Saccharothrix algeriensis NRRL B-24137, the first non-*Streptomyces* actinobacterium, produces holomycin after cystine feeding

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Abstract

Saccharothrix algeriensis NRRL B-24137 is an actinobacterium isolated from Algerian Saharan soil. This strain has the ability to produce several dithiolopyrrolone antibiotic derivatives depending on the precursors added to the culture medium. This group of antibiotics is known for their potent antimicrobial and anticancer activities. Holomycin is a member of the dithiolopyrrolone group of antibiotics, and has already been isolated from several species of actinobacteria belonging to the genus *Streptomyces* and also from some Gram-negative bacteria. In this study, holomycin was produced for the first time in the culture broth of a non-*Streptomyces* actinobacteria. This antibiotic was induced by adding 5 mM of L-cystine as precursor to the semi-synthetic fermentation broth of *Sa. algeriensis* NRRL B-24137 and then fully identified after HPLC purification. The minimum inhibitory concentrations (MIC) of holomycin were determined against several pathogenic microorganisms, including *Escherichia coli* ATCC 10536 *Klebsiella pneumoniae* CIP 82.91, *Listeria monocytogenes* CIP 82110, *Staphylococcus aureus* CIP 7625, *Aspergillus carbonarius* M333, *Fusarium culmorum* FC1, *Candida albicans* IPA 200. This antibiotic showed a broad-spectrum antimicrobial activity, inhibiting a variety of Gram-positive and Gram-negative bacteria, and micro-fungi.

Keywords Dithiolopyrrolone antibiotics · Holomycin · *Saccharothrix algeriensis* NRRL B-24137 · L-cystine · Antimicrobial activity

Introduction

Dithiolopyrrolones, which possess a unique pyrrolinono-dithiole skeleton linked to two variable acyl groups, are a group of natural antibiotics that have showed strong broad-spectrum activities towards various Gram-positive and Gram-negative bacteria, protozoa, yeast, pathogenic micro-fungi, and also against different cell lines involved in human cancers (Jimenez et al. 1973; Oliva et al. 2001; Li et al. 2014; Merrouche et al. 2017). Thiolutin was shown to potently inhibit developmental of angiogenesis in zebrafish and vascular outgrowth from tissue explants in 3D cultures by inhibition of endothelial cell adhesion (Jia et al. 2010). In the context of emerging antibiotic-resistant microorganisms, holomycin, one of the three subgroups of dithiolopyrrolones, received great interest regarding its activity against rifamycin-resistant bacteria as well as against methicillin-resistant *Staphylococcus aureus* N315 (Qin et al. 2013). This activity was determined to be involved in the inhibition of bacterial RNA polymerase

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(Oliva et al. 2001; Qin et al. 2013). Holomycin was first discovered in *Streptomyces griseus* (Ettlinger et al. 1959) and later was reported to be isolated from a number of *Streptomyces* species (Okamura et al. 1977; Kenig and Reading 1979; Ding et al. 2017; Wei et al. 2017; Zhang et al. 2017). The Gram-negative bacteria *Photobacterium halotolerans* and *Yersinia ruckeri* were also found to produce holomycin (Wietz et al. 2010; Qin et al. 2013).

Saccharothrix algeriensis NRRL B-24137 is a rare actinobacterium first isolated from the soil of a palm grove in Southern Algerian Sahara (Zitouni et al. 2004). This strain was shown to produce several dithiolopyrrolones depending on the incorporated precursors available in the culture medium. Five dithiolopyrrolone derivatives were produced in complex ISP2 medium (thiolutin, senecioyl-pyrrothine, tigloyl-pyrrothine, iso-butyryl-pyrrothine and butanoyl-pyrrothine) (Lamari et al. 2002a, b). Interestingly, the selection of appropriate sources of carbon and nitrogen was shown to have a significant effect on the production levels of these five known dithiolopyrrolones, and also on the induction of biosynthesis of new dithiolopyrrolone derivatives in fermentations that had been supplemented with valeric, benzoic or cinnamic acids (Bouras et al. 2006a, b, 2007, 2008). Thus, eight novel dithiolopyrrolones have been produced and characterized after addition of different carbon sources in culture broth of *Sa. algeriensis* (valeryl-pyrrothine, isovaleryl-pyrrothine, formyl-pyrrothine, crotonyl-pyrrothine, sorbyl-pyrrothine, 2-hexonyl-pyrrothine, 2-methyl-3-pentenyl-pyrrothine and benzoyl-pyrrothine) (Merrouche et al. 2010, 2011, 2019).

Through early feeding experiments, L-cystine appeared to be the precursor of dithiolopyrrolone biosynthesis (Furumai et al. 1982), but the biosynthesis pathway remained poorly understood until the last decade. Works on *Sa. algeriensis* NRRL B-24137 have determined that the transfer of the acyl group from acyl-CoA to a pyrrothine core, during dithiolopyrrolone biosynthesis, was linked to enzymatic activity of pyrrothine *N*-acyltransferase (Chorin et al. 2009; Saker et al. 2014). De la Fuente et al. (2002) has also shown that an *N*-acetyltransferase could be involved in the holomycin biosynthesis in cell-free extracts of the holomycin producer, *Streptomyces clavuligerus*. More recently, the gene cluster for holomycin biosynthesis was first characterized in *S. clavuligerus* ATCC 27064 (Li and Walsh 2010) and later in *Pseudoalteromonas* sp. (Fukuda et al. 2011) and *Yersinia ruckeri* (Qin et al. 2013).

The present study showed that the addition of 5 mM of L-cystine in a semi-synthetic medium as a nitrogen source has allowed the induction of holomycin biosynthesis by *Sa. algeriensis* NRRL B-24137. This antibiotic has been isolated for the first time from a non-*Streptomyces* genus member of actinobacteria.

Materials and methods

Producing strain

Saccharothrix algeriensis NRRL B-24137 was grown and maintained at 4 °C on slants of ISP2 (International *Streptomyces* Project 2) solid medium (Shirling and Gottlieb 1966). The pH of the medium was adjusted to 7.0 with 2-M NaOH before autoclaving at 120 °C for 20 min.

Fermentation conditions

The basal semi-synthetic medium (SSM) developed by Bouras et al. (2006a) was used for the antibiotic production. This medium consisted of 10-g D-glucose (Fisher Scientific, Illkirch, France), 2-g $(NH_4)_2SO_4$ (Prolabo, Paris, France), 2-g NaCl (Fisher Scientific), 0.5-g KH_2PO_4 (Acros, Geel, Belgium), 1-g K_2HPO_4 (Acros), 0.2-g $MgSO_47H_2O$ (Acros), 5-g $CaCO_3$ (Prolabo) and 2-g yeast extract (Difco, Detroit, MI, USA) in 1-L distilled water. The pH of the medium was adjusted to 7 using 2-M NaOH before autoclaving. L-cystine (Fluka, Buchs, Switzerland) was autoclaved separately and added to the basal SSM at a concentration of 5 mM which is the optimal concentration for antibiotic production as reported by Bouras et al. (2007). A mature slant culture of *Sa. algeriensis* was used to inoculate autoclaved 500-mL Erlenmeyer flasks, containing 100 mL of basal SSM. Concurrently, a control (SSM without L-cystine) was also prepared. The cultures were incubated on a rotary shaker (240 rpm) at 30 °C for 10 days.

Kinetics of growth, pH, antimicrobial products and antibiotics production

The changes in dry cell weights (DCWs), antimicrobial activity, pH, and antibiotic production were examined daily during 10 days of fermentation. DCWs were determined as described by Bouras et al. (2006a), briefly, the DCWs were determined by centrifuging (Microlitre Centrifuges, Heraeus Instruments, Biofuge, Hanau, Germany) 4 mL of homogenized culture broth in pre-weighed Eppendorf tubes for 10 min at 16,000×*g*. The pellet was washed three times with HCl (0.35 N, Sigma-Aldrich, Saint-Quentin-Fallavier, France) to eliminate $CaCO_3$, followed by distilled water. The Eppendorf tubes containing pellet were dried at 105 °C for 24 h, cooled in a desiccator, and weighed. The results were expressed as gram per liter. The pH value was measured with a pH meter (Consort C 832, Consort, NY, USA). The activity against *Bacillus subtilis* ATCC 6633 and *Umbelopsis ramanniana* NRRL 1829, used as test microorganisms, was regularly recorded each day by the agar diffusion

method. Briefly, the microbial suspensions were prepared in sterile distilled water and adjusted at a concentration of 1.2×10^8 CFU/mL and 0.4×10^6 CFU/mL for bacteria and micro-fungi, respectively. A volume of 20-mL nutrient agar (Difco) was inoculated with 20 µL of the considered microbial suspension and then poured into a Petri dish. After solidification, wells of 10 mm diameter were aseptically bored into the culture medium, and 200 µL of the culture supernatant was loaded in each well. The antimicrobial activities were evaluated by measuring the diameter of inhibition corresponding to the clear zones around the wells after 24 h and 48 h of incubation for bacteria and micro-fungi, respectively.

Concomitantly, the quantification of the induced dithiolopyrrolones was performed daily using a thiolutin standard calibration curve, since the molar extinction coefficient (ε) of thiolutin is nearly the same for all others dithiolopyrrolones ($\varepsilon 390 = 8317–9333$ L/mol/cm) as described by Lamari et al. (2002b). All tests were repeated two times from two separate cultures.

HPLC analysis and purification of the induced compound

The analysis of antibiotics induced by addition of L-cystine (at 5 mM) in the SSM was carried out according to the procedure described earlier by Merrouche et al. (2011). After mycelium was separated, the culture filtrate broth was extracted with an equal volume of dichloromethane (Fisher Scientific) and the organic layer was dried with anhydrous sodium sulfate (Acros). The extract was concentrated to dryness under vacuum on a Rotavapor (Laborata 4000, Heidolph, Illkirch, France), and then re-dissolved in 1 mL of methanol (Fisher Scientific) as crude extract. The analysis of antibiotics induced by addition of L-cystine in the SSM was carried out by a HPLC system equipped with a C18 reverse phase column (Zorbax SB, 150 × 4.6 mm; BioTek Instruments, Milan, Italy). The crude extracts were analyzed by linear gradient elution using methanol–water as mobile phase. The methanol separation gradient was 0–30% for 5 min, then 30–100% for 25 min with a flow rate of 0.8 mL/min. The formation of the new dithiolopyrrolone analog was monitored by comparison of the peak retention times and UV spectra with those of already known dithiolopyrrolone standards (produced by *Sa. algeriensis*) for distinction, since appearing dithiolopyrrolone products could be easily detected by HPLC analysis due to their intense absorption at 390 nm (Lamari et al. 2002b; Bouras et al. 2008).

To obtain a sufficient pure quantity of the induced new compound, for subsequent characterization, cultures were combined to obtain 15 l. The culture broth was centrifuged to remove the biomass and extracted with an equal volume of dichloromethane on the seventh day of fermentation. 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, 227 Waters, Milford, MA). The analysis was done using a linear gradient of methanol–water (20–100% for 30 min), as the mobile phase, with a flow rate of 1.5 mL/min. The detection of compounds was carried out at 390 nm.

Chemical characterization of induced compound

The UV spectrum was determined with a Shimadzu UV1605 spectrophotometer (Shimadzu, Kyoto, Japan). The molecular weight of holomycin was obtained by electron impact MS (EI-MS) recorded at 70 eV with a Nermag R-10-10C spectrometer. The nuclear magnetic resonance (NMR) sample was prepared by dissolving the pure molecule cyst1 in 600 mL of CD_2Cl_2. 1D ^1H experiment was 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 are in comparison with tetramethylsilane (TMS) using ^1H (residual) of the solvent as a secondary standard. The temperature was set at 298 K.

Antimicrobial MIC of induced compound

The MIC of the induced antibiotic was determined by a conventional agar dilution method (Oki et al. 1990). Twenty target micro-organisms (Table 1) were used and inoculated onto nutrient agar medium containing different concentrations of the purified induced antibiotic compound (1, 2, 3, 5, 8, 10, 15, 20, 25, 30, 40, 50, 60, 80 and 100 µg/mL). The antimicrobial activity was observed after a 24–48-h incubation at 37 °C for bacteria and a 48–72-h incubation at 28 °C for micro-fungi and yeasts. Medium without the antibiotic compound and inoculated with target microorganisms was used as a control.

All the chemicals used were supplied at a purity level ≥ 99%.

Results and discussion

Effect of L-cystine addition on growth, pH, antibiotics production and antimicrobial activity in *Sa. algeriensis*

To evaluate the effect of 5-mM L-cystine supplementation to SSM, the kinetic evolution of fermentation parameters (antibiotics production, DCW and pH) was monitored in comparison to non-supplemented control medium (Fig. 1) along with the analysis of the produced antibiotics by HPLC (Fig. 2).

Table 1 Minimum inhibitory concentrations (μg/mL) of holomycin antibiotic produced by *Saccharothrix algeriensis* against several microorganisms

Target organism	MIC (μg/mL)
Agrobacterium tumefaciens 2410 LB*	80
Escherichia coli ATCC 10536	75
Klebsiella pneumoniae CIP 82.91	8
Salmonella enterica CIP 81.3	80
Pseudomonas aeruginosa CIPA22	60
Bacillus subtilis ATCC 6633	8
Bacillus coagulans CIP 6625	8
Listeria monocytogenes CIP 82110	2
Micrococcus luteus ATCC 9314	5
Staphylococcus aureus CIP 7625	3
Aspergillus carbonarius M333*	8
Fusarium oxysporum f. sp. *lini* Foln 3*	20
Fusarium moniliforme FM1*	8
Fusarium equiseti FE1*	10
Fusarium culmorum FC1*	30
Fusarium graminearum FG1*	30
Umbelopsis ramanniana NRRL 1829	10
Penicillium expansum PE1*	80
Candida albicans IPA 200	80
Saccharomyces cerevisiae ATCC 4226	60

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

Compared to the control, the antimicrobial activity started earlier, at the 4th day of fermentation, in the presence of L-cystine against both *Bacillus subtilis* ATCC 6633 and *Umbelopsis ramanniana* NRRL 1829. Moreover, the *Sa. algeriensis* antimicrobial activity seemed better after the addition of L-cystine, and reached its maximum between the 7th and 8th days of fermentation (Fig. 1a).

With regard to retention times and UV characteristics of HPLC-obtained fractions profile from the 7-day-old crude fermentation extract, *Sa. algeriensis* NRRL B-24137 was shown to produce five known dithiolopyrrolones (thiolutin, iso-butyryl-pyrrothine, butanoyl-pyrrothine, tigloyl-pyrrothine and senecioyl-pyrrothine) in the basal control SSM (without addition of precursors) (Fig. 2a) as reported by Lamari et al. (2002b). Remarkably, addition of 5 mM of L-cystine in SSM induced the appearance of a new peak named Cyst1 (Fig. 2b), which has a different retention time of 11.10 min in comparison to those of known dithiolopyrrolone produced in the control.

The production of the induced compound Cyst1 started at the 2nd day and reached a maximum after 4 days of fermentation, with a production of 0.21 mg/L (Fig. 1b). The L-cystine supplementation triggered also modification in growth kinetic and pH in comparison with the control (Fig. 1c, d). In fact, without supplementation (control

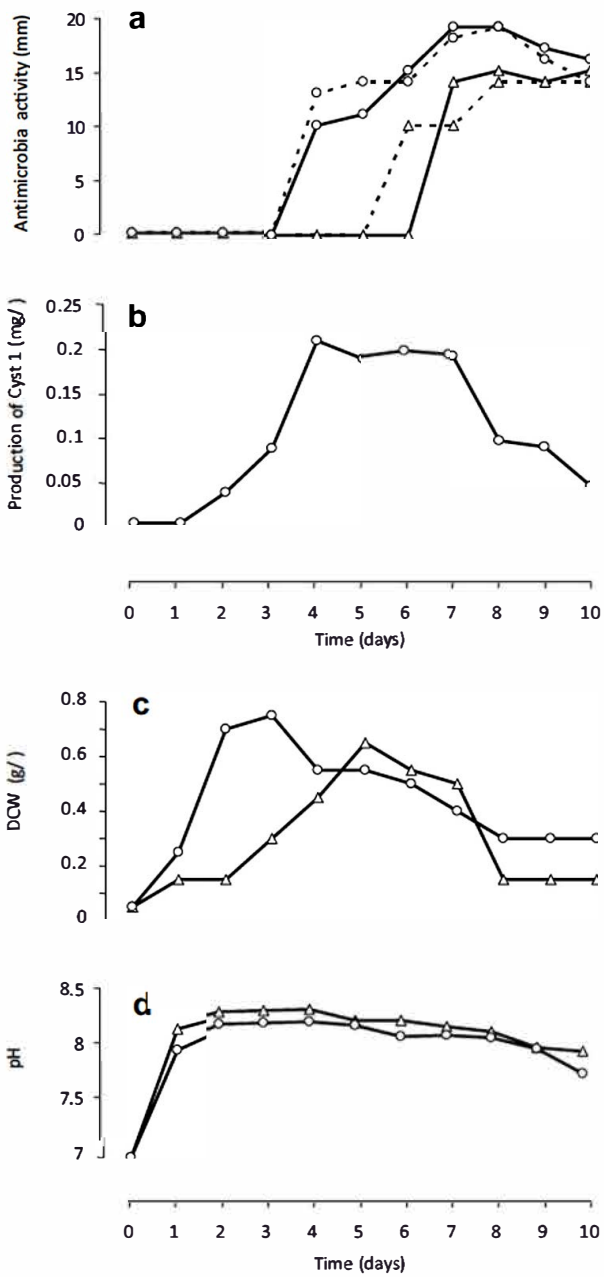

Fig. 1 Effect of L-cystine addition to the SSM on evolution of: **a** antimicrobial activity; **b** production of a new induced dithiolopyrrolone (Cyst1); **c** biomass; and **d** pH. *Umbelopsis ramanniana* NRRL 1829 (line), *Bacillus subtilis* ATCC 6633 (dotted line); (open triangle) SSM without adding L-cystine (control), (open circle) SSM with L-cystine at 5 mM

SS medium), the dry cell weight reached a maximum of 0.65 ± 0.05 g/L after 5 days of fermentation then continuously decreased up to the end of fermentation (10 days). While, addition of L-cystine permitted to obtain a greater maximal biomass (0.75 ± 0.08 g/L) after a fermentation time of 3 days. Moreover, the final pH at the end of

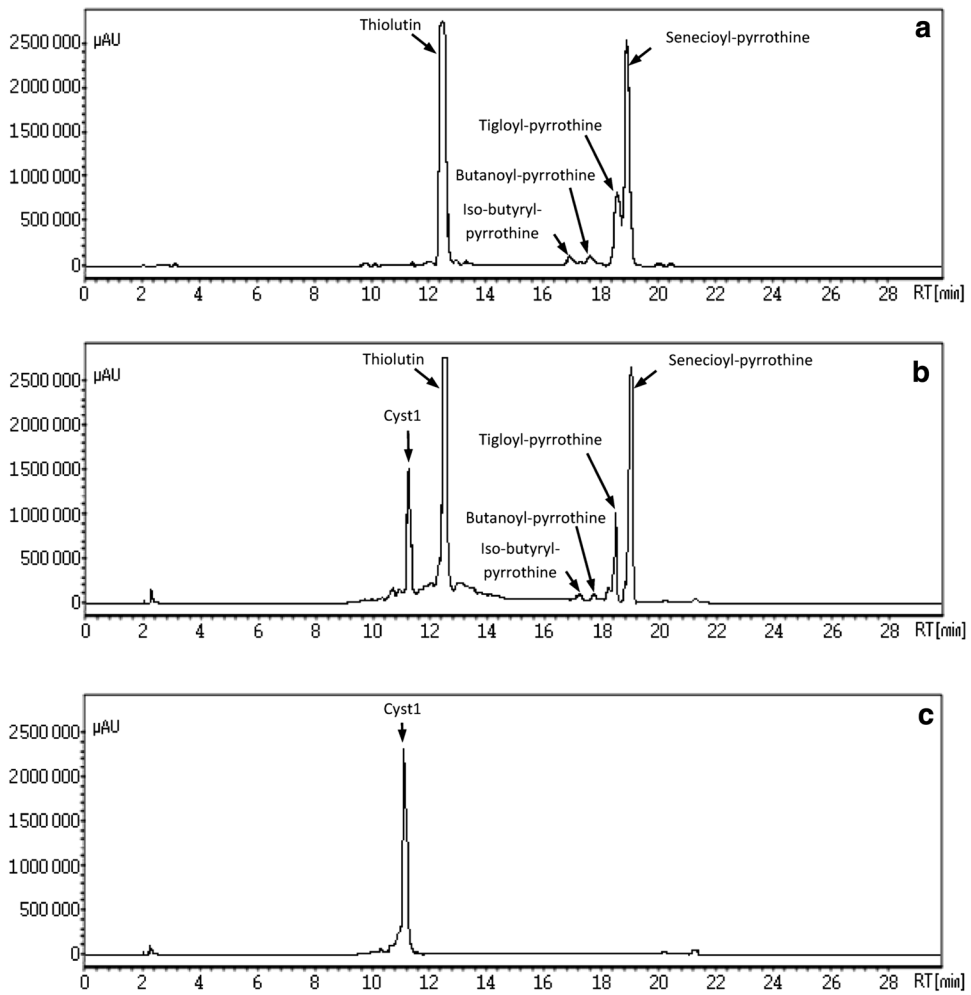

Fig. 2 Effect of L-cystine addition to the SSM on dithiolopyrrolones production displayed by HPLC analysis. **a** On SSM without L-cystine (control). **b** After addition of 5-mM L-cystine to SSM. **c** Purification of the compound Cyst1 after 2 successive re-injections. HPLC analysis at 390 nm was done on dichloromethane extract obtained from an 8-day-old *Sa. algeriensis* culture. Formation of dithiolopyrrolone analogs was monitored by comparison of the peak retention times and UV (390 nm) spectra with those of known dithiolopyrrolone standards. The retention times were as follows: Cyst1, 11.10 min; thiolutin, 12.50 min; iso-butyryl-pyrrothine, 16.80 min; butanoyl-pyrrothine, 17.60 min; tigloyl-pyrrothine, 18.50 min; senecioyl-pyrrothine, 18.80 min

fermentation seemed slightly less alkaline for L-cystine supplemented conditions in comparison to control.

These results were in agreement with the previous findings that demonstrated for all the described *Sa. algeriensis*-derived dithiolopyrrolones a production in the decline phase of growth with alkaline pH condition (Lamari et al. 2002a; Merrouche et al. 2011, 2019).

Purification and characterization of induced compound

After 7 days of fermentation in L-cystine-supplemented SSM, 15 L of the culture broth was extracted by dichloromethane and the yellow organic phase was concentrated to dryness. Through semi-preparative HPLC, the new Cyst1 compound was repetitively recovered and then finally purified after two successive re-injections in the HPLC system (Fig. 2c). This induced compound was subject to spectroscopic and spectrometric analyses to elucidate its structure.

The UV–visible spectrum of Cyst1 showed three absorption maxima at 245, 300 and 384 nm (Supplementary Fig. 1).

Through EI-MS analysis, compound Cyst1 exhibited a mass unit of *m/z* 214 (by EI-MS) (Fig. 3), that is similar to the molecular weight of formyl-pyrrothine, a known dithiolopyrrolone produced by *Sa. algeriensis* (Merrouche et al. 2010). However, in contrast to formyl-pyrrothine, which exhibits a prominent fragment ion at *m/z* 186, Cyst1 showed a prominent fragment ion at *m/z* 172, indicating an extra hydrogen in the heterocyclic ring as reported for other dithiolopyrrolones (Okamura et al. 1977; De la Fuente et al. 2002). While, a prominent fragment ion at *m/z* 186 indicated an extra methyl group in the heterocyclic ring instead of a hydrogen (Merrouche et al. 2010).

By the analysis of the ^{1}H NMR spectrum (Supplementary Fig. 2), it is possible to discern one olefinic group (δ^{H} 6.83) and NH groups (δ^{H} 8.03/7.93) typical of dithiolopyrrolone derivatives. Furthermore, compound Cyst1 shows one additional ^{1}H methyl signal (δ^{H} 2.14). However, a signal at 3.4 ppm corresponding to a methyl linked to Nitrogen of the cycle (N–CH_3) was not observed. The methyl was replaced by a hydrogen (N–H). The ^{1}H chemical shifts of the compound were as follows: ^{1}H NMR (CD_2Cl_2, 500 MHz) δ

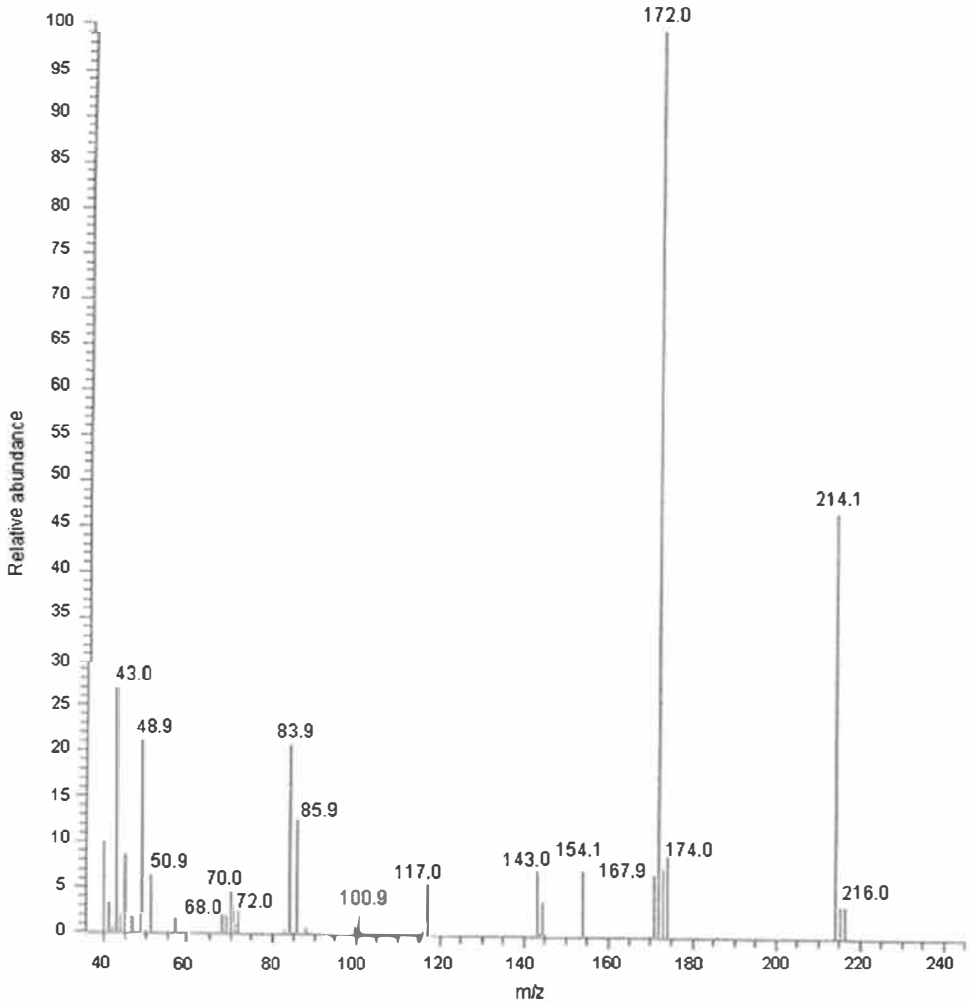

Fig. 3 Electron impact mass spectrum of the purified Cyst1 antibiotic compound

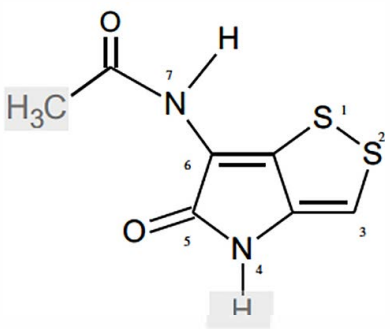

Fig. 4 Structure of dithiolopyrrolone Cyst 1 induced by adding L-cystine. Chemical groups specific to holomycin and discriminating from other dithiolopyrrolones have been indicated with a gray background

8.03/7.93 (1H, br s, N–H), δ 6.83 (1H, s, H3), δ 2.14 (3H, s, CH_3).

Based on the UV, MS and NMR results, the compound Cyst1 was identified as holomycin (Fig. 4), a dithiolopyrrolone derivative identified for the first time in *Sa. algeriensis* and more generally in a non-*Streptomyces* strain.

Accordingly, this study showed that the addition of 5 mM of L-cystine in SSM as precursor induced biosynthesis of holomycin by *Sa. algeriensis*. Bouras et al. (2006a, b) have already suggested that sulfur-containing amino acids, such L-cystine, can serve as a tool for studying the mechanism of dithiolopyrrolones biosynthesis in *Sa. algeriensis*.

As known, *Sa. algeriensis* has a remarkable ability to produce a wide range of dithiolopyrrolone derivatives depending on the composition of the culture medium, and both the nature and concentration of precursors added (Lamari et al. 2002b; Bouras et al. 2006a, b, 2007). This extraordinary flexibility of the dithiolopyrrolone biosynthetic pathway in *Sa. algeriensis* may be rendered by the enzymatic system, which was reported to be responsible for this diversity (Saker et al. 2014). This property was explored by introduction of various organic acids into fermentation media and led to generate 10 new dithiolopyrrolone analogs by

precursor-directed biosynthesis (PDB) process in *Sa. algeriensis* (Bouras et al. 2008; Merrouche et al. 2010, 2011, 2019).

The PDB is the simplest derivatization of a secondary metabolite by feeding biosynthetic precursor analogs into the fermentation broth of the producing microorganisms (Thiericke and Rohr 1993). Moreover, PDB provides a potent method to introduce non-native starting materials into biosynthetic pathways. It should be possible to reprogram the biosynthesis of natural products to produce new molecules with altered and possibly improved biological activities. PDB has proven to be a powerful tool for the rational synthesis of structural analogs of erythromycin and sansanmycin (Cane et al. 2002; Zhang et al. 2016) and for the production of borrelidin analog in *Streptomyces rochei* MB037, which presented low cytotoxicity and a potent inhibition of angiogenesis (Li et al. 2018).

MIC of induced compound

The antimicrobial activity of the pure antibiotic compound Cyst1 is shown in Table 1. This antibiotic showed a broad antibacterial spectrum, inhibiting a variety of Gram-positive and Gram-negative bacteria tested, except *Agrobacterium tumefaciens* 2410 LB, *Salmonella enterica* CIP 81.3 and *Pseudomonas aeruginosa* CIPA22 which were resistant. The latter Gram-negative bacterium has been previously noted to be resistant to holomycin (Oliva et al. 2001; Qin et al. 2013). Cyst1 antibiotic showed a strong to moderate activity against all filamentous fungi tested, the most sensitive micro-fungi were *Aspergillus carbonarius* M333 and *Fusarium moniliforme* FM1 (CMI = 8 µg/mL). This antibiotic lacked activity against the yeasts *Saccharomyces cerevisiae* ATCC 4226 and *Candida albicans* IPA 200, in accordance with Oliva et al. (2001) results, that already reported an absence of holomycin activity against *S. cerevisiae* and *Candida kefyr*.

In conclusion, this work demonstrates that *Saccharothrix algeriensis* NRRL B-24137 produced holomycin, as the first known non-*Streptomyces* actinobacteria exhibiting this ability, in a cystine-amended semi-synthetic culture medium.

Given the great *Sa. algeriensis* ability to produce a wide range of dithiolopyrrolone derivatives depending on the composition of the culture medium, our study used the "one strain many compounds (OSMAC)" approach as a simple and effective method to generate a maximum number of bioactive secondary metabolites from a single well-characterized microbial genome. In accordance, we have observed that *Sa. algeriensis* produces other unknown dithiolopyrrolones when supplied with other different amino acids such as arginine (data not shown). Further studies on the characterization of these new dithiolopyrrolones are currently underway.

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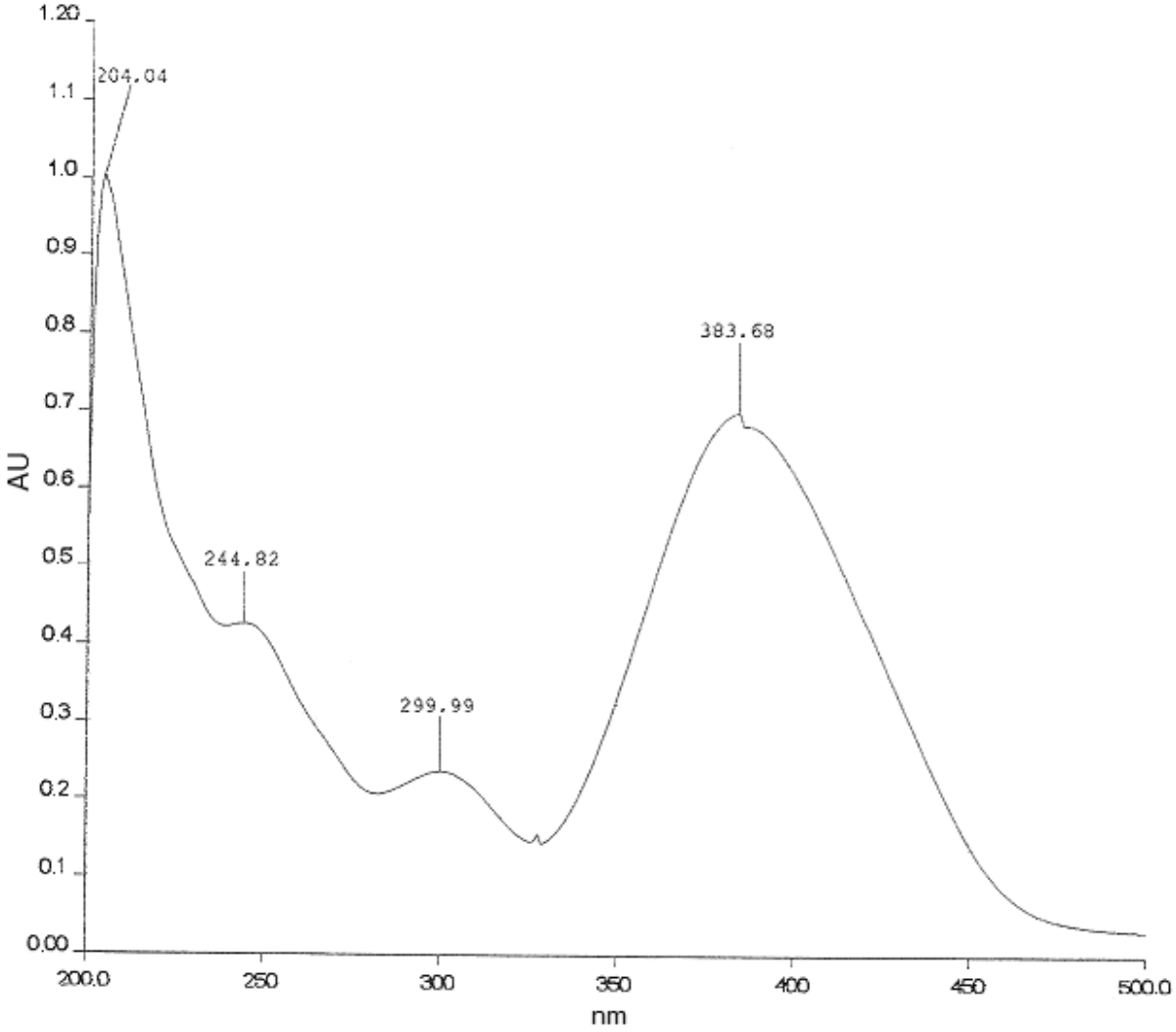
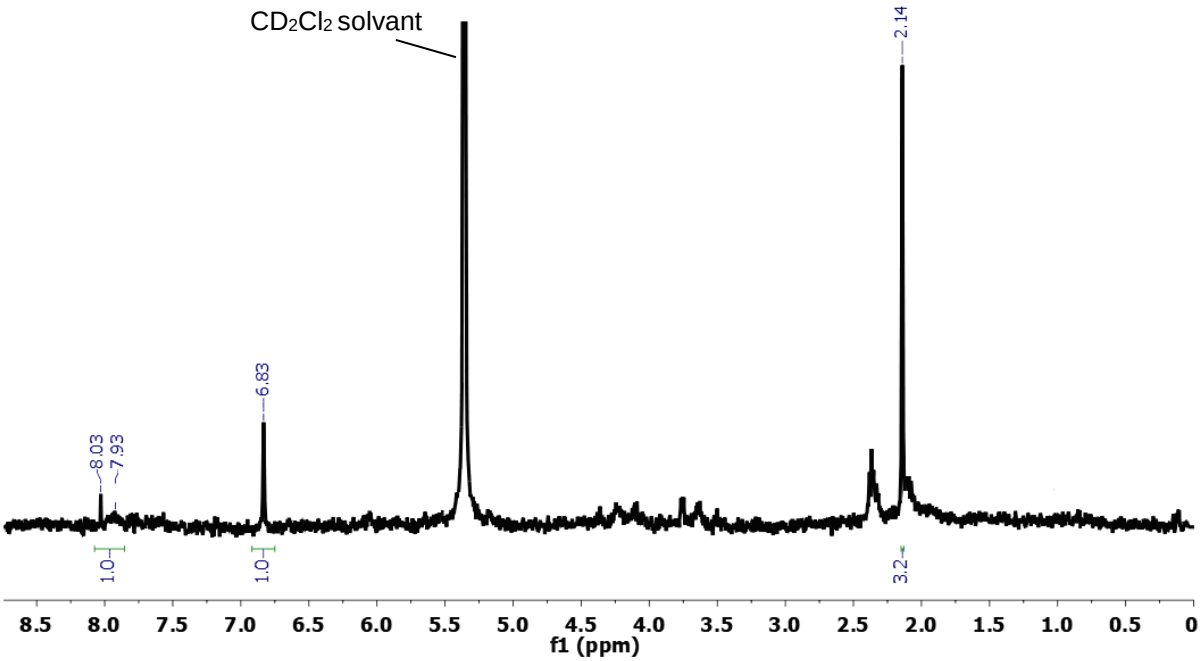
Supplemental Fig. 1 UV spectrum of compound Cyst1.

Supplemental Fig. 2 ^{1}H NMR spectrum of compound Cyst1.



Supplemental Fig. 1



Supplemental Fig. 2