Purification and structure elucidation of three naturally bioactive molecules from the new terrestrial *Streptomyces* sp. TN17 strain

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Thirty litres of fermentation broth was extracted from the newly isolated *Streptomyces* sp. strain TN17 and various separation and purification steps led to the isolation of three pure bioactive compounds (**1**–**3**). Compound **1**: cyclo (L-Leu-L-Arg), a diketopiperazine 'DKP' derivative; **2**: di-(2-ethylhexyl) phthalate, a phthalate derivative; and **3**: cyclo 1-[2-(cyclopentanecarbonyl-3-phenyl-propionyl]-pyrrolidine-2-carboxylic acid (1-carbamoyl-propyl)-amide, a cyclic tetrapeptide derivative. The chemical structure of these three active compounds was established on the basis of spectroscopic studies (MS and NMR) and by comparison with data from the literature. According to our biological studies, the pure compounds (**1**–**3**) possess antibacterial and antifungal activities.

Keywords: *Streptomyces* sp. TN17; purification; chemical structure; cyclo (L-Leu-L-Arg); phthalate derivative; cyclic tetrapeptide

1. Introduction

The increase in the frequency of multi-resistant pathogenic bacteria has created an urgent demand in the pharmaceutical industry for more rational approaches and strategies in the screening of new antibiotics. Currently available bioactive molecules can be classified in different ways based on the bacterial spectrum, the type of activity (bactericidal or bacteriostatic) and the chemical structure. Using chemical structure classification, several classes of antibiotics can be distinguished: β-lactams, chemically characterised by a β-lactam ring; chloramphenicol, a nitro benzene derivative of dichloroacetic acid; macrolides, macrocyclic lactones divided in two main subgroups – polyene macrolide and non-polyenic macrolide antibiotics; glycopeptides, which consist of glycosylated cyclic or polycyclic nonribosomal peptides; polyethers, which contain a number of cyclic ether and ketal units and have

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a carboxylic acid group; aminoglycosides, which characteristically contain amino sugars; tetracyclines, characterised as containing a polyhydronaphthacene nucleus; the cyclic tetrapeptides family; diketopiperazine derivatives 'DKP' (piperazine 2,5-diones, 2,5-dioxopiperazines or cyclodipeptides); phthalate derivatives; and so on.

It has been well established that microorganisms are an unlimited source of natural products, many of which have potential therapeutic applications. Filamentous soil bacteria belonging to the genus *Streptomyces* are widely recognised as industrially important microorganisms because of their ability to produce many kinds of secondary metabolites, including antibiotics and bioactive compounds valued in human and veterinary medicine and agriculture (Williams et al., 1983). These bacteria produce about 75% of commercially and medically useful antibiotics, and approximately 60% of antibiotics which have been developed for agricultural use were isolated from *Streptomyces* species (Miyadoh, 1993; Tanaka & Mura, 1993). This genus of bacteria represents at least 90% of actinomycetes isolated from soil (Anderson & Wellington, 2001). Active molecules of *Streptomyces* species are generally extracellular, and their isolation in the 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.

From Tunisian soil we have isolated a new actinomycete strain called *Streptomyces* sp. TN17, producing diverse biological activities (data not shown). The present article describes the extraction, purification (using different chromatographic techniques) and the structure elucidation of three bioactive molecules from a liquid culture broth of this strain. The biological activity of these pure compounds is also addressed.

2. Results and discussion

2.1. *Structure elucidation of the active compounds*

2.1.1. *Cyclo (L-Leu-L-Arg) (**1**)*

Compound **1** was obtained as a yellowish UV-absorbing solid. In DMSO, the ^{13}C and HSQC spectra showed 11 carbon signals. From the ^{13}C data, it was possible to discern two carbonyl groups (δ_c 170.8 and 167.0), three sp^3-hybridised carbons bearing an electronegative heteroatom (δ_c 59.0, 53.1 and 45.3), four sp^3-hybridised carbon (δ_c 38.3, 27.9, 24.6 and 22.9) and two methyl groups (δ_c 23.3 and 22.4). The 2D ^1H–^1H and ^1H–^{13}C experiments permitted assignment of two fragments to leucine and arginine. The presence of a NH proton (δ_H 7.96) and observed HMBC and NOE correlations enabled the identification of compound **1** as a (L-Leu-L-Arg) dipeptide. Based on these data, two possible structures were suggested for this compound. The first one is a linear structure where the corresponding molecular weight (MW) will be 287, and the second one is a cyclic structure where the MW will be 269 (Figure 1(a) and (b)). To distinguish between these two situations, we have determined the mass of compound **1** using the LC/MS technique. The obtained result, $[M–H]^+ = 268.5$, shows that the corresponding MW is 269. Consequently, we can deduce that compound **1** possesses a cyclic structure (Figure 1(b)), and the corresponding molecular formula is $C_{12}H_{23}O_2N_5$.

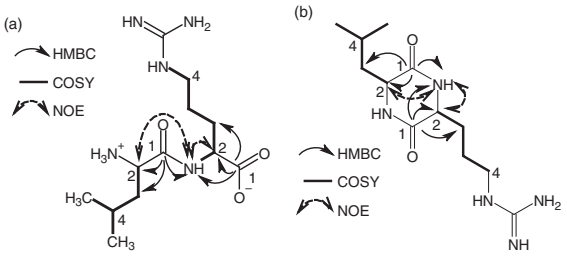

Figure 1. HMBC, COSY and NOE correlations of the two proposed structures (a and b) of compound **1**.

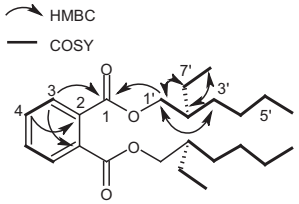

Figure 2. HMBC and COSY correlations of compound **2**.

2.1.2. *Di-(2-ethylhexyl) phthalate (**2**)*

Compound **2** was obtained as a white coloured solid. In CD_2Cl_2, the ^{13}C and HSQC spectra showed 12 carbon signals. From the ^{13}C data, it was possible to discern one carbonyl group (δ_c 162.5), three sp^2-hybridised carbons (δ_c 132.5, 130.9 and 128.7), one sp^3-hybridised carbon bearing an electronegative heteroatom (δ_c 68.0), five sp^3-hybridised carbons (δ_c 35.8–23.8) and two methyl groups (δ_c 13.8 and 10.7). The ^1H NMR spectrum showed a characteristic AA′BB′ system at 7.74 and 7.59 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 has a di *ortho*-substituted aromatic ring. Based on the revealed spectral data and a search in AntiBase, compound **2** was identified as di-(2-ethylhexyl) phthalate (Figure 2). The corresponding molecular formula is $C_{24}H_{38}O_4$, with a MW of 390.

2.1.3. *Cyclo 1-[2-(cyclopentanecarbonyl-3-phenyl-propionyl]-pyrrolidine-2-carboxylic acid (1-carbamoyl-propyl)-amide (**3**)*

Compound **3** was obtained as a white coloured solid. In CD_3OD, the ^{13}C and HSQC spectra showed 12 carbon signals. From the ^{13}C data, it was possible to discern two carbonyl groups (δ_c 169.5 and 165.5), four sp^2-hybridised carbons (δ_c from 137.7 to 126.8), three sp^3-hybridised carbons bearing an electronegative heteroatom (δ_c 58.9, 56.2 and 45.0) and three sp^3-hybridised carbons (δ_c 35.8, 28.2 and 22.4). The 2D ^1H–^1H and ^1H–^{13}C experiments permitted assignment of two fragments to proline and phenylalanine. The HMBC correlations from the α-protons and proline CH_2-5 protons to the respective amide carbonyls established the cyclic tetrapeptide. The structure of compound **3** was determined to be 1-[2-(cyclopentanecarbonyl-3-phenyl-propionyl]-pyrrolidine-2-carboxylic acid (1-carbamoyl-propyl)-amide (Figure 3). The corresponding molecular formula is $C_{28}H_{32}O_4N_4$, with a MW of 488.

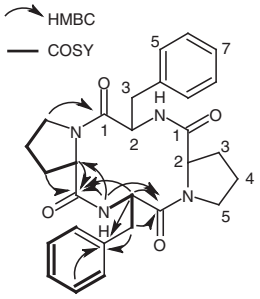

Figure 3. HMBC and COSY correlations of compound **3**.

2.2. *Biological activities of the three characterised compounds*

The new isolated *Streptomyces* sp. strain TN17 produced simultaneously three active compounds belonging to three different structure types.

The first active compound (**1**) is the cyclo (L-Leu-L-Arg), 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, Zhang, Abdi, Kohn, & Widger, 1999). Several DKP derivatives have been purified and characterised, especially from *Streptomyces* species (Ben Ameur-Mehdi, Mellouli, Chabchoub, Fotso, & Bejar, 2004; Ben Ameur-Mehdi, Sioud, Fourati Ben Fguira, Bejar, & Mellouli, 2006; Rhee, 2002). Compound **1** has been previously described as a natural product from *Streptomyces* species (Tatsuta, Tsuchiya, Umezawa, Naganawa, & Hamao, 1972) or obtained by chemical synthesis (Sasaki et al., 1982). According to our antimicrobial activity studies, we have observed that compound **1** possesses antibacterial activities against Gram-positive and Gram-negative bacteria, as well as antifungal activities (Table 1).

The second active compound produced by the *Streptomyces* sp. TN17 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, J. Kim, Lim, & C. Kim, 2000), marine algae (Chen, 2004), and fungal (Amade, Mallea, & Bouaicha, 1994) and bacterial culture broths, especially those belonging to the genus *Streptomyces*. Compound **2** has been already described from *Streptomyces bangladeshiensis* (Al-Bari, Abu Sayeed, Sazedur Rahman, & Ashik Mossadik, 2006). Other phthalate derivatives have been isolated from *Streptomyces* species, such as the dibutyl phthalate (El-Naggar, 1997; Lee, 2000; Roy, Laskar, & Sen, 2006). Phthalate derivatives which possess several antimicrobial activities are also effective compounds against demodicidosis (Yuan, Guo, Qin, Deng, & Huang, 2001), as well as endocrine disruptors with estrogenic activity (Marchetti et al., 2002) and drug channelling agents (Makhija & Vavia, 2003). Our antimicrobial studies show that compound **2** possesses antibacterial activities against Gram-positive bacteria and fungi (Table 1).

Table 1. Antimicrobial activities of compounds **1**–**3**.

	Diameter of inhibition zones (mm)		
Test organism	**1**	**2**	**3**
M. luteus LB 14110	20	17	19
S. aureus ATCC 6538	14	12	15
E. coli ATCC 8739	11	ND	ND
Fusarium sp.	21	14	17

Notes: A total of 50 μg per platelet, diameter of inhibition zones in mm. ND: activity not detected. For each pure active compound and indicator microorganism, the experiment was carried out simultaneously three times in the same conditions. In each case, all obtained diameters of inhibition zones were quite similar and the reported inhibition zones (mm) are the average of the three experiments.

The third active compound (**3**) produced by the studied TN17 strain is the cyclo 1-[2-(cyclopentanecarbonyl-3-phenyl-propionyl]-pyrrolidine-2-carboxylic acid (1-carbamoyl-propyl)-amide, a cyclic tetrapeptide derivative. Cyclic tetrapeptides are a class of natural products that have been shown to possess broad-ranging biological activities and good pharmacokinetic properties (Horton et al., 2008). Several groups of cyclic tetrapeptides have been described, such as rhodopeptins isolated from *Rhodococcus* species having antifungal activities (Chiba, Agematu, Dobashi, & Yoshioka, 1999), cyclic tetrapeptides group of protein synthesis inhibitors described from *Streptomyces* genus (Brandi et al., 2006), and apicidin tetrapeptides group acting as antitumour agents that can induce cell cycle arrest and apoptosis in various cancer cells (Okada et al., 2006). Compound **3** has been isolated from *Streptomyces barakatei* J2 and a patent for the use of this active molecule in plant treatment has been deposed (Lebrihi, Errakhi, & Barakate, 2008). According to our microbiological tests, compound **3** shows inhibitory activities against Gram-positive bacteria and fungi (Table 1).

3. Experimental

An NMR sample was prepared by dissolving the pure compounds (**1–3**) in 600 μL of DMSO, CD_2Cl_2 and CD_3OD, respectively. 1D and 2D ^1H and ^{13}C NMR experiments were recorded on a Bruker Avance 500 spectrometer equipped with a 5 mm triple resonance inverse Z-gradient probe (TBI ^1H, ^{31}P, BB) or a Bruker Avance 600 spectrometer equipped with a 5 mm triple resonance inverse (TCI ^1H, ^{13}C, ^{15}N) Z-gradient cryoprobe. All chemical shifts for ^1H and ^{13}C are relative to TMS using ^1H (residual) or ^{13}C chemical shifts of the solvent as a secondary standard. The temperature was set at 298K. All the ^1H and ^{13}C 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–^{13}C HMQC and ^1H–^{13}C HMBC experiments.

The LC/MS analysis of compound **1** was performed using an LC/MSD Trap XCT – Electrospray (Agilent Technologies), equipped with an HPLC Agilent 100

DAD detector (C_{18} column Zorbax 300 2.1×150 mm). $[M-H]^+$ of **1** is 268.5, which involves a corresponding MW of 269.

3.1. *Microorganisms*

The *Streptomyces* sp. TN17 strain was isolated and selected as a producer of potent antimicrobial activities. Bacterial strains: *Micrococcus luteus* LB 14110, *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8739 were used as indicator microorganisms for the antibacterial activity essays. Antifungal activity was determined against the filamentous fungus, *Fusarium* sp.

3.2. *Culture conditions and biological assays of antimicrobial activities*

Indicator microorganisms were grown overnight in LB medium at 30°C for *M. luteus* LB14110 (Gram-positive bacteria) and at 37°C for *S. aureus* ATCC 6538 (Gram-positive bacteria) and *E. coli* ATCC 8739 (Gram-negative bacteria), then diluted 1 : 100 in LB medium and incubated for 5 h under constant agitation of 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 by 40 µL of a 5-h-old culture of the corresponding microorganism. For antifungal activity against *Fusarium* sp., 100 µL of spores suspension were added to the 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 three pure compounds (**1**–**3**) 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 inhibition of growth of the corresponding indicator microorganisms around the paper disk.

3.3. *Extraction and purification of active compounds*

Spores at $10^7\,L^{-1}$ of *Streptomyces* strain TN17 were used to inoculate 1000 mL Erlenmeyer flasks with four indents, containing 200 mL of TSB (tryptic soy broth) medium at $30\,gL^{-1}$ supplemented with 1% (w/v) of glucose and potassium phosphate at $1\,mmolL^{-1}$. After incubation at 30°C for 24 h in an orbital incubator with shaking at 200 rpm, this pre-culture was used to inoculate (5% v/v) a total volume of 30 L culture medium having the same composition of the pre-culture. The shaker culture broth was harvested after 3 days to separate mycelium and supernatant. The resulting supernatant was extracted two times by ethyl acetate (v/v). The obtained organic phases were evaporated to dryness under vacuum, to give a brown crude extract (2.8 g) which was dissolved in 5 mL of dichloromethane–methanol (DCM/90% MeOH/10%) and then subjected to a column chromatography on Sephadex LH-20 using a dichloromethane–methanol (DCM/90%

MeOH/10%) giving seven fractions, which were reduced to three fractions (FI–FIII) by monitoring of TLC.

3.3.1. *Fraction FI*

The biologically active fraction FI was then subjected to a series of chromatographic systems: a column chromatography on silica gel eluted with a gradient of 100%DCM–0%MeOH to 0%DCM–100%MeOH. An active sub-fraction was obtained at 85%DCM–15%MeOH. This sub-fraction was then fractioned by HPLC (Waters: controller 600, pump 600, dual λ absorption detector 2487, linear recorder); column C_{18} (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. 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. Only the compound corresponding to the peak having a retention time of 39 min (**1**) possessed biological activities.

Compound (**1**): Cyclo (L-Leu-L-Arg). ^{1}H NMR (DMSO-d_6, 600 MHz) δ 7.96 (1H, br s, Arg-NH), δ 4.19 (1H, dd, J = 8.1, 8.0 Hz, Arg-H2), δ 4.01 (1H, dd, J = 6.9, 5.9 Hz, Leu-H2), δ 3.41 (1H, m, Arg-H5), δ 3.28 (1H, m, Arg-H5), δ 2.13 (1H, m, Arg-H3), δ 1.92 (1H, m, Arg-H3), δ 1.90 (2H, m, Leu-H4), δ 1.79 (2H, m, Arg-H4), δ 1.77 (1H, m, Leu-H3), δ 1.37 (1H, m, Leu-H3), δ 0.88 (3H, d, J = 7.0 Hz, Leu-H5), δ 0.87 (3H, d, J = 7.0 Hz, Leu-H5′); ^{13}C NMR (DMSO-d_6, 150MHz) δ 170.8 (C, Arg-C1), δ 167.0 (C, Leu-C1), δ 59.0 (CH, Arg-C2), δ 53.1 (CH, Leu-C2), δ 45.3 (CH_2, Arg-C5), δ 38.3 (CH_2, Leu-C3), δ 27.9 (CH_2, Arg-C3), δ 24.6 (CH, Leu-C4), δ 23.3 (CH_3, Leu-C5), δ 22.9 (CH_2, Arg-C4) and δ 22.4 (CH_3, Leu-C5′).

3.3.2. *Fractions FII and FIII*

These two active fractions were subjected separately to two other purification steps: preparative thin-layer chromatography (PTLC) and HPLC fractionation, using the conditions similar to those for compound **1** to obtain the two pure active compounds (**2** and **3**) having retention times of 11.2 and 23.5 min, respectively.

Compound (**2**): Di-(2-ethylhexyl) phthalate. ^{1}H NMR (CD_2Cl_2, 500 MHz) δ 7.74 (1H, m, H3), δ 7.59 (1H, m, H4), δ 4.25 (1H, dd, J = 10.8, 5.6 Hz, H1′), δ 4.21 (1H, dd, J = 10.8, 6.0 Hz, 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′, H5′), δ 0.96 (3H, t, J = 7.3 Hz, H8′), δ 0.94 (3H, t, J = 7.0 Hz, H6′); ^{13}C NMR (CD_2Cl_2, 125 MHz) δ 162.5 (C, C1), δ 132.5 (C, C2), δ 130.9 (CH, C4), δ 128.7 (CH, C3), δ 68.0 (CH_2, C1′), δ 38.8 (CH, C2′), δ 30.4 (CH_2, C3′), δ 28.9 (CH_2, C4′), δ 23.8 (CH_2, C7′), δ 23.0 (CH_2, C5′), δ 13.8 (CH_3, C6′) and δ 10.7 (CH_3, C8′).

Compound (**3**): Cyclo 1-[2-(cyclopentanecarbonyl-3-phenyl-propionyl]-pyrrolidine-2-carboxylic acid (1-carbamoyl-propyl)-amide. ^{1}H NMR (CD_3OD, 500 MHz) δ 7.98 (1H, br s, Phe-NH), δ 7.27 (4H, m, Phe-H5, Phe-H6), δ 7.20 (1H, m, Phe-H7), δ 4.36 (1H, t, J = 5.1 Hz, Phe-H2), δ 4.36 (1H, dd, J = 9.0, 7.0 Hz, Pro-H2), δ 3.40

(1H, m, Pro-H5), 3.28 (1H, m, Pro-H5), δ 3.08 (1H, dd, $J = 14.2$, 5.1 Hz, Phe-H3), 3.02 (1H, dd, $J = 14.2$, 5.1 Hz, Phe-H3), 2.01 (1H, m, Pro-H3), 1.73 (2H, m, Pro-H4), 1.43 (1H, m, Pro-H3); ^{13}C NMR (CD_3OD, 125MHz) δ 169.5 (C, Pro-C1), δ 165.5 (C, Phe-C1), δ 137.7 (C, Phe-C4), δ 130.2 (CH, Phe-C5), δ 128.4 (CH, Phe-C6), δ 126.8 (CH, Phe-C7), δ 58.9 (CH, Pro-C2), δ 56.2 (CH, Phe-C2), δ 45.0 (CH_2, Pro-C5), δ 35.8 (CH_2, Phe-C3), δ c28.2 (CH_2, Pro-C3) and δ 22.4 (CH_2, Pro-C4).

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