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Taxonomy and chemical characterization of new antibiotics produced by *Saccharothrix* SA198 isolated from a Saharan soil

D. Boubetra^a, N. Sabaou^{a,*}, A. Zitouni^a, C. Bijani^{b,c}, A. Lebrihi^{d,e}, F. Mathieu^e

^a Laboratoire de Biologie des Systèmes Microbiens, Ecole Normale Supérieure de Kouba, Alger, Algeria

^b Laboratoire de Chimie de Coordination (LCC), CNRS, Toulouse, France

^c Université de Toulouse, UPS, INPT, LCC, 205 Route de Narbonne, 31077 Toulouse, France

^d Université Moulay Ismail, Marjane 2, BP 298, Meknes, Morocco

^e Université de Toulouse, INPT-ENSAT, Laboratoire de Génie Chimique, UMR 5503 (CNRS/INPT/UPS), 1 Avenue de l'Agrobiopôle, BP 32607, Auzeville Tolosane 31326, Castanet-Tolosan, France

ABSTRACT

Actinomycete strain SA198, isolated from a Saharan soil sample of Algeria, exhibited antimicrobial activity against Gram-positive and Gram-negative bacteria, and phytopathogenic and toxinogenic fungi. The morphological and chemotaxonomic characteristics of the strain were consistent with those of the genus *Saccharothrix*. Analysis of the 16S rRNA gene sequence of strain SA198 showed a similarity level ranging between 97.2 and 98.8% within *Saccharothrix* species, *S. australiensis* being the most closely related. Two new active products were isolated by reverse HPLC using a C18 column. The ultraviolet-visible (UV-VIS), infrared (IR), mass, and ¹H and ¹⁴C nuclear magnetic resonance (NMR) spectra showed that these products were new bioactive compounds. The minimum inhibitory concentrations of these antibiotics showed a strong activity against fungi and moderate activities against Gram-positive and Gram-negative bacteria.

Keywords:

Actinomycetes

Saccharothrix

Taxonomy

Antibiotics

Antimicrobial activity

1. Introduction

As a result of the increasing prevalence of antibiotic resistant pathogens and the high incidence of fungal infections, the search for novel antibiotics has become more urgent. Among microorganisms, actinomycetes continue to be one of the groups the most investigated by screening for new bioactive molecules. Of all the antibiotics used in practice, more than 90% originate from actinomycetes, which produce about two-thirds of all the bioactive substances of microbial origin discovered (Berdy 2005).

In recent years, the rate of discovery of new antibiotics has decreased in the genus *Streptomyces*, from which a large number of antibiotics was obtained and studied in the past. Therefore, the search for novel compounds can also be concentrated on the isolation of non-*Streptomyces* actinomycetes. The rare actinomycetes thus constitute one of the most attractive sources of new bioactive metabolites and become crucial microorganisms in this context. The relevance of the non-*Streptomyces* actinomycetes in this regard can also be demonstrated by the fact that they also produce many interesting antibiotics, such as *Micromonospora*, *Nocardia*,

Nocardioopsis, *Saccharothrix*, *Actinomadura*, *Actinoplanes*, *Amycolatopsis*, *Saccharopolyspora* and *Streptosporangium* (Genilloud et al. 2011). Selective isolation methods have therefore been developed for taxa that are rarely isolated by conventional dilution plate methods (Hayakawa et al. 1995; Sabaou et al. 1998). The development and massive application of genus-oriented selective isolation methods, mainly applied by industrial researchers, has given significant impetus to the discovery of new microbial products of medical importance (Lazzarini et al. 2001). On the other hand, the exploration of new soils and habitats has been remarkably successful, and several rare actinomycetes have been noted as new sources of antibiotics (Sanglier et al. 1996; Lamari et al. 2002; Zitouni et al. 2004a; Bull and Stach 2007).

The Saharan soils of Algeria, exposed to an arid climate, contain an abundance of actinomycetes, and several rare genera have been detected. The presence of *Saccharothrix* genus was signaled on many samples of these soils (Sabaou et al. 1998; Zitouni et al. 2004b; Zitouni et al. 2005). It has also been found that *Saccharothrix* strains of these soils produce new antibiotics, such as dithiolopyrrolones (Lamari et al. 2002; Merrouche et al. 2011) and anthracyclines (Zitouni et al. 2004a). These promising results emphasize the need to continue the research in this direction.

In the present work, we describe the isolation, from a Saharan soil sample, of a *Saccharothrix* strain designated SA198, and its

* Corresponding author.

E-mail address: nsabaou@yahoo.fr (N. Sabaou).

identification by conventional and molecular methods, and also the production, isolation and characterization of the new antibiotics it provides.

2. Methods

2.1. Strain isolation

The actinomycete strain SA198 was isolated from a Saharan soil sample collected at Tamanrasset in southern Algeria (latitude 22°49'07"N, longitude 5°25'56"E, altitude 1370 m). The dry soil sample was suspended in sterile distilled water and diluted. Aliquots (0.2 ml) of each dilution were spread on the surface of chitin–vitamins agar (Hayakawa and Nonomura 1987) supplemented with actidione (50 µg ml⁻¹). The plates were incubated at 30 °C for 2 weeks.

2.2. Taxonomic studies

2.2.1. Morphological and cultural characteristics

The morphological and cultural characteristics were investigated on various ISP media from the International *Streptomyces* Project (ISP) (Shirling and Gottlieb 1966): yeast extract–malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts starch agar (ISP4), tryptic soy agar (TSA), nutrient agar and Bennett agar (Waksman 1961). The degree of growth, aerial mycelia, pigmentation and other features were recorded after 7, 14 and 21 days of incubation at 30 °C from naked eye examinations. Micromorphological observations were made with light and electron microscopy. The colors of substrate and aerial mycelia and any soluble pigments produced were determined according to the ISCC-NBS centroid color charts (Kelly and Judd 1976).

2.2.2. Chemotaxonomic analysis

Biomass for chemotaxonomic analysis was obtained from culture grown in shake ISP2 medium (Shirling and Gottlieb 1966) and incubated at 30 °C for 5 days. Cells of SA198 strain were harvested by centrifugation, washed in distilled water and hydrolyzed for diaminopimelic acid isomers (Becker et al. 1964), whole-cell sugar pattern (Lechevalier and Lechevalier 1970) and phospholipids (Minnikin et al. 1977) analysis.

2.2.3. Physiological characteristics

The media and procedures used for the determination of physiological features and carbon source utilization were those described by Locci (1989). Strain SA198 was examined for its ability to utilize three amino acids as sole nitrogen sources (alanine, proline and serine), and 22 carbohydrate compounds and nine organic acids as sole carbon sources. Degradation activities of strain SA198 were observed on various media containing 12 organic compounds (adenine, arbutin casein, esculin, gelatine guanine, hypoxanthine, starch, testosterone, tyrosine, Tween 80 and xanthine). Melanoid pigment production was tested on peptone yeast extract-iron agar (ISP6) and tyrosine agar (ISP7) media (Shirling and Gottlieb 1966). The production of nitrate reductase and the growth at different temperatures (37 °C and 45 °C) and pH (5.0 and 9.0), and in the presence of chloramphenicol (30 µg ml⁻¹), erythromycin (15 µg ml⁻¹) and novobiocin (5 µg ml⁻¹), were also studied.

2.2.4. DNA preparation, PCR amplification and sequence analysis

DNA was extracted using the procedure recommended by Liu et al. (2000). PCR amplification of the 16S rRNA gene sequence of strain SA198 was performed using two primers: 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTGTACGACTT-3'). The 16S rRNA gene sequence was amplified by PCR using an Invitrogen Kit. The final 50 µl volume

of reaction mixture contained 1X PCR buffer (10 mM of Tris–HCl, 50 mM of KCl, pH 9.0 at 25 °C), 1.5 mM of MgCl₂, 200 µM of each dNTP, 1 µM of each primer, 1.25 U of Taq DNA polymerase and 1 µl (500 ng) of purified DNA. The amplification was performed on a thermal cycler (STRATAGENE RoboCycler Gradient 96) according to the following profile: an initial denaturation step at 98 °C for 3 min, after which taq polymerase was added, followed by 30 amplification cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min, and a final extension step of 72 °C for 10 min. The PCR product was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining.

The PCR products obtained were sent to the MilleGen Company (Toulouse, France) for sequence determination. The same primers as above and an automated sequencer were used for this purpose. The sequences obtained were compared with sequences present in the public sequence databases and with the EzTaxon-e server (<http://eztaxon-e.ezbiocloud.net/>; Kim et al. 2012), a web-based tool for the identification of prokaryotes based on 16S rRNA gene sequences from type strains.

2.2.5. Phylogenetic analysis

Phylogenetic analyses were conducted using MEGA version 5 (Tamura et al. 2011). The 16S rRNA gene sequence of strain SA198 was aligned using the CLUSTAL W program (Thompson et al. 1994) against corresponding nucleotide sequences of representatives of the *Saccharothrix* genus retrieved from GenBank. Phylogenetic tree was inferred by the maximum-likelihood method (Felsenstein 1981) with Kimura 2-parameter (Kimura 1980) model. Tree topologies were evaluated by bootstrap analysis (Felsenstein 1985) based on 1000 resamplings.

2.3. Antimicrobial activity

The potential activity of the SA198 strain against various microorganisms (bacteria, filamentous fungi and yeasts) was evaluated on ISP2 medium by the streak method. Plates were inoculated with strain culture by a single streak and incubated at 30 °C for 10 days. Later, target microorganisms were seeded in streaks perpendicular to the actinomycete strain (a single streak for each at 90° to actinomycete strain). The antimicrobial activity was evaluated by measuring the distance of inhibition between target microorganisms and actinomycete colony margins, after incubation at 30 °C for 24 h for bacteria and yeasts, and 36 h for filamentous fungi.

2.4. Time course of growth and antibiotic production

Fermentation was carried out in ISP2 broth medium for 10 days in order to select the culture time favorable for antibiotic production from strain SA198. The 500-ml Erlenmeyer flasks containing 100 ml of medium were inoculated with 3 ml of a pre-culture broth of strain SA198 prepared with the same medium and incubated at 30 °C for 2 days. The cultures were incubated on a rotary shaker (250 rpm) at 30 °C for 10 days. Changes in pH, growth (dry weight of mycelium), and antibiotic production were examined daily. The antimicrobial activity in the culture broth was monitored by the conventional agar diffusion assay (well technique) using *Bacillus subtilis*, *Aspergillus carbonarius* and *Mucor ramannianus*. Each 10-mm-diameter well was filled with 0.2 ml of supernatant.

To purify the antibiotics, repeated fermentations were carried out to obtain a total of 7.0 L of culture broth.

2.5. Extraction and purification of antibiotics

The culture broth was centrifuged to remove the biomass. The cell-free supernatant was extracted with an equal volume of

dichloromethane. The organic extract was concentrated to dryness. The resulting dry extract was recovered in 1 ml of methanol and bioassayed against *B. subtilis*, *A. carbonarius* and *M. ramaniannus*.

Preparative chromatography with silica gel plates (Merck ART. 5735, Kiesselgel 60F 254) was used for the partial purification of the antimicrobial products. The crude extract was dissolved in methanol, spotted and developed with an ethyl acetate-methanol mixture (100:15, v/v). The active spots were detected by bioautography (Betina 1973) on silica gel plates seeded with *B. subtilis*, *A. carbonarius* and *M. ramaniannus*. The active compounds were also examined with the naked eye and visualized under UV at 254 nm (absorbance) and 365 nm (fluorescence), and by chemical reagents.

The final purification of the semi-purified antibiotic compounds was determined by Waters high-performance liquid chromatography (HPLC) using a C18 (5 µm) column (200 mm × 10 mm, Waters XBridge). A linear gradient of methanol-H₂O (30–100% for 40 min) was used as the mobile phase. The elution rate was 2 ml/min and the detection was carried out at 220 and 261 nm. Two peaks were collected separately, concentrated and then tested against *B. subtilis* and *M. ramaniannus*.

2.6. Spectroscopy and spectrometry of antibiotics

These analyses were made with the pure antimicrobial compounds. The UV spectrum was determined with a Shimadzu UV1605 spectrophotometer. The infrared (IR) spectrum was measured on a Shimadzu IR 470 using KBr tablets. The mass spectrum was recorded on an LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA) with nanospray ion electro-spray ionization (ESI) source (positive and negative ion mode). The accurate mass spectrometry (HREIMS) was carried out on a GCT Premier System. Nuclear magnetic resonance (NMR) of the proton and carbon 13 was carried out with a Bruker Avance 500 spectrometer equipped with a 5 mm triple resonance inverse Z-gradient probe (TBI ¹H, ³¹P, BB). The NMR sample was prepared by dissolving 3 mg of compound A4 or A5 in 600 µl of CD₃OD. All chemical shifts for ¹H and ¹³C were relative to TMS using ¹H (residual) or ¹³C chemical shifts of the solvent as a secondary standard. The temperature was set at 298 K. All the ¹H and ¹³C signals were assigned on the basis of chemical shifts, spin-spin coupling constants, splitting patterns and signal intensities, and by using ¹H-¹H COSY45, ¹H-¹³C HSQC and ¹H-¹³C HMBC experiments. Gradient-enhanced ¹H COSY45 included 36 scans per increment. ¹H-¹³C correlation spectra using a gradient-enhanced HSQC sequence (delay was optimized for ¹J_{CH} of 145 Hz) were obtained with 120 scans per increment. A gradient-enhanced HMBC experiment was performed allowing 62.5 ms for long-range coupling evolution (240 scans were accumulated). Typically, 2048 t2 data points were collected for 256 t1 increments.

2.7. Minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations were quantified by incorporating known concentrations of antibiotic into solid growth medium using a conventional agar dilution method (Oki et al. 1990). Inocula of six bacteria (*B. subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes* and *Pseudomonas aeruginosa*), six fungi (*Ascochyta fabae*, *A. carbonarius*, *Fusarium culmorum*, *Fusarium equiseti*, *M. ramaniannus* and *Penicillium expansum*) and two yeasts (*Saccharomyces cerevisiae* and *Candida albicans*) were inoculated onto Mueller Hinton medium for bacteria and Sabouraud medium for fungi, containing different concentrations of active products (0.5, 1, 2, 5, 10, 20, 30, 50, 75 and 100 µg ml⁻¹). After a growth period of 24–48 h at 37 °C for bacteria and 48–72 h at 28 °C for fungi, the plates were examined for growth

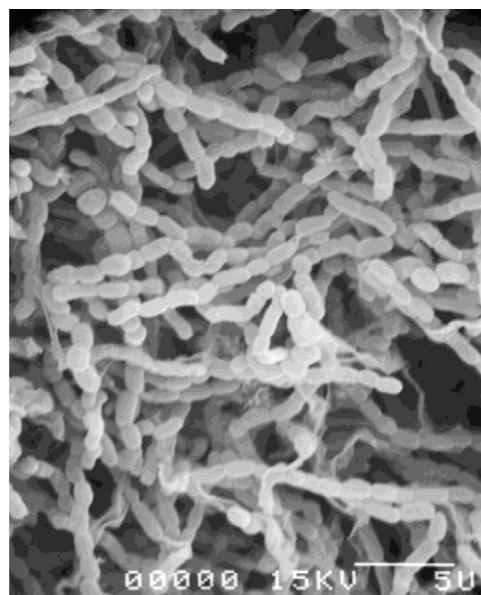


Fig. 1. Scanning electron micrography of spore chains of strain SA198 grown on ISP2 for 10 days at 30 °C. Bar, 5 µm.

and the lowest antibiotic concentration that inhibited the growth of each organism was determined. Mueller Hinton and Sabouraud media, without active products and inoculated with target organisms, was used as a control treatment.

Table 1
Physiological characteristics of the strain SA198.

Tests	Result	Tests	Result
Hydrolysis of		Trehalose	–
Adenine	–	Xylose	–
Arbutin	–		
Casein	+	Utilization of amino acids	
Esculin	+	Alanine	+
Gelatine	+	Proline	+
Guanine	–	Serine	–
Hypoxanthine	+		
Starch	–	Production of melanoid	
Testosterone	–	Pigments on ISP7 medium	+
Tween 80	+		
Tyrosine	+	Decarboxylation of sodium salts	
Xanthine	–	Acetate	+
		Benzoate	–
Utilization of glucides and		Butyrate	–
derivates		Citrate	–
Adonitol	–	Oxalate	–
Arabinose	–	Propionate	+
Cellobiose	+	Pyruvate	+
Fructose	+	Succinate	+
Galactose	–	Tartrate	–
Glucose	+		
Glycerol	–	Nitrate reduction	–
Inositol	–		
Lactose	–	Growth at	
Maltose	–	37 °C	+
Mannitol	–	45 °C	–
Mannose	–	pH 5.0	–
Melezitose	–	pH 9.0	+
Melibiose	–		
Raffinose	–	Growth in the presence of	
Rhamnose	–	antibiotics	
Ribose	+	Chloramphenicol (30 µg ml ⁻¹)	–
Salicin	–	Erythromycin (15 µg ml ⁻¹)	–
Sorbitol	–	Novobiocin (5 µg ml ⁻¹)	+
Sucrose	+		

+, positive test; –, negative test.

3. Results

3.1. Taxonomy

The strain SA198 showed very good growth with production of yellowish white aerial mycelium (MA) on ISP2, ISP3, ISP4 and Bennett media, and moderate to good growth on TSA and nutrient agar media without MA, after 14 days of incubation at 30 °C. The color of substrate mycelium (MS) was strong reddish brown on ISP2, strong yellowish brown on ISP3 and Bennett, light brown on ISP4, light yellow on TSA and light yellowish brown on nutrient agar.

Micromorphological characteristics of strain SA198 were consistent with those of the genus *Saccharothrix*. Its aerial hyphae were straight to irregularly curved, often with a zigzag shape. At the maturation state, these hyphae fragmented irregularly into long non-motile spore chains (Fig. 1). The MS was fragmented into rod-shaped and coccoid elements.

Strain SA198 contained *meso*-diaminopimelic acid but not glycine. Whole-cell hydrolysates contained galactose, rhamnose, ribose and traces of mannose, which are typical of cell-wall type IIIE (Stackebrandt et al. 1994). Diagnostic phospholipids detected were phosphatidylethanolamine and phosphatidylhydroxyethanolmine, corresponding to type PII phospholipids (Lechevalier et al. 1977).

The physiological properties of strain SA198 are shown in Table 1. This strain produced melanoid pigments on the ISP7 medium but not on the ISP6 medium. It was able to degrade casein esculin, gelatine, hypoxanthine, Tween 80 and tyrosine but did not degrade adenine, arbutin, guanine, starch, testosterone, or xanthine. Growth occurred at 37 °C, at pH 9.0 and in presence of novobiocin (5 µg ml⁻¹), but not at 45 °C, at pH 5.0 or in presence of chloramphenicol (30 µg ml⁻¹) or erythromycin (15 µg ml⁻¹). The strain SA198 was able to utilize alanine and proline as nitrogen sources, but not serine. Among the compounds tested, only cellobiose, fructose, glucose, ribose, sucrose, acetate, propionate, pyruvate and succinate were utilized as carbon sources.

The alignment of the 16S rRNA gene sequence (1368 nucleotides) of strain SA198, deposited in GenBank under accession number JN036396, with those of *Saccharothrix* reference species available in the GenBank database can be seen in the maximum-likelihood dendrogram (Fig. 2). The similarity level ranged from 97.2 to 98.8%, with *Saccharothrix australiensis* NRRL B-11239^T (Labeda et al. 1984) having the closest match.

Strain SA198 could also be distinguished from *S. australiensis* by phenotypic properties, such as the color of aerial and substrate mycelia, the production of melanoid pigments on ISP7 medium, the degradation of hypoxanthine and sucrose, the inability to use glycerol, maltose, mannose, sorbitol, or trehalose, and the incapacity to reduce nitrates or to grow at 45 °C.

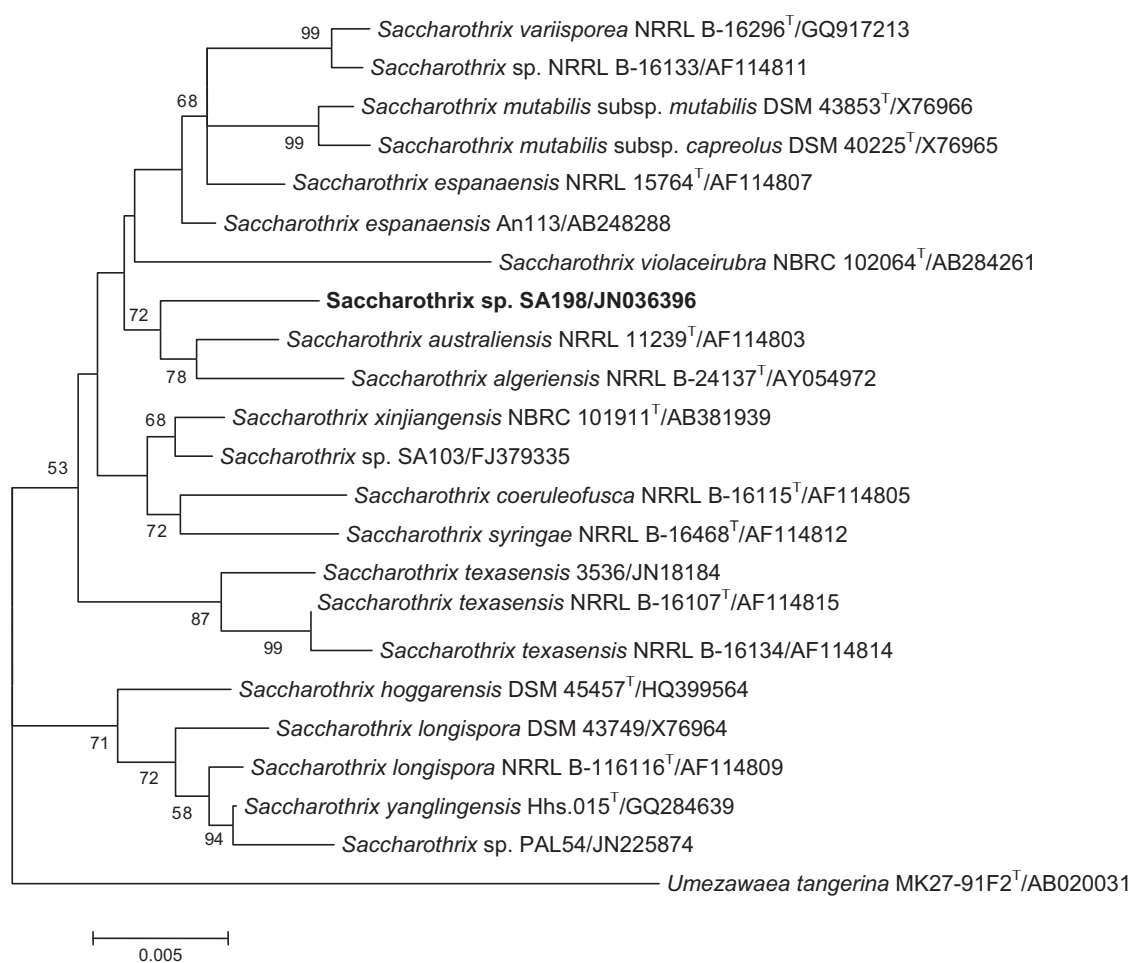


Fig. 2. Maximum-likelihood tree based on 16S rRNA gene sequence showing the relations between strain SA198 and type species of the genus *Saccharothrix*. The numbers at the nodes indicate the levels of bootstrap support based on maximum-likelihood analyses of 1000 resampled data sets (only values >50% are shown). Bar, 0.005 nt substitution per nt position. *Umezawaea tangerina* MK27-91F2^T has been used as outgroup.

Table 2
Antimicrobial activity of the strain SA198 on ISP2 medium.

Test organisms	Distance of inhibition (mm)
Gram-positive bacteria	
<i>Bacillus subtilis</i>	20
<i>Enterococcus faecalis</i>	25
<i>Listeria monocytogenes</i>	09
Gram-negative bacteria	
<i>Escherichia coli</i>	09
<i>Klebsiella pneumoniae</i>	02
<i>Pseudomonas aeruginosa</i>	08
Filamentous fungi	
<i>Ascochyta fabae</i>	27
<i>Aspergillus carbonarius</i>	40
<i>Fusarium culmorum</i>	20
<i>Fusarium equiseti</i>	20
<i>Mucor ramannianus</i>	43
<i>Penicillium expansum</i>	45
Yeasts	
<i>Saccharomyces cerevisiae</i>	00
<i>Candida albicans</i>	00

3.2. Antimicrobial activity

The strain SA198 showed broad-spectrum antimicrobial activity on ISP2 medium (Table 2). It was active against Gram-positive and Gram-negative bacteria and filamentous fungi, but not against yeasts. It showed strong to very strong activity against fungi such as *A. fabae*, *A. carbonarius*, *F. culmorum*, *F. equiseti* and *P. expansum*, and against bacteria such as *B. subtilis* and *Enterococcus faecalis*.

3.3. Time course of growth and antibiotic production

The kinetics of antibiotic production, growth and pH were monitored in ISP2 broth cultures, as shown in Fig. 3. The activities were detected on the first day of fermentation against *B. subtilis* and *M. ramannianus*, and on the second day against *A. carbonarius*, reaching a maximum the 8th day. The antifungal activity against *M. ramannianus* was stronger than that detected against *A. carbonarius* and *B. subtilis*. The antimicrobial activity was found to be correlated with the cell growth, so the biomass increased from the first day to the 8th day, and then decreased after the 9th day. The pH increased gradually to an alkaline state with a maximum (8.35) recorded on the second day. It decreased after the third day and small variations were recorded thereafter.

3.4. Isolation and purification of antibiotics

The cell-free supernatant of the 7.0L shake culture was extracted by dichloromethane. The yellow organic phase was concentrated to dryness. The TLC plate analysis showed the presence of an active yellow spot designated A, detected by bioautography at $R_f=0.37$, which exhibited antibacterial and antifungal activities. The preparative silica gel plates were then used to spot dichloromethane crude extract in bands in order to collect an appreciable quantity. The active bands were recovered and desorbed in methanol, concentrated to dryness and used for the final purification by HPLC using a reverse-phase column.

The HPLC profile obtained showed two peaks with antibacterial and antifungal activities, which were designated A4 (retention time: RT, 18.50 min) and A5 (RT, 20.7 min). Each peak was collected separately and re-injected into the HPLC system until total purification was achieved. The pure antibiotics had a strong yellowish color.

Chromogenic reactions for A4 and A5 compounds were negative with FeCl_3 , ninhydrine and naphtorescorcinol- H_2SO_4 .

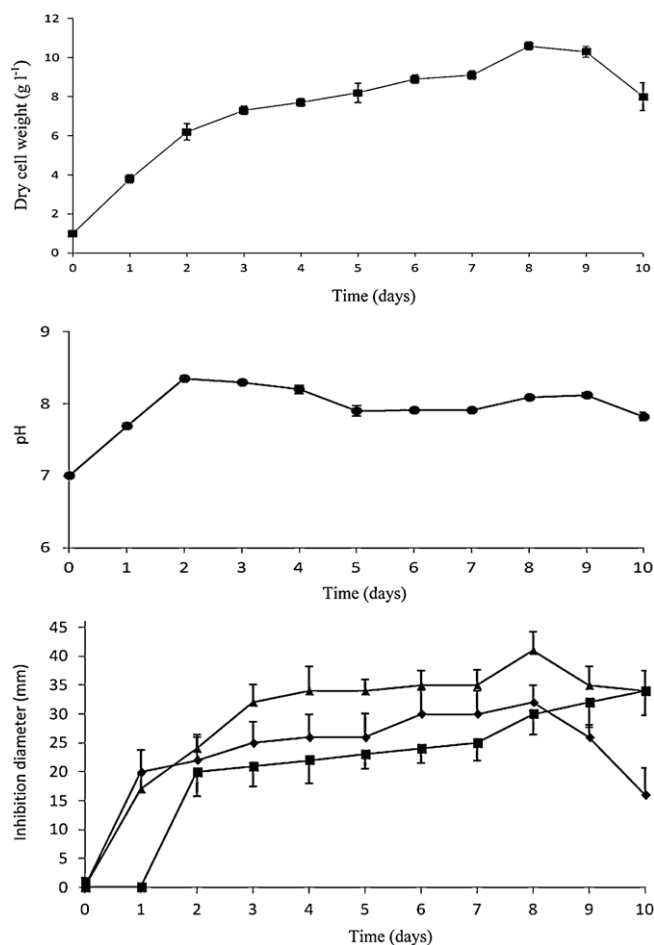


Fig. 3. Time course of growth, pH and antimicrobial activity in ISP2 medium against *Bacillus subtilis* (◆), *Aspergillus carbonarius* (■) and *Mucor ramannianus* (▲). Bars indicate standard deviation of the mean.

3.5. Spectroscopy and spectrometry analyses of antibiotics

The UV-visible spectra in methanol (data not shown) exhibited a maximal absorption at 262.5 and 343.7 nm for A4 and at 263.3 and 343.3 nm for A5. The infrared spectra of A4 and A5 molecules showed the presence of bands at 3323–3453 cm^{-1} (hydroxyl groups), 2850–2951 and 1434–1464 cm^{-1} (aliphatic C–H), 1685–1735 cm^{-1} (C=O), 1517–1634 cm^{-1} (C=C of aromatic ring) and 1151–1156 cm^{-1} (C–O). The ESI-MS spectra contained an ion peak at m/z 505.55 $[\text{M}-\text{H}]^-$ for A4 and at m/z 519.75 $[\text{M}-\text{H}]^-$ for A5, and their molecular formulas were determined by HRESIMS analysis as $\text{C}_{29}\text{H}_{30}\text{O}_8$ [calcd. 506.1838, found 506.1819] and $\text{C}_{30}\text{H}_{32}\text{O}_8$ [calcd. 520.1995, found 520.1983] for A4 and A5 respectively. Optical rotation of the two antibiotics were $[\alpha]_{\text{D}}^{21} +8$ (c 0.05, MeOH) for A4 and $[\alpha]_{\text{D}}^{21} +33.9$ (c 0.07, MeOH) for A5.

The ^1H and ^{13}C chemical shifts of A4 and A5 compounds are given in Table 3 and their structures can be seen in Figs. 4 and 5.

The ^{13}C and HSQC spectra show 29 carbon signals for compound A4 and 30 carbon signals for molecule A5. From the ^{13}C data, it was possible to discern one ketone group (δ_c 198.5), two enolic groups (δ_c 166.5), one carboxylate group (δ_c 189.2) one hydroxyl group (δ_c 71.0), one epoxy group (δ_c 52.5 and 56.9), 13 sp^2 -hybridized carbons (δ_c from 121.5 to 150.5), and five sp^3 -hybridized carbons (δ_c 20.8–31.4) for molecule A4, and six sp^3 -hybridized carbons (δ_c 10.7–38.8) for compound A5. The hydrogens of the hydroxyl and enol groups were not observed due to rapid exchange with MeOD. The 2D $^1\text{H}-^1\text{H}$ and $^1\text{H}-^{13}\text{C}$ experiments and, especially, the

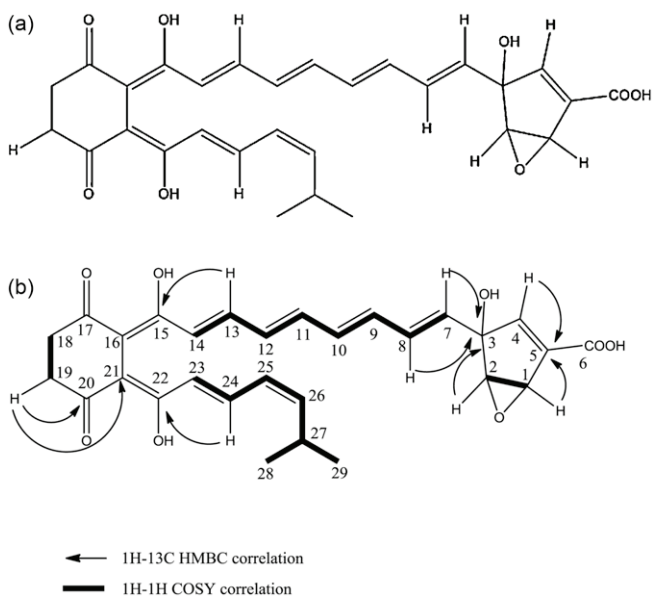
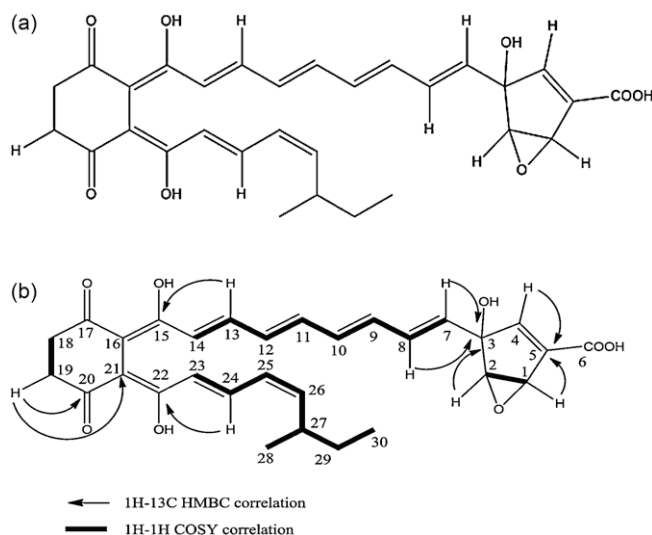
Table 3

¹H and ¹³C NMR data assignments of A4 and A5 compounds in CD₃OD at 298 K. See Figs. 4 and 5 for numbering of hydrogen and carbon atoms.

¹ H and ¹³ C number	¹ H chemical shift, ppm		¹³ C chemical shift, ppm	
	A4	A5	A4	A5
1	3.62	3.62	52.5	52.5
2	3.69	3.70	56.9	56.9
3	–	–	71.0	71.0
4	7.22	7.22	129.2	129.1
5	–	–	128.1	128.1
6	–	–	189.2	189.2
7	5.84	5.85	134.9	134.9
8	6.57	6.58	131.5	131.5
9	6.48	6.48	134.3	134.3
10	6.46	6.47	133.9	133.9
11	6.61	6.61	138.4	138.3
12	6.47	6.47	131.6	131.6
13	7.23	7.22	139.7	139.7
14	6.21	6.21	124.6	124.6
15–22	–	–	166.5	166.5
16–21	–	–	110.7	110.8
17–20	–	–	198.5	198.5
18–19	2.36	2.36	30.4	30.3
23	6.18	6.17	121.5	121.4
24	7.21	7.22	142.9	142.9
25	6.24	6.25	125.6	126.9
26	6.15	6.06	150.5	149.5
27	2.45	2.21	31.4	38.8
28	1.08	1.07	20.8	18.6
29	1.08	1.42	20.8	29.00
30	–	0.91	–	10.7

long range ¹H–¹³C couplings observed in the HMBC spectrum (see Figs. 4 and 5) established the connectivity between all the groups of molecules A4 and A5.

The structure of the A4 and A5 compounds was determined by NMR and mass spectrometry as respectively 4-hydroxy-4-{9-hydroxy-9-[2-(1-hydroxy-6-methyl-hepta-2,4-dienylidene)-3,6-dioxo-cyclohexylidene]-nona-1,3,5,7-tetraenyl}-6-oxa-bicyclo[3.1.0]hex-2-ene-2-carboxylic acid and 4-hydroxy-4-{9-hydroxy-9-[2-(1-hydroxy-6-methyl-octa-2,4-dienylidene)-3,6-dioxo-cyclohexylidene]-nona-1,3,5,7-tetraenyl}-6-oxa-bicyclo[3.1.0]hex-2-ene-2-carboxylic acid.

**Fig. 4.** Structure of antibiotic A4 (a) and HMBC and COSY correlations (b).**Fig. 5.** Structure of antibiotic A5 (a) and HMBC and COSY correlations (b).**Table 4**

Minimum inhibitory concentrations (MIC) of new antibiotics A4 and A5 produced by SA198 strain.

Test organism	Antibiotics ($\mu\text{g ml}^{-1}$)	
	A4	A5
<i>Bacillus subtilis</i>	20	10
<i>Enterococcus faecalis</i>	10	10
<i>Listeria monocytogenes</i>	30	30
<i>Escherichia coli</i>	20	30
<i>Klebsiella pneumoniae</i>	50	50
<i>Pseudomonas aeruginosa</i>	30	30
<i>Ascochyta fabae</i>	20	10
<i>Aspergillus carbonarius</i>	10	02
<i>Fusarium culmorum</i>	40	20
<i>F. equiseti</i>	30	30
<i>Mucor ramannianus</i>	05	01
<i>Penicillium expansum</i>	02	02
<i>Saccharomyces cerevisiae</i>	>100	>100
<i>Candida albicans</i>	>100	>100

3.6. Minimum inhibitory concentration

The antimicrobial activity of compounds A4 and A5 is shown in Table 4. A4 and A5 showed high activity against fungi such as *M. ramannianus* (MIC: 5–1 $\mu\text{g ml}^{-1}$), *A. carbonarius* (MIC: 10–2 $\mu\text{g ml}^{-1}$) and *P. expansum* (MIC: 2–2 $\mu\text{g ml}^{-1}$). Moderate activity was also recorded against other filamentous fungi such as *A. fabae*, *F. culmorum* and *F. equiseti* (MIC: 40–10 $\mu\text{g ml}^{-1}$) as well as Gram-negative bacteria (*E. coli*, *K. pneumoniae* and *P. aeruginosa*) and Gram-positive bacteria (*B. subtilis*, *E. faecalis* and *L. monocytogenes*). However, the two yeast strains (*C. albicans* and *S. cerevisiae*) have shown resistance (MIC: >100). The activity of A5 was more potent than that of A4.

4. Discussion

On the basis of the morphological and chemical characteristics described above, strain SA198 was classified in the genus *Saccharothrix* (Labeda et al. 1984). Comparison with the *Saccharothrix* species showed differences in the cultural characteristics and in the physiological properties. The 16S rRNA gene sequence of strain SA198 was compared with those of other *Saccharothrix* species. The similarity level ranged from 97.2 to 98.8%, *S. australiensis* (Labeda et al. 1984) being the most closely related species. However, it is

clear from phylogenetic analysis that strain SA198 represents a distinct phyletic line suggesting a new genomic species.

Our results showed that the antifungal activity was stronger than the antibacterial one. The same observations were announced by Igarashi et al. (1997). These authors reported that the formamycin produced by a strain of *Saccharothrix* sp. exhibited broad, strong antifungal activity against phytopathogenic fungi and moderate antibacterial activities against Gram-positive bacteria. Although the activity of *Saccharothrix* species against Gram-positive bacteria has been widely published (Takeuchi et al. 1992; Sabaou et al. 1998; Wang et al. 2001; Zitouni et al. 2005), its activity against Gram-negative bacteria and fungi has been reported only rarely (Suzuki et al. 1991; Kinochita et al. 1999).

The two compounds purified by HPLC showed antibacterial and antifungal activity. Compound A5 was more potent than A4.

It is very interesting to note that the activity of the two compounds is strong to moderate against filamentous fungi, but without any activity against yeasts. Indeed, the work of Andriole (1999) and Lass-Flörl et al. (2008) on many antifungal compounds tested against a very large number of strains of yeasts and filamentous fungi showed activity directed against these two groups of microorganisms, which is also the case in almost all works cited in the literature. In contrast, the studies showing activity against filamentous fungi, with no activity against yeasts, are very rare (Hosoe et al. 2004) or almost non-existent.

The data relative to chemical revelations and to the infrared spectrum suggested that our antibiotics did not contain sugar, free amine or phenol.

On the basis of NMR and HRESI-MS analysis, the molecular formulas were determined as $C_{29}H_{30}O_8$ for A4 and $C_{30}H_{32}O_8$ for A5. No resemblance was noted when the structures of compounds A4 and A5 were compared with those reported in the literature such as databases www.sciencefinder.com and www.chemspider.com, and also antibiotics described in dictionary of antibiotics and related substances (Bycroft 1988), in dictionary of natural products (Buckingham 1997), and in the review of bioactive microbial metabolites (Berdy 2005).

Saccharothrix species have been reported to produce antibiotics belonging to the aminoglycoside and benzoquinone (Takahashi et al. 1986), glycopeptide (Takeuchi et al. 1992), carboxylic nucleoside (Bush et al. 1993), dithiopyrrolone (Lamari et al. 2002), heptadecaglycoside (Singh et al. 2000), anthracyclin (Zitouni et al. 2004a), macrolide (Murakami et al. 2009) and angucycline (Kalinovskaya et al. 2010) families. No species of *Saccharothrix* is known to produce antibiotics close to compounds A4 and A5.

In conclusion, the results of this study show that the strain SA198 is different from known species of *Saccharothrix*, and produced new antimicrobial compounds.

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