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Self-Assembly to Function: Design, Synthesis and Broad Spectrum Antimicrobial Properties of Short Hybrid *E*-Vinylogous Lipopeptides

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Abstract: Non-ribosomal *E*-vinylogous γ-amino acids are widely present in many peptide natural products and have been exploited as inhibitors for serine and cysteine proteases. Here, we are reporting the broad spectrum antimicrobial properties and self-assembled nanostructures of various hybrid lipopeptides composed of 1:1 alternating α-and E-vinylogous residues. Analysis of the results revealed that self-assembled nanostructures also play a significant role in the antimicrobial and hemolytic activities. In contrast to the α-peptide counterparts, vinylogous hybrid peptides displayed excellent antimicrobial properties against various bacterial and fungal strains. Peptides that adopted nanofiber structures displayed less hemolytic activity, while peptides that adopted nanoneedle structures displayed highest hemolytic activity.

Introduction

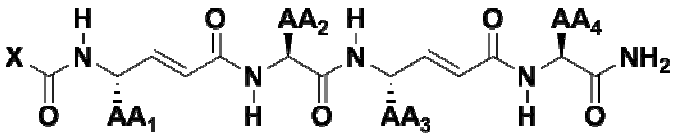
As a consequence of evolution, a large number of bacterial and fungal strains have developed resistance towards the conventional antibiotics, creating an urgent need for the design of new antibiotics with different mode of action. Indeed an overwhelming body of literature over the past several years demonstrated that naturally occurring antimicrobial peptides (AMPs) are evolutionary conserved components of the innate immune response capable of targeting wide range of "multidrug resistant" strains.[1] However, potential therapeutic applications of AMPs have been suffering with several inherent problems including selectivity, toxicity and bioavailability. In this regard, various approaches have been adopted to mimic the cationic and amphiphilic nature of natural AMPs including synthetic peptides,[2] β-peptides, [3] peptoids,[4, 5] polypyridines,[6] polynorboranes,[7] polysaccharides,[8] peptidopolysaccharides,[9] and so on. Lipopeptides are a recently emerged class of AMPs and the broad spectrum of natural lipopeptides has paved way for the development of new class of synthetic analogues.[10] Most of the native lipopeptides isolated from bacteria and fungi are non-specific and highly toxic. However, after the approval of drug daptomycin and the re-emergence of Polymyxin B as potential chemotherapeutic agents for the treatment of microbial infections, these lipopeptides have attracted considerable attention of medicinal chemists.[11] Previous studies of many natural and synthetic hybrid lipopeptides revealed that the antimicrobial activity of lipopeptides mainly depends on the sequence of peptides, length of fatty acid chain and overall hydrophobicity of the peptides.[12] These lipopeptides permeate rapidly and disintegrate the membrane morphology of pathogens.[13] In addition to this, many *N*-terminal fatty acid acylated amphiphilic peptides are self-assembled into cylindrical nanofibers with diameters ranging from nanometers to micrometers, lead to the formation of hydrogels, nanotubes, nanobelts and nanoribbons.[14, 15] The

applications of self-assembled nanostructures of amphiphilic peptide based materials have been found in various fields like bone regeneration and enamel formation.[16]

α, β-Unsaturated γ-amino acids (*E*-vinylogous amino acids) are important components of many biologically active peptides [17] and also have been used as potential candidates for design of inhibitors of serine and cysteine protease.[18] We have been interested in the synthesis and conformational analysis of hybrid peptides containing *E*-vinylogous amino acids.[19, 20] The structural analysis of the *E*-vinylogous amino acids in hybrid peptides reveal that due to the geometrical constraints of the double bonds they prefer to adopt extended β-sheet type of structures.[20, 21] Here, we sought to explore the antimicrobial properties of short hybrid lipopeptides containing geometrically constrained *E*-vinylogous amino acids. Herein, we are reporting the design, antimicrobial activities, self-assembling properties and the mode of action of short 1:1 alternating alpha and *E*-vinyologus hybrid lipopeptides. Systematic investigation of the mode of action reveals that the nature of the self-assemblies of these hybrid lipopeptides play a crucial role in their antimicrobial activities and the mechanism of action. The self-assembly properties associated with the mode of action can be controlled by tuning the hydrophobic threshold of lipopeptides.

Results and Discussion

In order to understand their antimicrobial activity, we designed two series of short hybrid *E*-vinylogous lipopeptides containing C_{12} and C_8 fatty acids. The schematic representation of the short hybrid lipopeptides is shown in Scheme 1. The design is based on the analysis of the natural lipopeptides which generally constitute equal proportions of hydrophobic and hydrophilic residues. The α-amino acids were chosen to be hydrophilic residues while *E*-vinylogous amino acids as hydrophobic residues in short tetrapeptide sequences. All *E*-vinylogous amino acids



Peptide	X	AA1	AA2	AA3	AA4	t_R
S1	$C_{11}H_{23}$	dgAla	Lys	dgAla	Lys	41.14
S2	C_7H_{15}	dgAla	Lys	dgAla	Lys	31.96
S3	$C_{11}H_{23}$	dgTyr	Lys	dgTyr	Lys	38.61
S4	C_7H_{15}	dgTyr	Lys	dgTyr	Lys	30.65
S5	$C_{11}H_{23}$	dgAla	Arg	dgAla	Arg	33.30
S6	C_7H_{15}	dgAla	Arg	dgAla	Arg	31.63
S7	$C_{11}H_{23}$	dgPhe	Lys	dgPhe	Lys	48.19
S8	C_7H_{15}	dgPhe	Lys	dgPhe	Lys	35.60
S9	C_7H_{15}	dgLeu	Lys	dgVal	Lys	37.50
S10	CH_3	dgPhe	Lys	dgPhe	Lys	25.25
S11	C_7H_{15}	Leu	Lys	Val	Lys	39.22

Scheme 1. Schematic representation of the hybrid *E*-vinylogous lipopeptides (dg stands for α, β-dehydro γ-amino acids).

were synthesized using Wittig reaction as reported earlier.[19, 20] All peptides were synthesized by solid phase method on Knorr amide resin and purified through reverse phase HPLC on C_{18} column. Based on the reverse phase HPLC retention time, the order of hydrophobicity of the E-vinylogous hybrid lipopeptides was found to be S7> S1> S3> S9>S8> S5> S2> S6> S4. The lipopeptide with α-amino acids (S11) and *E*-vinylogous hybrid peptide without fatty acid (S10) displayed the retention times 39.22 and 25.25, respectively.

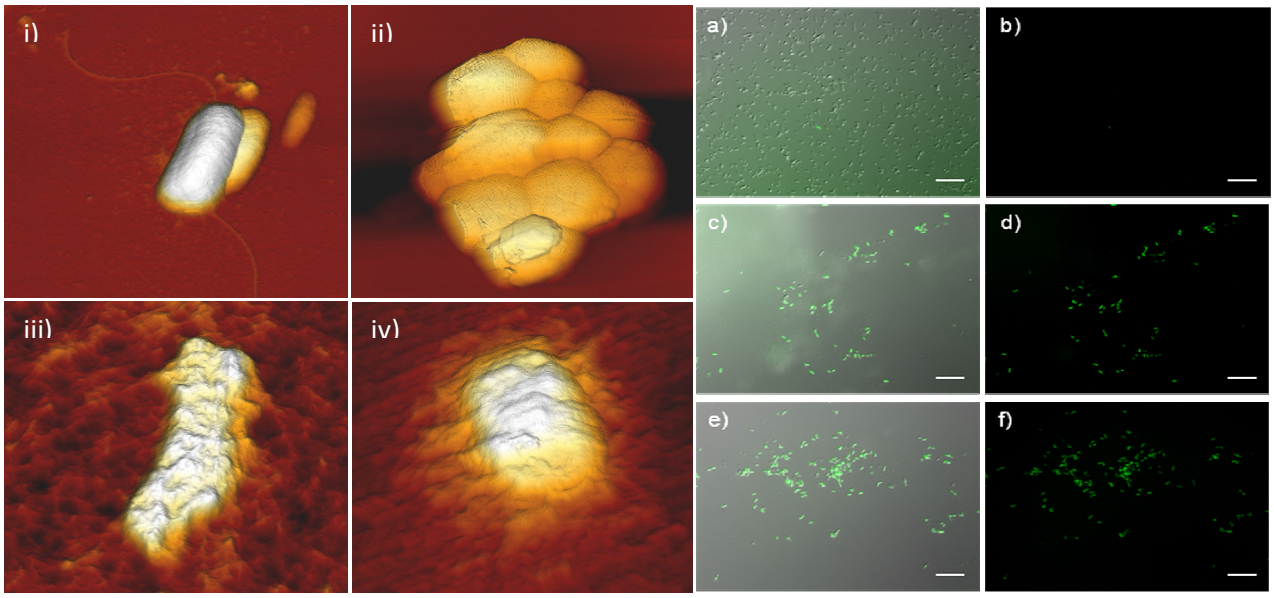
All *E*-vinylogous hybrid peptides with N-terminal C_{12} fatty acid (S1, S3, S5, and S7) and C_8 fatty acid (S2, S4, S6, S8 and S9) along with α-peptide control S11 and the hybrid peptide with N-acetyl group (S10) were subjected to antimicrobial studies. Instructively, except S7, all peptides with C_{12} fatty acids, displayed potent antimicrobial activity with various Gram-negative

Table 1: MICs of vinylogous hybrid lipopeptides (μg/ml)

Microorganisms	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
Escherichia coli	6.25	>100	6.25	25	12.5	>100	25	1.56	12.5	>100	>100
Escherichia coli K12	6.25	>100	6.25	25	12.5	>100	25	1.56	-	>100	>100
Klebsiella pneumoniae	3.12	>100	3.12	50	6.25	>100	25	3.12	25	>100	>100
Pseudomonas aeruginosa	6.25	>100	3.12	50	3.12	>100	25	3.12	50	>100	>100
Staphylococcus aureus	6.25	>100	3.12	50	6.25	>100	25	3.12	50	>100	>100
Salmonella typhimurium	6.25	>100	3.12	50	3.12	>100	25	3.12	50	>100	>100
Candida albicans	3.12	>100	1.56	12.5	3.12	>100	3.12	3.12	25	>100	>100
Candida glabrata	6.25	50	3.12	25	6.25	>100	6.25	3.12	50	>100	>100
Cryptococcus neoformans	6.25	>100	6.25	25	12.5	>100	6.25	3.12	6.25	>100	>100
Saccharomyces cerevisiae	3.12	>100	3.12	12.5	3.12	>100	6.25	3.12	50	>100	>100
Hemolytic HD_{10}	48	58	2.5	54	22	52	<1.56	16	-Nil-	-Nil-	-Nil-

and Gram-positive bacterial strains as well as various fungal strains. Among all lipopeptides in the series of C_8 fatty acid containing hybrid peptides, S8 displayed potent activity against all bacterial and fungal strains while moderate activities were observed for S4, S7 and S9. The MIC values of all the peptides are given in the Table 1. Similar MIC values of the individual peptides

against Gram-positive, Gram-negative and fungal strains suggest the unique mode of action of these lipopeptides. Peptides which showed strong inhibitory action against bacteria also displayed the strong inhibitory action against fungi (S1, S3, S5 and S8). In comparison with S7, S3 containing phenolic –OH groups displayed excellent antimicrobial activity. Interestingly, S8 showed more potent activity than S7, while S4 showed lower potency than that of the C_{12} analogue S3. In order to understand the role of basic residues, we replaced Lys with Arg in the peptides S5 and S6. Results reveal that the peptides containing Arg also displayed similar antimicrobial activities. In comparison with S8, S9 showed moderate activity against both bacteria and fungi. Further the control peptides, hybrid peptide with *N*-acetyl group (S10) and



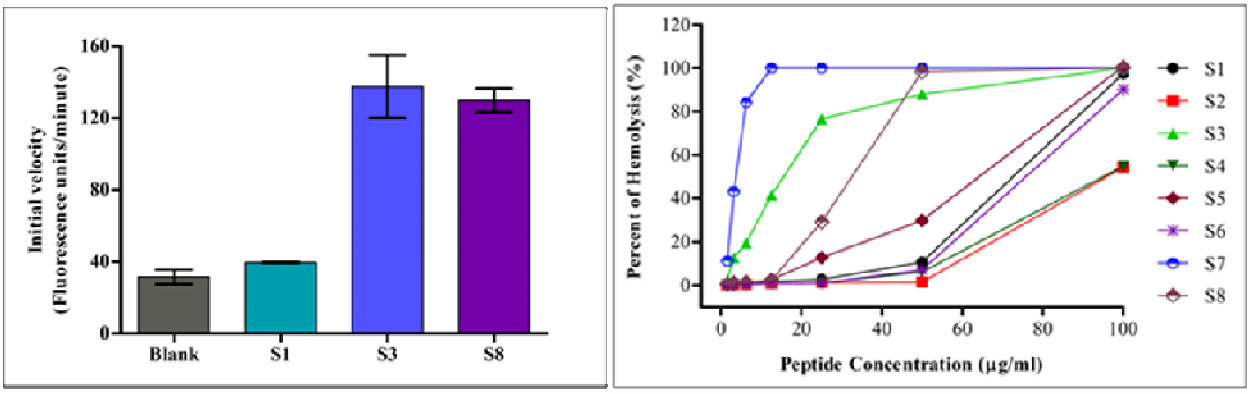
(A) **(B)**

Figure 1*:* A) Atomic Force Microscopy images of *Escherichia coli K12* (**i**) and *Candida albicans* (**ii**) before the treatment of lipopeptides, **iii** and **iv** after the treatment lipopeptide S1, respectively. B) Fluorescence images (FITC) of *E.coli* without peptides (**a**) and (**b**). Fluorescence

images (FITC) of the *E. coli* after the treatment of S3 (**c** and **d**) and S8 (**e** and **f**) lipopeptides (Scale bar 10 µm).

the α-peptide with C_8 fatty acid (S11) showed no activity even at higher concentration. These results suggest that *E*-vinylogous hybrid lipopeptides can be used as potential antimicrobial candidates. In addition, the antimicrobial activity further depends on the side-chains of amino acids as well as length of the fatty acids. We find it difficult to correlate the disparity in the antimicrobial activity of these hybrid lipopeptides with their hydrophobicity. However, the hybrid lipopeptide with higher hydrophobicity S7 or lower hydrophobicity S2 and S6 peptides displayed moderate or no activity against the microorganisms. All active peptides showing similar MIC values promoted us to probe the plausible mechanism of action of these lipopeptides.

The atomic force microscopy images of *Escherichia coli* (*E. coli*) and fungus *Candida albicans* (*C.albicans*) before and after the treatment of S1 lipopeptide are shown in Figure 1. The change in the morphology of both the organisms clearly indicates that the possible action of these peptides is through the disruption of cell membrane. Similar results were also observed for



(A) (B)

Figure 2: A) Peptide induced leakage of *β*-galactosidase from *Escherichia coli* Top10 (Invitrogen) cells. The graph represents relative amount of β-galactosidase present in the medium after treating with the peptides (20 μg/ml). Error bars are based on triplicate experiments for each peptide. B) Hemolytic activity of hybrid lipopeptides with 4% hRBCs. Curves were fitted based on the average of two independent replicates.

other peptides (data not shown). Though the cell wall components are different for bacteria and fungi, the mode of action of these peptides indeed appears to be the perturbation of the cell membrane. We further validate the ability of cell membrane disruption by the lipopeptides using FITC (MW 389.4 Da) uptake assay using *E.coli*.[22] Since the entry of FITC requires significant membrane damage, the cell membrane disruption was studied using S3 and S8. The fluorescent image of *E.coli* before and after the treatment of the lipopeptides is shown in Figure 1. No fluorescence was observed in *E.coli* cells in the absence of hybrid peptides. The observed fluorescence behavior of the cells suggests that lipopeptides render *E. coli* cells permeable to FITC.

Further, to understand the mechanism of the action of these peptides on the inner cell membrane, we studied the enzyme β-galactosidase leakage assay [23] using peptides S1, S3 and S8 on *E.coli* (pUC19 plasmid transformed Top10 (Invitrogen) competent cells). The leakage of *β*-galactosidase (MW≈116kDa) from *E.coli* was used as an indicator of the disruption of the cytoplasmic membrane. *β*-Galactosidase release was measured using the initial velocity of the reaction by standard fluorogenic substrate. Results reveal very intriguing information regarding the action of lipopeptides (Figure 2A). No reaction of *β*-galactosidase with the substrate observed in the case S1 suggests that the peptide probably disrupts the outer cell membrane by binding through the negatively charged lipopolysaccharides. In contrast, S3 and S8 displayed

equal amount of fluorescence intensity indicating the release of β-galactosidase upon the treatment of lipopeptides. This experiment suggests that these lipopeptides act on both outer and inner membrane and facilitate the release of β-galactosidase. Though the peptides S1, S3 and S8 display nearly similar MIC values, these results suggest their different mode of action.

The encouraging results of the antimicrobial activities of these peptides inspired us to subject them for hemolytic activity. Results of the hemolytic activities are shown in Figure 2B. Out of all the peptides tested, S7 and S3 (both highly hydrophobic) showed the highest hemolytic activity followed by S8. In contrast the highly potent antimicrobial peptide S1 displayed comparatively lesser hemolytic activity. Other peptides which show poor activity against the

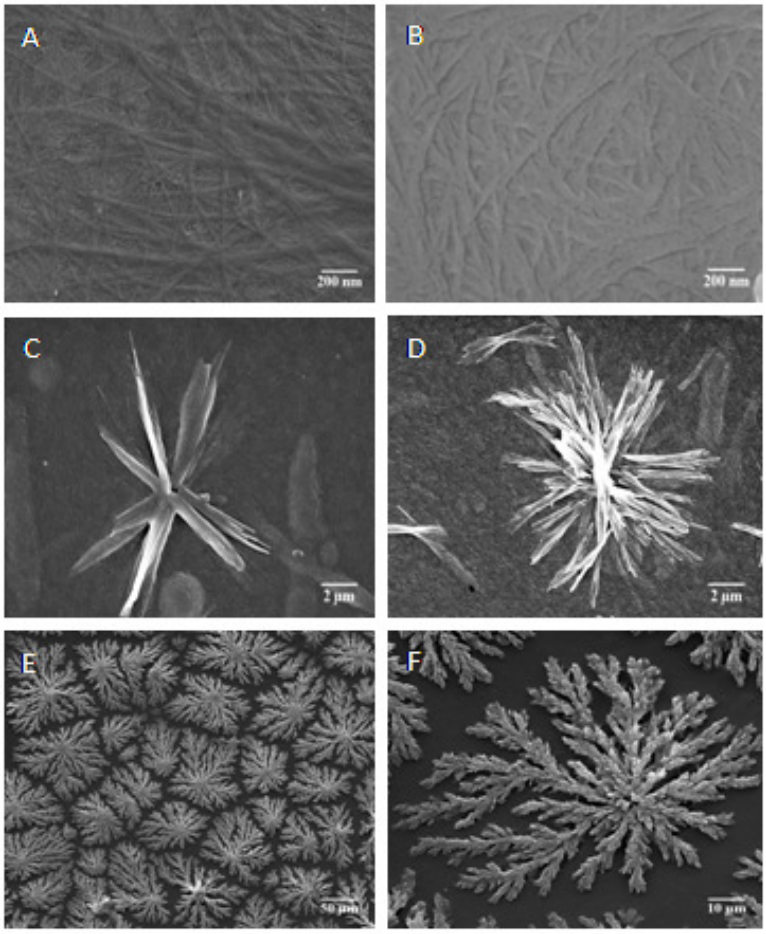


Figure 3: FE-SEM images of S1 (A), S5 (B), S3 (C, D) and S8 (E, F) hybrid lipopeptides.

bacteria and fungi have shown lesser hemolytic activity. Analysis of these results reveals that peptides containing aromatic side chains have shown greater hemolytic activity than those containing aliphatic side chain amino acids. Even though S1 is more hydrophobic than S8, it displayed lower hemolytic activity. Except S3 and S7 all other peptides which showed antimicrobial activities are non-hemolytic at their MICs. The control peptides S10 and S11 which are inactive in the antimicrobial studies displayed no hemolytic activities.

The disparity in the antimicrobial activities upon addition or truncation of N-terminal fatty acid between S8 and S7, also the different mode of action between S1, S3 and S8 promoted us to speculate the role of self-organization of these lipopeptides. To address this possibility and gain further knowledge regarding their mode of action we analyzed the self-assembly patterns of lipopeptides in both solution and on the surface.

Dynamic light scattering (DLS) experiments confirmed that all lipopeptides undergo a spontaneous self association to give nano-assemblies in solution. Mono dispersed self-assemblies of peptides in DLS are shown in supporting information. Intriguingly, the size of mono dispersed self-assemblies are ranging from 124 to 950 nm. Lipopeptides S1, S3, S7 and S8 displayed average size of the nano-assemblies 164, 220, 615 and 950 nm, respectively. Interestingly, the zeta-potential values of these self-assembled lipopeptides were found to be 54 ± 3, 55 ± 1, 47 ± 3 and 43 ± 4 respectively. Irrespective to the size of assembly, these peptides showed excellent surface potential charges. In order to understand the pattern of self-association, we subjected them to FE-SEM (Field Emission Scanning Electron Microscope) analysis. The FE-SEM images of S1, S3, S5, and S8 are shown in Figure 3. Peptides S1 and S5 containing dehydro alanine (dgA) adopted nanofibers type of morphology similar to the amyloid fibrils.[24] Peptide S3 and S8

with aromatic amino acid side chains underwent diffusion limited aggregation, however, with a different morphology. Peptide S3 was found to be composed of fractals with a needle shaped nano-structures. Ironically, S8 displayed spectacular fractal trees similar to the polyelectrolyte complexes and peptide containing aromatic amino acids.[25-27] S7 display fractals different than that of S8. Though the higher concentrations are required for the SEM analysis, however, the different morphologies observed in the self-assembly of these hybrid lipopeptides partially explains the disparity in their biological activity. Even though electric potential of self-assemblies are very similar, substantial change in the self-assembly pattern is observed between the peptides S1 and S3. The robust growth of fractals and needle shaped nano-structures from S3 and S8 may be influencing their antimicrobial and high hemolytic activities. As S7 showed poor antimicrobial activity, we anticipate that this peptide might have adopted different mechanism from that of S3 and S8. The low hydrophobicity and low surface charges of S2, S4 and S6 is reflected in their poor antimicrobial and lower hemolytic activity values.

Conclusion

In conclusion, we demonstrated the broad spectrum antimicrobial properties of short hybrid lipopeptides composed α- and *E*-vinylogous amino acids. Due to their geometrical restriction of the backbone double bonds, these short hybrid peptides with C_{12} and C_8 N-terminal fatty acids displayed better activity than the α- as well as β-lipopeptides containing C_{16} fatty acid at the N-terminal.[12c, 12d] The different nano-structures and assemblies of these lipopeptides may be influencing the disparity in their biological activities. The hybrid peptides with moderate hydrophobicity and high electrical potential of self-assemblies showed better antimicrobial and low hemolytic activities (S1). These peptides may possibly bind to the negatively charged outer membrane of the microorganisms and affecting the transmembrane electrical potential. The

robust growth of nano needles from the hybrid peptides containing aromatic amino acids may be responsible for the disruption of both outer and inner membranes. The antimicrobial activity, the mode of action and the self-assembly patterns of these short *E*-vinylogous hybrid lipopeptides, particularly S1 and S8 provide a unique opportunity to further design potent antimicrobial candidates as well as to explore their utility as smart biomaterials.

Experimental section

General. All amino acids, DCC, PPh_3 were purchased from Aldrich. The solvents THF, DCM, Toluene were purchased from Merck. Ethyl bromoacetate, di-tert-butyl dicarbonate , Fmoc-OSu were purchased from Spectrochem and used without further purification. Column chromatography was performed on Merck silica gel (100-200 mesh). Peptides were filtered and purified through reverse phase HPLC on C18 column using MeOH/H_2O gradient. ^1H NMR spectra were recorded on Jeol 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 were obtained from the MALDI-TOF/TOF. All HPLC purified peptides with the purity > 95% were used for the biological activities.

Synthesis of *N*-Fmoc-protected α, β-unsaturated γ-amino acids: *N*-Fmoc-protected α,β-unsaturated γ-amino acids were synthesized using reported procedure.[19, 20] Briefly, the Boc-amino aldehyde (10 mmol) and ylide (15 mmol, 5.22g) were dissolved in 30 mL of dry THF at room temperature and stirred for about 5hrs. Completion of reaction was monitored by TLC. The reaction mixture was concentrated under reduced pressure to give crude product, which was further purified on silica gel column chromatography using EtOAc/Pet-ether (1:5) to get pure

ethyl ester of Boc-protected α, β-unsaturated γ-amino acid. To a solution of ethyl ester of Boc-α, β-unsaturated γ- amino acid (2 mmol) in 5 mL of ethanol, mL of 1*N* NaOH was added dropwise. After completion of the reaction (~30 min), ethanol was evaporated and the residue was acidified using 10 mL of 5% HCl (5% volume in water) at cold conditions. The product was extracted with ethyl acetate (3× 40 mL). Combined organic layer was washed with brine (30 mL) and dried over anhydrous Na_2SO_4. The solvent was concentrated under reduced pressure to give Boc-α, β-unsaturated γ-amino acid as gummy product in a quantitative yield.

The Boc-α, β-unsaturated γ-amino acid (1 mmol) was dissolved in 5 mL of DCM and cooled to 0 °C in ice bath followed by addition of 5 mL neat TFA to the reaction mixture. After 30 min, TFA was removed from reaction mixture under *vacuum*. Residue was dissolved in 15 mL of water (15 mL) and the pH was adjusted to ~9-10 by the slow addition of solid Na_2CO_3. The solution of Fmoc-OSu (1 mmol) in 10 mL of THF was added slowly to the reaction mixture and stirred overnight at room temperature. After completion of the reaction, the reaction mixture was acidified with 20 mL of 20% HCl (20% volume in water) in cold condition. Product was extracted with ethyl acetate (3× 50 mL). Combined organic layer was washed with brine (30 mL) and dried over anhydrous Na_2SO_4. The solvent was concentrated under reduced pressure to give gummy product, which was recrystallized using EtOAc/Pet-ether. The pure solid Fmoc- α, β-unsaturated γ-amino acid was subjected for SPPS.

SPPS peptide synthesis and Purification

Peptides were synthesized at 0.25 mmol scales on Knorr amide resin using standard Fmoc-chemistry. HBTU/HOBt was used as coupling reagents. The coupling reactions were monitored by Kaiser Test. After completion of the synthesis, peptides were cleaved from the resin using cocktail mixture of TFA: water: thioanisole (98:1:1). After cleavage, the resin was filtered and

washed with TFA. The cleavage mixture was then evaporated under reduced pressure to give gummy product and purified through reverse phase HPLC on C_{18} column using MeOH/H_2O gradient. The purity of the peptides was further confirmed by analytical C_{18} column with the same gradient system.

Antibacterial activity:

The bacteria used in these experiments were collected from National Collection of Industrial Microorganisms (NCIM) *Escherichia coli* (NCIM 2065), *Escherichia coli K12* (NCIM2563), *Klebsiella pneumoniae* (NCIM 2957), *Pseudomonas aeruginosa* (NCIM 5029), *Salmonella typhimurium* (NCIM 2501), and *Staphylococcus aureus* (NCIM 5021).

The antibacterial activities of lipopeptides were carried out in 96-well microtiter plate using broth dilution method. The bacterial cultures were grown over night at 37 °C and serially diluted to a concentration of 10^6 colony forming units/mL with sterile MHB (Mueller-Hinton broth) medium. Two-fold serial dilutions with MHB medium were performed triplicate for each lipopeptide in a sterile 96-well plate to a final volume of 50 µl in each well. The final volume was made to 100 µl by adding an aliquot of 50 µl bacterial suspension to each well, and the plate was incubated at 37 °C for 18-20 h. Controls were done without peptide and the MIC of peptide was defined as the lowest concentration of the peptide required for the complete inhibition or killing the bacterial inoculum. The growth inhibition was monitored by measuring the absorbance at 492 nm.[12b] The MIC values reported were reproducible between three independent triplicates.

Antifungal activity:

The antifungal activity of lipopeptides was performed using 96 well plate methods (TPP brand) as follows: The 50 µl of water containing peptide was serially two fold diluted in PDB (Potato

dextrose broth, Hi-Media) medium. The final volume was adjusted to 100 µl by adding 50 µl of yeast suspension containing a concentration of 2×10^3 colony forming units/ml. A culture without peptide was also grown parallel as a control. Inoculated plates were incubated at 25 ºC for 48-72 h and the growth inhibition was determined by measuring the absorbance at 620 nm. The yeasts used were *Candida albicans* (NCIM 3471), *Candida glabrata* (NCIM 3237), *Cryptococcus neoformans* (NCIM 3542), and *Saccharomyces cerevisiae* (NCIM 3454). Experiments were carried out in triplicate. The MIC was determined as the lowest concentration of peptide required for the complete growth inhibition of each organism after 48-72 h incubation.

Hemolysis assay:

Fresh hRBCs (Human Red Blood Cells) were collected with EDTA. The hRBC were washed with Tris buffered saline (10 mM Tris, 150 mM NaCl, and pH 7.2) four times and diluted to a final concentration of 4% v/v. The assay was performed in sterile 96-well plate in a final volume of 100 µl as follows: aliquot of 50 µl of hRBC suspension were added to 50 µl (100 µg/ml) of two-fold serial diluted lipopeptide in Tris buffer. The plate was incubated at 37 ºC for 1 h and then centrifuged for 15 min at 3000 rpm. The supernatant (50 µl) from each well was transferred to a fresh 96-well plate containing 50 µl of water and release of hemoglobin was monitored by measuring the absorbance at 540 nm. The experiments were performed in duplicate and the hRBCs suspensions with Tris buffer and 1% Triton-X was consisted as negative and positive controls. Percentage of hemolysis was defined as $(A\text{-}A_N)/(A_P\text{-}A_N) \times 100$, where A is the absorbance of test well, A_N is the absorbance of the negative control and A_P is the absorbance of the positive control (100% hemolysis).

Atomic Force Microscopy Imaging:

A culture of *E. coli K12* (NCIM 2563) were grown in MHB medium and *Candida albicans* (NCIM 3471) were grown in potato dextrose broth. The cells were centrifuged and resuspended in deionized distilled water. The bacteria were incubated with 50 µM of S1 and S8 lipopeptide at 37 °C for 1 h. After incubation, 50 µl droplets of each sample were applied onto Poly-L-Lysine (PLL) pretreated glass slides and allowed to dry for 30 min at 25 °C. For control, cells without peptide were similarly prepared and imaged within 2 h of being resuspended in distilled water.

For AFM analysis of peptides the samples were applied on the freshly cleaved mica surface and dried for 10 min at 25 °C. The AFM images were acquired using a JPK Nano Wizard (Berlin, Germany) mounted on a Zeiss Axiovert 200 inverted microscope. Measurements were carried out in Intermittent contact (IC) mode using Tap 300 E-G (Cr/Pt Conductive coating) cantilevers from silicon AFM probes (Spring constant, k = 40 N/m, Resonance Frequency around 300 KHz, Radius <10 nm). Height and size information were acquired using JPK data processing software.

FITC uptake assay:

To analyze the membrane permeabilization of lipopeptides impermeant fluorescent probe Fluorescein isothiocyanate (FITC) was used. The assay was done as previously described (2) Culture of *E.coli K12* (NCIM 2563) were grown to absorbance OD 0.6 in MHB medium. The bacteria were washed and suspended (2×10^7 cfu /ml) in 10 mM sodium phosphate buffer (pH 7.4). The bacterial suspension was incubated with 50 µM of S1, S3 and S8 lipopeptides at 37 °C for 60 min. The microorganisms were immobilized on Poly-L-Lysine (PLL) coated glass slide for 50 min at 30 °C, followed by addition of 100 µl FITC (10 µg/ml) suspended in the same buffer. The slides were washed with 10 mM sodium phosphate buffer (pH 7.4) and viewed under Zeiss Axiovert apotome microscope equipped with an AxioCam camera. The images were

processed with Axiovision 4.7 software. The samples with peptide solvents were taken as a control.

Beta-galactosidase leakage from *E.coli* cells:

The PUC19 plasmid containing LacZ reporter gene was transformed in to the chemically competent Escherichia coli (TOP10, Invitrogen) cells. *E.coli* cells were grown in Luria Bertani broth with 100 µg/ml of ampicillin at 37 °C to an absorbance 0.6 at 660 nm. The cells were centrifuged and washed with fresh medium for three times to remove extracellular *β*-galactosidase. The 10 µl of water containing peptide of stock solution (200 µg/ml) were added in a sterile 96-well plate. The final volume was adjusted to 100 µl by adding an aliquot of 90 µl bacterial suspension to each well, and the plate was incubated at 37 °C for 1 h. After 1 h, the plate was centrifuged at 4000 rpm for 10 min to remove cell debris and 80 µl of supernatant was removed and separated in a fresh well. An aliquot 20 µl (0.4 mg/ml) of fluorescent indicator 4-Methylumbelliferyl-*β*-galactosidase (MUG, Sigma) was added to the well and *β*-galactosidase release was monitored for 1 h. Initial velocities of enzyme reaction were taken from the linear plot of fluorescence versus time. The samples with water were taken as a blank.

Scanning Electron Microscopy imaging:

The peptides were dissolved in water of concentration (50 µg/ml) was spotted on silicon wafers and air dried for 45 min. The samples were sputter coated with gold and observed in Zeiss FE-SEM. The sample was spotted on silicon wafers and recorded on FESEM. The images were converted to binary file and fractal dimension were analyzed by using fractal box count of Image J software.

Dynamic light scattering:

For DLS measurements, lipopeptides of concentration 1mg/ml were dissolved in water and by using poly propylene glass cuvette measurements were performed in Zeta sizer (Malvern Instrument). The average count rate at each time point was recorded.

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Abbreviations Used

AMP's, Antimicrobial peptides; MIC, Minimum inhibitory concentration; FITC, Fluorescein isothiocyanate; hRBC's, Human red blood cells; dgX, α, β- dehydro γ-amino acid (ex. dgA, α,β-dehydro γ-alanine); FESEM, Field emission scanning electron microscopy; AFM, Atomic force microscopy; DLS, Dynamic light scattering, DCC, *N*, *N*'-dicyclohexylcarbodiimide, PPh_3, Triphenylphosphine; Fmoc-OSu, 9-fluorenylmethylsuccinimidyl carbonate; HBTU, *N*,*N*,*N*',*N*'-Tetramethyl-O -(1*H*-benzotriazol-1-yl)-uronium hexafluorophoshate; NCIM, Nation collection of industrial microorganisms; MHB, Mueller Hinton broth; MALDI-TOF, Matrix assisted laser desorption ionisation-time of flight; HOBt, *N*-Hydroxybenzotriazole.

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Supporting Information Available: ^{1}H NMR and ^{13}C NMR for all E-vinylogous amino acids, mass spectra for all compounds, images of AFM and FE-SEM, DLS histograms and HPLC profiles. This material is available free of charge via the Internet at htpp:// pubs.acs.org.

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