

## 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-isopentenylloxyphenyl)-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 <sup>1</sup>H NMR (Epifano et al. 2011).

*p*-Isopentenylloxybenzaldehyde **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-isopentenylloxybenzyl alcohol **3** has been extracted as angelic acid ester from the roots of *Erechtites hieracifolia* (L.) Raf ex DC. (Asteraceae) (Bohlmann and Abraham 1980), 3-(4-geranyloxyphenyl)-1-ethanol **4** is a juvenile hormone of several insect species (Hammock et al. 1973), 3-(4-isopentenylloxyphenyl)-1-propanol **5** has been extracted from the roots of *Fagara zanthoxyloides* Lam. (Chaaib et al. 2003) and *Zanthoxylum wutaiense* Chen

(Rutaceae) (Huang et al. 2008), (2*E*)-3-(4-((*E*)-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-isopentenylxyeugenol **7** has been obtained from *Illicium anisatum* L. (Illiciaceae) (Shibuya et al. 1978), and finally 4-isopentenylxyisoeugenol **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., Cockeysville, Md., USA) at logarithmic phase, were incubated on microtiter plates at a concentration of  $5 \times 10^5$  CFU ml<sup>-1</sup>, with several dilutions (50–3200 µg ml<sup>-1</sup>) 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  $1-5 \times 10^5$  CFU ml<sup>-1</sup> 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<sup>-1</sup>. 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:

Hemolysis (%) = [(OD<sub>405</sub> sample - OD<sub>405</sub>0% lysis control)/(OD<sub>405</sub>100% lysis control - OD<sub>405</sub>0% lysis control)] X 100.

### ***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<sup>5</sup> CFU ml<sup>-1</sup>. 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<sub>600</sub> 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<sup>5</sup> CFU ml<sup>-1</sup> 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<sub>492</sub> 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  $\mu\text{g ml}^{-1}$  against planktonic microbial strains

Derivatives	<i>S. aureus</i>		<i>S. epidermidis</i>		<i>E. coli</i>		<i>P. aeruginosa</i>		<i>C. albicans</i>	
	ATCC 29213		ATCC 35984		ATCC 8739		ATCC 9027		ATCC 10231	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
<b>1</b>	> 3200	> 3200	> 3200	> 3200	> 3200	> 3200	> 3200	> 3200	200	3200
<b>2</b>	400	800	800	3200	3200	> 3200	3200	> 3200	200	400
<b>3</b>	3200	> 3200	3200	> 3200	3200	> 3200	> 3200	> 3200	200	1600
<b>4</b>	400	800	200	800	200	400	200	400	100	200
<b>5</b>	400	800	400	400	200	400	200	400	100	100
<b>6</b>	3200	> 3200	800	3200	1600	> 3200	1600	> 3200	200	400
<b>7</b>	1600	3200	400	3200	1600	1600	800	1600	200	800
<b>8</b>	1600	1600	800	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:  $0.24\mu\text{g ml}^{-1}$ ; *S. epidermidis* ATCC 35984, Levofloxacin MIC:  $0.12\mu\text{g ml}^{-1}$ ; *E. coli* ATCC 8739, Amoxicillin MIC:  $16\mu\text{g ml}^{-1}$ ; *P. aeruginosa* ATCC 9027, Levofloxacin MIC:  $1\mu\text{g ml}^{-1}$ ; *C. albicans* ATCC 10231, AmphotericinB MIC:  $0.12\mu\text{g ml}^{-1}$ .

**Table S2** Effect of compounds **4** and **5** on preformed biofilm against planktonic microbial strains (values are expressed in  $\mu\text{g ml}^{-1}$ )

Derivatives	<i>S. aureus</i>		<i>S. epidermidis</i>		<i>E. coli</i>		<i>P. aeruginosa</i>		<i>C. albicans</i>	
	ATCC 29213		ATCC 35984		ATCC 8739		ATCC 9027		ATCC 10231	
	BIC	BEC	BIC	BEC	BIC	BEC	BIC	BEC	BIC	BEC
<b>4</b>	1600	> 3200	1600	> 3200	3200	> 3200	3200	> 3200	800	> 3200
<b>5</b>	3200	> 3200	3200	> 3200	> 3200	> 3200	> 3200	> 3200	1600	> 3200

BIC: Biofilm Inhibitory Concentration; BEC: Biofilm Eradication Concentration

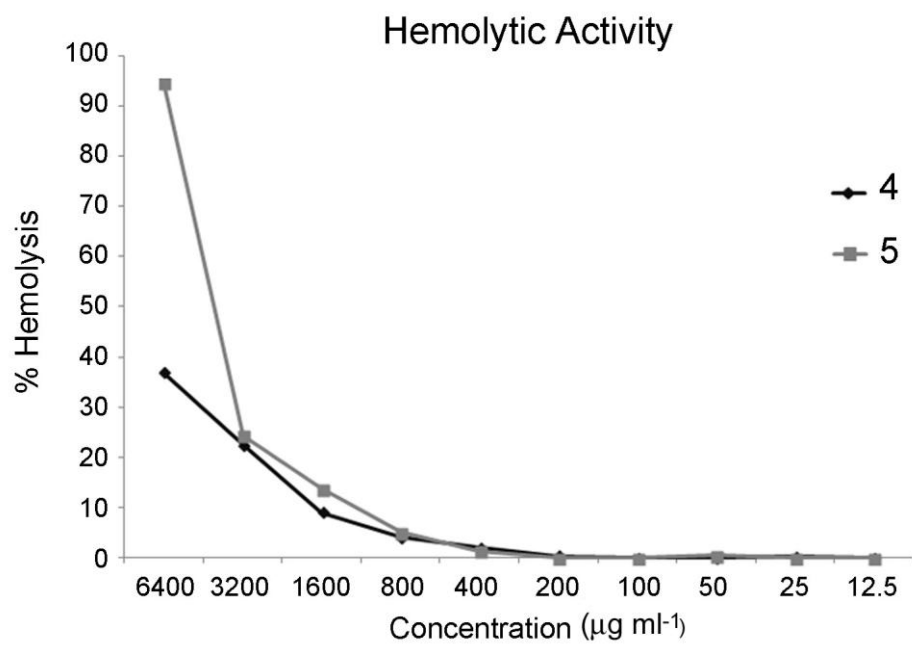


Figure S2. Dose–response curve of hemolytic activity of compounds **4** and **5** against h-RBCs

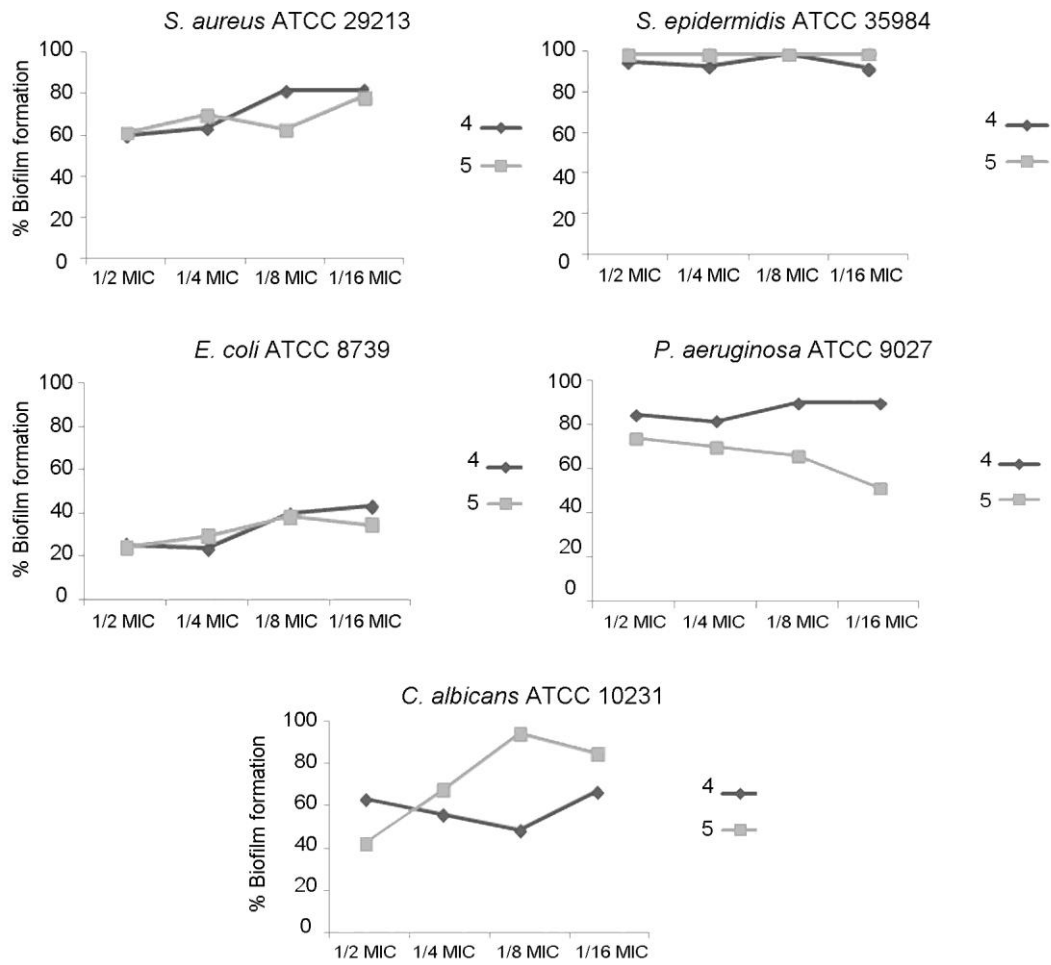


Figure S3. Effect of compounds 4 and 5 on microbial biofilm formation

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