Article

Comparative Study on Antistaphylococcal Activity of Lipopeptides in Various Culture Media

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Abstract: *Staphylococcus aureus* bacteria are one of the leading microorganisms responsible for nosocomial infections as well as being the primary causative pathogen of skin and wound infections. Currently, the therapy of staphylococcal diseases faces many difficulties, due to a variety of mechanisms of resistance and virulence factors. Moreover, a number of infections caused by *S. aureus* is connected with biofilm formation that impairs effectiveness of the therapy. Short cationic lipopeptides that are designed on the basis of the structure of antimicrobial peptides structure are likely to provide a promising alternative to conventional antibiotics. Despite the fact that many research groups have proved a high antistaphylococcal potential of lipopeptides, the lack of unified protocols in determination of antimicrobial activity may be the reason of inconsistency of the results. The aim of this study was to learn how the use of various bacteriological media as well as solvents may affect activity of lipopeptides and their cyclic analogs.

Keywords: antimicrobial peptides; lipopeptides; cyclic lipopeptides *Staphylococcus aureus*; biofilm; culture media

1. Introduction

Staphylococcus aureus is one of the major pathogens responsible for variety of community- and hospital-acquired infections ranging from topical and harmless skin infections to severe systemic disorders such as endocarditis, necrotizing pneumonia, osteomyelitis or even sepsis [1,2]. Prevalence of staphylococcal diseases is related to the fact that some strains of the genus *Staphylococcus* such as *S. aureus* and *S. epidermidis* are typical skin colonizers. It is stated that between 10 and 35% of healthy individuals are persistent carriers of *S. aureus*, and anterior nares are indicated as the most consistent sites from which these organisms can be cultured [3]. Moreover, there is a strong link between nasal carriage and increased risk of nosocomial infections [4]. Despite the fact that *S. aureus* and *S. epidermidis* are likely to belong to skin physiological flora, in some cases they can lead to opportunistic diseases. Moreover, those two strains have also been shown to be the most frequent causative factors of nosocomial infections on indwelling devices [5]. Treatment of staphylococcal diseases faces many difficulties, mainly because of a facile transmission (*e.g.* through exposure of the hands of healthcare workers), a variety of virulence factors, development of resistance to antibiotics, and the ability to adhere and form biofilm [4,6]. Biofilm can be defined as a microbial sessile community embedded within self-produced matrix of extracellular polymeric substances that can be attached to biotic and abiotic surfaces. It seems likely that bacteria in some conditions prefer to form biofilm, firstly because extracellular matrix is capable to sequestrate and concentrate environmental nutrients, secondly because it provides protection from exogenous stress factors such as antimicrobial substances or activity of immune system of invaded the organism. Another characteristic feature of the biofilm is a quorum-sensing. This term is referred to the ability of bacteria to secrete low-molecular-weight signaling molecules that affect transcription patterns throughout local biofilm population. As a result of that activity, a better adaptation to rapid environmental changes is provided [7]. Since approximately 80% of all human bacterial infections are associated with biofilm,

it is reasonable to consider antibiofilm activity of new antimicrobials [8]. Recently, various approaches to treat biofilm-related infections have been developed including antibodies, anti-adhesion strategies, the use of bacteriophages or quorum-sensing inhibitors [9]. Antimicrobial peptides (AMPs) are compounds with proven *in vitro* and *in vivo* antibiofilm activities [10]. These compounds are evolutionarily conserved molecules of innate immune system of almost all organisms, playing important role in warding off invading microbial pathogens [11]. To date, AMPs have been studied for their antimicrobial properties as they are targeting a broad spectrum of pathogens including bacteria, fungi, viruses, and protozoa [12,13]. Naturally occurring AMPs can vary in size and most of them do not have more than 50 amino acids in sequence. In majority, they are cationic molecules containing about 50% of hydrophobic amino acids [14]. Such properties allow them to electrostatically interact with a negatively charged bacterial surface, this leading to disruption of a cell membrane. Many attempts have been made to improve the activity and bioavailability of AMPs such as synthesis of short synthetic analogs, the use of D- or unnatural amino acids, fatty acid acylation or cyclization. Short cationic lipopeptides are compounds designed on the basis of AMPs amphiphilic character to imitate their antimicrobial properties. Net positive charge of these compounds as well as amphipathicity was obtained by combining the hydrophobic fragment of a fatty acid with a cationic peptide chain. The antimicrobial properties of short cationic lipopeptides have also been proven in several studies as a promising alternative for currently used antimicrobials [15,16]. Broth microdilution method is one of the leading procedures used in the *in vitro* susceptibility testing for bacteria, whereas the protocol issued by Clinical and Laboratory Standards Institute (CLSI) is being commonly used in scientific studies [17]. Owing to this assay the minimum inhibitory concentration (MIC) can be determined. However, since some peptides have tendency to precipitate or bind to polystyrene, methods for evaluation their antimicrobial activity are being discussed. For instance, the most popular one was described by Hancock (University of British Columbia, Vancouver, BC, Canada) which involves different preparation of the peptide stock solution [18]. Despite the fact that there are reference protocols for MIC determination (which is actually conducted for planktonic forms of bacteria), there are no unified procedures used to determine antibiofilm activity. The aim of this study was to evaluate and to compare the results of antistaphylococcal activity (against both planktonic and biofilm forms) of short cationic lipopeptides and their cyclic analogs in three different microbiological media: Mueller Hinton Broth (which is used in CLSI and Hancock protocol), Brain-Heart Infusion Broth, and Tryptic Soy-Broth. Stock solutions of lipopeptides were prepared in both PBS and aqueous solution of BSA and AcOH.

2. Results

Short cationic lipopeptides and their cyclic analogs were active against all tested strains of *S. aureus*. However the antistaphylococcal activity depended on the type of medium as well as the solvent used for compound solubilization. MIC values for linear lipopeptides were lower in the BHI and TSB media than in MH (Figure 1). Only in the case of non-cyclized Pal-CKKKRC-NH$_2$ this dependence was not observed. In case of cyclic lipopeptides, the influence of medium type was irrelevant (Pal-CKKRKC-NH$_2$ and Pal-CRKKKC-NH$_2$ were slightly more active in MH). Despite this fact, all cyclic compounds dissolved in acetic acid/BSA were as a rule more active in comparison to those dissolved in PBS. The antibiofilm activity was highest in the MH medium (Figure 2) and cyclic analogs were as active as the linear forms or twofold more active (but only with compounds dissolved in BSA/acetic acid solution). Interestingly, the activity of cyclic lipopeptides was related to the medium and stock solution type. The antibiofilm activity increased in the order MH < TSB < BHI. Moreover, cyclic lipopeptides were even fourfold more active when dissolved in the AcOH/BSA solution. With linear lipopeptides the type of stock solution seems to be less relevant. Biofilms formed in BHI exhibited resistance to almost all tested compounds, while in the case of TSB medium, cyclic analogs dissolved in PBS did not eradicate the staphylococcal biofilm.
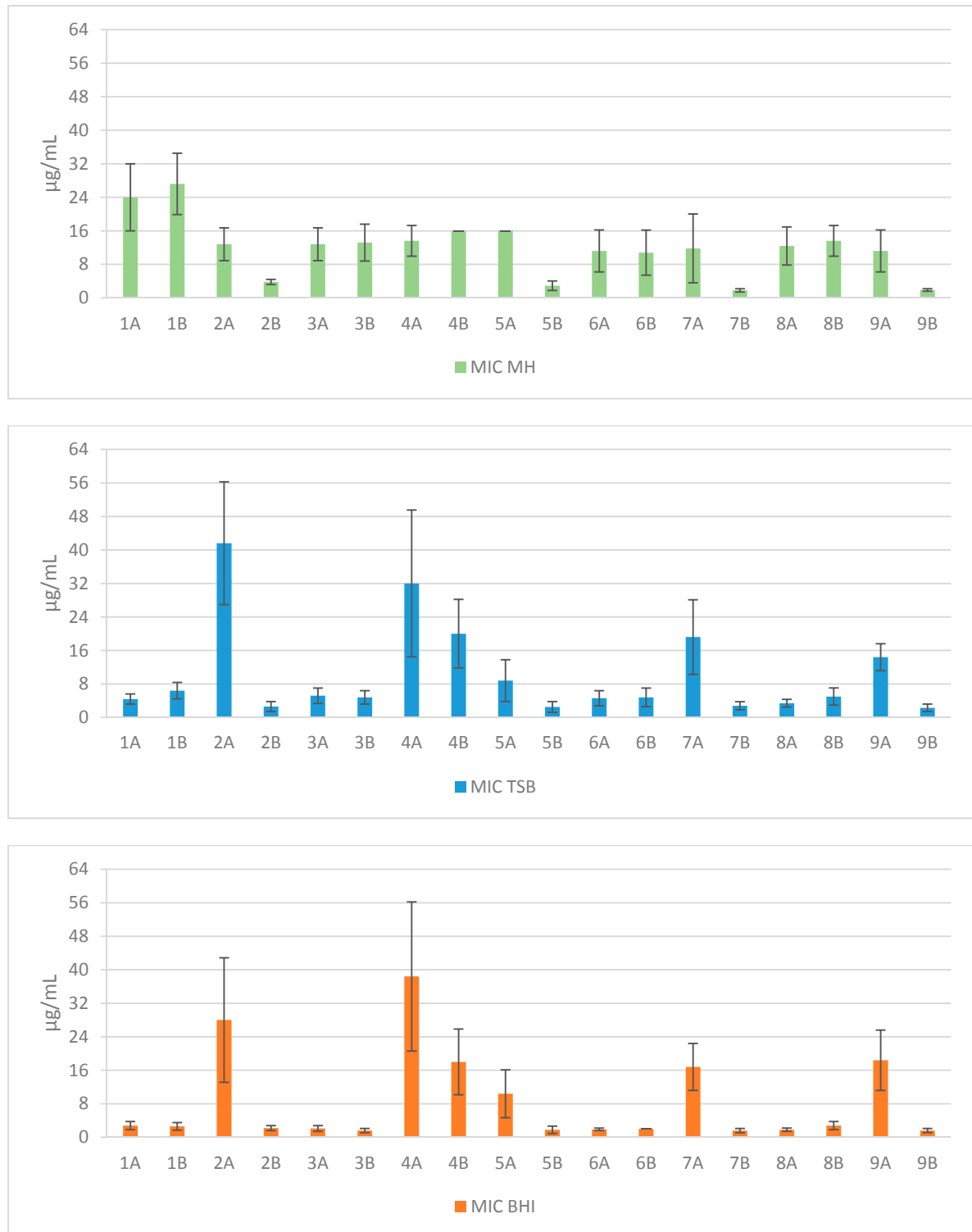
Figure 1. Mean MIC values against clinical and reference strains of Staphylococcus aureus. 1- Pal-KKK-NH2; 2- Pal-CKKKC-NH¬2 (cyclic); 3- Pal-KKKR-NH2;   4 – Pal-CKKKRC-NH2 (linear); 5- Pal-CKKKRC-NH2 (cyclic); 6- Pal-KKRK-NH2; 7- Pal-CKKRKC-NH2 (cyclic); 8- Pal-RKKK-NH2; 9- Pal-CRKKKC-NH2 (cyclic); Solvents: A – PBS; B – AcOH/BSA

Figure 2. Mean MBEC values against clinical and reference strains of Staphylococcus aureus. 1- Pal-KKK-NH2; 2- Pal-CKKKC-NH¬2 (cyclic); 3- Pal-KKKR-NH2;   4 – Pal-CKKKRC-NH2 (linear); 5- Pal-CKKKRC-NH2 (cyclic); 6- Pal-KKRK-NH2; 7- Pal-CKKRKC-NH2 (cyclic); 8- Pal-RKKK-NH2; 9- Pal-CRKKKC-NH2 (cyclic); Solvents: A – PBS; B – AcOH/BSA; * - concentration > 256µg/mL

3. Discussion

Such a comprehensive research on antimicrobial activity of lipopeptides has not been conducted so far. In this study, many factors were taken into account, including the type of microbiological medium, the use of various peptide stock solution solvents, and the impact of lipopeptide cyclization

as well. Moreover, the verification of the activity conducted on 10 strains of *S. aureus* (5 reference and 5 clinical ones) revealed a significant difference in antimicrobial activity of the lipopeptides in miscellaneous microbiological media. The reason of this difference may be associated with environment-dependent plasticity of lipid composition of *S. aureus* membranes. Sen *et al.* (2016) proved that the membranes of staphylococci grown in MH were less fluid than cells grown in BHI or TSB, which was related to a higher content of branched-chain fatty acids (BCFAs) and carotenoids [19]. These findings may be much more interesting as the staphyloxanthin, a carotenoid pigment, is one of the most potent virulence factors of *S. aureus* [20]. Staphyloxanthin provides integrity of the cell membrane and improves the resistance to reactive oxygen species this enhancing bacterial survival [21–23]. Furthermore, the influence of carotenoid content on susceptibility to host defense peptides was also investigated. In an *in vitro* study conducted by Mishra *et al.* (2011) the carotenoid-overproducing strains were less susceptible to daptomycin and cationic antimicrobial peptides such as human neutrophil defensin-1 (hNP-1), platelet microbicidal proteins (PMPs), and polymyxin B [24]. These results are consistent with the findings that higher carotenoid concentrations in *S. aureus* cell membrane cultivated in MH medium might suppress the activity of the tested compounds. In our study, the susceptibility of *S. aureus* biofilms in various growth media was examined. Biofilms formed in the BHI and TSB mediums were insensitive toward lipopeptides. The reason for this situation might be related to the impact of the glucose content on biofilm formation. In contrast to the MH medium (starch 1.50 g/L), BHI and TSB are supplemented with D-glucose (2.00 g/L and 2.50 g/L, respectively). Some studies proved the role of glucose in adhesion induction and modulation of biofilm formation [25,26]. Waldrop *et al.* (2014) have found that the mass of *S. aureus* biofilms rises with an increasing concentration of glucose with a threshold at 2.00 – 2.40 g/L [27]. Moreover, the catabolite control protein A (CcpA), which is responsible for regulation of various staphylococcal virulence determinants and resistance to antibiotics is also regulated by the presence of the glucose [28]. Furthermore, it should be noticed that conclusions drawn from the *in vitro* studies are not always consistent with those determined *in vivo*. Nevertheless, these "not-ideal" conditions may reveal an unexpected activity of new compounds. For example, the study conducted by Citterio *et al.* (2016) showed the synergy between human plasma and antimicrobial peptidomimetics, AMPs, and antibiotics against several bacterial pathogens [29]. In this case, the MIC values decreased down to sixteenfold in the presence of plasma. The synergistic effect may be connected with complement proteins or clotting factors, as the activity of all compounds in heat-inactivated plasma were significantly decreased. Another factor included in our study was the use of various solvents for peptide stock solution. Interestingly, lipopeptides dissolved in aqueous solution of acetic acid and bovine serum albumin (compounds marked with the letter B) were generally more effective than those dissolved in PBS. This approach was supported by a hypothesis that the type of solvent used in stock solution might have an impact on peptide solubility even after serial dilution in culture medium. For example, Steinberg *et al.* (1997) conducted a study on antimicrobial properties of protegrin-1 using a reference protocol issued by NCCLS (formerly CLSI) and modified a method, wherein the peptide was prepared in aqueous solution of acetic acid and BSA. Interestingly, the MIC values obtained using this modification were significantly lower; 5-times against S. *aureus* and *Enterococcus faecalis* and up to 30-times against *Escherichia coli* and *Pseudomonas aeruginosa* [30]. Similar observations were reported by Giacometti *et al.* (2000), who compared several methods for the determination of the activity of AMPs such as buforin II, cecropin, indolicidin, magainin II, nisin, and ranalexin [18]. Generally, our observation is inconsistent with the previously determined impact of BSA which is known to reduce lipopeptide antibacterial activity due to strong binding of fatty acids [31,32]. However, in our study the concentration of BSA was relatively low and thus lipopeptides remained active. BSA may have a negative influence on the antimicrobial activity of lipopeptides and in our study its application gives even better results than PBS stock solution, this showing that phosphate ion should be considered as an activity suppressor especially in the case of cyclic lipopeptides. Hypothetically, rigid cyclic structure and amino acids side chain orientation promote electrostatic interactions between amine and guanidine moieties with phosphate ion. Cyclization of AMPs is a popular approach to improve the activity and/or selectivity of the parent molecule [33].

For example, in a research conducted by Unger *et al.* (2001) the cyclization of melittin analogs resulted in an increased antibacterial activity. Interestingly, the cyclization resulted also in decreased hemolytic properties, this suggesting a lower toxicity towards mammalian cell lines [34]. It has been shown that disulfide cyclization may result in an enhanced peptide activity, selectivity, and stability [35]. In our study, cyclic lipopeptides were found to be more effective against all tested staphylococci, but only if they were dissolved in aqueous solution of acetic acid and BSA. Interestingly, the cyclization did not improve the antibiofilm activity in BHI or TSB. Nevertheless, it remains difficult to indicate which method provides more reliable data. Protocols utilized for the determination of antimicrobial activity should be standardized and the influence of different factors such as solvents for stock solutions, culture media used for *in vitro* studies should be taken into account. The relevance of each variable may be different, despite the overall chemical similarities between lipopeptides.

4. Materials and Methods

4.1. Synthesis of lipopeptides

The compounds were obtained by manual synthesis on solid support (polystyrene AM-RAM resin) using Fmoc/t-Bu strategy. Coupling reactions were carried out in DCM and DMF mixture using DIC as a coupling reagent and OxymaPure as racemisation suppressant. All reagents were used in fourfold excess based on the resin. Deprotection was performed with 20% piperidine solution in DMF for 15 minutes. Cleavage from the resin was accomplished in TFA and scavengers mixture – TFA:TIS:water, 96:2:2 (v/v) for 90 minutes with agitation. Additionally, EDT was applied as a scavenger if the peptide contained cysteine residues. Cyclic analogs were obtained by intramolecular disulphide bridge formation through oxidation with iodine. The lipopeptides were purified using RP-HPLC. The purity and identity of the peptides were confirmed by LC-MS analysis. The structures of selected lipopeptides used in the study are presented in Figures 3-5.

Table 1. List of lipopeptides used in this study.

| Sequence	Structure	Solvent	Signature
Pal-KKK-NH2	Linear	PBS	1A
Pal-KKK-NH2	Linear	AcOH/BSA	1B
Pal-CKKKC-NH2	Cyclic	PBS	2A
Pal-CKKKC-NH2	Cyclic	AcOH/BSA	2B
Pal-KKKR-NH2	Linear	PBS	3A
Pal-KKKR-NH2	Linear	AcOH/BSA	3B
Pal-CKKKRC-NH2	Linear	PBS	4A
Pal-CKKKRC-NH2	Linear	AcOH/BSA	4B
Pal-CKKKRC-NH2	Cyclic	PBS	5A
Pal-CKKKRC-NH2	Cyclic	AcOH/BSA	5B
Pal-KKRK-NH2	Linear	PBS	6A
Pal-KKRK-NH2	Linear	AcOH/BSA	6B
Pal-CKKRKC-NH2	Cyclic	PBS	7A
Pal-CKKRKC-NH2	Cyclic	AcOH/BSA	7B
Pal-RKKK-NH2	Linear	PBS	8A
Pal-RKKK-NH2	Linear	AcOH/BSA	8B
Pal-RKKK-NH2	Cyclic	PBS	9A
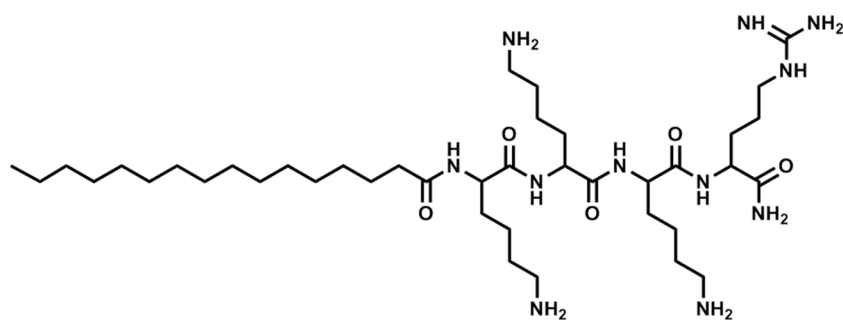
Figure 3. Structure of Pal-KKKR-NH2
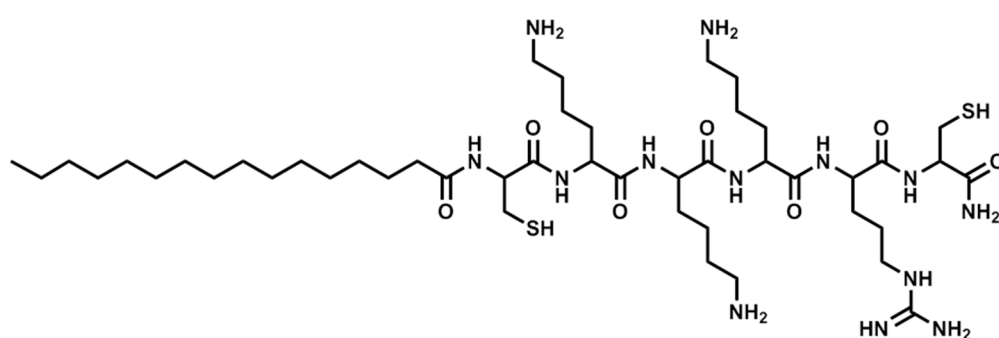
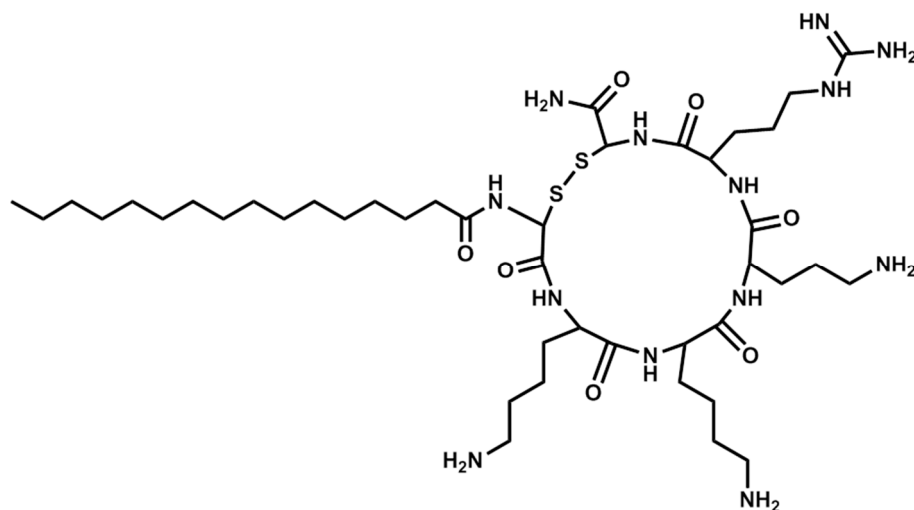**Figure 4.** Structure of Pal-CKKKRC-NH2 (linear)

Figure 5. Structure of Pal-CKKKRC-NH2 (cyclic)

4.2. Antimicrobial assays

4.2.1. MIC assay

Antimicrobial activity was determined against 5 reference strains: *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *S. aureus* ATCC 6538/P, *S. aureus* ATCC 9144, and *S. aureus* ATCC 12598, which

were obtained from the Polish Collection of Microorganisms (Polish Academy of Sciences, Wroclaw), and against 5 clinical strains of *S. aureus*, which were provided by the Department of Oral Microbiology, Medical University of Gdańsk (Poland). These strains were also characterized in terms of methicillin resistance. Preliminary identification and detection was conducted on the ChromID MRSA/ChromID *S. aureus* biplate (bioMérieux) for simultaneous detection of *S. aureus* and methicillin-resistant *S. aureus* (MRSA). All tested strains (both reference and clinical *S. aureus*) were stored in cryo-vials (Roti®-Store, Carl Roth) and recultivated before all microbiological tests. All strains were cultured in three different media: Brain-Heart Infusion Broth (BHI, Biocorp), Mueller Hinton Broth (MH, Biocorp), and Tryptic-Soy Broth (TSB, Biocorp). Moreover, the stock solutions of lipopeptides were prepared in two different solvents: PBS and an aqueous solution of acetic acid/BSA (0.01% and 0.2% respectively). The complete list of compounds used in this study and their signatures is presented in Table 1. MIC was determined by broth microdilution method on polystyrene 96-well plates. Bacteria at initial inoculums of 5×10^5 CFU/mL were exposed to lipopeptides at concentrations ranging from 0.5 to 256 µg/mL and incubated for 18 h at 37°C. The MIC value was taken as the lowest concentration of the compound that inhibited the growth of microorganism. Assay were performed in triplicate

4.2.2. MBEC assay

S. aureus biofilms were cultured on polystyrene 96-well flat bottom plates. Bacteria at initial inoculum of 5×10^8 CFU/mL in BHI, MH or TSB were added to the plates and incubated at 37°C. After 24 h of incubation all *S. aureus* cultures were rinsed three times with PBS (pH 7.4) and a fresh medium was added. Subsequently, all biofilms were exposed to lipopeptides at concentrations ranging from 0.5 to 256 µg/mL for 24 h at 37°C. After incubation, resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide, Sigma Aldrich) was added as a cell viability reagent and the MBEC was read. Assay were performed in triplicate.

Abbreviations

AcOH	– acetic acid
AMPs	– antimicrobial peptides
BSA	– bovine serum albumin
CFU	– colony forming unit
CLSI	– Clinical and Laboratory Standards Institute
DCM	– dichloromethane
DIC	– *N,N′*-diisopropylcarbodiimide
DMF	– *N,N*-dimethylformamide
EDT	– 1,2-ethanedithiol
Fmoc	– 9-fluorenylmethoxycarbonyl
MBEC	– minimum biofilm eradication concentration
MIC	– minimum inhibitory concentration
Pal	– palmitic acid
PBS	– phosphate buffer saline
TIS	– triisopropylsilane
TFA	– trifluoroacetic acid

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