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ORIGINAL ARTICLE

Biological and surface-active properties of double-chain cationic amino acid-based surfactants

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Abstract Cationic amino acid-based surfactants were synthesized via solid phase peptide synthesis and terminal acylation of their α and ε positions with saturated fatty acids. Five new lipopeptides, *N*-α-acyl-*N*-ε-acyl lysine analogues, were obtained. Minimum inhibitory concentration and minimum bactericidal (fungicidal) concentration were determined on reference strains of bacteria and fungi to evaluate the antimicrobial activity of the lipopeptides. Toxicity to *eukaryotic* cells was examined via determination of the haemolytic activities. The surface-active properties of these compounds were evaluated by measuring the surface tension and formation of micelles as a function of concentration in aqueous solution. The cationic surfactants demonstrated diverse antibacterial activities dependent on the length of the fatty acid chain. Gram-negative bacteria and fungi showed a higher resistance than Gram-positive bacterial strains. It was found that the haemolytic activities were also chain length-dependent values. The surface-active properties showed a linear correlation between the alkyl chain length and the critical micelle concentration.

Keywords Lipopeptides · Surfactants · Antimicrobial activity · Critical micelle concentration

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Abbreviations

Boc	*tert*-Butoxycarbonyl
CFU	Colony forming units
CMC	Critical micelle concentration
DCM	Dichloromethane
DIC	Diisopropylcarbodiimide
DMF	*N*,*N*-dimethylformamide
Fmoc	9-fluorenylmethoxycarbonyl
HOBt	1-Hydroxybenzotriazole
MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
TFA	Trifluoroacetic acid
TIS	Triisopropylosilane

Introduction

Essentially, any kind of drug formulation containing an aqueous phase requires the application of preservatives to maintain microbiological purity and prevent secondary microbial contamination during storage and usage of drugs from multiple usage packages. Emulsions are a large group of water-rich formulations and because they are multiphase systems, their composition involves surface-active compounds. Reciprocal interactions often appear between these surface-active compounds and preservatives. Generally, they are based on embedding a preservative in the structures formed by the surfactant. The result of such an incompatibility is a decrease in the preservative concentration in the aqueous phase and consequent insufficient protection against the growth of microorganisms. Therefore, an adequate form of the preservative for multiphase medicines constitutes a current issue for pharmaceutical technology (Denyer and Baird 2007).

The main objective of this work was to design and obtain via chemical synthesis new, cationic lipopeptide compounds of simple alkyl chains, which would simultaneously fulfil the roles of the preservative and the surface-active agent. The main assumption during the design process was to imitate the properties of the secondary structure of endogenous peptides (Zasloff 2002) responsible for their antimicrobial activity, i.e. amphipathicity (Tossi et al. 2000) and the positive net charge of the molecule (Chen et al. 2007; Jiang et al. 2008; Shai 2002), and translate them into the primary structure. It was assumed that basic amino acid residues of lysine could deliver the positive charge of the designed molecules. The amphipathicity condition was achieved via attachment of hydrophobic fragments of hexadecanoic, tetradecanoic, dodecanoic, decanoic and octanoic fatty acids. The amphiphilic structure of such compounds would ensure that the lipopeptides would demonstrate surface-active properties.

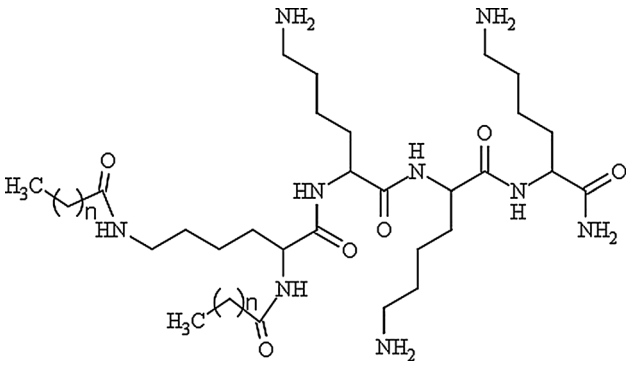

Fig. 1 Molecular structure of lipopeptide double chain surfactants, *N*-α-acyl-*N*-ε-acyl lysine analogues: $n = 6$ $(C_8)_2$-KKKK-NH_2, $n = 8$ $(C_{10})_2$-KKKK-NH_2, $n = 10$ $(C_{12})_2$-KKKK-NH_2, $n = 12$ $(C_{14})_2$-KKKK-NH_2, $n = 14$ $(C_{16})_2$-KKKK-NH_2

Materials and methods

Peptide synthesis and purification

The following five new amino acid-based cationic surfactants were obtained: $(C_8)_2$-KKKK-NH_2 (*N*-α-octanoyl-*N*-ε-octanoyl-Lys-Lys-Lys-Lys-NH_2), $(C_{10})_2$-KKKK-NH_2 (*N*-α-decanoyl-*N*-ε-decanoyl-Lys-Lys-Lys-Lys-NH_2), $(C_{12})_2$-KKKK-NH_2 (*N*-α-dodecanoyl-*N*-ε-dodecanoyl-Lys-Lys-Lys-Lys-NH_2), $(C_{14})_2$-KKKK-NH_2 (*N*-α-tetradecanoyl-*N*-ε-tetradecanoyl-Lys-Lys-Lys-Lys-NH_2), and $(C_{16})_2$-KKKK-NH_2 (*N*-α-hexadecanoyl-*N*-ε-hexadecanoyl-Lys-Lys-Lys-Lys-NH_2) (Fig. 1). The lipopeptides were assembled following the solid-phase procedure (Merrifield 1963) on an Fmoc-Rink Amide AM resin (0.59 mmol/g, Iris Biotech, Germany) employing the 9-fluorenylmetoxycarbonyl (Fmoc) methodology (Fields and Noble 1990). The Fmoc group of each amino acid was detached by 20 % piperidine in *N*,*N*-dimethylformamide (DMF) (Carpino 1987; Fields 1994). The coupling reactions were carried out with a two-fold excess of Fmoc-protected amino acid in an *N*,*N*-dimethylformamide/dichloromethane (DCM) mixture (1:1 v/v) in the presence of Triton X-100 using diisopropylocarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) as coupling reagents (König and Geiger 1970). Completion of the coupling reaction was monitored with the chloranil test (Christensen 1979). Removal of the side chains protecting groups and simultaneous cleavage from the solid support was achieved using 95 % trifluoroacetic acid (TFA) with 2.5 % triisopropylsilane (TIS) as a scavenger and 2.5 % water for 2 h (Chan and White 2004). The cleaved crude lipopeptides were precipitated with diethyl ether and lyophilized. They were then purified via semi-preparative reverse-phase high performance liquid chromatography (RP-HPLC) on a Knauer K1001 two-pump system (Knauer, Germany) with a Nucleodur C8ec column, 10 × 250 mm, 5 µm, 100 Å (Macherey–Nagel, Germany). The synthesized compounds were eluted with a linear gradient with 20–60 % of phase B (where phase A was 0.1 % TFA in water and phase B was 0.1 % TFA in acetonitrile), at a flow rate of 3 mL/min, at 214 nm.

Reversed-phase analysis of lipopeptides

The peptides were analysed via RP-HPLC on a Chromolith Performance RP-18 (100 × 4.6 mm) monolithic column (Merck, Germany) using a Knauer K1001 two-pump system with a linear gradient 0–100 % phase B (where phase A was 0.1 % TFA in water and phase B was 0.1 % TFA in acetonitrile), at a flow rate of 2 mL/min, at 214 nm. Fractions with a purity greater than 95 % were pooled and lyophilized. The peptides were further characterized via the matrix assisted laser desorption ionization time-of-flight mass spectrometry MALDI-TOF—Biflex III (Bruker, Germany).

Organisms and antimicrobial assay

The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined according to the procedure recommended by the Clinical Laboratory Standards Institute (CLSI 2009, 2010). The following reference strains were tested: Gram-negative *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Proteus vulgaris* (PCM 2668), *Pseudomonas aeruginosa* (ATCC 9027), Gram-positive *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (PCM 2118), *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis*

(ATCC 29212) and fungi *Candida albicans* (ATCC 10231), *Candida tropicalis* (PCM 2681) and *Aspergillus niger* (ATCC 16404). All the microorganisms were purchased from the Polish Collection of Microorganisms, Polish Academy of Science, Institute of Immunology and Experimental Therapy, Wrocław, Poland.

The MIC was determined using a microbroth dilution method with Mueller–Hinton (MH) broth (Becton–Dickinson, Le Pont de Claix, France). An initial inoculum of 10^5 cfu/mL for bacteria and 10^3 for fungi was used. Polypropylene 96-well plates (Nunc GmbH & Co. KG, Germany) were incubated for 18 h at 37 °C for bacteria and for 48 h at 25 °C for the tested fungi, MIC (μg/mL) was taken as the lowest concentration of lipopeptide at which the observable growth was inhibited. All the MIC wells which did not reveal turbidity were cultured on solid media, a Mueller–Hinton II Agar for bacteria and a Sabouraud 2 % Glucose Agar for fungi. The lowest concentrations of lipopeptides that did not show any visible growth on the plates after 24 h of incubation at 37 °C and 48 h of incubation at 25 °C were recorded as the MBCs and MFCs, respectively. The experiments were performed in triplicate on three different days (CLSI 2009, 2010).

Haemolytic activity

The haemolytic activity of the lipopeptides was determined by exposing human red blood cells (4 % v/v) to tested compounds at graded concentrations. Red blood cells were obtained from a healthy donor. EDTA was used in order to prevent the blood clotting. The plasma was removed via centrifugation and the erythrocytes were washed three times with PBS. The red blood cells were suspended in PBS and incubated with different concentrations of lipopeptides at 37 °C for 1 h and centrifuged for 5 min at 1,000*g*. The supernatants were transferred to a sterile 96-well plate and haemoglobin release was measured with Epoch microplate spectrophotometer (BioTek, USA) by recording the absorbance at 550 nm. A 0.1 % Triton X-100 solution was used as the positive control and pure PBS as the negative (Findlay et al. 2012).

CMC determination

The critical micelle concentration of each lipopeptide surfactant was determined from surface tension measurements on an EasyDyne (Krüss, Germany) tensiometer. Solutions of known concentration were progressively diluted and examined by the Du Noüy ring method. Temperature was kept at 25 ± 0.2 °C using a thermostated circulating water bath. The CMC value was calculated by plotting the surface tension against the log of the concentration of an amino acid surfactant. All measurements were conducted in unbuffered aqueous solutions.

Results and discussion

Antimicrobial assay

Bacterial and fungal susceptibility tests for the lipopeptide surfactants were performed by determination of MIC and MBC/MFC. Results of the microbiological tests are summarized in Tables 1 and 2. Data on MICs for selected microorganisms revealed that lipopeptides made up of four lysine residues, acylated at α and ε positions of the first lysine in the sequence, demonstrate different antimicrobial activities.

The data demonstrate that, among the five lipopeptides, the most active are the analogues $(C_{10})_2$-KKKK-NH_2 and $(C_{12})_2$-KKKK-NH_2. They already inhibit the growth of *B. subtillis* at a concentration of 2 μg/mL and of *S. epidermidis* at 2 and 4 μg/mL, respectively. A slightly lower activity was noticed against *E. fecalis* and *S. aureus* (MIC 8–16 μg/mL). It was found that those compounds modified with the octanoyl, tetradecanoyl and hexadecanoyl residues revealed a much lower antibacterial activity against the reference strains. The MIC against Gram-positives for these was noted in range from 64 to 512 μg/mL. Some lipopeptidomimetic oligo-acyl-lysine (OAK) analogues were also found to be highly effective against *E. fecalis* and *S. aureus* (Sarig et al. 2008).

Analogues $(C_{10})_2$-KKKK-NH_2 and $(C_{12})_2$-KKKK-NH_2 are also highly active against Gram- negative bacteria. They inhibit the growth of *E. coli*, *K. pneumoniae* and *P. aeruginosa* at concentrations of 16–64 μg/mL. Only *P. vulgaris* showed a much weaker susceptibility. Other analogues, $(C_8)_2$-KKKK-NH_2, $(C_{14})_2$-KKKK-NH_2 and $(C_{16})_2$-KKKK-NH_2, were poorly effective against all the Gram-negative strains tested. The MIC values for these compounds range from 256 to 512 μg/mL.

The bactericidal activity of analogues $(C_{10})_2$-KKKK-NH_2 and $(C_{12})_2$-KKKK-NH_2 was also potent against *S. epidermidis* and *B. subtillis*. The MBC concentration in this case was noted in a range from 2 to 4 μg/mL. However, they exhibit a slightly weaker bactericidal activity against Gram-negative strains, namely in a range from 16 to 128 μg/mL. *Proteus vulgaris* turned out to be resistant to the decanoyl- and dodecanoyl-acylated lipopeptides. For the other tested analogues, a weak bactericidal activity was observed both against the Gram-positive as well as the Gram-negative bacterial strains.

The activity of the compounds against fungi is much weaker than their antibacterial effectiveness. The most

Table 1 Minimum inhibitory concentrations (μg/mL) for compounds $(C_8)_2$-KKKK-NH_2, $(C_{10})_2$-KKKK-NH_2, $(C_{12})_2$-KKKK-NH_2, $(C_{14})_2$-KKKK-NH_2 and $(C_{16})_2$-KKKK-NH_2

Microorganism		MIC μg/mL (μM)				
		$(C_8)_2$-KKKK-NH_2	$(C_{10})_2$-KKKK-NH_2	$(C_{12})_2$-KKKK-NH_2	$(C_{14})_2$-KKKK-NH_2	$(C_{16})_2$-KKKK-NH_2
Gram-positives	*S. aureus*	256 (323)	8 (9.5)	16 (17.9)	256 (269)	512 (509)
	S. epidermidis	32 (40.9)	2 (2.4)	4 (4.4)	128 (135)	>512 (>509)
	B. subtillis	64 (81.8)	2 (2.4)	2 (2.2)	128 (135)	*512 (509)*
	E. fecalis	512 (654)	16 (19)	8 (8.9)	64 (67)	512 (509)
Gram-negatives	*E. coli*	512 (654)	16 (19)	64 (71.5)	512 (539)	512 (509)
	K. pneumoniae	256 (323)	32 (38)	32 (35.8)	512 (539)	512 (509)
	P. aeruginosa	256 (323)	16 (19)	32 (35.8)	512 (539)	512 (509)
	P. vulgaris	>512 (>654)	512 (610.6)	>512 (>572.5)	512 (539)	>512 (>509)
Fungi	*C. albicans*	256 (323)	128 (152.6)	256 (286)	256 (269)	512 (509)
	C. tropicalis	512 (654)	256 (305)	256 (286)	512 (539)	512 (509)
	A. niger	128 (163.7)	256 (305)	256 (286)	512 (539)	256 (254)

The figures in brackets give the concentrations in μM.

Table 2 Minimum bactericidal concentrations (μg/mL) for compounds (C8)2-KKKK-NH2, (C10)2-KKKK-NH2, (C12)2-KKKK-NH2, (C14)2-KKKK-NH2 and (C16)2-KKKK-NH2

Microorganism		MBC μg/mL (μM)				
		$(C_8)_2$-KKKK-NH_2	$(C_{10})_2$-KKKK-NH_2	$(C_{12})_2$-KKKK-NH_2	$(C_{14})_2$-KKKK-NH_2	$(C_{16})_2$-KKKK-NH_2
Gram-positives	*S. aureus*	>512 (>654)	16 (19)	32 (35.8)	512 (539)	>512 (>509)
	S. epidermidis	64 (81.8)	4 (4.8)	4 (4.4)	256 (269)	>512 (>509)
	B. subtillis	64 (81.8)	4 (4.8)	2 (2.2)	256 (269)	>512 (>509)
	E. fecalis	512 (654)	16 (19)	16 (17.9)	256 (269)	>512 (>509)
Gram-negatives	*E. Coli*	512 (654)	16 (19)	64 (71.5)	>512 (>539)	>512 (>509)
	K. pneumoniae	512 (654)	128 (152.6)	32 (35.8)	512 (539)	>512 (> 509)
	P. aeruginosa	512 (654)	16 (19)	32 (35.8)	512 (539)	>512 (>509)
	P. vulgaris	>512 (>654)	512 (610.6)	>512 (>572.5)	>512 (>539)	>512 (>509)
Fungi	*C. albicans*	512 (654)	256 (305)	512 (572.5)	512 (539)	>512 (>509)
	C. tropicalis	>512 (>654)	512 (610.6)	512 (572.5)	>512 (>539)	>512 (>509)
	A. niger	512 (654)	512 (610.6)	512 (572.5)	>512 (>539)	512 (509)

The figures in brackets give the concentrations in μM.

potent compound is $(C_{10})_2$-KKKK-NH_2, but its MIC and MFC value are no lower than 128 μg/mL. Slightly less active against fungi were proved to be the analogues $(C_8)_2$-KKKK-NH_2 and $(C_{12})_2$-KKKK-NH_2. Both cause the growth inhibition of *C. albicans* at a concentration of 256 μg/mL. $(C_8)_2$-KKKK-NH_2 inhibits the growth of *A. niger* at a concentration of 128 μg/mL and $(C_{12})_2$-KKKK-NH_2 at 256 μg/mL. The growth inhibition activity of the remaining compounds is rather poor and falls within the range of 256–512 μg/mL. A higher activity against *Candida* strains is demonstrated by anionic surfactants of the type of N^{α},N^{ε}-dioctanoyl lysine (Sánchez et al. 2007).

All the determined MIC values were compared with minimum inhibitory concentrations of conventional preservatives. Based on published data (Meyer et al. 2007), it can be stated that the synthesized lipopeptides exhibit a significantly higher antimicrobial activity.

Haemolysis

To study the surfactant—eukaryotic cell membrane interaction, haemolysis assays were performed. The results of haemolytic activity are presented in the Fig. 2. The cationic lysine-derived surfactants are characterized by different haemolytic properties. However, all tested

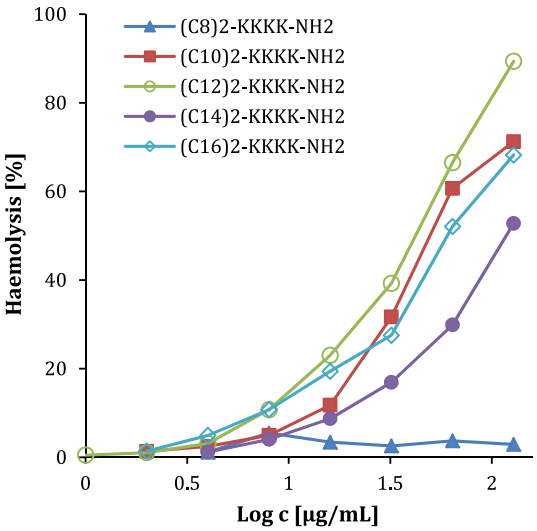

Fig. 2 Haemolytic activities of lipopeptide compounds $(C_8)_2$-KKKK-NH_2, $(C_{10})_2$-KKKK-NH_2, $(C_{12})_2$-KKKK-NH_2, $(C_{14})_2$-KKKK-NH_2 and $(C_{16})_2$-KKKK-NH_2. The concentrations are presented as Log c (μg/mL)

lipopeptides at concentrations of 2 μg/mL caused haemolysis <3 %. Above this concentration haemolytic activity of four compounds consecutively increases but in non-linear manner. An analogue with a dodecanoyl residue proved to be the most toxic one. At a concentration of 32 μg/mL $(C_{12})_2$-KKKK-NH_2 induced almost 40 % lysis of human erythrocytes. Somewhat weaker haemolytic properties were noticed for lipopeptides containing two hexadecanoic and decanoic chains. After application of these compounds at a concentration of 32 μg/mL, ca. 30 % of lysis of the exposed red blood cells occurred. In the case of ananalogue containing two octanoic chains, no significant haemolytic activity was observed over the whole tested concentration range (up to 512 μg/mL, data not shown in the Fig. 2). Although the haemolytic activity of the compounds is dependent on the alkyl chain length, the relationship being non-linear. Indeed, upon contact with an erythrocyte's lipid bilayer a surfactant molecule induces its solubilisation. More efficient are surfactants with hydrophobic chains similar to bilayer lipid hydrocarbon chains. An erythrocyte membrane is rich in C16 and C18 lipids (Brito et al. 2009), so it can be destroyed by $(C_{16})_2$-KKKK-NH_2 more easily than by $(C_8)_2$-KKKK-NH_2.

Micellization properties

The micellization process for amino acid-based surfactants containing two hydrophobic chains was studied by measuring solutions with lipopeptide concentrations above and below CMC. The surface tension vs. Log c plots for the five studied surfactants are shown in Fig. 3. The CMC and

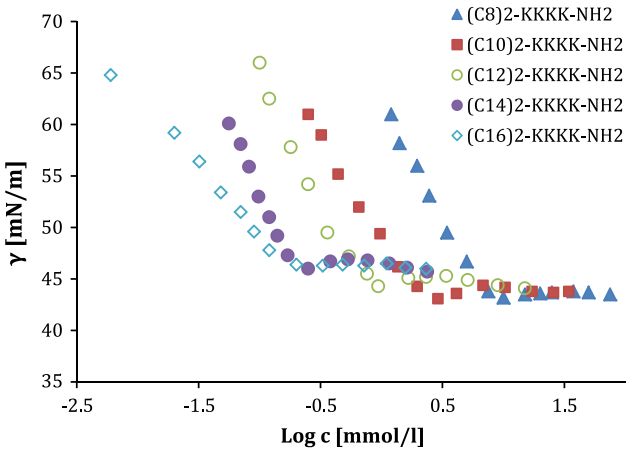

Fig. 3 Surface tension vs. logarithm of concentration curves for the cationic double-chained surfactants. All measurements are at 25 °C

Table 3 Critical micellar concentrations and surface tension at the critical micellar concentrations for the studied surfactants (C8)2-KKKK-NH2, (C10)2-KKKK-NH2, (C12)2-KKKK-NH2, (C14)2-KKKK-NH2 and (C16)2-KKKK-NH2

Compound	CMC		γ CMC (mN/m)
	(mM)	(μg/mL)	
$(C_8)_2$-KKKK-NH_2	6.8	5.32×10^3	43.5
$(C_{10})_2$-KKKK-NH_2	1.76	1.47×10^3	43.8
$(C_{12})_2$-KKKK-NH_2	0.66	590.2	45.1
$(C_{14})_2$-KKKK-NH_2	0.16	152.06	46.8
$(C_{16})_2$-KKKK-NH_2	0.11	110.7	46.5

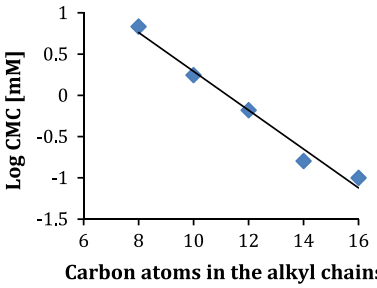

Fig. 4 CMC vs. carbon atoms in the hydrophobic chains for the *N*-α-acyl-*N*-ε-acyl lysine-based surfactants

surface tension values at CMC, γ_{CMC}, are summarized in Table 3. As expected, the CMC strongly depends on the length of the hydrophobic chains attached to the α and ε amino groups. The CMC decreases with an increase in the alkyl chain. A linear relationship of log CMC vs. carbon atoms in the hydrophobic chains is presented in Fig. 4.

The surface tension values at CMC (γ_{CMC}), an important parameter describing interfacial efficiency, slightly increases when the alkyl chain length increases. The most efficient is $(C_8)_2$-KKKK-NH_2 and the least is $(C_{16})_2$-

KKKK-NH_2. Amino acid-based surfactants based on serine studied by Brito demonstrate the same trend (Brito et al. 2011). This reduction in interfacial efficiency could be explained by an accelerated tendency for surfactants with larger hydrophobic molecules towards aggregation in aqueous media.

Concluding remarks

In conclusion, it is well known that a positive net charge and amphipathicity are responsible for the antimicrobial activity of endogenous, host defence peptides. The selected lipopeptide surfactants studied in this work exhibited excellent antimicrobial properties, better than most of the antimicrobials currently used as preservative agents. It was found that the studied compounds displayed a weaker activity against Gram-negative strains and fungi than against Gram-positive bacteria. The highly hydrophilic and anionic external membrane of the Gram-negative bacteria is more likely to imprison cationic aggregates, thus preventing their progression towards an internal target. It was also noticed that the antimicrobial activity of the lipopeptides was strongly dependent on the length of the hydrophobic chains. The excess of the hydrophobic moiety is probably responsible for reduced antibacterial and antifungal potency. Lipopeptides with longer hydrophobic chains are more susceptible to aggregation in bulk. CMC is also chain-length dependent, but it has no influence on MIC, because the activity of lipopeptide surfactants is dependent on the concentration of monomers in bulk and independent of aggregate formation concentration. Despite the fact that the selected lipopeptides exhibited excellent antimicrobial activity, it is unjustified to consider them as potential agents for systemic therapeutic applications due to their significant haemolytic activity. However, it is still possible to use lipopeptides in cosmetics, toiletry and pharmaceutical products for topical treatment.

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Conflict of interest The authors declare that they have no conflict of interest.

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