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Original Article

The new *Streptomyces* sp. TN605 Strain Secretes Simultaneously three active compounds and a High Level of the Interesting Pharmaceutical Industry Intermediate: 2-Hydroxyphenylacetic Acid

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ABSTRACT

An actinomycete bacterium isolated from south Tunisian soil was selected for its antimicrobial activities against Gram-positive and Gram-negative bacteria and fungi. Based on the results of cultural characteristic studies of this strain, the nucleotide sequence of the corresponding 16S rRNA gene (1391 pb accession n° FR853280) and the phylogenetic analysis, we propose the assignment of our new isolate bacterium as Streptomyces sp. TN605 strain. Ethyl acetate extract of a 20 litres culture broth of the Streptomyces sp. TN605 delivered three active compounds which are: brevianamide F, tryptophol and Cis-cyclo (Leucyl-Prolyl) and a fourth highly secreted molecule, 2-Hydroxyphenylacetic Acid, which is an aromatic compound of a high-added-value. To our knowledge, it is the first time that this compound is described from terrestrial Streptomyces bacterium.

INTRODUCTION

In human therapy, the development of resistance to multiple drugs is a major problem in the treatment of infections by pathogenic microorganisms. This antimicrobial resistance is presently an urgent focus of research and new bioactive compounds are necessary to combat these pathogens. Moreover, in agriculture domain, the use of chemical fungicides not only may pollute the atmosphere but also can be environmentally harmful as the chemicals spread out in the air and accumulate in the soil. Furthermore, the repeated use of such chemicals has increased the development of chemical resistance in the target organisms [1]. Fungicides of microbial origin, which are synthesized biologically, have been demonstrated to be effective on the target organisms and also inherently biodegradable [2]. Filamentous soil bacteria belonging to the genus *Streptomyces* are rich sources of a variety of bioactive natural products having great functional diversity [3, 4], which are extensively used as pharmaceuticals and agrochemicals [5, 6]. During our search program for bioactive compounds, a new actinomycete bacterium was isolated from south Tunisian soil and selected for its antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi. The present paper describes the identification and the phylogenetic determination of this bacterium, named *Streptomyces* sp. TN605 strain, as well as the influence of different nutritional additives on the biosynthesis of the bioactive molecules by this strain. Large scale fermentation (20 litres) of the *Streptomyces* sp. TN605 strain and extraction of yielded bioactive constituents in supernatant with ethyl acetate was carried out. An application of the crude extraction to purification using a series of chromatographic techniques yielded three bioactive compounds: brevianamide F, tryptophol and *cis-Cyclo* (Leucyl-Prolyl) and a fourth molecule: 2-Hydroxyphenylacetic Acid, an intermediate compound frequently used in the pharmaceutical industry [7]. Structures of these four obtained compounds were addressed using intensive studies of NMR spectroscopy and mass spectrometry as well.

MATERIAL AND METHODS

^{1}H NMR spectra: Varian Unity 300 (300 MHz), Bruker AMX 300 (300 MHz) or Varian Inova 500 (499.8 MHz). ^{13}C NMR spectra: Varian Unity 300 (75.5 MHz) or Varian Inova 500 (125.7 MHz). Chemical shifts were measured relatively to tetramethylsilane as internal standard. Mass spectra: EI MS with Varian MAT 95 Finigan (70 eV), high resolution with perflurokerosine as standard, ESI MS LCQ (Finnigan), DCI with Varian MAT 95 and NH3 as reactand gas. UV-VIS spectra were recorded on a Perkin-Elmer Lambda 15 UV/VIS spectrometer. High performance liquid chromatography (HPLC): Instrument I: Analytical: Jasco multiwavelength detector MD-910, two pumps type Jasco Intelligent Prep. Pump PU-987 with mixing chamber, injection valve (type Rheodyne) with sample loop 20 µl, Borwin-HPLC-software. Flash chromatography was carried out on silica gel (230-400 mesh). Thin layer chromatography (TLC) was performed on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.). $\boldsymbol{R_f}$ values were measured on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.). For preparative TLC (PTLC) we have used (glass plates, 1.5 mm silica gel 60 F_{254}, merck, Darmstad, Germany. Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia).

Isolation and identification of the new Streptomyces sp. TN605 strain

This strain was isolated from south Tunisian soil and selected for its antimicrobial activities against Gram-positive and Gram-negative bacteria and fungi. Transformation of *E. coli* DH5 with pIJ2925 derivatives was carried out according to [8]. The TN605 strain was grown in tryptic soy broth (TSB) at 30 g/l for the preparation of genomic DNA. Cultural characteristics of the studied strain were compared on the bases of observations made after 7, 14 and 21 days incubation on nutrient agar, Sabouraud agar and yeast malt agar media.

PCR amplification of the 16S rRNA gene of TN605 strain was performed in an automated thermocycler (Perkin Elmer) using two primers 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3' according to [9]. Nucleotide sequence of the 16S rRNA gene was determined on both strands by an automated 3100 Genetic Analyser (Applied Biosystems) using specific primers. Homology search was performed using Blast Search algorithm. The nucleotide sequence of the whole 16S rRNA gene 1391 pb of TN605 strain has been assigned GenBank (EMBL) under accession number n° FR853280. Multiple sequence alignment was carried out using clustal W [10] at the European Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw/). Phylogenetic analyses were performed using programs from the PHYLIP package [11] and phylogenetic tree was constructed by the neighbour joining (NJ) algorithm [12] using Kimura 2-parameter distance. The robustness of the inferred tree was evaluated by bootstrap (100 replication).

Cultural optimization of the new Streptomyces sp. TN605 strain

To investigate the influence of the medium on antimicrobial production, spores at 10^7 g/l were used to inoculate 500-ml Erlenmeyer flasks with four indents, containing 100 ml of TSB medium (30 g/l). After incubation at 30°C for 24 h, this pre-culture was used to inoculate at 1/10 (v/v) 1000-ml Erlenmeyer flasks with four indents, containing 200 ml of modified Bennett medium (peptone 2 g/l, yeast extract 1 g/l, beef extract 1 g/l) supplemented at 1% (w/v) with one of the five tested carbon sources (starch, fructose, glycerol, glucose and saccharose). After incubation at 30°C for 72 h in an orbital incubator with shaking at 200 rpm, biological activity was assayed for each culture supernatant. Influence of magnesium, potassium and trace mineral oligoelements on active molecules production was also investigated by addition of these chemical additives to the culture medium, additive by additive, the combination of two additives and the addition of all three additives. A culture without chemical additives was used as control. The final magnesium and potassium concentration was 3.5 and 1 mM respectively. For trace mineral oligoelements (40 mg $ZnCl_2$, 200 mg $FeSO_4{\cdot}7H_2O$, 6.5 mg H_3BO_3 and 13.5 mg $MoNa_2O_4{\cdot}2H_2O$ per 100 ml distilled water), 7.5 ml were added to 1 l of growth medium. Eight different concentrations (% w/v) of glycerol were tested: 0.2; 0.5; 0.75; 1; 1.25; 1.5; 1.75 and 2. The effect of culture conditions: temperatures (25, 30, 35 and 40°C), incubation times (18, 30, 48, 64, 78, 90, 110, 120 and 130 h) and rotary shaker (100, 150, 200, 250 and 300 rpm) on growth and antimicrobial activity production was studied. Biomass of the *Streptomyces* sp. TN605 strain was determined by measuring the dry weight after drying at 105°C. Antibacterial activity against *Micrococcus luteus* LB 14110, *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8739, and antifungal activity against *Fusarium* sp. *Verticillium dahlia* and *Candida tropicalis* were performed according to [6].

Fermentation and working up

For the purification of the active molecules from the *Streptomyces* sp. TN605 strain, spores at 10^7/ml were used to inoculate 1000-ml Erlenmeyer flasks, each containing 200 ml of TSB medium at 30 g/l. After incubation at 30°C for 24 h in an orbital incubator shaking at 200 rpm, the pre-culture was used to inoculate 20 l of modified Bennett medium, supplemented with glycerol at 0.75% (w/v). For this realisation, we have prepared four cultures in 7 l (working capacity of 5 l) fermentor (INFORS AG CH-4103 Bottmingen/Switzerland). After 64 h fermentation (30°C, 200 rpm), the culture broths were filtered and centrifuged. The afforded supernatant was extracted twice by an equal volume of ethyl acetate. The obtained organic extract was concentrated *in vacuo* to dryness, affording 2.51 g of a reddish brown crude extract.

Isolation and chemical characterization

The resulting crude extract (2.51 g) was fractionated on silica gel using CH_2Cl_2-MeOH as eluent system. After monitoring with TLC, six fractions (FI-FVI) were obtained. Guided bioassay of the afforded fractions showed an antimicrobial importance for fractions FII, FIII and FIV.

Purification of fraction FII (695 mg) using gel filtration on a Sephadex LH-20 column (CH_2Cl_2/MeOH 6:4) afforded three sub-fractions (FII_1-FII_3). Sub-fraction FII_1 (385 mg) was purified with the aid of PTLC (CH_2Cl_2/MeOH 95:5) to deliver a colourless solid of brevianamide F (**M1**, 22.5 mg). Further purification of the same sub-fraction (FII_1) using Sephadex LH-20 column (H_2Cl_2/MeOH 6:4) afforded 2-hydroxyphenyl acetic acid (**M2**, 48.1 mg**)** as colourless solid. A similar fractionation of fraction FIII (425 mg) using Sephadex LH20 column (CH_2Cl_2/MeOH 6:4) produced three sub-fractions ($FIII_1$-$FIII_3$). Sub-fraction $FIII_3$ (187 mg) was subjected to purification on silica column and eluted with CH_2Cl_2-MeOH gradient and followed by PTLC (hexane/ethyl acetate 94:6) to afford tryptophol (**M3**, 14.8 mg**)** as colourless solid. Finally, Fraction FIV (335 mg) was purified by gel filtration on a sephadex LH-20 column (MeOH) to produce two sub-fractions (FIV_1-FIV_2). Purification of sub-fraction FIV_2 (121 mg) using Sephadex LH-20 (CH_2Cl_2/MeOH 6:4) and PTLC (CH_2Cl_2/MeOH 95:5) led to a colourless solid of *cis-cyclo* (Leucyl-*P*rolyl) **(M 4**, 12.3 mg**)**.

Brevianamide F (M1): C16H17N3O2 (283.3)**,** isolated from fraction II, colourless solid, UV absorbing turned dark red by spraying with anisaldehyde/sulphuric acid, pink with Ehrlich's reagent and blue with chlorine/*o*-anisidine. **-*R***f = 0.47 (CHCl3/MeOH: 9:1). **- ^{1}H NMR (**CDCl3, 300 MHz): δ = 8.20 (s, 1H, NH), 7.61-7.14 (m, 5 H, 12-H, 16-H, 17-H, 18-H, 19-H), 4.39 (dd, *J* = 11, 3.5 Hz, 1H, 8-H), 4.08 (t, *J* = 7.5 Hz, 1 H, 5-H), 3.76 (dd, *J* = 15, 3.5 Hz, 2-Ha), 3.64 (m, 2 H, 10-CH2), 2.97 (dd, *J* = 10.9 Hz, *J* = 15.1 Hz, 1 H, 2-Hb), 2.36-1.90 (m, 4H, 3- CH2, 4-CH2). **- ^{13}C/APT NMR** ($CDCl_3$, 75.478 MHz): δ = 169.48 (Cq-9), 165.69 (Cq-6), 136.81 (Cq-14), 126.85 (Cq-15), 123.39 (CH-12), 122.81 (CH-16), 120.04 (CH-17), 118.55 (CH-18), 111.61 (Cq-11), 109.99 (CH-19), 59.18 (CH-5), 54.60 (CH-8), 45.33 (CH_2-10), 28.19 (CH_2-2), 26.76 (CH_2-4), 22.48 (CH_2-3). **- EI MS (**70 eV): *m/z* (%) = 283 ($[M]^+$, 17), 154 (10), 130 (100).

2-hydroxyphenyl acetic acid (M2): $C_8H_8O_3$ (152.15), isolated from FII, colourless solid, UV absorbing. - R_f = 0.8 ($CHCl_3$/MeOH 9:1). - **^{1}H NMR** ($CDCl_3$, 300 MHz): δ = 7.18 (dd, *J* = 8.8, 1.1 Hz, 1 H, 6-H), 7.08 (td, *J* = 8.8, 1.0 Hz, 1 H, 4-H), 6,85 (d, *J* = 8.8 Hz, 1 H, 5-H), 6.78 (td, *J* = 8.8, 1.0 Hz, 1 H, 3-H), 3.62 (s, 2 H, 7-CH_2). **- ^{13}C/APT NMR** ($CDCl_3$, 75.478 MHz): δ = 183.57 (Cq-8), 156.98 (Cq-1), 133.82 (CH-6), 130.93 (CH-5), 127.02 (Cq-2), 123.39 (CH-4), 118.87 (CH-3), 42.70 (CH_2-7). **- EI MS** (70 eV): *m/z* (%) = 152.1 ($[M]^+$, 98), 134.1 ($[M-H_2O]^+$, 75), 106.1 ($[M-HCOOH]^+$, 100) 78.0 (49), 43.1 (23).

Tryptophol (M3): $C_{10}H_{11}NO$ (161.2), afforded from fraction FIII, colorless solid, UV absorbing turned to intense violet by anisaldehyde/sulphuric acid. **- *R***f = 0.28 (CHCl3/ 10% MeOH). **- ^{1}H NMR (**CDCl3, 500 MHz): δ = 8.09 (brs, 1 H, NH), 7.54 (d, *J* = 7.9 Hz; 1 H, 4-H), 7.32 (d, *J* = 7.9 Hz, 1 H, 7-H), 7.14 (ddd, *J* = 7.9, 7.9, 1.2 Hz, 1 H, 6-H), 7.06 (ddd,*J* = 7.9, 7.9, 1.2 Hz, 1 H 5-H), 7.01 (d, J = 2.4, 1 H, 2-H), 3.93 (t, *J* = 6.6 Hz, 2 H, 9-CH2), 3.05 (t, *J* = 6.6 Hz, 2 H, 8-CH2) **- ^{13}C/APT NMR (**CDCl3, 125.707 MHz): δ = 136.41 (Cq-7a), 127.36 (Cq-3a), 122.47 (CH-2), 122.19 (CH-6), 119.45 (CH-5), 118.81 (Cq-4), 112.23 (Cq-3), 111.19 (CH-7), 62.58 (CH2- 9), 28.71 (CH2-8). **– EI MS** (70 eV): *m/z* (%) = 161 ($[M]^{+\cdot}$, 29), 130 (100), 103 (11), 77 (16).

Cis-cyclo*(Leucyl-Prolyl) (M4)**: $C_{11}H_{18}N_2O_2$ (210.27)**,** delivered from fraction IV, colourless solid, UV absorbing turned to violet by anisaldehyde/sulphuric acid. **- *Rf = 0.6 ($CHCl_3$/MeOH 95:5). **- ^{1}H NMR (**$CDCl_3$, 300 MHz): δ (ppm) = 6.39 (brs, 1 H, NH), 4.15 (t, *J* = 8.1 Hz, 1 H, 9-H), 4.04 (dd, *J* = 10.2, 6.2 Hz, 1 H, 6-H), 3.64-3.54 (m, 2 H, 3-CH_2, 10-Hb), 2.37 (m, 1 H, 10-Ha), 2.22-1.98 (m, 3 H, 4-H_2, 5-Ha), 1.75

(m, 1 H, 5-Hb), 1.55 (m, 1 H, 11-H), 1.03 (d, *J* = 8.1 Hz, 3 H, 12-CH_3), 0.96 (d, *J* = 8.1 Hz, 3 H, 13-CH_3). **- ^{13}C/APT NMR** ($CDCl_3$, 75,487 MHz): δ = 170.3 (Cq-1), 166.1 (Cq-7), 59.0 (CH-6), 53.4 (CH-9), 45.5 (CH_2-3), 38.6 (CH_2-5), 28.1 (CH_2-10), 24.7 (CH-11), 23.3 (CH_3-12), 22.7 (CH_2-4), 21.2 (CH_3-13). **- CI MS** (NH_3): *m/z* (%) = 438 ($[2M + NH_4]^+$, 7), 421 ($[2M + H]^+$, 4), 245 ($[M + NH_3 + NH_4]^+$, 64), 228 ($[M + NH_4]^+$, 100), 211 ($[M + H]^+$, 40).

RESULTS AND DISCUSSIONS

Isolation, selection and identification of the TN605 strain

A new actinomycete, named TN605 strain was isolated from south Tunisian soil and selected for its antimicrobial activities against Gram-positive and Gram-negative bacteria and fungi. Strain TN605 grew well in nutrient agar medium and yeast malt agar media and the colours of the vegetative and aerial mycelia were whitish and greyish, respectively. The spore chains were greyish in nutrient agar medium and whitish in yeast malt media. Total nucleotide sequence of 1391 pb (accession n° FR853280), of the whole 16S rRNA gene of the TN605 strain was determined in both strands. Based on the cultural characteristics of TN605 strain, the nucleotide sequence of the corresponding 16S rRNA gene and the phylogenetic analysis (Figure 1), we propose the assignment of our new isolate bacterium as *Streptomyces* sp. TN605 strain.

Optimization of nutritional and cultural conditions

It has been reported by several works that the composition and the concentrations of the constituents of the media are closely linked with the metabolic capacities of the producing organism and greatly influence the biosynthesis of the bioactive molecules [6, 9]. Equally, the effect of other factors including medium volume, oxygen transfer rate, temperature, initial pH of the medium and the incubation time on secondary metabolites production were frequently described [6, 9]. For the optimization of nutritional and cultural conditions of the studied strain, five carbohydrate sources (starch, fructose, glycerol, glucose and saccharose) were tested as sole carbon source at 1% (w/v) in the modified Bennett liquid medium. The studied strain exhibited the ability to grow on all tested carbon sources. The maximum biological activity production was afforded when glycerol was served as carbon source. To further optimize the culture conditions, three compounds potassium at 3.5 mM, magnesium at 1 mM and trace mineral oligoelements were tested using glycerol at 1% (w/v) as carbon source. The obtained results showed that the antimicrobial activity against the tested microorganisms was not affected by the addition of these three chemical compounds. We have also investigated the effect of the glycerol concentration. To do so, eight concentrations (% w/v) were tested (0.2, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2.0). Obtained data showed that the biological activity was correlated with biomass production. This biomass increased with increasing glycerol concentrations up to 0.75% (w/v) and remained nearly constant up to 1.25% (w/v) with a maximum at 0.75% (w/v). Any further increase of this substrate level resulted in a decrease in biomass and consequently in active molecules production. *Streptomyces* sp. TN605 strain showed a narrow range of incubation temperature for good growth and active molecules production. The optimum temperature was 30 °C. Concerning incubation time, antimicrobial activity appeared to be pronounced after 30 h of growth with a maximum at 64 h of incubation. This activity remained stable between 64 and 90 h and then decreased and disappeared after 130 h of incubation. Agitation rates of 100, 150 and 300 rpm gave a low production of active molecules, while the best result was noticed at 200 and 250 rpm.

Structure elucidation of the active compounds

Brevianamide F (M1). This Compound was obtained as a colourless solid from fraction II showing an UV absorbing band. An exposing of this band to spraying with anisaldéhyde/sulphuric acid lead to dark red staining , while it was turned to pink with Ehrlich's reagent and blue with chlorine/*o*-anisidine. The latter reaction established amide bonds in compound **M1**. The ^{1}H NMR spectrum of **(M1)** showed five aromatic protons (δ 7.61-7.14), beside to a broad 1H singlet (δ 8.20) for an NH. This fits on an indole skeleton substituted at 3-position. In the aliphatic region, another broad 1H singlet of an acidic proton at δ 5.75 due to NH of an amide, two oxy-or amino-methines at δ 4.39 (dd) and 4.08 (t) were visible. Moreover, three different resonances each of 1H, 2H, and 1H, respectively, were observed at δ 3.76 (dd), 3.64 (m) and 2.97 (dd). In addition, three multiplets of 4H, representative for two methylene groups, were visible between δ 2.36 and 1.90. This pointed to an

additional prolyl residue. The ^{13}C NMR spectrum exhibited four signals in the region between 22.48 and 45.33 ppm attributed to four CH_2. The rest of the spectrum displayed 12 signals, among them seven CH (123.4-110.0) and five quaternary carbons (169.5-111.6), two of them belong to amide carbonyls (169.5 and 165.7).

This was supported by the molecular weight of 283 Dalton (EI mass spectrum) and by a fragment at *m/z* 154 due to a glycinyl-proly residue, and a base peak at *m/z* 130 assigned to the indolyl-3-methylene ion. Based on these features, search in Antibase [13] and comparison with literature [14, 15], compound **M1** was established as brevianamide F (Figure 2). The class brevianamide (from A to F) has been reported for the first time in 1969 [16]. It has been reported that brevianamides possess nematocidal activity [17], anti-inflammatory [18] insecticidal properties [19] and antibacterial activity [20]. Brevianamide F is mostly described from fungi of the genera *Aspergillus* and *Penicillium*, and recently, this compound has been isolated from a culture of a *Streptomyces* species [20].

2-Hydroxyphenylacetic Acid (M2). This compound was further isolated from fraction II, exhibiting a middle polar UV absorbing band on TLC. The ^{1}H NMR spectrum displayed four proton signals (δ 7.18-6.78) of a 1,2-disubstituted aromatic ring. In addition, one 2H singlet of a methylene group was found at δ 3.62. The ^{13}C NMR spectrum contains 8 signals. The first at 42.70 ppm attributed to the CH_2 carbon. Four other signals (δ 118.87, 123.39, 130.93, 133.82) were attributed to 4 CH (CH-3,6). The rest of the signals were related to 3 Cq (δ 127.02, 156.98, 183.57). The EI mass spectrum delivered the molecular weight of **M2** as 152 Dalton. The molecular ion exhibited two further fragments at *m/z* 134 and 106 due to elimination of H2O and expulsion of acetic acid, respectively.

Table 1. ^{1}H ($CDCl_3$, 300; 500MHz); ^{13}C ($CDCl_3$, 75.478; 125.707 MHz) data of compounds **M1**-**M4**

Atom No	Chemical Shifts (ppm)							
	M1		M2		M3		M4	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	-	-	156.98	-	-	8.09 NH	170.30	-
2	28.19	3.76 Ha 2.97 Hb	127.02	-	122.47	7.01	-	-
3	22.48	2.36-1.90	118.87	6.78	112.23	-	45.50	3.64-3.54
3a	-	-	-	-	127.36	-	-	-
4	26.76	2.36-1.90	123.39	7.08	118.81	7.54	22.70	2.22-1.98
5	59.18	4.08	130.93	6.85	119.45	7.06	38.60	2.22-1.98
6	165.68	-	133.82	7.18	122.19	7.14	59.00	4.04
7	-	5.75 NH	42.70	3.60	111.19	7.32	166.10	-
7a	-	-	-	-	136.41	-	-	-
8	54.60	4.39	183.57	-	28.71	3.05	-	6.39 NH
9	169.48	-	-	-	62.58	3.93	53.40	4.15
10	45.33	3.64	-	-	-	4.5	28.10	2.37 2.22-1.98
11	111.61	-	-	-	-	-	24.70	1.55
12	123.39	7.61-7.14	-	-	-	-	23.30	1.03
13	-	8.20 NH	-	-	-	-	21.20	0.96
14	136.81	-	-	-	-	-	-	-
15	126.85	-	-	-	-	-	-	-
16	122.81	7.61-7.14	-	-	-	-	-	-
17	120.04	7.61-7.14	-	-	-	-	-	-
18	118.55	7.61-7.14	-	-	-	-	-	-
19	109.99	7.61-7.14	-	-	-	-	-	-

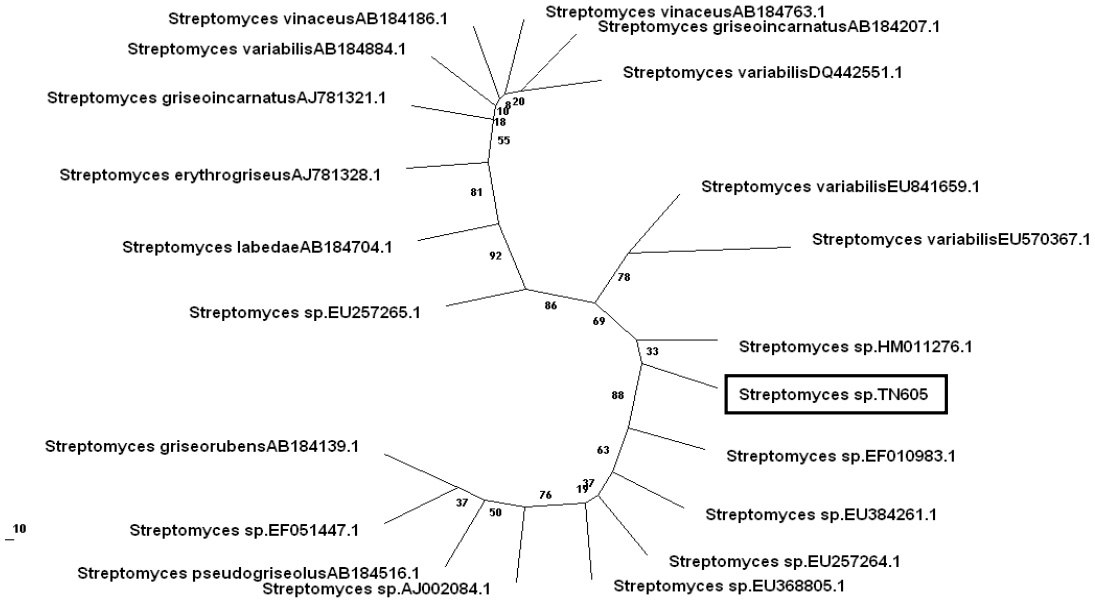
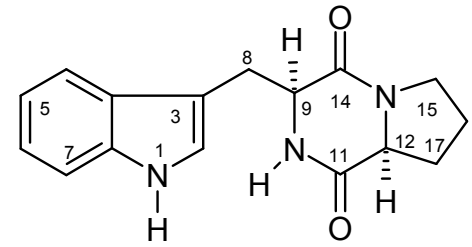
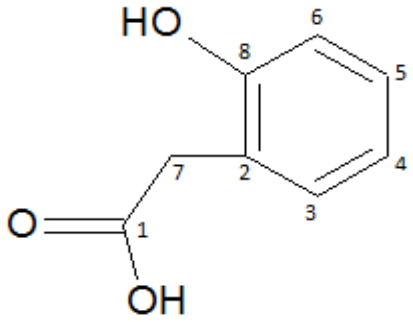


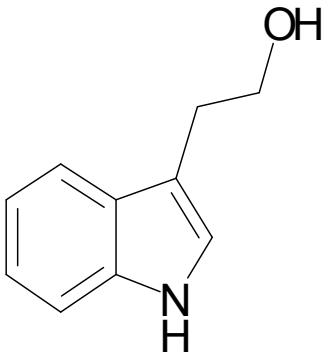
Figure 1. Phylogenetic tree of the *Streptomyces* sp. TN605 strain



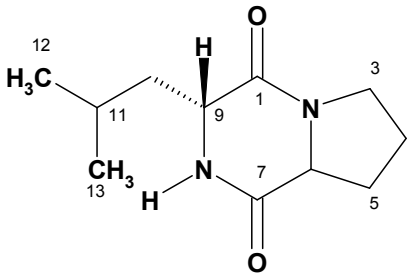
(Brevinamide F)
(2-hydroxyphenyl acetic acid)
M1



M2



Tryptophol
M3



***cis*-cyclo (Leucyl-prolyl)**
M4

Figure 2. Chemical structure of compounds **M1-M4**

In accordance, compound **M2** was recognized as 2-hydroxyphenylacetic acid, which was further confirmed by search in Antibase and comparison with the literature [21]. 2-hydroxyphenylacetic acid (Figure 2), was found as plant metabolite, e.g. from *Hydrangea macrophylla* [22] as well as fungal e.g. from *Phomopsis cassiae* [23] and bacterial e.g. from *Micomonospora* [24]. In our

knowledge, compound **(M2)** was isolated from terrestrial *Streptomyces* for the first time in this work. This compound stand out as aromatic molecule of a high-added-value. In fact, this product is frequently used in the pharmaceutical industry as intermediate in the preparation of some biologically active compounds such as antihypertensive agents [7]. Moreover, it should be noted that the new studied strain secretes the interesting compound **(M2)** with a high rate (48.1 mg), which is almost equivalent to the sum of the other three produced compounds (**M1**, **M3** and **M4**).

Tryptophol (M3). This Compound was obtained from fraction III as further colourless solid, showing violet colouration on spraying with anisaldehyde/sulphuric acid. The ^{1}H NMR spectrum showed two signals at 3.05 ppm (2H) and 3.93 ppm (2H) representing two CH_2 groups in addition to one signal at 8.09 ppm attributed to NH proton. Furthermore, five resonance signals were visible in aromatic region between 7.54-7.01 corresponding to 3-substituted indole moiety.

The ^{13}C NMR spectrum contains 10 signals; two among them were at 28.7 and 62.5, which were attributed to the two CH_2 carbons (C-8 and C-9) which were flanked by sp^2 and hydroxyl group, respectively. In the aromatic region eight carbon signals were shown in the region of 140~110, recognizing the 3-susbstituted indolyl moiety of tryptophol (**3**). The EI-MS gave the molecular ion peak [M^+] at m/z 161 which allowed identifying the molecular formula as $C_{10}H_{11}NO$ combining with the NMR data. Tryptophol (Figure 2), a well-known constituent of terrestrial plants and microorganisms, has so far been isolated from tryptophan fermentations, plant seedlings, *Aspergillus niger*, fungi *Balansia epichloe*, *Dreschslera nodulosum*, *Agrobacterium tumefaciens*, *Ceratocystis* spp., *Rhizobium* spp., marine sponge *Ircinia spinulosa*, and bacteria belonging to the *Streptomyces* genus [4]. This molecule was reported to have antibacterial activity against Gram-positive bacteria and antifungal activity against *Candida albicans* [4].

***Cis-cyclo* (Leucyl-Prolyl) (M4)**. This compound was isolated from fraction IV as middle polar colourless solid, which turned to violet by anisaldehyde/sulphuric acid and blue with chlorine/*o*-anisidine, pointing to its peptide property. The ^{1}H NMR spectrum showed a broad 1H singlet of an amide at δ6.39 ppm, two methane protons at δ4.15 (t), and 4.04 ppm (dd). Furthermore, a 3H multiplet between δ3.54-3.64 ppm (3-H_2, 10-H_b), a 1H multiplet at δ2.37 ppm (10-H_a), a multiplet between δ-2.22 ppm of (4- H_2, 5-H_a) and a multiplet at δppm of 5-H_b. In addition, a multiplet of 1 H at δ1.55 ppm (11-H) and two doublets each of 3H as of two equivalent methyl groups were found at δ1.03 and 0.96 ppm, delivering an isopropyl system. The ^{13}C/APT NMR spectra displayed two quaternary carbons of two CO groups at δ170.3 and 166.1 ppm. In the aliphatic region, two methine carbons at δ59.0 and 53.4 ppm which are linked most likely to nitrogen. Four methylene carbons at δ45.5, 38.6, 28.1, and 22.7 ppm, as well as a third methine carbon at δ24.7 ppm were observed. It showed finally two methyl carbons at δ23.3 and 21.2 ppm of the previously mentioned isopropyl group. The molecular weight was determined as 210 Dalton by EI mass spectra. According to the discussed chemical and spectroscopic data, compound **(M4)** was established as *Cis-cyclo* (Leucyl-Prolyl) (Figure 2). This molecule, a diketopiperazine (DKP) derivative, has been already described from bacteria, such as *Streptomyces* species [9]. DKP molecules constitute a family of secondary metabolites with diverse and interesting biological activities such as antibacterial, fungicidal, herbicidal, immunosuppressor, antitumors, and antiviral [25]. However, besides these biological activities, it has been reported that the compound **(M4)** inhibits the aflatoxin production. Aflatoxins are highly toxic, carcinogenic, and teratogenic secondary metabolites that are produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus* [26].

The ^{1}H and ^{13}C NMR data of the compounds **(M1-M4)** were summarized in Table 1.

CONCLUSION

A new actinomycete strain named TN605 was isolated from south Tunisian soil and selected for its antimicrobial activities. Based on the cultural characteristics of TN605 strain, the nucleotide sequence of the corresponding 16S rRNA gene and the phylogenetic analysis we propose the assignment of our new isolate bacterium as *Streptomyces* sp. TN605 strain. The maximum biological activity production by this strain was afforded under the following conditions: glycerol at 0.75% (w/v) as carbon source, incubation at 30°C for 64h and an agitation rate of 200 or 250 rpm. Ethyl acetate extract of a 20 litres culture broth of the *Streptomyces* sp. TN605 delivered three active compounds which are: brevianamide F, tryptophol and *Cis*-cyclo (Leucyl-Prolyl) and a fourth highly secreted molecule, 2-

Hydroxyphenylacetic Acid, which is an aromatic compound of a high-added-value. This molecule is frequently used in the pharmaceutical industry as intermediate in the preparation of some biologically active compounds such as antihypertensive agents. It should be noted that the *Streptomyces* sp. TN605 secretes the interesting compound with a high rate equivalent to the sum of the other three produced compounds. To our knowledge, it is the first time that this compound is described from terrestrial *Streptomyces* bacterium.

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