

Antimicrobial Activity of *N*-Alkoxycarbonylmethyl-*N*-alkyl-piperidinium Chlorides

Edyta Woźniak^{a,*}, Anna Mozrzyńska^a, Anna Czarny^b, Maja Kocięba^b,
Bożenna Różycka-Roszak^a, Zofia Dega-Szafran^c, Ewa Dulewicz^c, and
Magdalena Petryna^c

^a Department of Physics and Biophysics, Agricultural University, Norwida 25,
50-375 Wrocław, Poland. Fax: +48-71-3205172. E-mail: GUCIO@OZI.AR.WROC.PL

^b Institute of Immunology and Experimental Therapy, Polish Academy of Sciences,
53-114 Wrocław, Poland

^c Faculty of Chemistry, Adam Mickiewicz University, 60-780 Poznań, Poland

* Author for correspondence and reprint requests

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The aim of the study was to assay antibacterial and antifungal activity of newly synthesised *N*-alkoxycarbonylmethyl-*N*-alkyl-piperidinium chlorides. The compounds tested were found to inhibit the growth of some Gram-negative bacteria, Gram-positive strains and some representatives of yeast-type *Candida*. From microbiological experiments two of the compounds tested, *N*-dodecyloxycarbonylmethyl-*N*-methyl-piperidinium chloride (**3**) and *N*-dodecyl-*N*-ethoxycarbonylmethyl-piperidinium chloride (**6**), emerged as more active than the other compounds. Since the resistance of biofilms to biocides should be noted during the design and testing of new antimicrobial agents therefore, we have analysed antibacterial properties of the most active compounds towards biofilms. Our study focused on strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus* that served as main model organisms for the biofilm studies.

Key words: Piperidinium Chlorides, Antimicrobial Activity, Molar Volume

Introduction

Nosocomial infections of immunocompromised patients are difficult to treat and result in significant morbidity and mortality. It is important to define the role of opportunistic pathogens, which may be part of the normal human bacterial flora and cause the diseases especially when the host immunity becomes impaired (Gransolen, 1997). Unfortunately, in recent years a permanent increase in the number of bacterial strains resistant to disinfectants has been observed. The first step to reduce the risk of nosocomial infections is prophylactics. Disinfection of medical equipment by using antimicrobial agents with a broad spectrum of activity is necessary for the prevention of opportunistic fungal and bacterial infections. In view of the above, further efforts are needed to develop a new group of antimicrobial compounds (Russel, 1998). Cationic surfactants are of interest because of their membrane-disruptive and rapid antimicrobial activities. These agents are often active against a broad range of bacteria and other cells (Pinna-duwage *et al.*, 1989). This is the reason why the compounds belonging to this group are still syn-

thesised. In order to get some insight into the relationship between their biological activity and structure, a group of newly synthesised quaternary ammonium salts (QAS) has been tested on bacterial strains. QAS of heterocyclic and alicyclic bases show antibacterial properties (Shelton *et al.*, 1946; Smith *et al.*, 1951). Apart from the antibacterial properties, investigations of the antifungal activity of QAS have been carried out on the species *Candida* genus which are the primary etiologic agent of candidiasis, a disease that remains a major complication among immunocompromised patients following nosocomial infections. In the hospital environment microorganisms may be less susceptible to many commonly used disinfectants than culture collection strains. Therefore, some bacterial and *Candida* strains, used in this study, were clinical isolates. Also, it is well known, that antimicrobial agents of most type are much less effective on organisms embedded in a biofilm, in comparison to the planctonic bacteria in suspension (Gransolen, 1997). Microbial biofilms have been observed on surfaces in many aquatic ecosystems being widespread not only in nature but also in medical

devices. The ability of microorganisms to adhere to different materials can be a source of serious nosocomial infections. The increased resistance of biofilms to antimicrobial treatment suggests that new disinfectants should be developed against biofilms. Our study was centred on the strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which are important opportunistic pathogens involved in nosocomial infections and new quaternary piperidinium salts were tested as antibacterial and antifungal means.

Materials and Methods

Chemicals

The *N*-alkoxycarbonylmethyl-*N*-alkyl-piperidinium chlorides **1–6** were prepared as follows. *N*-Alkyl-piperidines (except *N*-methyl- and *N*-ethyl-piperidine; supplied from Aldrich) were prepared as described earlier (Stross and Evans, 1942) and purified by vacuum distillation. To *N*-alkyl-piperidine (1 M) in ether solution (50 cm³), alkylchloroacetate (Smith *et al.*, 1951; Nasimento *et al.*, 1996; Baniel *et al.*, 1948) in ether solution (50 cm³) was added while cooling and stirring, according to the procedure given by Barczyński *et al.* (2000). The mixture was kept at room temperature up to solidification. The precipitate was filtered off and washed with ethyl ether. The general structure of the tested compounds, particular substituents and melting points are documented in Table I. Dodecyltrimethylammonium chloride (DTAC; Aldrich) was used as a reference substance in the microbiological tests.

Bacterial and yeast-like strains

The bacterial and *Candida* strains used in this study were clinical isolates obtained from patients: two strains of *Pseudomonas aeruginosa*, one strain of *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*. The bacteria studied included the following standard strains from Polish Collection of Microorganisms (Institute of Immunology & Experimental Therapy, Polish Academy of Sciences, Wrocław): *Pseudomonas aeruginosa* PCM 2058, *Escherichia coli* PCM 2057, *Staphylococcus aureus* PCM 2054, *Bacillus subtilis* PCM 2021. In addition, the type strains of *Candida parapsilosis* and *Candida guilliermondii* (clinical isolates) were used. The bacteria strains were maintained on nutrient agar slants at 4 °C, whereas *Candida* strains were stored on Sabour-

aud agar slants at 4 °C. For the experiments described here, the species tested were cultured for 18 h at 37 °C (or 28 °C for *Candida*) in liquid growth medium. Bacterial growth was monitored by measuring the optical density at 600 nm on Specol (for *Candida* 560 nm) and the numbers of CFU (colony-forming units) were quantified by plating serial dilutions (1/10 dilutions) of suspensions onto nutrient or BHI agar plates (*Candida*-Sabouraud agar plate).

Media

Nutrient broth and nutrient agar (Difco, Detroit, USA) were used for cultivating Gram-negative strains, Brain Heart Infusion (BHI) and BHI agar (Oxoid, Basingstoke, UK) for Gram-positive bacterial strains. Sabouraud medium for culturing of *Candida* strains was used. PBS (phosphate buffered saline) for diluting was used.

Antimicrobial activity assay

4 h before the experiment, freshly made media were inoculated with an overnight culture of all tested strains and grown at 37 °C (or 28 °C for *Candida*). In this method, a culture of a known concentration was spread on an appropriate agar medium (100 µl). Biocides were prepared as stock solutions in water. Working solutions of compounds were prepared in PBS to a concentration of 5000 µg/ml and diluted. A predetermined concentration of antimicrobial agents (in PBS; 5000, 2500, 1250, 625, 300, 150 µg/ml) was applied dropwise on the seeded agar plate in a set pattern (10 µl). Then the plates were incubated for 24 h to 48 h at 37 °C (or 28 °C for *Candida*). Compound-free samples were used as controls for all tests. When the sample solution contained antimicrobial activity, a clear zone of inhibition on the agar was observed. Each test was performed in triplicate.

Bacterial biofilm

Pseudomonas aeruginosa and *Staphylococcus aureus* (clinical isolates) were used throughout the study. The biofilm formation involved housing plastic (polyethylene) disks (0.5 cm²) in respective bacterial suspension, 10⁷–10⁸ CFU/ml (in one of the 24-well Falcon tissue culture plate, with 1 ml of nutrient broth). Following incubation of disks for 24, 48, 72 h at 37 °C, the growth medium was removed from each well and fresh growth medium was added. After 72 h the medium was removed

and replaced with 1 ml of the tested compound solution. The biofilms (10^{10} – 10^{11} CFU/ml) were incubated for further 5 h at 37 °C. After 0.5 h, 1 h, 2 h, 3 h, 4 h and 5 h the samples of bacterial biofilm were gently rinsed in sterile PBS to remove any unattached planktonic microorganisms and the biofilm was removed from both sides of disks by scraping with a sterile cell scraper into 1 ml PBS. The bacteria were suspended by vortexing. The biofilm samples were serially diluted and cultured on appropriate agar plates. The dishes were incubated for 24 h at 37 °C, then the bacterial time-dependent sensitivity was measured on the basis of seeding results (no growth).

Results

The experiment started by comparing the killing activities of the compounds tested against selected bacteria species and two representatives of *Candida*-type yeast. The microorganisms were exposed to the compounds (Table I) investigated in different concentrations. The activity for the tested compound was estimated on the basis of the lowest concentration giving a lethal effect. The results presented in Table II indicate that the group of quaternary ammonium salts had growth-inhibitory activity. The strains assessed exhibited the highest sensitivity to compound **6**, and the activity of **3** was slightly lower (different killing activities towards the strains of *P. aeruginosa* and *E. coli* clinical isolates were observed). **2** revealed weak lethal activity. By comparing the antimicrobial activities of the tested compounds against the whole panel of the microorganisms studied it was shown that DTAC exerted a similar antimicrobial capability

as **2**. A further shortening of the long-chain hydrophobic substituent led to the loss of the activity. Compounds **1**, **4**, **5** were much less effective. These compounds in the range of concentrations tested were inactive against some strains of bacteria. The data have also shown that two species of *Candida* are sensitive to all substances tested. The results presented in Table II indicate that the overall order of antimicrobial activity is **6** > **3** > **2** > **5** > **1** > **4**.

Since a biofilm is much more resistant to biocides than the free bacteria we further explored the antimicrobial activity of the tested compounds by proposing a *in vitro* model of a bacterial biofilm. The data indicated that derivatives **6** and **3** are good antimicrobial compounds for planktonic bacteria in suspension. Therefore, the activities of **3** and **6** were analysed from 30 min to 300 min to a *P. aeruginosa* and *S. aureus* biofilm obtained on a polyvinyl surface. The results obtained clearly indicate the difference in sensitivity to biocides between free and biofilm bacterial population. The compounds at MIC concentration (Table II) cannot eradicate the biofilm. The bactericidal effect was not observed for a concentration lower than 5000 µg/ml. The results (Table III) show that the bactericidal activity of the compounds tested was time- and strain-dependent. The *Staphylococcus* biofilm was found to be much more sensitive to the investigated biocides than the *Pseudomonas* biofilm. As shown in Table III there was a significant difference in the antimicrobial activity of the substances tested. Considering the bactericidal activity of **6** and **3** towards a *Pseudomonas* biofilm the most active derivative appeared to be **6**. The bactericidal effect was already detected after 30 min.

Table I. The general structure and substituent groups of the compounds studied.

No	R ¹	R ²	M.p. [°C]	V ^b
1	CH ₃	C ₈ H ₁₇	152–153	333.86
2	CH ₃	C ₁₀ H ₂₁	158–159	365.19
3	CH ₃	C ₁₂ H ₂₅	157–158 ^a	396.51
4	C ₂ H ₅	C ₂ H ₅	165–169	255.07
5	C ₈ H ₁₇	C ₂ H ₅	112–116	349.36
6	C ₁₂ H ₂₅	C ₂ H ₅	109–114	412.01

^a M.p. 156–157 °C from Smith *et al.* (1951).

^b Molar volume.

Discussion

In the present study we used strains which are important opportunistic pathogens involved in nosocomial infections. The high resistance of biofilms to commonly used disinfectants suggests that the biofilm should be used for testing new biologically active compounds. Different sensitivities of the bacterial biofilms studied to QAS result from a few factors. For example, one of the biofilm resistance mechanisms is the presence of an exopolysaccharide matrix that can slow down the diffusion of antimicrobial agents. On the other hand, the differences in composition of the cell wall of Gram-negative (*P. aeruginosa*) and Gram-positive

Table II. Activity of the compounds studied with microorganisms.

Microorganism tested	Minimum lethal concentration of the compound						$\frac{[\mu\text{g/ml}]}{[\text{mM}]}$
	1	2	3	4	5	6	DTAC
<i>B. subtilis</i> PCM	5000 18.5	1250 4.2	150 0.5	>5000 >21.3	5000 15.7	150 0.4	1250 4.7
<i>P. aeruginosa</i> W*	>5000 >18.5	5000 16.8	5000 15.3	>5000 >21.3	10000 31	2500 6.6	5000 19
<i>P. aeruginosa</i> 219*	>5000 >18.5	5000 16.8	5000 15.3	>5000 >21.3	10000 31	2500 6.6	5000 19
<i>P. aeruginosa</i> PCM	>5000 >18.5	1250 4.2	300 1	>5000 >21.3	5000 15.7	300 0.8	1250 4.7
<i>K. pneumoniae</i> 64*	>5000 >18.5	625 2.1	300 1	>5000 >21.3	5000 15.7	300 0.8	625 2.4
<i>E. coli</i> PCM	5000 18.5	625 2.1	150 0.5	>5000 >21.3	5000 15.7	150 0.4	300 1.2
<i>E. coli</i> 0111*	5000 18.5	625 2.1	625 2	>5000 >21.3	5000 15.7	150 0.4	625 2.4
<i>S. aureus</i> PCM	5000 18.5	1250 4.2	150 0.5	5000 21.3	5000 15.7	150 0.4	1250 4.7
<i>E. faecalis</i> *	5000 18.5	1250 4.2	150 0.5	>5000 >21.3	5000 15.7	150 0.4	1250 4.7
<i>C. parapsilosis</i> *	5000 18.5	1250 4.2	150 0.5	5000 21.3	5000 15.7	150 0.4	2500 9.5
<i>C. guilliermondii</i> *	5000 18.5	1250 4.2	150 0.5	5000 21.3	5000 15.7	150 0.4	2500 9.5

* Clinical isolates.

Table III. Antimicrobial properties of compounds **3** and **6** at the concentration 5000 $\mu\text{g/ml}$ against bacterial biofilms.

Compound	<i>P. aeruginosa</i>								<i>S. aureus</i>						
	Time [h]														
3 (15.3 mM)	0	0.5	1	2	3	4	5	0	0.5	1	2	3	4	5	
6 (13.2 mM)	–	–	–	–	–	–/+	+	–/+	–/+	–/+	+	+	+	+	+
DTAC (19 mM)	–	+	+	+	+	+	+	–/+	+	+	+	+	+	+	+
	–	–	–	–	–	–	–	–	–	–	–	–/+	+	+	+

+ No growth of bacteria.
– Growth of bacteria.

bacteria (*S. aureus*) cause different resistance to killing by antimicrobial agents. Thus, the relatively low permeability of the outer membrane of Gram-negative microorganisms plays a key role in the defence strategy of bacteria since it slows down the penetration of many substances.

The compounds studied may be divided into two homologous series: **1**, **2** and **3** belong to the series with the methyl group as one substituent and **4**, **5** and **6** to the series with a long hydrophobic alkyl chain as a substituent of the piperidine derivatives. In both series the killing effect of the studied com-

pounds correlates well with the length of the chain.

As follows from earlier studies, the -COO-group in proximity of the head group can be considered equivalent to the -CH₂-group (Różycka-Roszak and Fisicaro, 1993). Therefore, the hydrophobic chain of **3** can be treated as an equivalent to an alkyl chain of 14 carbon atoms. Having the above in mind, compounds **6**, **2** and DTAC can be considered as having the same length of alkyl chains of 12 carbon atoms. So, in agreement with chain length-dependence of the lethal effect, the activity

of these compounds should be approximately similar while that of **3** higher than that of **6**. The activities of **2** and DTAC seem to be quite similar and **6**, not the expected **3**, proved to be the most active compound among the tested QAS. This indicates that the activity depends not only on the length of the longer substituent, but also on the presence of the second one that also contributes to the hydrophobicity of the whole molecule. Since all the compounds studied have the same polar part, the changes in the molar volume depend only on the structure of the hydrophobic part. Therefore we decided to compare the molar volumes of the

compounds studied with their biological activity. Molar volumes are calculated by the method described by Kier and Hall (1986) and listed in Table I. From the obtained results, we tentatively assume that the activity may relate to the molar volume. However, more analogues have to be compared to establish a proper structure-activity relationship.

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