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Lipidated Peptidomimetics with Improved Antimicrobial Activity

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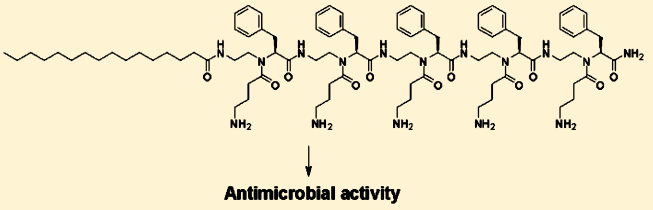
Supporting Information

ABSTRACT: We report a series of lipidated α-AApeptides that mimic the structure and function of natural antimicrobial lipopeptides. Several short lipidated α-AApeptides show broad-spectrum activity against a range of clinically related Gram-positive and Gram-negative bacteria as well as fungus. Their antimicrobial activity and selectivity are comparable or even superior to the clinical candidate pexiganan as well as previously reported linear α-AApeptides. The further development of lipidated α-AApeptides will lead to a new class of antibiotics to combat drug resistance.



KEYWORDS: *antimicrobial peptides, peptidomimetics, drug resistance, lipidation, α-AApeptides*

Antimicrobial peptides (AMPs) are found in most living organisms as an integral component of their innate defense against invading pathogens.[1,2] Unlike conventional antibiotics that target specific substrates involved in bacterial metabolism, AMPs are believed to kill bacteria via a nonspecific interaction with their membranes, which has less chance of inducing the development of resistance by bacteria.[1,2] Short cationic amphiphilic AMPs tend to interact with the negatively charged phospholipids on the bacterial membrane, which accounts for their selectivity for bacteria against eukaryotic cells whose membranes are more zwitterionic.[3,4] Because of their selectivity for bacteria, low propensity for development of drug resistance, and broad-spectrum antimicrobial potency, AMPs are considered an emerging class of antimicrobial agents.[1−3] Nevertheless, the therapeutic application of AMPs is impeded by their intrinsic instability in the context of proteolytic degradation.[1,2] Bactericidal peptidomimetics comprised of unnatural amino acids were thereby developed to circumvent the drawbacks of AMPs, which are protease-resistant and of improved bioavailability.[5] In recent years, several peptidomimetic analogues of AMPs, such as β-peptides,[6−9] arylamides,[10,11] and peptoids,[3,12,13] have received significant research interest.

Lipidated peptides such as polymyxin B[14] and daptomycin[15] are lipo-antibiotics, which possess fatty acid tails that are integral to their bactericidal activities. It has been shown that attachment of saturated linear fatty acids to peptide termini greatly enhanced AMPs' antimicrobial activities toward both Gram-positive and Gram-negative strains.[16−19] More recently, short peptoid mimetics alkylated with lipids of 10 or 13 carbons were demonstrated to bear improved selectivity, without losing any antimicrobial activities.[13] It was suspected that lipid alkylation improves the hydrophobicity of charged peptides[18] and facilitates the interaction with cytoplasmic membranes.[18] It is thereby envisioned that the development of lipidated peptidomimetics may overcome some of the drawbacks associated with current lipopeptide antibiotics. Herein, we report the development of lipidated α-AApeptides as potential antimicrobial agents. We have recently developed a novel class of peptidomimetics based on the α-chiral PNA (peptide nucleic acid) backbone, which is termed "α-AApeptides" (Figure 1), as

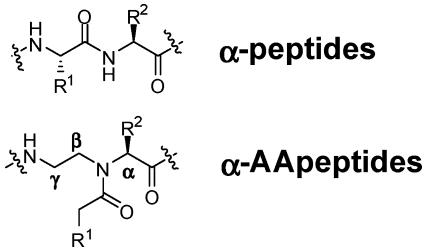


Figure 1. General structures of conventional α-peptides and α-AApeptides.

they contain N-acylated-N-aminoethyl amino acid residues. Chiral side chains of α-AApeptides are directly connected to the α-carbon of the building blocks, whereas the other half of the side chains are attached to backbone through acylation of the central N of the building blocks.

As compared to regular α-peptides, each building block of α-AApeptide is comparable to a dipeptide residue, and α-AApeptides of the same chain length display an identical number of side chains as regular peptides. As such, they can potentially be developed to mimic the structures and functions of peptides. There are a few advantages associated with α-

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AApeptides. Besides their resistance to proteolysis, α-AApeptides also feature limitless potential for diversification, which makes it a promising agent for biological applications, such as the inhibition of protein−protein interactions.[20] Notably, a 7-mer α-AApeptide **α1** was identified to kill both Gram-negative and Gram-positive bacteria and fungi, with a minimum hemolytic activity.[21] To further explore the potential antimicrobial application of α-AApeptide, as well as to develop shorter peptide mimics for synthetic and pharmacological benefits,[22−26] we now report our efforts in the development of lipidated antimicrobial α-AApeptides. Recently, we reported lipidated antimicrobial γ-AApeptides,[27] a closely related class of peptidomimetics to α-AApeptides. Therefore, it will be very interesting to find out the impact of lipidation of α-AApeptides on their antimicrobial activity.

The design of α-AApeptides follows the previously demonstrated principle[21,28] that a global distribution of cationic and hydrophobic side chains is key to antimicrobial activity and a defined secondary structure is unnecessary, as long as side groups can segregate into hydrophobic and cationic clusters upon interaction with bacterial membranes. As such, α-AApeptide building blocks were designed to be either amphiphilic or cationic, which were assembled and alkylated with C-16 hydrophobic lipid tail at the N terminus of the oligomers (Figure 2). The synthesis of lipidated α-AApeptides was carried out following a previous reported protocol,[29] and the N terminus of the last building block was capped with palmitic acid to achieve a final lipidation.

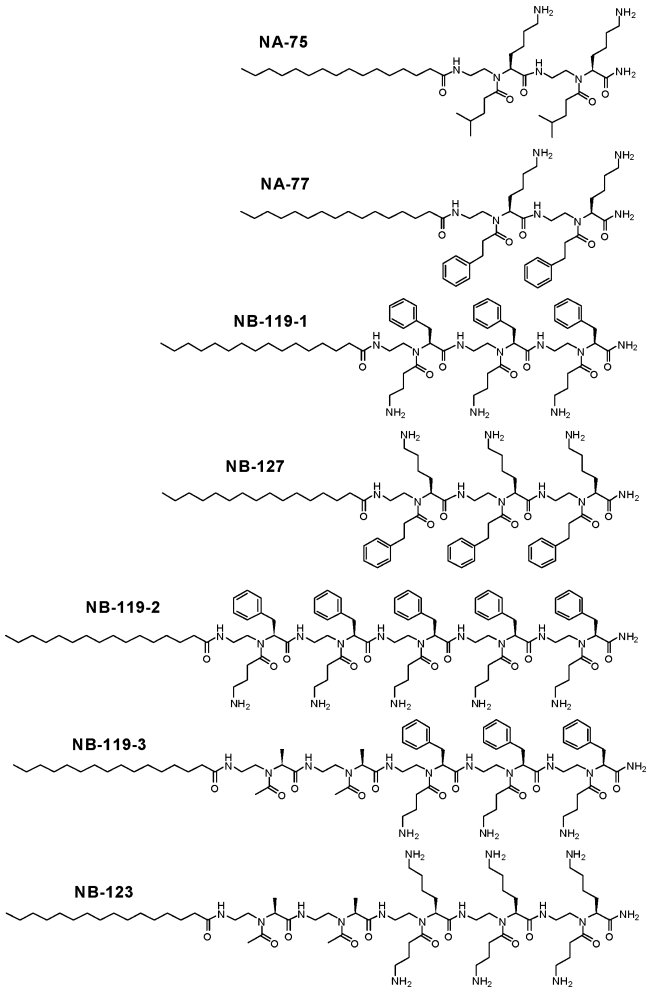

Figure 2. Structures of synthesized lipidated α-AApeptides.

These prepared lipidated α-AApeptides were tested for their antimicrobial activities against a range of bacterial strains including Gram-positive *Bacillus subtilis*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *Staphylococcus aureus*, Gram-negative *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, and fungus *Candida albicans*, many of which are multidrug-resistant strains. For the purpose of comparison, the previously reported most active linear α-AApeptide **α1**[21] as well as pexiganan, an AMP drug candidate,[3,13,30,31] were employed as positive controls.

The results have demonstrated the potential of lipidated α-AApeptides as a new generation of antibiotics with broad-spectrum activity. Being very short lipidated α-AApeptides, NA-75 and NA-77 (Figure 2) appeared to be ineffective against Gram-negative strains; however, they are very active against Gram-positive strains, including multidrug-resistant strains VREF (vancomycin-resistant *E. faecalis*) and MRSA (methicillin-resistant *S. aureus*), and fungus *C. albicans* (Table 1). Meanwhile, they almost had no hemolytic activity (Table 1), which augments their potential development as antifungal agents and antibacterial agents to treat Gram-positive bacterial infections. The activity toward Gram-negative bacterial strains was achieved by introducing additional α-AApeptide building blocks. For instance, NB-119-1 and NB-127 are 3-mer oligomers. Although NB-127 was still not active toward Gram-negative bacteria, NB-119-1 displayed an adequate activity against both Gram-positive and Gram-negative strains. In addition, NB-119-1 possessed a much lower toxicity (weaker hemolytic activity) than NB-127. Furthermore, the lipidated 3-mer NB-119-1 already displayed an improved activity than the linear 7-mer **α1**[21] made from the same building block, indicating that lipidation significantly enhanced the antimicrobial activity of α-AApeptides. Broad-spectrum and potent antimicrobial activity was further improved by the addition of more α-AApeptide building blocks. NB-119-2, NB-119-3, and NB-123 are all active toward all tested strains and are more potent than both pexiganan and linear **α1**. The results are consistent with general research findings that a certain degree of hydrophobicity has to be reached for an antimicrobial agent to be active. Both lipidation and addition of more α-AApeptide building blocks increase the hydrophobicity of sequences and thereby enhance the broad-spectrum activity against Gram-negative stains. It is noticeable that hemolytic activity is not always related to the overall hydrophobicity. For instance, NB-119-3 contains more hydrophobic building blocks than NB-127; however, it is much more active toward Gram-negative bacteria and less hemolytic. Generally, a longer sequence of cationic charges and hydrophobic groups can lead to improved overall activity, which is similar to previous observations.[21] They are also selective because none of them has toxicity toward red blood cells (Table 1).

As AMPs usually employ a membrane disruption mechanism to circumvent drug resistance occurring in the use of conventional antibiotics, it is essential to evaluate whether lipidated α-AApeptides can also adopt a similar bactericidal mechanism. Lipopeptide antibiotics polymyxin B and daptomycin do not adopt such mechanisms because they are only active toward either Gram-negative or Gram-positive bacterial strains, respectively. As such, one of the most potent lipidated α-AApeptide, NB-119-2, was assessed by fluorescence microscopy. Both Gram-negative *E. coli* and Gram-positive *B. subtilis*

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Table 1. Antimicrobial and Hemolytic Activities of Oligomers[a]

organism	MIC (μg/mL)								
	NA-75	NA-77	NB-119-1	NB-127	NB-119-2	NB-119-3	NB-123	pexiganan[3,13,30,31]	linear **α1**[21]
Gram-positive									
B. subtilis	2	4	2	2	4	2	4	4	2
S. epidermidis (MRSE)	10	15	8	20	10	4	10	8	10
E. faecalis (VREF)	1	3	4	10	4	4	20	32	>50
S. aureus (MRSA)	5	8	4	8	8	4	8	16	>50
Gram-negative									
E. coli	>50	>50	8	>50	4	30	4	8	4.5
K. pneumoniae	>50	>50	>50	>50	8	8	20	8	>50
P. aeruginosa	>50	>50	20	>50	12	8	10	8–16	>50
fungus									
C. albicans	2	2	3	>50	4	20	10	124	20–30
hemolysis (H_{10}/H_{50})	>500/>500	>500/>500	50/300	15/150	20/250	40/400	100/ >500	181/495	400/>500

[a]The minimum inhibitory concentration (MIC) for bacteria is the lowest concentration that completely inhibits growth after 24 h, and the MIC for fungus *C. albicans* is the lowest concentration that completely inhibits growth after 48 h. Pexiganan and Linear **α1** were used as controls. H_{10} is the concentration of α-AApeptides at which 10% hemolysis was observed, and H_{50} is the concentration of α-AApeptides at which 50% hemolysis was observed.

were treated with NB-119-2 and then stained by 4′,6-diamidino-2-phenylindole (DAPI), which stains all bacterial cells, and propidium iodide (PI) dye, which selectively penetrates dead or injured cells with damaged membranes[32,33] (Figure 3). Without incubation with NB-119-2, both *E. coli* and *B. subtilis* were stained in blue by DAPI, while little of them had PI staining (red fluorescence). However, both bacteria showed a strong red fluorescence as a result of PI staining after incubation with NB-119-2 for 2 h, suggesting significant membrane damage. The aggregation of injured or dead cells in both oligomer-treated *E. coli* and *B. subtilis* is consistent with previous reports,[32] which indicates a loss of membrane potential. Collectively, the lipidated α-AApeptide effectively inhibits bacteria, via a membrane-lysis mechanism similar to natural AMPs but different from lipo-antibiotics such as polymyxin B[34] and daptomycin.[35] This result further augments the potential of lipo-α-AApeptide as a new class of peptidomimetics to combat drug resistance occurring in bacteria during treatment of conventional antibiotics.

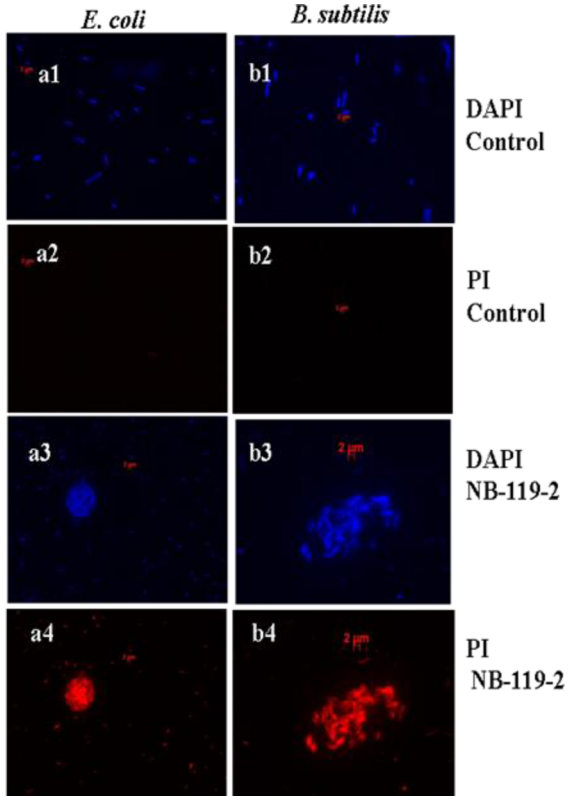

Figure 3. Fluorescence micrographs of *E. coli* and *B. subtilis* treated with 10 μg/mL of NB-119-2 for 2 h. a1–a4, *E. coli*; b1–b4, *B. subtilis*. A1 and b1, control, no treatment, DAPI stained; a2 and b2, control, no treatment, PI stained; a3 and b3, NB-119-2 treatment, DAPI stained; and a4 and b4, NB-119-2 treatment, PI stained.

Taken together, we have reported a series of lipidated α-AApeptides that feature significant antimicrobial activities. Through preliminary structure and activity studies, several short lipidated α-AApeptides, such as NB-123, NB-119-2, and NB-119-3, have been identified to have comparable or even superior activity and selectivity to the clinical candidate pexiganan, as well as the previously reported linear α-AApeptide **α1**. Because of the activity enhancement by lipidation, these oligomers have a relatively shorter backbone than the traditional linear **α1**. Mechanistic study shows that these lipidated α-AApeptides can mimic AMPs by disrupting bacterial membranes, which potentially circumvents drug resistance. Among all of the tested lipidated and regular α-AApeptides, NB-123, NB-119-2, and NB-119-3 bear the most potent and broad-spectrum antimicrobial activity and the highest selectivity. They are better than pexiganan in terms of the overall performance and thus could be promising novel antibiotic candidates in the future.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental procedure and characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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