Development of Norspermidine-based Lipopeptide Mimics to Tackle Bacterial Infection

A Thesis

Submitted in partial fulfillment for the degree of

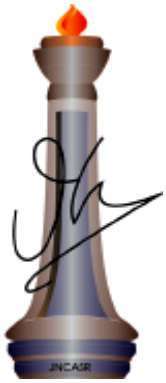
Master of Science

as a part of

Integrated Ph.D Programme (Chemical Science)

By

Mr. Mohini Mohan Konai



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MARCH-2014

Dedicated to My Family

Declaration

I hereby declare that the matter embodied in the thesis entitled "***Development of Norspermidine-based Lipopeptide Mimics to Tackle Bacterial Infection***" is the result of investigations carried out by me at the New Chemistry Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India under the supervision of Dr. Jayanta Haldar and that it has not been submitted elsewhere for the award of any degree or diploma.

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Mr. Mohini Mohan Konai

Certificate

I hereby certify that the matter embodied in this thesis entitled "***Development of Norspermidine-based Lipopeptide Mimics to Tackle Bacterial Infection***" has been carried out by Mr. Mohini Mohan Konai at the New Chemistry Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India under my supervision and that it has not been submitted elsewhere for the award of any degree or diploma.

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Dr. Jayanta Haldar

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Preface

Infectious diseases account for a large number of human deaths every year. This is mainly caused by microorganisms like bacteria, viruses, parasites and so on. Among them bacteria contribute highest to the total number of deaths. Antibiotics continue to save millions of lives, but bacteria are quick to develop resistance against them. Furthermore, there is a continual decrease in the number of antibiotics being approved for clinical use. The problem of bacterial resistance compounded by the declining rate of approval of antibiotics has left many human lives at risk. Throughout the world, various research groups have focused their interest towards the development of antibacterial drugs with novel mechanisms of action. Antimicrobial peptides and their mimics have received a lot of attention in the recent past but clinical success has not been achieved as yet. Lipopeptides and their mimics are another interesting class of compounds which are currently receiving a lot of attention. The problem with these compounds is that they have activity towards a narrow-spectrum of bacteria and thecomplexity of their synthesis makes them expensive. In this thesis we have develop a novel series of lipopeptide mimics following a simple three step synthetic procedure. In the chapter 2 we have described the development of a series of aliphatic norspermidine analogues (**ANAs**) consisting of two charges and a saturated aliphatic group. The efficacy of antibacterial potency and selectivity towards bacterial cells is also subjected in that chapter alongside synthesis and characterization of the compounds. Chapter 3 describes the development of the novel **PANA** derivatives which are conjugated with an additional phenylalanine amino acid. These derivatives portrayed improved antibacterial activity and selective toxicity towards Gram-positive bacteria. Chapter 4 consists of the development of **LANA** derivatives, wherein phenylalanine moieties were replaced by lysine. These derivatives showed broad-spectrum antibacterial activity and were non-toxic to red blood cells.These LANA derivatives were thus an improvement over PANA derivatives and have immense potential as clinically approved antibiotics against multi drug-resistant bacteria.

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Chapter 1

Introduction

1.1 Infectious diseases: global health concern

According to the world health report, published in 2013 by the World Health Organization (WHO), infectious diseases lead to more human deaths than any other disease throughout the world.[1] Infectious diseases are caused by the microorganisms like bacteria, viruses, parasites and so on. It has been found, that in 2011, about 8.2 million (>25% of total) of the total deaths (28.3 million) were directly associated with infectious diseases. Bacterial infections contributed to 6.6 million deaths alone (Figure 1.1).[1] Daszak *et al*. have reported an emergence of 335 infectious diseases between 1940 and 2004 among humans worldwide and the number is increasing day by day.[2]

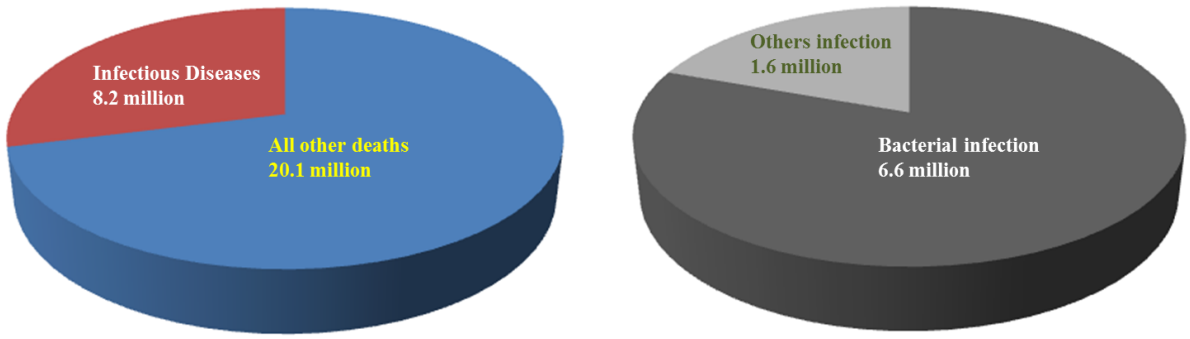


Figure 1.1: Statistics of deaths in 2011 (Figure was generated from reference 1)

Indians face a greater risk of being affected by infectious diseases.[3] Recently, The Global Antibiotic Resistance Partnership (GARP) has reported that India has occupied the highest position in bacteria diseases among the world.[4] *Streptococcus pneumoniae* causes death of 4,10,000 lives each year.[5] Many children die due to this bacterium, especially those from economically impaired families.[6]

Bacteria are responsible for causing various diseases like respiratory infections, chronic diseases (such as gastric ulcers and gastric cancer), tuberculosis, diarrhoea, pneumonia etc. The Gram-positive bacteria *Staphylococcus aureus* is responsible for most of the hospital-acquired bacterial infections. Some other Gram-positive bacteria such as *Enterococcus sp.* and *Streptococcus sp.* and Gram-negative bacteria such as *Klebsiella sp.*, *Escherichia sp.*, *Enterobacter sp.*, *Pseudomonas sp.*; known as 'nosocomial bacteria', are also responsible for hospital associated infections.[7] The condition of sepsis in patients is mainly a result of Gram-negative bacterial infection. *Pseudomonas aeruginosa* is the major contributor towards sepsis.

This bacterium also plays a crucial role in cystic fibrosis and cancer.[7]

With the advent of easily transferable New Delhi Metallo-β-lactamase 1 (NDM-1 gene), resistance to β-lactams is rampant.[8-9] Only tigecycline and colistin remain as active drugs against this strain of bacteria. Thus development of novel antibacterial agents against this kind of bacteria is imperative.

1.2 Antibiotics and bacterial resistance

1.2.1 Major classes of antibiotics

Antibiotics are the drugs approved for treatment of bacterial infections. Antibiotics have saved millions of lives since the discovery of penicillin by Alexander Fleming. Later, many antibiotics have been discovered and developed to treat the bacterial infections.[10-14] Mainly two different types of strategies have been followed, one involves isolation of antibiotics from natural sources and the other is based on chemical synthetic approaches designed by the human mind. Among the naturally occurring antibiotics, β-lactams were introduced for clinical use in 1941, followed by aminoglycosides in 1944. In the decade that followed, novel branches of antibiotics entered in the pipeline in order to tackle bacterial infection. Tetracyclines came in 1950, macrolides in 1952 followed by glycopeptides (vancomycin) in 1956. Alongside, man-made synthetic antibiotics like sulphonamide and quinolones were also launched in 1935 and 1962 respectively.

During the period of 1940-1960, most of the new classes of antibiotics were approved for clinical application. After this 'golden era' of discovery of antibiotics, not a single effective class of antibiotic has entered the antibiotic pipeline until 2000.[13] This gap period, known as 'innovation gap' in the antibiotics history has only aggravated the problem. Recently, daptomycin was approved by the Food and Drug Administration (FDA) in 2003[15] and the first oxazolidinone antibiotic, linezolid was approved in 2000. Very recently, another two antibiotics, fidaxomicin and bedaquiline were approved in 2011and 2012, respectively.[16] The antibiotic pipeline is illustrated in Figure 1.2.

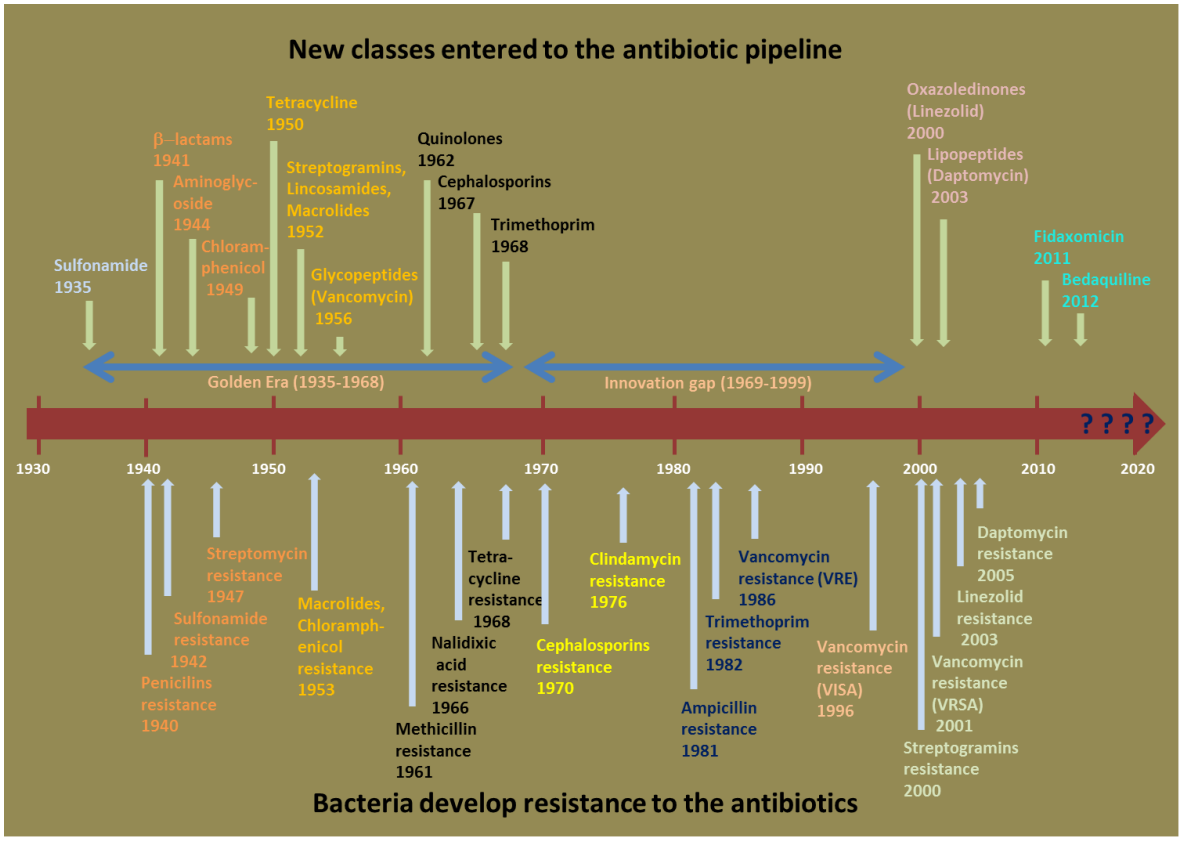


Figure 1.2: Antibiotic pipeline and development of bacterial resistance. (Figure was adopted from reference 13)

1.2.2 Antibiotics and their targets

Most antibiotics target a specific site in bacteria, and act by either inhibiting bacterial growth (bacteriostatic) or direct killing (bactericidal) mechanism. Five major validated target sites in bacteria (Figure 1.3) have been discovered.[17] β-lactams and glycopeptides act by targeting bacterial cell wall biosynthesis.[13] Ribosomes are primarily targeted by the aminoglycosides, macrolides, chloramphenicols and oxazolidinones classes of antibiotics. Nucleic acids such as DNA and RNA are also targeted by various antibiotics such as fluoroquinolones and rifamycins.[18] Another target of antibiotics is the pathway of bacterial folic acid synthesis.[19] Sulfonamides class of antibiotics are one such class, which stop folic acid production in bacteria.

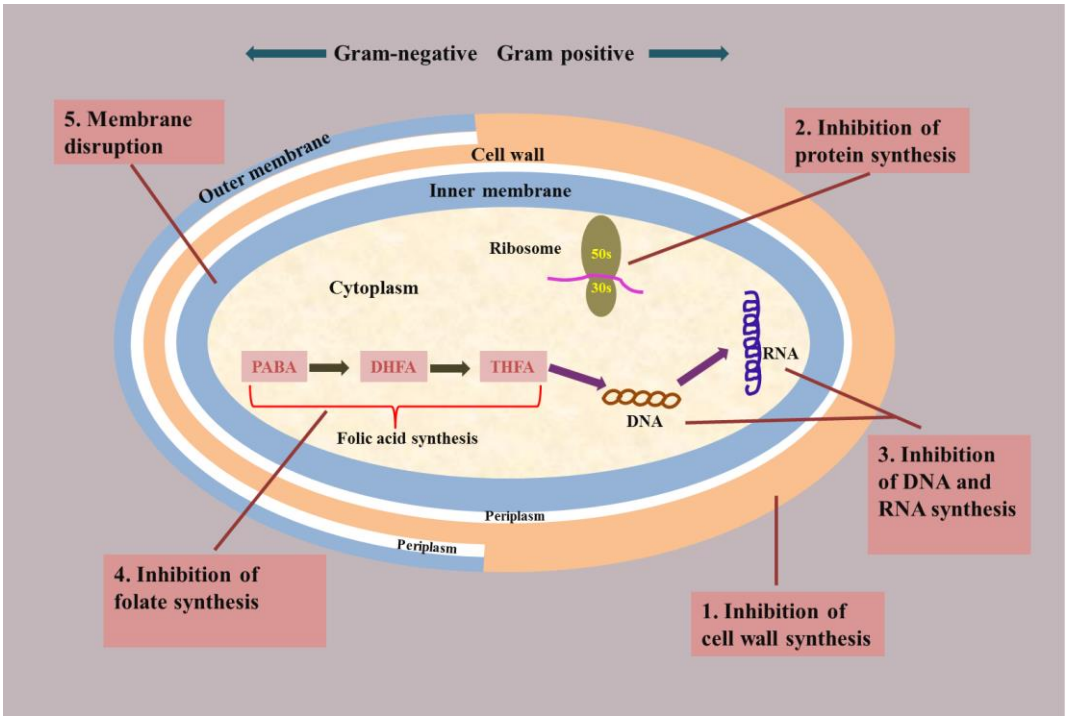


Figure 1.3: Five major targeted sites of antibiotic. (Figure was adopted from reference 13)

Some natural products such as antimicrobial peptides and lipopeptides act by targeting the bacterial cell membrane.[20-21] Daptomycin, approved in 2003, is a lipopeptide antibiotic whose mechanism of action involves disruption of bacterial cell membrane integrity.

1.2.3 Bacterial resistance to antibiotics

Although the first generation of antibiotics were called "wonder drugs", with time, bacteria have developed resistance against almost all classes of antibiotics. There are several strategies by which bacteria develop resistance (Figure 1.4); first involves targeting the antibiotics themselves. Bacteria produce enzymes for example β-lactamases that degrade the β-lactam (penicillin) class of antibiotics and render them ineffective.[22] Bacteria develop resistance to chloramphenicol and aminoglycosides by producing enzymes that can alter their chemical structure and make them inactive.[22]

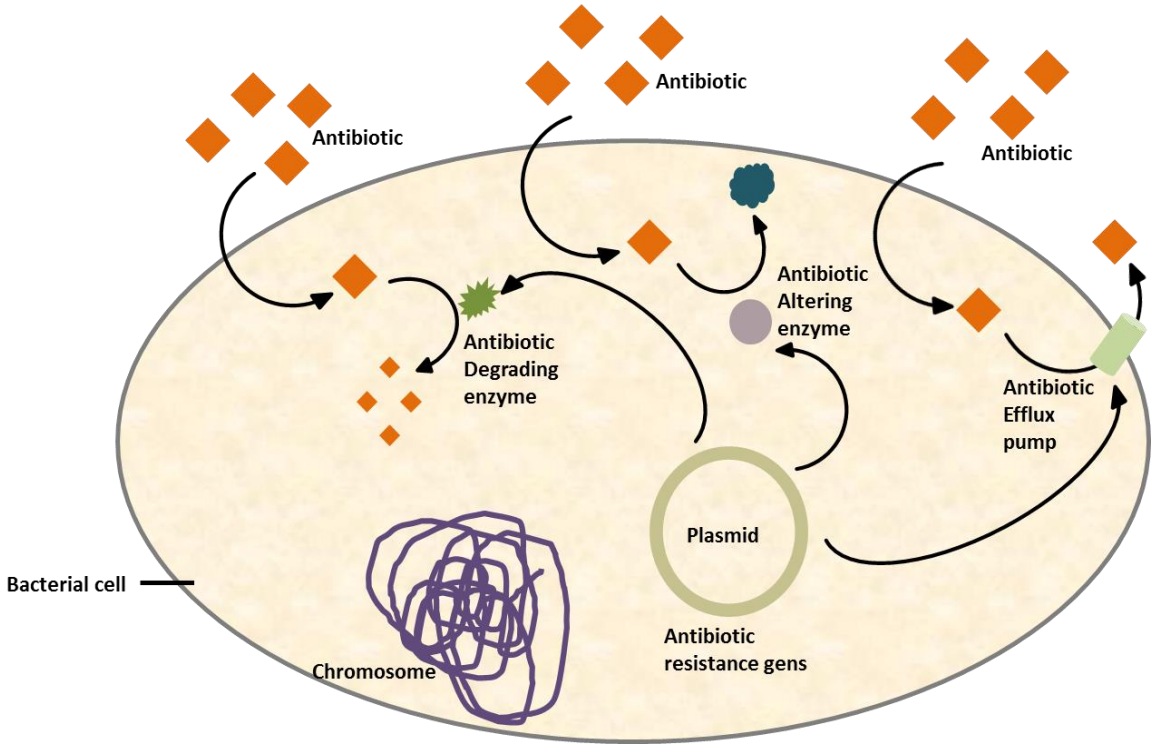


Figure 1.4: Mechanism of resistance. (Figure was adopted from reference 22)

Another strategy used by bacteria to develop resistance involves pumping out the antibiotics using efflux pumps.[23-24] Bacteria develop resistance to the tetracyclines, chloramphenicol and the fluoroquinolones using efflux pumps. Another strategy (not shown in Figure 1.4) involves the modification of drug binding site. Antibiotics such belonging to the class of glycopeptides (vancomycin), macrolides (azithromycin) lose potency as a result of modified target site.

In the history of antibiotics, bacteria start developing resistance towards an antibiotic within a couple of months or years of their approval (Figure 1.2).[25-26] Once a bacterium gets resistant, it starts transferring the mutated genes to the other bacteria; thus a chain reaction is initiated which leads to worldwide spread of bacterial resistance.[22] The first report of resistance to penicillin was documented in 1940; but presently almost all bacteria have developed resistance against this antibiotic. Methicillin, an example of later generation of β-lactam antibiotics, was introduced in the clinics in 1959, but methicillin resistant *Staphylococcus aureus* (MRSA) arose in 1961. This particular bacterium was untreatable by all the existing antibiotics except vancomycin. Consequently, between 1970 and 1980, MRSA associated diseases have resulted in uncountable numbers of deaths around the world. At present the

situation is even worse, as MRSA has developed resistance against the antibiotic of last resort, vancomycin. Vancomycin resistant *Enterococcus faceium* (VRE) was reported in 1986 followed by vancomycin intermediate resistant *Staphylococcus aureus* (VISA) and vancomycin resistant *Staphylococcus aureus* (VRSA) in 1996 and 2001, respectively. At last when the antibiotic pipeline was almost about to dry out, linezolid and daptomycin were launched in the year 2000 and 2003 respectively. However, bacteria had developed resistance to linezolid and daptomycin in 2003 and 2005 respectively.

Recently two antibiotics fidaxomicin and bedaquiline have been approved by FDA in 2011 and 2012, respectively, but these are not enough to fight against all kinds of bacterial infection. The problem associated with traditional antibiotics lies with the fact that they have specific targets for carrying out their antibacterial action. Consequently, simple mutation of that target site, or production of antibiotic degrading enzymes is easy and energetically inexpensive strategies for bacteria to follow. Hence, some alternative strategy towards the design of antibiotics needs to be developed to tackle this impending problem of bacterial resistance. Recently, the focus has been shifted from traditional antibiotics towards antimicrobial peptides (AMPs) and lipopeptides. As these peptides primarily target the bacterial cell membrane, the development of resistance towards them is energetically very expensive for bacteria. Thus natural antimicrobial peptides, lipopeptides and their mimics provide a novel approach towards tackling bacterial resistance.

1.3 Antimicrobial peptides and peptidomimetics

Antimicrobial peptides (AMPs) are small protein molecules (less than 50 amino acid residues in length) ubiquitously distributed throughout the plant and animal kingdom as part of their innate defence system.[27-28] The important characteristic features include the presence of cationic residues and facial amphiphilicity which brings about selective toxicity towards microorganisms over mammalian cells.[28] Optimal hydrophobic/hydrophilic balance also allows them to adopt an amphipathic structure which enables efficient insertion into bacterial membrane (Figure 1.5).[28] Upon insertion several of the AMPs assemble together to form pores through the cell membrane, which ultimately leads to death of the microorganism .[29-31]

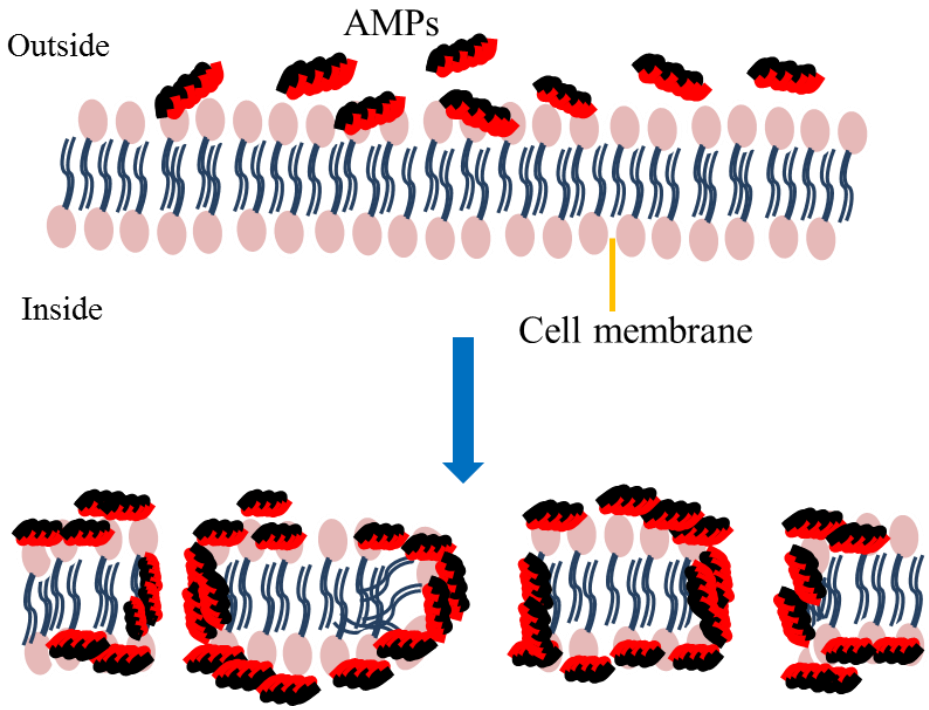


Figure 1.5: Mechanism of action of AMPs. (Figure was adopted from the reference 28)

Although AMPs have several advantages over traditional antibiotics, such as broad spectrum of activity, inability of bacteria to develop resistance against them,[29] they suffer from inherent problems. The clinical uses of AMPs are limited by their in vivo toxicity, propensity towards proteolytic degradation and difficulty in large scale manufacture.[27, 32-34] However, since bacterial resistance to traditional antibiotics has created an urgent need to develop alternative antibacterial agents, and various research groups have directed their interest towards natural AMPs and their synthetic mimics as future therapeutic agents.

Several synthetic AMPs such as Pexiganan acetate, Omiganan, Iseganan are undergoing Phase III clinical trials as topical antibiotics.[35] However, these synthetic AMPs are not completely devoid of the problems associated with natural AMPs. Thus, synthetic designs which maintain the antimicrobial properties of these peptides but effectively circumvent the problems associated with them are desirable. Several groups around the world have dedicated their research towards the development of such antimicrobial peptidomimetics. These peptidomimetic approaches involve subtle variations in the structure, albeit improve on

potency, stability and toxicity of AMPs. Several examples of successful synthetic mimics of AMPs (SMAMPs) exist in the literature.

In the first example of its kind, Gellman *et al.* have reported β-peptides as mimics to address the stability which lacks in natural AMPs.[36] Recently, they have shown that these poly-β-peptides (based on Nylon-3 polymers) are effective against both bacteria and fungi. DeGrado *et al.* over the years have developed several SMAMPs including β-peptides, amphiphilic polymethacrylate derivatives and arylamide foldamers.[37-40] The most successful amongst them are the arylamide foldamers which are undergoing Phase II clinical trials. Mor *et al.* have designed oligo acyl lysines which show potent *in-vitro* selective activity towards bacteria.[41] Barron *et al*. have designed very efficient antibacterial peptoids which bear much promise as SMAMPs.[42] Ceragenins reported by Savage *et al*., is another successful SMAMP, which bears immense promise and has a variety of applications.[43] Tew *et al.* have made generations of aromatic ring based successful SMAMPs which are not only promising antibacterials agents but also have immunomodulatory properties.[44] Yang *et al*. have been developing several antimicrobial materials which mimic the properties of AMPs.[45] The tripeptides created by Svendsen *et al.* showed excellent potency *in-vitro* and one of the compounds LTX109 is undergoing clinical trials.[46-47] Recently we have also reported a series of peptoid mimics with broad-spectrum antibacterial activity.[48] Thus peptidomimetic approaches towards development of antibacterial agents bear significant advantages and bear immense potential as clinically successful candidates.

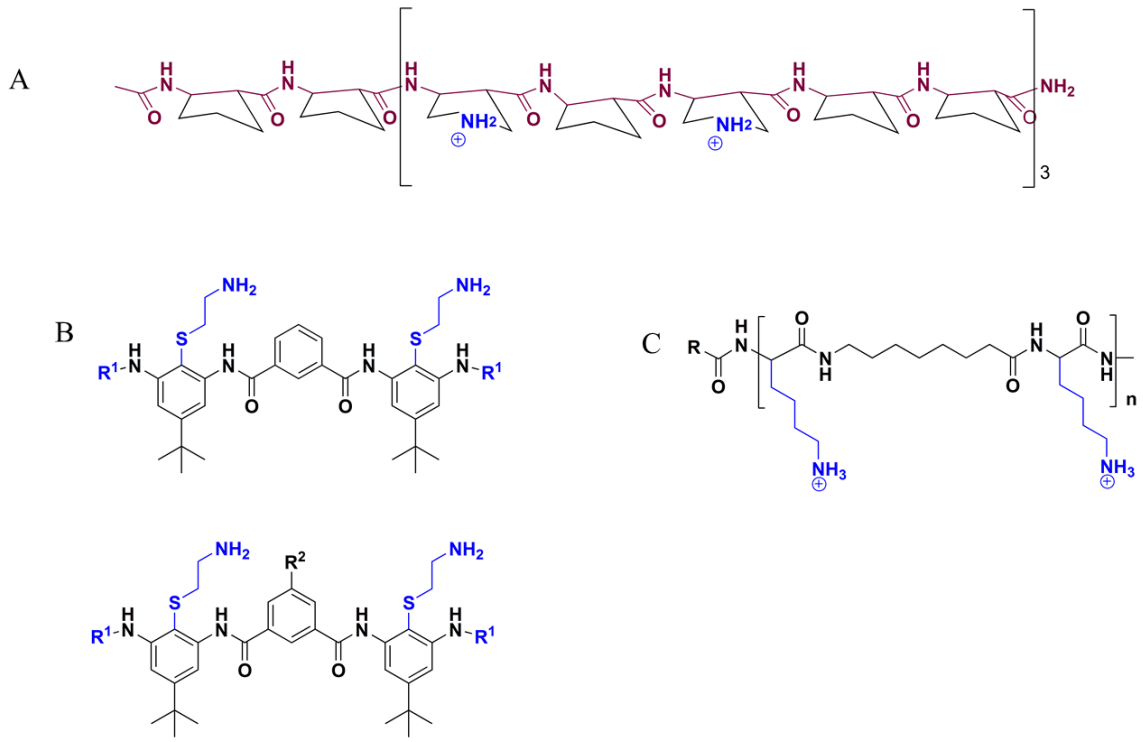


Figure 1.6: Examples of AMPs mimics. A) β-peptides reported by Gellman *et al.*, B) Arylamide foldamers reported by DeGrado *et al.*, C) Oligo acyl lysines reported by Mor *et al.*

1.4 Antibacterial lipopeptides and their mimics

Like AMPs, another class of amphipathic peptides are present in living organisms; these are known as lipopeptides which are characterized to have a net cationic/anionic charge along with lipophilic aliphatic chain.[49-50] The aliphatic tail in the lipopeptides plays a crucial role towards its activity by facilitating bacterial membrane interaction. Lipopeptides also show a promising direction towards development of novel antibacterial agents.

1.4.1 Natural lipopeptide antibiotics

Naturally occurring lipopeptide antibiotics include both the cationic and anionic lipopeptides. The cationic class such as polymixins were isolated from the bacterium *Bacillus polymyxa* in 1940 (Figure 1.7).[51] They have an aliphatic group conjugated to a cyclic peptide core and mainly act by binding to the anionic lipopolysaccharide (component of outer membrane of

Gram-negative bacteria). They are known to displace the membrane balancing cations like calcium (Ca^{+2}) and magnesium (Mg^{+2}), displayed selective activity towards Gram-negative bacteria.[52] Two representatives of this class, polymixin B and colistin became available for clinical use in 1960, however the uses were limited due to toxicity.[53-54] Several strategies have been developed to reduce toxicity of polymixins. Among them, sulfomethylation of free amino groups of colistin resulted in colistin methanesulfonate (CMS) with dramatically reduced toxicity albeit with reduction of *in-vitro* activity.[50] However, as multi-drug resistant Gram-negative bacterial infections became increasingly untreatable with the available arsenal of antibiotics, the toxicity issue of colistin was revisited and a better safety profile has been observed.[55] Currently, it is being used as the last resort for Gram-negative bacterial infections including those caused by the NDM-1 producing bacteria.

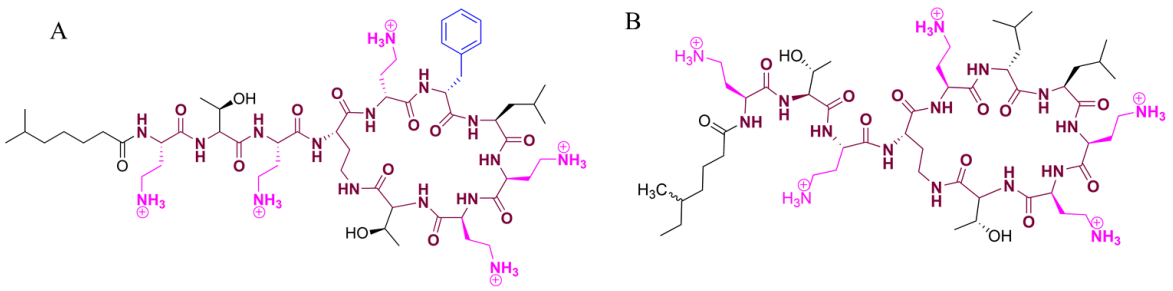


Figure 1.7: A) Structure of polymixin B. B) Structure of colistin (polymixin E).

In the anionic class, the representative compound daptomycin, was discovered in 1980. This compound is nonribosomally synthesised by *Streptomyces roseosporus*.[56] Like polymixins, daptomycin consists of a cyclic peptide core with an aliphatic group appended to it. However, it bears an overall negative charge.[50] The peptide core is composed of 13 amino acids, where 10 residues actually build the cyclic frame and the remaining three residues extend out from the cyclic part through which the aliphatic group is attached (Figure 1.8). The peptide sequence included some of non-natural amino acids also such as ornithine, D-alanine, D-serine, L-threo-3-methylglutamic acid etc. Daptomycin has a decyl chain coupled to the peptide sequence.[57] At neutral pH condition, it has a net negative charge contributed by the carboxylic acid group of amino acid residues whereas the hydrophobicity is contributed by the alkyl chain and the hydrophobic amino acid residues.[50] As a result of this amphipathic behaviour it can

form aggregates (micelles) in aqueous environment at a higher concentration of 2 mM. This property of aggregation formation is crucial for daptomycin to exhibit antibacterial activity.

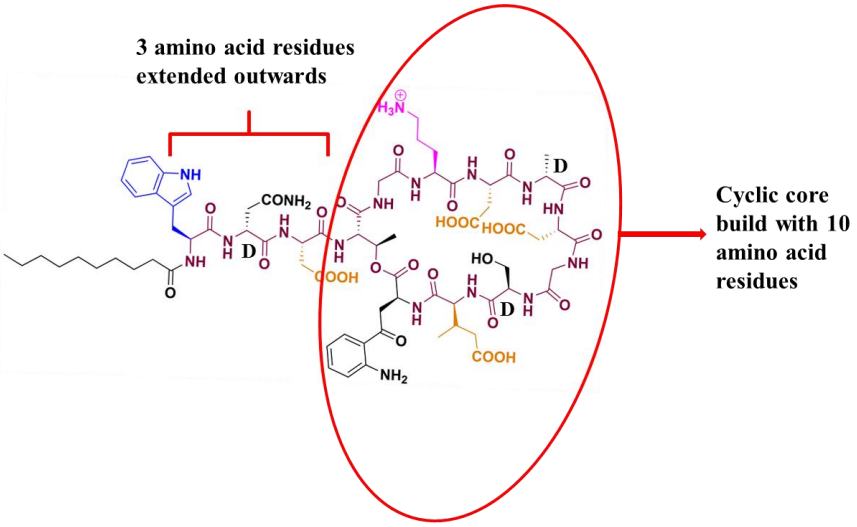


Figure 1.8: Structure of daptomycin.

It is proposed that daptomycin interacts with the bacteria through insertion of aliphatic tail into the bacterial membrane. This results in rapid membrane depolarization and potassium ion efflux which leads to cell death.[58-64] There are three hypothetical steps which are involved in the mechanism of action of daptomycin (Figure 1.9).[62] First, it gets inserted to the cytoplasmic membrane in a calcium-dependent manner; secondly it undergoes oligomerization followed by disruption of the membrane in the third step. This results in the release of intracellular ions (K^+) and rapid cell death ultimately. FDA has approved daptomycin for treatment of complicated skin and skin structure infections in 2003.[15] Unfortunately, bacterial resistance against daptomycin has already been reported.[65]

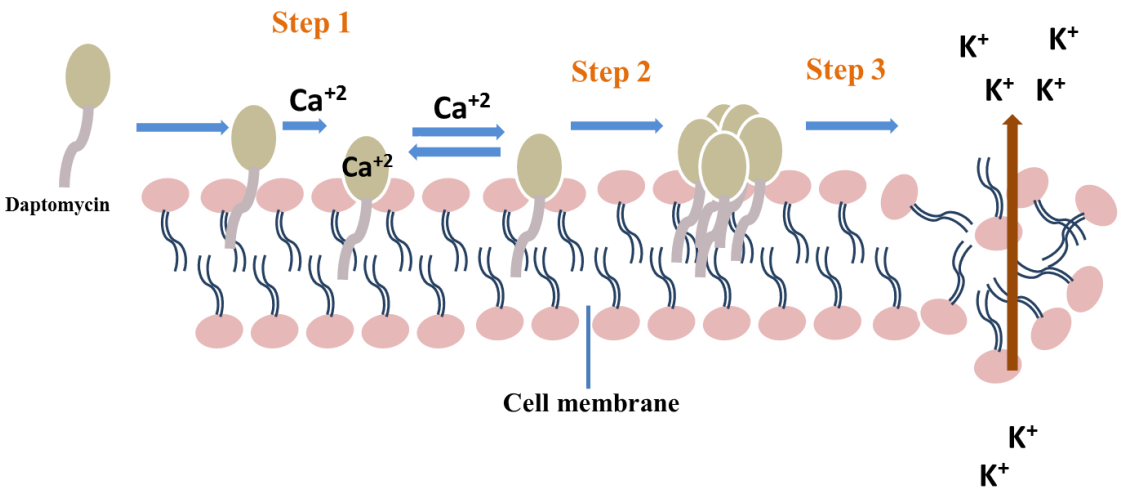


Figure 1.9: Mechanism of action of daptomycin. (Figure was adopted from reference 62)

Another important class of lipopeptides are echinocandins, which were first identified in 1974 as potent antifungal agents.[66] Echinocandins consist of a cyclic peptide core with an N-acyl fatty acid chain which is either branched or unbranched.[50, 67] The chain length varies from 14 to 18 carbons. In some examples, the lipophilic group can also consist of multiple aromatic rings. It has been observed that both the cyclic core and the pendant lipophilic group are important for activity. The spectrum of activity of echinocandins is limited to fungi especially *Candida sp.* and *Aspergillus sp.*[67-69] The mechanism of action of Echinocandins involves inhibiting the activity of enzyme 1-3-β-D-glucan synthase, which disrupts the synthesis of 1-3-β-D-glucan (a critical component of the fungal cell wall)[70] disruption of cell wall integrity followed by osmotic imbalance results in fungicidal activity. Three compounds belonging to this class of lipopeptides have already been approved; namely, Capsofungin, micafungin and anidulafungin. [67].

1.4.2 Synthetic lipopeptides and mimics of lipopeptides

Although natural liopeptide antibiotics found to be promising drug candidates for bacterial infections, they suffer from several disadvantages; firstly they display a narrow spectrum of antibacterial activity. For example, daptomycin is only active against Gram-positive bacteria;[15, 71] polymixin (polymixin B and colistin) is active only toward Gram-negative bacteria.[72-74]

Further limitations of lipopeptides mainly lie in their *in-vivo* toxicity, structural complexity, difficult synthesis and high cost of manufacture. Hence, various research groups have focused their interest to develop synthetic lipopeptides and lipopeptide mimics in order to address the disadvantages associated with natural lipopeptide antibiotics.

HB1345, is a synthetic lipopeptide, which is undergoing clinical trials as an agent for the treatment of skin infections.[75] This compound falls under a category known as lipohexapeptides. These compounds possess broad spectrum antibacterial activity and also show immune modulatory, angiogenesis and wound healing properties. These synthetic lipopeptides show low emergence of bacterial resistance [50]

Synthetic mimics of lipopeptides have also been attempted and some of the significant examples are covered below. Shai *et al.* have reported a family of ultrashort lipopeptides with potent antibacterial activity against a variety of bacterial strains.[76] These were synthesised by conjugating aliphatic groups of different chain lengths to a sequence of short tetrapeptides with various D/L amino acid residues (Figure 1.10A). The potency of antibacterial activity of these lipopeptides was governed not only by the nature of amino acid residues in sequence but also the length of the aliphatic groups. It was later reported that conjugation of palmitic acid to very short cationic dipeptides or tripeptides improved the antimicrobial activity.[77] The most potent activity was achieved when all the amino acid residues in the tripeptide sequence were lysine. It was interesting that they did not observe any deleterious effect in the antibacterial activity of the corresponding lipopeptides upon replacing an L-amino acid with its D-isomer.

David *et al.* have reported a series of spermine-based mono and bis-acyl polyamines with various aliphatic chain lengths.[78] From the structure-activity relationship (SAR) it was observed that these acyl polyamines displayed a strong dependence on the acyl chain length for antimicrobial efficacy.

A series of lipopeptides were reported by Gilmore *et al.* by conjugation of various saturated aliphatic groups to the N-terminal of the tetrapeptide sequence H-Orn-Orn-Trp-Trp-NH_2, these lipopeptides exhibited excellent antimicrobial activity over a broad-spectrum of bacterial strains.[79] The lipopeptide consisting of C-12 aliphatic group (Figure 1.10C) displayed potent antibacterial activity. The lipopeptide C_{12}-Orn-Orn-Trp-Trp-NH_2 exhibited MIC values in the range of 0.95-7.81 μg⁄mL against various bacteria.

Recently, Cai *et al.* have reported a series of lipidated γ-AApeptides which displayed potent and broad-spectrum antibacterial activity against various Gram-positive and Gram-negative bacteria.[80] The most potent derivative consisted of a C-16 satutrated aliphatic group (Figure 1.10D) and displayed MIC values in the range of 2.5-5 µg/mL. Structure-activity relationship studies revealed that the introduction of unsaturated aliphatic group (derivatives having oleyl group instead of C-16 chain) significantly reduced the toxicity towards human erythrocytes (RBCs) without losing the antibacterial activity. After γ-AApeptides series, a series of lipidated α-AApeptides as lipopeptide mimics were also reported that showed broad-spectrum antibacterial activity comparable to the clinical candidate, pexiganan.[81]

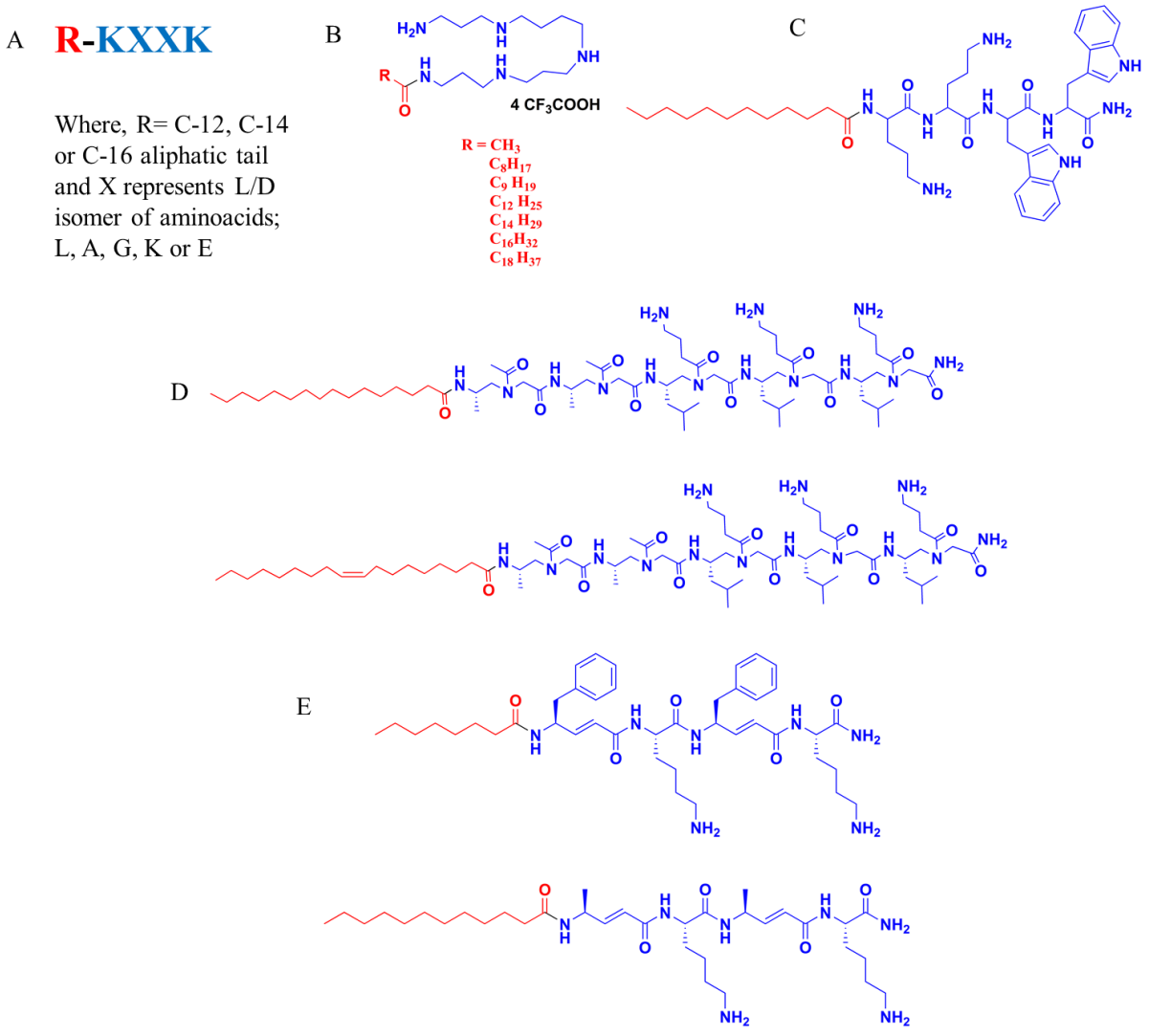


Figure 1.10 Examples of lipopeptide mimics. A) Ultrashort lipopeptides reported by Shai *et al*., B) Spermine-based mono acyl polyamines by David *et al.* C) Lipopeptide reported by Gilmore *et al.,* D) Lipidated γ-AApeptides reported by Cai *et al.,* E) Lipopeptide reported by by Gopi *et al.*

Recently, Gopi *et al.* have described a series of lipopeptides consist of alternating α-and α, β-unsaturated γ-amino acids (E-vinylogous amino acids) residues in 1:1 ratio (Figure 1.10E).[82] These compounds have displayed broad spectrum antimicrobial property. These compounds also could self-assemble and form nanostructures. Upon detailed investigation it was found that these self-assembled nanostructures displayed a significant role in both antimicrobial and haemolytic activities.

1.5. Scope of the thesis

Presently, development of bacterial resistance to conventional antibiotics is one of the most pressing problems of global health.[22, 25-26, 83] This is a matter of major concern in medicine as there has been a steady decline in the rate of development of new potent antibacterial drugs. Hence, the primary focus in the field of infectious diseases research has been directed towards development of new potent antibacterial drugs. As mentioned earlier, various research groups have focused their interest towards antimicrobial peptides (AMPs) and their synthetic mimics. AMPs are advantageous over conventional antibiotics because they act by causing lysis of cell membrane which prevents bacteria from developing resistance against them. But, unfortunately no AMP has been approved for clinical use due to several disadvantages as mentioned earlier. Parallel to AMPs, another class of amphipathic peptides, i.e. lipopeptides and mimics of lipopeptides were developed in order to address such unmet goal. But, again these too have associated limitations such as narrow-spectrum antibacterial activity. For example, daptomycin is active only against Gram-positive bacteria and polymixin (polymixin B and colistin) is effective only toward Gram-negative bacteria. However, FDA has approved daptomycin for treatment of complicated skin infections. But unfortunately resistance to daptomycin has already been observed. Initially the use of polymixins was limited by its toxicity but recently it has been reintroduced as a drug of last resort for Gram-negative bacterial infections. Several lipopeptides mimics have also been developed with increased antibacterial activity and

selectivity. But, limitation of these lipopeptide mimics lie in their synthetic design. The synthesis of previously reported lipopeptide mimics mainly involved the solid-phase peptide synthesis which increases the cost of synthesis. The aim of this thesis is to develop a novel series of lipopeptide mimics based on simple three step synthetic procedure. Chapter 2 describes a series of aliphatic norspermidine analogues (**ANAs**) with two charges and a saturated aliphatic tail. Evaluation of antibacterial activity and selectivity towards bacterial cells over human erythrocytes (RBCs) is also subjected in that chapter. In chapter 3, novel **PANA** derivatives, wherein additional phenylalanine residues were conjugated to the norspermidine backbone in order to address the limitation of **ANA** derivatives. These **PANA** derivatives exhibited higher antibacterial activity and improved selectivity compared to **ANA** derivatives. Finally chapter 4 introduces the rationality of increasing the number of charges from two to four. This leads to a discovery of novel series of lipopeptide mimics, **LANA** wherein the phenylalanine residues were replaced by lysine residues. The derivatives displayed activity against both Gram-positive and Gram-negative bacteria including the clinically isolated multi-drug resistant (MDR) strains. The lipopeptide mimics were found to be highly selective towards bacterial cells compared to human erythrocytes. These uniquely designed mimics of lipopeptides can be developed as clinically usable antibacterial agents to tackle bacterial infections.

Chapter 2

Synthesis and Antibacterial Properties of Aliphatic Norspermidine Analogues

Abstract

This chapter describes synthesis, characterization and antibacterial activity of a series of aliphatic cationic norspermidine derivatives as lipopeptide mimics. Antibacterial efficacy has been investigated against various Gram-positive (*Staphylococcus aureus* and *Enterococcus faecium*) and Gram-negative (*Escherichia coli*) bacteria including drug-resistant bacteria methicillin-resistant *Staphylococcus aureus* (MRSA), vacomycin-resistant *Enterococcus faecium* (VRE) and β-lactam resistant *Klebsiella pneumoniae*. The compounds were synthesised from three simple steps. The sequence of reactions consist of selective Boc protection of primary amine group of norspermidine, then incorporation of aliphatic groups of various lengths through a tertiary amide bond and finally deprotection of Boc groups using trifluoroacetic acid (TFA). The most potent compound **ANA4** with the hexadecyl aliphatic chain found to be active against VRE at a MIC value of 4 μM, while vancomycin did not display any potency even at very high concentration of 500 μM. Compound **ANA4** also found to be selectively active against bacterial cell compared to human erythrocytes (RBCs). With respect to the drug resistant bacteria VRE, compound **ANA4** exhibited a selectivity ratio (HC_{50} / MIC) of 22.

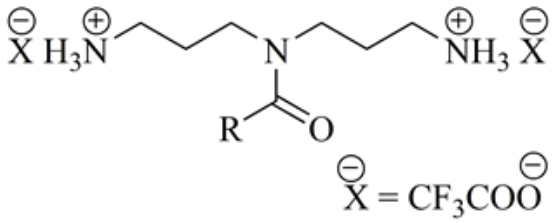
2.1 Introduction

Lipopeptides are a class of amphipathic peptides with a net cationic/anionic charge and lipophilic aliphatic tail.[49-50, 84] The aliphatic tail of the lipopeptides plays a crucial role towards its activity owing to hydrophobic interactions with the bacterial cell membrane. Lipopeptides generally show antimicrobial activity towards narrow spectra of microorganisms. For example, daptomycin, a naturally occurring lipopetide, is active only against Gram-positive bacteria; [15, 71] the polymixins (polymixin B and colistin), lipopeptides approved for topical use, are active only towards Gram-negative bacteria, [72-74] and echinocandins function only as antifungal drugs.[67-69] Limitations of lipopeptides mainly lie in their *in-vivo* toxicity, structural and synthetic complexity, high cost of manufacture and so on. Hence, chemists and biologist have focused their research towards the development of strategies which can counter the aforementioned problems of lipopeptides. Initial efforts were directed towards appending an alkyl chain to the naturally occurring cationic peptides.[85-91] Later synthetic chemists tried to create non-natural lipopeptides with improved antimicrobial activities.[41, 92-98] In an exemplary work, Shai *et al.* made a series of compounds wherein long alkyl chains were appended to a tetrapeptide sequence of naturally occurring amino acids.[99] These compounds were found to have potent antibacterial activity. However, preparation of these kinds of compounds required use of solid-phase peptide synthesis which increases the cost of synthesis. In another example, David *et al.* have developed a series of simple lipopolyamines, in which peptides were replaced by a spermine moiety.[78] In all these studies, the minimum number of positive charges required for antibacterial activity were three.[100] We sought to develop simplest mimics of lipopeptides by incorporating only two charges in the design and by addition of a single aliphatic long chain. The synthesis, characterization, antibacterial properties and toxicity of these cationic amphiphilies built on a norspermidine backbone is the subject of this chapter.

2.2 Structure and Design

This series of compounds were designed on the premise that a single aliphatic chain and a minimum of two cationic charges were necessary for obtaining lipopeptide mimics having potent antibacterial activity. Through this design, it was also envisaged to obtain selective toxicity against bacterial cells over human erythrocytes. To design the molecules we chose

to have backbone which would serve the following purposes. Firstly, provide an abiotic platform for the entire molecule. Secondly, it contains at least two positive charges. Finally, it has functional groups which would provide easy reaction centre for synthetic modification. We chose norspermidine because it is not expensive and is easily available. Furthermore there are three reactive amine groups for functionalization and the primary amines can itself provide cationic charges in physiological conditions. The secondary amine was considered to be an interesting centre for modification as it not only provides symmetry to the compounds but also allows the two positive charges (contributed by the two primary amine groups) to be separated by a distance of 8 bonds. Also coupling of this centre to a long aliphatic acid would not only produce an abiotic tertiary amide bond, but also provide a point of attachment of the lipophilic moiety (Figure 2.1). In order to ascertain the optimum lipophilicity required for obtaining a selective but potent antibacterial agent, hydrophobicity was varied through incorporation of different lengths of the aliphatic chains.



R= -C_9H_{19} **ANA1**
-$C_{11}H_{23}$ **ANA2**
-$C_{13}H_{27}$ **ANA3**
-$C_{15}H_3$ **ANA4**
-$C_{17}H_{35}$ **ANA5**

Figure 2.1: Structure of aliphatic norspermidine analogues (**ANAs**).

2.3 Synthesis and Characterization

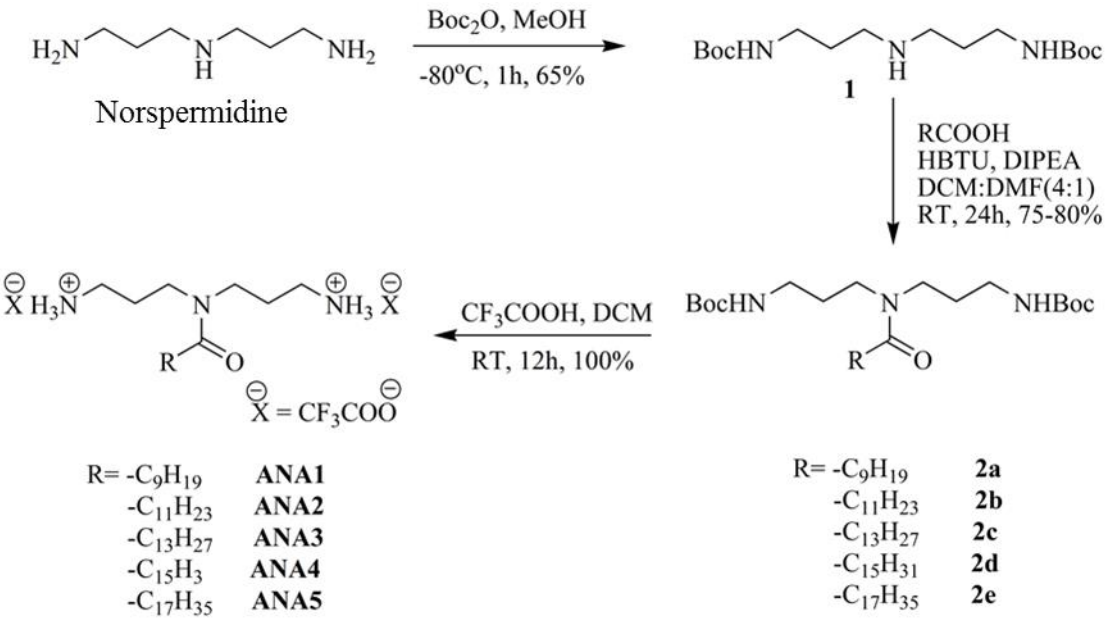
2.3.1 Materials and Methods

All the solvents were of reagent grade, which were distilled and dried before its uses. All the reagents were purchased from Sigma-Aldrich, Alfa-Aesar, S.D. Fine, Avra and

Spectrochem, were used without further purification. Analytical thin layer chromatography (TLC) was performed on E. Merck TLC plates pre-coated with silica gel 60 F_{254} and visualization was carried out using UV light and Iodine. Column chromatography was performed on silica gel (60-120 mesh) using different ratio of chloroform and methanol solvent system. Nuclear magnetic resonance spectra were recorded on Bruker (AV-400) 400 MHz spectrometer in deuterated solvents. Mass spectra were obtained using 6538-UHD Accurate mass Q-TOF LC-MS instrument. Infrared (IR) spectra of the compounds (in Chloroform or Methanol) were recorded on Bruker IFS66 V/s spectrometer using NaCl crystal. For optical density measurement Tecan InfinitePro series M200 Microplate Reader was used. Bacterial strains, *S. aureus* (MTCC 737) and *E. coli* (MTCC 443) were purchased from MTCC (Chandigarh, India). Methicillin-resistant *S. aureus* (ATCC 33591), *Enterococcus faecium* (ATCC 19634), vancomycin-resistant *Enterococcus faecium* (ATCC 51559) and *Klebsiella pneumoniae* (ATCC 700603) were obtained from ATCC (Rockville, MD, USA).

2.3.2 Reaction Scheme

Synthesis of the compounds was achieved in three steps (Scheme 2.1). First, the primary amine groups of norspermidine were selectively Boc protected. This was achieved by carrying out the reaction at -80ºC using Boc_2O for 1 h. Then an aliphatic chain was introduced through HBTU coupling at the secondary amine of Boc-protected norspermidine by reacting with various aliphatic acids. Finally, deprotection of Boc groups with trifluoroacetic acid (TFA) yielded the final compounds (**ANA1**-ANA**5**).



Scheme 2.1: Synthesis of aliphatic norspermidine analogues (**ANAs**).

2.3.3 Synthetic protocol and Characterization

Procedure for synthesizing N^1-Boc-N^3-(3-(Boc-amino)propyl)propane-1,3-diamine (1): 10 gm (1 equivalent) of norspermidine was dissolved in MeOH (50 mL) and the solution was kept at -80ºC. Then 24.94 gm (1.5 equivalents) of Boc_2O was dissolved in MeOH (50 mL) and added to the reaction mixture drop wise. The reaction was continued for 1 h at -80ºC. Then the reaction mixture was allowed to come at RT. MeOH was removed and purification was done through column chromatography on silica gel (60-120 mess) using methanol and chloroform (7:93) as eluent to afford the product with 65% yield. FT-IR (NaCl): 3344 cm^{-1} (-NH- str.), 2974 cm^{-1} (-CH_2- asym. str.), 2871 cm^{-1} (-CH_2- sym. str.), 1696 cm^{-1} (C=O str.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.182 (s, NH(-CH_2-CH_2-CH_2-*NH*Boc)$_2$, 2H), 3.190-3.175 (m, NH(-*C*H_2-CH_2-CH_2-NHBoc)$_2$, 4H), 2.649-2.616 (m, NH(-CH_2-CH_2-*C*H_2-NHBoc)$_2$, 4H), 1.860 (s, *NH*(-CH_2-CH_2-CH_2-NHBoc)$_2$, 1H), 1.666-1.602 (m, NH(-CH_2-*C*H_2-CH_2-NH-Boc)$_2$, 4H), 1.417 (s, NH(-CH_2-CH_2-CH_2-NH-COO-C(*C*H_3)$_3$)$_2$, 18H).

General procedure for synthesizing 2a- 2e: About 15 mmol (1.5 equivalents) of saturated aliphatic acid (decanoic, dodecanoic, tetradecanoic, hexadecanoic or octadecanoic) were dissolved in dry DCM (12 mL) at 0ºC. In the reaction mixture 4 equivalents of DIPEA was added followed by 1.5 equivalents of HBTU. Then DMF (3 mL) was added to the reaction mixture. After 10 minutes, 1 equivalent of **1** in dry DCM (1 mL) was added drop wise. The reaction mixture was brought to RT and allowed to stir for 24 h. Solvent was evaporated and residue was diluted in ethyl acetate (50 mL). Then work-up was carried out at first with 1N HCl (50 mL, 3 times) followed by saturated Na_2CO_3 solution (50 mL, 3 times). The crude product was extracted in ethyl acetate layer. Finally purification was accomplished through column chromatography on silica gel (60-120 mess) using methanol and chloroform (5:95) as eluent to afford **2a-2e** with 75-80% yield.

***N,N*-bis(3-(Boc-amino)propyl)decanamide (2a):** Yield-78%**;** FT-IR (NaCl): 3340 cm^{-1} (-NH- str.), 2929 cm^{-1} (-CH_2- asym. str.), 2860 cm^{-1} (-CH_2- sym. str.), 1705 (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.388-4.707 (d, R-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>Boc)$_2$, 2H), 3.386-3.016 (m, R-CO-N(-*C*<u>*H*$_2$</u>-CH_2-*C*<u>*H*$_2$</u>-NHBoc)$_2$, 8H), 2.293-2.248 (t, CH_3-$(CH_2)_6$-CH_2-*C*<u>*H*$_2$</u>- of R group, 2H), 1.760-1.611 (m, CH_3-$(CH_2)_6$-*C*<u>*H*$_2$</u>-CH_2-CO-N(-CH_2-*C*<u>*H*$_2$</u>-CH_2-NHBoc)$_2$, 6H), 1.408 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-COO-C*(C*<u>*H*$_3$</u>*)*$_3$)$_2$, 18H), 1.241 (bs, CH_3-*(C*<u>*H*$_2$</u>*)*$_6$-CH_2-CH_2- of R group, 12H), 0.871-0.837 (t, *C*<u>*H*$_3$</u>-$(CH_2)_6$-CH_2-CH_2- of R group, 3H).

***N,N*-bis(3-(Boc-amino)propyl)dodecanamide (2b):** Yield-80%; FT-IR (NaCl): 3288 cm^{-1} (-NH- str.), 2928 cm^{-1} (-CH_2- asym. str.), 2859 cm^{-1} (-CH_2- sym. str.), 1699 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.379-4.629 (d, R-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>Boc)$_2$, 2H), 3.400-3.029 (m, R-CO-N(-*C*<u>*H*$_2$</u>-CH_2-*C*<u>*H*$_2$</u>-NHBoc)$_2$, 8H), 2.296-2.258 (t, CH_3-$(CH_2)_8$-CH_2-*C*<u>*H*$_2$</u>- of R group,, 2H), 1.772-1.734-1.594 (m, CH_3-$(CH_2)_8$-*C*<u>*H*$_2$</u>-CH_2-CO-N(-CH_2-*C*<u>*H*$_2$</u>-CH_2-NHBoc)$_2$, 6H), 1.422 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-COO-C*(C*<u>*H*$_3$</u>*)*$_3$)$_2$, 18H), 1.249 (bs, CH_3-*(C*<u>*H*$_2$</u>*)*$_8$-CH_2-CH_2- of R group, 16H), 0.887-0.853 (t, *C*<u>*H*$_3$</u>-$(CH_2)_8$-CH_2-CH_2- of R group, 3H).

***N,N*-bis(3-(Boc-amino)propyl)tetradecanamide (2c):** Yield-75%; FT-IR (NaCl): 3337 cm^{-1} (-NH- str.), 2926 cm^{-1} (-CH_2- asym. str.), 2852 cm^{-1} (-CH_2- sym. str.), 1693 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.377-4.643 (d, R-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>Boc)$_2$, 2H), 3.400-3.030 (m, R-CO-N(-*C*<u>*H*$_2$</u>-CH_2-*C*<u>*H*$_2$</u>-NHBoc)$_2$, 8H), 2.299-2.261 (t, CH_3-$(CH_2)_{10}$-CH_2-*C*<u>*H*$_2$</u>- of R group, 2H), 1.774-1.592 (m, CH_3-$(CH_2)_{10}$-*C*<u>*H*$_2$</u>-CH_2-CO-N(-CH_2-*C*<u>*H*$_2$</u>-CH_2-NHBoc)$_2$, 6H), 1.422 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-COO-C*(C*<u>*H*$_3$</u>*)*$_3$)$_2$,

18H), 1.246 (bs, CH_3-*(C<u>H</u>$_2$)$_{10}$*-CH_2-CH_2- of R group, 20H), 0.887-0.853 (t, *C<u>H</u>$_3$*-$(CH_2)_{10}$-CH_2-CH_2- of R group, 3H).

***N,N*-bis(3-(Boc-amino)propyl)hexadecanamide (2d):** Yield-76%; FT-IR (NaCl): 3337 cm^{-1} (-NH- str.), 2922 cm^{-1} (-CH_2- asym. str.), 2854 cm^{-1} (-CH_2- sym. str.), 1699 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.377-4.643 (d, R-CO-N(-CH_2-CH_2-CH_2-*N<u>H</u>*Boc)$_2$, 2H), 3.400-3.030 (m, R-CO-N(-*C<u>H</u>$_2$*-CH_2-*C<u>H</u>$_2$*-NH-Boc)$_2$, 8H), 2.299-2.261 (t, CH_3-$(CH_2)_{12}$-CH_2-*C<u>H</u>$_2$*- of R group, 2H), 1.774-1.592 (m, CH_3-$(CH_2)_{12}$-*C<u>H</u>$_2$*-CH_2-CON(-CH_2-*C<u>H</u>$_2$*-CH_2-NH-Boc)$_2$, 6H), 1.422 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-COO-C*(C<u>H</u>$_3$)$_3$*)$_2$, 18H), 1.246 (bs, CH_3-*(C<u>H</u>$_2$)$_{12}$*-CH_2-CH_2- of R group, 24H), 0.887-0.853 (t, *C<u>H</u>$_3$*-$(CH_2)_{12}$-CH_2-CH_2- of R group, 3H).

***N,N*-bis(3-(Boc-amino)propyl)octadecanamide (2e):** Yield-78%; FT-IR (NaCl): 3340 cm^{-1} (-NH- str.), 2929 cm^{-1} (-CH_2- asym. str.), 2854 cm^{-1} (-CH_2- sym. str.), 1708 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.376-4.628 (d, R-CO-N(-CH_2-CH_2-CH_2-*N<u>H</u>*Boc)$_2$, 2H), 3.400-3.029 (m, R-CO-N(-*C<u>H</u>$_2$*-CH_2-*C<u>H</u>$_2$*-NHBoc)$_2$, 8H), 2.299-2.260 (t, CH_3-$(CH_2)_{14}$-CH_2-*C<u>H</u>$_2$*- of R group, 2H), 1.773-1.592 (m, CH_3-$(CH_2)_{14}$-*C<u>H</u>$_2$*-CH_2-CO-N(-CH_2-*C<u>H</u>$_2$*-CH_2-NHBoc)$_2$, 6H), 1.421 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-COO-C*(C<u>H</u>$_3$)$_3$*)$_2$, 18H), 1.243 (bs, CH_3-*(C<u>H</u>$_2$)$_{14}$*-CH_2-CH_2- of R group, 28H), 0.889-0.852 (t, *C<u>H</u>$_3$*-$(CH_2)_{14}$-CH_2-CH_2- of R group, 3H).

General procedure for synthesizing ANA1-ANA5: Aabout 7.5 mmol (1 equivalent) of **2a-2e** was dissolved in DCM (3 mL). To the intensely stirred solution 4 equivalents (excess amount) of trifluoroacetic acid (TFA) was added and allowed to stirring at RT for 12 h. Then solvent and unused TFA were removed to afford pure **ANA1-ANA5** with 100% yield.

***N,N*-bis((3-amino)propyl)decanamide bis(trifluoroacetate) (ANA1):** FT-IR (NaCl): 3367 cm^{-1}(-NH- str.), 2929 cm^{-1} (-CH_2- asym. str.), 2866 cm^{-1} (-CH_2- sym. str.), 1671 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 7.971-7.828 (d, R-CO-N(-CH_2-CH_2-CH_2-*N<u>H</u>*$_3^+$)$_2$, 6H), 3.326-3.267 (m, R-CO-N(-CH_2-CH_2-*C<u>H</u>$_2$*-NH_3^+)$_2$, 4H), 2.832-2.709 (m, R-CON(-*C<u>H</u>$_2$*-CH_2-CH_2-NH_3^+)$_2$, 4H), 2.297-2.260 (t, CH_3-$(CH_2)_6$-CH_2-*C<u>H</u>$_2$*- of R group, 2H), 1.804-1.486 (m, CH_3-$(CH_2)_6$-*C<u>H</u>$_2$*-CH_2-CO-N(-CH_2-*C<u>H</u>$_2$*-CH_2-NH_3^+)$_2$,, 6H), 1.242 (bs, CH_3-*(C<u>H</u>$_2$)$_6$*-CH_2-CH_2- of R group, 12H), 0.868-0.835 (t, *C<u>H</u>$_3$*-$(CH_2)_6$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 286.2880 [$(M+H)^+$] (Observed), 286.2858 [$(M+H)^+$] (Calculated).

***N,N*-bis((3-amino)propyl)dodecanamide bis(trifluoroacetate) (ANA2):** FT-IR (NaCl): 3375 cm^{-1} (-NH- str.), 2928 cm^{-1} (-CH_2- asym. str.), 2861 cm^{-1} (-CH_2- sym. str.), 1681 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 7.932-7.797 (d, R-CO-N(-CH_2-CH_2-CH_2-$N\underline{H_3}^+$)$_2$, 6H), 3.326-3.268 (m, R-CO-N(-CH_2-CH_2-$C\underline{H_2}$-NH_3^+)$_2$, 4H), 2.832-2.688 (m, R-CO-N(-$C\underline{H_2}$-CH_2-CH_2-NH_3^+)$_2$, 4H), 2.299-2.262 (t, CH_3-$(CH_2)_8$-CH_2-$C\underline{H_2}$- of R group, 2H), 1.820-1.469 (m, CH_3-$(CH_2)_8$-$C\underline{H_2}$-CH_2-CO-N(-CH_2-$C\underline{H_2}$-CH_2-NH_3^+)$_2$, 6H), 1.242 (bs, CH_3-$(C\underline{H_2})_8$-CH_2-CH_2- of R group, 16H), 0.871-0.837 (t, $C\underline{H_3}$-$(CH_2)_8$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 314.3172 [$(M+H)^+$] (Observed), 314.5297 [$(M+H)^+$] (Calculated).

***N,N*-bis((3-amino)propyl)tetradecanamide bis(trifluoroacetate) (ANA3):** FT-IR (NaCl): 3394 cm^{-1} (-NH- str.), 2922 cm^{-1} (-CH_2- asym. str.), 2869 cm^{-1} (-CH_2- sym. str.), 1680 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 7.946-7.940 (d, R-CO-N(-CH_2-CH_2-CH_2-$N\underline{H_3}^+$)$_2$, 6H), 3.324-2.685 (m, R-CO-N(-$C\underline{H_2}$-CH_2-$C\underline{H_2}$-NH_3^+)$_2$, 8H), 2.296-2.259 (t, CH_3-$(CH_2)_{10}$-CH_2-$C\underline{H_2}$- of R group, 2H), 1.800-1.473 (m, CH_3-$(CH_2)_{10}$-$C\underline{H_2}$-CH_2-CO-N(-CH_2-$C\underline{H_2}$-CH_2-NH_3^+)$_2$, 6H), 1.234 (bs, CH_3-$(C\underline{H_2})_{10}$-CH_2-CH_2- of R group, 20H), 0.865-0.831 (t, $C\underline{H_3}$-$(CH_2)_{10}$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 342.3505 [$(M+H)^+$] (Observed), 342.5829 [$(M+H)^+$] (Calculated).

***N,N*-bis((3-amino)propyl)hexadecanamide bis(trifluoroacetate) (ANA4):** FT-IR (NaCl): 3403 cm^{-1} (-NH- str.), 2924 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1680 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 7.923-7.912 (d, R-CO-N(-CH_2-CH_2-CH_2-$N\underline{H_3}^+$)$_2$, 6H), (m, R-CO-N(-$C\underline{H_2}$-CH_2-$C\underline{H_2}$-NH_3^+)$_2$, 8H), 2.293-2.252 (t, CH_3-$(CH_2)_{12}$-CH_2-$C\underline{H_2}$- of R group, 2H), 1.795-1.465 (m, CH_3-$(CH_2)_{12}$-$C\underline{H_2}$-CH_2-CO-N(-CH_2-$C\underline{H_2}$-CH_2-NH_3^+)$_2$, 6H), 1.231 (bs, CH_3-$(CH_2)_{12}$-CH_2-CH_2- of R group, 24H), 0.867-0.832 (t, $C\underline{H_3}$-$(CH_2)_{12}$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 370.3787 [$(M+H)^+$] (Observed), 370.6360 [$(M+H)^+$] (Calculated).

***N,N*-bis((3-amino)propyl)octadecanamide bis(trifluoroacetate) (ANA5):** FT-IR (NaCl): 3382 cm^{-1} (-NH- str.), 2928 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1679 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 7.897-7.773 (d, R-CO-N(-CH_2-CH_2-CH_2-$N\underline{H_3}^+$)$_2$, 6H), 3.321-2.706 (m, R-CO-N(-$C\underline{H_2}$-CH_2-$C\underline{H_2}$-NH_3^+)$_2$, 8H), 2.295-2.258 (t, CH_3-$(CH_2)_{14}$-CH_2-$C\underline{H_2}$- of R group, 2H), 1.817-1.482 (m, CH_3-$(CH_2)_{14}$-$C\underline{H_2}$-CH_2-CO-N(-CH_2-$C\underline{H_2}$-CH_2-NH_3^+)$_2$, 6H), 1.227 (bs, CH_3-$(C\underline{H_2})_{14}$-CH_2-CH_2- of R group, 28H), 0.859-0.825 (t, $C\underline{H_3}$-$(CH_2)_{14}$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 398.4083 [$(M+H)^+$] (Observed), 398.6892 [$(M+H)^+$] (Calculated).

2.4 Experimental procedure

2.4.1 Antibacterial assay

The antibacterial activity of the compounds is reported as their Minimum Inhibitory Concentrations (MIC), which is the lowest concentration of the antibacterial agent required to inhibit the growth of microorganism after overnight incubation. A glycopetide antibiotic, vancomycin and a lipopetide, colistin were also used in this study to compare the result. All synthesized compounds (**ANA1-ANA5**) were assayed in a micro-dilution broth format as described in CLSI guideline.[101] The bacterial freeze dried stock samples were stored at -80ºC. About 5 µL of these stocks were added to 3 mL of the respective broth and the culture was grown for 6 h at 37ºC with prior to the experiments.[102-103] This 6 h grown culture gives about 10^9 cfu/mL in case of *S. aureus* (MTCC 737), MRSA (ATCC 33591), and 10^8 cfu/mL in case of *E. coli* (MTCC 443), *Enterococcus faecium* (ATCC 19634), VRE and *Klebsiella pneumoniaea* (ATCC 700603) which were determined by spread plating method. This 6 h grown culture was diluted to give effective cell concentration of 10^5 cfu/mL which was then used for determining MIC. Compounds were serially diluted, in sterile millipore water (as 2 fold manner) and 50 µL of these serial dilutions were added to the wells of 96 well plate followed by the addition of about 150 µL of bacterial solution. The plates were then incubated for 24 h at 37ºC. The O. D. value at 600 nm was recorded using TECAN (Infinite series, M200 pro) Plate Reader. Each concentration had triplicate values and the whole experiment was done at least twice and the MIC value was determined by taking the average of triplicate O. D. values for each concentration and plotting it against concentration. The data was then subjected to sigmoidal fitting. From the curve the MIC value was determined, as the point where the O. D. was similar to that of control having no bacteria.

2.4.2 Hemolytic assay

Compounds were serially diluted in millipore water and 50 µL of these serial dilutions were added to the wells of 96 well plates. Human erythrocytes were centrifuge dawn from the fresh heparinised blood and suspended to 5 vol% in PBS (pH 7.4). In the compound containing 96 well plates, 150 µl of the erythrocyte suspension was added.[102-103] Two

controls were made, one without compound as negative control and the other as a positive control by addition with 50 µl of 1 vol% solution of Triton X-100 instead of compound. After that the plate was incubated at 37ºC for 1 h. Then it was centrifuged at 3,500 rpm for 5 minutes, 100 µL of the supernatant was transferred to another 96-well plate to measure the absorbance at 540 nm (A_{540}). To determine the percentage of hemolysis the following formula has been used.

$$(A - A_0)/(A_{total} - A_0) \times 100$$

Where, A is the absorbance of the compound containing well, A_0 the absorbance of the negative controls (without compound), and A_{total} the absorbance of the Triton X-100 containing well. Each concentration had triplicate values and the HC_{50} was determined by taking the average of triplicate O. D. values and plotted it against concentration fitted with sigmoidal plot. From the curve the values were determined corresponding to 50% hemolysis.

2.5 Results

2.5.1 Antibacterial activity

In order to estimate the efficacy of the aliphatic norspermidine derivatives as antibacterial agents their MIC was determined (Table 2.1) against different Gram-positive (*S. aureus* and *E. faecium*) and Gram-negative (*E. coli*) bacteria including drug resistant bacteria MRSA, VRE and *K. pneumoniae*.

Most of the compounds showed antibacterial activity against all the bacteria tested. Compound **ANA1,** which contains a decyl chain, was found to be least effective against all tested bacteria. The dodecyl analogue, compound **ANA2**, displayed slightly improved antibacterial activity with MIC values of 83 and 200 µM against *S. aureus* and *E. coli*, respectively. Further increase in the length of the aliphatic chain yielded a compound, **ANA3** (tetradecyl analogue) that displayed considerably better MIC values (31 µM and 50 µM against S. *aureus* and *E. coli*) respectively. The antibacterial efficacy was enhanced even further as the length of the aliphatic chain was increased to hexadecane (**ANA4**) as it displayed MIC values of 20 µM and 24 µM against *S. aureus* and *E. coli*, respectively. However, further increase in the length of the aliphatic chain to octadecyl, compound **ANA5** resulted in decrease in antibacterial activity. Compound ANA**5** displayed MIC values of 34

μM and 42 μM against *S. aureus* and *E. coli*, respectively. Thus, compound **ANA4** with C-16 aliphatic chain was found to be most potent antibacterial agent among the compounds in the series. The highlight of compound **ANA4** was its activity against *E. faecium* (MIC = 4.5 μM), whereas colistin did not display any activity even at a concentration of 199 μM. Compounds **ANA3** and **ANA5** also exhibited considerable activity against *E. faecium* with the MIC values of 47 μM and 33 μM. In fact, the activity of compound **ANA4** against VRE emphasizes the efficacy of the design as it exhibited an MIC value of 4 μM, while vancomycin remained inactive till a concentration of 500 μM. And **ANA4** was almost 200 times more potent against this drug resistant bacterium than vancomycin, the drug of last resort. A moderate activity against VRE was observed for compound **ANA3** and **ANA5**, with the MIC values of 41 μM and 17 μM, respectively.

Table 2.1: Antibacterial activity of the **ANA** derivatives

	Minimum Inhibitory Concentration (μM)					
	Drug sensitive strains			Drug resistant strains		
Compounds	*S. aureus*	*E. faceium*	*E. coli*	MRSA	VRE	*K. pneumonie*
ANA1	467	>487	450	266	487	>487
ANA2	83	115	200	97	106	300
ANA3	31	47	50	26	41	82
ANA4	20	4.5	24	19	4	109
ANA5	34	33	42	31	17	170
Colistin	20	>199	0.4	54	>199	1.2
Vancomycin	0.63	0.6	ND	0.63	750	ND

ND stands for "not determined". MRSA is methicillin-resistant *S. aureus*, VRE is vancomycin-resistant *E. faecium* and *K. pneumoniae* is resistant to β-lactam antibiotics.

2.5.2 Toxicity

Toxicity of these compounds (**ANA1**-**ANA5**) was evaluated (Table 2.2) against human erythrocytes (RBCs). The ability of the compounds to lyse RBCs was expressed as their HC_{50} values (the concentration at which 50% of the red blood cells are lysed).

Table 2.2: Hemolytic activity of aliphatic norspermidine derivatives.

Compounds	HC_{50} (μM)
ANA1	>1000
ANA2	198
ANA3	126
ANA4	87
ANA5	67

In general, the HC_{50} values of these compounds displayed a decreasing trend with increasing in aliphatic chain length. The values ranged from >1000 μM to 67 μM. The compound with the shortest alkyl chain in the series, **ANA1** did not show any haemolysis even at 1000 μM. Compound **ANA4** displaying most potent antibacterial efficacy, was found to have HC_{50} value of 87 μM.

2.6 Discussion

Lipopeptides are a promising class of antibacterial agents and some of them are already being used in the clinics for treatment against bacterial infection. However, due to several inherent problems, the clinically approved lipopeptides are limited to topical use only. Synthetic mimics of lipopeptides thus have opened up a promising new avenue towards the development of better antibiotics. Herein, we have designed simple mimics of lipopeptides, based on a norspermidine backbone and a single long aliphatic chain, involving only three synthetic steps. In the synthetic design two positive charges are contributed by the primary amine functionalities of the norspermidine and an aliphatic chain has been assembled into the system by a tertiary amide linkage with the secondary amine of the norspermidine backbone. In the field of antimicrobial peptidomimetics, it has been observed that the minimum necessary parameters required for potent antibacterial activity are two hydrophobic bulks and two positive charges.[104] It was thus surmised that the compounds designed by us might bear moderate antibacterial activity. In order to establish our hypothesis we have varied the length of appended aliphatic chain (from decyl to octadecyl) keeping the two positive charges constant. Indeed the compounds showed considerable antibacterial activity. Antibacterial efficacy was found to increase initially with increase in aliphatic chain (till the hexadecyl analogue). This might be as a result of improved

hydrophobic interaction with the bacterial cell membrane. An optimum condition was reached in compound **ANA4** (bearing hexadecyl chain), which displayed most potent antibacterial activity. Antibacterial potency was compromised on further increase in long chain, as was observed in the octadecyl analogue, compound **ANA5**. It was observed that this compound had low solubility at higher concentration. This might limit the activity of the compounds, as was observed for **ANA5**. Hence, a parabolic pattern (Figure 2.2A) of chain length dependent antibacterial activity was observed in this series of compounds. Vancomycin, the last resort antibiotics for the treatment of MRSA infection was found to be not-active against VRE at a very high concentration of 500 μM. The compound **ANA4** displayed almost 200 times more potency than vancomycin (Figure 2.2B) against this drug resistant strain displaying the MIC value of 4 μM. All the compounds displayed selective toxicity towards bacterial cell compared to human erythrocytes. The selective activity towards bacteria can be expressed as the ratio of HC_{50} and MIC values. For example, **ANA4** exhibited selectivity ratios of 19 and 22 (Figure 2.2C) towards *E. faceium* and VRE respectively over red blood cells.

The content of this chapter is thus a preliminary study based on the norspermidine scaffold. Although moderate antibacterial activity was observed in case of **ANA4**, much needs to be achieved. The beauty of the design also lies in the fact that it allows further modification through the primary amine groups. Further studies would be taken up in the subsequent chapters to establish these norspermidine based lipopeptides as novel antibacterial agents.

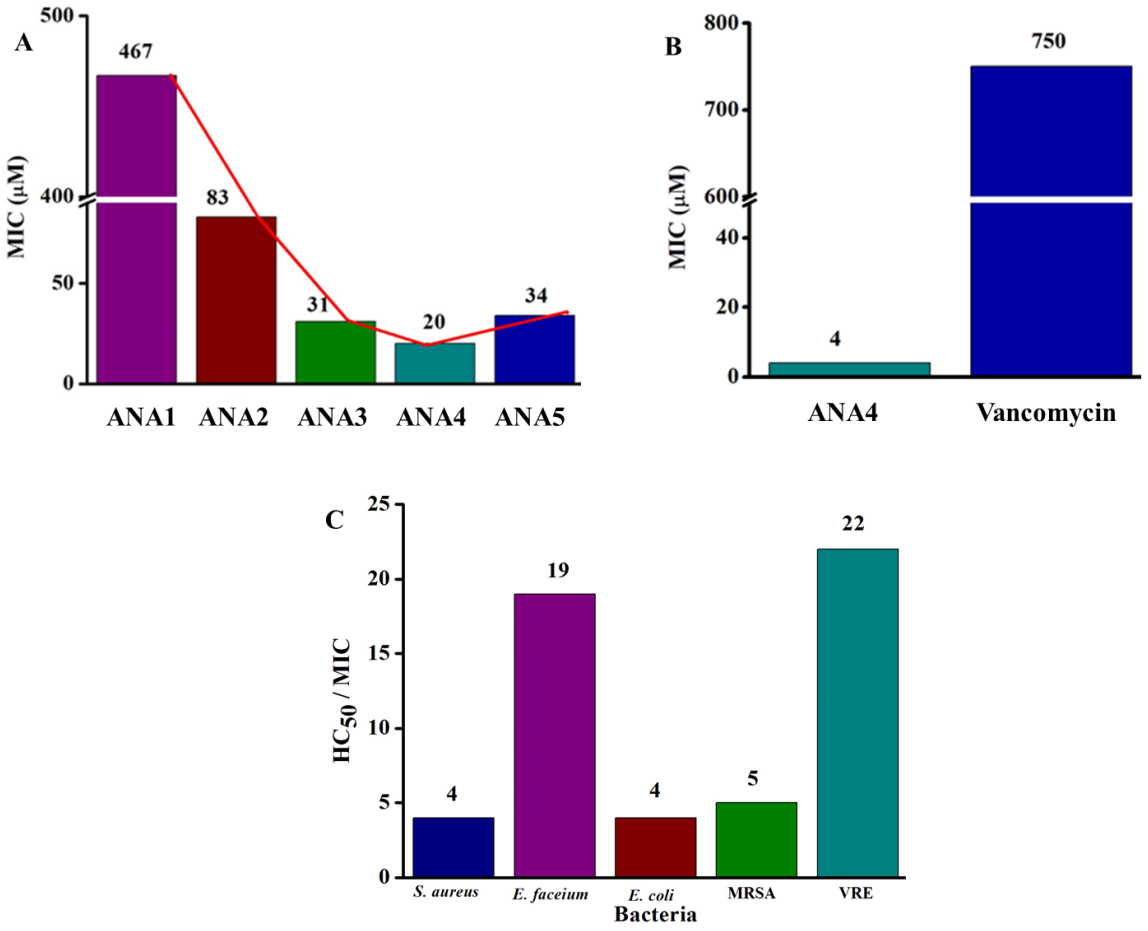


Figure 2.2: A) Variation of MIC values of the compounds against *S. aureus* (Numbers in the figure represent MIC values). B) Comparison of MIC values of the compound **ANA4** and vancomycin against VRE (Numbers in the figure represent MIC values). C) Selectivity ratio of compound **ANA4** against various bacteria (Numbers in the figure represent selectivity ratios).

2.7 Conclusion

In conclusion, a series of aliphatic cationic norspermidine derivatives have been developed through a simple synthetic strategy as lipopeptide mimics. Compound **ANA4** (bearing a hexadecyl chain) not only displayed almost 200 fold more antibacterial activity against VRE, but also was non-haemolytic at that concentration. Although these compounds displayed moderate and selective activity towards bacterial cells, there is much scope for improvement with respect to potency as well as the spectrum of activity. Further functionalization of this scaffold may yield more potent and selective antibacterial agents.

Chapter 3

Synthesis and Antibacterial Properties of Phenylalanine Conjugated Aliphatic Norspermidine Analogues

Abstract

As a further advancement to the norspermidine based lipopeptide mimics disclosed in Chapter 2, conjugation of amino acids was rationalised to be an effective way to improve antibacterial activity. This chapter constitutes synthesis, characterization, antibacterial activity and mechanism of antibacterial action of phenylalanine conjugated aliphatic norspermidine analogues (**PANAs**). Both D- and L-forms of phenylalanine were conjugated to the aliphatic norspermidine analogues to obtain a thorough structure-activity relationship study. These derivatives exhibited potent activity against various gram-positive (*S. aureus* and *E. faecium*) and gram-negative (*E. coli*) bacteria including various drug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vacomycin-resistant *Enterococcus faecium* (VRE) and β-lactam resistant *Klebsiella pneumoniae*. These **PANA** derivatives inflicted their bactericidal activity not only by permeabilization and depolarization of bacterial membrane but also by causing a leakage of K^+ ions from the cytoplasm. Proteolytically stable **PANA6**, also exhibited selective toxicity against VRE over human erythrocytes (HC_{50}/MIC = 86). An additional bonus of these compounds is that bacteria find it difficult to develop resistance against them even after 20 passages. These novel bactericidal agents can thus be optimized to have clinical applications towards the treatment of diseases caused by bacterial infections.

3.1 Introduction

In the previous chapter we developed **ANA** compounds as mimics of lipopeptides and identified the most potent analogue by varying the aliphatic chain. Among the **ANA** derivatives, the hexadecyl analogue (**ANA4**) displayed the best antibacterial efficacy. Although, **ANA4** showed excellent activity against VRE, potency against *S. aureus* and MRSA was only moderate. Moreover, these compounds were considerably toxic towards human erythrocytes. There could be several possible reasons for the low antibacterial activity displayed by the compounds. All lipopeptides have three physicochemical properties by which they interact with bacterial cell membrane such as lipophilic interactions, electrostatic interactions, and hydrogen bonding interactions.[105-108] We surmised that the **ANA** compounds might not be hydrophobic enough and the positive charges might just be too close to each other to impart significant activity. There were also no functional moieties which could be a source of hydrogen bonding. Hydrogen bonding interactions with the lipid head groups of the bacterial cell membranes often play an important role towards antibacterial activity.

On surveying the literature we observed a redundancy in the use of aromatic moieties in several effective antibacterial peptidomimetic designs. Svendsen *et al.* reported several potent cationic antibacterial tripeptides most of which involved aromatic moieties in their designs.[104] All of the antibacterial small molecules reported from the groups of DeGrado and Tew are based on aryl scaffolds.[38-39, 44] Recently we have reported from our group several potent antibacterial compounds based on fused aromatic rings.[48] Barron *et al.* have also used aromatic moieties in the design of antibacterial peptoids.[42] Cai *et al.* have reported a class of AMP mimics known as "AApeptides" which exhibited potent antibacterial efficacy over a broad spectrum of bacterial strains.[80, 109] In these derivatives too they have used phenyl groups in order to impart hydrophobicity to achieve potent antibacterial activity. Although according to our knowledge there is no example in literature correlating the importance of aromatic rings towards antibacterial activity, the overwhelming number of reports advocating their use cannot be a coincidence.

We thus wanted to conjugate some functional moieties into our design which would provide an aromatic moiety, space out the positive charges even further and also provide an option for hydrogen-bonding interactions. The presence of the lipophilic aliphatic chain completes

all the necessary criteria for development of an effective antibacterial mimic of lipopeptides. Amino acids seemed to be the best class of compounds which would be able to provide all of the properties sought. We narrowed down on phenylalanine as it was simple and posed no synthetic complexity. Also, since natural antimicrobial peptides and lipopeptides are known to be labile to proteases, we wanted to make our design as abiotic as possible. Hence, we also conjugated both L-Phenylalanine and D-Phenylalanine into our design. We hypothesized that this inclusion of the non-natural D-isomer might impart additional plasma stability to the compounds.

3.2 Structure and Design

This series of compounds (**PANA**) were designed to improve upon the properties of the previously reported **ANA** compounds (Chapter 2). Appendage of phenylalanine residues on both the primary amine functionalities of norspermidine was surmised to bring about several changes in physical properties of the compounds, thereby prompting a change in the biological properties too. The positive charges of the compounds were kept the same at two, only the difference was that it was now contributed by the amine group of phenylalanine. Like in the previous case, hydrophobicity was varied through incorporation of different lengths of the aliphatic chain (Figure 3.1). To investigate the effect of replacement of aliphatic hydrophobicity to corresponding aromatic hydrophobicity at the secondary amine position, a compound **PNP** was synthesised by coupling a α-naphthyl acetic acid group. Compound **PPP** was made by introducing another phenyl alanine amino acid instead of long chain aliphatic acid at the secondary amine of norspermidine backbone. Total charges in the **PPP** compound were three; the additional one was contributed by the third phenyl alanine. In order to impart additional plasma stability **PANA6** was synthesized through incorporation of “D” phenylalanine instead of “L” isomer. Finally, to understand the importance of aliphatic group, compound **PP** was made as a control compound which was devoid of the aliphatic tail.

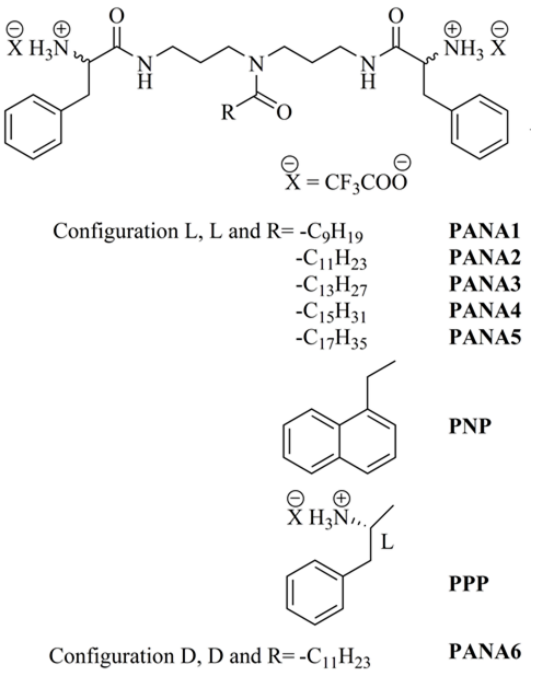


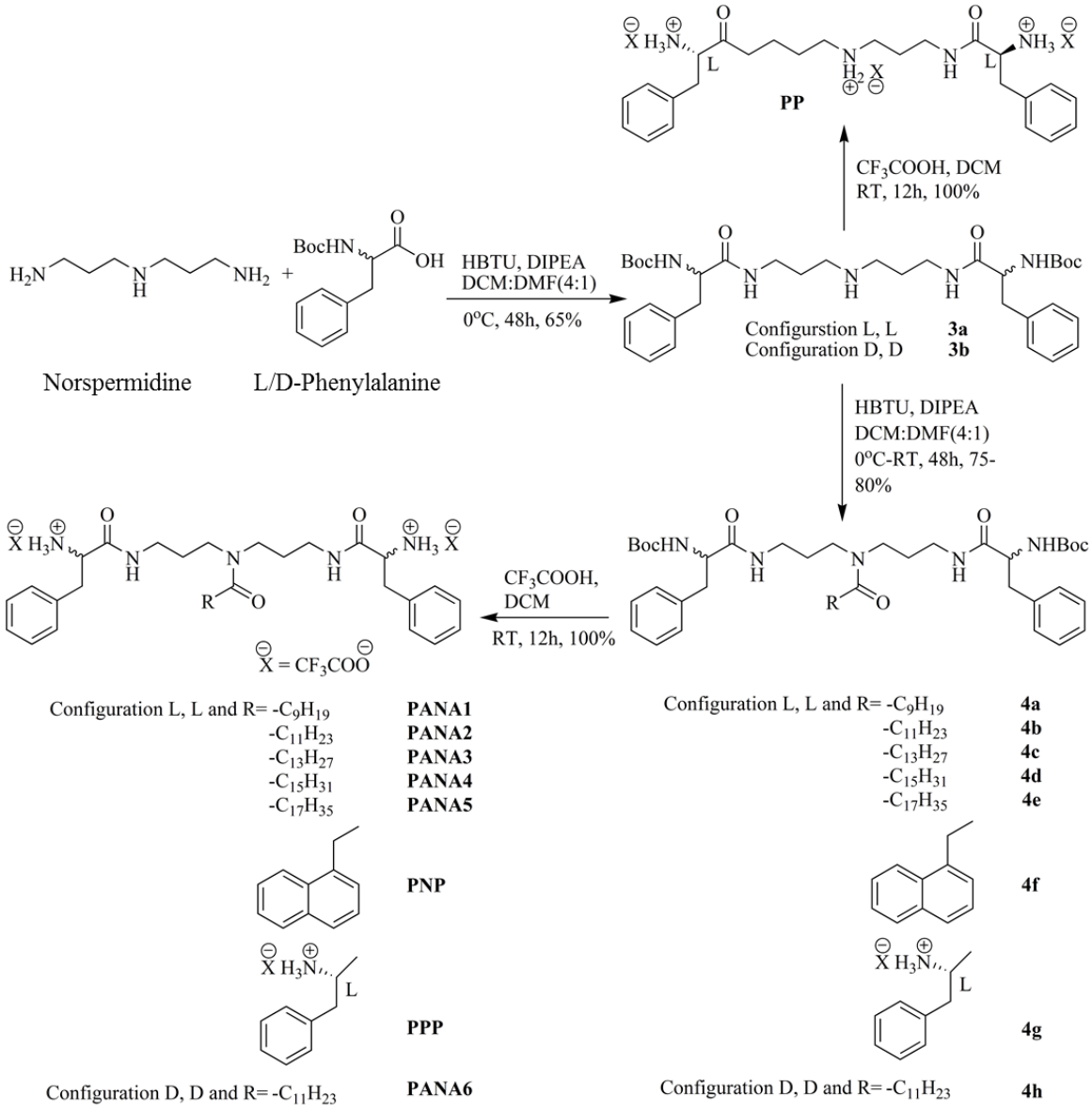
Figure 3.1: Structure of phenylalanine conjugated aliphatic norspermidine analogues (**PANAs**).

3.3 Synthesis and Characterization

3.3.1 Materials and Methods

The chemicals *N*-Boc-L-Phenyalanine and *N*-Boc-D-Phenyalanine were obtained from Spectrochem and Sigma-Aldrich, respectively. The rest of materials and methods are same as described in the section of 2.3.1 in Chapter 2.

3.3.2 Reaction Scheme



Scheme 3.1: Synthesis of phenylalanine conjugated aliphatic norspermidine analogues (**PANAs**).

Synthesis of the compounds was achieved in three steps (Scheme 3.1). First step involved the selective coupling of the primary amine groups of norspermidine with *N*-Boc-L/D-phenylalanine. This was achieved by carrying out amide coupling reaction at 0°C using HBTU as coupling agent for 48 h. Then an aliphatic chain was introduced at the secondary amine through another amide coupling step with various aliphatic acids. Finally,

deprotection of Boc groups with trifluoroacetic acid (TFA) yielded the final compounds (**PANA1-PANA6**). In order to study the importance of the aliphatic chain, α-naphthylacetic acid and *N*-Boc-phenylanine were used to couple to the secondary amine instead of aliphatic acids to generate compounds **PNP** and **PPP** respectively. The control compound **PP,** which is devoid of the long chain, was made directly adding TFA to the first step product.

3.3.3 Synthetic protocol and characterization

General procedure for synthesizing 3a and 3b: 5 g (2 equivalents, 18.85 mmol) of *N*-Boc-L/D- Phenylalanine was dissolved in dry DCM (30 mL) at 0ºC. To the reaction mixture DIPEA (9.8 mL, 6 equivalents, 56.55 mmol) was added followed by 7.2 g of HBTU (2 equivalents, 18.85 mmol). Then DMF (8 mL) was added to solubilize the reaction mixture. After 10 minutes, 1.2 g of norspermidine (1 equivalent, 9.43 mmol) was added drop wise. The reaction mixture was allowed to stir for 48 h at 0ºC. At the end of 48 hours, the solvent was evaporated under reduced pressure and residue was diluted with ethyl acetate (100 mL). It was then washed with 1N HCl (100 mL, 3 times) followed by saturated Na_2CO_3 solution (100 mL, 3 times). The crude product was collected in ethyl acetate layer and was passed through anhydrous sodium sulphate. Finally purification was done through column chromatography on silica gel (60-120 mess) using methanol and chloroform as eluent (5:95) to obtain the product with 65% yield.

***N*^{1}-(Boc-^{L}Phe)-*N*^{3}-[{3-(Boc-^{L}Phe)amido}propyl]propane-1,3-diamine (3a):** Yield-65%; FT-IR (NaCl): 3311 cm^{-1} (-NH- str.), 3033 cm^{-1} (aromatic C-H str.), 2929 cm^{-1} (-CH_2- asym. str.), 2854 cm^{-1} (-CH_2- sym. str.), 1700 cm^{-1} and 1684 cm^{-1} (C=O str.), 1654 cm^{-1}, 1635 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.300-7.204 (m, NH(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph*H*)$_2$, 10H), 7.194 (s, NH(-CH_2-CH_2-CH_2-*NH*-CO-CH(NHBoc)-CH_2-Ph)$_2$, 2H), 5.368 (s, NH(-CH_2-CH_2-CH_2-NH-CO-CH(*NH*Boc)-CH_2-Ph)$_2$, 2H), 4.314-4.298 (t, NH(-CH_2-CH_2-CH_2-NH-CO-*CH*(NHBoc)-CH_2-Ph)$_2$, 2H), 3.370-2.985 (m, NH(-*CH*$_2$-CH_2-*CH*$_2$-NH-CO-CH(NHBoc)-*CH*$_2$-Ph)$_2$, 12H), 2.570 (s, *NH*(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph)$_2$, 1H), 1.687-1.676 (m, NH(-CH_2-*CH*$_2$-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph)$_2$, 4H), 1.386 (s, NH(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(CH*$_3$*)*$_3$)-CH_2-Ph)$_2$, 18H); HRMS (m/z): 626.3891 [$(M+H)^+$] (Observed), 626.3918 [$(M+H)^+$] (Calculated).

***N*^{1}-(Boc-^{D}Phe)-*N*^{3}-[{3-(Boc-^{D}Phe)amido}propyl]propane-1,3-diamine (3b):** Yield-65%; FT-IR (NaCl): 3315 cm^{-1} (-NH- str.), 3027 cm^{-1} (aromatic C-H str.), 2932 cm^{-1} (-CH_2- asym. str.), 2856 cm^{-1} (-CH_2- sym. str.), 1704 cm^{-1} and 1682 cm^{-1} (C=O str.), 1652 cm^{-1}, 1637 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.303-7.201 (m, NH(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph*H*)$_2$, 10H), 7.197 (s, NH(-CH_2-CH_2-CH_2-*NH*-CO-CH(NHBoc)-CH_2-Ph)$_2$, 2H), 5.365 (s, NH(-CH_2-CH_2-CH_2-NH-CO-CH(*NH*Boc)-CH_2-Ph)$_2$, 2H), 4.319-4.294 (t, NH(-CH_2-CH_2-CH_2-NH-CO-*CH*(NHBoc)-CH_2-Ph)$_2$, 2H), 3.367-2.983 (m, NH(-*CH*$_2$-CH_2-*CH*$_2$-NH-CO-CH(NHBoc)-*CH*$_2$-Ph)$_2$, 12H), 2.569 (s, *NH*(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph)$_2$, 1H), 1.684-1.673 (m, NH(-CH_2-*CH*$_2$-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph)$_2$, 4H), 1.383 (s, NH(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C(*CH*$_3$)$_3$)-CH_2-Ph)$_2$, 18H); HRMS (m/z): 626.3891 [$(M+H)^+$] (Observed), 626.3918 [$(M+H)^+$] (Calculated).

General procedure for synthesizing 4a-4h: About 2.4 mmol (1.5 equivalents) of saturated aliphatic acid (decanoic, dodecanoic, tetradecanoic, hexadecanoic, or octadecanoic), α-Naphthylacetic acid, *N*-Boc-L-Phenylalanine were dissolved in dry DCM (12 mL) at 0ºC. To the reaction mixture 4 equivalents of DIPEA was then added followed by 1.5 equivalents of HBTU. Then DMF (3 mL) was added to the reaction mixture. After 10 minutes, 1 equivalent of **3a** or **3b** in dry DCM (2 mL) was added drop wise. The reaction mixture was brought to RT and allowed to stir for 24 h. At the end of 24 h, solvent was evaporated and residue was diluted in ethyl acetate (50 mL). Then work-up was done at first with 1N HCl (50 mL, 3 times) followed by saturated Na_2CO_3 solution (50 mL, 3 times). The crude product was extracted in ethyl acetate layer and dried over anhydrous sodium sulphate and finally purification was done through column chromatography on silica gel (60-120 mess) using methanol and chloroform as eluent (3:97) to afford **4a-4h** with 75-80% yield.

***N*,*N*-bis-[{3-(Boc-^{L}Phe)amido}propyl]decanamide (4a):** Yield-76%; FT-IR (NaCl): 3304 cm^{-1} (-NH- str.), 3029 cm^{-1} (aromatic C-H str.), 2927 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1699 cm^{-1} and 1684 cm^{-1} (C=O str.), 1652 cm^{-1}, 1635 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.246-7.097 (m, R-CO-N(-CH_2-CH_2-CH_2-*NH*-CO-CH(NHBoc)-CH_2-Ph*H*)$_2$, 12H), 5.350-5.229 (m, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*NH*Boc)-CH_2-Ph)$_2$, 2H), 4.355-4.315 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*CH*(NHBoc)-CH_2-Ph)$_2$, 2H), 3.384-2.871 (m, R-CO-N(-*CH*$_2$-CH_2-*CH*$_2$-NH-CO-CH(NHBoc)-*CH*$_2$-Ph)$_2$, 12H), 2.215-2.195 (t, CH_3-$(CH_2)_6$-CH_2-*CH*$_2$- of R group, 2H), 1.597-1.463 (m, CH_3-$(CH_2)_6$-

C$\underline{H_2}$-CH_2-CO-N(-CH_2-*C*$\underline{H_2}$-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph)$_2$, 6H), 1.353 (s, R-CO-NH(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*$\underline{H_3}$*)*$\underline{_3}$)-CH_2-Ph)$_2$, 18H), 1.255 (bs, CH_3-*(C*$\underline{H_2}$*)*$\underline{_6}$-CH_2-CH_2- of R group, 12H), 0.889-0.855 (t, *C*$\underline{H_3}$-$(CH_2)_6$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 780.5268 [$(M+H)^+$] (Observed), 780.5275 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(Boc-^{L}Phe)amido}propyl]dodecanamide (4b):** Yield-78%; FT-IR (NaCl): 3300 cm^{-1} (-NH- str.), 3030 cm^{-1} (aromatic C-H str.), 2928 cm^{-1} (-CH_2- asym. str.), 2854 cm^{-1} (-CH_2- sym. str.), 1699 cm^{-1} and 1683 cm^{-1} (C=O str.), 1652 cm^{-1}, 1635 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.215-7.096 (m, R-CO-N(-CH_2-CH_2-CH_2-*N*$\underline{H}$-CO-CH(NHBoc)-CH_2-Ph$\underline{H}$)$_2$, 12H), 5.417-5.277 (m, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*N*$\underline{H}$Boc)-CH_2-Ph)$_2$, 2H), 4.360-4.328 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*$\underline{H}$(NHBoc)-CH_2-Ph)$_2$, 2H), 3.356-2.869 (m, R-CO-N(-*C*$\underline{H_2}$-CH_2-*C*$\underline{H_2}$-NH-CO-CH(NHBoc)-*C*$\underline{H_2}$-Ph)$_2$, 12H), 2.209-2.189 (t, CH_3-$(CH_2)_8$-CH_2-*C*$\underline{H_2}$- of R group, 2H), 1.714-1.463 (m, CH_3-$(CH_2)_8$-*C*$\underline{H_2}$-CH_2-CO-N(-CH_2-*C*$\underline{H_2}$-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph)$_2$, 6H), 1.351 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*$\underline{H_3}$*)*$\underline{_3}$)-CH_2-Ph)$_2$, 18H), 1.244 (bs, CH_3-*(C*$\underline{H_2}$*)*$\underline{_8}$-CH_2-CH_2- of R group, 16H), 0.885-0.851 (t, *C*$\underline{H_3}$-$(CH_2)_8$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 808.5593 [$(M+H)^+$] (Observed), 808.5588 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(Boc-^{L}Phe)amido}propyl]tetradecanamide (4c):** Yield-75%; FT-IR (NaCl): 3301 cm^{-1} (-NH- str.), 3028 cm^{-1} (aromatic C-H str.), 2926 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1699 cm^{-1} and 1684 cm^{-1} (C=O str.), 1654 cm^{-1}, 1637 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.215-7.100 (m, R-CO-N(-CH_2-CH_2-CH_2-*N*$\underline{H}$-CO-CH(NHBoc)-CH_2-Ph$\underline{H}$)$_2$, 12H), 5.495-5.345 (m, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*N*$\underline{H}$Boc)-CH_2-Ph)$_2$, 2H), 4.352-4.333 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*$\underline{H}$(NHBoc)-CH_2-Ph)$_2$, 2H), 3.451-2.861 (m, R-CO-N(-*C*$\underline{H_2}$-CH_2-*C*$\underline{H_2}$-NH-CO-CH(NHBoc)-*C*$\underline{H_2}$-Ph)$_2$, 12H), 2.202-2.182 (t, CH_3-$(CH_2)_{10}$-CH_2-*C*$\underline{H_2}$- of R group, 2H), 1.644-1.547 (m, CH_3-$(CH_2)_{10}$-*C*$\underline{H_2}$-CH_2-CO-N(-CH_2-*C*$\underline{H_2}$-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph)$_2$, 6H), 1.345 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*$\underline{H_3}$*)*$\underline{_3}$)-CH_2-Ph)$_2$, 18H), 1.235 (bs, CH_3-*(C*$\underline{H_2}$*)*$\underline{_{10}}$-CH_2-CH_2- of R group, 20H), 0.879-0.845 (t, *C*$\underline{H_3}$-$(CH_2)_{10}$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 836.5908 [$(M+H)^+$] (Observed), 836.5901 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(Boc-^{L}Phe)amido}propyl]hexadecanamide (4d):** Yield: 80%; FT-IR (NaCl): 3299 cm^{-1} (-NH- str.), 3031 cm^{-1} (aromatic C-H str.), 2927 cm^{-1} (-CH_2- asym. str.), 2854 cm^{-1} (-CH_2- sym. str.), 1699 cm^{-1} and 1684 cm^{-1} (C=O str.), 1656 cm^{-1}, 1635 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.211-7.097 (m, R-CO-N(-CH_2-CH_2-CH_2-

*N*H-CO-CH(NHBoc)-CH_2-Ph*H*)$_2$, 12H), 5.515-5.357 (m, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*N*HBoc)-CH_2-Ph)$_2$, 2H), 4.356-4.337 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*H(NHBoc)-CH_2-Ph)$_2$, 2H), 3.425-2.861 (m, R-CO-N(-*C*H$_2$-CH_2-*C*H$_2$-NH-CO-CH(NHBoc)-*C*H$_2$-Ph)$_2$, 12H), 2.200-2.181 (t, CH_3-$(CH_2)_{12}$-CH_2-*C*H$_2$- of R group, 2H), 1.690-1.435 (m, CH_3-$(CH_2)_{12}$-*C*H$_2$-CH_2-CO-N(-CH_2-*C*H$_2$-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph)$_2$, 6H), 1.343 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*H$_3$*)*$_3$)-CH_2-Ph)$_2$, 18H), 1.233 (bs, CH_3-*(C*H$_2$*)*$_{12}$-CH_2-CH_2- of R group, 24H), 0.877-0.843 (t, *C*H$_3$-$(CH_2)_{12}$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 864.6227 [$(M+H)^+$] (Observed), 864.6214 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(Boc-^{L}Phe)amido}propyl]octadecanamide (4e):** Yield-77%; FT-IR (NaCl): 3302 cm^{-1} (-NH- str.), 3028 cm^{-1} (aromatic C-H str.), 2927 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1699 cm^{-1} and 1684 cm^{-1} (C=O str.), 1652 cm^{-1}, 1635 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.245-7.100 (m, R-CO-N(-CH_2-CH_2-CH_2-*N*H-CO-CH(NHBoc)-CH_2-Ph*H*)$_2$, 12H), 5.338-5.216 (m, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*N*HBoc)-CH_2-Ph)$_2$, 2H), 4.338-4.319 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*H(NHBoc)-CH_2-Ph)$_2$, 2H), 3.484-2.802 (m, R-CO-N(-*C*H$_2$-CH_2-*C*H$_2$-NH-CO-CH(NHBoc)-*C*H$_2$-Ph)$_2$, 12H), 2.214-2.194 (t, CH_3-$(CH_2)_{14}$-CH_2-*C*H$_2$- of R group, 2H), 1.631-1.3578 (m, CH_3-$(CH_2)_{14}$-*C*H$_2$-CH_2-CO-N(-CH_2-*C*H$_2$-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph)$_2$, 6H), 1.354 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*H$_3$*)*$_3$)-CH_2-Ph)$_2$, 18H), 1.250 (bs, CH_3-*(C*H$_2$*)*$_{14}$-CH_2-CH_2-, 28H), 0.894-0.860 (t, *C*H$_3$-$(CH_2)_{14}$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 892.6535 [$(M+H)^+$] (Observed), 892.6527 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(Boc-^{L}Phe)amido}propyl]α-naphthylacetamide (4f):** Yield-75%; FT-IR (NaCl): 3301 cm^{-1} (-NH- str.), 3033 cm^{-1} (aromatic C-H str.), 2933 cm^{-1} (-CH_2- asym. str.), 2856 cm^{-1} (-CH_2- sym. str.), 1700 cm^{-1} and 1684 cm^{-1} (C=O str.), 1654 cm^{-1}, 1635 cm^{-1} (aromatic C=C str.). ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.775-7.082 (m, Naph*H*-CH_2-CO-N(-CH_2-CH_2-CH_2-*N*H-CO-CH(NHBoc)-CH_2-Ph*H*)$_2$, 19H), 5.365-5.217 (m, Naph-CH_2-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*N*HBoc)-CH_2-Ph)$_2$, 2H), 4.334-4.298 (m, Naph-*C*H$_2$-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph)$_2$, 2H), 4.105-4.070 (t, Naph-CH_2-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*H(NHBoc)-CH_2-Ph)$_2$, 2H), 3.217-2.832 (m, Naph-CH_2-CO-N(-*C*H$_2$-CH_2-*C*H$_2$-NH-CO-CH(NHBoc)-*C*H$_2$-Ph)$_2$, 12H), 1.512-1.446 (m, Naph-CH_2-CO-N(-CH_2-*C*H$_2$-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph)$_2$, 4H), 1.346 (s, Naph-CH_2-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*H$_3$*)*$_3$)-CH_2-Ph)$_2$, 18H); HRMS (m/z): 794.4512 [$(M+H)^+$] (Observed), 794.4493 [$(M+H)^+$] (Calculated).

***N*^{1},*N*^{3}-bis-(Boc-^{L}Phe)-*N*^{1}-[{3-(Boc-^{L}Phe)amido}propyl]propane-1,3-diamine (4g):** Yield-78%; FT-IR (NaCl): 3311 cm^{-1} (-NH- str.), 3029 cm^{-1} (aromatic C-H str.), 2929 cm^{-1} (-CH_2- asym. str.), 2854 cm^{-1} (-CH_2- sym. str.), 1699 cm^{-1} and 1686 cm^{-1} (C=O str.), 1655 cm^{-1}, 1637 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.236-7.093 (m, Ph*H*-CH_2-CH(NHBoc)-CO-N(-CH_2-CH_2-CH_2-*NH*-CO-CH(NHBoc)-CH_2-Ph*H*)$_2$, 17H), 5.346-5.217 (m, PhH-CH_2-CH(*NH*Boc)-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*NH*Boc)-CH_2-Ph)$_2$, 3H), 4.347-4.307 (t, Ph-CH_2-*CH*(NHBoc)-CO-N(-CH_2-CH_2-CH_2-NH-CO-*CH*(NHBoc)-CH_2-Ph)$_2$, 3H), 3.387-2.869 (m, Ph-*C*H_2-CH(NHBoc)-CO-N(-*C*H_2-CH_2-*C*H_2-NH-CO-CH(NHBoc)-*C*H_2-Ph)$_2$, 14H), 1.537-1.455 (m, Ph-CH_2-CH(NHBoc)-CO-N(-CH_2-*C*H_2-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph)$_2$, 4H), 1.343 (s, Ph-CH_2-CH(NH-COO-C*(C*H_3*)*$_3$)-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*H_3*)*$_3$)-CH_2-Ph)$_2$, 27H). HRMS (m/z): 873.5239 [$(M+H)^+$] (Observed), 873.5126 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(Boc-^{D}Phe)amido}propyl]dodecanamide (4h):** Yield: 75%; FT-IR (NaCl): 3300 cm^{-1} (-NH- str.), 3030 cm^{-1} (aromatic C-H str.), 2928 cm^{-1} (-CH_2- asym. str.), 2854 cm^{-1} (-CH_2- sym. str.), 1699 cm^{-1} and 1683 cm^{-1} (C=O str.), 1652 cm^{-1}, 1635 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.260-7.094 (m, R-CO-N(-CH_2-CH_2-CH_2-*NH*-CO-CH(NHBoc)-CH_2-Ph*H*)$_2$, 12H), 5.555-5.442 (m, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*NH*Boc)-CH_2-Ph)$_2$, 2H), 4.353-4.334 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*CH*(NHBoc)-CH_2-Ph)$_2$, 2H), 3.452-2.892 (m, R-CO-N(-*C*H_2-CH_2-*C*H_2-NH-CO-CH(NHBoc)-*C*H_2-Ph)$_2$, 12H), 2.205-2.185 (t, CH_3-$(CH_2)_8$-CH_2-*C*H_2- of R group, 2H), 1.647-1.434 (m, CH_3-$(CH_2)_8$-*C*H_2-CH_2-CO-N(-CH_2-*C*H_2-CH_2-NH-CO-CH(NHBoc)-CH_2-Ph)$_2$, 6H), 1.343 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*H_3*)*$_3$)-CH_2-Ph)$_2$, 18H), 1.243 (bs, CH_3-*(C*H_2*)*$_8$-CH_2-CH_2- of R group, 16H), 0.878-0.844 (t, *C*H_3-$(CH_2)_8$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 808.5593 [$(M+H)^+$] (Observed), 808.5588 [$(M+H)^+$] (Calculated).

General procedure for synthesizing PANA1-PANA6, PNP, PPP and PP: About 1.2 mmol of **4a-4h (**1 equivalent) and 1.6 mmol of **3a (**1 equivalent) were dissolved in DCM (3 mL). To the intensely stirred solution 4 equivalents (excess amount) of trifluoroacetic acid (TFA) was added and the reaction mixture was stirred at RT for 12 h. Then solvent and unused TFA were removed under reduced pressure to obtain the pure product in quantitative yield.

***N,N*-bis-[{3-(L-Phe)amido}propyl]decanamide bis(trifluoroacetate) (PANA1):** FT-IR (NaCl): 3270 cm^{-1} (-NH- str.), 3034 cm^{-1} (aromatic C-H str.), 2930 cm^{-1} (-CH_2- asym. str.),

2859 cm^{-1} (-CH_2- sym. str.), 1673 cm^{-1} (C=O str.), 1618 cm^{-1}, 1577 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.473-8.231 (m, R-CO-N(-CH_2-CH_2-CH_2-*N<u>H</u>*-CO-CH*(N<u>H</u>$_3^+$)*-CH_2-Ph)$_2$, 8H), 7.339-7.219 (m, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph*<u>H</u>*)$_2$, 10H), 3.939 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C<u>H</u>*(NH_3^+)-CH_2-Ph)$_2$, 2H), 3.187-2.889 (m, R-CO-N(-*C<u>H</u>$_2$*-CH_2-*C<u>H</u>$_2$*-NH-CO-CH(NH_3^+)-*C<u>H</u>$_2$*-Ph)$_2$, 12H), 2.200-2.165 (t, CH_3-$(CH_2)_6$-CH_2-*C<u>H</u>$_2$*- of R group, 2H), 1.527-1.446 (m, CH_3-$(CH_2)_6$-*C<u>H</u>$_2$*-CH_2-CO-N(-CH_2-*C<u>H</u>$_2$*-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph)$_2$, 6H), 1.221 (bs, CH_3-*(C<u>H</u>$_2$)$_6$*-CH_2-CH_2- of R group , 12H), 0.864-0.830 (t, *C<u>H</u>$_3$*-$(CH_2)_6$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 171.81, 167.81, 167.59, 158.54, 158.22, 135.04, 129.44, 128.47, 127.09, 53.64, 45.00, 42.79, 37.15, 37.09, 36.57, 36.29, 32.07, 31.30, 29.04, 28.96, 28.80, 28.69, 28.36, 27.28, 25.09, 22.09, 13.94; HRMS (m/z): 580.4225 [$(M+H)^+$] (Observed), 580.4243 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(^{L}Phe)amido}propyl]dodecanamide bis(trifluoroacetate) (PANA2):** FT-IR (NaCl): 3275 cm^{-1} (-NH- str.), 3034 cm^{-1} (aromatic C-H str.), 2927 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1680 cm^{-1} (C=O str.), 1620 cm^{-1}, 1576 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.479-8.235 (m, R-CO-N(-CH_2-CH_2-CH_2-*N<u>H</u>*-CO-CH*(N<u>H</u>$_3^+$)*-CH_2-Ph)$_2$, 8H), 7.341-7.218 (m, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph*<u>H</u>*)$_2$, 10H), 3.940 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C<u>H</u>*(NH_3^+)-CH_2-Ph)$_2$, 2H), 3.171-2.924 (m, R-CO-N(-*C<u>H</u>$_2$*-CH_2-*C<u>H</u>$_2$*-NH-CO-CH(NH_3^+)-*C<u>H</u>$_2$*-Ph)$_2$, 12H), 2.199-2.165 (t, CH_3-$(CH_2)_8$-CH_2-*C<u>H</u>$_2$*- of R group, 2H), 1.526-1.444 (m, CH_3-$(CH_2)_8$-*C<u>H</u>$_2$*-CH_2-CO-N(-CH_2-*C<u>H</u>$_2$*-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph)$_2$, 6H), 1.221 (bs, CH_3-*(C<u>H</u>$_2$)$_8$*-CH_2-CH_2- of R group , 16H), 0.865-0.831 (t, *C<u>H</u>$_3$*-$(CH_2)_8$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 171.80, 167.79, 167.58, 158.38, 158.07, 135.01, 129.42, 128.47, 127.09, 53.63, 44.98, 37.14, 37.09, 36.56, 36.28, 31.29, 29.03, 29.01, 28.80, 28.70, 28.35, 27.28, 25.08, 22.09, 13.94; HRMS (m/z): 608.4510 [$(M+H)^+$] (Observed), 608.4540 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(^{L}Phe)amido}propyl]tetradecanamide bis(trifluoroacetate) (PANA3):** FT-IR (NaCl): 3275 cm^{-1} (-NH- str.), 3033 cm^{-1} (aromatic C-H str.), 2927 cm^{-1} (-CH_2- asym. str.), 2856 cm^{-1} (-CH_2- sym. str.), 1679 cm^{-1} (C=O str.), 1620 cm^{-1}, 1576 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.506-8.257 (m, R-CO-N(-CH_2-CH_2-CH_2-*N<u>H</u>*-CO-CH*(N<u>H</u>$_3^+$)*-CH_2-Ph)$_2$, 8H), 7.333-7.218 (m, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph*<u>H</u>*)$_2$, 10H), 3.944 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C<u>H</u>*(NH_3^+)-CH_2-

Ph)$_2$, 2H), 3.184-2.905 (m, R-CO-N(-*C*$\underline{H_2}$-CH$_2$-*C*$\underline{H_2}$-NH-CO-CH(NH_3^+)-*C*$\underline{H_2}$-Ph)$_2$, 12H), 2.197-2.163 (t, CH$_3$-(CH$_2$)$_{10}$-CH$_2$-*C*$\underline{H_2}$- of R group, 2H), 1.525-1.443 (m, CH$_3$-(CH$_2$)$_{10}$-*C*$\underline{H_2}$-CH$_2$-CO-N(-CH$_2$-*C*$\underline{H_2}$-CH$_2$-NH-CO-CH(NH_3^+)-CH$_2$-Ph)$_2$, 6H), 1.222 (bs, CH$_3$-*(C*$\underline{H_2}$*)$_{10}$*-CH$_2$-CH$_2$-, of R group , 20H), 0.865-0.831 (t, *C*$\underline{H_3}$-(CH$_2$)$_{10}$-CH$_2$-CH$_2$- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d$_6$) δ/ppm: 171.80, 167.80, 167.59, 135.03, 129.43, 128.46, 127.07, 53.63, 45.00, 42.79, 37.14, 37.08, 36.56, 36.29, 32.07, 31.30, 29.05, 29.02, 28.81, 28.71, 28.35, 27.27, 25.09, 22.10, 13.93. HRMS (m/z): 636.4843 [(M+H)$^+$] (Observed), 636.4853 [(M+H)$^+$] (Calculated).

***N,N*-bis-[{3-(L-Phe)amido}propyl]hexadecanamide bis(trifluoroacetate) (PANA4):** FT-IR (NaCl): 3277 cm^{-1} (-NH- str.), 3034 cm^{-1} (aromatic C-H str.), 2928 cm^{-1} (-CH$_2$- asym. str.), 2857 cm^{-1} (-CH$_2$- sym. str.), 1676 cm^{-1} (C=O str.), 1618 cm^{-1}, 1577 cm^{-1} (aromatic C=C str.). ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ/ppm: 8.500-8.254 (m, R-CO-N(-CH$_2$-CH$_2$-CH$_2$-*N*$\underline{H}$-CO-CH*(N*$\underline{H_3}^+$*)*-CH$_2$-Ph)$_2$, 8H), 7.334-7.218 (m, R-CO-N(-CH$_2$-CH$_2$-CH$_2$-NH-CO-CH(NH_3^+)-CH$_2$-Ph$\underline{H}$)$_2$, 10H), 3.944 (t, R-CO-N(-CH$_2$-CH$_2$-CH$_2$-NH-CO-*C*$\underline{H}$(NH_3^+)-CH$_2$-Ph)$_2$, 2H), 3.202-2.891 (m, R-CO-N(-*C*$\underline{H_2}$-CH$_2$-*C*$\underline{H_2}$-NH-CO-CH(NH_3^+)-*C*$\underline{H_2}$-Ph)$_2$, 12H), 2.197-2.163 (t, CH$_3$-(CH$_2$)$_{12}$-CH$_2$-*C*$\underline{H_2}$- of R group, 3H), 1.525-1.444 (m, CH$_3$-(CH$_2$)$_{12}$-*C*$\underline{H_2}$-CH$_2$-CO-N(-CH$_2$-*C*$\underline{H_2}$-CH$_2$-NH-CO-CH(NH_3^+)-CH$_2$-Ph)$_2$, 6H), 1.226 (bs, CH$_3$-*(C*$\underline{H_2}$*)$_{12}$*-CH$_2$-CH$_2$- of R group, 24H), 0.866-0.832 (t, *C*$\underline{H_3}$-(CH$_2$)$_{12}$-CH$_2$-CH$_2$- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d$_6$) δ/ppm:171.78, 167.76, 167.55, 158.44, 134.99, 129.39, 128.43, 127.06, 53.61, 44.97, 37.12, 36.55, 36.28, 35.75, 32.05, 31.26, 30.75, 29.01, 28.97, 28.78, 28.66, 28.34, 27.26, 25.05, 22.05, 13.89; HRMS (m/z): 664.5156 [(M+H)$^+$] (Observed), 664.5166 [(M+H)$^+$] (Calculated).

***N,N*-bis-[{3-(L-Phe)amido}propyl]octadecanamide bis(trifluoroacetate) (PANA5):** FT-IR (NaCl): 3275 cm^{-1} (-NH- str.), 3034 cm^{-1} (aromatic C-H str.), 2926 cm^{-1} (-CH$_2$- asym. str.), 2854 cm^{-1} (-CH$_2$- sym. str.), 1679 cm^{-1} (C=O str.), 1619 cm^{-1}, 1576 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ/ppm: 8.500-8.253 (m, R-CO-N(-CH$_2$-CH$_2$-CH$_2$-*N*$\underline{H}$-CO-CH*(N*$\underline{H_3}^+$*)*-CH$_2$-Ph)$_2$, 8H), 7.334-7.217 (m, R-CO-N(-CH$_2$-CH$_2$-CH$_2$-NH-CO-CH(NH_3^+)-CH$_2$-Ph$\underline{H}$)$_2$, 10H), 3.943 (t, R-CO-N(-CH$_2$-CH$_2$-CH$_2$-NH-CO-*C*$\underline{H}$(NH_3^+)-CH$_2$-Ph)$_2$, 2H), 3.201-2.891 (m, R-CO-N(-*C*$\underline{H_2}$-CH$_2$-*C*$\underline{H_2}$-NH-CO-CH(NH_3^+)-*C*$\underline{H_2}$-Ph)$_2$, 12H), 2.196-2.163 (t, CH$_3$-(CH$_2$)$_{14}$-CH$_2$-*C*$\underline{H_2}$- of R group, 2H), 1.524-1.443 (m, CH$_3$-(CH$_2$)$_{14}$-*C*$\underline{H_2}$-CH$_2$-CO-N(-CH$_2$-*C*$\underline{H_2}$-CH$_2$-NH-CO-CH(NH_3^+)-CH$_2$-Ph)$_2$, 6H), 1.226 (s, CH$_3$-*(C*$\underline{H_2}$*)$_{14}$*-CH$_2$-CH$_2$- of R group , 28H), 0.866-0.832 (t, *C*$\underline{H_3}$-(CH$_2$)$_{14}$-CH$_2$-CH$_2$- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d$_6$) δ: 171.80, 167.81, 167.59, 158.57, 158.26, 135.05, 129.44,

128.45, 127.07, 53.64, 45.01, 42.80, 37.15, 37.09, 36.57, 36.29, 32.08, 31.30, 29.06, 29.02, 28.82, 28.72, 28.35, 27.27, 25.09, 22.10, 13.93; HRMS (m/z): 692.5433 [$(M+H)^+$] (Observed), 692.5479 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(^{L}Phe)amido}propyl]α-naphthylacetamide bis(trifluoroacetate) (PNP):** FT-IR (NaCl): 3273 cm^{-1} (-NH- str.), 3036 cm^{-1} (aromatic C-H str.), 2924 cm^{-1} (-CH_2- asym. str.), 2857 cm^{-1} (-CH_2- sym. str.), 1678 cm^{-1} (C=O str.), 1618 cm^{-1}, 1580 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.535-8.316 (m, Naph-CH_2-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>-CO-CH(*N*<u>H_3^+</u>)-CH_2-Ph)$_2$, 8H), 7.931-7.206 (m, Naph<u>*H*</u>-CH_2-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph<u>*H*</u>)$_2$, 17H), 4.061-4.018 (t, Naph-CH_2-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NH_3^+)-CH_2-Ph)$_2$, 2H), 3.963-3.947 (d, Naph-*C*<u>*H*$_2$</u>-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph)$_2$, 2H), 3.533-2.954 (m, Naph-CH_2-CO-N(-*C*<u>*H*$_2$</u>-CH_2-*C*<u>*H*$_2$</u>-NH-CO-CH(NH_3^+)-*C*<u>*H*$_2$</u>-Ph)$_2$, 12H), 1.648-1.487 (m, Naph-CH_2-CO-N(-CH_2-*C*<u>*H*$_2$</u>-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph)$_2$, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 170.10, 167.88, 167.66, 158.75, 158.43, 135.09, 133.40, 132.93, 132.17, 129.48, 128.50, 127.12, 125.98, 125.66, 125.49, 124.16, 118.62, 115.65, 53.68, 45.60, 43.25, 36.69, 36.45, 28.26, 27.35; HRMS (m/z): 594.3456 [$(M+H)^+$] (Observed), 594.3444 [$(M+H)^+$] (Calculated).

***N*^{1},*N*^{3}-bis-(^{L}Phe)-*N*^{1}-[{3-(^{L}Phe)amido}propyl]propane-1,3-diamine tris(trifluoroacetate) (PPP):** FT-IR (NaCl): 3275 cm^{-1} (-NH- str.), 3036 cm^{-1} (aromatic C-H str.), 2923 cm^{-1} (-CH_2- asym. str.), 2856 cm^{-1} (-CH_2- sym. str.), 1680 cm^{-1} (C=O str.), 1617 cm^{-1}, 1579 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.444-8.300 (m, Ph-CH_2-CH(*N*<u>H_3^+</u>)-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>-CO-CH(*N*<u>H_3^+</u>)-CH_2-Ph)$_2$, 11H), 7.323-7.201 (m, Ph<u>*H*</u>-CH_2-CH(NH_3^+)-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph<u>*H*</u>)$_2$, 15H), 4.428-3.936 (t, Ph-CH_2-*C*<u>*H*</u>(NH_3^+)-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NH_3^+)-CH_2-Ph)$_2$, 3H), 3.072-2.645 (m, Ph-*C*<u>*H*$_2$</u>-CH(NH_3^+)-CO-N(-*C*<u>*H*$_2$</u>-CH_2-*C*<u>*H*$_2$</u>-NH-CO-CH(NH_3^+)-*C*<u>*H*$_2$</u>-Ph)$_2$, 14H), 1.438-1.290 (m, Ph-CH_2-CH(NH_3^+)-CO-N(-CH_2-*C*<u>*H*$_2$</u>-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph)$_2$, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 167.86, 167.83, 167.74, 158.50, 158.21, 135.04, 134.98, 134.36, 129.66, 129.47, 129.44, 128.61, 128.55, 128.52, 127.44, 127.18, 53.63, 53.59, 50.15, 44.77, 43.61, 37.36, 37.16, 36.67, 36.23, 27.80, 26.76; HRMS (m/z): 573.3569 [$(M+H)^+$] (Observed), 573.3553 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(^{D}Phe)amido}propyl]dodecanamide bis(trifluoroacetate) (PANA6):** FT-IR (NaCl): 3275 cm^{-1} (-NH- str.), 3034 cm^{-1} (aromatic C-H str.), 2927 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1680 cm^{-1} (C=O str.), 1620 cm^{-1}, 1576 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.483-8.240 (m, R-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>-

CO-CH(*N*$\underline{H_3}^+$)-CH_2-Ph)$_2$, 8H), 7.345-7.222 (m, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph$\underline{H}$)$_2$, 10H), 3.945 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*$\underline{H}$(NH_3^+)-CH_2-Ph)$_2$, 2H), 3.191-2.893 (m, R-CO-N(-*C*$\underline{H_2}$-CH_2-*C*$\underline{H_2}$-NH-CO-CH(NH_3^+)-*C*$\underline{H_2}$-Ph)$_2$, 12H), 2.203-2.169 (t, CH_3-$(CH_2)_8$-CH_2-*C*$\underline{H_2}$- of R group, 2H), 1.531-1.449 (m, CH_3-$(CH_2)_8$-*C*$\underline{H_2}$-CH_2-CO-N(-CH_2-*C*$\underline{H_2}$-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph)$_2$, 6H), 1.225 (bs, CH_3-*(C*$\underline{H_2}$*)*$_{\underline{8}}$-CH_2-CH_2- of R group , 16H), 0.853-0.836 (t, *C*$\underline{H_3}$-$(CH_2)_8$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 171.78, 167.78, 167.56, 158.33, 158.02, 135.00, 129.41, 128.45, 127.08, 53.61, 44.97, 42.76, 37.13, 37.07, 36.55, 36.27, 32.05, 31.28, 29.01, 29.00, 28.88, 28.78, 28.68, 28.53, 28.34, 27.26, 25.06, 24.48; HRMS (m/z): 608.4510 [$(M+H)^+$] (Observed), 608.4540 [$(M+H)^+$] (Calculated).

***N*^{1}-(**^{L}**Phe)-*N*^{3}-[{3-(**^{L}**Phe)amino}propyl]propane-1,3-diamine tris(trifluoroacetate) (PP):** FT-IR (NaCl): 3273 cm^{-1} (-NH- str.), 3036 cm^{-1} (aromatic C-H str.), 2924 cm^{-1} (-CH_2- asym. str.), 2857 cm^{-1} (-CH_2- sym. str.), 1678 cm^{-1} (C=O str.), 1617 cm^{-1}, 1579 cm^{-1} (aromatic C=C str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.502-8.242 (m, *N*$\underline{H_2}^+$(-CH_2-CH_2-CH_2-*N*$\underline{H}$-CO-CH(*N*$\underline{H_3}^+$)-CH_2-Ph)$_2$, 10H), 7.360-7.214 (m, NH_2^+(-CH_2-CH_2-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph$\underline{H}$)$_2$, 10H), 3.927-3.912 (t, NH_2^+(-CH_2-CH_2-CH_2-NH-CO-*C*$\underline{H}$(NH_3^+)-CH_2-Ph)$_2$, 2H), 3.171-2.719 (m, NH_2^+(-*C*$\underline{H_2}$-CH_2-*C*$\underline{H_2}$-NH-CO-CH(NH_3^+)-*C*$\underline{H_2}$-Ph)$_2$, 12H), 1.644-1.614 (m, NH_2^+(-CH_2-*C*$\underline{H_2}$-CH_2-NH-CO-CH(NH_3^+)-CH_2-Ph)$_2$, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 171.07, 161.54, 161.21, 135.98, 130.43, 129.94, 128.58, 119.21, 116.27, 113.34, 55.59, 45.85, 37.12, 26.38, 23.85; HRMS (m/z): 426.2879 [$(M+H)^+$] (Observed), 426.2869 [$(M+H)^+$] (Calculated).

3.4 Experimental procedure

3.4.1 Antibacterial assay

The same protocol was followed as described in the section of 2.4.1 in Chapter 2.

3.4.2 Time-kill kinetics assay

The bactericidal activity of the compounds was evaluated by performing time kill kinetics assay.[103] This gives the information about the rate at which the compounds are acting on bacteria. Briefly, *S. aureus* was grown in yeast-dextrose broth at 37ºC for 6 h. The compounds **PANA2** and **PANA6** were added to the bacterial solution (*S. aureus* of approximately 1.8×10^5 cfu/mL) with the working concentration of 25 μM (about 6×MIC). This was incubated at 37°C. At different time intervals (0, 1, 2, 3, 4, 6, 12 and 24 h) 20 μL of aliquots from that solution were serially diluted 10-fold in 0.9 % saline. Then from the dilutions, 20 μL was plated on yeast-dextrose agar plates and incubated at 37ºC for 24 h. The bacterial colonies were counted and results are represented in logarithmic scale, i.e. $\log_{10}$ (cfu/mL). The second experiment has been performed using a similar protocol at a shorter time gap of 0, 5, 15, 30, 45 and 60 min to find out the exact time required to show bactericidal activity.

3.4.3 Hemolytic assay

The same protocol was followed as described in the section of 2.4.2 in Chapter 2.

3.4.4 Antibacterial assay in presence of human plasma

To examine the susceptibility of the **PANA** derivatives towards plasma proteases, the antibacterial activity was tested in presence of 50% of plasma.[103] Briefly, 250 μL of **PANA2** and **PANA6** were added into 250 μL of fresh human plasma and incubated at 37ºC. At

specified time intervals of 0 h, 2 h, 6 h and 24 h the aliquot was 2-fold diluted in 0.9% saline. Now antibacterial activities were tested against *S. aureus* by following the same protocol as described above for antibacterial assay. The MIC values were determined and result has been compared with the experiment in absence of plasma.

3.4.5 Enzyme stability

In order to find out the stability of **PANA** derivatives towards enzymatic degradation, the most potent compound **PANA2** and its corresponding "D" analogue **PANA6** were dissolved in 0.1 M of NH_4HCO_3 to a final concentration 1 mg/mL. Then trypsin solution was prepared by dissolving 1 mg in 50 mL of 0.1 M NH_4HCO_3 buffer.[48] This freshly prepared trypsin solution (200 μL) and 200 μL of compounds (1 mg/mL) solution were incubated in 1600 μL of 0.1 M NH_4HCO_3 buffer at 37ºC. At a specified time intervals (0 min, 15 min, 1 h, 6 h and 24 h), 200 μL of aliquots were taken and diluted with 200 μL of milipore water. These diluted aliquots were then analysed by using LC-MS. For negative control, same concentration of compounds was made without trypsin. Enzymatic degradation of the compounds can be observed by monitoring the signal of total ion current (TIC) in the chromatogram. A decrease in TIC signal indicates the degradation of the compound.

3.4.6 Mechanism of Action

3.4.6.1 Membrane permeabilization assay

The 6 h grown culture (midlog phase) of *E. coli and S. areus* were harvested (3500 rpm, 5 min), washed, and resuspended in 5 mM glucose and 5 mM HEPES buffer at pH 7.2 (1:1).[48, 103] Then 10 μL of test compounds **PANA2** and **PANA6** were added to a cuvette containing 2 mL of bacterial suspension and 10 μM of propidium iodide (PI). In case of *E. coli* bacteria colistin was also included to compare the potential of the compounds in inner membrane permeabilization. Fluorescence has monitored at excitation wavelength of 535 nm (slit width: 10 nm) and emission wavelength of 617 nm (slit width: 5 nm). As a measure of inner membrane permeabilization the uptake of PI was monitor by the increase in fluorescence for 10 min.

3.4.6.2 Cytoplasmic membrane depolarization assay

The 6 h grown culture (midlog phase) of *S. areus* were harvested (3500 rpm, 5 min), washed in 5 mM glucose and 5 mM HEPES buffer (pH 7.2) in 1:1 ratio. And resuspended in 5 mM HEPES buffer, 5 mM glucose and 100 mM KCl solution in 1:1:1 ratio. [48, 103] Then 2 μM of 3, 3′-Dipropylthiadicarbocyanine iodide ($DiSC_3$) was added to a cuvette containing 2 mL of bacterial suspension and preincubated for 20 min. The fluorescence was monitored at excitation wavelength of 622 nm (slit width: 10 nm) and emission wavelength of 670 nm (slit width: 5 nm). Then 10 μL of test compounds **PANA2** and **PANA6** were added to the cuvette containing bacterial suspension and $DiSC_3$ after 2 min of fluorescence measurement. As a measure of membrane depolarization fluorescence was monitor for another 13 min.

3.4.6.3 K^+ leakage assay

The 6 h grown culture (midlog phase) of *E. coli and S. areus* were harvested (3500 rpm, 5 min), washed, and resuspended in 10 mM HEPES buffer and 0.5% glucose in 1:1 ratio. Then 2 mL of the bacterial suspension was placed in a cuvette. Then fluorescence was measured at excitation wavelength of 346 nm (slit width: 10 nm) and emission wavelength of 505 nm (slit width: 5 nm) for 50 sec at room temperature. Then PBFI-AM dye (1 μM) was added and fluorescence was monitored for another 150 sec. Finally the 10 μL of test compounds **PANA2** and **PANA6** were added to the cuvette containing bacterial suspension and PBFI-AM dye and fluorescence were monitored for another 800 sec. As a measure of K^+ leakage the increase in fluorescence signals was measured.

3.4.7 Resistance study

For resistance study the control antibiotics norfloxacin and colistin were chosen for *S. aureus* and for *E. coli*, respectively. At first, the MIC value of compound **PANA6** was determined against *S. aureus* and *E. coli* as described above in antibacterial activity. In case of norfloxacin and colistin also the initial MIC values were determined against respective bacteria. After the initial MIC experiment, serial passaging was initiated by harvesting bacterial cells growing at the sub-MIC concentration of the compounds (at [MIC]/2) and was subjected to another MIC assay. After 24 h incubation period, cells growing in the sub-MIC concentration of the compound (at [MIC]/2) from the previous passage were once again harvested and assayed for the MIC.[80] The process was repeated for 20 passages. The

fold of MIC increased for test compound and control antibiotics were plotted against the number of days.

3.4.8 Biofilm inhibition assay

6 h grown culture (midlog phase) of *S. aureus* (10^9 cfu/mL) were diluted to the concentration of 10^5 cfu/mL into the nutrient broth with a 0.5% glucose to make the bacterial stock solution.[110] Compound **PANA6** was serially (2-fold) diluted in sterile millipore water and 25 µL of these serial dilutions were added to the wells of 96-well plates. Then the 75 µL of this bacterial stock solution (10^5 cfu/mL) were added into compound containing 96-well plates. The plates were then incubated under stationary conditions for 24 h at 37ºC. After incubation, the medium was removed from the wells and washed for single time with 1×PBS buffer (pH = 7.4). Then 0.1% of crystal violet (CV) solution was prepared in sterile milipore water and 100 µL were added into the wells to stain the bacteria. The stained plates were incubated for 30 min at 37ºC. Then crystal violet solution was discarded and the plates were washed again with 1×PBS (pH = 7.4) buffer. The residual stain was solubilized with 200 µL of 95% ethanol solution. An amount of 10 µL of solubilized stain was then transferred from each well to the corresponding wells of another 96-well plate containing 90 µL of 95% ethanol and O. D. at 540 nm was recorded using TECAN (Infinite series, M200 pro) Plate Reader. Biofilm inhibition was quantified by subtracting the O. D. values of background where no biofilm was formed.

3.5 Results

3.5.1 Antibacterial activity

Like in the previous case, the activity of the phenylalanine conjugated aliphatic norspermidine analogues (**PANAs**) and other control compounds were determined (Table 3.1) against different Gram-positive (*S. aureus* and *E. faecium*) and Gram-negative (*E. coli*) bacteria including drug resistant bacteria MRSA, VRE and *K. pneumoniae*.

Table 3.1: Antibacterial activity of the phenylalanine conjugated aliphatic norspermidine analogues (**PANAs**).

	Minimum Inhibitory Concentration (μM)					
	Drug sensitive strains			Drug resistant strains		
Compounds	*S. aureus*	*E. faceium*	*E. coli*	MRSA	VRE	*K. pneumonie*
PANA1	19	36	24	25	17	70
PANA2	4.3	4.8	12	4	2.7	9.7
PANA3	7	5.2	16	4.8	2.8	18
PANA4	10	8.5	255	5.2	3	70
PANA5	12	11	270	5.6	3.2	95
PNP	>304	>304	>304	ND	>304	>304
PPP	>318	>318	>318	ND	>318	>318
PANA6	4.2	3.3	12	5	2.9	7.8
PP	>325	>325	>325	ND	>325	>325
Colistin	20	>199	0.4	54	>199	1.2
Vancomycin	0.63	0.6	ND	0.63	>500	ND

ND stands for "not determined". MRSA is methicillin-resistant *S. aureus*, VRE is vancomycin-resistant *E. faecium* and *K. pneumoniae* is resistant to β-lactam antibiotics.

All the **PANA** derivatives showed potent antibacterial activity against all the bacteria tested. The MIC values reflect an immense improvement in antibacterial efficacy over the previously reported **ANA** compounds (Chapter 2). Among the **PANA** derivatives, **PANA1** which contains a decyl chain was found to be least effective against all tested bacteria. **PANA1** has MIC values of 19 μM and 24 μM against *S. aureus* and *E. coli*, respectively. The dodecyl analogue, compound **PANA2** displayed excellent antibacterial activity with MIC values of 4.3 μM and 12 μM against *S. aureus* and *E. coli*, respectively. However, further increase in long chain to the tetradecyl analogue, compound **PANA3** resulted in a decrease in antibacterial activity. Compound **PANA3** displayed MIC values of 7 μM and 16 μM against *S. aureus* and *E. coli*, respectively. Further increase in long chain yielded **PANA4** (hexadecyl analogue); which displayed a decreased efficacy (MIC values of 12 μM and 270 μM against *S. aureus* and *E. coli*, respectively). The highest long chain analogue (octadecyl) **PANA5** followed the same trend and was found to be least active against gram-negative bacteria. Thus, compound **PANA2** with C-12 aliphatic chain was found to be most potent antibacterial agent among the compounds in the series.

Activity of **PANA2** against *E. faecium* (MIC = 4.8 μM), needs special mention because it was 40 fold more active than colistin. Compounds **PANA3**, **PANA4** and **PANA5** also exhibited considerable activity against *E. faecium* with the MIC values of 5.2 μM, 8.5 μM and 11 μM, respectively.

The fact that introduction of phenylalanine moiety was indeed an improvement to the existing design is reflected in the activity of compound **PANA2** against MRSA (MIC value of 4 μM) while unconjugated analogue **ANA4** had 19 μM (Chapter 2). Among the others, the compounds **PANA3**, **PANA4** and **PANA5**, displayed excellent activity with the MIC values of 4.8 μM, 5.2 μM and 5.6 μM, respectively against MRSA.

PANA2 also showed superior activity against VRE (MIC 2.7 μM), highlighting the effectiveness of the design. Compound **PANA2** exhibited >180 times better potency than vancomycin which was inactive till a concentration of 500 μM. **PANA3**, **PANA4** and **PANA5**, with the MIC values of 2.8 μM , 3 μM and 17 μM respectively also exhibited potent activity against VRE. The most potent analogue **PANA2** also showed good activity against the β-lactum resistant *K. pneumoniae* with the MIC value of 9.7 μM whereas the other compounds in the series showed moderate activity against this bacterium.

The compound **PNP** consisting of a naphthalene moiety in the place of aliphatic chain did not show any antibacterial activity even at 304 μM against all the tested bacteria. Similarly, **PPP** having a phenylalanine group instead of the aliphatic chain, also remained inactive even at concentration of 318 μM. But, the derivative **PANA6** (dodecyl long chain**)** consisting of "D" isomers of phenyl alanine displayed similar antibacterial efficacy compared to most potent compound **PANA2** (Table 3.1). Finally, the compound **PP** lacking the aliphatic chain showed complete loss of activity emphasizing on the importance of aliphatic chain of **PANA** derivatives.

3.5.2 Bactericidal kinetics

To shed some light on the nature of the compounds time-kill kinetics of **PANA2** and **PANA6** against *S. aureus* was performed. The compounds showed rapid-bactericidal activity, typical of mimics of antimicrobial peptides or lipopeptides. From the initial assay (Figure 3.2) both the tested compounds revealed approximately 5 $\log_{10}$ (cfu/mL) reduction in the number of viable bacteria within an hour at a concentration of 6 × MIC. In order to find out the exact time required to exhibit bactericidal activity, experiment was done for

PANA6 in a smaller time gap ('min'scale) with the same concentration 6 × MIC of compound. Compound **PANA6** showed ~4 $\log_{10}$ (cfu/mL) reduction within 15 minutes only (inset of Figure 3.2B).

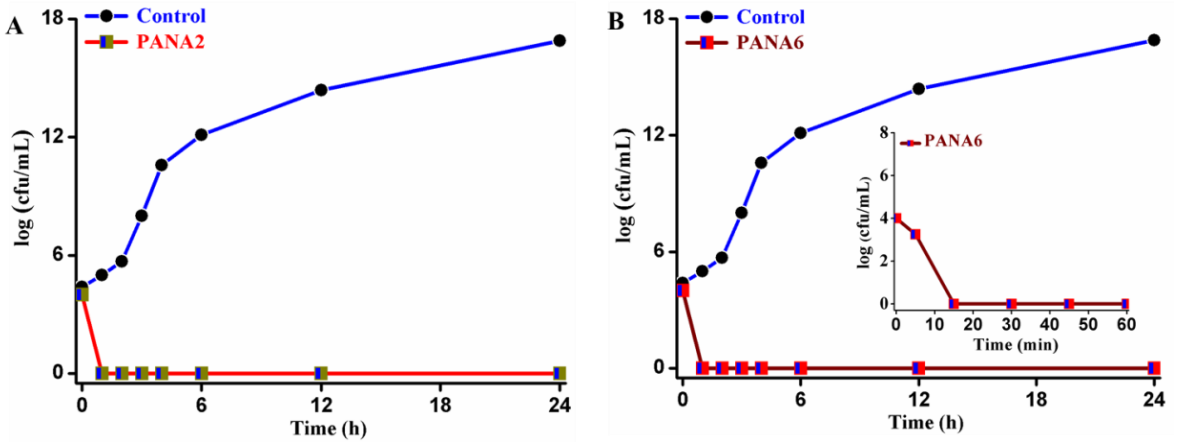


Figure 3.2: Time-kill kinetics against *S. aureus* of (A) **PANA2 (**6 × MIC) (B) **PANA6 (**6 × MIC).

3.5.3 Toxicity

In order to establish selective activity of the compounds towards bacterial cells over mammalian cells, their HC_{50} values were determined.

Table 3.2: Hemolytic activity of phenylalanine conjugated aliphatic norspermidine analogues (**PANAs**).

Compounds	HC_{50} (μM)
PANA1	232
PANA2	205
PANA3	144
PANA4	83
PANA5	71
PANA6	250

The HC_{50} values of these **PANA** derivatives ranged from 250 μM to 71 μM. The compound with the shortest alkyl chain in the series, **PANA1** had an HC_{50} value of 232 μM.

Compound **PANA2**, which displayed most potent antibacterial efficacy, was found to have HC_{50} value of 205 μM. The corresponding "D" isomer, compound **PANA6** showed lesser toxicity with the HC_{50} value of 250 μM. Further increase in aliphatic chain also prompted an increase in haemolytic activity.

3.5.4 Antibacterial efficacy in human plasma

Protease degradation is responsible for instability of antibacterial peptides (AMPs and lipopeptides), in which subsequently results in a decrease in antibacterial activity in the plasma condition. In order to determine the stability of **PANA** derivatives, antibacterial activity in presence of 50% plasma was determined. The model compounds selected for the study were the most potent derivatives **PANA2** and **PANA6**. In presence of 50% human plasma, a decrease in antibacterial activity was observed in case of compound **PANA2** up to 6 h of incubation (Figure 3.3A), but the corresponding "D" analogue **PANA6** did not reveal any loss in activity even at 24 h incubation (Figure 3.3). This indicates that corresponding "D" analogues of **PANA** derivatives are stable in plasma and do not lose activity.

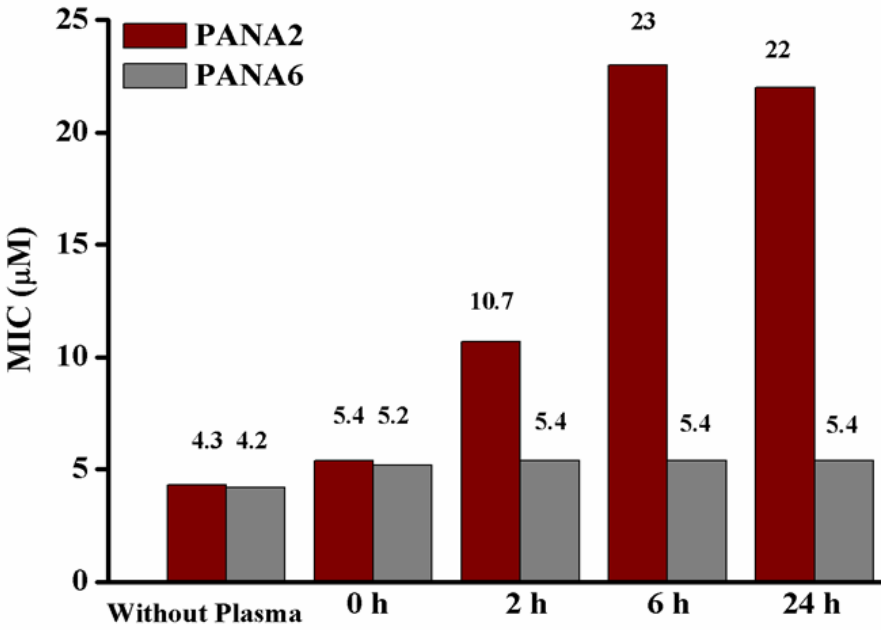


Figure 3.3: Antibacterial activity in plasma of **PANA2** and **PANA6** against *S. aureus* (Numbers in the figure represent the MIC values).

3.5.5 Stability towards enzymatic degradation

To investigate the decrease in antibacterial potency of **PANA2** (consisting of L phenyl alanine) in presence of human plasma further, the enzyme stability study was performed using the protease trypsin. The compounds were incubated with trypsin for 24 hours and at several time intervals, aliquots were taken out for analysis in LC-MS. In case of **PANA2,** it was found that total ion current (TIC) initially decreased from 0 min to 15 min. Further incubation resulted in no further decrease and the TIC remained constant even up to 24 h. This indicated that the compound degraded in the time interval of 0-15 minutes after incubation with trypsin. But, the corresponding "D" isomer **PANA6** did not show any decrease in TIC signal even up to 24 h incubation. This is confirmed that "D" isomer containing analogues are resistant to proteases degradation. Result obtained from this experiment thus supports the observation that antibacterial efficacy decreases in human plasma for the "L" analogue.

3.5.6 Mechanism of action

To probe the mechanism by which **PANA** derivatives act on bacteria, investigations were carried out to ascertain the membrane depolarization, K^+ ions leakage and membrane permeabilization abilities of the compounds using spectroscopic techniques. For these studies, the most potent analogues **PANA2** and **PANA6** were selected as model compounds. The bacterial membrane permeabilization was studied using the fluorescent probe propidium iodide (PI). Propidium iodide is a DNA intercalating agents and fluoresces only upon binding to DNA. Since it cannot enter intact bacterial cells, initially it does not show any fluorescence. However, if any compound causes bacterial membrane disruption, PI finds entry inside the cell and fluoresces upon binding to the DNA. As can be observed from Figure 3.4A and 3.4B, both the compounds were efficient in permeabilizing the membranes of both gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria.

Membrane depolarization properties of the compounds were studied using the membrane-potential sensitive dye $DiSC_3$. This dye, depending upon the membrane potential gradient, distributes itself between the cell interior and the medium. Upon initial intake of the dye by the bacterial cells, self-quenching occurs and thus there is a subsequent loss of fluorescence. If addition of compounds leads to loss of membrane potential, the fluorescence intensity increases. Figure 3.4C shows that both the compounds were able to depolarize the membranes of *S. aureus*. In order to investigate the ability of the compounds to cause

leakage of K^+ ions fluorescence spectroscopic studies with PBFI-AM dye carried out. The free dye is not fluorescent; however upon K^+ binding, there is an increase in fluorescence. As can be observed in Figure 3.4D and Figure 3.4E, both the compounds caused significant leakage of K^+ ions.

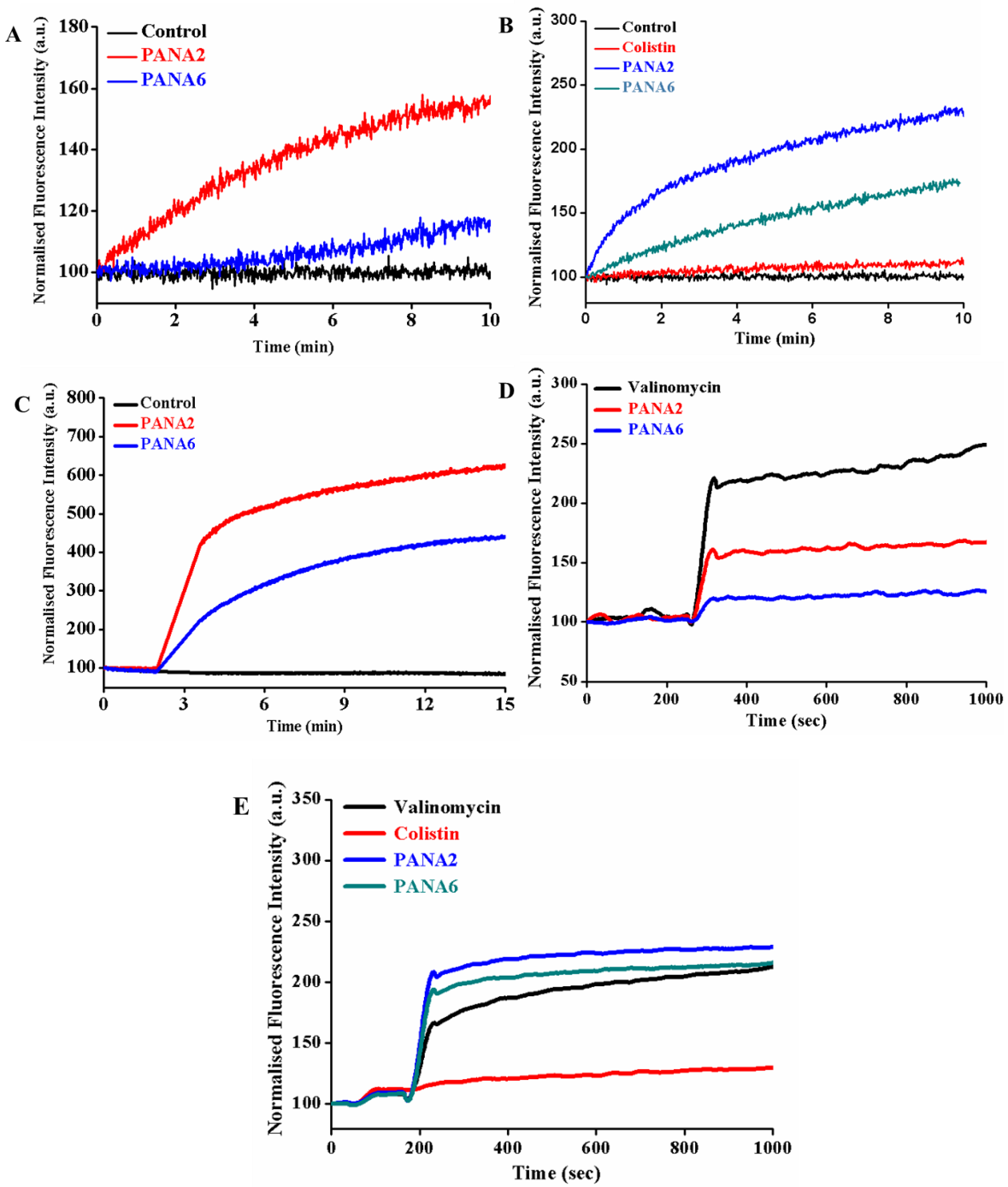


Figure 3.4: A) Membrane permeabilization of *S. aureus* by **PANA2** (12 × MIC) and **PANA6** (12 × MIC). B) Membrane permeabilization of *E. coli* by **PANA2** (12 × MIC) and **PANA6** (12 × MIC). C) Membrane depolarization of *S. aureus* by **PANA2** (12 × MIC) and

PANA6 (12 × MIC). D) K^+ leakage of *S. aureus* by **PANA2** (12 × MIC) and **PANA6** (12 × MIC). E) K^+ leakage of *E. coli* by **PANA2** (12 × MIC) and **PANA6** (12 × MIC).

3.5.7 Propensity to induce bacterial resistance

The emergence of antibiotic-resistant bacteria is a major problem to global health. Hence, to investigate the potential of **PANA** derivatives as an antibacterial agent with sufficient longevity, the ability of *S. aureus* (Gram-positive representative) and *E. coli* (Gram-negative representative) bacteria to develop resistance against these compounds were investigated. For this study compound **PANA6** has chosen as the model compound. Norfloxacin was used as a positive control for *S. aureus*, whereas colistin was used in case of *E. coli*. Results show no change in the MIC for the compound **PANA6** against both the bacteria even after 20 passages (Figure 3.5A and 3.5B) whereas around 800 fold increase in MIC was observed in case of norfloxacin and 250 fold in case of colistin. This study suggests that bacteria find it difficult to develop resistance against these **PANA** derivatives.

3.5.8 Biofilm inhibition

PANA6 also revealed an ability to inhibit the formation of bacterial biofilm (*S. aureus*). Complete inhibition of biofilm formation was observed at a concentration as low as 10 μM (almost double of MIC). In comparison to 100% viability (as observed in control), only 2-4% viability was observed at this compound concentration (Figure 3.5C).

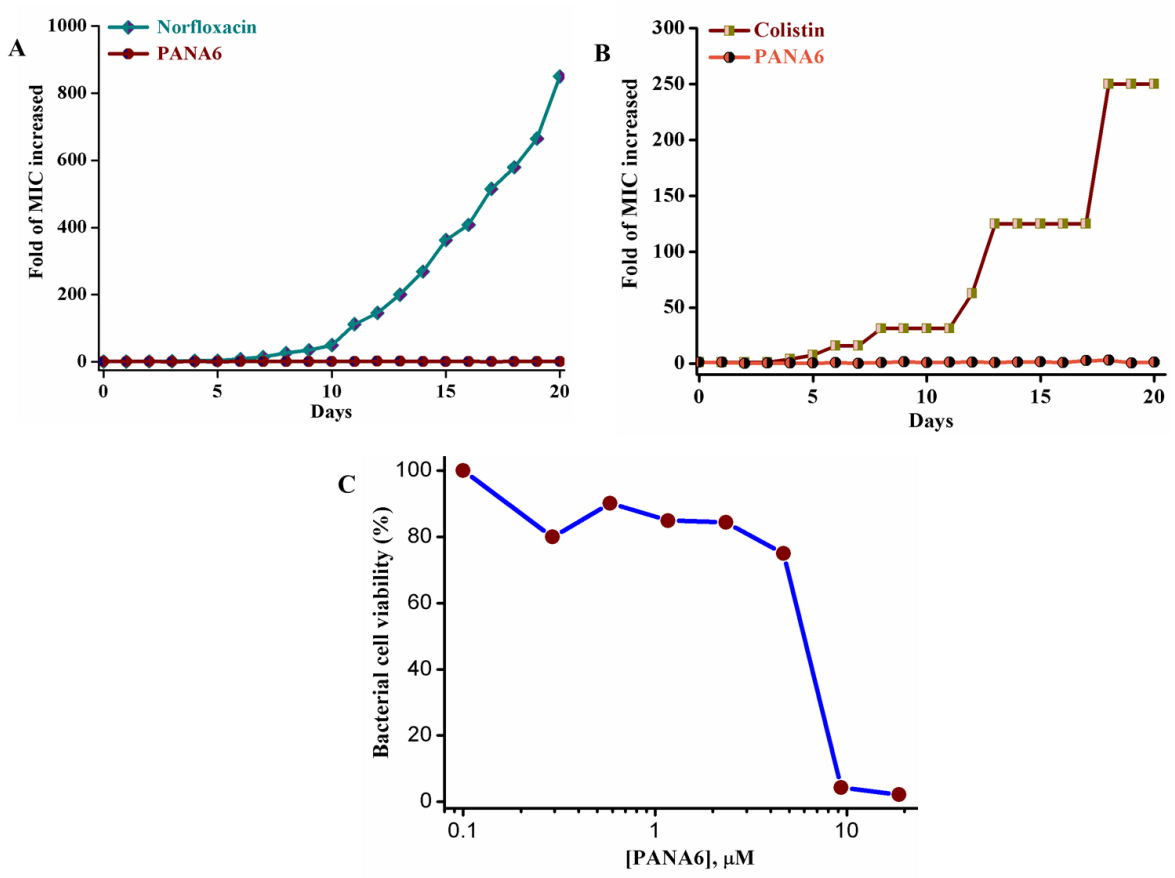


Figure 3.5: A) Development of resistant of *S. aureus* against norfloxacin and **PANA6. B)** Development of resistant of *E. coli* against colistin and **PANA6**. C) Inhibition of biofilm formation of *S. aureus* with **PANA6.**

3.6 Discussion

The previously (Chapter 2) developed **ANA** lipopeptide mimics exhibited a narrow spectrum of antibacterial activity, and considerable amount of toxicity towards human erythrocytes. To address the limitation, we designed of mimics of lipopeptide based on phenylalanine coupled norspermidine derivatives bearing a single long aliphatic chain, involving only three synthetic steps.

The introduction of phenylalanine moiety into the design was expected to bring about significant changes in the physical properties of the compounds. The positive charges now are contributed by the α-NH_2 groups of the phenylalanine moiety. There is a significant difference in the pKa values of the two NH_2 groups[111] This difference sometimes plays an important role towards improving antibacterial activity. What it also does is that it spaces out the positive charges a little further away from each other. This significantly changes the

properties of the compounds. The two amide linkages resulting from the coupling of the amino acids also provides a possibility of H-bonding interactions. These interactions are also essential for high antibacterial activity as it allows better interaction of the compounds with the lipid head groups in the bacterial cell membrane. As mentioned earlier, several groups around the world have used aromatic moieties in order to bring about improved hydrophobicity. We have observed in this study too that introduction of such aromatic moieties indeed plays an important role towards improvement of antibacterial activity. Whether the improved antibacterial activity is specifically because of the introduction of the aromatic moiety or is culmination of all the properties put together is a matter of debate. However, we strongly believe that all the properties put together play an important part towards achieving such potent antibacterial activity.

On observing the antibacterial activities closely we found that the SAR shows parabolic pattern of chain length dependant antibacterial activity (Figure 3.6). This is a well-known fact in the literature as optimization of amphiphilicity is the most important factor directing selective antibacterial activity. Indeed, the fact that the replacement of L-isomer with D-isomer brings about no significant change in antibacterial activity is hardly a matter of surprise. Such small molecules, with only two amino acid residues are not expected to change much with respect to structure in solution. However, if the molecules rely on specific interactions for antibacterial action other than membrane damaging abilities, then such changes can affect antibacterial activity significantly. However, subtle variations cannot be ruled out entirely.

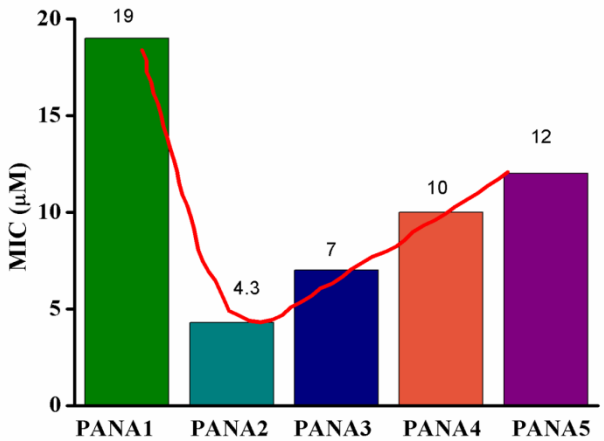


Figure 3.6: Variation of antibacterial activity against *S. aureus* with aliphatic chain length. (Numbers in the graph represent the MIC values).

The importance of aliphatic chain is established by the fact neither **PPP** (phenylalanine group in place of aliphatic group), **PNP** (1-ethylnaphthalene group in place of aliphatic group) nor **PP** (lacks aliphatic group) shows any significant antibacterial activity. The long flexible chain allows better interaction with the bacterial cell membrane. Even the naphthalene moiety with ten carbon atoms (PNP) was not able to show any significant activity. This is probably because structural flexibility is an essential condition for potent antibacterial activity.[38-39. 48]

Coming to activity against various classes of bacteria, we observe that in all the cases the activity against gram-positive bacteria is around 1.5 to 2 fold better than the corresponding activity against gram-negative bacteria. In fact the difference is much more prominent in case of **PANA4** and **PANA5**, which do not show activity at 250 μM. This might be due to the difference in the cell membrane of Gram-positive and Gram-negative bacteria. Another interesting observation is that almost all the compounds showed good antibacterial activity against drug-resistant species. As there is a dearth of antibiotics against drug-resistant bacteria, this important observation becomes a highlight of this series of compounds.

The rapid bactericidal properties of the compounds suggests that the possible mechanism of action of the compounds might be through disruption of bacterial membrane integrity It was also observed that these compounds kill bacteria in less than 15 minutes of exposure. The membrane lytic properties of the compounds were confirmed by the membrane de-stabilization experiments carried out with the compounds. Although concentration of compounds to show the membrane permeabilization of bacteria was higher than their corresponding MIC, even a small perturbation is enough to bring about significant damage. What is interesting in this study is the difference in fluorescence intensity brought about by the two compounds **PANA2** and **PANA6**. Similar observation was also made in their abilities to depolarize the cell membrane. This opens up the possibility of **PANA6** having alternative modes of action, as in all the cases the membrane damaging property of **PANA2** is more, but there is no significant difference in antibacterial activity.

The fact that bacteria find it difficult to grow resistance against these compounds in as many as 20 passages significantly enhances the potential of these compounds as possible

clinical candidates. Since the compounds also possess an ability to inhibit bacterial biofilm formation, an additional application of the compounds as coatings on medical devices has been brought forth. Although several toxicity studies, both *in-vitro* and *in-vivo* need to be performed in order to ascertain the actual potential of the compounds as future antibiotics, it is beyond doubt that they bear much promise.

3.7 Conclusion

In conclusion, a series of phenylalanine conjugated aliphatic norspermidine analogues have been developed through a simple synthetic strategy as lipopeptide mimics, which displayed improved antibacterial efficacy compared to **ANA4**. The most potent compound of this series **PANA2** (dodecyl analogue) and its "D" isomer analogue **PANA6** not only displayed >170 more antibacterial activity against VRE, but also was non-haemolytic at that concentration. These derivatives kill the bacteria rapidly, primarily by damaging the cell membrane, without allowing the bacteria to develop resistance against them. Although these compounds displayed improved and more selective activity towards bacterial cells, activity against Gram-negative bacteria leaves a lot of scope for improvement. However, these compounds are a much better improvement of the **ANA** derivatives and bear much promise as future antibiotics.

Chapter 4

Synthesis and Antibacterial Properties of Lysine Conjugated Aliphatic Norspermidine Analogues

Abstract

Although efficient antibacterial potency was achieved for the **PANA** derivatives reported in Chapter 3, the antibacterial activity against Gram-negative bacteria was not satisfactory. In order to achieve better activity against Gram-negative bacteria, we rationalised that the number of positive charges must be increased. Herein, we describe the synthesis, characterization, bactericidal activity and mechanism of action of lysine conjugated aliphatic norspermidine analogues (**LANAs**). The **LANA** derivatives not only exhibited potent antibacterial activity against all the Gram-positive *Staphylococcus aureus*, *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus* (MRSA), vacomycin-resistant *Enterococcus faecium* (VRE) bacteria, but also distinct activity were observed against Gram-negative *E. coli* and β-lactam resistant *Klebsiella pneumoniae*. Toxicity towards human erythrocytes was minimal. Membrane depolarization and leakage of K^+ ions from the cytoplasm were found to be the primary mechanism of action. Bacteria were also not able to develop resistance towards these derivatives. These improved mimics of lipopeptides bear immense potential as broad-spectrum antibacterial agents.

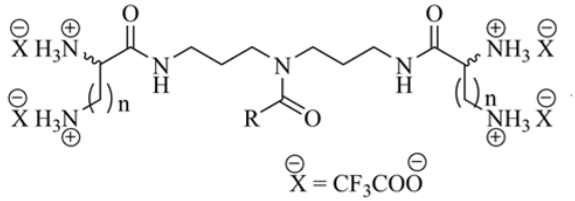
4.1 Introduction

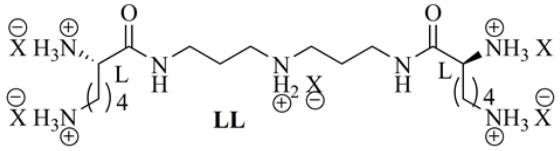
Although **PANA** set of compounds could achieve considerable antibacterial potency compared to the **ANA** derivatives, activity against Gram negative bacteria still needed improvement. Moreover, the compounds (both **ANAs** and **PANAs**) were reasonably toxic. As Gram-negative bacteria have negatively charged lipopolysaccharide as a major constituent of their outer membrane the overall negative charge density is much higher. [112-115] Only two positive charges do not seem to be enough to have a strong electrostatic interaction with the Gram-negative bacteria. Hence, potent activity against Gram-negative bacteria could not be achieved. To address the problem we thought to design the derivatives with more number of charges. Upon increasing the number of charges, electrostatic interaction would be increased. Additionally, there would be an increased possibility of formation of hydrogen bonding interactions. Amino acids with cationic side chains were envisioned to be the most suitable candidates for increasing the positive charges on the aliphatic norspermidine system. Lysine is ubiquitously found in natural AMPs and lipopeptides.[116-118] Use of lysine in the field of antimicrobial peptidomimetics is also abundant primarily because lysine provides the necessary positive charges required for the initial electrostatic interactions. For example, Mor *et al.* have reported several oligomers based on lysine.[41, 93] Bremner *et al.* have reported cationic antibacterial peptoids which also includes lysine moiety in the design.[119] Svendsen *et al.* have used arginines instead of lysine as cationic moieties in their designs.[120] In one of our successful designs of small molecular mimics of AMPs, lysine provided two cationic charges and the compounds showed excellent activity against both Gram-positive and Gram-negative bacteria (including drug-resistant strains).[48] Thus use of lysine instead of phenylalanine seemed to be an effective way of increasing the efficacy of the compounds.

4.2 Structure and Design

These series of derivatives (**LANAs**) were designed to improve the antibacterial activity towards Gram-negative bacteria and reduce the toxicity over the **PANA** derivatives. It was clear from the previous study that additional hydrophobicity was not sufficient. There were two alternatives. Firstly, to chemically modify the existing **PANA** structure, for example to

couple one more amino acid to the α-NH_2 group and secondly, to completely replace phenylalanine by another amino acid. To save synthetic complexities, we chose to replace phenylalanine by lysine moiety. Consequently, the number of positive charges of the compounds increased to four and the hydrophobicity was somewhat reduced to the different aliphatic long chain appendages led to different analogues of **LANAs** (Figure 4.1). To investigate the effect of unsaturation of aliphatic hydrophobicity, the compound **LANA6** and **LANA7** were synthesised by introducing of oleyl and lionleyl group. Like in the previous case, (**PANAs** in Chapter 3) in order to investigate the effect of stereoisoisomer, D-lysine was coupled instead of L-lysine in **LANA8** and **LANA9**. Also, to understand the importance of aliphatic group, in these derivatives too, the control compound **LL** was made which lacked the aliphatic tail. In order to investigate the importance of number of bonds between the charges, lysines were replaced by ornithines to yield **OANA1** (tetradecyl analogue) and **OANA2** (hexadecyl analogue), which changes the ε-NH_2 of **LANAs** as δ-NH_2 in **OANAs**. Moreover we also wanted to study if presence of non-proteogenic amino acid imparted any additional stability.





LL

$X = CF_3COO$

n = 4, Configuration L, L and R=	$-C_9H_{19}$	**LANA1**
	$-C_{11}H_{23}$	**LANA2**
	$-C_{13}H_{27}$	**LANA3**
	$-C_{15}H_{31}$	**LANA4**
	$-C_{17}H_{35}$	**LANA5**
	$-C_{17}H_{33}$	**LANA6**
	$-C_{17}H_{31}$	**LANA7**
n = 4, Configuration D, D and R=	$-C_{13}H_{27}$	**LANA8**
	$-C_{15}H_{31}$	**LANA9**
n = 3, Configuration L, L and R=	$-C_{13}H_{27}$	**OANA1**
	$-C_{15}H_{31}$	**OANA2**

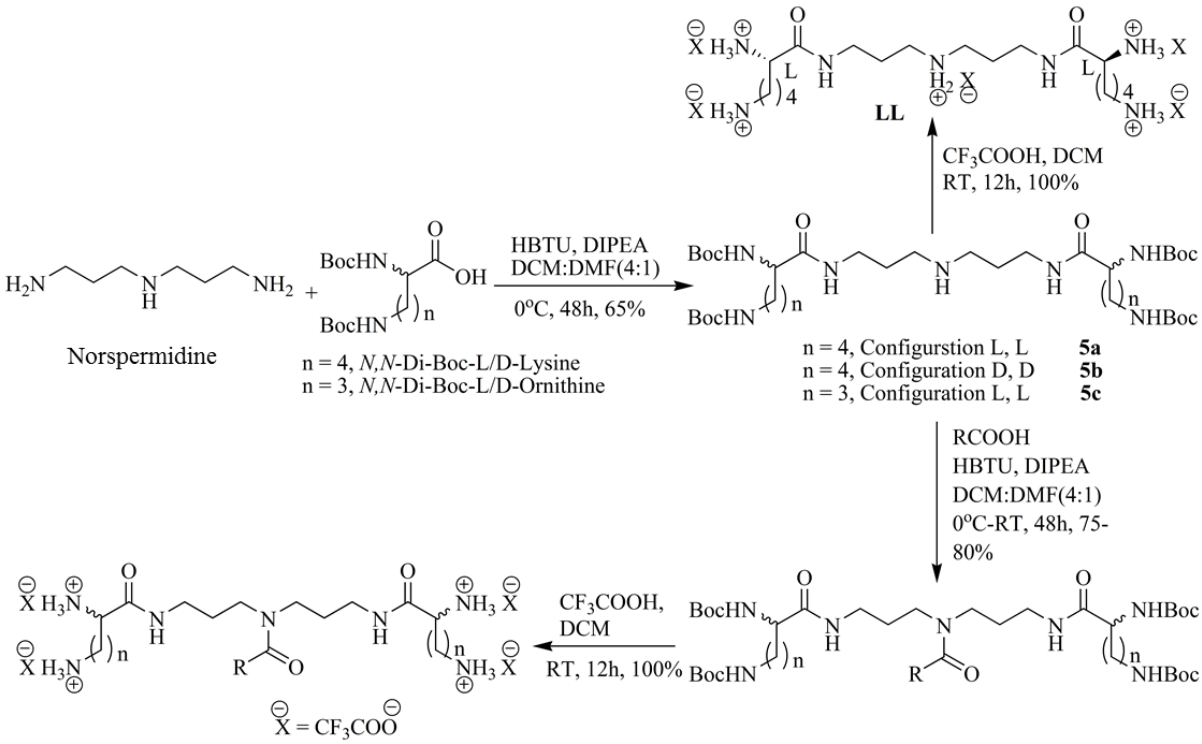
Figure 4.1: Structure of lysine and ornithine conjugated aliphatic norspermidine analogues.

4.3 Synthesis and Characterization

4.3.1 Materials and Methods

The chemicals L-Lysine, D-Lysine and L-ornithine were obtained from Spectrochem and Sigma-Aldrich, respectively. The rest of materials and methods are same as described in the section of 2.3.1 in Chapter 2.

4.3.2 Reaction Scheme



n = 4, Configuration L, L and R=	$-C_9H_{19}$	**LANA1**
	$-C_{11}H_{23}$	**LANA2**
	$-C_{13}H_{27}$	**LANA3**
	$-C_{15}H_{31}$	**LANA4**
	$-C_{17}H_{35}$	**LANA5**
	$-C_{17}H_{33}$	**LANA6**
	$-C_{17}H_{31}$	**LANA7**
n = 4, Configuration D, D and R=	$-C_{13}H_{27}$	**LANA8**
	$-C_{15}H_{31}$	**LANA9**
n = 3, Configuration L, L and R=	$-C_{13}H_{27}$	**OANA1**
	$-C_{15}H_{31}$	**OANA2**

n = 4, Configuration L, L and R =	$-C_9H_{19}$	**6a**
	$-C_{11}H_{23}$	**6b**
	$-C_{13}H_{27}$	**6c**
	$-C_{15}H_{31}$	**6d**
	$-C_{17}H_{35}$	**6e**
	$-C_{17}H_{33}$	**6f**
	$-C_{17}H_{31}$	**6g**
n = 4, Configuration D, D and R =	$-C_{13}H_{27}$	**6h**
	$-C_{15}H_{31}$	**6i**
n = 3, Configuration L, L and R =	$-C_{13}H_{27}$	**6j**
	$-C_{15}H_{31}$	**6k**

Scheme 4.1: Synthesis of lysine and ornithine conjugated aliphatic norspermidine analogues.

Like the **PANA** derivatives, the compounds were synthesized through three steps (Scheme 4.1) reaction. First step involving the selective coupling of the primary amine groups of norspermidine with *N,N*-Di-Boc-L/D-lysine or *N,N*-Di-Boc-L-ornithine. This was accomplished by carrying out amide coupling reaction at 0°C using HBTU as coupling agent for 48 h. Then various aliphatic acids were coupled with the secondary amine through another amide coupling step. In the third step, deprotection of Boc groups was carried out with trifluoroacetic acid (TFA) to get the final compounds (**LANA1-LANA9, OANA1 and OANA2**). Here also the control compound **LL (**without the aliphatic group**)** was made directly adding TFA to the first step product.

4.3.3 Synthetic protocol and characterization

General procedure for synthesizing *N,N*-Di-Boc-L/D-Lysine or *N,N*-Di-Boc-L-Ornithine: About 27 mmol of L/D-Lysine or L-Ornithine was dissolved in H_2O (50 mL), and to it $NaHCO_3$ (3 equivalents) was added and allowed to stirred. Then di-*t*-butylpyrocarbonate (Boc_2O) (1.2 equivalents) in 50 mL of tetrahydrofuran (THF) was added at 0°C. Now the reaction mixture was stirred at room temperature for 12 h. After 12 h, another same amount of Boc_2O (1.2 equivalents) was added at 0°C and the mixture was allowed to stirred for additional 12 h at room temperature. At the end of the reaction, THF was removed under reduced pressure and the aqueous layer was acidified to pH 4-5 using citric acid solution. The aqueous layer was then extracted with dichloromethane (DCM). Finally the organic layer was then washed with brine and dried over anhydrous Na_2SO4. The organic layer was removed under reduced pressure to obtain the compound in 95-98% yield.

***N,N*-Di-Boc-L-Lysine:** Yield- 98%; FT-IR (NaCl): 3341 cm^{-1} (-NH- str.), 2980 cm^{-1} (-CH_2- str.), 1704 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.6 (d,CH(*NH*Boc)(COOH)-CH_2-CH_2-CH_2-CH_2-NHBoc, 1H), 4.9 (s, CH(NHBoc)(COOH)-CH_2-CH_2-CH_2-CH_2-*NH*Boc, 1H), 4.15 (*t*, *CH*(NHBoc)(COOH)-CH_2-CH_2-CH_2-CH_2-NHBoc, 1H), 3.09 (d, CH(NHBoc)(COOH)-CH_2-CH_2-CH_2-*CH*$_2$-NHBoc, 2H), 1.8 -1.67 (m, CH(NHBoc)(COOH)-*CH*$_2$-CH_2-CH_2-CH_2-NHBoc, 2H), 1.54-1.32 (m, CH(NH-COO-*C*(*CH*$_3$)$_3$)(COOH)-CH_2-*CH*$_2$-*CH*$_2$-*CH*$_2$-NH-COO-*C*(*CH*$_3$)$_3$, 22H); HRMS (m/z): 369.2137 [$(M+Na)^+$] (Observed), 369.2002 [$(M+H)^+$] (Calculated).

***N,N*-Di-Boc-D-Lysine:** Yield- 95%; FT-IR (NaCl): 3344 cm^{-1} (-NH- str.), 2982 cm^{-1} (-CH_2- str.), 1703 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.4 (d, CH(*NH*Boc)(COOH)-CH_2-CH_2-CH_2-CH_2-NHBoc, 1H), 4.8 (s, CH(NHBoc)(COOH)-CH_2-CH_2-CH_2-CH_2-*NH*Boc, 1H), 4.17 (*t*, *CH*(NHBoc)(COOH)-CH_2-CH_2-CH_2-CH_2-NHBoc, 1H), 3.05 (d, CH(NHBoc)(COOH)-CH_2-CH_2-CH_2-*CH*$_2$-NHBoc, 2H), 1.82 -1.65 (m, CH(NHBoc)(COOH)-*CH*$_2$-CH_2-CH_2-CH_2-NHBoc, 2H), 1.55-1.31 (m, CH(NH-COO-*C*(*CH*$_3$)$_3$)(COOH)-CH_2-*CH*$_2$-*CH*$_2$-*CH*$_2$-NH-COO-*C*(*CH*$_3$)$_3$, 22H); HRMS (m/z): 369.2088 [$(M+Na)^+$] (Observed), 369.2002 [$(M+H)^+$] (Calculated).

***N,N*-Di-Boc-L-Ornithine:** Yield- 96%; FT-IR (NaCl): 3342 cm^{-1} (-NH- str.), 2981 cm^{-1} (-CH_2- str.), 1705 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.3 (d, CH(*NH*Boc)(COOH)-CH_2-CH_2-CH_2-NHBoc, 1H), 4.6 (s, CH(NHBoc)(COOH)-CH_2-CH_2-CH_2-*NH*Boc, 1H), 4.14 (*t*, *CH*(NHBoc)(COOH)-CH_2-CH_2-CH_2-NHBoc, 1H), 3.02 (d, CH(NHBoc)(COOH)-CH_2-CH_2-*CH*$_2$-NHBoc, 2H), 1.84 -1.62 (m, CH(NHBoc)(COOH)-*CH*$_2$-CH_2-CH_2-NHBoc, 2H), 1.54-1.33 (m, CH(NH-COO-*C*(*CH*$_3$)$_3$)(COOH)-CH_2-*CH*$_2$-*CH*$_2$-NH-COO-*C*(*CH*$_3$)$_3$, 22H); HRMS (m/z): 355.1867 [$(M+Na)^+$] (Observed), 355.1845 [$(M+H)^+$] (Calculated).

General procedure for synthesizing 5a-5c: About 16.6 mmol (2 equivalents) of *N,N*-Di-Boc-L/D-Lysine or *N,N*-Di-Boc-L-Ornithine were dissolved in dry DCM (30 mL) at 0ºC. Then 6 equivalents of DIPEA were added to the reaction mixture followed by 2 equivalents of HBTU. Then DMF (8 mL) was added to the reaction mixture. After 10 minutes, 1 equivalent of norspermidine was added to the reaction mixture drop wise. Next it was allowed to stir for 48 h at 0ºC. Then solvent was evaporated and residue was diluted in ethyl acetate (100 mL). Then work-up was carried out at first with 1N HCl (100 mL, 3 times) followed by saturated Na_2CO_3 solution (100 mL, 3 times). The crude product was collected in ethyl acetate layer. Finally column was done on silica gel (60-120 mess) using methanol and chloroform (5:95) as eluent to isolate the product with 62-65% yield.

***N*^{1}-(Boc-^{L}Lys-Boc)-*N*^{3}-[{3-(Boc-^{L}Lys-Boc)amido}propyl]propane-1,3-diamine (5a):** Yield- 65%; FT-IR (NaCl): 3315 cm^{-1} (-NH- str.), 2929 cm^{-1} (-CH_2- asym. str.), 2866 cm^{-1} (-CH_2- sym. str.), 1700 cm^{-1}, 1673 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.475 (s, NH(-CH_2-CH_2-CH_2-*NH*-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$ 2H), 5.521 (s, NH(-CH_2-CH_2-CH_2-NH-CO-CH(*NH*Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.786 (s, NH(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-*NH*Boc)$_2$, 2H), 4.034 (s,

NH(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.514-3.008 (m, NH(-*C*<u>*H*$_2$</u>-CH_2-*C*<u>*H*$_2$</u>-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*C*<u>*H*$_2$</u>-NHBoc)$_2$, 12H), 2.049 (s, *N*<u>*H*</u>(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 1H), 1.773-1.486 (m, NH(-CH_2-*C*<u>*H*$_2$</u>-CH_2-NH-CO-CH(NHBoc)-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-CH_2-NHBoc)$_2$, 16H), 1.425 (s, NH(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*<u>*H*$_3$</u>*)*$_3$)-CH_2-CH_2-CH_2-CH_2-NH-COO-C*(C*<u>*H*$_3$</u>*)*$_3$)$_2$, 36H); HRMS (m/z): 788.5493 [$(M+H)^+$] (Observed), 788.5497 [$(M+H)^+$] (Calculated).

***N*^{1}-(Boc-^{D}Lys-Boc)-*N*^{3}-[{3-(Boc-^{D}Lys-Boc)amido}propyl]propane-1,3-diamine (5b):** Yield-63%; FT-IR(NaCl): 3316 cm^{-1} (-NH- str.), 2931 cm^{-1} (-CH_2- asym. str.), 2863 cm^{-1} (-CH_2- sym. str.), 1701 cm^{-1}, 1670 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.475 (s, NH(-CH_2-CH_2-CH_2-*N*<u>*H*</u>-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.521 (s, NH(-CH_2-CH_2-CH_2-NH-CO-CH(*N*<u>*H*</u>Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.786 (s, NH(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-*N*<u>*H*</u>Boc)$_2$, 2H), 4.034 (s, NH(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.514-3.008 (m, NH(-*C*<u>*H*$_2$</u>-CH_2-*C*<u>*H*$_2$</u>-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*C*<u>*H*$_2$</u>-NHBoc)$_2$, 12H), 2.049 (s, *N*<u>*H*</u>(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 1H), 1.773-1.486 (m, NH(-CH_2-*C*<u>*H*$_2$</u>-CH_2-NH-CO-CH(NHBoc)-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-CH_2-NHBoc)$_2$, 16H), 1.425 (s, NH(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*<u>*H*$_3$</u>*)*$_3$)-CH_2-CH_2-CH_2-CH_2-NH-COO-C*(C*<u>*H*$_3$</u>*)*$_3$)$_2$, 36H); HRMS (m/z): 788.5497 [$(M+H)^+$] (Observed), 788.5593 [$(M+H)^+$] (Calculated).

***N*^{1}-(Boc-^{L}Orn-Boc)-*N*^{3}-[{3-(Boc-^{L}Orn-Boc)amido}propyl]propane-1,3-diamine (5c):** Yield-62%; FT-IR (NaCl): 3311 cm^{-1} (-NH- str.), 2927 cm^{-1} (-CH_2- asym. str.), 2856 cm^{-1} (-CH_2- sym. str.), 1700 cm^{-1}, 1678 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.472 (s, NH(-CH_2-CH_2-CH_2-*N*<u>*H*</u>-CO-CH(NHBoc)-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.524 (s, NH(-CH_2-CH_2-CH_2-NH-CO-CH(*N*<u>*H*</u>Boc)-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.776 (s, NH(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*N*<u>*H*</u>Boc)$_2$, 2H), 4.134 (s, NH(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NHBoc)-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.504-3.018 (m, NH(-*C*<u>*H*$_2$</u>-CH_2-*C*<u>*H*$_2$</u>-NH-CO-CH(NHBoc)-CH_2-CH_2-*C*<u>*H*$_2$</u>-NHBoc)$_2$, 12H), 2.139 (s, *N*<u>*H*</u>(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-NHBoc)$_2$, 1H), 1.765-1.445 (m, NH(-CH_2-*C*<u>*H*$_2$</u>-CH_2-NH-CO-CH(NHBoc)-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-CH_2-NHBoc)$_2$, 12H), 1.395 (s, NH(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*<u>*H*$_3$</u>*)*$_3$)-CH_2-CH_2-CH_2-NH-COO-C*(C*<u>*H*$_3$</u>*)*$_3$)$_2$, 36H); HRMS (m/z): 760.5172 [$(M+H)^+$] (Observed), 760.5184 [$(M+H)^+$] (Calculated).

General procedure for synthesizing 6a-6k: About (2.4 mmol) (1.5 equivalents) of saturated aliphatic acid (decanoic, dodecanoic, tetradecanoic, hexadecanoic or octadecanoic acid), oleic acid or linoleic acid were dissolved in dry DCM (12 mL) at 0ºC. Then 4 equivalents of DIPEA were added to the reaction mixture followed by 1.5 equivalents of HBTU. Then DMF (3 mL) was added to the reaction mixture. After 10 minutes, about 1 equivalent of **5a-5c** in dry DCM (2 mL) was added drop wise. The reaction mixture was then brought to RT and allowed to stir for 24 h. Then solvent was evaporated and residue was diluted in ethyl acetate (50 mL). Then work-up was done at first with 1N HCl (50 mL, 3 times) followed by saturated Na_2CO_3 solution (50 mL, 3 times). The crude product was collected in ethyl acetate layer. Finally column was accomplished on silica gel (60-120 mess) using methanol and chloroform (3:97) as eluent to isolate the pure product (**6a-6k**) with 75-80% yield.

***N,N*-bis-[{3-(Boc-^{L}Lys-Boc)amido}propyl]decanamide (6a):** Yield-77%; FT-IR (NaCl): 3316 cm^{-1} (-NH- str.), 2928 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1700 cm^{-1}, 1661 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.320 (s, R-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.364-5.300 (d, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*N*<u>*H*</u>Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.713 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-*N*<u>*H*</u>Boc)$_2$, 2H), 4.152-4.134 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.549-2.991 (m, R-CO-N(-*C*<u>*H*$_2$</u>-CH_2-*C*<u>*H*$_2$</u>-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*C*<u>*H*$_2$</u>-NHBoc)$_2$, 12H), 2.272-2.233 (t, CH_3-(CH_2)$_6$-CH_2-*C*<u>*H*$_2$</u>- of R group, 2H), 1.886-1.461 (m, CH_3-(CH_2)$_6$-*C*<u>*H*$_2$</u>-CH_2-CO-N(-CH_2-*C*<u>*H*$_2$</u>-CH_2-NH-CO-CH(NHBoc)-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-CH_2-NHBoc)$_2$, 18H), 1.419 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*<u>*H*$_3$</u>*)*<u>$_3$</u>)-CH_2-CH_2-CH_2-CH_2-NH-COO-C*(C*<u>*H*$_3$</u>*)*<u>$_3$</u>)$_2$, 36H), 1.249 (bs, CH_3-*(C*<u>*H*$_2$</u>*)*<u>$_6$</u>-CH_2-CH_2- of R group, 12H), 0.882-0.848 (t, *C*<u>*H*$_3$</u>-(CH_2)$_6$-CH_2-CH_2- of R group, 3H). HRMS (m/z): 942.6830 [$(M+H)^+$] (Observed), 942.6855 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(Boc-^{L}Lys-Boc)amido}propyl]dodecanamide (6b):** Yield-75%; FT-IR (NaCl): 3320 cm^{-1} (-NH- str.), 2929 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1695 cm^{-1}, 1661 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.349 (s, R-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.398-5.331 (d, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*N*<u>*H*</u>Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.728 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-*N*<u>*H*</u>Boc)$_2$, 2H), 4.151-4.134 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$,

2H), 3.545-2.988 (m, R-CO-N(-*C*$\underline{H_2}$-CH_2-*C*$\underline{H_2}$-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*C*$\underline{H_2}$-NHBoc)$_2$, 12H), 2.269-2.231 (t, CH_3-$(CH_2)_8$-CH_2-*C*$\underline{H_2}$- of R group, 2H), 1.737-1.458 (m, CH_3-$(CH_2)_8$-*C*$\underline{H_2}$-CH_2-CO-N(-CH_2-*C*$\underline{H_2}$-CH_2-NH-CO-CH(NHBoc)-*C*$\underline{H_2}$-*C*$\underline{H_2}$-*C*$\underline{H_2}$-CH_2-NHBoc)$_2$, 18H), 1.415 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*$\underline{H_3}$*)*$_3$)-CH_2-CH_2-CH_2-NH-COO-C*(C*$\underline{H_3}$*)*$_3$)$_2$, 36H), 1.242 (bs, CH_3-*(C*$\underline{H_2}$*)*$_8$-CH_2-CH_2- of R group, 16H), 0.880-0.846 (t, *C*$\underline{H_3}$-$(CH_2)_8$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 970.7135 [$(M+H)^+$] (Observed), 970.7168 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(Boc-^{L}Lys-Boc)amido}propyl]tetradecanamide (6c):** Yield-78%; FT-IR (NaCl): 3311cm^{-1} (-NH- str.), 2927 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1697 cm^{-1}, 1661 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.327 (s, R-CO-N(-CH_2-CH_2-CH_2-*N*$\underline{H}$-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.65-5.302 (d, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*N*$\underline{H}$Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.710 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-*N*$\underline{H}$Boc)$_2$, 2H), 4.156-4.139 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*$\underline{H}$(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.560-2.966 (m, R-CO-N(-*C*$\underline{H_2}$-CH_2-*C*$\underline{H_2}$-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*C*$\underline{H_2}$-NHBoc)$_2$, 12H), 2.275-2.236 (t, CH_3-$(CH_2)_{10}$-CH_2-*C*$\underline{H_2}$- of R group, 2H), 1.667-1.464 (m, CH_3-$(CH_2)_{10}$-*C*$\underline{H_2}$-CH_2-CO-N(-CH_2-*C*$\underline{H_2}$-CH_2-NH-CO-CH(NHBoc)-*C*$\underline{H_2}$-*C*$\underline{H_2}$-*C*$\underline{H_2}$-CH_2-NHBoc)$_2$, 18H), 1.423 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*$\underline{H_3}$*)*$_3$)-CH_2-CH_2-CH_2-NH-COO-C*(C*$\underline{H_3}$*)*$_3$)$_2$, 36H), 1.247 (bs, CH_3-*(C*$\underline{H_2}$*)*$_{10}$-CH_2-CH_2- of R group, 20H), 0.888-0.854 (t, *C*$\underline{H_3}$-$(CH_2)_{10}$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 998.7462 [$(M+H)^+$] (Observed), 998.7481 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(Boc-^{L}Lys-Boc)amido}propyl]hexadecanamide (6d):** Yield-80%; FT-IR (NaCl): 3337cm^{-1} (-NH- str.), 2929 cm^{-1} (-CH_2- asym. str.), 2869 cm^{-1} (-CH_2- sym. str.), 1697 cm^{-1}, 1661 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.360-7.309 (d, R-CO-N(-CH_2-CH_2-CH_2-*N*$\underline{H}$-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.422-5.351 (d, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*N*$\underline{H}$Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.750 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-*N*$\underline{H}$Boc)$_2$, 2H), 4.139-4.123 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*$\underline{H}$(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.526-2.985 (m, R-CO-N(-*C*$\underline{H_2}$-CH_2-*C*$\underline{H_2}$-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*C*$\underline{H_2}$-NHBoc)$_2$, 12H), 2.258-2.220 (t, CH_3-$(CH_2)_{12}$-CH_2-*C*$\underline{H_2}$- of R group, 2H), 1.714-1.452 (m, CH_3-$(CH_2)_{12}$-*C*$\underline{H_2}$-CH_2-CO-N(-CH_2-*C*$\underline{H_2}$-CH_2-NH-CO-CH(NHBoc)-*C*$\underline{H_2}$-*C*$\underline{H_2}$-*C*$\underline{H_2}$-CH_2-NHBoc)$_2$, 18H), 1.403 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*$\underline{H_3}$*)*$_3$)-CH_2-CH_2-CH_2-CH_2-NH-COO-C*(C*$\underline{H_3}$*)*$_3$)$_2$, 36H), 1.228 (s, CH_3-*(C*$\underline{H_2}$*)*$_{12}$-CH_2-CH_2-

of R group, 24H), 0.870-0.836 (t, $C\underline{H_3}$-$(CH_2)_{12}$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 1026.7787 [$(M+H)^+$] (Observed), 1026.7794 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(Boc-^{L}Lys-Boc)amido}propyl]octadecanamide (6e):** Yield-75%; FT-IR (NaCl): 3321 cm^{-1} (-NH- str.), 2927 cm^{-1} (-CH_2- asym. str.), 2853 cm^{-1} (-CH_2- sym. str.), 1699 cm^{-1}, 1663 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.367-7.310 (d, R-CO-N(-CH_2-CH_2-CH_2-$N\underline{H}$-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.436-5.372 (d, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH($N\underline{H}$Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.768 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-$N\underline{H}$Boc)$_2$, 2H), 4.116-4.099 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-$C\underline{H}$(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.513-3.055 (m, R-CO-N(-$C\underline{H_2}$-CH_2-$C\underline{H_2}$-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-$C\underline{H_2}$-NHBoc)$_2$, 12H), 2.252-2.216 (t, CH_3-$(CH_2)_{14}$-CH_2-$C\underline{H_2}$- of R group, 2H), 1.722-1.443 (m, CH_3-$(CH_2)_{14}$-$C\underline{H_2}$-CH_2-CO-N(-CH_2-$C\underline{H_2}$-CH_2-NH-CO-CH(NHBoc)-$C\underline{H_2}$-$C\underline{H_2}$-$C\underline{H_2}$-CH_2-NHBoc)$_2$, 18H), 1.398 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C($C\underline{H_3}$)$_3$)-CH_2-CH_2-CH_2-CH_2-NH-COO-C($C\underline{H_3}$)$_3$)$_2$, 36H), 1.223 (bs, CH_3-($C\underline{H_2}$)$_{14}$-CH_2-CH_2- of R group, 28H), 0.865-0.830 (t, $C\underline{H_3}$-$(CH_2)_{14}$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 1054.8097 [$(M+H)^+$] (Observed), 1054.8107 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(Boc-^{L}Lys-Boc)amido}propyl]oleylamide (6f):** Yield:77%; FT-IR (NaCl): 3321 cm^{-1} (-NH- str.), 2927 cm^{-1} (-CH_2- asym. str.), 2853 cm^{-1} (-CH_2- sym. str.), 1699 cm^{-1}, 1663 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.359 (s, R-CO-N(-CH_2-CH_2-CH_2-$N\underline{H}$-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.481-5.292 (m, CH_3-$(CH_2)_6$-CH_2-$C\underline{H}$=$C\underline{H}$-CH_2-$(CH_2)_4$-CH_2-CH_2-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH($N\underline{H}$Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 4H), 4.764 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-$N\underline{H}$Boc)$_2$, 2H), 4.130-4.117 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-$C\underline{H}$(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.436-3.049 (m, R-CO-N(-$C\underline{H_2}$-CH_2-$C\underline{H_2}$-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-$C\underline{H_2}$-NHBoc)$_2$, 12H), 2.250-2.212 (t, CH_3-$(CH_2)_6$-CH_2-CH=CH-CH_2-$(CH_2)_4$-CH_2-$C\underline{H_2}$- of R group, 2H), 1.980-1.949 (t, CH_3-$(CH_2)_6$-$C\underline{H_2}$-CH=CH-$C\underline{H_2}$-$(CH_2)_4$-CH_2-CH_2- of R group, 4H), 1.717-1.438 (m, CH_3-$(CH_2)_6$-CH_2-CH=CH-CH_2-$(CH_2)_4$-$C\underline{H_2}$-CH_2-CO-N(-CH_2-$C\underline{H_2}$-CH_2-NH-CO-CH(NHBoc)-$C\underline{H_2}$-$C\underline{H_2}$-$C\underline{H_2}$-CH_2-NHBoc)$_2$, 18H), 1.394 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C($C\underline{H_3}$)$_3$)-CH_2-CH_2-CH_2-CH_2-NH-COO-C($C\underline{H_3}$)$_3$)$_2$, 36H), 1.2267-1.232 (bs, CH_3-($C\underline{H_2}$)$_6$-CH_2-CH=CH-CH_2-($C\underline{H_2}$)$_4$-CH_2-CH_2- of R group, 20H), 0.861-0.827 (t, $C\underline{H_3}$-$(CH_2)_6$-CH_2-CH=CH-CH_2-$(CH_2)_4$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 1052.7944 [$(M+H)^+$] (Observed), 1052.7950 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(Boc-^{L}Lys-Boc)amido}propyl]linoleylamide (6g):** Yield:76%; FT-IR (NaCl): 3321 cm^{-1} (-NH- str.), 2927 cm^{-1} (-CH_2- asym. str.), 2853 cm^{-1} (-CH_2- sym. str.), 1699 cm^{-1}, 1663 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.348 (s, R-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.459-5.257 (m, CH_3-$(CH_2)_3$-CH_2-*C*<u>*H*</u>=*C*<u>*H*</u>-CH_2-*C*<u>*H*</u>=*C*<u>*H*</u>-CH_2-$(CH_2)_4$-CH_2-CH_2-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*N*<u>*H*</u>Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 6H), 4.760 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-*N*<u>*H*</u>Boc)$_2$, 2H), 4.115 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.514-2.973 (m, R-CO-N(-*C*<u>*H*$_2$</u>-CH_2-*C*<u>*H*$_2$</u>-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*C*<u>*H*$_2$</u>-NHBoc)$_2$, 12H), 2.750-2.718 (t, CH_3-$(CH_2)_3$-CH_2-CH=CH-*C*<u>*H*$_2$</u>-CH=CH-CH_2-$(CH_2)_4$-CH_2-CH_2- of R group, 2H), 2.251-2.212 (t, CH_3-$(CH_2)_3$-CH_2-CH=CH-CH_2-CH=CH-CH_2-$(CH_2)_4$-CH_2-*C*<u>*H*$_2$</u>- of R group, 2H), 2.038-1.987 (m, CH_3-$(CH_2)_3$-*C*<u>*H*$_2$</u>-CH=CH-CH_2-CH=CH-*C*<u>*H*$_2$</u>-$(CH_2)_4$-CH_2-CH_2- of R group, 4H), 1.704-1.442 (m, CH_3-$(CH_2)_3$-CH_2-CH=CH-CH_2-CH=CH-CH_2-$(CH_2)_4$-*C*<u>*H*$_2$</u>-CH_2-CO-N(-CH_2-*C*<u>*H*$_2$</u>-CH_2-NH-CO-CH(NHBoc)-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-CH_2-NHBoc)$_2$, 18H), 1.394 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C(*C*<u>*H*$_3$</u>)$_3$)-CH_2-CH_2-CH_2-CH_2-NH-COO-C(*C*<u>*H*$_3$</u>)$_3$)$_2$, 36H), 1.237 (bs, CH_3-(*C*<u>*H*$_2$</u>)$_3$-CH_2-CH=CH-CH_2-CH=CH-CH_2-(*C*<u>*H*$_2$</u>)$_4$-CH_2-CH_2- of R group, 14H), 0.871-0.836 (t, *C*<u>*H*$_3$</u>-$(CH_2)_3$-CH_2-CH=CH-CH_2-CH=CH-CH_2-$(CH_2)_4$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 1050.7785 [$(M+H)^+$] (Observed), 1050.7794 [$(M+H)^+$] (Calculated).

***N,N*-bis-[{3-(Boc-^{D}Lys-Boc)amido}propyl]tetradecanamide (6h):** Yield-76%; FT-IR(NaCl): 3311cm^{-1} (-NH- str.), 2927 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1697 cm^{-1}, 1661 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.326 (s, R-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.345-5.288 (d, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*N*<u>*H*</u>Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.708 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-*N*<u>*H*</u>Boc)$_2$, 2H), 4.158-4.141 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.569-2.984 (m, R-CO-N(-*C*<u>*H*$_2$</u>-CH_2-*C*<u>*H*$_2$</u>-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*C*<u>*H*$_2$</u>-NHBoc)$_2$, 12H), 2.276-2.237 (t, CH_3-$(CH_2)_{10}$-CH_2-*C*<u>*H*$_2$</u>- of R group, 2H), 1.747-1.465 (m, CH_3-$(CH_2)_{10}$-*C*<u>*H*$_2$</u>-CH_2-CO-N(-CH_2-*C*<u>*H*$_2$</u>-CH_2-NH-CO-CH(NHBoc)-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-CH_2-NHBoc)$_2$, 18H), 1.424 (s, CH_3-$(CH_2)_{10}$-CH_2-CH_2-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C(*C*<u>*H*$_3$</u>)$_3$)-CH_2-CH_2-CH_2-CH_2-NH-COO-C(*C*<u>*H*$_3$</u>)$_3$)$_2$, 36H), 1.247 (bs, CH_3-(*C*<u>*H*$_2$</u>)$_{10}$-CH_2-CH_2- of R group, 20H), 0.889-0.855 (t, *C*<u>*H*$_3$</u>-$(CH_2)_{10}$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 998.7415 [$(M+H)^+$] (observed), 998.7481 [$(M+H)^+$] (calculated).

***N,N*-bis-[{3-(Boc-^{D}Lys-Boc)amido}propyl]hexadecanamide (6i):** Yield-78%; FT-IR (NaCl): 3311cm^{-1} (-NH- str.), 2927 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1697 cm^{-1}, 1661 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.361-7.309 (s, R-CO-N(-CH_2-CH_2-CH_2-*NH*-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.435-5.363 (d, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*NH*Boc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.53 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-CH_2-*NH*Boc)$_2$, 2H), 4.120 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*CH*(NHBoc)-CH_2-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.529-2.978 (m, R-CO-N(-*CH*$_2$-CH_2-*CH*$_2$-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*CH*$_2$-NHBoc)$_2$, 12H), 2.256-2.218 (t, CH_3-$(CH_2)_{12}$-CH_2-*CH*$_2$- of R group, 2H), 1.712-1.446 (m, CH_3-$(CH_2)_{12}$-*CH*$_2$-CH_2-CO-N(-CH_2-*CH*$_2$-CH_2-NH-CO-CH(NHBoc)-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH_2-NHBoc)$_2$, 18H), 1.402 (s, CH_3-$(CH_2)_{12}$-CH_2-CH_2-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(CH*$_3$*)*$_3$)-CH_2-CH_2-CH_2-CH_2-NH-COO-C*(CH*$_3$*)*$_3$)$_2$, 36H), 1.226 (s, CH_3-*(CH*$_2$*)*$_{12}$-CH_2-CH_2- of R group, 24H), 0.868-0.834 (t, *CH*$_3$-$(CH_2)_{12}$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 1026.7742 [(M+H)$^{+}$] (observed), 1026.7794 [(M+H)$^{+}$] (calculated).

***N,N*-bis-[{3-(Boc-^{L}Orn-Boc)amido}propyl]tetradecanamide (6j):** Yield-77%; FT-IR (NaCl): 3318cm^{-1} (-NH- str.), 2928 cm^{-1} (-CH_2- asym. str.), 2854 cm^{-1} (-CH_2- sym. str.), 1698 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.350 (s, R-CO-N(-CH_2-CH_2-CH_2-*NH*-CO-CH(NHBoc)-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.523-5.548 (d, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*NH*Boc)-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.879 (s, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*NH*Boc)$_2$, 2H), 4.199-4.181 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*CH*(NHBoc)-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 3.479-3.025 (m, R-CO-N(-*CH*$_2$-CH_2-*CH*$_2$-NH-CO-CH(NHBoc)-CH_2-CH_2-*CH*$_2$-NHBoc)$_2$, 12H), 2.256-2.218 (t, CH_3-$(CH_2)_{10}$-CH_2-*CH*$_2$- of R group, 2H), 1.731-1.491 (m, CH_3-$(CH_2)_{10}$-*CH*$_2$-CH_2-CO-N(-CH_2-*CH*$_2$-CH_2-NH-CO-CH(NHBoc)-*CH*$_2$-*CH*$_2$-CH_2-NHBoc)$_2$, 14H), 1.397 (s, CH_3-$(CH_2)_{10}$-CH_2-CH_2-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(CH*$_3$*)*$_3$)-CH_2-CH_2-CH_2-NH-COO-C*(CH*$_3$*)*$_3$)$_2$, 36H), 1.224 (bs, CH_3-*(CH*$_2$*)*$_{10}$-CH_2-CH_2- of R group, 20), 0.864-0.8350 (t, *CH*$_3$-$(CH_2)_{10}$-CH_2-CH_2- of R group, 3H); HRMS (m/z): 970.7175 [(M+H)$^{+}$] (observed), 970.7168 [(M+H)$^{+}$] (calculated).

***N,N*-bis-[{3-(Boc-^{L}Orn-Boc)amido}propyl]hexadecanamide (6k):** Yield-75%; FT-IR (NaCl): 3318cm^{-1} (-NH- str.), 2927 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1700 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.284 (s, R-CO-N(-CH_2-CH_2-CH_2-*NH*-CO-CH(NHBoc)-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 5.459-5.387 (d, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(*NH*Boc)-CH_2-CH_2-CH_2-NHBoc)$_2$, 2H), 4.849 (s, R-CO-N(-CH_2-

CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*N*<u>*H*</u>Boc)$_2$, 2H), 4.216-4.199 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NHBoc)-CH_2-CH_2-CH_2-*N*<u>*H*</u>Boc)$_2$, 2H), 3.501-3.040 (m, R-CO-N(-*C*<u>*H*$_2$</u>-CH_2-*C*<u>*H*$_2$</u>-NH-CO-CH(NHBoc)-CH_2-CH_2-*C*<u>*H*$_2$</u>-NHBoc)$_2$, 12H), 2.270-2.232 (t, CH_3-$(CH_2)_{10}$-CH_2-*C*<u>*H*$_2$</u>- of R group, 2H), 1.767-1.541 (m, CH_3-$(CH_2)_{10}$-*C*<u>*H*$_2$</u>-CH_2-CO-N(-CH_2-*C*<u>*H*$_2$</u>-CH_2-NH-CO-CH(NHBoc)-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-CH_2-NHBoc)$_2$, 14H), 1.415(s, CH_3-$(CH_2)_{12}$-CH_2-CH_2-CO-N(-CH_2-CH_2-CH_2-NH-CO-CH(NH-COO-C*(C*<u>*H*$_3$</u>*)*$_3$)-CH_2-CH_2-CH_2-NH-COO-C*(C*<u>*H*$_3$</u>*)*$_3$)$_2$, 36H), 1.241 (bs, CH_3-*(C*<u>*H*$_2$</u>*)*$_{12}$-CH_2-CH_2- of R group, 24H), 0.882-0.848 (t, *C*<u>*H*$_3$</u>-$(CH_2)_{12}$-CH_2-CH_2- of R group, 3H). HRMS (m/z): 998.7415 [$(M+H)^+$] (observed), 998.7481 [$(M+H)^+$] (calculated).

General procedure for synthesizing LANA1-LANA9, OANA1, OANA2 and LL: At first 1 equivalent of **6a-6k** (0.6 mmol) and **5a** (0.8 mmol) were dissolved in DCM (10 mL). To the intensely stirred solution 4 equivalents (excess amount) of trifluoroacetic acid (TFA) was added and allowed to stir at RT for 12 h. After that reaction solvent and unused TFA was removed to afford the products with 100% yield.

***N,N*-bis-[{3-(^{L}Lys)amido}propyl]decanamide tetrakis(trifluoroacetate) (LANA1):** FT-IR (NaCl): 3277cm^{-1} (-NH- str.), 2928 cm^{-1} (-CH_2- asym. str.), 2858 cm^{-1} (-CH_2- sym. str.), 1677 cm^{-1} (C=O str.). ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.619-7.877 (m, R-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>-CO-CH*(N*<u>*H*$_3^+$</u>*)*-CH_2-CH_2-CH_2-CH_2-*N*<u>*H*$_3^+$</u>)$_2$, 14H), 3.709-3.653 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NH_3^+)-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 3.646-2.760 (m, R-CO-N(-*C*<u>*H*$_2$</u>-CH_2-*C*<u>*H*$_2$</u>-NH-CO-CH(NH_3^+)-CH_2-CH_2-CH_2-*C*<u>*H*$_2$</u>-NH_3^+)$_2$, 12H), 2.241-2.222 (t, CH_3-$(CH_2)_6$-CH_2-*C*<u>*H*$_2$</u>- of R group, 2H), 1.709-1.312 (m, CH_3-$(CH_2)_6$-*C*<u>*H*$_2$</u>-CH_2-CO-N(-CH_2-*C*<u>*H*$_2$</u>-CH_2-NH-CO-CH(NH_3^+)-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-*C*<u>*H*$_2$</u>-CH_2-NH_3^+)$_2$, 18H), 1.241 (bs, CH_3-*(C*<u>*H*$_2$</u>*)*$_6$-CH_2-CH_2- of R group, 12H), 0.871-0.837 (t, *C*<u>*H*$_3$</u>-$(CH_2)_6$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 171.92, 168.45, 168.25, 158.75, 158.43, 118.45, 52.13, 45.02, 42.86, 39.52, 38.46, 36.69, 36.45, 32.10, 31.31, 30.51, 29.02, 28.98, 28.81, 28.71, 28.49, 27.38, 26.57, 26.49, 25.09, 22.11, 21.30, 21.22, 13.95; HRMS (m/z): 542.4752 [$(M+H)^+$] (observed), 542.4758 [$(M+H)^+$] (calculated).

***N,N*-bis-[{3-(^{L}Lys)amido}propyl]dodecanamide tetrakis(trifluoroacetate) (LANA2):** FT-IR (NaCl): 3264 cm^{-1}(-NH- str.), 2929 cm^{-1} (-CH_2- asym. str.), 2869 cm^{-1} (-CH_2- sym. str.), 1676 (C=O str.). ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.608-7.862 (m, R-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>-CO-CH*(N*<u>*H*$_3^+$</u>*)*-CH_2-CH_2-CH_2-CH_2-*N*<u>*H*$_3^+$</u>)$_2$, 14H), 3.709 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NH_3^+)-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 3.274-2.761(m, R-CO-

N(-*C*<u>*H*₂</u>-CH_2-*C*<u>*H*₂</u>-NH-CO-CH(NH_3^+)-CH_2-CH_2-CH_2-*C*<u>*H*₂</u>-NH_3^+)$_2$, 12H), 2.254-2.221 (t, CH_3-$(CH_2)_8$-CH_2-*C*<u>*H*₂</u>- of R group, 2H), 1.688-1.289 (m, CH_3-$(CH_2)_8$-*C*<u>*H*₂</u>-CH_2-CO-N(-CH_2-*C*<u>*H*₂</u>-CH_2-NH-CO-CH(NH_3^+)-*C*<u>*H*₂</u>-*C*<u>*H*₂</u>-*C*<u>*H*₂</u>-CH_2-NH_3^+)$_2$, 18H), 1.239 (bs, CH_3-*(C*<u>*H*₂</u>*)*<u>*8*</u>-CH_2-CH_2- of R group, 16H), 0.871-0.837 (t, *C*<u>*H*₃</u>-$(CH_2)_8$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 171.94, 168.47, 168.27, 158.81, 158.50, 118.41, 52.14, 45.03, 42.89, 39.52, 38.48, 36.71, 36.47, 32.12, 31.33, 30.52, 29.09, 29.05, 28.83, 28.75, 28.51, 27.39, 26.59, 26.50, 25.11, 22.12, 21.31, 21.24, 13.95. HRMS (m/z): 570.5016 [$(M+H)^+$] (observed), 570.5071 [$(M+H)^+$] (calculated).

***N,N*-bis-[{3-(^{L}Lys)amido}propyl]tetradecanamide tetrakis(trifluoroacetate) (LANA3):** FT-IR (NaCl): 3316cm^{-1} (-NH- str.), 2978 cm^{-1} (-CH_2- asym. str.), 2867 cm^{-1} (-CH_2- sym. str.), 1697 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.599-7.841(m, R-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>-CO-CH*(N*<u>*H*₃</u>$^+$*)*-CH_2-CH_2-CH_2-CH_2-*N*<u>*H*₃</u>$^+$)$_2$, 14H), 3.706 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NH_3^+)-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 3.285-2.759 (m, R-CO-N(-*C*<u>*H*₂</u>-CH_2-*C*<u>*H*₂</u>-NH-CO-CH(NH_3^+)-CH_2-CH_2-CH_2-*C*<u>*H*₂</u>-NH_3^+)$_2$, 12H), 2.240-2.220 (t, CH_3-$(CH_2)_{10}$-CH_2-*C*<u>*H*₂</u>- of R group, 2H), 1.686-1.324 (m, CH_3-$(CH_2)_{10}$-*C*<u>*H*₂</u>-CH_2-CO-N(-CH_2-*C*<u>*H*₂</u>-CH_2-NH-CO-CH(NH_3^+)-*C*<u>*H*₂</u>-*C*<u>*H*₂</u>-*C*<u>*H*₂</u>-CH_2-NH_3^+)$_2$, 18H), 1.236 (bs, CH_3-*(C*<u>*H*₂</u>*)*<u>*10*</u>-CH_2-CH_2- of R group, 20H), 0.869-0.835 (t, *C*<u>*H*₃</u>-$(CH_2)_{10}$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 171.98, 168.50, 168.30, 158.91, 158.59, 118.41, 115.47, 52.18, 45.07, 42.93, 39.52, 38.50, 36.73, 36.49, 32.15, 31.36, 30.54, 29.12, 29.08, 28.87, 28.77, 28.52, 27.40, 26.60, 26.51, 25.13, 22.15, 21.34, 21.26, 13.96. HRMS (m/z): 598.3578 [$(M+H)^+$] (observed), 598.5384 [$(M+H)^+$] (calculated).

***N,N*-bis-[{3-(^{L}Lys)amido}propyl]hexadecanamide tetrakis(trifluoroacetate) (LANA4):** FT-IR (NaCl): 3337cm^{-1} (-NH- str.), 2979 cm^{-1}, 2929 cm^{-1} (-CH_2- asym. str.), 2869 cm^{-1} (-CH_2- sym. str.), 1697 cm^{-1}, 1661 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.600-7.842 (m, R-CO-N(-CH_2-CH_2-CH_2-*N*<u>*H*</u>-CO-CH*(N*<u>*H*₃</u>$^+$*)*-CH_2-CH_2-CH_2-CH_2-*N*<u>*H*₃</u>$^+$)$_2$, 14H), 3.706 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NH_3^+)-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 3.288-2.759 (m, R-CO-N(-*C*<u>*H*₂</u>-CH_2-*C*<u>*H*₂</u>-NH-CO-CH(NH_3^+)-CH_2-CH_2-CH_2-*C*<u>*H*₂</u>-NH_3^+)$_2$, 12H), 2.258-2.221 (t, CH_3-$(CH_2)_{12}$-CH_2-*C*<u>*H*₂</u>- of R group, 2H), 1.687-1.325 (m, CH_3-$(CH_2)_{12}$-*C*<u>*H*₂</u>-CH_2-CO-N(-CH_2-*C*<u>*H*₂</u>-CH_2-NH-CO-CH(NH_3^+)-*C*<u>*H*₂</u>-*C*<u>*H*₂</u>-*C*<u>*H*₂</u>-CH_2-NH_3^+)$_2$, 18H), 1.237 (s, CH_3-*(C*<u>*H*₂</u>*)*<u>*12*</u>-CH_2-CH_2- of R group, 24H), 0.870-0.836 (t, *C*<u>*H*₃</u>-$(CH_2)_{12}$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 171.94, 168.84, 168.27, 158.71, 158.39, 118.66, 115.6, 52.14, 45.02, 42.87, 38.47, 36.69, 36.46,

32.12, 31.31, 30.53, 29.08, 29.03, 28.84, 28.72, 28.49, 27.37, 26.57, 26.49, 25.10, 22.11, 21.30, 21.23, 13.95;HRMS (m/z): 626.5669 [(M+H) $^{+}$] (observed), 626.5697 [(M+H) $^{+}$] (calculated).

***N,N*-bis-[{3-(^{L}Lys)amido}propyl]octadecanamide tetrakis(trifluoroacetate) (LANA5):** FT-IR (NaCl): 3272 cm^{-1} (-NH- str.), 2979 cm^{-1} (-CH_2- asym. str.), 2854 cm^{-1} (-CH_2- sym. str.), 1676 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.599-7.846 (m, R-CO-N(-CH_2-CH_2-CH_2-*NH*-CO-CH*(*NH_3^+*)*-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 14H), 3.708 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*CH*(NH_3^+)-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 3.274-3.033 (m, R-CO-N(-*CH*$_2$-CH_2-*CH*$_2$-NH-CO-CH(NH_3^+)-CH_2-CH_2-CH_2-*CH*$_2$-NH_3^+)$_2$, 12H), 2.254-2.220 (t, CH_3-$(CH_2)_{14}$-CH_2-*CH*$_2$- of R group, 2H), 1.687-1.324 (m, CH_3-$(CH_2)_{14}$-*CH*$_2$-CH_2-CO-N(-CH_2-*CH*$_2$-CH_2-NH-CO-CH(NH_3^+)-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH_2-NH_3^+)$_2$, 18H), 1.235 (bs, CH_3-*(CH*$_2$*)*$_{14}$-CH_2-CH_2- of R group, 28H), 0.869-0.835 (t, *CH*$_3$-$(CH_2)_{14}$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 171.71, 168.24, 168.04, 158.54, 158.22, 118.24, 115.63, 51.92, 44.81, 42.65, 38.68, 38.25, 36.24, 31.89, 31.10, 30.30, 28.81, 28.77, 28.59, 28.50, 28.28, 27.17, 26.36, 26.28, 24.88, 21.90, 21.09, 21.01, 13.73; HRMS (m/z): 654.5973 [(M+H) $^{+}$] (observed), 654.6010 [(M+H) $^{+}$] (calculated).

***N,N*-bis-[{3-(^{L}Lys)amido}propyl]oleylamide tetrakis(trifluoroacetate) (LANA6):** 3272 cm^{-1} (-NH- str.), 2979 cm^{-1} (-CH_2- asym. str.), 2854 cm^{-1} (-CH_2- sym. str.), 1676 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.621-7.884 (m, R-CO-N(-CH_2-CH_2-CH_2-*NH*-CO-CH*(*NH_3^+*)*-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 14H), 5.350-5.010 (m, CH_3-$(CH_2)_6$-CH_2-*CH*=*CH*-CH_2-$(CH_2)_4$-CH_2-CH_2- of R group, 2H), 3.704 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*CH*(NH_3^+)-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 3.245-2.755 (m, R-CO-N(-*CH*$_2$-CH_2-*CH*$_2$-NH-CO-CH(NH_3^+)-CH_2-CH_2-CH_2-*CH*$_2$-NH_3^+)$_2$, 12H), 2.245-2.212 (t, CH_3-$(CH_2)_6$-CH_2-CH=CH-CH_2-$(CH_2)_4$-CH_2-*CH*$_2$- of R group, 2H), 1.687-1.224 (m, CH_3-*(CH*$_2$*)*$_6$-*CH*$_2$-CH=CH-*CH*$_2$-*(CH*$_2$*)*$_4$-*CH*$_2$-CH_2-CO-N(-CH_2-*CH*$_2$-CH_2-NH-CO-CH(NH_3^+)-*CH*$_2$-*CH*$_2$-*CH*$_2$-CH_2-NH_3^+)$_2$, 42H), 0.854-0.821 (t, *CH*$_3$-$(CH_2)_6$-CH_2-CH=C*H*-CH_2-$(CH_2)_4$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 172.05, 168.50, 168.30, 158.78, 158.46, 118.55, 115.58, 80.51, 69.70, 52.22, 45.07, 42.94, 38.55, 37.27, 36.76, 36.53, 32.90, 32.17, 31.37, 31.26, 30.57, 29.31, 29.18, 28.78, 28.55, 27.41, 26.63, 26.55, 25.34, 25. 14, 24.40, 22.14, 21.36, 21.29, 13.97; HRMS (m/z): 652.5846 [(M+H)$^{+}$] (observed), 652.5853 [(M+H)$^{+}$] (calculated).

***N,N*-bis-[{3-(^{L}Lys)amido}propyl]linoleylamide tetrakis(trifluoroacetate) (LANA7):** 3272 cm^{-1} (-NH- str.), 2979 cm^{-1} (-CH_2- asym. str.), 2854 cm^{-1} (-CH_2- sym. str.), 1676 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.604-7.850 (m, R-CO-N(-CH_2-CH_2-CH_2-*N*H-CO-CH(*N*H_3^+)-CH_2-CH_2-CH_2-CH_2-*N*H_3^+)$_2$, 14H), 5.499-5.050 (m, CH_3-$(CH_2)_3$-CH_2-*C*H=*C*H-CH_2-*C*H=*C*H-CH_2-$(CH_2)_4$-CH_2-CH_2- of R group, 4H), 3.707 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*H(NH_3^+)-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 3.252-2.758 (m, R-CO-N(-*C*H_2-CH_2-*C*H_2-NH-CO-CH(NH_3^+)-CH_2-CH_2-CH_2-*C*H_2-NH_3^+)$_2$, 12H), 2.280-2.219 (t, CH_3-$(CH_2)_3$-CH_2-CH=CH-CH_2-CH=CH-CH_2-$(CH_2)_4$-CH_2-*C*H_2- of R group, 2H), 1.977-1.239 (m, CH_3-*(C*H_2*)*$_3$-*C*H_2-CH=CH-*C*H_2-CH=CH-*C*H_2-*(C*H_2*)*$_4$-*C*H_2-CH_2-CO-N(-CH_2-*C*H_2-CH_2-NH-CO-*C*H(NH_3^+)-*C*H_2-*C*H_2-*C*H_2-CH_2-NH_3^+)$_2$, of R group, 38H), 0.854-0.821 (t, *C*H_3-$(CH_2)_3$-CH_2-CH=CH-CH_2-CH=CH-CH_2-$(CH_2)_4$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 172.05, 168.50, 168.30, 158.78, 158.46, 118.55, 115.58, 80.51, 69.69, 52.21, 45.06, 42.93, 38.55, 37.26, 36.76, 36.53, 32.90, 32.17, 31.37, 31.25, 30.57, 29.31, 28.85, 28.77, 28.55, 27.40, 26.63, 26.55, 25.33, 25. 14, 24.40, 22.13, 21.36, 21.29, 13.97; HRMS (m/z): 650.5688 [(M+H) $^{+}$] (observed), 650.5697 [(M+H) $^{+}$] (calculated).

***N,N*-bis-[{3-(^{D}Lys)amido}propyl]tetradecanamide tetrakis(trifluoroacetate) (LANA8):** FT-IR (NaCl): 3268 cm^{-1} (-NH- str.), 2927 cm^{-1} (-CH_2- asym. str.), 2857 cm^{-1} (-CH_2- sym. str.), 1676 cm^{-1} (C=O str.), ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 3.983-3.976 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*H(NH_3^+)-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 3.352-2.984 (m, R-CO-N(-*C*H_2-CH_2-*C*H_2-NH-CO-CH(NH_3^+)-CH_2-CH_2-CH_2-*C*H_2-NH_3^+)$_2$, 12H), 2.350-2.314 (t, CH_3-$(CH_2)_{10}$-CH_2-*C*H_2- of R group, 2H), 1.923-1.445 (m, CH_3-$(CH_2)_{10}$-*C*H_2-CH_2-CO-N(-CH_2-*C*H_2-CH_2-NH-CO-CH(NH_3^+)-*C*H_2-*C*H_2-*C*H_2-CH_2-NH_3^+)$_2$, 18H), 1.280 (s, CH_3-*(C*H_2*)*$_{10}$-CH_2-CH_2- of R group, 20H), 0.894-0.862 (t, *C*H_3-$(CH_2)_{10}$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 171.92, 168.44, 168.24, 158.79, 158.49, 118.21, 115.27, 51.12, 45.01, 42.86, 38.46, 36.69, 36.44, 32.10, 31.31, 30.50, 29.08, 29.03, 28.82, 28.72, 28.49, 27.37, 26.57, 26.49, 25.09, 22.10, 21.29, 21.22, 13.93; HRMS (m/z): 598.5378 [(M+H) $^{+}$] (observed), 598.5384 [(M+H) $^{+}$] (calculated).

***N,N*-bis-[{3-(^{D}Lys)amido}propyl]hexadecanamide tetrakis(trifluoroacetate) (LANA9):** FT-IR (NaCl): 3272 cm^{-1} (-NH- str.), 2926 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1677 cm^{-1} (C=O str.); ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 4.007-3.979 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*H(NH_3^+)-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 3.370-3.002 (m, R-

CO-N(-*C*<u>*H*₂</u>-CH_2-*C*<u>*H*₂</u>-NH-CO-CH(NH_3^+)-CH_2-CH_2-CH_2-*C*<u>*H*₂</u>-NH_3^+)$_2$, 12H), 2.372-2.336 (t, CH_3-$(CH_2)_{12}$-CH_2-*C*<u>*H*₂</u>- of R group, 2H), 1.924-1.462 (m, CH_3-$(CH_2)_{12}$-*C*<u>*H*₂</u>-CH_2-CO-N(-CH_2-*C*<u>*H*₂</u>-CH_2-NH-CO-CH(NH_3^+)-*C*<u>*H*₂</u>-*C*<u>*H*₂</u>-*C*<u>*H*₂</u>-CH_2-NH_3^+)$_2$, 18H), 1.300 (bs, CH_3-*(C*<u>*H*₂</u>*)*<u>*12*</u>-CH_2-CH_2- of R group, 24H), 0.913-0.880 (t, *C*<u>*H*₃</u>-$(CH_2)_{12}$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 171.89, 168.42, 168.22, 158.62, 158.31, 118.60, 115.63, 51.11, 44.98, 42.84, 38.45, 36.68, 36.44, 32.09, 31.29, 30.49, 29.06, 29.01, 28.82, 28.70, 28.48, 27.36, 26.56, 26.48, 25.08, 22.09, 21.28, 21.20, 13.94; HRMS (m/z): 626.5688 [(M+H) $^+$] (observed), 626.5697 [(M+H) $^+$] (calculated).

***N,N*-bis-[{3-(^{L}Orn)amido}propyl]tetradecanamide tetrakis(trifluoroacetate) (OANA1):** FT-IR (NaCl): 3266 cm^{-1} (-NH- str.), 2928 cm^{-1} (-CH_2- asym. str.), 2855 cm^{-1} (-CH_2- sym. str.), 1675 cm^{-1} (C=O str.). ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 4.048-4.010 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NH_3^+)-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 3.367-3.043 (m, R-CO-N(-*C*<u>*H*₂</u>-CH_2-*C*<u>*H*₂</u>-NH-CO-CH(NH_3^+)-CH_2-CH_2-*C*<u>*H*₂</u>-NH_3^+)$_2$, 12H), 2.373-2.236 (t, CH_3-$(CH_2)_{10}$-CH_2-*C*<u>*H*₂</u>- of R group, 2H), 2.001-1.576 (m, CH_3-$(CH_2)_{10}$-*C*<u>*H*₂</u>-CH_2-CO-N(-CH_2-*C*<u>*H*₂</u>-CH_2-NH-CO-CH(NH_3^+)-*C*<u>*H*₂</u>-*C*<u>*H*₂</u>-CH_2-NH_3^+)$_2$, 14H), 1.294 (bs, CH_3-*(C*<u>*H*₂</u>*)*<u>*10*</u>-CH_2-CH_2- of R group, 20H), 0.906-0.873 (t, *C*<u>*H*₃</u>-$(CH_2)_{10}$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 172.03, 168.19, 167.97, 158.60, 158.29, 118.58, 115.60, 51.68, 44.91, 42.90, 38.30, 38.19, 36.57, 36.41, 32.01, 31.24, 29.02, 28.97, 28.76, 28.66, 28.38, 28.13, 27.34, 24.98, 22.56, 22.49, 22.04, 13.93; HRMS (m/z): 570.5063 [(M+H) $^+$] (observed), 570.5071 [(M+H) $^+$] (calculated).

***N,N*-bis-[{3-(^{L}Orn)amino}propyl]hexadecanamide tetrakis(trifluoroacetate) (OANA2):** FT-IR (NaCl): 3267 cm^{-1} (-NH- str.), 2924 cm^{-1} (-CH_2- asym. str.), 2854 cm^{-1} (-CH_2- sym. str.), 1673 cm^{-1} (C=O str.). ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 4.044-4.011 (t, R-CO-N(-CH_2-CH_2-CH_2-NH-CO-*C*<u>*H*</u>(NH_3^+)-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 3.369-3.039 (m, R-CO-N(-*C*<u>*H*₂</u>-CH_2-*C*<u>*H*₂</u>-NH-CO-CH(NH_3^+)-CH_2-CH_2-*C*<u>*H*₂</u>-NH_3^+)$_2$, 12H), 2.372-2.235 (t, CH_3-$(CH_2)_{12}$-CH_2-*C*<u>*H*₂</u>- of R group, 2H), 2.001-1.579 (m, CH_3-$(CH_2)_{12}$-*C*<u>*H*₂</u>-CH_2-CO-N(-CH_2-*C*<u>*H*₂</u>-CH_2-NH-CO-CH(NH_3^+)-*C*<u>*H*₂</u>-*C*<u>*H*₂</u>-CH_2-NH_3^+)$_2$, 14H), 1.298 (bs, CH_3-*(C*<u>*H*₂</u>*)*<u>*12*</u>-CH_2-CH_2- of R group, 24H), 0.911-0.878 (t, *C*<u>*H*₃</u>-$(CH_2)_{12}$-CH_2-CH_2- of R group, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 172.06, 168.23, 168.01, 158.59, 158.28, 118.64, 115.66, 51.72, 44.95, 38.34, 38.24, 36.61, 36.45, 32.05, 31.28, 29.05, 29.00, 28.82, 28.69, 28.42, 28.18, 27.38, 25.02, 22.61, 22.53, 22.08, 13.93; HRMS (m/z): 598.5378 [(M+H) $^+$] (observed), 598.5384 [(M+H) $^+$] (calculated).

***N*^{1}-(^{L}Lys)-*N*^{3}-[{3-(^{L}Lys)amido}propyl]propane-1,3-diamine pentakis(trifluoroacetate) (LL)** FT-IR (NaCl): 3321 cm^{-1} (-NH- str.), 2927 cm^{-1} (-CH_2- asym. str.), 2846 cm^{-1} (-CH_2- sym. str.), 1710 cm^{-1}, 1678 cm^{-1} (C=O str.). ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 8.613-7.866 (m, *N*$\underline{H_2}$CO-N(-CH_2-CH_2-CH_2-*N*$\underline{H}$-CO-CH(*N*$\underline{H_3^+}$)-CH_2-CH_2-CH_2-CH_2-*N*$\underline{H_3^+}$)$_2$, 16H), 4.011 (t, NH_2^+(-CH_2-CH_2-CH_2-NH-CO-*C*$\underline{H}$(NH_3^+)-CH_2-CH_2-CH_2-CH_2-NH_3^+)$_2$, 2H), 3.512-3.014 (m, NH_2^+(-*C*$\underline{H_2}$-CH_2-*C*$\underline{H_2}$-NH-CO-CH(NH_3^+)-CH_2-CH_2-CH_2-*C*$\underline{H_2}$-NH_3^+)$_2$, 12H), 1.713-1.416 (m, NH_2^+(-CH_2-*C*$\underline{H_2}$-CH_2-NH-CO-CH(NH_3^+)-*C*$\underline{H_2}$-*C*$\underline{H_2}$-*C*$\underline{H_2}$-CH_2-NH_3^+)$_2$, 16H). HRMS (m/z): 388.3409 [$(M+H)^+$] (Observed), 388.3400 [$(M+H)^+$] (Calculated).

4.4 Experimental procedure

4.4.1 Antibacterial assay

In case of clinically isolated multidrug-resistant strains (MDR *E. Coli* R250, MDR *A. Baumanni* R676, *Klebsiella pnuemoniae* NDM-1 R3421), the working solution(1×10^5 cfu/mL) were made by purifying the bacterial strains from a clinical sample. The rest of the procedure is same as described in the section of 2.4.1 in Chapter 2.

4.4.2 Time-kill kinetics assay

The same protocol was followed as described in the section of 3.4.2 in Chapter 3.

4.4.3 Hemolytic assay

The same protocol was followed as described in the section of 2.4.2 in Chapter 2.

4.4.4 Mechanism of Action

4.4.4.1 Membrane permeabilization assay

The same protocol was followed using the test compounds **LANA3** and **LANA8** as described in the section of 3.4.6.1 in Chapter 3.

4.4.4.2 Cytoplasmic membrane depolarization assay

The same protocol was followed using the test compounds **LANA3** and **LANA8** as described in the section of 3.4.6.2 in Chapter 3.

4.4.4.3 K^+ leakage assay

The same protocol was followed using the test compounds **LANA3** and **LANA8** as described in the section of 3.4.6.3 in Chapter 3.

4.4.5 Resistant study

Same as described in the section of 3.4.7 in chapter 2. But, test compound in this case was **LANA8**.

4.4.6 Biofilm inhibition assay

Same as described in the section of 3.4.7 in chapter 2. But, test compound in this case is **LANA8**.

4.5 Results

4.5.1 Antibacterial activity

The antibacterial activity of the lysine conjugated aliphatic norspermidine analogues (**LANAs**) and other compounds (**OANA** derivatives and control compound **LL**) were determined (Table 4.1) against different drug sensitive Gram-positive (*S. aureus* and *E. faecium*) and Gram-negative (*E. coli*) bacteria as well as drug-resistant bacteria MRSA, VRE and *K. pneumoniae*.

All the **LANA** derivatives found to have potent activity against all the bacteria under tested. Unlike the compounds of other two series (**ANAs** and **PANAs**) these series of derivatives (LANAs) exhibited excellent antibacterial efficacy against Gram-negative bacteria as well. The compound **LANA1** which consist of a decyl chain showed low activity against all bacteria tested. The MIC values of **LANA1** were 53 μM and 40 μM against *S. aureus* and *E. coli* respectively. The next analogue **LANA**2 with a dodecyl chain displayed a

little enhanced activity with the MIC values of 37 μM and 14 μM against *S. aureus* and *E. coli* respectively. Upon increasing the chain length further to tetradecyl, the compound **LANA 3** exhibited even better antibacterial efficacy with the MIC values of 7.8 μM and 5.9 μM against the bacteria *S. aureus* and *E. coli*, respectively. The hexadecyl analogue, **LANA4** was the most potent antibacterial agent in this series displaying MIC values of 4.7 μM and 5.4 μM against *S. aureus* and *E. coli* respectively. However, the next homologue **LANA5**, with octadecyl aliphatic chain showed a slight decrease in antibacterial activity (MIC value of 7.7 μM and 8.4 μM against *S. aureus* and *E. coli*, respectively).

Table 4.1: Antibacterial activity of lysine-based lipopeptide mimics

	Minimum Inhibitory Concentration (μM)					
	Drug sensitive strains			Drug resistant strains		
Compounds	*S. aureus*	*E. faceium*	*E. coli*	*MRSA*	*VRE*	*K. pneumonie*
LANA1	53	60	40	65	58	98
LANA2	37	32	14	45	30	40
LANA3	7.8	2	5.9	14	1.5	9.3
LANA4	4.7	1.1	5.4	4.8	1	6.5
LANA5	7.7	4.5	8.4	14	2.2	7.5
LANA6	5.2	7	8	15	5.2	ND
LANA7	13.5	20	65	75	12	ND
LANA8	5.7	3.8	5.4	5.8	2.8	9
LANA9	3.9	2.7	4.8	4.2	2.2	5
OANA1	7.9	5.2	6.6	13.5	5.3	9.4
OANA2	6.4	3.2	5.2	9	3	4.8
LL	>261	>261	>261	>261	>261	>261
Colistin	20	>199	0.4	54	>199	1.2
Vancomycin	0.63	0.6	ND	0.63	750	ND
ANA4	20	4.5	24	19	4	109
PANA2	4.3	4.8	12	4	2.7	9.7

ND stands for "not determined". MRSA is methicillin-resistant *S. aureus*, VRE is vancomycin-resistant *E. faecium* and *K. pneumoniae* is resistant to β-lactam antibiotics.

In comparison to other bacteria, activity of **LANA3** and **LANA4** against *E. faecium* was superior with the values of 2 μM and 1.1 μM, respectively. Among the other derivatives **LANA 5** also exhibited significant activity with MIC of 4.5 μM. Compounds **LANA1** and **LANA2** were devoid of significant activity against *E. faecium*.

These derivatives were also found to have good antibacterial activity against the drug-resistant superbug MRSA. The most potent derivatives **LANA4** with MIC value of 4.8 µM, was almost as active as the most potent **PANA** derivative, **PANA**2. However, the highlight of these compounds was their superior activity against VRE. The activity of compounds **LANA3** and **LANA4** (MIC values of 1.5 µM and 1 µM respectively) accentuates the promise of these compounds as future antibacterial agents. Another derivative **LANA5** also showed potent activity against VRE (MIC value of 2.2 µM). An additional important feature of these compounds was the superior potency against the Gram-negative β-lactam resistant *K. pneumoniae*. **LANA4** displayed excellent activity with MIC of 6.5 µM against this bacterium. Among the other compounds **LANA3** and **LANA5** also showed decent potency, the MIC values being 9.3 µM and 7.5 µM respectively.

The compound **LANA6** consisting of an oleyl group in place of octadecyl group revealed similar antibacterial activity compare to its saturated analogue **LANA5.** Compound **LANA6** has MIC values of 5.2 µM and 8 µM against *S. aureus* and *E. coli* respectively. But**,** upon increasing the number of double bonds in the aliphatic group further, antibacterial activity was found decrease. The derivative **LANA7** consisting of linoleyl group (9-8 and 12-13 olefin analogue of octadecyl group) displayed a decreased antibacterial efficacy with MIC values of 13.5 µM and 65 µM against *S. aureus* and *E. coli* respectively. Like the case of **PANA** series; the derivatives **LANA8** and **LANA9** (consisting of D-Lysine) revealed similar antibacterial efficacy as the corresponding "L" analogues **LANA3** and **LANA4**, respectively (Table 4.1). Incorporation of ornithine in place of lysine showed a little decrease in antibacterial activity against most of the bacteria tested. The compounds **OANA1 (**tetradecyl analogue) and **OANA2** (hexadecyl analogue) displayed very good activity against VRE with MIC value of 5.3 µM and 3 µM respectively (Table 4.1). The control compound **LL** (lacking the aliphatic chain) did not show any activity up to 250 µM against all the bacteria tested.

A significant feature of these compounds was their superior activity against multi-drug resistant (MDR) clinical isolates (Table 4.2). For example, both **LANA4** and **LANA5** showed MICs of 3.5 µM against the clinical isolate of *E. coli*. Similar activity was also observed in case of *A. baumannii* but the activity against *K. pneumoniae* was somewhat compromised; MIC of **LANA 4** was 7µM while that of **LANA5** was 3.5µM.

Table 4.2: Antibacterial activity against multi-drug resistant (MDR) clinically isolated bacteria.

Minimum Inhibitory Concentration (μM)			
	Multi Drug Resistant (MDR) strains		
Compounds	*E. Coli*	*A. Baumanni*	*K. pneumonie*
LANA1	>250	>250	>250
LANA2	62.5	125	>250
LANA3	31	7	>250
LANA4	3.5	3.5	7
LANA5	3.5	3.5	3.5
LANA8	62	15	>250
LANA9	3.5	3.5	7
OANA2	3.5	3.5	4
LL	>250	250	>250

4.5.2 Bactericidal kinetics

These **LANA** derivatives, like the PANA derivatives were bactericidal agents (Chapter 3). The time-kill kinetics of the compounds **LANA3** and **LANA8** against *S. aureus* showed bactericidal activity within the first hour (Figure 4.2). Both the compounds were able to reduce the number of viable bacteria (approximately 5 $\log_{10}$ (cfu/mL) reduction) rapidly at a concentration of 6 × MIC. On performing the experiment in a smaller interval of time it was found that **LANA8** could reduce bacteria by 4 $\log_{10}$ (cfu/mL) within 30 minutes (inset of Figure 4.2B).

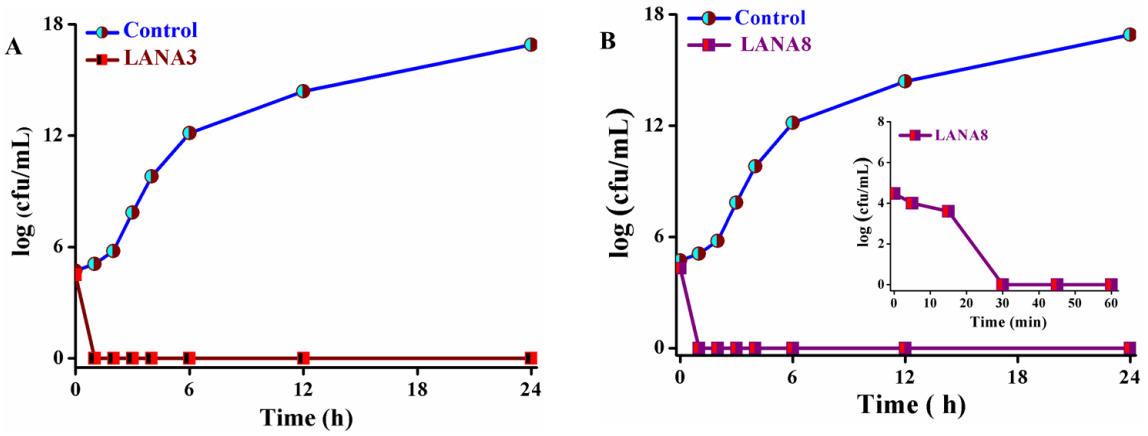


Figure 4.2: Time-kill kinetics against *S. aureus* of (A) **LANA3** (6 × MIC) and (B) **LANA8** (6 × MIC).

4.5.3 Toxicity

The effective antibacterial agent is one which has selective potency towards bacterial cell. As a preliminary investigation into the effectiveness of **LANA** derivatives as selective antibacterial agents, toxicity towards human erythrocytes (RBCs) was determined similar to the previous derivatives (**ANAs** and **PANAs**). The HC_{50} values of the compounds were showed in the Table 4.3.

Table 4.3: Haemolytic activity of lysine-based lipopeptide mimics.

Compounds	HC_{50} (μM)	Selectivity[a]
LANA1	>1000	>17
LANA2	>1000	>33
LANA3	588	392
LANA4	264	264
LANA5	108	49
LANA6	950	183
LANA7	>4000	> 333
LANA8	730	260
LANA9	283	129
OANA1	525	99
OANA 2	224	75
ANA4	87	22
PANA2	205	76

[a]Selectivity is calculated based on HC_{50}/the MIC against VRE.

From the HC_{50} values of these **LANA** derivatives it was found that most of the derivatives required a very high concentration to cause haemolysis. The derivatives with the decyl and dodecyl aliphatic group, compounds **LANA1** and **LANA2,** did not show any haemolytic activity till a concentration of 1000 μM. The derivative with tetradecyl chain (**LANA3**) revealed the HC_{50} of 588 μM which was comparably higher than those of compounds in **ANA** and **PANA** series. Due to the presence of more lipophilic chains,

compounds **LANA4** (hexadecyl analogue) and **LANA5** (octadecyl analogue) had lesser HC_{50} values of 264 µM and 105 µM respectively, as expected. The unsaturated derivatives **LANA6** and **LANA7** displayed very less toxicity towards RBCs. Compound **LANA6** having single olefin unsaturation had a HC_{50} of 950 µM, which is much higher than its corresponding saturated analogue (**LANA5**). The derivative with two unsaturated groups in the aliphatic chain, compound **LANA7,** showed only 30% haemolysis even at a concentration as high as 4000 µM. Like the **PANA** derivatives (Chapter 3) corresponding D-isomers, **LANA8** and **LANA9** showed lesser toxicity (HC_{50} values of 730 µM and 283 µM respectively). The derivatives consisting of ornithine showed a little higher toxicity compared to its lysine analogues; the HC_{50} values being 525 µM and 224 µM.

4.5.4 Mechanism of action

The **LANA** derivatives, like the PANAs, were expected to act by causing membrane depolarization, membrane permeabilization and leakage of K^+ ions. For carrying out the spectroscopic studies towards investigation of mechanism of action the most selective analogues **LANA3** and **LANA8** were selected as model compounds. But, surprisingly none of the compounds showed membrane permeabilization against either *S. aureus* (Figure 4.3A) or *E. coli* (Figure 4.3B). Although the compounds did not have ability to permeabilize the bacterial membrane, both the compounds were able to depolarize the membranes of *S. aureus* (Figure 4.3C). These derivatives also possessed the ability to cause the leakage of K^+ ions in both *S. aureus* and *E. coli* (Figure 4.3D and 4.3E). As colistin is active only against Gram-negative bacteria, this was included in the experiment against *E. coli.* However it had neither caused membrane permeabilization nor did it have the ability to cause leakage of K^+ ions.

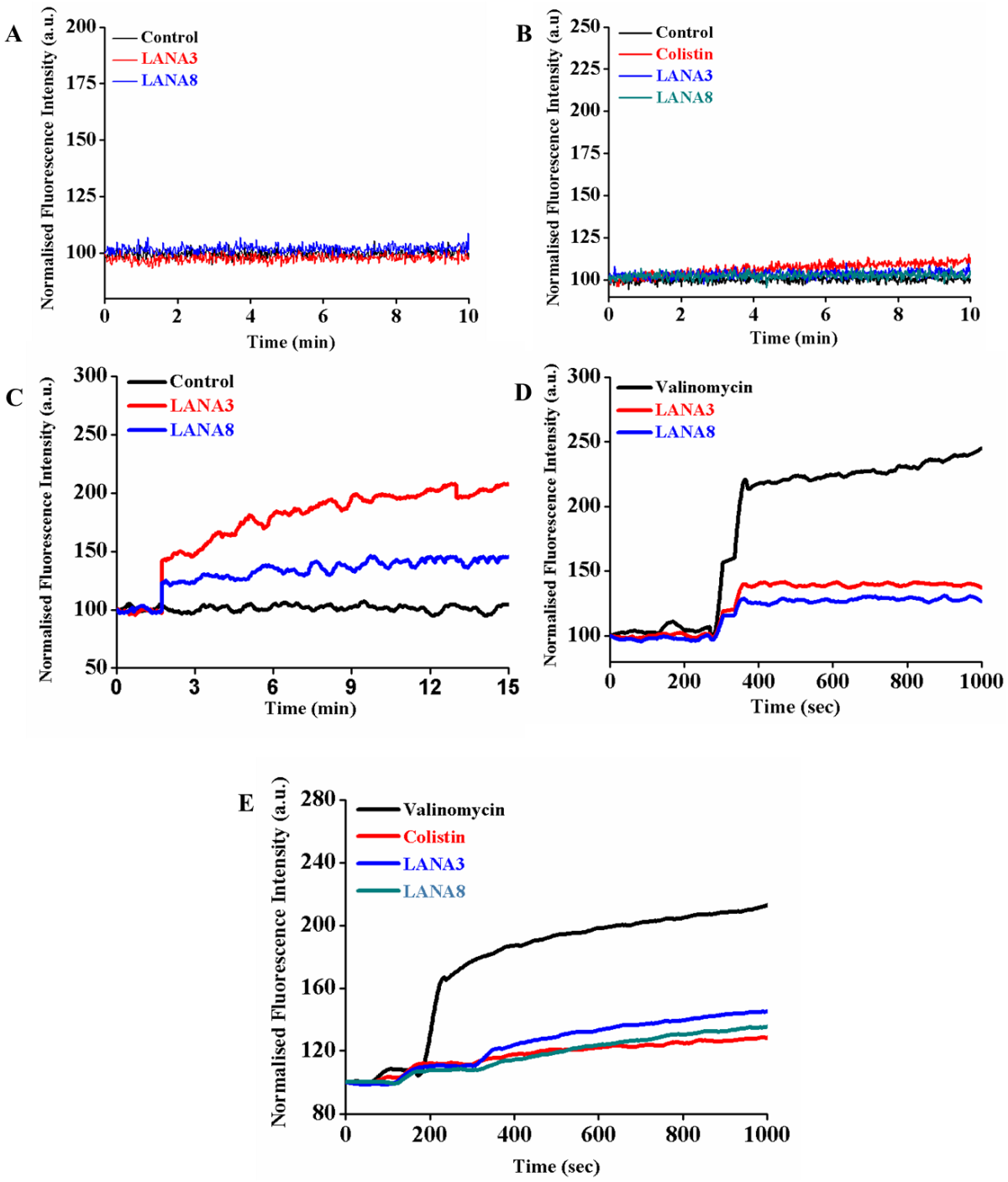


Figure 4.3: (A) Membrane permeabilization of *S. aureus* by **LANA3** (12 × MIC) and **LANA8** (12 × MIC). (B) Membrane permeabilization of *E. coli* by **LANA3** (12 × MIC), **LANA8** (12 × MIC) and **colistin** (12 × MIC). (C) Membrane depolarization of *S. aureus* by **LANA3** (12 × MIC) and **LANA8** (12 × MIC). (D) K^+ leakage of *S. aureus* by **LANA3** (12 × MIC), **LANA8** (12 × MIC). (E) K^+ leakage of *E. coli* by **LANA3** (12 × MIC), **LANA8** (12 × MIC) and colistin (12 × MIC).

4.5.4 Propensity to induce bacterial resistance

In order to investigate the efficiency of **LANA** derivatives as long-lasting antibacterial agents, the capability of *S. aureus* (Gram-positive representative) and *E. coli* (Gram-negative representative) bacteria to develop resistance against these derivatives were examined. The compound **LANA8** was selected as the model compound for this experiment. Similar to the previous chapter (Chapter 3) norfloxacin and colistin were selected a positive control for *S. aureus* and *E. coli* respectively. None of the bacteria were able to develop resistance towards the test compound **LANA8** as indicated by the constancy in MIC value even after 20 passages (Figure 4.4). Norfloxacin, on the other hand, was found to have around 800 fold increases in MIC value after 20 passages against *S. aureus* (Figure 4.4A). Similarly colistin was found to have around 250 fold increased MIC against *E. coli* (Figure 4.4B). This study suggests that like in the case of **PANA** derivatives (Chapter 3), bacteria faced difficulty to develop resistance against these **LANA** derivatives.

4.5.5 Biofilm inhibition

Similar to the previous derivatives (**PANAs**) biofilm inhibition experiment was performed for these compounds. Compound **LANA8** was selected as the model compound for this experiment. Test compound revealed an ability to inhibit the formation of bacterial biofilm (*S. aureus*) quite efficiently. This compound could bring down cell viability to 5% from 100%. The complete inhibition of biofilm formation was observed at a concentration of 10 μM (almost double of MIC of **LANA8**) as observed in Figure 4.4C.

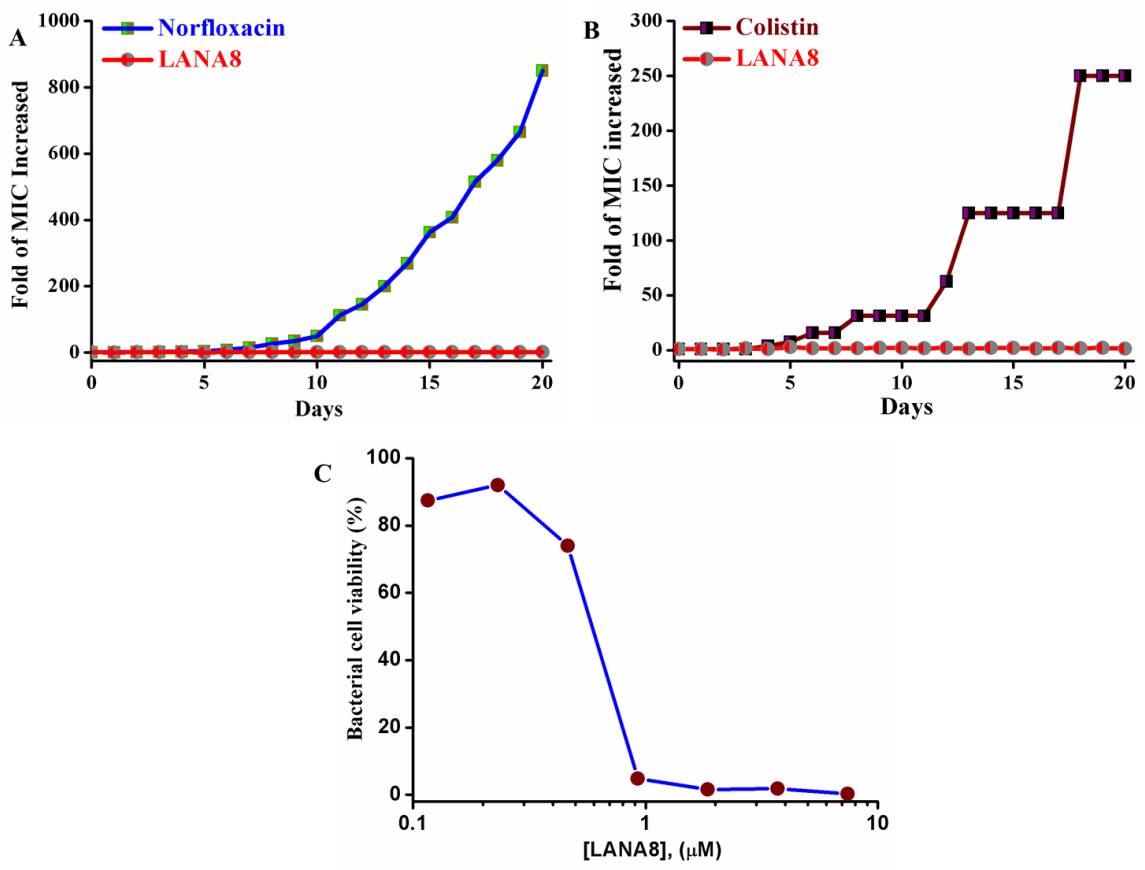


Figure 4.4: (A) Development of resistant of *S. aureus* against norfloxacin and **LANA8.** (B) Development of resistant of *E. coli* against colistin and **LANA8**. (C) Inhibition of biofilm formation of *S. aureus* with **LANA8**.

4.6 Discussion

In the previous chapters (Chapter 2 and Chapter 3), dicationic norspermidine based lipopeptide mimics were reported. Chapter 2 described the design and synthesis of **ANA** derivatives which showed moderate antibacterial activity within a narrow range of Gram-positive bacteria. The toxicity of those compounds towards human erythrocytes (RBCs) was considerable. In order to address such limitation associated with **ANA** derivatives, the **PANA** derivatives have been developed with the introduction of phenylalanine moiety into the design. The positive charges were then contributed by the α-NH_2 groups of the phenylalanine moiety, which spaced out the positive charges and also introduced the possibility of H-bonding interactions through the amide bond introduced into the design. As expected, it resulted in improved antibacterial activity against Gram-positive bacteria. But unfortunately these **PANA** derivatives only displayed moderate activity against Gram-

negative bacteria. In addition to that these derivatives also revealed considerable amount of toxicity towards RBCs.

As a further improvement on the design, **LANA** derivatives which were prepared in which lysine replaced the phenylalanine moieties. The presence of lysine is common in natural antimicrobial peptides and lipopeptides as well as their synthetic mimics, as mentioned earlier. The advantage that lysine provides in these derivatives is that it provides additional two positive charges. Thus, the design includes four cationic charges in total. Two were contributed by the α-NH_2 groups and the other two were from ε-NH_2 groups of lysine. This introduction of more cationic charges in the compounds allows them to have greater electrostatic interaction with the bacterial cell membrane. Additionally, it might provide additional hydrogen bonding interactions. This is more beneficial in case of Gram-negative bacteria as they have high negative charge density on their outer membrane. The lipophilic aliphatic chain is also present to facilitate better interaction with the membranes. Thus now in the design, all the necessary criteria for achieving efficient antibacterial activity have been incorporated, namely, electrostatic interaction, hydrogen bonding ability and presence of optimum hydrophobicity.

Our hypothesis was validated by the antibacterial activities displayed by these derivatives. The compounds showed potent activity against both Gram-positive and Gram-negative bacteria. The spectrum of activity was also extended over the clinically isolated multi-drug resistant bacterial strains. This outstanding activity towards multiple bacterial strains makes the **LANA** derivatives superior antibacterial agents over PANAs and ANAs derivatives.

Following the already established trend, antibacterial activity maintained a parabolic relationship with increasing aliphatic chain lengths (Figure 4.5A). Against all the bacteria, the maxima with respect to antibacterial efficacy were reached at **LANA4** (hexadecyl long chain). In order to establish an efficient SAR, L-lysine was replaced by L-ornithine in some cases. But no significant difference in activity was observed on replacing the lysine with ornithine, proving that the number of charges play a more vital role than the number of bonds between the charges. Additionally, unsaturation was introduced in the octadecyl chain in order to understand the importance of aliphatic geometry towards selective antibacterial activity. Again, introduction of a single unsaturation in the lipid tail (**LANA6**) does not create a significant difference in activity. However, on introduction of another unsaturation, the antibacterial activity is compromised, as was observed in **LANA7**. As in the previous

studies, the effect of stereoisomerism was also studied by replacing L-Lysine with D-Lysine, but no significant difference in antibacterial activity was observed.

With respect to toxicity, these **LANA** derivatives were far superior to **PANA** derivatives (Table 4.3). The most active compound (**LANA4**) consisting of hexadecyl aliphatic group was found to have HC_{50} of 264 μM; with respect to VRE the selectivity index was 264 which is an excellent value. The compound**LANA3** consisting of tetradecyl long chain was found to display an even higher selectivity (SI of 392) towards VRE (Figure 4.5B). In comparison the most selective compounds in the previous series had SI of 76 (**PANA2**) and 22 (**ANA2**) only. This fact implies that significant improvement in the design of lipopeptide mimics was achieved by the introduction of lysine moieties. Replacement of lysine by ornithine reflects a slight increase in haemolytic activity. But the difference in selectivity was superior on moving from **LANA5** to **LANA6** to **LANA7**. Compounds with linoleyl group (**LANA7**) showed no haemolysis while oleyl analogue LANA 6 had negligible toxicity. This proves that geometry and flexibility of the aliphatic chain has immense importance towards toxicity. No significant difference in toxicity was noticed once L-Lysine was replaced D-Lysine.

These derivatives also displayed a rapid killing efficacy of *S. aureus* bacteria, as was observed even in the **PANA** series. However, from the experiments performed towards mechanism of action, these compounds seemed to act only by causing depolarization of bacterial membrane. They definitely did not cause any membrane permeabilization, nor were they efficient in causing leakage of K^+ ions. Further investigations need to be carried to efficiently establish the mechanism of action of these rapidly bactericidal, broad-spectrum antibacterial agents. These derivatives did not induce resistance in neither Gram-positive, nor Gram-negative bacteria even after 20 passages. This proves the long-lasting antibacterial efficacy of the compounds and enhances their chances as potent clinical candidates. Additionally these derivatives also have the ability to inhibit bacterial biofilm formation at a low concentration, which extends the application of compounds towards coatings on medical implant and devices.

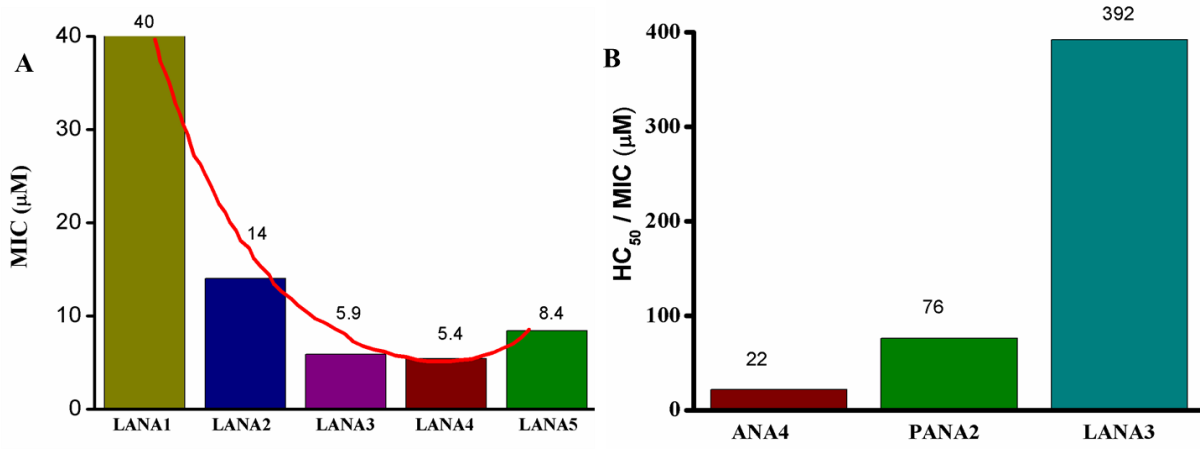


Figure 4.5: (A) Variation of antibacterial activity against *E. coli* with aliphatic chain. (Numbers in the graph indicate MIC values) (B) Comparison of selectivity ratio of **ANA4**, **PANA2** and **LANA3** against VRE. (Numbers in the graph indicate MIC values).

4.7 Conclusion

In summary, a novel series of lysine conjugated aliphatic norspermidine analogues (**LANAs**) have been designed and synthesised with improved antibacterial activity and selectivity. These compounds were active over a broad spectrum of bacteria strains (including clinically isolated multi-drug resistant) also. The best selective antibacterial compound found to consisting of tetradecyl aliphatic group which displayed a 392 fold selective activity towards drug resistant bacteria VRE. Rapidly bactericidal, these compounds do not allow the bacteria to develop resistance against them. Hence, these derivatives bear immense potential as therapeutic agents to tackle multi-drug resistant bacterial infection.

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Patents and Publications

1. **Konai, M. M.**; Haldar, J. Polyamine based antimicrobial conjugates, method for production and uses thereof. *Patent application no* 1345/CHE/2014.

2. Yarlagadda, V.; Manjunath, G. B.; **Konai, M. M.**; Haldar, J. Vancomycin-sugar conjugates and uses thereof. *Patent application no* 4314/CHENP/2013.

3. Ghosh, C.; Manjunath, G. B.; Akkapeddi, P.; Yarlagadda, V.; Hoque, J.; Uppu, D. S. S. M.; **Konai, M. M.**; Haldar, J. Small molecular antibacterial peptoid mimics: The simpler the better! *J. Med. Chem.* **2014**, 57, 1428-1436.

4. Yarlagadda, V.; Manjunath, G. B.; **Konai, M. M.**; Haldar, J. Sweetening of vancomycin to reinstate activity against vancomycin-resistant bacteria through enhanced binding affinity. *Manuscript under revision*.

5. Yarlagadda, V.; **Konai, M. M.**; Manjunath, G. B.; Haldar, J. Synthesis and evaluation of vancomycin-sugar conjugates to tackle vancomycin-resistant bacteria. *Manuscript under revision*.

6. Yarlagadda, V.; Manjunath, G. B.; **Konai M. M.**; Shome, B. R.; Krisnamurthy, P.; Haldar, J.; *In-vivo* antibacterial activity and toxicology of membrane active glycopeptide antibiotic. *Manuscript under submission*.

7. Yarlagadda, V.; **Konai M. M.**; Shome, B. R.; Krisnamurthy, P.; Haldar, J.; *In-vivo* analysis of lipovancomycin-sugar conjugates. *Manuscript under submission*.

8. **Konai M. M.**; Yarlagadda, V.; Ghosh, C.; Haldar, J. Synthesis and antibacterial properties of phenylalanine conjugated aliphatic norspermidine analogues. *Manuscript under preparation*.

9. **Konai M. M**.; Ghosh, C.; Yarlagadda, V.; Manjunath, G. B.; Haldar, J. Lysine-based lipopeptide mimics: A novel approach to tackle multidrug-resistant bacteria. *Manuscript under preparation*.