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Rational Design of Dimeric Lysine *N*-Alkylamides as Potent and Broad-Spectrum Antibacterial Agents

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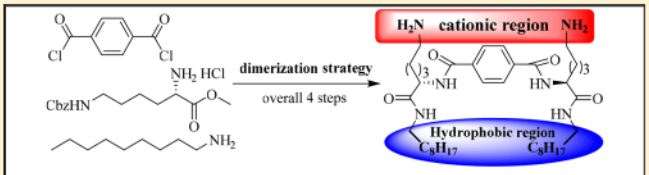
Supporting Information

ABSTRACT: Antibiotic resistance is one of the biggest threats to public health, and new antibacterial agents hence are in an urgent need to combat infectious diseases caused by multidrug-resistant (MDR) pathogens. Utilizing dimerization strategy, we rationally designed and efficiently synthesized a new series of small molecule dimeric lysine alkylamides as mimics of AMPs. Evaluation of these mimics against a panel of Gram-positive and Gram-negative bacteria including MDR strains was performed, and a broad-spectrum and potent compound **3d** was identified. This compound displayed high specificity toward bacteria over mammalian cell. Time−kill kinetics and mechanistic studies suggest that compound **3d** quickly eliminated bacteria in a bactericidal mode by disrupting bacterial cell membrane. In addition, lead compound **3d** could inhibit biofilm formation and did not develop drug resistance in *S. aureus* and *E. coli* over 14 passages. These results suggested that dimeric lysine nonylamide has immense potential as a new type of novel small molecular agent to combat antibiotic resistance.



MIC and HC_{50} (μg/mL)

MRSE	MRSA	VRE	*E.coli.*	*P.aeruginosa*	HC_{50}
1.5-3	**0.75-1.5**	**3-6**	**0.75-1.5**	**3-6**	**180**

INTRODUCTION

The increasingly emerging resistance to antibiotics has become one of the most pressing public concerns all over the world.[1] Multidrug resistant (MDR) bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis* (MRSE), and vancomycin-resistant *enterococciare* (VRE) are major cause of hospital and community acquired infection, leading to high mortality rate and much additional heath care costs. Pandrug resistant (PDR) strains of Gram-negative such as *E. coli*, *Pseudomonas aeruginosa* are now invulnerable to most antibiotics, even resistant to carbapenem drugs, the antibiotics of last resort, thereby posing substantial threats to public health.[2] Meanwhile, new conventional drugs being approved for entering the clinical pipeline are steadily declining.[3,4] Consequently, antibacterial entities with new structures and novel action modes are in highly urgent demand for combating antibiotic resistance.

Antimicrobial peptides (AMPs), which occur in virtually all organisms, are the first line of nonspecific defense system against a variety of pathogens including bacteria, fungi, and virus.[5,6] Out of 1000 known antimicrobial peptides (AMPs) with various secondary or tertiary structures, vast majority of them are generally short and overall positive charged peptides, and they share inducible amphiphilic confirmations where cationic residues and hydrophobic side chains are segregated onto opposite regions, which is believed to be responsible for their ability to kill bacterial cells. Unlike conventional antibiotics targeting specific cell wall or intracellular components of bacteria, AMPs are thought to mainly act on bacterial membranes in which cationic residues of peptides are initially attracted by negatively charged bacterial membranes, and then lipophilic side chains insert into the lipid bilayer to compromise membrane stability and integrity through multiple modes, leading to eventual bacterial death. This is also believed to be the main reason why AMPs are less prone to eliciting resistance.[7−10] In addition, AMPs display high specificity toward bacterial membranes over mammalian cell membranes because the components of the two types of membranes are different. Bacterial membranes are rich in negative charges, and the mammalian cell membranes are composed of largely zwitterionic sphingomyelin and phosphatidylcholine; therefore cationic antibacterial peptides could selectively recognize bacterial cytoplasmic membranes instead of mammalian cell membranes through electrostatic attraction and hydrophobic effects. The combination of low propensity to induce antibiotic resistance and high specificity against bacteria made AMPs the potential sources for discovery of new anti-infectious agents.[11,12] However, AMPs so far have not been widely applied to practice due to their in vivo enzymatic instability and moderate efficacy, potential immunogenicity, and high production costs.[13−15] To overcome these problems, a variety

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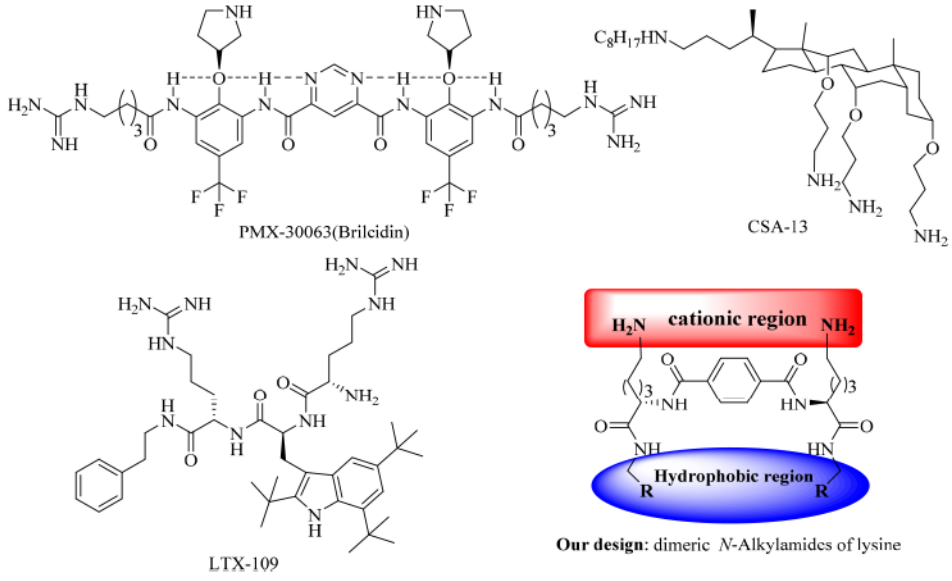
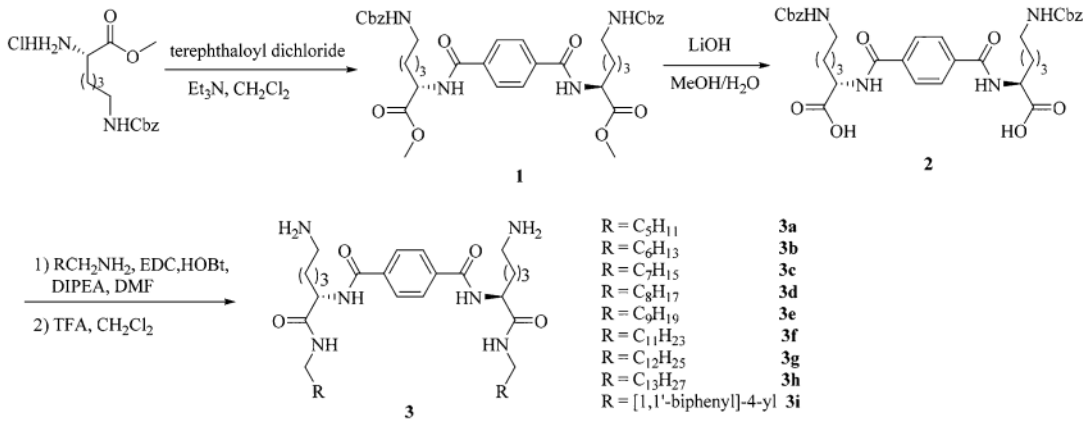


Figure 1. Compounds in clinic trials and our design of AMPs mimic based on dimeric lysine *N*-alkylamides.

Scheme 1. General Scheme for Synthesis of Dimeric Lysine *N*-Alkylamides 3a−i



of strategies have been adopted. Strategies of peptidomimetics including α-peptides,[16−18] β-peptides,[19−21] peptoids,[22] AApeptides,[23−29] and others[30−32] have been intensively explored in the past several decades. However, preparation of these oligomeric peptidomimetics still faces challenges of tedious synthesis and high cost. In recent years, small molecular mimics of AMPs, which were generally designed based on molecular scaffolds carrying hydrophobic patch and usually two to four cationic groups with inducible amphiphilic conformations, aroused significant interest. Examples include arylamide foldamers,[33] poly(phenyleneethynylene)s,[34,35] cholic acid scaffold,[36] xanthone,[37,38] norspermidine,[39−42] small peptoids,[43,44] aminoglycosides,[45−48] and so on.[49−56] These small molecules retain the advantages of macromolecular mimetics of AMPs such as mechanism of membrane disruption and high potency; in addition, due to their straightforward synthesis and more drug-like properties, they demonstrate higher promise in the development of antibacterial therapeutics. Their recent success is highlighted by three mimics (LTX-109, PMX-30063, and CSA-13) in clinical trials.[57−59] Being interested in discovering novel agents to overcome antibiotic resistance, we continue our exploration on the synthesis and evaluation of small molecular AMPs mimics.

RESULTS AND DISCUSSION

Design and Synthesis of Mimics. Previously, we have synthesized two series of γ-AApeptide-based AMP mimics, and some of those mimics displayed strong activities against drug resistant strains with elevated stability and low probability to elicit drug resistance.[60−63] However, molecular complexity of these peptidomimetics-based mimics and the number of steps involved in their synthesis hampered their further optimization and practical applications. As such, we sought to design small molecular mimics of AMPs, emphasizing on simple structures, easy synthesis, and low production cost without jeopardizing antibacterial properties. It is well-known that dimerization strategy is widely used by researchers to make compounds for therapeutic[64−66] and material applications.[67,68] Since known mimics of AMPs have at least two cations, an amphipathic molecular scaffold could be constructed readily through dimerization of lysine utilizing a terephthaloyl linker through the formation of two amide bonds (Figure 1). The balance of hydrophilicity and hydrophobicity could be easily adjusted by reacting two carboxylic groups from lysines with different commercially available alkylamines (Figure 1). With the fine-tune of cationic amphipathicity, we anticipated that potent antimicrobial molecules could be developed.

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Table 1. Antibacterial and Hemolytic Activities of the Compounds 3a−i

		MIC[a] (μg/mL)					
		Gram-positive			Gram-negative		
compd	AlogP/log D[b]	MRSE[c]	MRSA	VRE	*E. coli*	*P.A.*	HC_{50} (μg/mL)[a,e]
3a	3.66/1.65	NA[d]	NA[d]	NA[d]	NA[d]	NA[d]	ND[f]
3b	4.57/2.57	NA[d]	NA[d]	NA[d]	6−12	NA[d]	ND[f]
3c	5.48/3.48	3−6	1.5−3	NA[d]	0.75−1.5	3−6	>250
3d	5.63/3.62	1.5−3	0.75−1.5	3−6	0.75−1.5	3−6	180
3e	7.31/5.31	6−12	1.5−3	3−6	3−6	NA[d]	250
3f	9.13/7.13	6−12	3−6	NA[d]	3−6	12−25	>250
3g	10.04/8.04	6−12	6−12	NA[d]	NA[d]	NA[d]	ND[f]
3h	10.96/8.95	NA[d]	NA[d]	NA[d]	NA[d]	NA[d]	ND[f]
3i	5.38/3.38	6−12	3−6	NA[d]	3−6	12−25	>250

[a]All measurements were performed three times. [b]AlogP and log *D* calculated by software Pipeline Pilot 7.5. [c]MRSE, methicillin-resistant *S. epidermidis* (RP62A); MRSA, methicillin-resistant *S. aureus* (ATCC 33591); VRE, vancomycin-resistant *E. faecalis* (ATCC 25922); *E. coli* (ATCC 25922); *P.A.*, *P. aeruginosa* (ATCC 27853). [d]NA: not active. [e]Selectivity is calculated based on HC_{50}/MIC of *S. aureus*. [f]ND: not determined.

On the basis of this design, our synthesis of dimeric mimics of AMPs began with the reaction of terephthaloyl chloride with *N*-(epsilon)-Cbz-L-lysine methyl ester hydrochloride using *N,N*-diisopropylethylamine as a base (Scheme 1), leading to compound **1** in 95.1% yield. Then compound **1** was hydrolyzed in condition of LiOH (aq) in THF, furnishing diacid **2** which was subsequently coupled with various *n*-alkylamine under conditions of EDC, HOBt, DIPEA in DMF. After purification by flash column chromatography, the resulting product was subjected to the deprotection of Cbz group in TFA/CH_2Cl_2. The solvent was removed and the residue was purified by C-18 reversed-phase column chromatography to give final products **3a−i** with more than 95% purity (Supporting Information Table S1). The final compounds were characterized by ^{1}H NMR, ^{13}C NMR, and HRMS (Supporting Information). Our synthetic route of dimeric lysine *N*-alkylamides **3a−i** was short and straightforward, and all reagents used in the synthesis were common and cheap, and the conditions for each step are very mild.

Biological Evaluation of Dimeric Lysine *N*-Alkylamides 3a−i. *In Vitro Antibacterial and Hemolytic Activities.* Having obtained the compounds, we started to evaluate the antibacterial efficacy of compounds **3a−i** against a panel of Gram-positive and Gram-negative bacteria including MDR strains, such as methicillin-resistant *S. epidermidis* (MRSE) (RP62A), methicillin-resistant *S. aureus* (MRSA) (ATCC 33591), vancomycin-resistant *E. faecalis*, *E. coli* (ATCC 25922), and *P. aeruginosa* (ATCC 27853). Hemolytic activity (HC_{50}) was also determined (Table 1) to assess the selectivity of the compounds. As expected, the data from **3a−i** suggest a correlation between alkyl chain length and activity. Dimeric hexylamide of lysine **3a** did not show activity against any of bacterial strains tested up to 25 μg/mL, and heptyl-group-containing mimic **3b** only showed moderate activity (6−12 μg/mL) against *E. coli* and was not active toward other bacterial strains. Mimics **3a** and **3b** were not active mainly because hydrophobicity of two *n*-hexyl or *n*-heptyl groups is not strong enough. This is also consistent with the findings that **3c** has the increased hydrophobicity compared to **3a** and **3b** because of two *n*-octyl groups appended in this compound, exhibiting far more potent activity toward most tested bacteria than that of **3a** and **3b**. As shown in Table 1, **3c** showed an MIC value of 3−6 and 1.5−3 μg/mL against MRSE and MRSA, respectively, and also very strong activity against *E. coli* at 0.75−1.5 μg/mL. The HC_{50} value for this compound is more than 250 μg/mL, indicating excellent selectivity to bacteria over mammalian cells. Further enhancement of hydrophobicity by coupling 1-nonylamine to the core produced **3d** which turned out to be the most potent in the series and strongly inhibited the panel of all bacterial growth. As shown in Table 1, the compound **3d** was very effective against MRSA and MRSE with the MIC values of 0.75−1.5 μg/mL and 1.5−3 μg/mL, respectively. This compound also displayed excellent potency against VRE, *E. coli*, and *P. aeruginosa* in the ranges of 3−6, 0.75−1.5, and 3−6 μg/mL, respectively. Moreover, the HC_{50} value of **3d** was 180 μg/mL against hRBCs, indicating the compound has high specificity toward bacteria. Interestingly, with further increase in alkyl chain from decyl group to tetradecyl group, antibacterial activities of compounds **3e**, **3f**, **3g** and **3h** were gradually decreased in tested conditions; compound **3h** was even not active against any bacteria at 25 μg/mL. Intriguingly, we found that dimeric *N*-([1,1′-biphenyl]-4-ylmethyl)amide of lysine **3i**, the one with different side chain, had good to moderate activities against some of tested bacterial strains.

To further investigate the impact of hydrophobicity on overall activity profile, we measured HPLC retention time (RT) (Table S1) and determined AlogP and log *D* values (Table 1) of compounds **3a−i**. From the correlation of antibacterial activity against *E. coli* and MRSA with RT value (Figure S1), we could observe that when RT value is shorter than 21.6 min for compound **3d**, antibacterial activity of compounds increases with longer RT value, and when RT value is longer than 21.6 min, antibacterial activity against MRSA and *E. coli* decreases. For example, RT value of compound **3c** (18.6 min) is longer than that of compound **3b** (14.9 min); compound **3c** therefore showed higher efficacy against MRSR and *E. coli* than compound **3b** (Table 1); RT values of **3e** and **3f** are 26.4 and 35.7 min, respectively, and anti-MRSA activity of **3e** is more potent than that of **3f**. This rule can be applied to compounds **3f** and **3g**. There is an exception for **3i**. RT value for **3i** is 19.7 min, and RT value for **3b** is 18.6 min; however anti-MRSA and anti-*E. coli* activity of **3i** is less effective than that of **3b**. We believed that anomaly is ascribed to different properties of phenyl ring and *n*-heptyl chain. Same trend can be obtained from the correlation of antibacterial activity against *E. coli* and MRSA with AlogP value (Figure S2). From all these quantitative analysis, we could conclude that antibacterial activity and selectivity based on our scaffold could be improved by introduction of aromatic-ring-containing lipophilic side chain or by switching lysine to arginine and other cationic

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groups or even changing terephthalamide to other amide spacers. More importantly, we could say that there is a balance between hydrophobicity and hydrophilicity for optimum antibacterial activity, and if a compound is too hydrophobic or too hydrophilic, antibacterial activity of this compound will be jeopardized. This quantitative study will help us on design AMPs mimics with more potent antibacterial agents in the future.

Bactericidal Time–Kill Kinetics. It is believed that AMPs exerted their antibacterial effect in a bactericidal manner. To explore the action mode of the synthetic mimic, the kinetics of killing against MRSA was investigated for the most effective compound **3d** at four different concentrations. As shown in Figure 2, at lower concentrations such as 6.25, 12.5 μg/mL

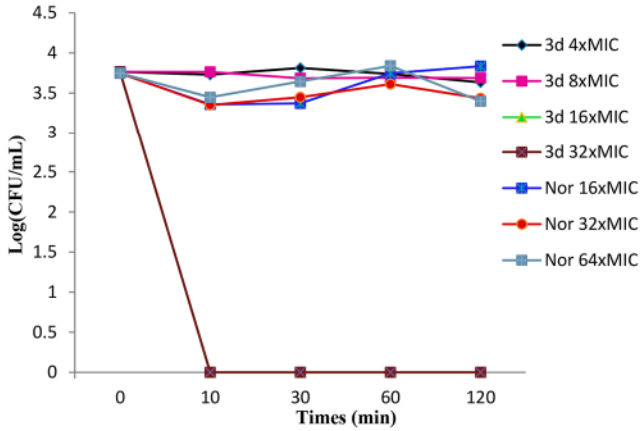

Figure 2. Curve of time–kill kinetics of **3d** and norfloxacin against MRSA. The killing activity against MRSA was monitored for 2 h. The concentrations of **3d** used in the experiments were 4 × MIC, 8 × MIC, 16 × MIC, and 32 × MIC, respectively. The concentrations of norfloxacin used were 16 × MIC, 32 × MIC, and 64 × MIC. The experiments were repeated three times.

which equals 4 and 8 × MIC of **3d**, lead compound cannot eradicate bacteria. However, the bacteria can be removed completely and irreversibly at higher concentration such as 16 and 32 × MIC (3.74 log CFU/mL reduction) within 10 min, suggesting it is bactericidal. In comparison, norfluoxacin cannot kill bacteria even at 64 × MIC, demonstrating it is bacteriostatic. These results indicated that mimic **3d** has a similar manner of action as AMPs.

Mechanism of Action. *Bacterial Membrane Permeability.* It was established that AMPs kill bacteria by primarily disrupting bacterial cell membrane. Since our compounds are mimic of AMPs, they are supposed to mainly act on bacterial membrane. Several spectroscopic techniques were performed to prove this theory. We first investigated bacterial membrane permeability by carrying out experiments of fluorescent microscopy using two dyes of propidium iodide (PI) and 4′,6-diamidino-2-phenylindole (DAPI) as shown in Figure 3. PI dye is commonly used to stain the dead or injured cells with compromised membrane, and DAPI dye can pass through cell membrane irrespective of their viability. The ability of compound **3d** to disintegrate the bacterial membrane of MRSA and *E. coli* was evaluated, and the results were shown in Figure 3. Without treatment with compound **3d**, *E. coli* and MRSA were stained with DAPI (a1 and b1) but not stained with PI (a2 and b2), demonstrating that membrane were intact for both bacterial strains. When tested bacterial cells were incubated with mimic **3d** for 2 h, fluorescence was observed in bacterial cells *E. coli* and MRSA (a3, b3, a4, and b4) under both channels, suggesting that bacterial membranes have been compromised (a4 and b4). The results indicated that the compounds are membrane-active molecules.

Bacterial Membrane Depolarization. To further assess that the antimicrobial mechanism of lead compound **3d** involves disruption of bacterial integrity, we performed membrane depolarization assay employing DiSC3(5) as a potentiometric probe, and results were presented in Figure 4. DiSC3(5) dye generally accumulates on hyperpolarized membranes, leading to weak fluorescence intensity due to self-quenching. When membrane potential is lost, fluorescence intensity would increase dramatically. As shown in Figure 4, when MRSA cells were treated with lead compound **3d** at the concentration of 5 μg/mL, dramatic enhancement of fluorescent intensity was observed. The result showed that membrane potential was lost, demonstrating the disruption of bacterial membrane disruption.

TEM Study. The effect of compound **3d** on the bacterial cell membrane was also investigated by transmission electron micrographs which provide a direct way to visualize cell membrane morphology. Without antibacterial treatment, cell membrane of mid log phase *E. coli*, MRSA had intact

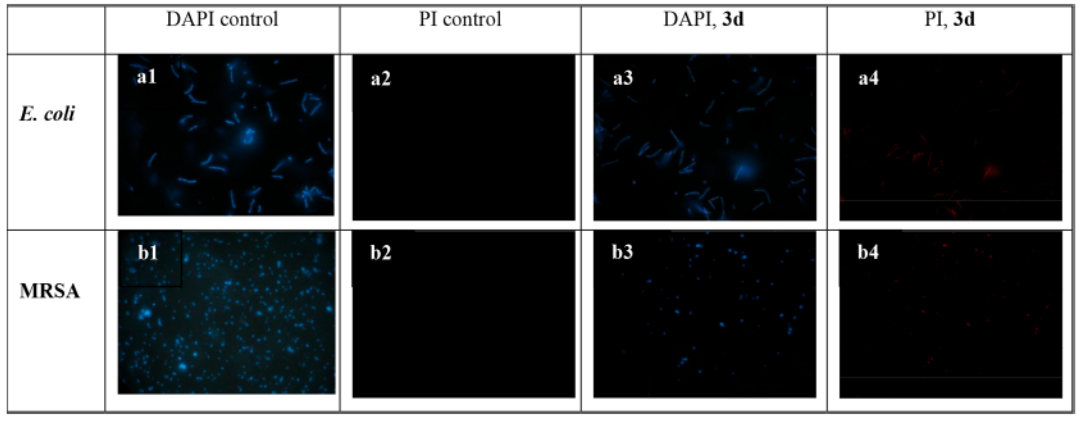

Figure 3. Fluorescence micrographs of *E. coli* and MRSA treated with **3d** at 2 × MIC for 2 h: (a1–a4) *E. coli*; (a1) control, no treatment, DAPI stained; (a2) control, no treatment, PI stained; (a3) 3d treatment, DAPI stained; (a4) 3d treatment, PI stained; (b1–b4) MRSA; (b1) control, no treatment, DAPI stained; (b2) control, no treatment, PI stained; (b3) 3d treatment, DAPI stained; (b4) 3d treatment, PI stained.

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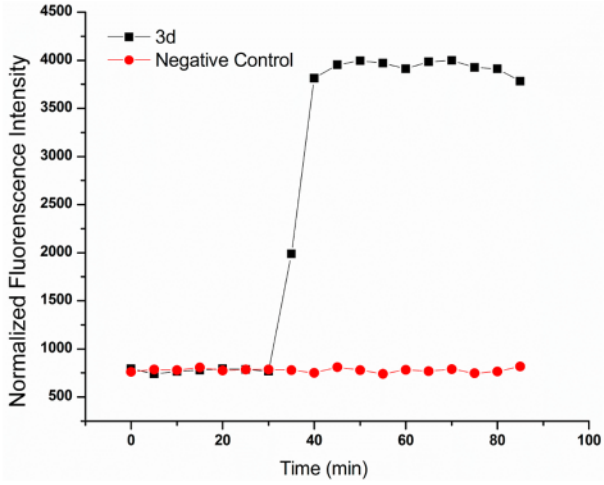

Figure 4. Membrane depolarization against MRSA using DiSC3(5) dye as fluorescence probe. Negative control is the culture without antibacterial treatment. The experiment was repeated three times with duplicates each time.

membrane structures after incubation (Figure 5A and Figure 5C). After exposure to lead 3d at the concentration of 5 μg/mL, we can directly observe that bacterial cells lost membrane integrity, even some cells dispersed (Figure 5B and Figure 5D), indicating that the lead compound disintegrated cell membranes.

Biofilm Inhibition. Most bacterial infections are accompanied by the biofilm formation, and bacterial biofilm contributes to three-quarters of microbial infections in humans such as endocarditis, periodontitis, and chronic lung infections in cystic fibrosis and so on.[69] In addition, due to intrinsic diffusion barrier of extracellular matrix, bacteria within biofilm are much more resistant to conventional antibiotic treatment, which would further lead to acquired antibiotics resistance after long-term antibacterial treatment. Previously, Haldar[40,51] and Gellman's[70] group reported that norspermidine derivatives and nylon-3 polymers can disrupt the biofilm or even disperse mature biofilm. We here evaluated the inhibitory effect of compound 3d on log phase *E. coli* biofilm formation (Figure 6).

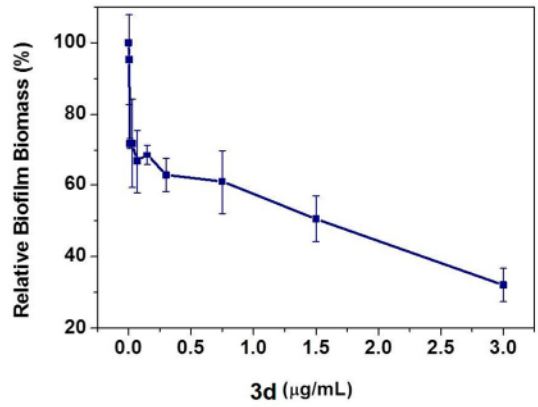

Figure 6. Inhibition of *E. coli* biofilm by compound 3d. Data were reported as an average of three independent experiments.

At a concentration of 1.5 μg/mL, 50% of biofilm formation could be blocked by compound 3d, and inhibition of 70% biofilm formation was observed at the concentration of 3.0 μg/mL.

Propensity To Develop Bacterial Resistance. The emergence of bacterial resistance to conventional antibiotics is a major problem for treatment of infectious diseases. To show the potential of lead 3d as a prospective solution to resistance development, studies on bacterial resistance by Gram-positive bacteria and Gram-negative bacteria toward 3d as well as norfloxacin against *S. aureus* were performed. MICs of 3d toward *S. aureus* and *E. coli* and of norfloxacin toward *S. aureus* were first determined, and then the compounds were incubated with bacteria in the well of the half-MIC and tested for their activity again (Figure 7). MIC values of 3d against two

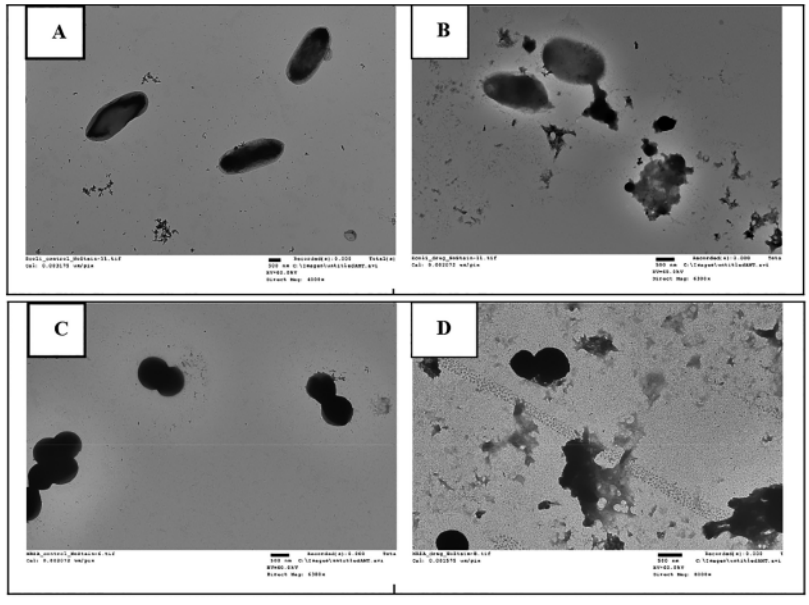

Figure 5. Observation of cell membrane damage by transmission electron micrographs (TEM): (A) *E. coli* cells without any antibacterial treatment (control); (B) *E. coli* cells treated with compound 3d at 5 μg/mL; (C) MRSA cells without any antibacterial treatment (control); (D) MRSA after treatment with 3d at 5 μg/mL. The experiment was repeated three times.

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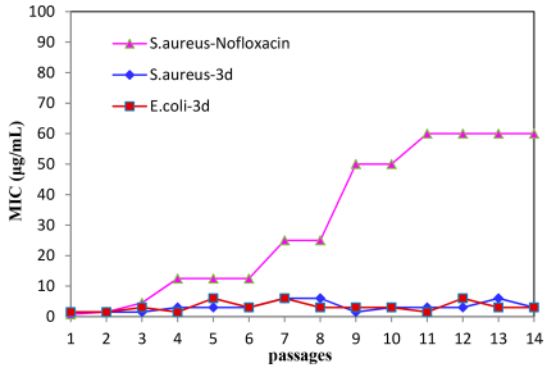

Figure 7. Development of resistance by *S. aureus* (ATCC 33592) and *E. coli* (ATCC 25922) toward **3d** and nofloxacin against *S. aureus* (ATCC 33592). The results were obtained as the average of three independent experiments.

strains remained almost unchanged after 14 passages, whereas norfloxacin induced resistance quickly, over an 80-fold increase in the MIC values. These results demonstrated that lead compound **3d** had minimum propensity to induce bacterial resistance.

CONCLUSION

In summary, a series of dimeric alkylamides of lysine, as mimics of AMPs, have been rationally designed and synthesized through dimerization strategy in a very straightforward way. Biological evaluation of these compounds led to the discovery of a lead compound **3d** that displayed very potent and broad-spectrum antibacterial activities against a series of Gram-positive and Gram-negative bacteria including MRSA, MRSE, and VRE and also showed high specificity toward bacteria. Further performance of other assay demonstrated that **3d** primarily targeted the bacterial cell membranes and killed the bacteria very rapidly. More importantly, mimic **3d** can inhibit biofilm formation and did not readily induce drug resistance. Therefore, this lead compound, combined with the dimerization strategy, would lead to the development of a new class of therapeutic agents to combat the bacterial resistance. Further optimization of dimeric lysine derivatives and in vivo studies with lead compound **3d** are ongoing in our lab.

EXPERIMENTAL SECTION

General Information. All chemicals were purchased as reagent grade without further purification unless especially noted. Dichloromethane was distilled over calcium hydride (CaH_2), and DMF was stirred with CaH_2 and distilled under reduced pressure. All reactions were monitored by thin layer chromatography (Merck silica gel 60 F_{254}). Visualization was accomplished by using a UV (254 nm) lamp and charring with acidic ceric ammonium molybdate solution. Column chromatography was carried out with silica gel (200−300 mesh). All reactions were carried out in oven-dried glassware under argon unless otherwise noted. ^1H and ^{13}C NMR spectra were recorded on Bruker III-400 using $CDCl_3$ or CD_3OD as the solvent. Chemical shifts (in ppm) were referenced to tetramethylsilane (δ = 0 ppm) in deuterated chloroform or CD_3OD. Coupling constants (J) are reported in Hz with the following splitting abbreviations: s = singlet, d = doublet, t = triplet. High-resolution mass spectra were recorded using a Waters Xevo G2 Q-TOF spectrometer. The purity of the compounds was determined to be >95% by analytical HPLC (1 mL/min flow, 25−95% linear gradient of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 60 min) on Agilent 1200 series. TEM images were obtained on a FEI Morgagni 268D TEM with an Olympus MegaView III camera on the microscope

Synthesis and Characterization. Dimethyl 2,2′-(Terephthaloylbis(azanediyl))(2*R*,2′*R*)-bis(6-(((benzyloxy)-carbonyl)amino)hexanoate) (1). To a solution of methyl N^6-((benzyloxy)carbonyl)-L-lysinate hydrochloride (10 g, 30.2 mmol) in CH_2Cl_2 (150 mL) and *N*,*N*-diisopropylethylamine (6.3 mL, 36.2 mmol) at 0 °C, terephthaloyl dichloride (2.9 g, 13.7 mmol) was added in portions, and then the reaction was stirred at room temperature for 2 h. After the reactant had been completely consumed, the mixture was neutralized with HCl (0.1 M) and diluted with H_2O (200 mL). Two layers were separated, and aqueous phase was extracted with CH_2Cl_2 (100 mL × 2). The combined organic phase was washed with water (400 mL), brine, dried over Na_2SO_4, and filtered. After the solvent was removed under reduced pressure, the residue was purified by column chromatography on silica gel (petroleum ether: ethyl acetate = 3:1) to afford the pure product **1** (9.76 g, 95.1%) as a white solid. ^1H NMR (400 MHz, $CDCl_3$) δ 7.85 (s, 2H), 7.34−7.31 (m, 5H), 6.85 (d, J = 6.5 Hz, 1H), 5.06 (d, J = 12.7 Hz, 1H), 5.01 (d, J = 12.7 Hz, 1H), 4.86 (br.s 1H), 4.82−4.75 (dd, J = 6.0, 11.0 Hz, 1H), 3.78 (s, 3H), 3.21−3.19 (m, 2H), 2.04−1.97 (m, 1H), 1.85−1.83 (m, 1H), 1.56−1.40 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.93, 166.32, 156.61, 136.57, 136.48, 128.48, 128.06, 127.98, 127.41, 66.58, 52.55, 52.50, 40.35, 31.83, 29.37, 22.37. HRMS (ESI-TOF) m/z calcd for $C_{38}H_{50}N_5O_{10}$ $[M + NH_4]^+$: 736.3558. Found: 736.3556.

(2*R*,2′*R*)-2,2′-(Terephthaloylbis(azanediyl))bis(6-(((benzyloxy)carbonyl)amino)hexanoic Acid) (2). To a suspension of compound **1** (8.7 g, 12.3 mmol) in MeOH (100 mL) at ice−water bath, LiOH (13.5 mL, 27 mmol, 2 M in water) was added dropwise, and the reaction mixture was stirred at room temperature overnight. After reaction was completed, the pH value of solution was adjusted to 3 by addition of citric acid at 0 °C and white solid was collected by filtration. The filter cake was thoroughly washed with ice−water until the pH value of filtrate reached 7, and then the solid was washed with minimum MeOH, dried using vacuum pump to give product **2** (7.04 g, 84.3%) as a white solid. ^1H NMR (400 MHz, CD_3OD) δ 7.94 (s, 2H), 7.32−7.27 (m, 5H), 5.09−5.03 (m, 2H), 4.59 (dd, J = 4.5, 9.2 Hz, 1H), 3.15−3.12 (m, 2H), 2.01−1.99 (m, 1H), 1.92−1.86 (m, 1H), 1.62−1.49 (m, 4H); ^{13}C NMR (101 MHz, CD_3OD) δ183.18, 175.41, 165.60, 146.78, 145.84, 137.84, 137.22, 137.18, 136.93, 74.59, 62.33, 49.57, 39.76, 38.54, 32.72. HRMS (ESI-TOF) m/z calcd for $C_{36}H_{42}N_4NaO_{10}$ $[M + Na]^+$: 713.2799. Found: 713.2800.

General Procedure for Synthesis of Final Products 3a−i. To a suspension of compound **2** (1 equiv) in dry DMF (5 mL) at 0 °C, EDC (2.4 equiv), HOBt (3.6 equiv), and *N*,*N*-diisopropylethylamine (2.88 equiv) were added. The reaction mixture was stirred at this temperature for 30 min, then a variety of amines (2.4 equiv) and *N*,*N*-diisopropylethylamine (2.4 equiv) were added, and the resulting mixture was kept stirring at 30 °C for 1 day. After starting material **2** was consumed, the solvent was removed and the residue was subjected to flash column chromatography (petroleum ether/ethyl acetate = 1:1 to CH_2Cl_2/MeOH = 15 to 10:1) to afford crude product which was dissolved in CH_2Cl_2 (4 mL) and trifluoroacetic acid (8 mL). The reaction was run at reflux for 4−6 h. After the solvent was removed, the residue was subjected to C-18 reversed-phase column chromatography (H_2O/MeOH = 1:1 to 1:10) to give the product as a white solid after lyophilization.

***N*^{1},*N*^{4}-Bis((*R*)-6-amino-1-(hexylamino)-1-oxohexan-2-yl)-terephthalamide (3a).** The title compound was prepared from **2** (100.0 mg, 0.14 mmol) and hexan-1-amine (34.4 mg, 0.34 mmol) according to the general procedure affording product **3a** (45.5 mg) in 55.2% yield as a white solid with 96.4% purity. ^1H NMR (400 MHz, CD_3OD) δ 8.00 (s, 2H), 4.54 (dd, J = 6.4, 11 Hz, 1H), 3.21 (t, J = 7.09 Hz, 2H), 2.96−2.91 (m, 2H), 1.98−1.80 (m, 2H), 1.77−1.68 (m, 2H), 1.57−1.45 (m, 4H), 1.37−1.29 (m, 6 H), 0.90 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.99, 169.25, 138.07, 128.77, 55.39, 40.53, 40.51, 32.61, 32.58, 30.30, 28.13, 27.61, 23.60, 14.33. HRMS (ESI-TOF) m/z calcd for $C_{32}H_{57}N_6O_4$ $[M + H]^+$: 589.4441. Found: 589.4442.

***N*^{1},*N*^{4}-Bis((*R*)-6-amino-1-(heptylamino)-1-oxohexan-2-yl)-terephthalamide (3b).** The title compound was prepared from **2**

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(100.0 mg, 0.14 mmol) and heptan-1-amine (39.1 mg, 0.34 mmol) according to the general procedure affording product 3b (34.0 mg) in 39.3% yield as a white solid with 96.7% purity. ^{1}H NMR (400 MHz, CD_3OD) δ 7.98 (s, 2H), 4.55 (dd, J = 5.8, 8.7 Hz, 1H), 3.31 (t, J = 7.1 Hz, 2H), 3.06−2.92 (m, 2H), 1.99−1.80 (m, 2H), 1.77−1.68 (m, 2H), 1.61−1.45 (m, 4H), 1.34−1.31 (m, 8 H), 0.90 (t, J = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ174.09, 169.26, 138.04, 128.84, 55.52, 40.53, 32.96, 32.59, 30.39, 30.10, 28.16, 27.94, 24.20, 23.67, 14.47. HRMS (ESI-TOF) m/z calcd for $C_{34}H_{61}N_6O_4$ $[M + H]^+$: 617.4754. Found: 617.4744.

N^1,N^4-Bis((*R*)-6-amino-1-(heptylamino)-1-oxohexan-2-yl)-terephthalamide (3c). The title compound was prepared from 2 (100.0 mg, 0.14 mmol) and octan-1-amine (44.0 mg, 0.34 mmol) according to the general procedure affording product 3c (41.3 mg) in 45.6% yield as a white solid with 97.3% purity. ^{1}H NMR (400 MHz, CD_3OD) δ 8.00 (s, 2H), 4.56 (dd. J = 6.0, 11.0 Hz, 1H), 3.22 (t, J = 7.2 H, 2H), 2.96−2.91 (m, 2H), 1.99−1.80 (m, 2H), 1.77−1.69 (m, 2H), 1.60−1.47 (m, 4H), 1.31−1.28 (m, 10 H), 0.89 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.99, 169.25, 138.08, 128.79, 55.35, 40.51, 32.96, 32.57, 30.37, 28.16, 27.96, 24.12, 23.70, 14.43. HRMS (ESI-TOF) m/z calcd for $C_{36}H_{65}N_6O_4$ $[M + H]^+$: 645.5067. Found: 645.5052.

N^1,N^4-Bis((*R*)-6-amino-1-(nonylamino)-1-oxohexan-2-yl)-terephthalamide (3d). The title compound was prepared from 2 (100.0 mg, 0.14 mmol) and nonan-1-amine (49.0 mg, 0.34 mmol) according to the general procedure affording product 3d (41.0 mg) in 43.5% yield as a white solid with 99% purity. ^{1}H NMR (400 MHz, CD_3OD) δ 7.96 (s, 2H), 4.52 (dd. J = 6.0, 11.0 Hz, 1H), 3.18 (t, J = 7.2 H, 2H), 2.94−2.90 (m, 2H), 1.97−1.80 (m, 2H), 1.77−1.68 (m, 2H), 1.60−1.45 (m, 4H), 1.35−1.28 (m, 12 H), 0.89 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.96, 169.22, 138.09, 128.78, 55.35, 40.54, 40.52, 33.00, 32.59, 30.62, 30.39, 30.34, 28.15, 27.94, 24.10, 23.67, 14.40. HRMS (ESI-TOF) m/z calcd for $C_{38}H_{69}N_6O_4$ $[M + H]^+$: 673.5380. Found: 673.5377.

N^1,N^4-Bis((*R*)-6-amino-1-(decylamino)-1-oxohexan-2-yl)-terephthalamide (3e). The title compound was prepared from 2 (100.0 mg, 0.14 mmol) and decan-1-amine (53.5 mg, 0.34 mmol) according to the general procedure affording product 3e (38.0 mg) in 38.6% yield as a white solid with >99% purity. ^{1}H NMR (400 MHz, CD_3OD) δ 7.98 (s, 2H), 4.54 (dd. J = 6.4, 11 Hz, 1H), 3.21 (t, J = 7.2 Hz, 2H), 2.96−2.91 (m, 2H), 1.99−1.68 (m, 2H), 1.77−1.69 (m, 2H), 1.58−1.45 (m, 4H), 1.37−1.27 (m, 14 H), 0.89 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.99, 169.25, 138.08, 128.80, 55.34, 40.52, 33.05, 32.58, 30.71, 30.69, 30.45, 30.42, 30.37, 28.16, 27.96, 24.13, 23.73, 14.44. HRMS (ESI-TOF) m/z calcd for $C_{40}H_{73}N_6O_4$ $[M + H]^+$: 701.5693. Found: 701.5700.

N^1,N^4-Bis((*R*)-6-amino-1-(dodecylamino)-1-oxohexan-2-yl)-terephthalamide (3f). The title compound was prepared from 2 (200.0 mg, 0.28 mmol) and decan-1-amine (126.0 mg, 0.68 mmol) according to the general procedure affording product 3f (54.8 mg) in 25.8% yield as a white solid with 98.5% purity. ^{1}H NMR (400 MHz, CD_3OD) δ 7.97 (s, 2H), 4.54 (t. J = 6.75 Hz, 1H), 3.21 (m, 2H), 2.94 (t, J = 7.2 Hz, 2H), 1.97−1.81 (m, 2H), 1.74−1.69 (m, 2H), 1.61−1.45 (m, 4H), 1.32−1.28 (m, 18 H), 0.89 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.98, 169.23, 138.07, 128.75, 55.34, 40.49, 33.05, 32.58, 30.76, 30.72, 30.70, 30.68, 30.45, 30.40, 30.35, 28.14, 27.95, 24.10, 23.71, 14.43. HRMS (ESI-TOF) m/z calcd for $C_{44}H_{81}N_6O_4$ $[M + H]^+$: 757.6319. Found: 757.6306.

N^1,N^4-Bis((*R*)-6-amino-1-(tridecylamino)-1-oxohexan-2-yl)-terephthalamide (3g). The title compound was prepared from 2 (200.0 mg, 0.28 mmol) and tridecan-1-amine (136.0 mg, 0.68 mmol) according to the general procedure affording product 3g (42.5 mg) in 19.3% yield as a white solid with 98.2% purity. ^{1}H NMR (400 MHz, CD_3OD) δ 8.01 (s, 2H), 4.59 (t. J = 6.75 Hz, 1H), 3.26(m, 2H), 2.99 (t, J = 7.2 Hz, 2H), 2.03−1.84 (m, 2H), 1.79−1.73 (m, 2H), 1.57−1.52 (m, 4H), 1.32−1.29 (m, 20 H), 0.91 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.93, 169.24, 138.13, 128.76, 55.27, 40.43, 33.06, 32.62,30.76, 30.75, 30.70, 30.68, 30.45, 30.40, 30.36, 28.18, 27.96, 24.08, 23.72, 14.42. HRMS (ESI-TOF) m/z calcd for $C_{46}H_{85}N_6O_4$ $[M + H]^+$: 785.6632. Found: 785.6613.

N^1,N^4-Bis((*R*)-6-amino-1-(tetradecylamino)-1-oxohexan-2-yl)terephthalamide (3h). The title compound was prepared from 2 (200.0 mg, 0.28 mmol) and tetradecan-1-amine (145.0 mg, 0.68 mmol) according to the general procedure affording product 3h (36.0 mg) in 15.8% yield as a white solid with 97.1% purity. ^{1}H NMR (400 MHz, CD_3OD) δ 7.97 (s, 2H), 4.54 (t. J = 6.9 Hz, 1H), 3.21 (t, J = 6.3 Hz, 2H), 2.93 (t, J = 7.5 Hz, 2H), 1.94−1.82 (m, 2H), 1.72−1.70 (m, 2H), 1.52−1.50 (m, 4H), 1.31−1.27 (m, 22H), 0.90 (t, J = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 173.94, 169.24, 138.11, 128.76, 55.27, 40.51, 33.07, 32.60, 30.79, 30.77, 30.76, 30.72, 30.70, 30.48, 30.42, 30.36, 27.18, 27.96, 24.09, 23.73, 14.44. HRMS (ESI-TOF) m/z calcd for $C_{48}H_{89}N_6O_4$ $[M + H]^+$: 813.6945. Found: 813.6913.

N^1,N^4-Bis((*R*)-1-(([1,1′-biphenyl]-4-ylmethyl)amino)-6-amino-1-oxohexan-2-yl)terephthalamide (3i). The title compound was prepared from 2 (200.0 mg, 0.28 mmol) and [1,1′-biphenyl]-4-ylmethanamine (124.0 mg, 0.68 mmol) according to the general procedure affording product 3i (16.0 mg) in 7.5% yield as a white solid with 97.9% purity. ^{1}H NMR (400 MHz, CD_3OD) δ 7.99 (s, 2H), 7.59−7.56 (m, 4H), 7.44−7.37 (m, 4H), 7.34−7.30 (m, 1H), 4.62 (dd. J = 7.0, 11.0 Hz, 1H), 4.46 (s, 2H), 2.95−2.90 (m, 2H), 2.02−1.86 (m, 2H), 1.76−1.68 (m, 2H), 1.62−1.45 (m, 2H); ^{13}C NMR (101 MHz, CD_3OD) δ 174.14, 139.47, 142.04, 141.48, 138.96, 138.12, 129.87, 129.06, 128.82, 128.36, 128.10, 127.87, 55.43, 43.83, 40.52, 32.48, 28.15, 24.13. HRMS (ESI-TOF) m/z calcd for $C_{46}H_{53}N_6O_4$ $[M + H]^+$: 753.4128. Found: 753.4111.

Biological Evaluation. In Vitro Antibacterial Activity. The bacterial strains employed in the experiment were methicillin-resistant *S. epidermidis* (RP62A), methicillin-resistant *S. aureus* (ATCC 33591), vancomycin-resistant *E. faecalis* (ATCC 25922), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853). The antimicrobial activities of compounds 3a−i were determined by broth microdilution method, and the procedures were used as previously reported.[59] The minimum inhibitory concentration (MIC) was determined by a lack of turbidity when bacterial growth was completely inhibited during course of incubation time. All measurements were performed three times.

Hemolysis Activity. The hemolysis assay was carried out as described in the previous protocol.[58,59] Briefly, hRBCs were isolated via centrifugation at 3000 rpm for 10 min and then suspended in 1× PBS to make a 5% v/v suspension. The suspension (50 μL) was incubated with 50 μL of compounds 3a−i of different concentrations at 37 °C for 1 h. The mixture was centrifuged at 3500 rpm for 10 min. Subsequently, the supernatant (30 μL) was added to 100 μL of PBS, and the absorbance was taken at 540 nm on a Biotek Synergy HT plate reader. The positive control was 2% Triton X-100, and the negative control was 1× PBS alone. The hemolysis activity was calculated by the formula % hemolysis = $[(Abs_{sample} - Abs_{PBS})/(Abs_{Triton} - Abs_{PBS})] \times 100$. The experiment was repeated three times with duplicates each time.

Time−Kill Kinetics Study. The rate of kill bacteria by compound 3d was evaluated by performance of the time−kill kinetics.[58,59] Generally, the bacteria MRSA were cultured in suitable growth medium such as tryptic soy broth (TSB) medium for several hours to make suspension of 10^6 colony-forming units per milliliter (CFU/mL). This suspension was incubated with 12.5, 25, 50 μg/mL of 3d at different time intervals (10 min, 30 min, 1 h, respectively). 20 μL aliquots were diluted by 10^2- to 10^4-fold and placed on TSB agar plates. The plates were incubated for 24 h at 37 °C. The number of colonies growth on plates was counted. All measurements were replicated, and the results were plotted against the incubation time.

Bacterial Membrane Permeability.[51,58,59] Staining experiments using DAPI and PI as dyes were conducted to distinguish viable from dead bacterial cells. *E. coli* or MRSA were cultured in suitable medium until they reached ~2 × 10^6 cells, and then bacteria were incubated with 3d at a concentration of 2 × MIC for 2 h. The bacterial suspension was centrifuged at 3000*g* for 15 min, and the supernatant was removed. The bacterial cells were washed several times with 1× PBS and then incubated with PI (5 μg/mL) in the dark for 15 min at 0 °C. After PI was removed thoroughly, the bacterial cells were incubated with DAPI (10 μg/mL in water) for 15 min in the dark at 0 °C. The DAPI solution was removed, and cells were washed with 1×

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PBS at least three times. Controls were carried out with the exact same procedure for bacteria without adding compound. The bacterial cells were then visualized and analyzed by employing the Zeiss Axio imager Z1 optical microscope with an oil-immersion objective (100×). The experiment was repeated three times with duplicates each time.

Bacterial Membrane Depolarization Assay.[61] Mid log phase MRSA cells were collected and washed with 5 mM HEPES and 5 mM glucose, respectively. Then the bacteria were resuspended in 5 mM glucose, 5 mM HEPES buffer, and 100 mM KCl solution in 1:1:1 ratio (10^8 CFU/mL). 200 μL of bacterial suspension and 2 μM DiSC3(5) were included in a 96-well plate, and the fluorescence of the suspension was monitored at room temperature for 30 min at excitation wavelength of 622 nm and emission wavelength of 670 nm. After the minimum value of the fluorescence (after 20 min) was reached, the lead compound was added to the wells, and the decrease in potential was monitored by the increase in fluorescence. The experiment was repeated three times with duplicates each time.

TEM Study.[71] Mid log phase bacterial cells were incubated with appropriate concentration of compound 3d in fresh broth for 2 h. Then the mixture was centrifuged, and collected bacteria were redissolved in deionized water, and samples were prepared. Control bacterial samples were obtained without adding drugs. Above samples were applied to TEM grids by adding a 10 μL sample solution, and the grids were allowed to dry for about 1 h. After being dried, the grids were stained with 10 μL of 1% (w/w) uranyl acetate aqueous solution, and extra solution was removed by immersion using wet filter paper after 30 s. The grids were again allowed to dry for TEM study. TEM images were obtained on a FEI Morgagni 268D TEM with an Olympus MegaView III camera on the microscope. The microscope uses AnalySiS software to run the camera. The microscope was operated at 60 kV.

Biofilm Inhibition Assay.[52] Bacteria (grown for 12 h) were inoculated into fresh 10% MHII broth at a ratio of 1:100. Incubation of 100 μL of inoculated culture with an appropriate amount of compound 3d was carried out in wells of 96-well plate. The plate was incubated at 37 °C overnight. Optical density of each well was recorded at a wavelength of 600 nm, and then the biofilm biomass was recorded by the crystal violet method. Biofilm biomass was presented as CV OD/OD of growth. Relative biofilm biomass values were normalized by the biomass value of control (without adding compound). Experiments were performed in triplicate, and the data were obtained as the mean ± STDEV.

Propensity To Develop Bacterial Resistance.[51,58,59] The initial MIC values of 3d against *S. aureus* and *E. coli* were measured following the exact same procedure for in vitro antibacterial assay. Bacteria in wells at a concentration of $^1/_2$ MIC were then employed to make the bacterial suspension (approximately 10^6 CFU/mL) for the next experiment. These bacterial suspensions were then incubated with 3d at 37 °C for 24 h, and then new MIC was measured. The experiment was repeated each day for 14 passages.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b01704.

Correlation of antibacterial activity (MIC) against MRSA and *E. coli* vs AlogP of compounds, ^{1}H NMR and ^{13}C NMR spectral data of compounds **1**, **2**, **3a**−**i**, and HPLC analysis of compounds **3a**−**i** (PDF)

Molecular formula strings and some data (CSV)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

MDR, multidrug-resistant;; PDR, pandrug resistant;; AMP, antimicrobial peptide;; *S. aureus*, *Staphylococcus aureus*; *E. coli*, *Escherichia coli*; MRSA, methicillin-resistant *Staphylococcus aureus*; MRSE, methicillin-resistant *Staphylococcus epidermidis*; VRE, vancomycin-resistant *Enterococci*; *P.A.*, *Pseudomonas aeruginosa*; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt, hydroxybenzotriazole; DIPEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; TFA, trifluoroacetic acid; MIC, minimum inhibitory concentration; HC_{50}, 50% hemolytic concentration; RT, retention time; UV, ultraviolet; DAPI, 4′,6-diamidino-2-phenylindole; PI, propidium iodide; PBS, phosphate buffered saline; CFU, colony-forming unit; hRBC, human red blood cell; TSB, tryptic soy broth; TEM, transmission electron microscopy; DiSC3(5), 3,3′-dipropylthiadicarbocyanine iodide; OD, optical density

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