Chemistry of Natural Compounds, Vol. 40, No. 5, 2004

PURIFICATION AND STRUCTURE ELUCIDATION OF TWO BIOLOGICALLY ACTIVE MOLECULES FROM A NEW ISOLATED *Streptomyces* sp. US 24 STRAIN

R. Mehdi-Ben Ameur,[1,2] L. Mellouli,[1]
F. Chabchoub,[2] S. Fotso,[3] and S. Bejar[1]

UDC 547.944

Two compounds having biological activities, 3-indolethanol and (L-phe, L-pro) diketopiperazine, have been isolated from a new filamentous soil bacterium called Streptomyces *sp. US24 strain.To our knowledge, it is the first time that these two active molecules are described from* Streptomyces *species. The structures of these two compounds were established on the basis on their spectral data such as IR, EI-MS, and ESI-MS spectrometry, as well as NMR 1D and 2D (COSY, HMQC, HMBC).*

Key words: *Streptomyces* sp. US 24, (L-phe, L-pro) diketopiperazine, 3-indolethanol, biological activities, spectroscopic methods.

In the course of screening of new biological active molecules, several research studies are currently oriented towards isolation of new *Streptomyces* bacteria from different soil and water samples. In fact, the soil bacteria *Streptomyces* produce a large number of secondary metabolites, including antibiotics, antifungals, immunosuppressants, herbicides, enzyme inhibitors, and other physiologically active substances [1]. These active molecules are generally extracellular. Their isolation in highest purity from the complex fermentation broth and their structure elucidation needs the application of a combination of various separation steps and several spectroscopic methods.

We have previously reported the isolation from Tunisian soil of a new actinomycete strain called *Streptomyces* sp. US 24 producing biological activities [2]. We report here the purification as well as the structure determination of two active molecules from this new bacterium.

The *Streptomyces* sp. US 24 strain was cultivated in tryptic soy broth (TSB) medium supplemented with starch at 1% (w/v) and trace mineral oligoelements [2]. After various purification steps of the resulting crude extract, two natural compoun ds having biological activities were purified and characterized: 3-indolethanol and (L-phe, L-pro) diketopiperazine.

3-Indolethanol. This molecule belongs to the tryptophol family and possesses biological activities against Gram-positive bacteria and *Candida albicans* [3]. It should be noticed that many indolethanol derivatives having interesting applications were prepared by semisynthetic pathways [4, 5].

The structure of this compound was determined by a combination of one- and two-dimensional spectra (COSY, HMQC, HMBC). The ^{1}H, ^{13}C, and HMBC NMR data of this molecule are summarized in Table 1. 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.

The ^{1}H-NMR spectrum showed two signals with chemical shifts 3.05 and 3.93 ppm for the two CH_2 groups and one signal at 8.09 ppm attributed to the N-H proton.

The ^{13}C NMR spectrum contains 10 signals, the first at 28.7 ppm and the second at 62.5 ppm. These two signals were attributed to the two CH_2 carbons (C-8 and C-9). In the region from 110 to 140 ppm we noted the presence of 8 signals attributed to the different carbon atoms.

1) Laboratory of Prokaryotic Enzymes and metabolites of the Centre of Biotechnology of Sfax, P.O. ′K′, 3038 Sfax, Tunisia, fax: 216 74 275 970, e-mail: lotfi.mallouli@cbs.rnrt.tn; 2) Laboratory of Applied Chemistry of the Faculty of Sciences of Sfax, P.O. ′K′, 3038 University of Sfax, Tunisia; 3) Department of Organic Chemistry, University of Goettingen, Tammannstrasse 2, D-37077 Goettingen, Germany. Published in Khimiya Prirodnykh Soedinenii, No. 5, pp. 419-421, September-October, 2004. Original article submitted March 26, 2004.

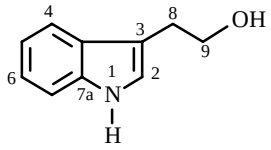
 0009-3130/04/4005-0510

TABLE 1. Chemical Shifts of ^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125.707 MHz, $CDCl_3$)

Atom	Chemical shifts		
	^{13}C	^{1}H (J/Hz)	HMBC coupled C No.
1	-	8.09 (1H, bs, NH)	-
2	122.47	7.01 (1H, d, J = 2.4)	3, 3a, 7a
3	112.23	-	-
3a	127.36	-	-
4	118.81	7.54 (1H, d, J = 7.9)	3, 6, 3a, 7a
5	119.45	7.06 (1H, ddd, J = 7.9, 7.9, 1.2)	7, 4, 6, 3a
6	122.19	7.14 (1H, ddd, J = 7.9, 7.9, 1.2)	7, 4, 7a
7	111.19	7.32 (1H, d, J = 7.9)	5, 6, 3a
7a	136.41	-	-
8	28.71	3.05 (2H, t, J = 7.1)	9, 3, 2, 3a
9	62.58	3.93 (2H, t, J = 7.1)	8, 3
10	-	4.5 (bs)	-

The 2- or 3- bound correlations in the HMBC spectrum between H-2 and C-3, C-3a, and C-7a, between H-4 and C-3, C-6, C-3a, and C-7a, between H-5 and C-7, C-4, C-6, and C-3a, between H-6 and C-7, C-4, and C-7a, and between H-7 and C-5, C-6, and C-3a unambiguously established the indolic moiety.

The position of the CH_2–CH_2–OH group in the indolic moiety was proved by ^{3}J correlation between H-8 and C-3a, and between H-9 and C-3 observed in the HMBC spectrum.



3-Indolethanol

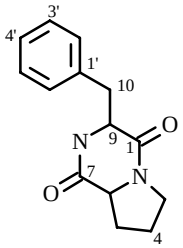
(L-phe, L-pro) diketopiperazine. The (+)-ESI spectrum showed quasi-molecular peaks at *m/z* 267 ($[M + Na]^+$) and 511 ($[2M + Na]^+$), respectively, which fixed the molecular weight at 244. High resolution at EI ionization afforded a molecular formula $C_{14}H_{16}N_2O_2$.

The IR spectrum revealed absorptions attributable to free N–H (3300 cm^{-1}) and carbonyl groups (1640 cm^{-1}).

The ^{1}H-NMR spectrum showed a singlet at 5.57 ppm assigned to the NH proton. Signals from 1 to 4.28 ppm were attributable at CH and CH_2 groups and those from 7.2 to 7.39 ppm to aromatic protons.

The ^{13}C NMR spectrum (Table 2) displayed signals of two carbonyls at 165.2 and 169.6 ppm. The ^{13}C signals in the aliphatic region were assigned by a DEPT experiment to four CH_2 (28.4, 22.6, 36.9, and 45.8 ppm) and two CH (56.2 and 59.2 ppm), which indicate the presence of two tertiary carbons attributable to (C-H pro) and (C-H phe), respectively.

Diketopiperazine derivatives constitute a family of secondary metabolites with diverse and interesting biological activities including antibiotics, immunosuppressants, and antitumors [6–8].



(L-phe, L-pro) diketopiperazine

TABLE 2. Chemical Shifts of ^{1}H (300 MHz, $CDCl_3$) and ^{13}C (50.306 MHz, $CDCl_3$)

Atom	Chemical shifts	
	^{13}C	^{1}H (J/Hz)
1	165.2	-
3	45.8	3.6-3.7 (2H, m, Pro 3-H_2)
4	22.6	1.9-2.1 (2H, m, Pro 4-3-H_2)
5	28.4	1.9-21 (1H, m, Pro 5-H)
		2.3-2.4 (1H, m, Pro 5-H)
6	56.2	4.08 (1H, m, Pro 6-H)
7	169.6	-
8	-	5.57 (1H, bs, NH)
9	59.2	4.28 (1H, dd, J = 9.2, 2.8, Phe 9-3-H_2)
10	36.9	3.6-3.7 (1H, m, Phe 10-H)
		2.77 (1H, dd, J = 14.4, 9.2, Phe 10-H)
1′	136	-
2′	129.4	
3′	129.2	
4′	127.9	7.2-7.39 (5H, m, Phe)

These molecules are mainly produced by microorganisms. Although the number of new, naturally isolated DKP derivatives has increased during the last decade, the biosynthetic pathways of these molecules remain largely unexplored especially those produced by soil bacteria belonging to the genus *Streptomyces*. The structural elucidation of DKP derivatives from these bacteria constitute a large contribution to the understanding of their biosynthesis phenomena. This fact will permit the production of new hybrid DKP derivatives with interesting biological properties.

EXPERIMENTAL

Description of the Producer Strain. *Streptomyces* sp. US 24 strain was newly isolated from Tunisian soil and possesses biological activities [2]. Permissive temperature ranges for growth of this strain were 30 to 40°C with an optimum at 37°C. In liquid media, active molecule production by the *Streptomyces* sp. US 24 strain was negatively affected by four carbohydrates (fructose, glycerol, glucose, and saccharose) and by potassium and magnesium. Starch at 1% (w/v) and trace mineral oligo-elements positively affected this production.

Culture Conditions and Purification of Active Compounds. For the purification of the active molecules from the *Streptomyces* sp. US 24 strain, spores at 10^7/mL were used to inoculate fifty 1000 mL Erlenmeyer flasks with four indents, each one containing 200 mL of TSB medium (30 g tryptic soy broth plus 5 g yeast extract per 1000 mL distilled water), supplemented at 1% (w/v) with starch and oligo-elements (40 mg $ZnCl_2$, 200 mg $FeSO_4 \times 7H_2O$, 6.5 mg H_3BO_3 and 13.5 mg $MoNa_2O_4 \times 2H_2O$ per 100 mL distilled water: 1.5 mL was added to 200 mL of growth medium). Cultures were incubated for 3 days at 37°C in an orbital incubator with shaking at 200 r.p.m.

The mycelia cake and the supernatant of 10 liter culture broth of the *Streptomyces* sp. US 24 strain were separately extracted three times each with ethyl acetate (7 liters). Since the chemical compositions of both organic phases were similar, they were combined and concentrated under reduced pressure to yield 2 g crude extract. The crude extract was dissolved in 2 mL dichloromethane-methanol 1:1 and applied to a column of Sephadex LH-20 packed in CH_2Cl_2–MeOH (1:1). Using this same solvent for the development of the column, we obtained one active fraction which was passed through a silica gel chromatography column eluted with a CH_2Cl_2–MeOH gradient. From the five obtained fractions, two possess biological activities. The first active fraction was submitted to preparative TLC (PTLC) (glass plates, 0.5 mm silica gel 60 F_{254}, Merck, Darmstad, Germany). After development in CH_2Cl_2–MeOH (9:1) the PTLC plate showed a blue fluorescence band under 254 nm yield to 3-indolethanol. Final purification of the second active fraction, (L-phe, L-pro) diketopiperazine, was

accomplished by reverse phase semipreparative HPLC (C18 column 7 mm, 4.6 mm inner diameter × 25 cm length). The elution was at a flow rate of 1 mL/min with a gradient of two solutions A (water – 0.1% formic acid) and B (acetonitrile – 0.1% formic acid). After injection of the sample, the column was eluted with a linear gradient from 100% buffer A to 50% buffer A and 50% buffer B over the first 40 min, followed by a linear gradient to 100% buffer B from 40 to 60 min, and then a steady flow of 100% buffer A through 10 min.

General. NMR spectra were measured on Bruker AMX (300.135 MHz) and Varian Inova 500 (499.876 MHz) spectrometers. HPLC/ESI-MS was recorded on a Finnigan LCQ with quaternary pump Rheos 4000 (Flux Instrument) and nucleosil column EC 125/2, 100-5, C18 (Macherey-Nagel & Co., Duren, Germany). EIMS was recorded on a Finnigan MAT 95 (70 ev) and perfluorokerosene was used as reference substance in HREIMS. IR spectra were recorded on a Jasco FT-IR 420 spectrometer (KBr pellets). Flash chromatography was carried out on silica gel (30–60 mm, J. T. Baker). Thin layer chromatography (TLC) was performed on polygram SILG/UV_{254} (Macherey–Nagel & Co.). R_f values were measured on polygram SILG/UV_{254} (Macherey–Nagel & Co.). Size exclusion chromatography was done on Sephadex LH-20 (pharmacia).

3-Indolethanol, R_f 0.28 (CH_2Cl_2 5%, MeOH); PMR spectrum (δ, ppm,$CDCl_3$, TMS): 3.05 (2H, t), 3.93 (2H, t), 7.54 (1H, d), 7.32 (1H, d), 7.14 (1H, t), 7.06 (1H, t), 7.01 (1H, d); ^{13}C NMR spectrum: 28.71 (C-8), 62.58 (C-9), 111.19 (C-7), 118.81 (C-4), 122.19 (C-6), 119.45(C-5), 122.47 (C-2), 136.41 (C-7a), 112.20 (C-3), 127.36 (C-3a); EIMS: *m/z* (%): 161 (M^+, 25), 147 (26), 130 (100), 120 (30), 91 (27), 77 (22), 43 (20); Molecular formula $C_{10}H_{11}NO$.

(L-phe, L-pro) diketopiperazine, R_f 0.73 (CH_2Cl_2 9%, MeOH). UV/vis (MeOH): λ_{max} 275 nm; IR spectrum (KBr, ν, cm^{-1}) 1600, 1630, 3400 cm^{-1}; PMR spectrum (δ, ppm, $CDCl_3$, TMS): 4.08 (1H, m, Pro), 3.6–3.70 (2H, m, Pro), 1.9–2.1(1H, m, Pro), 2.3–2.4 (1H, m, Pro), 1.9–2.1 (2H, m, Pro), 4.27 (1H, dd, Phe), 3.6–3.70 (1H, m, Phe), 2.77 (1H, dd, Phe), 5.57 (1H, bs), 7.2–7.39 (5H, m, Phe); ^{13}C NMR spectrum: 56.2 (C-6), 28.4 (C-5), 22.6 (C-4), 45.8 (C-3), 59.2 (C-9), 36.9 (C-10), 169.6 (C-7), 165.2 (C-1), 135.9 (C-1′), 129.4 (C-2¢), 129.2 (C-3′), 127.9 (C-4′); EI-MS: *m/z* (%) = 244 (M^+, 100), 153 (34), 130 (12), 125 (100), 91 (74), 70 (64), 41 (20); (+)- ESI-MS: *m/z* (%) = 511 ($[2M+Na]^+$, 100), 267 ($[M+Na]^+$, 65); molecular formula $C_{14}H_{16}N_2O_2$; EI-HRMS = 244.1212 (calc. 244.1191).

ACKNOWLEDGMENT

This work was supported by the Tunisian government (Contract Programme CBS-LEMP) and the Third World Academy of Sciences (RGA No. 02-173 RG/BIO/AF/AC). We are grateful to Prof. Dr. H. Laatsch (Department of Organic Chemistry, University of Goettingen, Germany) for his generous help and useful conversations.

REFERENCES

1. M. Suwa, H. Sugino, A. Sasaoka, E. Mori, S. Fujii, H. Shinkawa, O. Nimi, and H. Kinashi, *Gene*, **246**, 123 (2000).
2. L. Mellouli, R. Ben Ameur-Mehdi, S. Sioud, M. Salem, and S. Bejar, *R. Microbiol*., **154**, 345 (2003).
3. P. Waring and J. Beaver, *Gen. Pharmacol*., **27**, 1311 (1996).
4. L. Novak, G. Hornyanszk, J. Rohaly, P. Kolonits, and C. Szantay, *Liebigs Ann. Chem.*, 1877 (1995).
5. S. Armand, S. Drouillard, M. Schulein, B. Henrissat, and H. Driguez, *J. Biol. Chem*., **272**, 2709 (1997).
6. M. Chu, I. Truumess, M. L. Rothofsky, M. G. Patel, F. Gentile, P. R. Das, M. S. Puar, and S. L. Lin, *J. Antibiot*., **48**, 1440 (1995).
7. A. A. Willium, M. B. Lois, F. Meow-Chen, O. Helena, and S. G. Hossein, *Can. J. Chem.*, **64**, 904 (1995).
8. A. Magyar, X. Zhang, F. Abdi, H. Kohn, and W. Widger, *J. Biol. Chem.*, **274**, 7316 (1999).