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Authors: Sushil Benke, Hirekodathakallu Thulasiram, and Hosahudya Gopi

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Potent Antimicrobial activity of Lipidated Short α, γ-hybrid peptides

Mr. Sushil N. Benke,[a] Dr. Hirekodathakallu V. Thulasiram,[b] and Dr. Hosahudya N. Gopi*[a]

[a]Department of Chemistry, Indian Institute of Science Education and Research, Dr. Homi Bhabha Road, Pune-41108, [b] CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pune-411008, INDIA.

***Corresponding author**: Dr. Hosahudya N. Gopi, Department of Chemistry, Indian Institute of Science Education and Research, Dr. Homi Bhabha Road, Pune-41108; E-mail: hn.gopi@iiserpune.ac.in

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Abstract: We are reporting potent antimicrobial activity of α,γ-hybrid lipopeptides constituted with 1:1 alternating α- and γ-amino acids. Along with their potent antimicrobial activity against various Gram positive and Gram negative bacteria, these hybrid lipopeptides were found to be less haemolytic. The mechanism of action revealed that these short cationic lipopeptides bind and disrupt the bacterial cell membrane. Further, the time kill kinetics analysis revealed that the potent α,γ-hybrid lipopeptides completely inhibit the bacterial growth in less than 20 minutes. Overall, the promising antimicrobial activity along with less haemolytic activity displayed by the α,γ-hybrid lipopeptides can be further explored to design potent lipopeptide antibiotics.

Keywords: Hybrid lipopeptides; Microbial resistance; Antibiotics; Mode of action; Membrane disruption.

Introduction

The emergence of drug resistant microorganisms poses a great worldwide threat and created an immediate need for the development of novel antibiotics.[1] Antimicrobial resistance can affect any one irrespective of the age and the country, and also jeopardise the progress in medical and health sciences. In this context, the broad spectrum antimicrobial activity shown by the cationic host-defence antimicrobial peptides (AMPs) and native lipopeptides have attracted considerable attention.[2] The AMPs have been serving as defensive agents against various pathogens in plant and animal kingdoms.[3] However, many natural AMPs have been associated with various inherent limitations including higher haemolytic activity, poor selectivity and bioavailability.[4] These intrinsic problems hindered the potential therapeutic applications of natural AMPs. In contrast to AMPs, lipopeptides are produced only by bacteria, fungi and yeast.[5] The native lipopeptides are proved to be non-specific and also associated with high toxicity. The mechanism of action suggested that the cationic AMPs and lipopeptides bind to the bacterial membrane through charge interactions and disrupt the membrane.[6] Extensive efforts have been made to improve the specificity and activity of AMPs and lipopeptides.[7] Shai and colleagues[8] and others[9] have designed a variety of short lipopeptides and examined their potential antimicrobial properties. In addition to the lipopeptides consisting of completely L-amino acids as well as alternating L- and D-amino acids,[10] Schweizer and colleagues demonstrated the antimicrobial activity of short lipo-β-peptides.[11] Besides the utility of β-peptides to mimic protein secondary structures,[12] they have also been explored to design potent antimicrobial candidates.[13] In addition to the β-peptides, potential of γ-peptides built from γ-amino acids have been examined to derive protein secondary structure mimetics.[14] In contrast to β-peptides, the antimicrobial activity of γ-peptides have not been systematically investigated. The advantage

of β- and γ-peptides compared to the α-peptides is that they are proteolytically and metabolically stable.[15] We hypothesize that the conformationally flexible γ- and hybrid γ-peptides can be explored to design potent antimicrobial candidates. Recently, we demonstrated the supramolecular assembly mediated antimicrobial activity of hybrid lipopeptides containing α, β-unsaturated γ-amino acids.[16] Herein, we are reporting the design, synthesis and antimicrobial activity of short α,γ-hybrid lipopeptides, their mechanism of action and haemolytic activity. In comparison with α-peptide counterparts, these short α,γ-hybrid lipopeptides showed potent antimicrobial activity against various bacterial strains and lower hemolytic activity. In addition, the study of time kill kinetics assay revealed that the α,γ-hybrid lipopeptides completely eradicate the bacteria in less than 20 minutes.

Results and Discussion

To examine the antimicrobial activity of short α,γ-hybrid lipopeptides, we have designed nine α,γ-hybrid peptides (**P1**-**P9**) and an α-peptide **P10**. The sequences of these lipopeptides are shown in the Scheme 1. We have utilized both octanoic acid and dodecanoic acid to understand the importance of lipids in antimicrobial activity. Required γ-amino acids (γ-Ala, γ-Leu, γ-Tyr and γ-Phe) were synthesized starting from commercially available α-amino acids as reported earlier.[17] Using solid phase synthesis we have synthesized all peptides on Knorr amide resin. All α,γ-hybrid peptides and the control α-peptide were purified using reverse phase-HPLC on a C_{18} column. The α,γ-lipopeptides and the control α-peptide were subjected to antimicrobial and haemolytic activity.

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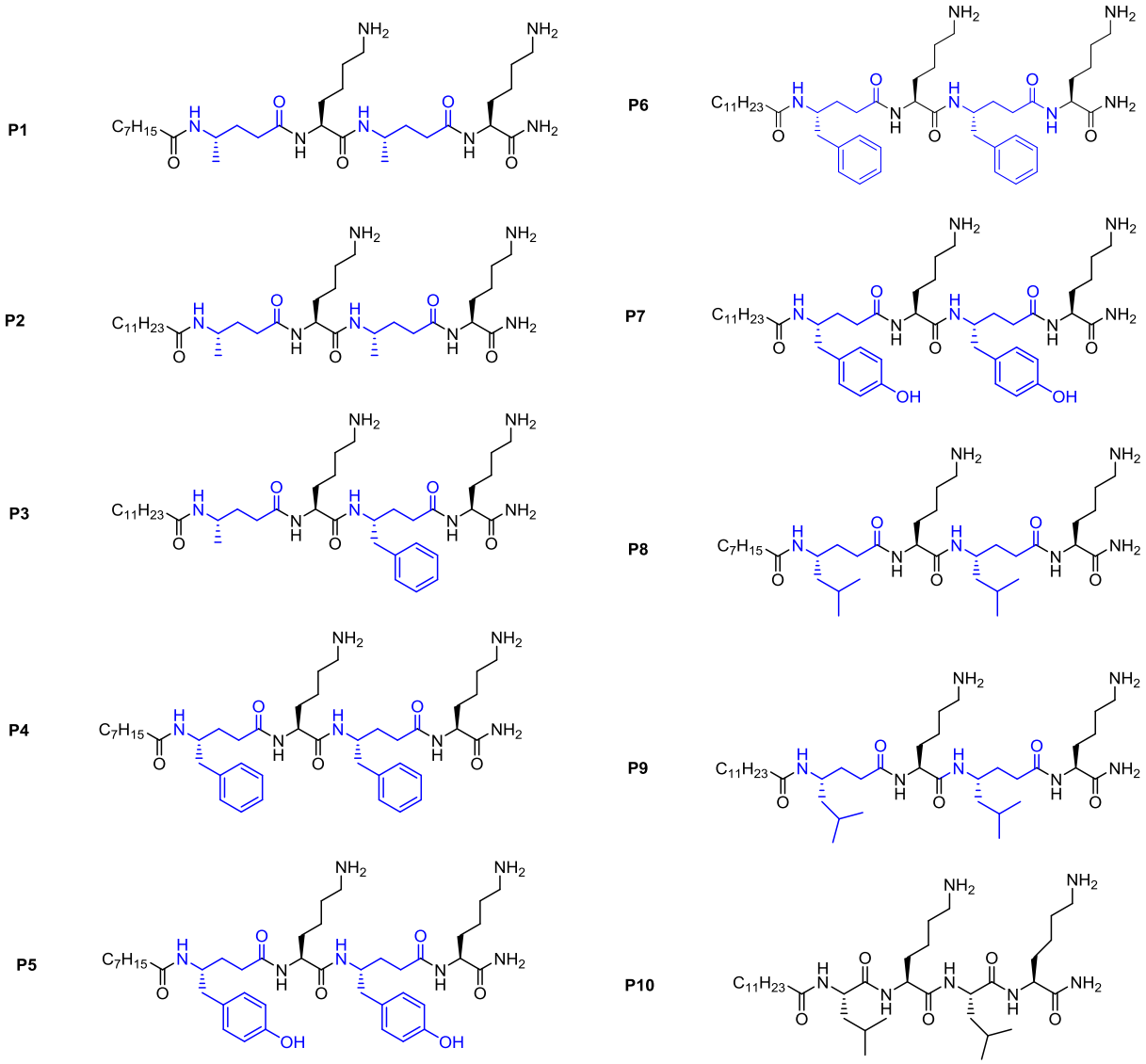

Scheme1. Sequences of α,γ-hybrid lipopeptides (**P1-P9**) and control α-lipopeptide (**P10**)

Table 1. Minimum inhibitory concentration* of α,γ-hybrid lipopeptides in μg/mL (μM)

	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**P10**
E. coli	>250 (418)	163 (249)	32 (43)	250 (333)	>250 (>320)	13 (16)	26 (31)	170 (249)	6 (8.1)	>250 (>366)
E. coli K12	>250 (418)	163 (249)	32 (43)	250 (333)	>250 (>320)	26 (32)	26 (31)	85 (124)	12 (16.2)	>250 (>366)
K. pneumoniae	>250 (418)	81 (124)	16 (21)	125 (166)	97 (124)	6 (8)	3.3 (3.9)	85 (124)	3 (4)	>250 (>366)
P. aeruginosa	>250 (418)	163 (249)	16 (21)	62 (83)	97 (124)	13 (16)	13 (15.5)	42 (62)	6 (8.1)	>250 (>366)
S. aureus	>250 (418)	81 (124)	16 (21)	125 (166)	195 (249)	13 (16)	13 (15.5)	85 (124)	6 (8.1)	>250 (>366)
S. typhimurium	>250 (418)	81 (124)	8 (10.5)	62 (83)	195 (249)	13 (16)	6 (7.8)	42 (62)	6 (8.1)	>250 (>366)
Hemolysis HD_{10}	>250 (418)	135 (206)	93 (127)	>250 (>333)	>250 (>320)	136 (168)	26 (31)	>250 (>367)	183 (248)	>250 (>366)

*The experiments were performed in triplicates and the least concentration of peptide required for complete killing of bacteria is reported as minimum inhibitory concentration (MIC).

We examined the antibacterial activity of short lipopeptides, **P1**-**P10** against Gram negative bacteria *Escherichia coli* (NCIM 2065), *Escherichia coli K12* (NCIM2563), *Klebsiella pneumoniae* (NCIM 2957), *Pseudomonas aeruginosa* (NCIM 5029), *Salmonella typhimurium* (NCIM 2501) and Gram positive *Staphylococcus aureus* (NCIM 5021). The antibacterial activity

of all peptides were carried out using microbroth dilution method in a 96-well microtiter plate. The minimum inhibitory concentration (MIC) was measured in triplicate and average values are given in the Table 1. Among all α,γ-hybrid lipopeptides, peptides coupled with dodecanoic acid showed better antimicrobial activity than the peptides coupled with octanoic acid. Among the dodecanoic acid coupled α,γ-hybrid peptides (**P2**, **P3**, **P6**, **P7** and **P9**), peptide **P9** with γ-Leu residues was found to be the best antimicrobial candidate. It inhibits various Gram –ve and Gram +ve bacteria with low MIC (6 μg/mL) value. The excellent activity of **P9** among all the hybrid peptides motivated us to design α-peptide analogue **P10**. More interestingly, the α-peptide counterpart displayed much higher MIC value under identical conditions. Replacing dodecanoic acid by octanoic acid (**P8**) leads to drastic decrease in the antimicrobial activity of the peptide. Among the C_8-fatty acid containing peptides, **P8** was found to be the best. The α-peptide counterpart of **P9**, peptide **P10** showed poor antimicrobial activity under identical conditions. It is worth mentioning that both amino acids side-chains and fatty acids play crucial role in the antimicrobial activity.

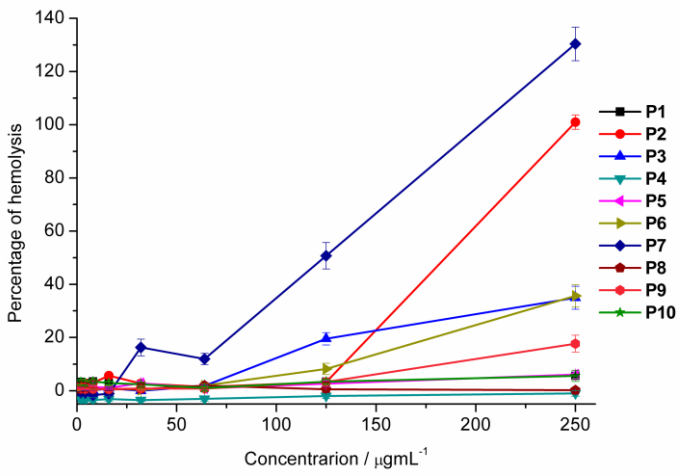

Figure 1. Hemolytic activity of short lipopeptides (The experiments were performed in triplicate and average value of % hemolysis is reported).

The encouraging antimicrobial activity of short α,γ-hybrid lipopeptides motivated us to investigate their haemolytic activity. We subjected all lipopeptides including control α-peptide **P10** for the haemolytic assay. Results of haemolytic activity of all lipopeptides are shown in Figure 1. The HD_{10} values are given in the Table 1. Among the active peptides, peptide **P7** with γ-Tyr residues and **P3** with γ-Ala and γ-Phe were found to most haemolytic in the series. Highly active peptide **P9** showed comparatively less haemolytic activity. The active peptide **P6** with γ-Phe was found to be relatively less haemolytic than **P7**. More importantly, at their MICs these peptides are not haemolytic. Further, the peptides which displayed weak antimicrobial activity (**P1**, **P4**, **P5** and **P8**) were also found to be less haemolytic.

As α,γ-hybrid lipopeptides **P6** and **P9** inhibit the growth of Gram positive and Gram negative bacteria with comparable MIC values across the panel motivated us to examine their mode of action. In order to understand whether these peptides act through membrane disruption similar to other native antimicrobial peptides,[6] we undertook Field-emission scanning electron microscopy analysis (FE-SEM). Figure 2A depicting the FE-SEM images of the bacteria before and after the treatment of peptides **P6** and **P9**. The FE-SEM images clearly suggested the change in morphology of the microorganisms after the treatment of α,γ-hybrid lipopeptides. These results indeed indicate that the α,γ-hybrid lipopeptides disrupt the bacterial membrane. In order to authenticate whether these peptides really disrupt the bacterial membrane, β-galactosidase leakage experiment was performed using *E. coli* containing LacZ reporter gene.[18] The *β*-galactosidase is expected to leak out from the *E-coli* upon disruption of the bacterial cell wall. The peptides **P6** and **P9** were incubated with *E. coli* (TOP 10) and the release of *β*-galactosidase was measured using standard fluorogenic substrate, 4-methylumbelliferyl-*β*-galactosidase. The results are shown in the Figure 2B. Increased fluorescence intensity

indicating the release of β-galactosidase upon the treatment of lipopeptides. In contrast, no β-galactosidase release was observed in the control experiment without lipopeptides. The enzyme leakage assay suggested that these lipopeptides bind and disrupt the bacterial cell membrane.

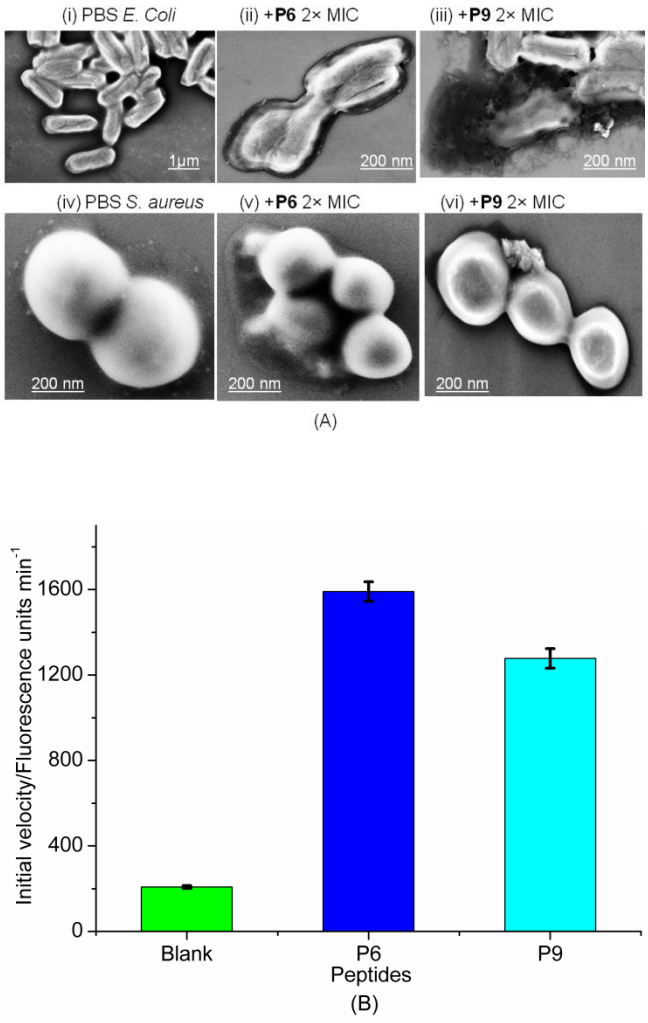

Figure 2. A) FE-SEM images of *bacteria E. coli K12* **(**i) and *S. aureus* (iv) without lipopeptides. Images of *E. coli* after the treatment with lipopeptides **P6**(ii) and **P9**(iii). Images of *S. aureus* after the treatment with lipopeptides **P6**(v)and **P9**(vi). B) Peptide mediated β-galactosidase leakage. The height of the graphs indicates the relative amount of β-galactosidase present in the medium after the treatment of peptides.

The potent antibacterial activity of lipopeptide **P9** inspired us to further evaluate its activity through time kill kinetics assay. This experiment gives the information about the rate at which the lipopeptide is acting on the bacteria. We selected *K. pneumoniae* for time kill kinetics experiment as the peptide displayed better MIC values against this microorganism. The peptide was incubated with the bacterial solution at 2 × MIC (6 μg/mL) and 20 μL of the peptide treated bacterial solution was drawn with increasing time intervals and diluted to ten times using 0.9% saline and plated again on Mueller Hinton agar plates. After incubating for about 24 h, the bacterial colonies were counted. These results were represented in CFU/mL scale. Results of time kill kinetics assay are shown in Figure 3. These results clearly suggested that the lipopeptide **P9** completely eradicate the bacteria within 20 min.

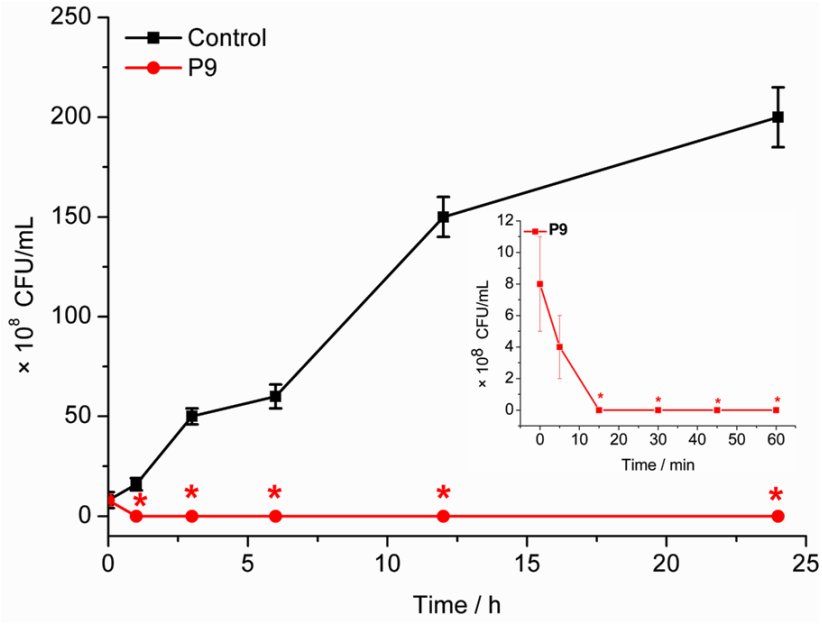

Figure 3. Time−kill kinetics of peptide **P9** (2 × MIC) against *K. pneumoniae* (asterisks correspond to <50 CFU/mL). (The experiments were performed in triplicates and the average CFU values are reported.)

In conclusion, we have shown the potent antibacterial activity and the mechanism of action of short α,γ-hybrid lipopeptides. The hybrid peptides coupled with dodecanoic acids showed better activity compared to the octanoic acid coupled hybrid peptides. Among the active lipopeptides, peptides composed of aromatic residues showed higher haemolytic activity compared to peptides with aliphatic residues. The mechanism of action suggested that these cationic lipopeptides bind and disrupt the bacterial cell wall. Informatively, lipopeptide **P10** with α-amino acid showed weaker antimicrobial activity compared to the α,γ-hybrid lipopeptides. Moreover, the time kill kinetics assay suggested that the lipopeptide **P9** completely inhibit the growth of bacteria within 20 min. Overall, the potent antibacterial activity, mechanism of action, and fast killing of bacteria, less haemolytic activity displayed by the short α,γ-hybrid peptides, particularly peptide **P9**, provided an unique opportunity to further design peptide antibiotics.

Experimental section

General. All reagents, amino acids, solvents were obtained from commercial sources and used without further purification. Peptides were purified through reverse phase HPLC on a C_{18} column using MeOH/H_2O gradient. ^{1}H NMR spectra were recorded on 400 MHz and ^{13}C NMR on100 MHz spectrometer using residual solvent as internal standard (DMSO-d_6 δ_H, 2.5 ppm, δc 39.51 ppm) The chemical shifts (δ) were reported in ppm and coupling constant (*J*) in *Hz*. Mass spectra of the peptides were obtained from the MALDI-TOF/TOF.

Synthesis of *N*-Fmoc-protected γ-amino acids: *N*-Fmoc-protected γ-amino acids were synthesized using reported procedure.[17] Briefly, the *N*-Boc-α, β-unsaturated γ-amino acid (2 mmol) dissolved in ethanol was subjected to double bond reduction using 20% Pd/C and

hydrogen. The progress of the reaction was monitored by TLC. After completion (approximately 2 h), Pd/C was filtered and ethanol was evaporated from the reaction mixture to give *N*-Boc-γ-amino acid as gummy product in a quantitative yield. Further, the Boc group from *N*-Boc-γ gamma amino acid (1 mmol) was deprotected using 5 mL of neat TFA and the free amine was protected again by Fmoc group using Fmoc-OSu (1 mmol) in basic conditions (10% $NaHCO_3$ and THF). The Fmoc-γ-amino acid was utilized for solid phase peptide synthesis without further purification.

Synthesis of peptides

All peptides were synthesized on Knorr amide resin at 0.25 mmol scale using standard Fmoc-chemistry. All coupling reactions were performed using HBTU along with HOBt and monitored by Kaiser Test. Finally, peptides were cleaved from the resin using the combination of TFA: water: thioanisole (98:1:1) and filtered. The cleavage solution was evaporated under reduced pressure to obtain crude product. The crude peptides were purified through reverse phase HPLC on C_{18} column using MeOH/H_2O gradient.

Antibacterial activity

The bacterial strains *Escherichia coli* (NCIM 2065), *Escherichia coli K12* (NCIM2563), *Klebsiella pneumoniae* (NCIM 2957), *Pseudomonas aeruginosa* (NCIM 5029), *Salmonella typhimurium* (NCIM 2501), *Staphylococcus aureus* (NCIM 5021) used for the antibacterial activity were obtined from National Collection of Industrial Microorganisms (NCIM).

The antibacterial activity of peptides were carried out in a 96-well microtiter plate using microbroth dilution method. The cultures of bacteria were incubated over night at 37 °C, and the solution was diluted with sterile MHB (Muller-Hinton broth) medium to a concentration of 10^6

colony forming units/mL. Increasing concentration of peptides **P1-P10** with the volume of 50μL was added to the 50μL bacterial suspension to each well, and incubated for about 18 h at 37 °C. Control experiment was performed without peptides. The inhibition of the growth of bacteria was monitored through measuring the absorbance at 492 nm. The lowest peptide concentration required for the complete killing was defined as the MIC value.

Hemolysis assay:

Hemolytic activity was performed on human red blood cells (hRBCs). The fresh hRBCs collected with EDTA were washed four times with Tris-buffered saline (10 mM Tris, 150mM NaCl, and pH 7.2). The hRBCs were again diluted to 4% v/v with Tris buffer. To the solution of hRBCs (50 μL), increasing concentration of the peptides in Tris buffer were added by keeping the total volume 100 μL. The combined hRBCs and peptides solution was incubated at 37 °C for about 1 h. After 1 h, the solution was centrifuged for about 15 min at 3000 rpm. The supernatant (50 μL) of the each well was transferred to another 96-well plated and diluted with 50 μL of water and measure the absorbance of released hemoglobin at 540 nm. Simple Tris buffer without peptides was used as a negative control and 1% Triton-X was used as a positive control. All experiments were performed in triplicate.

β-Galactosidase leakage from *E.coli* cells:

Membrane deformation studies were performed using *Escherichia coli* (TOP10, Invitrogen) cells containing LacZ reporter gene as reported earlier. Briefly, 90 μL of Luria Bertani broth containing the *E.coli* cells producing β-galactosidase were incubated separately with 10 μL (200 μg/mL) of peptides **P6** and **P9**. After incubating for about 1h, the bacterial solution was centrifuged for10 min at 4000 rpm. The clear supernatant solution (80 μL) was treated with 20

μL (400 μg/mL) of 4-methylumbelliferyl-*β*-galactosidase indicator. The release of the β-galactosidase was monitored for about 1 h by recording the fluorescence emission at 445 nm after exciting the solution at 365 nm. The linear plot of fluorescence versus time gave the initial velocity of the enzyme reaction. The control experiment was performed without peptides.

Time kill kinetics

To understand the rate at which the peptides can completely inhibit bacterial growth we have performed the time kill kinetics assay. The potent peptide **P9** (6 μg/mL, 2 × MIC)was added to the solution of *K. peumoniae* grown in Mueller Hinton broth (approximately 1.8×10^5 CFU/mL) and incubated at 37 °C. From this solution, 20 μL were drawn at 0, 1, 3, 6, 12, and 24 h intervals and diluted 10 times with 0.9% saline. The solution extracted at different time intervals were plated again on Mueller Hinton agar plates and incubated at 37 ^{o}C. Similar experiments were performed at shorter time intervals 0, 10, 20, 30, 45, and 60 min. After 24 h incubation, the bacterial colonies were counted.

FE-SEM Analysis

Peptides **P6** and **P9** (100 μL) at 2 × MIC values were pipetted into a 96-well cell culture plate. To this peptide solution, 100 μL of either *E. coli* or *S. aureus* bacterial solution in Mueller Hinton broth was added. The bacterial concentration was adjusted to 0.1-0.2 optical density at 600 nm. The peptide treated bacterial solution was incubated for about 2 h at 37 °C. The bacterial cells were centrifuged and the pellet was washed with PBS. Using 2.5% glutaraldehyde the cells were fixed and washed with PBS. The bacterial cells were centrifuged and washed with 30%, 50%, 70%, 95%, and 100% graded ethanol solution and finally with water. Finally, the

dried bacterial cells were mounted on a silicon wafer and imaged under a field emission scanning electron microscope after the gold coating.

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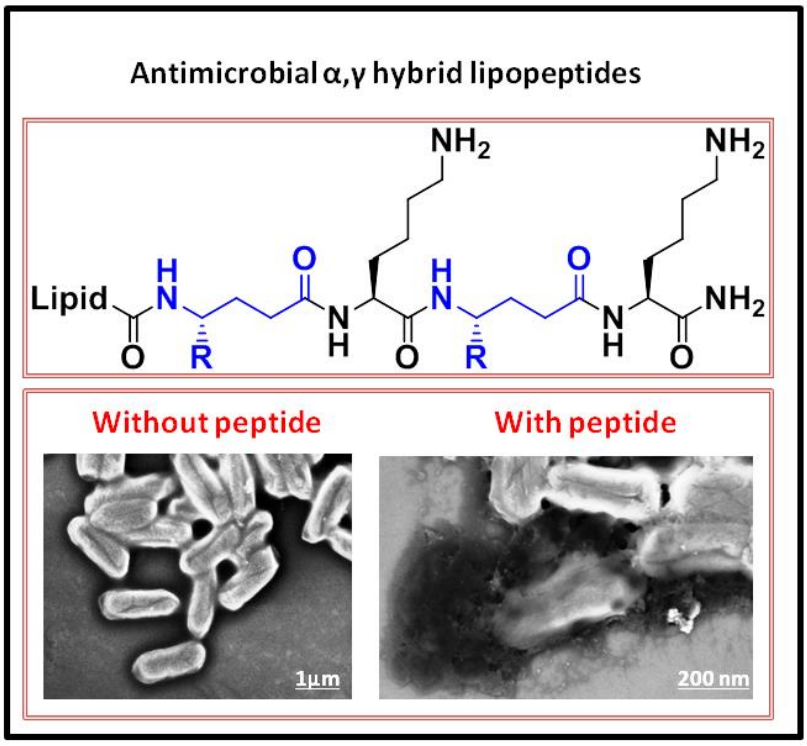

We are reporting the design, synthesis and potent antimicrobial activity of short α,γ-hybrid lipopeptides composed of 1:1 alternating α- and γ-amino acids. Investigation of the mechanism

of action revealed that these short cationic lipopeptides bind and disrupt the bacterial cell membrane.