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Oligomycins A and E, major bioactive secondary metabolites produced by *Streptomyces* sp. strain HG29 isolated from a Saharan soil

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KEYWORDS

Streptomyces;
Taxonomy;
Antifungal activity;
Oligomycins;
Minimal inhibitory concentrations

Summary An actinobacterial strain, HG29, with potent activity against pathogenic, toxigenic and phytopathogenic fungi was isolated from a Saharan soil sample of Algeria. On the basis of morphological and chemotaxonomic characteristics, the strain was classified in the genus *Streptomyces*. Analysis of the 16S rRNA gene sequence showed a similarity level of 99.3% with *Streptomyces gancidicus* NBRC 15412^T. The comparison of its cultural and physiological characteristics with this species revealed significant differences. Moreover, the phylogenetic tree showed that strain HG29 forms a distinct phyletic line within the genus *Streptomyces*. Production of antifungal activity was investigated by following kinetics in shake broth. The highest antifungal activity was obtained after five days of fermentation, and in the dichloromethane extract. Two active compounds, NK1 and NK2, were purified by HPLC using a C18 column. Their chemical structures were identified through nuclear magnetic resonance experiments and mass spectrometry as oligomycins E and A, respectively, which have not been reported to be produced

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by *S. gancidicus*. The two bioactive compounds exhibited significant antifungal activity *in vitro* showing minimal inhibitory concentrations (MICs) values between 2 and 75 µg/mL.

Introduction

Filamentous fungi cause a wide range of infectious diseases in humans, animals and plants [1,2]. In addition, their ability to produce mycotoxins such as aflatoxins, ochratoxins and patulin can be harmful to humans and animals at certain concentrations after ingestion of contaminated food and feed [3]. Current antifungal compounds are limited in their ability to treat infections due to the increase of the resistance in fungi and the toxicity of a wide range of antifungal molecules in use such as polyenes [4,5]. This phenomenon motivates the search for new bioactive compounds with strong activity against pathogenic fungi. In order to find new solutions, several approaches were developed, including the search for new antifungal compounds from microorganisms [6]. The actinobacteria are known as the most attractive source of several types of bioactive metabolites, especially members of the genus *Streptomyces*, which produce over two-thirds (70%) of the clinically useful antibiotics of natural origin [7]. The Algerian Saharan soils are rich and diversified in actinobacteria with interesting antimicrobial properties [8–13].

In this paper, we describe the taxonomy of an actinobacterial strain isolated from a Saharan soil sample, during a primary screening program to search for antimicrobial compounds. The strain, designated HG29, showed an interesting antifungal producing potential active against several pathogenic and toxigenic species of filamentous fungi. Two active compounds were purified, and their structures and activities were determined.

Materials and methods

Strain isolation and maintenance

The actinobacterial strain HG29 was isolated from a Saharan soil sample collected from Hoggar, Tamanrasset (Southern Algeria, 22°49'N, 5°25'E), by serial dilution agar plating method using chitin-vitamin B agar [14], supplemented with actidione (80 µg/mL). Pure culture of the strain was maintained at 4 °C on ISP4 medium slants.

Taxonomic studies of HG29 strain

The morphological characteristics were investigated using the media of the International *Streptomyces* Project (ISP2, ISP3 and ISP4) [15], Bennett medium and nutrient agar [16] after 7, 14 and 21 days of incubation at 30 °C. The mycelia organization and sporulation were observed by light microscopy.

For the chemotaxonomic analysis, biomass was obtained from cultures grown in shake flasks (250 rpm) using ISP2 medium. After 5 days of incubation at 30 °C, cells of strain HG29 were harvested by centrifugation, washed twice with

distilled water and hydrolyzed for diaminopimelic acid isomers [17], whole-cell sugar pattern [18] and phospholipids analyses [19].

For the physiological characteristics, 79 tests were performed according to the methods of Locci [20]. They concerned the production of melanoid pigments on ISP6 and ISP7 media and nitrate reductase, the assimilation of 26 carbohydrates and derivatives as sole carbon source, the utilization of 9 amino acids as sole nitrogen sources, the degradation of 7 organic acids on ISP9 medium, hydrolysis of 11 organic compounds (adenine, guanine, xanthine, casein, Tween 80, gelatin, starch, cellulose, esculin, arbutin and urea), the sensitivity to 5 different inhibitory compounds including violet crystal (0.05%), sodium azide (0.02%), potassium tellurite (0.01%), phenol (0.1%) and lysozyme (0.005%), the growth in the presence of different concentrations of NaCl (0, 2, 3, 5, 7, 9 and 10% w/v), at different temperatures (25, 30, 35, 40, 45 and 50 °C) and various pH values (3, 5, 7, 9 and 10). All the tests were performed at 30 °C (except for temperature tests) and observations were recorded at 7, 14 and 21 days.

For molecular analysis, the DNA of the strain HG29 was extracted with a DNA extraction kit (MasterPure™ Gram Positive DNA Purification Kit, Epicentre® Biotechnologies, Madison, WI). The 16S rRNA gene was amplified by PCR using two primers, 10–30F (5'-GAGTTTGATC-CTGGCTCA-3') and 1500R (5'-AGAAAGGAGGTGATCCAGCC-3'), as described by Rainey et al. [21].

Amplification was carried out in a 50 µL reaction volume containing 1.5 U of AmpliTaq Gold Taq polymerase (Applied Biosystems, Foster City, CA), dNTPs (0.25 mM each), 1 µM of each primer, and 100 ng of genomic DNA. Reaction conditions were: 97 °C for 4 min, followed by 35 cycles of 97 °C for 45 s, 52 °C for 45 s, and 72 °C for 45 s, with a final elongation step at 72 °C for 10 min. The amplified products were visualized on a 0.8% (w/v) agarose gel stained with ethidium bromide. PCR products were purified with a purification kit (Qiagen, Hilden, Germany). The primers used for sequencing are listed in Coenye et al. [22].

For the 16S rRNA gene phylogenetic analyses, the sequence obtained was compared for similarity with sequences present in the EzTaxon-e server (<http://www.eztaxon-e.ezbiocloud.net/>; [23]). The sequences were aligned with reference sequences of representatives of the genus *Streptomyces* retrieved from the GenBank database. Phylogenetic analyses were conducted using Molecular Evolution Genetics Analysis (MEGA) software version 5.0 [24]. Phylogenetic trees were constructed using the neighbor-joining method of Saitou and Nei [25]. The resulting topologies of trees were evaluated by bootstrap analyses [26] on 1000 replicates.

Primary screening for antimicrobial activity

Antimicrobial activity of the strain HG29 against fungi and bacteria was detected using the agar cross streak method.

The actinobacterium was streaked as a dense straight line on ISP2 medium plates and incubated at 30 °C for 10 days. After good growth, target strains were streaked at a right angle to HG29 strain, and then incubated at 30 °C for 48 h. The antimicrobial activity was evaluated by measuring the distance of inhibition between the target-microorganisms and the HG29 strain colony margin. Tests were conducted in triplicate.

The target-fungi tested were mostly pathogenic or toxigenic for humans, or phytopathogenic strains. They included twenty filamentous fungi (*Aspergillus carbonarius* M333, *A. niger* OT304, *A. parasiticus* CBS 100926, *A. westerdijkiae* NRRL 3174, *A. nidulans* KE202, *A. terreus* CT290, *A. fumigatus* CF140, *A. flavus* NRRL 3251, *Fusarium culmorum*, *F. equiseti*, *F. proliferatum*, *F. graminearum* Fg3, *F. sporotrichioides*, *F. moniliforme*, *F. oxysporum* f. sp. *lini*, *Fusarium oxysporum* f. sp. *albedinis*, *Penicillium expansum*, *P. glabrum*, *Botrytis cinerea* and *Umbelopsis ramanniana* NRRL 1829) and five strains of *Candida albicans* (M1, M2, M3, IPA200 and IPA988). In addition, four strains of bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* E40 and *Pseudomonas aeruginosa* ATCC 27853) were tested. The strains without accession number resulted from our laboratory collection.

Time course of antifungal activity production and growth

Fermentations were carried out in liquid medium containing glucose 1%, peptone 0.5% and MgCl₂ 0.1% [27]. The seed culture was prepared using a spore suspension in sterile distilled water with a final optical density (OD₆₀₀) of 0.1, and from a fresh culture of HG29 strain grown on ISP4 medium at 30 °C for 10 days. One milliliter of the spore suspension was inoculated into Erlenmeyer flasks (250 mL) containing 50 mL of the seed medium consisting of (g/L): 4 beef extract; 1 yeast extract; 4 peptone; 2.5 NaCl and 10 glucose, pH 7.2 [27]. The Erlenmeyer flasks were prepared and incubated on a shaker (250 rpm) at 30 °C for 48 h. Aliquots (5%, v/v) of this seed culture were transferred into 100 mL of the fermentation medium in 500-mL Erlenmeyer flasks. The cultures were incubated on a rotary shaker (250 rpm) at 30 °C for 10 days. The antifungal activity production, the pH and the dry cell weight (DCW) were measured every 24 h.

The antifungal activity was evaluated by agar well diffusion method against *Aspergillus carbonarius* M333. The spore suspension was prepared in sterile distilled water and adjusted as inoculum to a final concentration of 1 to 2 × 10⁶ CFU/mL. An amount of 20 mL of Sabouraud agar (10 g/L agar) was inoculated with 15 µL of spore suspension and then poured into a Petri dish. The plates were kept at room temperature for 30 min to allow the medium to solidify. Four wells of 10 mm diameter were aseptically bored into the culture medium, and 200 µL of each culture supernatant were loaded in each well. The plates were incubated at 30 °C for 48 h after a diffusion process of 2 h at 4 °C. Diameters of the inhibition zones around the wells were measured. The results were expressed as mean ± standard deviation (SD) from triplicate experiments. Growth was studied by determining the DCW using the method of Bouras et al. [28].

Extraction and purification of antifungal compounds

For the production of antibiotics, repeated fermentations were carried out to obtain a total of 4.0 L of culture broth. Extraction of antifungal compounds from the culture filtrate was carried out on the day of optimal antifungal activity production. The culture broth was centrifuged at 5000 g for 15 min. The culture filtrate obtained was extracted with dichloromethane solvent (1:1 v/v). The organic phase was concentrated with a rotary evaporator at 40 °C. The dry crude extract was dissolved in methanol and bioassayed against *A. carbonarius* M333 by the paper disk diffusion method. Each paper disk (6 mm diameter) was impregnated with 40 µL of the crude extract. All paper disks prepared were then air dried, sterilized under UV and then placed on the surface of Sabouraud agar, previously inoculated with *A. carbonarius* M333 as the target-fungus. Then, the plates were kept at 4 °C for 2 h to allow the diffusion of antifungal substances in the medium. Afterwards, the plates were incubated at 30 °C for 48 h. The antifungal activity was determined by measuring the diameters of inhibition zones around each paper disk.

The purification and the analysis of the crude extract were carried out by HPLC (Agilent Technologies). The equipment consisted of a variable wavelength detector (UV-Vis G1315D detector array), a pump system G1311C, and an auto-sampler G1329B. Data were acquired using Agilent ChemStation (Rev. B 04.03) software. The stationary phase consisted of a C18 reverse-phase column (Zorbax SB, 5 µm, 9.6 × 250 mm). The samples were analyzed by continuous linear gradient solvent system from 40 to 100% methanol in water during 25 min, using a flow rate of 1 mL/min and ultra violet detection at 220 nm. Fractions were collected separately, concentrated and bioassayed against *A. carbonarius* M333 by the paper disk diffusion method.

Spectroscopic and spectrometric analyses

The UV-visible of the active compounds was determined in methanol solution with a Perkin-Elmer Lambda 20 UV/Vis spectrophotometer. The mass spectrum was recorded with a LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, California, USA) equipped with a nanospray ion electro-spray ionization ESI source (positive and negative ion mode). ¹H and ¹³C NMR spectroscopy were used for the characterization of the active molecules. NMR sample was prepared by dissolving 2 mg of each purified compound in 600 µL of MeOD. All spectra were recorded on a Bruker Avance 500 spectrometer equipped with a 5 mm triple resonance inverse z-gradient probe (TBI ¹H, ³¹P, BB). All chemical shifts for ¹H and ¹³C are 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, ¹H–¹³C HMBC and ¹³C–¹H experiments. Gradient-enhanced ¹H COSY45 was realized included 24 scans per increment. ¹H–¹³C correlation spectra using a gradient-enhanced HSQC sequence (delay was optimized for ¹J_{CH} of 145 Hz) was obtained with 48 scans per increment.

Gradient-enhanced HMBC experiment was performed allowing 62.5 ms for long-range coupling evolution (340 scans were accumulated). Typically, 2048 t2 data points were collected for 320 t1 increments.

Minimal inhibitory concentrations

The minimal inhibitory concentrations (MICs) were determined by the conventional agar dilution method [29]. The target-microorganisms included fifteen filamentous fungi (*Aspergillus carbonarius* M333, *A. niger* OT304, *A. parasiticus* CBS 100926, *A. westerdijkiae* NRRL 3174, *A. nidulans* KE202, *A. terreus* CT290, *A. fumigatus* CF140, *A. flavus* NRRL 3251, *Fusarium culmorum*, *F. equiseti*, *F. proliferatum*, *F. oxysporum* f. sp. *lini*, *Penicillium expansum*, *P. glabrum* and *Umbelopsis ramanniana* NRRL 1829), one yeast (*Candida albicans* C200) and four bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* E40 and *Pseudomonas aeruginosa* ATCC 27853). Suspensions of target-microorganisms were prepared in sterile distilled water with an inoculum size of 1×10^6 to 3×10^6 CFU/mL for fungi and 10^8 CFU/mL for bacteria. The stock solutions were prepared by dissolving 3 mg of each antifungal powder in 3 mL of methanol. The agar media (Mueller Hinton for bacteria and Sabouraud for fungi) were supplemented with different concentrations of the antifungal compound (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 20, 30, 40, 50, 75 and 100 μ g/mL) and then poured into Petri dishes. Two standard antifungal drugs, amphotericin B (AMB) and itraconazole (ITR) were used as the positive controls. A negative control was included in the test by inoculating the target-microorganisms onto the media without the antifungal compounds. The agar surface of the plates containing a concentration of antifungal agent and the control plates were spot inoculated with 1 μ L of the suspension of each target-microorganism. The plates were incubated for 24–48 h at 37 °C for bacteria and 48–72 h at 28 °C for fungi. Tests were performed in duplicate. The MICs were determined as the lowest concentration of the antifungal compound

that inhibited the growth of the target-microorganism, as determined by the lack of visual growth compared to the negative control.

Results and discussion

Taxonomy

Good growth of strain HG29 was observed on ISP2, ISP4 and Bennett media. The growth was moderate on ISP3 and weak on nutrient agar medium. At maturity, the aerial mycelium was dark greenish blue and produced spirals chains with 3–8 coils, each chain borne by a short sporophore and containing 10–50 spores (ESM Fig. 1). The substrate mycelium was pale beige and non-fragmented, producing a brownish soluble pigment on ISP2 and Bennett media.

Strain HG29 contained LL-diaminopimelic acid isomer and glycine. Whole-cell hydrolysates contained galactose, glucose, ribose and traces of mannose, which are typical of cell-wall type IC [18]. Diagnostic phospholipid detected was phosphatidylethanolamine corresponding to type PII phospholipids [30]. Based on the chemotaxonomic and morphological properties, strain HG29 was identified as a member of the genus *Streptomyces* [31].

The physiological characteristics of strain HG29 are given in Table 1. The strain produced melanoid pigments on the ISP6 and ISP7 media and nitrate reductase. Growth occurred at temperatures between 25 and 45 °C, at pH 5, 7 and 9 and at a salt concentration of 9% NaCl (w/v). It can utilize all major organic compounds tested except dulcitol, erythritol, melezitose, sorbose, trehalose, glycine, histidine, tryptophane, valine, adenine, cellulose, guanine, benzoate and tartrate. It was unable to grow in the presence of crystal violet (0.05%), potassium tellurite (0.01%) and sodium azide (0.02%).

The phylogenetic analysis using the 16S rRNA gene sequence (1406 nucleotides) confirmed that HG29 strain belonged to the genus *Streptomyces*. The sequence was deposited in GenBank under the accession number

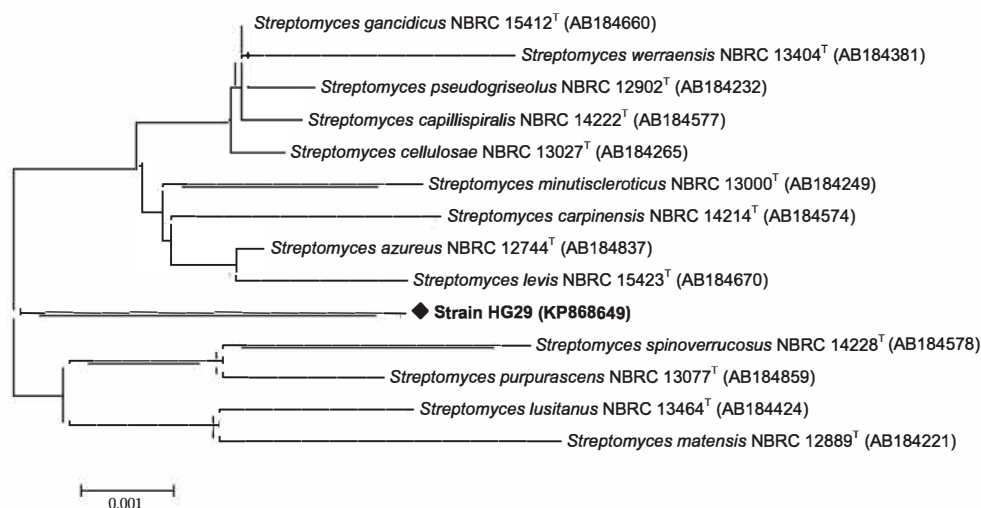


Figure 1 Phylogenetic tree derived from nearly complete 16S rRNA gene sequences showing relationships between the strain HG29 and the related type species of the genus *Streptomyces*. The tree was constructed using the neighbor-joining method. Bootstrap values are indicated at nodes ($\geq 50\%$). Bar 0.001 substitutions per nucleotide position.

Table 1 Physiological characteristics of strain HG29.

Test	Result
Hydrolysis of	
Adenine	—
Arbutin	±
Casein	+
Cellulose	—
Esculin	+
Guanine	—
Starch	+
Tween 80	+
Gelatin	+
Urea	+
Xanthine	+
Carbone source utilization (1% w/v)	
Adonitol	+
Arabinose	+
Cellobiose	+
Dextrin	+
Dulcitol	—
Erythritol	—
Fructose	+
Fucose	+
Galactose	+
Glucose	+
Glycerol	+
Inositol	+
Lactose	+
Maltose	+
Mannitol	+
Mannose	+
Melezitose	—
Melibiose	+
Raffinose	+
Rhamnose	+
Ribose	+
Sucrose	+
Sorbitol	+
Sorbose	—
Trehalose	—
Xylose	+
Nitrogen source utilization (0.1% w/v)	
L-Alanine	+
L-Asparagine	+
Glycine	—
L-Histidine	—
L-Proline	+
L-Serine	+
L-Threonine	+
L-Tryptophane	—
L-Valine	—
Decarboxylation of sodium salts	
Acetate	+
Benzoate	—
Citrate	+
Oxalate	+
Propionate	+
Pyruvate	+
Tartrate	—

Table 1 (Continued)

Test	Result
Growth in presence of (% w/v)	
Crystal violet (0.05%)	—
Phenol (0.1%)	+
Potassium tellurite (0.01%)	—
Sodium azide (0.02%)	—
Lysozyme (0.005%)	+
Growth at	
pH 3	—
pH 5, 7, 9	+
pH 10	±
T 25, 30, 35, 40, 45 °C	+
T 50 °C	—
NaCl 0–9%	+
NaCl 10%	±
Nitrate reductase	+
Production of melanoid pigments	
ISP6, ISP7	+

+: positive test; —: negative test; ±: doubtful.

Table 2 Antimicrobial activity of *Streptomyces* sp. HG29 by the agar cross streak method.

Target-fungi	Distance of inhibition (mm)
<i>Aspergillus carbonarius</i> M333	39.0 ± 1.0
<i>A. niger</i> OT304	37.3 ± 0.6
<i>A. parasiticus</i> CBS 100926	22.5 ± 0.5
<i>A. westerdijkiae</i> NRRL 3174	18.3 ± 0.6
<i>A. nidulans</i> KE202	5.7 ± 1.1
<i>A. terreus</i> CT290	4.5 ± 0.5
<i>A. fumigatus</i> CF140	3.3 ± 0.6
<i>A. flavus</i> NRRL 3251	2.1 ± 0.8
<i>Fusarium equiseti</i>	34.0 ± 1.0
<i>F. moniliforme</i>	32.6 ± 0.6
<i>F. sporotrichioides</i>	27.7 ± 0.6
<i>F. culmorum</i> FC200	26.0 ± 1.0
<i>Fusarium oxysporum</i> f. sp. <i>albedinis</i>	26.8 ± 0.8
<i>F. oxysporum</i> f. sp. <i>lini</i>	25.0 ± 1.0
<i>F. graminearum</i> Fg3	20.0 ± 1.0
<i>F. proliferatum</i>	20.3 ± 0.6
<i>Penicillium glabrum</i>	27.6 ± 0.6
<i>P. expansum</i>	27.0 ± 1.0
<i>Botrytis cinerea</i>	27.0 ± 1.0
<i>Umbelopsis ramanniana</i> NRRL 1829	4.8 ± 1.2
<i>Candida albicans</i> IPA200	3.8 ± 0.8
<i>C. albicans</i> IPA988	3.0 ± 1.0
<i>C. albicans</i> M1	2.8 ± 0.8
<i>C. albicans</i> M2	5.2 ± 0.8
<i>C. albicans</i> M3	6.2 ± 1.2
<i>Bacillus subtilis</i> ATCC 6633	20.0 ± 1.0
<i>Staphylococcus aureus</i> ATCC 25923	13.5 ± 1.3
<i>Klebsiella pneumoniae</i> E40	0.0
<i>Pseudomonas aeruginosa</i> ATCC 27853	0.0

Values are mean ± SD of three independent experiments.

KP868649. The highest similarity level was 99.3% with *Streptomyces gancidicus* NBRC 15412^T. The phylogenetic tree illustrated in Fig. 1 showed that the HG29 strain forms a distinct phylogenetic position with closely related species of the genus *Streptomyces*. However, our strain could be distinguished from *S. gancidicus* by some phenotypic properties such as the color of aerial mycelium (gray for *S. gancidicus*), the production of melanoid pigments on ISP6 and ISP7 media, the utilization of cellobiose, raffinose, sucrose and tryptophane, the decarboxylation of sodium citrate and growth in presence of lysozyme [32].

Primary screening of antimicrobial activity

The results of the screening for antimicrobial activity of strain HG29 are displayed in Table 2. *Streptomyces* sp. HG29 showed an antifungal activity against all the fungi tested. The antifungal activity was significantly strong (> 30 mm) against *Aspergillus carbonarius* M333, *A. niger* OT304, *Fusarium equiseti* and *F. moniliforme*; strong to moderate (21–30 mm) against *F. sporotrichioides*, *Penicillium glabrum*, *P. expansum*, *Botrytis cinerea*, *A. parasiticus* CBS 100926, *F. culmorum* FC200, *F. oxysporum* f. sp. *albedinis* and *F. oxysporum* f. sp. *lini*, and moderate (10–20 mm) against *A. westerdijkiae*, *F. proliferatum* and *F. graminearum*. However, a weak antifungal activity was detected (< 10 mm) against *A. nidulans* KE202, *A. terreus* CT290, *A. fumigatus* CF140, *A. flavus* NRRL 3251, *Umbelopsis ramanniana* NRRL 1829 and all tested yeasts.

The antibacterial activity was moderate against *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923, but no activity was found against *E. coli* ATCC 10536 and *Klebsiella pneumoniae* E40.

Consequently, *A. carbonarius* M333, the most sensitive strain, was selected as the indicator fungus for determining the antifungal activity in shake culture media.

Time course of antifungal activity production and growth

The kinetics of antibiotic production, growth and pH were investigated in shake medium, as shown on Fig. 2. The antifungal activities were detected on the first day of fermentation against *A. carbonarius* M333, reaching a maximum the 5th day. The antifungal activity production was found to be correlated with the cell growth. The pH decreased to an acid state until the 3rd day and then increased gradually and reached its maximum (pH 8.2) on the 10th day of fermentation.

Isolation and purification of antifungal compounds

Streptomyces sp. HG29 was grown for five days. The antifungal substances were extracted with dichloromethane from the culture filtrate. The active dichloromethane extract was subjected to HPLC analysis to determine the antifungal metabolite profiles. The HPLC profile showed two major peaks active against *A. carbonarius* M333, and were designed as NK1 (retention time (RT) = 14.99 min) and NK2 (RT = 15.77 min). These two peaks were collected and re-injected into the HPLC system until total purification was achieved (ESM Fig. 2). The pure bioactive compounds had a light yellowish color.

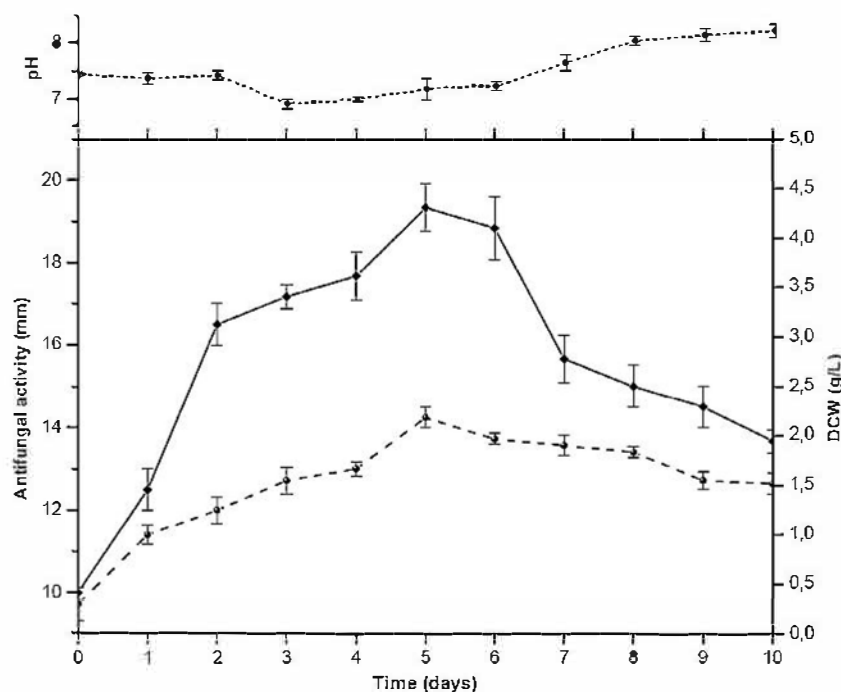


Figure 2 Time course of pH, growth (dashed curve) and antifungal activity production (solid curve) against *Aspergillus carbonarius* M333. Values include the diameter of the well (10 mm). Each measure represents average \pm standard deviation from three replicates per treatment.

Spectroscopic studies

The UV-visible spectra of the pure products NK1 and NK2 exhibited λ_{max} at 240 nm (NK1), and 232 nm (NK2) in MeOH. The mass spectra were obtained in positive and negative mode. The molecular weights of the active compounds are $M = 820$ (NK1) and $M = 790$ (NK2).

Table 3 ^1H and ^{13}C NMR data assignments of NK1 and NK2 compounds in CD_3CN at 298 K (See Figs. 3 and 4 for numbering of hydrogen and carbon atoms).

^1H and ^{13}C number	^1H chemical shift, ppm		^{13}C chemical shift, ppm	
	NK1	NK2	NK1	NK2
1	1.25	1.23	23.60	23.69
2	3.99	3.98	63.60	63.79
3	1.47	1.37	41.60	42.23
4	4.58	4.05	67.35	67.23
5	2.25	1.59	36.95	30.37
6	0.98	0.95	11.64	10.16
7	2.18–3.04	1.45–2.16	43.55	26.28
8	—	1.19–1.97	207.8	25.60
9	—	—	97.55	98.90
10	1.28	0.99	20.57	10.76
11	—	1.79	73.9	37.67
12	4.13	3.87	70.39	68.33
13	2.10	2.06	35.90	36.19
14	1.11	0.94	8.37	5.87
15	5.05	5.01	73.98	76.26
16	1.68	1.58	41.17	42.37
17	1.44	1.44–1.66	30.97	31.24
18	1.93	1.90	46.00	46.00
19	1.31–1.46	1.31–1.44	28.50	28.50
20	0.88	0.89	11.16	11.14
21	5.21	5.22	136.45	136.68
22	6.03	6.02	131.39	131.22
23	6.13	6.15	132.50	132.56
24	5.46	5.47	130.18	130.11
25	2.05–2.12	2.07–2.14	38.17	38.49
26	1.86	1.88	33.71	33.81
27	1.03	1.04	13.70	13.80
28	3.75	3.78	72.90	72.90
29	—	—	82.80	82.90
30	1.11	1.10	21.12	21.15
31	—	—	221.71	220
32	1.14	1.17	13.60	13.75
33	3.71	3.75	42.39	42.36
34	4.10	4.08	72.78	72.84
35	2.63	2.63	47.54	47.65
36	1.07	1.06	7.43	7.43
37	—	—	217.50	217
38	1.04	1.04	7.60	8.13
39	2.80	2.80	44.30	44.45
40	3.86	3.87	72.88	72.81
41	2.46	2.45	41.13	41.23
42	1.19	1.18	17.00	16.97
43	6.85	6.81	149.63	149.30
44	5.91	5.89	121.94	122.14
45	—	—	165.33	165.46

The ^1H and ^{13}C chemical shifts of NK1 and NK2 compounds are given in Table 3 and structures in Figs. 3 and 4. The ^{13}C , HSQC and HMBC spectra show 45 carbon signals for NK1 and NK2 molecules. It was possible to discern 3 ketone groups (δ_c 207.8 to 221.7), 6 hydroxyl group (δ_c 63.6 to 82.8), 1 ester function (δ_c 165.33), 6 sp^2 -hybridized carbons (δ_c from 121.9 to 146.63) and 25 sp^3 -hybridized carbons (δ_c 7.4 to 47.5) for molecule NK1, 2 ketone groups (δ_c 217.0 and 220.0), 1 ester function (δ_c 165.46) and 5 hydroxyl group (δ_c 63.79 to 82.90), 6 sp^2 -hybridized carbons (δ_c from 122.1 to 149.3) and 27 sp^3 -hybridized carbons (δ_c 7.4 to 47.7) for compound NK2. The hydrogens of the hydroxyl group are not observed due to rapid exchange with MeOD. The 2D ^1H – ^1H permitted the establishment of connectivity between the groups of the NK1 and NK2 molecules.

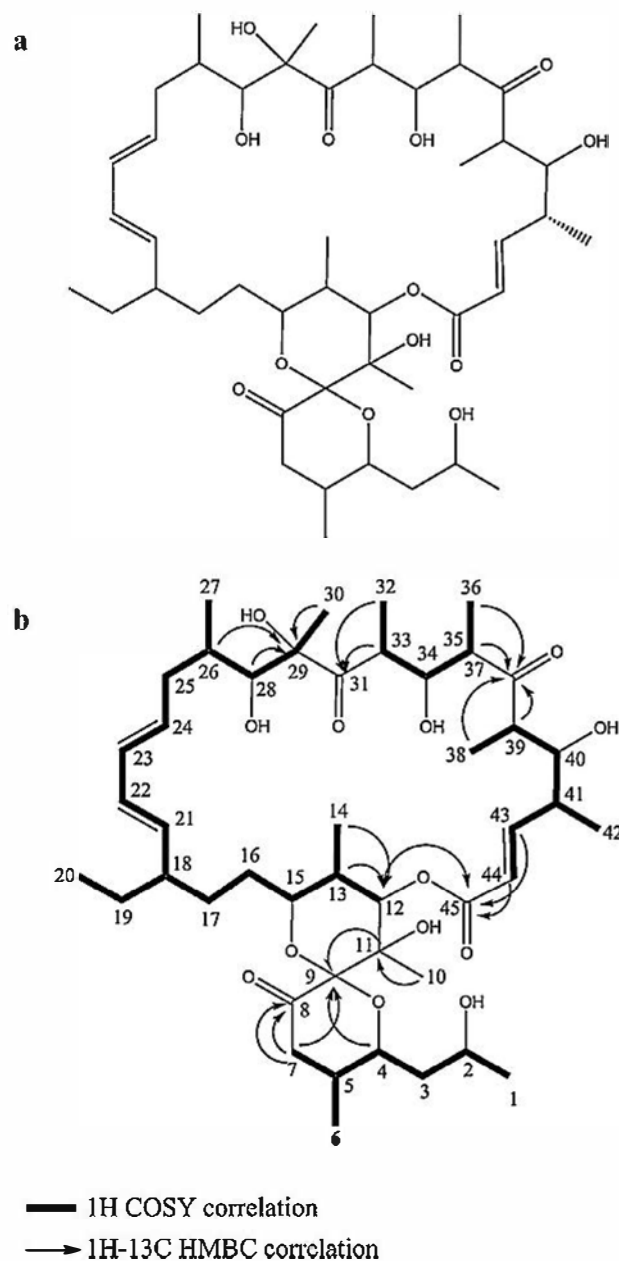


Figure 3 Structure of bioactive compound NK1 (a) and HMBC and COSY correlations (b).

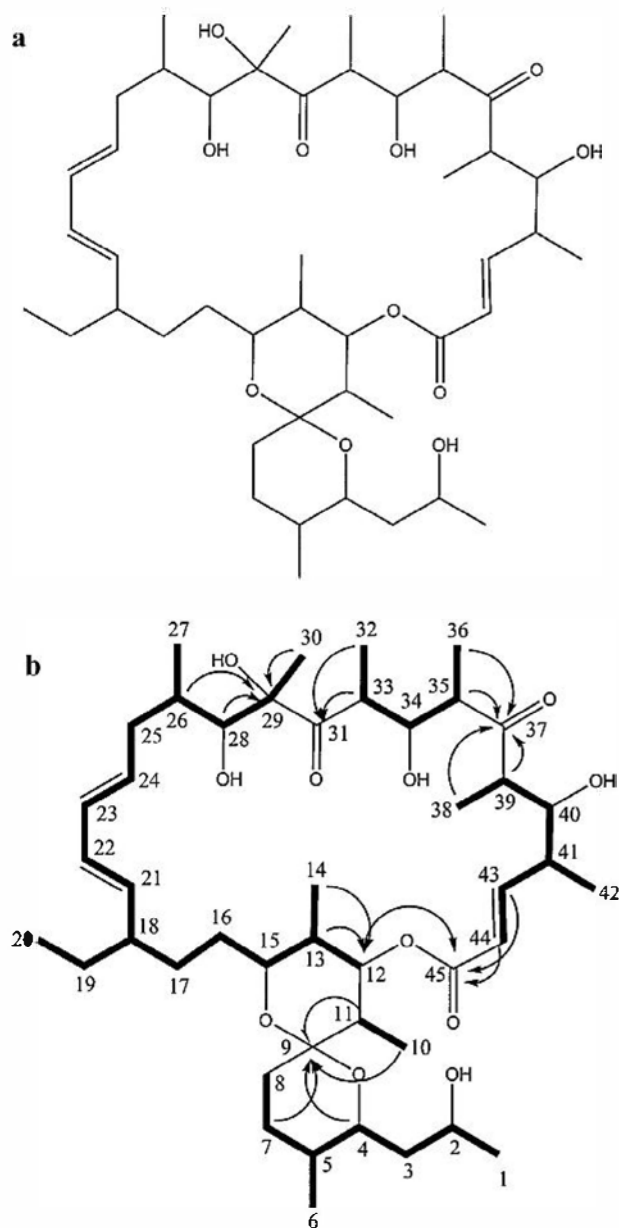


Figure 4 Structure of bioactive compound NK2 (a) and HMBC and COSY correlations (b).

The structures of the NK1 and NK2 compounds were determined by NMR and mass spectrometry to be oligomycin E [33] and oligomycin A [34], respectively.

Oligomycins are a subfamily of the macrolides class. The first oligomycin was described as a new antifungal antibiotic produced by *Streptomyces diastatochromogenes* [35]. Later, nine isomers named A, B, C, D, E, F, G, H and I were identified. They refer to a closely related molecular species whose basic structure consists of a macrolide ring of ketide units [36]. The oligomycins A and E differ in two side substituents [33,34] linked to the carbon in positions 8 and 11, as shown on Figs. 3 and 4. Oligomycin A is a highly specific inhibitor of mitochondrial ATP synthase that induces apoptosis in various cell types [37]. The oligomycin antibiotics are also produced by other species of *Streptomyces* including *S. bottropensis* [38], *S. ostreogriseus* [39], *S. libani* [40],

S. griseolus [41], *S. avermitilis* [42] and *S. diastaticus* [43]. However, their isolation has so far not been reported from *S. gancidicus*. This species produced the gancidin (molecular weight 210), a non-ribosomal peptide with antitumoral, antifungal and antibacterial properties [44,45]. The search for a possible phylogenetic relationship between the strain HG29 and the strains of oligomycins-producing *Streptomyces* was realized by the analysis and comparison of 16S rRNA gene sequences. The constructed phylogenetic tree (ESM Fig. 3) indicated that the strain HG29 constituted a distinct phyletic line with similarity levels ranging from 98 to 95%.

In addition to their antifungal activity, the oligomycins were reported to have other biological activities, including insecticidal and nematicidal activities [46] and immunosuppressive properties [39]. Oligomycins have not found clinical applications as antifungal agents because of their high toxicity to eukaryotic cells. The animal toxicity of oligomycins was reported since 1954 by Smith et al. [35]. The toxic levels by intravenous or intraperitoneal administration lied between 1.25 and 2.5 mg per kg of body weight. As a consequence of these toxic effects, eukaryotic cells may develop serious syndromes (including neurological disorders) that may degenerate into life-threatening diseases [47]. Consequently, oligomycins are not used therapeutically. They are used only in the research for studying mitochondrial function and dysfunction, and to modulate ATP synthesis in studies of cell or organ functions. They are also used for understanding the pathological mechanisms of some diseases such as Parkinsonian syndrome or immunodeficiency diseases, where mutations in genes encoding subunits of the ATP synthase are known to be responsible for human diseases [48,49]. In recent years, several methods were developed by chemical modification of the oligomycin A (at the side chain), which resulted in compounds with lower toxic properties than oligomycin A [50,51].

Determination of the minimum inhibitory concentrations

The obtained results are illustrated in Table 4. The two antifungal compounds showed almost the same biological activities against the majority of filamentous fungi. The anti-*Aspergillus* effects of NK1 and NK2 (MIC, 2–10 µg/mL) determined with *A. carbonarius* M333, *A. niger* OT304, *A. parasiticus* CBS 100926 and *A. westerdijkiae* NRRL 3174 were comparable or better than those of amphotericin B (AMB) and itraconazole (ITR). In contrast, their anti-*Fusarium* effects (MIC, 2–30 µg/mL) determined with *F. culmorum*, *F. equiseti*, *F. proliferatum* and *F. oxysporum* f. sp. *lini* were comparable to AMB, but much better than ITR. The anti-*Penicillium* effect (MIC, 3 µg/mL) determined with *P. expansum* and *P. glabrum* was similar to AMB and better than that of ITR. However, the MIC values of the two compounds against *C. albicans* C200 and all tested bacteria were superior to 100 µg/mL.

Regarding the MIC values determined with *Aspergillus* species (MIC, 2–10 µg/mL), *Fusarium* species (MIC, 2–30 µg/mL) and *Penicillium* species (MIC, 3 µg/mL), the effects of NK1 and NK2 are very important against these molds which are pathogenic and/or toxigenic for humans or phytopathogenic. The obtained values are comparable with AMB and better than those of ITR. These standard drugs used

Table 4 Minimum inhibitory concentrations (MIC) of the two antifungal compounds NK1 and NK2 produced by the strain HG29 and standard antifungal compounds against target-microorganisms.

Target-microorganisms	MIC (µg/mL) ^a			
	NK1	NK2	AMB	ITR
<i>Aspergillus carbonarius</i> M333	2	2	1	10
<i>A. westerdijkiae</i> NRRL 3174	8	10	> 100	7
<i>A. flavus</i> NRRL 3251	> 100	> 100	1	5
<i>A. parasiticus</i> CBS 100926	4	3	1	0.5
<i>A. nidulans</i> KE202	75	75	2	3
<i>A. niger</i> OT304	4	3	1	6
<i>A. terreus</i> CT290	75	75	> 100	5
<i>A. fumigatus</i> CF140	100	100	1	10
<i>Penicillium expansum</i>	3	3	3	5
<i>P. glabrum</i>	3	3	1	5
<i>Botrytis cinerea</i>	3	3	3	5
<i>Fusarium culmorum</i>	4	2	2	> 100
<i>F. equiseti</i>	2	2	1	> 100
<i>F. moniliforme</i>	2	2	2	> 100
<i>F. proliferatum</i>	20	30	> 100	> 100
<i>F. oxysporum</i> f. sp. <i>lini</i>	8	10	3	> 100
<i>Umbelopsis ramanniana</i> NRRL 1829	> 100	> 100	0.2	> 100
<i>Candida albicans</i> C200	> 100	> 100	1	> 100
<i>Bacillus subtilis</i> ATCC 6633	> 100	> 100	> 100	> 100
<i>Staphylococcus aureus</i> ATCC 25923	> 100	> 100	> 100	> 100
<i>Klebsiella pneumoniae</i> E40	> 100	> 100	> 100	> 100
<i>Pseudomonas aeruginosa</i> ATCC 27853	> 100	> 100	> 100	> 100

AMB: amphotericin B; ITR: itraconazole.

^a MIC values represent the average of two repetitions.

as positive controls are potent antifungal compounds [29]. The lowest MIC value found was 2 µg/mL against *A. carbonarius* M333 and *F. equiseti* which were included in the tests due to their ability to produce mycotoxins [52,53]. The antimicrobial spectrum of oligomycins discovered by Smith et al. [35] was similar to our obtained results showing a strong activity against several fungal species. Moreover, at 100 µg/mL, no activity was observed against Gram-positive and Gram-negative bacteria or yeasts. They are also similar in solubility properties (soluble in organic solvents, but insoluble in water). In our study, oligomycin A (NK2) showed an ultraviolet absorption maxima at 232 nm, similar to finding of Smith et al. [35].

Comparison between the distance of inhibition in the primary screening (Table 2) and MIC values of oligomycins A and E showed a direct correlation, whenever there is a strong distance of inhibition, the MIC value is lower and vice versa. Except for the antibacterial activity against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, detected by the streak method with moderate distances of inhibition, while the MICs were > 100 µg/mL. This result could be explained by

the presence of another antibiotic or more, showing antibacterial activity in the fermentation broth of strain HG29.

Conclusion

The results of the present study showed that the strain HG29 produced oligomycins A and E and shared 99.3% similarity with *Streptomyces gancidicus*. However, these two antibiotics are not produced by this nearest species. DNA–DNA hybridization experiments between strain HG29 and the closely related species *S. gancidicus* should determine the originality of strain HG29 or its assignment to the *S. gancidicus* species.

Despite the toxicity of oligomycins against eukaryotic cells, their extremely high potency against mycotoxigenic and phytopathogenic fungi, as well as their antitumor activity make these antibiotics an attractive scaffold for rational design of new chemotherapeutic agents. Further development of oligomycin derivatives (chemical modification or semi-synthesis) could be potentially useful for reducing the toxic effect and improvement of their therapeutic potential. On these bases, the possible use of oligomycins to treat infectious and non-infectious diseases would be an interesting perspective. Therefore, availability of oligomycins in substantial quantities by fermentation could be useful for such investigations. Based on the results presented in this paper, we propose a potent producer strain of oligomycins A and E.

Disclosure of interest

The authors declare that they have no competing interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mycmed.2017.10.007>.

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