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# Modular Design of Membrane-Active Antibiotics: From Macromolecular Antimicrobials to Small Scorpionlike Peptidomimetics

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abscess model of methicillin-resistant *Staphylococcus aureus* (MRSA) infections. The lead compound **12** showed impressive in vivo therapeutic efficacy with ~99.998% (4.7log) reduction in skin MRSA burden, a significantly higher bactericidal efficiency than ciprofloxacin, and good biocompatibility. These results highlight the potential of these HDP mimics as novel antibiotic therapeutics.

# INTRODUCTION

According to the 2019 Antibiotic Resistance Threats report from the Centers for Disease Control and Prevention, antibiotic resistance posed an ever-increasing threat to global health and caused more than 35,000 deaths in the United States annually. Serious human health threatening strains including multidrugresistant Pseudomonas aeruginosa (P. aeruginosa) and methicillinresistant Staphylococcus aureus (MRSA) account for substantial morbidity and mortality worldwide.<sup>1</sup> In addition to these strains, other pandrug-resistance bacteria such as methicillin-resistant Staphylococcus epidermidis (MRSE), Escherichia coli (E. coli), vancomycin-resistant Enterococcus faecalis (VREF), and Klebsiella pneumoniae (K. pneumoniae) are all making substantial contribution to lethal infections.<sup>2</sup> Against this background, antimicrobial peptides, sometimes termed host defense peptides (HDPs), have been developed as a promising alternative to conventional antibiotics because of their broad antibacterial spectrum, low resistance rates, and unique antibacterial mechanism.<sup>3,4</sup>

HDPs usually exert microbial killing activity through direct membrane-disruptive effects against cells in a phospholipiddependent manner based on their cationic and amphipathic structures.<sup>5,6</sup> A membranous environment can induce folding of HDPs into a secondary structure, such as  $\alpha$ -helix or  $\beta$ -sheet, with global segregation of cationic and lipophilic side chains (or segregation of hydrophilic and hydrophobic faces).<sup>7–11</sup> Unfortunately, most well-known HDPs are natural macromolecular products with complex structures, and their applications are limited owing to their high cost, poor efficacy, low in vivo stability, and nonspecific toxicity to mammalian cells.<sup>12</sup>

Recently, many synthesized amphiphilic polymers with HDPmimicking designs have been reported to have great potential in antibacterial applications.<sup>7,8,13–23</sup> However, due to the large molecular weight, large steric hindrance induced by bulky side chains, and lack of a well-defined structure, the synthesized macromolecular antimicrobials usually depend on uncontrolled polymeric self-aggregation to achieve irregular facial amphiphilicity without helical structures. This conformation would be difficult to manipulate, suffers from a very high entropic penalty from a whole macromolecule, and is unfavorable for adequate interactions with bacterial cell membranes.<sup>7</sup> Therefore, synthesized macromolecular antimicrobials had more difficulty in achieving good antimicrobial performance compared with small-molecular amphiphilic antimicrobials.

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In this research study, we hypothesized that small-molecular amphiphilic peptidomimetics, which have low molecular weight, reduced steric hindrance, and thus improved conformational flexibility, could mediate enhanced interactions with bacterial cell membranes. Therefore, a new class of HDP-mimetic amphiphilic compounds based on a small-molecular scorpionlike skeleton was established (Figure 1A) using biomimetic and



**Figure 1.** Design of the peptidomimetics. (A) Schematic illustrations for the structure of small scorpionlike HDP mimics, where their cationic modules acted as the grasping pincers of the scorpionlike skeleton to seize bacterial membranes and their hydrophobic modules constituted the body and tail of the scorpionlike molecule to facilitate membrane insertion. (B) General structures of peptidomimetics.

modular design strategies: (1) the positively charged domain of HDP mimics can electrostatically interact with anionic charges on the surfaces of bacteria, while their hydrophobic segments are helpful to insert into the hydrophobic core of the lipid bilayer.<sup>24</sup> We found that the modes of action of HDP or HDP mimics have great similarity to the dual modes of attack of a scorpion, which seizes its prey by a pair of grasping pincers and/or stings it by a tail tipped with a venomous stinger. We predicted that HDPmimetic amphiphilic compounds with a scorpionlike skeleton may be a promising method to simulate the predation ability of scorpion. (2) To achieve facial amphiphilicity, a modular design strategy was applied to combine hydrophobic and hydrophilic modules to obtain HDP mimics. Due to the convenience of replacing each module by groups with similar physicochemical properties, the modular design strategy could accelerate the development of antimicrobial compounds and facilitate their structure-activity relationship (SAR) studies.<sup>25</sup>

### RESULTS AND DISCUSSION

Design and Antimicrobial Activity of Peptidomimetics. As our previous studies demonstrated that cationic  $\gamma$ -AApeptide building blocks can efficiently bind to bacterial membranes,<sup>26–28</sup> we have designed the framework of a dimeric positively charged  $\gamma$ -AApeptide building block (cationic module shown in Figure 1A)<sup>29</sup> conjugated with 3,5-diaminobenzoic acid, providing four cationic amines as the "grasping pincers of scorpion" and three alkyl lipophilic moieties as the "tail" (Figure 1B). Our recent dimeric design has led to potent antibiotic agents that mimic HDPs.<sup>2,12</sup> However, the modification of the molecular scaffold for the adjustment of amphiphilicity is not straightforward. In the current study, we believe that dimeric  $\gamma$ -AApeptides bearing a hydrophobic portion in the center of the molecular scaffold could lead to manipulation of amphiphilicity at ease, thereby resulting in more potent and selective HDP-mimicking agents. In these derivatives, the modification was made with various alkyl substitutions, while cationic groups were not changed. Briefly, bulky alkyl groups with different sizes and linear alkyl groups with diverse lengths were explored to optimize for the best activity.

To evaluate the antibiotic activity of the compounds against Gram-positive bacteria and Gram-negative bacteria, a panel of six strains, including MRSA, MRSE, VREF, E. coli, K. pneumoniae, and P. aeruginosa, was selected to determine the minimal inhibitory concentrations (MICs). Ciprofloxacin, a common marketed antibiotic, was tested at the same time as a positive control. Hemolysis, which represents the degree of human blood being lysed by candidates, was also evaluated using HC<sub>50</sub> values. To our delight, many compounds were active against these strains and most of the compounds had HC<sub>50</sub> values exceeding the tested concentration,  $250 \,\mu g/mL$ . Since the cationic groups have not been changed in compounds 1-15, it was quite straightforward to establish the SAR. To begin with, compounds 1 and 2, which only have linear hydrophobic groups, were synthesized and tested. Compound 1 did not show activity up to 25  $\mu$ g/mL against MRSA and *E. coli*. After an increase in the length of fatty acids on the  $R_1$  position from hexanoic acid to octanoic acid, compound 2 started to exhibit potent activity against all strains except for K. pneumoniae. This result indicated that when keeping octanoic acid unchanged on the R<sub>2</sub> position, the R<sub>1</sub> position should have at least eight carbons to provide sufficient hydrophobic interaction with the bacterial cell membranes. We also explored a few other hydrophobic groups on  $R_1$  through acylation with 2-naphthoic acid (compound 3), [1,1'-biphenyl]-4-carboxylic acid (compound 4), 4-chlorobenzoic acid (compound 5), 3-chlorobenzoic acid (compound 6), and 1-adamantaneacetic acid (compound 7). It is interesting to note that compound 3 had less potent activity toward most bacterial strains than 2 bearing a linear hydrophobic group. However, 3 has a MIC of 4.5  $\mu$ g/mL against K. pneumoniae, while 2 showed no inhibitory activity, which may imply that both hydrophobicity and the nature of the group are all important for antimicrobial activity. Subsequent exploration showed that a more flexible and longer biphenyl group (4) led to a better activity than 3 for most of the strains, as seen for the MICs against three Gram-positive bacterial strains being decreased twofold or more, albeit its activity against K. pneumoniae was lost. Then, we considered chlorobenzoic acid to assess the effect of using smaller bulky lipophilic groups on R<sub>1</sub>. Small hydrophobic groups on  $R_1$  (5 and 6) completely abolished the antibiotic activity, demonstrating once again that groups of sufficient hydrophobicity and bulkiness are necessary for the maximum broad antibacterial activity. It is known that adamantanyl group is a commonly used alkyl substitute to improve the antibacterial activity.<sup>12,30</sup> As such, compound 7 was designed and synthesized. As expected, 7 turned out to be the best compound among 1-7. Thus, 7 was chosen as the lead compound for further modification. Switching octanoic acid to decanoic acid and dodecanoic acid on position R<sub>2</sub> led to

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#### Table 1. MICs and Therapeutic Index of Compounds $1-15^a$

			1	MIC ( $\mu g/r$	mL)			
	Gram-positive			Gram-negative				
compound #	MRSA	MRSE	VREF	E. coli	P. aeruginosa	K. pneumoniae	$HC_{50}$ ( $\mu g/mL$ )	the rapeutic index (HC $_{50}$ /MIC against MRSA)
1	>25	NT	NT	>25	NT	NT	>250	
2	4.5	4.5	4.5	4.5	4.5	>25	>250	>55.5
3	9	9	>25	4.5	>25	4.5	>250	>27.8
4	4.5	4.5	4.5	9	4.5	>25	>250	>55.5
5	>25	NT	NT	>25	NT	NT	>250	
6	>25	NT	NT	>25	NT	NT	>250	
7	2	2	4.5	4.5	4.5	4.5	>250	>125
8	1	2	4.5	19	2	4.5	>250	>250
9	4.5	4.5	4.5	>25	>25	>25	250	55.5
10	4.5	9	>25	>25	>25	>25	>250	>55.5
11	>25	NT	NT	>25	NT	NT	>250	
12	2	2	4.5	2	4.5	4.5	250	125
13	2	2	2	9	4.5	9	125	62.5
14	2	4.5	4.5	9	19	9	125	62.5
15	2	9	2	>25	>25	>25	125	62.5
ciprofloxacin	0.24	0.45	0.9	0.12	0.45	0.9	NT	
<sup>a</sup> NT: not tested.								

compounds 8 and 9, respectively. However, both compounds were less active in contrast to 7. This result suggested that increasing the carbon number of alkyl chain on the  $R_2$  position was not helpful in improving the activity. Indeed, a similar trend was observed when comparing the activity of 2 to 10. Also, it is expected that 11, without a  $R_1$  group, exhibited a worse activity than 10, again demonstrating the importance of  $R_1$  being a hydrophobic group. Interestingly, when replacing the linear lipophilic chain with a bulky adamantanyl group (compound 12), the best activity against all strains was reached.

Compounds 13, 14, and 15 with alkyl amines directly conjugated with 3,5-diaminobenzoic acid were also prepared. With the tail length increasing from 6 carbons to 10 carbons, the overall activity against six bacterial strains decreased. It is worth noting that compounds 14 and 15 were not as active as compounds 7 and 8, although 14 and 15 bear the same alkyl tails as compounds 7 and 8. This phenomenon provided the evidence that the linker, ethane-1,2-diamine, coupled between 3,5diaminobenzoic acid and the R2 group, benefited the antibacterial effect. Compound 12 had a HC<sub>50</sub> value of 250  $\mu$ g/mL, whereas the therapeutic index (HC<sub>50</sub>/MIC against MRSA) was 125. This result indicated that these compounds were unlikely to lyse human blood cells when being used at MIC concentrations. Interestingly, the compounds without the ethane-1,2-diamine linker, compounds 14 and 15, had lower therapeutic index than 7 and 8. As known, increasing the hydrophobicity will lead to an increased hemolytic activity. Compounds 7 and 14 showed retention times of 21 mins and 23.5 mins on a high-performance liquid chromatograph (HPLC), respectively. Compounds 8 and 15 showed retention times of 22.5 mins and 25 mins, respectively. Therefore, ethane-1,2-diamine was an effective linker that decreased the hemolytic activity of a compound while increasing its antibiotic activity.

The salt tolerance of antibiotics is a critical parameter that maintains the antimicrobial activity of peptides for biological applications.<sup>31</sup> To investigate whether the antimicrobial activity of compounds is impacted by salt or not, MICs of the lead compound **12** against all six strains were determined in a tryptic soy broth (TSB) in the presence of different salts. As shown in Table 2, monovalent (Na<sup>+</sup> and K<sup>+</sup>) and divalent (Ca<sup>2+</sup>) cations

# Table 2. MICs of Compound 12 in the Presence of Physiological Salts

	MIC ( $\mu g/mL$ )								
	Gr	am-positi	ve	Gram-negative					
compound #	MRSA	MRSE	VREF	E. coli	P. aeruginosa	K. pneumoniae			
12 (Na <sup>+</sup> and K <sup>+</sup> )	4.5	4.5	4.5	9	9	9			
12 (Ca <sup>2+</sup> )	2	4.5	4.5	4.5	19	9			

were added into the TSB medium. All MIC values remained the same or increased slightly twofold. It is therefore concluded that the antimicrobial activity of compound **12** did not change significantly in the presence of some physiological salts, indicating its high salt tolerance.

**Time-Kill Kinetics Study.** The lead compound 12 was studied regarding its efficacy of eradicating MRSA by measuring the time-kill kinetics. Ciprofloxacin was tested at the same time. As shown in Figure 2A, at a concentration of 8  $\mu$ g/mL (4 × MIC), compound 12 could eliminate all MRSA cells within 60 mins. Increasing the concentration to 16  $\mu$ g/mL (8 × MIC) reduced the time to as short as 30 mins. Although bacteria were not totally eradicated at 1 × MIC and 2 × MIC concentrations, the decreasing tendency was obvious when compared with a negative control. However, ciprofloxacin did not show a noticeable MRSA eliminating trend even at an 8 × MIC concentration within 2 h, delineating its different mechanism in contrast to 12, which is analogous to HDPs. However, the time-kill kinetics of compound 12 against *E. coli* were not as efficient as ciprofloxacin (Figure S1).

**Biofilm Inhibition.** A bacterial biofilm plays an active role in producing drug resistance and is strongly associated with the majority of bacterial infections.<sup>32</sup> Therefore, the inhibitory effect of compound **12** on MRSA biofilm formation was evaluated. As shown in Figure 2B, with the increase of the compound concentration, a decreasing trend of biofilm formation was observed. Nearly 40% biofilm was inhibited at 1.5  $\mu$ g/mL of compound **12**. The *E. coli* biofilm inhibition effect of compound **12** could also be observed at low concentrations (Figure S2).

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Figure 2. Time-kill kinetics study, biofilm inhibition, and drug resistance of compound 12. (A) Time-kill kinetic curves of compound 12 and ciprofloxacin. Controls were untreated MRSA. (B) MRSA biofilm inhibition by compound 12. (C) Propensity of developing drug resistance of compound 12 and ciprofloxacin against MRSA.

**Drug Resistance Study.** Since the emergence of penicillinresistant bacteria after only few years of penicillin discovery, it has been widely acknowledged that drug resistance is becoming a global threat.<sup>33</sup> Therefore, it is significant to assess the potential resistance development of new biocides. Figure 2C shows that the MIC values of compound **12** for MRSA did not change after 14 passages, whereas a 33-fold increase was observed in the case of ciprofloxacin. Similar results were also observed for *E. coli* (Figure S3). Thus, this result indicated that this type of antibiotic made it hard for bacteria to develop drug resistance.

Antimicrobial Mechanism. To confirm that the designed HDP mimics also act through a membrane-interrupting mechanism, we conducted a few experiments. Two dyes, 4',6diamidino-2-phenylindole (DAPI) and propidium iodide (PI), were first applied to carry out fluorescence microscopy. PI is not membrane-permeable. Therefore, it is commonly used to stain dead or injured cells. In contrast, DAPI can pass through the bacterial membrane regardless of its integrity. MRSA and E. coli were stained with both dyes after being treated or untreated with compound 12. As shown in Figure 3A, the negative control of MRSA and E. coli had blue color in the DAPI channel, while no fluorescence was observed in the PI channel. This result indicates that bacteria were alive in the negative control. For bacteria treated with compound 12, not only blue fluorescence was observed in the DAPI channel but red fluorescence was also observed in the PI channel, demonstrating that the bacterial membranes have been compromised by compound 12.

As TEM microscopy is a direct way to visualize the bacterial membrane,<sup>2</sup> imaging of MRSA and *E. coli* after being incubated with compound **12** was further conducted (Figure 3B). Obviously, without the treatment of compound **12**, MRSA and *E. coli* had an intact membrane. After incubation with

compound **12** at a concentration of  $6 \mu g/mL$ , both MRSA and *E. coli* lost their membrane integrity (Figure 3B).

It is known that the *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) hydrolysis assay is also a common method to detect whether the inner membrane of Gram-negative bacteria is intact or not. After membrane destabilization, ONPG interacts with the cytoplasmic enzyme  $\beta$ -galactosidase to form *o*-nitrophenol, which can be measured at 420 nm.<sup>34</sup> Therefore, this assay was performed on *E. coli* to further explore the inner membrane interruption effect of compound **12**, with melittin being included as the positive control. As shown in Figure 3C, compound **12** exhibited effective concentration- and time-dependent inner membrane interruption. Interestingly, the OD<sub>420</sub> value also showed an increasing trend when *E. coli* was treated with compound **12** at a 0.5 × MIC concentration, which indicated the effective inner membrane-interrupting ability of compound **12**.

The integrity of the cytoplasmic membrane of Gram-positive bacteria was evaluated using a dye, 3,3'-dipropylthiadicarbocyanine iodide [DiSC<sub>3</sub>(5)].<sup>35</sup> Generally, this dye enters into the bacterial cells and aggregates as a nonfluorescent conjugation. Once the cytoplasmic membrane is disturbed by biocides, the increasing fluorescence will be detected due to the formation of a dye monomer.<sup>36</sup> As shown in Figure 3D, MRSA treated with compound **12** was observed at different concentrations. Bacteria mixed with 0.5 × MIC showed a similar fluorescence intensity trend as the negative control, which was untreated MRSA. However, when the concentration changed from 1 × MIC to 8 × MIC, the increase of the fluorescence intensity was dose-dependent.

Effectiveness Evaluation Using a Mouse Model. As compound 12 demonstrated the most potent and broadspectrum activity in vitro, its in vivo performance was next



**Figure 3.** Evidence for the membrane-interrupting antimicrobial mechanism. (A) Fluorescence microscopy of MRSA and *E. coli* treated or untreated with 6  $\mu$ g/mL of compound **12** for 2 h. Scale bar =10  $\mu$ m. Controls were bacteria without treatment. (B) Transmission electron microscopy (TEM) images of MRSA and *E. coli* treated or untreated with 6  $\mu$ g/mL of compound **12**. Controls were bacteria without treatment. Scale bar is 2  $\mu$ m. (C) Inner membrane permeability of compound **12** and melittin against *E. coli*. The control was untreated *E. coli*. (D) Cytoplasmic membrane potential variation of MRSA. The control was untreated MRSA.

assessed using a murine abscess model of MRSA infections following a reported method.  $^{37,38}$  Ciprofloxacin (5 mg/mL) was used as the positive control. Low-dose groups (12-L) were administered with 100  $\mu$ L of 0.5 mg/mL compound 12 solution, while high-dose groups (12-H) were administered with 100  $\mu$ L of 5 mg/mL compound 12 solution. As shown in Figure 4B, there is a marked swelling on the dorsal skin surface and skin abscess in the subcutaneous tissues of the control mice, indicating the formation of skin abscess after 48 h. Notably, no visual lesions were observed in the mice that were treated with high doses of compound 12, whereas visible abscess or erythema still can be observed in the subcutaneous tissues of mice treated with ciprofloxacin or low doses of compound 12. As shown in Figure 4C, hematoxylin and eosin (H&E) histological examinations showed massive infiltration with acute and chronic inflammatory cells, primarily neutrophils, in the subcutaneous connective tissues of control mice. Encouragingly, the inflammatory cells were reduced after being treated with ciprofloxacin and even a low dose of compound 12. Moreover, the skin sections of mice treated with a high dose of compound 12 were nearly identical to those of healthy mice.

Compared with the control group, treatment with both ciprofloxacin and compound **12** had significantly decreased MRSA-induced production of proinflammatory cytokines including TNF- $\alpha$  (p < 0.001) and IL-6 (p < 0.001) and a high dose of compound **12** exhibited the best therapeutic effect (p < 0.001)

0.001 vs compound 12 and p < 0.001 vs ciprofloxacin) (Figure 4E,F). This result may explain the better in vivo performance of compound 12 than that of ciprofloxacin. The quantitative assessment of microbial burden in tissues revealed that ciprofloxacin and compound 12-L groups achieved, respectively, 81.1 and 98.7% (0.7 and 1.9log) reduction of MRSA, while compound 12-H treatment groups achieved 99.998% (4.7log) reduction of bacteria (Figure 4D,G,H). To the best of our knowledge, there are few studies demonstrating that HDPs or HDP mimics can achieve a ~99.998% reduction in skin bacterial load after a single treatment.<sup>39,40</sup> Furthermore, the number of bacteria remaining on the skin of compound 12 group was ~9100 times lower than those remaining on ciprofloxacintreated mice (p < 0.001). The impressive antimicrobial activities make compound 12 a promising alternative to conventional antibiotics in clinical applications.

**Biocompatibility Evaluation Using a Mouse Model.** To demonstrate the in vivo biocompatibility, the local and systemic toxicity of compound **12** was evaluated by examining the pathological changes of the mice in the skin, heart, liver, spleen, lungs, and kidneys. As shown in Figure 5A, topical application of compound **12** showed a good skin compatibility profile in mice. It is worth noting that ciprofloxacin treatment induced liver injury characterized by moderate cellular degeneration, but no obvious histopathological abnormalities or lesions to major organs were observed in the compound **12** treatment group



**Figure 4.** Effectiveness evaluation using a mouse model. (A) Schematic of the experiment protocol for the murine abscess model of MRSA infections. (B) Digital photographs of MRSA infection sites with various treatments 48 h after infection. Scale bar is 0.5 cm. (C) Histological photographs of infected skin that received various treatments. Scale bar is 200  $\mu$ m. (D) Images of MRSA colonies cultured from the homogenate of infected skin after appropriate dilution. Levels of (E) tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in the infected skin and (F) interleukin-6 (IL-6) in the infected skin. (G) Microbial burden of each group after 48 h of MRSA injection. (H) Bacterial survival of each group after 48 h of MRSA injection. " $\sigma$ " represents 0.002%. \*\*p < 0.01, \*\*\*p < 0.001. 12-L represents compound 12 at a low concentration (100  $\mu$ L, 0.5 mg/mL). 12-H represents compound 12 at a high concentration (100  $\mu$ L, 5 mg/mL). The concentration of ciprofloxacin is 5 mg/mL.

under the same experimental conditions (Figure 5B). Hence, compound 12 may be a promising candidate to be investigated as a safe antimicrobial agent.

# CONCLUSIONS

Battle against antibiotic resistance that we are facing is still uphill currently. Many macromolecular antimicrobials, based on the HDP-mimicking design, have already been developed with high activity and low potential of inducing drug resistance against multidrug-resistance bacteria. These compounds act via compromising the bacterial membrane by their facial amphiphilicity, which is difficult to control due to the unpredictable self-aggregation.<sup>7,41</sup> Cytotoxicity and hemolysis of human blood cells remain another issue for these antimicrobials. Considering the many advantages of small-molecular drug development, such as lower molecular weight



**Figure 5.** Biocompatibility evaluation using a mouse model. (A) Representative H&E-stained sections from mouse skin. (B) Representative H&E-stained sections from major organs after various treatments. Scale bar is 200  $\mu$ m. 12-L represents compound **12** at a low concentration (100  $\mu$ L, 0.5 mg/mL). 12-H represents compound **12** at a high concentration (100  $\mu$ L, 5 mg/mL). The concentration of ciprofloxacin is 5 mg/mL.

and easier administration, small-molecular antimicrobials could be an alternative solution to the drug resistance threat.

In this work, a new class of small scorpionlike peptidomimetics with excellent antimicrobial activity against both Grampositive and Gram-negative bacteria was developed via biomimetic and modular design strategies. These molecules also exhibited persistent bacterial killing activity in the presence of physiological salts. Biofilm formation could be inhibited by these molecules at a low concentration. Eradicating bacteria with low concentrations in a short time without developing drug resistance and hemolysis is another advantage of these peptidomimetics. Mechanism studies revealed their potential membrane-interrupting action mode. Significantly, the lead compound exhibited a highly impressive in vivo anti-infection activity and high in vivo biocompatibility, which made it a potential candidate for clinical applications. On the basis of the efficacy of the lead compound 12, the antimicrobial peptidomimetics based on the small-molecular scorpionlike skeleton are expected to serve as promising broad-spectrum antimicrobial agents in the treatment of drug-resistant infections. It should be noted that although the small scorpionlike peptidomimetic design is successful, only a limited number of hydrophobic  $R_1$ 

and  $R_2$  groups and  $\gamma$ -AApeptide building blocks were explored to demonstrate the design strategy. The comprehensive study and exploration of other  $R_1$  and  $R_2$  groups, although out of the scope of the current report, could lead to antibiotic agents with a better potent antimicrobial activity and a higher therapeutic index to combat drug resistance.

# EXPERIMENTAL SECTION

**General Information.** Solvents and other reagents were purchased from Fisher Scientific, Sigma-Aldrich, TCI America, or Chem-Impex and were used directly. All final products were purified using a Waters Breeze 2 HPLC system and lyophilized using a Labconco lyophilizer. Analytical HPLC (1 mL/min flow, 5–100% linear gradient of acetonitrile with 0.1% trifluoroacetic acid (TFA) and water with 0.1% TFA in water over 40 min) was conducted. The nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Avance NEO 400 instrument. High-resolution mass spectra of compounds were obtained using an Agilent Technologies 6540 UHD accurate-mass Q-TOF LC/MS spectrometer. A BioTek multimode microplate reader Synergy H4 was used in antimicrobial assays and mechanism of action studies. MRSA (ATCC 33591), MRSE (RP62A), VREF (ATCC 700802), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 13383), and *P. aeruginosa* (ATCC 27853) were applied in antimicrobial assays. Animal

experiments were performed according to the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-Sen University (approved protocol number SYSU-IACUC-2019-000203), and all animal care procedures were performed according to the Guide for Care and Use of Laboratory Animals. Healthy female ICR mice were obtained from the Animal Center of Sun Yat-Sen University. Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Dakewe in Shenzhen, China.

Synthesis of the Compounds. Synthesis of 3,5-bis((((9Hfluoren-9-yl)methoxy)carbonyl)amino)benzoic acid. A volume of 1 g (6.58 mmol) of 3,5-diaminobenzoic acid was suspended in 50 mL of THF/H<sub>2</sub>O = 1:1 solution. With stirring, 3.49 g (32.9 mmol) of Na<sub>2</sub>CO<sub>3</sub> and 3.75 g (14.48 mmol) of Fmoc-Cl were added into the above solution and then the reaction mixture was stirred at room temperature overnight. A 1 M HCl solution was added to quench the reaction, and the product was extracted with ethyl acetate (EA,  $100 \text{ mL} \times 3 \text{ times}$ ). After being dried with sodium sulfate, the EA solution was evaporated, and a solid crude product was left. To purify the crude product, dichloromethane (DCM) was added to it and then sonicated. The product (3.52 g) was obtained after filtering and washing with DCM. The yield was 90%. Then, 500 mg (0.84 mmol) of 3,5-bis((((9Hfluoren-9-yl)methoxy)carbonyl)amino)benzoic acid was dissolved in 50 mL of dimethylformamide (DMF) solution. A volume of 382 mg (1.00 mmol) of 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) and 173  $\mu$ L (1.00 mmol) of N,N-diisopropylethylamine (DIPEA) was then added into the above solution separately. Following this, 177 mg (1.00 mmol) of tert-butyl (2-aminoethyl)carbamate was added into the mixture. After the completion of addition, the reaction mixture was stirred at room temperature for 2 h. 1 M HCl (150 mL) was added into the reaction and then EA was used to extract the product. Silica gel chromatography was employed to purify this product. An intermediate (506 mg) was obtained with a yield of 82%. Then, at room temperature, 15 mL of DCM:TFA = 1:1 mixture was added into the intermediate to cleave the Boc protection group. The reaction lasted for 1.5 h. After the reaction was complete, TFA and DCM were evaporated and the crude product was directly used for the next step without purification. HATU (312 mg, 0.82 mmol) and DIPEA (141  $\mu$ L, 0.82 mmol) were applied as coupling reagents to conjugate them with 118 mg (0.82 mmol) of octanoic acid following the same conjugation reaction conditions in the first step. After chromatography purification, 461 mg of product was obtained. The yield was 88%. Finally, 20 mL of diethanolamine  $(DEA):CH_3CN = 1:1$  was used to cleave the Fmoc protection group to obtain 3,5-diamino-N-octylbenzamide. 3,5-diamino-N-(2aminoethyl)benzamide conjugated with other fatty acids was obtained following the same procedure.

 $\gamma$ -AApeptide building blocks (cationic module shown in Figure 1) with different R<sub>1</sub> groups were synthesized using a method reported before.<sup>29</sup> For compound 1, a  $\gamma$ -AApeptide building block (195 mg, 0.32 mmol) with a side chain of hexanoic acid in 20 mL of DMF solution was added to HATU (150 mg, 0.40 mmol) and DIPEA (69  $\mu$ L, 0.4 mmol) followed by the addition of 51 mg (0.16 mmol) of 3,5-diamino-*N*-(2-octanamidoethyl)benzamide for a 2 h reaction at room temperature. After being quenched by 1 M HCl, EA was used to extract the product (50 mL× 3 times), and the organic layer was dried with sodium sulfate. The product (143 mg) with a yield of 59% was obtained after column purification. Then, the product was treated with DEA:CH<sub>3</sub>CN = 1:1 and TFA:DCM = 1:1, respectively, to cleave all protection groups. An HPLC was used to purify compound 1, and 55 mg was obtained. Other compounds were prepared using the same method. All compounds have purity >95%.

tert-Butyl(S)-(2-((((9H-Fluoren-9-yl)Methoxy)Carbonyl)Amino)-6-((tert-Butoxycarbonyl)Amino)Hexyl) Glycinate. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ 7.88–7.90 (d, J = 8 Hz, 2H), δ 7.69–7.71 (d, J = 8 Hz, 2H), δ 7.40–7.44 (t, J = 16 Hz, 2H), δ 7.31–7.35 (t, J = 16 Hz, 2H), δ 7.05–7.07 (d, J = 8 Hz, 1H), δ 6.73–6.76 (t, J = 12 Hz, 1H), δ 4.28– 4.30 (d, J = 8 Hz, 2H), δ 4.20–4.23 (t, J = 12 Hz, 1H), δ 3.42–3.47 (q, J = 20 Hz, 1H), δ 3.19 (s, 2H), δ 2.86–2.91 (q, J = 20 Hz, 2H), δ 1.43– 1.49 (m, 1H), δ 1.41 (s, 9H), δ 1.37 (s, 9H), δ 1.25–1.29 (m, 2H), δ 1.16–1.20 (t, J = 16 Hz, 2H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>): δ 172.00, 156.44, 156.03, 144.45, 141.21, 128.05, 127.49, 125.69, 120.57, 80.51, 77.75, 65.58, 53.11, 51.57, 51.23, 47.30, 32.38, 29.92, 28.74, 28.24, 23.31. HRMS (ESI)  $C_{32}H_{45}N_3O_6$  [M + Na]<sup>+</sup> calcd = 590.3206; found [M + Na]<sup>+</sup> = 590.3218.

 $\begin{array}{l} Di(9H\mbox{-}Fluoren\mbox{-}9\mbox{-}yl)(5\mbox{-}((tert\mbox{-}Butoxycarbonyl)\mbox{-}Amino\mbox{-}Ethyl)\mbox{-}Carbamoyl\mbox{-}1,3\mbox{-}Phenylene\mbox{-}Dicarbamate. \ ^1H\mbox{-}NMR\mbox{-}(400\mbox{-}MHz,\mbox{-}DMSO\mbox{-}d_6): \delta\mbox{-}9.91\mbox{-}(s, 2H), \delta\mbox{-}8.33\mbox{-}8.36\mbox{-}(t, J\mbox{=}14\mbox{-}Hz, 1H), \delta\mbox{-}7.91\mbox{-}7.93\mbox{-}(d, J\mbox{=}8\mbox{-}Hz, 4H), \delta\mbox{-}7.88\mbox{-}(s, 2H), \delta\mbox{-}7.46\mbox{-}(t, J\mbox{=}16\mbox{-}Hz, 4H), \delta\mbox{-}7.38\mbox{-}(t, J\mbox{=}16\mbox{-}Hz, 4H), \delta\mbox{-}6.88\mbox{-}(t, J\mbox{=}16\mbox{-}Hz, 4H), \delta\mbox{-}6.88\mbox{-}(t, J\mbox{=}16\mbox{-}Hz, 2H), \delta\mbox{-}3.25\mbox{-}3.29\mbox{-}(q, J\mbox{=}16\mbox{-}Hz, 2H), \delta\mbox{-}3.06\mbox{-}3.11\mbox{-}(q, J\mbox{=}20\mbox{-}Hz, 2H), \delta\mbox{-}3.38\mbox{-}(s, 9H). \ ^{13}C\mbox{-}NMR\mbox{-}(101\mbox{-}MHz, DMSO\mbox{-}d_6)\mbox{-}\delta\mbox{-}16.18\mbox{-}153.87\mbox{-}144.22\mbox{-}141.25\mbox{-}139.88\mbox{-}136.59\mbox{-}128.20\mbox{-}127.61\mbox{-}125.70\mbox{-}120.68\mbox{-}112.47\mbox{-}78.17\mbox{-}66.25\mbox{-}47.03\mbox{-}28.69\mbox{-}HRMS\mbox{-}(ESI)\mbox{-}C_{44}H_{42}N_4O_7\mbox{-}[M\mbox{+}H]^+\mbox{-}calcd\mbox{=}739.3132\mbox{-}found\mbox{-}[M\mbox{+}H]^+\mbox{=}739.3147\mbox{-}. \end{array}$ 

$$\begin{split} &N,N'-(((5-((2-Octanamidoethyl)Carbamoyl)-1,3-Phenylene)Bis-(Az an e diyl))Bis (2-Ox oeth an e-2, 1-Diyl))Bis (N-((S)-2, 6-Diaminohexyl)Hexanamide) (1). <sup>1</sup>H NMR (400 MHz, DMSO-d_6): <math>\delta$$
 10.52–10.55 (m, 2H),  $\delta$  8.47–8.49 (m, 1H),  $\delta$  8.06–8.17 (m, 3H),  $\delta$  7.75–7.94 (m, 13H),  $\delta$  4.22–4.33 (dd, J = 20 Hz, 2H),  $\delta$  4.02–4.18 (dd, J = 16 Hz, 2H),  $\delta$  3.35–3.38 (m, 3H),  $\delta$  3.26–3.29 (m, 2H),  $\delta$  3.17–3.21 (m, 2H),  $\delta$  2.75–2.80 (d, J = 20 Hz, 4H),  $\delta$  2.34–2.46 (m, 1H),  $\delta$  2.19–2.22 (t, J = 12 Hz, 2H),  $\delta$  2.03–2.07 (t, J = 12 Hz, 2H),  $\delta$  1.41–1.54 (m, 18H),  $\delta$  1.21–1.28 (m, 16H),  $\delta$  0.82–0.87 (m, 9H). <sup>13</sup>C NMR (101 MHz, DMSO-d\_6):  $\delta$  174.99, 173.73, 173.01, 169.79, 168.44, 166.86, 166.72, 159.03, 158.70, 139.31, 136.51, 136.43, 114.12, 113.20, 52.12, 51.14, 50.46, 49.85, 49.70, 36.55, 31.37, 30.26, 29.06, 28.92, 27.20, 26.10, 24.70, 24.43, 23.03, 21.97, 15.05, 13.78, 12.88. HRMS (ESI) C<sub>45</sub>H<sub>82</sub>N<sub>10</sub>O<sub>6</sub> [M + H]<sup>+</sup> calcd = 859.6497; found [M + H]<sup>+</sup> = 859.6491.

$$\begin{split} &N,N'-(((5-((2-Octanamidoethyl)Carbamoyl)-1,3-Phenylene)Bis-(Azanediyl))Bis(2-Oxoethane-2,1-Diyl))Bis(N-((S)-2,6-Diaminohexyl)Octanamide) (2). ^{1}H NMR (400 MHz, DMSO-d_6): <math>\delta$$
 10.51–10.54 (m, 2H),  $\delta$  8.46–8.49 (m, 1H),  $\delta$  8.05–8.15 (3H),  $\delta$  7.71–7.95 (m, 13H),  $\delta$  4.21–4.32 (dd, J = 16 Hz, 2H),  $\delta$  4.02–4.18 (dd, J = 16 Hz, 1H),  $\delta$  3.36–3.46 (m, 3H),  $\delta$  3.26–3.29 (m, 2H),  $\delta$  3.18–3.20 (m, 2H),  $\delta$  2.73–2.8 (m, 4H),  $\delta$  2.36–2.44 (m, 1H),  $\delta$  2.18–2.22 (t, J = 16 Hz, 4H),  $\delta$  2.03–2.07 (t, J = 16 Hz, 2H),  $\delta$  1.39–1.52 (m, 18H),  $\delta$  1.20–1.26 (m, 24H),  $\delta$  0.79–0.86 (m, 9H). <sup>13</sup>C NMR (101 MHz, DMSO-d\_6):  $\delta$  174.99, 173.73, 173.01, 169.79, 168.44, 166.86, 166.72, 159.03, 158.70, 139.31, 136.51, 136.43, 114.12, 113.20, 52.12, 51.14, 50.46, 49.85, 35.89, 32.63, 32.36, 31.62, 31.37, 31.32, 30.26, 29.91, 29.06, 28.92, 27.20, 25.69, 24.70, 24.43, 22.53, 22.46, 21.97, 14.41, 14.37, 14.32. HRMS (ESI) C<sub>49</sub>H<sub>90</sub>N<sub>10</sub>O<sub>6</sub> [M + H]<sup>+</sup> calcd = 915.7123; found [M + H]<sup>+</sup> = 915.7115.

N,N'-(((5-((2-Octanamidoethyl)Carbamoyl)-1,3-Phenylene)Bis-(Azanediyl))Bis(2-Oxoethane-2,1-Diyl))Bis(N-((S)-2,6-Diaminohexyl)-2-(Naphthalen-1-yl)Acetamide) (3). <sup>1</sup>H NMR (400 MHz, DMSO $d_6$ ):  $\delta$  10.64–10.69 (d, J = 20 Hz, 1H),  $\delta$  10.51–10.56 (d, J = 20 Hz, 1H), δ 8.50-8.52 (m, 1H), δ 8.24-8.33 (m, 2H), δ 7.92-8.07 (m, 9H),  $\delta$  7.76–7.84 (m, 10H),  $\delta$  7.41–7.53 (m, 6H),  $\delta$  7.29–7.31 (d, J = 8 Hz, 1H),  $\delta$  4.48–4.61 (m, 2H),  $\delta$  4.23–4.37 (m, 2H),  $\delta$  4.06–4.18 (m, 3H),  $\delta$  3.72–3.81 (m, 2H),  $\delta$  3.38–3.41 (m, 2H),  $\delta$  3.17–3.32 (m, 5H),  $\delta$ 2.73–2.77 (m, 4H),  $\delta$  2.03–2.07 (t, J = 16 Hz, 2H),  $\delta$  1.38–1.67 (m, 14H),  $\delta$  1.15–1.24 (m, 8H),  $\delta$  0.81–0.84 (t, J = 12 Hz, 3H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>): δ 173.13, 171.97, 169.74, 168.45, 166.93, 159.00, 158.67, 139.38, 136.59, 133.74, 132.90, 132.76, 132.69, 132.61, 128.76, 128.45, 127.72, 126.35, 126.13, 126.01, 125.82, 125.04, 118.68, 114.34, 52.55, 51.66, 50.75, 50.07, 49.74, 35.89, 31.61, 30.30, 30.04, 29.06, 28.91, 27.25, 27.17, 25.69, 22.52, 22.03, 21.97, 14.41. HRMS (ESI)  $C_{57}H_{78}N_{10}O_6 [M + H]^+$  calcd = 999.6184; found  $[M + H]^+$  = 999.6175.

N,N'-(((5-((2-Octanamidoethyl)Carbamoyl)-1,3-Phenylene)Bis-(Azanediyl))Bis(2-Oxoethane-2,1-Diyl))Bis(N-((S)-2,6-Diaminohexyl)-[1,1'-Biphenyl]-4-Carboxamide) (4). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>):  $\delta$  10.25 (s, 1H),  $\delta$  8.41–8.49 (m, 1H),  $\delta$  7.93–8.06 (m, 7H),  $\delta$  7.78–7.81 (m, 6H),  $\delta$  7.71–7.73 (d, *J* = 8 Hz, 4H),  $\delta$  7.65–7.67 (d, *J* = 8 Hz, 4H),  $\delta$  7.36–7.55 (m, 9H),  $\delta$  4.15–4.44 (m, 4H),  $\delta$  3.67–3.70 (m, 4H),  $\delta$  3.17–3.26 (m, 5H),  $\delta$  2.79–2.83 (m, 3H),  $\delta$  2.02–2.09 (m, 2H),  $\delta$  1.54–1.64 (m, 6H),  $\delta$  1.43–1.49 (m, 7H),  $\delta$  1.18–1.21 (m, 9H),

$$\begin{split} &\delta \ 0.75-0.82 \ (t, \ J=28 \ Hz, \ 3H). \ ^{13}\text{C} \ \text{NMR} \ (101 \ \text{MHz}, \ \text{DMSO-}d_6): \ \delta \\ &173.31, \ 173.04, \ 168.14, \ 158.57, \ 141.81, \ 139.55, \ 139.18, \ 135.27, \ 129.50, \\ &128.75, \ 128.46, \ 127.91, \ 127.34, \ 127.22, \ 126.95, \ 113.91, \ 112.94, \ 53.95, \\ &49.68, \ 35.89, \ 31.60, \ 30.47, \ 29.05, \ 28.91, \ 27.25, \ 25.69, \ 22.52, \ 22.00, \\ &14.40. \ \text{HRMS} \ (\text{ESI}) \ \text{C}_{59}\text{H}_{78}\text{N}_{10}\text{O}_6 \ [\text{M} + \text{H}]^+ \ \text{calcd} = 1023.6184; \ \text{found} \\ &[\text{M} + \text{H}]^+ = 1023.6175. \end{split}$$

N,N'-(((5-((2-Octanamidoethyl)Carbamoyl)-1,3-Phenylene)Bis-(Azanediyl))Bis(2-Oxoethane-2,1-Diyl))Bis(4-Chloro-N-((S)-2,6-Diaminohexyl)Benzamide) (5). <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$ 10.15 (s, 1H), δ 8.36-8.46 (m, 1H), δ 7.94-7.98 (m, 5H), δ 7.85-7.88  $(d, I = 12 Hz, 2H), \delta 7.72 - 7.74 (m, 6H), \delta 7.57 - 7.58 (d, I = 4 Hz, 1H),$  $\delta$  7.50–7.53 (t, J = 12 Hz, 1H),  $\delta$  7.39–7.45 (m, 6H),  $\delta$  4.18–4.30 (dd, J = 20 Hz, 1H),  $\delta 4.01 - 4.13$  (q, J = 48 Hz, 3H),  $\delta 3.56 - 3.57$  (m, 4H),  $\delta$ 3.18-3.22 (m, 3H),  $\delta 3.10-3.13$  (m, 2H),  $\delta 2.59-2.75$  (m, 4H),  $\delta$ 1.96–2.00 (t, J = 16 Hz, 2H),  $\delta$  1.46–1.57 (m, 6H),  $\delta$  1.13–1.41 (m, 7H),  $\delta$  1.12–1.14 (m, 9H),  $\delta$  0.73–0.77 (t, J = 16 Hz, 3H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>): δ 173.06, 172.56, 167.96, 166.33, 163.76, 158.94, 158.61, 139.11, 137.13, 135.18, 134.87, 131.15, 129.99, 129.13, 128.89, 113.96, 110.68, 53.82, 49.61, 35.90, 31.61, 30.42, 29.85, 29.07, 28.92, 27.23, 25.70, 22.52, 21.99, 21.76, 14.41. HRMS (ESI)  $C_{47}H_{68}Cl_2N_{10}O_6 [M + H]^+$  calcd = 939.4779; found  $[M + H]^+$  = 939.4768

N.N'-(((5-((2-Octanamidoethyl)Carbamoyl)-1.3-Phenylene)Bis-(Azanediyl))Bis(2-Oxoethane-2,1-Diyl))Bis(2-((3S,5S,7S)-Adamantan-1-yl)-N-((S)-2,6-Diaminohexyl)Acetamide) (7). <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  10.55–10.56 (d, J = 4 Hz, 1H),  $\delta$  10.50 (s, 1H),  $\delta$ 8.48–8.51 (t, J = 12 Hz, 1H),  $\delta$  8.01–8.11 (m, 3H),  $\delta$  7.88–7.95 (m, 4H),  $\delta$  7.72–7.81 (m, 8H),  $\delta$  4.24–4.36 (dd, J = 20 Hz, 2H),  $\delta$  4.01– 4.17 (dd, J = 16 Hz, 2H),  $\delta$  3.65–6.71 (m, 2H),  $\delta$  3.25–3.27 (m, 2H),  $\delta$ 3.17-3.20 (m, 3H), δ 2.76-2.78 (m, 4H), δ 2.33-2.38 (m, 1H), δ 1.98-2.06 (m, 5H), δ 1.89-1.92 (m, 6H), δ 1.37-1.66 (m, 38H), δ 1.18–1.24 (m, 8H),  $\delta$  0.83–0.85 (t, J = 8 Hz, 3H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>): δ 173.18, 173.03, 171.77, 169.77, 168.65, 165.34, 158.84, 158.52, 139.30, 137.41, 113.74, 112.42, 52.90, 50.41, 49.93, 49.78, 45.42, 45.15, 36.89, 36.85, 35.89, 33.78, 33.20, 31.63, 30.37, 29.84, 29.07, 28.93, 28.49, 28.46, 27.25, 25.70, 22.54, 22.09, 21.97, 14.44. HRMS (ESI)  $C_{57}H_{94}N_{10}O_6 [M + H]^+$  calcd = 1015.7436; found  $[M + H]^+ = 1015.7425.$ 

N,N'-(((5-((2-Decanamidoethyl)Carbamoyl)-1,3-Phenylene)Bis-(Azanediyl))Bis(2-Oxoethane-2,1-Diyl))Bis(2-((3S,5S,7S)-Adamantan-1-yl)-N-((S)-2,6-Diaminohexyl)Acetamide) (8). <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  10.56–10.58 (d, J = 8 Hz, 1H),  $\delta$  10.50 (s, 1H),  $\delta$ 8.47–8.50 (t, J = 12 Hz, 1H),  $\delta$  8.11–8.21 (m, 3H),  $\delta$  7.90–7.94 (m, 4H),  $\delta$  7.73–7.87 (m, 8H),  $\delta$  4.25–4.36 (dd, J = 20 Hz, 2H),  $\delta$  4.02– 4.17 (dd, J = 16 Hz, 2H),  $\delta$  3.66–3.72 (m, 1H),  $\delta$  3.34–3.38 (m, 2H),  $\delta$ 3.25–3.29 (m, 2H),  $\delta$  3.17–3.22 (m, 2H),  $\delta$  2.73–2.82 (m, 4H),  $\delta$ 2.35-2.38 (d, I = 12 Hz, 1H),  $\delta 1.98-2.07$  (m, 5H),  $\delta 1.90-1.94$  (m, 6H),  $\delta$  1.37–1.67 (m, 38H),  $\delta$  1.16–1.26 (m, 12H),  $\delta$  0.83–0.87 (t, J = 12 Hz, 3H). <sup>13</sup>C NMR (101 MHz, DMSO- $d_6$ ):  $\delta$  173.17, 171.78, 169.75, 168.63, 165.68, 165.26, 158.97, 158.65, 139.46, 136.59, 114.18, 113.14, 52.91, 51.87, 50.40, 49.94, 49.78, 45.42, 45.14, 42.21, 36.89, 36.85, 35.88, 33.77, 33.20, 31.73, 30.35, 29.82, 29.36, 29.25, 29.13, 28.50, 28.46, 27.23, 25.69, 22.57, 22.08, 21.97, 14.44. HRMS (ESI)  $C_{59}H_{98}N_{10}O_6 [M + H]^+$  calcd = 1043.7749; found  $[M + H]^+$  = 1043.7739.

N,N'-(((5-((2-Dodecanamidoethyl)Carbamoyl)-1,3-Phenylene)-Bis(Azanediyl))Bis(2-Oxoethane-2,1-Diyl))Bis(2-((3S,5S,7S)-Adamantan-1-yl)-N-((S)-2,6-Diaminohexyl)Acetamide) (9). <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  10.50–10.51 (d, J = 4 Hz, 1H),  $\delta$  10.43 (s, 1H),  $\delta$  8.42–8.44 (t, J = 8 Hz, 1H),  $\delta$  8.08 (s, 2H),  $\delta$  7.83–7.88 (m, 3H),  $\delta$  7.74–7.76 (m, 4H),  $\delta$  4.18–4.29 (dd, J = 16 Hz, 2H),  $\delta$  3.95– 4.10 (dd, J = 16 Hz, 2H),  $\delta$  3.59–3.63 (m, 1H),  $\delta$  3.48–3.49 (m, 2H)  $\delta$ 3.26-3.28 (m, 2H),  $\delta$  3.18-3.21 (m, 2H),  $\delta$  3.11-3.12 (m, 2H),  $\delta$ 2.69–2.71 (d, J = 8 Hz, 4H),  $\delta$  2.28–2.32 (dd, J = 4 Hz, 1H),  $\delta$  1.91– 1.99 (m, 5H),  $\delta$  1.83–1.84 (t, J = 4 Hz, 6H),  $\delta$  1.37–1.60 (m, 39H),  $\delta$ 1.16–1.21 (m, 17H),  $\delta$  0.76–0.8 (t, J = 16 Hz, 3H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>): δ 173.13, 172.92, 171.75, 169.74, 168.61, 166.88, 158.88, 158.57, 139.31, 136.61, 114.18, 113.94, 113.09, 52.89, 51.87, 50.36, 49.90, 49.75, 45.41, 45.14, 42.21, 36.90, 36.85, 35.88, 33.77, 33.19, 31.78, 30.36, 29.82, 29.49, 29.41, 29.27, 29.21, 29.14, 28.50, 28.46, 27.24, 25.69, 22.59, 22.08, 21.97, 14.46. HRMS (ESI)  $C_{61}H_{102}N_{10}O_6 [M + H]^+$  calcd = 1071.8062; found  $[M + H]^+$  = 1071.8050

*N*,*N*′-(((5-((2-Dodecanamidoethyl)Carbamoyl)-1,3-Phenylene)-Bis(Azanediyl))Bis(2-Oxoethane-2,1-Diyl))Bis(N-((Ś)-2,6-Diaminohexyl)Octanamide) (10). <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  $10.50-10.51 (d, J = 4 Hz, 1H), \delta 10.48 (s, 1H), \delta 8.43-8.45 (m, 1H), \delta$ 8.11-8.14 (m, 2H), δ 8.04 (s, 1H), δ 7.88-7.91 (m, 5H), δ 7.71-7.80 (m, 8H),  $\delta$  4.21–4.33 (dd, J = 20 Hz, 2H),  $\delta$  4.02–4.18 (dd, J = 16 Hz, 2H),  $\delta$  3.26–3.29 (m, 3H),  $\delta$  3.17–3.21 (m, 2H),  $\delta$  2.75–2.80 (q, J = 20 Hz, 4H), δ 3.26–3.46 (m, 1H), δ 2.19–2.22 (m, 2H), δ 2.03–2.07 (t, I = 16 Hz, 2H),  $\delta$  1.39–1.55 (m, 18H),  $\delta$  1.21–1.27 (m, 32H),  $\delta$ 0.79-0.88 (m, 9H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>): δ 175.03, 173.73, 173.02, 169.80, 168.46, 166.87, 158.88, 158.56, 139.30, 136.56, 136.45, 114.13, 113.25, 52.19, 51.18, 50.54, 49.94, 35.91, 32.69, 32.44, 31.75, 31.68, 31.61, 30.28, 29.94, 29.45, 29.38, 29.23, 29.17, 29.10, 29.00, 27.21, 25.68, 25.04, 24.75, 22.55, 22.49, 21.98, 14.42, 14.35. HRMS (ESI)  $C_{53}H_{98}N_{10}O_6 [M + H]^+$  calcd = 971.7749; found  $[M + H]^+$  $H^{+} = 971.7755.$ 

*N*,*N*'-(5-((2-Dodecanamidoethyl)Carbamoyl)-1,3-Phenylene)Bis-(2-(((S)-2,6-Diaminohexyl)Amino)Acetamide) (11). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 10.15 (s, 1H), δ 8.46–8.50 (q, *J* = 16 Hz, 1H), δ 8.14–8.16 (q, *J* = 8 Hz, 1H), δ 7.93–7.96 (t, *J* = 12 Hz, 1H), δ 7.74–7.79 (m, 5H), δ 7.71–7.72 (d, *J* = 4 Hz, 2H), δ 4.21–4.23 (m, 2H), δ 4.01(s, 2H), δ 3.57–3.60 (m, 4H), δ 3.51–3.53 (m, 4H), δ 3.26–3.29 (m, 4H), δ 3.17–3.20 (m, 2H), δ 2.78 (s, 4H), δ 2.68–2.70 (m, 2H), δ 2.03–2.06 (t, *J* = 12 Hz, 2H), δ 1.61–1.69 (m, 4H), δ 1.53–1.60 (m, 4H), δ 1.45–1.48 (m, 2H), δ 1.36–1.39 (m, 4H), δ 1.16–1.27 (m, 16H), δ 0.83–0.87 (t, *J* = 12 Hz, 3H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>): δ 172.99, 166.84, 158.84, 158.51, 139.19, 123.37, 123.19, 114.32, 112.51, 56.83, 55.73, 35.88, 31.77, 30.51, 29.48, 29.40, 29.26, 29.20, 29.12, 27.07, 25.69, 23.31, 22.58, 14.46. HRMS (ESI) C<sub>37</sub>H<sub>70</sub>N<sub>10</sub>O<sub>4</sub> [M + H]<sup>+</sup> calcd = 719.5660; found [M + H]<sup>+</sup> = 719.5648.

$$\begin{split} &N,N'-(((5-((2-(2-((1s,3s)-Adamantan-1-Yl)Acetamido)Ethyl)-Carbamoyl)-1,3-Phenylene)Bis(Azanediyl)) Bis(2-Oxoethane-2,1-Diyl))Bis(2-((35,55,75)-Adamantan-1-yl)-N-((5)-2,6-Diaminohexyl)-Acetamide) (12). <sup>1</sup>H NMR (400 MHz, DMSO-d_6): <math>\delta$$
 10.60–10.61 (d, J = 4 Hz, 1H),  $\delta$  10.55 (s, 1H),  $\delta$  8.48–8.50 (m, 1H),  $\delta$  8.10–8.17 (m, 3H),  $\delta$  7.91–7.96 (m, 4H),  $\delta$  7.78–7.87 (m, 7H),  $\delta$  4.30–4.42 (dd, J = 20 Hz, 2H),  $\delta$  4.07–4.24 (dd, J = 16 Hz, 2H),  $\delta$  3.71–3.77 (m, 1H),  $\delta$  3.40–3.44 (m, 3H),  $\delta$  3.32–3.37 (m, 3H),  $\delta$  3.26–3.29 (m, 2H),  $\delta$  2.79–2.86 (m, 4H),  $\delta$  2.40–2.44 (m, 1H),  $\delta$  1.43–1.51 (m, 3H),  $\delta$  1.87–1.96 (m, 11H),  $\delta$  1.57–1.73 (m, 42H),  $\delta$  1.43–1.51 (m, 4H). <sup>13</sup>C NMR (101 MHz, DMSO-d\_6):  $\delta$  173.18, 171.78, 171.17, 169.77, 168.64, 158.65, 157.90, 139.30, 113.78, 133.19, 32.58, 30.37, 29.84, 28.50, 28.45, 27.24, 22.09, 21.97. HRMS (ESI) C<sub>61</sub>H<sub>96</sub>N<sub>10</sub>O<sub>6</sub> [M + H]<sup>+</sup> calcd = 1065.7593; found [M + H]<sup>+</sup> = 1065.7583.

N,N'-(((5-(Hexylcarbamoyl)-1,3-Phenylene)Bis (Azanediyl))Bis(2-Oxoethane-2,1-Diyl))Bis(2-((35,55,75)-Adamantan-1-yl)-N-((5)-2,6-Diaminohexyl)Acetamide) (13). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>):  $\delta$  10.51–10.52 (d, *J* = 4 Hz, 1H),  $\delta$  10.45 (s, 1H),  $\delta$  8.39–8.42 (m, 1H),  $\delta$  8.10 (s, 3H),  $\delta$  7.89 (s, 3H),  $\delta$  7.72–7.83 (m, 8H),  $\delta$  4.24–4.35 (dd, *J* = 20 Hz, 2H),  $\delta$  4.10–4.18 (dd, *J* = 16 Hz, 2H),  $\delta$  3.65–3.76 (m, 2H),  $\delta$  3.14–3.23 (m, 2H),  $\delta$  2.75–2.84 (m, 4H),  $\delta$  2.34–2.38 (d, *J* = 20 Hz, 1H),  $\delta$  1.91–2.07 (m, 10H),  $\delta$  1.37–1.63 (m, 38H),  $\delta$  1.28–1.32 (m,

6H),  $\delta$  0.85–0.88 (t, *J* = 12 Hz, 3H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  173.22, 171.79, 169.74, 168.65, 166.09, 157.95, 157.58, 139.22, 136.71, 114.24, 113.29, 52.97, 51.91, 50.47, 50.01, 49.85, 45.45, 45.17, 42.25, 36.89, 33.79, 33.22, 31.48, 30.38, 29.86, 29.49, 28.52, 28.48, 27.25, 26.59, 22.52, 22.08, 21.97, 14.40. HRMS (ESI) C<sub>53</sub>H<sub>87</sub>N<sub>9</sub>O<sub>5</sub> [M + H]<sup>+</sup> calcd = 930.6908; found [M + H]<sup>+</sup> = 930.6900.

N,N'-(((5-(Octylcarbamoyl)-1,3-Phenylene)Bis (Azanediyl))Bis(2-Oxoethane-2,1-Diyl))Bis(2-((3S,5S,7S)-Adamantan-1-yl)-N-((S)-2,6-Diaminohexyl)Acetamide) (14). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ 10.54–10.56 (d, J = 8 Hz, 1H),  $\delta$  10.47–10.48 (d, J = 4 Hz, 1H),  $\delta$ 10.44–10.45 (m, 1H), δ 8.11–8.12 (m, 2H), δ 8.03 (s, 1H), δ 7.91 (s, 3H),  $\delta$  7.68–7.81 (m, 7H),  $\delta$  4.25–4.36 (dd, J = 16 Hz, 2H),  $\delta$  4.01– 4.17 (dd, J = 16 Hz, 2H),  $\delta$  3.65–3.72 (m, 1H),  $\delta$  3.50–3.56 (m, 4H),  $\delta$ 3.31-3.33 (m, 2H), δ 3.17-3.22 (m, 2H), δ 2.73-2.79 (m, 4H), δ 2.35-2.39 (m, 1H), δ 1.98-2.04 (m, 3H), δ 1.90-1.96 (m, 6H), δ  $1.37-1.67 (m, 35H), \delta 1.25-1.28 (m, 10H), \delta 0.84-0.87 (t, J = 12 Hz, J = 12 Hz)$ 3H). <sup>13</sup>C NMR (101 MHz, DMSO- $d_6$ ):  $\delta$  173.20, 171.79, 169.75, 168.66, 165.41, 159.90, 159.20, 139.25, 137.08, 116.29, 114.22, 52.93, 51.89, 50.42, 49.96, 49.79, 42.22, 36.89, 36.85, 33.79, 33.20, 31.74, 30.36, 29.83, 29.52, 29.23, 29.14, 28.50, 28.46, 27.24, 26.93, 22.57, 22.08, 21.96, 14.45. HRMS (ESI)  $C_{55}H_{91}N_9O_5 [M + H]^+$  calcd = 958.7221; found [M + H]<sup>+</sup> = 958.7207.

N,N'-(((5-(Decylcarbamoyl)-1,3-Phenylene)Bis (Azanediyl))Bis(2-Oxoethane-2,1-Diyl))Bis(2-((3S,5S,7S)-Adamantan-1-yl)-N-((S)-2,6-Diaminohexyl)Acetamide) (15). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ 10.56–10.58 (d, J = 8 Hz, 1H),  $\delta$  4.48–4.49 (d, J = 4 Hz, 1H),  $\delta$  8.42– 8.45 (t, J = 12 Hz, 1H),  $\delta$  8.12–8.17 (m, 3H),  $\delta$  7.91–7.95 (m, 3H),  $\delta$ 7.82–7.85 (m, 5H), δ 7.69–7.77 (m, 2H), δ 4.26–4.36 (dd, J = 16 Hz, 2H),  $\delta$  4.02–4.17 (dd, J = 20 Hz, 2H),  $\delta$  3.66–3.73 (m, 1H),  $\delta$  3.17– 3.22 (m, 2H),  $\delta$  2.89–2.97 (q, J = 32 Hz, 2H),  $\delta$  2.75–2.80 (m, 4H),  $\delta$ 2.35–2.39 (d, J = 16 Hz, 1H),  $\delta$  1.98–2.05 (m, 3H),  $\delta$  1.90–1.95 (m, 6H), δ 1.34–1.67 (m, 37H), δ 1.24–1.27 (m, 14H), δ 1.14–1.18 (t, J= 16 Hz, 3H),  $\delta$  0.83–0.87 (t, J = 16 Hz, 3H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>): *δ* 173.19, 171.79, 169.72, 168.64, 164.89, 158.88, 158.56, 139.25, 137.04, 114.20, 111.31, 52.91, 51.89, 49.93, 45.42, 45.16, 42.30, 42.21, 36.89, 36.85, 33.79, 33.20, 31.77, 30.35, 29.82, 29.49, 29.27, 29.18, 28.50, 28. HRMS (ESI)  $C_{57}H_{95}N_9O_5$  [M + H]<sup>+</sup> calcd = 986.7534; found  $[M + H]^+ = 986.7537$ .

**MIC.** Briefly, MRSA, MRSE, VREF, *E. coli, K. pneumoniae*, and *P. aeruginosa* were grown in a TSB medium at 37 °C for 16 h while shaking. Then, 100  $\mu$ L of the bacterial solution was transferred into 4 mL of a fresh TSB medium and incubated for another 6 h to reach a mid-log phase. Following this, 50  $\mu$ L of bacteria with a concentration of 10<sup>6</sup> CFU/mL was added into 50  $\mu$ L of compounds with concentrations from 0.75 to 25  $\mu$ g/mL in 96-well plates. The mixture was incubated at 37 °C for 16 h. A multimode microplate reader was used to determine the MICs.

**Salt Sensitivity.** The same method of evaluating MICs was applied here to test the salt sensitivity. For salt sensitivity assay against Na<sup>+</sup> and K<sup>+</sup>, the medium we applied to dilute bacteria and compounds was TSB powder in a phosphate-buffered saline (PBS) buffer. Similarly, for the salt sensitivity assay against Ca<sup>2+</sup>, the medium was TSB powder in deionized (DI) water with 2 mM CaCl<sub>2</sub>. Then, MICs were tested as normal.

**Hemolytic Activity.** The fresh human red blood cells were washed with 1 × PBS buffer and centrifuged at 700 g for 10 min until the supernatant was clear. After removing the supernatant, the red blood cells were diluted into 5% suspension with 1 × PBS buffer. A volume of 50  $\mu$ L of suspension was added into 50  $\mu$ L of compounds with different concentrations from 250 to 1.95  $\mu$ g/mL in 96-well plates, and then the mixture was incubated at 37 °C for 1 h. Following this, the plates were centrifuged at 3500 rpm for 10 min. A volume of 30  $\mu$ L of supernatant was transferred into another well with 100  $\mu$ L of 1 × PBS buffer. Subsequently, the absorbance at 540 nm was read using a microplate reader. 2% Triton-100 was used as a positive control. The hemolysis activity was calculated using the formula % hemolysis = [(Abssample – AbsPBS)/(AbsTriton – AbsPBS)] × 100.

**Time-Kill Kinetics Study.** Mid-log phase MRSA and *E. coli* at a concentration of  $10^6$  CFU/mL in TSB solution were first obtained using the method mentioned in the MIC test. Compound **12** (300  $\mu$ L)

and ciprofloxacin with different concentrations were mixed with 300  $\mu$ L of the bacterial solution. The mixture was incubated at 37 °C for 0, 10, 30 min, 1, and 2 h, respectively. At each time point, the solution was diluted 1000-fold for *E. coli* and 100-fold for MRSA, respectively, and then 100  $\mu$ L was spread on TSB agar plates. After incubating at 37 °C for 16 h, colonies were enumerated and CFUs were calculated.

**Biofilm Inhibition.** Bacteria at a concentration of  $10^6$  CFU/mL was added to compound **12** with different concentrations. The mixture was incubated for 48 h. Subsequently, the suspension was discarded, and the biofilm was washed gently. After drying in air, 0.1% crystal violet in DI water was applied to stain the biofilm for 15 min. The dye solution was discarded, and the biomass was washed with DI water for several times. A volume of 200  $\mu$ L of 30% acetic acid was used to dissolve the colored biomass for 15 min after drying. The solution (125  $\mu$ L) was transferred into another 96-well plate to be read under 595 nm. The relative biofilm biomass values were normalized by the biomass value of the control (no addition of compounds). The data were presented as mean  $\pm$  STDEV.

**Drug Resistance Study.** The first-generation MIC data of compound 12 and ciprofloxacin against MRSA were obtained as described in the MIC study mentioned above. Then, MRSA in the well next to the last clear well was diluted to  $10^6$  CFU/mL to determine the MICs again at 37 °C incubation for 12 h. The step was repeated until 14 passages. Drug resistance of compound 12 and ciprofloxacin against *E. coli* was conducted with the same method.

**Fluorescence Microscopy.** After MRSA and *E. coli* were grown into the mid-log phase in the TSB medium, 30  $\mu$ L of bacterial solution was diluted to 3 mL in the TSB medium with 6  $\mu$ g/mL of compound **12**. The mixture was incubated at 37 °C for 2 h. Negative controls were untreated bacteria. Subsequently, the bacterial cells were collected and centrifuged at 3000 rpm for 10 min. The cell pellets were washed with 1 × PBS buffer three times and incubated with PI (5  $\mu$ g/mL) and DAPI (10  $\mu$ g/mL) for 15 min sequentially on ice in the dark. The bacterial cells were washed with 1 × PBS three times after dying procedure with both PI and DAPI. Next, after the final wash, 100  $\mu$ L of 1 × PBS was used to suspend the bacterial cells and 10  $\mu$ L of solution was dropped on to the slides to be observed using a Zeiss Axiovert 200 inverted microscope.

**TEM.** A volume of 30  $\mu$ L of mid-log phase MRSA and *E. coli* was diluted to 3 mL in a fresh TSB medium with 6  $\mu$ g/mL of compound **12** and was then incubated for 2 h, respectively. The bacterial pellets were centrifuged at 3000 rpm for 10 min. 1 × PBS was used to wash them for three times. The negative control was untreated bacterial cells. Then, resuspended bacterial samples were dropped on the surface of grids. The grids were dried in a vacuum oven at 45 °C for 30 s. TEM images were obtained using an 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.

**Inner Membrane Permeability.** *E. coli* was grown to the mid-log phase in a Mueller Hinton broth containing 2% lactose at 37 °C. Bacterial cells were centrifuged at 3000 rpm for 10 min at 4 °C and then were washed with 5 mM HEPES buffer containing 20 mM glucose and 1.5 mM ONPG once. Subsequently, the bacterial cells were diluted to  $OD_{600} = 0.1$  using the buffer mentioned above. The diluted bacterial cells (50  $\mu$ L) were added into 50  $\mu$ L of compound **12** and melittin with different concentrations in the buffer, respectively. The  $OD_{420}$  measurements of the mixture were carried out every 6 min at 37 °C until the fluorescence reached the highest value. The experiment was conducted independently in duplicate with two biological replicates.

**Cytoplasmic Membrane Assay.** Mid-log MRSA was collected and then washed with 5 mM HEPES:5 mM glucose = 1:1. Following this, MRSA cells were resuspended to OD<sub>600</sub> = 0.1 in 5 mM HEPES/5 mM glucose/100 mM KCl (1:1:1) buffer with 2  $\mu$ M DiSC<sub>3</sub>(5). The mixture was kept at room temperature for 30 min. The fluorescence of the suspension was then monitored at room temperature for 8 min with an interval of 2 min at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Compound **12** with different concentrations was added into the above suspension. The fluorescence was read continuously for 12 min. The negative control was untreated MRSA.

In Vivo Antibacterial Assessment and Biocompatibility Evaluation. The experiments were performed according to IACUC of Sun Yat-Sen University (approved protocol number SYSU-IACUC-2019-000203), and all animal care procedures were performed according to the Guide for Care and Use of Laboratory Animals. Healthy female ICR mice (16–20 g each), which were obtained from the Animal Center of Sun Yat-Sen University, were employed and randomly divided into five groups, including normal group, control group, ciprofloxacin group, low-dose compound 12 group (12-L), and high-dose compound 12 group (12-H). While a ciprofloxacin hydrochloride solution was prepared at a concentration of 5 mg/mL, compound 12 was dissolved in sterile PBS at concentrations of 0.5 and 5 mg/mL, respectively. The mice were anesthetized, and their back hair was removed with a shaving knife and depilation cream. Subsequently, a subcutaneous abscess was created by subcutaneously injecting MRSA  $(1 \times 10^8 \text{ CFU/mL})$  with a dose of 100  $\mu$ L into the middle dorsum of mice. After 30 min, 100 µL of sterile PBS, ciprofloxacin hydrochloride solution, or compound 12 solution was injected into the bacterial infection site. After 48 h, the mice were euthanized, and the infected skins were collected. To evaluate the antibacterial affect, the bacteria in the abscess were counted using the standard plate counting assays, and the visible bacterial colonies on the plates were imaged. The infected skins were fixed in 4% paraformaldehyde, embedded in paraffin, and stained with H&E for assessing the antimicrobial effectiveness of various agents. IL-6 and TNF- $\alpha$  levels in the skin homogenate solutions were measured using commercial ELISA kits (Dakewe, Shenzhen, China) following the manufacturer's instructions. The major organs of mice were also harvested and further analyzed by H&E staining for the systemic toxicity assessment. In a local toxicity study, the dorsal skin of ICR mice was injected subcutaneously with a peptide solution (100  $\mu$ L, 5 mg/mL) and collected for histological analysis after 5 days postinjection.

### ASSOCIATED CONTENT

### **Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00312.

Time-kill kinetics of compound **12** and ciprofloxacin against *E. coli*; *E. coli* biofilm inhibition by compound **12**; drug resistance study of compound **12** against *E. coli*; and <sup>1</sup>H NMR, <sup>13</sup>C NMR, and HPLC trace information (PDF) Molecular formula strings (CSV)

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### **Author Contributions**

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### ABBREVIATIONS

 $\gamma$ -AA peptides,  $\gamma$ -substituted-*N*-acylated-*N*-aminoethyl peptides; AMPs, antimicrobial peptides; CDC, Centers for Disease Control and Prevention; DAPI, 4',6-diamidino-2-phenylindole; DEA, diethanolamine; DiSC<sub>3</sub>(5), 3,3'-dipropylthiadicarbocyanine iodide; *E. coli, Escherichia coli*; HATU, 1-[bis-(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxide hexafluorophosphate; HDPs, host defense peptides; H&E, hematoxylin and eosin; IL-6, interleukin-6; *K. pneumoniae, Klebsiella pneumoniae*; MRSE, multiresistance *Staphylococcus epidermidis*; ONPG, *o*-nitrophenyl- $\beta$ -D-galacto-pyranoside; *P. aeruginosa, Pseudomonas aeruginosa*; PDR, pandrug resistance; PI, propidium iodide; PNA, peptide nucleic acid; TEM, transmission electron microscopy; TSB, tryptic soy broth; VREF, vancomycin-resistant *Enterococcus faecalis*.

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