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# 3,4,5,3′,5′-Pentabromo-2-(2′-hydroxybenzoyl)pyrrole: a potential lead compound as anti-Gram-positive and anti-biofilm agent

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**Abstract**

The activity against Gram-positive bacteria of 3,4,5,3′,5′-pentabromo-2-(2′-hydroxybenzoyl)pyrrole **I**, a synthetic anti-bacterial compound related to pyrrolomycins, was tested in vitro using seven reference bacterial strains and *Staphylococcus epidermidis* and *Staphylococcus aureus* preformed biofilms. Compound **I** was active against all strains tested, with minimum inhibitory concentration (MIC) values ranging from 0.002 to 0.097 mg/l and minimum bactericidal concentrations (MBCs) from 0.37 to 12.5 mg/l. Compound **I** was also active at low concentrations against preformed *S. epidermidis* and *S. aureus* biofilms.


*Keywords:* Gram-positive bacteria; Biofilms; Anti-bacterial activity

## 1. Introduction

Gram-positive bacteria are a significant cause of hospital-acquired and community infections and may induce diseases associated with serious levels of morbidity and mortality. Moreover, antibiotic resistance of important Gram-positive pathogens, such as *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterococcus faecalis* has become one of the major worldwide health problems. The recent appearance in many countries of vancomycin-intermediate resistant (VISA) and vancomycin-resistant *S. aureus* isolates (VRSA) [1] is the latest development in antibiotic resistance. Staphylococcal biofilms play an important role in human pathology because they represent an intrinsic form of resistance to antibiotics and colonize the surface of indwelling medical devices [2].

Biofilm-forming staphylococci cause infections that are very difficult to treat [3], and there is an urgent need to develop new agents to halt these pathogens. One approach is to improve the activity of natural anti-microbial substances by synthesis of analogous compounds. For this reason, we turned our attention to naturally produced organohalogens [4], and in particular to the halogenated pyrroles from natural sources, such as pentabromopseudilin and pyoluteorin [5,6], respectively produced by bacteria *Pseudoalteromonas luteoviolaceus* and *Pseudomonas aeruginosa* and pyrrolomycins [7] produced by some fungi. All these metabolites show good anti-microbial activity especially against Gram-positive bacteria.

We have previously described the synthesis of a series of bioactive brominated compounds related to monodeoxypyoluteorin [8], and we have reported the activity of these compounds against antibiotic sensitive and resistant *S. aureus* clinical strains [9].

In this paper, we report new biological activities of 3,4,5,3′,5′-pentabromo-2-(2′-hydroxybenzoil)pyrrole **I**, which was the most active of the brominated compounds series, against Gram-positive bacteria and as a potential anti-biofilm agent.

## 2. Materials and methods

### 2.1. Synthesis

3,4,5,3′,5′-Pentabromo-2-(2′-hydroxybenzoil)pyrrole **I** (Fig. 1) was synthesized by methods previously described [8].

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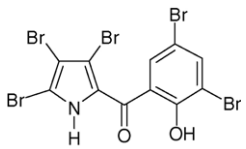


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Fig. 1. 3,4,5,3′,5′-Pentabromo-2-(2′-hydroxybenzoil)pyrrole **I** molecular structure.

### 2.2. Microorganisms

The strains used were *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *S. epidermidis* DSM 3269, *Streptococcus agalactiae* ATCC 13813, *E. faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633 and *Listeria monocytogenes* NCTC 7973.

### 2.3. Determination of minimum inhibitory concentrations (MICs) by broth dilution micro-method

A series of solutions was prepared with a range of concentrations from 50 to 0.001 mg/l (obtained by two-fold serial dilution). The serial dilutions were made in Mueller–Hinton broth (Merck) in a 96-wells plate, starting from a stock solution of 5 mg/ml in 1:1 (v/v) DMF/sodium hydroxide 0.1 M. To each well, 10 μl of a bacterial suspension from a 24 h culture containing $\sim 10^6$ cfu/ml was added [10]. The plate was incubated at 37 °C for 24 h; after this time, the MICs were determined by a microplate reader (ELX 800, Bio-Tek Instruments) as the lowest concentration of compound whose o.d., read at 570 nm, was comparable with the negative control wells (broth only, without inoculum). Vancomycin was used for comparative purposes and quality control of the method.

### 2.4. Determination of minimum bactericidal concentrations (MBCs)

The minimum bactericidal concentration was obtained by subculturing 0.1 ml from each negative well and from the positive control of MIC determination, onto substance-free Mueller–Hinton agar plates. The plates were incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration of substance, which produced subcultures growing no more than three colonies.

### 2.5. Biofilm susceptibility testing, methylthiazotetrazolium (MTT) method

*S. epidermidis* DSM 3269 and *S. aureus* ATCC 29213 were grown and diluted as described previously [11]. After cultivation for 24 h at 37 °C in 96-well plates, the wells were washed three times with 200 μl of sterile phosphate-buffered saline (PBS). The plates were air-dried at 37 °C and to each well was added 100 μl of Mueller–Hinton supplemented with several concentrations, ranging from 1.5 to 0.047 μg/ml, of substance **I**, except in the case of positive (growth) controls. The plates were incubated at 37 °C for 24 h; after this incubation time, the medium was removed, the plates were air-dried and then each well filled with 100 μl of PBS supplemented with 5 μl of a 5 mg/ml MTT (methylthiazotetrazolium) solution and incubated for 1 h at 37 °C. The insoluble purple formazan, obtained by cleavage of MTT made by dehydrogenase enzymes of living cells, was dissolved with a mixture of acidic isopropyl alcohol and Triton X-100 (Sigma). The o.d. of each well was read by a microplate reader (ELX 800, Bio-Tek instruments) at 570 nm with background subtraction at 630 nm. Comparison of the absorbance of positive control wells with absorbance of sample wells enabled the calculation of inhibition percentages of substance **I** at several concentrations.

### 2.6. Biofilms susceptibility testing, crystal violet method

*S. epidermidis* DSM 3269 and *S. aureus* ATCC 29213 were grown, diluted and wells were washed as previously described for the MTT method. The plates were air-dried at 37 °C and to each well was added 100 μl of Mueller–Hinton supplemented with several concentrations, ranging from 1.5 to 0.047 μg/ml, of substance **I**, except in the case of positive controls. The plates were incubated at 37 °C for 24 h; after this incubation time, the medium was removed, the plates were air-dried and then each well was filled with crystal violet solution (0.1%) for 15–20 min. The plate was then washed three times with water, and the crystal violet was dissolved in 150–200 μl of ethanol by pipetting up and down. The plate was read at 570 nm using a microplate reader. Inhibition percentages at several concentrations of substance **I** were obtained by comparing the o.d. of control wells with that of the sample wells.

## 3. Results and discussion

### 3.1. Anti-microbial activity

#### 3.1.1. MIC, MBC and anti-biofilm results

Compound **I** was tested for its in vitro anti-bacterial activity on a group of Gram-positive bacteria.

The anti-bacterial activities of compound **I**, expressed as minimum inhibitory concentration and minimum bactericidal concentration, are shown in Table 1 along with the activity of vancomycin for comparison. Compound **I** was found to be active against all strains tested with MIC values ranging from 0.002 to 0.097 mg/l and MBCs ranging from 0.37 to 12.5 mg/l.

Compound **I** showed considerable activity against a biofilm-positive strain of *S. epidermidis* DSM 3269, with inhibition percentages ranging from 100 to 49.4% or 82 to 46.5% at concentrations ranging from 1.5 to 0.047 mg/l (Fig. 2) using, respectively, MTT or crystal violet method for staining biofilms grown in microtitre plates. Inhibition against *S. aureus* ATCC 29213 biofilm ranged from 89 to 58% at concentrations 1.5–0.047 mg/l using the MTT method or 51.5–28.7% at the same concentrations but using crystal violet method (Fig. 3).

Table 1
MIC and MBC values in vitro expressed in mg/l for all strains tested

| | **I** | | Vancomycin | |
|---|---|---|---|---|
| | MIC | MBC | MIC | MBC |
| *S. aureus* ATCC 29213 | 0.01 | 3.1 | 1 | >8 |
| *S. aureus* ATCC 25923 | 0.005 | 1.5 | 1 | 8 |
| *S. epidermidis* ATCC 12228 | 0.094 | 6.2 | 1 | >8 |
| *S. epidermidis* DSM 3269 | 0.003 | 0.37 | 2 | 4 |
| *E. faecalis* ATCC 29212 | 0.19 | 12.5 | 4 | >8 |
| *S. agalactiae* ATCC 13813 | 0.04 | 1.5 | 1 | 4 |
| *L. monocytogenes* NCTC 7973 | 0.002 | 0.37 | 1 | >8 |
| *B. subtilis* ATCC 6633 | 0.094 | 1.5 | 1 | 2 |

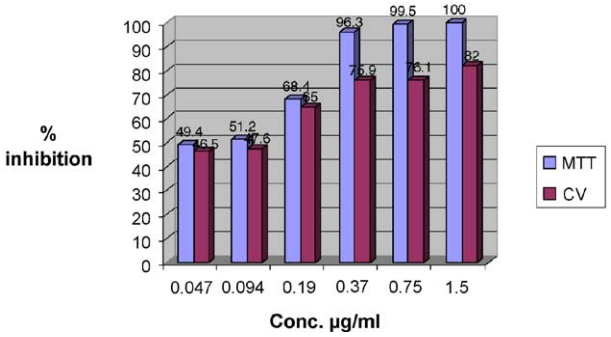


Fig. 2. In vitro percentages inhibition on *S. epidermidis* DSM3269 biofilm. MTT and crystal violet (CV) methods. Values are the average of at least three independent determinations. Coefficient of variation was less than 15%.

This discrepancy in data, depending on the method used for staining biofilms, is due to different information derived from these methods; MTT is a respiratory indicator and detects killing activity by compound **I**, whereas crystal violet staining, as an indicator of total attached biomass, shows removal of bacteria by compound **I**. Compound **I** killed microorganisms in the biofilm rather than removing the biofilm itself.

As far as we know, synthetic analogues of natural halogenated pyrroles have never been tested before as anti-biofilm agents. Although the MIC and MBC values were low for all tested strains, there was low activity against *S. epidermidis* DSM 3269 and *S. aureus* ATCC 29213 biofilms, especially when compared with the MIC values obtained against the planktonic form of these strains. Preformed biofilms were inhibited at 15× MIC or more for *S. epidermidis* DSM 3269 and at 4× MIC or more for *S. aureus* ATCC 29213, although there was good killing on the biofilms at the MBC for planktonic *S. epidermidis* DSM 3269, 0.37 mg/l and at 1.5 mg/l for *S. aureus* ATCC 29213, a concentration just below the MBC (Figs. 2 and 3).

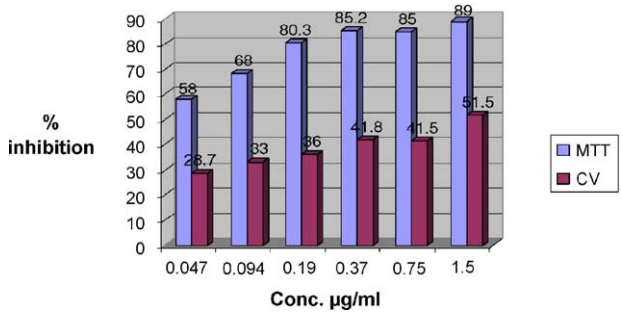


Fig. 3. In vitro percentages inhibition on *S. aureus* ATCC 29213 biofilm. MTT and crystal violet (CV) methods. Values are the average of at least three independent determinations. Coefficient of variation was less than 15%.

In conclusion, these data of the anti-bacterial activity of compound **I**, specifically its activity against bacteria in a protective physiological form intrinsically refractory to antibiotics, such as biofilms, are worthy of interest. Anti-biofilm activity should be considered an important factor in evaluating compounds as candidates for further development stages in anti-microbial research [12]. For this reason, we think that compound **I** might be a potential lead compound in the discovery of new anti-Gram-positive and anti-biofilm agents.

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