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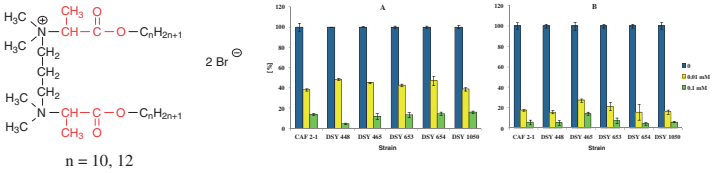

Chemodegradable Gemini Alanine-based Cationic Surfactants: Synthesis and Antifungal Activity

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The aim of the present study was to synthesize and examine the biological properties of soft gemini bis-quaternary surfactants, derivatives of bis-alanine esters. As a model system for the investigation of the activity of gemini surfactants *Candida albicans* strains with deletions of gene encoded ABC transporters were used. TMPAL-12Br was more active than TMPAL-10Br in dislodging of the *C. albicans* biofilms. The results of the present study may prove useful in antifungal applications.

Among many architectures cationic "gemini" (or "dimeric") surfactants constitute an important class of surface active compounds which can easily interact with cellular membranes of microorganisms. Consequently, they manifest high antimicrobial and antifungal activity.[1] *Candida albicans* is one of the opportunistic fungal pathogens that cause various mycoses of mucous membranes or internal organs of people with suppressed immunity.[2]

Frequent applications of antifungal drugs lead to a growing resistance of *C. albicans*.[3] Intensive investigations concerning *C. albicans* induced mycoses mainly concentrate on activity inhibition of ABC transporters and suppression of biofilms. *Candida* has two ABC transporters: Cdr1 and Cdr2, as well as MFS (major facilitator superfamily) transporter, Mdr1, which are responsible for resistance to, e.g., azole antifungals.[4] Another form of *Candida* resistance to antifungal agents consists in the formation of biofilms-multicellular structures immersed in polymeric matrix. Biofilms are formed on both synthetic and natural substrates.[5]

A series of soft gemini bis-quaternary surfactants, derivatives of bis-alanine esters, were synthesized in a two-step reaction constituting the appropriate *n*-alkyl α-bromopropionates (ABPs) synthesis and in the next step-quaternization of *N*,*N*,*N*′,*N*′-tetramethyl-1,3-propanediamines with ABPs (for the structure see Figure 1, abbreviated as TMPAL-*n*Br; $n = 10$ and 12).

n-Alkyl α-bromopropionates (ABPs) were synthesized in a reaction of *n*-decyl or *n*-dodecyl alcohol with 2-bromopropionyl bromide in dichloromethane as a solvent. Thus, 0.5 mol of the respective primary alcohol, dissolved in 400 cm^3 of dichloromethane, was stirred under reflux and 0.7 mol of 2-bromopropionyl bromide in 100 cm^3 of dichloromethane was added stepwise, while the hydrobromide formed was trapped in a NaOH solution. The reaction mixture was additionally refluxed for 8 h, then cooled and neutralized with sodium hydrocarbonate, and washed with water several times. After evaporation of the solvent, *n*-alkyl α-bromopropionates were obtained in 80% yield and they were immediately used for the quaternization step of *N*,*N*,*N*′,*N*′-tetramethyl-1,3-propanediamine. In the latter step 0.1 mol of the diamine in acetonitrile was heated at 80 °C and 0.2 mol of a given alkyl α-bromopropionate also in acetonitrile was added dropwise to the reaction mixture. The mixture was refluxed additionally for 30 h at 80 °C, then cooled in a refrigerator and the precipitated product filtered off (yield 15%). The crude product was recrystallized several times from a variety of mixed solvent systems.

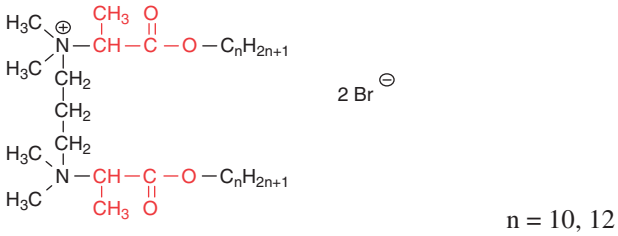

Figure 1. Structure of tested quaternary "gemini" surfactants derivatives of *N*,*N*,*N*′,*N*′-tetramethyl-1,3-propanediamine (TMPAL-*n*).

The surfactants were purified by repeated crystallization until no impurities could be detected by NMR spectra. The chemical structures of the compounds were determined by their ^{1}H NMR (Bruker Avance 300 MHz, $CDCl_3$, internal standard TMS, δ). The obtained results indicate that the gemini quaternary ammonium bromides under study were at least 98 mol % pure, which was confirmed by the very narrow range of melting points. ^{1}H NMR spectra of the compounds: **TMPAL-10Br**: 0.834–0.878 [6H, t, 2(–CH_3)$_2$], 1.242 [28H, d, 2(–CH_2–)$_7$], 1.553–1.654 [4H, m, 2(–O–CH$_2$–CH_2–)], 1.774–1.794 [6H, t, 2(CH–CH_3)], 2.679 [2H, s, N–CH$_2$–CH_2–CH$_2$], 3.499–3.540 [12H, d, 2(N–CH_3)$_2$], 3.911 [4H, t, N–CH_2–CH$_2$–CH_2], 4.131–4.267 [4H, d, 2(–O–CH_2)], 4.655 [2H, s, 2(–N–CH)]. **TMPAL-12Br**: 0.819–0.864 [6H, t, 2(–CH_3)$_2$], 1.225 [36H, d, 2(–CH_2–)$_9$], 1.565–1.664 [4H, m, 2(–O–CH$_2$–CH_2–)], 1.775–1.800 [6H, t, 2(CH–CH_3)], 2.690 [2H, s, N–CH$_2$–CH_2–CH$_2$], 3.500–3.539 [12H, d, 2(N–CH_3)$_2$], 3.911 [4H, t, N–CH_2–CH$_2$–CH_2], 4.116–4.250 [4H, d, 2(–O–CH_2)], 4.663 [2H, s, 2(–N–CH)].

Conventional cationic surfactants have a high chemical stability, associated with a poor chemical and biological degradability, thus, their use can be limited by ecological requirements.[6–8] An interesting and useful strategy is the synthesis of surfactants based on natural products, such as amino acids comprising a labile moiety in their structure.[9,10]

In biological investigations *C. albicans* strains with deletions of gene encoded multidrug resistance transporters were used (Table 1). The strain survival tests were carried out under oxygen and microaerophilic conditions at 30 °C. Antiadhesive and antibiofilm tests were performed for two and four hours at a temperature of 37 °C.

Strain survival tests were carried out under both aerobic and microaerophilic conditions because this fungus can colonize not only the skin but also the internal organs. Under certain conditions *C. albicans* can induce both superficial and serious

Table 1. *C. albicans* strains used in this work

Strain	Genotype
CAF 2-1–wild type	*Δura3::imm434/URA3*
CAF 4-2	*Δura3::imm434/Δura3::imm434*
DSY 448	*Δcdr1::hisG-URA3-hisG/Δcdr1::hisG*
DSY 653	*Δcdr2::hisG/Δcdr2::hisG-URA3-hisG*
DSY 465	*Δmdr1::hisG-URA3-hisG/Δmdr1::hisG*
DSY 654	*Δcdr1::hisG/Δcdr1::hisG Δcdr2::hisG-URA3-hisG/Δcdr2::hisG*
DSY 1050	*cdr1::hisG/Δcdr1::hisG Δcdr2::hisG/Δcdr2::hisG Δmdr1::hisG-*

life threatening systemic diseases, as well as be the cause of gastrointestinal infections.[11,12] Both type of surfactants at a concentration of 1 mM suppressed the viability of all strains in 100%. The 0.001 mM concentration of compounds was not active against *C. albicans* strains. In a concentration of 0.1 mM the tested compounds decreased in 80–90% the viability of strains. In microaerophilic conditions, gemini surfactants were less active than in aerobic conditions. Strains with deletions of all three ABC transporters and strains with deletion of Cdr1 and Cdr2 transporters were more sensitive to the TMPAL-12Br surfactant than other strains. It can suggest that this type of compound can be the substrate for Cdr1 or Cdr2 pump.

The first step for colonization of tissue or plastic is adhesion.[13] The influence of gemini surfactants on *C. albicans* adhesion on a 96-well polystyrenic plate was tested. The surfactant TMPAL-12Br at 0.1 mM decreased the adhesion of all strains with ABC transporters deletions by 20%. The adhesion of a *C. albicans* CAF 2-1 strain without deletions of pumps was not affected by a 0.1 mM concentration of the tested compound. The TMPAL-10Br compound was inactive in all cases.

Two kinds of biofilms formed from 10 (early logarithmic phase) and 24 h (stationary phase) cultures of *C. albicans* strains were investigated. In both cases TMPAL-12Br was more active than TMPAL-10Br (Figures 2 and 3). The investigated strains did not differ as to the sensitivity to gemini surfactants (Figures 2 and 3).

The studied gemini surfactants exhibited different activity against *C. albicans*. The TMPAL-10Br showed higher antifungal activity that did not depend on the presence or absence of ABC transporters, in contrast to the TMPAL-12Br. The TMPAL-12Br surfactant demonstrated much higher antiadhesive activity and stronger dislodging of *C. albicans* biofilms. According to literature data antifungal drugs are usually more active against planktonic cells than biofilms,[14,15] but the studied gemini esterquats TMPAL-10Br and TMPAL-12Br dislodged *C. albicans* biofilms at lower concentrations than these used for killing planktonic cells. Thus, the studied compounds can be used as surface coating agents against fungal colonization.

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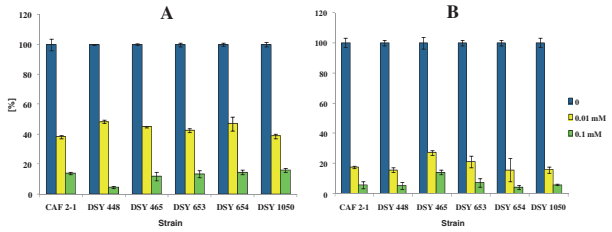

Figure 2. *C. albicans* biofilm dislodging by TMPAL-10Br (A) and TMPAL-12Br (B). Biofilm formed from a 24 h culture. The 96-well flat-bottomed plates were incubated for 2 h on a rotary shaker with 100 µL of *C. albicans* suspension in PBS. Unattached cells were removed by washing the wells three times with PBS. Next, 100 µL of different concentrations of the tested surfactants was added to each well and incubated at 37 °C for 2 h on rotary shaker. The plates were washed three times, adherent cells were fixed with 100 µL of 0.1% crystal violet for 5 min and again washed three times with PBS. The adherent cells were permeabilized and the dye was resolubilized with 150 µL of isopropanol–0.04 M HCl and 50 µL of 0.25% SDS per well. The crystal violet optical density of each well was measured at 590 nm.

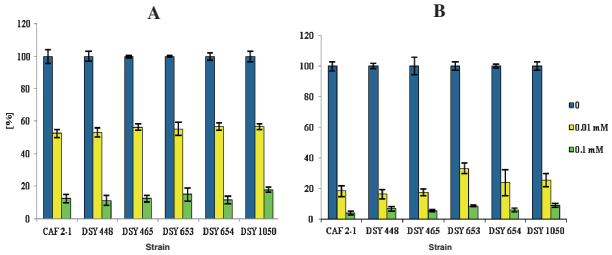

Figure 3. *C. albicans* biofilm dislodging by: TMPAL-10Br (A) and TMPAL-12Br (B). Biofilm formed from a 10 h culture. In this study we have described the synthesis and antifungal activity of gemini alanine-based cationic surfactants.

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