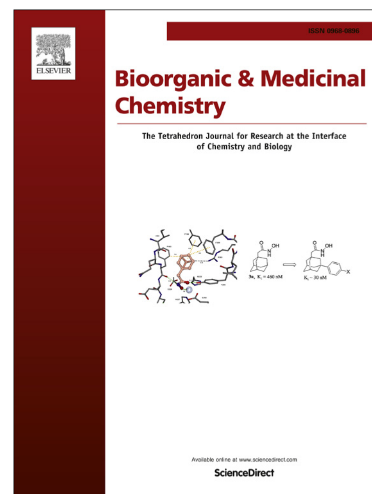
Accepted Manuscript



Development of novel membrane active lipidated peptidomimetics active against drug resistant clinical isolates

Sandeep Lohan, Arneesh kalanta, Praveen Sonkusre, Swaranjit Singh Cameotra, Gopal Singh Bisht

PII: S0968-0896(14)00557-4
DOI: http://dx.doi.org/10.1016/j.bmc.2014.07.041
Reference: BMC 11736

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 30 May 2014
Revised Date: 24 July 2014
Accepted Date: 26 July 2014

Please cite this article as: Lohan, S., kalanta, A., Sonkusre, P., Cameotra, S.S., Bisht, G.S., Development of novel membrane active lipidated peptidomimetics active against drug resistant clinical isolates, *Bioorganic & Medicinal Chemistry* (2014), doi: http://dx.doi.org/10.1016/j.bmc.2014.07.041

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Development of novel membrane active lipidated peptidomimetics active against drug resistant clinical isolates

Sandeep Lohan[a], Arneesh kalanta[a], Praveen Sonkusre[b], Swaranjit Singh Cameotra[b], Gopal Singh Bisht[a]*

[a]*Department of Pharmacy, Jaypee University of Information Technology, Solan-173234, India.*

[b]*Environmental Biotechnology & Microbial Biochemistry, Institute of Microbial Technology, Chandigarh-160036, India.*

[*]**Corresponding Author:** Dr. Gopal Singh Bisht

Department of Pharmacy, Jaypee University of Information Technology, Solan, India.

Email: bishtchem2005@gmail.com

Tel: 91-1792-239336

Fax: +91-1792-245362

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Abstract:

A new series of small cationic lipidated peptidomimetics have been synthesized and found to be highly active against several susceptible as well as drug resistant clinical isolates of bacteria and fungi. All lipidated peptidomimetics do not cause significant lysis of human erythrocytes (HC_{50} > 200 μg/mL). Calcein dye leakage experiment revealed membranolytic effect of LPEP08 which was further confirmed by scanning electron microscopy (SEM). The involvement of intracellular targets as an alternate mode of action was precluded by DNA retardation assay. Additionally, LPEP08 exhibit high proteolytic stability and dose not elicit resistance against drug resistant clinical isolate of *S. aureus*, even after 16 rounds of passaging. These results demonstrate the potential of lipidated peptidomimetics as biocompatible anti-infective therapeutics.

Keywords: peptidomimetics; antimicrobial peptides; lipopeptides; drug resistance; membranolytic

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

1. Introduction

The dramatically increased frequency of infections caused by multi-drug resistant bacterial and opportunistic fungal strains has driven research to expand the arsenal of anti-infective agents. Recently, WHO has recognized the infection caused by multidrug-resistant pathogens viz. methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Staphylococcus aureus* (VRSA), drug resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*as major causes of morbidity and mortality [1, 2]. In addition, majority of the invasive fungal infections caused by *Aspergillus* and *Candida* species emerged as major threat to public health [3]. These trends have accentuate to develop new class of antibiotics possessing novel mode of action as well as different cellular targets compared to conventional antibiotics in order to decrease the possibility of resistance development.

Antimicrobial peptides (AMPs) are found in virtually all multicellular organisms and functionally act as weapons to ward off pathogenic microbes in order to survive and thrive on this planet [4, 5]. In general, AMPs are typically composed of 20-50 amino acid residues and carrying a net positive charge (provided by Arg and Lys residues) with ≈50% hydrophobic residues. The mode of action of AMPs is of particular interest, as it is thought to be non-specific unlike conventional antibiotic drugs (usually directed against a specific cellular receptor) [6, 7]. Mechanically majority of AMPs are bind and permeate cell membranes and others have found to modulate the immune response or have targets within the cell. Taking these findings together, AMPs display unique mode of action that could not deriving the development of resistance [8, 9].

Lipopeptides constitute another class of native AMPs, which are produced non-ribosomally in bacteria and fungi during cultivation on various carbon sources [10-12].Structurally, native lipopeptides are complex molecules composed of aliphatic acid attached to the N-terminus of cationic or anionic peptidic moiety [13]. The mode of lytic action of lipopeptides is via

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

perturbation of the cell membrane by unknown mechanisms which is similar to most of the AMPs [14, 15]. Mechanistically electrostatic interaction between cationic lipopeptides and negative membrane surface charge of bacteria is the initial step of their bactericidal activity. On the other hand in the fungi lipopeptides bind to the negatively charged membrane phosphatidylinositol (PI) and to the negatively charged terminal sialic acid moieties [16, 17]. Clinically used members of this novel class of antimicrobials includes daptomycin (active only toward Gram-positive bacteria), polymyxin B (active only toward Gram-negative bacteria), and echinocandins (β-1,3-D-glucan synthase inhibitors; active only toward fungi) [18]. The major drawback associated with this class of antimicrobials is that the toxic dose is close to the therapeutic dose [18].

It is well documented that conjugation of linear fatty acids to small cationic AMPs resulted into enhancement of antimicrobial potential against Gram-negative and Gram-positive bacteria [19-21]. In addition, chongsiriwatana *et al.* demonstrated that lipidation of small peptoid sequences renders them more selective towards microbes without losing antimicrobial activity [22]. The aliphatic tail region of lipopeptides was found to be essential for their antimicrobial action as it may improves the hydrophobic interaction with cytoplasmic membrane [23]. Thus, the development of small synthetic congeners which mimics integral structural features may overcome some of the drawbacks associated with current lipopeptide antibiotics.

Herein, we reported a new series of small cationic lipidated peptidomimetics synthesized by incorporating 3-amino benzoic acid (3-ABA) as a turn motif between peptidic and aliphatic tail region of the molecules. We confirmed the broad-spectrum antimicrobial potential and low hemolytic action of lipidated peptidomimetics. For an initial investigation of bactericidal mechanism of the lead molecules, calcein leakage experiments on model membranes were performed and which was complemented by observing their effect on intact cells. Finally, the

ACCEPTED MANUSCRIPT

proteolytic stability even in human blood plasma and inability of drug resistant *S. aureus* to develop resistance aid in the future development of small lipidated peptidomimetics to treat fatal infections.

2. Results and discussion

2. 1. Design and synthesis

In a drug development perspective, proteolytic enzyme susceptibility is one of the biggest issues associated with the clinical applicability of peptide based therapeutics. Thus, with aim to develop novel potent biocompatible anti-infective agents, we carried out the synthesis of lipidated peptidomimetics using non-natural amino acid ornithine (Orn) as cationic charge residue. To synthesize lipidated molecules, small peptide scaffold composed of 3 and 4 Orn residues were acylated with fatty acid tail varying from 10 to 18 carbon atoms. It is well known fact about AMPs that the antimicrobial activity is somewhat depends on their secondary structure. For that reason, incorporation of any suitable moiety in the molecular framework which provides a specific turn might boost up the antimicrobial action. The incorporation of constrainedaromatic amino acid (3-ABA) in peptide sequence resulted into the improvement of stability of folded conformation [24, 25] as well as antimicrobial potential [26]. On the basis of these findings, we incorporated 3-ABA as a linker between peptide and hydrophobic tail region (Scheme 1).

2.2. Antimicrobial activity

All synthesized lipidated peptidomimetics were screened against representative Gram-positive and Gram-negative bacteria and fungi, including antibiotic resistant clinical isolates. Lipidated peptidomimetics composed of 3 Orn residues and 10 carbon atoms long aliphatic tail (LPEP01) was found to be almost inactive as antimicrobial. LPEP01 exhibits MIC > 50 µg/mL against all tested pathogens with an exception of having MIC = 31.5 µg/mL against *S. aureus* MTCC 3160 (Table 1). A closer examination of the activity results revealed that

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

lipopeptides with aliphatic chain length ranging from 12 to 18 carbon atoms showed improved antimicrobial activity. Among the lipidated peptidomimetics composed of 3 Orn residues (LPEP01-LPEP05), highest activity was observed in case of lipopeptide having N-terminus myristic acid (LPEP03) with MIC values of 2.5 µg/mL for *E. coli* and *P. aeruginosa* and 3.1 µg/mL for *S. aureus*. In addition, LPEP03 showed good antibacterial activity against antibiotic resistant clinical isolates of *E. coli* (MIC = 4 µg/mL) and *S. aureus* (MIC = 4.5 µg/mL). LPEP03 display moderate activity against *B. subtilis* with MIC = 15.5 µg/mL. Further increase in the length of aliphatic tail resulted into decrease in antibacterial potential (LPEP04 & LPEP05; Table 1). LPEP06, composed of 4 Orn residues and 10 carbon atoms long aliphatic tail, displayed moderate activity (MIC values in the range of 17.5-35 µg/mL) against all tested bacterial strains with an exception of having minimum killing effect against *B. subtilis* (MIC = 100 µg/mL; Table 1). Similar impact of aliphatic chain length on antibacterial potential was observed in case of lipidated peptidomimetics comprised of 4 Orn residues as significant improvement in antibacterial activity was observed for lipidated peptidomimetics bearing bulky aliphatic tail. LPEP08 exhibits maximum antibacterial potential with MIC values of 1.5 µg/mL for *E. coli* and *S. aureus* and 2 µg/mL for *P. aeruginosa* (Table 1). Further increment in aliphatic tail was not fruitful as somewhat decrease in activity was observed in case of lipopeptides (LPEP09 & LPEP10) having 16 and 18 carbon atoms long aliphatic tail. It was interesting to note that all synthesized lipidated peptidomimetics exhibit broad antibacterial activity spectrum with insignificant difference between MIC values against susceptible pathogens and drug resistant clinical isolates.

Lipidated peptidomimetics with comparatively bulky aliphatic tail were found to be more active towards fungal strains. LPEP10 has highest antifungal activity with MIC values of 1.5 µg/mL against *C. albican* and *A. fumigatus,* 2.5 µg/mL for *A. niger*, and 5.5 µg/mL for *C. neoformans*. It was encouraging to observe similar antifungal potential of LPEP10 against

ACCEPTED MANUSCRIPT

drug resistant clinical isolates of *C. albican* (MIC = 1.5 µg/mL) and *A. fumigatus* (MIC = 2 µg/mL; Table 2).

Analysing the antimicrobial activity results of lipidated peptidomimetics, we conclude that activity is depends on the content of both cationic charge and hydrophobic bulk. Importantly, in contrast to the most AMPs or natural lipopeptides that are active either against bacteria or fungi alone [27], lipidated peptidomimetics reported here are highly potent against both bacteria and fungi. The broad-spectrum antimicrobial potential of this library of compounds reflected their candidature to develop as novel anti-infective agents.

2.3. Hemolytic activity

All synthesized lipidated peptidomimetics displayed batter selectivity towards microbial cells (HC_{50} > 200 µg/mL) as summarized in Table 1. Lipidated peptidomimetics with bulky aliphatic tail (16 &18 carbon atoms long aliphatic tail) showed higher affinity towards hRBC as compared to the molecules bearing small fatty acid chain (10, 12, and 14 carbon atoms long aliphatic tail). Thus, it seems that, as the length of aliphatic tail increases, the ability of lipidated peptidomimetics to discriminate between anionic bacterial surface and zwitterionic mammalian membrane decreases. These outcomes were in accordance with our earlier findings [28]. Noticeably, most potent antibacterial lipidated peptidomimetic (LPEP08) has significantly wider therapeutic index (SR = 333), which we defined as $HC_{50}/MIC_{E.c}$ and $HC_{50}/MIC_{S.a}$ (Table 1).

2.4. Bactericidal kinetic assay

In contrast to the majority of the conventional antibiotics, AMPs are usually bactericidal, rather than bacteriostatic [29]. To determine whether this ability is inherent to newly synthesized lipidated peptidomimetics we performed time-kill assay by exposing *E. coli* and *S. aureus* to various concentrations of LPEP08.The results clearly showed that LPEP08 was bactericidal at 4 × MIC and 8 × MIC against both *E. coli* (Figure 1A) and *S. aureus* (Figure

ACCEPTED MANUSCRIPT

1B). At lower concentrations (MIC and 2 × MIC) LPEP08 was able to inhibit the growth. The results also demonstrated the rapid killing effect of LPEP08 at higher concentration levels (4 × MIC and 8 × MIC), as nearly 5-log reduction in the growth of *E. coli* and *S. aureus* was observed within 30 min of incubation (Figure 1A & 1B).

2.5. Biomembrane interaction study using model membranes

In the calcein leakage experiment, LPEP08 and LPEP10 at 4 µg/mL induced a rapid increase in the fluorescence intensity, leading to a 58% and 44% dye release from bacterial membrane mimicking liposomes, respectively. At 20 µg/mL, LPEP08 and LPEP10 caused 93% and 84% leakage, respectively. Whereas, LPEP06 at 4 µg/mL caused only 11% of dye leakage from bacterial membrane mimicking liposomes and it was increased to nearly 67% at highest used experimental concentration (20 µg/mL; Figure 2A). The better membrane interactions of LPEP08 and LPEP10 were also in concord with our activity results. However, at 4 µg/mL LPEP10 caused maximum dye leakage (57%) when incubated with fungal membrane mimicking liposomes. In contrast, at 4 µg/mL LPEP06 and LPEP08 were able to induce 17% and 29% leakage, respectively. At a concentration of 20 µg/mL the percentage of calcein leakage reached 89% for LPEP10 and 77% and 45% dye leakage was observed for LPEP08 and LPEP06, respectively (Figure 2B). Thus, in agreement with earlier literature as well as our activity and selectivity results these outcomes are indicative of the comparatively higher affinity of bulky aliphatic tail conjugated lipidated peptidomimetic (LPEP10) toward zwitterionic fungal membrane [17, 18, 28]. The outcomes of calcein dye leakage experiment indicated that lipidated peptidomimetics damage bacterial and fungal cell membrane mimicking liposomes in somewhat concentration dependant manner.

2.6. Surface disruption effect of lead lipopeptides in intact microbial cells

After knowing the membrane disruption effect of representative lipidated peptidomimetics (LPEP08 & LPEP10), we further confirmed the mechanism of action by visualizing the effect

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

on intact bacterial (*E. coli* and *S. aureus*) and fungal (*A. fumigatus*) cells. SEM, a microscopic technique has been used extensively for the elucidation of interaction of membrane active peptides [30]. Morphological alterations caused by LPEP08 and LPEP10 when incubated with bacterial and fungal cells respectively were observed under SEM. In SEM images we visualized that untreated (control; Figure 3(A1-A3)) microbial cells exhibited bright and smooth surface whereas treatment with lipidated peptidomimetics (LPEP08 and LPEP10) similar to many other membrane active peptides such as melittin [31] resulted into surface blebs and aggregation (Figure 3(B1-B3)). Therefore, SEM data suggested the membrane perturbation effect of LPEP08 and LPEP10 on intact microbial cells which was analogues to model microbial mimicking membranes (as evidenced by calcein dye leakage assay).

2.7. DNA binding assay

It is well documented that native lipopeptide antibiotic polymyxin B mainly active toward Gram-negative bacterial strains [23]. The selective action of polymyxin B reflects the possibility of their intracellular targets. These trends promoted us to determine the involvement of any intracellular targets for antimicrobial action of lipidated peptidomimetics. With this aim we compared the relative affinity of LPEP08 and polymyxin B to bind plasmid DNA. In DNA binding assay, LPEP08 do not caused retardation in the movement of DNA even at the highest used experimental concentration (12 µg/mL). On the other hand, polymyxin B showed DNA binding at 12 µg/mL (Figure 4). These findings suggested a predominant membrane disrupting mode of action for LPEP08, which is different from native lipo-antibiotic polymyxin B.

2.8. Resistance development study

The efficacy of conventionally used antibiotics in treating infections caused by drug resistant pathogens has been diminished as a result of pathogens ability to switch on to an alternate metabolic pathway. It has already been reported that low propensity of resistance

ACCEPTED MANUSCRIPT

development was observed in case of therapeutics that kill bacteria by targeting cell membrane [32]. Therefore, the potential of drug resistant clinical isolate of *S. aureus* to develop resistance was evaluated by serial passages of the bacterial cultures againstLPEP08. Results in figure5confirmedthat bacterial pathogen was unable to develop resistance against LPEP08 and native lipo-antibiotic polymyxin B when compared with ciprofloxacin as there was insignificant change in the MIC after 16 passages.

2.9. Evaluation of proteolytic stability

AMPs composed of genetically coded amino acids can be easily targeted by proteolytic enzymes. Therefore, proteolytic degradation is one of the biggest hurdles in the development of peptide based therapeutics. To investigate the proteolytic stability of our novel structural framework, we examined degradation of LPEP08 by representative protease enzymes trypsin and α-chymotrypsin. To further ensure the proteolytic stability, we tested LPEP08 in human blood plasma also. The results from the stability experiment showed that no degradation was observed for LPEP08 even after 4 days when incubated with α-chymotrypsin. It is well documented that α-chymotrypsin cleaves the peptide bond on the C-terminal side of large lipophilic amino acids (Phe, Trp) [33]. Consequently, the high stability of LPEP08 against α-chymotrypsin might be due to the absence of any bulky hydrophobic residues in the structural framework. Furthermore, LPEP08 was seemingly stable against proteolytic degradation by trypsin, and after 4 days, 90% of the parent molecule was still intact. Mechanistically, trypsin cleaves C-terminal to native cationic amino acids such as Lys and Arg [34]. Thus, the incorporation of non-natural amino acid ornithine as cationic charged residues renders LPEP08 immune toward tryptic degradation. It was further interesting to note that LPEP08 was found to be stable in plasma up to 24 h of incubation and after that it was slowly degraded with approximately 76% of the peptidomimetic remaining after 4 days (Figure 6).

ACCEPTED MANUSCRIPT

The results, thereby collectively demonstrated that the incorporation of ornithine could render LPEP08 immune against proteolytic enzymes.

3. Conclusions

Overall, the present study adds to the repertoire of peptide based anti-infectives with lack of mammalian toxicity and broad activity spectrum against several clinically relevant bacterial and fungal strains. The pore-forming tendency of LPEP08 and LPEP10 in artificial membrane along with weaker DNA-binding suggests their predominant membrane disrupting mode of action. More importantly, LPEP08 was found immune to proteolytic degradation in plasma and even drug resistant clinical isolate of *S. aureus* was unable to develop resistance. In vivo efficacy and pharmacokinetic evaluation of lead molecules is currently under progress. Therefore, taken together the advantageous features such as cell selectivity, broad-spectrum antimicrobial properties, proteolytic stability, and most noticeably, no sign of resistance development underscore the potential of LPEP08 for the development of novel antimicrobial agent to treat infections caused by resistant pathogens in near future.

4. Experimental section

4.1. Materials

Rink amide MBHA resin and Fmoc-Protected ornithine (Fmoc-Orn(Boc)-OH)were purchased from Novabiochem (Mumbai, India). Aromatic amino acid 3-amino benzoic acid and Fmoc-Cl were obtained from Spectrochem (Mumbai, India). Fatty acids used for acylation are caprylic acid, lauric acid, palmitic acid (Loba chemie), Myristic acid, and stearic acid (Fluka). Other reagents used for solid phase synthesis of peptidomimetic molecules included N-hydroxybenzotrizole (HOBt), N,N'-diisopropylcarbodiimide (DIC), Piperidine, N,N-dimethylformamide (DMF) (Spectrochem, Mumbai, India), dimethylsulphoxide (DMSO), dichloromethane (DCM), 1,2-Ethanedithiol (Merck, Mumbai, India), and Trifluoro acetic acid (TFA; Loba chemie, Mumbai, India). Calcein, propidium

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

iodide (PI), 4',6-diamidino-2-phenylindole (DAPI) and buffer material were purchased from Sigma-Aldrich (India). All the solvents used for purification were of HPLC grade and obtained from Merck (Mumbai, India). Buffers were prepared in double-distilled water.

4.2. General procedure for the Fmoc protection of 3-amino benzoic acid

To a solution of 3-amino benzoic acid (1.37 g, 10 mmol) in water (35 mL), was added sodium hydrogen carbonate (2.52 g, 30 mmol), and the resulting mixture was cooled to 5°C and it was slowly added with Fmoc-Cl (3.87 g, 15 mmol) as a solution in *para*-dioxane. The resulting mixture was stirred at 0 °C for 1 h and allowed to warm to room temperature overnight. Completion of reaction was monitored by precoated TLC plate. After the completion of reaction water was added to the reaction mixture and the aqueous layer was extracted with ethyl acetate. Then the organic layer was extracted twice with saturated aqueous solution of sodium bicarbonate. The combined aqueous layers were acidified to a pH of 2 with 10% HCl, and then extracted three times with ethyl acetate. The combined organic layers were removed under reduced pressure to isolate title compound. The crude material was used without any further purification.

4.2.1. Fmoc-3-ABA

^{1}H NMR (400 MHz, DMSO d_6) δ: 12.21 (s, 1H), 9.81 (s, 1H), 7.91 - 7.66 (m, 6H), 7.52 - 7.27 (m, 6H), 4.47 - 4.45 (d, J = 8Hz, 2H), 4.26 - 4.23 (t, J = 12Hz, 1H). MALDI-TOF: calcd for $C_{22}H_{17}NO_4$: 359.12, found 360.39.

4.3. General procedure for solid phase synthesis of lipidated peptidomimetics

Lipidated peptidomimetics were synthesized manually following standard Fmoc solid phase protocols using Rink amide-4-methylbenzhydrylamine hydrochloride salt (MBHA) resin (loading 0.79 mmol/g) as solid support [35]. Rink amide resin (100 mg) was washed in CH_2Cl_2 (3 × 2 mL), which is followed by swelling in DMF (3 mL) for 25 min. The Fmoc protecting group of resin was removed by treating with piperidine/DMF (20% v/v) mixture

ACCEPTED MANUSCRIPT

for 10 min, followed by extensive washes with DMF (5 × 2 mL). Ornithine coupling was performed by treatment of deprotected resin with Fmoc-Orn (Boc)-OH (0.14g; 4equiv) in the presence of DIPC/HOBt in DMF. Each successive ornithine coupling step was followed by Fmoc deprotection with piperidine/DMF (20% v/v) mixture. After coupling of 3 (LPEP1-LPEP5) or 4 (LPEP6-LPEP10) ornithine residues, coupling of Fmoc protected 3-amino benzoic acid was performed in the presence 2 equiv of DIPC/HOBt in DMF. Before acylation of amino acid sequence with specific fatty acid fmoc deprotection was performed to generate free amino group. Fatty acid acylation was performed by treatment of fatty acid (4equiv) in the presence 2 equiv of DIPC/HOBt in DMF. After the desired sequences were assembled, the peptidomimetic molecules were cleaved with a 5 mL solution of TFA/H_2O/1,2-Ethanedithiol (95:2.5:2.5) from solid support. The overview of the different synthetic steps involved in the synthesis of lipopeptides is given in Scheme 1.

4.4. Purification

All crude peptidomimetics were analysed on a reversed-phase high performance liquid chromatography (RP-HPLC) using a C_{18}waters column (Spherisorb®, ODS2, 5µm, 4.6 mm × 250mm) at room temperature. A linear gradient of 0.5-60% solvent B (0.05% TFA in acetonitrile) in solvent A (0.05% TFA in water) over 35 min, followed by 60-0.5% solvent B over 10 min was used at a flow rate of 0.5 mL/min. Preparative RP-HPLC was then performed on a Waters column (Spherisorb®, ODS2, 5µm, 20 mm × 250 mm) using 0.5-60% linear gradient of solvent B (0.05% TFA in acetonitrile) in solvent A (0.05% TFA in water) over 35 min, followed by 60-0.5% solvent B over 10 min at a flow rate of 5 mL/min. Electrospray mass spectroscopy (ESI-MS) was used to confirm their molecular weight. Purified HPLC fractions were than lyophilized. See the supporting information for analytical data (Mass spectra, ^{1}H NMR, and HPLC chromatograms).

4.4.1. $CH_3(CH_2)_8$CO-3-ABA-NH-Orn-Orn-Orn-NH_2 (LPEP01)

ACCEPTED MANUSCRIPT

^{1}H NMR (400 MHz, DMSO-d$_6$) δ 8.17 (s, 3H), 7.51 (d, J = 4.0 Hz, 1H), 7.36-7.27 (m, 3H), 7.12 (s, 2H), 4.22 (t, J = 20.0 Hz, 3H), 3.60 (s, 6H), 2.79-2.67 (m, 6H), 2.09 (t, J = 20.0 Hz, 2H), 1.72-1.42 (m, 12H), 1.22-1.11 (m, 16H), 0.82 (t, J = 20.0 Hz,3H). ESI-MS: calcd for $C_{32}H_{56}N_8O_5$: 632.44, found 633.43; Purity (determined by RP-HPLC): 99.67%.

4.4.2. $CH_3(CH_2)_{10}$CO-3-ABA-NH-Orn-Orn-Orn-NH_2(LPEP02)

^{1}H NMR (400 MHz, DMSO-d$_6$) δ 8.26 (s, 3H), 7.81 (d, J = 4.0 Hz, 1H), 7.48-7.42 (m, 3H), 7.14 (s, 2H), 4.22 (t, J = 24.0 Hz, 3H), 3.45 (s, 6H), 2.76-2.63 (m, 6H), 2.11-2.03 (m, 2H), 1.68-1.43 (m, 13H), 1.27-1.15 (m, 19H), 0.82 (t, J = 24.0 Hz,3H). ESI-MS: calcd for $C_{34}H_{60}N_8O_5$: 660.47, found 661.45; Purity (determined by RP-HPLC): 97.34%.

4.4.3. $CH_3(CH_2)_{12}$CO-3-ABA-NH-Orn-Orn-Orn-NH_2(LPEP03)

^{1}H NMR (400 MHz, DMSO-d$_6$) δ 8.47 (s, 3H), 7.62 (d, J = 4.0 Hz, 1H), 7.48-7.38 (m, 3H), 7.12 (s, 2H), 4.25 (t, J = 20.0 Hz, 3H), 3.64 (s, 6H), 2.88-2.73 (m, 7H), 2.09 (t, J = 12.0 Hz, 2H), 1.74-1.43 (m, 14H), 1.28-1.15 (m, 23H), 0.81 (t, J = 8.0 Hz,3H). ESI-MS: calcd for $C_{36}H_{64}N_8O_5$: 688.50, found 689.48; Purity (determined by RP-HPLC): 97.65%.

4.4.4. $CH_3(CH_2)_{14}$CO-3-ABA-NH-Orn-Orn-Orn-NH_2(LPEP04)

^{1}H NMR (400 MHz, DMSO-d$_6$) δ 8.17 (s, 3H), 7.51 (d, J = 4.0 Hz, 1H), 7.36-7.27 (m, 3H), 7.12 (s, 2H), 4.24-4.17 (m, 3H), 3.51 (s, 6H), 2.81-2.77 (m, 6H), 2.10 (t, J = 28.0 Hz, 2H), 1.74-1.53 (m, 15H), 1.27-1.09 (m, 27H), 0.81 (t, J = 12.0 Hz, 3H). ESI-MS: calcd for $C_{38}H_{68}N_8O_5$: 716.53, found 717.51; Purity (determined by RP-HPLC): 99.61%.

4.4.5. $CH_3(CH_2)_{16}$CO-3-ABA-NH-Orn-Orn-Orn-NH_2(LPEP05)

^{1}H NMR (400 MHz, DMSO-d$_6$) δ 8.29 (s, 3H), 7.79 (d, J = 4.0 Hz, 1H), 7.71-7.63 (m, 3H), 7.16 (s, 2H), 4.27-4.17 (m, 3H), 3.49 (s, 6H), 2.87-2.71 (m, 6H), 2.13 (t, J = 24.0 Hz, 2H), 1.78-1.46 (m, 16H), 1.31-1.11 (m, 30H), 0.82 (t, J = 12.0 Hz, 3H). ESI-MS: calcd for $C_{40}H_{72}N_8O_5$: 744.56, found 745.53; Purity (determined by RP-HPLC): 99.62%.

4.4.6. $CH_3(CH_2)_8$CO-3-ABA-NH-Orn-Orn-Orn-Orn-NH_2(LPEP06)

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (s, 4H), 7.84 (d, J = 4.0 Hz, 1H), 7.63-7.58 (m, 3H), 7.14 (s, 2H), 4.27-4.18 (m, 4H), 3.46 (s, 8H), 2.76-2.67 (m, 8H), 2.15-2.05 (m, 2H), 1.73-1.43 (m, 16H), 1.27-1.15 (m, 16H), 0.80 (t, J = 8.0 Hz, 3H). ESI-MS: calcd for $C_{37}H_{66}N_{10}O_6$: 746.52, found 747.48; Purity (determined by RP-HPLC): 98.77%.

4.4.7. $CH_3(CH_2)_{10}$CO-3-ABA-NH-Orn-Orn-Orn-Orn-NH_2(LPEP07)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (s, 4H), 7.93 (d, J = 8.0 Hz, 1H), 7.48-7.43 (m, 3H), 7.13 (s, 2H), 4.27-418 (m, 4H), 3.47 (s, 8H), 2.82-2.66 (m, 8H), 2.16-2.03 (m, 2H), 1.76-1.41 (m, 17H), 1.29-1.09 (m, 19H), 0.82 (t, J = 12.0 Hz, 3H). ESI-MS: calcd for $C_{39}H_{70}N_{10}O_6$: 774.55, found 775.51; Purity (determined by RP-HPLC): 99.14%.

4.4.8. $CH_3(CH_2)_{12}$CO-3-ABA-NH-Orn-Orn-Orn-Orn-NH_2(LPEP08)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.28 (s, 4H), 7.87 (d, J = 8.0 Hz, 1H), 7.73-7.67 (m, 3H), 7.12 (s, 2H), 4.27-4.23 (m, 4H), 3.62 (s, 8H), 2.81-2.74 (m, 8H), 2.17-2.05 (m, 2H), 1.77-1.46 (m, 17H), 1.27-1.12 (m, 22H), 0.79 (t, J = 12.0 Hz, 3H). ESI-MS: calcd for $C_{41}H_{74}N_{10}O_6$: 802.58, found 803.53; Purity (determined by RP-HPLC): 99.06%.

4.4.9. $CH_3(CH_2)_{14}$CO-3-ABA-NH-Orn-Orn-Orn-Orn-NH_2(LPEP09)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (s, 4H), 7.89 (d, J = 4.0 Hz, 1H), 7.48-7.43 (m, 3H), 7.12 (s, 2H), 4.27-4.19 (m, 4H), 3.44 (s, 8H), 2.79-2.73 (m, 8H), 2.13-2.04 (m, 2H), 1.76-1.46 (m, 18H), 1.27-1.13 (m, 25H), 0.80 (t, J = 12.0 Hz, 3H). ESI-MS: calcd for $C_{43}H_{78}N_{10}O_6$: 830.61, found 831.37; Purity (determined by RP-HPLC): 99.62%.

4.4.10. $CH_3(CH_2)_{16}$CO-3-ABA-NH-Orn-Orn-Orn-Orn-NH_2(LPEP10)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (s, 4H), 7.84 (d, J = 4.0 Hz, 1H), 7.64-7.57 (m, 3H), 7.13 (s, 2H), 4.26-4.17 (m, 4H), 3.49 (s, 8H), 2.81-2.73 (m, 8H), 2.19-2.05 (m, 2H), 1.73-1.46 (m, 19H), 1.26-1.11 (m, 28H), 0.81 (t, J = 12.0 Hz, 3H). ESI-MS: calcd for $C_{45}H_{82}N_{10}O_6$: 858.64, found 859.61; Purity (determined by RP-HPLC): 99.68%.

4.5. Antibacterial activity

ACCEPTED MANUSCRIPT

The antibacterial susceptibility testing of lipidated peptidomimetics was carried out by using a modification of the Clinical Laboratory Standard Institute (CLSI) micro dilution broth assay [36]. Briefly, the inoculums were prepared from mid-logarithmic phase bacterial cultures. Each well of 96-well polypropylene microtiter plate (SIGMA) was inoculated with 90 μL of bacterial suspension (10^5 CFU/mL) in Mueller-Hinton broth (HIMEDIA). Then 10 μL of serial 2-fold diluted test samples in 0.001% acetic acid and 0.2% bovine serum albumin (SIGMA) was added to the wells of microtiter plate. The microtiter plates were incubated overnight with agitation at 37 °C and absorbance was read at 600 nm after 18 h. Cultures (approximately 10^5 CFU/ mL) without test sample were used as positive control. Uninoculated Mueller-Hinton broth was used as negative control. The tests were carried out in triplicate. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of peptide that completely inhibits growth.

4.6. Antifungal Activity

The antifungal activity of the lipidated peptidomimetics was examined using the conditions of the National Committee for Clinical Laboratory Standards (NCCLS) document M27-A. The lipidated peptidomimetics were tested in sterile 96-well plates (BD Falcon Microtest tissue culture plate) in a final volume of 200 μL as follows: 100 μL of a suspension containing fungi at a concentration of 2.5×10^3 cfu/mL in Roswell Park Memorial Institute (RPMI) 1640 medium (with L-glutamine, without glucose and $NaHCO_3$, buffered to pH 7.0 with 0.165 M MOPS) was added to 100 μL of water containing lipidated peptidomimetics in serial 2-fold dilutions.. The fungi were incubated for 24 h (*Aspergillus fumigatus* and *Aspergillus niger*) or 48-72 h (*Candida albicans* and *Cryptococcus neoformans*) at 37 °C in a new brunswick scientific incubator shaker. Growth inhibition was determined by measuring the absorbance at 600 nm in a microplate autoreader (Bio-tek Instruments). The antifungal activity is expressed as the MIC, the concentration at which no growth was observed.

ACCEPTED MANUSCRIPT

4.7. Hemolysis assay

Hemolytic activity measurements were performed on all synthesized lipidated peptidomimetics. Freshly drawn human red blood cells (hRBC) were centrifuged for 15 min at 1500 rpm to remove the buffy coat and washed three times with phosphate buffer saline (35 mM phosphate buffer, 150 mM NaCl pH 7.2). 100 µL of the hRBC suspended 4% (v/v) in PBS was plated into sterilized 96-well plates and then 100 µL of lipidated peptidomimetic solutions (serial 2-fold dilution in PBS) were added to each well to reach a final volume of 200 µL on 96-well plates. The plates were incubated for 1 h at 37 °C without agitation and centrifuged at 1000 g for 5 min. The supernatant (100 µL) in each well was transferred to new 96-well plates. Hemolysis was monitored by measuring the absorbance of the released hemoglobin at 440 nm using ELISA plate reader (Bio-Rad). Percent hemolysis was calculated by the following formula:

Percentage hemolysis = $100[(A-A_0)/(A_t-A_0)]$

Where, A represents absorbance of peptide sample at 440 nm and A_0 and A_t represent zero percent and 100% hemolysis determined in PBS and 1% Triton X-100, respectively.

4.8. Bactericidal kinetics

The bactericidal kinetics studies of lipidated peptidomimetics was performed on *S. aureus* (MTCC 3160) and *E. coli* (MTCC 723). The bacterial cells were grown in MHB to mid-log phase (2×10^5 CFU/mL). The cells were either untreated (control) or treated to LPEP08 at MIC, 2 × MIC, 4 × MIC, 8 × MIC and then incubated at 37 °C. Aliquots were removed at different time interval (0, 15, 30, 60, 120, 240 min) and serial 10-fold dilutions were spread on the Mueller-Hinton agar plate and incubated at 37 °C for 18 h to determine the viable bacterial colony. To determine the log reduction of bacterial growth compared to untreated control viable cell counts were plotted on a log scale graph (CFU/mL vs time; Figure 1).

4.9. Calcein dye leakage assay

ACCEPTED MANUSCRIPT

In order to assess ability of the lipidated peptidomimetics (LPEP08 and LPEP10) to cause leakage of liposomal content, calcein encapsulated bacterial (PE/PG; 7:3; w/w) fungal PC/PE/PI/ergosterol (5:2.5:2.5:1, w/w), and hRBC (PC/cholesterol; 10:1; w/w) membrane mimicking large unilamellar vesicles (LUVs) were used (See supplemental data). The induced calcein dye leakage from the LUVs was monitored by measuring the fluorescence intensity at an excitation wavelength of 490 nm and an emission wavelength of 520 nm on a spectrofluorimeter. Lipidated peptidomimetics at various concentrations ranging from 1-20 µg/mL were incubated with liposome for 5 min before fluorescence measurement. For the determination of 100% dye leakage 10 µL of 10% Triton X-100 in 10 mM Tris-HCl buffer (150 mM NaCl, 0.1 mM EDTA) was added to dissolve the liposome. The percentage of dye leakage caused by the peptides was calculated using the formula;

$$\% \text{ Dye leakage} = 100\,[(F-F_0)/(F_t-F_0)]$$

Where F is the fluorescence intensity achieved by the lipopeptides and F_0 and F_t are fluorescence intensities in buffer and with Triton X-100, respectively. All measurements were made in triplicate.

4.10. Scanning electron microscopy (SEM)

Bacterial culture of *E. coli* (MTCC 0723) and *S. aureus* (MTCC 3160) were suspended at 1×10^6 CFU/mL in 10 mM PBS, pH 7.4 supplemented with 100 mM NaCl, and incubated at 37 °C with LPEP08 at 4 × MIC. Intact cells of *A. fumigatus* (2.5×10^7 CFU/mL) were incubated with LPEP10 (4 × MIC) at 30°C for 30 min. Control were run in the absence of test samples. After 30 min the cells were fixed with an equal volume of 4% glutaraldehyde in 0.2 MNa-cacodylate buffer, pH 7.4, for 3 h at 4 °C followed by dehydration with graded series of ethanol and dried the sample in HMDS (hexamethyl disilazane). Coating was done with gold approximately 20 nm thicknesses and observed under scanning electron microscope (Leo 435 VP).

ACCEPTED MANUSCRIPT

4.11. DNA-binding assay

Gel retardation experiments were performed for lead lipidated peptidomimetic (LPEP08) and polymyxin B (as a standard) as described previously [37]. Briefly, 100 ng of the plasmid DNA isolated from *E. coli* PGI/DML02292 (Supplemental data) was mixed with various concentrations of LPEP08 in 20 µL of binding buffer (5% glycerol, 10 mMTris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 20 mM KCl, and 50 µg/mL bovine serum albumin). Reaction mixtures were incubated at room temperature for 1 h. Subsequently, 4 µL of native loading buffer was added (10% Ficoll 400, 10 mM Tris-HCl, pH 7.5, 50 mM EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol), and a 20 µL aliquot subjected to 1% agarose gel electrophoresis in 0.5 × Tris borate-EDTA buffer (45 mMTris-borate and 1 mMEDTA, pH 8.0).

4.12. Resistance development study

We determine the potential of resistant clinical isolates of *S. aureus* to develop resistance against LPEP08. Native lipopeptide antibiotic polymyxin B and conventionally used fluoroquinolon antibiotic ciprofloxacin were also included in the study. The initial MIC values of LPEP08, polymyxin B, and ciprofloxacin against *S. aureus* was obtained as described above. Serial passage and MICs determination were performed in 96 well microtiter plate containing test compound, each over a range of doubling dilution concentrations. After the incubation period of 18 h the entire content of the triplicate wells with concentration of test compound permitting visible growth were used to prepare the bacterial dilution (approximately 2 × 10^5 CFU/mL) for the successive exposure. The experiment was repeated for 16 days.

4.13. Proteolytic digestion assay

The stability testing of lead peptidomimetics (LPEP08) against trypsin and α-chymotrypsin were conducted using a modified version of earlier reported protocol [33]. Briefly, each test

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

sample was dissolved in a 0.1 M NH_4HCO_3 buffer (pH 8.2) to a final concentration of 1 mg/mL. The enzymes (trypsin and α-chymotrypsin) solutions were prepared by dissolving 1 mg of enzyme to 50 mL of 0.1 M NH_4HCO_3 buffer (pH 8.2). The test sample solution (150 µL), enzyme solution (150 µL), and 0.1 M NH_4HCO_3 buffer (1200 µL) were combined and incubated at 37 ºC. Samples of 15 µL were collected at different time intervals, and 100 µL 10% (v/v) formic acid was added to stop the enzyme activity. For every test, a negative control without enzyme was incubated to ensure that whether the degradation was due to the enzyme or other factors. Quantitative analyses of remaining amount of the test samples were performed by using RP-HPLC using a C_{18} waters column (Spherisorb®, ODS2, 5µm, 4.6 mm × 250 mm) at room temperature. Solvents used in this system were: Solvent A, purified water with 0.05% TFA, and solvent B, HPLC grade acetonitrile with 0.05% TFA. The gradient chosen for separation started with an isocratic elution with 95% A and 5% B for 2 min, then a linear gradient to 40% A and 60% B after 3 min. The gradient was increased linearly to 10% A and 90% B after 10 min and was kept isocratic for 2 min. Flow speed was maintained at 0.2 mL/min for all set of experiments.

4.14. Plasma stability study

In vitro stability study of LPEP08 in human blood plasma was performed by using RP-HPLC as described previously [38]. A stock solution of LPEP08 (1 µg/mL) was made by dissolving in water. Freshly collected heparinized blood plasma (1 mL) was added with 50 µL of test sample (LPEP08) stock solution and incubated at 37 °C. Dilution of the human plasma was made in such a way that renders the proteolytic enzymes the limiting factor of the catalytic reaction. After different time intervals 100 µL of the reaction solution was removed and added to 200 µL of 95% ethanol for precipitation of plasma proteins. The cloudy reaction sample is cooled at 4 ºC for 15 min and then centrifuge (18,000 g) for 2 min to pellet the precipitated proteins. The clear supernatant was then analysed using RP-HPLC using a

ACCEPTED MANUSCRIPT

C_{18}waters column (Spherisorb®, ODS2, 5μm, 4.6 mm × 250 mm) at room temperature as described above.

Acknowledgments

GSB thanks Department of science and Technology for financial support (SR/FT/CS-61/2010). We acknowledge the Sophisticated Analytical Instrumentation Facility (SAIF), Punjab University, Chandigarh, India for providing the NMR facility.

ACCEPTED MANUSCRIPT

References:

1) Choi, S.; Isaacs, A.; Clements, D.; Liu, D.; Kim, H.; Scott, R. W.; Winkler, J. D.; DeGrado, W. F. *Proc. Natl Acad. U.S.A.* **2009**, *106*, 6968.
2) Tew, G. N.; Scott, R. W.; Klein, M. L.; Degrado, W. F. *Acc. Chem. Res.* **2009**, *43*, 30.
3) Brakhage, A. A.*Curr. Drug Targets* **2005**, *6*, 875.
4) Zasloff, M. *Nature* **2002**, *415*, 389.
5) Mangoni, M.L.*Cell Mol. Life Sci.* **2011**, *68*, 2157.
6) Chongsiriwatana, N. P.; Patch, J. A.; Czyzewski, A. M.; Dohm, M. T.; Ivankin, A.; Gidalevitz, D.; Zuckermann, R. N.; Barron, A.E. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 2794.
7) Shai, Y.*Biochim. Biophys. Acta* **1999**, *1462*, 55.
8) Fuertes, G.; Gimιnez, D.; Esteban-Martνn, S.; Sanchez-Muρoz, O.L.; Salgado, J. *Eur. Biophys. J.* **2011**, *40*, 399.
9) Lohner, K. *Gen. Physiol. Biophys.* **2009**, *28*, 105.
10) Balkovec, J. M. *Expert Opin. Invest. Drugs* **1994**, *3*, 65.
11) De Lucca, A.J.; Walsh, T. J. *Antimicrob. Agents Chemother.* **1999**, *43*, 1.
12) Raaijmakers, J. M.; De Bruijn, I.; De Kock, M. J. *Mol. Plant Microbe. Interact.* **2006**, *19*, 699.
13) Jerala, R. *Expert Opin. Invest. Drugs* **2007**, *16*, 1159.
14) Straus, S.K.; Hancock, R. E. *Biochim. Biophys. Acta* **2006**, *1758*, 1215.
15) Epand, R. M. *Biopolymers* **1997**, *43*, 15.
16) Toniolo, C.; Crisma, M.; Formaggio, F.; Peggion, C.; Epand, R. F.; Epand, R. M. *Cell. Mol. Life Sci.* **2001**, 58, 1179.
17) Avrahami, D.; Shai, Y. *Biochemistry* **2003**, *42*, 14946.
18) Makovitzki, A.; Avrahami, D.; Shai, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 15997.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

19) Majerle, A.; Kidric, J.; Jerala, R. *J. Antimicrob. Chemother.* **2003**, *51*, 1159.

20) Mak, P.; Pohl, J.; Dubin, A.; Reed, M. S.; Bowers, S. E.; Fallon, M. T.; Shafer, W. M. *Int. J. Antimicrob. Agents* **2003**, *21*, 13.

21) Lockwood, N. A.; Haseman, J. R.; Tirrell, M. V.; Mayo, K. H. *Biochem. J.* **2004**, *378*, 93.

22) Chongsiriwatana, N. P.; Miller, T. M.; Wetzler, M.; Vakulenko, S.; Karlsson, A. J.; Palecek, S. P.; Mobashery, S.; Barron, A. E. *Antimicrob. Agents Chemother.* **2011**, *55*, 417.

23) Hu,Y.; Amin, M. N.; Padhee, S.; Wang, R. E.; Qiao, Q.; Bai, G.; Li, Y.; Mathew, A.; Cao, C.; Cai, J. *ACS Med. Chem. Lett.* **2012**, *3*, 683.

24) Ramana Rao, M. H. V.; Kiran Kumar, S.; Kunwar, A. C. *Tetrahedron Lett.* **2003**, *44,* 7369.

25) Lengyel, G. A.; Eddinger, G. A.; Horne, W. S. *Org. Lett.* **2013**, *15*, 944.

26) Lundya, F. T.; Nelson, J.; Lockhart, D.; Greer, B.; Harriott, P.; Marley, J. J. *Mol. Immunol.* **2008**, *45*, 190.

27) Lohan, S.; Bisht, G. S. *Mini Rev. Med. Chem.* **2013**, *13*, 1073.

28) Lohan, S.; Cameotra, S. S.; Bisht, G. S. *Chem. Biol. Drug Des.* **2013**, *82*, 557.

29) Niu,Y.; Wang, R. E.; Wu, H.; Cai, J.*Future Med. Chem.* **2012**, *4*, 1853.

30) Exposito, I. L.; Amigo, L.; Recio, I. *Biochim. Biophys. Acta* **2008**, *1778*, 2444.

31) Park, S. C.; Kim, J. Y.; Shin, S. O.; Jeong, C. Y.; Kim, M. H.; Shin, S. Y.; Cheong, G. W.; Park, Y.; Hahm, K. S. *Biophys. Res. Commun.* **2006**, *343*, 222.

32) Wimley, W. C. *ACS Chem. Biol.* **2010**, *5*, 905.

33) Karstad, R.; Isaksen, G.; Brandsdal, B. O.; Svendsen, J. S.; Svenson, J. *J. Med. Chem.* **2010**,*53*, 5558.

ACCEPTED MANUSCRIPT

34) Svenson, J.; Stensen, W.; Brandsdal, B. O.; Haug, B. E.; Monrad, J.; Svendsen, J. S. *Biochemistry* **2008**, *47*, 3777.

35) Merrifield, R. B. *Science* **1986**, *232*, 341.

36) Wikler, M.A.; Low, D. E.; Cockerill, F. R.; Sheehan, D. J.; Craig, W. A.; Tenover, F. C.; Dudley, M. N. *CLSI (formerly NCCLS)* **2006** M7-A7.

37) Joshi, S.; Bisht, G. S.; Rawat, D. S. Kumar, A.; Kumar, R.; Maiti, S.; Pasha, S. *Biochim. Biophys. Acta* **2010**, *1798*, 1864.

38) Haug, B. E.; Stensen, W.; Kalaaji, M.; Rekdal, Ø.; Svendsen, J. S. *J. Med. Chem.* **2008**, *51*, 4306.

Figure legends

Scheme 1. **Overview of the solid phase synthesis of lipidated peptidomimetics.**

Reagents and reaction conditions (a) Deprotection; 20% piperidine. (b) Coupling; Fmoc-Orn(Boc)-OH, HOBt, DIPC, DMF. (c) Fmoc protection; Fmoc-Cl, $NaHCO_3$ (d) Acylation; Capric acid, Lauric acid, Myristic acid, Palmitic acid, and Stearic acid, HOBt, DIPC, DMF. (e) Cleavage; TFA : 1, 2-Ethanedithiol : H_2O (95:2.5:2.5).

Figure 1: Bactericidal kinetics.

Bactericidal kinetic study of LPEP08 against *S. aureus* (A) and *E. Coli* (B).The data obtained are from two independent experiments performed in triplicate.

Figure 2: **Calcein dye leakage effect of lipidated peptidomimetics.**

LPEP06, LPEP08, and LPEP10 incubated with bacterial membrane mimicking LUVs (A) and fungal membrane mimicking LUVs (B).The values are plotted as mean ± SD obtained from two independent experiments.

Figure 3: Scanning electron micrographs.

E. coli untreated (A1); *E. coli* treated with LPEP08 at 4 × MIC (B1); *S. aureus* untreated (A2); *S. aureus* treated with LPEP08 at 4 ×MIC (B2). *A. fumigatus* untreated (A3); *A. fumigatus* treated with LPEP10 at 4 × MIC (B3).

ACCEPTED MANUSCRIPT

Figure 4: Gel retardation assay.

Binding was assayed by the inhibitory effect of LPEP08 and polymyxin B on the migration of plasmid DNA bands. Various amounts of test samples were incubated with 100 ng of plasmid DNA at room temperature for 1 h and the reaction mixtures were applied to 1% agarose gel electrophoresis.

Figure 5: Resistance development study.

Evaluation of the ability of clinical isolate of Methicillin resistant *S. aureus* (PGI/DML03149) to develop resistance against LPEP08.

Figure 6: In vitro proteolytic digestion assay.

The lipidated peptidomimetic (LPEP08) was incubated with trypsin, α-chymotrypsin and human blood plasma. LPEP08 without enzyme was used as control. Percentage of the remaining LPEP08 was measured using analytical RP-HPLC.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

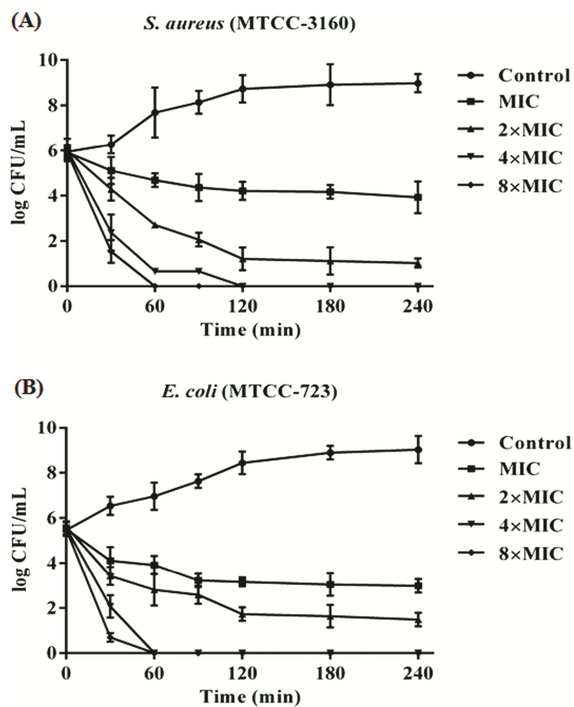


Fig. 1

ACCEPTED MANUSCRIPT

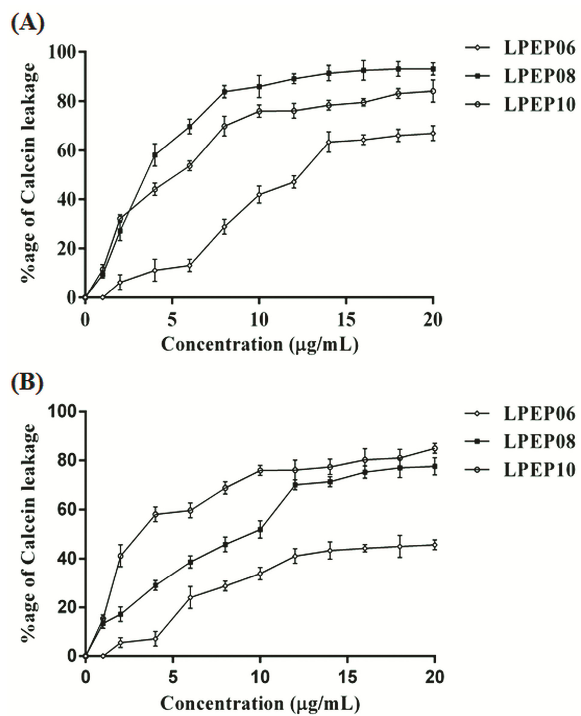


Fig. 2

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

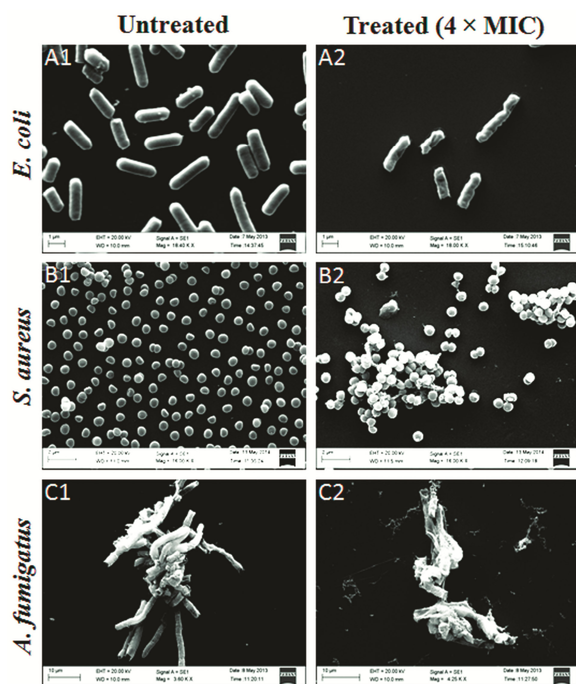


Fig. 3

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

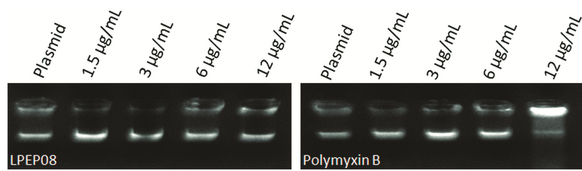


Fig. 4

ACCEPTED MANUSCRIPT

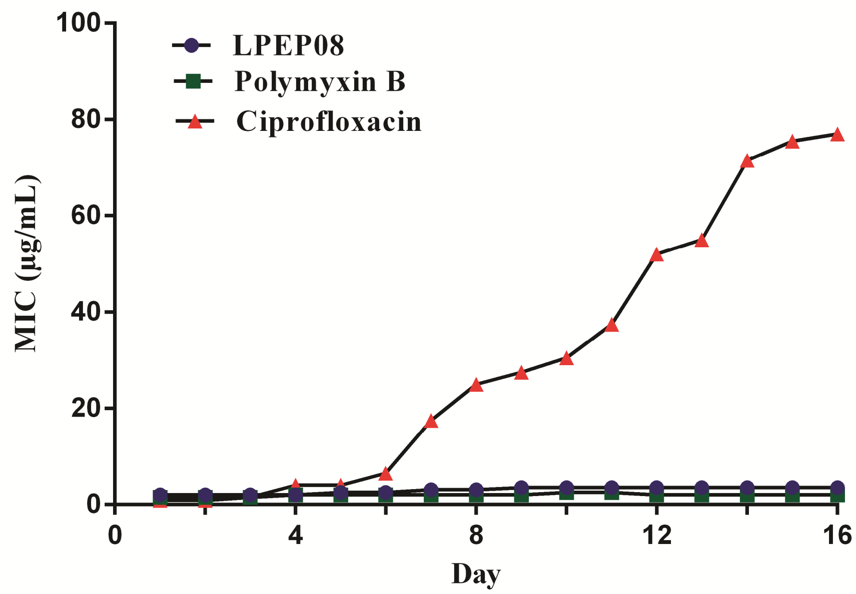


Fig. 5

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

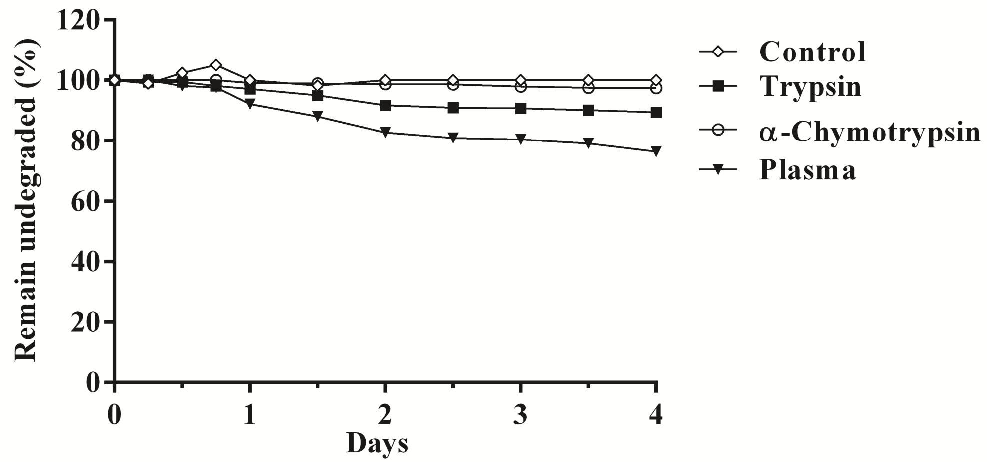
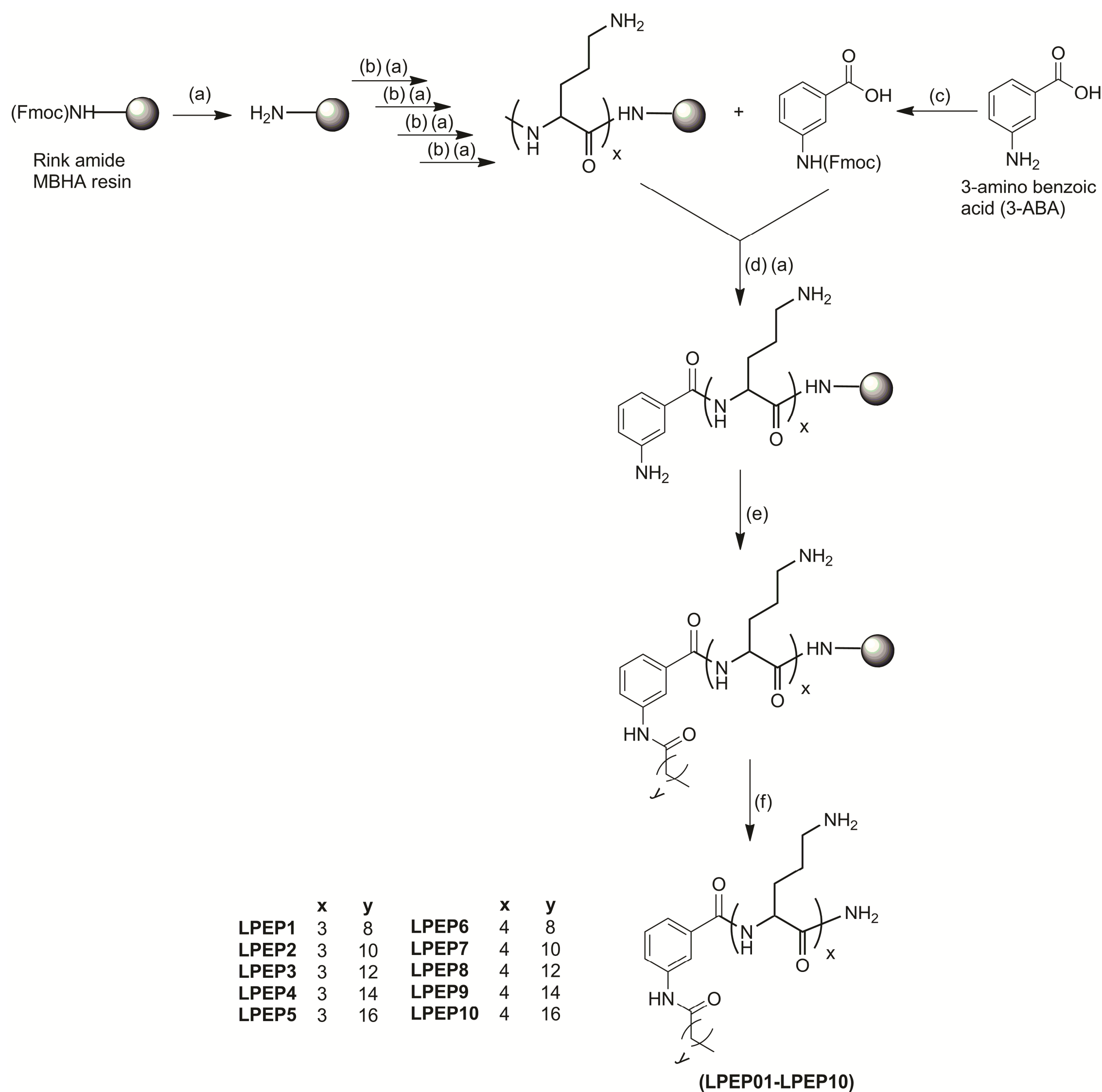


Fig. 6

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT



Scheme 1

ACCEPTED MANUSCRIPT

Code	MIC (µg/mL)						[c]HC_{50} (µg/mL)	Theraputic index	
	E. coli (MTCC 0723)	[a]*E. coli* (PGI/DML02292)	*P. aeruginosa* (MTCC 2295)	*S. aureus* (MTCC 3160)	[b]*S. aureus* (PGI/DML03149)	*B. subtilis* (MTCC 2763)		$HC_{50}/MIC_{E.\,c.}$	$HC_{50}/MIC_{S.\,a.}$
LPEP01	62.5	70	50	31.2	50	125	>1000	-	-
LPEP02	12.5	10.5	4.5	6.25	12	100	900	72	144
LPEP03	2.25	4	2.5	3.1	4.5	15.5	750	333.33	241.93
LPEP04	3.5	4.5	5.5	5	5	20	450	128.57	90
LPEP05	6.25	7.5	10	12.5	10.5	25	250	40	20
LPEP06	17.5	25	25	20	35	100	1000	57.14	50
LPEP07	1.5	5.5	1.5	2.5	3.25	15.5	750	500	300
LPEP08	1.25	3.1	2	1.5	2	10	500	400	333.33
LPEP09	2	3.5	3.1	3	5	20	350	175	116.67
LPEP10	3.5	5	6.25	10	10.5	12.5	200	57.14	20
Ciprofloxacin	0.5	0.75	0.3	0.2	0.9	0.5	ND	ND	ND
Polymyxin B	0.3	0.2	0.15	1.25	1.5	1.5	ND	ND	ND

Table 1: Antibacterial activity and hemolytic activity of lipidated peptidomimetics.

[a]Imipenem resistant clinical isolate of *E. coli*.
[b]Methicillin resistant clinical isolate of *S. aureus*.
[c]HC_{50} is the concentrations of lipidated peptidomimetic molecules at which 50% hemolysis was observed.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Table 2: Antifungal activity of lipidated peptidomimetics

Code	MIC (µg/mL)					
	C. albican (ATCC 24433)	[a]***C. albican*** (PGI/DML0109)	*C. neoformans* (ATCC 2344)	*A. fumigatus* (ATCC 42203)	[a]***A. fumigatus*** (IGMC/LM1/0336)	*A. niger* (ATCC 64028)
LPEP01	62.5	75	75	31.5	30	50
LPEP02	15.5	15	12.5	10	12.5	15
LPEP03	5.5	6.25	10	4.5	6.25	5
LPEP04	3	3.5	3.5	3.5	5	4.25
LPEP05	2.25	2	6.5	2	2.5	3.5
LPEP06	50	50	50	25	25	31.5
LPEP07	5.5	7.5	10.5	5	5.5	6.25
LPEP08	3.1	4	6.25	2.25	4	3
LPEP09	2	3.5	5	1.5	3.5	3.1
LPEP10	1.5	2	5.5	1.5	2	1.5
Amphotericin B	0.5	0.7	0.3	0.3	0.9	0.5

[a]Fluconazole resistant clinical isolates

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

$CH_3(CH_2)_{12}CONH$-3ABA-Orn-Orn-Orn-NH_2
(LPEP08)

SEM

Untreated | Treated (4 × MIC)

E. coli

S. aureus

A. fumigatus

RESISTANCE DEVELOPMENT STUDY

LPEP08
Polymyxin B
Ciprofloxacin

MIC (µg/mL)

Day

Graphical abstract

ACCEPTED MANUSCRIPT