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Pyrrolomycins as potential anti-staphylococcal biofilms agents

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With the goal of discovering new anti-infective agents active against microbial biofilms, this investigation focused on some natural pyrrolomycins, a family of halogenated pyrrole antibiotics. In this study the anti-staphylococcal biofilm activity of pyrrolomycins C, D, F1, F2a, F2b, F3 and of the synthesized related compounds I, II, III were investigated. The susceptibility of six staphylococcal biofilms was determined by methyltiazotetrazolium staining. Most of the compounds were active at concentrations of 1.5 μg ml^{-1} with significant inhibition percentages. A few of the compounds were active at the lowest screening concentration of 0.045 μg ml^{-1}. The population log reduction of activity against the two best biofilm forming *Staphylococcus aureus* strains as determined by viable plate counts is also reported. In order to adequately assess the utility of these compounds, their toxicity against human cells was evaluated. It is concluded that pyrrolomycins and synthetic derivatives are promising compounds for developing novel effective chemical countermeasures against staphylococcal biofilms.

Keywords: staphylococcal biofilms; anti-biofilm agents; pyrrolomycins

Introduction

Staphylococcal biofilms are a leading cause of device-related infections of medical relevance. *Staphylococcus aureus* is an important cause of metal-biomaterial, bone joint, and soft tissue infections. *Staphylococcus epidermidis*, on the other hand, is seen more often in polymer associated infections (Götz 2002). Together, the Gram-positive pathogens *S. aureus, S. epidermidis*, and *Enterococcus faecalis* represent more than 50% of the species isolated from patients with medical device-associated infections (Donelli et al. 2007). The ability of *S. aureus* to form a biofilm is probably the virulence factor that contributes the most to the development of the chronic and persistent forms of infectious diseases like osteomyelitis (Brady et al. 2008). A similar situation is seen in dairy cattle. *S. aureus* is a major pathogen of mastitis, which is one of the most common diseases in dairy cattle. Although it has good *in vitro* antimicrobial susceptibility, the therapy used to treat animals affected by mastitis is often disappointing and results in recurrent clinical and chronic sub-clinical infections. It has been suggested that these recurrent and chronic staphylococcal infections can be attributed to the growth of bacteria as biofilms (Melchior et al. 2006).

Currently, no therapies that effectively target microbial biofilms exist. This is in part because biofilms are intrinsically resistant to conventional antibiotics (Gilbert et al. 2002). There is undoubtedly an urgent need for new antibacterial drugs active against not only planktonic bacteria but also biofilms. With this aim, the present study focused on the halogenated pyrroles (Gribble 2003), pyrrolomycins B-F, which are naturally produced by *Actinosporangium vitaminophylum*. These compounds have been described in the past as significantly active agents against Gram-positive bacteria (Ezaki 1983).

The authors recently reported anti Gram-positive bacteria and the anti-staphylococci biofilm activities of 3,4,5,3′,5′-pentabromo-2-(2′-hydroxybenzoil) pyrrole **I** (Schillaci et al. 2005), a synthetic compound related to pyrrolomycin D. Furthermore, the pyrrolomycin biosynthetic gene cluster has been recently cloned and characterized (Zhang and Parry 2006).

This article aims to compare the anti-staphylococcal biofilm activity of some natural pyrrolomycins and some related synthetic derivatives. To better understand the utility of such compounds in the development of novel anti-staphylococcal biofilm agents, the toxicity towards a human primary cell culture and the selectivity indexes of these compounds were also determined.

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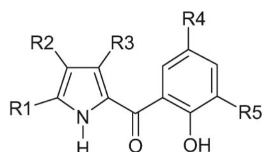
Materials and methods

Synthesis of pyrrolomycins and derivatives

Pyrrolomycins and derivatives were synthesized and characterized as described (Raimondi et al. 2006). The chemical structures of such molecules are reported in Figure 1.



	R1	R2	R3	R4	R5
C	Cl	Cl	H	Cl	Cl
D	Cl	Cl	Cl	Cl	Cl
F1	Br	Br	Br	Br	H
F2a	Br	Cl	Br	Br	H
F2b	Cl	Br	Br	Br	H
F3	Cl	Cl	Br	Br	H
I	Br	Br	Br	Br	Br
II	Br	Br	Br	Cl	Cl
III	Cl	Cl	Br	Cl	Cl

Figure 1. Chemical structures of pyrrolomycins and derivatives.

Bacterial strains

A group of biofilm forming staphylococcal reference strains, viz. *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. epidermidis* DSM 3269, and methicillin resistant *S. epidermidis* RP62A was used. Two staphylococcal isolates of veterinary interest were also used: *S. aureus* 657 and *S. aureus* 702. Strain 657 was isolated from a milk sample from an individual sheep affected by mastitis. Strain 702 was isolated from a bulk milk sample from a sheep flock.

Determination of MICs

Minimum inhibitory concentrations (MICs) against planktonic strains were determined as previously described by a broth dilution micromethod (Schillaci et al. 2005). Briefly, a series of solutions with a range of concentrations from 50 to 0.001 μg ml^{-1} (obtained by twofold serial dilution) was made in Mueller Hinton broth (Merck) in a 96-well plate. To each well 10 μl of a bacterial suspension, obtained from a 24 h culture, containing $\sim 10^6$ colony forming units (CFU) ml^{-1} were added. The plate was incubated at 37°C for 24 h. After this time the MIC values were determined by a microplate reader (ELX 800, Bio-Tek Instruments) as the lowest concentration of compound whose optical density (OD) at 570 nm, was comparable to the negative control wells (broth only).

Molecular characterization

The two *S. aureus* strains of veterinary interest were investigated for the presence of the intercellular adhesion (*ica*) locus, which encodes polysaccharide intercellular adhesin (PIA) and the biofilm-associated protein (*BAP*) gene, by site specific polymerase chain reaction (PCR) with the primers used in a previous study (Cucarella et al. 2004). The following PCR conditions were adopted: 95°C for 4 min, then 40 cycles (94°C × 30 s; 50°C × 30 s; 72°C × 50 s) at 72°C for 5 min and then held at 4°C.

The methicillin resistance gene (*mecA*) was also detected by a multiplex PCR using the gene amp 9700 (Applied Biosystem) as described by Mehrotra et al. (2000). DNA was extracted using QIAamp DNA mini kit spin columns according to the manufacturer's instructions (Quiagen SpA, Milan, Italy).

Biofilm forming evaluation

All the staphylococcal strains were tested for their ability to form biofilms. Briefly, bacteria were grown overnight in a shaking bath at 37°C in Tryptic Soy Broth (TSB, Sigma) containing 2% glucose and then diluted 1:200 to a suspension with an OD of $\sim$0.040 at 570 nm. Polystyrene 24-well tissue culture plates were filled with 1 ml of diluted suspension and incubated for 24 h at 37°C. Then, the wells were washed three times with 1 ml of sterile phosphate-buffered saline (PBS) and stained with 1 ml of safranin (0.1% v/v) for 1 min. The excess stain was removed by placing the plates under running tap water. Plates were dried overnight in an inverted position at 37°C. Safranin-stained adherent bacteria in each well were re-dissolved to homogeneity in 1 ml of 30% v/v glacial acetic acid, and the OD was read at 492 nm. Each assay was performed in triplicate and repeated at least twice.

Biofilm susceptibility testing: the methylthiazotetrazolium (MTT) method

Staphylococcal strains were grown in TSB containing 2% glucose, for 24 h at 37°C in a shaking bath and then diluted 1:200 in order to obtain a suspension whose OD at 570 nm was $\sim$0.015. The diluted suspension was added to the wells (100 μl per well) of a polystyrene microtiter plate and incubated for 24 h at 37°C. Then the wells were washed three times with 200 μl of sterile PBS and finally air-dried in an inverted position at 37°C. Each of the wells was then filled with 100 μl of Mueller-Hinton broth. With the exception of the positive (growth) control wells, the Mueller-Hinton broth was supplemented with concentrations, obtained by dilution, of 1.5 μg ml^{-1} or

0.045 μg ml^{-1} for each compound. Finally, the plates were incubated at 37°C for 24 h. Following this incubation period, the medium was removed, the plates were air-dried in an inverted position, and each well was filled with 100 μl of PBS and 5 μl of a 5 mg ml^{-1} MTT (methylthiazotetrazolium) solution and incubated for 1 h at 37°C. The insoluble purple formazan obtained by cleavage of MTT made by the dehydrogenase enzymes of living cells, was dissolved by a mixture of isopropyl alcohol (9 ml), Triton X-100 (Sigma) (1 ml), and HCl 37% v/v (300 μl). The OD of each well was read by a microplate reader (ELX 800, Bio-Tek instruments) at 570 nm with background subtraction at 630 nm. Comparing the average OD of the growth control wells with that of sample wells the following formula was used to calculate the inhibition percentages for each concentration of the compound:

$$(\text{OD growth control} - \text{OD sample}) / \text{OD growth control} \times 100$$

Experiments were performed at least in triplicate.

Biofilm susceptibility testing: viable plate counts

Staphylococcal strains were grown in TSB containing 2% glucose, incubated and diluted as in the MTT method. The diluted suspension was added to the wells (1000 μl per well) of a polystyrene microtiter 24-well plate. Glass discs (12 mm diameter) were immersed in each well and incubated for 24 h at 37°C. Following this incubation period the wells were washed three times with 200 μl of sterile PBS and filled with 1000 μl of Mueller-Hinton broth (except untreated growth control wells) supplemented with concentrations of 0.045 μg ml^{-1} of each compound. For comparative and quality control purposes, other wells were supplemented with rifampicin (Sigma) at a concentration of 0.045 μg ml^{-1}. Plates were incubated at 37°C for 24 h, the medium was removed, and the glass discs were scraped three times. The inocula were put in test tubes with 10 ml of NaCl (0.9% w/v solution) and sonicated for 2 min. Six 10-fold dilutions were prepared and 100 μl aliquots of each dilution were plated onto Plate count agar (Biokar Diagnostics, Beauvais, France). Plates were then incubated at 37°C, and CFU ml^{-1} were counted after 18 h. Each assay was performed in triplicate and repeated at least twice.

Mammalian cell cytotoxicity

A suspension (1×10^5 ml^{-1}) of a human primary cell culture (Human Derm) was seeded into 96-well plates and incubated for 72 h in a humidified incubator (95% air, 5% CO_2) until confluence. At the time of the assay, culture medium (MEM, Sigma-Aldrich, supplemented with fetal calf serum and antibiotics) was replaced with fresh RPMI medium (Sigma-Aldrich) without red phenol. The test compounds were added at various concentrations starting with a maximum concentration of 75 μg ml^{-1}. The microtiter plate was incubated for another 24 h and the toxicity was determined by MTT assay (Mosmann 1983). The cytotoxicity was estimated in terms of percent growth inhibition. IC_{50} values were defined as the test concentration at which the cell proliferation was inhibited by 50% with respect to the untreated growth control.

Results and discussion

Preliminary data were obtained by testing the antibacterial activity of compounds against planktonic staphylococcal strains. The results, expressed as MICs and the geometric means of these MIC values are listed in Table 1. The toxicity of these compounds against a human normal cell culture (Human Derm) and the selectivity index (ratio of cytotoxicity in terms of IC_{50} in μg ml^{-1} to antibacterial activity expressed as the geometric mean of the MICs) are also reported in Table 1. All compounds, except pyrrolomycin C, were

Table 1. Antistaphylococcal activity of pyrrolomycins and related compounds against planktonic strains.

	C	D	F1	F2a	F2b	F3	I	II	III
S. aureus ATCC 29213	3.2	≤0.001	≤0.001	0.001	0.03	0.01	0.01	0.2	0.1
S. aureus ATCC 25923	0.2	≤0.001	0.02	0.005	0.01	0.02	0.005	≤0.001	≤0.001
S. aureus 657	3.2	≤0.001	0.006	0.05	0.01	≤0.001	0.1	0.01	0.01
S. aureus 702	6.2	≤0.001	0.02	0.1	0.05	0.006	0.2	0.01	0.02
S. epidermidis DSM 3269	12.5	≤0.001	≤0.001	0.05	0.1	0.05	0.003	0.05	0.01
S. epidermidis RP62A	3.2	0.002	0.002	≤0.001	≤0.001	≤0.001	0.4	≤0.001	≤0.001
Geometric means of MICs	2.82	1.12×10^{-3}	4.11×10^{-3}	0.01	0.016	6.25×10^{-3}	0.033	0.01	7.65×10^{-3}
Mammalian cell cytotoxicity IC_{50} (μg ml^{-1})	≥75	≥75	60	≥75	≥75	60	≥75	≥75	40
Selectivity index (IC_{50}/MIC)	26.6	>10000	>10000	7500	4688	9600	2273	7500	5228

MIC expressed in μg ml^{-1}.

effective against all the tested strains with low MIC values. In particular, pyrrolomycin D showed a geometric mean of the MIC close to 0.001 μg ml^{-1} for all staphylococcal strains. These results are in accordance with previous reports that pyrrolomycin D is one of the most active naturally produced organo-halogens against Gram-positive pathogens (van Pee and Ligon 2000). The data in Table 1 indicate that most of the tested pyrrolomycins and related compounds possessed a very interesting ''safety margin'' or selectivity index as antibacterial agents. All of the compounds, except pyrrolomycin C, showed a selectivity index >1000 and in two cases (pyrrolomycins D and F1) >10000.

Site-specific PCR were used to determine whether or not the *ica* locus and *BAP* gene were present in the field isolates of *S. aureus* 657 and *S. aureus* 702. The PCR results indicate that these strains contain both the *ica* locus and the *BAP* gene. The *ica* locus can promote biofilm formation (Otto 2008) and the *BAP* gene, which encodes a cell-wall-bound surface protein, plays a role in *ica*-independent biofilm mechanisms (O'Gara 2007) and infection of mammary glands (Cucarella et al. 2004). The presence of the *mecA* gene was detected only in strain 657.

Biofilm formation was estimated for all strains used in this study by staining adherent cells on polystyrene surfaces with safranin and reading the ODs. The tested reference strains were observed to form more dense biofilms than the two staphylococcal isolates of veterinary interest (Table 2).

The anti-biofilm activity of pyrrolomycins and derivatives against all staphylococcal strains was evaluated using MTT for detecting live and adherent bacteria (Walencka et al. 2007). Tables 3 and 4 show the activities, in terms of percentage inhibition, at the concentrations of 1.5 and 0.045 μg ml^{-1}. The compounds, with the exception of pyrrolomycin C, were active against all reference and field isolate staphylococcal biofilms at the highest tested concentration of 1.5 μg ml^{-1}. The inhibition percentages were $>60\%$ for all the compounds and in many cases $>80\%$ (Table 3). To better understand the utility of such

Table 2. Biofilm production in test staphylococcal strains.

	OD at 492nm
S. aureus ATCC 29213	2.190 ± 0.20
S. aureus ATCC 25923	1.956 ± 0.17
S. aureus 657	0.646 ± 0.019
S. aureus 702	0.732 ± 0.021
S. epidermidis DSM 3269	3.380 ± 0.085
S. epidermidis RP62A	3.010 ± 0.35

Safranin method. Values are the average ± SD of at least three independent determinations.

Table 3. Anti-biofilm activity of pyrrolomycins and related compounds.

	C	D	F1	F2a	F2b	F3	I	II	III
S. aureus ATCC 29213	73.3	75.6	87	81	77	78.6	89	67.9	67.3
S. aureus ATCC 25923	62.8	70	68.3	75.3	75.8	76.5	68.5	84	84
S. aureus 657	78.5	75	85.3	84	80.5	85.6	85.3	69.5	81
S. aureus 702	63.5	79.5	84.3	82	79.3	84.3	85.6	74.5	71
S. epidermidis DSM 3269	NS	79.5	78.8	82.5	81	79.5	100	86.7	84.4
S. epidermidis RP62A	69.5	84.5	83	84.5	84.5	87.5	78	85	81
Mammalian cell cytotoxicity IC_{50} (μg ml^{-1})	≥75	≥75	60	≥75	≥75	60	≥75	≥75	40
Selectivity index (IC_{50}/anti-biofilm conc.)	NT	50	40	50	50	40	50	50	26.6

MTT method. Screening at 1.5 μg ml^{-1}. Activity expressed as percentage inhibition. Values are the average of at least three independent determinations. The variation coefficient was $<15\%$; NS, not significant because below 15% inhibition; NT, not tested.

Table 4. Anti-biofilm activity of pyrrolomycins and related compounds.

	C	D	F1	F2a	F2b	F3	I	II	III
S. aureus ATCC 29213	26	28	39	69	35	63	58	63	51
S. aureus ATCC 25923	24	37	46	53	40	72	27	60	57
S. aureus 657	NS	74	62	59.3	59.6	72	48.5	68.3	76
S. aureus 702	NS	60	41	39	30	65	28	70	69
S. epidermidis DSM 3269	NS	63	54	52	62	64	49.4	71	71
S. epidermidis RP62A	NS	25	38	41	25	73	26	56	65
Mammalian cell cytotoxicity IC_{50} (μg ml^{-1})	≥75	≥75	60	≥75	≥75	60	≥75	≥75	40
Selectivity index (IC_{50}/anti-biofilm conc.)	NT	NT	NT	NT	NT	1333	NT	1666	889

MTT method. Screening at 0.045 μg ml^{-1}. Activity expressed as percentage inhibition. Values are the average of at least three independent determinations. The variation coefficient was $<15\%$; NS, not significant because below 15% oinhibition; NT, not tested.

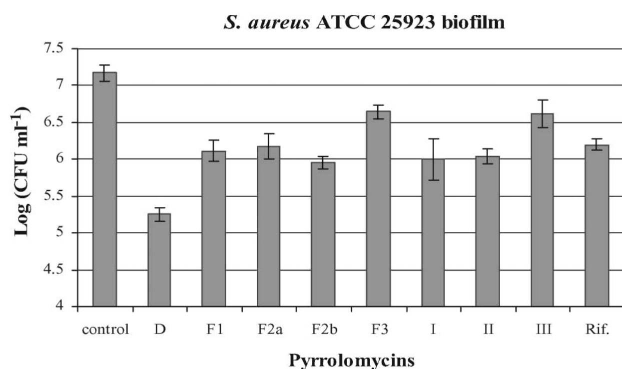


Figure 2. Numbers (CFU ml^{-1}) of *S. aureus* ATCC 25923 in untreated biofilm and in the presence of pyrrolomicins and rifampicin at 0.045 μg ml^{-1}. Data are reported as means $\pm$ SD.

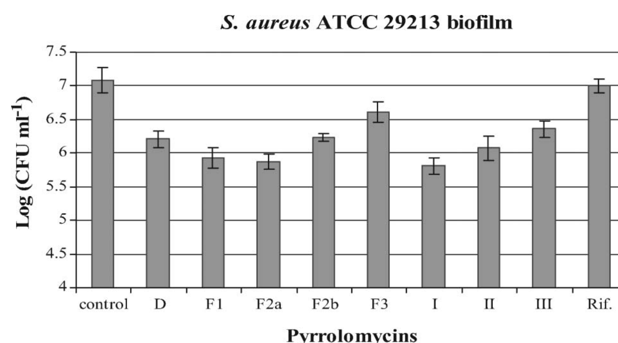


Figure 3. Numbers (CFU ml^{-1}) of *S. aureus* ATCC 29213 in untreated biofilm and in the presence of pyrrolomicins and rifampicin at 0.045 μg ml^{-1}. Data are reported as means $\pm$ SD.

compounds as potential anti-staphylococcal biofilm agents, the selectivity index (ratio of IC_{50} to anti-biofilm concentration) for the 1.5 μg ml^{-1} treatment was also determined and reported in the same Table. Due to the high concentration used, the selectivity indexes as anti-biofilm agents were very low (50 or less).

Test compounds were also evaluated at a minimum screening concentration of 0.045 μg ml^{-1}. In this case, pyrrolomycin F3 and the synthetic derivatives II and III were effective as anti-biofilm agents, showing inhibition percentages $>50\%$ against all tested strains. Significant selectivity indexes for the 0.045 μg ml^{-1} treatment were also found for these compounds. These were >1000 for pyrrolomycin F3 and compound II, and close to 900 for compound III (Table 4).

The effectiveness of pyrrolomycins against the two best *S. aureus* biofilm formers at the lowest screening concentration of 0.045 μg ml^{-1} was also evaluated in terms of log reductions based on viable plate count. Their effectiveness was compared to rifampicin, which has been described as an excellent agent alone or in combination with other antibiotics in the treatment of staphylococcal biofilms (Trampuz and Zimmerli 2006). The CFU ml^{-1} for adherent cells grown on glass discs was assessed after scraping and sonicating and is reported in Figures 2 and 3. At 0.045 μg ml^{-1}, rifampicin had weak activity against *S. aureus* ATCC 25923 (0.97-log reduction). However, stronger activity was observed in the presence of pyrrolomycin F2b (1.22-log reduction) and pyrrolomycin D (1.92-log reduction). No anti-biofilm activity was observed in the presence of rifampicin at 0.045 μg ml^{-1} against *S. aureus* ATCC 29213. In contrast, pyrrolomycin F2a and synthetic compound I showed a significant log reduction (around 1.20 logs) against this strain.

In conclusion, natural pyrrolomycins and synthetic derivatives are promising compounds for developing novel effective chemical countermeasures against staphylococcal biofilms. Some compounds showed anti-biofilm properties at 0.045 μg ml^{-1}, a concentration that can be considered a good starting point for novel potential anti-biofilm agents. Moreover, considering the human cell cytotoxicity, their selectivity indexes are very interesting, ie >1000. A selectivity index value of >200 can be considered a good "safety margin" to select a compound as a candidate for potential therapeutic development as antimicrobial agent (Suto et al. 1992). Nonetheless, further investigations are needed. The authors plan to evaluate and compare the anti-biofilm properties of each compound using different biofilm growth models including animal models to better understand and explain their potential therapeutic use.

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