1.0 ml of HoBt
26. Mix both for 8.0 minute(s) on speed 5
27. Empty collection vessel
28. Repeat from step 25, 1 time(s)
29. Fill CV with 5.0 ml of DMF
30. Mix both for 2.0 minute(s) on speed 5
31. Empty collection vessel
32. Fill CV with 5.0 ml of DCM
33. Mix both for 2.0 minute(s) on speed 5
34. Empty collection vessel
35. Fill CV with 5.0 ml of DMF
36. Mix both for 2.0 minute(s) on speed 5
37. Empty collection vessel
38. End

FOR AMINO ACIDS 11-37:-

1. Fill CV with 100 ml of DMF
2. Mix for 20.0 minute(s) on speed 5
3. Empty collection vessel
4. Fill CV with 2.0ml of AA
5. Fill CV with 2.0ml of DIC
6. Mix both for 60.0 minute(s) on speed 5
7. Empty collection vessel
8. Fill CV with 5.0 ml of DMF
9. Mix both for 2.0 minute(s) on speed 5
10. Empty collection vessel
11. Fill CV with 2.0ml of AA
12. Fill CV with 2.0ml of DIC
13. Mix both for 60.0 minute(s) on speed 5
14. Mix both for 60.0 minute(s) on speed 5
15. Empty collection vessel
16. Fill CV with 5.0 ml of DMF
17. Mix both for 60.0 minute(s) on speed 5
18. Empty collection vessel
19. Fill CV with 5.0 ml of DCM
20. Mix both for 2.0 minute(s) on speed 5
21. Empty collection vessel
22. Fill CV with 2.0 ml of NMM
23 Fill CV with 2.0 ml of HBTU ⟶(this is acetic anhydride in the bottle)
24. Mix both for 10.0 minute(s) on speed 5
25. Repeat from step 18, 1 time(s)
26. Empty collection vessel
27. Fill CV with 5.0 ml of DMF
28. Mix both for 60.0 minute(s) on speed 5
29. Empty collection vessel
30. Fill CV with 5.0 ml of DCM
31. Mix both for 2.0 minute(s) on speed 5
32. Empty collection vessel
33. Fill CV with 3.0 ml of PIP
34. Fill CV with 1.0 ml of HoBt
35. Mix both for 8.0 minute(s) on speed 5
36. Empty collection vessel
37. Repeat from step 25, 1 time(s)
38. Fill CV with 5.0 ml of DMF
39. Mix both for 60.0 minute(s) on speed 5
40. Empty collection vessel
41. Fill CV with 5.0 ml of DCM
42. Mix both for 2.0 minute(s) on speed 5
43. Empty collection vessel
44. Fill CV with 5.0 ml of DMF

45. Mix both for 2.0 minute(s) on speed 5
46. Empty collection vessel
47. End

A.2. Files used for MD simulations

LeuA.mdp (parameter file)

313 K

```
;
;  User spoel (236)
;  Wed Nov  3 17:12:44 1993
;  Input file
;
title               =  Yo
cpp                 =  /lib/cpp
;define            =  -DPOSRES
constraints         =  all-bonds
integrator          =  md
pbc                 =  xyz
tinit               =  0.0
dt                  =  0.002 ; ps !
nsteps              =  5000000  ; total 5000 ps.
nstcomm             =  1
nstxout             =  25000
nstvout             =  25000
nstfout             =  0
nstlog              =  25000
nstenergy           =  25000
table-extension     =  3
nstlist             =  10
ns_type             =  grid
deltagrid           =  2
coulombtype         =  PME
rlist               =  1.2
rcoulomb            =  1.2
rvdw                =  1.2
fourierspacing      =  0.12
pme_order           =  4
ewald_rtol          =  1e-5
; Temperature coupling is on in two groups
Tcoupl              =  yes
tc-grps       =  Protein       TFE        Na          Cl
tau_t               =  0.1           0.1        0.1         0.1
ref_t               =  313      313        313         313
; Energy monitoring
```

```
energygrps          =  Protein       TFE
; Pressure coupling is now on in two groups
Pcoupl              =  isotropic
tau_p               =  4.0
compressibility     =  1.34e-4
ref_p               =  1.0
; Generate velocites is off at 300 K.
gen_vel             =  no
gen_temp            =  313
gen_seed            =  173529
```

278 K

```
;
;  User spoel (236)
;  Wed Nov  3 17:12:44 1993
;  Input file
;
title               =  Yo
cpp                 =  /lib/cpp
;define              =  -DPOSRES
constraints         =  none
integrator          =  md
pbc                 =  xyz
tinit               =  0.0
dt                  =  0.002 ; ps !
nsteps              =  10000000; total 5000 ps.
nstcomm             =  1
nstxout             =  25000
nstvout             =  25000
nstfout             =  0
nstlog              =  25000
nstenergy           =  25000
nstlist             =  10
ns_type             =  grid
deltagrid           =  2
coulombtype         =  PME
rlist               =  1.2
rcoulomb            =  1.2
rvdw                =  1.2
fourierspacing      =  0.12
pme_order           =  4
ewald_rtol          =  1e-5
; Temperature coupling is on in two groups
Tcoupl              =  yes
tc-grps      =  Protein       TFE       Na         Cl
tau_t               =  0.1           0.1       0.1        0.1
ref_t               =  278     278       278        278
; Energy monitoring
energygrps          =  Protein       TFE
; Pressure coupling is now on in two groups
```

```
Pcoupl              =  isotropic
tau_p               =  4.0
compressibility     =  1.22e-4
ref_p               =  1.0
; Generate velocites is off at 300 K.
gen_vel             =  no
gen_temp            =  278.0
gen_seed            =  173529
```