SYNTHESIS, CHARACTERIZATION, AND ANTIBACTERIAL EVALUATION OF PEPTIDES AND THEIR POLY-*N*-SUBSTITUTED GLYCINE CONGENERS

Thesis submitted in fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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TABLE OF CONTENTS

DECLARATION BY THE SCHOLAR

I, **Deepika Sharma**, hereby declare that the work reported in the Ph.D. thesis entitled **"Synthesis, characterization, and antibacterial evaluation of peptides and their poly-*N*-substituted glycine congeners"** submitted at the **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Gopal Singh Bisht**. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. thesis.

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Synthesis, characterization, and antibacterial evaluation of peptides and their poly-*N*-substituted glycine congeners"** submitted by **Deepika Sharma (Enrollment No. 136753)** at the **Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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ABSTRACT

With the emergence of multi drug resistant bacterial strains, it is the need of hour to develop new antimicrobial therapeutic agents which are less prone to development of resistance. Antimicrobial peptides have emerged as one such class of antimicrobials that have been proposed to be less prone to development of resistance due to their membrane disruptive action on bacteria. However antimicrobial peptides suffer from few limitations, like poor proteolytic stability and high cost of production. To overcome these limitations, poly-*N*-substituted glycines are usually designed and synthesized. In the present work, numerous peptides and their poly-*N*-substituted glycine congeners were synthesized and tested against various bacterial strains. Synthesized compounds exhibited minimum inhibitory concentration in micro molar range (3-100 μg/ml) against bacterial strains tested. LP-23 and DP-23 emerged as lead compounds against *M. smegmatis*. On the other hand SA4 and SPO emerged as leads against biofilm forming susceptible and multidrug resistant clinical isolates of *A. baumannii*. These compounds have the potential to inhibit *A. baumannii* biofilms. All lead compounds LP-23, DP-23, SPO, and SA4 were found to be selective towards bacteria, as their HC_{50} values were high in comparison to their MIC values. The disruptive effects of lead compounds on bacterial cell membranes were visualized by SEM analysis.

From the SEM microphotographs we could visualize disruptive effects of short peptide based compounds SA4 and SPO on cell membranes of *A. baumannii* cells. Similarly membrane disruption effects were also observed in DP-23 and LP-23 treated *M. smegmatis* cells, DP-23 was found to form pores on cell membranes. This non specific membrane disruptive mode of action of these compounds makes them suitable candidates for antimicrobial drug development. In addition the stability of lead compounds to proteolysis was evaluated in human serum; SPO was found to be most stable in human serum. The present work affords four lead compounds LP-23, DP-23, SA4, and SPO with potent activity against representative Gram-positive and Gram-negative bacterial strains including multidrug resistant clinical isolates of biofilm forming *A. baumannii*. *N*-substituted glycine congeners DP-23 and SPO are more stable to proteolysis then LP-23 and SA4 peptides. All four active compounds did not cause any significant damage to human erythrocytes up to a concentration of 200 μg/ml. Based on SEM analysis, it may be proposed that the mode of action of these compounds is membrane lysis and further their selectivity towards bacterial cells makes them suitable candidates for antimicrobial drug development in future.

LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
º C	Degree Celsius
mg	Microgram
µl	Microliter
µm	Micromole
ml	Milliliter
AMPs	Antimicrobial peptides
hRBCs	Human red blood cells
HPLC	High performance liquid chromatography
H	Hour
OD	Optical density
PBS	Phosphate Buffer Saline
SD	Standard Deviation
WHO	World Health Organization
MOA	Mechanism of action
HDP	Host defense peptides
MIC	Minimum inhibitory concentration
MBEC	Minimum biofilm eradication concentration
rpm	Rotation per minute
AUC	Area under curve
nm	Nanometer
MDR	Multi drug resistant
AUC	Area under curve
TCA	Trichloroacetic acid
E. coli spp	*Escherichia coli* species
S. aureus	*Staphylococcus aureus*
P. aeruginosa	*Pseudomonas aeruginosa*
K. pneumonia	*Klebsiella pneumonia*
E. cloacae	*Enterobacter cloacae*
S. epidermidis	*Staphylococcus epidermidis*

LIST OF FIGURES

LIST OF SCHEMES

LIST OF TABLES

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1. INTRODUCTION

Antibiotics were developed in 20th century [1] and ever since the discovery of penicillin in 1928 by Sir Alexander Fleming, antibiotics have saved countless lives. A medical revolution concerning plant, animal, and human prophylaxis was brought and antibiotics were taken as for granted. Ease of availability of antibiotics led to rampant use of these medicines that resulted into emergence and spread of antibiotic resistance [2]. Antimicrobial resistance (AMR) can be defined as the ability of microbes to stop an antimicrobial agent from working against it. As a consequence, medicines become ineffective and infections persist in the body and may spread to others. As resistant pathogenic bacteria are spreading rapidly, the common infections like pneumonia, wound infections, and urinary tract infections are becoming more difficult to treat [3].

Incidences of infections due to multidrug-resistant (MDR) pathogens in immuno-compromised patients are high in comparison to healthy individuals [4]. In addition, these pathogens cause various hospital acquired infections, like ventilator-associated pneumonia [5], bloodstream infections [6], surgical site infections [7], and implant-associated infections [8]. Since very few new anti-infective agents have entered into the market over the last three decades; the problem of microbial resistance is still a challenge [9]. Drug resistant nosocomial bacteria have not only become major global healthcare issue, but on the other hand treatment of infections caused by such pathogens imposes an economic burden on society [10, 11].

Resistant pathogens are of great clinical concern as they are associated with enhanced virulence and transmissibility [12] [4]. Recently, WHO has published a list of antibiotic resistant “priority pathogens” for which new antibiotics are urgently needed. This list is a catalogue of bacteria from 12 families that pose a great threat to humanity. The bacteria have been categorized into three main categories (critical, high, and medium priority) depending on the urgency of need for new antibiotics (**Table 1.1**).

The critical group includes MDR that have become resistant to a large number of antibiotics, including the best available antibiotics (carbapenems and third generation

cephalosporins) for treating MDR bacteria. The second and third categories in the list are the high and medium priority categories. These categories include other increasingly drug-resistant bacteria that are the causative agents of more common diseases, like gonorrhoea and food poisoning caused by *Salmonella* [259]. WHO has made a nerve-racking prediction that by the year 2050, infections caused by drug resistant pathogens will kill about 10 million people per year [13].

Table 1.1: WHO priority pathogens list for R&D of new antibiotics [259]

Priority 1: CRITICAL
• *Acinetobacter baumannii*, carbapenem-resistant • *Pseudomonas aeruginosa*, carbapenem-resistant • *Enterobacteriaceae*, carbapenem-resistant, ESBL-producing
Priority 2: HIGH
• *Enterococcus faecium*, vancomycin-resistant • *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant • *Helicobacter pylori*, clarithromycin-resistant • *Enterococcus faecium*, vancomycin-resistant • *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant • *Helicobacter pylori*, clarithromycin-resistant • *Campylobacter* spp., fluoroquinolone-resistant • *Salmonellae*, fluoroquinolone-resistant • *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant
Priority 3: MEDIUM
• *Streptococcus pneumoniae*, penicillin-non-susceptible • *Haemophilus influenzae*, ampicillin-resistant • *Shigella* spp., fluoroquinolone-resistant

Moreover, a new concern has emerged nowadays with respect to biofilm-associated infections. Several biofilm-producing bacteria have become refractory to the presently available antimicrobial arsenal, leaving limited therapeutic options [14]. Sadly, no ideal antibiotics exist. Most of the antibiotics that exist possess antibacterial activity against

Gram-positive bacteria and some broad-spectrum antibacterial agents are active against certain Gram-negative bacteria [15].

Along with this problem various pharmaceutical companies, like Allergan, Novartis, Bristol-Myers Squibb, Sanofi, and Astra Zeneca have either turned away from participating in antimicrobial drug development programs or have dropped their antimicrobial research programs. The biggest reason behind this trend is big financial risk [16]. Huge investments are required to cover the expenses involved in the research and development of new drug candidate. Irrespective of the type of drug candidate, organizations have to bear big financial risks during clinical trials of drugs [17] [18].

Antibiotic development has now shifted from large firms to smaller biotechnology startup companies that have preferential interests in antimicrobial agents [19] [20] [21]. Therefore, there is a pressing need to discover new therapeutics to treat MDR bacterial strains [22]. This lack of treatment options highlights the need for novel strategies. One such strategy is the utilization of antimicrobial peptides (AMPs) as the promising alternative compounds in the treatment of microbial infections [23].

Host defense peptides (HDPs) have emerged as the potential antimicrobial candidates [24]. These natural antimicrobial peptides (AMPs) are integral components of the host innate immune system in organisms. They are present in various species, like plants, animals, and humans [25], [26]. AMPs exhibit high bactericidal activities [27] and majority of AMPs are cationic in nature, this positive charge plays a key role in antimicrobial action [28]. The cationic peptides with variable length, amino acid composition, and secondary structure have gained enormous attention as antimicrobial agents during the past decades [23].

Variety of AMPs have been discovered and isolated from different organisms, like bacteria, fungi, plants, and animals. These AMPs are capable of producing their antimicrobial action against susceptible as well as drug-resistant microbes. Mechanisms of antimicrobial action of AMPs are different from conventional antibiotics. The majority of them cause lysis of bacterial cell membrane [29]. They have broad-spectrum of activity, low toxicity, and low propensity of resistance development [30]. AMPs that have been isolated from nature have proven to be very useful templates for the design and synthesis of synthetic AMPs with improved properties [31] [32].

1.2. REVIEW OF LITERATURE

1.2.1. Role of AMPs in living organisms

In majority of organisms, AMPs are expressed on those surfaces that have constant exposure to pathogens. A few examples of such surfaces are the mucosal lining and skin of vertebrates [33] [34]. These AMPs can directly kill microorganisms and produce immunomodulatory actions during infection [35] [36]. AMPs have been isolated from amphibians, marine animals, and terrestrial animals [37]. In amphibians, AMPs are produced in the glands of the dermal skin layer and released upon exposure to pathogens, where they induce a microbicidal action through membrane disruption [38] [39].

While in humans, AMPs are continuously secreted by the sweat glands and secreted AMPs form a barrier against pathogens [40]. In addition, AMPs also play important roles in defense mechanisms of plants against pathogens. Various peptides have been isolated from different parts of the plants (seeds, leaves, and roots) [41]. Plant defensins are cationic in nature and exhibit potent antibacterial activities against pathogens [42] [43]. Mechanism of action (MOA) of plant based peptides is somewhat similar to MOA of AMPs from vertebrates. They disrupt the cell membrane and act on intracellular targets [44] [45]. On the whole, AMPs play multifunctional roles in providing immunity to organisms from invading pathogens [46] [47] [48].

1.2.2 Classification of AMPs

Based on their secondary structures, AMPs have been classified into 3 classes (**Figure 1.2.2**). These classes are α-helical, β-sheet, and extended-coiled peptides [49] [50]. The α-helical AMPs are the ones that remain unordered in aqueous solution but once they come in contact with a biological membrane, they assume an amphipathic α-helical structure [35]. For example, LL-37 from humans [51], aurein from the granular dorsal glands of the frog *Litoria aurea* [52], melittin from honey-bee venom, and cecropin from the hemolymph of *Hyalophora cecropia* [53].

While on the other hand, the β-sheet peptides constitute the largest group of AMPs. They are produced in several organisms like plants, marine organisms, amphibians, and animals [46] [47] [48] [54]. They have been found to exhibit antibacterial, antiviral, anti

inflammatory and antifungal properties [55] [35] [56]. When compared to α-helical peptides, these peptides remain ordered in aqueous solution [57]. Selected examples of few natural antibacterial peptides that attain β-Helix structure are protegrin [58] and polyphemusin [59]. Third class is an extended-coil structure, this class of AMPs lack secondary structures present in α-helical and β-sheet peptides. These AMPs in most of the cases have been observed to contain specific amino acids, like arginine, proline, and tryptophan [36]. They exhibit antibacterial activity and have been found to act against Gram-negative bacteria by membrane disruption or by targeting internal processes [60]. Selected examples of few natural antibacterial peptides that attain extended structure are indolicidin [61] and PR-39 [62].

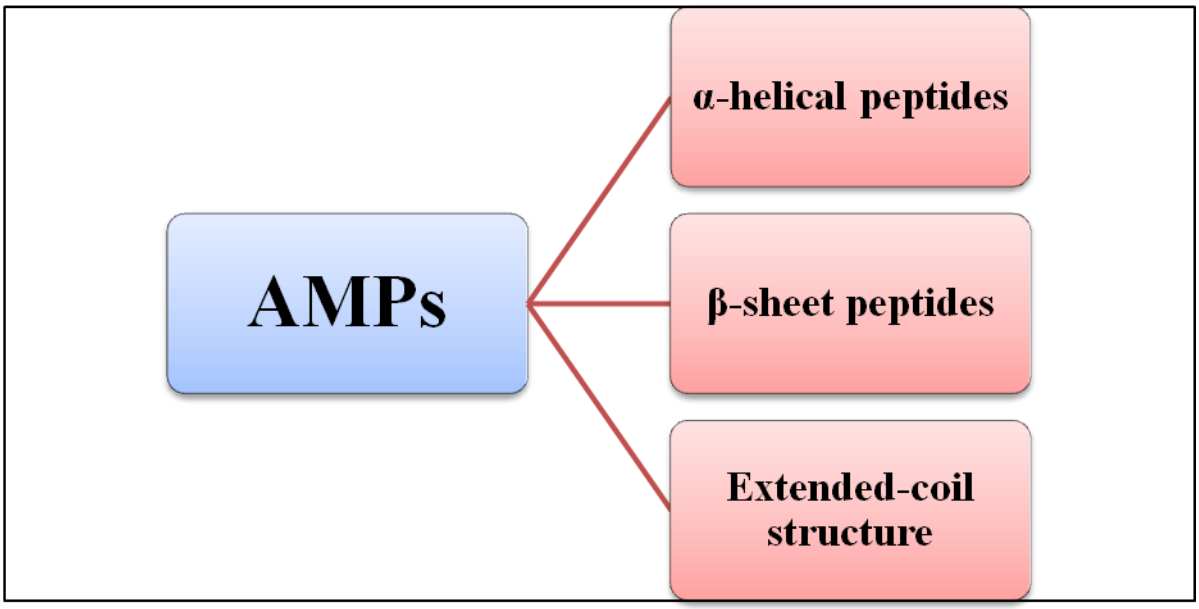


Figure 1.2.2: Classification of AMPs based on structure

1.2.3. Mechanism of action (MOA) of AMPs

Precise mechanism of AMPs is not clear but as per literature, AMPs kill pathogens either through membrane disruptive mechanisms or non-membrane disruptive mechanisms or combination of both [63] [64] [65]. Membrane disruptive killing action of AMPs is governed by targeting and disrupting the bacterial plasma-membrane through permeabilization, which results in leakage of intracellular contents and hence, causes cell death [66]. This type of action is dependent on the concentration threshold of peptide molecules on the surface of bacterial membranes [67] [68]. To explain this MOA of membrane disruption three models have been proposed, in general all models suggest that, the MOA of AMP begins with the accumulation of AMP molecules parallel

to the bacterial membrane. This accumulation is followed by electrostatic interaction between the cationic charges of AMPs and anionic charges on the surface of bacterial membrane [31].

MOA of AMPs has a close correlation with their net positive charge and hydrophobicity [69] [70]. The net positive charge of an AMP is influenced by the amino acid residues present in specific sequence of peptide. Amino acid residues, like arginine and lysine are cationic in nature and such residues help in selective attraction of an AMP to negatively charged bacterial plasma membranes. This electrostatic attraction between an AMP and anionic bacterial membranes is high in comparison to weak electrostatic attractions with zwitterionic membrane in eukaryotes [63].

While non-membrane disruptive mechanisms involve targeting physiological processes, proteins, and nucleic acids [71] [72]. Firstly, the AMP interacts with the plasma membrane before penetrating (*e.g.*, activity of buforin II on *E. coli*) and accumulates within the cell, where it targets and acts on the key processes, such as transcription and translation, protein synthesis, enzymatic activity, and microbial death [73] [74]. Few more examples of AMPs that act on intracellular targets are human α-defensin 1, human α-defensin 5, human β-defensin 4, and indolicidin [60] [75] [76].

1.2.4. Proposed trans-membrane pore models to explain MOA

There are various models (**Figure 1.2.4**) that have been proposed to explain membrane disruptive mechanisms of action of AMPs. These models are toroidal pore model, barrel stave pore model, detergent model, and carpet model. Description of each model is given below.

1.2.4.1 Barrel stave model

As per this model, peptides initially interact laterally with each another to form a specific structure resembling membrane protein ion channel [77]. The number of peptides in the pore will determine the size of the channel, followed by the addition of more number of monomers to increase pore size. These pores are irregular in shape and size. An increase in the peptide's positive charge reduces pore stability due to enhanced electrostatic repulsion between the side chains of peptides. This model holds good for cationic AMPs that are short and not able to cover the width of the membrane [78]. Typical for α-helical AMPs, the barrel-stave model constitutes the formation of

hydrophilic pores on the hydrophobic core of the bacterial membrane structure, resulting in membrane disruption and leakage of extracellular content [35].

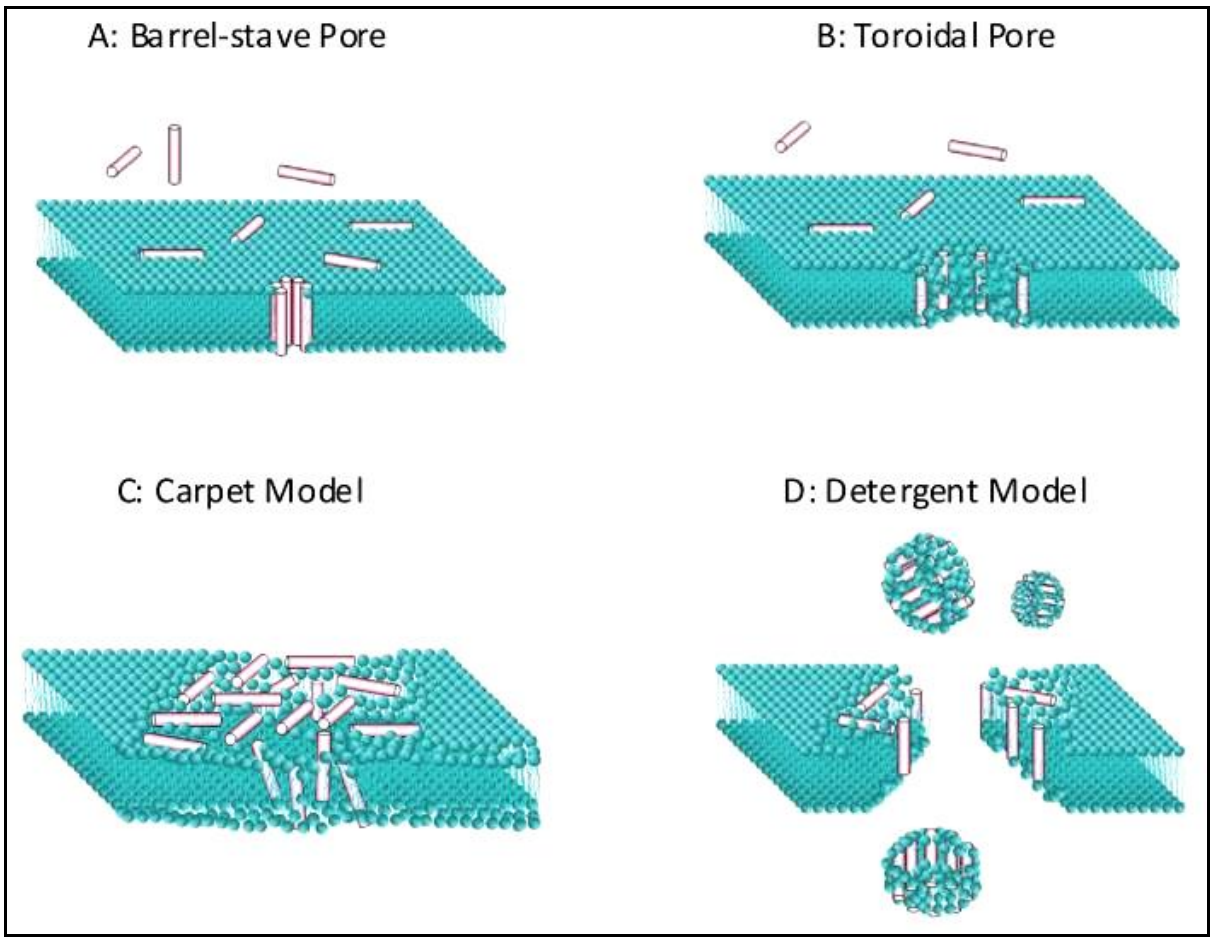


Figure 1.2.4: Proposed transmembrane pore models to explain MOA of AMPs
(Source: Wimley *et al.*, 2010; [77])

1.2.4.2. Toroidal pore model

Hypothesis behind the toroidal pore model is that the insertion of CAMPs into the membrane induces lipids to bend around peptide aggregates. Due to this, a continuous channel between the outer and inner leaflet is formed that is lined by both peptide and lipid head groups [78]. In this model, peptides do not interact with one another, instead they affect the local curvature of the bilayer in such a manner that a toroid of high curvature is formed [77]. In toroidal pore models, peptide cause membrane disruption by perpendicular insertion into the lipid bilayer [68] [77].

1.2.4.3. Carpet model and detergent model

In the carpet type model, peptides permeabilize membranes by carpeting the bilayer. Based on the utilization of peptides at high concentrations; the carpet model peptides

can behave like detergents [77]. In this model, peptide covers the surface of the cytoplasmic membrane like a carpet and further causing a complete detergent-like disruption of the membrane. This model differs from other two models as in this model membrane integrity is completely disrupted but no distinct pores are formed [78]. For the carpet-like mechanism, peptide molecules accumulate parallel to the membrane surface in a carpet-like fashion, followed by penetration into the membrane and disruption of the lipid bilayer [73] [31] [79].

1.2.5. AMPs as a hope against MDR nosocomial bacterial pathogens

Antimicrobial resistance is spreading rapidly among nosocomial pathogens. Emergence of AMR has become a clinical challenge worldwide. Especially, nosocomial pathogens have become recalcitrant to conventional antibiotics. Hospital acquired infections due to such pathogens have become a growing cause of concern in developing as well as developed countries. Treatment of nosocomial infections often lead to prolonged hospital stay, which further leads to economic losses. So, the time has arrived to discover and develop alternative therapies that can solve this issue. A number of AMPs have been studied so far, these studies have indicated that AMPs possess potent microbicidal activity against different bacteria, and there is a low propensity of resistance development against these compounds. All these desirable qualities of AMPs have created hope in winning the war against MDR nosocomial pathogens [80] [81] [82]. A well-known example of AMP used against MDR pathogens is Colistin. Colistin is often used as a last resort of treatment option against MDR strains. It is a combination of two polypeptides (polymyxin A and B) [83] [84]. Colistin is isolated from *Paenibacillus polymyxa* and used extensively in human prophylaxis [85]. AMPs that are broad acting, have high selectivity towards bacteria and possess low toxicity on mammalian cells are desirable [69]. At present, a range of AMPs are under clinical trials; but, there are few factors that hamper their utilization as therapeutic agents [31] [50] [54]. Limitations associated with AMPs have been discussed below.

1.2.6. Limitations associated with AMPs

1.2.6.1. Susceptibility to proteolysis

There are a variety of proteolytic enzymes present in gastrointestinal tract, intestinal mucosa, and blood plasma that can degrade AMPs. This affects the *in-vivo* stability and

pharmacokinetic behavior of peptides [86] [87]. This is the reason that most of AMPs are administered topically or intravenously. Additionally, some bacteria up-regulate the secretion of proteolytic enzymes and metalloproteases for partial or complete degradation of AMPs [88] [89].

1.2.6.2. Poor *in vitro-in vivo* correlation

Another major drawback associated with AMPs is poor *in vitro-in vivo* correlation [90] [91] [92]. The majority of AMPs tend to produce their antimicrobial effects via the electrostatic interactions between cationic peptides and anionic plasma membranes [63]. These electrostatic interactions can be affected by physiological conditions of the host [93]. Thus, AMPs should be screened under different physiological conditions of host to affirm their activity.

1.2.6.3. Cost of production

Cost of production of AMPs is another major limitation associated with AMPs; production of AMPs is expensive in comparison to other conventional antimicrobial agents. For that reason, various efforts have been aimed at designing shorter peptides that show enhanced stability under different physiological conditions and fewer side effects. Designing shorter peptide sequences automatically cuts down the cost of production [94] [95].

1.2.7. Strategies to reduce or overcome limitations

Different strategies that have been utilized so far aim to enhance the *in vivo* efficacy of AMPs, increase the selectivity towards microbial cells, reduce the selectivity towards mammalian cells, enhance peptide stability, and increase the stability to proteolysis. These strategies include, chemical modifications like utilization of D-amino acids, acetylation or cyclization to improve the stability of AMPs, and minimize their susceptibility to proteolysis [96] [97]. Other strategies include, cutting down the cost of production of AMPs by designing and synthesizing shorter sequences of peptides without compromising their desirable properties [98] [94]. Whereas to improve the selectivity of an AMP towards bacterial membranes, positively charged amino acid residues, like arginine and lysine are generally used [99] [100]. It has been found in various studies that cationic charge of an AMP has direct correlation with its

antimicrobial activity; increase in cationic charge of AMPs generally improves the antimicrobial activity against bacteria [101] [102] [103]. Though, this is not always the case, some studies suggest that increase in net cationic charge may increase hemolytic toxicity of peptides [104] [105].

Another important factor that governs the selective behavior of AMPs is their hydrophobicity. Optimum hydrophobicity is crucial for the selectivity of AMP against bacteria. Amino acid residues that impart hydrophobicity to peptide sequences are valine, methionine, leucine, alanine, isoleucine, tryptophan, phenylalanine, and tyrosine. An increase in hydrophobicity in AMPs beyond a certain level may increase their hemolytic toxicity as the extremely hydrophobic AMPs have a strong ability to penetrate the hydrophobic components present in erythrocytes [67] [106].

Moreover, different peptide delivery systems can be designed for improving the efficacy and stability issues associated with AMPs [107]. A drug delivery system that has received much attention during the past few decades is the utilization of nanocarrier based drug delivery system to prevent self-aggregation, improve chemical stability, and release profiles of AMPs to target sites. Various biodegradable nanocarriers, like lipids, gels, fiber, and cellulose have been used for designing such drug delivery carriers [108] [36] [109].

HDPs fulfill most of requirements required by the pharmaceutical industry, like broad-spectrum of activity, ability to neutralize endotoxins, low propensity of resistance development, and synergistic action with conventional antibiotics. Their use is still limited as many natural peptides are not suitable for drug development due to low stability, low bioavailability, and high production cost [110]. Unfortunately, because of their low proteolytic and chemical stability, the majority of AMP formulations are topical formulations. Numerous strategies are being vigorously considered to overcome the limitations that restrain the success of AMPs. The development of peptidomimetics is one such approach [111]. Peptidomimetics are mimics of peptides with improved properties that retain the basic features of natural AMPs like cationic charge and the amphipathic structure [112]. They mimic the mechanism of action of HDPs, and

effectively inhibit the growth of bacteria. Since, they are built on unnatural backbone due to which they are resistant to proteolytic degradation [113].

1.2.8. Peptoids

Peptidomimetics that were structurally different from peptides but could mimic the biological function of peptides were introduced in 1982 and were named as peptoids [114]. Definition of peptoids was made more specific by Barlett and his co-workers, as they defined peptoids as oligomeric *N*-substituted glycines [115]. Peptidomimetics with a modular structure of *N*-alkylated glycine oligomers are known as α-peptoids and ones with *N*-alkylated β-alanine oligomers are known as β-peptoids. These peptoids are of particular interest as these possess potent antimicrobial activity, offer possibility for versatile designs, and can be produced conveniently by standard solid-phase methods [116]. Structures of peptoids is different from α-peptides, as the side chains in peptoids are attached to the backbone amide nitrogen while in case of α-peptides side chains are appended to α-carbon (**Figure 1.2.8**) [117].

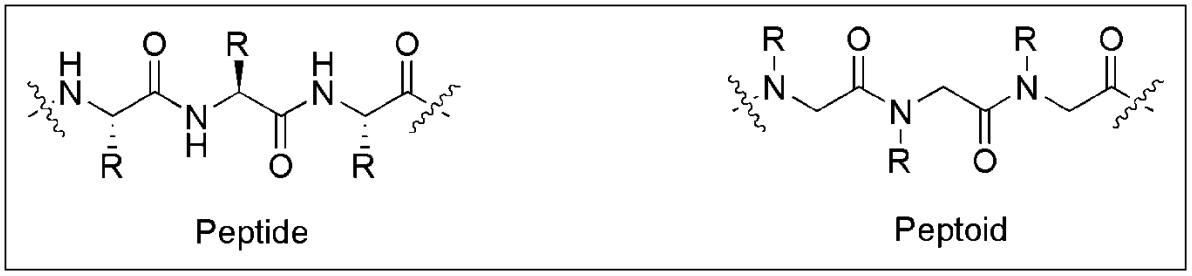


Figure 1.2.8: Structural difference between α-peptoids and α-peptides

Peptoid oligomers were primarily developed as the measure to speed up the drug-discovery process in the biotechnology and pharmaceutical sectors. Miscellaneous libraries of short peptoid oligomers were designed in mid 1990s, and solid-phase submonomer method (SPSMM) for peptoid synthesis became so popular that it became feasible to design longer polypeptoid chains and explore their applications in multiple areas beyond drug discovery. This was further followed with designing defined secondary or tertiary structures using non-natural sequence specific heteropolymers. This effort yielded the secondary structure mimetics that resembled their natural counterparts very closely. The ease of access of peptoid synthesis to many new investigators has prompted a fast development in the field of peptoid science [118].

It has been observed that peptoids are more stable to proteolysis than peptides [119] and studies have demonstrated increased cellular permeability of peptoids in comparison to α-peptides [120], [121]. Peptoids can be synthesized by solid-phase synthesis (SPS) and classical polymerization technique. SPS has advantage over classical polymerization technique as it allows better control over peptoid length and peptoid sequence in former technique [122]. This method is an efficient method for synthesis of *N*-substituted glycines and can be used to develop mimetics of *α*-peptides [123].

1.2.8.1. MOA of peptoids

MOA of peptoids have been deciphered by various research groups, these groups have indicated that the main MOA of the majority of peptoids against bacteria is membrane permeabilization [124] [125] [126] [127]. Though, a report on the mode of antibacterial action of peptoids suggests that such compounds act on intracellular targets of bacteria [128]. Diverse peptoids and their MOA against different bacteria have been discussed below.

Mojsoska *et al*., 2017 investigated the mechanism of action of two short peptoids (peptoid 1 and peptoid 2; **Figure 1.2.8.1a**). These peptoids were cationic and contained lysine and tryptophan as the main side chain functionalities. MIC for both peptoids against *E. coli* was found to be between 16-32 μg/ml. To confirm membrane permeabilization effect on bacterial membrane mimics, the dye leakage experiments were carried out. This dye leakage study confirmed the slow and low membrane permeabilization of bacterial membrane mimics treated with peptoid 1. Further *E. coli* cells were treated with 4×MIC of peptoids and analyzed by SEM. The SEM analysis results revealed that the peptoids acted by formation of pores, filamentation, and leakage of cytoplasmic content. Observed degree of lysis of bacteria by peptoid 2 was higher than the lysis produced by peptoid 1 [125].

Likewise, Smith *et al*., 2015 investigated the mechanism of action of peptoids as mimetic of AMPs against *Staphylococcus aureus* (MRSA). MRSA was treated with peptoids and mechanism of action of peptoids was elucidated by SEM. From results of SEM it was concluded that peptoids caused disruption of cell membrane that resulted in a loss of membrane integrity [124]. In an another study, Shyam *et al*., 2018 synthesized peptoid oligomer containing 1,2,3-triazolium side chains by SPS. This triazolium side

chain was incorporated as a cationic moiety as well as to induce helix formation. Antibacterial evaluation of these oligomers was done against *E. faecalis, S. aureus,* and *E. coli*. A number of these compounds were found to be selective towards bacteria in comparison to mammalian cells. SEM analysis of *S. aureus* cells treated with peptoids revealed their membrane damaging potential, and longer peptoids were even found to form pores in bacterial membrane [126].

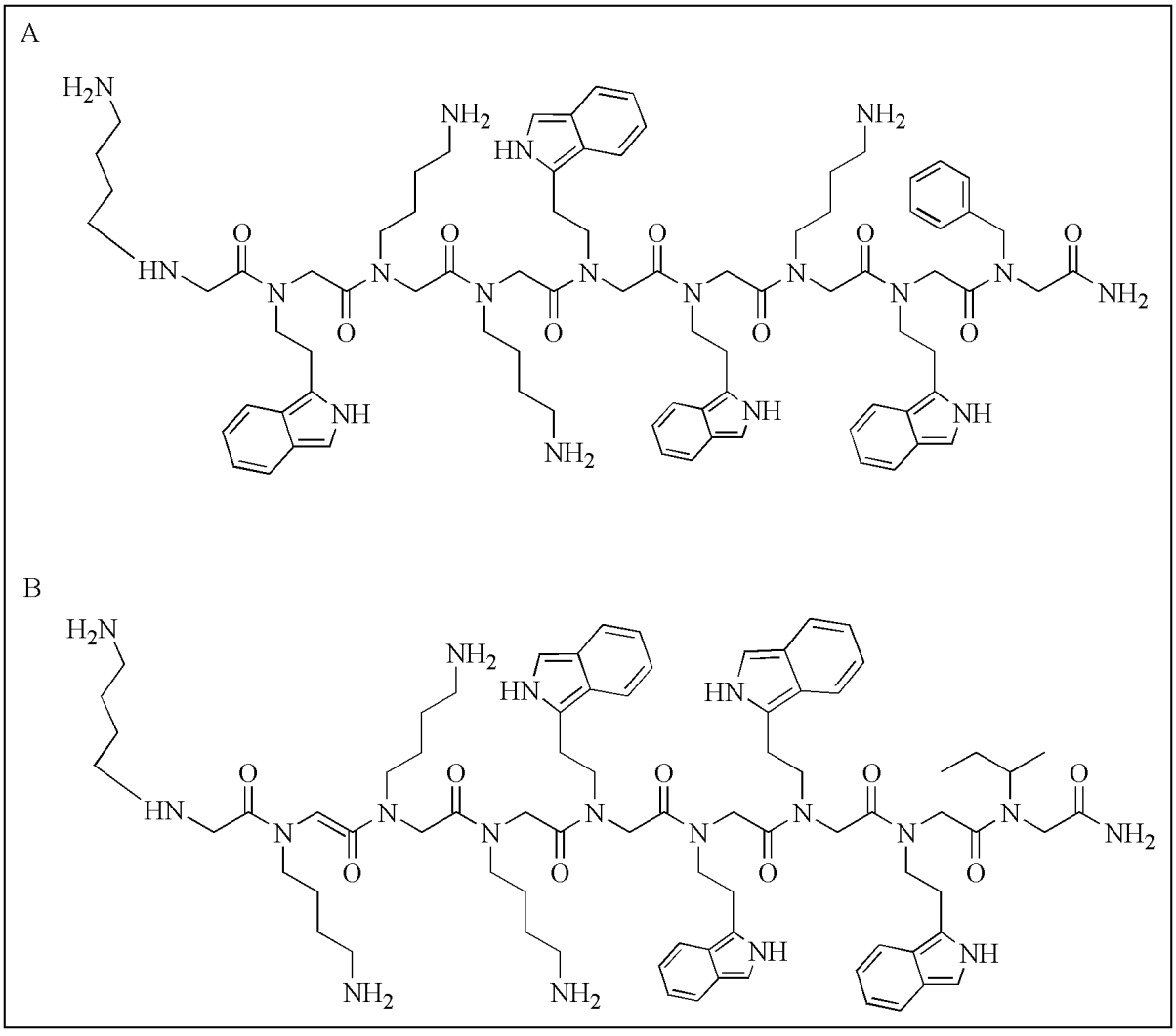


Figure 1.2.8.1a: Structures of peptoids (A: peptoid 1 and B: peptoid 2)

Aspiring to develop antibacterial agents against *P. aeruginosa,* Ghosh *et al.*, 2014 reported a range of antibacterial peptoids (Series 1, Series 2, and Series 3; **Figure 1.2.8.1b**), which exhibited high *in vitro* antibacterial activity against a variety of susceptible and drug resistant bacteria. These compounds possessed superior antibacterial activity against *P. aeruginosa,* a major nosocomial pathogen. These peptoids acted against *P. aeruginosa* by mechanism of bacterial membrane

permeabilization and depolarization. In addition these compounds were found to have rapid bactericidal action and were nontoxic toward mammalian cells [127]. Further in a recent study, Chongisiriwatana and coworkers, 2017 developed antimicrobial *N*-substituted glycine oligomers (ampetoids) based on AMPs and evaluated their mode of action. They showed that ampetoids are "rapid acting", which rapidly aggregate bacterial ribosomes *in vitro* and *in vivo* [128].

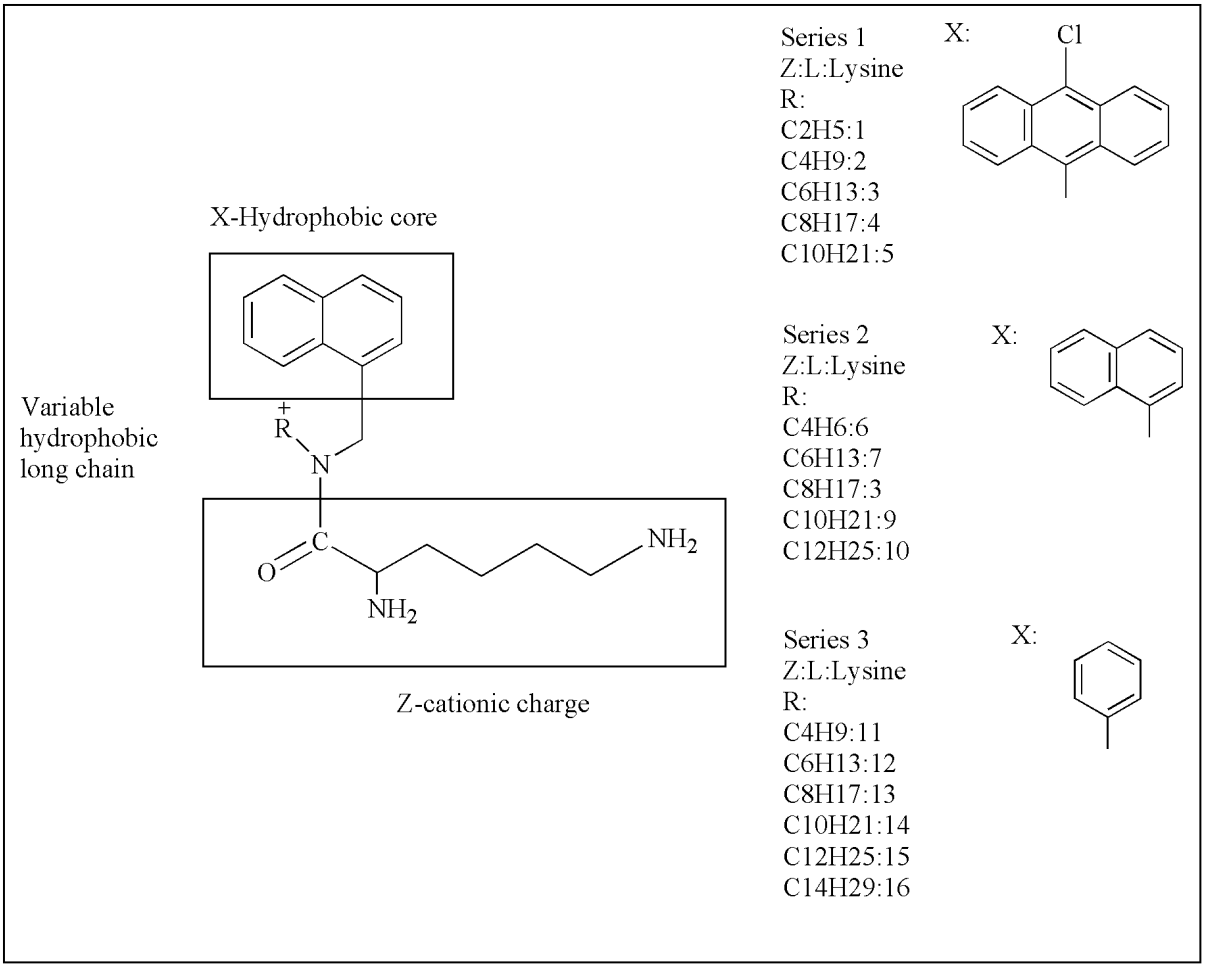


Figure 1.2.8.1b: Structures of peptoids (Series 1, Series 2, and Series 3)

1.2.8.2. Factors affecting antibacterial activity and selectivity of peptoids

Hydrophobicity and cationicity are two important factors that play an important role in antibacterial activity of peptoids. The influence of hydrophobicity and cationicity on antibacterial activity of peptoids has been investigated by Molchanova *et al.*, 2017. They tested a total of 22 peptidomimetics. Compounds rich in hydrophobic residues were found to be more active against Gram-positive bacteria. Arrangement of hydrophobic

and cationic moiety in an alternate way (**Figure 1.2.8.2a**) was more found to be superior to other distribution patterns [129].

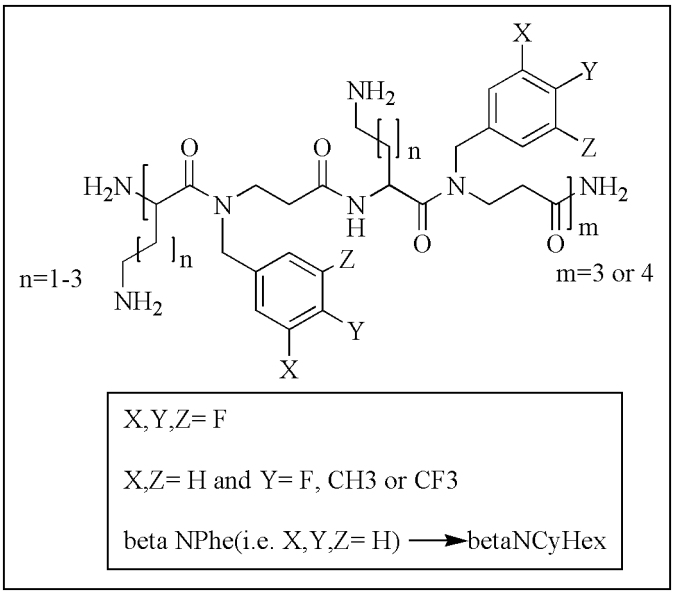


Figure 1.2.8.2a: Arrangement of hydrophobic and cationic moiety in an alternate way in different peptidomimetics.

Another attempt was made by Kapoor *et al*., 2011 to correlate the effect of alkylation of peptoids on their antibacterial activity. This group tested some peptoid analogues for their antibacterial activity against *Mycobacterium tuberculosis*. The unalkylated analogue of peptoid 1(4mer), was found to be inactive against *M. tuberculosis* whereas, alkylated cationic peptoid (1-C13(4mer)) was found to be the least toxic to mammalian cells [130].

In an additional study, Chongsiriwatana and fellow workers, 2011 created alkylated peptoids to mimic AMPs. The alkyl chains, 5-13 carbons long were used to produce these analogs. Various alkylated peptoids were found to be more selective and no loss in their antimicrobial activity was observed. Based on these observations, this group synthesized a 5-monomer peptoid. This peptoid was found to be selective, potent, and displayed broad-spectrum antimicrobial activity against microbes [131].

Design of peptoid is crucial for its antibacterial activity; Goodson *et al*., 1999 reported antibacterial activities of various peptoids from a library of peptoids. These peptoids were discovered by screening combinatorial chemistry for the inhibition of bacterial growth. Results of *in vitro* antibacterial activity of a CHIR29498 (**Figure**

1.2.8.2b) and some of its analogues against *S. aureus* indicated that these compounds are rapid-acting. Furthermore, these compounds were found to target the cell membrane of bacteria. In fact, the results of *in vivo* antibacterial evaluation of peptoid CHIR29498 in a simple mice infection model supported its antibacterial effects [132].

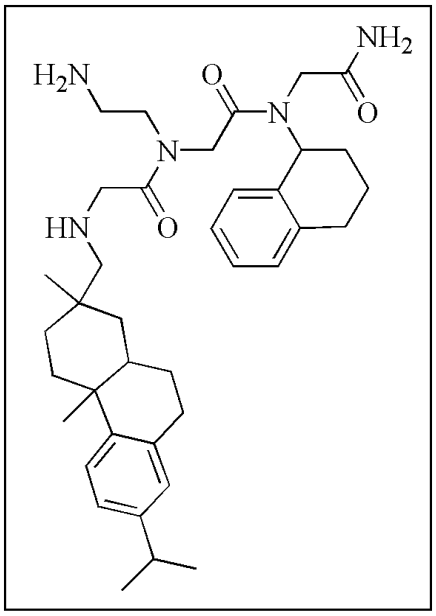


Figure 1.2.8.2b: Structure of peptoid CHIR29498

In another study, Patch and Barron, 2003 designed various peptoids (**Figure 1.2.8.2c**) based on magainin-2 amide antibacterial peptide. Designed peptoids were cationic, helical, and facially amphipathic. Circular dichroism spectroscopy was used to determine conformation of these peptoids in two different systems, such as aqueous buffer and lipid vesicles. These lipid vesicles were used as bacterial membrane-mimetics. This group indicated that lipophilicity of peptoids affects their hemolytic toxicity while a minimum length of peptoid residues (12 residues) and a helix length (24-34 Å) is optimum for antibacterial activity [133].

Similarly, Mojsoska, 2015 designed peptoids based on sequence of GN2 peptide (RWKRWWRWI-NH_2) to target bacteria. These peptoids were 8 to 9 residues long, cationic, and ampipathic in nature. The charge distribution and hydrophobicities were varied along the abiotic backbone. The majority of peptoids exerted low toxicity towards mammalian cells. Two peptoid candidates, 3 and 4 were optimized as lead compounds with high selectivity ratios, which acted by bactericidal mode of action against Gram-negative *E. coli* in a concentration-dependent manner [134].

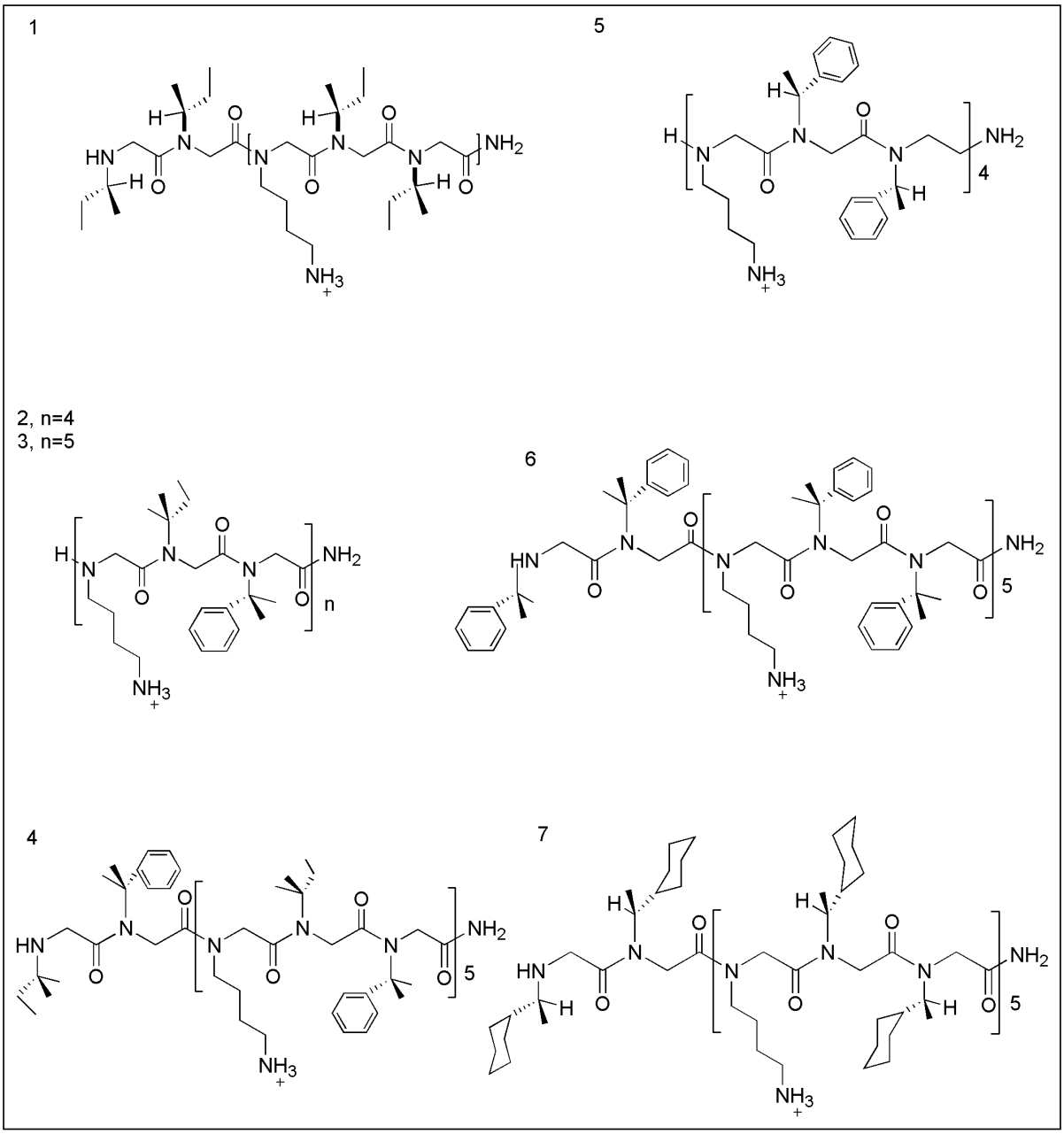


Figure 1.2.8.2c: Structures of various peptoids based on magainin-2 amide antibacterial peptide

Peptoids that are selective towards bacteria are desired; therefore Lee *et al*., (2018) modified previously developed antibacterial peptoid 1, to improve its selectivity. The new analogs (**Figure 1.2.8.2d**) which contained hydrophobic residues were found to possess non-specific cytotoxicity, on the other hand the analogues containing additional cationic residue showed comparable antibacterial activity and improved the selectivity of previous compound. Particularly, peptoid 7 was least toxic compared to peptoid 1, and retained the antibacterial activity of peptoid 1 [135].

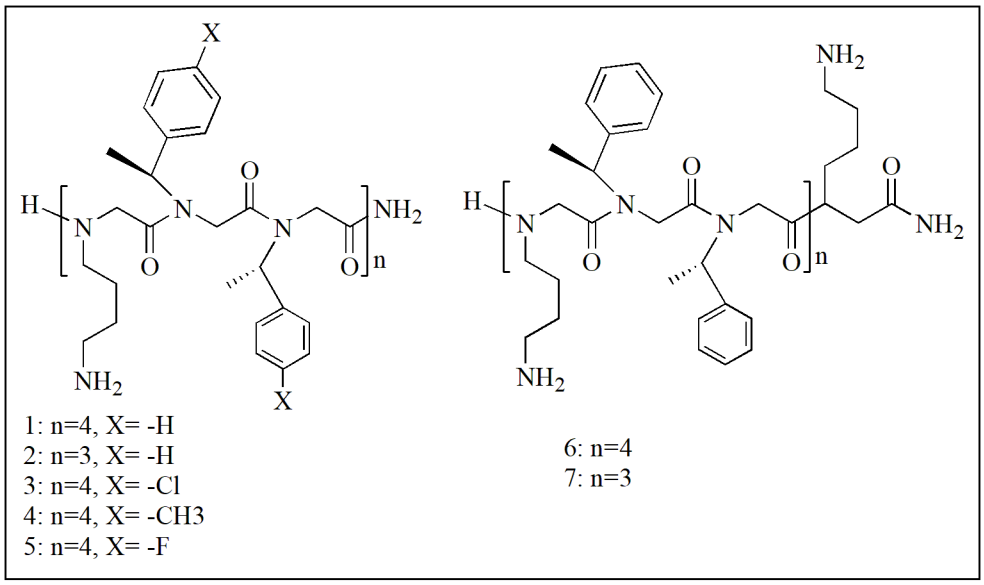
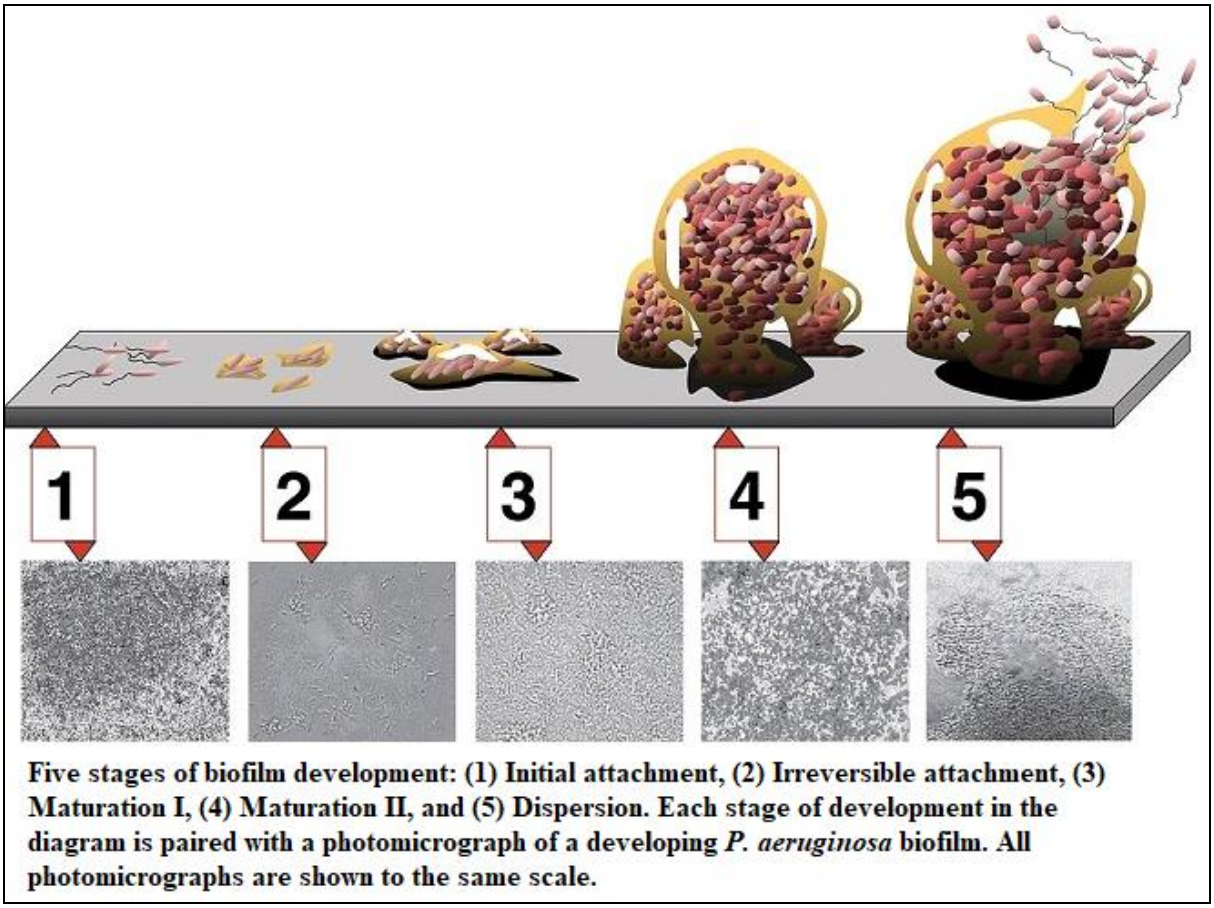


Figure 1.2.8.2d: Structures of various peptoids (**1-7**)

1.2.9. Antibacterial activity of peptoids against biofilm producing bacteria

Formation of biofilms is an important virulence factor of microbes that increase the resistance to antimicrobials [136]. Biofilm is an architecture made up of bacterial colonies within an extracellular polymer matrix produced by bacteria themselves [137]. Biofilms are ubiquitous in nature and are formed on biotic as well as abiotic surfaces [138]. Important steps involved in biofilm formation (**Figure 1.2.9**) are; initial attachment to a surface, formation of micro-colony, formation of three dimensional structure, biofilm formation, maturation, and finally dispersal of microbial cells [138]. It is very common for bacteria to live in biofilms [139] as biofilm protect such microbes from harsh environmental and stress conditions [140].

Common biofilm forming bacteria are *E. coli spp, S. aureus, P. aeruginosa, K. pneumonia, E. cloacae,* and *S. epidermidis* [141] [142] [143]. Major components of biofilm matrix are proteins, extracellular deoxy ribonucleic acid, polysaccharides, and lipids. Water is the major part of biofilm that allows the flow of nutrients inside biofilm matrix. Microbes that form biofilm are more likely to tolerate and neutralize antimicrobial agents. These components of biofilms provide integrity to biofilm architecture and thus, makes it resistant against stress [144] [145].



Five stages of biofilm development: (1) Initial attachment, (2) Irreversible attachment, (3) Maturation I, (4) Maturation II, and (5) Dispersion. Each stage of development in the diagram is paired with a photomicrograph of a developing *P. aeruginosa* biofilm. All photomicrographs are shown to the same scale.

Figure 1.2.9: Different stages of the biofilm development (Source: D. Monroe, 2007)

Biofilm associated infections (BAIs) have become a major threat to humanity as biofilms can easily adapt to changing environmental conditions, and hence, show resistance to conventional antibiotics. This is the reason that various BAIs are difficult to treat with a single antimicrobial drug. Currently, there is no specific antimicrobial agent that can tackle BAIs, and the lack of suitable therapeutic agent highlights the urgent need for new strategies to fight these biofilm producing pathogens. So, a combination of antibiotics is usually given by physicians to address this problem [146, 147].

The state of a microbial cell depends on the nutrients available, as the high concentration of nutrients at periphery of biofilms result into the metabolically active cell and low concentration of nutrients within interior result into the inactive cells. This results in difference in the growth of microbial cells involved in the biofilm formation [148]. Slowly growing cells become problematic for antibiotics, like penicillin and ampicillin, as these antibiotics attack rapidly growing microbial cells. While other

antibiotics, like aminoglycosides, β-lactams, fluoroquinolones, and cephalosporin attack microbial cells in their stationary phase [138].

We have reached an era where pathogens are becoming increasingly resistant to conventional antibiotics and at same time the antibiotic drug development pipeline is drying out [149]. Antibiotic resistance in clinical settings has become a worldwide problem and existence of pathogenic bacteria that form biofilms has further worsened the situation [150]. It is one of the reasons that lead to negative outcome of treatment and recurrence of infections in humans [151].

AMPs have displayed their antimicrobial potential against various biofilms [152]. A number of AMPs have even shown their ability in eradication of biofilms [153]. As AMPs are well-known to permeabilize the bacterial cell membranes, such peptides have been proposed to have the ability to overcome predictable mechanisms of antibiotic resistance [154]. Various AMPs have emerged as the antibiofilm compounds. The selected AMPs, their amino acid sequences, source, length, % hydrophobic content, and net charge are shown in **Table 1.2.9.1**. This data has been taken from the APD (http://aps.unmc.edu/AP).

Table 1.2.9.1: Selected AMPs, their amino acid sequences, source, length, % hydrophobicity and net charge

Peptide name with APD ID	Amino acid sequence	Source	Length	Hydrophobic content (%)	Net charge	Reference
Temporin B (AP00095)	LLPIVGNLLKSLL	Frog, *Rana temporaria*	13	61	2	[155]
Indolicidin (AP00150)	ILPWKWPWWPWRR	Bovine neutrophils, *Bos Taurus*	13	53	4	[156]
SMAP-29 (AP00155)	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG	Sheep leukocytes; *Ovis aries*	29	37	9	[157]
Citropin 1.1 (AP00351)	GLFDVIKKVASVIGGL	Frog, *Litoria citropa*	16	56	2	[158]
Nisin A (AP00205)	ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK	Bacteria, *Streptococcus lactis*, reclassified as *Lactococcus lactis*	34	44	3	[159]
BMAP-27 (AP00366)	GRFKRFRKKFKKLFKKLSPVIPLLHLG	Cow, *Bos Taurus*	27	40	10	[160]
Temporin 1Ola (AP00871)	FLPFLKSILGKIL	Frog, *Rana okaloosae*	13	61	3	[161]
NA-CATH (AP00897)	KRFKKFFKKLKNSVKKRAKKFFKKPKVIGVTFPF	Snake, *Naja atra*	34	38	15	[162]
Temporin-PT (AP01434)	FFGSVLKLIPKIL	Frog *Hylarana picturata*	13	61	3	[163]
Phylloseptin-1 (AP01581)	FLSLIPHIVSGVASIAKHF	Frog, *Phyllomedusa sauvagei*	19	57	2	[164]
Colistin (AP02204)	KTKKKLLKKT	Bacteria *Paenibacillus polymyxa* var. colistinus; Also known as *Bacillus polymyxa*	10	20	6	[165]
Dermaseptin-PH (AP02924)	ALWKEVLKNAGKAALNEINNLV	Frog, *Pithecopus (Phyllomedusa) hypochondrialis*	22	54	2	[166]
Con10 (AP02761)	FWSFLVKAASKILPSLIGGGDDNKSSS	Scorpion, *Opisthacanthus cayaporum*	27	40	1	[167]
Paracentrin 1 (AP02624)	EVASFDKSKLK	Sea urchin, *Paracentrotus lividus*	11	36	1	[168]
Phylloseptin-PHa (AP03057)	FLSLIPAAISAVSALANHF	Frog, *Pithecopus hypochondrialis*	19	68	2	[169]

(B represents α-aminoisobutyric acid and X represents 2,4-diaminobutanoic acid)

AMPs might be proposed as the solution to biofilm producing bacteria, but utilization of natural peptides as antibiofilm agents is limited due to their poor proteolytic stability and poor pharmacokinetics [147]. For this reason, the recent research on AMPs is focused on overcoming limitations associated with natural AMPs [170]. Hence, peptidomimetics are being explored as alternative antibiofilm agents to mimic the biological actions of peptides and simultaneously minimizing limitations associated with peptides [163]. Some of the recent works related to antibiofilm potential of peptoids have been discussed below.

Saporito *et al*., 2019 designed a series of peptoids and peptides based on a previously reported antimicrobial peptide GN-2. They evaluated the antibiofilm and antimicrobial activity of these compounds against *E. coli ATCC* 25922. The data indicated that with the decrease in hydrophobicity, the antibacterial activities of peptoids against biofilm forming cells were enhanced in comparison to their peptide counterparts. These observations suggested that the characteristic flexibility of peptoids might be the reason behind the bacterial membrane penetration [164].

Kapoor *et al*., 2011 screened a number of alkylated and non-alkylated acyclic amphipathic peptoid analogs based on AMP structures against biofilm forming *P. aeruginosa*. The alkylated peptoids were found to be more active than unalkylated ones against planktonic cells. Anti-biofilm effects of peptoids were studied at their respective MICs. Peptoid 1 and peptoid 1-C13(4mer) were the most effective antibiofilm agents at their respective MICs. This study suggested that these peptoids should be further explored in future [157].

Liu *et al*., 2013 prepared various *β*-peptoid-peptide hybrid acyclic oligomers (**Figure 1.2.9.1**) of different chain lengths and different amino acid/peptoid compositions. These oligomers were evaluated for their antimicrobial activity against planktonic and biofilm forming *S. epidermidis* cultures. The antimicrobial effects were compared with the standard antibiotic drug vancomycin. All compounds were found to inhibit the formation of biofilms at their MIC ranging between 80-160 μg/ml. However 6 h old biofilms were eradicated by these peptidomimetics at concentrations above the MIC and for complete eradication of mature biofilms (24 h old), even higher concentrations were needed.

Chirality and guanidinylation of hybrids were found be favorable for antibiofilm properties. Cytotoxicity assays showed that cell toxicity within each subclass of peptides depends on oligomer length [158].

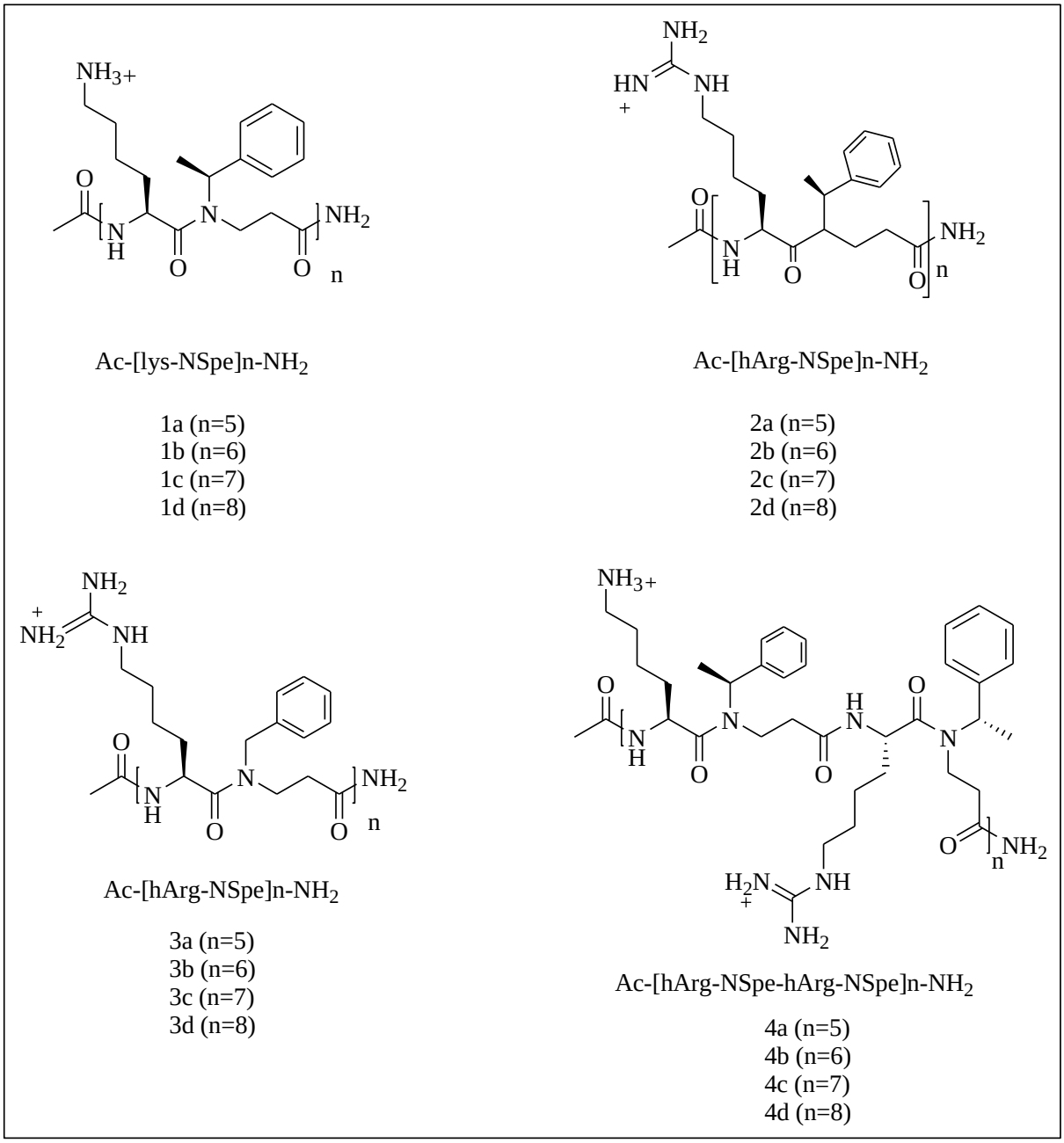


Figure 1.2.9.1: Structures of various *β*-peptoid-peptide hybrid acyclic oligomers

Although, a lot of work has been done on antimicrobial *N*-substituted glycines, but at present the information on toxicity of these compounds is somewhat limited due to which rational design of effective antimicrobial peptoids might be challenging for researchers. So an initiative was taken by Bolt *et al.*, 2017 to provide a brief insight into valuable data that may aid in the future rational design of antimicrobial peptoids. In

their work, they screened a series of linear peptoids for their toxicity against mammalian cell lines HepG2 and HaCaT and presented the toxicity profiling data of these peptoids. They not only correlated the cytotoxicity of the peptoids with their antibacterial properties against Gram-positive and Gram-negative bacteria, but on the other hand they have described the importance of the role of hydrophobicity in the peptoid sequences [160].

From the review of literature, it is clear that antibiotic drug development pipeline is drying and needs urgent attention from researchers to discover novel anti-infective agents which can combat the infections caused by MDR strains. AMPs have emerged as one such class of anti-infective agents that have the potential to fight these problematic issues associated with MDR bacteria, as most of the cationic antimicrobial peptides exert their antimicrobial action through membrane disruption. This property recommends their therapeutic application in treating diseases caused by MDR bacterial strains, since there is a low propensity of resistance development against membrane active AMPs. This makes them suitable candidates for the development of new generation antibacterials. However AMPs suffer from few limitations and it is evident from literature that peptidomimetics have the ability to tackle limitations associated with AMPs quite efficiently. Therefore in this research work we have synthesized peptides and their poly-*N*-substituted glycine congeners and evaluated them for their antibacterial activities against various bacterial strains.

Present dissertation is classified into following sections:

- **Chapter 1:** Introduction and review of literature.
- **Chapter 2:** Objectives and plan of work.
- **Chapter 3:** Synthesis of a series of peptide-based compounds and their preliminary biological evaluation.
- **Chapter 4:** *In vitro* biological evaluation of lipopeptide LP-23 and its poly-*N*-substituted glycine congener DP-23 against *M. smegmatis.*
- **Chapter 5:** *In vitro* biological evaluation of peptide SA4 and its poly-*N*-substituted glycine congener SPO against *A. baumannii*.
- **Conclusion**
- **Bibliography**
- **Appendices**

CHAPTER 2

OBJECTIVES AND PLAN OF WORK

2.1 OBJECTIVES

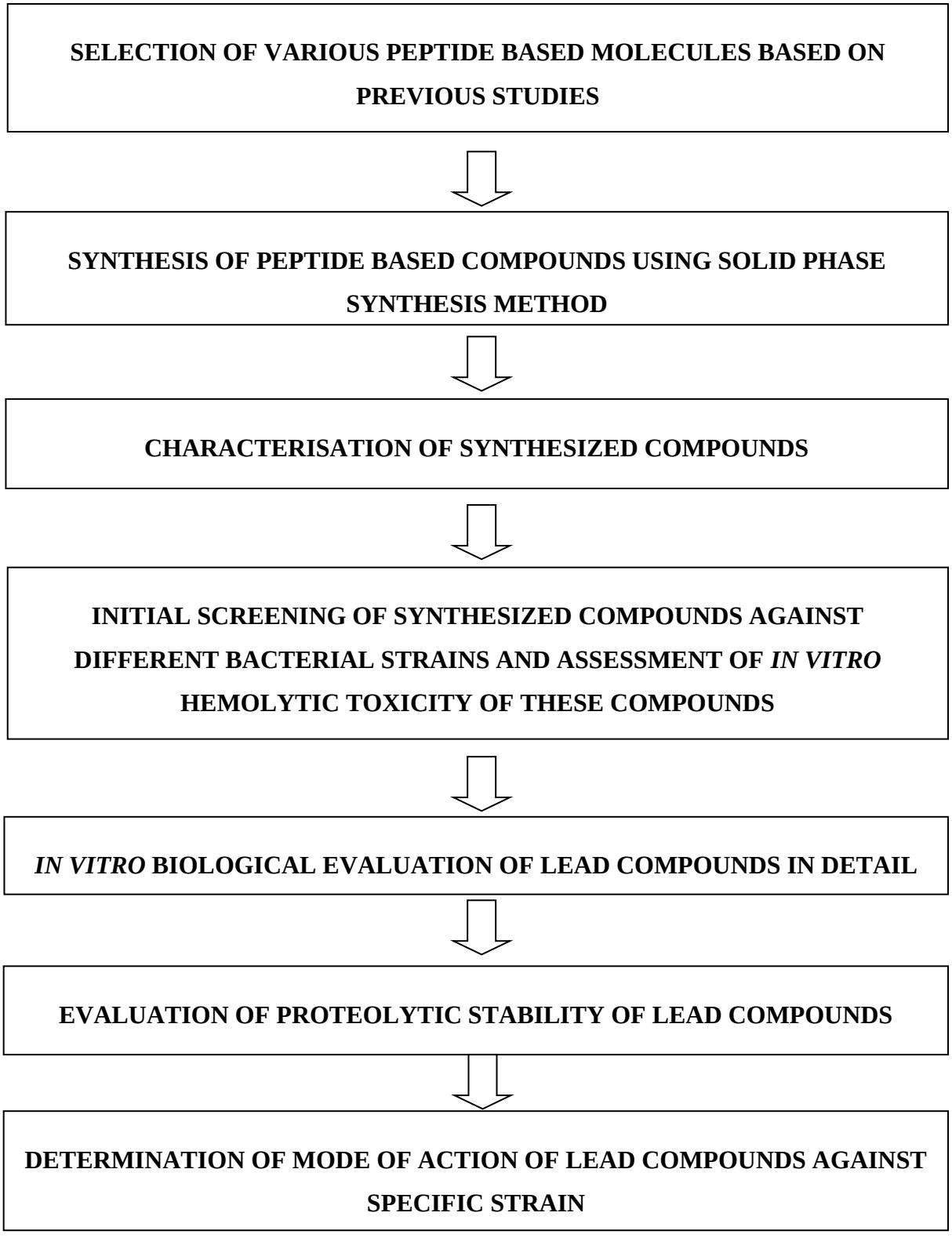
The emergence of AMPs provides us a prospective chance to develop these potential compounds as alternatives of conventional antibiotics. With this in view, the present work on **"Synthesis, characterization, and antibacterial evaluation of peptides and their poly-*N*-substituted glycine congeners"** was undertaken with following objectives.

Objective 1: Synthesis and characterization of peptides and their poly-*N*-substituted glycine congeners.

Objective 2: Evaluation of antibacterial activity and hemolytic toxicity of synthesized compounds.

Objective 3: *In vitro* biological evaluation of screened antibacterial lead compounds.

2.2 PLAN OF WORK



CHAPTER 3

SYNTHESIS OF SERIES OF PEPTIDE-BASED COMPOUNDS BY SOLID-PHASE SYNTHESIS AND THEIR PRELIMINARY BIOLOGICAL EVALUATION

3.1 INTRODUCTION

Host defense peptides (HDPs) are the components of innate immunity that have been proposed to provide the first line of mucosal host defense against numerous pathogens [171]. Up till now, more than 19000 AMPs both natural isolates and synthetic derivatives have been reported; though, most AMPs have direct antimicrobial activity but their exact mechanisms of action and their structure-activity relationships, are still not known [172]. Research data of the last decade indicate that antimicrobial peptides have broad-spectrum of activity, rapid killing effect, multiple mechanisms of actions and potentially low-resistance rate when compared to conventional antibiotics [173]. These peptides have attracted the attention of various medicinal chemists owing to their advantages. However, along with these advantages, AMPs are also associated with certain limitations like *in vivo* instability and lack of selectivity; leading to side effects and cytotoxicity [174]. The existence of natural HDPs in a multicellular organism provides motivation to design synthetic compounds based on natural AMP sequences with improved properties [175]. Most of the peptides used for research purpose or therapeutic applications are usually synthesized by solid-phase peptide synthesis (SPPS) methods [176]. The solid-phase synthesis (SPS) technique for the synthesis of peptides was introduced by Bruce Merrifield in the year 1963 [177]. SPS strategy (**Figure 3.1**) is based on the repetitive coupling of the C-terminus of protected amino acids to an insoluble solid support. Various amino acids can be attached to a solid support as per desired peptide sequence. These protected amino acids are attached to a solid support via a cleavable linker. The excess reagents used in synthesis can be washed away after each step and finally, the peptide sequence attached to the resin can be cleaved off from resin to get a pure product [178], [179], [180], [181], [182]. Successful SPPS is dependent on the convenient selection of solid support, linkers, amino acid derivatives,

protecting groups and coupling reagents [176]. The role of each component in SPS of peptides has been described below.

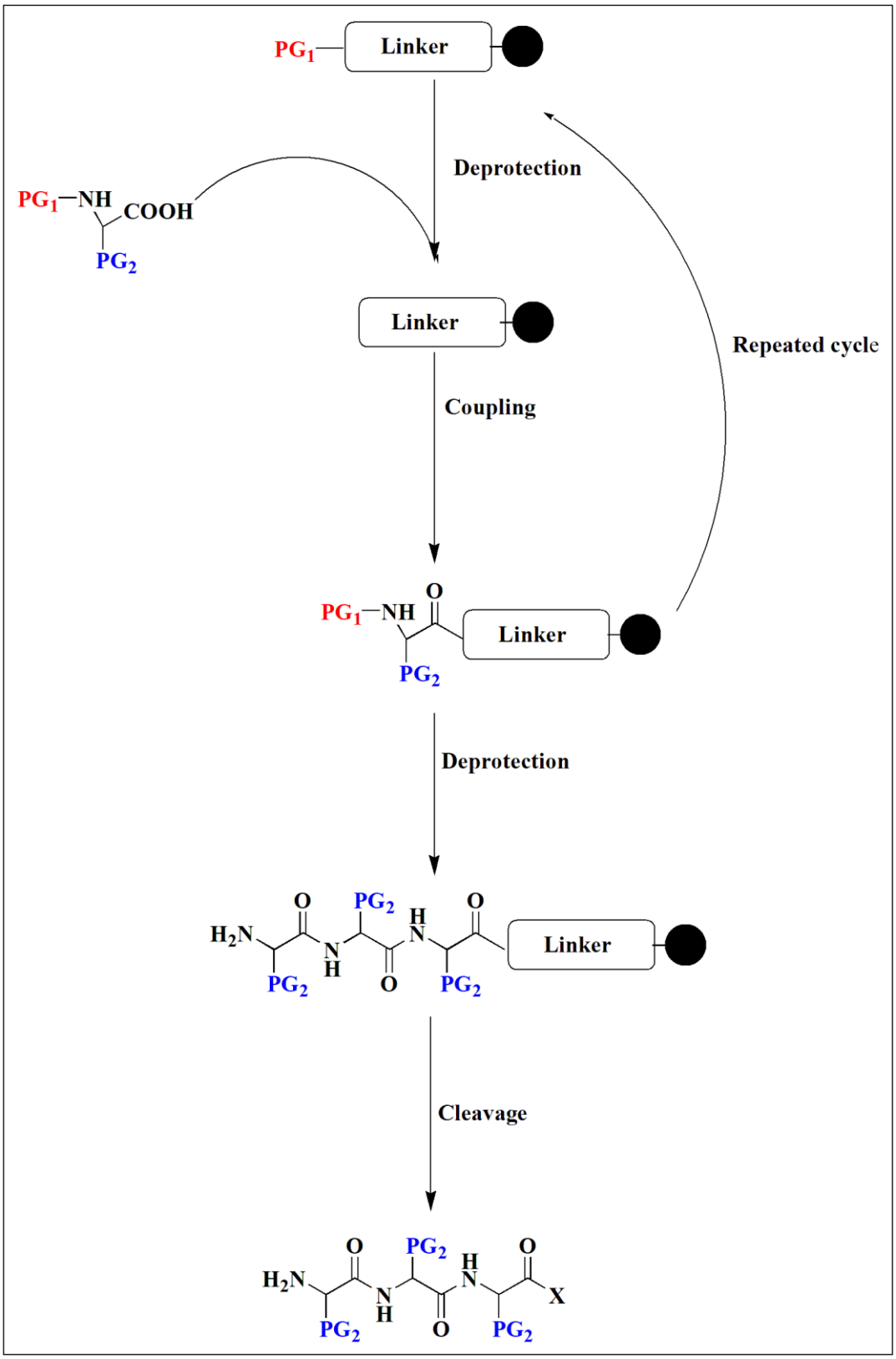


Figure 3.1: Schematic representation of SPS (Source: Munzker *et al.*, 2017 [183]); PG1: protecting group 1 and PG2: protecting group 2.

3.1.1 Solid supports

The solid support used for SPPS needs to have chemical, mechanical, and physical stability. Resins that are used as solid supports have been divided into three main categories [184] such as polystyrene resins; polystyrene resin functionalized with polyethylene glycol resins, and cross-linked polyethylene glycol resins. Polystyrene type of solid supports contain matrix cross-linked with 1–2 % divinylbenzene, this type of resin beads provide the best compromise between mechanical stability and swelling properties in low to middle polarity solvents, such as DMF and DCM. Polystyrene resins are most suitable for the synthesis of small peptides, as the high hydrophobic environment of the resin may amplify the aggregation of longer peptides [185].

3.1.2 Linker

Initial step of synthesis of peptides using SPS strategy consists of the attachment of the first amino acid (usually by its carboxyl function) to the resin via a linker [186]. A linker is a functional moiety that allows a cleavable link between the compound to be synthesized and the solid support. The conditions required for cleavage of a peptide from solid support depend on the particular linker used. There are a variety of linkers that are compatible with Fmoc SPPS. Commonly used linkers are 2-chlorotrityl [187], Wang linker [188] and Rink amide linker [189]. 2-Chlorotrityl is used for the synthesis of peptide acids [187], Wang linker is used for the synthesis of protected peptides [188] and Rink amide linker is used for the generation of peptide amides [189] (**Figure 3.1.2**).

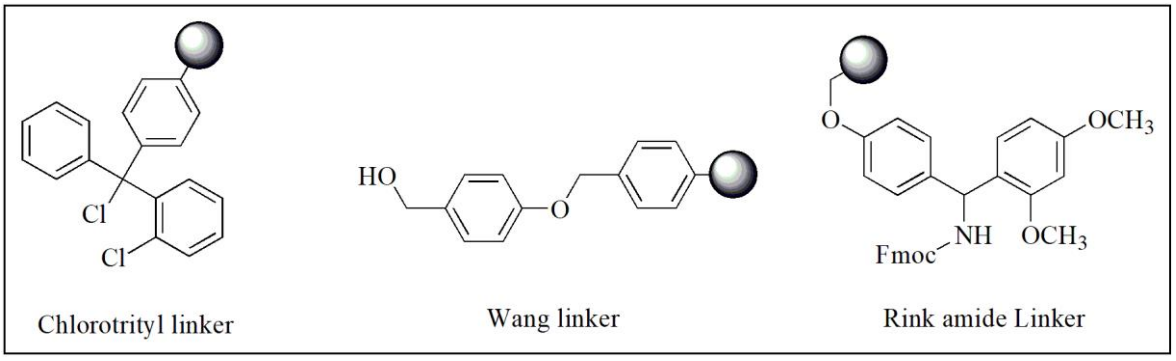


Figure 3.1.2: Commonly used linkers in Fmoc SPPS

3.1.3 Protecting groups

Amino acids used in SPPS are protected with different types of protecting groups.

These protecting groups shield the α-amino moiety and reactive side-chain functionalities to avoid polymerization reactions. There are two types of orthogonal protecting groups, acid-labile and base-labile. Depending on the composition of deprotection reagent used, one can selectively remove the N^{α}-protecting group with no effect on the side-chain protecting groups [186]. The release of the protected peptide from resin and removal of the side-chain protecting groups depend on the linker and the protection strategy adopted [190]. For example, acid-labile Boc groups (**Figure 3.1.3**) can be removed with 50 % trifluoroacetic acid (TFA) [191] and base-labile Fmoc groups (**Figure 3.1.3**) can be removed with piperidine [184]. Protecting groups are required to shield the nucleophilic functions of the amino acid side chains [192].

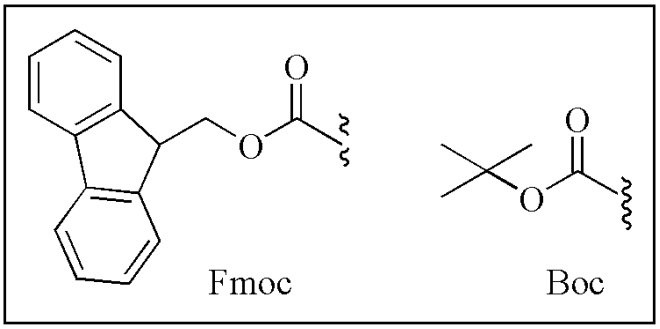


Figure 3.1.3: Commonly used protecting groups

3.1.4 Coupling reagents

Coupling reagents are required for the formation of an amide bond between two amino acids [193]. Coupling reagents have been divided into three categories: carbodiimides, aminium salts and phosphonium salts. Carbodiimides include DCC and DIC (**Figure 3.1.4a**) [194]; DIC is a preferred coupling reagent over DCC as it forms a urea by-product that is soluble in DMF and less toxic than that formed by DCC. Carbodiimides form a reactive species called *O*-acylisourea, this *O*-acylisourea then undergoes aminolysis by the N-terminus of the resin-bound amino acid, and result in the formation of an amide bond. However, this may also lead to several undesirable side reactions, like racemization and termination of the chain elongation. Therefore, to suppress these undesired side reactions, carbodiimides are commonly used with *N*-hydroxy derivatives (**Figure 3.1.4b**), such as HOAt [195], HOBt [196], and Oxyma [197].

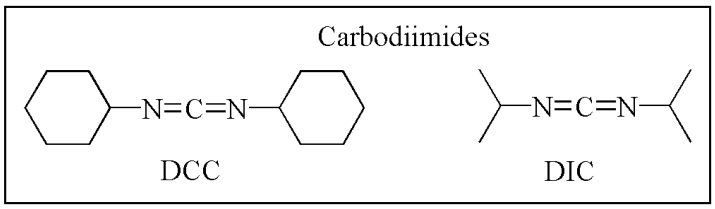


Figure 3.1.4a: Most common coupling reagents used in SPS

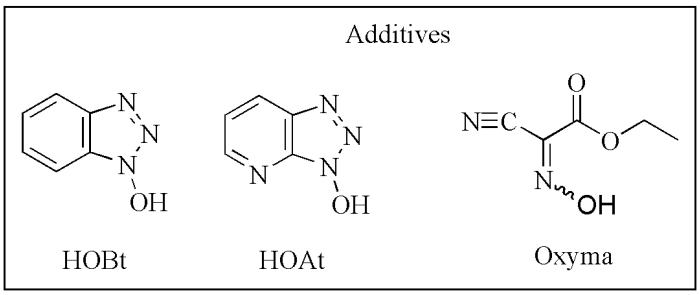


Figure 3.1.4b: Most common additives used along with coupling reagents used in SPS

3.1.5 Design of ultra-short peptide-based compounds

LP-16, LP-17, LP-22, LP-23, and LP-24 are antibacterial lipopeptides from a previous study, these lipopeptides contained *N*-ornithine as cationic charged residue and unsaturated fatty acids (myristic acid, palmitic acid and stearic acid) as hydrophobic moieties [198]. So to mimic these lipopeptides, we designed a library of ultra-short poly-*N*-substituted glycine congeners (DP-16, DP-22, DP-23 and DP-24) by utilizing hydrophobic alkyl tails and cationic charged residues. Thus, to provide structural amphipathicity to these poly-*N*-substituted glycine congeners, different lipophilic fatty acids (myristic acid, stearic acid, and palmitic acid) were conjugated to positively charged peptoid sequences made up of *N*-Boc-1,3-diaminopropane. All compounds were synthesized on rink amide MBHA resin. Synthesis schemes of ultra-short peptides and their poly-*N*-substituted glycine congeners are depicted in **Scheme 1** and **Scheme 2.** Characterization of lyophilized compounds was done by RP-HPLC and ESI-MS. Synthesized compounds (LP-16, DP-16, LP-22, DP-22, LP-23, DP-23, LP-24, and DP-24) with HPLC purity >90% were further evaluated for antibacterial activity.

3.1.6 Design of short peptide based compounds

In addition, few more compounds (SPA, SMO and SPO) were synthesized. The design of these compounds was based on minimum amphipathic template (H+HHG+HH+HH+NH_2) of lead antibacterial peptide SA4 (IOWAGOLFOLFO-NH_2)

from a previous study; where, H represents hydrophobic amino acid, G represents glycine, and "+" represents charged hydrophilic amino acid [199]. Among these compounds, SPA is a peptide that contains alanine in place of glycine at 8^{th} position, SPO is a 12-residue peptoid based on SA4 while SMO is a truncated 7-residue peptoid of SPO. For the synthesis of SPO and SMO, various amines were used to mimic different amino acids of SA4. Different synthetic amines that were used as side chains to mimic ornithine, phenylalanine, tryptophan, leucine, isoleucine, alanine were *N*-Boc-1,3-propanediamine, phenylethylamine, naphthyl ethylamine, butylamine, isopentylamine, and methylamine, respectively. All compounds were synthesized on rink amide MBHA resin. Synthesis strategy of all short peptides and their poly-*N*-substituted glycine congeners are depicted in **Scheme 3** and **Scheme 4**. Characterization of lyophilized compounds was done by RP-HPLC and ESI-MS. Synthesized compounds (SMO, SA4, SPO, and SPA) that were found to be >90% pure were further evaluated for antibacterial activity.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals and reagents

Rink amide MBHA resin, Fmoc-Orn(Boc)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Phe-OH, and Fmoc-Gly-OH were purchased from Novabiochem, India. Hydroxybenzotriazole (HOBt) was purchased from Spectrochem, India. *N*,*N*'- diisopropylcarbodiimide (DIC) and Resazurin sodium salt were purchased from Sigma-Aldrich, India. Triisopropylsilane (TIPS), 4-methylpiperidine, *N*,*N*-dimethylformamide (DMF), trifluoroacetic acid (TFA), *N*-methylpyrrolidinone (NMP), *N*-Boc-1,3-diaminopropane, butylamine, phenylethylamine, 1(1-naphthyl)ethylamine, methylamine and isoamylamine were purchased from Alfa Aeser, India. HPLC grade acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Merck, India. Glutaraldehyde, myristic acid, palmitic acid, and stearic acid were purchased from Loba Chemie, India. Tetracycline HCl, vancomycin, meropenem, Luria Broth (LB), Muller Hinton Broth (MHB), Middlebrook MB7H9 media, and RPMI medium were purchased from HiMedia, India.

3.2.2 Bacterial strains

Mycobacterium smegmatis MC^2155, *Bacillus subtilis* MTCC 121, *Acinetobacter baumannii* ATCC 19606 and multidrug-resistant clinical isolates of *Acinetobacter baumannii* (MDR1, MDR2, MDR3 and MDR4) were used in this study. See appendix for more details (**Appendix A**).

3.2.3 Synthesis and characterization of compounds

3.2.3.1 Synthesis of peptides

Peptides were synthesized by standard Fmoc SPS protocol (**Scheme 1** and **Scheme 3**) using Rink MBHA resin as solid support [200]. Deprotection of Fmoc protected resin was done using 20% piperidine in DMF. Once the Fmoc group was removed, first Fmoc-protected amino acid (4 equivalent), HOBt (2 equivalent) and DIC (2 equivalent) in 2 ml DMF was coupled. The completion of coupling was confirmed by the ninhydrin test. After completion of the first coupling, the Fmoc group of coupled amino acid was deprotected. This deprotection was followed by the coupling of the second Fmoc protected amino acid. Deprotection and coupling steps were repeated until the desired peptide sequence was attached to solid support. Finally, the Fmoc group from last Fmoc-protected amino acid coupled to resin was deprotected and the peptide was cleaved from the solid support using a TFA cocktail (TFA:water:TIPS in the ratio of 95:2.5:2.5). The cleaved peptide was precipitated in chilled diethyl ether. Diethyl ether was decanted and 6 ml solution of ACN:water; 1:1 was added and kept at -80 °C for 4 h before lyophilization. Preliminary characterization of synthesized lyophilized compounds was done using the RP-HPLC; see appendix for HPLC details (**Appendix B**).

3.2.3.2 Synthesis of peptoids

Peptoids were synthesized manually following the submonomer peptoid synthesis protocol (**Scheme 2** and **Scheme 4**), using Rink amide MBHA resin as the solid support [201]. Rink amide resin (100 mg) was washed in CH_2Cl_2 (3×2 ml), which was followed by swelling in DMF (3 ml) for 60 min. The Fmoc protecting group of linker on the resin was removed by treating with piperidine/DMF (20% v/v) mixture for (2×20 min), followed by extensive washes with DMF. The deprotected resin was further acylated with 0.6 M bromoacetic acid, DIC in DMF for 1 h. Extensive washings were given (5×2 ml), followed by displacement with 2 M Boc-protected amines/DIC in DMF for 4-5 h. Acylations and displacements were repeated desired number of times followed by final

acylation with fatty acid and DIC in DMF. Finally, TFA cocktail (TFA:water:TIPS in the ratio of 95:2.5:2.5) was used for the cleavage of peptoids from resin. Cleaved peptoid was precipitated in chilled diethyl ether. Diethyl ether was decanted and 6 ml solution of ACN:water; 1:1 was added and kept at -80 °C for 4 h before lyophilization. Preliminary characterization of synthesized lyophilized compounds was done using RP-HPLC technique on C18 Column using ACN (0.1 % TFA) and water (0.1% TFA) as a mobile phase. Samples were detected at 220 nm or 280 nm wavelength.

3.2.3.3 Preliminary antibacterial activity studies of synthesized compounds

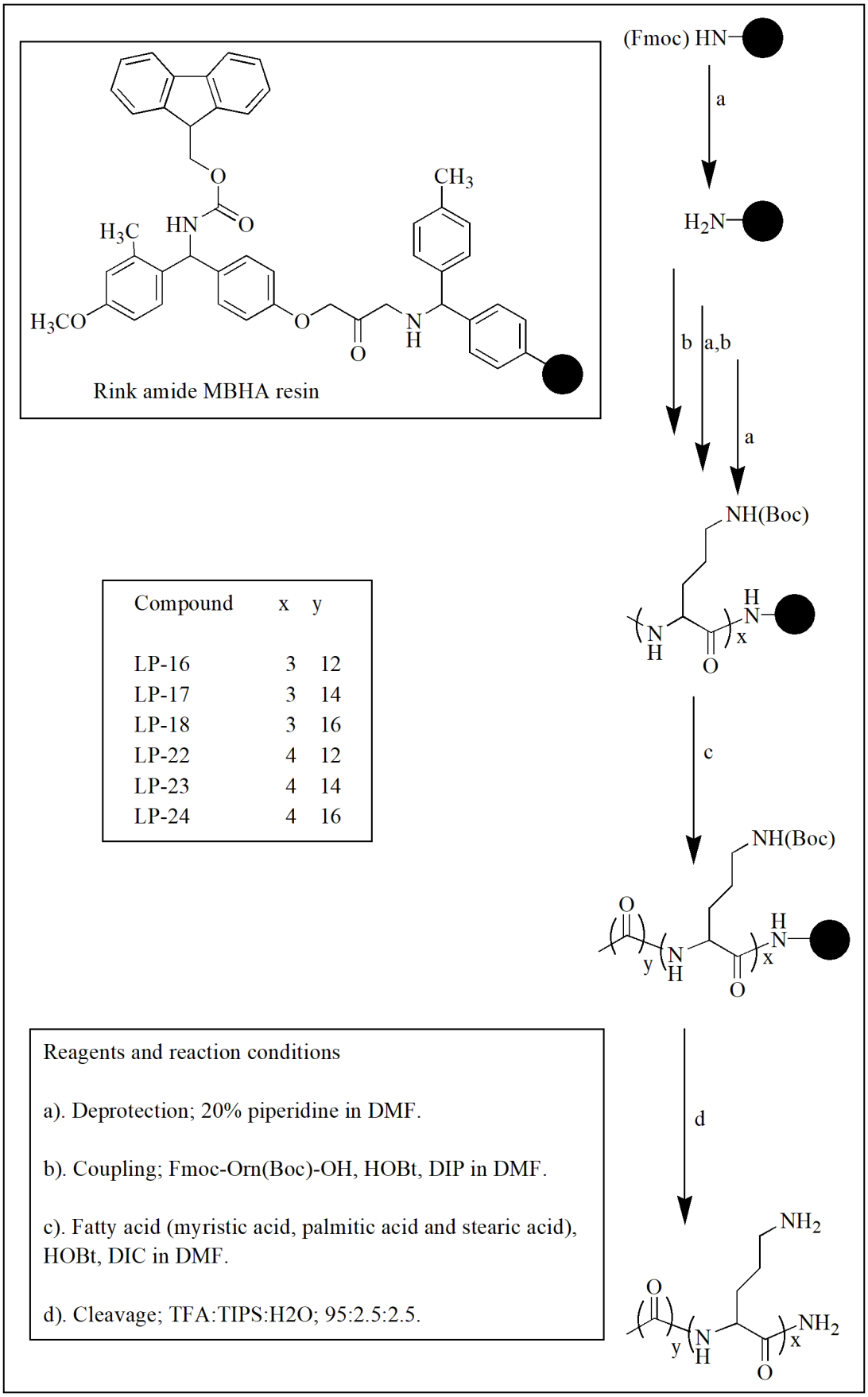
All synthesized compounds were screened for antibacterial activity against various Gram-positive and Gram-negative bacteria as per CLSI 2015 guidelines (Clinical Laboratory Standard Institute). A pure colony of bacteria was inoculated in respective growth media (LB/MHB/MB7H9) and incubated overnight at 37 °C. Overnight grown bacterial culture was adjusted to 0.5 McFarland standards, diluted 20-fold and placed into 96-well microtiter plates. Standard antibiotics (vancomycin/meropenem/tetracycline HCl) and synthesized compounds were placed into plates and incubated at 37 °C for 16-24 h for all strains, except *Mycobacterium smegmatis* which was incubated for 48 h. Optical density (OD) was observed at 600 nm and minimum inhibitory concentration (MIC) was determined according to CLSI 2015 [260].

3.2.3.4 Hemolytic toxicity study

Erythrocytes from healthy human volunteer were collected and washed thrice in normal saline using centrifuge (at 2000 rpm for 10 min). 100 µl of diluted suspension of erythrocytes in normal saline was added to 100 µl of diluted peptide based compound in normal saline to obtain particular concentrations of peptides and peptoids. These vials were kept in the incubator for 1 h (at 37 °C). Subsequently, the contents of the respective wells were centrifuged to separate supernatant and OD of supernatant was recorded at 570 nm. These OD values were used to calculate percent hemolysis caused by test compounds [202]. Suspension of human red blood cells (hRBCs) with normal saline was used as blank and suspension of hRBCs incubated with 1% Triton X-100 was used for 100 % hemolysis (positive control).

Following formula was used to calculate % hemolysis:

Percent hemolysis= 100(TC- B)/(TX- B); where B stands for absorbance of supernatant from blank vial, TC stands for absorbance of supernatant from test compound vial, and TX stands for absorbance of supernatant from Triton X treated vial.



Compound	x	y
LP-16	3	12
LP-17	3	14
LP-18	3	16
LP-22	4	12
LP-23	4	14
LP-24	4	16

Reagents and reaction conditions

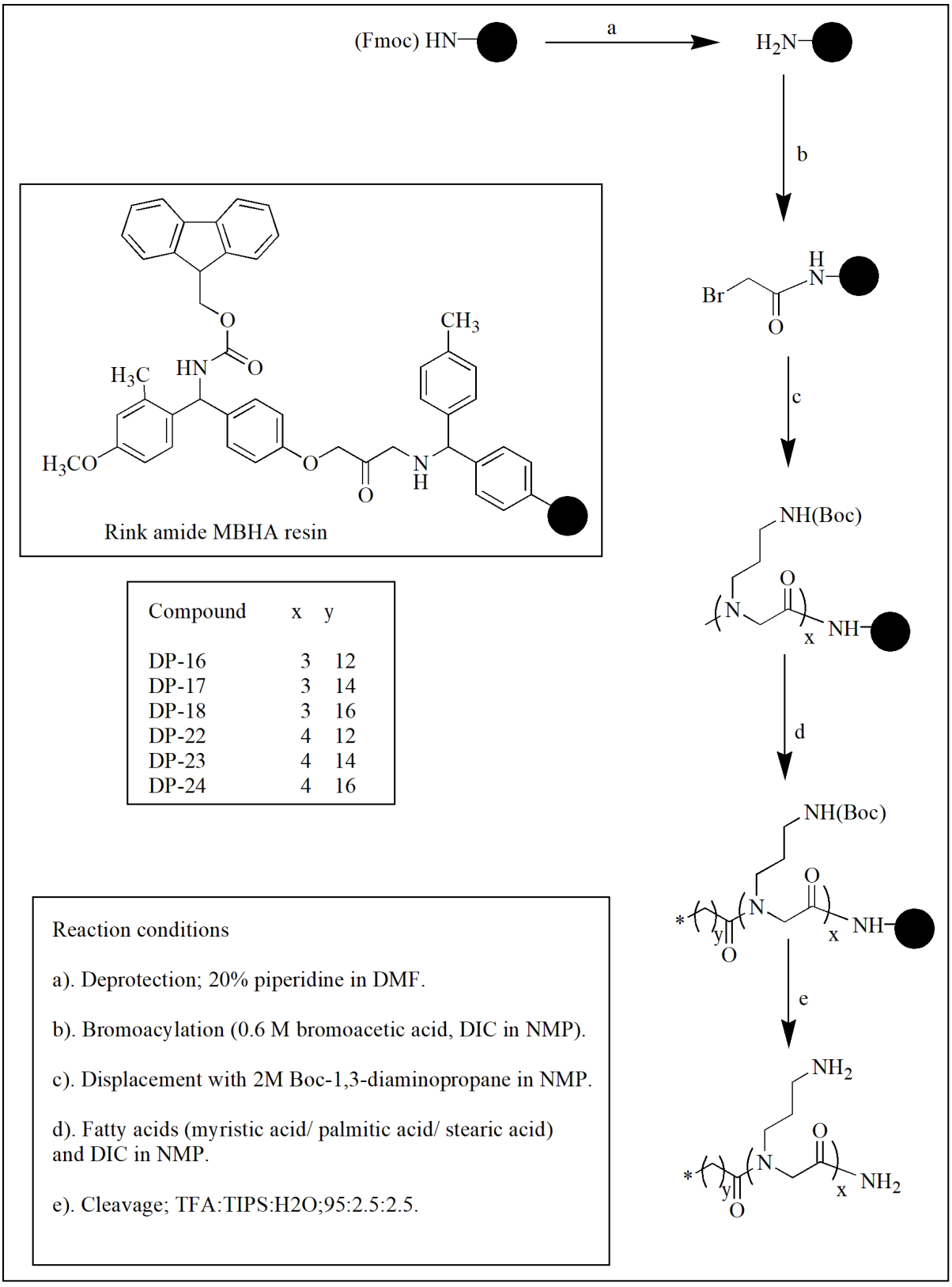
a). Deprotection; 20% piperidine in DMF.

b). Coupling; Fmoc-Orn(Boc)-OH, HOBt, DIP in DMF.

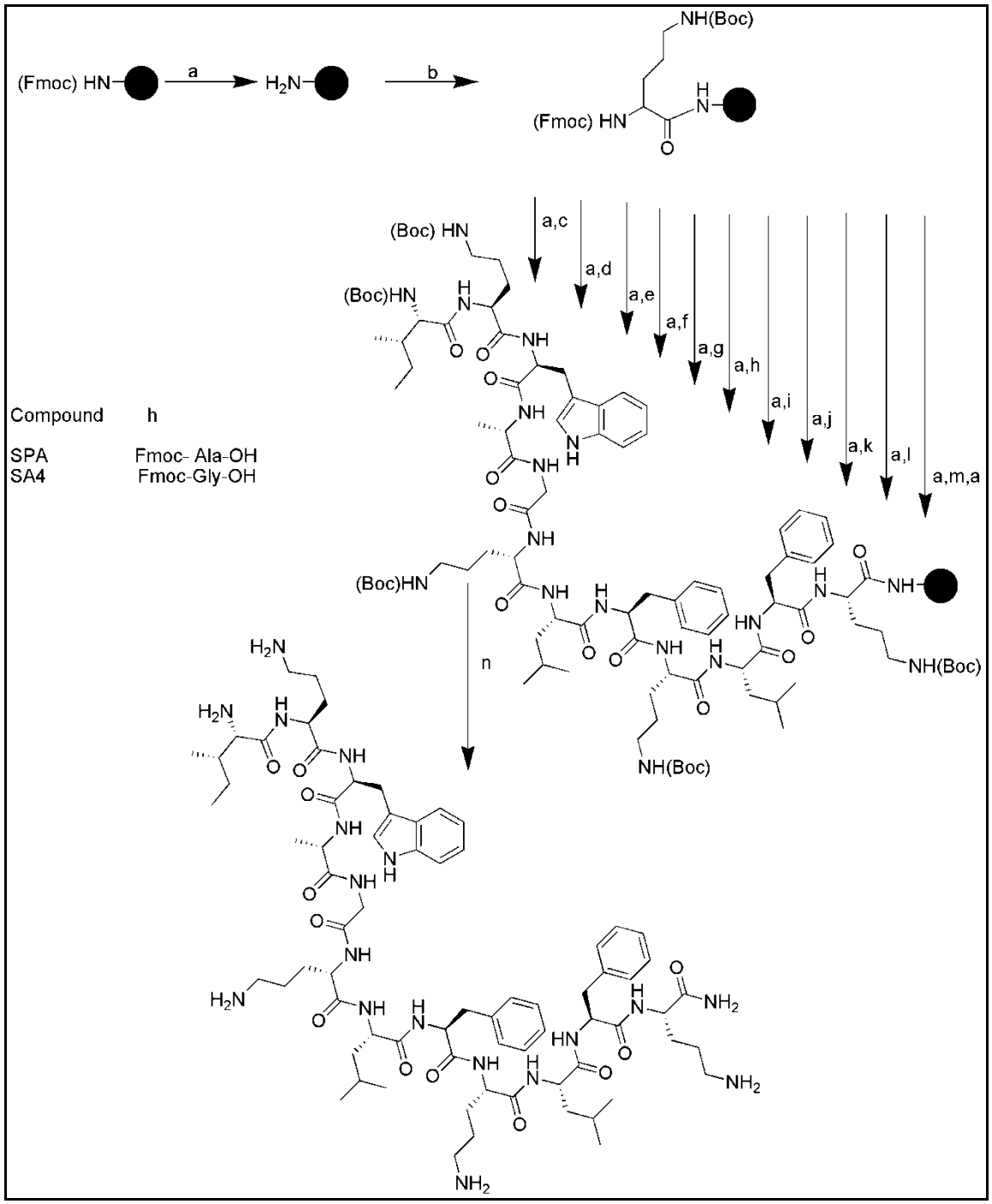
c). Fatty acid (myristic acid, palmitic acid and stearic acid), HOBt, DIC in DMF.

d). Cleavage; TFA:TIPS:H2O; 95:2.5:2.5.

Scheme 1: Synthesis of ultra-short peptides

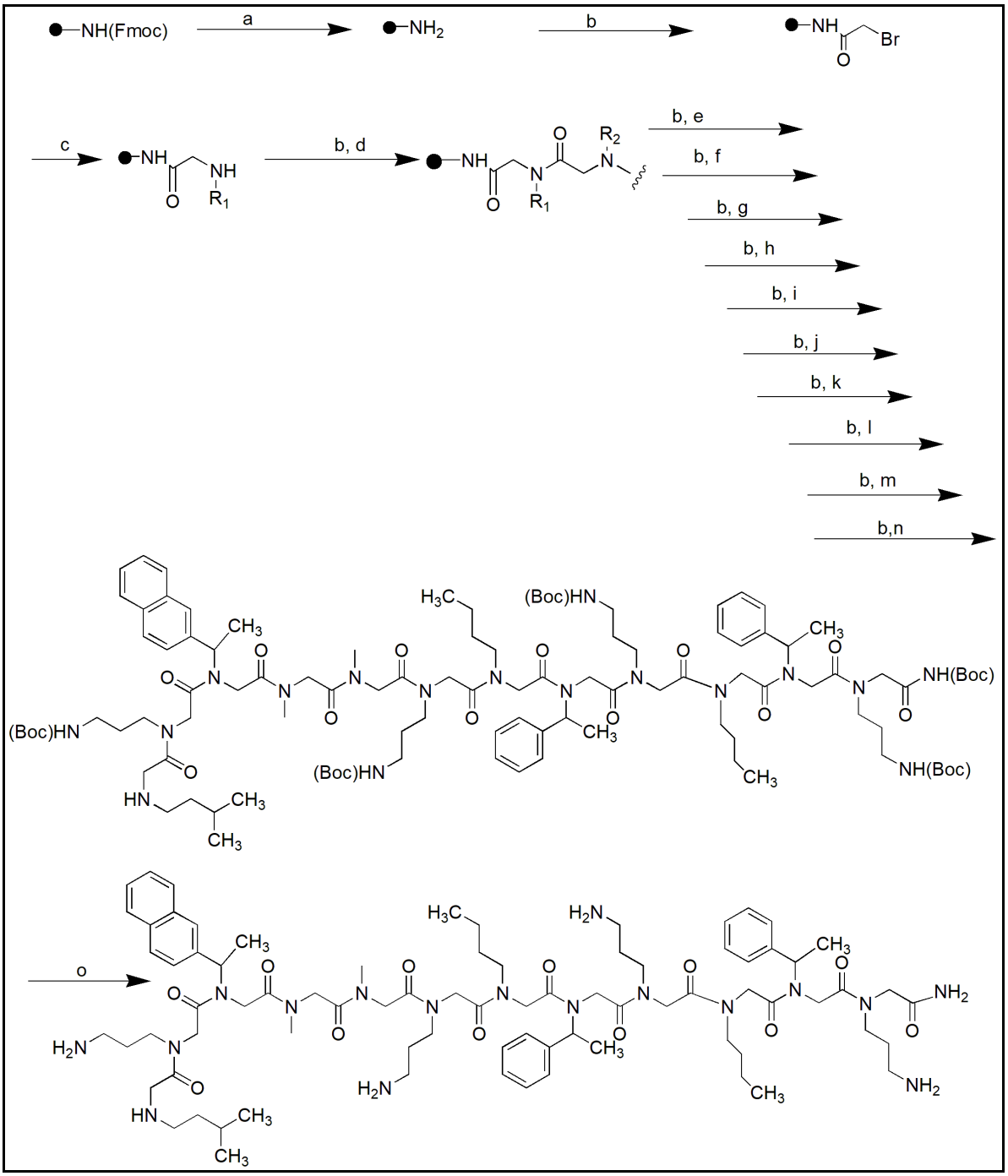


Scheme 2: Synthesis of ultra-short peptoids



Reagents and reaction conditions: *a). Deprotection; 20% piperidine in DMF, b). Coupling; Fmoc-Orn(Boc)-OH, HOBt, DIC in DMF, c). Coupling; Fmoc-Phe-OH, HOBt, DIC in DMF, d). Coupling; Fmoc-Leu-OH, HOBt, DIC in DMF, e). Coupling; Fmoc-Orn(Boc)-OH, HOBt, DIC in DMF, f). Coupling; Fmoc-Phe-OH, HOBt, DIC in DMF, g). Coupling; Fmoc-Leu-OH, HOBt, DIC in DMF, h). Coupling; Fmoc-Orn(Boc)-OH, HOBt, DIC in DMF, i). Coupling; Fmoc-Gly-OH, HOBt, DIPC in DMF, j). Coupling; Fmoc-Ala-OH, HOBt, DIC in DMF, k). Coupling; Fmoc-Trp(Boc)-OH, HOBt, DIC in DMF, l). Coupling; Fmoc-Orn(Boc)-OH, HOBt, DIC in DMF, m). Coupling; Fmoc-Ile-OH, HOBt, DIC in DMF, n). Cleavage; TFA:TIPS:H_2O;95:2.5:2.5.*

Scheme 3: Synthesis of 12-residue peptides



Reagents and reaction conditions: *a). Deprotection; 20% piperidine in DMF, b). Acylation; bromoacetic acid and DIC in DMF, c). Displacement with Boc-1,3-diaminopropane in DMF, d). Displacement with phenylethylamine in DMF, e). Displacement with butylamine in DMF, f). Displacement with Boc-1,3 diaminopropane in DMF, g). Displacement with phenylethylamine in DMF, h). Displacement with butylamine in DMF, i). Displacement with Boc-1,3 diaminopropane in DMF, j). Displacement with methylamine in DMF/THF, k). Displacement with methylamine in DMF/THF, l). Displacement with napthylethylamine in DMF, m). Displacement with Boc-1,3 diaminopropane in DMF, n) Displacement with isopentylamine in DMF, o). Cleavage; TFA:water:TIPS (95:2.5:2.5).*

Scheme 4: Synthesis of 12-residue peptoid

3.3 RESULTS

3.3.1 Compounds synthesized

Table 3.3.1: Name, sequence, HPLC retention time, HPLC purity, calculated mass and observed mass of synthesized compounds.

S. No.	Compound	Sequence	HPLC Retention Time	HPLC Purity (%)	Calc. Mass	Obs. Mass
1.	SPA	IOWAAOLFOLFO-NH_2	4.150	93.7	1434.89 (M)	359 $(M+4H)^{4+}$
2.	SPO	nInOnWnAnGnOnLnFnOnLnFnO-NH_2	4.055	>95	1501.95 (M)	752.35 $(M+2H)^{2+}$
3.	SA4	IOWAGOLFOLFO-NH_2	4.172	>95	1420.87 (M), 1534.89 $(M+TFA)^{+}$	1535.05 $(M+TFA)^{+}$
4.	SMO	nOnLnFnOnLnFnO-NH_2	23.710	91.96	907.60 (M)	908.5 $(M+H)^{+}$
5.	LP-16	$CH_3(CH_2)_{12}$CO-NH-OOO-NH_2	4.101	91.73	569.46 (M)	570.84 $(M+H)^{+}$
6.	DP-16	$CH_3(CH_2)_{12}$CO-NH-nOnOnO-NH_2	4.113	90.83	569.46 (M)	571.3 $(M+H)^{+}$
7.	LP-17	$CH_3(CH_2)_{14}$CO-NH-OOO-NH_2	4.167	88.78	597.49 (M)	598.6 $(M+H)^{+}$
8.	LP-18	$CH_3(CH_2)_{16}$CO-NH-OOO-NH_2	4.169	86.10	625.53 (M)	626.56 $(M+H)^{+}$
9.	LP-22	$CH_3(CH_2)_{12}$CO-NH-OOOO-NH_2	2.438	94.85	683.54 (M)	684.5 $(M+H)^{+}$
10.	DP-22	$CH_3(CH_2)_{12}$CO-NH-nOnOnOnO-NH_2	4.125	93.88	683.54 (M)	684.6 $(M+H)^{+}$
11.	LP-23	$CH_3(CH_2)_{14}$CO-NH-OOOO-NH_2	5.103	>95	711.57 (M)	712.5 $(M+H)^{+}$
12.	DP-23	$CH_3(CH_2)_{14}$CO-NH-nOnOnOnO-NH_2	5.073	>95	711.57 (M)	712.5 $(M+H)^{+}$
13.	LP-24	$CH_3(CH_2)_{16}$CO-NH-OOOO-NH_2	4.086	>95	739.60 (M)	740.6 $(M+H)^{+}$
14.	DP-24	$CH_3(CH_2)_{16}$CO-NH-nOnOnOnO-NH_2	4.085	>95	739.60 (M)	740.7 $(M+H)^{+}$

1. **Compound name:** LP-16

   Mol. formula: $C_{29}H_{59}N_7O_4$

   Calculated mass: 569.46 (M)

   Observed mass: 570.84 $(M+H)^+$

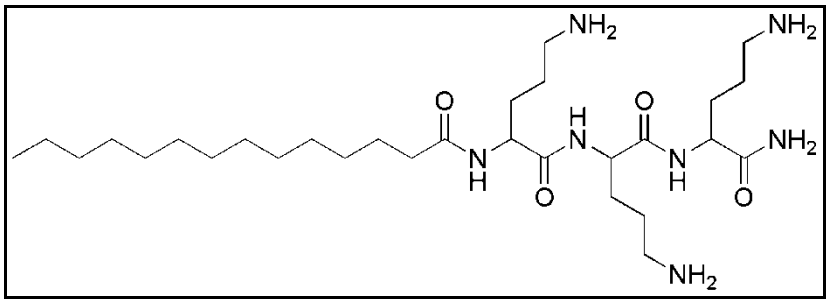


Figure 3.3.1(1): Chemical structure of LP-16

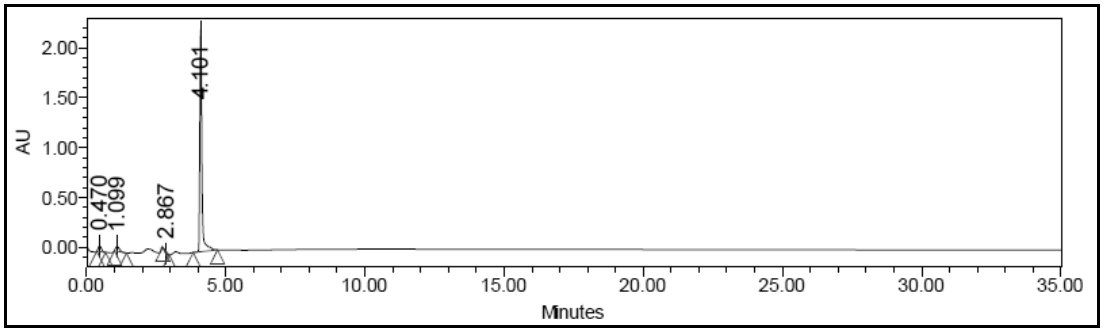


Figure 3.3.1(2): HPLC chromatogram of LP-16

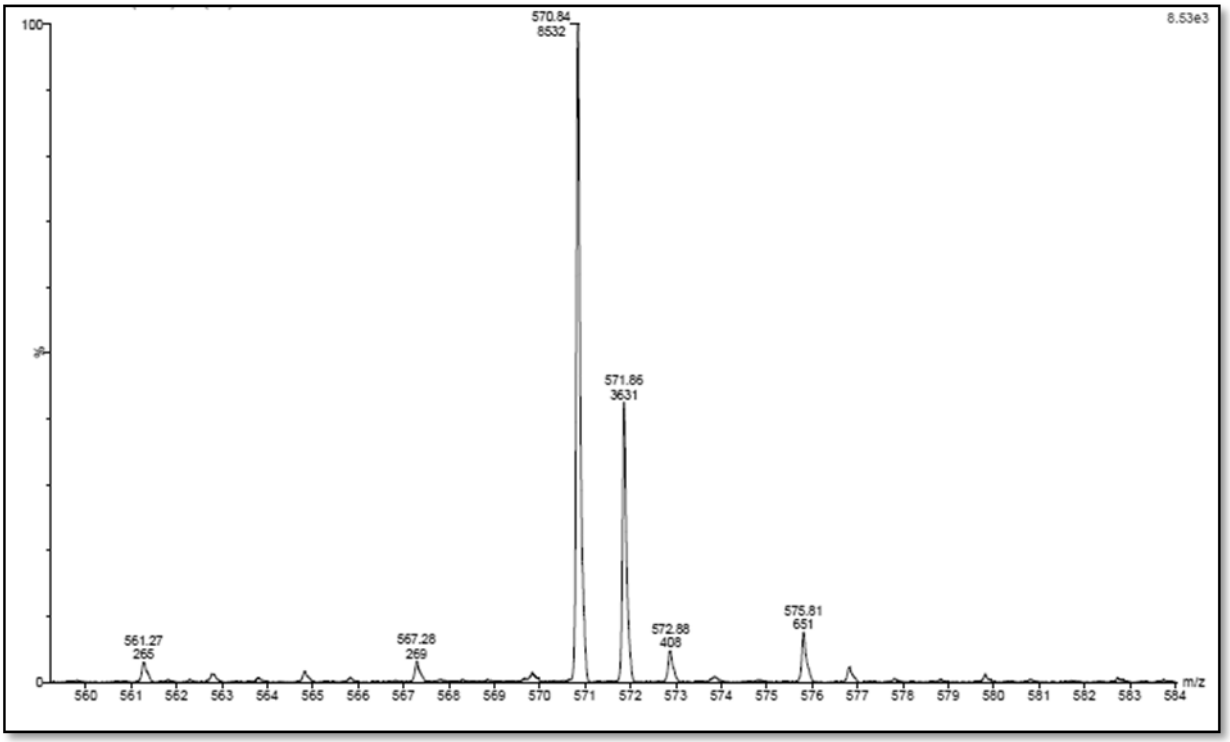


Figure 3.3.1(3): Mass spectrum of LP-16

2. **Compound name:** DP-16

Mol. formula: $C_{29}H_{59}N_7O_4$

Calculated mass: 569.46 (M)

Observed mass: 570.54 $(M+H)^+$

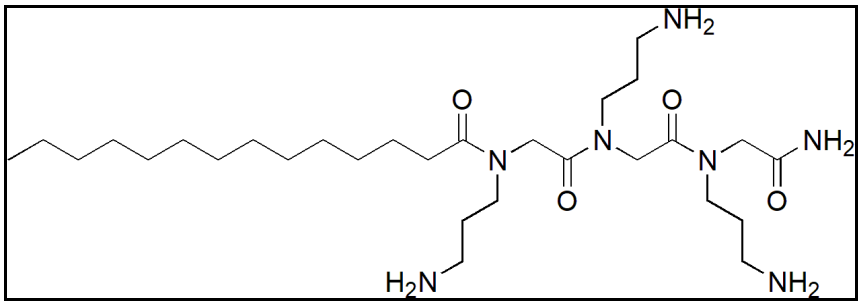


Figure 3.3.1(4): Chemical structure of DP-16

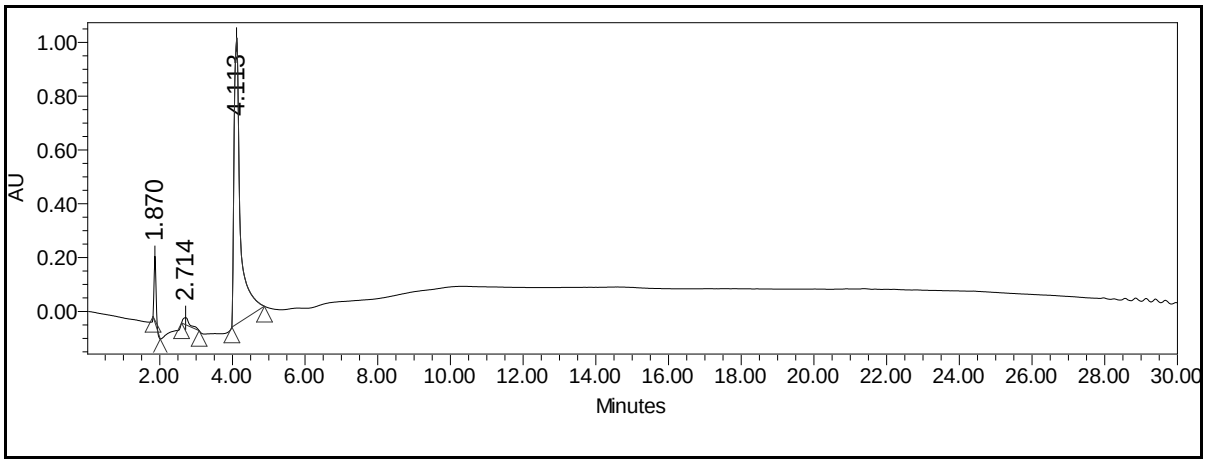


Figure 3.3.1(5): HPLC chromatogram of DP-16

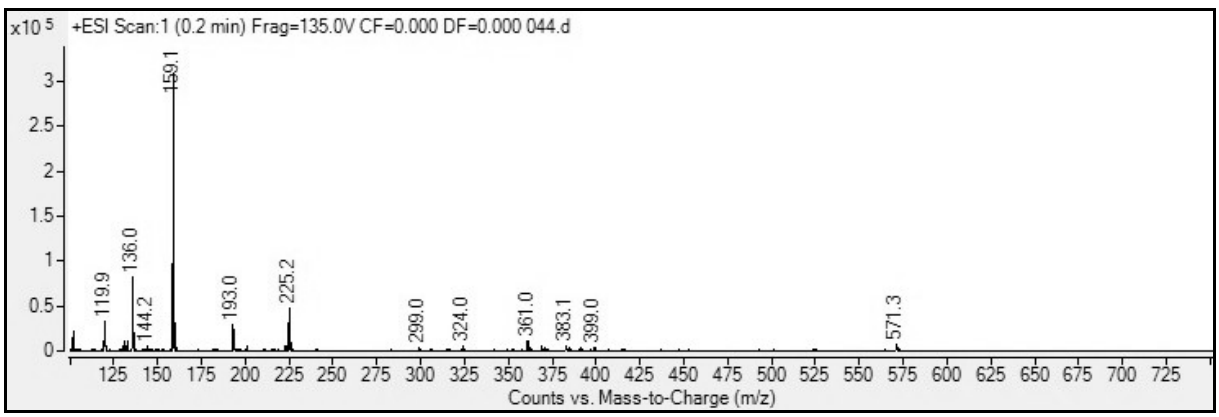


Figure 3.3.1(6): Mass spectrum of DP-16

3. **Compound name:** LP-17

   Mol. formula: $C_{31}H_{63}N_7O_4$

   Calculated mass: 597.49 (M)

   Observed mass: 598.6 $(M+H)^+$

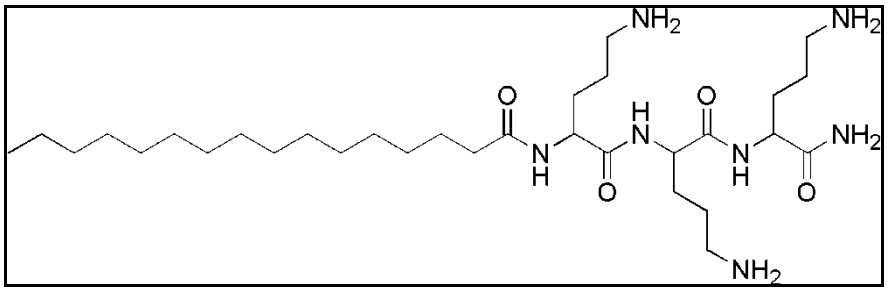


Figure 3.3.1(7): Chemical structure of LP-17

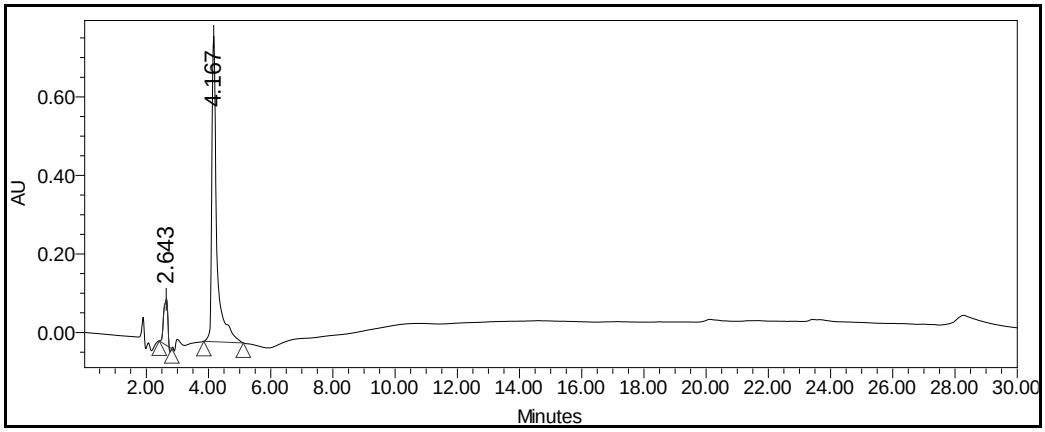


Figure 3.3.1(8): HPLC chromatogram of LP-17

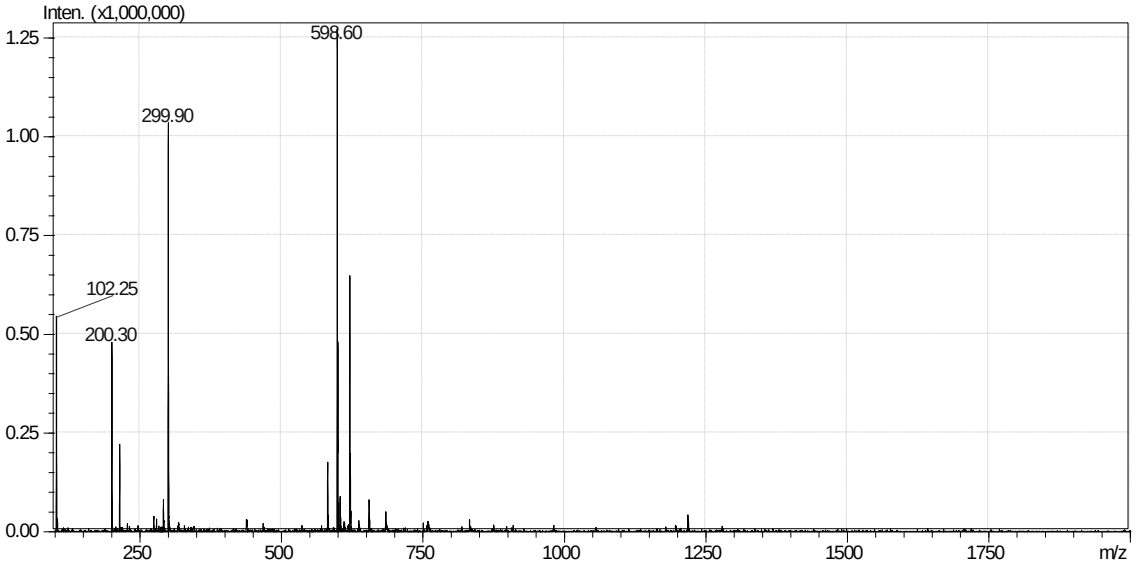


Figure 3.3.1(9): Mass spectrum of LP-17

4. **Compound name:** LP-18

Mol. formula: $C_{33}H_{67}N_7O_4$

Calculated mass: 625.53 (M)

Observed mass: 626.56 $(M+H)^+$

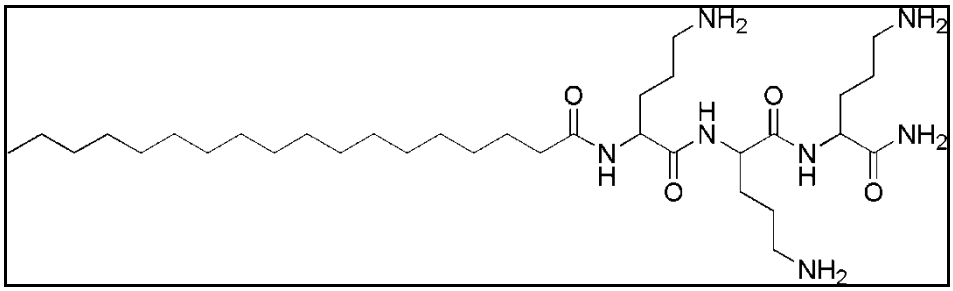


Figure 3.3.1(10): Chemical structure of LP-18

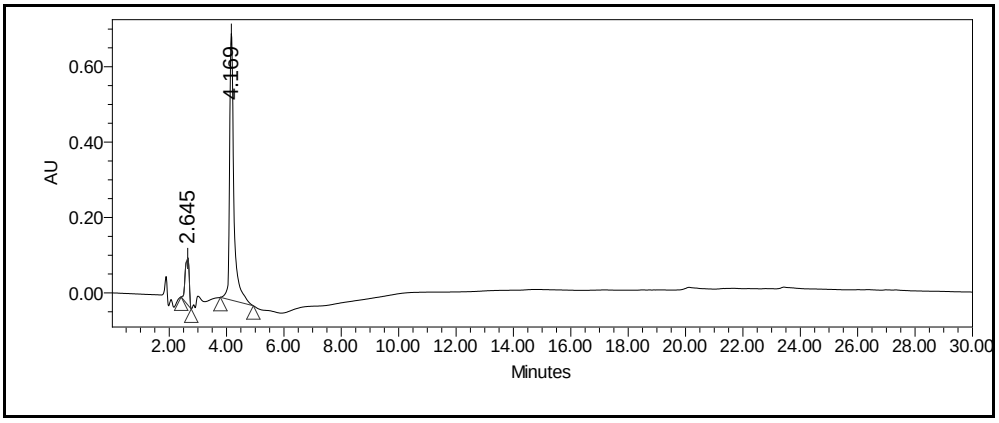


Figure 3.3.1(11): HPLC chromatogram of LP-18

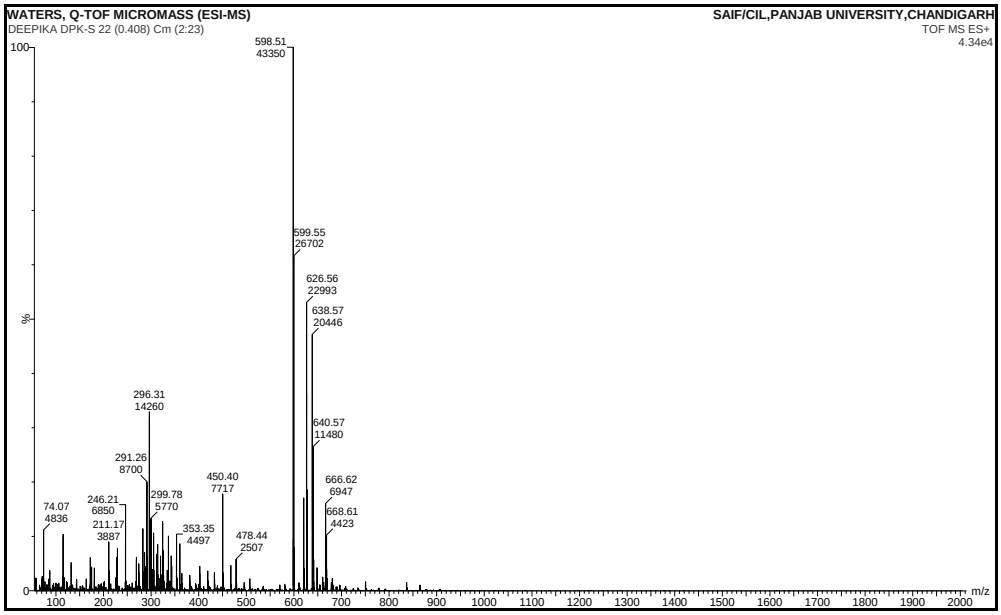


Figure 3.3.1(12): Mass spectrum of LP-18

5. **Compound name:** LP-22

   Mol. formula: $C_{34}H_{69}N_9O_5$

   Calculated mass: 683.54 (M)

   Observed mass: 684.5 $(M+H)^+$

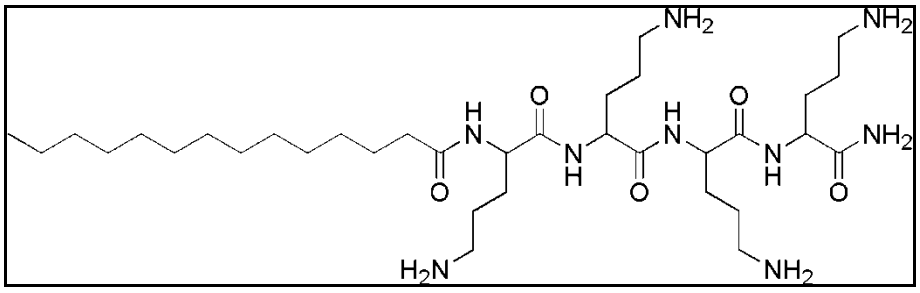


Figure 3.3.1(13): Chemical structure of LP-22

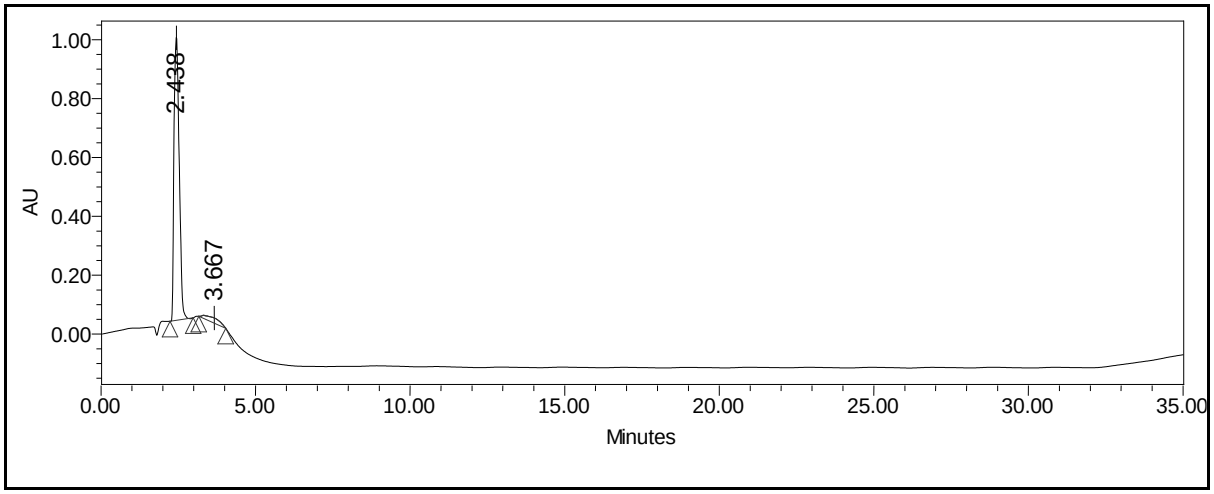


Figure 3.3.1(14): HPLC chromatogram of LP-22

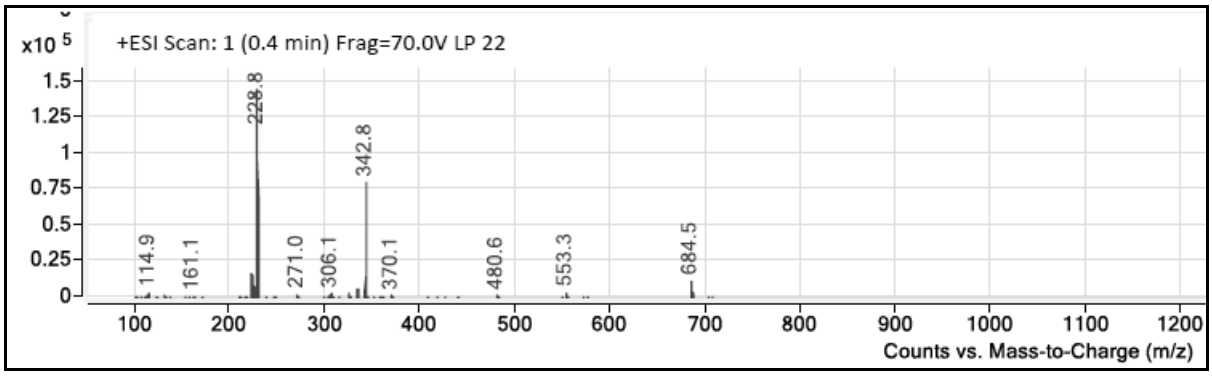


Figure 3.3.1(15): Mass spectrum of LP-22

6. **Compound name:** DP-22

   Mol. formula: $C_{34}H_{69}N_9O_5$

   Calculated mass: 683.54 (M)

   Observed mass: 684.7 $(M+H)^+$

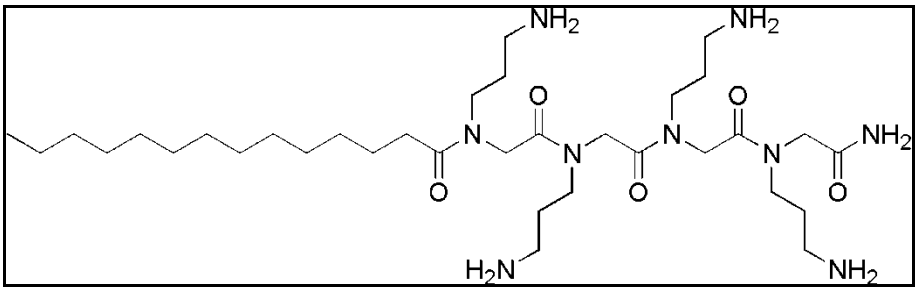


Figure 3.3.1(16): Chemical structure of DP-22

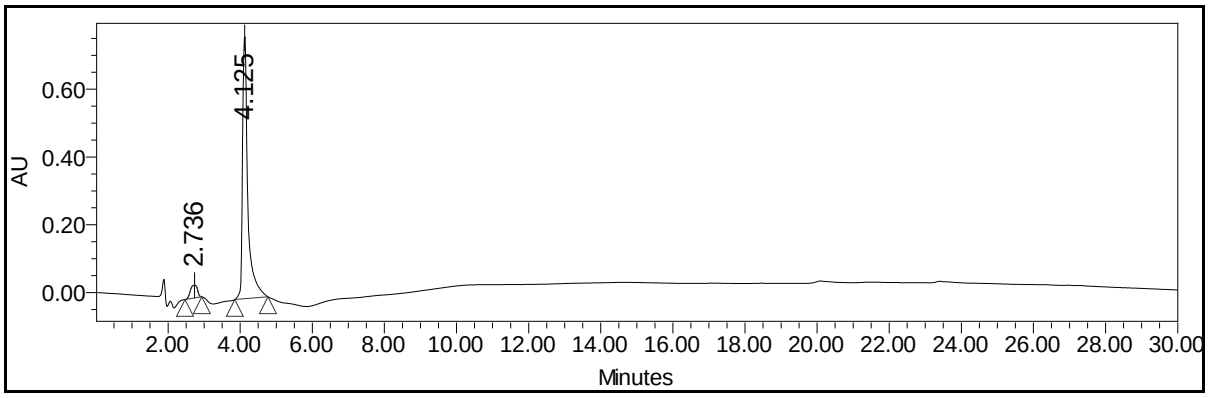


Figure 3.3.1(17): HPLC chromatogram of DP-22

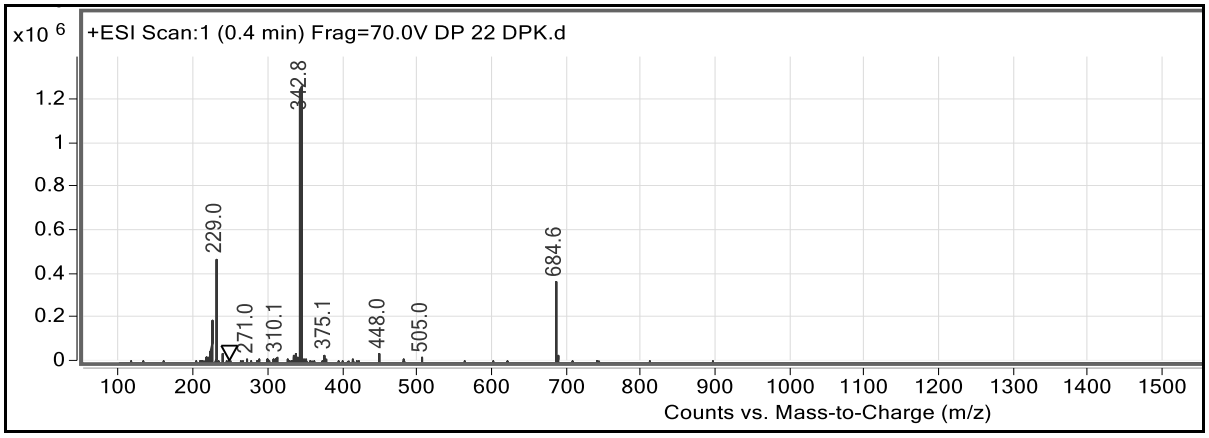


Figure 3.3.1(18): Mass spectrum of DP-22

7. **Compound name:** LP-23

   Mol. formula: $C_{36}H_{73}N_9O_5$

   Calculated mass: 711.57 (M)

   Observed mass: 712.5 $(M+H)^+$

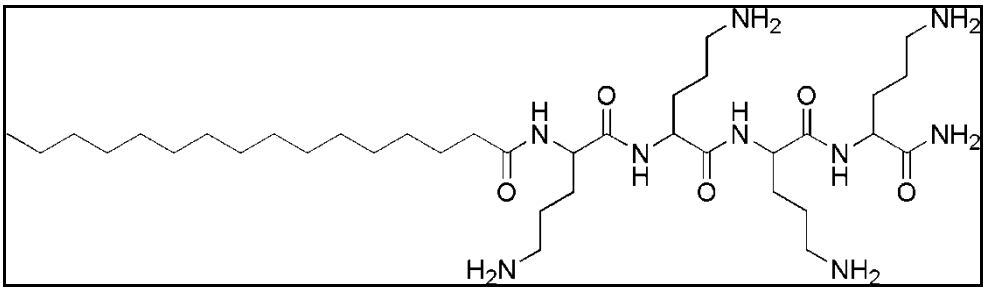


Figure 3.3.1(19): Chemical structure of LP-23

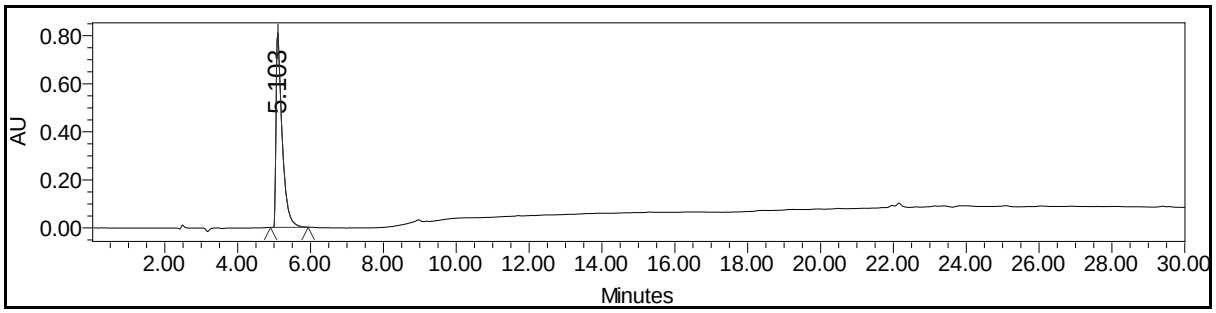


Figure 3.3.1(20): HPLC chromatogram of LP-23

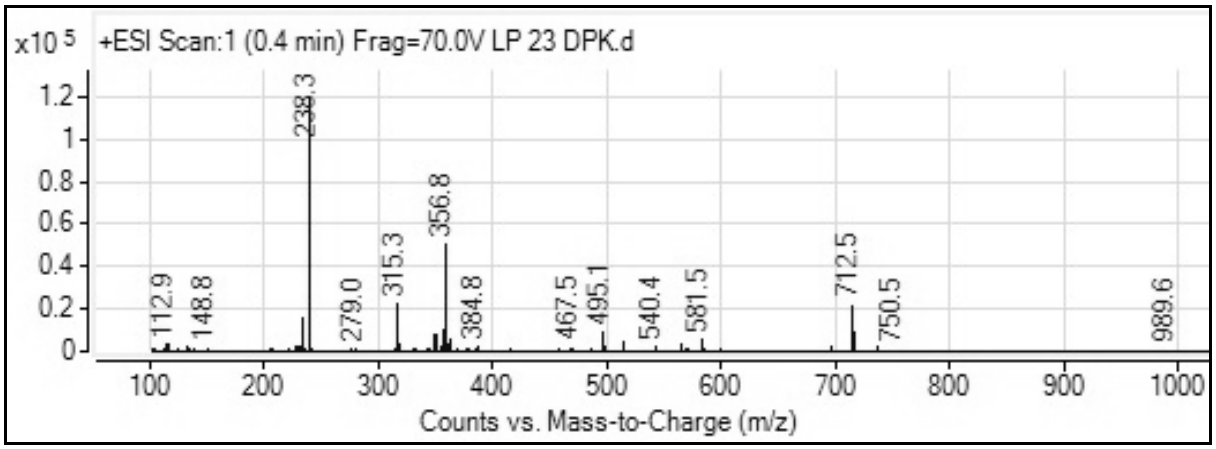


Fig 5.1(21): Mass spectrum of LP-23

8. **Compound name:** DP-23

   Mol. formula: $C_{36}H_{73}N_9O_5$

   Calculated mass: 711.57 (M)

   Observed mass: 712.5 $(M+H)^+$

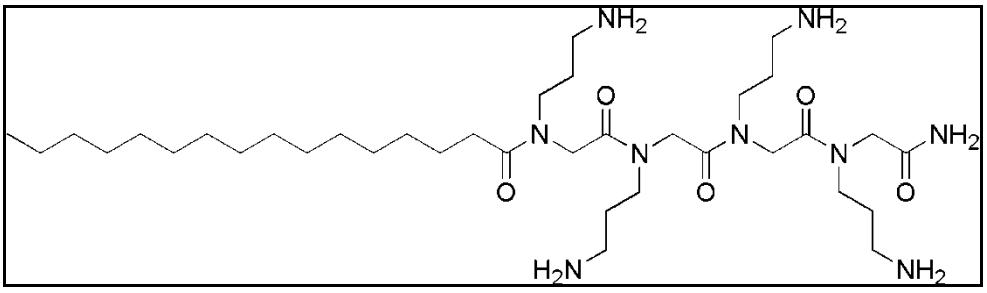


Figure 3.3.1(22): Chemical structure of DP-23

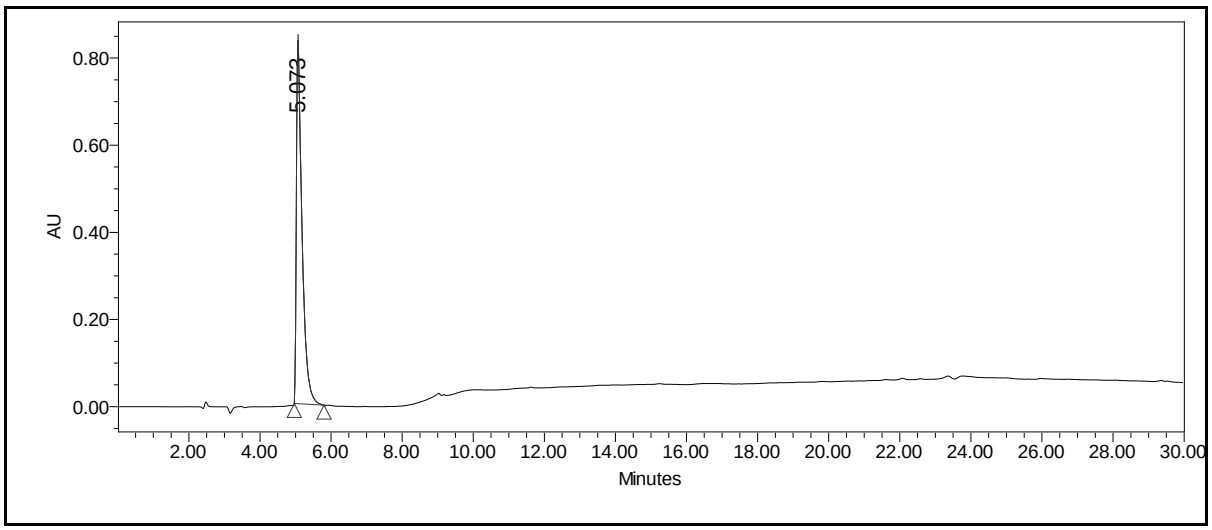


Figure 3.3.1(23): HPLC chromatogram of DP-23 at 220 nm

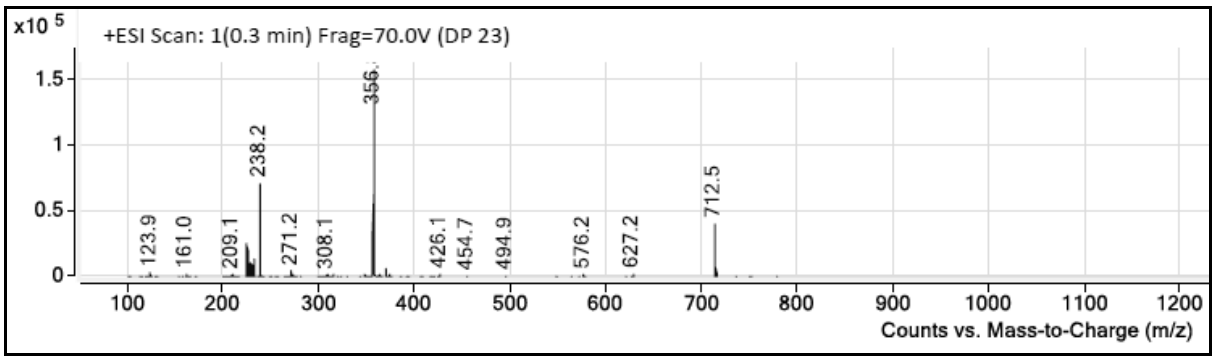


Figure 3.3.1(24): Mass spectrum of DP-23

9. **Compound name:** LP-24

   Mol. formula: $C_{38}H_{77}N_9O_5$

   Calculated mass: 739.6 (M)

   Observed mass: 740.6 $(M+H)^+$

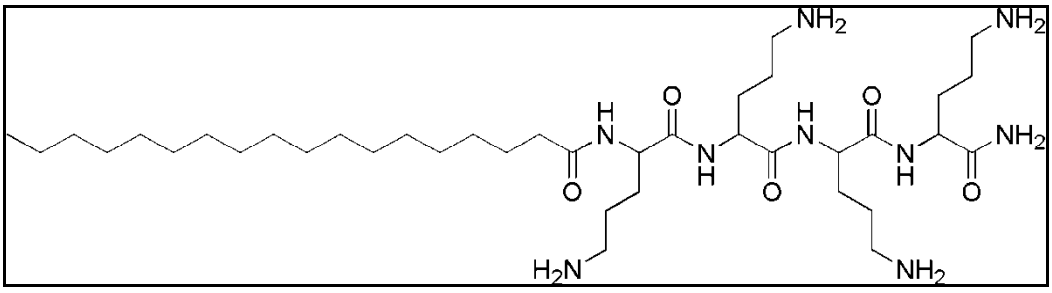


Figure 3.3.1(25): Chemical structure of LP-24

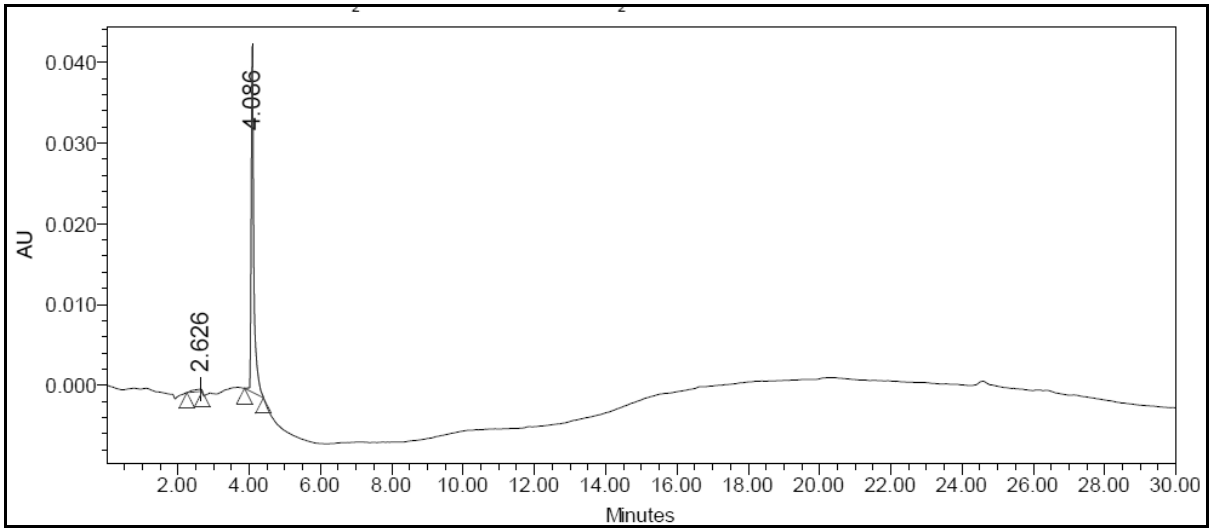


Figure 3.3.1(26): HPLC chromatogram of LP-24

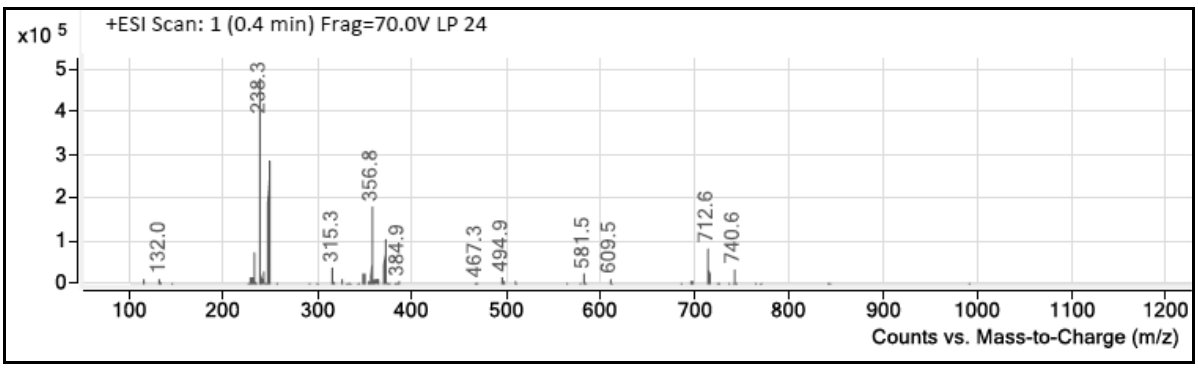


Figure 3.3.1(27): Mass spectrum of LP-24

10. Compound name: DP-24

Mol. formula: $C_{38}H_{77}N_9O_5$

Calculated mass: 739.60 (M)

Observed mass: 740.7 $(M+H)^+$

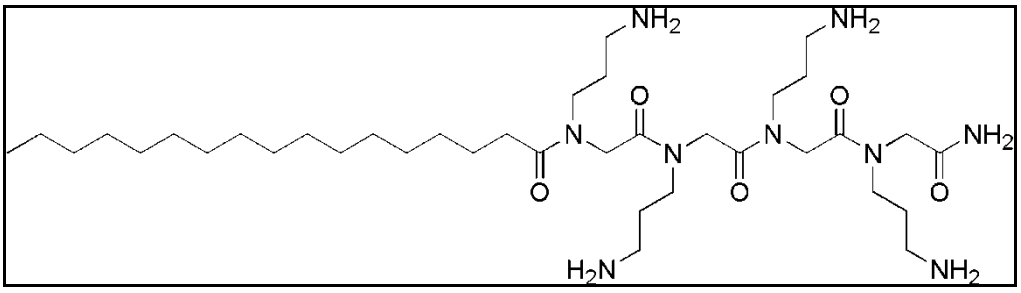


Figure 3.3.1(28): Chemical structure of DP-24

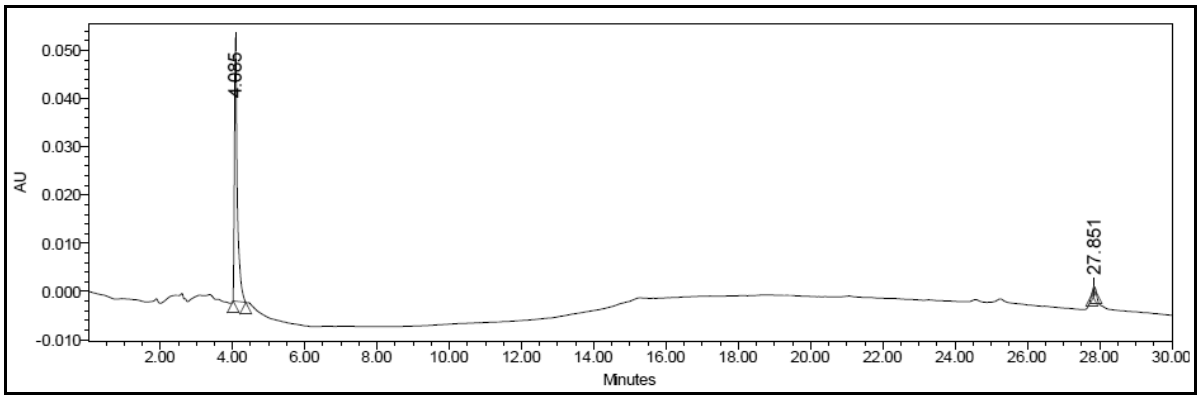


Figure 3.3.1(29): HPLC chromatogram of DP-24

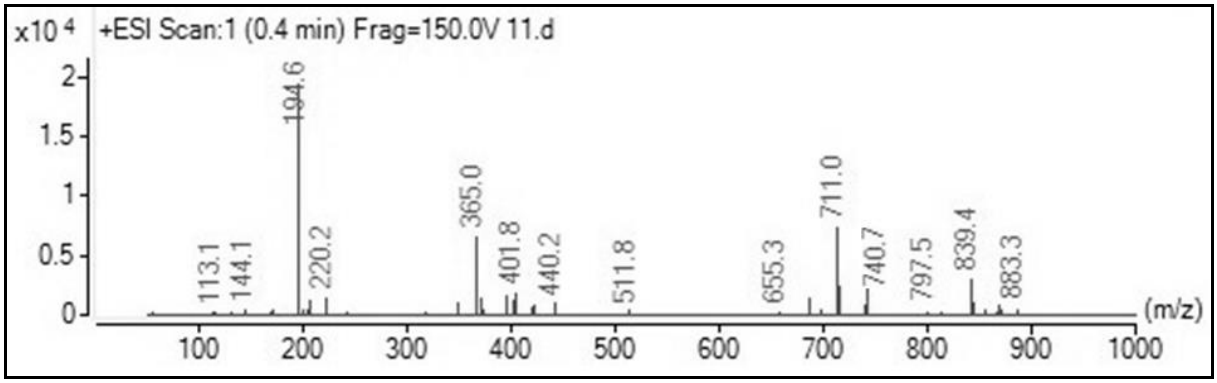


Figure 3.3.1(30): Mass spectrum of DP-24

11. Compound name: SPA

Mol. formula: $C_{73}H_{114}N_{18}O_{12}$

Calculated mass: 1434.89 (M)

Observed mass: 359 $(M+4H)^{4+}$

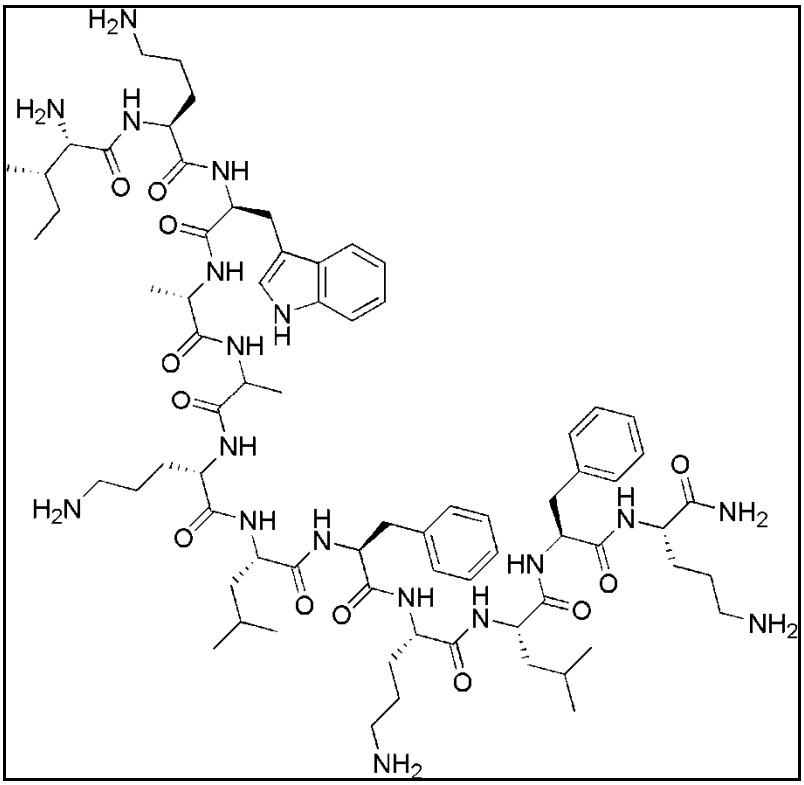


Figure 3.3.1(31): Chemical structure of SPA

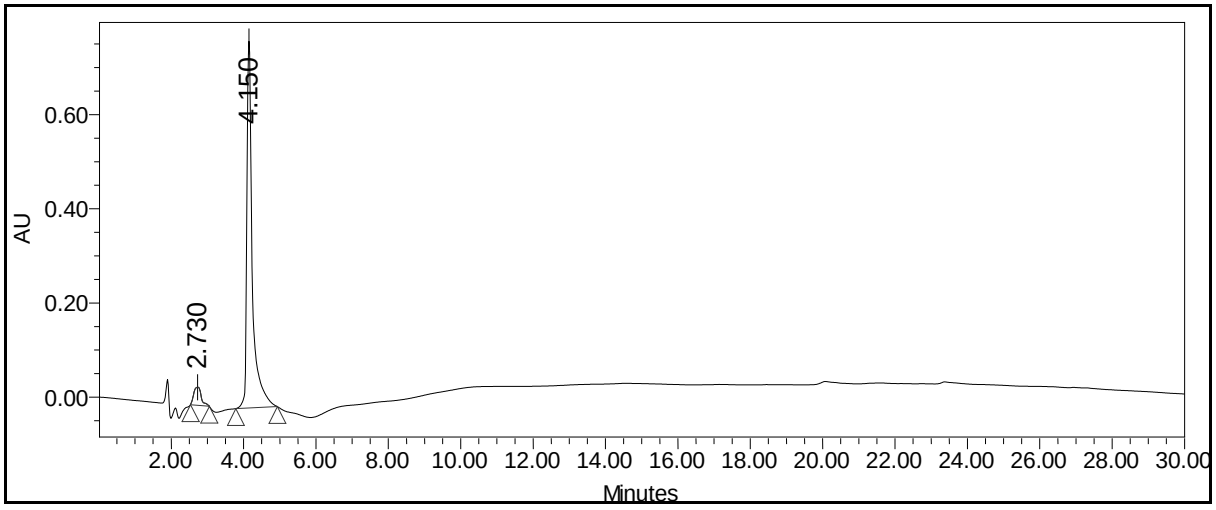


Figure 3.3.1(32): HPLC chromatogram of SPA

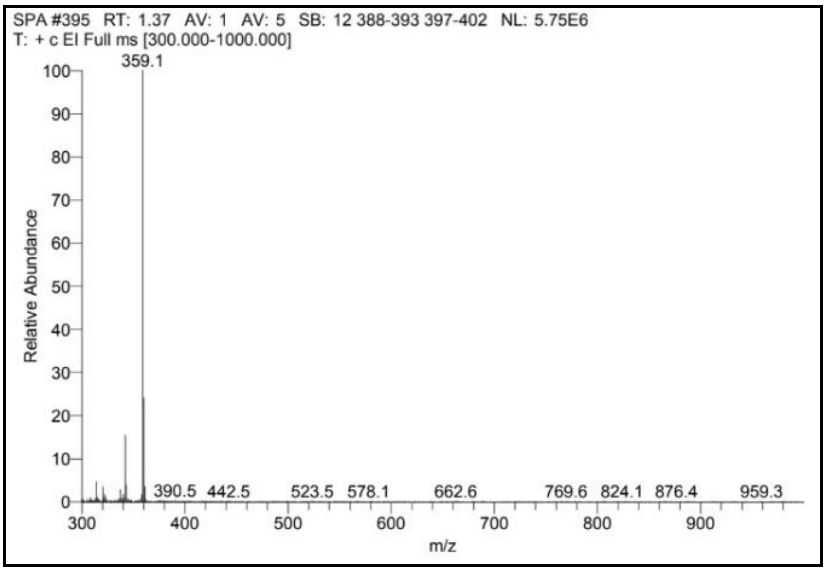


Figure 3.3.1(33): Mass spectrum of SPA

12. Compound name: SMO

Mol. formula: $C_{47}H_{77}N_{11}O_7$

Calculated mass: 907.60 (M)

Observed mass: 908.5 $(M+H)^+$

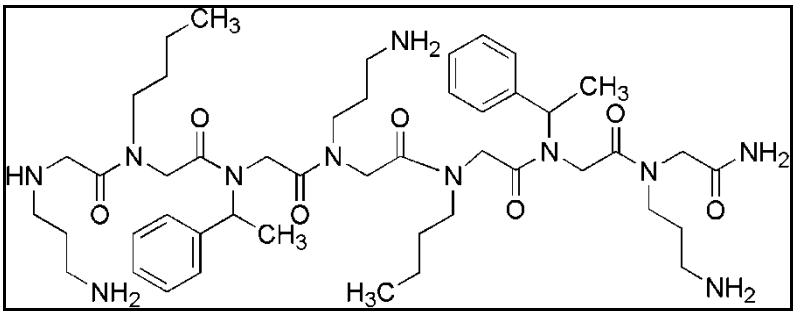


Figure 3.3.1(34): Chemical structure of SMO

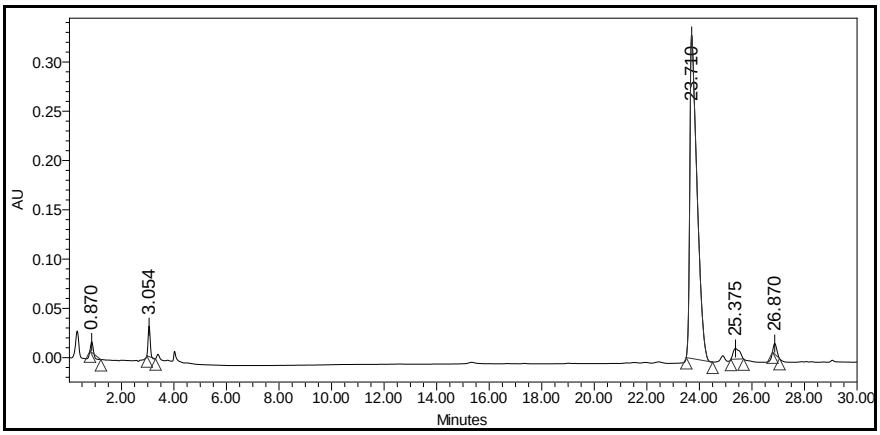


Figure 3.3.1(35): HPLC chromatogram of SMO

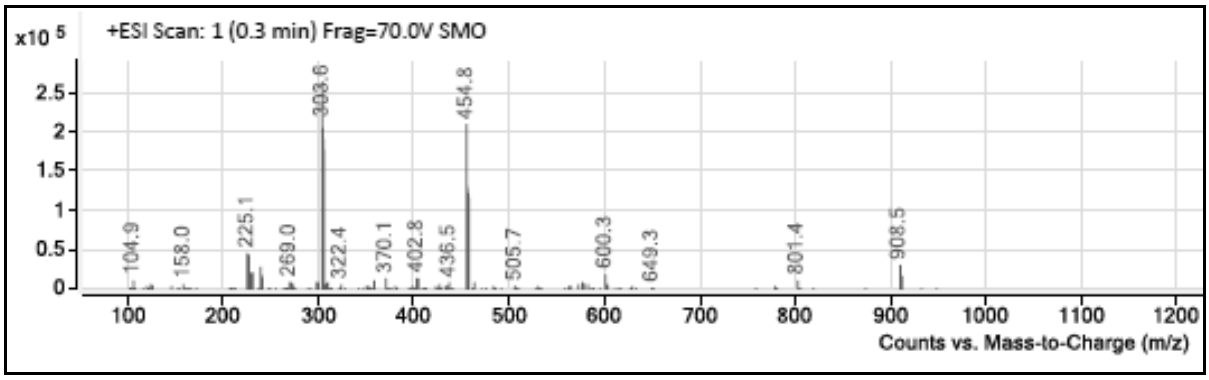


Figure 3.3.1(36): Mass spectrum of SMO

13. Compound name: SPO

Mol. formula: $C_{79}H_{123}N_{17}O_{12}$

Calculated mass: 1501.95 (M)

Observed mass: 752.35 $(M+2H)^{2+}$

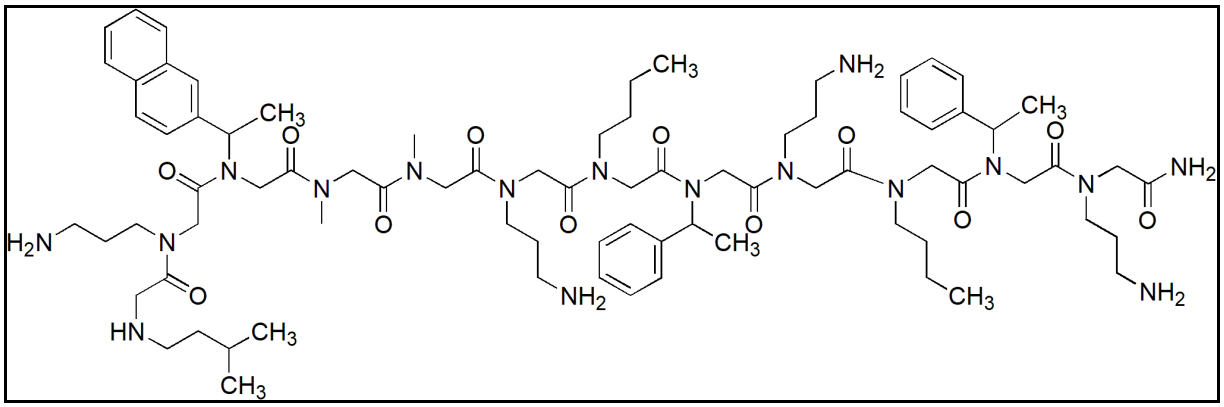


Figure 3.3.1(37): Chemical structure of SPO

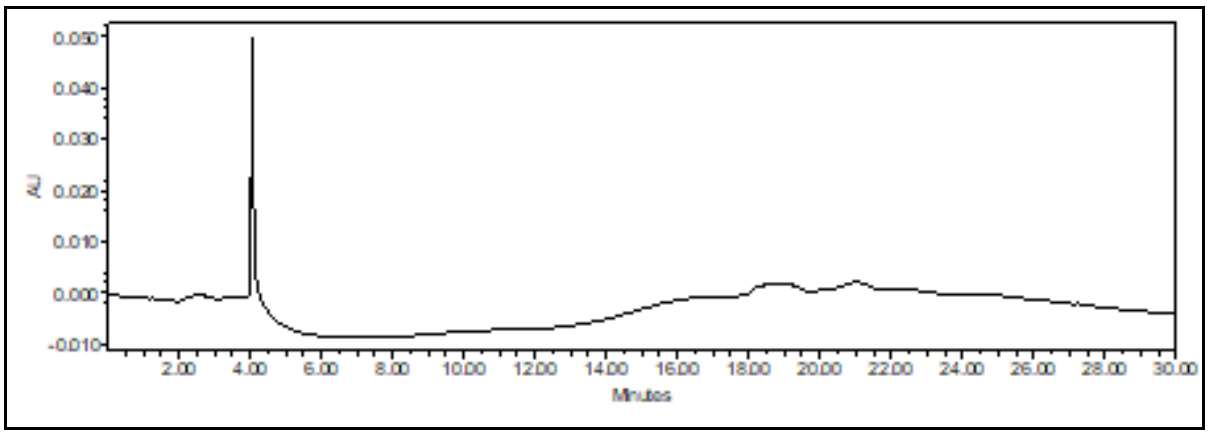


Figure 3.3.1(38): HPLC chromatogram of SPO at 280 nm

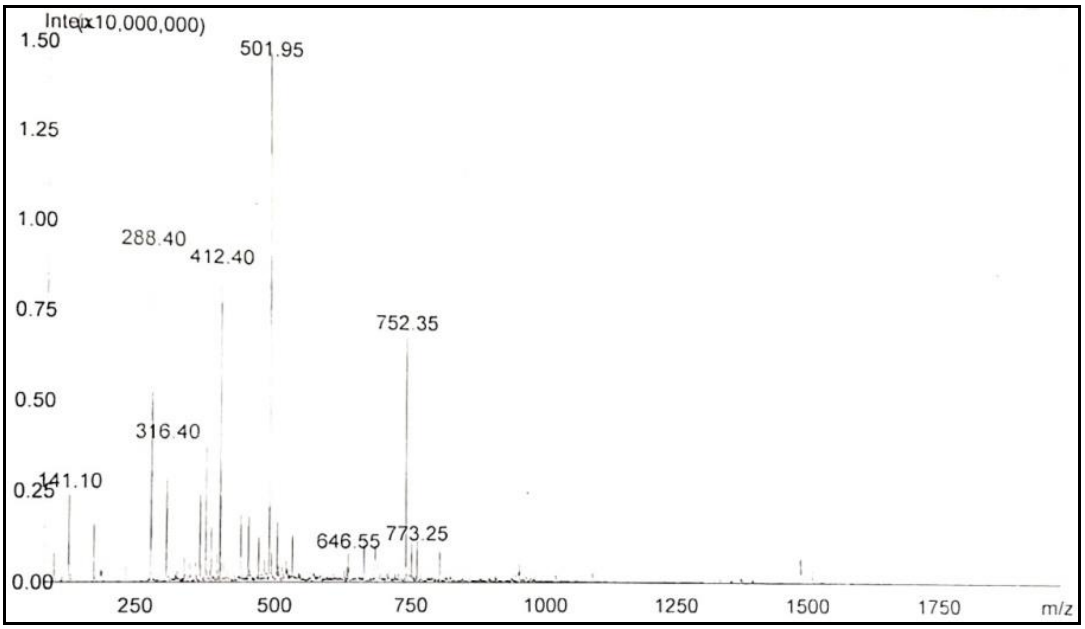


Figure 3.3.1(39): Mass spectrum of SPO

14. Compound name: SA4

Mol. formula: $C_{72}H_{112}N_{18}O_{12}$

Calculated mass: 1420.87 (M), 1534.89 $(M+TFA)^+$

Observed mass: 1535.05 $(M+TFA)^+$

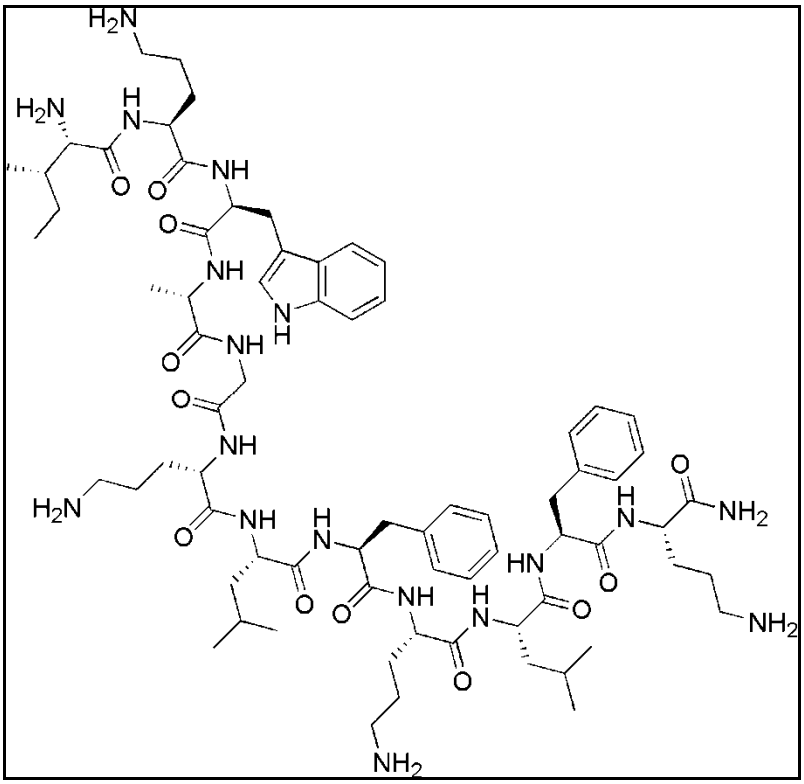


Figure 3.3.1(40): Chemical structure of SA4

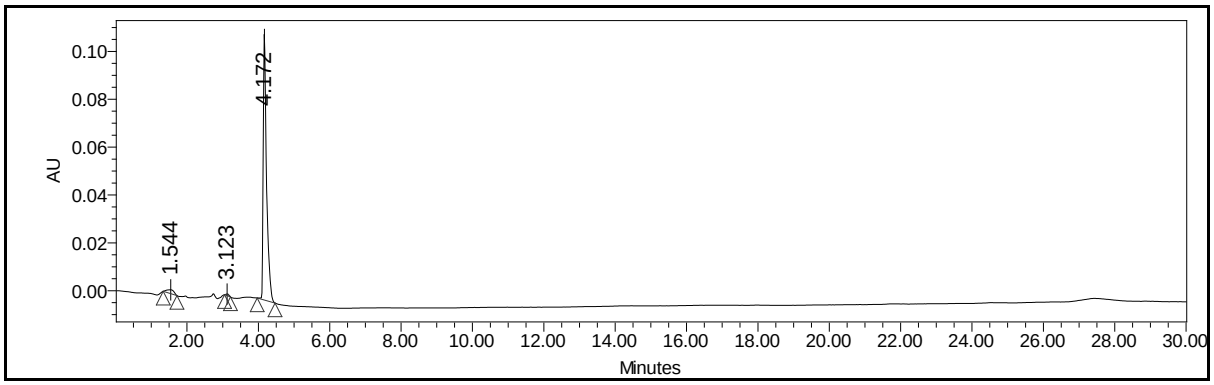


Figure 3.3.1(41): HPLC chromatogram of SA4 at 280 nm

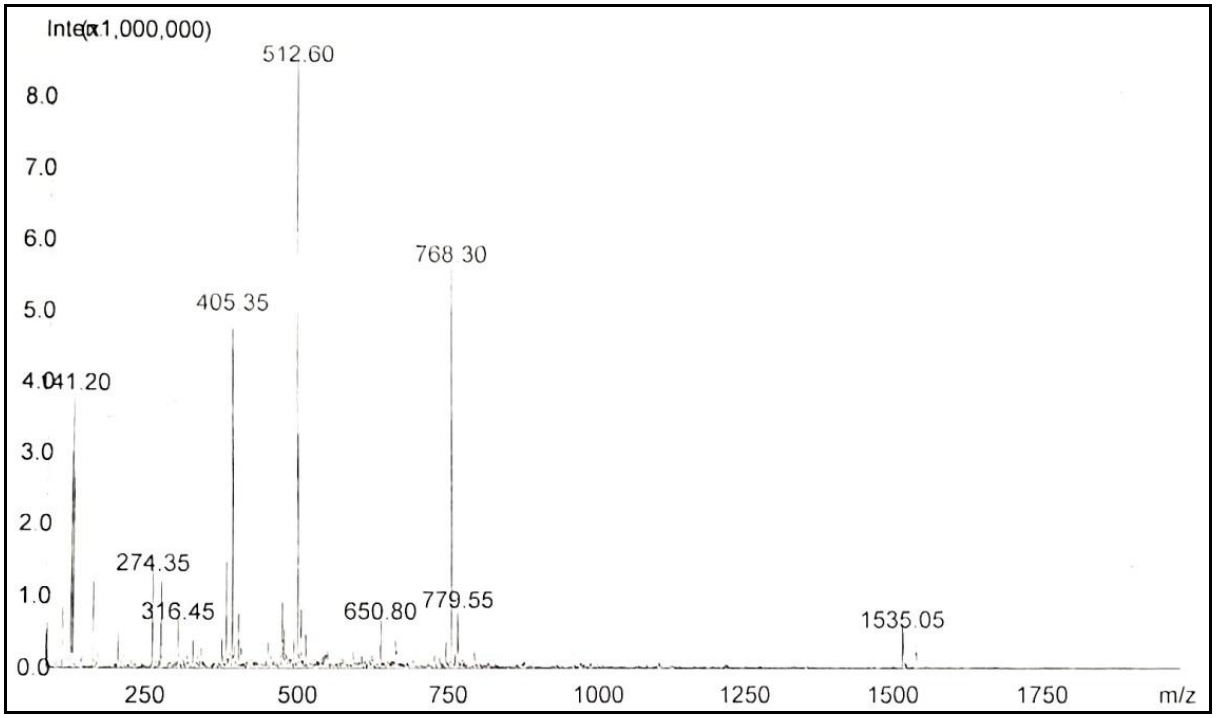


Figure 3.3.1(42): Mass spectrum of SA4

3.3.2 Preliminary biological evaluation

Antibacterial compounds that are toxic to bacterial cells and non-toxic to mammalian cells are always desirable. Therefore, for the initial biological evaluation of synthesized compounds, antibacterial activity and hemolytic toxicity study were carried out. Results of antibacterial activity against bacterial strains (**Table 3.3.2a, Table 3.3.2b)** suggested that most active compounds against Gram-positive bacteria were ultra-short peptide-based compounds (LP-16, DP-16, LP-23, and DP-23) while most active compounds against Gram-negative bacteria were short peptide-based compounds (SA4 and SPO). MIC of LP-23 and DP-23 against *M. smegmatis* was 6.25 µg/ml, while SPO and SA4

emerged as the lead compounds against *A. baumannii* strains with MIC ranging from 50-100 µg/ml against these strains.

Further results of HC_{50} values (**Table 3.3.2c**) of synthesized compounds at the highest evaluated concentration (250 µg/ml) against human erythrocytes suggested that all compounds possessed low toxicity (HC_{50} >250 µg/ml) to human erythrocytes except LP-24 and DP-24. HC_{50} value of LP-24 and DP-24 against hRBCs was found to be 250 µg/ml. Among all ultra-short peptide-based compounds that contained 4 ornithine amino acids attached to different fatty acid chains (stearic, palmitic and myristic acids), LP-24 and DP-24 emerged as the most hemolytic compounds. Since these two compounds contained stearic acid as a fatty acid chain and stearic acid is the most hydrophobic fatty acid among all hydrophobic acids attached. This might be the reason for the high hemolytic toxicity of LP-24 and DP-24.

Overall results of antibacterial activity and hemolytic toxicity of these compounds indicated that the majority of compounds tested were more toxic to bacterial cells in comparison to mammalian cells. The selectivity of these peptides may be attributed to the fact that the surface of bacterial membranes is negatively charged and synthesized peptides were rich in cationic charges; hence, displayed more selectivity towards bacteria. This result is supported by another study, in which bacteria rich in anionic lipids were found to be are more susceptible to cationic peptide [203]. Secondly, the outer membranes of bacterial cell walls also contain anionic molecules, while the outer leaflets of mammalian cells are mainly composed of zwitterionic substances [204]. Additionally, mammalian cells are rich in cholesterol, which acts as a membrane-stabilizing substance and protects the mammalian cells from attack by AMPs [100].

Table 3.3.2a: Antibacterial activity of synthesized compounds

S. No	Compound	MIC (µg/ml)		
		***B. subtilis* MTCC 121**	***M. smegmatis* MC ²155**	***A. baumannii* ATCC 19606**
1	LP-16	3.1	>50	100
2	DP-16	3.1	50	>100
3	LP-23	6.25	6.25	100
4	LP-24	12.5	50	>100
5	DP-23	6.25	6.25	>100
6	DP-24	6.25	25	>100
7	SMO	25	ND	>100
8	SPA	12.5	ND	100
9	SPO	50	50	100
10	SA4	12.5	50	100
11	VAN	ND	12.5	ND
12	TET	0.7	ND	0.35
13	MER	ND	ND	0.75

VAN; Vancomycin, TET; Tetracycline HCl, MER; Meropenem, ND; Not determined

Table 3.3.2b: MIC of lead compounds against MDR clinical isolates of *A. baumannii*

S. No	Compound	MIC (µg/ml) against *A. baumannii* strains			
		MDR1	**MDR2**	**MDR3**	**MDR4**
1	SA4	50	50	50	50
2	SPO	50	50	50	50
3	MER	>256	>256	>256	>256

Table 3.3.2c: Hemolytic toxicity of synthesized compounds

Compound	LP-16	DP-16	LP-23	LP-24	DP-23	DP-24	SMO	SPA	SPO	SA4
HC_{50} (µg/ml)	> 250	> 250	> 250	250	> 250	250	> 250	> 250	> 250	> 250

3.4 SUMMARY (CHAPTER 3)

A series of peptide-based compounds were synthesized on Rink amide MBHA resin using SPS methods. All compounds were characterized by RP-HPLC and mass spectrometry. Characterized compounds were screened against various bacterial strains, such as *M. smegmatis* MC^2155, *B. subtilis* MTCC 121, *A. baumannii* (ATCC 19606), and *A. baumannii* MDR clinical isolates (MDR1, MDR2, MDR3, and MDR4). Further, the hemolytic toxicity of synthesized compounds was carried out in human erythrocytes. Based on the results of hemolytic toxicity and antibacterial activity of all screened compounds, four lead compounds (LP-23, DP-23, SPO and SA4) were obtained. LP-23 and DP-23 emerged as the lead compounds against *M. smegmatis* while SA4 and SPO emerged as the lead compounds against *A. baumannii* bacterial strains. Screened lead compounds were further subjected to various *in-vitro* biological studies to determine their therapeutic potential. The *in-vitro* biological studies that were undertaken have been described in upcoming chapters (**Chapter 4** and **Chapter 5**), where **Chapter 4** deals with *in-vitro* biological evaluation of LP-23 and its poly-*N*-substituted glycine congener DP-23 while **Chapter 5** deals with biological evaluation of SA4 and its poly-*N*-substituted glycine congener SPO.

CHAPTER 4

IN VITRO BIOLOGICAL EVALUATION OF LEAD COMPOUNDS LP-23 AND DP-23 AGAINST *M. SMEGMATIS*

4.1 INTRODUCTION

Tuberculosis is amongst the major cause of public health concern worldwide [205]. Its causative agent, *M. tuberculosis* has the ability to escape mechanisms that provide immunity to humans [206]. It can survive within the unfavorable conditions offered by macrophages of host and hence, is able to infect the host [207]. Most of the conventional antibacterial drugs have become ineffective against *M. tuberculosis.* Therefore alternative therapeutic strategies are required to deal with this problematic issue. One such alternative strategy is the utilization of AMPs; research data of the last decade indicate that antimicrobial peptides have broad-spectrum of activity, rapid killing effect, multiple mechanisms of actions, and potentially low-resistance rate when compared to conventional antibiotics [173]. There are numerous AMPs but only a few of them have been tested against mycobacteria [4].

AMPs are components of the immune system and play a vital role in the protection of living organisms from bacterial pathogens [5]. MOA of AMPs has a close correlation with their net positive charge and hydrophobicity [69] [70]. As AMPs have not been extensively explored as antibacterial compounds against mycobacteria, thus its MOA against mycobacteria is still unclear [9]. Though, a study shows that synthetic cationic AMPs modified with different hydrophobic amino acids act via a lytic mechanism on the mycobacterial membrane [10]. Synthetic analogs of short cationic AMPs have shown promising results against *M. tuberculosis* [7]. Few AMPs even have the ability to kill susceptible and drug-resistant *M. tuberculosis* [8]. In spite of all these advantages, the utilization of AMPs as therapeutic agents is limited due to poor proteolytic stability [11]. Enormous efforts have been made to enhance the proteolytic stability of peptides, and designing their *N*-substituted glycine congeners (peptoids) is one such approach. Peptoids belong to the class of compounds that are prepared by the submonomer SPS by sequential haloacetylation and subsequent displacement of halogen with an amine.

Peptoids not only mimic peptides but they are more resistant to proteolysis than peptides [12].

Since *M. tuberculosis* is highly pathogenic; therefore, *Mycobacterium smegmatis* is commonly used as its substitute for *in vitro* biological studies [208]. Few advantages associated with *M. smegmatis* are its fast-growing and non-pathogenic nature, which makes this bacterium suitable for *in vitro* studies. Moreover, this bacterium shares several features with pathogenic *M. tuberculosis;* hence, used as a model organism for antitubercular studies [209]. Based on this fact, we screened our synthesized peptide-based compounds against this model organism. Among all tested compounds, LP-23 and DP-23 emerged as the most potent compounds against *M. smegmatis*. LP-23 (**Figure 4.1a**) is a lipopeptide made up of three ornithine residues and palmitic acid as lipid moiety. While DP-23 (**Figure 4.1b**) is a poly-*N*-substituted glycine congener based on LP-23, where 1,3-diaminopropane was used as a mimetic of *N*-ornithine. To evaluate whether DP-23 retained the antibacterial activity of LP-23; resazurin reduction assay against *M. smegmatis* was carried out. Further proteolytic stability of these two compounds was evaluated in human serum and the effect of these leads on the morphology of *M. smegmatis* was determined via scanning electron microscopy (SEM) analysis.

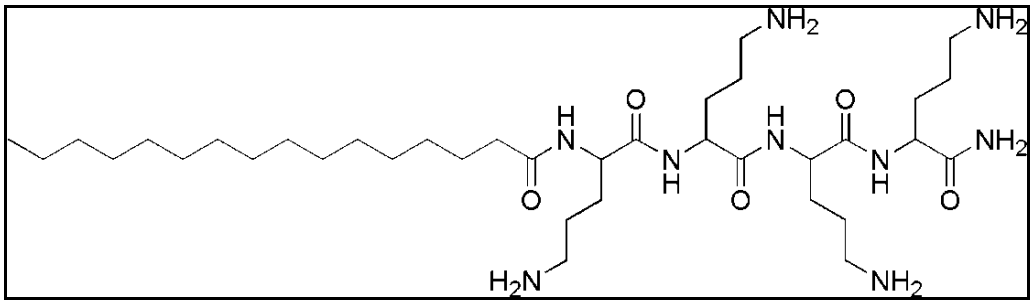


Figure 4.1a: Lipopeptide LP-23

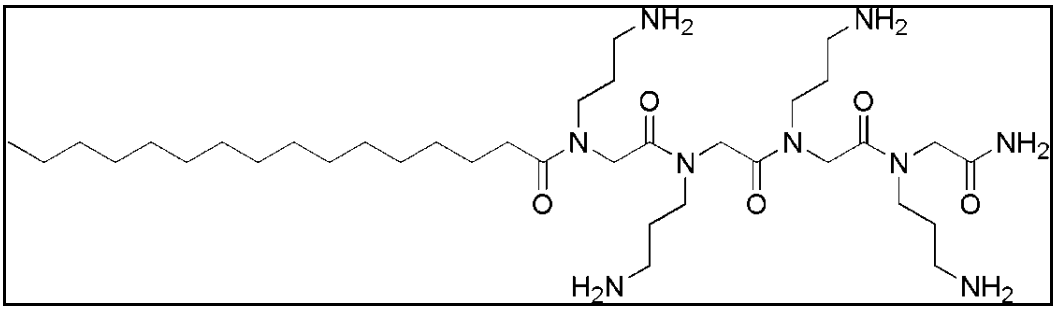


Figure 4.1b: Poly-*N*-substituted glycine congener of LP-23

4.2 MATERIALS AND METHODS

4.2.1 Materials

Resazurin sodium salt was purchased from Sigma, India. Middlebrook MB7H9, RPMI medium, nutrient agar, vancomycin, glutaraldehyde, sodium chloride, Triton X-100, trichloroacetic acid (TCA), and trisodium citrate were purchased from HiMedia, India. Potassium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate were purchased from Loba Chemie, India. Ethanol, HPLC grade TFA, and HPLC grade ACN were purchased from Merck, India.

4.2.2 Antibacterial activity and hemolytic toxicity

4.2.2.1 Resazurin reduction assay of screened leads against *M. smegmatis*

Leads (peptide LP-23 and peptoid DP-23) from preliminary antibacterial screening were subjected to resazurin reduction assay against *M. smegmatis* [210]. Overnight grown *M. smegmatis* culture was adjusted to 0.5 McFarland standards, diluted 20-fold and placed into 96-well microtiter plates and incubated for 72 h. Resazurin dye was added into culture and kept in the incubator at 37 °C for another 24 h. After 24 h of incubation, the plate was observed for color changes. The reduction of resazurin to resorufin was observed in the form of color change. The pink color indicated the presence of viable bacterial cells. Results were obtained in triplicates. The lowest concentration of LP-23 and DP-23 that prevented color change was considered as MIC of the tested compound.

4.2.2.2 Hemolytic toxicity study

Erythrocytes from healthy human volunteer were collected and washed thrice in normal saline using centrifuge at 2000 rpm for 10 min. 100 µl of diluted suspension of erythrocytes in 0.9% NaCl was added to 100 µl of diluted peptide based compound in normal saline. These vials were kept in the incubator for 1 h at 37 °C. Subsequently, the contents of the well were centrifuged to separate supernatant and the optical density (OD) of supernatant was recorded at 570 nm. These OD values were further used to calculate percent hemolysis caused by test compounds [202]. Suspension of human red blood cells (hRBCs) with normal saline was used as a blank and suspension of hRBCs incubated with 1% Triton X-100 was used for 100% hemolysis (positive control). Following formula was used to calculate % hemolysis:

Percent hemolysis= 100(TC- B)/ (TX- B)

Where B stands for absorbance of supernatant from blank vial, TC stands for absorbance of supernatant from test compound vial, and TX stands for absorbance of supernatant from triton X treated vial.

4.2.3 Serum stability study

A 25% v/v suspension of human serum was prepared in RPMI medium. To this suspension, stock solution of test compounds (LP-23 and DP-23) was added to get a final concentration of 50 μg/ml. 100 μl of this reaction mixture was removed and its serum proteins were precipitated with 200 μl of 6% TCA solution. A cloudy reaction mixture was formed, which was cooled and further centrifuged at 18000 g for 2 min. Supernatant was analyzed at 220 nm by RP-HPLC on C18 column. Area under curve (AUC) at 0 h was assumed as 100% intact peptide [211].

4.2.4 Effect of lead compounds on the growth of bacteria

Effect of DP-23 and LP-23 on the growth of *M. smegmatis* was studied as per resazurin reduction assay [212]. Overnight grown *M. smegmatis* culture was diluted, 100 μl of this culture was added to the equal volume of diluted solution of LP-23 and DP-23 in MB7H9 broth to attain 8×MIC as final concentration. The plate was incubated at 37 °C for 48 h, followed by addition of resazurin dye. The plate was further incubated at 37 °C for 24 h. OD was measured at 620 nm at different time points. OD of untreated and treated *M. smegmatis vs*. time was plotted. The experiment was performed in triplicates and readings are expressed as mean ± SD.

4.2.5 Effect of lead compounds on bacterial cell morphology

SEM was used to visualize the membrane damaging effects of lead compounds on *M. smegmatis* cell morphology as per the method reported previously [213]. Briefly, 10^8-10^{10} CFU/ml of bacterial culture was incubated with supra-MIC of lead compounds in small glass tubes at 37 °C. PBS treated bacterial cultures served as a negative control. After 0.5 h of incubation, the contents of these tubes were centrifuged to pellet down bacterial cells. The pellet was washed further three times with PBS. Supernatant was discarded and the formed pellet was fixed with glutaraldehyde. Extensive washings were given with phosphate buffer saline (PBS); bacterial samples were dried with increasing percentage of aqueous ethanol and subjected to SEM imaging.

4.3 RESULTS AND DISCUSSION

4.3.1 Determination of MIC by resazurin reduction assay against *M. smegmatis*

Resazurin reduction assay is a visual method that is used to determine the cell viability. Solution of resazurin in physiological buffers is deep blue in color; this solution is directly added to cells in a culture. Conversion of blue colored resazurin into pink colored resorufin indicates presence of viable bacterial cells [214-217]. The lowest concentration of test compound that prevented the formation of pink color was considered as MIC. Results of the resazurin reduction assay (**Figure 4.3.1**) indicated that the MIC of both compounds against *M. smegmatis* was 6.25 µg/ml. The same MIC of both compounds suggested that the poly-*N*-substituted glycine congener DP-23 retained the antibacterial activity of parent peptide LP-23. It may be because of the equal number of cationic charges and similar chain length of fatty acid present in the structures of these compounds.

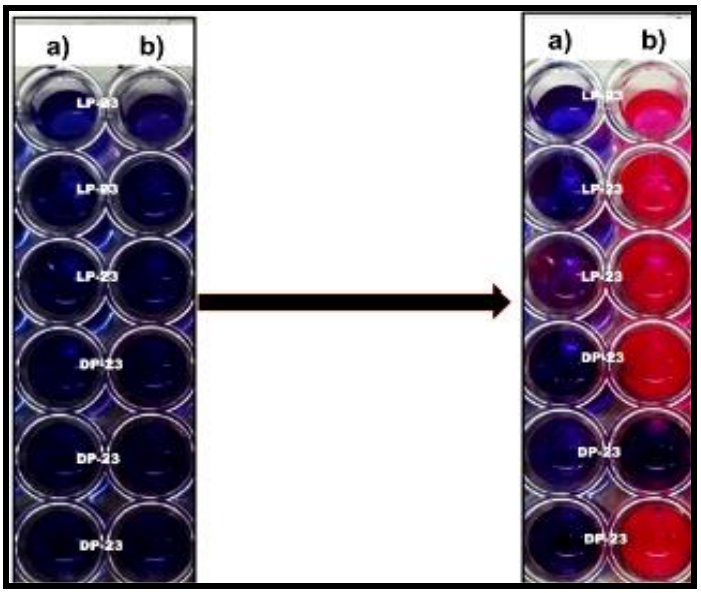


Figure 4.3.1: Reduction of resazurin dye by DP-23 and LP-23 against *M. smegmatis*
[**a well** (6.25µg/ml) and **b well** (3.125µg/ml), to the left of arrow indicate resazurin before incubation; to the right of arrow indicate color change after incubation]

4.3.1.2 Hemolytic toxicity

Hemolytic toxicity of AMPs is carried out to determine cytotoxic effects of peptides on mammalian cells, with this in view hemolytic toxicity of LP-23 and DP-23 was carried out against human erythrocytes. Preliminary hemolytic toxicity study of LP-23 and DP-23, as mentioned in **Chapter 3** suggested that both compounds have HC_{50}>250 µg/ml.

Therefore to find out HC_{50} of LP-23 and DP-23, hemolytic toxicity study of these compounds was carried out at concentrations ranging between 250-500 µg/ml; HC_{50} values (**Table 4.3.1**) of LP-23 and DP-23 against human erythrocytes were found to be 290 (µg/ml) and 390 (µg/ml), respectively. Results of this study show that both compounds were less toxic to mammalian cells in comparison to bacterial cells. These results are in accordance with results of Armas *et al.*, 2019, where ultra short cationic lipopeptides were found to be more selective towards bacteria [218].

Table 4.3.1: HC_{50} values of lead compounds LP-23 and DP-23

S. No	Peptide	Sequence	MIC (µg/ml)	HC_{50} (µg/ml)
1.	LP-23	$CH_3(CH_2)_{16}CO$-NH-OOOO-NH_2	6.25	290
2.	DP-23	$CH_3(CH_2)_{16}CO$-NH-nOnOnOnO-NH_2	6.25	390

4.3.2 Serum stability study

Serum stability study is used to evaluate the ability of compounds to resist proteolytic degradation in serum, as proteolytic degradation of peptide-based drugs is often considered as one of the major limitations associated with systemic therapeutic applications [219]. Therefore, huge efforts are typically devoted to stabilize sequences against proteases present in serum or plasma. Both compounds lipopeptide (LP-23) and its peptoid (DP-23) were evaluated for their stability to proteolysis in human serum.

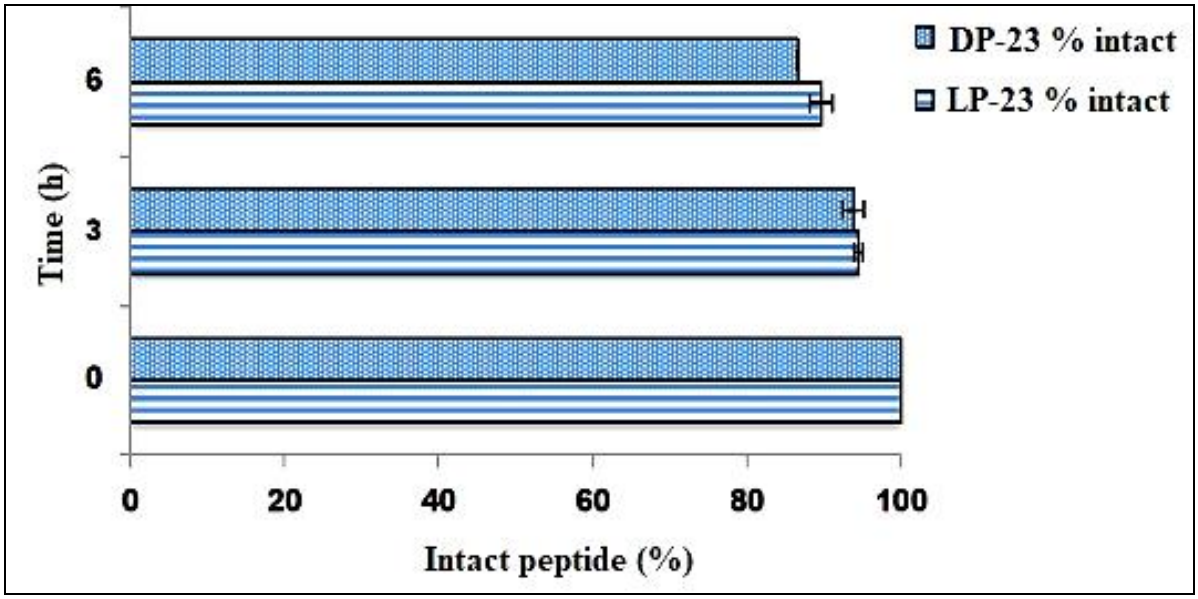


Figure 4.3.2: Stability of DP-23 and LP-23 in human serum; the results are presented as mean±SD.

Percent degradation of both leads at the end of the 6th h of incubation was <20% (**Figure 4.3.2**). The reason for stability of these compounds lies in the fact that LP-23 contains non-natural amino acid ornithine, whereas in case of DP-23 the reason could be attributed to stable form of amide bond due to shifting of side chain on to the nitrogen atom of the amide bond instead of usual position of side chain on the α-carbon [115, 220].

4.3.3 Effect of LP-23 and DP-23 on growth kinetics of bacteria

Effect of lead compounds and standard drug vancomycin on the growth kinetics of *M. smegmatis* was evaluated using resazurin reduction method. From 24th h (**Figure 4.3.3**) onwards till 48 h there were only slight increase in OD values of bacterial culture treated with LP-23, DP-23 and vancomycin. LP-23 and DP-23 were able to arrest the growth of bacterial culture even at 48th h of incubation.

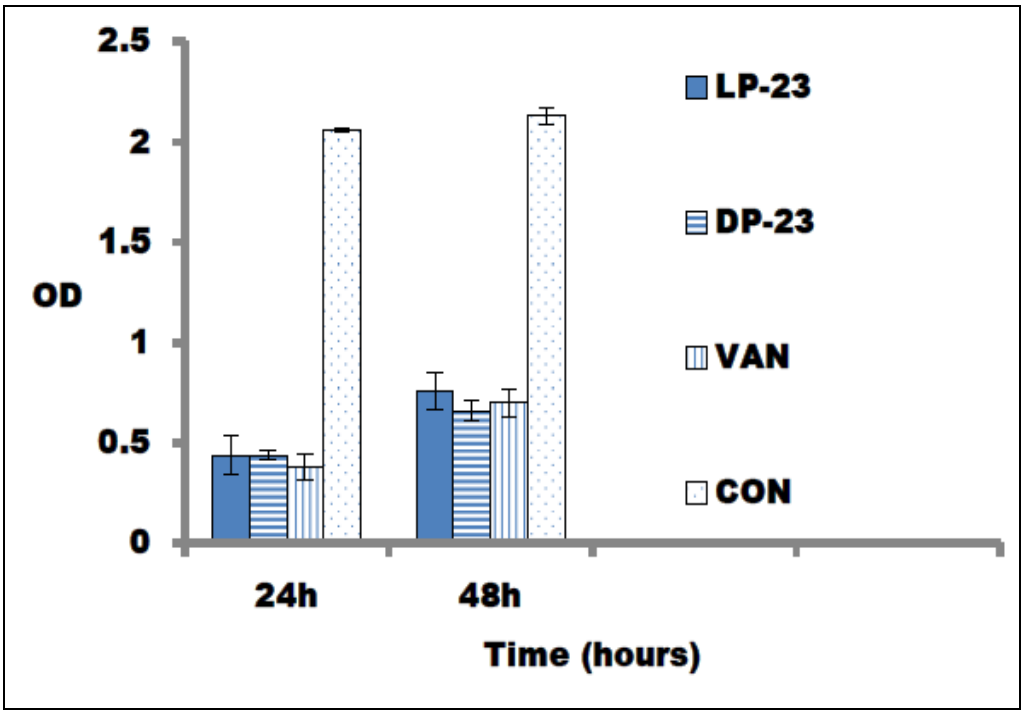


Figure 4.3.3: Growth kinetics spectrum of *M. smegmatis* treated with LP-23, DP-23, vancomycin and normal saline as a control; the results are presented as mean±SD.

It was observed that growth kinetics spectrum of *M. smegmatis* treated with LP-23 and DP-23 was comparable. The reason could be the fact that side chain made up of *N*-1,3-diaminopropane in DP-23 has a closer resemblance to the side chain formed by *N*-ornithine in LP-23, and these ultra-short peptide-based compounds were made up of

repeating units of single type of amino acid or amine, *i.e.*, ornithine or 1,3-diaminopropane. Moreover, the number of cationic charges and type of lipid moiety present in the chemical structures of LP-23 and DP-23 are also the same.

4.3.4 Effect of LP-23 and DP-23 on cell morphology of bacteria

AMPs are known to induce morphological changes in bacteria and SEM is a commonly used technique that is used to visualize such morphological changes [213]. Therefore, the effects of LP-23 and DP-23 on morphology of *M. smegmatis* were visualized by SEM. Both compounds were found to alter the morphology of *M. smegmatis* in comparison to control (**Figure 4.3.4a, Figure 4.3.4b** and **Figure 4.3.4c**).

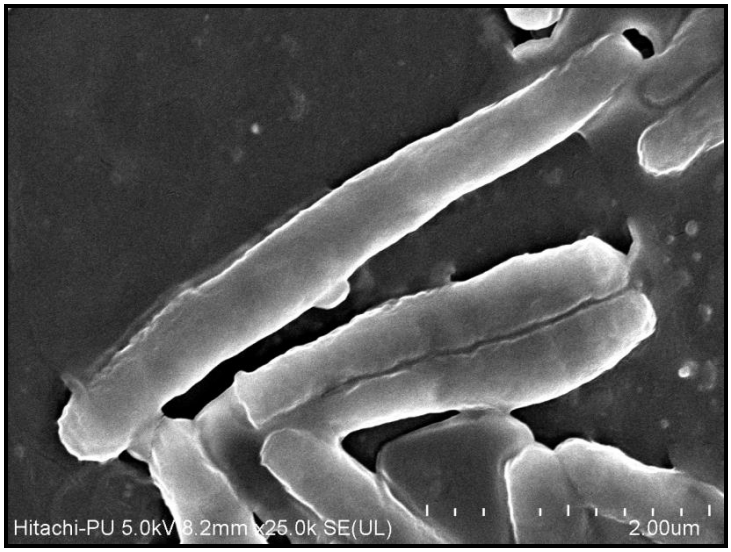


Figure 4.3.4a: SEM of normal saline treated *M. smegmatis* cells

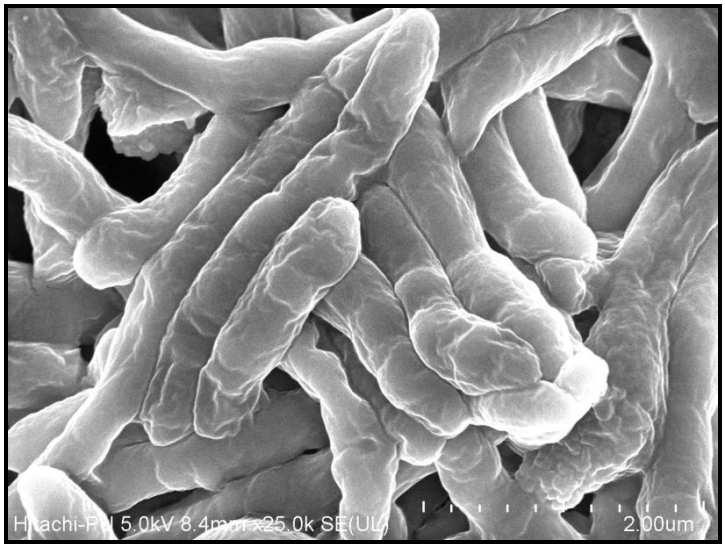


Figure 4.3.4b SEM of LP-23 treated *M. smegmatis* cells

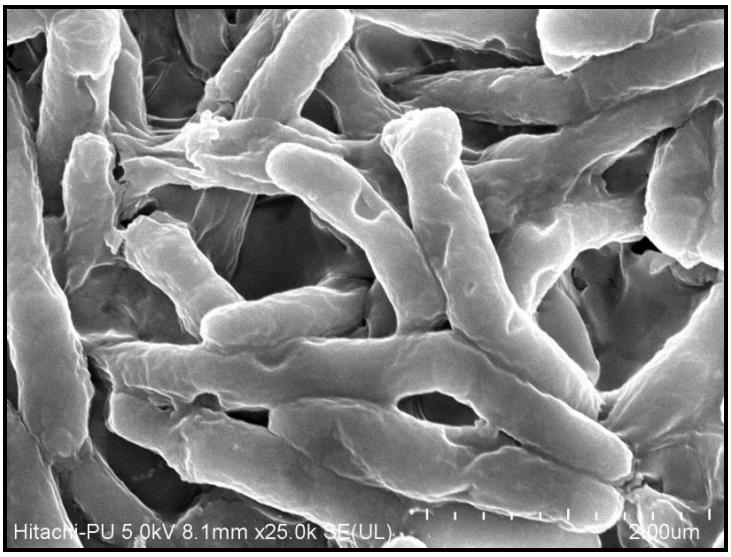


Figure 4.3.4c: SEM of DP-23 treated *M. smegmatis* cells

Various pores were observed on the surfaces of *M. smegmatis* cells treated with DP-23 (**Figure 4.3.4c**), while in case of LP-23 treated *M. smegmatis* cells, roughness in outer surfaces of bacterial cells was observed (**Figure 4.3.4b**). *M. smegmatis* cells treated with LP-23 and DP-23 were irregular in shapes in comparison to normal saline treated bacterial cells. Normal saline treated cells (**Figure 4.3.4a**) appeared to be smooth in comparison to LP-23 and DP-23 treated bacterial cells. Similar to this, it has been observed that host defense peptides (HDPs) at concentrations higher than MIC have the potential to disrupt the cell membranes of bacteria. These membrane disruption effects, like altered membrane surfaces or formation of pores have been microscopically observed for membrane-active HDPs via electron microscopy [221, 222].

4.4 SUMMARY (CHAPTER 4)

M. tuberculosis is one of the most problematic pathogens worldwide. Majority of the conventional antibiotics have become ineffective against this bacterium. Therefore, new therapeutic agents are urgently required against this pathogen. Since this pathogen is highly infectious; therefore, *M. smegmatis*, a non-pathogenic mycobacterium, which is generally used as a standard model organism for research purposes was utilized for evaluation of antibacterial activity of synthesized peptide-based compounds. In this study, we carried out the antibacterial evaluation of two peptide-based compounds LP-23 and DP-23 against *M. Smegmatis* by resazurin reduction assay. The MIC of both LP-23 and DP-23 against *M. smegmatis* against was found to be 6.25 μg/ml. Further for determining the toxic potential of these compounds against mammalian cells, hemolytic toxicity study was carried out using human erythrocytes. Results suggested that both compounds were more selective towards bacteria in comparison to mammalian cells. To determine the stability of these compounds against proteolysis, a serum stability study was carried out. Results of this study suggested that peptide LP-23 and its poly-*N*-substituted glycine congener DP-23 were stable to proteolysis in human serum. Mode of action of these compounds against *M. smegmatis* was visualized using SEM. From SEM results it may be proposed that mode of action of these compounds against *M. smegmatis* is membrane disruption.

CHAPTER 5

IN VITRO BIOLOGICAL EVALUATION OF LEADS SA4 AND SPO AGAINST *A. BAUMANNII*

5.1. INTRODUCTION

Acinetobacter baumannii is a causative agent of hospital-acquired infections and has emerged as one of the major global issues [223], [224]. *A. baumannii* has the ability to form biofilms on living as well as non-living materials [225]. This ability of *A. baumannii* helps it to survive in unfavorable environment [226]. Phenotypes associated with biofilms are intrinsically resistant to antibacterial drugs [227]. It is a common phenomenon for most of the nosocomial pathogens to produce biofilms. Biofilm production complements disease pathogenicity as well as resistance mechanisms [228] [229].

Few biofilm-producing *A. baumannii* have become resistant to nearly all antibiotics in comparison to non-biofilm-producing *A. baumannii*. Biofilm forming bacterial cells produce a protective coating around them, thus impairing the action of antibiotics [230]. The presence of dormant cells, slow bacterial growth within the biofilm, and adaptableness to stressful conditions contribute to resistance in biofilm-producing organisms [231] [232].

For *A. baumannii species*, the emergence of multidrug resistance to common classes of antibiotics, like aminoglycosides, fluoroquinolones, penicillins, and cephalosporins has increased over the years due to wide use of these antibiotics [233] [234]. Great efforts have been spent on the discovery of novel antibacterials active against multidrug-resistant (MDR) bacteria. Presently, numerous AMPs are under clinical trials as alternative strategies against MDR pathogens [91] [235] [236].

In comparison to traditional antibiotics, AMPs possess various properties that make them suitable candidates as alternative antibiotics. For instance, AMPs act by diverse mechanisms, such as membrane disruption, inhibitory action on intracellular targets, and impairment of key cellular processes [35] [57] [77]. This diversity in MOA reduces the propensity of resistance development in pathogens in comparison to most conventional antibiotics that only act on distinct targets unless used in combination [237] [238]. In

addition, most AMPs exhibit a broad spectrum of activity against bacteria, fungi, and viruses [239] [240] [241] and are rapid-acting agents [91] [242].

Colistin is an important last-resort peptide drug used against various MDR nosocomial pathogens. Though its use is limited to some extent due to its undesirable side effects [83] [243] [244], such as nephrotoxicity and neurotoxicity [83] [84]. It has been observed that some strains exhibit low susceptibility to colistin, thus toughens this war against MDR strains [245] [246] [84]. Hence, novel therapeutic agents that have the ability to fight against biofilm-forming MDR *A. baumannii* pathogens are required. In this study, we designed various peptide-based compounds and screened them against different *A. baumannii* strains.

From preliminary antibacterial screening, two lead compounds SA4 and SPO were obtained. SPO is a poly-*N*-substituted glycine congener of cationic and ampiphathic peptide SA4. In order to evaluate the therapeutic potential of these lead compounds, different *in vitro* biological studies were carried out, which include biofilm inhibition assay, biofilm eradication assay, and serum stability study. In addition, the effect of SA4 and SPO on the cell morphology of *A. baumannii* was also studied.

5.2. MATERIALS AND METHODS

5.2.1. Antibiotics

Meropenem and tetracycline HCl were used as the reference standards.

5.2.2. Bacterial strains

A. baumannii ATCC 19606 and different MDR clinical isolates of *A. baumannii* (MDR1-MDR4) were used; see appendix for details of MDR clinical isolates (**Appendix A**).

5.2.3. Biofilm inhibition assay

Biofilm inhibition activity of synthesized compounds was determined with slight modifications in the crystal violet staining method [247]. Overnight grown culture of bacteria was adjusted to 0.5 McFarland standards with normal saline. This was further diluted to 20-fold using MHB medium. 180 µl of this culture was mixed with 20 µl of varying concentration of compounds to achieve 100 to 3.125 µg/ml of test compound within the wells. The plate was kept in the incubator for 24 h at 37 ˚C to allow biofilm

formation. Un-inoculated MHB medium was used as the negative control while tetracycline HCl and meropenem treated cultures served as the drug control. Plates were taken out from incubator after 24 h and washed three times with normal saline to remove planktonic cells. The biofilms formed were stained with 200 µl of crystal violet solution for 20 min at 37 °C and wells were washed gently with PBS to remove excess dye. This step was followed by the addition of 200 µl of 95% ethanol in water into each well. The optical density of each well was measured at 570 nm using multiskan plate reader to calculate percent inhibition. Results were expressed as the mean ±SD of triplicates.

5.2.4. Biofilm eradication assay (BEA)

BEA was carried out in polystyrene 96-well microtiter plates [248]. Overnight grown *A. baumannii* bacterial cultures were adjusted to approximately 5×10^6 CFU/ml. 100 µl/well of adjusted cultures of bacteria were incubated at 37 °C for 24 h for the formation of biofilms. On the subsequent day, these biofilms were washed with PBS to get rid of planktonic cells and then incubated with 6 mg/ml concentrations of synthesized compounds diluted in fresh MHB medium for another 24 h at 37 °C under static conditions. After 24 h of incubation, biofilms were washed twice and fresh MHB was added and incubated for another 24 h. On the next day, the biomass formed was washed twice with PBS and further stained with 100 µl of crystal violet solution for 20 min at 37 °C and wells were washed gently with PBS to remove excess dye. 100 µl of 96% ethanol in water was added to wells for 10 minutes and OD values of these wells were obtained using multiskan reader at 570 nm. The minimum concentration of tested compounds required for biofilm eradication was determined as per Ceri *et al.*, 1999 [248].

5.2.5. Hemolytic toxicity study

Erythrocytes from healthy human volunteer were collected and washed thrice in normal saline using centrifuge at 2000 rpm for 10 min. 100 µl of diluted suspension of erythrocytes in normal saline was added to 100 µl of the diluted compound in normal saline to obtain particular concentrations of peptides and peptoids. These vials were kept in an incubator for 1 h at 37 °C. Subsequently, the contents of the well were centrifuged to separate supernatant and the OD value of supernatant was recorded at 570 nm. These OD values were further used to calculate percent hemolysis caused by test compounds [202]. Suspension of human red blood cells (hRBCs) with normal saline was used as

blank and suspension of hRBCs incubated with 1% Triton X-100 was used for 100 % hemolysis (positive control). Following formula was used to calculate % hemolysis:

Percent hemolysis= 100(TC- B)/ (TX- B)

Where B stands for absorbance of supernatant from blank vial, TC stands for absorbance of supernatant from test compound vial and TX stands for absorbance of supernatant form triton X treated vial.

5.2.6. Serum stability study

Evaluation of proteolytic stability of peptide based drugs in serum or plasma is an essential requirement for determining the systemic therapeutic application of any peptide based drug [219]. Therefore for this study, peptide based compounds were incubated in human serum; a 25% (v/v) suspension of human serum was prepared in RPMI medium. To this suspension, a stock solution of test compounds (SA4 and SPO) was added to get a final concentration of 50µg/ml. This reaction mixture (100 µl) was removed and its serum proteins were precipitated with 200 µl of 6% TCA solution. A cloudy reaction mixture was formed which was cooled and further centrifuged at 18000g for 2 min. The processed supernatant was analyzed by RP-HPLC using C18 column. Area under the curve at 0 min was considered as 100% intact compound and was further used for calculation of % intact compound at different time points [211].

5.2.7. Effect of lead compounds on the growth kinetics and cell morphology of bacteria

To determine the effect of SA4 and SPO on growth kinetics of *A. baumannii* cells, overnight grown *A. baumannii* culture was diluted in fresh in MHB medium. 100 µl of this culture was added to an equal volume of diluted solution of SA4 and SPO in MHB medium to attain 5xMIC as final concentration. Plates were incubated for 37 °C and OD was observed at 2 h, 4 h, 8 h, and 24 h at 600 nm. OD of control and test compound treated *A. baumannii vs*. time was plotted. Results were expressed as the mean ± SD of OD in triplicates. While on the other hand, to study the effect of lead compounds on bacterial cell morphology, SEM was used to visualize the membrane damaging effects of these compounds on *A. baumannii* cell morphology. This study was carried out as per the method described by M. Hartmann *et al.*, 2010 [213]. Briefly, 10^8-10^{10} CFU/ml of bacterial culture was incubated with supra MIC of lead compounds in small glass tubes

at 37 °C and 200 rpm. PBS treated bacterial cultures served as a negative control. After 1 h of incubation, the contents of these tubes were centrifuged to pellet down bacterial cells. Further three times washings were given with PBS. Supernatant was discarded and the formed pellet was fixed with glutaraldehyde. Extensive washings were given with PBS and bacterial samples were processed for capturing SEM images.

5.3. RESULTS AND DISCUSSION

Synthesis, characterization and preliminary antibacterial activity of SA4, a 12-residue peptide and its poly-*N*-substituted glycine congener SPO have already been discussed in **Chapter 3**. These two compounds emerged as the most potent antibacterial compounds against *A. baumannii* bacterial strains. Since *A. baumannii* is a notorious nosocomial pathogen that has become a cause of concern worldwide, as it has the ability to form biofilms which helps *A. baumannii* to survive in harsh environmental conditions [223-226]. Therefore, SA4 (**Figure 5.3a**) and SPO (**Figure 5.3b**) were evaluated for their antibiofilm potential against *A. baumannii* biofilms. Results of various *in vitro* biological studies that were carried out have been described below.

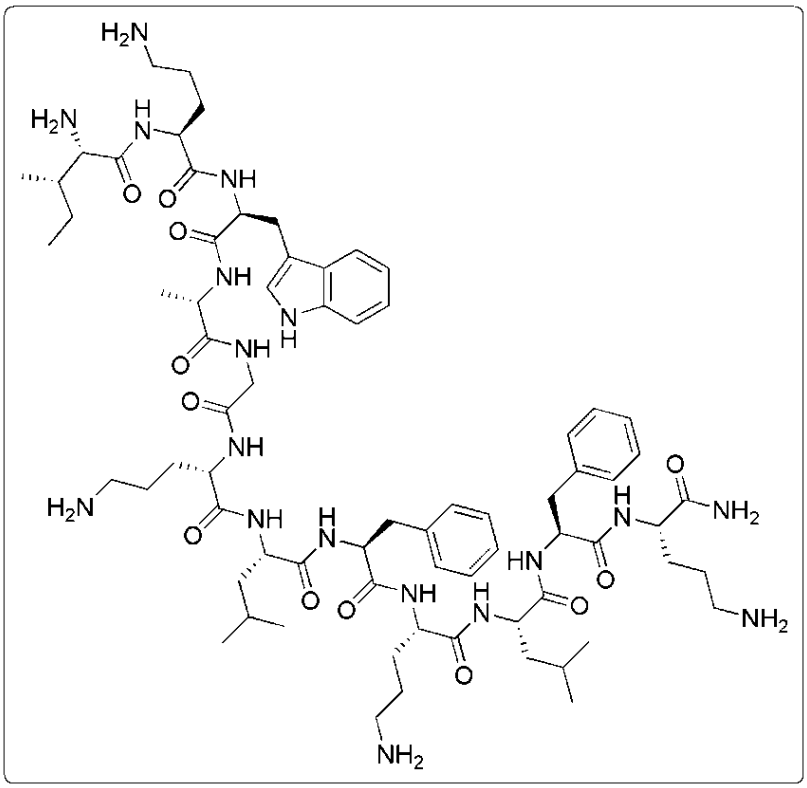


Figure 5.3a: 12-residue peptide SA4

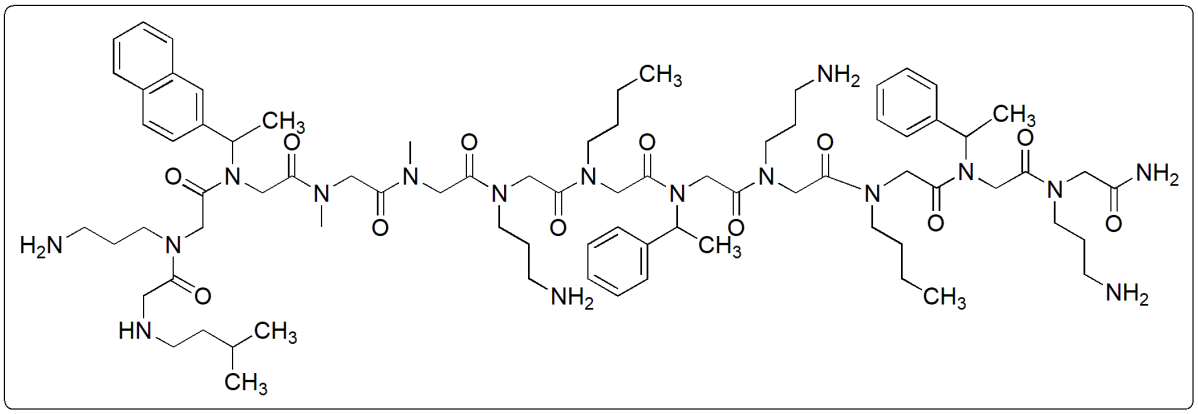


Figure 5.3b: Poly-*N*-substituted glycine congener of SA4

5.3.1. Hemolysis assay

Preliminary hemolytic toxicity study of SA4 and SPO, as mentioned in **Chapter 3** (**Table 3.2c**) suggested that both compounds have HC_{50}>250 µg/ml. Therefore, to find out HC_{50} of SA4 and SPO, hemolytic toxicity study of these compounds was carried out at 500 µg/ml; HC_{50} of SPO and SA4 was found to be >500 µg/ml, indicating that lead compounds possessed low toxicity to human erythrocytes. On the contrary, preliminary antibacterial activity study (**Table 3.2a** and **Table 3.2b; Chapter 3),** against *A. baumannii* strains revealed that even at low concentrations (50-100 µg/ml), these compounds were highly toxic to bacterial strains. This suggests that obtained leads were more toxic to tested bacterial strains than mammalian cells. This selectivity of SA4 and SPO to bacteria could be attributed to the cationic nature of these compounds. SA4 was rich in positively charged *N*-ornithine and SPO was rich in positively charged 1,3-diaminopropane. Cationic charge of AMPs is a major driving force in the initial attraction of peptides with anionic bacteria, whereas this electrostatic attraction is weak between zwitterionic mammalian cells and cationic peptides [249].

5.3.2. Biofilm inhibition assay and biofilm eradication study

Antibiofilm activity of synthesized compounds was determined against different *A. baumannii* strains using crystal violet assay. Percentage biofilm inhibition (**Figure 5.3.2**) by SPO and SA4 against these *A. baumannii* strains ranged between 20% to 80%. The values of these results revealed that percent biofilm inhibition by SPO and SA4 is almost comparable against different *A. baumannii* strains. On the other hand, a biofilm eradication study was carried out to determine the ability of antibacterial agents to eradicate the pre-established biofilms of *A. baumannii* 19606 and MDR3. The results of

this study (**Table 5.3.2**) show that minimum biofilm eradication concentrations (MBECs) of tested compounds against *A. baumannii* ATCC 19606 and MDR3 strains were found to be 6 mg/ml for SA4, >6 mg/ml for SPO, 2 mg/ml and >2 mg/ml for the reference standard tetracycline HCl, respectively.

Table 5.3.2: MBEC of SA4 and SPO against *A. baumannii* ATCC 19606 and MDR3

S. No	Compound	MBEC (mg/ml)	
		A. baumannii ATCC 19606	*A. baumannii* MDR3
1.	**SA4**	6	6
2.	**SPO**	>6	>6
3.	**Tetracycline HCl**	2	>2

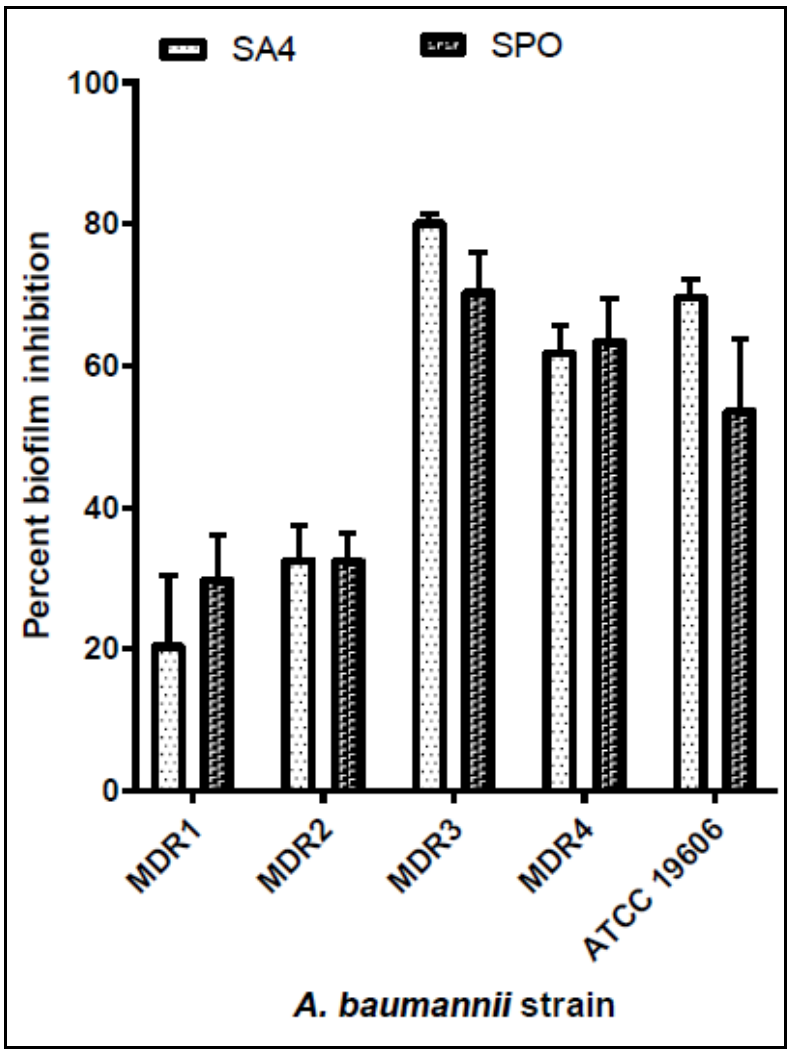


Figure 5.3.2: Antibiofilm activity of SA4 and SPO against different *A. baumannii* strains at their MIC. The results are presented as mean ± SD.

Biofilm eradication percentages varied for all compounds against tested bacteria, but overall these results suggest that high concentrations of compounds are required for eradication of pre-formed biofilms. Moreover, it is a well-known fact that for biofilm eradication 10×MIC-1000×MIC concentrations are usually required, where MIC is the minimum concentration that is required to inhibit the planktonic form of bacteria [248, 250].

5.3.3. Serum stability study

Stability study of SA4 peptide and SPO peptoid in human serum was used to determine the ability of these compounds to resist degradation by proteolytic enzymes present in human serum. Degradation kinetics of both compounds in serum was evaluated for 6 h. As per RP-HPLC analysis, percent of compounds that remained intact after 1 h of incubation was 53% and 97% for SA4 and SPO, respectively. Thereafter till 3rd h of incubation there were only minute changes in these values, whereas after 6 h the amount of intact compound dipped to 49.31% and 82.23% for SA4 and SPO, respectively.

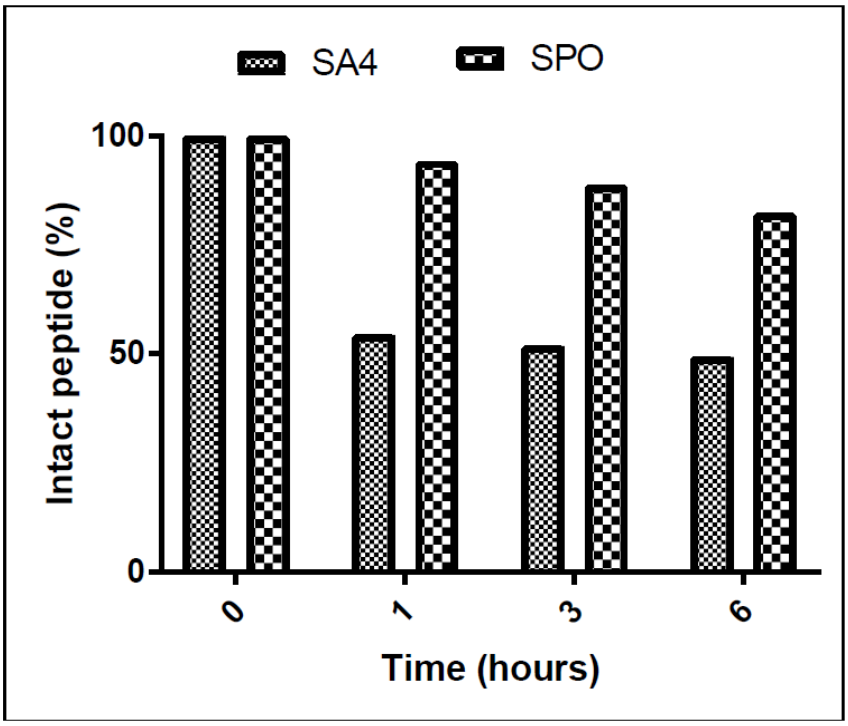


Figure 5.3.3: Serum stability profile of SA4 and SPO. The results presented are mean of duplicates.

Results of proteolytic stability study in human serum confirmed that overall loss in percent of intact SPO even after 6 h was less than 20% (**Figure 5.3.3**). On the other hand, the overall loss in percent of intact SA4 after 6 h was almost 50%. The reason

behind differential stabilities of SPO and SA4 in human serum could be high resistance of peptoids to proteolytic degradation in comparison to proteolysis of peptides [251] [252].

5.3.4. Effect of SA4 and SPO on growth kinetics of bacteria

It has been found that HDPs have a rapid mode of action on bacteria [72]. Therefore to evaluate the effect of the lead compounds SA4 and SPO on growth kinetics of *A. baumannii* cells, we incubated SA4 and SPO with *A. baumannii* ATCC 19606. Effect on growth kinetics was determined by measuring OD of treated bacteria at different time points.

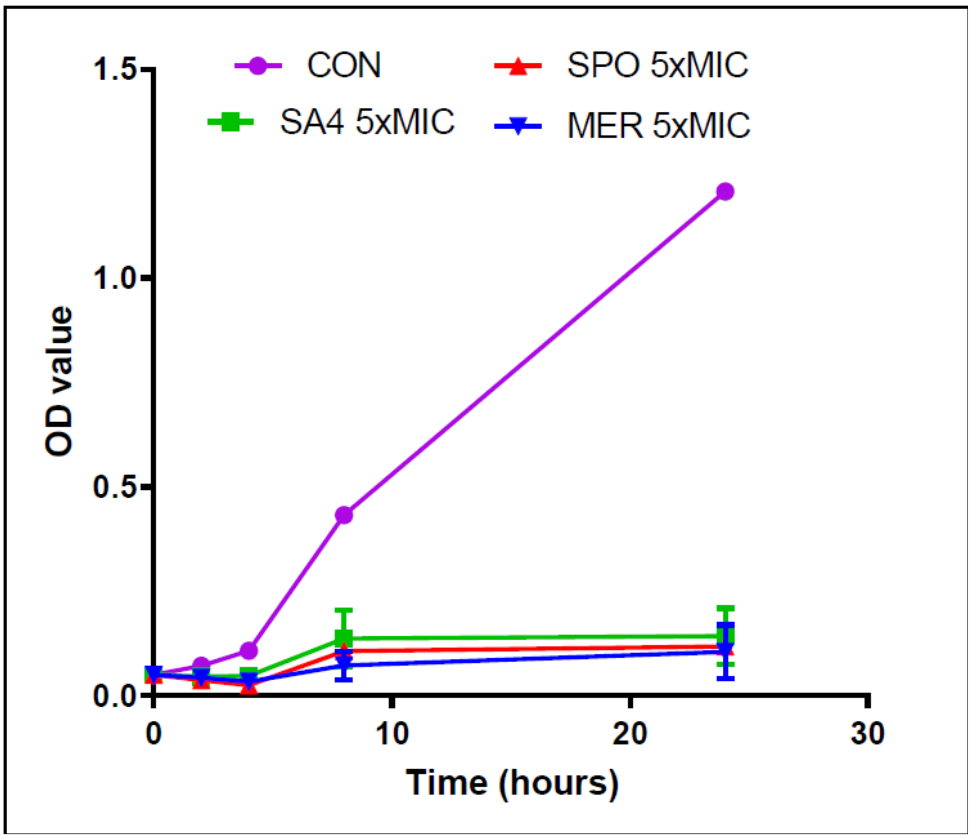


Figure 5.3.4: Effect of SA4, SPO, meropenem and control (no treatment) on growth kinetics of *A. baumannii* ATCC 19606. The results are mean of triplicates ±SD.

All three compounds SA4, SPO and meropenem inhibited the growth of bacteria within 2 h (**Figure 5.3.4**) of incubation and bacterial growth remained arrested till 8th h at 5×MIC. After 8th h to 24th h, there was a slight increase in OD values of all three compounds treated culture. However, this increase was too low in comparison to control. Thus, these lead compounds SA4 and SPO are capable of inhibiting *A.*

baumannii growth within a few hours of initial interactions. None of the test compounds could eradicate complete bacterial growth up to 24 h. One of the possible reasons for this might be the inherently resistant phenotypes associated with biofilm-producing bacteria [227].

5.3.5. Effect of lead compounds on bacterial cell morphology

To investigate effects of leads on morphology of *A. baumannii* cells; morphological damages caused by these compounds on bacteria were visualized using SEM.

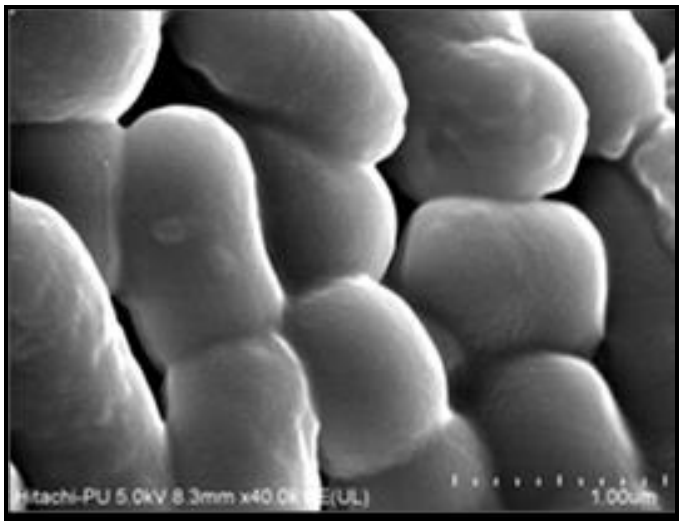


Figure 5.3.5a: SEM of control (normal saline-treated) ATCC 19606 cells.

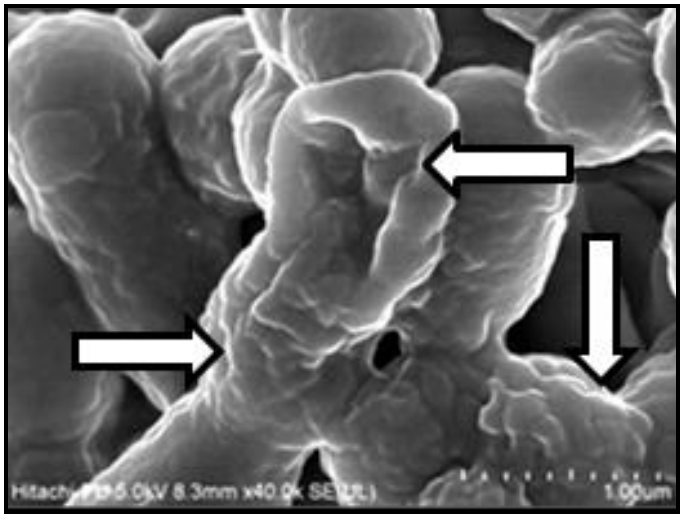


Figure 5.3.5b: SEM of SA4-treated ATCC 19606 cells. The arrows indicate morphological changes.

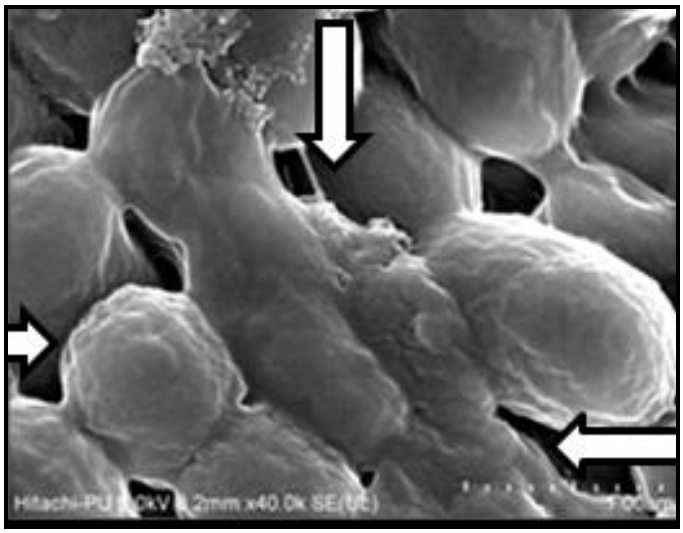


Figure 5.3.5c: SEM of SPO-treated ATCC 19606 cells. The arrows indicate morphological changes.

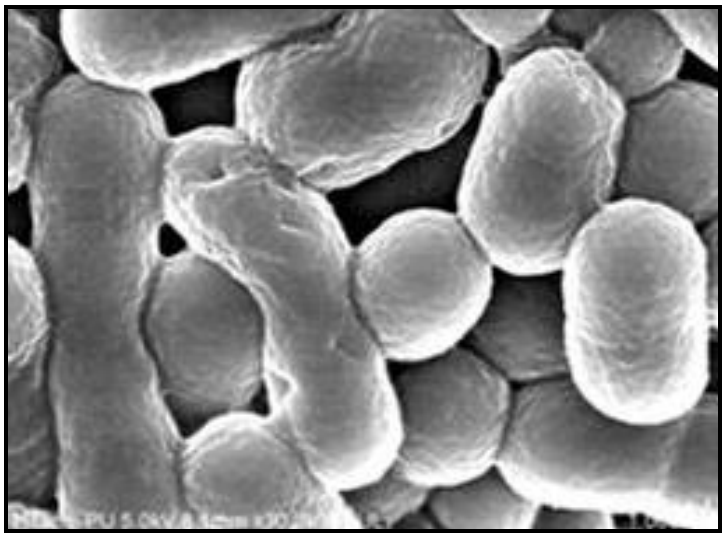


Figure 5.3.6a: SEM of control (normal saline-treated) treated MDR3 cells.

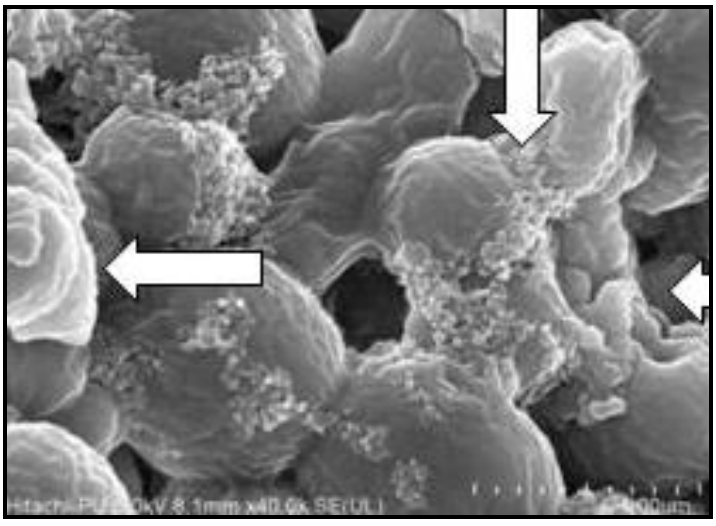


Figure 5.3.6b: SEM of SA4-treated MDR3 cells. The arrows indicate morphological changes.

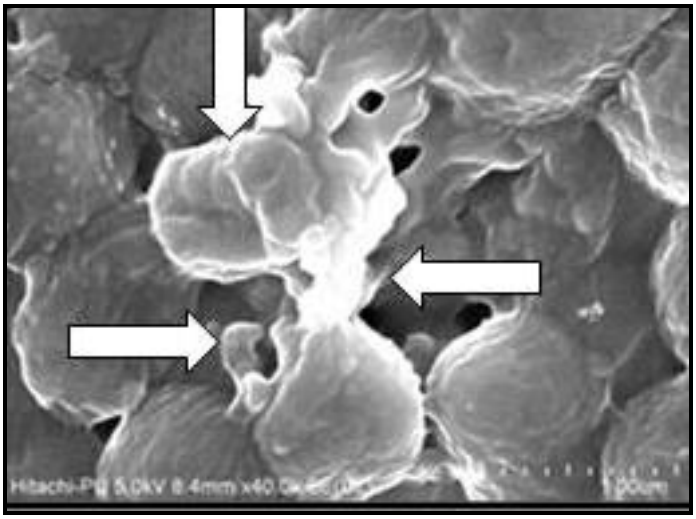


Figure 5.3.6c: SEM of SPO-treated MDR3 cells. The arrows indicate morphological changes.

SEM microphotographs (**Figure 5.3.5a, Figure 5.3.5b**, **Figure 5.3.5c, Figure 5.3.6a, Figure 5.3.6b** and **Figure 5.3.6c**) of SA4 and SPO treated bacterial cells show changes in cell morphology of treated bacterial cells in comparison to untreated bacteria. The SEM images are suggestive of membrane disruptive action of these leads on bacteria. SA4 and SPO caused alterations on the cell membrane of *A. baumannii* cells. Cellular debris was observed in SA4-treated and SPO-treated *A. baumannii* cells, whereas no such cell debris was observed in control *A. baumannii* cells (**Figure 5.3.5a** and **Figure 5.3.6a**). Moreover, minute cell protrusions can be seen in compounds treated *A. baumannii* ATCC 19606 (**Figure 5.3.5b, Figure 5.3.5c**) and MDR3 cells (**Figure 5.3.6b, Figure 5.3.6c**) These results are in accordance with the results observed by Hartmann *et al.*, 2010, where they observed protrusions on the surface of bacterial cells caused by AMPs [253]. Another study supports these results, where transmission electron microscopy was utilized to determine the effect of peptide Cec4 on the cell membrane of *A. baumannii* cells. The results of this study suggested that this peptide was found to act on the cell membrane of *A. baumannii* cells and damaged the morphology of these cells [254]. Though, detailed studies are required to comprehend the precise MOA of SA4 and SPO against *A. baumannii* cells.

5.4. SUMMARY (CHAPTER 5)

A. baumannii is responsible for the formation of biofilms on living as well as non-living materials [255]. It can form biofilms on polystyrene, glass, epithelial cells and fungal filaments [225]. It causes a wide-range of biofilm-associated infections (BAIs) [256]. Biofilm forming *A. baumannii* has evolved resistance to almost all conventional antimicrobial drugs [257]. Lack of novel antibacterial agents has further hampered the treatment of *A. baumannii* infections [258]. Thus to fight such biofilm-associated *A. baumannii* infections, antimicrobial peptides (AMPs) might be an alternative to conventional antibiotics [256]. With this in view, a peptoid SPO was designed based on previous 12-residue lead antibacterial peptide SA4 [199]. SA4 and SPO were evaluated for their antibacterial potential against *A. baumannii* biofilms. These compounds were found to possess antibacterial activity against *A. baumannii* biofilm-forming bacterial cells. These compounds possess biofilm inhibition as well as biofilm eradication potential. The results of the hemolytic toxicity study also showed the selectivity of SA4 and SPO towards bacterial cells over eukaryotic cells. Moreover, the SEM images of *A. baumannii* bacterial cells treated with these compounds indicated that bacterial cell membrane may be the target of antibacterial action of these compounds. However, detailed studies are required to understand the exact mechanism of action. Furthermore, the serum stability study of these compounds suggested that both compounds have different proteolytic stability. This information may be used in the future for deciding the route of administration and therapeutic application of these peptides. Overall results demonstrate the antibacterial potential of the peptide-based compounds SPO and SA4 against biofilm-forming *A. baumannii* cells.

CONCLUSION AND OUTLOOK

Conclusion

Research detailed in this thesis deals with synthesis, characterization and antibacterial evaluation of peptides and their poly-*N*-substituted glycine congeners. We successfully synthesized peptides (LP-16, LP-17, LP-18, LP-22, LP-23, LP-24, SA4, and SPA) by solid-phase synthesis on Rink amide MBHA resin. Similarly, *N*-substituted glycine congeners (DP-16, DP-22, DP-23, DP-24, SMO, and SPO) based on amphipathic templates of these peptides were also synthesized by submonomer peptoid synthesis approach. Synthesized compounds with HPLC purity >90% were screened for their antibacterial activity against a series of Gram-positive and Gram-negative bacteria, including multidrug-resistant clinical isolates of *A. baumannii*. From preliminary antibacterial screening four lead compounds LP-23, DP-23, SA4, and SPO were identified.

LP-23 is a 5-residue lipopeptide, with 4 cationic charges and palmitic acid as a lipid moiety while DP-23 is a poly-*N*-substituted glycine congener of LP-23 with 1,3-diaminopropane as a cationic charged amine. LP-23 lipopeptide and DP-23 lipopeptoid emerged as the lead compounds against *M. smegmatis* MC^2155 with MIC of 6.25 μg/ml. Similarly, SA4 is a 12-residue cationic peptide based on the minimum amphipathic template (H+HHG+HH+HH+NH_2), where H represents hydrophobic amino acid and "+" represents charged hydrophilic amino acid. SPO is a poly-*N*-substituted glycine congener of SA4 that was synthesized using various synthetic amines, like 1,3-diaminopropane, phenylethylamine, butylamine, methylamine, napthylethylamine, and isopentylamine as side chains. Peptide SA4 and peptoid SPO were found to be most effective against *A. baumannii* ATCC 19606 and multidrug-resistant clinical isolates of *A. baumannii* with MIC ranging between 50-100 μg/ml. SA4 and SPO were even found to possess biofilm inhibition and biofilm eradication potential against *A. baumannii*.

Synthesized leads LP-23, DP-23, SA4, and SPO demonstrated more selectivity towards bacteria than mammalian cells. Their HC_{50} values against human red blood cells were high in comparison to their MIC values against various bacterial strains. Further, the results of the serum stability study of antibacterial lead compounds revealed that SPO peptoid is the most stable, reflecting its high biocompatibility. Additionally, scanning

electron microscopy was used to collect the visible pieces of evidence of the membrane-active mode of action of these compounds. All four lead compounds damaged the integrity of bacterial cells in comparison to control bacterial cells. Based on the outcomes of this study, we propose that all four lead compounds may have a membrane damaging mode of action against bacteria. However, detailed studies are required to comprehend the precise mode of antibacterial action of these compounds.

Outlook

Design and synthesis of synthetic derivatives of natural peptide sequences is increasingly finding a place among the principal strategies in the antibacterial drug discovery domain to combat the limitations associated with the natural peptides. Taking this fact into consideration, various peptides and their *N*-substituted glycine congeners were synthesized using solid-phase synthesis. From this research work, ultra-short compounds (LP-23 & DP-23) and short compounds (SA4 & SPO) were found to be the most potent antibacterial agents. Hence, further *in vivo* antibacterial evaluation of these compounds in mouse models could be an encouraging step toward the development of these compounds as therapeutic antibacterial agents in the future.

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LIST OF PUBLICATIONS

Research Articles

1. **D. Sharma**, M. Choudhary, J. Vashistt, R. Shrivastava, and G. S. Bisht*, "Cationic antimicrobial peptide and its poly-*N*-substituted glycine congener: Antibacterial and antibiofilm potential against *A. baumannii*," *Biochem Biophys Res Commun*, vol. 518, pp. 472-478, 2019. [SCOPUS, SCI, IF- 2.985].

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4. **D. Sharma,** L. Sharma, G.S. Bisht* "Design, synthesis, characterization and evaluation of antimycobacterial activity of peptidomimetics," 3rd Himachal Science Congress National, 22-23rd Oct, 2018, at IIT Mandi. Proceeding of Himachal Pradesh Science Congress [Oral presentation].

5. **D. Sharma,** N. Thakur, J. Vashishtt & G.S. Bisht.* "Antimicrobial evaluation of copper oxide nanoparticles synthesized using leaf extract of *Terminalia chebula*," International conference on Advances in Plant & Microbial Biotechnology PMB, 2-4 Feb, 2017 at JIIT, Noida, India [Poster presentation].

6. Samriti, A. Gupta, **D. Sharma,** Gopal Singh Bisht.* "To study the antimicrobial properties of cuprous oxide synthesized using *Hypericum perforatum* plant extract," International conference on Pharmacovigilance and Clinical Research, 28-29 March 2018 at Bahara University, Waknaghat, India [Poster presentation].

7. P. Gupta, T. Gyeltshen, **D. Sharma,** G.S. Bisht.* "Evaluation of antibacterial activity of zinc oxide synthesized via *Terminalia chebula* leaf extract," International conference on Pharmacovigilance and Clinical Research, 28-29 March 2018 at Bahara University, Waknaghat, India [Poster presentation].

8. L. Sharma, D. Sharma, G.S. Bisht.* "Antimicrobial peptides as novel drug candidates for treatment of microbial infections," 3rd Himachal Science Congress National, 22-23rd Oct, 2018, at IIT Mandi. Proceeding of Himachal Pradesh Science Congress [Poster presentation].

APPENDIX A

Table (Appendix A): Antimicrobial susceptibility profiles of *A. baumannii* isolates against tested compounds

S. No	Antimicrobial drug	ATCC 19606	MDR1	MDR2	MDR3	MDR4
1.	Ceftazidime	12 µg (I)	>256 µg (R)	>256 µg (R)	>256 µg (R)	>256 µg (R)
2.	Levofloxacin	0.25 µg (S)	12 µg (R)	>32 µg (R)	>32 µg (R)	>32 µg (R)
3.	Amikacin	6 µg (S)	>256 µg (R)	>256 µg (R)	>256 µg (R)	12 µg (S)
4.	Piperacillin-tazobactam	4 µg (S)	>256 µg (R)	>256 µg (R)	>256 µg (R)	>256 µg (R)
5.	Tetracycline	0.35 µg (S)	>256 µg (R)	>256 µg (R)	>256 µg (R)	48 µg (R)
6.	Cefepime	6 µg (S)	>256 µg (R)	>256 µg (R)	>256 µg (R)	>256 µg (R)
7.	Imipenem	0.19 µg (S)	>32 µg (R)	>32 µg (R)	>32 µg (R)	>32 µg (R)
8.	Meropenem	0.75 µg (S)	>32 µg (R)	>32 µg (R)	>32 µg (R)	>32 µg (R)
9.	Cefoperazone-sulbactum	1.5 µg (S)	48 µg (I)	>256 µg (R)	>256 µg (R)	>256 µg (R)

APPENDIX B

Table (Appendix B): HPLC details

HPLC Instrument	Waters India
Column used	C_{18} (Spherisorb, ODS2, 5µm, 4.6 mm×250 mm)
Injection volume	20 µl
Flow rate	1 ml/min
Detector	Waters 2996 PDA detector
HPLC pump	PCM/15XXX
Temperature	Room Temperature
Autosampler	Waters 717 plus