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Amphiphilic Cationic β^{3R3}-Peptides: Membrane Active Peptidomimetics and Their Potential as Antimicrobial Agents

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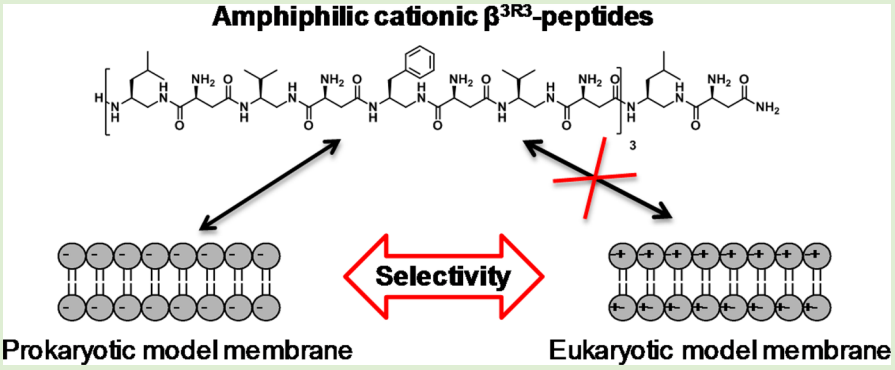
Supporting Information



ABSTRACT: We introduce a novel class of membrane active peptidomimetics, the amphiphilic cationic β^{3R3}-peptides, and evaluate their potential as antimicrobial agents. The design criteria, the building block and oligomer synthesis as well as a detailed structure−activity relationship (SAR) study are reported. Specifically, infrared reflection absorption spectroscopy (IRRAS) was employed to investigate structural features of amphiphilic cationic β^{3R3}-peptide sequences at the hydrophobic/hydrophilic air/liquid interface. Furthermore, Langmuir monolayers of anionic and zwitterionic phospholipids have been used to model the interactions of amphiphilic cationic β^{3R3}-peptides with prokaryotic and eukaryotic cellular membranes in order to predict their membrane selectivity and elucidate their mechanism of action. Lastly, antimicrobial activity was tested against Gram-positive *M. luteus* and *S. aureus* as well as against Gram-negative *E. coli* and *P. aeruginosa* bacteria along with testing hemolytic activity and cytotoxicity. We found that amphiphilic cationic β^{3R3}-peptide sequences combine high and selective antimicrobial activity with exceptionally low cytotoxicity in comparison to values reported in the literature. Overall, this study provides further insights into the SAR of antimicrobial peptides and peptidomimetics and indicates that amphiphilic cationic β^{3R3}-peptides are strong candidates for further development as antimicrobial agents with high therapeutic index.

INTRODUCTION

Bacterial resistance to conventional antibiotics has been recognized as one of the greatest public health threats in the 21st century.[1] Thus, alternative strategies and new molecules for antimicrobial treatment are a major focus of synthetic and pharmaceutical chemistry. Cationic antimicrobial peptides (AMPs) form part of the innate immune response to microbial and viral infection in many organisms and are considered a potential source of future antibiotics because of their particular mechanism of action compared to conventional antibiotics. AMPs are a major class of membrane-active peptides (MAPs) which strongly interact with negatively charged microbial surfaces leading to perturbation and disruption of membranes. Thereby, and in contrast to conventional antibiotics, AMPs can rapidly kill a broad-spectrum of microbes with a decreased likelihood of resistance development.[2] Besides antivirulence strategies that are pursued to avoid rapid developemt of resistances,[3,4] the development of novel antibiotics with new modes of action, such as antimicrobial peptides, is required. However, despite intense research efforts aimed at developing AMPs as therapeutic agents, limited clinical outcome has resulted so far.[5,6] A major disadvantage of natural AMPs is their protease liability related to the peptide formula per se that dominates their unfavorable pharmacokinetic profile.[7,8] In addition, most of natural AMPs display either modest direct antimicrobial activity or indiscriminant toxicity to prokaryotic and eukaryotic cells.[7,9] In order to overcome these major limitations and to realize the therapeutic potential of AMPs, different classes of antimicrobial peptidomimetics have been developed with promising results.[10−14] In particular, amphi-

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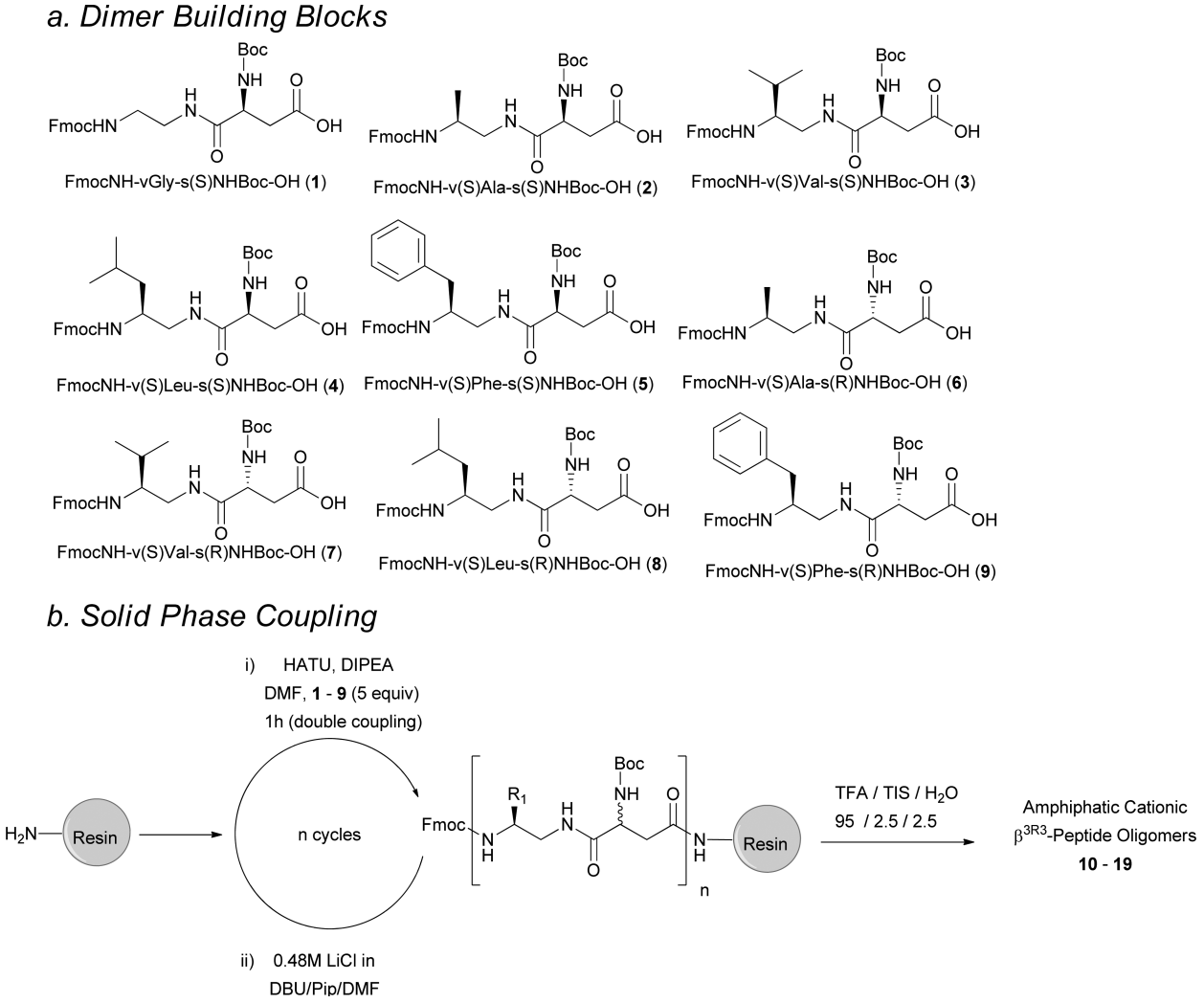

Figure 1. Synthetic strategy applied for the synthesis of β^{3R3}-peptides. (a) β-diamine and β-diacid monomers were synthesized and coupled in solution affording a small library of dimer building blocks (**1**–**9**). (b) These building blocks were employed for the solid phase synthesis of amphiphilic cationic β^{3R3}-peptide oligomers (**10**–**19**).

philic cationic β-peptides with helical conformation have prompted expectations,[13−15] as they can reproduce the physicochemical features of AMPs but exhibit improved proteolytic stability.[16,17] Such β-peptides have proven good antimicrobial activity,[18−22] but still exhibit significant hemolytic activity,[18,20−22] that indicates indiscriminant cytotoxicity and substantially hampers the development of these molecules as therapeutics. Recently, we established the β^{3R3}-peptides as a novel class of peptidomimetics introducing alternating directions of the amide bonds along β-peptide sequences and extending the structural space available to β-peptides while maintaining their enzymatic stability.[23] Here, we extend this class of peptidomimetics and present for the first time amphiphilic cationic β^{3R3}-peptides. The novel qualities of the β^{3R3}-peptide backbone and the combination of proteinaceous side chains allow for fine-tuning of the physicochemical properties and thus also should allow for fine-tuning of their membrane activity. Therefore, such amphiphilic cationic β^{3R3}-peptides can be expected to have great potential as membrane active peptidomimetics and antimicrobial agents. Complete control over the building blocks' chemical features as well as oligomer assembly allows us to perform a detailed structure−activity relationship (SAR) study aiming at disclosing correlations between primary sequence, physicochemical and biological properties of the amphiphilic cationic β^{3R3}-peptides and to derive a first set of design rules for the next generation of antimicrobial peptidomimetics. Specifically, we report on the design criteria, the building block and oligomer synthesis, and the physicochemical and biological characterization of amphiphilic cationic β^{3R3}-peptide sequences and investigate their potential as membrane active peptidomimetics and antimicrobial agents.

MATERIALS

Commercial grade reagents and solvents were used as purchased without further purification, except as indicated below. Fmoc-α-amino acids, Boc-L-Asp-OBn, and Boc-D-Asp-OBn were purchased from Iris Biotech, HATU, PyBOP and HOBt from Nova Biochem, and all other reagents from Aldrich; all were used without further purification. All solvents were HPLC grade. Regarding DCM, amylene-stabilized HPLC grade was chosen. The solid-support resins were purchased from Rapp Polymers. Tentagel S RAM resin (loading 0.23 mmol/g) (Fmoc-protected) was used as purchased.

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RESULTS AND DISCUSSION

Building Block Design and Synthesis. Despite their diversity in primary sequence and secondary structures, AMPs generally share a cationic and globally amphiphilic character[24] that is essential for the mode of action of AMPs and the ability of membrane active peptidomimetics to interact with a hydrophobic and negatively charged biomembrane.[2,25] Therefore, these physicochemical properties are also key features of amphiphilic cationic β^{3R3}-peptides. The synthesis of β^{3R3}-peptides is generally based on the solid phase coupling of dimer building blocks of β-diamines and β-diacids.[23] Here, we introduce a novel set of building blocks suitable for standard solid phase peptide chemistry introducing both, cationic and hydrophobic side chains. In total nine different dimer building blocks were synthesized presenting variations on their side chains and chiral configurations (Figure 1).

Earlier we presented an efficient synthetic protocol to produce Fmoc protected chiral β-diamines with proteinaceous side chains.[26] This route was applied using L-Ala, L-Val, and L-Leu as starting materials.[23] Here, this protocol was extended to L-Phe. The nomenclature of β^{3R3}-peptides monomers refers to the *v*/*s* prefix system in which *v*Xaa symbolizes the vicinal diaminoalkyl analogue of the indicated amino acid residue Xaa and *s*Xaa symbolizes succinyl analogues. All diamine subunits were synthesized in 30 g scale with overall yield ranging from 39% (*v*Ala) to 44% (*v*Phe). In addition, the nonchiral analogue of Gly (*v*Gly) was prepared via a novel, convenient route that afforded FmocNH-*v*Gly-NH_2·TFA in large scale (50 g) and with high yield (75%). A major synthetic advantage of using β-diamines and β-diacids as building blocks is the pool of commercially available chiral β-diacids with functionalized side chains, such as aspartic acid (Asp). Commercially available Boc-L-Asp-OBn and Boc-D-Asp-OBn were exploited as precursors of the succinyl analogues *S*-amino succinic acid ((S)*s*NH_2) and *R*-amino succinic acid ((R)*s*NH_2), as Boc and Benzyl (Bn) groups are orthogonal to the Fmoc-protecting group of the β-diamine counterparts. Referring to our dimer strategy,[26−29] the Fmoc protected chiral β-diamines and the monoprotected diacid Boc-Asp-OBn were combined in solution using a PyBOP/HOBt-mediated coupling reaction in DMF with DIEA as base. Notably, we developed a novel purification procedure based on sequential precipitations that removed hydrophilic and hydrophobic impurities efficiently to provide the products in high purity while avoiding time-consuming chromatographic purification. Thus, this procedure allows for performing different reactions in parallel and easy scale up. In the next step, the benzyl ester (OBn) was deprotected via catalytic hydrogenation with Pd on carbon (Pd/C) in DMF. Finally, precipitation in Et_2O/Hex mixture afforded the desired products. Conclusively, all synthetic routes fulfill the requirements for the synthesis of building blocks suitable for solid phase synthesis, employing readily available starting materials and giving high yields on multigram scale. These procedures rely exclusively on simple and convenient purification methods and no chromatographic purification was needed during the 8 steps synthesis. Finally, all building blocks (**1**−**9**) were obtained in high purity (>99%), as shown by NMR and HPLC (see the Supporting Information).

Oligomer Synthesis. The library of dimer building blocks (**1**−**9**) was then directly employed for solid phase synthesis of ten different amphiphilic cationic β^{3R3}-peptide sequences (**10**−**19**) (Figure 2) differing in their chain length, side chain pattern and chiral configuration. The β^{3R3}-peptides were synthesized via automated solid-phase synthesis on a standard peptide synthesizer and Fmoc amino acid coupling protocols using double coupling with five equivalents building blocks and coupling times of 1 h with HATU as an activation agent. At first, the use of 25% piperidine in DMF resulted in inefficient Fmoc deprotection, which could be attributed to a strong tendency of the hydrophobic residues to aggregate on the solid support.[21] Extending the time of deprotection did also not result in complete removal of Fmoc groups (see the Supporting Information for the Fmoc cleavage UV-patterns and HPLC traces). This challenge was successfully overcome by the use of LiCl in 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), piperidine, and DMF as mixture for Fmoc deprotection.[30] In the last step, the oligomers were cleaved from the resin using acidic conditions (95% TFA, 2.5% triisopropylsilane, and 2.5% water) releasing C-terminal carboxamides and simultaneously deprotecting the Boc groups on the *s*NH_2 units. All oligomers of up to 13 dimer building blocks, a chain length corresponding to 26 β-amino acids, were obtained in very high purity (>95%) after precipitation in cold Et_2O (see the Supporting Information for HPLC traces). Noteworthy, for the parent β^3-peptides, the successful synthesis of peptides made up of 24 amino acids is considered to be a good indication that every desired peptide sequence should be accessible.[31] Thus, it is remarkable that the developed synthetic strategy gives access to long and highly pure β^{3R3}-peptide sequences directly from natural α-amino acids without the need for any chromatographic purification along the complete synthetic route.

H_2N-(*v*Gly-(S)*s*NH_2)$_9$-$CONH_2$ (**10**)

H_2N-(*v*Ala-(S)*s*NH_2-*v*Val-(S)*s*NH_2-*v*Phe-(S)*s*NH_2-*v*Val-(S)*s*NH_2)$_2$-*v*Ala-(S)*s*NH_2-$CONH_2$ (**11**)

H_2N-(*v*Gly-(S)*s*NH_2-*v*Leu-(S)*s*NH_2-*v*Phe-(S)*s*NH_2-*v*Leu-(S)*s*NH_2)$_2$-*v*Gly-(S)*s*NH_2-$CONH_2$ (**12**)

H_2N-(*v*Ala-(S)*s*NH_2-*v*Val-(S)*s*NH_2-*v*Leu-(S)*s*NH_2-*v*Val-(S)*s*NH_2)$_2$-*v*Ala-(S)*s*NH_2-$CONH_2$ (**13**)

H_2N-(*v*Leu-(S)*s*NH_2-*v*Val-(S)*s*NH_2-*v*Phe-(S)*s*NH_2-*v*Val-(S)*s*NH_2)$_2$-*v*Leu-(S)*s*NH_2-$CONH_2$ (**14**)

H_2N-(*v*Leu-(S)*s*NH_2-*v*Val-(S)*s*NH_2)$_4$-*v*Leu-(S)*s*NH_2-$CONH_2$ (**15**)

H_2N-(*v*Phe-(S)*s*NH_2-*v*Val-(S)*s*NH_2)$_4$-*v*Phe-(S)*s*NH_2-$CONH_2$ (**16**)

H_2N-(*v*Leu-(R)*s*NH_2-*v*Val-(R)*s*NH_2-*v*Phe-(R)*s*NH_2-*v*Val-(R)*s*NH_2)$_2$-*v*Leu-(R)*s*NH_2-$CONH_2$ (**17**)

H_2N-*v*Leu-(S)*s*NH_2-*v*Val-(S)*s*NH_2-*v*Phe-(S)*s*NH_2-*v*Val-(S)*s*NH_2-*v*Leu-(S)*s*NH_2-$CONH_2$ (**18**)

H_2N-(*v*Leu-(S)*s*NH_2-*v*Val-(S)*s*NH_2-*v*Phe-(S)*s*NH_2-*v*Val-(S)*s*NH_2)$_3$-*v*Leu-(S)*s*NH_2-$CONH_2$ (**19**)

Figure 2. Amphiphilic cationic β^{3R3}-peptide oligomers synthesized and investigated in this study. First, 7 homochiral (S,S) oligomers made of 9 dimer building blocks and different primary sequence (**10**−**16**) have been synthesized. The heterochiral (*S*,*R*) analogue (**17**) of sequence **14** was prepared. Lastly, shorter (**18**) and longer (**19**) analogues of sequence **14** have been synthesized made of 5 and 13 dimer building blocks, respectively.

Oligomer Design. Besides their general cationic and amphiphilic character, other physicochemical and structural factors determine the membrane activity and selectivity of natural AMPs.[2] These include sequence length, net charge, hydrophobicity, presence of aromatic side chains, as well as conformation and aggregation properties.[22] All of these parameters were also addressed for our β^{3R3}-peptides by choosing different primary sequences specifically varying, for example, the number of aromatic side chains. The development of membrane active amphiphilic cationic β^{3R3}-peptides with potential antimicrobial activity relies on a rational, hierarchical

tuning of these parameters by varying the residue composition of the primary sequence. Therefore, 10 different sequences of amphiphilic cationic β^{3R3}-peptides (**10**−**19**) were designed and synthesized. All the oligomers feature a C-terminal carboxamide moiety that generally enhances the activity of both natural and synthetic antimicrobial peptidic agents.[19,21] First, seven homochiral (*S*,*S*) sequences (**10**−**16**) made of nine dimer building blocks and the same number of net charges were synthesized (Figure 2). Similar chain lengths are usually targeted for short AMPs with potent antimicrobial activity and β-peptides investigated as antimicrobial peptidomimetics.[18−22,24] Within these oligomers, the diamine units *v*Gly, *v*Ala, *v*Val, *v*Leu, and *v*Phe were systematically interchanged to produce amphiphilic cationic β^{3R3}-peptide oligomers with variegated combinations of different proteinaceous side chains, aliphatic and aromatic, and thus different physicochemical features. Primarily, this set of side chains features a fine scale of hydrophobicity as one of the major requirements for the effective interaction with membranes and resulting antimicrobial activity. However, excessive hydrophobicity can also lead to unspecific cytotoxicity and increased hemolytic activity.[19,20] Furthermore, this hydrophobicity may lead to aggregation in aqueous media and result in reduced antimicrobial activity.[10] Thus, it is crucial to truly fine-tune the hydrophobic properties of the oligomers and find the right balance of hydrophobicity as demonstrated by the detailed SAR studies of this first set of oligomers.

The incorporation of aromatic residues is another important factor that influences the membrane activity of peptides and peptidomimetics. Therefore, sequences **10**−**16** also present different numbers of *v*Phe units that can result in $\pi-\pi$ interactions with unsaturated fatty acids to potentially differentiate microbial and human cytoplasmic membranes.[32]

In addition, structural factors can influence biological properties of AMPs as their membrane activity requires nascent or induced structural motifs producing amphiphilic macromolecular folds with spatial segregation of charges and hydrophobic moieties.[2,22] Recently, we have shown that the β^{3R3}-peptide backbone has an intrinsic propensity for an extended conformation at the air/water interface.[23] However, homochiral amphiphilic cationic β^{3R3}-peptides cannot segregate cationic and hydrophobic moieties in an extended conformation and are expected to fold in order to adopt globally amphiphilic macromolecular folds typical for cationic AMPs. In contrast, a heterochiral sequence is expected to adopt a globally amphiphilic structure in extended conformation. Therefore, the heterochiral (*S*,*R*) analogue (**17**) of sequence **14** was prepared. Lastly, shorter (**18**) and longer (**19**) analogues of sequence **14**, made of 5 and 13 dimer building blocks, respectively, have been synthesized to evaluate the influence of chain length on the biological activity of amphiphilic cationic β^{3R3}-peptides.

Physicochemical Characterization of Amphiphilic Cationic β^{3R3}-Peptides. After the synthesis and chemical characterization of the different amphiphilic cationic β^{3R3}-peptide sequences, we investigated their physicochemical properties. Specifically physicochemical parameters such as hydrophobicity, hydrodynamic radius, aggregation behavior and membrane activity will be determined and then correlated with the antimicrobial properties of the amphiphilic cationic β^{3R3}-peptides in dependence of their primary sequence.

Hydrophobicity is perhaps the most critical parameter for membrane activity and selectivity of AMPs.[2,25] Therefore, the hydrophobicity of the amphiphilic cationic β^{3R3}-peptides was determined via RP-HPLC as percent of acetonitrile required to elute each oligomer from a C18 analytical column (Table 1).

Table 1. Chemical Features, Hydrophobicity, and Hydrodynamic Diameter of the Amphiphilic Cationic β^{3R3}-Peptide Oligomers

oligomer	.	*[b]	Ar	H[c]	Ø[e]	π_{eq}[g]	amide I[h]
10	9	*s*S	0	0[d]	//[f]	0.2	1666.5 ± 1.1
11	9	*s*S	2	23.2	//	1.1	1656.1 ± 1.6
12	9	*s*S	2	26.6	//	3.6	1653.6 ± 1.5
13	9	*s*S	0	22.1	//	1.7	1661.2 ± 1.6
14	9	*s*S	2	29.9	0.7	6.8	1653.1 ± 1.2
15	9	*s*S	0	28.7	0.7	8.6	1661.3 ± 1.2
16	9	*s*S	4	29.9	2.5	5.4	1654.0 ± 1.0
17	9	*s*R	2	27.9	0.9	13.3	1648.2 ± 0.4
18	5	*s*S	1	23.2	0.8	0.4	1665.4[i] ± 1.5
19	13	*s*S	3	33.9	0.8	11.3	1654.0 ± 0.9

[a]*n* stands for number of building blocks. [b]Asterisk (*) refers to the absolute configuration of stereocenter on the diacid sNH_2 units. [c]Hydrophobicity measure determined as percentage acetonitrile eluent in RP-HPLC analysis 5% to 95% MeCN in 60 min, using a C18 column. [d]Oligomer **10** is eluted within the injection peak. [e]Hydrodynamic diameter in nm measured by DLS. [f]The oligomer was not characterized. [g]Equilibrium surface pressure in mN/m obtained by surface tension measurements. Error bars: ±1 mN/m for all pressures. [h]Amide I band position in cm^{-1} observed by IRRAS. [i]Note the very weak intensity of the band to be univocally characterized.

This approach has been established to measure the lipophilicity of various molecules,[33] including antimicrobial β-peptides.[21] In general, within homochiral sequences of the same chain length (**10**−**16**), the relative values achieved can be linearly correlated to the hydrophobicity of the proteinaceous side chains on the diamine residues *v*Xaa. The most hydrophobic oligomers are compounds **14** and **16** that feature the same values of hydrophobicity (H = 29.9), sequence length, and net charge, and exclusively differ in the number and density of aromatic *v*Phe units, with three *v*Leu units of oligomer **14** replaced by three *v*Phe in oligomer **16** (Figure 2). Therefore, the direct comparison of compounds **14** and **16** allows us to evaluate the effect of $\pi-\pi$ interactions on the conformation and aggregation properties, and thus on the membrane and biological activity of amphiphilic cationic β^{3R3}-peptides.

Interestingly, the RP-HPLC analysis showed higher hydrophobicity for the homochiral (*v*S,*s*S) oligomer **14** than the heterochiral (*v*S,*s*R) analogue **17** presenting the same primary sequence. Longer retention time generally correlates with a greater propensity to adopt a globally amphiphilic conformation, as observed for α- as well as β-peptides.[21,34] Thus, this increase in retention time can be considered an indication that homochiral sequences might fold to result in longer retention times, while their heterochiral analogues might adopt a more extended conformation.

In addition, the chain length proved to have a strong influence on the hydrophobicity of amphiphilic cationic β^{3R3}-peptides as increasing the chain length leads to an increase in hydrophobicity (compare oligomers **18** (H = 23.2), **14** (H = 29.9) and **19** (H = 33.9) featuring the same side chain pattern and exclusively differing in the number of repeats). Furthermore, DLS was employed to determine the size distribution profile of the oligomers in solution (Table 1) and their potential aggregation in aqueous media. Oligomer **14**

Table 2. Surface Tension Experiments with Oligomers 14, 17, and 19 and Model Membranes

		oligomer[a]		
		14	17	19
bare	π_{eq}[c]	6.8	13.3	11.3
POPG $\pi_{inj}0$[b]	$\Delta\pi$[d]	11.4	16.6	14.0
POPC $\pi_{inj}0$	$\Delta\pi$	7.9	13.9	12.5
POPG $\pi_{inj}30$	ΔA[e]	11.8 (12.7%)	13.8 (14.4%)	20.3 (20.3%)
POPC $\pi_{inj}30$	ΔA	1.6 (1.8%)	−0.7 (−0.8%)	−1.0 (−1.1%)

[a]Injected into the subphase to obtain a concentration of 500 nM. [b]πinjX stands for the initial surface pressure at X mN/m of the lipid monolayer before the peptide injection. [c]π_{eq} stands for surface pressure in mN/m of the equilibrated oligomer monolayer. [d]$\Delta\pi$ is the surface pressure change. [e]ΔA stands for the change in surface area (cm^2) and percent of the equilibrated mixed oligomer−lipid monolayer to the lipid monolayer before the peptide injection. Error bars: ± 1mN/m for all pressures; ±2% for areas.

served as reference sequence with proper hydrophobicity and altered pattern of side chains. In comparison, oligomers **18** and **19** present the same side chain pattern but different chain length. In contrast, oligomer **15** features the same chain length but different side chains. Lastly, oligomer **17** (*v*S,*s*R) differs from oligomer **14** (*v*S,*s*S) by the chiral configuration of the diacid *s*NH_2 units. Regardless of the chain length and the side chains, the oligomers **14**, **15**, **17**, **18**, and **19** produce similar profiles with average hydrodynamic diameters of around 0.7−0.8 nm. In contrast, oligomer **16** displays a significantly increased hydrodynamic diameter of around 2.5 nm that can be attributed to the formation of aggregates. Such aggregation in aqueous media could derive from the sequence density of aromatic *v*Phe side chains that can generate both hydrophobic and $\pi-\pi$ interactions. Oligomer **14** features the same value of hydrophobicity (H = 29.9) as oligomer **16** (Table 1) but contains a lower number and density of aromatic *v*Phe units (Figure 2), therefore we mainly attribute the aggregation behavior to $\pi-\pi$ interactions of oligomer **16**. Lastly, surface tension measurements and IRRAS were employed to evaluate quantitative and qualitative physicochemical determinants of the amphiphilic cationic β^{3R3}-peptides at the air/water interface (Table 1). Indeed, the air/water interface as the simplest hydrophobic/hydrophilic interface can mimic the interface of the cell membrane and thus gives a first indication on the potential membrane activity of the β^{3R3}-peptides.[35−47]

First, the equilibrium surface pressure of a 500 nM solution in buffer (10 mM PBS at pH 7.4 with 150 mM NaCl) was measured as an alternative mean to estimate the hydrophobicity of the oligomers **10**−**19** (Table 1). A general trend parallel to the values of hydrophobicity obtained by RP-HPLC is found. However, a stronger influence of the chain length and number of charges is observed compared to the hydrophobicity values achieved by RP-HPLC. In particular, the short oligomer **18** is not surface active. In addition, oligomer **16** shows a reduced equilibrium surface pressure. This could correlate with the tendency of oligomer **16** to form aggregates in solution, as seen in DLS experiments that are less surface active. In contrast, the heterochiral (*v*S,*s*R) oligomer **17** displays an extraordinary high equilibrium surface pressure. This observation can be explained by the potential to adopt an extended conformation segregating charges and hydrophobic moieties. Therefore, oligomer **17** could form strands that can assemble and align at the air/water interface and increase the surface concentration.

To further confirm this observation and provide qualitative structural information, the position of the amide I band in the IRRA spectra was analyzed (Table 1). All homochiral (*v*S,*s*S) oligomers **10**−**16**, **18**, and **19** present the amide I band at around 1660 (±6) cm^{-1} that is characteristic for conformations indicating intramolecular hydrogen bond networks, such as helices. In contrast, the heterochiral (*v*S,*s*R) oligomer **17** displays the amide I band at around 1648 cm^{-1}, typical for intermolecular hydrogen bond networks at the air/water interface.[38,42,44,48,49] Indeed, similar values have been observed for the hydrophobic β^{3R3}-peptide oligomers forming strands that self-assemble into amorphous β-sheet-like structures.[23]

Interaction of Amphiphilic Cationic β^{3R3}-Peptides with Model Membranes. Both surface tension and IRRAS shed light on the interactions with model membranes and thus the ability of the amphiphilic cationic β^{3R3}-peptides to penetrate lipid membranes.[50] As model membranes, the anionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (POPG) and the zwitterionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipids were employed to mimic prokaryotic and eukaryotic membranes, respectively. Both lipids have one unsaturated acyl chain and thus form stable monolayers in the liquid disordered state even at high surface pressures. Two sets of experiments were performed at different surface pressures using different amounts of lipid. The experiments were carried out on 10 mM PBS at pH 7.4 with 150 mM NaCl as aqueous subphase. In the first set of experiments, 25 μL of a 1 mM lipid solution in $CHCl_3$ were spread on the air/buffer interface and the monolayer was compressed to 30 mN/m. This surface pressure is discussed as the internal pressure in biological membranes.[51] Thereafter, the surface pressure was kept constant and changes in the surface area were monitored. In the second set of experiments, only 13 μL of the lipid solutions were spread and compressed to the same trough surface as in the previous experiment but with the surface pressure still at 0 mN/m close to the lift-off point of the isotherm. The area was kept constant and changes in the surface pressure were monitored. The amphiphilic cationic β^{3R3}-peptide oligomers were injected under the lipid film to obtain a concentration of 500 nM in the subphase and the resulting surface pressure and changes in surface area were detected, respectively (Table 2). Injection of the same amount of pure buffer did not create any changes in the monolayer characteristics.

In general, the equilibrium surface pressure after the injection of the oligomers below the uncompressed zwitterionic eukaryotic-like POPC monolayer at 0 mN/m is almost equal to the equilibrium surface pressure of the oligomers alone showing that the oligomer adsorption is solely driven by their surface activity. Moreover, the area of the compressed POPC monolayer at 30 mN/m does not change after the injection of the oligomers, which means that no insertion of the amphiphilic cationic β^{3R3}-peptide into the lipid layer or adsorption to the hydrophilic lipid head groups occurs. Differently, in the case of

a POPG monolayer at 0 mN/m, the equilibrium surface pressure observed after injection of the oligomers is significantly higher than the value previously registered at the bare buffer surface pointing to specific interactions between the oligomers and POPG. Additionally, the amphiphilic cationic β^{3R3}-peptides also insert into the lipid monolayer at a surface pressure of 30 mN/m and the area of the equilibrated mixed peptide-lipid monolayer is increased. Specifically, oligomer **19** gives the largest effect with an overall increase in surface area of 20%. Thus, the amphiphilic cationic β^{3R3}-peptides and especially oligomer **19** produce strong interactions with the negatively charged prokaryotic model membrane. In contrast, no interaction was observed with the zwitterionic eukaryotic membrane model. Therefore, the amphiphilic cationic β^{3R3}-peptides can be expected to display high and selective antibacterial activity without cytotoxicity versus eukaryotic host cells.

In addition, the position of the amide I band in the IRRA spectra in the presence of the model membranes was analyzed. Interestingly, an exclusive shift toward a higher wavenumber is observed in the amide I band of the heterochiral (*v*S,*s*R) oligomer **17** in the presence of the bacterial membrane mimicking POPG indicating a change in conformation (see the Supporting Information). Thus, similar to several natural AMPs,[2] lipid−peptide interactions seem to change the conformational state of the heterochiral amphiphilic cationic β^{3R3}-peptide. For the homochiral peptide of the same sequence, no shift of the amide I band was observed. Current studies are further evaluating this finding.

Antibacterial Activity of Membrane Active β^{3R3}-Peptides. After the comprehensive physicochemical characterization proving the general potential of amphiphilic cationic β^{3R3}-peptides for high and selective activity toward prokaryotic membranes, antibacterial growth assays have been performed for all oligomers (**10**−**19**). The Minimal Inhibitory Concentration (MIC) values were determined against *E. coli* (strain BL21) and *M. luteus* (type strain DSM 20030) as representative strains of both, Gram-negative and Gram-positive bacteria. Gram-positive bacteria have a thick peptidoglycan layer on the outside of the cell membrane while Gram-negative bacteria have a thick or thin peptidoglycan layer that is located between two cell membranes. Thus, differences can be expected for the membrane interactions with AMPs in dependence of the bacterial strain. Indeed, we see a strong dependency of the monomer sequence as well as the targeted organism on the resulting antibacterial activity (Table 3).

In general, the amphiphilic cationic β^{3R3}-peptides show higher activity against Gram-positive *M. luteus*. Within sequences of the same chain length of nine repeating units (**10**−**17**), differences in hydrophobicity showed a strong effect on the antibacterial activity. The most hydrophobic oligomers are **14**, **15**, and **16** (Table 1). Indeed, **14** is the most active antibacterial oligomer with a MIC of 10.7 μg/mL against *M. luteus*. In contrast, oligomerS **15** and **16** display reduced activity with MIC against *M. luteus* of 16.6 and 25.3 μg/mL, respectively. Obviously, the number of aromatic side chains has to be considered as well. The higher MIC value of oligomer **16** could derive from the aggregation in aqueous media related to the higher density of aromatic side chains which was observed in DLS experiments (Table 1). Indeed, self-assembly in aqueous media may result in decreased antimicrobial activity.[10] In contrast, oligomer **15** is slightly less hydrophobic, presents no aromatic side chains and is less active against Gram-positive *M. luteus* than **14** (Tables 1 and 3). However, oligomer **15** shows higher activity against Gram-negative *E. coli* (Table 3). Thus, the aromatic *v*Phe units seem to play an important role driving the membrane selectivity of amphiphilic cationic β^{3R3}-peptides.

Table 3. MIC Values of the Amphiphilic Cationic β^{3R3}-Peptide Oligomers 10−19[a]

oligomer	*M. luteus*	*E. coli*
10	no activity	no activity
11	no activity	no activity
12	no activity	no activity
13	no activity	87.4 μg/mL
14	10.7 μg/mL	101.9 μg/mL
15	16.6 μg/mL	64.2 μg/mL
16	25.3 μg/mL	108.8 μg/mL
17	29.2 μg/mL	130.6 μg/mL
18	no activity	no activity
19	5.3 μg/mL	43.2 μg/mL

[a]The MIC values have been extrapolated from the plots of antimicrobial activity using linear fit (for plots of antimicrobial activity, see the SI).

From the physicochemical characterization of the oligomers we know that also the chiral configuration of the sNH_2 diacid units seems to influence the conformation as well as the effective hydrophobicity of the oligomers. We expect to see a difference in antibacterial activity. Indeed, the heterochiral (*v*S, *s*R) oligomer **17** is less active than its homochiral (*v*S, *s*S) analogue **14** against both, M. luteus and *E. coli* (Table 3). This is again in agreement with a reduced hydrophobicity and could be also attributed to intermolecular sheet assembly of strands of oligomer **17** resulting in reduced antibacterial activity. We believe that this finding is in agreement with the differences observed for helical or non helical antimicrobial peptides and the differences in their mode of action. To draw further conclusions, a more detailed structural analysis will be required.

We observed that the chain length and number of charges of the amphiphilic cationic β^{3R3}-peptides extensively influence their antimicrobial activity. Generally antimicrobial activity increases with increasing numbers of repeating units and charges (Tables 1 and 3). Indeed, oligomers **14**, **18**, and **19** that feature the same side chain pattern and differ exclusively in the number of repeats (Figure 2) show very different antimicrobial activity. While the shortest oligomer **18** is not active, the longest oligomer **19** is the most active amphiphilic cationic β^{3R3}-peptide against both, *M. luteus* and *E. coli*, with MIC of 5.3 μg/mL (1.9 μM) and 43.2 μg/mL (15.4 μM), respectively. Such values are comparable to biologically important natural AMPs, such as mammalian defensins[52] or the most active antimicrobial β-peptides known from literature.[12] Therefore, this data clearly shows that amphiphilic cationic β^{3R3}-peptides can display potent antimicrobial activity in correlation to their primary sequence.

In order to further investigate the antimicrobial activity also in dependence of the targeted bacterial strain, antimicrobial peptides that showed activity against Gram-negative *E. coli* and Gram-positive *M. luteus* were then evaluated for their antibacterial activity against two additional, clinically relevant bacterial pathogens of both classes, *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* Newman, respectively (Figure 3). Furthermore, the antimicrobial peptide MAX1[53] was used as a control. Here, none of the tested peptides showed measurable

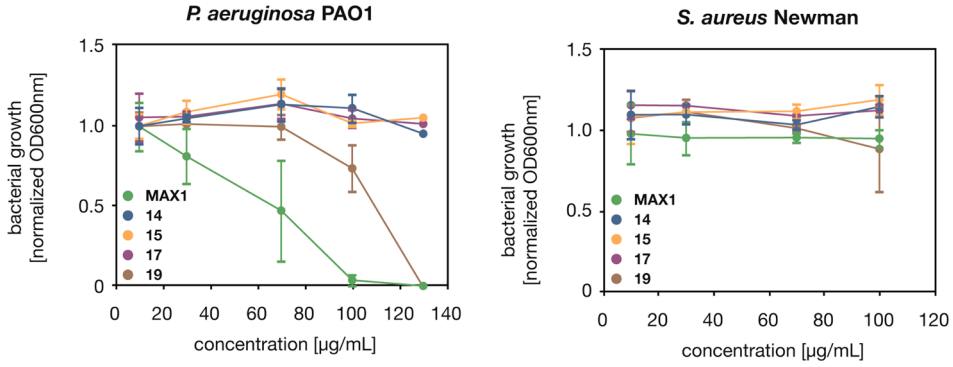

Figure 3. Antimicrobial peptide MAX1 and β^{3R3}-peptide **19** show antimicrobial activity against *P. aeruginosa* but not against *S. aureus*. Growth of Gram-negative *P. aeruginosa* PAO1 and Gram-positive *S. aureus* Newman was measured after growing 14 h in the presence of different concentrations of AMP MAX1 and β^{3R3}-peptides **14**, **15**, **17**, and **19**.

antibacterial activity up to 100 μg/mL against Gram-positive *S. aureus*. However, amphiphilic peptidomimetic **19** as well as the control MAX1 showed good antimicrobial activity against *P. aeruginosa*. The other amphiphilic β^{3R3}- peptides **14**, **15**, and **17** did not show activity against *P. aeruginosa* up to 130 μg/mL. These results highlight the potential of peptidomimetic **19** and control MAX1 as potential therapeutics against infections with *P. aeruginosa*. In contrast to the increased antibacterial activity of **19** for the Gram-positive *M. luteus*, certain mechanisms of resistance in *S. aureus* seem to prevent toxicity against this Gram-positive organism. *S. aureus* is known to covalently modify its cell envelope with L-lysine or D-alanine catalyzed by the bacterial enzymes MprF and DltA, respectively.[54] These mechanisms introduce positive charges on the surface, which leads to electrostatic repulsion of cationic antimicrobial peptides, such as the ones reported here, rendering them ineffective.

Cytotoxicity of Antibacterial Amphiphilic Cationic β^{3R3}-Peptides. Both, natural AMPs and β-peptides with high antimicrobial activity often display indiscriminant cytotoxicity and high hemolytic activity,[7,9,18,20−22] which substantially hampers the development of these systems as therapeutics. Therefore, the hemolytic activity of the amphiphilic cationic β^{3R3}-peptide sequences has been evaluated by testing hemoglobin release as a result of human red blood cell (hRBC) lysis upon incubation with the oligomers. Moreover, the integrity of the hRBC was controlled by microscopic analysis before and after treatments with amphiphilic cationic β^{3R3}-peptide oligomers. In addition, the toxicity toward human foreskin fibroblasts (HFF) was tested by monitoring fibroblast growth in the presence or absence of the oligomers after 24, 48, and 72 h. All oligomers **10**−**19** show no cytotoxicity toward HFF and hemolytic activity at a concentration of 80 μM, which is in the order of magnitude of the MIC values (see the Supporting Information). In addition, the hemolytic activity of selected oligomers was tested in a concentration range between 0 to 2000 μM. The oligomers **14**, **15**, and **19** were chosen as they display the highest antimicrobial activity. Furthermore, the hemolytic activity of the heterochiral (*v*S,*s*R) oligomer **17** was investigated due to its peculiar conformational properties, which could potentially influence the hemolytic activity of antimicrobial peptidomimetics. In general, all oligomers tested show negligible hemolytic activity. In agreement with the IRRAS and surface tension experiments carried out in the presence of the model eukaryotic membrane POPC, all four tested oligomers do not interact with eukaryotic cell membranes and do not produce hemolysis at the concentration required for antimicrobial activity (Table 4) regardless of their physicochemical differences (Table 1) as well as their different activities against prokaryotic cell membranes (Tables 2 and 3). For all compounds, the concentration required for the lysis of 50% of the red blood cells (HD50) is significantly higher than the lowest reported HD50 values for β-peptides with antimicrobial activity so far and range from 1315.5 μM (**17**) to 1714.4 μM (**15**).[18−22] In addition, only the heterochiral sequence **17** shows hemolytic activity at a concentration of 200 μM (390 μg/mL). Thus, by comparing the oligomers **14** (*v*S,*s*S) and **17** (*v*S,*s*R) that differ exclusively in the configuration of their chiral centers on the sNH_2 units, we can conclude that homochiral sequences (*v*S,*s*S) are superior as antimicrobial peptidomimetics combining high antibacterial activity with extremely low cytotoxicity.

Table 4. MIC, HD50, and Selectivity Index of the Amphiphilic Cationic β^{3R3}-Peptide Oligomers 14, 15, 17, and 19

oligomer	*M. luteus*[a]	*E. coli*[a]	GM[ab]	HD50[a]	SI[c]
14	10.7	101.9	33.0	3065.2	92
15	16.6	64.2	32.6	3223.7	98
17	29.2	130.6	61.7	2563.2	41
19	5.3	43.2	15.1	4013.7	265

[a]Expressed μg/mL. [b]Geometric mean (GM) of MIC values for the M. luteus and E. coli. [c]Selectivity index (SI) is calculated as HD50/GM.

Conclusively, the amphiphilic cationic β^{3R3}-peptides can display an extremely high selectivity index (Table 4) that is defined as the ratio between the HD50 and the MIC values. Furthermore, oligomers **17** and **19** as exemplary structures for amphiphilic cationic β^{3R3}-peptides showed high stability against enzymatic degradation (see the SI). Overall, this indicates the great potential of amphiphilic cationic β^{3R3}-peptides for the future development as novel antimicrobial agents. In particular, oligomer **19** is the best candidate for further research and a good starting point for the development as antimicrobial agent due to its high antimicrobial activity and unique selectivity.

CONCLUSIONS

In summary, we have introduced amphiphilic cationic β^{3R3}-peptides as a novel class of membrane active peptidomimetics and evaluated their potential as antibacterial agents. Novel building blocks were synthesized combining hydrophobic

diamine units with proteinaceous side chains and aspartic acid as chiral β-diacid units providing cationic charges in α-position. Different amphiphilic cationic β^{3R3}-peptide sequences were prepared varying primary sequence, chirality and chain length. All oligomers were characterized for their physicochemical properties via HPLC, DLS, surface tension, and IRRAS experiments. Additionally, the interactions of three selected oligomers with model prokaryotic and eukaryotic membranes have been tested by surface tension and IRRAS measurements. Thereby, the relationship between the building block and backbone features was elucidated, as well as the primary sequences and the overall physicochemical properties that conclusively determine membrane activity and selectivity of the oligomers.

The amphiphilic cationic β^{3R3}-peptides were tested for their potential as antimicrobial peptidomimetics. Cell viability experiments showed that specific amphiphilic cationic β^{3R3}-peptides with appropriate physicochemical properties have very high antimicrobial activity especially against Gram-positive bacteria. In addition, the amphiphilic cationic β^{3R3}-peptides show an extraordinary selectivity index (SI up to 265) with high antimicrobial and yet extremely low hemolytic activity. This study provided new insights into the SAR of AMPs and proved that the amphiphilic cationic β^{3R3}-peptides are strong candidates for further development as antimicrobial agents with high therapeutic index. Future studies will further elucidate the mechanisms of selectivity targeting different bacteria and cell types.

■ ASSOCIATED CONTENT

Supporting Information

Analytical data of building blocks and oligomers, NMR spectra of intermediates and building blocks, HPLC traces of building blocks and oligomers, CD and IRRAS spectra of all oligomers, experimental details on antibacterial growth and hemolysis assays. 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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