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Anti-Staphylococcal Biofilm Activity of Miconazoctylium Bromide

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We designed and synthesized miconazole analogues containing a substituted imidazolium moiety. The structural modification of the miconazole led to a compound with high potency to prevent the formation and disrupt bacterial biofilms, as a result of accumulation in the matrix biofilm, permeabilization of the bacterial membrane and generation of reactive oxygen species in the cytoplasm.

Introduction

Bacterial biofilms are microbial communities held together by an extracellular matrix[1] that is composed of exo-polymeric substances (EPS),[2] polysaccharides, proteins, lipids, extracellular DNA and other bacterial decomposition substances. The polymers are held together by a complex network of hydrogen bonds, ionic and van der Waals interactions that are crucial for the integrity of the biofilm.[3]a-c. Bacterial biofilms can be found in numerous infections including the lungs of cystic fibrosis patients, burns, teeth and ear infections, etc.[4] Their presence has led to a dramatic enhancement in bacterial resistance to antibiotics. They are a serious health concern as 17 million new biofilm-associated infections occur each year in the United States alone, resulting in up to 550,000 deaths annually.[5], [6] Therefore, there is an increasing interest in developing new strategies and agents to prevent the formation of biofilms or to destroy already formed biofilms.

Biofilm formation is a multistep process as shown in Figure 1. Planktonic bacteria are first thought to attach to an abiotic surface; the attachment becomes irreversible in a second stage. Subsequent steps involve biofilm maturation and growth, which results in the formation of the three-dimensional bacterial community. Bacterial detachment occurs in the last step, when planktonic bacteria are released from the biofilm in order to colonize new sites. Among the different interactions responsible for the formation of biofilms, electrostatic interactions are the earliest forces affecting bacterial adherence to surfaces (Figure 1).[7] Since the outer surfaces of biofilms consist of an anionic matrix, it is not surprising that cationic compounds may act as early stage biofilm inhibitors.[8] Indeed, during the past decade, quaternary ammonium compounds (QACs) have been shown to be potent inhibitors for biofilm formation,[9] but they have also been shown to be toxic to mammalian cells.[10]

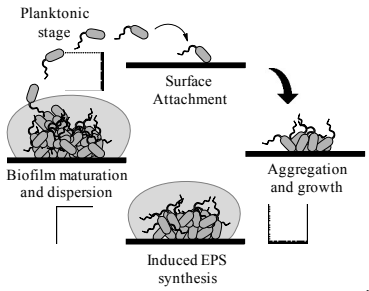

Figure 1. Formation, maturation, and dispersion of bacterial biofilms.[1]

Staphylococcus aureus (*S. aureus*) is a Gram-positive, biofilm forming bacteria responsible for over 50,000 deaths annually in the United States. Furthermore, the emergence of antibiotic-resistant strains such as methicillin-resistant *S. aureus* (MRSA) has become a serious concern in clinical establishments since it was associated with viral infections and high levels of mortality.[11] The appearance of MRSA resistant to benzalkonium chloride (**BAC**),[12] a QAC widely used as disinfectant in hospitals, is now being reported on a regular basis.[13]

Miconazole, an imidazole-containing compound, is a potent antimycotic agent used against a wide range of pathogenic fungi that also possesses antistaphylococcal activity.[14], [15] It was proposed that miconazole inhibits bacterial and fungal growth by binding to flavohemoglobin, an enzyme involved in

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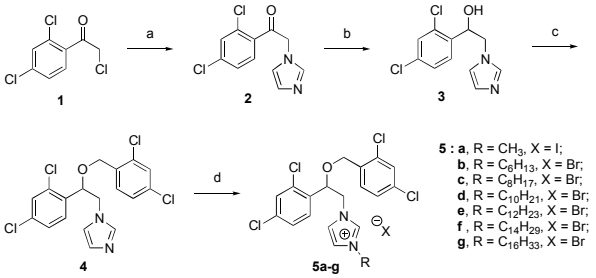
the bacterial defense against nitrosative stress. Miconazole's action was then related to the production of endogenous reactive oxygen species (ROS) that led to significant cellular structural damage, resulting in cell death.[16] The imidazole ring was proposed to bind the iron centre of the heme moiety of flavohemoglobin resulting in the generation of intracellular ROS that act as nitric oxide dioxygenase inhibitors.[17] Unfortunately, microorganisms rapidly develop resistance to compounds that act on one specific receptor as in the case of miconazole, especially in mixed biofilms.[18] We show here that changing the imidazole ring of the known drug miconazole into an imidazolium cation results in a compound that has both antibacterial activity and also anti-biofilm properties.

Results and discussion

As mentioned above, QACs in general, and **BAC** in particular, can act as anti-biofilm agents by disrupting the interactions of the extracellular DNA and other components of the bacterial EPS. QACs can alter the properties of abiotic surfaces, decreasing their surface tension and therefore preventing the formation of biofilms.[19] With this information in mind, we transformed the imidazole unit of a miconazole into a quaternary amphiphilic imidazolium that should facilitate its penetration of EPS, due to the presence of the positive charge and the lipophilic alkyl chain. All the studied alkylmiconazolium salts (Scheme 1) are also different from miconazole in terms of mechanism of action, because they do not possess the capacity to bind to flavohemoglobin. Also, the longer alkyl chains possess the amphiphilic balance of a QAC and can act as anti-biofilm agent. The antibacterial activity of the alkylmiconazolium bromides was first tested against planktonic Gram-positive, Gram-negative bacteria and fungi and compared to miconazole and **BAC** (Table 1).

Scheme 1. Synthesis of alkylmiconazolium salts[a]



[a] Reagents and conditions: (a) 1*H*-imidazole (3.0 equiv), CH_2Cl_2, 0 °C to rt, 72 %; (b) $NaBH_4$, MeOH/CH_2Cl_2, 0 °C to rt, 77 %; (c) NaH, 2,4-dichlorophenylmesylate, DMF, 0 °C, 87 %; (d) Alkyl halide, MeCN, 70 °C, 70-95 %.

The minimal inhibitory concentrations (MIC)[20] of alkylmiconazolium salts **5a-g**, corresponding to the lowest concentration of antimicrobial that resulted in the reduction of the visible growth of planktonic *MRSA* (ATCC 43300), *E. coli* (MG1655) and *C. albicans* (SC5314) are reported in **Table 1**. Miconazoctylium bromide (**5c**) was our lead compound since it showed a 12-fold increased inhibition activity compared to miconazole (**4**) and a 7-fold increased activity compared to **BAC** *MRSA*. The higher activity of miconazoctylium bromide (**5c**) on *MRSA* cannot be attributed only to its surfactant properties, as it is more active than **BAC**.[21] At the same time, the absence of free electrons on the imidazole's nitrogen atom prevents its complexation of the heme. However, the transformation of the imidazole group into an imidazolium cation is not the only parameter responsible for the higher activity of **5c**, as analog **5a** was 8-fold less active than **5c**. The hydrophilic/hydrophobic balance responsible for the penetration and permeabilization of the bacterial phospholipid membrane is also an important factor, as salts **5b**, **5c** and **5d** were the most active compounds on Gram-positive, Gram-negative bacteria and fungi.

Table 1. Minimal concentrations (μM) required to inhibit the growth of different organism.

	Methycillin-resistant S. aureus[a] (ATCC 43300)	*E. coli*[b] (MG1655)	*C. Albicans*[c] (SC5314)
Miconazole (4)	> 550	> 115	25
(5a)	345	> 345	20
(5b)	85	50	10
(5c)	45	25	2.5
(5d)	45	> 345	2.5
(5e)	150	> 345	10
(5f)	> 345	> 345	> 200
(5g)	> 345	> 345	> 200

[a] Gram-positive bacteria. [b] Gram-negative bacteria. [c] Yeast. Each value is the mean of at least three independent experiments, each including three replicates for each antimicrobial concentration.

With these results in hand, we studied the ability of the most active miconazoctylium bromide (**5c)** to prevent the formation of *MRSA* biofilms, using a commercially available fluorescent assay (Baclight Live/Dead) that differentiates between alive intact cells (green) and permeablized dead cells (red) populations.[22] Miconazoctylium bromide (**5c**) showed a remarkable biofilm-destruction activity, being able to completely inhibit the biofilm formation at its MIC value (45 μM). In contrast, at the same concentration, miconazole (**4**) and **BAC** induced the formation of thicker biofilms, likely due to an increased environmental stress on the bacteria (**Figure 2** and **Table 2**). Moreover, as shown in **Figure 2**, miconazoctylium bromide **5c** not only inhibit the formation of the biofilm in 24h at its MIC concentration, but also at sub-MIC concentrations.

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Table 2. Bacterial mortality after 24 h at different antimicrobial concentrations

Antimicrobial	Concentration (μM)	% Mortality [a]
(**4**)	50	27
(**5c**)	50	87
	25	76
	12.5	51

[a] Dead surface area/Live surface area

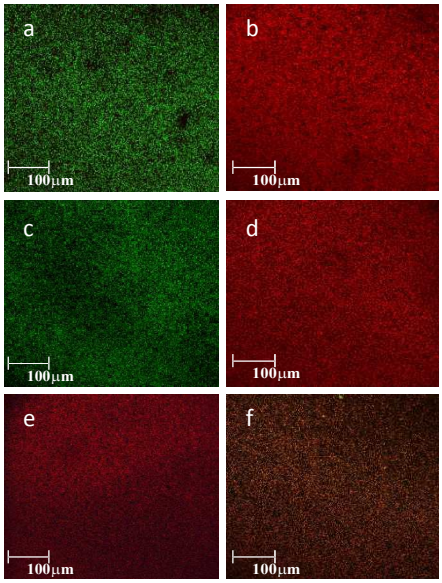

Figure 2. *S. aureus* biofilms labeled with *Live/Dead* stains after a 24 h incubation in growth media (LB broth). a) Negative control (DMSO). b) Positive control (70 % ethanol). c) Miconazole (**4**) at 50 μM. d) Miconazoctylium bromide (**5c**) at 50 μM. e) Miconazoctylium bromide (**5c**) at 25 μM. f) Miconazoctylium bromide (**5c**) at 12.5 μM.

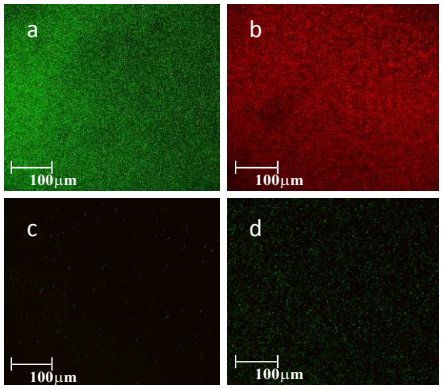

Figure 3. *MRSA* biofilms labeled with *Live/Dead* stains after a 48 h incubation in growth media (LB broth). a) Negative control (DMSO). b) Positive control (70 % ethanol). c) Miconazoctylium bromide (**5c**) at 25 μM. d) Miconazoctylium bromide. (**5c**) at 12.5 μM.

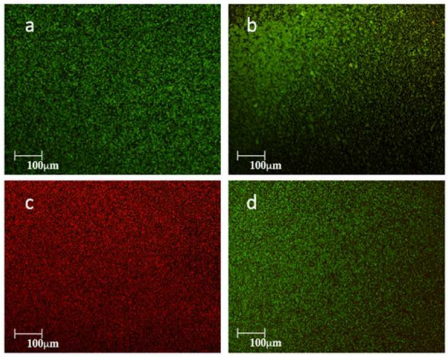

Figure 4. Pre-formed *MRSA* biofilms treated with 50 μM antimicrobials in 0.9% NaCl. a) Negative control (DMSO only, after 24h). b) Miconazole (**4**) (after 24 h). c) Miconazoctylium bromide (**5c**) (after 5 min). d) Miconazole (**4**) (after 48 h).

Even if some planktonic bacteria were still visible in the aqueous growth media after 24h, biofilm inhibition was achieved even at 12.5 μM (1/4xMIC), concentration at which 50% biofilm inhibition was observed. Moreover, miconazoctylium bromide **5c** at sub-MIC concentrations completely inhibit the formation of the biofilm after 48h (**Figure 3**). This time-dependent activity can be correlated to an accumulation of **5c** into the biofilm due to its lipophilic character. This property could be useful for in the treatment of biofilm-related diseases, since our compound will mostly accumulate into the biofilm matrix due to its hydrophobicity and will not induce the activation of resistance mechanisms on planktonic bacteria. At lower concentrations (1/8xMIC) miconazoctylium bromide **5c** did not show significant biofilm-destruction activity, even after 48h.

The minimal bactericidal concentration (MBC) of **5c** was determined by re-culturing broth dilutions that inhibited the growth of the bacteria at and above the MIC. The MBC for **5c** was 100 μM.

In contrast to inhibitors, which prevent the formation of biofilms, compounds that eradicate established biofilms at low concentrations are rare. Miconazoctylium bromide (**5c**) showed a strong capacity to disrupt pre-formed *MRSA* biofilms, being able to completely permeate bacterial membranes and disrupt the biofilm after 5 minutes at 50 μM. At the same concentration, miconazole (**4**) had no effect on the pre-formed biofilm, even after 48 h of incubation (**Figure 5**). Miconazoctylium bromide (**5c**) also showed an excellent ability to disrupt the *MRSA* biofilm at sub-MIC concentrations. For example, about 50% of the bacteria in the biofilm were dead after a 24 h exposure to 3 μM concentration of **5c** (1/16 x MIC). Complete biofilm disruption was observed at 12 μM (1/4 x MIC) after 30 min and at 6 μM (1/8 x MIC) after 6 h of incubation (**Figure 5** and **Table 3**)**.** Exposure to 6 or 12 μM of miconazoctylium bromide (**5c**) induced the formation of thicker stripes of colonies in the biofilm, indicating an increased stress on bacteria and increased ROS production (**Figure 5e** and **Figure S1,** Supporting Information).[23]

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Once again, at sub-MIC concentrations, the biofilm disruption was time-dependent, indicating the slow penetration and accumulation of **5c** in the matrix of the biofilm over time.

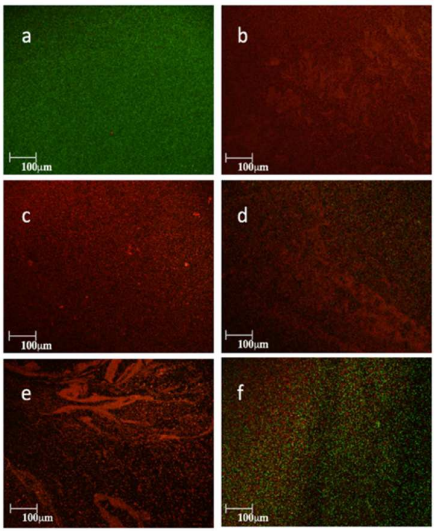

Figure 5. Preformed *S. aureus* biofilms treated with different concentrations of miconazoctylium bromide (**5c**) over 24 h monitored in 0.9 % NaCl solution. a) Negative control (DMSO only, after 24 h). b) Positive control (70 % ethanol). c) 25 µM (1/2 x MIC after 5 min). d) 12 µM (1/4x MIC after 30 min). e) 6 µM (1/8x MIC after 6 h). f) 3 µM (1/16 x MIC after 24 h).

Table 3. Bacterial mortality in pre-formed biofilms at different antimicrobial concentrations.

Antimicrobial	Concentration (µM)	Time (h)	Mortality [a] (%)
Miconazole (**4**)	50	24	4
Miconazoctylium bromide (**5c**)	50	0.1	98
	25	0.1	93
	12	0.5	87
	6	6	96
	3	24	57

[a] Dead surface area/Live surface area

If new compounds are identified to be useful as anti-biofilm agents and are in contact with the human body, it is important that they are not toxic to human cells at concentrations they possess anti-biofilm activity. The toxicity of miconazoctylium bromide (**5c**), evaluated by monitoring the hemolysis of red blood cells and haemoglobin release, is very low, as only 20 % hemolysis was observed even at concentrations 10-fold higher than its MIC (Figure 6). This low toxicity is very encouraging for the use of this compound for further *in vivo* studies.

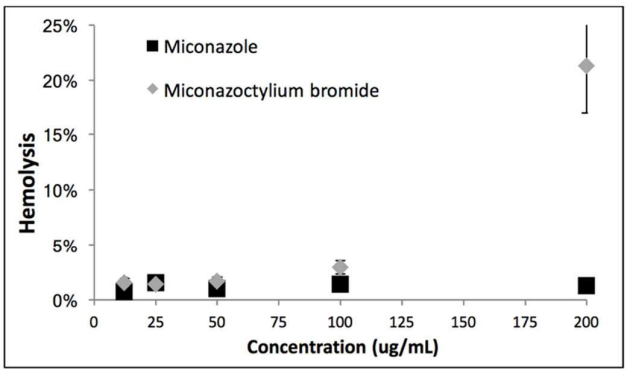

Figure 6. Red blood cells hemolysis of compound (**5c**) and miconazole (**4**).

Experimental

Materials and methods. All starting material, benzalkonium chloride (**BAC**), and 2,7-dichlorofluorescein diacetate (H_2DCFDA) were purchased from Sigma-Aldrich. ^{1}H and ^{13}C NMR spectra were recorded in deuterated solvents at 400 and 100 MHz, respectively, on Bruker spectrometers. The purity of final compounds used in biological assays was determined by ESI/ LC-MS analysis (≥ 95%).

1-(2-((2,4-dichlorobenzyl)oxy)-2-(2,4-dichlorophenyl)ethyl)-*1H*-imidazole (4). A suspension of sodium hydride (15 mg, 0.36 mmol) in DMF (0.5 mL) was treated with a solution of (**3**) (see ESI for the synthesis of **3**) (100 g, 0.33 mmol) in DMF (0.5 mL) at 0 °C and the resulting mixture was stirred at 0 °C for 2 h before a solution of the crude 2,4-dichlorophenylmesylate (125 mg, 0.36 mmol) in DMF (0.5 mL) was added dropwise. The resulting mixture was stirred for 12 hours at room temperature before water (1 mL) was added. The aqueous layer was extracted three times with CH_2Cl_2, dried over Na_2SO_4 and purified using silica gel chromatography (6/4 EtOAc/hexanes) to afford miconazole as a white solid (118 mg, 0.29 mmol, 87 %).

1-(2-((2,4-dichlorobenzyl)oxy)-2-(2,4-dichlorophenyl)ethyl)-3-methyl-1*H*-imidazol-3-ium iodide (5a). A solution of **(4)** (100 mg, 0.24 mmol) and methyliodide (45 µL, 0.72 mmol) was refluxed 12 hours in MeCN (1 mL). The solvent was evaporated and the residue was suspended into EtOAc to remove any trace of methyliodide. Evaporation of the residual solvent afforded the salt **(5a)** as a white powder (95 mg, 0.17 mmol, 71 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.96 (s, 1H), 7.49 (s, 1H), 7.36 (d, *J* = 7.3 Hz, 3H), 7.28 – 7.15 (m, 3H), 7.05 (s, 1H), 5.15 – 5.03 (m, 1H), 4.59 – 4.31 (m, 4H), 3.64 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.64, 135.94, 135.02, 134.29, 133.77, 132.85, 132.22, 131.50, 130.15, 129.39, 129.30, 128.37, 127.72, 122.57, 121.28, 75.91, 68.45, 53.07, 50.47; HRMS: (ESI) calcd. for [M+] $C_{19}H_{17}Cl_4N_2O$: 429.0081, found 429.0095; IR (neat, cm^{-1}) 3170.6, 3105.7, 1585.9, 1474.3; Melting point: 165-168 °C.

1-(2-((2,4-dichlorobenzyl)oxy)-2-(2,4-dichlorophenyl)ethyl)-3-hexyl-1*H*-imidazol-3-ium bromide (5b). A solution of **(4)** (100 mg, 0.24 mmol) and hexylbromide (101 µL, 0.72 mmol) was

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refluxed 12 hours in MeCN (1 mL). The solvent was evaporated and the residue was triturated with EtOAc to remove any trace of hexyliodide. Evaporation of the residual solvent afforded the salt **(5b)** as a white powder (124 mg, 0.216 mmol, 90 %). ^{1}H NMR ($CDCl_3$,400 MHz) δ 10.53 (s, 1H), 7.63 – 7.39 (m, 3H), 7.39 – 7.27 (m,3H), 7.24 (d, *J* = 6.9 Hz, 2H), 5.24 (dd, *J* = 6.9, 4.3 Hz, 1H), 4.80 – 4.61 (m, 2H), 4.54 (d, *J* = 12.0 Hz, 1H), 4.45 (d, *J* = 11.9 Hz, 1H), 4.33 (dh, *J* = 20.9, 7.4, 6.9 Hz, 2H), 1.95 – 1.84 (m, 2H), 1.32 (s, 6H), 0.91 (t, *J* = 6.2 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.64, 135.94, 135.02, 134.29, 133.77, 132.85, 132.22, 131.50, 130.15, 129.39, 129.30, 128.37, 127.72, 122.57, 121.28, 76.18, 68.46, 53.24, 50.43, 31.18, 30.33, 26.00, 22.50, 14.06; HRMS: (ESI) calcd. for [M+] $C_{24}H_{27}Cl_4N_2O$: 499.0876, found 499.0877; IR (neat, cm^{-1}) 3146.9, 3051.6, 2943.9, 2864.9, 1650.4, 1587.3, 1468.8, 1379.4; Melting point: 106-109 °C.

Miconazoctylium bromide, 1-(2-((2,4-dichlorobenzyl)oxy)-2-(2,4-dichlorophenyl)ethyl)-3-octyl-1H-imidazol-3-ium bromide (5c). A solution of **(4)** (100 mg, 0.24 mmol) and octylbromide (125 µL, 0.72 mmol) was refluxed 12 hours in MeCN (1 mL). The solvent was evaporated and the residue was triturated with EtOAc to remove any trace of octylbromide. Evaporation of the residual solvent afforded the salt **(5c)** as a white powder (135 mg,0.22 mmol, 93 %). ^{1}H NMR ($CDCl_3$,400 MHz) δ 10.51 (s, 1H), 7.49 (d, *J* = 8.3 Hz, 1H), 7.46 – 7.43 (m, 2H), 7.34 – 7.26 (m, 3H), 7.23 – 7.19 (m, 2H), 5.21 (dd, *J* = 7.5, 4.3 Hz, 1H), 4.74 – 4.60 (m, 2H), 4.51 (d, *J* = 11.9 Hz, 1H), 4.42 (d, *J* = 12.0 Hz, 1H), 4.29 (dh, *J* = 20.9, 7.3, 6.9 Hz, 2H), 1.91 – 1.80 (m, 2H), 1.26 (d, *J* = 20.4 Hz, 10H), 0.87 (t, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) d 138.64, 135.93, 135.02, 134.28, 133.77, 132.86, 132.22, 131.49, 130.14, 129.39, 129.30, 128.37, 127.71, 122.57, 121.27, 76.18, 68.46, 53.25, 50.44, 31.80, 30.38, 29.14, 29.05, 26.36, 22.71, 14.19; HRMS: (ESI) calcd. for [M+] $C_{26}H_{31}Cl_4N_2O$: 527.1185, found 527.1194; IR (neat, cm^{-1}) 3007.9, 2927.5, 2926.5, 2854.5, 1646.7, 1587.7, 1561.3, 1467.4, 1379.8, 1338.7; Melting point: 131-134 °C.

3-decyl-1-(2-((2,4-dichlorobenzyl)oxy)-2-(2,4-dichlorophenyl) ethyl)-1*H*-imidazol-3-ium bromide (5d). A solution of **(4)** (100 mg, 0.24 mmol) and decylbromide (150 µL, 0.72 mmol) was refluxed 12 hours in MeCN (1 mL). The solvent was evaporated and the residue was triturated with EtOAc to remove any trace of decylbromide. Evaporation of the residual solvent afforded the salt **(5d)** as a white powder (130 mg, 0.2 mmol, 85 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 10.59 (s, 1H), 7.53 – 7.41 (m, 3H), 7.36 – 7.27 (m, 3H), 7.18 – 7.09 (m, 2H), 5.21 (dd, *J* = 7.3, 4.2 Hz, 1H), 4.77 – 4.58 (m, 2H), 4.52 (d, *J* = 12.0 Hz, 1H), 4.43 (d, *J* = 12.0 Hz, 1H), 4.31 (dh, *J* = 20.9, 13.7, 7.4 Hz, 2H), 1.87 (t, 2H), 1.28 (d, *J* = 21.3 Hz, 14H), 0.88 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.76, 135.96, 135.05, 134.30, 133.77, 132.85, 132.20, 131.52, 130.17, 129.41, 129.32, 128.39, 127.73, 122.52, 121.16, 76.19, 68.47, 53.26, 50.46, 31.98, 30.39, 29.59, 29.50, 29.38, 29.11, 26.38, 22.80, 14.25; HRMS: (ESI) calcd. for [M+] $C_{28}H_{35}Cl_4N_2O$: 555.1498, found 555.1503; IR (neat, cm^{-1}) 3055.12, 3009.9, 2926.8, 2854.1, 1640.9, 1589.3, 1466.5, 1379.8, 1338.8; Melting point: 81-84 °C.

1-(2-((2,4-dichlorobenzyl)oxy)-2-(2,4-dichlorophenyl)ethyl)-3-dodecyl-1*H*-imidazol-3-ium bromide (5e). A solution of **(4)** (100 mg, 0.24 mmol) and dodecylbromide (173 µL, 0.72 mmol) was refluxed 12 hours in MeCN (1 mL). The solvent was evaporated and the residue was triturated with EtOAc to remove any trace of dodecylbromide. Evaporation of the residual solvent afforded the salt **(5e)** as a white powder (144 mg, 0.22 mmol, 90 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 10.45 (s, 1H), 7.51 – 7.41 (m, 3H), 7.34 – 7.28 (m, 3H), 7.25 – 7.20 (m, 2H), 5.42 – 5.10 (m, 1H), 4.70 – 4.62 (m, 2H), 4.50 (d, *J* = 12.0 Hz, 1H), 4.41 (d, *J* = 12.0 Hz, 1H), 4.30 (dh, *J* = 16.8, 13.7, 6.4 Hz, 2H), 2.03 – 1.73 (m, 2H), 1.25 (d, *J* = 17.6 Hz, 18H), 0.86 (t, *J* = 6.7 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.52, 135.90, 134.98, 134.23, 133.77, 132.85, 132.22, 131.44, 130.12, 129.37, 129.28, 128.35, 127.70, 122.61, 121.35, 77.16, 68.43, 53.21, 50.42, 32.02, 30.39, 29.71, 29.63, 29.49, 29.45, 29.10, 26.36, 22.80, 14.24. HRMS: (ESI) calcd. for [M+] $C_{30}H_{39}Cl_4N_2O$: 583.1823, found 583.1816; IR (neat, cm^{-1}) 3148.6, 3106.1, 3052.7, 2920.4, 2852.1, 1649.8, 1589.0, 1468.5, 1380.9, 1342.6; Melting point: 71-74 °C.

1-(2-((2,4-dichlorobenzyl)oxy)-2-(2,4-dichlorophenyl)ethyl)-3-tetradecyl-1*H*-imidazol-3-ium bromide (5f). A solution of **(4)** (100 mg, 0.24 mmol) and tetradecylbromide (214 µL, 0.72 mmol) was refluxed 12 hours in MeCN (1 mL). The solvent was evaporated and the residue was triturated with EtOAc to remove any trace of tetradecylbromide. Evaporation of the residual solvent afforded the salt **(5f)** as a white powder (140 mg, 0.20 mmol, 85 %). ^{1}H NMR ($CDCl_3$,400 MHz) δ 10.63 (s, 1H), 7.57 – 7.47 (m, 3H), 7.39 – 7.30 (m, 3H), 7.20 – 7.08 (m, 2H), 5.24 (dd, *J* = 7.3, 4.3 Hz, 1H), 4.75 – 4.64 (m, 2H), 4.55 (d, *J* = 12.0 Hz, 1H), 4.46 (d, *J* = 11.9 Hz, 1H), 4.34 (ddt, *J* = 26.9, 13.9, 7.5 Hz, 2H), 1.90 (s, 2H), 1.31 (d, *J* = 20.1 Hz, 22H), 0.91 (t, *J* = 6.7 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.89, 135.99, 135.08, 134.34, 133.77, 132.84, 132.19, 131.56, 130.19, 129.42, 129.33, 128.40, 127.74, 122.46, 121.06, 76.20, 68.49, 53.27, 50.48, 32.07, 30.39, 29.83, 29.79, 29.74, 29.65, 29.51, 29.12, 26.39, 22.84, 14.27. HRMS: (ESI) calcd. for [M+] $C_{32}H_{43}Cl_4N_2O$: 611.2128, found 611.2129; IR (neat, cm^{-1}) 3149.2, 3054.9, 2922.5, 2852.5, 1589.7, 1561.5, 1467.7, 1378.8, 1340.6; Melting point: 100-104°C.

1-(2-((2,4-dichlorobenzyl)oxy)-2-(2,4-dichlorophenyl)ethyl)-3-hexadecyl-1*H*-imidazol-3-ium bromide (5g). A solution of **(4)** (100 mg, 0.24 mmol) and hexadecylbromide (220 µL, 0.72 mmol) was refluxed 12 hours in MeCN (1 mL). The solvent was evaporated and the residue was triturated with EtOAc to remove any trace of hexadecylbromide. Evaporation of the residual solvent afforded the salt **(5g)** as a white powder (166 mg, 0.23 mmol, 95 %). ^{1}H NMR ($CDCl_3$,400 MHz) δ 10.41 (s, 1H), 7.62 – 7.41 (m, 3H), 7.37 – 7.23 (m, 5H), 5.23 (s, 1H), 4.68 (s, 2H), 4.52 (d, *J* = 12.1 Hz, 1H), 4.44 (d, *J* = 11.9 Hz, 1H), 4.38 – 4.23 (m, 2H), 1.86 (s, 2H), 1.26 (s, 26H), 0.89 (s, 3H).^{13}C NMR (126 MHz, $CDCl_3$) δ 138.22, 135.75, 134.82, 134.07, 133.66, 132.75, 132.13, 131.27, 129.97, 129.23, 129.15, 128.21, 127.58, 122.59, 121.42, 76.04, 68.29, 53.08, 50.27, 31.92, 30.28, 29.69, 29.66, 29.61, 29.53, 29.39, 29.36, 29.00, 26.24, 22.69, 14.13. HRMS: (ESI) calcd. for [M+] $C_{34}H_{47}Cl_4N_2O$: 639.2447, found 639.2442; IR (neat, cm^{-1}) 3148.1, 3051,6,

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3010.2, 2921.3, 2851.4, 1590.4, 1561.4, 1467.2, 1380,5, 1341.6; Melting point: 106-110°C.

Bacterial strains, culture conditions and viability. MICs were determined on 96-well microtiter plates. Assays were conducted in Lauria Broth (LB) medium at 37 °C in triplicate, for three different bacterial inoculations and preculture. Bacterial cell density (OD at 600 nm) was measured using a Fischer Scientific cell density meter model 40. UV-vis and fluorescence spectroscopy experiments were performed on a Tecan Infinite M200 microplate reader. Bacterial cell lysis was performed by sonication using a Sonics & Materials inc. Vibra-Cells VCX-500 Ultrasonic Processor (5 to 15 rounds of 30 seconds sonication until the OD_{600nm} = 0).

Biofilm inhibition. *S. aureus* cells were incubated in LB medium at 37 °C for 5 h and rediluted in LB medium to the desired final concentration (OD_{600nm}= 0.1-0.15). *S. Aureus* biofilms were labeled with *Live/Dead* stain after 12 h incubation with antibiotics in growth media (LB broth). Each experiment was performed at least three times.

Biofilm disruption. *S. aureus* cells were incubated in LB medium at 37 °C for 5 h and rediluted in LB medium to the desired final concentration (OD_{600nm}= 0.1-0.15). After an incubation of 12 h in 8-well chambers, the growth media was removed via pipetting and the resulting biofilms were washed 2 times with 0.9 % NaCl solution to remove the remaining planktonic cells. Formed *S. aureus* biofilms were treated with antibiotics over a 24 h monitoring in 0.9 % NaCl solution. Each experiment was performed at least three times.

Biofilm staining and confocal laser scanning microscopy (LSM) Analysis. Biofilms stained with FilmTracer™ LIVE/DEAD® Biofilm viability kit (Molecular Probes, Life Technologies Ltd.). Briefly, a working solution of fluorescent stains was prepared by adding 1 µL of SYTO® 9 stain and 1 µL of PI stain to 1 mL of filter-sterilized water. Two hundred µL of staining solution were deposited on each well of a 8-well chambered coverglass, after 15 min at room temperature in the dark, samples were washed with sterile saline (0.9% NaCl) from base of the support material. Then, biofilms were examined with a confocal laser microscope (Leica model TCS SP5; Leica Microsystems CMS GmbH, Mannheim, Germany) using a 20x dry objective (HC PL FLUOTAR 20.0 x 0.50 DRY). A 488 nm laser line was used to excite SYTO® 9, while the fluorescent emission was detected from 500 to 540 nm. PI was sequentially excited with 561 nm laser line and its fluorescent emission was detected from 600 to 695 nm. Each experiment was performed at least three times.

Minimal Bactericidal Concentration (MBC). MBCs were determined using LB-agarose plates at 37 °C in triplicate, from three independent innoculations. The MBC was determined by re-culturing broth dilutions (from 96-wells plates assay) that inhibited growth of the bacteria (at and above the MIC). The broth dilutions were streaked onto LB-agar plates and incubated for 24h. The MBC is the lowest broth dilution of antimicrobial that prevents growth of the organism on the agar plate. The sterility tests used in the MIC assay (LB only) were streaked onto LB-agar plates as an overall negative-control and the bacterial growth tests used in the MIC assay were streaked onto LB-agar plates as a Positive-control.

Hemolysis. Fresh human red blood cells (blood type O) purchased from Innovative Research in Alserver's solution were centrifuged 10 min at 300 x g, washed 3 times with PBS buffer and resuspended in PBS at 2% v/v. In a 96 wells plate were added 195 µL of red blood cells solution and 5 µL of compound in DMSO, the plate was incubated with gentle agitation for 1 h at 37 °C. The plate was the centrifuged for 10 min at 300 x g and 50 µL of the supernatant solution of each well was transferred to another plate. The absorbance was measured at λ = 405 nm.

Conclusions

We show here that the transformation of miconazole into a miconazoctylium cation resulted in a more potent compound that could disrupt pre-formed biofilms and inhibit the formation of new *S. aureus* biofilms. Miconazoctylium bromide was shown to be effective on *MRSA*, even at very low concentrations (6 µM). Work is underway in our group to further study the activity of this cationic version of miconazole against a broad spectrum of Gram-positive and Gram-negative bacteria. Although they belong to distinct phylogenetic kingdoms, *S. aureus* and fungus *C. albicans* usually co-exist as complex polymicrobial biofilms within the human host.[25] As we showed here that **5c** was also active on *C. albicans*, studies are underway to identify the capacity of miconazoctylium bromide **5c** to inhibit and destry polymicrobial biofilms.

Conflicts of interest

There are no conflicts to declare.

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