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Activity of 2,4-Di-tert-butylphenol produced by a strain of *Streptomyces mutabilis* isolated from a Saharan soil against *Candida albicans* and other pathogenic fungi

Activité du 2,4-Di-tert-butylphenol produit par une souche de Streptomyces mutabilis isolée d'un sol Saharien contre Candida albicans et d'autres champignons pathogènes

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KEYWORDS

Streptomyces;
Taxonomy;
Antifungal activity;
Candida albicans;
2,4-Di-tert-butylphenol

Summary In a search for new antifungal antibiotics active against *Candida albicans* and others pathogenic fungi, a strain of actinobacteria, designated G61, was isolated from a Saharan soil and tested for its activity against these microorganisms. The analysis of its 16S rDNA sequence showed a similarity level of 100% with *Streptomyces mutabilis* NBRC 12800^T. The highest anticandidal activities produced by the strain G61 were obtained on Bennett medium in the fourth day of incubation. The active product, extracted by *n*-butanol, contained one bioactive spot detected on thin layer chromatography plates. It was purified by HPLC and its chemical structure was determined by spectroscopic analyses as 2,4-Di-tert-butylphenol. The minimum inhibitory concentrations (MIC) of this product against several strains of pathogenic microorganisms are interesting.

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MOTS CLÉS

Streptomyces ;
Taxonomie ;
Activité antifongique ;
Candida albicans ;
2,4-Di-tert-butylphénol

Résumé Dans le cadre de la recherche de nouveaux antifongiques actifs contre *Candida albicans* et contre d'autres champignons pathogènes, une souche d'actinobactérie, nommée G61, a été isolée à partir d'un sol saharien et testée pour son activité contre ces microorganismes. L'analyse de sa séquence d'ADNr 16S a montré un taux de similarité de 100 % avec la souche-type *Streptomyces mutabilis* NBRC 12800^T. Les plus fortes activités anticandidosiques produites par la souche G61 ont été obtenues sur le milieu Bennett au quatrième jour d'incubation. Le produit actif, extrait par le *n*-butanol, contient une tache active détectée par chromatographie de couche mince. Elle a été purifiée par HPLC et sa structure chimique a été déterminée par analyses spectroscopiques en tant que 2,4-Di-tert-butylphénol. Les concentrations minimales inhibitrices (CMI) de ce produit contre diverses souches de microorganismes pathogènes sont intéressantes.

Introduction

Fungi are responsible for a large number of diseases in humans, animals and plants, and cause damage to wood products, fruits, vegetables and other foodstuff [3]. Diseases or mycoses are classified as fungal infections caused by eumycotic organisms such as opportunistic and also pathogenic *Candida* spp., *Aspergillus* spp., *Fusarium* spp., *Cryptococcus* and some other fungal species [22]. These invasive diseases have assumed a greater importance because of their increasing incidence in persons with haematological malignancies and in many other immunocompromised patients. Most infections are caused by *Candida* spp., but the emergence of *Aspergillus* spp. and other fungi as etiological agents is continuously changing the spectrum of diseases [39].

Candida albicans is the most important fungal opportunistic pathogen of humans [49]. It belongs to the normal microbiota of individual's mucosal oral cavity, gastrointestinal tract and vagina [44], and it is responsible for various clinical manifestations from mucocutaneous colonization to blood stream infections [36]. It alone accounts for 60% of nosocomial fungal infections [33]. In addition, *C. albicans* causes systemic candidiasis associated with high mortality despite the availability of treatment [4]. The high mortality rates occurring during invasive fungal infections cases may be due to delayed diagnosis and treatment, development of resistance or severity of illness [37].

The antifungal drugs currently used for the treatment include amphotericin B, nystatin, fluconazole, itraconazole, ketoconazole, voriconazole, 5-fluorocytosine and caspofungin [56]. However, the choice of suitable antifungal agents remains relatively limited due to the toxicity and low solubility of some of them, emergence of resistant fungal strains, delayed diagnosis of fungal infection, variable drug bioavailability in immunocompromised patients, lack of either oral or intravenous preparations and drug interaction infections [10]. There is a need to explore novel therapeutic approaches and to develop new antifungal compounds to treat fungal infections [45].

The antibiotics for treatment of infections are derived either directly from natural sources, semisynthesized from a natural product parent, or completely synthesized but modeled after a natural product lead compound [9]. It has been estimated that about two-third of the natural antibiotics have been isolated from actinobacteria, especially from the genus *Streptomyces* [6].

Previous studies have reported the abundance and diversity of actinobacteria in Algerian Saharan soils [40,58]. In our laboratory, it has been demonstrated that many strains of actinobacteria isolated from these soils were found to produce bioactive compounds with interesting activities [2,3,13,52].

In the present study, we describe the taxonomy of an actinobacteria strain isolated from a Saharan soil. In addition, we report on production and characterization of its antifungal compounds.

Materials and methods

Isolation of the actinobacteria strain

The strain G61 was isolated from soil sample collected from Metlili (Ghardaïa, 32°16'N, 3°37'E), province in Sahara of Algeria, by the dilution agar plating method using a chitin-vitamin agar medium [17] consisting of (per liter of distilled water): 2 g chitin, 0.35 g K₂HPO₄, 0.15 g KH₂PO₄, 0.2 g MgSO₄ 7H₂O, 0.3 g NaCl, 0.02 g CaCO₃, 10 mg FeSO₄ 7H₂O, 1 mg ZnSO₄ 7H₂O, 1 mg MnCl₂ 4H₂O and 18 g agar. The medium was supplemented with penicillin (25 mg/L) and cycloheximide (50 mg/L) to inhibit the growth of undesirable bacteria and fungi, respectively. The plates were incubated at 30 °C for 3 weeks.

Morphological characteristics

The morphology of the strain G61, grown on various media at 30 °C for 10 days, was examined under light microscopy for the mycelia organization and sporulation.

Cultural characteristics observed on media from the International *Streptomyces* Project (ISP), ISP-1, ISP-2 and ISP-4 media [47], nutrient agar and Bennett agar [55] were recorded after 7–14 days incubation at 30 °C. Colors were determined according to the ISCC–NBS centroid color chart [23].

Chemotaxonomic study

For chemotaxonomic analyses, strain G61 was grown in ISP-2 broth medium at 30 °C for 4 days on a rotary shaker (250 rpm). The biomass was harvested by centrifugation at 6000 rpm and washed twice with distilled water [47]. The determination of diaminopimelic acid isomer and the presence or absence of

glycine were carried out using the method of Becker et al. [5]. Ten milligrams of dry mycelium strain were hydrolyzed for 18 h at 100 °C with 1 mL of hydrochloric acid (6 N). The hydrolyzate was filtered through glass membrane and concentrated to dryness in a bath of hot sand. The dry residue was dissolved in 0.3 mL of distilled water and analyzed by descending chromatography using Whatman No. 1 paper. Standard aqueous solutions containing a mixture of DL and LL-isomers of diaminopimelic (Fluka) (0.01 M) and glycine amino acid (0.2% w/v) were chromatographed as a control treatment. The whole-cell sugar pattern was determined using the method of Lechevalier and Lechevalier [27]. Fifty milligrams of dry mycelium were hydrolyzed with 1 mL of sulfuric acid (1 N) at 100 °C for 2 h. The acid is neutralized with saturated solution of barium hydroxide until a neutral pH. The supernatant was collected, evaporated to dryness at 40 °C, dissolved in 0.3 mL of distilled water and analyzed by descending chromatography using Whatman No. 1 paper. Standard aqueous solutions (0.1% w/v) containing sugars arabinose, galactose, glucose, mannose, rhamnose, ribose and xylose were chromatographed as a control treatment. The phospholipids were analyzed according to the procedures developed by Minnikin et al. [34]. One gram of mycelium was dissolved in a mixture of chloroform-methanol (2/1 v/v) at 25 °C overnight. After filtration, the extract was evaporated to dryness at 40 °C. The residue was dissolved in 0.3 mL of the same solvent and analyzed by ascending thin layer chromatography using silica gel G60 (Merck). Ten µL of phosphatidylethanolamine were used as a control treatment.

Physiological characteristics

The media and procedures used for the determination of the physiological features were described by Locci [30]. The strain G61 was examined for its ability to utilize six amino acids as sole nitrogen sources, and 20 carbohydrate compounds and 7 organic acids as sole carbon sources. The degradation activities of the strain G61 were observed on various media containing adenine, esculin, gelatin, guanine, hypoxanthine, starch, tyrosine and xanthine. The melanoid pigment production was tested on peptone yeast extract-iron agar (ISP-6) and tyrosine agar (ISP-7) media [47]. The production of nitrate reductase, the sensitivity to phenol (0.001% w/v), sodium azide (0.001% w/v), sodium chloride (4 and 7% w/v) and crystal violet (0.05% w/v), and the growth at pH 5 and 9 and at 40 and 45 °C, and in the presence of seven antibiotics were also studied.

Molecular study

The molecular study is based on extraction, PCR amplification of DNA coding for 16S ribosomal RNA (16S rDNA) and determination of its sequence. Once determined, this sequence is compared with those of reference species.

DNA preparation, PCR amplification and sequence analysis

The DNA was prepared according to the method of Liu et al. [29]. The strain G61 was grown at 30 °C for 7 days in a shake flask containing 100 mL of ISP-2 medium supplemented with

5% of NaCl. PCR amplification of the 16S rDNA of the strain was performed using two primers: 27f (5'-AGTTT-GATCCTGGCTCAG-3') and 1492r (5'-GGTACCTTGTTAC-GACTT-3'). The 16S rDNA was amplified by PCR using Invitrogen kit. The final volume of reaction mixture of 50 µL 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 the purified DNA. The amplification was performed on a 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 fluorescence after ethidium bromide staining. The sequencing reaction was performed by MilleGen Company (Toulouse, France). The same primers as before and an automated sequencer were used for this purpose.

Phylogenetic analyses

The sequence determined was compared for similarity level with the reference species of bacteria contained in genomic database banks, using the EzTaxon-e server (<http://eztaxon-e.ezbiocloud.net/>; [24]). Phylogenetic and molecular evolutionary analyses were conducted using software included in MEGA version 6.0 [50] package. The 16S rDNA sequence of the strain G61 was aligned using the CLUSTAL W program [51] against corresponding nucleotide sequences of representative species of the genus *Streptomyces* retrieved from GenBank. Evolutionary distance matrices were generated as described by Kimura [25] and a phylogenetic tree was inferred by the neighbor-joining method [41]. Tree topologies were evaluated by bootstrap analysis [15] based on 1000 resamplings of the neighbor-joining dataset.

Antifungal activity and target microorganisms

Screening of the antifungal activity was carried out by cross streak plate method. The strain G61 was inoculated as a single streak on the Petri dish containing ISP-2 medium and incubated at 30 °C for 10 days to permit growth and antibiotic production. Later, the target yeasts were inoculated by streaking perpendicular to the growth of strain. The plates were incubated for 48 h at 30 °C. The antifungal activity was evaluated by measuring the distance of inhibition between target strain and *Streptomyces* colony margins. The used target microorganisms were five strains of *C. albicans* (M1, M2, M3, IPA200 and IPA988), seven strains of filamentous fungi (*Fusarium culmorum* FC200, *Fusarium graminearum* Fg3, *Aspergillus carbonarius* M333, *Aspergillus westerdijkiae* ATCC 3174, *Aspergillus parasiticus* CBS 100926, *Aspergillus flavus* AF3 and *Umbelatosia ramanniana* NRRL 1829), and two strains of bacteria just to know the spectrum of our bioactive compounds (*Staphylococcus aureus* ATCC 43300 and *Escherichia coli* ATCC 10536). These microorganisms were mostly pathogenic or toxicogenic for humans as noted by Aouiche et al. [1].

Antifungal compounds production

The growth of the strain G61 was carried out in 2 broth media: yeast extract–malt extract–glucose (ISP-2) [47], and meat extract–yeast extract–peptone–glucose (Bennett medium) [55]. The pH of each medium was adjusted to 7.2 prior to autoclaving. Each flask containing 100 mL of medium was inoculated with 3% v/v of actinobacteria culture grown in the same medium for 72 h at 30 °C. The flasks were incubated at 30 °C for 10 days with shaking at 250 rpm. Two milliliters aliquots were collected regularly to estimate antibiotic activity by the agar well method against *C. albicans* strain M3. Well (10 mm in diameter) made in the ISP-2 agar plates (containing 10 g/L agar) were filled with 0.2 mL of the supernatant samples. The growth (dry weight of mycelium) and the pH were also measured.

Isolation and purification of antifungal compounds

The culture broth (from Bennett medium) was centrifuged to remove biomass. The cell-free supernatant was extracted with an equal volume of organic solvent. Four extraction solvents were tested for effectiveness, including *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. Each organic extract was concentrated to dryness. The resulting dry extract was recaptured in 1 mL of methanol and bioassayed against *C. albicans* strain M3 and *A. carbonarius* (M333), by paper disk diffusion method [21]. Paper disks were impregnated with 80 µL of extract. They were dried to evaporate the solvent and sterilized under UV at 254 nm for 30 min. The disks were then deposited on the surface of ISP-2 medium (10 g/L agar) pre-inoculated with the target germ (10⁸ CFU/mL). The zones of growth inhibition around each disk were measured. Preparative chromatography with silica gel plates (Merck Art. 5735, Kiesselgel 60F 254) was used for the partial purification of antifungal product. A dry crude extract, dissolved in methanol, was spotted and developed in the solvent system ethyl acetate-methanol (100:15 v/v). The developed TLC plates were air dried overnight to remove all traces of solvents. The separated compounds were visualized under UV at 254 nm (absorbance) and at 365 nm (fluorescence) and the active spot was detected by bioautography [7,20,38]. The TLC plates were placed in a plastic bioassay dish (23 cm × 23 cm × 2.2 cm, Fisher Scientific Labosi, Elancourt, France) and overlaid with 150 mL of ISP-2 medium (containing 7 g/L agar) inoculated with *C. albicans* strain M3 and *A. carbonarius* (M333) as target organisms, and incubated at 30 °C. Clear areas due to the inhibition of the growth of target organism indicated the location of antibiotic compound. The retention factor (RF) of the active spot was measured. Three hundred microliters of extract were deposited progressively on a continuous line in the lower edge of the silica gel plates. The plates were then developed in the same solvent system and the active fractions were scraped, desorbed in methanol and filtered from silica gel. The filtrate was evaporated to dryness and the residue was dissolved in 1 mL of methanol. The final purification was carried out by reverse phase HPLC using a C18 column (5 µm, 200 × 10 mm, WATERS). The elution was at a flow rate of 1 mL/min with a continuous gradient solvent system from

20 to 100% methanol in water. The detection was by UV at 220 nm. The peaks were collected separately, concentrated and bioassayed by disk diffusion method against the indicator organisms *C. albicans* strain M3 and *A. carbonarius* (M333).

Spectroscopic studies

The absorption spectrum of the active molecule in methanol was determined with a JASCO V-660 k spectrophotometer. Mass spectrometry was performed using an LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA). Nano electrospray analysis were performed using a commercial nanospray ESI source (The Protein Analysis Co., Odense, Denmark) using glass capillaries (Proxeon Biosystems, Odense, Denmark) that were positioned using a stereomicroscope, directly at a distance of 1 mm from the entrance hole of the heated transfer capillary. The capillaries were palladium and gold-coated for electrical contact. The glass capillaries were filled with 5 µL of analyte solution. A nebulizer gas was not necessary in this spray mode. The nanospray needle voltage was set to 700 V. The heated transfer capillary was kept at a temperature of 150 °C. Samples were analyzed in the positive ion mode. The software used for the analysis was Xcalibur 1.2 from Thermo Electron (San Jose, CA). ¹H and ¹³C NMR spectroscopy were used for the characterization of the molecule. NMR sample was prepared by dissolving 2 mg of compound in 600 µL of CD₃OD. 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 and ¹H-¹³C HMBC experiments. Gradient-enhanced ¹H COSY45 was realized included 4 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 16 scans per increment. Gradient-enhanced HMBC experiment was performed allowing 62.5 ms for long-range coupling evolution (64 scans were accumulated). Typically, 2048 t2 data points were collected for 256 t1 increments.

Minimum inhibitory concentrations

The minimum inhibitory concentrations were quantified by incorporating known concentrations of pure antibiotic into solid growth medium using a conventional agar dilution method [35]. The 14 target microorganisms (yeasts, filamentous fungi and bacteria) cited above were used. The inoculums of the microorganisms were prepared in sterile aqueous suspensions, adjusted by a JASCO V-660 k spectrophotometer to match the turbidity of 0.5 McFarland standard (equivalent to 10⁸ CFU/mL) and inoculated by point (1 µL) on Sabouraud medium for yeasts or filamentous fungi and on Mueller Hinton medium for bacteria. These media contain different concentrations of the active product dissolved in methanol (0.5, 1, 2, 3, 4, 5, 10, 20, 30, 50, 75 and 100 µg/mL). The same concentrations of three standard antifungal

Table 1 Physiological characteristics of strain G61.
Caractéristiques physiologiques de l'isolat G61.

Test	Result
Hydrolysis of organic compounds	
Adenine	+
Esculin	+
Gelatine	+
Guanine	—
Hypoxanthine	+
Starch	+
Tyrosine	+
Xanthine	+
Utilization of glucides (1% w/v)	
L-Arabinose	+
Adonitol	—
Cellobiose	+
Erythritol	—
D-Fructose	+
Galactose	+
Glucose	+
myo-Inositol	+
Lactose	+
Maltose	+
D-Mannitol	—
D-Mannose	—
Melibiose	—
D-Raffinose	—
L-Rhamnose	+
D-Ribose	+
Sucrose	+
Sorbitol	—
Trehalose	+
D-Xylose	+
Utilization of aminoacids (0.1% w/v)	
L-Proline	+
L-Serine	+
L-Alanine	+
L-Phenylalanine	+
L-Arginine	+
L-Histidine	+
Decarboxylation of sodium salts	
Acetate	+
Benzoate	—
Citrate	—
Oxalate	—
Pyruvate	+
Succinate	—
Tartrate	+
Resistance to antibiotics (µg/mL)	
Chloramphenicol (25)	+
Erythromycin (10)	+
Kanamycin (25)	—
Penicillin (25)	+
Rifampicin (5)	+
Streptomycin (10)	+
Vancomycin (5)	+
Growth with (% w/v)	
Crystal violet (0.05)	—
Phenol (0.001)	—

Table 1 (Continued)

Test	Result
Sodium azide (0.001)	—
NaCl (7)	+
Growth at	
pH 5	—
pH 9	—
40 °C	+
45 °C	—
Nitrate reduction	+
Production of melanoid pigments	—

+: positive test; —: negative test.

compounds (nystatine, amphotericin B and tioconazole) were dissolved in methanol and used for comparison. After a growth period of 48–72 h at 30 °C, the plates were examined for growth and the lowest antibiotic concentration that completely inhibited the growth of each organism (MIC) was determined. Mueller Hinton and Sabouraud media with methanol alone and inoculated with target microorganisms were used as a control treatment.

Results

Morphological, physiological and chemotaxonomic characteristics

The strain G61 showed a good growth on ISP-1, ISP-2 and Bennett media and moderate growth on ISP-4 and nutrient agar media, after 14 days of incubation at 30 °C. The aerial mycelium was whitish grey on all media used. It was moderately branching and fragmented into hook and closed loops. Spore chains of 3 to 10 non-motile spores were produced on short sporophores. Substrate mycelium was not fragmented and its color was yellowish grey on ISP-1 and Bennett media, light grey on ISP-2 and ISP-4, and light yellow on nutrient agar. No diffusible pigment was produced on any of the media used. Melanoid pigments were not produced on ISP-6 and ISP-7 media. Endospores, sclerotic granules, synnema and flagellated spores were not observed.

The physiological properties of the strain G61 are shown in [Table 1](#). The optimal growth of the strain was obtained at 30 °C and at pH 7. It was able to grow at 7% of NaCl. It failed to grow at pH 5 and 9, at 45 °C, and in the presence of crystal violet (0.05%), phenol (0.001%) and sodium azide (0.001%). The strain was able to degrade all organic compounds tested except guanine, adonitol, erythritol, D-mannitol, D-mannose, melibiose, D-raffinose, sorbitol, benzoate, citrate, oxalate and succinate. It was resistant to all antibiotics used except kanamycin. The strain G61 was found to contain LL-diaminopimelic acid isomer and glycine in the cell-wall. The whole-cell hydrolysates contained non-characteristic sugars (ribose, glucose and galactose), typical of cell-wall type IC [27]. The phospholipid profile was of type PII, characterized by the presence of phosphatidylethanolamine [28].

Phylogenetic analyses

The nearest complete 16S rDNA sequence (1474 nucleotides) of strain G61 was determined and deposited in GenBank

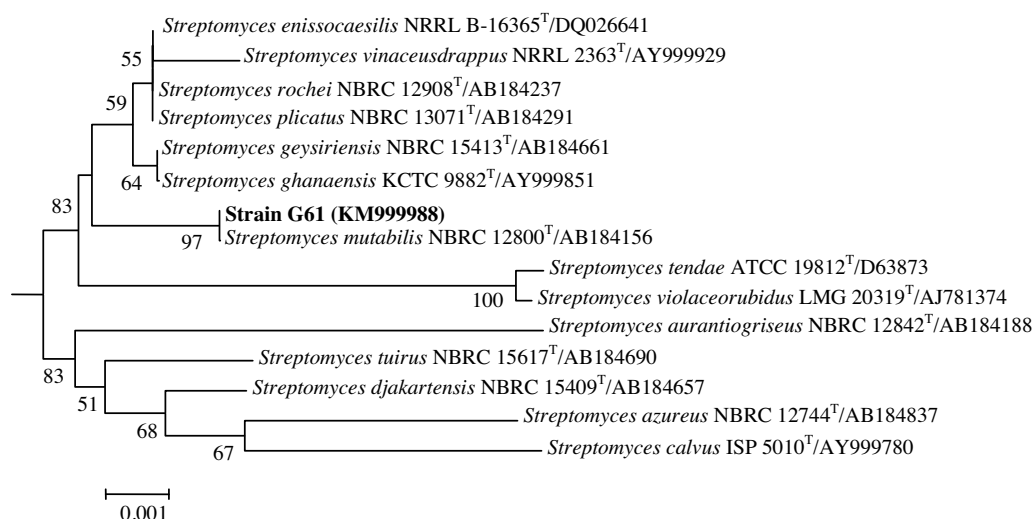


Figure 1 Neighbor-joining tree based on 16S rDNA sequences showing the relationship between the strain G61 and the related type-species of the genus *Streptomyces*. The numbers at the nodes indicate the levels of bootstrap support ($\geq 50\%$) based on neighbor-joining analyses of 1000 resampled data sets. Bar: 0.001 nt substitution per nt position.

Arbre phylogénétique basé sur l'analyse des séquences de l'ADNr 16S montrant la relation entre l'isolat G61 et les souches-types des espèces les plus proches du genre *Streptomyces*.

under the accession number [KM999988](#). Phylogenetic analysis using this sequence confirmed that the isolate G61 belonged to the genus *Streptomyces*. Its position in the 16 rDNA *Streptomyces* tree is shown on [Fig. 1](#). The similarity level was 100% with *Streptomyces mutabilis* NBRC 12800^T [46].

Antifungal activity

The isolate G61 showed a broad-spectrum antifungal activity ([Table 2](#)). It exhibited strong activity against *C. albicans* M3 and strong to moderate activity against *C. albicans* M1,

Table 2 Antimicrobial activities of strain G61 on ISP-2 medium.

Activité antimicrobienne de l'isolat G61 sur milieu ISP-2.

Target yeasts	Distance of inhibition (in mm) ^a
<i>Candida albicans</i> M1	23.0 ± 1.0
<i>C. albicans</i> M2	17.6 ± 0.6
<i>C. albicans</i> M3	25.6 ± 1.1
<i>C. albicans</i> IPA200	22.0 ± 1.0
<i>C. albicans</i> IPA988	22.0 ± 1.0
<i>Fusarium culmorum</i> (FC200)	17.0 ± 1.0
<i>F. graminearum</i> (Fg3)	18.3 ± 0.6
<i>Aspergillus carbonarius</i> (M333)	26.3 ± 1.5
<i>A. westerdijkiae</i> (ATCC 3174)	7.6 ± 1.1
<i>A. parasiticus</i> (CBS 100926)	5.6 ± 0.6
<i>A. flavus</i> (NRRL 3251)	8.6 ± 1.1
<i>Umbelatosia ramanniana</i> (NRRL 1829)	25.0 ± 1.0
<i>Staphylococcus aureus</i> (ATCC 43300)	21.3 ± 0.6
<i>Escherichia coli</i> (ATCC 10536)	5.3 ± 0.6

^a Average ± standard deviation from three replicates per treatment.

IPA200, IPA988 and M2. The activity against filamentous fungi was moderate with *F. culmorum* (FC200) and *F. graminearum* (Fg3), strong with *U. ramanniana* (NRRL 1829) and *A. carbonarius* (M333) and moderate to low against the other species of the genus *Aspergillus*. Strong activity was detected against *S. aureus* (ATCC 43300), but low activity was found against *E. coli* (ATCC 10536). According to these results, the strains *C. albicans* M3 and *A. carbonarius* (M333) are the most susceptible. For this reason, they have been chosen for the further work.

Time course of growth and antifungal compounds production

The kinetics of antibiotic production, growth and pH were monitored in ISP-2 and Bennett broth cultures, as shown on [Fig. 2](#). The activities were detected on the first day of fermentation against *C. albicans* M3 in the broth Bennett medium and on the second day in the ISP-2 broth medium, reached a maximum after 4 days on Bennett and 5 days on ISP-2. The antifungal activity was found to be correlated with the cell growth, so the biomass increased from the first day, reached the maximum at day 4, and then decreased after the 8th day. The pH varied between 6.8 and 7.8 during the incubation.

Isolation and purification of the bioactive compounds

The antifungal compounds were extracted from broth culture (Bennett medium) with *n*-butanol, but not with *n*-hexane, dichloromethane and ethyl acetate. The active extract was concentrated to dryness using the rotavapor, recuperated in methanol and analyzed by TLC developed in ethyl acetate-methanol system (100:15 v/v). One active band, named fraction G, was detected by bioautography

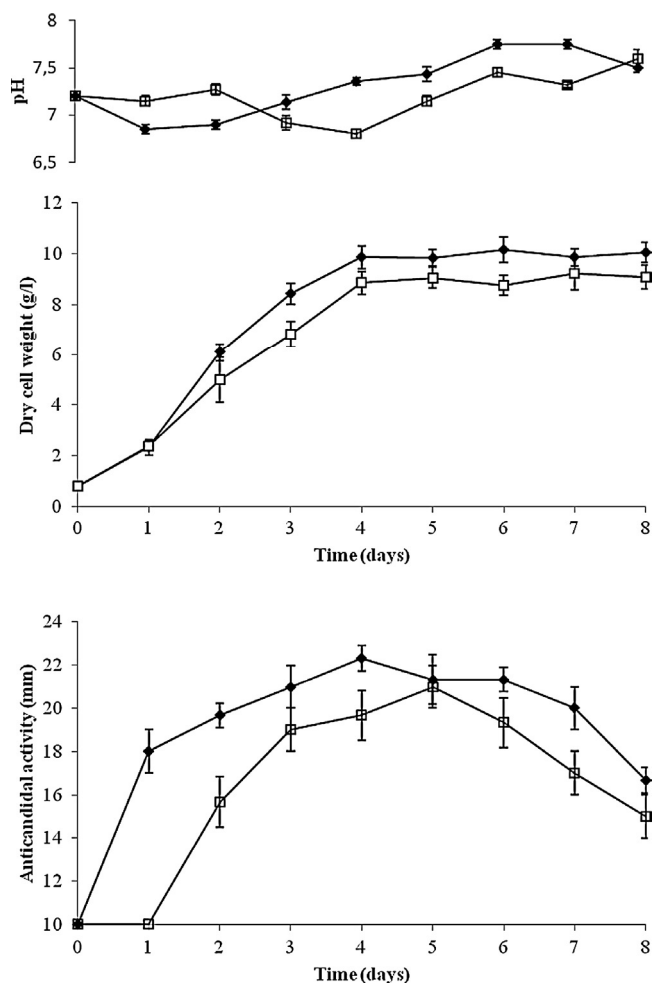


Figure 2 Time course of pH, growth and activity against *Candida albicans* M3 on ISP-2 and Bennett broth media ISP-2 (□), Bennett (◆). Measurements of activity against yeast represent the diameters of inhibition with including the diameter of wells (10 mm). Each measure represents average \pm standard deviation from three replicates per treatment.

Cinétiques de l'évolution du pH, de la croissance et des activités contre Candida albicans M3 dans les milieux ISP-2 et Bennett.

($R_f = 0.44$). This band showed a strong activity against *C. albicans* M3 and *A. carbonarius* (M333). The active band was 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 one active peak (retention time, 50.0 min), which was designated GB61. The active peak was collected separately and reinjected into the HPLC system until total purification was achieved. The pure compound had a pale yellowish color.

Spectroscopic studies

The UV–visible spectrum of the bioactive compound GB61 showed maxima at 221, 228 and 280 nm. The molecular weight of the active compound is 206.3. The ^{13}C , HSQC and HMBC spectra showed 10 carbon signals. It was possible to discern 1 phenol group (δ_c 153.38), 4 sp^3 –hybridized carbons (δ_c 28.67 to 34.31) and 5 sp^2 –hybridized carbons (δ_c 115.19 to 140.96) for GB61 molecule. The hydrogen of the phenol group is not observed due to rapid exchange with MeOD. The 2D ^1H – ^1H and ^1H – ^{13}C experiments and especially the long-range ^1H – ^{13}C couplings observed in the HMBC

spectrum (Fig. 3 and Table 3) permitted to establish the connectivity between all the groups of the molecule. The structure of the GB61 compound was determined by NMR to be 2,4-Di-tert-butylphenol (2,4-DTBP).

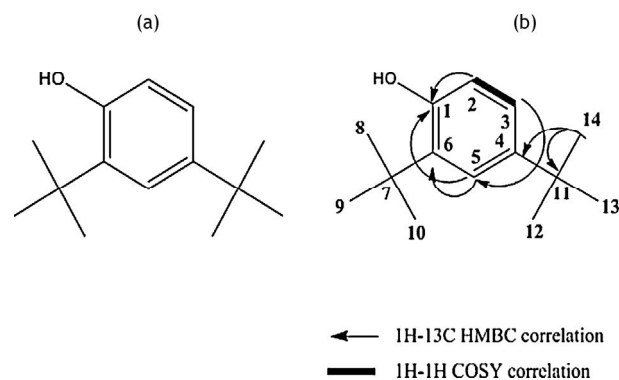


Figure 3 Structure of the compound GB61 (a) and COSY and HMBC correlation (b).

Structure du composé G61 (a) et corrélation COSY et HMBC (b).

Table 3 ^1H and ^{13}C NMR data assignments of GB61 compound in CD_3OD at 298 K (see Fig. 3 for numbering of hydrogen and carbon atoms).

Données RMN ^1H et ^{13}C du composé GB61 dissout dans du CD_3OD à 298 K.

^1H and ^{13}C number	^1H chemical shift, ppm	^{13}C chemical shift, ppm
1	–	153.38
2	6.64	115.19
3	7.02	122.82
4	–	140.96
5	7.24	122.91
6	–	134.77
7	–	34.31
8–9–10	1.40	28.67
11	–	33.58
12–13–14	1.29	30.76

Minimum inhibitory concentration

The minimum inhibitory concentrations (MIC) of the compound GB61 is shown in Table 4. It can be seen that this compound has high activity against *C. albicans* M3 (MIC: 5 $\mu\text{g}/\text{mL}$) and moderate activity against the other strains: *C. albicans* M1, IPA200, IPA988 (MIC: 10 $\mu\text{g}/\text{mL}$) and M2 (MIC: 15 $\mu\text{g}/\text{mL}$). Moderate to low activity is shown against filamentous fungi (MIC between 10 and 100 $\mu\text{g}/\text{mL}$). The comparison of GB61 MIC values with the standard antifungal compounds used in the experience indicates that this compound showed potent activity against *C. albicans* strains (range of 5–15 $\mu\text{g}/\text{mL}$) with approximate values to nystatin MIC values (10 $\mu\text{g}/\text{mL}$), more than the tioconazole (75 $\mu\text{g}/\text{mL}$) but less than the amphotericin B (range of 1–3 $\mu\text{g}/\text{mL}$). The comparison of GB61 MIC values with those of the three

standard antifungal compounds against the filamentous fungi showed a lowest activity.

Discussion

On the basis of its morphological and chemical properties, the strain G61 was classified in the genus *Streptomyces* [18]. This genus contained 663 species including 38 subspecies [14]. The morphological and physiological properties of strain G61 are similar to those of *S. mutabilis* NBRC 12800^T [18] except for the utilization of D-mannitol for growth. The 16S rDNA sequence of strain G61 was compared with those of other *Streptomyces* species. The similarity level was 100% with *S. mutabilis* NBRC 12800^T the most closely related species. This result confirms the previous phenotypic studies, and indicates that the strain G61 is *S. mutabilis*. Our isolate showed a broad-spectrum antifungal activity and exhibited strong activity against *C. albicans* M3 and *A. carbonarius* (M333). It is reported that other strains of *S. mutabilis* were found to have antifungal activity such as *S. mutabilis* NRP1–14 [42] and *S. mutabilis* MS-6-6 [57]. One active fraction with interesting antifungal activity was detected in the butanolic extract by silica gel thin layer chromatogram. This fraction was found to contain one active compound by HPLC.

The UV–visible maxima were 221, 228 and 280 nm and the molecular weight was 206.3. ^{13}C NMR spectra showed the presence of 10 carbon signals, of which six downfield carbon signals between 115.19 and 153.38 ppm were in the aromatic region, whereas the remaining four carbon signals were in the upfield region between 28.67 and 34.31 ppm. Of six aromatic carbon signals, three were quaternary carbons (134.77, 140.96 and 153.38 ppm), suggesting that the aromatic substitutions were at these carbons. The most downfield quaternary carbon at 153.38 ppm indicated the presence of a phenolic OH group at this carbon in the

Table 4 Minimum inhibitory concentrations (MIC) of 2,4-DTBP produced by strain G61 and standard antifungal compounds against target microorganisms.

CMI du composé 2,4-DTBP produit par l'isolat G61 et des antifongiques standards contre les microorganismes cibles.

Target microorganisms	MIC ($\mu\text{g}/\text{mL}$) ^a			
	GB61	N	A	T
<i>Candida albicans</i> M1	10	10	3	75
<i>C. albicans</i> M2	15	10	3	75
<i>C. albicans</i> M3	5	10	3	75
<i>C. albicans</i> IPA200	10	10	3	75
<i>C. albicans</i> IPA988	10	10	1	75
<i>Fusarium culmorum</i> (FC200)	30	5	3	10
<i>F. graminearum</i> (Fg3)	30	10	3	10
<i>Aspergillus carbonarius</i> (M333)	20	5	3	1
<i>A. westerdijkiae</i> (ATCC 3174)	> 100	10	3	40
<i>A. parasiticus</i> (CBS 100926)	> 100	5	10	10
<i>A. flavus</i> (NRRL 3251)	> 100	3	10	10
<i>Umbelatosia ramanniana</i> (NRRL 1829)	10	3	3	15
<i>Staphylococcus aureus</i> (ATCC 43300)	30	ND	ND	ND
<i>Escherichia coli</i> (ATCC 10536)	> 100	ND	ND	ND

N: nystatin (97%); A: amphotericin B (80%); T: tioconazole (96.5%); ND: not determined.

^a MIC values represent the average of two repetitions.

molecule. Among the four upfield carbon signals, the two at 33.58 and 34.31 ppm were tertiary carbons and the remaining two at 28.67 and 30.76 ppm were methyl signals. This clearly suggested the presence of two tert-butyl substitutions on the two remaining quaternary carbons at 134.77 and 140.96 ppm in the aromatic ring. Based on these spectroscopic studies, it can be predicted that the molecule is 2,4-Di-tert-butylphenol (2,4-DTBP). The 2,4-DTBP belongs to the alkylphenols family [53]. It is produced by bacteria such as *Sphingobium fuliginis* [53], *Flavobacterium johnsoniae* [43], a cyanobacteria [8], and *Pseudomonas putida* [12], and also by higher fungi and plants such as *Termitomyces heimi*, *Pleurotus sajor-caju* and *Hericium erinaceus* [31], *Pereskia bleo* [48], *Balanites aegyptiaca* [26], *Piper nigrum* [16], and *Pennisetum purpureum* [11]. 2,4-DTBP has been reported to have many bioactive properties: anti-carcinogenic agent [32], antimalarial activity [26], biocontrol agent against *Phytophthora capsici* [43], antioxidant effect [8], herbicidal substance [11] and antifungal compound against *Fusarium oxysporum* [12]. 2,4-DTBP is also widely used in the plastics industries [31] and in manufacturing of pharmaceuticals and fragrances [19]. The proposed mechanism of 2,4-DTBP activity as described by Dharni et al. [12] was the inhibition of spore germination by preventing the emergence of normal germ tube leading to abnormal swelling and branching of hyphae. It has been demonstrated that 2,4-DTBP inhibits the assembly of spindle microtubules and disturbs the chromosomal alignment at the metaphase plate and microtubule–kinetochore interactions, causing chromatid loss, which may reduce the mycelia growth and the germination of spores [54]. The cytotoxic property of 2,4-DTBP has been reported by different research groups. This molecule has a cytotoxic activity against the human cancer cell lines KB (IC₅₀ value of 0.8 µg/mL) and against MCF7 (IC₅₀ 5.75 µg/mL), A549 (IC₅₀ 6 µg/mL), Caski cells (IC₅₀ 4.5 µg/mL), normal human cell line MRC-5 (IC₅₀ 20 µg/mL) [31] and HeLa cell lines (IC₅₀ 10 µg/mL) [54]. It is the first time that this compound is reported in actinobacteria and the genus *Streptomyces*, and it has never been described as anticancer compound.

Disclosure of interest

The authors declare that they have no competing interest.

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