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Meroparamycin Production by Newly Isolated *Streptomyces* sp. Strain MAR01: Taxonomy, Fermentation, Purification and Structural Elucidation

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Twelve actinomycete strains were isolated from Egyptian soil. The isolated actinomycete strains were then screened with regard to their potential to generate antibiotics. The most potent of the producer strains was selected and identified. The cultural and physiological characteristics of the strain identified the strain as a member of the genus *Streptomyces*. The nucleotide sequence of the 16S rRNA gene (1.5 kb) of the most potent strain evidenced a 99% similarity with *Streptomyces* spp. and *S. aureofaciens* 16S rRNA genes, and the isolated strain was ultimately identified as *Streptomyces* sp. MAR01. The extraction of the fermentation broth of this strain resulted in the isolation of one major compound, which was active *in vitro* against gram-positive, gram-negative representatives and *Candida albicans*. The chemical structure of this bioactive compound was elucidated based on the spectroscopic data obtained from the application of MS, IR, UV, ^{1}H NMR, ^{13}C NMR, and elemental analysis techniques. Via comparison to the reference data in the relevant literature and in the database search, this antibiotic, which had a molecular formula of $C_{19}H_{29}NO_2$ and a molecular weight of 303.44, was determined to differ from those produced by this genus as well as the available known antibiotics. Therefore, this antibiotic was designated Meroparamycin.

Keywords: meroparamycin, purification, *Streptomyces* sp. MAR01, structural elucidation, biological activity, 16S rRNA

It has been well established that microorganisms are a virtually unlimited source of natural products, many of which have potential therapeutic applications. Filamentous soil bacteria of the genus *Streptomyces* are remarkable, and merit special consideration with regard to the morphological and metabolic differentiation phenomena they manifest during later stages of development. *Streptomyces* species generally synthesize a sizeable number of diverse natural secondary metabolites, the best known of which are antibiotics currently used worldwide as pharmaceutical and agrochemical products (El-Naggar *et al.*, 2003; Pamboukian and Facciotti, 2004; Ben-Fguira *et al.*, 2005). Two-thirds of commercially available antibiotics (Miyadoh, 1993) and approximately 60% of those used for agricultural purposes were isolated originally from *Streptomyces* species (Tanaka and Mura, 1993).

Antibiotic resistance is increasing globally, and may make the long list of currently available antimicrobial agents that are now insufficient for the control of microbial infections (Bhavanani and Ballow, 2000). The increase in the frequency of multi-resistant pathogenic bacteria is created an urgent demand in the pharmaceutical industry for more rational approaches and strategies to the screening of new antibiotics with a broad spectrum of activity, which resist the inactivation processes exploited by microbial enzymes (Saadoun and Gharaibeh, 2003; Motta *et al.*, 2004). However, it appears that these niches, which should be first screened for bacteria that generate new antibiotics, are not exotic places, but rather, established collections of *Streptomyces* species.

In the present work, we describe the isolation of an actinomycete strain from Egyptian soil, which generates an antibacterial compound. The identification of this strain, based on the cultural and physiological characteristics, as well as 16S rRNA methodology, is also reported. The primary bioactive substance was isolated, purified, and its structure was elucidated.

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Materials and Methods

Microorganisms and culture conditions

12 actinomycete strains were isolated from the Burg-Arab region, in Alexandria, Egypt. Soil samples were taken to a 10 cm depth into the soil surface after the removal of approximately 3 cm of the soil surface. Actinomycetes were isolated using Starch-casein agar (Okazaki and Okami, 1972) containing cycloheximide and nystatin (50 μg/ml each) in order to minimize fungal contamination. One gram of soil was diluted (10^{-2} to 10^{-4}) with sterile saline and plated on starch-casein agar supplemented with rifampicin (10 μg/ml). Unsupplemented starch-casein agar plates were used as a control. The plates were incubated for 4 weeks at 28°C. The isolated actinomycete strains were then screened with regard to their potential to generate bio-active compounds. The most potent of the producer strains was then selected and identified. The cultures were maintained on starch-nitrate agar slants. The inoculated agar medium was incubated for 7 days at 30°C, and then maintained at 4°C until further use.

Cultural and physiological characteristics

The cultural and physiological characteristics of the most potent producer strain were studied in accordance with the guidelines established by the International *Streptomyces* Project (Shirling and Gottlieb, 1966). Colors were assessed on the scale developed by Kornerup and Wanscher (1978). The electron microscope study was conducted using a scanning electron microscope (JEOL JSM 5300, JEOL Technics Ltd., Japan).

Dry weight determination

The *Streptomyces* strain cells were separated from the culture filtrate via 15 min of centrifugation at 5000 rpm, washed twice in distilled water, then dried at 70°C until reaching a constant weight.

Antibiotic bioassay

The antibacterial potential of the culture filtrates was assayed using 30 μl of culture filtrate to fill agar-wells (6 mm i.d.) punched in Mueller-Hinton agar plates which had been freshly seeded with 0.3 ml of *Staphylococcus aureus* ATCC 29737 (3×10^6 cfu/ml) as the test organism. The petri-dishes were maintained for 2 h in a refrigerator at 4°C to allow for the diffusion of the bioactive compound. The diameter of the inhibition zone was measured (in mm) after 24 h of incubation at 37°C.

The minimum inhibitory concentration (MIC) of the purified antibiotic was determined via agar dilution. Mueller-Hinton and Sabouraud agar media were utilized for bacteria (*Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 10536, *Staphylococcus aureus* ATCC 29737, *Staphylococcus epidermidis* ATCC 12228, *Micrococcus luteus* ATCC 9341) and yeast (*Candida albicans* ATCC 10231), respectively.

Stock cultures of the test organisms were maintained on Tryptic soya agar slants. The inoculated agar medium was incubated for 24 h at 37°C for *E. coli*, *S. aureus*, and *S. epidermidis*, and at 30°C for *B. subtilis*, *M. luteus*, and *C. albicans*, then maintained at 4°C until further use.

DNA isolation and manipulation

The locally isolated actinomycete strain was grown for 7 days on a starch agar slant at 30°C. Two ml of a spore suspension were inoculated into the starch-nitrate broth and incubated for 3 days on a shaker incubator at 200 rpm and 30°C to form a pellet of vegetative cells (pre-sporulation). The preparation of total genomic DNA was conducted in accordance with the methods described by Sambrook *et al.* (1989).

Amplification and sequencing of the 16S rRNA gene

PCR amplification of the 16S rRNA gene of the local actinomycete strain was conducted using two primers, StrepF; 5′-ACGTGTGCAGCCCAAGACA-3′ and Strep R; 5′-ACAAGCCCTGGAAACGGGGT-3′, in accordance with the method described by Edwards *et al.* (1989). The PCR mixture consisted of 30 pmol of each primer, 100 ng of chromosomal DNA, 200 μM dNTPs, and 2.5 units of Taq polymerase, in 50 μl of polymerase buffer. Amplification was conducted for 30 cycles of 1 min at 94°C, 1 min of annealing at 53°C, and 2 min of extension at 72°C. The PCR reaction mixture was then analyzed via agarose gel electrophoresis, and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA).

The 16S rRNA gene was sequenced on both strands via the dideoxy chain termination method, as described by Sanger *et al.* (1977). The 16S rRNA gene (1.5 kb) sequence of the PCR product was acquired using a Terminator Cycle Sequencing kit (ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA). The sequence data were deposited in the GenBank database, under the accession number DQ386119.

Sequence similarities and phylogenetic analysis

The BLAST program (www.ncbi.nlm.nih.gov/blst) was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluated using BioEdit software (Hall, 1999). The phylogenetic tree was displayed using the TREE VIEW program (Page, 1996).

Production and purification of the active compound, Meroparamycin

A loopful of the actinomycete strain from the 14-day

slant culture was transferred to each of fifty Erlenmeyer flasks (500 ml). Each of the flasks contained 200 ml of sterile starch-nitrate medium adjusted to an initial pH of 7.0. The flasks were incubated for 7 days at 30°C.

The culture broth (ca. 10 L) was filtered with a Büchner funnel *in vacuo* using Whatman filter paper No. 1, and the filtrate was extracted with 10 L of chloroform, which was considered to be the best solvent. The latter was concentrated to approximately 1 L under reduced pressure using a rotary evaporator (Büchi, R-114, Switzerland at a temperature not exceeding 50°C), after which n-hexane (500 ml) was added and the chloroform layer was re-separated, dried with anhydrous sodium sulphate, and filtered and evaporated under reduced pressure, yielding a yellowish oily material as a product (1.9 g).

The mycelium cake, however, was soaked in ethyl acetate (7 L) and stirred overnight at room temperature and filtered, and the ethyl acetate extract was concentrated to approximately 1 L and added to n-hexane (250 ml), after the ethyl acetate layer had been separated. Ethyl acetate extract was dried as described above, and evaporated, yielding a yellowish oily material (0.9 g).

The filtrate and mycelium extracts were tested via thin layer chromatography (TLC) and ^{1}H NMR, and determined to be identical. Therefore, they were combined and dissolved in 5 ml of chloroform and subjected to column chromatography using silica gel (3 i.d. × 25 cm) as a stationary phase. The column was developed with a solvent mixture of chloroform : methanol (15:1 - to - 1:1). Ten fractions were collected (500, 200, 100, 30, 50, 10, 60, 15, 10 and 15 mg, in order). The TLC plates revealed that the first three fractions were identical and active against *S. aureus* and were, therefore, pooled. Final purification was achieved using a Sephadex LH-20 column (3 i.d. × 25 cm) using chloroform: methanol (1:1) as the eluent. Ten fractions were collected and only 2 of them (400 and 30 mg, respectively) were determined to be active against *S. aureus* with different R_f values (0.4, 0.3, respectively) when the chloroform: methanol (95:5) mixture was used. The purity of the first fraction was verified via high performance liquid chromatography (HPLC), via the injection of 20 µl into an analytical (C_{18}) column (25.0 × 0.46 cm) packed with 5 µg of hypersile octadecyl silane (ODS), at a flow rate of 1.0 ml/min. The mobile phase was an aqueous solution of 75% acetonitrile. The peaks were detected solely by a UV detector at 220 nm (Cynkotek UVD 170S, Germany).

The pure fraction was then subjected to spectroscopic analyses: 300 MHz ^{1}H NMR, 75 MHz ^{13}C NMR (Varian mercury VX-300 NMR spectrophotometer). Elemental analysis, IR (Shimadzu FT-IR 8101 PC infrared spectrophotometer), UV absorption (Shimadzu spectrophotometer). High and low-resolution mass spectra were recorded on a JEOL HX 100A-HX 100A tandem mass spectrometer, using the electron impact (EI) method to elucidate its structure.

Results

Selection and identification of the producer strain

Twelve actinomycete strains were isolated and screened with regard to their potential to generate bioactive substance(s), using the following test organisms: *B. subtilis*, *E. coli*, *S. aureus*, *S. epidermidis*, *M. luteus*,

Table 1. Screening of the three biologically active actinomycete isolates for the antibacterial activity

Time (days)	Antibacterial activity[a] and growth[b] profiles of the actinomycete isolates					
	Isolate No. 1		Isolate No. 9		Isolate No. 12	
	IZ	DW	IZ	DW	IZ	DW
1	00	0.0	00	0.0	9	0.0
2	23	0.4	12	0.3	11	0.4
3	23	0.8	13	0.6	11	0.7
4	25	1.0	15	0.8	11	1.0
5	26	1.4	16	1.1	11	1.4
6	28	2.2	15	1.8	11	1.8
7	26	2.3	14	2.0	11	2.1
8	25	2.4	14	2.1	12	2.5
9	25	2.4	14	2.2	12	2.5
10	22	2.4	14	2.2	12	2.5
11	22	2.4	13	2.2	11	2.4
12	22	2.4	12	2.2	11	2.4

[a]Antibacterial activity expressed as an inhibition zone diameter (IZ, in mm) against *S. aureus* as a test organism and [b]the growth profile expressed as dry weight (DW, in mg/ml).

Table 2. Cultural characteristics of the locally isolated *Streptomyces* sp. MAR01

Medium	Growth	Aerial Mycelium	Reverse side
Yeast extract-malt extract agar	Good	Abundant, gray	Yellowish brown
Oatmeal agar	Good	Abundant, gray	Light brown
Inorganic salts-starch agar	Good	Abundant, gray	Grayish brown
Glycerol-asparagine agar	Moderate	Moderate, white	Light brown
Peptone-yeast extract iron agar	Moderate	Moderate, white	Yellowish brown
Tyrosine agar	Poor	Moderate, white	Brown
Sucrose nitrate agar	Good	Abundant, gray	Light brown
Sucrose asparagines agar	Good	Abundant, gray	Light brown

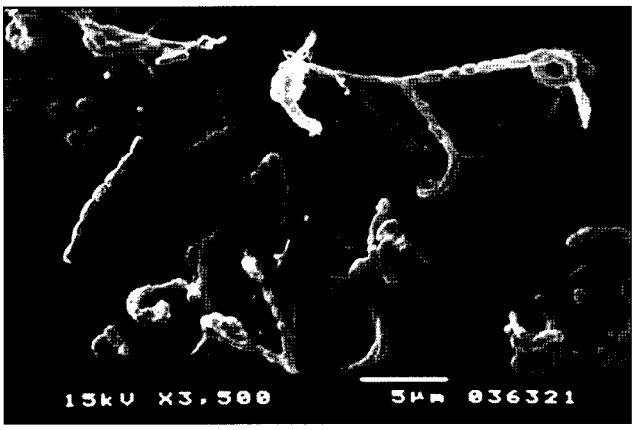

Fig. 1. Scanning electron micrograph (SEM) showing the spore chains of the locally isolated *Streptomyces* sp. MAR01 grown on yeast- malt extract agar for 14 days at 30°C.

and *C. albicans* (data not shown). Only three actinomycetes strains (1, 9 and 12) evidenced antibacterial potential, and this antibacterial activity was detected after the second day of incubation. The maximum antibacterial zones were obtained after days 5, 6 and 7 for isolates No. 9, 1, and 12, respectively (Table 1). Actinomycete isolate No. 1 was found to be the most potent antibiotic producing strain, and was therefore selected for strain identification and for the isolation of its bioactive metabolite.

The cultural characteristics of the local actinomycete strain are provided in Table 2. This strain grew well on most of the tested organic and synthetic media. Typically, the colony was elevated, spreading, and was covered with gray aerial mycelia and spores. Diffused melanoid pigments were absent. The aerial mycelium branched monopodially (Fig. 1) and formed spore chains (10 spore/chain). The spores evidenced smooth surfaces, and were morphologically spherical to cylindrical, with a size of 0.6-0.8 × 0.6-1.0 μm. The physiological properties of this strain, as well as the carbon and nitrogen compound utilization characteristics of the strain, were compared with the data published for *S. aureofaciens* (Lechevalier *et al.*, 1989). Strain No. 1 exhibited positive histidine and phenylalanine utilization, as compared to the negative response observed for *S. aureofaciens*.

Molecular phylogeny

The 16S rDNA sequence of the local isolate was compared to the sequences of 22 *Streptomyces* spp. in order to determine the relatedness of the local isolate to these *Streptomyces* strains. The phylogenetic tree (as displayed by the Tree View program) revealed that the locally isolated strain is closely related to *Streptomyces* sp., rather than to *S. aureofaciens* (Fig. 2). Multiple sequence alignment was conducted between the sequences of the 16S rDNA genes of *S. aureofaciens* and *Streptomyces* spp. (CHR and SNG) and the local isolate. Computer assisted DNA searches against bacterial database similarly revealed that the 16S rDNA sequence was 99% identical with both *Streptomyces* spp. and *S. aureofaciens* strains, and gave a 98% result for the rest of the *Streptomyces* strains.

Structural elucidation

The purified yellow active compound yielded by the combination of both filtrate and mycelial extracts was subjected to spectroscopic analyses in an effort to elucidate its structure.

The Infrared spectrum (KBr) of the pure compound evidenced a diagnostic peak at 3726 cm^{-1}, which is indicative of the $-NH_2$ group. However, the peak at 1736 cm^{-1} was assigned to the carbonyl (C=O) group of the amide functional group. The peak appearing at 1735 cm^{-1} was assigned to the carbonyl (C=O) group of the ketone functional group.

The UV visible spectrum of the purified active compound (dissolved in methanol) indicates the presence of an aromatic nucleus with a maximum absorption at a λ_{max} of 240 nm.

The molecular formula of the antibiotic was deduced as $C_{19}H_{29}NO_2$ based on the results of elemental analysis (Anal Cal. for $C_{19}H_{29}NO_2$: C 75.21%, H 9.63%, N 4.62%, O 10.55%; Found C 75.2%, H 9.8%, N 4.6%), and in accordance with the number of

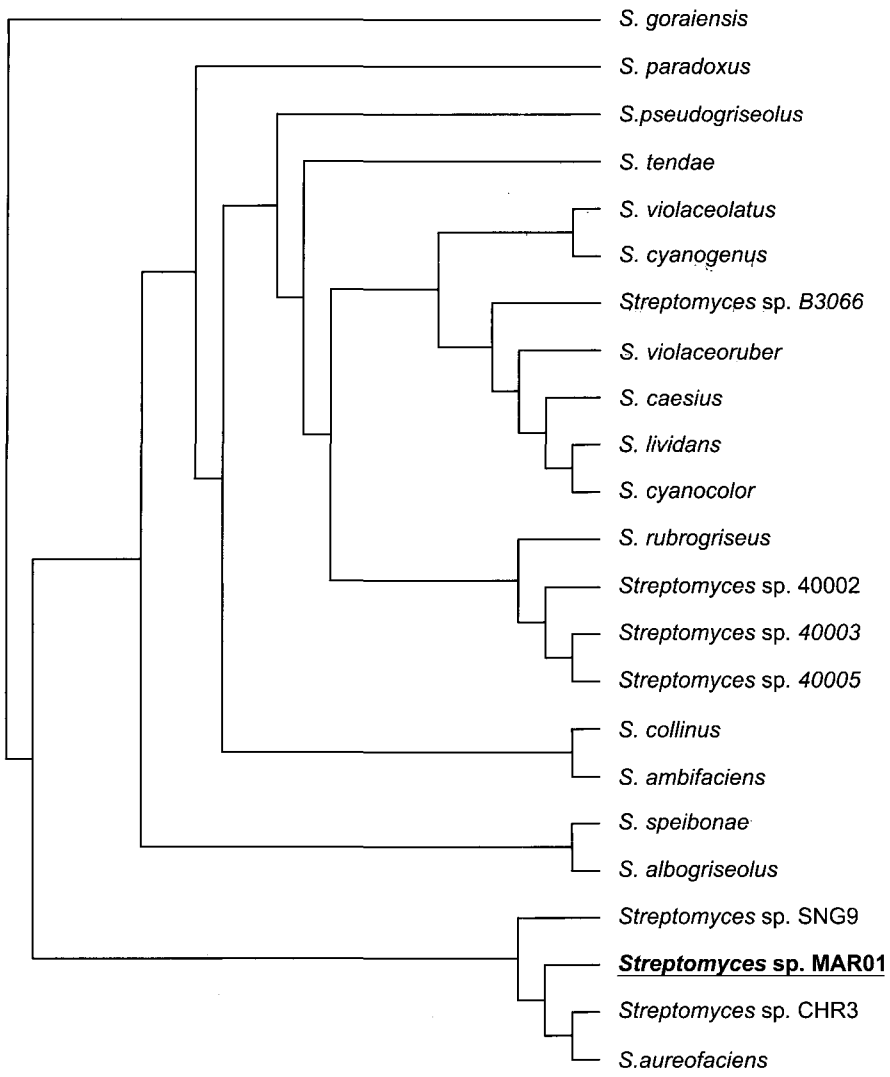

Fig. 2. The phylogenetic position of the local *Streptomyces* sp. strain MAR01 among neighboring species. The phylogenetic tree was based on the pairwise comparisons of 16S rDNA sequences.

Table 3. ^{1}H NMR chemical shifts ($CDCl_3$, ppm) of the antibiotic Meroparamycin

Hydrogen No.	Hydrogen type	ppm (multiplicity)
1, 1'	CH_3	0.97 (t)
2, 2'	CH_2	1.25 (m)
3, 3'	CH_2	1.26 (m)
4	CH	1.46 (m)
5	CH_2	1.53 (m)
6	CH_2	2.28 (t)
7	-	-
8	CH_2	2.24 (t)
9	CH_2	4.15 (t)
10	-	-
11, 11'	CH	7.63-7.64 (m)
12, 12'	CH	7.43-7.45 (m)
13	-	-
14	NH_2	6.7 (br s)

carbon atoms observed on ^{13}C NMR. The electron impact (EI) mass spectrum confirmed that the molecular weight of the antibiotic was 303.44.

The proton nuclear magnetic resonance (^{1}H NMR) spectrum of the isolated compound appears to evidence two identical sets of aromatic protons, which were observed as multiplets at 7.43-7.45 and 7.63-7.64 ppm, which is indicative of a para substituted aromatic structure. The multiplet centered at 4.15 ppm is indicative of benzylic protons. The aliphatic protons appearing at 0.97, 1.25, 1.26, 1.46, 1.53, 2.28, and 2.24 ppm were assigned to the rest of the aliphatic chain (Table 3).

The ^{13}C NMR nuclear magnetic resonance spectrum reveals alkyl carbons in the region between 14-40 ppm (Table 4). It also reveals the presence of two peaks at 128.76 and 132.43 ppm, which were assigned

Table 4. ^{13}C NMR chemical shifts ($CDCl_3$, ppm) of the antibiotic Meroparamycin

Carbon No.	Carbon type	ppm
1	CH_3	14
2	CH_2	19.19
3	CH_2	38.75
4	CH	34.38
5	CH_2	29.07
6	CH_2	39.04
7	C=O	205.34
8	CH_2	38.75
9	CH_2	31.90
10	C	obscured
11, 11'	CH aromatic	128.76
12, 12'	CH aromatic	132.43
13	C	130.82
14	C=O	167.73

Table 5. Antimicrobial activities of the antibiotic Meroparamycin produced by *Streptomyces* sp. MAR01

Test organism (ATCC Number)	MIC (mg/ml)
Bacillus subtilis (6633)	6.25
Escherichia coli (10536)	50
Staphylococcus aureus (29737)	6.25
Staphylococcus epidermidis (12228)	12.5
Micrococcus luteus (9341)	3.13
Candida albicans (10231)	25

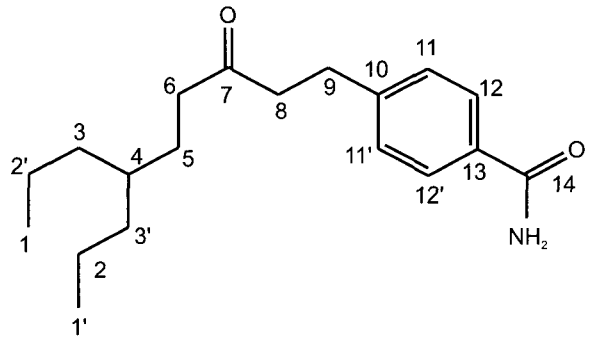

Fig. 3. The molecular structure of the antibiotic Meroparamycin produced by *Streptomyces* sp. MAR01.

to aromatic carbons. Furthermore, one aromatic quaternary carbon was obscured. In addition, another two peaks were observed at 167.73, which is assigned to the carbon atom of the carbonyl of the amide ($-CONH_2$) and the peak at 205 ppm is assigned to the carbonyl of the ketone group. Furthermore, the number of peaks in the ^{13}C NMR supported the proposed structure as a para-substituted, rather than an ortho-substituted, aromatic compound.

The results of MS, elemental analysis, IR, ^{1}H NMR, and ^{13}C NMR confirmed the proposed structure of the isolated compound, which was assigned the name Meroparamycin, as is shown in Fig. 3.

The antimicrobial activity of the pure Meroparamycin compound is shown in Table 5. The pure compound inhibited the growth of both gram-positive and gram-negative representative bacteria, as well as *C. albicans*.

Discussion

It has long been known that some of the actinomycete strains of the same species could generate different antibiotics, whereas some other strains belonging to different species generated the same antibiotics (Lechevalier, 1975). The production of antibiotics by actinomycetes, therefore, may not be species-specific, but rather strain-specific. Antibiotics of actinomycete origin evidence a wide variety of chemical structures, including aminoglycosides, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyketides, actinomycins, and tetracyclines (Okami and Hotta, 1988; Baltz, 1998).

The locally-isolated *Streptomyces* strain was identified as *Streptomyces* sp. as the result of 16S rDNA techniques, as did the cultural and physiological characteristics of the strain. The cultural and physiological properties of the isolated strain were compared to those of the actinomycetes as described in Bergey's Manual of Determinative Bacteriology, and determined that this strain belongs to the genus *Streptomyces* (Lechevalier *et al.*, 1989). The comparison of the physiological characteristics of the local isolate to those published in Bergey's Manual of Determinative Bacteriology for *S. aureofaciens* clearly showed that these two strains are significantly different. These differences disallowed the consideration of *S. aureofaciens* as the proper name for the local isolate. However, despite the profound similarity (99%) between the nucleotide sequence of the 16S rRNA gene of the locally isolated *Streptomyces* and *S. aureofaciens* and *Streptomyces* spp., but we can only hypothesize that any two *Streptomyces* strains are identical when a 100% similarity is detected. Therefore, the isolated strain was identified as *Streptomyces* sp. MAR01.

Moreover, *S. aureofaciens* is an industrially important microorganism, and a producer of both chlortetracycline and tetracycline (Yang and Ling, 1989). Numerous *S. aureofaciens* strains have been identified as antibiotic producers, and the antibiotics generated by this species are tetracyclines (Stryzhkova *et al.*, 2002), macrolides (White *et al.*, 2001), and quinocyclines (Furumai *et al.*, 2002). In the present work, the antibiotic generated by the local isolate, after conducting the separation and purification procedures, is a primary bioactive constituent isolated from the culture filtrate, and its structure was determined via spectro-

scopic techniques. Neither the proposed molecular structure of the isolated antibiotic, nor its molecular weight, resembles any of the antibiotics generated by any of the strains of *S. aureofaciens*. Finally, by conducting a search using the AntiBase database (Laatsch, 2003) as well as a survey of the available literature, the structure of the antibiotic generated by this local strain in the present investigation proved not to be identical to any other reported antibiotics. Therefore, the antibiotic was assigned the name Meroparamycin. This antibiotic evidenced an *in vitro* antimicrobial activity against gram-positive and gram-negative bacteria, as well as *C. albicans*.

In conclusion, the data obtained in this study strongly suggests that the isolated strain is related to *Streptomyces* sp., rather than to *S. aureofaciens,* and that the bioactive compound differs from those microbially-produced antibiotics. The possible routes of antibiotic synthesis and its antitumor activity are currently under investigation.

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