Synthetic 3-Arylideneflavanones as Inhibitors of the Initial Stages of Biofilm Formation by *Staphylococcus aureus* and *Enterococcus faecalis*

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The antimicrobial activity of twenty two synthetic flavonoids is reported. Among them three 3-arylideneflavanones, **2b**, **2c**, and **2i**, were shown to be highly active against *Staphylococcus aureus*, *S. epidermidis*, and *Enterococcus faecalis* reference strains, with MIC (minimal inhibitory concentration) values ranging from 4.68 μg/ml (14.3 μM) to 37.5 μg/ml (119.7 μM). The synergy of oxacillin and vancomycin with **2c**, evaluated as fractional inhibitory concentration index (FICI) was shown (against planktonic culture of *S. aureus* A3 and *E. faecium* 138/09 clinical strains). The presence of **2c** in the culture medium diminished the initial adhesion of bacteria to an abiotic surface. Such an effect resulted in a decrease in biofilm formation during prolonged culture. Unfortunately, **2c** failed to eradicate the *S. aureus* mature biofilm which was already preformed, however, decreased the number of live biofilm cells. The biofilm of *E. faecalis* was more susceptible to the action of 3-arylideneflavanone **2c** than the *S. aureus* biofilm. The finding that 3-arylideneflavanones are lipophilic, cause bacterial aggregation, and influence the integrity of membranes making them permeable to SYTO 9/propidium iodide dyes may implicate the cytoplasmic membrane as a target site for these compounds activity.

Key words: 3-Arylideneflavanones, Biofilm, *Staphylococcus*, *Enterococcus*

Introduction

Bacterial and fungal pathogens have developed numerous resistance mechanisms against antimicrobial agents. Moreover, microorganisms tend to form biofilm communities which are very difficult to control because of their extremely high resistance to antibiotics and the host defense system. It therefore seems to be necessary to search for alternative strategies to combat infections of biofilm nature and the best solution being the prevention of their adhesion (Rice, 2006). Currently, natural plant compounds are in the focus of attention as a source or, which is no less interesting, as molecular patterns for new drugs (Dürig *et al.*, 2010). Usually, their structures differ from those of microbes-derived antibiotics and also their action proceeds *via* different mechanisms. These dissimilarities greatly increase the chance of avoiding rapid development of microbial resistance. One of the large interesting groups of phytochemicals is the class of flavonoids which are ubiquitously present and perform a protective function in abiotic and biotic stress (Gibbons, 2008). They have been shown to display a wide range of medicinal properties including antibacterial, antifungal, anti-inflammatory, and anticancer effects, respectively (Cushnie and Lamb, 2005; Deng *et al.*, 2000; Sato *et al.*, 1994; Konieczny *et al.*, 2007; Chikhalia *et al.*, 2008). Some of these phytochemicals, due to their simple structure and potentially facile *de novo* synthesis, are worth further investigation (Prasad *et al.*, 2008; Mughal *et al.*, 2006; Andersen and Markham, 2006).

Based on the published data showing that chromone derivatives like chalcones and flavanones show antimicrobial activity (Gatto *et al.*, 2002; Lahtchev *et al.*, 2008; Avila *et al.*, 2008), the aim of our study was to search among the compounds

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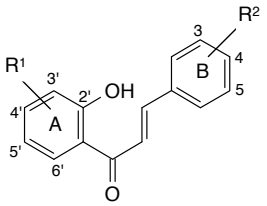
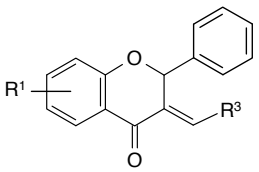
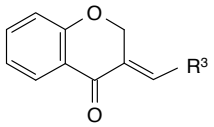
Table I. Chemical structures of the flavonoids studied.



2′-Hydroxychalcones



3-Arylideneflavanones



3-Arylidenechromanones

Compound	R^1	R^2	Compound	R^1	R^3	Compound	R^3
1a	4′–OH	H	**2a**	H		**3a**	
1b	H	4–OH	**2b**	H		**3b**	
1c	5′–OH	H	**2c**	H		**3c**	
1d	H	4–Cl	**2d**	6–OH			
1e	H	4–Br	**2e**	H			
1f	H	4–NO_2	**2f**	H			
1g	H	4–OC_2H_5	**2g**	H			
1h	H	3,4,5–$(OCH_3)_3$	**2h**	H			
1i	5′–Cl	4–$CH(CH_3)_2$	**2i**	H			
1j	3′–Br, 5′–CH_3	4–OCH_3					

synthesized and owned by us for compounds with such potency. We focused our attention on the possibility to fight the two main members of the "alert" pathogens group, *i.e.* the staphylococci *Staphylococcus aureus* and *S. epidermidis* (methicillin-resistant, MRSA, MRSE) and enterococci (including the vancomycin-resistant *Enterococcus faecium*, VRE). These pathogens were chosen as targets because of the availability of epidemiological data on the incidence of infections they cause or contribute to (Rice, 2006). *S. aureus* and *S. epidermidis* are common types of bacteria but one major problem is that occasionally a mild skin infection can spread to almost any other organ in the body and become life-threatening. Moreover, the resistance of staphylococci to methicillin means that they are resistant to all *β*-lactam antibiotics as well, which implies the need to search for new therapeutics. Enterococci can often cause harmless infections of the urinary tract and wounds. If such infections are caused by strains resistant to vancomycin and/or affect the bloodstream, they are very difficult to treat (Rice, 2006; Goetz, 2002; Fux *et al.*, 2005; Mohamed and Huang, 2007).

Thus, in the first part of this work, synthetic 2'-hydroxychalcones, 3-arylideneflavanones, and 3-arylidenechromanones were screened for their bacteriostatic/bactericidal effect against reference and clinical *Staphylococcus* and *Enterococcus* strains. The susceptibility of *S. aureus* and *E. faecium* multidrug-resistant strains, to oxacillin or vancomycin alone or in combination with selected compounds, was also tested. Then, compounds showing the highest activity were tested for their anti-adhesion and anti-biofilm potency. For comparison, the effects of reference antibiotics and phytochemicals were determined.

Material and Methods

Synthesis and chemical characteristics of flavonoids

The 2'-hydroxychalcones **1a**–**1j** were prepared according to the procedure given in the literature (Prasad *et al.*, 2008). The 3-arylideneflavanones **2a**–**2i** were obtained by a three-step synthesis. In the first step, chalcones were synthesized by the Claisen-Schmidt reaction (Raval and Shah, 1956). The substituted flavanones were obtained from precedent chalcones according to the procedure reported in the literature (Geissman and Clinton, 1946). The flavanones were then condensed with dedicated aldehydes in the presence of piperidine as a catalyst (Pijewska *et al.*, 1993). The 3-arylidenechromanones **3a**–**3c** were also synthesized by the above-mentioned condensation method using commercially available chromanone and appropriate aldehydes.

The compounds were finally purified by repeated crystallization from ethanol. The chemical

structures of the flavonoids studied are presented in Table I. IR analysis revealed the presence of carbonyl groups in the typical positions: 1644–1634 cm^{-1} for chalcones, 1668–1664 cm^{-1} for 3-arylideneflavanones, 1667–1637 cm^{-1} for 3-arylidenechromanones. The chemical structures of **2a**, **2c**, **2d**, **2h**, and **3a** were confirmed on the basis of the spectral analysis data presented in a previous publication (Pijewska *et al*., 1993).

*Evaluation of lipophilicity of 3-arylideneflavanones **2b, 2c, and 2i***

The log *P* values for the tested compounds were calculated using the Gaussian G09 program package (Frisch *et al*., 2009). Structures of the compounds were optimized on the DTF level of theory using the B3LYP (Becke, 1988; Lee *et al*., 1988) functional and 6-31++G(d,p) (Ditchfield *et al*., 1971; Hehre *et al*., 1972; Hariharan and Pole, 1973) basis sets. The following calculation of energies of the compounds in water and *n*-octanol were performed on the same level of theory using the SMD solvation model (Merenich *et al*., 2009). The values of the partition coefficient of each compound were calculated from the difference of the compound's energy in water and *n*-octanol using the equation developed by Leo *et al*. (1971).

Evaluation of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the compounds

The reference *Staphylococcus aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212 strains, nine *Staphylococcus* spp. clinical strains (from our own collection), and 10 *Enterococcus* spp. strains (a gift from the collection of Prof. W. Hryniewicz, National Medicinal Institute, Warsaw, Poland) were used as targets for the tested synthetic flavonoids. Sterile stock solutions of each compound at the concentration of 60.0 mg/ml were prepared in DMSO (dimethyl sulfoxide). The agent concentration range used in the antimicrobial tests was 0.29–300.0 μg/ml prepared in Mueller-Hinton broth (Difco, Detroit, USA). DMSO alone was always used as a negative control. Prior to the assay, the strains were evaluated for antibiotic susceptibility, using the standard disc diffusion and/or broth microdilution MIC tests, according to CLSI (2006) (M7-A5).

MIC values were specified as the concentrations of compounds at which microbial growth was inhibited during a 24-h culture; the turbidity was thus equal to A_{600} of the culture medium used as a blank control (0.06). The solubilizer DMSO at a concentration of 10 ml/l (the same as the highest final concentration used for the dilution of compounds) had no antimicrobial activity itself.

The lowest concentration of compounds bactericidal to ≥99.9% of the original inoculum was determined by subculturing 10 μl from the wells of MIC, 2 MIC, and 4 MIC to the broth media without antimicrobial agent. The concentration at which no visible growth after a subsequent 24-h incubation is observed means MBC. The compound is regarded bactericidal if the MBC value is not greater than four times the MIC value. Another 10 μl from the well of MIC, 2 MIC, and 4 MIC were subcultured onto agar media without compounds, for evaluation of CFU (colony-forming units) and colony appearance.

In the reference experiments, the following standard antibiotics were used: ampicillin (Serva, Heidelberg, Germany), ofloxacin (Sigma, Munich, Germany), penicillin, methicillin, oxacillin, gentamicin, vancomycin, erythromycin, tetracycline, teicoplanin, ciprofloxacin, chloramphenicol, linezolid, rifampicin, imipenem (Mast Diagnostics, Merseyside, UK). For comparison, four phytochemicals, quercetin (3,3',4',5,7-pentahydroxyflavone), naringin (4',5,7-trihydroxyflavanone-7-rhamnoglucoside), naringenin [5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one], and thymol (2-isopropyl-5-methylphenol), purchased from Sigma were used.

*Determination of bacterial growth rate in the presence of 3-arylideneflavanones **2b** and **2c***

The growth rates of *S. aureus* ATCC 29213 (reference MSSA-methicillin-sensitive), *S. aureus* A3 (clinical methicillin-resistant), *E. faecalis* ATCC 29212 (reference vancomycin-sensitive), and *E. faecium* 138/09 (clinical vancomycin-resistant), cultured for 4, 8 or 24 h at 37 °C in a 96-well microplate in the presence of 3-arylideneflavanones **2b** or **2c** at MIC, were evaluated. The results were compared with the bacterial growth rate in the presence of oxacillin MIC (for staphylococci) or vancomycin MIC (for enterococci). After the incubation, compound/antibiotic concentrations causing 50% or 90% reduction, respectively, in microbial growth at each time point were deter-

mined (turbidity at A_{600}) and compared. Experiments were performed in duplicate to confirm the reproducibility of the results. Results are presented as the mean A_{600} ± S.D.

*Enumeration of viability and aggregation of bacterial cells treated with 3-arylideneflavanone **2c***

The potential effect of 3-arylideneflavanone **2c** on bacterial cell membranes was assessed by **2c**-mediated uptake of SYTO 9 and propidium iodide (LIVE/DEAD BacLight Bacterial Viability kit, L7007, Molecular Probes, Invitrogen, Eugene, USA). Cell suspensions of *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 in PBS ($1.0 \cdot 10^6$ CFU/ml) were incubated with MIC, ½ MIC or ¼ MIC of **2c** at 37 °C for 4 h with shaking. After incubation, bacteria were washed with 5 ml sterile PBS, and stained using the LIVE/DEAD kit according to the manufacturer's recommendations. Cells with LIVE/DEAD and no **2c** served as negative controls. After the incubation, all samples were washed and resuspended in PBS, and a drop of each suspension was examined with a confocal laser scanning microscope for green/red fluorescence using fluorescein and Texas red band-pass filters to visualize SYTO 9 and propidium iodide, respectively. Six areas of each of the triplicate samples were photographed with an integrated Hamamatsu digital camera (C4742-95; Nikon, Tokyo, Japan).

Additionally, in order to test whether the compound induces aggregation of the cells, 25 μl of bacterial suspension treated as described above were placed on a glass microscopic slide and gently smeared. After air-drying, heat-fixation, and Gram staining, slides were examined by light microscopy for the presence of clusters, in comparison to the control (non-treated bacteria), according to the score established by Cushnie *et al.* (2007).

*Effects of combinations of 3-arylideneflavanone **2c** and antibiotics on S. aureus and E. faecium cells*

Two-fold dilutions of one antibiotic (oxacillin for *S. aureus* A3 or vancomycin for *E. faecium* 138/09) in combination with ½ MIC or MIC of **2c** were used in the test for potential synergy. The fractional inhibitory concentration (FIC), which is an interaction coefficient indicating whether the combined inhibitory effect is synergistic, additive or antagonistic, was calculated for each combination that inhibited growth. The following formula was used: FIC = (MIC of antibiotic in combination with **2c**/MIC of antibiotic alone) + (MIC of **2c** in combination/MIC of **2c** alone). The average FIC index (FICI) was calculated from individual FIC values. FICI ≤ 0.5 was defined as synergistic, >0.5 – 1 as additive, >1 – <2 as indifferent, and ≥2 as antagonistic (EUCAST, 2000).

*Bacterial adherence, biofilm formation, and their suppression under the influence of 3-arylideneflavanone **2c***

Bacteria (*S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212) were grown for 24 h at 37 °C in 5 ml of tryptic-soy broth (TSB) supplemented with 0.25% D-(+)-glucose (TSBGlc). The cultures were diluted 1:40 in TSBGlc and 200 μl were added to the wells of a 96-well tissue culture-treated polystyrene plate (Nunclon™ Surface No. 167008; Nunc, Roskilde, Denmark). The adherence and biofilm formation in the absence or presence of **2c** (at ½ MIC and MIC) were evaluated after 2 h or 24 h of incubation, respectively, at 37 °C. The influence of 3-arylideneflavanone **2c** (at a concentration equal to MIC, 2–8 MIC) on preformed biofilms was determined. For this, **2c** was added to 24-h-old biofilms for further 24–48 h of incubation at 37 °C (total biofilm age was 72 h). After the above-mentioned treatments, adherent bacterial cells/biofilm mass was stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, USA] as described earlier (Walencka *et al.*, 2005, 2007). In this assay, live bacteria with an active electron transport system reduce the tetrazolium salt to a water-soluble purple formazan product, and optical density values are directly dependent on their cell numbers. Results are presented as the mean A_{550} ± S.D.

Results

Twenty two synthetic flavonoids, *i.e.* 2'-hydroxychalcones, 3-arylideneflavanones, and 3-arylidenechromanones (structures shown in Table I), were screened for their *in vitro* minimal inhibitory concentration (MIC) against an important group of "alert" human pathogens – *Staphylococcus* and *Enterococcus* spp. The concentration range of each compound was 0.29–300.0 μg/ml (eleven two-fold dilutions). The 2'-hydroxychalcones **1a**, **1b**, and **1c** showed an unsatisfactory effect (MIC

Table II. Sensitivity of *S. aureus* and *S. epidermidis* reference and clinical strains, respectively, to 3-arylideneflavanones **2b** and **2c**, in comparison to antibiotics.

Strain (reference, clinical)	P	Antibiotic break-points[a] MT	OXA	VAN	E	GM	T	IM	MIC[b] [μg/ml] AM	OXA	OFX	MIC[b] [μg/ml (μM)] **2b**	**2c**
Staphylococcus aureus													
ATCC 29213	R	S	S	S	S	S	S	S	1.0	0.5	0.5	18.75 (59.9)	4.68 (14.3)
ATCC 6538	S	S	S	S	S	S	S	S	nt	0.25	nt	18.75 (59.9)	4.68 (14.3)
hVISA 1474/1	R	R	R	I	R	R	R	R	nt	>128.0	nt	9.37 (29.9)	4.68 (14.3)
A3	R	R	R	S	R	R	R	R	nt	>128.0	nt	18.75 (59.9)	4.68 (14.3)
A7	R	R	R	S	R	R	R	R	nt	>128.0	nt	18.75 (59.9)	4.68 (14.3)
D5	R	R	R	S	R	R	R	R	nt	>128.0	nt	18.75 (59.9)	4.68 (14.3)
E1	R	R	R	S	R	R	R	R	nt	>128.0	nt	18.75 (59.9)	4.68 (14.3)
E4	R	R	R	S	R	R	R	R	nt	>128.0	nt	18.75 (59.9)	9.37 (28.6)
E7	R	R	R	S	R	R	R	R	nt	>128.0	nt	18.75 (59.9)	4.68 (14.3)
Staphylococcus epidermidis													
ATCC 12228	R	S	S	S	S	S	R	S	8.0	0.5	0.25	18.75 (59.9)	4.68 (14.3)
C10	R	R	R	S	R	R	S	R	nt	16.0	nt	18.75 (59.9)	9.37 (28.6)
C27	R	R	R	S	R	R	S	R	nt	128.0	nt	18.75 (59.9)	4.68 (14.3)

P, penicillin; MT, methicillin; OXA, oxacillin; VAN, vancomycin; E, erythromycin; GM, gentamicin; T, tetracycline; IM, imipenem; AM, ampicillin; OFX, ofloxacin; nt, not tested.

[a] S, susceptible; R, resistant; I, intermediate susceptible (break-points by disc-diffusion test, according to CLSI).

[b] MIC, minimal inhibitory concentration; MIC of antibiotics in μg/ml; MIC of **2b** and **2c** in μg/ml (μM).

150–300 μg/ml; 624.4–1248.8 μM) against reference strains. On the other hand, three out of nine members of the 3-arylideneflavanones group (**2b**, **2c**, and **2i**) showed potent activity, with MIC values ranging from 4.68 μg/ml (14.3 μM) to 37.5 μg/ml (119.7 μM) (Tables II, III).

The main goal of the next part of the study was to see whether **2b** [(*E*)-2,3-dihydro-2-phenyl-3[(pyridin-4-yl)-methylene]chroman-4-one], **2c** [(*E*)-3-(4-hydroxybenzylidene)-2,3-dihydro-2-phenylchroman-4-one] and **2i** [(*E*)-3-(3-hydroxybenzylidene)-2,3-dihydro-2-phenylchroman-4-one] are equally potent against antibiotic-susceptible and multidrug-resistant strains. For this experiment, we selected a range of clinical isolates from blood, cerebrospinal fluid, catheter swab, and stool of hospitalized patients with local or systemic infections. Strong activity of these compounds was indeed observed, regardless of the antibiotic susceptibility profiles of the strains. Active concentrations ranged from 4.68 μg/ml to 75.0 μg/ml (Tables II, III). MIC values of these compounds were much lower than those of the natural flavonoids quercetin (>300.0 μg/ml) and naringenin (300.0 μg/ml), and lower or equal to the MIC of thymol, a compound with known antibacterial activity (18.75 μg/ml for *S. aureus*; 75.0 μg/ml for *E. faecalis*) and lower than MIC values of the reference antibiotics. In Tables II and III, MIC values of compound **2b** and **2c** are presented in both micrograms per ml and in micromolar concentration, however, later in the text micrograms per ml were used, as for the reference antibiotics. Compound **2i**, differing from **2c** only in the OH substituent at the phenyl ring and showing similar MIC values, was not included in the further study.

The experiment designed to test the kinetics of the compounds' action showed that the 3-arylideneflavanones **2b** and **2c** at MIC (18.75 and 4.68 μg/ml, respectively) efficiently reduced the number of staphylococci by approximately 50% within 4 h and by 90% within 8 h. The time-dependent effect was comparable to the action of the standard β-lactam antibiotic, oxacillin, used against MSSA at a concentration of 0.5 μg/ml (Fig. 1A). Compounds **2b** and **2c** used at these relatively low MIC values, also efficiently reduced the multiplication of MRSA, while as much as 128.0 μg/ml of oxacillin was needed to obtain the same effect (Fig. 1B). Similar time-dependent antibacterial activity of these compounds was found

Table III. Sensitivity of *E. faecalis* and *E. faecium* reference and clinical strains, respectively, to 3-arylideneflavanones **2b** and **2c**, in comparison to antibiotics.

Strain (reference, clinical)	MIC[a] [μg/ml]									MIC[a] [μg/ml (μM)]	
	P	AM	CIP	CH	GM	LZD	RIF	T	TP	VAN	**2b**	**2c**
Enterococcus faecalis												
ATCC 29212	nt	1.0	nt	nt	nt	nt	nt	nt	nt	2.0	37.5 (119.7)	9.37 (28.6)
1480/06	2.0	0.5	0.5	64.0	512.0	1.0	8.0	64.0	0.38	2.0	75.0 (239.4)	9.37 (28.6)
1610/06	1.0	1.0	64.0	64.0	1024.0	2.0	0.5	128.0	0.38	2.0	37.5 (119.7)	9.37 (28.6)
2003/06	1.0	0.5	0.5	64.0	512.0	1.0	8.0	64.0	0.25	2.0	37.5 (119.7)	9.37 (28.6)
2041/06	1.0	0.5	1.0	8.0	1.0	1.0	2.0	0.5	0.38	2.0	37.5 (119.7)	9.37 (28.6)
2681/06	1.0	0.5	2.0	16.0	2.0	1.0	32.0	32.0	0.38	2.0	75.0 (239.4)	9.37 (28.6)
348/07	1.0	1.0	64.0	128.0	>1024.0	1.0	8.0	64.0	0.25	2.0	75.0 (239.4)	9.37 (28.6)
351/07	2.0	1.0	64.0	128.0	1024.0	1.0	0.5	64.0	0.38	2.0	75.5 (239.4)	9.37 (28.6)
683/07	1.0	1.0	1.0	8.0	512.0	1.0	8.0	64.0	0.25	2.0	37.5 (119.7)	9.37 (28.6)
Enterococcus faecium												
138/09	nt	nt	nt	nt	nt	nt	nt	nt	1.0	64.0	37.5 (119.7)	9.37 (28.6)
988/09	nt	nt	nt	nt	nt	nt	nt	nt	32.0	>256.0	37.5 (119.7)	9.37 (28.6)

P, penicillin; AM, ampicillin; CIP, ciprofloxacin; CH, chloramphenicol; GM, gentamicin; LZD, linezolid; RIF, rifampicin; T, tetracycline; TP, teicoplanin; VAN, vancomycin; nt, not tested.

[a] MIC, minimal inhibitory concentration; MIC of antibiotics in μg/ml; MIC of **2b** and **2c** in μg/ml (μM).

when *E. faecalis* and *E. faecium* were used as target microorganisms and confronted with the action of the reference antibiotic vancomycin. This antibiotic was used for sensitive and resistant strains at a concentration of 2 μg/ml or 64.0 μg/ml, respectively (Figs. 2A, B).

The answer to the question of whether **2b** and **2c** are bacteriostatic or bactericidal was provided by two parallel tests. A CFU counting test revealed ≥99.9% reduction of the original inoculum by 2–4 MIC of **2b** and **2c** which indicated their bactericidal effect. However, subculturing of the same samples on broth media showed bacterial regrowth. This observation would rather indicate that **2b** and **2c** are not bactericidal but bacteriostatic. This interpretation was supported by the appearance and characteristics of the colonies recovered on the solid medium. They were differentiated (small and large) which reflects the influence of **2b** and **2c** on the cell wall and/or on the cell membrane integrity. This was also proved by experiments for **2c**-mediated SYTO 9/propidium iodide uptake, evaluated by fluorescent confocal microscopy. As assessed by the emission of green/red fluorescence, non-treated staphylococcal as well as enterococcal cells showed only green fluorescence (live with undamaged cell wall), except for very few red cells. However, **2c**-treated cells (¼ MIC, ½ MIC, MIC) exhibited increased uptake of propidium iodide (leaking cell wall and membrane), proportional to the increase in its concentration. When samples were examined by light microscopy, the majority of bacteria treated with **2c** were found in small and large aggregates, as compared to pairs and small clusters in control samples. Also, the size and shape of the cells was changed under the influence of the compounds applied (data not shown). Our observations indicate that this effect is reversible, since bacteria from colonies of different sizes subcultured onto medium without antimicrobial chemicals had the correct parental morphology.

The synergistic activity against the *S. aureus* A3 clinical strain was observed for combinations of **2c** used at MIC and oxacillin (FICI = 0.01). **2c** used at ½ MIC also increased significantly (64-fold) the oxacillin susceptibility of *S. aureus*, however, the FICI value was on the border between synergy and additive effect (FICI = 0.58). The similar observation was made concerning vancomycin susceptibility changes of the *E. faecium* 138/09 clinical strain in the presence of **2c**.

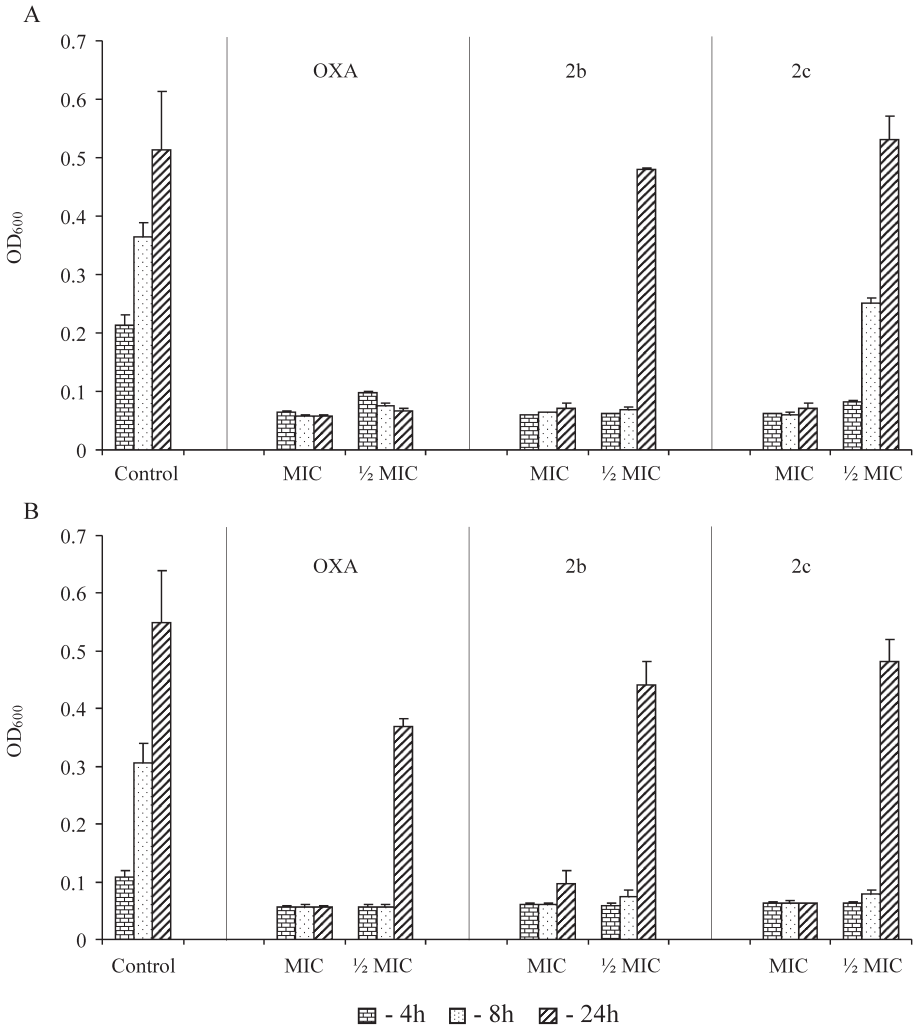


Fig. 1. Growth rate of (A) reference *S. aureus* ATCC 29213 and (B) clinical A3 strains, cultured for 4, 8 or 24 h in the presence of 3-arylideneflavanones **2b** and **2c** and compared with the growth rate in the presence of oxacillin (OXA). Results are presented as the mean A_{600} ± S.D. MIC of **2b** = 18.75 μg/ml for both strains; MIC of **2c** = 4.68 μg/ml for both strains; MIC of OXA = 0.5 μg/ml for *S. aureus* ATCC 29213 (MSSA); MIC of OXA = 128.0 μg/ml for *S. aureus* A3 (MRSA).

Synergy of vancomycin and **2c** used at MIC was observed (FICI = 0.003), whereas an antibiotic in combination with ½ MIC of **2c** showed an additive effect, reducing the value of the vancomycin MIC to ⅛ (FICI = 0.97) (Table IV).

The effect of **2c** on adhesion to and formation of a biofilm by *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 strains on a polystyrene surface was evaluated. After MTT staining, the mean A_{550} value for *S. aureus* after 2 h was 0.55 ± 0.02, and after 24 h it was 2.96 ± 0.01. The mean A_{550} value for *E. faecalis* after 2 h was 0.40 ± 0.12, and after 24 h it was 1.68 ± 0.06. Therefore these strains were recognized as a strong biofilm producer. The inhibition of their initial adhesion ranged from 47.8% (½ MIC) to 54.5% (MIC) and from 42.9% (½ MIC) to 54.5% (MIC), respectively, for *S. aureus* and *E. faecalis*.

Such a strong inhibitory effect of **2c** resulted in a decrease in *E. faecalis* biofilm formation by 27.9% (mean A_{550} 1.21 ± 0.06 at ½ MIC) and by 49.4% (mean A_{550} 0.83 ± 0.06 at MIC). Unfortu-

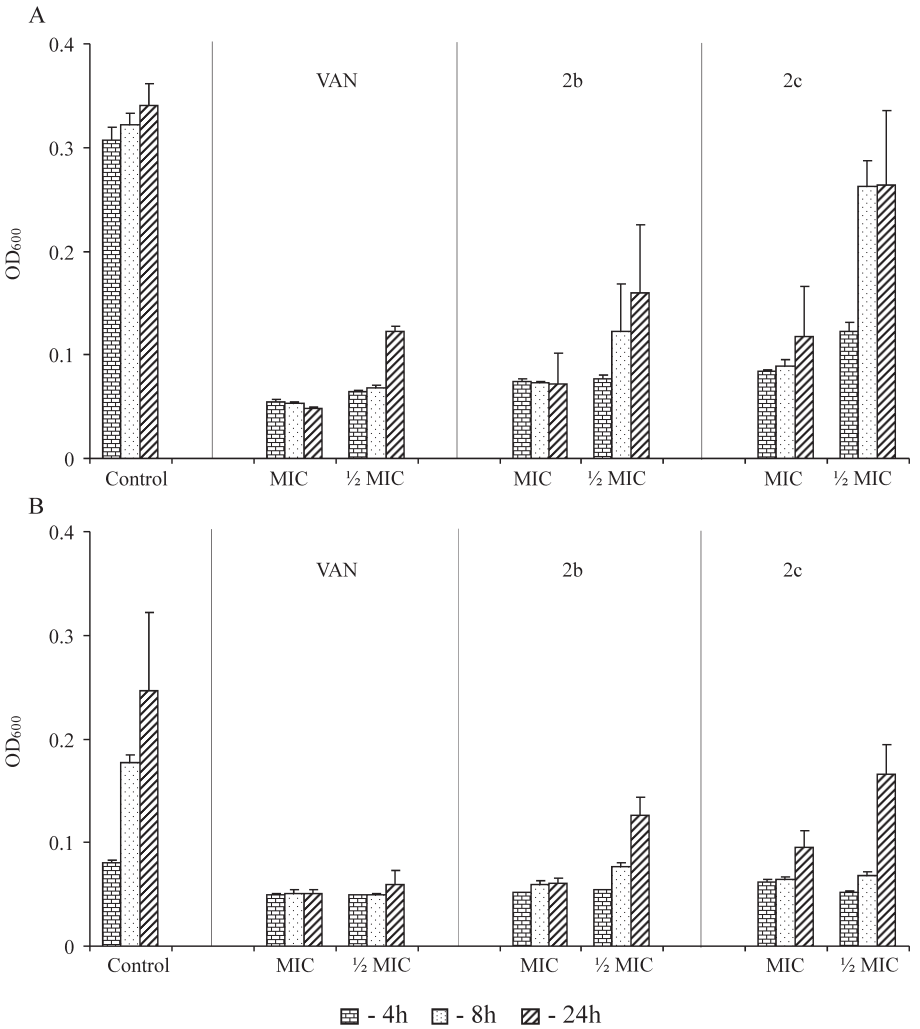


Fig. 2. Growth rate of (A) reference *E. faecalis* ATCC 29212 and (B) clinical *E. faecium* 138/09 strains, cultured for 4, 8 or 24 h in the presence of 3-arylideneflavanones **2b** and **2c** and compared with the growth rate in the presence of vancomycin (VAN). Results are presented as the mean A_{600} ± S.D. MIC of **2b** = 37.5 μg/ml for both strains; MIC of **2c** = 9.37 μg/ml for both strains; MIC of VAN = 2.0 μg/ml for *E. faecalis* ATCC 29212 (VSE); MIC of VAN = 64.0 μg/ml for *E. faecium* 138/09 (VRE).

nately, although the early adhesion of staphylococci in the presence of **2c** (½ MIC) was reduced by nearly 50%, it did not stop biofilm formation during a 24-h co-incubation. However, when **2c** was used for this purpose at MIC, the *S. aureus* biofilm formation was inhibited by 50% (mean A_{550} dropped from 2.96 ± 0.01 to 1.47 ± 0.12). On the other hand, **2b** and **2c** used at MIC as well as at 4–8 MIC, failed to cause complete eradication of the *S. aureus* and *E. faecalis* biofilms which had been already preformed. The degree of biomass reduction obtained was not significant (5–10%).

Discussion

Our study has revealed the potent antibacterial activity of some synthetic 3-arylideneflavanones. Similarly to our results, flavanones were often shown to be most active against Gram (+ve) bacteria (Cushnie and Lamb, 2005; Alcaraz *et al.*, 2000; Nishino *et al.*, 1987; Jeong *et al.*, 2009).

Table IV. MIC values of oxacillin or vancomycin alone and in combination with **2c** against *S. aureus* A3 (MRSA) and *E. faecium* 138/09 (VRE) clinical strains.

Bacterial strain	A* MIC_A [μg/ml]	**2c** MIC_{2c} [μg/ml]	A+½MIC **2c** MIC_A [μg/ml]	A+MIC **2c** MIC_A [μg/ml]
S. aureus A3 (MRSA)	128.0	4.68	2.0	0.062
E. faecium 138/09 (VRE)	64.0	9.37	8.0	0.031

*A, antibiotic: oxacillin for *S. aureus*, vancomycin for *E. faecium*.
The experiment was repeated two times, each sample in triplicate, and no MIC values variation was noted.

For example, remagiflavanone A and B, isolated from the methanolic extract of dried leaves of *Physena madagascariensis* demonstrated activity against *S. aureus* at 3.75 μg/ml and against *E. faecalis* at 7.5 μg/ml, whereas against various Gram (-ve) bacteria the active concentration was as high as 125.0 μg/ml (Deng *et al*., 2000). Thus, our results concerning the antibacterial activity of compounds **2c** and **2i** are satisfactory. Other authors, who tested twelve commercially available natural flavanones, identified six of them as good candidates to be antimicrobials despite their MIC values ranging from 128.0 to 512.0 μg/ml (Jeong *et al*., 2009). According to many authors, natural, semi-synthetic or synthetic compounds capable of inhibiting bacterial growth at a concentration lower than 10 μg/ml are worth further study (Gibbons, 2008; Cushnie and Lamb, 2005). Such requirements were met by our three compounds (**2b**, **2c**, and **2i**) showing MIC values ranging from 4.68 μg/ml to 37.5 μg/ml; however, these were not bactericidal.

Among Gram (+ve) bacteria, increasing concerns have been voiced about nosocomial and community-associated staphylococci (MRSA), and vancomycin-resistant enterococci (VRE) (Rice, 2006). However, biofilm formation by these bacteria is a major medical problem and the most important one to be resolved (Goetz, 2002; Mohamed and Huang, 2007). Due to the high resistance of biofilms to antibiotics and host-derived immune factors, the ideal solution to avoid their engagement in the pathogenesis process would be to prevent their development (Hoiby *et al*., 2010; Dürig *et al*., 2010).

Thus, one of the main questions we posed was whether these compounds may be considered effective agents in anti-biofilm therapy. Indeed, we have demonstrated that these compounds were effective against staphylococcal and entrococcal adhesion and biofilm formation. Unfortunately, the reduction in biomass and disruption of mature biofilm structure by our synthetic flavonoids was not significant. There is general agreement that complete eradication of biofilm-embedded bacteria by medical therapy is hard to achieve, but it is possible that their pretreatment with natural phytochemicals or synthetic analogues, though not sufficient for eradication, could increase the antibiotic effect by disruption of the biofilm integrity. So, our findings suggest that 3-arylideneflavanones, and in particular compound **2c**, alone or in combination with antibiotics, might be useful in controlling MRSA and VRE infections. Compound **2c**, used even in the sub-inhibitory concentration, reduced MRSA resistance to the β-lactam antibiotic (oxacillin) and VRE resistance to the glycopeptide antibiotic (vancomycin). In view of the rising prevalence of multi-resistant staphylococci and enterococci, such a combined therapy may be useful in improving the efficacy of "old" antibiotics.

When comparing the chemical structures of more and less biologically active compounds, two features seem to be characteristic. For high activity the first one is the presence of the phenyl group of the most powerful 3-arylideneflavanones **2b** and **2c**. The 3-arylidenechromanones **3b** and **3c**, which are closely related to them (albeit-inactive), do not have this substituent. The second one is the importance of the 3- or 4-hydroxyphenyl group, respectively, at R^3 of **2c** and **2i**. In this case, the two compounds are equally efficient. The related compound **2a** with an unsubstituted phenyl ring at this site was inactive. The requirement of at least one hydroxyphenyl group and a certain degree of lipophilicity for flavonoids to display antimicrobial activity was reported earlier (Cushnie and Lamb, 2005; Mughal *et al*., 2006; Avila *et al*., 2008). It should also be noted that other sub-

stituents in the 3-arylideneflavanone phenyl ring such as halogen atoms (F, Cl, Br) in **2e**–**2g** and the -CN group in **2h** do not increase the antimicrobial efficacy of these derivatives.

For certain natural or modified flavonoids, the mechanisms underlying their antimicrobial action are already known (Gibbons, 2008; Cushnie and Lamb, 2005). It is suggested, however, that an individual compound may have multiple cellular targets, rather than one specific site of action. Although it is difficult to predict the pharmacodynamic features of such agents, their good penetration, direct membrane damage, and lipophilicity seem to be the most important properties of potential drugs. It is also possible that they affect the hydrophobicity of the bacterial cell surface. By decreasing the expression of surface-exposed hydrophobins, these chemicals could interfere with the adhesion step essential in biofilm development. Such an effect observed in our study could be the result of the relatively high lipophilicity of synthetic flavanones (partition coefficient log *P* for **2b** = 1.54, for **2c** = 2.13, for **2i** = 2.30, calculated by mathematical analysis). It suggests that synthetic compounds tested in the present work may easily penetrate biological membranes, probably without any help of active transport mechanisms, thus being promising as future alternative therapeutic agents.

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