

Dithiopyrrolone Antibiotic Formation Induced by Adding Valeric Acid to the Culture Broth of *Saccharothrix algeriensis*

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Three new antibiotics were isolated from the fermentation broth of *Saccharothrix algeriensis* NRRL B-24137 and characterized as the dithiopyrrolone derivatives valerylpyrrothine (**1**), isovalerylpyrrothine (**2**), and formylpyrrothine (**3**) as well as the known antibiotic aureothricin. The production of the dithiopyrrolone derivatives was induced by adding valeric acid to the culture medium. The compounds exhibited moderate antimicrobial activity in vitro.

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-dithio[4,3-*b*]pyrrole. Dithiopyrrolone derivatives were previously identified from the culture broth of certain species of bacteria such as *Streptomyces*,^{1,2} *Xenorhabdus*,³ and *Alteromonas*.⁴ *Saccharothrix algeriensis* NRRL B-24137 (Actinomycetales) isolated as a new species from Algerian Saharan soil in our laboratory^{5,6} produces five dithiopyrrolone derivatives: thiolutin, seneciopyrrothine (SEP), tigloylpyrrothine (TIP), isobutyrylpyrrothine (ISP), and butanoylpyrrothine (BUP).⁷

Dithiopyrrolone antibiotics have strong activities against a variety of Gram-positive and Gram-negative bacteria, yeasts, filamentous fungi, and protozoa.^{5,8–10} Furthermore, this class of antibiotics exhibits protozoicidal, larvicidal, and insecticidal activities.^{9–11} Dithiopyrrolones also have strong activity against several human cancer cell lines and are especially useful in the treatment of malignant mammary cells.^{12–15} However, it is clear that the prospects for development of dithiopyrrolone derivatives into potential pharmaceuticals will depend upon elucidating their mode of action and determining whether they exert adverse effects on human health.

The search for new bioactive compounds is one of the central subjects of industrial and academic natural products discovery.¹⁶ Several studies have reported the generation of novel bioactive molecules by metasythesis, semisynthesis, bio-organic synthesis, and also precursor-directed biosynthesis, which is considered a promising approach.^{17,18} Several microorganisms can be fed by uncommon and unusual precursors, generating derivatives of expected new natural products that are not easily obtainable by chemical synthesis.¹⁹ The antimicrobial and antitumoral activities of dithiopyrrolones are related to their variable acyl groups. Consequently, the obtained uncommon dithiopyrrolone derivatives could lead to improvement and discovery of new biological activities. In the present work, the formation of new dithiopyrrolone antibiotics from *S. algeriensis* has been induced and the new antibiotics have subsequently been purified and characterized.

The actinomycete strain *S. algeriensis* NRRL B-24137 afforded a yellow culture broth with antimicrobial activity. The strain was cultivated in a semisynthetic medium (SSM) in 12 L Erlenmeyer flasks. The culture broth was separated from the mycelium by

filtration and extracted with DCM on the fifth day of fermentation. Valeric acid was added to the medium at a concentration of 5 mM prior to inoculation. The crude extract was concentrated under vacuum (3.8 g from 12 L) and partially purified on preparative silica gel 60 plates followed by semipreparative reversed-phase HPLC. The latter afforded compounds **1–3**, and their concentrations reached levels of 1.31, 0.35, and 0.08 mg/L, respectively, at day 5. These compounds showed antimicrobial activity. Furthermore, the known antibiotic aureothricin (propionylpyrrothine) was also isolated from the fermentation broth of *S. algeriensis*. The latter compound was previously identified from the culture broth of several actinomycetes belonging to the genus *Streptomyces* such as *S. kasugaensis*.^{8,10}

HPLC analysis of the partially purified extract revealed new peaks in fermentations that were supplemented with valeric acid. The retention times of these peaks (**1–3**) were recorded at 9.51, 25.02, and 28.37 min, respectively. The UV spectra of compounds **1–3** showed three absorption maxima. Compound **1** absorbs at 207, 307, and 392 nm, compound **2** at 204, 305, and 388 nm, and compound **3** at 203, 311, and 389 nm. The molecular weights of these compounds were obtained by EIMS. Compounds **1** and **2** have the same molecular weight, 270, suggesting isomeric compounds. Compound **3** (molecular weight 214) has the same molecular weight of a known antibiotic, holomycin, produced by some species of *Streptomyces* such as *S. clavuligerus*, *S. pimprina*, and *S. griseus*.^{1,2}

The three compounds (**1–3**) showed a prominent fragment ion at *m/z* 186, indicating an extra methyl group in the heterocyclic ring as reported for other dithiopyrrolones.^{3,7}

On the basis of NMR and MS data the molecular formula of compound **1** was determined as C₁₁H₁₄N₂O₂S₂, suggesting incorporation of the intact valeric acid into the pyrrothine ring (Figure 1). Compound **2** showed the same molecular weight as compound **1** (*m/z* 270). Compound **3** was found to have a molecular formula of C₇H₆N₂O₂S₂ (*m/z* 214). This compound is an isomer of the holomycin antibiotic. The latter showed a fragment ion of *m/z* 172 (corresponding to the empirical formula C₅H₄N₂OS₂) in place of *m/z* 186 for compound **3**, corresponding to a hydrogen instead of a methyl group in the pyrrothine moiety.³

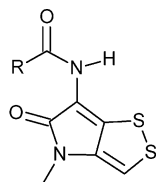
Compounds **1**, **2**, and **3** show common ¹H and ¹³C NMR features: one carbonyl group (δ_C 167.0–166.5), three sp²-hybridized quaternary carbons (δ_C from 136.9 to 114.5), one olefinic group (δ_H 6.74–6.70 and δ_C 108.8–108.5), one N-CH₃ group (δ_H 3.37–3.34 and δ_C 27.6–27.5), and one NH group (δ_H 8.02–7.55). These ¹H and ¹³C NMR signals are typical of dithiopyrrolone derivatives.

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1 R = CH₂CH₂CH₂CH₃

2 R = CH(CH₃)(CH₂CH₃)

3 R = H

Figure 1. Structure of induced dithiopyrrolones (**1–3**).

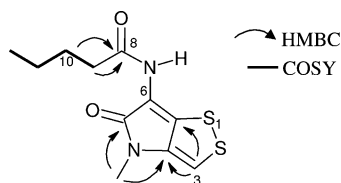


Figure 2. HMBC and COSY correlations of **1**.

Furthermore, compound **1** shows one additional carbonyl group (δ_C 167.0) and four additional sp^3 -hybridized carbons (δ_C 35.9, 27.4, 22.3, and 13.5). The 2D 1H - 1H and 1H - ^{13}C COSY experiments confirmed the presence of the pentanoyl side chain (Figure 2). Compound **2** shows one additional carbonyl group (δ_C 166.8) and four additional sp^3 -hybridized carbons (δ_C 42.8, 27.2, 17.0, and 11.6), accounting for a 2-methylbutanoyl side chain. Compound **3** shows one additional carbonyl group (δ_C 166.8) and one proton at δ_H 8.19, characterizing a formyl side chain. From an analysis of MS and 1H and ^{13}C NMR data, as well as by comparison with those reported, the structures of the three dithiopyrrolones (**1–3**) were characterized as *N*-acyl derivatives of 6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo[4,3-*b*]pyrrole. The new dithiopyrrolones **1–3** were named, respectively, valerylpyrrothine, isovalerylpyrrothine, and formylpyrrothine.

Dithiopyrrolones are known to be produced by several species of *Streptomyces* and *Xenorhabdus* and also by *Alteromonas rava*. The actinomycete *S. algeriensis* produces in basic medium (without precursors) five dithiopyrrolones (thiolutin, ISP, TIP, BUP, and SEP). The addition of valeric acid at a concentration of 5 mM to the medium as a precursor induced the production of new dithiopyrrolones derivatives (**1–3**).

The antimicrobial activity of compounds **1–3** and aureothricin produced by *S. algeriensis* is shown in Table 1. Compound **3** showed higher activity than compounds **1** and **2** against Gram-positive bacteria and the majority of filamentous fungi. Compound **2** was less active than its isomer **1** and aureothricin. Except for compound **2**, which is not active against the phytopathogenic fungi *Fusarium moniliforme* and *F. graminearum*, all other compounds showed a moderate to strong activity against all filamentous fungi and yeasts tested. All compounds showed no (or weak) activity against Gram-negative bacteria. Similar results were observed with other known dithiopyrrolones produced by our strain.⁵

Experimental Section

General Experimental Procedures. The optical rotation was measured on a Perkin-Elmer (model 241) polarimeter. UV spectra were measured on a Shimadzu UV 1605 spectrophotometer. The NMR sample was prepared by dissolving compounds **1**, **2**, and **3** in 600 μ L of CD_2Cl_2 . 1D and 2D 1H and ^{13}C NMR 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). The proton and carbon chemical shifts are relative to TMS using 1H (residual) or ^{13}C chemical shifts of the solvent as a secondary standard. The temperature

Table 1. Antimicrobial MIC (μ g/mL) Values of Dithiopyrrolones **1–3** and Aureothricin

test organism ^a	compound			
	1	2	3	aureothricin
<i>Bacillus subtilis</i> (ATCC 6633)	20	40	2	20
<i>Bacillus coagulans</i> (CIP 6625)	20	40	2	20
<i>Micrococcus luteus</i> (ATCC 9314)	40	100	3	20
<i>Staphylococcus aureus</i> (CIP 7625)	75	100	2	100
<i>Listeria monocytogenes</i> (CIP 82110)	>100	>100	20	>100
<i>Escherichia coli</i> (ATCC 10536)	>100	>100	40	75
<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>Agrobacterium tumefaciens</i> (no. 2410 LB)	>100	>100	>100	>100
<i>Mucor ramannianus</i> (NRRL 1829)	10	30	10	20
<i>Penicillium expansum</i>	20	40	3	20
<i>Aspergillus carbonarius</i> (M333)	20	75	50	40
<i>Fusarium oxysporum</i> f.sp. <i>lini</i> (Foln 3–5)	50	75	30	40
<i>Fusarium moniliforme</i>	75	>100	75	75
<i>Fusarium equiseti</i>	20	50	10	20
<i>Fusarium culmorum</i>	20	40	10	20
<i>Fusarium graminearum</i>	75	100	40	75
<i>Candida albicans</i> (IPA 200)	20	75	75	75
<i>Saccharomyces cerevisiae</i> (ATCC 4226)	10	20	75	40

^a The target microorganisms without accession number resulted from our laboratory collection.

was set at 298 K. The 1H and ^{13}C NMR signals were assigned on the basis of chemical shifts, coupling constants, splitting patterns, and signal intensities and by using 1H - 1H COSY45, 1H - ^{13}C HMQC, and 1H - ^{13}C HMBC experiments. EIMS were recorded at 70 eV with a Nermag R-10-10C spectrometer. The accurate mass spectrometry (HREIMS) was carried out on a GCT Premier System. Semipreparative HPLC was run on a Waters system using a C18 column (UP50DB, 250 \times 7.8 mm). The samples were analyzed by linear gradient elution using MeOH as solvent A and ultrapure H₂O 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 220 and 390 nm.

Producing Strain. *Saccharothrix algeriensis* NRRL B-24137 (=DSM 44581) was isolated from a Saharan soil sample collected in 1992 at a palm grove in Adrar (southwest of Algeria).⁶ This strain was grown and maintained at 4 $^{\circ}C$ on slants of ISP 2 (International *Streptomyces* Project 2) solid medium containing (in grams per liter of distilled water): D(+)-glucose 4.0, malt extract 10.0, yeast extract 4.0, and agar 18.0. The pH of the medium was adjusted to 7.0 with a 2 M NaOH solution before autoclaving at 120 $^{\circ}C$ for 20 min.

Fermentation and Isolation. A mature slant culture of the strain *S. algeriensis* NRRL B-24137 was inoculated into 500 mL Erlenmeyer flasks each containing 100 mL of a basal semisynthetic medium (SSM) consisting of (in g/L of distilled H₂O) D(+)-glucose (10.0), (NH₄)₂SO₄ (2.0), NaCl (2.0), KH₂PO₄ (0.5), K₂HPO₄ (1.0), MgSO₄·7H₂O (0.2), CaCO₃ (5.0), and yeast extract (2.0). The pH of the medium was adjusted to 7.0 using a 2 M NaOH solution prior to autoclaving. The valeric acid was added at a concentration of 5 mM to the medium prior to inoculation. The culture was incubated on a rotary shaker (240 rpm) at 30 $^{\circ}C$ for 5 days. The fermentation procedure was repeated to obtain a total of 12 L of culture broth. These cultures were centrifuged and filtered to remove mycelium. The culture filtrate was extracted with

an equal volume of DCM, and the organic layer was dried with Na₂SO₄ and concentrated under vacuum to generate a crude extract. The latter was partially purified on preparative silica gel 60 [MeOH–EtOAc (15:100)]. Two active bands were obtained as yellow (AJ) and yellow-orange (PS) bands at *R_f* values of 0.52 and 0.59, respectively. After elution with MeOH, crude AJ and crude PS were obtained and purified by HPLC using a continuous grade from 20% to 100% MeOH in H₂O. AJ was composed of two components, thiolutin and **3**. PS contained the five compounds **1**, **2**, aureothricin, TIP, and SEP. However, ISP and BUP were not obtained under these conditions. The three yellow compounds **1–3** showing the main antimicrobial activity were further purified by HPLC and characterized as new dithiolopyrrolone derivatives.

Bioassay. MIC values of the antibiotics **1–3** were determined by a conventional agar dilution method using ISP2 medium. For each test, the experiments were repeated four times. The antimicrobial activity was observed after 24–48 h incubation at 37 °C for bacteria and 48–72 h incubation at 28 °C for filamentous fungi and yeasts.

Valerylpyrrothine (1): orange-yellow powder; UV (MeOH) λ_{\max} (log ϵ) 207 (4.1), 307 (3.7), 392 (3.9) nm; ¹H NMR (CD₂Cl₂, 500 MHz) δ 8.02 (1H, br s, N7–H), 6.70 (1H, s, H-3), 3.34 (3H, s, NCH₃), 2.38 (1H, t, *J* = 7.6, H-9), 1.67 (1H, m, H-10), 1.41 (1H, m, H-11), 0.96 (3H, t, *J* = 7.3, H-12); ¹³C NMR (CD₂Cl₂, 125 MHz) δ 171.6 (C, C-8), 167.0 (C, C-5), 136.8 (C, C-6a), 132.1 (C, C-3a), 114.7 (C, C-6), 108.8 (CH, C-3), 35.9 (CH, C-9), 27.6 (CH₃, NCH₃), 27.4 (CH₂, C-10), 22.3 (CH₃, C-11), 13.5 (CH₃, C-12); EIMS *m/z* 270 (M⁺; 28), 186 (100), 83 (17), 57 (8); HREIMS *m/z* 270.0489 (calcd, C₁₁H₁₄N₂O₂S₂, 270.0497).

Isovalerylpyrrothine (2): yellow-orange powder; [α]_D²⁵ –0.7 (c 0.3, MeOH); UV (MeOH) λ_{\max} (log ϵ) 204 (4.1), 305 (3.7), 388 (3.9) nm; ¹H NMR (CD₂Cl₂, 500 MHz) δ 7.55 (1H, br s, N7–H), 6.70 (1H, s, H-3), 3.34 (3H, s, NCH₃), 2.34 (1H, dq, *J* = 6.8, 7.1, H-9), 1.72 (1H, m, H-10), 1.51 (1H, m, H-10), 1.20 (3H, d, *J* = 6.8, H-12), 0.96 (3H, t, *J* = 7.4, H-11); ¹³C NMR (CD₂Cl₂, 125 MHz) δ 174.8 (C, C-8), 166.8 (C, C-5), 136.9 (C, C-6a), 132.0 (C, C-3a), 114.5 (C, C-6), 108.5 (CH, C-3), 42.8 (CH, C-9), 27.6 (CH₃, NCH₃), 27.2 (CH₂, C-10), 17.0 (CH₃, C-12), 11.6 (CH₃, C-11); EIMS *m/z* 270 (M⁺; 49), 186 (100), 83 (7), 57 (11); HREIMS *m/z* 270.0503 (calcd, C₁₁H₁₄N₂O₂S₂, 270.0497).

Formylpyrrothine (3): yellow powder; UV (MeOH) λ_{\max} (log ϵ) 203 (4.2), 311 (3.5), 389 (3.9) nm; ¹H NMR (CD₂Cl₂, 500 MHz) δ 8.19 (1H, s, H), 7.68 (1H, br s, N7–H), 6.74 (1H, s, H-3), 3.37 (3H, s, NCH₃); ¹³C NMR (CD₂Cl₂, 125 MHz) δ 166.5 (C, C-5), 159.3 (C, C-8), 136.9 (C, C-6a), 108.8 (CH, C-3); 1D ¹³C NMR data were not recorded for **3** due to sample limitation; C-3a and C-6 were not detected in the HMBC experiment; EIMS *m/z* 214 (M⁺; 72), 186 (100), 86 (11), 84 (8); HREIMS *m/z* 213.9881 (calcd, C₇H₆N₂O₂S₂, 213.9871).

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