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New dithiopyrrolone antibiotics induced by adding sorbic acid to the culture medium of *Saccharothrix algeriensis* NRRL B-24137

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Abstract

Dithiopyrrolone antibiotics, produced by several microorganisms, are known for their strong antimicrobial activities. This class of antibiotics generated new interest after the discovery of their anticancer and antitumor properties. In this study, four new antibiotics were purified from the fermentation broth of *Saccharothrix algeriensis* NRRL B-24137 and characterized as dithiopyrrolone derivatives. These new dithiopyrrolone antibiotics were induced by adding sorbic acid, as precursor, at a concentration of 5 mM to the semi-synthetic medium. The analysis of the induced antibiotics was carried out by HPLC. The maximal production of the antibiotics PR2, PR8, PR9 and PR10 was 0.08 ± 0.04 , 0.21 ± 0.04 , 0.13 ± 0.03 and $0.09 \pm 0.00 \text{ mg L}^{-1}$, respectively, obtained after 8 days of fermentation. The chemical structures of these antibiotics were determined by ¹H- and ¹³C-nuclear magnetic resonance, mass and UV-visible data. The four new dithiopyrrolone antibiotics – PR2, PR8, PR9 and PR10 – were characterized, respectively, as crotonyl-pyrrothine, sorbyl-pyrrothine, 2-hexonyl-pyrrothine and 2-methyl-3-pentenyl-pyrrothine. The minimum inhibitory concentrations of the new induced antibiotics were determined.

Keywords

new dithiopyrrolone antibiotics; *Saccharothrix algeriensis*; precursors; sorbic acid; fermentation; antimicrobial activity.

Introduction

Actinomycetes are filamentous bacteria that naturally inhabit soils. They are of great importance in biotechnological process because of their ability to produce a large number of antibiotics and other bioactive secondary metabolites. *Saccharothrix algeriensis* NRRL B-24137 (= DSM 44581) is an actinomycete that produces bioactive compounds belonging to the dithiopyrrolone class of antibiotics (Lamari *et al.*, 2002a, b; Zitouni *et al.*, 2004). Dithiopyrrolones are members of the pyrrothine class of naturally occurring antibiotics that contain *N*-acyl derivatives of 6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo[4,3-*b*]pyrrole. Dithiopyrrolone derivatives were previously identified from the culture broth of certain *Streptomyces* spp. (Okamura *et al.*, 1977; De la Fuente *et al.*, 2002) and from other microorganisms such as the symbiotic bacteria *Xenorhabdus* spp. (McInerney *et al.*, 1991) and the marine bacterium *Alteromonas rava* (Shiozawa *et al.*, 1997).

Dithiopyrrolone antibiotics have strong activities against a variety of Gram-positive and Gram-negative bacteria, yeasts, filamentous fungi and amoeboid parasites (Celmer & Solomons, 1955; Webster *et al.*, 2002; Lamari *et al.*, 2002a). Furthermore, this class of antibiotics exhibits protozoicidal, larvicidal and insecticidal activities (Šturdíková *et al.*, 1990; Webster *et al.*, 2002), and possess outstanding antiallergic action (Stahl *et al.*, 1988). Dithiopyrrolones also have strong activity against several human cancer cell lines and are especially useful in the treatment of malignant mammary cells (Webster *et al.*, 2000; Minamiguchi *et al.*, 2001).

The previous studies showed that *S. algeriensis* produces five dithiopyrrolone derivatives characterized by their different *N*-acyl groups (R): acetyl-pyrrothine (thiolutin), iso-butyril-pyrrothine, butanoyl-pyrrothine, seneciroyl-pyrrothine and tigloyl-pyrrothine (Lamari *et al.*, 2002a, b; Bouras *et al.*, 2006a) (Fig. 1). Furthermore, the addition of precursors to the culture medium led to the modification in production levels of known dithiopyrrolones (Bouras *et al.*, 2006a, b)

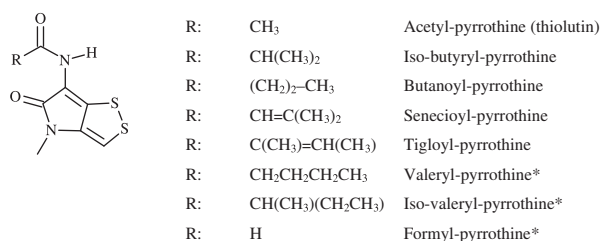


Fig. 1. Structure of dithiopyrrolone antibiotics produced by *Saccharothrix algeriensis* NRRL B-24137. *Dithiopyrrolones induced by adding valeric acid alone.

and also to precursor-directed biosynthesis of new dithiopyrrolone analogues (Bouras *et al.*, 2007, 2008). Consequently, *S. algeriensis* has the ability to produce a wide range of dithiopyrrolones based on different acyl-CoA depending on the precursors added (Bouras *et al.*, 2007, 2008). Recently, Merrouche *et al.* (2010) showed that the addition of valeric acid at a concentration of 5 mM induced the production of three new dithiopyrrolone derivatives: formyl-pyrrothine, valeryl-pyrrothine and iso-valeryl-pyrrothine (Fig. 1).

In the present work, new dithiopyrrolone antibiotics from *S. algeriensis* have been induced by adding sorbic acid and subsequently purified and characterized. The minimum inhibitory concentrations (MIC) of the new induced antibiotics against several microorganisms were determined.

Materials and methods

Producing strain

Saccharothrix algeriensis NRRL B-24137 (Zitouni *et al.*, 2004) was grown and maintained at 4 °C on slants of International *Streptomyces* Project 2 (ISP 2) medium (Shirling & Gottlieb, 1966).

Fermentation studies of *S. algeriensis* with and without sorbic acid

Culture conditions

A mature slant culture of the strain *S. algeriensis* was inoculated into 500 mL Erlenmeyer flasks, each containing 100 mL of a basal semi-synthetic medium (SSM) consisting of 10 g glucose D⁺ (Fisher Labosi), 2 g (NH₄)₂SO₄ (Prolabo), 2 g NaCl (Fisher Labosi), 0.5 g KH₂PO₄ (Acros), 1 g K₂HPO₄ (Acros), 0.2 g MgSO₄ · 7H₂O (Acros), 5 g CaCO₃ (Prolabo) and 2 g yeast extract (Difco), in 1 L distilled water. The pH of the medium was adjusted to 7 using a 2 M NaOH solution before autoclaving. The sorbic acid (Fluka), at a concentration of 5 mM, was supplied to the basal SSM prior inoculation. The inoculated cultures were put on a rotary shaker at 240 r.p.m. at 30 °C for 10 days.

Kinetics of antimicrobial products, growth and pH

All kinetics was assessed on SSM supplemented with sorbic acid (at 5 mM) and control (without sorbic acid). The activity against *Bacillus subtilis* and *Mucor ramannianus*, used as test microorganisms, was regularly recorded each day by the agar diffusion method (well technique; each well of 10 mM in diameter made in the ISP 2 agar plate was filled with 200 µL of supernatant).

Dry cell weights were determined as described by Bouras *et al.* (2006a) and expressed as gram per litre. The pH value was measured with a pH meter (Consort C 832, Consort, NY). All tests were repeated two times from two separate cultures.

Kinetics of antibiotic production and HPLC analysis

The culture filtrate was extracted with an equal volume of dichloromethane and the organic layer was dried with anhydrous sodium sulphate and concentrated under vacuum to generate a crude extract. The extract was concentrated to dryness under vacuum on a Rotavapor, and dissolved in 1 mL of methanol as crude extract. The analysis of antibiotics induced by addition of sorbic acid in the SSM was carried out by a HPLC system equipped with a C₁₈ reverse phase column (Uptisphere UP5ODB, 150 × 4.6 mm; BioTek). The samples were analysed and quantified as described by Lamari *et al.* (2002b) and Bouras *et al.* (2006a).

Purification of induced antibiotics

The bacteria were cultivated in 500 mL Erlenmeyer flasks, each containing 100 mL of SSM supplemented with sorbic acid (5 mM). For the purification, cultures were combined to obtain 15 L. The mycelium was separated, and the culture broth was extracted with dichloromethane on the eighth day of fermentation. The concentrated extracts were partially purified on preparative silica gel 60 plates (Merck Art 5735, Kieselgel 60F 254) and separated by a mixture of ethyl acetate and methanol (100:15 v/v). Two active bands were obtained as yellow (AJ) and yellow-orange (PS) bands at retention factor (*R_f*) values of 0.52 and 0.59, respectively. After elution with methanol, crude AJ and crude PS were obtained and purified by HPLC. Semi-preparative HPLC was performed on a Waters system using a C₁₈ column (UP5ODB, 250 × 7.8 mm). The samples were analysed by linear gradient elution using methanol as solvent A and ultra pure water as solvent B. The separation gradient started with 40% solvent A and 60% solvent B, and reached 100% solvent B and 0% solvent A in 30 min, using a flow of 1.5 mL min⁻¹. The detection of compounds was carried out at 390 and 220 nm.

Chemical characterization of induced antibiotics

UV-visible absorption spectra of induced antibiotics were determined with a Shimadzu UV 1605 spectrophotometer. The molecular weights of the compounds were obtained by electron impact MS (EIMS) recorded at 70 eV with a Nermag R-10-10C spectrometer. The nuclear magnetic resonance (NMR) sample was prepared by dissolving the pure molecules (PR2, PR8, PR9 and PR10) in 600 μL of CD_2Cl_2 . One- and two-dimensional (2D) ^1H and ^{13}C experiments were recorded on a Bruker Avance 500 spectrometer equipped with a 5 mM triple resonance inverse Z-gradient probe (TBI ^1H , ^{31}P , BB). All chemical shifts for ^1H and ^{13}C are relative to tetramethylsilane (TMS) using ^1H (residual) or ^{13}C chemical shifts of the solvent as a secondary standard. The temperature was set at 298 K. All the ^1H and ^{13}C signals were assigned on the basis of chemical shifts, spin-spin coupling constants, splitting patterns and signal intensities in ^1H - ^1H COSY45, ^1H - ^{13}C HMQC and ^1H - ^{13}C HMBC experiments.

Antimicrobial MIC of purified antibiotics

The MIC of antibiotics were determined by a conventional agar dilution method using ISP 2 medium. 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.

Results

Kinetics of antimicrobial products of *S. algeriensis*

The results of evolution of antimicrobial products of *S. algeriensis* are shown in Fig. 2. The antimicrobial activity started earlier in the presence of sorbic acid (third day of fermentation against *M. ramannianus* and fourth day against *B. subtilis*) as compared with control (seventh day of fermentation against *M. ramannianus* and sixth day against *B. subtilis*). *Saccharothrix algeriensis* exhibited better antimicrobial activity after addition of sorbic acid compared with the control. The maximal antifungal activity (25 mM diameter inhibition after 9 days of fermentation) was greater than the maximal antibacterial activity (15 mM diameter of inhibition after 7 days of fermentation).

Effect of addition of sorbic acid on pH of the medium, growth and antibiotic production in *S. algeriensis*

The actinomycete *S. algeriensis* produces five known dithiolopyrrolones (thiolutin, iso-butyryl-pyrrothine, butanoyl-pyrrothine, senecioid-pyrrothine and tigloyl-pyrrothine) in the SSM (without precursors) as reported by Lamari *et al.*

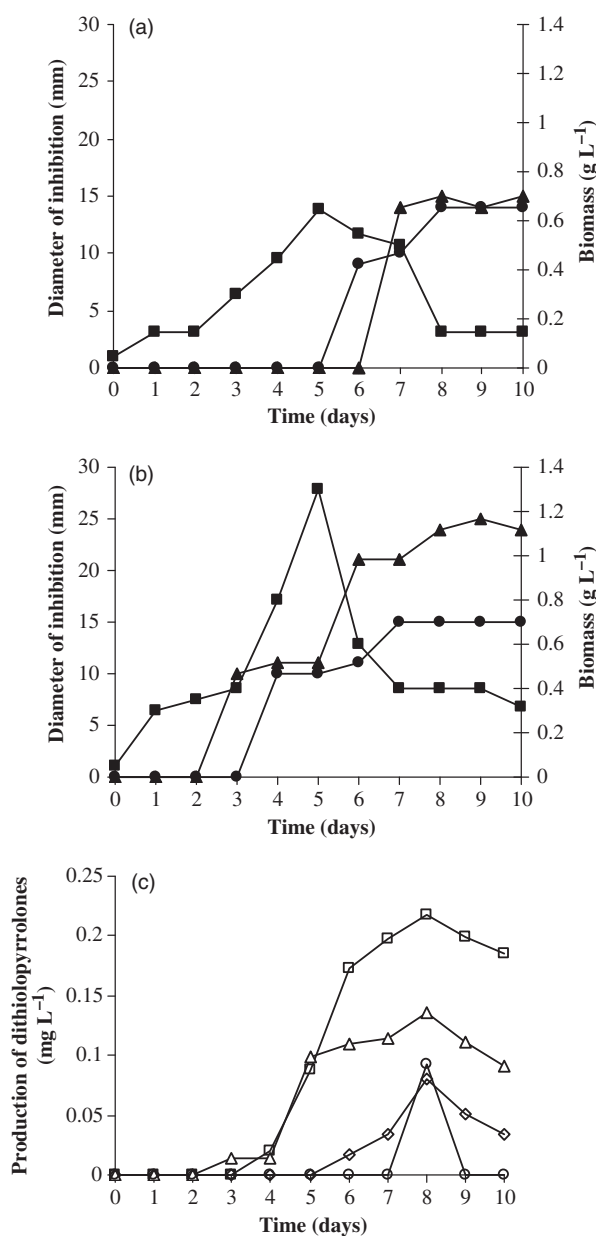


Fig. 2. Effect of addition of sorbic acid to the SSM on evolution of biomass, antimicrobial activity and production of new dithiolopyrrolones by *Saccharothrix algeriensis*: (a) SSM without adding sorbic acid (control); (b) SSM with sorbic acid (at 5 mM). ■, Evolution of biomass; ●, activity against *Bacillus subtilis*; ▲, activity against *Mucor ramannianus*; values do not include the diameter of wells (10 mm). (c) Production of new obtained dithiolopyrrolones. ◇, PR2; □, PR8; △, PR9; and ○, PR10.

(2002b). Importantly, the addition of sorbic acid to the SSM induced the production of four new dithiolopyrrolones (PR2, PR8, PR9 and PR10). The retention times of these new induced compounds (PR2, PR8, PR9 and PR10) were recorded at 28.24, 36.86, 37.16 and 37.82 min, respectively. The growth of *S. algeriensis* was influenced by the addition

of sorbic acid. In SSM broth (control), the dry cell weight reached a maximum after 5 days of fermentation ($0.65 \pm 0.05 \text{ g L}^{-1}$) and then decreased to reach a value of $0.15 \pm 0.03 \text{ g L}^{-1}$ at the end of fermentation (after 10 days). However, by addition of sorbic acid, the dry cell weights reached a maximum of $1.30 \pm 0.08 \text{ g L}^{-1}$ (also obtained after 5 days of fermentation) and then decreased $0.32 \pm 0.06 \text{ g L}^{-1}$ at the end of fermentation.

Moreover, the sorbic acid allowed a high specific growth rate (μ_{max}) of $0.074 \pm 0.004 \text{ h}^{-1}$, in comparison with $0.045 \pm 0.002 \text{ h}^{-1}$ with control. In addition, the optimal production of new dithiopyrrolones PR2, PR8, PR9 and PR10 was observed during the idiophase and was generally dissociated from growth. The maximal production of the antibiotics PR2, PR8, PR9 and PR10 was 0.08 ± 0.04 , 0.21 ± 0.04 , 0.13 ± 0.03 and $0.09 \pm 0.00 \text{ mg L}^{-1}$, respectively, recorded on the eighth day of fermentation. The production of thiolutin was reduced three times more after addition of sorbic acid ($0.29 \pm 0.08 \text{ mg L}^{-1}$) than with the control ($0.89 \pm 0.09 \text{ mg L}^{-1}$). Moreover, the final pH at the end of fermentation (after 10 days) was 7.92 ± 0.06 in the presence of sorbic acid as compared with 8.16 ± 0.04 with the control.

Purification and characterization of induced antibiotics

The culture broth with antimicrobial activity was partially purified, and the thin-layer chromatography plates showed two bands (AJ and PS). The analysis by semi-preparative reversed-phase HPLC showed that the AJ band was composed of one compound (thiolutin); however, the PS band contained eight compounds: iso-butyryl-pyrrothine, butanoyl-pyrrothine, seneciroyl-pyrrothine, tigloyl-pyrrothine (Lamari *et al.*, 2002a) and four induced unknown compounds. These last four compounds were purified by HPLC, and all appear yellow and exhibit antimicrobial activity. The UV-visible spectra of each of the induced compounds showed three absorption maxima. Compound PR2 absorbed at 203, 304 and 395 nm, PR8 at 202, 270 and 413 nm, PR9 at 204, 303 and 402 nm and PR10 at 202, 304 and 398 nm. The molecular weights of PR2 and PR8 are m/z 254 and 280, respectively. PR9 and PR10 have the same molecular weight (m/z 282).

Compounds PR2, PR8, PR9 and PR10 show common ^1H - and ^{13}C -NMR spectral features: two carbonyl groups (δ_{c} 167.0~166.6 and δ_{c} 164.8~163.8), two sp^2 -hybridized quaternary carbons (δ_{c} 137.4~136.9 and δ_{c} 132.1~131.6), one olefinic group (δ_{H} 6.71~6.66 and δ_{c} 108.7~108.3), one N- CH_3 group (δ_{H} 3.36~3.35 and δ_{c} 28.0~27.4), and one NH group (δ_{H} 7.60~7.43). These ^1H and ^{13}C signals are typical of dithiopyrrolone derivatives. Compound PR2 showed two additional sp^2 methines (δ_{H} 6.99 and 5.98 and δ_{c} 142.8 and 123.2) and one additional methyl group (δ_{H} 1.93 and δ_{c} 17.4).

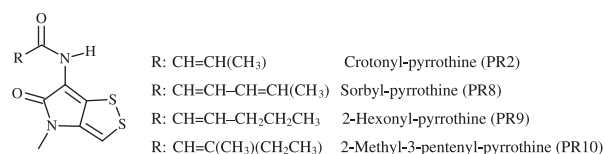


Fig. 3. Structure of new dithiopyrrolones PR2, PR8, PR9 and PR10 induced by adding sorbic acid.

The 2D ^1H - ^1H and ^1H - ^{13}C experiments made it possible to confirm the presence of a 2-butenamide side chain (Fig. 3). The E-geometry of the double bond was obtained on the basis of the coupling constant of H9-H10 (15.2 Hz). Compound PR8 showed four additional sp^2 methines (δ_{H} 7.30, 6.27, 6.26 and 5.92 and δ_{c} 143.2, 140.0, 129.3 and 119.3) and one additional methyl group (δ_{H} 1.90 and δ_{c} 18.4). The 2D ^1H - ^1H and ^1H - ^{13}C experiments clearly revealed that PR8 contained a 2,4-hexadienamide side chain (Fig. 3). The E,E-geometry of the double bond was deduced from the coupling constant of H9-H10 (15.0 Hz) and of H11-H12 (15.1 Hz, obtained from simulation). Compound PR9 showed two additional sp^2 methines (δ_{H} 6.98 and 5.95 and δ_{c} 147.5 and 121.9), two additional sp^3 methylenes (δ_{H} 2.25 and 1.54 and δ_{c} 34.1 and 13.4) and one additional methyl group (δ_{H} 0.98 and δ_{c} 13.4). The 2D ^1H - ^1H and ^1H - ^{13}C experiments established the presence of a 2-hexenamide side chain (Fig. 3). The E-geometry of the double bond was obtained on the basis of the coupling constant of H9-H10 (15.2 Hz). Compound PR10 showed one additional sp^2 methine (δ_{H} 5.72 and δ_{c} 115.7), one sp^3 methylene (δ_{H} 2.21 and δ_{c} 34.2) and two additional methyl groups (δ_{H} 2.24 and 1.12 and δ_{c} 19.1 and 12.1). The 2D ^1H - ^1H and ^1H - ^{13}C experiments made it possible to confirm the presence of 2-pentenamide, 3-methyl side chain (Fig. 3). The geometry of the double bond was assigned as E by the long-range $^3\text{J}_{\text{HC}}$ coupling constants between H9 and C11 ($< 2 \text{ Hz}$) and between H9 and C13 ($\sim 7 \text{ Hz}$).

Antimicrobial activity of purified dithiopyrrolone antibiotics

The antimicrobial activity of the new dithiopyrrolone antibiotics (PR2, PR8, PR9 and PR10) is shown in Table 1. The antibiotic PR8 showed higher activity than other compounds against Gram-positive bacteria. The antibiotics PR2 and PR9 were not active against *Aspergillus carbonarius* and the phytopathogenic fungi *Fusarium oxysporum* f. sp. *lini*, *Fusarium graminearum* and *Fusarium moniliforme*. However, the antibiotics PR8 and PR10 showed a moderate activity against all fungi and yeasts tested. None of the new induced antibiotics showed activity against Gram-negative bacteria.

Discussion

Dithiopyrrolones are known to be produced by several species of *Streptomyces*, *Xenorhabdus* and *Alteromonas*. The

Table 1. Antimicrobial MIC ($\mu\text{g mL}^{-1}$) of new dithiopyrrolone antibiotics produced by *Saccharothrix algeriensis*

Test organism*	New dithiopyrrolones			
	PR2	PR8	PR9	PR10
<i>Bacillus subtilis</i> (ATCC 6633)	75	20	> 100	20
<i>Bacillus coagulans</i> (CIP 6625)	75	30	> 100	20
<i>Listeria monocytogenes</i> (CIP 82110)	40	10	20	10
<i>Micrococcus luteus</i> (ATCC 9314)	30	10	75	75
<i>Staphylococcus aureus</i> (CIP 7625)	30	10	> 100	100
<i>Agrobacterium tumefaciens</i> (no. 2410 LB)	> 100	> 100	> 100	> 100
<i>Escherichia coli</i> (ATCC 10536)	> 100	> 100	> 100	> 100
<i>Klebsiella pneumoniae</i> (CIP 82.91)	> 100	> 100	> 100	> 100
<i>Salmonella enterica</i> (CIP 81.3)	> 100	> 100	> 100	> 100
<i>Pseudomonas aeruginosa</i> (CIP A22)	> 100	> 100	> 100	> 100
<i>Aspergillus carbonarius</i> (M333)	> 100	30	> 100	2
<i>Fusarium oxysporum</i> f. sp. <i>lini</i> (Foln 3)	> 100	75	> 100	50
<i>Fusarium moniliforme</i>	> 100	75	> 100	5
<i>Fusarium equiseti</i>	50	20	75	30
<i>Fusarium culmorum</i>	50	50	50	30
<i>Fusarium graminearum</i>	> 100	50	> 100	30
<i>Mucor ramannianus</i> (NRRL 1829)	50	20	40	5
<i>Penicillium expansum</i>	75	20	> 100	40
<i>Candida albicans</i> (IPA 200)	> 100	50	50	2
<i>Saccharomyces cerevisiae</i> (ATCC 4226)	30	50	30	10

*The test microorganisms without an accession number were from our laboratory collection.

actinomycete *S. algeriensis* produces five dithiopyrrolones in the basic medium (without precursors): thiolutin, isobutyryl-pyrrothine, butanoyl-pyrrothine, seneciyl-pyrrothine and tigloyl-pyrrothine (Lamari *et al.*, 2002b). This actinomycete has a great ability to produce a wide range of dithiopyrrolone derivatives that, depending on the composition of the culture medium, nature and concentration of precursors added and an enzymatic system, are involved in attaching a variety of radicals (R) into pyrrothine ring (Bouras *et al.*, 2006a, b, 2007, 2008; Chorin *et al.*, 2009). The data presented above show that the addition of sorbic acid at a concentration of 5 mM to the SSM as a precursor has induced the production of four new peaks, as revealed by HPLC analysis. These induced compounds did not correspond to known dithiopyrrolones with respect to retention time, but they were identified as dithiopyrrolone derivatives by their spectral characteristics (UV spectra, EIMS and NMR).

From MS and ^1H - and ^{13}C -NMR spectroscopic analyses, as well as by comparison with all dithiopyrrolone derivatives reported in the literature, the structures of the four new dithiopyrrolones (PR2, PR8, PR9 and PR10) were characterized as *N*-acyl derivatives of 6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo[4,3-*b*]pyrrole. The four compounds showed a prominent fragment ion of m/z 186 and indicated by the EIMS spectrum an extra methyl group in

the heterocyclic ring (corresponding to the empirical formula $\text{C}_6\text{H}_6\text{N}_2\text{OS}_2$) as reported for other dithiopyrrolones (McInerney *et al.*, 1991; Lamari *et al.*, 2002b).

On the basis of NMR and MS data, the molecular formula of PR2 was determined as $\text{C}_{10}\text{H}_{10}\text{N}_2\text{O}_2\text{S}_2$ (Fig. 3). The antibiotic PR8 was determined as $\text{C}_{12}\text{H}_{12}\text{N}_2\text{O}_2\text{S}_2$, suggesting an intact direct incorporation of the sorbic acid into pyrrothine ring. The results of Bouras *et al.* (2008) showed that addition of precursors into the culture medium, such as organic acids, led to precursor-directed biosynthesis of new dithiopyrrolone analogues. In the same context, Chorin *et al.* (2009) suggest that the enzymatic reaction of pyrrothine acylation takes part in the dithiopyrrolone biosynthetic pathway in *S. algeriensis*, which is able to use acyl-CoA with different structures (acetyl-CoA and benzoyl-CoA) as substrates to produce corresponding dithiopyrrolones (acetyl-pyrrothine and benzoyl-pyrrothine). Furthermore, the antibiotic PR9 showed the same molecular weight as PR10 (m/z 282) with the same molecular formula $\text{C}_{12}\text{H}_{14}\text{N}_2\text{O}_2\text{S}_2$, suggesting isomeric compounds.

The new dithiopyrrolones (PR2, PR8, PR9 and PR10) were named, respectively, crotonyl-pyrrothine, sorbyl-pyrrothine, 2-hexonyl-pyrrothine and 2-methyl-3-pentenyl-pyrrothine.

Our results showed that the antibacterial and antifungal activities of the newly obtained dithiopyrrolones are related to their variable acyl groups. The antibiotic PR8 (sorbyl-pyrrothine) showed higher activity than other compounds against Gram-positive bacteria. The new dithiopyrrolone antibiotics showed a moderate activity against all fungi and yeasts tested (except for PR2 and PR9, which are not active against *A. carbonarius*, *F. oxysporum* f. sp. *lini*, *F. graminearum* or *F. moniliforme*). Interestingly, the antibiotic 2-methyl-3-pentenyl-pyrrothine (PR10) showed higher activity against *A. carbonarius* and *Candida albicans*, than showed by any of the other dithiopyrrolones produced by *S. algeriensis*. In fact, the biological activity of dithiopyrrolones is strongly influenced by the nature of variable acyl groups, as reported previously (Oliva *et al.*, 2001; Li *et al.*, 2007; Guo *et al.*, 2008). Furthermore, none of the newly obtained antibiotics showed any activity against Gram-negative bacteria; similar results have been obtained with other dithiopyrrolones produced by *S. algeriensis* (Lamari *et al.*, 2002a; Merrouche *et al.*, 2010).

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