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Antitumor Activity of the Cyclo (L-Phenyl, L-Prolyl) Diketopiperazines Produced By a Newly Isolated *Streptomyces Sp*. A4.4

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Abstract

This paper describes the identification of antitumor Cyclo (L-phenyl, L-prolyl) Diketopiperazines in a newly isolated Streptomyces sp. Strptomyces sp strain A 4.4 was isolated from soil samples collected from Sudan, purified and screened for their antimicrobial activity against pathogenic microbes, and taxonomically characterized on the basis of morphological and physiological characteristics, phylogenetic analysis and genotypic data. Cultural characteristic studies strongly suggested that these strains are members of the genus *Streptomyces*. The comparison of the 16S rRNA sequence of the A 4.4 isolate with those sequences submitted to GenBank demonstrated that this strain was 94% similar to the 16S rRNA sequence to number of isolates all of which were *Streptomyces* species, However, there are many differences between strain A 4.4 and those strains according to the published data. The phylogenetic tree, suggested that Streptomyces sp A 4.4 arose from different node which suggested that this organism was not a closely related species. A 4.4 was proposed as a novel *Streptomyces* species. The pure active antibiotics were isolated from the culture supernatant and from the biomass using reverse phase HPLC-DAD. Spectroscopical analysis employing Mass, ^{1}H and ^{13}C NMR spectra data the compound was chemically characterized as Cyclo-(Proline--Phenylalnine). cyclo (pro-phe), produced by these identified Streptomyces strains showed significant anticancer effect against human cervical carcinoma cells and Glioma UG-87 cells using MTT assay. The two antibiotics at different concentrations (1µg ml^{-1}, 10µg ml^{-1}, 20µg, 30µg ml^{-1} and 40µg ml^{-1}) of the compounds for 48 hour incubation. caused death of 90% of cancer cells in comparison to the control cells. In conclusion, we have isolated and identified a new cyclo (phenylalanine-proline) producing streptomyces from Sudanese soil and characterized as Streptomyces A4.4 by 16S rRNA homology. This Streptomyces sp. A 4.4 was the new species of the genus actinomycetes that had the highest level of antibiotic activity showing promising anticancer activity on cervical carcinoma cells and Glioma cells UG- 87.

Keywords: Streptomyces sp; Cyclo (L-phenyl, L-prolyl); Anti-cancer activity.

1. Introduction

While soil actinomycetes have been extensively studied for their antibiotic production, Sudanese soil samples have been relatively poorly investigated. Due to the large degree of geographical variation, there are a wide variety of soil types found throughout Sudan, many of which are rich in flora and fauna and in microbial diversity. Thus we have focused on Sudanese soil samples as a potential source of microbial diversity and hence new molecules of better therapiotic effects. 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 Currently, actinomycetes bacteria considered as one of the most attractive sources of antibiotics and other biologically active substances of high commercial value and they are attracting considerable interest from bacteriologists, biotechnologists, geneticists and ecologists. Streptomycetes are the source of several useful antibiotics that are used not only in the treatment of various human and animal diseases but also as biological control and biochemistry as metabolic poisons [1]. At least 70 of the approximately 100 marketed antibiotics used for the treatment of infections in humans are derived from substances produced by *Streptomyces* spp. Streptomycetes are known producers of industrial enzymes and medically important compounds, e.g. polyketides, tetracyclines , antitumor agents and the best known are antibiotics currently used worldwide as pharmaceutical and agrochemical products [2-4]. Diketopiperazine and its derivatives constitute a family of secondary metabolites with diverse and interesting biological activities including antibiotics, immunosuppressant, and antitumors. Diketopiperazines (DKPs) corresponding to cyclic dipeptides has been isolated

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from microorganisms and sponges and from a variety of tissues and body fluids [5, 6]. These heterocyclic compounds display pharmacological effects in various mammals. Moreover, it has been suggested that several DKPs may play an important role as chemical mediators of bacterial quorum-sensing and signaling systems. This paper describes identification of antitumor Cyclo (L-phenyl, L-prolyl). Diketopiperazines in a newly isolated *Streptomyces* sp. Material and MethodsIsolation, characterization and identification of Streptomyces A4.4. Strain A4.4 was isolated after a screen of soil samples collected from different locations in the Sudan. Isolation of the strains was performed by soil dilution plate technique usingstarch-casein nitrate agar (SCNA) (starch 10.0 g, casein 0.3 g, KNO_3 2.0 g, NaCl 2.0 g, K_2HPO_4 2.0 g, $MgSO_4.7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4.7H2_O$ 0.1 g, Agar 18 g, $H2_O$ 1.0 l), supplementedwith 10 mg/ml cyclohexamide. The antimicrobial activity of the cell cultures was screened against a range of microbes. The pure isolates were maintained as lyophils and as spore suspensionsat -80°C as described by Hopwood and co-workers [7].Molecular Genomic fingerprint of bioactive Streptomyces isolate A total DNA was isolated from Streptomyces A 4.4 strain by using the method of Nikodinovic, *et al.* [8]. PCR amplification and sequencing of 16S rRNA gene was carried out as described previously [9] using a Peltier thermal cycler (BIO-RAD). The PCR product was cleaned up using a Qiagen Qlaquick PCR purification kit according to the manufacture's instruction. Sequencing was performed commercially by MWG-Biotech (Germany). Sequenced gene fragments were compared to those in Genebank using the BLAST program on the website of the National Center for Biotechnology information [10]. Sequencing of the other Streptomyces spp. were obtained from Genebank and the sequences were aligned with similar sequences using the Clustal X program [11]. Phylogenetic analysis was conducted by the neighbour-joining method using the TREECON program. Extraction of Bioactive MetabolitesThe culture broth (1 L) was centrifuged at 6,000 rpm for 15 min to separate the mycelial biomass. Activities against test organisms using the agar well diffusion method. Mycelium and culture supernatants were beastly extracted with ethyl acetate; the antibiotic activity remained in the organic phase. Ethyl acetate was removed using flash evaporator (Buchi, Switzerland) to dryness at 40°C under vacuum. 1 gram crude Ethyl acetate extract that possess activity against test organisms, was dried and separated by solid phase extraction (SPE) on a Hypersil C18 column. Fraction containing highest antibiotic activity was collected and purified further by HPLC (Varian Prostar system) using an isocratic elution (80% methanol-water) on a Zorbax StableBond column. Further purification was achieved by reversed phase HPLC. Diode Array Detector DAD using an isocratic elution (80% methanol-water) on a Zorbax StableBond column. NMR analyses were performed using Varian Inova 300 MHz spectrometer. Samples for H^1 NMR and C^{13} NMR spectroscopy were dissolved in chloroform ($CDCl_3$). NMR data for the compound was assigned by 2D NMR analysis and comparison to literature data. Antitumor activity of Cyclo (L-phenyl, L-prolyl Various cell lines, such as cervical carcinoma cells and Glioma cells UG- 87, were used to evaluate the in-vitro anti-tumor effects of the cyclo (pro- phe) produced by *Streptomyces* sp. A 4.4. MTT assay were performed in the UCD Conway institute of biomolecular and biomedical research. Growth inhibitory effect of glioma cells (UG- 87) (brain) and hela (cervical) cells with various treatments was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co) assay as as described elsewhere [12]. '

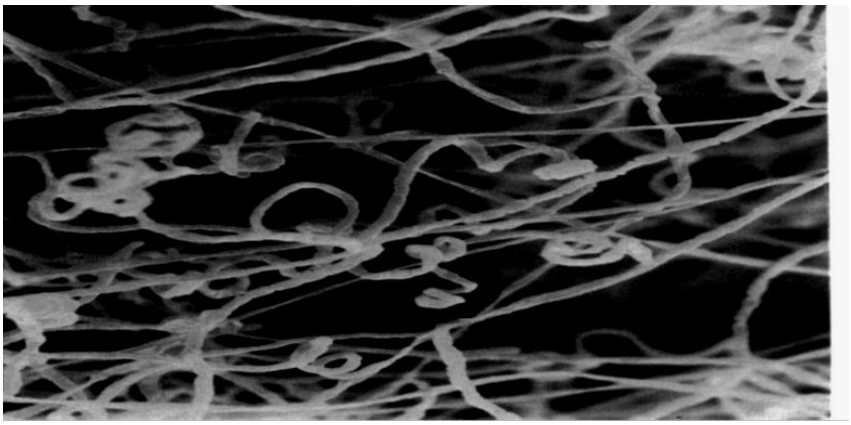
2. Results and Discussion

2.1. Cultural Characteristics of Biologically Active Streptomyces Sp

Based on the prominent antimicrobial activities, the morphological and cultural characteristics of the selected isolate was observed after 10 days incubation on the International Streptomyces Project (ISP) media figure (1, 2) and various other media and they showed considerable variations.

Fig-1. Morphological type of Colonies of *Streptomyces* spp. In ISP4 Medium after 10 Days Incubation at 30°C

Fig-2. Scanning Electron Micrographs of the Spore Chains of the Bioactive *Streptomyces* sp. A 4.4



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, Figure (3). *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*. However, there are many differences between strain A 4.4 and these strains according to the published data. Phylogenetic analysis of the strains with the most closely related *Streptomyces* spp. and those that are known to produce Cyclo (L-phenyl, L-prolyl). The 16*S* rDNA sequence of this isolate was submitted to GenBank (accession number GU013558).

From the phylogenetic tree, *Streptomyces* sp A 4.4 arose from different node and this suggested that this organism was not closely related species. A 4.4 is proposed as the novel species of Streptomyces, Figure (3).

The 16S rRNA gene sequence is highly conserved within living cells and has been widely used for evolutionary studies in bacteria [13], placing organisms in the framework of phylogenetic relationships [9]. However, 16S rRNA gene sequences may be insufficient to define phylogenetic relationships among closely related species and among strains belonging to a species because of evolutionary conservation of the 16S rRNA gene [13]. It has been suggested that the 16S–23S rRNA internally transcribed spacer (ITS) region is a powerful tool for phylogenetic analysis of Gram-negative bacteria, but not of Gram-positive bacteria, especially *Streptomyces* species [14, 15]. To overcome these problems, it has now become common practice to delineate novel *Streptomyces* species using a combination of genotypic and phenotypic data [16] in a so called polyphasic taxonomic study, which is expected to lead to well-described species and a stable nomenclature [17]. In addition, 16S rRNA sequence data have proved invaluable in streptomycetes systematic, in which they have been used to identify several newly isolated Streptomyces species [18].

Fig-3. Amplified 16S rRNA gene fragment total genomic DNA. Lanes 1-2 (1= 1.5 kb DNA ladder).*Streptomyces* sp. A 4.4. (1288 bp)

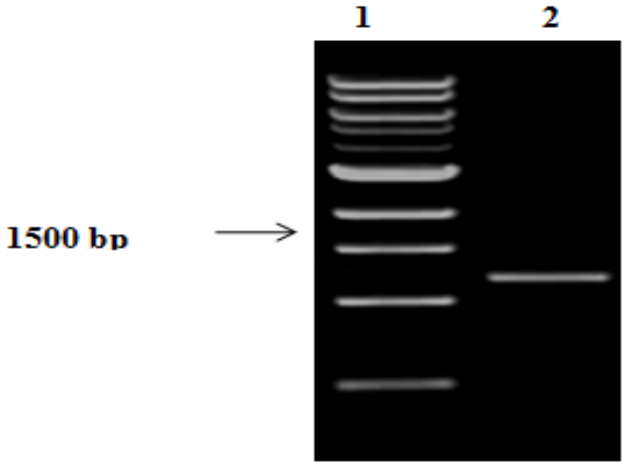
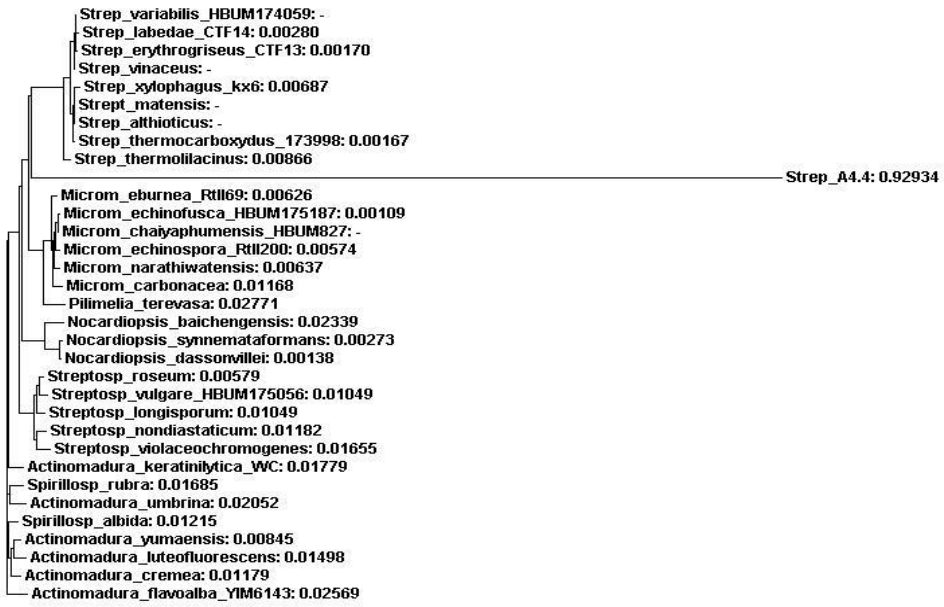


Fig-4. Phylogenetic Tree Showing the Relationship Between strain A4.4 and Representative Species of the Genus *Streptomyces* and other Taxa Based on Nearly Complete 16S rRNA Gene Sequences



2.2. Purification and Structure Elucidation of the Cyclo (L-Phenyl, L-Prolyl) Molecule

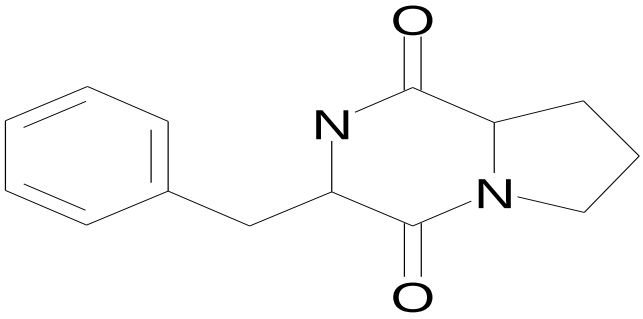
The purification of the active colorless amorphous solid compound (144 mg) was carried out using reverse phase SPE and HPLC-DAD analysis. This compound showed a strong absorption under UV light at 212 nm. Compound ESI [+] *m/z* 245, had a molecular formula of $C_{14}H_{16}N_2O_2$. . NMR analyses were performed using Varian Inova 300 MHz spectrometer. Samples for H1 NMR and C13 NMR spectroscopy was dissolved in chloroform ($CDCl_3$). NMR data for the compound was assigned by 2D NMR analysis table (1) and compare to literature data. The chemical structure of this compound is shown in Figure (5).

The cyclo (L-phenyl, L-prolyl) molecule which is a diketopiperazine (DKP) derivatives. Diketopiperazine molecules constitute a family of secondary metabolites with diverse and interesting biological activities such as antibacterial, fungicidal, herbicidal, immunosuppressor, antitumors, antiviral [19, 20]. The DKP derivative, produced by the *Streptomyces* sp. A4.4 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) [21, 22], and from a Norcardia species for the cis-cyclo (Leucyl-Prolyl) [21], 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. [23]*, and from *Alcaligenes faecalis* for the Cyclo-(L-Pro-L-Phe) and Cyclo-(L-Pro-L-Leu) [24]. DKPs comprise an important family of the secondary metabolites that are mainly produced by microorganisms [23]. The Sudanese bacterium Strptomyces sp. A4.4 is a new source of bioactive DKPs.

Table-1. NMR data of the ^{1}H (300 MHz) and ^{13}C NMR (300 MHz) of cyclo (phenylalanine-proline) in $CDCl_3$ from Streptomyces sp. A4.4

Position	Proton H	Position	Carbon 13
1	-	1	165.20
2	-	2	-
3	3.60-3.70	3	45.80
4	1.90-2.10	4	22.6
5	2.3-2.4	5	28.40
6	4.08	6	56.20
7	-	7	169.60
8	5.57	8	-
9	4.27	9	59.20
10	3.60-370	10	36.90
1’	-	1’	135.90
2’	7.20-7.39	2’	129.40
3’	7.20-7.39	3’	129.40
4’	7.20-7.39	4’	129.40
5’	7.20-7.39	5’	129.40
6’	7.20-7.39	6’	129.40

Fig-5. Chemical Structure Cyclo-(phe-pro) Produced by *Streptomyces* sp. A4.4



2.3. Antitumor Activity of the Cyclo (Phe-Pro) Compound

Activity of the cyclo (phenyl-prolyl) compound against human cervical carcinoma cells and Glioma cells UG-87 as antitumor was assessed in microtiter plate using MTT assay. Figure (xxxxx) showed that cyclo (phe-pro) in concentrations of 1µg ml $^{-1}$, 10µg ml $^{-1}$, 20µg ml $^{-1}$, 30µg ml $^{-1}$ and 40µg ml $^{-1}$ showed significance activity to the human cervical carcinoma cells and Glioma cells UG- 87. Microscopically analysis of the isolated compound suggested antitumor response, because they caused 90% death of the treated cells in compression to the control cells. These results agreed with those of Laura Hernández-Padilla, *et al.* [25] his showed that PAO1-CDPs affected the viability of HeLa cells in a dose-dependent manner. Cell cultures showed 75% of dead cells after treatment with PAO1-CDPs, using either individual (purified) CDPs and the crude CDP mixture at 1.0 mg/mL after 24 h, being the cyclo (L-Pro-L-Phe) slightly more active against HeLa cells than the other CDPs. These result encourages further research on cyclo (phe-pro) and It is plausible that strains A 4.4 can be used to produce this antibiotics to enable studies of medicinal applications.

3. Conclusions

In conclusion, we have isolated and identified a new cyclo(phenylalanine-proline) producing streptomyces from Sudanese soil and characterized as Streptomyces A4.4 by 16S rRNA homology. This Streptomyces sp. A 4.4 was the new species of the genus actinomycetes that had the highest level of antibiotic activity showing promising anticancer activity on cervical carcinoma cells and Glioma cells UG- 87.

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