

ANTIBACTERIAL ACTIVITY OF 24 L-PHENYLALANINE DERIVED SURFACE-ACTIVE IONIC LIQUIDS (SAILS) TOWARDS TWO CLINICALLY RELEVANT PATHOGENS

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Abstract

Ionic liquids - low melting point salts - are attractive for a wide range of applications, from material science to medicinal chemistry. The vast number of possible combinations of different cations and anions enables researchers to fine-tune the physico-chemical and/or biological properties (e.g., hydrophobicity or toxicity). This paper systematically analyses the antimicrobial potency of a library of 24 L-phenylalanine derived surface-active ionic liquids (SAILS; C₂-C₁₆) and provides EC₅₀, MIC and MBC values for these compounds towards two clinically relevant pathogenic bacterial models – Escherichia coli and Staphylococcus aureus using standard broth microdilution method ISO 20776-1:2006. We demonstrated that the antimicrobial potency of SAILS containing different cationic headgroups (pyridinium, imidazolium, and cholinium) increased with the length of the alkyl ester chain from C₂ to C₁₂ and then similar values obtained for C₁₄ followed by a decreased toxicity for C₁₆. This trend was not dependent on the type of headgroup. The minimum inhibitory concentration (MIC) ranged from 8000 mg/L (C₂ SAILS) down to 4 mg/L (C₁₂ SAILS). Most potent were C₁₂ SAILS that inhibited the growth of S. aureus and E. coli at concentration of few milligrams per liter: EC₅₀ values ~2 and ~15 mg/L (or ~4 and ~27 µM), respectively. These data are comparable to the antimicrobial efficiency of benzalkonium chloride, which is widely used antimicrobial compound. As a rule, gram-positive S. aureus was 7-fold more susceptible to the SAILS than gram-negative E. coli. In addition, the results obtained in this study on medically relevant bacteria were in agreement with our previous data on Vibrio fischeri – a naturally luminescent marine bacterium. We hypothesize that the toxic effects of studied SAILS was manifested via disturbing the bacterial membranes. To summarise, the SAILS are promising antimicrobials which toxicity towards bacteria can be tuned by modifying the alkyl ester chain properties.

Keywords: Surface active ionic liquids, alkyl ester chain length, Escherichia coli, Staphylococcus aureus, ISO 20776-1:2006

1. INTRODUCTION

Due to the continuously increasing occurrences of antibiotic resistant bacteria and hospital-acquired infections [1], scientists have examined alternative approaches to solve this problem, e.g. developing new antimicrobial chemicals. Within the vast library of chemical compounds that can be envisaged, numerous ionic liquids (ILs), including surface active ionic liquids (SAILS), with intrinsic antimicrobial properties and highly tuneable nature, have been developed to meet this need [2, 3].

SAILS can be considered analogues of native lipopeptides as they are cationic amphiphiles consisting of one or two amino acids connected to a hydrophobic part. In consequence, they show the toxic properties towards microorganisms and are of low potency to induce resistance [4]. At least two functional groups, carboxylic groups, and amino groups are inherently present in amino acids. These substances can be transformed into single chain surfactants with reactive sites in their hydrophobic chain, for example, fatty acids, fatty esters, fatty amines, and fatty alcohols. The hydrophobic chain can be linked to the structure of amino acid through alkyl ester or amide linkages [5].

Various studies on the biological activity of ILs on bacteria have been carried out, including gram-negative and gram-positive bacteria [6-10]. In addition, several test methods for measuring toxicity

towards bacteria have been introduced. One of the most recognized antibacterial activity tests is a standard broth microdilution method described in ISO 20776-1:2006 "Reference method for testing the *in vitro* activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases" [11]. The method is based on the serial dilution of tested chemical and incubating the dilutions with test bacteria for determination of minimum inhibitory concentration (MIC). In addition, half-effective concentration (EC_{50}) values of test chemicals can be calculated from the test data. Moreover, as a prolongation of the test, the incubation mixtures can be plated out on agarized nutrient medium to determine minimal bactericidal concentrations of the tested chemicals (MBC).

Escherichia coli (gram-negative bacterium) and *Staphylococcus aureus* (gram-positive bacterium) were used in this study as model organisms that may cause skin and soft tissue infections (SSTIs) [12]. Moreover, within these bacteria there are strains that have developed resistance towards common antibiotics. The latter motivates the studies for discovery of novel compounds (including ionic liquids) with antimicrobial properties [13].

In general, research on the antibacterial activity of ILs has been limited to determination of MIC [14] and comprehensive data on bacterial growth inhibition, MIC and MBC, are still rare. The current study aimed to explore the antibacterial activity of 24 L-phenylalanine derived SAILs, including cyclic cation (pyridinium and imidazolium) and acyclic cation (cholinium), all as bromide salts, containing the same linker (L-phenylalanine), and different alkyl ester chain lengths (C_2 to C_{16}) (see Fig. 1) against *E. coli* and *S. aureus*.

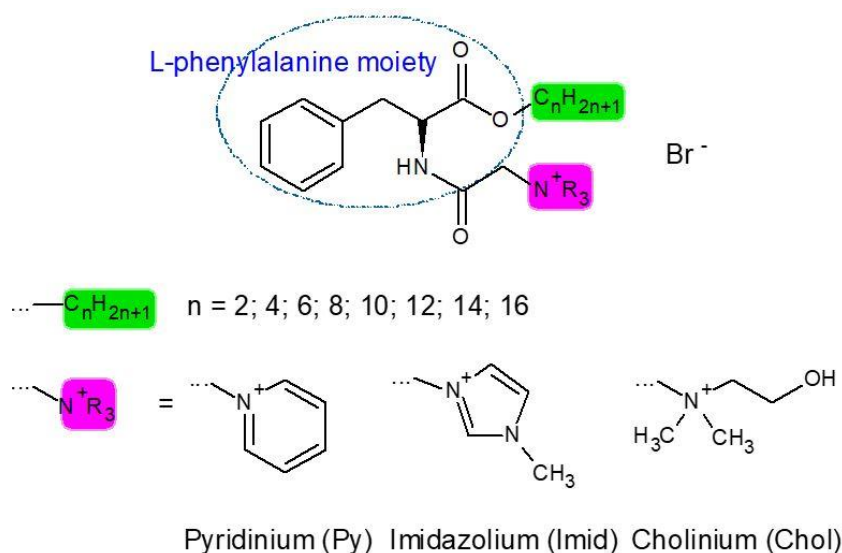


Fig. 1. A library of 24 L-phenylalanine derived SAILs with three different cations (pyridinium, imidazolium and pyridinium), all paired with bromide-anion and varying alkyl chain (C_2 to C_{16}) studied in this work: PyC_nPhe , $ImidC_nPhe$ and $CholC_nPhe$. Figure modified from Kusumahastuti et al. [15].

2. MATERIALS AND METHODS

2.1. Materials

A series of 24 L-phenylalanine derived SAILs was synthesised in the Green chemistry laboratory of Tallinn University of Technology, Estonia. The description of synthesis and characterization data is described in Kapitanov et al. [3]. The stock solutions of SAILs (1 to 8000 mg/L; soluble concentrations) were prepared in sterile MilliQ water, stored at ambient temperature in the dark and tested for toxicity in nominal concentrations within 1-2 weeks.

2.2. Methods

2.2.1. Antimicrobial activity evaluation of SAILs

Antibacterial activity evaluation was adapted from the standard broth microdilution method ISO 20776-1:2006 for antimicrobial susceptibility testing. Altogether 24 L-phenylalanine derived SAILs were evaluated using 2 bacterial strains: *Staphylococcus aureus* 6538 and *Escherichia coli* MG1655. Prior to testing, bacteria were first cultivated overnight in 3 ml of Mueller-Hinton Broth (MHB, Oxoid CM 405, Oxoid Microbiology Products; containing per 1 litre: dehydrated infusion from beef 300 g, casein hydrolysate 17.5 g, starch 1.5 g, Ca^{2+} 4.19 mg and Mg^{2+} 5.6 mg) at 30 °C with shaking at 200 rpm, and then diluted with fresh medium to a bacterial density of $\sim 5 \times 10^5$ CFU/mL in the test (verified by viable plate counts on agarized medium). For toxicity testing, bacteria were incubated with different concentrations of SAILs by combining the bacterial suspension (50 μL) and test compound (SAILs) solution (50 μL) in 96 well transparent microtiter plates (BD Falcon) at 37 °C for 20 hours in the dark in aerobic conditions with minor shaking every 15 min. Wells containing 50 μL of control broth and 50 μL of bacterial culture in MHB were included for each tested strain and served as non-treated controls. The growth of bacteria was evaluated by the increase of the absorbance at 600 nm in 15 min intervals using microplate Spectramax Paradigm spectrophotometer (Molecular Devices, USA). Based on the bacterial growth data two parameters were determined: 1) minimum inhibitory concentration (MIC) as the lowest tested concentration of SAILs where no visible growth of bacteria was observed and 2) half effective concentration (EC_{50}) of the SAILs that reduced bacterial growth by 50%, calculated from the dose-effect curves of bacterial log-phase growth (between 3 h and 10 h of incubation, depending on the bacterial strain, i.e. inhibition of the growth rates of chemical exposed bacteria versus bacterial growth in the control broth) using MS Excel macro Regtox [16]. In addition, minimum bactericidal concentration (MBC) was determined in the end of the experiment (20 hours). For that 3 μL of each sample was removed from the microplate and pipetted as a spot onto Muller Hinton Agar (MHA) growth medium. The inoculated agar plates were incubated at 30 °C in the dark for 24 hours and MBC was designated as the lowest tested concentration of SAILs which totally inhibited the ability of the test bacteria to yield visible colonies on toxicant-free MHB plates.

2.2.2. Statistical analysis

All tests were performed in at least three independent experiments and duplicates in every single experiment. The data are expressed as mean $\pm$ standard deviation. To define statistically significant differences between samples, the data were analyzed by SPSS 21.0 Edition. Data were analyzed by one-way analysis of variance and Tukey's test was used to compare differences among all samples. A p value < 0.05 was considered statistically significant difference.

3. RESULTS

In this study the toxicity of 24 SAILs with cationic headgroups (pyridinium, imidazolium, and cholinium) and varying alkyl ester chain (C_2 - C_{16}) described in Kapitanov et al. (2019) were analysed against 2 different bacteria *S. aureus* and *E. coli* that are models for clinical pathogens. The heat-map of colour-coded toxicity data (mg/L) is presented in Table 1: the SAILs that according to their EC_{50} , MIC or MBC values are presumably non-hazardous (> 100 mg/L; green); harmful (>10 -100 mg/L; yellow), toxic (>1 -10 mg/L; pink) or very toxic (≤ 1 mg/L; red). This simplified hazard ranking system has been initially used to heat-map the ecotoxicological results by Aruoja et al. [17]. The same toxicity ranking criteria were recently used by us for the same library of SAILs towards naturally luminescent marine bacterium *Vibrio fischeri* (also known under the name of *Photobacterium phosphoreum* NRRL-B-11177 and *Aliivibrio fischeri*) which is a well-known model organism for ecotoxicity studies [15]. For the comparison *V. fischeri* data are also presented in Table 1.

Table 1. Toxicity data (EC₅₀, MIC, and MBC) of studied 24 SAILs towards *E. coli* and *S. aureus*. Benzalkonium chloride (BAC) was used as a positive control. For the comparison, 30-min EC₅₀ values for the same SAILs towards bacteria *Vibrio fischeri* [15] are presented. All toxicity values are expressed in both, mg/L and µM (in brackets).

Compound	<i>E. coli</i> (20 h of incubation at 37°C in MHB)			<i>S. aureus</i> (20 h of incubation at 37°C in MHB)			<i>V. fischeri</i> (30 min of incubation at ~20°C in 2%NaCl)
	EC ₅₀ mg/L (µM)	MIC mg/L (µM)	MBC mg/L (µM)	EC ₅₀ mg/L (µM)	MIC mg/L (µM)	MBC mg/L (µM)	EC ₅₀ mg/L (µM)
PyC ₂ Phe	>3933 (>10000)	>3933 (>10000)	>3933 (>10000)	1426±21.6 (3625±54.8)	7866 (20000)	7866 (20000)	866±110 (2203±281)
ImidC ₂ Phe	>3969 (>10000)	>3969 (>10000)	>3969 (>10000)	2474±8.67 (6234±21.9)	7926 (20000)	7926 (20000)	1227±112 (3096±282)
CholC ₂ Phe	>4033 (>10000)	>4033 (>10000)	>4033 (>10000)	1580±10.7 (3917±10.7)	8066 (20000)	8066 (20000)	1153±109 (2858±271)
PyC ₄ Phe	418±1.15 (993±2.73)	843 (2000)	843 (2000)	418±19.6 (991±43.7)	527 (2000)	527 (2000)	81.0±13.4 (192±31.8)
ImidC ₄ Phe	176±44.2 (415±95.1)	849 (2000)	849 (2000)	187±49.7 (442±117)	424 (1000)	424 (1000)	91.7±10.7 (216±25.2)
CholC ₄ Phe	265±17.8 (613±41.2)	863 (2000)	863 (2000)	201±54.2 (466±126)	431 (1000)	431 (1000)	90.1±14.8 (209±34.2)
PyC ₆ Phe	233±14.6 (519±32.6)	449 (1000)	899 (2000)	64.8±2.98 (144±6.63)	112 (250)	112 (250)	12.2±0.73 (27.3±1.62)
ImidC ₆ Phe	285±27.6 (629±61.0)	905 (2000)	905 (2000)	73.0±14.7 (161±32.4)	113 (250)	113 (250)	11.0±1.58 (24.3±3.50)
CholC ₆ Phe	456±35.1 (996±76.5)	916 (2000)	916 (2000)	113±5.03 (246±11.0)	229 (500)	229 (250)	14.8±2.02 (32.3±4.40)
PyC ₈ Phe	34.8±2.93 (72.9±6.14)	59.7 (125)	59.7 (125)	8.64±0.48 (18.1±1.02)	14.9 (31.3)	14.9 (31.3)	9.32±1.21 (19.5±2.53)
ImidC ₈ Phe	56.1±0.08 (117±0.16)	60.1 (125)	60.1 (125)	8.72±0.38 (18.2±0.79)	15.0 (31.3)	15.0 (31.3)	9.30±1.90 (19.4±3.95)
CholC ₈ Phe	30.9±1.48 (63.4±3.03)	60.9 (125)	60.9 (125)	12.2±1.15 (25.1±2.35)	15.2 (31.3)	15.2 (31.3)	20.5±1.23 (41.9±2.51)
PyC ₁₀ Phe	24.2±6.48 (47.9±12.8)	63.2 (125)	63.2 (125)	4.74±0.24 (9.37±0.47)	7.89 (15.6)	7.89 (15.6)	4.40±0.62 (8.71±1.23)

ImidC ₁₀ Phe	31.7±1.50 (62.3±2.9)	63.6 (125)	63.6 (125)	4.72±0.10 (9.28±0.21)	7.93 (15.6)	7.93 (15.6)	4.52±0.42 (8.89±0.83)
CholC ₁₀ Phe	32.2±3.25 (62.5±6.31)	64.4 (125)	32.2 (62.5)	5.00±0.08 (9.69±0.15)	8.04 (15.6)	8.04 (15.6)	5.72±0.77 (11.1±1.49)
PyC ₁₂ Phe	15.4±2.14 (28.8±4.00)	33.3 (62.5)	33.4 (62.5)	1.39±0.20 (2.61±0.37)	4.16 (7.80)	4.16 (7.80)	4.17±0.44 (7.82±0.82)
ImidC ₁₂ Phe	13.1±2.42 (24.4±4.50)	33.5 (62.5)	33.5 (62.5)	2.41±0.09 (4.49±0.17)	4.19 (7.80)	4.19 (7.80)	4.48±0.33 (8.36±0.62)
CholC ₁₂ Phe	15.1±1.20 (27.8±2.22)	34.0 (62.5)	34.0 (62.5)	2.87±0.58 (5.28±1.07)	4.24 (7.80)	4.24 (7.80)	4.29±0.44 (7.90±0.81)
PyC ₁₄ Phe	27.2±1.29 (48.4±2.29)	>70.2 (>125)	>70.2 (>125)	2.25±0.49 (4.01±0.87)	4.38 (7.80)	4.38 (7.80)	5.14±1.22 (9.16±2.17)
ImidC ₁₄ Phe	21.1±6.28 (37.3±11.12)	>70.6 (>125)	>70.6 (>125)	2.28±0.31 (4.05±0.55)	4.40 (7.80)	4.40 (7.80)	4.64±0.47 (8.22±0.83)
CholC ₁₄ Phe	>71.4 (>125)	>71.4 (>125)	>71.4 (>125)	2.62±0.08 (4.58±0.14)	4.46 (7.80)	4.46 (7.80)	4.59±0.41 (8.03±0.73)
PyC ₁₆ Phe	>73.7 (>125)	>73.7 (>125)	>73.7 (>125)	11.1±1.45 (18.8±2.46)	18.4 (31.3)	18.4 (31.3)	>36.9 (>62.5)
ImidC ₁₆ Phe	>74.1 (>125)	>74.1 (>125)	>74.1 (>125)	12.1±1.59 (20.4±2.69)	18.5 (31.3)	18.5 (31.3)	>37.05 (>62.5)
CholC ₁₆ Phe	>74.9 (>125)	>74.9 (>125)	>74.9 (>125)	12.1±2.99 (20.3±4.98)	18.7 (31.3)	18.7 (31.3)	>37.47 (>62.5)
BAC	13.9±1.36 (49.03±4.80)	25.0 (88)	25.0 (88)	1.92±0.05 (6.76±1.86)	3.12 (11)	3.12 (11)	0.94±0.18 (3.31±0.62)

Colour code: ≤ 1 mg/L (■) = very toxic; 1 mg/L < toxic ≤ 10 mg/L (■); 10 mg/L < harmful ≤100 mg/L (■); > 100 mg/L (■) = not classified/not harmful.

4. DISCUSSION

4.1. Antimicrobial potency of 24 SAILs to *E. coli* and *S. aureus*.

Table 1 shows that, as a rule, the toxicity of studied SAILs was not depending on the cationic headgroup but on the length of the alkyl ester chain: the longer the chain the more toxic the compound. This tendency was observed also in our previous study concerning toxic effects of SAILs to *V. fischeri* [15]. As expected, all the C₂ SAILs, independent of the headgroup (pyridinium, imidazolium or cholinium), did not inhibit the bacterial growth (*E. coli* or *S. aureus*) even at very high concentrations (exceeding 1000 mg/L) (Table 1), i.e. these SAILs are not good candidates for antimicrobial use. C₄ and C₆ SAILs were of low to intermediate antimicrobial potency, i.e. inhibited bacterial growth from 65 to 916 mg/L concentrations, depending on toxicity endpoint and bacterium. C₈ and especially C₁₀-C₁₄ SAILs proved efficient inhibiting the growth of bacteria at relatively low concentrations, from 1.39 to 64.4 mg/L, depending on toxicity endpoint and bacterial strain. These inhibitory values were already close to antimicrobial efficiency of benzalkonium chloride (BAC) - a well-known and widely used antimicrobial compound: EC₅₀ values of BAC ranged from 1.9-13.9 mg/L and MIC and/or MBC from 3-25 mg/L (Table 1; Fig. 2). As a rule, the MIC and/or MBC values of studied SAILs were between 4 mg/L to > 8000 mg/L, up to 5-fold higher compared to respective EC₅₀ values and dependent on the length of the alkyl ester chain (Table 1). Analogous results have been obtained with polyoxometalate ionic liquids (POM-ILs) by Kubo et al. [18].

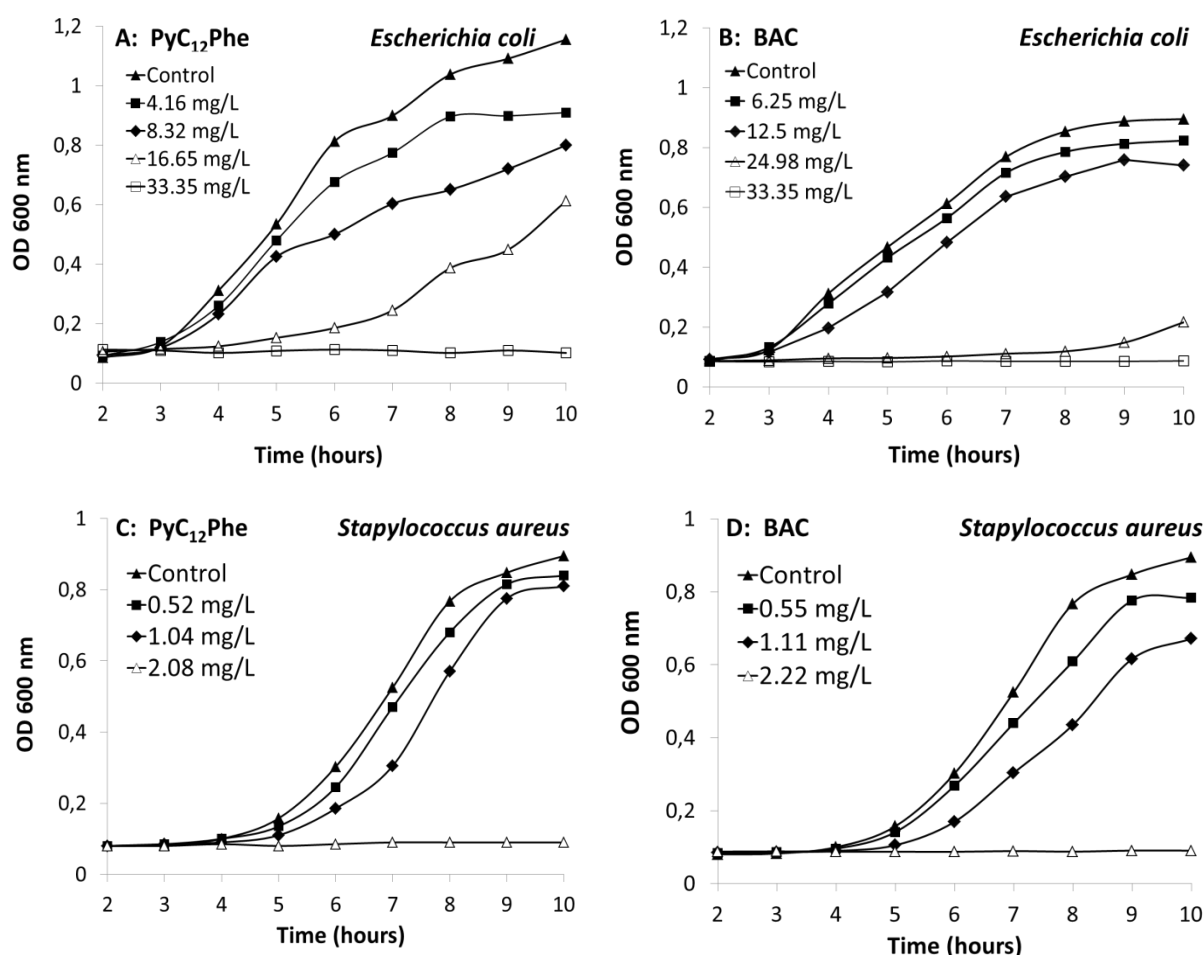


Fig. 2. The representative dose-response growth curves of *S. aureus* and *E. coli* for pyridinium C₁₂ SAILs (A, C) and positive control benzalkonium chloride (BAC) (B, D).

The EC₅₀ values for 24 SAILs tested on *E. coli* and *S. aureus* were in good correlation (log-log R²=0.93) (Fig. 3A) although gram-positive *S. aureus* analysed in the growth inhibition assay in the same conditions and using the same toxicity endpoint was as average 7-fold more sensitive towards SAILs than gram-negative *E. coli*. Higher sensitivity of *S. aureus* compared to *E. coli* towards different type of ILs - polyoxometalate (POM) ionic liquids - was also observed by Kubo et al. [18]. The gram-positive bacterium *S. aureus* is more susceptible to studied SAILs than *E. coli* most probably due to different structure of cell envelope of these bacteria (see 4.2). Interestingly, in the recent work of Kubo et al. [19] where we compared the sensitivity of *E. coli* and *S. aureus* towards nanosilver, the results were opposite, i.e. *E. coli* was more susceptible than *S. aureus*. Thus, the susceptibility of bacteria to different types of antimicrobials depends on type of the chemical and it is highly recommended that the testing for antimicrobials must be conducted with both, gram-positive and gram-negative bacteria.

As it was shown in the work of Kubo et al. [18], the antimicrobial potency of studied POM-ILs was dependent on the alkyl chain length of the cation, i.e. the longer the carbon chain the higher the antimicrobial potency. The same pattern was observed in case of library of SAILs analysed in the current work (Table 1; Fig. 3). Concerning the antimicrobial potency of C₁₄ and C₁₆ SAILs (Table 1) it could be noted that in the toxicity tests made in the current study they are less toxic than C₁₂, i.e. the elongation of the carbon chain was not accompanied by the increased toxicity. That is probably due the limited solubility of this compound and lower bioavailable fraction. Indeed, we observed that C₁₄ and C₁₆ were less soluble in the water than C₁₂. Therefore, the C₁₂ SAILs are probably most suitable candidates for antimicrobial ILs with relatively high antimicrobial potency (Fig. 2) and reasonable solubility in water.

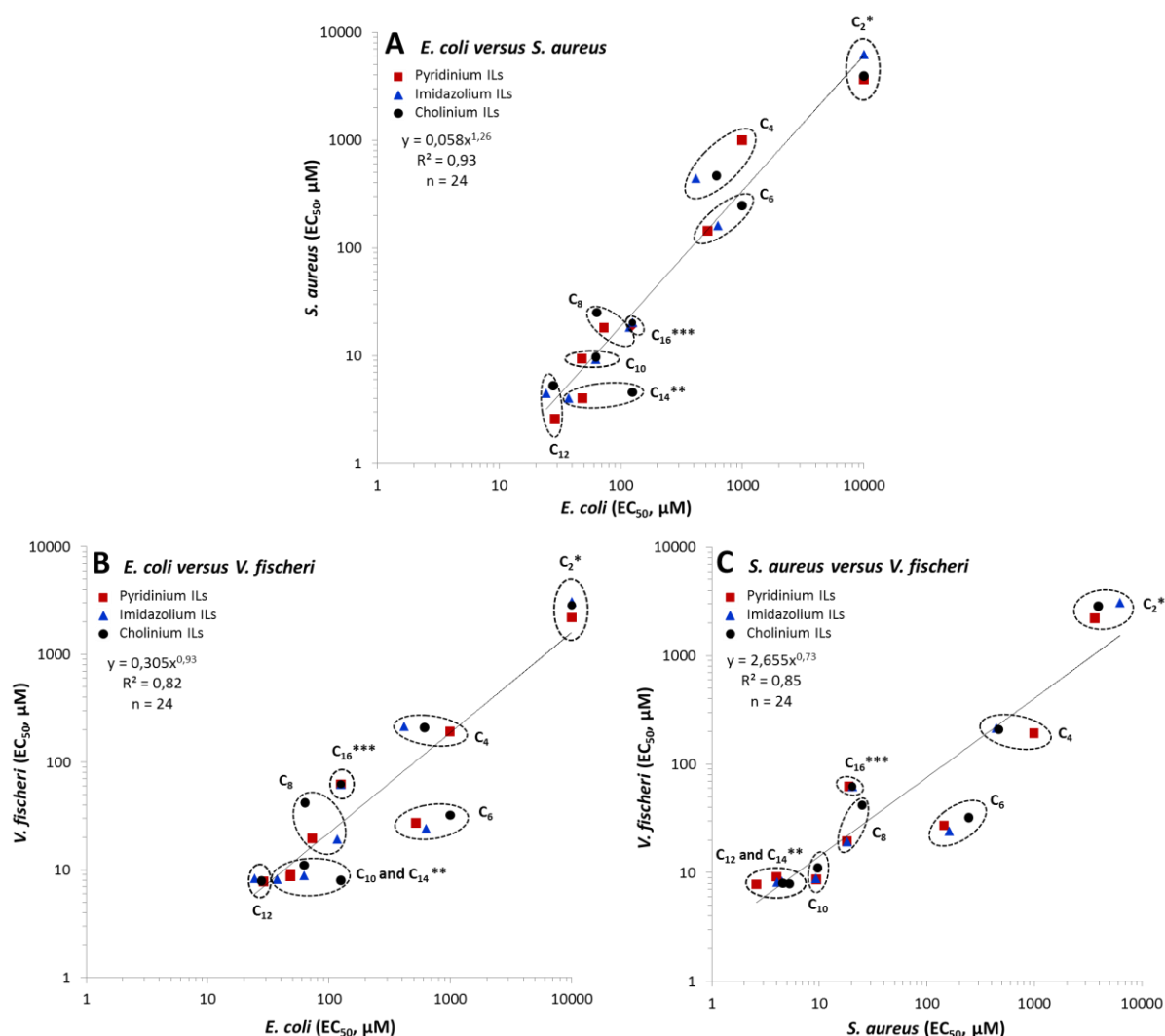


Fig. 3. The comparison of the toxicity of 24 SAILs towards different bacteria. A: *E. coli* versus *S. aureus*. Log-log $R^2=0.93$; B: *E. coli* versus *V. fischeri*. Log-log $R^2=0.82$; C: *S. aureus* versus *V. fischeri*. Log-log $R^2=0.85$. Data are plotted from Table 1. * EC_{50} of studied C_2 SAILs to *E. coli* > 10 000 μM ; ** EC_{50} of Chol C_{14} Phe to *E. coli* > 125 μM ; *** EC_{50} of studied C_{16} SAILs to *E. coli* > 125 μM and *V. fischeri* > 62 μM .

4.2. Mechanism of antibacterial action: effect on cellular membranes

Bacteria are unicellular organisms that are separated from the surrounding environment by cell envelope consisting of various layers (cell wall, membrane) depending on the bacterial type (gram-negative versus gram-positive bacteria). However, the central barrier between the living and non-living is a biological membrane which basic structural unit is a lipid bilayer. As mentioned above, the type of the headgroup of studied 24 SAILs had a negligible effect on their antibacterial efficiency. However, the toxic effect was depending on alkyl ester chain length (Table 1). This is known as side chain effect [20] and the toxicity is assumed to be a manifestation of the interaction of SAILs with cell membranes. SAILs with longer alkyl ester chains probably more efficiently penetrate the cellular membrane's lipid bilayer, and the cell structure of the microorganism can be disrupted [21]. According to Yoo et al. [22], when the ILs cationic part begins to penetrate the cell membrane, the bending of the membrane starts yielding cell damage.

Based on the report of Ibsen et al. [2] there are two main stages for interaction of cationic amphiphiles with microorganisms: (i) first, the attachment of amphiphile to the target membrane - a process

governed by electrostatic interactions between the positively charged polar head of the surfactants and the negatively charged molecules of the bacterial membranes (lipopolysaccharides in gram-negative and lipoteichoic acid in gram-positive bacteria) [23]; (ii) following that, the hydrophobic alkyl chain of cationic amphiphiles interacts with the lipid bilayers of membranes. For that stage there is an optimum relationship between the hydrophobicity and polarity of the surfactant to facilitate the diffusion of the surfactant in the non-polar environment of lipid bilayer. Hence, cationic surfactants are generally more potent against gram-positive bacteria, which contain high amounts of negatively charged lipids [24]. Furthermore, it has been reported that there is an interaction between imidazolium heterocycle and the lipid head group in the cell membrane, while the side chain will interact with the lipid tail. This interaction can lead longer alkyl ester side chains to penetrate deeper into lipid bilayer [25]. Research by Cornmell et al. [26] has shown that the accumulation of ILs [P_{6,6,6,14}] [NTf₂] was found in the membrane of *E. coli* and not in the cytoplasm of bacteria. Therefore, cell membrane damage is assumed to be the main reason for SAILs antimicrobial action.

The effect of SAILs on biological membranes as a toxicity mechanism is supported also by the fact that growth inhibition data (EC₅₀; 20 h of incubation) for *E. coli* and *S. aureus* correlated well with 30-min bioluminescence inhibition data of *Vibrio fischeri* (Fig. 3B and 3C). The bioluminescence of *V. fischeri* is tightly connected to the vitality of its cellular membrane and the membrane-related processes such as energy production [27] and the decrease of the luminescence is proportional to the toxicity of chemical compound. Therefore, these data indicate that if toxic, SAILs mostly affect biological membranes and thus probably act universally on all types of microorganisms [28].

Research on this library of SAILs to refine design principles to enable the development of low microbial toxicity ILs has also been performed by Kapitanov et al. [3]. This included a preliminary toxicity evaluation where they used various microorganisms, including *E. coli* and *S. aureus*. In general, the trends observed were in agreement to our results reported herein: (i) the toxicity increased with the increase of the alkyl ester chain and was not dependent on the headgroup; (ii) the more efficient in inhibiting bacterial growth were C₁₀-C₁₄ SAILs; (iii) *S. aureus* was more susceptible than *E. coli* to SAILs tested whereas the test format and test strains were different of these used in the current study.

Due to the importance of the membranes in "guarding" the entrance of toxicants into the living cell, the octanol/water partition coefficient (K_{ow}) of studied 24 SAILs was compared to their antimicrobial potency. Indeed, the ability of a chemical to dissolve in lipids (a proxy for ability to cross the biological membrane's lipid bilayer and enter the cell) is in correlation with its toxicity. According to Bradbury and Lipnick [29] about 70% of industrial organic chemicals are estimated to act *via* the narcosis mechanism the toxicity. Narcotic effects are estimated by the ability of a compound to interact with cellular membranes. Narcosis is "baseline" toxicity of chemicals i.e. the toxicity which correlates with their hydrophobicity (for example, octanol/water partition coefficient, K_{ow}) and this parameter is widely used as a descriptor for QSARs [30, 31]. Therefore, consistent experimental toxicity data for different organisms are crucial in order to develop respective QSARs. As the research conducted by Stepnowski and Storonik [32] has shown the relationship between the lipophilic properties of ionic liquids and their ability to penetrate cells, we compared the log K_{ow} values (taken from Kusumahastuti et al. [15]) with toxicity data (EC₅₀ values; Table 1) of 24 SAILs to *E. coli* and *S. aureus* (Fig. 4). All log K_{ow} values of the studied ILs were estimated to be <4 and there was a positive correlation between the toxicity and log K_{ow} up to C₁₂ (Fig. 4) but not further (for C₁₄ and C₁₆). Also, the toxicity pattern of studied SAILs (Table 1; Fig. 5) indicates a hysteresis: toxicity of C₁₄ and C₁₆ was lower than that of C₁₂ SAILs. Analogous pattern was observed in our previous study by Kapitanov et al. [3] and Kusumahastuti et al. [15] that all studied the toxicity of the same library of SAILs but with different bioassays. Also some previous studies [20, 33] have shown that the elongation of alkyl chain length up to specific lengths could decrease their toxicity; this phenomenon has been labelled as a "cut-off effect".

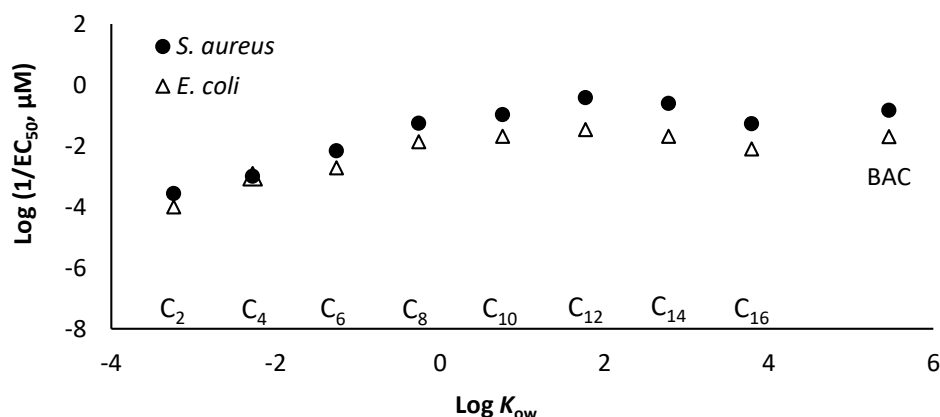


Fig. 4. Relation between $\text{Log } K_{ow}$ (X-axis) and $\log (1/EC_{50})$ (Y-axis) of 24 SAILs. Data of EC_{50} (μM) are taken from Table 1. The K_{ow} values were calculated using open-access online tool (<https://www.molinspiration.com/services/logp.html>) and taken from Kusumahastuti et al. [15].

To explain the latter irregularity, a contribution to the observed toxicity due to the critical micelle concentration of SAILs may be operational. Specifically, Kapitanov et al. [3] state that the effectiveness of surfactants as antimicrobial agents can be linked to critical micelle concentration (CMC). They found that the MIC_{95} values of C_{14} and C_{16} were above the CMC value. Therefore, their toxicity decreased. They proposed theories that the elongation of the side alkyl chain length affects the formation of micelles rather than their tendency to move to the surface of bacterial cells. This hypothesis was in agreement with Pernak et al. [34] who studied a series of alkoxyethylimidazolium ILs.

In summary, a systematic study of the antimicrobial potency of a library of 24 L-phenylalanine derived surface-active ionic liquids (SAILs; C_2 - C_{16}) towards *E. coli* and *S. aureus* bacteria was performed using standard broth microdilution method ISO 20776-1:2006. The main results on the overall antimicrobial potency of studied SAILs to *E. coli* and *S. aureus* are depicted in Fig. 5: the short and medium chain length (C_2 to C_6) showed low toxicity but the SAILs with longer chain length (C_8 to C_{12}) exhibited antimicrobial activity to the bacteria screened, with the C_{12} SAILs being recommended as the most suitable candidates of our series for antimicrobial use.

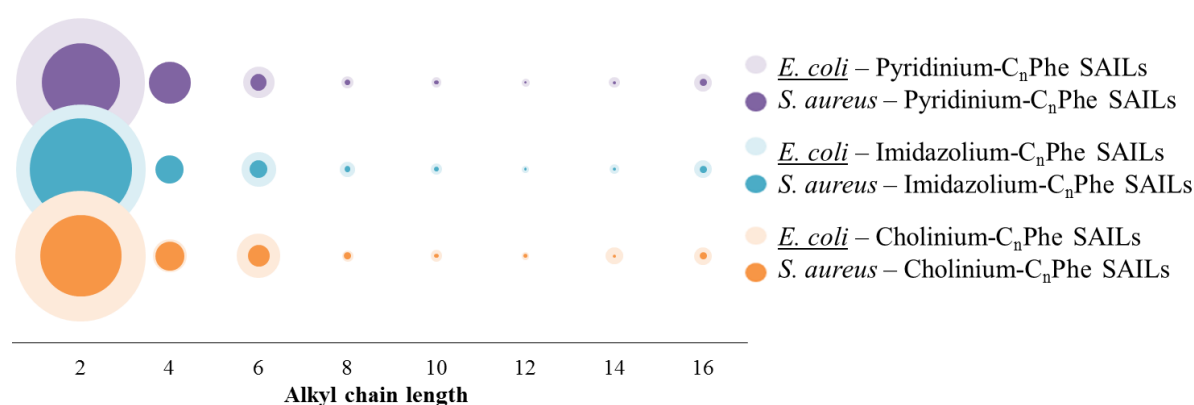


Fig. 5. Effect of the alkyl chain length (C_2 - C_{16}) of studied SAILs (X-axis) on the overall antimicrobial potency (expressed as EC_{50} values, μM ; Table 1) to *E. coli* and *S. aureus* bacteria (Y-axis). The smaller the bubble the more antimicrobial SAILs. Pyridinium, imidazolium and cholinium head groups are indicated with different colours.

5. CONCLUSIONS

In this paper the antimicrobial activity of 24 L-phenylalanine based SAILs against clinically important gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria are reported. The following conclusions can be drawn from the present study:

1. The antimicrobial potency of SAILs containing different cationic headgroups (pyridinium, imidazolium, and cholinium) increased with the length of the alkyl ester chain (from C₂ to C₁₂) and then levelled up (C₁₄) or decreased (C₁₆) and was not dependent on the type of head group.
2. The minimal inhibitory concentration (MIC) range varied from up to 8000 mg/L (C₂ SAILs) down to ~4 mg/L (C₁₂ SAILs). Most efficient were C₁₂ SAILs that inhibited the growth of gram-positive bacterium *S. aureus* and gram-negative bacterium *E. coli* at concentration of few milligrams per liter that is comparable to the antimicrobial efficiency of benzalkonium chloride - a well-known and widely used antimicrobial compound.
3. As a rule, gram-positive *S. aureus* bacteria were more susceptible to the SAILs than gram-negative *E. coli*.
4. The growth inhibition data (EC₅₀) for *E. coli* and *S. aureus* correlated well. Also, the EC₅₀ values for above mentioned bacteria correlated well with 30-min bioluminescence inhibition data of *Vibrio fischeri* showing that the toxicity of these SAILs mostly affects biological membranes and thus probably affects universally all types of microorganisms.

Overall, the SAILs are promising antimicrobials, which toxicity towards bacteria can be tuned by modifying the alkyl ester chain properties.

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