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Synthesis of an isotopically labelled analog of the antimicrobial peptide ll-37

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Synthesis of an isotopically labelled analog of the antimicrobial Peptide LL-37

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ABSTRACT

LL-37 is a cationic cathelin-associated broad spectrum antibiotic peptide of human neutrophils. Its mechanism of action is by disruption of the bacterial cell membrane structure. LL-21 is a simplified form of the peptide that contains only the core portion of LL-37, which retains antimicrobial activity and is easier to synthesize. We synthesized an ^{15}N –Val-labeled LL-21 peptide which allows us to perform antimicrobial assays to know the antimicrobial activity of the new peptide LL-21 on various gram positive and gram negative bacteria. We found that our new peptide LL-21 shows good minimum inhibitory concentrations on various gram positive and gram negative bacteria except in the case of *Bacillus subtilis* and when compared with the parent analog, the new peptide requires slightly higher concentrations to kill the bacteria.

Solid state NMR studies on LL-21 help to measure helix tilt in the bacterial cell membranes. This will provide information on its precise mode of action, either detergent-like activity or pore formation. In the future, the replacement of some of the amino acids in LL-21 with ^{19}F and deuterium labels in specified positions will give more information on the orientation, dynamics, and rotation of the peptide in bacterial cell bilayers.

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CHAPTER 1

INTRODUCTION

1.1 Antibiotics

Currently, antibiotics are very important drug agents used to treat bacterial infections. Antibiotics show bactericidal or bacteriostatic effects by acting on specific targets in the bacterial cell. They act by interfering with protein synthesis, DNA synthesis, cell wall synthesis, cell membrane permeability, enzymes and by modifying cell membranes. So antibiotics act on specific targets to affect their activity. For example, penicillin acts by interfering with the formation of glycopeptides in the cell wall, which are responsible for the rigidity and shape of the bacteria.[1]

1.2 Bacterial resistance toward antibiotics

Excessive use of these antibiotics can cause an increase in the resistance of bacteria to the drug and nullification of the antibiotic effect. Bacteria develop resistance toward modern antibiotics because these drugs interfere with protein, DNA, or cell wall synthesis, or by inhibiting a specific enzyme. Consequently, bacteria can easily develop resistance towards antibiotics by, for example, modifying the production of proteins, DNA, or the cell wall. Bacteria develop resistance toward antibiotics through enzymatic degradation or modification that inactivates the antibiotic drug molecule, through mutation to alter the targets, through overproduction of targets to dilute the drug action, and through preventing the access of drug to its targets.[2]Through all these mechanisms of resistance, bacteria overcome the action of antibiotics and render them useless in the

prevention of bacterial infections. So this antibiotic resistance is a major issue in current days that necessitates the development of new drugs like antimicrobial peptides.

1.3 Antimicrobial Peptides

Unlike the antibiotics mentioned above which act on specific targets, antimicrobial peptides act by a non-specific mechanism of action. In this case, antimicrobial peptides act on bacterial cell membranes, thereby leading to cell death by disruption of membrane integrity. In a bacterial cell, the hydrophobic core of the lipid bilayer acts as a barrier between the inside and outside of the cell environment, thereby protecting the cell contents and preventing leakage. This hydrophobic bilayer is disrupted by insertion of the peptide, decreasing the ability of the bilayer to act as a barrier and leading to formation of pores or local defects in the cell membrane and ultimately causing cell death. The extent of bacterial cell damage by the peptide depends upon the interaction of hydrophobic bilayer with the peptide.[3]

In every kingdom and phylum ranging from prokaryotes to humans, antimicrobial peptides are produced. The observed composition and functions of these antimicrobial peptides observed in different organisms is varied. For example LL-37, magainin 2, and cecropin B peptides contain no cysteine and are linear, whereas tachyplesin has cysteines and disulfide bonds, making it cyclic. Their exact functions also are different from each other. Some peptides are very effective on bacteria, viruses, and fungi, and they are not harmful to mammalian cells, whereas some peptides are toxic to both mammalian and bacterial cells.[10] Bacterial outer cell membranes contain 20 to 25% negatively charged lipids. Phosphatydyl ethanolamine (PE) is the most common zwitterionic lipid. At physiological pH, most of the amphipathic, α-helical antimicrobial

peptides are positively charged. So the electrostatic attraction between positively charged peptides and negatively charged bacterial outer membranes is the driving force for the selectivity of these antimicrobial peptides toward the bacterial outer membranes. The extent of peptide insertion and disruption of membrane integrity mainly depends on the hydrophobic moment and the angle created by the hydrophobic face of the helix.[4]

1.4 Basic mechanisms of action

Antimicrobial peptides (specifically, those that have alpha-helical structures) show activity first by binding to the bacterial cell membrane and then interacting with the cell membrane. They mainly act by three mechanisms of action: the barrel-stave mechanism; the toroid pore or wormhole mechanism; and the carpet mechanism.

1.4.1 *Barrel-Stave Mechanism*

In this mechanism, the peptide forms a barrel-like ring and creates an aqueous pore in the bacterial cell membrane. The peptide binds to the bacterial cell membrane surface by the hydrophobic portion of the peptide facing outward and toward the acyl chains of the cell membrane lipids, and the hydrophilic portion forms the pore lining. Phase transition takes place after the binding of the peptide to the cell membrane, and the hydrophobic portion of the peptide inserts into the bacterial cell membrane outer layer, leading to the formation of the pore in the membrane. Through aggregation of the peptide as concentration increases, the pore expands, leading to the leakage of cell contents and, thereby, cell death. This mechanism is shown in Figure 1 (the A' to B' path way).

1.4.2 *Toroid Pore or Wormhole Mechanism*

In this mechanism of action the peptide binds parallel to the surface of the bacterial cell membrane and creates a positive curvature strain, destabilizing the

membrane integrity and leading to the formation of a toroidal lipid-peptide pore in the bacterial cell membrane. Peptide with more positive charge creates a toroid pore with shorter life span, so there is an inverse relationship between stability and charge of the peptide.[2]

1.4.3 *Carpet Mechanism*

The carpet mechanism (shown in Figure 1), also called the detergent mechanism, uses the diffusion principle. In this mechanism, the bacterial cell membrane is disrupted by changes in membrane fluidity by phospholipid displacement or by decreasing membrane barrier properties by accumulation of a high density of peptides on the bacterial cell membrane. At high peptide concentrations, micelles are formed and the bacterial lipid bilayer is disrupted through a detergent-like effect.[4] The non-polar portions of the membrane are enclosed by the amphipathic peptide, effectively pulling them out into water.

Bacterial cell death may result from all of the mechanisms through leaking of ions and metabolites, depolarization, and membrane-coupled respiration.[2] Therefore these antimicrobial peptides act by nonspecific mechanisms like forming a pore in the cell wall, thereby leading to lysis and cell death, or by acting like a detergent.

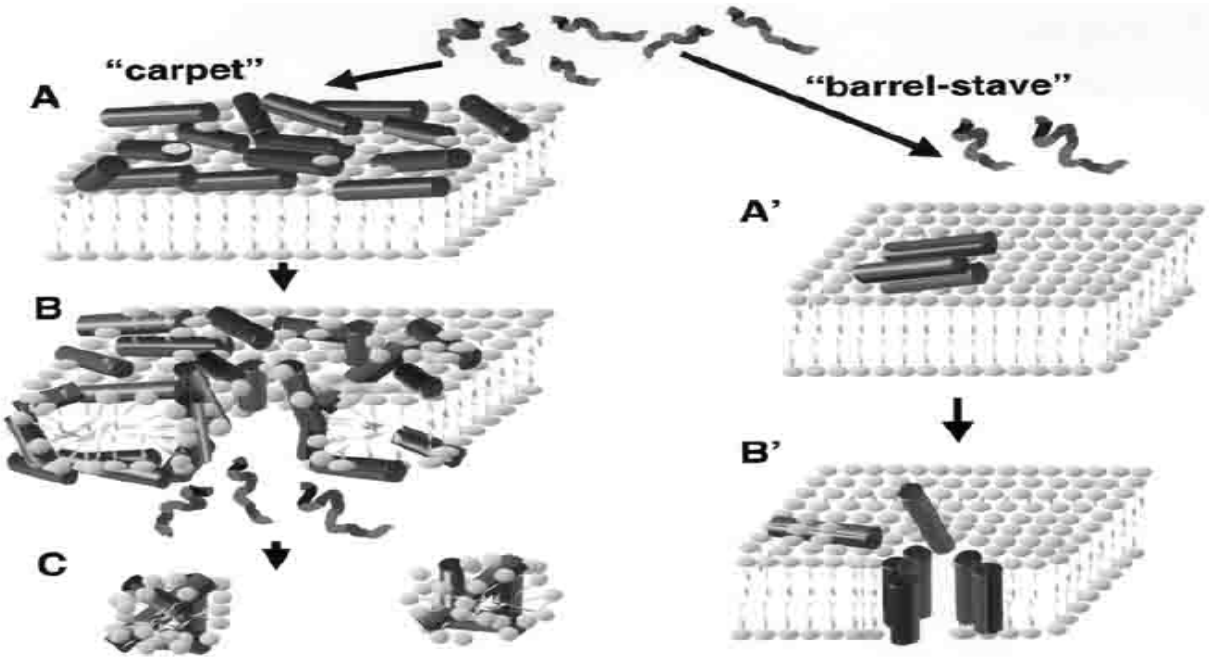

Figure1. Carpet and barrel-stave mechanisms of action of antimicrobial peptides.[10]

1.5 NMR Studies on Antimicrobial Peptides

To better understand the mechanism of action and secondary structure of antimicrobial peptides, orientation of the peptides in the bacterial lipid bilayer membrane, and dynamics of phospholipids bilayer membranes, nuclear magnetic resonance (NMR) spectroscopy is the best technique available for researchers. The lipid head group shape and the effects of peptides on the macroscopic phase properties of the membranes can also be obtained from NMR studies. NMR studies have been useful in determining dynamic and conformational information of site-specifically labeled short membrane peptides like human LL-37, magainin, protegrin-1, colicin Ia, and subtilosin A.

To study the perturbation of the phospholipid bilayers by peptides, two techniques in solid state NMR called static ^{31}P and ^{2}H are used. ^{15}N chemical shift anisotropy of a single static site will give information on the orientation of an antimicrobial peptide in the bacterial phospholipid bilayer membranes.[5]

^{15}N solid state NMR is used to give information about the orientation of the peptide with respect to the bilayer. Most solid state spectra are broad due to anisotropy and the absence of averaging (Figure 2). Due to the different alignment of the amide bond with respect to the magnetic field, peptides oriented parallel to the lipid chains in the membrane bilayer give a peak at about 220 ppm; those oriented perpendicular to the fatty acid chains give a peak at about 50 to75 ppm (Figure 3). A peptide that lies parallel to the lipid chains is most likely forming a channel for ions to destroy the membrane (barrel stave mechanism). A peptide lying on the surface, perpendicular to the chains, is more likely destroying the membrane through the toroidal pore or detergent/micellization mechanism (carpet mechanism). Therefore this information is valuable in determining the mode of action of the antimicrobial peptide.

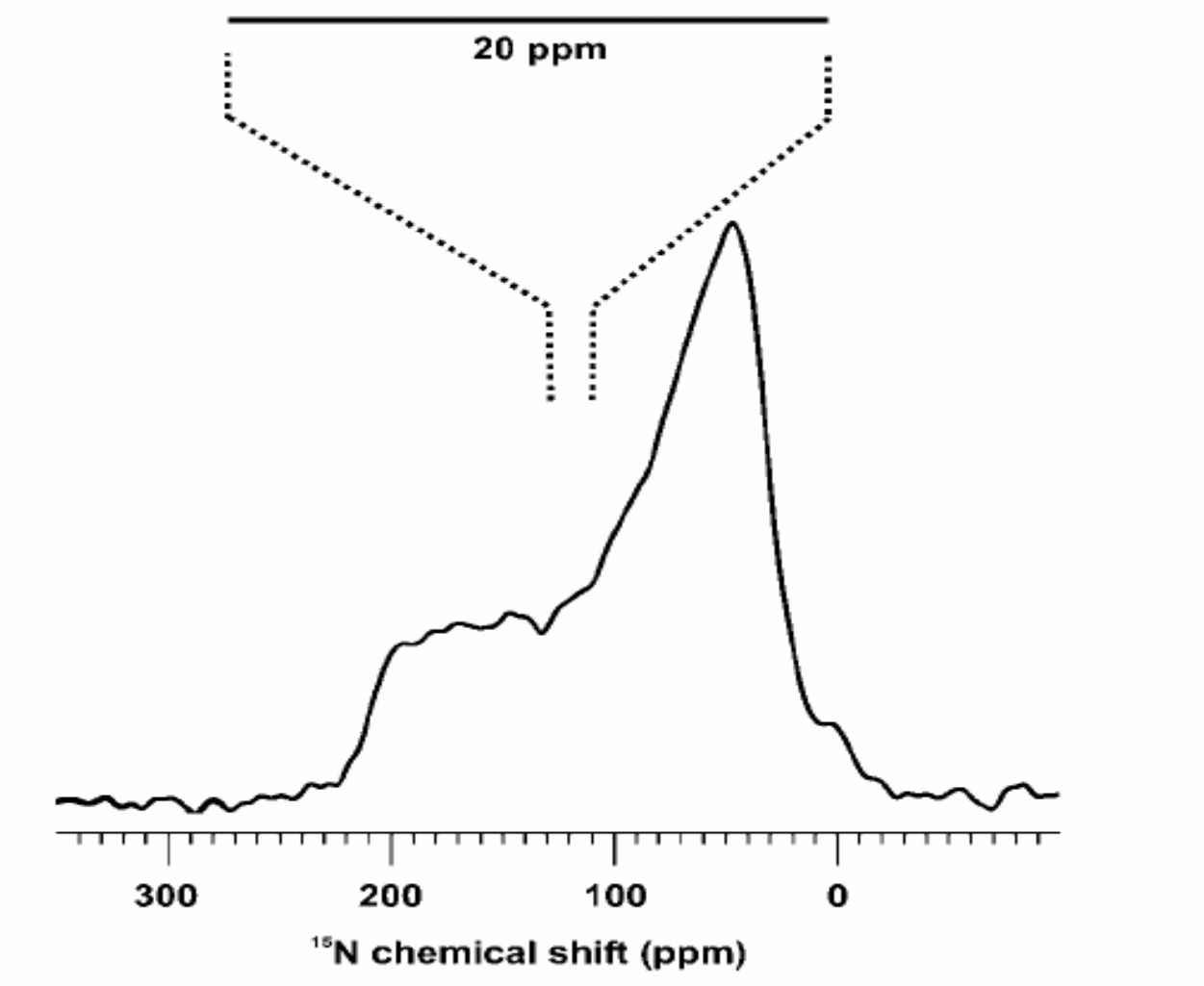

Figure 2. ^{15}N solid state NMR spectrum, showing broadening due to anisotropy and absence of averaging.

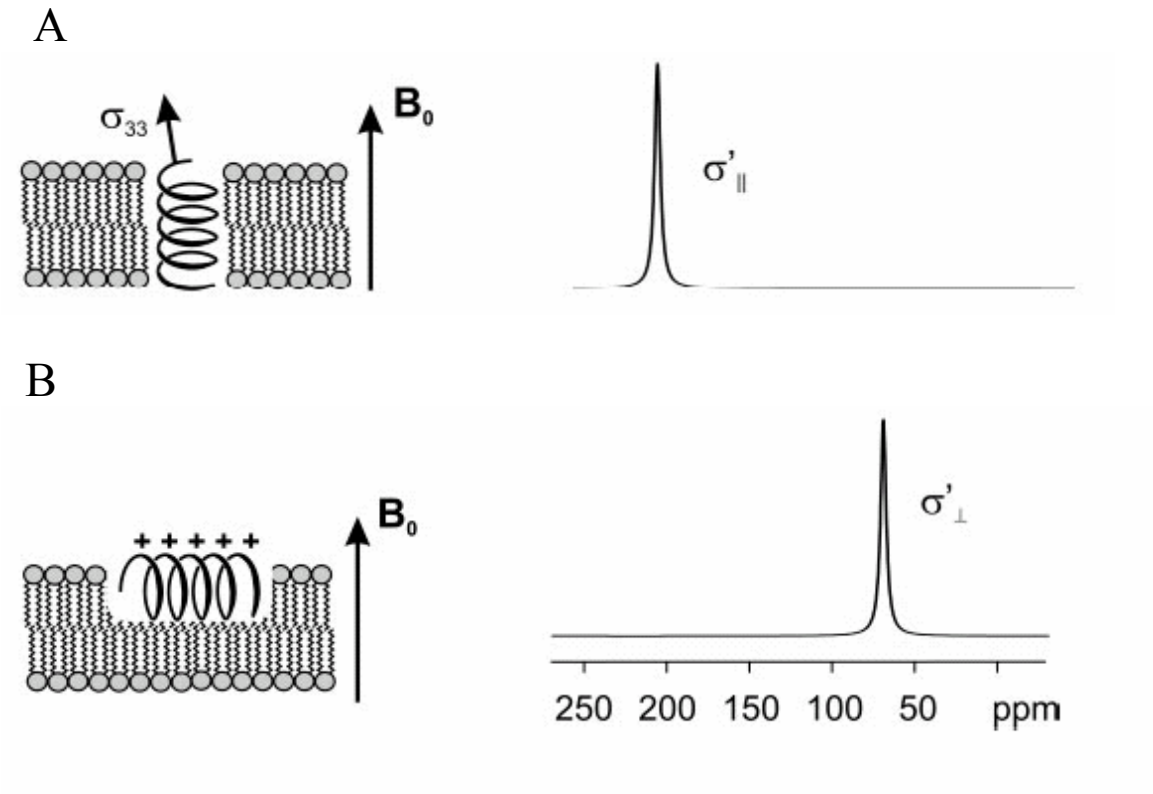

Figure 3. Chemical shift anisotropy (CSA) of peptide with respect to the bilayer. (A) Peptides that orient parallel to the lipid chains give a peak at about 220 ppm. (B) Peptides that orient perpendicular to the lipid chains give a peak at about 50-75 ppm.

Three dimensional structural studies of peptide interactions would be useful in understanding the molecular level mechanism of action, broad spectrum antimicrobial activity, and potencies, and also in designing synthetic pharmaceutical peptides that show similar antimicrobial activity and are readily synthesized and easy to administer.[5]

1.6 Cathelicidin family peptides

More than 20 members of a variety of species like cow, pig, sheep, rabbit, and mouse are included in this cathelicidin family. Cathelicidins are inactive precursors of the mammalian antimicrobial peptide gene family and are stored in cytoplasmic granules of neutrophil leukocytes. Upon activation of leukocytes, these antimicrobial peptides are

activated and released from the cytoplasmic granules. The structure and mechanism of action of killing bacteria varies from one member to another in this cathelicidin family. Antimicrobial peptides, which contain α-helical structures, act by permeabilizing bacterial cell membranes, and peptides, which do not have α-helical structure, act by inhibiting the DNA or protein synthesis or by inhibiting the incorporation of necessary precursor molecules into protein or RNA.[5]

1.7 Introduction to LL-37

LL-37 is an important human antimicrobial peptide that belongs to the Cathelicidin family, with cationic charge and amphipathic α-helical character that kills bacterial cells through disruption of their membranes. LL-37 acts as a first line of defense mechanism against various gram positive and gram negative bacteria and is effective against pathogens that cause both systemic and local invasions.[4] The sequence of LL-37 is shown in Figure 4.

LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES - amide

Figure 4. Sequence of LL-37 [4]

It contains 16 charged residues (five negative and eleven positive), so it is a highly positively charged peptide. It also contains 14 non-polar amino acids that can interact with membranes through hydrophobic interactions.

1.8 Minimum inhibitory concentrations (MIC)

The minimum inhibitory concentration is the lowest concentration of peptide at which there is no bacterial growth. The minimum inhibitory concentration of LL-37 is 1 to 10 μM for several species, which means that this peptide can effectively kill gram positive and gram negative bacteria at this concentration. In eukaryotic cells, 13 to 25 μM concentrations are cytotoxic to eukaryotic cells. This is an undesirable effect in that it indicates harm to the host. A good drug is one that destroys bacteria but does not significantly affect host cells. Concentrations between 1 to 10 μM fit this profile. LL-37 aggregates more in zwitterionic phosphatydyl choline (PC) bilayers than in negatively charged bilayers containing PC and phosphatydyl serine, so this suggests that LL-37 is very sensitive to the composition of the lipid bilayer.[4]

1.9 Structure and activity of LL-37

LL-37 contains an α-helical secondary structure. At physiological pH and ion concentrations, this secondary structure aggregates. In pure water LL-37 forms a random coil and at millimolar anion concentrations it becomes α-helical, so LL-37 is suggested to be very sensitive to salt concentrations. In a wide range of buffer conditions including high salt concentrations and in the presence of lipids, the helical structure of LL-37 was stable.[4] LL-37 forms an amphipathic α-helix from residues 11 to 32, and this helix is involved in its activity and aggregation in the presence of micromolar anion concentrations. Usually antimicrobial peptides with α-helical structure retain an α-helix when in contact with the membrane and are monomeric and less structured in solution. Therefore the conformational change from random coil to α-helix is the driving force for the aggregation and insertion of peptide in the bacterial cell membranes. However, LL-37

does not undergo any conformational changes because it is in a helical structure in both physiological and buffer conditions.[4]

1.10 Mechanism of Action of LL-37

LL-37 is highly selective towards bacterial lipid bilayers and is hydrophobic enough to penetrate into the lipid bilayer core of the bacteria cell wall. Membrane composition and aqueous environmental conditions mainly influence the interaction of LL-37 with the bacterial lipid bilayer membranes. Solid-state NMR and differential scanning calorimetry studies show that in water LL-37 exhibits a random coil structure; and in the presence of lipid or anion at micromolar concentrations it exhibits helical structure. As mentioned previously, LL-37 contains primarily an α-helical secondary structure at different peptide/lipid ratios in both water and phosphate buffer.[4]

The antibiotic mechanism of LL-37 includes several steps,which will be discussed in the next five sections.

1.10.1 *Orientation of peptide in the lipid bilayer*

Orientation of the peptides in the lipid bilayer is an important step for the action of peptides. The peptides can be oriented parallel along the length of the bacterial bilayer or oriented perpendicular to the lipid bilayer membrane to exert its activity.

The ^{15}N labeled group in the middle of the peptide was used to study the orientation of the α-helix of the peptide in lipid bilayer. Its parallel orientation indicates that it works by a carpet (detergent-like) or toroidal pore mechanism and not by creating a channel (barrel-stave).

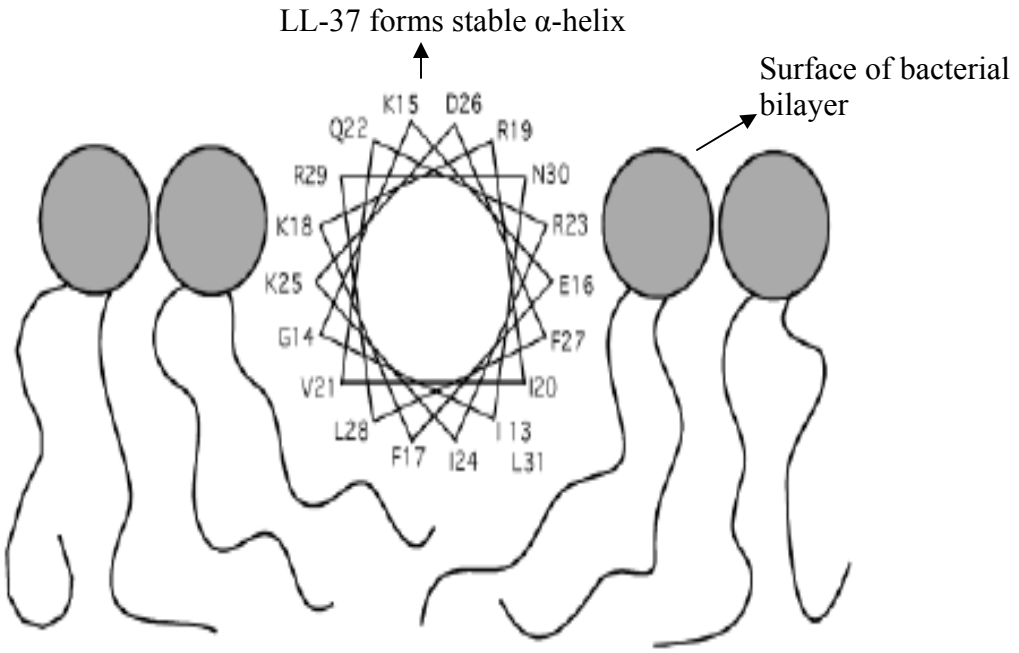

Figure 5. LL-37 forms a stable α-helix structure and located at the polar/non polar interface on the surface of bacterial lipid bilayer.[4]

LL-37 is more stable with its α-helical structure, and this is located at the polar/nonpolar interface on the surface of bilayer (Figure 5). In lipid bilayers, LL-37 orients parallel along the surface, and this orientation is stable over wide changes in pH, lipid compositions, temperature, or presence of aqueous ions.

1.10.2 *Perturbation of lipid bilayer by LL-37*

^{31}P solid-state NMR spectra was used to study the perturbation of the lipid head group by LL-37. In rigid and fully saturated bilayers containing a higher percentage of lipids, LL-37 shows greater head group distortion or disruption of the bilayer. With the increase in the temperature, disruption of the bilayer decreases due to increases in fluidity. The peptide effect was much greater in the presence of cholesterol because of the increase in the rigidity of the fluid lamellar bilayer. The head group size of a major component of the bilayer affects the activity of LL-37 in both anionic and zwitterionic

lipids. In lipids containing large unoriented components, at intermediate concentration LL-37 shows maximum activity. So LL-37 induces perturbation of the lipid bilayer, which leads to disruption of the lipid bilayer but does not form any micelles when reacting with the lipid bilayer. So it is reported that LL-37 does not act through a detergent-like mechanism with the bacterial lipid bilayers.[3]

1.10.3 *Induction of positive curvature strain in lipid bilayers*

The ability of LL-37 to induce positive curvature strain in the lipid bilayer was studied by using ^{31}P NMR and differential scanning calorimetry (DSC). Positive curvature strain was important to form a toroidal pore in the bacterial cell membrane. With an increase in the peptide concentration at the lipid bilayer, positive curvature strain was increased on the lipid head group of bacterial bilayer membranes. This is followed by insertion of the peptide into the bilayer, leading to formation of a toroidal-lipid pore (Figure 6).

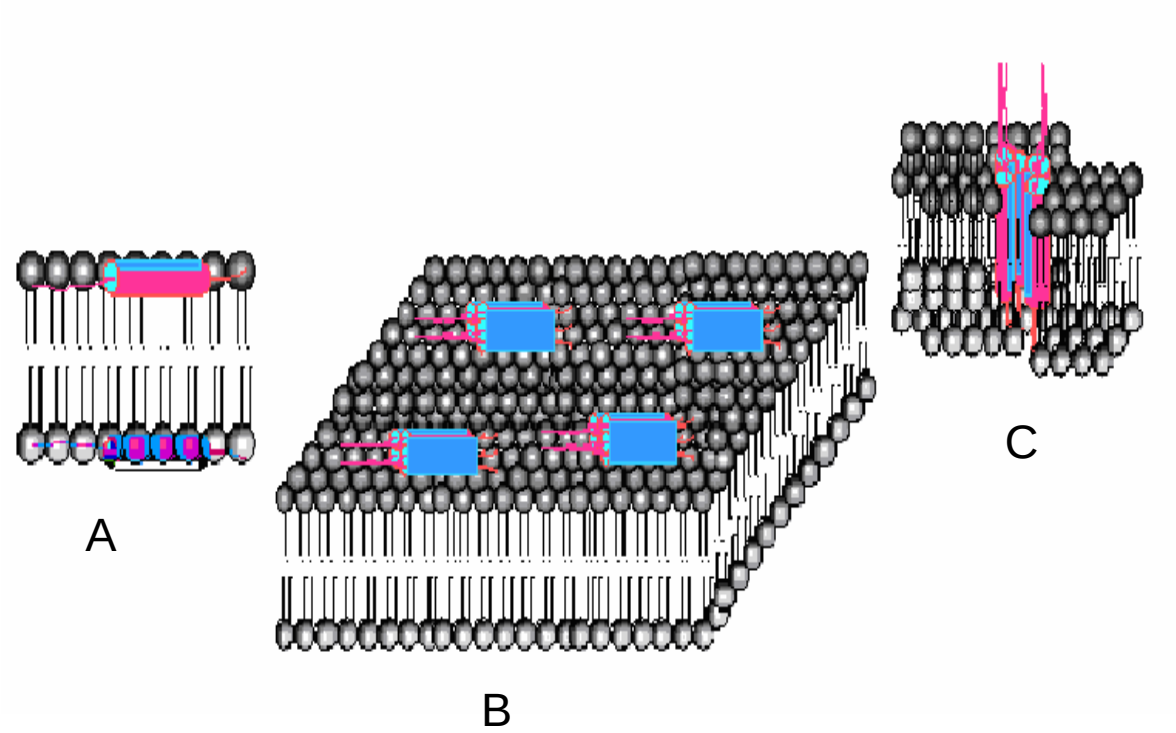

Figure 6. Membrane binding and pore formation of LL-37. (A) Peptide binds along the length of the bacterial lipid Bilayer. (B) Peptide binding and perturbing the membrane by creating positive curvature strain. (C) Insertion of peptide in the lipid Bilayer membrane by creating a pore in the membrane.[6]

It was reported that LL-37 does not act through a detergent mechanism because of a lack of micelle formation. The net positive charge of LL-37 is +6, so the electrostatic attraction between this positively charged peptide and the negatively charged anionic lipid bilayers is the driving force for aggregation of peptide. The positive curvature strain induced by LL-37 supports the toroidal-pore mechanism of membrane disruption. In this mechanism, the pores allow leakage of ions and molecules from the bacterial cell and lead to the death of bacterial cells.[4]

1.10.4 *Antimicrobial activity of LL-37*

On various gram positive and gram negative bacteria, LL-37 is active in the 1 to 10 μM concentration range. *In vitro*, LL-37 shows hemolytic activity at 25 μM concentration.[3] Besides antimicrobial activity, LL-37 can act as a chemotoxin for mast cells, which play an important role in inflammatory reactions. At 5 μg/mL concentrations, LL-37 can effectively promote migration of the mast cells in the body and shows its chemotoxic action by stimulating the degranulation of mast cells.[7] A concentration of 5 μM is the minimum inhibitory concentration required for LL-37 to effectively kill *E. coli* bacteria. LL-37 shows cytotoxic activity at 13-25 μM against various eukaryotic cells.[8] The minimum inhibitory concentrations of LL-37 on various gram positive and gram negative bacteria at 0mM and 100mM salt concentrations were shown in Table 1 and Table 2.

Table 1. Activity of LL-37 against various gram-positive bacteria [9]

Organism	MIC (μg/ml) at 0 mM salt concentration	MIC (μg/ml) at 100 mM salt concentration
L.monocytogenes EGD	1.5	2.2
S.epidermidis	7.6	43.9
S.aureus 930918-3	12.5	9.2
S.aureus 67395	2.9	5.4
S.aureus 68721	3.2	9.0
S.aureus 502A	3.6	7.2
MRSA 1083	6.5	>79.1
MRSA 30371	10.5	>79.1
MRSA 54424-1	3.0	25.0
MRSA ATCC 33591	3.4	>79.1
B.subtilis	2.7	0.5
VREF 94.132 (*E.faecium*)	0.7	0.6
VREF CDC 21 (*E.faecalis*)	3.5	2.0
C.albicans 820	> 250	> 250

At low salt concentrations, LL-37 can effectively kill various gram positive bacteria at very low concentrations, while a slightly higher concentration is required at higher salt concentrations.

Table 2. Activity of LL-37 against various gram-negative bacteria [9]

Organism	MIC (μg/ml) at 0 mM salt concentration	MIC (μg/ml) at 100 mM salt concentration
E.coli ML-35p	0.6	7.6
E.coli ATCC 9637	0.1	2.1
E.coli ATCC 11775	1.9	2.7
E.coli MCR 106	1.6	2.1
S. typhimurium 7953s	0.4	3.6
S. typhimurium 14028s	1.2	2.9
P.aeruginosa MR 3007	4.7	3.8
P.aeruginosa MR 53647-1 CF	2.5	3.7
P.aeruginosa AML 654 (mucoid)	5.7	3.6
P.aeruginosa CL79	1.4	0.9
P.aeruginosa SBI-N	1.3	4.0
B.cepacia 96-11	>79.1	>250
B.cepacia ATCC 25416	>79.1	>250
S.maltophilia 411 A-15	1.9	5.1
P.mirabilis ATCC 7002	5.7	>25.0
P.vulgaris ATCC 13315	2.5	5.4

LL-37 was effective against most of the gram negative bacteria, and it was shown that it was very effective against *E. coli* bacteria at very low concentrations. At high salt concentrations, LL-37 needs more time and concentration to kill bacteria than at low salt concentrations, but it is still effective.[9] Therefore the antimicrobial activity of LL-37 would be helpful in treating various gram positive and gram negative bacterial infections. It would be an effective drug in the treatment of various respiratory tract infections, even in cystic fibrosis, where high salt concentrations exist in the lung due to a chloride ion channel defect.

CHAPTER 2

RESEARCH GOAL

Our research goal was to develop a simplified peptide with good antimicrobial activity from the core portion of the peptide LL-37 and study its mechanism of action. Previous truncation studies have shown this segment as the minimal sequence that retains antimicrobial activity. The structures of these peptides are shown in Figure 7.

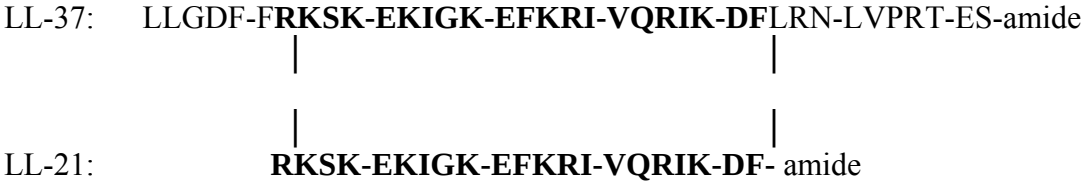

Figure 7. The structure of the peptide LL-37, compared to that of our analog, LL-21.

Since this peptide contains the core portion of the peptide LL-37 that includes more positively charged amino acids and is made up of a total of 21 amino acids, it was labeled LL-21. The small peptide LL-21 has several advantages, outlined in the following sections.

2.1 Simplified form is easier to synthesize

LL-21 contains only 21 amino acids, so it is a simplified version of peptide LL-37. Since LL-21 contains fewer amino acids than the parent chain, it is very easy to synthesize this peptide. Longer sequences, especially those over 20 amino acids, often result in lower yields and more difficult purifications. This is due to more side reactions and, often, lower aqueous solubility.

2.2 Economically advantageous

Because it contains fewer amino acids than the parent chain, more time can be saved in synthesizing the peptide, and it is less expensive to buy materials for the synthesis, including amino acids, coupling agents, deprotection reagents, and solvents.

2.3 Isotopic labelling of LL-21

Our other research goal was to introduce isotopic labeling into LL-21 for NMR structural studies. This can help determine if LL-21 works by the same mechanism as proposed for LL-37. This can be done by replacing the 15^{th} position, valine, with radiolabelled valine, ^{15}N-Val.

So the main aims of the project were to synthesize the peptide LL-21, check its antimicrobial activity by determining the minimum inhibitory concentration (MIC) of LL-21 against various gram positive and gram negative bacteria and compare the results to those of LL-37, determine the orientation of LL-21 peptide in the bacterial lipid bilayer through structural NMR studies, and therefore discover its mechanism of action, which may be different from that of LL-37.

CHAPTER 3

EXPERIMENTAL METHODS

3.1 Materials and Methods Summary, EMU Lab

LL-21 peptide was prepared by using double coupling of the desired amino acid sequence with the help of resin, coupling agent, and deprotection solvents, and the resin was removed from the peptide by acidolysis using trifluoroacetic acid (TFA). Reverse phase high performance liquid chromatography was used to purify the peptide from other impurities, and the purity of the peptide was confirmed by using analytical HPLC and MALDI-TOF mass spectrometry.

3.2 Solid phase peptide synthesis overview

In solid phase peptide synthesis, one end of the peptide chain is attached to the solid support resin, and any desired sequence of N- α amino acids can be attached. After the completion of the required peptide sequence, the solid support resin can be detached from the peptide sequence.

In this process the carboxyl end of the amino acid and the –OH or $–NH_2$ end of the solid support resin react together to form a covalent bond by eliminating a water molecule. In the next step, the fluorenyl-methoxy-carbonyl (Fmoc) group from the amino acid-resin complex can be removed by deprotection, and the α-amine is available to react with another amino acid. In the coupling step, the sequence would react with another amino acid, forming a covalent bond by eliminating a water molecule. This is aided by a coupling agent. This process will continue until the desired sequence of the peptide is obtained. In the final step the polymer resin can be detached from the peptide sequence by acidolysis, using weak acids such as TFA (10mL) and some scavengers like

anisole (0.5mL), thioanisole (0.5mL), and phenol (1 crystal). The automatic peptide synthesizer used for the synthesis of peptide was shown in Figure 8.

Figure 8. Automated PS3 peptide synthesizer having automatic carousel with N,N-dimethylformamide (DMF) as pressurized solvents, 20% piperidine in DMF as a deprotecting agent, and 0.4 M N,N-diisopropylethylamine in DMF as an activator.

3.3 Peptide Synthesis of LL 21

Protected amino acids, resins, and coupling agents were purchased from Bachem California, Midwest Biotech, and Anaspec, Inc. The labeled protected 15N-Val was purchased from Cambridge Isotope Laboratories, Inc. Solvents and deprotecting agents were obtained from Fisher Scientific and Aldrich Chemical Co.

Standard solid phase techniques for N-α-fluorenylmethyloxycarbonyl (Fmoc) protected amino acids on Rink amide *p*-methylbenzhydrylamine (MBHA) resin (0.6 mmoles/g) were used to prepare the peptide using a PS3 Automated Peptide Synthesizer from Protein Technologies. A 20% piperidine solution in N,N-dimethylformamide (DMF) was used for deprotection. O-(Benzotriazol-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was used as a coupling agent, activated by 0.4 M N,N-diisopropylethylamine in DMF.

Amino acids were weighed for about 0.4 milli moles equivalent weight and transferred into vials. HBTU (0.152gm) was weighed and transferred into each amino acid vial. HBTU works by forming activated benzotriazolyl esters with the incoming amino acids.

All vials of desired amino acids were placed in the order of appearance in the peptide in the automated PS3 peptide synthesizer shown in Figure 8. FMOC-synthesis continued, and the desired sequence was attained. Both LL-21 and [^{15}N Val]-labeled LL-21 were synthesized.

3.4 Peptide Cleavage

After FMOC-synthesis was completed, the peptide was cleaved from the polymer resin by acidolysis using TFA (trifluoroacetic acid) and scavengers. First the peptide–resin mixture was washed using DMF and methylene chloride, and dried under vacuum for 30 min to 1 hr. The cleavage solution containing 0.5 mL of anisole, 0.5 mL of water, 0.5 mL of thioanisole, one unit of crystal phenol, and 10 mL of trifluoroacetic acid was added to the peptide-bound resin at 0°C in an ice bath. The reaction mixture was then stirred in a small beaker for 2 to 3 hr at room temperature. The dissolved peptide in the solution was then precipitated by adding 50 mL cold diethyl ether and collected by

filtration. The precipitated peptide was then dissolved in 70% acetontrile/water mixture, and an equal amount of distilled water was added. This solution was frozen at –70°C and lyophilized overnight.

3.5 HPLC Purification and Analysis

Reverse phase high performance liquid chromatography was used to purify the peptide. In reverse phase HPLC, compounds are separated based on their hydrophobic nature and eluted when the eluent hydrophobicity increases. In this reverse phase HPLC, 0.1% TFA-water and 0.1% TFA-acetonitrile solutions were mixed with a linear gradient program and run through a C18 column. The HPLC used for the purification is shown in Figure 9.

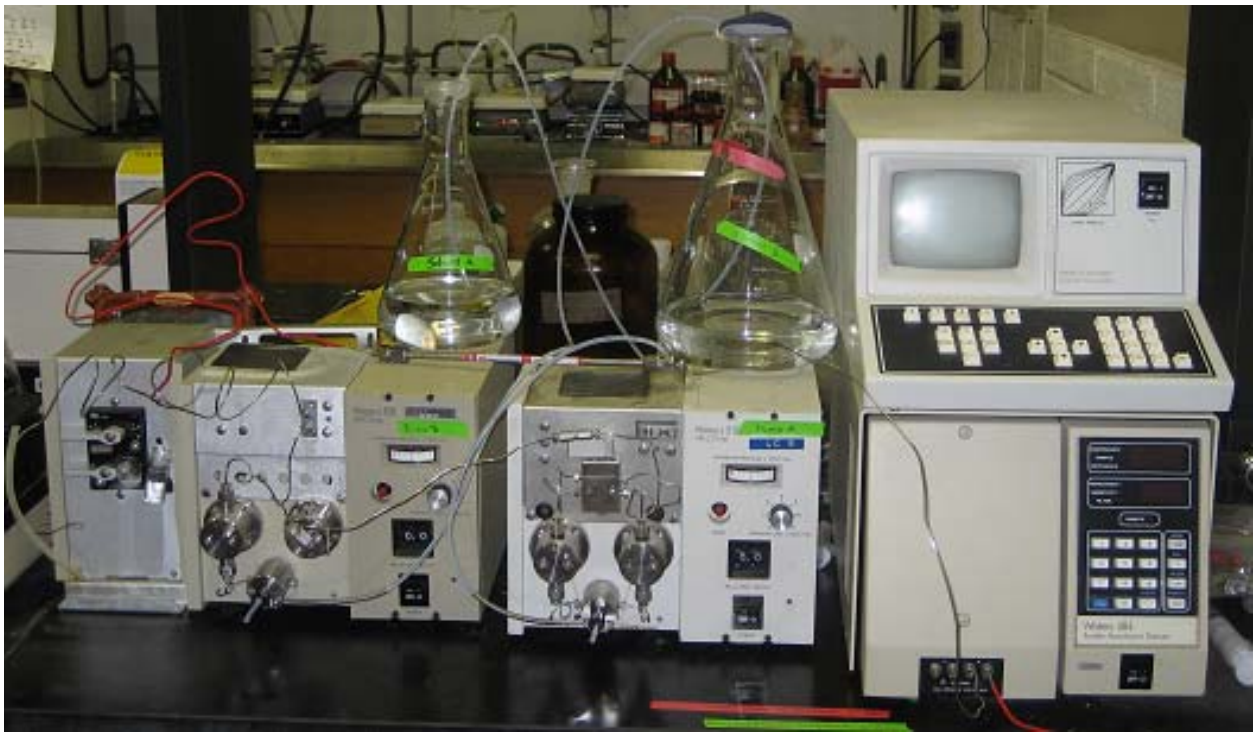

Figure 9. Waters 717 HPLC with 484 UV absorbance detector having Phenomenex Jupiter C18 column used for the separation of pure peptide using 0.1% TFA-water and 0.1% TFA-acetonitrile mobile phases.

3.5.1 *Preparative HPLC*

Preparative HPLC was used to separate the pure peptide from the remaining components. In this preparative HPLC, Waters 501 and 510 pumps and a Waters 484 tunable absorbance detector was used. A Phenomenex Jupiter C18, 10μm, 250 x 21.20 mm, 300A°column was used to separate the peptide from impurities. A mobile phase made of 0.1% TFA-water and 0.1% TFA-acetonitrile solutions were run with gradient programming with a 10 mL/min flow rate. Pure peptide was collected based on major peak absorbance at 254nm and the collected solution was frozen and lyophilized.

3.5.2 *Analytical HPLC*

In analytical HPLC, the peptide was tested for purity by using an analytical column. In this run, a Phenomenex Jupiter 5 μm, C18, 250 x 4.6mm column was used to test the purity of the peptide. The gradient was generally 0 to 66% organic component over 22 minutes, using the same solvent system as the preparative run. Peaks were monitored at 254 nm. Integration of peak areas showed the peptide to be 95% pure.

3.6 Mass spectral analysis

Mass spectrometry was used to confirm the correct weight and purity of the peptide. Matrix associated laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry was used to check the purity of the peptide. These experiments were carried out by Rammamoorthy's group at the University of Michigan.

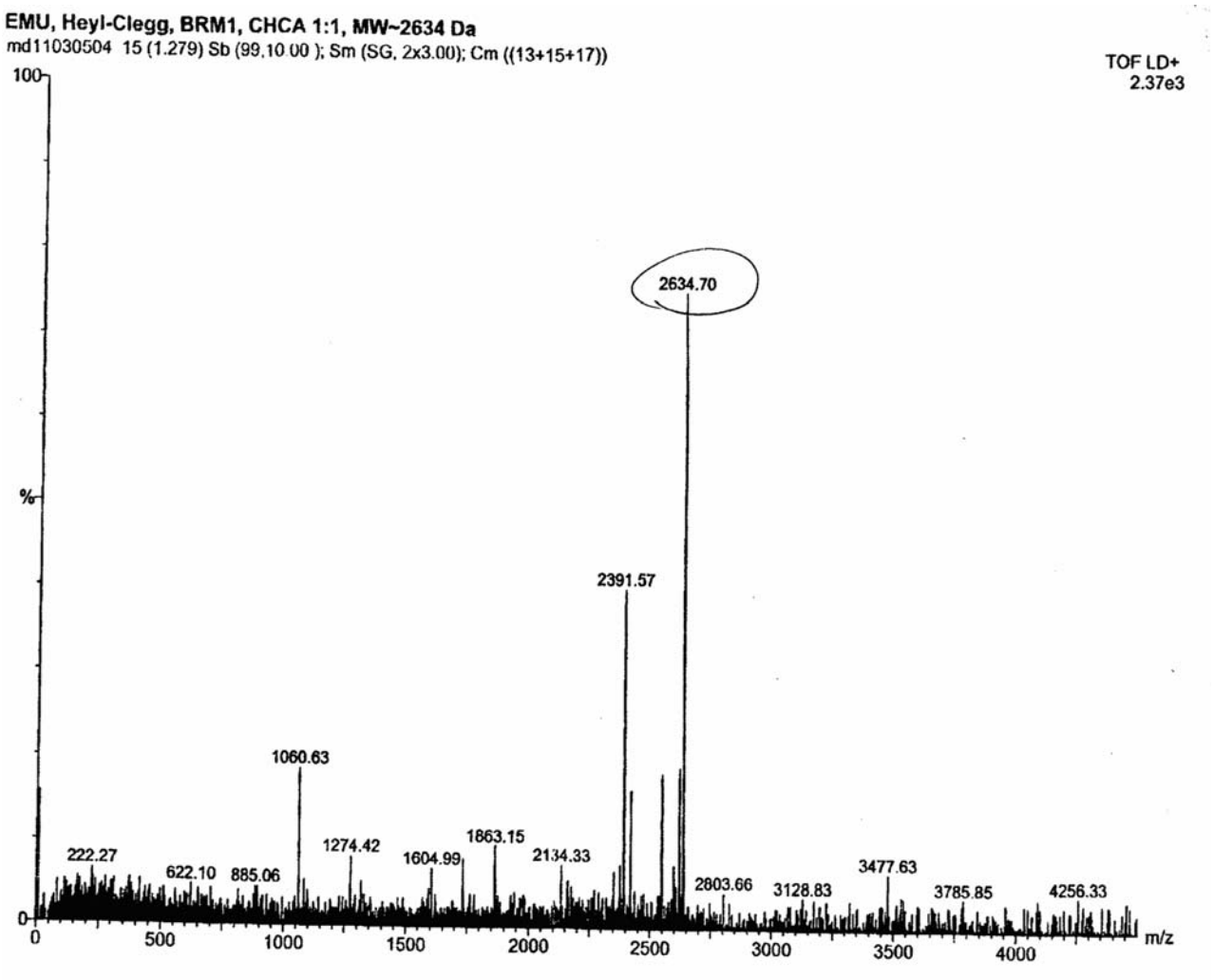

Figure 10. MALDI-TOF mass spectrum of LL-21

From the MALDI-TOF results, shown in Figure 10, the molecular weight of the peptide LL-21 was shown to be 2634.70 Daltons. This matches with the expected molecular weight of the added amino acids, considering the water eliminated for each amide bond formed.

3.7 Antimicrobial assay

For determining antimicrobial activity, minimum inhibitory concentrations (MIC) of LL-21 on various gram positive, gram negative, aerobic and anaerobic bacteria were determined using broth dilution methods. In this method, cultures of bacterial strains were grown in well plates using Todd-Hewitt broth. A series of dilutions of peptide were added to the well plates, and optical density of all well plates was measured after incubating the plates for 24 hrs at 37°C. The protocol is described in more detail elsewhere.[11] This assay was performed in a collaborator's lab.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Antimicrobial activity/Minimum inhibitory concentrations (MIC)

The minimum inhibitory concentration is the lowest concentration of the peptide at which no bacterial growth was observed. The minimum inhibitory concentrations of LL-21 on various gram positive, gram negative, aerobic, and anaerobic bacteria are shown in Table 3.

Table 3. Minimum inhibitory concentration of LL-21 with reference to literature reported values of LL-37 activity on various gram positive, gram negative, aerobic and anaerobic bacteria.[9]

Bacterial Strain	**LL21 Activity (μg/mL)**	**LL37 Activity (μg/mL)**
Porphyromonas gingivalis W83	12.5	< 10
Staphylococcus aureus	50	2.9
Salmonella enterica	50	< 10
Pseudomonas aeruginosa	100	12
S. gordonia	6.25	< 10
Enterococcus feacalis FA2-2	12.5	3.5
Enterococcus feacalis OG1X	50	3.5
Escherichia coli	0.4	0.1
Bacillus subtilis	Enhancer (0.1)	2.7

From these findings it can be stated that LL-21, a fragment of LL-37, also shows good antimicrobial activity against various strains of gram positive and gram negative bacteria except in the case of *Bacillus subtilis*. When treated with different gram positive

and gram negative bacteria, LL-21 effectively kills most of these strains below 50 μg/ml concentration range, so the peptide LL-21 was acting effectively on various gram positive and gram negative strains of bacteria and showing good antimicrobial activity. When compared with the parent LL-37, slightly higher concentrations of LL-21 are required to inhibit bacterial growth for most species. LL-37 kills most of the bacterial strains below 10 μg/ml concentrations, while LL-21 requires up to 50 μg/ml concentrations, so LL-21 is slightly less effective than the parent chain LL-37. However, LL-21 is actually just as effective as LL-37 against *E. coli* since LL-21 needs a concentration of 0.4 μg/ml concentration to kill this strain. So LL-21 was shown to be most effective against the gram negative bacteria *E. coli*.

In the case of *Bacillus subtilis*, LL-21 shows an enhancing effect, i.e., it increases the growth of the bacteria at higher concentrations. LL-37 can effectively kill the strain *Bacillus subtilis* at 2.7 μg/ml concentration, but the peptide LL-21 proved to be ineffective against this strain. Even though *Staphylococcus aureus* is gram positive and *Salmonella enterica* is gram negative, they show similar effects when treated with LL-21 as shown in Figure 11. At concentrations lower than 50 μg/ml, neither bacteria strain was inhibited in growth, and there was a sudden decrease in bacterial growth at 50 μg/ml concentration.

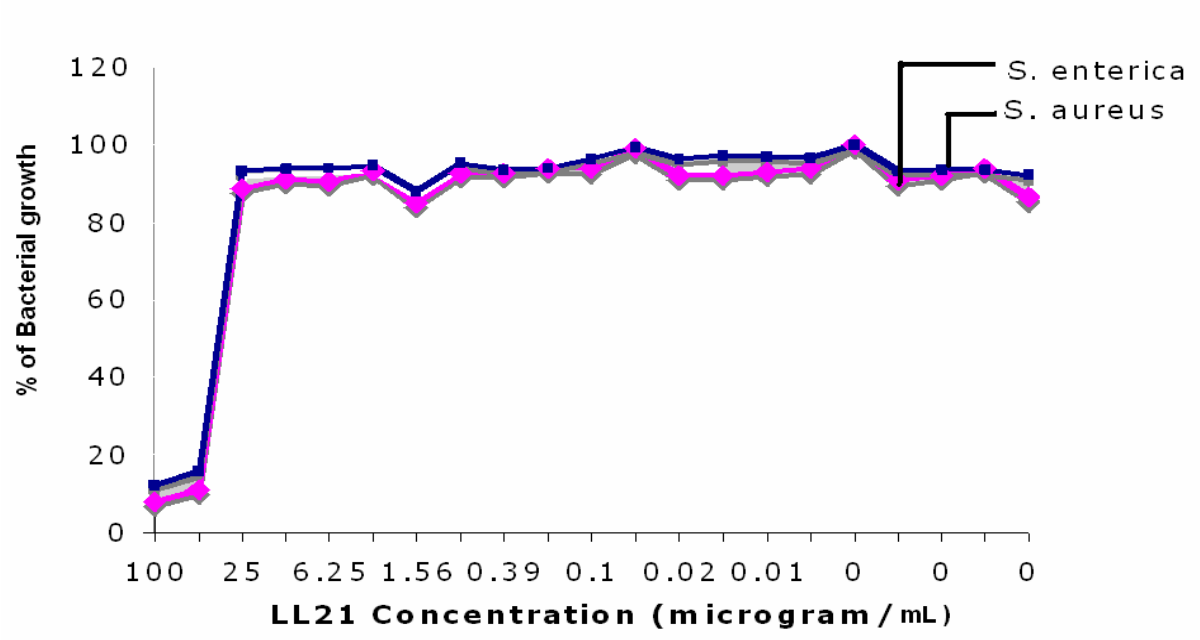

Figure 11. Typical antimicrobial activity comparison of LL-21 when treated with *Staphylococcus aureus,* which is gram positive bacteria, and *Salmonella enterica,* which is gram negative bacteria.

NMR studies that are currently underway will give more information on the orientation of LL-21 in the bacterial lipid bilayer, providing more information on the exact mechanism of action of LL-21.

4.2 NMR Data (provided by Dr. Ullrich Darr, University of Michigan)

The data from solid state NMR experiments show an expectedly broad peak in the 50 to 100 ppm region (Figure 12). This indicates that the peptide LL-21, like LL-37, lies perpendicular to the fatty acid chains of the membrane bilayer. This means that it lies on the surface and most likely works through a toroidal pore or detergent-like mechanism, rather than forming a pore.

It also should be noted that at 5 mole percent and at 35°C (chosen as a temperature at which to observe the peptide in 'physiological' liquid-crystalline lipid phase), spectra show the co-existence of the liquid-crystalline and gel phases. This may

explain why there are two components in the ^{15}N spectra. Also, at both 2 mole percent and 5 mole percent peptide, clearly different ^{15}N chemical shift positions are observed at high temperatures. This may indicate a major re-alignment of the peptide on the surface at high temperature. This effect is being investigated further.

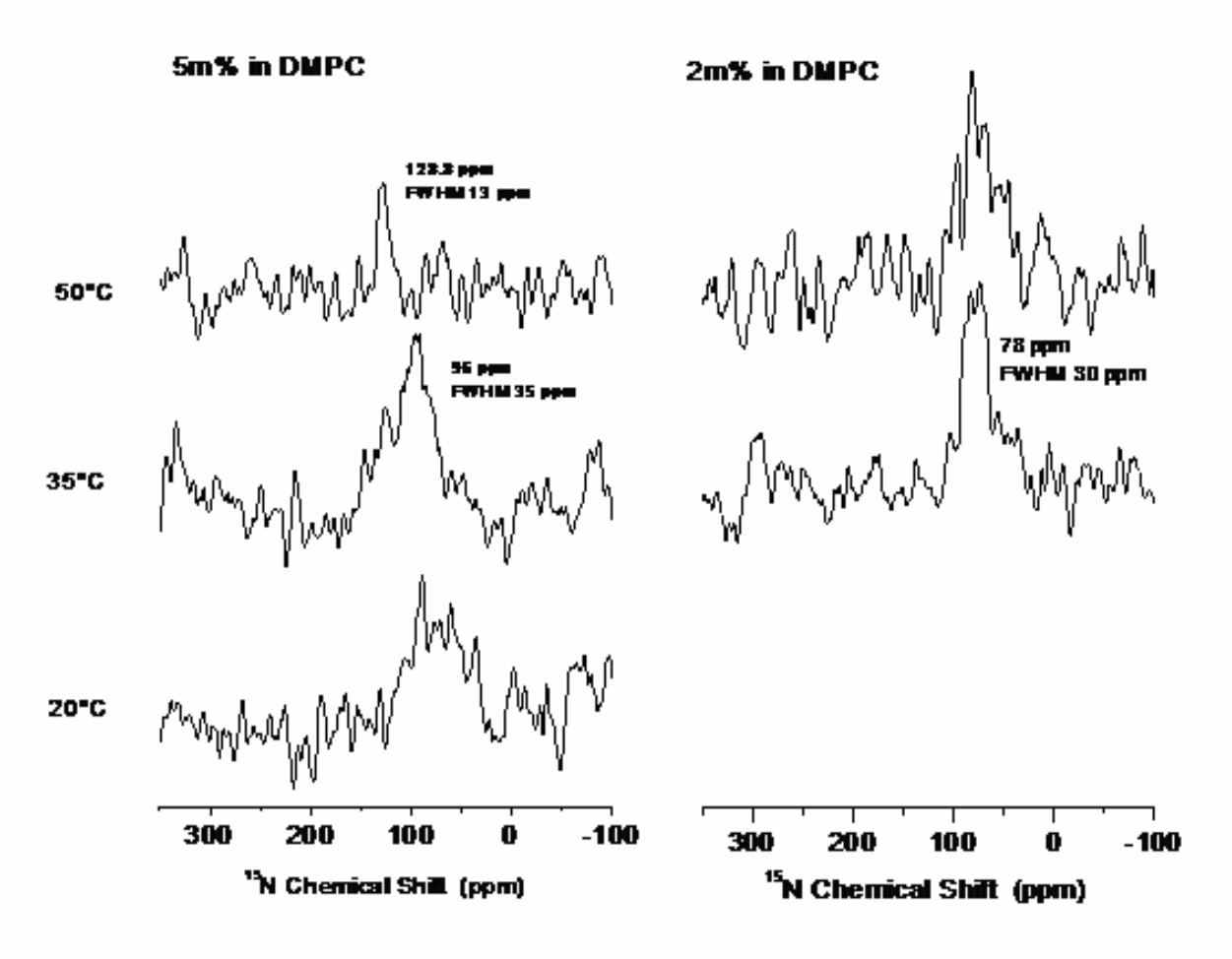

Figure 12. Solid state NMR spectra of LL-21 treated with 2 and 5 mole % of Dimyristoylphosphatidylcholine (DMPC)-based lipid bilayers.

CHAPTER 5

CONCLUSIONS

In conclusion, our new sequence LL-21 shows good antimicrobial activity against most of the gram positive and gram negative bacterial strains and is more effective against gram negative bacteria *E. coli*, when compared with the parent chain LL-37. Slightly higher concentrations of LL-21 are required to inhibit bacterial growth for most species. So LL-21 is slightly less effective than the parent LL-37. However, LL-21 is actually just as effective as LL-37 against *E. coli*. NMR data suggest that LL-21 lies perpendicular to the fatty acid chains of bacterial membrane bilayer and most likely works through a toroidal pore or detergent-like mechanism rather than forming a pore.

This information will be very useful in studies of synergy and biophysical studies of different types of antimicrobial peptides and how they interact with membranes. The data on the antimicrobial activity and mechanism of action of LL-21 will be helpful in developing other short chain analogues from LL-37.

CHAPTER 6

FUTURE WORK

- Introducing 4-^{19}F-Phe label by replacing phenylalanine at the 11th position in LL-21 (Figure 13) to investigate dimerization in the lipid bilayer, which would provide information on orientation and dynamics of the peptide. Dimerization of peptide gives valuable information on the aggregation of peptide, orientation, and the exact mechanism of action.
- Introducing ^{2}H-Ala (deuterium) label by replacing isoleucine at the 7th position in LL-21 (Figure 13) to measure helix rotation (angle of helix with respect to bacterial lipid bilayer).

RKSKEK<u>I</u>GKE<u>F</u>KRIVQRIKDF-amide

Figure 13. The structure of the peptide LL-21.

The aggregation, orientation, and helix rotation of the peptide obtained from the new analogues of LL-21 will be compared with parent chain LL-37, and this information will be very useful for designing short sequence peptides with good antimicrobial peptides, which are economically advantageous.

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