Chapter 4

Design, Synthesis and Mode of Action of Novel Linoleic Acid Tagged di-peptide Spermidine Conjugates

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Synthesis, antibacterial activity and mode of action of novel linoleic acid–dipeptide–spermidine conjugates

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4.1 Introduction

Emergence of multiple drug resistant bacterial strains causes millions of deaths worldwide [1,2]. The rise in morbidity and mortality related to microbial infections has lead to tremendous pressure on health care systems [3]. Cationic antimicrobial peptides (CAMPs) also sometimes called as Host defense cationic peptides (HDCPs) are known for their wide range of activity against microorganisms including bacteria, fungi, viruses and even cancerous cells. With multifaceted role in innate immunity and a direct cell lytic mode of action, it is difficult for a microorganism to endure resistance against HDCPs [4,5]. Therefore, HDCPs are being explored as promising alternative to conventional drugs to combat drug resistant bacterial strains [6,7].

However, at present, only few antimicrobial peptides are under clinical trials due to their high manufacturing costs, poor pharmacokinetic properties and associated toxicity issues [8]. To address these issues, efforts are being made to explore the characteristic features of HDCPs, such as, net positive charge at physiological pH and hydrophobic bulk [9] to develop economically viable membrane active antimicrobial peptidomimetics. Many classes of compounds such as ceragenins [10,11], oligoacyllysines [12,13], arylamides [14], peptoid based scaffolds [15] and lipopeptides [16,17] that mimic HDCPs are being developed as alternative antimicrobial agents with low susceptibility for development of resistance. Among the various classes of potent antibacterial agents some of the promising scaffolds are oligoacyllysines and ceregenins.

Oligoacyllysines (OAKs) are a group of antimicrobial copolymers, comprising of repeats of acyllysine unit to mimic the primary structure and function of natural antimicrobial peptides [18]. This class of compounds is based on the assumption that acyl moieties might be able to substitute extensive sequences within the peptide...
backbone, thus enabling a positive charge and hydrophobicity balance to elicit antimicrobial activity. Structure of representative OAKs is shown in Figure 4.1(a).

![Figure 4.1: Representative structures of oligoacyllysine (a) and ceregenin (b)](image)

Another promising class of antimicrobial peptidomimetics is ceragenins. The ceragenins were designed to mimic the facially amphiphilic morphology of antimicrobial peptides. Keeping the marine natural antibiotic squalamine (dog fish shark) and lipopeptide antibiotic polymyxin B (*Bacillus polymyxa*) in mind, Savage and co-workers designed this template to show a broad range of activity against Gram-positive as well as Gram-negative bacterial strains [19]. Representative structure of ceregenins is shown in Figure 4.1(b). Interestingly, the most active ceragenin analogue CSA-13 has been established to show potent antiviral activity against vaccinia virus in *Eczema vaccinatum* which is a potentially fatal, disseminated, viral skin infection that develops in individuals with atopic dermatitis after exposure to the vaccinia virus [20]. With excellent cell selectivity ceragenin, CSA-54 and other members of the Ceragenin™ family are being forwarded as preclinical compounds for use as potential systemic and topical therapies in the treatment and prevention of microbial diseases [21,22].
A number of lipopeptides with clinical applications as an antimicrobial agents, such as polymyxin B, daptomycin and echinocandins [23-25] have spurred the design of lipopeptides to achieve net positive charge (at least +2) and hydrophobic bulk making use of varied chemical moieties [26-28]. However, cationic lipopeptides with saturated fatty acids are found to be difficult to optimize for cell selectivity [29]. Long-chain free fatty acids (FFAs) are known to be present at skin surfaces, maintaining an acidic pH which helps to prevent colonization of various microorganisms including methicillin-resistant *S. aureus* and *H. pylori* [30-32] (Figure 4.2). The mode of bacterial killing for FFAs has not been unambiguously determined, however, the prime target is believed to be microbial membranes where direct lysis, perturbation of electron transport chain and oxidative phosphorylation have been reported as some of the probable causes leading to bacterial cell death [33]. Interestingly, Zheng and co-workers found that linoleic acid inhibited bacterial enoyl-acyl carrier protein reductase (FabI), an essential component of bacterial fatty acid synthesis, making linoleic acid a suitable tag for promising antibacterial activity [34].

![Figure 4.2: Structures of known lipids with antibacterial activity](image-url)
Therefore, with multiple non specific modes of action, broad range of activity and minimum toxicity, long chain FFAs were chosen to be incorporated into designed peptidomimetics as hydrophobic moiety.

The cationic polyamines putrescine (1,4-diaminobutane), spermidine (1,8-diamino-4-azaoctane), and spermine (1,12-diamino-4,9-diazadodecane) are ubiquitous polycations that are found in significant amounts in nearly every prokaryotic and eukaryotic cell type [35] (Figure 4.3). However, recently it was reported that *S. aureus* lacks identifiable polyamine biosynthetic genes and consequently produces no spermine/spermidine or their precursors [36]. These, low molecular weight polycations are known to be required for optimal cell growth. Spermidine and spermine primarily exist in aqueous solution at pH 7.4 as fully protonated polycations and possess the pKa values >8. This high degree of positive charge is an important factor in the multiple biological functions of these molecules [37].

![Figure 4.3: Structures and pKa values of polyamines](image)

The intracellular concentration of polyamines to support a wide variety of cellular functions is in mM range. However, the free polyamine concentrations are considerably
lower, as they are bound to anionic groups in DNA, RNA, proteins and phospholipids [38]. It has been reported that, insufficient levels of polyamines result in sub optimal growth and high levels of polyamines can lead to malignant transformation [39,40]. A number of modified/conjugated polyamines have been reported with various biological activities such as LPS sequestration [41], anti-parasitic activity [42,43], anticancer activity [44,45], as well as nucleic acid carriers for DNA transfection [46]. Considering medicinal potential of polyamines, we anticipated that incorporation of spermidine as positive charged moiety to hydrophobic-dipeptides may lead to small peptidomimetics with better antibacterial activity.

### 4.2 Present work

- To design and synthesize short peptidomimetics with broad range of antibacterial activity and cell selectivity using economically viable building blocks.
- To evaluate interaction studies of designed peptidomimetics with artificial membranes and intact bacterial cells to delineate factors responsible for activity and selectivity.

### 4.3 Design of template based antimicrobial agents

Encouraged by clinical potential of facial amphiphilic molecules we designed and synthesized a small library of lipophilic-dipeptide-spermidine template based sequences consisting of cationic polyamine spermidine and FFAs (Figure 4.4). We dissected the template into two parts to evaluate role of hydrophobic moiety-dipeptide or cationic-dipeptide part of the template in imparting activity and/or selectivity to these sequences.
Figure 4.4: Design of template

For the di-peptide portion, we made use of three different combinations of tryptophan (Trp) and ornithine (Orn) amino acids \textit{i.e.} Trp-Trp, Trp-Orn and Orn-Orn. Hydrophobic Trp amino acid was chosen because of its well documented membrane anchoring property \cite{47}. Cationic residue Orn was used to impart protease stability to designed sequences due to its non ribosomal origin. Based on various biological activities associated with spermidine conjugates as outlined above, we incorporated spermidine at C-terminus of the dipeptides to give rise to sequences 1-3 (Scheme 1). N-Terminal hydrophobic tagging of dipeptide sequences was done to evaluate role of FFAs such as linoleic acid and stearic acid in imparting activity leading to sequences 4-11.

To have an appropriate balance between charge and hydrophobicity the designed complete template consisting of hydrophobic tag at N-terminal with cationic spermidine at the C-terminus resulting into sequences 12-19 were also synthesized. To evaluate the role of lipidation as a control an aromatic moiety 3-(4-hydroxyphenyl)-propionic acid (HPPA) was also conjugated. The HPPA was used based on our observations that covalent hybridization of HPPA to a tetra peptide template led to the discovery of some potent tetra peptidomimetics \cite{48}. 
4.4 Synthetic strategy for peptidomimetics

The di-peptides were synthesized on 2-chlorotrityl chloride resin as solid support using Fmoc chemistry as reported previously [49]. The terminal amino group of dipeptides was Boc protected on solid support before cleavage from resin under mild conditions (TFE: CH₃COOH: CH₂Cl₂ cocktail 1: 1: 8) to retain Boc group. The cleaved dipeptides were coupled with N₁,N⁴-bis(boc)spermidine (SIGMA) using HOBt and DIPCDI in dry tetrahydrofuran under N₂ atmosphere at 0 °C for 30 min, followed by 18 h at room temperature as reported previously [50]. The obtained product was dissolved in CHCl₃ (15 mL) and washed with 1% aqueous NaHCO₃ (50 mL), 1% aqueous HCl (50 mL), and...
brine (50 mL). The organic layer was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure to give crude dipeptide spermidine conjugates. Boc groups were removed from conjugates using 50% TFA in CH₂Cl₂ to give sequences 1-3 (Scheme 1). On solid support, N-terminal end tagging of dipeptides was achieved by coupling 4 equivalents of 3-(4-hydroxyphenyl)-propionic acid (HPPA)/linoleic acid/stearic acid overnight with HOBT and DIPCDI. Kaiser test was performed to check completion of reactions on solid support [51]. The N-terminal tagged di-peptidomimetics were cleaved from solid support under two different conditions. For synthesis of 4-11, cleavage was effectuated using 50% TFA in DCM. For synthesis of 12-19, cleavage was performed under mild conditions (TFE: CH₃COOH: CH₂Cl₂ cocktail 1: 1: 8). Further Boc protected N-terminal tagged conjugates were coupled with N¹,N⁴-bis(boc) spermidine as described for dipeptides earlier in the text. Finally Boc groups were removed with 50% TFA resulting into sequences 12-19.

4.5 Experimental methods

4.5.1 Cytotoxicity

Since HDCPs mimics are considered more suitable as topical agents, we evaluated effects of designed sequences on human keratinocytes.

Principle of method

The toxicity was studied by MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay. It is used for the quantitative determination of cellular proliferation, activation and toxicity evaluation. The MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings
of the pale yellow MTT and form dark blue formazan crystals, which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Formazan crystals may be dissolved in a solubilization buffer containing detergent/dimethylsulphoxide (DMSO). The solubilized formazan product can be photometrically quantified using an ELISA reader. A living cell with metabolic activity leads to color production which is indicative of cell viability.

Method

A previously defined method was used for cytotoxicity experiment [52]. HaCaT keratinocytes, 3000 cells/well, were seeded in 96-well plates in DMEM HAMS F12 media supplemented with 10% serum (FBS) to grow over night. Next day media was aspirated and fresh incomplete media was added (50 µL per well). To the wells serial two fold dilutions of different test sequences (50 µL) were added and the plates were incubated at 37 ºC with 5% CO$_2$ for 18 h. After 18 h, the media was aspirated and 100 µL of MTT solution was added to each well. The plates were further incubated for 4 h in CO$_2$ at 37 ºC. After 4 h MTT-containing medium was removed by aspiration. Blue formazan product generated was dissolved by the addition of 100 µL of 100% DMSO per well. The plates were then gently swirled for 2-3 min at room temperature to dissolve the precipitate. The absorbance was monitored at 540 nm. Percentage viability was calculated based on the following formula

$$\% \text{ Cell viability} = \frac{A}{A_{\text{control}}} \times 100$$

where, $A$ represent sample absorbance at a given concentration and $A_{\text{control}}$ represent untreated cells. The experiment was repeated thrice and results are given as mean ±S.D.
4.5.2 Membrane depolarization

After study on model membranes for the mode of action on intact MRSA cells, membrane depolarization experiment was performed. Since the sequences showed potent activity against MRSA, this strain was chosen to evaluate depolarization ability of designed sequences.

Principle of method

3,3’-Dipropylthiadicarbocyanine iodide [DiSC$_3$(5)], a potential sensitive dye, is used to monitor changes in plasma membrane integrity in response to any membrane potential destabilizing agent [53]. The dye is able to gain access to the cytosol after permeation through the outer membranes in both Gram-positive and Gram-negative cells. In the cytosol the fluorescence of DiSC$_3$(5) would be quenched due to self-association. A dissipation of membrane potential on account of disruption of membrane integrity will release DiSC$_3$(5) into solution leading to an increase in fluorescence intensity. So if the designed antimicrobial agents are able to alter membrane potential as a result of pore formation/membrane destabilization an increase in fluorescence intensity is observed.

Method

For the evaluation of membrane depolarization a previously defined method was used [54]. Briefly, overnight grown MRSA was subcultured into MHB for 2-3 h at 37 °C to obtain midlog phase cultures. The cells were centrifuged at 4000 rpm for 10 min at 25 °C, washed and re-suspended into respiration buffer (5 mM HEPES, 20 mM glucose, pH 7.4) to obtain a diluted suspension of OD$_{600}$ ≈ 0.05. DiSC$_3$(5), 0.18 µM (prepared in DMSO) was added to 500 µL aliquot of the re-suspended cells and allowed to stabilize for 1 h. Baseline fluorescence was acquired using a Fluorolog (Jobin Yvon, Horiba)
spectrofluorometer by excitation at 622 nm and emission at 670 nm. Bandwidth of 5 nm was employed for excitation and emission. Subsequently, increasing concentrations of test sequences between 2-19.2 µg/mL were added to the stabilized cells and the increase of fluorescence on account of the dequenching of DiSC₃5 dye was measured after every 2 min to obtain the maximal depolarization. Percent depolarization was calculated by using the formula

\[
\% \text{ depolarization} = \frac{F - F_0}{F_m - F_0} \times 100
\]

where, F is the fluorescence intensity 2 min after addition of sequences, F₀ is the initial basal fluorescence intensity, and Fₘ is the maximum fluorescence intensity obtained after addition of 10 µg/mL gramicidin. Percentage depolarization, mean ± S.D. of two independent experiments was plotted versus increasing concentrations of different sequences.

4.5.3 Scanning electron microscopy (SEM)

Method

For electron microscopy samples were prepared as described previously [55]. Briefly, freshly inoculated methicillin resistant \textit{S. aureus} (ATCC 33591) was grown on MHB up to an OD₆₀₀ of 0.5 (corresponding to 10⁸ CFU/mL). Bacterial cells were then spun down at 4000 rpm for 15 min, washed thrice in PBS (20 mM, 150 mM NaCl) and re-suspended in an equal volume of PBS. The cultures were then incubated with test sequences 14, 15 or 16 at 10 X MIC for 30 min. Controls were run in the absence of sequences. After 30 min, the cells were spun down and washed with PBS thrice. For cell fixation the washed bacterial pallet was re-suspended in 1 mL of 2.5% glutaraldehyde in PBS and was incubated at 4 °C for 4 h. After fixation, cells were spun
down and washed with PBS twice. Further the samples were dehydrated in series of graded ethanol solutions (30% to 100%), and finally dried in desiccators under a vacuum. An automatic sputter coater (Polaron OM-SC7640) was used for coating the specimens with 20 nm gold particles. Then samples were viewed via a scanning electron microscope (EVO 40, Carl Zeiss, Germany).

4.6 Results and discussion

The synthesized sequences were purified using RP-HPLC and characterized with LC-HR-MS (Table 4.1).

Table 4.1: Sequence, composition, molecular mass and % of acetonitrile at RP-HPLC elution

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Composition</th>
<th>Mass [M+H]⁺</th>
<th>Charge</th>
<th>% Acetonitrile at RP-HPLC elution</th>
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<td></td>
<td></td>
<td>Calc.</td>
<td>Obser.</td>
<td></td>
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<tr>
<td>1</td>
<td>NH₂-WW-Spermidine</td>
<td>518.3238</td>
<td>518.3228</td>
<td>+3</td>
</tr>
<tr>
<td>2</td>
<td>NH₂-WO-Spermidine</td>
<td>446.3238</td>
<td>446.3226</td>
<td>+4</td>
</tr>
<tr>
<td>3</td>
<td>NH₂-OO-Spermidine</td>
<td>374.3238</td>
<td>374.3235</td>
<td>+5</td>
</tr>
<tr>
<td>4</td>
<td>HPPA-WW-COOH</td>
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<td>539.2277</td>
<td>-1</td>
</tr>
<tr>
<td>5</td>
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<td>467.2278</td>
<td>0</td>
</tr>
<tr>
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<td>653.62</td>
<td>-1</td>
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<tr>
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<td>581.4063</td>
<td>0</td>
</tr>
<tr>
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<td>509.4054</td>
<td>+1</td>
</tr>
<tr>
<td>9</td>
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<td>657.68</td>
<td>-1</td>
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<tr>
<td>10</td>
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<td>585.4366</td>
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<td>666.3762</td>
<td>+2</td>
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<tr>
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<td>594.3760</td>
<td>+3</td>
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<tr>
<td>14</td>
<td>LIN-WW-Spermidine</td>
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<td>780.5537</td>
<td>+2</td>
</tr>
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<td>708.5544</td>
<td>+3</td>
</tr>
<tr>
<td>16</td>
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<td>636.5540</td>
<td>+4</td>
</tr>
<tr>
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<td>784.5858</td>
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<td>18</td>
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<td>712.5847</td>
<td>+3</td>
</tr>
<tr>
<td>19</td>
<td>STER-OO-Spermidine</td>
<td>640.5848</td>
<td>640.5853</td>
<td>+4</td>
</tr>
</tbody>
</table>

HPPA: 3-(4-Hydroxy phenyl)-propionic acid, LIN: linoleic acid; STER: stearic acid, * ESI-MS data
4.6.1 Structures of designed active sequences

Sequence 14

Sequence 15

Sequence 16
Sequence 17

Sequence 18

Sequence 19
4.6.2 Purification and characterization of sequences

Synthesized sequences (Table 4.1) were purified using RP-HPLC column (7.8 x 300 mm, 125 Å, 10-µm particle size) with either gradient of 10 to 90% buffer 2 where, buffer 1 was water (0.05% TFA) and buffer 2 was acetonitrile (0.05% TFA) over 45 min or 30 to 100% buffer 2 gradients over 45 minutes where buffer 1 was water (0.1% TFA) and buffer 2 was acetonitrile (0.1% TFA). The correct sequences after purification were confirmed by LC-MS/MS (using Quatro micro API, Waters/ LTQ Orbitrap XL (Thermo Fisher Scientific, USA) mass determination.

4.6.2.1 RP-HPLC traces of designed active sequences

RP-HPLC profiles of sequences 14-19 (absorbance at 220 nm). A linear gradient of 30 to 100% buffer 2 was run over 45 minutes for all the sequence except 16 where, buffer 1 was water (0.1% TFA) and buffer 2 was acetonitrile (0.1% TFA). For sequence 16, HPLC gradient of 10 to 90% buffer 2 was run where, buffer 1 was water (0.05% TFA) and buffer 2 was acetonitrile (0.05% TFA) over 45 min.
Figure 4.5.1: RP-HPLC chromatogram of sequence 14

Figure 4.5.2: RP-HPLC chromatogram of sequence 15

Figure 4.5.3: RP-HPLC chromatogram of sequence 16
Figure 4.5.4: RP-HPLC chromatogram of sequence 17

Figure 4.5.5: RP-HPLC chromatogram of sequence 18

Figure 4.5.6: RP-HPLC chromatogram of sequence 19
4.6.2.2 HR-ESI-MS spectra of sequences 1-5, 7, 8 and 10-19 using LC-ESI-HRMS

The data was acquired using UHPLC (Dionex, Germany) and LTQ Orbitrap XL (Thermo Fisher Scientific, USA). LC-MS method where LC conditions were, column BEH shields RP C18 (2.1 X 100 mm X 1.7 µM), flow rate: 0.3 mL/min, solvent gradient as described for RP-HPLC, run time: 6 min. The MS was run in positive ion mode where the parameters were, capillay voltage: 3.0 kV, cone voltage: 20 V, desolvation gas: 350 L/hr, source temperature: 100 °C, cone gas: 50 L/hr

Figure 4.6.1: HR-MS of Sequence 1
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Figure 4.6.2: HR-MS of Sequence 2

Figure 4.6.3: HR-MS of Sequence 3
Figure 4.6.4: HR-MS of Sequence 4

Calculated [M+H]$^+$ = 539.2289

Figure 4.6.5: HR-MS of Sequence 5

Calculated [M+H]$^+$ = 467.2289
Figure 4.6.6: HR-MS of Sequence 7

Figure 4.6.7: HR-MS of Sequence 8
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Figure 4.6.8: HR-MS of Sequence 10

Figure 4.6.9: HR-MS of Sequence 11
Figure 4.6.10: HR-MS of sequence 12

![HR-MS of sequence 12](image)

Calculated [M+H]$^+$ = 666.3762

Figure 4.6.11: HR-MS of Sequence 13

![HR-MS of Sequence 13](image)

Calculated [M+H]$^+$ = 594.3762
Figure 4.6.12: HR-MS of Sequence 14

Figure 4.6.12a: Isotopic peaks for molecular ion peak of 14

Calculated [M+H]$^+$ = 780.5535
Figure 4.6.13: HR-MS of Sequence 15

Calculated [M+H]$^+$ = 708.5535

Figure 4.6.13a: Isotopic peaks for molecular ion peak of 15
Figure 4.6.14: HR-MS of Sequence 16

Figure 4.6.14a: Isotopic peaks for molecular ion peak of 16
Figure 4.6.15: HR-MS of Sequence 17

Figure 4.6.15a: Isotopic peaks for molecular ion peak of 17

Calculated [M+H]$^+$ = 784.5848
Figure 4.6.16: HR-MS of Sequence 18

Calculated [M+H]$^+$ = 712.5848

Figure 4.6.16a: Isotopic peaks for molecular ion peak of 18
Figure 4.6.17: HR-MS of Sequence 19

Figure 4.6.17a: Isotopic peaks for molecular ion peak of 19

Calculated [M+H]^+ = 640.5848
4.6.2.3 ¹H-NMR based characterization of selective sequences

Sequence 11: \([\text{C}_{28}\text{H}_{56}\text{N}_{4}\text{O}_{4}]\cdot\text{2CF}_3\text{COOH}; ¹H \text{ NMR (400 MHz, DMSO-}d_6\text{)}: 8.04 (d, J = 8 \text{ Hz, 1H}), 7.96 (d, J = 8 \text{ Hz, 1H}), 7.78 (\text{brs, 4H}), 4.27-4.29 (m, 1H), 4.04-4.05 (m, 1H), 2.75 (\text{brs, 4H}), 2.10-2.06 (m, 2H), 1.73-1.67 (m, 2H), 1.57-1.52(m, 6H), 1.46 (t, J = 6.6 \text{ Hz, 2H}), 1.25-1.21 (\text{brs, 30H}), 0.83 (t, J = 6.5 \text{ Hz, 3H}).

Sequence 16: \([\text{C}_{35}\text{H}_{69}\text{N}_{7}\text{O}_{3}]\cdot\text{4CF}_3\text{COOH}; ¹H \text{ NMR (400 MHz, DMSO-}d_6\text{)}: 8.72 (\text{brs, 2H}), 8.04-8.01 (m, 3H), 7.84 (\text{brs, 9H}), 5.34-5.30 (m, 2H), 4.24-4.18 (m, 4H), 3.08-3.05 (m, 2H), 2.97-2.94 (m, 2H), 2.88-2.85 (m, 4H), 2.75-2.70 (\text{brs, 4H}), 2.11-2.09 (m, 2H), 2.02-1.97 (m, 2H), 1.91-1.86 (m, 2H), 1.66 (\text{brs, 2H}), 1.52-1.44 (m, 8H), 1.44-1.38 (m, 6H), 1.22 (\text{brs, 16H}), 0.83 (t, J = 6.5 \text{ Hz, 3H}).

Sequence 17: \([\text{C}_{47}\text{H}_{73}\text{N}_{4}\text{O}_{3}]\cdot\text{2CF}_3\text{COOH}; ¹H \text{ NMR (400 MHz, DMSO-}d_6\text{)}: 10.8 (\text{brs, 2H}), 7.91 (d, J = 8 \text{ Hz, 1H}), 7.79 (d, J = 8 \text{ Hz, 1H}), 7.72-7.69 (m, 1H), 7.49 (\text{dd, } J = 8 \text{ Hz, }
2H), 7.29 (d, J = 8 Hz, 2H), 7.06 (d, J = 4Hz, 2H), 7.04-7.0 (m, 2H), 6.95-6.91 (m, 2H), 4.47-4.41 (m, 2H), 3.02-2.96 (m, 6H), 2.83-2.80 (m, 3H), 2.74 (t, J = 7.3 Hz, 2H), 2.61-2.59 (m, 2H), 1.96 (t, J = 7.3, 2H), 1.72 (pentet, J = 7.3 Hz, 2H), 1.33-1.29 (m, 2H), 1.27-1.21 (m, 4H), 1.12 (brs, 22H), 1.12-1.05 (m, 6H), 0.98 (d, 4H), 0.83 (t, J = 6.5 Hz, 3H).

Sequence 18: [C$_{47}$H$_{73}$N$_4$O$_3$].3CF$_3$COOH; $^1$H NMR (400 MHz, DMSO-$d_6$): 10.82 (s, 1H), 8.03 (d, J = 8Hz, 1H), 7.94 (d, J = 7.3 Hz, 1H), 7.76 (m, J = 5.3 Hz, 1H), 7.55 (d, J = 8 Hz, 1H), 7.29 (d, J = 8 Hz, 1H), 7.1 (s, 1H), 7.03 (t, J = 6.6 Hz, 1H), 6.96-6.92 (m, J = 7.3 Hz, 1H), 4.48 (m, 1H), 4.19 (m, 1H), 3.14-3.12 (m, 2H), 3.10-3.08 (m, 4H), 3.01-2.99 (m, 2H), 2.85-2.81 (m, 4H), 2.76-2.72 (m, 4H), 2.02 (t, J = 7.3 Hz, 2H), 1.79-1.75 (m, 2H), 1.74-1.68 (m, 2H), 1.54-1.44 (m, 4H), 1.39-1.36(m, 4H), 1.31 (brs, 22H), 1.25-1.21 (m, 6H), 1.14-1.07 (m, 4H), 0.83 (t, J = 6.9 Hz, 3H).

Sequence 19: [C$_{35}$H$_{73}$N$_7$O$_3$].4CF$_3$COOH; $^1$H NMR (400 MHz, DMSO-$d_6$): 7.99-7.97 (m, 3H), 7.82 (brs, 9H), 4.23-4.17 (m, 2H), 3.2 (brs, 2H), 3.07-3.01 (m, 2H), 2.93 (t, J = 7.3 Hz, 2H), 2.87-2.83 (t, J = 8Hz, 4H), 2.74 (brs, 4H), 2.44 (brs, 2H), 2.11-2.08 (m, 2H), 1.85 (pentet, J = 8.0 Hz, 2H), 1.65 (brs, 2H), 1.51 (brs , 6H), 1.47-1.39 (m , 4H), 1.27-1.21 (brs, 28H), 0.83 (t, J = 6.5 Hz, 3H).
4.6.3 Antimicrobial activity of designed sequences

Antibacterial activity of designed sequences against a range of Gram-positive and Gram-negative bacterial strains was determined using serial broth dilution method (Table 4.2).

Table 4.2: Antibacterial activity of sequences against Gram-positive and Gram-negative bacterial strains

<table>
<thead>
<tr>
<th>Sequences</th>
<th>MIC (µg/mL)</th>
<th>Gram-negative bacteria</th>
<th>Gram-positive bacteria</th>
<th>% hemolysis (at 62.5 µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E.coli</td>
<td>P.aeruginosa</td>
<td>A. baumannii</td>
<td>S.aureus</td>
</tr>
<tr>
<td>1</td>
<td>&gt;227.2</td>
<td>ND</td>
<td>ND</td>
<td>113.6</td>
</tr>
<tr>
<td>2</td>
<td>&gt;227.2</td>
<td>ND</td>
<td>ND</td>
<td>&gt;227.2</td>
</tr>
<tr>
<td>3</td>
<td>&gt;227.2</td>
<td>ND</td>
<td>ND</td>
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<td>&gt;227.2</td>
<td>&gt;227.2</td>
<td>&gt;227.2</td>
<td>&gt;227.2</td>
</tr>
<tr>
<td>5</td>
<td>&gt;227.2</td>
<td>&gt;227.2</td>
<td>&gt;227.2</td>
<td>&gt;227.2</td>
</tr>
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<tr>
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<td>&gt;227.2</td>
<td>&gt;227.2</td>
<td>&gt;227.2</td>
<td>113.6</td>
</tr>
<tr>
<td>8</td>
<td>28.4</td>
<td>113.6</td>
<td>&gt;227.2</td>
<td>56.8</td>
</tr>
<tr>
<td>9</td>
<td>&gt;227.2</td>
<td>ND</td>
<td>&gt;227.2</td>
<td>227.2</td>
</tr>
<tr>
<td>10</td>
<td>&gt;227.2</td>
<td>113.6</td>
<td>ND</td>
<td>113.6</td>
</tr>
<tr>
<td>11</td>
<td>113.6</td>
<td>ND</td>
<td>56.8</td>
<td>7.1</td>
</tr>
<tr>
<td>12</td>
<td>56.8</td>
<td>113.6</td>
<td>ND</td>
<td>56.8</td>
</tr>
<tr>
<td>13</td>
<td>&gt;227.2</td>
<td>&gt;227.2</td>
<td>&gt;227.2</td>
<td>113.6</td>
</tr>
<tr>
<td>14</td>
<td>22.7</td>
<td>7.1</td>
<td>28.4</td>
<td>7.1</td>
</tr>
<tr>
<td>15</td>
<td>7.1</td>
<td>14.2</td>
<td>56.8</td>
<td>3.5</td>
</tr>
<tr>
<td>16</td>
<td>3.5</td>
<td>7.1</td>
<td>56.8</td>
<td>3.5</td>
</tr>
<tr>
<td>17</td>
<td>&gt;227.2</td>
<td>113.6</td>
<td>56.8</td>
<td>7.1</td>
</tr>
<tr>
<td>18</td>
<td>28.4</td>
<td>14.2</td>
<td>28.4</td>
<td>3.5</td>
</tr>
<tr>
<td>19</td>
<td>7.1</td>
<td>14.2</td>
<td>28.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>&gt;227.2</td>
<td>&gt;227.2</td>
<td>&gt;227.2</td>
<td>113.6</td>
</tr>
<tr>
<td>Spermidine</td>
<td>&gt;227.2</td>
<td>&gt;227.2</td>
<td>&gt;227.2</td>
<td>113.6</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.3</td>
<td>0.3</td>
<td>ND</td>
<td>0.3</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>0.7</td>
<td>0.7</td>
<td>ND</td>
<td>15.5</td>
</tr>
</tbody>
</table>

Out of the sequences 1-3, sequence 1 exhibited MIC at 113.6 µg/mL against S. aureus. Sequences 4, 5 and 9 were devoid of antibacterial activity, whereas sequences 6 and 7
showed MIC at 113.6 µg/mL against *S. aureus* and *B. subtilis*. Sequence 8 with +1 charge showed moderate activity against tested strains with MIC in the range of 28.4-113.6 µg/mL. Sequence 11 with +1 charge and stearic acid tagging showed good antibacterial activity against Gram-positive bacterial strains with MIC in the range of 7.1-14.2 µg/mL. Sequence 12 with HPPA tagging showed moderate activities against almost all the tested strains with MIC in the range of 56.8-113.6 µg/mL. Sequence 13 showed activity against *S. aureus* and *B. subtilis* with MIC at 113.6 µg/mL. Percentage of acetonitrile from RP-HPLC elution profile data as indicator for hydrophobicity along with MIC data showed that the designed sequences with a threshold of acetonitrile (50%) with single positive charge (8 and 11) showed good antibacterial activity (Table 4.2).

Similar to a previously reported concept of hydrophobicity window for HDCPs [56], we proposed a window of 50% to 70% acetonitrile at RP-HPLC elution (Table 1) below or above this threshold the sequences were either less active or inactive. MIC data on these sequences thus ensured that lipidation with a single positive charge may lead to activity (8 and 11) however; charge alone (1-3) without lipidation is not sufficient to impart appreciable activity. Sequences 14-19 showed broad range of activity against Gram-positive as well as Gram-negative bacterial strains with MIC values in the range of 0.88-28.4 µg/mL. Against *E. coli* and *P. aeruginosa*, MIC values of these sequences were found to be in the range of 3.5-28.4 µg/mL and 7.1-14.2 µg/mL respectively (except sequence 17). Against the four Gram-positive strains tested, all the sequences showed MIC values below 15 µg/mL. Interestingly MIC of these sequences against MRSA was found to be comparable or even better as compared to *S. aureus*. Sequence 17 with 76% acetonitrile at RP-HPLC elution was above the hydrophobicity threshold of 70%, above which the sequences were found to be less active against tested strains.
Linoleic acid and spermidine showed activity against *S. aureus* and *B. subtilis* with MIC at 113.6 µg/mL. It is important to note that in comparison to standard antibiotics tetracycline and polymyxin B, sequences 14-19 showed better MIC against MRSA (except 17), where 14 and 15 were very promising with MIC at 0.88 µg/mL.

### 4.6.4 Evaluation of toxicity of designed sequences

The efficacy of designed sequences as safe antibacterial agents was established based on their interaction with enucleated hRBCs and human keratinocytes (HaCaT cells). Most of the sequences (1-7, 9, 12, 13, 16 and 17) were found to be non-hemolytic up to 62.5 µg/mL. Sequences 8 and 11 were found to be lytic to hRBCs, however on conjugation with spermidine, reduction in hemolysis was observed for N-terminal tagged dipeptides (Table 4.2). Sequences 14 and 16 showed negligible hemolytic activity whereas sequence 15 caused 40% hemolysis at concentrations corresponding to 18-78 times its MIC values against Gram-positive bacterial strains tested in the study. Sequences with stearic acid tagging (18 and 19) were found to be more lytic to hRBC than the linoleic acid counter sequences (15 and 16). Similar to our results, experimental evidences are present in literature that report cationic lipopeptides with saturated fatty acid chain length to be more lytic as compared to their unsaturated counterparts [29].

**Cytotoxicity:** Upon 18 h incubation with designed active sequences 14-19, HaCaT cells showed ~60% viability at 62.5 µg/mL. At the same concentration, sequences 14, 16 and 17 showed ~70%, 100% and 65% cell survival respectively (Figure 4.7). Even up to a high concentration of 250 µg/mL 65% and 78% cell survival was observed for sequences 14 and 16 respectively. In concord with the hemolytic activity data stearic
acid conjugated sequences 17, 18 and 19 showed lower cell survival as compared to
linoleic acid tagged sequences. This data thus confirms no detrimental effects of
sequences 14 and 16 on human keratinocytes even upon incubation for 18 h.

Figure 4.7: Percentage viability of cells upon treatment with different concentrations of
sequences based on MTT assay. Error bar represents mean ±S.D.

4.6.5 Mode of action studies

4.6.5.1 Bactericidal kinetics

HDCPs with a predominant membrane active mode of action are endowed with the
potential to kill bacterial cells within minutes at concentration higher than MIC. In order
to monitor the rapidity of mode of action of designed sequences, we incubated
sequences 14, 15 and 16 with log-phase MRSA at 37 ºC and monitored the course of
change in optical density (OD$_{600}$) at different time intervals. The results showed
inhibition of bacterial growth after 2 h at MIC for sequences 15 and 16 (Figure 4.8.1).
Sequence 14 was found to be less effective at MIC even up to 5 h.
At 4 X MIC all three sequences inhibited bacterial growth from 2h onwards keeping growth arrested till 5 h (Figure 4.8.2). However complete eradication of bacterial growth by any of the tested sequences was not observed up to 5h. At 4 X MIC 16 exhibited the most potent inhibition of growth compared to 14 or 15. Thus in line with HDCPs the designed sequences are capable of inhibiting bacterial growth within hours of initial interactions.
4.6.5.2 Calcein leakage

To examine role of membrane interactions in mode of action for designed sequences 14-19, we first compared their membrane pore forming ability using artificial LUVs comprising of bacterial mimic membranes with composition POPC: POPG [7:3, w/w]. For calcein leakage a similar method was used as described in chapter-3. For sequences 14 and 16, graded release of encapsulated calcein dye up on increasing concentration of sequence was observed where; at the highest concentration tested (7.87 µg/mL) partial leakage of vesicle contents was observed (Figure 4.9). For 15 and 18 rapid increase in fluorescent intensity due to a preferential pore forming ability was observed, sequence 18 caused a burst release of calcein dye at 1.99 µg/mL causing almost 72% leakage instantly. For sequence 17 and 19 moderate levels of leakage were observed where; the maximum extent of leakage reached up to 60% and 51% respectively, at the highest concentration tested.

![Figure 4.9: Concentration-dependent leakage of encapsulated calcein dye from bacterial mimic POPC/POPG LUVs (180 µM) at pH 7.2 measured after 5 min of incubation with different concentrations of designed sequences. Sequences are presented as (▲) 14, (●) 15, (■) 16, (△) 17, (◆) 18, (◇) 19 and (○) tetracycline.](image-url)
This data makes it clear that sequences with stearic acid conjugation lead to a better pore forming ability as compared to linoleic acid tagged sequences. Consistent with previous reports on HDCPs, the Trp-Trp dipeptide sequences 14 and 17 showed a preference to reside at the membrane inter-phase, causing relatively lower levels of calcein leakage [47]. Overall out of the active sequences, sequences 15 and 18 caused formation of large enough pores for complete leakage of encapsulated calcein dye whereas sequences 14 and 16 showed partial leakage and sequences 17 and 19 caused moderate levels of leakage. Standard antibiotic tetracycline showed less than 15% leakage up to the highest concentration tested.

4.6.5.3 Membrane depolarization

The data shows concentration dependent effects and no perfect co-relation between MIC values and extent of membrane depolarization was observed (Figure 4.10).

Figure 4.10: Membrane depolarization ability of designed sequence. MRSA was grown to log phase (OD 600 ~ 0.05) and treated with different concentrations of desired sequences. Sequences are presented as (△) 14, (●) 15, (■) 16, (□) 17, (×) 18, (■□) 19 and (●) tetracycline.
The changes in fluorescence were instant with maximum increase within 2 min after treatment at all concentrations. Only 48% increase in fluorescence intensity was caused by active sequences 14 and 17 even up to a concentration of 19.2 µg/mL. Sequences 15, 16, 18 and 19 lead to significant depolarization of membrane potential leading to almost 78-96% increment in fluorescence at a concentration of 14.5 µg/mL.

Concomitant with depolarization experiment a propidium iodide (PI) uptake experiment was set up to evaluate if depolarization was a lethal event. Similar method was used for PI uptake experiment as described in Chapter-3. The PI uptake data showed loss of viability upon treatment of MRSA with sequences at 19.2 µg/mL (Figure 4.11).

![Figure 4.11: PI uptake experiment on MRSA upon treatment with sequences 14-19. The values represent 5 of PI positive cells upon incubation with different sequences at 19.2 µg/mL. Background values obtained with untreated samples were subtracted from each peptide-treated sample. Events (5000) were counted for each experiment.](image-url)
4.6.5.4 DNA binding

Antimicrobial potency of various classes of DNA binding agents is well reported in literature [57, 58]. To understand mode of action of designed active sequences we accessed DNA binding ability of sequences 14-19 as role of spermidine conjugation in membrane translocation as well as DNA complexation is well known [59].

Sequences 14 and 16 showed DNA retardation at a concentration of 12.5 µg/mL whereas, for 16 slight retardation was observed even at 6.25 µg/mL (Figure 4.12). Sequence 15 showed excellent DNA binding ability with complete retardation at 3.12 µg/mL. Sequences 18 and 19 showed good DNA retardation ability with complete binding at 6.25 µg/mL. Noticeably Trp-Trp containing sequences 14 and 17 showed poor DNA complexation which may in part be ascribed to lower charge density in these sequences.

Figure 4.12: Gel retardation assay, binding was assayed by the inhibitory effect of conjugates on the migration of DNA bands. Various amounts of conjugates were incubated with 100 ng of plasmid DNA at room temperature for 1 h and the reaction mixtures were applied to a 1% agarose gel electrophoresis.
However it was intriguing that DNA binding was influenced by overall structure of the sequences as Trp-Orn dipeptide containing sequences 15 and 18 showed most potent DNA retardation ability as compared to Orn-Orn analogues 16 and 19. Recently it was shown that a novel class of DNA minor groove binders based on benzophenone tetra amide scaffolds showed strong DNA binding ability with membrane active bactericidal mode of action [60]. Therefore unexpectedly DNA binding was not directly involved with mode of action of this class of compounds [61]. On similar lines in the present study upon comparing DNA binding and antimicrobial potency (sequence 14 showed lesser DNA binding though was equipotent as 15) it was evident that there were no direct correlations between DNA retardation and antimicrobial potency/mode of action.

**4.6.5.5 SEM on MRSA**

To have visual evidences for membrane active mode of action, we incubated sequences 14, 15 and 16 with MRSA at concentrations higher than MIC. Disintegration of bacterial membranes due to pore formation or surface swelling have been microscopically observed for membrane active HDCPs at concentrations higher than MIC [62, 63]. Control MRSA cells exhibit bright smooth appearance with intact cell membrane (Figure 4.13.1).
Figure 4.13.1: Control MRSA cells

Figure 4.13.2: MRSA upon treatment with Sequence 14 at 10X MIC
Figure 4.13.3: MRSA upon treatment with Sequence 14 at 10X MIC

Figure 4.13.4: MRSA upon treatment with Sequence 15 at 10X MIC
Figure 4.13.5: MRSA upon treatment with Sequence 15 at 10X MIC

Figure 4.13.6: MRSA upon treatment with Sequence 16 at 10X MIC
Membrane damage and cellular debris as small and round structures were apparent upon treatment of MRSA with sequence 14 (Figure 4.13.2 and 4.13.3). In sequence 15 treated S. aureus cells, cellular protrusions as well as flattened cells due to complete leakage of cellular contents were visible (Figure 4.13.4 and 4.13.5). For sequence 16 deformed outer membranes were observed where, surface blabbing was visible without much leakage of cellular contents (Figure 4.13.6 and 4.13.7). Appearance of such protrusions on the surface of S. aureus caused by HDCPs Gramicidin S and PGLa have recently been reported as well [64]. This data is in agreement with calcein leakage and membrane depolarization experiments where sequence 15 caused cellular damage in the form of membrane disruption whereas 14 and 16 were found to show lesser leakage of encapsulated dye.
4.7 Summary

Antimicrobial peptidomimetics with the desirable features of small size, lower proteolytic cleavage, cell selectivity and reasonable production costs are being looked upon as suitable for clinical optimization. Here out of the designed 19 sequences, we obtained 6 sequences 11, 14, 15, 16, 18 and 19 with potent antibacterial activity. Upon dissecting the designed template to identify features responsible for activity and selectivity, we found that the cationic charge imparted to dipeptides by spermidine in sequences 1-3 was not sufficient per se to show bactericidal properties. Lipidation alone with neutral or negative charge in sequences 6, 7, 9 and 10, also lead to low activity. With unit positive charge, lipidated sequences 8 and 11 exhibited improved activity though with compromised cell selectivity (Table 4.2). However, conjugation of spermidine in sequences 14-19 improved potency as well as cell selectivity. With minimum +2 charges, lipidated sequences 14-19 showed broad range of antibacterial activity.

Since these sequences showed potent activity against MRSA, to have better insights into mode of action of designed sequences we characterized their interactions with S. aureus mimic artificial membranes, intact MRSA and DNA.

Mode of action studies revealed a predominant role of dipeptide sequence in initial binding, bactericidal kinetics and membrane disrupting abilities of designed sequences. For sequence 14 low leakage causing ability, lower levels of membrane depolarization as well as reduced DNA binding ability makes membrane destabilization a less probable mode of action at MIC. A slower bactericidal kinetics of 14 at MIC might be due to different modes of action operative at low concentrations. Slower bactericidal kinetics has previously been reported for HDCPs mimics interfering with vital functions in bacterial cells other than membrane disruption [65]. However in SEM studies at
concentrations 10 X MIC cellular debris and diffused outer bacterial membranes were evident for 14 potentiating a membrane disruptive mode of action (Figure 4.13.2 and 4.13.3). Sequence 15 showed faster leakage of calcein along with membrane depolarization, rapid killing kinetics and excellent DNA binding ability. Therefore this sequence showed clean membrane perturbing mode of action at MIC as well as higher concentrations as was evidenced in SEM images of the treated MRSA (Figure 4.13.4 and 4.13.5). Sequence 16 with rapid bactericidal kinetics, good DNA binding ability caused appreciable damage to membrane potential in MRSA at tested concentrations however moderate levels of leakage causing ability showed that either transient pores were formed or the pores were not large enough to cause leakage of calcein which is evident by surface blabbing observed in SEM studies (Figure 4.13.6 and 4.13.7). A low leakage causing ability in spite of potent membrane depolarization has previously been reported for analogues of HDCP indolicidin [66].

Recalcitrant drug resistant Gram-positive bacterial strains *S. aureus* is a frequent cause of skin and soft tissue infections, sepsis, endocarditis or pneumonia in nosocomial environments as well as in communities. Using simple chemistry and economically viable building blocks FFAs (linoleic acid/ stearic acid), Trp, Orn and spermidine, we obtained six active sequences with broad range of activity against Gram-positive as well as Gram-negative bacterial strains including clinically relevant pathogen MRSA. Sequences 14 and 16 showed excellent cell selectivity and membrane perturbing mode of action at concentrations higher than MIC. These sequences were also able to alter the electrophoretic mobility of DNA which although was not directly related to activity may as well be responsible for further enhanced potency of these sequences due to intracellular mode of action.
4.8 References


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