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# Effective biosynthesis of benzoyl-pyrrothine dithiopyrrolone antibiotic by cinnamic acid-precursor addition in culture of *Saccharothrix algeriensis* NRRL B-24137

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**Significance and Impact of the Study:** Dithiopyrrolone antibiotics, known for their strong antimicrobial activities, gained greater interest after the discovery of their antitumor properties. Depending on precursors added, *Saccharothrix algeriensis* NRRL B-24137 has the ability to produce several dithiopyrrolones derivatives. Since biological activities of dithiopyrrolones are related to their variable structure, discover of new natural analogues to be therapeutically explored remains a significant framework of research. In this study, a new dithiopyrrolone derivative was purified from the fermentation broth of *S. algeriensis* NRRL B-24137. This new antibiotic, characterized as benzoyl-pyrrothine dithiopyrrolone, was induced by adding cinnamic acid, as precursor, to a semi-synthetic medium.

## Keywords

antimicrobial activity, cinnamic acid, dithiopyrrolone antibiotics, precursor, *Saccharothrix algeriensis*.

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## Abstract

Dithiopyrrolone antibiotics, produced by several micro-organisms, are known for their strong antimicrobial and antitumor activities. Among of this micro-organisms, *Saccharothrix algeriensis* NRRL B-24137, a rare actinobacterium, has the ability to produce several dithiopyrrolones derivatives depending on precursors added in the culture medium. After 10 days of strain fermentation on semi-synthetic medium supplemented with cinnamic acid and HPLC purification, biosynthesis of benzoyl-pyrrothine dithiopyrrolone was evidenced through complete spectroscopic (UV-visible and <sup>1</sup>H and <sup>13</sup>C NMR) and spectrometric (electron impact mass spectrum) analyses. The pure molecule showed appreciable minimum inhibitory concentration values against several Gram-positive bacteria and filamentous fungi.

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## Introduction

Dithiopyrrolones are a group of antibiotics that possess the unique pyrrolinodithiole (4H-[1,2] dithiolo [4,3-b] pyrrol-5-one) skeleton linked to two variable acyl groups (Jiang *et al.* 2012). Because of their strong activities against a variety of Gram-positive and Gram-negative bacteria, eukaryotic micro-organisms (yeast, filamentous fungi and amoeboid parasites) and tumour cells, dithiopyrrolone derivatives gained great interest (Webster *et al.* 2002; Li

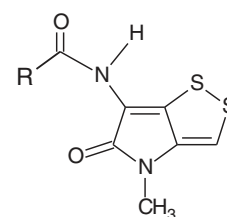
*et al.* 2014; Merrouche *et al.* 2017). To date, approximately 30 naturally occurring dithiopyrrolone compounds were listed and divided into three subgroups: N-methyl, N-acylpyrrothine (pyrrothine type), N-acylpyrrothine (holomycin type) and thiomarinol (Qin *et al.* 2013). Holomycin appeared to be active against rifamycin-resistant bacteria as well as against methicillin-resistant *Staphylococcus aureus* N315 (Qin *et al.* 2013) and thiolutin potently inhibits developmental of angiogenesis in zebrafish and vascular outgrowth from tissue explants in 3D cultures (Jia *et al.* 2010).

In the context of screening novel dithiopyrrolones-producing micro-organisms, *Saccharothrix algeriensis* NRRL B-24137, a rare actinobacterium isolated from the soil of a palm grove in Southern Algeria (Lamari *et al.* 2002a) and published as a novel species by Zitouni *et al.* (2004), was shown to commonly produce in complex ISP2 broth (glucose-yeast extract–malt extract) (Shirling and Gottlieb 1966) at least five dithiopyrrolone derivatives characterized by their different N-acyl groups, namely acetyl-pyrrothine (thiolutin), senecieryl-pyrrothine, tigloyl-pyrrothine, isobutyrylpyrrothine and butanoyl-pyrrothine (Fig. 1) (Lamari *et al.* 2002a,b). Furthermore, the addition of amino acids and organic acids as precursors to the basal semi-synthetic (SS) medium containing glucose, yeast extract and several mineral sources permitted to modify the production levels of this known dithiopyrrolones (Bouras *et al.* 2006a,b, 2007). The so called precursor directed biosynthesis (PDB) method also permitted the production of several uncommon new analogues, among which seven of them were fully characterized by mass and RMN (Fig. 1) (Bouras *et al.* 2008; Merrouche *et al.* 2010, 2011). In fact, addition of valeric acid in the culture medium induced the production of three new dithiopyrrolone derivatives: formyl-pyrrothine, valeryl-pyrrothine and iso-valeryl-pyrrothine (Merrouche *et al.* 2010). Likewise, addition of sorbic acid allowed formation of four new dithiopyrrolone derivatives: crotonyl-pyrrothine, sorbyl-pyrrothine, 2-hexonyl-pyrrothine and 2-methyl-3-pentenyl-pyrrothine (Merrouche *et al.* 2011).

Thus, depending on precursors added, which determine the activated organic acid (acyl-CoA) type incorporated into the pyrrothine nucleus, *S. algeriensis* has the ability to produce several dithiopyrrolones derivatives. The transfert of the acyl group from acyl-CoA to pyrrothine core during dithiopyrrolone biosynthesis was determined as linked to enzymatic activity of pyrrothine N-acyltransferase (Chorin *et al.* 2009; Saker *et al.* 2013).

Since biological activities of dithiopyrrolones are related to their variable acyl groups, the obtained structurally novel analogues could lead to discover new biologically active natural products that remain to be therapeutically exploited (Oliva *et al.* 2001; Li *et al.* 2007, 2014).

In this work, through spectroscopic and spectrometric analyses, biosynthesis of benzoyl-pyrrothine antibiotic (PR5 compound) was evidenced after addition of cinnamic acid in the culture medium of *S. algeriensis*. After subsequent production, purification and chemical characterization, the benzoyl-pyrrothine minimum inhibitory concentrations (MICs) were evaluated towards several human and plant pathogenic micro-organisms.



R: CH <sub>3</sub>	Acetyl-pyrrothine (thiolutin)
R: CH(CH <sub>3</sub> ) <sub>2</sub>	Iso-butyryl-pyrrothine
R: (CH <sub>2</sub> ) <sub>2</sub> -CH <sub>3</sub>	Butanoyl-pyrrothine
R: CH=C(CH <sub>3</sub> ) <sub>2</sub>	Senecieryl-pyrrothine
R: C(CH <sub>3</sub> )=CH(CH <sub>3</sub> )	Tigloyl-pyrrothine
R: CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	Valeryl-pyrrothine*
R: CH(CH <sub>3</sub> )(CH <sub>2</sub> CH <sub>3</sub> )	Isovaleryl-pyrrothine*
R: H	Formyl-pyrrothine*
R: CH=CH(CH <sub>3</sub> )	Crotonyl-pyrrothine**
R: CH=CH-CH=CH(CH <sub>3</sub> )	Sorbyl-pyrrothine**
R: CH=CH-CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	2-Hexonyl-pyrrothine**
R: CH=C(CH <sub>3</sub> )(CH <sub>2</sub> CH <sub>3</sub> )	2-Methyl-3-pentenyl-pyrrothine**

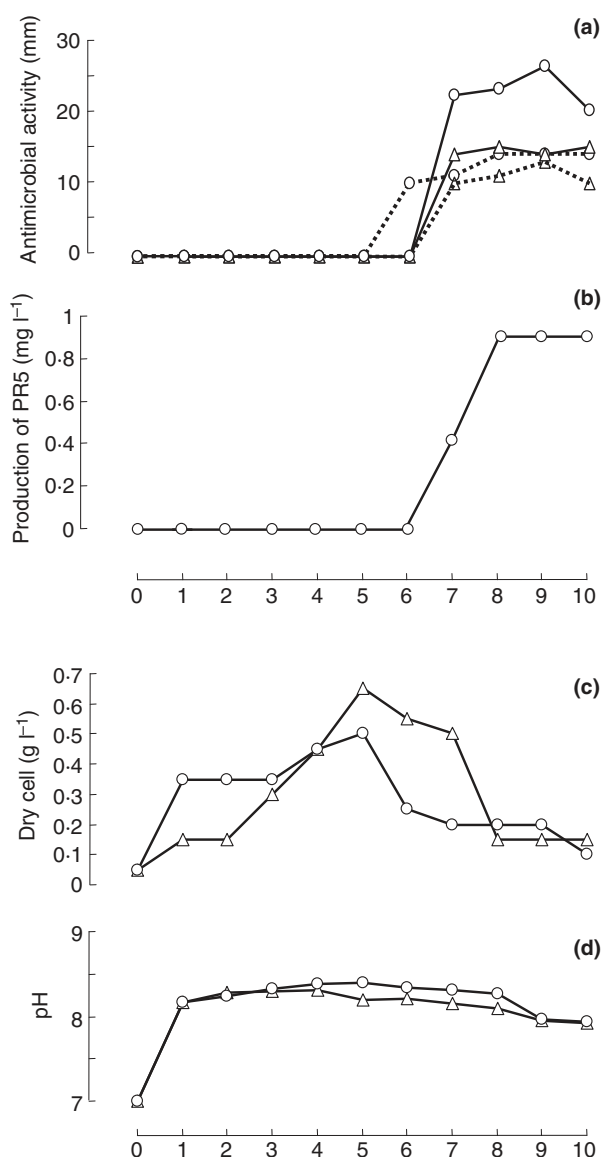
**Figure 1** Structure of characterized dithiopyrrolone antibiotics produced by *Saccharothrix algeriensis* NRRL B-24137. \*Dithiopyrrolones induced by adding valeric acid to semi-synthetic (SS) medium. \*\*Dithiopyrrolones induced by adding sorbic acid to SS medium. The other antibiotics are produced in both ISP2 and SS media with or without addition of valeric or sorbic acids.

## Results and discussion

### Effect of cinnamic acid addition on kinetic of growth, antibiotics production and antimicrobial activity in *S. algeriensis*

During the time course of fermentation in SS medium supplemented with cinnamic acid or not (control), antibiotics production, dry cell weight (DCW) and pH parameters were monitored (Fig. 2) along with the analysis of produced antibiotics (Fig. 3).

The overall antimicrobial activity expressed by the actinobacterium strain towards *Bacillus subtilis* ATCC 6633 and *Umbelopsis ramanniana* NRRL1829 started at the 7th day of fermentation (except against *B. subtilis* in presence of cinnamic acid which started at the 6th day) and reached a maximum between the 8th to the 10th



**Figure 2** Effect of cinnamic acid addition to the semi-synthetic medium on evolution of: (a) antimicrobial activity; (b) production of a new induced dithiopyrrolone (PR5); (c) biomass and (d) pH. (—) *Umbeopsis ramanniana* NRRL1829; (••••) *Bacillus subtilis* ATCC 6633; (Δ) SSM without adding cinnamic acid (control) and (○) SSM with cinnamic acid at  $5 \times 10^{-3}$  mol.

day (Fig. 2a). The production of compound PR5 also started at the 7th day and reached a maximum after 8 to 10 days of fermentation, with a production of  $0.93 \text{ mg l}^{-1}$  observed during the decline phase (Fig. 2b). This supplementation also triggered modification in biomass kinetic in comparison to control (Fig. 2c). The pH increased rapidly to an alkaline state after 24 h of culture (Fig. 2d).

The HPLC profile shown in Fig. 3a indicated that *S. algeriensis* NRRL B-24137 produces five known dithiopyrrolones (thiolutin, iso-butyryl-pyrrothine, butanoyl-pyrrothine, tigloyl-pyrrothine and seneciroyl-pyrrothine) in the basal SS medium (control without addition of precursors) as reported by Bouras *et al.* (2008). Importantly, addition of cinnamic acid to the SS medium induced the production of a new compound named PR5 at a retention time of 19.09 min (Fig. 3b). These findings were in agreement with previous studies, which have shown for all *S. algeriensis*-synthesized dithiopyrrolones (common or PDB induced) an optimal production during the idiophase with alkaline pH condition (Lamari *et al.* 2002a; Bouras *et al.* 2008; Merrouche *et al.* 2011).

Globally, *S. algeriensis* exhibited better antimicrobial activity after addition of cinnamic acid than the control (without cinnamic acid) with a greater anti-*U. ramanniana* activity (Fig. 2a). This was probably due to the effect of cumulative production of PR5 with the other known dithiopyrrolones.

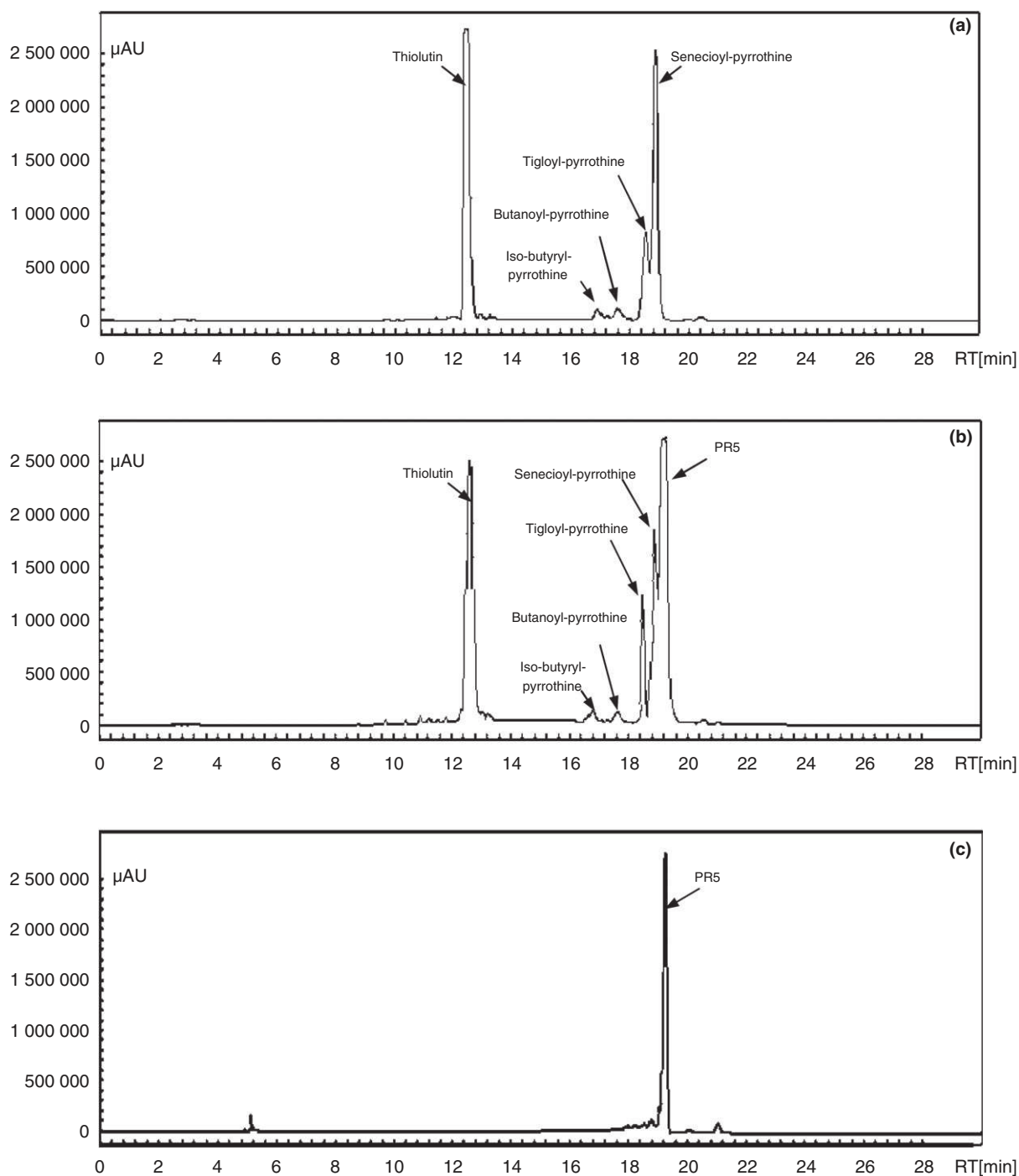
#### Isolation and purification of induced PR5 compound

The cinnamic acid-supplemented SS culture broth of 10 days (12 l) was extracted by dichloromethane and the yellow organic phase was concentrated to dryness. The analysis by semi-preparative HPLC showed the presence of the compound PR5, in addition to the other four dithiopyrrolones (iso-butyryl-pyrrothine, butanoyl-pyrrothine, seneciroyl-pyrrothine and tigloyl-pyrrothine). The compound PR5, which was purified after two successive re-injections in the HPLC system, appeared yellow and exhibited significant antimicrobial activity against *U. ramanniana* and *B. subtilis*. This induced compound was subject to spectroscopic and spectrometric analyses to elucidate its structure.

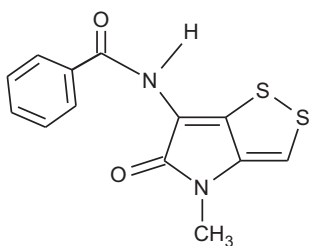
#### Elucidation of the structure of induced compound PR5

The UV-visible spectrum of the induced compound PR5 showed three absorption maxima at 207, 308 and 398 nm. The molecular weight of PR5 is  $M = 290$  (Fig. S1).

The <sup>1</sup>H and <sup>13</sup>C NMR chemical shifts of the compound were as follow: <sup>1</sup>H NMR (CD<sub>2</sub>Cl<sub>2</sub>, 500 MHz) δ 8.20 (1H, br s, N7-H), δ 7.92 (2H, m, H10), δ 7.54 (2H, m, H11), δ 7.63 (1H, tt, J = 7.4, J = 1.3, H12), δ 6.75 (1H, s, H3), δ 3.40 (3H, s, NCH<sub>3</sub>); <sup>13</sup>C NMR (CD<sub>2</sub>Cl<sub>2</sub>, 125 MHz) δ 166.7 (C, C5), δ 164.6 (C, C8), δ 137.1 (C, C3a), δ 132.8 (C, C9), δ 132.4 (C, C6a), δ 132.4 (CH, C12), δ 128.8 (CH, C11), δ 127.2 (CH, C10), δ 108.8 (CH, C3), and δ 27.6 (CH<sub>3</sub>, NCH<sub>3</sub>). Carbon C6 was not detected on the



**Figure 3** Effect of cinnamic acid addition to the semi-synthetic medium on dithiopyrrolones production. (a) Under standard conditions for dithiopyrrolone production (control). (b) Addition of cinnamic acid to the fermentation broth. (c) Purification of the new compound PR5. HPLC analysis at 390 nm was done on crude extract from a 8 days-old *Saccharothrix algeriensis* culture. Formation of dithiopyrrolone analogs was monitored by comparison of the peak retention times and UV (390 nm) spectra with those of known dithiopyrrolone standards. The retention times were as follows: thiolutin, 12.50 min; iso-butyryl-pyrrothine, 16.80 min; butanoyl-pyrrothine, 17.80 min; tigloyl-pyrrothine, 18.50 min; senecieryl-pyrrothine, 18.80 min; PR5, 19.09 min.



**Figure 4** Structure of dithiopyrrolone PR5 induced by adding cinnamic acid.

HMBC experiment (see Fig. 4 for numbering of hydrogen and carbon atoms). Through  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral features it is possible to discern two carbonyl groups ( $\delta_c$  166.7/171.5 and  $\delta_c$  164.6/166.8), one olefinic group ( $\delta_H$  6.75/6.68 and  $\delta_c$  108.8/108.0), one N-CH<sub>3</sub> group ( $\delta_H$  3.40/3.35 and  $\delta_c$  27.6/27.7) and one NH group ( $\delta_H$  7.92/7.50). In addition, compound PR5 presents two  $\text{sp}^2$ -hybridized quaternary carbons signal ( $\delta_c$  137.1 and 132.4). These  $^1\text{H}$  and  $^{13}\text{C}$  signals are typical of dithiopyrrolone derivatives. Furthermore, compound PR5 show additional  $^1\text{H}$  and  $^{13}\text{C}$  signals typical of a benzoyl group ( $\delta_H$  7.54 (2H), 7.62 (1H) and 7.93 (2H);  $\delta_c$  132.8 (quaternary), 132.4 (CH), 128.8 (CH) and 127.2 (CH)) that was confirmed with the 2D  $^1\text{H}$ - $^1\text{H}$  and  $^1\text{H}$ - $^{13}\text{C}$  experiments. The dithiopyrrolone PR5 was identified as a benzoyl-pyrrothine (Fig. 4). The details of the NMR data of the PR5 compound are found in the supporting information (Figs S2–S6).

Through partial mass spectrum (MS) analysis, Bouras *et al.* (2008) have already hypothesized a possible production of benzoyl-pyrrothine by using benzoic acid and cinnamic acid in the culture medium of *S. algeriensis* NRRL B-24137 but this putatively deduction was never confirmed. These authors also found that the production of this compound was significantly enhanced by addition of cinnamic acid in comparison to benzoic acid.

Most of the isolated dithiopyrrolones are carboxyl-pyrrothine analogues of the added carboxylic acids but additionally, some unexpected dithiopyrrolones were obtained as a result of biotransformation and the operation of new biosynthesis pathways (Merrouche *et al.* 2010, 2011). In our study, the addition of cinnamic acid induced production of benzoyl-pyrrothine. As a whole, these findings suggested a transformation of cinnamic acid to benzoic acid that led to benzoyl-pyrrothine production. This result agrees with those of Brunati *et al.* (2004), who reported the same observation about biotransformation of cinnamic acid to benzoic acid by some strain of actinomycetes. Conversion of cinnamic acid to benzoyl-CoA has been reported for the first time in *Streptomyces maritimus* (Noda *et al.* 2012); this may

explain the stimulation of benzoyl-pyrrothine production in *S. algeriensis* when SS medium was supplemented by cinnamic acid. Furthermore, other works have suggested an enzymatic activity catalysing the incorporation of cyclic acyl group to the pyrrothine core responsible for a putative formation of benzoyl-pyrrothine in *S. algeriensis* (Chorin *et al.* 2009; Saker *et al.* 2013). However, these results were never confirmed since no study with RMN evidences has resulted in the final structure of the compound.

### Minimum inhibitory concentrations

The MICs values of the pure antibiotic compound PR5 are shown in Table 1. The antibiotic PR5 exhibited moderate activity against fungi, yeasts and Gram-positive bacteria tested (except *Staphylococcus aureus*, MIC > 100  $\mu\text{g ml}^{-1}$ ) with MICs ranging from 20 to 40  $\mu\text{g ml}^{-1}$ . *Listeria monocytogenes* was the most sensitive (MIC = 4  $\mu\text{g ml}^{-1}$ ). However, the compound PR5 showed no activity against Gram-negative bacteria (MIC > 100  $\mu\text{g ml}^{-1}$ ). Similar antimicrobial spectrum has been observed with other *S. algeriensis*-produced dithiopyrrolones derivatives (Lamari *et al.* 2002a; Merrouche *et al.* 2010, 2011).

In conclusion, from the results presented in this paper, effective *S. algeriensis*-induced biosynthesis of benzoyl-pyrrothine dithiopyrrolone, that displayed antibacterial activities, was evidenced by adding cinnamic acid in the culture medium as precursor.

**Table 1** Minimum inhibitory concentrations (MICs) of dithiopyrrolone antibiotic PR5 produced by *Saccharothrix algeriensis*

Target micro-organisms	MIC ( $\mu\text{g ml}^{-1}$ )
<i>Bacillus subtilis</i> (ATCC 6633)	40
<i>Bacillus coagulans</i> (CIP 6625)	20
<i>Listeria monocytogenes</i> (CIP 82110)	4
<i>Micrococcus luteus</i> (ATCC 9314)	30
<i>Staphylococcus aureus</i> (CIP 7625)	>100
<i>Agrobacterium tumefaciens</i> (no. 2410 LB)	>100
<i>Escherichia coli</i> (ATCC 10536)	>100
<i>Klebsiella pneumoniae</i> (CIP 82.91)	>100
<i>Salmonella enterica</i> (CIP 81.3)	>100
<i>Pseudomonas aeruginosa</i> (CIPA22)	>100
<i>Aspergillus carbonarius</i> (M333)	40
<i>Fusarium oxysporum</i> f. sp. <i>lini</i> (Foln 3)	40
<i>Fusarium moniliforme</i> (Fm1)	40
<i>Fusarium equiseti</i> (Fe1)	20
<i>Fusarium culmorum</i> (Fc1)	30
<i>Fusarium graminearum</i> (Fg1)	40
<i>Umbelopsis ramanniana</i> (NRRL 1829)	20
<i>Penicillium expansum</i> (Pe1)	20
<i>Candida albicans</i> (IPA 200)	30
<i>Saccharomyces cerevisiae</i> (ATCC 4226)	20

## Materials and methods

### Producing strain

The actinobacterium *S. algeriensis* NRRL B-24137 used throughout this study was grown and maintained at 4°C on slants of ISP (International *Streptomyces* Project) medium 2 composed of (per litre of distilled water): 4 g glucose, 10 g malt extract, 4 g yeast extract, 18 g agar and pH 7.

### Culture condition

Fermentation was carried out in SS medium for antibiotic production by strain *S. algeriensis* NRRL B-24137. This medium consisted of (per litre of distilled water): 10 g glucose (Fisher Labosi, Elancourt, France), 2 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Prolabo, Paris, France), 2 g NaCl (Fisher Labosi), 0.5 g KH<sub>2</sub>PO<sub>4</sub> (Acros, Geel, Belgium), 1 g K<sub>2</sub>HPO<sub>4</sub> (Acros), 0.2 g MgSO<sub>4</sub>·7H<sub>2</sub>O (Acros), 5 g CaCO<sub>3</sub> (Prolabo) and 2 g yeast extract (Difco, Detroit, MI). The 500-ml Erlenmeyer flasks containing 100 ml of medium were inoculated with 3 ml of a preculture broth of strain *S. algeriensis* prepared with the same medium and incubated at 30°C for 2 days. The initial pH of the medium was adjusted to 7 using 2 mol l<sup>-1</sup> NaOH prior to autoclaving. The cinnamic acid (Fluka, Buchs, Switzerland), at a concentration of 5 × 10<sup>-3</sup> mol, was supplied to the basal SS medium prior inoculation. The cultures were incubated on a rotary shaker (240 rev min<sup>-1</sup>) at 30°C for 10 days.

### Assessment of pH, DCW, antibiotics production and antimicrobial activity

During the 10-days fermentation time course, changes in pH, DCWs of mycelium, and antibiotic production were examined daily. The pH value of fermentation broth was measured with a pH meter (Consort C 832; Consort, New York, NY). DCWs were determined as described by Bouras *et al.* (2006a) and expressed as gram per litre. The analysis of antibiotics induced by addition of cinnamic acid (at 5 × 10<sup>-3</sup> mol) in the SS medium was carried out by a Waters HPLC system equipped with a C18 reverse phase column (Uptisphere UP5ODB, 150 × 4.6 mm; BioTek Instruments, Milan, Italy). The samples were analysed as described by Lamari *et al.* (2002b) and Bouras *et al.* (2006a). Briefly, the formation of new dithiopyrrolone analog was monitored by comparison of the peak retention times and UV spectra with those of known dithiopyrrolone standards since appearing dithiopyrrolone products could be easily detected by HPLC analysis due to the intense absorption

at 390 nm. Quantification of PR5 compound was performed using thiolutin standard calibration curve. The molar extinction coefficient ( $\epsilon$ ) of thiolutin is nearly the same for all others dithiopyrrolones ( $\epsilon_{390} = 8317\text{--}9333 \text{ l mol}^{-1} \text{ cm}^{-1}$ ) as described by Lamari *et al.* (2002b).

Concomitantly, the antimicrobial activity in the culture broth was monitored daily during the 10-days fermentation time by the conventional agar diffusion assay (well technique) using *B. subtilis* ATCC 6633 and *U. ramanniana* NRRL1829. Each 10-mm-diameter well was filled with 0.2 ml of supernatant. All tests were repeated two times from two separate cultures.

### Extraction and purification of new induced antibiotic

Repeated fermentations (10 days) were carried out to obtain a total of 12 l of culture broth then purified according the procedure described earlier (Merrouche *et al.* 2011).

Briefly, the culture broth was centrifuged to remove the biomass and extracted with dichloromethane (v/v) on the tenth day of fermentation (determined through the kinetic assay as the day of optimal production of new compound). The organic extract was concentrated to dryness. The crude extract was dissolved in methanol and subjected to semi-preparative HPLC purification on a Waters system using a C18 column (UP5ODB, 250 × 7.8 mm; Waters, Milford, MA). A linear gradient of methanol–water (50–100% for 30 min) was used as the mobile phase. The elution rate was 1.5 ml min<sup>-1</sup> and the detection was carried out at 390 nm.

### Spectroscopy and spectrometry of new induced antibiotic

These analyses were made with the pure antimicrobial compound PR5. The UV spectrum was determined with a Shimadzu UV1605 spectrophotometer (Shimadzu, Kyoto, Japan). The molecular weight of the compound was obtained by electron impact MS recorded at 70 eV with a Nermag R-10-10C spectrometer. NMR sample was prepared by dissolving the pure molecule PR5 in 600  $\mu$ l of CD<sub>2</sub>Cl<sub>2</sub>. 1D and 2D <sup>1</sup>H and <sup>13</sup>C experiments were recorded on a Bruker Avance 500 spectrometer equipped with a 5 mm triple resonance inverse Z-gradient probe (TBI <sup>1</sup>H, <sup>31</sup>P, BB). All chemical shifts for <sup>1</sup>H and <sup>13</sup>C are relative to TMS using <sup>1</sup>H (residual) or <sup>13</sup>C chemical shifts of the solvent as a secondary standard. The temperature was set at 298 K. All the <sup>1</sup>H and <sup>13</sup>C signals were assigned on the basis of chemical shifts, spin-spin coupling constants, splitting patterns and signal intensities, and by using <sup>1</sup>H-<sup>1</sup>H COSY45, <sup>1</sup>H-<sup>13</sup>C HSQC and <sup>1</sup>H-<sup>13</sup>C HMBC experiments.



## MICs of new induced antibiotic

The MICs of the purified dithiopyrrolone antibiotic were estimated by the conventional agar dilution method (Oki *et al.* 1990) towards a selection of 20 target micro-organisms. These micro-organisms were inoculated onto nutrient agar medium containing different concentrations of active compounds (1, 2, 3, 5, 8, 10, 15, 20, 25, 30, 40, 50, 60, 80 and 100  $\mu\text{g ml}^{-1}$ ). The antimicrobial activity was observed after 24–48 h incubation at 37°C for bacteria and 48–72 h incubation at 28°C for fungi and yeasts. Medium without active compound and inoculated with target micro-organisms was used as control.

## Conflict of Interest

The authors declare no conflict of interest.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

**Figure S1** Electron impact mass spectrum of PR5 compound.

**Figure S2**  $^1\text{H}$  NMR of PR5 compound.

**Figure S3**  $^{13}\text{C}$  NMR of PR5 compound.

**Figure S4** Result of  $^1\text{H}$ - $^1\text{H}$  COSY45 of PR5 compound.

**Figure S5** Result of  $^1\text{H}$ - $^{13}\text{C}$  HMBC of PR5 compound.

**Figure S6** Result of  $^1\text{H}$ - $^{13}\text{C}$  HSQC of PR5 compound.