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1 Yields enhancement strategy of dithiolopyrrolone from *Saccharothrix algeriensis* by aliphatic alcohols
2 supplementation.

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Abstract

The production of dithiolopyrrolones by *Saccharothrix algeriensis* was investigated after supplementing the culture medium with ethanol and/or 1-butanol. Optimized Optimal conditions for the addition of ethanol to the culture medium provided a maximal dithiolopyrrolone titer of about 200 mg·L⁻¹ after 5 days of culture, roughly corresponding to a 600%-increase. ~~The involvement of ethanol in enzyme-mediated reactive oxygen species generation, using diphenyleneiodonium, NAD(P)H oxidase inhibitor, was observed.~~ Using NAD(P)H oxidase inhibitor (diphenyleneiodonium) or reactive oxygen species scavenger (para-aminobenzoic acid), we suppose that ethanol promotes the formation ~~generation~~ of reactive oxygen species in *Saccharothrix algeriensis*, which, in turn, ~~act as elicitors for~~ could induce biomass decline and dithiolopyrrolone overproduction. However, the underlying mechanisms remain to be elucidated. These results may be helpful for the ~~optimization~~ control of dithiolopyrrolone yields from *Saccharothrix algeriensis* cultures. ~~and thus pave the way toward their large-scale production.~~

Keywords

Saccharothrix algeriensis, dithiolopyrrolones, reactive oxygen species, alcohols addition

1. Introduction

In the search for substitutes to the current production processes of chemicals, microorganisms can be considered as an attractive alternative. Dithiopyrrolones (DTP) are a promising class of antimicrobial agents found in the culture broths of some *Streptomyces*, and other microorganisms such as the symbiotic bacteria, *Xenorhabdus* spp [1], or the marine bacterium *Alteromonas rava* [2]. This class of antibiotics encompasses about thirty compounds, including holomycin, thiolutin, aureothricin and thiomarinols, that are characterized by a unique pyrrolinodithiole (4*H*-[1, 2]-dithiolo-[4, 3-*b*]-pyrrol-5-one) nucleus substituted with two variable alkyl groups (Fig. 1) [3]. These compounds exhibited a broad-spectrum antibacterial activity, especially against Gram-positive bacteria, mediated by RNA and protein synthesis inhibitions [4, 5]. Moreover, holomycin and thioluthin have been reported as potential antitumor agents [6, 7].

The antimicrobial activities of ~~dithiopyrrolones~~ DTP can be of interest in the agro-forestry ~~alimentary~~ industry ~~for managing of plant pathogens~~. For example, the DTP of the xenorhabdin group have shown potential in crop protection against plant diseases, such as the phytophthora blight or the grey mould [8]. ~~DTP derivatives from *Saccharothrix algeriensis* could be used to control development of several strains of *Fusarium oxysporum* [9].~~

Thiolutin-like DTP are produced by various bacterial strains such as *Saccharothrix algeriensis*, a filamentous ~~actinobacterium~~ isolated from the soil of the palm groves of Adrar in southern Algeria [9 10]. Several research groups have focused their attention on this bacterium because of its ability to produce new DTP from organic acids through precursor-directed biosynthesis [~~10-12-11-13~~]. However, few studies aimed at improving DTP production; therefore our objective is the development of new approaches to stimulate the production of secondary metabolites in *S. algeriensis*. ~~In this study, we report the role of two aliphatic alcohols, ethanol and 1-butanol, as inducers of DTP biosynthesis.~~

In the natural environment, the microbial production of secondary metabolites is linked to many external factors and physiological signals. In liquid cultures, the production of secondary metabolites by Gram-positive bacteria is generally confined to the stationary phase and is frequently associated with the depletion of nutrient pools. Thus, adding a chemical elicitor inducing stress conditions to the culture medium is a rather convenient strategy to enhance the synthesis of secondary metabolites [14]. Among many other chemicals, ethanol supplementation to the culture medium of some antibiotic-producing

bacterial strains has a positive impact on antibiotic production. This phenomenon has been described for validamycin A production by *Streptomyces hygroscopicus* 5008 [13 15] or jadomycin B production by *Streptomyces venezuelae* ISP5230 [14]. The microbial secondary metabolism stimulation triggered by ethanol some alcohols may be mediated by ROS. Previous studies showed that one important generator of ROS were a family of membrane bound enzymes, NAD(P)H oxidases (NOX) [15]. Moreover, it was recently proven that ethanol exposure increased NOX expression [16]. Besides their well-known toxicity toward living cells, ROS are also involved in complex physiological processes such as cell signaling [17] and could activate genes transcription of secondary metabolites biosynthesis [15]. Stress from aliphatic alcohols, like ethanol or 1-butanol, has been studied in various bacteria showing the ROS overproduction [17], but no reports were available about the use of 1-butanol for secondary metabolism elicitation. In this work, we focused on the effects of low concentration levels of two aliphatic alcohols (ethanol and 1-butanol) on cell growth and DTP biosynthesis in *S. algeriensis*. The culture conditions were modified by adding those alcohols at different concentrations and at different times of incubation. In addition, Experiments were also conducted under conditions preventing the ROS overproduction (in presence of ROS scavenger or ROS producing enzyme inhibitor) [18] in order to demonstrate a putative link between ROS generation and DTP production in *S. algeriensis*. Diphenyleneiodinium (DPI) has frequently been used to inhibit reactive oxygen species ROS production mediated by flavoenzymes, particularly NAD(P)H oxidase [18]. Para-aminobenzoic acid (PABA) is described as ROS scavenger [19]. These results may be helpful for the control of secondary metabolism and optimization of DTP yields, obtained from *S. algeriensis* cultures and thus pave the way toward their large scale production.

2. Materials and Methods

2.1 Microorganism and culture conditions

Saccharothrix algeriensis NRRL B-24137 (= DSM 44581) was used in this study. *S. algeriensis* was cultured on International *Streptomyces* Project 2 (ISP2) agar plates for 7 days at 30°C, and the mycelium was then harvested, transferred into a cryoprotective medium (0.1% Tween 80 and 1:1 (v/v) glycerol-water mixture) and stored at -20°C. The titer of the suspension expressed as colony-forming units per milliliter (CFU·mL⁻¹) of suspension was determined by enumeration on ISP2 agar plates.

ISP2 solid medium contains (per liter of distilled water): 4 g yeast extract (Biomérieux), 4 g D-(+)-glucose (Prolabo), 10 g malt extract (Bacto) and 18 g agar (Biomérieux). The pH of medium was adjusted to 7.0 with a 2 M NaOH solution, and the medium was autoclaved at 121°C for 20 min.

For precultures and cultures, we used a basal semi-synthetic medium (SSM) [10] containing, per liter of distilled water: 2 g (NH₄)₂SO₄ (Prolabo), 2 g NaCl (Prolabo), 2 g yeast extract (Biomérieux), 5 g CaCO₃ (Prolabo), 1 g K₂HPO₄ (Fluka), 0.5 g KH₂PO₄ (Fluka), 0.2 g MgSO₄·7H₂O (Prolabo), and 15 g of D-(+)-glucose (Prolabo). Before autoclaving, the pH of the medium was adjusted to 7.0 using 2 M NaOH solution.

Precultures of *S. algeriensis* were grown from 1 mL aliquots of frozen bacterial suspension (10⁸ CFU·mL⁻¹) inoculated into 250 mL Erlenmeyer flasks containing 50 mL of SSM each. These precultures were incubated at 30°C under shaking at 250 rpm for 48 h. Thereafter, cultures were prepared in 250 mL Erlenmeyer flasks containing each 50 mL of culture medium inoculated with 5 mL of homogenized preculture. Cultures were incubated at 30°C under shaking at 250 rpm for no longer than 120 h (5 days).

The cultures were supplemented with either ethanol or 1-butanol (Acros) to achieve final concentrations ranging from 10 mM to 680 mM (0.06 - 4 % v/v) of ethanol, or 10 mM to 100 mM (0.09 - 0.9% v/v) of 1-butanol. Alcohols were added to the medium at 0 h, or 24 h or 48 h of incubation. In parallel, a non-supplemented control culture was used for comparison under each experimental condition.

In our experiments, to evaluate the involvement of ethanol in ROS generation, two kinds of ROS inhibitors were used: DPI and PABA. Diphenylpicrylhydrazonium (DPI) has frequently been used to inhibit reactive oxygen species ROS production mediated by flavoenzymes, particularly NAD(P)H oxidase [18]. The culture medium was supplemented with 1 or 2 µM of DPI (Sigma) or with 5 or 10 µM of PABA (Acros) in absence or presence of ethanol (300 mM, 1.74% v/v).

2.2 Measurement of cell-growth and DTP production

For biomass measurement, the whole volume of culture broth was filtered using a vacuum filtration system. The obtained biomass was treated with 30 mL of 0.3 M HCl to remove CaCO₃, the pellets thus collected were dried at 98°C for 24 h and weighed. The results were expressed as g·L⁻¹ of culture broth.

Culture supernatants were filtered through a 0.2 µm filter and the analysis of DTP content was carried out by High Performance Liquid Chromatography (HPLC). Holothin was used as an external standard for the

plotting of the calibration curve and the quantification of DTPs, since the molar extinction coefficient (ϵ) of holothin at 390 nm is nearly identical to the extinction coefficient of all other known pyrrothines (ϵ_{390} ranging from 8317 to 9333 L·mol⁻¹·cm⁻¹) [9]. The results were expressed as mg·L⁻¹ of culture.

The analytical HPLC equipment consisted of a Hitachi (La Chrom, Merck) instrument, equipped with an isocratic pump and UV detector. A Hypersil BDS C18 RP Column (Thermo Scientific) was used (250 mm × 4.6 mm, 5 μ m particle size) fitted with a guard column (Thermo Scientific). The column was maintained at 30°C and the mobile phase consisted in a 3:7 (v/v) distilled water/acetonitrile mixture; the flow rate was set to 0.8 mL·min⁻¹. The injection volume was 20 μ L. DTPs were detected at 390 nm.

~~The evaluated~~ To evaluate the amount of DTPs that might be trapped within the cells, the biomass pellets were re-suspended in 1 mL of ethanol and sonicated for 10 minutes. After centrifugation, the resulting supernatant was analyzed by HPLC. No DTP signal was detected.

2.3 Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of ethanol and 1-butanol

~~The MIC of ethanol and 1-butanol were determined after incubation of *S. algeriensis* culture broth, in 96 well-plates using the Müller-Hinton medium, in presence of serially diluted alcohols. The MIC of ethanol and 1-butanol against *S. algeriensis* were determined using the broth microdilution method described in the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Tenth Edition. CLSI document M07-A10. Wayne, PA: Clinical and Laboratory Standards Institute; 2015). Briefly, alcohols were loaded into the wells of a sterile 96-wells microtiter plate and subjected to two-fold serial dilutions with Müller Hinton medium (Biomérieux, Craponne, France) to achieve final concentrations of alcohols ranging from 64% to 1% v/v.~~

~~The microtiter plates were inoculated with *S. algeriensis* preculture (10⁶ CFU·mL⁻¹), to obtain a final concentration of 10⁵ CFU·mL⁻¹. The 96-well plates were incubated for 5 days at 30°C. Cell suspensions were added into all wells except outer wells which were used as sterility control. Growth wells (with bacteria and without test solution) were also included. The plates were prepared in triplicate.~~

~~MBC were determined by sub-culturing the *S. algeriensis* culture broth from the MIC determination experiment (wells without visible growth) on ISP2 agar plates. MBC was determined by sampling 100 μ L~~

of culture broth from every well in which no bacterial growth could be observed during the MIC determination experiment and then plating the aliquots onto ISP2 agar plates. MBC for each alcohol was determined as the lowest alcohol concentration for which no bacterial growth could be observed in those subcultures after a 5-day incubation at 30°C.

~~The MIC corresponds to the lowest alcohol dilution at which no bacterial growth was visible after 5 days incubation on micro-plates at 30°C. MBC is the lowest dilution of alcohol that prevents the growth of sub-cultured *S. algeriensis* after 5 days incubation on the ISP2-plate at 30°C.~~

2.4 Statistical analysis

All results are expressed as the mean values of three independent experiments carried out in triplicate. The error bars indicate the standard deviation (SD) from the mean value. Data were analyzed using Wilcoxon signed-rank test and **Duncan's multiple comparison analysis** on R Statistical Software Version 1.0.136 2009-2016 © RStudio, Inc. The difference between contrasting treatments was considered significant when $p < 0.05$.

3. Results and discussion

3.1 Effect of non-cytotoxic ethanol concentrations on biomass and DTP production

First, the MIC and MBC of ethanol were determined, corresponding to 16% and 32% v/v respectively (equivalent to 2.74 M and 5.48 M). Subsequent experiments were carried-out with sub-MIC concentrations of ethanol, ranging from 10 to 685 mM, added initially to the culture medium. The effect of ethanol concentrations on biomass and DTP production, after 5 days of incubation, are reported in Fig. 2a. The weight of biomass decreased significantly when ethanol concentration increased. When the ethanol concentration was 4% v/v (685 mM) the yield of biomass was decreased five-fold (to 0.30 ± 0.07 g·L⁻¹), as compared to the non-supplemented control culture (1.50 ± 0.45 g·L⁻¹). There is also a dose-dependent relationship between ethanol concentrations and DTP production, which rises from 31 ± 7 mg·L⁻¹ of culture (control) to reach a maximum at ~~190 ± 45~~ 231.2 ± 32 mg·L⁻¹ of culture in the presence of 3% v/v of ethanol (514 mM). At higher ethanol concentrations (4% v/v), the DTP production was further diminished, to 115 ± 11 mg·L⁻¹ of culture, evidencing the negative impact of high ethanol concentration levels on DTP biosynthesis.

Similar dose-dependent inductions of antibiotics in presence of ethanol under the appropriate conditions were observed in cultures of other bacterial strains such as *Streptomyces hygroscopicus* 5008 or *Streptomyces venezuelae* ISP5230 [13–14] [15].

3.2 Effect of non-cytotoxic 1-butanol concentration levels on biomass and DTP production

The MIC / MBC were also determined: 4 and 8 % v/v, respectively, which correspond to 430 mM and 870 mM). These values are much lower than the ones found with ethanol. In the subsequent experiences *S. algeriensis* was incubated for 5 days in presence of 1-butanol at final concentrations of 10, 50 and 100 mM, which correspond to 0.09, 0.45 and 0.9 % v/v, respectively (Fig 2b).

At all concentrations tested, 1-butanol had a significantly negative impact on biomass production, yields being about 30% lower than in control cultures. At 10 mM and 50 mM the DTP concentration remains stable, at concentration level similar to that of the control (about 30 mg·L⁻¹) despite the biomass decline. However, at 100 mM, DTP concentration drops dramatically to 4.4±0.4 mg·L⁻¹. These observations may indicate that 1-butanol is more toxic to *S. algeriensis* than ethanol, resulting in a decline of metabolic activity. As 1-butanol is more hydrophobic than ethanol, it may penetrate easily into the cytoplasmic membranes and alter the membrane structure, interfering with vital functions of the bacterium [19 20].

~~3.3 Effect of simultaneous addition of ethanol and 1-butanol on biomass and DTP production~~

The simultaneous addition of 300 mM of ethanol and 10 mM or 50 mM of 1-butanol to the culture medium at 0 h was tested for a potential synergistic effect of both alcohols on the DTP production (see Fig. S1 in Supplementary data). ~~After the simultaneous addition of both alcohols, the production of biomass was reduced, as compared with the control unexposed to any alcohol, but was similar to yield obtained from the culture supplemented with 300 mM ethanol only (Fig. 3a). The DTP production increased slightly only in presence of 10 mM of 1-butanol with 300 mM ethanol, as compared to the unsupplemented control (Fig. 3b). In conclusion,~~ The combination of both alcohols entails no observable benefit in respect to the production of DTP. These results highlight the detrimental effect of 1-butanol on *S. algeriensis* secondary metabolism.

~~3.4~~ 3.3 Effect of the time of ethanol supplementation on biomass and DTP production

Under standard culture conditions, *S. algeriensis* growth includes two exponential phases of growth interrupted by two stationary phases related to the DTP biosynthesis [20 21]. The DTP production starts after the first growth period (*i.e.*, after about 15 h of culture) when the first amino acid pools are depleted, and persists for approximately 20 h. After 40 h of culture, the second growth phase takes place, and the second phase of DTP production coincides with the depletion of the glucose pool (after about 90 h of culture). Hence, in order to investigate the impact of ethanol on the different growth phases, the alcohol was added to the medium at 0 h (at the start of the first exponential phase), at 24 h (during the first phase of DTP biosynthesis) or at 48 h (at the early stage of the second exponential phase) after the beginning of culture fermentation.

For these experiments, a final ethanol concentration of 300 mM was used. First, to assess if ethanol evaporated or was enzymatically catabolized during the incubation, ethanol concentration in culture broth was monitored at various time (0 h, 24 h, 48 h, 72 h, 96 h, 120 h) of incubation, both in the presence and in the absence of *S. algeriensis*. In both experiments, the concentration of ethanol in the medium remained stable over 72 h, and decreased slightly afterward (see Fig S2 in supplementary data).

After a 72 h-incubation, the biomass production was significantly altered when ethanol was added at 0 h and 48 h (0.62 ± 0.14 g·L⁻¹ and 0.90 ± 0.10 g·L⁻¹ respectively) and slightly impacted when ethanol was added at 24 h (1.30 ± 0.10 g·L⁻¹) as compared to the control (1.50 ± 0.30 g·L⁻¹) (Fig. 3a and 4b).

Hence, ethanol addition seems to exert a significant positive effect on DTP production if is added at an early stage of culture (at 0 h) (Fig. 3b). In this conditions, after 48 h of culture, the DTP concentration reached 37 ± 7 mg·L⁻¹, more than twice the DTP concentration level observed in control (14.5 ± 2 mg·L⁻¹), and peaked at 148 ± 23 mg·L⁻¹ after 72 h of culture (Fig. 4b 3b). However, the latest additions of ethanol (24 h or 48 h) had no significant effect on DTP production after 72 h of incubation.

Consequently, if ethanol is added at an early stage of culture (at 0 h) the first growth phase could be shortened and the metabolism could be shifted sooner toward the production of secondary metabolites, leading to enhancement of DTP biosynthesis.

The addition of ethanol only after 24 h of culture, during the stationary phase, leads to a slight decrease in biomass yields and to a progressive increase in DTP production after 72 h of incubation as compared to the control (Fig. 4a and 4b 3a and 3b). Thus, we hypothesize that the second growth phase may be either delayed or inhibited. This supposition is supported by the results of the experiments in which ethanol was

added after 48 h of culture, i.e. during the second growth phase. The addition of ethanol at this stage after 48 h of culture, i.e. during the second growth phase, seems to have a deleterious effect on cells, as we observed decrease in biomass without positive effect on DTP production. The inhibition of cell growth and decrease of microbial viability, caused by low concentration levels of ethanol exposure, has already been described for *Staphylococcus aureus* [22]. A similar phenomenon has already been described for *Staphylococcus aureus* cultures exposed to low concentration levels of ethanol [21]. Ethanol inhibited cells growth and decreased the viability of the microorganisms. Moreover, this effect persisted long after ethanol in the culture medium had been.

These findings indicate that the cellular physiological state modifications in *S. algeriensis*, and DTP biosynthesis, seem to be closely related to the time of ethanol addition.

3.5 3.4 Modulation of the inducing effect of ethanol by ROS inhibitors

Finally, we studied a putative mechanism involving ROS by which ethanol may stimulate DTP production. In order to establish whether ethanol promotes ROS production mediated by oxidases or peroxidases, experiments were conducted by combining ethanol with an inhibitor of key ROS-generating enzymes, DPI, which is widely used as an uncompetitive inhibitor of NAD(P)H oxidases [18].

We determined the concentrations of DPI which did not affect cell growth of *S. algeriensis*. DPI was added to the culture medium in final concentrations ranging from 1 μ M to 10 μ M. After a 5-day incubation, no effect on either biomass production or DTP production could be observed for DPI concentrations $\leq 2 \mu$ M.

Then, 1 or 2 μ M of DPI were added to the culture medium at 0 h, in the absence or presence of 300 mM ethanol. As shown in Fig. 5 4 (a, b, white bars) in absence of ethanol, neither biomass nor DTP production were affected in cultures containing DPI. These parameters were similar to those of the control cultures. In accord with previous observations, the biomass yield was reduced by 60% in cultures supplemented with ethanol without ROS inhibitor, and the addition of DPI did not lead to almost complete biomass recovery (Fig. 5a 4a grey bars). As we observed above, the presence of ethanol supports the microbial DTP production. But, the addition of the ROS inhibitor to culture containing ethanol canceled its positive effect on DTP production in dose-dependent manner, resulting in similar level of DTP in culture containing ethanol and 2 μ M of DPI as those observed in non-supplemented control

cultures without ethanol (Fig 5b 4b grey bars). ~~We can assume that~~ DPI could thus prevent at least enzymatic ROS production, induced by ethanol.

Supplementary experience was conducted in presence of ROS scavenger PABA. PABA, at 5 or 10 μ M, was added to the culture medium at 0h, in the absence or presence of 300 mM ethanol. No effect was observed on the biomass and DTP production in cultures containing PABA alone (Fig. 5 a and b, white bars). In the cultures with PABA and ethanol, the biomass yield is more important than in the culture containing ethanol without this ROS scavenger, this yield is close to those of control culture without ethanol (Fig. 5a grey bars).

Presence of PABA in culture medium supplemented with ethanol reduces DTP concentration by about 60 %, with respect to the culture with ethanol alone (Fig. 5 b grey bars). The positive effect on biomass grown could be related to PABA function as a protector of cells against ROS radicals [19]. In this way, PABA by reacting with ROS, the decline in DTP production might be due to the low level of ROS in culture medium.

~~Moreover,~~ These results suggest several roles of ethanol in *S. algeriensis* culture: the deleterious effect on cell growth and elicitation of DTP biosynthesis, via ROS. These species have been shown to be toxic but also have role as signalling molecules to control microbial behavior, allowing for adaptation to stress injury [17] [16].

4. Conclusion

This study revealed that sub-toxic concentrations of ethanol ($\leq 3\%$ v/v) could be beneficial to the DTP production by *S. algeriensis*, in a dose-dependent relationship. Ethanol promotes the generation of ROS via NAD(P)H oxidases, and, as a result, microbial stress, leading to DTP overproduction. Further research should allow us to evaluate the impact of ethanol on the expression of genes implicated in DTP biosynthesis.

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Figures captions

Fig. 1

Structures of some dithiolopyrrolones

Fig. 2

Effect of ethanol (a) or 1-butanol (b) addition on *S. algeriensis* biomass (white bars) and DTP concentrations (grey bars) after 5 days of incubation. Results are expressed as means and standard deviations of triplicate experiments. Statistical differences (*, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$) between test compound and its respective control (n=9)

Fig. 3

~~Comparison between effect of 1 butanol alone (white bars) and a combination of 1 butanol with 300 mM ethanol (grey bars) on *S. algeriensis* (a) biomass and (b) DTP concentrations after 5 days of incubation. Results are expressed as means and standard deviations of triplicate experiments. Statistical differences (*, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$) between test compound and its respective control (n=9)~~

Fig. 4 Fig. 3

Effect of ethanol time addition on *S. algeriensis* (a) biomass and (b) DTP concentration after 3 days of incubation. Ethanol final concentration 300 mM (1.74% v/v). Results are expressed as means and

standard deviations of triplicate experiments. Statistical differences (*, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$) between test compound and control (n=9)

Fig. 4

Effect of ethanol alone (white bars) or in the presence of ROS-inhibitor (DPI) (grey bars) on *S. algeriensis* (a) biomass and (b) DTP concentrations after 5 days of incubation. Results are expressed as means and standard deviations of triplicate experiments. Statistical differences (*, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$) between test compound and control, similar letters over the bars represent homogeneous mean groups (n=9)

Fig. 5

Effect of ethanol alone (white bars) or in the presence of ROS scavenger (PABA) (grey bars) on *S. algeriensis* (a) biomass and (b) DTP concentrations after 5 days of incubation. Results are expressed as means and standard deviations of triplicate experiments. Statistical differences (*, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$) between test compound and control, similar letters over the bars represent homogeneous mean groups (n=9)

Fig. 1

R1: H	R2: H	Holothin
R1: H	R2: CH ₃	Holomycin
R1: CH ₃	R2: CH ₃	Thiolutin

Fig. 2

Fig. 3

Figure deleted:

Fig. 4 Fig. 3

Fig. 5

Fig. 5