SUPPLEMENTARY MATERIAL

Antimicrobial evaluation of selected naturally occurring oxyprenylated secondary metabolites

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Abstract

This study tested the antimicrobial activity of eight selected naturally occurring oxyprenylated secondary metabolites against Staphylococcus aureus ATCC 29213, S. epidermidis ATCC 35984, Escherichia coli ATCC 8739, Pseudomonas aeruginosa ATCC 9027 and Candida albicans ATCC 10231. Results showed a moderate antimicrobial activity. The most active compounds were: 3-(4-geranyloxyphenyl)-1-ethanol (4) and 3-(4-isopentenyloxyphenyl)-1-propanol (5) that were tested on mature and in-formation biofilms of all microorganisms, moreover the cytotoxic activity was evaluated. Except for S. epidermidis, both compounds reduced significantly (p<0.05) the microbial biofilm formation at 1/2 MIC and 1/4 MIC, in particular, compounds 4 and 5 at each concentration, inhibited E. coli biofilm formation to a greater extent, the biofilm formation was never more than 44% respect to the control, moreover both compounds showed a low cytotoxic effect. Oxyprenylated derivatives may be of great interest for the development of novel antimicrobial therapeutic strategies and the synthesis of semi-synthetic analogues with anti-biofilm efficacy.

Keywords: antimicrobial activity; biofilm formation; Gram negative and Gram positive bacteria; oxyprenylated secondary metabolites

Experimental

Compounds

Compounds 1-8 were chemically synthesized as already reported and their purity (>97.5%) assessed by GC/MS and 1H NMR (Epifano et al. 2011).

p-Isopentenyloxybenzaldehyde 1 has been isolated from the essential oil of the leaves of Clausena anisata Hook f. (Rutaceae) (Garneau et al. 2000), geranyloxyvanillin 2 has been obtained from the apolar extracts of Crithmum maritimum L. (Apiaceae) (Cunsolo et al. 1993), 5-dimethoxy-4-isopentenyloxybenzyl alcohol 3 has been extracted as angelic acid ester from the roots of Erechtites hieracifolia (L.) Raf ex DC. (Asteraceae) (Bohllmann and Abraham 1980), 3-(4-geranyloxyphenyl)-1-ethanol 4 is a juvenile hormone of several insect species (Hammock et al. 1973), 3-(4-isopentenyloxyphenyl)-1-propanol 5 has been extracted from the roots of Fagara zanthoxyloides Lam. (Chaaiab et al. 2003) and Zanthoxylum wutaiense Chen
(Rutaceae) (Huang et al. 2008), (2E)-3-(4-((E)7,3,7-Dimethylocta-2,6-dienyloxy)-3-methoxyphenyl)acrylaldehyde 6 has been isolated from the bark of F. rhetza (Roxb.) DC (Fam. Rutaceae) (Shibuya et al. 1992), 4-isopentenyloxeyeugenol 7 has been obtained from Illicium anisatum L. (Illiciaceae) (Shibuya et al. 1978), and finally 4-isopentenyloxyisoeugenol 8 has been isolated from I. verum Hook. f. (Song et al. 2007). The synthesis of compounds 1-8 was accomplished following the already reported methodologies (Epifano et al. 2012).

**Antimicrobial activity**

Two Gram-positive (S. aureus ATCC 29213 and S. epidermidis ATCC 35984) and two Gram-negative (E. coli ATCC 8739 and P. aeruginosa ATCC 9027) strains, and one fungal strain C. albicans ATCC 10231 were used for the evaluation of the antimicrobial activity of the compounds 1-8.

The compounds effect on planktonic cells were evaluated by Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) using the broth microdilution method according to EUCAST guidelines (European Committee for Antimicrobial Susceptibility Testing, 2003). Bacterial suspensions, grown in cation-adjusted Mueller-Hinton II broth (CA-MHB, Becton Dickinson & Co., Cockeisville, Md., USA) at logarithmic phase, were incubated on microtiter plates at a concentration of 5 X 10⁵ CFU ml⁻¹, with several dilutions (50–3200 μg ml⁻¹) for 24 h at 37°C. The MIC was defined as the lowest concentration of substances giving a complete inhibition of visible growth in comparison with a control well, and the MBC was determined as the lowest concentration at which no bacterial growth occurred on Mueller–Hinton Agar (Oxoid, Milan, Italy) plates.

The MICs detection of C. albicans ATCC 10231 was performed using the broth microdilution method according to EUCAST guidelines (Arendrup et al. 2012) in RPMI 1640 plus 2% glucose with a final inoculum of 1-5 X 10⁵ CFU ml⁻¹ for 24–48 h at 37°C. The Minimum Fungicidal Concentration (MFC) was determined as the lowest concentration of substances at which no fungal growth occurred on Sabouraud agar (Oxoid, Milan, Italy) plates.

Data were obtained from at least three independent experiments performed in duplicate. For the internal quality control, S. aureus ATCC 29213, S. epidermidis ATCC 35984 and P. aeruginosa ATCC 9027 were tested against levofloxacin, E. coli ATCC 8739 was tested against amoxicillin and amphotericin B was used against C. albicans ATCC 10231.

**Hemolytic Activity**

Hemolytic activity of the substances was tested against human red blood cells (h-RBCs) (BIOSIGMA S.r.l., Cona, Venice) following the methodology previously described (Baldassarre et al. 2011). Fresh human blood, collected with EDTA, was centrifuged at 3000 rpm for 5 min then washed three times with PBS pH 7.3. Red blood cells were diluted to 4% in PBS and incubated with different substances dilutions ranging from 6400 to 12.5 μg ml⁻¹. After 1 h of incubation at 37°C, the suspensions were sedimented by centrifugation and the release of hemoglobin was determined by absorbance measurement at 405 nm and
compared with a 0% hemolysis control (PBS) and a 100% hemolysis control (PBS with 1% v/v Triton X-100). The percentage of hemolysis was calculated using the following equation:

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\text{Hemolysis (\%)} = \left( \frac{\text{OD}_{405 \text{sample}} - \text{OD}_{405 \text{0\% lysis control}}}{\text{OD}_{405 \text{100\% lysis control}} - \text{OD}_{405 \text{0\% lysis control}}} \right) \times 100.
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**Effect on microbial mature biofilm**

The efficacy on established biofilm was evaluated by determining Biofilm Inhibitory Concentration (BIC) and the Biofilm Eradication Concentration (BEC) according to the method described by Johnson et al. (2002) with some modifications. Bacterial suspensions, grown in Tryptic soy broth (TSB, Oxoid, Milan, Italy) supplemented with 0.5% (v/v) glucose at logarithmic phase as well as *C. albicans* grown in RPMI 1640 plus 2% glucose, were incubated on flat-bottomed microtiter plates at a concentration of 5 X 10^5 CFU ml^-1. After 24 h of incubation at 37°C for bacteria and 48 h at 37°C for *C. albicans*, the planktonic cells were gently removed and the wells were washed with sterile phosphate-buffered saline (PBS) pH 7.3 and filled with compound dilutions ranging from the MIC values to a maximum concentration 16MIC. The OD_{600} was measured at time 0 and after incubation for 24 h at 37°C. The BIC values were determined as the lowest concentrations where no growth occurred in the supernatant fluid, confirmed by no increase in optical density compared with the initial reading. The BEC values were determined as the lowest concentrations at which no bacterial growth occurred on Tryptic Soy Agar for bacteria and on Sabouraud agar for *C. albicans*. Data were obtained from at least three independent experiments performed in duplicate.

**Effect on microbial biofilm formation**

The effect of different concentrations of each compound (ranging from MIC to 1/16MIC) on bacterial and *C. albicans* biofilm-forming ability was tested on polystyrene flat-bottomed microtiter plates, as previously reported (Nostro et al. 2014) with some modifications. Briefly, bacterial cultures were grown overnight in TSB, diluted in TSB plus 0.5% (v/v) glucose to 0·5 McFarland and 100 μl was dispensed into each well of 96-well polystyrene flat-bottomed microtiter plates in the presence of 100 μl subinhibitory concentrations (subMIC) of each compound (diluted in TSB plus 0.5% glucose) or 100 μl TSB plus 0.5% glucose (control). *Candida albicans*, grown in RPMI 1640 (Sigma-Aldrich Co., St Louis,MO, USA) plus 2% glucose, was incubated on flat-bottomed microtiter plates at a concentration of 5 X 10^5 CFU ml^-1 and 100 μl was dispensed into each well of 96-well polystyrene flat-bottomed microtiter plates in the presence of 100 μl subinhibitory concentrations (subMIC) of each compound (diluted in RPMI 1640 plus 2% glucose) or 100 μl RPMI 1640 plus 2% glucose (control). After incubation for 24 h at 37°C for bacteria and 48 h at 37°C for *C. albicans*, each well was washed twice with sterile PBS (pH 7.4), dried, stained for 1 min with 0.1% safranin and washed with PBS. The stained biofilms were resuspended in 200 μl ethanol and OD_{492} was measured by spectrophotometry using an ELISA reader.

Each assay was performed in duplicate for three independent experiments.
**Statistical analyses**

All obtained data were summarized using the mean and standard deviation. The *t*-Student test was performed to reveal the statistical significance. For all statistical tests, a *p* value <0.05 was considered to be as statistically significant.

**Supplementary Results**

**Table S1** Antimicrobial activity of compounds 1–8, expressed in μg ml⁻¹ against planktonic microbial strains

| Derivatives | *S. aureus* ATCC 29213 | *S. epidermidis* ATCC 35984 | *E. coli* ATCC 8739 | *P. aeruginosa* ATCC 9027 | *C. albicans* ATCC 10231 |
|-------------|-------------------------|-----------------------------|---------------------|---------------------------|------------------------|
|             | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** |
| 1           | > 3200 | > 3200 | > 3200 | > 3200 | > 3200 | > 3200 | > 3200 | > 3200 | 200    | 3200    |
| 2           | 400    | 800    | 800    | 3200    | 3200    | > 3200 | > 3200 | > 3200 | > 3200 | 200    | 400    |
| 3           | 3200   | > 3200 | 3200   | > 3200 | 3200   | > 3200 | 3200   | > 3200 | > 3200 | 200    | 1600   |
| 4           | 400    | 800    | 400    | 800    | 200    | 400    | 200    | 400    | 100    | 200    |
| 5           | 400    | 800    | 400    | 800    | 200    | 400    | 200    | 400    | 100    | 100    |
| 6           | 3200   | > 3200 | 800    | 3200   | 1600   | > 3200 | 1600   | > 3200 | > 3200 | 200    | 400    |
| 7           | 1600   | 3200   | 400    | 3200   | 1600   | 1600   | 800    | 1600   | 200    | 800    |
| 8           | 1600   | 1600   | 800    | 1600   | 1600   | 1600   | 3200   | 800    | 1600   | 100    | 100    |

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; MFC: Minimum Fungicidal Concentration.

Internal Quality Control: *S. aureus* ATCC 29213, Levofloxacin MIC: 0.24 μg ml⁻¹; *S. epidermidis* ATCC 35984, Levofloxacin MIC: 0.12 μg ml⁻¹; *E. coli* ATCC 8739, Amoxicillin MIC: 16 μg ml⁻¹; *P. aeruginosa* ATCC 9027, Levofloxacin MIC: 1 μg ml⁻¹; *C. albicans* ATCC 10231, AmphotericinB MIC: 0.12 μg ml⁻¹.

**Table S2** Effect of compounds 4 and 5 on preformed biofilm against planktonic microbial strains (values are expressed in μg ml⁻¹)

| Derivatives | *S. aureus* ATCC 29213 | *S. epidermidis* ATCC 35984 | *E. coli* ATCC 8739 | *P. aeruginosa* ATCC 9027 | *C. albicans* ATCC 10231 |
|-------------|-------------------------|-----------------------------|---------------------|---------------------------|------------------------|
|             | **BIC** | **BEC** | **BIC** | **BEC** | **BIC** | **BEC** | **BIC** | **BEC** | **BIC** | **BEC** |
| 4           | 1600    | > 3200 | 1600   | > 3200 | 3200    | > 3200 | 3200    | > 3200 | 800    | > 3200 |
| 5           | 3200    | > 3200 | 3200   | > 3200 | > 3200  | > 3200 | > 3200  | > 3200 | 1600   | > 3200 |

BIC: Biofilm Inhibitory Concentration; BEC: Biofilm Eradication Concentration
Figure S2. Dose–response curve of hemolytic activity of compounds 4 and 5 against h-RBCs
Figure S3. Effect of compounds 4 and 5 on microbial biofilm formation
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