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Full Length Research Paper

Cyclo (L-phenyl, L-prolyl). Diketopiperazines from a newly isolated *Streptomyces sudanensis*. A4.4

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A new actinomycete strain designated A4.4 possessing strong antibiotic properties was isolated from soil samples collected from different geographical areas in Sudan. The isolate was taxonomically characterized on the basis of morphological, physiological and phylogenetic analyses, which have properties similar to Streptomyces strain. In order to confirm the identified isolate, 16S rRNA gene (1.277 k p) analysis of the isolate was studied. The comparison of the 16S rRNA sequence of the A 4.4 isolate with those sequences submitted to GenBank demonstrated that the strain was 94% similar to the 16S rRNA sequence to number of isolates all of which were *Streptomyces* species, but phylogenetic analysis demonstrated that the Sudanese strain was on a different node to previously identified strains. Also it was readily distinguished strain A4.4 from its closest phylogenetic neighbors of these species by using a combination of phenotypic properties; these species are distinct from the known producers of Cyclo (L-phenyl, L-prolyl). On the basis of these results, strain A4.4 is proposed as the type strain of the novel species, for which the name *Streptomyces sudanensis*. A4.4 is proposed. The bioactive compound was isolated from the strain and was revealed to be cyclo (L phenyl, L-prolyl) by UV, ^{1}H- and ^{13}C-NMR and MS analyses and by comparison with reference data from literature.

Keywords: *Streptomyces sudanensis*, A4.4, cyclo(L-phenyl, L-prolyl), 16S rRNA.

INTRODUCTION

The emergence of antibiotic resistance among pathogenic bacteria has become a serious problem worldwide. The overuse of antibiotics in a number of settings is contributing to the increase in antibiotic-resistant in microorganisms, so the need for the discovery and development of new and effective antibiotics is a priority.

Actinomycetes are one of the most attractive sources of antibiotics and other biologically active substances of high commercial value. Among the Actinomycetes, most antibiotics, enzymes, enzyme inhibitors, antitumour agents and antifungal compounds have been isolated from *Streptomyces* spp (Kim and Hwang, 2003; Ozgur et al., 2008). Screening and isolation of promising strains of Actinomycetes with potential antibiotics is still a significant area of research and is suggested that the explorations of materials from new areas and habitats have a pivotal role to play in the search for new microbes and novel metabolites.

Diketopiperazine and its derivatives constitute a family of secondary metabolites with diverse and interesting biological activities including antibiotics, immunosuppressant, antitumor, antimutagenic and antiviral properties (Rhee et al., 2004, Niege et al., 2007). Diketopiperazines (DKPs) have been isolated from microorganisms, and from marine sponges (De Rosa *et al.,* 2003). These heterocyclic compounds display pharmacological effects in various mammals, including antimicrobial activity, cytotoxicity, anti-dinoflagellate

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activity and inhibitory activity against enzyme sortase B (Cueto et al., 2000; Lee et al., 2005; Chen et al., 2012). This paper describes the isolation of a new *Streptomyces sp*. from Sudanese soil and also the major bioactive compound produced by this strain: cyclo (L-phenyl, L-prolyl).

MATERIAL AND METHODS

Isolation of Streptomyces strain.

Streptomyces strain A4.4 was isolated from soil samples collected from different locations in the Sudan (15^{o} 36^{-} N and 32^{o} 33^{-} E). Isolation was performed by soil dilution plate technique using starch-casein nitrate agar (SCNA) (Starch 10.0 g, casein 0.3 g, KNO_3 2.0g, NaCl 2.0 g, K_2HPO_4 2.0 g, Mg $SO_4.7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4$. 7 H_2O 0.1. H_2O 1.0 L). The medium was supplemented with 10 µg/ml cyclohexamide and agar (18 g/l). The pure isolate was maintained as lyophilized and as spore suspensions at -80 °C as described by Hopwood and Chater (1985).

Cultural, physiological and biochemical tests

The isolate was characterized morphologically and physiologically following the directions given by the International Streptomyces Project (ISP), (Shirling and Gottlieb, 1966) and Bergey's Manual of Systematic Bacteriology. Microscopic characterization was carried out by cover slip culture method as described by (Shirling and Gottlieb, 1966), and formation of aerial and substrate mycelium and spores was observed under light microscope.

Scanning electron microscopy was performed using a JEOLJSM 5410LV scanning electron microscope at 35 kV. A plug of agar containing the culture was removed and fixed with glutaraldehyde (2.5 % v/v), washed with water and post-fixed in osmium tetroxide (1 %w/v) for 1 h. The sample was washed with water and dehydrated in ascending ethanol before drying in a critical drying point apparatus (Polaron E3000) and coated in gold.

Cultural characteristics (growth, colouration of substrate mycelia, and formation of soluble pigment) were tested according to the procedures of ISP. Biochemical tests including degradation of tyrosine, aesculin, casein and xanthine, production of synnemata and sclerotium were performed as recommended by ISP.

The effects of temperature (45 °C), pH (6-8) and salinity (NaCl 7%), growth in the presence of inhibitory compounds (phenol (0.1%), crystal violet (0.05%), sodium azide (0.01%) and lysozyme (0.005%) were also examined. Tests of antibiotic sensitivity against streptomycin (10 mg/l), kanamycin (25 mg/l), vancomycin (5 mg/l), and ampicillin (20 mg/l) were also performed.

Chemical composition of the cell wall

Cell walls were purified and analysed using the method of Lechevalier and Lechevalier (1980). The procedures of Becker et al. (1964) and Lechevalier and Lechevalier (1980) were used for analysis of whole-cell chemical composition.

16S rRNA gene sequence determination and analysis

The isolate was grown in nutrient broth for the preparation of genomic DNA which was extracted according to methods described by Nikodinovic et al. (2003). PCR amplification and sequencing of 16S rRNA gene was carried out as described previously (Stackebrandt et al., 1997) using a Peltier thermal cycler (BIO-RAD). The reaction mixture included the universal primers 27f (5'-CCG TCG ACG AGC TCA GAG TTT GAT CCT GGC TCA G-3') and 1392r (5'-CCC GGG TAC CAA GCT TAA GGA GGT GAT CCA GCC GCA-3'). To improve the denaturation of the genomic DNA, 5 µl DMSO was added to the reaction mixture. Amplification of the 16S rRNA gene was performed according to the following temperature profile: 95 °C for 2 min, followed by 30 cycles consisting of denaturing (40 sec), primer annealing at 50 °C (40 sec) and at 70 °C extension (1 min). A final extension step at 70 °C was included (10 min). Amplified DNA was detected by electrophoresis on a 1% agarose gel and visualized by UV fluorescence after ethidium bromide staining. Amplified fragments were purified using Qiaquick PCR cleanup kit (Qiagen) according to the manufacturer's instructions, and sequenced commercially by MWG. Trees were generated using CLUSTAL X programme (Larkin et al., 2007).

Extraction, purification and identification of active metabolite

0.5 ml spores suspension of *Streptomyces* sp. A4.4 strain, were used to inoculate 250 mL Baffled Erlenmeyer flasks containing 50 mL TSB medium, after incubation at 30^{o} C for 48 hours at 200 rpm, the cultured broth (1 l) was centrifuged at 6000 rpm for 15 minutes to remove the biomass. The supernatant was extracted twice with an equal volume of ethyl acetate and then evaporated on a Rotavapor and concentrated under vacuum at 40 ° C to eliminate the organic solvent and to obtain the crude extract which was dissolved in 1 ml of methanol. The mycelia cake were firstly soaked in methanol for 5 hours at room temperature and then extracted with dichloromethane (DCM) to give crude antibiotic extract dissolved in 1 ml methanol. Activities against test organisms (*Staphylococcus aureus, Bacillus subtilis, Streptococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Aspergillus niger,* and *A. flavus*) were monitored during the isolation, using the antibiotic disk method.

The crude organic extract was separated by solid phase extraction (SPE) on a Hypersil C18 column, and eluted with a stepwise gradient of methanol (20-100 %). Fractions containing highest antibiotic activity were purified further by HPLC (Varian Prostar system) using an isocratic elution (80% methanol-water) on a Zorbax Stable Bond column. Peak purity was assessed by analytical HPLC with a gradient elution of acetonitile using a Thermo Hypersil C18 column (4.6 x 150 mm 5 µm). This compound showed a strong absorption under UV light at 254 nm and gives a violet colouration on spraying with anisalde-hyde/sulphuric acid.

For the structure elucidation of pure bioactive compound ^{1}H NMR spectra were measured on a Varian Inova (300.135 MHz) spectro- meter. ^{13}C NMR spectra were measured on a Varian Inova (75 MHz) ESI-MS was recorded on a Quattro Triple Quadrupole Mass Spectrometer, Finnigen TSQ 7000 with nano-ESI-API-ion source. ESI-HRMS was measured on Micromass LCT mass spectrometer coupled with a HP 1100 HPLC with a

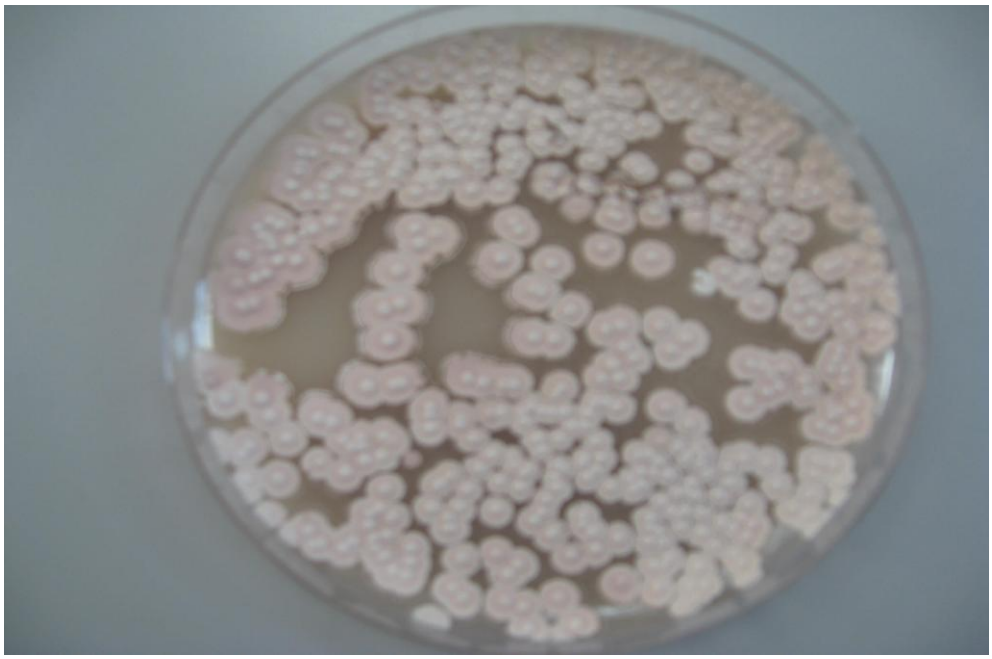

Figure 1. Morphological types of colonies of Streptomyces sp A 4.4 in ISP4 medium after 10 days incubation at 30°C.

Diode Array Detector. Reserpin (MW = 608) and Leucin-Enkephalin (MW = 555) were used as standards in positive and negative mode. EI-MS was recorded on a Finnigan MAT 95 (70 eV). Flash chromatography was carried out on silica gel (230–400 mesh).

RESULTS AND DISCUSSION

Cultural, physiological characteristics and identification of the A4.4 strain

A new Streptomyces designated A 4.4 isolated from Sudanese soil produced antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi. A4.4 colonies in ISP4 medium as shown in Figure 1. Based on the prominent antimicrobial activities, the A 4.4 strain was taken for the taxonomical studies. The morphological and cultural characteristics **(**Growth characteristics, reverse colour and spores colour) of the isolate was observed after 10 days incubation on the International Streptomyces Project (ISP) media and various other media and they showed considerable variations, isolate A 4.4 showed good growth on most media with exception of ISP 5. The colour of reverse mycelium were yellowish and brown and the spore chains were pink and rose.

It is evident that different physiological characteristics influence the growth rate of the actinomycetes (Kim *et al.,* 1999; Shimizu *et al.,* 2000). In the present study, the assessment of physiological characteristics of the strain A 4.4 revealed that it could grow well at 45^{o}C and pH 6.0 to 8.0 respectively. However, the strain had maximum growth rate at a NaCl 7%, no production of diffusible pigment was observed on all media tested, and the growth on inhibitory compounds patterns against the test isolate was also observed. The culture characteristics are summarized in Table 1.

Light and scanning electron microscopy revealed that isolate A4.4 is Gram positive, filamentous and spore forming. The isolate A 4.4 spore chains are primitive open spirals (Figure 2). In order to confirm the identified isolates, 16S rRNA gene analysis of the isolate was done.

The comparison of the 16S rRNA sequence of the A 4.4 isolate with those sequences submitted to GenBank demonstrated that the strain was 94% similar to the 16S rRNA sequence to number of isolates all of which were *Streptomyces* species. According to the sequence alignment and phylogenetic tree based on the 16S rRNA genes,. *Streptomyces* sp A 4.4 was closest to *Streptomyces thermolilacinus, Streptomyces fradiae* strain HBUM174185*, Streptomyces* sp. SD 534*, Streptomyces* sp. A554 Ydz-TA*, Streptomyces rubrolavendulae* and *Streptomyces coeruleoprunus*.

It is evident from Table 1 and Figure 3 that strain A 4.4 can be distinguished from the type strains of its most immediate phylogenetic neighbors of the genus *Streptomyces* by using a combination of phenotypic properties. Additional phenotypic properties are cited in the species description. In this regard, the morphological features and degradation of organic compounds were

Table 1. Characteristics that separate strain A 4.4 from the type strains of phylogenetically closely related Streptomyces species

	1	2	3	4	5	6	7
Morphology							
Aerial spore mass	pink	Grey	White	Yellow to grey	Grey	Yellow-pink	Grey
Substrate Mycelium	Yellowish brown	Yellow	Grayish	Yellow to brown	Light grayish	Yellow to brown	Grayish
Diffusible Pigment	None	None	Yellow	None	None	ND	Brown
Production of							
Synnemata	-	+	-	-	+	-	-
Sclerotium	-	-	+	-	+	+	-
Degradation of							
Tyrosine	+	-	-	-	+	+	NT
Aesculin	+	-	-	D	-	-	-
Casein	+	+	+	-	NT	+	+
Xanthine	-	+	-	+	NT	-	-
Growth at/in							
7 % NaCl	+	-	-	NT	+	-	-
45^{o} C	+	+	-	D	-	+	D
6-8 pH	+	-	+	-	+		
Growth on inhibitory compounds							
Phenol 0.1%	-	+	-	-	-	-	-
Lysozyme 0.005%	+	-	-	+	-	+	-
Sodium azide 0.01%	-	-	-	+	-	-	+
Crystal violet 0.05%	-	-	+	-	-	-	-
Streptomycin 10 mg/L	+	-	+	-	-	+	+
Kanamycin 25 mg/L	+	-	-	-	+	-	-
Vancomycin 5 mg/L	-	+	-	-	+	+	+
Ampicillin 20 mg/L	+	-	-	-	-	-	+

Strains: {1,AH4.4; 2, S *thermolilacinus*; 3, S. *fradiae* strain HBUM174185; 4, S. sp. SD 53; 5, S. sp. A554 Ydz-TA; 6, S. *rubrolavendulae*; 7, S. *coeruleoprunus*. Data for the type strains of the species indicated were taken from Shirling & Gottlieb (1968a, b, 1969, 1972), Williams et al.(1983) and Kim et al. (1999). +, Positive; –, negative; NT, not tested; D, doubtful}.

very significant in distinguishing strain A 4.4 from its phylogenetic neighbors. It was reported in previous studies that differential morphological and pigmentation features are especially significant for the delineation of members of phylogenetically related *Streptomyces* species (Labeda and Lyons, 1991; Kim et al., 2000; Manfio et al., 2003). It is clear from the genotypic and phenotypic data that strain AH 4.4 should be recognized as the type strain of a novel species in the genus *Streptomyces.* The cell wall of the A4.4 strain contained L- diaminopimelic acid and glycine. The whole-cell hydrolysates contained galactose.

Extraction, purification and structure elucidation of the active compound

Extraction, purification and identification steps to the supernatant and the mycelium of 1l culture broth of the *Streptomyces* sp. A4.4 strain, with ethyl acetate led to isolation of pure molecule having biological activities (144 mg/L). The purification of the active colourless amorphous solid compound was carried out using reverse phase SPE and HPLC-DAD analysis.

The chemical structure of the active compound purified from the *Streptomyces sp*. A4.4 is shown in Figure 4. Cyclo (L -phenyl, L -prolyl). The (+)-ESI/MS spectrum delivered quasi-molecular peaks at m/z 267 ([M + Na]+) and 511 ([2M + Na]+), respectively, which fixed the molecular weight to 244. High resolution at EI ionization afforded a molecular formula $C_{14} H_{16} N_2 O_2$. The ^{1}H NMR spectrum showed a singlet at 5.57 ppm assigned at 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 at aromatic protons. The ^{13}C NMR spectrum displayed signals of two carbonyls at 165.2 and 169.6 ppm. The ^{13}C

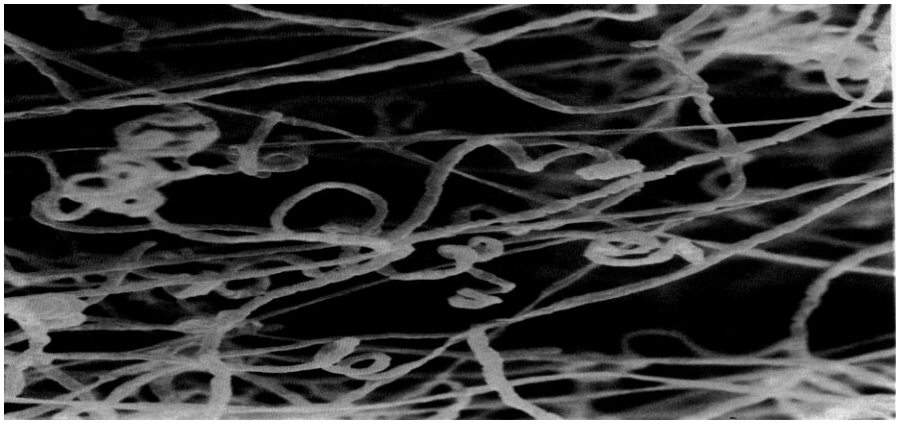

Figure 2. Scanning electron micrographs of the spore chains of Streptomyces sp. A4.4.

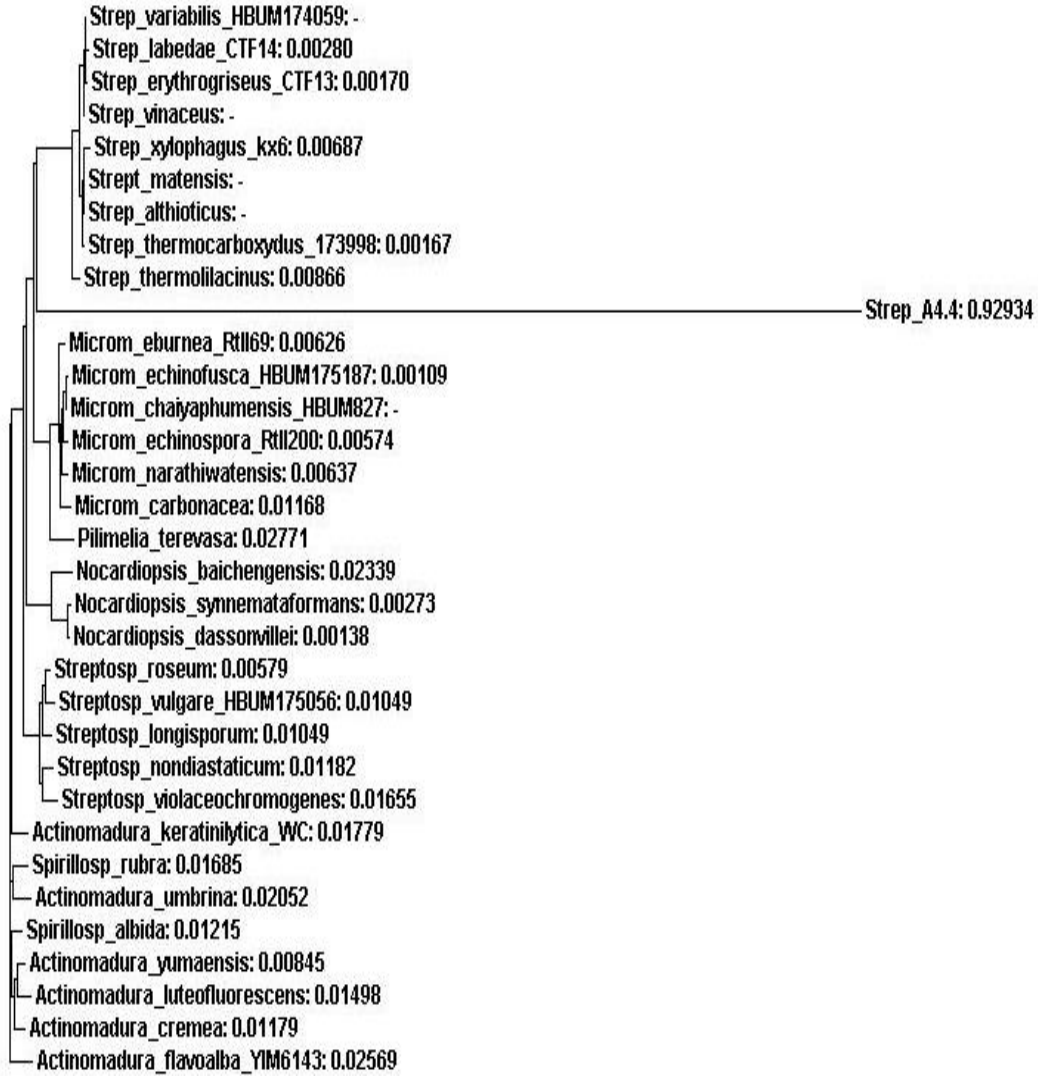

Figure 3. Phylogenetic tree showing the relationship between strain A4.4 and representative species of the genus *Streptomyces* and other taxa based on nearly complete 16S rRNA gene sequences.

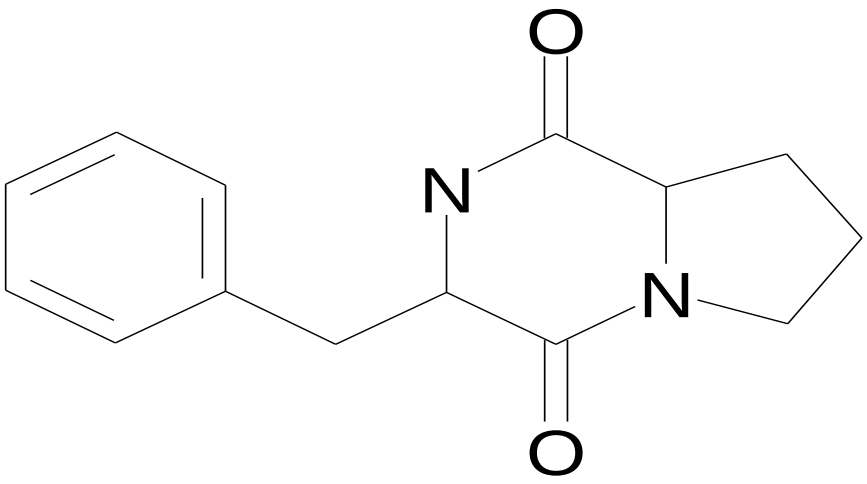

Figure 4. The structure of Cyclo (L-phenyl, L-prolyl)

signals in the aliphatic region were assigned by APT as 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.

DKPs comprise an important family of the secondary metabolites that are mainly produced by microorganisms (Niege *et al.,* 2007). The DKP derivative, produced by the *Streptomyces* sp. A 4.4 strain, were previously described from the North Sea bacterium *Cytophaga marinoflava* strain Am13,1 and the actinomycete strain A8 for the molecule cis-cyclo (L-phenyl, L-prolyl) (Shaaban, 2004; Arunrattiyakorm et al., 2006), and from a Norcardia species for the cis-cyclo (Leucyl-Prolyl) (Shaaban, 2004) and from *Aspergillus fumigates* for the (L)- Pro-(L)-Gly , (L)-Pro-(L)-Leu, (L)-4-OH-Pro-(L)-Leu, (L)-Pro-(L)-Phe and 4-OH-(L)-Pro-(L)-Phe. (Niege *et al.,* 2007), and from *Alcaligenes faecalis* for the Cyclo-(L-Pro-L-Phe) and Cyclo-(L-Pro-L-Leu) (Zhiyong.*et al.,* 2008).

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