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Taxonomy, purification and chemical characterization of four bioactive compounds from new *Streptomyces* sp. TN256 strain

S. Smaoui · F. Mathieu · L. Elleuch · Y. Coppel · G. Merlina · I. Karray-Rebai · L. Mellouli

Abstract A new actinomycete strain designated TN256, producing antimicrobial activity against pathogenic bacteria and fungi, was isolated from a Tunisian Saharan soil. Morphological and chemical studies indicated that strain TN256 belonged to the genus *Streptomyces*. Analysis of the 16S rDNA sequence of strain TN256 showed a similarity level ranging between 99.79 and 97.8% within *Streptomyces microflavus* DSM 40331^T and *Streptomyces griseorubiginosus* DSM 40469^T respectively. The comparison of its physiological characteristics showed significant differences with the nearest species. Combined analysis of the 16 S rRNA gene sequences (FN687758), fatty acids profile, and results of physiological and biochemical tests indicated that there were genotypic and phenotypic differentiations of that isolate from other *Streptomyces* species neighbours. These date strongly suggest that strain TN256 represents a novel species with the type strain *Streptomyces* TN256 (=CTM50228^T). Experimental validation by DNA–DNA hybridization would be required for conclusive confirmation. Four active products (1–4) were isolated from the culture broth of *Streptomyces* TN256 using various separation and purification steps and procedures. **1**: N-[2-(1H-indol-3-yl)-2 oxo-ethyl] acetamide 'alkaloid' derivative; **2**: di-(2-ethylhexyl) phthalate, a phthalate derivative; **3**: 1-Nonadecene and **4**: Cyclo (L-Pro-L-Tyr) a diketopiperazine 'DKP' derivative. The chemical structure of these four active compounds was established on the basis of spectroscopic studies NMR and by comparing with data from the literature. According to our biological studies, we showed in this work that the pure compounds (1–4) possess antibacterial and antifungal activities.

Keywords *Streptomyces* sp. TN256 · Taxonomy · Bioactive compounds · Purification · Chemical characterization

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Introduction

Actinomycetes are gram-positive, free living and saprophytic bacteria widely distributed in soil, water and colonizing plants. They show marked chemical and morphological diversity and form a distinct evolutionary line of organisms. They constitute a potential source of many bioactive compounds (Vining 1992). The species belonging to the *Streptomyces* genus represent 50% of the total population of soil actinomycetes. Moreover, 75–80% of the commercially and medicinally useful antibiotics have been derived from this genus (Miyadoh 1993). The classification of the genus *Streptomyces* in the current edition of Bergey's Manual of Systematic Bacteriology (Williams et al. 1989) is not based on the combination of genotypic and phenotypic properties, but on the extensive numerical taxonomic survey

of Williams et al. (1983). Given the presence of a phylogenetic branching pattern, a combination of properties such as wall chemotype, peptidoglycan type, whole-cell sugars, fatty acid and phospholipid profiles, and menaquinones facilitate the delineation of actinomycete genera from neighboring taxa (Kroppenstedt et al. 1990). The isolation of novel *Streptomyces* species is in great need as they are very potent producers of secondary metabolites (Mellouli et al. 2003). *Streptomyces* have been the most fruitful source of microorganisms for all types of bioactive metabolites that have important applications in human medicine and in agriculture (Watve et al. 2001). The great number of diverse antibiotics that produce these bacteria can be classified in different ways based on bacterial spectrum, the type of biological activity and the chemical structure. This latter way is the most useful and several classes of antibiotics can be distinguished, as: alkaloid derivatives which possess a diverse range of biological activities, *e.g*. anticancer and antimicrobial (Chatterjee et al. 1995; Gebhardt et al. 2002); phthalate derivatives which are biologically active compounds (Marchetti et al. 2002); nonadecene compounds known for their antioxidant (Yassa et al. 2009) and antifungal activities (El-Sakhawy et al. 1998); diketopiperazine (DKP) derivatives having useful biological properties (Ben Ameur Mehdi et al. 2004, 2006), etc. Despite the large number of species with validly published names, the genus *Streptomyces* as a whole is underspeciated (Kim and Goodfellow 2002). Through molecular systematic data some established species described using phenotypic criteria should be reduced to synonyms of previously described species (Lanoot et al. 2004; Liu et al. 2005). Active molecules of *Streptomyces* species are generally extracellular and their isolation in highest purity from the complex fermentation broth needs the application of a combination of various separation steps such as solvent extraction, chemical precipitation, ion exchange chromatography, HPLC purification, etc.

In the present work, we describe the identification of a *Streptomyces* strain, designated TN256, isolated from a Tunisian Saharan soil sample by conventional and molecular methods as well as the description of extraction, purification (using different chromatographic techniques) and structure elucidation of four different bioactive molecules (1–4) from a liquid culture broth of this strain. The biological activities of these pure compounds are also addressed.

Materials and methods

Organisms

Strain TN256 was isolated from Saharan soil of Tunisia. The strain was maintained by cultivation on ISP 2 agar medium that contained: 4 g l^{-1} glucose, 4 g l^{-1} yeast extract, 5 g l^{-1} malt extract and vitamin-amino acid mixture (1 mg l^{-1} vitamin B1; 1 mg l^{-1} vitamin B2; 1 mg l^{-1} vitamin B6; 1 mg l^{-1} biotin; 1 mg l^{-1} nicotinic acid; 1 mg l^{-1} phenylalanine; 0.3 g l^{-1} alanine) at pH 7.2, incubated at 28°C for 2 weeks. The strain was maintained on yeast extract-malt extract-dextrose (YMD) agar medium at 4°C (Williams and Cross 1971). The isolate is screened for its ability to produce antimicrobial agents against Gram-positive and Gram-negative bacteria and filamentous fungi. Broth from the isolate was cultured in Bennett, medium that contained: 2.0 g l^{-1} peptone, 10.0 g l^{-1} glucose, 1.0 g l^{-1} yeast extract and 1.0 g l^{-1} beef extract at pH 7.5 for 3 days at 28°C.

Antimicrobial activity determination

Indicator microorganisms were grown overnight in LB medium at 30°C for *Micrococcus luteus* LB14110 (Gram-positive bacterium) and at 37°C for *Staphylococcus aureus* ATCC 6538 (Gram-positive bacterium), *Salmonella enterica* ATCC43972 and *Escherichia coli* ATCC 8739 (Gram-negative bacteria), then diluted 1:100 in LB medium and incubated for 5 h under constant agitation on 200 rpm at the appropriate temperature. *Fusarium sp*. was grown in potato dextrose agar (PDA) for 7 days at 30°C. Spores were collected in sterile distilled water and then adjusted to a spore density of approximately 10^4 spores l^{-1}. Antimicrobial activities were determined by the agar diffusion test: a paper disk (8 mm Ø) was impregnated with 50 µl of the corresponding sample and then laid on the surface of an agar plate containing 3 ml of top agar inseeded with 40 µl of a 5-h-old culture of the corresponding microorganism. For antifungal activity against the *Fusarium* sp., 100 µl of spore suspension were added to 3 ml of top agar. After 2 h at 4°C, plates containing *M. luteus* LB 14110 and *Fusarium* sp. were incubated at 30°C and those inoculated with *S. aureus* ATCC 6538 and *E. coli* ATCC8739 were incubated overnight at 37°C. The antimicrobial activities of the four pure compounds (1–4) were determined under the same conditions mentioned above. The quantity used for each pure active compound was 50 µg per disk. Plates were examined for evidence of antimicrobial activities represented by a zone of growth inhibition of the corresponding indicator microorganisms around the paper disk. As negative control (NC), we have used the dry extract of the culture medium without inoculation with the studied *Streptomyces* sp. TN256 strain. This culture was incubated and treated exactly in the same conditions of those of the inoculated culture. As positive control (PC), we have used the active dry extract of the *Streptomyces* sp. TN262 strain. This strain has been isolated, identified and studied in our laboratory (Elleuch et al. 2010). The corresponding dry extract possesses inhibitory

activity against the five used indicator microorganisms: *Micrococcus luteus* LB14110, *Staphylococcus aureus* ATCC 6538, *Salmonella enterica* ATCC43972, *Escherichia coli* ATCC 8739 and *Fusarium* sp. (Elleuch et al. 2010).

Phenotypic characterization

The cultural characteristics and the colours of mature sporulating aerial and substrate mycelia were monitored on 7, 14 and 21 day-old cultures of the isolate TN256. These characteristics were tested on the bases of the observations made on International *Streptomyces* Project (ISP) media recommended by Shirling and Gottlieb (1966), Bennett agar, nutrient agar and Sabouraud agar. Colour determination was carried out using ISCC-NBS colour charts (Kenneth 1958). Melanin production was tested in peptone-yeast extract- iron (ISP medium 6) agar and trypsine (ISP medium 7) agar.

Chemotaxonomic studies

Biomass for most of chemotaxonomic studies was obtained after incubation at 28°C for 3 days by growing in shake flasks of ISP 2 broth. The isomeric form of Diaminopimelic acid (DAP), glycine and sugars in the whole cell hydrolysates were analysed by TLC (Staneck and Roberts 1974). Phospholipids were examined by two-dimensional TLC and identified using several spray reagents and through co-migration with authentic standards (Collins and Jones 1980; Minnikin et al. 1979). Biomass for a quantitative fatty acid analysis was prepared by scraping cell mass from plates containing trypticase soy broth (BBL; 3% w/v) and Bacto agar (Difco; 1.5% w/v), that had been incubated for 3 days at 28°C. Fatty acids were extracted, methylated and analysed by gas chromatography (GC) using the standard Microbial Identification System (MIDI) (Sasser 1990).

Physiological tests

The ability of the strains to use 15 compounds as sole carbon sources and 16 compounds as sole nitrogen sources for energy and growth was examined on ISP medium 9 for 3 days at 28°C. Each source was added at a final concentration of 1% (w/v) and 0.1% (w/v) respectively. The utilization of sole carbon and sole nitrogen sources was investigated according to Shirling and Gottlieb (1966) and Tsukamura (1966). Glycerol was used as carbon source when testing nitrogen sources (Tsukamura 1966). Sodium salts (acetate, alginate, benzoate, butyrate, citrate, desoxycholate, hydrogen carbonate, nitrate, oxalate, perchlorate, propionate, pyruvate, succinate, sulfite, tartrate, tetraborate and thiosulfate) were added to a final concentration of 0.1% (w/v) for testing the ability to use a sole carbon source (Gordon et al. 1974). The purpose of testing sodium salts was to study their effect in cellular growth and active secondary metabolites production. In this context, Voelker and Altaba (2001) reported that the addition of sodium nitrate to the culture medium of the *Streptomyces pristinaespiralis* affect positively the pristinamycin production.

Esculin and arbutin (1.0% w/v) degradations were determined by the methods of Gordon et al. (1974) at 28°C and examined after 3 days. The degradation of casein (1.0% w/v) was determined by the method of Gordon et al. (1974) at 28°C and examined after 3, 7 and 14 days; clearing of the areas under and around the growth was scored as positive. Gelatin (0.4% w/v) and starch (1.0% w/v) degradations were read after 3 days in nutrient agar at 28°C by flooding plates with trichloroacetic acid (3.0% v/v) and iodine solutions (Covan and Steel 1974) respectively, and scoring zones of clearing as positive.

The degradation of tyrosin, hypoxanthin, xanthin, adenine and guanine (1.0%, w/v) was investigated according to Gordon et al. (1974) at 28°C, and observed at 14 and 21 days. The hydrolysis of Tween 80 was measured using the method of Sierra (1957).

The effects of salt on growth were determined in TSB media supplemented with graded doses of sodium chloride (1, 4, 5, 7 and 10% w/v) at 28°C and examined after 3 days. Maximum sodium chloride concentration in the medium allowing any growth was recorded by Williams et al. (1983).

Tolerance to temperatures (4, 10, 15, 25, 30, 37, 40, and 45°C) and pH (4.0 ∼ 10.0 in 0.5 unit increments) were tested on ISP 2 agar plates incubated for 7–14 days.

Tolerance to lysozyme was examined using glycerol broth containing 0.005% lysozyme. After 2 and 4 weeks of incubation at 28°C, the cultures were observed for growth (Gordon and Barnett 1977). Tolerance to phenol (0.05, 0.1, 0.2, 0.5 and 1.0%), and sodium azide (0.001 and 0.01%) was tested using GYEA media (Athalye et al. 1985) at 28°C and examined after 3 days.

Resistance to antibiotics was examined using: erythromycin, streptomycin, penicillin at (10 mg l^{-1}), rifampicin, gentamicin, vancomycin at (5 mg l^{-1}) and chloramphenicol, oxytetracyclin, kanamycin at (25 mg l^{-1}) incorporated into glucose yeast extract agar (Lechevalier and Lechevalier 1970a, b) as a basal medium. Readings were taken at 28°C and examined after 1, 3, 7 and 14 days: organism is scored as resistant (+) when growth on the test plates was greater or equal to that of positive control plates lacking inhibitors and inhibition zones observed were scored as negative.

Genotypic characterization

DNA isolation and manipulation

Total DNA preparation from TN256 strain was carried out according to Hopwood et al. (1985). Small-scale plasmid preparations from *E. coli* were performed as described by Sambrook et al. (1989). Digestion with restriction endonucleases, separation of DNA fragments by agarose gel electrophoresis, dephosphorylation with alkaline calf intestinal phosphatase, ligation of DNA fragments, and transformation of *E. coli* were done according to Sambrook et al. (1989).

PCR amplification of the 16S rRNA gene of TN256 strain

PCR amplification of the 16S rRNA gene of TN256 strain was performed using two primers 5′-AGAGTTTGATCCTGG CTCAG-3′ and 5′-AAGGAGGTGATCCAGCCGCA-3′ as described by Edwards et al. (1989). Approximately 200 ng of the genomic template DNA was used with 150 pmol of each primer per 50 µl reaction volume. To improve the denaturation of the DNA, 5% (v/v) DMSO was added to the reaction mixture. Amplification was performed in an automatedthermoclycer (Perkin Elmer) using 1 U Pfu DNA polymerase (Stratagene) and the recommended buffer system according to the following amplification profile: 94°C (3 min) followed by 45 cycles of denaturation at 94°C (30 s), annealing at 60°C (1 min), and extension at 72°C (3 min). The PCR reaction mix was analyzed by agarose gel electrophoresis, and the DNA of the expected size was purified and then cloned into pCR-Blunt vector yielding pLE1.

DNA sequencing and analysis

Nucleotide sequence of the 16S rRNA gene of TN256 strain was determined on both strands by an automated 3100 Genetic Analyzer (Applied Biosystems) using specific primers. Homology search was performed using BLAST Search algorithm. The nucleotide sequence of the whole 16S rRNA gene (1.430 pb) of TN256 strain has been assigned in the GenBank (EMBL) under accession number FN687758. Multiple sequence alignment was carried out using CLUSTAL W (Thompson et al. 1997) at the European Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw/). Phylogenetic analyses were performed using programs from the PHYLIP package (Felsentein 1985) and phylogenetic tree was constructed by the neighbour joining (NJ) algorithm (Saitou and Nei 1987) using Kimura 10-parameter distance. The robustness of the inferred tree was evaluated by bootstrap (100 replications).

Isolation and purification of antimicrobial products

Thirty litres of Bennett culture broth was centrifuged to remove the biomass. The cell-free supernatant was extracted with ethyl acetate (2×) and the obtained organic extract was concentrated in vacuum to dryness, affording 3.0 g of a brown crude extract. The resulting dry extract was recuperated in 2 ml ethyl acetate and assayed against indicator microorganisms, by paper disk diffusion method. Preparative chromatography with silica gel plates (Merck Art. 5735, Kiesselgel 60F 254) was used for the partial purification of antimicrobial products. A dry crude extract, dissolved in ethyl acetate, was spotted and developed in the solvent system dichloromethane– methanol (96:4 v/v). The developed plates were air dried overnight to remove all traces of solvents. Seven well separated bands were visualized under UV at 254 nm (absorbance) and at 365 nm (fluorescence) and for each band the corresponding compounds were recovered yielding seven fractions (FI-FVII). These fractions were tested for their biological activity against indicator microorganisms using the paper disk diffusion method. Four fractions possess biological activity (FI, FIII, FV and FVII) and their retention factor (Rf) were measured. The active fractions of all prepared plates were collected separately, concentrated (FI 228 mg; FIII 133 mg; FV 189 mg and FVII 205 mg) and then fractioned by HPLC (waters: controller 600, pump 600, dual λ absorption detector 2487, Linear Recorder); column C18 (250 × 7) 8 mm UP ODS). Elution was at a flow rate of 1 ml min^{-1} with a linear gradient of two solutions A (water) and B (acetonitrile) from 100% buffer A to 50% buffer A and 50% buffer B over the first 35 min, followed by a linear gradient to 100% buffer B from 35 to 45 min. Detection was carried out by using a wavelength of 280 nm. For each active fraction, different well-developed peaks having a retention time between 5 min and 45 min were collected separately, concentrated and then tested for the inhibitory activity against the used indicator microorganisms. The four pure active products (1–4) were obtained as follow. **1** (58.33 mg) from FI; **2** (18 mg) from FIII; **3** (23.8 mg) from FV and **4** (33 mg) from FVII.

Characterization of compounds

NMR sample was prepared by dissolving the pure compounds (1–4) in 600 µl of CD_3OD. 1D and 2D ^{1}H and ^{13}C experiments were recorded on a Bruker Avance 500 spectrometer equipped with a 5 mm triple resonance inverse Z-gradient probe (TBI ^{1}H, ^{31}P, BB) or a Bruker Avance 600 spectrometer equipped with a 5 mm triple resonance inverse (TCI ^{1}H, ^{13}C, ^{15}N) Z-gradient cryoprobe. All chemical shifts for ^{1}H and ^{13}C are relative to TMS using ^{1}H (residual) or ^{13}C chemical shifts of the solvent as

a secondary standard. The temperature was set at 298 K. All the ^{1}H and ^{13}C signals were assigned on the basis of chemical shifts, spin–spin coupling constants, splitting patterns and signal intensities, and by using ^{1}H–^{1}H COSY, ^{1}H–^{1}H NOESY, ^{1}H–^{13}C HMQC and ^{1}H–^{13}C HMBC experiments.

Results and discussion

Morphology characteristics

Strain TN256 was a Gram-positive bacterium. Morphological observation of the 7–15 day old culture of strain TN256 grown on yeast -malt extract agar (ISP2) (Shirling and Gottlieb 1966) revealed that both aerial and vegetative hyphae were abundant. The isolate developed well on several media including ISP2, ISP3, ISP4, ISP5, Bennett agar and nutrient agar. The detailed cultural characteristics of strain TN256 are given in Table 1. Aerial mycelium was abundant, well-developed and varied from white to yellow on all media tested. The substrate hyphae varied from yellowish white to yellowish brown. Diffusible pigments were not produced on any tested media and melanin was not produced. Strain *Streptomyces* sp. TN256 (TN256) has been deposited in the CTM under the number CTM50228^{T}.

Chemotaxonomic analysis

The cell wall peptidoglycan of strain TN256 contained only LL-diaminopimelic acid and glycine, indicating that strain TN256 has a chemotype cell-wall type I (Lechevalier and Lechevalier 1970a, b). The diagnostic phospholipids was phosphatidylethanolamine (PE) (phospholipids type II sensu Lechevalier and Lechevalier 1970b). No diagnostic sugars were found in the cell wall constituents. The fatty acid profile included mainly saturated iso- and anteiso-branched-chain and straight-chain fatty acids (fatty acid type 2c sensu Kroppenstedt 1985). The major cellular fatty acids were $C_{14:0}$ 2-OH (12.08%), $C_{14:0}$ 3-OH (24.19%) and $C_{16:0}$ (43.12%), and smaller amounts of $C_{15:0}$ (2.84%), anteiso $C_{15:0}$ (1.18%), iso $C_{16:0}$ (3.52%), $C_{16:0}$ 2-OH (6.81%), $C_{16:1}$ *w* 9 (2.45%) and iso $C_{17:0}$ (2.03%) were also present. The chemical and morphological properties of strain TN256 are clearly consistent with its assignment to the genus *Streptomyces* (Williams et al. 1989).

Phylogenetic analysis

The almost complete 16S rRNA gene sequence of strain TN256 (1.430 nucleotides) was determined in this study and has been deposited in the GenBank database (FN687758). This sequence was subjected to similarity searches against public databases to infer possible phylogenetic relationships of strain TN256. The phylogenetic tree (Fig. 1) from representative strains of the related species indicated that strain TN256 should be placed in the genus *Streptomyces*. In the comparison of 16S rRNA gene sequences, TN256 was mostly related with *Streptomyces microflavus* DSM 40331^{T} (NBRC 13062^{T}) (99.79%) and *Streptomyces griseorubiginosus* DSM 40469^{T} (NBRC 13047^{T}) (99.71%). Strain TN256^{T} also shared relatively high 16S rRNA gene sequence similarity values with the type strains of *Streptomyces griseus* NBRC 13350^{T} and *Streptomyces erumpens* NBRC 15403^{T} (99.36%).

Physiological characteristics

A comparative study between strain TN256^{T} and closely related species of the genus *Streptomyces* revealed that it differed from *Streptomyces microflavus* DSM 40331^{T} and *Streptomyces griseorubiginosus* DSM 40469^{T} in morphological and cultural characteristics as summarised in Table 2.

The three strains of *Streptomyces* share some physiological characters like utilization of sole carbon and nitrogen sources: L-arabinose, D-galactose, glucose, glycerol, *Meso*-Inositol, D-xylose, sucrose, D-trehalose and D-ribose (sole carbon sources) and L-aspartic acid, L-alanine, L-tryptophane, L-tyrosine, L-histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine and L-glutamic acid (sole nitrogen sources).

Table 1 Culture characteristics of strain TN256^{T} on various media

Medium	Growth	Sporulation	Aerial mycelium	Substrate mycelium
Yeast -malt extract agar (ISP^{T} medium 2)	Good	Good	Yellow	Soft yellowish brown
Oatmeal agar (ISP^{T} medium 3)	Good	Good	White	Pale yellow
Inorganic salt-starch agar (ISP^{T} medium 4)	Moderate	Good	White	Soft yellowish white
Glycerol-asparagine agar (ISP^{T} medium 5)	Good	Good	Yellow	Moderate brown
Bennett agar	Good	Good	Yellow	Pale yellow
Nutrient agar	Good	Moderate	Yellow	Soft yellow
Sabouraud agar	Moderate	Moderate	Yellow	Pale yellow

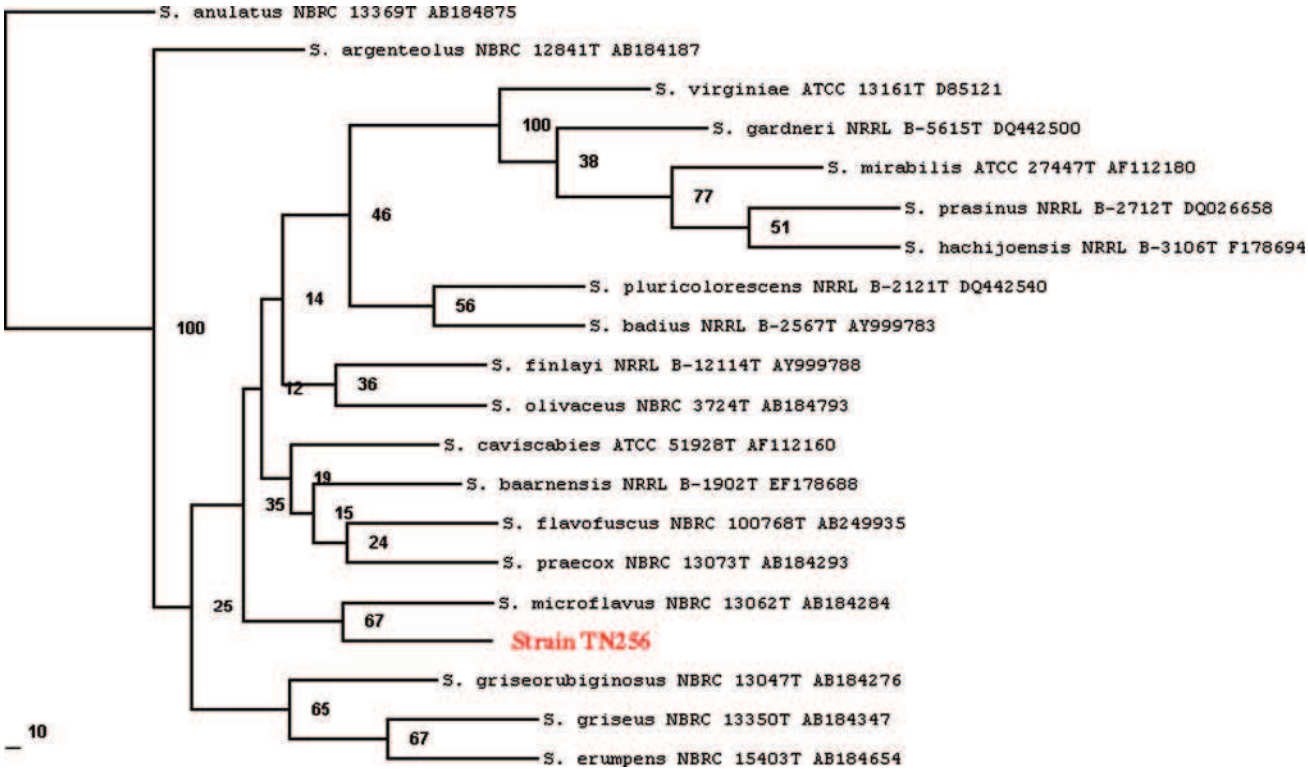

Fig. 1 Phylogenetic trees derived from 16S rDNA sequence of *Streptomyces* sp. TN256 strain. This strain was mostly related with *Streptomyces microflavus* DSM 40331^T and *Streptomyces griseorubiginosus* DSM 40469^T. All the sequences used here were from *Streptomyces* type strains. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1,000 resampled datasets. Bar 10 substitutions per nucleotide position. The sequence of *Streptomyces anulatus* NBRC 13369T (AB 184875) was used as root and the root position of the neighbour-joining tree was estimated using this strain as the outgroup

The differences consisted of the degradation of D-fructose, D-lactose, L-rhamnose, D-mannose, D-raffinose and maltose (sole carbon sources). L-asparagine, L-leucine, L-cysteine and L- arginine (sole nitrogen sources).

The aerial mycelium of strain TN256^T varied from yellow to white. In contrast, the substrate mycelium of strain TN256^T is yellow, *Streptomyces microflavus* DSM 40331^T and *Streptomyces griseorubiginosus* DSM 40469^T are greyish yellow. In any cases, soluble pigments were not produced.

Growth of the three *Streptomyces* strains (TN256^T, DSM 40331^T and DSM 40469^T) was observed at a wide range of temperatures (30–40°C) and concerning the TN256^T strain, the optimal temperature of growth was ranged between 30 and 35°C. The pH range for the growth of the *Streptomyces microflavus* DSM 40331^T and the *Streptomyces griseorubiginosus* DSM 40469^T was between pH 5–8, however for the studied TN256^T strain, the pH range for the growth was between 5 and 9 with an optimum at 7.5. The two strains TN256^T and *Streptomyces griseorubiginosus* DSM 40469^T were capable to grow in the presence of 7% NaCl and 0.2% of phenol while *Streptomyces microflavus* DSM 40331^T, grow only in the presence of maximum 5% NaCl but tolerate 0.5% of phenol. In addition, the three *Streptomyces* strains reduced nitrate to nitrite and degraded starch, casein, gelatin and adenine. Contrary to strains DSM 40331^T and DSM 40469T, the studied TN256^T strain was not able to degrade guanine.

The detailed fatty acid profile of strain TN256^T given in Table 3 was clearly different from that of *Streptomyces microflavus* DSM 40331^T and *Streptomyces griseorubiginosus* DSM 40469^T. For *Streptomyces microflavus* DSM 40331^T, the major cellular fatty acids were $C_{14:0}$ 3-OH (28.93%) and $C_{16:0}$ (33.64%), and smaller amounts of $C_{14:0}$ 2-OH (7.39%) and iso $C_{16:0}$ (9.87%). For *Streptomyces griseorubiginosus* DSM 40469^T, The major cellular fatty acids were $C_{14:0}$ 2-OH (23.27%) and $C_{14:0}$ 3-OH (35.71%) and smaller amounts of iso $C_{16:0}$ (11.79%) and $C_{16:0}$ 2-OH (12.78%).

Genotypic and phenotypic results strongly suggest that strain TN256 is a new species of the genus *Streptomyces*, for which the name *Streptomyces* sp. TN256. Nevertheless, experimental validation by DNA–DNA hybridization would be required for conclusive confirmation.

Table 2 Phenotypic properties separating strain TN256^T from related *Streptomyces* species

Characteristics	1	2	3
Colony color on ISP2	Yellow	Greyish yellow	Greyish yellow
Production of diffusible pigment	−	−	−
Melanin production on ISP6	−	−	−
Melanin production on ISP7	−	−	−
Melanoid pigment on tryptone-yeast extract broth	−	−	−
Nitrate reduction	+	+	+
Growth on sole carbon sources (1%, w/v)			
L-Arabinose	−	−	−
D-Fructose	+	−	−
D-Galactose	+	+	+
Glucose	+	+	+
Glycerol	+	+	+
Meso-Inositol	−	−	−
D-Lactose	−	+	−
Maltose	+	−	+
D-Mannose	+	−	−
D-Raffinose	−	+	−
L-Rhamnose	−	+	+
D-Ribose	+	+	+
Sucrose	+	+	+
D-Trehalose	−	−	−
D-Xylose	−	−	−
Decarboxylation of sodium (0.1%, w/v)			
Acetate	+	+	+
Alginate	+	+	−
Benzoate	−	−	−
Butyrate	−	−	−
Citrate	+	+	+
Desoxycholate	−	−	−
Hydrogen carbonate	−	−	−
Nitrate	+	−	+
Oxalate	−	+	−
Operchlorate	−	+	+
Propionate	−	−	+
Pyruvate	−	−	+
Succinate	+	−	+
Sulfite	−	+	−
Ttartrate	−	−	−
Tetraborate	−	−	−
Thiosulfate	+	−	+
Growth on sole nitrogen sources (0.1%, w/v)			
L-Asparagine	+	−	−
L-Aspartic acid	−	−	−
L-Alanine	+	+	+
L-Arginine	+	−	−
L-Cysteine	+	−	+
L-Glutamic acid	−	−	−
L-Histidine	−	−	−
L-Isoleucine	+	+	+
L-Leucine	+	−	+
L-Methionine	+	+	+
L-Phenylalanine	+	+	+
L-Proline	+	+	+
L-Serine	+	+	+
L-Threonine	+	+	+
L-Tryptophane	−	−	−
L-Tyrosine	−	−	−
Degradation activity			
Adenine	+	+	+
Arbutin	−	−	−
Casein	+	+	+
Esculin	−	+	−
Gelatin	+	+	+
Guanine	−	+	+
Hypoxanthine	−	+	−
Starch	+	+	+
Tween 80	−	−	+
Xanthine	−	−	−
Growth temperature (°C) range	30–40	30–40	30–40
Growth pH range	5–9	5–8	5–8
Growth in the presence of:			
Lyzozyme (0.005%)	+	+	+
Phenol (0.05%)	+	+	+
(0.1%)	+	+	+
(0.2%)	+	+	+
(0.5%)	−	−	+
(1.0%)	−	−	−
Sodium azide (0.001%)	−	+	+
(0.01%)	−	−	−
Growth sodium chloride range (%)	1–7	1–7	1–5
Resistance to antibiotics (μg ml^{-1})			
Erythromycin (10)	−	−	−
Streptomycin (10)	+	+	+
Penicillin (10)	−	−	−
Gentamicin (5)	−	+	−
Rifampicin (5)	+	−	+
Vancomycin (5)	−	−	−
Chloramphenicol (25)	−	−	−
Oxytetracyclin (25)	+	+	+
Kanamycin (25)	+	+	−

Strains: (**1**) TN256; (**2**) *Streptomyces griseorubiginosus* DSM 40469^T; (**3**) *Streptomyces microflavus* DSM 40331^T

Table 3 Cellular fatty acid contents (%) of (**1**) TN256; (**2**) *Streptomyces griseorubiginosus* DSM 40469^T; (**3**) *Streptomyces microflavus* DSM 40331^T

Fatty acids	1	2	3
$C_{10:0}$ 2-OH	–	–	4.755
$C_{11:0}$	–	–	1.032
$C_{12:0}$	–	3.101	1.576
$C_{12:0}$ 2-OH	0.316	–	–
$C_{12:0}$ 3-OH	–	–	1.001
$C_{13:0}$	0.579	–	1.78
$C_{14:0}$	–	–	2.673
$C_{14:0}$ 2-OH	12.082	23.277	7.396
$C_{14:0}$ 3-OH	24.192	35.708	28.937
$C_{15:0}$	2.846	4.104	1.277
Iso $C_{15:0}$	–	–	–
Anteiso $C_{15:0}$	1.184	4.639	–
$C_{16:0}$	43.124	–	33.644
Iso $C_{16:0}$	3.523	11.79	9.875
$C_{16:0}$ 2-OH	6.812	12.782	3.836
$C_{16:1}$ *w* 9	2.54	4.209	–
$C_{17:0}$	2.035	–	2.212
Iso $C_{17:0}$	0.515	–	–
Anteiso $C_{17:0}$	–	–	–
$C_{18:0}$	–	–	–
$C_{18:1}$ *w* 9 (cis)	0.064	0.384	–
$C_{18:1}$ *w* 9 (trans)	0.182	–	–
$C_{18:2}$ *w* 9,12	–	–	–
$C_{19:0}$	–	–	–
Iso $C_{19:0}$	–	–	–
$C_{20:0}$	–	–	–

Isolation and structural elucidation of antimicrobial compounds

Compound 1: N-[2-(1H-indol-3-yl)-2 oxo-ethyl] acetamide (1), was obtained as a white coloured solid. In CD_3OD, the HSQC and HMBC spectra show 10 carbon signals. From the ^{13}C data, it was possible to discern one ketone (δ_c 190.3), one carbonyl group (δ_c 172.2), six sp^2–hybridized carbons (δ_c from 132.5 to 111.3), one sp^3-hybridized carbon bearing an electronegative heteroatom (δ_c 45.5) and one methyl group (δ_c 21.1). From observed HMBC and NOE correlations (Fig. 2) compound 1 and based on the revealed spectral data and search in AntiBase was identified as N-[2-(1H-indol-3-yl)-2 oxo-ethyl] acetamide (Fig. 2). The corresponding molecular formula is $C_{12}H_{12}O_2N_2$ with a MW of 216.

^1H NMR (CD_3OD, 600 MHz) δ 8.56 (1H, br s, NH-4), δ 8.25 (1H, s, H5), δ 8.24 (1H, d, J = 7.8, H6), δ 7.26 (1H, m, H8), δ 7.23 (1H, m, H7), δ 7.48 (1H, d, J = 7.9, H9), δ 4.61 (2H, s, H11), δ 2.09 (3H, s, H15); ^{13}C NMR (CD_3OD, 150 MHz) δ 190.3 (C, C10), δ 172.2 (C, C14), δ 132.5 (CH, C5), δ 122.9 (CH, C8), δ 121.6 (CH, C7), δ 121.3 (CH, C6), δ 114.3 (C, C1), δ 111.3 (CH, C9), δ 45.5 (CH_2, C11), δ 21.1 (CH_3, C15).

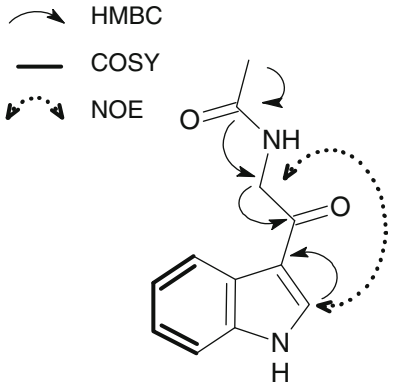

Fig. 2 HMBC, COSY and NOE correlations of compound 1

Compound 2: Di-(2-ethylhexyl) phthalate (2) was obtained as a white coloured solid. In CD_3OD, the ^{13}C and HSQC spectra show 12 carbon signals. From the ^{13}C data, it was possible to discern one carbonyl group (δ_c 168.0), three sp^2–hybridized carbons (δ_c 132.3, 130.9 and 128.3), one sp^3-hybridized carbon bearing an electronegative heteroatom (δ_c 67.6), five sp^3-hybridized carbon (δ_c 38.7–23.5) and two methyl groups (δ_c 12.9 and 10.0). The ^1H NMR spectrum showed a characteristic AA'BB' system at 7.74 and 7.64 ppm ($J_{AA'} = 0.7$ Hz, $J_{AB} = J_{A'B'} = J_{BB'} = 7.8$ Hz and $J_{AB'} = J_{A'B} = 1.1$ Hz obtained from simulation). These data established a compound that have a di *ortho*-substitued aromatic ring. The 2D ^1H–^1H and ^1H–^{13}C correlations and based on the revealed spectral data and search in AntiBase permitted to determine that the compound is Di-(2-ethylhexyl) phthalate (Fig. 3). The corresponding molecular formula is $C_{24}H_{38}O_4$ with a MW of 390.

^1H NMR (CD_3OD, 500 MHz) δ 7.74 (1H, m, H3), δ 7.64 (1H, m, H4), δ 4.25 (1H, dd, J = 10.8, 5.6, H1'), δ 4.22 (1H, dd, J = 10.8, 6.0, H1'), δ 1.71 (1H, m, H2'), δ 1.46 (2H, m, H7'), δ 1.40 (2H, m, H3'), δ 1.36 (4H, m, H4',

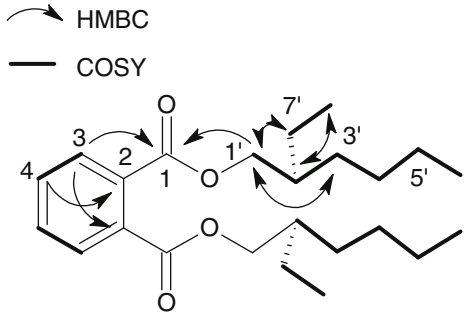

Fig. 3 HMBC and COSY correlations of compound 2

H5'), δ 0.97 (3H, t, J = 7.3, H8'), δ 0.94 (3H, t, J = 7.0, H6'); ^{13}C NMR (CD_3OD, 125 MHz) δ 168.0 (C, C1), δ 132.3 (C, C2), δ 130.9 (CH, C4), δ 128.3 (CH, C3), δ 67.6 (CH_2, C1'), δ 38.7 (CH, C2'), δ 30.3 (CH_2, C3'), δ 28.5 (CH_2, C4'), δ 23.5 (CH_2, C7'), δ 23.4 (CH_2, C5'), δ 12.9 (CH_3, C6'), δ 10.0 (CH_3, C8').

Compound 3: 1-Nonadecene (3) was obtained as a white coloured solid. In CD_3OD, the combined analysis of ^{1}H, ^{13}C and HSQC spectra showed that compound 3 contain two sp^2–hybridized carbons (δ_c 139.2 and 113.2), 16 sp^3-hybridized carbon (δ_c 33.4–22.7) and one methyl group (δ_c 12.9). The structure of compound 3 was identified as 1-Nonadecene (Fig. 4). The corresponding molecular formula is $C_{19}H_{38}$ with a MW of 266.

^{1}H NMR (CD_3OD, 500 MHz) δ 5.82 (1H, m, H2), δ 4.99 (1H, dm, J = 17.2, H1), δ 4.93 (1H, dm, J = 10.2, H1'), δ 2.07 (2H, dt, J = 7.3, 6.8, H3), δ 1.40–1.28 (30H, m, H4 to H18), δ 0.92 (3H, t, J = 7.3, H19); ^{13}C NMR (CD_3OD, 125 MHz) δ 139.2 (CH, C2), δ 113.2 (CH_2, C1), δ 33.4 (CH_2, C2), δ 32.3–22.7 (15 CH_2, C4 to C18), δ 12.9 (CH_3, C19).

Compound 4: Cyclo (L-Pro-L-Tyr) (4) was obtained as a yellowish UV-absorbing solid. In CD_3OD, the ^{13}C and HSQC spectra show 12 carbon signals. From the ^{13}C data, it was possible to discern two carbonyl groups (δ_c 169.2 and 165.5), four sp^2–hybridized carbons (δ_c from 156.4 to 114.7), three sp^3-hybridized carbons bearing an electronegative heteroatom (δ_c 58.7, 56.4 and 44.4), three sp^3-hybridized carbon (δ_c 36.3, 27.9 and 21.2). The 2D ^{1}H–^{1}H and ^{1}H–^{13}C experiments permitted to assigned two fragments to proline and tyrosine. The structure of compound 4 was determined to be cyclo (L-Pro-L-Tyr) (Fig. 5). The corresponding molecular formula is $C_{14}H_{16}O_3N_2$ with a MW of 260.

^{1}H NMR (CD_3OD, 500 MHz) δ 7.06 (2H, d, J = 8.6, Tyr-H5), δ 6.72 (2H, d, J = 8.6 Tyr-H6), δ 4.38 (1H, dd, J = 5.2, 4.7, Tyr-H2), δ 4.08 (1H, dd, J = 11.0, 6.3, Pro-H2), δ 3.58 (1H, m, Pro-H5), 3.38 (1H, m, Pro-H5), δ 3.10 (1H, dd, J = 14.0, 5.2, Tyr-H3), 3.05 (1H, dd, J = 14.0, 4.7, Tyr-H3), 2.12 (1H, m, Pro-H3), 1.83 (2H, m, Pro-H4), 1.25 (1H, m, Pro-H3); ^{13}C NMR (CD_3OD, 125 MHz) δ 169.2 (C, Pro-C1), δ 165.5 (C, Tyr-C1), δ 156.4 (C, Tyr-C7), δ 130.7 (CH, Tyr-C5), δ 126.1 (C, Tyr-C4), δ 114.7 (CH, Tyr-C6), δ 58.7 (CH, Pro-C2), δ 56.4 (CH, Tyr-C2), δ 44.4 (CH_2, Pro-C5), δ 36.3 (CH_2, Tyr-C3), δ 27.9 (CH_2, Pro-C3), δ 21.2 (CH_2, Pro-C4).

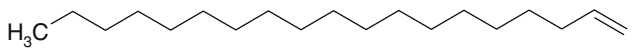

Fig. 4 Molecular structure of compound 3

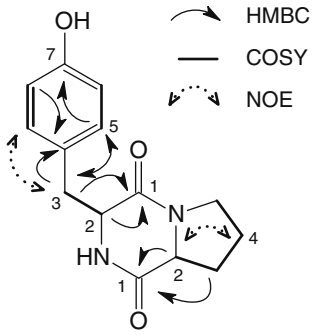

Fig. 5 HMBC, COSY and NOE correlations of compound 4

Biological activities of the four characterised compounds

The new isolated *Streptomyces* sp. strain TN256 produced simultaneously four active compounds belonging to four different structure types.

The first active compound (**1**) is the N-[2-(1H-indol-3-yl)-2 oxo-ethyl] acetamide, an alkaloid indole derivative. This active molecule has been already described from *Streptomyces* sp. (Chatterjee et al. 1995; Gebhardt et al. 2002) and terrestrial *Pseudomonas* sp. (Ge et al. 2004) as well as marine Streptomycete (Pusecker et al. 1997). Besides its antitumor activity, compound (**1**) possesses antimicrobial activities (Chatterjee et al. 1995; Gebhardt et al. 2002). According to our antimicrobial tests, we have observed that this compound presents antibacterial activities against Gram-positive and Gram-negative bacteria as well as antifungal activities (Table 4).

The second active compound produced by the *Streptomyces* sp. TN256 is the di-(2-ethylhexyl) phthalate (**2**). Phthalate compounds are petrochemicals used as plasticisers or solvents in a variety of industrial products. Nevertheless, many phthalate derivatives have been isolated from terrestrial and marine organisms including plants (Lee et al. 2000), fungal and bacterial culture broths, especially those belonging to the genus *Streptomyces*. Compound 2 has been already described from *Streptomyces bangladeshiensis* (Al-Bari et al. 2006). Other phthalate derivatives have been isolated from *Streptomyces* species, such as the dibutyl phthalate (El-Naggar 1997; Roy et al. 2006). Phthalate derivatives which possess several antimicrobial activities are also effective compounds against demodicidosis, as well as endocrine disruptors with estrogenic activity (Marchetti et al. 2002) and drug channelling agents (Makhija and Vavia 2003). Our antimicrobial studies show that compound 2 possesses antibacterial activities against Gram-positive bacteria and fungi (Table 4).

The third active molecule (**3**) is the 1-Nonadecene. This molecule was extracted from the plant *Rosa damascene* and possesses antioxidant activities (Yassa et al. 2009). The essential oils of fresh flowers, leaves and fruits of *Murraya exotica* L., contained the 1-Nonadecene,

Table 4 Antimicrobial activities of compounds: (**1**) the N-[2-(1H-indol-3-yl)-2 oxo-ethyl] acetamide, (**2**) the di-(2-ethylhexyl) phthalate, (**3**) the 1-Nonadecene and (**4**) the Cyclo (L-Pro-L-Tyr)

Test organism	Diameter of inhibition zones (mm)					
	1	2	3	4	NC	PC
M. luteus LB 14110	21 ± 2.0	16 ± 1.0	17 ± 1.0	20 ± 1.0	ND	30 ± 2.0
S. aureus ATCC 6538	15 ± 1.0	13 ± 0.5	14 ± 0.5	15 ± 0.5	ND	22 ± 1.0
S. enterica ATCC43972	14 ± 0.5	12 ± 1.0	11 ± 1.0	13 ± 1.0	ND	11 ± 0.5
E. coli ATCC 8739	12 ± 1.0	10 ± 0.5	10 ± 0.5	11 ± 0.5	ND	19 ± 1.0
Fusarium sp.	20 ± 0.5	11 ± 0.5	17 ± 1.0	20 ± 2.0	ND	22 ± 2.0

50 μg/platelet, diameter of inhibition zones in mm. For each pure active compound and indicator microorganism, the experience was carried out simultaneously three times in the same conditions. In each case, all obtained diameter of inhibition zones were quite similar and the reported inhibition zones (mm) are the average of the three experiences (Mean ± SD; n = 3)

ND activity not detected, *NC* negative control, *PC* positive control

exhibited strong antifungal activity against *Candida albicans* (El-Sakhawy et al. 1998). As shown in Table 4, we have observed that compound (**3**), in addition to its antifungal activity, this compound possesses antibacterial activities against Gram-positive bacteria.

The fourth active compound (**4**) is the Cyclo (L-Pro-L-Tyr) a diketopiperazine (DKP) derivative. DKP derivatives, produced naturally by many organisms and microorganisms, display a very wide diversity of structures and biological functions, making them useful chemical entities for the discovery and development of new drugs. Useful biological properties have already been demonstrated for some of them, such as antibacterial, fungicidal, herbicidal, antiviral, immunosuppressor, antitumour activities, etc. (Magyar et al. 1999). Several DKP derivatives have been purified and characterised especially from *Streptomyces* species (Rhee 2002; Ben Ameur Mehdi et al. 2004, 2006). Concerning compound 4, it has been previously described as a natural product from *Streptomyces* species or obtained by chemical synthesis. According to our antimicrobial studies, compound **4** possesses antibacterial activities against Gram-positive and Gram-negative bacteria as well as antifungal activities (Table 4).

Conclusions

A new aerobic bacterium TN256 was isolated from Tunisian Saharan soil and has been selected for its antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi. Based on the results of cellular morphology, physiological and chemotaxonomic characterization, nucleotide sequence analysis (1,430 pb) of the whole 16S rRNA gene (accession no. FN687758) of the TN256 strain and phylogenetic analysis, this isolate has been assigned as *Streptomyces* sp. TN256 strain (=CTM50228^T). Four active compounds from the culture supernatant of this strain where extracted, purified. The corresponding chemical structure of these active compounds was established by different spectroscopic techniques (HPLC, LCMS/MS and NMR) and compared with reference data. **1**: N-[2-(1H-indol-3-yl)-2 oxo-ethyl] acetamide; **2**: di-(2-ethylhexyl) phthalate; **3**: 1-Nonadecene and **4**: cyclo (L-Pro-L-Tyr). This study reveals that the strain TN256 is promising microorganism producing antibiotics and anti-tumor active compounds.

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