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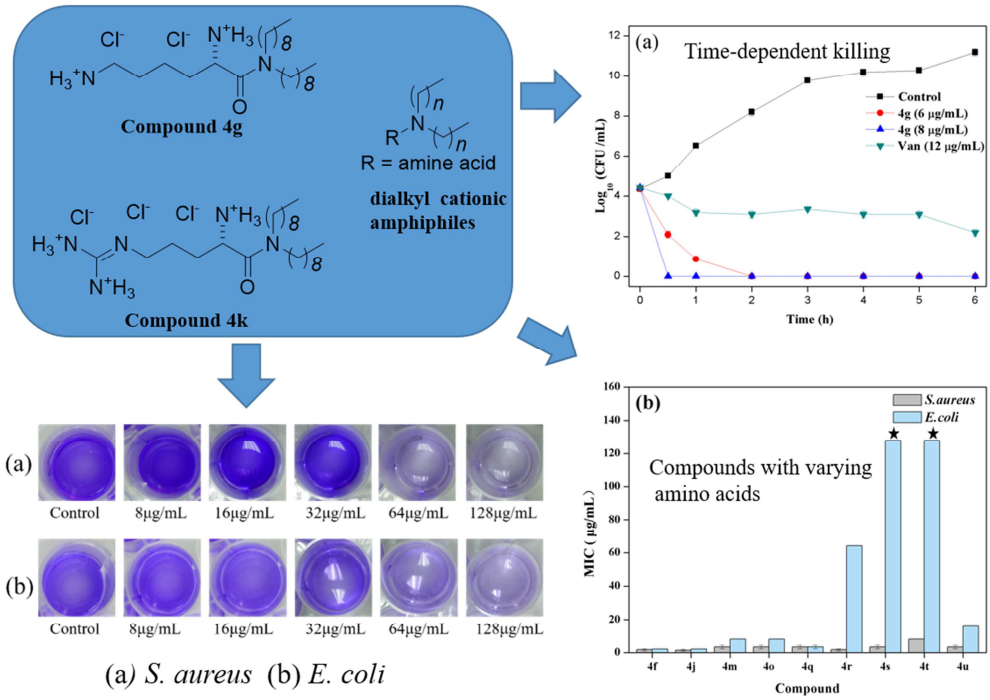
Graphical abstract



(a) *S. aureus* (b) *E. coli*

Synthesis and bioactivities study of new antibacterial peptide mimics: the dialkyl cationic amphiphiles

En Zhang*[1,2], Peng-Yan Bai[1], De-Yun Cui[1], Wen-Chao Chu[1],Yong-Gang Hua[1], Qin Liu[1], Hai-Yang Yin[1], Yong-Jie Zhang[1], Shangshang Qin*[1,2], Hong-Min Liu*[1,2]

[1]School of Pharmaceutical Sciences; Institute of Drug Discovery and Development; Key Laboratory of Advanced Pharmaceutical Technology, Ministry of Education of China; Zhengzhou University, Zhengzhou 450001, PR China. [2]Collaborative Innovation Center of New Drug Research and Safety Evaluation, Henan Province, Zhengzhou 450001, PR China.

E-mail: zhangen@zzu.edu.cn; qinshangshang@126.com; liuhm@zzu.edu.cn

ABSTRACT

The emergence of infectious diseases caused by pathogenic bacteria is widespread. Therefore, it is urgently required to enhance the development of novel antimicrobial agents with high antibacterial activity and low cytotoxicity. A series of novel dialkyl cationic amphiphiles bearing two identical length lipophilic alkyl chains and one non-peptidic amide bond were synthesized and tested for antimicrobial activities against both Gram-positive and Gram-negative bacteria. Particular compounds synthesized showed excellent antibacterial activity toward drug-sensitive bacteria such as *S. aureus*, *E. faecalis*, *E. coli* and *S. enterica*, and clinical isolates of drug-resistant species such as methicillin-resistant *S. aureus* (MRSA), KPC-producing and NDM-1-producing carbapenem-resistant Enterobacteriaceae (CRE). For example, the MIC values of the best compound **4g** ranged from 0.5 to 2 μg/mL against all these strains. Moreover, these small molecules acted rapidly as bactericidal agents, and functioned primarily by permeabilization and depolarization of bacterial membranes. Importantly, these compounds were difficult to induce bacterial resistance and can potentially combat drug-resistant bacteria. Thus, these compounds can be developed into a new class of antibacterial peptide mimics against Gram-positive and Gram-negative bacteria, including drug-resistant bacterial strains.

Keywords: Antimicrobial; dialkyl cationic amphiphiles; non-peptidic amide bonds;

drug-resistant; permeabilization; depolarization

1. Introduction

Antimicrobials are probably one of the most successful forms of chemotherapy in the history of medicine. These compounds have saved many lives and contributed significantly to the control of infectious diseases.[1] However, prevailing bacterial resistance to antibiotics combined with stagnant antibiotic discovery has created an urgent need for the development of new antimicrobial agents that exert novel mechanisms of action.[2, 3]

Because of the widespread use and misuse of antimicrobial agents, the multidrug-resistance of bacteria has become a severe threat to public health.[4] There are many pathogenic bacteria that evade antibiotics, notably the "ESKAPE" bacteria: *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumanii*, *P. aeruginosa* and *Enterobacter* species.[5] Since MRSA was first reported[6] in the 1950s, it has gradually become the most commonly identified drug-resistant bacteria worldwide.[7] Quaternary ammonium compounds (QACs) were widely used to eradicate bacteria, but the presence of *qac* genes in MRSA led to the emergence of QACs resistance.[8-12] The carbapenem-resistant strains are also very problematic. Such as New Delhi metallo-β-lactamase 1 (NDM-1) producing isolates, which are highly resistant to nearly all antibiotics, except colistin and tigecycline.[13] And KPC-2 carbapenemase producing isolates were also exhibited resistance to all β-lactam agents and other antimicrobials.[14] Although the number of bacteria showing antibiotic resistance grows, between 1983 and 2012 the number of new systemic antibacterial agents approved by the US Food and Drug Administration continued to decrease.[15] Thus, there is an urgent need to develop novel antibacterial agents against such bacteria.

Antimicrobial peptides (AMPs) have received substantial attention in recent years owing to their membrane targeting mechanism, broad-spectrum activities and minimum cytotoxicity.[16-19] AMPs display a diverse range of primary peptide sequences and secondary structures. Additionally, AMPs have two characteristics: a large number of cationic residues and hydrophobic domains.[20] Several AMPs are currently being validated in preclinical and clinical settings for the treatment of

infections caused by antimicrobial-resistant bacteria.[21] However, AMPs have some disadvantages, including in vivo toxicity, protease instability and high manufacturing costs.[20] Therefore, a variety of structurally diverse synthetic membrane-active compounds have also been developed as an alternative to antibacterial peptides, such as peptidomimetics[22], oligomers[23], antimicrobial polymers (e.g., cationic derivatives of polymethacrylate[24, 25], polyarylamide[26] and polystyrene[27]), cationic amphiphiles[28, 29] and β-2,2-amino acid derivatives[30].

Several small amphiphilic molecules based on natural product skeletons of xanthone[31, 32] and binaphthyl[33] with potent antibacterial and antibiofilm activities have been developed.[34-39] Additionally, amphiphilic derivatives based on approved antibiotics such as vancomycin[40-42] and kanamycin B[43],[44] were reported to have potent antimicrobial activity. There are already many synthetic antibacterial peptidomimetics currently undergoing clinical trials, such as the membrane-disrupting compound LTX 109[45] (Fig. 1), the cationic steroid compound CSA-13[46] (Fig. 1) and the novel peptide mimetic brilacidin[47] (Fig. 1). These synthetic analogues, possessing excellent antimicrobial activity and high selectivity, were relatively facile and inexpensive to prepare in large quantities. The diversity of structures of these molecules requires further exploration.[26]

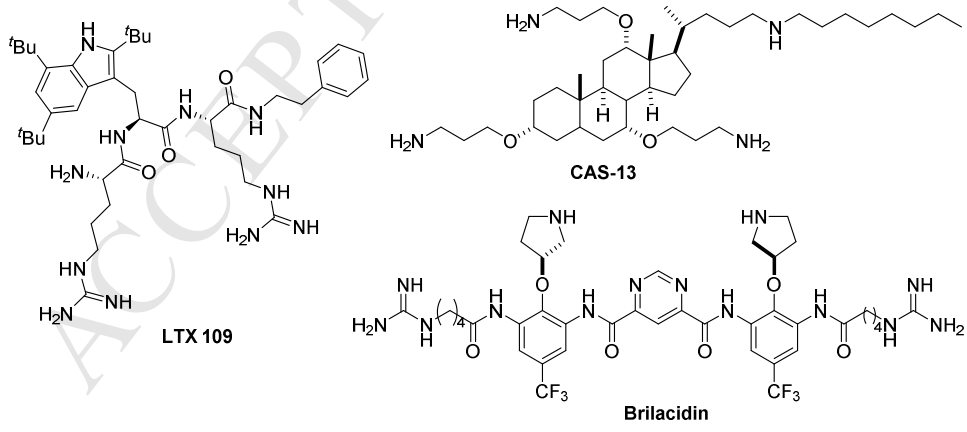
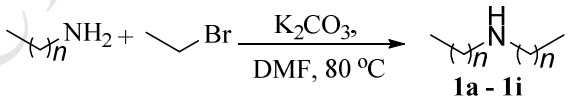


Fig. 1. Structures of some representative AMPs.

During the course of our recent investigations for new structures with good biological activity,[48, 49] we disclosed several heterocyclic derivatives with good antimicrobial activity.[50-52] Inspired by these exciting results, we further showed

through preliminary results the broad antibacterial activity of small amphiphilic molecules[29, 39] against several important drug-resistant strains.

Recently, a series of small molecular cationic compounds with membrane-active and selective broad-spectrum activity were designed and synthesized. The preparation of these new antibacterial amphiphiles was through an effective and reliable synthetic method. The final compounds were obtained by two or three step processes.[35] Moreover, these compounds possessed several positive charges, had two lipophilic alkyl chains and one nonpeptidic amide bond, and displayed good solubility in water. Herein, we reported the activity of these compounds against drug-sensitive bacteria, including *S. aureus*, *E. coli*, *E. faecalis* and *S. enterica*, and drug-resistant bacteria such as methicillin-resistant *S. aureus* (without of *qac* genes), NDM-1-producing *Enterobacteriaceae* (NDM) and carbapenemase-producing *K. pneumonia*e (KPC). The bactericidal kinetics and the propensity of bacteria to develop resistance against these compounds were studied. The mechanism of action of the compounds to disrupt bacterial biofilms was investigated. Additionally, stability and activity of these molecules in complex mammalian fluids such as plasma, serum and whole blood were evaluated to understand the optimal conditions that enhanced the potency of these small molecules. The toxicity of the compounds was studied using HeLa cells and mammalian red blood cells. Interestingly, using fluorescence and electron scanning spectroscopy, these amphiphilic compounds did not lead to cell death at the minimal inhibitory concentration.

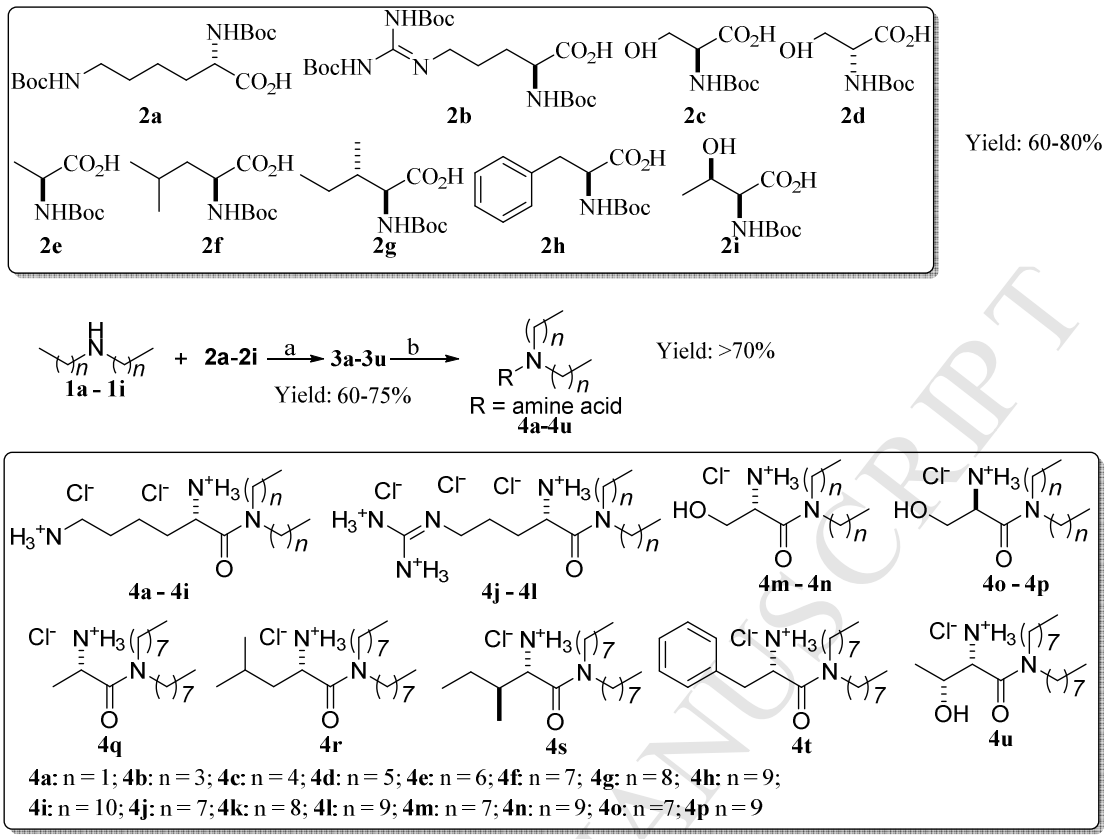


1a - 1i

Yield: 30-40%

1a: n = 1; **1b**: n = 3; **1c**: n = 4; **1d**: n = 5; **1e**: n = 6;
1f: n = 7; **1g**: n = 8; **1h**: n = 9; **1i**: n = 10

Scheme 1. Synthesis of symmetric dialkyl amine intermediates (**1a–1i**).



(a) DIPEA, HBTU in DMF/$CHCl_3$ (5:2), RT, 24 h; (b) CH_3COCl/CH_3OH, 0 °C–RT, 24 h.

Scheme 2. Synthesis of dialkyl cationic amphiphiles (**4a–4u**).

2. Results and discussion

2.1. Synthesis and characterization

The molecules **4a–4u** were synthesized only in three steps using amines as starting materials (Schemes 1 and 2). To assess the importance of the amphiphilic balance, the length of the lipophilic alkyl chains was varied from two to 11. To further fine-tune the structure-activity relationship of the compounds, hydrophilic moieties (amino acids) and the configuration of the amino acids were also varied on these lipid frameworks. To synthesize these compounds, the various intermediates **2a, 2e–2i**,[39] **2b**,[53] **2c** and **2d**[54] were prepared by known methods. The general syntheses of *N*,*N*-dialkylamines (**1e**, **1g** and **1i**; Scheme 1) were accomplished by a conventional procedure[55] and other *N*,*N*-dialkylamines were commercial available. Key amide coupling compounds (**3a–3u**; Scheme 2) were synthesized

using N-Boc-protected amino acids and **1a–1i**. Finally, target compounds (**4a–4u**; Scheme 2) were obtained individually from **3a–3u** with acetyl chloride in methanol. The products were obtained without further chromatography purification in high yield. All final compounds were characterized by ^{1}H NMR, ^{13}C NMR and HRMS. Finally, 21 dialkyl cationic small molecules were synthesized using different lipophilic secondary amines with the same alkyl chain and nine different amino acids.

2.2. Antibacterial activity

The antibacterial efficacy of these compounds was determined in suitable culture medium and expressed as the minimum inhibitory concentration (MIC) (Table 1). The glycopeptide antibiotic vancomycin and the β-lactam antibiotic meropenem were also used to compare the MIC results with our compounds. These compounds displayed preferable antibacterial potency against a wide spectrum of drug-sensitive bacteria such as *S. aureus*, *E. coli*, *E. faecalis* and *S. enterica*, and drug-resistant bacteria such as methicillin-resistant *S. aureus* (MRSA), NDM-1-producing *Enterobacteriaceae* and carbapenemase-producing *K. pneumoniae*.

In general, the compounds **4e–4m**, **4o**, **4q** and **4u** showed good activities that were comparable to vancomycin against all the bacteria tested, and these cationic small molecules were found to be more active toward Gram-positive bacteria than Gram-negative bacteria. The range of MIC values for the small molecules (**4e–4m**, **4o**, **4q** and **4u**) were 0.5–8 μg/mL against Gram-positive drug-sensitive and drug-resistant bacteria, whereas the range of MIC values was 1–32 μg/mL for Gram-negative bacteria. The MICs of compounds **4e–4m**, **4o**, **4q** and **4u** were comparable to MSI-78 (an AMP currently undergoing phase III clinical trials as a topical antibiotic).[56, 57] The amphiphilic balance of the small molecules was evaluated by ClogP and was found to correlate very well with their antibacterial activity. The ClogP values of these compounds ranged from 0.96 to 9.48 (Table 1). The antibacterial activity was good when the ClogP values ranged between 5 and 8. For example, the span of MIC values for the compounds **4m** and **4o** were 2–32 μg/mL and their ClogP value was 6.62. In contrast, the range of MIC values for molecules **4n** and **4p** were 8–128 μg/mL and even >128 μg/mL, and their ClogP value was 8.73. Additionally, the

configuration of the amino acid showed negligible influence to the biological activity. **4m** and **4o** with L-serine had similar activity compared with that of **4n** and **4p** with D-serine residues, which is consistent with literature.[37]

Table 1. MIC and hemolytic activity (HC_{50}) of small molecules.

	MIC μg/mL							ClogP[e]	HC_{50} (μg/mL)
	Drug-sensitive bacteria				Drug-resistant bacteria[a]				
compd	*S. aureus*	*E. faecalis*	*E. coli*	*S. enterica*	MRSA	KPC	NDM		
4a	>128	>128	>128	>128	-[b]	-[b]	-[b]	0.96	>1000
4b	>128	128	>128	128	-[b]	-[b]	-[b]	2.07	743
4c	128	128	128	128	-[b]	-[b]	-[b]	3.13	576
4d	64	64	64	64	-[b]	-[b]	-[b]	4.19	496
4e	8	8	8	16	4	16	16	5.25	434
4f	2	2	2	4	0.5	1	4	6.31	225
4g	1	1	2	2	0.5	2	2	7.36	133
4h	4	2	2	2	1	2	4	8.42	98
4i	4	2	16	8	4	8	32	9.48	89
4j	1	2	2	8	0.5	4	4	4.89	124
4k	1	2	2	4	2	4	4	5.95	119
4l	2	2	8	4	2	4	4	7.01	87
4m	4	2	8	8	2	32	16	6.62	>1000
4n	8	8	>128	>128	16	-[b]	-[b]	8.73	>1000
4o	4	4	8	8	4	32	16	6.62	367
4p	16	32	>128	>128	32	-[b]	-[b]	8.73	>1000
4q	4	8	4	32	4	16	64	7.57	306
4r	2	2	64	>128	4	-[b]	-[b]	8.02	>1000
4s	4	4	>128	>128	4	-[b]	-[b]	8.02	293
4t	8	128	>128	>128	32	-[b]	-[b]	8.98	>1000
4u	4	8	16	64	8	64	64	6.92	268
VAN[c]	2	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]
MEM[d]	-[b]	-[b]	<0.125	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]
MSI-78	8-16[f]	64[f]	16-32[f]	-[b]	16-32[f]	-[b]	-[b]	-[b]	120[g]

[a]MRSA (methicillin-resistant *S. aureus*), KPC (carbapenemase-producing *K. pneumoniae*), NDM-1 (NDM-1-producing *Enterobacteriaceae*), [b]not determined. [c]VAN (vancomycin), [d]MEM (meropenem), [e]ClogP (calculated with ChemBioDraw software), [f]literature values[57], [g]literature values[58].

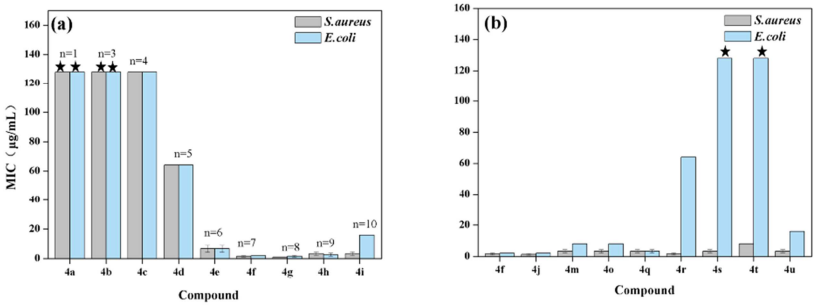


Fig. 2. MICs of cationic small molecules: (a) with varying alkyl chain lengths (**4a–4i**); (b) with different amino acids (**4f**, **4j**, **4m**, **4o** and **4q–4u**). The black star represents MIC >128 μg/mL.

The length of the alkyl chain of compounds **4a–i (**Fig. 2a**)** was extremely important to their antibacterial activity. The MICs were good when the lipophilic chain was C_7H_{15}, C_8H_{17} and C_9H_{19}. This is consistent with reported results.[37, 39] Therefore, the antibacterial activity of these compounds was affected strongly by the length of the alkyl chain.

The kinds of amino acids attached to the compounds had an important influence on antibacterial activity. For example, compounds **(4f**, **4j**, **4m**, **4o**, **4q–4u**; Fig. 2b**)** with the same lipophilic moieties and different hydrophilic amino acids displayed significant differences in their antibacterial activity. Compounds **4r**, **4s** and **4t** showed no activity toward Gram-negative bacteria, but molecule **4r** had good MICs against Gram-positive bacteria (2 μg/mL against *S. aureus*, 2 μg/mL against *E. faecalis*), which was comparable to vancomycin activity (2 μg/mL against *S. aureus*) against Gram-positive bacteria. Compound **4s** also had similar selectivity, and this is the first report on small cationic antimicrobial peptides with an L-leucine residue (**4r**). The antimicrobial activity of compounds **4r**, **4s** and **4t** was reduced when the amino acid residues were L-leucine, L-isoleucine, or L-phenylalanine. The structure-activity relationship studies of this series of dialkylamine cationic small molecules revealed that an amphiphilic structure was critical for broad-spectrum activity, especially against Gram-negative bacteria.

Table 2. MIC of small molecules against drug-resistant MRSA.

compound	MRSA

	M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-8	M-9	M-10
4e	8	16	16	16	8	4	4	8	4	4
4f	1	1	1	1	1	0.5	0.5	1	1	0.5
4g	1	1	1	2	1	1	1	1	1	0.5
4h	1	1	2	2	2	1	1	1	1	1
4i	2	1	2	2	4	4	4	4	4	4
4j	1	1	0.5	1	1	0.5	0.5	1	1	0.5
4k	2	1	1	1	2	2	2	2	2	2
4l	2	1	2	2	2	2	2	2	2	2
4m	4	4	4	4	4	4	2	4	4	2
4n	16	4	4	4	16	8	8	16	16	16
4o	4	4	4	4	4	4	4	4	4	4
4p	32	32	32	32	32	32	32	32	32	32
4q	4	4	4	4	4	4	4	4	4	4
4r	4	2	2	2	4	4	4	4	4	4
4s	4	4	4	4	4	4	4	4	4	4
4t	32	16	16	16	32	32	16	32	32	32
4u	8	4	4	4	8	8	8	8	8	8

The antibacterial activity (Table 2) of selected small molecules against drug-resistant MRSA (10 clinical isolated strains) was further investigated. All selected molecules had good MIC values. Among the 17 compounds in this experiment, **4j** was most effective. In addition, this series of small cationic amphiphiles were extremely active against MRSA with MICs of ~0.5–4 μg/mL. The MIC of **4j** (0.5–1 μg/mL) was comparable to vancomycin (0.9 μg/mL)[39] and better than MSI-78 (16–32 μg/mL)[57] (Table 1).

Table 3. MIC of small molecules against drug-resistant bacteria KPC.

Compound	KPC									
	K-1	K-2	K-3	K-4	K-5	K-6	K-7	K-8	K-9	K-10
4e	16	16	16	16	16	16	16	16	16	16
4f	2	2	2	2	2	2	2	1	2	2
4g	2	2	2	2	2	2	2	2	2	2
4h	4	4	4	4	4	4	4	2	4	4
4i	32	16	16	8	32	32	32	8	32	32
4j	8	4	4	4	8	8	8	4	8	4
4k	4	4	4	2	4	4	4	4	4	4
4l	4	4	4	4	4	4	4	4	4	4
4m	32	32	32	32	32	32	32	32	32	32
4o	32	32	32	32	32	32	32	32	32	32
4q	16	16	16	32	32	32	32	16	16	16
4u	64	64	64	64	64	64	64	64	64	64

The antibacterial activity (Table 3) of small molecules against KPC (10 clinical isolated strains) was tested. The MICs of these compounds ranged from 1–64 μg/mL. The MIC of the most effective compounds **4f–4h** and **4j–4l** were 2–8 μg/mL. Most importantly, compounds **4f–4h** and **4j–4l** were even better than MSI-78 (8–16 μg/mL).

Table 4. MIC of small molecules against drug-resistant bacteria NDM-1.

Compound	NDM-1									
	N-1	N-2	N-3	N-4	N-5	N-6	N-7	N-8	N-9	N-10
4e	32	32	32	32	16	16	32	32	16	16
4f	8	8	8	8	4	4	8	8	8	8
4g	2	2	2	2	2	2	2	2	4	4
4h	4	4	4	4	4	4	4	4	4	4
4i	32	32	32	32	32	32	16	16	32	32
4j	8	8	8	8	4	4	4	4	8	8
4k	4	4	4	4	4	4	2	2	4	4
4l	4	4	4	4	4	4	4	4	8	8
4m	32	32	16	16	16	16	32	32	32	32
4o	32	16	16	16	16	16	32	32	16	16
4q	64	64	64	64	64	64	64	64	64	64
4u	>64	>64	64	64	64	>64	>64	>64	>64	>64

Moreover, the antibacterial activity (Table 4) of small molecules against NDM-1 (10 strains) was investigated. The MICs ranged from 2 to 64 μg/mL and even >64 μg/mL. The MIC of the most effective compounds **4f–4h** and **4j–4l** were 2–8 μg/mL.

From the MIC values (Tables 1–4), compounds **4f–4h** and **4j–4l** not only had potent activity against both drug-sensitive bacteria and drug-resistant bacteria, but also had comparative activity against both Gram-positive and Gram-negative bacteria. Furthermore, the antibacterial activities of compounds **4f–4h** and **4j–4l** were better than the corresponding activity of MSI-78. Compounds **4f–4h** and **4j–4l** showed broad-spectrum antibacterial activity, whereas other molecules had better activities against Gram-positive bacteria than against Gram-negative bacteria. The structures of molecules **4f–4h** and **4j–4l** have two alkyl chains (-C_7H_{15}, -C_8H_{17}, -C_9H_{19}) with an L-lysine or L-arginine residue. The ClogP values of amphiphiles **4f–4h** and **4j–4l** ranged between 5 and 8.4. The optimum amphiphilicity may be an essential element to achieve the maximum antibacterial activity of these amphiphilic small molecules.

Noteworthy, compounds **4r** and **4s** had selective antibacterial activity against Gram-positive bacteria.

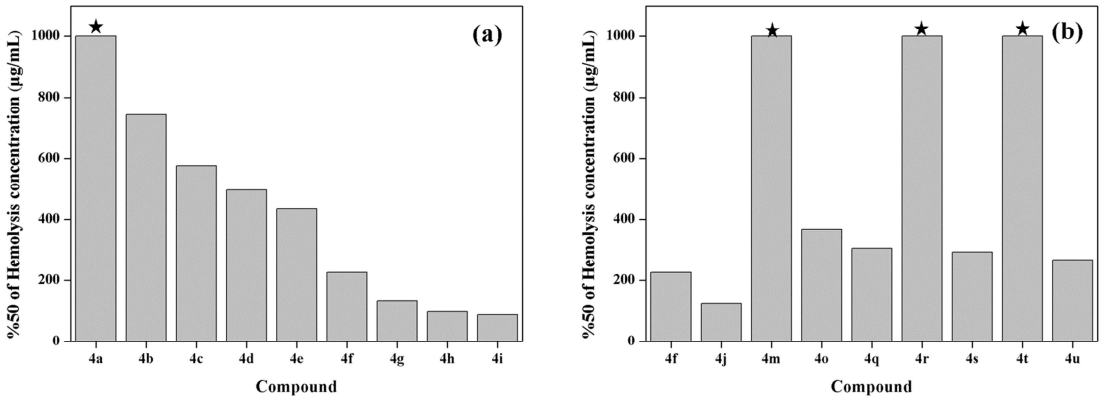


Fig. 3. 50% hemolysis concentration of cationic small molecules: (a) with varying lipophilic alkyl chain (compounds **4a–4i**); (b) with varying amino acids (compounds **4f**, **4j**, **4m**, **4o** and **4q–4u**). The black star represents >1000 μg/mL.

2.3. Hemolytic activity

The hemolytic activity of the cationic small molecules **4a–4u** was represented as their HC_{50} values. The ability of compounds to lyse red blood cells (RBCs) was the toxicity evaluation of compounds toward mammalian cells. HC_{50} values of the small molecules ranged between 87 and >1000 μg/mL (Table 1). In general, HC_{50} values of the cationic small molecules **4a–4i** with different lipophilic alkyl chains were found to increase as the alkyl chain length increased (Fig. 3a). For example, the HC_{50} values of compounds **4a–4i** were >1000, 743, 576, 496, 434, 225, 133, 98 and 89 μg/mL, respectively. To our delight, compounds **4a–4i** showed minimal toxicity toward red blood cells. On the other hand, the hemolytic activity of compounds **4f**, **4j**, **4m**, **4o** and **4q–4u** with different amino acids and the same alkyl chain length were found to vary considerably (Fig. 3b). For example, the HC_{50} values of compounds **4m, 4r** and **4t** were >1000 μg/mL, whereas compounds **4f**, **4j**, **4o**, **4q**, **4r**, **4s** and **4u** ranged between 124 and 367 μg/mL. However, compound **4g**, one of the most potent molecules, had an HC_{50} value of 133 μg/mL, giving good selectivity ($S = HC_{50}/MIC$) against *S. aureus* (133) and *E. coli* (66.5) respectively (Table 1). Compound **4r** had excellent selective antibacterial activity (MIC = 2 μg/mL against both *S. aureus* and *E.*

coli) against Gram-positive bacteria and low hemolytic activity (HC_{50} >1000 μg/mL). The hemolytic toxicity study revealed that these compounds had minimal toxicity toward mammalian red blood cells and the above results indicate that the activity of these compounds was selective toward bacteria.

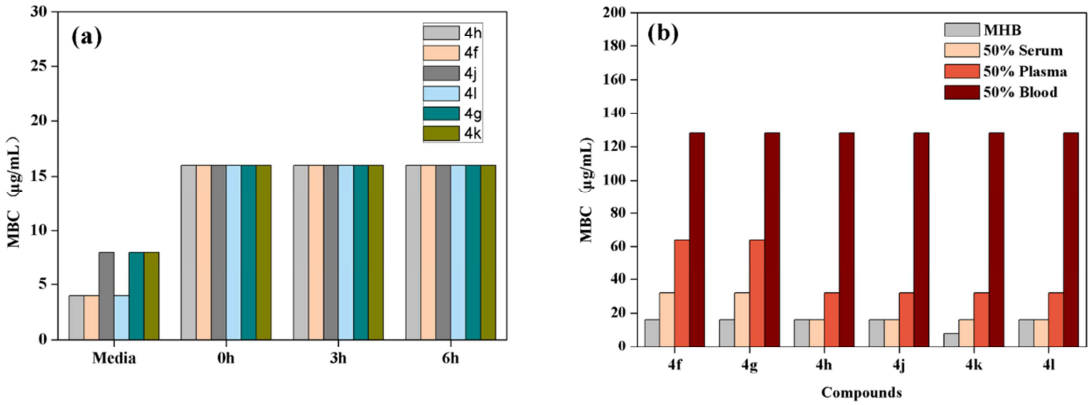


Fig. 4. Plasma stability and antibacterial activity in complex mammalian fluids of small molecules: (a) antibacterial efficacy of compounds **4f**, **4g**, **4h**, **4j**, **4k** and **4l** against *S. aureus* after preincubating in 50% plasma for different periods of time (0, 3 and 6 h); (b) minimum bactericidal concentrations (MBCs) of **4f**, **4g**, **4h**, **4j**, **4k** and **4l** in 50% serum, 50% plasma and 50% blood against MRSA.

2.4. Plasma stability

Protease degradation is a major disadvantage of natural antibacterial peptides, which results in reduced antibacterial activity of these peptides in mammalian fluids.[59] To determine the stability of the dialkylamine cationic amphiphilic molecules under plasma conditions, antibacterial efficacy of compounds **4f**, **4g**, **4h**, **4j**, **4k** and **4l** against *S. aureus* after preincubating in 50% plasma for different periods of time (0, 3 and 6 h) was evaluated. The MBC values of compounds **4f**, **4g**, **4h**, **4j**, **4k** and **4l** increased from 4 or 8 μg/mL (100% media) to 16 μg/mL (50% plasma) after 0, 3 and 6 h treatment (Fig. 4a). The above results indicate that compounds **4f**, **4g**, **4h**, **4j**, **4k** and **4l** lost only a small amount of antibacterial efficacy upon pretreatment in plasma.

2.5. Antibacterial activity in complex mammalian fluids

The loss of antibacterial activity of the compounds in the presence of complex

mammalian fluids was another serious concern. Antibacterial activity of compounds **4f**, **4g**, **4h**, **4j**, **4k** and **4l** was investigated by determining the minimum bactericidal concentration (MBC) in 50% serum, 50% plasma and 50% blood supplemented with 50% MHB against MRSA. The compounds **4f**, **4g**, **4h**, **4j**, **4k** and **4l** were found to be active in 50% serum and 50% plasma, but were not active in 50% blood. The MBC values of compounds **4f**, **4g**, **4h**, **4j**, **4k** and **4l** increased 1-fold or 2-fold in 50% serum and 2-fold or 4-fold in 50% plasma, whereas the MBC values increased 8-fold or 16-fold in 50% blood (Fig. 4b). The 2–4-fold increase of MBC values could be due to negatively charged proteins and macromolecules in human serum, plasma, or blood that tightly bind to the cationic molecules, thereby inhibiting their activity toward bacterial membranes. The above results indicate that compounds **4f**, **4g**, **4h**, **4j**, **4k** and **4l** were active in complex mammalian fluids like serum and plasma, but these compounds were not able to retain their antibacterial activity in blood.

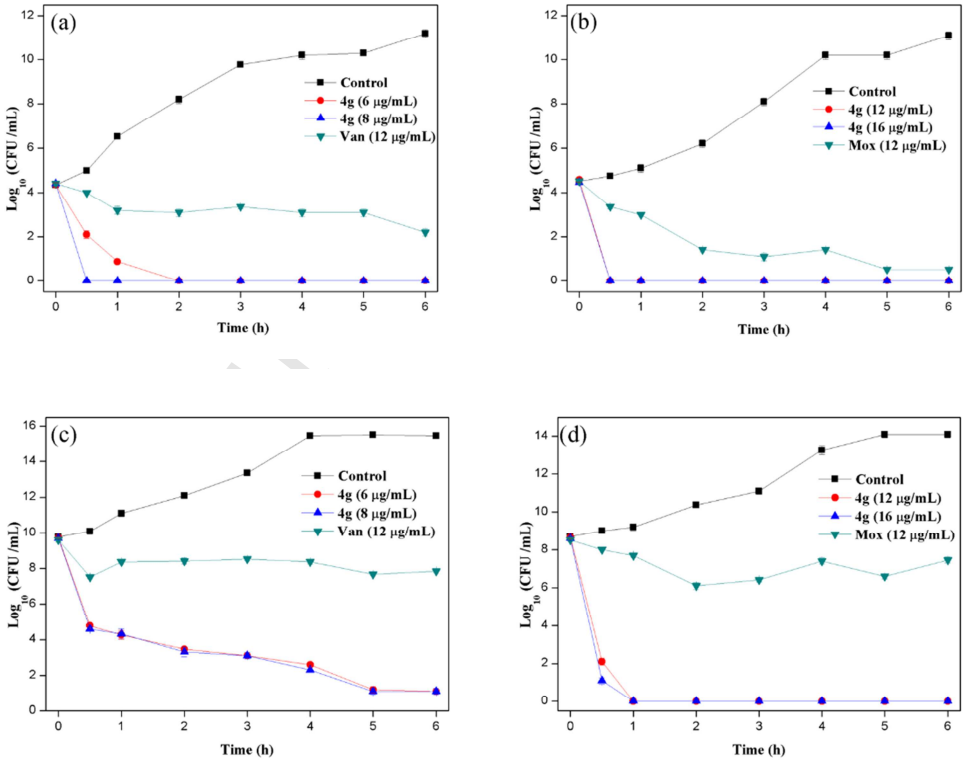


Fig. 5. Time-dependent killing of pathogens by compound **4g**. (a), (c) *S. aureus* were grown to early (a) and late (c) exponential phase, and challenged with compound **4g** (at 6× MIC and 8× MIC) and Van (vancomycin); (b), (d) *E. coli* were grown to early

(b) and late (d) exponential phase, and challenged with compound **4g** (at 6× MIC and 8× MIC) and Mox (moxalactam). The control was treatment with sterile water. Data are representative of three independent experiments.

2.6. Bactericidal time-kill kinetics

The time-kill kinetics of compound **4g** was performed to determine the rate of bactericidal action. This investigation was carried out against *S. aureus* and *E. coli*. *S. aureus* and *E. coli* were grown to early and late exponential phase and challenged with compound **4g** (at 6× MIC and 8× MIC), vancomycin (*S. aureus*) and moxalactam (*E. coli*). Compound **4g** had excellent bactericidal activity against *S. aureus*, showing superior activity to vancomycin in killing early and late exponential phase populations (Fig. 5a, c). Moreover, **4g** killed early exponential phase *S. aureus* at 0.5 h at 8× MIC and 120 min at 6× MIC, and **4g** killed late exponential phase *S. aureus* at 6 h at 8× MIC and 6× MIC, while vancomycin did not kill *S. aureus* at 6 h. Compound **4g** displayed rapid bactericidal activity against *E. coli*, and was superior to moxalactam in killing early and late exponential phase bacteria (Fig. 5b, d). **4g** killed early and late exponential phase *E. coli* at 0.5 h and at 1 h, respectively, while moxalactam did not kill early or late exponential phase *E. coli* at 6 h. Clearly, compound **4g** had excellent antibacterial activity against *S. aureus* and *E. coli* (Fig. 6), and after 6 hours treatment the control group samples were cloudy, but the groups of **4g** (at 6× MIC and 8× MIC) were both clear. Thus, the results suggest that the dialkyl cationic amphiphile **4g** killed both Gram-positive and Gram-negative bacteria rapidly.

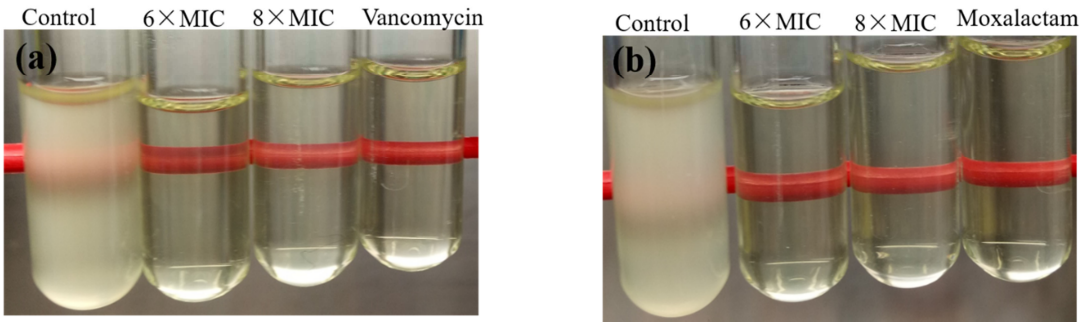


Fig. 6. Time-kill kinetics of compound **4g**. (a), *S. aureus* were grown to early exponential phase and challenged with compound **4g** (at 6× MIC and 8× MIC) and vancomycin; (b), *E. coli* were grown to early exponential phase and challenged with

compound **4g** (at 6× MIC and 8× MIC) and moxalactam. The control was sterile water. Pictures taken after treatment with compounds for 6 h.

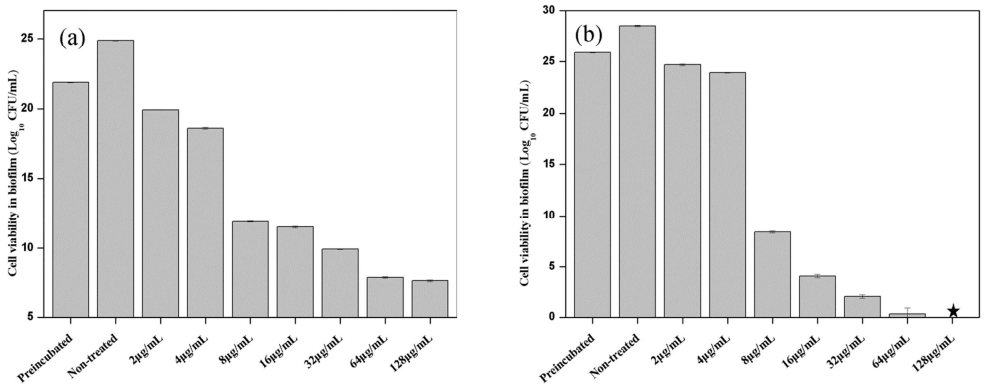


Fig. 7. Antibiofilm activity of small molecule **4g**. (a) Cell viability in non-treated and treated biofilms of *S. aureus* grown on cover slips for 24 h and after treating the biofilms with **4g** at different concentrations. (b) Cell viability in non-treated and treated biofilms of *E. coli* grown on cover slips for 72 h and after treating the biofilms with **4g** at different concentrations. The black star represents <50 CFU/mL.

2.7. Biofilm disruption activity

To evaluate the efficiency of this class of compounds to eradicate preformed biofilms, one of the best compound **4g** was used against established *S. aureus* and *E. coli* biofilms. Mature *S. aureus* biofilms in a 96-well plate (grown for 24 h) with an initial count of 21.9 $\log_{10}$ CFU/mL per well of bacteria were treated with **4g** at seven different concentrations. **4g** was found to reduce cell viabilities in the biofilms (19.9, 18.6, 11.9, 11.5, 9.9, 7.9 and 7.6 $\log_{10}$ CFU per well at 2, 4, 8, 16, 32, 64 and 128 μg/mL, respectively), whereas the cell viability in the non-treated biofilm increased to 24.9 $\log_{10}$ CFU per well (Fig. 7a). **4g** was also able to reduce the cell viabilities in mature *E. coli* biofilms (developed for 72 h) from an initial count of 25.9 $\log_{10}$ CFU per well to 24.7, 23.9, 8.4, 4.1, 2.1 and 0.3 $\log_{10}$ CFU per well at 2, 4, 8, 16, 32, 64 and 128 μg/mL, respectively, whereas the cell viability in the non-treated biofilm increased to 28.5 $\log_{10}$ CFU per well (Fig. 7b). Biofilm disruption activity of

compound **4g** was visually shown by crystal violet staining (Fig. 8a, b). Moreover, the $MBIC_{80}$ values of **4g** were 18.0 μg/mL against *S. aureus* and 53.9 μg/mL against *E. coli* (Table 5). And the MBEC values of **4g** were 64 μg/mL and 128μg/mL against *S. aureus* and *E. coli*, respectively. That were 64-fold of MIC values. The above results and images indicate that compound **4g** can inhibit bacterial biofilm formation and eradicate established biofilms at 64 and 128 μg/mL.

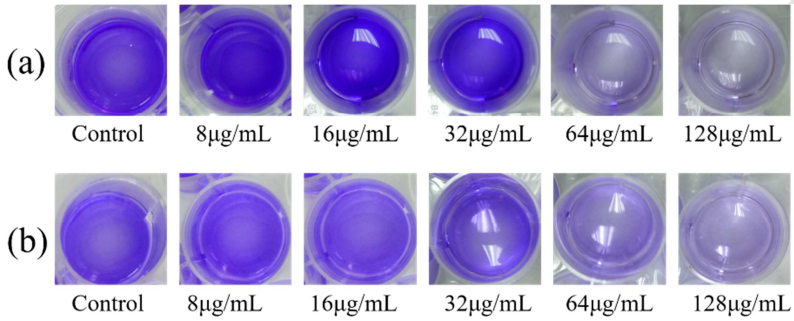


Fig. 8. Antibiofilm activity of small molecule **4g**. (a) Images of the small molecule treated and non-treated biofilms of *S. aureus* after staining with crystal violet. (b) Images of the small molecule treated and non-treated biofilms of *E. coli* after staining with crystal violet.

Table 5. MBIC and MBEC of compound **4g**.

Compound	$MBIC_{80}$ / $MBIC_{90}$ (μg/mL)		MBEC (μg/mL)	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
4g	18.0/26.9	53.9/73.6	64	128

2.8. Mechanism of action

The detailed molecular mechanism of action was investigated to confirm whether these dialkyl cationic amphiphilic small molecules acted by disrupting the integrity of the bacterial cell membrane. Gram-positive *S. aureus* and Gram-negative *E. coli* were used with all compounds to assess the structure-activity relationship of this series of compounds. The mechanism of action can be confirmed by the following three experiments (membrane depolarization, and inner membrane and outer membrane permeabilization, Fig. 9).

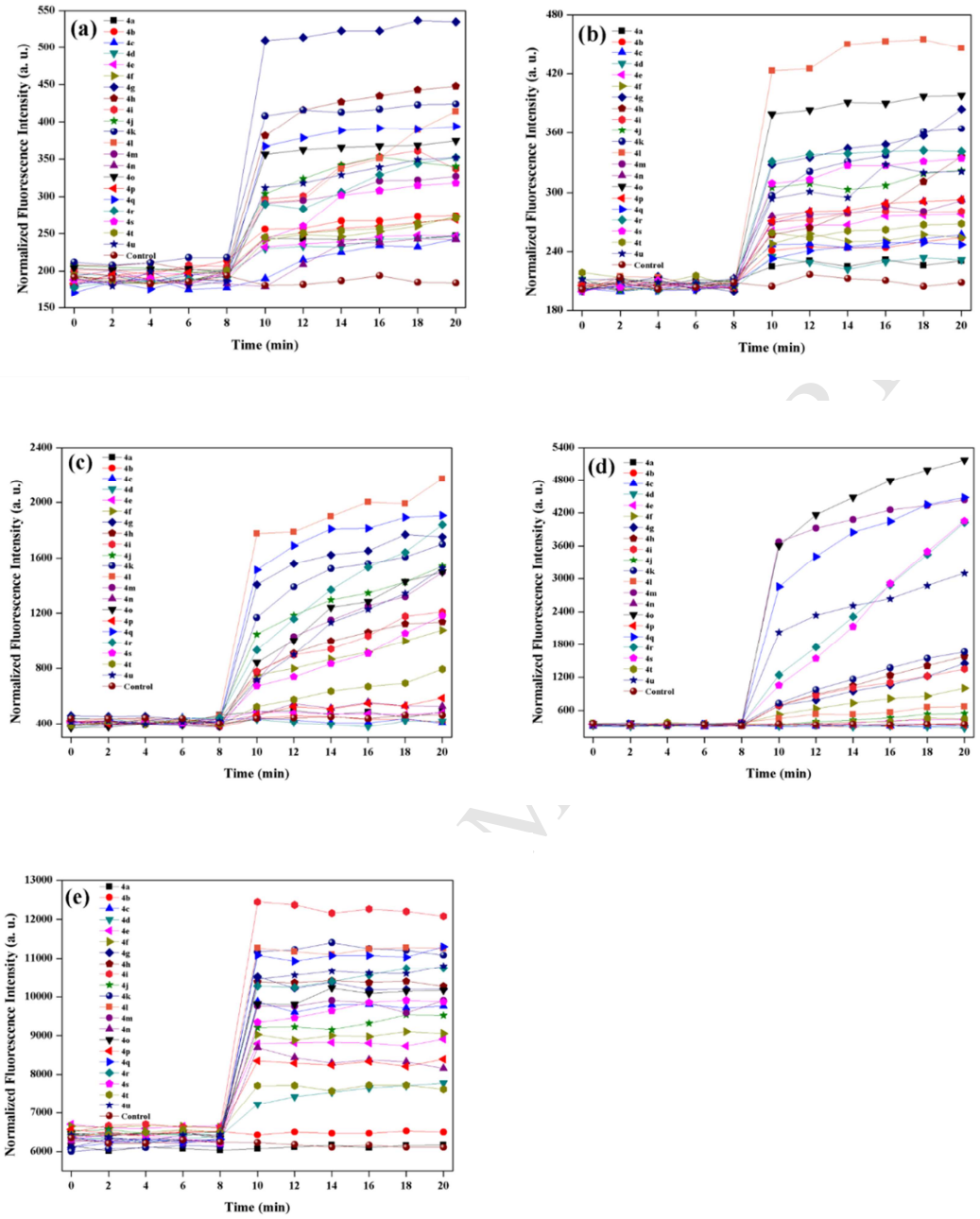


Fig. 9. Mechanism of antibacterial action of the cationic small molecules **(4a–4u)**. Cytoplasmic membrane depolarization of *S. aureus* (a) and *E. coli* (b). Inner membrane permeabilization of *S. aureus* (c) and *E. coli* (d). (e) Outer membrane permeabilization of *E. coli* in the presence of amphiphilic small molecules at 10 μg/mL.

2.8.1. Cytoplasmic membrane depolarization

The dialkyl amphiphilic small molecules were found to dissipate the membrane potential of both Gram-positive and Gram-negative bacteria, as monitored by the

membrane-potential fluorescence sensitive dye diSC35. Furthermore, different compounds showed different levels of dissipation of the membrane potential. Compounds **4g**, **4k**, **4l**, **4h** and **4o** (final concentration was 20 μg/mL) showed maximum membrane depolarization, whereas compounds **4a–4e**, **4n** and **4p** exhibited less membrane depolarization against both *S. aureus* and *E. coli* (Fig. 9a, b). The results further indicate that the ability of the compounds **4a–4u** to dissipate the membrane potential are well correlated with their MIC values.

2.8.2. Inner membrane permeabilization

Bacterial cytoplasmic membrane permeabilization was studied using the fluorescent probe propidium iodide (PI). PI enters bacteria only through compromised membranes and fluoresces upon binding to cellular DNA. After treatment with the small molecules **4a–4u** (final concentration was 20 μg/mL), an enhancement in the fluorescence intensity was observed in *S. aureus* and *E. coli* (Fig. 9c, d). Thus, the results show that compounds **4a–4u** permeabilize the membrane of both Gram-positive and Gram-negative bacteria.

2.8.3. Outer membrane permeabilization

Outer membrane permeabilization was studied using the hydrophobic dye N-phenyl naphthylamine (NPN). NPN was generally excluded from the outer membrane of Gram-negative bacteria. When the outer membrane was damaged, NPN accessed the impaired outer membrane, exhibiting an increase in fluorescence intensity. After treatment with the small molecules **4a–4u** (final concentration was 20 μg/mL), an enhancement in the fluorescence intensity was observed in *E. coli*; compounds were able to permeabilize the outer membrane (Fig. 9e). The above results indicate that compounds **4a–4u** can permeabilize the membrane of Gram-negative bacteria.

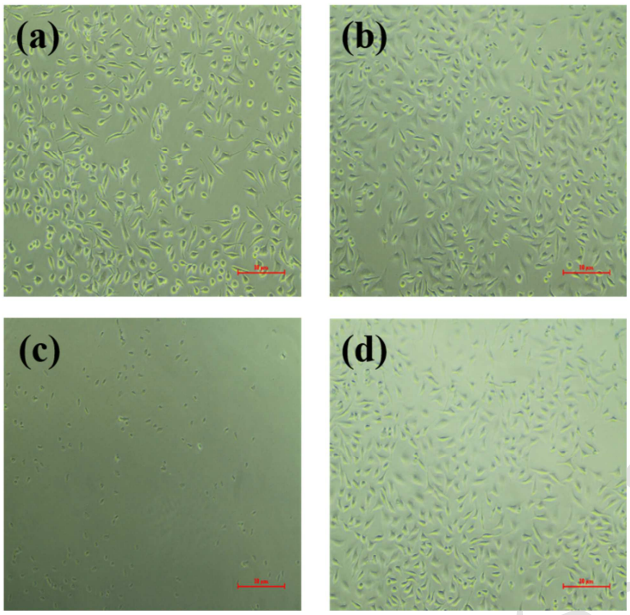


Fig. 10. Electron scanning microscopy images of HeLa cells following treatment with small molecules **4g** and **4k** for 24 h. (a) Cells treated with **4g** (1× MIC); (b) cells treated with **4k** (2× MIC); and (c) cells treated with 0.1% Triton-X (positive control) and (d) non-treated cells (negative control). Scale bar is 10 μm.

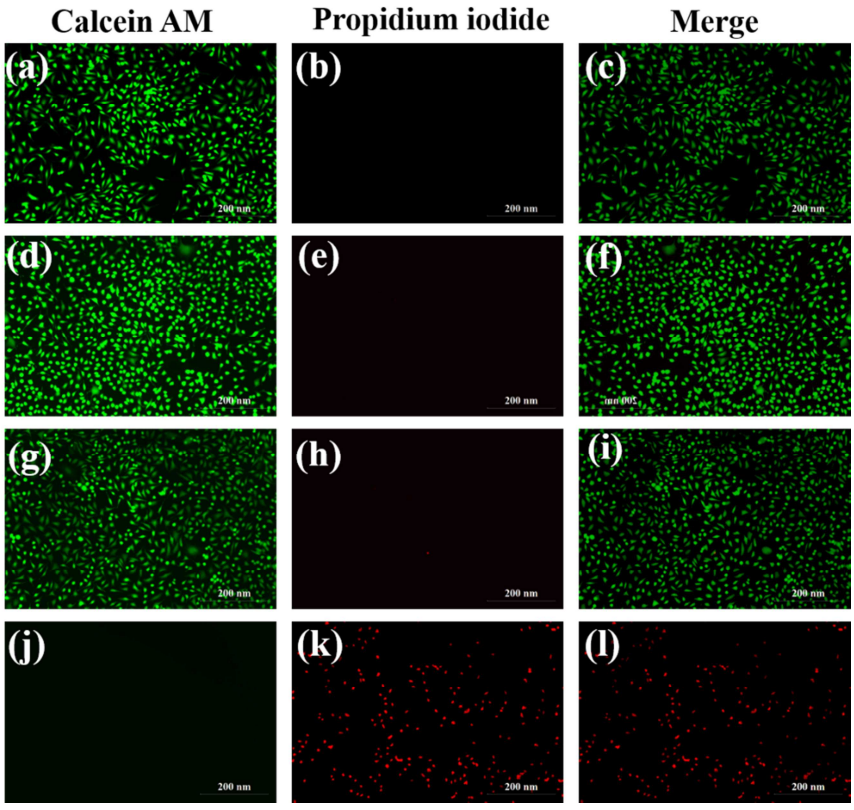


Fig. 11. Fluorescence microscopy images of HeLa cells after treatment with small molecules **4g** and **4k** for 24 h and staining with calcein AM and propidium iodide (PI). (a-c) Non-treated cells (negative control); (d-f) cells treated with **4g** (1× MIC); (g-i) cells treated with **4k** (2× MIC); and (j-l) cells treated with 0.1% Triton-X (positive control). Scale bar is 200 nm.

2.9. Fluorescence and electron scanning microscopy

Cells were seeded into the wells of a 12-well plate and then treated with compounds (**4g** and **4k**) at various concentrations (1, 2 and 4 μg/mL). The treated cell lines were imaged by optical microscopy to visualize the morphology (Fig. 10). The treated cells were found to have normal morphology at 1× MIC and 2× MIC, (Fig. 10a, b), and they were similar to the untreated cell lines (Fig. 10d). In contrast, cells treated with Triton-X were found to have a completely damaged shape (Fig. 10c). Fluorescent microscopy studies using the live/dead staining method showed that cells treated with compounds **4g** and **4k** showed green fluorescence even at 1× MIC, 2× MIC (Fig. 11d-f, g-i) and were similar to the untreated cells (Fig. 11a-c). In contrast, cells treated with Triton-X were found to have completely red fluorescence (Fig. 11j-l). The above results indicate that these small molecules were indeed nontoxic toward mammalian cells at their MIC values.

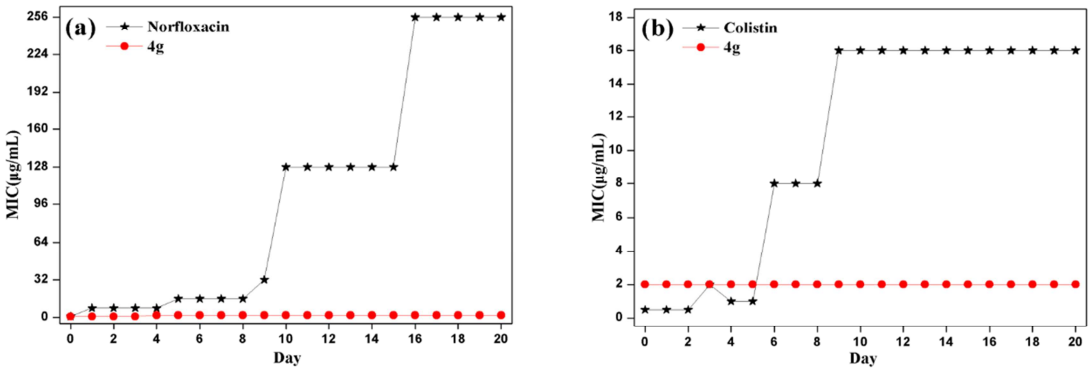


Fig. 12. Propensity of development of bacterial resistance against compound **4g**. (a) For *S. aureus* where antibiotic norfloxacin was used as the control; (b) for *E. coli* where lipopeptide colistin was used as the control.

2.10. Propensity to induce bacterial resistance

It is important to evaluate the potential emergence of bacterial resistance against these small molecules. We took one of the most active compounds (i.e., **4g**) as a representative to evaluate the ability of these compounds to suppress the development of resistance against both Gram-positive *S. aureus* and Gram-negative *E. coli*. Two control antibiotics norfloxacin and colistin were chosen for *S. aureus* and for *E. coli*, respectively. In the case of norfloxacin and colistin, the initial MIC values were determined against respective bacteria. After the initial MIC experiment, serial passaging was initiated by transferring the growing bacterial suspension at sub-MIC of the compound/antibiotics (at MIC/2), and was subjected to another MIC assay and the process was repeated for 20 passages. The cationic compound **4g** showed no change in the MIC against both *S. aureus* and *E. coli* even after 20 passages, whereas 256-fold and 32-fold increases in MIC were observed for norfloxacin and colistin, respectively (Fig. 12a, b). The above results indicate that these small molecules are difficult to induce bacterial resistance and they represent potential compounds to combat drug-resistant bacteria. In contrast, resistance to the two positive control antibiotics norfloxacin and colistin by *S. aureus* and *E. coli*, respectively, was easily developed.

3. Conclusions

Twenty-one small molecules using different secondary amines with the same alkyl chain and nine different amino acids were synthesized. These compounds displayed potent broad-spectrum and membrane-active antibacterial activity against various drug-sensitive and drug-resistant bacteria (both Gram-positive and Gram-negative). Variation of the amino acids and amphiphilic nature of the dialkyl cationic amphiphiles strongly affected their antibacterial activity as well as hemolytic activity. The structure-activity relationship studies of this series of dialkyl cationic small molecules revealed that an amphiphilic structure was critical for broad-spectrum activity, especially against Gram-negative bacteria. Additionally, the small molecules were found to be stable under plasma conditions and showed excellent activity in various complex mammalian fluids such as serum and plasma, but these compounds were not able to retain antibacterial activity when incubated in blood. Moreover, these

small molecules acted on bacteria rapidly and did not allow bacteria to develop resistance. Importantly, various spectroscopic and microscopic studies revealed that depolarization and disruption of the bacterial cell membrane are the primary mechanisms describing the bactericidal action of the compounds. Furthermore, the optimized molecule was found to be nontoxic toward mammalian red blood cells and showed negligible toxicity against mammalian cells. Therefore, these molecules hold great promise to combat drug-resistance pathogens and have the ability not only to inhibit bacterial biofilm formation but also to eradicate established biofilms. Thus, these compounds can be potentially used to tackle infections caused by various bacteria.

4. Experimental section

General: Reagents and solvents were purchased from commercial sources and were used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 MHz and 101 MHz spectrometer respectively, and TMS as internal standard reference. Coupling constants (J) are given in hertz (Hz). High resolution mass spectra (HRMS) were recorded on a Waters Micromass Q-T of Micromass spectrometer. The compounds were purified by reverse phase HPLC (Waters Symmetry C-18 4.5×250mm, 5μm) using sodium dihydrogen phosphate buffer (PH=3 or 7.5) /acetonitrile (0−100%) as mobile phase to more than 95% purity. Analytical thin layer chromatography (TLC) was performed on glass plates pre-coated with silica gel (5–40um, Qingdao Marine Chemical Factory (China)) to monitor the reactions. Visualization was accomplished using UV light and 5% phosphomolybdic acid ethanol solution. Column chromatography was performed on silica gel. For optical density and fluorescence measurements, Tecan Infinite Pro series M200 Microplate Reader was used. The biological experiments were performed with a 1300 series A_2 Biological Safety Cabinet. TDL-5M Desktop Low-speed Refrigerated Centrifuge was used in antibacterial studies. All the solvents were of reagent grade. Dimethylformamide, Dichloromethane, 1,2-Dichloroethane, Tetrahydrofuran, Diethyl ether, Chloroform, Methanol, NaOH, K_2CO_3, and other solvents were

supplied by chemical reagent company (China). Di-*tert*-butylcarbonate, Diisopropylethylamine, HBTU, L-lysine, L-arginine, L-serine, D-serine, L-alanine, L-leucine, L-isoleucine, L-phenylalanine and L-threonine were purchased from Aladdin (China). N-n-heptylamine, n-nonylamine, n-undecylamine, Di-n-ethylamine, di-n-butylamine, di-mylamine, di-n-hexylamine, di-n-octylamine and di-n-decylamine were purchased from Chembee reagent company (China). All the chemicals were used as supplied. Drug-sensitive bacterial strains, *E.coli* (ATCC 25922), *S.aureus* (ATCC 29213), *E.faecalis* (ATCC 29212) and *S.enterica* (ATCC 8387). Drug-resistant bacteria, Methicillin-resistant *S. aureus* (MRSA), Carbapenemase-producing *K. pneumonia* (KPC) and NDM-1-producing Enterobacteriaceae (NDM-1) were clinical isolated strains. Sheep RBCs were used for hemolytic assay.

General procedure for the synthesis of N, N-di-n-alkylamines (1e, 1g, 1i): Compounds **(1a-1d, 1f, 1h)** were from commercial resources. The general synthesis of other di-n-alkylamines **(1e, 1g, 1i)** are accomplished by conventional chemical synthesis procedure as described below. Mixture of n-ethyl-amine (1 g, 1equiv), n-ethyl bromide (1equiv) and anhydrous potassium carbonate (1equiv) in 7.5 mL dimethyl sulfoxide was kept under stirring for 12 h at 80 ºC.[55] And the reaction was monitored by TLC, R_f = 0.2~0.3 (PE: EA: TEA = 5: 1: 0.05, v/v/v) and the visualization was accomplished using UV light and 5% phosphomolybdic acid ethanol solution. PE represents petroleum ether, EA represents ethyl acetate and TEA is triethylamine. The reaction mixture was taken in 25 mL chloroform, washed with water (3 × 25 mL), dried over anhydrous magnesium sulfate and filtered. Chloroform was removed from the filtrate on a rotary evaporator and the resulting residue was purified by Silica gel column chromatography (PE: EA: TEA = 10: 1: 0.05, v/v/v), then afforded the intermediate secondary amine.

N, N-di-n-heptylamine (1e): Colourless liquid, yield: 36.4%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.62 – 2.54 (m, 4H), 1.55 – 1.41 (m, 4H), 1.29 (d, *J* = 3.3 Hz, 16H), 0.88 (t, *J* = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 50.18, 31.83, 30.23, 29.27, 27.40, 22.62, 14.07.

N, N-di-n-nonylamine (1g): Light yellow liquid, yield: 32.6%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.61 – 2.54 (m, 4H), 2.21 (s, 1H), 1.47 (dd, *J* = 13.7, 6.8 Hz, 4H), 1.28 (d, *J* = 6.0 Hz, 24H), 0.88 (t, *J* = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 50.11, 31.86, 30.16, 29.57, 29.55, 29.26, 27.40, 22.63, 14.02.

N, N-di-n-undecylamine (1i): White solid, yield: 32.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.61 – 2.56 (m, 4H), 1.48 (dd, *J* = 13.9, 7.0 Hz, 4H), 1.27 (d, *J* = 8.2 Hz, 32H), 0.88 (t, *J* = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 50.11, 31.92, 30.10, 29.63, 29.62, 29.60, 29.35, 27.43, 22.69, 14.11.

General procedure for the synthesis of intermediate molecules (2a, 2e-2i): L-lysine, L-alanine, L-leucine, L-isoleucine, L-phenylalanine, L-threonine (5 g, 1 equiv) was dissolved in H_2O (100 mL), and to it NaOH (3 equiv) was added and stirred. To this, di-*tert*-butyl dicarbonate (Boc_2O) (2.4 equiv) in 50 mL of tetrahydrofuran (THF) was added at 0 °C. [39] Then the reaction mixture was stirred at room temperature for 24 h. At the end of the reaction, THF was removed under reduced pressure and the aqueous layer was washed with diethyl ether to remove organic impurities. Then the aqueous layer was acidified to pH 4~5 using 1M H_2SO_4 aqueous solution. The aqueous layer was then extracted with dichloromethane (DCM). The organic layer was then washed with brine and dried over anhydrous Na_2SO_4. The organic layer was removed under reduced pressure to obtain the compound.

Boc-Lys(Boc)-OH (2a): Light yellow solid, yield: 64.3%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.30 (s, 1H), 4.73 (s, 1H), 4.21 (d, *J* = 73.0 Hz, 1H), 3.12 (d, *J* = 5.1 Hz, 2H), 1.78 (d, *J* = 51.3 Hz, 2H), 1.55 – 1.37 (m, 22H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.90, 125.01, 80.08, 79.36, 53.32, 40.08, 32.02, 29.56, 28.43, 28.36, 22.42.

N-Boc-L-alanine (2e): White solid, yield: 89.4%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.29 (s, 1H), 4.26 (d, *J* = 72.0 Hz, 1H), 1.44 (d, *J* = 8.1 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.91, 155.46, 80.26, 49.13, 28.31, 18.42.

N-Boc-L-leucine (2f): Colourless viscous oily liquid, yield: 75%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (s, 1H), 4.23 (d, *J* = 72.5 Hz, 1H), 1.81 – 1.53 (m, 3H), 1.45 (s, 9H),

1.00 (t, *J* = 30.9 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.75, 80.13, 52.08, 41.45, 28.29, 24.78, 22.83, 21.76.

N-Boc-L-isoleucine (2g): Colourless viscous oily liquid, yield: 78.8%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (s, 1H), 4.37 – 3.99 (m, 1H), 1.91 (s, 1H), 1.44 (d, *J* = 8.6 Hz, 10H), 1.23 (ddd, *J* = 21.1, 13.5, 4.8 Hz, 1H), 1.02 – 0.90 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.75, 155.79, 80.06, 57.87, 37.75, 28.30, 24.88, 15.49, 11.60.

N-Boc-L-phenylalanine (2h): Colourless viscous oily liquid, yield: 72.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 – 7.16 (m, 5H), 6.95 (s, 1H), 4.59 (d, *J* = 5.6 Hz, 1H), 3.24 – 3.01 (m, 2H), 1.49 – 1.25 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.85, 155.43, 136.03, 129.44, 128.56, 127.02, 80.21, 54.37, 37.86 28.30.

N-Boc-L-threonine (2i): Colourless viscous oily liquid, yield: 72.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.63 (d, *J* = 8.2 Hz, 1H), 4.70 (s, 3H), 4.40 (s, 1H), 4.27 (d, *J* = 7.5 Hz, 1H), 1.53 – 1.38 (m, 9H), 1.27 (dd, *J* = 11.5, 5.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.31, 156.66, 80.54, 67.82, 58.64, 28.30, 19.29.

General procedure for the synthesis of intermediate molecules (2b): L-arginine (8.7 g, 50 mmol) was added into a solution of tert-butanol (150 mL) and water (150mL) in a 500 mL round-bottom flask. The mixture was cooled to 0 °C in an ice bath and NaOH (7.0 g, 175 mmol) was added. The solution was stirred for 5 min at 0 °C and to it was added di-*tert*-butyl dicarbonate (43.7 g, 200 mmol) in portions.[53] The reaction mixture was stirred for 48 h at room temperature and monitored by TLC (CH_2Cl_2/MeOH/CH_3COOH, 40:1:0.2). The organic solvent (150 mL) was evaporated under reduced pressure and the residue was extracted with ethyl ether. The extracted solution was divided into three layers in the separating funnel, the middle layer was collected and carefully acidified with citric acid to pH 3~4 and was extracted with ethyl acetate (3 × 60 mL). The extract was dried with anhydrous sodium sulfate and evaporated in a vacuum. The white solid was obtained after dried in a vacuum oven.

Boc-Arg(Boc)$_2$-OH (2b): White solid, yield: 61.1%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.71 (s, 1H), 4.33 (s, 1H), 3.88 (s, 2H), 3.42 (s, 1H), 1.73 (dd, *J* = 37.7, 31.2 Hz, 5H), 1.52 (d, *J* = 5.0 Hz, 9H), 1.49 (s, 9H), 1.44 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.29, 156.20, 154.75, 153.17, 84.32, 83.19, 79.71, 79.45, 77.39, 77.07, 76.75, 60.43, 44.35, 40.51, 29.02, 28.36, 28.18, 28.02, 27.96, 27.89, 24.67, 21.02, 14.16.

General procedure for the synthesis of intermediate molecules (2c, 2d): To a stirred solution of L-serine and D-serine (5.2 g, 50.0 mmol) in 1M NaOH aqueous solution (50 mL) and 1, 4-dioxane (100 mL), di-tert-butyl dicarbonate (13.1 g, 60.0 mmol) was slowly added at 0 °C. The mixture was warmed to room temperature and stirred for 24h. After evaporation of 1, 4-dioxane, the aqueous layer was wash with Et_2O (50 mL). The aqueous layer was acidified with 1M H_2SO_4 aqueous solution to give pH 2-3 and extracted with ethyl acetate (3×50 mL). The combined organic layer was dried over anhydrous Na_2SO_4, filtrated, and concentrated under reduced pressure to give the product N-Boc-L-serine.

N-Boc-L-serine (2c): White solid, yield: 53.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.63 (d, *J* = 46.7 Hz, 2H), 4.30 (d, *J* = 60.7 Hz, 1H), 4.16 – 3.80 (m, 2H), 1.36 (d, *J* = 77.2 Hz, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.93, 156.30, 80.67, 62.99, 55.50, 28.30.

N-Boc-D-serine (2d): White solid, yield: 53.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.04 (s, 2H), 4.36 (s, 1H), 4.05 (s, 1H), 3.91 – 3.81 (m, 1H), 1.45 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.90, 156.28, 80.68, 62.99, 55.47, 28.30.

General procedure for the synthesis of amide coupling compounds (3a-3u): To a stirred solution of N, N-di-n-alkylamines **(1a-1i)** (200mg, 1 equiv) in 5:2 DMF/$CHCl_3$ (8.4 mL) was added N, N-di-isopropyl-ethylamine (DIPEA, 3 equiv) at 0 °C. To this solution was then added HBTU (1.25 equiv). This mixture was stirred for 5 min at 0 °C, and subsequently the Boc-protected amino acids **(2a-2i)** (1.25 equiv) were added to it. The mixture was stirred at 0 °C for 30 min and subsequently at RT for 24 h typically[39]. At the end, $CHCl_3$ was evaporated under reduced pressure and the resulting solution was diluted to 2 times its original volume by addition of ethyl

acetate. This mixture was subsequently washed with 0.5 M $KHSO_4$, H_2O (three times), and brine. The combined organic layer was dried over anhydrous Na_2SO_4, filtrated, and concentrated under reduced pressure and the residue was purified using column chromatography (petroleum ether: ethyl acetate= 5:1 v/v and with 5%triethylamine) to obtain the product in 40%-80% yield. The purified compound was subsequently characterized using ^{1}H NMR and ^{13}C NMR.

Di-tert-butyl (6-(diethylamino)-6-oxohexane-1,5-diyl)(S)-dicarbamate (3a): Colourless oily liquid, yield: 51.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40 (d, *J* = 8.7 Hz, 1H), 4.66 (s, 1H), 4.53 (d, *J* = 4.6 Hz, 1H), 3.52 (dd, *J* = 13.6, 7.0 Hz, 1H), 3.43 – 3.31 (m, 2H), 3.23 (dd, *J* = 13.6, 7.0 Hz, 1H), 3.10 (dd, *J* = 12.8, 6.5 Hz, 2H), 1.70 – 1.48 (m, 4H), 1.43 (s, 18H), 1.41 – 1.35 (m, 2H), 1.23 (t, *J* = 7.1 Hz, 3H), 1.12 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.56, 156.05, 155.62, 79.52, 49.85, 41.89, 40.35, 33.68, 28.44, 28.39, 22.52, 14.63, 12.94.

Di-tert-butyl (6-(dibutylamino)-6-oxohexane-1,5-diyl)(S)-dicarbamate (3b): Yellow oily liquid, yield: 72.0%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (d, *J* = 8.3 Hz, 1H), 4.67 (s, 1H), 4.53 (d, *J* = 4.3 Hz, 1H), 3.54 – 3.45 (m, 1H), 3.26 (dd, *J* = 15.1, 8.2 Hz, 2H), 3.17 – 3.03 (m, 3H), 1.63 (dd, *J* = 11.5, 7.2 Hz, 2H), 1.59 – 1.47 (m, 6H), 1.43 (s, 18H), 1.40 – 1.27 (m, 6H), 0.94 (dt, *J* = 12.6, 7.4 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.90, 156.03, 155.57, 79.44, 60.39, 49.84, 47.54, 45.76, 33.80, 31.37, 29.72, 28.44, 28.38, 22.56, 20.18, 20.07, 13.85, 13.82.

Di-tert-butyl (6-(dipentylamino)-6-oxohexane-1,5-diyl)(S)-dicarbamate (3c): Yellow oily liquid, yield: 72.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (d, *J* = 7.4 Hz, 1H), 4.66 (s, 1H), 4.53 (d, *J* = 4.5 Hz, 1H), 3.48 (d, *J* = 7.7 Hz, 1H), 3.25 (dd, *J* = 15.3, 8.2 Hz, 2H), 3.17 – 3.04 (m, 3H), 1.62 (dd, *J* = 13.5, 9.2 Hz, 2H), 1.58 – 1.46 (m, 6H), 1.44 (s, 18H), 1.31 (ddd, *J* = 24.0, 14.3, 7.0 Hz, 10H), 0.90 (dd, *J* = 14.7, 7.3 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.90, 156.03, 155.55, 79.44, 49.83, 48.31, 47.75, 45.98, 40.41, 33.82, 29.71, 29.50, 29.12, 28.98, 28.44, 28.38, 22.57, 22.44, 14.03, 13.98.

Di-tert-butyl (6-(dihexylamino)-6-oxohexane-1,5-diyl)(S)-dicarbamate (3d): Yellow oily liquid, yield: 71.9%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (d, *J* = 8.4 Hz, 1H), 4.65 (s, 1H), 4.53 (d, *J* = 4.4 Hz, 1H), 3.53 – 3.44 (m, 1H), 3.25 (dd, *J* = 15.3, 8.6 Hz, 2H),

3.15 – 3.06 (m, 3H), 1.64 (dd, *J* = 13.5, 5.1 Hz, 2H), 1.59 – 1.48 (m, 6H), 1.43 (s, 18H), 1.33 – 1.25 (m, 14H), 0.89 (q, *J* = 6.5 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.88, 156.02, 155.55, 79.44, 49.83, 47.79, 46.01, 40.40, 33.83, 31.55, 31.53, 29.51, 29.27, 28.44, 28.38, 27.54, 26.63, 26.54, 22.59, 14.02, 13.98.

Di-tert-butyl (6-(diheptylamino)-6-oxohexane-1,5-diyl)(S)-dicarbamate (3e): Yellow oily liquid, yield: 62.6%.^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (d, *J* = 8.3 Hz, 1H), 4.66 (s, 1H), 4.53 (d, *J* = 3.7 Hz, 1H), 3.52 – 3.43 (m, 1H), 3.25 (dd, *J* = 15.5, 9.0 Hz, 2H), 3.15 – 3.06 (m, 3H), 1.62 (dd, *J* = 13.3, 8.1 Hz, 2H), 1.58 – 1.48 (m, 6H), 1.43 (s, 18H), 1.28 (dd, *J* = 8.4, 6.0 Hz, 18H), 0.87 (dd, *J* = 6.8, 4.1 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.01, 155.54, 79.42, 49.82, 47.77, 45.99, 40.38, 33.81, 31.77, 31.73, 29.30, 29.02, 28.42, 28.36, 27.58, 26.92, 26.82, 22.57, 22.54, 14.04.

Di-tert-butyl (6-(dioctylamino)-6-oxohexane-1,5-diyl)(S)-dicarbamate (3f): Yellow oily liquid, yield: 67.8%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (d, *J* = 8.4 Hz, 1H), 4.66 (s, 1H), 4.53 (d, *J* = 4.2 Hz, 1H), 3.47 (d, *J* = 6.6 Hz, 1H), 3.25 (d, *J* = 6.5 Hz, 2H), 3.11 (dd, *J* = 12.0, 6.6 Hz, 3H), 1.62 (dd, *J* = 13.1, 8.1 Hz, 2H), 1.58 – 1.47 (m, 6H), 1.43 (s, 18H), 1.27 (dd, *J* = 13.5, 6.6 Hz, 22H), 0.91 – 0.85 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.87, 156.02, 155.55, 79.43, 49.83, 47.79, 46.01, 40.40, 33.83, 31.80, 31.76, 29.34, 29.25, 29.22, 28.44, 28.38, 27.60, 26.99, 26.89, 22.63, 22.56, 14.09, 14.07.

Di-tert-butyl (6-(dinonylamino)-6-oxohexane-1,5-diyl)(S)-dicarbamate (3g): Yellow oily liquid, yield: 66.8%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38 (d, *J* = 8.5 Hz, 1H), 4.69 (s, 1H), 4.53 (dd, *J* = 12.7, 8.3 Hz, 1H), 3.48 (dt, *J* = 15.0, 7.6 Hz, 1H), 3.33 – 3.19 (m, 2H), 3.16 – 3.04 (m, 3H), 1.66 – 1.59 (m, 2H), 1.58 – 1.47 (m, 6H), 1.43 (s, 18H), 1.26 (s, 26H), 0.91 – 0.85 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.86, 156.01, 155.53, 79.40, 78.95, 49.80, 47.77, 46.00, 40.36, 33.77, 31.82, 31.81, 29.67, 29.51, 29.48, 29.34, 29.28, 29.22, 29.19, 28.41, 28.35, 27.57, 26.95, 26.85, 22.63, 22.61, 22.53, 14.06.

di-tert-butyl (6-(didecylamino)-6-oxohexane-1,5-diyl)(S)-dicarbamate (3h): Yellow oily liquid, yield: 74.0%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 (d, *J* = 8.6 Hz, 1H), 4.62 (d, *J* = 17.5 Hz, 1H), 4.53 (d, *J* = 4.2 Hz, 1H), 3.46 (s, 1H), 3.32 – 3.17 (m, 2H), 3.16

– 3.03 (m, 3H), 1.62 (dd, *J* = 13.0, 8.0 Hz, 2H), 1.58 – 1.46 (m, 6H), 1.43 (s, 18H), 1.26 (d, *J* = 2.9 Hz, 30H), 0.90 – 0.85 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.86, 156.02, 155.55, 79.43, 79.01, 49.83, 47.80, 46.02, 40.42, 33.83, 31.90, 31.88, 29.60, 29.56, 29.53, 29.39, 29.31, 28.44, 28.39, 27.60, 27.00, 26.90, 22.69, 22.56, 14.11.

di-tert-butyl (6-(diundecylamino)-6-oxohexane-1,5-diyl)(S)-dicarbamate (3i): Light yellow oily liquid, yield: 70.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 (d, *J* = 8.1 Hz, 1H), 4.65 (s, 1H), 4.53 (s, 1H), 3.48 (dt, *J* = 15.1, 7.5 Hz, 1H), 3.32 – 3.18 (m, 2H), 3.10 (s, 3H), 1.59 (s, 2H), 1.58 – 1.47 (m, 6H), 1.43 (s, 18H), 1.26 (d, *J* = 2.4 Hz, 34H), 0.88 (dd, *J* = 6.8, 5.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.03, 155.55, 79.43, 78.99, 49.87, 47.80, 46.02, 40.40, 33.82, 31.92, 29.60, 29.57, 29.39, 29.34, 29.32, 28.44, 28.39, 27.62, 27.00, 26.93, 26.90, 22.69, 22.56, 14.12.

(3j): Yellow oily liquid, yield: 46.7%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.37 (s, 1H), 9.20 (s, 1H), 5.40 (d, *J* = 8.7 Hz, 1H), 4.52 (s, 1H), 3.88 (s, 2H), 3.47 (dt, *J* = 15.1, 7.7 Hz, 1H), 3.32 – 3.17 (m, 2H), 3.16 – 3.04 (m, 1H), 1.69 – 1.56 (m, 6H), 1.51 (s, 9H), 1.49 (s, 9H), 1.43 (d, *J* = 11.5 Hz, 9H), 1.30 (dd, *J* = 21.4, 13.0 Hz, 22H), 0.91 – 0.84 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.62, 163.86, 160.61, 155.37, 155.00, 83.67, 79.34, 78.66, 50.13, 47.82, 46.08, 44.39, 31.81, 31.76, 31.18, 29.32, 29.25, 29.23, 28.38, 28.35, 28.05, 27.60, 27.01, 26.90, 24.92, 22.63, 14.09, 14.07, -0.00.

(3k): Yellow oily liquid, yield: 44.6%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.37 (s, 1H), 9.20 (s, 1H), 5.39 (d, *J* = 8.6 Hz, 1H), 4.52 (s, 1H), 3.88 (s, 2H), 3.47 (dt, *J* = 15.1, 7.7 Hz, 1H), 3.34 – 3.17 (m, 2H), 3.14 – 3.04 (m, 1H), 1.60 (dd, *J* = 20.6, 14.1 Hz, 6H), 1.51 (s, 9H), 1.49 (s, 9H), 1.42 (s, 9H), 1.26 (s, 26H), 0.88 (dd, *J* = 6.9, 5.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.62, 163.86, 160.61, 155.37, 155.01, 83.67, 79.33, 78.65, 50.13, 47.83, 46.09, 44.39, 31.85, 31.19, 29.71, 29.54, 29.53, 29.36, 29.26, 29.22, 28.38, 28.36, 28.05, 27.61, 27.01, 26.91, 24.92, 22.67, 22.65, 14.10.

(3l): Yellow oily liquid, yield: 42.6%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.37 (s, 1H), 9.20 (s, 1H), 5.39 (d, *J* = 8.6 Hz, 1H), 4.51 (s, 1H), 3.88 (s, 2H), 3.52 – 3.42 (m, 1H), 3.26 (dd, *J* = 17.3, 10.2 Hz, 2H), 3.14 – 3.04 (m, 1H), 1.62 (d, *J* = 21.2 Hz, 6H), 1.51 (s, 9H), 1.49 (s, 9H), 1.42 (s, 9H), 1.26 (s, 30H), 0.88 (t, *J* = 6.6 Hz, 6H).^{13}C NMR (101 MHz, $CDCl_3$) δ 171.61, 163.86, 160.61, 155.36, 155.00, 83.67, 79.33, 78.66,

50.12, 47.82, 46.09, 44.39, 31.90, 31.87, 31.19, 29.60, 29.58, 29.57, 29.52, 29.37, 29.30, 28.38, 28.36, 28.05, 27.61, 27.02, 26.92, 24.91, 22.68, 14.11.

Tert-butyl (S)-(1-(dioctylamino)-3-hydroxy-1-oxopropan-2-yl)carbamate (3m): Yellow oily liquid, yield: 62.9%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.62 (d, *J* = 8.0 Hz, 1H), 4.65 – 4.53 (m, 1H), 3.74 (dt, *J* = 11.0, 9.0 Hz, 2H), 3.51 – 3.23 (m, 4H), 1.65 – 1.48 (m, 4H), 1.44 (s, 9H), 1.29 (d, *J* = 7.7 Hz, 20H), 0.95 – 0.83 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.79, 155.80, 80.09, 64.79, 51.25, 47.88, 46.13, 31.78, 31.74, 29.69, 29.35, 29.32, 29.21, 29.19, 28.30, 27.51, 26.94, 26.83, 22.61, 22.60, 14.06, 14.05.

Tert-butyl (S)-(1-(didecylamino)-3-hydroxy-1-oxopropan-2-yl)carbamate (3n): Yellow oily liquid, yield: 49.8%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.61 (d, *J* = 7.6 Hz, 1H), 4.59 (s, 1H), 3.77 (t, *J* = 14.9 Hz, 2H), 3.51 – 3.21 (m, 4H), 1.55 (dd, *J* = 16.7, 7.5 Hz, 4H), 1.44 (s, 9H), 1.26 (s, 28H), 0.88 (t, *J* = 6.3 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.80, 155.80, 80.10, 64.83, 51.25, 47.89, 46.14, 31.88, 31.86, 29.69, 29.56, 29.54, 29.51, 29.37, 29.29, 29.28, 28.30, 27.52, 26.95, 26.84, 22.67, 14.09.

Tert-butyl (R)-(1-(dioctylamino)-3-hydroxy-1-oxopropan-2-yl)carbamate (3o): Yellow oily liquid, yield: 67.3%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.63 (d, *J* = 8.2 Hz, 1H), 4.66 – 4.55 (m, 1H), 3.83 – 3.67 (m, 2H), 3.33 (ddd, *J* = 25.2, 15.7, 8.0 Hz, 4H), 1.61 – 1.49 (m, 4H), 1.44 (s, 9H), 1.31 – 1.25 (m, 20H), 0.91 – 0.85 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.79, 155.77, 80.06, 64.75, 51.24, 47.88, 46.13, 31.78, 31.74, 29.69, 29.32, 29.21, 29.19, 28.29, 27.50, 26.93, 26.82, 22.61, 22.60, 14.06, 14.05.

Tert-butyl (R)-(1-(didecylamino)-3-hydroxy-1-oxopropan-2-yl)carbamate (3p): Yellow oily liquid, yield: 52.6%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.62 (d, *J* = 8.1 Hz, 1H), 4.67 – 4.54 (m, 1H), 3.75 (ddd, *J* = 29.3, 11.3, 4.1 Hz, 2H), 3.46 – 3.20 (m, 4H), 1.61 – 1.48 (m, 4H), 1.47 – 1.41 (m, 9H), 1.26 (t, *J* = 7.1 Hz, 28H), 0.88 (dd, *J* = 6.9, 5.5 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.80, 155.80, 80.10, 64.81, 60.41, 51.24, 47.90, 46.15, 31.89, 31.87, 29.58, 29.55, 29.52, 29.39, 29.31, 29.29, 28.31, 27.53, 26.96, 26.85, 22.68, 14.11.

tert-butyl (S)-(1-(dioctylamino)-1-oxopropan-2-yl)carbamate (3q): Yellow oily liquid, yield: 64.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.46 (d, *J* = 7.8 Hz, 1H), 4.67 – 4.47 (m, 1H), 3.45 (dt, *J* = 14.8, 7.6 Hz, 1H), 3.25 (t, *J* = 7.7 Hz, 2H), 3.19 – 3.11 (m, 1H), 1.63 – 1.46 (m, 4H), 1.43 (s, 9H), 1.29 (d, *J* = 6.8 Hz, 23H), 0.93 – 0.82 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.50, 155.06, 79.34, 47.67, 46.21, 45.92, 31.81, 31.75, 29.36, 29.31, 29.27, 29.24, 29.21, 28.39, 27.56, 26.94, 26.88, 22.64, 22.63, 19.81, 14.09, 14.07.

tert-butyl (S)-(1-(dioctylamino)-4-methyl-1-oxopentan-2-yl)carbamate (3r): Yellow oily liquid, yield: 72.1%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.21 (d, *J* = 9.1 Hz, 1H), 4.60 (t, *J* = 8.0 Hz, 1H), 3.54 – 3.42 (m, 1H), 3.27 (t, *J* = 7.7 Hz, 2H), 3.19 – 3.06 (m, 1H), 1.84 – 1.67 (m, 2H), 1.66 – 1.57 (m, 2H), 1.53 – 1.48 (m, 3H), 1.44 (d, *J* = 15.9 Hz, 9H), 1.33 – 1.23 (m, 20H), 0.99 (d, *J* = 6.5 Hz, 3H), 0.93 (d, *J* = 6.7 Hz, 3H), 0.88 (td, *J* = 6.6, 2.6 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.72, 155.53, 79.24, 48.63, 47.77, 46.06, 43.46, 31.80, 31.77, 29.71, 29.36, 29.30, 29.24, 28.36, 27.60, 26.96, 24.64, 23.55, 22.65, 22.62, 21.82, 14.09, 14.07.

tert-butyl ((2S,3S)-1-(dioctylamino)-3-methyl-1-oxopentan-2-yl)carbamate (3s): Yellow oily liquid, yield: 64.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.21 (d, *J* = 9.4 Hz, 1H), 4.48 – 4.36 (m, 1H), 3.66 – 3.54 (m, 1H), 3.45 – 3.30 (m, 1H), 3.28 – 3.18 (m, 1H), 3.09 – 2.97 (m, 1H), 1.74 – 1.63 (m, 2H), 1.55 (ddd, *J* = 21.2, 10.5, 5.0 Hz, 4H), 1.43 (d, *J* = 6.5 Hz, 9H), 1.27 (s, 20H), 1.17 – 1.04 (m, 1H), 0.96 – 0.82 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.02, 155.62, 79.23, 54.41, 48.03, 45.98, 38.76, 31.80, 29.37, 29.27, 29.21, 28.35, 27.59, 27.05, 26.86, 24.07, 22.64, 15.77, 14.08, 11.43.

tert-butyl (S)-(1-(dioctylamino)-1-oxo-3-phenylpropan-2-yl)carbamate (3t): Yellow oily liquid, yield: 64.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 – 7.16 (m, 5H), 5.33 (d, *J* = 8.8 Hz, 1H), 4.74 (d, *J* = 6.6 Hz, 1H), 3.48 – 3.33 (m, 1H), 3.08 – 2.80 (m, 5H), 1.74 – 1.33 (m, 13H), 1.26 (t, *J* = 15.9 Hz, 20H), 0.88 (dt, *J* = 7.0, 3.3 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.21, 154.93, 136.78, 129.56, 128.36, 126.76, 79.48, 51.50, 47.72, 46.36, 40.56, 31.82, 31.77, 29.37, 29.28, 29.24, 29.18, 28.98, 28.33, 27.52, 27.05, 26.77, 22.65, 22.61, 14.09, 14.08.

tert-butyl ((2S,3R)-1-(dioctylamino)-3-hydroxy-1-oxobutan-2-yl)carbamate (3u): Yellow oily liquid, yield: 58.1%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.46 (d, *J* = 9.6 Hz, 1H), 4.41 (d, *J* = 9.7 Hz, 1H), 4.04 (d, *J* = 6.2 Hz, 1H), 3.53 (dd, *J* = 13.3, 6.9 Hz, 1H), 3.28 (tdd, *J* = 19.4, 13.8, 5.6 Hz, 3H), 1.62 – 1.49 (m, 4H), 1.45 (d, *J* = 7.0 Hz, 9H), 1.28 (s, 20H), 1.17 (d, *J* = 6.3 Hz, 3H), 0.88 (t, *J* = 5.9 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.58, 156.02, 79.86, 68.04, 52.63, 48.01, 46.26, 31.80, 31.77, 29.53, 29.37, 29.36, 29.23, 29.21, 28.29, 28.19, 27.50, 26.98, 26.81, 22.63, 18.67, 14.08.

General procedure for the synthesis of amphiphilic small molecules (4a-4u): This was the reaction about deprotection of Boc Groups. The amide coupling compounds **(3a-3u)** (1 equiv) were dissolved in methanol and then stirred at 0°C, then dropwise addition of a known amount of acetyl chloride (6 equiv) usually via a weighed syringe to an ice cold solution of excess amount of methanol.[60] Ice cold solutions are used in order to increase the solubility of the HCl and prevent its escape, the initial generation of the HCl being exothermic. In cases where simple esterifications are carried out, excess acetyl chloride may be used without detrimental effects, since the workup involves simple evaporation of the solvent and excess HCl. The solutions were allowed to warm to room temperature and the reactions were completed within 24 h. The final compounds were characterized by ^{1}H NMR, ^{13}C NMR, and mass spectrometry.

(S)-6-(diethylamino)-6-oxohexane-1, 5-diaminium chloride (4a): Dark yellow foamed solid, yield: 42.5%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.31 (s, 3H), 8.13 (s, 3H), 4.16 (d, *J* = 5.0 Hz, 1H), 3.41 – 3.25 (m, 3H), 3.21 – 3.14 (m, 1H), 2.74 (d, *J* = 5.8 Hz, 2H), 1.75 – 1.65 (m, 2H), 1.63 – 1.53 (m, 2H), 1.47 – 1.33 (m, 2H), 1.14 (t, *J* = 7.0 Hz, 3H), 1.05 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.52, 49.17, 41.20, 38.15, 30.14, 26.37, 20.82, 14.27, 12.67. HR-MS (ESI) Calcd for $C_{10}H_{23}N_3O$ $[M+H]^+$: 202.1914, found: 202.1918.

(S)-6-(dibutylamino)-6-oxohexane-1, 5-diaminium chloride (4b): Light yellow foamed solid, yield: 31.7%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.32 (d, *J* = 48.6 Hz, 6H), 4.12 (s, 1H), 3.47 (dd, *J* = 13.7, 7.4 Hz, 1H), 3.34 – 3.24 (m, 1H), 3.21 – 3.11 (m, 1H), 3.01 (dt, *J* = 13.3, 6.8 Hz, 1H), 2.71 (s, 2H), 1.70 (d, *J* = 6.2 Hz, 2H), 1.46 (ddd,

J = 26.1, 20.1, 13.9 Hz, 8H), 1.25 (ddd, *J* = 21.3, 13.7, 7.0 Hz, 4H), 0.87 (dd, *J* = 16.6, 7.3 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.87, 49.20, 48.49, 46.58, 44.83, 38.14, 30.65, 30.14, 29.02, 26.27, 20.87, 19.49, 19.29, 13.71, 13.69. HR-MS (ESI) Calcd for $C_{14}H_{31}N_3O$ $[M+H]^+$: 258.2540, found: 258.2545.

(S)-6-(dipentylamino)-6-oxohexane-1, 5-diaminium chloride (4c): Light yellow foamed solid, yield: 79.8%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.21 (d, *J* = 79.2 Hz, 6H), 4.14 (s, 1H), 3.46 – 3.41 (m, 1H), 3.38 – 2.97 (m, 3H), 2.74 (s, 2H), 1.70 (d, *J* = 6.6 Hz, 2H), 1.65 – 1.15 (m, 16H), 0.89 (ddd, *J* = 9.4, 7.6, 2.1 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.42, 167.89, 49.28, 46.74, 45.04, 38.19, 32.26, 30.16, 28.42, 28.22, 28.14, 26.50, 26.36, 26.20, 25.47, 22.43, 22.21, 21.88, 21.78, 16.72, 16.46, 13.87, 13.85, 11.32, 11.19. HR-MS (ESI) Calcd for $C_{16}H_{35}N_3O$ $[M+H]^+$: 286.2853, found: 286.2860.

(S)-6-(dihexylamino)-6-oxohexane-1, 5-diaminium chloride (4d): Dark yellow viscous solid, yield: >95%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.15 (d, *J* = 82.2 Hz, 6H), 4.14 (t, *J* = 5.6 Hz, 1H), 3.41 – 2.95 (m, 4H), 2.74 (t, *J* = 6.9 Hz, 2H), 1.70 (dd, *J* = 14.5, 7.4 Hz, 2H), 1.59 – 1.54 (m, 2H), 1.44 (dd, *J* = 15.2, 7.1 Hz, 2H), 1.36 (s, 2H), 1.32 – 1.18 (m, 14H), 0.85 (dd, *J* = 8.9, 4.5 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.89, 49.31, 46.77, 45.08, 38.22, 30.97, 30.88, 30.19, 28.51, 26.79, 26.40, 25.87, 25.65, 22.00, 21.96, 20.85, 13.87, 13.84. HR-MS (ESI) Calcd for $C_{18}H_{39}N_3O$ $[M+H]^+$: 314.3166, found: 314.3171.

(S)-6-(diheptylamino)-6-oxohexane-1, 5-diaminium chloride (4e): Light yellow viscous solid, yield: >95%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.19 (d, *J* = 77.4 Hz, 6H), 4.14 (d, *J* = 4.9 Hz, 1H), 3.51 – 3.45 (m, 1H), 3.35 – 3.23 (m, 1H), 3.16 (s, 1H), 3.02 (dd, *J* = 9.2, 4.2 Hz, 1H), 2.83 – 2.64 (m, 2H), 1.70 (dd, *J* = 14.2, 7.5 Hz, 2H), 1.60 – 1.50 (m, 4H), 1.44 (dd, *J* = 13.9, 6.9 Hz, 2H), 1.34 – 1.15 (m, 18H), 0.85 (dd, *J* = 6.8, 3.9 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.88, 49.29, 46.79, 45.09, 38.19, 31.16, 31.13, 30.17, 28.56, 28.42, 28.31, 26.83, 26.37, 26.17, 25.94, 21.99, 21.97, 20.85, 13.91, 13.90. HR-MS (ESI) Calcd for $C_{20}H_{43}N_3O$ $[M+H]^+$: 342.3479, found: 342.3484.

(S)-6-(dioctylamino)-6-oxohexane-1, 5-diaminium chloride (4f): Dark yellow viscous solid, yield: 73.3%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.19 (d, *J* = 79.3 Hz, 6H), 4.14 (s, 1H), 3.29 (dd, *J* = 15.2, 7.0 Hz, 1H), 3.17 (dd, *J* = 14.9, 5.9 Hz, 1H), 3.08 – 2.99 (m, 1H), 2.74 (s, 2H), 1.70 (dd, *J* = 14.2, 7.5 Hz, 2H), 1.61 – 1.49 (m, 4H), 1.44 (dd, *J* = 13.2, 6.5 Hz, 2H), 1.40 – 1.36 (m, 1H), 1.25 (d, *J* = 5.5 Hz, 22H), 0.89 – 0.83 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.89, 49.28, 46.79, 45.11, 38.17, 31.18, 31.16, 30.16, 28.73, 28.59, 28.55, 26.82, 26.37, 26.22, 25.99, 22.03, 20.84, 13.91. HR-MS (ESI) Calcd for $C_{22}H_{47}N_3O$ $[M+H]^+$: 370.3792, found: 370.3797.

(S)-6-(dinonylamino)-6-oxohexane-1, 5-diaminium chloride (4g): Dark yellow viscous solid, yield: 63.8%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (d, *J* = 70.4 Hz, 6H), 4.14 (d, *J* = 4.2 Hz, 1H), 3.46 – 3.43 (m, 1H), 3.29 (dd, *J* = 14.6, 6.7 Hz, 1H), 3.21 – 3.13 (m, 1H), 3.03 (dd, *J* = 9.1, 4.2 Hz, 1H), 2.74 (d, *J* = 5.7 Hz, 2H), 1.74 – 1.67 (m, 2H), 1.60 – 1.48 (m, 4H), 1.43 – 1.38 (m, 2H), 1.25 (s, 26H), 0.86 (t, *J* = 6.1 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.89, 49.26, 46.81, 45.15, 38.15, 31.24, 31.22, 30.15, 28.89, 28.85, 28.77, 28.65, 28.62, 28.59, 26.83, 26.35, 26.23, 25.99, 22.05, 20.84, 13.91. HR-MS (ESI) Calcd for $C_{24}H_{51}N_3O$ $[M+H]^+$: 398.4105, found: 398.4112.

(S)-6-(didecylamino)-6-oxohexane-1, 5-diaminium chloride (4h): Light yellow foamed solid, yield: 92.5%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.21 (d, *J* = 74.4 Hz, 6H), 4.14 (s, 1H), 3.43 (s, 1H), 3.28 (dd, *J* = 14.8, 6.7 Hz, 1H), 3.21 – 3.11 (m, 1H), 3.09 – 2.99 (m, 1H), 2.74 (s, 2H), 1.70 (dd, *J* = 14.2, 7.5 Hz, 2H), 1.54 (ddd, *J* = 27.6, 13.2, 7.3 Hz, 4H), 1.41 (dd, *J* = 15.1, 7.5 Hz, 2H), 1.24 (s, 30H), 0.85 (t, *J* = 6.6 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.89, 49.27, 46.82, 45.17, 38.16, 31.24, 30.15, 28.92, 28.90, 28.76, 28.64, 28.54, 26.83, 26.35, 26.22, 25.98, 22.05, 20.84, 13.90. HR-MS (ESI) Calcd for $C_{26}H_{55}N_3O$ $[M+H]^+$: 426.4418, found: 426.4425.

(S)-6-(diundecylamino)-6-oxohexane-1, 5-diaminium chloride (4i): Rice white viscous solid, yield: 45.4%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.24 (t, *J* = 46.6 Hz, 6H), 4.15 (s, 1H), 3.43 (s, 1H), 3.35 – 3.25 (m, 1H), 3.17 (dd, *J* = 14.8, 5.8 Hz, 1H), 3.09 – 3.00 (m, 1H), 2.74 (s, 2H), 1.70 (dd, *J* = 14.2, 7.5 Hz, 2H), 1.55 (ddd, *J* = 18.6, 12.6, 7.0 Hz, 4H), 1.41 (dd, *J* = 15.4, 7.7 Hz, 2H), 1.24 (s, 34H), 0.86 (t, *J* = 6.7 Hz,

6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.89, 49.28, 46.83, 45.19, 38.16, 31.25, 30.15, 28.96, 28.95, 28.91, 28.89, 28.75, 28.66, 28.64, 28.54, 26.83, 26.36, 26.23, 25.97, 22.05, 20.83, 13.90. HR-MS (ESI) Calcd for $C_{28}H_{59}N_3O$ $[M+H]^+$: 454.4731, found: 454.4735.

(4j): Light yellow foamed solid, yield: 79.6%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.32 (d, *J* = 13.5 Hz, 3H), 7.97 (t, *J* = 5.4 Hz, 1H), 7.65 – 6.90 (m, 3H), 4.19 (d, *J* = 4.2 Hz, 1H), 3.36 – 3.27 (m, 1H), 3.16 (dt, *J* = 15.2, 7.0 Hz, 2H), 3.09 – 2.99 (m, 1H), 1.74 (d, *J* = 5.6 Hz, 2H), 1.64 – 1.39 (m, 6H), 1.25 (s, 20H), 0.94 – 0.75 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.83, 157.00, 49.14, 46.83, 45.12, 31.19, 31.18, 28.72, 28.61, 28.55, 27.96, 26.83, 26.23, 25.99, 23.76, 22.04, 13.91. HR-MS (ESI) Calcd for $C_{22}H_{47}N_5O$ $[M+H]^+$: 398.3853, found: 398.3858.

(4k): Light yellow foamed solid, yield: 81.8%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.18 (s, 1H), 8.99 (s, 2H), 8.44 (d, *J* = 61.4 Hz, 3H), 8.10 – 6.77 (m, 1H), 4.21 (s, 1H), 3.33 (s, 2H), 3.21 – 3.12 (m, 1H), 3.01 (d, *J* = 5.7 Hz, 1H), 1.73 (d, *J* = 23.9 Hz, 2H), 1.62 (s, 2H), 1.46 (s, 8H), 1.17 (d, *J* = 49.7 Hz, 24H), 0.84 (d, *J* = 6.6 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.79, 153.46, 151.60, 83.54, 49.06, 46.84, 45.07, 31.24, 31.23, 28.91, 28.85, 28.74, 28.66, 28.63, 28.61, 28.47, 27.70, 27.48, 26.81, 26.23, 25.99, 23.10, 22.05, 13.86. HR-MS (ESI) Calcd for $C_{24}H_{51}N_5O$ $[M+H]^+$: 426.4166, found: 426.4171.

(4l): Light yellow foamed solid, yield: 78.8%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.32 (d, *J* = 13.5 Hz, 3H), 7.95 (t, *J* = 5.6 Hz, 1H), 7.70 – 6.85 (m, 3H), 4.20 (s, 1H), 3.35 – 3.28 (m, 1H), 3.20 – 3.11 (m, 2H), 3.08 – 2.98 (m, 1H), 1.72 (t, *J* = 14.7 Hz, 2H), 1.63 – 1.37 (m, 8H), 1.24 (s, 28H), 0.86 (t, *J* = 6.5 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.83, 156.98, 49.14, 46.85, 45.15, 31.25, 28.91, 28.75, 28.66, 28.52, 27.96, 27.50, 26.84, 26.23, 25.97, 23.75, 22.06, 13.91. HR-MS (ESI) Calcd for $C_{26}H_{55}N_5O$ $[M+H]^+$: 454.4479, found: 454.4487.

(S)-1-(dioctylamino)-3-hydroxy-1-oxopropan-2-aminium chloride (4m): Yellow foamed solid, yield: 94.5%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (s, 3H), 4.16 (s, 1H), 3.70 (dd, *J* = 11.5, 4.4 Hz, 1H), 3.55 (dd, *J* = 11.5, 7.0 Hz, 1H), 3.42 (dd, *J* = 15.8, 7.6 Hz, 2H), 3.22 – 3.02 (m, 2H), 1.61 – 1.36 (m, 4H), 1.25 (d, *J* = 8.0 Hz, 20H),

0.92 – 0.76 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.23, 60.32, 52.37, 46.70, 45.11, 40.12, 39.91, 39.71, 39.50, 39.29, 39.08, 38.87, 31.20, 28.76, 28.68, 28.63, 28.57, 26.92, 26.18, 26.01, 22.05, 13.91. HR-MS (ESI) Calcd for $C_{19}H_{40}N_2O_2$ [M+H]$^+$: 329.3163, found: 329.3163.

(S)-1-(didecylamino)-3-hydroxy-1-oxopropan-2-aminium chloride (4n): Light yellow foamed solid, yield: 97.2%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.18 (s, 3H), 5.56 (s, 1H), 4.23 – 4.08 (m, 1H), 3.70 (d, *J* = 10.2 Hz, 1H), 3.45 – 3.33 (m, 2H), 3.10 (ddd, *J* = 13.4, 11.4, 5.1 Hz, 2H), 1.58 – 1.38 (m, 4H), 1.24 (d, *J* = 4.6 Hz, 28H), 0.85 (t, *J* = 6.5 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.14, 60.22, 52.35, 46.73, 45.15, 31.24, 28.89, 28.74, 28.65, 28.46, 26.88, 26.13, 25.96, 22.05, 13.90. HR-MS (ESI) Calcd for $C_{23}H_{48}N_2O_2$ [M+H]$^+$: 385.3789, found: 385.3793.

(R)-1-(dioctylamino)-3-hydroxy-1-oxopropan-2-aminium chloride (4o): Dark yellow viscous solid, yield: 96.3%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (s, 3H), 4.16 (s, 1H), 3.71 (d, *J* = 4.4 Hz, 1H), 3.53 (d, *J* = 7.1 Hz, 1H), 3.42 (dt, *J* = 15.2, 7.1 Hz, 3H), 3.18 – 2.99 (m, 2H), 1.57 – 1.36 (m, 4H), 1.23 (d, *J* = 7.5 Hz, 20H), 0.83 (dd, *J* = 6.6, 2.6 Hz, 6H).^{13}C NMR (101 MHz, DMSO-d_6) δ 166.21, 60.25, 52.32, 46.74, 45.16, 39.90, 39.69, 39.49, 39.28, 39.07, 38.86, 38.65, 31.18, 28.72, 28.64, 28.58, 28.53, 28.48, 26.88, 26.15, 25.97, 22.03, 13.86. HR-MS (ESI) Calcd for $C_{19}H_{40}N_2O_2$ [M+H]$^+$: 329.3163, found: 329.3177.

(R)-1-(didecylamino)-3-hydroxy-1-oxopropan-2-aminium chloride (4p): Dark yellow viscous solid, yield: >95%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (s, 3H), 4.11 (s, 1H), 3.59 – 3.45 (m, 4H), 3.36 (d, *J* = 6.0 Hz, 2H), 1.53 – 1.27 (m, 4H), 1.16 (s, 28H), 0.78 (t, *J* = 6.0 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.24, 60.25, 52.28, 48.48, 46.78, 45.21, 31.21, 28.89, 28.86, 28.72, 28.63, 28.45, 26.87, 26.12, 25.93, 22.02, 13.86. HR-MS (ESI) Calcd for $C_{23}H_{48}N_2O_2$ [M+H]$^+$: 385.3789, found: 385.3795.

(S)-1-(dioctylamino)-1-oxopropan-2-aminium chloride (4q): Dark yellow viscous oily liquid, yield: 96.8%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.34 (s, 3H), 4.20 – 4.07 (m, 1H), 3.54 – 2.97 (m, 4H), 1.59 – 1.38 (m, 4H), 1.32 (d, *J* = 6.8 Hz, 3H), 1.25 (d, *J* = 7.2 Hz, 20H), 0.92 – 0.80 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.94, 46.49,

45.85, 44.82, 31.18, 28.72, 28.66, 28.61, 28.56, 28.53, 26.82, 26.14, 25.99, 22.04, 16.84, 13.90. HR-MS (ESI) Calcd for $C_{19}H_{40}N_2O$ $[M+H]^+$: 313.3213, found: 313.3216.

(S)-1-(dioctylamino)-4-methyl-1-oxopentan-2-aminium chloride (4r): Dark yellow viscous oily liquid, yield: 90.2%.^{1}H NMR (400 MHz, DMSO-d_6) δ 8.33 (d, J = 3.0 Hz, 3H), 4.11 – 4.02 (m, 1H), 3.56 – 2.99 (m, 4H), 1.91 – 1.74 (m, 1H), 1.70 – 1.36 (m, 6H), 1.26 (d, J = 11.0 Hz, 20H), 0.88 (ddd, J = 12.4, 10.4, 6.0 Hz, 12H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.51, 48.06, 46.83, 45.13, 31.17, 31.16, 28.64, 28.63, 28.57, 28.55, 28.41, 26.83, 26.18, 26.12, 23.32, 23.11, 22.05, 22.01, 21.25, 13.90. HR-MS (ESI) Calcd for $C_{22}H_{46}N_2O$ $[M+H]^+$: 355.3683, found: 355.3689.

(2S, 3S)-1-(dioctylamino)-3-methyl-1-oxopentan-2-aminium chloride (4s): Dark yellow viscous oily liquid, yield: 91.2%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.27 (s, 3H), 4.06 – 3.99 (m, 1H), 3.67 – 3.55 (m, 1H), 3.14 (ddd, J = 83.0, 11.1, 6.6 Hz, 3H), 1.78 (d, J = 5.2 Hz, 1H), 1.49 (ddd, J = 17.9, 9.7, 5.1 Hz, 5H), 1.33 – 1.19 (m, 20H), 1.17 – 1.07 (m, 1H), 0.96 (d, J = 6.9 Hz, 3H), 0.90 – 0.82 (m, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.62, 53.53, 47.03, 44.97, 36.28, 31.20, 31.15, 28.69, 28.65, 28.59, 28.57, 28.45, 26.80, 26.23, 25.99, 23.10, 22.04, 14.83, 13.90, 13.88, 11.31. HR-MS (ESI) Calcd for $C_{22}H_{46}N_2O$ $[M+H]^+$: 355.3683, found: 355.3687.

(S)-1-(dioctylamino)-1-oxo-3-phenylpropan-2-aminium chloride (4t): Dark yellow viscous oily liquid, yield: 92.0%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.53 (s, 3H), 7.41 – 7.14 (m, 5H), 4.30 (dd, J = 8.9, 5.0 Hz, 1H), 3.34 (s, 1H), 3.17 (dd, J = 13.1, 4.9 Hz, 1H), 3.00 – 2.68 (m, 4H), 1.19 (dt, J = 36.0, 23.4 Hz, 24H), 0.91 – 0.82 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.28, 134.78, 129.55, 128.40, 127.24, 50.14, 46.82, 45.58, 37.29, 31.20, 31.18, 28.68, 28.55, 28.52, 28.13, 26.81, 26.30, 25.99, 22.06, 22.02, 13.90. HR-MS (ESI) Calcd for $C_{25}H_{44}N_2O$ $[M+H]^+$: 389.3526, found: 389.3531.

(2S, 3R)-1-(dioctylamino)-3-hydroxy-1-oxobutan-2-aminium chloride (4u): Dark yellow viscous oily liquid, yield: 95.3%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.25 (d, J = 3.0 Hz, 3H), 4.09 – 4.01 (m, 1H), 3.93 – 3.86 (m, 1H), 3.59 – 3.48 (m, 3H), 3.20 – 3.10 (m, 1H), 3.05 – 2.96 (m, 1H), 1.49 (dd, J = 20.6, 13.5 Hz, 4H), 1.26 (d, J = 5.3

Hz, 20H), 1.12 (d, *J* = 6.4 Hz, 3H), 0.92 – 0.80 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.35, 66.39, 54.45, 47.23, 45.36, 31.20, 31.18, 28.74, 28.66, 28.61, 28.56, 26.86, 26.26, 26.06, 22.04, 19.07, 13.91. HR-MS (ESI) Calcd for $C_{20}H_{42}N_2O_2$ $[M+H]^+$: 343.3319, found: 343.3325.

Microorganisms and culture conditions: The antibacterial activity of all the dialkyl cationic amphiphilic small molecules was evaluated against both Gram-positive bacteria (*S. aureus*, *E. faecalis* and MRSA) and Gram-negative bacteria (*E. coli*, *S.enterica*, KPC, NDM-1). All the bacteria were cultured in Muller-Hinton broth (5.0 g of beef extract, 17.5 g of casein hydrolysate, and 1.5 g of starch in 1000 mL of distilled water) Brain-heart infusion broth (5.0 g beef heart infusion form, 12.5 g of calf brains infusion form, 2.5 g Na_2HPO_4, 2.0 g D-glucose, 10 g of peptone and 5.0 g NaCl in 100 mL of sterile distilled water) was used for stock samples of bacteria, the freeze dried stock samples of bacteria in 33.3% glycerol were stored at -80 ºC. For solid media, Mueller-Hinton agar (5.0 g of beef extract, 17.5 g of casein hydrolysate, 1.5 g of starch and 12.5 g of agar in 1000 mL of distilled water) was used as growth medium.

Cell culture: Human cervical carcinoma cell line (HeLa cell), maintained in complete DMEM media (Bioind) supplemented with 10% FBS (Zeta Life), at 37 ºC in a humidified atmosphere of 5% CO_2 in air. All the cells were mycoplasma free. The cells were trypsinized, counted and seeded in 96-well plates for viability studies or in 12-well plates for other studies. The cells were allowed to adhere overnight before they were used for experiments.

Antibacterial assay (minimum inhibitory concentration): Minimum inhibitory concentration (MIC) of all the small molecular compounds (**4a-4u**) were determined by broth microdilution method according to CLSI guidelines. The test medium for most species was cation-adjusted Muller-Hinton broth (MHB). The 4-6 h grown culture as described in the microorganism and culture condition section gives about 10^8 CFU/mL of bacteria. The bacterial cultures were then diluted to give approximately 10^6 CFU/mL in Muller-Hinton broth media which were then used for determining antibacterial efficacy. All the final compounds were water soluble at room temperature. Stock solutions of the final compounds were prepared with sterile Milli-Q water. Then the

stock solutions were serially diluted to different concentration (256 μg/mL, 128 μg/mL, 64 μg/mL, 32 μg/mL, 16 μg/mL, 8 μg/mL, 4 μg/mL, 2 μg/mL, 1 μg/mL, 0.5 μg/mL) by using Muller-Hinton broth media. These dilutions (100 μL) were added to the wells of 96 well plate followed by the addition of 100 μL of bacterial suspension (10^6 CFU/mL). Two controls were made: one containing 200 μL of media (negative contrast) and the other containing 200 μL of bacterial solution (10^6 CFU/mL, positive contrast). The plates were then incubated at 37 ºC for 16-20 h. After the incubation, read the results. Each concentration was determined in twice and the whole experiment was repeated at least twice. The antibacterial activity was thus expressed as minimum inhibitory concentration (MIC). A glycopeptides antibiotic vancomycin and a β-lactams antibiotic meropenem were used to compare the antibacterial efficacy in this study. To determine the minimum bactericidal concentration (MBC), the bacterial suspension that appeared to have less/little turbidity in the MIC experiment was plated (20 μL) and the agar plates were incubated for 20-24 h at 37 °C. Concentration at which no bacterial growth (no bacterial colony) was observed was taken as the MBC of the respective compounds.

Antibacterial activity in plasma (Plasma stability): Bacteria (*S. aureus*) was grown in a similar way as mentioned in the microorganism and culture condition and finally diluted in the respective media to give 10^6 CFU/mL. The fresh sterile defiber sheep blood (from commercial resource) was centrifuged at 3500 rpm for 10 min. The plasma, separated from the blood cells after centrifugation, was carefully collected. The test compound **(4g)** was dissolved in 50% sterile Milli-Q water and 50% plasma at a concentration of 512 μg/mL. Three such test samples were preincubated at 37 °C in 50% plasma for 0 h, 3 h and 6 h respectively. Then the three samples were serially diluted to several concentration (256 μg/mL, 128 μg/mL, 64 μg/mL, 32 μg/mL, 16 μg/mL, 8 μg/mL, 4 μg/mL). After that, 50 μL of the above solutions was added to wells of a 96-well plate and 150 μL of the bacterial suspension (10^5 CFU/mL) was added to wells. The plate was then incubated for 20-24 h at 37 °C and antibacterial efficacy (minimum bactericidal concentration) of the test compound was determined as described in the previous section (antibacterial assay).

Antibacterial assay in complex mammalian fluids: The fresh sterile defiber sheep

blood was bought from a biochemical reagent company (China). Plasma was obtained as mentioned above. Serum was isolated by using serum tube containing sheep blood and then centrifuging the blood at 3,500 rpm for 10 min. Methicillin-resistant S. aureus (MRSA) was grown in way as mentioned in the microorganism and culture conditions. Finally, MRSA was diluted with 50% Muller-Hinton broth (MHB) medium and 50% mammalian media (serum, plasma, blood), individually in a way to give 10^5 CFU/mL of MRSA in 50% serum, 50% plasma, and 50% blood (having 50% MHB medium). The test molecule **(4g)** was dissolved in sterile water with the serial dilution method at the concentration of (512 μg/mL, 256 μg/mL, 128 μg/mL, 64 μg/mL, 32 μg/mL, 16 μg/mL, 8 μg/mL, 4 μg/mL). Then 50 μL of the dilutions was added to the wells of a 96-well plate and 150 μL of the bacterial suspension (10^5 CFU/mL) was added separately to the wells containing the dilutions of the compound **(4g)**. The plate was then incubated for 20-24 h at 37 °C and minimum bactericidal concentration of the test compound was determined by plating the bacterial suspension (20 μL) directly from the wells onto Muller-Hinton agar (MHA) plate. The agar plates were incubated at 37 °C for 20-24 h and colonies were observed to determine the MBC.

Time-dependent killing: An overnight culture of bateria *S. aureus* (ATCC 29213) and *E.coli* (ATCC 25922) was diluted 1:10,000 in MHB medium and incubated at 37 °C with aeration at 225 rpm for 2 h (early exponential) or 5 h (late exponential). Bacteria were then challenged with compounds **(4g)** at 6×MIC and 8×MIC (a desirable concentration at the site of infection) and two antibiotics vancomycin (12 μg/mL, *S.aureus*) or moxalactam (12 μg/mL, *E.coli*) in culture tubes at 37 °C and 225 rpm. At different intervals, 100 μL bacteria solution were removed to 96-well plate, centrifuged at 4,000 rpm for 3 min (TDL 5M centrifuge) and resuspended in 100 μL of sterile phosphate buffered saline (1×PBS). Ten-fold serially diluted suspensions were plated on MHA plates and incubated at 37 °C overnight. Colonies were counted and CFU per mL was calculated. Experiments were performed with biological replicates.

Biofilm disruption assay (determination of viable count and imaging): The bacteria *S. aureus* and *E. coli* (4-6 h grown, mid-log phase) were diluted to ~10^5

CFU/mL into suitable media (MHB for *S. aureus* and M9 media supplemented with 0.02% casamino acid and 0.5% glycerol for *E. coli* respectively). The 96-well plates containing 100 µL of these suspensions were incubated under stationary conditions (for about 24 h for *S. aureus* and 72 h for *E. coli*). After incubation, the bacteria suspensions were centrifuged at 3,500 rpm for 5 min, the medium was removed and the wells were washed with 1×PBS once. Compound **(4g)** (100 µL at 2, 4, 8, 16, 32, 64, and 128 µg/mL) was then added to the wells containing preformed bacterial biofilms and allowed to incubate for 24 h at 37 °C. A control was made where 100 µL of the above medium was added. After 24 h, medium was discarded and the planktonic cells were removed by washing with 1×PBS. Then 100 µL of trypsin-EDTA solution was added to the treated biofilm to make the suspension of bacterial cells present within the biofilm. Cell suspension was then assessed by plating the 10-fold serial dilutions of the suspension on suitable agar plates. After 24 h of incubation, bacterial colonies were counted and cell viability was expressed as log10 (CFU/well) along with the control. For visualizing the disruption of biofilm by the small molecules, Crystal Violet (CV) staining[61] was done as described previously except that the cells were grown in 24-well plates. The 24-well plates containing 1.5 mL/well of these suspensions were incubated at 37 °C for about 72 h. Wells were stained with 300 mL of 1.0 % crystal violet dye, rinsed twice with 2 ml deionized water and thoroughly dried. For quantification, 0.5 mL of 95 % ethanol were added to each well. Plates were incubated for one hour at room temperature with shaking. Then take photos for these wells.

Minimum biofilm eradication concentration (MBEC) assay[62]**:** Biofilm eradication assays involve three phases separated by wash steps, including (i) initial biofilm establishment on well surfaces without test compound; (ii) biofilm treatment with test compound; and (iii) recovery of viable biofilms in fresh medium alone. Both assays were used to demonstrate the biofilm eradication activities of compound **4g**. Biofilm eradication assays were performed in 96-well plates, and microtitre wells were inoculated with 100 µL of a 1:1000-fold exponential-phase *S. aureus* and *E. coli* (10^8 CFU/mL) and were incubated for 24 h at 37 °C. After 24 h, medium and planktonic

cells were removed and 100 μL of two-fold serial dilutions of test compound was added to the wells in fresh medium and was incubated for 24 h at 37 °C (phase 2). After this time, the contents were removed and 100 μL of fresh medium only was added to allow viable biofilms to recover and to disperse planktonic bacteria into the medium resulting in a turbid microtitre well (24 h incubation at 37 °C; phase 3). After this final phase, microtitre plates were examined for visible bacterial growth (turbidity) and the MBEC was recorded as the lowest concentration at which no turbidity could be observed (due to eradicated biofilms).

Minimum biofilm inhibitory concentration (MBIC) determination [62]**:** In 96-well plates, two-fold serial dilutions of test compound **4g** was added in MHB medium. Then, 1:1000-fold exponential-phase *S. aureus* and *E. coli* (10^8 CFU/mL) in MHB was added to each well and was allowed to incubate at 37 °C for 24 h. After this time, the con-tents from the 96-well plates were removed and the wells were rinsed with water, followed by the addition of 100 μL of 0.1% crystal violet to stain the biofilms (10 min incubation at room temperature). The plates were then rinsed and 100 μL of ethanol was added to dissolve the crystal violet stained biofilms. Minimum concentrations required to inhibit 80% of biofilm formation ($MBIC_{80}$) were determined (OD_{540}) by comparing compound treated versus untreated wells and the resulting data were used to generate dose–response curves using Spss 20.0. Note: these experiments were performed to determine whether the antibiofilm activities of compound **4g** were dependent on or independent of their antibacterial activities.

Cytoplasmic membrane depolarization assay: The 4-6 h grown bacteria (mid-log phase) were harvested (3,500 rpm, 5 min), washed and resuspended with 1×PBS (*S. aureus*) and 5 mM HEPES buffer, 5 mM glucose and 100 mM KCl solution at 1:1:1 ratio (*E. coli*). Then the bacterial suspension (~10^8 CFU/mL, 150 μL) was added to the wells of a 96-well plate (Black plate, clear bottom with lid). Then 3, 3′-dipropylthiadicarbocyanine iodide (diSC35) (10 μM, 50 μL) was added to the wells containing bacterial suspension and pre-incubated for about 30 min for *S. aureus* and 40 min for *E. coli* (additional 50 μL of 200μM of EDTA was also added in case of *E. coli*). After the incubation, fluorescence was measured for the next 8 min at every 2 min

interval at an excitation wavelength of 622 nm (slit width: 10 nm) and emission wavelength of 670 nm (slit width: 5 nm). Bacterial suspensions were then transferred to another well-plate containing 10 μL of 420 μg/mL of small molecules (**4a-4u**) and fluorescence intensity was monitored immediately for another 12 min at every 2 min interval, the final concentration of small molecules (**4a-4u**) was 20 μg/mL. A control experiment was performed by treating the preincubated bacterial suspension and dye solution only with sterile Milli-Q water (50 μL).

Outer membrane permeabilization assay: The outer membrane permeabilization activity of the small molecules **(4a-4u)** was determined by the N-phenylnapthylamine (NPN) assay. Mid-log phase bacteria (*E. coli*) were harvested similarly as mentioned in earlier experiments, washed and resuspended similarly as the previous method. Bacterial suspension (~10^8 CFU/mL, 150 μL) was transferred into the wells of a black 96-well plate. Then NPN dye (10 μM, 50 μL) was added to the wells containing bacterial suspension and pre-incubated for about 30 min for *S. aureus* and 40 min for *E. coli*. After the incubation, fluorescence was monitored for next 8 min at every 2 min interval at an excitation wavelength of 350 nm (slit width: 10 nm) and emission wavelength of 420 nm (slit width: 5 nm). Then, the bacterial suspensions were transferred to another black well-plate containing 10 μL of 420 μg/mL of small molecules (**4a-4u**) and fluorescence intensity was monitored immediately for another 12 min at every 2 min interval, the final concentration of small molecules (**4a-4u**) was 20 μg/mL. A control experiment was performed by treating the preincubated bacterial suspension and dye solution only with sterile Milli-Q water (50 μL).

Inner membrane permeabilization assay: The 4-6 h grown bacteria (mid-log phase) were harvested (3500 rpm, 5 min), washed and resuspended similarly as the previous method. Then the bacterial suspension (~10^8 CFU/mL, 150 μL) was added to the wells of a 96-well plate (Black plate, clear bottom with lid). Then propidium iodide (PI) (10 μM, 50 μL) was added to the wells containing bacterial suspension and pre-incubated for about 30 min for *S. aureus* and 40 min for *E. coli*. After the incubation, fluorescence was measured for the next 8 min at every 2 min interval at an excitation wavelength of

535 nm (slit width: 10 nm) and emission wavelength of 617 nm (slit width: 5 nm). Bacterial suspensions were then transferred to another well-plate containing 10 μL of 420 μg/mL of small molecules and fluorescence intensity was monitored immediately for another 12 min at every 2 min interval, the final concentration of small molecules (**4a-4u**) was 20 μg/mL. A control experiment was performed by treating the preincubated bacterial suspension and dye solution only with sterile Milli-Q water (50 μL).

Propensity of bacterial resistance development: In order to evaluate the propensity of developing bacterial resistance towards the compounds, one of the potent compound **(4g)** was used in the study. First, MIC of compound **(4g)** was determined against *S. aureus* and *E. coli*, and subsequently the compound was challenged repeatedly at the 1/2 MIC level. Two control antibiotics norfloxacin and colistin were chosen for *S. aureus* and for *E. coli*, respectively. In case of norfloxacin and colistin, the initial MIC values were determined against respective bacteria. After the initial MIC experiment, serial passaging was initiated by transferring bacterial suspension grown at the sub-MIC of the compound/antibiotics (at MIC/2) and was subjected to another MIC assay. After 24 h incubation period, cells grown at the sub-MIC of the test compound/antibiotics were once again transferred and assayed for MIC experiment. The process was repeated for 20 passages for both *S. aureus* and *E. coli* respectively. The MIC for test compound to the control antibiotics was plotted against days to determine the propensity of bacterial resistance development.

Hemolytic activity: Red blood cells (RBCs) were isolated from sheep blood and resuspended in 1×PBS (5%). RBC suspension (150 μL) was then added to solutions of serially diluted small molecules **(4a-4u)** at the concentration of (5120, 2560, 1280, 640, 320, 160, 80, 40, 20, 10 μg/mL) in a 96-well plate (50 μL). Two controls were prepared, one 50 μL RBC suspension (5%) and the other with 50 μL of 0.1% solution of Triton X-100. The plate was then incubated for 1 h at 37 °C. After the incubation, the plate was centrifuged at 3,500 rpm for 5 minutes. Supernatant (100 μL) from each well was then transferred to a fresh 96-well plate and absorbance at 540 nm was measured. Percentage of hemolysis was determined as $(A–A_0) / (A_{total}–A_0) \times 100$, where A is the

absorbance of the test well, A_0 is the absorbance of the negative control (5% RBC suspensions), and A_{total} the absorbance of wells with 0.1% Triton X-100).

Cytotoxicity study: Cytotoxicities of the small molecules were evaluated by the Cell Counting Kit-8 (CCK-8). Briefly, 5×10^3 cells in 100 μL medium were seeded to each of 96-well plates. After 24 h incubation at 37 °C, the culture medium was removed and replaced with fresh medium (100 μL) containing the candidate compounds (**4g** and **4k**) in different concentration. And only media was used as negative control. At the end of the treatment (24 h), the medium was discarded and washed twice with the new culture medium, then added 100 μL new medium (with 5% CCK-8) to each well. Cells were incubated at 37 °C for a further 4 h and then the absorbance at 450 nm was measured using a Microplate Reader. Results were expressed as percent viability = $[A\text{-}A_0 / A_{nc}\text{-}A_0] \times 100\%$, where A is the absorbance of the treated cells, A_{nc} is the absorbance of the negative control and A_0 is absorbance of the background (new medium containing 5% CCK-8). The average 50% inhibitory concentration (IC_{50}) was determined from the dose-response curves according to the inhibition ratio for each concentration. Each concentration was analyzed in triplicate and the experiment was repeated three times.

Fluorescence and electron scanning microscopy: As mentioned above for the cytotoxicity study, cells were seeded into the wells of a 12-well plate and then treated with compounds (**4g** and **4k**) at various concentrations (1, 2, 4, 8 and 16μg/mL). For positive control 0.1% Triton X-100 was used. All the treated and untreated cells (as negative control) were washed once with 1×PBS (the images were captured with a 10× objective in electron microscope) and stained with 2 μM calcein AM (Fluka) and 4.5 μM propidium iodide (PI) (Sigma-Aldrich) (500 μL of 1:1 calcein AM:PI) for 15 min at 37 °C under 5% CO_2-95% air atmosphere. Finally, the images were captured with a 10× objective in fluorescence microscope using a band-pass filter for calcein AM at 500-550 nm and a long-pass filter for PI at 590-800 nm.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at

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1) A series of dialkyl cationic amphiphiles bearing two same length lipophilic alkyl chain and one non-peptidic amide bonds, were synthesized and tested for antimicrobial activities.
2) The MIC values of the best compound **4g** ranged from 0.5 μg/mL to 2 μg/mL against all these strains.
3) Preliminary mechanistic studies suggested that these compounds were rapidly bactericidal agents primarily acted by permeabilization and depolarization of bacterial membrane.
4) Compound **4g** was difficult to induce bacterial resistance and potential to combat drug-resistant bacteria.