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Structure–Activity Relationship of Amino Acid Tunable Lipidated Norspermidine Conjugates: Disrupting Biofilms with Potent Activity against Bacterial Persisters

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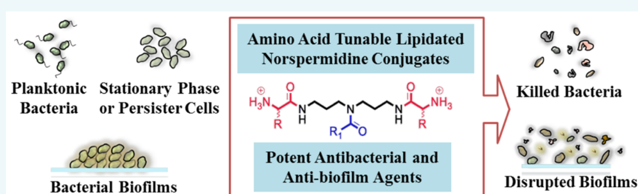
Supporting Information

ABSTRACT: The emergence of bacterial resistance and biofilm associated infections has created a challenging situation in global health. In this present state of affairs where conventional antibiotics are falling short of being able to provide a solution to these problems, development of novel antibacterial compounds possessing the twin prowess of antibacterial and antibiofilm efficacy is imperative. Herein, we report a library of amino acid tunable lipidated norspermidine conjugates that were prepared by conjugating both amino acids and fatty acids with the amine functionalities of norspermidine through amide bond formation. These lipidated conjugates displayed potent antibacterial activity against various planktonic Gram-positive and Gram-negative bacteria including drug-resistant superbugs such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*, and *β*-lactam-resistant *Klebsiella pneumoniae*. This class of nontoxic and fast-acting antibacterial molecules (capable of killing bacteria within 15 min) did not allow bacteria to develop resistance against them after several passages. Most importantly, an optimized compound in the series was also capable of killing metabolically inactive persisters and stationary phase bacteria. Additionally, this compound was capable of disrupting the preformed biofilms of *S. aureus* and *E. coli*. Therefore, this class of antibacterial conjugates have potential in tackling the challenging situation posed by both bacterial resistance as well as drug tolerance due to biofilm formation.



INTRODUCTION

Bacterial resistance to conventional antibiotics along with the problems posed by the formation of biofilms has brought forth an alarming situation.[1−4] Within the confines of a biofilm, bacteria are known to lose their planktonic identity and act as multicellular entities, conferring them immunity against conventional antibiotics as well as host immune systems.[3−6] By existing in a quiescent state, bacteria within a biofilm are responsible for causing persistent infections such as burn wound infections, catheter infections, chronic wound infections, bacterial endocarditis, and lung infections of cystic fibrosis (CF) patients.[3] Not only are these infections almost untreatable with conventional antibiotics therapy, some antibiotics of the aminoglycoside class have also been reported to induce biofilm formation.[7−10] In order to resolve this situation several antibiofilm agents have been recently reported in the literature, but clinical success is still elusive.[11−16] Unfortunately, this problem is gaining further complexity with time due to the occurrence of bacterial resistance. In the face of these unmet clinical problems, several approaches have been taken to tackle bacterial biofilms, such as through a nanoparticle-based strategy more recently.[17] There is still a pressing need to develop a novel class of antibacterial molecules that are capable of combating bacterial resistance and eradicating bacterial biofilms in tandem.

In the recent past, membrane active antimicrobial peptides (AMPs) and lipopeptides have been recognized as effective antibacterial agents.[18,19] This class of molecules offers a ray of hope against the juggernaut of resistance as they have been known to possess a low propensity for triggering the development of bacterial resistance. A sign of further promise is that some of these antibacterial peptides have already been reported to have antibiofilm properties in addition to their potent activity against planktonic bacteria.[20−22] However, most of these classes of compounds suffer from limitations such as low selectivity, proteolytic degradation, and steep costs of development.[23] Even within the lipopeptide class, although some noteworthy members such as Colistin and Daptomycin have entered the antibiotic pipeline, success in general for this class of compounds is largely shrouded by their narrow spectrum of activity.[19,24−26] In addition, resistance development against them in clinics has already begun to be reported.[27] Recent years, therefore, have seen the emergence of a plethora of new classes of antibacterial molecules.[28−43] Despite their effectiveness, however, the evident complexity in most synthetic designs and the obvious expenses entailing solid-phase synthesis in many cases have left the scope in favor of

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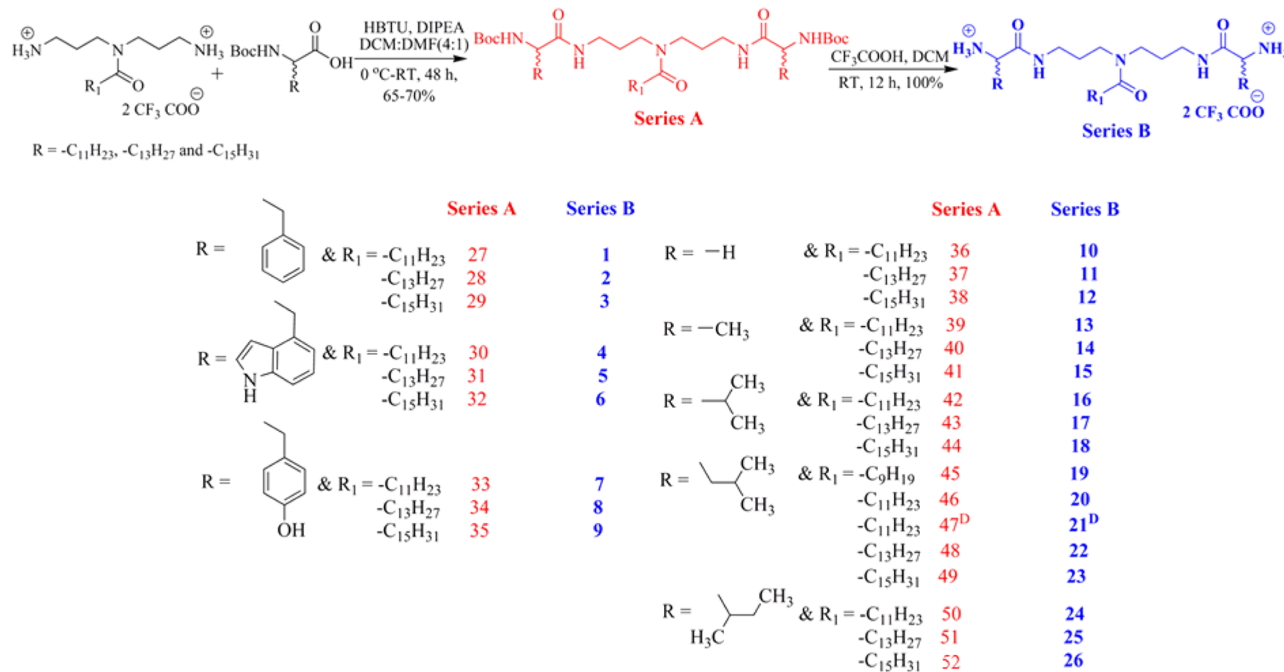
Scheme 1. Synthesis of Amino Acid Tunable Lipidated Norspermidine Conjugates[a]



[a]D indicates that compounds **21** and **47** consists of D-amino acid. All other compounds consist of L-amino acids.

development of much simpler designs. Our group has been involved in developing new antibacterial molecules through such simple synthetic designs.[16,35,44,45]

In this work we followed a simple two-step synthetic protocol to generate a library of amino acid tunable lipidated conjugates in order to determine the effect of hydrophobicity on the overall antibacterial activity and toxicity, keeping the total number of positive charges constant at two. In our previous work on phenylalanine based norspermidine conjugates bearing two charges, good antibacterial activity was observed due to the presence of the hydrophobic phenyl moiety.[44] However, in the previous report we described only the effect of long chain variation toward antibacterial activity. Therefore, here for the first time we were specifically interested in exploring how the antibacterial activity varied due to side chain variation of hydrophobic amino acids. Additionally, our goal was to arrive at the lead compounds through a detailed structure−activity relationship. Our premise was based on the rationale that various research groups in diverse fields of chemistry have directed their research interest to the variation of hydrophobic amino acids in optimizing the chemical structure of the most effective compound.[46,47] Importantly, variation of hydrophobic amino acids in a synthetic design has already been reported to play a crucial role in dictating antibacterial properties.[48−50] One of the obvious logical approaches toward newer compounds was to incorporate aromatic amino acids within the molecules which were achieved by introducing tryptophan and tyrosine residues in place of phenylalanine. In order to further ascertain whether it is indeed the aryl scaffold or the overall hydrophobicity of the amino acid side chain that plays a role toward the enhancement of antibacterial activity, we also synthesized analogous compounds by incorporating other amino acids having nonaromatic hydrophobic side chains such as alanine, valine, leucine, and isoleucine. We further went on to prepare glycine conjugated norspermidine derivatives as well, to investigate the effect of an absence of side chain hydrophobicity. Furthermore, the lipophilicity of these molecules was also varied by changing the lipophilic moiety from the dodecanoyl to tetradecanoyl and finally to hexadecanoyl groups. This entire library of compounds was then tested for antibacterial activity against a number of planktonic bacteria, including drug resistant strains. Furthermore, experiments to determine the toxicity against human red blood cells (hRBCs) and mammalian cells (RAW 264.7 TIB-71 cell line), bactericidal kinetics, and membrane active mechanism of action were also performed. Because persistent infections caused by dormant bacteria are of particular relevance at present, the antibacterial activity of the optimized compound against persister cells and stationary phase bacteria were also tested. Finally, the ability to disrupt the preformed biofilms and the propensity to develop bacterial resistance were evaluated.

RESULTS AND DISCUSSION

Synthesis. The synthesis of the amino acid tunable lipidated norspermidine conjugates (compounds **1**−**26**) was achieved by following the reaction scheme as outlined in Scheme 1. All the compounds were synthesized through two-step reactions, starting from previously reported lipophilic norspermidine derivatives.[44] In the first step of the reactions, the carboxylic acid group of Boc-protected amino acids (phenylalanine, tryptophan, tyrosine, glycine, alanine, valine, leucine, and isoleucine) were coupled with the two primary amine groups of the lipophilic norspermidine derivatives through an amide bond using HBTU as coupling agent. In the second step of the reaction, all the final compounds (**1**−**26**) were achieved through deprotection of Boc groups by using trifluoroacetic acid (TFA). Finally, all the compounds were characterized by ^{1}H NMR and HR-MS (Supporting Information Figure S1−S52) and their purity was determined by HPLC (Supporting Information Figure S53−S63) which proved that the compounds were more than 95% pure. In order to achieve

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Table 1. Antibacterial and Hemolytic Activity of the Amino Acid Tunable Lipidated Norspermidine Conjugates

compd	minimum inhibitory concentration (μg/mL)						HC$_{50}$ (μg/mL)
	drug-sensitive bacteria			drug-resistant bacteria			
	S. aureus	*E. faecium*	*E. coli*	MRSA[a]	VRE[b]	*K. pneumoniae*[c]	
1	3.6	4	10	3.3	2.3	8	171
2	6	4.5	14	4.1	2.4	16	124
3	9	7.6	>100	4.6	2.7	62	74
4	3.5	2.8	12.5	2.3	1.8	25	133
5	2.5	2.5	39	3.7	2.7	80	104
6	6.5	5.4	>100	4.7	5.6	>100	81
7	23	26	49	38	22	>100	276
8	4.9	5	6.4	5.7	4.2	17.8	115
9	5.8	4.8	12	6	3.5	>100	75
10	50	56	>100	40	73	>100	400
11	8.5	6	11	7.3	8.9	>100	157
12	4.6	2.8	4.2	7.1	23	3.3	90
13	65	50	>100	91	56	>100	562
14	13.5	6.4	12	12	5.4	>100	93
15	4.1	2.2	3	4.4	22	8.9	68
16	21	18.5	13.3	30	18.5	43	264
17	3	2.8	5.4	2.6	3	5.6	134
18	2.1	1.4	8	1.5	1.1	>100	86
19	36	45	30	60	56	>100	530
20	4.5	6.6	6.8	6.8	3.8	13.8	305
21	5.1	6.2	5.8	3.5	3.4	12.5	310
22	2.9	3.3	4.3	2.1	2	88	118
23	3.1	2.8	16.5	2.4	2.8	>100	95
24	6.1	6.1	8.4	4.4	8.7	21.5	95
25	2.4	3.2	4	2.3	2	>100	64
26	3.3	2.5	21	2.1	3.4	>100	60
colistin	25	>250	0.5	68	>250	1.5	ND.[d]
vancomycin	0.8	0.8	N.D.	0.8	>1000	N.D.	N.D.

[a]Methicillin-resistant *S. aureus*. [b]Vancomycin-resistant *E. faecium*. [c]β-lactam-resistant *K. pneumoniae*. [d]N.D. stands for "not determined".

the optimized compounds through a detailed structure–activity relationship (SAR) the hydrophobicity was tuned by varying the amino acids as well as by varying the lipophilic moiety. Compounds **1**–**9** comprise the hydrophobic amino acids bearing an aromatic side chain, namely, phenylalanine (**1**–**3**), tryptophan (**4**–**6**), and tyrosine (**7**–**9**), in the structure with the long chain varying from the dodecanoyl to hexadecanoyl groups. Compounds **10**–**12** consist of glycine (lacking a hydrophobic side chain) as the amino acid whereas compounds **13**–**26** comprise the other hydrophobic amino acids with an aliphatic side chain, namely, alanine (**13**–**15**), valine (**16**–**18**), leucine (**19**–**23**), and isoleucine (**24**–**26**). Compound **19** which contains leucine bears a decanoyl group in its structure. Except compound **21** which bears a D-amino acid, all the compounds consist of L-amino acids.

Antibacterial Activity. In order to understand any possible correlation borne out of the side chain variation of the conjugated amino acids and the antibacterial effectiveness of this class of compounds (**1**–**26**), the activity was tested against various drug-sensitive (*S. aureus*, *E. faecium* and *E. coli*) and drug-resistant (MRSA,VRE, and β-lactam-resistant *K. pneumoniae*) bacteria. The antibacterial activities thus investigated are reported as their minimum inhibitory concentration (MIC) values (Table 1). From the MIC values, it is clear that almost all the compounds displayed appreciable activity against both classes (Gram-positive and Gram-negative) of bacteria, with most of the compounds showing antibacterial activity at low concentrations (in the range of 1–10 μg/mL). From this analysis therefore, we observed that not only the previously reported phenylalanine series of compounds but rather the entire family of this class of compounds show potent antibacterial activity. However, it should be noted that the compounds displayed less activity against Gram-negative bacteria compared to Gram-positive bacteria. This compromised activity of the compounds probably due to less interaction with the complex cell envelope of Gram-negative bacteria.

On preliminary analysis of the aromatic amino acid analogues (compounds **1**–**9**), it was observed that the tyrosine derivatives (compounds **7**–**9**) were the least active compounds (MIC values in the range of 3.5–38 μg/mL against Gram-positive bacteria), in comparison to the phenylalanine (**1**–**3**) and the tryptophan (**4**–**6**) derivatives. Against Gram-positive bacteria, compounds **1**–**3** displayed MIC values mainly in the range of 2.3–9 μg/mL, whereas compounds **4**–**6** were found to have in the range of 1.8–6.5 μg/mL. Therefore, a general observation suggested that the tryptophan derivatives were the most potent series of compounds, followed closely by the phenylalanine derivatives. The importance of hydrophobicity is clearly established here by the observation that the least hydrophobic (tyrosine) among the three aromatic amino acids leads to the least activity. A more detailed analysis on this library of compounds reveals that the higher chain length derivative of tyrosine, compound **9**, was found to be more potent compared

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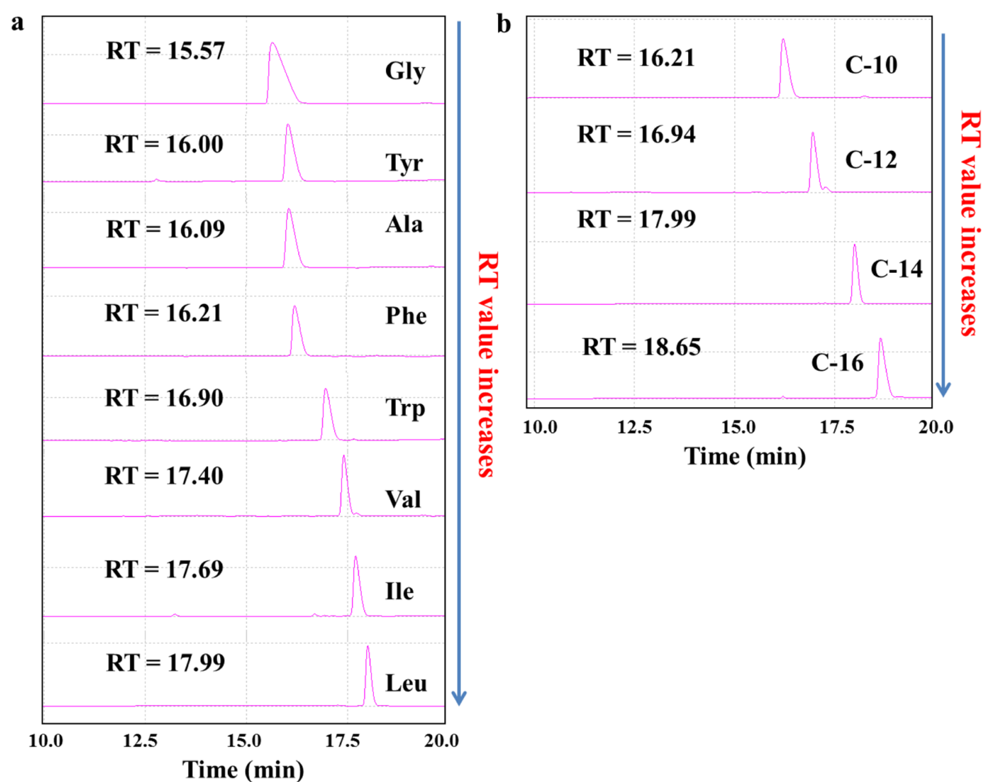

Figure 1. Variation of RT values: (a) HPLC trace of compounds by varying the amino acids of the tetradecanoyl analogues; (b) HPLC trace of leucine-based compounds by varying lipophilic long chain.

to shorter long chain analogues (compounds **7** and **8**). However, while considering the cases for phenylalanine and tryptophan series of compounds, the shortest long chain analogues (compound **1** and compound **4**) displayed the most potent antibacterial efficacy. This is indicative of the effect of the overall hydrophobicity of an antibacterial compound on its concomitant biological activity.

We next analyzed the antibacterial activity of the non-aromatic amino acid containing compounds (**10**−**26**). Among these compounds, it was observed that the set of glycine conjugated derivatives (**10**−**12**) were the least potent against all the bacteria tested. In fact, compound **10** (consisting of dodecanoyl long chain) was found to be the least active compound in the entire set of compounds, having MIC values greater than 30 μg/mL against all the tested bacteria. Therefore, such an observation confirms the necessity of side chain hydrophobicity of the amino acids to exhibit potent antibacterial activity. The set of compounds (**13**−**15**) consisting of alanine as the amino acid also showed a similar activity profile like the glycine series of compounds with the least activity displayed by the compound bearing the dodecanoyl long chain. However, the hexadecanoyl analogue was found to be most potent compound having MIC values in the range of 2.2−22 μg/mL. On transition from alanine derivatives to valine conjugated compounds (**16**−**18**), a noticeable improvement in activity was noted for the compounds containing shorter aliphatic long chain (dodecanoyl analogue). But even here, the highest long chain derivative (compound **18**) displayed the best antibacterial activity among this series of compounds, exhibiting MIC values of 2.1 μg/mL and 8 μg/mL against *S. aureus* and *E. coli*, respectively. The highlight of compound **18**, however, was its potent activity against the drug resistant bacteria MRSA (MIC = 1.5 μg/mL) and VRE (MIC = 1.1 μg/mL). Unfortunately, this compound was not active against β-lactam resistant *K. pneumoniae* (MIC > 100 μg/mL). On further progressing to the derivatives with a larger hydrophobic side chains containing amino acids, such as leucine (compounds **19**−**23**) and isoleucine (compounds **24**−**26**), an overall increasing trend in antibacterial activity was observed. Compound **20**, the dodecanoyl analogue in the leucine series of compounds, displayed potent activity against all the tested bacteria with the MIC values in the range of 3.8−13.8 μg/mL. This compound exhibited MIC values of 4.5 μg/mL and 6.8 μg/mL against *S. aureus* and *E. coli*, respectively. This compound also showed good activity against the drug resistant superbugs MRSA, VRE, and β-lactam resistant *K. pneumoniae* with MIC values of 6.8 μg/mL, 3.8 μg/mL, and 13.8 μg/mL, respectively. The other compounds of the leucine series (Compounds **22** and **23**) also showed high antibacterial activity against most of the bacteria (except *K. pneumoniae*) tested with MIC values in the range of 2−16.5 μg/mL. Finally, the compounds containing isoleucine as the amino acid (Compounds **24**−**26**) showed a similar activity profiles as the leucine containing compounds with MIC values lying in the range of 2−21 μg/mL (except *K. pneumoniae*). In order to determine the optimized compound from this entire SAR study we synthesized compound **19**, the decanoyl derivative of the leucine containing compounds. This compound showed compromised activity with the MIC values in the range of 30−100 μg/mL. This probably suggests that the optimum hydrophobicity to show appreciable biological activity cannot be furnished by the compounds with long chains shorter than dodecanoyl. However, to investigate whether any difference is revealed by changing the stereochemisty of the amino acids, compound **21** was tested for antibacterial activity. It was found that the compound displayed similar activity when compared to

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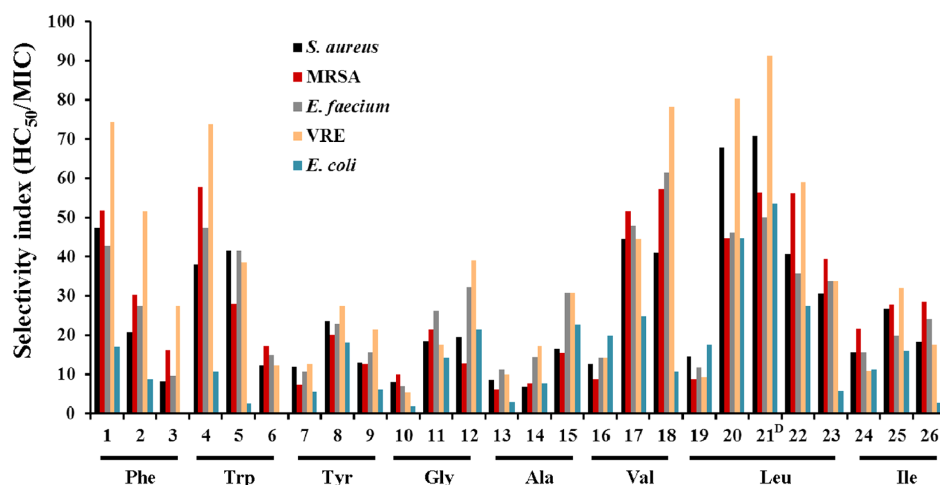

Figure 2. Selectivity ratio (HC_{50}/MIC) of the compounds against various bacteria.

its L-isomeric analogue (compound **20**). This observation shows that stereochemical changes to the compound do not bring about any significant differences in its activity profile. Instead, it is the overall hydrophobicity which plays a greater role in dictating antibacterial efficacy.

Furthermore, to understand the implications of hydrophobicity on the overall activity profile, an analysis was performed with respect to HPLC retention times (RT). RT values of the compounds were compared with antibacterial activity of the tetradecanoyl analogues to understand how it varies with the amino acid side chain. We further considered the RT values for the leucine conjugated compounds to have an idea of the activity trend with varying long chain lipophilicity. In general, we observed increased activity for higher RT values in both cases (Figure 1 and Table 1). For example, in the tetradecanoyl series of compounds, the compounds showing lower retention times (RT values < 17 min) showed more antibacterial activity than those with RT values greater than 17 min. Similarly, on increasing the chain length of the leucine analogues, an overall increase in antibacterial efficacy was observed.

Hemolytic Activity. In order to have a preliminary idea about the toxicity of this family of conjugates (**1**−**26**), the ability of these compounds to lyse the human erythrocytes (hRBCs) was tested. The extent of hemolytic activity was determined as their HC_{50} values, which refers to the concentration that causes lysis of 50% of the hRBCs. The HC_{50} values for the entire library of compounds ranged from 60 μg/mL to 562 μg/mL. The most active compound, **18**, which is the hexadecanoyl analogue of the valine series of compounds, showed HC_{50} of 86 μg/mL. Compound **20**, which is the dodecanoyl derivative of the leucine series of compounds with HC_{50} value of 305 μg/mL, was much less toxic, while it showed good activity against all the bacteria tested. The corresponding D analogue **21** showed a similar HC_{50} value of 310 μg/mL (Table 1). Based on the observed toxicity values, we performed an initial screening of compounds in order to properly identify promising compounds for further biological studies. As a quantitative measure of the suitability of the compounds to progress for these further studies, we plotted the selectivity ratios (HC_{50}/MIC) of all the compounds against all the bacteria (Figure 2). The results suggested that compounds containing phenylalanine, tryptophan, valine, and leucine in the structure showed higher selectivity compared to other series of compounds. The tyrosine, glycine, and alanine series of compounds showed the least selectivity, with the isoleucine analogues showing moderate selectivity. Among the compounds which showed good selectivity, the leucine series of compounds emerged as the most promising candidates for further study. Figure 2 illustrates that compound **20** and its D-isomeric analogue compound **21** showed a broad spectrum of selectivity including even Gram-negative bacteria (*E. coli*). But the highlight of this work was compound, **21**, with a high selectivity (∼40−90) for all the bacteria tested (except *K. pneumoniae*). Therefore, these two best compounds (compound **20** and **21**) were chosen for further studies. The studies reveal that an optimum hydrophobicity is an important parameter for showing selective activity against bacteria, which can be achieved by varying either the long chain lipophilicity or the side chain hydrophobicity of the conjugated amino acids.

Antibacterial Efficacy in the Presence of Human Plasma and Serum. One of the primary setbacks faced by antimicrobial peptides, as we mentioned previously, is their susceptibility to protease degradation. This translates to an overall decrease in antibacterial activity under in vivo conditions. A sustained antibacterial efficacy even in such conditions would be a significant advantage. These compounds were therefore further investigated by studying their antibacterial activity in the presence of human plasma and serum. It was found that compound **21** (containing D-isomeric amino acids) showed no loss in antibacterial activity in the presence of plasma as well as serum (Supporting Information Figure S64a and b). However, compound **20** (containing L-isomeric amino acids) was found to suffer almost an 8-fold loss in activity after 3 h incubation against either bacteria (*S. aureus* and *E. coli*) in both plasma and serum conditions.

Cytotoxicity. For evaluation of this class of conjugates as selective antibacterial agents, a further toxicity study was performed against mammalian cells (RAW 264.7 TIB-71). The cytotoxicity experiments with the most effective compound **21** were performed by using LDH assay. Results suggested that the compound did not show any toxicity at its bactericidal concentration (Supporting Information Figure S65a and b). It can be clearly visualized from the bright field images of the cells that the morphology of the compound-treated cells was similar compared to that of nontreated control even at a concentration of 16 μg/mL. The EC_{50} value, which corresponds to

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concentration at which 50% of treated cells are viable, was determined as 28 μg/mL. This value is more than 8-fold higher compared to its MIC against the drug resistant superbug VRE and considerably higher compared to the MIC values against all other bacteria.

Bactericidal Kinetics. The kinetics of killing against both *S. aureus* and *E. coli* was investigated for the most effective compound **21**. The results suggested that the compound had a rapid bactericidal activity against both bacteria. The compound revealed complete killing (>4 Log CFU/mL reduction) of *S. aureus* within 15 min at a concentration of 20 μg/mL (Supporting Information Figure S66a). Against *E. coli*, compound **21** showed complete killing (>4 Log CFU/mL reduction) within 30 min at concentration of 20 μg/mL (Supporting Information Figure S66b). However, at a higher concentration of 40 μg/mL, compound **21** displayed rapid killing kinetics, showing complete killing within 15 min.

Antibacterial Activity against Metabolically Inactive Bacteria. After demonstrating the potent antibacterial activity against planktonic bacteria, we investigated whether this class of conjugates could also display potency against metabolically inactive bacteria such as persister cells and stationary phase bacteria. The most effective compound **21** was chosen as model conjugate for these studies. Figure 3 clearly illustrates that

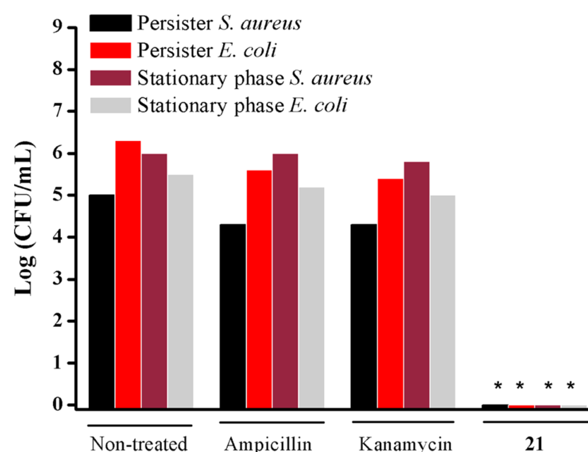

Figure 3. Antibacterial activity of compound **21** against persister cells and stationary phase bacteria. Potency of the compound was compared with the antibiotics ampicillin and kanamycin. Concentration of 20 μg/mL was used for compound **21**, and the control antibiotics were 20 μg/mL and 100 μg/mL for *S. aureus* and *E. coli*, respectively (asterisks correspond to <50 CFU/mL).

compound **21** showed high activity against persister cells of both *S. aureus* and *E. coli*. It revealed a complete reduction of cell viability of *S. aureus* and *E. coli* persisters (>4 Log CFU/mL against *S. aureus* and >5 Log CFU/mL against *E. coli*) at a concentration of 20 μg/mL, whereas the antibiotics ampicillin and kanamycin remained completely inactive against *S. aureus* and *E. coli* at concentrations of 20 μg/mL and 100 μg/mL, respectively (Figure 3). Additionally, compound **21** was also found to have equally high potency against stationary phase bacteria. Here too, complete reduction of cell viability (>5 Log CFU/mL against both the bacteria) was observed at a concentration of 20 μg/mL while the antibiotics (both ampicillin and kanamycin) did not display any activity (Figure 3). Therefore, this class of conjugates holds immense potential to treat chronic infections associated with metabolically inactive bacteria, which are almost impossible to treat with conventional antibiotics.

Disruption of Preformed Biofilms. Having established the antibacterial potency of this class of conjugates against both planktonic as well as metabolically inactive bacteria, we next investigated their efficacy as the antibiofilm agents. We performed the experiment with the most effective compound **21**. The effectiveness of this compound as an antibiofilm agent was compared with the antibiotics vancomycin and erythromycin. Our results suggested that this compound displayed a significant reduction of cell viability in the preformed biofilms of pathogenic *S. aureus* (∼3 Log CFU/mL) as well as *E. coli* (∼3 Log CFU/mL) at its MIC, whereas vancomycin or erythromycin displayed hardly ∼1 Log CFU/mL reduction in cell viability even at a higher concentration of 50 μg/mL (Figure 4a and c). At a concentration of 10 μg/mL (∼2 × MIC), compound **21** displayed around 10 Log (CFU/mL) and 4 Log (CFU/mL) reduction in cell viability in *S. aureus* and *E. coli* biofilms, respectively. At a high concentration of 80 μg/mL (∼16 × MIC), the compound completely reduced the cell viability in both biofilms (>17 Log CFU/mL for *S. aureus* and >7 Log CFU/mL for *E. coli*). Furthermore, in order to have a quantitative idea about the concentration of compound required to completely eradicate the biofilms, we also performed the Minimum Biofilm Eradication Concentration (MBEC) assay. The MBEC for this compound was determined to be 125 μg/mL against *S. aureus* (Supporting Information Figure S67). Finally, to visualize the extent of disruption we analyzed the disrupted biofilms by using confocal imaging after staining with Syto-9 dye. Figure 4b,d illustrates that compound **21** displayed a significant reduction in preformed biofilm mass compared to the nontreated control against both bacteria *S. aureus* and *E. coli*. Therefore, this class of conjugate shows promise as novel therapeutic agents for treating infections associated with bacterial biofilms which are difficult to combat with conventional antibiotics.

Membrane Targeted Mechanism of Action. In order to establish the fact that this class of conjugates kill bacteria primarily by acting on their cell membrane, we performed several experiments by using spectroscopic techniques. For example, experiments using the dye propidium iodide (PI) revealed that compound **21** caused permeabilization of the membrane of both *S. aureus* and *E. coli* even at concentration of 5 μg/mL (∼MIC). The increase in fluorescence intensity due to exposure of compound proved the fact that PI had indeed passed through the membrane of the compromised bacterial cells. It was observed that the compound showed concentration dependent membrane permeabilization for both bacteria as demonstrated in Figure 5a and b. In another experiment with the membrane potential sensitive dye $DiSC_3(5)$, this compound showed rapid membrane depolarization of both *S. aureus* and *E. coli*. Under normal membrane potential gradient, this dye can distribute itself between the cell interior and the external medium. Any disturbance in this normal membrane potential causes the leakage of dye into the external medium which leads to increase in fluorescence. An enhancement in fluorescence intensity was observed due to the compound exposure at 5 μg/mL (∼MIC) and further increase was observed with increasing concentration (Figure 5c and d). These results suggested that the compound induced a disturbance in the membrane potential of the bacterial cells. Additionally this compound was found to cause K^+ leakage, the extent of which, however, was less compared to the positive

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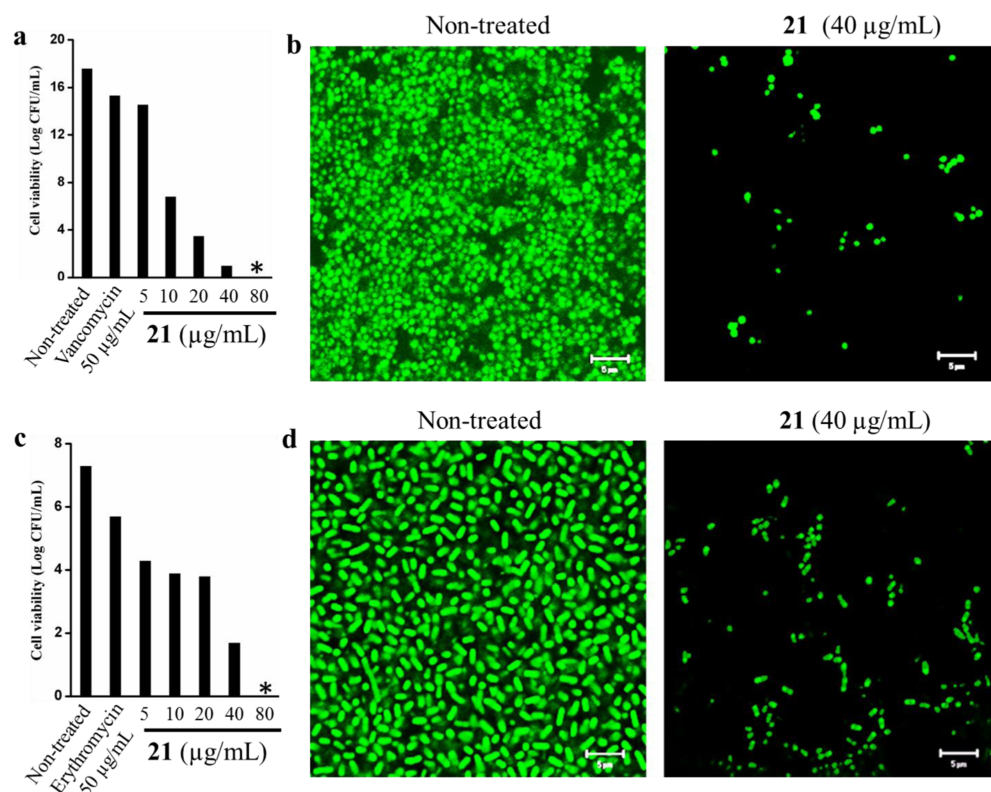

Figure 4. Biofilm disruption: (a) quantification of cell viability in *S. aureus* biofilms; (b) confocal microscopy images of *S. aureus* biofilms after staining with SYTO-9; (c) quantification of cell viability in *E. coli* biofilms; (d) confocal microscopy images of *E. coli* biofilms after staining with SYTO-9 (asterisks correspond to <50 CFU/mL).

control valinomycin (Figure 5e and f). Therefore, these experiments collectively reaffirm that this class of conjugates primarily act on both classes of bacteria by membrane targeting modes of action.

Susceptibility to Induce Bacterial Resistance. The development of long-lasting antibacterial agents is important in an era when bacterial resistance is posing serious challenges to global health. In order to demonstrate the potential of these conjugates as a prospective permanent solution against bacteria, bacterial resistance studies were performed where we determined the relative ability of the conjugates to induce bacterial resistance against both Gram-positive bacterial strain *S. aureus* and Gram-negative bacterial strain *E. coli*. The control antibiotics chosen for these studies were norfloxacin (for *S. aureus*) and colistin (for *E. coli*). Results suggested that compound **21** showed no propensity to induce bacterial resistance, and its MIC value remained almost unchanged over 14 passages (Supporting Information Figure S68a and b). However, in the case of the corresponding control antibiotics, bacterial resistance development was observed. The control antibiotics norfloxacin and colistin showed 400-fold and 250-fold increments, respectively, in their MIC values. This inference conclusively establishes that this class of conjugates are in fact incredibly immune to bacterial resistance, highlighting the effectiveness of the synthetic design.

CONCLUSIONS

A library of amino acid tunable lipidated norspermidine conjugates was developed through a simple synthetic strategy. This class of conjugates showed potent antibacterial activity against drug sensitive as well as drug resistant bacteria. The optimized compound was shown to be highly bactericidal, capable of killing both classes of bacteria within 15 min. Additionally, this class of compounds was also active against metabolically inactive bacteria such as persister cells and stationary phase *S. aureus* and *E. coli*, whereas antibiotics ampicillin and kanamycin were completely ineffective. Most importantly, the compound disrupted preformed biofilms of both Gram-positive *S. aureus* and Gram-negative *E. coli* bacteria. Finally these membrane targeting compounds did not trigger bacterial resistance even after several passages. Therefore, this class of conjugates holds significant promise in tackling the present challenges associated with bacterial resistance as well as drug tolerance.

EXPERIMENTAL SECTION

Materials and Bacterial Strains. Dichloromethane (DCM), *N,N*-dimethylformamide (DMF), and methanol were obtained from Spectrochem (India) and were dried before their use. Norspermidine was purchased from Sigma-Aldrich. All saturated fatty acids, dodecanoic, tetradecanoic, and hexadecanoic acids, were obtained from Alfa-Aesar. *N,N,N′,N′*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) and *N,N*-diisopropylethylamine (DIPEA) were purchased from Spectrochem (India). All these chemicals were used for reaction directly without any further purification. Analytical thin layer chromatography (TLC) was performed on E. Merck TLC plates precoated with silica gel 60 F_{254} and visualization was carried out using iodine. Column chromatography was performed on silica gel (60−120 mesh) using different ratios of chloroform and methanol solvent system as eluent. Nuclear magnetic resonance (NMR) spectra were

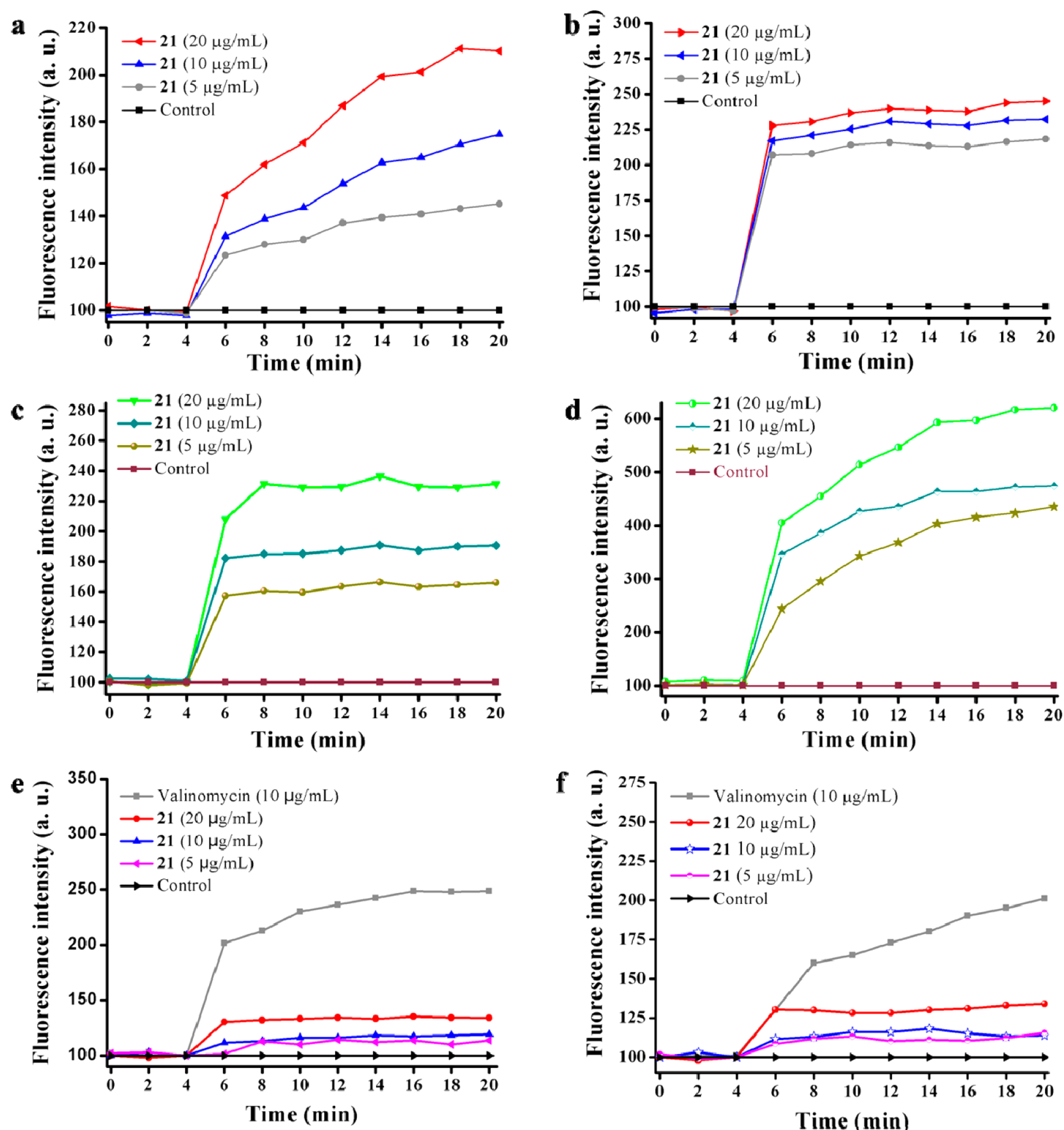

Figure 5. Membrane active mechanism of action of compound **21**: (a) membrane permeabilization of *S. aureus*, (b) membrane permeabilization of *E. coli*, (c) membrane depolarization of *S. aureus*, (d) membrane depolarization of *E. coli*, (e) K^+ leakage of *S. aureus*, (f) K^+ leakage of *E. coli*. Membrane permeabilization studies were performed by using propidium iodide (PI) as fluorescence probe, membrane depolarization studies using $DiSC_3$ (5) dye as fluorescence probe, and K^+ leakage experiment using the dye PBFI-AM as fluorescent probe.

recorded on Bruker (AV-400) 400 MHz spectrometer in deuterated solvents. Mass spectra were obtained using 6538-UHD Accurate Mass Q-TOF LC-MS instrument. For optical density (O.D.) measurement, Tecan Infinite Pro series M200 Microplate Reader was used. Bacterial strains, *S. aureus* (MTCC 737) and *E. coli* (MTCC 443), were purchased from MTCC (Chandigarh, India). MRSA (ATCC 33591), *E. faecium* (ATCC 19634), VRE (ATCC 51559), and *β*-lactam-resistant *K. pneumoniae* (ATCC 700603) were obtained from ATCC (Rockville, MD, USA). *E. coli* was cultured in Luria−Bertani broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl in 1000 mL of sterile distilled water), while *S. aureus*, MRSA, and *K. pneumoniae* were grown in yeast-dextrose broth (1 g of beef extract, 2 g of yeast extract, 5 g of peptone, and 5 g of NaCl in 1000 mL of sterile distilled water). For *E. faecium* and VRE, brain heart infusion broth (BHI) was used as growth medium. For solid media, 2.5% agar was used along with the above-mentioned growth medium.

Antibacterial Assay.[44,45] The antibacterial assay was performed following a previously reported protocol. Briefly, about 5 *μ*L of bacterial freeze stock (at −80 °C) was added to 3 mL of the respective broth and the culture was grown for 6 h at 37 °C prior to the experiments. This 6 h grown culture gives about 10^9 CFU/mL in the case of *S. aureus*, MRSA, and 10^8 CFU/mL in the case of *E. coli*, *K. pneumoniae*, *E. faecium*, and VRE which were determined by spread plating method. The 6 h grown culture was diluted to give approximate cell concentration of 10^5 CFU/mL which was then used for determining MIC. Compounds were serially diluted, in sterile Millipore water (in a 2-fold manner), and 50 *μ*L aliquots of these serial dilutions were added to the wells of a 96 well plate followed by the addition of about 150 *μ*L of bacterial solution. The plates were then incubated for 24 h at 37 °C. The O.D.

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value at 600 nm was recorded using TECAN (Infinite series, M200 pro) plate reader. Each concentration had triplicate values and the whole experiment was done at least twice and the MIC value determined by taking the average of triplicate O.D. values for each concentration.

Hemolytic Assay.[44,45] Briefly, compounds (**1**−**26**) were serially diluted in Millipore water and 50 μL aliquots of these serial dilutions were added to the wells of 96-well plates. Two controls were made, one without compound as negative control and the other as a positive control by addition with 50 μL of 1 vol % solution of Triton X-100 instead of compound. Freshly collected hRBCs were then centrifuged down from the heparinized blood and suspended to 5 vol % in 1 × PBS (pH = 7.4). Next, 150 μL of the erythrocyte suspension was added to the compound-containing plates. After that the plate was incubated at 37 °C for 1 h. It was then centrifuged at 3500 rpm for 5 min, and 100 μL of the supernatant was then transferred to another 96-well plate to measure the absorbance at 540 nm. To determine the percentage of hemolysis, the following formula was used: $(A_{\text{tret}} - A_{\text{nontret}})/(A_{\text{TX-tret}} - A_{\text{nontret}}) \times 100$, where A_{tret} is the absorbance of the compound-treated well, A_{nontret} the absorbance of the negative controls (without compound), and $A_{\text{TX-tret}}$ the absorbance of the Triton X-100-containing well. Each concentration had triplicate values and the HC_{50} was determined by taking the average of triplicate O.D. values.

Antibacterial Assay in the Presence of Human Plasma and Serum.[44] To examine the susceptibility of the new compounds toward plasma proteases, the antibacterial activity was tested in the presence of 50% of human plasma and serum. Briefly, 250 μL of compounds **20** and **21** was added into 250 μL of fresh human plasma and serum and incubated at 37 °C. After 3 h of incubation, the aliquot was 2-fold diluted in 0.9% saline. Then antibacterial activities were determined against *S. aureus* and *E. coli* by following the same protocol as described above for antibacterial assay.

LDH Assay. Cytotoxicity of compound **21** was assessed against the RAW cell line through LDH assay by using cytotoxicity assay kit (CytoTox 96 Non-Radioactive, Promega). Briefly, the cells were grown in a 96-well plate in DMEM media (supplemented with 10% fetal bovine serum and 5% penicillin−streptomycin) until they reached around 70−80% confluency. The cells were then treated with 50 μL of serially diluted compound. Two controls were made: one containing no compound (nontreated cells) and the other one treated with 0.5 vol % Triton-X solution. The plate was incubated for 24 h at 37 °C under 5% CO_2 atmosphere. The plate was then centrifuged at 1100 rpm for 5 min. The supernatants from respective wells were transferred and the assay was performed according to the manufacturer's protocol. The supernatant (100 μL) was transferred to a fresh 96-well plate and absorbance at 490 nm was measured using a Tecan Infinite Pro series M200 Microplate Reader. Percentage of cell death was determined as $(A \times A_0)/(A_{\text{total}} \times A_0) \times 100$, where A is the absorbance of the test well, A_0 is the absorbance of the negative controls, and A_{total} is the absorbance of Triton-X treated wells, all at 490 nm. Percentage of LDH release, i.e., cell death was plotted as a function of concentration of the compound, and the half-maximal inhibitory concentration (EC_{50}) was defined as the compound concentration that caused 50% LDH release relative to the positive control. For bright-field microscopic images, a 20× objective was used and images were captured using a Leica DM2500 microscope.

Bactericidal Time-Kill Kinetics.[44,45] The bactericidal activity of the compounds was evaluated by performing time kill kinetics assay. Briefly, *S. aureus* and *E. coli* were grown in respective broth at 37 °C for 6 h to their mid log phase. The test compound **21** was then added to the bacterial solution (approximately 10^5 CFU/mL) with the working concentration of about 4 × MIC and 8 × MIC. For the control, the same experiment was performed with same volume of saline instead of the compound. At different time intervals corresponding to 15, 30, 60, 120, and 180 min, 20 μL of aliquots from that solution were serially diluted 10-fold in 0.9% saline. From the dilutions, 20 μL was plated on yeast−dextrose agar plates and incubated at 37 °C for 24 h. The bacterial colonies were counted and results were represented in logarithmic scale, i.e., Log (CFU/mL).

Antibacterial Activity against Metabolically Inactive Bacterial Cells.[45] *Stationary Phase Bacteria.* Stationary phase bacterial culture was achieved by following a procedure in the literature with little modification. Briefly, mid log phase bacterial culture was diluted to 1:1000 in respective medium and allowed to grow for 16 h to reach stationary phase. These bacterial suspensions were harvested (9000 rpm, 2 min), washed in MEM (*S. aureus*) or M9 media (*E. coli*). It was then resuspended and diluted to $\sim 10^5-10^6$ CFU/mL in MEM (*S. aureus*) or M9 media (*E. coli*). Compound **21** and control antibiotics (ampicillin and kanamycin) were then added to a 96-well plate containing 150 μL of these bacterial suspensions. In the case of the control experiment, the same volume of saline was added instead of compound or antibiotics. The plate was then incubated at 37 °C for 2 h. After 2 h, the bacterial suspension was serially diluted (10-fold) and spot-plated on agar plates. The viable colonies (<100) were counted after 48 h incubation at 37 °C and the cell viability were represented as Log CFU/mL.

Persister Cells. Persister cells were prepared from the stationary phase bacterial cells. Briefly, 1 mL of stationary phase culture was treated with 100 μg/mL (for *S. aureus*) and 300 μg/mL (for *E. coli*) of ampicillin sodium for 3 h at 37 °C to generate the persister cells. After 3 h, the bacteria were centrifuged, washed three−four times, and resuspended in M9 media (*E. coli*) or MEM (*S. aureus*) to remove the traces of the antibiotic. Then the bacterial suspension was diluted to $\sim 10^6$ CFU/mL in M9 media (*E. coli*) or MEM (*S. aureus*). Compound **21** and the control antibiotics ampicillin and kanamycin were added to a 96-well plate containing 150 μL of these persister cells. It was then incubated at 37 °C for 2 h. One control was made where the same volume of saline was added instead of compound or antibiotics. At the end of 2 h, it was serially diluted in a 10-fold manner and spot-plated on agar plates. The viable colonies (<100) were counted after 24 h incubation at 37 °C and the cell viability were represented as Log CFU/mL and the result was compared with nontreated control.

Biofilm Disruption Assay.[45] *Quantification of Cell Viability.* Mid log phase (6 h grown) culture of bacteria (*S. aureus* and *E. coli*) was diluted to a concentration of $\sim 10^5$ CFU/mL to make the bacterial stock solution. *S. aureus* suspension was prepared in nutrient broth supplemented with 1% glucose and 1% NaCl. For *E. coli*, M9 media supplemented with 0.02% casamino acid and 1% glycerol was used. Then 2 mL of the suspension was added into 6 well plates containing a sterile 18 mm glass coverslip. The plates were then incubated under stationary conditions for 24 and 72 h for *S. aureus* and *E.*

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coli, respectively. The biofilm-containing coverslips were then carefully taken out and washed once with 1× PBS and placed into another 6-well plate. After that, 2 mL of the test compound **21** diluted in the respective medium (at various concentrations) were added to the preformed biofilms. It was then allowed to incubate for 24 h. The control experiments were performed where the same volume of respective medium was added instead of compound. At the end of 24 h, the coverslips were then taken out and washed carefully with 1× PBS and placed into another 6-well plate. Two mL of trypsin−EDTA solution diluted in saline (1:4 ratios) was added to the coverslip containing 6-well plate and allowed to incubate for 15 min. Cell suspension was then assessed by plating serial 10-fold dilutions of biofilm on respective agar plates. After 24 h of incubation the plates were counted and cell viability was expressed as Log (CFU/mL) and compared with the nontreated control.

Confocal Laser-Scanning Microscope (CLSM) of Biofilms. Coverslips (18 mm) were first sterilized by soaking them in ethanol followed by drying in flame and then placing in well of a 6-well plate. Mid log phase (6 h grown) culture of *S. aureus* and *E. coli* were then diluted to approximately 10^5 CFU/mL in a nutrient broth supplemented with 1% glucose and 1% NaCl (for *S. aureus*) or in M9 medium supplemented with 0.02% casamino acid and 1% glycerol (for *E. coli*). 2 mL of this bacterial suspension were then added into 6 well plates containing the sterile coverslips. The plate was then incubated under stationary conditions for 24 and 72 h for *S. aureus* and *E. coli*, respectively. After that, the medium was removed and planktonic bacteria were carefully washed out with 1 × PBS (pH = 7.4). Biofilm containing coverslips were placed into other 6-well plates and 2 mL aliquots of test compound **21** (8 × MIC) diluted in the respective medium were then added to it and allowed to incubate for 24 h. In the case of the control, 2 mL of respective medium was added instead of compound. At the end of 24 h, the medium was removed. Then, planktonic cells were removed by washing with 1 × PBS. Coverslips containing biofilm were carefully removed from the well and stained with SYTO-9 (3 μM) and imaged using a confocal laser-scanning microscope.

MBEC Assay.[45] In order to determine the minimum concentration required to completely eradicate the preformed biofilms, we have also performed the MBEC assay for the optimized compound. Briefly, mid log phase culture of *S. aureus* was diluted to a concentration of $\sim 10^5$ CFU/mL in nutrient broth supplemented with 1% glucose and 1% NaCl. Then, 100 μL of the suspension was added into the wells of 96-well plates. The plates were then allowed to incubate under stationary conditions for 24 h. At the end of 24 h, the medium and planktonic bacteria were removed and the plate was carefully washed with 1 × PBS. After that, 100 μL of the test compound **21** diluted in the medium (at various concentrations) was added to the preformed biofilms. It was then allowed to incubate for 24 h. The control experiments were performed where 100 μL of media was added instead of the compound. At the end of 24 h, the media along with disrupted biofilms were removed and the plate carefully washed with 1 × PBS. 200 μL of fresh media was then added to the disrupted biofilms and allowed to regrow for 24 h. The O.D. values (at 600 nm) of the regrown biofilms were then recorded by using TECAN (Infinite series, M200 pro) plate reader. This experiment was performed in triplicate and the MBEC value was considered to be the lowest concentration at which the O.D. (average of the triplicate values) was found to be below 0.1.

Membrane Active Mechanism of Action. *Cytoplasmic Membrane Permeabilization Assay.*[44] Briefly, the 6 h grown cultures (mid log phase) of *S. aureus* and *E. coli* were harvested (3500 rpm, 5 min), washed in 5 mM glucose and 5 mM HEPES buffer (pH = 7.4) in 1:1 ratio. It was then resuspended in 5 mM HEPES buffer, 5 mM glucose solution in 1:1 ratio. After that, 10 μM of propidium iodide (PI) was added to black-bottom 96-well plates containing 200 μL of bacterial suspension. The fluorescence was monitored for 4 min at excitation wavelength of 535 nm and emission wavelength of 617 nm. Next, 10 μL of test compound **21** (at various concentrations) was added to the plate containing bacterial suspension and dye. As a measure of the extent of membrane permeabilization, increase in fluorescence was monitored for another 16 min.

Cytoplasmic Membrane Depolarization Assay.[44,45] The 6 h grown cultures (mid log phase) of *S. aureus* and *E. coli* were harvested (3500 rpm, 5 min), washed in 5 mM glucose and 5 mM HEPES buffer (pH = 7.4) in 1:1 ratio and resuspended in 5 mM HEPES buffer, 5 mM glucose, and 100 mM KCl solution in 1:1:1 ratio. Then, 2 μM of 3,3′-Dipropylthiadicarbocyanine iodide ($DiSC_3$) was added to 96-well plates containing 200 μL of bacterial suspension. It was then allowed to incubate for 20 min in the case of *S. aureus* and about 45 min for *E. coli*. The fluorescence was monitored for 4 min at excitation wavelength of 622 nm and emission wavelength of 670 nm. After that, 10 μL of test compound **21** (at various concentrations) was added to the plate containing bacterial suspension and dye. As a measure of membrane depolarization, increase in fluorescence was monitored for another 16 min.

K^+ Leakage Assay.[44,45] This assay was performed by following the previously reported protocol with a little modification. Briefly, mid log phase *S. aureus* and *E. coli* were harvested (3500 rpm, 5 min), washed, and resuspended in 10 mM HEPES buffer and 0.5% glucose in 1:1 ratio. Then, 200 μL of the bacterial suspension with PBFI-AM dye (1 μM) was added in the wells of a black-bottom 96-well plate. Fluorescence of the plate was measured at excitation wavelength of 346 nm and emission wavelength of 505 nm (slit width of 5 nm) for 4 min. After that, 10 μL aliquots of test compound **21** (at various concentrations) and valinomycin (10 μg/mL) were added to the bacterial suspension containing PBFI-AM dye, and fluorescence was monitored for another 16 min. As a measure of K^+ leakage, the increase in fluorescence signals was measured and data were compared with the positive control valinomycin.

Resistance Studies.[44,45] For resistance study the antibiotics norfloxacin and colistin were chosen as the positive control. Briefly, the MIC values of compound **21** against both the bacteria *S. aureus* and *E. coli* and for the control antibiotics norfloxacin (against *S. aureus*) and colistin (against *E. coli*) were determined as described in the antibacterial assay. For the next day MIC experiment, the bacterial dilution was made by using the bacteria from sub-MIC (MIC/2) concentration of the compound **21** and control antibiotics. After 24 h incubation period, again bacterial dilution was prepared like the previous day by using the bacterial suspension from sub-MIC concentration and assayed for the next MIC experiment. The process was repeated for 14 passages. The fold of MIC increase for the test compound **21** and the control antibiotics were plotted against the number of days.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00494.

Details of characterization of the final compounds, spectral data (NMR and HR-MS spectra) and figures for microscopy images of RAW cells of cytotoxicity assay, bactericidal kinetics, activity in the presence of plasma and serum, resistance studies (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

HBTU, *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)-uronium hexafluorophosphate; $(Boc)_2O$, di-*tert*-butyldicarbonate; DIPEA, *N,N*-diisopropylethylamine; DCM, dichloromethane; DMF, *N,N*-dimethylformamide; MRSA, Methicillin-resistant *S. aureus*; VRE, Vancomycin-resistant *E. faecium*; MIC, minimum inhibitory concentration; HC_{50}, 50% hemolytic concentration; MBEC, Minimum Biofilm Eradication Concentration; CFU, colony forming units; PI, propidium iodide; $DiSC_3(5)$, 3, 3′-dipropylthiadicarbocyanine iodide

■ REFERENCES

(1) Bush, K., Courvalin, P., Dantas, G., Davies, J., Eisenstein, B., Huovinen, P., Jacoby, G. A., Kishony, R., Kreiswirth, B. N., Kutter, E., et al. (2011) Tackling antibiotic resistance. *Nat. Rev. Microbiol. 9*, 894−896.

(2) Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., Scheld, M., Spellberg, B., and Bartlett, J. (2009) Bad Bugs, No Drugs: No ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis. 48*, 1−12.

(3) Stewart, P. S., and Costerton, J. W. (2001) Antibiotic resistance of bacteria in biofilms. *Lancet 358*, 135−138.

(4) Parsek, M. R., and Singh, P. K. (2003) Bacterial biofilms: an emerging link to disease pathogenesis. *Annu. Rev. Microbiol. 57*, 677−701.

(5) Davies, D. (2003) Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug Discovery 2*, 114−122.

(6) Whiteley, M., Bangera, M. G., Bumgarner, R. E., Parsek, M. R., Teitzel, G. M., Lory, S., and Greenberg, E. P. (2001) Gene expression in *Pseudomonas aeruginosa* bioflms. *Nature 413*, 860−864.

(7) Mah, T. F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S., and O'Toole, G. A. (2003) A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature 426*, 306−310.

(8) Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., and Ciofu, O. (2010) Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Agents 35*, 322−332.

(9) Savage, V. J., Chopra, I., and O'Neill, A. J. (2013) *Staphylococcus aureus* biofilms promote horizontal transfer of antibiotic resistance. *Antimicrob. Agents Chemother. 57*, 1968−1970.

(10) Hoffman, L. R., D'Argenio, D. A., MacCoss, M. J., Zhang, Z., Jones, R. A., and Miller, S. I. (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature 436*, 1171−1175.

(11) Worthington, R. J., Richards, J. J., and Melander, C. (2012) Small molecule control of bacterial biofilms. *Org. Biomol. Chem. 10*, 7457−7474.

(12) Böttcher, T., Kolodkin-Gal, I., Kolter, R., Losick, R., and Clardy, J. (2013) Synthesis and activity of biomimetic biofilm disruptors. *J. Am. Chem. Soc. 135*, 2927−2930.

(13) Jennings, M. C., Ator, L. E., Paniak, T. J., Minbiole, K. P. C., and Wuest, W. M. (2014) Biofilm eradicating properties of quaternary ammonium amphiphiles: simple mimics of antimicrobial peptides. *ChemBioChem 15*, 2211−2215.

(14) de la Fuente-Núñez, C., Reffuveille, F., Mansour, S. C., Reckseidler-Zenteno, S. L., Hernández, D., Brackman, G., Coenye, T., and Hancock, R. E. (2015) D-Enantiomeric peptides that eradicate wild-type and multidrug-resistant biofilms and protect against lethal *Pseudomonas aeruginosa* infections. *Chem. Biol. 22*, 196−205.

(15) Garrison, A. T., Bai, F., Abouelhassan, Y., Paciaroni, N. G., Jin, S., and Huigens, R. W. (2014) Bromophenazine derivatives with potent inhibition, dispersion and eradication activities against *Staphylococcus aureus* biofilms. *RSC Adv. 5*, 1120−1124.

(16) Hoque, J., Konai, M. M., Gonuguntla, S., Manjunath, G. B., Samaddar, S., Yarlagadda, V., and Haldar, J. (2015) Membrane active small molecules show selective broad spectrum antibacterial activity with no detectable resistance and eradicate biofilms. *J. Med. Chem. 58*, 5486−5500.

(17) Duncan, B., Li, X., Landis, R. F., Kim, S. T., Gupta, A., Wang, L. S., Ramanathan, R., Tang, R., Boerth, J. A., and Rotello, V. M. (2015) Nanoparticle-stabilized capsules for the treatment of bacterial biofilms. *ACS Nano 9*, 7775−7782.

(18) Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. *Nature 415*, 389−395.

(19) Pirri, G., Giuliani, A., Nicoletto, S. F., Pizzuto, L., and Rinaldi, A. C. (2009) Lipopeptides as anti-infectives: a practical perspective. *Cent. Eur. J. Biol. 4*, 258−273.

(20) Kapoor, R., Wadman, M. W., Dohm, M. T., Czyzewski, A. M., Spormann, A. M., and Barron, A. E. (2011) Antimicrobial peptoids are effective against *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother. 55*, 3054−3057.

(21) De Zoysa, G. H., Cameron, A. J., Hegde, V. V., Raghothama, S., and Sarojini, V. (2015) Antimicrobial peptides with potential for biofilm eradication: synthesis and structure activity relationship studies of battacin peptides. *J. Med. Chem. 58*, 625−639.

(22) Nagant, C., Pitts, B., Stewart, P. S., Feng, Y., Savage, P. B., and Dehaye, J. P. (2013) Study of the effect of antimicrobial peptide mimic, CSA-13, on an established biofilm formed by *Pseudomonas aeruginosa*. *MicrobiologyOpen 2*, 318−325.

(23) Haug, B. E., Stensen, W., Kalaaji, M., Rekdal, O., and Svendsen, J. S. (2008) Synthetic antimicrobial peptidomimetics with therapeutic potential. *J. Med. Chem. 51*, 4306−4314.

(24) Steenbergen, J. N., Alder, J., Thorne, G. M., and Tally, P. (2005) Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. *J. Antimicrob. Chemother. 55*, 283−288.

(25) Falagas, M. E., and Kasiakou, S. K. (2005) Colistin: The revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin. Infect. Dis. 40*, 1333−1341.

(26) Deris, Z. Z., Swarbrick, J. D., Roberts, K. D., Azad, M. A. K., Akter, J., Horne, A. S., Nation, R. L., Rogers, K. L., Thompson, P. E., Velkov, T., et al. (2014) Probing the penetration of antimicrobial polymyxin lipopeptides into Gram-negative bacteria. *Bioconjugate Chem. 25*, 750−760.

(27) Jones, T., Yeaman, M. R., Sakoulas, G., Yang, S. J., Proctor, R. A., Sahl, H. G., Schrenzel, J., Xiong, Y. Q., and Bayer, A. S. (2008) Failures in clinical treatment of *Staphylococcus aureus* infection with daptomycin are associated with alterations in surface charge, membrane phospholipid asymmetry, and drug binding. *Antimicrob. Agents Chemother. 52*, 269−278.

DOI: 10.1021/acs.bioconjchem.5b00494
Bioconjugate Chem. 2015, 26, 2442−2453

(28) Chen, Y. X., Mant, C. T., Farmer, S. W., Hancock, R. E. W., Vasil, M. L., and Hodges, R. S. (2005) Rational design of alpha-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index. *J. Biol. Chem. 280*, 12316−12329.
(29) Porter, E. A., Wang, X., Lee, H. S., Weisblum, B., and Gellman, S. H. (2000) Non-haemolytic beta-amino-acid oligomers. *Nature 405*, 298−298.
(30) Violette, A., Averlant-Petit, M. C., Semetey, V., Hemmerlin, C., Casimir, R., Graff, R., Marraud, M., Briand, J. P., Rognan, D., and Guichard, G. (2005) *N,N′*-Linked oligoureas as foldamers: chain length requirements for helix formation in protic solvent investigated by circular dichroism, NMR spectroscopy, and molecular dynamics. *J. Am. Chem. Soc. 127*, 2156−2164.
(31) Choi, S., Isaacs, A., Clements, D., Liu, D. H., Kim, H., Scott, R. W., Winkler, J. D., and DeGrado, W. F. (2009) De novo design and in vivo activity of conformationally restrained antimicrobial arylamide foldamers. *Proc. Natl. Acad. Sci. U. S. A. 106*, 6968−6973.
(32) Ilker, M. F., Nusslein, K., Tew, G. N., and Coughlin, E. B. (2004) Tuning the hemolytic and antibacterial activities of amphiphilic polynorbornene derivatives. *J. Am. Chem. Soc. 126*, 15870−15875.
(33) Colomer, A., Pinazo, A., Manresa, M. A., Vinardell, M. P., Mitjans, M., Infante, M. R., and Pérez, L. (2011) Cationic surfactants derived from lysine: effects of their structure and charge type on antimicrobial and hemolytic activities. *J. Med. Chem. 54*, 989−1002.
(34) Radzishevsky, I. S., Rotem, S., Bourdetsky, D., Navon-Venezia, S., Carmeli, Y., and Mor, A. (2007) Improved antimicrobial peptides based on acyl-lysine oligomers. *Nat. Biotechnol. 25*, 657−659.
(35) Ghosh, C., Manjunath, G. B., Akkapeddi, P., Yarlagadda, V., Hoque, J., Uppu, D. S. S. M., Konai, M. M., and Haldar, J. (2014) Small molecular antibacterial peptoid mimics: The simpler the better! *J. Med. Chem. 57*, 1428−1436.
(36) Nederberg, F., Zhang, Y., Tan, J. P. K., Xu, K. J., Wang, H. Y., Yang, C., Gao, S. J., Guo, X. D., Fukushima, K., Li, L. J., et al. (2011) Biodegradable nanostructures with selective lysis of microbial membranes. *Nat. Chem. 3*, 409−414.
(37) Haldar, J., Kondaiah, P., and Bhattacharya, S. (2005) Synthesis and antibacterial properties of novel hydrolyzable cationic amphiphiles. Incorporation of multiple head groups leads to impressive antibacterial activity. *J. Med. Chem. 48*, 3823−3831.
(38) Uppu, D. S. S. M., Akkapeddi, P., Manjunath, G. B., Yarlagadda, V., Hoque, J., and Haldar, J. (2013) Polymers with tunable side-chain amphiphilicity as non-hemolytic antibacterial agents. *Chem. Commun. 49*, 9389−9391.
(39) Niu, Y., Padhee, S., Wu, H., Bai, G., Qiao, Q., Hu, Y., Harrington, L., Burda, W. N., Shaw, L. N., Cao, C., et al. (2012) Lipo-γ-AApeptides as a new class of potent and broad-spectrum antimicrobial agents. *J. Med. Chem. 55*, 4003−4009.
(40) Makovitzki, A., Avrahami, D., and Shai, Y. (2006) Ultrashort antibacterial and antifungal lipopeptides. *Proc. Natl. Acad. Sci. U. S. A. 103*, 15997−16002.
(41) Balakrishna, R., Wood, S. J., Nguyen, T. B., Miller, K. A., Kumar, E. V. K. S., Datta, A., and David, S. A. (2006) Structural correlates of antibacterial and membrane permeabilizing activities in acylpolyamines. *Antimicrob. Agents Chemother. 50*, 852−861.
(42) Worley, B. V., Slomberg, D. L., and Schoenfisch, M. H. (2014) Nitric Oxide releasing quaternary ammonium-modified poly-(amidoamine) dendrimers as dual action antibacterial agents. *Bioconjugate Chem. 25*, 918−927.
(43) Schmidt, M., Harmuth, S., Barth, E. R., Wurm, E., Fobbe, R., Sickmann, A., Krumm, C., and Tiller, J. C. (2015) Conjugation of ciprofloxacin with poly(2-oxazoline)s and poly ethylene glycol via end groups. *Bioconjugate Chem. 26*, 1950.
(44) Konai, M. M., Ghosh, C., Yarlagadda, V., Samaddar, S., and Haldar, J. (2014) Membrane active phenylalanine conjugated lipophilic norspermidine derivatives with selective antibacterial activity. *J. Med. Chem. 57*, 9409−9423.
(45) Konai, M. M., and Haldar, J. (2015) Lysine based small molecules that disrupt biofilms and kill both actively growing planktonic and non-dividing stationary phase bacteria. *ACS Infect. Dis. 1*, 469−478.
(46) Joly, J. P., Mata, G., Eldin, P., Briant, L., Vive, F. F., Duca, M., and Benhida, R. (2014) Artificial nucleobase-amino acid conjugates: a new class of TAR RNA binding agents. *Chem. - Eur. J. 20*, 2071−2079.
(47) Lehrman, J. A., Cui, H., Tsai, W. W., Moyerb, T. J., and Stupp, S. I. (2012) Supramolecular control of self-assembling terthiophene−peptide conjugates through the amino acid side chain. *Chem. Commun. 48*, 9711−9713.
(48) Chongsiriwatana, N. P., Patch, J. A., Czyzewski, A. M., Dohm, M. T., Ivankin, A., Gidalevitz, D., Zuckermann, R. N., and Barron, A. E. (2008) Peptoids that mimic the structure, function, and mechanism of helical antimicrobial peptides. *Proc. Natl. Acad. Sci. U. S. A. 105*, 2794−2799.
(49) Irwansyah, I., Li, Y. Q., Shi, W., Qi, D., Leow, W. R., Tang, M. B. Y., Li, S., and Chen, X. (2015) Gram-Positive antimicrobial activity of amino acid-based hydrogels. *Adv. Mater. 27*, 648−654.
(50) Roy, S., and Das, P. K. (2008) Antibacterial hydrogels of amino acid-based ctionic amphiphiles. *Biotechnol. Bioeng. 4*, 756−764.

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